Multiplicity of *Clostridium histolyticum* Collagenases*

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Two distinct collagenolytic enzyme fractions have been separated from a crude *Clostridium histolyticum* collagenase preparation by gradient elution from a DEAE-Sephadex A50 column. The first of these fractions, though very active against native collagen and synthetic collagenase substrates, shows no activity whatsoever against the unspecific denatured collagen substrate azocoll and negligible activity only against gelatin. Both collagenase fractions are free of activity against casein, hemoglobin, elastin, leucylglycylglycine, or similar peptides, as well as benzoylarginine amide or benzoylarginine naphthylamide with and without potential activators. Ninhydrin tests for the number of peptide bonds cleaved show that both fractions have approximately the same activity against undenatured collagen whether insoluble, salt soluble, or acid soluble. Both fractions break down substrates such as Cbz-Gly-Pro-Gly-Gly-Pro-Ala; but based on the same activity against collagen, fraction I is far more active against the synthetic substrate. Against gelatin on the same basis fraction I shows only 0.01 the activity of fraction II. Further comparison of the two fractions indicates no significant differences in *p*H optimum (approximately 7), electrophoretic mobility, or amino acid composition. The enzymes therefore do not appear to be isozymes but rather multiple forms of collagenases with different specificities.

Collagenases by definition are enzymes capable of digesting native undenatured collagen under physiological conditions of *p*H and temperature (Mandl, 1961). True collagenases are very rare and have been uncontroversially confirmed only in culture filtrates of certain clostridia, in particular several strains of *Clostridium histolyticum*. Since collagenases are associated with many other proteolytic enzymes of lesser specificity but similar physical and chemical characteristics, their purification has at best been incomplete. Separation from nonspecific caseinolytic activity was achieved for the first time by De Bellis *et al.* (1954). As newer methods of purification became available and were applied, refinements in analytical techniques led to the realization that the number of constituents present in the crude collagenase preparations were far greater than originally assumed. A variety of substrates was found susceptible to what must now be considered distinct enzymatic activities. About a year ago we were able to develop two new methods of purification (Keller and Mandl, 1963) which allowed the preparation, reproducibly and in good yield, of relatively pure *Cl. histolyticum* collagenase. The enzyme so obtained, although completely free from previously described activities against casein, hemoglobin, benzoylargininamide, peptides, and the like, showed an elution pattern which indicated the possibility that more than one collagenolytic enzyme might be present. We have now modified one of the two methods, ion-exchange chromatography on a DEAE-Sephadex A50 column, substituting a different gradient-elution system, and thus obtained at least two well-separated fractions with collagenolytic activity. Although both digest native collagen at approximately the same rate, there are differences in their specificity against synthetic collagenase substrates on the one hand and denatured collagen on the other.

Since denatured and altered collagen (gelatin, azocoll) have the same amino acid composition as the native protein it has always been assumed that any true collagenase would be capable of digesting these less-specific substrates, though the reverse was by no means true. After the discovery of synthetic substrates with susceptible peptide bonds (Nagai and Noda, 1959; Heyns and Legler, 1959) it was further believed that the enzyme capable of hydrolyzing these substrates at the fastest rate would be the same one which digested collagen itself at greatest speed. Comparison of the fractions obtained by the method described in this paper showed that these assumptions were incorrect and that a collagenase (I) does exist which will not attack the unspecific substrate azocoll, has minimal activity against gelatin, is free from all known proteolytic activities associated with crude collagenase, and has in terms of its collagenolytic activity a much higher specific rate of synthetic substrate hydrolysis than another collagenase (II), equally free of nonspecific (caseinolytic) activities, but very active against both azocoll and gelatin.

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MATERIALS AND METHODS

Starting material for most of our experiments was a crude *Cl. histolyticum* collagenase, K 264, prepared in our laboratory some 12 years ago (Mandl *et al.*, 1953) by solid ammonium sulfate precipitation of a strain 230–2 culture filtrate. This preparation had been kept in the form of a lyophilized powder at +4° without apparent loss in activity and was somewhat more active than similar preparations from H 4 strain filtrates prepared in our laboratory or obtainable from commercial sources.

This crude enzyme (100 mg) was applied to a 1.2 × 100–cm column of DEAE-Sephadex A50 and a gradient-elution system of Tris-calcium acetate buffers set up in a Varigrad. The first buffer, *p*H 7.2, ionic strength 0.01 M, was replaced, after 1000 ml (100 tubes) had been collected, by the gradient produced by a 4-chamber Varigrad device. Each chamber contained 350 ml of the following buffers: (I) Tris-HCl, 0.01 M; calcium acetate, 0.01 M, *p*H 7.2; (II and III) Tris-acetic acid, 0.02 M; calcium acetate, 0.02 M, pH 6.2; (IV) sodium acetate, 0.06 M; calcium acetate, 0.1 M, *p*H 5.6. Fractions were collected in 10-ml amounts.

The eluted material was tested as follows: (a) ultraviolet absorption at 265 μ for each tube, (b) azocoll test on every tube, (c) synthetic-substrate test against Cbz-Gly-Pro-Gly-Gly-Pro-Ala of every second tube, and (d) casein test for every second tube. On the basis of the graphs resulting from plotting the values

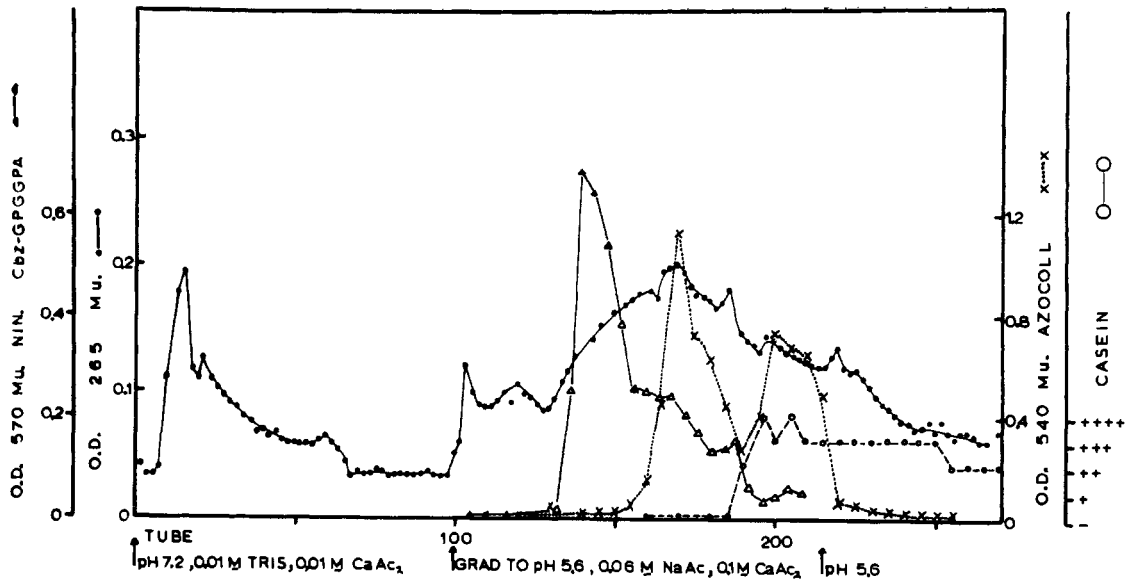

FIG. 1.—Elution pattern from a DEAE-Sephadex A50 column of a *Cl. histolyticum* collagenase (run S 53). For details see text.

so obtained (see Fig. 1), tubes which appeared to form part of one peak were pooled and tests (b) (c) (d) were repeated for each pooled fraction. In addition each of the fractions was now tested for activity against (e) benzoylargininamide in the presence of cysteine activator (f) benzoylarginine-β-naphthylamide, by itself and in the presence of CN-activator (g) Leu-Gly-Gly as a typical peptidase substrate, (h) orcein-elastin, (i) gelatin, (j) native insoluble Achilles-tendon collagen, (k), and other susceptible synthetic collagenase substrates, in particular Cbz-Gly-Pro-Leu-Gly-Pro kindly supplied by Dr. H. Noda. Results are shown in Table I. Test conditions for all except (k) have been described in previous publications from this laboratory

TABLE I

RELATIVE PROTEOLYTIC ACTIVITIES OF FRACTIONS ELUTED FROM DEAE-SEPHADEX A50–COLUMN PURIFICATION OF COLLAGENASE K 264 (RUN S 69)

Substrate	Fraction (mg substrate/mg enzyme/hr) I	II	III
Collagen	85	42	0
Azocoll	0	3600	930
Casein	0	0	>28
Elastin-orcein	0	0	~5
Cbz-Gly-Pro-Gly-Gly-Pro-Ala	710	166	23
Cbz-Gly-Pro-Leu-Gly-Pro	90	10	5
Benzoylarginine-β-naphthylamide	0	0	0
Benzoylargininamide	0	0	0
Leu-Gly-Gly	0	0	0

(Mandl *et al.*, 1953; Keller and Mandl, 1963). Reaction (k) was performed analogously to reaction (c) with the sole exception that incubation was prolonged to at least 2 hours. Experiments were also performed to compare the effect of the fractions on different collagen substrates, substituting (l) neutral salt-soluble collagen and (m) acid-soluble collagen for the insoluble substrate in test (j). To demonstrate possible synergism of fraction I and II tests against collagen were run as in (j) with 1 ml fraction I, 1 ml fraction II, and also 0.5 ml of fraction I plus 0.5 ml of fraction II. The relative number of peptide bonds split was determined by ninhydrin test (Rosen, 1957) of the supernatant and compared with the proportion of collagen solubilized as indicated by both the ninhydrin test for total amino acids and a specific hydroxyproline test (Stegemann, 1958) performed on the acid-hydrolyzed supernatant. This juxtaposition indicates the relative chain length of the products.

Amino acid analyses were performed on a Technicon amino acid analyzer essentially by the method of Piez and Morris (1960) after complete acid hydrolysis for 18 hours with 5.6 N HCl under nitrogen in sealed tubes. N-Terminal amino acid residues were determined by the Konigsberg and Hill (1962) modification of the Edman degradation (Edman, 1950).

Two-dimensional paper chromatograms were made of the DNP derivatives of tryptic degradation products of collagenases I and II to find possible differences between the peptides produced. The solvents used were *sec*-butanol–3% NH_3 (3:1) in the first dimension, phosphate buffer, *p*H 6.5, 1.5 M, in the second dimension. Electrophoretic mobility of the fractions was measured in acrylamide gel in the E-C apparatus (E–C Apparatus Corp., Philadelphia, Pa.) according to Raymond (1962). Approximate molecular weights were estimated after 17-hour runs at 40,000 *g* in sucrose gradients in a Spinco Model L 2 ultracentrifuge with swinging-bucket rotor.

Differences in serological activity were determined by the method of Oakley and Warrack (1950) and Mandl and Zaffuto (1958) comparing the amounts of six multivalent *Cl. histolyticum* antisera of known anticollagenase titer that were required for complete inhibition of collagenolytic activity of fractions I and II, respectively. Immunodiffusion on agar plates against the same six sera was tested by the Ouchterlony (1953) procedure. To reaffirm their identities fractions I and II were rechromatographed on columns of Sephadex A50. The column eluates corresponding to these fractions were diluted with three volumes of the starting buffer, Tris-HCl, *p*H 7.2, 0.01 M–calcium acetate, 0.01 M. This provided the necessary low ionic strength for adsorption of the enzymes to the Sephadex ion exchanger. Each sample was then applied to a column and eluted in the same manner as in the original fractionation. Eluates were tested for activity against Cbz-Gly-Pro-Gly-Gly-Pro-Ala, azocoll, and collagen.

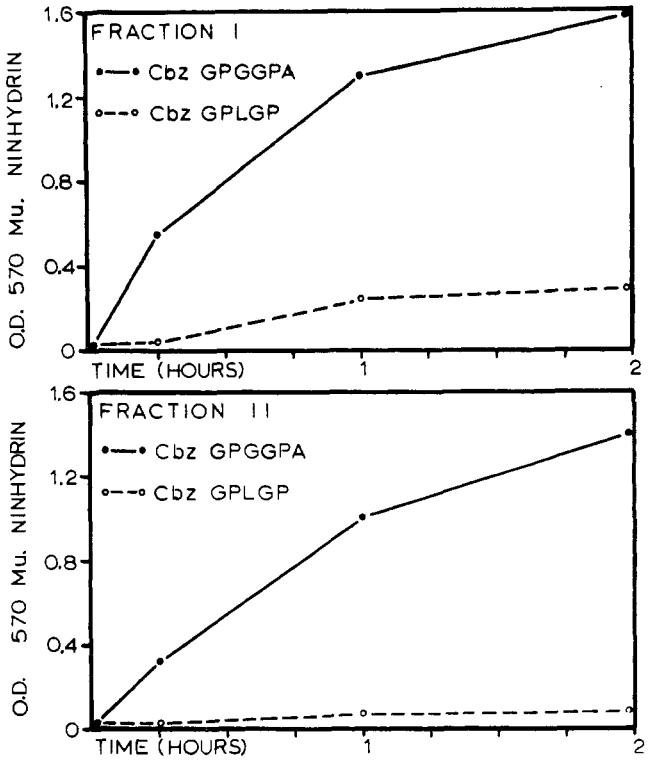

FIG. 2.—Comparison of reaction rates against two synthetic substrates (run S 69).

RESULTS

Figure 1 shows a typical separation using K 264, an ammonium sulfate–precipitated crude-collagenase preparation, as the starting material. These runs are reproducible and have been repeated with essentially the same results many times. Invariably there are two well-defined collagenolytic zones, fractions I and II. Neither has any activity against casein, hemoglobin, elastin, benzoylargininamide, benzoylarginine-β-naphthylamide, or peptide substrates. Both are free from pigment. Both have approximately the same activity against native collagen as measured by ninhydrin reaction though there are slight differences between runs. Activity against synthetic substrates is considerably higher for fraction I than for fraction II. Similar relative ratios are found against other synthetic substrates, though the absolute rate against Cbz-Gly-Pro-Leu-Gly-Pro is much lower (see Fig. 2). Activity against the unspecific substrate azocoll (an azo dye coupled to denatured collagen) which is broken down by trypsin, papain, chymotrypsin, and numerous other proteolytic enzymes, as well as by crude collagenase and fractions II and III, is completely absent from fraction I. Gelatinase activity, though present, is not more than 0.01 of that observed in fraction II. Fraction III has no collagenase activity but has all the caseinolytic activity and all the elastase activity, as well as azocoll and gelatinase activity (see Table I). No peptidase, amidase, or naphthylamidase activity was observed in either fraction. We assume that these activities are retained on the column under our experimental conditions or else, though less likely, irreversibly inactivated. Further comparison of the two collagenolytic fractions showed no significant difference in *p*H optimum, though the maximum was broad both against collagen and synthetic substrates (Fig. 3). Both enzymes require calcium for stability as well as for activity. EDTA and also exhaustive dialysis irreversibly inactivate the purified collagenases; unlike the stabler crude enzyme,

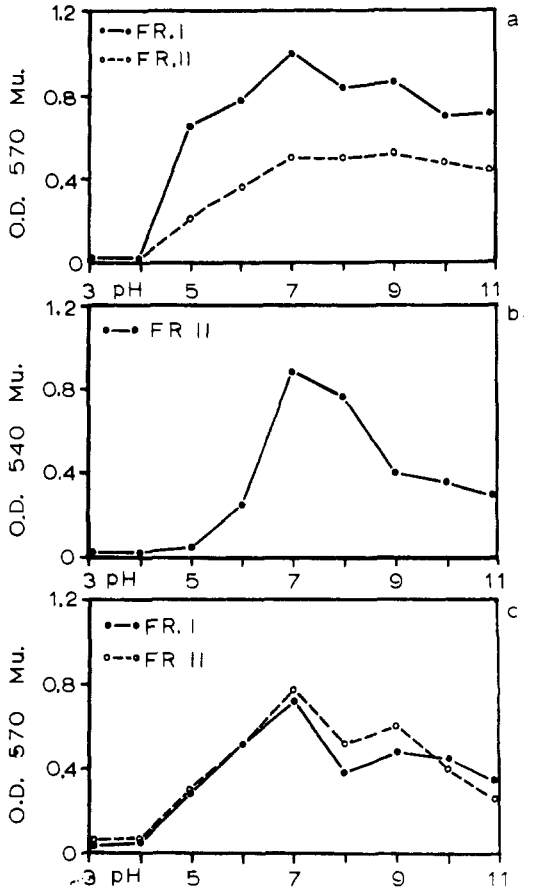

FIG. 3.—pH-activity curves against various substrates. (a) Cbz-Gly-Pro-Gly-Gly-Pro-Ala, (b) azocoll, (c) collagen; (run S 53).

they can not be reactivated by addition of calcium or other metal ions. Cysteine also inactivates both enzymes irreversibly. In contrast to reports by Heyns and Legler (1960), who used a DEAE-cellulose purified collagenase, we found that synthetic-substrate hydrolysis by the fractions was also inhibited by cysteine. Both fractions hydrolyzed native Achilles-tendon collagen to the same extent, producing peptides with an average chain-length of four to five amino acids. Soluble collagens were also susceptible to both fractions to an equal extent but the absolute hydrolysis rate was considerably higher for the neutral salt–soluble collagen. Acid-soluble collagen was digested to a greater extent by fraction II, the enzyme capable of digesting gelatin and azocoll (see Table II). Synthetic substrates are digested considerably faster by fraction I. Figure 2 shows the relative digestion rate of Cbz-Gly-Pro-Gly-Gly-Pro-Ala and also Cbz-Gly-Pro-Leu-Gly-Pro. The latter substrate is digested at a much slower rate in spite of the fact that R_1 = leucine instead of glycine, which has been reported to increase susceptibility ap-

TABLE II

RELATIVE ACTIVITIES OF FRACTIONS I AND II (RUN S 53) AGAINST DIFFERENT COLLAGEN SUBSTRATES[a]

Substrate	Fraction I	Fraction II
Insoluble tendon	0.76	0.70
Neutral salt soluble	3.2	2.9
Acid soluble	0.2	0.3

[a] Results are based on ninhydrin tests after 18-hours hydrolysis and calculated in μmoles leucine equivalents released per mg collagen.

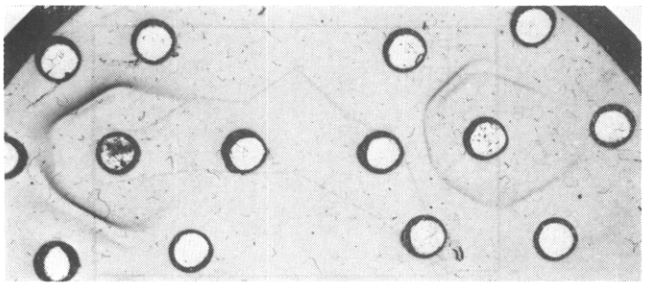

FIG. 4.—Section of Ouchterlony plate showing precipitin lines of fraction I (left) and fraction II (right) with six multivalent horse antisera (run S 53).

TABLE III

SYNERGISTIC ACTION OF COLLAGENOLYTIC FRACTIONS (RUN S 69) AGAINST COLLAGEN[a]

	Fraction I (1 ml)	Fraction II (1 ml)	Fraction I (0.5 ml) + Fraction II (0.5 ml)
Ninhydrin	0.8 μmole	1.0 μmole	1.6 μmoles
Hydroxyproline	16 μg	12 μg	28 μg

[a] Results are based on tests after 24-hours hydrolysis and calculated in μg hydroxyproline and in μmoles leucine equivalents released per mg collagen, respectively.

TABLE IV

RELATIVE DILUTIONS OF SIX MULTIVALENT HORSE ANTISERA COMPLETELY INHIBITING COLLAGEN HYDROLYSIS BY FRACTIONS I AND II (RUN S 53)[a]

Serum No.	Inhibitor Dilution		Relative Antititer		
	Fraction I	Fraction II	Fraction I	Fraction II	β
2001	25	50	100	100	100
2005	75	175	300	350	350
2008	90	200	380	400	370
2035	60	125	225	250	230
8003	50	100	200	200	260
8006	40	75	190	150	180

[a] Antititers calculated from these dilutions are compared with the β-antitoxin activity of each serum (Oakley and Warrack, 1950).

proximately 20-fold (Heyns and Legler, 1960). This is probably owing to the decrease in rate with unprotected rather than blocked terminal proline. The relative activities of the two fractions, however, were the same against either substrate. It appears that fraction I is primarily involved in the splitting of the susceptible peptide bond whereas fraction II contains another, less specific component. That there is a difference in the mode of action of the enzymes gains support from the results of experiments proving the synergistic effect of the two fractions. Table III shows that equal amounts of fractions I and II hydrolyzed collagen at a faster rate than equivalent amounts of either fraction alone.

To further test the identity of the enzymes serological titrations with multivalent immune sera were made. Table IV shows that the same relative values were obtained with both fractions and that the antititers are those determined for *Cl. histolyticum* β-toxin (collagenase) (Oakley and Warrack, 1950). The absolute amounts required to completely inhibit fraction I, however, are twice those necessary to inhibit fraction II. Ouchterlony plates (Fig. 4, 5) made with the same six sera indicated cross reactivity of one component present in both fractions but showed additional components that were identical. It thus appears that even fraction I, the most specific collagenase ever prepared, is not homogeneous. Physical measurements therefore reflect only the composition of the particular preparations.

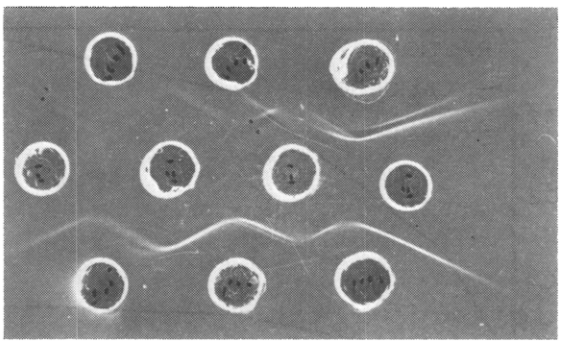

FIG. 5.—Section of Ouchterlony plate showing cross reactions of identity and nonidentity of fractions I and II (alternating in center row) against six antisera (top and bottom rows) (run S 53).

TABLE V

AMINO ACID COMPOSITION OF COLLAGENOLYTIC FRACTIONS (RUN S 69)

	Fraction I	Fraction II
Aspartic acid	7.14	9.30
Glutamic acid	8.97	7.40
Serine	8.71	8.57
Threonine	4.86	4.90
Glycine	12.44	11.96
Alanine	11.86	9.33
Proline	8.34	5.34
Valine	4.70	5.21
Leucine	4.61	4.70
Isoleucine	3.32	4.07
Methionine	1.42	1.82
Tyrosine	3.39	4.23
Phenylalanine	3.26	3.93
Lysine	6.37	8.63
Histidine	5.77	5.81
Arginine	4.88	4.81

Amino acid composition is shown in Table V. It will be noted that there are differences between the values given here and those published for the collagenase purified with Sephadex (Keller and Mandl, 1963). The high lysine content of the latter, however, has meanwhile been shown to be caused by an artifact. The isoelectric points of the two fractions described in this paper are near neutral. There does not seem to be a significant difference in the composition of the two fractions except possibly the higher content of certain neutral amino acids, notably proline and alanine in fraction II. The N-terminal amino acids could not be established with certainty. Probably owing to the inhomogeneity of the preparations, several amino acids appeared to be present in N-terminal positions; of these glutamic acid predominated in fraction I, serine in fraction II.

DNP-peptide maps of tryptic digests of both fractions showed essentially the same breakdown products, indicative of similar configurations at least in the region of the susceptible basic amino acid residues. Molecular-weight estimations by ultracentrifugation gave an average of 112,000. There was no significant difference between fraction I and fraction II. The electrophoretic mobilities of the collagenolytic fractions in acrylamide gel were indistinguishable. This similarity in physical properties is not surprising in view of the fact that electrophoresis as well as other methods had failed to reveal the presence of more than one enzyme. Since only DEAE derivatives of supporting media

(Sephadex or cellulose) effect separation of the collagenases their most likely difference would be one of charge. Although we have definitely identified two collagenolytic enzymes only, a look at the elution diagram (Fig. 1), in particular the shoulder associated with fraction II, and also the evidence of the Ouchterlony plates (Figs. 4, 5) makes us suspect that more components may be present. Since their specificity is not the same we do not think that they can be considered isozymes, but prefer to consider them as multiple forms of closely related enzymes.

The fractions I and II which were rechromatographed on Sephadex A50 showed up as single peaks in the expected locations in the elution diagrams. Fraction I rerun showed activity against Cbz-Gly-Pro-Gly-Gly-Pro-Ala and collagen and none against azocoll. Fraction II rerun showed activity against azocoll, Cbz-Gly-Pro-Gly-Gly-Pro-Ala, and collagen all in one well-defined peak with the maxima coinciding at the same point. This, along with the observed lack of caseinolytic activity in fraction II, indicates that the activities found in fraction II were not carried over from adjacent fractions.

Discussion

The presence of more than one collagenolytic enzyme in crude *Cl. histolyticum* collagenase has been suspected before. Grant and Auburn (1959) obtained three fractions with different electrophoretic mobilities after batchwise elution from DEAE-cellulose columns, and Schuytema (1956) reported collagenases with different isoelectric points using Ivalon-sponge electrophoresis. At this time the specificity requirements of collagenase (Nagai and Noda, 1959; Heyns and Legler, 1959) had not been determined and no difference in the susceptibility of substrates to the fractions was suspected.

After we had eluted the two collagenases and reported our unexpected finding at the Fall, 1963, American Chemical Society meeting (Mandl *et al.*, 1963) we heard that essentially similar results had been obtained with DEAE-cellulose by Noda and his co-workers in Japan (Noda *et al.*, 1964) and at Worthington Biochemical Corp. (R. Egan, personal communication, 1963). Both these groups found differences in the susceptibility of synthetic substrates to the two fractions. We have tested the Worthington preparation and found that fraction I, like our own, will not attack azocoll. Yoshida and Noda (1964) recently reported that their fractions, unlike ours, showed similar activity against gelatin. Like ourselves they detect large differences in activity against synthetic substrates, but though they too find that azocoll is split faster by the fraction showing less activity against synthetic substrates, they do not completely eliminate the azocoll activity. Presumably their separation is less complete. They also feel that the small differences observed in molecular weight and amino acid composition are significant but that the enzymes cannot be distinguished immunochemically. Our immunochemical results as well as the synergistic action of the two fractions against collagen indicate that more than one component may be involved in collagenolysis, but the actual mechanism of action remains obscure.

We are aware that unfortunately these new enzymes, in spite of their great specificity, are by no means pure. We know that inorganic ions and carbohydrate material derived from the column contribute to the impurity. On the other hand, the greater the purification the lower is the stability of the enzyme. Whereas the crude enzyme can be dialyzed and lyophilized without loss of activity, the purified preparations are easily inactivated by these processes. The enzymes appear to be stable indefinitely in calcium-Tris buffer solutions but removal of calcium irreversibly inactivates them, possibly by a collapse in the tertiary structure. Lyophilization also leads to almost complete loss of activity. We are trying to overcome this by concentration through ultrafiltration and are confident that eventually extraneous impurities can be eliminated. In the meantime we feel that the lack of activity of fraction I against the unspecific substrate azocoll and the minimal effect against gelatin require a revision of our concepts of the mode of action of collagenase. The great specificity of this fraction should furthermore allow new approaches to the study of the mechanism of collagenolysis and, through investigation of the breakdown products, the microstructure of collagen.

Acknowledgments

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