Collagenase and Protease Activity

in

Bacteroides melaninogenicus

by

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ABSTRACT

A rapid sensitive collagenase assay has been developed using ^{14}C-acetyl-ated collagen as the substrate. Acid-soluble calfskin collagen was labelled with acetic-1-^{14}C-anhydride at pH 8. The radioactively labelled collagen remained susceptible to <u>Clostridium histolyticum</u> collagenase but resistant to nonspecific proteolysis indicating that denaturation of the collagen had not occurred as a result of the acetylation procedure. The rate of release of ^{14}C from labelled collagen by <u>C</u>. <u>histolyticum</u> collagenase was proportional to enzyme and substrate concentrations. The results of the ^{14}C assay correlated with those of other collagenase assays tested.

Collagenolytic activity of <u>Bacteroides melaninogenicus</u> was examined both <u>in vitro</u> and the guinea pig model system using known collagenase-produc-ing strains and recent isolates. In contrast to the reported results of pre-vious workers, only two of the thirty <u>B</u>. <u>melaninogenicus</u> strains isolated were found to have collagenase activity. Collagenase was found in washed cells of positive strains of <u>B</u>. <u>melaninogenicus</u> as early as day one. No activity was ever observed in culture supernatants. Studies of material aspirated from guinea pigs infected with pathogenic <u>B</u>. <u>melaninogenicus</u> demonstrated the presence of collagenase in the infectious exudates. The enzyme was associated with the bacterial cells, was dependent on reducing conditions and was stimulated by a factor in filtered exudate.

Partial characterization of cell-free protease activity in <u>B</u>. <u>melanino-genicus</u> has shown the protease to be dependent on reducing agents, requiring 5×10^{-3} M cysteine for activity. Other reducing agents were also effective in stimulating protease activity. The activity was stable at 4°C, resistant to autodigestion, and sensitive to EDTA at concentrations of 10^{-3}M and above. The pH optimum for activity against Azocoll was 8.0.

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I. INTRODUCTION

Infections characterized by mixed populations of nonsporulating anaerobes have been known for some time (1,5,30). Examples include brain abscesses, lung abscesses, appendicitis, human bite wounds and periodontal disease. A characteristic mixed bacterial population, the so-called "fusospirochaetal complex" was observed in these infections. Experimental mixed infections containing the same "fusospirochaetal complex" could be produced by subcutaneous inoculation of experimental animals with human gingival debris or material from a variety of abscesses (21,46,49). Rosebury and co-workers (40), in a study of experimental mixed infections produced with gingival debris, found that the mixture retained its infectivity through several animal passages or through several transfers as a mixed culture in vitro, but that recombination of pure cultures isolated from the mixture failed to produce the "typical" fusospirochaetal infection.

The criteria used for defining a typical transmissible infection as used by these workers were summarized by Socransky and Gibbons (47); a typical infection is described by Macdonald et al. (28). Experimental mixed infections in the guinea pig model system, as described by these workers, can be of two types. One is a walled-off localized abscess containing foul-smelling exudate which can be used to transmit the infection to a second animal. The other is a rapidly spreading necrotic infection which perforates the abdominal wall or the skin. The animal loses hair and necrosis of the skin occurs in the abdominal region. The exudate readily infects other animals. Positive infectivity is indicated by death, development of spreading necrotic lesions, or development of a localized transmissible abscess. Hard, nodular, nontransmissible caseous abscesses or mild inflammation indicate noninfectivity. Thus a typical transmissible infection is one that (a) is infective according to

the above description and (b) can be passaged via the exudate to another animal.

Macdonald et al. (28) succeeded in producing typical transmissible mixed infections in guinea pigs when seventeen pure cultures isolated from an infection were recombined and injected into an animal. By successively eliminating various organisms from the original combination of seventeen, he was able to reproduce the typical "fusospirochaetal" infection with a combination of four organisms, none of which were fusiforms or spirochaetes (29). Inoculation of an animal with the "pathogenic quartet," which included two strains of Bacteroides, a motile gram-negative rod and a facultative diphtheroid, always produced the typical transmissible lesion. One of the Bacteroides species was identified as B. melaninogenicus, and further studies on the mixed infection (27) showed that this organism was an essential component of the system. In the study of one mixed anaerobic infection, it was found that no infection occurred when B. melaninogenicus was omitted from the recombination mixture. Other members of the infective combination could be replaced or omitted depending on the strain of B. melaninogenicus used (48).

In the four-organism system the role of the diphtheroid was found to be the production of a naphthoquinone compound required by the B. melaninogenicus strain. Substitution of a naphthoquinone-independent B. melaninogenicus strain for the original strain used showed that the diphtheroid was then no longer required for infectivity and was in fact eliminated from the mixture (27). Recombination of B. melaninogenicus with populations of organisms from which it had been eliminated restored infectivity to a number of systems (48). Evidence thus implicated B. melaninogenicus as the primary pathogen in mixed infections, and the organism closest to being an overt pathogen of all the normal inhabitants of the oral cavity (27,48).

On the assumption that elucidation of the pathogenic mechanisms involved

in experimental mixed infections might provide some understanding of the mechanisms involved in the pathogenicity of clinical anaerobic infections, investigators became concerned with the production of potentially damaging metabolites, toxins, or other factors which would explain how B. melaninogenicus and associated organisms were able to cause infection. Although Macdonald had suggested earlier that perhaps mixed infections were bacterially nonspecific but biochemically specific in terms of toxins, lytic enzymes and other damaging factors produced by the mixed population (26,29), the demonstration of the essential role of B. melaninogenicus in the experimental system suggested that the "nonspecific" infections were in fact dependent on the presence of B. melaninogenicus and that the role of other organisms was one of supporting and enhancing the in vivo growth of the primary pathogen (27,48). Consequently B. melaninogenicus was examined for pathogenic properties.

B. melaninogenicus is a gram-negative, nonmotile, nonsporulating anaerobic rod. The species is somewhat loosely defined, comprising those organisms producing black colonies when incubated anaerobically on blood agar. The organism can isolated from the oral cavity, feces, and a number of naturally occurring clinical infections (5,23,51,53). Most strains require hemin and many require vitamin K or a related naphthoquinone for growth (12,13). Although significant differences have been found among strains in carbohydrate fermentation patterns, menadione requirement and colony morphology (42), investigators have been unable to find any useful patterns dividing them into taxonomic groups and find the "species" to represent a biochemically and immunologically diverse group of organisms (6).

Macdonald and Gibbons (27) had shown that the organism produces H_2S, DNase, indole, and NH_3, and it was known that the organism contained endotoxins (31). B. melaninogenicus was found to be active against a number of protein substrates (12,27,42). All strains tested were able to substitute

for the original strain of B. melaninogenicus in the mixed infection (27).

While differences were found between strains with regard to protease, colla-

genase, DNase, RNase, NH_3, indole, H_2S and fermentation patterns, no correla-

tion between any of the above and pathogenicity could be found (27) until

Kestenbaum (20) demonstrated a positive correlation between collagenase ac-

tivity and infectivity for four B. melaninogenicus strains in a guinea pig

system. Collagen degradation is a feature of periodontal disease (24,46,47),

and although B. melaninogenicus is the only organism indigenous to the oral

cavity known to produce a collagenase (12), the relationship between collagen-

ase production and the pathogenicity of the organism either in oral lesions

or in other mixed anaerobic infections remains unclear. (Whether factors

other than collagenase were involved in Kestenbaum's system is not known.)

Enhancement of a fusobacterial infection in rabbits by simultaneous injection

of a crude cell-free preparation of B. melaninogenicus collagenase was demon-

strated by Kaufman (19). Thus there is evidence that B. melaninogenicus

collagenase plays a role in the organism's pathogenicity.

The unique properties of collagen and the collagenolytic enzymes have

resulted in the formulation of specific criteria for the definition of

collagenases. A thorough discussion of the collagen molecule has been edited

by Ramachandran (37), and a recent review of collagen biosynthesis has been

written by Bornstein (4); while excellent reviews of the collagenolytic

enzymes have been written by Seifter and Harper (44,45); Eisen and co-workers

(9) and Nordwig (34). The collagen molecule is characterized by its sensi-

tivity to collagenases but resistance to other proteolytic enzymes. The

basic unit or tropocollagen molecule consists of three polypeptide chains

intertwined into a rigid helical structure, the unusual nature of which is

partly due to its amino acid composition. In most of the polypeptide chain every third amino acid is glycine, and the molecule contains a large amount of proline and hydroxyproline. Because collagen is one of the few proteins containing appreciable amounts of hydroxyproline, the purity of a collagen preparation can be determined by assaying the amount of this amino acid present (12,14). Solutions of native collagen have high viscosities and form a rigid, opaque gel when incubated at 37°C. Both properties are absent in gelatin, the denatured form of collagen. Gelatin is also susceptible to a number of proteases which are incapable of attacking native collagen. Native collagen can thus be distinguished from its denatured state by physical properties as well as enzyme susceptibility.

The collagenases constitute a class of unique proteases capable of attacking native collagen in the helical portion of the molecule. Because of the susceptibility of gelatin to a number of proteases, valid assays for collagenase activity must use undenatured collagen as the substrate and must be performed under non-denaturing conditions. Many assays for collagen-olytic activity are based on the measurement of physical changes in collagen preparations, such as a decrease in the viscosity of collagen solutions (43,44) or the dissolution of collagen fibers in suspensions or gels (3, 15,44). These procedures require a considerable amount of substrate and are difficult to quantitate. Synthetic peptides with sequences identical to portions of the collagen molecule have been used with Clostridium histolyticum collagenase but are unsatisfactory for collagenases of different specificities or for enzymes of questionable purity, as a number of peptidases incapable of attacking native collagen can cleave the synthetic peptides, and a number of true collagenases have no activity against the peptide substrates (16). A ^{14}C assay based on the release of ^{14}C-glycine-containing peptides from ^{14}C label-led guinea pig skin collagen was developed by Gross and co-workers (32) and

is widely used for tissue collagenases (7,10,39). ^{14}C-proline and ^{3}H-proline have been used to label chick embryo collagen (35,41). In all of these systems the radioactive collagen is prepared by injecting the animal with the ^{14}C-amino acid shortly before extracting collagen from the tissue. Although small amounts of enzyme can be assayed quantitatively, the specific activity of the collagen preparations is variable, and the purity of the ^{14}C-protein must be determined after labelling.

The collagenase of B. melaninogenicus differs from other microbial collagenases in that it is cell-bound and requires cysteine or some other reducing agent for activity (12). The enzyme is released when the cells lyse. Hausman and Kaufman (17) succeeded in isolating collagenase activity in a particulate fraction obtained by centrifuging the autolysate supernatant at 100,000 x g for one hour. Further attempts to purify the collagenase from the particulate fraction, or to separate it from the caseinolytic activity with which it is associated have been unsuccessful.

This thesis is concerned with the study of collagenase activity in B. melaninogenicus both in vitro and in the guinea pig model system. Partial characterization of protease activity in B. melaninogenicus culture supernatants will also be discussed. Specific points to be covered include:

(1) Development of a rapid and sensitive collagenase assay based on the release of ^{14}C-peptides from labelled collagen.

(2) Studies on collagenase activity in B. melaninogenicus cells

    (a) in vitro

    (b) in the guinea pig model system.

(3) Studies on protease activity in B. melaninogenicus culture supernatants.

II. MATERIALS AND METHODS

A. Organisms.

Bacteroides melaninogenicus strains K110 and CR2A, obtained from Dr. P.A. Mashimo, were collagenolytic strains originally isolated by Macdonald and co-workers (27,28). Other strains were isolated in the laboratory from gingival scrapings taken from persons in varying states of oral health. The organisms were isolated from cultures grown on blood agar plates (Difco blood agar base to which was added 5% laked human blood). The plates were incubated either in anaerobic jars evacuated and flushed with $H_2:CO_2$ (95:5) (Matheson) or in an anaerobic glove box (Coy Mfg., Ann Arbor, Michigan) containing an atmosphere of $N_2:H_2:CO_2$ (85:10:5). After 4-7 days incubation at $37^\circ C$, black colonies were picked from the plates and subcultured on the same medium until pure cultures were obtained.

Staphylococcus aureus was a laboratory stock strain. Cultures of both organisms were maintained by weekly transfer or lyophilization.

B. Growth of organisms.

Liquid cultures of B. melaninogenicus were maintained in the trypticase-yeast extract medium described by Gibbons and Macdonald (12). Glucose was routinely omitted from the medium as were menadione and $NaHCO_3$. Liquid cultures were incubated at $37^\circ C$ either in the anaerobic chamber or in stoppered tubes gassed with $H_2:CO_2$ (95:5).

Since neither vitamin K_1 nor menadione appeared to enhance the growth of vitamin K-requiring strains when added to the medium, vitamin K requirers were grown on blood agar plates with a streak of S. aureus. Liquid medium for vitamin K-requiring strains was prepared as follows: filter-sterilized vitamin K_1 (0.5% in 95% ethanol) was added to sterile 2% agar which had been cooled to $50^\circ C$. The mixture was dispensed into tubes in 1 ml quantities and

allowed to solidify. Ten ml of the standard liquid medium then was added.

Thioglycolate, was not included in the medium as vitamin K_1 is inactivated by

reducing compounds.

Table I lists the vitamin K requirements and infectivity of the B. melan-

inogenicus strains most frequently used. Strains not listed in the table did

not require vitamin K and were noninfective.

C. Chemicals and chromatographic materials.

Acetic-1-^{14}C anhydride was obtained from New England Nuclear. Azocoll

was purchased from Calbiochem. Clostridium histolyticum collagenase, trypsin,

subtilisin, subtilopeptidase, pronase, vitamin K_1, bovine serum albumin (BSA)

and hydroxyproline were obtained from Sigma. Chymotrypsin was obtained from

Worthington. Bio-Gel P-60 was supplied by Bio-Rad laboratories, and Sephadex

G-75 was purchased from Pharmacia.

D. Preparation of bacterial cells for enzyme assays.

Cells for enzyme assays were harvested by centrifugation and resuspended

in the assay buffer at a concentration 10x that of the original culture.

Cells used for viscosity assays were dialyzed against Tris-HCl buffer (0.1M,

pH 7.2) containing 10^{-2}M cysteine for 4 hours before the assay.

E. Preparation of culture supernatants for enzyme assays and for column chro-

matography.

Cells were sedimented from 48 hour cultures by centrifugation. The

supernatant was filtered through a 0.45 μ filter (Millipore Corp.) to remove

any remaining bacterial cells and was then concentrated to 1/10-1/20 the

original volume using an ultrafiltration apparatus (Amicon) fitted with a

PM-10 membrane. The concentrated supernatant was diluted to the original

volume with distilled water and re-concentrated twice before use.

F. Extraction of collagen.

Neutral salt-soluble collagen was extracted from guinea pig skin accord-

Table I

Properties of B. melaninogenicus Strains

| strain | vitamin K | collagenase | infectivity | |
			pure culture	mixed culture
CR2A	-	+	-	-
K110	-	+	-	?
2D	-	+	+	+
GP14	+	+	-	+
GP2	-	-	-	-
MA-R	-	-	-	-

ing to the method of Gross (14). Acid-soluble collagen was extracted from fresh fetal calfskin as described by Gallop and Seifter (11), except that ultracentrifugation of the pooled citrate supernatants was replaced by filtration through fine sintered glass to remove particulate matter. Lyophilized collagen was stored in stoppered flasks at -20°C.

G. Acetylation of collagen.

Lyophilized acid-soluble collagen was solubilized in 0.01% cold acetic acid by stirring overnight at 4°C. The concentration of collagen was 2 mg/ml. Immediately prior to the addition of the acetylating agent the pH of the collagen solution was brought to 8.0 by the addition of 1M K_2HPO_4. The acetylating agent, acetic-1-^{14}C anhydride in benzene, was added dropwise over a period of 2 hours. A typical preparation consisted of 250 mg of collagen and 10.2 mg of anhydride (100 μCi/mg). The temperature of the reaction mixture was maintained at 10°C in a water-ice bath, and the mixture was stirred continuously on a magnetic stirrer. The pH was monitored by a pH meter and readjusted to 8.0 if necessary by the addition of 1N NaOH. When all of the anhydride had been added, stirring of the mixture was continued for another hour in the cold. The mixture was acidified with glacial acetic acid to solubilize any collagen which had gelled and to facilitate removal of the benzene. The acetylated collagen was dialyzed against 15 l of distilled water at 4°C to remove ^{14}C-acetic acid, a by-product of the reaction. Dialysis was continued until no further ^{14}C was detected in the dialysate, which usually required 5 days with frequent changes of distilled water. The acetylated collagen was lyophilized and stored at -20°C.

H. Assays for collagenase activity.

The ^{14}C-collagen substrate was prepared by solubilizing lyophilized, acetylated collagen in 0.01% acetic at a concentration of 1 mg/ml by stirring overnight at 4°C, The resulting clear, viscous solution was stored at 4°C

for periods of up to two months. Typical reaction mixtures for both C. histo-
lyticum collagenase and B. melaninogenicus collagenase are shown in Table II.
Commercial enzyme from C. histolyticum was used in the assay buffer at a con-
centration of 30 units/ml. The reducing agent employed with the B. melanino-
genicus enzyme was cysteine hydrochloride, made up to a concentration of
5×10^{-2}M in the assay buffer and neutralized by adding 5N NaOH before use.
The cysteine solution was always prepared immediately prior to use. The
assay components were incubated for fifteen minutes before the addition of
substrate and incubation was continued at room temperature. The reaction was
stopped and unreacted collagen precipitated by the addition of 0.04N phospho-
tungstic acid (PTA) and 2N HCl to final concentrations of 0.01N and 0.5N
respectively. A 0.1 ml aliquot of the reaction mixture was added to a "micro-
fuge" tube (Beckman) containing 50 µl each of PTA and HCl. The samples were
left at room temperature for ten minutes before centrifugation for five minutes
in a Beckman-Spinco 152 microfuge (Beckman Instruments). 100 µl of the
supernatant was counted. The control sample for each assay contained buffer
in place of enzyme.

Viscosity assays were performed as described by Seifter and Gallop (43)
using a viscometer with a flow rate of 80-100 seconds for distilled water.
When B. melaninogenicus cells were used as the source of enzyme, the dialyzed
cell suspensions were preincubated with 5×10^{-2}M cysteine for 15 minutes at
room temperature.

I. Guinea pig infections.

Cells for inoculation of animals were harvested from blood agar plates
or from broth cultures. Colonies from plates were resuspended in approxi-
mately 1 ml of phosphate-buffered saline (PBS), pH 7.0, and a portion of
the suspension was injected into the animal. Cells from broth cultures were
harvested by centrifugation and resuspended in the desired volume of PBS

Table II

Protocol for Collagenase Assay

component	volume (ml)	
	B. melaninogenicus	C. histolyticum
^{14}C-collagen (0.1%)	0.2	0.2
Tris-HCl buffer (.05M, pH 7.2) with $CaCl_2$ (.005M)	0.1	0.2
cysteine (.005M)	0.1	-
collagenase (30 units/ml)	-	0.1
cell suspension (10x)	0.1	-

unless otherwise stated. Guinea pigs used for the studies were 200-250 grams

in weight. The animals were shaved and inoculated subcutaneously in the groin

area. They were observed for up to six weeks. Exudate was aspirated from

infected animals using a sterile disposable syringe while the animal was under

light ether anesthesia. The exudate was examined for microbial purity by

plating on blood agar.

J. Assays for proteolytic activity.

Proteolytic activity was determined by measuring activity against Azocoll

or casein. The reaction mixture for the Azocoll assay contained: Tris-HCl

buffer (0.05M, pH 7.2), 4.8 ml; neutralized cysteine hydrochloride in the

same buffer (0.05M), 1.2 ml; and enzyme, 0.5 ml. The reaction components were

incubated at $37^{o}C$ for 15 minutes before the addition of Azocoll (20 mg), and

incubation was continued at the same temperature in a shaking water bath.

Two-ml samples were removed at desired time intervals, chilled immediately in

ice, and centrifuged or filtered to remove insoluble material. The amount of

dye released was determined by measuring the absorbance of the supernatant at

520 nm.

The substrate for the casein assay was prepared by dissolving 1 g of

casein (Hammarsten) in 100 ml of phosphate buffer (pH 7.4), and heating 15

minutes in a boiling water bath. The reaction mixture contained 2.5 ml casein,

1.0 ml of neutralized cysteine hydrochloride ($5 \times 10^{-2}M$) when required, 0.5-1.0

ml of enzyme, and buffer to 5.0 ml. The mixture was incubated at $37^{o}C$ and the

reaction terminated by the addition of 5.0 ml of 10% trichloroacetic acid.

After 30 minutes at room temperature the contents of the tubes were filtered

and the A_{280} of the filtrates determined.

K. Polyacrylamide gel electrophoresis.

Gel electrophoresis was performed using the method of Nagai et al (31).

Samples were denatured prior to electrophoresis by heating to $50^{o}C$ for 15 min-

utes.

L. Hydroxyproline assay.

Hydroxyproline was assayed by the method of Leach (22). Citrate-extract-
ed collagen (5 mg/ml) was hydrolyzed in vacuo in 6N HCl by heating to 108°C
for 18 hours. The hydrolyzed samples were neutralized with NaOH, diluted to
100 μg protein/ml and filtered prior to assaying for hydroxyproline.

M. Protein determination.

Protein was determined by the method of Lowry et al. (25) using BSA
as a standard.

III. RESULTS

A. Development of a new collagenase assay.

The main criterion for any valid collagenase assay is the use of unde-
natured collagen as the substrate. Acetylation of the epsilon amino groups
of the lysine residues of collagen was the labelling procedure chosen because
the reaction can be carried out under mild conditions minimizing the possi-
bility of denaturation of the collagen molecule. The validity of the ^{14}C
assay was examined by determining (1) the purity and properties of the citrate
extracted collagen, (2) the properties of the acetylated collagen and (3) the
sensitivity of the detection method to changes in enzyme and substrate concen-
tration.

1. <u>Analysis of the citrate-extracted collagen.</u>

a. <u>Hydroxyproline</u>. The purity of the citrate-extracted collagen was
determined by measuring the amount of hydroxyproline present in a weighed
sample. A value of 12 μg/100 μg protein, or 12%, was obtained when the
citrate-extracted collagen was analyzed for hydroxyproline. This agrees with
the value of 13% given for the hydroxyproline content of calfskin collagen (8).

b. <u>Polyacrylamide gel electrophoresis</u>. When subjected to gel elec-
trophoresis according to the method of Nagai (32), the heat-denatured collagen
stained as three bands corresponding to the α-, β-, and γ-components.
This is in agreement with the expected results.

c. <u>Viscosity assays; enzyme susceptibility</u>. Citrate-extracted
collagen was susceptible to collagenase but resistant to trypsin when assayed
viscometrically. The viscosity of the collagen preparation was evidence that
the collagen was not denatured.

d. <u>Gel formation at 37°C</u>. Formation of the typical rigid, opaque
gel was observed when collagen was prepared according to the method of Bennick

and Hunt (2).

2. Acetylation of collagen.

a. Results of a typical acetylation. Table III shows the results of a typical acetylation of acid-soluble calfskin collagen. The amount of label incorporated ranged from 4-7%. The proportion of lysine residues labelled was calculated using a value of 4.0% for the lysine content of collagen (8) and assuming that the epsilon amino group of lysine was the principal group labelled under these conditions (38).

b. Effect of pH on the efficiency of acetylation. The acetylation procedure was carried out in phosphate buffer at pH values of 7.0, 7.5, and 8.0. The substrate, neutral salt-soluble collagen at a concentration of 0.2%, was prepared by stirring overnight at $4^{o}C$ in the appropriate buffer. Equal amounts of the acetylating agent were added to each sample. The acetylated samples were dialyzed against 0.1M phosphate buffer, pH 7.0, for three days before analysis. As shown in Table IV, the acetylation procedure was most effective at pH 8.0 of the pH values tested.

3. Analysis of acetylated collagen.

a. Precipitation of collagen. Collagen was precipitated in initial experiments by adding ethanol to a final concentration of 50% (43). Since 8-10% of the radioactivity remained in the supernatant after ethanol treatment, a number of common protein precipitants were tested in an attempt to reduce background counts. Two types of collagen preparation were used in these experiments. "Insoluble" collagen was a fine suspension of collagen fibers prepared by stirring the ^{14}C-collagen overnight at $4^{o}C$ in Tris buffer (.05M, pH 7.0), with added $CaCl_2$ (.005M); "soluble" collagen was prepared by solubilizing the acetylated collagen in 0.01% acetic acid, then neutralizing the solution. "Soluble" collagen was a more viscous, gel-like preparation than was "insoluble" collagen. Table V summarizes the results obtained with

Table III

Acetylation of Collagen

protein added (mg)	250
protein recovered (mg)	211
specific activity (dpm/mg collagen)	6.25×10^5
label incorporated (%)	7.0
lysine residues labelled (%)	20.2
^{14}C in solution after addition of PTA and HCl (%)	1.4

Table IV

Effect of pH on Efficiency of Acetylation

pH	specific activity (cpm x 10^{-4}/mg)
7.0	1.5
7.5	0.45
8.0	5.9

Each reaction mixture contained : collagen, 5 mg; phosphate buffer, pH as indicated, 1 m mole; acetic-1-^{14}C-anhydride (5 μCi/μmole), 5 μmoles. Total volume 2.5 ml, temperature 10°C, reaction time 1 hr.

Table V

Non-precipitable ^{14}C

collagen preparation	precipitating reagent	concentration	soluble ^{14}C (%)
insoluble	acetone	30%	10.3
"	"	50%	66.2
"			
"	BSA	2 mg/ml	
	EtOH	50%	9.9
"	ZnCl$_2$	1.0%	
	EtOH	50%	9.1
"	polyethylene glycol	15%	
	EtOH	50%	9.2
"	EtOH	50%	8.7
"	PTA	.01N	
	HCl	.5N	6.0
"	none	--	40.5
soluble	none	--	90.0
"	PTA	.01N	
	HCl	.5N	0

several of the precipitating reagents tested. The precipitating agents were added to the collagen samples. After 15 minutes at ice bath temperature the samples were centrifuged 5 minutes in the Microfuge. Control samples were centrifuged 5 minutes without addition of any precipitating agent. Since the lowest backgrounds were obtained using "soluble" collagen and a combination of PTA and HCl (44), ^{14}C-collagen was always prepared for assays by solubilization in 0.01% acetic acid. Even in later experiments when collagen of higher specific activity was obtained, background counts remained around 1-2% of the total counts.

b. Susceptibility of ^{14}C-collagen to collagenase and other proteolytic enzymes. Table VI shows the percentage of radioactivity released from ^{14}C-collagen by collagenase and a number of proteolytic enzymes. The substrate remains susceptible to the action of both C. histolyticum and B. melaninogenicus collagenases but resistant to attack by a number of common proteases. Some release of ^{14}C due to nonspecific proteolytic attack on the non-helical portions of the molecule would be expected (36). The release of a small percentage (10%) of counts by trypsin and other proteases was presumed to reflect this and is in agreement with the results of others (38).

c. Protease-treated collagen. ^{14}C-acetylated collagen in 0.01% acetic acid (1 mg/ml) was incubated with subtilisin. The reaction mixture contained 5 mg collagen, 37 units of enzyme, and Tris-HCl buffer (0.05M, pH 8, with .005M CaCl$_2$) to a final volume of 10 ml. Incubation was continued for 6 hr at room temperature, after which the reaction mixture was dialyzed overnight against distilled water at 4°C to precipitate the collagen. The precipitated collagen was centrifuged and washed with distilled water until no protease activity (as detected by measuring dye release from Azocoll) remained in the pellet. The pellet was solubilized in 0.01% acetic acid. Preliminary incubation of the ^{14}C-collagen with subtilisin produced a substrate

Table VI

^{14}C Solubilized by Proteolytic Enzymes

enzyme	concentration (mg/ml)	^{14}C solubilized (%)[*]
collagenase	0.2	75.0
trypsin	0.1	9.7
subtilisin	17.5	3.2
subtilopeptidase	17.5	7.1
pronase	1.25	1.0
chymotrypsin	6.25	1.3
B. melaninogenicus strain CR2A	20.0	46.6

[*]Incubation time 2 hr. for CR2A, 1 hr. for other enzymes.

Table VII

Release of ^{14}C from Untreated and Pre-digested ^{14}C-collagen

by Subtilisin and Collagenase

collagen	enzyme	soluble ^{14}C (%)
untreated	collagenase	
	C. histolyticum	61.0
	B. melaninogenicus	46.5
	subtilisin	7.4
pre-digested	collagenase	
	C. histolyticum	44.0
	B. melaninogenicus	47.5
	subtilisin	0.95

which remained susceptible to attack by both clostridial and B. melaninogeni-cus collagenases but was less susceptible to attack by proteases (Table VII). This indicated that acetylation had not denatured the main helical portion of the molecule. The results obtained also indicate that the ^{14}C has actually labelled the helical, collagenase-susceptible portion of the molecule rather than only the non-helical, protease-susceptible ends of the collagen molecule.

4. Kinetic studies of the collagenase assay.

a. Release of radioactivity from labelled collagen by clostridial collagenase. The time course of release of radioactivity from ^{14}C-collagen by C. histolyticum collagenase is plotted in Figure 1. The rate of release of ^{14}C was linear during the early part of the reaction. Two rates of reaction were observed when low concentrations of collagenase were used, indicating that a number of less reactive sites are available to the enzyme after the initial cleavage of the molecule.

b. Effect of substrate concentration. The release of ^{14}C from labelled collagen when substrate concentration was varied followed a typical saturation kinetics pattern as shown in Figure 2. When higher concentrations of substrate were used, however, accurate sampling was made impossible by the high viscosity of the reaction mixture.

c. Effect of enzyme concentration. The rate of release of radioactivity from ^{14}C-collagen during the earliest part of the reaction varies directly with enzyme concentration, as shown in Figure 3. In this case a concentration of 1.1 μg/ml of C. histolyticum collagenase produced significant release of ^{14}C. Smaller amounts of enzyme could be detected if the reaction mixture was incubated for longer periods of time.

Figure 1. Release of ^{14}C from acetylated collagen by <u>C</u>. <u>histolyticum</u> collagenase.

The reaction mixture contained: ^{14}C-collagen, 1.9 x 10^5 cpm/mg, 200 µg; Tris buffer, pH 7.2, 160 µmoles; CaCl$_2$, 4 µmoles; <u>C</u>. <u>histolyticum</u> collagenase, 1.5 units. Total volume, 1.05 ml; temperature, 37°C.
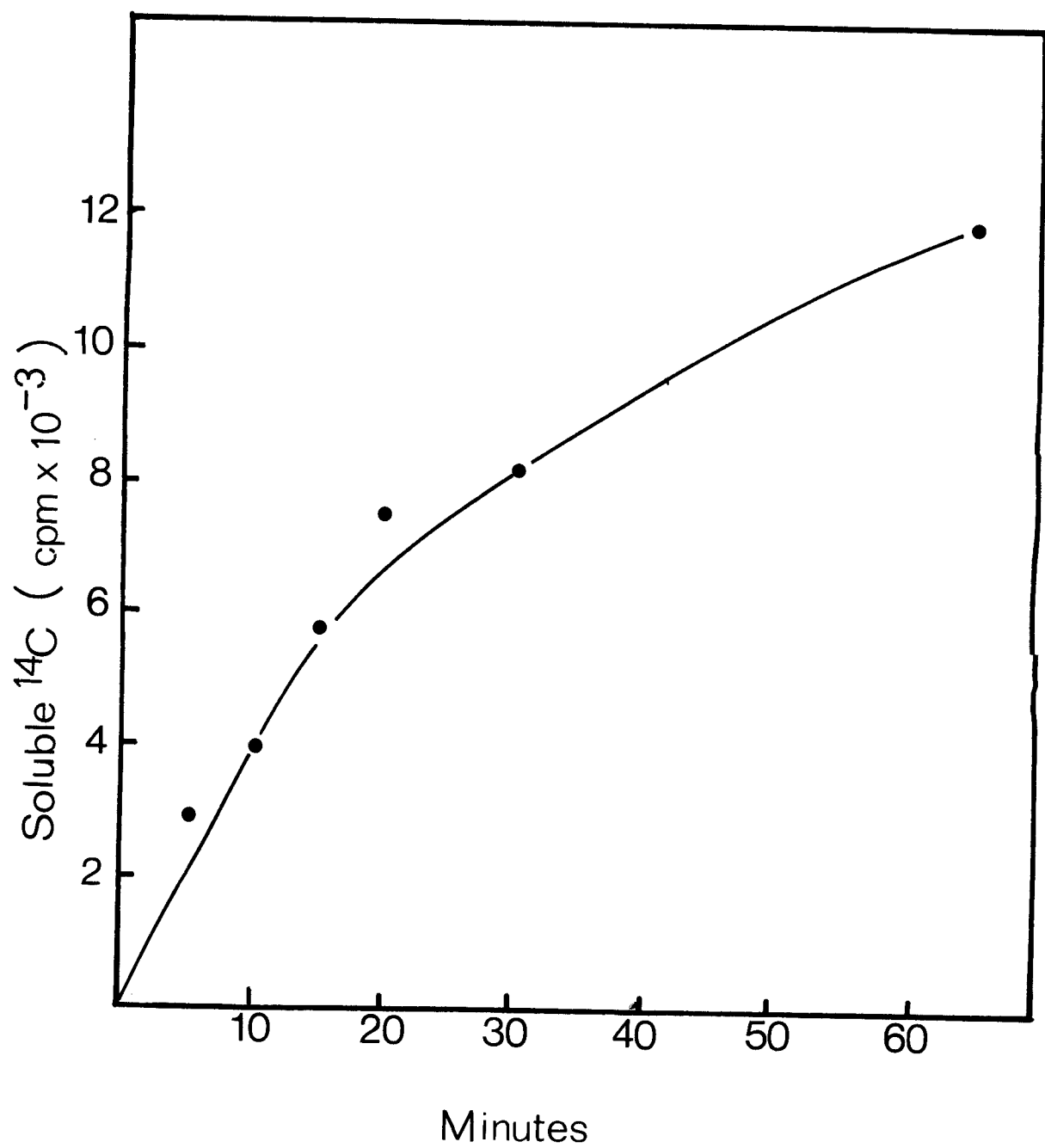
Figure 2. Release of ^{14}C from acetylated collagen with increasing substrate concentration.

Each reaction mixture contained: ^{14}C-collagen, 1.9 x 10^5 cpm/mg, as indicated; Tris buffer, pH 7.2, 480 µmoles; CaCl$_2$, 12 µmoles; C. histolyticum collagenase, 0.375 units. Total volume 2.45 ml, temperature 37°C, reaction time 30 minutes.
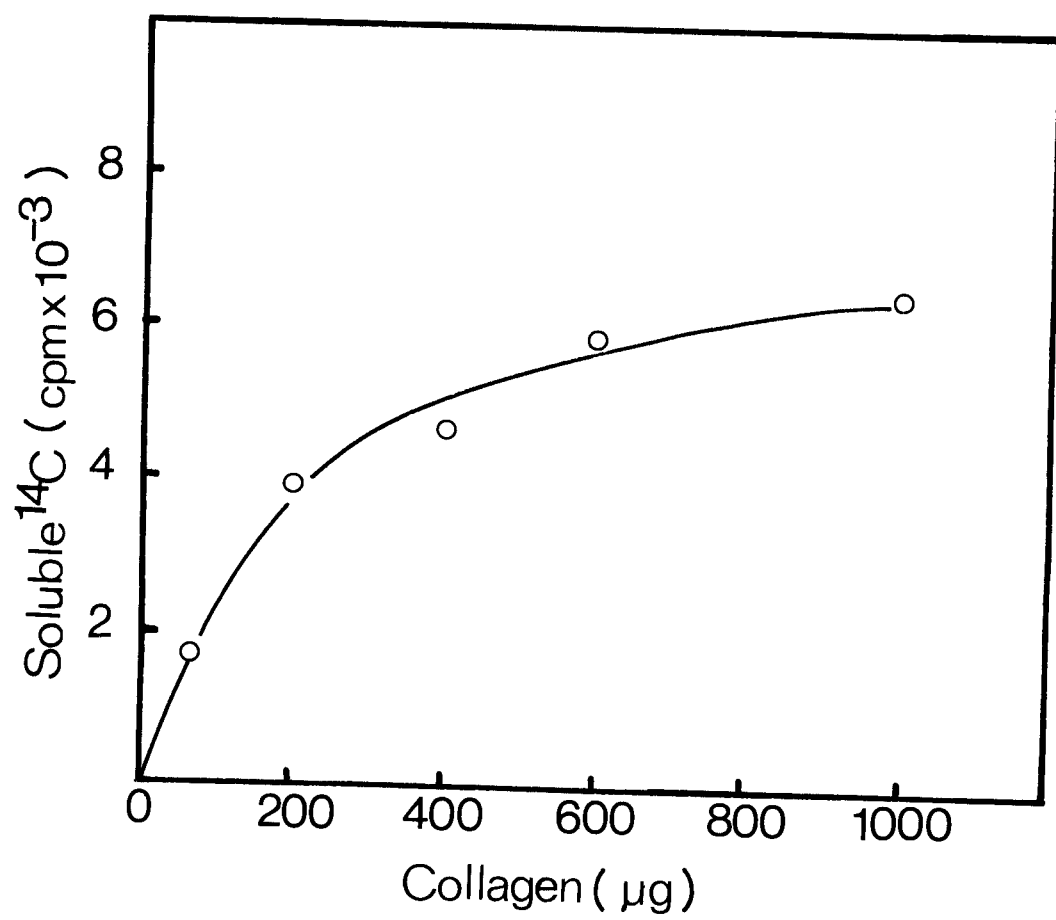
Figure 3. Release of ^{14}C from acetylated collagen as a function of col-

lagenase concentration.

Each reaction mixture contained: ^{14}C-collagen, 1.9 x 10^5 cpm/mg.

200 µg; Tris buffer, pH 7.2, 160 µmoles; CaCl$_2$, 4 µmoles; enzyme as indi-

cated. Total volume 1.05 ml, temperature 37°C.
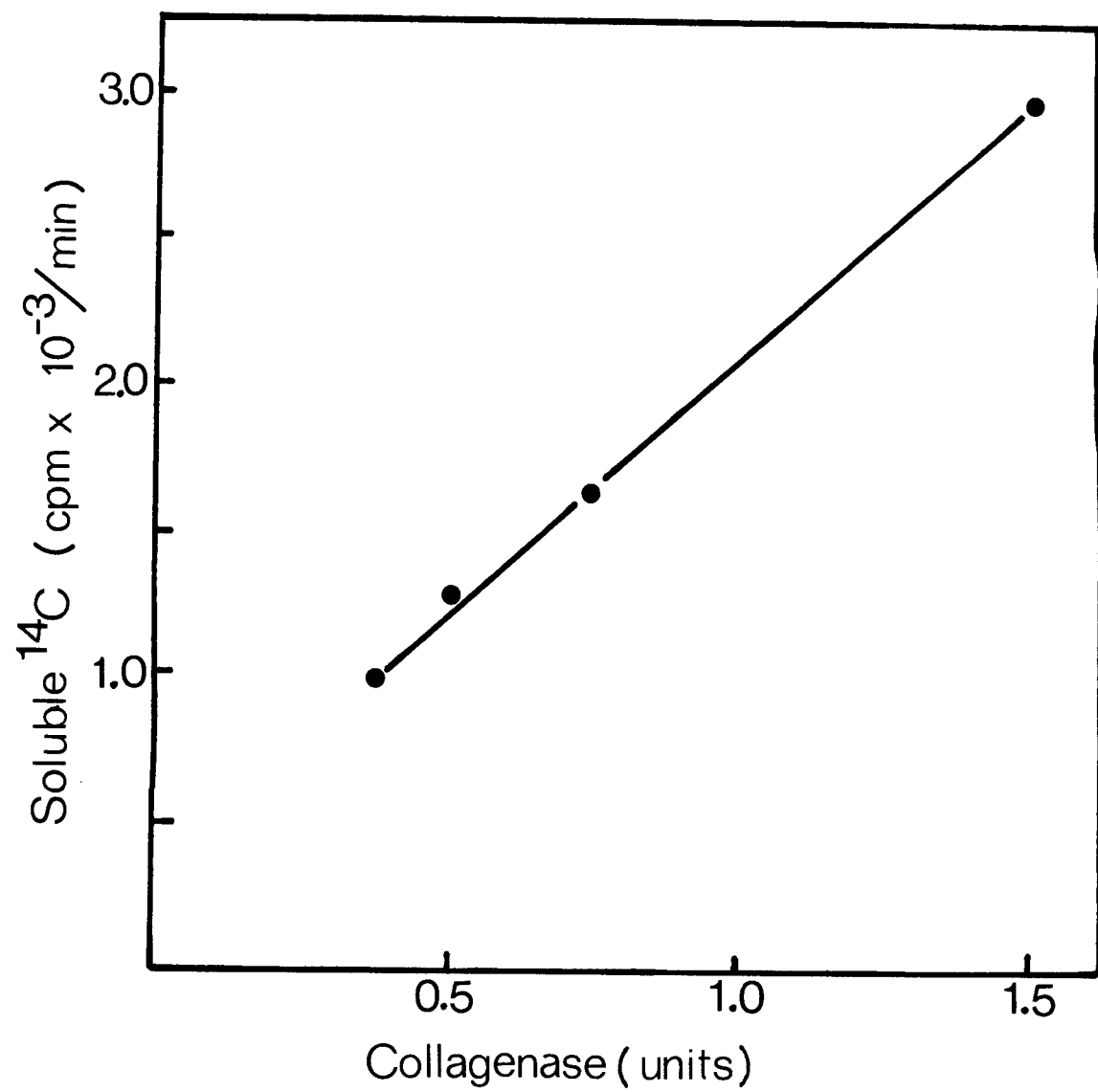
B. Collagenase in B. melaninogenicus: in vitro studies.

    1. Attempts to isolate collagenase-producing strains. Thirty strains

of B. melaninogenicus isolated from different gingival samples were tested for

activity against ^{14}C-collagen. The results are shown in Table VIII. Since

the experiments were done on separate occasions the incubation times and ages

of the cells vary somewhat. From the data it can be seen, however, that only

two of the thirty strains were active against ^{14}C-collagen. No activity was

found in any of the supernatants of the samples tested. Assays for collagenase

activity using the viscometric method gave results identical to those obtain-

ed with the ^{14}C assay. The lack of collagenase activity in the majority of

strains tested contrasts with the findings of others (12,42) who detected

collagenase activity in all strains tested.

    2. Attempts to increase collagenase activity. Macdonald et al. (27)

found that collagenolytic activity was apparently increased in B. melaninogen-

icus cells grown in dilute medium, which suggested that the enzyme might be

repressed in the normal growth medium. In an effort to either de-repress or

to induce collagenase, a number of B. melaninogenicus strains from the above

group of thirty were grown in medium containing 1/10 the normal amount of

trypticase and supplemented with insoluble collagen. Growth in collagen-

supplemented medium was about 1/10 that in normal medium, as estimated by

absorbance at 660nm, and counts released from ^{14}C-collagen remained at back-

ground levels for organisms grown both in standard and in collagen-supplemen-

ted media. Substitution of acid-soluble collagen for the insoluble collagen

had no effect.

    Because cobalt ions have been found to stimulate C. histolyticum collagen-

ase and Zn^{++} is an intrinsic part of the enzyme structure (44,45), both ions

were tested on B. melaninogenicus cells. Neither ion had any activating

effect when added to the reaction mixture.

Table VIII

Collagenase Activity in Thirty B. melaninogenicus Isolates

isolate	culture age (days)	incubation time (hrs)	^{14}C released (%)	collagenase activity
2	2	1	4.4	−
13	2	1	2.6	±
8	2	1	0.7	−
12	2	1	6.9	−
15	2	1	3.0	−
9	2	1	9.8	?
6	2	1	4.9	−
14	2	1	0.25	±
11	2	1	2.1	−
10	2	1	1.6	−
16	7	2	4.3	−
17	7	2	3.7	−
DG	7	2	3.7	−
TP	7	2	4.5	−
BW	7	2	3.5	−
PN	7	2	4.3	−
MA−R	7	2	4.6	−
H−1	7	2	3.9	−
DW	7	2	5.3	−
R6	7	2	3.9	−
3D	3	2	0.25	±
2D	3	2	23.0	+
AF	3	2	1.9	−
FC−1	3	2	1.3	−
CP	3	2	0.13	−
JG	3	2	0.38	−
GP2	5	2	4.7	−
GP14	3	3	44.0	+
FC	3	2	1.2	−
TN	3	2	0.13	−

3. <u>Collagenolytic activity in collagenase producing strains CR2A and K110</u>.

    a. Viscosity assays. Figure 4 illustrates the reduction in viscosity of a collagen solution when incubated with cells of <u>B</u>. <u>melaninogenicus</u> strains CR2A and K110. Both <u>B</u>. <u>melaninogenicus</u> strains were active against collagen when assayed by this method.

    b. ^{14}C assays. Both strains were active against ^{14}C-collagen as illustrated in Table IX. All activity was found in the cell suspensions rather than in the supernatants.

    c. <u>Study of collagenase production by K110 as a function of culture age</u>. K110 cells and supernatants were assayed for activity against ^{14}C-collagen over a period of 10 days. Cells were grown in standard trypticase-yeast extract medium, centrifuged and resuspended in buffer at 20x their original concentration. Collagenase activity was detectable as early as day one and was found exclusively in the cells (Table X). CR2A produced similar results. Assays for collagenase activity using the viscometric method also gave positive results at day one. This contrasts with the finding of Gibbons and Macdonald (12), who found that collagenolytic activity was not evident until the culture began to lyse (72 hrs). However, their assay method, based on the release of hydroxyproline from collagen gels, may not have been sensitive enough to detect activity during earlier stages of growth.

C. Collagenase in <u>B</u>. <u>melaninogenicus</u> infectious exudates.

    1. <u>Studies of the infection</u>. Although Macdonald and co-workers (27) established <u>B</u>. <u>melaninogenicus</u> as an essential component of the mixed anaerobic infection in the guinea pig system, and Kestenbaum demonstrated a correlation between collagenase activity and infectivity (20), little else is known about the relationship of <u>B</u>. <u>melaninogenicus</u> collagenase to pathogenicity. Some of the objectives in attempting to establish the infective system in guinea pigs were to (1) determine whether collagenase activity of the organisms

Figure 4. Viscometric assay of collagenolytic activity.

The reaction mixtures contained: collagen, 8 mg; Tris buffer, pH 7.0, 320 μmoles; $CaCl_2$, 2 μmoles,; cysteine 72 μmoles; 10^9 bacterial cells. Total volume, 5.2 ml, temperature $25^{\circ}C$.

ΔT is the elapsed time (flow time) for each reading. The midpoint is defined as the time elapsed from the beginning of the assay to the beginning of the particular reading, plus 1/2 the elapsed time for that reading (1/2 ΔT).
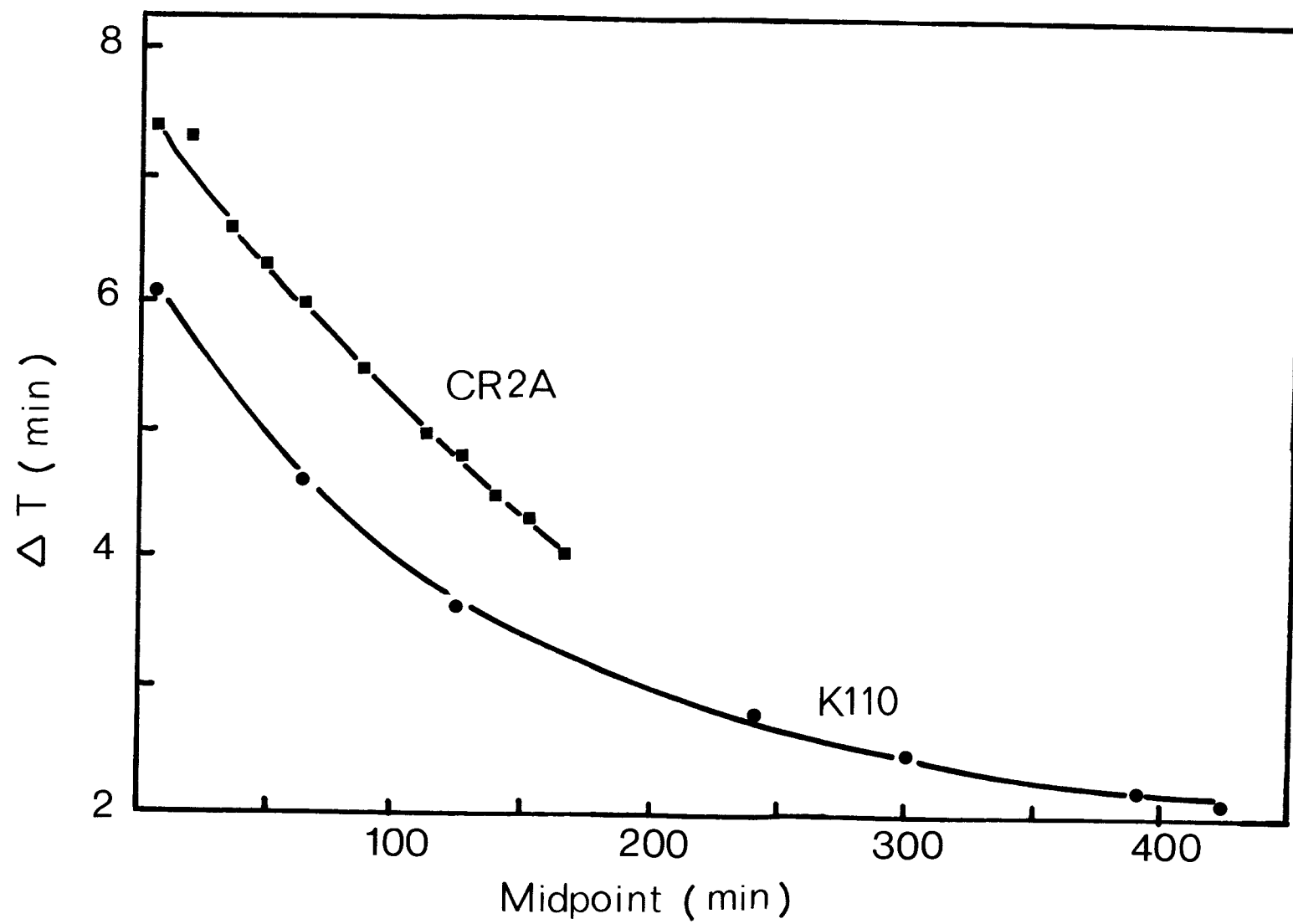
Table IX

Collagenase Activity in <u>B.</u> <u>melaninogenicus</u> CR2A and K110

addition	soluble ^{14}C (cpm)[*]	% total ^{14}C
none	0	0
K110 cells	5074	56.5
supernatant	262	2.9
CR2A cells	3411	38.0
supernatant	248	2.8

[*]Corrected for background
Incubation time 3 hr.

Table X

Collagenase Activity as a Function of Culture Age

age of culture (days)	absorbance (660 nm)	^{14}C solubilized (%)[*]	
		cells	supernatant
1	0.95	50	0
2	1.2	64	0.6
3	1.2	60	3.0
4	1.5	49	0
7	0.85	96	1.5
8	0.62	36	0.6
9	0.45	33	3.7
10	0.37	11	0.7

[*]Incubation time 5 hr.

correlated with infectivity, (2) determine whether collagenase could be induced by animal passage, and (3) determine whether collagenase was produced in the infection itself.

a. <u>Establishment of a mixed infection</u>. Initial attempts to establish a transmissible mixed infection in guinea pigs were unsuccessful. Gingival scrapings from a number of persons in varying states of oral health were cultured on blood agar plates for one week, following which the colonies were scraped from the plates, resuspended in sterile saline and injected into animals. The response was at best a small nodular lesion confined to the site of injection, from which a small amount of thick pustular material could sometimes be aspirated. In no instance could an infection be produced in a second animal by injection of the exudate.

A transmissible mixed infection was finally established with a mixed culture obtained from a periodontal patient. The day following the injection the animal developed an abscess containing watery, dark coloured, foul-smelling exudate which contained large numbers of coccobacilli, spirochaetes, long rods, and motile organisms. Inoculation of a second animal with 0.1 ml of exudate produced a rapidly spreading infection. Autopsy of the infected animals showed marked tissue necrosis in the area of injection, with perforation of the peritoneal wall in one animal. The characteristics of the infection were similar to those described by Macdonald (28) for very acute mixed anaerobic infections.

b. <u>Organisms from the infectious mixture</u>. A serial dilution of exudate from an infected animal was prepared and samples were plated on blood agar. Saline suspensions of colonies scraped from the plates were injected into animals with the purpose of establishing a typical infection with a minimum number of species. Organisms removed from the 10^{-7} dilution of exudate were able to produce the typical infection; the <u>B</u>. <u>melaninogenicus</u> strain

isolated from this mixture was designated GP 14. The infective mixture was maintained as a mixed culture on blood agar and was also transferred several times on trypticase soy agar to eliminate the B. melaninogenicus from the mixture. The mixed culture without B. melaninogenicus was designated GP 25 and was maintained as a mixed culture on blood agar. The mixed culture alone was not infective, but infectivity was restored when GP 14 was added back to the culture. Strain GP 14 was not infectious by itself.

c. Recombination of collagenase-producing strains with GP 25. Four known collagenase-producing strains of B. melaninogenicus were recombined with the noninfectious "helper organisms" of the GP 25 mixture in an attempt to produce infection. The B. melaninogenicus strains used were GP 14, originally isolated from the mixture; 2D, which was isolated from a periodontal patient; and K110 and CR2A, which were collagenase producing strains originally isolated by Gibbons and Macdonald (27,28). The B. melaninogenicus strains were grown with the mixture on blood agar plates prior to injection into animals. Guinea pigs weighing 200 g were injected with 0.6 ml of a 2 ml saline suspension of organisms scraped from the plates. The results in Table XI show that infections were produced by both GP 14 and 2D but not by CR2A and K110. Strain K110 has subsequently been found to be infective when inoculated in combination with one organism from the GP 25 mixture.

d. Production of a transmissible infection with a single strain of B. melaninogenicus. B. melaninogenicus strain 2D was tested for its ability to produce an infection without the support of other organisms. Cells from a liquid culture were washed, resuspended in sterile PBS and injected into a guinea pig. Within 18 hours the animal had developed symptoms of a rapidly spreading infection: marked edema in the thoracic area, darkening of skin and loss of hair in the thoracic area, and considerable weight loss. Material aspirated from the animal was dark in colour, watery and foul-smelling, and

Table XI

Infections Produced by Recombination of B. melaninogenicus

with a Mixed Culture

B. melaninogenicus strain added to GP25	infection (5 days)	transmissibility
K110	localized response	−
CR2A	localized response (slight)	−
GP14	rapidly spreading abscess, burst to outside	+
2D	very rapidly spreading infection; animal dead by day 5	+

when examined by phase-contrast microscopy appeared to contain a pure culture of B. melaninogenicus along with red blood cells, white blood cells and pus cells. Fatty acid analysis revealed that acetic, propionic, isobutyric, butyric and isovaleric acids were present. These acids are produced by B. melaninogenicus in in vitro culture. Inoculation of the exudate onto blood agar showed that it contained a pure culture of B. melaninogenicus. Strain 2D was thus one of the few B. melaninogenicus strains capable of producing an infection without the support of other organisms (27,49,50). The infection produced symptoms similar to those described in the literature for infections produced by CR2A (27). However, CR2A was never found to produce an infection, either alone or in combination with other organisms. Thus it would appear to have lost some factor required for infectivity.

e. Attempts to characterize the infection produced by 2D. Since 2D was infective by itself, the 2D infection was chosen as the simplest system to study in an attempt to delineate some of the requirements for infectivity. However, because of the variability among animals and the fact that 2D lost infectivity with continued culture, the results of this study can only be noted as general trends. The following observations were made:

(1) Guinea pigs weighing less than 250 g were more susceptible to infection than larger guinea pigs.

(2) The production of a successful infection usually required 10^9 cells as determined by direct count. However, since viable counts (as determined by plate count and Most Probable Number methods) were often less than 10% of the total counts, the number of viable cells required to produce an infection may have been much less than 10^9.

(3) 48-hour cells produced successful infections in a greater percentage of cases (66%) than did 24-hour cells (30%). 72-hour cells were as effective as 48-hour cells.

Figure 5. ^{14}C released from collagen by whole exudate.

Each reaction mixture contained: ^{14}C-collagen, 4.5 x 10^5 cpm/mg, 200 µg; Tris buffer, pH 7.0, 20 µmoles; CaCl$_2$, 1 µmole; cysteine where indicated, 10 umoles; exudate, aspirated 48 hrs. after infection, 1.5 x 10^9 bacterial cells. Total reaction volume, 0.5 ml, temperature, 25°C.
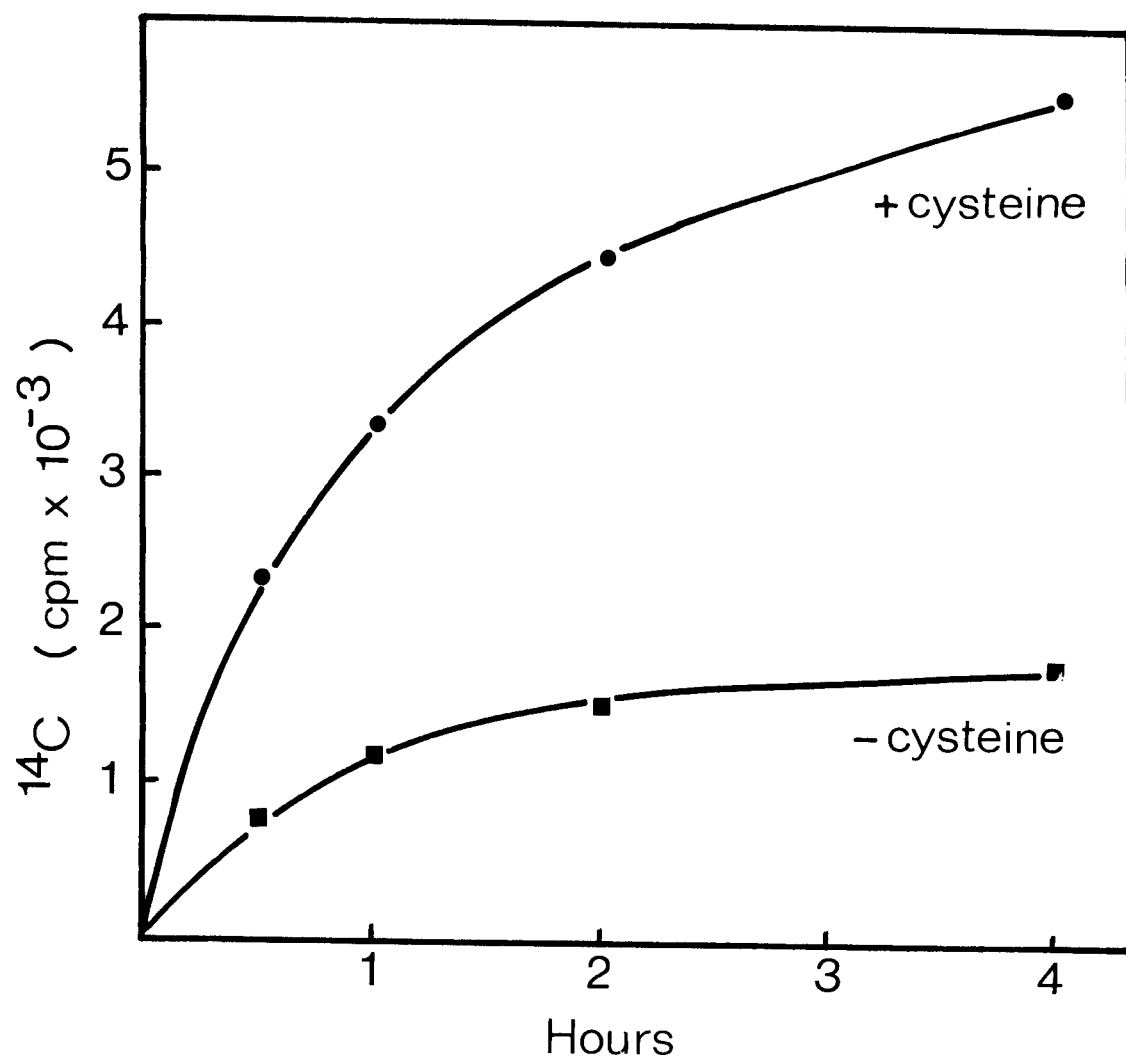
(4) Successful infections could usually be produced with cells from lyophilized cultures when the cultures maintained in the laboratory did not infect. This supports the idea that the strain lost some property required for infectivity.

2. <u>Studies of collagenase activity in material aspirated from infected animals</u>. Material aspirated from guinea pigs infected with 2D was studied to determine if collagenase was: (1) present in the infection, (2) cell-associated or free, and (3) dependent on reducing conditions. Since there was considerable fluid accumulation in infected animals (5-10% of the animal's body weight could often be aspirated), 10-15 ml of exudate could be aspirated from an infected animal without difficulty.

a. <u>Demonstration of collagenolytic activity in exudate from an infected animal</u>. Figure 5 shows the release of counts from ^{14}C-collagen by a sample of exudate taken from an infected animal. The exudate contained 1.5×10^{10} cells/ml by direct count. Cysteine stimulated collagenase activity in the exudate.

b. <u>Association of collagenase activity with bacterial cells in exudate</u>. In order to determine whether the collagenase activity observed in the guinea pig exudate was associated with the bacterial cells, the following experiment was done. Exudate was fractionated according to the following scheme:

Exudate ⟶ Fraction A

270 x g
15 min

Low speed pellet   Low speed supernatant → Fraction C

resuspend in Tris   17,000 x g
                                                     15 min
Fraction B   High speed pellet   High speed
                                                 supernatant
                          resuspend
                        Fraction D
                                                   Fraction E
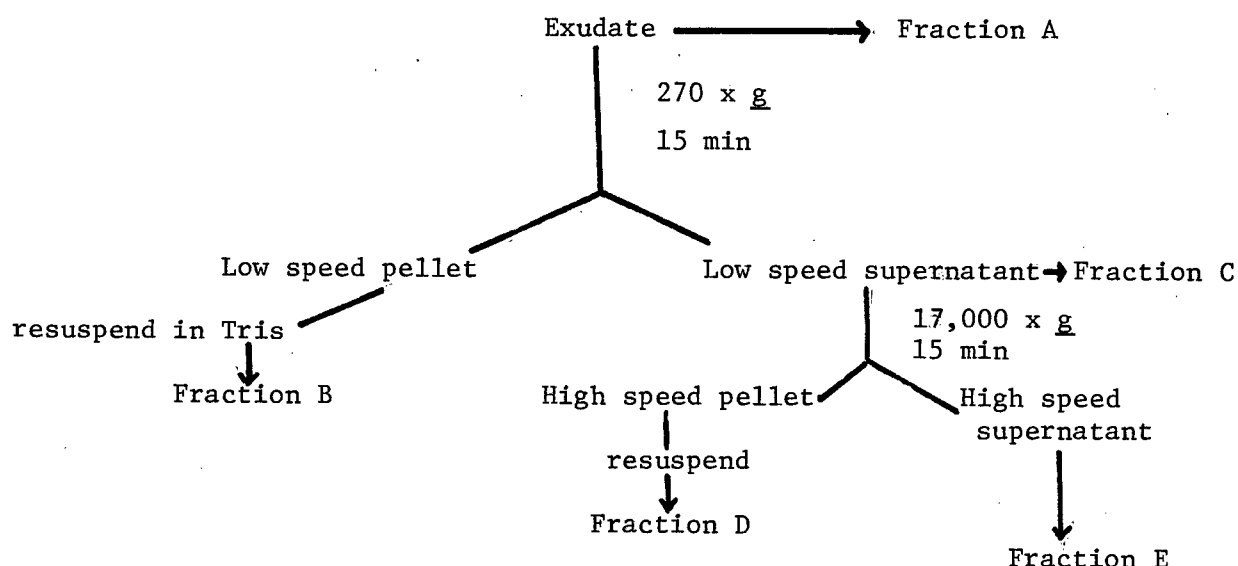
The following fractions were assayed in the presence and absence of cysteine:
(a) whole exudate, (b) low speed pellet, (c) low speed supernatant, (d) high
speed pellet, and (e) high speed supernatant. All fractions were adjusted to
their original volumes. As shown in Table XII, collagenase activity was found
in fractions A, B, C, and D (slight). No activity was apparent in the cell-
free supernatant. Definite dependence on cysteine was observed in the active
fractions. Since activity in the low speed supernatant was much higher than
that of the bacterial cells (high speed pellet) resuspended in buffer, it was
felt that some factor in the high speed supernatant was required for or stimu-
lated collagenase activity in the bacterial cells. In an effort to demonstrate
activity in the bacterial cells, a sample of exudate was fractionated accord-
ing to the above scheme and the high speed pellet resuspended either in buf-
fer or in high speed supernatant prior to assay. Figure 6 shows that acti-
vity was much greater in the cells resuspended in supernatant than in those
resuspended in buffer. No activity was apparent in the supernatant alone. In
all cases collagenolytic activity was dependent on cysteine. Activity was
higher in cells resuspended in high speed supernatant than in the whole exu-
date, which suggests that the whole exudate contains an inhibitor which is

Table XII

Collagenolytic Activity in Fractionated Guinea Pig Exudate

fraction	cysteine	^{14}C released[*] (cpm x 10^{-4})
A	−	1.8
	+	2.6
B	−	.16
	+	1.7
C	−	.95
	+	2.6
D	−	.14
	+	.76
E	−	.15
	+	.25

[*]Incubation time 3 hr.
All values corrected for background.

Figure 6. Stimulation of collagenase activity by high speed supernatant.

Each reaction mixture contained: ^{14}C-collagen, 4.5 x 10^5 cpm/mg, 200 µg; Tris buffer, pH 7.0, 100 µmoles; cysteine, 10 µmoles; CaCl$_2$, 1 µmole; 2D cells resuspended as indicated. Total volume, 0.5 ml; temperature, 25°C.
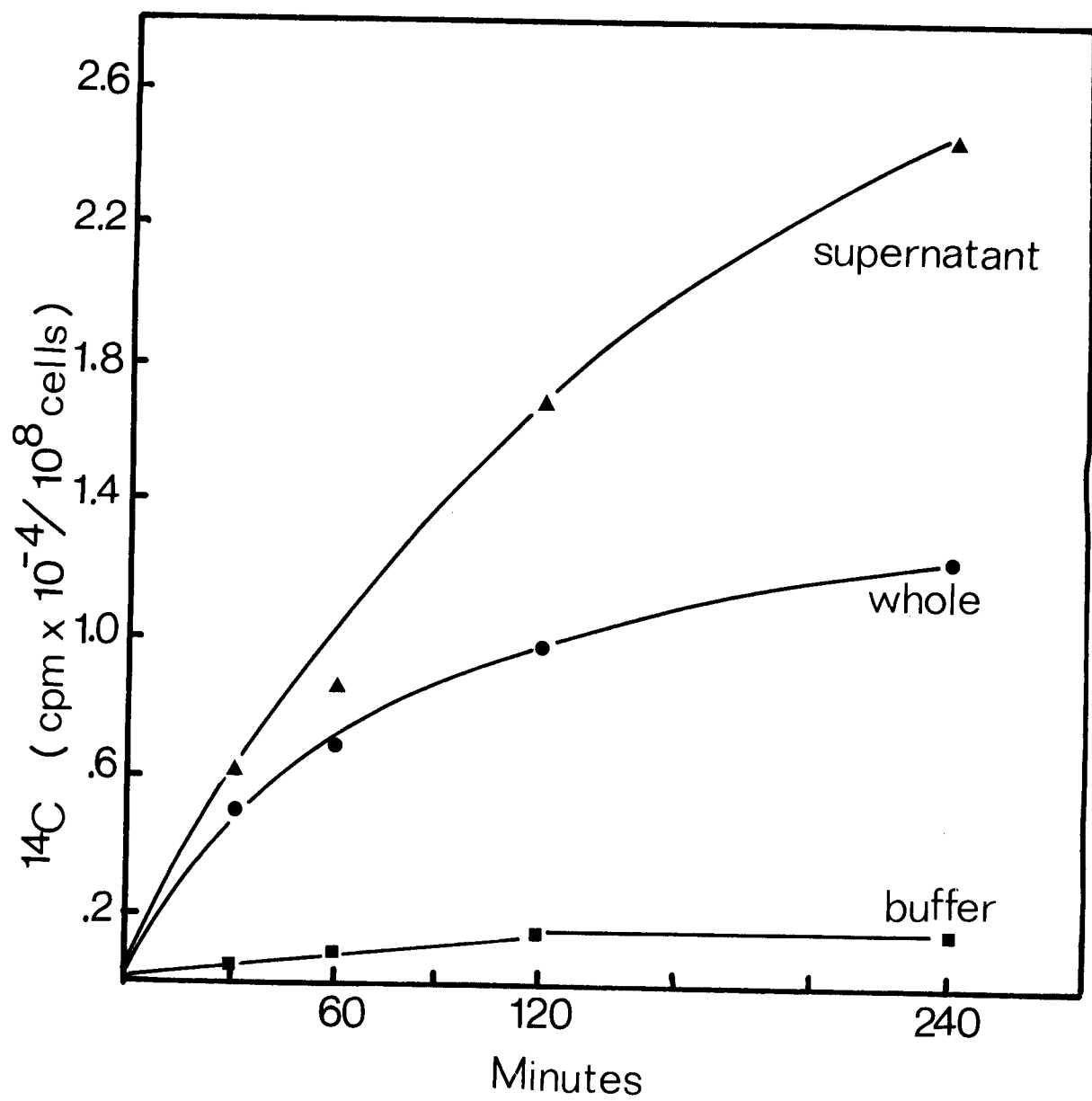
removed by low speed centrifugation.

  c. <u>Characterization of the stimulatory factor</u>. Collagenase activity in exudates from guinea pig infections was now known to be (1) associated with the bacterial cells, (2) dependent on cysteine and (3) stimulated by a factor in the high speed supernatant. Stimulation might be due to a factor such as a protease, which would act on partially digested collagen to increase the rate of release of counts, or to some other molecule or ion which stimulated collagenase activity.

  (1) <u>Effect of ultrafiltration on the high speed supernatant</u>. In order to roughly determine the size of the stimulatory factor in the high speed supernatant, a sample of supernatant was filtered using an Amicon ultra-filtration apparatus fitted with UM-10 and UM-05 membranes, which retain globular molecules of 10,000 and 500 molecular weight, respectively. Bacterial cells from a sample of exudate were harvested as previously described and resuspended either in buffer, high speed supernatant, UM-10 filtered supernatant or UM-05 filtered supernatant. As is shown in Table XIII, very little difference was found between the whole supernatant and the filtered supernatants, indicating that the stimulatory factor was a small molecule.

  (2) <u>Stability of the filtrate</u>. Bacterial cells from a sample of exudate, collected as previously described, were resuspended in (1) UM-10 filtered supernatant, (2) filtered supernatant which had been placed at 100°C for 15 minutes, (3) filtered supernatant which had been stored at -20°C for three weeks, and (4) sample #3 which had been placed at 100°C for 15 minutes. No significant differences were observed as shown in Table XIV.

  To determine whether the stimulatory factor was a small organic molecule or a metal ion, a sample of the UM-10 filtrate was ashed by placing it at 550°C for 3 hours. The ashed sample was resuspended in the original volume. As shown in Table XV, ashing the filtrate had no effect on its

Table XIII

Filtration of High Speed Supernatant

resuspension treatment	^{14}C released (cpm x 10^{-3}/10^8cells/30 min)
buffer	0.81
supernatant	2.1
UM-10 filtrate	1.8
UM-05 filtrate	2.5

Table XIV

Stability of UM-10 Filtrate

resuspension treatment	^{14}C released (cpm x $10^{-3}/10^8$cells/30 min)
UM-10 filtrate	1.8
boiled filtrate	2.1
frozen filtrate	1.7
" " , boiled	2.2

Table XV

Ashing

resuspension treatment	^{14}C released (cpm x 10^{-3}/10^8 cells/hr)
buffer	1.9
UM-10 filtrate	4.2
ashed filtrate	5.7

stimulatory activity, which suggested that the stimulatory factor was probably an ion. Addition of Ca^{++} or Na^{+} to the reaction mixture had no stimulatory effect.

D. Proteolytic activity in B. melaninogenicus culture supernatants.

1. Demonstration of an extracellular protease. Proteolytic activity against Azocoll in the culture supernatants of B. melaninogenicus strains 2D and CR2A is shown in Figure 7. A definite increase in the rate of dye release occurs when 0.01M cysteine is included in the reaction mixture. A lag was observed before dye release could be measured; this may have been due to the fact that the Azocoll had to be reduced before enzymic attack would occur. Activity against Azocoll was lost when the supernatant was bubbled with air for 15 minutes (Figure 8), but was restored with cysteine. The concentrated supernatant was active against casein but showed very little activity against BSA. No activity could be demonstrated against ^{14}C-collagen. The proteolytic activity was retained by an Amicon PM-30 membrane which retains globular molecules of 30,000 MW. Most of the activity was retained by an XM-50 membrane (50,000 MW retention), suggesting that the protease was either about 50,000 MW or associated with a large molecule or cell fragment.

2. Protease activity as a function of culture age. Protease activity was assayed in culture supernatants of 2D prepared at various stages in growth. Supernatants were assayed without concentration. Protease activity against Azocoll, shown in Figure 9, could be measured after 10 hours of growth and increased with increasing cell numbers until 48 hours when a further increase in protease activity was noted. Similar experiments also demonstrated an increase in proteolytic activity in mid- to late stationary phase which continued as the cells began to lyse.

3. Purification of the protease. Attempts at fractionation of the concentrated supernatant with the object of purifying the protease produced

Figure 7. Protease activity in CR2A and 2D supernatants.

Each reaction mixture contained: Tris buffer, pH 7.2, 300 μmoles;

cysteine, where indicated, 100 μmoles; supernatant, 2.5 mg protein;

Azocoll, 30 mg. Total volume 10 ml, temperature 37°C.

Figure 8.   Proteolytic activity in 2D supernatants.

Each reaction mixture contained: Tris buffer, pH 7.2, 300 μmoles; cysteine, where indicated, 100 μmoles; supernatant, 2.4 mg protein; Azocoll, 30 mg.   Total volume 10 ml, temperature 37$^{\text{o}}$C.

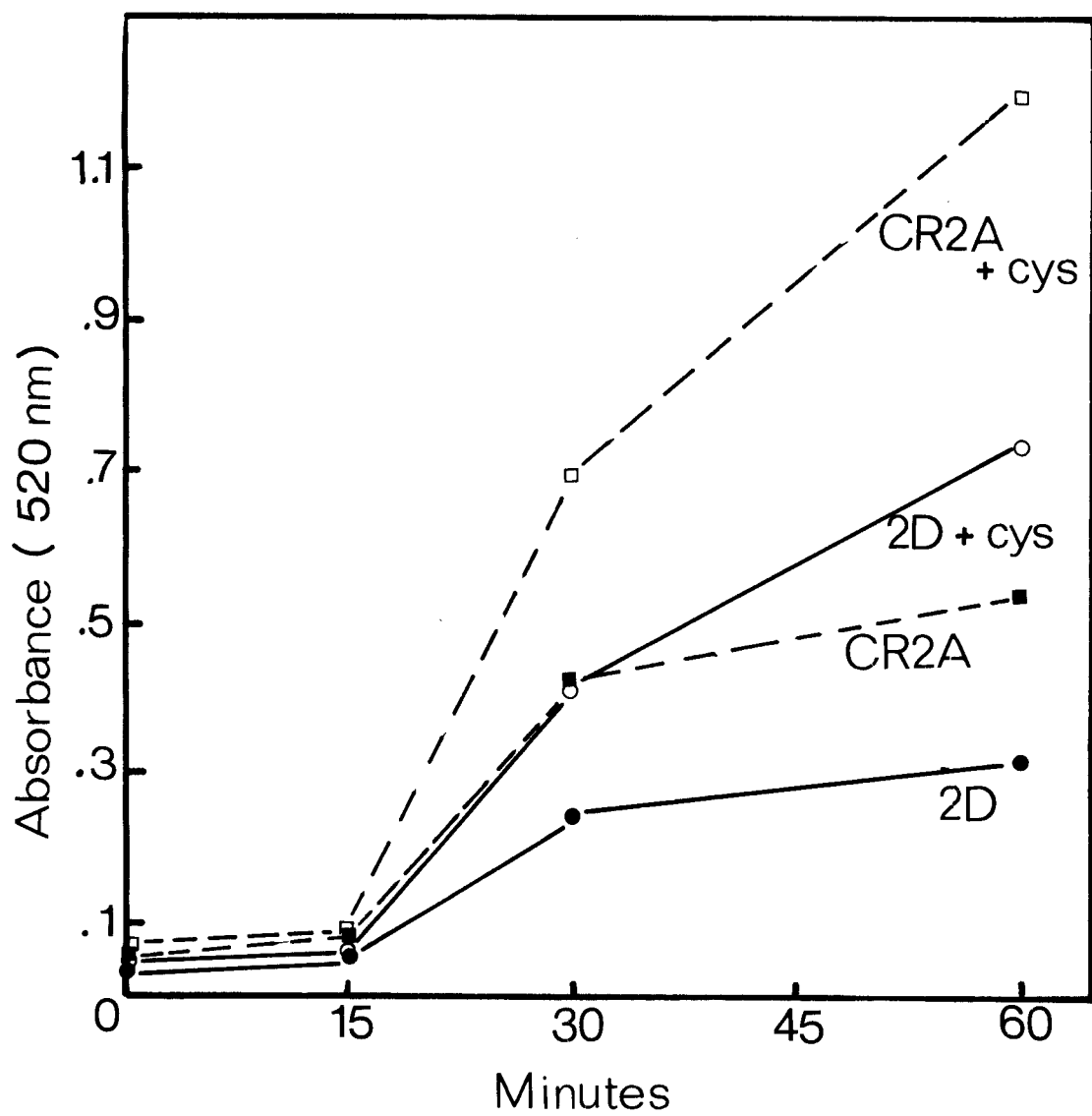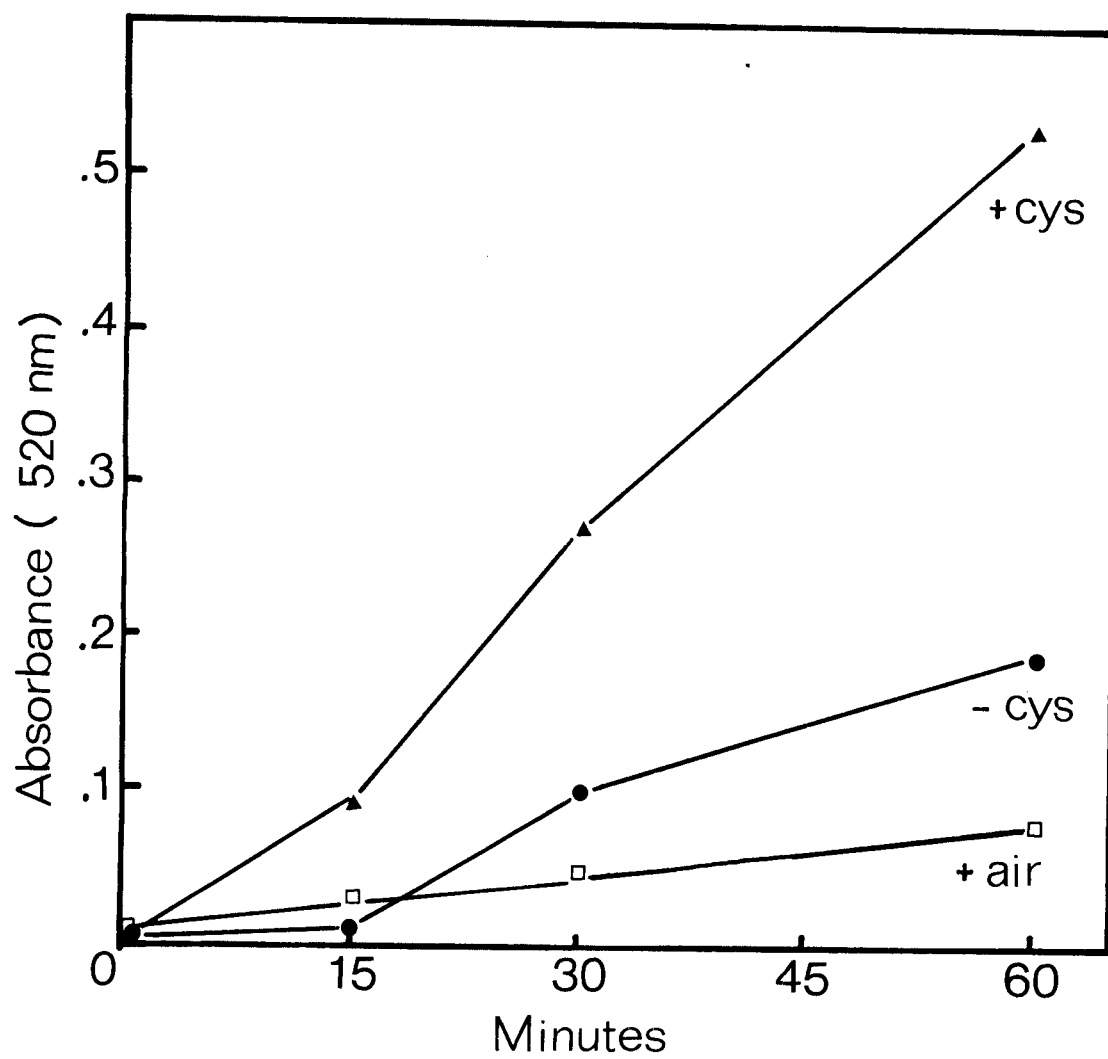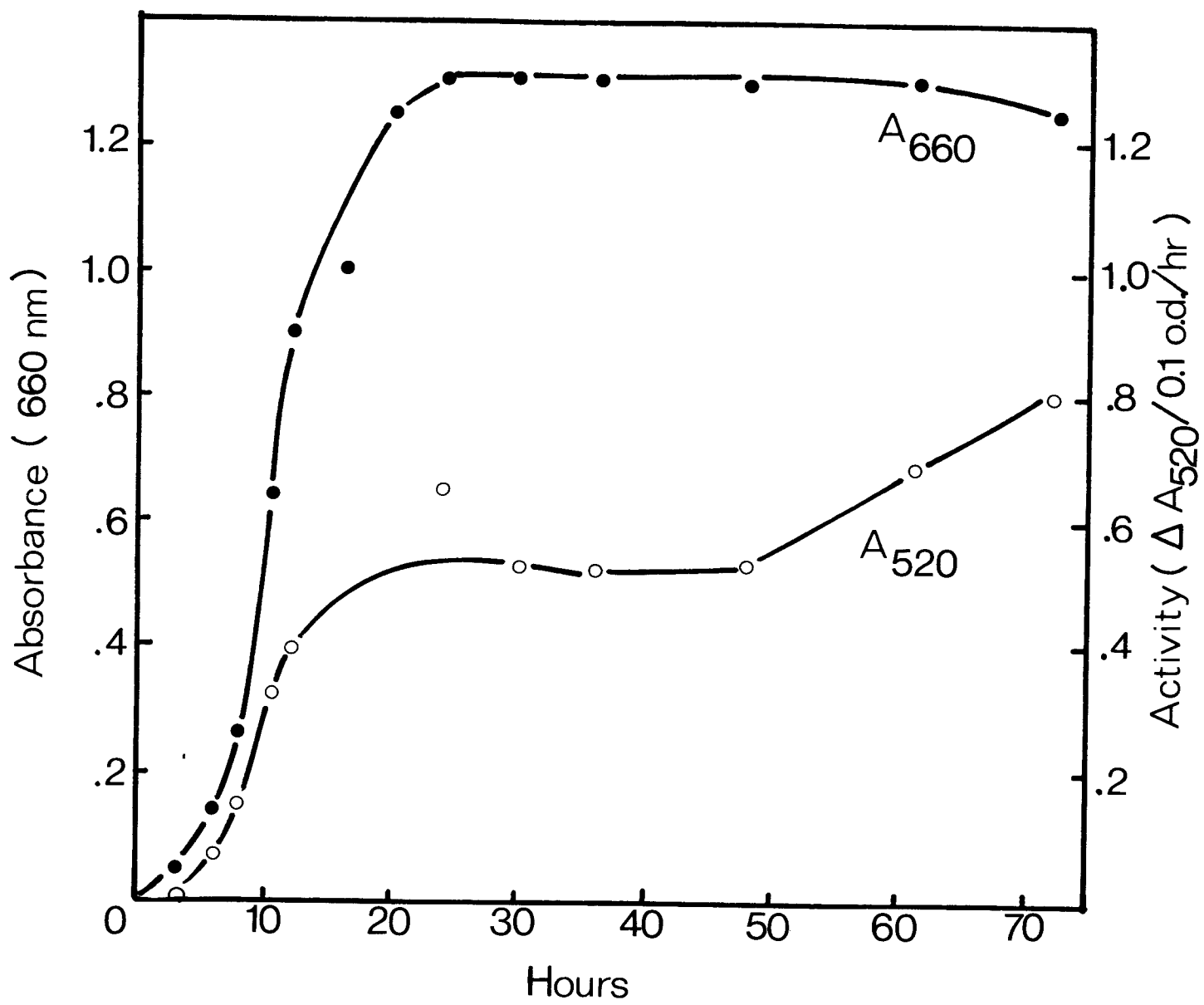Figure 9. Protease activity as a function of culture age.

Each reaction mixture contained: Tris-HCl buffer, pH 7.2, 240 μmoles;

cysteine, 80 μmoles; supernatant, 9.3 mg protein; Azocoll, 25 mg. Total

volume 8.0 ml, temperature 37°C, reaction time 60 minutes.

variable results. Supernatant from a 2D culture was concentrated and low molecular weight molecules removed as described in Methods. Chromatography on Bio-Gel P-60 resulted in the recovery of only one third of the material applied to the column (as estimated by A_{280}). Activity against Azocoll was not detected in the effluent. When a sample of supernatant was chromatographed on a Sephadex G-75 column (100 cm x 2.5 cm), only about one half of the material applied to the column was recovered, and activity against Azocoll was detected in many fractions, suggesting that the protease activity might be associated with another constituent of the supernatant.

Since the protease activity appeared to be either sticking nonspecifically to the longer column or to associate with some other component of the concentrated supernatant, it was felt that chromatography of the supernatant in the presence of a large carrier protein such as BSA might allow the protease to complex with the carrier and elute with it. Figure 10 shows the elution profiles of concentrated supernatant, BSA, and a combination of supernatant and and BSA from a G-75 column. A complex has apparently formed between the BSA and some constituent of the supernatant, as the BSA peak has been shifted to the left, indicating a larger molecule. Protease activity eluted with the complex. Rechromatography of the complex, however, produced a pattern similar to the original elution profile of the concentrated supernatant in Figure 10. Considerable variation was encountered among the elution patterns of different samples or of the same sample chromatographed at different times. The reason for the variation is not known although it may have been due to breakdown of some of the components of the supernatant with storage, or to proteolytic action on some of the protein material. Another possibility is that the protease may have been associated with a component of the cell wall, which would result in the association of proteolytic activity with random fragments when the cells lysed.

Figure 10. Effect of BSA on chromatography of supernatant on G-75.

Column dimensions, 46.6 x 1.2 cm; flow rate, 30 ml/hr; fraction size, 1.5 ml; eluant, Tris-HCl, 0.05M, pH 7.0.

Samples applied: (1) concentrated supernatant (2.5 mg protein/ml), 1.0 ml, > 2 O.D./cm, ▼··············▼. (2) BSA (1 mg/ml), 1.0 ml, 0.6 O.D./cm, □————□. (3) BSA + concentrated supernatant (1:1, v:v), 1.5 ml, 1.9 O.D./cm, ●————● . Azocoll activity, ————————.
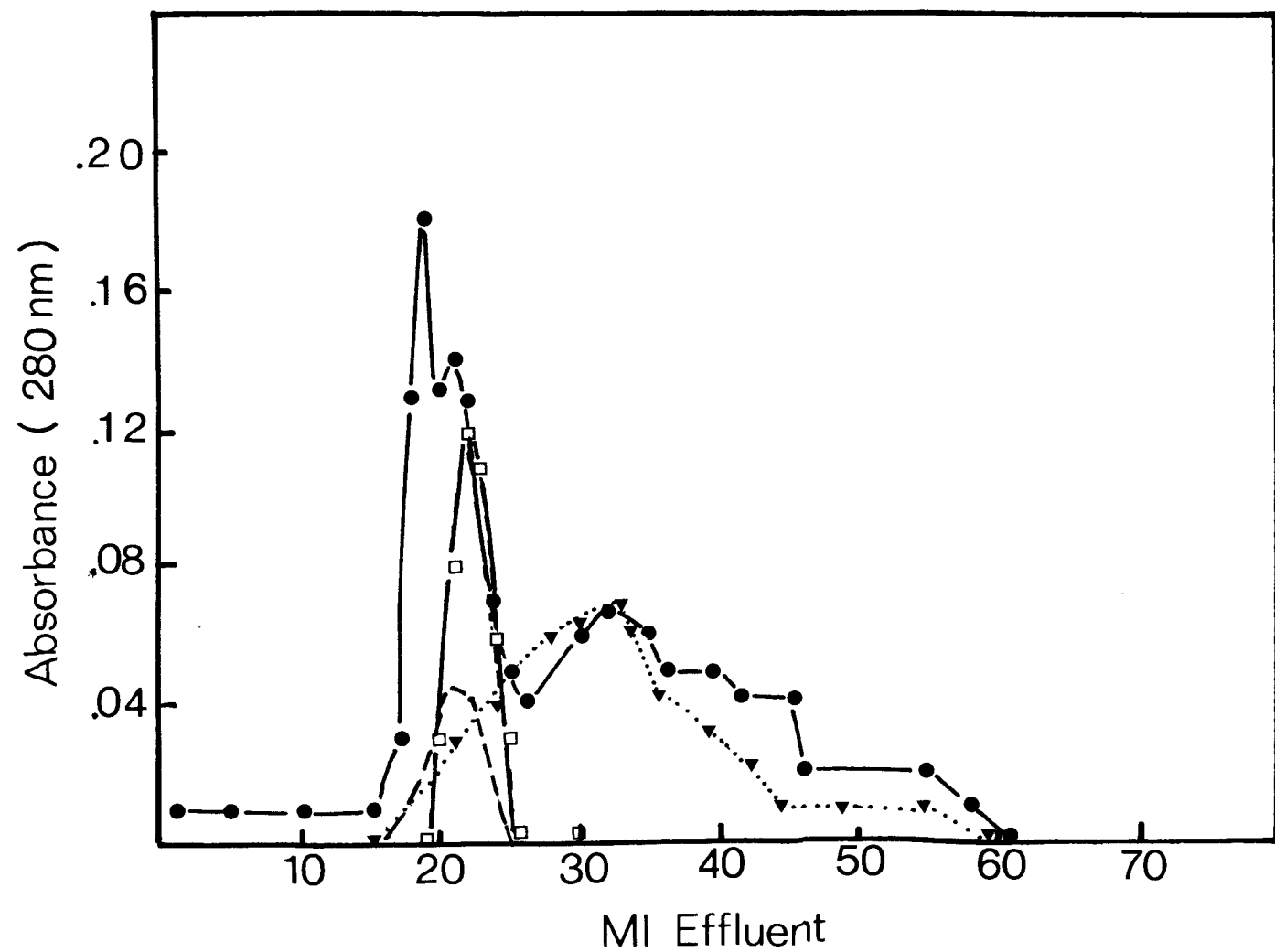
4. <u>Partial characterization of protease activity</u>.

    a. <u>Stability of the protease</u>. Aliquots of a concentrated supernatant of 2D were placed in tubes. Cysteine (final concentration 10^{-2}M) was added to half the samples. The samples were stored at 4°C and at -20°C; those containing cysteine were under an atmosphere of $H_2:CO_2$ (80:20). Samples were removed and assayed at intervals over a period of seven weeks. The results indicated that proteolytic activity against Azocoll is stable under these conditions for at least seven weeks although a slight amount of activity may be lost on storage at 4°C without cysteine.

    The protease was inactivated by boiling, a temperature of 100°C for five minutes reducing the rate of dye release to 1/3 that of the control. No activity was present after treatment at 100°C for ten minutes.

    Resistance of the protease to autodigestion was indicated by the following experiment. Samples of the concentrated supernatant were incubated at room temperature with and without cysteine. Aliquots were assayed at intervals for protease activity. As illustrated in Table XVI, no decrease in protease activity was apparent in any of the samples, indicating that the protease is resistant to autodigestion for at least a period of four hours.

    b. <u>Effect of cysteine concentration on protease activity</u>. In order to determine the concentration of cysteine required for protease activity, cysteine concentrations of the reaction mixture were varied from 10^{-5}M to 10^{-1}M. A concentration of at least 5×10^{-3}M cysteine was required for activity (Table XVII). With lower concentrations of cysteine the rate of dye release never exceeded that of the control. Increasing the cysteine concentration resulted only in a slight increase in protease activity.

    c. <u>Effect of other reducing agents on protease activity</u>. Mercaptoethanol, dithiothreitol and sodium thioglycolate were tested for their effectiveness in stimulating protease activity. The effectiveness of the

Table XVI

Resistance to Autodigestion

time at 25°C (hrs)	cysteine (10^{-2}M)	activity ($A_{520}/15'$)
0	−	0.13
	+	−−
1	−	0.13
	+	0.17·
2	−	0.18
	+	0.14
4	−	0.15
	+	0.15

Table XVII

Effect of Cysteine Concentration

cysteine added (μmoles)	final concentration (M)	activity ($A_{520}/10'$)
none	0	.03
.065	10^{-5}	.01
.65	10^{-4}	.02
6.5	10^{-3}	.04
32.5	5×10^{-3}	.38
65.0	10^{-2}	.32
325.0	5×10^{-2}	.49
650.0	10^{-1}	.44

reducing agents was greater at pH 8.0 than at pH 7.0. Dithiothreitol was the most effective of those tested (Table XVIII).

d. <u>Effect of pH on protease activity</u>. Protease activity was measured at pH values form 4.0 to 9.0. Acetate buffers were used from pH 4.0 to 5.5, phosphate buffers from pH 6.0 to 7.5, and Tris-HCl buffers from pH 7.0 to 9.0. The results shown in Figure 11 indicate that the optimum pH for activity against Azocoll is 8.0. Good activity was always obtained at pH values between 7.0 and 8.5.

e. <u>Effect of EDTA on protease activity</u>. EDTA was tested for its effect on protease activity by adding it to the reaction mixture to give final concentrations ranging from 10^{-2}M to 10^{-5}M. The protease showed some sensitivity to EDTA at concentrations of 10^{-2}M and 10^{-3}M, which reduced the rate of dye release to about 40% that of the control. EDTA at 10^{-4}M had no effect and 10^{-5}M EDTA was if anything slightly stimulatory (Figure 12).

Table XVIII

Effect of Reducing Agents

reducing agent	μmoles	final concentration (M)	pH	activity ($A_{520}/10'$)
none	0	0	8.0	.21
dithiothreitol	650	10^{-1}	"	.56
	65	10^{-2}	"	.53
	6.5	10^{-3}	"	.41
mercaptoethanol	650	10^{-1}	"	.59
	65	10^{-2}	"	.33
	6.5	10^{-3}	"	.17
thioglycolate	650	10^{-1}	"	.44
	65	10^{-2}	"	.25
	6.5	10^{-3}	"	.17
none	0	0	7.0	.02
cysteine	65	10^{-2}	"	.17
dithiothreitol	65	10^{-2}	"	.12
mercaptoethanol	65	10^{-2}	"	.10
thioglycolate	65	10^{-2}	"	.02

Figure 11.   Effect of pH.

Each reaction mixture contained: buffers, pH as indicated, acetate, 300 μmoles, phosphate, 300 μmoles, Tris 300 μmoles; cysteine, 100 μmoles, supernatant 1.25 mg protein; Azocoll, 20 mg. Temperature, 37°C, pre-incubation 10 minutes. Total volume, 6.5 ml.
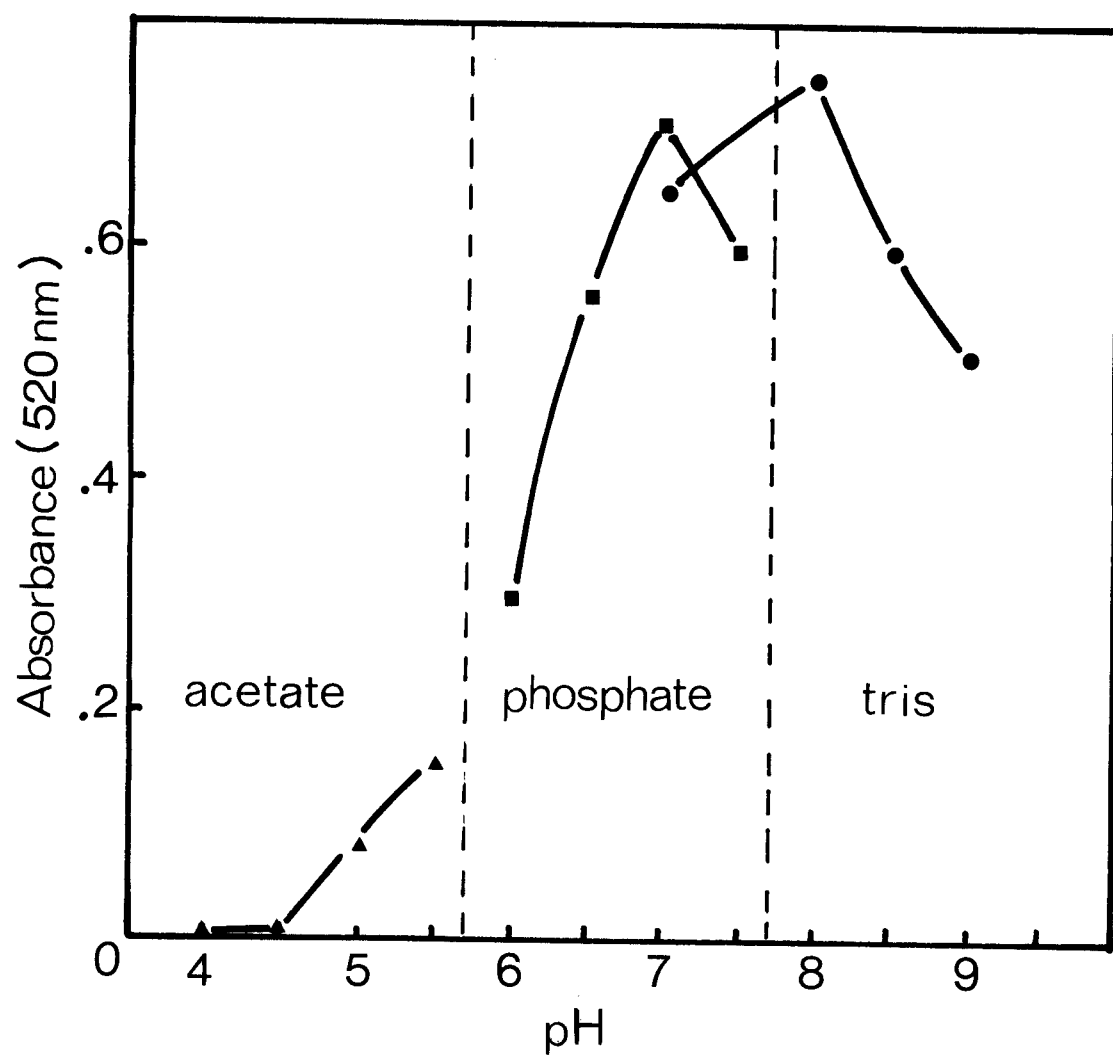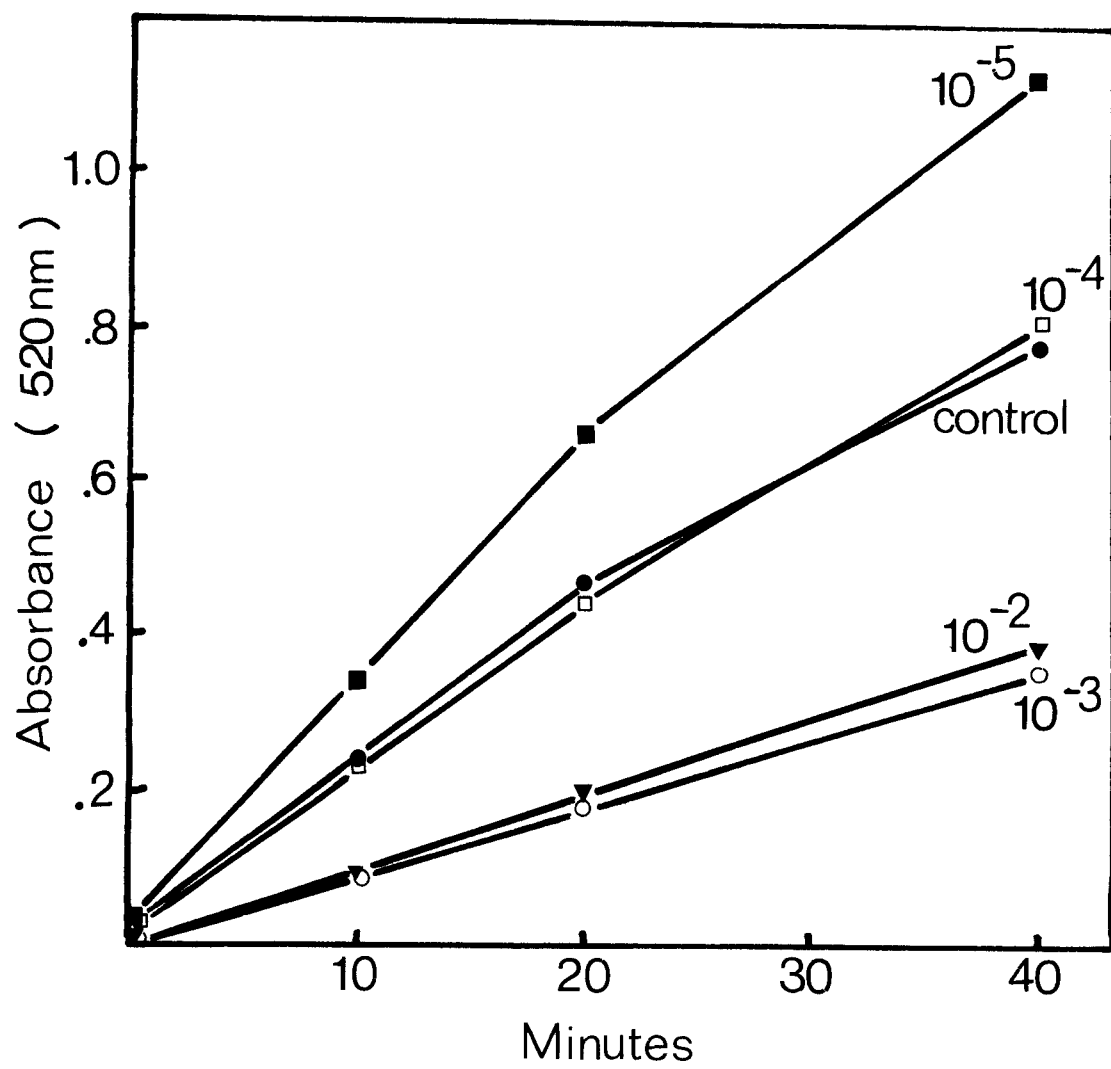
Figure 12.   Effect of EDTA.

Each reaction mixture contained: Tris buffer (pH 8), 300 μmoles;

cysteine, 60 μmoles; EDTA, as indicated; supernatant, 1.25 mg protein;

Azocoll, 20 mg.   Total volume 6.5 ml, temperature 37°C, preincubation 10 min.

IV. DISCUSSION

The development of a rapid and sensitive assay for collagenolytic acti-

vity should facilitate collagenase studies not only in bacterial systems but

in mammalian systems as well. The advantage of using ^{14}C-acetylated collagen

would appear to be the speed of the assay relative to the viscometric method

and the ease of preparation of large amounts of substrate of high specific

activity relative to the ^{14}C-glycine method. A possible disadvantage of

using acetylated collagen rather than ^{14}C-glycine labelled collagen is the

fact that the radioactive label in the former is located in a functional group

which has been chemically attached to the collagen molecule, whereas in the

latter the label occurs in an amino acid intrinsic to the collagen molecule.

Studies done on the assay, however, have not revealed any differences between

the behaviour of the labelled and the unlabelled substrates. The acetylated

collagen does not appear to have been denatured by the labelling procedure,

as is evidenced by its continued resistance to nonspecific proteolytic attack,

and appears to be a suitable assay substrate as evidenced by treatment with

collagenase. To date, perfect correlation has been obtained between the

results of the ^{14}C-collagen assay and those of other collagenase assays used.

In contrast to the findings of other workers (12,42), studies in this

laboratory have shown that only a small percentage of human oral isolates

of B. melaninogenicus are collagenolytic. In addition, the presence of

collagenolytic activity in B. melaninogenicus strains studied so far has been

found to correlate with the presence of other characteristics in the organism

including a specific fatty acid profile and hemagglutinating activity (T. Ed-

wards, personal communication).

The collagenase of B. melaninogenicus was found to be associated with the

cells and dependent on reducing conditions for activity, as reported by

others (12). Gibbons and Macdonald (12) found that degradation of collagen gels by B. melaninogenicus cultures was not evident until culture autolysis began at 72 hours, which suggested to them that the enzyme was intracellular and was released with lysis of the cells. Similarly, Hausman and Kaufman found no evidence of collagenase activity in culture supernatants until autolysis began (17). In contrast to the findings of these workers, collagenase activity was found in washed cells of B. melaninogenicus as early as day 1. No activity was ever detected in the culture supernatants even after autolysis had occurred.

The characteristics of the transmissible mixed infection produced by the injection of guinea pigs with organisms cultured from gingival material were similar to those of the rapidly spreading infections described by Macdonald and co-workers (28). Results obtained when the B. melaninogenicus strain was eliminated from and subsequently recombined with the mixture were in agreement with those reported by others. However, in contrast to previous reports that a number of B. melaninogenicus strains could replace the original in the infectious mixture, typical infections were obtained only when certain of the collagenase producing strains were recombined with the mixture. This may be due to the fact that the B. melaninogenicus strains used by previous workers were all collagenolytic. Kestenbaum (20) found a positive correlation between collagenase activity of the B. melaninogenicus strains used and the severity of the infection. Whether or not the B. melaninogenicus strains differed in other respects such as protease production was not established, thus it cannot be said that the differences in the severity of the infections were dependent solely on differences in collagenase activity. The data obtained using the experimental mixed infection system support the idea of a correlation between collagenase activity and infectivity insofar as successful infections were produced with collagenolytic B. melaninogenicus strains and

noncollagenolytic strains were never infective. The determination of whether collagenase activity is essential for a successful infection will require a mutant of an infective, collagenase-producing strain differing from the parent strain only in the absence of collagenase activity. It evident, however, that collagenase activity alone is not sufficient for infectivity, as one collagenase-positive strain (CR2A) was never found to produce any infection either alone or in combination with other organisms. Assuming that it is the same organism described by Macdonald (27), it can be presumed that CR2A has lost some "infectivity factor" required for pathogenicity. Of interest also is the observation that 2D, which originally produced infections similar to those described for CR2A, appeared also to be losing an "infectivity factor" and becoming noninfective both in pure and in mixed culture. Both strains, however, remained strongly collagenolytic. The observation that infections were produced more frequently with older cells of 2D indicates that a factor required for infectivity is produced during the stationary phase of growth. Studies of material aspirated from guinea pig infections have shown that 2D elaborates collagenase in the infection as well as _in_ _vitro_. The activity is definitely associated with the bacterial cells. The nature of the stimulatory factor in filtered exudate remains unclear; however, due to the fact that it was not destroyed by ashing it is probably a metal ion.

_B._ _melaninogenicus_ has been shown to possess proteolytic activity but the activity has not been characterized. An organism dependent on peptides for growth (52) would be expected to be actively proteolytic. The organism may elaborate more than one protease, as activity has been demonstrated in the washed cells as well as in the supernatant, and Hausman and Kaufman have found caseinolytic activity associated with a particulate fraction from the autolysate supernatant (17). The role of the protease if any in infection cannot be established yet; conceivably proteolytic activity could enhance collagenolytic

activity to promote the spread of infection as well as being of nutritional significance to the organism. Preliminary studies in this laboratory indicate that dermonecrotic activity in <u>B</u>. <u>melaninogenicus</u> supernatants may be associated with protease activity.

The role of collagenase in <u>B</u>. <u>melaninogenicus</u> infections and its relationship to pathogenicity remains unclear. <u>B</u>. <u>melaninogenicus</u> has been isolated for years from a number of clinical infections, is known to comprise 5% of the cultivable flora of the gingival crevice, and it is known that some strains produce a collagenolytic enzyme. However, the common speculation that collagenase production by <u>B</u>. <u>melaninogenicus</u> is largely responsible for the pathogenesis of periodontal disease and other infections may be an oversimplification in view of the finding that considerable differences exist between strains with regard to collagenase activity and infectivity. In experimental infections produced with collagenolytic strains of the organism, <u>B</u>. <u>melaninogenicus</u> has been established as the primary pathogen and has been demonstrated to produce collagenase <u>in</u> <u>situ</u>. However, in naturally occurring infections the relative proportions of "pathogenic"and "nonpathogenic" <u>B</u>. <u>melaninogenicus</u> strains is not known. Collagenase-positive organisms obviously exist in such situations as they have been isolated from infections, but their contribution to the total infectivity of the bacterial population is unknown. The finding that the majority of human oral isolates of <u>B</u>. <u>melaninogenicus</u> are noninfective and collagenase-negative suggests that if <u>B</u>. <u>melaninogenicus</u> collagenase is to be implicated in the pathogenesis of periodontal disease it must be postulated either that the non-collagenolytic organisms are induced to produce collagenase in the disease state or that collagenolytic <u>B</u>. <u>melaninogenicus</u> strains are introduced into the population.

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