EXTRACELLULAR ANTIGENS IN STEADY-STATE CULTURES OF THE HEMOLYTIC STREPTOCOCCUS: PRODUCTION OF PROTEINASE AT LOW pH[1]

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Received for publication March 4, 1958

Concentrates of β-hemolytic streptococcal culture filtrates have been shown by gel precipitation to contain at least 7 antigens, in recent studies by Halbert *et al.* (1955) and in this laboratory (Harris *et al.*, 1955). Subsequently, electrophoretic analysis of concentrates of streptococcal culture supernatants also indicated the presence of a number of proteins in such preparations. In the course of experiments directed at the study of some properties of these proteins it became apparent that their relative concentration varied substantially among successive cultures. In the present study the conventional method of bulk culture used in the earlier work was replaced by the cultivation of β-hemolytic streptococci under steady-state conditions in the chemostat, with the use of a completely defined medium. The chemostat provides a means of cultivating bacteria with continuous dilution of the culture at constant volume so that the pH and composition of the medium in which the cultivation of bacteria occurs are kept constant. Some characteristics of steady-state cultures of a strain of group A β-hemolytic streptococci have been described elsewhere (Karush *et al.*, 1956).

In the present study culture supernatant concentrates were examined as obtained from steady-state cultivation of strain H44 (type 4) at pH values in the range of 6.8 to 5.95. Preliminary examination indicated that in the upper part of this range a number of proteins were produced at concentrations, relative to the total macromolecular material in such concentrates, which were below the threshold of detection by paper electrophoresis, whereas concentrates of supernatants from cultivation in the lower part of this range showed one constituent to be present in relatively high concentration, as compared with other proteins present. This constituent was chosen for the first study. Subsequent study of the properties of this constituent indicated that it was in all probability identical with the streptococcal proteinase described by Elliott (1945, 1950), Elliott and Dole (1947), and Shedlovsky and Elliott (1951). This report will present the results of studies on the production in the chemostat of this substance, attempts to characterize and purify it, and its relationship to the streptococcal proteinase referred to above.

MATERIALS AND METHODS

Cultivation of streptococci. Organism. The β-hemolytic streptococcus used for the greater part of this work was strain H44 of group A, type 4, which was made available through the courtesy of Dr. Karl Meyer. Some work was also done with strain 5797, for which we are indebted to Dr. Rebecca Lancefield. Each strain was maintained in the lyophilized state, from which it was cultivated in brain heart infusion broth, then in the defined medium described below to which small quantities of a casein hydrolyzate had been added. The concentration of the casein hydrolyzate was gradually reduced in serial cultures until the organism grew in the chemically defined medium and could be stored in it at 4 C.

Medium. The experiments reported here were done using a chemically defined medium developed in this laboratory by Dr. William L. Landau (1958). This medium was adapted from that of Bernheimer *et al.* (1942) by the substitution of amino acids and biotin for the casein hydrolyzate, and with other modifications required for its use in continuous culture. Several

[1] This study was supported by Research Grant H-869 of the National Heart Institute, National Institutes of Health, U. S. Public Health Service.

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strains of streptococci have been cultivated in this medium, in bulk culture, the turbidity of overnight cultures and the extent of production of some extracellular proteins being in the same range as in the case of cultivation in meat extract broth.

The composition of the medium is shown in table 1. In making up solution A, the designated volumes of the individual amino acid solutions were mixed. To this were added, in order, the phosphates, thioglycolic acid (11 ml of a 68 per cent assay preparation), and 24 ml of the salt mixture. The solution was then brought to a pH of 7.48 and a volume of 4 L, and passed through

TABLE 1

Composition of the chemically defined medium

Solution	Component	Concentration in Stock Solution g/L	Volume of Stock Solution or g, per 12 L	Final Concentration in Medium, moles/L
A	Amino acid:			
	1. DL-Alanine	14.22	150 ml	1.99×10^{-3}
	2. L-Arginine·HCl	32.00	150 ml	1.90×10^{-3}
	3. L-Cysteine·HCl	25.17	150 ml	1.99×10^{-3}
	4. Glycine	4.80	150 ml	7.95×10^{-4}
	5. L-Histidine·HCl	24.00	150 ml	1.57×10^{-3}
	6. DL-Isoleucine	10.47	150 ml	9.96×10^{-4}
	7. L-Lysine·HCl	8.40	150 ml	5.96×10^{-4}
	8. DL-Methionine	3.57	150 ml	2.96×10^{-4}
	9. DL-Phenylalanine	6.60	150 ml	4.96×10^{-4}
	10. L-Proline	1.22	150 ml	1.36×10^{-4}
	11. DL-Threonine	19.03	150 ml	2.10×10^{-3}
	12. DL-Valine	25.57	150 ml	2.72×10^{-3}
	13. L-Asparagine	26.40	300 ml	4.97×10^{-3}
	14. L-Glutamic acid·HCl	36.50	300 ml	6.20×10^{-3}
	15. DL-Leucine	7.85	300 ml	1.49×10^{-3}
	16. DL-Serine	50.40	300 ml	1.19×10^{-2}
	17. DL-Tryptophan	0.9780	300 ml	1.19×10^{-4}
	18. L-Tyrosine	0.960	600 ml	2.64×10^{-4}
	K_2HPO_4	—	104.5 g	5.00×10^{-2}
	Na_2HPO_4	—	85.2 g	5.00×10^{-2}
	Thioglycolic acid	—	11 ml	8.76×10^{-3}
	Salt Mix:			
	$CuSO_4 \cdot 5H_2O$	0.500		4.00×10^{-6}
	$ZnSO_4 \cdot 7H_2O$	0.500		3.46×10^{-6}
	$FeSO_4 \cdot 7H_2O$	0.500	24 ml	3.58×10^{-6}
	$MnCl_2 \cdot 4H_2O$	0.200		2.02×10^{-6}
B	$MgSO_4$	15.00		4.17×10^{-3}
	Uracil	0.300	400 ml	8.90×10^{-5}
	Adenine	0.300		4.30×10^{-5}
C	Glucose	270.0		5.00×10^{-2}
	$CaCl_2 \cdot 2H_2O$	0.221	400 ml	5.00×10^{-5}
D	Niacin	0.150		1.01×10^{-5}
	Pyridoxine·HCl	0.150		7.41×10^{-6}
	Calcium pantothenate	0.600		2.10×10^{-5}
	Thiamin·HCl	0.150	100 ml	3.73×10^{-6}
	Riboflavin	0.075		1.58×10^{-6}
	Biotin	1.2×10^{-4}		4.00×10^{-9}
E	$NaHCO_3$	100.0	240 ml	2.50×10^{-2}

a Seitz filter. Solution A and the other 4 solutions were added to 7.2 L of sterile distilled water, bringing the final volume of the medium to 12 L. Solutions B and C were sterilized by heat; solutions A, D, and E were passed through a Seitz filter.

Steady-state culture apparatus. The apparatus used for the continuous culture technique has been described in a previous report (Karush *et al.*, 1956).

Preparation of culture supernatant concentrates. The culture was collected from the chemostat in 9 L bottles submerged in ice. In many experiments 0.1 M sodium iodoacetate solution was placed in the bottle before collection (10 ml per L). The collected culture was run through a Sharples centrifuge at 50,000 rpm. The organisms were discarded and 900 to 950 ml portions of the supernatant were placed in 36/32 in Visking cellophane bags. The bags were hung before a large electric fan overnight, and the contents, after being concentrated approximately 10-fold by the pervaporation, were transferred to 22/32 in cellophane bags for dialysis against running tap water (24 hr at 4 C). The material was further concentrated by 2 more cycles of pervaporation and dialysis, with one centrifugation at 11,000 rpm, to a final 60-fold concentration. At this stage the material was lyophilized and stored at 4 C with the designation culture supernatant concentrate ("CSC"). An alternate method of CSC preparation was to treat a 50-fold concentrate of the culture supernatant (after the second pervaporation) with $(NH_4)_2SO_4$ to 80 per cent saturation. The precipitate was collected by centrifugation for 30 min at 5000 rpm, dialyzed 24 hr against running distilled water and lyophilized.

Paper electrophoresis. Paper electrophoresis was carried out in two kinds of apparatus of the pressure plate type, one modified from that described by Smith and Conley (1953) and the other manufactured by the EC Apparatus Company, Swarthmore, Pennsylvania. After an electrophoretic run at 4 C of 4 to 6 hr, the strips were placed immediately in a 110 C oven for 15 to 20 min and then for 10 min in a solution of 0.5 per cent bromphenol blue which was dissolved in 95 per cent ethyl alcohol saturated with mercuric chloride. After 2 washings in a 5 per cent solution of glacial acetic acid, the strips were dried at 110 C and exposed briefly to ammonium hydroxide fumes.

Test for proteinase activity. This procedure was essentially that described by Elliott and Dole (1947). Serial dilutions of the preparation were made in 0.4 ml volumes, in 0.85 per cent buffered saline solution containing 0.1 per cent of normal rabbit serum at pH 7.3. To each tube was added 0.4 ml of a 4:1 mixture of skimmed milk and 0.5 M neutral sodium thioglycolate. The tubes were shaken, incubated overnight at 37 C and read. The end point was taken as the highest dilution in which clotting was observed. Commercially available liquid skimmed milk gave the most consistent results.

Gel precipitation technique. The technique used in the present study was that of double diffusion in tubes, originally described by Oakley and Fulthorpe (1953), as modified by Preer (1956). In this procedure approximately 0.17 ml of an appropriate antiserum was placed in the bottom of a soft glass tube (3 mm internal diameter, 76 mm high). Then 0.1 ml of a 0.3 per cent solution of washed agar was placed over the serum and allowed to harden. Finally, 0.17 ml of the solution to be tested was placed over the agar. The tube was covered with adhesive tape and incubated for 3 days at room temperature (25 C). The tubes were then examined for the number of bands of precipitate in the agar zone, their position and relative density.

RESULTS

1. Paper electrophoretic examination of culture supernatant concentrates (CSC). (a) Static cultures:—Early electrophoretic examinations were made on culture supernatant concentrate obtained from conventional bulk cultures. For these, 6-L volumes of defined medium of essentially the same composition as that described above were seeded with 6 ml of a 6-hr culture of strain H44. The culture was allowed to incubate overnight (18 hr) at 37 C. The turbidity reading in the Klett colorimeter (filter no. 56) was 230 to 240, and the pH had changed from the initial value of 7.6 to 6.2 or 6.3. Paper electrophoretic examinations were done on samples of such concentrates, each at 50 to 100 mg of dry weight per ml (solutions of lower concentrations did not yield any zones which were detectable on staining with bromphenol blue). The electrophoretic

pattern of a sample run 4 hr in 0.05 M veronal buffer pH 8.6 usually showed one dense zone that migrated towards the anode somewhat more rapidly than human serum albumin. (This component was called "A.") Two faint zones that moved less rapidly towards the anode were often present, and frequently another zone that migrated slowly toward the cathode. In repetitions of this experiment under presumably identical conditions, substantial variations were found in the consistency with which the various electrophoretic bands appeared and in their apparent relative concentrations.

(b) Steady-state cultures:—Concentrates of supernatants were examined from cultures grown in the chemostat at a number of levels of pH, between 6.8 and 5.95, as indicated in table 2. Paper electrophoretic examination of concentrates from cultures obtained at pH 6.5 and 6.8 yielded no zones detectable at the threshold of sensitivity of paper electrophoresis with the present method of staining. From cultures at steady-state pH values of 6.3 to 5.95, however, paper electrophoresis of the concentrates at 50 mg/ml, in 0.05 M veronal buffer pH 8.6, showed one very dense zone that moved slowly towards the cathode; no other zones were observed. This zone was found to have the same rate of migration, under these conditions, in the culture supernatant concentrate of all cultures within this pH range which were tested, and was designated component "B," to differentiate it from the anode-approaching zone (component "A") found earlier in static-culture preparations.

Paper electrophoretic examinations of component "B"-containing concentrates (steady-state pH range 6.3 to 5.95) in the range from 100 mg/ml to 1 mg/ml yielded a single zone, which was wide, very dense, and somewhat blurred, in the case of the higher concentrations, quite clear at 5 mg/ml and distinguishable even at 2 mg/ml. At no concentration tested, including 100 mg/ml, was any other constituent present in sufficient concentration for detection by the techniques of electrophoresis and staining described above.

It was of interest at this time to attempt to find component "B" in static cultures by using the same medium as that which was used in the chemostat. Because of the low range of operating pH of the chemostat at which this component had been produced the pH of the medium was lowered from 7.6 to 6.7. Conventional bulk cultures seeded in medium at this pH and incubated overnight were processed in the usual way, and the culture supernatant concentrate showed an electrophoretic band which moved toward the cathode at the same rate as component "B" of the chemostat cultures. A greater amount of the component was found in the case of culture supernatant concentrate from medium initially at pH 6.7 than the usual 7.6, and increases in the amount of seed from 0.1 per cent, as it was originally used, to 1.6 per cent and to 10 per cent caused concomitant increases in the yield of component "B."

2. Gel-precipitin examination of culture supernatant concentrate from steady-state cultures. Concentrates from chemostat cultures of the same steady-state pH values as those described above were tested at various concentrations by gel

TABLE 2

Number of zones on paper electrophoresis and gel-precipitin bands found in culture supernatant concentrate preparations from chemostat cultures at various values of steady-state pH

Preparation	Steady-State pH	Yield, mg/L	Paper Electrophoresis, No. of Zones	No. of Gel-Precipitin Bands at Various Concentrations of the Preparation, mg/ml							
				6.0	3.0	1.0	0.5	0.25	0.125	0.05	0.025
D_{34}	6.8	8.6	0	4	3	3	0	0	0	0	0
D_{29}	6.52	17.7	0	6	6	3–5	1–3	0	0	0	0
D_{30}	6.31	15.3	1	1	2	2	1	1	2	1	1
D_{47}	6.28	18.5	1	—	3	2	—	—	—	2	1
D_{31}	6.20	21.4	1	2	3	1	1	1	0	1	1
D_{47}	6.12	15.0	1	—	3	2	—	—	—	2	1
D_{32-33}	6.04	17.6	1	3	3	2	2	2	2	2	1
D_{35}	5.95	32.4	1	3	2	2	1	1	1	1	1

— = not tested at this concentration.

precipitation against a pool of sera from patients with acute rheumatic fever. This serum pool had been found to yield as many as 7 bands against pooled concentrates from bulk cultures of this strain of the hemolytic streptococcus (Harris *et al.*, 1955). The data obtained in such examinations again showed differences between concentrates from cultures of steady-state pH 6.5 and 6.8 and those cultivated in the pH range of 6.3 and below. As can be seen in table 2, concentrates from cultures of higher pH range gave evidence of between 4 and 6 antigens at 6 mg/ml or less, whereas those from cultures of lower pH showed no more than 3 bands in this range of concentration. Also preparations from higher-pH cultures yielded no gel-precipitin bands at any concentration tested below 0.5 mg/ml, whereas those from lower-pH cultures yielded a band at the lowest concentration tested, 0.025 mg/ml.

3. Identification of the component yielding a gel-precipitin band at low concentrations of culture supernatant concentrate with component "B." In table 2 it can be seen that the concentrates which gave electrophoretic evidence of containing component "B" were the same as those which yielded a gel-precipitin band in solutions of low concentration. In order to determine whether the constituent which gave rise to the gel-precipitin band when the concentrate was used in the low range of concentration was component "B," experiments of the following type were done: In preliminary experiments a concentrate known to contain component "B" was run at relatively high concentration on several strips of Whatman no. 1 filter paper in the paper electrophoresis apparatus. After the run, one such strip was removed and stained with bromphenol blue. The eluate of corresponding portions of the unstained strips was then dialyzed and lyophilized, and the dried material was tested at various concentrations by gel precipitation against the same serum pool. In no tube was more than one band present. A concentration of the lyophilized eluate was then chosen which gave a sharply defined band, and a concentration was chosen for each of the several concentrates which had given a sharply defined band in the low range of concentration. Gel precipitin tests done on mixtures of equal volumes of the eluate and each of the culture supernatant concentrate solutions, each at twice the concentration indicated above, yielded only one band in each case.

4. Some properties of component "B." (a) Distilled water insolubility:—In the course of preparing culture supernatant concentrate from low-pH steady-state cultures it was observed that a precipitate appeared during the second dialysis against running tap water of the partially concentrated material. The sediment, redissolved in 0.8 per cent NaCl and tested by paper electrophoresis, showed the zone characteristic of component "B." The supernatant showed no zones by paper electrophoresis. Gel-precipitin examination of the redissolved sediment (table 3) indicated the presence of a band of precipitate at 0.01 mg/ml, presumably due to component "B."

TABLE 3

Gel-precipitin data of an original lyophilized culture supernatant concentrate (CSC), a water-insoluble fraction, and a fraction insoluble in both 40 per cent saturated ammonium sulfate and water; comparison of sediment and supernatant from 40 per cent saturation with ammonium sulfate

STRAIN H44

Conc	CSC Original		Water-Insoluble Fraction		40 Per Cent $(NH_4)_2SO_4$-Water-Insoluble Fraction	
	No. of Bands	Position	No. of bands	Position	No. of bands	Position
mg/ml						
6.0	3		1	5	1	7
3.0	3		1	7.5	1	6
1.0	1	6	1	6.0	1	4
0.5	1	4.5	1	6.5	0	
0.05	*		*		*	
0.01	1	6.0	1	6.0	1	5.5

STRAIN 5797

Conc	CSC Original		40 Per Cent $(NH_4)_2SO_4$ Precipitate		40 Per Cent $(NH_4)_2SO_4$ Supernate	
	No. of bands	Position	No. of bands	Position	No. of bands	Position
mg/ml						
6.0	4		2		4	
3.0	3		1	3.5	3	
1.0	2		0		1	5.5
0.5	Not tested		0		1	6.5
0.05	1	4.5	1	7.0	0	
0.01	0		1	4.9	0	

* Dense precipitate.

Gel precipitin examination at higher concentrations, also shown in table 3, indicated that other proteins were present in lower concentration in the water-insoluble fraction than in the original culture supernatant concentrate.

(b) Isoelectric range:—Determinations of direction and relative rates of migration of component "B" in paper electrophoresis were carried out in buffers of various pH in order to obtain some approximation of the isoelectric point. In order to correct for electroosmotic effect, parallel determinations were made of the migration rate of an uncharged macromolecular substance, dextran. The migration rate of the latter was in all cases subtracted from that observed for component "B." In this manner, the isoelectric point of the component was estimated at 9.0 to 9.1 in 0.1 M glycine or barbital buffer. However, because of the relatively large corrections for electroosmotic effect which were involved, solutions of component "B" were examined by moving-boundary electrophoresis (in the Perkin-Elmer apparatus) in 0.1 M barbital buffer at pH 8.4 and 8.7. Migration at low rates and in opposite directions was observed at these two levels of pH, indicating an isoelectric point between 8.4 and 8.7 for this substance in the buffer indicated.

(c) Effect of ammonium sulfate:—Fractional precipitation with ammonium sulfate was carried out at pH 8, near the isoelectric range indicated above. Increasing amounts of precipitate were obtained between 10 and 40 per cent of saturation with this salt, then no further precipitate appeared until 80 per cent of saturation. The 40 per cent-saturation precipitate was collected, dissolved and dialyzed against distilled water. The precipitate thus obtained was dissolved in saline solution, lyophilized, and tested by gel precipitation against the same serum. The results of this examination are shown in the last pair of columns in the upper half of table 3. It can be seen that this fraction again yields a gel-precipitin band at a concentration of 0.01 mg/ml, and the data obtained at higher concentrations indicate further purification of the material.

Gel-precipitin data obtained with a low-pH-range steady-state culture of another strain of group A μ-hemolytic streptococcus, strain 5797, are shown in the lower part of table 3. Here component "B" appears to constitute a lower percentage of the culture supernatant concentrate than in the case of the H44 material shown in the upper half of the table, the ratio of concentrations being of the order of 10. Here also it can be seen that a band of precipitate is given by a low concentration of the 40 per cent ammonium sulfate precipitate. This is, again, presumably due to component "B," which in the case of this preparation is apparently in a substantially higher concentration in the ammonium-sulfate-precipitated fraction than in the original concentrate.

5. Identification of component "B" with the streptococcal proteinase described by Elliott. The properties described above for component "B"—production at a low pH range, water insolubility, high isoelectric point, precipitability at low concentrations of ammonium sulfate—were similar to those described by Elliott (1945) for the streptococcal proteinase studied by him, which may, in turn, have been the same enzyme as that described by Frobisher (1926) and Seegal and Seegal (1936). The following experiments were done:

(a) Enzymatic activity of preparations containing component "B":—Elliott (1947) had found that the proteinase caused clotting of casein in skimmed milk, in the presence of thioglycolic acid or similar reducing substance, and that the enzyme was irreversibly inactivated by iodoacetate. Such tests set up with materials from low-pH chemostat cultures showed milk-clotting activity in original supernatants up to a dilution of 200, and in 1 mg/ml solutions of concentrates up to 1000. Milk clotting was inhibited by iodoacetate or iodoacetamide in low concentration.

(b) Adsorption of antisera with graded amounts of culture supernatant concentrate: Effects on the relative concentrations of antiproteinase and on antibody to component "B" as determined by gel precipitation:—Sera of rabbits which had been injected with culture supernatant concentrate were found to inhibit milk clotting by preparations of this concentrate as well as to yield gel-precipitin bands *vs.* component "B." To portions of such a serum were added various amounts of the partially purified concentrate. After suitable incubation and removal of the precipitates which had formed, the supernatants were tested for anti-component-"B," as judged by gel precipitation, and anti-proteinase (milk-clotting inhibition) in comparison with the original, unadsorbed,

TABLE 4

Gel-precipitin and milk-clotting-inhibition data of anti-culture supernatant concentrate serum adsorbed with various amounts of culture supernatant concentrate

Conc. of Antiserum Used (Rabbit 989 12/56)*	Conc. of Antigen Added,	Milk-Clotting-Inhibition Titer	Position of Gel-precipitin Band,
	*mg/ml**		*mm*
1:4	0	256	3.5
1:4	0.01	192	3.5
1:4	0.05	96	4.2
1:4	0.10	32	—
1:4	0.5	24	—

* These reagents added in equal volumes

serum. The data obtained in such an experiment are shown in table 4. At the final dilution of 1:8 the antiserum had a titer of 256 in the milk clotting inhibition test, and produced a gel-precipitin band 3.5 mm from the antigen zone, against the concentration of culture supernatant concentrate employed. The smallest amount of antigen used for adsorption caused a small decrease in milk clotting inhibition titer without an observed change in the position of the gel-precipitin band. The next level of addition of antigen caused a further reduction by 50 per cent in the titer of milk-clotting inhibition and a change of 0.7 mm in the position of the gel-precipitin band, which is in the direction and range of magnitude corresponding to a loss of antibody of the order of one half. The next step in amount of antigen added caused a further decrease of almost a power of 2 in milk clotting inhibition titer, and this preparation gave no gel-precipitin band, presumably because the concentration of anticomponent "B" was now too low.

(c) Cultivation of a known proteinase-producing strain of the hemolytic streptococcus:—Strain 5797 of the hemolytic streptococcus, which had been used by Elliott in his work on streptococcal proteinase, was adapted to the synthetic medium and to the chemostat, and cultivated at a steady-state pH of 6.2. The turbidities of the chemostat cultures were approximately the same as that of H44. However, in the cultures of this strain the milk-clotting titers were approximately one-tenth of those obtained with H44, relative to volume of culture supernatant or to dry weight of concentrate. Gel-precipitin examination indicated that component "B" was in considerably lower concentration in culture supernatant concentrate from strain 5797 than in that from H44, and the gel-precipitin test indicated higher concentrations of other proteins. However, fractionation by ammonium sulfate gave a partially purified preparation such as that shown in table 3, which resembled analogous preparations from H44 cultures in electrophoretic pattern, gel precipitation, and milk-clotting titer.

(d) Proteolytic effect of the proteinase on other antigens in the culture supernatant:—The proteolytic property of proteinase was used for its partial purification, as suggested by Elliott, as follows: Partially concentrated and dialyzed preparations of low-pH chemostat cultures containing the active form of streptococcal proteinase were incubated overnight (18 hr) in the presence of 10^{-2} M thioglycolate and 0.85 per cent NaCl at a pH of 7.3, dialyzed overnight versus distilled water, and then lyophilized. Gel precipitin tests of such preparations indicated a decrease of the order of 100-fold in the concentration of the contaminating antigens, relative to that of component "B," or proteinase.

(e) Experimentally induced changes in the ratio between enzymatic activity of proteinase in culture supernatant concentrate and concentration of component "B" as determined by gel precipitation:—A change in this ratio was observed in two instances: the first of these was dialysis against tap water of culture supernatant concentrate containing active proteinase. This caused a marked decrease in the milk clotting titer of the concentrate, without observable effect on the activity in gel precipitation. The second was the addition of iodoacetate to the concentrate at a concentration sufficient to inactivate the enzyme. This again did not affect the behavior of the concentrate in gel precipitation. These two observations were not considered as giving evidence against the identity of component "B" and proteinase. Rather, it was considered likely that the dialysis had caused a degree of denaturation of the protein which affected the enzymatic activity but not the antigenic sites, or a loss of some trace ion necessary for the enzymatic activity, and that in the second instance the presumed binding of the SH groups by iodoacetate had inactivated the enzymatic sites

without affecting the antigenic determinant groups on the protein molecule.

6. Partial purification of the proteinase. A comparison was made between the degree of purification of proteinase which could be attained by the use of its enzymatic property with that resulting from precipitation by ammonium sulfate. In preliminary experiments it was found that the degree of purification of the proteinase was increased by a second precipitation with ammonium sulfate but not by a third one. It was also found that when such precipitation was applied to concentrates with proteinase in the active form (i. e. from a culture collected without iodoacetate), nonresoluble material was present in the precipitate, and the degree of purification was less than in the case of material from cultures collected with iodoacetate. Accordingly, a concentrate from an H44 culture was twice precipitated with ammonium sulfate at 40 per cent saturation (pH 8), with the removal, also, of distilled-water-insoluble material. Another preparation of culture supernatant concentrate, from a culture collected without iodoacetate, was brought to 80 per cent saturation with ammonium sulfate. The redissolved precipitate was incubated with sodium thioglycolate and 0.001 M merthiolate and processed as described above. The resulting material, as well as material obtained after the first precipitation with ammonium sulfate, were examined by gel-precipitation; the results of such tests are shown in table 5. Here it can be seen that in all 3 partially purified preparations the gel-precipitin band, presumably due to proteinase, was found at a concentration of 0.01 mg/ml, and that contaminating antigens had been removed to such an extent that substantially higher concentrations of the partially purified preparations were required for the demonstration of any of these contaminating antigens. In the case of the two more highly purified preparations, those obtained by two precipitations with ammonium sulfate or by proteolysis of contaminating antigens, it can be seen that in both cases a concentration of 20 mg/ml was required to produce, presumably by the most concentrated contaminating antigen, a band at approximately the same position as that attributed to proteinase in the 0.01 mg/ml solution. This implies for either of these two partially purified preparations a ratio of the order of 2000 between the concentration of proteinase and that of the most concentrated contaminating antigen to which antibody is present in the serum used.

Several attempts were made to crystallize proteinase from the culture supernatant of low-pH chemostat cultures, or from appropriate preparations of culture supernatant concentrate, using the procedure described by Elliott. These attempts were unsuccessful.

7. Spontaneous loss of proteinase production in low-pH steady-state culture of strain H44. The studies described above involved a considerable number of experiments in the chemostat, with a total of over 2000 generations of steady-state cultivation of the streptococci, most of these being in the low range of pH. During this time there were two episodes in which the pH and turbidity of the culture spontaneously underwent a series of changes, involving a rapid change of pH (6.1 to 6.5) and turbidity (290 to 200) with loss of proteinase production, and return of pH and turbidity to the previous levels, but without restoration of production of proteinase. These two episodes occurred in the course of the two longest experiments in the chemostat, after 252 and 270 consecutive generations, respectively (there was no other experiment as long as 200 generations). Concentrates prepared from the culture collected during 40 generations of growth without proteinase production were tested in gel precipitation. No bands of precipitate appeared at 0.5 mg/ml or less, and 6 bands at 2.5 mg/ml. Such gel precipitin data had been found to be characteristic of nonproteinase producing cultures (see table 2).

DISCUSSION

The application of steady-state culture to the production of streptococcal antigens. As was stated above, the conventional bulk cultures of hemolytic streptococci had produced antigens at concentrations which varied substantially among successive cultures. In the series of low-pH steady-state cultures described here there was a substantially greater degree of uniformity in the production of antigens. However, one could not generalize from the experience thus far because of the singular circumstance that the antigen produced in greatest amount was itself a proteolytic enzyme which could cause lysis of other proteins produced by the organisms. In fact, evidence that the low concentration range of

TABLE 5

Comparative data by gel precipitation of the products of two procedures for purification of proteinase: precipitation with ammonium sulfate and proteolysis of contaminating antigens

Conc.	40 Per Cent $(NH_4)_2SO_4$ 1× ppt		40 Per Cent $(NH_4)_2SO_4$ 2× ppt		Proteolysis	
	No. of bands	Position	No. of bands	Position	No. of bands	Position
mg/ml						
20	Not tested		1	5.8	1	5.4
10	1	5.8	1	5.0	±	
5.0	1	3.5	0		0	
2.5	0		0		0	
1.0	0		0		0	
0.5	0		0		0	
0.2	0		0		0	
0.1	0		0		0	
0.05	0		0		0	
0.025	1	7.0	1	7.0	Not tested	
0.01	1	5.8	1	6.0	1	5.4
0.005	Not tested		Not tested		1	4.3

antigens other than proteinase was not due necessarily to a low rate of production of those proteins at low pH was available from the two instances of sudden change of pH of the culture and loss of proteinase production described above. Here the production of proteinase was not resumed on the return of the culture to its low pH value, and other antigens were present in higher concentration in the completed culture than before.

Aspects of this work of interest in relation to protein separation. In the work reported above there are three points of interest for the general problem of analysis and separation of protein mixtures. The first of these is the substantially greater sensitivity of gel precipitation than paper electrophoresis for the detection of proteins. Several preparations have been referred to above in which only one electrophoretic zone was detected or even none, but in which as many as 7 bands appeared in gel precipitation. This difference is not surprising if one recalls that the threshold of concentration for detectable proteins is of the order of 1 mg/ml, in either moving-boundary or paper electrophoresis, whereas proteins can be detected by gel precipitation at a concentration of 0.005 mg/ml or even less.

The other two points of interest for protein separation referred to above lie in two of the technical procedures used in this work. One of these is the identification of an antigen associated with a particular gel-precipitin band by gel precipitation of a mixed antigen preparation including an eluate of paper electrophoresis. In the present work this procedure was used to identify by electrophoresis an antigen which produced a gel-precipitin band at a given concentration range of the mixture; in other situations it might be useful for identifying a given constituent in order to determine the results of a fractionation procedure. Other procedures for associating gel-precipitin bands with the substances giving rise to them have been described, in one-dimensional gel precipitin systems, by Oudin (1952), Telfer (1953) and others. In those cases the association was within the gel-precipitin system itself, involving combinations of antigen mixtures in various ratios and observing the positions of the bands on testing the mixtures. The procedure described here is directed at identifying with a given gel-precipitin band a protein recognized by some other procedure, as by electrophoresis or fractionation.

The other technique referred to is that of estimating the effectiveness of fractionation procedures by gel-precipitin tests of the original mixture and of the fraction. The numbers of gel-precipitin bands produced by fractions of mixtures has been used to observe increases in purification of a protein in other studies, such as that of Pope *et al.* (1951) in the case of diphtheria toxin. In the present procedure the degree of relative purification has been estimated by comparison of the minimum concentrations of the original mixture and of the fraction, respectively, which are required for band formation by the protein being purified and by contaminating proteins. In this study the use of gel precipitation as an indicator of relative purity was facilitated by the fact that the constituent being purified was more concentrated than any other protein in the original mixture. In a situation in which this was not the case the technique could be used to guide the fractionation, at least after the early stages of fractionation had brought the concentration of the desired constituent to this point.

SUMMARY

β-Hemolytic streptococci have been grown in a steady-state by continuous dilution of the culture at constant volume, using a chemically defined medium. Concentrates of macromolecular material of supernatants of such cultures were

examined by paper electrophoresis and gel precipitation against rabbit antisera. In concentrates of filtrates of cultures at steady-state pH 6.5 to 6.8 several antigens could be detected by gel precipitation, none being sufficiently concentrated relative to the others to be detected by paper electrophoresis. Similar concentrates from cultures of steady-state pH 6 to 6.3 were found by paper electrophoresis to contain one protein in relatively high concentration, and, by gel precipitation, two or three other antigens.

Some properties of the chief component of the low-pH cultures were determined, and these suggested its identity with the streptococcal proteinase described by Elliott. Further experiments gave additional evidence of this identity.

In the case of strain H44, the proteinase was found to consist of about 90 per cent of the proteins of the concentrated supernatants of steady-state cultures of pH 6.2. In such cultures of strain 5797 the concentration of proteinase was not so high, but was greater than that of any other protein. Repeated precipitation with ammonium sulfate near the isoelectric point of the proteinase or application of the proteolytic property of this substance resulted in approximately similar degrees of relative purification of the proteinase.

There were two instances of interruption in the production of proteinase in the course of continuous culture, with concomitant spontaneous changes in the turbidity and pH. The latter returned spontaneously to their normal values, but the production of proteinase was not resumed. These episodes occurred after the two longest consecutive experiments with steady-state culture (more than 250 generations).

Applications of one-dimensional gel precipitation are described for identifying a constituent of a mixture of antigens with the same substance identified by other means (in this case, paper electrophoresis) and for estimating changes in the degree of relative purification of one constituent in a mixture of antigens.

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