THE ACTION OF COLLAGENASE ON SKIN AND THE ANTI-COLLAGENASE FACTOR IN HUMAN SERUM*

RICHARD B. STOUGHTON, M.D. AND ALLAN L. LORINCZ, M.D.

This report concerns observations of collagenase activity on human skin, in vitro, and inhibition of this enzyme by human serum albumin. Preliminary studies of collagenase inhibition by the sera of three patients with subacute lupus erythematodes and one patient with dermatomyositis are presented.

HISTORICAL

Maschmann (1), in 1937, described the liquefaction of gelatin and disintegration of collagen by a filtrate of Clostridium welchii. He attempted to purify the active principle (2) and named it collagenase (3). In 1945, MacLennan and MacFarlane (4), demonstrated softening of muscle by collagenase activity. Robb-Smith (5) in 1945, studied tissues microscopically after treatment with collagenase. In 1946, Oakley, Warrach and van Heyingen (6) showed collagenase to be distinct from hyaluronidase and from the alpha and theta toxins of Cl. welchii filtrates. Bidwell and van Heyingen (7), in 1948, reported that collagenase was inactive on many protein substances and that it was inactive on amino acid complexes similar to those found in collagen. This point was further clarified when Gersh and Catchpole (8), in 1949, demonstrated, on freeze-dry preparations, that collagenase removes polysaccharide substances from basement membranes and connective tissue but does not attack purified collagen. These authors also demonstrated the sporadic presence of collagenase in malignant and benign tumors and even in normal tissue. Bidwell (9), in 1949, differentiated two separate enzymes in the fraction known as collagenase.

PART I

EFFECT OF CLOSTRIDIUM WELCHII TYPE A FILTRATE ON NORMAL HUMAN SKIN

Methods

a) Small pieces of normal human skin (biopsy and autopsy specimens) from non-exposed areas were fixed in cold acetone for 24 hours transferred to fresh acetone at room temperature for 12 hours and treated with petroleum ether (two changes of 30 minutes each) before infiltrating and embedding in paraffin. Six μ sections were cut and mounted on albumenized slides. These sections were covered with a solution containing Cl. welchii type A filtrate[1] diluted one part in four with M/10 phosphate buffer (pH 7.4), and incubated at 37°C. for 16 hours. The sections were then individually stained with the McManus procedure (10) for polysaccharides, acid orcein for elastic fibers and routine hematoxylin and eosin.

b) In a second series, the same procedure was followed, but instead of the

* From the Section of Dermatology, School of Medicine, University of Chicago, Chicago, Illinois (Chief of Service: Stephen Rothman, M.D.)

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[1] Supplied through the courtesy of Dr. I. S. Danielson of the Lederle Laboratories.

original bacterial filtrate, an ammonium sulfate precipitate of this filtrate was used.

To 200 grams of ammonium sulfate, 200 cc. of Cl. welchii type A filtrate were added. The precipitate was dissolved in 50 cc. of phosphate buffer (pH 7.4).

c) In a third series the heat stability of the enzyme preparation was tested by exposing samples for 10 minutes in a water bath at the following temperatures: 52°C., 54°C., 56°C., 58°C., 60°C. These samples were then tested individually for collagenase activity by applying them to mounted sections as already described and subsequent staining with the McManus method.

d) In order to establish whether collagenase liberates microchemically demonstrable amounts of polysaccharides from human connective tissue, the following procedure was adopted. Shreds of cutaneous connective tissue from cadavers were first treated with ½ saturated calcium hydroxide for 48 hours in order to remove the alkali-extractable mucinous material, the so-called coriomucoid. Subsequently glycogen was removed by treatment with amylase.[2] The residue was filtered, washed with saline and divided into two parts. To one were added 10 cc. of precipitated collagenase, prepared as in (b), and to the other was added the same amount of enzyme solution which had been inactivated with heat. These were incubated 16 hours at 37°C. and filtered. The filtrates were each tested with the Hotchkiss spot testing method (11) and the intensity of color compared grossly. The filtrates were also tested with Benedict's solution and dinitrobenzene for reducing substances.

e) To differentiate the effect of collagenase from that of some other enzymes acting on polysaccharides, solutions of hyaluronidase[3] in M/10 phosphate buffer (pH 6.5) (150 TRU per 10 cc.) and crystalline lysozyme in saline (14 mgm. per cc.) were applied to acetone fixed sections and incubated 24 hours at 37°C. These sections were then stained with the McManus method and compared with control sections treated with buffer only.

Results

a) After incubation with collagenase, as reported by Gersh and Catchpole (9) for sections fixed with the freeze-dry method, the polysaccharide substances in the basement membrane, vessel walls and collagen disappear from acetone-fixed sections of the skin leaving behind morphologically well preserved collagen bundles and epidermis (Fig. 1a, b).

Despite the morphologically intact appearance of the collagen bundles, they obviously have undergone changes. They have lost their eosinophilia (Fig. 1, c, d) and, if after treatment with collagenase, the sections are subjected to hot water or hot 70% alcohol (60°C.–70°C.), the collagen bundles are readily solubilized (12) leaving behind only epidermis and elastic fibers (Fig. 2).

Acid orcein stains of sections incubated with collagenase reveal the elastic fibers to be perfectly intact and apparently unaltered.

[2] Supplied through the courtesy of Rhom & Haas Co., Washington Square, Phila., Penna.

[3] Supplied through the courtesy of the Schering Corp.

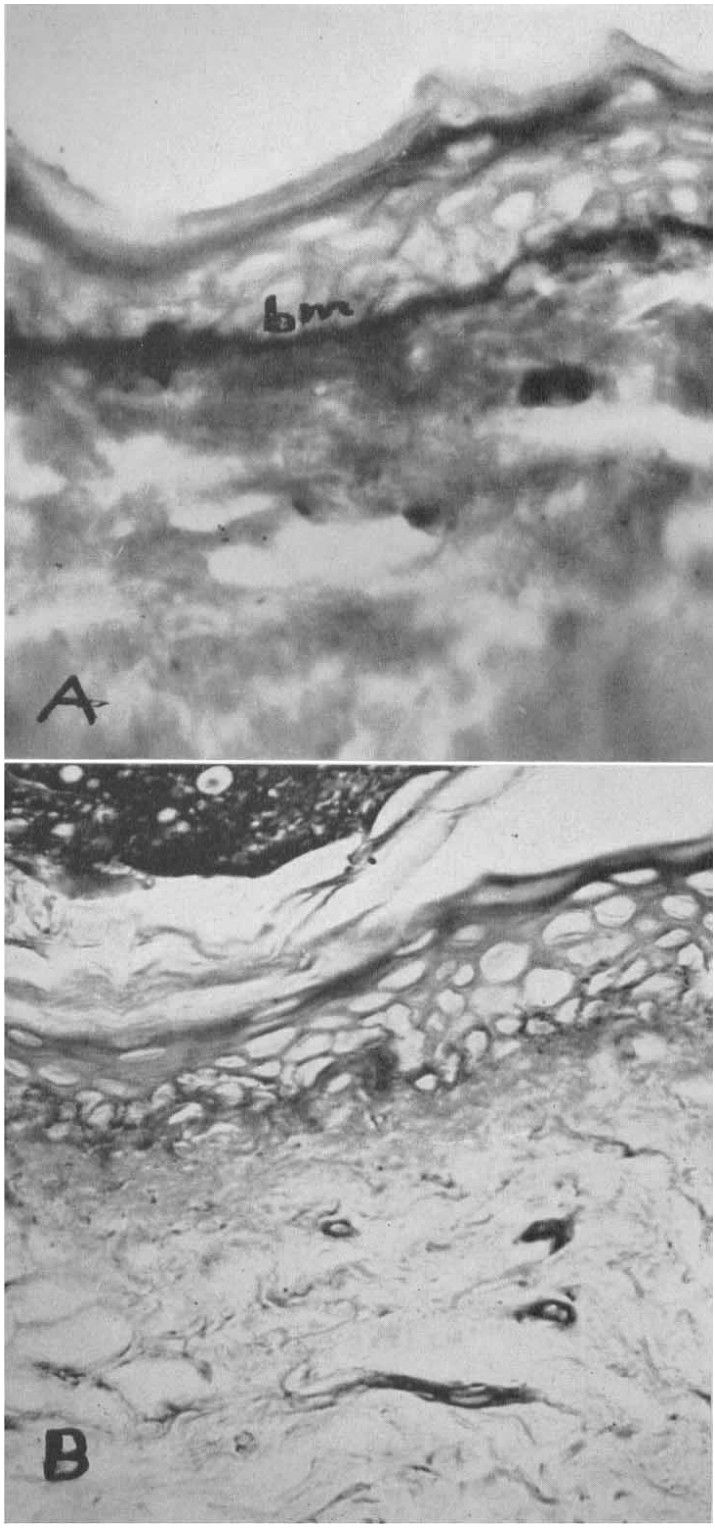

FIG. 1 A&B.

FIG. 1 a) McManus stain of control section showing normal basement membrane (b.m.) and polysaccharides of corium (800 ×). b) McManus stain of collagenase treated section. Note absence of basement membrane and loss of polysaccharides in corium (630×). c) H & E stain: control section (140 ×). d) H & E stain: serial section with (c) and previously exposed to collagenase. Note loss of eosinophilia in collagen (140 ×).

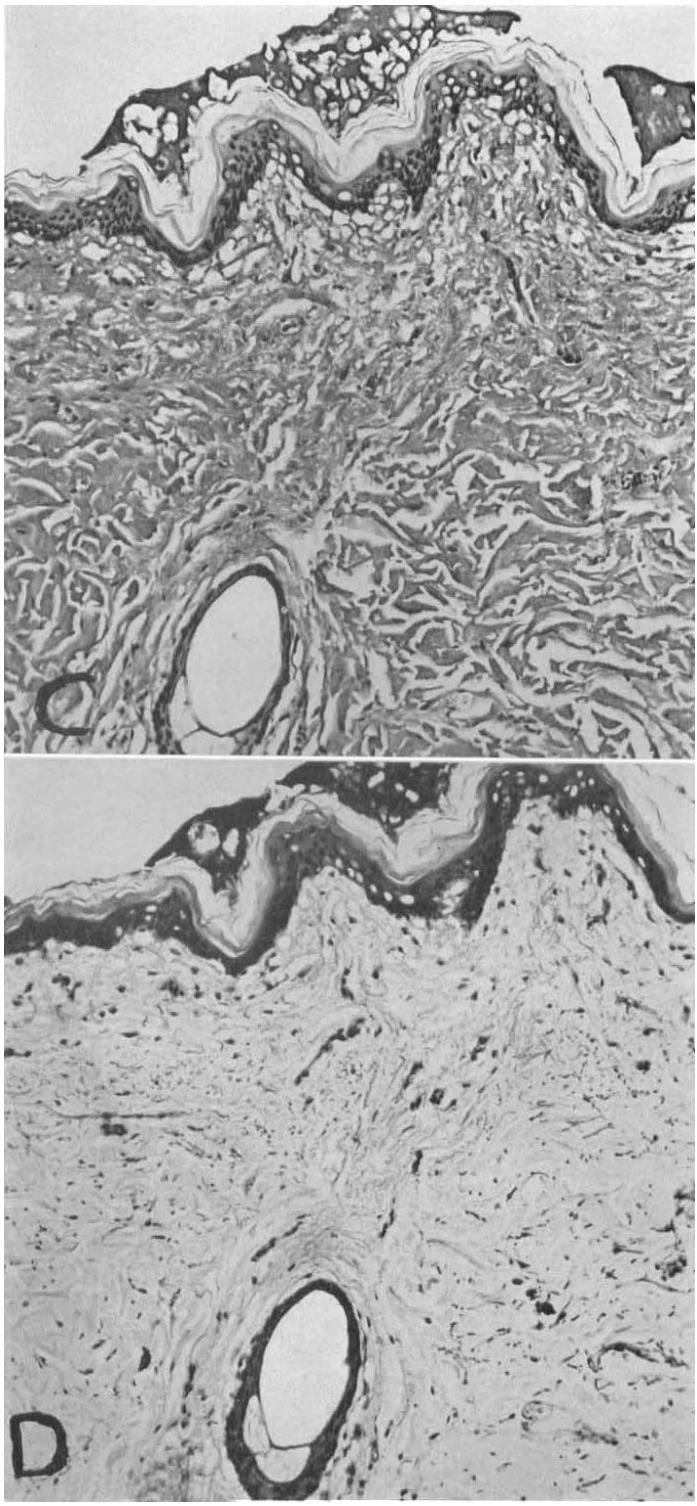

FIG. 1 C&D.

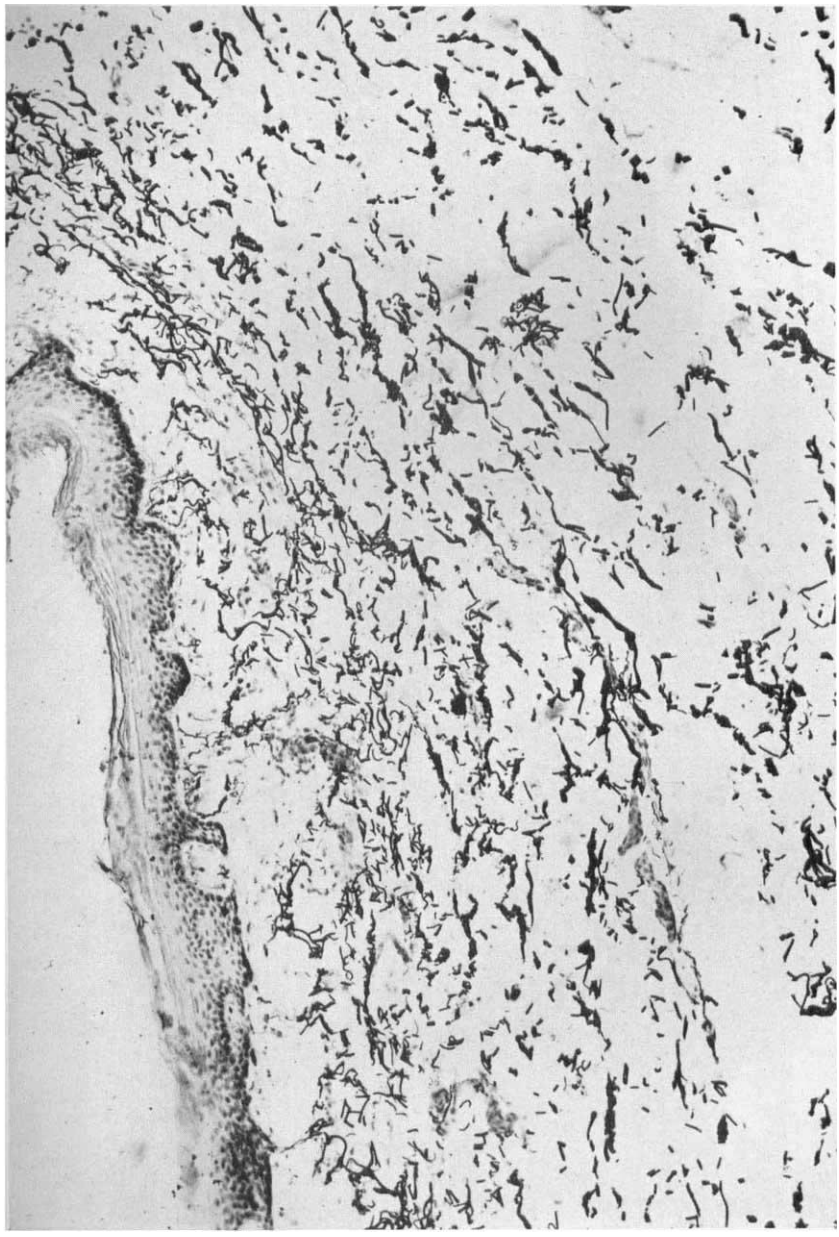

FIG. 2 Acid orcein stain of section treated with collagenase followed by exposure to hot water (65°–70°C.). Only elastic fibers and epithelial cells remain (140 ×).

Sections of tissue previously fixed in aqueous formalin reveal after incubation with collagenase no alterations of the polysaccharides such as those found with acetone-fixed sections. The different behavior of formalin-fixed sections could be due to a denaturation process which renders the susceptible groups unresponsive to collagenase.

b) The ammonium sulfate precipitate fraction of Cl. welchii filtrate has the same action as the original filtrate.

c) The action of collagenase is prevented by heat inactivation at 56°C. for 10 minutes. This degree of heat-lability corresponds with that of the muscle disintegrating enzyme which was separated by Bidwell and which she thought to be distinct from the more heat-stable enzyme which disintegrates "azocoll."

d) Filtrates of fresh normal human collagen previously treated with collagenase show a very strong reaction for polysaccharides as tested with the Hotchkiss spot testing method. Filtrates of collagen treated in the same manner only with heat-inactivated collagenase give a mild color reaction in comparison. Neither of the filtrates revealed free reducing sugars. This would indicate that collagenase liberates, from connective tissue, water insoluble polysaccharides in a water soluble form. However, no reducing sugars are formed.

e) Hyaluronidase and lysozyme had no apparent effect on the polysaccharides of the sections.

Part II

Effect of human serum on the action of collagenase

a) In order to establish the minimal concentration of the bacterial filtrate at which the polysaccharides of the basement membrane disappear completely or almost completely, the following procedure was adopted.

Varying amounts of Cl. welchii filtrate were added to tubes containing 1 cc. of M/10 of phosphate buffer (pH 7.4). The volumes were made equal. One cc. of each of these solutions of varying concentrations of filtrate was applied to sections and incubated for 16 hours at 37°C. They were washed with phosphate buffer and all stained simultaneously with the McManus method. The least amount of filtrate per cc. which caused complete or almost complete loss of the polysaccharides from the basement membrane was arbitrarily designated as 50 units. The same lot of filtrate was used in all quantitative estimations and the base line frequently checked. With the lot used in these experiments, the least amount of filtrate in 1 cc. of M/10 phosphate buffer (pH 7.4) required to give this complete or almost complete loss was consistently 0.02 cc.

b) In order to quantitate the inhibitory action of human serum on collagenase activity, constant volumes of sera were mixed with graded amounts of the enzyme. The tests for collagenase activity were carried out on sections in the manner described above, and the end point read in the same way (i.e. the least amount of filtrate necessary to accomplish a complete or almost complete loss of polysaccharides from the basement membrane). Two or three slides were run concurrently with Cl. welchii filtrate in phosphate buffer to check the base line.

c) In order to establish whether the inhibitory fraction of serum is dialysable

or not, 20 cc. of normal human serum were put in a dialysis bag and immersed in 20 cc. of saline. This was allowed to stand at 4°C. for 36 hours. In the manner already described, Cl. welchii filtrate in varying amounts was tested for collagenase activity when mixed with 1 cc. portions of dialysate and the nondialysable fraction.

d) To test the heat-stability of the inhibitory principle, 1 cc. portions of normal human citrated plasma were heated at 50–54°C., and at 58–62°C. respectively for 10 minutes. 0.12 cc. of Cl. welchii filtrate was added to each tube containing 1 cc. of heated plasma and the collagenase activity measured on fixed sections as outlined above.

e) When the question arose which non-dialysable fraction of plasma carries the anti-collagenase activity, the following experiment was carried out. To 100 cc. of human plasma, 100 cc. of saturated ammonium sulfate solution was added. This material was filtered and the residue redissolved in 100 cc. of M/10 phosphate buffer (pH 7.4). To the original filtrate ammonium sulfate was added to saturation. This material was then filtered and the residue redissolved in 100 cc. of M/10 phosphate buffer (pH 7.4).

Each protein fraction was then tested for its inhibitory effect on collagenase by the same method used in testing non-fractionated serum. Also commercial immune gamma globulin in the approximate concentration found in human serum and 15% egg albumen in distilled water were tested for collagenase inhibition with this method.

Results

Normal human serum has a definite and readily quantifiable amount of anti-collagenase activity. This is shown to reside in the albumin fraction of serum. Commercial gamma globulin and egg albumen have no demonstrable inhibition of collagenase. The anti-collagenase factor in human serum albumin is inactivated by heating for 10 minutes at 55°C.–60°C. (See table).

TABLE

TEST SUBSTANCE	COLLAGENASE PER CC. No. of units						
	25	50	100	200	300	400	500
Serum albumin	0	0	0	0	0	+	+
Serum globulin	0	0	+	+	+	+	+
Immune gamma globulin	0	+	+	+	+	+	+
Egg albumin	0	+	+	+	+	+	+
Normal human serum	0	0	0	0	0	0	+
Phosphate buffer pH 7.4	0	+	+	+	+	+	+
Dialysate of serum	0	+	+	−	−	+	−
Non-dialysable fraction	−	0	−	−	0	+	−

0.02 cc. of filtrate equal to 50 units.

+ represents maximal action of collagenase.

0 represents no action of collagenase.

Estimation of collagenase and anti-collagenase activity can be made surprisingly well with this method. With the same lot of Cl. welchii filtrate, the minimal amount necessary to cause disappearance of polysaccharides from the basement membrane is, on many trials, consistently 0.02 cc. per 1 cc. of phosphate buffer (pH 7.4).

If the amount of collagenase in 0.02 cc. of Cl. welchii type A filtrate is designated as 50 units, it can be stated that 1 cc. of serum from 5 healthy adults inhibited in each case 400–500 units of collagenase. These tests were run with the serum within 4 hours after it was drawn from the patient.

The sera of three patients with subacute lupus erythematodes and one patient with dermatomyositis were tested individually and in all cases the anticollagenase action was below 400 units and in one case of lupus erythematodes was only 200–300 units. These results represent only preliminary observations. It does not seem practicable to run mass studies with this method. At present a simpler method is being worked out to measure collagenase activity.

Discussion

The question of just how collagenase acts on collagen is not yet clear. Gersh and Catchpole (8) found that collagenase has no action on purified collagen and on teased collagen fibers which were observed under the microscope. In summary these authors say: "The reactive materials of the ground substance may be selectively separated from the enclosed fibrillar structures. These remain morphologically intact, but are fragile. It seems as if the action of the enzymes is to remove cement substances whose presence strengthens the reticular and collagenous structures." They believe that the cement substance is depolymerized into water soluble units. Bidwell and vanHeyingen (7) found collagenase to be inactive on many purified proteins and on polypeptides, similar to those found in collagen. They demonstrated free amino groups after action of collagenase on collagen but apparently not enough to account for the amount of solubilization of collagen and liquefaction of gelatin. They were unable to demonstrate to their satisfaction that collagenase is a peptidase. Thus the nature of the chemical linkage on which collagenase acts is still unknown.

Admittedly when one works with a filtrate of Cl. welchii or even a 'purified' preparation, one is dealing with material which contains numerous enzymes. Collagenase has been definitely distinguished from hyaluronidase, lecithinase and the theta toxin which occur in Cl. welchii filtrates (6). The entity collagenase has been further analyzed by Bidwell (9) and shown to have at least two distinct enzymes, one of which is active on muscle and is destroyed by heating at 56°C. for 10 minutes and another which is still active on 'azocoll' after such heat treatment. The enzyme responsible for the loss of polysaccharides in skin is destroyed by heating at 56°C. for 10 minutes in a phosphate buffer (pH 7.4) and corresponds therefore with the agent found by Bidwell to be responsible for muscle disintegration and is, perhaps, a separate entity from the enzyme which disintegrates 'azocoll.'

Our results with the spot testing method of Hotchkiss indicate that collagenase liberates polysaccharide components from human skin but that these components

are not further hydrolyzed to reducing sugars. The mechanism of this action is not understood but one might speculate that water insoluble complex sugars are depolymerized to water soluble components as Gersh and Catchpole (8) have assumed, or that chemical linkages which bind these complex sugars to the polypeptides of collagen have been broken.

Specific anti-collagenase substance has been shown to be present in sera of animals previously injected with the antigen, collagenase (6). However, as far as we know, no one has referred to an anti-collagenase component occuring in the sera of normal animals or man. Our results show that there is an anti-collagenase component in the albumin fraction of normal human serum. What could be the significance of such a substance? Gersh and Catchpole (8) have shown that many malignant and benign tumors of man as well as some normal tissues contain a substance, collagenase, which disintegrates 'azocoll.' One could postulate a balanced mechanism in which collagenase and anti-collagenase are in equilibrium and if under abnormal conditions this equilibrium is disturbed, alterations in the polysaccharide components may result. In a previous paper (13) definite polysaccharide changes were shown in the so-called collagen diseases: lupus erythematodes, dermatomyositis, lichen sclerosis et atrophicus and poikiloderma (Jacobi).

Preliminary studies on three patients with subacute lupus erythematodes and one patient with dermatomyositis show a small but definite decrease in the anti-collagenase power of their sera as compared with the normal.

It is well known that serum albumin is decreased in patients with lupus erythematodes and dermatomyositis. Possibly this decrease of anti-collagenase activity is a quantitative rather than qualitative factor.

Because our method is not adapted to mass analysis, and we therefore have too few controls, no definite conclusions can be made regarding the significance of the latter findings.

Summary

1. Filtrates of Cl. welchii Type A attack the polysaccharide components of acetone fixed skin leaving morphologically intact collagen bundles and normal appearing elastic fibers.

This action is not present on sections of tissue fixed in aqueous formalin.

2. A method of quantitative estimation of collagenase activity is described.

3. Normal human serum albumin has a definite anti-collagenase action whereas normal human serum globulin, commercial gamma globulin, egg albumin and serum dialysate have no such effect. The anti-collagenase effect of human blood plasma is destroyed by heating to 55°C.–60°C., for 10 minutes.

4. Preliminary studies of the sera of three cases of lupus erythematodes and one of dermatomyositis indicate a decreased anti-collagenase activity as compared with the sera of 5 normal adult subjects.

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DR. STOUGHTON: 6 mm. punch biopsies of normal skin subjected to small amounts of collagenase for 24 hours before fixation and sectioning revealed a very small loss of polysaccharide substances from the basement membrane and collagen bundles. This loss is small compared to that found in 6 μ sections previously fixed in acetone. These results may be explained by the fact that the concentration of enzyme per unit of tissue was much less when the whole biopsy specimen was used.