ENHANCEMENT OF ANTIBIOTIC ACTIVITY OF COLICINE K AND ALTERATION OF SEROLOGICAL PROPERTIES OF COLICINE K AND ENDOTOXINS*

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Colicine K, isolated from *Escherichia coli* K 235, has been shown to be a lipocarbohydrate-protein complex that is serologically and chemically similar to the O antigen of this strain of bacteria (1–3). Colicine K differs from other O antigens in that it possesses antibiotic activity toward susceptible *E. coli* (4). The antibiotic activity, which resides in the protein moiety of the complex is closely associated with the lipocarbohydrate but can be extracted with organic solvents, and thus does not appear to be covalently linked (5). Colicine K was thought to be related to the tail antigen of T6 bacteriophage (4). Since the neutralization of T2, T4, and T6 bacteriophage by normal human serum was under investigation at that time (6), an attempt to demonstrate a phage-related structure in colicine K by neutralization of its antibiotic activity by normal human serum was made. Neutralization of antibiotic activity was not observed. Instead, its antibiotic activity toward susceptible *E. coli* was enhanced many fold (7). Subsequently, it was shown that no serological relationship exists between colicine K and bacteriophage T6 (2). Enhancement of antibiotic activity of colicine K by serum had been observed earlier (8, 9, 2) but no attempt at purification of the factor responsible for the observed enhancement had been reported.

Our earlier investigations on the nature of the colicine-enhancing factor (CEF) in serum showed it to be present at highest concentrations in fraction IV-1 obtained by subjecting outdated human plasma to the ethanol fractionation method of Cohn *et al.* (7). However, it was subsequently found that lysates of red blood cells were far more potent in enhancing the antibiotic activity of colicine K and that hemoglobin and its constituent chains were the active components (10). Since colicine K is a good antigen, the interaction

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of hemoglobin and its subunits with colicine K was also studied by observing alteration of serological properties. This latter manifestation of the colicine K–hemoglobin interaction, *i.e.* alteration of serological properties, was also observed with other O antigens lacking antibiotic activity.

This communication describes studies in which the effects of environmental factors on the interaction of hemoglobin and its chains with colicine K and O antigens were investigated. Some of the experiments with serum and tissue fractions and their relationship to the hemoglobin system are also considered.

Materials and Methods

Colicine K and O Antigens.—Colicine K was the generous gift of Dr. W. F. Goebel of The Rockefeller Institute. *Escherichia coli* endotoxin (from strain 0111:B4) and its antisera were given to us by Dr. J. Fine, Beth Israel Hospital, Boston. *Shigella flexneri* and *Salmonella typhimurium* endotoxins and antisera were given to us by Dr. S. B. Formal, Walter Reed Army Institute of Research, Washington, D.C. The lipopolysaccharides of *Serratia marcescens*, *Salmonella enteritidis* and *Salmonella typhosa* were purchased from Difco Laboratories, Inc., Detroit.

Hemoglobin and Its Chains.—The twice crystallized human hemoglobin was obtained from Pentex Inc., Kankakee, Illinois; the alpha and beta chains of human hemoglobin, purchased from Gallard-Schlesinger Chem. Mfg. Co., Carle Place, New York, had been prepared by countercurrent distribution (11). Amino acid analysis of the alpha chain agreed with the published values; analysis of the beta chain revealed the presence of 0.15 residue of isoleucine which may indicate contamination by fetal hemoglobin or a non-heme protein (11). Since the chains were not easily soluble and tended to aggregate on prolonged standing in aqueous solutions, a stock solution (2 mg/ml) was made by dissolving them in 2 M urea.

Assay for Enhancement of Colicine K Activity.—Prior to setting up the enhancement assay, it was necessary to determine the activity of the colicine K preparation using a modified zone inhibition assay on nutrient agar plates seeded with *E. coli* B (12). Twofold serial dilutions of colicine K were made in 0.005 M phosphate buffer, pH 6.8, and 0.01 ml from each reaction mixture was applied to the appropriate segment of the seeded plate. Under our conditions, the final dilution that gave a zone of inhibition contained 0.1 μg of colicine K in the 0.01 ml applied to the plate (10 μg colicine K/ml of dilution mixture). The amount required to give an end point can vary over a 2-fold range. The specific activity of the colicine K preparations was lower than that found by Dr. Goebel under his assay conditions, probably reflecting some differences in the two assays; *e.g.*, number of organisms applied to the plate and time of preincubating the plate prior to assay.

For measurement of the enhancement activity, six or eight 2-fold serial dilutions of the factor to be tested were made in 0.005 M phosphate buffer (pH 6.8) in a total volume of 0.2 ml. To each tube was then added 0.2 ml of a constant but suboptimal amount of colicine K. This concentration of colicine K was usually one-fourth to one-sixth the amount previously found to be necessary to give a zone of inhibition. After incubation for 1 hour at 37°C, 0.01 ml was taken from each tube and applied to the plate. The end point for the colicine-enhancing factor is that concentration of sample giving a complete zone of inhibition with the suboptimal amount of colicine K. As a control, this suboptimal concentration of colicine K was incubated under the identical assay conditions and did not give a zone of inhibition. The extent of enhancement of a given preparation of colicine can vary from 6- to 200-fold depending on whether the zone inhibition or viable cell assay is used. At least two factors responsible for this variation, the number of bacteria (8) and the growth phase of the bacteria, have been recognized.

Alteration of Serological Activity.—In general, the effects of hemoglobin, alpha and beta chains, sera, or tissue fractions on the serological activity of colicine K, *E. coli* endotoxin, fetuin, and pneumococcus polysaccharides were measured by incubating the antigen with the sample being tested in total volume of 3.0 ml of phosphate buffer. After incubation, an aliquot was removed and diluted into chilled buffer (0.01 M tris, pH 7.4, 0.140 M NaCl) and assayed for its C′-fixing capacity using a micro-C′ fixation assay (13). Variations of this general scheme will be presented in detail with the appropriate experiment.

Colicine K Antisera.—For production of rabbit antisera, 500 μg of colicine K in a volume of 1.0 ml was mixed with an equal volume of complete Freund's adjuvant and injected into the toe pads and intramuscularly. The rabbits were bled three weeks after the initial injection and were boosted with 500 μg of colicine K by the same procedure. After the second bleeding, the rabbits were given an additional immunizing dose of 500 μg. The antibodies formed against the colicine K complex are to a large degree oriented toward the lipocarbohydrate moiety (2).

Chromatographic Techniques.—The chromatographic techniques used will be referred to in the specific experiment.

RESULTS

Analyses of Sera, Plasma, Cohn Fractions, and Tissues for CEF.—The data presented in Table I show that CEF was present in the sera of all species tested; CEF was not affected by dialysis or by passage through a column of cation exchange resin (IRC-50) in the sodium cycle; or by heating at 90°C for 10 minutes. Ashing, however, completely destroyed its activity. Individual human sera varied somewhat in their activity and pools of sera and plasma varied within a 4-fold range.

Cohn fractions I, II, III, IV-1, V, and super V, which had been prepared by Method 6 from outdated plasma at the New York State Department of

TABLE I

Assay of Sera and Plasma for Ability to Enhance the Antibiotic Activity of Colicine K

Sample	Final dilution required for complete zone of inhibition	Sample	Final dilution required for complete zone of inhibition
Human serum		Human plasma, outdated pool	1/640
Individuals 1, 2, and 3	1/160, 1/320, and 1/640	Beef serum (1)	1/160
		Rabbit serum (1)	1/160
Pool of 6 specimens	1/160	Cow serum (1)	1/80
Individual 4	1/320	Hamster serum (1)	1/160
Individual 4 dialyzed	1/320	Chicken serum (1)	1/320
Individual 4 through cation exchange resin	1/320	Guinea pig serum (1)	1/160
		Rat serum (1)	1/40
Individual 4 heated at 90°C for 10 min. at a 1:10 dilution	1/320	Mouse serum (1)	1/320
Individual 4 ashed	>1/1		

Health, were assayed for CEF. Fraction IV-1 was the most potent fraction giving a complete zone of inhibition at 25 μg/ml. This fraction contains some albumin and beta globulins but is particularly rich in alpha globulins. CEF was stable on exposure to pH 2–11 for several hours at 0°C and to digestion by trypsin and chymotrypsin at pH 7.0. However, if fraction IV-1 was treated at 90°C for 10 minutes prior to enzymatic digestion, CEF activity was completely destroyed by trypsin and chymotrypsin. Electrophoretic analysis of sera and fraction IV-1 showed that CEF migrated mainly as an alpha 2 globulin. Some purification of CEF from fraction IV-1 was possible with $(NH_4)_2SO_4$ and ethanol precipitation and chromatography on DEAE cellulose. CEF activity emerged in two peaks. Rechromatography of the peaks gave additional splitting of the peaks (14). The most potent CEF fraction was active at a concentration of 10 to 15 μg/ml.

Analysis of fraction IV-1 obtained from four different laboratories showed variation in CEF activity of from 5- to 20-fold compared to the New York State Department of Health preparations. An attempt was therefore made to isolate CEF directly from sera using chromatography on DEAE under the conditions described by Sober *et al.* (15). The activity emerged as a peak coincident with the hemoglobin-haptoglobin complex (16).

Homogenates of various tissues were also made and tested for CEF and for their ability to alter the serological activity of colicine K. Every tissue tested possessed some activity but, in general, spleen, heart, kidney, liver, and muscle had the highest specific activity. Two procedures were developed for the isolation of CEF from spleen and heart. One consisted of homogenizing the tissue, centrifuging the insoluble material, adjusting the pH of the supernatant fluid to 2 and heating it at 100°C for 10 minutes. Upon cooling, the homogenate was neutralized and clarified by centrifugation. The supernatant fluid was extracted with saturated phenol and CEF was precipitated from the phenol layer by addition of acetone. It was redissolved in saline-phosphate buffer and the insoluble material was removed by centrifugation. The supernate contained the CEF.

The second procedure consisted of suspending the tissue homogenate in 8 M urea at pH 2, precipitating inactive protein with 2 per cent TCA and then precipitating active protein with 5 per cent TCA. Chromatography on the cation exchange resins XE-64 and carboxymethyl cellulose (CMC) using aqueous solvents proved difficult since the preparations tended to aggregate. Chromatography in urea appeared more promising.

CEF Activity of Hemoglobin, Its Subunits, and Myoglobin.—While the above studies were in progress, two hemolyzed bloods from duck and monkey were analyzed for CEF. Whereas the highest titer of sera or plasma was 1/640, these hemolyzed bloods were active at 1/60,000. In order to test the effect of the hemolytic products, fresh sheep blood was separated into its cellular and plasma components and assayed for CEF. While whole blood and plasma

were active at a 1/200 dilution, the lysed cells were active at 1/30,000 (10). Since there are 11 gm of hemoglobin in 100 ml of sheep blood, this dilution represents 3.7 μg of hemoglobin assuming quantitative recovery from the lysed blood cells. Twice crystallized sheep hemoglobin was active at a concentration of 1.6 μg while similar hemoglobin preparations from various species were found to be active at the following levels: human (1.6 μg), rabbit (1.6 μg), guinea pig (1.6 μg), sheep (1.6 μg), rat (6.4 μg), and pork (1.6 μg). Crystalline human hemoglobin was also chromatographed on CMC according to the procedure described by Reichlin *et al.* (17) and the effluent pattern of CEF activity was found to correspond to the major peak of optical density of 410 mμ (hemoglobin A_1). There was no appreciable loss in colicine enhancing activity when hemoglobin was treated with acetone-HCl to prepare globin (18) nor when globin was subjected to urea-HCl to split it into chains (19). A concentration of 1.6 μg of alpha and beta chain of hemoglobin was sufficient to enhance the activity of colicine K. Hemin was tested and found to be inactive. These experiments indicated that hemoglobin and its protein components were the active principles in lysates.

Since myoglobin resembles hemoglobin in its tertiary structure and has some similar areas of amino acid sequences (20), it was assayed for its ability to enhance the activity of colicine K. The twice crystallized horse myoglobin used in these studies had been shown by immunological and chromatographic techniques to be contaminated with approximately 3 per cent hemoglobin (17). It was therefore dialyzed in 0.01 M phosphate buffer, pH 6.4, and chromatographed on a CMC column equilibrated with the same buffer utilizing a pH gradient of 0.01 phosphate buffer, pH 8.3 for elution of the protein (17). Myoglobin A_2, accounting for 90 per cent of the material emerged at an effluent pH of 6.7. The colicine-enhancing activity coincided with the myoglobin elution pattern. The chromatographically purified myoglobin gave a complete zone of inhibition at a concentration of 6 μg, ml.

Other purified proteins had been tested for colicine-enhancing activity (10). Egg white lysozyme had activity, but was only 5 per cent as effective as hemoglobin and its subunits. Lysozyme, however, can enhance the activity of other antibiotics probably by causing changes in the cell wall (21). Ribonuclease, another small basic protein, showed no activity at 200 μg/ml. Incubation of colicine K with levels of poly-L-lysine and poly-L-glutamic acid of up to 50 μg/ml did not give enhancement of activity.

The effect of time and temperature on the ability of hemoglobin to enhance the activity of colicine K was tested. These results are only semiquantitative for the following reasons: (*a*) In our hands, the zone inhibition assay can vary by a factor of 2; (*b*) Since the plates have to be incubated to allow bacterial growth to take place, the reaction between colicine K and the factor may continue after the reaction mixture is applied to the seeded plate for assay.

Nevertheless, some general conclusions could be made from these studies.

The reaction was both temperature- and time-dependent. It did not proceed appreciably at 0°C and required progressively less hemoglobin to reach an end point when the reaction mixtures were incubated at 25° and 37°C. High concentrations of hemoglobin and alpha chain did not affect CEF activity as measured by the zone inhibition assay.

Effect of Hemoglobin and Its Subunits on the Serological Activity of Colicine K and O Antigens.—Colicine K or *E. coli* endotoxin, assayed with homologous antisera by C′ fixation gave a characteristic antigen-antibody response curve. When colicine K or *E. coli* endotoxin was incubated with serum, plasma, or

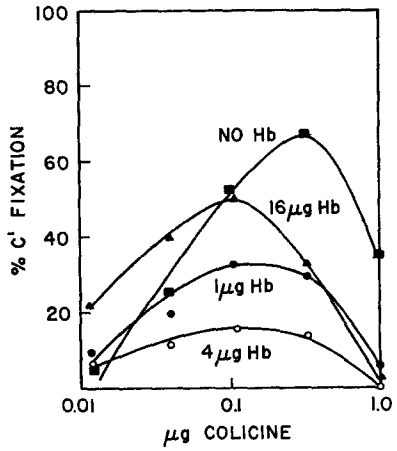

FIG. 1. The effect of various concentrations of hemoglobin on the serological properties of colicine K. Colicine K (1.65 μg/ml) was incubated with various concentrations of hemoglobin at 37°C for 30 minutes. Samples were diluted and assayed for C′ fixation using anticolicine Ra, No. 456-B2, at a 1/600 dilution.

tissue homogenates, alterations in their serological response were observed. Lysed red cells were again 2 to 3 orders of magnitude more effective at altering these serological activities than serum, plasma, or washed whole red blood cells (10). Twice crystallized human hemoglobin and the alpha chain decreased the fixation of C′ 50 per cent at levels of 1.6 μg, while 4.1 μg of beta chain was required for similar alterations. Since antisera to colicine K are directed mainly toward the lipocarbohydrate component (2), changes in the serological response probably reflect to a large extent alterations in the antigenic determinants of the lipocarbohydrate moiety. The quantitative immunological technique was used to study the effect of temperature, divalent cations, salt concentrations, time, and protein (hemoglobin and its subunits) concentration on the serological activity of colicine K and *E. coli* endotoxin.

The data shown in Fig. 1 represent the serological responses after incubation of a constant quantity of colicine K with various amounts of hemoglobin.

When measured as the per cent decrease in C′ fixation, preincubation of colicine K with 1 μg hemoglobin decreased serological activity approximately 60 per cent, while incubation with 4 μg hemoglobin resulted in a decrease in C′ fixation of 75 per cent. Incubation with 16 μg hemoglobin caused a reversal of this trend; the maximum C′ fixation was now within 25 per cent of the C′ fixed by the colicine control incubated with buffer. Fig. 2, in which the per cent decrease in C′ fixation is plotted against the amounts of hemoglobin reacting with a constant quantity of colicine K, summarizes the results of several experiments. Such a response curve resembles the antigen-antibody

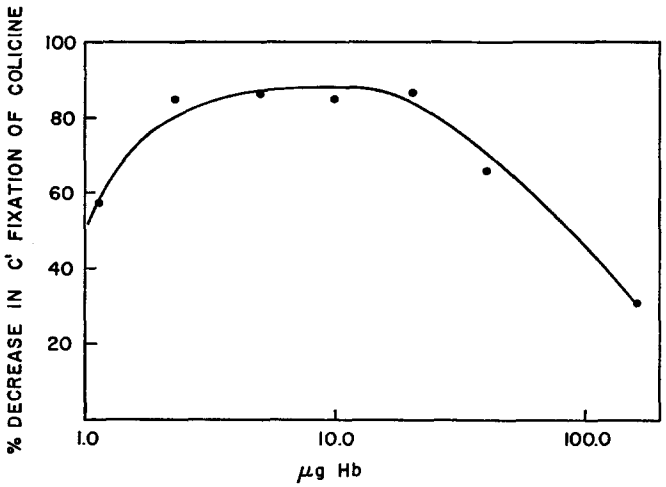

FIG. 2. The effect of various concentrations of hemoglobin on the serological properties of colicine K. Colicine K (1.65 μg/ml) was incubated with various amounts of hemoglobin under the conditions described in Fig. 1 and assayed with anticolicine.

precipitin or C′ fixation curve in that the interaction observed is inhibited by an excess of one of the reactants.

Fig. 3 shows the effect of NaCl concentration on the reaction. At 0.75 M NaCl, the colicine K–hemoglobin interaction was completely inhibited. The interaction of colicine K and hemoglobin was rapid, the reaction being complete in 10 minutes at 37°C (Fig. 4).

Fig. 5 shows the results obtained when *E. coli* endotoxin and the alpha chain of hemoglobin were incubated in the presence of varying amounts of Ca^{++}. It can be seen that in the presence of 3×10^{-3} M Ca^{++} the interaction was completely inhibited. Table II shows the effect of other divalent cations on the interaction of colicine K with alpha chain. Of the cations tested, Zn^{++} and Cd^{++} were the most potent inhibitors followed by Ba^{++}, Mn^{++}, Co^{++}, Ca^{++}, Mg^{++}, and Sn^{++}. The effects of Ca^{++}, Mg^{++}, and Mn^{++} on the inter-

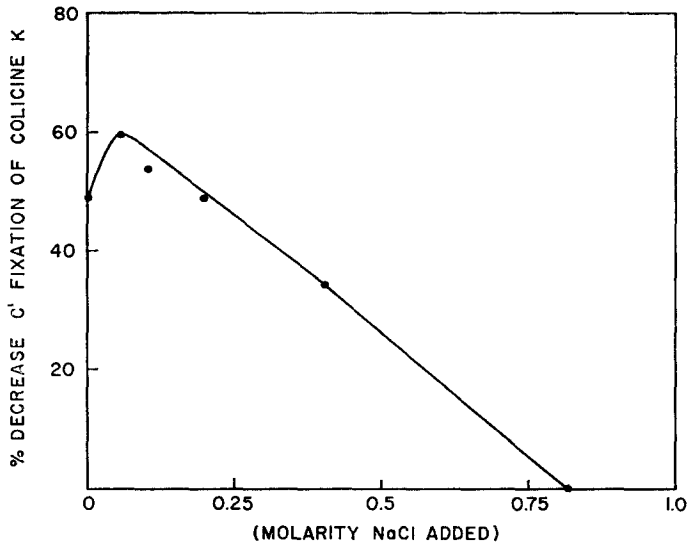

FIG. 3. The effect of ionic strength on the serological alteration of colicine K (1.6 μg/ml) incubated with alpha chain (3.0 μg/ml) in 0.005 M phosphate buffer, pH 6.8, for 30 minutes at 37°C. The molarity of NaCl was varied and all reaction mixtures were in a final volume of 3.0 ml.

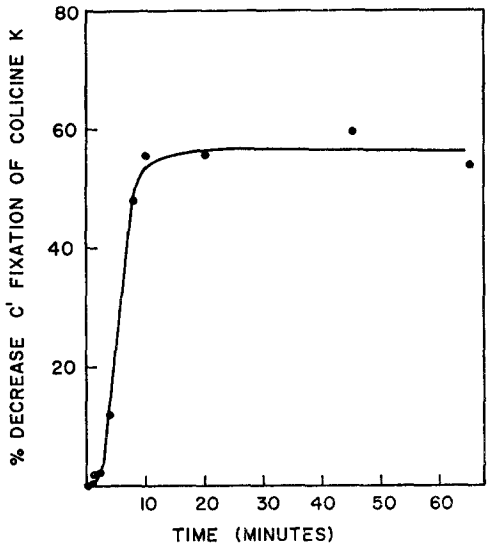

FIG. 4. The per cent decrease in C' fixation when colicine K (1.65 μg) and hemoglobin (2.0 μg) were incubated for different intervals, and then diluted and assayed with anticolicine.

action of *E. coli* endotoxin and alpha chain were of the same order as that found with the colicine K system.

The results obtained when a constant amount of colicine K was incubated at different temperatures with various concentrations of alpha chain are summarized in Table III. At weight ratios of alpha chain to colicine K below 2, the extent of reaction after incubation for 30 minutes was relatively slow at 20°C, reached a maximum at 60 to 80°C and decreased slightly at 80°C and 100°C. At certain temperatures, *e.g.* 60°C and at alpha chain to colicine K ratios above 5, the reaction was markedly inhibited.

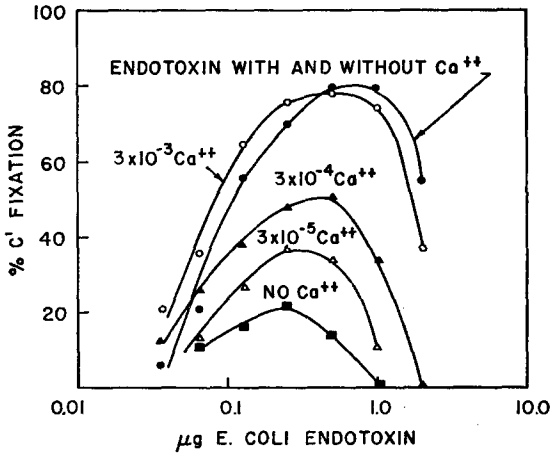

FIG. 5. The effect of varying concentrations of calcium on the C′ fixation of *E. coli* endotoxin with anti-*E. coli* endotoxin. Endotoxin (5.6 μg/ml) was incubated with 10 μg of hemoglobin in buffer containing various amounts of Ca^{++}.

Properties of the Colicine K Hemoglobin or Alpha Chain Complex.—Gel filtration on sephadex g 100 was used to determine whether colicine K underwent changes in molecular weight during its interaction with hemoglobin components. The eluted fractions were assayed by zone inhibition to determine the position in the chromatogram of the antibiotic activity and by immunological techniques to determine the location of the lipocarbohydrate moiety.

In experiment 1 (Table IV) colicine K was passed through a column of sephadex 100 and the fractions which emerged were assayed for their ability to prevent growth of *E. coli* B. Antibiotic activity was present in tubes 15 through 18. If aliquots were taken from these tubes and preincubated with CEF (in this experiment rabbit serum was used as a source of CEF) and reassayed by zone inhibition, activity was found in tubes 14 to 20. Therefore, the colicine K activity emerged in a discrete peak and further activation did not reveal any additional activity in other parts of the chromatogram. When

colicine K was preincubated with rabbit sera (Experiment 2, Table IV) and then passed through sephadex 100, the activity was found in tubes 15 to 39. The active moiety was therefore significantly retarded, suggesting that the activity resided in a smaller molecule(s).

TABLE II

Effect of Divalent Cations on Alteration of Serological Activity of Colicine K and E. coli Endotoxin by α-Chain

Cation	Molarity	Inhibition	
		Colicine K	*E. coli* endotoxin
		per cent	*per cent*
Calcium	3.3×10^{-3}	78	100
	3.3×10^{-4}	39	50
	3.3×10^{-5}	19	27
Magnesium	5×10^{-3}	68	74
	5×10^{-4}	31	53
	5×10^{-5}	22	17
Strontium	3.3×10^{-3}	79	—
	3.3×10^{-4}	36	—
	3.3×10^{-5}	14	—
Barium	3.3×10^{-3}	97	—
	3.3×10^{-4}	95	—
	3.3×10^{-5}	73	—
Manganese	5×10^{-3}	100	100
	5×10^{-4}	70	76
	5×10^{-5}	25	18
Cobalt	3.3×10^{-3}	82	—
	3.3×10^{-4}	58	—
	3.3×10^{-5}	46	—
Zinc	3.3×10^{-4}	100	—
	3.3×10^{-5}	100	—
	3.3×10^{-6}	81	—
Cadmium	3.3×10^{-4}	83	—
	3.3×10^{-5}	89	—
	3.3×10^{-6}	73	—

These experiments were repeated using alpha chain to enhance the activity of colicine K rather than sera. Essentially the same results were obtained (Experiments 3 and 4, Table IV). The antibiotic activity of colicine K was enhanced at least 6-fold in these experiments. Within the limitations of the

TABLE III

Effect of Temperature on the Alteration of Serological Activity of Colicine K by the α-Chain of Human Hemoglobin

α-Chain	Decrease in max. C′ fixation				
	20°C	40°C	60°C	80°C	100°C
μg	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	8	46	50	45	68
2	6	58	63	74	61
4	8	68	83	74	61
8	20	77	75	62	55
16	64	81	81	46	0
32	—	—	79	0	0

1.65 μg colicine K/ml is incubated with varying amounts of α-chain under standard assay conditions.

TABLE IV

The Elution Patterns of Antibiotic Activity of Colicine K and Colicine K Preincubated with Sera or Alpha Chain after Passage through a Sephadex G 100 Column

Exp. No.	Conditions	Tubes containing activity	
		A*	B‡
1	500 μg colicine K preincubated in 3.0 ml phosphate buffer, 37°C, 60 min.	15–18	14–20
2	500 μg colicine preincubated with 0.2 ml rabbit sera in a total volume of 3.0 ml buffer, 37°C, 60 min.	15–39	—
3	600 μg colicine K preincubated in 3.0 ml buffer, 37°C, 60 min.	15–18	15–21
4	600 μg colicine K preincubated with 500 μg α-chain in 3.0 ml buffer, 37°C, 60 min.	15–38	—

The sephadex column was 4 cm in diameter x 40 cm long, the effluent buffer was 0.005 M PO_4 (pH 6.8) and the fractions were collected in 3 ml aliquots.

* Aliquots (0.2 ml) were removed from each of the effluent tubes into 0.2 ml of phosphate buffer and then assayed directly by the zone inhibition assay.

‡ Aliquots (0.2 ml) were removed from each tube into 0.2 ml of phosphate buffer containing at 1/100 dilution of rabbit sera and preincubated for 60 minutes at 37°C prior to assay.

zone inhibition assay, approximately 50 per cent of the activity could be accounted for in the broad peak.

In comparable experiments, colicine K was passed through a sephadex 100 column and assayed immunologically. This activity was found to emerge

between tubes 25 to 35 (Fig. 6). Aliquots from the peak portion of the curve all showed the same C′ fixation. Using the extent of the lateral shift, the amount of material that emerged in each tube was quantitated. The C′ fixation curves obtained from tubes 23, 30, and 36 are shown in Fig. 6.

Preincubation of colicine K with alpha chain, followed by passage of the

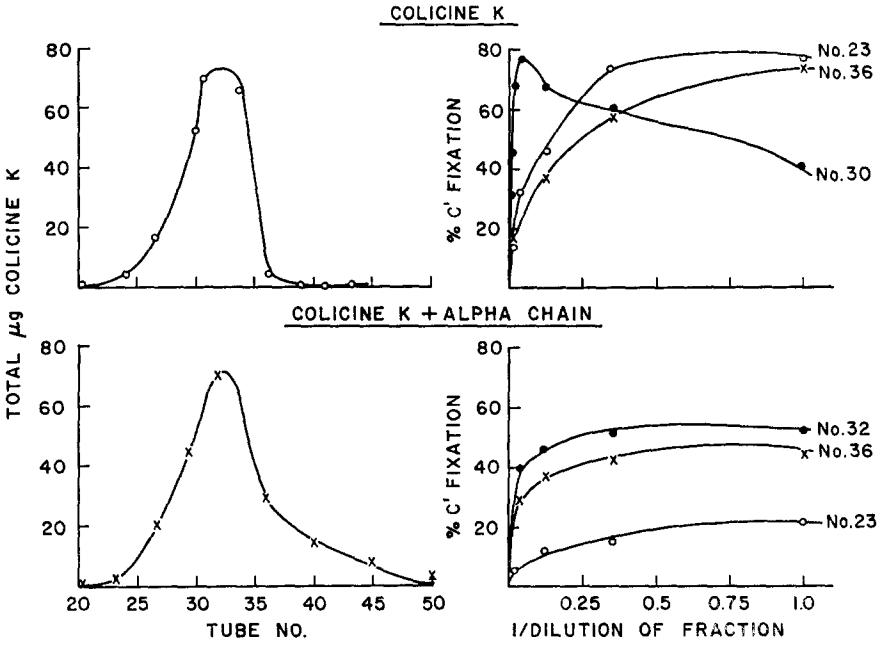

FIG. 6. Top left; the chromatogram resulting from the passage of 100 μg of colicine K through a sephadex 100 column (1.8 x 100 cm) equilibrated with 0.005 M phosphate buffer at pH 6.8. Top right; the C′ fixation curves obtained when aliquots of the individual tubes were assayed.

Bottom left; the chromatogram resulting from the passage of the preincubated reaction mixture containing 100 μg of colicine K and 200 μg of alpha chain through the same column. Bottom right; the C′ fixation curve obtained when aliquots of individual tubes were assayed.

reaction mixture through this same sephadex column revealed that the immunological activity emerged in essentially the same place. All tubes containing antigen showed a decreased maximum C′ fixation. C′ fixation curves obtained with known amounts of alpha chain were used as calibration curves to quantitate the altered colicine K that emerged from the column. The first tube in the eluting peak, No. 23, showed the greatest decrease in C′ fixation suggesting that the maximum interaction of alpha chains with colicine K had occurred in the early tubes. Indeed, because of such interaction, even larger molecular weight products may have been formed. The 2 to 1 ratio of alpha

chains to colicine K used in the experiment as well as the incubation temperature (37°C) preclude the possibility that serological activity in other tubes was being overlooked because of the reversal of inhibition (*e.g.* alpha chain at a concentration of 32 μg at 80°C, Table III). In addition, tubes along the chromatogram were assayed with increased concentrations of antibody but none outside of the peak fractions (25 to 35) possessed any C′ fixing capacity. Nor did they contain any fragments capable of inhibiting the intact antigen-antibody C′ fixation. Similar gel filtration properties were observed with *E. coli* endotoxin before and after incubation with hemoglobin.

Since the immunological activity was not appreciably displaced in the elution pattern of the chromatogram, the lipocarbohydrate moiety did not appear to have undergone fragmentation to units of smaller molecular weight. The significant retardation of the moiety having the antibiotic activity, however, suggested that it was now smaller in molecular weight. The broadness of the eluting peak may represent some heterogeneity in the molecular weight and/or in the charge of the active fragment. In any case, it appears that the lipocarbohydrate moiety and the alpha chains have interacted, and in so doing have changed the characteristics of the antibiotic moiety so that it is now retarded during gel filtration. Prior to carrying out the physical and chemical studies necessary to determine the properties of the antigenically and biologically active fragments, attempts are being made to determine why the antibiotic activity emerges in such a broad peak.

Interaction of Alpha Chains with Other Immune Systems.—The progressive loss of C′ fixation obtained by incubating colicine K and *E. coli* endotoxin with increasing amounts of alpha chain was also found with the *S. typhimurium* and *Sh. flexneri* endotoxin immune systems. Although antisera to endotoxins isolated from *S. typhosa*, *S. enteritidis*, and *S. marcescens* were not available, their interaction with alpha chain was demonstrated by their ability to compete with colicine K for the alpha chain. An experiment demonstrating this competition is summarized in Fig. 7. Various amounts of heterologous lipopolysaccharide were incubated with a constant amount of colicine K and alpha chain. *S. marcescens*, *S. typhosa*, and *S. enteritidis* lipopolysaccharides inhibited the serological alteration of colicine K caused by alpha chain. Although all the endotoxins tested showed this competitive capacity, the amount required to inhibit the alteration of C′ fixation 50 per cent varied with different preparations.

Incubation with alpha chain or hemoglobin did not alter the C′ fixation of three *Pneumococcus* polysaccharide immune systems. Nor did alpha chain alter the T4 DNA immune system in which the glucosylated hydroxymethylcytosine is part of the antigenic determinant (22). In agreement with these findings, the pneumococcus polysaccharides and T4 DNA did not inhibit the reaction of alpha chains with colicine K.

A small but reproducible alteration was seen with the fetuin immune system However, the interaction of alpha chain with this glycoprotein resulted in an

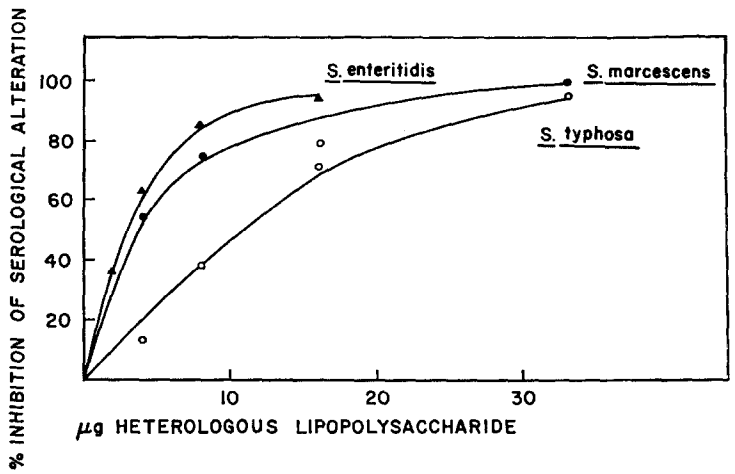

FIG. 7. Inhibition of the colicine K–alpha chain interaction by *S. enteritidis*, *S. mercescens* and *S. typhosa* endotoxins. A constant amount of colicine K (1.6 μg/ml) and varying amounts of the heterologous lipopolysaccharides were incubated with a constant amount of alpha chain (3.2 μg/ml) and assayed with anticolicine K.

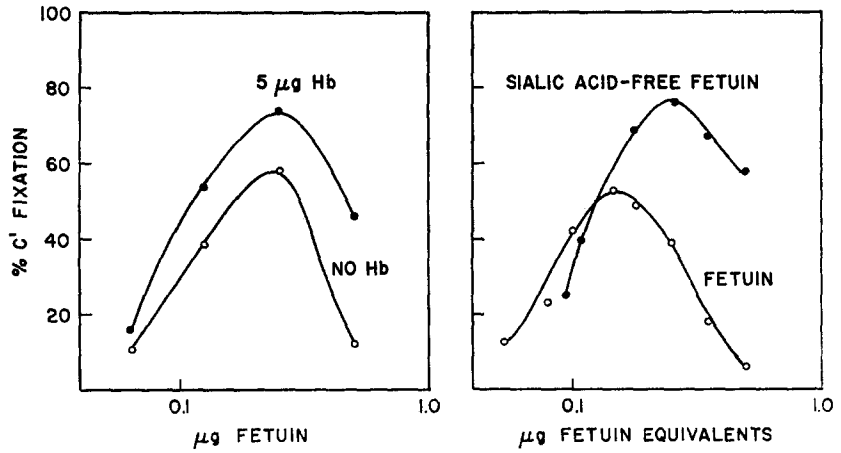

FIG. 8. The effect of hemoglobin on the serological activity of fetuin. Left, 5 μg of fetuin were incubated at 37°C for 30 minutes in phosphate buffer 0.005 M at pH 6.8 (bottom curve) and with 5 μg of hemoglobin (top curve). Right, from reference 23.

increase in C′ fixation (Fig. 8). A similar increase in C′ fixation as a result of removing sialic acid with neuraminidase had been noted in an earlier study of the fetuin amino system (23). Since antifetuin is directed toward the protein

moiety of this glycoprotein, this increase in serological activity with sialic acid–free fetuin was thought to be due to the removal of the strong negatively charged portion of the fetuin molecule, thus allowing better interaction of the protein moiety with the antibody. A similar explanation for the increase in C′ fixation of fetuin after incubation with hemoglobin is possible. Hemoglobin may complex with the sialic acid containing carbohydrate moiety of fetuin to neutralize its negative charge, and thus make possible better interaction of the protein moiety of fetuin with its antibody.

DISCUSSION

Two experimental results have been described in this paper. Hemoglobin and its chains can enhance the antibiotic activity of colicine K and are also able to interact with colicine K as well as other O antigens to alter their serological activities. Most likely, hemoglobin and its subunits complex with the lipopolysaccharide to mask some of its antigenic determinants. In the case of colicine K, dissociation of the antibiotic activity also occurs. Evidence to support this conclusion was obtained from experiments in which colicine K that had been incubated with the alpha chains of hemoglobin was subjected to gel filtration through sephadex. The elution pattern revealed that the antibiotic activity was significantly retarded but that there was no alteration in the location of the lipocarbohydrate moiety. The serological activity of the lipocarbohydrate moiety with anticolicine was altered, however. The possibility that the lipocarbohydrate fragmented to release the antibiotic activity (assumed to be protein) and repolymerized rapidly to form a large molecular weight complex cannot be definitely ruled out. The fate of the alpha chain could not be determined in these experiments. Antibodies specific for the alpha chain are currently being prepared for this purpose.

Some role for electrostatic forces in the interaction between hemoglobin and O antigens is suggested by its inhibition at high concentrations of salt and relatively low concentrations of divalent cations. Alterations in the serological activity of colicine K are also obtained with the basic proteins, ribonuclease and lysozyme, and polylysine but higher concentrations of these cationic molecules were required (10). Ribonuclease and polylysine do not enhance colicine K antibiotic activity and lysozyme is only one-twentieth as effective as hemoglobin. Thus, two reactions, one leading to complex formation with displacement of the antibiotic portion (as is seen with hemoglobin, its subunits and myoglobin) and the other leading to complex formation without displacement of the antibiotic moiety, may be occurring. Evidence for complex formation has also been obtained using a turbidometric assay (24).

While hemoglobin and its chains have been shown to interact with colicine K, *E. coli*, *Sh. flexneri*, *S. typhimurium*, *S. typhosa*, *S. enteriditis*, *S. abortus equi*, and *S. marcescens* lipopolysaccharides, they do not interact with other polysaccharide immune systems such as *Pneumococcus* Type II (composed of

rhamnose, glucose, and glucuronic acid), *Pneumococcus* Type VIII (composed of galactose, glucose, and glucuronic acid), *Pneumococcus* Type XIV (composed of glucose and galactose) and T4 DNA (glucosyl 5-OH methylcytosine, adenine, guanine, thymine, deoxyribose, and phosphate). A clue to the residue in the endotoxin mediating the interaction with hemoglobin may be seen from the increased C′ fixation of the fetuin immune system. Fetuin, preincubated with hemoglobin, gives an immunological response similar to fetuin in which the sialic acid was removed with neuraminidase (23). It should be noted that the haptoglobins, which avidly complex with the hemoglobin in the sera, also contain sialic acid. 2-Keto-3-deoxyoctonate (KDO) has recently been identified in the lipopolysaccharides of the *Escherichia-Salmonella* group (25). Whether the sialic acid in glycoproteins and KDO in O antigens are part of the hemoglobin binding sites remains to be answered, especially since colicine K has not been shown to contain KDO (3).

The inhibition with excess hemoglobin and its subunits resembles the inhibition seen with excess antigen in antigen-antibody precipitin or C′ fixation reactions. It is possible that the alpha chain–lipopolysaccharide complex, like the antigen-antibody reaction, involves multivalencies on both the alpha chain (or beta chain or hemoglobin) and the lipopolysaccharide. Preliminary experiments designed to identify the active amino sequence in hemoglobin and the chains showed that partial digestion by trypsin leads to a loss of serological alteration activity. The digested fragments, however, still can combine with portions of the lipopolysaccharide as measured by competition experiments. Continued digestion, however, results in small fragments unable to compete with hemoglobin or alpha and beta chains for the lipopolysaccharide binding sites.

The evidence is accumulating that complex formation between endotoxins and various proteins can alter the biological and serological properties of endotoxins. The ability of strong cationic macromolecules and proteins to inactivate the tumor-necrotizing activity of *S. marcescens* endotoxin could be reversed by treating the complexes with the polyanion, polyglucose sulfate (26, 27). Using proteolytic enzyme digestion, Rudbach and Johnson (28) have also been able to restore pyrogenic and immunological activities to *S. typhosa* endotoxin previously rendered inactive by treatment with Cohn fraction IV-1. Some humoral and tissue factors are thought to affect the biological and serological activities of endotoxins because they possess enzymatic activities. The possibility that complex formation may be responsible for the observed effects in some of these instances has been stressed (27, 28).

There are several reasons for believing that the hemoglobin components are also probably the factors in serum and tissue which are capable of enhancing the activity of colicine K and may be important factors in altering the serological activity of O antigens. Hemoglobin, globin, and its subunits do not exist free in serum or plasma since they form extremely strong complexes with the

haptoglobins, a molecularly heterogeneous group of glycoproteins which are under genetic control (29). While hemoglobin and globin have electrophoretic mobilities of beta globulins, they migrate when complexed with haptoglobins, as alpha globulins (30). In addition, chromatography of sera on DEAE cellulose showed that CEF activity emerged in the same area as the haptoglobin-hemoglobin complexes. Since haptoglobins are genetically different and complex with globin and its subunits as well as with hemoglobin, the multiple peaks of activity observed during the purification of CEF from fraction IV-1 prepared from pooled outdated plasma is an expected finding. The stability of CEF to heat, urea, and extremes in pH is also consistent with what has been observed with the hemoglobin components. As far as the distribution of the factor in tissues goes, it was apparent that those tissues which contained the greatest amount of blood had the highest specific activity. The isolation procedure that was being evolved was in fact similar to those used to produce and isolate hemoglobin chains (19).

SUMMARY

Hemoglobin, its chains, and myoglobin enhance the antibiotic activity of colicine K. These proteins also interact with colicine K and other O antigens to alter their serological activity. The hemoglobin proteins did not alter the serological activities of three *Pneumococcus* polysaccharides or T4 bacteriophage DNA antigens but did alter the antigenic activity of fetuin.

Interaction of hemoglobin and colicine K resulted in a retardation of colicine K antibiotic moiety as measured by gel filtration but did not affect the gel filtration properties of the lipopolysaccharide moiety.

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