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ANTIBACTERIAL ACTIVITY OF THE COELOMIC FLUID FROM THE POLYCHAETE, *GLYCERA DIBRANCHIATA*. II. PARTIAL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE ACTIVE FACTOR

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Abstract

The coelomic fluid of *Glycera dibranchiata* is bactericidal for the Gram-negative rod, *Serratia marcescens*. An active factor has been partially purified using gel chromatography and affinity chromatography on concanavalin A-Sepharose, and has been shown to be a glycoprotein with a molecular weight of $2.5–4.5 \times 10^5$ containing bound divalent cations and at least one disulfide bridge. The active molecule showed no biochemical similarities to other humoral bactericidal systems, either vertebrate or invertebrate, and would appear to define a new class of antibacterial molecules.

Introduction

We have previously described a bactericidal factor in the coelomic fluid of the polychaete *Glycera dibranchiata* (Anderson and Chain, 1982), and have studied the kinetics of its action against the Gram-negative rod, *Serratia marcescens* in the previous paper. These studies suggested, *inter alia*, that *Glycera* antibacterial factor (GAF) is a heat-labile protein, which acts by a 2-step process involving an initial reversible binding to the bacterial cell surface, followed by a "killing reaction" which perhaps involves damage to the cell membrane. In this paper we present some studies on the biochemical characterization of GAF. Only a partial purification of GAF has been achieved and many details of its structure remain unknown. However, the information which has been gained is sufficient to allow us to begin to draw comparisons with other humoral bactericidal systems.

Methods

Coelomic fluid from *Glycera dibranchiata* was obtained as described previously and antibacterial activity was measured using a turbidometric assay (Chain and Anderson, 1982). Briefly, coelomic fluid (CF) and bacteria (6×10^6 organisms from an overnight culture of *S. marcescens*) were incubated together in 0.5 ml artificial sea water (ASW) at room temperature for 30 min. 4 ml of tryptic soy broth (TSB) were then added to each tube, and the bacteria grown up for 4–5 h at 37°C. The optical density of the cultures (compared to controls without CF) could be converted directly to % bacteria killed.

Column chromatography was carried out at 4°C using gels obtained from Pharmacia Fine Chemicals; protein concentration in the eluants was continuously monitored by recording UV absorbance (280 nm). Gel filtration was carried out on 50 or 100 × 1.5 cm columns of Sephacryl S-300, molecular weight range 10^4–1.5

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$\times 10^6$, or Sepharose 4B, molecular weight range 6×10^4–20×10^6. Ion-exchange chromatography was carried out on DEAE-Sepharose-C1 6B. Coelomic fluid was dialyzed, using Spectrapor 2 tubing (Spectrum Medical Industry, Inc.), overnight against a large volume of Tris buffer (pH 8.5, 0.1 *M*), containing 10 m*M* calcium chloride. 3 ml of dialyzed coelomic fluid were run onto the column and unbound material was washed off using the same buffer. Elution was carried out with a linear 0–0.5 M gradient of sodium chloride in the same buffer. Affinity chromatography was carried out on a 8 × 1.5 cm column of Con A–Sepharose 4B. Coelomic fluid (10–20 ml) was run through the column, which was then washed with several volumes of ASW. To elute bound components a volume (equal to the void volume of the column) of a 0.5 *M* solution of 1-O-methyl α-D-glucopyranoside or α-methyl D-mannoside in ASW was run onto the column and left for 30 min to insure that all bound components were displaced by the sugar. The displaced glycoprotein was eluted with additional 0.5 *M* saccharide solution. Column fractions were concentrated for electrophoresis using immersible CX-30 Millipore filters (nominal M.W. retention >30,000).

Gel electrophoresis was carried out at 120 volts for 16 h on Pharmacia precast polyacrylamide gradient gels; gels useful in the molecular weight range 5×10^4–2×10^6 and 10^5–5×10^6 were used. Gels were stained for protein with Coomassie blue (1 h, 0.25%) or for glycoproteins using the periodate-Schiff stain according to the protocol of Smith (1976).

Artificial sea-water was made up using Instant Ocean (Aquarium Systems) to a total salinity of 38‰. Its ionic composition has been given previously (Chain and Anderson, 1982). Other chemicals were obtained from Sigma Chemical Company.

Results

Preservation of GAF activity

Two major problems have hindered the biochemical purification of GAF: the greatly accelerated loss of antibacterial activity occurring upon CF dilution, and the rather rigid ionic requirements of the active moiety. The effects of overnight dilution of CF in ASW (the optimum ionic medium for preserving GAF activity) are shown in Table I. As can be seen, substantial activity was irreversibly lost in 16 hours if CF was diluted 1:50–1:100. In contrast, other studies showed that undiluted CF retained full activity at 4°C for 2–3 days. The phenomenon of loss of activity on dilution was particularly apparent in the gel filtration experiments reported below. Various attempts at preserving the activity by inhibiting disulfide bond formation

TABLE I

Effects of dilution in artificial sea water on coelomic fluid activity

Volume of coelomic fluid (μl)	Antibacterial activity (% killing)	
	Diluted immediately before assay	Diluted 16 hours before assay
5	53 ± 12	3 ± 3
10	98 ± 1	70 ± 5

Antibacterial activity was measured using the turbidometric assay with a 30 min initial incubation period. The specified volume of coelomic fluid was diluted into 0.5 ml ASW for the assay. Results are expressed as % killing ± S.E.M., N = 3.

TABLE II

Antibacterial activity of Glycera *coelomic fluid after dialysis against solutions of differing ionic composition*

Dialysis solution	Activity (μl CF required for 50% decrease in optical density)
Artificial Sea Water	5.0 ± 0.4 (3)
10 m*M* $CaCl_2$ in Tris Buffer (0.01 *M*, pH 8)	6.5 ± 0.7 (6)
10 m*M* $MgCl_2$ in Tris Buffer (0.01 *M*, pH 8)	13.0 ± 2.6 (3)
1 *M* NaCl in Tris Buffer (0.01 *M*, pH 8)	6.0 ± 1 (3)
Tris Buffer (0.01 *M*, pH 8)	no activity
Phosphate Buffer (0.05 *M*, pH 7.5)	no activity
1 *M* NaCl + 1 m*M* EDTA in Tris Buffer (0.01 *M*, pH 8)	no activity
Undialyzed coelomic fluid	4.6 ± 0.5 (11)

1–2 ml of coelomic fluid was dialyzed overnight against 1 liter of the solutions indicated above. Various volumes of retentate were assayed after dilution in ASW for bactericidal activity, using the turbidometric assay, and the volume required for a 50% decrease of optical density was measured from the dose-response curve obtained. Results are given as $\bar{X}$ ± S.E.M., (N).

(−SH oxidation) with dithiothreitol, by introducing a protein carrier (BSA), or by cross-linking with very low concentrations of gluteraldehyde, were unsuccessful. Dithiothreitol totally destroyed GAF activity suggesting the presence of disulfide bonds in the GAF molecule.

Experiments illustrating the ionic requirements for preservation of GAF activity are shown in Table II. Overnight dialysis against solutions without divalent cations (Tris or phosphate buffers, or 1 *M* sodium chloride with 1 m*M* EDTA) irreversibly destroyed GAF activity. Native GAF activity was preserved by dialyzing against ASW. As little as 10 m*M* $CaCl_2$ preserved most of the activity, while the same concentration of $MgCl_2$ was less effective. High concentrations of sodium chloride were also sufficient even in the absence of added calcium, perhaps as a result of enhanced binding of intrinsic divalent cations in conditions of high ionic strength.

Although optimal preservation was obtained in ASW, other solutions (containing only the minimum requirement of 10 m*M* calcium chloride) had to be adopted in some experiments in order to use particular techniques, such as ion exchange chromatography, or polyacrylamide gel electrophoresis.

Gel filtration

Elution profiles of coelomic fluid eluted in ASW from two types of gels are shown in Figures 1 and 2. Antibacterial activity after frationation on Sepharose 4B (an agarose gel with an exclusion limit of 2×10^7 Daltons), or with Sephacryl S-300 (an alkyl dextran gel cross-linked with N,N′-methylene bis acrylamide, exclusion limit 1.5×10^6 Daltons) appeared as a single symmetrical peak associated with the major protein peak. A comparison of the elution volume of the peak of antibacterial activity to known protein standards gave molecular weight values of $4–5 \times 10^5$ Daltons on Sepharose 4B, and $2.5–3 \times 10^5$ on Sephacryl S-300.

Measurement of total protein concentration in each fraction (Lowry *et al.*, 1951) was used to compare the specific activity in the eluate to that in the original coelomic fluid. The fractions showing 90–100% bactericidal activity had a protein concentration of 0.4–0.6% mg/ml. With unfractionated coelomic fluid equivalent activity

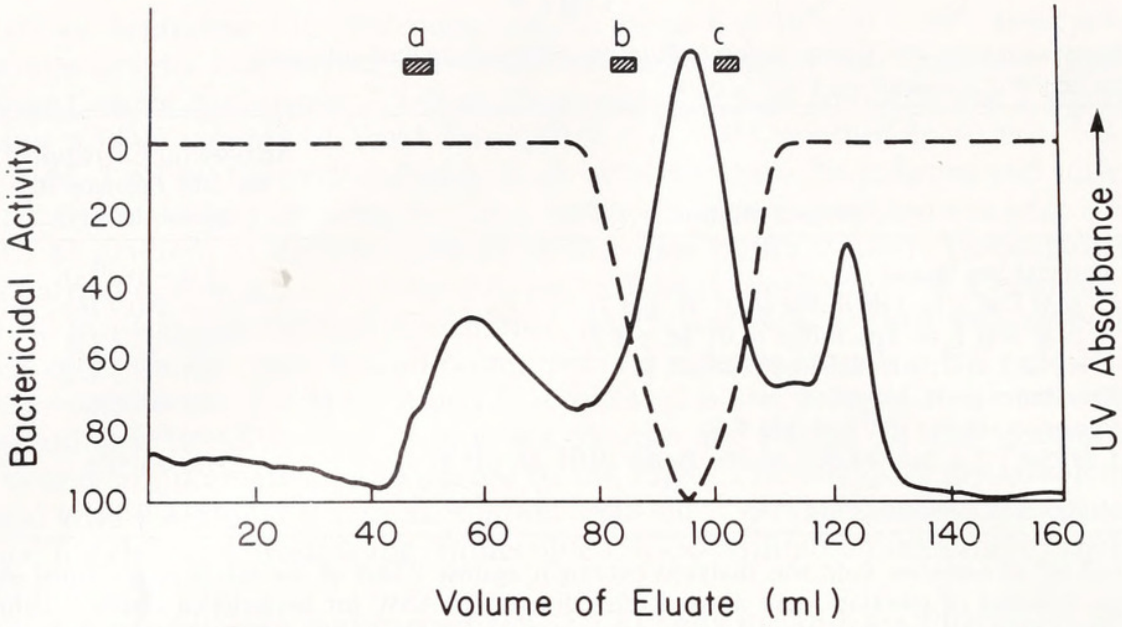

FIGURE 1. Gel filtration of 3 ml coelomic fluid on Sepharose 4B, elution with ASW. Three ml fractions were collected and 0.5 ml of each fraction was tested for bactericidal activity using the turbidometric assay.

a) Void volume; b) Thyroglobulin, M.W. 669,000; c) Aldolase, M.W. 158,000

—— UV absorbance (280 nm) - - - Bactericidal activity

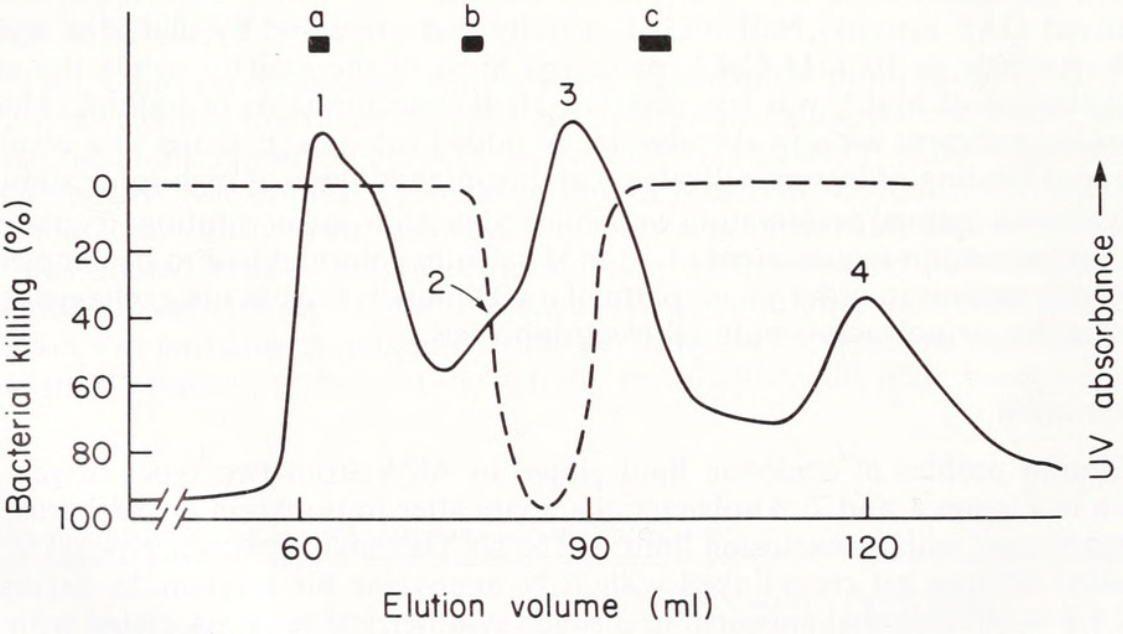

FIGURE 2. Gel filtration of concentrated coelomic fluid on Sephacryl S-300, elution with ASW. Fifteen ml of CF were concentrated to 4 ml by pressure ultrafiltration through Millipore Pellicon membrane ultrafilters (type PTHK). Three ml fractions were collected and 0.5 ml of each fraction was tested for bactericidal activity using the turbidometric assay. Numbered peaks are analyzed by electrophoresis as shown in Figure 3.

a) Void volume; b) Ferritin, M.W. 440,000; c) Aldolase, M.W. 158,000

—— UV absorbance (280 nm) - - - Bactericidal activity

was obtained with a protein concentration of 0.1–0.2 mg/ml, indicating a loss in specific activity of GAF during fractionation. One obvious possibility was that GAF was in reality composed of several different components, and that loss of activity was actually a direct result of their separation during purification. However, numerous attempts to reconstitute activity by combining fractions, both from within given peaks and from different peaks, were unsuccessful. It seemed probable, therefore, that a real loss in the biological properties of the active molecule occurred during gel filtration. In part this may be a consequence of protein dilution as was discussed above, and the degree of loss of activity was also correlated to the length of time the GAF remained on the column.

The fractions corresponding to the major protein peaks from the Sephacryl S-300 column were analyzed further by gel electrophoresis on polyacrylamide gradient gels which separate proteins of >150,000 Daltons (Fig. 3). As can be seen, each peak is made up of several components, and overlap is particularly high between peaks 2 (a shoulder on peak 3) and 3. Comparison of biological activity with S-300 protein peaks (Fig. 2) suggests that peak 3 contains the major portion of GAF activity. The main protein components of peak 3 correspond to the major protein band (A) of CF (Fig. 3) and the band just above it on the gel.

We attempted to localize GAF activity directly on gels after CF electrophoresis. Antibacterial activity was visualized by overlaying the gel with trypticase soy agar containing a suspension of *Serratia marcescens* followed by 18 h incubation at 37°C. Bactericidal activity appeared as areas of sparse growth in the otherwise uniform bacterial lawn (Fig. 4). Rather surprisingly, two zones of activity could be seen, one corresponding approximately to the major protein band (A) and the band imme-

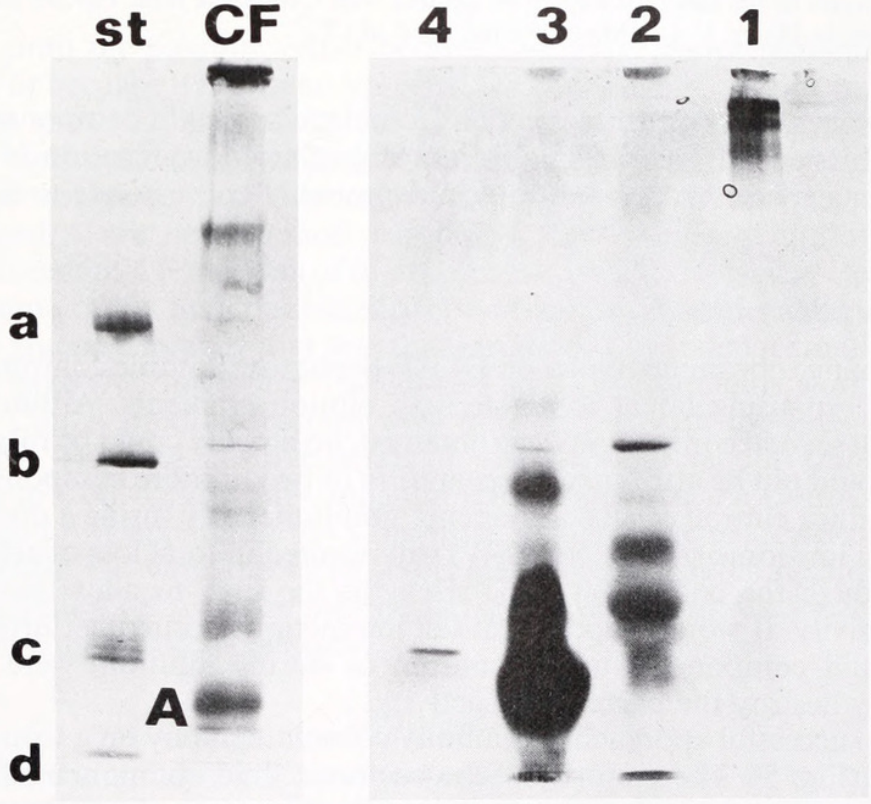

FIGURE 3. Polyacrylamide gradient gel electrophoresis of coelomic fluid (CF) and major protein peaks (1–4) of Sephacryl S-300 eluate. Numbers correspond to fractions shown in Figure 2. St = Standards a) Thyroglobulin, M.W. 669,000; b) Ferritin, M.W. 440,000; c) Catalase, M.W. 232,000; d) Lactate dehydrogenase, M.W. 140,000. A = Major protein band in CF.

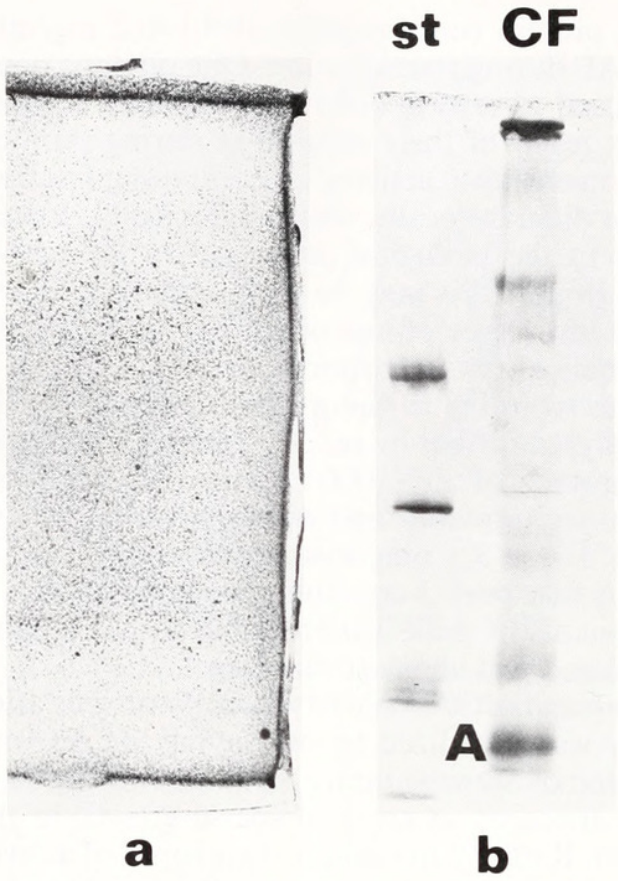

FIGURE 4. Antibacterial activity in coelomic fluid (CF) visualized after electrophoretic fractionation on a polyacrylamide gradient gel. a) Area of a gel overlayed with bacteria (6×10^5 *S. marcescens*/ml in TS agar). b) Portion of the same gel stained for protein with Coomassie blue. Protein standards (st) are the same as used in Figure 3; A = Major protein band of CF.

diately above it, and the other to a very high molecular weight component. It seemed likely that this second band of inhibition resulted from aggregation of GAF which occurs under experimental conditions and probably corresponds to S-300 protein peak 1.

Other purification methods

Ion exchange chromatography on DEAE-Sepharose anionic columns (at pH 8.5) was carried out using linear ionic strength elution gradients. Although excellent separation of several components was obtained, no activity could be recovered. Once again this could not be attributed to separation of two or more components required for activity since elution of all components simultaneously (using a one-step elution change from low ionic strength to ASW) still resulted in total loss of activity. Simple passage through the column at ionic strengths too high to allow binding had no effect on activity. It would appear that the interactions occurring during binding of coelomic fluid components to the column or during dilution, were sufficient to permanently destroy the bactericidal activity.

A more successful approach was affinity chromatography on a Con A Sepharose 4B column (Fig. 5). The majority of the coelomic fluid components failed to bind to the column and could be eluted with ASW. However, all antibacterial activity was adsorbed onto the column. A fraction eluted from the column with a high concentration of α-methyl D-glucopyrannoside (or mannoside) showed a bactericidal effect. However, this activity represented only a minute fraction of the total

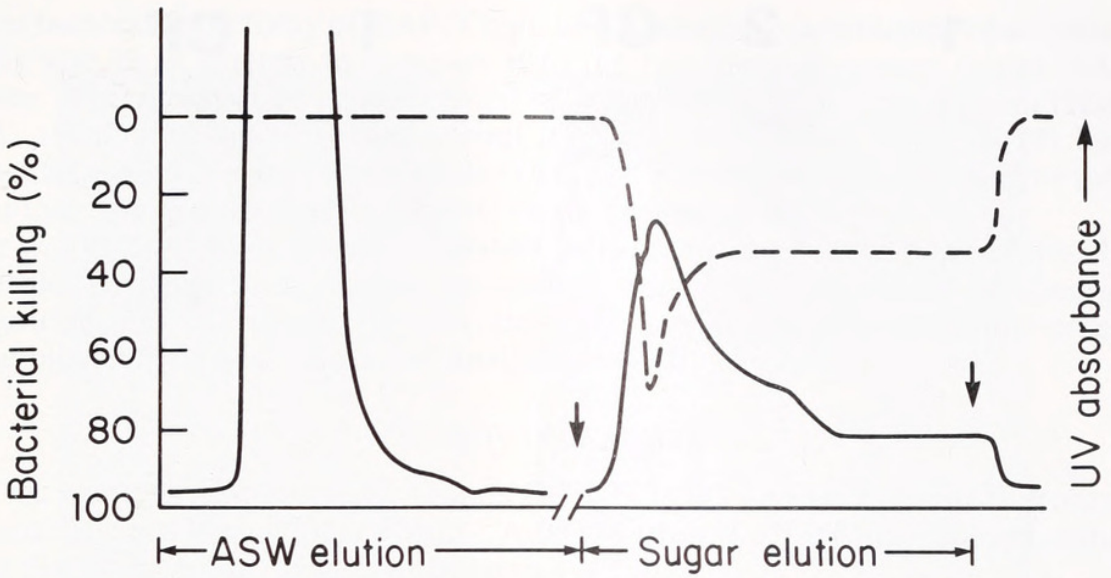

FIGURE 5. Affinity chromatography of CF on Con A-Sepharose 4B. Ten ml of coelomic fluid were run through a short (8 × 1.5 cm) column of Con A-Sepharose using artificial sea-water as the eluent. At arrow eluent was changed to a 0.5 *M* solution of 1-0-methyl-α-D-glucopyranoside in ASW. Three ml were run onto the column and flow was stopped for 30 min to allow time for the competing ligand to displace substances bound by the Con A. Glycoproteins were then eluted with more sugar solution. At second arrow eluent was changed back to ASW. Three ml fractions were collected throughout the experiment and 0.5 ml of each fraction tested for bactericidal activity using the turbidometric assay. Note that the high sugar concentration alone inhibited bacterial growth to a certain extent.

—— UV absorbance (280 nm)

– – – Bactericidal activity

activity put onto the column, either because the active factor remained bound to the column, or because it had been irreversibly denatured by binding to the column.

Figure 6a shows an analysis of the Con A fractionation using polyacrylamide gradient gel electrophoresis. Although Con A selectively removes certain high molecular weight components of the coelomic fluid, most of the major proteins in the region of band A are not retained, suggesting that GAF is quantitatively a very minor constituent of CF proteins of this molecular weight. However, electrophoresis of a concentrate of the material initially bound to Con A, and subsequently eluted with methyl glucose, showed that this fraction indeed included a small component of the major band (A). Since Con A selectively binds glycoproteins, samples of whole CF and the Con A binding fraction were also stained with the PAS glycoprotein stain (Fig. 6b). The PAS-positive bands in whole CF correspond rather closely to those bound to Con A. The only apparent anomaly is that the Con A eluate component of band A is apparently PAS-negative, though there is a PAS-positive component of whole CF with the same molecular weight. The concentration of this component in the Con A eluate may be too low to stain with the PAS stain, which is less sensitive than Coomassie blue.

DISCUSSION

Glycera antibacterial factor appears to be a glycoprotein of molecular weight $\sim 2.5\text{–}4.5 \times 10^5$. There are also strong indications that the molecule contains bound divalent cations which are essential to its structural integrity and at least one disulfide bridge. Because of the loss in bactericidal activity, which occurred during all chromatographic separations, and the presence of multiple components in column frac-

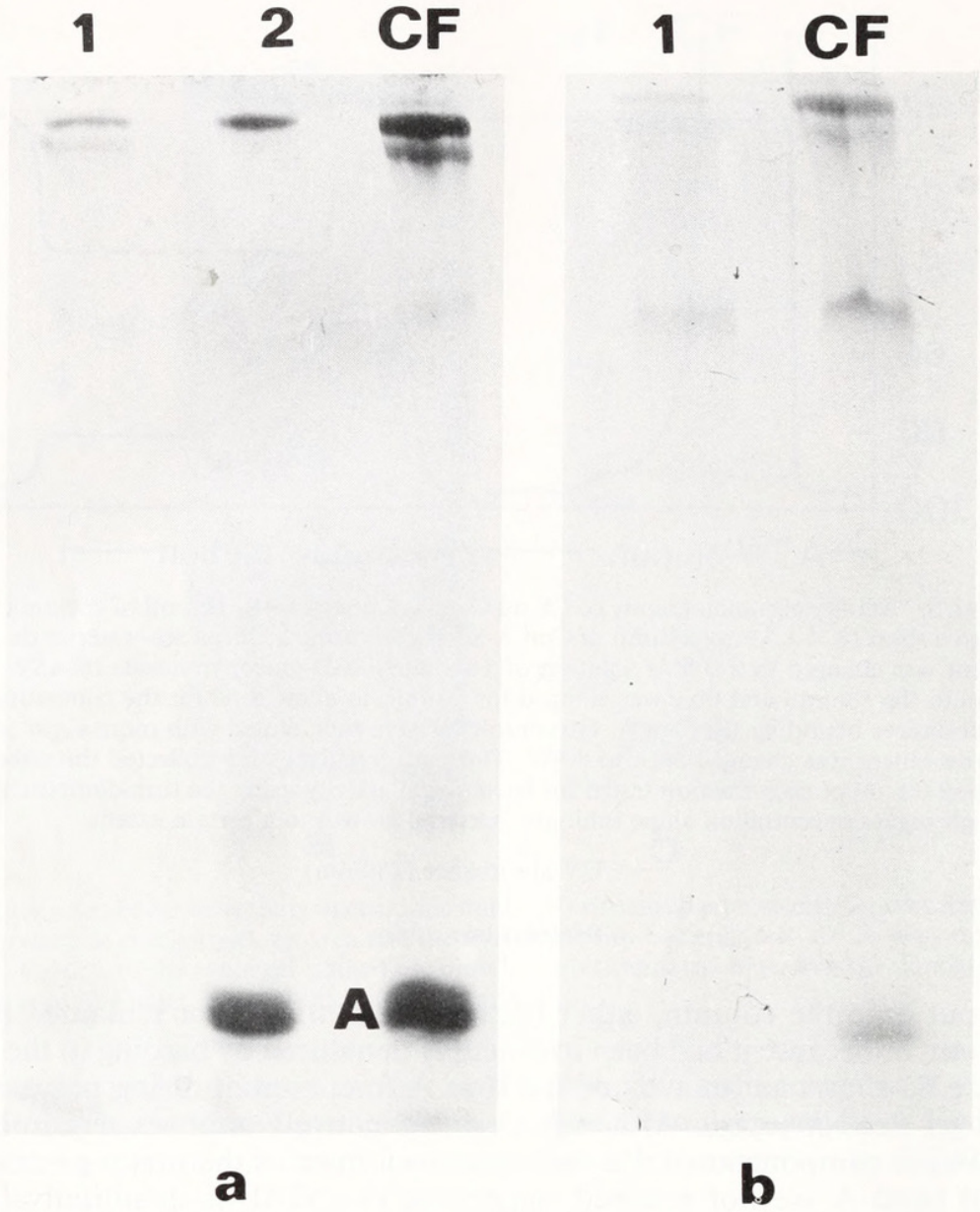

FIGURE 6a. Analysis of affinity chromatography experiment (see Fig. 5) using polyacrylamide gradient gel electrophoresis. Gel stained for protein with Coomassie blue. CF = untreated coelomic fluid; 1 = CF components bound by Con A and eluted with 0.5 *M* 1-0-methyl-α-D-glucopyranoside; 2 = CF after passage through Con A column; A = major protein band of CF.

b. Gel stained for glycoproteins using periodate-Schiff reagent. 1 = PAS staining of CF components bound by Con A column and eluted as in Figure 6a.

tions even after purification, as shown by gel electrophoresis, it is as yet impossible to rule out the possibility that several different molecules have GAF activity, or that GAF aggregates may exist. Therefore, the results obtained may only refer to one of the major antibacterial components of the coelomic fluid. The molecular weight estimates of GAF clearly differentiate it from any other antibacterial system found in either vertebrates or invertebrates. For example, it is clearly different from bactericidal proteins recently isolated from insects (Hültmark *et al.*, 1980) and oligochaetes (Roch *et al.*, 1980). Nor is there any evidence of any relationship to components of the vertebrate complement system (Müller-Eberhard, 1968; Müller-Eberhard and Schreiber, 1980), although the bactericidal mechanism of action of the two systems may show some similarities. In view of the probable cytotoxic, as

well as bactericidal activity of GAF (Chain and Anderson, unpublished observation), it may also be of interest to compare it to the lymphotoxin system found in vertebrates. The most active component(s) of lymphotoxin from both human (Harris *et al.*, 1981) and various other animal lymphoid cells (Ross *et al.*, 1979) has a molecular weight in the same range as GAF, and will also readily aggregate or break down into component parts with little or no biological activity.

It would be of great interest to dissect further the component parts of the GAF molecule, particularly in relation to separate functions such as cell binding and recognition, and cell killing. However, these studies will have to await improvements in the biochemical and functional analysis of GAF.

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