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Proteins Adhering to *Escherichia coli* after Exposure to Guinea-Pig Serum

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Summary. The constituents of guinea-pig serum that attach to the surface of a serum-sensitive strain of *Escherichia coli* have been determined. Antisera from rabbits immunized with bacteria exposed to serum ('sensitized' bacteria) were analysed by means of immunoelectrophoresis. It is concluded that at least seven constituents from the serum of the normal adult guinea-pig adhere to the *E. coli* during sensitization. These bind tightly enough to resist removal by at least six washings of the sensitized bacteria. Two of these constituents (IgG and IgM) are known to possess antibody activity. A third constituent, β_{1C}, is known to be the third component of the complement system of the guinea-pig. Another is probably the fourth component of this complement system. The other three serum components remain unidentified, although one of them is shown to possess esterase activity, and thus may be related to the first component of complement.

Evidence is presented suggesting that normal guinea-pigs may have little or no antibody against *E. coli* present in the IgG fraction of the serum.

If, before sensitization of the *E. coli*, the serum is heated to inactivate the activity of bactericidal complement, the serum does not kill this organism, but no change is detectable in the serum proteins adhering to these bacteria. Similarly, if, before sensitization of *E. coli*, the bactericidal antibody to the *E. coli* is absorbed from the serum, while bactericidal complement is left intact, there is no change in the serum proteins adhering to the bacteria. When the serum used for sensitizing *E. coli* is from guinea-pigs that have been actively immunized with the same strain of *E. coli*, rabbits immunized with these sensitized bacteria appear to form increased amounts of antibody directed against the 7S γ_2-globulin of the guinea-pig serum.

INTRODUCTION

Identification of the components of normal or immune serum that participate in the death of those Gram-negative enteric bacilli sensitive to serum has been carried out primarily by modifying the serum. Thus, components required for the killing of bacteria have been identified by absorbing them from serum. Alternatively, serum has been heated, or been altered by addition of reagents to inactivate or destroy components necessary for bactericidal activity. By such approaches, it can be shown that specific immune globulins, all major components of complement, and the divalent cations, calcium and magnesium, participate in the bactericidal activity of serum. Assuming that components of serum may attach to the bacterial cell in order to participate in its killing, we have attempted in the present study to identify proteins attaching to serum-sensitive *E. coli* when these are exposed to active serum.

Turner and Rowley (1963) immunized rabbits with opsonins from pig serum complexed with material from cell walls of *E. coli*. They reported the appearance in the rabbit of antibody directed against IgG, β_{2M} and β_{2A} globulins of the pig serum. Norins and Holmes (1964) detected by immunoelectrophoresis three serum proteins attached to Coombs-positive erythrocytes of mice. All three proteins were globulins, one was a γ-globulin and two were unidentified. The number of rabbits used in these studies was small, and the investigators recognized that the number of proteins detected represented a minimum, since antisera rarely contain antibodies directed against all the potential antigenic components of serum.

In this study antisera from rabbits immunized with *E. coli* exposed to serum from the guinea-pig were analysed by immunoelectrophoresis. We have compared *E. coli* exposed to normal serum with those exposed to the same serum after absorption of all detectable bactericidal activity, or after inactivation of bactericidal activity by heating. In addition, *E. coli* exposed to serum collected from guinea-pigs, after specific, active immunization, were studied.

MATERIALS AND METHODS

Exposure of E. coli *to serum*

Exposure of bacteria to serum will be referred to as 'sensitization' of bacteria; bacteria after exposure to serum will be referred to as 'sensitized' bacteria.

E. coli $P^4$27 (type OX8), sensitive to the bactericidal activity of serum and used by us previously (Steward, Collis and Roantree, 1964), were grown in brain–heart broth, killed by exposing a suspension to flowing steam in an Arnold sterilizer for 2 hours, and washed in isotonic saline. Approximately 10^{12} bacteria were sensitized by suspending them in 35 ml of guinea-pig serum for 12 hours at 4°. These sensitized bacteria were then washed six times in 35 ml of isotonic saline.

Serum for sensitization was pooled from a large number of normal guinea-pigs and frozen (obtained from Hyland Laboratories). This pool had a titre of bactericidal antibody against this strain of *E. coli* of 1:16,000 as determined by a procedure previously described (Roantree and Steward, 1965).

E. coli were sensitized with this serum, or with this serum heated at 56° for 60 minutes to destroy the activity of complement, or with this serum after absorption in the cold with *E. coli* to remove all detectable bactericidal antibody against this strain. The activity of bactericidal complement was not diminished measurably by the absorptions. In other experiments we used a pool of serum from only six normal guinea-pigs for sensitization. In addition, serum from eight guinea-pigs actively immunized with *E. coli* in incomplete Freund's adjuvant was pooled and used for sensitization of *E. coli*. This serum from immunized guinea-pigs had a titre of bactericidal antibody of 1:250,000 against this strain of *E. coli*.

These symbols will be used for the antigens:

E. coli-NS: *E. coli* sensitized with pooled serum from a large number of normal adult guinea-pigs.

E. coli-HS: *E. coli* sensitized with the same pool of serum used in the preparation of *E. coli*-NS, but the serum was heated at 56° for 60 minutes before sensitization of the bacteria.

E. coli-AS: *E. coli* sensitized with the same pool of serum used in the preparation of *E. coli*-NS, but all detectable bactericidal antibody was absorbed from the serum with *E. coli* before sensitization of the bacteria.

E. coli-IS: *E. coli* sensitized with pooled serum from eight guinea-pigs actively immunized with *E. coli*.

Immunization

At least three adult rabbits were used for immunization with each of the antigens listed above. Each rabbit received weekly for 3 weeks, either intravenously or subcutaneously, 3×10^{10} to 10×10^{10} *E. coli*, non-sensitized, or sensitized with serum as described. Antiserum was obtained 8 days after the last injection of antigen.

Antiserum directed against whole normal guinea-pig serum was obtained from rabbits which had received subcutaneous injections of guinea-pig serum twice weekly for at least 4 weeks. In immunoelectrophoretic analysis with guinea-pig serum this antiserum showed at least thirteen lines of precipitation.

To prepare antiserum against guinea-pig macroglobulin, serum from guinea-pigs was put through a column of Sephadex G-200. The eluate representing the first of three well-separated peaks of absorption at 280 μ was lyophilized and 0·25 mg of this in 1 ml of complete Freund's adjuvant was injected into each footpad of a rabbit. The rabbit was bled 3 weeks later.

Antisera

Globulin was precipitated from the rabbit antisera by 50 per cent saturation with ammonium sulphate, and the washed precipitate was dissolved in one-third the original volume in isotonic saline. It was then dialysed against saline until free of ammonium sulphate. Sodium ethylmercurithiosalicylate was added as a preservative.

Treatment of serum with 2-mercaptoethanol

One volume of serum was mixed with 9 volumes of 0·1 M mercaptoethanol in 0·2 M phosphate buffer at pH 7·1. This mixture was left for 24 hours at room temperature, then dialysed for 3 days at 4° against 0·02 M sodium iodoacetate. Another portion of the same serum was similarly processed except mercaptoethanol was omitted from the phosphate buffer.

Immunoelectrophoretic analysis

Electrophoresis of serum was performed according to Scheidegger's modification (1955) of the technique of Grabar and Burtin (1960). One per cent agar (Difco) was prepared in 0·05 M barbiturate buffer at pH 8·6. Serum was separated by electrophoresis for 1 hour employing a potential gradient of 20 V/cm. Forty-eight hours were allowed for the precipitin lines to develop before the slides were washed in buffered saline, dried and stained with amido black. The technique of Uriel (1961) using β-napthyl acetate as substrate was employed to detect esterase activity of precipitin lines.

Double diffusion in agar

The technique of Allison and Humphrey (1959) was used to estimate the size of the serum proteins detected immunologically in this study. This technique is based on the observation that when a mixture of antigens is placed in a trough at right angles to a trough containing its polyvalent antiserum, the precipitin lines which appear in the gel can be described by the angle formed between the precipitin line and the trough containing the source of antigens. The angle bears a definite relationship to the diffusion coefficients of corresponding antigen and antibody.

The technique of Ouchterlony for analysis of sera by double diffusion was performed on microscope slides covered with a 2 mm layer of 1 per cent agar (Difco) in barbiturate buffer at pH 8·6. The precipitin lines were allowed to develop for 36 hours before the slides were washed in buffered saline, dried under filter paper and stained with amido black.

RESULTS

I. IMMUNOELECTROPHORETIC ANALYSIS OF ANTISERA PRODUCED IN THE RABBIT AGAINST *E. coli* SENSITIZED WITH A POOL OF SERUM FROM A LARGE NUMBER OF NORMAL ADULT GUINEA-PIGS

The nine antisera obtained from three rabbits immunized with *E. coli*-NS, three rabbits immunized with *E. coli*-HS, and three rabbits immunized with *E. coli*-AS showed no marked differences when analysed immunoelectrophoretically with serum from normal adult guinea-pigs. Seven lines of precipitation have been identified, and these are seen in

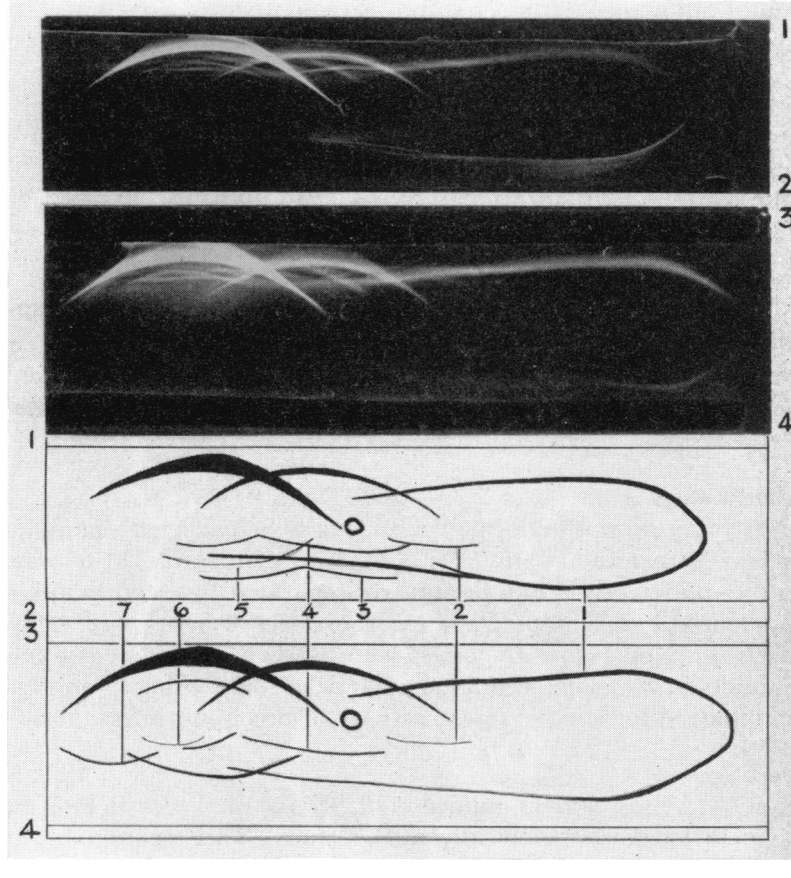

FIG. 1. Analysis of antisera from rabbits immunized with *E. coli* sensitized with pooled serum from normal adult guinea-pigs. Each well contains whole serum from a normal guinea-pig. Troughs 1 and 3 contain antiserum from a rabbit immunized with whole normal guinea-pig serum. Trough 2 contains antiserum from an individual rabbit immunized with *E. coli* sensitized in normal guinea-pig serum; trough 4 contains antiserum from a different rabbit, similarly immunized; antisera in troughs 2 and 4 were selected to allow presentation of all seven antigens detected with this technique.

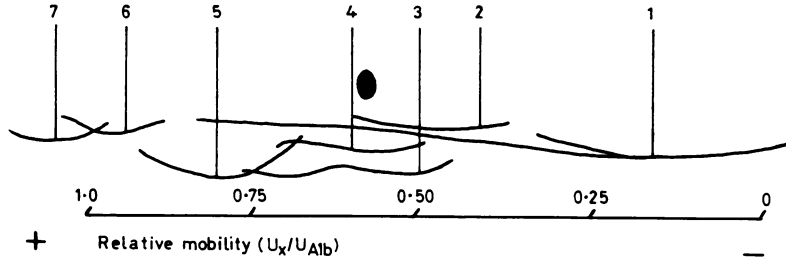

FIG. 2. Schematic diagram of a composite picture of the analysis of antisera prepared by immunizing rabbits with *E. coli* sensitized in pooled serum from normal adult guinea-pigs. The scale of mobility is relative to guinea-pig albumin. A mobility of zero is assigned to the position of migration of levan according to a technique described by Grabar, Uriel and Courcon (1960), and Uriel (1958). The antigens are numbered arbitrarily according to their position of migration from right to left.

Figs. 1 and 2. The antiserum from at least one of the three rabbits in each group showed all seven lines of precipitation. In Table 1 are listed the mobilities of each antigen relative to the mobility of albumin from guinea-pig serum, the identity of the antigen, if known, and

TABLE 1

RESULTS OF THE IMMUNOELECTROPHORETIC ANALYSIS OF NINE ANTISERA PRODUCED BY IMMUNIZING RABBITS WITH *E. coli* SENSITIZED WITH A POOL OF SERUM FROM A LARGE NUMBER OF ADULT GUINEA-PIGS

Antigen	Mobility (U_x/U_{alb})	Identification	No. antisera reacting with AG	Possible identity	Reference
1	0–1·0	IgG (γ_1 and γ_2)	9/9	–	–
2	0·3–0·5	IgM (γ_M; β_{2M})	9/9	–	–
3	0·5–0·8	–	3/9	C'_4	Peethoom and Pondman (1963)
4	0·6–0·8	$\beta_{1C} \rightarrow \beta_{1A}$ (C'_3)	9/9	–	–
5	0·8	–	5/9	C'_4	Cochrane (personal communication)
6	1·0	Esterase	4/9	C'_1	–
7	1·2	–	3/9	C'_1	Haines and LePow (1964)

the number of antisera in which antibody to a given antigen could be detected by immunoelectrophoretic analysis. Included is speculation on the identity of those antigens not identified. The antigens are labelled 1–7 and are described below:

Antigen No. 1

The precipitin line formed by this antigen on immunoelectrophoretic analysis extends from zero mobility into the zone of albumin and gives the classical appearance of γ-globulin (IgG). There is some spurring of this line at the mobility of 0·3 (see Fig. 2). This line was demonstrated with all nine antisera.

Antigen No. 2

The mobility of this antigen, found in all of nine antisera, extends from 0·35 to 0·6 and the precipitin line forms very close to the axis of the electrophoretic migration of the serum. This suggests that the antigen has a high molecular weight and is probably a macroglobulin.

From rabbits immunized with *E. coli*-NS an antiserum was selected that gave on immunoelectrophoretic analysis only three lines of precipitation, including the ones

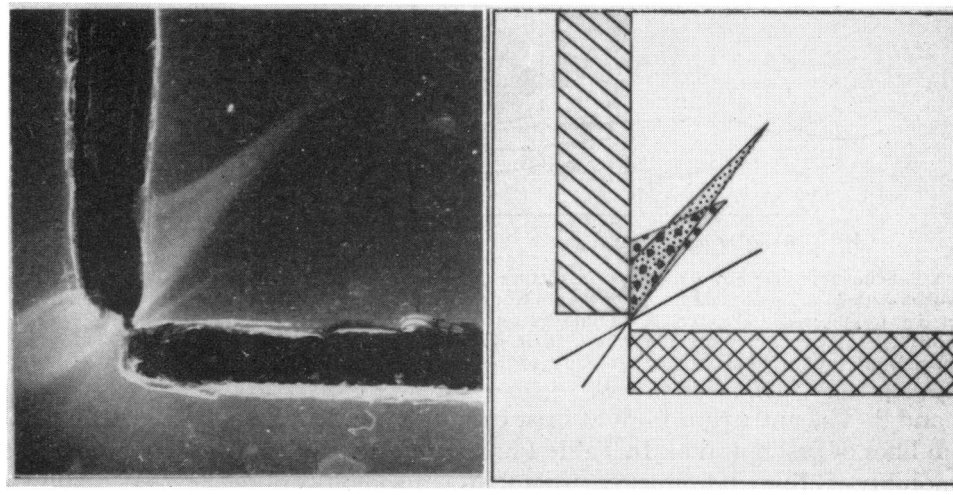

FIG. 3. Estimation of the diffusion coefficient of antigens Nos. 1, 2 and 4 by right angle diffusion according to the technique of Allison and Humphrey (1959). The horizontal trough contains serum from an adult guinea-pig. The vertical trough contains antiserum from a rabbit immunized with *E. coli* sensitized in normal guinea-pig serum; this antiserum contains antibody to antigens Nos. 1, 2 and 4. Three lines of precipitation are noted, one of which forms an angle of 30° with the horizontal. The diffusion coefficient for this line falls between 2·0 and $1{\cdot}0 \times 10^{-7}$ cm^2 sec^1, corresponding to a substance whose molecular weight is between 600,000 and 2,000,000.

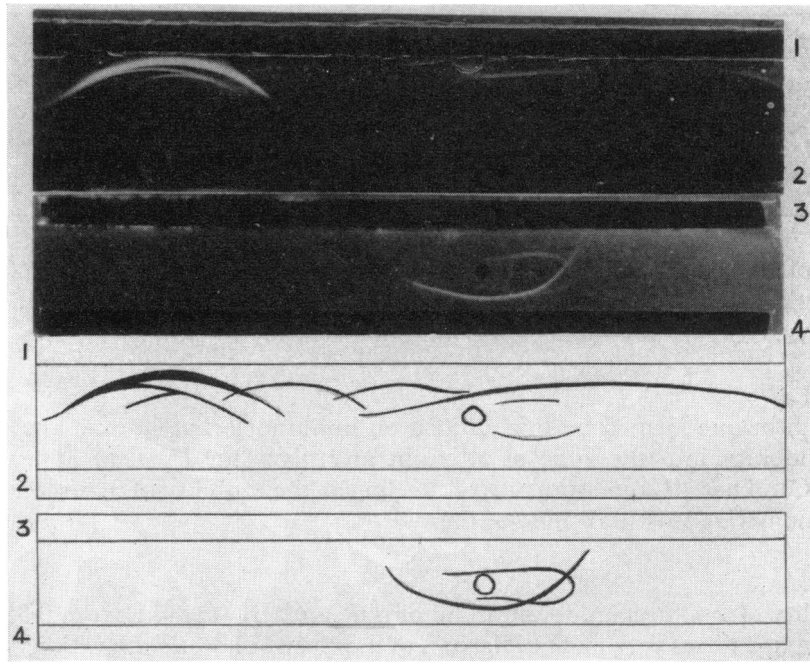

FIG. 4. Identification of antigen No. 2 as a macroglobulin by using an antiserum specifically directed against the beta macroglobulin of the guinea-pig. Both wells contain serum from the adult guinea-pig. Trough 1 contains an antiserum obtained from a rabbit immunized with whole serum of the guinea-pig. Troughs 2 and 3 contain antiserum from a rabbit immunized with the first peak of concentrated protein as eluted from Sephadex G-200 after filtration of whole guinea-pig serum. Trough 4 contains the antiserum from a rabbit immunized with *E. coli* sensitized in normal serum of the guinea-pig; this antiserum shows antibody only to antigens Nos. 2 and 4.

designated Nos. 1, 2 and 4 in Figs. 1 and 2. As seen in Fig. 3 using the technique of Allison and Humphrey, the diffusion coefficients for the three antigens were determined. Two were found to lie between 3·6 and 4·0 corresponding to substances with molecular weights between 100,000 and 200,000. The third diffusion coefficient was between 1·0 and 2·0, corresponding to a molecular weight between 600,000 and 2,000,000. This indicates that one of these three antigens (Nos. 1, 2 or 4) is macroglobulin.

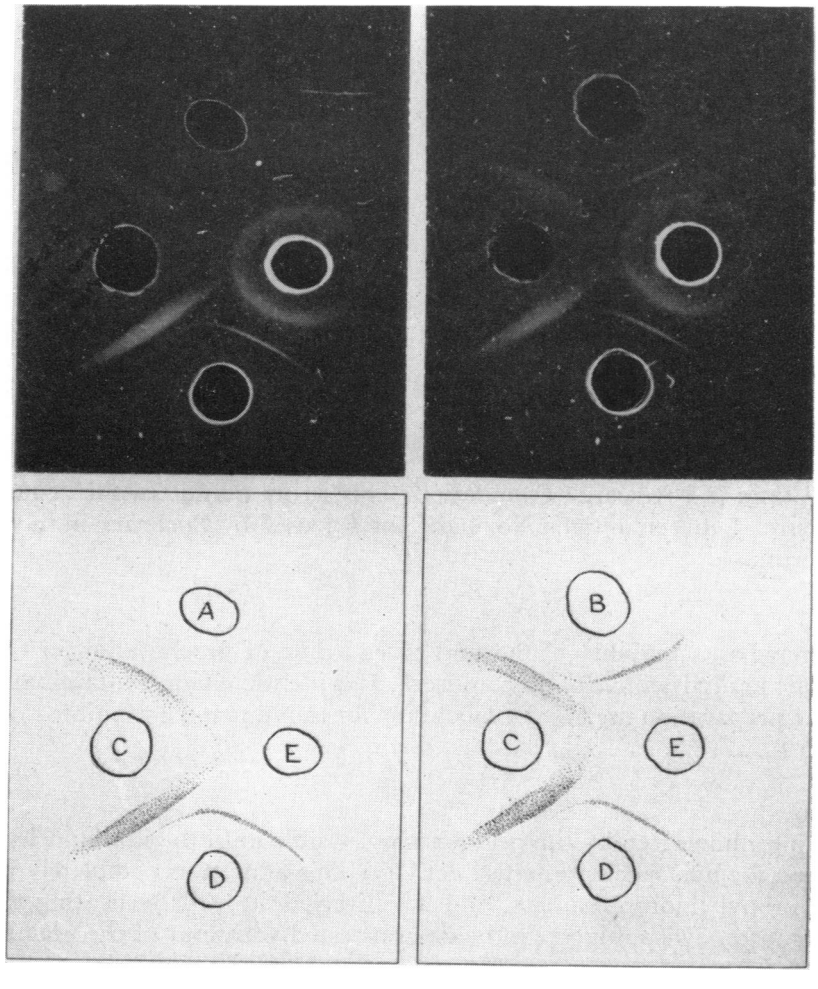

FIG. 5. Treatment of serum from the adult guinea-pig with mercaptoethanol resulting in the disappearance of antigen No. 2 as demonstrated with Ouchterlony's technique of analysis by double diffusion in gel. Normal serum from the guinea-pig was treated with mercaptoethanol and placed in well A. The control is contained in well B. Well C contains serum from a rabbit immunized with *E. coli*-NS; well D contains fresh serum from a guinea-pig; and well E contains an antiserum specific for the β macroglobulin of the guinea-pig (see Fig. 4). Note the absence of the macroglobulin line in the left plate.

Serum from normal guinea-pigs was put through a column containing Sephadex G-200 and that eluate giving the first peak of concentrated protein was used to immunize rabbits. This first peak of concentrated protein contains the α and β macroglobulins of serum (Flodin and Killander, 1962). One of the antisera obtained from these rabbits

produced on immunoelectrophoretic analysis with whole serum from the guinea-pig a single line of precipitation in the β_2 region. This is seen in Fig. 4 where also it can be seen to have identity with the precipitin line of antigen No. 2 in antiserum from a rabbit immunized with *E. coli*-NS.

As seen in Fig. 5, treatment of serum from the guinea-pig with mercaptoethanol resulted in the disappearance of antigen No. 2, while antigen No. 1 remained unchanged as determined with Ouchterlony analysis (Deutsch, 1957).

Antigen No. 3

The line of precipitation formed by this antigen lies near the trough containing antibody, indicating its relatively free diffusion, and takes the form of a double arc without spurring between the mobilities of 0·55–0·80. Antibody to this antigen was found in three of the nine antisera.

Antigen No. 4

All nine antisera contained antibody to this antigen whose mobility is between 0·65 and 0·8. The β_1 mobility of this component suggests its relationship to the C'_3 component of human serum designated β_{1C} by Müller-Eberhard (1960a). Upon heating fresh serum from a guinea-pig for varying times at 56°, the mobility of this component on electrophoresis in agar changed in a manner similar to that described for the $\beta_{1C} \rightarrow \beta_{1A}$ conversion in human serum (Müller-Eberhard, 1960b). Antiserum produced in a rabbit against the β_{1C} constituent of serum from the guinea-pig was obtained from Dr Charles Cochrane of the Scripps Clinic and Research Foundation. This serum permitted the demonstration of complete identity between antigen No. 4 and the β_{1C} used by Cochrane in the preparation of this antiserum.

Antigen No. 5

This antigen has a mobility of 0·8 and gives a line of precipitation in the immunoelectrophoretic analysis of five of nine antisera. It is identical to a 'contaminating' antigen present in the preparation used by Dr Cochrane for immunizing a rabbit to obtain specific antiserum to β_{1C}.

Antigen No. 6

The precipitin line given by this antigen is not visible until the immunoelectrophoretic slide is stained for non-specific esterase activity. This activity is completely inhibited by 10^{-4} M diisopropyl fluorophosphate, and is entirely destroyed by heating the serum at 56° for 20 minutes. This antigen can be demonstrated with four of the nine antisera and has a mobility of 1·0.

Antigen No. 7

This antigen has a mobility of 1·2 and was detected only with antisera from rabbits immunized by the subcutaneous route.

II. IMMUNOELECTROPHORETIC ANALYSIS OF ANTISERA OBTAINED FROM RABBITS IMMUNIZED WITH *E. coli* SENSITIZED WITH POOLED SERUM FROM A SMALL NUMBER OF NORMAL GUINEA-PIGS

Two rabbits were immunized with a vaccine prepared from *E. coli* as described for *E. coli*-NS except the washings following sensitization were carried out with distilled water,

instead of normal saline. It is of interest that antisera from these two rabbits both contained antibodies only to antigens Nos. 2, 4 and 5, whereas nine out of nine rabbits immunized with *E. coli*-NS prepared from the large commercial pool of serum and washed with saline after sensitization produced antibody to antigen No. 1.

III. IMMUNOELECTROPHORETIC ANALYSIS OF ANTISERA PRODUCED IN THE RABBIT AGAINST *E. coli* SENSITIZED WITH POOLED SERUM FROM ADULT GUINEA-PIGS ACTIVELY IMMUNIZED WITH *E. coli*

Six rabbits were immunized with *E. coli*-IS. Some of the rabbits received *E. coli* washed five times in distilled water instead of saline after sensitization, while others received *E. coli* washed eight times in distilled water after sensitization. The antigens detected with

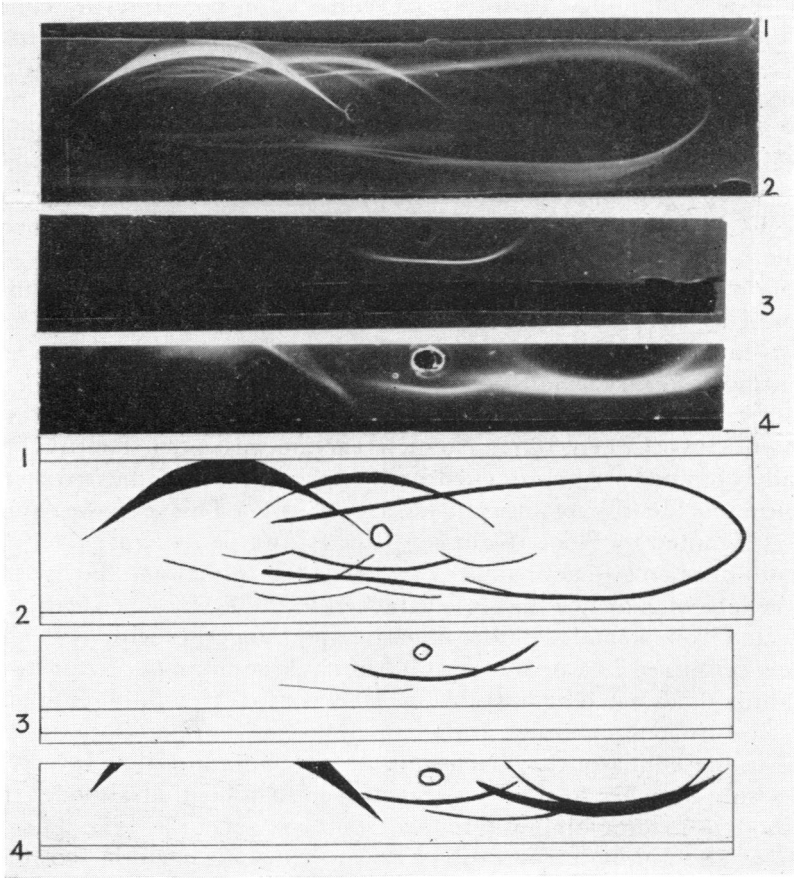

FIG. 6. Analysis of sera from rabbits immunized with *E. coli* sensitized in guinea-pig serum from different sources. All wells contain serum from the adult guinea-pig. Trough 1 contains antiserum from a rabbit immunized with whole serum from the guinea-pig. Trough 2 contains antiserum from a rabbit immunized with *E. coli* sensitized in a commercially-obtained pool of serum from a large number of normal guinea-pigs. Note precipitins to both 7S γ_1- and 7S γ_2-globulins. Trough 3 contains antiserum from a rabbit immunized with *E. coli* sensitized in a pool of serum from a small number of guinea-pigs bled in this laboratory. Note precipitins to neither 7S γ_1 nor 7S γ_2-globulins. Trough 4 contains antiserum from a rabbit immunized with *E. coli* sensitized in serum from guinea-pigs actively immunized with *E. coli*. Note that the predominating precipitins are directed against the 7S γ_2-globulin.

antisera from these six rabbits on analysis by immunoelectrophoresis using normal guinea-pig serum were essentially the same as for the antisera previously described. Present in all antisera were antibodies to antigens Nos. 1, 2 and 4, and present in three of them was antibody to antigen No. 5. The difference in the appearance of the IgG line produced by these antisera and that produced by the nine antisera discussed above is remarkable. The precipitin line for IgG (antigen No. 1) seen with antiserum against *E. coli*-IS has the form of a double arc with the slower migrating arc being very thick (see Fig. 6), and giving a single spur with the more rapidly moving arc. In contrast, antiserum against *E. coli*-NS forms with antigen No. 1 two arcs which are of uniform intensity and, often, seem to be part of a single, continuous line of precipitation.

DISCUSSION

All of nine rabbits immunized with *E. coli* sensitized in pooled serum collected from a large number of normal guinea-pigs recognized three globulins which we have identified as IgG, IgM and β_{1C}. This may indicate that these globulins are better antigens than the other proteins detected, or that they are altered during complexing with the bacterium in such a way that their antigenicity is enhanced. Perhaps these three globulins are present in more prominent steric positions on the bacterial surface than are the other globulins. C'_3 (β_{1C}) attaches to the antigen–antibody complex in amounts 100 times greater than that of the $C'_1C'_{4a}$ which activates it (Müller-Eberhard, 1965). This may account for the ease with which it is identified with immunological techniques.

The lack of detectable antibody to IgG in antisera from two rabbits immunized with *E. coli* sensitized in the pool of serum from a small number of guinea-pigs bled in this laboratory was surprising. This not only implies the absence of any IgG attaching to the *E. coli*, but antibody formed against IgM was probably directed against the heavy chain only. If antibody were directed against the light chains of the IgM, this might be expected to cross-react with IgG (Cohen, 1963). No such reaction was noted. That IgG from the pool of commercially-obtained serum attached itself to the *E. coli*, is indicated by the spur seen on the precipitin line identifying antigen No. 1 (see Fig. 2). This spur, we think, represents the slow IgG designated by Bloch, Kourilsky, Ovary and Benacerraf (1963), as 7S γ_2 and should not result from cross-reacting antibody formed in response to the light chains of the IgM. Thus, the lack of evidence that IgG attached to the *E. coli* when sensitized in a pool of serum collected from a small number of guinea-pigs, together with evidence that it did attach to *E. coli* sensitized in a pool of serum from a large number of guinea-pigs suggests that specific antibody directed against this *E. coli* is not found in the IgG fraction of serum of all apparently normal guinea-pigs. If the attachment of immune globulins to the bacterium is related to the killing of that bacterium, then our finding that IgG as well as IgM from normal serum attaches to the bacterial surface suggests that although most natural bacterial antibody is macroglobulin in nature, some may occur as IgG. The marked antibody response to 7S γ_2-globulin by rabbits vaccinated with bacteria sensitized in serum from guinea-pigs immunized with the *E. coli* is of interest in light of experiments showing the complement-fixing nature of this particular 7S immune globulin (Nussenzweig and Benacerraf, 1964).

That the IgG, IgM and β_{1C} globulins all play a role in the complement-dependent bactericidal activity of serum is known (Michael and Rosen, 1963; Rowley and Turner, 1964), and it seems probable that their presence on the bacterium is directly related to the bactericidal property of serum. Yet experiments using serum from which all detectable

bactericidal antibody to the *E. coli* had been absorbed, or which had been heated to inactivate bactericidal complement, gave results identical to those experiments using unaltered serum from the same pool. Since immunoelectrophoretic analysis is not quantitative, it is quite possible that in the experiment using heated serum, although detectable bactericidal activity of the complement system has been destroyed, trace amounts of complement remain which are sufficiently intact to adhere to the bacteria and induce formation of antibodies in the rabbit. On the other hand, it is possible that the presence of all of these globulins on the bacterium is non-specific and has no relationship to the bactericidal activity of the serum.

The detection on these sensitized bacteria of at least four other globulins raises further the question of non-specific fixation of protein or of contamination of the bacteria with non-fixed proteins. Both possibilities seem unlikely, since none of the antisera contained antibodies to albumin or transferrin. Albumin is present as more than half of the protein in normal serum, and transferrin is notorious for its non-specific fixation and its excellent antigenicity. Antibody to transferrin is known to appear first and in large quantity when animals are immunized with the whole serum of another species. Moreover, the detection of antibody to only one serum component possessing esterase activity gives further evidence that contamination of the bacteria with nonspecific globulins is unlikely. The technique used to identify esterase is extremely sensitive, since it is based on activity of the enzyme whereby many molecules of substrate are converted to a visible coloured substance by each molecule of enzyme. Thus, precipitates of antigen–antibody complexes that are not visible to the eye may be easily detected. That only one esterase is detectable in our immunoelectrophoretic analysis strongly suggests that it is specifically attached to the bacteria. If one esterase were non-specifically attached, we might expect one or more of the other three esterases we have demonstrated in whole serum from the guinea-pig to be similarly present.

The identity of the globulins designated as antigens Nos. 5, 3, 6 and 7 is not established. Antigen No. 5 is identical to one revealed by a 'contaminating' antibody in the specific anti-β_{1C} serum obtained from Dr Cochrane. This antigen behaves electrophoretically like β_{1E} (C'_4) of human serum, and Cochrane has presumed that this is the homologue in the guinea-pig for human β_{1E}. It is present in the pseudoglobulin fraction of the serum from the guinea-pig. Antigen No. 3 behaves immunologically and electrophoretically like an antigen in human serum described by Peethoom and Pondman (1963) which they identify as β_{1E_2}, a portion of the inactivated C'_4 molecule. The detection of the two α-globulins (antigens Nos. 6 and 7) with our antisera raises the possibility that these may be homologous with alpha globulins known to be in a fraction of human serum containing highly active C'_1 esterase (Haines and LePow, 1964). This conjecture is re-enforced by the detection in antigen No. 6 of esterase activity that is inactivated by diisopropyl fluorophosphate or by heating the serum at 56° for 20 minutes.

It is clear that none of the seven precipitin lines is due to the presence of antibody to antigenic groups present on the bacteria which might be shared with certain of the serum proteins of the guinea-pig, since antiserum from rabbits immunized with *E. coli* alone show no precipitin reactions with the serum from normal guinea-pigs.

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