Episome-carried Surface Antigen K88 of Escherichia coli

II. Isolation and Chemical Analysis

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ABSTRACT

The K88 antigen was carried by episomal transfer to D282, a nonmotile Escherichia coli strain without K antigen. D520, obtained by this episomal transfer, was used for the extraction of K88 antigen. It was shown by the agar gel precipitation technique that some K88 antigen was released from D520 into suspending aqueous medium. The amount of liberated material was increased by gentle heating (60°C) or treatment in a Waring Blendor. The antigen was obtained from the extracts in a purified form by making use of its insolubility between pH 3.5 and 5.5 and of its high sedimentation rate (S_{20,w} = 36.7S). The homogeneity of the material was demonstrated by agar gel precipitation with D520 antiserum, by analytical ultracentrifugation, and by moving-boundary electrophoresis. Chemical analysis revealed that K88 is a pure protein containing all the common amino acids with the exception of cysteine-cystine. Purified K88 selectively precipitated the K88 antibodies from D520 antiserum and was shown to be immunogenic in rabbits.

The antigen K88 of Escherichia coli was first described in 1961 (12). It was found in two strains isolated from cases of enteritis and edema in swine, and was shown to belong to the L type of heat-labile K antigens as defined by Kauffmann (7). In addition to K88, both strains carried another K antigen of the B type, one of which (K85) has been reported to be an acidic polysaccharide (11).

Since then it has been shown that K88 exists in at least two serologically different varieties (13), and many other strains of E. coli have been isolated which carry K88 in different antigenic combinations (13, 15, 22). Most of these strains were found in diseased swine, and it has been suggested that the presence of K88 antigen might have some bearing on the pathogenicity of E. coli in piglets (22).

G7 [O8:K87(B),K88(L):H19], one of the first two strains found to carry K88, proved to be a genetic donor. It could transfer the ability to form K88 antigen to other E. coli strains, and it could also transfer chromosomal markers with low frequency (10, 12). The strains which acquired the K88 antigen also generally received the ability to transfer chromosomal markers. Of these new donors, some segregants were isolated which retained the donor ability but lost the K88 antigen. Acridine was found to "cure" the bacteria of K88, and it was concluded that the determinant for this antigen was carried by an episome.

In view of these facts, the isolation and chemical analysis of K88 antigen seemed of interest. Preliminary experiments showed that the material was a protein, as reported in a brief communication (17).

MATERIALS AND METHODS

Strains. The E. coli strains listed in Table 1 were used.

Media. For differentiation of the ability to ferment lactose, modified Drigalski plates with lactose (1%) were used, with bromothymol blue (BTB) as indicator (7). For all other purposes, ox heart broth was used for plates (1.6% agar), and beef broth, for fluid medium. In both cases, 1% peptone, 0.3% NaCl, and 0.2% Na₂HPO₄ were added.

Preparation of antisera. Formalin (0.5%)-killed suspensions of agar plate cultures, containing about 10⁸ bacteria per milliliter, were used for the production of sera against whole cells. Rabbits were injected intravenously five times at 4- to 5-day intervals, with doses increasing from 0.25 to 2.0 ml. The animals were bled 8 days after the last injection.

Rabbits were also used for the preparation of sera.
against purified K88 antigen. Four different modes of administration were applied.

(i) Lyophilized antigen was suspended in saline to about 10 mg/ml and was dispersed thoroughly in a Thomas grinding vessel. The suspension was administered according to the procedure for whole bacteria as described above.

(ii) A 1% solution of K88 in pH 7.4 phosphate (0.05 M)-buffered saline was injected as described for hemocyanin by Campbell et al. (2). Since the animals became ill or died after the first injection of 1 ml, the dosage was reduced to 0.2 ml for the surviving rabbits.

(iii) In accordance with the general outlines given by Kabat and Mayer (6) and by Lennox (8) for aluminium hydroxide-adsorbed antigens, 1 ml of 3% aqueous alum [KAl(SO₄)₂ · 12H₂O] was added to each 10 ml of 0.3% antigen in buffered saline, and the pH was adjusted to 7 by addition of 0.1 N NaOH. The animals were injected for a period of 4 weeks, receiving 0.5 ml once subcutaneously in the shoulder and 0.5 ml four times intravenously each week. The first intravenous injection, however, was 0.2 ml and the last two were 1.0 ml each. Final bleeding was again carried out 8 days after the last administration of antigen.

(iv) Equal volumes of a 1.4% solution of K88 in buffered saline and of Complete Freund Adjuvant (Difco) were mixed thoroughly. Once a week for 3 weeks, 1 ml of the emulsion was injected subcutaneously at different sites in the shoulder. The rabbits were bled 7 weeks after the last injection.

Intravenous application of K88 antigen in the dosages employed generally weakened the animals, some of which died.

Agglutination test. Slide and tube agglutination tests were used. The details of these techniques were reported previously by Kauffmann (7) and Ørskov et al. (12).

Precipitation analysis by double diffusion in agar gel (Ouchterlony). A filter paper disc modification of the Ouchterlony technique was adopted: 1% agar (Behring "Reinagar") in 0.066 M phosphate buffer (pH 7) containing 0.065% sodium azide and 0.11% ethylenediaminetetraacetic acid (EDTA) was poured into petri dishes (14-cm diameter) to give a layer about 1 mm thick. Filter paper discs (6-mm diameter), as used for sensitivity tests, were soaked with sera or K88 antigen solutions, or they were placed on confluent bacterial plate cultures and then heated in an oven to 60 C for 0.5 hr to enhance antigen release. Finally, the discs were placed on the agar and were gently pressed to the surface. The closed petri dishes were stored in plastic bags to reduce evaporation and were incubated for 1 day at 37 C, and then for 4 to 10 days at room temperature. The development of the precipitation lines was inspected daily.

Absorption of K88 antibodies. After a preliminary precipitation test in tubes to find the concentration of purified K88 which could deplete serum D520 of K88 precipitins, 1 ml of D520 antiserum diluted 1:2 in saline was added to 1 ml of a 0.3% solution of K88 in phosphate (0.05 M pH 7.4)-buffered saline. This mixture was held at 37 C for 1 hr, and was placed in a refrigerator overnight. After removal of the precipitate by centrifugation, the supernatant fluid was assayed for agglutinins.

Conditions for transfer of K88. Transfer of K88 from donor D432 to a recipient not having this antigen has been previously described in detail (10).

Ultracentrifugation. Preparative ultracentrifugation was carried out in a Spincos model L centrifuge.

Analytical ultracentrifugation was performed in a Spincos model E centrifuge with the use of an analytical rotor (An-D) and a 12-mm analytical cell. The samples were analyzed at 20 C and 39,400 rev/min, and photographs were taken at intervals of 4 or 8 min.

Free electrophoresis. The procedure of Tiselius was carried out in an LKB apparatus with schlieren optics equipped with a phase plate. Veronal buffer of pH 8.6 (ionic strength, 0.1) and phosphate buffer of pH 7.0 (ionic strength, 0.1) were used.

Elemental analysis. Dumas nitrogen was determined with a Coleman analyzer, and phosphorus, by the procedure of Lowry et al. (9).

Amino acid analysis. A 13.7-mg amount of freeze-dried K88 antigen was dissolved in distilled 6 N
hydrochloric acid by gentle warming and stirring, to give 7.5 ml of solution after cooling. Six 1.0-ml samples, containing 1.83 mg of protein each, were pipetted into Pyrex glass tubes, which were then constricted at the top and cooled in a mixture of Dry Ice and alcohol; after evacuation with a water aspirator, the tubes were sealed. Two samples were hydrolyzed for 24, 48, or 2 hr at 110°C, and the solutions obtained were evaporated at 40°C by passing a stream of air over their surface. The samples were dissolved in 1.1 ml of citrate buffer (pH 2.2), and 0.5 ml of this solution was applied to each of the two ion-exchange columns of a Spinco model 120 amino acid analyzer. The analyses were carried out according to the instructions of the manufacturers; for further details, see Johansen and Ottesen (5).

Tryptophan was determined by the method of Spies and Chambers (16). A Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) was used throughout.

Color reactions for proteins. The biuret reaction as described by Campbell et al. (2) and the ninhydrin reaction as described by Kabat et al. (6) were used.

Estimation of neutral sugars. The indole and orcinol reactions as described by Kabat et al. (6) were adopted. Glucose was used as a standard.

Estimation of hexosamines. Hydrochloric acid hydrolysates of the material were evaporated and N-acetylated, and N-acetyl hexosamine was determined by the technique of Reissig, Strominger, and Leloir (14). A detailed description of the procedure is given by Jann (Ph.D. Thesis, Univ. Freiburg, Freiburg, Germany, 1965). Glucosamine hydrochloride was used as a standard.

Extraction of lipids. Freeze-dried protein was extracted by boiling with distilled methanol, followed by methanol-chloroform, as described by Weibull (18). The procedure of Weibull (18) was also followed for the purification of the lipids. The lipids were dried over P2O5, KOH, and paraffin filings in a vacuum desiccator, and were then weighed. The residue was tested for ester linkages by the hydroxamic acid method as described by Feigl (3).

RESULTS

Selection of K88 antigen strain D520. The original K88 donor strain G7 seemed less suitable for the extraction and isolation of K88 antigen because it carries two K antigens, the separation of which could be expected to cause additional difficulties.

Therefore, K88 was transferred from D432 to D282, which has neither K nor H antigens and which was also found to be nonfimbriate when grown on agar plates, as shown in the next paper of this series. The mating mixture was plated on lactose agar with bromothymol blue as indicator, the lactose-fermenting colonies were tested for K88 antigen by slide agglutination with K88 antisemum, and a few positive colonies were purified.

D520, one of the lactose- and K88-positive clones selected, was identical with D282 in all antigenic and fermentative characteristics, except for the presence of K88. D520 was used for the extraction of the antigen.

Identification of K88 antigen in agar gel precipitation. From earlier investigations, with different K88+ and K88− pairs, it was known that the K88 antigen precipitated as a single band in agar gel precipitation, by use of the Ouchterlony technique (F. Ørskov and I. Ørskov, unpublished data).

This finding was now confirmed with other pairs of K88 strains and their corresponding sera. Figure 1 shows the result of a typical experiment. Apart from strains D282 and D520 and their corresponding sera, another set was used, i.e., strains D627 and D628 and their sera. The K88 strain D628 was obtained by transfer of K88 from D432 to D627 according to the same procedure used for the development of D520. The sharp line near the antigen discs was clearly due to the precipitation of the K88 antigen.

Thus, the gel precipitation technique was used throughout this investigation as an isolation guide during the purification steps.

Extraction of K88 from D520 by heating in an aqueous medium. Recently, several E. coli K anti-

![Fig. 1. K88 antigen in Ouchterlony agar gel precipitation. Bacterial antisera: S1, D282; S2, D520; S3, D627; S4, D628. Antigens: 1, whole culture of D520 (O8:K88(L):H−); 2, D282 (O8:K+:H−); 3, D627 (O16:K1(L):H+); 4, D628 (O16:K1(L), K88(L):H+); 5, solution of purified K88 antigen; 6, crude extract of D520. Precipitation lines: a, K88 antigen; b, somatic antigens of respective strains. Some weak, unspecific precipitation lines involving some of the cultures and extracts, but not the purified K88 antigen, have not been drawn. Strain D527 is inagglutinable in O antisemum and contains the K1 antigen. No precipitation lines corresponding to the K1 specificity were detected, probably because the sera used had very low K1 titers.](http://jb.asm.org/Downloaded from http://jp.asm.org)
genic substances have been isolated by use of the phenol-water extraction procedure of Westphal, Lüderitz, and Bister (see 20). The K antigens were found in the water phase after removal of the O antigenic lipopolysaccharides by ultracentrifuga-
tion (4, 11; Jann, Ph.D. Thesis, 1965), and were shown to be acidic polysaccharides, which could be purified by Cetavlon precipitation. However, the aqueous phase obtained from a K88+ strain in this manner did not contain any polysaccharide responsible for the K88 specificity. Therefore, use was made of the observation that K88 antigenic material was easily released from the intact bacteria into a suspending aqueous medium as shown by the Ouchterlony tests with whole cultures. D520 was suspended in saline and heated to 60 C. The organisms were then centrifuged off, and the supernatant fluid was tested; it was found to give a dense K88 precipitation line in agar gel diffusion (Fig. 1).

Once it was recognized (see following paragraph) that K88 is a protein, insoluble at its isoelectric region between pH 3.5 and 5.5, the age of cultures, pH of the extraction medium, extraction time, and temperature were tested for their influence on the yield of the antigen. The different extracts were brought to pH 5 after removal of the organisms, and the antigen was sedimented, washed, and estimated by the biuret reaction. The following extraction conditions were found to give good results, i.e., about 2 to 4 mg of crude protein antigen per 14 cm of broth-agar plate culture of D520.

Confluent growth (16 to 30 hr) of D520 was harvested from the agar and suspended in a buffer (pH 6.5 to 9); e.g., 3 to 5 ml of 0.1 M phosphate buffer (pH 7) per plate. The mixture was heated to 60 to 65 C and was gently agitated for at least 15 min. The bacterial bodies were separated by centrifugation at 27,000 × g for 10 min, and the supernatant fluid was stored at 4 C for at least 3 days. To prevent bacterial growth, 0.05% sodium azide was added. Considerable amounts of material, but no K88 antigen, sedimented during this period and were removed by suction through a membrane DA filter (0.65-μm pore size; Millipore Filter Corp., Bedford, Mass.). Filtration was generally preceded by centrifugation to avoid clogging.

Treatment in a Waring Blender. Friction was also applied for the removal of K88 antigen from the bacterial surface. Agar plate cultures of D520 were harvested and suspended as above, and were then treated in a Waring Blender. At about one-third the maximal speed, 1 min of agitation was sufficient to yield extracts containing as much protein antigen as those obtained by heating.

Extraction and purification of K88 antigen are summarized in Fig. 2.

Isolation of K88 antigen from the extracts. It was observed early that the antigen was insoluble between about pH 3.5 and 5.5, and the first ultracentrifugal analysis indicated a sedimentation coefficient of approximately 35S. Use was made of these two properties for the isolation of K88.

Isoelectric precipitation. Extracts obtained by either of the two methods described were stirred and brought to about pH 5.3 by the slow addition

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I. Plate culture of *Escherichia coli* D520
   - a. Suspended in buffer, pH 6.5 to 9
   - b. Heated to 60°C or treated in a Waring Blender
   - c. Centrifuged at 27,000 × g, sediment discarded

II. Crude extract
   - a. Stored at 4°C
   - b. Filtered through a type DA Millipore membrane filter

III. Filtrate
   - a. pH → 5.3
   - b. Centrifuged at 3,000 × g, supernatant fluid discarded

IV. Sediment of crude antigen
   - a. Washed at pH 5.3
   - b. Dissolved at pH 6.5 to 9
   - c. Solution filtered
   - d. pH → 5.3, etc., complete procedure repeated two or three times

V. Solution of reprecipitated antigen
   - a. Ultracentrifuged at 115,000 × g for 10 min, sediment discarded
   - b. Ultracentrifugation continued for 200 min, supernatant fluid discarded
   - c. Pellets redispersed in buffer pH 6.5 to 9
   - d. Solution filtered
   - e. Ultracentrifuged at 115,000 × g for 10 min, etc., procedure repeated once or twice

VI. Solution of purified K88 antigen

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**Fig. 2. Extraction and isolation of K88 antigen from Escherichia coli D520.**
of dilute acetic acid. The precipitated protein was collected by centrifugation for 10 min at 3,000 × g and was washed twice with a dilute buffer of pH 5.3 (e.g., 0.01 M McIlvaine buffer). The material was redissolved at pH 6.5 to 9 (e.g., in 0.1 M phosphate buffer pH 7) by gentle stirring at 4°C, and the solution was again filtered through a type DA membrane filter (Millipore Filter Corp.).

To avoid high viscosities and resulting difficulties during filtration, 0.1 to 0.3% solutions of K88 were generally used. Sodium azide (0.05%) was added to all buffers.

The antigen was reprecipitated two or three times and dissolved again, and the solution was filtered. The product thus obtained (generally 1 to 2 mg per agar plate as determined by the biuret reaction) showed the K88 precipitation line only, when tested in a 0.1 to 0.3% solution by agar gel diffusion against D520 serum.

Preparative ultracentrifugation. In K88 antigen, purified by repeated isoelectric precipitation, two minor contaminants were detected, which proved difficult to remove by further precipitations. In the Ouchterlony test with D520 serum, a second, weakly developed precipitation line of a non-specific, common antigen appeared when 3% solutions of the material were applied. Furthermore, in the analytical ultracentrifuge, the main peak (Fig. 3) was preceded by some material of higher sedimentation velocity. When the antigen was sedimented from solution by preparative ultracentrifugation, there appeared a discolored tip of the otherwise transparent pellets.

For further purification, a solution of reprecipitated K88 antigen was centrifuged for 10 min at 115,000 × g in a 40.3 rotor of a Spinco model L preparative ultracentrifuge, and the small, brownish pellets were discarded. K88 was completely sedimented from the supernatant fluid by further centrifugation at 115,000 × g for another 200 min. The pellets were redispersed by gentle agitation at 4°C in a pH 7 phosphate buffer, and the solution was filtered again as above. The whole process was repeated once or twice.

The solution of purified K88 antigen thus obtained was used for all further experiments. Exhaustive dialysis against distilled water, followed by lyophilization, yielded the antigen in the form of a white powder.

The isolation and purification of K88 are summarized schematically in Fig. 2.

Filtration. K88 antigen in a 0.1 to 0.3% solution passed through a type DA membrane filter (0.65-μm pore size; Millipore Filter Corp.) with no difficulty. Filtration through an HA membrane filter (0.45 μm; Millipore Filter Corp.), however, proved difficult, and PH filters (0.3 μm; Millipore Filter Corp.) completely retained the material. Thus, filtration through a PH filter could also be used for the isolation of K88 antigen.

Criteria of homogeneity. Purified K88 antigen was tested for homogeneity as follows.

Gel precipitation. Solutions of 0.1 to 3% K88 antigen in a pH 7 phosphate buffer were tested against D520 sera from different rabbits by use of different distances between antigen and antibody discs. The plates were inspected daily for up to 10 days of incubation. In all cases, only the K88 precipitation line was observed (Fig. 1).

Analytical ultracentrifugation. Two preparations of K88 antigen dissolved in phosphate (0.05 M)-buffered saline (pH 7.38) were analyzed. Of each preparation, five concentrations in the range from 0.1 to 0.7% (w/v) were investigated. The protein sedimented as a symmetrical peak (Fig. 3), and only the higher concentrations revealed trace amounts of faster-sedimenting material.

Fig. 3. Two exposures from the analytical ultracentrifugation of a 0.4% solution of K88 in phosphate-buffered saline (pH 7.38) are shown. The pictures were taken 6 (A) and 62 (B) min after full speed (39,460 rev/min) was reached. Sedimentation from right to left.
More than 95% of the material sedimented within the main peak.

**Free electrophoresis.** Solutions of K88 antigen in Veronal buffer (pH 8.6) showed one main boundary when concentrations from 0.1 to 0.5% (w/v) were investigated. The boundary of the positive limb was always extremely narrow, and only trace amounts of slower-moving material were demonstrated. Electrophoresis of 0.5 and 0.8% solutions in phosphate buffer (pH 7.0) showed also that more than 93% of the material moved as a single component (Fig. 4).

**Properties of purified K88 antigen.** As mentioned above, the antigen is quantitatively retained by a PH membrane filter with a pore size of 0.30 ± 0.02 μ.

**Sedimentation constant.** The mean S rate was calculated for each of the analyses (see above). The sedimentation constant at zero concentration and the slope of the line 1/S vs w versus c were calculated to be 36.7 S and 7.7 ml g⁻¹ S⁻¹, respectively, by the method of the least squares.

**Mobility in moving-boundary electrophoresis.** The mobilities at pH 8.6 and 7.0 corresponded to the mobility of α₂ globulins of human serum, i.e., approximately 4.2 and 3.2 mobility units (−10⁻⁶ cm² sec⁻¹ v⁻¹), respectively.

**Solubility.** Undenatured K88 antigen was highly soluble in 0.01 to 1.0 M buffers of pH 6.5 to 9. Solutions above 5%, however, proved too gelatinous to be handled. When a solution of K88 was slowly acidified, precipitation occurred at pH 5.4 to 5.7, and an incomplete redissolution (partial denaturation) took place between about pH 3.0 and 3.5. The antigen was almost completely insoluble around pH 5; 100 ml of the supernatant fluid after precipitation contained about 0.3 mg of protein only. The antigen was soluble in concentrated hydrochloric acid and in aqueous sodium hydroxide. K88 was precipitated at pH 7 by the addition of ammonium sulfate to about 13% of saturation or more.

**Denaturation.** K88 antigen, which was dissolved in hydrochloric acid or sodium hydroxide solution, or which had been suspended in formamide or heated to above 70 C, did not give a precipitation line in agar gel diffusion. Prolonged, vigorous stirring in solution (rapid breaking of air-liquid or liquid-liquid surfaces), lyophilization, or treatment at pH 2 to 3 greatly reduced the solubility of the material in pH 6.5 to 9 buffers, but the suspensions still gave the gel precipitation line.

It should be mentioned that D520 bacteria, which had been treated with a 50% aqueous solution of urea for 24 hr at 37 C, were not agglutinated by K88 antiserum.

**Fig. 4.** Results of analysis by free electrophoresis of a 0.5% solution of K88 in Veronal buffer, pH 8.6 (A and B, positive and negative limb, respectively), and phosphate buffer pH 7.0 (C and D, positive and negative limb, respectively) are shown. The peak moved as the α₂ globulin region of human serum in both buffers. The zone sharpening is exceptionally pronounced in the positive limb.
Ultraviolet (UV)-absorption spectrum. The UV-absorption curve of a 0.05% solution of K88 in 0.1 N NaOH was measured in a Beckman DU spectrophotometer. The material showed the broad absorption band at about 280 m\(\mu\) characteristic for proteins, with a shoulder at 290 m\(\mu\) indicating the presence of tryptophan. No peak was observed at 260 m\(\mu\), where the absorption maximum of nucleic acids occurs.

Color reactions. The specific extinction of freeze-dried K88 in the biuret reaction was comparable to that of other proteins (e.g., edestine) used as standards. In the ninhydrin reaction as given by Kabat et al. (6), K88 yielded an extinction of 0.015 per \(\mu\)g of nitrogen per 2.6 ml in a 1-cm cell.

Chemical analysis of purified K88 antigen. Lyophilized K88 antigen was dried in vacuo at 105 C over phosphorous pentoxide. Constant weight, however, was not attained; thus, the moisture content was calculated from the loss of weight after 24 hr of drying (1). Elemental analysis indicated the presence of 14.0% nitrogen (15.5% calculated on a dry weight basis) and 0.04% phosphorus. The antigen contained 10.0% water, 2.1% inorganic ash, and 3 to 4% lipids, which together comprised 15 to 16% of the total weight.

The small amounts of purified lipids obtained by chloroform-methanol extraction of lyophilized K88 gave a positive hydroxamic acid test.

The percentages of amino acid residues (Table 2) were extrapolated as follows. The value of valine after 72 hr of hydrolysis was taken. With serine and threonine, the values decreased with increasing time of hydrolysis. These data were extrapolated to zero-time of hydrolysis. In all other cases, the means were calculated from the values obtained after 24, 48, and 72 hr of hydrolysis. No cysteine-cystine or uncommon amino acids were detected in the chromatograms. Amino acids comprised 81.2% of the total weight of the antigen.

The presence of 1.0% neutral sugars was detected in purified K88 antigen; the determination of hexosamines, however, yielded values below 0.1%. Thus, 97 to 98% of the total weight could be accounted for.

Serological analysis of purified K88 antigen; absorption of serum D520. Serum D520, known to contain O8 and K88 agglutinins, was saturated with purified K88 antigen and examined for remaining agglutinins. The K88 agglutinins were completely removed (Table 3) as evidenced by the fact that the unheated culture of the K88 strain D225 failed to react in the absorbed serum. D282, which has no K antigen, agglutinated in both absorbed and unabsorbed serum almost to the same extent whether heated or unheated. In other words, purified K88 antigen can remove K88 agglutinins, while the O agglutinins remain.

Absorption of another K88 antiserum, produced by an entirely different strain, also resulted in removal of the K88 agglutinins.

Immunogenicity of purified antigen. The K88 titers of the sera are given in Table 4. K88 agglutinins were obtained in all cases.

In addition, it was demonstrated that the K88

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Hydrolysis time</th>
<th>Extrapolated values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
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<tr>
<td>Lysine</td>
<td>4.03</td>
<td>4.14</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.11</td>
<td>0.10</td>
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<tr>
<td>Arginine</td>
<td>3.85</td>
<td>3.88</td>
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<td>Aspartic acid</td>
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<tr>
<td>Threonine</td>
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</tr>
<tr>
<td>Serine</td>
<td>4.20</td>
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<tr>
<td>Glutamic acid</td>
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<td>Glycine</td>
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<tr>
<td>Alanine</td>
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<tr>
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<td>Methionine</td>
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<td>Isoleucine</td>
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<tr>
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<td>6.64</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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<td>4.53</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.5</td>
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</table>

Total | 81.2 |

*Results are expressed as the percentage of the total weight of the antigen. The results for each amino acid are the means of two determinations.

<table>
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<tr>
<th>Antigen</th>
<th>Serum D520</th>
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<tr>
<td></td>
<td>Unabsorbed</td>
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<tr>
<td>Formalized D225(K88)</td>
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</tr>
<tr>
<td>D282</td>
<td>1,024</td>
</tr>
<tr>
<td>Heated to 100 C D282</td>
<td>2,048</td>
</tr>
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</table>

*The titers are given as the reciprocal values of the highest serum dilutions displaying a macroscopic reaction in tube titration; 0 means negative in a dilution of 1:20.
TABLE 4. K88 rabbit antiserum obtained by four different methods*

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Sera</th>
<th>Purified antigen</th>
<th>Whole cells of D520</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23°</td>
<td>24</td>
</tr>
<tr>
<td>Formalinized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D520</td>
<td>2,560</td>
<td>640</td>
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<td>D214</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heated to 100°C</td>
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</tr>
<tr>
<td>D520</td>
<td>2,560</td>
<td>2,560</td>
<td>1,280</td>
</tr>
<tr>
<td>D214</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Test O8</td>
<td>1,280</td>
<td>2,560</td>
<td>160</td>
</tr>
</tbody>
</table>

- Titters are given as in Table 3. Methods were as follows: 1, suspension of lyophilized antigen in saline given iv, about 50 mg per rabbit; 2, solution of antigen in buffered saline given iv, 15 to 20 mg per rabbit; 3, aluminium hydroxide adsorbed antigen given iv, and subcutaneously, 25 to 30 mg per rabbit; 4, solution of antigen in buffered saline mixed with complete Freund Adjuvant and given subcutaneously, about 20 mg per rabbit.
- Number of rabbit.
- Heating destroys K88 specificity and renders O8 test strain O-agglutinable.

antigen provoked a production of O antibodies, since heated and unheated culture of D282 agglutinated in the sera. The test strain of O antigen 8 also agglutinated to a high titer after heating. This strain has a K antigen and is O-inagglutinable unless it is heated.

DISCUSSION

It was again shown that the ability to form K88-(L) antigen can be transferred from E. coli G7 to other strains of E. coli.

D520, used for the extraction of K88, is particularly suitable for this purpose, since it has no other K antigen and no H antigen.

In addition to bacterial agglutination, the presence or absence of K88 antigen in strains of E. coli can be shown by use of the Ouchterlony technique with whole cultures and K88 antiserum. K88 forms a sharp, characteristic precipitation line near the antigen discs (Fig. 1).

K88 is easily released into a suspending aqueous medium, especially if the bacteria are heated to 60 to 65°C or treated in a Waring Blender.

K88 can be isolated from such extracts of D520 by simple isoelectric reprecipitation and preparative ultracentrifugation (Fig. 2). A product of satisfactory homogeneity was obtained, as shown by gel precipitation with D520 antiserum (Fig. 1), analytical ultracentrifugation (Fig. 3), and moving-boundary electrophoresis (Fig. 4).

K88 is a pure protein of uncommon properties. It has a sedimentation coefficient of $S_{20,w} = 36.7S$, a maximal diameter of at least 0.3 μ, and is practically insoluble at pH 3.5 to 5.5, but readily soluble at pH 6.5 or more. It contains all the common amino acids, in particular, aspartic acid, threonine, and glutamic acid, but no cysteine-cystine (Table 2).

Purified K88 antigen will selectively remove the K88 antibodies from a D520 antiserum (Table 3) and is immunogenic in rabbits (Table 4).

The presence of 1% carbohydrates and of 3 to 4% lipids extractable with methanol-chloroform in the purified K88 antigen (Table 2) could be due to a small contamination with outer-layer cell wall material (19). This assumption is corroborated by the fact that the purified protein provokes O antibody formation in rabbits. The presence of mureine (19), however, is excluded since there are no hexosamines in the material. The UV-absorption spectrum and the phosphorus content of purified K88 antigen exclude the presence of more than trace amounts of nucleic acid.

The fact that purified K88 antigen yields sera with high O titters is not surprising, but supports the observation of other investigators that the actual amount of immunodeterminant material on a given carrier has no direct relation to the potency of the sera obtained. Thus, purified Salmonella flagellin preparations (above 99% protein) were reported to act as extremely good O antigens (G. Nossal, unpublished data). The high O titters obtained with purified K88 antigen might therefore indicate that this protein is a very good carrier.
A 2- to 4-mg amount of crude protein antigen was obtained from a 24-hr culture of D520 on a broth-agar plate (14-cm diameter). Thus, the material must amount to at least 1 to 2% of the bacterial dry weight, which is generally found to be about 200 mg per such a plate.

K88 is the first E. coli K antigen shown to be a protein. The K-specific substances which were previously isolated, serologically identified, and analyzed were all found to be (mostly acidic) polysaccharides (4, 11, 21; G. T. Barry and T. Tsai, Federation Proc., 22:206, 1963; Jann, Ph.D. Thesis, 1965).

K88 is a K antigen of the thermolabile L type as defined by Kauffmann (7). Upon boiling, cultures of E. coli carrying L antigens lose their L agglutinability, their ability to absorb L antibodies, and their L immunogenicity. For K88(L), all this can be explained by the denaturation of the protein at temperatures above 70 C.

D520 is nonmotile; thus, the possibility that K88 is a flagellar antigen can be excluded with great certainty. However, the question of whether it belongs to the group of bacterial appendages known as fimbrae or pili is very intriguing. C. C. Brinton, Jr., and M. J. Stone (Bacteriol. Proc., p. 96, 1961), for instance, have reported that the pili can be removed from E. coli Barm P+ by agitation of a bacterial suspension in a blender, that they can be purified by isoelectric precipitation at pH 4, and that they consist of pure protein. This problem is considered in detail in the following paper on the morphology of K88 antigen.

ACKNOWLEDGMENTS

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LITERATURE CITED