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large to be estimated spectrographically such as Ca, Mg, K, P, and Fe. It has been found that the iron content of vegetables from different parts of the state varies widely. Thus turnip greens grown on the poorer types of soil had 90 p.p.m. of iron while those on the better soils had 318 p.p.m.

A STUDY OF THE PATHOLOGIC CHANGES IN TISSUES AND ORGANS AFFECTED BY DEFICIENCY DISEASES OR BY TOXIC SUBSTANCES

Purnell Project No. 222 — C. F. Ahmann

Because of the reputed dietetic value of the papaya, an investigation was begun to determine the pharmacological and physiological action of some of the constituents of the papaya plant and fruit. The pharmacological action of papain isolated from the leaves and immature fruits was studied during the year. Experiments were carried out on pigeons to determine the antiemetic properties of the enzymes. When emetics were administered orally, subcutaneously, and intravenously, emesis took place in most instances regardless of the amount of papain given. In fact, in several cases the administration of papain seemed to hasten emesis and render the symptoms more acute.

Papain

The next series of experiments was designed to show the effect of papain as a cardiac stimulant. When isolated frog hearts were irrigated with 1% to 2% of papain it seemed to strengthen the contractions of both auricle and ventricle by lengthening the refractory period, diminishing fibrillation and other cardiac irregularities. Papain seemed to have no effect upon the amplitude of the heart beat. The properties of papain as a vermifuge for dogs are now being studied.

THE DEVELOPMENT OF QUANTITATIVE SPECTROGRAPHIC METHODS IN AGRICULTURAL RESEARCH

Purnell Project No. 256 — L. W. Gaddum, R. C. Williamson* and L. W. Rogers

The method for the quantitative determination of zinc in plant ashes was developed to the practical stage. A preliminary report of the method has been submitted for publication in the Analytical Edition of the Journal of Industrial and Engineering Chemistry. In connection with this work a number of zinc determinations were made on samples of leaf mould, weeds, etc., gathered in connection with Chemistry and Soils project 220.

A technique (based upon the ratio quantitative principle) for detection and estimation of 18 elements (barium, strontium, vanadium, chromium, aluminum, manganese, nickel, cobalt, silver, copper, cadmium, tin, titanium, bismuth, arsenic, antimony, zinc and beryllium) in biologic materials was developed. This technique was employed in analyzing samples of grasses in connection with forage crop work on Animal Husbandry project 133.

MISCELLANEOUS PROJECTS

A Study of the Physical and Chemical Properties of Eggs from Hens on Experimental Diets. (Cooperative with Agricultural Extension Service and the Florida National Egg-Laying Contest)

This cooperative project on the physical and chemical properties of eggs from hens on experimental diets has continued through two laying

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Ann. Otology, Rhinology, & Laryngology, v. 68, 1959

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Scientific Papers of the American Broncho-Esophagological Association

Papain

LXIII

PERFORATION OF THE ESOPHAGUS AFTER USE OF A DIGESTANT AGENT

REPORT OF CASE AND EXPERIMENTAL STUDY

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REPORT OF A CASE

A 27-year-old woman was brought to the Mayo Clinic desperately ill from an esophageal perforation with mediastinitis and empyema in both pleural spaces. Ten days earlier a bolus of meat had lodged in the cervical portion of her esophagus while she was eating her noon meal. Approximately three hours later a solution of caroid had been given to digest the meat and within two hours she had swallowed liquids satisfactorily. The next morning, approximately 12 hours after the ingestion of caroid, she had been discovered in a state of shock with subcutaneous emphysema in the neck. Cervical mediastinotomy and tracheotomy had been done and the administration of antibiotics had been started. During the ensuing two or three days empyema developed in each pleural cavity and bilateral intercostal drainage of these spaces with catheters had been established.

On transfer to this clinic ten days after her initial difficulty, little remained to be done except to assure adequate drainage from the mediastinotomy and both pleural spaces, administer proper antibiotics, and give sufficient supportive treatment with intravenously administered fluids and electrolytes. Micrococcus pyogenes, Pseudo-

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Read at the Thirty-ninth Annual Meeting of the American Broncho-Esophagological Association, Hot Springs, Va., March 1959.

monas and Proteus were cultured from purulent material from all drainage sites. On the twelfth day of her illness, two days after her arrival at the clinic, massive hemorrhage suddenly developed from the cervical mediastinotomy and the patient died almost instantaneously.

Postmortem examination revealed that the fatal hemorrhage came from erosion through the wall of the common carotid artery. The cervical portion of the esophagus was completely destroyed for a distance of approximately two inches. There were fistulous passages into each pleural space from the cervical mediastinum.

EXPERIMENTAL STUDY

In an attempt to determine the role caroid might have played in digesting and perforating the patient's esophagus we inserted a bolus of cooked meat perorally into the esophagus of each of several anesthetized dogs. The meat was tied to a whalebone staff and inserted into the esophagi of the dogs. After the meat was satisfactorily placed in each esophagus, the staff was fastened at the mouth so that the meat impaction would remain stationary. Caroid solution in the form of essence of caroid or half an ounce of caroid powder mixed with one ounce of water was introduced through a polyethylene catheter attached to the staff terminating at the upper level of the meat. The body of each dog was elevated approximately 25 degrees with the head above the feet.

Fourteen dogs were used in pairs (Table I). Initially the same size piece of meat was inserted into the esophagus of each of a pair of dogs, but only one dog received caroid. After three such pairs it soon became apparent that the meat alone produced no discernible damage to the esophagus, so controls were abandoned. Almost every dog receiving caroid showed some degree of damage to the esophageal wall with reddening of the mucosal wall and thickening of the wall at the site of the lodged bolus (Fig. 1). Microscopically epithelium was denuded in a patchy distribution without much inflammatory reaction. The meat impaction was friable and had about the same consistency as cooked fish. In addition the lungs of each dog receiving caroid showed the development of hemorrhagic edema which, in some instances, was sufficient to cause rapid death (Fig. 2).

COMMENT

Caroid is a proprietary digestant containing papain which is a proteolytic enzyme obtained from the tropical papaw or melon tree, Carica papaya. In general its action resembles animal pepsin or tryp-

TABLE I

DOG	CAROID, DOSE	FATE	ABNORMAL FINDINGS
1	10 cc once; 3 cc 1 hr. later	Died in 3 hr.	Esophageal wall reddened, edematous; extreme hemorrhagic pulmonary edema
2	None; control for dog 1	Sacrificed 3½ hr.	None
3	5 cc hourly; 6 doses	Sacrificed 7 hr.	Esophagus reddened, edematous; moderate hemorrhagic pulmonary edema
4	None; control for dog 3	Sacrificed 7 hr.	None
5	5 cc hourly; 6 doses	Sacrificed 6½ hr.	Esophagus normal; extreme hemorrhagic pulmonary edema
6	None; control for dog 5	Sacrificed 6½ hr.	None
7	5 cc 30-minute intervals; 7 doses	Died 3¼ hr.	Esophagus reddened; extreme hemorrhagic pulmonary edema
8	5 cc 30-minute intervals; 11 doses	Died 5¼ hr.	Esophagus reddened; extreme hemorrhagic pulmonary edema
9	5 cc 30-minute intervals; 9 doses	Sacrificed 5½ hr.	Periesophageal edema; moderate hemorrhagic pulmonary edema
10	5 cc 30-minute intervals; 9 doses	Sacrificed 5½ hr.	Periesophageal edema; slight hemorrhagic pulmonary edema
11	5 cc 30-minute intervals; 4 doses	Sacrificed 16½ hr. (was about to die)	Esophageal wall hemorrhagic; extreme hemorrhagic pulmonary edema
12	5 cc 30-minute intervals; 4 doses	Sacrificed 7 hr. (was about to die)	Esophageal wall hemorrhagic; extreme hemorrhagic pulmonary edema
13	5 cc 15-minute intervals; 4 doses	Died 1 hr.	Esophageal wall hemorrhagic; extreme hemorrhagic pulmonary edema
14	5 cc 15-minute intervals; 6 doses	Died 1¾ hr.	Esophageal wall hemorrhagic; extreme hemorrhagic pulmonary edema

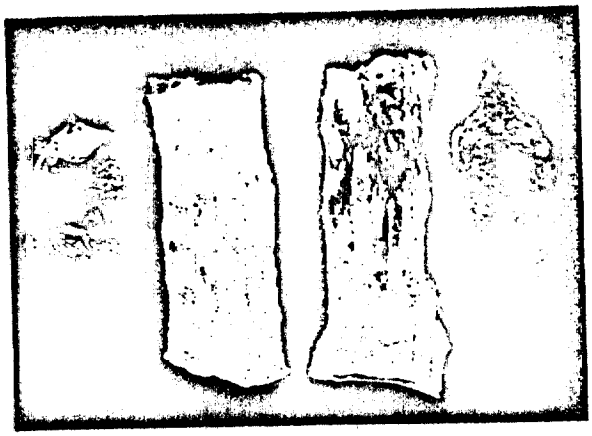

Fig. 1.—Bolus of meat and esophagus from control dog (left) and from dog 1 (right) which received caroid.

sin in its digestive action on proteins, and its medicinal use in this continent antedates the arrival of Columbus.

In 1945 Richardson[1] reported the use of this enzyme in the treatment of esophageal obstruction due to meat impaction. He narrated the histories of 16 patients whom he had treated with sips of a 5 per cent solution of papain successfully to digest a bolus of meat sufficiently to dislodge the obstruction in the esophagus. His treatment had failed in only one patient. Since then other reports[2-4] have appeared advocating the use of caroid for the treatment of meat impaction in the esophagus. We have not encountered a single report of an untoward reaction when caroid was used in this fashion. Tashiro and Schmidt[5] discovered no instance of harm to the gastrointestinal tract when papain was given. Supposedly this enzyme will not digest living, viable tissue except perhaps in a closed space. These authors demonstrated proteolytic damage when papain was injected intraperitoneally into rabbits.

We wondered whether an esophageal wall distended by a meat impaction might be sufficiently ischemic to allow the proteolytic action to attack the mucosa in addition to the cooked meat. We attempted to simulate clinical conditions in our experiment as closely

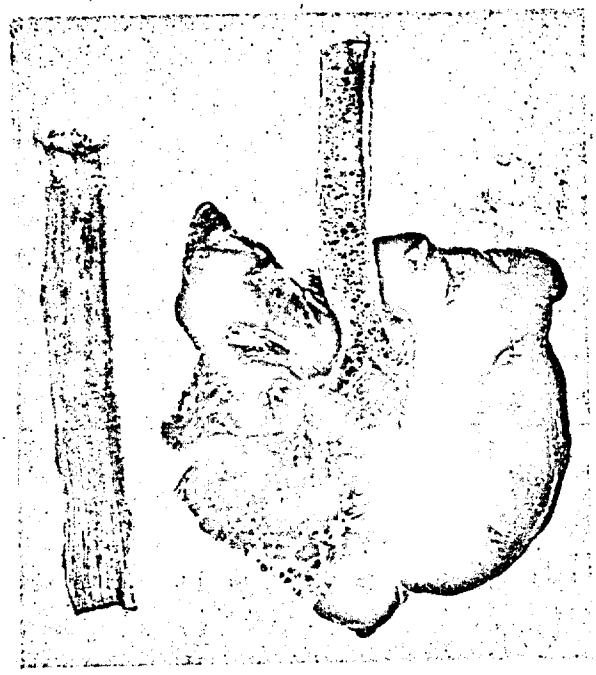

Fig. 2.—Lungs of dog 13 showing hemorrhagic pulmonary edema, more marked in the right or dependent lung.

as possible and, therefore, did not distend the esophagus of dogs with balloons or materials that would not invite the use of a protein digestant by a physician. In our opinion we have demonstrated that caroid is capable of damaging the wall of the esophagus. Even though we did not produce actual perforation of the esophagus, this is probably a matter of degree. We feel that prolonged contact of caroid with an ischemic esophageal wall is fraught with danger.

Godfrey and Miller[6] used several proteolytic enzymes on cooked meat and found that caroid was the most effective digesting agent. They thought crystalline papain was preferable, however, because it was sterile, whereas a gram-positive micrococcus was cultured from caroid. It is of interest to note that Micrococcus pyogenes was grown from the purulent drainage of our patient. We can only speculate about the role that this organism played in the course of her disease.

Hemorrhagic pulmonary edema was found in almost all dogs receiving caroid and was sufficient to cause the death of some. Our dogs were anesthetized and the pulmonary edema probably was due to aspiration of caroid into the tracheobronchial tree. In patients this complication probably would not be encountered because the person is usually advised to sip a solution and therefore must be awake with his cough reflex intact. The possibility of aspiration with esophageal obstruction is well recognized, however, and this serious pulmonary complication must be considered as a hazard.

SUMMARY

The death of a 27-year-old, previously healthy woman from perforation of the esophagus after the use of a proprietary digestant (caroid) derived from Carica papaya for a meat impaction prompted us to investigate this proteolytic agent. Approximately 12 hours after the ingestion of caroid the patient experienced rapidly progressive, fulminating mediastinitis which terminated in perforation of the common carotid artery and sudden massive hemorrhage.

We conducted experiments on dogs and attempted to simulate clinical conditions by obstructing and distending the esophagus with a bolus of meat. Caroid was introduced just above the obstruction in some of the dogs. Definite damage to the esophageal wall was noted in those receiving this substance. We postulated that stretching and compression of the esophageal wall caused sufficient ischemia for the proteolytic enzyme (papain) in the digestant to act. Advanced hemorrhagic pulmonary edema causing death also was encountered.

Extreme caution in the use of caroid for treatment of meat impaction of the esophagus is urged.

MAYO CLINIC

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possibly be involved, although citric acid produced no stabilization.[2] Balls *et al.*[4] have shown that the crystalline enzyme loses activity when mixed with latex. Inactivation also occurred when heated latex, having no activity itself, was substituted for fresh latex. This all points to a reaction of thymol and sodium bisulfite, with inhibiting factors present in fresh latex as the most probable explanation of their stabilizing action.

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DEMONSTRATION OF THE *IN VIVO* ACTIVITY OF PAPAIN

By Aaron Arnold, Jean R. Schmitz, and Harold Blumberg

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The use of plant proteinases for the softening of meats has led to investigations concerning the effect of their ingestion upon animals and man. Tashiro and Schmidt[1] reported they were unable to discern any adverse effect in animals due to the feeding of papain-containing extracts. In fact, they suggested that "the optimum conditions of peptic digestion destroy papain." Lineweaver and Schwimmer[2] indicated accord with this view by suggesting that "it is doubtful if it (papain) will survive the acidity and other destructive agents that may be in gastric juice."

General interest[3–9] in the oral administration of papain, however, indicated the desirability of determining whether papain increased the digestion of protein *in vivo*. In this report, therefore, are given the results of studies which demonstrate that such activity does in fact exist.

Experimental

Papain Source Material. The source of papain for these studies was commercial papain (dried papaya latex). It had 163 units per gram by the milk-clotting test before activation and 212 units after activation.[7] The heat-inactivated material referred to below was prepared by autoclaving a portion of the papain at 15 lbs. pressure for 30 minutes. After autoclaving, it was dried at 60°C. under vacuum and ground to a fine powder. Its lack of proteolytic activity was confirmed by incubation with gelatin under standard test conditions.[3]

Procedure. Young adult rats in individual cages were starved for 24 hours and were then offered the following food mixture:

Casein	0.95 gram
Papain, unheated or heat-inactivated	0.05 gram
Glucose	0.35 gram

The rats used for test were those which voluntarily consumed the mixture within 7 to 15 minutes. They were sacrificed 2.5 hours after being fed the mixture. At that time, the stomach and fore-stomach contents were washed into a beaker with a small amount (25 ml.) of distilled water. The pH of the water suspension was noted and then adjusted to pH 4.85 to precipitate the proteins. The suspension was centrifuged and the clear or slightly cloudy supernatant fluid was poured off. The supernatant fluid was adjusted to pH 6.0 with 0.1 N sodium hydroxide, and a calculated amount of formalin was added so as to yield a formaldehyde concentration of about 9 per cent.[10] The solution was then titrated with 0.1 N sodium hydroxide to pH 9.0 as a measure of the protein digestion products.

Results and Discussion

A. *Tests at the Natural pH of the Stomach Contents.* The results (means ± S.E.) of the studies are summarized in TABLE 1.

It is seen clearly in TABLE 1 that marked differences resulted from the titrations of stomach contents. Thus, after the addition of formalin, 3.56 ± 0.12 ml. of 0.1 N sodium hydroxide were required to increase the pH from 6.0 to 9.0 for the stomach and fore-stomach contents of the rats fed papain. Only 1.97 ± 0.09 ml. of 0.1 N sodium hydroxide were required, however, for the comparable fraction from the control group of rats fed the heat-inactivated papain. The significance of the difference obtained was confirmed by statistical treatment of the data which showed P = <0.0001 or, that the result would be a chance occurrence less than once in ten thousand times. Thus, the appearance of the alkali titratable groups indicates that the proteolytic activity of papain increased the essentially

TABLE 1

EFFECT OF PAPAIN IN AIDING PROTEIN DIGESTION IN RATS

Index	*Test group fed active papain*	*Control group fed inactivated papain*
No. Rats	20	21
Males	12	12
Females	8	9
Weights, grams	206 ± 7	204 ± 10
Time to consume food, min.	10.0 ± 0.66	10.5 ± 0.86
pH stomach contents	3.89 ± 0.06	3.61 ± 0.07
	p = 0.003	
0.1 N sodium hydroxide to pH 4.85, ml.	1.56 ± 0.12	1.57 ± 0.096
Volume of centrifuge solids, ml.	1.66 ± 0.069	1.71 ± 0.068
0.1 N sodium hydroxide to pH 6.0, ml.	0.70 ± 0.079	0.49 ± 0.11
0.1 N sodium hydroxide to pH 9.0, ml.	3.56 ± 0.12	1.97 ± 0.092
	p = <0.0001	

normal rate of digestion 1.8 fold. Under less favorable conditions, this might conceivably be even greater. It is thus clear that papain administered orally contributes to the digestion of proteins in the stomach.

In harmony with the data which demonstrate that papain aided protein digestion in the stomach is the observation that the pH of the stomach contents was slightly higher (pH 3.89 ± 0.06) in the presence of papain than when heat-inactivated papain was fed (pH 3.61 ± 0.07). Statistically, the difference was indicated to be significant, P = 0.003, in that it would occur by chance only three times in a thousand. This agrees with the known anti-acid properties of protein hydrolysis products. This indication of an increased content of hydrolysis products in the rats fed papain over that noted in the control group was in harmony with the results obtained by titration of the stomach contents discussed above.

B. Test in Conjunction with Anti-acid. The foregoing results demonstrated clearly that papain was active at the natural pH of the stomach. It was of interest also to determine whether a decrease in gastric acidity

would enable papain to exert a greater effect and thereby indicate, indirectly, that papain was interfered with by the low pH of the gastric contents or by other possible inhibitors as suggested by Lineweaver and Schwimmer.[2] Accordingly, this was put to test by feeding anti-acids along with the papain-containing test meal. Three anti-acid combinations were chosen for this purpose: (1) aluminum hydroxide, (2) sodium bicarbonate, and (3) a combination of anti-acid agents, as supplied by "Al-Caroid" powder, including sodium bicarbonate, calcium carbonate, bismuth subcarbonate, magnesium carbonate, and magnesium oxide. The amounts of anti-acids fed and the results (means ± s.e.) obtained are summarized in TABLE 2.

TABLE 2

EFFECT OF PAPAIN IN AIDING PROTEIN DIGESTION IN RATS GIVEN ANTI-ACIDS

Index	*Sodium bicarbonate [illegible] mg. per rat*		*Aluminum hydroxide [illegible] mg. per rat*		*Al-Caroid [illegible] mg. per rat*	
	Control group fed inactivated papain	*Test group fed papain*	*Control group fed inactivated papain*	*Test group fed papain*	*Control group fed inactivated papain*	*Test group fed papain*
No. Rats	14	17	13	11	19	15
Males	15	17	8	8	15	15
Females	5	0	5	3	1	0
Weights, grams	162 ± 4	17[illegible] ± [illegible]	159 ± 11	184 ± [illegible]	190 ± 7	191 ± [illegible]
Time to consume food, min.	14.1 ± 1.3	14.9 ± 1.3	11.8 ± 1.1	13.6 ± 1.3	13.[illegible] ± 1.0	12.[illegible] ± [illegible]
pH stomach contents	5.13 ± 0.05	4.77 ± 0.06	4.05 ± 0.10	3.97 ± 0.17	5.03 ± 0.06	5.07 ± [illegible]
Vol. of centrifuge solids, ml.	1.34 ± 0.07	0.94 ± 0.05	1.76 ± 0.08	1.32 ± 0.094	1.44 ± 0.08	0.9[illegible] ± 0.09
	p = <0.0001		p = 0.0014		p = 0.00015	
0.1 N Sodium hydroxide to pH 6.0, ml.	0.45 ± 0.02	0.50 ± 0.05	0.25 ± 0.017	0.145 ± 0.034	0.35 ± 0.02	0.55 ± [illegible]
0.1 N Sodium hydroxide to pH 9.0, ml.	2.25 ± 0.12	3.56 ± 0.03	1.40 ± 0.04	2.90 ± 0.17	2.[illegible] ± 0.03	3.72 ± [illegible]
	p = 0.0007		p = <0.0001		p = 0.[illegible]05	

In the presence of each of the anti-acids listed in TABLE 2, it is clearly evident that the ingestion of papain significantly increased the extent to which the casein had been digested. The significance of the differences was confirmed by statistical treatment of the data, which demonstrated that in all cases P was less than 0.001, a criterion usually accepted as highly significant.

The increased amount of protein digestion, due to the addition of papain to the protein test meal, varied somewhat from test to test. When aluminum hydroxide was added to the test meal, about 2.5 times as much protein digestion occurred in the group receiving papain as occurred in the test control group. Upon the addition of sodium bicarbonate to the test meal, about 1.5 times as much protein digestion resulted in the group receiving papain as noted in the control animals. In the test in which Al-Ca[illegible] anti-acids were employed, about 1.9 times as much protein digestion was observed when papain was included with the test meal as

occurred in the control rats. A comparison of these ratios with that which resulted (1.8) from feeding papain without an added anti-acid makes it evident that the anti-acids neither increased nor decreased the effectiveness of the papain in the stomach. Thus, no evidence was obtained to substantiate the view that the stomach contents would destroy papain activity because of gastric acidity, or the lack of it, or other adverse environmental factors present in the stomach.

In the presence of each of the three anti-acids, the volume of solids in the stomach after centrifuging was less in the presence of papain than when heat-inactivated papain was fed. In each case, the differences are statistically significant. The results are in the same direction as the formol titration data and can be interpreted only as confirming the *in vivo* activity of the papain in digesting the protein of the test meal.

Thus, from the evidence presented here, it is clear that *in vivo* papain activity, as supplied by average dried papaya latex preparations, is easily and convincingly demonstrated by studies on the extent of intragastric digestion.

Summary

1. Studies with rats are described in which the extent of *in vivo* gastric protein digestion was followed by examination of the stomach and forestomach contents 2.5 hours after the ingestion of a test meal.

2. From the results of formol titrations of the gastric contents and measurements of the volumes of undigested residues, a 1.5- to 2.1-fold greater protein digestion, produced by feeding papain, was clearly demonstrated.

3. The presence of anti-acids did not modify the effect of the papain on digestion.

4. On the basis of these results, it was concluded that orally ingested papain has *in vivo* digestive activity. No evidence was seen that the gastric contents adversely affect the digestive activity of the enzyme.

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THE EFFECT OF THE ACTIVITY STATE OF THE ENZYMES ON THE *IN VIVO* ACTIVITY OF PAPAIN

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In earlier papers,[1,2] *in vitro* methods for the estimation of the activity of the proteolytic enzymes of papaya latex were compared. As pointed out there, these proteolytic enzymes exist in two activity states, the native or active state and the reversibly inactive state. The former digests proteins and clots milk without prior activation, whereas the latter exhibits its proteolytic properties only after activation by such agents as HCN, H_2S, mercaptans, cysteine, *etc.* It has been shown, however, that certain proteinaceous substrates, particularly beef[1-3] and heat-coagulated egg albumin,[4] have the ability to activate this inactive form during *in vitro* proteolysis.

Recently, Arnold, Schmitz, and Blumberg[5] have shown that papain, when added to a high protein test meal, significantly increased the extent of protein digestion occurring in the rat's stomach. The papain these investigators fed had approximately equal quantities of the enzymes in each of the two activity states. Additional investigations have therefore been carried out concerning the digestive power of these two activity states of the enzymes *in vivo*.

The activity of two papain samples having widely different ratios of active-to-reversibly-inactive enzymes were compared to determine the extent of intragastric digestion, and, more particularly, whether the reversibly inactive forms were active *in vivo*. Casein was chosen as the protein for the test meal, since previous investigations[5] had shown that it is not acted upon *in vitro* by the reversibly inactive enzymes. Beef or heat-coagulated fresh egg albumin could not be used for this test, since their *in vitro* activating effect might mask any *in vivo* activation.

Experimental

1. *Comparison* in vitro. A papain preparation, Papain A, with the enzyme content largely in the active state, was prepared from frozen fresh papaya latex by treatment with 0.5 per cent sodium bisulfite prior to drying, as has been described elsewhere.[6,7] National Formulary Reference Papain was used as the low active, high reversibly inactive sample. A sample of commercial papain was irreversibly inactivated by autoclaving for 30 minutes at 15 lbs. to be used as a negative control. These three samples were tested by the Blau modification[2] of the Balls and Hoover[8] milk-clotting method, both with and without HCN activation. They were also tested by an egg digestion method[5] and a gelatin digestion-form[1] procedure. The results are given in TABLE 1.

[illegible] that there is a vast difference between the native [illegible] of the two active samples. The ratio of the native [illegible] activity plus reversibly inactive activity [illegible] papain and Papain A are 1:75 and 1:30, respectively, [illegible] by the milk-clotting procedure. The egg digestion method gives

The Determination of the Concentration of Hydrolytic Enzyme Solutions: α-Chymotrypsin, Trypsin, Papain, Elastase, Subtilisin, and Acetylcholinesterase[1]

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Received June 20, 1966

Abstract: The use of rate assays is contrasted with the use of titrants to determine the absolute concentration of active sites of hydrolytic enzymes. Arguments are given which lead to the preference of a stoichiometric titration for the determination of the concentration of enzyme active sites. The absolute standard in this method is the small organic titrant rather than the enzyme which must be the absolute standard in a rate assay procedure. The mathematical basis of four methods of determining absolute enzyme concentration is presented. Three of these four methods were tested using *p*-nitrophenyl acetate and 2,4-dinitrophenyl acetate as titrants for α-chymotrypsin, showing good agreement. Of five titrants investigated for the determination of the concentration of trypsin solutions, *p*-nitrophenyl N$^{\alpha}$-benzyloxycarbonyl-L-lysinate hydrochloride was the most precise, and the one most closely resembling a specific substrate of trypsin. *p*-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate served as a titrant for papain solutions. N-*trans*-Cinnamoylimidazole was a titrant for the enzyme subtilisin. For the titration of elastase, no suitable specific substrate was found and diethyl *p*-nitrophenyl phosphate was used. *o*-Nitrophenyl dimethylcarbamate was used for the titration of acetylcholinesterase. For each titration, it was demonstrated that the criteria necessary for a rigorous titration were met. For most enzymes, a rate assay, based on a k_{cat} calculated from the titration data, was developed as a secondary standard for the determination of enzyme concentration. A discussion of the purity of each enzyme preparation, possible impurities in the preparations, and the possible effect of these impurities on titrations and kinetics is given.

The assay of an enzyme solution historically has been tied to an operational phenomenon: an enzyme is present if catalysis of a particular chemical reaction can be detected, and the concentration of the enzyme is related to the rate of catalysis. Enzymes, however, may now be characterized by more than their catalytic behavior; namely, in terms of chemical constitution and in terms of stoichiometric chemical reactions. Thus, the usage of a "rate assay" to determine the concentration of a catalyst such as an enzyme may be questioned, a usage which is tantamount to using a rate assay for determining the concentration of a catalyst such as hydrochloric acid. This commentary is particularly true of the hydrolytic enzymes whose chemical constitution and stoichiometric chemical reactions have become reasonably well known in the past decade. It is the thesis of this paper that the accurate, simple, convenient procedures for the direct titration of the concentration of active sites of the hydrolytic enzymes chymotrypsin, trypsin, elastase, subtilisin, papain, and acetylcholinesterase, now available, offer a better approach to the determination of enzyme concentration than the rate assays of the past. Thus the definition of an enzyme used here is an intact active site which can undergo a specific stoichiometric reaction.

Methods will be presented for the determination of the concentration of active sites of certain hydrolytic enzymes. Because of the possible ambiguity with respect to the number of active sites per enzyme molecule, it is convenient to define these determinations in terms of normality of active sites. This is the object of this paper.

There are many other approaches to defining enzyme solutions. One may speak of enzyme units, enzyme activity, enzyme purity, or enzyme concentration.[3] One unit of enzyme is defined as that amount of enzyme which will catalyze the transformation of 1 μmole of substrate per minute under defined conditions.[3] The specific activity is expressed as units of enzyme per milligram of protein. The concentration of an enzyme solution is expressed as units of enzyme per milliliter. The purity of an enzyme solution is expressed as the specific activity of the preparation divided by the specific activity of the pure enzyme. These definitions were developed on the basis of the premise stated earlier that enzymes may be observed to catalyze reactions, but are not sufficiently characterized chemically for direct analysis. Of the above terms, we will not be concerned with units of enzyme nor with specific activity of an enzyme. We will, however, be concerned with the concentration of an enzyme solution which we will attempt to express in equivalents of enzyme per liter (normality). Knowing the equivalent weight of the enzymes from independent investigations and the

(1) This research was supported by grants from the National Institutes of Health.

(2) (a) Subtilisin; (b) National Institutes of Health Postdoctoral Research Fellow; (c) elastase; (d) papain; (e) trypsin; (f) chymotrypsin; (g) National Institutes of Health Predoctoral Research Fellow; (h) acetylcholinesterase.

(3) Report of the Commission on Enzymes of the International Union of Biochemistry, Pergamon Press, London, 1961, p. 7. Enzyme Nomenclature: Recommendations (1964) of the International Union of Biochemistry, Elsevier Publishing Co., New York, N. Y., 1965, Acta 150, 719 (1965).

weight of enzyme making up a given solution, purity of an enzyme solution can then be expressed in terms of the actual normality divided by the normality calculated on a weight basis.

Rate assays for the determination of the concentration of enzyme solutions in terms of units of enzyme per milliliter have been described for many of the enzymes considered here utilizing natural or synthetic substrates.[4,5] In certain instances these assays have been converted from units of enzyme per milliliter to equivalents of enzyme per milliliter, a true concentration, by making the assumption that a reference standard of 100% pure (active) enzyme was available. There is one fundamental problem and a number of subsidiary problems associated with such a procedure.

The fundamental problem is associated with our present inability to define a 100% pure (active) enzyme. Certainly enzymes have been crystallized, shown to be homogeneous chromatographically, electrophoretically, and by ultracentrifugation. But it is difficult to accept the use of a pure enzyme as an absolute analytical standard when enzymes can adsorb impurities, when crystalline enzymes, even pure ones, contain very appreciable amounts of water which cannot be removed without denaturation of the enzyme, and when crystalline α-chymotrypsin, one of the purest and best characterized enzymes extant, is often found to contain 20-30% of impurities (including water). Analytical chemistry demands that absolute reference standards be extremely well characterized. At the present time no enzyme meets the criteria that analytical chemistry demands for such a characterization.

In addition to this fundamental problem of the absolute reference standard, rate assays are plagued with many uncertainties which limit the accuracy of the determination of an enzyme concentration. These include uncertainties due to the large number of variables in the rate assay that must be controlled such as temperature, ionic strength, cofactors, inhibitors, activators, pH, etc. The recommended procedure[3] for carrying out a rate assay is to use initial rates "in order to avoid complications due, for instance, to reversibility of reactions or to formation of inhibitory products." Furthermore, conditions are recommended in which the enzyme is saturated with substrate so that the rate approaches zero-order kinetics and a maximal velocity may be observed, or alternatively that a maximal velocity be determined by a Michaelis-Menten treatment.[3] Many of the rate assays for the enzymes under discussion violate one or more of the recommendations given above. These recommendations, even if followed, beg the question of the use of initial rates, a risky, last-resort procedure because initial rates are, many times, hard to determine and may hide a multitude of kinetic sins; beg the question of impurities in the enzyme test solution which may completely invalidate the kinetics when compared to a pure enzyme solution; and beg the question of the problems of rate assay which in some instances will not allow agreement from two laboratories to better than a factor of two. Thus, both theoretical and practical problems plague the use of rate assays for the determination of enzyme concentration.

(4) M. Laskowski, *Methods Enzymol.*, 2, 8 (1955).
(5) H.-U. Bergmeyer, Ed., "Methods of Enzymatic Analysis," Academic Press Inc., New York, N. Y., 1963.

A number of the above problems may be obviated by the use of the "optical factor." In essence, this procedure involves a spectrophotometric determination of the concentration of protein in a given solution. Again the 100% pure (active) enzyme is used as absolute reference standard and again the problems inherent in such a procedure are not met. But in addition, the assumption must be made that the enzyme test solution under consideration is absolutely pure, using the same criteria as that for the 100% pure enzyme, and furthermore, that this solution is free from ultraviolet-absorbing contaminants, particularly of the peptide and protein variety. Since this assumption is ordinarily the point of issue in a determination of enzyme concentration, the use of the "optical factor" is a most unsatisfactory method for the determination of absolute enzyme concentration. The use of an "optical factor" involves not the determination of an *enzyme* concentration but rather the determination of a *protein* concentration. Since the two concentrations are not necessarily identical because of the usual presence of denatured enzyme, this procedure is unsatisfactory. Thus the use of an "optical factor" begs the question of the reactivity of the enzyme since the activity of the enzyme may easily be lost without any change in gross chemical structure. Thus analytical methods must be developed for the determination of the active sites of enzymes.

One of the knotty problems in dealing with enzymes is the possible presence of an enzymatically active impurity which can react with a substrate or titrant. Such extraneous reaction would be expected to occur at a rate different from that of the principal reaction, and thus should show up in the kinetics. However, it is advisable with any enzyme preparation to determine kinetics with at least two different degrees of purification against several substrates in order to determine if the observed activity is independent of purification. A further check may be made by comparing the rate of reaction of an inhibitor with the enzyme preparation to the rate of inhibition of its activity as determined with several different substrates.

The Stoichiometric Basis of the Titrations of Hydrolytic Enzymes. The stoichiometric reactions of hydrolytic enzymes with organophosphates provide the ultimate basis for the determination of the concentration of such enzyme solutions.[6] Following the discovery of these stoichiometric reactions, many other stoichiometric reactions have been observed with hydrolytic enzymes, some of which are readily adaptable for the present purposes. In general, what is required for the determination of enzyme concentration is a stoichiometric reaction which is readily and accurately observable.

But since an enzyme is a complex organic molecule, care must be taken to ensure that the stoichiometry observed and used is a meaningful stoichiometry. The primary requisite of the stoichiometry is that it must involve the active site of the enzyme. This requirement follows from the objective of these determinations, namely to titrate active sites as a true measure of active enzyme molecules. The stoichiometry may involve either a substrate,[7] an inhibitor,[8] or a coenzyme.[9]

(6) A. K. Balls and E. F. Jansen, *Advan. Enzymol.*, 13, 321 (1952).
(7) B. Chance in "Technique of Organic Chemistry," Vol. 8, S. L. Friess and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1953, p 627, and references therein.

However, the latter two reagents do not test the primary chemical reaction of the enzyme and thus are less desirable than the former. Thus, the optimal stoichiometric reaction is a reaction at the active site of something closely resembling a normal substrate of the enzyme.

The stoichiometric basis of the present determinations of hydrolytic enzyme concentrations may be represented by eq 1 which represents the over-all pathway for

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_1} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$+ P_1$$

α-chymotrypsin,[10] trypsin,[11] elastase,[12] subtilisin,[13] papain,[14] and acetylcholinesterase[15] reactions. The conversion of enzyme, E, into acyl-enzyme, ES′, and product one, P_1, can be considered a stoichiometric reaction with respect to an individual active site. If this process can be observed before the turnover (regeneration) of the enzyme occurs, then a direct measure of the enzyme concentration may be made.

A limiting example of the conversion of an enzyme into acyl-enzyme and P_1 without the regeneration of any enzyme is seen in the reaction of organophosphate and organosulfonate compounds with many hydrolytic enzymes, mentioned above. Such stoichiometric reactions for α-chymotrypsin, trypsin, elastase, subtilisin, acetylcholinesterase, and other cholinesterases were followed by the complete inhibition of these enzymes. Observations of the stoichiometry of the process were made by the amount of reactant (organophosphate) used up, by the liberation of P_1, in terms of hydrogen ion or *p*-nitrophenol, or by the attachment of phosphorus, isopropyl groups (from diisopropylphosphorofluoridate) or radioactivity (from a radioactively labeled organophosphate) to the protein.[4] Any of these observations of stoichiometry could be used as the basis of a procedure for the routine determination of enzyme concentration. Several disadvantages of using this approach may be noted. (1) The organophosphates are, in general, nonspecific compounds; for example, a stoichiometric reaction of bromelain and diisopropylphosphorofluoridate has been found but it does not involve the active site of this enzyme;[16] (2) except for the observation of *p*-nitrophenol (P_1), the experimental methods are both tedious and of limited accuracy.

The use of a specific substrate [illegible] not [illegible] the requirement that the stoichiometric [illegible] place at the active site, but [illegible] of being able to titrate the active [illegible] the presence of a number of other [illegible] titrant for any given enzyme should optimally be modeled as closely as possible on the substrate of the enzyme to ensure reaction only at the active site and to ensure maximum selectivity between different enzymes.

The stoichiometry to be used for the titrations presented here will depend not on a pure enzyme as absolute standard, but rather on (a change in a property of) the substrate as absolute standard. The stoichiometric transformation will involve the reaction $E{\cdot}S \rightarrow ES' + P_1$. The ultimate test of the stoichiometry, as mentioned above, is that ES′ is inactive whereas E is active enzymatically. With this test, the stoichiometry which is pertinent to an enzymatic process is demonstrated. In other words, this stoichiometry is an operational measure of the active site of the enzyme.

Probably the first direct observation of a stoichiometric production of P_1 using a substrate was in the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate.[17] Observation of the liberation of *p*-nitrophenol from this substrate led to the conclusion that the reaction proceeds in at least two distinct kinetic steps, specifically a fast initial liberation of approximately 1 mole of *p*-nitrophenol per mole of enzyme, followed by a slow turnover reaction. This observation, itself, indicated the possibility of determining the concentration of kinetically active sites on the enzyme in an easy and accurate manner. However, the [illegible] working conditions of a reliable experimental [illegible] method were not established.

The kinetic equations rigorously describing the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin proved to be complex,[18,19] and furthermore the amount of phenol liberated in the initial step was shown to be a complex function of the equilibrium and rate constants involved in the reaction.[20,21] Experimental confirmation of these equations was not demonstrated. Subsequently methods were developed to titrate α-chymotrypsin with the substrate *N-trans*-cinnamoylimidazole[22] and the inhibitor 4-nitrophenyl N,N-diphenylcarbamate.[23] These methods did not, however, test the validity of the equations referred to above describing the stoichiometry during the enzymatic hydrolysis of a specific substrate.

It is the object of the present paper to [illegible] number of titrations of hydrolytic enzymes which conform to the stoichiometry contained in eq 1 and which provide practical methods for the absolute determination of the normality of an enzyme solution. These determinations involve titrations of the concentrations of active sites of α-chymotrypsin, trypsin, elastase, subtilisin, papain, and acetylcholinesterase. A num-

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(11) M. L. Bender and E. T. Kaiser, *ibid.*, **84**, 2556 (1962), and references therein; M. L. Bender, F. J. Kézdy, and J. Feder, *ibid.*, **87**, 4953, 4955 (1965); M. L. Bender and F. J. Kézdy, *ibid.*, **87**, 4954 (1965); M. L. Bender, J. V. Killheffer, Jr., and F. J. Kézdy, *ibid.*, **86**, 5331 (1964); C. R. Gunter, Ph.D. Thesis, Northwestern University, 1966.

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(18) H. Gutfreund and J. M. Sturtevant, *J. Am. Chem. Soc.*, **78**, [illegible] (1956).

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these titrations involve substrates specific for the ular enzyme, and thus the titrations are accurate, perimentally facile, and specific.

Titration Theory. The kinetic equations describing q 1 have been solved.[17,18,24,25] These equations offer eral methods for the determination of the con- ntration of the (kinetically) active enzyme. Let us nsider some of these possibilities. The first of these es either a relative or absolute enzyme concentration, t the others give absolute enzyme concentration.

(1) The initial turnover rate of formation of nitrophenol in the enzymatic hydrolysis of a *p*-nitro- enyl ester following eq 1 may be expressed by eq 2[18]

$$V_0 = \frac{\frac{k_2k_3}{k_2 + k_3}[E]_0[S]_0}{[S]_0 + K_sk_3/(k_2 + k_3)} = \frac{k_{cat}[E]_0[S]_0}{[S]_0 + K_m(\text{app})} \quad (2)$$

here K_m(app) is the apparent Michaelis constant and $_{cat}$ is the catalytic rate constant, both operational rameters; K_s, k_2, and k_3 are defined by eq 1 and may operational in certain instances.

When $[S]_0 \gg K_m$(app), the rate reduces to

$$V_0 = k_2k_3[E]_0/(k_2 + k_3) \quad (3)$$

rthermore, when $k_2 \gg k_3$

$$V_0 = k_3[E]_0 \quad (4)$$

all cases V_0 is proportional to $[E]_0$ at a given $[S]_0$ and en experimental conditions. If the proportionality stant between V_0 and $[E]_0$ is known, the latter may easily calculated from the former; if the proportion- y constant is not known, a relative rate may still be lculated. Many rate assays of hydrolytic enzymes based on this proportionality. However, as dis- sed above, such rate assays have severe limitations.

One approach for determining the proportionality stant between V_0 and $[E]_0$ is to determine one V_0 and $[E]_0$. V_0 may be measured straightforwardly but 100% pure (active) enzyme must be obtained in der to know $[E]_0$. Thus, this method depends timately on using a pure enzyme as absolute reference ndard, with the limitations inherent in this proce- re, as outlined above.

Another approach for determining the proportional- y constant between V_0 and $[E]_0$ may be utilized when q 4 is operative. The proportionality constant of q 4, k_3, may be determined independently if the acyl- zyme, ES′, of either a good or poor substrate is pared. ES′ may be prepared by using an excess of strate to effect acylation, followed by dilution of the ation so that the substrate concentration drops ow K_m(app) and buildup of the acyl-enzyme stops. ternatively, acylation may be stopped by shifting the H and/or removing the excess substrate by means of a phadex filtration or other methods. The decay of S′ to E may be followed in many ways to determine first-order constant, k_3, the most rigorous being the neration of E as measured with a "true" substrate the enzyme; this reaction, is, of course, independent the original enzyme concentration. The constant, together with the maximal velocity, $V_0 = k_3[E]_0$, n gives the absolute enzyme concentration directly.

Essentially this procedure was used by Wilson and co-workers[26,27] for acetylcholinesterase, with the exception that the transformation from k_3 to $[E]_0$ was more complicated.

(2) The relationship between the slopes of the Lineweaver–Burk plots of the steady-state and presteady-state portions of reaction 1 leads to the determination of the absolute concentration of enzyme. Equation 2, describing the initial steady-state (turnover) reaction, may be transformed into

$$\frac{1}{V_0} = \frac{k_2 + k_3}{k_2k_3}\frac{1}{[E]_0} + \frac{K_s}{k_3[E]_0}\frac{1}{[S]_0} \quad (5)$$

which in terms of Michaelis parameters is identical with

$$\frac{1}{V_0} = \frac{1}{k_{cat}}\frac{1}{[E]_0} + \frac{K_m(\text{app})}{k_{cat}[E]_0}\frac{1}{[S]_0} \quad (6)$$

The comparable equation of the presteady state is

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_s}{k_2}\frac{1}{[S]_0} \quad (7)$$

if $(k_2 + k_3) > k_3K_m$(app)[18] (b is the first-order rate constant of the presteady state; see eq 10). Thus the slope of eq 5 (plotted as $1/V_0$ *vs.* $1/[S]_0$) is $K_s/k_3[E]_0$ while the slope of eq 7 is K_s/k_2; by dividing the former slope by the latter, we may obtain $[E]_0$, the absolute concentration of enzyme.[28]

(3) Under certain very stringent conditions, the kinetics of acylation ($d[P_1]/dt$) may be observed under second-order conditions.[24,25,29] Under these conditions, which require that $k_2 \gg k_3$ and that K_m(app) $> [S]_0 \cong [E]_0 < K_s$, the rate of appearance of *p*-nitrophenol may be represented by

$$V = d[P_1]/dt = (k_2/K_s)([E]_0 - [P_1])([S]_0 - [P_1]) \quad (8)$$

Equation 8 may be transformed into

$$\frac{V}{[S]_0 - [P_1]} = \frac{k_2}{K_s}[E]_0 - \frac{k_2}{K_s}[P_1] \quad (9)$$

A plot of $V/([S]_0 - [P_1])$ *vs.* $[P_1]$ gives a straight line whose intercept divided by the slope is $[E]_0$, the absolute concentration of enzyme.

(4) The most common set of conditions for observation of enzymatic process, and the most important set for the determination of enzyme concentration, utilizes $[S]_0 \gg [E]_0$. Under these conditions a presteady state (acylation) and a steady state (deacylation) may be observed, the former often being too fast to measure, and the concentration of P_1 liberated in the presteady state (π) may be related to the absolute enzyme concentration. For reaction 1, when $[S]_0 \gg [E]_0$, $[P_1]$ (conveniently *p*-nitrophenol) produced in time t may be described by[18]

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(27) I. B. Wilson and M. A. Harrison, *ibid.*, 236, [illegible] (1961).

(28) J. M. Reiner, "Behavior of Enzyme Systems," Burgess Publishing Co., Minneapolis, Minn., 1959, p 58; [illegible] determination of enzyme concentration [illegible] tion of enzyme concentration which [illegible] 5 and 7. This method is limited, however, [illegible] initial rates and only when $[E]_0 \ll [S]_0 \ll K_s$.

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(25) F. J. Kézdy and M. L. Bender, *Biochemistry*, 1, 1097 (1962).

$$[P_1] = \frac{k_{cat}[E]_0[S]_0 t}{[S]_0 + K_m(\text{app})} + [E]_0 \left[\frac{\left(\frac{k_2}{k_2 + k_3}\right)}{\left(1 + \frac{K_m(\text{app})}{[S]_0}\right)} \right]^2 \times \left(1 - \exp \frac{-(k_2 + k_3)[S]_0 + k_3 K_s}{K_s + [S]_0} t\right) \quad (10)$$

At high values of t, the exponential term approaches zero and the production of P_1 can be described as a linear function of t

$$[P_1] = \pi + At \quad (11)$$

where $A = (k_{cat}[E]_0[S]_0/([S]_0 + K_m(\text{app}))$ and π may be expressed by

$$\pi = [E]_0 \frac{\left(\frac{k_2}{k_2 + k_3}\right)^2}{\left(1 + \frac{k_m(\text{app})}{[S]_0}\right)^2} \quad (12)$$

π may be easily determined experimentally since it is the intercept of a plot of $[P_1]$ *vs.* t at $t = 0$. Equation 12 may be transformed to eq 13, which indicates that

$$\frac{1}{\sqrt{\pi}} = \frac{k_2 + k_3}{k_2} \frac{1}{\sqrt{[E]_0}} + \frac{(k_2 + k_3)K_m(\text{app})}{k_2\sqrt{[E]_0}} \frac{1}{[S]_0} \quad (13)$$

$\pi = [E]_0$ only if $k_2 \gg k_3$ and $[S]_0 \gg K_m(\text{app})$. If the latter condition does not hold, a series of experiments at constant $[E]_0$ but varying $[S]_0$ enables one to circumvent this condition. From eq 13, a plot of $1/\sqrt{\pi}$ *vs.* $1/[S]_0$ gives a straight line whose intercept is $((k_2 + k_3)/k_3(1/[E]_0))$. If the condition is met that $k_2 \gg k_3$, the intercept of such a plot will give $1/[E]_0$ and hence $[E]_0$ directly.

The reactions of diethyl *p*-nitrophenyl phosphate and related compounds with various hydrolytic enzymes is usually carried out under conditions in which $[S]_0 > [E]_0$. However, these reactions must be treated in a special manner since no evidence for an adsorptive enzyme–substrate complex has as yet been found. Hartley and Kilby[30a] who first investigated the reaction of this reagent with chymotrypsin considered that the reaction followed eq 14 where S is the phosphate ester, ES′, is the diethyl phosphoryl-enzyme, P_1 is nitrophenol, and k_{11} is the second-order rate constant of the reaction of enzyme and phosphate ester, rather than eq 1.

$$E + S \xrightarrow{k_{11}} ES' + P_1 \quad (14)$$

However, it is known that diethyl phosphoryl-trypsin and diethyl phosphoryl-chymotrypsin spontaneously dephosphorylate.[30b,c] Therefore, the reaction should be written

$$E + S \xrightarrow{k_{11}} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (15)$$

where the symbolism is the same as before with the addition of P_2, diethyl phosphate. If one assumes that the concentration of phosphate titrant, S, is much greater than that of the enzyme and that the reaction between enzyme and phosphate occurs only at the active site of the enzyme, the relationship between π, the amount of *p*-nitrophenol liberated in the pseudo-first-order reaction, and the true enzyme concentration may be derived. The derivation utilizes two differential equations describing eq 15, $d[P_1]/dt = k_{11}[S][E]$, and $d[ES']/dt = k_{11}[S][E] - k_3[ES']$, and the conservation equation $[E]_0 = [E] + [ES']$, and leads to eq 16 and 17

$$\sqrt{\pi/[E]_0} = \frac{1}{1 + k_3/k_{11}[S]_0} \quad (16)$$

or

$$\sqrt{\pi/[E]_0} = \frac{1}{1 + k_3/(k_{obsd} - k_3)} \quad (17)$$

where k_{obsd} (*b*) is the pseudo-first-order rate constant when the substrate is in great excess over the enzyme, corresponding to k_{11}. From eq 17, it may be seen that $\pi = [E]_0$ when k_{obsd}, the pseudo-first-order rate constant of the inhibition reaction, is much greater than the dephosphorylation rate constant, k_3. Thus, there are three requirements for a successful titration corresponding to eq 15: (1) $[S]_0 \gg [E]_0$; (2) $k_{obsd} \gg k_3$; and (3) the titration reaction must occur at the active site and only at the active site of the enzyme.

Experimental Section

Materials. *p*-Nitrophenyl acetate was recrystallized from chloroform–hexane, mp 79.5–80.0°. 2,4-Dinitrophenyl acetate was recrystallized from the same solvent, mp 71–71.5°. *p*-Nitrophenyl isobutyrate was prepared from *p*-nitrophenol and isobutyric anhydride in pyridine, and was recrystallized four times from pentane, mp 39.5–40.2° (lit.[31] mp 39–40°). *p*-Nitrophenyl trimethylacetate was prepared from *p*-nitrophenol and trimethylacetyl chloride in pyridine, and was recrystallized twice from ethanol, mp 93.5–94.5° (lit.[32a] mp 94–95°). Upon hydrolysis in 0.05 *M* potassium hydroxide, $\Delta\epsilon$ was 18,300 ± 840 (lit.[32c] of *p*-nitrophenoxide ion, 18,300).

o-Nitrophenyl dimethylcarbamate was synthesized from dimethylcarbamyl chloride and *o*-nitrophenol in pyridine. The crude yellow carbamate was crystallized twice from ether–heptane solution, mp 56.7–57.0°. *Anal.* Calcd for $C_9H_{10}N_2$: C, 51.43; H, 4.80; N, 13.33. Found: C, 51.63; H, 4.82; N, 13.17.[33a] *p*-Nitrophenyl dimethylcarbamate was synthesized from dimethylcarbamyl chloride and *p*-nitrophenol in pyridine. The carbamate was crystallized three times from hot heptane, mp 107.5–108.0°. *Anal.* Calcd for $C_9H_{10}N_2$: C, 51.43; H, 4.80; N, 13.33. Found: C, 51.55; H, 4.87; N, 13.03. Phenyl acetate was an Eastman Kodak Co. product. The material was either distilled directly at atmospheric pressure followed by distillation at reduced pressure or alternatively was first treated with 5% ice cold potassium carbonate solution followed by distillation at reduced pressure. The latter procedure gave a product which contained less than 0.1% [illegible]

Diethyl *p*-nitrophenyl phosphate (Paraoxon) was [illegible] the American Cyanamid Corp.[33] and used without further purification. Its ultraviolet and infrared spectra were [illegible] in the literature[34] although it had a faint [illegible] spectrum agreed with the designated structure and at 99 ± 1% of the theoretical amount of *p*-nitrophenol in 0.05 *M* KOH. The compound appeared to contain less than [illegible] *p*-nitrophenol impurity.

N-*trans*-Cinnamoylimidazole[35] was recrystallized from (C_6H_{12}-distilled) *n*-hexane, mp 111–113°. 2,4-Xylyl 1,2,3-[illegible] *p*-nitrophenyl ester[10] has been described previously.

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p-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, mp 155.0-156.4°, was a gift of Dr. C. J. Martin. α-N-Benzoyl-L-arginine ethyl ester hydrochloride was a Mann Research Labs product (lot 6024), mp 130-133°. Soybean trypsin inhibitor was a Mann Research Labs product (lot M1573), five times crystallized. *p*-Nitrophenyl N-benzyloxycarbonyl-L-valinate was purchased from the Cyclo Chemical Co. and was recrystallized from ether-petroleum ether, mp 63.5-64° (lit.[36] 63°). Sephadex G-25 and G-50 were obtained from Pharmacia Fine Chemicals, Inc. *p*-Nitrophenyl N-benzyloxycarbonyl-L-isoleucinate was a Cyclo Chemical Co. product, mp 57-58° (lit.[37] 60-62°). *p*-Nitrophenyl N-benzyloxycarbonyl-L-leucinate was a Cyclo Chemical Co. product, mp 92-93.5° (lit.[37] 95°). *p*-Nitrophenyl N-benzyloxycarbonylglycinate was a Cyclo Chemical Co. product, mp 128.5-130° (lit.[38] 130°). *p*-Nitrophenyl furoate was prepared from the acid chloride as described above, mp 164-165°. *Anal.* Calcd for $C_{11}H_7NO_6$: C, 56.65; H, 3.03; N, 6.01. Found: C, 56.77; H, 2.79; N, 6.07.

p-Nitrophenyl N-acetyl-L-leucinate was synthesized from N-acetyl-L-leucine and *p*-nitrophenol using ethyl chloroformate according to the method of Martin, Golubow, and Axelrod.[39,40] The product was recrystallized from hexane-methylene chloride, mp 116-117°. *Anal.* Calcd for $C_{14}H_{18}N_2O_6$: C, 57.17; H, 6.15; N, 9.26. Found: C, 57.14; H, 6.17; N, 9.52. Spectrophotometric analysis of the *p*-nitrophenol liberated on treatment with base indicated 100 ± 2% of the expected amount. Spectrophotometric analysis of the *p*-nitrophenol liberated on treatment with α-chymotrypsin indicated that the material contained about 25% of the D compound.

p-Nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride was prepared by selective removal of the *t*-butyloxycarbonyl group from *p*-nitrophenyl N^2-benzyloxycarbonyl-N^6-*t*-butyloxycarbonyl-L-lysinate.[41] The latter compound was prepared by the carbodiimide procedure.[37] The *t*-butyloxycarbonyl group was removed by treatment with excess anhydrous hydrogen chloride in ethyl acetate at room temperature for 20 min. After one recrystallization from acetonitrile, the hydrochloride melted at 150-152°, $[\alpha]^{20}D$ 22° (*c* 2.2, dimethylformamide). *Anal.* Calcd for $C_{20}H_{24}O_6N_3Cl$: C, 54.86; H, 5.52; N, 9.60. Found: C, 55.15; H, 5.74; N, 9.64. Hydrolysis of the titrant in 0.1 *N* sodium hydroxide produced 95-97% of the calculated amount of *p*-nitrophenol. Enzymatic hydrolysis at pH 6.83 gave 100 ± 0.5% of the amount of *p*-nitrophenol produced by nonenzymatic (alkaline) hydrolysis at the same pH, indicating the optical purity of the titrant. *p*-Nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride was also purchased from the Cyclo Chemical Co. (lot M-2135), recrystallized from acetonitrile containing 3% ethanol, and dried over P_2O_5, mp 150-151°.

All buffers and salts were of reagent grade. The water was doubly distilled water, using either a Corning all-Pyrex glass still or an alkaline permanganate still utilizing Pyrex. Buffer solutions were filtered before use if the 0.0-0.2 slide wire of the Cary spectrophotometer was to be used. Eastman Kodak Co. spectral grade acetonitrile was usually distilled repeatedly from phosphorus pentoxide. pH was measured with a Radiometer 4C pH meter, standardized against pH 4.01 phthalate buffer, and equipped with either a G 200B or a G 220B glass electrode. pH was determined at the conclusion of all experiments except as noted.

Enzymes. α-Chymotrypsin was a Worthington three times crystallized, salt-free product. In a few specified instances Worthington chymotrypsin purified by gel filtration through Sephadex G-50 was used. Enzyme solutions were made up in the appropriate buffer, and centrifuged for 30 min at 15,000 rpm.

Bovine trypsin was a Worthington twice crystallized, lyophilized product, lots TRL 6253 and TRL 6256. Porcine trypsin was a gift from the Armour Pharmaceutical Co., lot K172185A.[42] In a few instances, Worthington trypsin purified by gel filtration through Sephadex G-25 was used.

There are strong indications that the dissolution of lyophilized bovine trypsin in aqueous solution does not instantaneously yield a stable, well-defined trypsin solution. Previously it was observed that upon dissolution of this substance, the enzymatic activity of the solution, as measured at pH 7.8 toward α-N-benzoyl-L-arginine ethyl ester, shows an initial sharp decrease of the order of 25%, followed at higher pH's, *e.g.*, 7, by an irreversible denaturation at a much slower rate in a manner similar to the denaturation of α-chymotrypsin.[43] The rate of this initial, partial loss of activity appears to be pH dependent, and is accompanied by an observable decrease in the sedimentation rate of the protein.[43] The decrease in activity and the change in the apparent molecular weight thus seem to be different facets of the same fundamental change in the state of the protein.

For kinetic and titration studies, a stable and enzymatically well-defined trypsin solution is necessary. In search of the conditions necessary for stability, we have carried out experiments which have confirmed and extended the above observations.

Our observations yield the following points. (1) The initial, partial loss of activity, as measured by using α-N-benzoyl-L-arginine ethyl ester as substrate at pH 8.0, 0.05 *M* Tris buffer, 25.0°, is first order in trypsin; the rate constant is independent of the protein concentration from 0.5 to 2.0 mg/ml. (2) The initial loss of activity is first order in hydrogen ion concentration since the reaction has a half-life of approximately 120 min at pH 4 and approximately 10 min at pH 3. (3) The extent of the initial loss of activity (about 25% of the total activity) is independent of the pH of incubation of the enzyme between pH 2 and 5. (4) The amount and rate of initial loss of activity are independent of the substrate used to assay the system (α-N-benzoyl-L-arginine ethyl ester or *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate), or the presence or absence of calcium ion. (5) The loss of activity cannot be reversed by changing the pH of the solution. (6) At the end of the initial loss of activity the enzyme solution is stable for several hours at room temperature at pH 3 ($[E]_0$ = 0.5-2.0 mg/ml), whereas at higher pH's the enzyme slowly loses its activity, the rate of inactivation being dependent on the pH and on the total enzyme concentration. (7) When the rate assays are determined at pH's lower or equal to 6, no initial loss of activity is observed, hence the initial loss of activity mentioned above must involve a change in the pK_1 of the catalytic base of the enzyme. (8) Finally, none of these phenomena are observable with porcine trypsin.

Subtilisin (Bacterial Proteinase Novo) was purchased from the Novo Pharmaceutical Co., Copenhagen, Denmark. Enzyme solutions were prepared in pH 5.0, 0.05 *M* acetate buffer. A refrigerated enzyme solution containing approximately 80 mg/ml of subtilisin in this buffer loses about 15% of its activity in a week as measured by the N-*trans*-cinnamoylimidazole titration.

Porcine elastase, electrophoretically purified, lots LSLE 8691-923, 6507B, and 6501, was obtained from the Worthington Biochemical Corp. Crystalline elastase was also a Worthington product. Solutions of the enzyme were prepared in pH 4.8 or 4.7 acetate buffer, I = 0.05. The turbid solution was centrifuged at 9,700 rpm for 20 min in the cold. Using electrophoretically purified enzyme, the clear supernatant contained 96% of the original weight of protein as estimated by the optical factor (milligrams of protein per milliliter = OD_{280}/0.54).[44]

A solution of elastase (lot LSLE 6501) corresponding to 90 mg of elastase in ammonium acetate buffer (I = 0.05) was centrifuged at 14,000 rpm to give a clear supernatant and was chromatographed. The chromatography was carried out in a cold room at 4°. A carboxymethylcellulose column previously washed with distilled water, decanted 20 times, washed thoroughly in pH 4.8 ammonium acetate buffer (I = 0.05), and stored in the same buffer. The column[45] had a capacity of 200 mg of protein. A Gilford ultraviolet absorption meter and a Texas Instrument strip chart recorder were used to determine the protein content of the effluent at 280 nm. An approximately linear sodium chloride gradient was used. The chromatography procedure followed in general that of Naughton and Sanger.[46]

A similar chromatography was carried out with Worthington crystalline elastase which had been previously purified by a modification of the procedure of Smillie and Hartley,[47] which consisted of treatment of a solution of 400 mg of crystalline elastase in Tris

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buffer (adjusted to pH 9.0) with 50 ml of a settled suspension of DEAE Sephadex A-50 in Tris buffer. The resulting suspension was stirred at 4° for 4 hr and filtered. The filtrate was lyophilized, and the resulting solid was dissolved in 25 ml of 0.1 *M* ammonia and dialyzed against two 1-l. portions of 0.1 *M* ammonia and then against one 1-l. portion of 0.05 *M* ammonia. The contents of the dialysis sacs were lyophilized, giving 117 mg of a fluffy white solid, soluble in ammonium acetate buffer.

One sample of elastase used in this research (JRW-2) was a preparation of crystalline elastase chromatographed on carboxymethylcellulose in pH 8.0, 0.02 *M* Tris HCl buffer. A sodium chloride gradient was used.[48]

The acetylcholinesterase was a commerical preparation of the Sigma Chemical Co. The enzyme had been isolated from the electric eel and purified by the method of Lawler.[49] This enzyme preparation, designated type 3 by the supplier, was reported to have 16.7 mg of protein/ml and 5 mg of ammonium sulfate/mg of protein. The preparation was offered in lots of 0.4 ml in the form of a frozen soolution and was stored in this form before use. The solution had a light yellow color which did not disappear on dialysis. The enzyme solutions were always dialyzed to remove either the ammonium sulfate present in the original preparation or the products of reaction if the enzyme had been previously used. The dialysis was ordinarily carried out with 0.2 *M* sodium chloride and 10^{-4} *M* phosphate buffer, pH 6.8–7.0. On several occasions it was found desirable to concentrate the enzyme solution. This was most readily accomplished by dialysis against 29% ammonium sulfate solution which precipitated the enzyme. The precipitated enzyme was centrifuged and then redissolved in the buffer mentioned above. An attempt at concentrating an enzyme solution using Sephadex G-25 was unsuccessful. The presence of small particles (probably denatured protein) in the enzyme solution which scattered light created a considerable spectrophotometric problem. An attempt was made without complete success to remove these particles by centrifugation. However, the use of a Millipore filtration apparatus was successful. This apparatus consisted of a Swinny hypodermic adapter (Millipore) no. XX 30-012-00 equipped with a 22-μ filter. After this filtration, stirring the solution resulted only in a 0.0003 absorbance increase which returned to its original value.

Papain was purchased as a suspension of twice-crystallized material (lot No. 5588) from the Worthington Biochemical Corp. and converted to mercuripapain by a modification[50] of the method of Kimmel and Smith.[51] The mercuripapain (1–2 × 10^{-3} *M* in 0.05 *M* acetate buffer, pH 5.2) was shaken with a toluene solution of 4-methylbenzenethiol;[52] the aqueous layer was filtered through filter paper and chromatographed on a 12 × 1.6 cm column of Sephadex G-25. The active enzyme which was eluted between 8 and 14 ml eluate volume, was stored in a stoppered volumetric flask at 2°. When the solution was in use, the flask was kept in ice. After being used, the flask was flushed with nitrogen before it was returned to 2°. The normality of the stock solution varied from 1.5 to 4.0 × 10^{-4} *M*.

Kinetic and Titration Measurements. Kinetic and titration experiments were conducted using a Cary Model 14PM or CM recording spectrophotometer equipped with a thermostated cell compartment at 25.0 ± 0.2° and 0.0–2.0 and 0.0–0.2 slide wires. Ordinarily a 1-cm quartz cuvette containing 3.0 ml of buffer solution was thermostated for an appropriate period of time. The reference cell contained the corresponding buffer. Then an aliquot of substrate solution in purified acetonitrile (or other organic solvent) was added, and the spontaneous hydrolysis of the substrate was observed spectrophotometrically for 100–200 sec. The enzymatic reaction was then initiated by introduction of an aliquot (20–100 μl) of enzyme solution.

For some "burst" experiments, a Cary cell compartment cover with a 1-in. diameter hole directly above the cell position was used in order to provide the fastest initiation and thus the closest extrapolation to zero time for fast hydrolyses such as the reaction of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate with papain. Experiments showed that if no direct light from overhead fell on the hole, the recorded absorbance did not change when the hole was uncovered. With the spectrophotometer operating, 50 μl of enzyme solution was added on the end of a flat-tipped stirring rod through the hole in the cover. The rapid rise in absorbance caused by the interruption of the light beam by the stirring rod signaled the precise time of addition. This method of initiation permitted the observation of the reaction as soon as 2.5 sec after the addition of enzyme.

For most substrates, the steady-state phase of the reaction was determined by aligning a straight edge with the trace on the Cary chart, and the "burst" of *p*-nitrophenol was measured by extrapolating the steady-state line back to the time of enzyme addition. A correction was applied to the steady-state velocity by subtracting the velocity of the spontaneous reaction (the zero-order spontaneous reaction was assumed to remain unchanged after the usually small decrease in substrate concentration resulting from the burst).

Absorbance values for *p*-nitrophenol were calculated from the experimental values, ϵ_{400} 18,320 for phenolate ion and pK_a 7.14. The absorption of 2,4-dinitrophenol was measured in pH 8.0 buffer, by completely hydrolyzing 2,4-dinitrophenyl acetate, and was found to be 1.46 × 10^4.

The molar absorptivities of N-*trans*-cinnamoylimidazole used in this study are: ϵ_{315} 2.89 × 10^3 and ϵ_{335} 9.04 × 10^3 (pH 7.0, 0.025 *M* phosphate buffer, I = 0.1 (NaCl), 3.2% (v/v) acetonitrile–water). These values were obtained by extrapolating the absorption of slowly hydrolyzing solutions to zero time. Since the spontaneous hydrolysis of N-*trans*-cinnamoylimidazole is a minimum at pH 7.0, the extrapolation is not great. The values are believed accurate to ±2%. Since the pK_a of this tirant is 7.1, the ϵ's are not very pH dependent near pH 7. The absorption of N-*trans*-cinnamoyl-subtilisin (*vs.* subtilisin at 345 mμ is negligible, while it is very small at 335 mμ ($\Delta\epsilon$ 0.26 × 10^3) and is not pH dependent in the range pH 5–7.

For the trypsin titration using *p*-nitrophenyl N$^{\alpha}$-benzyloxycarbonyl-L-lysinate hydrochloride, $\Delta\epsilon_{340}$ 6150 in pH 3.0 citrate buffer (0.05 *M*) and $\Delta\epsilon_{320}$ 8171 in pH 3.71 formate buffer (0.01 *M*). The value of $\Delta\epsilon_{340}$ for the hydrolysis of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, at pH 3.2, the pH of the papain titrations, is 6220 ± 30, the average of ten determinations. Phenyl acetate was followed at 275.5 mμ close to the isosbestic point of phenol phenolate ion: $\Delta\epsilon_{275.5}$ 1.32 × 10^3 at pH 7.82 and 1.31 × 10^3 at pH 8.5.

The "burst" of *p*-nitrophenol in an enzyme-catalyzed hydrolysis of a *p*-nitrophenyl ester or decrease in absorbance in an enzyme-catalyzed hydrolysis of an acylimidazole substrate, when an aliquot of enzyme is added to a solution of the substrate, may be calculated from eq 18. The factor $A_0 - A_\infty$ is the measured burst since A_0 and A_∞ are the absorbancies extrapolated to time 0 from the enzyme steady-state turnover and from the spontaneous hydrolysis of the substrate, respectively. Two correction factors must be applied to the measured burst in order to determine the true burst, π: a factor accounting for the dilution of the substrate, (v_1/v_2) I_0, where v_1/v_2 is the ratio of volumes of the enzyme aliquot and the total volume of the reaction mixture; and (ϵ) a factor accounting for the absorbance of the enzyme, A_e (which is determined separately). The difference in extinction coefficients (products minus starting species) is $\Delta\epsilon$. The correction factors involving dilution of the substrate and absorbance of the enzyme tend to cancel one another. Ordinarily their compute correction to the measured burst does not exceed 10%. When the final absorbance of the burst is less than the initial absorbance, all the signs of eq 18 are reversed in the acylimidazole titrations.

$$\pi = \frac{A_0 - A_\infty + (v_1/v_2)A_e - A_e}{\Delta\epsilon} \quad (18)$$

When an aliquot of substrate is added to a solution of enzyme, the "burst" of *p*-nitrophenol is given by eq 19, where A_0 and A_2 are again the absorbancy extrapolated to time 0 from enzymatic steady-state turnover and the absorbance of the substrate, respectively.

$$\pi = (A_0 - A_2)/\Delta\epsilon \quad (19)$$

The titration of α-chymotrypsin by N-*trans*-cinnamoylimidazole has been described previously.[11] A variant of Bender's method, which has proved useful is the direct titration of 1 ml of enzyme at pH 7 *via* a method II titration (addition of a measured amount of

(48) This sample was kindly prepared by Professor J. R. Whitaker of the University of California, Davis.

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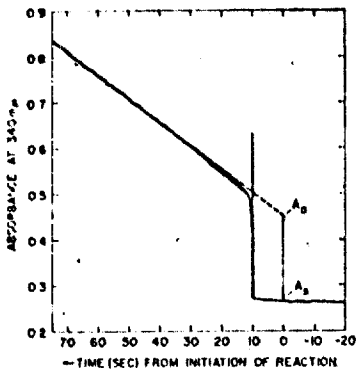


Figure 1. The reaction of *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate hydrochloride with bovine trypsin: $[E]_0 = 3 \times 10^{-5}$ *M*, $[S]_0 = 1.05 \times 10^{-3}$ *M*, pH 3.0, citrate buffer (0.05 *M*), 1.3% (v/v) acetonitrile-water, 25°.

titrant solution to the enzyme solution in the cuvette). This procedure has two advantages: (1) smaller concentrations of enzyme may be titrated; and (2) the molar absorptivity of N-*trans*-cinnamoylimidazole is not sensitive to pH as it is in the region around pH 5.05, previously suggested for the titration.[22]

The titration of the active sites of a trypsin solution is optimally carried out using *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate as substrate, using the two methods described for α-chymotrypsin.[22] Method A. A 6×10^{-2} *M* solution (50 μl) of the lysine ester in 3% (v/v) water–acetonitrile[54] was added to 3.0 ml of pH 3.0 citrate buffer (0.05 *M*). The slow spontaneous hydrolysis was observed. Then 50 μl of trypsin solution in pH 3.0 citrate buffer (0.05 *M*) was added. A typical burst using this method is shown in Figure 1. First, the slow spontaneous hydrolysis of the ester may be observed, then upon the addition of enzyme, the initial "burst" together with the end of the presteady state, and finally the linear (turnover) portion of the reaction. Burst titrations with chymotrypsin and trypsin show similar behavior. Method B. Trypsin–buffer solution (3 ml) was added to a 1.0-cm silica cuvette. Then 50 μl of titrant solution was added. Since the ester absorbs in the region of 340 mμ, it was necessary to determine its absorbance under the same conditions.

Trypsin solutions were assayed by measuring the initial rate of hydrolysis of α-N-benzoyl-L-arginine ethyl ester spectrophotometrically at 255 mμ.[55] The reaction was carried out in pH 8.0 Tris buffer (0.1 *M*) using 50 μl. of an 8.65×10^{-2} *M* stock substrate solution. This reaction is accelerated by the presence of calcium ions. Care must therefore be taken to prepare the enzyme in a citrate buffer so that any calcium ions present are chelated by citrate.

The concentration of a porcine trypsin solution was also measured by the liberation of *p*-nitrophenol from a reaction with excess diethyl *p*-nitrophenyl phosphate. In addition, the pseudo-first-order rate constant for this reaction was determined in two ways: (1) directly from the rate of liberation of *p*-nitrophenol, and (2) by rate assay of the remaining trypsin activity during phosphorylation using *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate as a monitor substrate. An aliquot of the substrate was added to 3 ml of pH 7.8 Tris buffer (0.1 *M*). The spontaneous hydrolysis was followed at 400 mμ. Then an aliquot of porcine trypsin solution was added and the reaction recorded until the liberation of nitrophenol was again linear. The pH of the solution was determined at the end of the reaction.

The optimal procedure for the titration of subtilisin by N-*trans*-cinnamoylimidazole involved the addition of an aliquot of the enzyme to a solution of the titrant in the spectrophotometric cuvette, analogous to that described as the "method A" titration of chymotrypsin by N-*trans*-cinnamoylimidazole.[22] The alternate procedure involving the addition of an aliquot of the titrant to a solution of the enzyme, described as "method B" in the titration of chymotrypsin, is less accurate in this case and is not recommended. With care the "method A" burst is reproducible to ±1%.

(54) A mixed aqueous solvent is necessary for dissolution of the substrate. This composition minimizes the rate of hydrolysis of the substrate.

(55) G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, 16, 570 (1955).

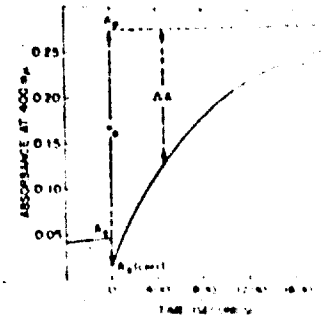


Figure 2. Titration of elastase with diethyl *p*-nitrophenyl phosphate: pH 7.74 Tris HCl buffer, 7.6% (v/v) acetonitrile-water, 25.0 ± 0.2°, $[E]_0 = 1.71 \times 10^{-5}$ *M*, $[S]_0 = 7.15 \times 10^{-5}$ *M*. The dashed line is extrapolated from the spontaneous hydrolysis of the phosphate after more than eight half lives of the reaction. Time zero is the time of addition of the enzyme. At negative times the spontaneous hydrolysis of the phosphate alone is seen. The noise level is 0.002 absorbancy unit.

In titrations of elastase, diethyl *p*-nitrophenyl phosphate and the buffer were placed in the cuvette, and the spontaneous hydrolysis of the phosphate ester was observed. An aliquot of a stock solution of elastase in buffer was added on the end of a flattened stirring rod, and the cell was covered to prevent evaporation. Recording was recommenced within 10 sec and continued for 20 half lives to facilitate extrapolation to the time of enzyme addition. A typical reaction of diethyl *p*-nitrophenyl phosphate with elastase is shown in Figure 2.

The elastase-catalyzed hydrolyses of *p*-nitrophenyl isobutyrate and trimethylacetate exhibit a presteady state "burst" followed by a steady-state liberation of *p*-nitrophenol. When the effect of diethyl *p*-nitrophenyl phosphate on the enzymatic activity was measured, an aliquot (100 μl) of the elastase phosphate reaction mixture was added to an assay cuvette containing 3.0 ml of substrate solution. The 30-fold dilution of the reaction mixture in the assay cuvette was sufficient to quench the phosphate reaction.

For papain titrations using *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, an aliquot of enzyme solution was added to the substrate solution in the cuvette using the technique of addition through a hole in the cell compartment cover. Before and after a given series of titration experiments, the activity toward α-N-benzoyl-L-arginine ethyl ester of the stock enzyme solution was determined. Although the enzyme flask was opened repeatedly during this time interval (2–4 hr), the enzymatic activity measured in this manner decreased by less than 2%.

In order to conserve acetylcholinesterase, quartz cells of the following inner dimensions were used in its titration: 10-mm light path with a 4-mm path width and a 30-mm height (Precision Cell Co.), and 10-mm light path with a 4.5-mm path width and 15-mm height (Pyrocell Manufacturing Co.). It was ascertained that the light path of the spectrophotometer is totally transmitted through the solution in the cell. The most critical procedure of this kind involved the Pyrocell cells used with a volume of [illegible] ml. A procedure was developed which demonstrates that the [illegible] completely transmitted during the titration experiments with this cell.[56] An aliquot of acetylcholinesterase solution was added to the cell. After the base line was determined, a 25-μl aliquot of the buffer solution (or water) was added, and the small decrease in absorbance, needed to correct for the decrease which the titrant was added, was recorded. A 25-μl aliquot of the enzyme solution diluted carbamate solution was added and stirred, and the liberation of nitrophenol was recorded. At the end of the steady state turnover reaction a 25-μl aliquot of nitrophenol in water was added to the enzyme solution, and the absorbance change was recorded on the 1.0 absorbance slide wire in order to obtain the molar absorptivity of the nitrophenol under the reaction conditions. Finally the pH of the solution was determined.

Figure 3 shows a "burst" titration of acetylcholinesterase with *o*-nitrophenyl dimethylcarbamate. The observed burst, #, was

(56) J. K. Stoops, Doctoral Dissertation, Northwestern University, 1966.

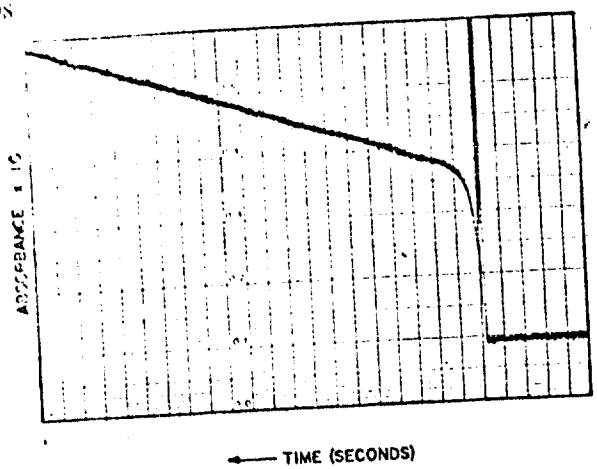


Figure 3. Titration 6 of acetylcholinesterase with *o*-nitrophenyl dimethylcarbamate at pH 7.70. The extrapolation of the absorbance to time zero, which is represented by the initial rise in absorbance, corresponds to an enzyme concentration of 7.45×10^{-6} *M*. The initial presteady-state reaction of approximately 1 min is followed by a steady-state (zero-order) reaction. The abscissa is 60 sec/division.

calculated according to eq 20[57,58]

$$\pi = \frac{A_3 - A_2 + F(A_1 - A_2) - A_4}{\Delta\epsilon} \qquad (20)$$

where A_1 is the initial absorbance of the enzyme solution, A_2 is the absorbance after the addition of an aliquot of water (or of the buffer solution), A_3 is the absorbance measured from the extrapolation of the absorbance to zero time, and A_4 is the absorbance of the carbamate. The difference of A_1 and A_2 is corrected for dilution by the factor F. The difference in extinction coefficients, $\Delta\epsilon$, is ϵ(phenol) − ϵ(ester). The factor F is given by eq 21[59]

$$F = \frac{1 - V_2/V_3}{V_2/V_1 - 1} \qquad (21)$$

where V_n corresponds to the volume of the solution at which the absorbance was measured. The corrections $F(A_1 - A_2)$ and A_4 *in toto* generally decrease the uncorrected burst, $A_3 - A_2$, by 5–20%.

Results for Chymotrypsin

α-Chymotrypsin (chymotrypsin A, EC 3.4.4.5)[3] has been titrated previously using a number of substrates, both specific and nonspecific, in this laboratory and elsewhere: however, several additional titrations are reported here. These titrations involve the substrates, *p*-nitrophenyl acetate, and 2,4-dinitrophenyl acetate using three methods of determining concentration: (1) a comparison of the slopes of the Lineweaver–Burk plots of the steady-state and presteady-state portions of the reaction (method 2); (2) second-order kinetics (method 3); and (3) measurements of the burst of *p*-nitrophenol in the presteady state (method 4). The three methods give results which are in essential agreement with one another.

Comparison of the Slopes of the Lineweaver–Burk Plots of the Steady-State and Presteady-State Portions of the Reaction. Previously results were presented concerning the turnover rates of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate at substrate concentrations sufficiently low that complications

(57) This equation follows that for a method B titration of α-chymotrypsin with N-*trans*-cinnamoylimidazole.[33]

(58) This equation was incorrectly given in a preliminary communication of this work: M. L. Bender and J. K. Stoops, *J. Am. Chem. Soc.*, **87**, 1622 (1965).

(59) Derivation of this formula is given in ref 56.

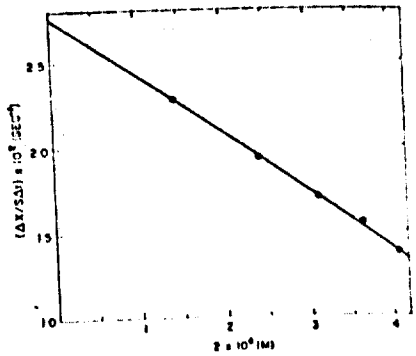


Figure 4. The titration of α-chymotrypsin by *p*-nitrophenyl acetate using second-order conditions: 25.0°, pH 7.8, 0.067 *M* phosphate buffer, $[S]_0 = 6.72 \times 10^{-6}$ *M*, $[E]_0 = 8.17 \times 10^{-6}$ *M*.

arising from extraneous reactions were avoided. From the slopes of (turnover) plots of $1/V_0$ vs. $1/[S]_0$ values of $[E]_0k_2/K_S$ were determined, following eq 4. In addition, the kinetics of the presteady state were determined, giving from eq 7 an independent value of k_2/K_s. Using these two sets of data, $[E]_0$ was determined as shown in Table I.

Table I. Determination of the Concentration of an α-Chymotrypsin Solution using the Steady-State and Presteady-State Portions of the Hydrolysis of *p*-Nitrophenyl Acetate[a]

Concn of enzyme from wt, g/l.	pH	$k_2[E]_0/K_s$, sec^{-1}	k_2/K_s, M^{-1} sec^{-1}	$[E]_0$, *M*	$[E]_0$,[b] *M*
1.61×10^{-2}	7.8	0.0017	3530	4.8×10^{-7}	$5.0 \times$ [illegible]
1.03×10^{-2}	7.7	0.00093	3410	2.7×10^{-7}	$3.1 \times$ [illegible]

[a] 1.6% acetonitrile–water at 25°. [b] Titration of the enzyme solution with N-*trans*-cinnamoylimidazole.

Second-Order Kinetics. The α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate was carried out under second-order conditions (K_m(app) > [S]$_0$ ≅ $[E]_0 < K_s$). The data were treated according to eq 9. Figure 4 shows such a plot. Values of V were obtained by calculating $V \sim \Delta[P_1]/\Delta t$ from experimental [illegible] with Δt approximately $0.25t_{1/2}$. From eq 9, it is [illegible] that the slope of this plot gives k_2/K_s (3380 M^{-1} sec^{-1} while a conventional second-order plot gives k_2/K_s 3630 M^{-1} sec^{-1} for the same reaction), while the intercept of this plot gives $k_2[E]_0/K_s$. The quotient of the intercept/slope = $[E]_0$ = 8.17×10^{-6} *M* which [illegible] N-*trans*-cinnamoylimidazole titration of the [illegible] solution gave $[E]_0$ = 7.85×10^{-6} *M*.

Measurement of the Burst of *p*-Nitrophenol in the Presteady State. The α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate was carried out under turnover conditions ($[S]_0 \gg [E]_0$). Plots of eq 10 ($1/\sqrt{\pi}$ vs. $1/[S]_0$) are shown in Figure 5 for two different enzyme concentrations. The experimental points [illegible] on fairly good straight lines, whose slopes are in good agreement with the calculated slopes based on values of the various constants of eq 14 given previously. The extrapolated π value at $[S]_0 = \infty$ ($1/[S]_0 = 0$) [illegible] good agreement with the N-*trans*-cinnamoylimidazole titration values, as shown in Table II. In addition, the α-chymotrypsin-catalyzed hydrolysis of 2,4-dinitrophenyl acetate was investigated under turnover conditions.

Table II. Titration of Some α-Chymotrypsin Solutions Calculated from the Burst of *p*-Nitrophenol from a *p*-Nitrophenyl Ester Substrate

Substrate	$[S]_0 \times 10^5$, *M*	$[E]_0$, g/l.	$[E]_0 \times 10^6$,[a] *M*	$[E]_0 \times 10^6$,[b] *M*	$[E]_0 \times 10^6$,[c] *M*	% purity
p-Nitrophenyl[d] acetate	3.4-67.7	0.694	27.98	20.42	21.08	75.3
p-Nitrophenyl[e] acetate	6.8-67.7	0.277	11.17	7.82	8.18	73.2
2,4-Dinitrophenyl acetate	13.94			2.82	2.86	
2,4-Dinitrophenyl acetate	6.97			2.82	2.82	
2,4-Dinitrophenyl acetate	14.02			1.14	1.15	

[a] Theoretical using weight of enzyme and a molecular weight of 24,800. [b] Titration with N-*trans*-cinnamoylimidazole. [c] This titration [d] pH 7.8, 1.6% acetonitrile-water, 25°. [e] pH 5.92, 1.6% acetonitrile-water, 25°.

Table III. Titrants for α-Chymotrypsin Solutions[a]

Titrant	Species measured	$[S]_0$[d]/ K_m (app)	k_2/k_3	Titration value × 100% using N-*trans*-cinnamoyl-imidazole	Ref
p-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate	*p*-Nitrophenol	100	1000 (?)	97–101	*h*
p-Nitrophenyl N-acetyl-DL-tryptophanate	*p*-Nitrophenol	10–100	1000 (?)	91–99	*h*
p-Nitrophenyl acetate	*p*-Nitrophenol	1–10[b]	600	88–104	19, 25, *b, f, l, n*
p-Nitrophenyl isobutyrate	*p*-Nitrophenol	...	...	~100 ± 10	*f*
p-Nitrophenyl trimethylacetate	*p*-Nitrophenol	10	2800	~100 ± 10	*f*
2,4-Dinitrophenyl acetate	*p*-Nitrophenol	30	600	101	*b*
N-*trans*-Cinnamoylimidazole		100	4000	100	22
Phenylmethanesulfonyl fluoride[c]	Protons	∞	∞	103	*g*
	^{14}C labeling of enzyme	∞	∞	103	*g*
3-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate[f]	3-Nitro-4-hydroxybenzoic acid	∞	∞	100 ± 10[e]	23
Diisopropylphosphorofluoridate[c]	Isopropyl groups of enzyme	∞	∞	100 ± 10[e]	6
	P of enzyme	∞	∞	100 ± 10[e]	6
	Protons	∞	∞	100 ± 10[e]	*l, m*
Diethyl *p*-nitrophenyl phosphate[c]	*p*-Nitrophenol	∞	∞	100 ± 10[e]	

[a] All titrations were carried out using method 4 (measurement of the burst of P_i or ES′ in the presteady state) except for *p*-nitrophenyl acetate where methods 2 + 3 were used. [b] Present investigation. [c] The rigorous alternative treatment using eq 17 gives the same result. [d] These ratios are taken from the references in the table together with references *j*, *k*, and *l*. [e] Estimate on basis of purity or derived molecular weight. [f] C. E. McDonald and A. K. Balls, *J. Biol. Chem.*, **227**, 727 (1957). [g] A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 787 (1964). [h] F. J. Kézdy, G. E. Clement, and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3690 (1964). [i] M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962). [j] G. A. Hamilton and M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2570 (1962). [k] G. E. Clement and M. L. Bender, *Biochemistry*, **2**, 886 (1963). [l] B. S. Hartley and B. A. Kilby, *Biochem. J.*, **50**, 672 (1952). [m] B. A. Hammond and H. Gutfreund, *ibid.*, **73**, 526 (1959). [n] J. R. Knowles, *ibid.*, **95**, 180 (1965).

Discussion. Solutions of α-chymotrypsin have been titrated many ways. Most of the titrants are listed in Table III together with the relationship of the titration value resulting from each titrant to that found with N-*trans*-cinnamoylimidazole, not because the latter is inherently the best titrant, but because it has been available for comparative purposes as an accessible standard for a longer period of time than any other titrant. All of the titrations in Table III meet the requirements set forth above for measurement of the stoichiometric conversion of enzyme to acyl-enzyme, namely that $[S]_0 \gg K_m(\text{app})$ and that $k_2 \gg k_3$. Furthermore, $[S] > [E]$ in all titrations, although in some titrations, e.g., with N-*trans*-cinnamoylimidazole, the $[S]/[E]$ ratio is low (*vide infra*). Finally, all the titrations including those with the nonspecific substrates such as *p*-nitrophenyl trimethylacetate, those with specific substrates such as *p*-nitrophenyl benzyloxycarbonyl-L-tyrosinate, and those with inhibitors such as 3-nitro-4-carboxyphenyl-N,N-diphenylcarbamate and diisopropylphosphorofluoridate give titrations which are essentially equivalent with one another (some of the comparisons are indirect ones, such as a calculation of molecular weight of the enzyme on the basis of the stoichiometry). Since the titrations are independent of

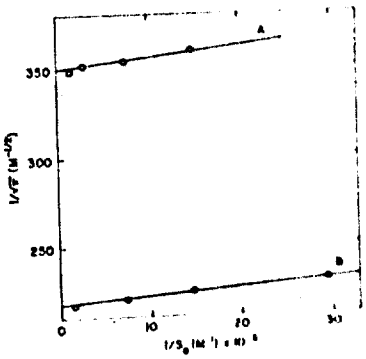


Figure 5. The titration of α-chymotrypsin by *p*-nitrophenyl acetate at 25.0°, pH 7.8, 0.067 *M* phosphate buffer, 1.6% acetonitrile-water (v/v); A, $[E]_0 = 8.2 \times 10^{-6}$ *M*; B, $[E]_0 = 21.1 \times 10^{-6}$ *M*.

substrate, a strong argument may be made for stating that in all titrations, one and the same active site

Table IV. Titration of Trypsin with Some *p*-Nitrophenyl Esters[a]

p-Nitrophenyl ester of	$[S]_0 \times 10^5$, *M*	K_m(app) $\times 10^5$, *M*	k_2/k_3	pH	μ	CH_3CN, % (v/v)	Titrated $[E]_0 \times 10^5$, *M*	Enzyme[b] % yield
			Porcine trypsin[c]					
Acetic acid	81–271	15.6	6	6.24		15	15.8	83
N-Acetyl-L-leucine	28–97	32	*d*	4.46	0.2	2.4	32.4	83
	16–42	25	*d*	4.55	0.2	1.6	32.4	81
N-Acetyl-DL-tryptophan	5.3–11.2	32	*d*	3.46	0.05	1.6	3.20	81
	5.3–11.2	7.1	*d*	3.32	0.5	1.6	5.50	80
N^2-Benzyloxycarbonyl-L-lysine	105	*e*	*d*	3.0	0.05	1.3	418	75
Diethylphosphoric acid[f]	305			7.8	0.05	6.1	181	63
			Bovine trypsin					
Acetic acid	10–124	7.7	15.2	7.16	0.5	1.6	22.4	63
	15–124	7.7	15.2	7.59	0.5	1.6	20.6	63
	15–124	7.7	15.2	8.00	0.5	1.6	16.9	64
	15–124	7.7	15.2	8.61	0.5	1.6	15.4	59
N-Acetyl-L-leucine	28–73	23		4.49	0.02	2.4		51
N-Acetyl-DL-tryptophan		131[g,h]					181	50
		104[g,h]					229	64
N^2-Benzyloxycarbonyl-L-lysine	5.9–29.3	7.9	*d*	3.46	0.05	1.6	1.95	41
	7–80	1.0	*d*	3.71	0.02	2.0	18.9	53
	105	1.27	27.6[i]	3.0	0.05	1.3	48	57.5

[a] 25°. [b] Calculated as the per cent of the titrated $[E]_0$ of the $[E]_0$ determined on a weight basis assuming a molecular weight of 23,000. [c] Armour Pharmaceutical Co. [d] Unknown; assumed to be ∞ in calculation of $[E]_0$. [e] Unknown; assumed to be 0 in calculation of $[E]_0$. [f] See text. [g] Using second-order conditions. [h] k_2/K_m, M^{-1} sec^{-1}. [i] *Cf.* ref 11.

is being titrated stoichiometrically, and further that no extraneous reaction is being observed. Titrations of the active site of α-chymotrypsin are also independent of pH, some of the titrations being carried out from pH 9.5 (*p*-nitrophenyl acetate) down to pH 2.5 (*p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate).

The most specific titrant for α-chymotrypsin is *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate; the least specific titrant is diethyl *p*-nitrophenyl phosphate. As an additional check to determine whether these two compounds, and by inference the other titrants of Table III, reflect the same site of the enzyme, the rate constant for the liberation of *p*-nitrophenol from the reaction of diethyl *p*-nitrophenyl phosphate with α-chymotrypsin was compared to the rate constant for the loss of enzymatic activity toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate during this reaction. The two rate constants were found to be identical,[60] within experimental error, confirming the coherence of the titrants of Table III.

Results for Trypsin

The stoichiometric basis of a titration procedure for the determination of the active sites of trypsin (trypsin, EC 3.4.4.4)[3] in terms of eq 1 is predicted on the basis of kinetic studies and isolation experiments.[11] However, unlike α-chymotrypsin, no stoichiometric procedures, other than the early phosphorylation experiments, have been developed for trypsin. On the other hand, as with many enzymes, a host of rate assay procedures have been developed, involving denatured hemoglobin, denatured casein, α-N-benzoyl-L-arginine ethyl ester, α-N-benzoyl-L-arginine amide, and α-N-benzoyl-L-lysine amide as substrates.[61,62] These rate assays suffer from the inadequacies of rate assays as discussed previously. Therefore, a search was made for a titrant for the active sites of trypsin, preferably based on a specific substrate of the enzyme.

Solutions of bovine and porcine trypsin have been titrated using five titrants and two methods of titration. The titrants include diethyl *p*-nitrophenyl phosphate, *p*-nitrophenyl acetate, *p*-nitrophenyl N-acetyl-L-leucinate, *p*-nitrophenyl N-acetyl-DL-tryptophanate, *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride. The methods of titration involve measurement of the "burst" of *p*-nitrophenol in the prestead state of the reaction and the use of second-order kinetics.

The Burst of *p*-Nitrophenol. The most extensive experiments determined the initial "burst" of *p*-nitrophenol liberated in the prestead state of the trypsin-catalyzed hydrolysis of the *p*-nitrophenyl esters. Representative results of the "burst" titrations of bovine trypsin with *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate are given in Figure 6, showing the dependence of the burst on the substrate concentration. The "bursts" in the reaction of trypsin with the other titrants (except diethyl *p*-nitrophenyl phosphate) are more heavily dependent upon substrate concentration than the lysine "burst." A summary of the burst results for both bovine and porcine trypsin is given in Table IV. Using acceptable concentrations of the titrants, *p*-nitrophenyl acetate, N-acetyl-L-leucinate, and N-acetyl-DL-tryptophanate, the burst of *p*-nitrophenol is far from the limiting burst because the $[S]_0/K_m$(app) and/or k_2/k_3 are not sufficiently high.

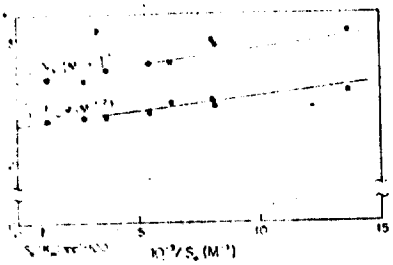
The burst produced in the reaction of porcine trypsin and diethyl *p*-nitrophenyl phosphate can be shown to be stoichiometric. The kinetics show that $k_2 = k_3$ = 1.65 × 10^{-4} sec^{-1} for reaction with 1.5 × 10^{-4} *M* phosphate and k_3 = 10^{-4} sec^{-1} [illegible]. Thus from eq 1 the enzyme concentration does not differ from the burst more than 5%.

The optimal titrant of trypsin on the basis of data

(60) This result is similar to the result of Hartley and Kilby[20a] who demonstrated the identity of the inhibition of amino acid esterase, amidase, and proteolytic activities with that of *p*-nitrophenol release in the reaction of α-chymotrypsin and diethyl *p*-nitrophenyl phosphate.

(61) See ref 4.

(62) See ref 5.



ure 6. The effect of substrate concentration on the burst titra- (1/$\sqrt{\pi}$) and the turnover (1/v) of bovine trypsin with p-nitro- yl N²-benzyloxycarbonyl-L-lysinate, hydrochloride, pH 3.71, M total formate buffer, I = 0.02, 25°, 2% acetonitrile-water

ichiometric burst and its specificity is p-nitrophenyl N²-benzyloxycarbonyl-L-lysinate hydrochloride. The ti- ation of trypsin with this substance has been investi- ted in detail. Figure 1 shows a typical titration of psin with this substance. Figure 6 shows that the st of p-nitrophenol in the presteady state of the ation reaction is not greatly affected by substrate ntration in the region of substrate concentration ly used. Thus, using a substrate concentration × 10^{-3} M, the usual concentration for the n, and the data of Table IV, eq 12 indicates that ratio $\pi/[E]_0$ = 0.909. That is, under these condi- ns, the burst corresponds to 91% of the true enzyme centration.[63]

Since, as indicated in the Experimental Section, solu- s of bovine trypsin at pH 3 undergo an initial loss of ity, as measured by rate assay with α-N-benzoyl-L- nine ethyl ester at pH 8, titrations of trypsin solu- s after variable amounts of time were carried out pH 3, using p-nitrophenyl N²-benzyloxycarbonyl-L- ate as titrant. From 4-min incubation to 3-hr incu- ion at room temperature, no change in the burst was rved.

The rate of a trypsin-catalyzed hydrolysis of α-N- zoyl-L-arginine ethyl ester is considerably increased the presence of calcium ion, 10^{-2} M calcium ion ne nearly 100% increase in rate (partially an ionic ngth effect). Therefore, the dependence of the ion on calcium ion was investigated. Bovine psin was prepared both in pH 2.92 citrate buffer d in pH 3.0 0.06 M calcium ion. The per cent of e trypsin determined in two titrations of the same psin stock using these two solutions were 57 and 60, pectively. Thus the effect of calcium ion on the burst d best to increase the value 5%.

Second-Order Conditions. Several experiments were formed to determine trypsin concentration in this ner, using N-acetyl-DL-tryptophan p-nitrophenyl r and p-nitrophenyl N²-benzyloxycarbonyl-L-lysi- hydrochloride as substrates. These determinations limited to the bovine enzyme since a large amount zyme is consumed in each experiment. Figure 7 s a plot of $V/([S] - [P_t])$ for the tryptophan sub- Although these experiments are subject to from both spontaneous hydrolysis and deacylation e acyl-enzyme, the data lead to results which are bly consistent with those of the burst titrations Table IV).

This correction was not made in the preliminary communication k: M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, Biophys. Res. Commun., 19, 161 (1965).

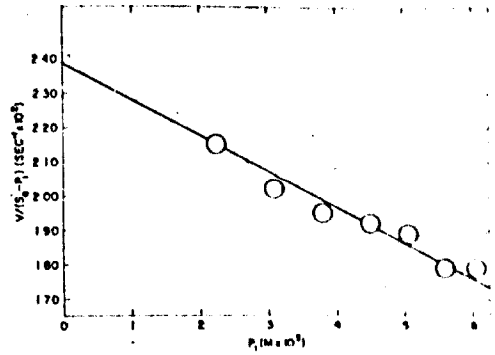


Figure 7. The trypsin-catalyzed hydrolysis of N-acetyl-DL-trypto- phan p-nitrophenyl ester under second-order conditions: $[S]_0$ = 2.71–7.43 × 10^{-5} M, $[E]_0$ = 1.81 × 10^{-5} M, pH 3.11, 25°, 0.05 M citrate buffer, 1.6% acetonitrile-water (v/v).

Discussion. All titrations of Table IV indicate that the bovine trypsin preparation used is 49–60% active enzyme. Those titrations of Table IV where both the k_2/k_3 and $[S]_0/K_m$(app) (or k_h/k_3) ratios are known indicate that the bovine trypsin preparation contains 59–69% active enzyme and that the porcine trypsin preparation contains 65–83% active enzyme. The purity of porcine trypsin may be appreciably improved by gel filtration using Sephadex G-25 (*vide infra*).

Although the agreement in Table IV between different titrants is not precise, it is certainly reasonable considering the uncertainties of the experiments and different conditions used. The question may still be asked as to whether the various titrants of Table IV reflect the same active site. Two approaches have been used to investigate this problem. The rate constant for the phosphorylation of porcine trypsin with diethyl p-nitrophenyl phosphate, as reflected by the release of p-nitrophenol, was compared to the rate constant for the inactivation of the enzyme, as reflected by the turnover of p-nitrophenyl N²-benzoyloxycarbonyl-L-lysinate at pH 6.55. The first-order rate constants of these two reactions were 1.77 × 10^{-4} and 1.65 × 10^{-4} sec^{-1}, respectively, indicating that the most specific titrant, the lysine ester, and the least specific titrant, the phosphate, react at the same site on the enzyme. Presumably the other intermediate titrants do also. At pH 6.28, the turnover rate of p-nitrophenyl N²-benzyloxycarbonyl-L-lysinate with porcine trypsin is inhibited 92% by 3.26 × 10^{-6} M soybean trypsin inhibitor. This experiment indicates that at least 92% of the burst of p-nitrophenol using the lysine substrate as titrant must arise from the active site as defined by soybean trypsin inhibitor.[64] Thus, the titrations of Table IV appear to be independent both of pH and substrate.

For trypsin solutions, a convenient secondary standard is the rate assay based on α-N-benzoyl-L-arginine ethyl ester as substrate. This reaction has been investigated intensively, and found to obey Michaelis-Menten kinetics.[65] The K_m(app) for this reaction at pH 8 is 2 × 10^{-5} M so that at a concentration of 10^{-3}

(64) Since the binding of soybean trypsin inhibitor diminishes greatly at lower pH, it is not possible to carry out this experiment at the pH of the titration: M. Kunitz, *J. Gen. Physiol.*, 30, 291 (1947); R. L. Steiner, *Arch. Biochem. Biophys.*, 49, 71 (1954).

(65) H. Gutfreund, *Trans. Faraday Soc.*, 51, 444 (1955); G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, 179, 665 (1949); T. Inagami and J. M. Sturtevant, *Biochim. Biophys. Acta*, 38, 63 (1960).

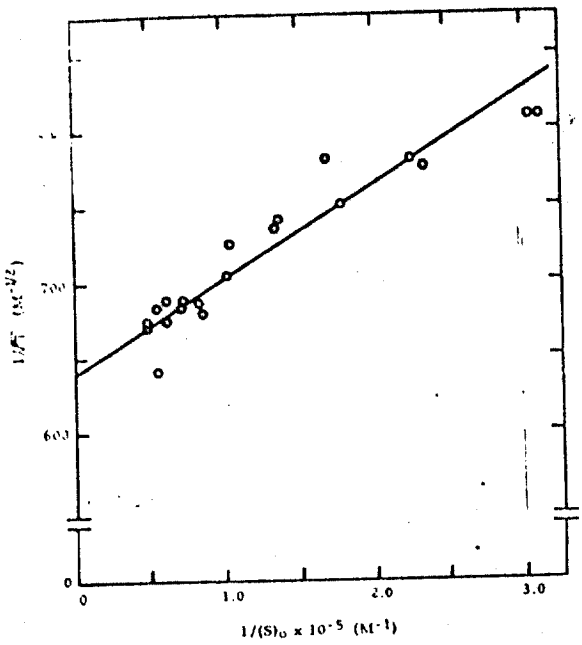


Figure 8. The dependence of the *p*-nitrophenol "burst" on the initial substrate concentration in the titration of papain by *p*-nitrophenyl α-N-benzyloxycarbonyl-L-tyrosinate: 1.6% (v/v) acetonitrile-water, pH 3.18, 0.05 *M* potassium hydrogen phthalate, 25.0°, $[E]_0 = 2.45 \times 10^{-6}$ *M*.

M, saturation conditions hold and the velocity may be expressed by $V_{max} = k_3[E]_0$. Using a trypsin titrant it is possible to determine $[E]_0$ and using the rate assay it is possible to determine V_{max}. Thus, the coefficient k_3 may be determined. The known k_3 may then be used in conjunction with V_{max} to determine the $[E]_0$ of an unknown solution. Using a trypsin solution incubated in 0.05 *M* citrate buffer, pH 3, for at least 20 min, and assay conditions of pH 8.0, 25.0°, and 10^{-3} *M* α-N-benzoyl-L-arginine ethyl ester, k_3 is found to be 1.32×10^4 absorbance units M^{-1} sec^{-1} at 253 mμ.

p-Nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride is the most specific substrate of trypsin known today, using as criterion that it has the highest rate constant (k_3(lim) = 170 sec^{-1}). It is interesting that this compound serves as the most convenient titrant of the active sites of trypsin.

Results for Papain

Papain (papain, EC 3.4.4.10)[3] is an enzyme which is distinguished from the enzymes discussed so far by the fact that it contains a sulfhydryl group in its active site, which acts as a nucleophile during the catalytic process converting the substrate first into an intermediate acyl-enzyme having the characteristics of a thiol ester; the acyl-enzyme then reacts with water giving product carboxylic acid, and regenerating the enzyme.[14] Although the important nucleophile of the active site is different from that of α-chymotrypsin and trypsin, the over-all pathway of the papain reaction appears to follow eq 1, which serves as the stoichiometric basis of the titration of the enzyme.

Burst Titration. The papain-catalyzed hydrolysis of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate shows an initial "burst" of *p*-nitrophenol when the zero-order portion of the plot of absorbance of *p*-nitrophenol *vs.* time is extrapolated to time zero. This observation is excellent confirmatory evidence for the mechanism eq 1.

A typical set of data for "bursts" at various subst[illegible] concentrations is shown in Figure 8 plotted accor[illegible] to eq 13. Such plots were analyzed by a weig[illegible] least-squares method using $[S]_0$ as the weighting fac[illegible] The results of three sets of experiments are listed [illegible] Table V.

Several semiquantitative arguments are pres[illegible] that k_2/k_3 is much greater than unity in this reacti[illegible] (1) A survey of reported K_m (K_s) data for papain s[illegible] strates shows values ranging from 10^{-3} *M* upw[illegible] The extremely low K_m(app) ($= k_3/(k_2 + k_3)K_s$) [illegible] *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, [illegible] μ*M*, suggests that the "normal" value of K_s ([illegible] *M*) is multiplied by a very small ratio of $k_3/(k_2 + [illegible]$ *i.e.*, that the ratio k_2/k_3 is very large. (2) In the p[illegible] ence of 30% methanol, the turnover rate incre[illegible] twofold, possibly because methanol is acting as [illegible] nucleophile in deacylation as it is known to do in t[illegible] deacylation of *trans*-cinnamoyl-papain. The turnov[illegible] rate constant is predominantly k_3 since a burst [illegible] observed and since there is independent evidence f[illegible] only one active site per molecule.[14] Hence 30[illegible] methanol must increase k_3 by a factor of two. Yet t[illegible] burst was found to be undiminished within the exper[illegible] mental error of 4%, indicating that the ratio $k_2/(k_2 + [illegible]$ k_3) is sufficiently close to unity so that a twof[illegible] increase in k_3 does not affect π more than 4% [illegible] $k_2/k_3 \geq 50$. (3) The substrate *p*-nitrophenyl N-acety[illegible] DL-tryptophanate shows a burst with papain, the [illegible] species only reacting. Although this substrate is m[illegible] soluble than the tyrosine substrate, the former has [illegible] much less favorable K_m(app), resulting in a m[illegible] steeper extrapolation of the $(\pi)^{-1}$ *vs.* $[S]_0^{-1}$ plot [illegible] thus in a less precise titration. The results with t[illegible] tryptophan substrate agree with those using the tyro[illegible] substrate to within 9%, which is considered fair agr[illegible] ment in view of the imprecision associated with t[illegible] tryptophan substrate. These results indicate that b[illegible] reactions must have k_2/k_3 ratios much greater t[illegible] unity, since the two substrates would be expected t[illegible] have different k_2/k_3 ratios. (4) A lower limiting va[illegible] of the k_2/k_3 ratio can be calculated from the experim[illegible] observation that the presteady state is complete in t[illegible] time that elapsed between the addition of enzyme a[illegible] the beginning of observation of the appearance of *p*-[illegible] trophenol. The presteady-state, first-order rate c[illegible] stant, *b*, is given by[23]

$$b = \frac{(k_2 + k_3)[S]_0 + k_3K_s}{K_s + [S]_0} \quad (1[illegible])$$

Since $K_s = ((k_2 + k_3)/k_3)K_m$(app), eq 4 may b[illegible] transformed into eq 23. The K_m(app) val[illegible]

$$k_2/k_3 = \frac{b}{k_3 - \frac{K_m(\text{app})}{[S]_0}(b - k_3)} - 1 \quad (2[illegible])$$

were obtained from a Lineweaver Burk plot [illegible] Table V); k_{cat} ($\sim k_3$, *vide supra*) was calculated [illegible] rectly from the slope of the zeroorder appe[illegible] of *p*-nitrophenol following the burst. Since the [illegible] steady state in the burst titration was "comp[illegible] before the first spectrophotometric observation [illegible] the addition of enzyme, at least four to six hal[illegible]

Table V. The Titration of Papain Using *p*-Nitrophenyl N-Benzyloxycarbonyl-L-tyrosinate[a]

$[S]_0 \times 10^6$, M	$K_m \times 10^6$, M	k_2/k_3[d]	$[E]_0^{expt} \times 10^6$,[b] M	$[E]_0^{calcd} \times 10^6$,[b,c] M	Enzyme [illegible] points	[illegible] 10^6, M [illegible]
4.0-21.5	0.63	100 ± 60	2.45 ± 0.03	6.22	39.4	[illegible]
3.4-21.8	0.63		1.67 ± 0.02	3.98	42.0	[illegible]
4.2-19.8[e]	0.39	190 ± 110	2.49 ± 0.03	6.22	39.9	[illegible]

[a] 16% acetonitrile-water (v/v), I = 0.05, pH 3.18, 0.05 M potassium hydrogen phthalate; 25.0° unless otherwise noted. [b] Coefficient [illegible]. [c] Determined spectrophotometrically at 280 mμ; see text. [d] Calculated lower limit; see text. [e] 11° [illegible] N-benzoyl-L-arginine ethyl ester assay was carried out in 0.05 M acetate, pH 5.17, μ = 0.038, 10^{-4} M ethylenediaminetetraacetic acid, 25.0°. [f] [S] [illegible] M.

[illegible]st have elapsed, depending on the magnitude of the [illegible]st and the accuracy with which one can read the [illegible]sorbances. Hence a lower limit can be calculated [illegible] b, and consequently for k_2/k_3. Table V shows [illegible]er limiting values (with the attendent experimental [illegible]certainties) for the k_2/k_3 ratio thus calculated. The [illegible]ta at 11° show the tail end of the presteady state at [illegible] lowest substrate concentrations, and thus the [illegible]try k_2/k_3 = 190 in Table V is based on the estimated [illegible]tual value of b, rather than its lower limit. Although [illegible] upper limit of these (lower limiting) k_2/k_3 estimates [illegible] quite uncertain, the lower limit of this ratio is [illegible]lieved to be quite certain. This lower limit is, of [illegible]urse, the point of importance here.

Special consideration must be given to the substrate [illegible]ncentrations used in these experiments. The lowest [illegible]tant concentrations in Table V are only some 60% [illegible]eater than the enzyme normality in the cell, in violation of the assumption implicit in the derivation of eq [illegible] that $[S]_0 \gg [E]_0$. Actually to be more correct, the [illegible]cessary assumption is that the rate of change of the [illegible]yl-enzyme ($d[ES']/dt$) is much less than the rate of [illegible]nge of the substrate ($d[S]/dt$).[66] In the present [illegible]periments, since the presteady state is essentially [illegible]stantaneous with respect to the steady state ($b \gg k_3$), [illegible] substrate concentration rapidly diminishes by an [illegible]ount equal to the concentration of acyl-enzyme [illegible]med, and then decreases in the steady-state reaction [illegible]ile the acyl-enzyme concentration remains sensibly [illegible]nstant.

The substrate concentrations reported in Table V [illegible] used throughout the calculations are in re[illegible] ($[S]_0 - \pi/2$). This corrected substrate concen[illegible]tion gives the closest approximation to the true [illegible]bstrate concentration during the burst, by taking an [illegible]erage of the substrate concentration during this [illegible]riod. The value of π so obtained differs by only [illegible]2% from that obtained when ($[S]_0 - \pi$) or $[S]_0$ are [illegible]ed, respectively. Thus, this correction is not a [illegible]jor consideration. The reason for the insensitivity [illegible] the burst to this correction is that the highest values [illegible] $[S]_0$ are about 35 times the value of K_m(app), these [illegible]periments giving values of π very near the maximum [illegible]lue.

Relationship between the Titration and Rate Assay. [illegible]he rate of the hydrolysis of α-N-benzoyl-L-arginine [illegible]hyl ester, a specific substrate for papain, was carried [illegible]t simultaneously with the titration experiments. [illegible]aus since K_m(app) ≫ $[S]_0$, a pseudo-first-order reac[illegible]n is observed whose rate constant is k_{BAEE} = (k_{cat}/K_m(app))$[E]_0$. This pseudo first-order rate constant was shown to be proportional to $[E]_0$ and independent of the substrate concentration provided that 10^{-4} M ethylenediamine tetraacetic acid was present. In the absence of the chelating agent, there is a small decrease in k_{BAEE} as the initial substrate concentration increases, possibly due to a small metal ion impurity in the substrate solution. Using $[E]_0$ from the titrations, the ratio $(K_m(app)/k_{cat})_{BAEE}$ (equal to $[E]_0/k_{BAEE}$) was determined as 8.84×10^{-4} M sec at pH 5.17 and 25°. Thus the enzyme concentration in the cell is given by $[E]_0 = k_{BAEE} \times 8.84 \times 10^{-4}$ M sec under the conditions specified in footnote *f* of Table V. By this means the normality of any papain solution can be readily determined.

Identification of the Site of Titration as the Active Site. To demonstrate that the titration arises from reaction only at the active site, the following experiments were carried out. Papain can be converted in approximately 90% yield to give the acyl-enzyme, *trans*-cinnamoyl-papain, and then freed of excess substrate by Sephadex filtration.[14] Under conditions where ethyl α-N-benzoyl-L-argininate is hydrolyzed by papain in a first-order reaction ($[(BAEE)_0] \ll K_m$(app)), a plot of log $[(BAEE)_0/(BAEE)]$ *vs.* time will, of course, give a straight line. If part of the enzyme is cinnamoylated, a similar first-order plot exhibits initial curvature until the *trans*-cinnamoyl-papain has completely decinnamoylated as indicated by eq 24

$$\log \frac{(BAEE)_0}{(BAEE)} = \frac{k_{cat}}{2.303K_m(app)}\left[\left([E]_0 + (CP)_0\right)t - \frac{(CP)_0}{k_3^{CP}}\left(1 - e^{-k_3^{CP}t}\right)\right] \quad (24)$$

where $(CP)_0$ and k_3^{CP} are the initial concentration and deacylation rate constant, respectively, of *trans*-cinnamoyl-papain, and $[E]_0$ is the initial concentration of free papain. The difference between the experimental curve and the extrapolated straight line portion of such a plot plotted logarithmically *vs.* time gave a value for k_3^{CP} which agreed within 19% of the independently determined value.[14]

The initial slope of the plot of eq 24 (which can be determined analytically) is proportional to the concentration of free papain at the time of initiating the reaction, and the final slope is proportional to the total concentration of papain, $[E]_0 + (CP)_0$. When a *trans*-cinnamoyl-papain solution prepared at pH 3.3 was used in a typical "burst" experiment, the "burst" indicated $0.37 \pm 0.13 \times 10^{-6}$ M free papain. A BAEE assay experiment at pH 5.17 was performed simultaneously. From the initial and final slopes of the first-

[illegible] The titration of α-chymotrypsin with *trans*-cinnamoylimidazole[23] [illegible]ates this point; it is successful as long as $[S]_0$ is only slightly [illegible]r than $[E_0]$. The deacylation rate is relatively so small that this [illegible]ition is met.

order plot for the total BAEE hydrolysis and the factor 8.84×10^{-4} M sec, it was calculated that the free papain concentration in the absorbance cell of the "burst" experiment was $0.32 \pm 0.14 \times 10^{-6}$ M, in good agreement with that determined from the "burst," while the total concentration of papain plus *trans*-cinnamoyl-papain in the cell was 2.12×10^{-6} M. Taking into account the limits of experimental error, 0.32×10^{-6} M $(-[(0.37 + 0.13) - (0.32-0.14)] \times 10^{-6})$ is the maximum amount of *p*-nitrophenol which could be released by a site on the enzyme other than the one occupied by the *trans*-cinnamoyl group. Thus at least 85% of the burst of *p*-nitrophenol must arise from reaction at the active site.

Discussion. The purity of the papain preparations as shown in Table V is calculated as the ratio of the titration to the spectrophotometric determination of total protein at 280 mμ. The value ϵ_{278} 51,800 (based on anhydrous, ash-free protein) may be calculated[67] assuming a molecular weight of 20,700. Thus an ϵ value of 51,100 was calculated for 280 mμ from a spectrum of papain. Recent sequence studies[68] suggest a molecular weight some 6% higher than 20,700. Such a change would have the effect of raising the purity by the same percentage. The data presented in Table V were obtained using an enzyme stock solution which was 15 (line 2) and 18 (lines 1, 3) days old, respectively. Enzyme stock solutions generally show a decrease in activity of about 2% per day, probably due to autolysis and oxidation. The α-N-benzoyl-L-arginine ethyl ester activity of the enzyme stock solution used for the titrations given in Table V was, however, also determined immediately after activation from mercuripapain; from this determination the freshly prepared enzyme was calculated to have been 58.0% pure. There is some variability among different batches of Worthington papain, and purities of 75% have been obtained regularly with two other Worthington lots, no. 5569-70 and 5573. It should be emphasized that the factor relating the pseudo-first-order rate constant for α-N-benzoyl-L-arginine ethyl ester hydrolysis to the titrated normality of the enzyme solution is independent of both the Worthington lot and the age of the activated enzyme stock solution.

The observation that papain is only 58–75% pure reconciles the data of Smith and Parker[69] with that of Whitaker and Bender.[14] The former workers found a maximal k_{cat} (or k_0) for α-N-benzoyl-L-arginine ethyl ester of 9.0 sec^{-1} assuming a 100% pure enzyme preparation, whereas the latter, who were able to titrate their papain solutions, obtained a value of 16.0 sec^{-1}. These two values are in agreement if Smith's enzyme solutions were, in fact, only 56% pure, a reasonable value in view of the findings presented here.

Presumably any stoichiometric reaction with the single sulfhydryl group of the active site may provide a titration for papain. In early studies of papain, Finkle and Smith[70] noted that papain reacted with less than 1 equiv of *p*-chloromercuribenzoate, a sulfhydryl reagent, when the latter was in twofold excess. The fraction of sulfhydryl groups so titrated was observed to be approximately proportional to the activity of the enzyme preparation; thus, this procedure appears to allow titration of the active site of papain. However, this titration has the disadvantage that a given enzyme preparation may contain protein molecules, or fragments thereof, which, though enzymatically inactive, may still contain free sulfhydryl groups capable of reacting with *p*-chloromercuribenzoate. In fact, experiments in this laboratory indicate that in the course of heat denaturation, papain loses its activity toward the substrate N-α-benzoyl-L-arginine ethyl ester much more rapidly than it loses its ability to react with *p*-chloromercuribenzoate.[71] Liener[72] found that the best preparations of ficin, an enzyme similar to papain, reacted with 0.90–0.95 equiv of N-ethylmaleimide based on an enzyme concentration calculated from a nitrogen analysis and a molecular weight of 26,000. The release of iodide ion in the reaction of iodoacetamide with ficin[74] indicated that the preparation used was 77% pure as determined on a dry weight basis and molecular weight of 26,000. However, these titrations suffer from the same disadvantages as those mentioned above for papain. Furthermore, the reactions of *p*-chloromercuribenzoate, N-ethylmaleimide, and iodoacetamide are undesirable for titrations because these substances are not related to papain substrates.

Results for Subtilisin

The stoichiometric basis of a titration procedure for the determination of the active sites of subtilisin (subtilopeptidase A, EC 3.4.4.16)[3] in terms of eq 1 is predicted on the basis of the stoichiometric reaction of the enzyme with diisopropylphosphorofluoridate[73] and the isolation of the acyl-enzyme, *trans*-cinnamoyl-subtilisin.[13] The latter work[13] mentioned a titration procedure for the active site of subtilisin using N-*trans*-cinnamoylimidazole, although no details were given.

Burst Titration. The subtilisin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole shows an initial (positive) "burst" in the disappearance of the substrate when the zero-order portion of the plot of absorbance vs. t is extrapolated to time zero. This observation is excellent confirmatory evidence for the mechanism of eq 1. A typical set of data for the "burst" at various substrate concentrations and enzyme concentrations is given in Table VI.

No presteady-state reaction was observed in any [illegible] tion when the substrate concentration greatly exceeded that of the enzyme, if the spectrophotometer was turned on 5 sec after the addition of enzyme to the substrate. One can thus conclude on a qualitative [illegible] that $k_2 \gg k_3$.

An attempt to make a quantitative estimate of the k_2/k_3 ratio by observation of the presteady state [illegible] stopped-flow spectrophotometer was of only [illegible] success because the highest substrate concentration possible was only one-tenth that of the apparent K_m. With these limitations, k_2 was found to be [illegible] at pH 7 indicating that $k_2 \gg k_3$, since k_{cat} at pH [illegible] (presumably k_3) = 1.45×10^{-4} sec^{-1}. An estimate

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Table VI. The Titration of Subtilisin Using N-*trans*-Cinnamoylimidazole[a]

Titration	Enzyme soln	$[S]_0 \times 10^4$, M[b]	π, absb unit	$[E]_{tot} \times 10^5$, M[c]	$[E]_{obsd} \times 10^5$, M[d]	% purity[e]	k_2, [illegible]
I[b]	1	1.89	0.507	6.02	1.93	57.1	1.32
II[b]	1	1.98	0.263	3.10	1.95	57.7	1.34
III	2	2.02	0.371	4.38	1.40	57.6	1.33
IV	3	2.02	0.285	3.36	1.08	55.7	1.30
V[c,d]	4	5.81	0.131	4.58	1.47 ± 0.07		1.32
VI[c,d]	4	2.87	0.131 ± 0.006	4.64	1.48 ± 0.07		1.35
VII	4	2.09	0.383 ± 0.006	4.51	1.44 ± 0.02		1.33

[a] 100 μl of enzyme solution 1. [b] 50 μl of enzyme solution 1. [c] 345 mμ, all others 335. [d] ± based on uncertainty of ± 0.003 absorbance unit in π. [e] Concentration at termination of "burst." [f] Calculated using eq 18. [g] Based on the weight of enzyme used and a molecular weight of 27,300. [h] $(V^{obsd} - V^{spont})/[E]_{total}$ (V^{spont} usually < 1% V^{obsd}). [i] pH 7.00 ± 0.05, 3.2% (v/v) acetonitrile, 0.025 M (μ) phosphate buffer, I 0.11 (NaCl).

the k_2/k_3 ratio can also be obtained from the presteady-state reaction using eq 22. Using stopped-flow spectrophotometry, b at pH 7 was found to be 2.7 ± 0.3 sec^{-1} at $[S]_0 = 4.93 \times 10^{-4}$ M. Since it was not possible to observe a saturation in the presteady state, we know that $K_s \gg [S]_0$. Thus eq 22 reduces to $b = ([S]_0/K_s) + k_3$. Using the known values of k_{cat}/K_m(app) $= k_2/K_s = 5.05 \times 10^3$ M^{-1} sec^{-1} and $k_{cat} = k_3 = 1.45 \times 10^{-2}$ sec^{-1}, the above equation is seen to reduce to $b = k_2[S]_0/K_s$. Once again since we know that $K_s \gg [S]_0$, then $k_2 \gg 2.7$ sec^{-1}. On the basis that $k_2 = 2.7 \pm 0.3$ sec^{-1}, the minimal k_2/k_3 ratio $= 186 \pm 20$, barely satisfying the requirement that $k_2 \gg k_3$.

The value of K_m(app) determined from a Lineweaver–Burk plot of the steady-state reaction under the conditions of the titration experiment was 2.87×10^{-6} M. The substrate concentrations ordinarily used in titration experiments are 100-fold greater than this value. The calculated values of the enzyme concentration reported in Table VI have utilized eq 12 with the known K_m(app)/$[S]_0$ ratios for each experiment. The use of this correction usually amounted to a factor of about 3.2% and in no case more than 4.2% in the over-all calculation.

The derivation of eq 12 requires that $[S]_0 \gg [E]_0$. Under the titration conditions $[S]_0/[E]_0$ was usually 10, which does not meet this requirement completely. Since the substrate concentration does change appreciably during the period of the burst, the $[S]_0$ listed in Table VI and used in eq 12 is the concentration of substrate after the burst. Since the k_2/k_3 ratio is so high in this titration, there is no theoretical ambiguity connected with the fact that the $[S]_0/[E]_0$ ratio is not very high.

N-*trans*-Cinnamoylimidazole is certainly not a "natural" substrate for subtilisin. In addition, it is a highly reactive acylating agent and is used in large excess. Therefore it is necessary to determine whether N-*trans*-cinnamoylimidazole reacts with the active site that is operative for other (natural) substrates of subtilisin and with no other groups.

Subtilisin has a broad specificity toward acylamino acid derivatives. When subtilisin is titrated with N-*trans*-cinnamoylimidazole at pH 7, a *trans*-cinnamoyl intermediate is formed.[13] Such a reaction was carried out and immediately afterward the pH of the solution was lowered to pH 4.0 (from 7.0) and the solution was passed through a Sephadex G-25 column. This treatment gave a solution of *trans*-cinnamoyl-subtilisin which had been separated from excess substrate and any hydrolysis products. When an aliquot of this acyl-enzyme is added to a solution containing *p*-nitrophenyl N-benzyloxycarbonyl-L-valinate and the release of *p*-nitrophenol followed, a sigmoid curve reflecting a first-order approach (deacylation of the cinnamoyl-enzyme) to the first-order ester hydrolysis reaction should be observed, if the enzyme is indeed cinnamoylated at the same active site as that responsible for the ester hydrolysis. The rate constant for the approach to the ester hydrolysis turnover should equal k_3, the deacylation rate constant for *trans*-cinnamoyl-subtilisin, according to eq 24. This phenomenon was observed and the rate constant so obtained was $1.52 \pm 0.15 \times 10^{-2}$ sec^{-1}, which agrees within experimental error with the value of k_3 determined directly (1.45×10^{-2} sec^{-1}). Thus the observed "burst" in the reaction of subtilisin with N-*trans*-cinnamoylimidazole represents acylation of the same active site as that involved in the hydrolysis of a substrate containing a naturally occurring amino acid.

In the above discussion, the applicability of eq 1 has been assumed. There is, however, one piece of information which may be interpreted as being inconsistent with eq 1; namely only a questionable saturation was observed in the presteady-state reaction. Thus, the experimental observations on this titration reaction may require that eq 15–17 be utilized. However, the above data also satisfy the requirements of eq 17 for a rigorous titration.

The Use of Rate Assay as Secondary Standard. Only stock solutions of rather high enzyme concentration ($\sim 1 \times 10^{-3}$ M) can be directly titrated by the procedure given above. Since more dilute stock solutions are frequently desired, it is convenient to have a secondary standard that can establish the concentrations in more dilute solutions. The reaction of N-*trans*-cinnamoylimidazole with subtilisin is, in fact, both a titration and a rate assay. The turnover constant k_{cat} ($= k_3$ in this instance since $k_2 \gg k_3$) has been determined under the conditions of the titration by dividing the velocity of the steady-state reaction by the enzyme concentration determined from the burst, as shown in Table VI. Using $k_3 = 1.45 \times 10^{-2}$ sec^{-1}, the enzyme concentration can then be determined from the equation $V_{max} = k_3[E]_0$. If the activity of the enzyme is not affected by dilution, the enzyme concentration calculated from the rate assay should equal the enzyme concentration calculated by dilution of the titrated stock enzyme solution. Such an identity does, in fact, hold over at least a 40-fold range in enzyme concentration from 1.7×10^{-6} to 4.8×10^{-5} M.

[13] See the discussion in the papain section.

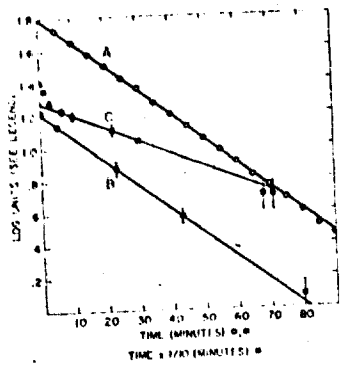

Figure 9. The reaction of diethyl *p*-nitrophenyl phosphate with elastase TSFF 6501 (fraction 29) chromatographed on carboxymethylcellulose and dialyzed against pH 7.8 ± 0.03 phosphate buffer (I = 0.1), $[E]_0 = 4 \times 10^{-5}$ M, $[S]_0 = 2.2 \times 10^{-3}$ M, 3.85% (v/v) acetonitrile–water: A, release of *p*-nitrophenol, ordinate, log ($10^3\Delta A$); B, loss of activity of enzyme toward *p*-nitrophenyl isobutyrate, ordinate log (10^3V_{net}) when 100 μl of phosphate solution was assayed; C, loss of activity of enzyme toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, ordinate, log (10^3V_{net}) when 200 μl of phosphate solution was assayed.

Discussion. Throughout this discussion the assumption has been made that only one enzyme exists in the test solutions. The possibility that more than one enzyme is being titrated seems unlikely since the two enzymes would have to be very similar indeed to give consistent first-order acylation kinetics and first-order deacylation kinetics and produce no effects on dilution. The titration indicates the presence of approximately 57% active enzyme and 43% (w/w) inert material in a typical sample of crystalline subtilisin. The possibility that other enzymatically active components are present in this material has not been excluded. The pronounced tendency of subtilisin to autolyze during purification has been noted by several workers[77] and it is possible that the inert material could consist largely of autodegradation products.

Results for Elastase

The stoichiometric basis of a titration procedure for the determination of the active sites of elastase (pancreatopeptidase E, EC 3.4.47)[3] is predicted on the basis of the stoichiometric inhibition of elastase by diisopropylphosphorofluoridate[78] and the observation of an initial burst of *p*-nitrophenol in the elastase-catalyzed hydrolysis of *p*-nitrophenyl isobutyrate and trimethylacetate (*vide infra*).

Burst Titration. With the other enzymes reported in this series, stoichiometric titrations, corresponding to step k_2 of eq 1, have been observed with specific and/or nonspecific substrates of the enzyme. With elastase, we have not as yet found a carboxylic ester that is satisfactory as a titrant. However, *p*-nitrophenyl diethyl phosphate (Paraoxon), which was previously shown to react with chymotrypsin and trypsin resulting in a completely inhibited enzyme and the liberation of approximately 1 mole of *p*-nitrophenol per mole of enzyme,[66,79] has been used here as a titrant of the active sites of elastase. Elastase reacts with excess diethyl *p*-nitrophenyl phosphate at pH 7.8 in a reaction which is characterized by a first-order liberation of *p*-nitrophenoxide ion (Figure 2). Before the reaction takes place and after the reaction has reached its half-lives, a slow spontaneous zero-order hydrolysis of diethyl *p*-nitrophenyl phosphate is evident, the rates of the spontaneous hydrolysis before and after reaction are identical within the experimental error of [illegible]. That the reaction is essentially irreversible was shown by inhibition of elastase with excess diethyl *p*-nitrophenyl phosphate, separation of the inhibited enzyme from the excess reactant by gel filtration using a Sephadex G-25 column, and observation of the activity of the enzyme toward *p*-nitrophenyl trimethylacetate. At the conclusion of the gel filtration, the enzyme had no activity (less than 1% of the original activity). After 65 hr at pH 7.8, the inhibited enzyme had regained no activity (less than 5%) while a blank, uninhibited enzyme retained 35% of its activity toward *p*-nitrophenyl trimethylacetate. These results indicate that there is probably no "turnover" of diethyl *p*-nitrophenyl phosphate during the course of a titration experiment.

By plotting the logarithm of ΔA, the difference between the absorbance at any time and the absorbance extrapolated from the spontaneous hydrolysis of the phosphate after the reaction is complete (see Figure 2), *vs.* time, the pseudo-first-order rate constant, b, of the reaction may be obtained (Figure 5). In addition, by extrapolating this plot to zero time, a measure of the *p*-nitrophenol liberated in this reaction and thus a measure of the enzyme consumed in the reaction, may be obtained, using eq 18 where A is defined as the extrapolation to time zero of the spontaneous hydrolysis reaction, whereas previously it was defined as the extrapolation of the steady-state turnover.

The *p*-nitrophenol produced from the reaction of diethyl *p*-nitrophenyl phosphate with elastase is proportional to the concentration of enzyme protein present over a tenfold range, while the pseudo-first-order rate constant of the reaction is independent of the enzyme protein concentration over this range. On the other hand, the pseudo-first-order rate constant of the reaction is directly proportional to the phosphate concentration. Both of these results are presented in Table VII.

Table VII. Effect of Enzyme and Substrate Concentrations on the Reaction of Elastase with Diethyl *p*-Nitrophenyl Phosphate[a]

Enzyme stock soln, μl	pH	Phosphate × 10^3, M	π, absorbance	[Enzyme stock] × 10^5, M	k × 10^3, [illegible]
50	7.50	7.48	[illegible]	[illegible]	[illegible]
250	7.74	7.48	[illegible]	[illegible]	[illegible]
200	7.79	7.48	[illegible]	[illegible]	[illegible]
25	7.87	7.48	[illegible]	[illegible]	[illegible]
50	7.92	2.99	[illegible]	[illegible]	[illegible]

[a] 25.0°, 7.6% (v/v) acetonitrile–water, 0.1 [illegible] ionic strength. [illegible] TSFF 5691 dissolved in pH 4.6 acetate buffer, total volume [illegible] ml. [b] Calculated from observed burst of *p*-nitrophenol.

Since the diethyl *p*-nitrophenyl phosphate–elastase reaction is stoichiometric within experimental error, π is a direct measure of the normality of active sites of elastase in the solution. Table VII records the results of a number of such reactions. These experiments

(77) G. Johansen and M. Ottesen, *Compt. Rend. Trav. Lab., Carlsberg*, 34, 199 (1965).
(78) M. A. Naughton and F. Sanger, *Biochem. J.*, 70, 4P (1958).
(79) B. A. Kilby and G. Youatt, *ibid.*, 57, 303 (1954).

Table VIII. The Reaction of Elastase with Diethyl *p*-Nitrophenyl Phosphate[a]

Enzyme	[CH_3CN], % (v/v)	[Phosphate] $\times 10^3$, *M*	pH	π, absb unit	[E] $\times 10^5$,[c] *M*	[E]$_0$(exptl)/[E]$_0$(calcd)	$k \times 10^4$, sec^{-1}
ESFF 6501[b]	3.85	2.2	~7.8[f]	0.65	5.2	0.79	8.78
Crystalline[c]	3.85	2.2	7.77	0.72	5.6	0.83	8.86
ESFF 5691	7.6	7.48	7.80 ± 0.1	0.025[g]	20.2	0.78	12.0
				0.250			

[a] 25.0 ± 0.2°. [b] Purified by carboxymethylcellulose chromatography. The fraction used corresponds to the peak of the main protein absorption of the effluent. [c] Material which had been purified with DEAE-Sephadex A-50 (see Experimental Section) and then subjected to carboxymethylcellulose chromatography. This fraction corresponds to the peak of the main protein absorption of the effluent. [d] [E]$_0$(exptl) is that determined in the reaction with diethyl *p*-nitrophenyl phosphate. [E]$_0$(calcd) is that calculated from the known protein absorbance, optical factor, and the assumption that the equivalent weight of the enzyme is 25,000, which probably has an uncertainty of 10%. [e] Concentration of enzyme stock solution. For the first two entries, this also corresponds to the concentration in the cuvette. [f] pH was not determined at the conclusion of this titration. [g] These titrations include the five experiments of Table VII.

which were carried out with three different enzyme preparations, two different phosphate concentrations, and three different enzyme concentrations) give results which are quite consistent with one another. The purity of the three enzyme preparations ([E]$_0$(exptl)/[E]$_0$(calcd)) (defined in Table VIII) is the same within experimental error (±10% maximum deviation). The pseudo-first-order rate constants for the three reactions are reasonably consistent considering the unknown effects of different acetonitrile concentrations and phosphate concentrations.

The titration of elastase with diethyl *p*-nitrophenyl phosphate follows eq 15–17. The condition that [S]$_0$ ≫ [E]$_0$ is easily met by the design of the experiments. The condition that $k_{obsd} \gg k_3$ is met since k_{obsd} is as high as 1.2×10^{-3} sec^{-1} (Table VII) whereas k_3 must be less than 3×10^{-6} sec^{-1} since the half-life of the attempted dephosphorylation of the inhibited enzyme must be greater than 65 hr.

The Relationship of the Reaction of Elastase with Diethyl *p*-Nitrophenyl Phosphate to Other Reactions of Elastase. The question may be asked as to whether the titration occurred at the active site of the enzyme and only at the active site of the enzyme. To this end, two sets of experiments were carried out. In the first experiments a determination was made of the rate of abolition of enzymatic activity toward different substrates during the phosphate inhibition. Three substrates were used: *p*-nitrophenyl trimethylacetate, isobutyrate, and N-benzyloxycarbonyl-L-tyrosinate. The results of these experiments are shown in Table IX and partially illustrated in Figure 9.

The rate constant of the liberation of *p*-nitrophenol in the reaction of elastase with diethyl *p*-nitrophenyl phosphate is equivalent to the rate constant of the loss of activity of elastase toward *p*-nitrophenyl trimethylacetate and isobutyrate. (Table IX). However, the rate constant of the loss of activity of elastase toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate is 23-fold slower than the rate constant of the titration reaction with elastase ESFF 5691 while the activity loss of elastase ESFF 6501 shows two rate constants, one of which agrees roughly with the rate constant of the titration reaction, and the other of which is 15-fold slower. In contrast, the rate constant of the loss of activity of chymotrypsin and trypsin toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate equals the rate constant of the reaction of these enzymes with diethyl *p*-nitrophenyl phosphate.

Table IX. Kinetics of the Loss of Activity of Elastase during Reaction with Diethyl *p*-Nitrophenyl Phosphate[a]

Enzyme	ESFF 5691[b]	ESFF 6501[c]
k(release of *p*-nitrophenol from diethyl *p*-nitrophenyl phosphate), sec^{-1}	1.22×10^{-3}	5.7×10^{-4}
k(loss of activity toward *p*-nitrophenyl trimethylacetate), sec^{-1}	1.3×10^{-3}[d]	
k(loss of activity toward *p*-nitrophenyl isobutyrate), sec^{-1}		5.5×10^{-4}
k(loss of activity toward N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester), sec^{-1}	5.6×10^{-5}	6.3×10^{-4}[e] 3.67×10^{-5}

[a] 25.0°. [b] 7.6% (v/v) acetonitrile water, 7.48×10^{-3} *M* diethyl *p*-nitrophenyl phosphate, pH 7.8 Tris HCl or phosphate buffer. [c] Fraction 29 of chromatography; 3.85% (v/v) acetonitrile water, 2.2×10^{-3} *M* diethyl *p*-nitrophenyl phosphate, pH 7.8 phosphate buffer (I = 0.1). [d] The actual pseudo-first-order rate constant was 5-fold less using a concentration of diethyl *p*-nitrophenyl phosphate which was 5-fold less. [e] This rate constant is an approximate value based on one point (see Figure 9).

In the second set of experiments, the second-order rate constant for the elastase-catalyzed hydrolysis of a considerable number of substrates of elastase was determined, using two enzyme preparations which had been purified in entirely different ways. Values of $k_{cat}[E]_0/K_m$(app) were determined either as observed pseudo-first-order rate constants or from Lineweaver–Burk plots. Since [E]$_0$ is known by titration, k_{cat}/K_m(app) is readily evaluated. As seen in Table X, the k_{cat}/K_m(app)'s determined for a given substrate with the two enzyme preparations were quite similar except for *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate. This result indicates that the titration of the active sites of elastase with diethyl *p*-nitrophenyl phosphate gives a consistent measure of the activity of the enzyme toward seven substrates of elastase, including both acylamino acid and carboxylic acid derivatives.

The Use of a Rate Assay as a Secondary Standard. Any of the reactions shown in Table X, which have been shown to give concordant results of k_{cat} or k_{cat}/K_m(app) with two different enzyme preparations (with the exception of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate), can be used as a rate assay to determine enzyme concentration. Using the equations $V_{max} = k_{cat}[E]_0$ or $k_{obsd} = (k_{cat}/K_m)[E]_0$ and the values of k_{cat} or k_{cat}/K_m(app) for a particular substrate under the

(30) U. J. Lewis, D. E. Williams and N. G. Brink, *J. Biol. Chem.*, 222, 705 (1956); Naughton and Sanger[28] report 28,500.

Table X. The Kinetics of Some Elastase-Catalyzed Reactions Using Two Elastase Preparations[a]

p-Nitrophenyl ester	pH	$[E]_0 \times 10^6$, *M*	$[S]_0 \times 10^5$, *M*	Kinetics	k_{cat}, K_m, k_{cat}/K_m			
					M			
Trimethylacetate	7.33	5	5.5	MM[b]	0.[illegible][c]	[illegible]	[illegible]	[illegible]
Acetate	7.43	7.0	16	MM[b]	0.[illegible]	[illegible]	[illegible]	[illegible]
Isobutyrate	7.68	0.7–2.6	0.7–15	MM[b]	[illegible][c]	[illegible]	[illegible]	[illegible]
Fourate	7.69	0.5–2.6	0.1–2.0	MM[b]	[illegible]	[illegible]	[illegible]	[illegible]
N-Benzyloxycarbonyl-L-isoleucinate	7.70	0.5–2.9	0.53	First order	0.[illegible]	[illegible]		
N-Benzyloxycarbonyl-L-leucinate	7.79	0.1–0.5	0.5	First order[e]	28.[illegible]	[illegible]		
N-Benzyloxycarbonyl-glycinate	7.85	9.6	1.0	First order	15.[illegible]	[illegible]		
N-Benzyloxycarbonyl-L-tyrosinate	7.79	0.53–5.6	0.95	First order	10.[illegible]	[illegible]		

[a] 25.0 ± 0.2°, phosphate buffer, $I = 0.05$. [b] Average of two or more determinations. [illegible] purified elastase ESFF 6507B. [d] Enzyme 2 (JRW-2) was a Worthington crystalline elastase purified [illegible] cellulose at pH 8.0 (see Experimental Section). [e] pH 7.52. [f] pH 7.72. [g] pH 7.39. [h] pH 7.26. [i] M[illegible]

conditions of reaction shown in Table X, $[E]_0$ may be calculated directly from V_{max} or k_{obsd}.

Discussion. Elastase appears to contain one active site per mole since the molecular weight determined by sedimentation (25,000)[80] agrees roughly with the equivalent weight determined by diisopropylphosphorofluoridate inactivation (28,500).[46] On this basis, titrations of both the electrophoretically purified and chromatographically purified elastases discussed here with diethyl *p*-nitrophenyl phosphate indicate that 62–80% of the protein in these solutions is active enzyme. The rate of loss of activity of the enzyme toward *p*-nitrophenyl trimethylacetate and isobutyrate (and N-benzyloxycarbonylglycinate) is essentially the same as the rate of the titration reaction, indicating a common active site for all processes. Furthermore, the second-order rate constants for seven substrates of elastase are essentially the same for two different elastase preparations, using the titrated value of the enzyme concentration, again indicating that the titration gives a consistent measure of the elastase active sites. Finally, one of these enzymes (ESFF 6507A has been shown to have high elastolytic activity.[81] Thus, it appears that the titration occurs at the active site of the enzyme.

However, with the substrate *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, the two tests indicated above gave inconsistent results. Chromatography of several elastase samples reveals that whereas the *p*-nitrophenyl isobutyrate activity parallels the ultraviolet protein absorption in the effluent, the *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate activity has a peak several fractions prior to (and overlapping with) the main protein peak. Since no protein peak is detected at the maximum tyrosine activity, it may be concluded that an extraneous enzyme, highly active toward tyrosine ester, is present in low concentration and is imperfectly separated from elastase under the purification conditions. The actual amount of this extraneous enzyme is unknown, although it is probably present to an extent less than 1–7%, and the actual amount may be much lower. An impurity has been identified by electrophoresis in the present preparation as [illegible] mucase,[83] which is known to have [illegible] toward tyrosine esters.[84] Another impurity [illegible] unusually high activity toward tyrosine substrates [illegible] been found in elastase and [illegible] elastase.[illegible] It is inactivated by diisopropylphosphorofluoridate. While these other enzymes would presumably react with diethyl *p*-nitrophenyl phosphate, the titration of elastase is still valid when the other enzymes are present in [illegible] amounts.

Results for Acetylcholinesterase

The enzyme acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) serves physiologically as a catalyst for the hydrolysis of acetylcholine. However, acetylcholinesterase is similar to the proteolytic enzymes in this series in that its kinetic pathway also follows eq 1.[85]

The following evidence supports this pathway. The enzyme is inhibited by certain organophosphorus compounds (for example, tetraethyl pyrophosphate and diisopropylphosphorofluoridate) by forming a phosphoryl enzyme.[87] Like the other enzymes of this series, the phosphoryl moiety is isolated on a serine hydroxyl group after the degradation of the protein. The phosphoryl enzyme can be reactivated by nucleophilic compounds.[88] Dimethylcarbamoylcholine chloride also inhibit the enzyme.[89] The enzyme is inhibited by two carbamates to zero activity in water at the same rate and is reactivated by hydroxylamine again at the same rate.[90] These results indicate the formation of acyl enzyme in the enzyme-catalyzed hydrolysis of these substrates. Finally, nonsingular inhibition[15] and pH dependence studies [illegible] esters are consistent with the three-step kinetic [illegible]

(81) Personal communication from Dr. W. A. Loeven, The Netherlands Institute for Preventive Medicine, Lieden, The Netherlands. The activity corresponded to 3.4 eu/mg of protein[82] when tested against alkali-treated elastin of pH 8.7. This enzyme is free of the α_2 globulin fraction which contains elastase inhibitor.

(82) W. A. Loeven, *Acta Physiol. Pharmacol. Neerl.*, **12**, 57 (1963).

(83) Dr. W. A. Loeven, [illegible]

(84) S. Ayrmcas and J. [illegible] **4**, [illegible]

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(86) K.-B. Augustinsson and [illegible] *Acta*, **129**, [illegible]

(87) H. O. Michel and [illegible] **120**, [illegible] (1951).

(88) F. Sanger, *Proc. Chem. Soc.*, [illegible] (1958).

(89) I. B. Wilson, *J. Biol. Chem.*, **190**, 111 (1951); I. B. Wilson, A. Ginsburg, and C. Quan, *Arch. Biochem. Biophys.*, **77**, [illegible] references therein.

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(91) I. B. Wilson and J. [illegible] *Acta*, **237**, 1123 (1962); R. [illegible] Krupka, *Biochemistry*, **3**, [illegible]

(92) R. M. Krupka, *Biochem. Biophys. Res. Commun.*, **19**, [illegible]

Table XI. The Titration of Acetylcholinesterase Solutions with *o*-Nitrophenyl Dimethylcarbamate (Numbers 1–4 and 6–8) and *p*-Nitrophenyl Dimethylcarbamate (Number 5)[b,c]

No.	[Titrant] × 10^3, *M*	pH	Dilution factor[a]	$\pi = [E]_0 \times 10^8$[b]	$V \times 10^{10}$, *M* sec^{-1}[b]	$k_3 \times 10^4$,[e] sec^{-1}	Buffer soln[d]
1[f]	6.64	8.20	3.750	2.0 ± 0.1	9.2 ± 0.28	4.8 ± 0.4	0.0525 *M* Tris,[g] 0.021 *M* HCl, 0.16 *M* NaCl, 1.2% (v/v) acetonitrile–water
2[f]	6.87	8.20	4.833	1.5 ± 0.1	6.8 ± 0.14	4.6 ± 0.4	0.0560 *M* Tris,[g] 0.025 *M* HCl, 0.15 *M* NaCl, 1.2% (v/v) acetonitrile–water
3[m]	10.03	7.73	3.750	3.6 ± 0.15	19.9 ± 0.6	5.5 ± 0.4	4.22×10^{-2} *M* KH_2PO_4, 1.51×10^{-2} *M* Na_2HPO_4, 5.33×10^{-2} *M* NaCl, 1.2% (v/v) acetonitrile–water
4[m,h]	5.02	7.72	3.750	3.6 ± 0.15	22.5 ± 0.3	6.2 ± 0.3	4.22×10^{-2} *M* KH_2PO_4, 1.51×10^{-2} *M* Na_2HPO_4, 5.33×10^{-2} *M* NaCl, 1.2% (v/v) acetonitrile–water
5[m,h]	10.33	7.78	7.500	1.8 ± 0.08[j]	5.15 ± 0.13	[g]	5.02×10^{-2} *M* KH_2PO_4, 5.37×10^{-2} *M* Na_2HPO_4, 2.67×10^{-2} *M* NaCl, 1.2% (v/v) acetonitrile–water
6[m]	5.94	7.70	1.100	7.45 ± 0.13	48.9 ± 0.4	6.57 ± 0.17	6.78×10^{-2} *M* Na_2HPO_4, 6.41×10^{-2} *M* KH_2PO_4
7[n]	6.01	9.00	1.375	3.46 ± 0.09	21.0 ± 0.2	6.08 ± 0.2	6.19×10^{-2} *M* Na_2CO_3, 3.18×10^{-2} *M* $NaHCO_3$, 0.3 *M* NaCl
8[n]	5.89	7.01	1.100	1.4 ± 0.4	6.77 ± 0.07	3.9 ± 0.2[k]	3.14×10^{-2} *M* KH_2PO_4 and 5.28×10^{-2} *M* Na_2HPO_4

[a] The number of times the enzyme was diluted in the titration. [b] The uncertainty has been estimated from the uncertainty in the absorbance values. [c] Calculated from the expression, $V = k_3[E]_0$. The uncertainty for these values represents the sum of the uncertainty estimated for V and $[E]_0$. [d] Buffers solutions were at ionic strength 0.2 except for no. 7 which was 0.35. [e] Tris(hydroxymethyl)aminomethane. [f] Enzyme concentration was calculated from concentration determined in no. 3 and 4. [g] It was not possible to calculate a value using the expression $V = k_3[E]_0$ since [S] was not much larger than K_m and k_2 is not much larger than k_3 (see later). [h] The uncertainty in this titration was large so that the concentration determined by rate assay 8 (1.74×10^{-8} *N*) was used in the calculation of k_3. The uncertainty given here is the sum of the uncertainties in V_m for rate assay 8, k_{cat} of 1.76×10^4 sec^{-1}, and the k_3 in column six. [i] *o*-Nitrophenoxide ion was followed at 415 mμ and *p*-nitrophenoxide ion was followed at 400 mμ. [j] Dust introduced into titrations 4 and 5 for at least 83 min produced the large uncertainty in these experiments. [k] A linear steady-state reaction was found in titrations 4 and 5 for at least 83 min. [l] Enzyme solution dialyzed and concentrated as described in the Experimental Section. [m] Enzyme solution dialyzed as described in the Experimental Section. [n] Enzyme solution dialyzed and filtered as described in the Experimental Section. [o] More details on the treatment of enzyme solutions may be found in ref 56.

involving the formation of an acyl-enzyme intermediate in the reaction pathway.

Three titrations of acetylcholinesterase have been made on the basis of the stoichiometry of eq 1. The enzyme has been phosphorylated with ^{32}P-labeled diisopropylphosphorofluoridate leading to a labeled enzyme from which the concentration of active sites could be calculated.[87] Similar titrations were carried out using diethoxyphosphorylthiolcholine and tetraethyl pyrophosphate.[93] In both these instances possible nonspecific phosphorylation was taken into account. A different approach utilized dimethylcarbamyl fluoride substrate. The ratio of the activity of the carbamylated enzyme solution to the original activity of the enzyme was followed nearly to completion of the carbamylation and decarbamylation reactions; from this determination and the decarbamylation rate constant, the normality of the enzyme solution could be evaluated.[94]

Here we report the direct spectrophotometric observation of a stoichiometric reaction in the eel acetylcholinesterase-catalyzed hydrolysis of a carboxylic ester and its use in the development of a procedure for the determination of a concentration (normality) of the active sites of this enzyme. *o*-Nitrophenyl dimethylcarbamate was used as the substrate.[95,96]

Titrations. The titrations of eel cholinesterase by *o*-nitrophenyl and *p*-nitrophenyl dimethylcarbamate are shown in Table XI. All the titrations except no. 5 are characterized by an initial rapid release of *o*-nitrophenol followed by a slow steady-state release of the phenol. The initial release of nitrophenol was usually of the order of 20×10^{-4} absorbance unit. In titration 6 shown in Figure 3 the initial release of nitrophenol was considerably greater than this value; in titrations 1, 2, 5, and 8 it was considerably less.

For titrations 1–4, k_2 was determined by using *p*-nitrophenyl acetate to assay the enzyme-catalyzed hydrolysis of *o*-nitrophenyl dimethylcarbamate under conditions similar to those in the titrations.[97] The k_2 was 0.5–0.7 sec^{-1} at pH 7.78 in 1.6% (v/v) acetonitrile–water, KH_2PO_4–Na_2HPO_4 buffer at I = 0.2. Values of k_3 determined in titrations 1–4 are in the range 4.6–6.2 × 10^{-4} sec^{-1} (Table XI). Thus for titrations 1–4, k_2/k_3 is 800–1500. For titrations 6–8 carried out in the absence of acetonitrile, the value of k_2 has not been determined directly. However, the carbamylation rate constant, b, was determined for titration 6 (Figure 3) by plotting log ΔA *vs.* time for the presteady-state reaction. The rate constant, b, is given by eq 22. From eq 22, the value of k_2 can be approximated using the experimental

(95) A preliminary communication of this work has been given: M. L. Bender and J. K. Stoops, *J. Am. Chem. Soc.*, 83, 1622 (1961).
(96) J. K. Stoops and M. L. Bender, to be published.

Table XII. The Values of K_m[a] for the Acetylcholinesterase-Catalyzed Hydrolysis of *o*-Nitrophenyl Dimethylcarbamate and a Comparison of the Values of k_3 for the Deacylation of the Dimethylcarbamyl-Enzyme Determined by the Rate Assay Method[b] with the Values Determined by the Titrations

From the rate assay method			From the titrations in Table XI		
pH	$k_3 \times 10^4$,[c] sec^{-1}	K_m(app) $\times 10^4$, *M*	$k_3 \times 10^4$, sec^{-1}	pH	Buffer soln[d]
7.85	6.7 ± 0.3		6.2 ± 0.3	7.72	5.92 × 10⁻³ *M* KH_2PO_4, 6.24 × 10⁻³ *M* Na_2HPO_4, 1.2%, (v/v) acetonitrile–water
7.98	7.3–7.6		4.8 ± 0.4	8.20	3.88 × 10⁻³ *M* KH_2PO_4, 6.56 × 10⁻³ *M* Na_2HPO_4
9.24	6.0 ± 0.1		6.1 ± 0.2	9.00	1.23 × 10⁻³ *M* $NaHCO_3$, 2.36 × 10⁻³ *M* Na_2CO_3, 1.7%, (v/v) acetonitrile–water
7.03	3.5 ± 0.0		3.9 ± 0.2	7.01	3.21 × 10⁻³ *M* KH_2PO_4, 5.42 × 10⁻³ *M* Na_2HPO_4, 1.2%, (v/v) acetonitrile–water
7.00	4.3,[e] 3.2[f]				0.1 *M* NaCl, 0.01 *M* $MgCl_2$, 0.02 *M* sodium phosphate[e]
7.04		2.9 ± 0.1			3.51 × 10⁻³ *M* KH_2PO_4, 5.55 × 10⁻³ *M* Na_2HPO_4
7.82		5.4 ± 0.2			5.46 × 10⁻³ *M* KH_2PO_4, 5.52 × 10⁻³ *M* Na_2HPO_4
7.82		3.0 ± 0.1			5.46 × 10⁻³ *M* KH_2PO_4, 5.52 × 10⁻³ *M* Na_2HPO_4, 1.7%, (v/v) acetonitrile–water
9.16		4.5 ± 0.1			3.17 × 10⁻³ *M* $NaHCO_3$, 6.13 × 10⁻³ *M* Na_2CO_3, 1.50 × 10⁻³ *M* NaCl

[a] *o*-Nitroacetanilide was used to determine the K_m for the enzyme-catalyzed hydrolysis of *o*-nitrophenyl dimethylcarbamate.[99] [b] *o*-Nitroacetanilide was used to assay the enzyme-catalyzed hydrolysis of *o*-nitrophenyl dimethyl carbamate.[96] [c] Reproducibility of two or more determinations is given after these values. [d] Buffer solutions were at ionic strength 0.2. These buffer solutions pertain to the rate assays. [e] See ref 99. [f] I. B. Wilson, M. A. Harrison, and S. Ginsburg, *J. Biol. Chem.*, 236, 1498 (1961).

value of *b*, a value of 2×10^{-3} *M* for K_s,[96] and 6.6×10^{-4} sec^{-1} for k_3 (Table XI). This calculation yields $k_2 \sim 0.3$ sec^{-1} and thus $k_2/k_3 \sim 400$. It is of interest that the value of k_2 in the absence of acetonitrile is approximately one-half the value in the presence of 1.7% (v/v) acetonitrile, indicating a peculiar solvent effect. In conclusion, for all titrations using *o*-nitrophenyl dimethylcarbamate, $k_2/k_3 \geqq 400$.

The K_m(app) for the acetylcholinesterase-catalyzed hydrolysis of *o*-nitrophenyl dimethylcarbamate, determined under conditions similar to the titrations, are listed in Table XII. By comparing the titrant concentrations in Table XI and the K_m(app)'s of Table XII it is seen that the $[S]_0/K_m$(app) ratio was greater than 100 for all titrations.

As seen in Table XI, the ratio $[S]_0/[E]_0 > 100$ for all the titrations and therefore the requirement that $[S]_0 < [E]_0$ is satisfied.

In the steady-state turnover of the titration experiments, $k_2 \gg k_3$ and $[S]_0 \gg K_m$(app) (except for titration 5). Thus $V = k_3[E]_0$. The values of k_3 so determined are shown in Tables XI and XII.

The enzyme preparations used in these titrations consisted of approximately 7% active enzyme based on a comparison of the activity per milligram of protein determined by Kremzner and Wilson[97] on the purest preparation to date and the activity per milligram of protein of the present lot 23B-7587.[98] Thus 7% is an upper limit. The corrections made in this comparison[98] are all small and therefore the value of 7% is probably correct within ±3%. Since the enzyme preparations consist of approximately 93% inactive protein,[99] it is important to demonstrate that the titrant does not react with this protein. Even if the enzyme preparations were 100% pure, the reliability of the titration depends on the demonstration that the carbamate reacts only at the active site. One argument against reaction by an impurity is the pure first-order nature of the carbamylation reaction. Furthermore, if the titrant were to react unspecifically with the enzyme or protein, k_3 determined from the titration would be lower than k_3 determined by the rate assay. The comparison shown in Table XII indicates that all values of k_3 except those determined in titrations 1–3 are within 10% of each other, which is within experimental error. The low value of k_3 in titrations 1–3 are probably due to the incursion of substrate inhibition (titration 1) or noncompetitive inhibition by the Tris buffer (titrations 1 and 2). Since the values of k_{cat}(lim) determined for phenyl acetate in all these titrations are in good agreement (Table XIII), the low values calculated in titrations 1–3 cannot be the result of some nonspecific reaction of the titrant.

An unlikely possibility exists that the titrant reacts unspecifically and decarbamylates at approximately

(97) L. T. Kremzner and I. B. Wilson, *J. Biol. Chem.*, 238, 1714 (1963).

(98) The activity reported by Kremzner and Wilson was multiplied by 1.124[91] to correct for substrate inhibition and was multiplied by 1.25 to calculate a pH-independent activity.[92] The corrected activity is 0.26 mmole of acetylcholine hydrolyzed per second per milligram of protein. The activity of lot 23B-7587, sample 1, determined at pH 7.62 with phenyl acetate was multiplied by 1.05 to calculate the pH-independent value, and multiplied by 0.90 to account for the difference between phenyl acetate and acetylcholine activities.[92]

(99) This inactive protein apparently has no effect on the kinetics of the acetylcholinesterase-catalyzed hydrolysis of substrates. The value of k_3 for the decarbamylation of the dimethylcarbamyl enzyme is constant in the range of 10^{-4} *M* (by titration) and 10^{-6} *M* (by rate assay) (Table XII) is independent of the protein concentration. Furthermore, the k_{cat} and K_m(app) for the enzyme-catalyzed hydrolysis of phenyl acetate (Table XIII) are also independent of the protein concentration.

Table XIII. Kinetics of the Acetylcholinesterase-Catalyzed Hydrolysis of Phenyl Acetate

Rate assay[a]	$V_m \times 10^7$,[a] M sec^{-1}	$[E]_0 \times 10^8$,[b] before dilution	Dilution factor $\times 10^{-8}$	$[E]_0 \times 10^{11}$,[c] after dilution	$k_{cat} \times 10^{-4}$,[d] sec^{-1}	K_m(app) $\times 10^3$ M	pH	k_{cat}(lim)[e] $\times 10^{-4}$ (sec^{-1})
1,2	10.0 ± 0.3	7.4[f]	122	6.07 ± 0.3	1.65 ± 0.14	1.9	7.62	1.7[g] ± 0.18
3,5	9.0 ± 0.2	13.5	244	5.53 ± 0.23	1.63 ± 0.10	1.8	7.82	1.71 ± 0.11
6	140 ± 2	8.21	10.32	79.4 ± 1.4	1.76 ± 0.06	2.1	8.57	1.76 ± 0.06
7	168 ± 2	4.76	5.16	92.2 ± 2.4	1.82 ± 0.07	2.2	8.52	1.82 ± 0.02
8	65.1 ± 1	1.91[g]	5.16	37.0 ± 0.18[h]		1.9	8.52	

[a] Determined from the intercepts of Eadie plots; uncertainty was estimated from the intercepts. [b] These values were calculated by multiplying the normality values in Table XI by the number of times the enzyme was diluted in the titrations. [c] The enzyme solution was diluted as shown in the preceding column for assay. This column then is the value in column three divided by the corresponding value in column four. The uncertainty in these values represents the uncertainty in the titrations, column five, Table XI. [d] Calculated using the expression $k_{cat} = V_m/[E]_0$ and the values in columns two and five. The uncertainty given here is the sum of the uncertainty of the values in columns two and five. [e] Calculated using $k_{cat} = k_{cat}(\lim)/(1 + (H^+/K_a))$, the values in column six and eight, and a pK_a of 6.5.[66] The uncertainty given here is the uncertainty in the values in column six. [f] This value is the average value in titration one and two (Table XI). [g] This value was calculated from the expression $[E]_0 = V_m/k_{cat}$ and the value in column two and a value of 1.76×10^4 sec^{-1} for k_{cat}. [h] See footnote g. The uncertainty given here is the sum of the uncertainty in k_{cat}(lim) for rate assay 6 and the V_m in rate assay 8. [i] In rate assays 1–5, the phenyl acetate was introduced in acetonitrile while in rate assays 6–8, it was dissolved in aqueous solution (see Experimental Section).

the same rate as it does at the active site, thus perturbing both V_m and $[E]_0$ in the same way and leading to an unperturbed k_3. Using a different titrant and a different titration method, Wilson and Harrison[94] were able to determine the k_{cat} for the acetylcholinesterase-catalyzed hydrolysis of acetylcholine which when extrapolated to the pH-independent value (k_{cat}(lim)) is 7×10^4 sec^{-1}.[100] This value is within experimental error of the values reported for k_{cat}(lim) for phenyl acetate, using the titration of the enzyme with *o*-nitrophenyl dimethylcarbamate and the phenyl acetate rate assay, and is another check on the specificity of this titrant.

In conclusion, the titration of the active sites of bovine acetylcholinesterase by *o*-nitrophenyl dimethylcarbamate meets all the criteria for a valid titration.

The results using *p*-nitrophenyl dimethylcarbamate as titrant were disappointing. Although the spectral properties of this reaction are favorable, the reaction has a low k_2/k_3 ratio (approximately 5) and a high K_m(app) (4×10^{-4} M).[96] Although it is still possible to utilize this substrate as a titrant under these conditions, the composite uncertainty which would be introduced in the calculation of $[E]_0$ using the reduction in the size of the burst ($\pi < [E]_0$, eq 12) and the very long pre-steady-state reaction of ~50 min indicate that *p*-nitrophenyl dimethylcarbamate is not a suitable titrant for acetylcholinesterase.

The titration with *o*-nitrophenyl dimethylcarbamate can be carried out from approximately pH 5 to 10, spanning the entire range where the enzyme is stable.[101] The lower limit of enzyme concentration usable in this titration is about 10^{-6} M, where the uncertainty would be about 20% if the titration was carried out above pH 8 in order to obtain the maximal spectral change.

The hydrolyses of *o*-nitrophenyl and *p*-nitrophenyl dimethylcarbamates are not catalyzed by chymotrypsin, subtilisin, trypsin, elastase, or papain, using a 10^{-5} M enzyme solution at pH 7–8 for 15–30 min. On the other hand, pig liver[102] and ox liver[103] carboxylesterases catalyze the hydrolyses of *o*- and *p*-nitrophenyl dimethylcarbamates. In fact, titrations of these enzymes using these substrates have been developed.[102,103]

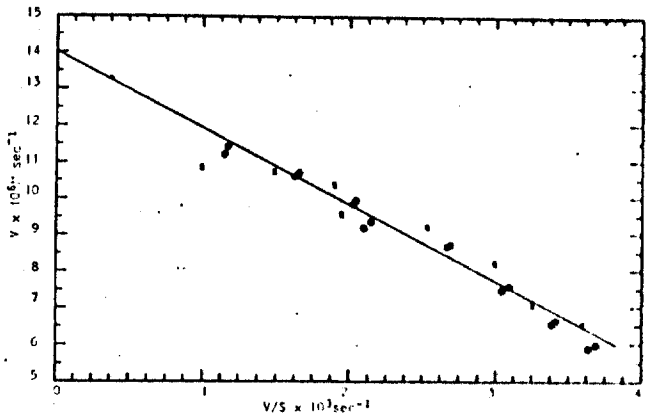

Figure 10. Eadie plots for the acetylcholinesterase-catalyzed hydrolysis of phenyl acetate for rate assay 6: x, first determination of the initial rates, ●, second determinations carried out 1 week later.

Rate Assay Using Phenyl Acetate. A spectrophotometric rate assay has been developed for determining enzyme concentration down to at least 5.5×10^{-11} M, using a value of k_{cat} based on the titration reported here. An aliquot of enzyme, which had been previously titrated, was diluted and the initial rate of the enzyme-catalyzed hydrolysis of phenyl acetate was determined over the range of concentration of the ester. These data were plotted in the form of Eadie plots in order to determine V_m, the maximal velocity. An example is given in Figure 10. The rate assay was carried out for every titrated enzyme sample. The acetylcholinesterase-catalyzed hydrolysis of phenyl acetate follows Briggs–Haldane kinetics up to 10^{-2} M where substrate inhibition becomes apparent. Thus $k_{cat} = V_m/[E]_0$. Using this relationship and the values for $[E]_0$ and V_m, k_{cat} can be calculated, as shown in Table XIII. The values of k_{cat}(lim) calculated from the various titrations agree well with those calculated from their corresponding rate assays, the greatest difference being about 6%. The rate assays were determined on enzyme solutions

(100) At pH 7.0, a k_{cat} of 1.4×10^4 sec^{-1} was reported;[94] k_{cat}(lim) calculated using the equation in footnote *e* of Table XIII, this value and a pK_a of 6.4.[93]

(101) The k_{on} of *o*-nitrophenyl dimethylcarbamate is quite low (8.7 M sec^{-1} at 25.0°), leading to negligible spontaneous hydrolysis in experiments.

(102) D. J. Horgan, E. C. Webb, and B. Zerner, *Biochem. Biophys. Res. Commun.*, 23, 18 (1966).

(103) M. Runnegar and B. Zerner, personal communication.

from 5.5×10^{-11} N to 9.2×10^{-10} N, and on enzyme solutions which had been diluted from 5160 to 244,000 times from solutions used in the titrations. The agreement among the k_{cat}(lim) values indicates that the enzyme can be diluted quantitatively to this low concentration range, and indicates that in this range any possible association-dissociation phenomena of the enzymes do not affect k_{cat}(lim).

Since k_{cat}(lim) for the acetylcholinesterase-catalyzed hydrolysis of phenyl acetate is found to be 1.76×10^4 sec^{-1}, the concentration of an enzyme solution can be determined by this rate assay, using the relationship, $[E]_0 = V_m/k_{cat}$(lim). The rate assay may be carried out over the pH range 6.4–9.0 where the pH dependency of k_{cat} is given by the equation in footnote *e* of Table XIII. Rate assays have been reported for the acetylcholinesterase-catalyzed hydrolysis of acetylcholine, leading to a k_{cat} of 1.4×10^4 sec^{-1} at pH 7, 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.02 M sodium phosphate at 25.0°,[94] and a k_{cat} of 1.2×10^4 sec^{-1} at pH 7.4, 38°, 3.4×10^{-2} M $MgCl_2$, 2.5×10^{-2} M $NaHCO_3$, and 1.5×10^{-2} M substrate.[87] Since the acetylcholinesterase-catalyzed hydrolysis of acetylcholine and of phenyl acetate are reported to give k_{cat}'s within 10% of one another,[92] they may be compared. The first of the acetylcholine rate constants gives a k_{cat}(lim) of 1.7×10^4 sec^{-1}, which is in good agreement with the rate constant of phenyl acetate reported here.

Discussion

Titrations. The titrations described above are based on the mechanistic pathways of eq 1 or eq 15. If these equations do not describe the titration reactions completely, for example, if another intermediate exists in finite amount, then, of course, the titration is no longer quantitatively valid. However, all the burst titrations described here may be quantitatively described by eq. 1 or 15, and are considered to be valid. Furthermore, the titrations meet the criteria for a rigorous determination of the concentration of active sites in terms of eq 12 and 17. These criteria require the determination of k_2 or k_{obsd}, k_3, and K_m(app), as well as the knowledge of $[S]_0$ and $[E]_0$. The former constants have been determined in a variety of ways. The constant k_2 was determined by: (1) measurement of the rate of loss of enzyme activity by an external assay (acetylcholinesterase); (2) the determination of b and from it an estimate of k_2 according to eq 22 (acetylcholinesterase and subtilisin); (3) an analysis of the presteady state at different substrate concentrations (chymotrypsin);[19,25] (4) an analysis of both the presteady state and steady state at different substrate concentrations (trypsin).[11] The constant k_3 was ordinarily determined from the steady-state reaction (k_{cat}), knowing that $k_2 \gg k_3$ and thus that $k_{cat} = k_3$ (acetylcholinesterase, elastase, subtilisin, and chymotrypsin).[19,25] For trypsin, a complete analysis of both the presteady state and steady state was used.[11] The constant b was determined directly. For papain, the k_2/k_3 ratio was considered directly by: (1) the determination of b and the use of eq 23, (2) the absolute magnitude of K_m(app), (3) the effect of methanol on the kinetics, and (4) the agreement of burst from two titrants of different k_2/k_3 ratio.

The titrations of α-chymotrypsin, trypsin, and pa[illegible] utilize specific substrates of these enzymes, while [illegible] titrations of elastase, subtilisin, and acetylcholinest[illegible] do not. However, the titrations of each of the [illegible] enzymes has been shown to occur at the active site [illegible] the enzyme, as defined by a specific substrate. [illegible] titrations of α-chymotrypsin and tryspin appear to [illegible] independent of both titrant, over a wide range of spe[illegible] ficity, and of pH of the titration. The titration [illegible] papain gives identical results with two substrat[illegible] The titration of elastase gives the same results w[illegible] *p*-nitrophenyl trimethylacetate[104] as with the dieth[illegible] *p*-nitrophenylphosphate described here. All of th[illegible] identities indicate the determination of a fundamen[illegible] quantity, namely the intact active site.

The optimal titrant should have the following ch[illegible] acteristics: (1) be a specific substrate, (2) give a titrat[illegible] in a few minutes in order to obviate denaturation, a[illegible] for convenience, (3) give a titration around neutral[illegible] in order to reproduce best physiological conditions, (4) be a stable, available, and soluble reagent whose st[illegible] chiometric reaction is easily detectable, and (5) give [illegible] titration over a wide range of enzyme concentrat[illegible] Not one of the titrants described fulfills all of the re[illegible] quirements listed above. However, each fits the[illegible] requirements most closely for a given enzyme.

Impurities. It is the thesis of this paper that t[illegible] concentration of active enzyme may be determined by [illegible] titration procedure in the presence of impurities a[illegible] furthermore that once this concentration is known [illegible] may be used for subsequent kinetic studies where [illegible] value is needed. The presence of impurities in [illegible] enzyme preparation could affect the titration procedu[illegible] by interfering with the active site and/or the substra[illegible] Impurities which reversibly block the active site wo[illegible] not be expected to influence a titration since it is [illegible] dependent of such problems. A competitive inhibit[illegible] such as a peptide impurity may raise the K_m(app) t[illegible] some apparent higher value, K_m(app)′, but it is lik[illegible] under such a perturbation that $[S]_0$ will still be mu[illegible] greater than K_m(app)′ for many titrants, and thus t[illegible] titration will remain stoichiometric. Since the pre[illegible] ence of a considerable amount of competitive inhibit[illegible] would not be expected to affect a titration, the pos[illegible] bility exists that it may lie undetected in the titrat[illegible] and later may affect the kinetics. However, if [illegible] enzyme is used in the determination of the kinetics of [illegible] specific substrate, the concentration of enzyme, a[illegible] thus the concentration of any competitive inhibit[illegible] associated with it, will be at such a low level that t[illegible] latter will probably be in too low concentration t[illegible] affect the former. Irreversible inhibitors will, of [illegible] affect both the titration and the kinetics in the [illegible] way. Finally, an impurity which is finitely but [illegible] reversible may complicate both the titration and t[illegible] kinetics.

In the titrations reported above, the enzyme pu[illegible] has varied from a low of 7% with acetylcholinest[illegible] to a high of 83% with porcine trypsin.[105] Even Worthington three-times-crystallized α-chymotrypsin titra[illegible]

(104) T. H. Marshall, Ph.D. Diss., Northwestern University, [illegible]

(105) The percentages of purity listed in Table [illegible] are [illegible] a different manner. The titrations [illegible] of sites per milligram of protein, which also [illegible] chemist (and the authors) may prefer the former, [illegible] latter. The two ways of expressing purity are in fact identical.

Table XIV. α-Chymotrypsin- and Trypsin-Catalyzed Hydrolysis of Specific Substrates

Enzyme	Batch no.	Substrate	% purity by wt	$[E]_0$ soln, M	$[S]_0$ soln, M	k_{cat}, sec^{-1}	K_m(app), M[c]
Chymotrypsin	CDI-6114-5 untreated	Methyl N-acetyl-L-tryptophanate	87.1[a]	1.411×10^{-7}[b]	2.011×10^{-3}	30.1[d]	13.0×10^{-3}[e]
	CDI-6114-5 Sephadexed through G-25 (fine)[f]	Methyl N-acetyl-L-tryptophanate	86.6[a]	2.117×10^{-7}[b]	2.011×10^{-3}	32.0[d]	9.3×10^{-3}[e]
	CDI-6110-1 untreated	Methyl N-acetyl-L-tryptophanate	66.1[a]	0.9869×10^{-7}[b]	2.011×10^{-3}	40.5[d]	13.8×10^{-3}[e]
Trypsin	TRL-6261 untreated	Ethyl α-N-benzoyl-L-argininate	57[f]	2.504×10^{-7}[g]	1.623×10^{-3}	16.2[h]	1.2×10^{-3}[h]
Trypsin	TRL-6261 Sephadexed using G-50	Ethyl α-N-benzoyl-L-argininate	77[f]	1.65×10^{-7}[g]	1.584×10^{-3}	13.2[h]	1.06×10^{-3}[h]

[a] Assuming mol wt 24,800. [b] Determined by an N-*trans*-cinnamoylimidazole titration using $\Delta\epsilon$ 8950 at 335 mμ and $[E]_0 = 2[I]_0$. [c] pH 8.33, Tris buffer, 0.05 M, 3.16% CH_3CN, 25.0°, 300 mμ, $\Delta\epsilon$ 251, methyl N-acetyl-L-tryptophanate, H and M Co., used as received. [d] Enzyme used 10 min after dissolution. For experimental procedure, see ref 10. [e] These values are slightly lower than others reported in the literature[105] because 3.6% acetonitrile was used here. [f] Reference 106 reports k_{cat} of 45 ± 0.9 and 28.0 ± 1.05 for untreated and Sephadexed CDI-6114-5 at pH 8.00 in aqueous solution. While the former number is compatible with the present results because of the solvent effect, the latter number is not. [g] Assuming mol wt 24,000. [h] Determined by titration with *p*-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate at pH 3.5, pH 9.88, 0.10 M carbonate buffer, 25°, 260 mμ, $I = 0.3$. [i] Gift of Dr. C. J. Martin.

at 90% purity only in certain instances (Table XIV). These numbers raise substantial doubts about either the purity of the various enzyme preparations or the titrations themselves. The validity of each titration has been discussed above. Therefore, let us look at the impurities.

The purities mentioned above have been calculated from the relationship of the titrated value to that calculated on the basis of the weight and molecular weight of the enzyme. A fraction of the impurities calculated on this basis can be accounted for by the moisture content of these preparations, perhaps 5%. The hygroscopic nature of the lyophilized proteins can possibly increase this percentage. Another fraction of the impurities in α-chymotrypsin[106] and trypsin[107] (and probably other proteolytic enzymes) consists of ninhydrin-sensitive materials, which may be identified most easily as degradation products of the enzyme such as amino acids and peptides. These materials can be separated from α-chymotrypsin or trypsin using Sephadex G-25 or G-50. An additional impurity has been separated from α-chymotrypsin using Sephadex G-25 and must be a small molecule since it is eluted from the column considerably after the enzyme and peptide fractions.[106] This material has not yet been identified.

Recently different batches of α-chymotrypsin have been found to give different results in experiments involving proton transfer from an indicator to the imidazole group of the active site of α-chymotrypsin.[106] As a consequence of these findings the gel filtration purification using Spehadex G-25 mentioned above was developed.[106] In order to test the effect of batch difference and Sephadex treatment on both the titration of active sites and the kinetics of enzymatic catalysis, the experiments in Table XIV were carried out. For one batch of α-chymotrypsin which gave disparate results in temperature-jump experiments,[106] Sephadex filtration changed neither the purity of the enzyme preparation, as determined by N-*trans*-cinnamoylimidazole titration, nor the kinetic constants in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester. Furthermore, two batches of α-chymotrypsin, different in purity, gave identical kinetic constants when their differences in purity were taken into account by determination of the concentration of active enzyme *via* titration. Finally, Sephadex filtration improved the purity of a bovine trypsin preparation, but its kinetic constants remained unchanged. This has been the history of the titration usage so far. For example, the k_{cat}(lim) for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester in *aqueous* solution carried out by four different workers over a period of 4 years using four different preparations of α-chymotrypsin were: (1) 57 sec^{-1},[108] (2) 55 sec^{-1},[109] (3) 53 sec^{-1},[110] and (4) 50 sec^{-1}.[111,112]

Acknowledgment. The authors gratefully acknowledge the most helpful suggestions of Dr. Burt Zerner in the acetylcholinesterase titration. We further thank Dr. S. P. Jindal and Dr. J. R. Whitaker for their enthusiastic assistance.

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(111) M. J. Gibian, unpublished observations in this laboratory using Worthington chymotrypsin CDI-6054-5.
(112) See exception in footnote *e* of Table XIV.

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JOURNAL OF INVESTIGATIVE DERMATOLOGY

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Vol. 56, No. 5
Printed in U.S.A.

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THE EFFECT OF INTRAVENOUS ~~PAPAIN~~ ON CARTILAGE GLYCOSAMINOGLYCANS*

N. BLUMENKRANTZ, A. BURGOYNE AND R. FLEISCHMAJER, M.D.

ABSTRACT

A time course effect of intravenous papain on the chondromucoprotein complex of rabbit ear cartilage was followed chemically. There was a decrease of chondroitin sulfate A and non-sulfated glycosaminoglycans (GAG) up to the moment of maximal collapse of the rabbits ears. This was shown by a decrease in the content of hexuronic acids and hexosamines. During the repair of the matrix, there was an increase in non-sulfated GAG, which preceded the synthesis of chondroitin sulfate A.

Papain has been shown to produce profound ...ero as well as microscopic changes in carti...e. These changes have been correlated with ...mical alterations of the matrix (1–3). Follow...g intravenous papain administration, depletion ... glycosaminoglycans (GAG) from the rabbit ...r cartilage has been demonstrated to be asso...ted with the characteristic "sheep-like rab...'s ear" (1). The ability of this enzyme to ...erate GAG from the chondromucoprotein ...mplex has been shown in both in vivo and in ...tro experiments. In conjunction with this ef...t (or as a consequence of it), it has been ...ggested that a concomitant increase in GAG ...osynthesis also occurs (4). Thus, the effect of ...pain on the cartilage matrix may be used as ...n experimental model to study the mechanisms ... biosynthesis and metabolism of GAG. Al...ough histochemical time sequential experi...ents have shown a depletion of chondroitin ...fate on rabbit ear cartilage following intrave...ous injections of papain, parallel chemical anal...s of the hexuronic acid and hexosamine moie...s of the GAG macromolecules has not been ...ported. The present study was designed to de...rmine the time course effect of papain on the ...ove mentioned parameters.

MATERALS AND METHODS

Eight male white adolescent rabbits (2-3 kg) ...re used in this experiment. The animals received, ... the ear vein, 30 mg of papain in 0.15M NaCl per kg of body weight (4). Rabbits were sacrificed by air embolism at the following time intervals: control (0 time), immediately after the injection, and at 2, 6, 17.5, 18, 24, 48, and 72 hours. The ears were removed immediately after death and stripped of skin and perichondrium. Wet weight of the cartilage was determined. The cartilage was cut into small pieces, defatted and dehydrated with 3 changes of acetone daily for three days, followed by one extraction with acetone-ether and a final extraction with ether. The samples were filtered and dried in a vacuum desiccator over phosphorous pentoxide to constant weight. Dry weight of the samples was then determined.

GAG were isolated according to the method of Schiller *et al.* (5). Samples of dried defatted cartilage were digested with papain for 18 hours and then treated with sodium hydroxide. After dialysis the samples were digested with trypsin, the undigested proteins precipitated with trichloracetic acid and the precipitate separated by filtration. The supernatants were dialyzed and lyophilized (extracts). The extracts were dried in a vacuum desiccator over phosphorous pentoxide and their dry weights determined. The samples were submitted to chemical analysis and to fractionation with cetylpyridinium chloride (CPC). Uronic acids were determined by the Bitter and Muir carbazole method (6) and by the orcinol method (7). Hexosamines were determined by the method of Schloss (8). The nitrogen content was analyzed by the micro-Kjeldahl method.

Identification of GAG was performed by a) assessing critical salt concentration required to break the GAG-CPC complex during fractionation (5); identifying b) infra-red spectra of the extracts, in a Beckman IR-8-spectrophotometer. Two mg of each sample were mixed with 350 mg of KBr, formed into a film and compared with a KBr blank. c) Electrophoresis: the identity of the GAG in the extracts, as well as those eluted with 0.4N and 1.2N NaCl during the CPC fractionation were studied by fractionation on cellulose acetate strips. The electrophoresis was performed after the CPC elimination. The strips were soaked in veronal buffer (Gelman #51104) pH 8.8, 0.025M, and the samples applied 1 cm off the cathode end. The

Received August 12, 1970; accepted for publi...ation December 12, 1970.

Supported in part by NIH Training Grant ...M 05622, and an Institutional grant from the ...merican Cancer Society.

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separation was performed on a horizontal electrophoresis chamber for 15 minutes at a constant current of 2 mAmp per strip. Purified samples of hyaluronic acid, chondroitin and chondroitin sulfate A (gifts of Dr. K. Meyer from Yeshivah University, New York, and Dr. M. B. Mathews from the University of Chicago) were run as standards. The strips were stained with Alcian Blue for 20-30 minutes and then destained with 5% acetic acid. d) The extracts were treated with testicular hyaluronidase and the effect was measured turbidimetrically (9). Decrease or absence of turbidity after hyaluronidase digestion in relation to undigested samples was the criteria used to evaluate activity of hyaluronidase. Thin-layer chromatography on Alusil-layers was performed according to Waldi (10).

TABLE

Chemical analysis of the cartilage extracts

Time (hours)	Uronic acids		Hexosamines	Nitrogen
	Carbazole	Orcinol		
			mg/100 mg dry extracts	
0	27.0	16.0	21.5	1.6
2	28.9	17.5	20.5	1.6
6	28.4	16.5	23.1	1.8
17.5	30.7	21.4	21.5	1.8
18	25.3	17.4	19.6	1.6
24	23.0	13.2	14.4	1.3
48	24.9	20.0	18.4	1.5
72	27.7	18.9	21.2	1.8

RESULTS

Maximum collapse of the rabbit ears occurs 24 hours after the injection of papain with subsequent restoration. There was a decrease of more than 20% in the weight of the cartilage at the time of maximum collapse of the ears, with gradual increase thereafter. There was a slight decrease in weight of the dry extracts between 2 and 6 hours, reaching a plateau between 17.5 and 72 hours. There was no difference in the water content of the different cartilage samples.

The Table shows the results of the chemical analysis of the extracts. At 24 hours there was a parallel decrease in the concentration of hexuronic acids and hexosamine followed by an increase to restoration of normal values by 72 hours. All samples were susceptible to testicular hyaluronidase. Hyaluronidase sensitivity was noted by the absence of turbidity after enzymatic digestion (9).

IR spectra of all extracts, except for the corresponding to the time of maximum depletion, presented bands at 928 cm^{-1}, 825 cm^{-1} and 725 cm^{-1}, which correspond to the sulfate group with axial configuration of CSA (11, 12). Results of the CPC fractionation of extracts are shown in the Figure. At the time of maximum collapse of the ears, there was a parallel decrease of non-sulfated GAG (eluted with 0.1N NaCl) and chondroitin sulfate (eluted with 1.2N

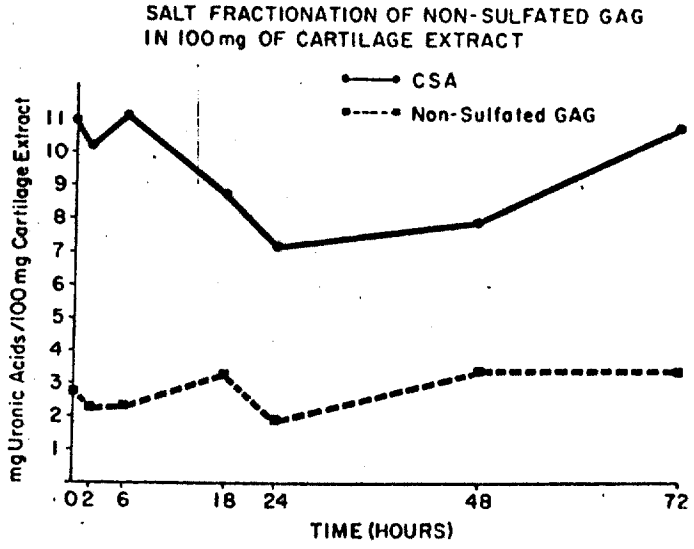

FIG. 1.

NaCl). At 48 hours, there was an increase of both fractions, the non-sulfated reaching a plateau at that stage while the CSA continued increasing up to 72 hours after the papain injection.

Samples eluted with 1.2N NaCl showed the same electrophoretic mobility as CSA, while samples eluted with 0.4N NaCl showed electrophoretic mobilities corresponding to hyaluronic acid and chondroitin. A previous experiment showed that the CPC-chondroitin complex can be eluted together with hyaluronic acid with 0.4N NaCl. After CPC elimination, samples eluted with 0.4N and 1.2N NaCl were hydrolyzed. Thin layer chromatography showed the presence of glucosamine and galactosamine in the former and only galactosamine in the latter.

DISCUSSION

This study showed a decrease of non-sulfated (hyaluronic acid and chondroitin) and sulfated (CSA) GAG in rabbit ear cartilage following the intravenous administration of papain. The maximum effect was noted at 24 hours. This was followed by morphological as well as chemical restoration of the ear. Our results confirmed those of Tsaltas (13) who used hexosamines as an index of GAG concentration in samples obtained after larger periods of observations. Since hexosamines are also part of glycoproteins, our study included the determination of both, hexosamine as well as hexuronic acid moieties. The higher values of hexosamines and lower values of nitrogen content in our extracts would suggest less contamination from proteins than those reported by Tsaltas and Greenwald (4). A real modification of the amorphous ground substance was observed at the moment of maximum collapse of the ear cartilage. At this stage, the IR spectra of the extract showed absence of the bands at 928, 852 and 725 cm^{-1}. Our finding of relative increase in non-sulfated and under-sulfated GAG at the stage of maximal depletion, suggest that, in conjunction with the liberation of GAG from the chondromucoprotein complex, there is also a reciprocal increase in GAG biosynthesis. It is well known that sulfation is the last step in the biosynthesis of GAG.

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CONTRIBUTION TO THE STUDY OF PHYSIOLOGICAL SYNTHESIS OF HEMOGLOBIN

IV. ROLE OF PAPAIN IN THE TREATMENT OF SIMPLE ANEMIAS

By

R. Bonnet and E. Meyer

Purpose of Study

In their earlier research on the physiological synthesis of hemoglobin in anemic animals, Bonnet, Mahler and Faber [1, 2] and Bonnet and Mahler [3] have shown two facts that seem to be essential for the regeneration of the blood pigment. In the first place, they demonstrated that the composition of the therapeutic mixture must take into account the hematopoietic requirements and bring together the components of hemoglobin with the factors catalyzing the synthesis reaction; in the second place, there is the nature of the anemia. A mixture consisting of various amino acids, iron, ascorbic acid and pantothenic acid was shown to be highly effective for simple anemias resulting from hemorrhaging; the same mixture however, produced only mediocre results against anemias induced by sulfamides.

While pursuing our own research regarding hemoglobin synthesis, we were impressed by the results obtained by the many investigators with combinations of iron and other substances capable of affecting hematopoiesis. Among others, Ventura [16] has pointed out the advantages of iron in association with other anti-anemic factors for the treatment of hypochromic anemia. However, we were particularly impressed by the mixture consisting of iron, papain, and ascorbic acid used successfully for the treatment of iron deficient hypochromic anemia by Mallarme, Tilquin and Tilquin [10].

What is the basic role of these factors which strengthen the hematopoietic activity of iron? In general, these substances are believed to improve both the absorption and the utilization of the metal. Thus, according to Gordonoff [5], vitamin C facilitates the assimilation of iron. The same opinion has been expressed by Prina and Barbieri [12], although they express a minor reservation. The latter investigators have shown that vitamin C promotes the intestinal absorption of iron but has no significant effect on the ability of the organs to accumulate the metal. In reference to papain, Tilquin [14] has formulated the hypothesis that the enzyme also regulates iron absorption and improves the absorption of the metal by the organism. However, since papain is both a proteolytic enzyme and a complex protein molecule, could it conceivably play a more direct role in the regeneration of hemoglobin? When subjected to diastasic hydrolysis, papain could act on the food protein to release amino acids that are essential to hematopoiesis. As a matter of fact, the studies of Roche and Mourgue [13] have shown that papain acts on the peptide bonds of casein for instance, to release leucine, valine and short chain polypeptides. On the other hand, Fruton [4] reported the role played by papain in the biosynthesis of polypeptide bonds; this fact was also reported by Kusin and Poliakova [7] and used by Hanson and Smith [6] to separate the d- and l- isomers of the various amino acids present in a mixture according to the optic specificity of this action. Furthermore, the digestion of the papain molecule itself would provide amino acids, or any other "hematopoietic factor", analogous to the "growth factor" whose presence was recently reported by Mohan and Sreenivasaya [11] in the acid papain hydrolyzate.

The object of the present report is to determine the role of papain on hematopoiesis.

METHODOLOGY

The general experimental schedule is based on the same technique used in the previous studies by Bonnet, Malher and Faber [1]. Thus, we also used adult rabbits receiving for the entire duration of the experiments a complete and balanced diet, in order to obtain the best conditions conducive to blood regeneration. The condition of the animals was kept under control by frequent body weight tests.

Each experimental series underwent three stages:

a) a short control period in order to obtain a constant blood composition as to the number of red blood cells and hemoglobin levels;

b) a period when a durable simple anemia was established by extracting regularly (every 2 days, on the average) 20 ml of blood from an ear vein;

c) a therapeutic stage when the animals were treated in order to induce hemoglobin regeneration. The intensity of hemoglobin synthesis was evaluated with respect to untreated control rabbits.

More than one therapeutic schedule was used. Thus, one lot of rabbits received only papain, in oral doses of 50, 75, 100 or 150 mg per day. A second group of rabbits received daily oral doses of a mixture consisting of 200 mg papain and 50 mg ascorbic acid. A third group of rabbits was treated with a complete mixture consisting of iron, papain and ascorbic acid; each rabbit was treated daily with 100 mg papain, 50 mg ascorbic acid and 20 mg ferrous sulfate.

The variation in the number of red blood cells was determined daily; the hemoglobin levels were determined according to the colorimetric method

described by Sahli.

EXPERIMENTAL RESULTS

The results obtained during the experiments carried out in the various groups of animals are summarized in the respective Tables.

DISCUSSION OF RESULTS

In order to evaluate the results obtained, let us examine first the three series of observations made subsequent to the treatment of rabbits according to the various therapeutic schedules described.

a) *Effect of papain alone. - (Table I).*

Following a 27 day period, all three animals (4, 5 and 9) exhibited a stable condition of hypochromic anemia. The loss of blood was not compensated despite the complete and balanced diet received by the animals. At the end of this time period, no more blood was removed from the three animals. Papain was added to the normal diet of rabbits 5 and 9, while rabbit 4 was used for control purposes.

Table I

	Rabbit No.	4		5		9	
	Average weight	2,300 g		2,520 g		3,010 g	
	Treatment	Control		Papain 150 mg		Papain 50 mg	
Period	Day	RBC millions per mm^3	Hb per 100 cc g	RBC millions per mm^3	Hb per 100 cc g	RBC millions per mm^3	Hb per 100 cc g
Induction of anemia, 20 cc of blood taken every 2 days							
Therapeutic period							
Average daily regeneration, %							

Regeneration of the blood pigment was evident in all three animals. In general, the increase of hemoglobin levels occurred at a higher rate than the number of red blood cells; this is indicative of an increase in the globular levels. However, a simple calculation demonstrates that, during the first 8 days of the therapeutic period, the hemoglobin levels of controls increased from 7.0 g per 100 cc of blood to 8.5 g, corresponding to a rise of 21 per cent; during the same time period, the rate of hemoglobin regeneration in the treated animals was 32 per cent and 49 per cent, respectively. The more active hematopoietic activity observed in the treated animals lasted for the entire duraction of the experiments. In order to obtain comparable values for the three rabbits, we determined the average rate of daily hemoglobin regeneration and we found it to be 3 per cent for the control rabbit and 4.3 per cent and 4.1 per cent respectively for rabbits 5 and 9. The results obtained in animals treated with daily doses of papain equal to 75 mg and 100 mg are of the same order of magnitude. On the average, in rabbits treated with papain alone the daily rate of papain regeneration was equal to 4.2 per cent as compared to only 2.6 per cent in controls. At this point we must point out the rate of hemoglobin regeneration does not seem to be proportional to the dose of papain administered to the rabbits. Thus, the response obtained was practically the same for all the treated rabbits while the dose ratio was 1 to 3.

b) *Effect of the papain-ascorbic acid mixture. -(Table II).*

The effectiveness of the mixture was established by performing a comparison similar to that applied to the previous series. At the end of 9 days of treatment, the rate of hemoglobin regeneration in rabbits 2 and 3 was 43% and 45%, respectively, as compared to only 17% in the control

animal; the respective average daily regeneration rates were 4.2%, 4.6% and].6%.

Table II.

	Rabbit No.	1		2		3	
	Avg. weight	2,750 g		2,300 g		2,400 g	
	Treatment	Control		Papain 200 mg+ ascorbic acid 50mg		Papain 200 mg+ ascorbic acid 50 mg	
Period	Day	RBC in millions per mm^3	Hb per 100 cc g	RBC in millions per mm^3	Hb per 100 cc g	RBC in millions per mm^3	Hb per 100 cc g
Induction of anemia, 20 cc of blood taken every 2 days							
Therapeutic period							
Average daily regeneration, %							

c) *Effect of the papain-ascorbic acid-iron mixture. - (Table III).*

Administration of the complete mixture to rabbits with induced anemia resulted in a rate of hemoglobin regeneration which was generally higher than that determined in the other groups of experimental animals. At the end of 10 days of treatment, the hemoglobin level of rabbit 12 increased from 4.5 g to 8.4 g per 100 cc of blood; the corresponding rate of increase is equal to 86%. A high rate was also determined in rabbit 13 (74%), while the respective increase over the same period of time for the control animal was only 34%. Average daily rate of hemoglobin regeneration was 3.3% for the control animal and 8.7% and 6.8% respectively for the treated rabbits.

Table III.

	Rabbit No.	11		12		13	
	Average weight	3,250 g		2,700 g		2,650 g	
	Treatment	Control		100 mg papain + 20 mg SO_4 Fe + 50 mg ascorbic acid		100 mg papain + 20 mg SO_4 Fe + 50 mg ascorbic acid	
Period	Day	RBC in millions per mm^3	Hb per 100 cc g	RBC in millions per mm^3	Hb per 100 cc g	RBC in millions per mm^3	Hb per 100 cc g
Induction of anemia, 20 cc of blood taken every 2 days							
Therapeutic period							
Average daily regeneration, %							

Let us now compare the results obtained in the three experimental series presented. Table IV summarizes the average daily rates of hemoglobin regeneration for each lot of treated rabbits, with respect to the controls (control levels were equated to unity). The figure obtained can be qualified as the coefficient of blood regeneration.

The first fact to emerge from these findings is that papain has a certain degree of hematopoietic activity, as demonstrated by the fact that when added alone to the normal diet of the animals it induced a rate of blood regeneration which is 1.5 times higher than that demonstrated by untreated animals.

The addition of ascorbic acid to papain results in only a slight increase of the effect induced by the latter alone; the slight improvement may be due to the better absorption of the hypothetical products derived from the enzyme itself. Based on the results obtained by us, we do not believe

that vitamin C has a direct effect on hematopoiesis. We reached the same conclusions following the examination of the results obtained by Lozner [9], and by Schulze and Morgan [15]. Nevertheless, we cannot exclude the possible activating effect of ascorbic acid, especially in the presence of iron.

Table IV

Controls		Papain		Papain + ascorbic acid		Papain + ascorbic acid + iron	
RBC	Hb	RBC	Hb	RBC	Hb	RBC	Hb

Accordingly, the combination of iron, asocrbic acid and papain could be expected to be highly effective especially since it has been shown that papain alone has direct hematopoietic activity. Our experiments confirm just that: the iron-papain-ascorbic acid mixture increases three-fold the regeneration rate of hemoglobin.

The determination of the hematopoietic activity of papain in combination with ascorbic acid and iron raises questions as to the mechanism of action of this effect. Does papain affect the synthesis of the blood pigment in its quality of proteolytic enzyme, or is its protein molecule responsible for the phenomenon? What are the factors generated by papain which, together with the synergistic action of iron and the assistance of ascorbic acid that are responsible for the encouraging results obtained in the treatment of hypochromic anemia? It could be that the iron complex formed is of the nature postulated by Lederer [8] in his studies regarding

the role of gastric juice in the regulation of iron metabolism, and that this complex increases greatly the utilization of the metal. We shall attempt in the future to investigate the hypotheses regarding the action mechanism of papain.

SUMMARY

Oral administration of papain alone to rabbits with anemia induced by bleeding resulted in a faster regeneration of the blood pigment than in untreated control animals. The results indicate that papain has a certain hematopoietic activity. This activity is significantly greater when papain is given in association with some other factors whose presence in a mixture increases the effect of the individual components. Such is the case for the iron-papain-ascorbic acid combination. The excellent clinical results were confirmed by the experimental findings obtained in the present study.

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CONTRIBUTION A L'ÉTUDE DE LA SYNTHÈSE PHYSIOLOGIQUE DE L'HÉMOGLOBINE.

IV. — LE ROLE DE LA PAPAÏNE DANS LE TRAITEMENT DES ANÉMIES SIMPLES.

par R. BONNET et E. MEYER.

(*Mémoire reçu le 30 juin 1951*).

OBJET DU TRAVAIL.

Dans leurs précédentes recherches sur la synthèse physiologique de l'hémoglobine chez les animaux anémiés, BONNET, MAHLER et FAUER [1, 2] et BONNET et MAHLER [3] ont mis en lumière deux faits fondamentaux, desquels semble dépendre la régénération du pigment sanguin : d'une part la composition du mélange thérapeutique, devant tenir compte des besoins hématopoïétiques et apporter à la fois les facteurs constituants de l'hémoglobine et les facteurs catalyseurs de la réaction de synthèse ; d'autre part la nature de l'anémie. Un mélange de différents acides aminés, de fer, d'acide ascorbique et d'acide pantothénique a manifesté sa plénitude d'action dans le cas des anémies simples provoquées par saignées, alors que ce même mélange n'a donné qu'un rendement médiocre dans les anémies sulfamidiques.

Poursuivant nos recherches sur la synthèse de l'hémoblobine nous avons retenu les résultats favorables obtenus par de nombreux chiniciens avec des combinaisons du fer et d'autres substances ayant une influence sur l'hématopoïèse. VENTURA [16], entre autres, signale les avantages de l'association du fer avec d'autres facteurs antianémiques pour le traitement des anémies hypochromes. Mais notre attention a été particulièrement attirée par le mélange fer, papaïne, acide ascorbique, employé avec succès comme thérapeutique des anémies hypochromes ferriprives par MALLARMÉ, TILQUIN et TILQUIN [10].

Quel est le rôle profond des facteurs qui viennent ainsi renforcer l'action hématopoïétique du fer ? D'une manière générale on attribue à ces substances celui de favoriser l'absorption et l'utilisation du métal. Ainsi, pour GORDONOFF [5] la vitamine C faciliterait l'assimilation du fer. Tel est aussi l'avis de PRINA et BARBIERI [12], quoique avec une petite restriction. Ces auteurs montrent qu'elle favorise bien l'absorption intestinale du fer, mais ne modifie pas notablement la capacité des organes de réserves à l'accumuler. Pour ce qui est de la papaïne, TILQUIN [14] émet l'hypothèse qu'elle régulariserait, elle aussi, la digestion du fer et permettrait sa meilleure absorption.

Mais la papaïne, en tant qu'enzyme protéolytique, en même temps que molécule protidique complexe, ne pourrait-elle pas jouer un

côté plus direct dans la régénération d
lyse diastasique elle pourrait libérer à p
des acides aminés indispensables à l'hé
d'après des travaux de ROCHE et MOUR
sede une action sur les liaisons peptidiq
la caséine de la leucine, de la valine c
courtes. Inversement, FRUTON [1] signa
la biosynthèse des liaisons peptidiques,
et POLIAKOVA [7] et utilisé par HANSON
cette optique de cette action à séparer
des aminés d— et l— dans un mélange.
la molécule de papaïne elle-même fourni
autre « facteur hématopoïétique », ana
nice » signalé récemment dans l'hydrol
MOHAN et SREENIVASAYA [11].

C'est dans le but de préciser le rôle
l'hématopoïèse, que nous avons réalisé la
l'objet du présent mémoire.

TECHNIQUE.

En ce qui concerne la conduite général
suivi la même technique que BONNET, MA
travaux précédents. Comme eux, nous a
adultes, recevant pendant toute la durée
lation complète et équilibrée, nous plaç
conditions quant à la régénération san
permettent de contrôler l'état du sujet.

Chaque série expérimentale comporte
a) une période de contrôle, courte, p
composition constante du sang au point
rouges et du taux d'hémoglobine ;
b) une période d'établissement d'une a
lèvements à intervalles réguliers (tous les
cm³ de sang à une veine de l'oreille ;
c) une période thérapeutique pendant l
les en vue de la régénération de l'hémog
thèse est évaluée par comparaison à des
de témoins.

Précisons les différentes modalités de
Un premier lot de lapins reçoit la pap
voie orale, mélangée à la nourriture. Le
50 mg, 75 mg, 100 et 150 mg par jour. Un
reçoit par la même voie quotidiennemen
papaïne et 50 mg d'acide ascorbique. Da
mentale enfin, nous étudions l'action du
païne, acide ascorbique. Chaque lapin re
païne, 50 mg d'acide ascorbique et 20 mg

Les variations du nombre des globules
ment par une numération globulaire ; le t
minée par la méthode colorimétrique de S

1851

UDE DE LA SYNTHÈSE PHYSIOLOGIQUE : L'HÉMOGLOBINE.

LA PAPAÏNE DANS LE TRAITEMENT ANÉMIES SIMPLES.

. BONNET et E. MEYER.

e reçu le 30 juin 1951).

JET DU TRAVAIL.

recherches sur la synthèse physiologique nimaux anémiés, BONNET, MAHLER et FABER [3] ont mis en lumière deux faits fon- le dépendre la régénération du pigment mposition du mélange thérapeutique, de- soins hématopoïétiques et apporter à la ts de l'hémoglobine et les facteurs catalyse thèse ; d'autre part la nature de l'anémie cides aminés, de fer, d'acide ascorbique et anifesté sa plénitude d'action dans le cas qué par saignées, alors que ce même mé- em médiocre dans les anémies sulfami-

es sur la synthèse de l'hémoblobine nous avorables obtenus par de nombreux ch- ons du fer et d'autres substances ayant une e. VENTURA [16], entre autres, signale les u fer avec d'autres facteurs antianémiques emies hypochromes. Mais notre attention rée par le mélange fer, papaïne, acide succès comme thérapeutique des anémies MALLARMÉ, TILQUIN et TILQUIN [10]. des facteurs qui viennent ainsi renforcer fer ? D'une manière générale on atten- e favoriser l'absorption et l'utilisation du FF [5] la vitamine C faciliterait l'assimi- l'avis de PRINA et BARBIERI [12], quoique es auteurs montrent qu'elle favorise bien er, mais ne modifie pas notablement la erves à l'accumuler. Pour ce qui est de et l'hypothèse qu'elle régulariserait, elle permettrait sa meilleure absorption. qu'enzyme protéolytique, en même temps mplexe, ne pourrait-elle pas jouer

rôle plus direct dans la régénération de l'hémoglobine ? Par hydrolyse diastasique elle pourrait libérer à partir des protides alimentaires des acides aminés indispensables à l'hématopoïèse. On sait, en effet, d'après des travaux de ROCHE et MOURGUE [13], que la papaïne possède une action sur les liaisons peptidiques, libérant p.ex. à partir de la caséine de la leucine, de la valine et des polypeptides à chaînes courtes. Inversement, FRUTON [1] signale le rôle de la papaïne dans la biosynthèse des liaisons peptidiques, fait rapporté aussi par KUSIN et POLIAKOVA [7] et utilisé par HANSON et SMITH [6], grâce à la spécificité optique de cette action à séparer les dérivés de différents acides aminés d— et l— dans un mélange. D'autre part, la digestion de la molécule de papaïne elle-même fournirait des acides aminés ou tout autre « facteur hématopoïétique », analogue au « facteur de croissance » signalé récemment dans l'hydrolysat acide de la papaïne par MOHAN et SREENIVASAYA [11].

C'est dans le but de préciser le rôle éventuel de la papaïne sur l'hématopoïèse, que nous avons réalisé la suite des recherches qui font l'objet du présent mémoire.

TECHNIQUE.

En ce qui concerne la conduite générale des expériences, nous avons suivi la même technique que BONNET, MALHER et FABER [1], dans leurs travaux précédents. Comme eux, nous avons expérimenté sur lapins adultes, recevant pendant toute la durée de l'expérience une alimentation complète et équilibrée, nous plaçant ainsi dans les meilleures conditions quant à la régénération sanguine. De fréquentes pesées permettent de contrôler l'état du sujet.

Chaque série expérimentale comporte également 3 périodes :

a) une période de contrôle, courte, permettant de s'assurer de la composition constante du sang au point de vue nombre de globules rouges et du taux d'hémoglobine ;

b) une période d'établissement d'une anémie simple, stable, par prélèvements à intervalles réguliers (tous les 2 jours en moyenne), de 20 cm³ de sang à une veine de l'oreille ;

c) une période thérapeutique pendant laquelle les animaux sont traités en vue de la régénération de l'hémoglobine. L'intensité de la synthèse est évaluée par comparaison à des lapins, non traités, servant de témoins.

Précisons les différentes modalités de cette période thérapeutique. Un premier lot de lapins reçoit la papaïne seule, administrée par voie orale, mélangée à la nourriture. Les doses sont respectivement 50 mg, 75 mg, 100 et 150 mg par jour. Un deuxième groupe de lapins reçoit par la même voie quotidiennement un mélange de 200 mg de papaïne et 50 mg d'acide ascorbique. Dans une troisième série expérimentale enfin, nous étudions l'action du mélange complet : fer, papaïne, acide ascorbique. Chaque lapin reçoit par jour 100 mg de papaïne, 50 mg d'acide ascorbique et 20 mg de sulfate ferreux.

Les variations du nombre des globules rouges sont suivies journellement par une numération globulaire ; le taux d'hémoglobine est déterminée par la méthode colorimétrique de SAHLI.

Résultats expérimentaux.

Les résultats des diverses séries expérimentales sont consignés dans les tableaux ci-après.

Discussion des résultats.

Pour l'interprétation des résultats mentionnés ci-dessus, examinons tout d'abord séparément les 3 séries d'observations, au cours desquelles les traitements administrés aux lapins ont été différents.

a) *Action de la papaïne seule.* — (Tableau I).

Après une période de 27 jours, les 3 sujets (4, 5 et 9) sont dans un état d'anémie hypochrome, stable. Malgré l'alimentation complète et équilibrée les pertes de sang ne sont pas contrebalancées. A ce moment, les saignées sont supprimées chez les 3 animaux. Les lapins 5 et 9 reçoivent alors en plus de la nourriture normale de la papaïne, le lapin 4 sert de témoin.

TABLEAU I.

	Lapin n°	4		5		9	
	Poids moyen	2300 g		2520 g		3010 g	
	Traitement	Témoin		Papaïne 150 mg		Papaïne 50 mg	
Période	Jour	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g
Etablissement de l'anémie, prise de 20 cm³ de sang tous les 2 jours	1	5,6	12,0	5,5	11,2	5,2	11,4
	6	4,4	9,8	4,3	9,8	4,4	9,4
	12	3,0	7,1	3,0	7,1	3,1	6,9
	24	3,1	7,0	2,9	5,7	3,0	6,8
	27	3,2	7,0	2,8	5,5	3,1	6,2
Période thérapeutique	29	3,7	7,6	2,9	6,0	2,9	6,8
	33	4,0	8,0	4,3	7,6	4,0	8,1
	35	4,8	8,5	4,5	8,2	4,6	8,2
	38	5,2	9,3	4,8	8,4	5,1	8,8
	41	5,2	9,9	4,8	8,8	5,0	9,8
Régénération moyenne quotidienne en p. 100		5,7	3,0	6,5	4,3	5,9	4,1

Chez les 3 animaux le pigment sanguin est régénéré. Dans l'ensemble le taux d'hémoglobine remonte plus vite que le nombre des globules rouges, traduisant ainsi une augmentation de la valeur globulaire. Mais un calcul simple montre que chez le témoin, et pendant les 8

premiers jours de la période thérapeutique, l'hémoglobine passe de 7,0 g p. 100 cm³ de sang à 8,5 g, correspondant à une augmentation de 21 p. 100, alors que les animaux traités, pendant le même temps, régénèrent 32 p. 100 et 49 p. 100 de leur hémoglobine. Cette hématopoïèse plus intense chez les animaux traités s'observe jusqu'à la fin de l'expérience. Pour avoir des valeurs comparables chez les 3 lapins, nous évaluons un pourcentage moyen de régénération de l'hémoglobine par jour : il est de 3 p. 100 pour le témoin et respectivement de 4,3 p. 100 et 4,1 p. 100 pour les lapins 5 et 9. D'autres animaux, recevant 100 mg et 75 mg de papaïne par jour, fournissent des résultats qui sont du même ordre de grandeur. En moyenne, les lapins traités avec la papaïne seule, régénèrent journellement 4,2 p. 100 de leur hémoglobine, par contre les témoins seulement 2,6 p. 100. Remarquons que l'intensité de cette action ne semble pas varier dans le même sens que la quantité de papaïne administrée ; les doses sont dans le rapport de 1 à 3, l'effet enregistré est pratiquement le même pour tous les lapins traités.

b) *Action du mélange papaïne-acide ascorbique.* — (Tableau II).

Une comparaison analogue entre témoin et traités démontre l'action du mélange administré. Après 9 jours de thérapeutique les lapins 2 et 3 régénèrent 43 p. 100 et 45 p. 100 de leur hémoglobine, le témoin seulement 17 p. 100, soit une régénération quotidienne moyenne de 4,2 p. 100, 4,6 p. 100 et 1,6 p. 100.

TABLEAU II.

	Lapin n°	1		2		3	
	Poids moyen	2750 g		2300 g		2500 g	
	Traitement	Témoin		Pap. 200 mg + ac. asc. 50 mg		Pap. 200 mg + ac. asc. 50 mg	
Période	Jour	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g
Etablissement de l'anémie, prise de 20 cm³ de sang tous les 2 jours	1	5,8	13,8	5,3	13,4	5,6	13,4
	6	4,3	10,0	4,5	9,8	4,0	9,2
	15	3,8	8,8	3,3	8,0	3,4	7,7
	20	3,8	8,7	3,4	8,1	3,5	7,6
Période thérapeutique	23	3,9	9,1	3,9	9,4	4,8	8,4
	25	4,1	9,6	4,6	9,6	5,3	9,7
	27	4,3	9,8	4,8	10,8	5,3	10,2
	29	4,9	10,2	5,0	11,6	5,2	11,0
	34	4,9	10,6	5,2	11,8	5,0	11,2
	38	5,0	10,8	5,3	12,1	5,2	11,1
Régénération moyenne quotidienne en p. 100.....		3,1	1,6	4,0	4,2	9,8	4,6

c) *Action du mélange papaïne-acide ascorbique-fer.* — (Tableau III).

L'administration aux lapins anémiés du mélange complet, provoque une régénération qu se révèle d'emblée être plus intense que dans les deux cas précédents. Après 10 jours de traitement le taux d'hémoglobine du lapin 12 passe de 4,5 g p. 100 cm³ de sang à 8,4 g, correspondant à une augmentation de 86 p. 100. Chez le lapin 13 elle est également très élevée : 74 p. 100, alors que chez le témoin elle est pour la même durée seulement de 34 p. 100 La régénération moyenne par jour est de 3,3 p. 100 pour le témoin et 8,7 p. 100 et 6,8 p. 100 pour les lapins traités.

TABLEAU III.

	Lapin n°	11		12		13	
	Poids moyen	3250 g		2700 g		2650 g	
	Traitement	Témoin		Pap. 100 mg + SO_4Fe 20 mg + ac. asc. 50 mg		Pap. 100 mg + SO_4Fe 20 mg + ac. asc. 50 mg	
Période	Jour	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g	Gl. r. en millions p. mm³	Hb. dans 100 cm³ g
Etablissement de l'anémie, prise de 20 cm³ de sang tous les 2 jours	1	5,6	11,4	5,3	9,8	5,0	11,2
	6	5,6	11,4	5,2	9,7	4,8	9,5
	15	4,3	8,6	3,3	6,3	3,2	9,4
	23	2,9	7,2	2,7	5,6	2,8	7,6
	31	2,8	5,8	2,4	4,5	2,7	5,0
Période thérapeutique	33	3,0	5,8	3,4	6,2	2,8	6,1
	35	3,1	5,8	3,9	6,3	3,3	7,3
	37	3,7	6,2	4,2	6,4	4,2	7,3
	41	4,2	7,8	4,6	8,4	4,7	8,8
	45	5,4	8,7	5,2	9,6	5,2	9,8
Régénération moyenne quotidienne en p. 100.		6,2	3,3	7,8	8,7	7,5	6,8

Confrontons maintenant les résultats de ces 3 séries expérimentales. Dans le tableau IV nous avons rapporté les pourcentages moyens de la régénération quotidienne de chaque lot de lapins traités à ceux, pris comme unité, des lapins témoins. On obtient ainsi un nombre que l'on pourrait qualifier de coefficient de régénération sanguine.

Un premier fait en découle : c'est l'évidence d'une certaine activité hématopoïétique de la papaïne, son adjonction à la nourriture normale suffit à provoquer une régénération sanguine environ 1,5 fois plus intense que chez les animaux non traités.

L'addition à la papaïne de l'acide ascorbique a pour conséquence un léger renforcement seulement de l'action de la papaïne, ce ren-

forcement pouvant être dû à une meilleure résorption des produits éventuels, encore hypothétiques, qui prendraient naissance à partir de l'enzyme. Nous ne croyons pas, en nous basant sur ces résultats, que la vitamine C ait une action directe dans l'hématopoïèse. D'ailleurs des observations de LOZNER [9] et de SCHULZE et MORGAN [15] nous amènent à la même conclusion. Néanmoins, cela n'exclut pas la possibilité pour l'acide ascorbique de jouer le rôle d'un facteur activant, surtout lorsqu'il se trouve en association avec le fer.

TABLEAU IV.

Témoins		Papaïne		Pap. + ac. asc.		Pap. + ac. asc. + fer	
Gl. r.	Hb.	Gl. r.	Hb.	Gl. r.	Hb.	Gl. r.	Hb.
5,0	2,6	5,5	4,2	6,9	4,4	7,6	7,7
1	1	1,1	1,6	1,4	1,7	1,5	2,9

Si nous combinons donc fer, acide ascorbique et papaïne, cette dernière substance possédant, comme nous venons de le voir, une activité directe dans l'hématopoïèse, on devra s'attendre à une haute efficacité de la part de ce mélange. C'est ce que confirme, en effet, notre expérience : le mélange fer-papaïne-acide ascorbique triple l'intensité de la régénération de l'hémoglobine.

La constatation du fait de l'activité hématopoïétique de la papaïne dans ses combinaisons avec l'acide ascorbique et le fer, nous conduit au problème du mécanisme de cette action. La papaïne intervient-elle dans la synthèse du pigment sanguin en tant qu'enzyme protéolytique ou bien agit-elle au titre de molécule protidique ? Quels sont le ou les facteurs qu'elle engendre et qui, grâce à l'action synergique du fer, épaulé utilement par l'acide ascorbique, conduisent à ces résultats encourageants dans le traitement des anémies hypochromes ? Peut-être aussi se trouverait-on en présence d'un complexe à base de fer du type de ceux entrevus par LEDERER [8] dans ses études sur le rôle du suc gastrique dans la régulation du métabolisme du fer, complexe qui augmenterait de beaucoup l'utilisation du métal. Autant d'hypothèses possibles du mode d'action de la papaïne que nous chercherons à préciser par la suite.

RÉSUMÉ.

Des lapins sont anémiés par saignées. Traités avec la papaïne seule, administrée par voie orale, ils régénèrent plus vite leur pigment sanguin que des animaux témoins. La papaïne possède donc un certain pouvoir hématopoïétique. Celui-ci exerce cependant sa plénitude d'action dans l'association de la papaïne à d'autres facteurs, la présence simultanée des divers constituants du mélange renforçant leurs actions individuelles. Tel est le cas du mélange fer-papaïne-acide as-

corbique, dont les excellents résultats, obtenus en clinique, sont co
firmés par nos expériences.

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RECHERCHES SUR LES « VITA
LE SCORBUT EXPÉRIMENTAL ET LA FR

DEUXIÈME MÉMOIRE.

par Jean Sevestre, Jean Fabianek, Joseph N

(*Mémoire reçu le 15 mai*

Les expériences précédemment décrites
olé vasculaire accompagnant le scorbu
pouvait être prévenue ou guérie par l'aci
Cependant les régimes alimentaires ut
étaient peu satisfaisants. Composés pou
ente de substances naturelles, ils appor
vonoïdes ayant des propriétés de « vitami
recevant ces régimes et de l'acide asc
curatif n'avaient pas survécu au delà de
Nous avons alors cherché à améliorer
ur des survies plus longues. Ne réussiss
régime entièrement artificiel et exemp
voulu savoir si le scorbut expérimental
ferente chez des cobayes ayant reçu
rices de végétaux verts (donc de flav
multanément à la carence en acide as

EXPÉRIENCE DU 8 J

1. — *Régime*

Pendant 14 jours, *huit* cobayes re
prenant pour 100 gr : avoine triée :
18 ; levure de bière fraiche : 15.8 ;
e soja : 2.25 ; lactate de Ca : 2.25.

Pendant ces 14 jours, les animaux
1) tous les jours, 1 cm³ de la s
.5 gr ; alun de fer amm. : 0.2 gr ;
Mn : 0.02 gr ; eau q.s.p. 100 cm³ ;
2) 10 mg de chlorhydrate de c
3) 3 mg d'inositol, deux fois pa
4) 111 gouttes d'huile de foie
maine ;
5) 5 *mg d'acide ascorbique, to*

A partir du 14e jour, la form
100 gr) : son de blé : 20 ; case
raisse de beurre : 12.5 ; huile
18.2 ; chlorure de Na : 2 ; méla

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 76, 122-130 (1958)

The Release of Chondroitin Sulfate from Rabbit Cartilage Following the Intravenous Injection of Crude Papain[1]

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Received October 21, 1957

Introduction

Thomas observed that the ears of young rabbits collapsed following the single intravenous injection of a solution of crude papain, and that they returned to normal shape and position 3 or 4 days later (1). He further noted the disappearance of the "basophilia" characteristic of cartilage following crude papain injection and its return at the time the ears resumed their normal shape. He established that these effects were not caused by crystalline papain and that the administration of cortisone prevented the return of the ears to normal shape.

These observations were extended by Spicer and Bryant (2) who described the extensive alterations in cartilaginous architecture, including transient swelling of interlacunar septae, crenation of chondrocytes, and disturbance of the pattern of elastic fibers. Particularly striking was the total disappearance, throughout the body, of the metachromatic staining characteristics of cartilage and the subsequent appearance of relatively large amounts of amorphous, aggregated, basophilic staining material in the pericartilaginous interstitial spaces, lymph vessels, and blood vessels. The material in the lymph vessels stained metachromatically (purple) with Azure A or toluidine blue dyes, while the material in the interstitial spaces and blood vessels stained orthochromatically (blue). The amorphous material was particularly prominent in sections of the

[1] Crude papain, as used here, refers to commercially prepared dried papaya latex extract.

[2] Fellow of the National Foundation for Infantile Paralysis.

liver and kidney where there were also changes suggestive of local toxic effects. None of these changes was elicited by the intravenous injection of crystalline papain (Worthington Biochemicals Corp.), nor by the intravenous injection of chondroitin sulfate commercially prepared from beef and pig cartilage.

The present report describes the appearance of chondroitin sulfate in the blood and urine of rabbits following the injection of crude papain. Chondroitin sulfate has been isolated from these sources and its physical and chemical characteristics determined.

Materials and Methods

Crude papain, obtained from the Fisher Chemical Co., was stirred for 30 min. at 3° with 50 vol. distilled water. The mixture was filtered, and the filtrate, containing approximately 10 mg. protein/ml., was used in subsequent experiments.

Beef and rabbit chondroitin sulfate were prepared from tracheal and ear cartilage, respectively (ear cartilage was obtained from New Zealand Albino rabbits weighing from 800 to 1000 g.), by the method of Einbinder and Schubert (3). The final steps of purification were modified to include treatment of the isolated chondroitin sulfate with trypsin and chymotrypsin.

Hexosamine was determined by the Elson-Morgan method as modified by Boas (4), uronic acid by the carbazole method of Dische (5), by the orcinol method (6), and by the hydroxamic method after conversion to the lactone (7). In the orcinol determination it was found that the maximum color development for both standard potassium glucuronate and polymer uronic acid was achieved after heating in boiling water for 60 min.

Sulfate was determined according to Alicino (8) as described by Meyer and Rapport (9).

For the purposes of calculation, molar values of hyaluronic acid and chondroitin sulfate are expressed on the basis of the repeating disaccharide unit having a molecular weight of 497.

Hyaluronic acid was obtained from Worthington Biochemicals Corp. Testicular hyaluronidase was obtained from Wyeth Institute for Medical Research. Bacterial hyaluronidase used was Varidase, Lederle.

Experimental and Results

In vitro experiments suggested that chondroitin sulfate was not degraded by crude papain; subsequent histochemical observations indicated that chondroitin sulfate might be released into the blood stream. Accordingly, the levels of mucopolysaccharide in the blood and urine, before and after the injection of crude papain, were studied.

Four New Zealand Albino rabbits weighing approximately 1 kg. were injected in an ear vein with 0.75 ml. of crude papain solution. Blood

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samples were obtained prior to injection and periodically, after injection, from the heart or contralateral ear, and the sera were assayed for uronic acid content by the carbazole method. The early appearance of circulating carbazole-reactive material, following crude papain injection, is shown in Fig. 1. Maximal values, approximately 50-fold greater than controls, were reached after 7 hr., and normal levels were restored after 48 hr.

Urine was collected under toluene from four rabbits in 24-hr. samples for 4 days prior to injection and 6 days after injection of crude papain. Five-milliliter aliquots of these samples were treated according to a modification of the method of DiFerrante (10). After precipitation at pH 5 with cetyltrimethylammonium bromide, the mucopolysaccharide was dissolved in alkaline solution, and the final product was cleared of interfering chromogens by passage through a Dowex 50 column in hydrogen form. The increase in urinary excretion of carbazole-reactive material is shown in Fig. 2. The average control value for total daily urinary excretion was 1.1 mg. uronic acid (16 samples). The maximum excretion was reached during the first 24-hr. period following injection in three of the four rabbits with an average increase of 3.3 mg. over the average control value. These control levels were considerably in excess of the normal values in human urine samples reported by DiFerrante (10). This species difference may be attributed to greater formation of mucoid substances in the genitourinary tract of the rabbit.

To determine the nature of the carbazole-reactive material in the blood and urine of injected rabbits, its isolation was undertaken. A number of

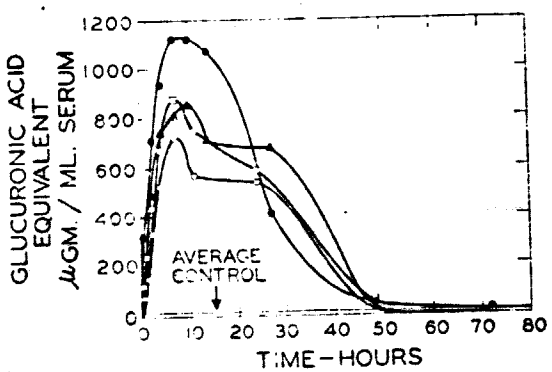

FIG. 1. The appearance of carbazole-reactive material in the serum of rabbits following the intravenous injection of crude papain.

rabbits weighing from 800 to 1200 g. was injected with 0.5–1 ml. of crude papain solution, and blood samples were obtained during the succeeding 3 days. The sera from these samples were pooled, trichloroacetic acid was added to a final concentration of 5%, and, after centrifugation, the supernatant solution was dialyzed against running tap water for 48 hr. Five per cent of solid calcium acetate was added and sufficient acetic acid to give a concentration of 0.5 *N*. The calcium salt of chondroitin sulfate was then precipitated by the addition of 2 vol. ethanol. The precipitate was washed with 80% ethanol, absolute ethanol, and ether, and dried. A portion of this was further purified by treatment with trypsin and chymotrypsin, and the impurities were adsorbed with kaolin. After several reprecipitations, this material was used for analysis.

The urinary mucopolysaccharide was isolated by precipitation with cetyltrimethylammonium bromide (11) from urine collected under toluene for 4 days following the injection of crude papain. After repeated washings with absolute ethanol saturated with sodium chloride, the precipitate was dissolved in alkaline solution, precipitated as the calcium salt from acetate buffer, and further purified as described above. This material was used for analysis.

It is known that testicular hyaluronidase will hydrolyze chondroitin sulfates A and C, whereas the bacterial hyaluronidases are not active against these chondroitin sulfates unless they have been desulfated (12). Chondroitin sulfate B, on the other hand, is resistant to the action of both enzymes. It was therefore of interest to compare the effects of these enzymes on the various isolated chondroitin sulfate preparations. The

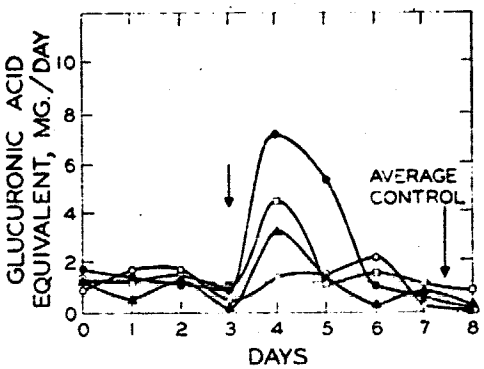

FIG. 2. The urinary excretion of carbazole-reactive material. Four rabbits were injected with crude papain on the third day as indicated by the arrow.

TABLE I

The Enzymic Hydrolysis of Isolated Chondroitin Sulfates

Hyaluronic acid and the various chondroitin sulfate preparations were incubated with testicular or bacterial hyaluronidase, and the extent of hydrolysis was measured by the increase in reducing sugar. Incubations were carried out at 37° for 120 min. in 0.1 *M* acetate buffer, pH 5.0; 0.2 ml. incubation mixtures contained hyaluronidase, 0.1 mg.; substrate, 0.07 μmole; NaCl, 0.015 μmole. Reducing sugar was determined by the method of Schales (13). Values are reported in micromoles glucose equivalent per micromole substrate.

Substrate	Control	Testicular hyaluronidase	Bacterial hyaluronidase
	glucose equivalent in μmoles		
Hyaluronic acid	0.15	0.89	0.51
Rabbit cartilage chondroitin sulfate	0.18	0.83	0.08
Beef cartilage chondroitin sulfate	0.21	0.86	0.01
Rabbit blood chondroitin sulfate	0.07	0.71	0.03
Rabbit urine chondroitin sulfate	0.16	0.76	0.04

results, presented in Table I, show that, while hyaluronic acid was degraded by both enzymes, the materials isolated from blood and urine, like chondroitin sulfate from cartilage, were hydrolyzed only by testicular hyaluronidase. These findings are compatible with the conclusion that the material appearing in blood and urine of rabbits after crude papain injection belongs to the same class of compounds as chondroitin sulfates A and C.

A comparison of certain of the chemical properties of the materials isolated from blood and urine with reference samples of chondroitin sulfate from rabbit and beef cartilage is presented in Table II. The nitrogen values are seen to be somewhat in excess of theoretical values, particularly in the material isolated from urine, probably due to contaminating amino acids. DiFerrante and Rich reported similar nitrogen values in chondroitin sulfate isolated from normal human urine (11).

The principal mucopolysaccharide constituent of cartilage is termed chondroitin sulfate A. A minor component, called chondroitin sulfate C, is also present. These two substances are closely related, being similar in most physical and chemical properties, differing from each other in solubility and optical rotation. The calcium salt of chondroitin sulfate A in acetate buffer, pH 5.0, precipitates at ethanol concentrations of 30–40%, while C precipitates at concentrations of 40–50%. In neutral solution chondroitin sulfate A has a specific optical rotation $[\alpha]_D^{27} = -30°$ to $-32°$, and C has $[\alpha]_D^{27} = -16°$ to $-22°$ (14). The structural difference

TABLE II

Properties of Isolated Chondroitin Sulfates

Preparations of chondroitin sulfate	Per cent nitrogen	Per cent sulfur	Per cent uronic acid: Carbazole	Per cent uronic acid: Orcinol	Per cent hexosamine	$[\alpha]_D^{27}$ C = 2 in water	Per cent ethanol fraction
						deg.	
Rabbit cartilage	2.9	3.4	31.9	40.1	32.9	−20	35–52
Beef cartilage	3.3		33.7	34.8	28.7	−19	37–53
Rabbit urine	3.8	4.3	31.5	37.0	32.8	−20	37–55
Rabbit blood	3.0	4.5	35.4	39.0	33.0	−16	40–51
Theoretical values[a]	2.8	6.4	35.4	35.4	36.0		

[a] Based on 497 as weight of repeating unit of calcium chondroitin sulfate; average water content of 14%.

between these two compounds is not known, and indeed, the classification of a given sample of chondroitin sulfate as A or C is at times doubtful. The solubilities and optical rotations presented in Table II would correspond to chondroitin sulfate C. This, however, in the case of the materials prepared from rabbit and beef cartilage, would be at variance with the presumed predominance of A in that tissue. The method of preparation of the polysaccharide from cartilage was that of Einbinder and Schubert (3, 15) who isolated crystalline chondroitin sulfate from beef cartilage, and the data presented here agree with their data. On the basis of the data at hand, classification of these materials as either A or C does not seem warranted.

That we are not dealing with chondroitin sulfate B, in which iduronic acid occurs in lieu of D-glucuronic acid, is apparent from the differences in optical rotation, solubility in aqueous ethanol, hydrolysis by testicular hyaluronidase, and the carbazole–orcinol ratio of approximately 1. The corresponding ratio in the case of chondroitin sulfate B is 0.5 (16).

Since galacturonic acid also gives a carbazole–orcinol ratio of approximately 1, the isolated polymer was hydrolyzed with Dowex 50 H^+ and the uronic acid assayed by the carbazole procedure, and, after lactonization with glacial acetic acid, by the hydroxamic acid method (7). The results, presented in Table III, effectively rule out galacturonic acid as the polymer uronic acid.

The hexosamine component of the isolated chondroitin sulfates was identified as galactosamine by the method of Stoffyn and Jeanloz (17),

TABLE III

Uronic Acid Color Reactions of Chondroitin Sulfate Hydrolyzates

One milligram samples of chondroitin sulfate, isolated from blood and urine were hydrolyzed on the steam bath with 60 mg. of Dowex 50 H^+ in a volume of 1.2 ml. The uronic acid was determined by the carbazole method of Dische and by the hydroxamic method after converting to the lactone (7). The results are expressed as optical density units with the carbazole values adjusted to give a ratio of 1.0 for pure glucuronic acid.

Substance	Carbazole	Hydroxamic	C/H ratio
Blood chondroitin sulfate	.58	.61	0.95
Urine chondroitin sulfate	.59	.62	0.95
Glucuronic acid	.50	.50	1.0
Galacturonic acid	.63	.46	4.0

in which the acid hydrolyzates of the material to be tested were treated with IR 4B anionic exchange resin and reacted with ninhydrin in the presence of pyridine. The reactants were chromatographed in butanol-ethanol-water 4:1:1, and the paper was developed with silver nitrate according to Trevelyan *et al.* (18). In this reaction with ninhydrin hexosamines are degraded to the corresponding pentoses. It was observed that the isolated chondroitin sulfates yielded spots of identical mobility to lyxose in contrast to glucosamine which gave rise to a spot corresponding to arabinose.

Discussion

The histological finding in rabbits treated with crude papain of amorphous aggregates of material in the pericartilaginous spaces, lymph vessels, and blood vessels, which stained metachromatically and orthochromatically at a time when the cartilage was losing its metachromasia (2), suggests that chondroitin sulfate, rather than being degraded *in situ*, is liberated from the cartilage as a polymer. The isolation and characterization of chondroitin sulfate from the blood and urine of animals treated with crude papain supports this suggestion.

There is an obvious disparity between the amount of chondroitin sulfate which appears in the blood stream and that which is excreted in the urine. In the four experimental rabbits, assuming an average blood volume of 55 ml., there was a minimum of 25 mg. of circulating glucuronic acid equivalent as chondroitin sulfate at the peak of concentration. The total mean increase in urinary excretion of glucuronic acid subsequent to injection was 4.4 mg. This difference in some manner reflects the metabolic utilization or degradation of the circulating chondroitin sulfate.

Thomas observed that the injection of crude papain would not cause the ear-drooping phenomenon in adult rabbits (1). We have found, in mature rabbits receiving near-lethal injections of crude papain, aggregates of basophilic material in the lymph and blood vessels but only a slight decrease in the metachromasia of the cartilage, in contrast to young rabbits where the metachromasia disappeared entirely and the ears became flaccid. It remains to be determined whether these striking differences reflect quantitative or qualitative alterations with age in the structure or composition of the cartilage. Another observation of considerable interest was made in mature rabbits weighing 3–4 kg. following the injection of crude papain, in which the venous clotting time was markedly prolonged and the serum was heavily lipemic. In young rabbits following papain the venous clotting time was consistently normal and the serum was clear. These variations may also reflect age differences and are under investigation.

The close correlation between the onset of ear collapse and the release of chondroitin sulfate suggests that chondroitin sulfate is intimately associated with the structural elements which lend rigidity to the cartilage.

The active factor in crude papain which is responsible for this effect has not been identified, nor has the mechanism of action been elucidated. It seems reasonable to propose that there is a proteolytic action at or near the points of attachment of chondroitin sulfate to cartilage protein, and that, following cleavage of this attachment, chondroitin sulfate diffuses into surrounding tissues.

Acknowledgments

The authors wish to thank Mrs. Helen Postman for her excellent technical assistance and Mr. Ignacio Smith, Jr. for his efficient cooperation in the animal studies.

Summary

The intravenous injection of crude papaya latex extract (crude papain) into young rabbits is followed by the appearance, in blood and urine, of a mucopolysaccharide, resembling in chemical and physical properties chondroitin sulfates A and C. The time course of appearance and disappearance correlates well with the gross and histological changes in cartilages of ear and elsewhere. Only a small fraction of the mucopolysaccharide which enters the blood stream is accountable in the urinary excretion.

Differences of response in old rabbits have been observed and possible relationships to the process of aging are discussed.

The possible activity in crude papaya extract responsible for the observed histological and chemical effects is discussed.

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The Flavonols of *Impatiens balsamina* L.[1]

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Received October 23, 1957

Introduction

The garden balsam, *Impatiens balsamina* L. (Balsaminaceae), lends itself well to the study of the biochemical-genetical relation of genes and flower pigments. The genes responsible for 24 different, easily identified phenotypes have been recognized (1), and the anthocyanin and leucoanthocyanin pigments present in these color types have been identified (2). Except for the flavonol kampherol reported as occurring in a few color varieties (3), no identification of the anthoxanthins present in these flower types has been made.

Visible color in *I. balsamina* is controlled by genes at four nonlinked loci (1). The gene L causes the production of lavender or purple flowers which contain the anthocyanin malvidin, while the gene H causes pink or red flowers to be produced in which pelargonidin is found (2). At the third locus, P, three alleles are recognized which influence the amount of color produced. The gene P^r causes the production of intensely colored flowers on plants with red stems. This gene is dominant over P^g which causes the production of less floral pigment on green-stemmed plants. The double recessive, pp, diminishes the intensity of the flower color to such an extent that the flowers appear to be almost white. Pure white flowers are produced when the genes H and L are also recessive. At a fourth locus, W, the recessive gene causes the production of cream sepals.

The petals and petaloid sepals differ somewhat in their visible pigment content, but not in the leucoanthocyanin complement (2). Both

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Contribution to the study of the local and general action of the basic proteins - papain and trypsin

B. A. Capri, P. Mastroeni and F. Orecchio

In a research study carried out at our Institute on the general and local action of some basic proteins it was proven that protamine sulfate causes a conspicuous leukotaxis if injected intracutaneously in doses of 50 - 100 mu g in 1 ml physiological solution in the rabbit and in the guinea pig (1) and that it causes a considerable leukocystosis if injected intravenously in microdoses (3 mg/kg) in the same animals.

Histone sulfate showed in the same doses similar effects (3). Recently it was also observed that lysozyme (both chloride and methyl inactivated) causes local leukotaxis and general leukocytosis, though of lesser intensity (4).

Since we wanted to extend the investigations to other basic proteins, we experimented with papain and trypsin, because there were no data available on the former, while the results obtained by the authors with the latter in preceding tests were controversial.

Recently it was shown that trypsin acts as an inhibitor in the edema from egg white, if administered parenterally. When injected intracutaneously together with diptheria toxin it inhibits the phlogistic effects quite considerably (5).

Some authors think that these antiphlogistic actions are related to the proteolytic activity of trypsin (6).

Material and technique - For our tests we used young rabbits weighing 1.500 to 2 kg; a solution in distilled water, free of pyrogens and sterile, of NaCl 0.9%; papain (Merck) and trypsin (Merck).

The samples were taken by puncturing the marginal vein of the ear and spontaneous bleeding. The counting was made with the Thomas camera before

the injection of the substance after 1,2,3,4,6,8,10 hours.

For the local treatment the substances were injected into the skin of the abdomen which had been depilated several days before.

The temperature was checked each time and a complete hemochromocytometric examination was made.

Intravenous treatment - The rabbits were divided into groups of 6 animals each. The first group was treated with a sterile 0.9% NaCl solution (2 ml); The second group was treated with 30 mg/kg papain in 2 ml solvent; The third group was treated with 7 mg/kg trypsin in 2 ml solvent;

Results: 1st group: No change in the content of leukocytes. 2nd group: one hour after the injection slight leukopenia, followed by a progressive increase in the number of leukocytes, which attained its maximum (three times the initial value) between 2 and 3 hours, to return to normal within 6 hours from the injection. 3rd group: after 1-2 hours discreet leukopenia, followed by marked leukocytosis (initial value quadrupled) with acme toward 4-6 hours, and return to normal after 10 hours.

The number of red globules and the rectal temperature remained unchanged; the leukocytal formula showed clear amphophilia. In orientative tests we saw that papain in smaller doses than considered optimum by us had a less intensive object.

Trypsin injection in doses of 70 mg/kg body weight caused an increase of the leukocyte content almost equal to that produced by doses of 7mg/kg body weight. The animals were sacrificed after the treatment and subjected to an autopsy which was always negative due to considerable macroscopic lesions in the various organs.

Local treatment: 50,100 and 200 mu g papain and trypsin resp. in 1 ml physiological solution were injected subcutaneously into single zones.

The injections were made in time intervals in order to observe the process in the various phases. Samples were taken from the inoculated

zones in any thickness after the animals were sacrificed by bleeding them white.

Results - The histologically most significant findings were obtained both for papain and for trypsin used in doses of 100 mg after 2½ and 3½ hours respectively. They consisted in: edema of moderate size, slight hyperemia, discreet intravasal leukotcystosis; perivascular infiltrate around the vessels and of gradually decreasing intensity, extending upward to the surface skin and below the fur. This infiltrate consisted primarily of well-preserved amphophils.

Conclusions - Papain and trypsin Merck injected into the rabbit intravenously in optimum doses of 30 and 7 mg/kg body weight resp. cause considerable amphophilic leukocytosis, similar to that found in other studies conducted at this Institute with protamine sulfate and histone sulfate. The only difference was noticed in the behavior of the leukocytal growth curve in time, which shows in fact, in the case of the substances used by us, a slower increase and in each case independent, within certain limits, of the injected doses, different from that observed for protamine and histone.

The local treatment with doses of 100 mg causes constant phenomena of leukocytosis, similar to these put in evidence in the course of the studies concerning this same field of work.

(Translated by Carl Demrick Associates,Inc/IE/t)

Boll. Soc. Ital. Soc. Biol. Sper., 37, 1961

L'attivazione della fosforilasi atriale ad opera della nicotina, e la scomparsa del suo effetto attivante contemporaneamente alla scomparsa dei suoi effetti stimolanti inotropo e cronotropo dopo trattamento reserpinico, è un'ulteriore prova a sostegno dell'interpetrazione che l'effetto stimolante di questa sostanza è mediato attraverso la liberazione di catecolamine.

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CONTRIBUTO ALLO STUDIO DELL'AZIONE LOCALE E GENERALE DELLE PROTEINE BASICHE — PAPAINA E TRIPSINA —.

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(*Dall'Istituto di Patologia Generale dell'Università di Messina*)*.

Sezione di Messina — Seduta del 24 febbraio 1961.

In un piano di ricerche, in corso di espletamento nel nostro Istituto, sulla azione generale e locale di alcune proteine basiche è stato messo in evidenza il fatto che il solfato di protamina determina una cospicua leucotassi, se iniettato per via intradermica, alla dose di 50 - 100 μg in ml 1 di sol. fisiol. nel coniglio e nella cavia (1) e che determina una imponente leucocitosi se inoculato in microdosi (mg 3/kg) per via venosa negli stessi animali (2).

Anche il solfato di istone ha presentato, alle medesime dosi, analoghe azioni (3). Recentemente è stato visto che anche il lisozima (sia cloruro che metil-inattivato) determina leucotassi locale e leucocitosi generale, sebbene di minore intensità (4).

Volendo estendere l'indagine ad altre proteine basiche, abbiamo sperimentato con la papaina e la tripsina, perchè, per quanto ci riguarda, sulla prima non esistono dati, mentre sulla seconda i risultati degli AA. che ci hanno preceduto sono controversi.

Recentemente è stato dimostrato che la tripsina agisce come inibitore dell'edema da bianco d'uovo, somministrata per via parenterale; essa, inoltre, iniettata per via intradermica assieme a tossina difterica ne inibisce notevolmente gli effetti flogistici (5).

Alcuni AA. ritengono che queste azioni antiflogistiche siano in relazione con l'attività proteolitica della tripsina (6).

Materiale e tecnica. — Per le nostre esperienze abbiamo adoperato conigli giovani, del peso di kg 1,500 - 2; soluzione in acqua distillata, priva di pirogeni e sterile, di NaCl 0,9%; papaina (Merck) e tripsina (Merck).

I prelievi sono stati eseguiti mediante puntura della vena marginale dell'orecchio ed a sanguinamento spontaneo. Le conte sono state

(*) Lavoro eseguito durante la direzione del prof. M. Teti.

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eseguite con la camera di Thomas prima dell'inoculazione della sostanza e dopo 1, 2, 3, 4, 6, 8, 10 h da essa.

Per il trattamento locale le sostanze sono state inoculate nella cute dell'addome depilata qualche giorno prima.

E' stata ogni volta controllata la temperatura ed è stato eseguito l'esame emocromocitometrico completo.

Trattamento endovenoso. — I conigli sono stati divisi in lotti di 6 animali ciascuno.

Il I lotto è stato trattato con soluzione NaCl 0,9% sterile (ml 2);

Il II lotto è stato trattato con mg 30/kg di peso di papaina in ml 2 di solvente;

Il III lotto è stato trattato con mg 7/kg di peso di tripsina in ml 2 di solvente.

Risultati. — I lotto: nessuna modificazione del tasso leucocitario. II lotto: dopo 1 h dall'inoculazione, lieve leucopenia, seguita da un progressivo incremento del numero dei leucociti che raggiunge il suo massimo (triplicandosi il numero iniziale) fra la 2 e la 3 h, per ritornare alla norma entro la 6ª h dall'inoculazione. III lotto: dopo 1-2 h discreta leucopenia, seguita da leucocitosi marcata (valore iniziale quadruplicato) con acme verso la 4-6ª h, con ritorno alla norma verso la 10 h.

Il numero dei globuli rossi e la temperatura rettale sono rimasti immutati; la formula leucocitaria ha dimostrato una netta anfofilia. In prove di orientamento, abbiamo visto che la papaina a dosi inferiori a quelle da noi ritenute ottimali aveva un'azione meno intensa.

La tripsina inoculata a dosi di mg 70/kg di peso ha apportato un incremento del tasso leucocitario pressochè uguale a quello ingenerato dalla dose di mg 7/kg di peso. Gli animali sacrificati dopo il trattamento venivano sottoposti ad esame autoptico, che è stato sempre negativo per lesioni macroscopicamente apprezzabili a carico dei vari organi.

Trattamento locale. — Sono state inoculate intracute in singole zone 50-100-200 μg di papaina, e rispettivamente di tripsina, in ml 1 di sol. fisiologica.

Le inoculazioni venivano eseguite ad intervalli di tempo, allo scopo di seguire il processo nelle varie fasi. Le zone inoculate venivano prelevate a tutto spessore dopo sacrificio dell'animale per dissanguamento.

Risultati. — I quadri istologici più significativi si sono avuti sia per la papaina che per la tripsina impiegate alla dose di 100 μg e rispettivamente dopo 2 h e 30 min e 3 h e 30 min. Essi consistono in: edema di modesta entità, lieve iperemia, discreta leucocitosi intravasale; infiltrato perivasale, fitto attorno ai vasi e via via meno intenso, estendentesi in alto verso il derma superficiale e fino al disotto del pellicciaio. Quest'infiltrato è costituito prevalentemente da anfofili ben conservati.

Conclusioni. — Papaina e tripsina Merck inoculate nel coniglio per via venosa alle dosi ottimali rispettive di mg/kg 30 e 7 hanno provocato sensibile leucocitosi anfofila, analoga a quella riscontrata in altre ricerche condotte in questo Istituto con protamina solfato ed istone solfato. La sola differenza si nota nel comportamento della curva d'incremento leucocitario nel tempo. Questa presenta, infatti, nel caso delle sostanze da noi impiegate, un aumento più tardivo ed in ogni caso

indipendente, entro certi limiti, dalle dosi inoculate a differenza di quanto si verifica per la protamina e per l'istone.

Il trattamento locale con dosi ottimali di μg 100 provoca costanti fenomeni di leucotassi, simili a quelli che sono stati messi in evidenza nel corso di ricerche appartenenti a questo stesso piano di lavoro.

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CONTENUTO IN GLICOGENE E BIOSINTESI DI COZIMASI NEL FEGATO DI CAVIE TRATTATE CON FORTI DOSI DI NICOTINAMIDE (*).

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(Dall'Istituto di Chimica Biologica dell'Università di Siena).

Sezione di Siena — Seduta del 4 maggio 1961.

Nel fegato di ratto trattato con nicotinamide, accanto ad una intensa biosintesi di piridinnucleotidi (PN) — soprattutto della forma ossidata del DPN e, in misura minore, del DPNH (1) e del TPN (2) — è stata osservata una intensa glicogenolisi (3), che, tra la 4ª e la 16ª h dal trattamento, raggiunge valori dell'ordine del 90%; tale glicogenolisi era stata messa in relazione con l'aumentata biosintesi di piridinnucleotidi, in quanto essa potrebbe fornire i composti necessari per la costruzione della parte glucidica della molecola del DPN.

Una relazione del genere è stata però esclusa in base a nostre ricerche (4) in cui si dimostrava che la biosintesi di PN da carico di nicotinamide si verifica anche in ratti tenuti a digiuno da 60 h, e aventi un contenuto pressochè nullo di glicogene epatico.

Allo scopo di studiare ulteriormente i rapporti tra contenuto in glicogene epatico e biosintesi di PN, abbiamo preso in considerazione la biosintesi di PN da carico di nicotinamide e il contenuto epatico di glicogene nella cavia, animale nel quale il metabolismo della nicotinamide ha un andamento diverso rispetto al ratto (5).

Abbiamo preso in esame 2 gruppi, ciascuno di 6 cavie adulte e del peso di 350 g circa. Le cavie di un gruppo erano trattate con nicotinamide per via intraperitoneale alla dose di 100 mg/100 g di peso.

12 h dopo il trattamento, periodo durante il quale tutti gli animali erano tenuti a digiuno, le cavie trattate e quelle di controllo erano uccise, i fegati rapidamente prelevati e suddivisi in due aliquote: su una di esse era dosato il glicogene con la reazione di Van der Kleij (6) e col metodo di Carrol e coll. (7), sull'altra erano estratti, come indicato da Spirtes e Eichel (8) DPN e DPNH che erano dosati spettrofotometricamente con alcooldeidrogenasi (9).

Dall'esame della tabella risulta che la somministrazione di nicoti-

(*) Lavoro eseguito con il contributo del C.N.R.

J. BACTERIOL.

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JOURNAL OF BACTERIOLOGY, May 1967, p. 1493-1499
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Vol. 93, No. 5

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Immunosuppressive Activity of the 3a-1 Fraction of Papain

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Received for publication 11 January 1967

The maximal nonlethal dose of the 3a-1 fraction of papain, determined by use of 9-week-old white albino rabbits, was 10 mg per injection, administered intravenously. The immunosuppressive activity of the 3a-1 fraction of papain was studied by its inhibition of sheep red blood cell hemolysin, bacterial agglutinin, and horse serum precipitin. Immune suppression was observed when papain injections preceded antigen injections by 12 to 18 hr. The enzyme preparation was analyzed by use of cellulose acetate and starch-gel electrophoresis and by the micro Kjeldahl method. Starch-gel electrophoresis of serum samples revealed qualitative alterations in the α^{-1} region 7 hr after papain administration. Changes were also observed in the urinary aminopolysaccharide content.

Halpern et al. (4) found that the injection of crystalline papain produced a collagen-like disease. His group observed glycoprotein thesaurosis in the liver, lesions in the epiphyseal plate of the long bones, and marked osteoporosis in young rabbits after 23 injections of papain during a 70-day period. Further, Morard et al. (8) reported autoantibodies against cartilage, renal papilla, and liver extracts. These autoantibodies caused lesions in the pleura, pericardium, and peritoneum. J. E. Fidler (Ph.D. Thesis, Univ. of Nebraska, Lincoln, 1966) reported that young rabbits given 500 μg of the 3a-1 fraction of papain failed to produce detectable streptococcal anti-M antibodies after exposure to viable streptococci 7 hr later.

Thomas (11) reported that solutions of papain (3a-1) caused ear collapse of rabbits weighing 1 kg. The ear collapse was associated with the depletion of the ear cartilage and disappearance of basophilia from the matrix. The effects of papain (3a-1) were not reproduced by separate injections of crystalline papain protease or crystalline plant lysozyme. The third constituent, chymopapain, of the 3a-1 fraction may be the causative agent.

This paper describes the immunosuppressive activity of the 3a-1 fraction of papain and concomitant findings in the urine and serum of the rabbit.

MATERIALS AND METHODS

Experimental animals. White albino rabbits, 9 weeks old and weighing 1 kg each, were used. They were maintained on a diet of antibiotic-free Purina Rabbit Chow and water ad libitum.

Preparation of 3a-1 fraction of papain. This fraction was prepared by the method of Kimmel and Smith (5). A 180 g amount of dried papaya latex (Wallerstein Co., Staten Island, N.Y.), 100 g of Celite, and 150 g of washed sand were mixed in a Waring Blendor with 200 to 300 ml of 0.04 M cysteine (6.3 g of cysteine hydrochloride in 1,000 ml of 0.05 N NaOH).

With the use of the supernatant fluid, the extraction and grinding procedures were repeated with the cysteine solution until a total volume of 1 liter had been used. The resulting suspension was mixed and filtered by use of a 0.5 cm layer of Hyflo Super Cel (Johns-Manville, New York, N.Y.) on Whatman no. 1 filter paper. The filtrate (fraction 1) was opalescent and greenish-yellow, with a *p*H of approximately 5.5.

Fraction 1 was adjusted to *p*H 9.0 with 1 N NaOH. The resulting gray precipitate was removed by centrifugation at 2,000 rev/min for 1 hr. The supernatant fluid (fraction 2) was clear.

Fraction 2 was brought to 0.4 saturation with solid ammonium sulfate (250 g per liter). After 1 to 2 hr at 4 C, the white precipitate (fraction 3) was again centrifuged and the supernatant solution (fraction 3a) was saved. Fraction 3a was brought to 0.8 saturation with ammonium sulfate and allowed to stand overnight at 4 C. The resultant precipitate (fraction 3a-1) was again collected by centrifugation. The material was dialyzed against deionized water until the dialysate was free from NH_4^+ ions (as detected by Nessler's reagent). The preparation was freeze-dried by use of the Virtis Freeze-Drier (Virtis Co., Inc., Gardiner, N.Y.).

Cellulose and starch gel electrophoresis of papain. The composition of the 3a-1 fraction was determined by cellulose acetate and starch gel electrophoresis. The modified method of Marsh, Jolbitt, and Payne (7) was used for starch gel electrophoresis. The starch

was manufactured by Connaught Medical Laboratories, Toronto, Canada. The concentration of starch was 13.6 g per 100 ml of diluent. Amido Black 10 B was used as the stain.

Cellulose and starch gels were scanned by use of a Photovolt Densiometer (model 542) equipped with a 525-mμ filter.

The nitrogen content of the 3a-1 fraction of papain was determined by the Kjeldahl method.

Rabbit tolerance test. The maximal nonlethal dose of the 3a-1 fraction was determined. The end point was that concentration which, when injected intravenously in six consecutive doses, every other day, caused no lethality. A dose of 10 mg per injection fulfilled these requirements.

Urinary aminopolysaccharide determination of papain-treated animals. Urinary aminopolysaccharides were isolated by the method of DiFerrante and Rich (1), and the hexuronide acid content was determined by the carbazole method of Dische (2). Animals were injected intravenously in the lateral ear vein with 10 mg of the 3a-1 fraction of papain. Urination of the animals was induced by two 50-mg injections of Diamox (acetazolamide, Lederle Laboratories, Pearl River, N.Y.) in the lateral ear vein.

Starch-gel electrophoresis of serum. The same procedure was used as that outlined under cellulose and starch gel electrophoresis of papain.

Identification of serum proteins by starch-gel electrophoresis. Tentative identification of rabbit serum proteins was kindly supplied by Pentex, Inc., Kankakee, Ill.

Determination of the immunosuppressive activity. For the studies of the suppressive effect of the 3a-1 papain fraction upon antibody synthesis, three antigens were used, i.e., sheep red blood cells (SRBC), horse serum, and *Salmonella choleraesuis*. All rabbits were injected intravenously. Blood was withdrawn after heart puncture.

Thirty-four rabbits were used for the hemolysin series. Seven rabbits were used as controls, and SRBC injections were given on day 0 and on days 2, 14, 20, and 28. Bleedings and antibody tests were completed on days 10, 19, 26, 34, and 52. The average titers of these seven sera are shown in Fig. 4A.

Another group of seven rabbits (Fig. 4B) and a third group of eight animals (Fig. 4C) were similarly immunized (same quantity of inoculum and same injection and bleeding schedule). With the smaller group, however, beginning 10 days after the last SRBC injection, five papain injections of 10 mg each were administered every other day for a period of 10 days. With the larger group, the rabbits were given five papain injections of 10 mg each every other day for a period of 10 days, followed by the SRBC injections 10 days after the last papain injection.

For the final papain study with the SRBC antigen, seven rabbits were immunized on day 0 and on days 2, 6, 12, 18, and 36. However, 12 to 18 hr before each SRBC injection (with the exception of the injection on the 36th day), 10 mg of papain was injected. Bleedings and hemolysin determinations were completed on days 9, 16, 20, and 38. Average titers of the seven serum samples are shown in Fig. 4D. A [illegible] group of five rabbits was similarly immunized [illegible] with the exclusion of the papain injections, [illegible]

A group of 22 rabbits were used for the [illegible] agglutinin studies. Five animals were used as [illegible] and were injected on day 0 and on days 2, 8, 12, [illegible] 38, and 58. Bleedings and agglutinin tests were [illegible] pleted on days 10, 16, 20, 40, and 62. The [illegible] representing the average of the five rabbit sera [illegible] shown in Fig. 5A.

Another group of five animals was similarly immunized, but, 8 days after the fifth antigen injection five papain injections of 10 mg each were injected every other day for a period of 10 days (Fig. 5B). The final two groups of six rabbits also received the same quantity of antigen and followed the same injection and bleeding schedule. One of the groups, however, received five papain injections of 10 mg each every other day for a period of 10 days, the last papain injection falling 10 days before the first bacterial antigen injection (Fig. 5C), and the other group (Fig. 5D) received the 10-mg papain injections 12 to 18 hr preceding each bacterial-antigen injection.

A group of 25 rabbits was immunized with horse serum. Six rabbits served as controls, and were inoculated on days 2, 8, 12, 17, and 42. Bleedings and precipitin tests were completed on days 11, 18, 26, and 46 (Fig. 6A). With the same quantity of inoculum and the same injection and bleeding schedule, six rabbits received an additional 10 mg of papain on alternate days after antigen injections (Fig. 6B), six received 10 mg of papain on alternate days before antigen injection (Fig. 6C), and seven were injected with 10 mg of papain 12 to 18 hr before each antigen was injected (Fig. 6D).

Antigens. SRBC were prepared from fresh defibrinated whole sheep blood; the final concentration was 5% (v/v).

Heat-inactivated horse serum was used as the soluble antigen. A 1-ml amount of Merthiolate (1:1,000) was added to 9.0 ml of serum.

The bacterial antigen *S. choleraesuis* was grown on nutrient agar at 37 C; physiological saline with 5% neutral Formalin was used to flush the bacterial growth from the surface. The final concentration was approximately 1.2×10^9 cells per milliliter.

Determination of antibody titers. Hemolysin titer was determined by the procedure of Smadel (9).

Bacterial agglutinin titer was determined by the cross-titration of 0.5 ml of dilutions of bacteria with 0.5 ml of heat-inactivated antisera dilutions. The results represent the reciprocal of the highest dilution of antisera reacting with 5.2×10^8 cells per milliliter. Mixtures were incubated for 1 hr at 37 C and overnight at 4 C.

Serum precipitin titer was determined by the cross-titration of 0.5 ml of antigen dilutions with 0.5 ml of heat-inactivated antisera dilutions. Mixtures were incubated for 1 hr at 37 C and overnight at 4 C. The results reflect the reciprocal of the highest dilution of antisera reacting with the 1:4 dilution of antigen. Controls were maintained for all tests.

Electrophoretic [illegible] of papain [illegible] papain by cell[illegible] trophoresis. Fig [illegible] Fund. The analy[illegible] Kunnel and Smi[illegible] removal of three [illegible] tons (Fig. 1B). [illegible] showed the nitro[illegible] to be 12.8 ± 0.5[illegible]

Rabbit tolerance [illegible] fraction caused [illegible] Necropsy revealed [illegible] arteries, and renal [illegible]

Determination [illegible] *saccharide (APS)* [illegible] tained approximately [illegible] during the course [illegible]

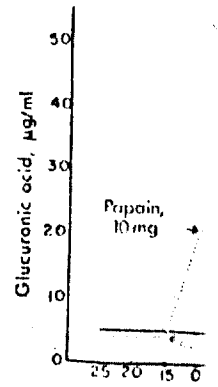

FIG. 1. (A) *Elec*[illegible] *papain fraction;* (B [illegible] *crude papaya latex a*[illegible]

FIG. 2. *Rabbit uri*[illegible] *after intravenous inje*[illegible] *fraction. Urination i*[illegible] *experimental urine;* [illegible]

RESULTS

Electrophoretic and nitrogen analysis of 3a-1 fraction of papain. Analysis of the 3a-1 fraction of papain by cellulose acetate and starch gel electrophoresis (Fig. 1A) revealed three protein bands. The analysis of the crude papaya latex by Kimmel and Smith (5) was included to show the removal of three other contaminating plant proteins (Fig. 1B). Results of 10 determinations showed the nitrogen content of the 3a-1 fraction to be 12.5 ± 0.5%.

Rabbit tolerance test. Lethal doses of the 3a-1 fraction caused death 2 min after injection. Necropsy revealed hemorrhagia in lungs, heart, arteries, and renal tissue.

Determination of the urinary aminopolysaccharide (APS) content. Urine controls contained approximately 6 to 7 μg of APS per ml during the course of the experiment (Fig. 2). Experimental urines contained 5 to 6 μg of APS per ml during the 10 hr prior to treatment. A 50 mg amount of Diamox was administered at 15 hr prior to treatment, which elevated the APS content fourfold (20 μg/ml). Test animals were injected intravenously with 10 mg of papain at zero-time. At 7 hr, test animals showed an eightfold increase (43 μg/ml) in APS compared with controls. By 14 hr, the urine APS content was normal.

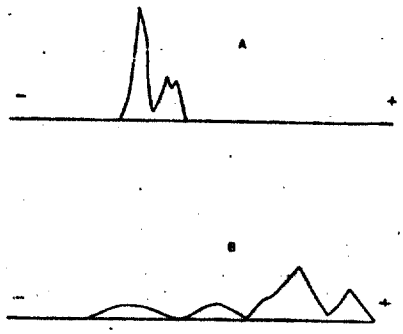

FIG. 1. *(A) Electrophoretic-scan patterns of 3a-1 papain fraction; (B) electrophoretic-scan pattern of crude papaya latex analyzed by Kimmel and Smith (5).*

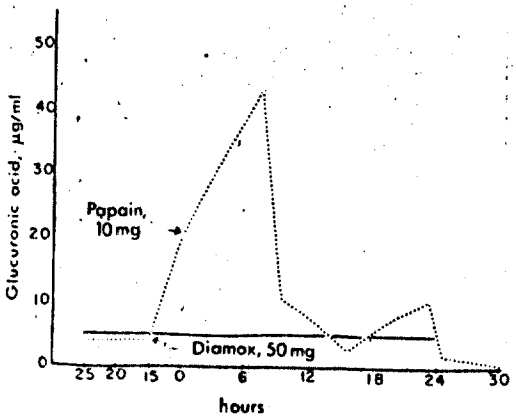

FIG. 2. *Rabbit urinary aminopolysaccharide analysis after intravenous injection of 10 mg of the 3a-1 papain fraction. Urination induced with Diamox. Dotted line, experimental urine; solid line, control urine.*

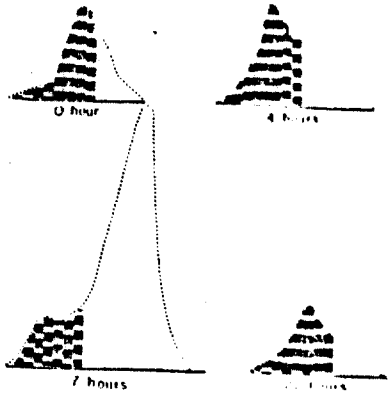

FIG. 3. *Starch-gel electrophoretic scan patterns showing α-1 lipoprotein and fetuin elevations after intravenous injection of 10 mg of 3a-1 papain fraction.*

Starch-gel electrophoresis of serum. Examination of the serum protein of 18 animals 72 hr after the injection of 10 mg of papain indicated that no abnormal proteins were present. (Identification of bands was kindly supplied by Pentex, Inc.) However, the serum proteins studied 12 to 18 hr after 10-mg papain injections indicated α-1 lipoprotein and fetuin elevations at 4 and 7 hr. By the 22nd hr, these fractions had returned to normal.

Comparison of results obtained from the urine assay (Fig. 2) and the starch-gel analysis (Fig. 3) between 4 and 7 hr showed fairly good correlation regarding synchrony of appearance of pathologies.

Immunosuppressive activity. The immunological response to the SRBC antigen is presented in Fig. 4A to E. The 12 control animals (Fig. 4A and E) had peak titers of 1:4,000. No apparent titer differences were observed when papain was administered 10 days after the last SRBC injection (Fig. 4B). For example, a 1:1,142 titer was observed with this papain group, whereas the control groups showed titers of 1:1,542 and 1:1,520.

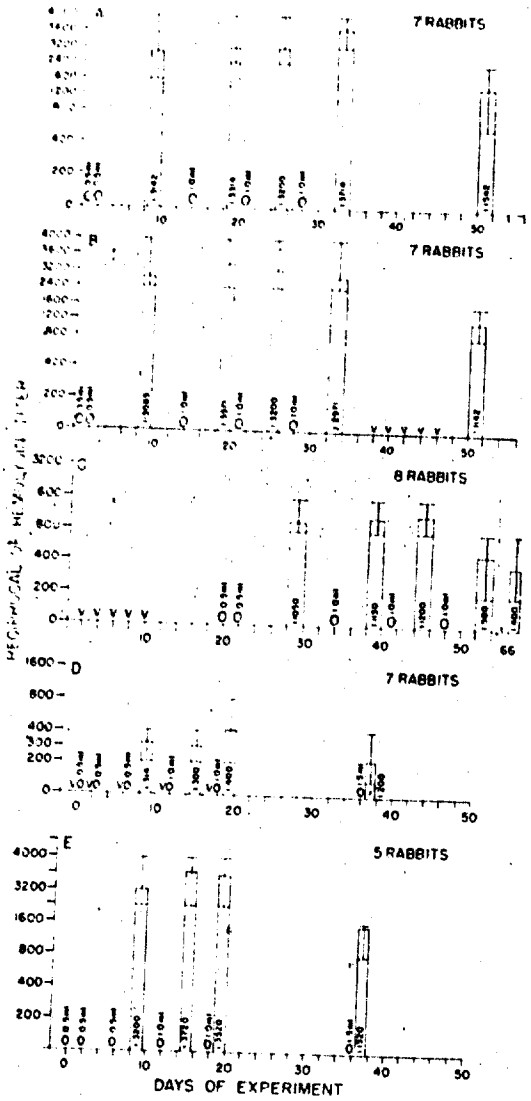

FIG. 4. *Average antibody titers representing 34 rabbits. (A, E) Rabbits receiving SRBC injections only; (B) rabbits receiving SRBC injections prior to injections of 3a-1 papain fraction; (C) rabbits receiving injections of 3a-1 papain fraction prior to SRBC injections; (D) rabbits receiving the injections of 3a-1 papain fraction 12 to 18 hr prior to SRBC injections. O = antigen, V = papain fraction, and vertical line joining two parallel lines at the top of each bar = range of titers.*

However, a 3-fold titer reduction was observed when the animals received papain 8 to 10 days before antigen inoculation (Fig. 4C), and a 10-fold decrease when papain was given 12 to 18 hr before the antigen (Fig. 4D).

The agglutinin response is indicated in Fig. 5A to D. The control group showed an average peak titer of 1:2,046 (Fig. 5A). Less than a two-fold increase in titer (1:1,152) occurred when papain followed the antigen injections (Fig. 5B). The average titers of these rabbits characteristically showed lower bacterial agglutination titers. When compared with the control group, therefore, this small difference was not significant. When papain preceded the antigen injections by 10 days (Fig. 5C) and by 12 to 18 hr (Fig. 5D), 25- and 50-fold titer reductions, respectively, were observed. Suppression of the precipitin antibody was also apparent (Fig. 6A-D), but only with rabbits which received papain 12 to 18 hr prior to the antigen. Peak titers in the control animals (Fig. 6A) occurred after the third horse serum injection (1:149). No significant titer differences were observed

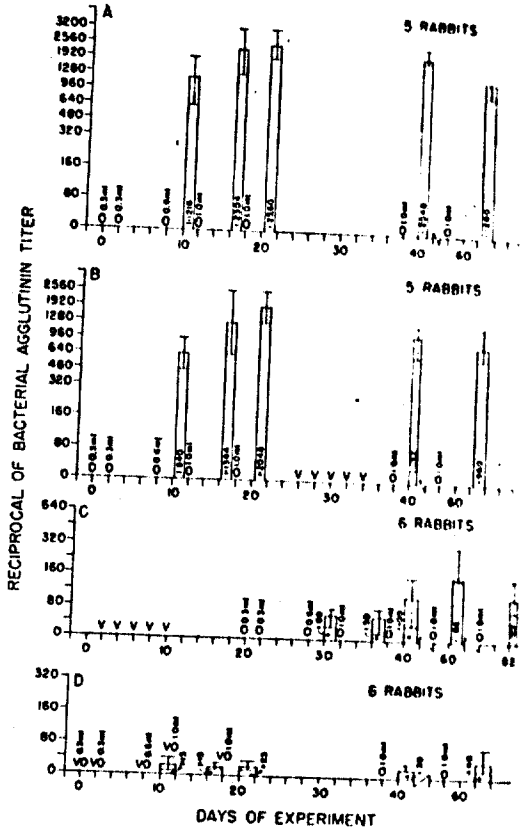

FIG. 5. *Average antibody titers representing 22 rabbits. (A) Rabbits receiving Salmonella choleraesuis injections only; (B) rabbits receiving Salmonella injections prior to injections of 3a-1 papain fraction; (C) rabbits receiving injections of 3a-1 papain fraction prior to Salmonella injections; (D) rabbits receiving injections of 3a-1 papain fraction 12 to 18 hr prior to Salmonella injections. O = antigen, V = papain fraction, and vertical line joining two parallel lines at the top of each bar = range of titers.*

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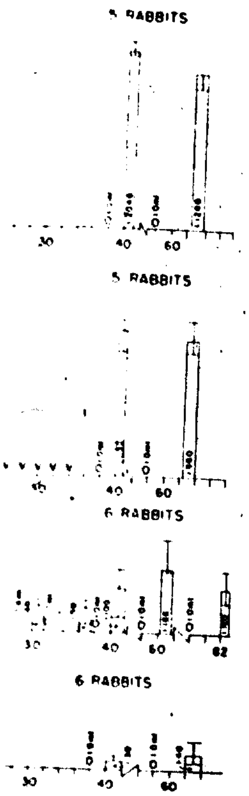

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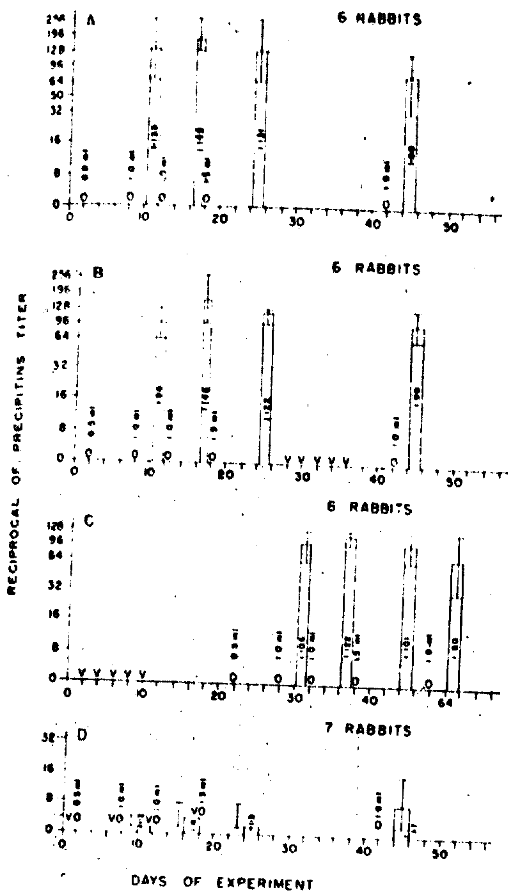

FIG. 6. *Average antibody titers representing 25 rabbits. (A) Rabbits receiving horse serum injections only; (B) rabbits receiving serum injections prior to injections of 3a-1 papain fraction; (C) rabbits receiving injections of 3a-1 papain fraction prior to serum injections; (D) rabbits receiving injections 3a-1 papain fraction 12 to 18 hr prior to serum injections. O = antigen, V = papain fraction, and vertical line joining two parallel lines at the top of each bar = range of titers.*

when the papain injections followed the antigen injections, or when the booster titers (Fig. 6B) were compared with the control-group titers. The two groups gave respective titers of 1:90 and 1:80. Similarly, no appreciable comparative titer differences were noticeable in the series in which papain was injected 10 to 12 days before the antigen injections (Fig. 6C). A 35-fold reduction, however, was again apparent when the papain was injected before the antigen (Fig. 6D).

DISCUSSION

The 3a-1 papain fraction was prepared from crude papaya latex. The predominant fractions prepared from papaya latex have been reported to be lysozyme (10), papain proper (8), and chymopapain (3). Fraction 3a-1 injected into our experimental animals contained all three substances, but was particularly rich in chymopapain.

Rabbits injected intravenously with the papain fraction, followed within 12 to 18 hr by antigen inoculations, showed the most marked immune suppression. Under the experimental conditions outlined, synthesis of hemolysin (SRBC), agglutinin (*S. choleraesuis*), and precipitin (horse serum) was impaired (Fig. 4D, 5D, and 6D). Furthermore, when papain was given 8 to 10 days after the antigen inoculations, normal antibody development occurred (Fig. 4B, 5B, and 6B). When the series of papain inoculations preceded two of the antigen injections (SRBC and *S. choleraesuis*) by 10 to 12 days, reductions in antibody titers were noticeable (Fig. 4C and 5C).

Although no adequate explanation is available at present for the effectiveness of the 3a-1 papain fraction in the suppression of the immune response, certain considerations appear to be worth mentioning: e.g., factors relative to the influence of papain upon the antigen, upon complete antibody molecules, and upon antibody forming cells.

In vitro mixtures of papain, particularly with SRBC and horse serum, would markedly alter the antigens, resulting in complete lysis of SRBC and proteolysis of horse serum proteins. However, we feel that the immunosuppressive effect is not the result of the in vivo action of papain upon the antigens. First of all, the in vivo indexes of papain activity must be considered. The aminopolysaccharide concentration (Fig. 2) and the α-1 lipoprotein and fetuin measurements (Fig. 3) indicate that maximal concentrations occurred 6 to 7 hr after intravenous inoculation. Antigens were introduced 10 to 11 hr after maximal papain activity occurred. Furthermore, previous studies (6) have shown that, after intravenous injection of papain, rather extensive depletion of tracheal, nasal, and auricular cartilage occurs, indicating saturation of these sites with papain. Admittedly, however, definite proof of papain alteration of antigen in vivo is not available.

Our observations also indicate that once antibody was demonstrated against SRBC, *S. choleraesuis*, and horse serum antigens, five series of intravenous papain injections on alternate days failed to destroy or modify the specificity of the antibodies. The antibody titer of these animals (Fig. 4B, 5B, and 6B) was essentially similar to the controls (Fig. 4A, 5A, and 6A). Furthermore, when the routine hemotoxylin and eosin staining technique was used after paraffin embedding of tissue, examination of experimental rabbit kidneys and spleen tissues (removed 6 to 8 hr after intra-

venous papain injections) exhibited no detectable histological alteration from similar control tissue.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service Predoctoral Traineeship No. 5 T1 GM 59803.

We are indebted to Harlan Papenfuss for his histological interpretations.

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PAPAIN AS AN ANTHELMINTIC FOR *HAEMONCHUS CONTORTUS* IN SHEEP.

By I. I. Drezancic.*

Introduction

Over the years there have been many attempts to control intestinal nematodes in man and animals with proteolytic enzymes as anthelmintics. Centuries ago, in Central and South America, fig sap from *Ficus glabrata* and *Ficus laurifolia*, known as lêche de higuerón, was taken internally as a cure for intestinal worms (Sumner and Sommers, 1947). Analysis of the fig sap showed that it contained a proteolytic enzyme now called ficin.

The proteolytic enzyme, papain, is obtained from the green fruit of the paw-paw, *Carica papaya* and its optimum pH for protein digestion is 7 to 7.5. Bromelin is a proteolytic enzyme obtained from the fruit and leaves of the pineapple, *Anana sativa* and its optimum pH for protein digestion is 7. These proteinases are unusually heat resistant and often maintain their activity at temperatures of 60° to 70°C. There are many other proteinases in plants, but their physiological function is unknown.

Certain of the plant proteolytic enzymes have technological uses. Commercially the most important is probably in tenderizing meat and other proteins by papain or bromelin (Colowick and Kaplan, 1955). These enzymes digest protein to amino-acids. The fresh latex quickly loses its activity by oxidation of its -SH groups but it can be restored by the addition of reducing agents (Liebmann, 1953).

Worms are able to exist in the alimentary tract without being digested because the nematode cuticle consists of keratin which is not attacked by intestinal enzymes. However, plant proteolytic enzymes can digest the keratinous cuticle of the nematodes, after which both the plant and alimentary enzymes continue the digestion of the internal structures of the parasite.

The plant proteolytic enzyme, papain, is marketed under such names as "Nematolyt," "Vermizym" and "Papain." The action of these enzymes on parasites was first investigated *in vitro* (Schmidt and Leidel, 1951; Liebmann, 1953). It was found that *Toxocara canis, Strongylus vulgaris, Dictyocaulus filaria, Protostrongylus rufescens, P. nigrescens, Haemonchus contortus* and *Heterakis gallinae* were macerated within one half to three hours after exposure to 0.5 to 4% papain suspension. Drezancic and Wikerhauser (1956) obtained the same result *in vitro* with *Ascaris lumbricoides (suis)*. Liebmann (1953) found that the eggs of *T. canis* were not affected in the same time *in vitro* but those of *S. vulgaris, D. filaria, P. rufescens* and *P. nigrescens* dissolved very quickly.

Experiments were then commenced *in vivo* in domestic and wild animals. It was usually recommended to withhold protein food for at least one day before treatment and that all food should be withheld for 12 to 24 hours. Boch (1954) treated foxes infested with ascarids and *Uncinaria stenocephala* with "Vermizym" and concluded that it was unsuccessful. Mendheim (1954) drew attention to good results with "Nematolyt" against ascarids and whipworms in monkeys. Schmidt and Leidl (1951) reported good results also in the control of ascarids in dogs and cats. Fraedrich (1953) claimed excellent results in the treatment of dogs infested with nematodes. Drezancic and Wikerhauser (1956) tested the action of "Vermizym" and "Nematolyt" against the ascarids of pigs. Results were checked by repeated worm-egg counts and by slaughtering the treated pigs. It was found that there had been no anthelmintic effect although in some pigs the dose was doubled. Subsequently "Vermizym" was used against ascarids in dogs but was successful in only 2 of the 13 dogs used. These results were contrary to those previously reported but were confirmed also in dogs by Enigk and Nickel (1955) and Holz and Pezenburg (1955).

Proteolytic enzymes were also used against intestinal nematodes in man. MacCarthy (1954) claimed success in 10 patients infested with ascarids and whipworms and Greif (1953) concluded that "Nematolyt" was successful against nematodes in man. Siegel (1954) considered that "Nematolyt" and "Vermizym" were effective against *A. lumbricoides* and *Enterobius vermicularis*. On the other hand, Hoekenga (1954) stated that "Nematolyt" was not successful for the treatment of ascaridosis in man and referred to Holz and Pezenburg (1955) who recorded only about 50 per cent. efficiency

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for the treatment of roundworms in man with "Nematolyt."

The cause of these discrepancies was investigated in the laboratory in Zagreb and the explanation was found to lie in the pH of the gastric juice. In dogs the pH is 1.5 to 2.0, in pigs 1.0 to 2.0 and in man 1.5 to 2.5; at this level of acidity the plant proteolytic enzymes are inactivated. It was found that the administration of sodium bicarbonate prior to the plant proteolytic enzymes produced good results in the control of ascarids in dogs.

No author has recorded any evidence of toxicity after the administration of papain and there is no evidence of any injury to the intestinal mucous membrane. Boch (1954) examined histologically the action of "Vermizym" at different doses on the intestinal mucosa of normal and artificially infested mice, guinea pigs and cats. Even in high concentrations there was no evidence of damage to the mucosa.

Drezancic and Wikerhauser (1956) in clinical trials with dogs and pigs found no evidence of any disturbances over a period of several weeks even after the dose was increased to 2 or 3 times that prescribed. In their experience there is no need to administer laxatives to animals treated with papain. It appears that papain is safe to use several times at short intervals and is safe for weak, sick and very young animals and for animals in advanced pregnancy.

Experiments with Sheep

While stationed at the McMaster Laboratory, the opportunity was taken to test the effect of papain on sheep experimentally infested with *H. contortus* and the results are recorded in Table 1. In the initial trials the dose was injected into the abomasum through the abdominal wall.

Sheep 16 was heavily infested with *H. contortus* and had a worm egg count of 114,000 per gram of faeces. The sheep was so weak it could not stand, the conjunctivae were white and it was apparent that it would die within a few hours. It was given 60 ml. of 10% sodium bicarbonate direct into the abomasum, followed after 1 minute by 10 g. "Nematolyt" in 60 ml. water. The sheep died 2 hours later and at post-mortem examination no worms were found in the abomasum or in the first part of the small intestine. Sheep 1 was given by mouth 10 g. "Nematolyt" in 60 ml. water without sodium bicarbonate after swabbing the mouth with 10% copper sulphate to stimulate closure of the oesophageal groove. Four days after treatment the worm egg count had fallen from 4,000 to 400 per g. and on the eighth day no eggs were found in the faeces.

Sheep 2 was given 60 ml. 10% sodium bicarbonate direct into the abomasum followed by 10 g. "Nematolyt" in 60 ml. water. On the fourth day after treatment the worm egg count had fallen from 4,000 per g. of faeces to 200 and, by the eighth day, to 0. Similar results were obtained with sheep 3, 5, 6 and 8. When the drug was given by mouth after swabbing with 10% copper sulphate the count dropped in sheep 9 from 11,000 to 200 and in sheep 10 from 17,000 to 400 and then rose to 1,000.

When the dose was reduced to 5 g., the egg count fell to 0 in sheep 11 and 13 and in sheep 14 it dropped from 8,000 to 1,400.

In sheep 4, 10 g. "Nematolyt" in 60 ml. water was given into the rumen through the abdominal wall and 14 days later the egg count had fallen to 0. In sheep 12, when 10 g. "Nematolyt" was administered after swabbing the mouth with copper sulphate the worm egg count dropped from 12,400 per gram to 400 after 13 days and it appears probable that in this sheep the drug entered the rumen instead of the abomasum. It would appear possible that when the drug enters the rumen its action is retarded but in the case of sheep 15, to which it was given into the rumen, the worm egg count fell on the third day from 14,800 to 2,200.

In sheep 7 the drug was given directly into the abomasum without sodium bicarbonate and the worm egg count fell on the fifth day from 32,000 per g. to 1,200.

Effect on *H. contortus* Eggs

H. contortus

The action of papain on the eggs of *H. contortus* was also investigated and it was found that 90% of the eggs were digested in a short time. In previous experiments on the treatment of ascarids in dogs, it was observed that when the worms were destroyed many more eggs were released in the faeces from the females and they were not destroyed by papain. In contrast, in sheep treated with "Nematolyt" both the female *H. contortus* and the eggs in the ingesta were destroyed.

Discussion

In 10 of 16 sheep treated with "Nematolyt" it appeared that all *H. contortus* were destroyed, in 4 sheep (7, 9, 10, 12) there was a great reduction in worm egg counts and in the remaining two sheep (14, 15) a considerable reduction took place.

TABLE 1.

Results of Treatment of H. contortus *Infestations with "Nematolyt."*

Sheep No.	Worm Egg Count Per Gramme of Faeces Before Treatment	Dose and Method of Administration	RESULT Worm egg count
1	4,000	10 g. in 60 ml. water; by mouth after throat swabbed with 10% $CuSO_4$.	Next day 3,400, 4th day 400, 8th day 0.
2	4,000	10 g. in 60 ml. water with 60 ml. $NaHCO_3$: by abomasal injection.	Next day 400, 4th day 200, 8th day 0.
3	20,000	10 g. in 60 ml. water: by abomasal injection.	Next day 8,200, 4th day 400, 14th day 0.
4	2,800	10 g. in 60 ml. water: by rumenal injection.	Next day 2,800, 4th day 3,600, 14th day 0, 16th day 0.
5	30,000	10 g. in 80 ml. water with 60 ml. 10% $NaHCO_3$: by abomasal injection	4th day 200, 6th day 0.
6	15,000	10 g. in 60 ml. water with 60 ml. 10% $NaHCO_3$: by abomasal injection.	4th day 0, 6th day 0.
7	32,000	10 g. in 60 ml. water: by abomasal injection.	5th day 1,200, 8th day 8,000, 13th day 12,000.
8	4,000	10 g. in 60 ml. water: by abomasal injection.	4th day 0, 6th day 0.
9	11,000	10 g. in 60 ml. water: by mouth after throat swabbed with 10% $CuSO_4$.	5th day, 1,200, 7th day 200.
10	17,000	10 g. in 60 ml. water: by mouth after throat swabbed with 10% $CuSO_4$.	5th day 400, 8th day 1,000.
11	2,800	5 g. in 80 ml. water: by mouth after throat swabbed with 10% $CuSO_4$.	3rd day 0, 7th day 0.
12	12,400	10 g. in 100 ml. water: by mouth after throat swabbed with 10% $CuSO_4$.	6th day 12,200, 13th day 400, 19th day 800.
13	9,000	5 g. in 80 ml. water: by mouth after throat swabbed with 10% $CuSO_4$.	3rd day 600, 8th day 0.
14	8,000	5 g. in 50 ml. water with 50 ml. 10% $NaHCO_3$: by mouth after throat swabbed with 10% $CuSO_4$.	3rd day 1,400, 9th day 1,800.
15	14,800	10 g. in 100 ml. water: by rumenal injection.	3rd day 2,200, 7th day 5,000, 14th day 3,000.
16	114,000	10 g. in 60 ml. water with 60 ml. 10% $NaHCO_3$: by abomasal injection.	PM after 2 hours. No worms or eggs in abomasum or first part of small intestine.

It is apparent that there is no need to administer sodium bicarbonate prior to "Nematolyt," because the pH of the abomasal contents is more favourable for the action of the enzyme than in man, dog or pig.

Papain has certain advantages over other anthelmintics. It is not toxic and is not absorbed from the intestine. Its destructive effect on the eggs of *H. contortus* is very valuable and may be of considerable importance in the epidemiology of nematode infestation by reducing the degree of contamination of the pastures by worm eggs.

Experiments with the treatment of *Trichostrongylus* spp. are in progress and early results are promising.

Summary

"Nematolyt" (papain) was a successful anthelmintic against *H. contortus*. It has some advantages over other anthelmintics as it is not toxic to the host and can be administered safely to young, sick or weak animals and to animals in advanced pregnancy. Its ability to destroy the eggs of *H. contortus* as well as the worms is of importance in the reduction of contamination of the pastures.

Acknowledgements

Thanks are due to Dr. D. F. Stewart for permission to work at the McMaster Laboratory and for assistance in the preparation of this paper. Special thanks are due to Mr. H. McL. Gordon, Mr. I. Pearson and Mr. B. Thomson for assistance in the work. I am grateful to the Food and Agricultural Organization of the United Nations Organization for the award of the Fellowship which enabled me to carry out this study.

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(*Received for publication 5th March*, 1957.)

ENZOOTIC METRITIS OF SOWS

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Introduction.

Sporadic cases of metritis in sows have occurred in our practice and have attracted little attention. In the past five years, twelve cases occurred in small groups of pigs maintained in association with dairies or to dispose of market garden refuse. During the same period in three commercial piggeries one case occurred in one herd and two in another but the third herd had eleven cases in a two year period. All cases were clinically identical and in the first instance were taken to be non-specific endometritis such as occurs sporadically in most species. The high incidence in one herd however gave rise to the suspicion that the disease might be a specific infectious disease. This report deals with the observations made on the one farm where the incidence was high.

Clinical Findings

Affected sows became ill within 17 to 36 hours after farrowing. Loss of appetite, cessation of milk flow indicated by continual sucking by piglets and their failure to fill out and recumbency were noted by the farmer. A severe temperature rise of 105 to 107°F occurred in all cases. Normal sows examined at the same time had temperatures of 102 to 102.5°F. Usually a purulent discharge was visible about the vulva; in some cases quite appreciable amounts (50-100 ml.) of thin, white, opaque, odourless material containing some mucus lay in a pool behind the sow. This discharge was not apparent after the third or fourth day. Heart rates were in the range of 100 to 150 per minute, in most cases being above 120. The udders usually

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Arch. Int. Pharm. Codyn Therap 159: 126-34 1966

THE ANALGESIC-ANTI-INFLAMMATORY ACTIVITY OF PAPAIN

BY

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(*Received for publication* 18-1-1965).

The use of proteolytic enzymes as therapeutic agents has received a degree of clinical acceptance during the last few years (1-3). These agents have been demonstrated to have some clinical efficacy in gastro-intestinal disorders (4), as local agents to debride or solubilize collections of proteinaceous material (5, 6), as thrombolytic (7, 8) and anti-inflammatory agents (9-12).

It was the purpose of this investigation to determine the analgesic-anti-inflammatory activity of papain [1] in the experimental animal.

METHODS

The following conditions were common to all of the methods: 1) drugs were administered orally as suspensions in 1 % gum tragacanth; 2) ten animals were used per dose, no animal was used more than once; 3) ED50 (that dose which was effective in 50 % of the experimental population) values were calculated according to probit analysis and confidence limits were established according to the method of LITCHFIELD and WILCOXON (13).

The drugs studied were papain and acetylsalicylic acid. The activity of papain is expressed in mg, one mg of the enzyme equals 1351 units. One unit is that quantity of proteolytic enzymes extracted from the Carica papaya which, under the conditions specified in the method of assay (14), will clot 2.64 microliters of milk substrate in 2 min at 45 C.

Randall-Selitto method. Manor Farms female rats weighing from 90 to 140 g were used. The RANDALL-SELITTO method (15) was modified as follows: inflammation was produced by the injection of 0.05 ml of a 20 % suspension of Brewers yeast into the

[1] The papain used in these studies was a highly purified extract of proteolytic enzymes from Carica papaya (Prolase 300 — Wallerstein Laboratories).

plantar surface of one hind paw of a rat. Pain threshold was measured in terms of mm Hg pressure applied to the inflamed paw requisite to the production of a pain reaction, i.e., withdrawal of the paw from under the pressure applicator.

The pressure apparatus is pictured in Figure 1. Pressure was applied through a fluid system at a constant rate of 20 mm Hg per sec by means of a motor driven unit which consisted of a motor (A) geared through a shaft to a metal extension of the plunger (B) of a 20 ml syringe (C); the outlet of the syringe was connected in series to a mercury manometer (D) and to a 10 ml syringe with a wooden peg with a hard rubber tip (E) attached to the plunger. Pressure contact was made between the hard rubber tip and the dorsal surface of the rat's hind paw. Pressure was recorded kymographically (F). The criteria for drug induced analgesia was an increase in the pain threshold of the inflamed paw.

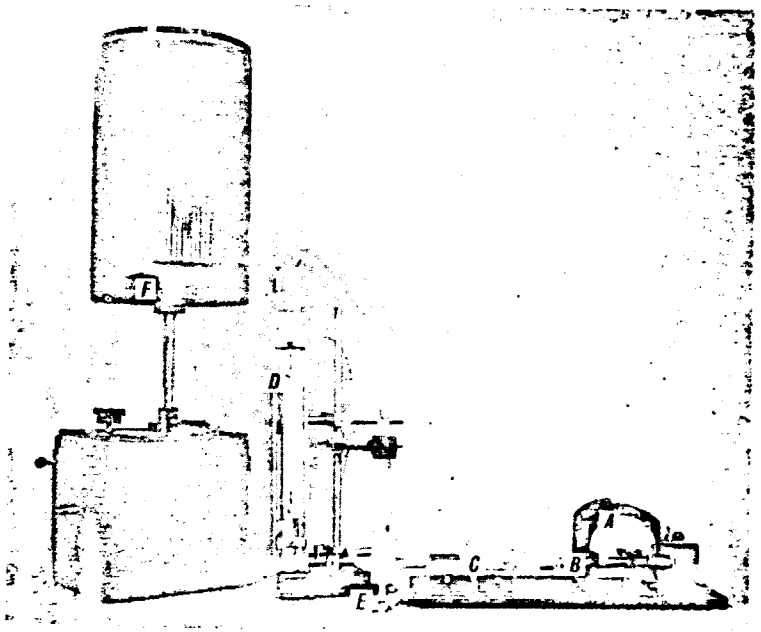

FIG. 1
Photograph of pressure apparatus

The circumference of the inflamed paw at the base of the toes was measured in mm by means of nylon thread attached to a mm scale. Drug induced anti-inflammatory activity was gauged by a decrease in the circumference of the inflamed paw following drug administration and was compared with that on the contralateral normal paw. Pain threshold and paw circumference were measured at 0, 1, 2, 4, 6 and 24 hours after drug administration.

The analgesic-anti-inflammatory activity of papain following acute and chronic ad-

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ministration was studied. In the acute studies the enzyme was given just prior to the drug. The chronic study was designed for the administration of papain, 400 mg/kg, twice a day for 2 consecutive days and once on the 3rd day; in these studies yeast-induced inflammation was produced on the first day of the study just prior to the first papain dose. The activity of the enzyme was studied one hour after the 4th and 5th dose.

Phenylquinone writhing syndrome. A modification (116) of the phenylquinone writhing syndrome described by SIEGMUND *et al.* (117) was used. Manor Farms female mice weighing from 18 to 22 g were kept separately in individual glass containers for critical examination. Each mouse was injected i.p. with 0.25 ml of an 0.02 % alcoholic solution of phenylquinone (Eastman Kodak #7106); all of the mice writhed within 10 min after the phenylquinone injection. Accordingly, a 10 min observation period was used as the cut-off time for these experiments. It has been established that 100 % of the estimated 9000 mice used during the past several years in these laboratories as controls in this method writhed within 10 min of the phenylquinone injection; therefore, in studying the effects of drugs, any deviation from the control of 100 % in an experimental population is statistically significant ($p < 0.01$) and is considered a true drug effect. The mice were challenged with phenylquinone at 15, 30, 60 and 120 min after drug administration. Dose response curves were established from an all or none response, i.e., the percentage of mice which did not writhe during the 10 min cutoff time after phenylquinone was plotted against the dose.

Bradykinin writhing. This method has been fully described (18). One hundred and eighty mice were used as controls. Each mouse was injected i.p. with 2.5 μg of synthetic bradykinin (1) in a total volume of 0.25 ml. The bradykinin induced writhing syndrome observed was similar to the phenylquinone writhing syndrome. The characteristic pattern consisted of abdominal torsion, drawing up of the hind legs to the body, marked contraction of the abdominal area and arching of the back so that the caudal ventral surface of the mouse touched the floor of the beaker. After the injection of bradykinin 5–10 % of the mice began to writhe within 10 min. The percentage of the animals writhing in the sample increased to a peak of 85 % at 20 min, plateaued at this percentage for the next 20 min and after this time the incidence of writhing gradually decreased. Therefore, the peak of writhing response occurred during the period of 20–40 min after the bradykinin injection. Groups of 10 mice per dose level were used. The drug was given orally, to the animals, 10 min later the mice were challenged with bradykinin and 20 min after the bradykinin injection the percentage of mice writhing in the group of mice was noted. The observation time during which the percentage of writhing was noted was 10 min. Drug activity was studied 30 min after drug injection. One writhe per mouse was considered a positive response.

Hot-plate method. A modification of the procedure described by EDDY and LEIMBACH (10) was used. Each mouse was its own control; a response time (that time between the placement of the mouse on a copper plate maintained at 55 ± 0.5 C and its blowing on its front paws or its fanning of its hind paws) to the thermal stimulus was measured, the drug to be studied was administered and again response times were measured at 15, 30, 60, 120, 240 and 360 min after drug administration. A 30 sec cut-off time was used; if the mouse did not respond on or before 30 sec it was removed from the hot plate. Drug activity was expressed in per cent increase of the response time of the drug treated mouse as compared to its control reaction time.

(1) San Ilar Pharmaceutical Company

RESULTS

Randall-Selitto method, acute studies. The injection of yeast into the plantar surface of the paw of a rat produced inflammation, descriptive by an average increase in the circumference of the paw of 7 to 8 mm within the first hour after the yeast injection. This degree of inflammation was maintained for 5 hours and then plateaued at a 5 to 6 mm increase for the next 48 hours. Concurrent with the inflammation was a decrease in the pain threshold of the paw; in a population of 100 rats the control pain threshold averaged 162 mm Hg, the peak decrease in this threshold averaged 66 mm Hg at 4 hours; at 24 hours the pain threshold averaged 90 mm Hg.

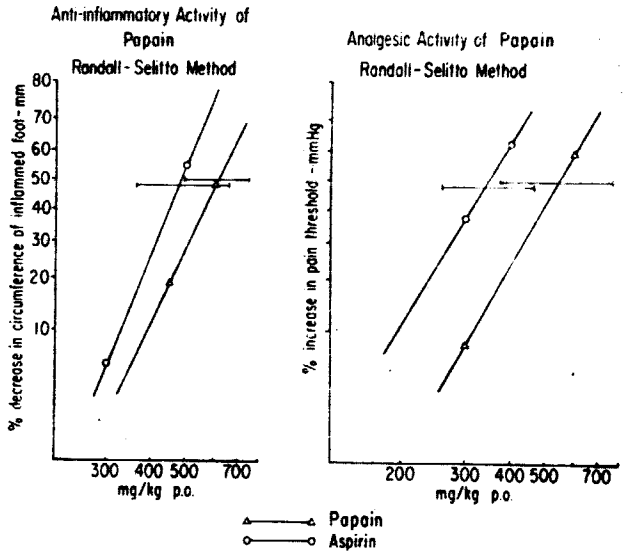

FIG. 2

Analgesic and anti-inflammatory activity of papain (Randall-Selitto method)

The administration of oral doses of papain ranging from 200 to 600 mg/kg produced a decrease in the circumference and a concurrent increase in the pain threshold of the yeast-induced inflamed hind paw of the rats. The onset of this effect was 1 hour and the peak effect was between 2 and 4 hours; the duration of action was greater than 24 hours. The effects seen with papain were also evident in the animals treated

with acetylsalicylic acid. The comparative analgesic and anti-inflammatory ED_{50}s of the two drugs presented in Figure 2 do not differ significantly (p .05) either between drugs or between pharmacological activities. The oral analgesic ED_{50} of papain was 540 (372-77) mg kg and that of acetylsalicylic acid was 345 (259-458) mg kg. The oral anti-inflammatory ED_{50} of papain was 610 (495-750) mg kg and that of acetylsalicylic acid was 485 (360-654) mg kg.

The rats which had been treated with the enzyme exhibited less edema and inflammation of the yeast injected paw than the non-treated animals. In Figure 3 the difference between the treated and non-treated animals can be visualized. Two hours after the yeast injection the paws of the control rats were markedly edematous and had a bluish-red discoloration;

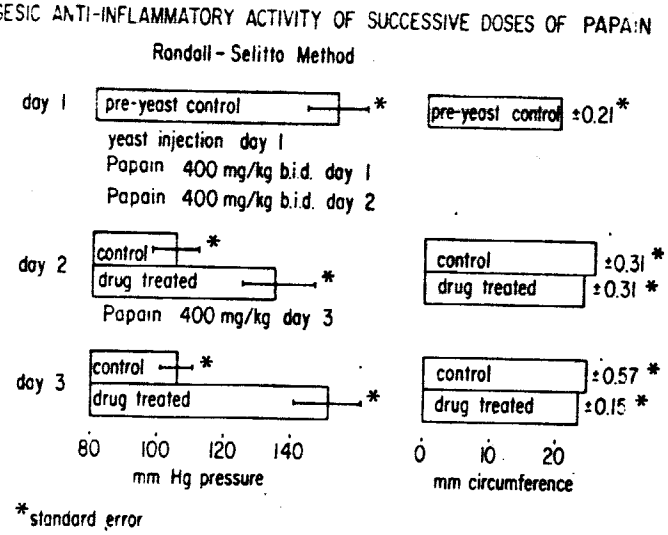

FIG. 4
Analgesic anti-inflammatory activity of successive doses of papain

in contrast the papain treated animals had only moderate edema of the paw and there was little if any discoloration. At 24 hours the paws of the papain treated animals more closely resembled those of the normal animal than those of the control yeast injected rats, the latter had little change in the edematous state of the paw. The treated rats exhibited greater mobility, were more active and had less alteration in gait than the non-treated controls.

Neither drug had any significant effect on the normal, uninflamed paw.

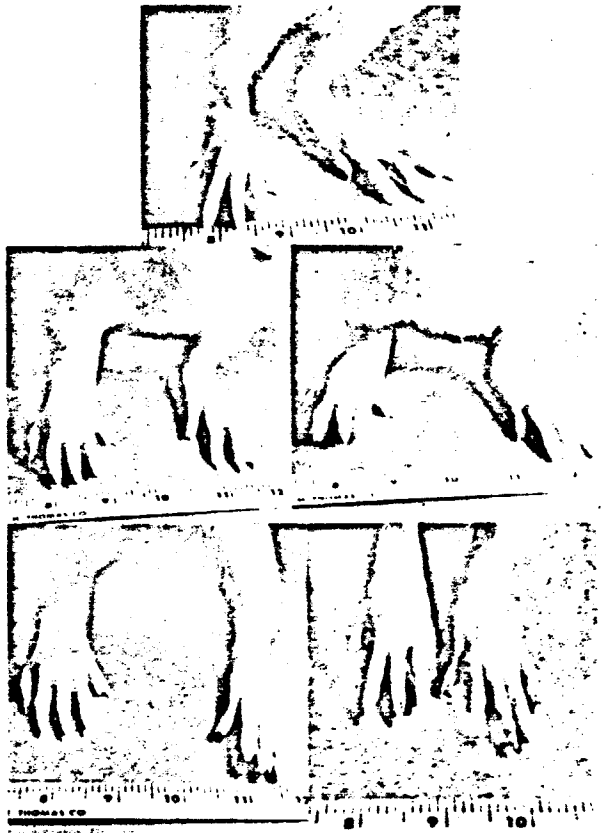

FIG. 3
Anti-inflammatory activity of papain.
A. Normal rat paws. — B. Control — 2 hours after yeast-induced inflammation of right hind paw. — C. Papain treated rat — 2 hours after yeast-induced inflammation of right hind paw. — D. Control — 24 hours after yeast-induced inflammation of right hind paw. — E. Papain treated rat — 24 hours after yeast-induced inflammation of right hind paw.

Randall-Selitto method, chronic studies. The results of this study are presented in Figure 4. The average pain threshold of the control rats was 153.6 ± 97 mm Hg; these rats had an average foot circumference of 20.65 ± 0.21 mm. Twenty-four hours after the yeast injection the pain threshold of the control rats was 105.6 ± 15.1 mm Hg compared to 134.[illegible] ± 20.5 mm Hg for the papain treated animals; the foot circumference of the controls at this time averaged 25.35 ± 0.31 mm compared to 23.9[illegible] ± 0.31 mm for the treated rats. At 48 hours the pain threshold for the control and treated rats was 106.8 ± 97 mm Hg and 151.6 ± 19.8 mm Hg respectively. The paw circumference was 24.5 ± 0.57 mm and 23.20 ± 0.15 mm respectively. The pain threshold of the drug treated rats was significantly greater than that of the control animals at 48 hours when the pain threshold of the treated rats was not significantly different from that of the normal, pre-yeast injected animals. The average circumference of the paw of the drug treated rats averaged 30 % less than that of the control non-treated animals.

Phenylquinone method. Papain produced no analgesia by the phenylquinone method; this is in contrast to the marked analgesic activity of acetylsalicylic acid which has an oral ED50 of 150 (121-186) mg kg.

Bradykinin method. Papain exhibited analgesic activity in this method; although the activity was inferior to that of acetylsalicylic acid. The comparative analgesic ED50 of papain and acetylsalicylic acid were 300 and 21 (11.9-37.2) mg kg, p.o., respectively.

TABLE I

Comparative Analgesic ED50s

Method	Papain	Acetylsalicylic acid
Phenylquinone Writhing	600	150 (129-174)
Bradykinin Writhing	300	21 (11.9-37.2)
Eddy's Hot Plate	630 (468-730)	1000
Randall-Selitto	540 (372-777)	345 (250-458)
LD50 mg kg, p.o.		800
Mice	3000	1050 (1302-2730)
Rats	4000	

Hot Foot method. It is accepted that this method is not sensitive to the analgesic activity of the salicylates or the weak analgesics; however, in contrast to the lack of activity of acetylsalicylic acid by this method, papain exhibited an oral ED50 of 630 (468-730) mg/kg.

The comparative analgesic ED50s and LD50s of papain and acetylsalicylic acid according to the various methods are presented in Table I. Because of the apparent lack of toxicity of papain the therapeutic ratio of this drug is much superior to that of acetylsalicylic acid.

Discussion

Papain, a highly purified extract of proteolytic enzymes from the Carica papaya, has been shown to effect oral analgesic and anti-inflammatory activity in laboratory animals.

The analgesic activity of papain has been demonstrated in the Randall-Selitto, Eddy hot plate and bradykinin writhing methods. The activity was demonstrated in the first method by the significant increase in the pain threshold of the inflamed paw of the treated rat requisite to the production of a pain reaction as compared to the pain threshold of the non-treated controls. The analgesic potency of papain, on a mg/kg basis, was not significantly different from that of acetylsalicylic acid. The similarity of papain to acetylsalicylic acid was extended to include the absence of analgesic effect on the normal, uninflamed contralateral hind paw of the rat. SELITTO (15) postulated that centrally acting analgesics affect both the inflamed and normal paw; but peripherally acting analgesics affect only the inflamed paw. It could be postulated on this basis that the mechanism of papain analgesia is a peripheral one possibly causally related to the anti-inflammatory effects.

It was of interest, however, that papain proved to have analgesic activity in the hot plate method, a method which does not detect the activity of weaker analgesics and a method which is divorced from the influence of attendant edema. Therefore, these results are suggestive of a mechanism of action of papain induced analgesia other than that associated with the anti-inflammatory property.

The inhibition of bradykinin induced writhing is suggestive of the efficacy of papain in pain and inflammation, inasmuch as bradykinin, a naturally occurring substance in both man and animals has been implicated in the physiological and pathological response of inflammation and associated pain (20). The anti-bradykinin activity of papain was not as potent on a mg/kg basis as was that of acetylsalicylic acid.

The lack of analgesic activity of papain in the phenylquinone method could be attributed to a type of pain, chemically induced, which is not affected by this enzyme extract. It is recognized in the field of analgesia that all such drugs are not applicable universally in algesic conditions.

The anti-inflammatory activity of papain was demonstrated by the decrease in the circumference of the inflamed paw of the treated rats as compared to those of the untreated controls. The rats which had received the enzyme exhibited less edema and inflammation of the yeast-infected paw and had a greater degree of articulation than the non-treated controls. There was no significant difference in the potency and apparent anti-inflammatory activity between papain and acetylsalicylic acid according to the Randall-Selitto method. There was no linear relation between the degree of analgesia and the degree of anti-inflammatory activity of either papain or acetylsalicylic acid. This lack of relationship between the attendant edema and the hyperesthesia has been reported by GILFOIL *et al.* (21) in his studies with acetylsalicylic acid.

Summary

The oral analgesic and anti-inflammatory activity of papain, a highly purified extract of proteolytic enzymes from the Carica papaya, has been demonstrated in laboratory animals. The potency of papain was not significantly different from that of acetylsalicylic acid in the Randall-Selitto method.

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MODIFICATION OF VASCULAR ACTIONS OF SYMPATHOMIMETIC DRUGS BY METHYLDOPA (1)

BY

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(*Received for publication* 22-1-1965)

Methyldopa is an effective inhibitor of DOPA decarboxylation as demonstrated *in vivo* and *vitro* by several workers (13, 11, 4, 12) It depletes catecholamines from tissue stores and also impairs the norepinephrine storage capacity of the tissues (8, 14) It also lowers tissue serotonin levels (1). The present work has been carried out to see how it modifies the vascular effects of catechols, sympathomimetics and sympathetic stimulation induced by central vagal stimulation and by carotid occlusion.

MATERIALS AND METHODS

The present study has been carried out in groups of five rabbits. The acute experi-ments with methyldopa did not give any significant results and the manner in which methyldopa modified the responses to central vagal stimulation, carotid occlusion and various drugs was observed in rabbits pretreated for 2 days, and compared with control rabbits 2 ml/kg of normal saline in control rabbits and methyldopa 100 mg/kg in test rabbits was given intravenously for two days. The experiments were carried out 16 to 24 hours after the last dose. The blood pressure recording and observations of the responses to various procedures were carried out after bilateral vagotomy in both groups of animals. Intravenous injections were given into the cannulated external jugular vein. A procedure was done or the drug was injected 10 minutes after the effect of previous procedures or drugs passed off. Central Vagal stimulation was done by stimulating both vagi with bipolar electrodes: 10 V square wave pulses of 5 millisecond duration were given at a frequency of 30 per second for an accurately timed 10 second interval. Maximum change during the 10 second period or few seconds thereafter was considered. Bilateral carotid occlusion was done for 30 seconds. In addition following drugs were injected: epinephrine 5 mcg/kg; norepinephrine 5 mcg/kg; dopa 1 mg/kg; dopamine 20 and 100 mcg/kg; tyramine 200 mcg/kg and amphetamine 250 mcg/kg.

(1) [illegible] work was included by one of us (RKS) in a thesis for the degree of M.Sc. [illegible] in Pharmacology of the University of Rajasthan.

months. Reproducible results have been obtained by 3 different research groups in laboratory, yielding a maximum patency of 4 weeks.

Summary. A technique is described for catheterization of the coronary sinus permitting a constant rate of sampling coronary sinus blood in states of normal and stress. This method, in combination with arterial blood samples and left coronary inflow, permits quantitative measurement of changes in left ventricular metabolism in chronic unanesthetized dogs.

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Received May 10, 1963. P.S.E.B.M., 1963, v113.

Submaxillary Gland Hypertrophy in Rats Fed Proteolytic Enzymes.* (28518)

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Previous studies(1) indicate that raw pancreas contains an orally active, heat-labile factor whose ingestion resulted in a marked hypertrophy of the submaxillary glands of rats. In this report data are presented indicating that a number of proteolytic enzymes of both animal and vegetable origin can induce a similar effect.

Procedure and results. Ninety male rats of the Long-Evans strain and 42 male rats of the Holtzman strain ranging between 50 and 62 g in body weight were divided into comparable groups of 6 animals each. One group of each strain was fed a basal natural food stock ration.† The remaining groups were fed the stock ration plus the various supplements indicated in Table I. Supplements were added in place of an equal amount of stock ration. Rats were placed in metal cages with raised screen bottoms (3 animals per cage) and were provided the various test diets and water *ad libitum*. Animals were sacrificed after 12 days of feeding and the submaxillary glands were removed and weighed. The latter were placed immediately thereafter in 10% neutral formalin solution, prepared for paraffin embedding in the routine manner, sectioned at 7 μ in thickness and stained with the periodic acid-Schiff technique and with hematoxylin and triosin. Data on body and submaxillary gland weights are summarized in Table I. In agreement with previous findings(1) desiccated and defatted raw pancreas at levels of 5% and 10% of the diet resulted in a highly significant increment in submaxillary gland weight. Papain and Protease P-6, enzyme preparations obtained from *Carica papaya* were also active in this regard, the former at levels of 5% and 10% of the diet and the latter at levels of 2½% and 5% of the ration. Crystalline chymotrypsin at levels of 0.2% and 0.5% of the diet and crystalline trypsin at levels of 0.1% and 0.2% of the ration also induced a marked increment in submaxillary gland weights. The parotid glands of rats fed the above supplements, however, did not differ significantly in weight from those of animals fed the unsupplemented stock ration. In contrast to findings with the proteolytic enzymes indicated above, pepsin when incorporated at levels of 2½% and 5% in the diet was without significant effect on submaxillary gland weight. In general supplements which induced a hypertrophy of the submaxillary glands also resulted in a reduction in body weight, degree of

*This investigation was supported in part by a research grant from Nat. Inst. of Dental Research, P.H.S., N.I.H.

†Staley's Rockland Rat Diet in Meal Form, A. E. Staley Manufacturing Co., Decatur, Ill.

TABLE I. Comparative Effects of Desiccated and Defatted Raw Pancreas and Various Proteolytic Enzymes on Weight Increment and Submaxillary Gland Weight of Immature Male Rats (6 animals per group).

Dietary group	Body wt (g) Initial	Final	Submaxillary wt (mg) Avg	Range	Submaxillary wt/100 g body wt (mg)
Long Evans rats					
Basal	57	129	321	224-375	248
Basal + following supplements:					
5% desiccated and defatted raw pancreas*	57	112	566	484-657	505
10% " " " " " *	57	86 (5)	629	566-703	729
5% papain†	57	103	406	303-512	391
10% " †	56	84	553	459-701	658
5% pepsin	57	140	329	297-383	234
2½% "	57	134	340	283-423	254
5% Protease P-6‡	57	73 (3)	808	738-864	1107
2½% " " ‡	57	96	564	444-784	586
.5 % alpha chymotrypsin§	56	58 (2)	638	622-654	1100
.25% " " §	56	126	444	416-496	352
.2 % trypsin, salt free‖	56	116	695	672-707	602
.1 % " " " ‖	57	119	465	434-504	390
.2 % " 50% $MgSO_4$¶	57	121	413	382-450	342
.1 % " " " ¶	57	125	382	355-407	305
Holtzman rats					
Basal	55	116	318	297-338	274
Basal + following supplements:					
5% desiccated and defatted raw pancreas*	56	97	829	698-978	857
10% " " " " " *	56	80 (4)	817	765-829	1022
.2% trypsin**	56	73 (4)	672	542-757	920
.1% " **	55	106	736	614-922	693
.5% chymotrypsin††	55	88	908	818-1046	1035
.2% " ††	55	113	768	687-893	682

The values in parentheses indicate the number of animals which survived and on which averages were based when this number was less than the original number per group.

* Viokase Powder 4 N.F. Pancreatin, VioBin Corp., Monticello, Ill.

† Papain Powder Purified Standard, Hathaway Allied Products, Los Angeles, Calif.

‡ Protease P-6 (a purified, standardized proteolytic preparation made from the latex of the green fruit of *Carica papaya* with 6,000 hemoglobin digestion units per g), Chas. Pfizer & Co., Inc., New York.

§ Alpha Chymotrypsin, General Biochemicals, Inc., Chagrin Falls, Ohio.

‖ Trypsin 2 × Crystalline, Salt Free, General Biochemicals, Inc., Chagrin Falls, Ohio.

¶ Trypsin, 2 × Crystalline, 50% $MgSO_4$, General Biochemicals, Inc., Chagrin Falls, Ohio.

** Trypsin, Crystallized (potency not less than 2500 Armour Units/mg), Armour Pharmaceutical Co., Kankakee, Ill.

†† Chymotrypsin, Crystallized (potency, 800-1200 Armour Units/mg), Armour Pharmaceutical Co., Kankakee, Ill.

growth retardation being proportional to the level of supplement fed. This relationship, however, was not invariable since chymotrypsin at a 0.2% level in the diet was without significant effect on body weight but did induce a marked hypertrophy of the submaxillary glands. Histologically, the submaxillary glands of rats fed the desiccated and defatted raw pancreas exhibited marked enlargement of the alveoli with pale, mucoid appearing, hypertrophied acinar cells, abundant PAS-staining cytoplasmic granules and a peripherally displaced, disfigured and hyperchromatic nucleus. Qualitatively similar changes were observed in the submaxillary glands of rats fed the papain, Protease P-6, chymotrypsin and trypsin supplements. In contrast to the above, the submaxillary glands of rats fed the unsupplemented stock ration had smaller alveoli, dense staining acinar cells and less PAS-staining cytoplasmic granules.

No data are available as to the *modus operandi* whereby trypsin and other proteolytic enzymes induced the effects noted above. It was not anticipated that significant amounts of these enzymes would have been absorbed from the intestinal tract or buccal cavity when administered as part of the diet although these possibilities have not been [illegible]

cluded. Further studies are indicated on the comparative effects of oral *vs* parenteral administration of proteolytic enzymes and of stomach tube feeding *vs* ingestion as a component in the diet. Such studies might indicate whether the effects of the proteolytic enzymes reported above were mediated at the buccal, intestinal or systemic level.

Summary. Immature male rats were fed a natural food stock ration supplemented with graded levels of trypsin, chymotrypsin or proteolytic enzymes derived from *Carica papaya*. A marked hypertrophy of the submaxillary glands occurred in rats fed the above diets in contrast to those of rats fed the unsupplemented stock ration.

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Received May 13, 1963. P.S.E.B.M., 1963, v113.

Activity of the Antibody-Complement System and Lysozyme Against Rough Gram Negative Organisms.* (28519)

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The killing of gram negative bacteria by [illegible] has been attributed generally to the [illegible]body-complement system(1). Once killed [illegible] the substances of this system, these bac[illegible] may be lysed by the action of lysozyme [illegible] Recent observations that purport to [illegible]fy this view, at least with rough organ[illegible], have been reported(3,4,5). In contrast [illegible]mooth strains of the gram negative en[illegible] bacteria, rough variants are extremely [illegible]tive to the bactericidal action of normal [illegible]m and this property constitutes one of [illegible] distinguishing characteristics of rough [illegible]nts(6). The bactericidal activity of pre[illegible]tral calf serum(3) and of the serum of [illegible]ts reared under sterile conditions with[illegible] maternal colostrum(4) against rough [illegible]ns has been attributed to non-specific [illegible]tances in the apparent absence of anti[illegible]. In another approach toward determin[illegible] the serum substances responsible for bac[illegible]dal action, with which this report is [illegible]ly related, the presence of antibody was [illegible]ered, but the interesting suggestion was [illegible] that it played no role in bactericidal [illegible] of normal serum(5). This was based [illegible] the finding that absorption of normal [illegible] with cells of a rough variant, *Escheri*[illegible] *coli* strain Lilly, resulted in a loss of lysozyme in the serum with a concomitant loss of bacteriolytic activity that was restored by addition of lysozyme to the absorbed serum. It was postulated that the killing and lysis of that organism resulted from the combined actions of complement and lysozyme without the mediation of antibody. The loss of activity resulting from the absorption was attributed to lysozyme rather than to antibody(5). This conclusion, which is incompatible with the classic concept that complement functions in the killing and lysis of bacteria sensitized by antibody, warranted further study.

Materials and methods. Egg white lysozyme, 2× crystallized, and histone, products of Worthington Biochemical Corp., were used. The cultures of *Escherichia coli* B and *Salmonella typhosa* Mrs. S were obtained from Mr. A. Abrams, Walter Reed Army Inst. of Research, and *Escherichia coli* Lilly from Dr. A. Wardlaw, Connaught Laboratories, Univ. of Toronto. These cultures satisfy most of the criteria used in defining rough variants(6). Sera were absorbed with 3× saline washed, heat killed (60°C for 1 hr) organisms using about 10^{11} cells per ml of serum. Antisera were prepared in rabbits by 2 subcutaneous injections of about 10^9 living organisms suspended in saline after 15 hours growth on meat extract. An interval of one week was allowed between injections and the

[illegible] work was aided by a grant from the Na[illegible] Inst. of Allergy and Infectious Diseases.

...e Toxici ...n injecte		...aine Hydro ...yaluronidase	
—With		...e—	
No. died	No. react.	...me of reactions, min	St. dev.
1	9	3.5	± 1.2
4	10	3.3	± 2.5
6	10	2.5	± .6
7	10	2.1*	± .6
10	10	1.8	± .5

36 mg/kg
28-45 mg/kg

...bability limits did hyaluronid... ...e toxicity of procaine injected s... ...y into mice. Hyaluronidase did ... ...e toxicity of tetracaine within th... ... From determinations of the p... ...ood of mice at various time interv... ...ub...neous injection, it was ... ...t ...locaine blood level increas... ...ginning with the second minute a... ...s peak at the eighth minute. T... ...blood level remained uniform ... ...eighth and twentieth minutes af... When hyaluronidase was mix... procaine injected subcutaneou... ...a more rapid rate of increase of ... ...blood level than that of the com... ...aximal procaine blood levels in ... ...were the same.

...ors wish to thank Dr. B. M. Benn... ... of Public Health and Preventive M... ...d in statistical analysis.

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Received May 6, 1952. P.S.E.B.M., 1952, v80.

Antifatty Liver Action of Papain and Ficin in Insulin-Treated, Depancreatized Dogs.* (19555)

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Previous work from this laboratory has shown that the fatty liver that develops in insulin-treated, depancreatized dogs can be prevented by the feeding of either methionine, choline, or hydrolysed casein(1,2). On the basis of these findings, it was proposed that the prevention of the fatty liver by raw pancreas, pancreatic juice and certain fractions derived from raw pancreas was the result of proteolytic enzymes furnished by the pancreatic substances(3). This view was later substantiated by the finding that crystalline trypsin, when added to the diet, also prevented the development of fatty livers(4).

The present report shows that prevention of fatty livers in depancreatized dogs can be achieved with plant preparations--papain and ficin--substances rich in proteolytic activity.

Experimental. The methods for assay of antifatty liver activity have been described elsewhere(5). Papain and ficin were fed twice daily to depancreatized dogs, for periods of 18-21 weeks, along with a diet consisting of 250 g of lean meat, 50 g of sucrose, 5 g of bone ash, and 1.5 g of salt mixture(4). Vitamin supplements(4) were fed once daily. Eight units of insulin were injected at each time of feeding. During the 3-4-week interval between pancreatectomy and the initiation of papain and ficin administration, the dogs were fed 25 g of raw pancreas with each meal in order to insure normal livers at the start of the assay period. At the end of the assay period, the dogs were anesthetized with nembutal. The entire liver was removed and thoroughly ground and mixed. Several samples were taken for the determination of total fatty acids(6).

Results. The fatty acid content of the livers of the control dogs fed no supplement ranged from 13.2 to 21.5% (Table I). The livers of the dogs fed papain were well within the normal range, *i.e.*, from 2.2 to 4.7%. Similar results were obtained with ficin-fed dogs; in these, the total fatty acid content of the liver varied from 1.6 to 2.5%. Throughout the course of the experiment the animals displayed a vigorous appetite. Ficin and papain did not prevent a loss in weight in these dogs.

Discussion. The capacity of papain to digest protein is well established(7), and its specificity, as determined on certain peptide linkages, is apparently similar to that of trypsin; indeed, papain is classified as a trypsinase (8). It is of interest to note that papain shows proteolytic activity over a wide range of temperature (10°-70°) and pH (3-12)(7). Although ficin has not been so extensively studied, it is nevertheless considered a papain-like enzyme(9).

Procedures for preventing the accumulation of fat in the liver of insulin-treated, depancreatized dogs can be classified into 1) those furnishing lipotropic substances in the free

* The work reported herein was supported by a contract from the Veterans Administration upon recommendation of the National Research Council's Committee on Veterans Medical Problems. We are indebted to Eli Lilly and Co. for the insulin used in this study.

† Parke Laboratories, Inc. Research Fellow.

TABLE I. Effect of Papain and Ficin Feeding on Fatty Acid Content of Liver of 16 Insulin-Treated Depancreatized Dogs.

Pre-operative wt, kg	Final wt, kg	Supplement Type	Supplement Amt daily, g*	Period fed, wk	Liver Wt, g	Liver Total fatty acids, % wet wt
9.5	7.2	None	—	20	670	21.5
6	5.5	"	—	24	580	13.3
7.9	6.5	"	—	19	310	37.3
9.2	8.2	"	—	12	720	17.2
13.1	9.2	Papain	.5	18	433	2.4
13.1	7.2	"	.5	18	443	4.5
12.8	9	"	1	21	433	3.1
12.8	11	"	1	21	382	2.5
10	9.4	"	1	21	432	3.1
9.7	6.3	"	1	21	356	3.5
13.8	10.7	"	4	21	630	4.7
6.4	6.6	"	4	20	463	2.2
14	8.7	"	4	20	495	2.8
8.3	6.6	Ficin	.5	21	400	1.6
9.1	7.8	"	.5	21	390	2.6
9.1	11.4	"	.5	18	420	2.5

* One-half of the amount recorded was fed with each meal.

form, and 2) those providing a means of liberating lipotropic substances from their bound form. There can be little doubt that the two plant enzymes, papain and ficin, act by replacing the loss of digestive capacity of the depancreatized dog. These results indicate that restoration of adequate digestive function, even with substances not specific to the animal organism, can prevent fat deposition in the liver.

Summary. It is shown that the development of fatty liver in insulin-maintained, depancreatized dogs can be prevented by the daily administration of the plant proteolytic enzymes, papain and ficin.

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Received May 6, 1952. P.S.E.B.M., 1952, v8[illegible]

Effect of β-propiolactone on Metabolism of *Pseudomonas aeruginosa* and Growth of Certain Fungi. (19556)

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From Duke University School of Medicine, Department of Physiology and Pharmacology, Durham, N. C.

β-propiolactone has been shown to be both bactericidal and virucidal and to have a relatively low toxicity(1-5). One of the organisms it is effective against; *Pseudomonas aeruginosa*, has been used in the following to study its possible mode of action. In aqueous solution the lactone hydrolyzes to β-hydroxypropionic acid, a fairly strong acid. Th

1582

from a micro-buret to a yellow end-point. Each ml. of 0.01 *N* sulfuric acid is equivalent to 0.75 mg. (750 mcg.) of aminopropanol.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Loss on drying, page 931. Dry at 56° for 4 hours in vacuum over phosphorus pentoxide.

Residue on ignition. Ignite a 1-gram sample as directed in the general method, page 945.

Packaging and storage. Store in tight containers.

Functional use in foods. Nutrient; dietary supplement.

PAPAIN

DESCRIPTION

Papain is a purified proteolytic substance derived from *Carica papaya* L. (Fam. *Caricacea*). It occurs as a white to light tan amorphous powder. It is soluble in water, the solution being colorless to light yellow and somewhat opalescent. It is practically insoluble in alcohol, in chloroform, and in ether. It may be adjusted, if necessary, to conform to the Codex requirement for papain activity by admixture with papain containing a higher or lower enzyme activity, or with lactose or other suitable diluents.

SPECIFICATIONS

Assay. Not less than 6000 N.F. Units of papain activity per mg.

(*Note:* One N.F. Unit of papain activity is the activity which releases the equivalent of 1 mcg. of tyrosine from a specified casein substrate under the conditions of the *Assay*, using the enzyme concentration which liberates 40 mcg. of tyrosine per ml. of *Standard Solution*.)

Loss on drying. Not more than 7 percent.

pH of a 1 in 50 solution. Between 4.8 and 6.2.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

TESTS

Assay

Sodium Phosphate Solution (0.05 *M*). Dissolve 7.1 grams of anhy-

drous dibasic sodium phosph
ml. with water, and add 1 dr

Citric Acid Solution (0.05
monohydrate, $C_6H_8O_7 . H_2O$, i
and add 1 drop of toluene as

Casein Substrate. On the
sten type casein in 50 ml. of t
in a boiling water bath for 30
room temperature, and add t
0.1, stirring rapidly and cont
precipitation of the casein. F

Phosphate-Cysteine-EDTA
transfer 3.55 grams of anhydr
into a 500-ml. volumetric fla
grams of disodium ethylened
$Na_2O_8 . 2H_2O$, and 3.05 grams
$C_3H_7NO_2S . HCl . H_2O$, and mi
hydrochloric acid or 1 *N* sodi
water.

Trichloroacetic Acid Solutio
acid, $C_2HCl_3O_2$, in 100 ml. of

Standard Solution. Transf
Standard, accurately weighed
in the *Phosphate-Cysteine-ED*
the solution, and mix. Pipet
metric flask, dilute to volume
within 30 minutes after prepa

Assay Solution. Dissolve
sample, equivalent to abou
Standard, in sufficient *Phosp*
make 100 ml. Dilute 2.0 ml.
solution.

Procedure. Into each of tw
ml. of the *Casein Substrate*, pl
10 minutes for the solutions to
of duplicate tubes, both of w
Standard Solution and 1 ml.
Solution (*P-C-EDTA Buffer*)
stopper, and replace in the bat
S_2, pipet 1.5 ml. of the *Standar*
Buffer, and proceed as with t
two tubes labeled S_3, to whic
added, and for two tubes lab
Solution and 0.5 ml. of the *P-C*
60 minutes, add to all twelve
Solution, and shake vigorous

Each ml. of 0.01 *N* sulfuric
of aminopropanol.
as directed for organic com-
senic Test, page 865.
2-gram sample as directed in
age 920, using 20 mcg. of lead

° for 4 hours in vacuum over

am sample as directed in the

containers.
ietary supplement.

stance derived from *Carica*
as a white to light tan amor-
the solution being colorless
It is practically insoluble in
ay be adjusted, if necessary,
papa ctivity by admixture
enzy. activity, or with lac-

f papain activity per mg.
rity is the activity which
yrosine from a specified
of the *Assay*, using the
40 mcg. of tyrosine per

ent.
and 6.2.

arts per million (0.0003 per-

n 10 parts per million (0.001

Dissolve 7.1 grams of anhy-

drous dibasic sodium phosphate, Na_2HPO_4, in water, dilute to 1000 ml. with water, and add 1 drop of toluene as preservative.

Citric Acid Solution (0.05 *M*). Dissolve 10.5 grams of citric acid monohydrate, $C_6H_8O_7 . H_2O$, in water, dilute to 1000 ml. with water, and add 1 drop of toluene as preservative.

Casein Substrate. On the day of use, disperse 1 gram of Hammersten type casein in 50 ml. of the *Sodium Phosphate Solution*, and heat in a boiling water bath for 30 minutes, stirring occasionally. Cool to room temperature, and add the *Citric Acid Solution* to a pH of 6.0 ± 0.1, stirring rapidly and continuously during the addition to prevent precipitation of the casein. Finally, dilute to 100 ml. with water.

Phosphate-Cysteine-EDTA Buffer Solution. On the day of use, transfer 3.55 grams of anhydrous dibasic sodium phosphate, Na_2HPO_4, into a 500-ml. volumetric flask, dissolve in 400 ml. of water, add 7.0 grams of disodium ethylenediaminetetraacetate dihydrate, $C_{10}H_{14}N_2Na_2O_8 . 2H_2O$, and 3.05 grams of cysteine hydrochloride monohydrate, $C_3H_7NO_2S . HCl . H_2O$, and mix. Adjust the pH to 6.0 ± 0.1 with 1 *N* hydrochloric acid or 1 *N* sodium hydroxide, and dilute to volume with water.

Trichloroacetic Acid Solution. Dissolve 30 grams of trichloroacetic acid, $C_2HCl_3O_2$, in 100 ml. of water.

Standard Solution. Transfer 100.0 mg. of N.F. Papain Reference Standard, accurately weighed, into a 100-ml. volumetric flask, dissolve in the *Phosphate-Cysteine-EDTA Buffer Solution*, dilute to volume with the solution, and mix. Pipet 2 ml. of this solution into a 50-ml. volumetric flask, dilute to volume with the buffer solution, and mix. Use within 30 minutes after preparation.

Assay Solution. Dissolve an accurately weighed amount of the sample, equivalent to about 100 mg. of N.F. Papain Reference Standard, in sufficient *Phosphate-Cysteine-EDTA Buffer Solution* to make 100 ml. Dilute 2.0 ml. of this solution to 50.0 ml. with the buffer solution.

Procedure. Into each of twelve 18 × 150-mm. test tubes, pipet 5 ml. of the *Casein Substrate*, place them in a 40° water bath, and allow 10 minutes for the solutions to reach the bath temperature. Into each of duplicate tubes, both of which are labeled S_1, pipet 1 ml. of the *Standard Solution* and 1 ml. of the *Phosphate-Cysteine-EDTA Buffer Solution* (*P-C-EDTA Buffer*), mix by swirling, note the zero time, stopper, and replace in the bath. Into each of two other tubes, labeled S_2, pipet 1.5 ml. of the *Standard Solution* and 0.5 ml. of the *P-C-EDTA Buffer*, and proceed as with the S_1 tubes. Repeat this procedure for two tubes labeled S_3, to which 2.0 ml. of the *Standard Solution* is added, and for two tubes labeled U_2, to which 1.5 ml. of the *Assay Solution* and 0.5 ml. of the *P-C-EDTA Buffer* are added. After exactly 60 minutes, add to all twelve tubes 3.0 ml. of the *Trichloroacetic Acid Solution*, and shake vigorously. With the four tubes to which no

standard or sample solutions were added, prepare blanks by pipetting, respectively, 1.0 ml. of the *Standard Solution* plus 1.0 ml. of the *P-C-EDTA Buffer*, 1.5 ml. of the *Standard Solution* plus 0.5 ml. of the *P-C-EDTA Buffer*, 2.0 ml. of the *Standard Solution*, and 1.5 ml. of the *Assay Solution* plus 0.5 ml. of the *P-C-EDTA Buffer*. Replace all tubes in the 40° bath for 30 to 40 minutes, allowing the precipitated protein to coagulate completely, and then filter through Whatman No. 40 or equivalent filter paper, discarding the first 3 ml. of filtrate. Determine the absorbance of each filtrate, which must be perfectly clear, at 280 mμ against its respective blank. Plot the readings for S_1, S_2, and S_3 against the enzyme concentration of each corresponding concentration. By interpolation from this standard curve, correcting for dilution factors, calculate the potency of the sample, in units per mg., by the formula $C \times (100/W) \times (50/2) \times (10/1.5) \times A$, in which C is the concentration, in mg. per ml., obtained from the standard curve, W is the weight of the sample, in mg., and A is the activity of the Reference Standard in units per mg.

Loss on drying, page 931. Dry in a vacuum oven at 60° for 4 hours.

pH of a 1 in 50 solution. Determine by the *Potentiometric Method*, page 941.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed under *Method II* in the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Packaging and storage. Store in tight, light-resistant containers in a cool, dry place.

Functional use in foods. Proteolytic enzyme.

PARSLEY SEED OIL

DESCRIPTION

The oil obtained by steam distillation of the ripe seed of *Petroselinum sativum* Hoffm. (Fam. *Umbelliferae*). It is a yellow to light brown liquid, having a rather harsh odor. It is soluble in most fixed oils, and in mineral oil. It is slightly soluble in propylene glycol, but it is insoluble in glycerin.

SPECIFICATIONS

Acid value. Not more than 4.0.

Angular rotation. Between −4° and −10°.

Refractive index. Between 1.513 and 1.522 at 20°.

Saponification value. Betwee

Solubility in alcohol. Passes

Specific gravity. Between 1.04

TESTS

Acid value. Determine as 893.

Angular rotation. Determin *Optical Rotation*, page 939.

Refractive index, page 945. fractometer of equal or greater a

Saponification value. De method, page 896, using 5 gram

Solubility in alcohol. Proc page 899. One ml. dissolves i sionally with slight haziness.

Specific gravity. Determine

Packaging and storage. Sto or other suitably lined container

Functional use in foods. Fl

P

DESCRIPTION

A hydrophyllic colloidal carbo extract of the inner portion of c Food-grade pectin is usually esterification, or methoxy conte for standardization of gel prop methylated polygalacturonic a glycan of α(1→4)-linked D-gala

In *low ester* pectin, the remai form of the free acid or as its a and in some types the acid amid the proportion of methoxyl a degree of polymerization.

In *high ester* pectin, portions esters, and the remaining carbox free acid or as its ammonium, properties may vary with the degree of polymerization.

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THE EFFECT OF PROTEOLYTIC ENZYMES ON ACID FORMATION IN THE MOUTH[1]

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It has been known for many years that fermentation is caused by the chemical action of enzymes rather than by the living organism, and that the enzymes are active even when the living organism which produces the enzyme is dead (1). It is now known that the production of acids in the mouth is also the result of enzyme action (2, 3) and that the mechanism involved is no exception to the general mechanism of fermentation and carbohydrate metabolism (4, 5). Most of the enzymes that have been isolated and identified are protein in nature (6, 7, 8, 9, 10, 11, 12) and if this is the case, it should be possible to inactivate them by means of substances that destroy the protein molecule. Thus, it might be possible to inhibit acid formation in saliva by the use of a proteolytic enzyme which would act on proteins under the normal conditions that exist in saliva. Among the proteolytic enzymes that are commercially available and are active in the reaction that prevails in normal saliva are papain, trypsin, and pancreatin. The effect of these enzymes in various concentrations is described in this paper.

EXPERIMENTAL PROCEDURE

Saliva was gathered by paraffin stimulation, well mixed, and divided into several different portions of 10 cc. each. One sample of the fresh saliva was analyzed for calcium. To each of the other samples were added 1 gram of glucose and 0.1 gram of 300 mesh powdered human tooth enamel. In addition to this, appropriate amounts of the test material were added to the samples, with the exception of 2 that were used as controls. Two samples of each concentration were made up in order to secure checks. The samples were then sealed and placed in a water bath and incubated at 37° C. with constant agitation. This constant shaking is essential in order to facilitate the solution of the enamel as fast as the acids are formed. After 4 hours of incubation the samples were removed and analyzed for calcium. This procedure is essentially the same as the chemical test for caries susceptibility (13). The acids formed were expressed as milligrams of calcium dissolved per 100 cc. of saliva. The results are in the Table I.

DISCUSSION

Contrary to expectations, the proteolytic enzymes did not cause an inhibition of acid formation. Trypsin and papain caused a marked stimulation, while

[1] This work was done under a grant from The Abbott Foundation for Medical Research.
Received for publication December 8, 1943.

pancreatin showed no appreciable effect. It is entirely possible that the protein of the enzymes concerned with carbohydrate degradation is more resistant to the action of proteolytic enzymes than is the normal salivary protein. No explanation as to the cause of the stimulation is offered.

TABLE I

The effect of proteolytic enzymes on acid production

TUBE	MG. ENZYME PER 100 CC.	MG. CALICUM PER 100 CC. SALVIA	
TRYPSIN			
0	control saliva before incubation	4.5	4.5
1	0	12.8	13.5
2	0	13.0	13.1
3	10	13.2	13.2
4	20	14.4	14.3
5	40	16.7	16.9
6	80	17.8	17.4
7	100	21.8	22.4
8	200	24.5	25.6
PANCREATIN			
0	control saliva before incubation	5.7	5.5
1	0	22.2	23.0
2	0	22.5	22.9
3	10	22.8	22.8
4	20	23.5	23.6
5	30	23.9	23.9
6	60	25.4	25.5
7	100	24.5	24.2
8	200	21.1	20.4
PAPAIN			
0	control saliva before incubation	5.5	5.4
1	0	13.2	13.2
2	0	13.0	13.2
3	14	13.4	13.8
4	28	14.1	14.2
5	56	15.4	15.5
6	112	16.0	17.9
7	224	17.9	18.1
8	448	22.5	23.5

SUMMARY

The effect of papain, trypsin, and pancreatin on the rate of acid formation in saliva has been studied. The trypsin and papain stimulate acid formation, while the pancreatin has no effect.

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sirable changes has led to their large-scale use as modifiers of food ingredients. Such commercial enzyme applications have grown from a relatively insignificant role to one of the most important aspects of food processing during the past quarter of a century.

The practical application of enzymes to accomplish certain reactions has been conducted for centuries. The fermentation of foods—beer, wine, bread, cheese—is older than recorded history. All fermentations are conversions produced enzymatically through the metabolism of living organisms, hence these food fermentations are examples of enzymatic modifications. Where a number of enzymes, constituting an enzyme system, are necessary to produce desired changes, it is advantageous to employ intact cells in fermentation processes. Where a single enzyme or a system of only two or three enzymes is involved in a desired reaction, isolated enzyme products are preferable to intact cells. A detailed treatment of fermentations is beyond the scope of this chapter.

Examples of ancient uses of enzymatic action, other than fermentations, are malt in brewing, stomach mucosa in milk clotting for cheese making, papaya juice for meat tenderization, and dung for leather bating. Of course, the ancient peoples using these natural products did not know there were such entities as enzymes. However, crude enzyme preparations extracted from animal tissues such as pancreas and stomach mucosa, or from plant tissues such as malt and papaya fruit, became articles of commerce.

The usefulness of the crude enzyme preparations was established, and when the biocatalytic enzymes responsible for their action became recognized, a search began for better, less expensive, and more readily available sources for such enzymes. Advances in enzymology and microbiology have resulted in more progress in enzyme production and application during the past 75 years than in the preceeding 5000 years. Early pioneers who laid the foundations for the production and use of industrial enzymes were Takamine,[167] Rohm[144] and Wallerstein.[192] The development of methods for large-scale production of enzymes, along with knowledge for controlling enzymatic processes and applications has resulted in a sizeable number of industrial enzyme products. These have been listed by deBecze by trade name, giving for each the producer, enzymes present, primary action on substrate, uses, and recommended pH and temperature ranges.[43]

Today, commercial enzyme preparations obtained from plant, animal and microbial sources are widely used by industry. Malt amylase and the proteases, papain, ficin and bromelain, from tropical plants are the best known enzymes from plant sources. Proteases, amylases and lipases from pancreas, pepsin and rennet from stomach mucosa, and catalase from liver are useful commercial enzymes from animal tissues. Certain microorganisms have been found to produce abundant amounts of useful enzymes, and these have become a major source for commercial enzymes.

Enzyme Theory

Before considering the specific applications for enzymes in present day food industries, it is desirable to consider briefly simple enzyme theory—what enzymes are, how they work, and factors affecting their action. This is necessary in order to understand and control the practical applications of enzymes.

catechuic acid, caffeic acid and hydroxygallic acid which are abundant in nature, through the action of enzymes originally called oxygenases. The preferred name is now polyphenol oxidase, although the terms phenolase and polyphenolase also are found in use. This entire class of enzymes has copper as the prosthetic group, and comprises a number of different enzymes distinguished by their specific substrates, such as tyrosinase, catecholase, laccase, etc.

The fruits apples, peaches, apricots, bananas, cherries, grapes, pears, strawberries, figs, and vegetables potatoes and red beets contain polyphenol oxidases and substrate compounds which cause enzymatic browning when the cut tissues are exposed to air. For the browning to occur three components must be brought together, enzyme, substrate and oxygen. If any of the three is missing, or prevented from reacting by some means, oxidation and browning will not take place. Natural control exists in many fruits and vegetables, as in cantaloupe and tomato which have neither enzyme or substrate, in the unique Sunbeam peach which has enzyme but no substrate, and most of the berries and citrus fruits which do not have the enzyme.

The polyphenol oxidases are quite sensitive to heat, and the blanching process is a means of control of enzymatic browning. Besides heat these enzymes can be inactivated by heavy metals, halogens, sulfites, cyanides. The heavy metals and cyanides cannot be used in foods because of their toxicity, but halogen salts and sulfites are very frequently used in the food industry. Sulfuring with sulfur dioxide or sulfites is the commonest method employed with most fruits and many vegetables before dehydration, to inhibit the browning reaction. Sodium chloride brines are used for a similar purpose for dipping sliced potatoes before dehydrating. The influence of pH in reducing the rate of browning is widely applied in the food industry. Apricots or peaches, after lye peeling, are immersed immediately into citric acid solution to reduce their pH far below the sharp optimum for polyphenol oxidase activity. A solution of 0.5% citric acid and 0.03% ascorbic acid may be used to prevent browning in cut fruits intended for freezing. In this case the ascorbic acid acts as an antioxidant.

Production of Industrial Enzymes

Industrial enzymes are produced from animal tissues, plant tissues and microorganisms. For example, commercial animal enzymes include pancreatin, trypsin, chymotrypsin and lipase obtained from pancreas, pepsin and rennet from stomach mucosa, and catalase from liver. Commercial plant enzyme products include ficin from fig latex, bromelain from pineapple, papain from papaya, and malt amylase from barley malt. A wide range of enzymes of all classes is obtained from a variety of species and strains of molds, yeasts and bacteria. Microbial production processes afford a degree of control of efficacy, quality and quantity which is difficult to achieve with plant or animal sources. For this reason, together with the greater number of enzymes readily available from microorganisms, microbial enzymes are assuming increasingly predominate roles as industrial enzyme products.

Enzyme preparations are obtained from animal and plant sources by collecting or pressing out the juices, or extracting the tissues with water. Microbial

is therefore so quickly inactivated during the baking process that the sticky-crumb problem is eliminated. According to unpublished reports the use of microbial protease in cracker doughs greatly decreases the mixing and holding time necessary to develop the gluten, promotes uniform rolling of the dough without stickiness or "bucking" at the edges, increases spread, gives exceptionally even baking, with uniform and improved browning, produces more open grain with enhancement of tenderness and flavor, and reduces the shortening requirements. Similar favorable results are obtained in baking of sugar wafers, vanilla wafers, and other cookies.

Another major application for proteases is in the brewing industry. A problem which has long been recognized in brewing is the tendency for an undesirable haze to develop in beer and ale when the beverages are cooled. The chill haze consists of a very fine precipitate which forms when beer is cooled below about 10°C. The formation of haze is accelerated by the action of light, heat, oxygen, or the presence of traces of copper or iron. The degree of haziness increases rapidly with extended storage or with higher storage temperatures. The chill haze has a variable composition, but generally contains protein, tannin and carbohydrates.[117] Since protein is a necessary component of chill haze, addition of protease enzymes for its prevention was the logical approach for solving the chill haze problem. Use of proteolytic enzymes has become the dominant method for chillproofing beers.

Almost any protease active at the normal pH of beer (about 4.5)—papain, pepsin, ficin, bromelain, fungal and bacterial proteases—can be used for chillproofing. Papain alone or in combination with other proteases is most commonly employed. The enzymes are added during the cellar operations. The amounts used vary depending upon the raw materials and processing method, and the anticipated length of storage and storage temperature. About 8 ppm of papain seems about an average level of addition to digest enough of the protein to prevent haze formation.[193] Foaming properties of the beer are not adversely affected by nominal levels of chillproofing enzymes.

There is no good correlation between enzyme potency as determined by conventional methods for protease assay, using hemoglobin, casein, gelatin or other proteins as substrates, and the performance of the enzyme in chillproofing. It is therefore necessary to evaluate enzymes for chillproofing by testing them at various levels on untreated, fresh beer as the substrate. Although quite laborious, methods have been developed and published for conducting such evaluations.[170,195] Once an evaluation has been made on beer to determine the proper use level, for a particular enzyme, this enzyme can then be used on the basis of its assay potency determined by a conventional method. For example, if 1 g of papain assaying 1000 units per gram is required per barrel, 2 g of papain assaying 500 units per gram would be necessary. However, with another enzyme such as bacterial protease assaying 1000 units per gram by the same method, no judgment can be made from this assay value as to the required amount to use. This can only be determined by actual evaluation with beer.

Still another use for proteolytic enzymes is in tenderizing meat, particularly beef. There is a great deal of variation in the toughness (or its opposite, tenderness) of beef depending upon the age of the animal and the part of the carcass from which it came. As discussed in a previous section of this chapter, beef

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growth when cobalamin is the growth factor, but not when methionine is supplied. It does not inhibit the growth of strains which do not require cobalamin for growth.

I am very grateful to Professor D. D. Woods, F.R.S., for his constant interest and advice, and to the Medical Research Council for a Training Scholarship. I am also indebted to Miss Jane Duncan for competent technical assistance. Dr E. Lester Smith kindly provided generous samples of the analogues of cobalamin. The research was aided by a grant for expenses from the Nuffield Foundation.

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Chemical Changes in Rabbit Aorta and Ear Cartilage Induced by Papain and Calciferol

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(*Received* 30 *October* 1959)

It has been shown both histochemically (Thomas, 1956) and chemically (Tsaltas, 1958) that the intravenous administration of a solution of crude papain decreases the mucopolysaccharide content of rabbit-ear cartilage.

With regard to the action of papain *in vivo*, it has been shown by McCluskey & Thomas (1958) that the injection of crystalline papain results in depletion of cartilage matrix only if the enzyme is inactivated by oxidation or sulphydryl blocking agents before administration. Cysteine-activated crystalline papain produces little effect on cartilage when injected. The changes produced by injection of inactivated crystalline papain were found to be indistinguishable from those produced by crude papain. These workers suggested that the reason for the failure of activated papain to enter cartilage after intravenous injection is that it preferentially reacts with one or more substrates present in [illegible]. Oxidized or inactivated papain, on the other [illegible], appears to be taken up by cartilage and [illegible] into the active form. In the present study a [illegible] tion of crude non-activated papain (L. L[illegible] Co. Ltd) in 0·9% sodium chloride solution [illegible] used.

It has also been shown that after toxic [illegible] calciferol in rats, there is an increase [illegible] hexose and hexosamine content of the aorta, [illegible] parallels the aortic mineralization which [illegible] (Gillman, Grant & Hathorn, 1960). Because [illegible] possibility that mucopolysaccharides may [illegible] important role in the process of calcification, [illegible] decided to test the effects of papain on the action [illegible] calciferol given simultaneously.

METHODS

Four young hybrid rabbits (three male and one female) with a mean weight of 1960±70 g. (S.E.) were randomly allocated to each of the following treatment groups (a total of 16 rabbits being employed): group I, these were injected intravenously in the left ear with a 1% solution of crude papain (L. Light and Co. Ltd.) in 0·9% NaCl solution (1 ml./kg. body wt.) on the first, fourth, seventh and tenth days of the experiment; group II, as in group I, but injected with 0·9% NaCl solution instead of papain solution; group III, papain injections were given as in group I, together with 0·5 mg. of calciferol (Petersen and Co., Cape Town)/kg. body wt. given orally daily for 10 days as a solution in arachis oil (15 mg./ml.); group IV, these were given injections of 0·9% NaCl solution as in group II together with calciferol as in group III.

The rabbits were killed after 10 days, and the cartilage of the right ear and the entire aorta was removed for chemical analysis. Freshly excised tissues were freed from connective tissue, rinsed in 0·9% NaCl solution, cut into small pieces and placed under dry acetone. After 24 hr. the acetone was renewed and allowed to stand for a further 24 hr. period. The acetone was then replaced with a mixture of ether and ethanol (1:1, v/v) and after standing overnight the solvent was removed and the tissues were dried to constant weight in a hot-air oven at 110°.

Hydrolysis. Portions of dry tissue (50 mg.) were heated with 4 ml. of 0·5 N-HCl for 4 hr. at 100° in sealed tubes. The solutions were then filtered and the volumes adjusted to 50 ml.

Hexoses. These were determined in 1 ml. portions of the hydrolysate with anthrone according to the method of Trevelyan & Harrison (1952), the results being expressed as glucose. Tests with glucose showed that the amount of destruction during the hydrolysis period was insignificant. The yield of hexose as determined by anthrone appeared to be maximal under these conditions.

Hexosamines. These were determined by a modification of the Elson & Morgan method (1933). The acetylacetone reagent was made by dissolving 1 ml. of redistilled acetylacetone in 25 ml. of M-Na_2CO_3 (A.R.) solution. To 1 ml. of hydrolysate 2 ml. of reagent was added and the mixture heated for exactly 15 min. in a boiling-water bath. After cooling, 6 ml. of diluted reagent [consisting of 1·6 g. of p-dimethylaminobenzaldehyde (Merck) dissolved in a mixture of 30 ml. of redistilled ethanol and 30 ml. of conc. HCl and diluted with 300 ml. of ethanol] was added. The tube was allowed to stand at room temperature for 10 min. with occasional shaking to remove CO_2 and the colour density of the solution was measured at 530 mμ in a Zeiss spectrophotometer, model P.M. QII. Each run included a duplicate standard (50 μg. of glucosamine). Tests with finely divided, acetone-dried and defatted aorta and cartilage and also with chondroitin sulphate showed that the hydrolysis conditions resulted in a maximum yield of hexosamine as determined by the Elson–Morgan reaction. Also, examination of the absorption spectra of the reaction products between 450 and 600 mμ showed a single well-defined peak with a maximum at 530 mμ, closely resembling the curve obtained with pure glucosamine. According to Rose (1953) this indicates that only small amounts of interfering chromogens are present, and it was not therefore considered necessary to separate the amino sugars by means of ion-exchange resin before analysis.

Calcium. Portions of hydrolysate (1 ml.) were diluted with 2 ml. of water and 1 ml. of 2·5 N-NaOH solution. The calcium was then titrated with 0·01 N-ethylenediaminotetra-acetic acid with the calcium indicator of Diehl & Ellingboe (1956), screened with thymolphthalein (Tucker, 1957).

Uronic acid. This was determined in 1 ml. portions of the hydrolysate by the method of Dische (1947). Glucuronolactone (L. Light and Co. Ltd.) was used for standardizing.

All chemical estimations were carried out in duplicate. No differences were found in the results from the male and female rabbits in each group.

RESULTS

The mean percentage composition of the ear cartilage is shown in Table 1, together with an analysis of variance of the individual results obtained in the 16 animals. It will be seen that papain significantly lowered the hexosamine and uronic acid content of the ear cartilage, irrespective of whether calciferol was administered simultaneously or not. In addition, calciferol resulted in a significant decrease in hexose, irrespective of whether papain was administered simultaneously. There was also an increase in calcium content of ear cartilage in the calciferol-treated rabbits, but the difference did not reach significant levels. Finally, the analysis shows that there was no significant interaction between papain and calciferol, i.e. they neither potentiated nor inhibited each other.

The weight of the fat-free aorta is expressed as dry wt./kg. body wt. As will be seen in Table 2, this weight was almost doubled in the calciferol-treated rabbits. Consequently, if the aortic constituents are expressed in terms of concentration, changes in their absolute values would be obscured. These values are therefore expressed as mg./aorta for a standard body weight (kg. body wt.).

Table 2 shows that calciferol resulted in significant increases in hexosamine and uronic acid (to just less than double) and an approximately 25-fold increase in the calcium content. These changes, induced by calciferol, were so marked that they tended, in the analysis of variance, to obscure changes induced by papain alone without simultaneous calciferol administration. A subsidiary analysis of the original data from groups I and II showed that papain significantly lowered the hexose, hexosamine and uronic acid content [Mann–Whitney U test (Siegal, 1956), $P < 0·05$].

Because of the marked differences in standard error (and hence variance) of the calcium results in Table 2, doubt may be cast on the applicability of an analysis of variance in this case. The original data for each group were therefore subjected to a non-parametric test. Similar conclusions were

Table 1. *Chemical composition of rabbit-ear cartilage*

NS, Not significant.

	Percentage of dry, fat-free tissue (mean ± S.E.*)			
	Hexose	Hexosamine	Uronic acid	Calcium
Papain	2·75 ± 0·37	1·64 ± 0·15	1·45 ± 0·13	0·39 ± 0·03
0·9% Sodium chloride	3·06 ± 0·04	1·99 ± 0·08	1·80 ± 0·08	0·42 ± 0·04
Papain + calciferol	2·15 ± 0·03	1·46 ± 0·19	1·43 ± 0·13	0·53 ± 0·11
Saline + calciferol	2·22 ± 0·18	2·04 ± 0·13	1·80 ± 0·16	0·63 ± 0·12

Analysis of variance

	F†	P	F	P	F	P	F	P
Main effects								
Papain	0·86	NS	8·72	<0·025	7·30	<0·025	0·56	NS
Calciferol	15·00	<0·01	0·10	NS	0·04	NS	4·52	>0·05
Interaction								
Papain + calciferol	0·29	NS	0·62	NS	0·04	NS	0·15	NS

* Standard error calculated as S.D./$\sqrt{(n-1)}$.
† Ratio of the variance due to the effect considered to the error variance. Tested with 1 and 12 degrees of freedom.

Table 2. *Chemical composition of rabbit aorta*

NS, Not significant.

	Aortic wt. (mg./kg. body wt.)	Weights [mg./aorta/kg. body wt. (mean ± S.E.*)]			
		Hexose	Hexosamine	Uronic acid	Calcium
Papain	30·2 ± 3·8	0·30 ± 0·02	0·15 ± 0·01	0·11 ± 0·01	0·29 ± 0·09
0·9% Sodium chloride	37·3 ± 3·9	0·41 ± 0·04	0·20 ± 0·02	0·19 ± 0·02	0·20 ± 0·04
Papain + calciferol	70·2 ± 9·7	0·46 ± 0·08	0·32 ± 0·04	0·25 ± 0·04	5·94 ± 1·05
0·9% Sodium chloride + calciferol	65·2 ± 4·6	0·44 ± 0·04	0·32 ± 0·02	0·25 ± 0·02	6·48 ± 1·53

Analysis of variance

	F†	P	F	P	F	P	F	P	F	P
Main effects										
Papain	0·01	NS	0·98	NS	0·91	NS	2·60	NS	0·06	NS
Calciferol	29·41	<0·001	4·03	NS	31·52	<0·001	15·65	<0·01	41·23	<0·001
Interaction										
Papain + calciferol	0·97	NS	2·05	NS	1·30	NS	2·02	NS	0·12	NS

* Standard error calculated as S.D./$\sqrt{(n-1)}$.
† Ratio of the variance due to the effect considered to the error variance. Tested with 1 and 12 degrees of freedom.

reached, namely that papain had no significant effect on aortic calcification whereas the differences induced by calciferol were highly significant (Mann–Whitney U test: $P < 0·001$).

DISCUSSION

The present findings in ear cartilage are consistent with previous reports (Thomas, 1956; Tsaltas, 1958; Spicer & Bryant, 1958) that papain decreases the mucopolysaccharide content of rabbit-ear cartilage. It should be noted that papain induced a similar decrease in the hexose, hexosamine and uronic acid contents of the rabbit aorta.

On the other hand, the effects of calciferol were different in the two tissues analysed here. Whereas calciferol induced a massive mineralization of the aorta, the calcium content of the ear cartilage was only slightly increased. Calciferol decreased the hexose in ear cartilage and did not affect hexosamine and uronic acid contents, whereas in the aorta it resulted in an increase in both hexosamine and uronic acid, unassociated with any significant change in aortic hexose.

In the rat, calciferol administration results in increases in aortic hexose and hexosamine (Gillman *et al.* 1960) whereas the present results show no significant change in the hexose content of the calciferol-treated rabbit aorta. These two experiments were performed under different conditions, however, and a species difference cannot therefore be inferred from our data.

We wish to draw attention to the remarkable difference in sensitivity of the rabbit and the rat to calciferol: on a body-weight basis rats tolerated a

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so approximately 12 times as great as that tolerated by the rabbit.

In conclusion, from these results it would appear that although papain decreases the hexosamine and uronic acid content of the rabbit aorta, it has no effect on the degree of mineralization induced by calciferol administered simultaneously; nor does papain interfere with the ability of calciferol to raise the content of these mucopolysaccharides in the calcifying aorta.

SUMMARY

1. Chemical changes induced by papain and calciferol in rabbit-ear cartilage and aorta have been determined.

2. Papain significantly lowered the hexosamine and uronic acid contents of ear cartilage whether calciferol was given simultaneously or not. Calciferol lowered the hexose content when it was given alone or with papain.

3. The weight of dry aortic tissue was almost doubled in the calciferol-treated rabbits owing to heavy mineralization. Aortic hexosamine, uronic acid and calcium were increased in the calciferol-treated animals, whereas the hexose content was unchanged.

4. In the papain-treated animals there was a decrease in aortic hexosamine and uronic acid. However, when given simultaneously with calciferol, papain had no effect on the extent of mineralization.

This study was aided by grants from the Wellcome Trust, the Nuffield Foundation, the C.S.I.R. and Mr Ronald Cohen.

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occurred in the control rats. A comparison of these ratios with that which resulted (1.8) from feeding papain without an added anti-acid makes it evident that the anti-acids neither increased nor decreased the effectiveness of the papain in the stomach. Thus, no evidence was obtained to substantiate the view that the stomach contents would destroy papain activity because of gastric acidity, or the lack of it, or other adverse environmental factors present in the stomach.

In the presence of each of the three anti-acids, the volume of solids in the stomach after centrifuging was less in the presence of papain than when heat-inactivated papain was fed. In each case, the differences are statistically significant. The results are in the same direction as the formol titration data and can be interpreted only as confirming the *in vivo* activity of the papain in digesting the protein of the test meal.

Thus, from the evidence presented here, it is clear that *in vivo* papain activity, as supplied by average dried papaya latex preparations, is easily and convincingly demonstrated by studies on the extent of intragastric digestion.

Summary

1. Studies with rats are described in which the extent of *in vivo* gastric protein digestion was followed by examination of the stomach and forestomach contents 2.5 hours after the ingestion of a test meal.

2. From the results of formol titrations of the gastric contents and measurements of the volumes of undigested residues, a 1.5- to 2.1-fold greater protein digestion, produced by feeding papain, was clearly demonstrated.

3. The presence of anti-acids did not modify the effect of the papain on digestion.

4. On the basis of these results, it was concluded that orally ingested papain has *in vivo* digestive activity. No evidence was seen that the gastric contents adversely affect the digestive activity of the enzyme.

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THE EFFECT OF THE ACTIVITY STATE OF THE ENZYMES ON THE *IN VIVO* ACTIVITY OF PAPAIN

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In earlier papers,[1,2] *in vitro* methods for the estimation of the activity of the proteolytic enzymes of papaya latex were compared. As pointed out there, these proteolytic enzymes exist in two activity states, the native or active state and the reversibly inactive state. The former digests proteins and clots milk without prior activation, whereas the latter exhibits its proteolytic properties only after activation by such agents as HCN, H_2S, mercaptans, cysteine, *etc.* It has been shown, however, that certain proteinaceous substrates, particularly beef[1-3] and heat-coagulated egg albumin,[4] have the ability to activate this inactive form during *in vitro* proteolysis.

Recently, Arnold, Schmitz, and Blumberg[5] have shown that papain, when added to a high protein test meal, significantly increased the extent of protein digestion occurring in the rat's stomach. The papain these investigators fed had approximately equal quantities of the enzymes in each of the two activity states. Additional investigations have therefore been carried out concerning the digestive power of these two activity states of the enzymes *in vivo*.

The activity of two papain samples having widely different ratios of active-to-reversibly-inactive enzymes were compared to determine the extent of intragastric digestion, and, more particularly, whether the reversibly inactive forms were active *in vivo*. Casein was chosen as the protein for the test meal, since previous investigations[1] had shown that it is not acted upon *in vitro* by the reversibly inactive enzymes. Beef or heat-coagulated fresh egg albumin could not be used for this test, since their *in vitro* activating effect might mask any *in vivo* activation.

Experimental

A. Comparison in vitro. A papain preparation, Papain A, with the enzyme content largely in the active state, was prepared from frozen fresh papaya latex by treatment with 0.5 per cent sodium bisulfite prior to drying, as has been described elsewhere.[6,7] National Formulary Reference papain was used as the low active, high reversibly inactive sample. A sample of commercial papain was irreversibly inactivated by autoclaving for 30 minutes at 15 lbs. to be used as a negative control. These three samples were tested by the Blau modification[1] of the Balls and Hoover[8] milk-clotting method, both with and without HCN activation. They were also tested by an egg digestion method[4] and a gelatin digestion-formol titration procedure.[1] The results are given in TABLE 1.

It may be seen that there is a vast difference between the native (not activated) activities of the two active samples. The ratios of the native activities and the total (native plus reversibly inactive) activities of the N. F. Reference papain and Papain A are 1:75 and 1:3.9 respectively, as measured by the milk-clotting procedure. The egg digestion method gives

a slightly lower ratio for the total activities, 1:1.5. It is apparent that if *in vivo* digestion is produced also by the reversibly inactive form of the enzymes, these two active preparations should show it quite clearly.

B. Comparison in vivo. Rats of about 200 gm. body weight were starved for 24 hours and then given a high protein test meal consisting of 1000 mg. of casein, 330 mg. of dextrose and the desired amount of papain added at the expense of the casein. Those animals that ate their test meal within 20 minutes were sacrificed two hours from the time the test meal was offered. At that time, their stomachs were removed and the contents washed into a suitable container. This suspension was adjusted to pH 4.85 with 0.1N NaOH and then centrifuged. The clear supernatant liquid was decanted and adjusted to pH 6.0 with additional 0.1N NaOH. Ten milliliters of freshly neutralized 40 per cent formaldehyde solution were then added and the solution titrated to pH 9.0 with 0.1N NaOH. The volume of alkali

TABLE 1

THE *In Vitro* ACTIVITY OF THREE PAPAINS

Sample	Milk clotting U.A. gm.*		Egg digestion, relative activity	Gelatin digestion, mEq.-NH_2†
	HCN activated	Not activated		
Papain A	361	361	1.52	3.14
NF reference papain	92.3	4.8	1.0	0.23
Autoclaved papain	0.0	0.0	0.0	0.0

* Units of Activity per gram.
† Milliequivalents of amino groups liberated by 100 mg. of unactivated papain digesting 20 ml. of citrate buffered (pH 5), 10 per cent gelatin solution for 2 hours at 40°C.

used in the last titration was a measure of the amount of amino groups present and, consequently, of the extent of protein digestion.[9]

For the first experiment, 50 mg. of the papain preparations were included in the test meals. The rats refused, however, to eat those containing this much Papain A. In the next experiment, 20 mg. of papain were used, but there was too much digestion in the Papain A group and a considerable portion of the digested material had left the stomach in the two-hour period. In two cases, the stomachs were practically empty. For the subsequent experiments, more nearly threshold doses of 1.25 mg. and 5 mg. of Papain A and 5 mg. and 20 mg. of N. F. Reference and autoclaved papains were fed.

Results

The results of the *in vivo* trials are given in TABLE 2. The autoclaved and completely inactive papain was used as a negative control to measure the amount of spontaneous digestion under the conditions. That it was completely inactive was shown by the lack of increase of digestion between the 5 mg. and 20 mg. doses respectively of this preparation. It may be seen that feeding active papain with the test meal materially increased the amount of digestion of the protein. This increase ranged from 34 to 101 per cent, depending on the dose of the papain given. The amount of digestion increased with the dose of papain in a regular manner. These

results, therefore, were clear confirmation that papain taken orally could increase significantly the amount of gastric digestion of protein.[5]

Since the values obtained with the 5 mg. and 20 mg. quantities of autoclaved papain are identical within the limits of variation of the method,

TABLE 2

In Vivo ACTIVITY OF PAPAIN AS MILLIEQUIVALENTS × 10 OF AMINO GROUPS LIBERATED DURING TWO HOURS' DIGESTION OF CASEIN IN THE RAT STOMACH

	Papain A		N. F. Reference papain		Autoclaved papain	
Dose	1.25 mg.	5 mg.	5 mg.	20 mg.	5 mg.	20 mg.
Number of animals	14	24	27	11	26	9
Mean	1.34	1.87	1.50	2.01	0.99	1.04
Standard errors of the mean	±0.048	±0.077	±0.070	±0.156	±0.040	±0.073

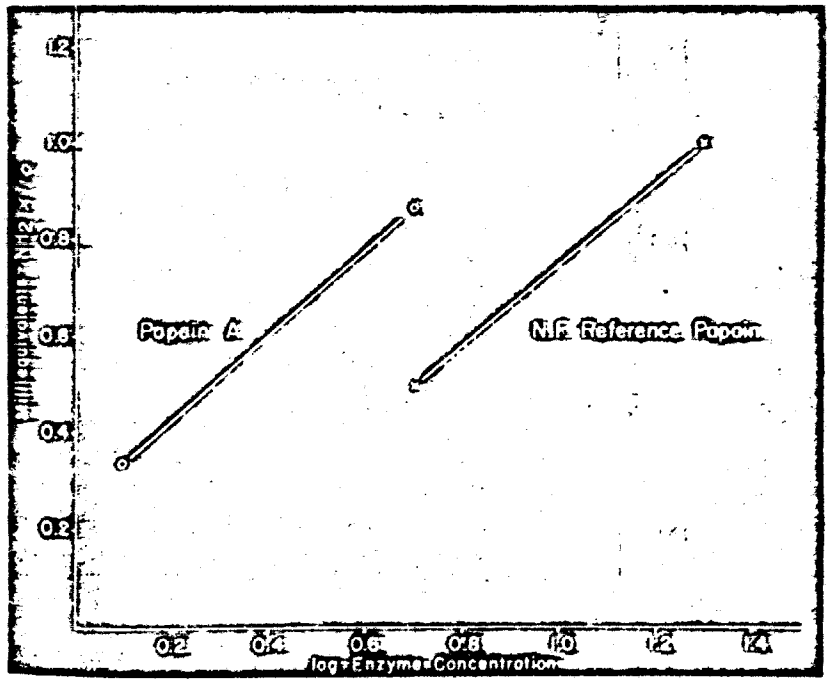

FIGURE 1. The average gain in amino groups liberated by papain during the *in vivo* digestion of casein in the rat's stomach.

they may be combined to give a control blank value for the other experiments. This yields an average control figure of 0.10 milliequivalents of liberated amino groups, which represents the amount of normal digestion. Subtraction of this value from the averages in TABLE 2 gives the average gains in liberated amino groups produced by the respective quantities of papain. These results are shown graphically in FIGURE 1.

Since the curves are substantially parallel, the ratio of activity of the two

samples may be readily calculated from them. The average ratio of *in vivo* potency of Papain A to the N. F. Reference papain, as well as the calculated ratios between the *in vitro* native and total activities of these samples are given in TABLE 3.

It is readily apparent that the *in vivo* ratio of activities of these papains agrees well with the *in vitro* activated milk-clotting value and with the egg-digestion figure, which is a spontaneously activated value. The native milk-clotting value, which does not measure the reversibly inactivated enzymes, widely diverges from the *in vivo* results. These ratios indicate that the reversibly inactive form of the enzymes contributes to the *in vivo* digestion of protein in substantially full measure.

It will also be noted in TABLE 3 that, although the *in vivo*, the activated milk-clotting, and the egg-digestion ratios of activity are of the same magnitude, there are apparently significant differences between them. The full significance of this is not clear, but it probably is connected with the activation phenomenon. Some indications for this are to be found in the literature on the activation of papain.[10, 11] Both Fruton and Bergmann[10] and Scott

TABLE 3

THE RELATIVE *In Vivo* AND *In Vitro* POTENCIES OF PAPAIN A AND N.F. REFERENCE PAPAIN

Test method	*Ratio of Papain A to N.F. Reference papain*
Rat, *in vivo*	2.7:1
Native, milk clotting	75:1
Activated, milk clotting	3.9:1
Egg digestion	1.5:1

and Sandstrom[11] have reported an increased activation of papain with thiols (H_2S, cysteine, or mercaptans) as compared with HCN. The Papain A used in our work contained none of the reversibly inactive enzymes and was therefore insensitive to activators. On the other hand, the N. F. Reference papain contains a high proportion of this form of the enzymes and is therefore amenable to activation. It may be that the activation of the N. F. Reference papain during the proteolysis of heat-denatured fresh egg albumin was due to either the original presence of, or the liberation of, thiols in the substrate. Thus, it would appear that the differences in the *in vitro* activity ratios resulted from differences in the activators involved. If this is the mechanism, then the *in vivo* activation can be assumed to be produced by an activator or activators having activating properties ranging between those of HCN and those in the egg albumin substrate. In any event, the data demonstrates that the reversible form of the enzyme contributes markedly to the digestive power *in vivo*.

Summary

The potency of the proteolytic enzymes of papaya latex for digestion of protein in the stomach has been examined by studies with rats. The extent

of digestion was estimated by formol titration of the stomach contents after test meals of casein plus various doses of papain preparations. Addition of papain to the test meal significantly increased the amount of gastric digestion.

A comparison of the activities of two preparations containing markedly different amounts of reversibly inactive enzymes demonstrated that this form of the enzymes, as well as the active form, is effective *in vivo*. From the results, it is evident that papain can facilitate the digestion of protein when taken orally, and that its *in vivo* activity is proportional to the total (active plus reversibly inactive) enzyme rather than to only the naturally active enzyme content.

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nis myopathy characteristically have been severe alcoholics, and one might speculate whether some of the patients in the cases reported were also imbibers of isopropyl alcohol.

Comment

Isopropyl alcohol has been reported as producing a wide variety of clinical manifestations, most of them common to ethyl alcohol intoxication.[1-3] However, certain features may serve to differentiate the two. Isopropyl alcohol is more toxic, probably because of its greater molecular weight, and this is felt to result in an intensified and prolonged course.[1] The presence of acetone in the breath, urine, serum, and gastric washing, in the absence of glycosuria or hyperglycemia, may prove an invaluable clue to diagnosis, since 15% of isopropyl alcohol is metabolized to acetone.[3,11]

The central nervous system is especially vulnerable to isopropyl alcohol and the patient is more likely to show deep refractory narcosis, areflexia, and depressed respirations.[2,3,12,13]

Isopropyl alcohol is much more irritating to the gastrointestinal tract and more likely to produce nausea, vomiting, and abdominal pain than ethyl alcohol.[2,3,14] Hematemesis and melena may appear early and they are related to ulceration of the gastric mucosa. The secretion of isopropyl alcohol by the salivary glands and the gastric mucosa tends to prolong the action of the alcohol.[1-3] This physiologic fact is of importance in therapy, since early and repeated gastric lavage may have considerable value.

Extensive hemorrhagic tracheobronchitis, bronchopneumonia, and hemorrhagic pulmonary edema have been found at postmortem examination. The mechanism of these changes is not clear, but may be related to exhalation of isopropyl alcohol by the lungs.

Generic and Trade Names of Drugs

Sodium chromate Cr 51—*Chromitope Sodium, Rachromate -51.*
Diphenylhydantoin sodium—*Danten, Denyl Sodium, Dilantin, Diphentoin, Diphenylan Sodium, Ekko.*

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Esophageal Perforation Following Meat Impaction and Papain Ingestion

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A patient had extensive destruction of the esophageal wall from the use of papain to soften and dislodge impacted meat from her esophagus. The sequence of events that occurred in this patient imply that this material, when it contacts a necrotic area in the esophageal wall, may induce further necrosis in the adjacent tissue which in turn leads to extensive destruction of the esophagus and surrounding structures. This method of treatment is contraindicated in treatment of impacted meat in the esophagus.

OBSTRUCTION of the distal part of the esophagus by impacted meat is a common cause of inability to swallow. The individual is frequently elderly and has ill-fitting dentures[1,2] though others may initiate this by bolting food. The endoscopic treatment may be difficult and hazardous, particularly after barium ingestion. In 1945 Richardson[1] advocated the use of papain for the digestion of impacted meat on the basis that this would not attack viable tissue. The enzyme, a popular meat tenderizer, is a powerful proteolytic enzyme obtained from the leaves of the *Carica papaya* tree.[2,4] It can digest 45 times its weight in lean meat.[2]

Clinical Material

From 1955 until the end of 1965, 27 patients received a suspension of papain at Duke University Medical Center for meat impacted in the esophagus. In the 28th patient, perforation and destruction of the entire esophagus occurred. The report of this case and the review of previous cases in which papain was utilized constitute the basis of this report.

Most of the patients treated at our hospital with papain usually either had esophageal disease or did not have teeth. The most common abnormalities of the esophagus were hiatus hernia (eight cases) and esophageal stricture (six cases). Ten patients had either no teeth or used dentures.

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Report of a Case

A 39-year-old woman was admitted to Duke University Medical Center with a history of acute esophageal obstruction for the past eight hours. This occurred while eating pork. Past history revealed that she had had symptoms of intermittent aphagia and epigastric pain during the preceding four to five years.

Physical examination revealed a markedly obese woman who occasionally gagged, spitting up moderate quantities of thick saliva. X-ray study showed an obstruction in the terminal region of the esophagus, which was thought to be a foreign body. For treatment of the obstruction, the patient was given sips of papain suspension. She received a total of 1.2 gm over the next 12 hours. After about six or seven hours of treatment, the patient noted relief of her dysphagia. Eight hours after the last dose, pain developed in the upper part of the abdomen and lower part of the chest. X-ray examination of the chest revealed bilateral pleural effusions, while thoracocentesis produced serosanguineous fluid. Though no free air was present in the thorax the diagnosis of a perforated esophagus was made. The patient immediately underwent a lateral thoracotomy on the left side. The esophagus was found to be perforated over a 2 × 3-cm area, with frank necrosis of most of the esophagus in the thoracic region. (Figure). The esophagus was then removed from the level of the superior thoracic inlet to the esophagogastric junction. The proximal region of the esophagus was brought out of the neck and a gastrostomy was performed. The patient did well until her tenth hospital day when she was found to be in shock, a large amount of blood having drained from her thoracotomy tube. Exploration of the chest revealed the chest tube had eroded the descending thoracic aorta. Resuscitative efforts were ineffectual and the patient died. Autopsy was refused.

Comment

In 1945 when Richardson[3] first used papain in the treatment of 17 patients with esophageal obstruction due to meat impaction, he utilized a 5% solution[2,4] which was dissolved in a 10% alcoholic vehicle. He recommended that 1 cc of this solution be administered every 15 minutes until the meat impaction passed. When as much as a teaspoonful could be taken, the meat was found to digest more rapidly. He found that 16 patients obtained prompt relief of symptoms with no untoward reactions and no complications from this therapy. However, in 1959, Andersen et al[5] reported a case history of a patient who was referred to the Mayo Clinic after having been treated with papain for esophageal obstruction. Within two hours after ingestion of papain this patient began swallowing liquid satisfactorily. However, approximately 12 hours after ingestion of papain the patient was discovered in a state of shock. During the ensuing two to three days, empyema developed in each pleural cavity and drainage was required. The patient was transferred to the Mayo Clinic and on the 12th day of her illness, two days after her arrival, massive hemorrhage developed. The patient died almost instantaneously from erosion of the wall of the common carotid artery. At postmortem examination most of the esophagus in the cervical region was found destroyed.

Andersen et al[5] carried out experimental studies on 14 dogs to determine the role papain might have played in digesting and perforating the esophagus of the patient seen at the Mayo Clinic. Almost

Section of esophagus with loss of esophageal epithelium and extensive hyaline-like necrosis of submucosal and muscular coats.

every dog receiving papain showed some degree of damage to the esophageal wall with reddening and thickening of the mucosal wall at the site of the lodged bolus. Microscopically, epithelium was denuded in a patchy distribution without much inflammatory reaction. However, perforation of the esophagus did not occur. Advanced hemorrhagic pulmonary edema was found to be the cause of death in these animals, which may have occurred from aspiration of the papain.

It is possible that in the patient reported here, the esophagus was perforated prior to the administration of papain, but the perforation could have been due to the proteolytic activity of the enzyme acting upon the ischemic wall of the esophagus in the area of meat impaction or upon an area of necrosis associated with the esophagitis. The continued administration of papain subsequent to the dissolution of the impacted meat may have contributed to the eventual perforation.

It is likely that the sequence of events was as follows: The meat, after being in place for a long period of time, caused necrosis of the esophageal wall. The papain digested this area. Perhaps the products of protein digestion caused other areas of necrosis which in turn were digested. Thus, a self-perpetuating reaction occurred. It is of interest that this patient, as well as the one reported from the Mayo Clinic, died from hemorrhage from a great vessel. In this patient there was some evidence that the hard rubber catheter may have eroded the aorta.

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Plasma Antithrombin Alterations Following Oral Papain. (26669)

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We have recently found that oral administration of papain* results in therapeutic activity in man of the same character as that resulting from buccal administration(1). Since this therapeutic efficacy was not accompanied by the untoward side-effects sometimes seen with intravenous enzyme therapy, it appeared that the enzyme was probably not absorbed, but that a plasminogen activator, released by the action of papain in the upper gastro-intestinal tract and absorbed from that site, could be implicated. The lack of systemic absorption of papain is further suggested by the fact that the oral mouse LD_{50} of this enzyme is in the neighborhood of 15,000 mg/kg(2).

Roche e Silva, Andrade, and Teixeira(3) have shown that intravenous infusion of proteolytic enzymes into animals results in the appearance of an antithrombin-like component in blood plasma. Niewiarowski and Kowalski confirmed this, using streptokinase and plasmin in the cat(4). The latter authors have described the anticoagulant as a high molecular weight product of fibrinogen digestion, which can be destroyed by further proteolysis(4). On the basis of this and their own work(5), Wallen and Bergstrom postulated that the protein fragments formed during fibrinogenolysis, and which are substrates for thrombin, can act as competitive inhibitors in the proteolytic reaction of thrombin on fibrinogen, thus contributing to antithrombin activity.

With this background, the present study has been carried out to ascertain whether or not oral papain administration can produce *in vitro* changes which will signal its activity. This work does not purport to show that oral papain alters *in vivo* clotting mechanism. In fact, in clinical application, no signs referable to changes in clotting mechanism have been observed with oral papain.

Methods. Fifteen hospitalized patients who were recovering from recent fractures served as subjects. Blood specimens were collected with 0.4 ml of 5% potassium oxalate for 5 ml of blood.

For assay of plasma antithrombin, the method of Fletcher, Alkjaersig, and Sherry (6) was employed. This test measures clotting time of a patient's plasma following *in vitro* addition of a standardized thrombin† solution. Euglobulin clot lysis determinations were made according to von Kaulla and Schultz(7).

As a low dose of papain, 10 mg were administered every 4 hours by mouth for 48 hours. As a high dose, 200 mg were administered every hour for 4 hours to the same patients. One control clotting time estimation served for both administrations in each patient. Papain was administered as 10 mg uncoated tablets, since enteric coated papain has been previously found to be clinically relatively ineffective(1).

Results. Table I clearly demonstrates that the small dose of papain caused a marked increase in plasma antithrombin activity. The high dose resulted in an equally consistent reduction in plasma antithrombin activity. Both sets of results differ from control levels in a statistically highly significant manner. The reduction in plasma thrombin clotting time below that of the control may be explained by the terminal hydrolysis not only of antithrombin formed from fibrinogen but also of the normally circulating antithrombin present in plasma. It is of further interest that patients with reduced thrombin clotting times showed marked acceleration in euglobulin clot lysis time. This finding implies that under the test conditions reduced thrombin clotting time seems to be related to enhanced proteolysis (Table II).

* Papase—Warner-Chilcott Laboratories.

† Topical Thrombin, Parke-Davis.

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TABLE I. Plasma Thrombin Clotting Time (Seconds).

Reaction mixture: .1 ml plasma + .1 ml thrombin			
Patient	Control	After 4 hourly doses of 200 mg each	After 12 doses of 10 mg q.4.h.
R.B.	17	10	45
D.L.	16	10	35
S.K.	18	9	35
T.G.	17	12	40
R.V.	17	10	>600
D.L.	18	11	24
Y.F.	17	10	>600
E.C.	16	10	27
M.V.	17	9	24
S.A.	17	9	>600
I.D.	17	12	30
S.L.	16	11	25
R.W.	19	12	27
H.J.	16	10	26
D.S.	17	10	31
Means	17	10	25–37*
		p = <.01	p = <.01†

* Calculated true mean is between 25–37 at p = .99.

† p = <.01 when values >600 were excluded. p = .02–.05 when values >600 were included. The paradoxical decrease in significance was due to the large increase of stand. error when the >600 figures were added.

TABLE II. Euglobulin Clot Lysis Time after Oral Papain (Hours).

Dose & frequency	Control	Treated
200 mg/hr for 4 hr	6	1
Idem	7	3
"	9	2
"	9	3
"	8	2
"	6	2
"	6	3.5
"	7	3
"	7	2
"	6	2
"	8	1
"	9	3
"	8	3
"	7.9	2
"	6	1
Mean	7.3 (6.7–7.9)*	2.2 (1.8–2.6)*

* 95% confidence limits p = <0.01.

More recent studies appear to show that by increasing the quantity of fibrinogen (plasma) in the thrombin-fibrinogen reaction mixture, a prolonged thrombin clotting time is produced with high papain dosage as well as with low doses. This is compatible with the postulate of Niewiarowski and Kowalski (4) that thrombin inhibition is regulated by concentration of fibrinogen-derived polypeptides in this system.

Summary. 1. Administration of small oral doses of papain to human patients results in production of a plasma antithrombin agent demonstrated by prolonged thrombin clotting time *in vitro*. 2. Administration of large oral doses of papain to human patients results in a decrease in thrombin clotting time, and an acceleration of euglobulin clot lysis time. 3. These experiments demonstrate that orally administered papain produces a systemic effect, although in all probability it is not itself absorbed.

We are indebted to Mr. N. Stasilli of the Warner-Lambert Research Inst. for statistical computations.

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Life Sciences No. 9, pp. 671-675, 1963. Pergamon Press, Inc. Printed in the United States.

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Papain

PEPTIDASE ACTIVITY IN RAT URINE FOLLOWING ORALLY GIVEN PROTEASES

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(Received 10 July 1963)

ANTAGONISM to bradykinin induced pain, vasodilatation, capillary permeability and smooth muscle contraction followed orally given proteases.[1,2] The underlying mechanism was unknown, but direct inactivation of bradykinin appeared unlikely since streptokinase, plasmin and trypsin do not hydrolyze bradykinin. The possibility was considered, therefore, that orally given streptokinase or proteases participate in the activation of peptidases which hydrolyze bradykinin.

The purpose of the present investigation, therefore, was to measure urinary peptidase activity following orally given kinases and proteases in rats.

Materials and Methods

This study comprised 80 Sprague Dawley rats. Leucine aminopeptidase was measured according to Goldbarg and Rutenberg.[3] Trypsin-like enzymes was measured according to Blackwood and Mandl.[4] Carboxypeptidase B activity was measured according to Folk et al.,[5] using hippuryl - L - arginine as substrate.

Procedure

Rats were fasted for 24 hours preceding the test and maintained on water ad libitum. Medication was dissolved in 1 ml. saline and introduced via stomach tube every two hours for six hours. 20 control rats received placebo tablets. Treated rats were divided into three groups of 20, and treated as follows: Group 1 received 200 mg papain per dose; group 2 received streptokinase plus human plasma, 90,000 units per dose; group 3 received 500 mg fungal

protease per dose. Sixteen hour samples of urine were collected under glacial acetic acid (0.01 ml/per rat).

Uterine Stimulating Activity

A rat uterus was suspended in an organ bath containing 17 ml Tyrode's solution. The temperature was 30°C and 100% O2 was bubbled through the bath. The following substances were introduced into the bath: bradykinin 1.5 μg leucine aminopeptidase* 2.5 mg control urine 0.1 ml treated urine 0.1 ml.

Results and Discussion

Rats treated with orally given kinases or proteases developed significantly greater urinary peptidase activity than placebo treated controls (Table 1). In control rats trypsin-like activity was 0.041 mg trypsin equivalents (Colorimeter readings were converted to μg naphthylamine released from a calibration curve and expressed as mg trypsin equivalents). The mg trypsin equivalents in treated rats were: fungal protease treated, 0.66; papain treated, 0.58; streptokinase treated, 0.61. In control rats leucine aminopeptidase activity was 473 units, average. The increases in treated rats were: streptokinase, 59%; papain treated, 102%; fungal protease treated, 92%. In control rats carboxypeptidase activity was 176 units. The increases in treated rats were: papain treated, 105%; fungal protease treated, 78%. It appears, therefore, that following orally given streptokinase, papain or fungal protease a broad spectrum of urinary peptidase activity is engendered with potential points of attack at C-terminal, N-terminal and intermediate linkages of peptide molecules. Augmented peptidase activity of this character and magnitude would be expected to yield peptides in different stages or degradation, with probable increase in peptides of sufficiently short structure to possess biologic activity. According to Specter[6], peptides comprising eight to fourteen amino acid residues are pharmacologically active. This activity was reflected in the enhanced uterotonic effects of treated rat urine over control rat urine on the isolated rat uterus (Fig. 1).

*Mann Research Biochemical New York City

Table 1

Urine Peptidase Activity In Control And Treated Rats

	Trypsin-Like Activity				Leucine Aminopeptidase Activity				Carboxypeptidase Activity			
	Average of 20 Det.	Range	S.D.	P	Average of 20 Det.	Range	S.D.	P	Average of 20 Det.	Range	S.D.	P
Control	238	206-292	±29		473	384-560	± 47		176	168-194	±13	
Fungal Protease	385	228-530	±89	<0.01	903	605-1200	±219	<0.01	307	264-370	±28	<0.01
Papain	336	325-457	±49	<0.01	975	910-1120	± 62	<0.01	362	340-374	±11	<0.01
Streptokinase	360	282-483	±51	<0.01	740	610-850	± 54	<0.01				

No. 9

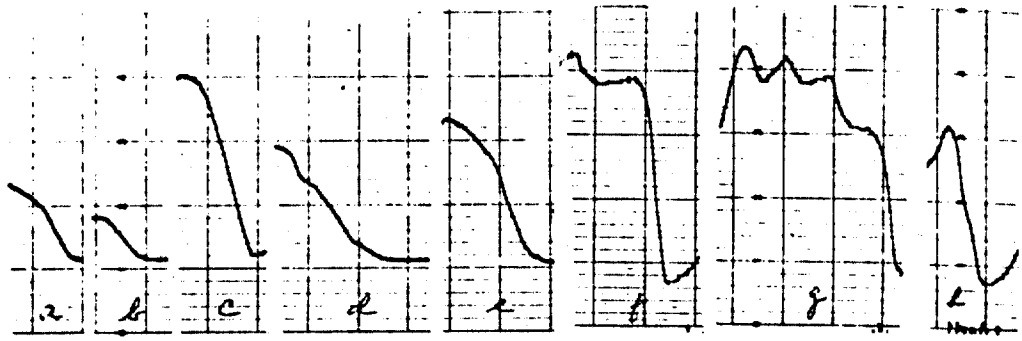

FIG. 1

Rat Uterus Contractions

(a) Normal Control (b) L A P (c) BK (d) BK + LAP (e) Control Urine (f) Control Urine + BK (g) Treated Urine (h) Treated Urine + BK

Uterine contracting activity of control urine was potentiated following the introduction of bradykinin (Fig. 1). There was decreased uterotonicity following the addition of bradykinin to treated urine (Fig. 1). This observation indicates bradykinin destroying action in treated urine, and suggests a relationship between augmented peptidase activity in treated urine and bradykinin inactivation.

It is of further interest that the uterotonic effect of bradykinin was decreased following incubation with leucine aminopeptidase (Fig. 1). Structural modification of bradykinin could exert just this type of effect. According to Erdos[7], however, leucine aminopeptidase does not attack bradykinin. Our findings, therefore may be due to impurities in the enzyme preparation employed.

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Life Sciences Vol. 3, pp. 267 - 275, 1964. Pergamon Press, Inc. Printed in the United States.

TISSUE PEPTIDASE ACTIVITY FOLLOWING ORALLY GIVEN PROTEASES

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(Received 6 January 1964)

In a recent study enhanced rat urinary peptidase and bradykinin inactivating effects followed orally given kinases or proteases (1). The inactivation of bradykinin was of considerable interest since none of the preparations employed (streptokinase, plasmin, trypsin) attack bradykinin.(2) The possibility was considered therefore, that orally given streptokinase or proteases participate in the activation of tissue pro-peptidases into kinin-inactivating peptidases. The site of this postulated zymogen activation was unknown.

The present investigation was undertaken to determine the level and mode of activation of peptidases in rat liver, spleen and kidney homogenates, and their relationship to orally given streptokinase and proteases.

Materials and Methods

This study was carried out on 80 Sprague Dawley rats. Leucine aminopeptidase was measured according to Goldbarg and Rutenberg.(3) Trypsin-like enzymes were measured according to Blackwood and Mandl,(4) using benzoylarginine-B-naphthylamide (Bana) as substrate. Colorimeter readings were converted into ug naphthylamine released and expressed as mg. trypsin equivalents. Carboxypeptidase B activity was measured according to Folk (5) *et al.*; the homogenate supernatant was 5 mg./ml.

Fibrin Plates

The fibrin plate method of Astrup and Mullertz,(6) as modified by von Kaulla(7) was used for evaluation of action. Activity was recorded as the diameter product (in mm^2.) of the lysed zone after 20 hours digestion at room temperature (average of 3 determinations). Heated and unheated fibrin plates were prepared immediately before use.

Thrombin

Fibrin plates were made by adding 12.5 N.I.H. units of Parke-Davis Topical Thrombin to fibrinogen.

Fibrinogen

The concentration of fibrinogen was 6 mg./ml. of buffer. Each plate contained 18 ml. of buffered fibrinogen.

Buffers

A stock of 0.03 M sodium phosphate buffer pH 7.5 was used to prepare all plates and to dilute samples.

Procedure

A. In Vivo Study

Rats were fasted for 24 hours preceding the test and maintained on water ad libitum. Medication and placebo tablets were dissolved in one ml. saline and introduced via stomach tube every half hour for one and one-half hours. Twenty control rats received placebo tablets. Treated rats were divided into three groups of 20 and treated as follows: Group 1 received 200 mg. papain per dose; group 2 received 90,000 units streptokinase plus 0.3 ml. human plasma per dose; group 3 received 500 mg. fungal protease per dose. Animals were sacrificed 30 minutes after the last dose. Rat liver, spleen and kidney homogenates 1 mg/ml) were used in the experiments. Organs were eigher immediately removed and homogenized, or were frozen after removal

from the animals and kept at -20° until ready for use. Pieces were cut from frozen blocks which were less than one week old, were weighed, and then homogenized in water or buffer with a motor-driven ground glass homogenizer.

B. In Vitro Study

Streptokinase (2,000 units) plus 0.1 ml. human plasma or fungal protease (2.5 mgs.) were incubated with 1 ml. kidney homogenates from control rats. Naphthylamidase and carboxypeptidase activities were determined in homogenate alone; enzyme alone; homogenate plus enzyme. Heat inactivation was carried out by boiling for 20 minutes. Heated and unheated fibrin plate determinations were made on 0.03 ml. samples of kidney homogenates from 6 control rats and 6 rats treated with streptokinase plus human plasma.

Results and Discussion

Significantly greater tissue peptidase activity developed in rats treated with orally given kinases or proteases than placebo treated controls (Table 1). Trypsin-like activity (Bana) was greater by 95%, 85%, and 139% respectively in liver, spleen and kidney homogenates obtained from rats treated with orally given streptokinase. Somewhat lower Bana values were observed in fungal protease or papain treated rats. Significant elevation in leucine aminopeptidase and carboxypeptidase activity developed (Table 1). These findings correlate well with a prior study in which a broad spectrum of urinary peptidase activity followed orally given kinases or proteases, with potential points of attack at C-terminal, N-terminal and intermediate linkages of peptide molecules.(1)

TABLE 1

Tissue Peptidase Activity In Control And Treated Rats

		Average of 20 Det.	Range	S. D.	P
Trypsin-Like Activity					
Control	Liver	285	131-384	±41	
	Spleen	288	214-346	±33	
	Kidney	302	168-480	±64	
Fungal Protease	Liver	556	510-629	±38	<0.01
	Spleen	510	409-520	±41	<0.01
	Kidney	722	510-1210	±272	<0.01
Papain	Liver	453	360-590	±58	<0.01
	Spleen	426	384-620	±64	<0.01
	Kidney	563	470-690	±56	<0.01
Streptokinase	Liver	431	351-602	±57	<0.01
	Spleen	406	381-627	±61	<0.01
	Kidney	718	601-792	±34	<0.01
Leucine Aminopeptidase Activity					
Control	Liver	793	650-780	±36	
	Spleen	665	654-671	± 4	
	Kidney	973	930-1010	±21	
Fungal Protease	Liver	946	730-1070	±142	<0.01
	Spleen	966	950-990	± 8	<0.01
	Kidney	1205	1130-1300	± 44	<0.01
Papain	Liver	970	790-1150	±110	<0.01
	Spleen	840	760-980	± 51	<0.01
	Kidney	1080	990-1210	±52	<0.01
Streptokinase	Liver	842	740-1010	±99	<0.01
	Spleen	734	604-780	±44	<0.01
	Kidney	1144	705-1280	±149	<0.01

continued/...

TABLE 1 /continued

Carboxypeptidase Activity					
		Average of 20 Det.	Range	S. D.	P
Control	Liver	160	142-175	±13	
	Spleen	121	112-145	±12	
	Kidney	220	214-246	±13	
Fungal Protease	Liver	290	206-370	±47	<0.01
	Spleen	351	249-401	±44	<0.01
	Kidney	339	304-516	±51	<0.01
Papain	Liver	192	186-210	±11	<0.01
	Spleen	181	175-205	±10	<0.01
	Kidney	314	296-341	±13	<0.01
Streptokinase	Liver	210	186-226	±14	<0.01
	Spleen	246	174-301	±49	<0.01
	Kidney	496	347-714	±81	<0.01

Data presented in Tables 2 and 3 indicate that renal peptidases exist in zymogen form and are activated by streptokinase or fungal protease. Table 2 lists range and average values of the naphthylamidase (trypsin-like) activity in rat kidney before and after incubation with streptokinase or fungal protease, and relates them to trypsin equivalents at pH 7.2. At the thirty minute incubation period aliquots of homogenate, enzyme and substrate showed significantly greater peptidase activity than the sum of aliquots comprising substrate and enzyme plus substrate and homogenate. Boiled homogenates or enzymes were devoid of naphthylamidase activity.

Table 3 lists similar increases in kidney carboxypeptidase activity with higher carboxypeptidase values in reaction mixtures consisting of enzyme, homogenate and substrate than enzyme and

TABLE 2

Effect of Proteases on Naphthylamidase Activity of Normal Rat Kidney

Kidney Homogenate	Enzyme	Substrate	Mg. Trypsin Equivalents (30 min. incubation at pH 7.2)
+	-	BANA	0.021
0	SK + HP*	BANA	0.011
0	FP**	BANA	0.010
+	SK + HP	BANA	0.058
+	FP	BANA	0.054
Heated	SK + HP	BANA	0.011
Heated	FP	BANA	0.012
+	Heated SK + HP	BANA	0.019
+	Heated FP	BANA	0.022

* Streptokinase plus human plasma
** Fungal Protease

TABLE 3

Effect of Streptokinase on Carboxypeptidase Activity of Normal Rat Kidney

Kidney Homogenate	Enzyme	Substrate	Average of 10 Determinations	Range	S. D.	P
+	-	H-L-A*	71	64-78	±4	
0	SK + HP**	H-L-A	82	79-86	±3	<0.01
+	SK + HP	H-L-A	342	204-517	±86	<0.01

* Hippuryl-L-Arginine
** Streptokinase + Human Plasma

substrate plus homogenate and substrate. These findings are consistent with the interpretation that pro-peptidases in rat kidney homogenates exist and may be converted to peptidases by streptokinase or the proteases used in this study. Other possibilities include removal of an inhibitor, activation of catheptic enzymes or increase in peptidase solubility.

Fibrin plate studies (Table 4) show a marked increase (79%) in the zones of lysis produced by kidney homogenates from treated rats on unheated plates. When heated fibrin plates were used, there were no significant differences in the zones of lysis produced by control or tissue homogenates. This finding documents a considerable increase in plasminogen activator activity in treated rat kidney homogenates over controls.

An earlier study utilizing similar fibrin plate methods showed that streptokinase converted gastric juice plasminogen pro-activator into plasminogen activator.(8)

TABLE 4

Plasminogen Activator Activity of Kidney Homogenates

Kidney Homogenate (ml.)	Zones of Lysis (average of D^2 in mm)* Unheated Plate		Heated Plate	
	Control	Treated	Control	Treated
0.03	33	77	2	3
0.03	38	58	3	1
0.03	44	89	1	4
0.03	39	53	4	2
0.03	36	37	3	3
0.03	41	102	2	3
AVERAGE:	38.5	69	2.6	2.5

* Average of Triplicate determination

* Hippuryl-L-Arginine
** Streptoki + Human Plasma

The present study indicates a rise in plasminogen activator activity in the kidneys of animals following oral doses of streptokinase. This increase may be due to (a) renal diffusion of plasminogen activator arising from gastric juice, or (b) intrinsic activation of renal plasminogen by streptokinase. The latter possibility can be dismissed, since recent studies make it unlikely that any orally given enzyme is directly absorbed per se. (9) (10) Relating increase in activator to renal diffusion of circulating plasminogen activator arising from gastric juice, while probable, is complicated by the lack of reliable blood assay technics sufficiently sensitive to detect anything but massive increases in circulating plasminogen activator.

It appears unlikely that one of the renal peptidase zymogens is the zymogen plasminogen. Augmented urinary peptidase and bradykininase activity was induced by oral streptokinase given to rats (1). Since plasmin does not attack bradykinin, (2) the kininase effect following oral streptokinase is more likely related to a pathway involving plasmin activation of carboxypeptidase B zymogen. Active carboxypeptidase B readily inactivates bradykinin. The steps involved in this process are as follows:

plasminogen activator + renal plasminogen ---> plasmin

plasmin + renal pro-carboxypeptidase B ---> carboxypeptidase B

carboxypeptidase B + bradykinin ---> bradykinin degradation

Plasmin activation of pro-carboxypeptidase B in kidney is shown in Table 3 wherein enhanced hippuryl-L-argininase (carboxypeptidase B) activity followed incubation of streptokinase and human plasma with rat kidney homogenate.

It appears, then, that experimental evidence accumulated in this study permits the following observations: in vivo peptidase activity in rat liver, spleen and kidney is considerably enhanced

following orally given streptokinase or protease; in vivo plasminogen activator activity of rat kidney is increased following orally given streptokinase; in vitro, rat kidney pro-peptidases are converted into peptidases by streptokinase or fungal protease.

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Life Sciences Vol. 4, pp. 1301-1305, 1965. Pergamon Press Ltd. Printed in Great Britain.

THE "FIBRIN TO CLOT" CLOTTING TIME AFTER ORAL STREPTOKINASE OR PAPAIN

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(Received 13 April 1965; in final form 28 April 1965)

THE DETERMINATION of blood coagulation has been termed the simplest, most informative and difficult of all common clotting procedures.[1] The clotting time of human blood in glass is between 8 and 20 minutes. In siliconized glassware, the usual clotting time range is 13 to 35 minutes. In doubly ligated vein segments, however, the blood coagulation time is several hours, or more.[2] The disparity between rapid clotting of blood in vitro and slow coagulation within human vein segments has been overcome by the method of Spaet.[3] Utilizing a plastic tube device, Spaet has reported in vitro clotting times of two hours or more.

In a recent study orally given streptokinase appeared to improve defective thromboplastin generation in patients with hemophilia or liver disease.[4] The purpose of this report is to determine the effect of orally given proteases on the length of time for the first appearance of fibrin compared with the time for complete clot formation, utilizing the plastic tube technic described by Spaet.[3]

Materials and Methods

The subjects were healthy male students, ages 21 to 25. Five subjects received one streptokinase tablet* (10,000 SK units) every half hour for six doses. Five subjects received one SK tablet (10,000 units) and one 500 mg. epsilon aminocaproic acid tablet** every half hour for six doses. Five subjects received one 7.5 mg. papain tablet*** every half hour for six doses. Under fast-

ing conditions blood specimens were collected in regular blood donor sets**** immediately before and one hour following treatment. These sets consist of plastic tubing 90 cm. in length and 3 mm. in inside diameter with 15 gauge silicone-coated needles at each end. Two end points were determined: (1) Fibrin Formation. This is the time when tiny strands or flakes of fibrin are visible either floating free in saline or adhering to the tubing; (2) Complete Clot Formation. This is the time for completion of the clotting process with the formation of a firm, adherent, solid clot. There are three criteria for this end point: adherence of the clot to the tube when held vertically; clot extrusion only following squeezing, or gentle pushing from above with a probe; a clot diameter of 3 mm. The "fibrin to clot" clotting time is the difference between the clot formation time and the fibrin formation time. One subject with pre-treatment fibrin to clot clotting time exceeding 50 minutes was excluded from this study.

Results

The data are presented in Table 1.

Under pre-treatment control conditions the first evidence of fibrin formation varied from 120 to 180 minutes, and averaged 146 minutes. The range post-treatment was 70-130, and averaged 94 minutes. In pre-treatment controls, the time for solid clot formation ranged from 150-220 minutes, and averaged 180 minutes. The range for post-treatment was 220-360 minutes, and averaged 277 minutes. The pre-treatment F-C (Fibrin to Clot) time ranged from 30-40 minutes, and averaged 34 minutes. Post-treatment the F-C varied from 110 to 280 minutes, and averaged 173.5 minutes.

---

*Oral Varidase, Lederle

**Amicar, Lederle

***Papase, Warner-Chilcott

****Saftedonor, Cutter Laboratories

TABLE 1

Effect of Oral SK or Papain on the "Fibrin to Clot" Clotting Time

		Control (Minutes)			Treated (Minutes)		
Patient	Treatment	Fibrin Formation	Clot Formation	Fibrin To Clot Time	Fibrin Formation	Clot Formation	Fibrin To Clot Time
1	SK	130	160	+30	80	280	+200
2	SK	140	180	+40	80	240	+160
3	SK	130	160	+30	110	220	+110
4	SK	180	210	+30	130	315	+185
5	SK	120	150	+30	70	280	+110
6	Papain	170	200	+30	100	240	+140
7	Papain	180	220	+40	130	300	+170
8	Papain	140	180	+40	80	360	+280
9	Papain	150	190	+40	90	250	+160
10	Papain	120	150	+30	70	290	+220
Mean Values:		146	180	34	94	277	173.5

Discussion

This study reveals two seemingly paradoxical in vivo effects of proteolytic enzymes on the phenomenon of whole blood clotting: reduced fibrin formation time and prolonged clot formation time. Recent studies by Sherry[5] and Bang,[6] however, show that these results are in accord with the biochemical activity of increased levels of plasminogen activator in the blood stream. The inhibitory effect of EACA (Table 2) provides evidence that plasminogen activator activity is related to oral SK therapy and is essential for the phenomenon of prolonged "fibrin to clot" clotting time. Plasmin has been show to accelerate blood coagulation and concomitantly cause a defect in the polymeriza-

TABLE 2

Effect of Oral EACA plus SK on the "Fibrin to Clot" Clotting Time

	Control (Minutes)			Treated (Minutes)		
Patient	Fibrin Formation	Clot Formation	Fibrin To Clot Time	Fibrin Formation	Clot Formation	Fibrin To Clot Time
1	120	160	40	120	150	30
2	130	170	40	130	170	40
3	180	210	30	160	195	35
4	140	175	35	150	180	30
5	130	165	35	130	160	30

tion and gelation of fibrin.[6] The accelerating action of plasmin on thromboplastin generation probably relates to enchanced Factor X and prothrombin activation, and could explain the more rapid initial appearance of fibrin observed in the present study. The well documented effects of plasmin on fibrinogen, characterized by alterations in the final stages of fibrinogen-fibrin conversion, defective fibrin polymerization and fibrinogen breakdown products with anticoagulant, antithrombin properties, could explain the vastly prolonged clot formation time observed in the present study (Table 1).[7]

According to Wessler[8] and others[9] small intravascular fibrin deposits occur constantly and are cleared by the removal or inactivating capacities of the liver and reticulo-endothelial system. Clinically, therefore, earlier intravascular fibrin formation would be without significance. On the tissue level, however, the earlier appearance of fibrin would not be vitiated by hepatic or RES mechanisms, and could serve a useful purpose in localizing infection and in wound healing. The role of proteolytic enzymes as wound healing and anti-inflammatory agents has been reported.[10, 11]

The delayed "fibrin to clot" clotting time observed in this study appears of considerable interest. Further studies on in vivo fibrin polymerization and

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gelation changes following orally given streptokinase or papain appear warranted.

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The evolution of papain emphysema in the rat

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This study demonstrates that the inhalation of papain produces a pulmonary lesion which resembles centrilobular emphysema. The initial lesion consists of widespread damage to the alveolar walls with apparent loss of such damaged tissue. Subsequently, the centrilobular airspaces further enlarge, probably as a consequence of the altered tissue compliance. This latter change correlates well with demonstrable alterations in the elastin and reticulin fibers. The papain lesion shows neither progression nor resolution past the first 30 days following papain exposure.

The significance of individual factors in the etiology of human emphysema is obscured by the diverse nature of potential lung insults which may occur over extended periods. Characterization of animal models of emphysema may help delineate the importance of these individual factors.

Many models of emphysema have demonstrated hyperinflated airspaces, but the loss of alveolar walls, the hallmark of human emphysema, has not been adequately documented.[1] Recently, the proteolytic enzyme, papain, has been used to produce a pulmonary lesion which resembles emphysema in a variety of experimental animals.[2-7] Whether administered by intratracheal injection or by aerosolization, papain has been reported to cause dilatation of the bronchioles and alveolar ducts in association with a minimal inflammatory response. Only Gross and associates[2] have reported their histologic findings in detail, and have used the shortening or disappearance of alveolar walls, which invaginate into alveolar ducts, as evidence of alveolar-septal destruction. A systematic study of the early stages of the lesion has not been reported.

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Supported in part by United States Public Health Service Grants AI 08664 and HE 06296.
Received for publication Dec. 11, 1970
Accepted for publication July 7, 1971.

*Dr. Johanson is the recipient of a United States Public Health Service Special Research Fellowship award.

The present study was undertaken to determine with more certainty that the papain lesion is associated with tissue destruction, and to investigate its histopathologic mode of action.

Methods

Male, white, Sprague-Dawley rats, which weighed 150 to 200 grams, were exposed to papain* which was aerosolized from a reservoir nebulizer as a 10 per cent solution in isotonic saline. The routine period of aerosol exposure was 4 hours, and at least 2 animals were killed at 0, 1, 2, 3, 4, 6, 8, 12, 19, and 24 hours, and at 2, 3, 4, 14, 33, 60, and 90 days after exposure. Some of the animals were removed from the chamber following 1, 2, and 3 hours of exposure and killed immediately. Nonaerosol-exposed animals were killed in parallel with the experimental group. The animals were anesthetized with intraperitoneal thiamylal, a tracheostomy was performed, and the chest opened widely. The lungs were immediately inflated in situ with iced glutaraldehyde under 20 cm. H_2O pressure. The trachea was ligated; the heart and lungs were carefully removed, and the specimen was immersed in glutaraldehyde.

After at least 24 hours of fixation, the lungs were cut in a coronal plane with the use of a double-bladed, oscillating electric carving knife which tended to prevent distortion of the inflated lungs. A midcoronal block of the specimen was dehydrated, embedded in paraffin, and sectioned for light microscopy. Sections of 5, 10, and 40 microns were stained with hematoxylin and eosin, Wilder's reticulin stain, Gomori's aldehyde-fuchsin stain for elastic tissue, and Gomori's trichrome stain for collagen. In addition to ordinary light microscopy, the sections were examined by Nomarsky's interference-phase microscopy (Reichart optics), which provided additional contrast, and a three-dimensional image of the alveolar walls.

Blocks which measured approximately 0.5 to 1.0 mm.^{3} were taken for electron microscopy from both the central and peripheral zones of the lung tissues which remained. These blocks were postfixed in Palade's buffered osmium tetroxide[8] for 1 hour, dehydrated in graded alcohol solutions, infiltrated with propylene oxide, and infiltrated and embedded in maraglass.[9] Sections were cut with a Porter-Blum II ultramicrotome, stained with lead citrate,[10] and examined with an RCA EMU-30 electron microscope. Maraglass sections (0.5μ) were stained with toluidine blue, and examined by ordinary light and interference-phase microscopy to insure proper orientation of the electron micrographs.

Lung morphometrics were performed on the lungs of a group of animals which were killed 60 days after exposure and similarly maintained control animals. Following fixation, the lungs were dissected free of the attached tissues and their volume (V_L) determined by water displacement. Midcoronal sections, 5 microns thick, of both lungs were prepared and stained with hematoxylin and eosin. A grid with 3 mm.^{2} spaces was applied to the slide and alternate squares were counted. The mean chord length (Lm) of the parenchymal airspaces and internal surface area (ISA) were determined by utilization of the techniques described by Weibel.[11]

Results

After 30 minutes of exposure to the papain aerosol, most of the animals exhibited respiratory distress manifested by tachypnea, intercostal retraction, cyanosis, and disappearance of spontaneous voluntary activity. Occasional deaths occurred during the 4-hour exposure period, and about 10 per cent of the animals died within 48 hours after exposure. Signs of respiratory distress disappeared within 2 to 3 days in the survivors.

Gross examination of the lungs of animals killed within 24 hours of exposure

*Papain, Difco, Difco Laboratories, Detroit, Mich.

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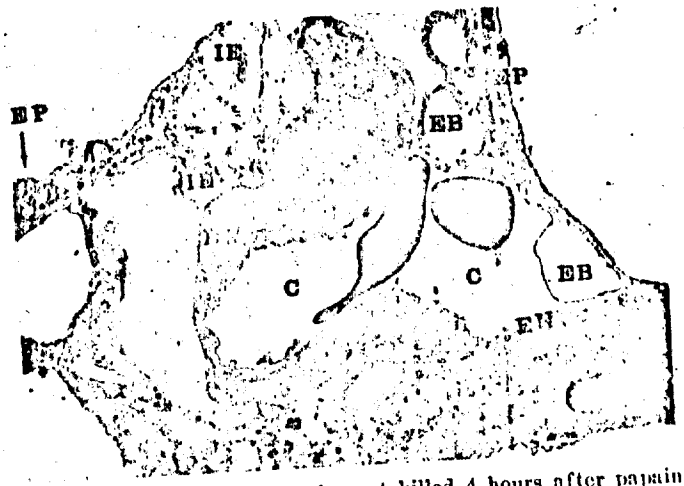

Fig. 1. Electron micrograph of the lung of a rat killed 4 hours after papain exposure. Normal structures labeled are the alveolar epithelium *(EP)*, capillary endothelium *(EN)*, and capillary lumen *(C)*. While the epithelium appears intact, the endothelium demonstrates focal blebbing *(EB)*. Interstitial edema *(IE)* is prominent and surrounds bundles of collagen. (×6,000.)

revealed heavy, edematous lungs with extensive focal subpleural and intraparenchymal hemorrhages. Microscopically, intra-alveolar hemorrhage was widely distributed and involved both the central and peripheral areas. Vascular damage was not apparent with light microscopy, but electron microscopy revealed a swelling of the cytoplasm of capillary endothelial cells and, in some areas, a striking blebbing of the endothelium (Fig. 1). These changes were frequently associated with interstitial edema. Occasional perivascular accumulations of fluid were seen with the light microscope.

A slight inflammatory cell infiltrate, composed primarily of polymorphonuclear leukocytes (PMN), was seen in the alveolar walls after 2 to 3 hours of papain exposure. Electron microscopic examination revealed that most of the PMN's were marginated within the alveolar capillaries. This cellular response progressed for 8 to 12 hours after exposure, but was never a prominent feature. After 12 hours, eosinophils were frequently found in the alveolar capillaries and later were observed in the perivascular edema fluid.

A marked alteration in the staining of reticulin and elastin fibers was noted within 3 hours of exposure to the papain aerosol. The initial alteration in reticulin was the loss of silver staining in the finest branches of the alveolar reticulin network, best demonstrated at 800 to 1,000 diameters of magnification. The disappearance of silver-stained reticulin fibers was progressive, and by 6 to 8 hours after the termination of exposure, numerous alveolar wall segments were completely devoid of these fibers (Fig. 2). The loss of affinity for silver stain was widespread but focal, and corresponded with alveolar wall segments which appeared hyalinized with hematoxylin and eosin stain. Although this lesion was more extensive in the areas of hemorrhage, it was not restricted to such areas. Many elastin fibers developed a beaded appearance which was most readily observed in the larger fibers (Fig. 3). In some areas, fragmentation of the fibers

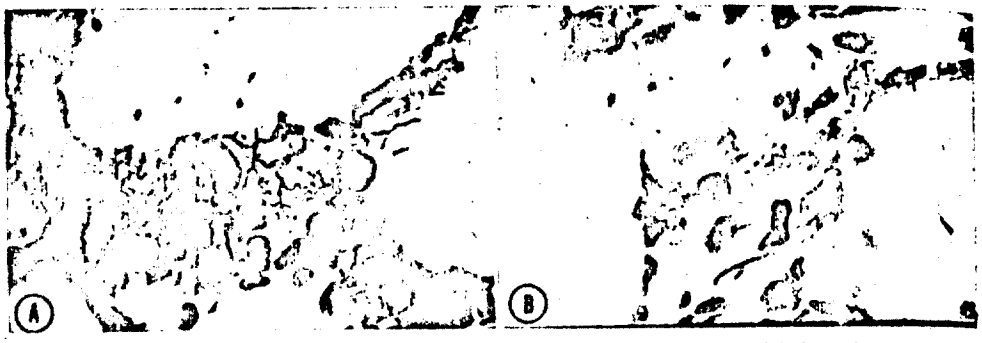

Fig. 2. (A) Alveolar wall of a normal rat lung photographed *en face* with interference phase microscopy. The fine, interlacing fibers of the reticulin network are demonstrated. The section is 10 microns thick. (Wilder's reticulin stain; ×1,400.) *(B)* Interference phase photomicrograph of an alveolar wall from a rat killed 4 hours after exposure to papain. The fine, branching network of reticulin fibers is absent. The section is 10 microns thick. (Wilder's reticulin stain; ×1,400.)

Fig. 3. (A) Photomicrograph of a normal rat lung which demonstrates normal elastin fibers. The section is 10 microns thick. (Aldehyde fuchsin stain; ×900.) *(B)* Photomicrograph of the lung of a rat 4 hours after exposure to papain which demonstrates the disruption of elastin fibers. The section is 10 microns thick. (Aldehyde fuchsin stain; ×900.)

occurred, and only clusters of granules remained. The perivascular, elastin fiber network and the elastic lamina of the adjacent vessels appeared normal.

Electron microscopic examination revealed edema around the collagen bundles, but no changes were demonstrated in the fibers which were examined. The elastin fibers stained poorly and were inconstantly demonstrated in controls and exposed animals.

Gross structural changes were associated with the alteration of connective tissue. Dilatation of the alveolar ducts appeared by 2 hours after the termination of exposure. Fewer alveolar septa were observed invaginating into the alveolar ducts, and many of the septa which remained appeared shortened.

The material examined by electron microscope demonstrated a relatively mild injury of the alveolar epithelium. While rare areas of necrosis and separation from the basement membrane were seen, the usual changes consisted of focal

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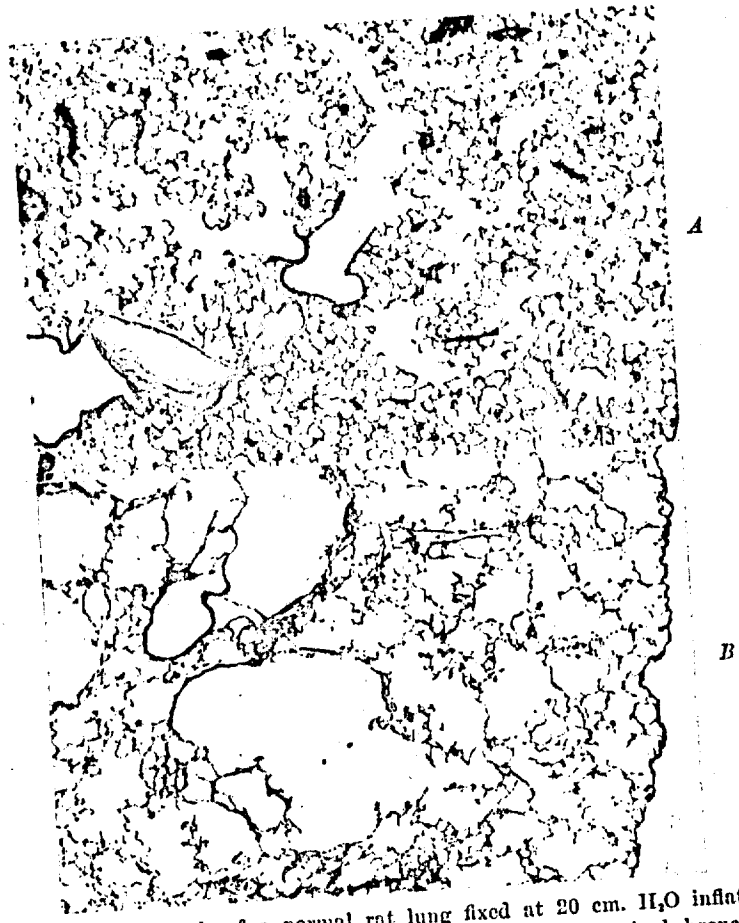

Fig. 4. (A) Photomicrograph of a normal rat lung fixed at 20 cm. H_2O inflation pressure. Numerous alveolar ducts originate directly from a branching terminal bronchiole without intervening respiratory bronchioles. The alveolar ducts are formed by invaginating alveolar septa. The section is 5 microns thick. (Hematoxylin and eosin stain; ×110.) *(B)* Photomicrograph of the lung of a rat fixed at 20 cm. H_2O inflation pressure 33 days after exposure to papain. Multiple, smooth-walled, cystic airspaces are present. Inflammation is not present and fibrosis is minimal. The section is 5 microns thick. (Hematoxylin and eosin stain; ×110.)

cytoplasmic swelling with a decrease in the pinocytotic vesicles in the involved areas (Fig. 1).

After 12 to 24 hours, further progression of these acute lesions was not seen, and evidence of healing appeared. Moderate numbers of macrophages and PMN entered the alveolar spaces; phagocytosis is of the erythrocytes transpired. By 2 weeks, all that remained of the intra-alveolar hemorrhages was an occasional fibrous plaque. The extent of focal interstitial fibrosis resultant from this process was minimal.

Maximum dilatation of the centrilobular airspaces was present in 2 to 4 weeks and then remained static without evidence of regression or progression. The disappearance of the alveolar septa which invaginated into the alveolar ducts caused these airways to appear as large, smooth-walled airspaces (Fig. 4). The

Table I. Lung morphometrics 2 months after papain exposure

	*Lung volume** (*ml.*)	*Mean chord length* (*cm.*)	*Internal surface area* (*cm.²*)
Controls	13.5	0.0068	7147
	16.0	0.0075	7680
	14.0	0.0067	7522
	14.5	0.0069	7565
	15.0	0.0080	6750
	14.6	0.0072	7333
Papain	15.5	0.0085	6565
	15.0	0.0094	5745
	17.0	0.0116	5276
	13.5	0.0078	6231
	15.3	0.0093	5954
		($p < 0.05$)†	($p < 0.01$)†

*Volume after fixation at 20 cm. H_2O pressure, uncorrected for shrinkage.
†Compared to control values, grouped t test.

mean chord length was significantly larger in the papain-treated animals ($p < 0.05$) (Table I). The internal surface area of these emphysematous lungs was reduced approximately 19 per cent from the value obtained in the controls ($p < 0.01$). Lung volumes, uncorrected for shrinkage during fixation, were slightly higher in the papain group, but the difference was not significant.

No evidence of injury to the bronchial epithelium was noted. The terminal bronchioles which communicate with the dilated airspaces were patent and appeared normal, even in animals killed 3 months after exposure.

Discussion

The initial evidence of lung damage following the administration of papain is the appearance of alveolar hemorrhage, the severity and extent of which is influenced by the route of papain administration, the concentration of the enzyme deposited, and possibly the species of animal which is utilized. In the study of Gross and associates,[2] small alveolar hemorrhages were described following single or multiple intratracheal injections of papain (1 or 2 mg.) in rats. In contrast, Goldring and associates[4] described extensive edematous, hemorrhagic pulmonary lesions in hamsters after exposure to an aerosol of 3 per cent papain for 4 hours. In the present study which utilized the aerosol administration of papain to rats, diffuse alveolar hemorrhage also occurred. Direct evidence of vascular damage was seen by electron microscopy in association with interstitial edema.

The early cellular response to papain has been variously reported. This variation may be, in part, explained by concomitant pulmonary infection. In the study of Gross and associates,[2] pneumonia was a constant feature of the acute reaction. Pushpakom and associates,[7] administered antibiotics to their dogs to control infection following papain, but histologic evidence of infection was present in 3 out of the 8 animals which were studied. In each of these studies, papain was administered by intratracheal injection. In neither the study of Goldring and associates[4] nor the present study, in which aerosol administration

was used, was inflammation a prominent feature. While the occurrence of inflammation may be influenced by the route of administration, the present study indicates that the papain lesion does not require an inflammatory response.

The pathologic criteria of emphysema include not only the enlargement of the airspaces, but also the loss of parenchymal lung tissue. The parameter used to evaluate such tissue loss by Gross and associates[2] was the shortening or disappearance of the septa which invaginate into the alveolar ducts; while these changes were also observed in the present study, it seemed likely that hyperinflation alone could cause a similar appearance. However, the extensive alterations which we observed in elastin, reticulin, and the capillary endothelium during the acute phase indicate that parenchymal damage had occurred. The subsequent finding of a significant decrease in the internal surface area of lungs exposed to papain is evidence that parenchymal tissue had been destroyed.

In the present study, the alveolar ducts were the primary site of injury and more peripheral alveoli usually remained normal. These lesions cannot be strictly interpreted as centrilobular as defined by Leopold and Gough[12] who have shown that the dilated airspaces in human centrilobular emphysema are markedly altered respiratory bronchioles. In the rat, terminal nonrespiratory bronchioles lead directly into branching alveolar ducts, and respiratory bronchioles are rarely demonstrated. However, the pattern of emphysema in the rat following an aerosol papain exposure conforms more closely to centrilobular than panlobular emphysema.

It is not clear which structures of the lung maintain the normal architecture of the centrilobular spaces. Pierce and Ebert[13] have maintained that the structural integrity of the lung is due to collagen. In our studies, no alterations in the collagen fibers were noted, and papain has been reported to have no collagenolytic activity.[14] Reticulin, generally regarded as immature collagen, was markedly altered by papain inhalation. The role of this connective tissue substance in the maintenance of the lung structure is unknown. Normal architecture has been reported in lungs fixed in a noninflated state, rendered virtually devoid of elastic fibers by the in vitro incubation with pancreatic elastase.[15] However, papain also has been reported to have elastolytic activity in vitro,[16] and in our studies which utilized inflated lung specimens, structural abnormality correlated well with an alteration in the elastic fibers.

It is of interest to compare the evolution of papain emphysema with the various theories of the pathogenesis of centrilobular emphysema in man. Airway obstruction has been suggested as the etiology of emphysema by clinicians and investigators.[17] Numerous animal models of emphysema induced by various forms of imposed airway obstruction have been reported, but uniform lung hyperinflation without alveolar wall destruction has been the usual result.[18, 19] McLean[20, 21] has followed the course of bronchiolitis in man, and has concluded that small airway obstruction is the primary lesion in centrilobular emphysema.[22] The papain lesion demonstrates that emphysema may follow a single, severe lung insult in the absence of airway obstruction. The interrelationship between a less severe, or chronic insult and airway obstruction in the production of emphysema is not clarified by this study. The lack of progression seen in the papain model may reflect the absence of airway disease.

Leopold and Gough[12] concluded that the primary lesion in emphysema was inflammatory tissue damage, initiated in the respiratory bronchioles, with subsequent dilatation of the altered tissue. In detailed studies of emphysematous human lungs, Anderson and Foraker[23] traced the alveolar damage from an initial interstitial inflammatory reaction with dissolution of some septa and marked alteration of others. This alteration was characterized by disruption of the elastin, obliteration of the capillaries, and fibrosis. These authors[12, 23] emphasized that the alveolus is the site of primary damage in the pathogenesis of emphysema. The association of these early lesions with accumulations of inflammatory cells is of particular interest, since such cells have been demonstrated to liberate proteolytic,[24] elastolytic,[25] and collagenolytic[26] lysosomal enzymes which are active at a physiologic pH. These observations, in concert with the clinical observation that individuals who are hereditarily deficient in alpha-1-antitrypsin are unusually susceptible to emphysema,[27] support the concept that lung damage in emphysema may be initially proteolytic in nature. The papain model cannot be used as proof of this concept in man because of differences in the enzymes, route of administration, and amount of enzyme available. However, the similarity between our observations in the rat lung, and those of Anderson and Foraker[23] in human lungs suggests that enzymatic attack on the structural proteins may be the common mode of pathogenesis for the changes in the alveolar walls.

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STUDIES ON THE ENZYMATIC DISSOLUTION OF KIDNEY STONES

By

L. Jozsa and Gy. Szederkenyi

The urinary colloids found in the largest quantities are the urinary proteins, the uromucoids and the acid mucopolysaccharides. The quantity of protein in the urine amount to approximately 40 mg/24 hours, corresponding to a concentration of 2-6 mg%. This protein is not uniform, but contains albumins, globulins and lipoproteins. The excretion of protein in the urine may amount to as much as 86-312 mg per day in patients suffering from kidney stones. The reason for this is the increase of α and β globulin fractions. The daily excretion of uromucoids in healthy persons is 40-45 mg, in patients with kidney stones, it is 147-1040 mg. The uromucoid/urinary protein level can thus be raised from a normal value of 1 to 2.5-14. The uromucoid that accumulates in renal stones contains two identifiable fractions. The more important one is that whose polymerism is lower. It forms 5/6 of the uromucoids and forms an indissoluble complex with Ca- and phosphate groups. Healthy males excrete more uromucoid in the urine than healthy females of the human species and according to some authors, this explains the more frequent occurrence of urinary calculi in men. The uromucoid stems partly from proteins in the tissues and the blood and is partly produced by the epithelium of the winding duct. It has been proven that the uromucoid either resembles the mucoprotein of the connective tissue and the basic substance of the bones or that it is the same and thus it becomes understandable that in hyperparathyreosis, when the basic substance of the bones and cartilage is mobilized to an increased degree, the markedly Ca-binding mucoids frequently cause the

development of calculi. Any external or internal factor interfering with the basic substance of the mesenchyma, means that there is a danger of the formation of calculi. Simultaneously, it was proven that the acid mucopolysaccharides excreted in the urine (chiefly hyaluronic acid) exercise the function of a protective colloid by preventing the precipitation of uromucoid and inorganic substances.

Renal stones consist partly of pure protein and most of them contain 2-6% protein and a considerable amount of uromucoid (UM). According to other authors and our observations, the protein and UM form the nucleus of the stone and the binding substance of individual layers. Using immunohistochemical methods, Keutel proved that the cement holding the stone together and the mucoids of the tubular epithelium are immunologically identical. Little is known of the chemical character and the enzymatic dissolution of the uromucoids but it is known that a part of the protein-splitting enzymes of the organism is excreted in the urine and that the enzymes entering the organism from the outside are likewise excreted in the urine.

Our experiments were based on the assumption that, provided the binding material of renal calculi is protein or UM, it can be controlled via a corresponding enzyme and that, if it were possible to dissolve the binding material, the stone would disintegrate. The following is a report on our observations made during the first phase of the in vitro experiments.

Material and Method.

Investigated was the dissolution of renal calculi obtained through autopsy. Our models were calcium oxalate and calcium-ammonium phosphate stones. We proceded as follows: 2-700 mg of the stones were weighed on an analytical scale. Stones whose weight had been exactly determined were

placed in the urine-enzyme solution. The control in each case was a stone placed in urine that did not contain any enzymes. The general formula of the solutions obtained in this manner, can be expressed as follows: 1. stone + 10 ml urine + 100 mg enzyme; 2. control: stone + 10 ml urine.

In our experiments we used urine that was obtained sterile from healthy male subjects. Initially, the pH was not modified, but later it was adjusted to a neutral or slightly acid pH. In each enzymatic solution 10 oxalate and phosphate stones were studied. The weight of the stones was determined by use of an analytical scale after 24, 48, 72 and 96 hours of enzymatic digestion and the weight changes were expressed partly in absolute figures and partly as a percentage of the initial value. Enzymatic digestion took place at 37°C in reagent tubes with ground-in glass stoppers. The following enzymes were used for our studies:

1. Elastase crudum (Pharmazeutische Fabrik Kobanya, Budapest), which an electrophoretic examination revealed to consist of three fractions (elastomucoproteinase, elastoproteinase, collagen mucoproteinase).
2. Trypsin lyophilic (Pharmazeutische Fabrik Kobanya, Budapest).
3. Testicular hyaluronidase (Hyason Organon, Holland).
4. Papain (Merck, Darmstadt).
5. Diastase (Pharmacopoeia Hungarica V).
6. Lysozyme (Renal, Budapest).

The enzymes were used uniformly at a concentration of 10 mg/ml urine. The concentration of hyaluronidase was 60 TRU/ml urine.

Results

As expected, some enzymes proved to be entirely ineffective concerning a dissolution of the stones. The stones did not dissolve in the solutions of

diastase, hyaluronidase and lysozyme enzyme. Papain and trypsin resulted in a moderate dissolution of the stones and elastase was the most effective. In the remaining enzymatic solutions, the weight loss of the stones amounted maximally to 10% and this loss can, at any rate, not be attributed to the activity of the enzyme. The weight loss of the stones obtained with the 3 enzymes is listed in Table 1 and Fig. 1. In the case of the phosphate stones, conditions of dissolution were somewhat different. This was demonstrated not only by the fact that at a given pH trypsin and papain were much more effective, but also insofar as the stones disintegrated completely and were pulverized under the effect of elastase. Hyaluronidase, diastase and lysozyme had no effect on the phosphate stones and the weight loss during the period of observation did not exceed 10%. A 100% weight loss from our point of view was indicated when the stones disintegrated completely to form a fine sand and when the grain size amounted to no more than 1 mm (Table 2, Fig. 2).

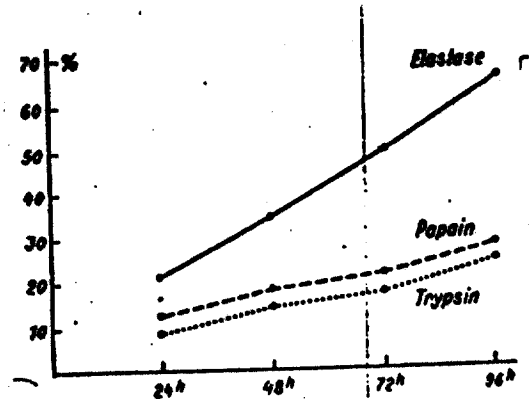

Fig. 1. Weight loss of oxalate stones (in %) in response to enzymatic activity

Table 1. Weight loss of oxalate stones (in %) of enzymatic activity

	24 hours	48 hours	72 hours	96 hours
Elastase	20,6 ± 4,4	35,3 ± 3,1	49,3 ± 3,4	66,2 ± 2,9
Papain	10,7 ± 2,0	17,2 ± 2,3	20,8 ± 1,9	26,6 ± 1,8
Trypsin	9,15 ± 3.3	14,5 ± 3,0	16,1 ± 2,8	24,9 ± 2,2

Table 2. Weight loss of phosphate stones (in %) of enzymatic activity

	24 hours	48 hours	72 hours	96 hours
Elastase	41,3 ± 3,7	62,5 ± 3,2	89,0 ± 2,9	100,0 ± 2,3
Papain	28,0 ± 2,5	40,3 ± 3,0	53,6 ± 3,1	62 2 ± 4,1
Trypsin	23,8 ± 2,7	35,1 ± 3,7	42,3 ± 2,7	54,7 ± 3,3

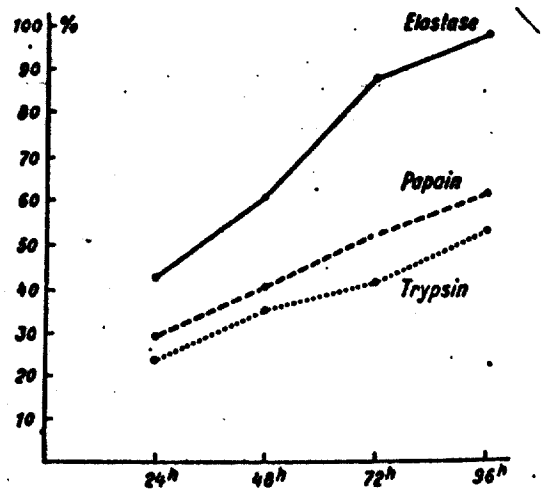

Fig. 2. Weight loss of phosphate stones (in %) of enzymatic activity

Discussion

In 1817, Marcel realized that renal stones contain protein and in 1884, Ebstein clearly defined these protein stones. Stones consisting of pure protein are rare, but later it became evident that protein can be found in

all renal calculi. On the basis of our own experiences, we attribute the role of a binding substance to the protein and uromucoid content of kidney stones. We feel that the mucoproteins are primarily responsible for the formation of the stones and for their growth. Our working hypothesis was the attempt to dissolve renal stones with enzymes containing protein and mucolytic elements in vitro. Of the number of enzymes usually used, pepsin could not be employed, since its optimal pH is so low that a similar pH value cannot be produced in the urinary paths and that, secondly, the pH is so low that the acid itself can dissolve the stones in a highly acid medium, so that one cannot determine how much of a possible dissolution or weight loss is attributable to the acid and how much to the enzyme. A protein-splitting effect of diastase could not be expected. We attempted to use this substance on the theory that it might free the sugar components from the protein molecule, upon which the impaired structural equilibrium of the mucoid molecules could lead to disintegration. Our expectations in this regard were not fulfilled: diastase proved to be completely ineffective. Just as ineffective was hyaluronidase, since renal calculi do not contain hyalase-sensitive acid mucopolysaccharides. Although lysozyme has a mucoprotein-splitting effect, it was found to be inactive with respect to kidney stones. There are several explanations for this, the most plausible being that the enzyme we used was not of animal, but of vegetable origin.

The optimal pH of trypsin and papain was approximately the same as that of the urine we used. Activators were not used (cysteine, EDTA, etc.) so that their effect could not interfere with the enzymatic activity. Papain and trypsin dissolved the matrix of renal stones, and the intensity of their effect was approximately the same. The main substrate of both enzymes is

protein and for this reason, the dissolution of UM can only be regarded as a side effect, which explains that they did not lead to the disintegration of the stones even at a relatively high concentration.

Elastase is primarily a mucolytic enzyme and can dissolve not only the mucoids of the basic substance of connective tissue and fibers, but also the mucoproteins of saliva and bronchial secretions. Of elastase, we expected especially a dissolution of uromucoid and this occurred. As far as we know, this effect of elastase and its use for the dissolution of stones has so far not been described.

The effect of the enzyme preparations was not the same on both the oxalate and the phosphate stones. The weight loss of the oxalate stones was less and there was no total disintegration during the experimental period. The weight loss of the phosphate stones was twice as large as that of the oxalate stones during the first 24 hours. Concerning both types of stones, we observed that the disintegration of renal stones is very much delayed in time and there was an increase of the weight loss every day. Our explanation of this was that the disintegrating effect of the enzymatic protein and UM takes place first on the surface, from where it proceeds and dissolves the connective substance of the crystal platelets layer by layer. The crystal structure of oxalate and phosphate stones differs, and the enzyme has better and faster access to the uromucoids of the phosphate stones. One can assume that the oxalate stones would have disintegrated if they had been left in the enzyme solution for a longer time.

This raises the question as to where to go from here. Numerous methods have been proposed during the past 50 years for the dissolution of renal calculi, but none of them was useful in the practice. It is our opinion that it might be worthwhile to administer trypsin and elastase under experimental

conditions and to attempt to dissolve the stones in this manner. Both perorally administered elastase and injectable trypsin are excreted in the urine at relatively high concentrations. It can be assumed that an appropriately maintained administration of enzymes would dissolve the renal stones in vivo.

Summary

The authors investigated the enzymatic dissolution of renal stones in vitro. Of the 6 enzymes that were tested, diastase, lysozyme and hyaluronidase did not have a disintegrating effect on the stones. Treatment with papain and trypsin led to a 24-26% weight loss of oxalate stones and to 56-62% weight loss of phosphate stones after incubation for 96 hours. During the same time, elastase led to the complete disintegration of the phosphate stones and to a 66% weight reduction of the oxalate stones.

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(Aus dem Krankenhaus des Komitatsrates Bács-Kiskun, Abteilung für pathologische Anatomie und Histologie, Chefarzt: Dr. G. Luaztig, Kecskemét, Ungarn)

Untersuchungen zur enzymatischen Auflösung von Nierensteinen

Von L. Józsa und Gy. Szederkényi

Mit 2 Abbildungen

Von den Harnkolloiden werden die Uroproteine (6), Uromukoide (6, 9) und die sauren Mukopolysaccharide (6, 9, 11) in der größten Menge gefunden. Die Uroproteinmenge beträgt etwa 40 mg/24 Std. und das entspricht einer Konzentration von 2—6 mg%. Das Uroprotein selbst ist nicht einheitlich, sondern beinhaltet neben Albuminen und Globulinen auch Lipoproteine (6). Die Uroproteinausscheidung kann bei Steinträgern täglich 86—312 mg betragen, und zwar infolge der Zunahme an α- und β-Globulinfraktionen. Die tägliche Uromukoidausscheidung (UM) beträgt bei Gesunden 40—45 mg, bei Steinträgern 147 bis 1040 mg. Dadurch kann der Uromukoid/Uroproteinwert vom normalen 1, auf 2,5—14 erhöht sein. Bei dem in den Harnsteinen angehäuften Uromukoid können 2 Fraktionen differenziert werden, und zwar ist die mit niedriger Polymerität wichtiger, welche fünf Sechstel des UM beträgt und mit den Ca- und Phosphatgruppen einen unlöslichen Komplex bildet. Im Harn von Männern — auch bei Gesunden — ist die UM-Ausscheidung höher und nach einigen Autoren wird das häufigere Vorkommen von Nierensteinen bei Männern dadurch erklärt. Das Uromukoid stammt teils aus den Gewebs- und Blutmukoproteinen, teils wird es vom gewundenen Kanalepithel erzeugt. Es wurde nachgewiesen (2, 6), daß das UM dem Mukoprotein des Bindegewebes und der Knochengrundsubstanz ähnlich oder gleich ist und so wird verständlich, warum bei Hyperparathyreose infolge der erhöhten Knochen- und Knorpelgrundsubstanz-Mobilisierung die stark Ca-bindenden Mukoide häufig eine Steinbildung verursachen. Jede äußere oder innere Einwirkung, welche die Grundsubstanz des Mesenchyms stört, bringt auch die Gefahr der Steinbildung mit sich. Gleichzeitig wurde nachgewiesen, daß die mit dem Harn ausgeschiedenen sauren Mukopolysaccharide (in erster Linie Hyaluronsäure) eine Schutzkolloidfunktion erfüllen, indem sie die Ausfällung von UM und anorganischen Substanzen hindern.

Ein Teil der Nierensteine besteht aus reinem Eiweiß (3, 5, 10), die Mehrzahl enthält 2—6% Eiweiß und eine bedeutende Menge an UM (8—12). Nach anderen Autoren und unseren Feststellungen bildet das Eiweiß und UM den Kern des

Steines und die Bindesubstanz einzelner Schichten. Keutel (7) konnte mit immunhistochemischen Methoden nachweisen, daß das Zementmaterial des Steines und die Mukoide des Tubulusepithels immunologisch gleich sind. Wir wissen wenig über den chemischen Charakter, über die enzymatische Lösung der Uromukoide, es ist jedoch bekannt, daß ein Teil der eiweißspaltenden Enzyme des Organismus mit dem Harn ausgeschieden und die von außen eingeführten Enzyme ebenfalls mit dem Harn entleert werden.

Bei unseren Experimenten gingen wir von der Annahme aus, daß, falls das Bindematerial der Steine Eiweiß bzw. UM ist, dann kann es mit entsprechenden Enzymen digeriert werden und, falls es gelingt, diese zu lösen, so zerfällt der Stein. Wir möchten im folgenden über die Beobachtungen während der ersten Phase der in-vitro-Versuche berichten.

Material und Methode

Wir untersuchten die Lösung der während der Sektion entfernten Nierensteine, und zwar verwendeten wir als Modell Kalziumoxalat und Kalzium-Ammoniumphosphatsteine. Das Verfahren war wie folgt: Von den Steinen wurde 2—700 mg mittels einer analytischen Waage abgemessen. In die Harn-Enzymlösung wurden Steine mit genau bestimmtem Gewicht gelegt. Als Kontrolle verwendeten wir neben jeder Untersuchung einen in enzymfreien Harn gelegten Stein. Die allgemeine Formel der in dieser Weise gewonnenen Lösungen kann folgendermaßen ausgedrückt werden: 1. Untersuchungsmaterial: Stein + 10 ml, Harn + 100 mg Enzym; 2. Kontrollmaterial: Stein + 10 ml Harn.

Für unsere Untersuchungen verwendeten wir steril gewonnenen Harn gesunder Männer; das pH wurde anfangs nicht modifiziert, später auf neutralen oder gering sauren pH (6,8—7,0) eingestellt. In jeder Enzymlösung wurden 10 Oxalat- und Phosphatsteine untersucht. Das Gewicht der Steine wurde nach 24-, 48-, 72-, 96stündiger Enzymdigestion auf analytischer Waage gemessen und die Gewichtsveränderungen teils in absoluten Zahlen, teils im Prozentsatz des Grundwertes ausgedrückt. Die Enzymdigestion erfolgte bei 37 °C in Reagenzgläsern mit geschliffenen Glaspropfen. Für unsere Untersuchungen verwendeten wir folgende Enzyme:

1. Elastase crudum (Pharmazeutische Fabrik Köbánya, Budapest), welches bei elektrophoretischer Untersuchung aus 3 Fraktionen (Elastomukoproteinase, Elastoproteinase, Kollagenmukoproteinase) bestand.
2. Trypsin lyophil (Pharmazeutische Fabrik Köbánya, Budapest).
3. Hoden-Hyaluronidase (Hyason Organon, Holland).
4. Papain (Merck, Darmstadt).
5. Diastase (Ph Hg. V).
6. Lysosym (Renal, Budapest).

Die Enzyme wurden einheitlich in einer Konzentration von 10 mg/ml Harn verwendet; die Konzentration von Hyaluronidase betrug 60 TRU/ml Harn.

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Ergebnisse

Wie zu erwarten, erwiesen sich einige Enzyme bezüglich der Steinlösung vollkommen wirkungslos. Wir konnten in den Diastase-, Hyaluronidase- und Lysosym-Enzymlösungen keine Steinauflösung beobachten. Die Steine wurden durch Papain und Trypsin mäßig gelöst, die meiste Wirkung hatte Elastase. In den übrigen Enzymlösungen betrug der Gewichtsverlust von Steinen höchstens 10% und das kann keinesfalls der Enzymwirkung zugeschrieben werden. Der bei den 3 Enzymen erfolgende Gewichtsverlust wird in Tabelle und Abbildung 1 angeführt. Im

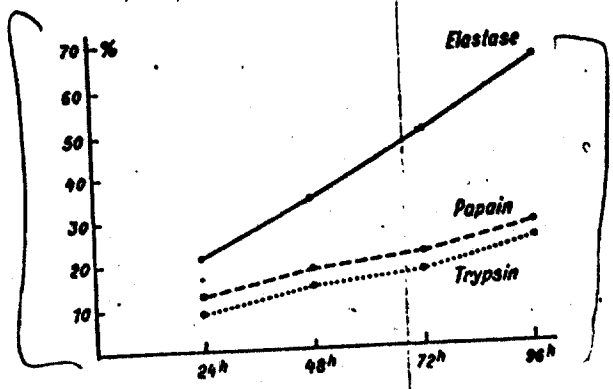

Abb. 1. Gewichtsverlust von Oxalatsteinen (in %) auf Enzymwirkung

Tabelle 1

	Gewichtsverlust von Oxalatsteinen (in %) auf Enzymwirkung			
	24 Std.	48 Std.	72 Std.	96 Std.
Elastase	20,6 ± 4,4	35,3 ± 3,1	49,3 ± 3,4	66,2 ± 2,9
Papain	10,7 ± 2,0	17,2 ± 2,3	20,8 ± 1,9	26,6 ± 1,8
Trypsin	9,15 ± 3,3	14,5 ± 3,0	16,1 ± 2,8	24,9 ± 2,2

Falle von Phosphatsteinen fanden wir etwas andere Verhältnisse der Steinlösung. Das zeigt sich nicht nur darin, daß bei gegebenem pH das Trypsin und Papain bedeutend wirkungsvoller war, sondern auch darin, daß infolge der Elastasewirkung die Steine ganz zerfielen, pulverisiert wurden. Die Hyaluronidase, Diastase und das Lysosym hatte keine Wirkung auf die Phosphatsteine, während der Untersuchungsperiode trat kein größerer Gewichtsverlust als 10% ein. Wir betrachteten es als 100%igen Gewichtsverlust, wenn die Steine zu feinem Sand zerfielen und die Körnchengröße 1 mm nicht übertraf (Tabelle 2, Abb. 2).

Tabelle 2

	Gewichtsverlust von Phosphatsteinen (in %) auf Enzymwirkung			
	24 Std.	48 Std.	72 Std.	96 Std.
Elastase	41,3 ± 3,7	62,5 ± 3,2	89,0 ± 2,9	100,0 ± 2,3
Papain	28,0 ± 2,5	40,3 ± 3,0	53,6 ± 3,1	62 2 ± 4.1
Trypsin	23,8 ± 2,7	35,1 ± 3,7	42,3 ± 2,7	54,7 ± 3,8

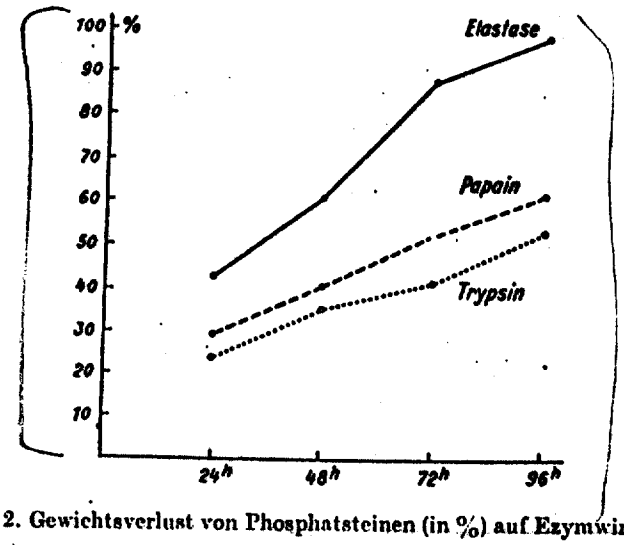

Abb. 2. Gewichtsverlust von Phosphatsteinen (in %) auf Ezymwirkung

Besprechung

1817 erkannte Marcel den Eiweißgehalt von Nierensteinen und 1884 hat **Ebstein** die Eiweiß-Steine genau definiert. Die reinen Eiweißsteine sind eine Seltenheit, doch später zeigte es sich, daß in allen Nierensteinen Eiweiß zu finden ist. Auf Grund unserer eigenen Erfahrungen (12) können wir dem Eiweiß- und Uromukoidgehalt von Nierensteinen eine bindesubstanzartige Rolle beimessen, und zwar machen wir in erster Linie die Mukoproteide für die Steinbildung und Steinvergrößerung verantwortlich. Unsere Arbeitshypothese bestand in dem Versuch, die Nierensteine mit Eiweiß- und mukolytischen Enzymen in vitro aufzulösen. Von den im allgemeinen Verkehr gebräuchlichen Enzymen konnten wir das Pepsin nicht verwenden, denn sein pH-Optimum ist so gering, daß einerseits ein ähnliches in den Harnwegen nicht zustande gebracht werden kann, andererseits besitzt es ein niedriges pH, d. h. in stark saurem Medium kann die Säure selbst die Nierensteine auflösen und so können wir nicht feststellen, wieviel bei einer eventuellen Auflösung bzw. Gewichtsverlust durch die Säure- bzw. die Enzymwirkung bedingt ist. Eine eiweißspaltende Wirkung der Diastase war nicht zu erwarten. Wir versuchten es mit der Zielsetzung, daß sie eventuell die Zucker-

komponenten aus dem Eiweißzuckermolekül auslöst und somit infolge des gestörten strukturellen Gleichgewichts der Mukoidmoleküle zum Selbstzerfall führen kann. Unseren diesbezüglichen Erwartungen wurden nicht erfüllt: die Diastase erwies sich als vollkommen wirkungslos. Genauso wirkungslos war auch die Hyaluronidase, denn die Nierensteine enthalten keine hyasesensitiven sauren Mukopolysaccharide. Das Lysosymenzym besitzt zwar einen mukoproteinspaltenden Effekt, wir fanden es aber für Nierensteine wirkungslos. Das kann verschiedene Erklärungen haben, am wahrscheinlichsten ist, daß das von uns verwendete Ferment nicht tierischen, sondern pflanzlichen Ursprungs war.

Das pH-Optimum von Trypsin und Papain war annähernd dasselbe wie das des von uns verwendeten Harns. Wir benutzten keine Aktivatoren (Zystein, EDTA, usw.); damit deren Eigenwirkung die Enzymwirkung nicht stören sollte. Papain und Trypsin lösten die Matrix von Nierensteinen und ihre Wirkungsstärke war annähernd gleich. Das Hauptsubstrat beider Fermente ist das Eiweiß, daher kann ihre UM-Lösung nur als Nebenwirkung betrachtet werden. So wird es verständlich, daß sie auch bei verhältnismäßig hoher Konzentration nicht zum Steinzerfall führten.

Die Elastase ist in erster Linie ein mukolytisches Ferment (1), sie kann nicht nur die Mukoide der Bindegewebe-Grundsubstanz und Fasern, sondern auch die Mukoproteide des Speichels und Bronchialsekrets lösen. Wir erwarteten von der Elastase vor allem eine Uromukoidlösung und das wurde auch bestätigt. Nach unserem Wissen wurde eine diesbezügliche Wirkung der Elastase und ihre Verwendung zur Steinlösung bis jetzt nicht beschrieben.

Die Enzympräparate hatten keine gleiche Wirkung auf die Oxalat- und Phosphatsteine. Der Gewichtsverlust der Oxalatsteine war geringer, während der Versuchsperiode erfolgte ein totaler Zerfall nicht. Der Gewichtsverlust von Phosphatsteinen war in den ersten 24 Stunden doppelt so hoch, als jener der Oxalatsteine. Bei beiden Steinarten konnten wir beobachten, daß der Zerfall von Nierensteinen zeitlich sehr verzögert ist (4), die Gewichtsverminderung nahm täglich immer mehr zu. Das erklärten wir damit, daß die Zerfallswirkung von Enzymeiweiß und UM zuerst an der Oberfläche zur Geltung kommt und schichtweise die Bindesubstanz der Kristallplättchen auflöst. Die Kristallstruktur von Oxalat- und Phosphatsteinen ist unterschiedlich, das Enzym kann besser und schneller zu den Uromokoiden der Phosphatsteine gelangen. Es kann angenommen werden, daß falls wir die Oxalatsteine längere Zeit in der Enzymlösung inkubiert hätten, es zu deren Zerfall gekommen wäre.

Es erhebt sich die Frage: wie weiter? Für die Lösung von Nierensteinen wurden während der vergangenen 50 Jahre zahlreiche Methoden vorgeschlagen, doch keine bewährte sich in der Praxis. Wir sind der Meinung, daß es sich lohnen würde, unter experimentellen Umständen Trypsin und Elastase einzuführen und dadurch die Steinlösung zu versuchen. Sowohl die perorale Gabe von Elastase, als auch das in Injektionsform verabreichte Trypsin entleert sich mit dem Urin, und zwar in einer verhältnismäßig hohen Konzentration. Es kann angenommen werden, daß eine entsprechend anhaltende Enzymgabe auch in vivo die Nierensteine lösen wird.

Zusammenfasung

Die Autoren untersuchten die enzymatische Lösung von Nierensteinen unter in-vitro-Bedingungen. Von den 6 untersuchten Fermenten hatten die Diastase, das Lysosym und die Hyaluronidase keine steinlösende Wirkung. Die Papain- und Trypsinbehandlung bewirkte bei Oxalatsteinen einen 24—26, bei Phosphatsteinen einen 56—62%igen Gewichtsverlust nach einer 96stündigen Inkubation. Die Elastase führte während der gleichen Zeit zum totalen Zerfall der Phosphatsteine und zu einem 66%igen Gewichtsverlust bei Oxalatsteinen.

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Anschrift d. Verff.: Dr. L. Józsa, Krankenhaus des Komitatsrates Bács-Kiskun, Abteilung f. path. Anatomie u. Histologie, Kecskemét (Ungarn)

dose at different periods.
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Mitotic Changes in Rat Liver After Intraperitoneal Papain Protease

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Crystalline papain protease induces striking mitotic changes in the liver without necrotic changes, when given intraperitoneally. The mitotic figures are in normal configuration and all stages of mitotic cycle are observed. The mitotic frequency in the cells parallels the activity of enzyme injected and its effect is much more significant than that of crude papain. Accordingly, the most important component of crude papain responsible for the mitosis may be a papain protease. The mitotic changes also are observed in the proximal convoluted tubules of the kidney and in the cardiac myofibers, though the effect of enzyme injected is apparently less marked. The enzyme has no influence on the mitosis in the small intestine and adrenal gland. It is thus evident that the papain protease is particularly effective for an induction of mitosis in the hepatic cells.

As DISCUSSED in recent reviews by swam,[1,2] knowledge of the mitotic mechanism in mammalian cells and of substances fecting the phenomenon is still very limited.

Hayashi et al[3-9] have shown that a specific skin sulfhydryl-dependent protease, responsible for the Arthus reactions, can induce mitotic changes in the cardiac and vascular smooth muscle cells of rabbits when given intramyocardially or intravenously. Similar observations were obtained with intravenous or intramyocardial papain.[10] Kasumido[11] found in Wistar strain rats that intravenously injected papain induces mitoses in hepatic cells as well as in muscle cells. Detailed observations by us[12,13] confirmed that papain showed a more striking mitotic activity for hepatic cells of rats in the absence of necrosis in the tissue when given intraperitoneally. The mitotic frequency after a single injection of the enzyme paralleled the activity of enzyme injected. In the same experiment, we also found that ficin and bromelin could enhance mitotic changes in the hepatic cells of rats, though the effects of the latter proteases were less marked. In contrast to sulfhydryl proteases as listed above, serine proteases such as α-chymotrypsin and trypsin had no mitosis-inducing effect on the cells.

In the experiments described above, crude preparations of papain, bromelin, and ficin were used. As is well known, crude papain preparations contain three crystallizable enzymes, ie, papain protease, chymopapain, and papain lyzozyme.[14-18] The former two are the sulfhydryl-proteases, while the latter has no proteolytic activity.[16] It is thus suggested that the protease(s) responsible for mitosis may be the papain protease or chymopapain, or both.[12,13]

The present paper describes the observations on the mitotic effect of crystalline papain protease, confirming that the protease is one of crude papain components responsible for the mitosis.

Materials and Methods

Animals.—Male rats of Wistar strain, weighing between 110 and 130 gm, were used. The animals were fed on semisynthetic diet (Oriental MF, Oriental Co., Tokyo) once a day at a fixed time. Throughout the experiments the animals had free access to water. The room temperature was kept between 15 and 20 C.

Enzyme Solution.—Papain protease crystallized two times; suspended in sodium acetate buffer; Nutritional Biochemicals Corp., Cleveland, was dissolved in deionized water to give desired con-

Accepted for publication Feb 28, 1966.
From the First Department of Pathology, Kumamoto University Medical School, Kumamoto, Japan.
Read before the 23rd general meeting of the Japanese Cancer Association, Tokyo, Nov 9, 1964 and the 54th general meeting of the Japanese Pathological Society, Nagasaki, April 6, 1965.
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TABLE 1.—*Effects of Intraperitoneal Crystalline Papain Protease on the Mitotic Index of Hepatic Parenchymal Cells*

Papain Activity * (E_{276})	Concentration of Papain (E_{280})	Mitoses Per 100,000 Cells: Individual Cases †					Av
0.6	11.6	596	636	481	458	323	579
0.4	7.3	681	495	477	339	271	453
0.25	4.6	488	477	436	341	302	409
0.1	1.8	314	218	217	187	124	212
0.05	1.1	90	68	30	19	18	45
None	—	14	14	10	8	3	10

* Papain activities of 50-fold diluted enzyme solutions.
† Average of six sections.

TABLE 2.—*Comparison of Mitotic Index in Each Lobe of Rat Liver by Intraperitoneal Crystalline Papain Protease* *

Rat No.	Lobes †: 1	2	3	4	5	6	Av
1	500	525	410	545	445	434	477
2	525	510	485	420	430	555	488
3	460	425	390	410	465	465	436
4	260	305	365	335	380	400	341
5	265	290	305	320	280	350	302
Average	402	411	391	406	400	441	409

* Caseinolytic activity of 50-fold diluted solution of injected material was 0.25 (E_{276}).
† Lobe numbers indicate: 1, right lateral posteriol lobe; 2, right lateral anteriol lobe; 3, right central lobe; 4, left central lobe; 5, left lateral lobe; and 6, caudate lobe.

centrations. Concentrations of the enzyme solutions were shown by the absorbency at 280 mμ. A Shimadzu's spectrophotometer, model QR-50, was used.

Unless stated in particular, 0.25 ml of papain solution/100 gm of body weight was injected intraperitoneally. To avoid any misleading interference resulting from the periodicity of the mitotic rate,[17] injections were performed between 10:00 AM and 12:00 noon.

Protease Assay.—The proteolytic activity of the enzyme solution was measured by the modified method of Kunitz.[18] One milliliter of 2% casein solution and 1 ml of phosphate buffer were mixed and held at 37 C for five minutes before adding 1 ml of 50-fold diluted enzyme solution and 1 ml of 0.15 M saline. The mixtures were kept at pH 7.1 and ionic strength 0.25. After incubation at 37 C for 30 minutes, the mixtures were added to 5 ml of trichloroacetic acid. The optical density at 276 mμ of the filtrate was estimated with a spectrophotometer, using the filtrate from the control preparation as a blank. Control flasks were identical with the reaction flasks, with the exception that the enzyme solution was added after the trichloroacetic acid.

Mitotic Counts.—The animals were killed by exsanguination 48 hours after the injection, since the maximum frequency of mitosis in the liver has been shown to be reached in 48 hours.[12] The tissues were fixed in 10% buffered formalin, and paraffin or frozen sections stained with hematoxylin and eosin. Fat was stained with Sudan III and glycogen with McManus's periodic acid-Schiff technique. The mitotic counts in the liver were made in 100 arbitrary microscopic fields, one field containing approximately 200 hepatic parenchymal cells. The mitotic index (MI) was expressed as the number of mitoses per 100,000 cells. As the other tissues, kidney, heart, small intestine and adrenal gland were examined. In the kidney, observation of mitoses was limited to the epithelial cells of the proximal convoluted tubules. A total of 5,000 cells in the fields selected at random was considered, and the MI was expressed as number of mitoses per 5,000 cells. In the heart, mitotic counts were made in 50 arbitrary microscopic fields of the left ventricle, and numbers of mitoses observed were expressed as MI. In the small intestine, mitotic counts were made on epithelial cells of three crypts of the upper jejunum, 10 cm distal to the pylorus, only crypts which were vertically oriented from the basement membrane of the lowest point in the crypt were selected for this purpose. In the adrenal gland, the MI was determined by counting the mitotic divisions per section that passed through the medulla of the gland.

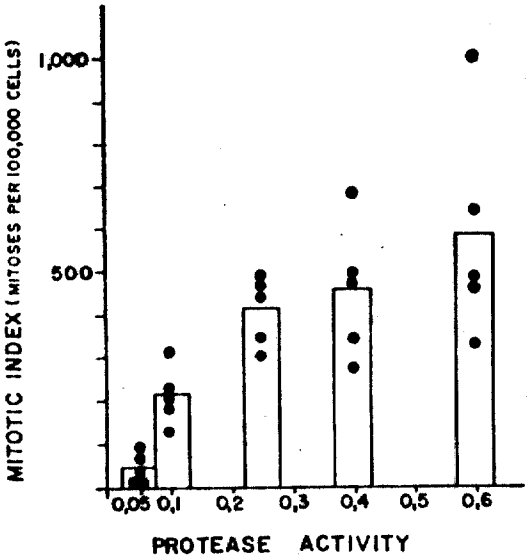


Fig 1.—Correlation between protease activity and MI, measured at 48 hours after intraperitoneal injection of crystalline papain protease. Protease activity was shown by caseinolytic activity (optical density at 276 mμ) of 50-fold diluted solution of injected material. Each column represents the average of the values.

Results

Effect of Crystalline Papain Protease on the Hepatic Cells.—Twenty-five rats were divided into five batches. The animals of each batch were given a single injection of each enzyme solution showing caseinolytic activity of 0.05, 0.1, 0.25, 0.4, and 0.6 in the 50-fold diluted solution. The amount injected was 0.25 ml/100 gm of body weight. The animals became slightly inactive within about ten minutes of the intraperitoneal injection, but one to two minutes later they

Fig 2.—Liver from rat r activity in 0.6 in 50-fold dilute degree of enlargement of hep cytoplasm of a few cells. Neer

Fig 3.—Liver from rat rece solution. Note five mitoses in r appearance, and decreased bas necrosis (hematoxylin and eosi

*Mitotic Index in [illegible] Intraperitoneal Crystalline [illegible] Protease**

Lobes†				
3	4	5	6	[illegible]
410	515	445	434	[illegible]
485	420	430	555	[illegible]
390	410	465	465	[illegible]
365	335	380	400	[illegible]
305	320	280	350	[illegible]
391	406	400	441	[illegible]

[illegible] of 50-fold diluted solution of [illegible] (Eco).

[illegible]ate: 1, right lateral posterior [illegible] lobe; 3, right central lobe; [illegible] eral lobe; and 6, caudate lobe.

[illegible]asks, with the excep[illegible] was added after the tri[illegible]

[illegible]—The animals were k[illegible] hours after the injection, s[illegible] ency of mitosis in the liver has [illegible]ached in 48 hours.[illegible] The tissues [illegible] buffered formalin, and para[illegible] stained with hematoxylin a[illegible]ed with Sudan III and glycog[illegible] periodic acid-Schiff technique [illegible] in the liver were made in [illegible]pic fields, one field containing [illegible] hepatic parenchymal cells. T[illegible]) was expressed as the num[illegible] cells. As the other tissues [illegible] intestine and adrenal gland [illegible] In the kidney, observation [illegible]ed to the epithelial cells of the [illegible]ed tubules. A total of 5,000 cells [illegible]ted at random was considered [illegible] expressed as number of mitoses [illegible] the heart, mitotic counts were [illegible]ary microscopic fields of the left [illegible]mbers of mitoses observed were [illegible]. In the small intestine, mi[illegible] on epithelial cells of three cry[illegible]num, 10 cm distal to the [illegible]h were vertically oriented [illegible]mbrane of the lowest point in [illegible]ed for this purpose. In the adre[illegible] was determined by counting [illegible] per section that passed [illegible]e gland.

Results

[illegible]rystalline Papain Prot[illegible] Cells.—Twenty-five [illegible] five batches. The ani[illegible]re given a single injecti[illegible] solution showing ca[illegible] 5, 0.1, 0.25, 0.4, and [illegible] l solution. The amount [illegible] 100 gm of body weig[illegible] me slightly inactive [illegible] of the intraperito[illegible] one to two minutes la[illegible]

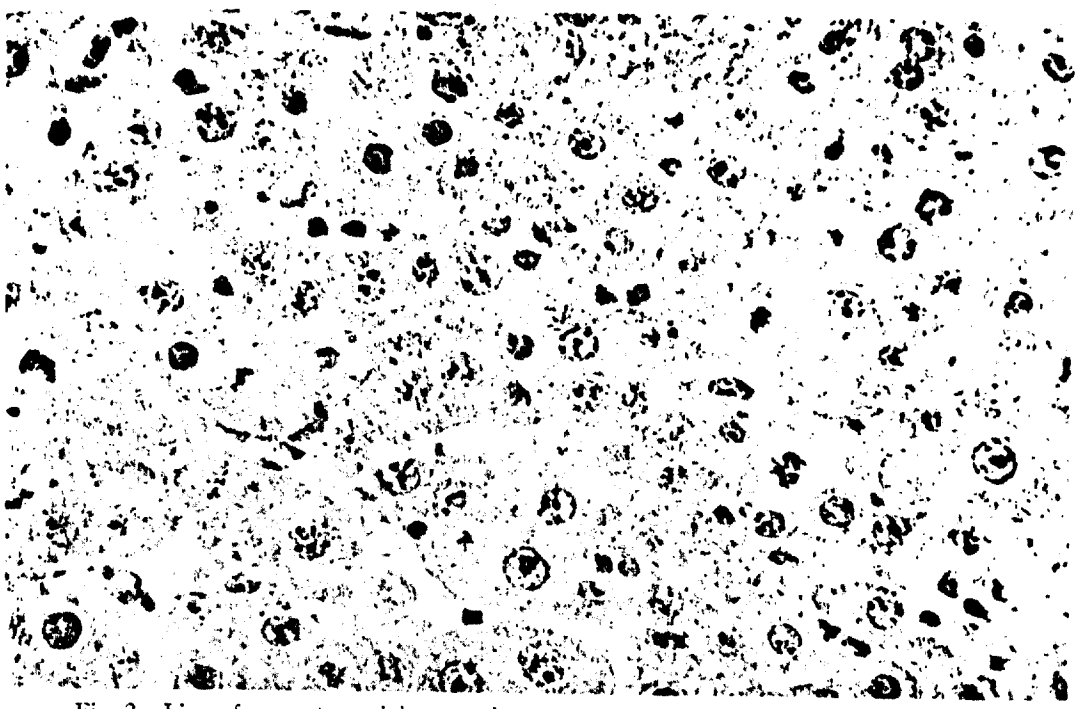


Fig 2.—Liver from rat receiving papain protease, 0.25 ml/gm of body weight, papain activity in 0.6 in 50-fold diluted solution. Note ten mitoses in the field. There is observed slight degree of enlargement of hepatic cells and their nuclei, and small hydropic vacuoles in the cytoplasm of a few cells. Necrosis is never obtained (hematoxylin and eosin, × 500).

Fig 3.—Liver from rat receiving papain protease, papain activity in 0.25 in 50-fold diluted solution. Note five mitoses in the field. Enlargement of hepatic cells and their nuclei, hydropic appearance, and decreased basophilia in the cytoplasm of the cells are observed. There is no necrosis (hematoxylin and eosin, × 800).

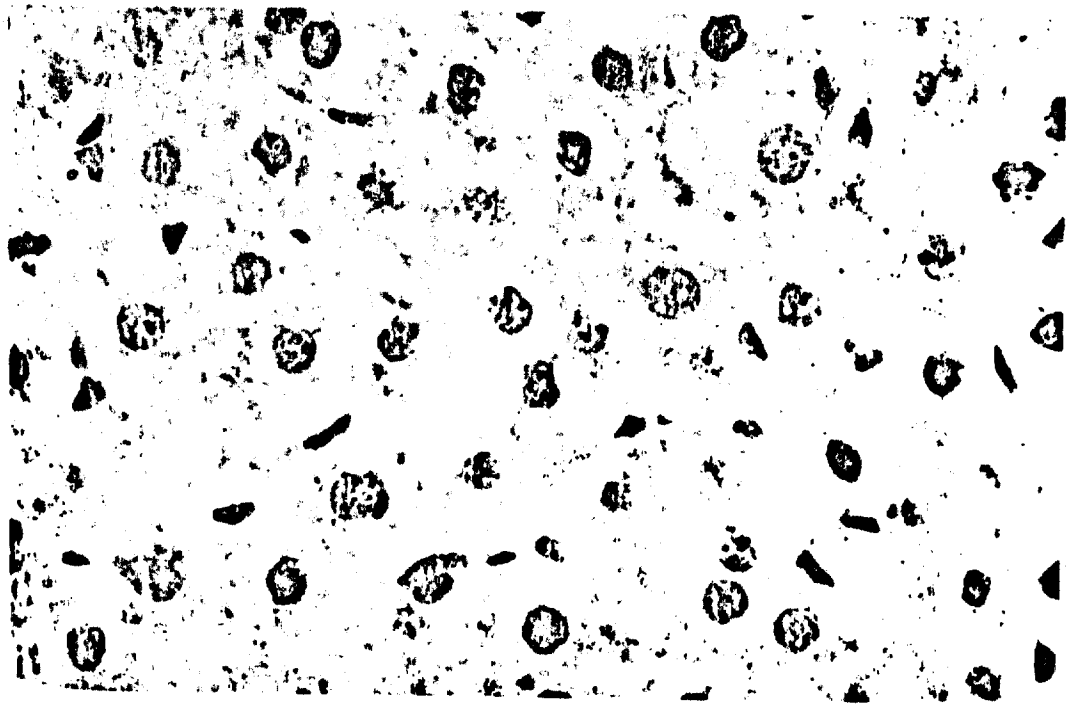


TABLE 3.—*Effect of Intraperitoneal Crystalline Papain Protease* on the Mitotic Activities of Various Tissues*

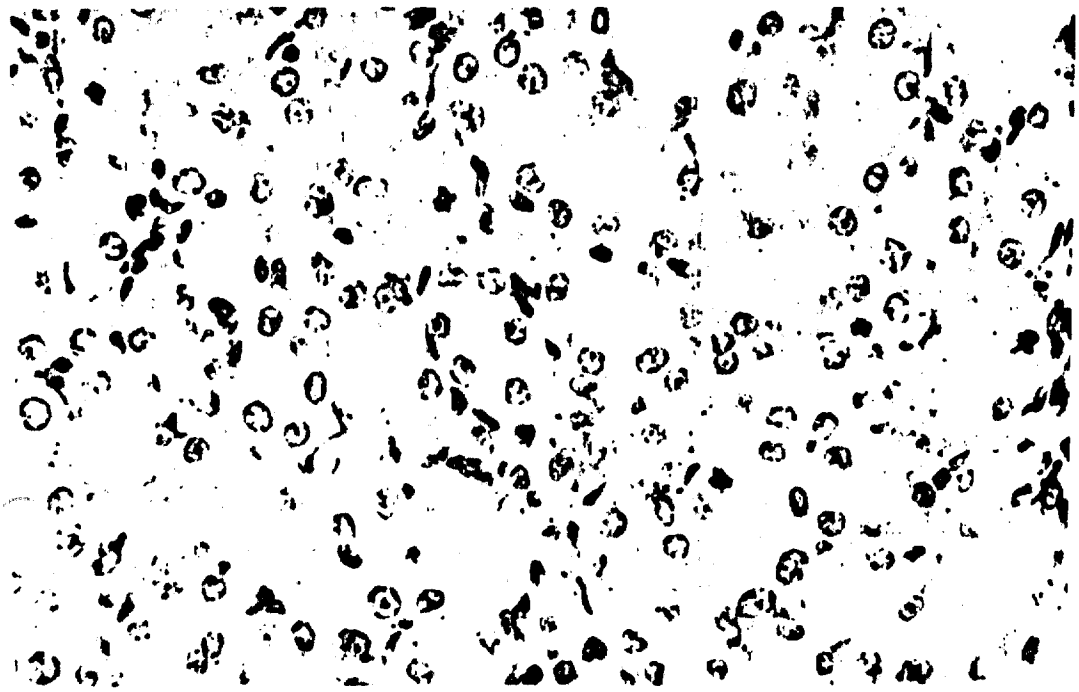
Tissues		Materials Injected	No. of Mitoses: Individual Cases					Av
Kidney	Proximal convolution, mitoses/5,000 cells	Papain	32	26	24	22	18	24.4
		Deionized water	7	6	6	3	2	4.8
Heart	Myofiber, mitoses/50 opt fields	Papain	3	3	2	1	1	2.0
		Deionized water	0	0	0	0	0	0.0
Small intestine	Crypt cells, mitoses/3 crypts	Papain	22	20	17	16	16	18.2
		Deionized water	25	23	19	15	13	19.0
Adrenal gland	Cortical cells, mitoses/section	Papain	0	0	0	0	0	0.0
		Deionized water	0	0	0	0	0	0.0

*Caseinolytic activity of 50-fold diluted solutions of injected enzyme was 0.25 (E_{280}).

became active. No pathological appearance was observed in all the animals injected.

No gross alterations were observed in the peritoneal cavity and liver of these animals. Microscopically, a striking feature was the ›earance of mitotic changes at all stages m the hepatic cells, as shown in Table 1 and Fig 1. The average MI of six lobes from each animal was 45, 212, 409, 453, and 579, respectively, and the values closely paralleled the activity of enzyme injected. Little difference in the MI was found in each lobe, as shown in Table 2. There were found all phases of mitotic changes; the percentage of prophase, metaphase, anaphase, and telophase was 21.5, 49.6, 5.3, and 23.5, respectively, in the rat liver received papain protease showing enzyme activity of 0.25. The mitotic figures were all of normal configuration and distributed diffusely throughout the liver lobules (Fig 2). It is important to note that the mitotic changes were not

Fig 4.—Kidney cortex from rat receiving papain protease, papain activity in 0.25 in 50-fold diluted solution. Ten mitoses are observed in the epithelial cells of the proximal convoluted tubules, and one in the Henle's loop. Pathological changes are not observed (hematoxylin and eosin, × 500).



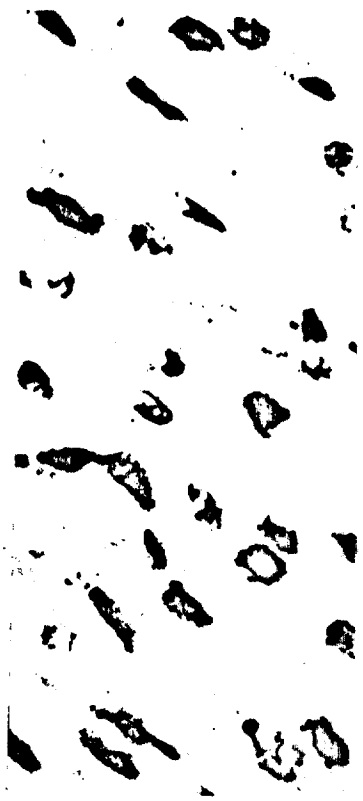


Fig 5.—Heart from rat receiving papain activity in 0.25 in 50-fold Note two mitoses in the field changes are observed (hematox × 900).

accompanied by any patholog in the tissue. Necrotic chang tissues were not found in any microscopic alterations, if pres slight degree of enlargement appearance of the hepatic c nuclei. The cytoplasm occasi a slight degree of hydropic vac decreased basophilia (Fig 2 an hydropic vacuoles showed neg and fat staining. Such chang marked than those in rats that papain.[12,13] It seems possible th mitotic changes produced by i papain protease may not be a process for damaged or nec tissue,[19,20] but associated with action of enzyme.

In addition, mitoses were in the Kupffer cells and in the epithelial cells, though the mit here was apparently less marke

Effect of Crystalline Papain Mitotic Rate of Various Organ observation was made on other ly, the kidney, heart, small i

Papain Protease
Tissues

No. of Mitoses			
Individual Cases			Av
24	22	18	24.4
6	3	2	4.8
2	1	1	2.0
0	0	0	0.0
17	16	16	[illegible]
19	15	13	[illegible]
0	0	0	[illegible]
0	0	0	[illegible]

0.25 (Eq).

MI was found in each lobe, as Table 2. There were found all mitotic changes: the percentage of metaphase, anaphase, and tel 21.5, 49.6, 5.3, and 23.5, respec the rat liver received papain showing enzyme activity of 0.25. tic es were all of normal con and distributed diffusely through er lobules (Fig 2). It is important hat the mitotic changes were not

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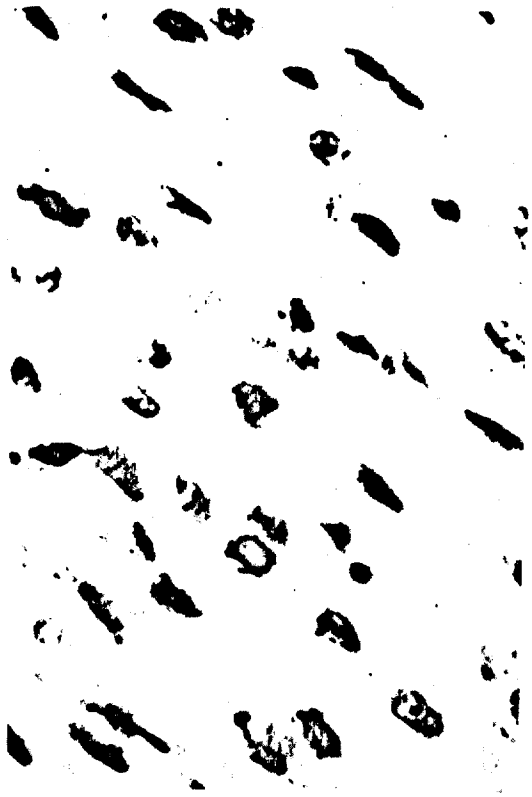

Fig 5.—Heart from rat receiving papain protease, papain activity in 0.25 in 50-fold diluted solution. Note two mitoses in the field. No pathological changes are observed (hematoxylin and eosin, ×900).

accompanied by any pathological alteration in the tissue. Necrotic changes of hepatic tissues were not found in any instance. The microscopic alterations, if present, were only slight degree of enlargement and hydropic appearance of the hepatic cells and their nuclei. The cytoplasm occasionally showed a slight degree of hydropic vacuolization and decreased basophilia (Fig 2 and Fig 3). The hydropic vacuoles showed negative glycogen and fat staining. Such changes were less marked than those in rats that received crude papain.[12,13] It seems possible that the striking mitotic changes produced by intraperitoneal papain protease may not be a compensatory process for damaged or necrotic hepatic tissue,[19,20] but associated with the primary action of enzyme.

In addition, mitoses were also observed in the Kupffer cells and in the cholangiolar epithelial cells, though the mitotic frequency here was apparently less marked.

Effect of Crystalline Papain Protease on Mitotic Rate of Various Organs.—The same observation was made on other organs, namely, the kidney, heart, small intestine, and adrenal gland. Macroscopically, no change was revealed in these organs and tissues.

Kidney: Mitotic figures in the epithelial cells of the proximal convoluted tubules of normal kidneys were rarely found; there were only from two to seven mitoses (4.8 in mean average) per 5,000 epithelial cells (Table 3). In contrast, in the animals injected with crystalline papain protease, the mitoses in the tubules were increased in number, 18 to 32 (24.4 in mean average), as seen in Table 3 and Fig 4. Such mitotic changes were occasionally observed in the epithelial cells of the Henle's loops. Microscopically, no pathological alterations in the tissues and epithelial cells were observed.

Heart: The changes in the cardiac myofibers in the left ventricles were examined. One to three mitoses in the myofibers were observed for 50 microscopic fields (ocular ×7, objective ×40) of each case (Table 3 and Fig 5). Necrotic changes were not observed in any heart. In the left ventricles of control animals no mitosis was observed.

Small Intestine: Microscopically, no alteration was observed in the tissues and mucous cells. The number of mitoses per 3 crypt was about 19.0 in the control animals, and 18.2 in papain-treated animals indicating that the enzyme had no mitotic effect on the cells.

Adrenal Gland: Microscopic examination showed that the enzyme did not induce any pathological or mitotic changes in the adrenal gland.

Comment

The results described demonstrate that crystalline papain protease can cause a significant increase in the number of mitotic figures in the liver of rats, when given intraperitoneally. The MI is considerably increased, depending upon the activity of enzyme injected (Table 1 and Fig 1). In spite of the fact that the incidence of the mitotic figures in the normal liver is very low and less than 15/100,000 cells,[12,14] there is no doubt that hepatic parenchymal cells possess a high ability to multiply, as shown in the liver tissues partially removed and damaged.[19-21] It is evident that the mitotic reaction described here is not correlated with a compensatory response for such damage, and with a mere prolongation of mitotic cycle or an accumulation of metaphase, as occurred, for example, after colchicine application. Colchicine is not used in

this experiment. Accordingly, the reaction may be associated with the primary action of papain protease.

Another protease of crude papain preparation, ie, chymopapain, was not tested because it was difficult to obtain a satisfactorily purified preparation of the enzyme. Since it is, however, shown that papain protease induces mitoses more significantly than crude papain, papain protease is considered to be the most important component of crude papain responsible for the mitosis.

There is some evidence of a papain inhibitor in serum of rabbit,[22] cow,[23] and man.[24] If such an inhibitor is present in rat serum, the striking induction of mitosis in the hepatic cells is due to activity of papain protease not inactivated by the inhibitor, because the occurrence of mitotic changes parallels the enzyme activity.

Mitotic changes are also found in the proximal convoluted tubules, but the occurrence by the enzyme is apparently less marked (Table 3). Kasumido[11] did not find such an increase in mitosis when crude papain was given intravenously. An increase in mitotic changes is also observed in the cardiac myofibers of these animals injected with papain protease (Table 3), though the occurrence is apparently less marked. No increase in rate of mitoses is observed in the small intestine and adrenal gland. Accordingly, it may be said that the enzyme, ie, papain protease, when given intraperitoneally, acts on the hepatic cells in a specific manner and results in the production of their mitotic changes. The question of whether such action of the protease may be directly associated with organ specificity remains to be studied.

Professor Hideo Hayashi, Kumamoto University Medical School, gave valuable instruction. Dr. Keiji Udaka provided helpful advice and Miss Machiko Shii gave technical assistance.

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Hyperthyr

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Accepted for publication March
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thin. In vitro lung slice studies were done on 1, 2, 4, 8, and 16 days after papain administration. Following the period of incubation, the slices were removed, washed with 2 ml of nonlabeled incubation media, and homogenized in 2:1 chloroform-methanol. Lipids were extracted using the method of Folch[7] and lecithin was isolated by silica-gel-thin-layer chromatography. Duplicate lanes were spotted with identical amounts of lipid suspended in chloroform. The lecithin from one lane was used for scintillation counting while the lecithin content from the other lane was determined by the method of Skipski et al.[8] Data were presented as disintegrations per minute (dpm) of choline labeled with radioactive carbon (^{14}C) per microgram of lecithin.

Similarly weighed slices of lung were incubated in Krebs-Ringer bicarbonate which contained 4 microcuries of a labeled mixture of arginine (312 millicuries/millimol), leucine (316 millicuries/micromol), lysine (301 millicuries/millimol) and valine (276 millicuries/millimol) to measure incorporation of these amino acids into trichloroacetic acid-insoluble material[9] at 15, 30, and 60 minutes. Data were collected as dpm ^{14}C amino acid per milligram of total lung protein; no attempt was made to quantitate the degree of incorporation of each individual amino acid. Lungs were studied from animals killed at 3, 8, and 16 days and compared to lung slices from saline-injected animals.

Total protein content was determined on similar slices of lung[7] and collagen and elastin contents were estimated after extraction of lipids, carbohydrates, and soluble proteins by the method of Lowry et al.[8]

Results

Morphologic Alterations.—The pleural surface of fresh lungs from hamsters given papain intratracheally appeared "bubbly," (Fig 1, *bottom left*) when compared to normal lungs (Fig 1, *top left* and *right*). When these lungs were cut after inflation fixation the pleural surface was scalloped instead of being smooth, (Fig 1, *bottom right*). This appearance was so consistent that it was used to diagnose the destructive lesion in lungs used in biochemical studies. Twelve hours after papain injection lungs had increased wet weight and were hemorrhagic. Many alveoli were filled with proteinaceous fluid and

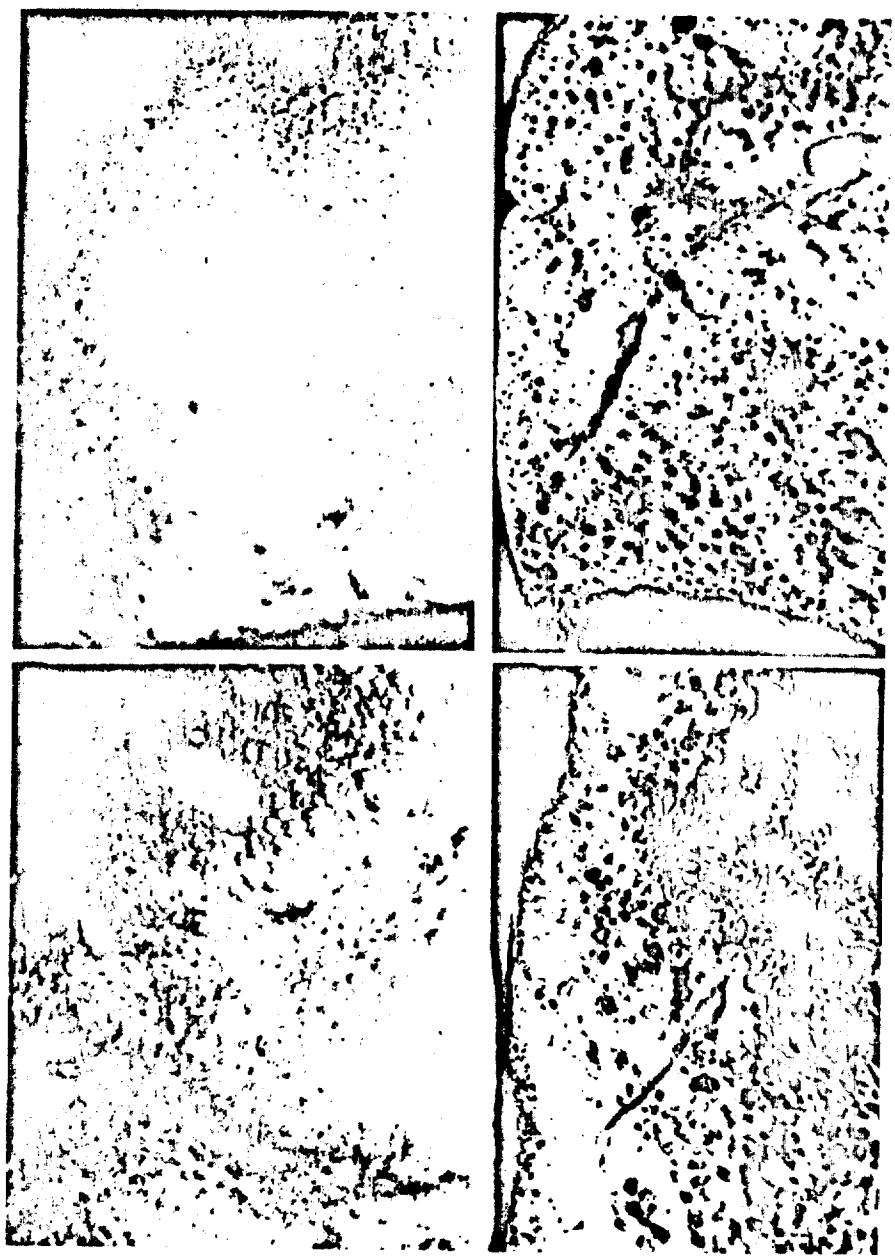

Fig 1.—Top left, *Control hamster lung shows smooth pleura and uniformly small alveoli (fixed by formaldehyde fumes. ×2).* Top right, *Cut surface of Fig 1, top left, shows regular alveoli and alveolar ducts branching from bronchioles.* Bottom left, *Pleural surface of hamster lung 16 days after papain administration has a bubbly or cobblestone appearance.* Bottom right, *Cut surface of Fig 1, bottom left, shows irregular pleural surface and a coarse appearance with air spaces of alveolar duct size or larger.*

Fig 2.—Top left, *Twelve hours after papain was administered intratracheally many alveoli were filled with edema and erythrocytes (inflation fixation, hematoxylin-eosin stain, ×25).* Top right, *Two days after papain administration edema had decreased but hemorrhage remained. Terminal bronchiole and alveolar ducts and alveoli are seen.* Bottom left, *Four days after papain administration alveolar loss has occurred and many airspaces are enlarged.* Bottom right, *Eight days after papain most airspaces are large; isolated alveolar strands are seen representing sectioned trabecule which crossed large departitioned spaces.*

erythrocytes (Fig 2, *top left*). Electron microscopy showed loss of definition of collagen fibrils in alveolar walls which were replaced by clear areas lacking in cellular detail (Fig 3, *top left*). Both epithelial and endothelial membranes were intact and there was neither edema nor blebs. Mitochondrial membranes of all cells were preserved.

One day after papain, hemorrhage and edema were still prominent features but heterophils had appeared in alveolar spaces. After two days, macrophages and scattered eosinophils had become prominent in alveolar spaces (Fig 2, *top right*). These macrophages were multivacuolated. Loss of collagen in alveolar walls was more apparent, and intact, well preserved collagen was seen rarely, (Fig 3, *top right*). Only ghosts of collagen fibrils remained. The other feature was the appearance of large amounts of endoplasmic reticulum, particularly in fibroblasts but also in type II alveolar cells. Alveolar type II cells were surrounded by four to seven capillaries which suggests that the capillaries had survived and coiled back on these cells after loss of intervening alveolar walls. This appearance suggested rosettes or trabeculae cut in cross section.

Four days after papain injection, departitioning of alveoli was clearly identified in histologic sections and in formalin fume-fixed lungs (Fig 2, *bottom left*). Collagen which remained had distinct fibrils. Capillary endothelium, particularly of those capillaries around type II cells, had become wide and hyperplastic obstructing the lumens (Fig 3, *bottom left*).

Eight days after papain administration, departitioning had reduced the numbers of alveoli in most areas to a small fraction of those present originally (Fig 2, *bottom right*). Mature fibroblasts had replaced the large numbers of similar cells with abundant, rough endoplasmic reticulum. The capillaries surrounding type II cells remained but many lumens

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Morphological and Biochemical Assessment of Papain-Induced Emphysema

Kaye H. Kilburn, MD; Anthony R. Dowell, MD; and Philip C. Pratt, MD, Durham, NC

A model for emphysema was produced in Syrian hamsters by one intratracheal injection of 1 mg of purified papain in saline solution. Papain produced pulmonary edema and hemorrhage initially but after four days collagen was lost from alveolar walls. Departitioning of alveoli occurred despite normal synthesis of lecithin and, probably, increased synthesis of protein. Total lung protein, collagen, and elastin did not change. Alveolar basement membrane connective tissue, particularly collagen, was digested but repair was stimulated as judged from cellular changes and protein synthesis rates. Two interpretations are possible: either collagen is redistributed in alveoli by digestion and synthesis with loss of interstitial connective tissue or the collagen content in alveolar walls is so small a proportion of total lung collagen that its disappearance was not measured.

Papain, an enzyme which attacks native protein, has been reported to produce a lung lesion in experimental animals which resembles centrilobular emphysema.[1] Administration of multiple doses intratracheally or by aerosol over several weeks to hamsters, rabbits, and dogs caused alveolar enlargement and destruction which was accompanied by an increased total lung capacity and decreased lung elastic recoil pressure.[2] The effect of a single dose of papain, time course of the development of the structural changes of the lesion, and the metabolic alterations in pulmonary connective tissue are not known. This study was designed to determine the time course of morphologic and biochemical effects of a single intratracheal dose of papain in the Syrian hamster. Particular attention was paid to the lung's ultrastructure, connective tissue content, and capacity for protein and phospholipid synthesis.

Received for publication Oct 21, 1970; accepted Feb 15, 1971.

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Read in part before the Tenth Annual Biology Symposium on Pollution and Lung Biochemistry, Richland, Wash, June 5, 1970, jointly sponsored by the Battelle Memorial Institute-Pacific Northwest Laboratories, National Air Pollution Control Administration, National Institute of Environmental Health Sciences, and the US Atomic Energy Commission.

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Methods

Sixty-four Syrian hamsters of both sexes, age 6 to 8 weeks, which weighed between 90 and 120 gm were anesthetized with 3 to 6 mg sodium pentobarbital administered intraperitoneally. One milligram of papain per 100 gm of body weight was dissolved in 0.5 ml of saline solution and introduced into the trachea either via a small polytef (Teflon) catheter passed through the larynx or directly by needle puncture of the trachea exposed through a small skin incision. Thirty-two control hamsters were injected with 0.5 ml saline solution. Two series of 32 treated and 16 control animals were studied. Four papain treated and two control animals were killed at ½, 1, 2, 4, 8, 16, 32, and 64 days for morphologic and biochemical studies. Animals were weighed daily.

Macrostructure of lungs of a treated and a control animal in each group was studied after fixation with formaldehyde vapor while inflated at 12 cm H_2O pressure. Histology and ultrastructure were analyzed in lungs fixed within the intact anesthetized animal with 1% osmium tetroxide in fluorocarbon via an endotracheal catheter.[3] Sagittal sections of whole lungs were dehydrated, embedded in paraffin, sectioned at 6μ, and stained with hematoxylin-eosin. Before dehydration, 1 mm cubes of osmium-fixed sections were selected, dehydrated in anhydrous alcohol and propylene oxide, embedded in resin (Epon), and cut with glass knives. Selected areas were sectioned with a diamond knife, stained with uranyl acetate and lead citrate, and examined in the electron microscope.

Hamsters for biochemical studies from the same group were killed by decapitation in the head-down position to prevent bleeding into the trachea, and the lungs were removed, blotted, and weighed. Weighed slices of lung were cut to approximately 2 mm thick and incubated in 0.05M tromethamine (TRIS) (pH 7.4) in Krebs-Ringer bicarbonate (pH 7.4) containing 2 microcuries of choline methyl-^{14}C chloride (7.6 millicuries/mmol) at 37 for three hours to determine the incorporation of choline into

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Fig 3.—Top left, Several capillaries identified by endothelial cells surround a fibrocyte 12 hours after papain adminis-tration. Partly cleared collagen ghosts (C) contrast with in-tact epithelial (Epi) and endothelial (Endo) membranes. The latter enclose erythrocytes (RBC) (osmium fixation, uranyl acetate and lead citrate stain, ×10,500). Top right, Two days after papain administration many erythrocytes are free in al-veoli together with granular debris, macrophages (M), and an eosinophil (Eos) Collagen "ghosts" are still obvious and large alveolar cells (LAC) are vacuolated (×5,250). Bot left, After four days departitioning is extensive, but survi collagen has become more distinct. Endothelium is ede tous and hyperplastic and a platelet (Pl) is attached to membrane (×17,500). Bottom right, After eight days s capillaries (Cap) are reduced to slits and many capill show endothelial hyperplasia. By 16 days remodeling is c plete and endothelial cells are normal (×17,500).

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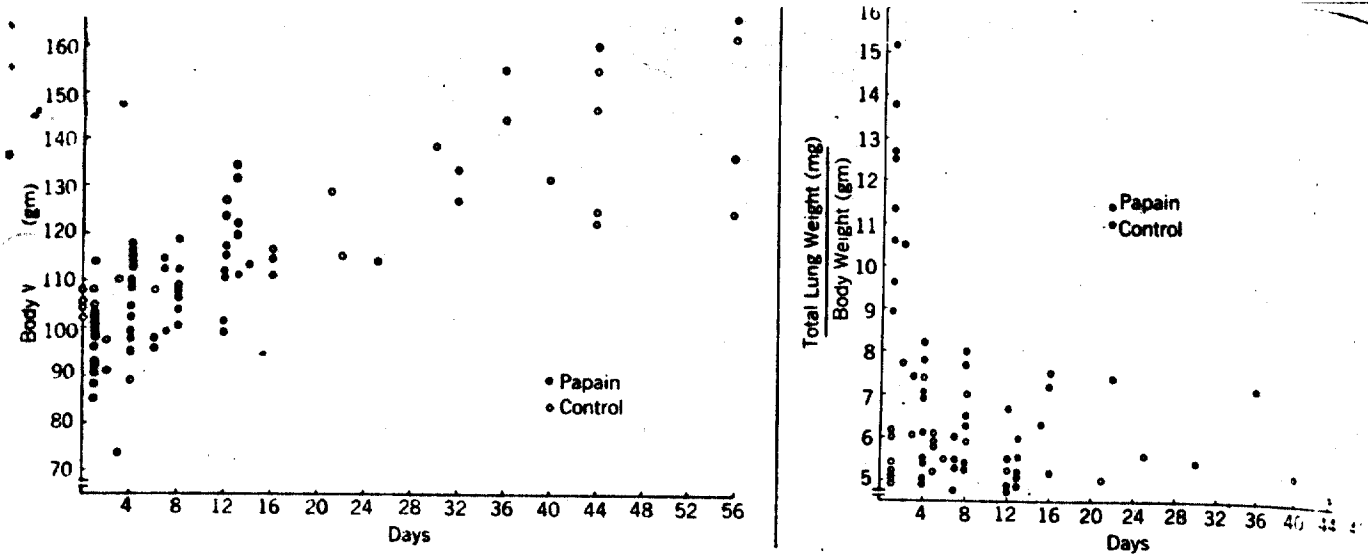


Fig 4.—Left, *Papain-treated and control hamsters had similar weight increments through 56 days.* Right, *The ratio of lung weight to body weight was high in papain-treated animals on the first and second days while pulmonary edema was present but became indistinguishable after four days.*

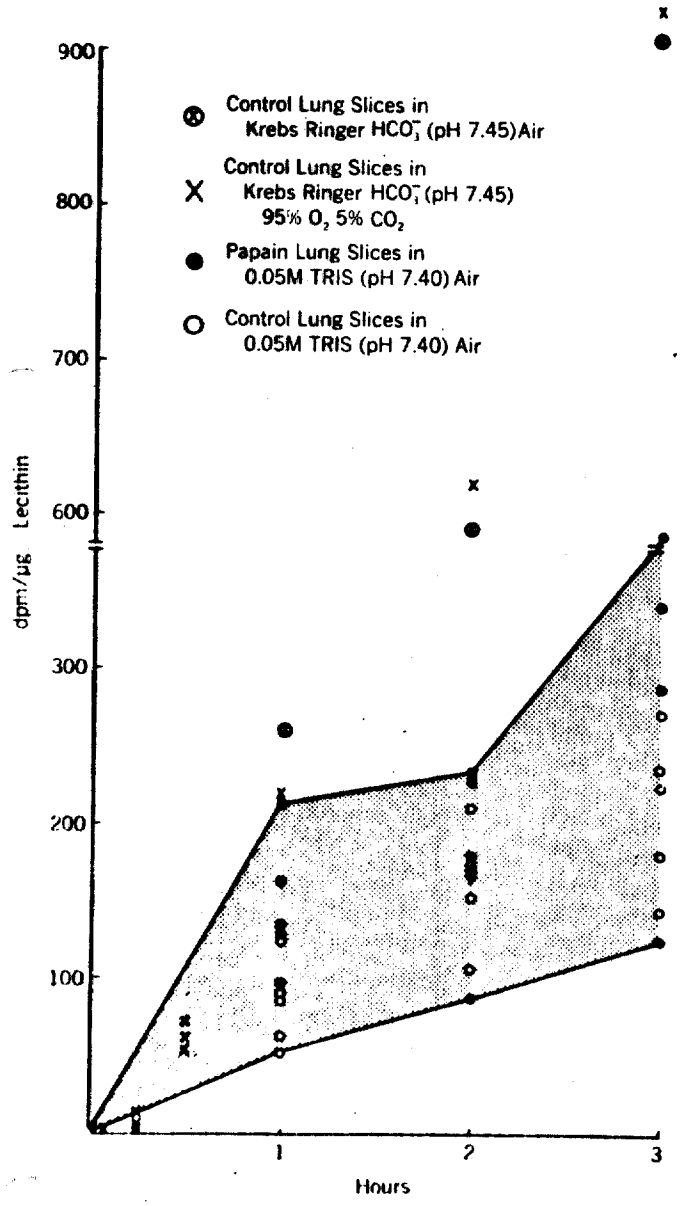
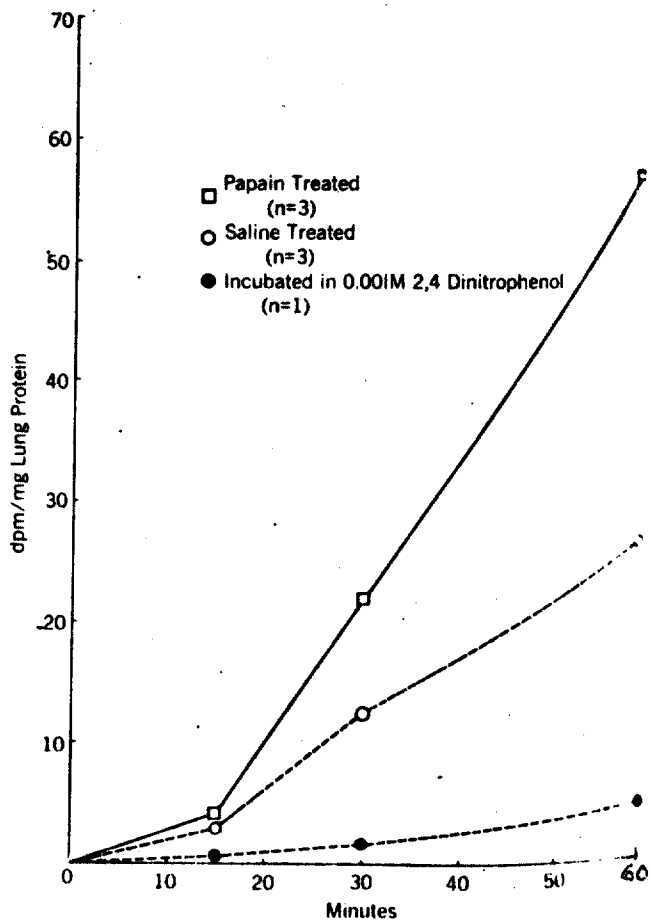


Fig 5.—*Lecithin synthesis measured by choline incorporation in lung slices (in vitro) from papain-treated hamsters was different from that in control animals. Shaded area represents range of data from control animals using tromethamine media.*

Fig 6.—*Protein synthesis by lung slices (in vitro) measured by incorporation of a mixture of four amino acids was greater in papain-treated hamsters than in control animals.*



were obliterated by hyperplasia of endothelial cells (Fig 3, *bottom right*).

The lesions did not show further changes at 16, 32, and 64 days when appraised on fume fixed macroscopic sections and histologic appearances. The prominence of rough endoplasmic reticulum in interstitial cells disappeared and the mature fibrocytes predominated in the ultrastructure of the later lesions. The endothelial swelling and lumen obliterating lesions disappeared but alveolar walls were thick around type II alveolar cells. Perhaps there were fewer capillaries in the trabeculae. Rosette lesions remained prominent. Collagen reappeared around these cells but alveolar septae were thickened and irregular. Collagen around arterioles, around bronchi, in pleura, and in septae appeared unaffected and intact at all stages of departitioning.

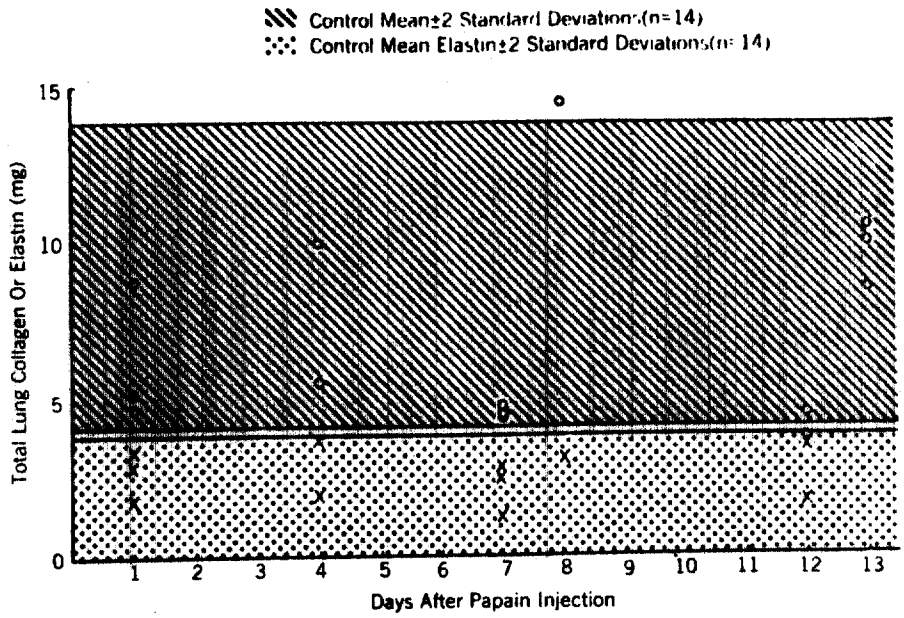


Fig 7.—Total lung collagen and elastin contents of papain-treated hamster lungs were within two standard deviations of means in control animals as indicated by diagonal striped and dotted areas.

Biochemical and Metabolic Studies.—*Growth.*—The papain treated animals grew at the same rate as the controls (Fig 4, *left*). The ratios of lung weight to body weight were unchanged except at ½, 1, and 2 days when pulmonary edema and hemorrhage were widespread (Fig 4, *right*).

Lecithin Synthesis.—Lecithin is a major component of lung surfactant obtained by lavage. In vitro choline incorporation of choline-^{14}C into lecithin by lung slices at one, two, and three hours from animals 2, 8, and 16 days after papain administration was measured to determine whether this vital component was affected by papain. Synthesis was not different from studies in lung slices taken from control (saline-injected) hamsters (Fig 5). The incorporation by control slices of lung was greater when incubated in Krebs-Ringer bicarbonate than when incubated in tromethamine buffer but use of 95% oxygen, 5% carbon dioxide did not change rates from those in air. Therefore, conditions were not optimal for choline incorporation for the lung slices in tromethamine buffer from either control or papain-injected hamsters. Further in vitro studies are being done to compare lecithin synthesis in papain-treated hamster lungs with the use of Krebs-Ringer bicarbonate media.

Protein Synthesis.—The incorporation of the four amino acids into protein was greater at 30 and 60 minutes in lung slices from three papain-treated compared to slices from three saline-treated animals (Fig 6). The animals which comprised this study were killed 2, 8, and 16 days after papain treatment. There was no overlap of dpm per milligram of protein at the 30 and 60 minute incubation periods between the papain-treated and control animals. However, no correction was made for amino acid pool size in the lung slices. The incorporation of amino acids was inhibited by adding 10 $^{-3}$M dinitrophenol to the incubation medium which showed that it was an aerobic synthesis. The finding of increased protein synthesis correlated with many interstitial cells which contained large amounts of smooth endoplasmic reticulum in the lungs of papain-treated hamsters. However, cell numbers were not measured.

Collagen and Elastin Contents.—Collagen and elastin contents of lung were unchanged from control at 1, 4, 8, and 12 or 16 days after papain administration (Fig 7). Collagen and elastin were expressed as milligrams per lung to prevent bias from edema and hemorrhage during the first four days in papain treated animals.

Comment

Alveolar departitioning occurred quickly after a single dose of papain in young Syrian hamsters. It was easily recognized in inflation-fixed lungs and in histologic sections by 4 days and appeared unchanged after 8 days to 64 days. The brief time course for development of this extensive lesion has not been appreciated previously.[7] The analysis of ultrastructural changes showed four changes. First was an early loss of collagen fibrils, followed by the disappearance of alveolar septa with

capillaries arranged in rosettes around type II alveolar cells. Third, there was stimulation of fibroblasts and type II cells evidenced by increased endoplasmic reticulum. Finally, there were endothelial changes at eight days which produced capillary obliteration in the rosettes by 16 days after papain. These rosettes resemble the vascular trabeculae described in human emphysema.[10] The papain-induced endothelial-obliterative changes may be related to vascular lesions observed in spontaneous emphysema in rabbits.[11]

After the first 48 hours the papain-treated hamsters appeared healthy and showed no signs of respiratory distress or cyanosis. They grew normally and ratios of lung wet weight of body weight were normal at four days and thereafter. Therefore, moderate to extensive alveolar loss did not stunt growth. In vitro incorporation of choline-^{14}C into lecithin was measured to ascertain whether phospholipid synthesis was changed when numbers of airspaces were reduced and their size increased in the lung. Choline incorporation rate into papain-treated lungs was no different from that in control lung slices. However, we cannot determine how near to maximal incorporation rates were achieved with the use of this system. In vivo lecithin synthesis rates should be measured to establish this point. More rapid in vitro incorporation of amino acids into total protein in lung slices from departitioned lungs may be explained by either easier access of synthesizing cells to substrate in departitioned lung or by increased protein synthesis per cell.[12] Alterations in amino acid pool size could also result in higher radioactive amino acid incorporation in slices from papain-treated animals. The finding of large numbers of interstitial cells with prominent, rough endoplasmic reticulum recommend the possibility that protein synthesis was increased after papain-induced alveolar departitioning. Total lung protein, collagen, and elastin were normal in the papain-treated hamsters. Because collagen and elastin were measured in the preliminary studies by insoluble residue of lung before and then after hot alkali extraction, this finding should be regarded as tentative until confirmed by a specific collagen method. Nevertheless, the finding suggests that collagen in alveoli is a small fraction of total lung collagen or perhaps that synthesis (repair) is proceeding at a pace consonant with destruction.

As an extension of previous studies, alveolar departitioning resembling centrilobular emphysema has been produced four days after a single intratracheal dose of papain in the hamster. The model should be useful to study further the morphologic and metabolic mechanisms in departitioning of lung. It is important to know that a single exposure of alveoli to a proteolytic enzyme causes collagen to disappear from alveolar walls within 48 hours. Following this change alveolar walls are destroyed and capillaries are rearranged around type II alveolar cells. Thereafter, these capillaries appear to involute. Repair apparently begins shortly after injury and this activity may explain the finding of no change in collagen and elastin contents of the lung following papain administration. Alternatively the proportion of total lung collagen in alveolar walls may be so small that changes are not detected by the method used. Also this model should permit experiments to determine whether the presence of alveolar damage increased the susceptibility of bronchiolar epithelial cells to goblet cell metaplasia (chronic bronchitis) after exposure to air pollutants such as sulfur dioxide, sulfuric acid mist or nitrogen dioxide.

This investigation was supported in part by Public Health Service research grants HE [illegible] and HE-07868 from the National Heart and Lung Institute and by contract 22-69-109 from the Air Pollution Control Organization.

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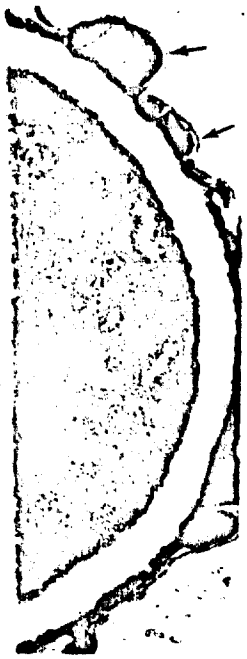
Studies in Permeability of Articular Cartilage in New Zealand Albino Rabbits

The Effect of Aging, Papain, and Certain Steroid Hormones

Andreas P. Kyriazis, MD, PhD and Theodore T. Tsaltas, MD



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IT HAS BEEN ESTABLISHED that cartilage constitutes an active metabolic tissue. Representing a specialized form of connective tissue, it is composed of two distinct components, extracellular matrix and chondrocytes.

A review of the histologic structure of cartilaginous tissues reveals that articular and certain other forms of cartilage are unique among all body tissues in that they are avascular. In avascular cartilage, the viability of chondrocytes, as well as their metabolic and morphologic integrity, depends exclusively on nutrients diffusing through the gelled intercellular substance that surrounds them. Consequently, the permeability of cartilage matrix is of paramount importance in determining the state of nutrition of cartilage cells.

Evidence from this and other laboratories indicates that the chemical composition of cartilage matrix is greatly influenced by the general aging process of the animal or the human and by various hormones.

Previous studies have dealt with changes in the chemical composition of cartilage matrix under certain conditions. The effect of an intravenous solution of papain on the matrix *in vivo*[1-4] and the effect of certain hormones upon its chemical composition have been studied.[5-7] It has also been shown that age brings about certain changes in the chemical composition of cartilage matrix.[8,9] We, therefore, thought it important to investigate the effect of the aging process and that of certain steroid hormones upon the permeability of cartilage matrix. The reasoning was that since cartilage tissue is avascular, its metabolism depends upon the permeation of nutrients through its matrix, and any changes in the permeability of the matrix will eventually affect the function of chondrocytes, the chemical composition and function of the matrix and, finally, the morphologic appearance of cartilage.

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Supported by Research Grant HD-01502 from the US Public Health Service.

Accepted for publication September 2, 1970.

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This paper presents data pertinent to a method for measuring, in New Zealand albino rabbits, the permeability of articular cartilage matrix to a colloidal substance, the influence of aging, and the effect of some steroid hormones on matrix permeability.

Materials and Methods

All experiments were performed on a total of 192 New Zealand albino rabbits. They were divided into two age groups, adolescents (6–7 months) and adults (not older than 24 months). Since, however, the precise age of all animals could not be determined, it was necessary to establish some uniform criteria for the age of the animals. For this purpose, tables obtained from commercial breeders, animal food manufacturers and the US Government Department of Agriculture showed that a direct relationship exists between the age and the weight of rabbits. Animals weighing less than 3.50 kg belong to the group of adolescents. Mature animals have a weight above 3.50 kg. This criterion was shown to be particularly useful in selecting the group of adult rabbits. The ossification of the epiphyseal cartilage of the tibia was an additional indication of the maturity of the animals. Each group contained 96 animals (48 males and 48 females).

Papain (purified powder papain, Sterling-Winthrop, Inc. New York) was injected intravenously, with a constant infusion pump, into the marginal vein of the ear as a 1.5% aqueous solution; 35 mg/kg were injected over a 15-minute period.

Adolescent animals were sacrificed at 48 hours and adults at 72 hours after the injection of papain, at the time the drug was maximally effective.[10] In animals that received hormones, the papain solution was injected 48 hours after the last injection of hormones.

Cortisone acetate (Intra Products, Inc. Dayton, Ohio) was injected intramuscularly in a dose of 5 mg/kg body weight, every other day, over a period of 24 days for a total of 12 injections.

Animals received diethylstilbestrol diphosphate (Dome Laboratories, a division of Miles Laboratories, Inc, New York, New York) 2 mg/kg body weight daily, intramuscularly, for 15 consecutive days.

Animals receiving testosterone propionate (Organon, Inc. West Orange, New Jersey) were injected with 10 mg/kg body weight, intramuscularly, four times a week for a total of 15 injections.

Each one of the two large groups (adolescent and adult) was divided into 8 subgroups (12 animals per group, 6 males and 6 females) as follows:

Group 1	Controls
Group 2	Animals injected with papain
Group 3	Animals injected with cortisone acetate
Group 4	Animals injected with cortisone acetate and papain
Group 5	Animals injected with diethylstilbestrol diphosphate
Group 6	Animals injected with diethylstilbestrol diphosphate and papain
Group 7	Animals injected with testosterone propionate
Group 8	Animals injected with testosterone propionate and papain.

The permeability of articular cartilage was tested by the method of Stockwell and Barnett.[11] All rabbits were killed by air-embolization and the head of the femur removed immediately, washed for 1 minute in 0.9% saline and immersed in a 6% aqueous Protargol solution (Protargol-S, Winthrop Lab, New York, New York) under continuous shaking for 6 minutes. The specimen was then fixed in 10% neutral formalin solution. The time lapse between death of the animal and fixation was kept to 7–8 minutes. In none of the experiments did the foregoing procedure exceed 10 minutes. This precaution was taken since reports indicated that the permeability of rabbit articular cartilage to various molecules diffusing through it decreases considerably after this time.[12] The fixed specimens were decalcified in a formic acid-sodium citrate solution[13] for 1 week, washed in running tap water for 4 hours and embedded in paraffin. Sections 7 and 10 μ thick were cut perpendicularly to the surface of the articular cartilage with a Spencer 820 microtome. The Protargol was visualized by the method of Bodian.[14] Sections were also stained with hematoxylin and eosin.

Measurements for Protargol permeability were performed with a Zeiss research microscope equipped with a microscale, positioned in the left eyepiece and calibrated before all measurements. After calibration with objective 10× and eyepiece 10×, each division of the microscale corresponded to 0.0087 mm. The total thickness of articular cartilage and the depth penetrated by Protargol were accurately measured in at least three different areas and the mean of these measurements was recorded. Points of readings were only those areas in which the section was perpendicular to the surface. When this condition was met, the variation within a given animal never exceeded 5% (range, 0–5%). The permeability index (PI) was obtained by dividing the depth of cartilage penetrated by Protargol by the total thickness of articular cartilage and then multiplying by 100.

Results

After all measurements had been completed, the collected data were classified according to age, sex and treatment of the animal group used. A complete factorial was carried out on a IBM 360/30-64K computer. The computer analysis showed that there was strong interaction between treatment and age of the tested animals and that significant differences existed among treatments. Since there was no significant difference in the values obtained from control and experimental groups in regard to sex, animals of both sexes were combined for further statistical evaluation.

Comparison of the results between the two age groups, adolescent and adult, showed that the permeability of articular cartilage decreases as the animals become older. The PI in adult rabbits was found to be 7.36 less than that of adolescents, representing a change of 20% ($P_{(t)} < 0.05$) (Table 1). In order to evaluate the significance of this change, the total thickness of cartilage in adolescent animals was compared statistically to that of the adult group. This was found necessary

Table 1. Comparison of Mean PI Values Obtained for Adolescent and Adult Rabbits and Their Statistical Evaluation

	PI*	Change (%)	F value	$P_{(t)}$
Adolescents	35.45 ± 2.8			
Adults	28.12 ± 4.6	20	5.48	<.05

* Mean ± SE.

since the PI is an expression of a ratio and this might vary with changes in the denominator as well as in the numerator. Consequently, a difference in the thickness of cartilage, associated with aging, could change this ratio without any appreciable difference in the unit permeability of cartilage. It was found that mean thickness of articular cartilage in adolescent animals (400 ± 11.9 μ), did not differ statistically from that of the adult group (410 ± 12.0 μ).

Injection of papain into adolescent rabbits decreased the PI by 10.25 when compared with the untreated group. This represented a change of 29% ($P_{(t)} < 0.001$). Adult rabbits did not respond to the injection of papain (Table 2). When permeability values in both control and papain-treated adolescent animals were compared with the corresponding adult groups, it was found that the permeability of articular cartilage in adolescent papain-treated animals dropped to the level of adult control or papain-treated (Text-fig 1) animals.

Administration of cortisone increased the permeability of articular cartilage both in adolescent and adult animals. The PI of the adolescent group showed a change of 62.5% ($P_{(t)} < 0.001$). The response of the adult group was much more pronounced, the change was 152%. Estrogen and testosterone also increased the permeability of articular cartilage. In adolescent animals, the PI increased by 13.3 ($P_{(t)} < 0.01$) after estrogen treatment and by 29 ($P_{(t)} < 0.001$) after testosterone. In the groups of adult animals similarly treated the changes were 10.7 ($P_{(t)} = 0.001$), and 7.69 ($P_{(t)} < 0.05$), respectively (Table 3).

Animals pretreated with steroid hormones responded to papain in a way largely determined by the hormonal treatment that preceded it. Table 2 summarizes the changes in permeability for the papain-treated groups. Both cortisone-treated and testosterone-treated adolescent animals were extremely sensitive to papain, as evidenced by the sharp decrease in permeability, with PIs equal to that of the control-papain group. The estrogen-treated group showed a considerable degree of resistance to papain, with a permeability much above that of the untreated control group (Text-fig 2). In adult animals, papain had no effect on the permeability of cartilage in animals treated with estrogen or testosterone. However, papain decreased the permeability of cartilage in animals pretreated with cortisone. In this group, the PI of articular cartilage decreased by 16, a change of 22.5% ($P_{(t)} = 0.01$) (Text-fig 3).

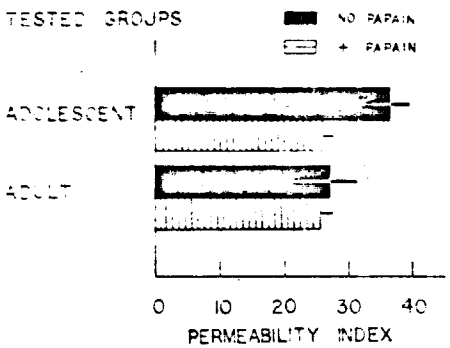
Discussion

In studying the permeability of avascular cartilage, certain factors that may affect the diffusion of a substance through its matrix should be

Table 2. Comparison of Mean PI Values Obtained for Control and Hormone-Treated Adolescent and Adult Rabbits Before and After Intravenous Papain and Their Statistical Evaluation

Treatment	Adolescents				Adults			
	PI*	Change (%)	F values	$P_{(t)}$	PI*	Change (%)	F values	$P_{(t)}$
Control	35.45 ± 2.8	29	11.78	<.001	28.12 ± 4.6	8.5	0.26	NS†
Control-papain	25.23 ± 1.44				26.73 ± 2.1			
Cortisone	57.68 ± 2.6	55	67.43	<.001	70.79 ± 2.9	22.5	6.63	.01
Cortisone-papain	25.53 ± 1.3				54.83 ± 4.4			
Estrogen	48.75 ± 2.7	20	4.55	<.05	38.82 ± 2.1	14.1	2.34	NS†
Estrogen-papain	39.45 ± 2.4				33.34 ± 1.4			
Testosterone	64.57 ± 3.2	59.7	84.38	<.001	35.81 ± 2.6	16.1	3.19	NS†
Testosterone-papain	25.94 ± 1.0				29.99 ± 1.9			

* Mean ± SE.
† Indicates statistically nonsignificant difference with $P_{(t)} > 0.05$.



TEXT-FIG. 1.—Permeability of articular cartilage of adolescent and adult rabbits before and after injection of papain. Horizontal lines represent standard error (SE).

taken into account—*ie*, the particle size of the reference substance and its affinity for various matrix components. It might be argued that a particle size larger than the mean pore diameter of cartilage matrix, estimated to be approximately 62 Å,[15] would exclude that substance from entering into the matrix. Also, a chemical interaction with the matrix components would be expected to confine the substance to the very superficial layers of the matrix.

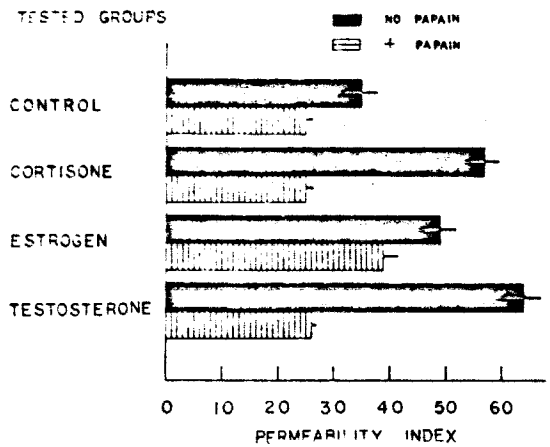
Protargol, with a particle size of about 40 Å,[16] has been shown to permeate articular cartilage easily;[11] this finding has been further confirmed by the present study. However, whether Protargol with a molecular size close to the mean pore diameter of cartilage matrix might block the pores in the deeper layers of matrix, thus interfering with its permeability, remains an open question. In addition, the authors of the present study have no information about the effect synovial mucin adsorbed on the surface of articular cartilage may have on the permeability of cartilage matrix.

Despite these limitations, the foregoing data clearly indicate that both aging and hormone administration are factors affecting the permeability of avascular cartilage. It seems that certain changes associated with aging occur at the early stages of maturation in animals and these changes persist throughout adulthood.[17] It may be postulated that such early changes might be the ones that most adversely affect the total metabolism of cartilage tissue, ultimately reflected in changes in the chemical composition and function of the cartilage matrix. During maturation there is a progressive decrease in the hexosamine content of cartilage matrix, followed by a decrease in

Table 3. Comparison of Mean PI Values Obtained for Adolescent and Adult Rabbits after Treatment with Steroid Hormones and Their Statistical Evaluation

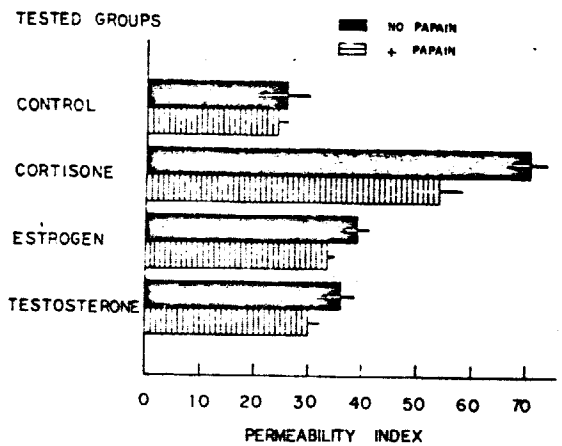
	Adolescents				Adults			
Treatment	PI*	Change (%)	F values	$P_{.05}$	PI*	Change (%)	F values	$P_{.05}$
Control	35.45 ± 2.8				28.12 ± 4.6			
Cortisone	57.68 ± 2.6	62.5	23.95	<.001	70.79 ± 2.9	152.0	86.52	<.001
Estrogen	48.75 ± 2.7	37.0	10.24	<.01	38.82 ± 2.1	38.0	10.54	.001
Testosterone	64.57 ± 3.2	81.2	36.37	<.001	35.81 ± 2.6	23.7	5.93	<.05

* Mean ± SE.



TEXT-FIG 2.—Permeability of articular cartilage of adolescent rabbits in relation to the treatment before and after injection of papain. Horizontal lines represent SE.

the nitrogen/hexosamine molar ratio.[15] Similar changes have been observed by Matthews[19] and Bollet *et al*[20] in the cartilage of persons with degenerative arthritis. They are much more pronounced in papain-treated animals and, as Tsaltas *et al*[15] have pointed out, there is a



TEXT-FIG 3.—Permeability of articular cartilage of adult rabbits in relation to the treatment before and after the injection of papain. Horizontal lines represent SE.

similarity between papain-induced changes and those due to either the aging process or degenerative arthritis. Since these changes represent an alteration in the chemical composition of cartilage matrix, the injected steroid hormones may have increased the permeability of cartilage matrix by restoring its chemical composition to that of an earlier stage of development. This hypothesis is supported by experiments carried out in this laboratory (the results of which will be published in a separate paper) showing that changes in the permeability of the cartilage matrix are closely related to changes in its total chemical composition, which is strongly affected by steroid hormone and/or papain treatment. It is better illustrated by the decreased PI in animals that received papain, which hydrolyzes the cartilage matrix *in vivo*: a large portion of the hexosamines is removed and the nitrogen/hexosamine molar ratio, is increased.

Among all steroids, cortisone appears to be the most effective in increasing the permeability of articular cartilage, particularly of adult animals. This observation seems to be of particular importance in promoting a better understanding of the mode of action of cortisone upon those pathologic conditions in which the primary effect on cartilage is matrix degeneration.

Pretreatment of adolescent rabbits with either cortisone or testosterone does not affect their response to papain. In the three groups, control-papain, cortisone-papain, and testosterone-papain, the PI values were 25.23, 25.53 and 25.94, respectively (Table 2). Estrogen, on the other hand, seems to have a relative protective effect against the action of papain. Here the decrease in permeability is considerably less than in the other three groups and the PI of the estrogen-papain group (39.45) is significantly higher compared to those of the previous groups.

Adult rabbits were resistant to papain. In our experiments, however, adult rabbits pretreated with cortisone showed an increased response to papain, as evidenced by a decrease in PI followed by changes in the chemical composition of cartilage matrix.

These differences may indicate that certain steroid hormones alter the response of the animals to papain. They may suggest a difference in the mode of action of the steroids studied, although the end result is the same, namely an increase in the permeability of cartilage matrix.

Summary

The effect of aging and that of papain and certain steroid hormones upon the permeability of articular cartilage was studied in 192 New

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we were unaware of the previous description of this type of device. We made no claim to originality, however, and, further, the description in the textbook to which Dr. Hobbs refers is only very brief. We would again stress the value of this type of device in the operating-theatre, where the level and tilt of the table are frequently altered."—ED.L.

DEXTRAN 40

SIR,—Prompted by your annotation on the new *B.P.* Addendum,[1] we should like to comment on the monographs on dextran with special reference to the one on dextran 40, a product which was originally developed and characterised, and the use of which was pioneered, by our company under the name of 'Rheomacrodex'.

We very much regret the inclusion of dextran 40 in the 1966 Addendum based on the monograph as it now stands, because we consider the methods given for its characterisation to be insufficient, and conducive to erroneous and misleading information on the factual average molecular weight and molecular-weight distribution in a given preparation. The determination of *intrinsic viscosity*, which the monograph prescribes as the only procedure for characterisation of molecular size, is an indirect method, and must depend on calibrated standards relating the viscosity to molecular size determined by an absolute method such as the *light-scattering analysis*.

It is our experience, based on a vast amount of determinations of samples from various sources, that there exists no satisfactory general correlation between viscosity and molecular size for the dextran fractions in clinical use. Substantial variations in both average molecular size and size of the important tail-end fractions can exist for samples well within the viscosity limits given in the *B.P.* specification. Accordingly a prescribing physician cannot be sure that a preparation of dextran 40, characterised only by the monograph in the *B.P.*, is in fact identical with one with which he has previously obtained the biological effect and the absence of side-reactions which led him to use the treatment in the first place.

It should be pointed out that the *light-scattering method* is by no means an esoteric novelty, since it is the procedure used to secure that dextran preparations correspond to the U.S. Defence Medical Purchase Description, a set of specifications which have gradually gained acceptance as an international standard. In addition, with regard to other indices, the new *B.P.* monograph fails to include up-to-date, adequate, and exhaustive methods now available for the characterisation of the relatively complex, polydisperse mixtures of polymers known as dextrans.

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CREAM

SIR,—Your annotation (Sept. 10, p. 580) and the report[2] on fresh cream in Worcestershire are timely. Colenso et al. are to be congratulated on an interesting and valuable survey. As you rightly point out, too little attention has been paid in the past to cream and its bacteriological quality. In addition to the well-known West Country clotted creams, a confusing variety of different liquid creams are available, and there is a tendency to assume that because most of these are heat-treated or pasteurised, all is well. This is not borne out by our experience, which corresponds generally with that described in Worcestershire; our findings will be published when our investigations are completed.

We have now examined more than 500 samples of various creams, and although we have some reservations about the phosphatase test, we consider that the methylene-blue-reduction test together with a semiquantitative coliform test are useful. Indeed only 99 samples (22%) of clotted creams were considered to be satisfactory in that they did not decolorise methylene blue in less than 2½ hours and were free from faecal coli. Of over 100 samples of heat-treated fresh liquid creams examined, 44% contained faecal coli, and most of these decolorised methylene blue in less than 30 minutes. In addition, we have isolated *Brucella abortus* from one cream cake, 11 samples of heat-treated liquid creams, and 4 samples of clotted creams.

Contamination after heat-treatment and bulk distribution will always remain potential hazards, but our findings suggest that there is still some room for improvement in cream-processing methods, and support the view that there should be a minimum bacteriological standard in order to give the consumer a reasonable degree of protection.

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1. *Lancet*, 1966, i, 867.
2. Colenso, R., Court, G., Henderson, R. J. *Mon. Bull. Min. Hlth*, 1966, 25, 153.

Yy CHROMOSOMAL CHIMÆRISM

SIR,—We have read with interest the letter of Dr. Naiman and his colleagues (Sept. 10, p. 590) in which they report an apparent foetal graft arising after intrauterine blood-transfusion for erythroblastosis fœtalis. In 1962 we published[1] results demonstrating the persistence of viable, mitotically-competent, lymphocytes in bank blood, and discussed the implications which lymphocyte grafts might have under specific clinical situations. We suggested that consideration should be given to the removal of leucocytes from blood to be administered to patients with incompetent immune mechanisms. The report of Dr. Naiman and his colleagues, in which they advance the finding of "Yy" chromosomal chimærism as evidence for such a graft, may represent the first firm evidence for lymphocyte graft arising from blood-tranfusion. We await with interest their detailed report.

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NICHOLAS L. PETRAKIS.

MITOGENIC ACTION OF PAPAIN

SIR,—We have found (July 23, p. 232) that trypsin and chymotrypsin have a mitogenic action on in-vitro short-term cultures of peripheral lymphocytes from normal persons. Such an effect seems to be related to the damaging action of these enzymes on the cell membrane. This finding supports the hypothesis that the mitogenic action of phytohæmagglutinin (P.H.A.) on lymphocytes from almost all normal persons is also non-specific, and depends on an irritative or damaging action on the cell surface, unrelated to immunological mechanisms. We report here analogous results with papain to those we have obtained with trypsin and chymotrypsin.

Cultures of lymphocytes incubated with papain showed higher incidence of blastic elements than controls. Lymphocytes from 5 normal persons were tested: at the 144th hour, in the cultures with papain, blastic elements ranged from 2 to 7% of cultured cells, while in control cultures without stimulating agents they ranged from 0·03 to 0·1%. But papain, at the concentration used (8–40 μg. per 5 ml. of medium), showed a weaker mitogenic action than trypsin and chymotrypsin (2–14% of blastic elements at the 96th hour), and than all the other blastogenic substances with a non-specific action, such as P.H.A.,[2] staphylococcal hæmolysins,[3] and anti-leucocyte sera.[4]

The demonstration of the mitogenic action of another proteolytic enzyme, in addition to trypsin and chymotrypsin,

1. Petrakis, N. L., Polithy, G. *New Engl. J. Med.* 1962, 267, 286.
2. Nowell, P. C. *Cancer Res.* 1960, 20, 462.
3. Ling, N. R., Husband, E. M. *Lancet*, 1964, i, 363.
4. Gräsbeck, R., Nordman, C., de la Chapelle, A. *ibid.* 1963, ii, 385.

is in keeping with the above-mentioned interpretation of a non-specific, non-immunological genesis of the blasts appearing in cultures in relation to agents which damage the cell surface.

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LYMPHOCYTES AND DRUG HYPERSENSITIVITY

SIR,—Dr. Vischer's report (Aug. 27, p. 467) is of great interest. We are not at all surprised that he failed to demonstrate a positive response to penicillin in cultures of cells from all his subjects with a history of reaction following penicillin therapy. We have been engaged in a study of persons presenting with skin eruptions, anaphylaxis, or serum-sickness-like reactions following penicillin therapy, or with histories of such reactions. These persons were tested for immediate and delayed skin sensitivity to penicillin and penicilloyl-polylysine and for the presence of circulating hæmagglutinating antibodies of 7S and 19S classes. Their lymphocytes were cultured in the presence of penicillin or phytohæmagglutinin and without additives. Control subjects were also studied. The results, to be published in greater detail elsewhere, may be summarised briefly.

Only 11 of the 17 subjects giving a history compatible with allergic reaction to penicillin had a positive in-vitro lymphocyte response to penicillin (a significant increase in the number of "blast" cells and mitoses present compared with control values). Several members of the control group having no history of reaction following penicillin therapy also had a positive lymphocyte response. The one factor common to all the persons who had a positive lymphocyte response was the presence of a positive reaction to intradermal injection of penicillin G or penicilloyl-polylysine. Almost all the subjects whose cells responded to penicillin in vitro had an immediate wheal-and-flare response to penicillin or the penicilloyl group. The others all exhibited delayed sensitivity to the drug before, or at the time of, the first positive lymphocyte response. Within a month of the first positive lymphocyte culture, all had a positive immediate skin-test reaction. There were no positive lymphocyte responses in subjects with negative immediate and delayed skin tests, irrespective of the clinical history. Furthermore, there was no consistent correlation with either the length of time elapsed since the last clinical reaction or the levels of circulating hæmagglutinating antibody.

We therefore conclude that a meaningful evaluation of in-vitro lymphocyte response to penicillin, or probably any antigenic substance, must take into account more objective and sophisticated criteria of allergy than a history of a reaction following exposure to the material. Examples of such criteria are immediate and delayed skin-test reactions with the proper antigenic determinants.

We agree completely with Dr. Vischer's comments on the difficulties of using morphological alterations for evaluating lymphocyte response. Only insistence on punctilious details of culture and preparative technique, acceptance of rigid definitions for reactive (blast) cells, and use of all appropriate controls can overcome these difficulties and yield satisfactory and reproducible results.

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EFFECT OF RESERPINE ON LYMPHOPOIETIC SYSTEM

SIR,—Having noted that reserpine produces gross and microscopic changes in the thymus and bursa of Fabricius of newly hatched white leghorn chicks, we designed an investigation to analyse the effect of this drug on the lymphopoietic system in chicks and in mammals—mice (CH_3), and *Meriones unguiculatus* (gerbil). The gerbil was chosen because of its very large thymus.[1] We give here the preliminary results of these investigations.

In chicks, a pronounced fall in the number of circulating lymphocytes followed the injection of 0·1 mg. of reserpine, and the thymus, bursa of Fabricius, and spleen were strikingly decreased in size and showed severe lymphoid depletion. Similar results were observed in adult mice (25–30 g.) and in *M. unguiculatus* (65–87 g.).

In chicks, the fall of circulating lymphocytes followed by a slow and progressive recovery and a short, lasting stimulation of heterophils are very much reminiscent of the haematological changes that follow thymectomy and bursectomy.

This investigation shows that reserpine administered in high doses can produce sustained depression of the entire lymphopoietic system and temporary depression of granulocytopoiesis. These results, we assume, provide an indication for a trial of this drug in experimental studies in immunodepression.

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FATAL POISONING WITH METHAQUALONE AND DIPHENHYDRAMINE

SIR,—A note in *Drug and Therapeutics Bulletin*[3] has drawn attention to the dangers of overdosage with methaqualone, available in this country as 'Mandrax' (Roussel) tablets (methaqualone 250 mg. and diphenhydramine 25 mg.). At that time, the only fatal cases recorded were from Germany, and were after methaqualone only, marketed there as 'Revonal'. We report here a fatal case of poisoning with mandrax in Great Britain.

A 62-year-old woman was admitted to this hospital, at 1 P.M., deeply unconscious and responding only to very painful stimuli, apparently having taken about 30 tablets of mandrax. A neighbour had seen her to be well the previous evening. On admission there were no focal neurological signs, and the pupils, although dilated, reacted normally to light. The stomach was washed out, but no drug residues were recovered, and an intravenous infusion was set up with the aim of promoting a forced diuresis. Shortly after admission generalised jactitations were noted, and an attempt was made to control these with intramuscular paraldehyde. For roughly 6 hours after admission her condition seemed to improve, in that spontaneous movements of the limbs occurred and she responded to less painful stimuli. At about 7 P.M., however, the patient had a generalised convulsion lasting roughly 10 minutes accompanied by central cyanosis, after which she became deeply unconscious and completely unrousable. An endotracheal tube was passed to maintain a good airway and to prevent the inhalation of vomit, and for the next 30 hours her condition remained unchanged, with an occasional mild convulsion controlled with intravenous phenytoin. 40 hours after admission she developed Cheyne-Stokes respiration and died shortly afterwards.

Postmortem examination showed that the immediate cause of death was severe acute purulent bronchitis, with mucosal ulceration, pulmonary œdema, and congestion. This had occurred despite antibiotic therapy with tetracycline from admission. There was a film of fresh subdural blood clot on the right middle fossa, and a few small cortical haemorrhagic infarcts on the kidneys. With the exception of these haemorrhagic complications, the picture was that of an acute respiratory infection after a sedative poison.

Biochemical investigations on blood taken on admission to hospital showed substances which gave a combined spectrophotometric curve of methaqualone and diphenhydramine. Liver and kidney specimens taken at necropsy contained methaqualone metabolites, estimated at 2·0 mg. and 0·6 mg. per 100 g., respectively.

1. Pay, D. L., Magdine, S. F., Mazzei, [illegible]
2. Masciolì, G. P., Magdine, S. F., [illegible], 54, 911
3. *Drug Ther. Bull.* 1966, 4, 24

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STUDY OF THE INTERRELATIONSHIP OF SERUM CHOLESTEROL LEVEL, AORTA CHOLESTEROL CONTENT, AND THE DEGREE OF ATHEROSCLEROSIS IN EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS

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Evidence has been accumulating over many years suggesting that excessive consumption of fats and hypercholesterolemia may be factors involved in etiology of arteriosclerosis (12, 14, 16, 20 and 22). Although it was formerly believed that large dietary intake of cholesterol caused an increase in serum cholesterol, it is now generally agreed that these two factors are not necessarily interdependent (12) and, furthermore, that dietary cholesterol may be absolutely without influence on blood cholesterol level. However, a diet rich in cholesterol is usually a hight-fat diet and the latter does appear to correlate with elevated serum cholesterol levels and presence of arteriosclerosis.

Further evidence linking cholesterol and fats to arteriosclerosis is derived from consideration of conditions associated with hypercholesterolemia and arteriosclerosis such as diabetes, nephrosis, hypothyroidism, and essential familial hypercholesterolemia. The opposite is seen in patients with idiopathic steatorrhea where impaired absorption of dietary fats is accompanied by low incidence of arteriosclerosis.

The theory that cholesterol and fats are related to arteriosclerosis is supported by post-mortem studies indicating that arteriosclerotic plaques are rich in cholesterol esters apparently derived from serum cholesterol (3, 4 and 5). On this basis, and because of close interrelations between the kind and amount of dietary fat and dietary cholesterol in hypercholesterolemia, influence of different types of dietary fats has been the subject of much recent investigation. Evidence was then put forward (10 and 25) that while dietary fat appears to be related to serum cholesterol the type of fats is even more important. Animal fats and hydrogenated vegetable oils tend to raise serum cholesterol, whereas marine or fish oils and natural vegetable oils act to lower the serum level (1, 2, 9, 13, 26 and 28). Difference in these two types of fats is to be found in the higher concentration of unsaturated fatty acids in the latter oils. Presumably, these unsaturated fatty acids act in some way to lower serum cholesterol. This, indeed, is claimed to be the explanation of a relatively low incidence of coronary artery disease among populations consuming fats of the unsaturated type.

Most of the serum cholesterol is believed to be combined with globulins in the form of a lipoprotein complex (8). Although extensive studies have been undertaken to determine the lipoprotein fraction most responsible for development of arteriosclerosis, results thus far have not been conclusive. However, in patients with arteriosclerosis, the alpha fraction is generally depressed and the beta fraction is elevated (7).

Solubility of the cholesterol-containing lipoproteins in blood, apparently, is maintained by plasmatic stabilizers such as heparin, fatty acids, and phosphatides (11). Experimental results indicate that when the level of these stabilizers is

lowered, solubility of lipoproteins decreases and arteriosclerotic plaques composed of cholesterol form. Therefore, it has been proposed that atherogenesis might be initiated by decreases in the level of heparin, fatty acids, and phosphatides.

It was the purpose of this experiment to study the interrelations between a highly unsaturated fatty acid, cis-oleic acid, its isomer, and dietary cholesterol in experimental hypercholesterolemia.

MATERIAL AND METHODS

The chemical analyses to be reported here were done at completion of the experiment described in Table I.

All information pertaining to bleeding of the animals, grading of visible lipemia, and techniques used for chemical analyses has been provided in a previous publication (15). They will be briefly summarized here for the benefit of the reader.

Determinations were made of the cholesterol content in the serum and the aorta. Although chemical analyses were done of the serum cholesterol at the mid-point of the experiment, only the values obtained at termination of the experiment will be considered here.

For the purposes of the analyses to be carried out, a quantity of ten millimeters of blood was removed from the ear according to the following technique. The underside of the left ear of the rabbit was first painted with xylol, followed in a few seconds by an application of 95 per cent alcohol. This procedure resulted in a sustained dilatation of the vessels which facilitated the flow of blood. A small incision with a sharp razor blade across the central vessels of the allowed adequate quantities of blood to lected. The blood was received in a dry centrifuge tube and allowed to clot. Th vided serum for the estimation of lipem serum cholesterol levels.

Bleeding from the ear, after the ne quantity of blood had been collected was s by application of digital pressure over the vessels for a few minutes.

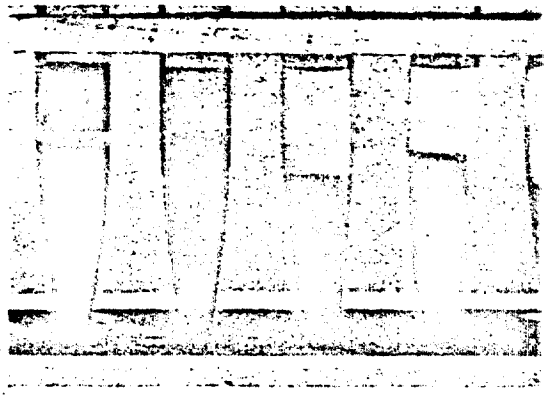


Figure 1 — Grading of lipemic serum, using a O to +++++ (readings from left to right : +++, ++++ and +++++).

The lipid content of the serum was estim a rough quantitative basis and graded fro to five, the former being clear and the latte white in color. The intermediate deg opacity were placed between these extren cording to the scale shown in Figure 1.

Determinations were then made of th and free cholesterol in both the serum a aorta by the Schœnheimer-Sperry di precipitation method (27), as modified

TABLE I

Protocol of experiment

GROUP	NUMBER OF ANIMALS	DIET	TREATMENT
M	18	Cholesterol ± corn oil	*Nil*
C	23	Cholesterol ± cis-oleic acid	*Nil*
P	17	Cholesterol ± cis-oleic acid	Papain : 1 mL of a 2% solution per Kg. body weight after 6 weeks.
D	21	Cholesterol ± cis-oleic acid	Vitamin D_2 : 2,000,000 U per day
T	21	Cholesterol ± trans-oleic acid	*Nil*

laboratory by our biochemist, Dr. B. I. Weigensberg (23 and 24). From the values obtained the er cholesterol was derived by merely subtracing the free from the total cholesterol.

Values for the total and free cholesterol were first obtained as calorimetric galvanometer readings. By applying these to their respective standard curves the cholesterol readings were converted to milligrammes per cent. Results, in each instance, were then expressed in milligrammes of cholesterol per 100 milliliters of serum, or per 100 milligrammes of frozen-dried aorta.

Statistical analysis of the results was made according to the following formula :

1) Determination of the standard deviation (S) :

$$S = \frac{\sqrt{\frac{\Sigma X^2}{N-1} - \bar{X}^2}}{\sqrt{N-1}}$$

where X is an individual reading ;
$\bar{X}$ is the average of individual readings ;
Σ is the sum of individual readings ;
N is the number of readings.

2) Determination of the standard error (t) :

$$t = \frac{D}{\sqrt{S_1^2 - S_2^2}}$$

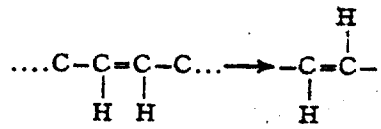
where D is the difference between means.

3) Determination of the probability (p) :

« p » value was obtained by considering the value of the standard error (« t ») in regards to the total number (N) of readings compared.

Computation of the standard error (« t » value) and of the probability (« p » value) was made from the tables compiled by Fisher and Yates (6). Only « p » values lower than 0.05 ($p<0.05$) have been considered of statistical significance.

The trans-oleic acid was prepared in the biochemistry laboratory of our department by elaidation of cis-oleic acid. The chemical reaction is concerned with the displacement of a hydrogen from its natural opposing position to a diagonal position, as shown by the following equation :



RESULTS

In order to provide a basis for comparison of the data obtained in this experiment, and to show the relative constancy of values found in normal sera, a table has been made of all the data previously obtained in this department from analyses of normal rabbit's serum (Tables II and III). All ratios usually referred to in the study of blood lipids have also been included in a table for quick reference (Table IV).

TABLE II

Normal range of serum lipids (male rabbits)

	Animals	Observations	Cholesterol mg. % *			Lipid Phosphorus mg. %*	Fatty Acid of Neutral Fat mg. %*
			Total	Free	Esterified		
GAINSBOROUGH	30	30	55.0	20.0	35.0	6.5	8.7
FLUHS	50	50	48.6	12.1	36.7	5.2	
HORLICK	95	95	41.6	8.9	27.9	4.3	
HUTCH	15	15	43.6	16.7	26.9		
BACH	76	168	42.1±23.4	15.2±9.85	26.9		
McMILLAN	40	143	35.7±19.6	11.5±5.4	24.8±16.7		
MEISENER	44	44	36.8	14.3	22.5	5.2	7.2
FAYNE	42	42	46.0±17.5	19.0±9.5	27.0	4.4	7.3±3.1
WHITESIDE	69	69	49.7±23.3	18.5±11.2	31.2	7.2±2.4	6.3±3.7

* Mean values ± standard deviation of the mean.

TABLE III

Normal range of serum lipids (male rabbits)

	Animals	Observations	Cholesterol mg. % *			Lipid Phosphorus mg. %*	Fatty Aci of Neutral F mg. %
			Total	Free	Esterified		
Gordon	72	144	51.6±7.4	11.6±3.0	40.6±5.6	5.0±0.4	8.7±0.9
Horlick	42	42	50.1±6.0	13.7±2.0	36.4±5.0	5.5±0.4	
McMillan	40	143	35.7±1.6	11.5±0.45	24.8±1.4		

* Mean values ± standard error of the mean.

TABLE IV

Ratios of the mean values of the serum lipids.

Serum Cholesterol /	Free / Esterified	0.22 – 0.37
Serum Cholesterol /	Free / Total	0.21
Serum Cholesterol /	Total / Esterified	1.2 – 1.4
Serum Total Cholesterol / Serum Lipid Phosphorus	.....	8.4 – 12.4
Fatty Acid / Serum Cholesterol Esterified	.....	0.20 – 0.26

Experimental results obtained in respec groups can be found in Tables V, VI, VII, V and IX.

Considering the experiment as a whole, following observations can be readily made :

1. The variations in individual serum c esterol levels are entirely unrelated to the t cholesterol ingested at the dose used.

2. There is but a rough general correlatio tween the degree of lesions and the amou cholesterol ingested, and there are wide indivi fluctuations.

TABLE V

Terminal serum cholesterol levels and severity of atherosclerosis in rabbits of group M.

Rabbit Number	Terminal Serum				Severity of Aortic Atherosclerosis						
	Visible Lipemia	mg. cholesterol per 100 ml. serum			% of intimal area involved with lesions				mg. cholesterol per 100 m frozen – dried aorta		
		Total	Free	Esterified	Whole	Arch	Thoracic	Abdominal	Total	Free	Est
1 U	3	803	92	711	27	85	20	7	5.869	0.804	5
6 U	2	283	85	198	14	30	13	4	2.243	0.082	2
11 U	0	199	77	122	8	13	8	3	3.510	0.261	3
16 U	3	1062	93	969	20	88	15	5	6.067	0.599	5
21 U	2	837	101	736	8	27	6	2	1.453	0.394	1
26 U	2	243	74	169	0	0	0	0	0.155	0.116	0
31 U	3	850	108	742	57	53	45	57	12.189	1.818	1
36 U	2	657	108	549	0	0	0	0	0.190	0.095	0
41 U	2	538	74	464	5	30	3	2	1.352	0.164	
46 U	2	558	80	478	4	13	2	1	1.002	0.356	0
51 U	4	949	170	779	6	38	3	2	3.008	0.608	
56 U	2	632	88	544	11	42	6	3	2.546	0.652	
71 U	0	462	141	321	0.5	0.8	0.5	0.8	1.402	0.709	1
76 U	4	2354	564	1790	4	30	2	1	0.842	0.447	0
81 U	4	2487	662	1825	3	13	2	1	1.464	0.766	
91 U	2	925	284	641	0.7	0.7	0.5	0.5	0.846	0.490	
46 V	0	139	45	94	0	0	0	0	0.979	0.683	
47 V	2	1099	329	770	3	5	3	1	1.209	0.747	
Mean (18)	2.17	838	176	662	10	26	7	5	2.573	0.555	
S.D. ±	1.24	646	176	204	14	27	11	13	2.94	0.405	

TABLE VI

Terminal serum cholesterol levels and severity of atherosclerosis in rabbits of group C

Rabbit Number	Terminal Serum				Severity of Aortic Atherosclerosis						
	Visible Lipemia	mg. cholesterol per 100 ml. serum			% of intimal area involved with lesions				mg. cholesterol per 100 mg. frozen - dried aorta		
		Total	Free	Esterified	Whole	Arch	Thoracic	Abdominal	Total	Free	Esterified
2 U	3	1033	120	913	7	25	3	4	1.05	0.74	0.31
12 U	5	2570	501	2069	12	85	11	6	4.45	0.74	3.71
17 U	2	1099	204	895	30	52	18	39	3.97	0.72	3.25
22 U	2	1266	275	991	4	14	7	4	1.85	0.88	0.97
37 U	5	2570	353	2217	77	94	73	70	5.44	0.80	4.64
42 U	3	1825	186	1639	3	6	1	1	0.97	0.85	0.12
47 U	5	983	171	812	4	15	2	2	1.33	0.85	0.48
52 U	3	1015	197	819	8	28	5	3	1.78	0.72	1.06
57 U	4	2198	366	1831	14	85	8	4	6.07	1.59	4.48
82 U	5	4081	1243	2838	90	97	83	92	4.93	2.43	2.49
92 U	2	1340	441	899	7	20	6	4	2.19	0.78	1.41
97 U	3	1568	573	995	22	50	14	28	2.77	0.95	1.82
48 V	0	180	76	104	1	1	1	1	0.76	0.62	0.14
49 V	0	377	153	224	34	22	45	5	3.37	1.17	2.20
50 V	4	1484	492	992	5	2	6	3	1.14	0.80	0.33
51 V	2	1099	386	713	11	35	10	1	2.38	0.66	1.72
54 V	2	1456	450	1006	7	21	9	1	1.75	0.67	1.07
Mean (17)	2.94	1538	364	1174	20	38	18	16	2.7176	0.9395	1.7781
S.D. ±	1.60	924	272	714	26	33	25	30	1.6882	0.4492	1.4830

TABLE VII

Terminal serum cholesterol levels and severity of atherosclerosis in rabbits of group P

Rabbit Number	Terminal Serum				Severity of Aortic Atherosclerosis						
	Visible Lipemia	mg. cholesterol per 100 ml. serum			% of intimal area involved with lesions				mg. cholesterol per 100 mg. frozen - dried aorta		
		Total	Free	Esterified	Whole	Arch	Thoracic	Abdominal	Total	Free	Esterified
10 U	5	1190	256	1734	25	87	38	3	7.2493	1.3486	5.9007
15 U	4	1389	87	1302	4	20	3	1	2.4895	0.0994	2.3901
20 U	3	1664	109	1555	13	55	10	5	3.4008	0.3982	3.0026
25 U	4	2067	205	1862	79	68	70	93	7.6752	1.3089	6.3663
30 U	4	2707	352	2355	28	98	25	22	6.9802	4.5052	2.4750
35 U	5	3181	689	2492	13	68	7	3	3.9282	0.2298	3.6984
40 U	5	2253	346	1907	4	27	1	1	3.2515	0.4944	2.7571
45 U	4	1910	194	1716	5	10	4	2	2.5519	1.0787	1.4732
50 U	5	3748	655	3093	43	37	37	48	8.7066	0.8564	7.8502
55 U	5	2497	655	1842	40	96	35	12	8.1909	2.3699	5.8210
60 U	5	2239	484	1755	20	92	23	8	7.6502	2.6807	4.9695
75 U	3	1630	605	1025	7.5	38	3	1.5	0.4355	0.4288	0.0067
80 U	0	382	122	260	5	30	2	0.5	0.5751	0.5036	0.0715
85 U	4	2466	708	1758	18	12	27	2	2.2395	0.0439	2.1956
90 U	4	2442	698	1744	26	82	24	22	8.2585	0.9104	7.3481
95 U	3	1658	466	1192	7	20	5	2	1.8799	0.0706	1.8093
100 U	5	2853	938	1915	7	87	6	2	1.9628	0.1898	1.7730
Mean (17)	4.0	2181	445	1736	20	52	19	13	4.5544	1.0304	3.5240
S.D. ±	1.3	761	259	616	20	31	19	24	2.96	1.18	2.42

TABLE VIII

Terminal serum cholesterol levels and severity of atherosclerosis in rabbits of group D

Rabbit Number	Terminal Serum				Severity of Aortic Atherosclerosis						
	Visible Lipemia	mg. cholesterol per 100 ml. serum			% of intimal area involved with lesions				mg. cholesterol per 100 mg. frozen – dried aorta		
		Total	Free	Esterified	Whole	Arch	Thoracic	Abdominal	Total	Free	Esterified
5 U	4	1561	400	1161	43	94	45	8	3.8889	0.4630	3.4259
48 U	5	2942	484	2458	28	94	40	5	6.2770	3.8001	2.4769
58 U	5	4129	1168	2961	—	—	—	—	—	—	—
73 U	5	4158	1211	2947	77	96	67	73	7.2626	2.8476	4.4150
78 U	4	3027	793	2234	65	95	73	48	6.8675	1.5793	5.2882
83 U	5	3760	1243	2517	8	50	4	2	5.7799	0.7275	5.0524
88 U	4	2230	711	1519	7	17	6	4	2.5176	0.3597	2.1579
2 V	5	2589	1067	1522	97	96	96	98	10.1484	3.9858	6.1626
Mean (8)	4.6	3050	885	2165	46	77	47	34	6.1059	1.9661	4.1398
S.D. ±	0.63	926	333	686	35	32	34	39	2.45	1.57	1.50

TABLE IX

Terminal serum cholesterol levels and severity of atherosclerosis in rabbits of group T

Rabbit Number	Terminal Serum				Severity of Aortic Atherosclerosis						
	Visible Lipemia	mg. cholesterol per 100 ml. serum			% of intimal area involved with lesions				mg. cholesterol per 100 mg. frozen – dried aorta		
		Total	Free	Esterified	Whole	Arch	Thoracic	Abdominal	Total	Free	Esterified
9 U	5	2918	224	2694	3	11	1	1	0.97	0.44	0.53
14 U	5	3007	602	2405	47	95	25	68	7.90	0.49	7.41
19 U	5	4498	1185	3313	18	97	13	1	7.44	0.44	7.00
24 U	5	4326	865	3461	35	63	33	43	7.17	4.25	2.92
34 U	5	2383	831	1552	19	91	14	1	11.95	5.65	6.30
39 U	5	2905	721	2184	10	77	7	2	3.75	0.45	3.30
44 U	—	—	—	—	20	70	12	4	4.99	0.52	4.47
49 U	5	2088	428	1660	14	82	7	2	5.52	0.90	4.62
79 U	5	2741	1085	1656	58	94	72	22	6.93	0.68	6.25
84 U	5	4010	1277	2733	85	85	87	89	5.32	3.21	2.11
89 U	5	3007	1330	1677	53	87	73	8	18.80	9.13	9.67
94 U	4	2930	925	2005	3	5	2	2	0.67	0.47	0.20
99 U	5	4312	1195	3117	18	57	17	3	3.76	0.91	2.85
52 V	5	3611	1259	2352	20	95	16	4	4.90	0.58	4.31
53 V	3	1799	659	1140	17	60	10	15	3.74	0.82	2.92
55 V	5	3412	1022	2390	20	60	20	4	4.83	0.60	4.23
4 U	—	—	—	—	82	95	72	78	4.40	1.60	2.80
Mean (15)	4.80	3196	907	2289	31	72	28	20	6.06	1.83	4.23
S.D. ±	0.46	822	331	688	25	28	29	30	4.21	2.42	2.48

3. A good correlation between the total cholesterol plasma level and the degree of aortic lesion is obtained in a small percentage of animals only.

When each group is considered separately and compared with the control or untreated group (group M), the following remarks apply :

1. Association of cis-oleic acid to cholesterol feeding increases twofold the terminal serum cholesterol levels and the severity of aortic athero-

sclerosis, without varying however the total cholesterol content of the aorta.

2. Addition of papain to cis-oleic acid and cholesterol feeding increases by half the serum cholesterol levels noted in animals fed cis-oleic acid only, but increases twofold the amount of cholesterol content in the aorta, and this without evidence of more extensive or severe aortic lesions.

3. Combination of vitamin D to the basic cis-oleic acid-cholesterol diet increases twofold the serum cholesterol levels found in the cis-oleic group, also increases twofold the severity of aortic lesions, but threefold the cholesterol content of the aorta. This is an indication of the pronounced injurious effect imposed by vitamin D on the circulatory system, in particular on the vessel wall.

4. Isomerization of the cis-oleic acid as trans-oleic acid transforms the relatively inocuous natural fatty acid into a very noxious agent which causes changes comparable to that of the vitamin D treatment. Feeding with trans-oleic id, however, also causes marked alterations in the spleen and liver, as observed at necropsy examination (7).

When comparison is made between various experimental groups, it is found that values are statistically significant ($p<0.05$) in :

1. Serum visible lipemia,
   between group D and M, D and C ($p<0.001$) ;
   between group T and M, T and C ($p<0.001$).

2. Total serum cholesterol level,
   between group C and M ($p<0.02$),
   group C and D ($p<0.01$) ;
   between group T and M, T and C ($p<0.001$).

3. Atherosclerotic involvement of aorta,
   between group C and D, C and T : arch only ($p<0.01$) ;
   between group T and M : whole and thoracic ($p<0.01$), arch ($p<0.001$).

4. Total cholesterol content of aorta,
   between group D and M ($p<0.02$),
   group D and C ($p<0.01$) ;
   between group T and M ($p<0.02$),
   group T and C ($p<0.01$) ;

DISCUSSION

It is agreed that a disturbance in cholesterol-lipid metabolism is a *sine qua non* for experimental atherogenesis. The high incidence of atherosclerotic lesions observed in this experiment must be attributed to the fact that all the foregoing experimental regimens effected a chronic hypercholesterolemic hyperlipemia. Analysis of experimental data, however, points out to two main findings.

The first observation is made evident when the serum cholesterol levels is correlated with the degree of aortic lesions. It clearly shows that the hypercholesterolemic effects of a given regimen varied depending on individual susceptibility.

The second finding concerns the marked enhancement of atherogenic activity conferred on cholesterol by combination with trans-oleic acid. When the necropsy observations of an enlarged, cirrhotic-like liver and of severe aortic atherosclerosis (20) are correlated with the high serum cholesterol level and decreased ratio of esterified to free cholesterol, it becomes evident that the cis-oleic acid isomer promotes the gastrointestinal absorption of cholesterol. Since exogenous cholesterol is absorbed in the intestine and reaches the systemic circulation via the lymphatics of the portal venous system, its increased rate of absorption affords an explanation for its heavy deposition in the liver which represents a sort of barrier to its free dissemination throughout the body.

Furthermore, since esterification also occurs in the liver, pathological deposition of cholesterol in this organ, either by interfering with the normal process or by overtaxing an already disturbed mechanism, will decrease rate of esterification and thus account for the low esterified free cholesterol

ratio encountered. Lastly, as endogenous cholesterol is synthesized in the liver, rate of cholesterol synthesis will also suffer from the pathological accumulation. As the liver cholesterol deposits are gradually discharged into the circulation, it appears correct to postulate that rate of cholesterol synthesis in the liver is inversely related to the level of cholesterol in the blood.

CONCLUSION

Although cholesterol has been shown to have a close association with arteriosclerosis (17, 18 and 19), it has not yet been demonstrated that it is the sole or even the most important etiologic factor in man.

Nevertheless, a number of investigators have been sufficiently impressed with the role of cholesterol in atherogenesis to endeavor to find methods of lowering serum cholesterol levels.

One of the methods mostly utilized to lower serum cholesterol involves modification of the diet, and more specifically reduction in the fat content. An interesting avenue opened to research is the search for substances that would prevent the gastrointestinal absorption of cholesterol, namely by forming a non absorbable complex with cholesterol in the intestine, thereby inhibiting hypercholesterolemia and atherogenesis.

As a negative counterpart for this, elimination from the diet of substances known to promote cholesterol absorption and, consequently, its visceral deposition is not a least contributory asset in this effort to inhibit hypercholesterolemia. In this respect, we think that the enhanced atherogenic activity afforded cholesterol by trans-oleic acid in the rabbit of sufficient interest that a close examination of its effects in primates and man should be undertaken. With better knowledge of cholesterol metabolism, or the finding of some anti-cholesterol metabolite, or the exclusion from the diet of major atherogenetic factors, or other observations like that described here, arteriosclerosis may ultimately prove preventable.

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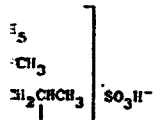
:N₂O₄..., yellow noncryst
.9N NaCl). Very sol in

ıgN₄[illegible].C₂H₆O, crystals
: +5 [illegible] c = 4 in water).
ıon max: 260-265 mμ (ε
:
N₂O₅S, crystals from ethyl
= 1 in ethanol). Soluble in

ₐO₅S, thick syrup. $[\alpha]_D^{27}$

ethyleneiminocarbonylethyl-
ibutyramide). [HOCH₂C-
NHCH₂CH₂S]₂; mol wt
%, H 7.63%, N 10.10%.
factor for *Lactobacillus*
l. Chem. 177, 933 (1949).
ible for the LBF activity
natase. Formed by oxida-
., *J. Biol. Chem.* 198, 375
Am. Chem. Soc. 72, 5349
ırmann, *Naturwiss.* 38, 384
m. Soc. 75, 1694 (1953);
37, 375 (1954); Bowman,
1; Shimizu *et al.*, *Chem.*
eview refs see pantetheine.
noncryst powder. $[\alpha]_D^{27}$
Microbiological activity:
water; less sol in ethanol;
ethyl acetate.

ethylpentyl p-aminobenzo-
zoic acid β-diethylamino-
-aminobenzoic acid *N,N-*
mate; *p*-aminobenzoyl-*N*-
methanesulfonate; 2-
p-amino benzoate. C₁₈-
4%, 30%, N 7.21%,
ebea [illegible] anot, *Traité de*
son [illegible]e, Paris, 1955-
iorid[illegible] arrer, U.S. pat.
Flora); see also Ger. pat.
: Flora).



3 parts water, alcohol;
r, ethyl acetate, acetone,
kg.
anesthetic.

actone; pantoyl lactone;
-dimethylbutyric acid γ-
γ-butyrolactone; 4,5-
H)-furanone. C₆H₁₀O₃;
. O 36.88%. A degrada-
n liver: Williams, Major,
intermediate in the syn-
be prepd by condensing
e yielding α,α-dimethyl-
condensed with hydro-
icium chloride to form
odifications of this pro-
46 (1904); Stiller *et al.*,
); Reichstein, Grüssner,
rter, Ney, *J. Am. Chem.*
terature, *e.g.*, Beckmann

o

OH

et al., U.S. pat. 2,967,869 (1961 to Nopco); Klein, U.S. pat. 3,024,250 (1962 to Nopco).

D(−)-Form: Hygroscopic crystals from benzene + petr ether, mp 92°. $[\alpha]_D^{25}$ −50.7° (c = 2.05 in H₂O). Can be purified by microsublimation.

L(+)-Form: Hygroscopic crystals from benzene, mp 91°. $[\alpha]_D^{25}$ +50.1° (c = 2 in H₂O).

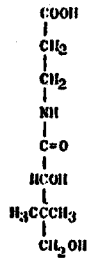
DL-Form: Hygroscopic rosettes of prisms, mp 80°, bp₁₅ 130°. Freely sol in water. Soluble in ether, benzene, chloroform, alcohol, carbon disulfide.

Pantopon[D]. "Concentrated opium"; Omnopon; Papaveretum. A mixture of the hydrochlorides of the opium alkaloids in approx the proportion in which they exist in Turkish opium. Morphine contents = about 50% as anhydr morphine, or 60% as morphine sulfate, and 29.9-34.2% of bases of secondary opium alkaloids. One part Pantopon = 5 parts opium, U.S.P.

Yellowish-gray, cryst powder. Freely sol in water.

MED USE: Narcotic. Analgesic, antitussive, sedative. *Dose:* i.m., s.c. 2.5-20 mg. *Side Effects:* Overdosage may produce nausea, vomiting, constipation, urinary retention, miosis, depressed respiration, coma, shock. *Antidote:* Analeptics, nalorphine hydrochloride, levallorphan tartrate. *Caution:* Drug dependence.

Pantothenic Acid. D(+)-*N*-(2,4-Dihydroxy-3,3-dimethylbutyryl)-β-alanine; chick antidermatitis factor. C₉H₁₇NO₅; mol wt 219.23. C 49.30%, H 7.82%, N 6.39%, O 36.49%. A member of the B complex vitamins. Also referred to as *vitamin B₅:* Malgras, Pax, *Ann. Inst. Pasteur* 93, 792 (1957). Occurs everywhere in animal and plant tissue. The richest common source is liver, but jelly of the queen bee contains 6 times as much as liver. Rice bran and molasses are other good sources. Pantothenic acid derivatives sold commercially are prepd by synthesis. Isoln from liver: Williams, Truesdail, Weinstock, Rohrmann, Lyman, McBurney, *J. Am. Chem. Soc.* 60, 2719 (1938). May be prepd by the direct condensation of β-alanine with the optically resolved form of the lactone of pantoic acid (2,4-dihydroxy-3,3-dimethylbutyric acid) which is prepd from isobutyraldehyde. Synthesis: Stiller, Harris, Finkelstein, Keresztesy, Folkers, *ibid.* 62, 1785 (1940); Reichstein, Grüssner, *Helv. Chim. Acta* 23, 650 (1940). Grüssner, Gätzi-Fichter, Reichstein, *ibid.* 1276. Only the natural, dextrorotatory form has vitamin activity.



Unstable, viscous oil. Extremely hygroscopic. Easily destroyed by acids, bases, heat. $[\alpha]_D^{25}$ +37.5°. Freely sol in water, ethyl acetate, dioxane, glacial acetic acid; moderately sol in ether, amyl alcohol; practically insol in benzene, chloroform. One gram of pantothenic acid equals 70,000-75,000 chick units.

Sodium salt, C₉H₁₆NNaO₅; *Panthoject;* very hygroscopic crystals, mp 122-124°. $[\alpha]_D^{25}$ +27.1° (c = 2). Can be stored in sealed ampuls only.

Calcium salt, *see* separate entry.

MED USE: Human requirement and deficiency syndrome not established. *Dose:* Oral, i.v. 5-50 mg (as the calcium or sodium salt).

VET USE: Nutritional factor for all species. Horses and ruminants normally need no dietary sources.

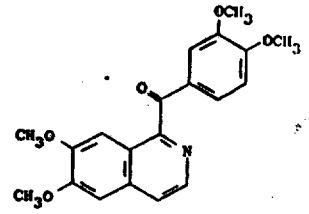
Papain. Papayotin; vegetable pepsin; Summetrin; Nematolyt; Vermizym; Tromasin; Velardon; Arbuz. From the juice of the fruit and leaves of *Carica papaya* L., *Caricaceae.* Contains enzymes similar to pepsin, but acting in acid, neutral, or alkaline media. The potency of papain of commerce varies according to the process of prepn, etc. The usual grade digests about 35 times its wt of lean meat. The best grades are claimed to render sol 200-300 times their wt of coagulated egg albumin in alkaline media. A temp range from 60 to 90°C is favorable for the digestive process, and 65°C is regarded as the optimum point. The best pH is 5.0, but it functions also in neutral or alkaline medium. The enzyme is activated by reduction (HCN, H₂S, etc.) and inactivated by oxidation (H₂O₂, iodoacetate). Has been crystallized: Balls, Lineweaver, *J. Biol. Chem.* 130, 669 (1939). Due to the economic factors involved, Africa and Ceylon are the principal sources of crude papain. Comprehensive *review and bibliography:* "The Properties of Papain" by J. R. Kimmel and E. L. Smith in *Advances in Enzymology* 19, 267-334 (1957). Prepn from commercial dried papaya latex and production in tropical countries: S. Becker, *Economic Botany* 12, 62-79 (1958). Purification: Gibian, Bratfisch, U.S. pat. 2,950,227 (1960 to Schering AG); Lesuk, U.S. pat. 3,011,952 (1961 to Sterling Drug, Inc.); Burdick, U.S. pat. 3,141,832 (1964).

White or grayish-white, slightly hygroscopic powder. Incompletely sol in water, glycerol; practically insol in most organic solvents. *Keep well closed.*

USE: For tenderizing meats; for clearing beverages; for bating skins.

MED USE: Has been used to prevent adhesions; in sloughing and infected wounds; intern. as protein digestant; as anthelmintic (nematodes). *Side Effects:* Allergic manifestations may occur.

Papaveraldine. *6,7-Dimethoxy-1-isoquinolyl 3,4-dimethoxyphenyl ketone;* 6,7-dimethoxy-1-veratroylisoquinoline; xanthaline. C₂₀H₁₉NO₅; mol wt 353.36. C 7.98%, H 5.42%, N 3.96%, O 22.64%. The old name papaveraldine is retained to avoid confusion. From opium; whether papaveraldine occurs as such in the poppy plant, or is formed during the process of extraction, has not been investigated. Oxidation of papaverine to papaveraldine by SeO₂: Menon, *Proc. Indian Acad. Sci.* 19A, 21 (1944). For older references *see:* Small, Lutz, "Chemistry of the Opium Alkaloids," *U.S. Public Health Reports,* Suppl. No. 103, Washington (1932). Synthesis from Reissert compds: Popp, McEwen, *J. Am. Chem. Soc.* 79, 3773 (1957).



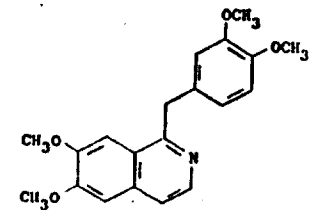
Crystals from abs ethanol, mp 208-209°. Soluble in benzene, chloroform; slightly sol in alcohol, ether, petr ether; nearly insol in water, alkalies or carbonates.

Hydrochloride, C₂₀H₁₉NO₅.HCl, yellow crystals from abs alcohol, mp 200°. Also a hemipentahydrate.

Methiodide, C₂₀H₁₉NO₅.CH₃I.2H₂O, yellow prisms, dec 133-135°.

Picrate, C₂₀H₁₉NO₅.C₆H₃N₃O₇, yellow needles, dec 208-209°.

Papaverine. *6,7-Dimethoxy-1-veratrylisoquinoline.* C₂₀H₂₁NO₄; mol wt 339.38. C 70.78%, H 6.24%, N 4.13%, O 18.86%. Found in opium (0.8-1.0%). Synthesis: Pictet, Gams, *Compt. Rend.* 149, 210 (1909); *Ber.* 42, 2943 (1909). Commercial syntheses have been developed: Goldberg, *Chem. Products* 17, 371-374 (1954), a review. Improved synthetic procedures: Braz, Chizhov, *Soviet Pharmaceutical Research* 3, 90-93 (New York, 1958).



Triboluminescent, orthorhombic prisms from alcohol + ether. mp 147°. Sublimes at 135-140° at 11 mm pressure

AN INVESTIGATION OF THE COAGULATION DEFECT BROUGHT ABOUT IN RABBITS AND DOGS BY THE INTRAVENOUS INJECTION OF PAPAIN*

By Frank C. Monkhouse

Abstract

The intravenous injection of papain causes the blood of dogs and rabbits to become incoagulable. In rabbits the incoagulability persists for many hours. In some animals there remained a mild disturbance in the coagulation system up to two weeks after the injection. The release of heparin can account for part of the effect, but there is evidence of some other contributing factor. Results indicate that the added factor affects the thrombin fibrinogen reaction. Calcium chloride in excess of that needed for normal coagulation will greatly inhibit the antithrombic effect of commercial beef heparin, and to a lesser extent, the antithrombic effect of an injection of papain.

Introduction

Eagle (3) and Ferguson and Ralph (4) investigated the action of papain on the coagulation system *in vitro*. They found that its action was similar to that of thrombin in that it could coagulate fibrinogen directly. Dyckeroff and Gigante (2) confirmed this and suggested the name "phyto-thrombin" to signify its plant origin.

In contrast to this *in vitro* coagulant action, Kellner *et al.* (6) showed that when papain was injected intravenously into rabbits the blood of these animals became completely incoagulable. They could not demonstrate abnormal amounts of antithrombin or anticoagulant. According to these authors the defect resembled hemophilia, and appeared to be due to a deficiency or inactivation of thromboplastin or a thromboplastin precursor.

In preliminary experiments it was found that papain, injected intravenously in doses of 20 to 30 mgm. per kilogram body weight, caused the blood of rabbits to become completely incoagulable. This incoagulability persisted for many hours after the injection. Contrary to the report of Kellner *et al.* (6), we found that, in every instance, there was an increase in antithrombin activity. A material could be extracted from the plasma and identified as heparin. There was some doubt in our minds, however, that the pronounced anticoagulant effect was entirely due to the presence of heparin. This was especially true in rabbits where the coagulation time was often increased out of proportion to the level of extractable heparin found in the blood. Even in dogs the heparin level had often returned toward normal, while the clotting time remained high.

These apparent discrepancies, and the suggestion by Kellner that the condition resembled hemophilia, encouraged us to undertake a further study of this interesting phenomenon. The following is a report of our study and the conclusions derived therefrom.

Manuscript received October 21, 1954.
Author's address: from the Department of Physiology, University of Toronto, Toronto, Canada.
This work was supported by grants from the Defence Research Board of Canada, Grant No. 331.

Methods and Materials

Collection of Blood Samples

Unless otherwise stated, all samples were taken into one-ninth their volume of 3.8% (w/v) sodium citrate.

Heparin Estimation

Heparin was extracted by the method of Monkhouse and Jaques (9). The plasma was deproteinized with phenol and the heparin precipitated from the aqueous layer by the addition of two to three volumes of 95% alcohol. The mixture was allowed to remain at 2° C. for several hours to ensure complete flocculation of the precipitate. The material was then centrifuged and the precipitate washed, first with 95% alcohol, then ether, and finally dried at room temperature. The heparin was extracted from the dried powder by means of physiological saline. The extracts were then assayed according to their ability to prevent the coagulation of citrated dog blood or horse plasma on the addition of dilute solutions of thrombin. The potency is expressed in terms of standard beef heparin kindly supplied by the Connaught Medical Research Laboratories.

Aluminum Hydroxide

This was prepared as the $C\alpha$ salt according to the method of Bertho and Grassman (1). In spite of careful attention to detail, different preparations varied in their ability to adsorb heparin from plasma. However, once a suitable product was obtained it was stable for several months when stored at a temperature of 2-4 degrees Centigrade. Each milliliter of plasma was treated with 0.15 ml. of the aluminum hydroxide. Adsorption was carried out at room temperature for a period of 15 min. with an occasional mixing during that time.

Thromboplastin Solutions

A full strength (F.S.) solution was prepared by extracting one ampoule of the Difco rabbit brain material with 5 ml. of physiological saline at 50° C. for 10 min. From this, dilute solutions were prepared as required and expressed as a fraction of the full strength solution.

Clotting Times

(*a*) *Whole Blood Clotting Times:* tests were carried out by a modified Lee and White method, two tubes being used for each determination.

(*b*) *Recalcified Clotting Times:* tests were carried out by adding 0.1 ml. of a 0.025 *M* solution of calcium chloride to 0.2 ml. of plasma.

(*c*) *Thrombin Clotting Times:* a concentrated solution containing 140 units of Parke Davis Topical Thrombin in 50% glycerol and 50% saline was used as a stock solution. For the test, dilutions of 1 : 10 and 1 : 20, and occasionally 1 : 40 were used.

To 0.2 ml. of plasma, 0.1 ml. of saline and then 0.1 ml. of the thrombin solution were added. The 0.1 ml. of saline was introduced as a medium in which to add heparin or other reagents to the system.

Papain

The papain used was a product manufactured by the Nutritional Biochemical Corporation Laboratories at Cleveland, Ohio, for chemical and investigational use only. It was given intravenously as a saline suspension. Rabbits were given 60-100 mgm. in 5 ml. and dogs 350-400 mgm. in 20 ml. The injection was carried out rapidly, varying in time from 20 to 45 sec.

Results

In Table I are shown typical results of adding varying concentrations of papain and trypsin to 0.4 ml. quantities of citrated blood. In all of our *in vitro* tests the addition of 0.1 ml. of 0.025 *M* calcium chloride greatly enhanced the coagulation properties of both papain and trypsin. Indeed it was only with the more concentrated solutions of papain that clotting took place at all in citrated blood without the addition of calcium chloride.

TABLE I

THE INFLUENCE OF PAPAIN AND TRYPSIN ON THE CLOTTING TIME OF CITRATED RABBIT'S BLOOD, BOTH WITH AND WITHOUT THE ADDITION OF CALCIUM CHLORIDE

Amount of enzyme added, mgm.	Clotting time, sec.		
	Papain	#	Trypsin
1.00	35 300*	17	135*
0.50	43 >600*	23	140*
0.25	115	25	300*
0.12	127	29	550*
0.06	240	35	>600*
0.03	270	55	
0.00	300	310	

* *Saline added in place of calcium chloride.*
\# *A 1 : 250 Difco standardized product.*

The *in vivo* action of papain is in direct contrast to its *in vitro* action. The blood of rabbits becomes completely incoagulable within a few minutes after the intravenous injection of 60-100 mgm. of papain. The results obtained from a group of 10 healthy male rabbits, ranging in weight from 2.5 to 3.0 kgm., are shown in Table II. All these animals survived the experiment. In every instance heparin could be detected in the blood within a few minutes after the injection. It often increased in concentration up to two hours and then gradually decreased. In most cases coagulation was still prolonged after 24 hr. As further proof that the extracted material was heparin, the following experiment was carried out. Four rabbits were injected with papain and one hour after the injection they were bled out and the plasma pooled. A total of 190 ml. of plasma was obtained and on treatment it yielded an extract containing 26 units of heparin by antithrombic titration. This

material showed metachromatic and anticoagulant potency in good agreement with that shown for the heparin extracted from rabbits in anaphylactic shock reported by Monkhouse *et al.* (8). This experiment was repeated, with similar results, on another group of four rabbits.

TABLE II

HEPARIN RELEASED IN RABBITS BY THE INTRAVENOUS INJECTION OF PAPAIN

Animal No.	Papain, mgm.	Heparin, units per ml. of blood			
		½ hr.	1 hr.	2 hr.	4 hr.
1	60		0.20	0.12	
39	60	0.30		0.22	0.20
	75	0.30	0.30		
48	75	0.27	0.25	0.12	
50	75	0.24	0.10		
52	75	0.10	0.12	0.12	0.15
	90		0.20	0.20	
55	60		0.05	0.10	0.06
90	100	0.02	0.08	0.08	
86	75	0.12	0.18		0.18
85	75	0.04	0.10	0.10	
89	60	0.07		0.14	

In Table III are shown typical results of an intravenous injection of 400 mgm. of papain in a 10 kgm. dog. As in the experiments on rabbits, it was found that heparin was released in every instance with doses of this order. With the exception of one animal, which suffered profound shock, the amounts released were only about 1/10th those found in the blood of dogs suffering severe anaphylactic or peptone shock. This is in contrast to the rabbits, which, if anything, released more heparin after papain injection than during anaphylactic shock.

TABLE III

EFFECT OF AN INTRAVENOUS INJECTION OF 400 MGM. OF PAPAIN IN THE DOG

Time after injection, min.	Thrombin Clotting Times, sec.				Heparin, units ml. blood
	Before Al(OH)$_3$		After Al(OH)$_3$		
	1 : 10	1 : 20	1 : 10	1 : 20	
0.0	17	30	22	34	0.01
15.0	210	*	21	36	0.35
90.0	93	213	22	35	0.18
150.0	35	94	23	36	0.07
320.0	33	52	22	37	0.02

* *Greater than 300 sec.*

Confirmation of the heparin titer is shown by the thrombin clotting times which vary in direct proportion to the amount of heparin extracted. Furthermore these times could be restored to normal by treating the plasma with aluminum hydroxide. This treatment has been shown to remove heparin from plasma (7). As will be shown later there is not always such a good agreement between heparin titer and the Thrombin Clotting Times.

In some experiments the Recalcified Clotting Times of citrated plasma taken after a papain injection were considerably shortened as compared to simultaneously taken Whole Blood Clotting Times. This was by no means a constant finding. However, addition of calcium chloride always resulted in a marked shortening of the Thrombin Clotting Times of these samples.

The influence of calcium chloride on the Thrombin Clotting Times is illustrated in Fig. 1. This animal received 400 mgm. of papain intravenously. It is clearly shown that calcium chloride caused a marked shortening of the Thrombin Clotting Times. The Whole Blood Clotting Times of all samples taken after the papain injection were greater than six hours. The Recalcified Clotting Times of all samples except the one taken at the 290 min. period were also greater than six hours.

This shortening of the Thrombin Clotting Times can be observed also when coagulation is prolonged by the injection of commercial beef heparin, provided of course the amount of heparin injected is not greatly in excess of one unit per ml. of plasma. Fig. 2 illustrates the effect of added calcium chloride on the Thrombin Clotting Times of citrated dog plasma at various times after the animal had received an intramuscular injection of 8000 units of heparin. Note the strong inhibitory action of calcium chloride on the antithrombic potency of heparin. The similarity of the results of these two

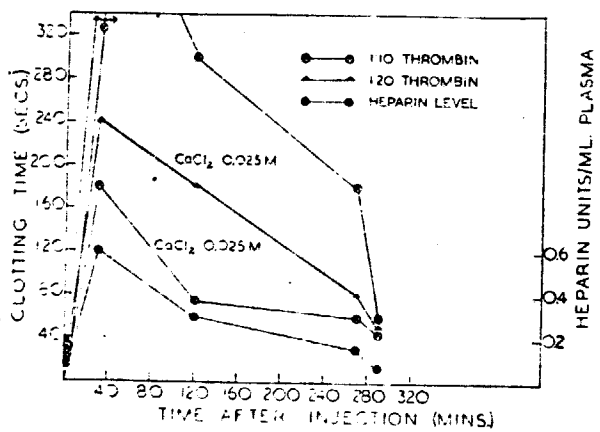


FIG. 1. The effect of calcium chloride on the Thrombin Clotting Time of plasma from a dog which had previously received an intravenous dose of 400 mgm. of papain. (Clotting time greater than 360 sec.)

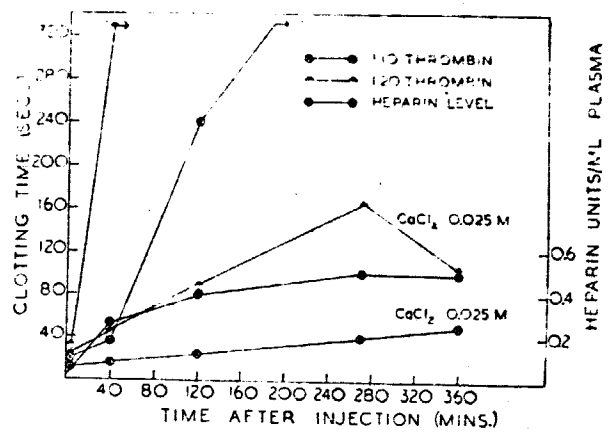


FIG. 2. The effect of calcium chloride on the Thrombin Clotting Times of plasma from a dog which had previously received an intramuscular injection of 8000 units (0.8 ml.) of beef heparin. (Clotting time greater than 600 sec.)

experiments provides further evidence that the presence of heparin in the blood accounts for at least part of the prolonged clotting time which follows an injection of papain.

Jacobsen and Plum (5) showed that calcium ions inhibited the anticoagulant effect of heparin, but did not carry their experiments further than this. Our experiments indicate that calcium chloride interferes with the antithrombic power of heparin. Indeed it may be that calcium chloride exerts its main action at this phase of clotting since the Thrombin Clotting Times of blood heparinized to a moderate degree could be brought much nearer normal with calcium chloride than could the Recalcified Clotting Times.

Further studies comparing the anticoagulant effect of an injection of papain with the anticoagulant action of commercial beef heparin were made on rabbits. The results of a typical experiment are shown in Table IV. Two samples of blood were taken prior to the injection of papain, one into one-ninth its volume of citrate and the other into one-ninth its volume of saline containing enough heparin to give a final concentration of 0.5 units per ml. of blood. Ninety minutes after the animal had received an intravenous dose of 75 mgm. of papain, three more samples were taken, one into citrate, one into saline, and the third into a clean dry siliconed tube. Thrombin Clotting Times were carried out (in ordinary pyrex clotting tubes) on the plasma of all samples using varying amounts of both thrombin and calcium chloride. The extractable heparin in the papain sample assayed at 0.2 units per ml. of blood, which is a concentration less than half that added to the normal sample. The coagulability, however, was affected to a much greater extent in the papain sample.

TABLE IV

A COMPARISON OF THE EFFECTS OF VARYING CONCENTRATIONS OF CALCIUM CHLORIDE AND THROMBIN ON THE THROMBIN CLOTTING TIME OF HEPARINIZED RABBIT PLASMA AND PLASMA OBTAINED AFTER AN INTRAVENOUS INJECTION OF PAPAIN

		Thrombin Clotting Times (sec.)				
		Normal plasma		Papain plasma		
Thrombin, % F.S.	$CaCl_2$, M	Citrate	Heparin and saline	Citrate	Saline	Silicone
10	0.000	24	62	600	63	300 ±
"	0.025	20	35	240 ±	51	270 ±
"	0.050	16	27	63	56	240 ±
"	0.100	14	21	45	59	115
5	0.000	40	210 ±	600	138	600
"	0.025	25	120 ±	330	110	600
"	0.050	21	75	120	100	600
"	0.100	21	75	120	100	540 ±
2.5	0.000	60	600	600	180	600
"	0.025	31	600	600	130	600
"	0.050	26	240 ±	240	122	600
"	0.100	25	57	180	110	600

± Partially clotted.
F.S. thrombin = 150 units per ml.

The addition of calcium chloride greatly reduced the Thrombin Clotting Times of the normal sample taken into heparin and the papain sample taken into citrate but had only slight effect on the other two papain samples. In this regard the coagulation defect brought on by papain differs from an

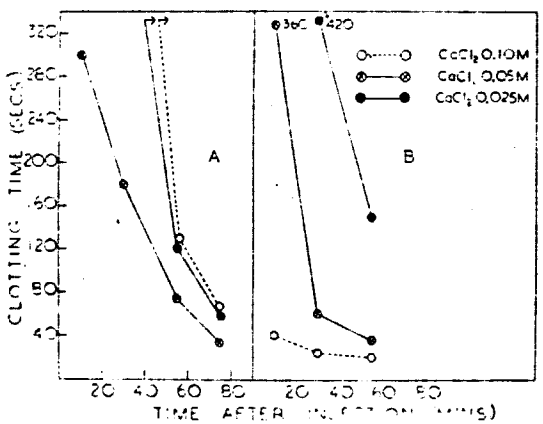


FIG. 3. The effect of calcium chloride on the Thrombin Clotting Times of plasma from a rabbit which had previously received an intravenous injection of 500 units of heparin. A = samples taken into citrate. B = samples taken into saline. → = Clotting time greater than 600 sec. A 1:10 thrombin dilution was used.

intravenous injection of heparin. In the latter case the calcium chloride has a greater effect on the inhibiting action of heparin when the sample is taken into saline than when it is taken into citrate. A typical result of an intravenous injection of heparin is shown in Fig. 3. This rabbit received 500 units of heparin and duplicate samples of blood were withdrawn at intervals thereafter. One sample was taken into citrate and the other into saline. It can be seen from the graph that calcium chloride lowered the Thrombin Clotting Times of the sample taken into saline to a greater degree than those taken into citrate. It is difficult to understand why this should be so. This experiment has been repeated however, with similar results.

Another point brought out in Table IV is that the Thrombin Clotting Times of the sample taken into saline were much shorter than those of the samples taken into the dry siliconed tube. This difference appeared to be due to the diluting effect of the saline.

Tocantins (11) has shown that dilution with saline shortens the clotting time of both normal and hemophilic plasma. We found that blood or plasma made incoagulable by an injection of papain often clotted when diluted 30% with saline. This result appeared to be due mainly to an effect on the thrombin fibrinogen reaction, since dilution had a greater relative effect on the Thrombin Clotting Time than on the Whole Blood Clotting Time.

Fig. 4 illustrates the results from a typical experiment in which varying concentrations of thromboplastin were added to rabbit plasma and the

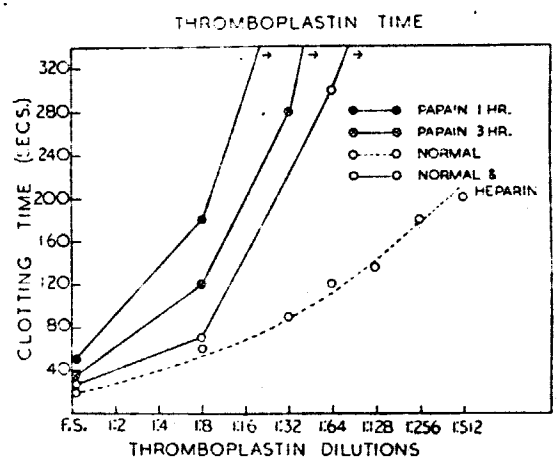


FIG. 4. The effect of adding varying concentrations of thromboplastin to the Recalcified Clotting Times of rabbit plasma. A comparison is made of normal and heparinized plasma and plasma after an intravenous injection of 75 mgm. of papain. → Clotting time greater than 600 sec.

Recalcified Clotting Times measured. The graph compares normal plasma, heparinized plasma, and plasma obtained after an injection of 75 mgm. of papain. Both samples of plasma taken after papain injection responded to dilute solutions of thromboplastin, in a manner similar to heparinized plasma. On the other hand, while the concentrated solutions reduced the clotting time to the normal in the heparinized samples, they failed to do so in the samples taken after injection of papain. It should be pointed out that the concentration of extractable heparin in the papain plasma was less than half of that in the heparinized plasma.

Thus again we observe that the effect of papain cannot be explained entirely on the basis of extractable heparin. The results shown in Fig. 4 indicate that the additional disturbance is not due to a deficiency of thromboplastin.

Conclusion

When papain is injected intravenously into rabbits or dogs their blood becomes incoagulable. This effect is most pronounced in the rabbit, persisting for many hours.

Heparin is released into the blood in all instances but the amount extractable does not satisfactorily account for all the anticoagulant effect.

As already mentioned, Kellner *et al.* (6) suggested that the defect resembled hemophilia. From the results of our experiments we can not agree with these authors, but are of the opinion that the defect is in the thrombin–fibrinogen reaction. We have been unable to show any quantitative change in the fibrinogen and no evidence of fibrinolysis. There may however be a change in the quality of the fibrinogen which renders it less reactive to thrombin. There also remains the possibility of an increase in one of the other antithrombins as described by Seegers *et al.* (10).

In two of the rabbits surviving for some time after the injection of papain there was evidence of a mild disturbance in the coagulation system lasting for as long as two weeks. In the light of this finding it may be of interest to investigate this problem further using repeated small doses of papain or slow injections over a prolonged period.

An interesting side issue which arose out of the experimental work on papain was the inhibiting action of calcium chloride on the anticoagulant action of heparin. This effect of calcium chloride appeared to be most powerful at the thrombin fibrinogen phase.

Acknowledgments

The author wishes to acknowledge the technical assistance of Mrs. L. Davidson and Mrs. D. Parrott.

The work was supported in part by Grant No. 334 from the Defence Research Board.

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Experimental Papain-Induced Emphysema in Dogs[1-3]

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P. T. MACKLEM, and W. M. THURLBECK

SUMMARY

Papain was injected intratracheally into 8 dogs after measurements had been made of oxygen tension, carbon dioxide tension and pH of arterial blood, subdivisions of lung volume, steady state diffusing capacity for carbon monoxide, fractional uptake of carbon monoxide, static pressure volume curves, and pulmonary resistance at different lung volumes, and measurements were repeated one to four weeks later. There were no significant changes in arterial blood gas tensions, acid-base balance, or pulmonary resistance, but the diffusing capacity decreased. Total lung capacity, functional residual capacity, and residual volume increased; elastic recoil decreased. Pulmonary resistance was partitioned when the dog was sacrificed; the resistance of airways smaller than 2 mm in diameter was increased whereas that of larger airways was decreased. Trivial to moderate panlobular emphysema was found in all animals on pathologic examination. Internal surface area was reduced, particularly when it was assessed at a standard lung volume. The mean linear intercept was increased. The disease induced by papain administered intratracheally to dogs was similar both pathophysiologically and morphologically to human panlobular emphysema.

Introduction

Gross and his associates (1) were the first to produce emphysema by intratracheal installation of papain. Since that time, a number of investigations have been performed to determine the pathophysiology of this condition (2, 3). The present study extends these investigations and presents the effects of papain-induced emphysema on subdivisions of lung volume, static pressure-volume relationships of the lung, resistance properties of airways, diffusing capacity, and arterial blood gas tensions.

Materials and Methods

Eight mongrel dogs weighing from 24 to 69 pounds were used. All animals were studied before injection with papain (control study) and at various intervals after treatment with papain. In the control study, the dogs were anesthetized with intravenous thiopental sodium (initial dose: 10 mg per pound). Light anesthesia was maintained throughout the experimental period with pentobarbital (50 mg to 100 mg), given intravenously at 30- to 60-minute intervals. The trachea was intubated with a wide bore, rubber, endotracheal tube of 9.0 mm internal diameter. Intrathoracic pressure was measured with an esophageal balloon 5 cm long, containing 0.5 ml air. Transpulmonary pressure was obtained by measuring the difference between tracheal pressure and esophageal pressure with a Sanborn 267B transducer; flow was measured at the endotracheal tube opening with a NIL pneumotachygraph and Sanborn 270 transducer; volume was measured with a volume displacement body plethysmograph (4). Pressure, volume, and flow signals were recorded on a 4-channel Sanborn recorder and on a Tektronix 564 storage oscilloscope. Functional residual capacity (FRC) was measured by the DuBois technique (5), which required that the airway be occluded at end-expiration while the animal made respiratory efforts. A complete pressure-volume diagram was constructed by using positive pressure to inflate the lungs to total capacity (transpulmonary pressure: 30 cm H_2O) and then deflating them quasi-statically to residual volume (RV); suction was used to deflate the lungs to less than FRC to a pressure at the trachea of −60 cm H_2O. Total lung capacity (TLC) was calculated by adding to the FRC the volume of air used to inflate the lungs from FRC to a transpulmonary pressure of 30 cm H_2O. Residual volume was calculated by subtracting from FRC the volume of air necessary to deflate the lungs from FRC to a tracheal pressure of −60 cm H_2O. Static compliance was taken from the slope of the static volume-pressure curve in tidal volume range after full inflation of the lungs. Pulmonary resistance was measured at various lung volumes by the forced oscillation technique (6).

Initially, arterial blood gases and diffusing capacity were measured in lightly anesthetized dogs that were allowed to breathe spontaneously. Because ventilation during the experimental study varied considerably from that during control studies on the same dog, however, it single intravenous dose of succinylcholine chloride (0.5 mg per pound); supplementary doses of 8 mg to 20 mg were given when necessary. They were ventilated with a respiratory pump[4] at a breathing frequency of 16 breaths per min and a tidal volume that was adjusted so the arterial carbon dioxide tension (Pa_{CO_2}) decreased between 30 mm Hg and 40 mm Hg in the control study. This ventilation was then used in all subsequent studies after the dogs had received papain.

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[2] Supported by grants from the Life Insurance Medical Research Fund and the Medical Research Council of Canada.

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[4] Harvard Apparatus Company, Dover, Massachusetts.

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TABLE 1

DOSAGE SCHEDULE OF PAPAIN USED IN 8 DOGS

Dog	Weight (pound)	Sex	Dose of Papain* (mg/pound)	No. of Injections	Total Dose (mg)	Time from Injection to Sacrifice (days)	No. of Studies after Initial Injection of Papain
1	24	F	2	1	48	7	1
2	27	M	2	1	54	14	1
3	27	M	2	1	54	14	1
4	69	M	1	1	69	14	1
5	50	M	1/week	4	200	28	2
6	45	M	1/week	4	180	28	2
7	44	M	1/week	4	176	28	2
8	48	M	1/week	4	192	28	2

* From papaya latex crystallized suspension in 0.05 M sodium acetate, pH 4.5 (Sigma Chemical Co.)

TABLE 2

STATIC LUNG COMPLIANCE IN DOGS BEFORE AND AFTER PAPAIN INTRATRACHEALLY

Dog	Period after Injection (days)	Static Compliance (liter/cm H_2O)
1	Control	0.07
	7	0.09
2	Control	0.08
	14	0.10
3	Control	0.02
	14	0.05
4	Control	0.09
	14	0.12
5	Control	0.05
	14	0.07
	28	0.10
6	Control	0.09
	14	0.10
	28	0.12
7	Control	0.07
	14	0.16
	28	0.18
8	Control	0.11
	14	0.15

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Diffusing capacity for carbon monoxide (CO) and fractional uptake of CO were measured by the steady state technique described by Bates and co-workers (7). Arterial oxygen tension (Pa_{O_2}), Pa_{CO_2}, and arterial pH were measured with an IL Model 133 pH/Gas Analyzer. The plasma bicarbonate concentration was determined by the Siggaard-Andersen nomogram (8). An oximeter[5] was used to measure the arterial oxygen saturation. The results reported for all measurements were the average of duplicate readings. After these data were obtained the dogs were allowed to awaken and were returned to their cages. Papain was administered a few days later when they had completely recovered. They were anesthetized again, and papain was injected intratracheally. To obtain different degrees of emphysema, some dogs were given papain in doses of 1 mg per pound body weight, others received 2 mg per pound. For the same reason, the number of doses was varied (table 1). Penicillin and streptomycin were given to control infection. Pulmonary function studies were repeated one week after the papain injection according to the schedule in tables 1 and 2.

The dogs were sacrificed with intravenous nembutal sodium after the final study was completed, and the lungs were removed. The retrograde catheter technique (6) was used to measure the resistance of the central and peripheral airways. The lungs were then fixed in inflation with 10 per cent formalin intrabronchially, at a constant pressure of 25 cm of formalin. After fixation for at least 18 hours, the lungs were cut saggitally into slices, which were impregnated with barium sulfate and examined under water with a dissecting microscope. Emphysema was assessed by measurement of the average intra-alveolar distance (mean linear intercept or Lm) and the internal (alveolar) surface area (ISA) in stratified, random, histologic sections. The internal surface area was corrected to an arbitrary total lung volume of 2 liter (ISA_2). The techniques used for these procedures were fully described elsewhere (9). In addition, paraffin sections of 50 μ to 100 μ thickness were stained and used to assess emphysema, particularly in examples of mild or equivocal emphysema. The control values of Lm and ISA were obtained from the excised lungs of normal dogs sacrificed by nembutal sodium given intravenously. The lung specimens were prepared in the same way as the papain-treated lungs. Sections from the main branches of both experimental and control animals were examined for evidence of hyperplasia of bronchial mucous glands.

Results

Results of measurements of total lung volume and its subdivision are shown in figure 1. Papain caused an increase in TLC and all its subdivisions in all experimental dogs. The increase in TLC ranged from 8 ml to 1,095 ml, which was significant ($P < 0.05$) when compared using a paired t test. The FRC and RV were also increased ($P < 0.05$); the increases ranged from 38 ml to 612 ml for FRC and 2.5 ml to 480 ml for RV.

Static compliance (table 2) was increased in all papain-treated dogs from 18.5 per cent to 146.6 per cent of control values ($P < 0.01$). The over-all volume-pressure relationship in all papain-treated dogs is illustrated in figure 2. The pressure-volume curve of the papain-treated lung was steeper and shifted upwards and to the left of the control curve.

Total pulmonary resistance is shown as a percentage of the control value in figure 3. The resistance was increased in some dogs but not in others. The difference between means of the control and the experimental values was not significant ($P > 0.05$). The partitioning of pulmonary resistance obtained from eight excised lungs is shown in figure 4, in which the resistance of airways smaller than 2 mm diameter (Rp, calculated as a percentage of the total resistance (R_L), is plotted against transpulmonary pressure. Rp was definitely increased when compared with that obtained from normal dogs in a previous study by Macklem and Mead (6). These authors found that Rp was too small to be detected above 80 per cent of vital capacity, but that it increased to 15 per cent of R_L at lower lung volumes. In the present report, 22 per cent to 90 per cent of the total resistance was comprised of Rp.

Resting diffusing capacity, fractional removal of CO, and arterial blood gases are shown in table 3 for the 4 dogs whose ventilation was controlled during both control and experimental studies. Diffusing capacity and the fractional removal of CO decreased progressively, as the papain dose was increased and the experimental time was prolonged. The reduction in diffusing capacity of CO was significant ($P < 0.01$).

The Pa_{O_2} and oxyhemoglobin saturation were decreased in all dogs when they were studied 28 days after papain administration. The Pa_{CO_2} and plasma bicarbonate varied between individual dogs, and the pH was unchanged (table 3). The changes in arterial blood gases, however, were not significant ($P > 0.1$).

[5] American Optical Company, Southbridge, Mass.

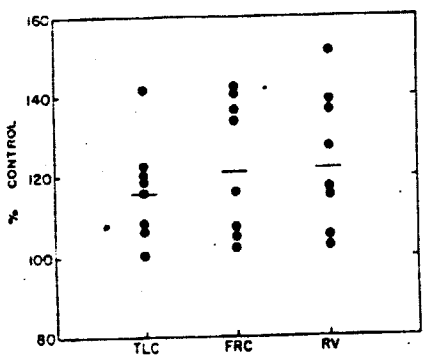

Fig. 1. Influence of experimental papain emphysema on the subdivisions of lung volume expressed as a percentage of the control value. Individual results are shown as solid circles. The height of the bar is the mean value. TLC = total lung capacity, FRC = functional residual capacity, RV = residual volume.

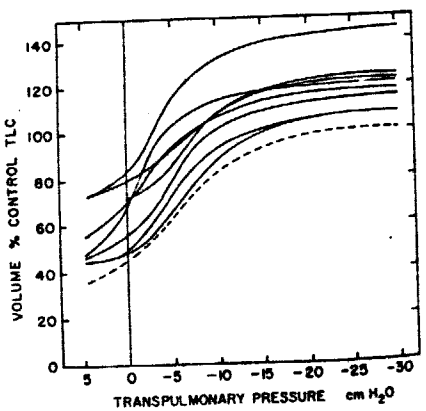

Fig. 2. Influence of experimental papain emphysema on the deflation static pressure-volume curve of the lung. The interrupted curve is the mean value of the control curves. Results in individual dogs after papain administration are shown by solid lines.

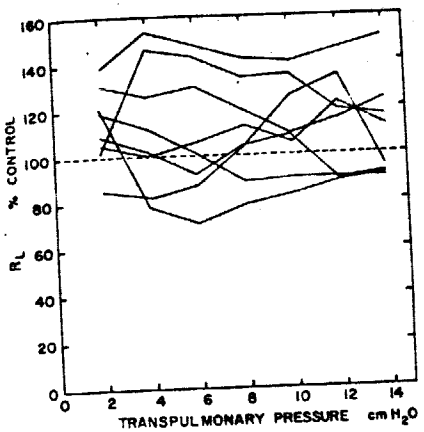

Fig. 3. Pulmonary resistance (R_L) expressed as a percentage of the control value in papain-treated dogs and plotted against transpulmonary pressure.

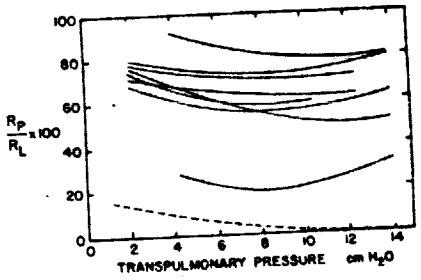

Fig. 4. Partition of pulmonary resistance in the excised lungs of papain-treated dogs. The resistance of the peripheral airways smaller than 2 mm in diameter (Rp) is expressed as a percentage of the total pulmonary resistance (R_L) at different transpulmonary pressures. Results in individual dogs are shown by the solid lines. The interrupted line is the mean value in normal dogs found by Macklem and Mead (6).

TABLE 3

DIFFUSING CAPACITY OF CARBON MONOXIDE (STEADY-STATE), FRACTIONAL CARBON MONOXIDE UPTAKE, AND ARTERIAL BLOOD GASES IN EXPERIMENTAL PAPAIN-INDUCED EMPHYSEMA IN DOGS

Dog	Period after Papain (days)	DLCO (ml/mm Hg/min)	Change (%)	Fractional CO Uptake	Change (%)	pH	Change (%)	$PaCO_2$ (mm Hg)	Change (%)	HCO_3^- (mEq/liter)	Change (%)	PaO_2 (mm Hg)	Change (%)	SaO_2 (%)	Change (%)	VE (liter/min)
5	Control	6.40	–	.454	–	7.37	–	32.8	–	18.7	–	76.5	–	91.3	–	3.77
	14	5.54	−13.4	.400	−11.9	7.39	0.2	35.0	6.9	20.5	10.0	77.0	0.7	90.3	−1.1	3.78
	28	3.51	−45.2	.380	−16.3	7.37	−0.1	37.0	13.0	21.0	12.6	69.4	−9.3	89.6	−1.8	3.73
6	Control	7.11	–	.402	–	7.43	–	36.0	–	23.0	–	93.0	–	93.8	–	3.82
	14	6.39	−10.1	.387	−3.7	7.33	−1.4	45.0	25.0	22.8	−1.1	77.0	−17.2	90.2	−3.8	3.80
	28	3.35	−52.9	.357	−11.2	7.39	−0.5	34.3	−4.9	20.3	−12.0	80.5	−13.4	91.0	−2.9	3.86
7	Control	8.47	–	.515	–	7.42	–	36.0	–	22.1	–	74.0	–	91.8	–	3.46
	14	3.67	−56.7	.417	−19.0	7.40	0.0	30.3	−13.4	19.3	−12.9	83.5	12.8	92.0	0.27	3.47
	28	2.59	−69.4	.301	−41.6	7.44	0.3	32.6	−7.0	21.5	−2.7	67.9	−8.2	91.6	−0.16	3.50
8	Control	5.17	–	.396	–	7.45	–	38.3	–	25.8	–	70.8	–	90.5	–	4.03
	28	2.38	−54.0	.310	−21.7	7.41	0.47	25.6	−33.1	15.5	39.9	38.7	−45.3	66.0	−27.1	4.00

Key: $PaCO_2$ = arterial carbon dioxide tension; HCO_3^- = plasma bicarbonate concentration; PaO_2 = arterial oxygen tension; SaO_2 = arterial oxyhemoglobin saturation; VE = ventilation; DLCO = pulmonary diffusing capacity for carbon monoxide.

Trivial to moderate panlobular emphysema was found in the animals and was usually fairly localized in extent (figures 5 to 7). The internal surface area of the lung, however, was reduced by papain, even though the lesions were not striking on gross examination in some dogs (table 4). Although there was some overlap between normal and emphysematous lungs for both Lm and ISA, the difference between means was significant ($P < 0.05$). Mild chronic inflammation was present in the bronchi of dogs 6 and 7 whereas the histologic sections from dog 8 revealed chronic inflammation of the bronchioles some of which contained infected mucous plugs (figure 6C). The other dogs showed no evidence of inflammation in either the bronchi or bronchioles. Mucous glands were not found consistently in the experimental animals, so mucous gland hyperplasia could not be assessed.

Discussion

The present study indicates that a disease strikingly similar to human panlobular emphysema, both morphologically and functionally, can be produced in dogs by papain introduced intratracheally. Although papain is a powerful proteolytic enzyme and might exert its effect by destroying the supporting matrix of the lung, the exact pathogenesis of the lesion cannot be determined from these experiments and might be fundamentally different from naturally occurring human emphysema. The changes in lung volume, diffusing capacity, pulmonary elasticity, and partitioning of airway resistance, however, closely resemble those found in human emphysema (10–13).

The resistance of small airways increased in papain-induced emphysema, but this change could not be detected by the measurement of total pulmonary resistance made at the mouth. This was because there was a concomitant reduction in the resistance of central airways, so that total pulmonary resistance was not significantly altered.

In all dogs, panlobular emphysema that closely resembled localized emphysema found [illegible]

Fig. 5. A. Paper-mounted whole lung section from a normal dog.

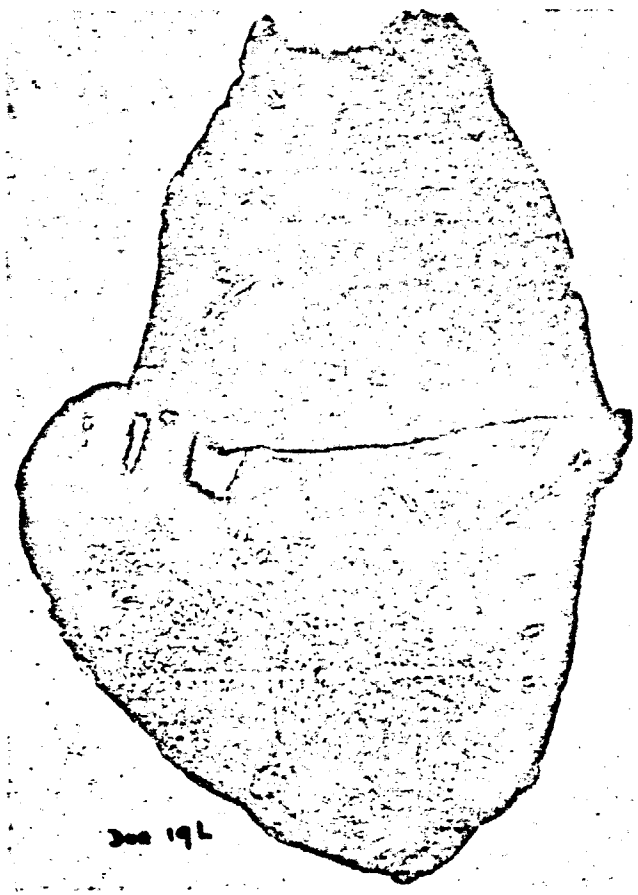

Fig. 5. B. Paper-mounted whole lung section from a papain-treated dog showing mild widespread panlobular emphysema (pale areas).

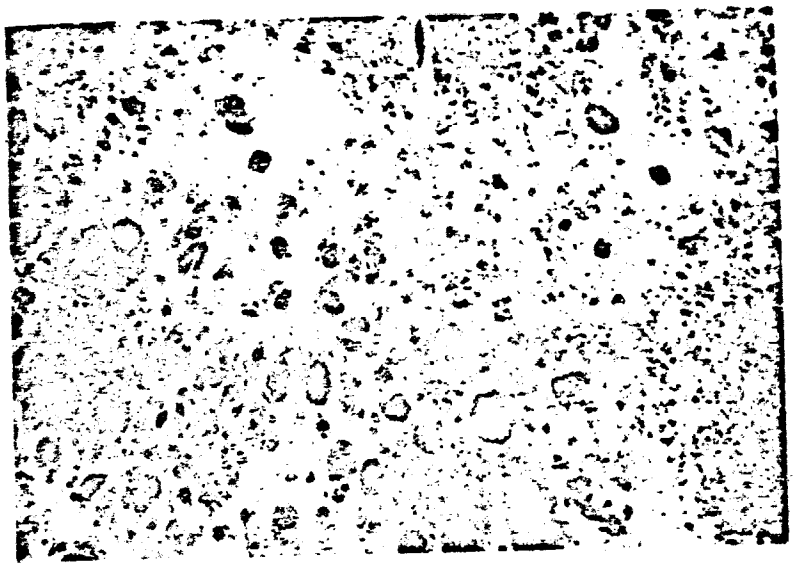

Fig. 6. A (*top*). Surface of slice of lung impregnated with barium sulfate. Normal lung is present above and to the right, and adjacent to this is a sharply demarcated area of panlobular emphysema. B (*bottom*). Close up of lesion in A. [illegible]

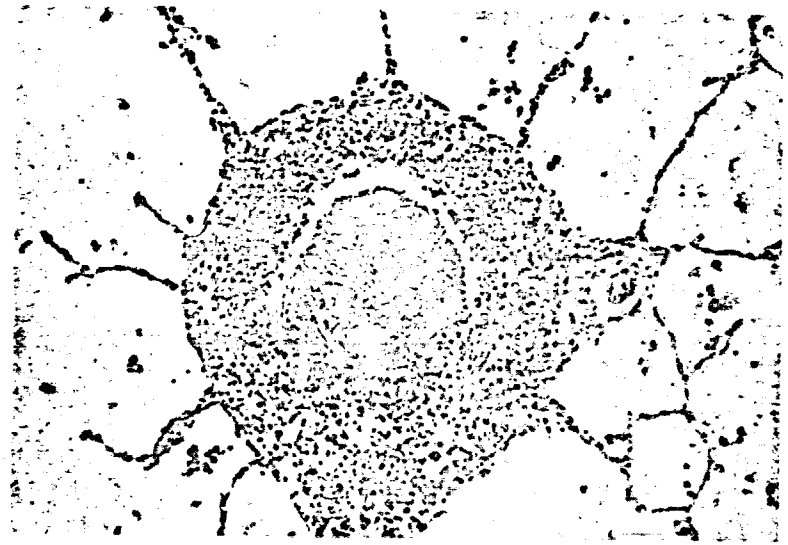

Fig. 6. C. Photomicrograph of a branchiole containing an infected mucous plug with inflammation of the bronchiolar wall (original magnification × 200).

Fig. 7A

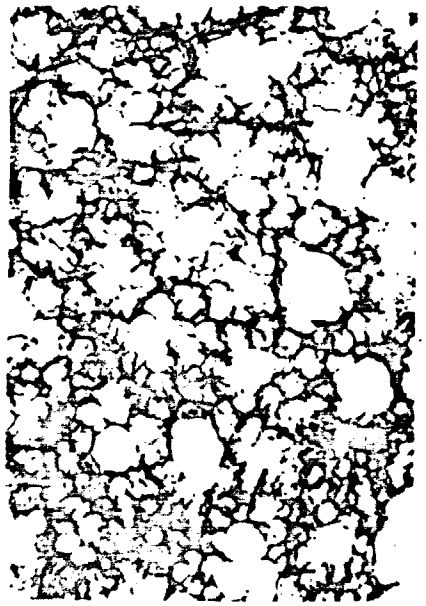

Fig. 7B

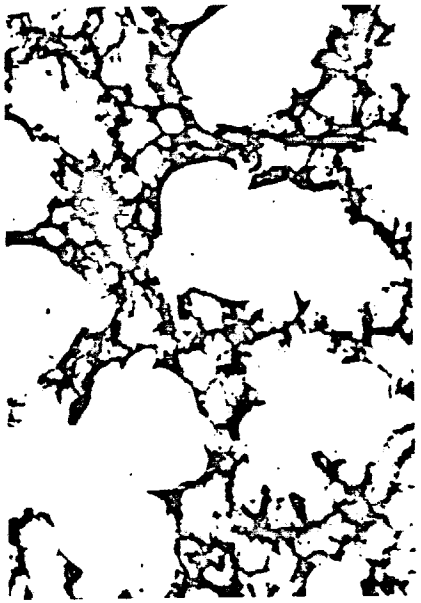

Fig. 7C

Fig. 7. A. 100 μ-thick section of control dog lung to be contrasted with B and C, which show mild panlobular emphysema. Striking enlargement of alveolar ducts can be seen in C. (All at same original magnification of approximately × 40).

was produced (14). The emphysema in the animals was generally not severe and was limited in extent compared with symptomatic human emphysema. At first sight, it is perhaps surprising that these mild changes were accompanied by any changes in pulmonary function. Similar changes, however, have been described in subjects with mild emphysema (15). Although fairly high correlation coefficients existed between a number of pulmonary function tests and the severity of the emphysema as assessed by ISA_2, only in the case of FRC did the correlations meet conventional levels of significance. The experimental group was so small and the amounts of emphysema so similar that significant correlations were not expected.

In conclusion, experimentally induced papain emphysema in the dog does appear to be very similar to panlobular emphysema both morphologically and functionally. It appears to be eminently suitable as a model for the study of the human disease.

TABLE 4

MORPHOLOGIC STUDIES IN DOGS WITH PAPAIN-INDUCED EMPHYSEMA AND IN NORMAL DOGS

Dog	Weight (lb)	TLV (liter)	Lm (mm)	ISA (m^2)	ISA_2 (m^2)
Normal Dogs					
1	44	2.43	0.106	79	69
2	45	2.10	0.098	73	71
3	42	2.20	0.110	71	67
4	45	2.53	0.116	78	67
5	30	1.81	0.122	55	59
6	61	4.00	0.146	98	61
7	60	3.09	0.132	82	62
Mean	46	2.59	0.119	77	65
Emphysematous Dogs					
1	48	1.48	0.165	32	39
2	49	1.60	0.144	41	48
3	27	1.51	0.140	37	41
4	69	2.40	0.140	65	57
5	50	2.88	0.150	68	54
6	45	2.12	0.138	54	52
7	44	2.21	0.152	59	56
8	48	3.21	0.258	46	33
Mean	48	2.18	0.161	50	48

Key: TLV = volume of lungs at a distending pressure of 25 cm formalin; Lm = average interalveolar distance; ISA = alveolar surface area; ISA_2 = alveolar surface area at standard lung volume of 2 liters.

(Received for publication May 21, 1970)

RESUMEN

Enfisema experimental inducido por la papaína en perros

Se inyectó papaína intratraqueal a 8 perros tras de determinar la tensión arterial de oxígeno, anhídrido carbónico, pH, valores de volúmenes pulmonares, capacidad difusora de monóxido de carbono, retención fraccionada del monóxido de carbono, curvas volumétricas de la presión estática y la resistencia pulmonar a distintos volúmenes pulmonares. Se repitieron las determinaciones de 1 a 4 semanas tras la inyección de la papaína. No hubo cambios apreciables en la tensión de gases arteriales el balance acido-alcalino o en la resistencia pulmonar, aunque la capacidad difusora [illegible] la capacidad

pulmonar total, capacidad residual y volumen residual, aumentaron: la elasticidad disminuyó. Tras de sacrificar el perro se determinó la resistencia pulmonar y se observó que la resistencia en conductos menores de 2 mm en diámetro aumentó, mientras que en los conductos mayores, disminuyó. Enfisema panlobular de leve a moderado fue observado en el estudio anatomopatológico en todos los animales. El área de la superficie interna disminuyó, especialmente al medirla durante un volumen pulmonar fijo. Se concluye que la enfermedad provocada mediante la administración intratraqueal de papaína a perros es similar, tanto patofisiológica como morfológicamente, al enfisema panlobular humano.

RESUME

Emphysème experimental induit par la papaïne chez des chiens

De la papaïne a été injectée à l'intérieur de la trachée chez 8 chiens après qu'on ait procédé à des mesures de la tension artérielle en oxygène, de la tension artérielle en dioxyde de carbone, du pH, des subdivisions du volume pulmonaire, de la capacité de diffusion (steady state diffusing capacity) pour le monoxyde de carbone, de la charge fractionnée (fractional uptake) en monoxyde de carbone, des courbes volumétriques à pression constante, et de la résistance pulmonaire à différents volumes du poumon. Les mesures ont été répétées une à quatre semaines après l'injection de papaïne. On n'a pas observé de différence significative dans les pressions artérielles en gaz sanguins, dans l'équilibre acido-basique, ou dans la résistance pulmonaire. La capacité de diffusion a toutefois diminué. La capacité pulmonaire totale, la pleine capacité résiduelle, et le volume résiduel, ont augmenté. Le "recoil" élastique a décru. La résistance pulmonaire a été étudiée par régions après que le chien eut été sacrifié. On a observé que la résistance des voies aériennes ayant un diamètre inférieur à 2 mm était accrue alors que celle des conduits aériens plus larges était réduite. De l'emphysème de divers degrés, de trivial à modérément panlobulaire, a été observé chez tous les animaux lors des examens du matériel pathologique. La superficie intérieure était réduite, et particulièrement lorsqu'on la comparait à un volume pulmonaire standard. La ligne d'interception moyenne (mean linear intercept) était augmentée. On en conclut que la maladie produite par la papaïne, administrée à l'intérieur de la trachée chez des chiens, est semblable à l'emphysème humain panlobulaire, tant sur le plan patho-physiologique que sur le plan morphologique.

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SOME PHARMACOLOGICAL AND TOXICOLOGICAL PROPERTIES OF THE ANTHELMINTIC ENZYME "VERMIZYM"

By

T. Takaoka

Papan

Introduction

Robbins et al. [2] demonstrated that the anthelmintic effect of fig juice was attributable to the fact that the juice contained a kind of emzyme capable of decomposing proteins and assigned the name "ficin" to the crude enzyme preparation precipitated from the juice by 3% acetone or 5% ethanol. He also showed that 0.1-0.2% solution of this enzyme preparation was capable of digesting and destroying live roundworms within a short period of time. This preparation was found to be an effective anthelmintic for the roundworms as well as other intestinal worms; it is especially effective in destroying *Trichocephalus trichiurus* which is difficult to eradicate with other drugs [3,4]. In 1938 Walti was able to crystallize ficin. However, the anthelmintic effect of this crystalline preparation as well as the crude juice preparation could be reduced markedly during storage if conventional preservation techniques were employed. Therefore, the use of these preparations was limited to the small area where the preparations were manufactured. It was also known that papain obtained from the unripened fruit of *Carica papaya* had enzymatic properties similar to those of ficin.

Recently, the Dr. Schwab Company in Germany marketed an anthelmintic called "Vermizym" which was said to be a papain preparation with enhanced anthelmintic effectiveness and stability. This product was kindly supplied to us by the Fujisawa Pharmaceutical Co., and therefore we decided to study its various pharmacological and toxicological properties in order to

determine its usefulness as an anthelmintic drug.

A summary of this paper was presented at the 26th Meeting of the Japanese Pharmacological Society (abstract: *Nippon Yakuri Zasshi* 49, 154, 1953) and the 22nd Meeting of the Japanese Parasite Society (abstract: *Kiseichyugaku Zasshi* 2, 57, 1953).

Experimental materials and methods

Verimzym (abbreviated as Verm in the following) is a tablet containing yellowish-brown materials coated with a thin sugar layer. The content showed characteristic odor and a slight acidic taste. It gradually produces an astringent or slightly irritating taste to the tongue. In our studies, the tablets were powdered by the use of a mortar, and emulsions were prepared by suspending the powder in distilled water, 0.9% saline, or Ringer solution. In some cases, the emulsion was filtered. The tablets were stored as dry as possible. Experimental procedures for each study will be described in each section.

Experimental Results

A. Anthelmintic action

1. *In vitro digestion of pig roundworms.* One pig roundworm each was placed in 30 cc of a 2% Verm-Ringer emulsion (pH 5.6) and was kept at 37°C. In about 1.5-2 hours, white erosion spots could be observed at various places on the skin surface. The spots gradually enlarged and became more distinct. In 2-3 hours, some perforations could be noted. Digestion then proceeded rapidly and, in about 4 hours, extrusion of internal organs and destruction of the body wall could be seen. In 5 hours, extensive digestion resulted in the disappearance of the external worm shape. At this moment, the roundworm was still capable of spontaneous movement despite the extrusion of some

internal organs. In other experiments, the emulsion was preheated at 65°, 70°, 75° or 80° for 5 min., cooled immediately to 37°C and was used in the experiment as mentioned above. The emulsion preheated to 80° or above for 5 min. completely lost its anthelmintic ability. At temperatures below 80°C, the decrease in the anthelmintic effectiveness depended on the preheating temperature. In other experiments, the pH of the 2% Verm-Ringer emulsion was adjusted to 3, 4, 5, 6, 7, 8, or 9 by the use of an AkIlvain buffer solution (to obtain the pH 9 solution, NaOH was used to adjust the pH of the pH 8 solution), and the experiment as described above was carried out. The optimum pH was found to be near 5. At pH below 3 or above 9, no anthelmintic effect could be observed. When Verm-Ringer solution was made strongly acidic (pH 2.0), and kept at 37°C for 1 hour, the roundworm-digesting ability could no longer be restored even after the emulsion was neutralized with NaOH. A similar result was observed with the addition of pepsin (5%) to the emulsion. These results are about the same as those obtained with ficin reported by Robbins with the exception of the result concerning the enzyme concentration.

Papayotin was used for comparative studies. To determine their corresponding concentration, their gelatin-digesting ability was compared. 1 ml each of 5% gelatin solution (buffered at pH 5.0) containing 0.1% thymol was placed in 10 test tubes, and various amounts of 0.1% papayotin (Merck preparation 1:200 and 1:80) or Verm ranging from 0 to 0.1 ml with 0.1 ml increments were added to the tubes. Various amounts of pH 5 buffer solution were then added to the tubes to make the final volume in each tube 2 cc. Liquefaction of gelatin was measured at 37°C. The results are shown in Fig. 1. With a 4-hour action time, the concentration ratio with the same digesting efficiency was found to be Verm 2% to papayotin (1:200) 6.6%. These concentrations were used to compare their pig roundworm digesting ability. For

Verm, 2 to 3 hours were needed to produce perforation in the worm body and the destruction of the worm body was seen in 5 hours, while papayotin merely produced erosion of the skin surface after 5 hours.

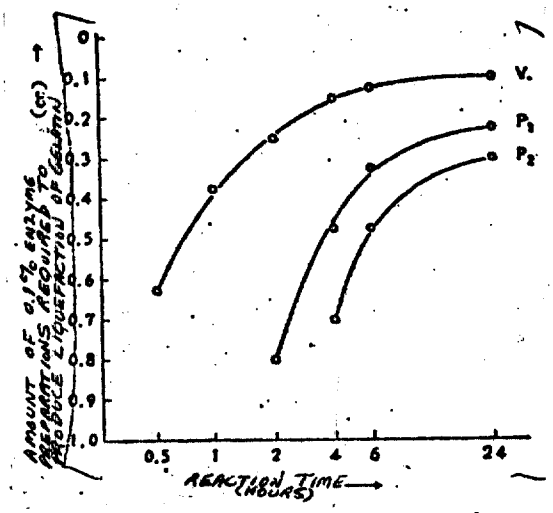

Fig. 1. Gelatin liquefaction effect of Vermizym and papayotin.

2. *Experiments with human roundworms.* 5 roundworm-infected patients with various degrees of infection (27-58 year old men) were studied. No meat was eaten by the patients one day before the study and in the evening, a light supper was given. Just before bed-time, some patients were asked to take a laxative (either a laxative provided with the anthelmintic or laxatol) and some did not receive a laxative. The next day, early in the morning, 5 tablets per dose of Verm were given with water 5 times at one-hour intervals before breakfast. The presence of roundworms in the stools collected during the following 7 days was examined. In addition, the numbers of roundworm eggs per g of stool (E.P.G) in the 7-day stool collections as well as in the stools collected prior to and 3 weeks after the drug administration were determined. Results are summarized in Table 1. 4 out of 5 patients studied excreted half-digested roundworms (1-6 worms) and 2 of them showed no presence of worm eggs after the drug. The other 2 patients showed a decrease in the egg count. The patient with no worm excretion also displayed no change

in the egg count. No side effects were noted in any of the patients examined. No abnormality was seen in the properties of the stool excreted by the patients who received the anthelmintic only. One patient (U.K.) also carried *Trichocephalus trichiurus*, and his egg count of this worm decreased somewhat but did not disappear completely.

Table 1. Results of experiments involving removal of roundworms from the human body by Vermizym.

Patients studied	E.P.G. before drug	Number of days elapsed and E.P.G.; numbers in ( ) indicate the number of worms excreted							Total No. of excreted worms	E.P.G. after drug (3 wks later
		1	2	3	4	5	6	7		
U.K.*	12500	(0)	38700(2)	12100(4)	50(0)		0(0)		(6)	0
K.K.*	10450	10200(0)	11550(0)	7600(0)		8600(0)	8000(0)	(1)	(1)	9300
S.D.	2600	(0)	2900(1)	2450(1)	1100(0)	1550(0)	1600(0)	1750(0)	(2)	1650
T.T.	1750	(0)	1250(0)		1850(0)		1400(0)		(0)	1450
K.H.	1500	(0)	500(1)	150(0)		0(0)		0(0)	(1)	0

* A laxative was given in the evening before the drug; dose and method of administration are given in the text.

It is of interest to note that the patient with the highest number of excreted worms (U.K.) showed a temporary but marked increase in the worm egg count around 2 to 3 days after drug administration. The fact that some of the eggs had thin egg shells suggests that the transient increase in the egg count could be caused by the digestive destruction of the inner reproductive tubes.

B. Toxicity

1. Toxicity in mice with the drug given through a stomach tubing. Male mice weighing 10-15 g were starved overnight. 0.25-0.75 cc/10 g of 25 and 50% Verm emulsion was given through a stomach tubing. The chief acute

toxic symptoms were goose flesh, breathing difficulty, diarrhea and weakness. Large dosages would cause death due to respiratory weakness. LD_{50} was about 12.5-18.7 g/kg. Difference in the toxicity due to different dosage concentrations, which is expected to occur with the preparation with enzymatic properties, was not observed (see Table 2). With the lethal dose or the doses close to it, diarrhea developed within several hours. For those mice which survived 1-2 days, blood could be seen in the stool and swelling, erosion and hemorrhage of the mucous membrane of the anus were observed. Most animals died within 24 hours, but some died 72-96 hours later. The lethal effect could not be determined in 24 hours. This observation is different from that with ficin reported by Molitor et al. [8].

Table 2. Toxicity in mice through the stomach.

Drug concentration	Dosage/10 g: Volume of drug solution used (cc)	Dosage/10 g: Amount of drug used (mg)	Number died/number studied*
50	0.25	125	0/6
	0.37	187	4/6
	0.50	250	6/6
	0.75	375	4/4
25	0.50	125	2/6
	0.75	187	4/6

*: A laxative was given in the evening before the drug; dose and method of administration are given in the text.

Results of biopsy: In the mice which died within 24 hours after drug administration, sporadic distribution of small hemorrhage spots could be seen on the serous membrane of the small intestine, especially the jejunum, but the membranes of those mice which died later than 24 hours showed reddish-purple color all over the small intestine. No blood was detected in the

intestinal contents of the former group of mice, but the intestinal contents of the latter group of mice looked like bloody purulent matter, and some of their intestinal walls became thinner. No distinct change could be seen with the naked eye in other organs. In those mice which survived more than one week, no marked change observable by the naked eye could be detected in the digestive tract.

When a 50% solution of this drug was heated to 95°C for 5 min. and more than 0.5 cc/10 g of this heat-treated solution was administered to mice, the mice developed toxic symptoms different from those observed in the previous experiments, i.e., clonic and stiffening spasms. Further studies are needed to determine whether this drug preparation contains toxic components which produced the toxic symptoms observed in the previous experiments or the new toxic components were produced as a result of heating.

2. *Toxicity produced by intravenous injection into guinea pigs.* 1 to 5% solution in 0.9% saline after filtration was used for injection. The solution was injected into the upper leg vein at a rate of 0.5 cc/min. When the amount equivalent to 25 mg Verm/kg was injected, no symptoms could be observed, or some animals developed very mild transient tachypnea which quickly disappeared. With a dose of 50 mg/kg, the animals developed marked dyspnea, lay down, and died from suffocation following bronchial obstruction-like convulsive respiration (2 out of 4 animals studied). With a dose of 100 mg/kg, 4 out of 5 animals died (3-5 min. after the injection) from suffocation after displaying symptoms similar to those mentioned previously. Their chests were opened immediately after they stopped breathing. The lungs showed marked expansion and filled the entire chest cavity. The heart beat continued for some time. These results are similar to those observed in histamine intoxication. When a drug solution, preheated at 80°C for 5 min.,

was used, a dose as high as 300 mg/kg failed to produce any toxic symptoms.

3. *Effect on the rat blood picture.* The same drug solution used in the intravenous injection of guinea pigs was also used in this study. The amount of solution equivalent to 60 mg of Verm/kg was injected into the rat tail vein. Marked decreases in the numbers of red blood cells and white blood cells were observed (the change in Hb was roughly parallel with that of red blood cell count). When the drug was given orally, a dose as large as 10 g/kg (more than 170 times the intravenous dose) failed to kill the rat and the blood cell counts did not decrease.

C. Local Action

1. *Action on the conjunctiva (rabbits).* 0.2 cc of 10% and 25% Verm emulsion in 0.9% saline or 0.05 g of Verm powder was applied on the conjunctiva of rabbits. One minute later, the drug was removed by washing the eye with physiological saline and the conjunctiva and the cornea were examined at a certain time interval. Closed eyes, tearing (lacrimation), a discharge from the eye, and bloodshot eyes etc. were noted, but no marked swelling or turbidity in the cornea could be seen. The irreversible impairments said to be produced by ficin [8] were not observed.

2. *Action on the skin (rabbits).* The hair on the rabbit's stomach was removed by use of barium sulfide one day before the experiment. A glass funnel with about 2.5 cm diameter was placed air-tight against the exposed skin and 25% and 50% Verm emulsions in 0.9% saline were poured into the funnel. The contact was maintained for 1 hour. The normal healthy skin only showed several mild hemorrhagic spots from the treatment using 50% solution. When the skin pre-cut randomly but without causing hemorrhage was treated with either 25% or 50% solution, the presence of blood in the solution could be seen several minutes after the treatment; one hour later, strong congestion

and hemorrhage were noted locally. If the Verm emulsion was preheated at 80°, the symptoms described above were not observed.

3. *Local action produced by subcutaneous injection (guinea pigs).* Hairs on the backs of guinea pigs were shaved and 0.3 cc of 5%, 10% and 25% Verm emulsion in 0.9% saline was injected subcutaneously. Even with 5% emulsion, the site of the injection gradually turned dark purple and resulted in necrosis of the skin, and 20 hours later a scar with 1.5 cm diameter remained. With the 25% emulsion, the necrotic change was more rapid and stronger and the area was as large as 3 × 4 cm. In some cases, the necrotic change was also seen in the stomach area and 24 hours later an abscess with 1.5 cm diameter could be noted under the stomach skin. The skin in this area also showed necrosis. Those guinea pigs which produced an abscess died 4 days after the injection, but other guinea pigs eventually showed healing of the necrotic change.

4. *Local action by intramuscular injection (dogs).* 0.3 cc of 25% Verm emulsion in 0.9% saline was injected into the thigh muscle as aseptically as possible. Shortly after the injection, the dogs refused to walk by lifting up their legs, expressing pain caused by pressure, but 3 hours later they started to walk normally and 48 hours later they expressed no pain. With 50% emulsion, besides the symptoms noted above, marked local swellings were observed. The swellings became as big as 5 × 12 cm within 3 to 5 hours. In addition to the pain, local fever could be noted in the infected area. However, 18 hours later, they walked normally and 96 hours later the pain disappeared.

5. *Action on the mucous membrane of the small intestine (dogs).* Dogs were anesthetized with urethane-morphine and ligation of the small intestine was carried out without impairing the mesentery blood stream insofar as

possible. The ligation was made on both ends of a 6 cm portion of the small intestine about 30 cm below the pylorus. 3 cc of 50% Verm emulsion were injected into the ligated small intestine or, in some cases, 1.5 g of Verm powders were placed inside the intestine by making a small incision. The intestinal mucous membrane was examined 1 hour and 3 hours after the drug application. A dog receiving a preheated 50% emulsion was used as control. One hour after the application of either emulsion or powders, the mucous membrane was somewhat congested and swollen. Three hours later, some dogs displayed detachment and erosion of the epithelium of the mucous membrane. These symptoms were stronger when powdered Verm was used, and when a Verm tablet as such was used, the symptoms were milder.

6. *Action on the stomach mucous membrane by oral administration (dogs).* The dogs fasted overnight, and 5 cc of 20% Verm emulsion/kg were introduced into the stomach by the use of a Nelaton catheter. 5 hours later, the dogs were killed by bleeding under chloroform anesthesia, and the stomach mucous membrane was examined. Grossly, no change in the membrane as compared to the control dog could be seen when Verm alone was given. However, careful examination revealed, in some dogs, sporadic millet-size white spots and a slight loss of luster on the mucous membrane. Lamson, Brown and Ward [9] reported that administration of ficin to the dog whose stomach mucous membrane was pre-treated with hexylresorcinol to induce erosion damages would exacerbate the damage. If this is true, it is dangerous to give ficin to a patient with organic disorders of the stomach. It is reported that the change in the mucous membrane is most distinct 5 hours after hexylresorcinol administration and 24 hours later, no trace of hexylresorcinol is present in the stomach [9, 10]. Therefore, in our studies, the same amounts of Verm as used in the previous experiment were given 5 hours and 24 hours after the dogs were

treated with hexylresorcinol. Dogs receiving hexylresorcinol alone were used as controls. The results of this experiment are summarized in Table 3. Verm was found to be without effect on the hexylresorcinol-induced impairments. The result of the study in which Verm was administered 24 hours after hexylresorcinol treatment suggest that the observed results shown in the Table below are not related to the enzyme-inhibiting effect of hexylresorcinol and thus our findings can be applied to general stomach mucous membrane lesions. Throughout this experiment, Verm caused no damage to the intestinal mucous membrane.

D. Other Pharmacological Actions

1. Effects on dog blood pressure. A filtrate of 5% Verm in 0.9% saline was injected in the leg vein of urethane-morphine anesthetized dogs. The amount of Verm injected was equivalent to 40-50 mg/kg. A marked decrease in the arterial blood pressure and a slight increase in the portal vein pressure were observed. The changes in the blood pressures were more distinct when Verm was injected into the portal vein. Repeated injection into the leg vein produced tachyphylaxis, whereas repeated injection into the portal vein caused a gradual increase in the drug effect. Pre-intravenous injection of 10 mg/kg of benadryl caused no effect. Preliminary intravenous injection of 10 mg/kg of benadryl caused no effect on the Verm solution, induced lowering of the arterial blood pressure and elevation of the portal blood pressure.

2. Effects on smooth muscle tissue. Application of more than $1:5 \times 10^5$ of Verm on the guinea pig intestinal tract removed by the Mognus technique, induced slow contraction, and washing caused gradual relaxation. Repeated treatments resulted in a gradual decrease in the degree of the drug effect on the contraction of the intestinal tract but the intestinal tract still reacted very well to histamine (see Figure 2). This effect of Verm was

Table 3. Action of orally administered Vermizym on dog stomach mucous membrane.

Drugs	Dog No.	Body weight	Sex	Stomach membrane: Congestion	Hemorrhage	Erosion	Ulcer	Remarks
Control	1	5.2	♀	-	-	-	-	
	2	2.8	♀	-	-	-	-	
Verm alone (biopsy 5 hrs. later)	3	2.6	♂	-	-	-	-	No. 4: a female roundworm parasitizing middle part of jejunum No. 5: a female roundworm parasitizing middle part of jejunum; white erosion on worm body and extrusion of internal organs were noted
	4	3.6	♂	-	-	-	-	
	5	3.0	♀	-	-	-	-	
H.R. alone (biopsy 10 hrs. later)	6	5.8	♂	+	+	+	-	
	8	4.4	♀	+	+	+	-	
Verm given 5 hrs. after H.R. administration (biopsy 5 hrs. later)	7	5.4	♂	++	+	+	-	No. 9:
	9	4.0	♂	+	+	+	-	
H.R. alone (biopsy 29 hrs. later)	10	4.0	♂	+	+	+	-	
Verm given 24 hrs. after H.R. adminis.	11	5.0	♂	+	+	+	-	

Notes: -: normal, ± very small change, +: relatively marked change, ++: very distinct change, H.R.: hexylresorcinol.

still observed in the presence of 5×10^{-8} atropine sulfate and 2×10^{-8} neoantergan maleate. Application of 1×10^{-4} Verm on the rat virgin uterus insensitive to histamine caused contraction, and the contraction was not affected by atropine pre-treatment. The results described above could not be observed with the preheated Verm solution.

Fig. 2. Isolated guinea pig ileum in Tyrode's solution medium containing 50 μg/cc of atropine sulfate.
V: Vermizym 1.0 mg, H: histamine hydrochloride 1.0 μg (representing the amount contained in 10 cc of medium), 1: washing.

Discussion

The experimental results described above indicate that the in vitro roundworm digesting ability of Vermizym is stronger than that of the equivalent amount of papayotin having the same gelatin-liquefaction power as Verm. Therefore, they are not identical substances. The optimum pH for in vitro pig roundworm digestion and the resistance to heat treatment are the properties almost identical to those of ficin reported by Robbins [1]. If the Verm solution is kept at acidic pH 2 for 1 hour at room temperature, neutralization with NaOH will no longer restore its ability to digest roundworms. Therefore, a decrease in the effectiveness due to gastric acid should be taken into consideration when Verm is used. Similar results were reported on ficin by Robbins [1], but Yoncya [11] reported restoration of the ficin effect under similar conditions.

In the clinical study on the removal of intestinal worms, Hannak [12,13] reported that 5 tablets 5 times a day resulted in negative egg counts in 71% of patients infected with roundworms, while the same treatments for 3 days were required to cure 70% of patients infected with *Trichocephalus trichiurus*.

Weise [14] reported that repeated treatments for 2 days were needed to cure patients infected with large numbers of roundworms. Even though the number of patients studied in our experiments was small, it is of no doubt that a one-day treatment with a dose of 5 tablets 5 times a day for roundworms will not give satisfactory results. The same is true for the treatment of patients infected with *Trichocephalus trichiurus*. In order to obtain an effective treatment, the dosage or the number of administrations has to be increased. Fortunately, no side effects were noted.

Toxicity in rats by oral administration is rather weak judging from the dose per kg body weight for producing toxicity. This fact, as well as various properties of Verm and the biopsy results of intoxicated mice suggest that the toxicity of Verm is probably not due to its absorption but due to its direct damaging effects on tissues or due to secondary toxic substances formed as a result of its direct damaging action. Intravenous injection of Verm into guinea pigs produces bronchial-constriction symptoms similar to those observed in histamine intoxication. This result also suggests that the direct action of Verm on damaged tissues is the main cause of its toxicity. Rocha and Silva et al. reported a release of histamine from rabbit blood cells by papain [15] and a histamine release from the guinea pig lung by trypsin [16]. These phenomena are very similar to those observed in our studies. In our studies with rats receiving intravenous injections, marked decreases in the leukocyte and erythrocyte counts were observed. These decreases are probably caused by the direct action of Verm on blood cells. Oral administration of Verm in a dose 150 times greater than that of the intravenous injection failed to produce any changes. Therefore, it is not probable that the orally administered enzymes could be absorbed into the blood stream in the form of active enzymes and the toxicological problems probably

stemmed entirely from the local damaging effects of the drug.

In order for an enzyme preparation to be effective as an anthelmintic, it must not damage the digestive tract of the host before it completely digests the skin proteins of intestinal parasites. In our studies on the dog stomach mucous membrane, the concentration of the enzyme many times that required for digesting roundworms failed to cause damage to the mucous membrane, suggesting that this preparation could be an effective anthelmintic. Mendel and Blood [17] reported that intestinal parasites possess antipepsin and antitrypsin enzymes but no antipapain enzymes. These enzymes are probably present in the mucous membrane of the host digestive tracts. It is of particular importance to know that the enzyme preparation failed to exert its digestive action on the artificially produced erosion on the stomach mucous membrane. However, rabbit conjunctiva and dog and guinea pig connective tissues are less resistant to this enzyme preparation than the stomach mucous membrane.

As mentioned before, the release of histamine from tissues by this enzyme preparation can be anticipated from the similarity of this preparation to trypsin and papain. However, its arterial pressure lowering and portal vein pressure elevating effects were not clearly depressed by antihistamines. It is also known that the marked histamine-releasing action of peptone is not inhibited by antihistamines [18]. The marked blood pressure lowering effect of this enzyme preparation injected into the portal vein is also observed in peptone and many other histamine-releasing substances [19]. That the repeated intravenous injection of this enzyme preparation gradually increases its effectiveness is also observed in organic hydrochloric acid salts of histamine-releasing substances as reported by MacIntosh and Paton [19]. The toxic symptoms observed in the guinea pigs receiving intravenous injections

also support the belief that the enzyme preparation is capable of causing histamine release. Its contractile effect on the isolated guinea pig ileum was not depressed by atropine or neoantergan. Therefore, this effect is different from the contraction effect of histamine; rather, it is similar to the action of the so-called "slowly reacting substance" described by Kellaway et al. [20]. The observation that repeated treatment reduces the contraction effect suggests that the reacting substance might be released slowly from tissues, and this slowly-released substance could be identical to the substance responsible for causing contraction of the rat virgin uterus. Therefore, the action of Verm is probably accompanied by the release of histamine and other pharmacologically-active substances including a smooth muscle contracting agent, but the release of secondary active substances by the damaging effect of the enzyme preparation will not produce adverse side effects in its anthelmintic action.

Summary

1. 2% Vermizym in Ringer solution (pH 5.6, 37°C) can perforate the skin of roundworms within 2 to 3 hours and can digest and destroy the worm body within 4 to 5 hours. The optimum pH of its activity (near 5) and its temperature resistance (inactivated at 80°C for 5 min.) are similar to those of ficin. The activity is destroyed irreversibly at pH 2 in 1 hour. The digestive action of Verm on live roundworms is stronger than the equivalent concentration of papayotin which has the same gelatin-liquefaction power as Verm.

2. Treatment by a dose of 2.5 g (5 tablets) for 5 times is effective in removing roundworms and *Trichocephalus trichiurus* from the human body but is not sufficient to eradicate them totally.

3. Oral administration of Verm produces little toxic effect (LD_{50} for mice 12.5-18.5 g/kg, and for rats more than 10 g/kg. Intravenous injection into guinea pigs of 50 mg/kg of Verm killed the animal due to bronchial contraction. Intravenous injection of 60 mg/kg caused a marked decrease in the erythrocyte and leukocyte counts in rats, but oral administration of as much as 10 g/kg did not produce any change.

4. Oral administration of the lethal dose for mice causes erosion and hemorrhage on the stomach and intestinal mucous membranes, but contact with the powdered preparation or highly concentration solution for 1 hour did not produce marked damage to the dog stomach and intestinal membranes. Oral administration of these doses did not markedly exacerbate the hexylresorcinol-induced damage to the stomach mucous membrane. However, this preparation had more pronounced damaging effect on the conjunctiva, the skin with detached epidermis, muscle and subcutaneous tissues than on the stomach and intestinal mucous membrane.

5. Intravenous injection of Verm caused lowering of the dog arterial blood pressure and elevation of the portal vein pressure. It also produced slow contraction of isolated guinea pig intestinal tract and the rat virgin uterus . These actions are probably caused by the release of histamine and other unknown active substances. However, the release of these substances bears no toxicologically important meaning as far as the anthelmintic action of this preparation is concerned.

6. The actions of Verm described above disappear when the preparation is heated at 80°C for more than 5 min.

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Nippon Yakubutsugaku Zasshi, 51, 1955

日薬理誌 51, 27～33 (1955)

酵素驅虫劑 Vermizym の二, 三薬理學的ならびに中毒學的性質について

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(昭和29年7月22日受付)

緒 言

無花果汁液の驅虫效果が、その含有する一種の蛋白分解酵素によることが *Robbins*[1] によつて知られ、彼は 3% Acetone 又は 5% Alcohol で沈澱する粗製酵素を Ficin と名ずけ、その0.1～0.2%液中で生きた蛔虫が短時間で溶化され死滅するとのべている。この乳汁は蛔虫その他一部の腸内寄生虫にも用いられて效果が認められているが、特に他の藥物の效かし難い鞭虫に效果を有する點が注目されている[2]。1938年 *Walt*[3] により結晶形 Ficin がとり出されたが、乳液及びこの Ficin ともに普通の保存法では效力が著しく低下するので、一般的利用は現地以外では廣く行われるに至つていない。一方、*Carica papaya* の未熟果實の汁液よりえられる Papain も酵素學的性質はこれと類似し、このものも腸内寄生虫溶化能をもつといわれる[3]。

最近、ドイツ *Dr. Schwab* 會社からこの Papain の效力を強調し且つ安定にしたと稱する驅虫劑が Vermizym の名稱で市販され、偶々藥品の厚意によりその提供をうけたので、このものについて特に驅虫劑としての用途に關連する二、三の藥理學的ならびに中毒學的性質について檢索を行つた。

本論文の要旨は第26回日本藥理學會（抄錄：日藥理誌 49, 154§, 1953）及び第22回日本寄生虫學會（抄錄：寄生虫學雜誌 2, 57, 1953）に發表した。

實驗材料及び實驗方法

Vermizym（以下 Verm. と略記）檢體は、薄く褐衣された內容黃褐色の錠劑で、內容は特有の臭氣と僅かな酸味があり舌に徐々に收斂味乃至極度の苦味を與える。實驗にはこの錠劑をその儘、乳鉢ですりつぶして蒸溜水、0.9%食鹽液又は *Ringer* 液などを加えて乳劑とし、又はこれを濾過して用いた。この錠劑の保存には特に濕氣をさけるよう注意した。個々の實驗方法については必要に應じてのべる。

實 驗 成 績

A. 驅 虫 作 用

1. *in vitro* 豚蛔虫溶化作用　2% Verm.-*Ringer* 乳劑 (pH5.6) 30cc中に元氣な豚蛔虫1匹づゝを投入し37°C保溫で觀察するに、約1.5～2^{h}で角皮表面の所々に溶化斑點の出現を生じ、蠕動消失しながら溶化が漸明となり、2～3^{h}でその一部に穿孔をきたす。この頃より溶化は急速に進行し約4^{h}で著明な穿孔部及び體壁の破裂をきたし、5^{h}に至れば虫體の溶化甚しく殆どその外形をとゞめなくなる。しかし、この溶解虫は一部腸壁出をともないつゝもある程度自發運動をつゞけた。この乳劑を液の65°, 70°, 75°及び80°C, 5^m 加熱後直ちに37°Cにもどしたものについて上記同樣の實驗を行つたところ、80°C, 5^m 以上の加熱ではその虫溶化作用は完全に失われ、それ以下では加熱溫度に應じて效力の減弱がみられた。*McIlwain* 緩衝液でpH3, 4, 5, 6, 7, 8及び9に調整した 2% Verm.-*Ringer* 乳劑（但し、pH9 は8を NaOH で修正）をつくり、上記同樣の實驗を行つた

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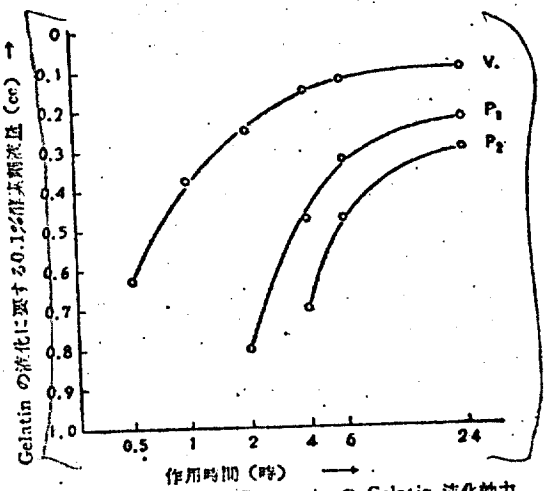
OF MEDICINE FOR PURPOSES OF STUDY OR RESEARCH IN LIEU OF LENDING THE ORIGINAL.

ところ，至適 pH は5附近で，3以下及び9以上ではその効力は発現されなかつた．Verm.-*Ringer* 乳剤を一応pH2の塩酸酸性とし37°C，1ʰ放置後，NaOH で中和した場合，蟯虫消化能はもはや認めなかつた．Pepsin (5%) を添加したものでもこの関係は同様であつた．以上の所見は酵素の種差は別として *Robbins* が Ficin について報告しているところと凡そ同様であつた．

次に Papayotin と効力を比較してみた．先ず，酵素濃度を揃えるため，Gelatin 消化能の比較を行つた．試験管10本に0.1% Thymol 加5% Gelatin 液 (pH 5 緩衝) 各1ccを入れ，Papayotin (*Merck* 製 1:200 及び 1:80) と Verm. の各0.1%液0～1.0ccを0.1cc宜で加えて更に蒸留水が2ccになるようにpH5の緩衝液を注加し，37°Cにおける Gelatin 液化力を比較したところ，第1図の成績をえた．これにより4ʰ後における同表面積に相当する Verm. 2%と Papayotin (1:200) 6.6%液をえらび，両者の蟯虫消化力を比較したところ，前者では2～3ʰで虫体に穿孔をみ，5ʰでその崩壊をきたすのに対し，後者では5ʰで表皮の膨潤をみるにすぎなかつた．



第1図．Vermizym 及び Papayotin の Gelatin 液化効力．
V.: Vermizym, P₁: Papayotin (1:200), P₂: 〃 (1:80).

2．人体蟯虫実験　感染程度の異る蟯虫症患者5名 (27～58歳，♂) に，前日より肉類の摂取を禁じ，前夕食を軽くとらしめ，就床前に下剤 (製剤に添付の下剤又はラキサトール) を服用さし，又は一切下剤を與えずに，当日早朝空腹時1ʰ間隔で Verm. 5錠づゝ5回，水で服薬させ，以後7日間の全排便についての排虫検査，ならびにこれらの糞便のほか服薬前及び3週間後の糞便g当り虫卵数 (E.P.G.) を検べた．その成績は第1表の如くである．即ち，被検者5名中の4名に生活化の虫体排出 (1～6匹) を認め，その中2名は駆虫され，他の2名も虫体減少を認めたが，排虫の認められなかつた1例では虫卵も殆ど変化をみなかつた．全員格別の副作用を認めず，糞便の性状も Verm. 単独投与例では殆ど全く異常を認めなかつた．駆虫保有の1例 (U.K.) では駆虫虫卵数に若干減少をみたが，除虫には至らなかつた．

第1表　Vermizym の人体蟯虫駆除実験成績．

被検者	前E.P.G.	経過日数とE.P.G.，( )内は排虫数							排虫総数	後E.P.G. (3週間後)
		1	2	3	4	5	6	7		
U.K.*	12500	(0)	38700(2)	12100(4)	50(0)		0(0)		(6)	0
K.K.*	10450	10200(0)	11550(0)	7600(0)		8600(0)	8000(0)	(1)	(1)	9300
S.D.	2300	(0)	2900(1)	2450(1)	1100(0)	1550(0)	1600(0)	1750(0)	(2)	1650
T.T.	1750	(0)	1250(0)		1850(0)		1400(0)		(0)	1450
K.H.	1500	(0)	500(1)	150(0)		0(0)		0(0)	(1)	0

(注)　*：服薬前夕下剤使用，その他の用量用法本文参照．

興味ある点は，排虫数の少かつた患者 (U.K.) で駆虫後2～3日頃の糞便に一過性の著明な虫卵数増加を認めたことである．虫卵の非常なものが含まれていたところからみて内生殖管の消化機能によるものであろうと想像される．

B．毒　性

1．マウス経胃投與による毒性　…
0.25～0.75cc/10gを胃内に注入した．
要注意のもとに観察し，異常はみられ

第2表．マウス経胃投與による…

薬液濃度 (%)	投與量/10g		死亡数
	液量 (cc)	薬量 (mg)	
50	0.25	125	0
	0.37	187	4
	0.50	250	6
	0.75	375	4
25	0.50	125	2
	0.75	187	4

(注)　*：投薬後の観察期間7日
には肉眼的に著変を認めない．1週間…
本剤を50%液を95°C，5ᵐ以上加熱…
する前記と異る中間症状が認められた．
新な有効成分の生成を推考すべきもの…

2．モルモット腹腔内注射の毒性　…
上腹部腹腔内に0.5cc/1ᵐの速度で注射した
の一過性呼吸麻痺ののち間もなく正常と
を思わせる軽微な呼吸ののち死亡する
4例が注射後3～5ᵐで殺れた．呼吸…
又は痙攣はその発現潜伏期長い例もあった．か
方，80°C，5ᵐ加熱したものでは300mg…

3．ラット血液像に及ぼす影響　モル
ラットの腹腔内に注射するに著変な…
たが，経口投与ではその170倍に当る10g…

1．結膜に対する作用(兎)　10%及び…
を兎の結膜嚢内に1ᵐ作用させ生理的食塩…
流涙，眼瞼及び結合膜が認められたが，…
ていろ様な不可逆性の重篤な傷害は認め…

2．皮膚に対する作用(兎)　前日脱毛…
せ，25%及び50% Verm.-0.9%食塩水乳剤…
軽微の紅斑浮腫を数ヶ所認めたにすぎな
数分後には全部の恢復をみ，1ʰ後には…
はかゝる作用を認めなかつた．

3．皮下注射の局所作用 (モルモット)
蒸水乳剤0.3ccを注射し，その局部温度を
20ʰ後には約1.5cmの部分が発赤した．25…
は腫脹に移行し24ʰ後には腫瘍部及び…

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1. マウス経胃投與による毒性　前夕より絶食させた体重10～15gの雄マウスに25及び50% Verm. 蒸水乳剤0.25～0.75cc/10gを胃内に注入した．主な急性中毒症状は立毛，呼吸困難，下痢及び衰弱で，大量の場合には呼吸衰弱のもとに斃れる．痙攣はみられない．LD_{50}は12.5～18.7g/kgの間で，酵素という性質から推量された開投與量についての毒性の差異は認められなかった（第2表参照）．致死量又はそれに近い用量では数時間内に下痢がみられ，1～2日以上生き延びたものでは漸次これに血液を混じた．かような例では腸内容の軟膏，腸粘膜の剥離，出血などがみられた．死亡例の多くは24h内に斃れたが，72～95h後の例もあり，*Molitor* らの Ficin についてのべているように24h内に生死を決定することはできなかった．

第2表．マウス経胃投與による毒性．

酵素濃度 (%)	投與量/10g 液量 (cc)	酵素量 (mg)	斃死數/動物數*
50	0.25	125	0/6
	0.37	187	4/6
	0.50	250	6/6
	0.75	375	4/4
25	0.50	125	2/6
	0.75	187	4/6

註）*：投與後の観察期間7日．

剖検所見：投與後24h内の早期死亡例では小腸特に空腸の粘膜面に極く微細な點状の溢血が播種状に散在したが，遅延死の場合にはむしろ全腸管に紫紅色を呈していた．腸内容は前者では血液を混じたものはないが，後者では血性粥状で且つ又小腸壁が肥厚となったものがみられた．他の臓器には肉眼的に著変を認めない．1週間以上の生存例では腸管にも肉眼的に著変を認めなかった．

本剤を50%液を95℃，5m以上加熱したものを投與するに0.5cc/10g以上では腸代謝及び腸管機能を主徴とする前記と似る中毒症状が認められた．本剤の酵素作用以外にかような有害成分を含有するか，或は加熱により新な有害成分の生成を推考すべきものか，尚検討を要する．

2. モルモット腹腔内注射の毒性　0.9%食塩水に 1～5% に溶かしたものを充分濾過したのちモルモットの上腹部腹腔に0.5cc/1mの速度で注射した．Verm. 相當量25mg/kg以下では全く症状をみないか又は極く軽度の一過性呼吸促迫のみ間もなく正常となるが，50mg/kgでは強度の呼吸困難をきたし極度となり痙攣発作を思わせる強直性呼吸ののち死亡するもの（4例中2例）を認め，又100mg/kgでは虚脱症状のもとに6例中4例[illegible]投與3～5mで斃れた．呼吸麻痺直後ちに開腹するに腹は著明に膨脹し，殆ど全腹膜をみたしており，又し[illegible]まその変相甚しい腸壁がみられた．かような所見は毒性類似した Histamine 中毒の場合に類似していた．一方，80℃，5m加熱したものでは300mg/kgの大量でも殆ど何ら中毒症状を呈しなかった．

3. ラット血液像に及ぼす影響　モルモット腹腔内注射に用いたと同様の濃度の Verm. 相當量60mg/kgをラットの腹腔内に注射するに著明な赤血球及び白血球数の減少（Hbの変化は赤血球数と略平行する）をきたしたが，経口投與ではその170倍に當る10g/kgの大量でも死亡せず，これら血球数の減少は全く認められなかった．

C. 局所作用

1. 結膜に対する作用（兎）　10%及び25% Verm.-0.9%食塩水乳剤0.2cc又は Verm. を標榜としたもの0.05gを兎の結膜嚢内に1m作用させ生理的食塩水で洗浄後，結膜及び角膜の状態を定時的に観察したところ，眼瞼，結膜，眼脂及び充血等が認められたが，著明な浮腫及び角膜の混濁をきたすものなく，Ficin について報告されている様な不可逆性の重症な障害は認められなかった．

2. 皮膚に対する作用（兎）　前日硫化 Barium で除毛した兎背部皮膚に直径約2.5cmのガラス漏斗を密着させ，25%及び50% Verm.-0.9%食塩水乳剤を漏斗内に注入し，1h作用させた．健常皮膚では50%液の場合にのみ軽度の點状溢血を数ヶ所認めたにすぎなかったが，潰瘍出血性皮膚に塗布した皮膚では両濃度液の場合とも数分後には血液の混入を認め，1h後には局部の強い充血及び出血を認めた．80℃，5m加熱した Verm. 乳剤ではかゝる作用を認めなかった．

3. 皮下注射の局所作用（モルモット）　剪毛したモルモット背部皮下に5%，10%及び25% Verm.-0.9%食塩水乳剤0.3ccを注射し，その局所変化を検べた．5%乳剤でも注射局部は暗紫色化し浮腫の発生をきたし，[illegible]h後には径1.5cmの部分が結痂した．25%液ではこの変化は一層強度に発現し変化部は径3×4cm，[illegible]部変化は周囲に進展し24h後には周囲皮膚皮下に径1.5cmの大の壊死結節をつくり，この部の皮膚にも壊死が現われた．

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腫瘍形成例は注射後4日目に確認したが，微塵のものはやがて局所の変化が結痂して治癒した．加温操作を加えた溶液にはこの作用はなかつた．

4．筋肉内注射の局所作用（犬）　25% Verm-0.9%食塩水乳剤0.3ccをなるべく無菌的に犬の二頭筋筋肉内に注射するに，暫くすると脚を引摺って歩行を始め，跛行を訴えたが3ʰ後には正常歩行をはじめ，48ʰ後には跛行の様子はみられなかつた．50%乳剤同液量ではこのほか局所の腫脹が著明で，3～5ʰで5×12cm大に達し，跛行のほか局所疼痛をふれた．但し，18ʰ後歩行正常となり96ʰで腫脹がとつた．

50%乳剤注射後5ʰで局所を切開した所見では注射部位の出血及び漿液による変化のほか，筋組織の筋間の結合組織及び皮下組織に黄褐色の浸潤液がみられたが，筋組織の内部的変化は認められなかつた．本質脂においても加熱の操作では殆ど全く変化を認めなかつた．

5．小腸粘膜に對する作用（犬）　Urethane-Morphine 麻酔犬の幽門部より約30cm下から長さ6cmつゝの長さに縦切つて刃及び刷毛で出血を防止せぬ様に注意して結紮した小腸腔内に，50% Verm.-蒸水乳剤3ccを注入し，又粉末粉末1.5gを小切開を加えて腸腔内に入れ，1ʰ及び3ʰ後の粘膜の傷害程度を検べた．1ʰ後は何れの場合も加熱した50%乳剤の例に比べて充血やゝ強く，心もち浮腫状の外観を呈したのみであつた．3ʰ後の所見では所々に粘膜上皮剥離及び壊死を認めたものがあつた．この変化は粉末の方が強かつた．錠剤そのまゝを入れた場合ではこれらの変化は粉末の場合よりも軽度であつた．

6．經口投與の胃腸粘膜に對する作用（犬）　前夕より絶食した犬に20% Verm.-蒸水乳剤5cc/kgを *Nélaton* カテーテルで胃内に注入し，5ʰ後クロロホルム麻酔の下に出血致死，剖検を行い，胃腸粘膜の傷害程度を検べたところ，Verm. 単独投與例では一般に對照に比べて特に著つた所見はみられなかつたが，よくみると胃粘膜が強かに光沢を失い数箇犬の白化斑点をまばらに認めたものがあつた．*Lamson, Brown & Ward*[9] は犬に Hexylresorcinol を與えて胃粘膜に壊死性胃炎を與えたのち Ficin を投與すると粘膜の傷がひどくなるとのべている．これが事実ならば胃の緩慢な炎症患者には Ficin は禁忌なわけである．Verm. についてこの點を検討するため，Hexylresorcinol 0.2g/kg 投與後及も粘膜の変化の著明といわれる5ʰ後[9,10]と，Hexylresorcinol の殺菌懸念のない24ʰ後にそれぞれ Verm. の上記用量を投與し，いずれも，Hexylresorcinol のみの投與對照と胃粘膜所見を對比してみた．これらの實驗成績の概略は第3表に示した如く，Verm. の Hexylresorcinol 傷害に對する影響は事實上認められなかつた．Hexylresorcinol 投與24ʰ後の Verm. 投與實驗からみて，この知見は前者の酵素阻害的効果に關係はないものと考えられるから，胃粘膜損傷の一般例に對しても適用できるものと考えられる．これらの實驗を通じて Verm. の胃粘膜に對する傷害的影響は全く認められなかつた．

第3表　經口投與 Vermizym の犬胃粘膜に對する作用．

薬剤	犬 No.	體重 (kg)	性	胃粘膜 充血	胃粘膜 滲出血	胃粘膜 糜爛	胃粘膜 潰瘍	備考
對照	1	5.2	♀	−	−	−	−	
	2	2.8	♀	−	−	−	−	
Verm. 単独(5ʰ後に剖検)	3	2.6	♂	−	−	−	−	No. 4：盲腸中部に蛔虫1匹寄生
	4	3.6	♂	−	−	−	−	No. 5：盲腸中部に蛔虫1匹寄生し，虫体に白化斑点，内臓充出あり．
	5	3.0	♀	−	−	−	−	
H.R.単独(10ʰ後に剖検)	6	5.8	♂	+	+	+	−	
	8	4.4	♀	+	+	+	−	
H.R.投與5ʰ後 Verm.(5ʰ後に剖検)	7	5.4	♂	+	++	+	−	No. 9：Verm. 投與後30ᵐᵢⁿ頃嘔吐．
	9	4.0	♂	+	+	+	−	
H.R.単独(29ʰ後に剖検)	10	4.0	♂	+	+	+	−	
H.R.投與24ʰ後 Verm.(5ʰ後に剖検)	11	5.0	♂	+	+	+	−	

（註）　−：正常，±：極く軽度の変化，+：比較的はつきりした変化，++：相當著明な変化，H.R.：Hexylresorcinol.

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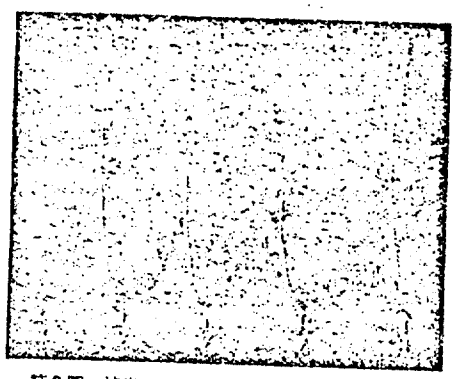
D. その他の薬理作用

1. 犬の血圧に対する作用　Urethane-Morphine 麻酔犬の股静脈内に5% Verm.-0.9% 食塩水溶液の溶液を Verm. 相当量40～50 mg/kg注射すると何等著明な動脈圧の下降と門脈圧の上昇を認めた．これらの作用は門脈内注射の場合股静脈内注射に比べ著明に強く現われ，又反復注射に際し前者では Tachyphylaxis 現象を認めるに對し，後者ではむしろ増大作用が増強の傾向を示した．Benadryl 10mg/kg 静脈内注射前処置は Verm. 液の動脈圧下降及び門脈圧上昇作用の何れにも顕著な抑制を與えなかつた．

2. 滑平筋組織に対する作用　*Magnus* 法摘出モルモット腸管に対して Verm. $1:5\times10^{-5}$ 以上では緩慢な収縮を起させ，後弛により徐々に弛緩した．かような適用を反復すると次第にその腸管の収縮反應の程度が減弱するが，この腸管は尚 Histamine にはよく反應した（第2図参照）．Verm. のこの作用は硫酸 Atropine 5×10^{-8} 及び Neoantergan maleate 2×10^{-8} 作用下に於ても依然出現した．又 Histamine に感じないラット妊女子宮に Verm. $1:10^{-4}$ を作用させると収縮反應を呈し，Atropine 前処置後でも同様であつた．上記の作用は一旦加熱した Verm. 液では認められなかつた．



第2図．摘出モルモット回腸．緩性 Atropine 50μg/l. 含有 Tyrode 液メチウム．28°C．V: Vermizym 1.0mg, H: 緩性 Histamine 1.0μg（いずれも10cc液槽中の薬物量），↓: 洗浄．

考　　察

上述の實驗成績によると，Vermizym の *in vitro* 蛔虫消化作用は Gelatin 液化力同等の濃度の Papayotin（Merck 製 1:250）よりも遙かに強力な點よりみて，両者は同一のものではない．*in vitro* 豚蛔虫消化の至適pH及び温度に対する関係は *Robbins*[1] の報告した Ficin の性質とほゞ一致する．一方HClで酸性pH2とし1時室温に放置後NaOHで中和したものはもはや蛔虫消化効力の復活は認められないので，胃液による効力の減少を多少考慮に入れる必要がある．*Robbins*[1] は Ficin に関してこれと同様の成績をえているが，*Yoneya*[11] はこの條件で Ficin の効力回復をのべており一致していない．

臨床駆虫實驗成績では，*Hannak*[10] の報告しているところによると5錠×5，1日では蛔虫症71%の駆除効，鞭虫症ではこの方法を3日間繰返し70%完全治癒したというが，*Weise*[4] は蛔虫でも感染量の大なる場合にはこの方法を2日繰返す必要があるといつている．私の實驗は少く用法についての吟味も充分といえないが，少くとも蛔虫症に対する効果は5錠×5，1日では満足でない．鞭虫症についても同様である．充分な効果をのぞむには，用量乃至投與回数の増加の必要があると思われる．副作用の殆どないのはこの點都合はよい．

マウス経口投與の毒性は實用量の数百倍からみれば非常に弱い．このことや，この物質の性質及び中毒マウスの剖検所見からみても，毒性の主要をなすのはこの物質自體の毒性によるというよりもそれによる組織崩壊乃至それにもとづく異物性物質の生成によることを考えやすい．モルモットで腸管に際して Histamine 中毒と類似の気管支痙攣症状をみたことも，そういう可能性を暗示している．*Rocha e Silva* らは Papain が血漿から Histamine 遊離をおこすのをみ[9]，又 Trypsin が摘出モルモット肺からやはり Histamine 遊離を起すのをみている[10]．これらの現象は互に類似性を暗示している．ラットに対する毒性が著明な赤血球及び白血球数の減少を示したのは血球に対する直接作用であろうが，経口投與では150倍以上の大量でもかゝる変化を認めないので，實際臨床に酵素が活性のまゝ血中に吸收されることはあるまいと思われる．從つて中毒學的問題は殆ど全く局所的腸管作用を基礎とするものであろう．

駆虫的目的にこの酵素が用いられるのは，宿主の消化管を侵害するまでに寄生虫の角皮蛋白を充分侵襲できるという假定にもとずくものである．この點に關して犬の胃液稀釈が蛔虫消化に必要な濃度の数十倍の酵

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果濃度に対しても抵抗を示したことはこの條件を満足させる知見である。*Mendel & Blood*[19]はこれら寄生虫が抗 Pepsin 及び抗 Trypsin 酵素はもつているのに抗 Papain 酵素をもたぬとのべているが、宿主の消化管粘膜にはこれがいずれも存在するためであろう。人工的に糜烂を起した胃粘膜に対しても、この酵素剤の消化作用のみられなかつたことは、臨床的上重要な意義がある。しかし、豚粘膜、犬及びモルモットの結合組織はこの酵素剤に対して、胃粘膜に比らべると抵抗が弱い。

この酵素酵素の Trypsin や Papain との類似性から上述の如く組織 Histamine 遊離作用を予想されたが、犬の動脈血圧降下と門脈圧上昇を起した Histamine 様の作用は、抗 Histamine 剤によつて明瞭には抑制されなかつた。しかし、Histamine 遊離の著明な Peptone のこれら作用も抗 Histamine 剤の抑制をうけないことが知られている[10]。門脈内注射の血圧降下作用が特に著明にみられたことは Peptone その他多くの Histamine 遊離物質に共通の性質[10]だし、又一般静脈内注射の場合反復刺激によつて効果の漸増するという所見も *MacIntosh & Paton*[10]が有機塩基 Histamine 遊離物質についてみているところと一致している。このほか、モルモット摘出中膜標本もこの物質の Histamine 遊離を支持する所見である。一方、摘出モルモット回腸に対する収縮反応は Atropine 及び Neoantergin で抑制されないし、Histamine による収縮とはちがつて、むしろ *Kellaway*[9]らの所謂"Slowly reacting Substance"の作用に類似している。そして、その反復作用の成績の様子からこの作用物質は腸壁に粘膜より遊離されるものであることが想像される。ラット遠女子宮の収縮をよぼす物質も恐らくこの未知物質と同一のものであろう。従つて、Verm. の作用下には恐らく Histamine とその他この種平滑筋収縮性物質を含む他の薬理学的活性物質の遊離も伴つて行われるものとみられる。但し、これらの傷害作用による二次的活性物質の遊離は駆虫剤としての實用上に殆ど副作用としての意味はもたないであろう。

総　　括

1. 2% Vermizym の *Ringer* 液乳剤（pH5.6, 37°C）は生きた蟯虫角皮に2～3^{h}で穿孔を生じ、4～5^{h}で虫体を消化崩壊する。この活性の至適 pH（5附近）及び温度に対する抵抗（80°C, 5^{m}で不活性化）は Ficin とほゞ同様である。pH 2, 1^{h} の処理により活性は不可逆的に除去される。この Verm. の生蟯虫消化効力は Gelatin 液化力に於て同等の濃度の Papayotin よりも強い。

2. Verm. の人蟯虫及び豚虫駆虫効果は2.5g（5歳）×5回でも認められるが充分ではない。

3. Verm. は経口投与では毒性が少い（マウスLD$_{50}$ 12.5～18.5g/kgの間、ラット10g/kg以上）。モルモット腹腔内注射では50 mg/kgで痙攣発作を起して死亡。ラット60mg/kgの腹腔内注射で赤血球及び白血球の著明な減少をきたしたが、経口投与では10g/kgでもこの変化はみられぬ。

4. マウス致死量の経口投与では胃粘膜の糜烂及び出血がみられるが、犬の胃粘膜はその粉末又は高濃度液の1^{h}接触によつて著明な損傷をうけない。その経口投与は Hexylresorcinol によつて生じた胃粘膜の損傷を著明に悪化しない。但し、結膜、表皮粘膜、筋及び皮下組織は胃粘膜よりも傷害をうけやすい。

5. Verm. は腹腔内注射により犬の動脈血圧の下降、門脈圧の上昇作用を示し、摘出モルモット腸管に緩慢な収縮をきたし、ラット遠女子宮を収縮する。これらの作用は Histamine のほか未知の活性物質の遊離によるものとみられる。但し、駆虫の目的での用法に際し中毒現象に重要な意味をもつものとは考えられぬ。

6. Verm. の以上の作用は殆どすべて80°C, 5^{m}以上の加熱によつて失われる。

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TOXICITY OF VARIOUS ENZYMES WHEN INOCULATED INTRACEREBRALLY INTO MICE

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Received 26 Nov 1956

THE effect of certain proteolytic enzymes on rabies virus has been the subject of a number of reports in the literature. In nearly every instance, experimental animals were inoculated intracerebrally with virus preparations that contained some quantity of active enzyme, but none of the writers reported toxic reactions attributable to the enzymes employed. Several investigators who worked solely with various proteolytic enzymes have described such responses as trypsin and peptone shock following the inoculation of experimental animals with these preparations.

The author is currently investigating the effects of certain enzymes on rabies virus. To determine the maximum concentration of each enzyme that could be employed without producing toxic reactions in experimental animals, a preliminary investigation was conducted. Mice were inoculated intracerebrally with serial dilutions of 12 proteolytic enzymes. These results are reported in the hope that they may be of some value to other investigators.

REVIEW OF PERTINENT LITERATURE

Few references appear in the literature concerning the effects manifested in laboratory animals following the injec-

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tion of proteolytic enzymes. Jobling and associates[4] inoculated dogs by intravenous and subcutaneous routes with varying quantities of active and inactive purified trypsin. The intravenous injection of active and inactive trypsin in dogs produces a condition similar in many respects to anaphylactic and peptone shock. The state of trypsin shock which they produced was not fatal to any of the dogs inoculated.

Weil,[12] in his investigation of the similarity between peptone and anaphylactic shock, found it necessary to inject approximately 0.5 g of Whitte's peptone per kg of body weight in order to kill a dog. By injecting a 13-pound dog with 2 ml of a 10 percent solution of Whitte's peptone via the left branch of the portal vein, he produced localized hepatic congestion, incoagulability of the blood, and intense shock within 15 minutes. He concluded that peptone and anaphylactic shock affect the hepatic mechanism in an identical manner.

Northrop[5] subjected living and dead tadpoles and *Arbacia* eggs to proteolysis with papain, ficin, and trypsin of approximately the same proteolytic activity. Tadpoles remained alive and active in all three enzyme solutions from 12 to 24 hours. Dead tadpoles were partly digested at the end of 24 hours. The concentration of papain in solution was not stated. In the case of trypsin and ficin, three tadpoles were placed in 10 ml of solution containing from 0.1 to 1 mg of crystalline enzyme per ml. In experiments with *Arbacia* eggs, freshly fertilized eggs developed normally in both trypsin and ficin solutions containing 0.1 mg of enzyme per ml. In papain, the eggs developed but the cells became separated.

METHODS OF PROCEDURE

Various manufacturers of biological products were asked to furnish samples of suitable proteolytic enzymes. Twelve samples were received. Trypsin, bromelain, and ficin were supplied by General Bio-Chemicals, Inc. Crystalline papain was prepared by Jensen-Salsbery Laboratories. Chymotrypsin was purchased from the Nutritional Biochemical Corp. "Rhozyme P-11" and "Protease 15," fungal and bacterial proteases, respectively, were furnished by Rohn and Haas Co.

"Protease (trypsin)" was supplied by Takamine Laboratories. Lysozyme was prepared and furnished by Dr. William P. Switzer of the Veterinary Medical Research Institute, Iowa State College. "Proteinase A," a crude preparation produced from the filtrates of a culture of an aerobic soil saprophyte, and pancreatic desoxyribonuclease were manufactured and supplied by Sharp and Dohme. Streptokinase-streptodornase was supplied by Lederle Laboratories.

One-g quantities of trypsin, bromelain, ficin, chymotrypsin, "Rhozyme P-11," "Protease 15," "Protease (trypsin)," and lysozyme were individually weighed out. Ten percent solutions were prepared of each of these enzymes, employing a diluent composed of 1 part antibiotic solution and 9 parts physiological salt solution to make a total volume of 10 ml. From the initial 10 percent solution of each enzyme serial dilutions were prepared with the same diluent to contain 0.5, 0.25, 0.125, 0.1, 0.0625, 0.04687, 0.03125, and 0.01562 g of enzyme. Each dilution was mixed by flushing it 10 times with a separate, sterile 5-ml pipette.

With papain, three additional dilutions were prepared. One of these dilutions contained 0.00020, another 0.00010, and another 0.00005 g of papain per 10 ml of diluent.

Serial dilutions of "Proteinase A" were prepared in the same manner except that measurement was by units rather than by weight. Separate solutions containing 25,000; 18,750; 12,500; and 1,560 units of the enzyme per 10 ml of diluent were prepared.

Pancreatic desoxyribonuclease and streptokinase-streptodornase were suspended full strength, as supplied by the manufacturer, in 10 ml of diluent. No serial dilutions of these two products were prepared.

Each enzyme preparation, described above, was inoculated into 20 g, Swiss mice, S-P strain. Four mice were used per dilution. Each mouse received 0.03 ml of inoculum intracerebrally while under the influence of deep ether anesthesia. The inoculated mice were observed for a period of 14 days.

Mixtures of the antibiotic solution, which contained 12,500 units of penicillin G and 25 mg of streptomycin sulfate per

ml, and physiological salt solution were prepared in 1:1, 1:1.5, 1:2.75, 1:4, and 1:9 proportions. As controls, four 20-g Swiss mice per dilution were inoculated and observed concurrently with the animals undergoing the actual toxicity tests.

EXPERIMENTAL RESULTS

One hundred and ten groups of four mice each were inoculated intracerebrally with 0.03 ml of serial dilutions of various enzymes. Certain of these enzyme dilutions produced toxic reactions. Approximately 80 percent of the mice affected by the enzymes exhibited reactions characterized by the onset of generalized clonic spasms of the skeletal muscles within 3 to 5 minutes, which invariably progressed to tonic spasms and death within 7 to 10 minutes of inoculation. The remainder of the affected mice exhibited extreme dyspnea with rapid breathing within 5 to 10 minutes following inoculation. Many of these mice breathed through the mouth, and a bloody froth appeared at the nostrils. Within 1 to 2 hours clonic spasms of the skeletal muscles developed, followed in a few minutes by tonic spasms and death.

None of the mice which exhibited clinical symptoms survived. The pattern of the toxic reactions, whether immediate or gradual in onset, did not appear to be associated with any particular enzyme. Both forms occurred with each enzyme tested with the exception of "Protease 15," pancreatic desoxyribonuclease, and streptokinase-streptodornase.

From the results shown in table I, it is evident that "Protease 15" was the least toxic of the 12 enzymes tested. As much as 0.03 ml of a 10 percent solution of the enzyme could be inoculated intracerebrally without producing any toxic effects in mice. Next in order of toxicity was lysozyme which was toxic in concentrations greater than 0.25 g per 10 ml of diluent. Trypsin and "Protease (trypsin)" were of approximately equal toxicity. Concentrations greater than 0.125 g per 10 ml of either enzyme were toxic to inoculated mice. "Rhozyme P-11" was twice as toxic as trypsin and "Protease (trypsin)." Mice inoculated with concentrations greater than 0.06 g per 10 ml exhibited toxic reactions.

Trypsin, "Rhozyme P-11," "Protease 15," "Protease (trypsin)," and lysozyme were considerably less toxic to mice than bromelain, ficin, chymotrypsin, or papain. All four of the latter enzymes were toxic in concentrations as low as 0.015 g per 10 ml of diluent. Since concentrations of less than 0.015 g per 10 ml were not tested, with the exception of papain, it was not possible to ascertain the final end point of the toxicity range of each of these enzymes. In the case of papain, three additional dilutions were prepared, which are not shown in table I, containing 0.0002, 0.0001, and 0.00005 g per 10 ml of diluent. Each of these additional dilutions proved to be 100 percent fatal to inoculated mice.

TABLE I

Toxicity of serial dilutions of nine enzymes when inoculated intracerebrally into mice

Enzymes	Grams of enzyme in 10 ml of diluent								
	1.0	0.5	0.25	0.12	0.10	0.06	0.04	0.03	0.015
Trypsin	4*	4	4	0	0	0	0	0	0
Bromelain	4	4	4	4	4	4	4	4	4
Ficin	4	4	4	4	4	4	3	3	3
Chymotrypsin	4	4	4	4	4	4	4	4	4
Rhozyme P-11	4	4	4	4	3	0	0	0	0
Protease 15	0	0	0	0	0	0	0	0	0
Protease (trypsin)	4	4	3	0	0	0	0	0	0
Lysozyme	3	1	0	0	0	0	0	0	0
Papain	4	4	4	4	4	4	4	4	4

* Number of mice, out of four mice in each group, which died after exhibiting symptoms suggestive of anaphylaxis.

Three enzymes, which are not shown in table I, were also tested. These enzymes were prepared in serial dilutions of units of measurement which were based upon the manufacturers' assay. Two of these enzymes, pancreatic desoxyribonuclease and streptokinase-streptodornase, exhibited no

toxic activity when 100,000 units of each were suspended in 10-ml quantities of diluent and inoculated into mice in 0.03-ml dosages. The third enzyme was "Proteinase A." Three-tenths-ml quantities of serial dilutions in 10 ml of diluent were inoculated into mice. The 25,000-, 18,750-, and 12,500-unit dilutions killed all of the mice inoculated. The 6,250- and 3,125-unit dilutions were fatal to half of each group of four inoculated mice. The 2,500-unit dilution killed one out of four inoculated mice. A 1,560-unit dilution was nontoxic to inoculated mice.

From the results obtained in this investigation, it was possible to calculate the minimal quantity of each enzyme required to produce fatal toxic reactions in inoculated mice. The minimal lethal mouse dose of each enzyme tested appears in table II.

As can be seen by a comparison of the minimal lethal mouse dosages shown in table II, bromelain, ficin, chymotrypsin, and papain were extremely toxic as compared with the other enzymes. The minimal lethal mouse dose of each of these enzymes was less than 0.00003 g.

The antibiotic solution employed as a bacteriostatic agent with each enzyme dilution did not appear to be associated with the symptoms manifested by mice following inoculation of the enzyme preparations. Inoculation of a solution containing the same quantity of antibiotics as the enzyme preparations failed to produce any symptoms in mice. No toxic symptoms were manifested by mice which received concentrations of antibiotics two and three times as great as in the enzyme preparations.

DISCUSSION

Although no attempt was made to determine whether the inactivation of any of the enzymes employed in this investigation would lessen their toxic effects upon mice when inoculated intracerebrally, it is assumed that the inactivation of an enzyme's proteolytic activity would not necessarily remove the cause of its toxic properties for mice. Jobling and associates found that active and inactive trypsin, when injected

intravenously, were equally capable of producing toxic reactions in dogs.

All mice which evidenced toxic reactions following inoculation with serial dilutions of proteolytic enzymes presented clinical symptoms which, although they resembled severe

TABLE II

Minimal lethal mouse dose of 12 proteolytic enzymes

Enzymes	Quantity of enzyme per 0.03-ml dose	
	Grams	Units by mfg. assay
Proteinase A	—	7.5
Pancreatic desoxyribonuclease	—	300
Streptokinase-streptodornase	—	300
Trypsin	0.0008	
Bromelain	0.00004	
Ficin	0.00003	
Chymotrypsin	0.00004	
Rhozyme P-11	0.0002	
Protease 15	0.001	
Protease (trypsin)	0.0005	
Lysozyme	0.003	
Papain	0.0000001	

anaphylactic shock, could more correctly be designated as proteolytic enzyme shock. Jobling and associates describe similar symptoms which resembled anaphylaxis in cases of peptone shock in dogs. In his discussion of syndromes similar to the anaphylactic reaction, Godlowski[3] likened peptone and trypsin shock to anaphylactic shock. He drew the distinction that no sensitization period precedes peptone or trypsin shock. It is believed that the same distinction should apply to the results obtained with the various enzymes employed in this investigation. In no instance was there any known period of sensitization with the enzymes tested. It is difficult to conceive how there could have been any natural prior sensitization of

an animal such as a mouse to the enzymes employed in this study, particularly with such enzymes as ficin, bromelain, and papain.

The action of most proteolytic enzymes, and enzymes in general, is specific. This specificity exists, according to Bodansky,[1] between a given enzyme preparation and the general chemical structure of the substances which react in its presence, and extends to specific atomic groups or linkages in the molecule of the substrate. This factor would have a tendency to restrict various enzymes to definite ranges of proteolytic activity. For example, lysozyme,[6] pancreatic desoxyribonuclease,[9] and streptokinase-streptodornase[2] possess rather limited ranges of proteolytic activity.

From the results obtained in the enzyme toxicity tests it appears that there is some relationship between the specificity of a proteolytic enzyme as to the substrate on which it acts and its toxicity in mice. In general, those enzymes with a limited range of proteolytic activity, as cited above, were comparatively nontoxic when inoculated intracerebrally in mice in comparatively concentrated dosages. In the same respect, enzymes such as trypsin, "Protease (trypsin),"[11] "Proteinase A,"[10] and "Rhozyme P-11,"[7] with somewhat wider ranges of proteolytic activity, were toxic for mice in concentrated dosages. The degree of toxicity became progressively greater with those enzymes which possessed the widest range of proteolytic activity, with the exception of "Protease 15."[8]

CONCLUSION

The toxicity of 12 proteolytic enzymes was determined by the intracerebral inoculation of mice with serial dilutions of solutions of each enzyme. Four enzymes, lysozyme, pancreatic desoxyribonuclease, "Protease 15," and streptokinase-streptodornase, were reatively nontoxic to mice. Four enzymes, trypsin, "Rhozyme P-11," "Protease (trypsin)," and "Proteinase A," were demonstrated to be toxic for mice when inoculated in high concentrations but relatively nontoxic when inoculated in low concentrations. Four enzymes, bromelain, ficin, chymotrypsin, and papain, were found to be extremely toxic to mice even in very low concentrations.

may also occur when pancreas is kept for a period in a medium of slight acidity. Trypsin hydrolyzes proteins at peptide linkages involving the carboxyl groups of arginine and lysine. The chymotrypsins are most active on peptide bonds involving phenylalanine, tyrosine, and tryptophan; the actions of the enzymes are additive, resulting in more complete degradation of proteins to small peptides. Carboxypeptidase A liberates carboxyl-terminal amino acid residues, while carboxypeptidase B acts on peptides having terminal arginine or lysine residues.

Pancreatic amylase hydrolyzes starches; while it resembles ptyalin in some respects, it differs from it in its ability to digest even uncooked starch grains.

Steapsin, also called pancreatic lipase, is the fat-splitting enzyme of pancreatic juice. It exists there in an inactive form, called *prolipase*; in the intestine it is activated by some means as yet not fully explained while *in vitro* it may be activated by boiled extracts of pancreas, by blood serum, or even by lead salts.

The official pancreatin contains all of these enzymes, with more or less extraneous matter.

Pancreatin is variously prepared, as by extracting minced pancreas with 25 percent alcohol and evaporating the alcohol. Preparation of pancreatin with a higher digestive power than officially required is described by Bauer and Vazakas (*J.A.Ph.A.*, **40**, 552, 1951).

Uses.—Pancreatin was first introduced into medicine as an emulsifying agent, especially for cod liver oil. It is no longer used for this purpose but rather for possible enzymic action in various digestive disturbances. However, most of it is destroyed in the stomach and even when enteric-coated, to ensure passage into the intestine, it is doubtful whether it significantly augments intestinal digestion. In cases of pancreatic achylia, Beazell *et al.* (*J.A.M.A.*, **116**, 2735, 1941) employed 4 Gm. before and 4 Gm. after each meal in the form of enteric-coated tablets; this dose represents about 15 percent of the normal daily secretion of pancreatic juice. It is unlikely that pancreatin would be useful in any other conditions than those due to deficient pancreatic output, as pancreatitis and cystic fibrosis.

Pancrelipase is a standardized preparation or whole hog pancreas containing amylase, trypsin, and lipase but with lipase activity much greater than in other pancreatic enzyme preparations. It is recommended to help control steatorrhea, specifically as replacement therapy in chronic pancreatitis, obstruction caused by cancer of the pancreas, in cystic fibrosis, and after pancreatic resection of gastrectomy. It is used in doses of 300 to 900 mg. with each meal and 300 mg. with food eaten between meals. Pancrelipase is available, under the trademark *Cotazym* (Organon), in capsules containing 300 mg.

Pancreatin has been used to predigest certain foods, such as milk; this was done by adding to a pint of milk about 5 grains of pancreatin and 20 grains of sodium bicarbonate in a fluidounce of warm water and maintaining the temperature at 110° F. for 20 to 60 minutes. Nutritive enemata have been similarly pancreatinized.

Dose.—The usual dose of N.F. Pancreatin is 500 mg. to 1 Gm. with meals.

Papain

"Papain is the dried and purified latex of the fruit of *Carica Papaya* Linné (Fam. *Caricaceae*). Papain possesses a digestive activity not less than that of the Reference Papain." *N.F. VIII.*

Papain is obtained commercially by slashing the fruits of *C. Papaya*, collecting the latex and allowing it to evaporate until a granular residue is obtained; this is the crude papain of commerce. Purification is effected by solution of the crude papain in water and precipitation with alcohol, followed by low-temperature drying of the precipitate.

Description.—Light brownish gray to weak reddish brown granules or yellowish gray to weak yellow powder with a characteristic odor and taste. Partially soluble in water, the solution being more or less opalescent or hazy; nearly insoluble in alcohol, in chloroform, and in ether.

A method for crystallizing papain having a molecular weight of about 20,000 has been described by Kimmel and Smith (*J. Biol. Chem.*, **207**, 515, 1954); the substance is a protein noteworthy for absence of methionine.

Actions and Uses.—Papain has long been known to have protein-digesting power. It is said to have been used by the Indians to tenderize meat by wrapping it in fresh leaves of the papaya plant. It is the active ingredient of present-day meat tenderizers. In the brewing industry it is used to prepare "chillproof" beer through its proteolytic action on proteins that would otherwise precipitate on cooling.

It was long popular as a digestant for protein foods. Other uses in medicine have been as a topical application for the treatment of sloughing wounds, carbuncles, and chronic purulent otitis media; also to dissolve the eschar of burns, to loosen the exudate in membranous tracheobronchitis, and to dissolve the membrane of diphtheria. It has been used also by intraperitoneal instillation for prevention of postoperative adhesions.

A preparation of proteolytic enzymes from *Carica papaya*, designated *Proteolytic Enzymes from Carica Papaya*, and available under the trademark *Papase* (Warner-Chilcott) was proposed for use for the reduction of soft tissue inflammation and edema associated with traumatic injury (contusions, sprains, ecchymoses, hematomas), localized inflammations (abscesses, varicose ulcers), induced trauma (dental, obstetric, and general surgical procedures), and for liquefaction of mucoid secretions of the upper respiratory tract (asthma, bronchitis, general pulmonary diseases). The postulated rationale for use of this enzyme preparation is that it has a depolymerizing action on soft fibrin deposits in inflamed areas, by virtue of which reduction of inflammation, drainage of fluids, and resolving of cellular debris occur to facilitate tissue repair. Clinical evaluations, which depend on subjective findings or impressions because of the impracticality of establishing well-controlled studies, are variable. Based on a review of Papase by the National Academy of Sciences–National Research Council and/or other information, FDA has classified the indications as follows: "Possibly" effective for relieving symptomatology related to episiotomy. Final

classification of the less-than-effective indication requires further investigation. Papase, which is standardized by a milk-clotting procedure, is administered buccally or orally in prophylactic doses of 10,000 or 20,000 units (1 or 2 tablets) an hour or two before a traumatic procedure such as surgery, or for treatment in doses of 20,000 units (2 tablets) 4 times a day for at least 5 days.

Contraindications.—Papase is contraindicated in patients being treated with anticoagulants, also in patients with generalized or systemic infections or blood coagulation disorders.

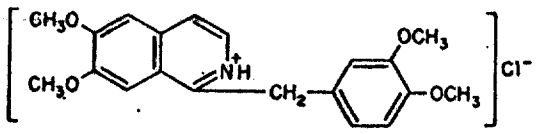
Untoward Effects.—Papain has caused allergy, probably as a result of inhalation of powder, manifested by severe paroxysmal cough, vasomotor rhinitis, and dyspnea.

Nausea, vomiting, diarrhea, dizziness, pruritus, rash, and urticaria have been reported from oral or buccal use of proteolytic enzymes from *Carica papaya;* a local tingling sensation has been noted in connection with buccal use of the tablet. It is advised that this preparation not be used simultaneously with anticoagulants; also, that it not be used in patients with generalized or systemic infections or disorders of blood coagulation.

Dose.—Papain has been used as a protein digestant in doses of 100 to 600 mg. For doses of proteolytic enzymes from *Carica papaya* see above.

Papaverine Hydrochloride

Papaverine hydrochloride is 6,7-dimethoxy-1-veratrylisoquinoline hydrochloride and contains, on the dry basis, not less than 98.0 percent of $C_{20}H_{21}NO_4.HCl$ (375.86).



Papaverine was discovered as an alkaloid of opium by Merck, in 1848; it occurs in the drug to the extent of about 1 percent. It may be obtained from opium in the solution remaining after separating morphine and codeine: Papaverine, a tetramethoxy-1-benzylisoquinoline, may be synthesized by several methods, one procedure utilizing *o*-dimethoxybenzene (veratrole) as the starting compound (Pictet and Gams, *Compt. rend. acad. sc.*, **149**, 210, 1909); it may also be synthesized from vanillin.

Description.—White crystals or white, crystalline powder. One Gm. dissolves in about 30 ml. of water and in 120 ml. of alcohol. Soluble in chloroform; practically insoluble in ether.

Actions and Uses. Papaverine, a member of the benzylisoquinoline group of opium alkaloids, lacks the narcotic properties of morphine; its chief effect is to diminish smooth muscle spasm.

Papaverine is completely absorbed from the gastrointestinal tract and undergoes almost complete conversion to glucuronide conjugates of phenolic metabolites, principally 4′-hydroxypapaverine; these are excreted in the urine (Axelrod *et al.*, *J. Pharmacol.*, **124**, 9, 1958). The principal metabolic site is the liver. Papaverine has a biologic half-life of about 100 minutes.

Papaverine relaxes the smooth muscle of the intestinal and biliary tracts, bronchial tree, ureter and blood vessels, including the coronary supply. In the vasculature it acts particularly where there is spasm. Jayne *et al.* (*J. Clin. Inv.*, **31**, 111, 1952) demonstrated that papaverine hydrochloride administered intravenously in doses of 200 mg. produced an increase of 13 percent in cerebral blood flow. It is believed that it increases cerebral blood flow and decreases cerebral vascular resistance by a direct vasodilating effect on cerebral vessels. With intravenous doses of 100 mg., McHenry *et al.* (*New Eng. J. Med.*, **282**, 1167, 1970) observed an 18 percent increase in mean cerebral blood flow in patients with focal cerebral vascular disease; there was no evidence, however, that papaverine alters or influences the clinical course of patients with stroke. Although Gross and Slaughter (*J. Pharmacol.*, **43**, 551, 1931) stated that the action on the intestines is too feeble to be of any significance, the experimental findings of Mulinos *et al.* (*J. Pharmacol.*, **66**, 27, 1939) and the clinical results of Bargen and Jackman (*Surg. Gynec. Obst.*, **68**, 749, 1939) indicated otherwise. It has been frequently prescribed in various forms of gastric or intestinal spasms, asthma, biliary colic, and other conditions of excitability of involuntary muscles. Papaverine has been used successfully in treatment of pyloric spasm in newborn and young nursing infants; it is of no avail if there is hypertrophic stenosis of the pylorus.

Much interest has centered on the effects of papaverine as an arterial dilator. McCall *et al.* (*Am. J. Obst. Gyn.*, **61**, 393, 1951) contributed a significant study on the effects of the drug on cerebral blood flow and related metabolic functions of the brain in women with preeclampsia and hypertensive toxemia of pregnancy. They reported significant lowering of mean arterial pressure, rapid decrease of cerebral vascular resistance to normal, increase of cerebral blood flow, and increased oxygen utilization by the brain, suggesting that it may be a valuable adjunct in therapy of toxemia of pregnancy. Russek and Zohman (*J.A.M.A.*, **136**, 930, 1948) found that it reduces severity and frequency of seizures of patients with cerebral angiospasm. Combination with phenobarbital permits of smaller dosage than when papaverine is used alone.

Papaverine has had wide popularity in therapy of coronary artery disease. Gray *et al.* (*New Eng. J. Med.*, **232**, 389, 1945), however, found no change in a standard exercise-tolerance test in patients with angina pectoris who had taken as much as 200 mg. of papaverine orally 4 times daily for a week. Larger doses are impractical because of gastric distress. Intravenous administration produces a more favorable response, but the effect is limited to about 1 hour. Studies by Simon *et al.* (*J. Lab. Clin. Med.*, **34**, 992, 1949) confirmed its ineffectiveness in this disorder; some of their patients exhibited constipation and drowsiness. Nyman (*Acta med. Scandinav.*, **131**, 565, 1948) found papaverine inferior to aminophylline as a coronary dilator. Russek *et al.* (*J.A.M.A.*, **153** 207, 1953) compared the ability of various drugs to modify the electrocardiogram

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Taste receptors and sialadenotrophic action of proteolytic enzymes in rats[1]

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HERBERT, ANGELA A. V. PERONACE, AND LAWRENCE W. STARK. The sialadenotrophic action of proteolytic enzymes. Am. J. Physiol. 208(5): 877–881. 1965.—Administration of proteolytic enzymes such as chymotrypsin, bacterial protease, or papain resulted in a increase in the weight of the submandibular salivary glands. Sympathectomy, but not parasympathectomy, of the glands to papain was partially reduced. After autonomic denervation the papain effect was complete. The enzymes retained their sialadenotrophic when administered as intra-oral drops, but were not administered by stomach tube. Although the papain was completely absent after section of the lingual nerves, section of the lingual nerves did not the response. The sialadenotrophic effect of repeated of lower incisor teeth was not altered by prior the glossopharyngeal or lingual nerves. The results that the sialadenotrophic action of proteolytic enzymes on a neural reflex arc. The afferent arm consists of receptors innervated by the glossopharyngeal nerves, the efferent arm is composed of both branches of the nervous supply to the glands.

submandibular salivary glands — lingual nerves
sympathectomy — hypertrophy — gustatory stimuli
glossopharyngeal nerves — incisor amputations
parasympathectomy

ADMINISTRATION OF A MIXTURE of pancreatic proteolytic enzymes, pancreatin, to the diet of rats results in a increase in the size and weight of the submandibular salivary glands. Histological examination that the increase in the weight of the glands is hypertrophy of the acinar cells (2, 3). The primary of pancreatin is the oral cavity (9) and it for a full expression of its sialadenotrophic the intact autonomic nervous supply to the

Received for publication 5 November 1964.
This work was supported, in part, by the National Institute of Health and Human Development (Research Grant and, in part, by the National Institute for Dental Research (Research Grant).
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The experiments summarized in the present report were designed to investigate further the mechanism of this effect of proteolytic enzymes. The results confirm that a wide variety of proteolytic enzymes are sialadenotrophic agents; that they depend for a full expression of this effect on both branches of the autonomic nervous system; and that the primary locus of action of these substances is on structures within the oral cavity, primarily the taste receptors.

METHODS

The rats were males (60–90 days of age) obtained from the Holtzman Company, Madison, Wisconsin. All rats in each experiment had been born on the same day, and they were assigned to groups at random. The regular diet consisted of Purina laboratory meal and water ad lib.

The following proteolytic enzymes were obtained from California Biochemical Corporation, Los Angeles, California: alpha chymotrypsin (2 × cryst.), trypsin (1 × cryst.), bacterial protease (2 × cryst.), and papain powder (crude). Alpha chymotrypsin inactivated with diisopropylfluorophosphate and a comparable preparation of active alpha chymotrypsin were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. For inclusion in the diet the enzymes were powdered and thoroughly mixed with Purina laboratory meal. Additional technical details as well as a description of the design of the various experiments are given under RESULTS.

The standard errors in the tables and figures were calculated from the residual error term of the analysis of variance except where the variances were heterogeneous by Bartlett's test. In such cases the standard errors were based on the distribution of values in each group separately.

RESULTS

The results of four separate experiments, performed on different days, are presented in Table 1. In the first three experiments an active proteolytic enzyme was added to the diet and fed for 3 or 4 days. Control rats ate the normal diet, and water was available ad lib. for all groups. In *experiment 4*, alpha chymotrypsin inac-

TABLE 1. *Effect of dietary proteolytic enzymes on submandibular gland weight*

Treatment	Body Initial Wt, g	Final Body Wt, g	Submandibular Gland Wt, mg
Exp. 1			
Normal diet (4)	152	157	450 ± 10
Alpha chymotrypsin, 0.2% (4)	149	153	655 ± 18
Trypsin, 0.4% (4)	150	148	615 ± 5
Exp. 2			
Normal diet (5)	152	150	433 ± 14
Bacterial protease, 0.1% (5)	150	156	5[illegible] ± [illegible]
Exp. 3			
Normal diet (5)	205	[illegible]	5[illegible] ± [illegible]
Papain, 2.0% (5)	204	[illegible]	704 ± [illegible]
Exp. 4			
Normal diet (4)	148	157	500 ± [illegible]
Alpha chymotrypsin, 0.4% (4)	151	164	600 ± [illegible]
Alpha chymotrypsin-DFP 0.4% (4)	149	163	6[illegible] ± [illegible]

Values are means ± SE. Numbers in parentheses = number of rats. Duration of experiments: *Exp. 1* was 4 days, *Exp. 2*, 3, and 4 were 3 days. DFP = diisopropylfluorophosphate. Normal diet = Purina laboratory meal.

tivated with diisopropylfluorophosphate was a dietary additive. At the end of an experiment the rats were killed with an overdose of ether and the submaxillary and major sublingual glands were removed and weighed together on a torsion balance as the "submandibular" glands. The results indicate that, in confirmation of Ershoff and Bajwa (2), the addition to the diet of alpha chymotrypsin, trypsin, bacterial protease, or papain resulted in a significant ($P < .01$) increase in the weight of the submandibular glands. In the case of trypsin and papain the increase in the weight of the glands occurred despite a loss in over-all body weight.

Since the principal characteristic which all of these enzymes have in common is their ability to hydrolyze proteins, it seemed likely that their effect on salivary glands was related to this common proteolytic action rather than to some other characteristic. This possibility was examined by testing the effect of alpha chymotrypsin inactivated by diisopropylfluorophosphate. Both the active and inactive forms of the enzyme were mixed separately with ground chow at a concentration of 0.4% by weight and fed for 3 days. The results, in Table 1, indicate that feeding this preparation of active chymotrypsin resulted in a significant ($P < .01$) increase in the weight of the glands, while feeding the inactivated enzyme had no effect on gland weight. Thus, the proteolytic center of this enzyme, which is blocked by diisopropylfluorophosphate, appears to be necessary for its sialadenotrophic action.

To determine whether changing the conditions of administration of the enzymes would alter their effect on the weight of the glands, two of the enzymes, alpha chymotrypsin and papain, were administered as intra-oral drops instead of as additives to the diet. The alpha chymotrypsin was dissolved in distilled water at a concentration of 250 mg/ml. Twice a day, 0.20 ml of this

TABLE 2. *Effect on submandibular gland weight of intra-oral alpha chymotrypsin and papain in the absence of food, and of papain administered by stomach tube*

Treatment	Initial Body Wt, g	Final Body Wt, g	Submandibular Gland Wt, mg
Exp. 1			
Water; food at night (4)	170	191	455 ± 9
Alpha chymotrypsin, 100 mg/day; food at night (4)	172	189	595 ± [illegible]
Exp. 2			
Water, food at night (10)	241	257	619 ± [illegible]
Papain, 400 mg/day; food at night (10)	243	262	7[illegible] ± [illegible]
Exp. 3			
Normal diet (6)	133	153	400 ± [illegible]
Normal diet, water by stomach tube (6)	136	151	3[illegible] ± [illegible]
Normal diet; papain by stomach tube, 400 mg/day (6)	135	153	410 ± 4
Normal diet; papain as intra-oral drops, 400 mg/day (6)	134	150	573 ± [illegible]

Values are means ± SE. Numbers in parentheses = number of rats. Duration of experiments was 3 days.

solution was administered by medicine dropper directly into the oral cavity of the rats. Each rat thus received a total dose of 100 mg/day for 3 days. Control rats each received two applications a day for 3 days of 0.20 ml of distilled water. The diet for both groups consisted of Purina meal. To insure that there was no food present when the enzyme was administered, the rats were allowed access to food only at night. On each day, therefore, the first dose of enzyme was administered in the morning (11 AM) 2 hr after the food had been removed from the cage and the second dose was administered at 4 PM, 2 hr before the feeding bowls were placed back in the cages. Free access to tap water was allowed at all times. In a second experiment the same procedure was followed except that papain instead of alpha chymotrypsin was administered. The papain powder was suspended in distilled water and each rat received 400 mg/day in two doses of 200 mg each. In both experiments, fresh aqueous enzyme preparations were made for each application. The results of both experiments, in Table 2, indicate that administration of these enzymes as intra-oral drops resulted in a significant ($P < .01$) increase in the weight of the submandibular glands, and that the enzymes retained their sialadenotropic action when administered at a time when food was not being ingested.

We have shown previously that the effect of pancreatin on the weight of the glands is lost when the material is administered by stomach tube instead of by addition to the diet (9). It was of interest to know whether this was also true for papain, a non-pancreatic proteolytic enzyme. For this purpose, a suspension of papain in distilled water was prepared and administered by stomach tube two 0.20-ml portions/day containing 200 mg papain each, for a daily total of 400 mg. Control rats received two 0.20-ml portions of distilled water. For comparison, another group of rats received 400 mg/day of the same papain suspension as

TABLE 3 *Effect of dietary papain on submandibular gland weight in intact, complete autonomic denervation*

Treatment	Final Body Wt.	Submandibular Gland Wt. mg		Diff. Between Glands
	g	Left	Right	mg
[illegible]	187	247 ± 11	253 ± 17	14 ± 8
	183	209 ± 4	217 ± 4	8 ± 4
	181	161 ± 5	251 ± 9	90 ± 8
P × (6)	182	165 ± 6	243 ± 9	80 ± 7
[illegible]				
[illegible] operations (6)	165	422 ± 24	429 ± 20	7 ± 8
	164	282 ± 28	434 ± 19	152 ± 20
	161	367 ± 37	459 ± 31	83 ± 28
P × (6)	155	166 ± 22	461 ± 14	295 ± 30

[illegible] are means ± SE. Snx = sympathectomy. Pnx = parasympathectomy. Mean initial body weight of groups was [illegible]. Operations on left side. Numbers in parentheses = [illegible] rats.

[illegible] drops in two doses of 200 mg each. Free [illegible] food was allowed for the duration of the experiment, which was 3 days. The results, in Table 3, *experiment* [illegible], indicate that the papain preparation with a [illegible] ($P < .01$) effect on the weight of the glands, [illegible] administered as intra-oral drops, was not effective [illegible] administered by stomach tube. These results [illegible] that, in order to produce an effect on gland [illegible], papain, like pancreatin, must come in contact [illegible] the oral cavity.

Previous studies (8) have indicated that the sialadenotrophic action of pancreatin depends on an intact autonomic nervous supply to the glands. In order to [illegible] further the sialadenotrophic actions of pancreatin and a nonpancreatic proteolytic enzyme, papain [illegible] added to the diet of rats with the autonomic innervation of the submandibular glands intact, partially [illegible], or completely denervated. For this purpose [illegible] submandibular glands of groups of rats were [illegible]ectomized, parasympathectomized, or both. [illegible] surgical techniques for sympathectomy (extirpation [illegible] superior cervical ganglion) and for preganglionic [illegible] sympathectomy (section of the chorda tympani [illegible]) have been described previously (6, 8). After the [illegible], including the appropriate sham operations, [illegible] completed, some of the rats were fed ground [illegible] alone or a ground chow diet to which papain had [illegible] added at a concentration of 1% by weight. The [illegible] and experimental diets were fed for 5 days, after [illegible] the rats were autopsied and the right and left [illegible]bular glands were removed and weighed [illegible]. The results in Table 3 show, in confirmation [illegible] results (8), that preganglionic parasympa[illegible] denervation resulted in a significant ($P < .01$) [illegible] in the weight of the ipsilateral gland. Post[illegible] sympathectomy alone had only a slight effect, [illegible] on ipsilateral gland weight. Complete denerva[illegible] greater an effect on the weight of the ipsi[illegible] gland than parasympathectomy alone. The

TABLE 4 *Effect of papain on submandibular gland weight after denervation of the taste receptors*

Treatment	Final Body Wt.	Submandibular Gland Wt.
	g	mg
Exp. 1		
Intra-oral water		
Sham operated (6)	231	690 ± 3
Glx (6)	226	602 ± 17
Intra-oral papain		
Sham operated (6)	231	708 ± 32
Glx (6)	227	663 ± 24
Exp. 2		
Intra-oral water		
Sham operated (7)	190	517 ± 8
Glx (7)	187	483 ± 6
Intra-oral papain		
Sham operated (7)	201	790 ± 14
Glx (7)	188	520 ± 2
Exp. 3 and *4*		
Intra-oral water		
Sham operated (11)	184	518 ± 11
Lx (12)	178	475 ± 21
Intra-oral papain		
Sham operated (11)	181	611 ± 18
Lx (12)	181	643 ± 22

Values are means ± SE. Numbers in parentheses = number of rats. Glx = bilateral section of glossopharyngeal nerves. Lx = bilateral section of lingual nerves. Mean initial body wt of the groups: *exp. 1* = 179 g; *exp. 2* = 179 g; *exp. 3* and *4* = 177 g.

addition of papain to the diet resulted in a marked increase in the weight of both glands. After unilateral sympathectomy the response of the ipsilateral gland to papain was reduced markedly. The parasympathectomized gland, on the other hand, still retained most of its response to papain. After complete denervation, however, the response to papain was absent completely. Therefore, the maximal sialadenotrophic action of papain, like that of pancreatin, can occur only if both autonomic branches are intact.

The results indicating that, to be effective sialadenotrophic agents, proteolytic enzymes must come in contact with the oral cavity, suggested that the primary receptors for this response must be within the oral cavity. One possibility was that the taste receptors could be the sensitive structures. To test this hypothesis, papain in suspension was administered to rats in which the glossopharyngeal or lingual nerves had been sectioned bilaterally. Details of the surgical procedure developed by one of us (J.H.P.) for section of the glossopharyngeal nerves will be supplied on request.

The results of administering papain after denervation of the taste receptors are shown in Table 4. In *experiment 1*, papain administration was not begun until 1 week had elapsed after bilateral section of the glossopharyngeal nerves. In *experiment 2*, enzyme administration commenced 24 hr after the surgery. In both experiments the dose of papain for each rat was 200 mg given twice a day (at approximately 10:00 AM and 4:00 PM), as intra-oral drops, for a total dose of 400 mg/rat per day. The duration of papain administration in both experiments was 3 days. The results indicate that, by itself, bilateral section of the glossopharyngeal nerves had no

TABLE 5. *Effect of incisor amputations on submandibular gland weight after denervation of the taste receptors*

Treatment	Final Body Wt, g	Submandibular Gland[1] Wt, mg
Exp. 1		
Sham operation (4)	226	587 ± 23
Glx (4)	218	574 ± 20
Sham operations, incisors amputated (6)	225	706 ± 24
Glx, incisors amputated (5)	216	707 ± 18
Exp. 2		
Sham operation (5)	200	[illegible]
Glx (6)	2[illegible]	[illegible]
Sham operation, incisors amputated (5)	[illegible]	[illegible]
Glx, incisors amputated (4)	2[illegible]	[illegible]
Exp. 3		
Sham operation (6)	222	541 ± 10
Lx (6)	208	4[illegible] ± 20
Sham operations, incisors amputated (6)	2[illegible]	752 ± [illegible]
Lx, incisors amputated (6)	[illegible]	767 ± 38

Values are means ± SE. Glx = bilateral section of glossopharyngeal nerves. Lx = bilateral section of lingual nerves. Incisors amputated 5 times in 8 days. Mean initial body wt of the groups: *Exp. 1* = 172 g, *Exp. 2* = 203 g, *Exp. 3* = 178 g. Numbers in parentheses = number of rats.

significant effect on the weight of the submandibular glands. After section of the nerves, however, the usual increase in the weight of the glands which follows the intra-oral administration of papain was completely absent.

This table also shows the results of two experiments in which the lingual nerves had been sectioned bilaterally without apparent damage to the efferent part of the chorda tympani nerves. The surgical procedure was generally the same as that described previously for section of the combined chorda tympani and lingual nerves (8). After the nerves had been identified with a dissecting microscope, the lingual nerve was sectioned between the point where the chorda branches to the submandibular gland and the lingual nerve enters the tongue. This technique allows the afferent fibers from the tongue to be cut without damage to the efferent parasympathetic nerves to the submandibular glands. The results of two experiments (*Exp.* 2 and 3) are combined for presentation in Table 4. In both experiments papain administration was begun after completion of the surgery and lasted for 3 days. The daily dose of papain was 400 mg/rat given as two doses of 200 mg each. The results indicate that, by itself, bilateral section of the lingual nerves had no significant effect on the weight of the glands. Prior section of the lingual nerves also had no significant effect on the response of the glands to papain.

A pronounced increase in the weight of the submandibular salivary glands also follows repeated amputation of the erupted portion of the lower incisor teeth (7, 10). Histological examination of the enlarged glands indicates that the increase in gland weight is due to hypertrophy of the acinar cells (4). This is the same structural alteration observed in glands enlarged following the addition of pancreatin to the diet (3). The evidence, to date, indicates that the enlargement of the glands following amputation of the incisor teeth depends on a neural reflex arc (9). Since the results of the experiments reported above indicated that the receptors in the oral cavity involved in the response to proteolytic enzymes are taste receptors, and the oral receptors involved in the response to incisor amputation are still unknown, it was of interest to determine whether prior denervation of the taste buds would affect the response of the submandibular glands to amputation of the teeth. For this purpose the incisor teeth were amputated in rats in which either the glossopharyngeal or lingual nerves had been sectioned bilaterally. The technique for amputation of the teeth has been described previously (10). The procedure was begun immediately after completion of the neurosurgery, and the teeth were amputated at 2-day intervals for a total of five times in 8 days. The rats were autopsied 48 hr after the last amputation. The results, in Table 5, of three separate experiments indicate that prior section of neither the glossopharyngeal nor lingual nerves had a significant effect on the increase in the weight of the glands which follows amputation of the incisor teeth.

DISCUSSION

In a previous report (9) it was suggested that there was, in pancreatin, some specific sialadenotrophic factor other than chymotrypsin and trypsin. The results of the present studies, however, confirm Ershoff and Berg (2) and indicate strongly that the increase in the weight of the submandibular salivary glands which follows ingestion of a diet containing pancreatin is attributable to the large amounts of chymotrypsin and trypsin contained in pancreatin.

Because the only obvious feature which all the enzymes possess in common is their proteolytic action, it seems likely that this is the critical factor determining their effectiveness as sialadenotrophic agents. The lack of effect of alpha chymotrypsin when the active serine center is inactivated by diisopropylfluorophosphate is further indication that proteolytic activity is associated with the property that enables these enzymes to cause the salivary glands to undergo hypertrophy.

Previous studies and the present results make it certain that the site of action of these substances is the oral cavity. They are effective only if they come in direct contact with the oral cavity. The experiments showing that the enzymes retain their activity when administered separately from the food indicate that probably the enzymes per se which are the active agents and not some other substance which results from the action of the enzymes on the food. We have yet, however, ruled out the possibility that an intermediate product, formed from the action of the enzymes on salivary proteins or oral bacteria, is the active agent. This possibility is currently under investigation.

The results indicating that denervation of some taste buds, by section of both of the glossopharyngeal nerves, will completely abolish the response of the

enzymes provides strong evidence that these [illegible] play an important role in the response. According to Pfaffman [illegible] the glossopharyngeal nerves [illegible] the taste buds on the posterior third of the [illegible] the anterior two-thirds of the tongue [illegible] innervation from a branch of the chorda [illegible] the lingual nerves. In man, moreover, [illegible] regions of the tongue are especially sensitive [illegible] taste components, with the posterior [illegible] particularly responsive to substances with a bitter [illegible] In man to rat, section of the glossopharyngeal nerves may deprive the rat of the ability [illegible] to react to substances with a strong, bitter taste. This would suggest that proteolytic enzymes [illegible] strong and bitter taste stimuli, and the [illegible] the authors, who have sampled some of [illegible], would bear this out. The fact that prior denervation of the taste receptors on the anterior two-thirds of the tongue does not interfere with the response [illegible] to the proteolytic enzymes emphasizes the specificity of action of the enzymes and adds further [illegible] these enzymes may possess a bitter taste [illegible]. Some preliminary results suggest, however, [illegible] taste alone is not sufficient to cause enlargement [illegible] the glands. When urea, a substance with a strong bitter taste, was administered in solution as intraoral [illegible] such that each rat received 2 doses/day of 200 mg [illegible] for 5 days, there was no significant effect on the [illegible] of the glands. Although this dose of urea may [illegible] been of insufficient strength to produce an effect [illegible] the weight of the glands, it did result in a fairly copious secretion of saliva. It was also perceived by the authors as having an intensely bitter taste. Possibly, then, the [illegible] of the enzymes is not strictly dependent on [illegible] bitter stimuli, and the exact mechanism of their [illegible] requires further investigation.

[illegible] present working hypothesis to explain the sialadenotrophic action of proteolytic enzymes is as follows. [illegible] enzymes cause hypertrophy of the acinar cells of the salivary glands by [illegible] which the afferent arm is the taste receptors and the innervation. The efferent arm of the reflex is the [illegible] nervous supply to the glands. Stimulation of the taste receptors by the enzymes results in an increased nervous stimulation of the glands by both the sympathetic and parasympathetic branches, resulting in hypertrophy of the acinar cells and an increase in the weight of the glands.

The response to incisor amputation also involves a nervous reflex arc. The afferent arm of the reflex, however, still remains to be elucidated. The results of the present experiments indicate that the taste receptors are not involved in the response to amputation. The fact that denervation of the taste receptors does not, by itself, have a significant effect on the weight or rate of growth of the glands indicates that other sensory conditions arising in receptors probably do not contribute much of the sensory input needed for maintenance of the normal weight of the glands.

Although it is well known that various substances stimuli are capable of eliciting copious salivary secretion, the only recent attempt to modify the weight of the salivary glands by means of taste stimuli is that of Cox et al. [illegible]. They added a variety of weak acids in low concentration (0.05 M) to the drinking water of rats for 28 days and found a significant increase in the gland weight-to-body weight ratio. The effect on gland weight observed by Cox et al. was quite small in comparison to that which follows even a brief period of proteolytic enzyme administration. However, because of the [illegible] difference in the concentration at which the acids and enzymes were tested, it is not possible on the basis of present data to draw a valid conclusion regarding the relative sialadenotrophic potency of these substances.

The authors are indebted to Weldon Lloyd and Patrick Gray for their excellent technical assistance. Elizabeth M[illegible] for [illegible] the statistical analyses.

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THE *IN VIVO* PROTEOLYTIC ACTIVITY OF PAPAIN AS DEMONSTRATED BY NITROGEN BALANCE STUDIES IN DOGS

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In earlier papers[1,2] in this series, it was shown that activity of orally ingested papain could be demonstrated *in vivo* in rats. This disproved earlier suggestions[3,4] that papain could not be expected to withstand gastric acidity and peptic digestion. To determine, further, how readily papain activity *in vivo* could be demonstrated in other animals, dog-feeding studies were instituted in which the effect of adding papain to soy bean meal diets was followed by nitrogen balance determinations. The results of these studies are given in this paper.

Experimental

Dogs. The adult mongrel dogs selected, some of whom had been used satisfactorily in previous feeding experiments, were of moderate size and parasite free. Their quarters were air conditioned and maintained at constant temperature. For these studies, the dogs were housed in metabolism cages which had sheet metal sides and wide mesh screen bottoms. There were three urine collection periods weekly, two 2-day samples during the week and a 3-day sample over the weekend. The completeness of urine collections was verified for each collection period by creatinine determinations. Nitrogen analyses were made by the macro-Kjeldahl procedure with a copper sulfate catalyst.

As in a previous publication from this laboratory,[5] the daily caloric needs of the dogs were based on the optimum body weight at a nutritive state of 0.3. This weight was estimated for each dog by the Cowgill and Drabkin[6] formula $W = (0.3L)^3$, where W = weight in kilograms and L = length in centimeters. The caloric allotments were subsequently fixed between 60 and 80 calories per kilogram for each dog, to minimize weight fluctuations. The experimental findings, however, have been expressed in terms of the actual weights of the animals rather than upon their calculated optimum weights.

Papain. The dried papain used in these studies was prepared from papaya latex.† This "active" papain contained approximately 460 milk-clotting units[7] per gram. The control diets containing an "inactive" papain were made using a dried, autoclaved (120°C. for 30 minutes) sample which no longer possessed any proteolytic activity. The papains were added to the diets in the amount of one per cent of the protein content of the ration. This constituted roughly 0.2 per cent of the diet or 250–400 mg. of papain powder per dog per day, dependent upon the weights of the dogs.

Soy Bean Meal. Unheated soy bean meal was selected as the protein

* The technical assistance of Daniel J. Bradley, Jr. is gratefully acknowledged.
† The papain used in these studies was supplied through the courtesy of E. T. Hinkel, Jr.

source because it is known to be incompletely digested. Two samples of soy bean meal were fed during the course of these studies. The first had been prepared earlier in this laboratory by ether extracting ground raw soy beans at room temperature. This sample contained 6.66 per cent nitrogen. The other was represented by the manufacturer to be unheated, defatted soy bean grits.* It contained 8.48 per cent nitrogen.

Procedure. The studies consisted of a comparison of the amounts of soy bean protein digested by dogs given active or inactive papain in the diet. The metabolic nitrogen values required for calculating percentage digestibility were based on the fecal nitrogens which resulted from the feeding of lactalbumin,† an essentially completely digestible protein. For calculations of protein biological values, estimations of the endogenous nitrogens were also required. These were based on the urinary nitrogen excretion during the same lactalbumin feeding periods.

The dogs were brought into comparable physiological states by equilibrating them at minimal nitrogen intakes. The method is well established from the studies of several investigators[8,9] It was accomplished by maintaining the dogs on a nitrogen-free diet, described earlier by Kade *et al.*,[10] supplemented with an amount of lactalbumin which would permit the dogs to come into nitrogen equilibrium at a low level of nitrogen intake.

When the dogs had been brought near or into nitrogen equilibrium, they were fed the unheated, defatted soy bean meal and papain-containing diets. The basal nitrogen-free diet to which the test materials were added was formulated to supply only a small amount of fat so that it would tend to pass more rapidly through the stomach upon ingestion and thus serve to minimize the action of pepsin. It had the following composition: glucose (Cerelose) 46, dextrin 24.5, hydrogenated vegetable oil (Primex) 3, salts (Jones and Foster[11]) 4, methyl cellulose 2.5, cod liver oil (2,000 units vitamin A, 250 units vitamin D per gram) 0.75, wheat germ oil (Fyvee) 0.25, liver extract (Wilson's fraction 0) 0.2, choline chloride 0.3, niacin 0.006, thiamine hydrochloride 0.0006, calcium pantothenate 0.0006, riboflavin 0.0006, pyridoxine hydrochloride 0.0006, and folic acid 0.0002. The diet supplied 3.83 calories per gram.

During the course of the study, the dogs were usually fed the diet containing either active or inactive papain for two or three weeks before they were fed the alternate experimental diet. At the end of this experimental period, they were re-equilibrated on the lactalbumin containing diet. This was done to re-establish the fact that they were at or near nitrogen equilibrium and to redetermine their metabolic and endogenous nitrogen excretion.

It became evident early in the course of the study that active papain in the diet improved protein digestion, presumably making more peptides and amino acids available to the body. Supplements of sulfur amino acids, therefore, at one per cent of the amount of soy bean protein, were subsequently added to some of the diets. This was done, in part, to permit

* Obtained from A. E. Staley Manufacturing Co. under the designation I-8-2.
† Borden's lactalbumin 15-42, 12.6 per cent nitrogen.

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addition of cystine and or methionine. In part, the amino acids were added to determine their quantitative effect on the utilization of the protein in the presence of active and inactive papain.

Results and Discussion

In FIGURE 1 are shown the changes in digestibility and in the calculated amount of nitrogen required to maintain dogs in nitrogen equilibrium resulting from the addition of active or inactive papain to soy bean meal-containing diets.

During the first seven weeks of the study, dogs 55, 62, and 66 received the laboratory extracted soy bean meal. Henceforth, all dogs were fed the commercially prepared material. In all calculations, no distinction has been made between the use of the two soy bean meals, since there was no evidence that there was any material difference in the results obtained under each of the feeding trials.

Effect of Papain on Digestibility. The extent to which the unheated, defatted soy bean protein was digested by the dogs was calculated by the formula[12]:

$$\text{Digestibility in per cent} = \frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})}{\text{Food N}} \times 100.$$

As indicated above, the metabolic nitrogen figure was based on the lactalbumin fecal nitrogen observed at the time the dogs were at or near nitrogen equilibrium. This more nearly represents the metabolic nitrogen than does the value obtained by feeding a nitrogen-free diet, as is sometimes done, since there is no additional demand on the tissues for nitrogen, as is the case when a nitrogen-free diet is fed. A single metabolic nitrogen figure was used throughout for each dog based on the average of all the values obtained for that dog.

It may be seen from FIGURE 1 and from the data in TABLE 1 that amino acid supplementation had little effect on digestibility, as might have been anticipated. Accordingly, single digestibility figures for active and for inactive papain were calculated for each dog and are summarized in TABLE 2.

It may be seen from the summary data in TABLE 2 that the presence of active papain in the diet was responsible for greater digestion of soy bean protein in all the dogs. In the case of six out of the nine dogs, the differences were beyond any probable limit of spontaneous variation ($P = 0.006$ or less).* Since these dogs did not have impaired digestive functions and

* The P values were derived from the corresponding t values, taking into account the number of weeks of test utilized in calculating the means and where

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\sigma^2_{\bar{x}_1} + \sigma^2_{\bar{x}_2}}}$$

Increased significance could have been ascribed to the means by basing the calculations on the 3- and 5-day collection periods. It was believed, however, that the more conservative procedure employed here, that of basing the calculations on the results of a week's observations, would be more meaningful where it was found to lead to significant differences.

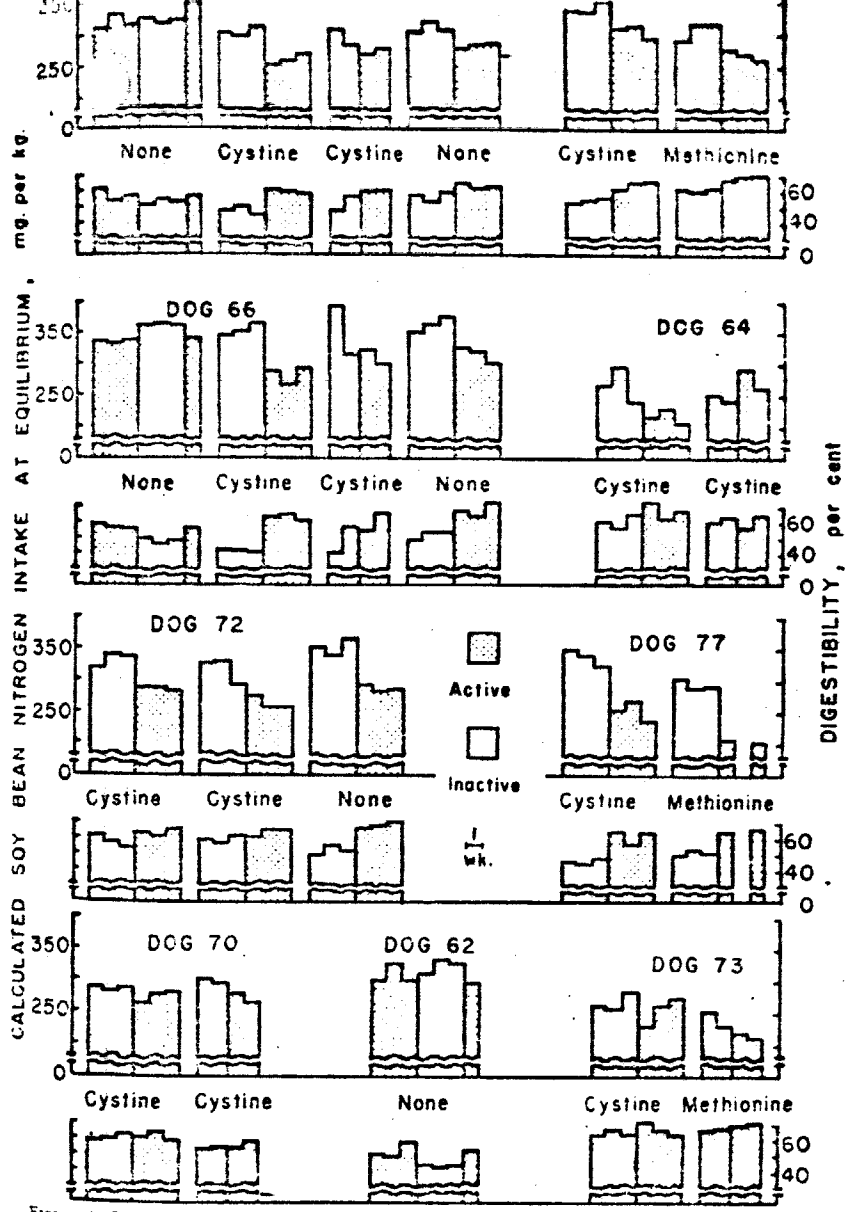

FIGURE 1. Graphic representation of the calculated soy bean nitrogen intakes required for equilibrium, together with the corresponding percentage digestibility values. The amino acid supplements were added, as indicated between the histograms. A one-week break which occurred during the feeding of active papain to dog 77 is due to a lost aspirated feces sample.

had not been subjected to any surgical procedures, the fact that active papain increased the digestion of protein from 54.7 per cent to 63.1 per cent, an increased utilization of 15.5 per cent, was still more noteworthy.

TABLE 1

WEEKLY NITROGEN BALANCE DATA OF DOGS FED LACTALBUMIN OR SOY BEAN MEAL DIETS*

Dog no.	Weight	Nitrogen source	Nitrogen intake: test protein	Nitrogen intake: basal diet	Total N intake	Amino acid supplement	Caroid	N excretion: urinary	N excretion: fecal	Nitrogen balance	Calculated N-intake at equilibrium
	kg.		mg./kg./day		mg./day			mg./day		mg./kg./day	mg./day
55	7.0	SBM	300	6	2140	none	active	1208	1020	−13	313
	6.9	SBM	304	6	2140	none	active	1164	1175	−29	333
	6.9	SBM	304	6	2140	none	active	1133	1095	−13	317
	6.9	SBM	304	6	2140	none	inactive	1055	1234	−22	326
	6.8	SBM	309	6	2140	none	inactive	1065	1148	−11	320
	6.8	SBM	309	6	2140	none	inactive	1055	1197	−16	325
	6.7	SBM	313	6	2140	none	active	1202	1123	−28	351
	7.2	Lact.	88	6	674			661	182	−23	111
	7.2	Lact.	88	6	674			545	183	−8	96
	7.2	SBM	330	6	2416	l-cystine	inactive	765	1469	+25	305
	7.2	SBM	330	6	2416	l-cystine	inactive	793	1403	+31	299
	7.2	SBM	330	6	2416	l-cystine	inactive	785	1519	+16	314
	7.2	SBM	292	6	2145	l-cystine	active	810	1037	+37	255
	7.2	SBM	292	6	2145	l-cystine	active	861	1052	+32	260
	7.1	SBM	297	6	2145	l-cystine	active	896	1082	+25	272
	6.8	Lact.	93	7	683			511	245	−11	104
	6.8	Lact.	93	7	683			472	197	+2	91
	6.8	SBM	329	6	2282	l-cystine	inactive	763	1405	+17	312
	6.8	SBM	329	6	2282	l-cystine	inactive	809	1170	+45	284
	6.9	SBM	325	6	2282	l-cystine	active	799	1110	+54	271
	6.9	SBM	325	6	2282	l-cystine	active	884	1105	+47	278
	6.9	Lact.	91	7	683			559	207	−12	103
	6.9	Lact.	91	7	683			480	199	+1	90
	6.9	SBM	315	7	2225	none	inactive	1014	1126	+12	303
	6.9	SBM	315	7	2225	none	inactive	1059	1217	−7	322
	6.9	SBM	315	7	2225	none	inactive	1094	1095	+5	
	6.8	SBM	297	8	2073	none	active	1043	906	+18	279
	6.8	SBM	297	8	2073	none	active	1020	966	+13	284
	6.8	SBM	297	8	2073	none	active	1041	959	+11	286
	6.7	Lact.	95	7	683			538	180	−7	102
	6.8	Lact.	93	7	683			464	228	−1	94
62	11.5	SBM	300	6	3513	none	active	1507	2047	−4	304
	11.4	SBM	303	6	3513	none	active	1690	2105	−25	328
	11.2	SBM	308	6	3513	none	active	1675	1781	+5	303
	11.2	SBM	308	6	3513	none	inactive	1312	2287	−8	316
	10.9	SBM	317	6	3513	none	inactive	1353	2341	−17	334
	10.8	SBM	319	6	3513	none	inactive	1320	2304	−10	329
	10.7	SBM	322	6	3513	none	active	1292	1967	+24	298
	11.3	Lact.	92	7	1120			774	359	−1	93
64	9.9	Lact.	91	7	973			715	315	−6	97
	10.0	SBM	340	7	3469	l-cystine	inactive	1103	1648	+72	268
	10.2	SBM	333	7	3469	l-cystine	inactive	1270	1800	+39	294
	10.2	SBM	275	7	2875	l-cystine	inactive	1207	1287	+37	238
	10.2	SBM	275	7	2875	l-cystine	active	1225	1072	+57	218
	10.3	SBM	233	8	2478	l-cystine	active	1198	1232	+5	228
	10.3	SBM	233	8	2478	l-cystine	active	1130	1099	+24	209
	10.3	Lact.	87	7	973			793	288	−10	97
	10.3	Lact.	87	7	973			718	281	−3	90

TABLE 1—*Continued*

Dog	Weight	Nitrogen source	Nitrogen intake: test protein	Nitrogen intake: basal diet	Total N intake	Amino acid supplement	Caroid	N excretion: urinary	N excretion: fecal	Nitrogen balance	Calculated N intake at equilibrium	Digestibility
	kg.		mg./kg./day		mg./day			mg./day		mg./kg./day	mg./kg./day	%
	10.5	SBM	267	7	2875	l-cystine	inactive	1301	1436	+13	254	60.3
	10.7	SBM	262	7	2875	l-cystine	inactive	1327	1332	+20	242	63.9
	10.6	SBM	264	7	2875	l-cystine	active	1638	1522	−27	291	57.3
	10.7	SBM	262	7	2875	l-cystine	active	1621	1283	−3	265	65.6
73	7.3	Lact.	99	6	767			636	275	−20	119	
	7.3	SBM	307	8	2301	l-cystine	inactive	894	1103	+42	265	63.9
	7.2	SBM	311	8	2301	l-cystine	inactive	939	995	+51	260	68.6
	7.1	SBM	316	8	2301	l-cystine	inactive	1018	1072	+30	286	65.2
	7.1	SBM	270	9	1983	l-cystine	active	894	814	+39	231	72.7
	7.0	SBM	274	9	1983	l-cystine	active	1001	911	+10	264	67.8
	6.9	SBM	278	9	1983	l-cystine	active	984	975	+3	275	64.5
	6.9	Lact.	104	8	775			508	269	0	104	
	7.0	SBM	280	9	2024	dl-methion.	inactive	897	953	+25	255	66.4
	7.0	SBM	280	9	2024	dl-methion.	inactive	784	916	+46	234	68.2
	7.1	SBM	256	9	1880	dl methion.	active	799	833	+35	221	70.2
	7.1	SBM	256	9	1880	dl-methion.	active	797	795	+41	215	72.2
	7.1	Lact.	102	8	775			530	321	−8	110	
66	9.8	SBM	306	6	3052	none	active	1773	1593	−32	338	59.3
	9.7	SBM	309	6	3052	none	active	1632	1653	−24	333	57.3
	9.6	SBM	312	6	3052	none	active	1640	1678	−28	340	56.5
	9.4	SBM	319	6	3052	none	inactive	1574	1883	−43	362	49.8
	9.4	SBM	319	6	3052	none	inactive	1503	1980	−46	365	46.6
	9.3	SBM	322	6	3052	none	inactive	1487	1930	−39	361	48.2
	9.3	SBM	322	6	3052	none	active	1538	1690	−19	341	56.1
	9.8	Lact.	92	6	962			714	328	−8	100	
	9.7	Lact.	93	6	962			671	396	−10	103	
	9.8	SBM	347	7	3469	l-cystine	inactive	1129	2333	+1	346	42.8
	9.8	SBM	347	7	3469	l-cystine	inactive	1178	2349	−6	353	42.4
	9.7	SBM	350	7	3469	l-cystine	inactive	1221	2393	−15	365	41.1
	9.8	SBM	347	7	3469	l-cystine	active	1255	1624	+60	287	63.3
	9.9	SBM	343	7	3469	l-cystine	active	1192	1545	+74	269	65.6
	10.0	SBM	340	7	3469	l-cystine	active	1316	1693	+46	294	61.3
	9.7	Lact.	93	7	973			759	320	−11	104	
	9.7	Lact.	93	7	973			655	387	−7	100	
	9.7	SBM	372	7	3680	l-cystine	inactive	1296	2517	−17	389	40.3
	9.8	SBM	368	7	3680	l-cystine	inactive	1133	2035	+52	316	57.5
	9.9	SBM	364	7	3680	l-cystine	active	1251	2033	+10	324	54.3
	10.0	SBM	361	7	3680	l-cystine	active	1215	1610	+86	275	65.8
	9.9	Lact.	91	7	973			642	333	0	91	
	10.0	Lact.	90	7	973			507	363	+10	80	
	10.1	SBM	351	7	3622	none	inactive	1157	2176	−1	352	49.6
	9.9	SBM	358	7	3622	none	inactive	1624	2024	−3	361	53.8
	9.8	SBM	362	7	3622	none	inactive	1736	2024	−14	376	53.8
	9.8	SBM	327	8	3271	none	active	1810	1121	+1	326	67.3
	9.9	SBM	323	8	3271	none	active	1727	[illegible]	+2	321	64.2
	10.0	SBM	320	7	3271	none	active	1810	1211	+19	301	72.7
	9.9	Lact.	91	7	973			763	308	−10	101	
	10.0	Lact.	90	7	973			804	362	−19	109	

TABLE 1—*Continued*

Dog no.	Weight	Nitrogen source	Nitrogen intake: test protein	Nitrogen intake: basal diet	Total N-intake	Amino acid supplement	Caroid	N-excretion: urinary	N-excretion: fecal	Nitrogen balance	Calculated N-intake at equilibrium	Digestibility
	kg.		mg./kg./day		mg./day			mg./day		mg./kg./day	mg./kg./day	%
70	7.3	Lact.	81	5	625			805	199	−52	133	
	7.4	SBM	247	6	1863	l-cystine	inactive	1189	992	−43	290	60.0
	7.2	SBM	253	6	1863	l-cystine	inactive	1125	973	−33	286	61.0
	7.1	SBM	257	6	1863	l-cystine	inactive	1177	910	−32	289	64.4
	7.1	SBM	257	6			active					
	7.0	SBM	261	6	1863	l-cystine	active	937	962	−5	266	61.6
	6.8	SBM	268	6	1863	l-cystine	active	1037	894	−10	278	65.3
	6.8	SBM	268	6	1863	l-cystine	active	951	994	−12	280	59.9
	6.7	Lact.	88	6	625			459	245	−12	100	
	6.9	SBM	305	8	2155	l-cystine	inactive	895	1221	+6	304	54.8
	6.8	SBM	310	8	2155	l-cystine	inactive	905	1200	+7	298	55.8
	6.9	SBM	305	8	2155	l-cystine	inactive	853	1175	+5	282	55.1
	7.0	SBM	287	8	2065	l-cystine	active	845	1086	+19	268	59.4
	7.0	SBM	287	8	2065	l-cystine	active	431	274	−3	95	
	6.9	Lact.	92	7	681			417	269	−1	92	
	7.0	Lact.	91	7	681			613	184	−20	131	
72	5.7	Lact.	111	8	681			861	1058	+17	320	61.5
	5.8	SBM	337	11	2016	l-cystine	inactive	876	1127	+2	341	58.1
	5.7	SBM	343	11	2016	l-cystine	inactive	845	1178	−1	338	55.6
	5.8	SBM	337	11	2016	l-cystine	inactive	782	919	+8	287	63.5
	5.7	SBM	295	11	1744	l-cystine	active	749	962	+6	284	61.0
	5.8	SBM	299	11	1744	l-cystine	active	770	870	+18	282	66.4
	5.5	Lact.	113	9	681			428	292	−4	120	
	5.5	Lact.	113	9	681			373	292	+3	112	
	5.6	SBM	349	11	2016	l-cystine	inactive	798	1095	+22	327	59.7
	5.7	SBM	343	11	2016	l-cystine	inactive	794	1135	+15	328	57.[illegible]
	5.7	SBM	343	11	2016	l-cystine	inactive	669	1055	+51	292	61.[illegible]
	5.8	SBM	286	11	1720	l-cystine	active	700	963	+10	276	60.4
	5.8	SBM	286	11	1720	l-cystine	active	688	871	+28	258	65.8
	5.8	SBM	286	11	1720	l-cystine	active	680	875	+28	258	65.5
	5.8	Lact.	109	8	681			455	334	−19	128	
	5.7	Lact.	111	8	681			342	330	+2	109	
	5.8	SBM	331	9	1970	none	inactive	840	1265	−23	354	50.1
	5.8	SBM	331	9	1970	none	inactive	879	1135	−8	339	56.7
	5.7	SBM	337	9	1970	none	inactive	931	1202	−29	366	53.3
	5.7	SBM	305	9	1793	none	active	852	866	+13	292	67.4
	5.8	SBM	300	9	1793	none	active	858	847	+15	285	68.5
	5.8	SBM	300	9	1793	none	active	911	808	+13	287	70.7
	5.6	Lact.	113	9	681			378	257	−8	105	
	5.7	Lact.	111	9	681			321	285	−13	98	
75	6.8	Lact.	105	9	780			555	289	−9	114	
	6.7	SBM	334	9	2301	l-cystine	inactive	988	1361	−7	341	53.5
	6.7	SBM	334	9	2301	l-cystine	inactive	1014	1319	5	339	55.3
	6.1	SBM	350	9	2301	l-cystine	inactive	1025	1293	−3	353	56.1
	6.2	SBM	310	10	1983	l-cystine	active	981	1011	−6	316	62.1
	6.2	SBM	310	10	1983	l-cystine	active	1088	943	−8	318	67.1
	6.1	SBM	315	10	1983	l-cystine	active	937	937	+18	297	67.4
	6.1	Lact.	117	10	780			576	284	−13	130	
	6.2	Lact.	115	10	780			462	270	+8	107	
	6.3	SBM	334	10	2165	dl-methion.	inactive	804	1118	+39	295	61.8
	6.2	SBM	340	10	2165	dl-methion.	inactive	918	1132	+19	321	61.1
	6.1	SBM	345	10	2165	dl-methion.	inactive	932	1087	+24	321	63.2

TABLE 1—*Concluded*

Dog no.	Weight	Nitrogen source	Nitrogen intake: test protein	Nitrogen intake: basal diet	Total N intake	Amino acid supplement	Caroid	N excretion: urinary	N excretion: fecal	Nitrogen balance	Calculated N intake at equilibrium	Digestibility
	kg.		mg./kg./day		mg./day			mg./day		mg./kg./day	mg./kg./day	%
	6.1	SBM	270	10	1711	dl-methion.	active	937	828	−9	279	68.6
	6.0	SBM	275	10	1711	dl-methion.	active	892	802	+3	272	70.1
	5.9	SBM	279	11	1711	dl-methion.	active	818	797	+16	264	70.4
	5.9	Lact.	121	10	780			540	309	−12	133	
	6.0	Lact.	119	10	780			443	300	+6	113	
77	9.5	Lact.	109	9	1130			747	460	−8	117	
	9.5	SBM	340	9	3313	l-cystine	inactive	1185	2220	−10	350	15.8
	9.5	SBM	340	9	3313	l-cystine	inactive	1078	2241	−1	341	45.1
	9.7	SBM	333	9	3313	l-cystine	inactive	1090	2142	+8	325	48.2
	9.7	SBM	284	9	2850	l-cystine	active	1142	1449	+30	251	65.1
	9.7	SBM	284	9	2850	l-cystine	active	1071	1645	+14	270	57.2
	9.8	SBM	282	9	2850	l-cystine	active	997	1430	+43	239	64.7
	9.9	Lact.	105	9	1130			582	400	+15	90	
	10.1	Lact.	103	9	1130			550	424	+15	90	
	10.3	SBM	330	8	3483	dl-methion.	inactive	1089	2157	+23	307	50.3
	10.3	SBM	330	8	3483	dl-methion.	inactive	1009	2055	+41	289	53.2
	10.3	SBM	330	8	3483	dl-methion.	inactive	994	2099	+38	292	51.9
	10.4	SBM	249	9	2679	dl-methion.	active	888	1361	+41	208	65.1
	10.4	SBM	249	9	2679	dl-methion.	active	924	—	—	—	—
	10.4	SBM	249	9	2679	dl-methion.	active	886	1314	+46	203	66.8
	10.5	Lact.	99	9	1130			571	440	+11	88	
	10.5	Lact.	97	9	1130			531	402	+18	79	

Montgomery *et al.*[13] reported that ingested trypsin exerted its activity in eight depancreatized dogs and noted, without giving their data, that under the same approach papain was also effective upon oral administration. They also observed positive effects in all their dogs. These two studies are, therefore, in essential agreement, since it was observed, in each instance, as was the case in the rat feeding trials referred to above,[1,2] that *in vivo* enzyme digestive activity can be demonstrated after oral ingestion of proteolytic enzymes.

Effect of Papain on the Soy Bean Nitrogen Intake Required for Equilibrium. The effect of papain on the amount of soy bean nitrogen required by the dogs for equilibrium (nitrogen intake = nitrogen output) was calculated in the following way:

nitrogen intake at equilibrium = nitrogen intake − nitrogen balance.

TABLE 2

THE INFLUENCE OF PAPAIN ON THE DIGESTIBILITY OF SOY BEAN MEAL PROTEIN FED TO DOGS

Dog no.	Endogenous nitrogen excretion	Metabolic nitrogen excretion	Amount of protein digested, means ± s.e.: inactive papain	Amount of protein digested, means ± s.e.: active papain	P
	mg./day	mg./day	%	%	
55	529	203	52.9 ± 1.5	60.5 ± 1.0	<0.001
62	774	359	44.4 ± 0.2	54.0 ± 2.0	0.006
64	742	295	60.7 ± 1.4	65.1 ± 2.6	0.15
66	689	350	47.8 ± 1.8	62.0 ± 2.0	<0.001
70	528	247	59.2 ± 1.7	60.3 ± 1.7	0.2
72	416	282	57.2 ± 1.3	65.5 ± 1.1	<0.001
73	558	272	66.5 ± 1.0	69.5 ± 2.3	0.12
75	515	290	58.6 ± 1.5	67.6 ± 1.3	0.001
77	596	425	49.1 ± 1.3	63.8 ± 1.6	0.001
Average			54.7 ± 1.1	63.1 ± 0.7	<0.001

Since amino acid supplementation had a pronounced effect upon the equilibrium nitrogen value, the data of TABLE 1 are averaged in TABLE 3 by groups in accordance with the supplement employed.

Considering all the results of TABLE 3, it may be seen that invariably less soy bean nitrogen was required to maintain the dogs in nitrogen equilibrium when their diet was supplied with active papain than when inactive papain was fed. In only five out of the fifteen individual dietary periods compared were the differences not far beyond the probable limits of spontaneous variability.

Considering the data by groups, it may be seen that eight per cent less nitrogen was required to maintain the dogs in equilibrium when active papain was fed along with the unsupplemented diets. Fed along with the cystine supplemented diets, active papain effected a 12 per cent reduction in the amount of nitrogen required to maintain the dogs in equilibrium. An even greater effect on nitrogen equilibrium was noted when the methionine supplemented diets were fed. In the latter instance, an 18 per cent re-

duction in the amount of nitrogen required to maintain the dogs in equilibrium resulted.

Thus, as in the case of the comparisons based upon the extent of protein digestion, it may be seen that papain activity may be demonstrated conclusively by this. The markedly improved responses of most of the dogs due to the active papain are the more significant because the increases were superimposed on normal digestive activities.

Effect of Cystine or Methionine Supplements on the Biological Value of Soy Bean Protein. While these tests had for their primary interest the

TABLE 3

CALCULATED SOY BEAN NITROGEN INTAKES REQUIRED FOR EQUILIBRIUM IN DOGS FED INACTIVE OR ACTIVE PAPAIN IN THEIR DIETS

Dog no.	Amino acid supplement	Soy bean nitrogen intake at equilibrium, means ± s.e.		P
		Inactive papain	Active papain	
		mg. N/kg./day		
55	none	318 ± 3	309 ± 10	0.4
62	none	326 ± 5	308 ± 7	0.9
66	none	363 ± 3	329 ± 5	<0.001
72	none	353 ± 8	288 ± 2	0.002
Average		340 ± 2	312 ± 1	<0.001
55	l-cystine	303 ± 4	267 ± 6	<0.001
64	l-cystine	261 ± 8	242 ± 16	0.3
66	l-cystine	354 ± 12	290 ± 10	0.003
70	l-cystine	293 ± 3	275 ± 3	0.006
72	l-cystine	324 ± 7	274 ± 5	<0.001
73	l-cystine	270 ± 8	257 ± 13	0.2
75	l-cystine	344 ± 4	310 ± 7	0.013
77	l-cystine	359 ± 7	254 ± 9	0.001
Average		310 ± 3	271 ± 2	<0.001
73	dl-methionine	245 ± 11	218 ± 3	0.13
75	dl-methionine	312 ± 9	271 ± 5	0.014
77	dl-methionine	296 ± 6	206 ± 3	0.001
Average		289 ± 7	237 ± 8	<0.001

effect of orally ingested papain on protein digestion, the data permit calculations of the biological value of soy bean protein by Mitchell's formula:[14]

$$\text{Biological Value} = \frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N}) - (\text{Urinary N} - \text{Endogenous N})}{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})} \times 100$$

When thus calculated, the biological value is apparently uninfluenced by papain ($P = 0.1$ or more). Consequently, the data for active and inactive papain were combined to give data on the biological value of the soy bean protein when fed unsupplemented, or when fed supplemented with cystine or methionine.

The biological value of the unsupplemented soy bean protein, as determined in dogs equilibrated at minimal nitrogen intakes, was 54.0 (s.e. ± 1.2, 4 dogs, 38 weeks of feeding). That for cystine supplemented soy bean meal was 69.1 (s.e. ± 1.1, 8 dogs, 70 weeks of feeding). That for the methionine supplemented protein was 70.3 (s.e. ± 1.6, 3 dogs, 15 weeks of feeding). These values were significantly different from each other ($P = <0.001$).

The value for raw soy bean protein found here is in good agreement with that of 57, given by Block and Mitchell in their extensive review.[14]

Lactalbumin Nitrogen Intake at Equilibrium. It is of interest to compare the amount of lactalbumin nitrogen found necessary to maintain the dogs in equilibrium with findings of other investigators. Melnick and Cowgill[15] observed that dogs require 175 to 200 mg. of lactalbumin nitrogen per kg. per day to maintain them in nitrogen equilibrium. More recently, Kade *et al.*[7] reported the requirement to be 90 mg. of lactalbumin nitrogen per kg. per day. These may be compared with the value derived from these studies (9 dogs, 44 weeks of observations) of 104 (s.d. ± 14) mg. lactalbumin nitrogen per kg. per day. The value is lower than the one submitted by Melnick and Cowgill for the probable reason that these investigators did not equilibrate their dogs at minimal nitrogen intakes before placing them on test. Kade *et al.* indicated that their procedure of calculating the minimal nitrogen intakes, based upon the calculated optimum weight of the dogs, rather than the actual weights, as used by Risser[7] and as used here, yielded values somewhat lower than would have been obtained if the actual weights of the dogs had been used. As stated above, we have preferred to calculate the caloric needs of the dogs on the basis of the Cowgill-Drabkin formula, but have expressed the results in terms of the actual weights of the animals, rather than upon calculated ones.

Summary

The *in vivo* activity of papain added to the diet has been studied in nine normal dogs maintained near or at nitrogen equilibrium after equilibration at minimal protein intakes. The protein source material in these studies was unheated, defatted soy bean meal.

On the basis of the results obtained, the following comparisons may be made:

Papain added to the diet significantly increased the digestion of soy bean protein, even above the normal digestive capacity of these animals. The difference in three of the nine dogs was barely significant statistically but was beyond the range of probable spontaneous variation in the remaining six. The overall average was an increase in the protein digested from 54.6 per cent to 63.1 per cent, or an improvement in digestibility of 15.5 per cent above the normal.

While papain added to the diet significantly [illegible] the amount of soy bean protein required to maintain the dogs [illegible] equilibrium. The results were the same, whether or not [illegible] cystine or methionine.

It was noted that the biological value [illegible] added to the diet. When [illegible] that the soy

bean protein had a biological value of 54.0 (s.e. ± 1.2). Supplementing the soy bean protein with one per cent cystine increased the biological value to 69.1 (s.e. ± 1.1). One per cent methionine increased it to 76.3 (s.e. ± 1.6).

An incidental observation of the study indicated that 104 (s.d. ± 14) mg. of lactalbumin nitrogen per kilogram per day were required to keep the dogs in nitrogen equilibrium.

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