C. xerose. The 1:50,000 concentration of potassium tellurite inhibited the 4 gram-negative bacteria but did not inhibit the 4 gram-positive organisms. Usually greater concentrations of the substances were required to produce the same effect in the liquid medium; basic fuchsin, 1:100,000, and crystal violet, 1:100,000 inhibited the 4 gram-positive organisms but permitted growth of the 4 gram-negative bacteria and the PPLO. Potassium tellurite, 1:50,000, inhibited *E. coli* and *A. aerogenes* but not *P. vulgaris* and *Ps. aeruginosa* among the gram-negative bacteria and in addition inhibited the gram-positive organisms except for *C. xerose.*

Discussion. To our knowledge the only other work on the susceptibility of PPLO to bacteriostatic agents is that of Edward(5). Whereas he observed that neither of his 2 strains isolated from mice was inhibited by 1:5,000 sodium azide, we observed that 5 of our 8 test strains were inhibited. The 3 of our strains not inhibited by 1:5,000 sodium azide were strains which had been on artificial media for some time. Our strains from humans, even the recently isolated strains, appear to be slightly more resistant to potassium tellurite and crystal violet than were the two mouse strains tested by Edward. Like Edward, we found that PPLO were inhibited by brilliant green in 1:100,000 concentration. In addition, Edward investigated thallium acetate but it appears to offer no advantages over potassium tellurite which is commonly used to inhibit gram-negative organisms.

Summary. Strikingly, the PPLO do not behave either as gram-positive or gram-negative microorganisms in that they are not inhibited on solid medium by a 1:250,000 dilution of crystal violet which normally inhibits gram-positive microorganisms nor by a 1:50,000 dilution of potassium tellurite which normally inhibits gram-negative organisms. By being able to inhibit both gram-positive and gram-negative microorganisms the isolation of PPLO from mixed cultures can be greatly facilitated. Generally, strains which have been cultivated on artificial media for some time are more resistant to the bacteriostatic substances than are the recently isolated strains.

5. Edward, D. G., ff., *J. Gen. Microbiol.,* 1947, v1, 238.

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Production, Purification, and Some Properties of *Clostridium histolyticum* Collagenase.* (17970)

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Jennison(1) reported that *Clostridium histolyticum* produced a proteinase that was able to digest "native" collagen. In a previous communication from this laboratory(2) the activity of *Cl. histolyticum* filtrates in the degradation of collagen was confirmed, as well as the lack of activity of other proteinases on unmodified collagen.

In this communication a reproducible procedure will be described for the isolation and partial purification of a proteinase(s) from *Cl. histolyticum* which is very active in the solubilization of native collagen. A method will be described wherein filtrates with little or no lethal toxin but adequate "collagenase" activity may be produced in large batches. The final product purified by the method to be described is 400 times as active per unit nitrogen as the original filtrate. The separation of a proteolytic enzyme which has no collagenase activity and which is activated

* This work was done under a contract between the U. S. Army and the University of Cincinnati.

1. Jennison, M. W., *J. Bact.,* 1945, v50, 349.

2. Neuman, R. E., and Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 409.

by Fe and cysteine (gelatin substrate) is described. This enzyme has been described previously(3,4).

Experimental. Stock and seed cultures. The strain of *Cl. histolyticum* used in these studies was CHT from the stock collection in this laboratory. The stock medium was prepared as previously described for *Cl. perfringens*(5). In carrying this culture on stock medium, no glucose was used. Just before transfer, 1 ml of a sterile sodium thioglycolate solution (4 mg) was added to each tube. The stock cultures were incubated at 37°C for 16-18 hours. Seed cultures were incubated for 8 hours.

Medium for collagenase production. Casein digest and medium were prepared as previously described(5). The bottles containing 3 liters of medium were steam sterilized at 15 lb. for 1 hour and cooled to approximately 38-40°C in running tap water. A sterile solution (75 ml) containing 3 g of sodium thioglycolate was added to each bottle immediately prior to incubation with 40 ml of seed culture. The bottles were then placed in a 37°C water bath for 18 hours.

Filtration. The cultures were cleared of most of the organisms by filtration with suction through a layer (1 cm) of supercel (Johns Manville Co.). Clear, sparkling filtrates suitable for the purification procedure were obtained by filtration through 12 inch Berkefeld "N" candles. The filtrates were tested for toxicity by the intravenous route (lethal toxin) in mice (16-18 g) and usually assayed at 1 to 2 LD_{50} per ml.

Dialysis. The filtrates were poured into Visking casing (1⅝″) and dialyzed against cooled running tap water (average temperature 15°C) in a continuous rocking dialyzer for 17-18 hours. If the dialysis was interrupted the sacks were placed in distilled water in the cold room at 4-6°C.

Precipitation with methanol. To each 1500 ml of dialyzed filtrate at 0°C, 1000 ml of methanol (pre-cooled) was added with constant stirring. The precipitate was allowed to form and flocculate for 24 hours during which time the precipitate settled to the bottom of the container. The supernatant fluid was removed by careful siphoning and the precipitate collected in a refrigerated centrifuge.

Precipitation with ammonium sulphate. The methanol precipitate from 1500 ml of dialyzed filtrate was taken up in 40 ml of water, and 20 ml of 0.1 M phosphate buffer at pH 7.2 was added. This yielded a clear pigmented solution. The solution was brought to 75% saturation by the addition of 180 ml of saturated ammonium sulphate at room temperature. The precipitate was allowed to form at room temperature for 24 hours before it was collected by centrifugation in a refrigerated centrifuge. This precipitate was then dissolved in 20 ml of distilled water. The cloudy solution was centrifuged in a refrigerated centrifuge to remove insoluble pigmented material which was discarded. The supernatant was then dried by lyophilization to a clean, white, easily soluble powder.

Measurement of collagenase activity. The activity of enzyme preparations was measured by the release of soluble nitrogen from collagen. The activity was expressed as mg collagen (N x 5.85) solubilized per mg enzyme nitrogen in 18 hours from 100 mg beef tendon collagen† at 37°C. The pH was 7.2; buffer 0.1 M phosphate. A typical experimental batch of 6 liters carried through the purification procedure is shown in Table I. Twelve such batches have been carried to this point with little or no variation.

Results. pH activity curve. The activity of *Cl. histolyticum* collagenase was measured in phosphate buffers from pH 4.6 to 8.9. The greatest activity was attained at pH 7.6 under the conditions of these experiments.

Heat inactivation. At an enzyme concentration of 4 μg nitrogen per ml the collagenase activity was destroyed to the extent of 98.5% in 20 minutes at 50°C. It is of interest to

3. Kocholaty, W., and Krejci, L. E., *Arch. Biochem.*, 1948, v18, 1.

4. van Heyningen, W. E., *Biochem. J.*, 1940, v34, 1540.

5. Boyd, M. J., Logan, M. A., and Tytell, A. A., *J. Biol. Chem.*, 1948, v174, 1013.

† This preparation is described in(2).

TABLE I.
Typical Data* on Enzyme Purification Experiment.

Preparations	mg collagen dissolved per mg N	Recovery % total original activity
Original filtrate	100	
Step 1—Dialyzed filtrate	636	—
" 2—Methanol ppt.	7430	58
" 3—$(NH_4)_2SO_4$ ppt.	38000	61.5

* Data from one of 12 batches of 6 liters each.

TABLE II.
Effect of Normal Sera, Specific Antisera, and Soy Bean Trypsin Inhibitor on *Cl. histolyticum* Collagenase. Substrate level 100 mg beef tendon collagen. Enzyme level 1 μg nitrogen. Incubation at 37°C for 18 hr.

Serum	Amt test, ml	Inhibition, %
Normal horse I*	.05	11
" " I*	.10	30
" " II*	.05	5
" " II*	.10	21
Horse antiserum I*	.001	0
(100 units antitoxin/ml)	.005	100
Horse antiserum II*	.001	91
(350 units antitoxin/ml)	.005	97
Normal guinea pig serum	.05	10
" " " "	.10	31
" rabbit serum	.05	0
" " "	.10	0
" " "	.20	23.5
Soy bean trypsin inhibitor† (crystalline)	150 μg	0
	300 μg	0

* Obtained through the courtesy of Dr. Irvin S. Danielson, Lederle Laboratories Division, American Cyanamid Co.

† Obtained through the courtesy of Dr. M. Kunitz, Rockefeller Institute.

note that this heated preparation retained 30% of its original activity on a casein substrate.

Effect of normal sera, specific antisera, and crystalline soy bean trypsin inhibitor. Normal horse, guinea pig, and rabbit sera were tested and showed only partial inhibition of collagenase activity at relatively high concentrations. Specific antisera inhibited collagenase activity at relatively low concentrations. The efficacy of the specific antisera was not proportional to the antitoxin potency. Under the conditions of the experiments collagenase activity was not inhibited by the crystalline soy bean trypsin inhibitor. A typical experiment is shown in Table II.

Effect of Fe^{++}, cysteine. Ferrous iron (as ferrous sulphate) and cysteine alone or together did not enhance collagenase activity (Table III). In fact in most instances these substances inhibited collagenase activity to a significant degree. These results are in

TABLE III.
Inhibitory Effect of Fe^{++} and Cysteine on *Cl. histolyticum* Collagenase.

Substance	mM	Inhibition, %
Fe^{++}	.01	53
"	.10	79
Cysteine	.01	17
"	.10	0
Fe^{++} and cysteine	.01 each	18.7
" " "	.10 "	40.7

agreement with those of Maschmann(6) but not in accord with the properties of the proteinase isolated by Kocholaty and Krejci(3). This discrepancy is explained by the separation of a second proteolytic enzyme from *Cl. histolyticum* filtrates as will be shown in a later section. These findings are in agreement with those of van Heyningen(4).

Action of specific inhibiting substances. The collagenase activity was inhibited by or-

6. Maschmann, E., *Biochem. Z.*, 1937, v295, 391.

TABLE IV.
Effect of Several Inhibitors on *Cl. histolyticum* Collagenase.

Substance	mM	Inhibition, %
Mercuric chloride	.05	100
Phenylmercuric acetate	.05	87
p-Chloromercuribenzoate	.008	100
Copper sulfate	.01	82.5
Formaldehyde	.30	100

ganic mercurials, copper sulphate, and formaldehyde in relatively low concentrations (Table IV). More extensive studies on this phase of the problem are in progress.

Separation of a proteolytic enzyme activated by Fe^{++} and cysteine. By precipitation from *undialyzed* filtrates with 66% methanol at 0°C an enzyme preparation was obtained which was active on gelatin but not on collagen. This preparation was further purified by precipitation from 0.05 M acetate buffer at pH 4.6. The product was activated by Fe^{++} and cysteine and its properties more closely resembled the proteinase described by Kocholaty and Krejci(3) and van Heyningen (4).

Summary. A consistently reproducible method is described for producing *Clostridium histolyticum* filtrates with good collagenase activity and little or no toxin. The proteolytic enzyme which attacks collagen has been purified 400 times based on activity per unit of nitrogen. The "collagenase" activity is not enhanced by Fe^{++} and cysteine. The collagenase activity is inhibited by specific *Cl. histolyticum* antisera in low concentrations. This inhibition is not proportional to the antitoxic value of the antiserum. Normal rabbit, guinea pig, and horse serum in high concentrations partially inhibit collagenase activity. Collagenase is not inhibited by crystalline soy bean trypsin inhibitor. Collagenase activity is inhibited by reagents supposedly specific for amino groups and sulphydryl groups. It is also sensitive to heavy metals. Collagenase activity is destroyed at 50°C in dilute solutions of enzyme. A proteolytic enzyme, activated by Fe^{++} and cysteine, active on gelatin and not active on collagen is also present in *Cl. histolyticum* filtrates.

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Pteroylglutamic Acid-like Effect of Dehydroisoandrosterone on Growth of Certain Microorganisms.* (17971)

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During routine testing of a number of compounds for ability to reverse aminopterin inhibition of *Streptococcus faecalis* it was found that dehydroisoandrosterone acetate† was especially active. Further trials revealed that this steroid has pteroylglutamic acid (PGA) like activity for both *S. faecalis* and *Lactobacillus casei* and is capable of stimulating *Escherichia coli* in the presence of inhibiting levels of sulfanilamide. Several other steroids and 2 carcinogens were tested and a few found to have very slight PGA-like activity. Although the activity was small when compared with that of pteroylglutamic acid the ability of compounds such as sex hormones and carcinogens or their derivatives to stimulate cell division in a manner similar to that of pteroylglutamic acid could hardly fail to have great biological significance. Further experiments on steroids are under way. The data presented here are representative of a large number of tests made to determine the

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† We would like to express our appreciation to the Schering Corporation for furnishing us with the dehydroisoandrosterone acetate used in this experimental work.