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Enterobacterial Common Antigen

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INTRODUCTION

The O, K, and H antigens of *Enterobacteriaceae* and several other gram-negative bacteria have been serologically recognized since early in this century (93, 205). Their location in the bacterial cell, serological properties, biological effects, and clinical implications have been extensively characterized; more recently their chemical structure, biosynthesis, and genetic determination have been the subject of much research (82, 84, 88, 89, 109, 129, 150, 163, 190). It is, then, rather surprising that another antigenic component, the enterobacterial "common antigen" or "Kunin antigen," was not discovered until 1962 (102) and has so far received rather limited attention. The diagnostic and eventual prophylactic possibilities of an antigen shared by many common pathogens are obvious. This was promptly recognized by Neter and his associates, who have since devoted much of their research to various aspects of the "common antigen" (135-149; 208-229). General interest in it may still be hampered by lack of knowledge of its chemical nature and by apparent confusion about its identity. Recent studies have uncovered new facts that may help to clarify some points of confusion and at the same time define subjects requiring further work. We hope that we can here describe what is hitherto known of the "common antigen" in a way that will stimulate further research.

DISCOVERY AND DEFINITIONS

The enterobacterial common antigen was found by Kunin in the course of studies of urinary tract infections, of *Escherichia coli* types responsible for them, and of the presence of anti-*E. coli* antibodies in the patients (99, 101,

102). He used indirect hemagglutination (as applied for use with enterobacterial [lipo]polysaccharides by Neter et al. [139, 144]) for detection of *E. coli* anti-O antibodies and was led to investigate the extent of serological cross-reactions between the various *E. coli* O serotypes.

When thus screening extracts of 145 *E. coli* strains representing almost all the known O serotypes for their ability to sensitize erythrocytes (RBC) to agglutination not only by homologous but also by heterologous antisera, it was found (102) that, unexpectedly, almost all these extracts were capable of such sensitization when certain heterologous antisera were used for the hemagglutination. The best serum for this hemagglutination was anti-*E. coli* O14, whereas anti-*E. coli* O56 and anti-*E. coli* O144 showed less reaction. The cross-reactive property of the anti-O14 serum could be removed by absorption with extracts of any *E. coli* strain, whereas the absorbed serum retained its capacity to agglutinate RBC coated with the homologous O14 extract. Thus the anti-O14 serum seemed to have antibodies to an antigen common to all *E. coli* strains and a different sort of antibody specific for the O14-type lipopolysaccharide (LPS) not shared by other strains. That most other sera did not contain the first kind of antibodies, namely those reactive with the common antigen, whereas all anti-O14 sera prepared by different immunization schemes did, was quite surprising. It could be accounted for by assuming that the common antigen in most bacteria is present in a haptenic form, whereas in the O14-type strains (and probably in some others as well) it is in an immunogenic form.

This apparent existence of the common antigen in two different forms has caused much confusion in further research. The chemical basis of these two forms has only recently started to be understood (see below, "Immunogenicity"). In both forms, the immunodeterminant part is apparently identical, but may be associated with different carrier structures.

The "immunogenic" form of the common antigen is present in only few bacterial strains, of which *E. coli* O14 is an example. It now seems that in this form the immunodeterminant structure of the common antigen is probably covalently linked to LPS. The "haptenic" form of the common antigen is probably present in all common antigen-positive bacteria, of which most are "non-immunogenic," i.e., do not elicit anti-common antigen antibodies when injected as whole heat-killed bacterial suspensions. This form is characterized by its ability to sensitize RBC for hemagglutination. Our current working hypothesis is that in this haptenic form the immunodeterminant structure is linked to a "carrier" molecule, which is not LPS. The carrier may be mostly responsible for the ability to coat RBC. (The haptenic form of the common antigen may under certain conditions also elicit an anti-common antigen response, and these conditions will be discussed later on in the section on "Immunogenicity".)

This common antigen was found, besides in *E. coli*, in several other members of the family *Enterobacteriaceae* (102). It was not present in other gram-negative bacteria such as *Pseudomonas* and *Brucella* or in any gram-positive organisms, and later studies have confirmed it as a property typical of the *Enterobacteriaceae* (see below, "Distribution of ECA"). We should thus define the enterobacterial common antigen in accordance with the original description by Kunin as a cross-reactive antigen detectable in several species of enteric bacteria by indirect hemagglutination when using anti-*E. coli* O14 sera. Other sera and other detecting methods can be used, but in each case the identity of the antigen detected must be confirmed by comparison with the original definition. We would also like to suggest the term ECA for the enterobacterial common antigen to indicate its restriction to enteric bacteria. So far the abbreviation CA has been generally used, but it is more ambiguous and can be confused with Ca, for cancer.

SEROLOGICAL METHODS

Hemagglutination (HA), Hemolysis, and Hemagglutination Inhibition (HAI)

Fresh human (blood group O) or sheep RBC have been used mostly, but probably other RBC and formalin-preserved cells work as well (22, 139, 216). These are coated by the sensitizing ECA preparations and thus become reactive with anti-ECA sera. This reaction can be visualized as agglutination of the RBC (HA). It can also be seen as lysis of the coated RBC in the presence of complement, but, peculiarly, there are restrictions as to the kind of cells used—human RBC are not lysed in this test (144, 216). Both reactions can in turn be inhibited by free ECA.

The usual sensitizing antigen is a supernatant of cultures grown in broth or synthetic media (61, 102), or suspended in saline or buffer from agar surfaces. The latter are more reactive if heated at 100°C (usually for 1 h)—presumably to release more antigen to the supernatant—before removing the bacteria by centrifugation (216). LPS prepared by the hot phenol-water extraction method or by trichloroacetic acid extraction as the Boivin antigen (109) is also reactive (102), whereas LPS ex-

tracted with the phenol-chloroform-petroleum ether method is not (126). Brodhage (20-22) used urea extracts of bacteria in a similar assay and detected a common antigen—probably ECA, although the exact correlation has not been studied (see also "Other cross-reactivities and related antigens").

The sensitization step in the standard assay takes place at 37°C (for 30 to 60 min), usually with a 2.5% suspension of washed RBC. After excess antigen is washed away, the sensitized cells are suspended to a concentration of approximately 2.5% in phosphate-buffered saline or other buffer. The agglutination proceeds at 37°C for 30 min and is preferably read by the naked eye after a light centrifugation (e.g., 3 min at 1,300 × *g*) (216). Hemolysis is a slightly more sensitive assay than HA (144, 216) when sensitized sheep RBC, guinea pig complement, and rabbit anti-ECA sera are used. It is dependent on the use of correct complement source and sera. A Jerne plaque modification of the hemolysis assay has also been developed (39). Hemolysis of sensitized cells also occurs in vivo in immunized animals (136, 193), a phenomenon that could be important in natural infection.

The classical antiserum for ECA detection is rabbit anti-*E. coli* O14 serum obtained by various immunization schedules using heated (100°C for 1 to 2 h) suspensions of whole bacteria as immunogen. Similar antisera are obtained using certain rough (R) *Shigella* or *E. coli* mutants or a semipurified ECA—"ethanol-soluble fraction"—isolated from any ECA-containing bacteria as immunogens (192) (see "Immunogenicity"). All these sera seem to measure ECA equally well. When used with a heterologous source of ECA, they also seem to be equal in specificity.

When measuring anti-ECA antibodies, most studies have used ECA from *Salmonella* or *E. coli* strains (O14, O111, O7, etc.) for sensitizing the RBC with essentially the same results, and it was expected that any ECA-containing strain would do as well. However, it now seems probable that extracts of the *Proteus rettgeri* strain 6572 used by McCabe et al. (110, 112, 113, 115) have predominantly another antigen (see "Chemical identity of ECA") also capable of sensitizing RBC for agglutination by anti-*E. coli* O14 serum (W. McCabe, personal communication). This other antigen may explain some differences in the findings of McCabe et al. as compared with most other studies. Therefore, it should be determined as soon as possible to what extent this other antigen may be responsible for some reactions ascribed to ECA. HAI, the ability of ECA to inhibit HA or hemolysis, is a good measure of its antigenic (immunodeterminant) part and should be the best way to look for the presence or absence of ECA in unknown materials. The inhibiting capacity is not affected by the ability or inability of ECA to sensitize RBC, to elicit an immune response, or to be precipitated. This inhibitory capacity has been successfully used in following ECA during purification steps (85, 100, 192). Until chemical identification of ECA has progressed further, HAI remains the principal method of quantitating ECA.

Other Serological Methods

Immunoprecipitation would be especially useful for monitoring an antigen during chemical purification and as a final test of purity and identity. It was therefore disappointing that in the initial studies of ECA the results were negative: no precipitation was seen in test tube or agar-gel diffusion (100-102). More recently it has become evident that the failure to detect precipitation is dependent on the antiserum used—some sera contain precipitating antibodies, others do not. The first negative results in precipitation had a more trivial cause: the antisera used were standard sera for routine agglutination tests and contained 50% glycerol. This made them very hygroscopic, and consequently diffusion in agar took place only toward the antiserum well, not away from it (85).

Mayer and Schmidt found two precipitating sera among several anti-R1-type sera tested (126). Both double-diffusion precipitation and immunoelectrophoresis have been used (85, 126, 211). The precipitation method is now a valuable help in following purification of ECA, as first shown by Johns et al. (85). It is obviously necessary to select suitable antisera for this purpose, and negative findings must be interpreted with caution; e.g., a change in the size of the ECA can alter its precipitability while the immunodeterminant remains intact. Specificity of the reaction must be ascertained by HAI.

An immunofluorescence (IF) test for detecting ECA was developed because of clinical interest, in hopes of detecting the antigen in tissue specimens. Using fluorescein-labeled goat anti-rabbit gamma globulin and anti-*E. coli* O14 rabbit serum, Aoki et al. (8) obtained strong fluorescence in smears of all ECA-positive bacteria and weak or no fluorescence with ECA-negative bacteria. The identity of the reactants in IF is based on these cross-reactions only and cannot therefore be considered as certain as recognition of ECA on the basis of HA.

Bacterial agglutination is not a usable method, since anti-ECA antibodies do not ag-

glutinate intact bacteria (102). The reason for this is not known. Intact bacteria seem able to bind anti-ECA (as suggested by IF and by their ability to remove anti-ECA from immune sera [219] or to become opsonized [38]). Anti-ECA antibodies are mostly immunoglobulin M (224) and would therefore be expected to be capable of causing agglutination.

Agglutination of coated latex particles does not take place either. Latex particles adsorb both ECA and LPS from crude bacterial extracts (100, 219), and the presence of both can be demonstrated by the ability of the coated particles to remove the corresponding antibodies from immune sera (37, 146) and to be opsonized for phagocytosis (38). However, the latex particles become agglutinable by anti-LPS sera only (217).

Anti-ECA antibodies obtained by immunizing rabbits with either *E. coli* O14 or various immunogenic preparations of *Salmonella* or *E. coli* O111 were bactericidal (in a heterologous complement-mediated system) to *E. coli* O14 but not to *E. coli* O1, O55, or O111 nor to *Shigella dysenteriae* or *Salmonella* species (38, 101). Accepting the notion that in *E. coli* O14 the ECA immunodeterminant is covalently linked to LPS, these findings can be interpreted to mean that the killing of *E. coli* O14 is caused by a reaction of anti-ECA antibodies with LPS, whereas such a reaction between the same antibodies and ECA (non-LPS linked) does not lead to killing. Unfortunately, we do not know the requirements for antibody + complement-mediated killing, although the topological location of the antigen in the bacterial cell is believed to be important (160). The reaction of ECA with its antibody can activate complement as evidenced by the hemolysis reaction, but whether this reaction takes place on the intact bacteria has not been tested.

Several anti-ECA sera could be shown to opsonize ECA-positive *E. coli* and *Salmonella* but not ECA-negative *Pseudomonas* for phagocytosis by rabbit peritoneal exudate cells; the homologous system *E. coli* O14–anti-*E. coli* O14 (containing both anti-O and anti-ECA antibodies) was only slightly more effective than a heterologous (ECA-dependent) system based solely on anti-ECA antibodies (38). The opsonizing capacity of the antisera could be removed by absorption with ECA-coated RBC (38). In a similar experiment, ECA-coated latex particles were opsonized for phagocytosis by anti-ECA serum but not by normal serum. Free LPS interfered with the reactivity of ECA, a phenomenon that will be discussed in detail in the section on immunogenicity.

CHEMISTRY

In spite of several recent studies on the immunochemical characterization of ECA, its chemical identity is still far from clear. Each group of workers has developed its own extraction and purification procedures, and they often use different strains as starting material. The purified products have not yet been sufficiently compared. Table 1 summarizes the isolation procedures used and gives a summary on the properties and characteristics of the final products.

It seems reasonable to discuss the work of Kunin (100) and of Hammarström et al. (70) first, since both studied, at least predominantly, the immunogenic strain *E. coli* O14:K7. We will then discuss the isolation procedures described by Johns et al. (85), Suzuki et al. (192), and McLaughlin and Domingue (116) and Marx and Petcovici (122), who used nonimmunogenic strains for ECA isolation. Finally, we will describe and discuss in more detail the recent work of D. Männel and H. Mayer (unpublished data) on genetically defined ECA-positive and -negative mutants of *Salmonella*.

Studies on Immunogenic Strains

All LPS preparations, no matter how extracted, obtained from the ECA-immunogenic strains elicit the synthesis of anti-ECA in rabbits, whereas LPS of nonimmunogenic strains does not. This strongly suggests that in the immunogenic strains the ECA immunodeterminant is part of the LPS molecule. In addition, these strains, like all other ECA-positive strains, probably contain non-LPS-linked ECA.

The first report on the isolation and immunochemical characterization of ECA goes back to Kunin (100), who used for ECA extraction the hot phenol-water method (207), which was originally designed for the isolation of O antigen. The aqueous-phase material obtained by this procedure was first dialyzed, then concentrated, and finally precipitated with 10 volumes of ethanol. The redissolved precipitate was subjected to diethylaminoethyl (DEAE)-cellulose chromatography, using a gradient of NaCl for elution.

The individual fractions were tested for the presence of ECA by HAI and for O antigen by both HAI and precipitability in the agar double-diffusion test. The main peaks of ECA or O antigen activity eluted at slightly different NaCl molarities (0.16 versus 0.21 to 0.28, respectively), indicating that both activities are at least partly separable. Similar results were obtained with extracts of *E. coli* O1, a nonde-

fined *E. coli* R mutant, *Salmonella typhosa*, *Shigella flexneri*, and *Proteus* OX19 (100). Several other methods, such as ethanol fractionation, preparative ultracentrifugation at 136,000 × *g*, or chromatography on columns of Sephadex G-100 or G-200, resulted in only incomplete separation of ECA from LPS (100), suggesting that these two antigens are complexed together or are of similar molecular weight.

Detailed chemical analyses were carried out only with the *E. coli* O14 material, because much more ECA-specific material could be obtained from this strain than from any of the others investigated (100). As shown in Table 1, the ECA material, purified by DEAE-cellulose chromatography, contained a small percentage of neutral sugars besides a fairly large amount of amino sugar (GlcN). Neither uronic acids nor 6-deoxyhexoses were detectable. The rather high nitrogen content was not accounted for by protein, which showed large fluctuation among individual preparations (1.7 to 8.4%). From the nature of the principal amino acids identified, i.e., glutamic acid, alanine, glycine, aspartic acid, and (probably) diaminopimelic acid, it was concluded that ECA was most likely of cell wall origin (100). The ECA activity of the purified material was not affected by periodate oxidation or by trypsin digestion. From the elution of ECA into the V_0 fraction of a Sephadex G-100 column, the molecular weight of ECA was assumed to exceed 40,000.

This purified ECA material failed to coat RBC in spite of its good HA-inhibiting capacity, nor did it engender ECA-specific antibodies in rabbits by intravenous immunization. In both these respects it differed from the phenol-water extract used as starting material. In the light of present knowledge, it seems probable that the purified product represented the haptenic, non-LPS-linked form of ECA, which is the minor form of ECA in this strain (*E. coli* O14). The lack of activity in HA is more difficult to explain; it may indicate a loss of the carrier part of ECA.

The immunochemical studies on ECA of *E. coli* O14 reported by Hammarström et al. (70) seem to contradict the above findings in essential points. They also used the hot phenol-water method (207) for ECA extraction from carefully washed *E. coli* O14 bacteria, and again both ECA and O antigen were demonstrated in the aqueous phase. After removal of coextracted RNA by treatment with pancreatic ribonuclease, the residual material was found to be of high molecular weight (in the V_0 fraction in Sepharose 4B column chromatography). Analysis of the material before further separation showed the presence of sugars of the LPS core (see Fig. 7): glucose, galactose, heptose, ketodeoxyoctonic acid (KDO), and some glucosamine beside a large amount of *O*-acetyl. Most of the latter could be removed by treatment with alkali (0.25 N NaOH, for 3 h at 50°C) without affecting the serological ECA specificity. Alkali-treated material was subjected to mild acid hydrolysis (1% acetic acid for 1.5 h at 100°C), which cleaved the ketosidic linkage between KDO and lipid A. After removal of the lipid precipitate, the acid-soluble fraction was passed over Sephadex G-50 with pyridine-acetic acid of pH 5.4 as eluting buffer. A major peak in the molecular weight region of about 2,000 to 3,000 showed inhibition of an ECA HA system in contrast to a number of minor peaks eluted either before or after this major peak. Sugar analysis of the inhibiting fraction showed a ratio of galactose-glucose-heptose of 2:1:5:1. The glucosamine content was low; the nitrogen content, however, was higher than expected. The authors concluded that in *E. coli* O14 the ECA specificity is related to the O14 core region.

The data obtained by Hammarström et al. (70) are in agreement with immunogenic ECA being LPS linked. They show in addition that this linkage is not cleaved by hydrolysis with 1% acetic acid used for splitting the KDO-lipid A linkage (109). Kunin (100) apparently was working with a mixture of the free and the LPS-linked ECA of *E. coli* O14, judging from the reported chromatographic separation of ECA and O14 specificities. A comparison of the elution patterns given by Kunin (100) shows a wide overlap of both specificities in *E. coli* O14 and a clear separation of ECA and O specificities in the nonimmunogenic strains.

Studies on Nonimmunogenic Strains

Salmonella typhosa strain O:901 was selected by Johns et al. (85) for ECA extraction because of its lack of possibly interfering Vi, H, and K antigens. Acetone-killed bacteria were extracted at room temperature with distilled water. The aqueous extract obtained was then mixed with picric acid to 90% saturation, resulting in removal of several protein antigens as followed by agar-gel precipitation with a homologous antiserum. Two volumes of acetone was added to the picric acid supernatant to precipitate residual antigenic material and to remove most of the picric acid. The precipitate was dissolved in bicarbonate buffer, dialyzed, concentrated, passed over a Sephadex G-200 column, and thereby separated into four fractions, which were tested for O antigen and ECA specificity by immunoelectrophoresis and im-

TABLE 1. *Summary of methods used for the isolation of ECA*[a]

Reference and ECA source	Determination																			
	Immunogenicity of heat-killed bacteria	Extraction (solvent and temp [°C])	Purification steps			Purification followed by:	ECA form isolated	Constituents (%)									Biological properties			
			Water phase	Saline-soluble material	Ethanol-soluble material			Hexose	Heptose	Hexosamines	KDO	Protein (Lowry)	Lipids	Nitrogen	Phosphorus	Others	RBC coating	Inhibition capacity[b]	Gel precipitation	Immunogenicity[c]
Kunin (100); *E. coli* O14	+	Phenol-water, 68	Ethanol precipitation; DEAE-cellulose chromatography			HAI	LPS-ECA and) ECA[d]	2.3	NR	12.6 (GlcN)	NR	4.4	0	6.5	0.5		–	++	–[e]	–
Hammerström et al. (70); *E. coli* O14	+	Phenol-water, 68	Sepharose 4B; partial hydrolysis; Sephadex G-50			HAI	LPS-ECA	43		1.1 (GlcN)	18.4	NR	NR	1.9	NR	O-acetyl: 0.4	–	+	–	NR
Johns et al. (85); *S. typhosa* O901	–	Water, 25	Picric acid precipitation; supernate fraction; acetone precipitated; Sephadex G-200; preparative gel electrophoresis			Precipitation	ECA[d]	42	0	1.26 (GlcN)	0	4.45	NR	1.7	0		–	+	+	–
McLaughlin and Domingue (116); *E. coli* O6 and O75	–	Saline, 100		Ethanol fractionation; 85% ethanol-soluble material collected		HA, HAI	ECA	0.2	NR	NR	NR	15–20	1–2	NR	NR	RNA:10–17	+	+	+	+

Marx and Petcovici (122); *S. typhimurium* TV149 (Ra)	–	Ethanol, 60			85% ethanol-soluble material; acetone precipitation; Sephadex G-75	HA, HAI	ECA	3.4 (Glc)	0	4.1 (GlcN)	0	15.0	C_{14}: 0 = 0.6; C_{16}: 0 = 14.8; C_{18}: 0 = 4.9	NR	1.9	Ethanolamine, glycerol: 1.3; acetyl: 4.07	+	++	NR	+
Männel and Mayer (unpublished); *S. montevideo* SH94 (S) and *S. minnesota* (S and R)	–	Phenol-water, 68	PCP extraction; phenol-soluble fraction; DEAE-cellulose chromatography			HA, HAI, precipitation	ECA	Trace	0	20–25 GlcN; 20–25 ManNUA	0	Trace	Fatty acids present, especially C_{16}: 0	+	Trace	Acetyl (total): 13.0; O-acetyl: 1.3	+	++	+	+ (on carrier)

[a] Abbreviations: KDO, 2-keto-3-deoxyoctonate; GlcN, glucosamine; Glc, glucose, ManNUA, mannosaminuronic acid; RNA, ribonucleic acid; PCP, phenol-chloroform-petroleum ether mixture; NR, not reported.
[b] HAI has been measured, but values of different authors are so differently based that only crude quantitation (+ to ++) can be given.
[c] Intravenously in rabbits.
[d] Does not sensitize RBC.
[e] Antiserum used probably did not contain precipitating antibodies.

munoprecipitation. The third fraction contained no O antigen but two other antigens; one reacted only with the homologous anti-*S. typhi* antiserum, and the other reacted with anti-*E. coli* O14 serum. The latter was taken to represent ECA, and was further purified by preparative gel electrophoresis. Sodium chloride extraction of the appropriate gel fractions yielded a material that strongly precipitated with an *E. coli* O14 antiserum. It was homogeneous on the basis of a single precipitation arc and had a high negative charge (fast anodic migration) at pH 8.6. From the rather high mobility in agargel diffusion, its molecular weight was assumed to be far below the 40,000 reported by Kunin (100). Identical materials were demonstrable in similar extracts of *Salmonella minnesota* S, Ra, and Rb chemotypes, but not in the mutants of Rc to Re chemotypes (see Fig. 4; all the *S. minnesota* mutants of chemotypes Rc to Re investigated also contain a *rfe* mutation [see below "Genetic determination"]).

Chemical analyses of the electrophoretically pure material revealed a high percentage of hexose, mostly galactose, but also some glucose (M. A. Johns, Ph.D. thesis, Boston Univ., Boston, Mass., 1973) and a lower percentage of both hexosamines (GlcN) and protein. Phosphorus, heptose, and KDO were completely absent. The isolated material failed to sensitize human RBC; it was, however, active in inhibiting an ECA hemagglutinating system, although a rather high dose (200 μg/ml) of the inhibitor was required. No anti-ECA antibodies were engendered by intravenous immunization of rabbits with the purified product (85; Johns, Ph.D thesis).

Making use of the important discovery of Neter and his group (192) that LPS and ECA are separable by ethanol fractionation, McLaughlin and Domingue (116) obtained a partly purified ECA preparation from *E. coli* O6 and O75 and *Klebsiella pneumoniae*. Cells were harvested with phosphate buffer of pH 7.3, and the resulting suspension was heated for 1 h at 100°C. The supernatant fluid containing both ECA and O-antigenic material was mixed with 95% ethanol to give a final ethanol concentration of 85%. Ethanol-soluble and -insoluble materials were separated by centrifugation. The former contained ECA, whereas most of the O antigen was in the ethanol-insoluble fraction. After removal of the ethanol at room temperature, the resulting residue was dissolved in water. A partial chemical characterization of the ethanol-soluble, non-desalted material showed mainly protein and nucleic acids; some carbohydrate material (about 1% by the phenol-sulfuric acid method) and some chloroform-methanol-soluble lipid were also present (see Table 1). The material was active in HAI as well as in coating RBC for HA by heterologous anti-ECA sera. It also engendered ECA antibodies by intravenous immunization of rabbits. The heat stability of ECA was considered to contradict its being a protein, contrary to the analytical data presented.

In a recent study by Marx and Petcovici (122) heated ethanol was used as extractant of ECA from *Salmonella typhimurium* TV149 (a rough mutant of type Ra; see Fig. 4). A suspension of 200 g of wet bacteria in 2,000 ml of 96% ethanol was heated at 60°C for 20 min. After centrifugation of the cooled suspension, the supernatant was collected, evaporated to near dryness, and then taken up in 40 ml of 85% aqueous ethanol and precipitated with 3 volumes of acetone. The sediment collected by centrifugation was redissolved in 4 ml of water and applied to a Sephadex G-75 column. ECA was found mainly in the V_0 fraction and was recovered in a yield of 5 mg from the 200 g of bacterial wet weight. Purified ECA obtained by this technique was of high molecular weight (excluded from Sepharose 2B), retained its RBC-coating ability, and was immunogenic in rabbits using intravenous immunization. Chemical analyses (see Table 1) revealed mainly protein, glucosamine, and glucose, as well as constituents characteristic of a cephaline-type phosphoglyceride, i.e., glycerol, ethanolamine, phosphorus, and fatty acids (mostly palmitic acid, but also some stearic, myristic, and unidentified fatty acids). The highly acidic character of the isolated product was documented by its ready absorption to various anion exchangers and by its precipitation with lysozyme (the latter as a basic protein is known to precipitate polyanions, e.g., ribonucleic acid [182]). The material, in accordance with an earlier report, lost its RBC-sensitizing ability during incubation with phospholipase A (121). In contrast to the earlier report, phospholipase A was found to also destroy the capacity of ECA to inhibit HA, suggesting that the immunodeterminants were also destroyed. The ECA materials tested in these two studies were admittedly different, yet it is difficult to reconcile the conflicting findings. The action of phospholipase A was accompanied by a release of about half of the fatty acids present, presumably from the phosphoglyceride.

A new extraction and purification method (Fig. 1) for ECA was developed by Männel and Mayer (D. Männel, thesis, Universität Freiburg i.Br., in preparation). It is based on earlier observations showing that phenol-water-extracted LPS usually contains the ECA determinant, whereas a phenol-chloroform-petro-

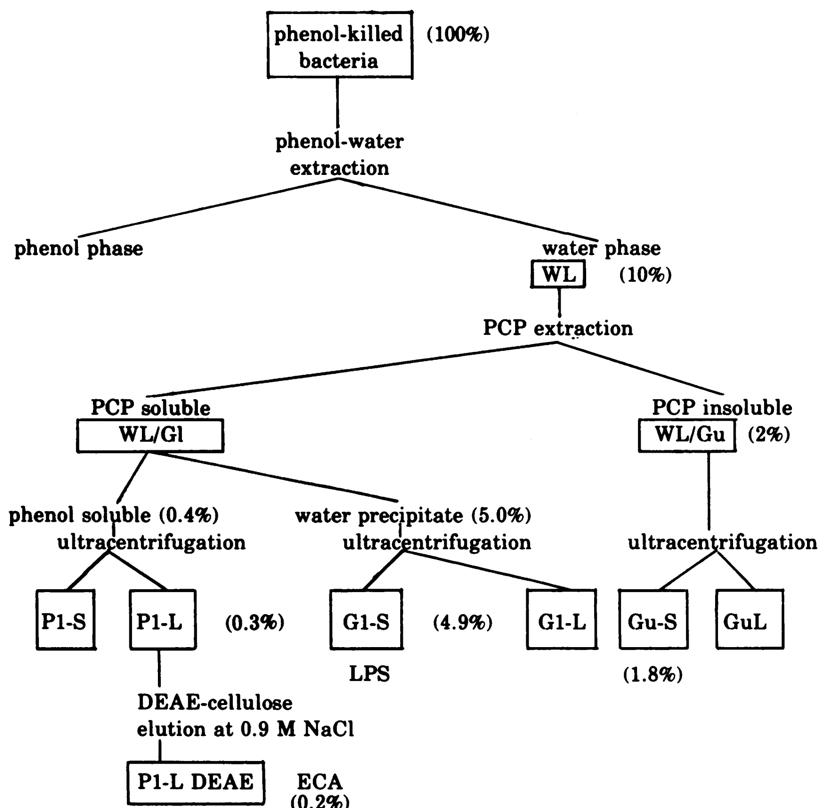


FIG. 1. *Extraction procedure (D. Männel and H. Mayer, to be published) for ECA and ECA-free LPS. ECA is accumulated in fractions P1-S and P1-L; P1-L further purified gives P1-L DEAE. ECA-free LPS is present in G1-S. The relative yields as percentages of dry weight of bacteria (the smooth strain Salmonella montevideo SH94 in this case) are given in parentheses.*

leum ether (PCP)-extracted LPS (51) does not (126). Subsequent extraction with phenol-water of previously PCP-extracted R mutants gives an aqueous-phase material that is considerably enriched for ECA (126). The newly devised method uses the same two extractants but in a reverse order, and this makes it possible to extract ECA from both S and R forms (PCP does not extract LPS from S-form bacteria [51]). C. Galanos (personal communication) obtained highly purified LPS of *Salmonella* S forms by treating the aqueous phase of phenol-water extracts with the PCP mixture. ECA and LPS are both soluble in this mixture. However, on addition of some drops of water to the phenol phase (obtained after removal of the volatile chloroform and petroleum ether), only LPS precipitates, whereas ECA remains in solution. Extensive dialysis and a final lyophilization step results in the so-called P1 fraction (see Fig. 1), which can be separated by ultracentrifugation to give a sediment (P1-S) and a supernatant fraction (P1-L).

Although both fractions showed high ECA activity (HAI), only the P1-L fraction was used for further purification. P1-L material was dissolved by ultrasonic treatment in a small amount of 0.5 M ammonium acetate-methanol buffer (31) and was then applied to a DEAE-cellulose column previously equilibrated with the same buffer. Stepwise elution with ammonium acetate-methanol of increasing molarity (0.5, 1.0, and 1.6) eluted ECA in the middle fraction. Rechromatography of this fraction with 0.9 M buffer yielded ECA as a symmetrical peak as assayed by differential refractometry. Investigation of individual fractions by HAI and gel precipitation showed that the peak eluted with 0.9 M buffer contained the entire ECA specificity. Corresponding fractions were combined, dialyzed, and electrodialyzed (50) and were then used for chemical analysis.

The P1-L DEAE-purified ECA formed a precipitate in immunoelectrophoresis in agar gels at pH 8.6, usually in the form of a bird-wing pattern similar to that observed with acidic capsular polysaccharides of *E. coli* (152). Mild alkali treatment resulted in a single precipita-

tion arc, thus indicating that ester linkages might have been cross-linking individual chains of ECA. The alkali-treated, more homogeneous P1-L DEAE fraction was used for determining the sedimentation coefficient. It showed a single peak in analytical ultracentrifugation; the sedimentation coefficient of 0.5S suggests a rather small molecular weight, far below 10,000.

Chemical characterization of this purified ECA material is being continued (D. Männel and H. Mayer, manuscript in preparation). Thus far, it has been shown that as the main component this material contains a heteropolymer composed of two amino sugars, D-glucosamine and D-mannosaminuronic acid (ManNUA), which are partly esterified by palmitic acid. After hydrolysis with hydrochloric acid, the ManNUA is present almost completely in the lactone form (124), which is easily demonstrated by high-voltage electrophoresis (Fig. 2 and 6). Its unequivocal identification was achieved by reduction of the esterified material with $NaBH_4$, resulting in the formation of mannosamine. Its D-configuration was proven by phosphorylation of the newly formed mannosamine with hexokinase-adenosine 5'-triphosphate (124).

The importance of ManNUA for the antigenicity of ECA was indicated by complete disappearance of the precipitation line (Männel and Mayer, unpublished data) upon esterification of its carboxylic groups with diazomethane. Subsequent saponification of the esterified ECA restored its precipitability. Also, reduction of the purified ECA material changed its precipitation. An independent line of proof of ManNUA being part of ECA is provided by mutants which, because of genetic blocks, lack ECA: they all also lack ManNUA (see "Genetic determination of ECA"). Third, ManNUA-containing polymers are found in ECA-positive enterobacterial strains of different genera. Figure 2 shows an electrophoretic separation of hydrolysates of semipurified ECA fractions from different strains.

The palmitic acid is apparently not part of the immunodeterminant of ECA, but is important for its RBC-coating ability: the removal of the palmitic acid by alkali treatment does not affect the ability of the material to inhibit HA, but completely abolishes the sensitizing ability. This observation is analogous to the demonstration by Hämmerling and Westphal (72) that lipid-free polysaccharides—with the exception of several acidic capsular polysaccharides of high molecular weight (67, 84)—usually are not attached to the surface of RBC, whereas their artificially prepared O-stearoyl derivatives are.

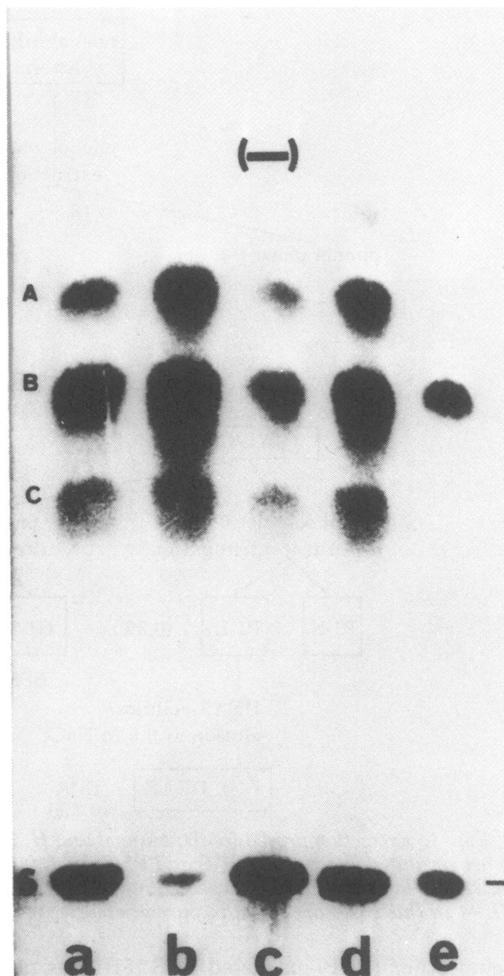


FIG. 2. *Electrophoretic separation of hydrolysates (N HCl, 4 h, 100°C) of semipurified ECA-containing fractions (pH 2.8, 50 V/cm, 1 h, $AgNO_3$/NaOH). (A) ManNU (lactone of mannosaminuronic acid); B, glucosamine; C, oligosaccharide of A and B. (a) Citrobacter 1658; (b) Salmonella arizonae F628 (O9a⁻, O9c⁻); (c) S. minnesota R1; (d) E. coli F870 (K-12); (e) standards of glucosamine and glucose.*

Other components, reported previously as constituents of ECA, like protein, ribonucleic acid, or neutral sugars (see Table 1), are present in this material at most in minimal amounts. It is not certain, however, that all constituents of this ECA material have been discovered.

Chemical Identity of ECA

As a whole, the data summarized in Table 1 vary widely, making it difficult to draw conclusions as to the chemical identity of ECA.

Most workers have found ECA to be highly negatively charged. Its antigenicity is not de-

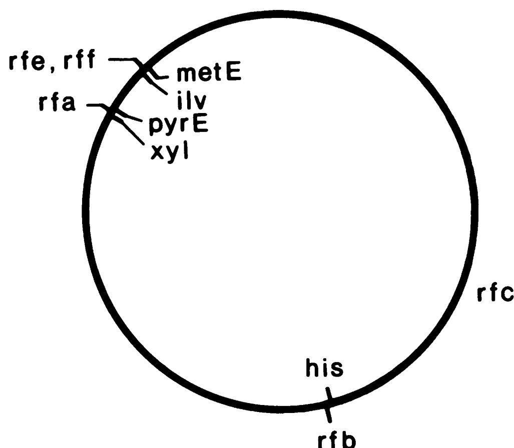


FIG. 3. *Simplified chromosome map of Salmonella showing the positions of rf. genes (190; P. H. Mäkelä, G. Schmidt, H. Mayer, H. Nikaido, H. Y. Whang, and E. Neter, J. Bacteriol., in press). rfa, rfb, rfc, and rfe genes participate in the synthesis of LPS, rff, and rfe, and probably some rfb genes participate in the synthesis of ECA.*

stroyed by heat, even 120°C (216), or by trypsin or Pronase treatment (219), suggesting that it is not a protein; however, protein constituents of the outer membrane of enteric bacteria are extremely resistant to such treatments (e.g., references 16, 77, 105, 150, 165). Since ECA activity is also resistant to periodate oxidation (100, 121) and is present in mutants that are unable to synthesize uridine 5'-diphosphate (UDP)-glucose, UDP-galactose, or guanosine 5'-diphosphate (GDP)-mannose (see "Genetic determination of ECA"), ECA is probably not a carbohydrate composed of the usual common hexoses. Acid hydrolysis (0.1 N HCl) destroys ECA only slowly compared with LPS (100). A *Pseudomonas* "factor," probably an enzyme, which unfortunately has not been further characterized, destroyed ECA as measured by HAI but did not affect the LPS-linked ECA (219, 221).

The data of Männel and Mayer described above suggest that ECA is a heteropolymer composed predominantly of *N*-acetylated amino sugars, the ManNUA providing the negative charge and being part of the immunodeterminant structure. The strongest evidence for this view is the lack of ManNUA in mutants lacking ECA and its presence in various strains having ECA. A partial esterification by palmitic acid adds hydrophobic groups to the otherwise hydrophilic polymer. Such amphiphilic polymers are expected to be surface active (75, 231), and ECA can easily attach to all kinds of surfaces (RBC, lymphocytes, and latex particles as demonstrated by the acquisition of serological ECA specificity). Alkali treatment destroys the sensitizing capacity of ECA in crude extracts (58, 126) as well as in the P1-L DEAE-purified preparation, from which it removes palmitic acid. In contrast, the ability of LPS to sensitize RBC for agglutination by anti-O antibodies is enhanced by and often requires this alkali treatment (145). Some agents that inhibit or destroy the capacity of LPS to sensitize RBC for agglutination with anti-O antibodies have much less effect on the sensitizing capacity of ECA, e.g., normal rabbit serum and polymyxin B (60, 143, 227). Treatment with phospholipase A (but not C or D) destroyed the sensitizing capacity of ECA (121). ECA purified by certain methods (DEAE chromatography of phenol-water extracts [100], Sephadex G-200 chromatography of picric acid-soluble water extracts [85]), although active in HAI or precipitation, does not sensitize and may have lost the hydrophobic part of the molecule.

The proposed structure could also well account for the apparent presence of ECA as aggregates of different sizes in crude bacterial extracts as well as in purified preparations. These aggregates could be micelles of ECA and also co-micelles with other amphiphilic compounds, e.g., LPS. The possible importance of aggregate size will be discussed below under "Immunogenicity." However, how can this structure be reconciled with the results of chemical analysis of the other ECA preparations?

One obvious possibility is that what is called ECA is in actuality not one antigen but rather several (at least two) independent immunologically different antigens that have the common property of being shared by several species of enteric bacteria. Another possibility may be the relative impurity of most ECA preparations. Some discrepancies can be accounted for by association of the ECA immunodeterminant groups with different carrier structures in the various preparations.

A heteropolymer built mostly of amino sugars could easily be overlooked, especially when present in rather small amounts: the usual colorimetric assays for carbohydrates (orcinol or phenol-sulfuric acid, etc.) do not measure amino sugars, nor do aminouronic acids react with carbazole-sulfuric acid (154), which is normally used for quantitation of unsubstituted uronic acids (35). Glucosamine has been found as a constituent of ECA by nearly all investigators (Table 1), although to various extents. High values were reported by Kunin (100) and by Marx and Petcovici (122). The latter authors have kindly provided us with a

sample of their purified ECA preparations, which we could show to contain appreciable amounts of ManNUA in addition to glucosamine (122).

We also studied a sample of the material isolated by Johns et al. (85) in which, however, we found no or only trace amounts of either amino sugar. Reciprocally, McCabe and Johns (personal communication) compared the P1-L DEAE material of Männel and Mayer prepared from *Salmonella montevideo* SH 94 with their ECA in immune precipitation with *E. coli* O14 antiserum. This comparison revealed that the two ECA preparations are serologically different. Whereas the Johns et al. preparation gave one sharp precipitation band with the anti-*E. coli* O14 serum in double diffusion in agar, the P1-L DEAE material gave two bands, only one of which showed a reaction of identity with the Johns et al. material. The other one was completely separate, closer to the antigen well. In immunoelectrophoresis the same picture was retained, the Johns et al. material giving one sharp band with fast movement to the anode and the P1-L DEAE material giving two bands, both less sharp, one in the same position as the band of the Johns et al. preparation and the other closer to the antigen well. A further difference between these two preparations is less activity in HA and HAI of the Johns et al. material. Obviously, the two preparations are different both serologically and chemically. The genetic evidence speaks for the ManNUA polymer being ECA. For future work it will be necessary to establish the identity of both antigens.

GENETIC DETERMINATION OF ECA

Mutations blocking the biosynthesis of a component of ECA would be expected to block its synthesis and result in strains lacking ECA. Conversely, determining the ECA content of known mutants might help to deduce the composition of ECA. Unfortunately, no way of selecting ECA-negative mutants is known, and all such mutants we now know were either selected indirectly or found accidentally.

Mutants defective in the synthesis of UDP-glucose, UDP-galactose, and GDP-mannose (*pmi*), which interfere with the synthesis of LPS, were readily available in *Salmonella* and *E. coli* (134, 158, 190). They were studied for the presence of ECA (121; our unpublished data). They all were positive, suggesting that the corresponding hexoses are not a part of ECA. If ManNUA is a constituent of ECA, the presence of ECA in *pmi* mutants indicates that ManNUA is not synthesized via the GDP-mannose pathway. This agrees with the demonstrated mode of biosynthesis of ManNUA via glucosamine nucleotides (see below, "Other cross-reactivities and related antigens").

When the extensively studied series of rough (R) mutants of *S. minnesota* (108, 109, 173, 175) were tested for the presence of ECA, a suggestive pattern emerged (85, 120): the R mutants with the most defective LPS were devoid of ECA, whereas the smooth parent strain and the R mutants with more complete LPS core structures were ECA positive. This indicated a relationship between the synthesis of ECA and that of LPS and suggested even structural similarities (85). However, at that time it was not possible to propose a coherent hypothesis because there were no reliable data on the chemical composition of ECA. The finding that another type of R mutant, those with a complete LPS core but lacking O-specific side chains because of deletion of a long chromosomal segment in the *his-rfb* region, were also ECA negative (P. H. Mäkelä, G. Schmidt, H. Mayer, H. Nikaido, H. Y. Whang, and E. Neter, J. Bacteriol., in press) caused further difficulties in interpretation.

A genetic study of these and other mutants has now established a connection between the biosynthesis of ECA and LPS. First, *rfe* genes, close to *ilv* (Fig. 3) on the genetic map, are required for the synthesis of ECA as well as for the synthesis of the LPS side chains of some chemotypes. Second, *rff* genes, located very close to *rfe*, are required for ECA synthesis but differ from *rfe* by not participating in LPS synthesis. Third, some so-far undefined part of the *rfb* gene cluster also participates in the synthesis or regulation of synthesis of ECA. Furthermore, the linkage of ECA to LPS in immunogenic strains requires the function of *rfa* genes to produce the correct complete LPS core and to transfer the ECA immunodeterminant to it.

The *rfe* Genes

A genetic analysis of the *S. minnesota* R mutant series (mR3, mR5, mR8, etc.) mentioned above revealed that all of the more defective mutants (LPS chemotypes Rc, Rd_1 or Rd_2, and Re; Fig. 4) that were also ECA negative in fact had two separate *rf.* mutations, both of which affected LPS synthesis (87). One mutation in each strain was of the known *rfa* type, i.e., affecting a specific transferase of a core constituent (sugar or phosphate) and thereby responsible for the LPS chemotype of each mutant (190). The other mutation, however, was of the less well-understood *rfe* type, which prevents the synthesis of the O side-chain polysaccharide in certain *Salmonella* groups (118): *rfe*

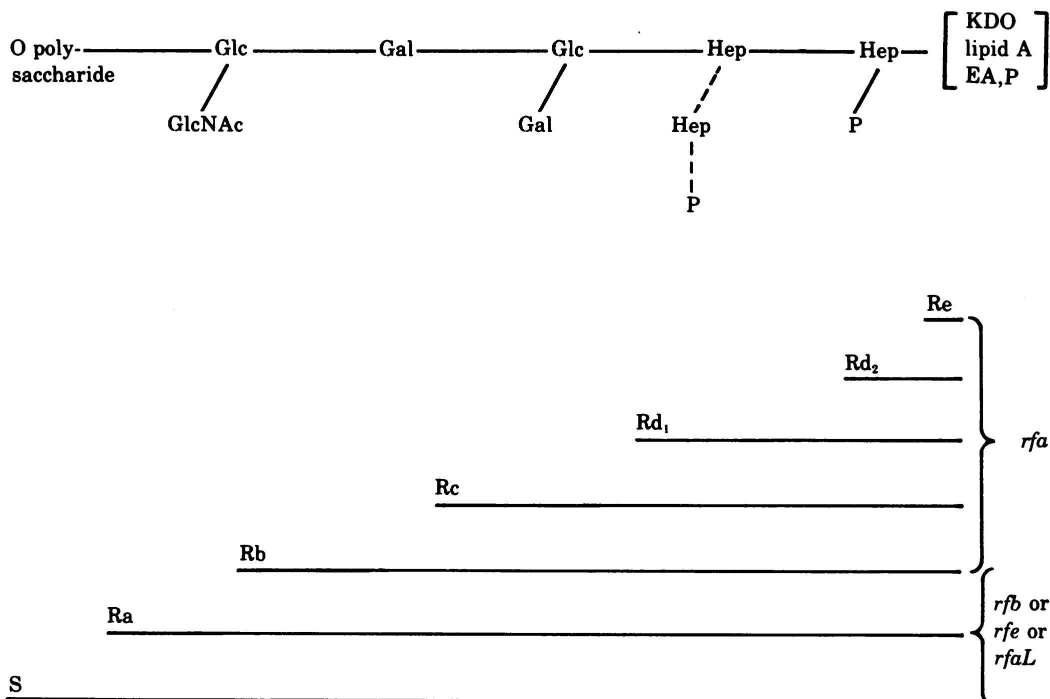


FIG. 4. *Schematic structure of the LPS in smooth (S) and rough (R) strains of Salmonella, the R chemotypes Ra to Re, and the rf. mutations leading to them (84, 109, 190). EA, ethanolamine; Gal, D-galactose; Glc, D-glucose; GlcNAc, N-acetylglucosamine; Hep, heptose, KDO, ketodeoxyoctonic acid; P, phosphate.*

mutants have a complete LPS core of chemotype Ra. Operationally, *rfe* mutants can be distinguished from *rfa*$^-$ mutants by the location of the affected gene (Fig. 3): the *rfe* genes are close to *ilv* at min 122, and the *rfa* genes are close to *pyrE* at min 116.

It turned out that the lack of ECA in the *S. minnesota* R strains was associated with the *rfe* type of mutation (120): all the single *rfa* mutant derivatives of chemotypes Re, Rd_2, Rd_1, and Rc were ECA positive, contrary to the *rfe*$^-$ derivatives, which all were ECA negative. A clear implication of this finding was that the LPS core could not be part of ECA, contrary to previous hypotheses (70, 85).

It was less clear what the requirement of an intact *rfe* function for ECA synthesis meant, because the function of *rfe* genes is still unknown. However, more proof of the role of *rfe* genes in ECA synthesis has been accumulating. *rfe* mutants had been originally found as R mutants in *S. montevideo* (O-6,7 of group C_1) as well as in *S. minnesota* (O-21 of group L) (118). All these *rfe* mutants were devoid of ECA or contained it in minimal amounts only (119, 120). These trace amounts could be explained by assuming that the *rfe* mutation in question had not completely inactivated the *rfe* gene function and that the remaining function was detectable as "leakiness" of the mutation. Such leaky mutations are very common among other genes of LPS synthesis (103, 158, 190).

In *Salmonella* of O group B (O-4,12), *rfe* mutations were not known, and it was not likely that a comparable *rfe* function would be required for the synthesis of the O-4,12-type polysaccharide. Hybrid strains derived from *S. typhimurium* O group B by introducing into them by conjugation a mutant *rfe* (C-*rfe*$^-$) gene from *S. montevideo* were smooth with LPS of type O-4,12 as determined by the group B *rfb* and *rfc* genes (119). These hybrids were nevertheless ECA negative, indicating that there are in *S. typhimurium* genes allelic to *rfe* of group C and that these are also necessary for its ECA synthesis. This hypothesis was tested by further genetic manipulations. The C-*rfe*$^-$ genes of the ECA-negative hybrid were replaced in further crosses by the C-*rfe*$^+$ genes from *S. montevideo* or by the corresponding (B-*rfe*$^+$) region from *S. typhimurium* (119); in both cases the recombinants had become ECA positive.

A further study of such hybrids, obtained by conjugation or transduction and containing various combinations of *rfe* and *rfb* plus *rfc* from groups B and C_1, either functional or mu-

tated, has made it clear that the *rfe* genes of either group B or group C are required for ECA synthesis (118, 119; P. H. Mäkelä, unpublished observations). They are not necessary for the synthesis of the O-4,12 polysaccharide but participate in the synthesis of the O-6,7 LPS of group C_1. In all these cases the *rfe* mutants were obtained by selecting for R mutants in strains having the rfb^+ genes of group C. Thus it could be concluded that all *rfe* mutations that impair the synthesis of O-6,7-type polysaccharide also impair the production of ECA. At that time it was not clear, however, whether the reverse was true, i.e., whether all mutations in group C that prevent the production of ECA would also affect LPS; no method was available for a direct selection of ECA-negative mutants.

To find out whether *rfe* genes are also present in *E. coli*, rfe^- genes were transferred from *S. montevideo* or *S. typhimurium* into *E. coli* strains (G. Schmidt, H. Mayer and, P. H. Mäkelä, J. Bacteriol., in press). These recombinants were all ECA negative and rough, with the complete R core. This suggested that the function of *rfe* genes is required for the production of ECA and for the synthesis of the *E. coli* O9, O8, and O100 polysaccharide in much the same way as it is needed in group C *Salmonella*. When R mutants with the complete LPS core were selected in a strain of *E. coli* O8:K27$^-$, some of them were also ECA negative and *ilv* linked (P1 transduction), and therefore probably of *rfe* type.

Some F′ episomes of *E. coli* K-12 carry the *ilv* genes and probably also the nearby *rfe* genes. When *E. coli* K-12 strains with the F′ factors F14 or F133 were used as genetic donors to several ilv^- ECA-negative *rfe* (or *rff*; see below), mutants of *S. typhimurium*, ilv^+ recombinants were ECA positive, indicating that F14 and F133 carry *rfe* (*rff*) genes that are functionally homologous to *Salmonella rfe* (G. Schmidt et al., J. Bacteriol., in press).

Role of the *rfb* Region

A series of R mutants with various lengths of their chromosome in the *rfb-his* region deleted has been isolated and characterized in *S. typhimurium* (151). By analyzing several of the enzyme products of the *rfb* genes, it was possible to deduce how far the deletion in each mutant strain extended into the *rfb* cluster. When this series was tested for the presence of ECA (Mäkelä et al., in press), a clear pattern emerged: the strains with the longer deletions were ECA negative, and those with shorter deletions were

rfb genes
his operon
MAN RHA ABE MAN
2 2 Y 1 3 1 2 3 3 2
+
+
+
+
—
—
— —
— —

FIG. 5. *Presence (+) or absence (−) of ECA in deletion mutants of the his-rfb region of Salmonella typhimurium (151; Mäkelä et al., J. Bacteriol., in press). MAN 2, etc., refer to enzymes determined by the rfb genes (MAN 2 = phosphomannomutase; MAN 3 = GDP-mannose pyrophosphorylase; ABE 3 = "CDP-abequose synthetase"; ABE 2 = CDP-glucose oxidoreductase; ABE 1 = CDP-glucose pyrophosphorylase; RHA-3 "thymidine diphosphate [TDP]-rhamnose synthetase"; RHA 1 = TDP-glucose pyrophosphorylase; RHA 2 = TDP-glucose oxidoreductase; Y = unidentified, probably regulatory, function concerning another rfb gene).*

ECA positive (Fig. 5). This finding suggested the possibility of defining at least one of the enzymes required for ECA biosynthesis. When, however, some of these long-deletion strains were used in genetic crosses, in which the deleted *rfb* region together with *his* was replaced by its wild-type allele (as evidenced by the production of a smooth O-4,12-type LPS), these recombinants were still ECA negative.

It was therefore suspected that their ECA-negative phenotype was due to a second mutation outside the *rfb* cluster. A likely possibility was an *rfe* type of mutation. This was tested by first isolating ilv^- mutants of these deletion strains and then crossing them with an ECA-positive *S. typhimurium* donor. Many ilv^+ and therefore probably also rfe^+ recombinants were tested and found to be still ECA negative. However, recombinants in which both the *rfe* and *rfb* chromosomal regions came from an ECA-positive *S. typhimurium* donor were ECA positive (P. H. Mäkelä, G. Schmidt, H. Mayer, H. Nikaido, H. Y. Whang, and E. Neter, J. Bacteriol., in press). The inevitable conclusion was that the long-deletion strains contained mutations in (at least) two separate genes, one in the *his-rfb* region and the other close to *ilv*, both required for the synthesis of ECA.

That the *rfb* region of *S. typhimurium* was playing a role in the production of ECA had been suggested by the absence of ECA in hybrids between *S. typhimurium* and *S. montevideo* in which the group B *rfb* region of the former was replaced by the group C *rfb* region of the latter (119). This also suggested a difference between *Salmonella* groups B and C in this respect: only the group B *rfb* region furnished the function required for ECA synthesis. When deletions of the *his-rfb* region were studied in group C (*S. montevideo*), they all were ECA positive, supporting the notion that C-*rfb* does not participate in ECA synthesis (Mäkelä et al., in press).

The mutant and hybrid strains described above, which were missing a function of the group B *rfb* region required for the production of ECA, were, on closer analysis, not completely devoid of ECA. Instead, trace amounts of ECA could be found in each case in strains that did not have additional mutations affecting ECA (119). This trace reaction is probably true ECA, since it is detected by HAI as well as by the ability of bacterial extracts (especially after concentration) to sensitize RBC for HA. Also, some ManNUA-containing material can be isolated from these strains (results to be published). It cannot be caused by conventional leakiness of a mutation, since it is found in strains with large chromosomal deletions. It could, however, result from a situation in which another enzyme, specified by a separate gene, was providing the same function, although in a less efficient form; examples of this are known for some rfb^--determined enzymes (134, 151). Another possibility is that the role of B-*rfb* genes in ECA synthesis is regulatory.

Another clue to the function of the *rfb* genes may be provided by the phenotype of the *rfb* deletion mutants. Many of these mutants are slightly more sensitive to a detergent, deoxycholate, than are normal strains. When their derivatives, which contained only the *rfb* deletion mutation, were prepared, they turned out to have more dramatic phenotypic defects. Such strains grew poorly on ordinary media and were sensitive not only to deoxycholate but also to sodium dodecyl sulfate at concentrations to which wild-type *Salmonella* are completely resistant. These changes in detergent sensitivity suggest a defect in the organization of the cell wall, probably the outer membrane, of which LPS is a part. Generalized defects of the outer membrane have been described in deep LPS mutants (those with a Re type core; Fig. 4): the protein content of their outer membrane is reduced and they are highly sensitive to deoxycholate and many other toxic agents (3, 170, 171, 185, 230).

Mutants of a New, "*rff*," Type

A mutant strain of *S. minnesota*, smooth but ECA negative, was accidentally found, and showed immediately that not all mutations preventing the synthesis of ECA also affect LPS (52). The mutation in this strain was closely linked to *ilv*, like *rfe*. Whether this locus should be called *rfe* is disputable: it shares two properties (map position, ECA-negative mutant phenotype) with *rfe* but differs in a third, participation in LPS synthesis. We propose to call it *rff* until it is further characterized. It seems likely that the function and mode of action of the genes of the *rfe* cluster will soon be clarified, and this problem can then be solved.

More mutations of the above-mentioned "*rff*" type have been found recently (Mäkelä et al., in press). The *ilv*-linked mutation found in many *his-rfb*-deletion strains seems to be of this type. When the *his-rfb* region of such strains is replaced by the wild-type allele of either group B or even group C_1, the recombinants are smooth (O-4,12 or O-6,7) but ECA negative. The *ilv*-linked mutation therefore behaves like *rff*: it prevents the biosynthesis of ECA without interfering with the synthesis of the O polysaccharide.

The *rff* mutation in the *his-rfb*-deletion strains alleviates the phenotypic effect of the

deletion on detergent sensitivity of the bacteria. The *rff*$^-$ *rfb*$^-$-deletion mutants are less sensitive to deoxycholate than are the *rfb*-deletion mutants, and single *rff*$^-$ mutant bacteria derived from them are as resistant as wild-type strains. New *rff* mutations could be easily selected from *his-rfb*-deletion strains by selecting for resistance to sodium dodecyl sulfate. How the *rff* mutations counteract the pleiotropic effects of the *rfb* deletion has not been clarified yet, and a closer study of the outer membrane of these various mutant types is needed.

Genetics of ECA in Immunogenic Strains

For immunogenicity, the ECA immunodeterminant must be linked to LPS. As described in the section on immunogenicity, this requires a certain type of LPS, essentially the complete LPS core of unusual type present only in a few enterobacterial strains. A *rfa* mutant of such a strain (*E. coli* O14) was found to be ECA positive but nonimmunogenic, presumably because ECA could not be linked to an incomplete core (123).

The gene *rfaL* codes for a translocase, which is required for the attachment of O side chains to LPS core (190). *rfaL* mutants of immunogenic strains of *E. coli* and *Shigella* also have ECA but are not immunogenic, suggesting that the *rfaL* function is required for the translocation of ECA to LPS (174).

Genes required for the synthesis of the ECA immunodeterminant are also expected to be required for immunogenicity. This has been tested for *rfe*: *rfe* mutants of an immunogenic *E. coli* strain had no detectable ECA and did not elicit the production of anti-ECA (G. Schmidt et al., in press).

Conclusions

What we now know of the role of each of the genes of ECA and/or LPS synthesis can be described as summarized in Table 2. The table contains two main columns, one for the *Salmonella* O group B, as exemplified by *S. typhimurium*, and the other for several other *Salmonella* and *E. coli* serogroups. These differ in the role of *rfe* genes, required for the synthesis of O polysaccharide in most O groups but not in *S. typhimurium*. They also differ in the role of *rfb* genes, which apparently participate in ECA synthesis only in *S. typhimurium*.

In all strains so far examined, *rfe* and *rff* genes are required for the synthesis of ECA. The immediate products of any of these genes have unfortunately not yet been isolated and identified, and consequently only hypotheses can be presented of their mode of function. The *rfe* genes should concern a step common to the synthesis of ECA as well as to T1 and O side chains of so many different O groups that common components to all are not known. The hypothesis best fitting the existing data is that *rfe* genes are involved in the synthesis or modification of a carrier molecule used in the assembly of a number of polymers, such as ECA, T1, and several types of O side chains. This carrier would have to be different from the "ACL" carrier lipid in the assembly of O side chains of group B *Salmonellae*, since these do not require the *rfe* function. Some evidence for a different mode of synthesis for the O side chain of *S. montevideo*, O group C_1, has indeed been presented (54). The *rff* genes not participating in LPS synthesis could be involved in the synthesis and assembly of such ECA-specific compounds as ManNUA (at least two enzymes re-

TABLE 2. *ECA and LPS characteristics of mutants in different rf. genes*

Reference	Mutation	*Salmonella montevideo* of group C;[a] *Salmonella minnesota* of group L; *Escherichia coli* O8, O9, O100			*Salmonella typhimurium* of group B		
		ECA	O hapten[b]	LPS[c]	ECA	O hapten	LPS
	None	+	(+)	HS	+	(+)	S
119, 120	*rfb*	+	−	R, complete core	+	−	Ra
A[d]	*rfb* long deletion	+[e]	−	R, complete core	Trace	−	Ra
119, 120, B[d]	*rfe*	−	−	R, complete core	−	(+)	S
A	*rff*	−	(+)	S	−	(+)	S
119, 120, 123, B	*rfaL* to *rfaE*	+	+	R, complete or incomplete core	+	+	Ra to Re

[a] Most but not all points tested for each species/serotype.

[b] O-specific polysaccharide detected as hapten if not transferred to the LPS core; small amounts present, even in smooth forms as intermediates of biosynthesis.

[c] See Fig. 4 for different R structures in *rf.* mutants.

[d] A, P. H. Mäkelä, G. Schmidt, H. Mayer, H. Nikaido, H. Y. Whang, and E. Neter, J. Bacteriol., in press. B, G. Schmidt, H. Mayer, and P. H. Mäkelä, J. Bacteriol., in press.

[e] Several *his-rfb*-deletion strains tested, but length of deletion was not known.

quired for its synthesis; see "Other cross reactivities and related antigens").

ECA, then, behaves in many ways like both the O and T1 polysaccharides (190). All these antigens (ECA, O, and T1) are linked to LPS, provided the core is complete and of the correct type and the product of the *rfaL* gene (apparently part of translocase) is present. If they are not linked to LPS, they stay as "hapten," linked to the carrier molecule on which they were synthesized. They all (in the case of O polysaccharide, this applies to certain O groups only) require the function of the *rfe* gene, which may be providing the correct form of the carrier. In addition, there are genes involved in the synthesis of structures specific for each one of them: *rff* (and possible other types, not yet found) for ECA, *rfb* for O, and *rft* for the T polysaccharide.

The ECA-negative mutants have provided the best opportunity so far to test the chemical identity of ECA. The presence or absence of the ManNUA-containing material has been tested in all the three mutant classes (*rfe*, *rff*, and *rfb* deletion) found ECA negative by serological HA and HAI tests (D. Männel, H. Mayer, and P. H. Mäkelä, manuscript in preparation). In each case a complete correlation was found between lack of ManNUA and the ECA-negative phenotype. Figure 6 shows the lack of ManNUA (lactone of ManNUA) in all three ECA-negative mutants of *S. minnesota* studied (R5 and R8 = rfe^- mutants; SH 3786 = a rff^- mutant) and its presence in a *S. minnesota* wild-type strain (S1114) as well as in $ilv^+rfe^+rff^+$ recombinants of the three ECA-negative mutants. Their P1-L fractions (preparation as in Fig. 1) were also compared serologically (Table 3). Similar results were obtained with ECA-negative mutants of *S. typhimurium* and *S. montevideo*, and they are in our opinion a very strong proof of the identity of ECA with the ManNU/GlcN polymer.

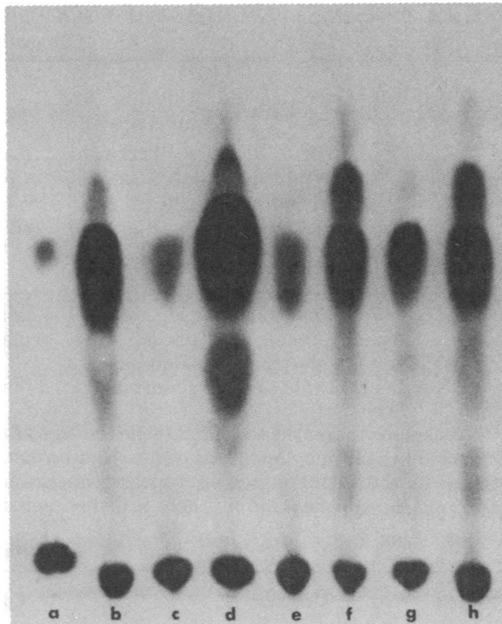


FIG. 6. *Electrophoretic separation of hydrolysates (4 N HCl for 2 h at 100°C) of P1-L fractions (250 μg) from Salmonella minnesota strains (120; D. Männel, H. Mayer, and P. H. Makela, manuscript in preparation) compared with glucosamine and glucose standards (a). Electrophoretic conditions and staining as in Fig. 2. (b) ilv^+ rfe^+ recombinant of R5, ECA positive; (c) R mutant R5 (rfa^- rfe^-), ECA negative; (d) ilv^+ rfe^+ recombinant of R8, ECA positive; (e) R mutant R8 (rfa^- rfe^-), ECA negative; (f) ilv^+ rff^+ recombinant of SH3786, ECA positive; (g) SH3786, a rff^- mutant, ECA negative; (h) parent strain S1114, smooth, ECA positive.*

IMMUNOGENICITY

Immunogenic Strains

Anti-ECA was originally (102) found in the sera of rabbits that had been immunized with a few *E. coli* serotypes (O14, O56, O124, O144), whereas numerous other *E. coli* serotypes were apparently unable to immunize the rabbits to ECA. When whole killed bacteria are used as immunogens, very few strains (although ECA positive by various serological tests) are able to elicit the production of anti-ECA antibodies (Table 4). Although it was later found possible to prepare immunogenic ECA also from nonimmunogenic strains as a fraction soluble in 85% ethanol (192), the immunogenic strains remained different: part of their immunogenic ECA was ethanol insoluble and present in the same fraction with LPS.

Although the overall chemical composition of the LPS (= O antigen) of the two hitherto investigated immunogenic serotypes (O14 and O56) shows that they both belong to the most simple chemotype I (84, 109, 153), as also do complete R-type mutants, this did not readily suggest why they were immunogenic. A clue to the chemistry of immunogenicity was found when Mayer et al. discovered (126–128) that certain rough mutants of *E. coli* and *Shigella* were immunogenic in the same sense as was *E. coli* O14. Heat-killed bacteria of these R types were immunogenic, and most of their immunogenic ECA was ethanol insoluble (214). Immunogenicity was limited to certain core types. The complete *Salmonella*-type core is described as Ra, whereas among *E. coli* and *Shigella* strains several different core types are found, designated from R1 to R4, plus the core type found in the geneticist's favorite strain, *E. coli* K-12 (84;

TABLE 3. *Comparison of Pl-L fractions from ECA-positive and ECA-negative smooth strains of Salmonella minnesota*[a]

Strain	Derivation	Pl-L (mg)	Titer in HA[b]	Minimal inhibiting dose (μg/ml) in HAI	Precipitation in IE, pH 8.6	ManNUA, HVE
S1114	Wild type	35.5 (2.0)[c]	1:5120	7.8	+	+
SH3786	*rff* mutation	10.7 (0.9)	1:1280[d]	250	−	Trace[d]
SH5657	ilv^+ rff^+ recombinant of SH3786	28.5 (2.0)	1:5120	3.9	+	+

[a] Based on reference 12D and D. Männel, H. Mayer, and P. H. Mäkelä, manuscript in preparation. Abbreviations: HA, hemagglutination; HAI, hemagglutination inhibition; IE, immunoelectrophoresis; HVE, high-voltage electrophoresis on paper.

[b] ECA antiserum was rabbit antiserum against heat-killed whole cells of *Shigella boydii* type 3^- (F3140) (128).

[c] Numbers in parentheses indicate percentage of aqueous phase.

[d] Probably caused by leakiness of the *rff* mutation, which allows the synthesis of a small amount of ECA detected in the Pl-L fraction in which it is enriched. No ECA activity was detected in this strain by examining the supernatant of a heat-killed suspension for HA.

TABLE 4. *ECA-immunogenic strains*[a]

Organism	Core type LPS	ECA immunogenicity	Reference
Escherichia coli O14:K7	R4	++	102, 123, 172, 214
E. coli O56		+	102, 112
E. coli O124		+	102, 112
E. coli O144		+	102
E. coli K-12	R-K-12	+	174, A, B[b]
Shigella sonnei phase II	R1	++	44, 126
R mutant of *Shigella boydii* type 3	R1	++	214
R mutant of *E. coli* O8:$K27^+$	R1	++	214
R mutant of *E. coli* O8:$K27^-$	R1	++	126, 214
R mutant of *E. coli* O9:$K29^-$	R1	++	126
R mutant of *E. coli* O9:$K31^-$	R1	++	126

[a] I. e., strains that elicit an anti-ECA response when injected, as heat-killed suspensions, intravenously in rabbits.

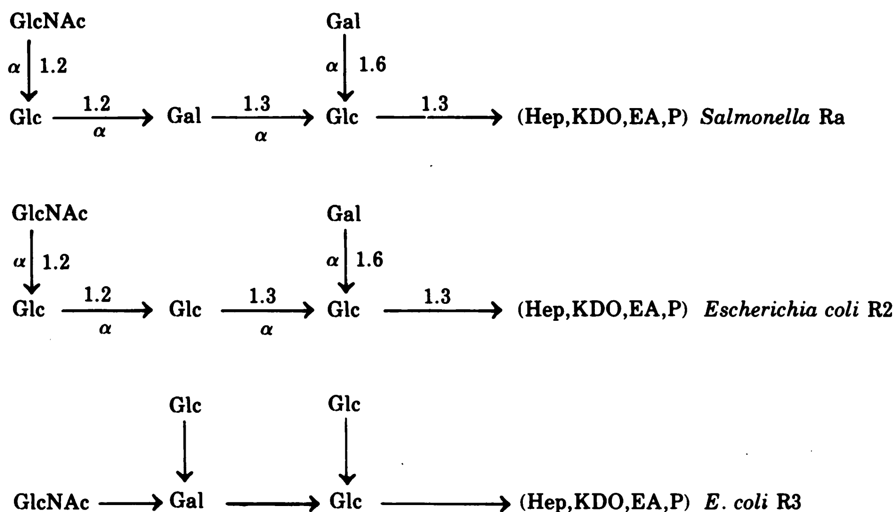
[b] A, P. Prehm, S. Stirm, B. Jann, K. Jann, and H. G. Boman, Evr. J. Biochem. 66:369-377, 1976. B, H. Mayer, A. M. C. Rapin, G. Schmidt, and H. G. Boman, Eur. J. Biochem. 66:357-368, 1976.

Fig. 7). Of these, only R1, R4, and at least partly K-12 are associated with ECA immunogenicity (126-128, 172, 214). The latter mutants are chemically very similar and even serologically cross-reacting to some degree (127). The Ra, R2, and R3 core types have a terminal *N*-acetylglucosamine, whereas the R1 and R4 cores end in an unsubstituted glucose. In the K-12-type core, the terminal glucose is partially substituted by glucosamine. The immunogenic and nonimmunogenic core types seem to differ also in the anomeric linkages of the terminal or subterminal glucose: it is probably α in the nonimmunogenic strains and β in the immunogenic ones (125). Perhaps this is the reason why a *Salmonella* mutant of core type Rb (Fig. 4) was not ECA immunogenic (H. Mayer, unpublished observations).

The classically immunogenic *E. coli* O14 was recently found to fit into this scheme (172) insofar as it was shown to be in fact a rough strain whose cultural roughness was masked by the capsule (K antigen). Its acapsular mutants are rough, and LPS extracted from either the capsulated strain or acapsular mutants is R, corresponding to the new R type R4. The serological specificity called O14 would then represent the specificity of the R4 core. In fact, F. Kauffmann (Copenhagen), who established the *E. coli* typing scheme, hesitated to identify *E. coli* O14 as a smooth strain since it had features of an R mutant (215). The other immunogenic O types have not been studied in equal detail; knowing how easily R mutants arise and accumulate in preserved cultures of the enteric bacteria, it would not be surprising if they, too, were R or partly R. The anti-ECA titers obtained when using them as immunogens are rather low (102, 85, 126).

We may conclude that the immunodetermi-

NONIMMUNOGENIC STRAINS



ECA-IMMUNOGENIC STRAINS

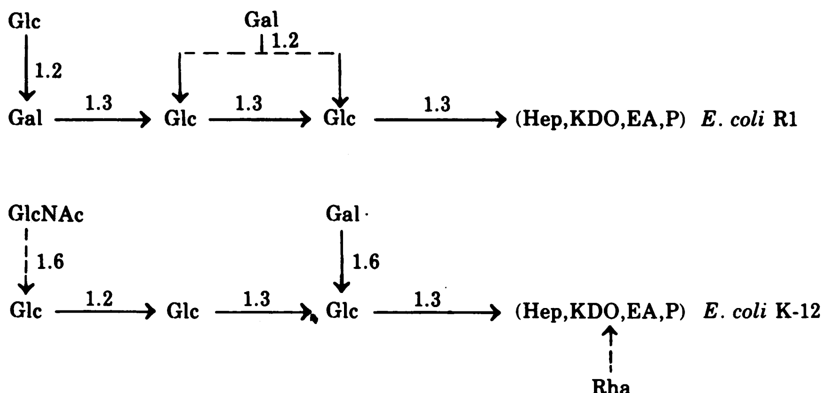


Fig. 7. *Schematic structure of the LPS core types in ECA-nonimmunogenic strains Salmonella Ra (109), E. coli R2 (71), and E. coli R3 (86), and in ECA-immunogenic strains E. coli R1 (84) and E. coli K-12 (P. Prehm, S. Stirm, B. Jann, K. Jann, and H. G. Boman, Eur. J. Biochem., 66:369–377; H. Mayer, A. M. C. Rapin, G. Schmidt, and H. G. Boman, Eur. J. Biochem. 66:357–368.*

nant of ECA can be linked to the LPS core, providing the core is of a suitable configuration, and in this linkage it is immunogenic for the ECA determinant. We would like to believe that this is a covalent linkage because of the association of ECA with LPS in the following procedures (126, 128): (i) in phenol-water and PCP extraction (the PCP extract of nonimmunogenic strains does not contain ECA); (ii) in ultracentrifugation; (iii) in the purification scheme combining these principles and presented in Fig. 1, in which LPS and ECA from nonimmunogenic strains can be separated; (iv) in the fraction insoluble in 85% ethanol (192); and (v) in retaining its RBC-coating ability after alkali treatment (126). These tests certainly establish a close association between LPS and ECA, but a direct proof of covalent linkage is still missing.

Some further information has been provided by studies of mutants of the immunogenic strains (see "Genetics of ECA in immunogenic strains"). The core must be free, not substituted by O side chains: heat-killed suspensions of

smooth forms of *E. coli* O8:(K27) or *Shigella boydii* type 3+, core type R1, were not immunogenic (128, 215). Schmidt et al. (172) prepared genetic hybrid strains in which the R4-type O14 core was combined with O side chains of specificity O8. Such hybrids were stated to be "at best slightly immunogenic." The core must also be complete; a *rfa* mutant of *E. coli* O14 in which the core was incomplete, lacking 1 mol each of galactose and glucose, contained ECA in ethanol-soluble but not in ethanol-insoluble (LPS-linked) form, and its heat-killed cells did not elicit the synthesis of anti-ECA in rabbits, contrary to the behavior of the parent strain (123).

These very exact requirements for the LPS structure suggest that linkage of ECA to LPS is enzyme mediated and covalent. The fact that the function of a *rfaL* gene, probably a translocase, is also required for ECA immunogenicity provides a further argument for the view that ECA is covalently linked to the LPS core, probably to the terminal glucose in the same way as are the O side chains of smooth forms. For a final proof of this, the linkage region should, however, be isolated and its structure determined.

Interference by LPS

Although covalent or other specific linkage of LPS to ECA in immunogenic strains results in immunogenicity of ECA, it has, largely through the work of E. Neter and his associates, become clear that a different sort of interaction is responsible for the lack of immunizing ability in nonimmunogenic strains. This interaction is probably a nonspecific aggregation of ECA with LPS, and this ECA can be rendered immunogenic when fractionated away from LPS.

Suzuki et al. (192) did just this: they separated extracts of nonimmunogenic strains of *E. coli* or *Salmonella* into ethanol-soluble and -insoluble fractions, of which the ethanol-soluble fraction was immunogenic for ECA, whereas the whole extract or the ethanol-insoluble fraction (which contained LPS) was not. Mixing the ethanol-soluble with the ethanol-insoluble fraction resulted in a marked decrease in its immunogenic capacity.

It must be stressed at this point that when speaking of immunogenicity and nonimmunogenicity, we in fact only state something of quantitative differences, and it might be more appropriate to talk of strong and weak immunogenicity. For example, in many cases a "nonimmunogenic" preparation of ECA could be made to immunize when administered properly. Subcutaneous immunization with a crude bacterial extract containing ECA + LPS, especially with complete or incomplete Freund adjuvant, resulted in an anti-ECA antibody response (65). The difference between the immunogenic and nonimmunogenic forms is best seen when weak immunization methods (intravenous injection as opposed to subcutaneous injection with adjuvant) are used. And even the "immunogenic" or "strongly immunogenic" ethanol-soluble ECA is a rather weak antigen, usually requiring several injections a few days apart to produce good anti-ECA titers.

LPS is generally recognized as an adjuvant of immune responses (137). It increases antibody production to weak antigens, prevents the development or maintenance of tolerance (28), and converts T-dependent antigens to T-independent antigens (29, 30). These functions of LPS are thought to correlate with its mitogenicity to B cells (5, 27, 183). Suppression of immunity has, however, been reported in certain specific instances (2, 137, 177).

The inhibitory effect of LPS has been mostly studied in rabbits, partly because mice respond very poorly to ECA immunization (see "Prevalence of antibodies to ECA"). The necessity of using rabbits has obvious disadvantages, since large-scale immunizations are not possible. An exact quantitation of the parameters of the LPS effect is missing, partly because of this and partly because of difficulties of quantitating ECA. The immune response has been measured as titers in indirect hemagglutination, which is not a very exact measure either. And although LPS can now be obtained in fairly pure form, special care must be taken to obtain it free of ECA (the best method probably being the ECA preparation method of Fig. 1, which gives ECA-free LPS as a water-precipitated fraction, G1-S), and this has usually not been done.

In summary, ECA coaggregated with LPS by mixing in the test tube, coextracted from bacteria or present together on heat-killed bacteria, gives rise to a minimal anti-ECA response (HA), if any at all, when injected intravenously. Nevertheless, it has had an apparent priming effect on the animal, as seen when a subsequent intravenous injection of ECA (free of LPS) results in a secondary type of response. After this single booster injection, the antibody level rises more rapidly and reaches a much higher peak (e.g., HA titer of 1:6,000 compared with 1:34) than without the priming (39, 147). Both inhibition of the response by LPS and the booster effect can also be seen by measuring the numbers of lymph node or spleen cells synthesizing anti-ECA and giving hemolytic plaques

with RBC sensitized with ECA (39). The inhibition cannot be due to direct toxicity of LPS since it is not seen if LPS is injected at a separate site from ECA (194).

The quantitative and kinetic aspects of the system were studied by Whang and Neter (222). The extract of *E. coli* O111 could be used for priming over a 1,000-fold range of dilutions. Similarly, the booster antigen (= the ethanol-soluble fraction) could be used undiluted or down to a dilution of 1:1,000. LPS was effective for priming at 100 μg or higher doses. The interval between the priming and booster injections had to be 2 or more days to produce the secondary response. The strength and kinetics of the secondary response remained unaltered when the interval was increased up to 8 weeks (the maximum tested). The specificity of the priming was tested by using such ECA-negative organisms as *Pseudomonas* or *Staphylococcus* for the primary immunization; a subsequent injection of ethanol-soluble ECA caused only a minimal response (= weak primary response to a single suboptimal dose of antigen).

Many LPS preparations have been tested for their immunosuppressive effect towards ECA. The LPS present in the crude extracts or in their ethanol-insoluble sediment, as well as several purified LPS preparations, act as immunosuppressants (194, 195, 222). The lipid A part of LPS, which is responsible for the many biological effects of endotoxin (= LPS), was also found to be responsible for this specific immunosuppression (212, 222). If the lipid A in some experiments (194) was less active than LPS, the lipid A was probably poorly soluble.

The ethanol-insoluble LPS from the immunogenic strain *E. coli* O14–probably LPS linked with ECA–was immunogenic for ECA, i.e., did not show the immunosuppressive effect (192, 195). When LPS from other sources was mixed (coaggregated) with it, its capacity to elicit the production of anti-ECA was reduced (195).

Importance of the Form of Aggregation

Besides LPS, several other materials have been found to have an immunosuppressive effect toward ECA immunization. Cardiolipin in 54- to 540-μg/ml amounts, mixed with ethanol-soluble ECA, prevents the primary response but not immunological priming to ECA (226). It also has the same effect on the LPS-linked ECA of *E. coli* O14. Whole serum from various animal species (227); membranes of a *Mycoplasma* (13) and chlorphenesin (228) (3-*p*-chlorphenoxy-1,2-propanediol, a compound shown to cause immunosuppression when injected together with sheep RBC [12]); gangliosides; methyl palmitate; and detergents such as Triton X-100 and Tween 20 (1) have a similar effect, in many cases dependent on the dose of the immunogen and/or inhibitor. Several other lipids (glyceryl trioleate, sphingomyelin, and cerebrosides) have been reported to be without effect on the immunogenicity of ECA (1). Cholesterol was shown to prevent the immunosuppressive effect of LPS (crude bacterial extracts) (225) and of Triton X-100 (1).

Most of the above-mentioned immunosuppressants are known to be membrane active compounds, i.e., lipids or ampholytes. As such they can be expected to complex readily with cellular membranes on the one hand and with lipidic or amphiphilic compounds (such as ECA may well be) on the other (75). The first mechanism may affect early events in the immune response (stimulation of the precursors of antibody-forming cells probably occurs as a membrane reaction), whereas the second may be expected to modify the antigenic properties of ECA. This modification could involve masking of antigenic groups by steric hindrance or alteration of the solubility properties and thereby the aggregate size or the conformation of ECA.

Some experimental data bear on these possibilities. LPS (195), serum (227) and cardiolipin (226) were shown to interfere only minimally or not at all with the ability of ECA to coat RBC or to react with anti-ECA in HAI. LPS or lipid A inhibited antibody-enhanced phagocytosis of latex particles sensitized with mixtures of these and ECA, although they did not reduce the amount of ECA available on the latex particles to adsorb anti-ECA antibodies (37). These data suggest that the antigenic groups of ECA were exposed to soluble antibodies and that the inhibitory action could take place at a stage of active cell surface events such as occur in phagocytosis (or stimulation of cells).

The importance of the particle size of the ECA-containing immunogen was best demonstrated by Whang et al. (208). The ethanol-soluble ECA fraction was further divided by centrifugation (23,500 × *g* for 20 min) into a pellet (larger aggregates) and the supernatant. Of these, only the pellet was a good immunogen, although both the resuspended pellet and the supernatant contained equal amounts of ECA measured by HAI activity. Reducing the particle size of the ethanol-soluble fraction by passing it through membrane filters reduced and finally abolished its immunogenicity (208).

A change in particle size or in conformation could be responsible for the effects of heating, freezing, and alkali treatment. Heating (at

100°C for 1 h) of the immunogenic ethanol-soluble ECA reduces its immunogenicity, which can be restored by repeated freezing and thawing (213). This freeze-thaw procedure also restores the lost ECA immunogenicity of membrane filtrates (208). Alkali treatment (0.1 N NaOH for 1 h at 37°C) also abolishes ECA immunogenicity, which cannot be restored by freezing and thawing. This treatment probably removes part of the palmitic acid and thereby reduces the hydrophobicity and aggregation tendency of ECA. LPS, treated similarly by heat or alkali, also loses its immunogenicity, and poor immunogenicity of phenol-water-extracted LPS preparations can be improved by repeated freezing and thawing (148, 213). Varying mitogenic efficiencies of different LPS preparations can be similarly explained (184). The sensitivity of ECA and LPS to these procedures was similar but not exactly the same, except for the LPS-linked ECA of immunogenic strains. All these nonimmunogenic preparations nevertheless primed the animals to give a secondary response to either ECA or O antigens (LPS) after a booster injection of immunogenic material (148, 208, 213).

Intimate contact between ECA and the inhibitory compound is necessary for the inhibition. Both inhibitor and ECA must be injected simultaneously at the same site (1, 194, 227). A more clear-cut inhibition is obtained if they are mixed thoroughly, in ethanol (in which they at least partly solubilize) for example, and incubated (allowing time for rearrangement) before injection. It is this requirement of intimate contact that led Neter et al. to propose the term "antigen-associated" immunosuppression (227) for this effect.

If it is more the form of antigen presentation than the presence or absence of a specific inhibitor that determines the immunogenicity of ECA, some apparently contradictory findings fall in place. Among these are the following. (i) The suppression of ECA immunogenicity is seen most clearly with heat-killed cells, whereas living bacteria or bacteria killed with formalin, merthiolate, or phenol do elicit the production of anti-ECA antibodies (214). (ii) Several fractions of disrupted (French press) bacteria were isolated from nonimmunogenic strains without prior heating (36). Most of these fractions, as well as the crude disrupted material, elicited a good anti-ECA response when injected intravenously into rabbits. Heating reduced their ECA immunogenicity only to a minor extent. Significantly, they all contained LPS (as judged by their ability to evoke an anti-O antibody response), which apparently did not suffice to suppress the immunogenicity of ECA in this situation. (iii) The physicochemical state of ECA and LPS as well as their relative amounts can easily be expected to be abnormal in spheroplasts, which lack the rigid cell wall; this could account for the results (whole spheroplasts immunogenic, their supernatants or lysates not) obtained with these cells (220). (iv) RBC or fibroblasts coated with ECA from crude extracts of nonimmunogenic strains are immunogenic for ECA in rabbits (58, 149).

The immunosuppression by LPS and other similar immunosuppressants has also been shown to affect the "common antigen" of gram-positive bacteria (226, 227). As discussed below ("Other cross-reacting or related antigens"), this antigen is ill defined but may be a teichoic or lipoteichoic acid. Teichoic acids have been shown to have aggregation tendencies with several compounds, e.g., polysaccharides and albumin (41). In any case, the immunosuppression with priming is very similar to that seen with ECA coaggregated with LPS. The O antigen (the antigenic property of the polysaccharide part of LPS) is not affected by addition of LPS from the same or other source or by addition of serum or detergents (1, 39, 147, 195, 213). When injected intravenously as a heat- or alkali-treated preparation, it results, however, in immunosuppression with priming. The above reasoning of reduced aggregate size as the basis for this immunosuppression could fit the case of LPS too, although the requirements for a suitable form of presentation may be different for LPS than for ECA. We are not aware of other antigens that have been tested for this effect.

The observations described above speak for a decisive role of the proper form of ECA for immunogenicity. The aggregation tendency is consistent with the probable chemical structure of ECA as an amphiphilic compound (75, 231). However, it is not possible to define the exact requirements for good immunogenicity. In several cases, it is clear that the small size of the aggregates is responsible for poor immunogenicity. Whether LPS and other similarly acting compounds always reduce particle size or whether they also or under other circumstances modify the conformation in other ways, which are incompatible with immunogenicity, cannot be decided at this time. An experimental possibility may be offered by the recent work of Galanos et al. (49, 50), showing that different salt forms (e.g., triethylamine, sodium, calcium) of LPS have very different solubility properties. The size of the LPS aggregates—vesicles or micelles—is altered, and it is very possible, although not studied in detail yet, that the affinity of the LPS to animal cell membranes or to ECA could be altered as well.

Immunological Tolerance?

Another obvious possibility to account for the described immunosuppression is the well-documented phenomenon of immunological tolerance or paralysis (42, 80). Reduction of the molecular size reduces immunogenicity of most antigens, both proteins and polysaccharides. The smaller molecules (below a molecular weight of 10,000) of fairly monotonous polysaccharides such as glucans or levan are nonimmunogenic, but produce a state of tolerance or unresponsiveness to further injections of the antigen even in its immunogenic form. The tolerance may fade with time, but requires much longer periods, even weeks in mice, than the intervals used before the booster injections of ECA. Also, the animal species plays an important role in determining the outcome – an immune response or not – of immunization (e.g., reference 66).

Tolerance production is favored by weak immunization, such as intravenous injection of antigen (where the ECA immunosuppression is best seen). The LPS preparations, with which the ECA immunosuppression was demonstrated, were obtained by phenol-water extraction and contained ECA. It is possible that coaggregates of ECA and LPS are especially favorable for tolerance induction, even with very small amounts of ECA. One experimental observation (215) speaks strongly for a tolerogenic mechanism: whereas LPS isolated from ECA-positive strains of *Salmonella* produced the above-described immunosuppression to ECA, LPS from ECA-negative mutant strains did not. This finding is difficult to explain on the hypothesis of aggregate size but easy on the hypothesis of tolerance: the LPS from ECA-negative strains is not expected to have any ECA immunodeterminants for tolerance induction. However, the mode of action of the *rfe-rff* mutations responsible for the ECA negativity of the strains is not known. Therefore it is possible that these mutations also affect LPS, for instance in a way that would alter its conformation or affinity to ECA or the size of the aggregates produced.

Experiments using whole bacteria (215) also contradict the simple hypothesis that the aggregate size is the sole basis of immunosuppression. When immunogenic *E. coli* or *Shigella* and nonimmunogenic *Salmonella* were injected together at the same site, anti-ECA production was suppressed. This did not happen if the two kinds of bacteria were injected in separate ear veins. Under these conditions, most of the immunizing ECA would be expected to be bound to the bacterial cells and not affected by ECA or LPS on other bacteria. On the other hand, if the role of the *Salmonella* bacteria was tolerogenic, it is difficult to see why they would not have the same effect when injected at separate sites.

What speaks most strongly against the tolerogenic effect of LPS-ECA mixtures is the fact that no tolerance to a subsequent injection of immunogenic ECA is produced, but instead the animals are primed to give a heightened "secondary" response. In conventional tolerance, some kind of priming is sometimes seen with either protein or polysaccharide antigens on recovery from the tolerant state, and called "overshoot" (42, 181). The time required for the transition from unresponsiveness to an increased response is, however, longer than the mere 2 days required before a booster dose of ECA.

In several cases, a T-cell-dependent antigen caused an apparent priming effect in animals that were lacking functional T cells and were therefore unable to respond with antibody production (32, 164, 176, 180, 196). These results suggested that the antigen had nevertheless succeeded in stimulating B cells to proliferation and production of memory cells. A comparable situation was described by Chiller and Weigle (28) in mice tolerant to a T-cell-dependent antigen (human gamma globulin). At a certain stage of recovery, the tolerance could be broken by injecting the antigen in a T-cell-independent form (together with LPS), and this resulted in a secondary response type of increased antibody synthesis. They suggested that because of different sensitivities of T and B cells to a tolerizing antigen, it may be possible that at a certain dose T cells become tolerant whereas B cells are immunized (primed), although no antibody production takes place since it would require T-cell participation. If these B cells then meet an immunogenic, T-cell-independent stimulus, they respond with rapid antibody production.

These examples have attractive similarities to the ECA case. However, so far there are no data to demonstrate the presence of memory cells to ECA, and the fact that anti-ECA (measured, it is true, mainly by HA) even after this kind of priming is almost exclusively 19S (224) speaks against memory. Nor do we know whether ECA or coaggregates of ECA and LPS would stimulate B cells in rabbits, although the fact that LPS alone can be a T-cell-independent antigen in mice suggests that this could be the case. Furthermore, little is known of rules of immunity with such amphiphilic materials as ECA is likely to be. True tolerance to ECA could never be demonstrated, for example by varying the dose of the antigen (208). Unfortunately, the poor immunogenicity of ECA in

mice precludes experiments with T-cell-deficient mice (166) and the C3H/HeJ strain, which is unresponsive to many effects of LPS (191), experiments which would help to clarify these problems and to demonstrate how far the observed "antigen-associated" immunosuppression represents a new immunological principle.

In view of the many seemingly irreconcilable observations, we believe that both theories may turn out to be partly correct. The form of aggregation (or conformation) may be decisive in some situations, and some sort of tolerance may operate in others.

ECA IN THE BACTERIAL CELL

Very little is known of the localization of ECA in bacteria. At least parts of it must reside on the surface of the cell; some of it is very easily eluted into the surrounding medium (102), and anti-ECA antibodies attach to intact bacteria, as shown by fluorescence elicited by subsequent treatment with fluorescent antigamma globulin (8). The "ECA-dependent" fluorescence is less intense than that caused by LPS-anti-LPS, which may suggest that ECA is less exposed than is LPS or (which is more likely in view of the large amounts of LPS in the outer membrane) that there is less ECA present. However, the specificity of the IF reaction for ECA is not finally established.

Whang and Neter (220) have looked for ECA in glycine-induced spheroplasts of *Salmonella typhi* and have detected some activity by HA, by HAI, and by immunization. Unexpectedly, they could demonstrate ECA only in spheroplast suspensions, not in the supernatant or in lysed suspensions, perhaps suggesting that ECA was present in reduced amounts. This could be taken to support the localization of ECA in the outer membrane, which is partly disintegrated in spheroplasts, but the evidence is weak and circumstantial.

The fact that anti-ECA cannot kill bacteria in the presence of complement may suggest that ECA is not embedded in the outer membrane in the way LPS is. On the other hand, antibodies towards capsular polysaccharides can be bactericidal (66), suggesting that the molecule mediating a bactericidal reaction does not need to be an integral component of the bacterial membrane. (It must be admitted, though, that the location of the capsular polysaccharides is not well defined either. However, the absence of any lipid-soluble part in the polysaccharides makes it unlikely that they would be embedded in the predominantly lipidic membrane.)

Domingue and Johnson (36) and Johnson, Domingue, and Klein (submitted for publication) have made the most direct attempts at localizing ECA. They used *Salmonella* strains, *E. coli* O14, and *Klebsiella* and isolated different fractions, including the outer membrane and the cytoplasmic membrane. The ECA activity of *E. coli* O14 was more clearly present in the cell envelope fraction, but in most other cases all fractions, also the cytoplasm, contained ECA. It seems likely that the location of the LPS-linked ECA in *E. coli* O14 followed the location of LPS, whereas the haptenic ECA of the other strains was solubilized in the process and contaminated all fractions.

CLINICAL IMPLICATIONS

Many of the bacteria of the family *Enterobacteriaceae* are important pathogens of man, animals, and plants. In some disease groups (urinary tract infections) they are the main source of infection, and in others they are very common causative organisms (all infections of the peritoneal cavity and its organs). A component common to all *Enterobacteriaceae*, therefore, would have great promise as a means of developing generally applicable diagnostic methods for these diseases or for measuring immunity to them. Questions of the possible role of ECA in pathogenesis are obvious. Is it a virulence factor (by direct toxic action, by protecting bacteria from host defenses, by affecting tissue affinity of the bacteria)? Do antibodies to ECA protect the host animal (man)? Are there antigenically similar components in the host tissues and, if so, how does this affect the acute infection or its sequelae?

Immediate Biological Effects of ECA

Endotoxin (LPS) has well-documented toxic effects, which can be demonstrated in the whole animal and partly also at the cellular level and which profoundly affect the course of infections caused by gram-negative bacteria (89).

Kunin (100) tested his DEAE-purified ECA from *E. coli* O14 for pyrogenic and lethal effects in rabbits; at a dose of 500 μg it produced fever but no other harmful effects, whereas LPS was lethal at the tested dose of 25 μg. The same observation was made by Johns et al. (85) with their picric acid-Sephadex G-200-purified material from *S. typhi*. It produced fever at a dose of 250 but not 100 μg; LPS was stated to be about 1,000 times more active as pyrogen. This purified material was also 1,000-fold less active than LPS in the limulus coagulation assay, which is another, apparently rather unspecific but extremely sensitive, test for endotoxin activity. It may be remembered that in both these

cases the ECA preparations were not in the "native" state a shown by their lack of RBC-sensitizing capacity.

In a mouse toxicity test (material injected intraperitoneally into mice kept at 37°C), the ethanol-soluble ECA (192) was nonlethal at a dose of 1,000 μg, whereas LPS still showed 20% lethality at a dose of 1 μg (96). The same preparation was 100- to 1000-fold less efficient than LPS in several other endotoxin assays: promotion of a Shwartzman-like reaction by epinephrine in rabbits, immediate production of nonspecific resistance to *Salmonella* infection in mice, and direct cytotoxicity on monolayers of guinea pig peritoneal macrophages (96).

In all these tests, the doses of ECA required to produce any of the pyrogenic or other toxic effects were so high that the effects could be accounted for by contaminating LPS present in the ECA preparations. A different toxic mechanism has been suggested for ECA: in vivo hemolysis of RBC coated with ECA released during infection (136, 193), but we have no data bearing on this possibility in human infections.

Is ECA a Virulence Factor?

A role of ECA in the pathogenic properties of enteric bacteria was recently demonstrated. ECA-positive and ECA-negative (rfe^-) derivatives of the mouse pathogen *S. typhimurium* were injected intraperitoneally in mice (202). Whereas the LD_{50} (dose lethal to 50% of the test animals) of ECA-positive strains was 10^4, the LD_{50} of their ECA-negative sisters (all strains smooth, O-4,12, constructed by conjugation) was 10-fold higher, indicating that ECA is needed for the expression of the full pathogenic capacity of the bacteria.

The mode of action of the ECA as a virulence factor has also been studied, so far with negative results (202; unpublished observations). There was no difference in the growth rate between ECA-positive and -negative sister strains. After intravenous injection, both variants were removed slowly from the blood—a test presumed to measure phagocytosis by the reticuloendothelial system. In this test nonvirulent rough (R) bacteria disappear more rapidly than virulent smooth (S) bacteria (98). However, it is likely that the test is not sensitive enough to detect small differences in bacterial susceptibility to phagocytosis; e.g., no difference was seen between S bacteria, which had only small differences in their LPS but differed approximately 10-fold in their virulence (200, 201).

Conversely, low content of ECA (measured by HAI) has been reported as a characteristic of *E. coli* strains causing diarrhea in young infants in Mexico, as compared with strains isolated before or after the diarrheal episode (24a, 98a). This was equally true of enteropathogenic and nonenteropathogenic serotypes; a correlation with enterotoxin production has not been reported.

More studies of the role of ECA in pathogenicity of enteric bacteria are obviously needed. As a surface component it can be expected to react with the defense mechanisms of the host and thereby influence the outcome of infection. The possible antigenic interrelationships of ECA and some tissue components (see below) suggest a role in specific tissue affinity. The possibility of preparing ECA-negative mutants of strains to be studied should now make such studies more meaningful.

Prevalence of Antibodies to ECA

A low level of anti-ECA antibodies seems to be present in normal human sera. Kunin (99–101) measured hemagglutinin titers of a large number of human sera using RBC sensitized with several *E. coli* extracts; this would measure both anti-O and anti-ECA antibodies. When the sera were absorbed with an ECA preparation (*E. coli* O14 extract), the titers to many other strains were reduced by a factor of 2 to 4, suggesting that as a rule the sera contained both anti-ECA and many different anti-O activities. The presence of anti-ECA deduced by this means in various age groups would be parallel to the presence of anti-O. There was a low level of anti-ECA in cord blood and very little at 2 to 6 months of age, with increasing titers above that age. The levels in cord blood were much lower than in the maternal serum. In colostrum, both anti-O and anti-ECA levels were high (100). Similarly, Whang and Neter (218) found anti-ECA in the cord blood of 8 out of 18 infants tested.

Pools of sera from healthy blood donors were shown to regularly contain anti-ECA (again by comparing heterologous hemagglutinin titers before and after adsorption by ECA-containing extracts of several enteric bacteria or a non-ECA-containing extract of *Staphylococcus aureus*) (218). For example, an initial titer of 1:40 was reduced by ECA-absorption to 1:10. Several gamma globulin preparations from Japan, South Africa, India, and the U.S.A. could likewise be shown to have anti-ECA as well as anti-O antibodies to several indicator bacteria (218).

Pools of sera from horses, cattle, dogs, and pigs all contained appreciable amounts of anti-ECA antibodies (101). Normal rabbit serum, on the other hand, was practically free of anti-

ECA activity (tested at a dilution of 1:10 with several *E. coli* preparations) (101). Since it seems reasonable to believe that the anti-ECA activity in normal sera is the result of continuous immunization caused by products of intestinal bacteria, the absence of anti-ECA in rabbit serum may reflect the predominantly gram-positive flora of the rabbit gut.

Active immunization with a suitable immunization schedule (see above, "Immunogenicity") leads to production of relatively high levels of anti-ECA antibody, although animal species and strains differ in responsiveness.

Most of the studies with ECA have used rabbit sera. Fractionation of rabbit anti-ECA sera by filtration through Sephadex G-200 has been performed (224). Several sera obtained with different immunization schedules showed anti-ECA (HA) in the 19*S* fraction only. A "late" serum, taken at day 14 of a secondary response when antibody levels were maximal, had 90% of its anti-ECA activity at 19*S*, the rest distributed among a large number of fractions. All antibody in all these sera was susceptible to 2-mercaptoethanol. No hemagglutinating (or blocking) antibodies were detected in the 7*S* fractions. The anti-O antibodies (measured by bacterial agglutination) in the same sera were found in both the 19*S* and 7*S* fractions, and all were susceptible to mercaptoethanol. When pregnant rabbits had been immunized, considerable amounts of anti-ECA were found in the pooled sera of the fetuses at days 27 to 28 of pregnancy. Again, this anti-ECA was mainly 19*S*. Further proof of transplacental transfer of anti-ECA antibodies was obtained by passive immunization of the mother, which resulted in measurable anti-ECA titers in the newborn but no anti-ECA titer in the amniotic fluid.

Mice respond relatively poorly to ECA, be it the LPS-linked, immunogenic form in *E. coli* O14 or the ethanol-soluble, non-LPS-linked ECA (56, 58, 62, 63; E. A. Gorzynski and E. Neter, Bacteriol. Proc., p. 86, 1968). The best responder among mouse strains was C57Bl/6HA; the CBA/St strain was fairly good, whereas DBA/2Jax and outbred Swiss albino mice were very poor responders. In mice, the non-LPS-linked ECA was usually a better immunogen, eliciting higher titers and a response in more mice than did an *E. coli* O14 extract. Mice also differed from rabbits in not showing a secondary response (booster injection of ethanol-soluble ECA after a rest period of 52 days).

Contrary to this experience, McCabe and Greely (112) obtained good anti-ECA titers in the mouse strain CH1 after immunization with heat-killed *E. coli* O14. The antibody was present in both 7*S* and 19*S* fractions. The difference may be related to their use of an extract of *Proteus rettgeri* strain 6572 to sensitize the RBC for HA (see "Serological methods").

Guinea pigs have been immunized by both *E. coli* O14 extracts and ethanol-soluble ECA in Freund adjuvant and tested for cutaneous delayed hypersensitivity and antibody production (132). No or only minimal anti-ECA activity was detected in the sera 9 days after a single dose of immunogen. A booster on day 20 resulted in low anti-ECA titers in all animals. A delayed-type hypersensitivity reaction was elicited on day 9 as well as on day 30 by intradermal injection of various ECA preparations. A *Pseudomonas* extract not containing ECA also caused erythema and induration in the animals immunized with ethanol-soluble ECA, and it was not completely clarified whether the delayed hypersensitivity reactions were caused by ECA or by small amounts of LPS contaminating the ECA preparations.

Some immunizations have been performed in humans with ethanol-soluble ECA from *E. coli* O111 (64). The anti-ECA titers (HA) in preimmunization sera ranged from below 1:10 to 1:40; those after immunization ranged from 1:160 to 1:1,280 (nine out of ten immunized subjects; one did not respond). The post-immunization sera had also more opsonic activity than did the pre-immunization sera (203).

ECA and Anti-ECA Antibodies in Relation to Disease

The possible role of anti-ECA antibodies in various infections usually caused by enteric bacteria has been tested. The general impression has been rather negative: the levels of anti-ECA are low, and only a fraction of the patients respond with a rise (Table 5). The titer value may, however, not be very significant; only HA has been used, and therefore (almost) only IgM antibody has been measured. In the case of anti-O, only IgG antibodies have been shown to be protective in human disease (114). When anti-O titers (to the O antigen of the causative organism) have been measured in the same samples as ECA, they have been consistently higher (4, 33, 114, 141, 146, 204, 218).

Diseases in which anti-ECA responses have been found only occasionally, and at a low titer, are enteritis (*Salmonella* or *E. coli*), bacteremia, and acute urinary tract infections (Table 5). The only conditions in which high anti-ECA levels and/or a rise in anti-ECA have been consistent features are shigellosis (33, 218), peritonitis (141, 146), and chronic urinary tract infection (4, 167, 218). In these, over 50% of the patients had high anti-ECA titers. In relation

TABLE 5. *Anti-ECA in human infections caused by gram-negative bacteria*

Infection	No. of patients	Anti-ECA	Anti-ECA in controls	Reference
Urinary tract	Chronic, 6	1:80–1:320 in 5/6	1:40	218
	Acute, 13	1:160, no rise		204
	Acute, 10 below 1 yr old and 10 above 1 yr old; chronic, 2	0–1:8 1:8–512 1:128	0–1:16	4
	Acute or chronic, 50	18% – 1:160 or more 16% – fourfold rise	1:40	33
	Children, 11	1 – 1:160 or more		141
	?	No difference to control[a]		113
	152	Elevated in 63%	Not elevated	167
Salmonellosis or other enteritis	1	Fourfold rise to 1:160	1:40	218
	50	24% – 1:160 or more 16% – fourfold rise	1:40	33
	10	7 – below 1:40 0 – above 1:80		141
Shigellosis	50	56% – 1:160 or more 38% – fourfold rise	1:40	33
Bacteremia	3	No change		33
	108 with fatal underlying disease, 65 of these followed	Initial titers low 29% – fourfold rise	1:160–1:2,560[a]	113, 115
	98 without fatal underlying disease, 43 of these followed	Initial titers not different from controls 36% – fourfold rise		
Peritonitis	27	8% – below 1:40 55% – 1:160 or more 82% – fourfold rise		141
	1 (rough *E. coli*, core type R1)	Rise from 0 to 1:640		146

[a] Cells sensitized for HA by an extract of *Proteus rettgerii* 6572; for discussion see "Serological methods."

to shigellosis, one is reminded of the frequency of R mutants with the R1 type of core in *Shigella* strains, especially *Shigella sonnei* (44, 127) – such R strains belong to the strains that are "immunogenic" for ECA. In the other two conditions a long-continuing or especially strong antigenic stimulus is probable. Over 60% of peritonitis patients showed a fourfold or greater rise in antibody (141), indicating an active process of immunization. Over 80% of patients with chronic or repeated urinary tract infections (4, 167, 218) had high and persistent anti-ECA titers. In view of the clinical importance and frequency of this condition, this diagnostic possibility should be studied further.

ECA was detected by IF in kidney tissue in confirmed pyelonephritis (six cases out of six) and in six cases out of seven of pyelonephritis in which bacteria had not been cultivated, but not in patients with other types of chronic renal disease (7). However, other workers could find ECA in kidney tissue in only one out of nine cases of chronic pyelonephritis and three out of five cases of acute pyelonephritis (178). In experimental pyelonephritis of the rat, ECA could be demonstrated in acute infection but not later on (197a). The reasons for the different findings are not apparent; the techniques and antisera were very similar, and proper IF controls with bacteria (8, 178) or tissue from experimental animal infections (9, 178) were performed (however, see discussion of the IF method in "Other serological methods").

McCabe et al. (113, 115) have studied a large series of patients with bacteremia compared with healthy blood donors in an attempt to find correlations between anti-O, anti-R, and anti-lipid A and anti-ECA antibodies with the occurrence or prognosis of the infection. The bacteremia patients had lower anti-ECA titers than the controls, but this difference was almost entirely accounted for by the approximately half of the patients who had an ultimately fatal underlying disease. A rise (fourfold or more) in anti-ECA was seen in one-third of the patients irrespective of the basic disease or the species of

TABLE 6. *Presence (+) or absence (−) of ECA in extracts of various bacteria as tested by hemagglutination and/or inhibition of hemagglutination*[a]

Name	ECA	No. of strains	Reference
Gram-negative facultatively anaerobic rods			
Family *Enterobacteriaceae*			
Escherichia	+[b]	166	8, 102, 106, 229
"alcalescens-dispar"	+	4	106
Shigella	+	44	102, 106, 229
Edwardsiella	+	33	106, 229
Salmonella	+[b]	73	102, 106, 229
S. arizona (=*Arizona*)	+	12	229
Klebsiella	+	26	8, 102, 106, 229
Enterobacter	+	56	8, 34, 102, 106, 229
Citrobacter (including *Levinea*)	+	33	106, 229
Serratia	+[b]	26	34, 106, 229
Proteus	+[b]	50	8, 102, 106, 229
Providencia	+	19	106, 229
Yersinia	+	70	106, 117
Pectobacterium	+	15	34, 229
Erwinia (*herbicola, carotovora, amylovora*)	+	24	102, 106
Erwinia chrysanthemi	−	6	106
Family *Vibrionaceae*			
Plesiomonas? (Vibrio? Aeromonas?)shigelloides	+	15	106, 210
Aeromonas	−	46	106, 210
Vibrio	−	17	106
Genera of uncertain affiliation			
Flavobacterium	−	19	34
Haemophilus	−	1	106
Pasteurella	−	6	106, 117
Gram-negative aerobic rods and cocci			
Family *Pseudomonadaceae*			
Pseudomonas	−	14	8, 102, 106, 117
Genera of uncertain affiliation			
Alcaligenes faecalis	−	4	106
Brucella	−	4	102, 106, 117
Bordetella	−	2	102, 106
Francisella tularensis	−	1	117
Other gram-negative bacteria			
Acinetobacter calcoaceticus	−	1	117
Rhodopseudomonas capsulata	−	1	126
Gram-positive bacteria			
Staphylococcus	−	2	102, 216
Streptococcus	−	2	102, 216
Sarcina	−	1	102
Bacillus	−	2	102, 216

[a] Classification according to *Bergey's Manual* (23).

[b] Occasional ECA-negative strains have been reported: one strain of *Serratia marcescens* (102), six *Proteus* strains (*P. vulgaris, morgani, rettgeri, mirabilis*) (8, 106), and mutants of *E. coli* and *Salmonella* (119, 120; G. Schmidt, H. Mayer, and P. H. Mäkelä; J. Bacteriol., in press; P. H. Mäkelä, G. Schmidt, H. Mayer, H. Nikaido, H. Y. Whang, and E. Neter, J. Bacteriol., in press) among many positive strains of the same species.

enteric bacteria (*E. coli*, *Klebsiella* group, *Proteus*, or *Salmonella*) causing the infection. The level of anti-ECA was not correlated with prognosis when patients were examined in separate groups depending on whether or not they had a fatal basic disease. By the same methods, poor prognosis (death or development of shock) could be correlated with low anti-O titer (immunoglobulin G only), low anti-R (of the Re type of basal core, Fig. 4; see also "Other cross-reactivities and related antigens") and less clearly low anti-lipid A. Despite the careful analysis made and the clearly important division of the patients in groups of basically different capacities to resist infection (those with versus those without an underlying fatal disease), these conclusions of the correlation of the different antibody levels with prognosis should probably not yet be accepted as the final truth. First, the correlations are not of very high level of signifi-

cance, and larger groups are still needed – nonhomogeneity of the patient group has, as demonstrated, a large influence on the prognosis. Second, the use of a *Proteus rettgeri* extract to sensitize the RBC for HA (113) has not been completely correlated with ECA standards (see above, "Serological methods"). And third, since a correlation of anti-O with prognosis in human bacteremia could be shown only when immunoglobulin G antibodies were examined (114), although anti-O has been repeatedly shown to protect in experimental infection in mice (95, 107, 163), it may be questionable to study only HA titers (predominantly, at least, immunoglobulin M) of the other antibodies.

Anti-ECA in Experimental Infection

The possible protection afforded by ECA immunization has been studied in either generalized bacteremic infection of mice, caused by intravenous or intraperitoneal injection of *S. typhimurium*, *E. coli*, or *Klebsiella*, or pyelonephritis of rabbits, caused by retrograde or hematogenous infection by *E. coli*, *Klebsiella*, or *Proteus mirabilis*. The results in mice have been equivocal and those in rabbits have been more promising, but it is not at all clear whether this difference is related to the different infection types or to the animal species, mice being much poorer responders to immunization with ECA than rabbits (see above, "Prevalence of antibodies to ECA"). In some cases, an ECA-negative *Pseudomonas* strain was used as control challenge (40), increasing the significance of results obtained. It can be argued, however, that *Pseudomonas* differs from enteric bacteria in so many ways that it cannot be regarded as a valid control. ECA-negative mutants are now available, and they should provide better controls.

Gorzynski et al. (56, 58, 63; Gorzynski and Neter, Bacteriol. Proc., p. 86, 1968) used mice immunized with *E. coli* O14 or the ethanol-soluble ECA of *E. coli* O111 and challenged with *S. typhimurium*. Both C57Bl/Ha6 mice, shown to be relatively good at producing anti-ECA, and poorly responding Swiss albino mice (62) gave almost identical protection results after intraperitoneal immunization (63; Gorzynski and Neter, Bacteriol. Proc., p. 86, 1968); anti-ECA was not recorded. RBC (of horse origin; mouse RBC did not work) sensitized with ECA could also be used for immunization (58). Passive immunization with rabbit anti-ECA gave similar results (56). The protection was never complete. A significant difference could be demonstrated between the ECA-immunized and control animal (receiving normal rabbit serum [56] or extracts of *Staphylococcus aureus* or *Pseudomonas*) only by following the kinetics of the infection. Survival rates in the two groups were compared each day, a 50% comparison value indicating equality between the groups. In this way the ECA-immunized group got approximately 60 to 70% scores, which in most cases were significant ($P < 0.001$).

McCabe and Greely (112) immunized mice (CFI) intraperitoneally with *E. coli* O14 and obtained good anti-ECA titers (measured by HA with *Proteus rettgeri* strain 6572 as sensitizing agent). Upon challenge with *Klebsiella pneumoniae* or *E. coli* (unrelated strain), the LD_{50} values were exactly the same in the immunized and the control groups, whereas immunization with the challenge organism was clearly protective, increasing the LD_{50} 30- to over 1,000-fold. Passive immunization with two rabbit anti-ECA sera gave no protection either, but a third serum did. This protective serum was shown to contain anti-Re antibodies (Fig. 4 and "Other cross-reactivities and related antigens") in addition to anti-ECA; absorption of the serum with ECA did not abolish its protective capacity, whereas absorption with Re did. This finding strongly suggests that anti-Re antibodies may be important as cross-reactive protecting factors. This same conclusion has been reached from other studies with Re immunization in mice (114) and from examination of the correlation of different antibodies with prognosis of human bacteremia caused by enteric bacteria (114, 115).

These findings make it necessary to examine the possible effect of Re and lipid A antibodies in each case where protection by anti-ECA is claimed. For instance, in the above-mentioned studies by Gorzynski et al. (56, 58, 63; Gorzynski and Neter, Bacteriol. Proc., p. 86, 1968), Re antibodies could apparently be involved. Although ethanol-soluble and therefore relatively LPS-free ECA was often used as immunizing agent, it contains enough LPS to elicit the production of anti-O antibodies, although not very efficiently (192). On the other hand, since mice are poor anti-ECA producers, the heavy immunization necessary could have magnified the anti-LPS responses (no values are given).

Domingue et al. (40, 48, 116) used the rabbit pyelonephritis model (infection either via the bladder supported with a foreign body or intravenously after local kidney damage caused by oxamide feeding). They immunized the rabbits with the ethanol-soluble ECA, in one case (116) partly purified, and obtained good anti-ECA titers. They found definite protection from pyelonephritis irrespective of route of infection and irrespective of whether the diagnosis was estab-

lished on the basis of bacteremia or histopathology. Both active immunization of the rabbits and rabbit anti-ECA antiserum (but not antiserum absorbed with ethanol-soluble ECA coated on sheep RBC) were effective when the challenge organism was ECA-positive *Proteus mirabilis* but not when it was ECA-negative *Pseudomonas* (40). A capsulated *Klebsiella* strain, which was not sensitive to opsonization by anti-ECA, was not sensitive to the anti-ECA-mediated protection either. It may be worth mentioning that anti-ECA antibodies did not increase in any of the animals during the course of their kidney infection, although anti-O antibodies did.

Antigenic Similarities between ECA and Animal Tissues

Antigenic cross-reactions between microorganisms and host tissues are believed to be involved in the pathogenesis of certain autoimmune diseases, of which the best-known example may be rheumatic fever with cross-reactions between streptococcal antigens and heart tissue (91). Sera from patients with ulcerative colitis, a chronic disease in which autoimmune phenomena are suspected of being important, have antibodies reacting with antigen(s) of colon tissue. This was demonstrated also with colon tissue of germfree animals, since otherwise many bacterial antigens contaminating extracts of normal colon would have interfered with interpretations (43, 104, 155, 156). These sera, as well as sera of rats made autoimmune by injections of rabbit colon, also react with ECA (*E. coli* O14 extract) in HA, HAI, and the test for a leucocyte migration inhibition factor (24, 43). An equally high frequency of antibodies apparently against ECA, although their identity has not been finally established, was present in liver cirrhosis (43) but was not found in other intestinal or specific colon diseases (43, 104). The cross-reacting tissue antigen was fairly specific to colon, and resembled ECA in being present in phenol-water extracts of the tissue, in its heat resistance, and in its ability to adsorb to RBC (104).

These findings obviously suggest a possible role of ECA of intestinal bacteria in triggering an autoimmune reaction, which could lead to symptoms such as those of ulcerative colitis. The reactivity of the cross-reacting antigen in the migration inhibition factor test points at its possible participation in reactions of cellular immunity as well.

Gorzynski and Krasny (55, 57, 59) looked for possible tissue components cross-reacting with ECA in mice. They found cross-reacting material, when measured by HAI, in extracts of livers and to a less extent of spleens and kidneys. When a more sensitive assay, the ability of the extracts to prime rabbits for a low dose of immunogenic ethanol-soluble ECA, was used, many tissues of most mouse strains seemed to contain cross-reactive material. It was suggested that the presence of the cross-reactive material in mouse tissues could account for the generally poor response of mice to immunization with ECA, and also for differences between mouse strains (62). However, other animals have not yet been studied in the same way, although the authors apparently plan to do so. In their tests colon was never a very good source of the cross-reacting material, contrary to the studies in rats and humans by Perlmann et al. (155).

Holmgren et al. (79) have found cross-reactivities between human kidney tissue and *E. coli* and suggest that these may have a role in the pathogenesis of renal lesions in pyelonephritis. Antisera to several (but not all) *E. coli* serotypes precipitated extracts of kidney tissue but not of liver or spleen. Anti-human kidney serum gave precipitation reactions with extracts of the same *E. coli* types. One of the precipitation lines showed a reaction of identity between the three *E. coli* strains, one of which was O14. With the data given, the possibility that this could be ECA is not excluded, although it is made somewhat unlikely by the absence of precipitation with several other *E. coli* strains.

OTHER CROSS-REACTIVITIES AND RELATED ANTIGENS

Various "Common" Antigens

Other common or "cross-reactive" antigens between different bacteria have been described, some of which may be identical with ECA whereas others can be clearly differentiated from it.

Brodhage (20–22) described a serological cross-reactivity probably caused by ECA, which he called a common, or "C," antigen. It was detected in enteric bacteria by indirect HA using urea extracts of the bacteria. The antiserum showing these cross-reactions was an anti-*Shigella sonnei* serum probably containing anti-ECA; *S. sonnei* cultures usually have a proportion of R mutants (44), and these have the R1 type of LPS core, connected with immunogenic form of ECA.

The outer membrane of all gram-negative bacteria has a similar overall composition of LPS, (lipo)proteins, and lipids (16, 105, 150). The LPS is responsible for the O-antigenic differences that form the basis of the hundreds of

serotypes in *E. coli*, *Salmonella* ("species"), etc. (109). These O-antigenic specificities reside in the distally located polysaccharide chains of LPS. The other end of the LPS molecule constitutes of a lipid, designated lipid A, which is probably embedded in the outer membrane. The lipid A has, with a few exceptions, a very similar structure in all gram-negative bacteria (161) and could thus represent a common antigen. This is, however, not easily demonstrable—whole bacteria do not elicit anti-lipid A antibodies, and such antibodies react with isolated lipid A and only to a small extent with whole bacteria or with the complete LPS (52). The polysaccharide chains are attached to lipid A via the R-core oligosaccharide region. This core is identical in all *Salmonella* and rather similar (but not always serologically cross-reacting) in all members of *Enterobacteriaceae*. Best conserved in phylogeny is the "deep core," the part closest to lipid A. This deep-core region is represented by the Re LPS (or rather Re glycolipid) of the most deficient still viable R mutants (*rfaE* or "heptoseless") (108, 109, 173, 175). The isolated Re structure as well as the *rfaE* mutant bacteria are immunogenic. The resulting anti-Re antibodies react with Re-type LPS and the *rfaE* mutant bacteria. They do not agglutinate normal smooth bacteria, but stain them for indirect IF (233). This method gave positive reactions with many *Enterobacteriaceae* strains but also with several other gram-negative organisms. The specificity of the reaction was not controlled so as to exclude participation of other cross-reacting antigens. Anti-lipid A and anti-Re antibodies seem to occur in normal human sera, and their levels are increased in gram-negative infections (49, 113–115, 141). Their possible role in either enhancing (206) or, hopefully, protecting against (111, 114, 133) infection is being studied.

The outer-membrane proteins have only recently become amenable to detailed studies, which are showing even more similarities between all these bacteria. A lipoprotein (15) linked to peptidoglycan on the one hand and exposed enough on the cell surface to be accessible to antibodies on the other hand is a component apparently common to many gram-negative bacteria. Rabbit antisera prepared against whole bacteria of *E. coli*, *Salmonella*, and *Shigella* all have antibodies to this lipoprotein (17). It remains to be seen how far its fine structure is conserved among gram-negative organisms to support immunological cross-reactivity.

Other proteins of the outer membrane may also be fairly similar between different species, although differences in the molecular weights of the major proteins are known (77). Cross-reactivity due to such a major outer-membrane protein has been described between separate groups (grouping being based on the capsular polysaccharides) of *Neisseria meningitidis* (47).

Cross-reactivity extending from a wide range of gram-negative organisms to the gram-positive *Bacillus subtilis* and *Staphylococcus aureus* detectable with an anti-*Hydrogenomonas facilis* phospholipoprotein serum has been described (162). However, according to present knowledge, the exterior surfaces of gram-negative and gram-positive bacteria are so different that such a cross-reactivity is rather unexpected and should be carefully ascertained; so far no details of absorption studies have been given.

Seltmann found an acidic, thermolabile antigen common to all gram-negative bacteria and possibly glycoprotein in nature (179). Holmgren et al. and Kaijser (78, 79, 90) used immunodiffusion, immunoprecipitation, and immunoelectrophoresis techniques in screening gram-negative bacteria for common antigens. They detected (i) several components common between many *E. coli* strains and (ii) an electrophoretically fast-moving acidic antigen (probably the same described by Seltmann [179]) apparently common to *E. coli* and some *Proteus*, *Pseudomonas*, and *Neisseria meningitidis* strains but not to *Staphylococci*. This specific distribution seemed to preclude its identity with ECA. Also the fact that it was demonstrable with antisera other than anti-*E. coli* O14 speaks against it being ECA.

Several common antigens detectable by precipitation have been described in water extracts (85) or high-salt extracts (25, 26, 159) of *Salmonella* strains. They are apparently often caused by lipoprotein antigens and separate from ECA.

A phenomenon apparently similar to that observed with ECA was described in gram-positive organisms by Rantz et al. (157) and confirmed by Neter and others (6, 138, 140, 142). Simple extracts of many gram-positive bacteria (including *Streptococcus*, *Staphylococcus*, *Listeria*, and *Bacillus* species), but not of gram-negative ones, sensitized RBC for HA in certain human sera or in sera of rabbits that had been immunized with gram-positive but not with gram-negative organisms. The non-species-specific substance was characterized only very incompletely but appeared to behave like a polysaccharide. Whether the non-species-specific substance was a teichoic acid has so far not been clarified, but teichoic acids have since emerged as substances shared in identical or closely similar forms by many gram-positive bacteria and responsible for many of their

cross-reactions (for review, see reference 97). The staphylococcal common antigen is susceptible to the antigen-associated immunosuppression by LPS and many other substances as described for ECA (see "Immunogenicity").

Quite apart from these cross-reactive antigens shared by many phylogenetically related bacteria are another sort of cross-reactivities which we would like to call accidental. These are exemplified by the cross-reaction between the capsular polysaccharides of group B meningococci and *E. coli* capsular type K1 based on a similar polysaccharide, poly-*N*-acetylneuraminic acid (11, 14, 68, 92). It can be assumed that identical structural features among polysaccharides arise by chance with a reasonable frequency; the possible numbers of monosaccharides to be used as their building blocks are limited. Such cross-reactions are known not only between different bacteria but also between bacteria and animal or human cells: the Forssman antigen present in humans, guinea pigs, horses, sheep, chickens, etc., and also found in bacteria (19), and the immunodeterminants of several human blood group antigens shared also by plants and bacteria (83, 187–189). Such cross-reactions have been ascribed roles in the pathogenesis of infectious diseases, and especially of their reactive sequelae (79, 91). Many of these cross-reactions have been summarized in a review (84).

ManNUA-Containing Bacterial Antigens

Although ManNUA is a very rare sugar, it is not restricted to ECA. ManNUA was first discovered by Perkins (154) as a constituent of a polysaccharide antigen of *Micrococcus lysodeikticus*, in which it replaces the usual teichoic acids as teichuronic acid (73). ManNUA has also been found in other bacterial antigens. Together with glucose it forms the above-mentioned teichuronic acid of *M. lysodeikticus* (73, 74, 199) and also the capsular K7 antigen of *E. coli* O7 and O14 (81, 94, 124). In combination with fucosamine, it is found in a surface antigen of the H variant of *Staphylococcus aureus* (232). ManNUA also occurs in the K15 antigen of *Vibrio parahaemolyticus* (168, 198).

The apparent presence of two independent ManNUA-containing antigens in *E. coli* O14:K7, i.e., in the strain in which Kunin (100) first found the ECA, is surely noteworthy. Preliminary investigations carried out with acapsular mutants of *E. coli* O14 and O7 (i.e., with *E. coli* O14:K7$^-$ and O7:K7$^-$) revealed that both mutants still contain the ECA determinant in fair amounts (G. Schmidt, D. Männel, and H. Mayer, unpublished data); however, K$^-$ mutations are often leaky, and therefore this evidence is not very strong. ManNUA-containing ECA preparations are obtained from many *Salmonella*, which have no K antigens, and in them the ECA could not have been mixed up with a capsular polysaccharide. Absorption of an ECA-antiserum with heat-killed *M. lysodeikticus* showed no lowering of the ECA titer, suggesting that no cross-reaction exists between ECA and the teichuronic acid of *M. lysodeikticus*, although ManNUA is also part of the serological determinant of the latter (199; our unpublished results).

The biosynthesis of ManNUA starting from UDP-*N*-acetylglucosamine (UDPGlcNAc) was recently elaborated in *E. coli* O14 by Kawamura et al. (94), who were able to separate the enzymes responsible for epimerization and dehydrogenation. UDPGlcNAc is apparently (94) first epimerized to UDPManNAc and then converted to UDPManNAcUA, the activated form of ManNUA. These findings are expected to facilitate future studies of the biosynthesis of ECA as well as interpretation of genetic data (see P. Kiss, thesis, University of Freiburg, 1975).

DISTRIBUTION OF ECA: TAXONOMIC IMPLICATIONS

Kunin (101) defined the distribution of ECA in a way that turned out to be precisely correct: he found it in a "large variety of groups belonging to *Enterobacteriaceae*" but not in other gram-negative organisms (*Brucella*, *Pseudomonas*, *Bordetella pertussis*) nor in gram-positive bacteria (*Staphylococcus*, *Streptococcus*, *Sarcina*, *Bacillus*). Table 6 shows the bacteria that have been tested for the presence of ECA in buffer extracts. In most cases the ability of the extract to sensitize RBC for HA and in some cases also the inhibition of HA was tested.

Many strains from all known genera of *Enterobacteriaceae* (23, 45) have been shown to contain ECA. Gram-positive strains and many gram-negative strains not classified as *Enterobacteriaceae* have been recognized as ECA negative. Representatives of some families that may be closest to *Enterobacteriaceae* (such as *Azotobacteraceae*, *Rhizobiaceae*, *Halobacteriaceae*, *Nitrobacteraceae*, and *Bacteroidaceae*) are unfortunately missing from the list of examined bacteria, which is medically oriented.

In some instances the presence or absence of ECA may be an additional taxonomic criterion as discussed by Le Minor et al. (106) and Bader (10). *Yersinia* have recently been recognized on the basis of their biochemical properties as separate from *Pasteurella* (186, 197) and tentatively transferred to *Enterobacteriaceae* (130, 131); this transfer will very probably be ac-

cepted. The presence of ECA in *Yersinia* and its absence in *Pasteurella* supports this classification.

The positions of *Erwinia* and *Pectobacterium* are not completely clear; they are included in the same genus of *Enterobacteriaceae* in the 8th edition of *Bergey's Manual* (23). It is quite possible that the group is very heterogeneous and contains members close to *Enterobacteriaceae* (also ECA positive) and others that should not be so classified (six strains of "*Erwinia chrysanthemi*" examined were all ECA negative) (18, 53, 169).

Although *Aeromonas* resembles *Enterobacteriaceae* in some of its fermentative properties, it is classified in *Vibrionaceae* (23) and is also ECA negative. Several *Vibrio* strains have also been found to be ECA negative. In contrast, bacteria variously classified as *Plesiomonas* or *Aeromonas* or *Vibrio shigelloides* (46, 69, 76) are ECA positive. The specific name *shigelloides* refers to antigenic cross-reactivity with *Shigella sonnei*, which seems to involve the O antigen (46). This applies to both the O-specific polysaccharide, which is serologically similar to that of *S. sonnei* (phase I, the smooth form) and to the R core, which is of the R1 type (T. Kontrohr, Pécs, personal communication). The ECA was apparently not LPS linked, and only the ethanol-soluble fraction was immunogenic (210), as expected of ECA-positive smooth strains. We propose that the taxonomic position of *Plesiomonas* be reexamined for inclusion in *Enterobacteriaceae* as originally suggested (46).

ECA-positive bacteria can understandably lose through mutation their capacity to produce ECA. Such ECA-negative mutants have been described in *Salmonella* (119, 120) and *E. coli* (see "Genetic determination of ECA"). A few ECA-negative strains have been encountered in surveys among mainly ECA-positive strains of a species (e.g., one *Serratia*) (101) and some *Proteus* strains (106) (Table 6), and it is likely that they also represent such mutants.

SUMMARY

It has become clear that all strains (with the exception of defective mutants) of bacteria belonging to the family of *Enterobacteriaceae* share at least one antigenic component—the enterobacterial common antigen, ECA—not present in other gram-negative or gram-positive organisms. This antigen is demonstrable by indirect hemagglutination and by other means. A pure anti-ECA serum is not yet available, and tests on ECA are always based on demonstrating cross-reactivity between different genera. Whenever doubt of the identity of ECA arises, rabbit anti-*E. coli* O14 serum should be the standard anti-ECA and HA and HAI should be the standard methods, (using RBC coated with extracts of several different enterobacterial strains) as defined by Kunin (101). There may be more than one antigen shared by the enteric bacteria, and this should be clarified as soon as possible. We tend to believe that the ECA defined as above is one antigenic entity, but the evidence is mostly indirect, and a final chemical identification is urgently needed.

It seems that the ECA in most bacteria is present in a "haptenic" form. This form of ECA has a propensity to aggregate with various hydrophobic structures, for instance with LPS in cell extracts or with the RBC membranes to sensitize them for hemagglutination. In some bacteria, however, ECA is linked, probably covalently, to LPS.

Recently a preparation of ECA free of LPS and active in various serological tests for ECA has been obtained. Even if it is not completely pure (two lines are detected by immunoprecipitation), at least 50% of its weight is accounted for by a heteropolymer of GlcNAc and ManNAcUA. The latter sugar appears essential for its serological specificity. This material is present in various ECA-positive species of enteric bacteria but absent from their ECA-negative mutants. The behavior of these mutants and ECA-positive recombinants obtained from them constitutes the strongest evidence for this material representing ECA. Some fatty acid, mainly palmitic, is linked to the GlcN-ManNUA polymer. ECA therefore seems to be an amphiphilic molecule in which the sugar units (at least the ManNUA) are responsible for the immunological specificity and the hydrophobic part is responsible for the aggregation tendency.

The LPS-linked form of ECA has the same immunological specificity and therefore should contain ManNUA. This is probably linked to the nonreducing terminus of the LPS core, since the requirements for this linkage are very strict. An unusual type of core structure (without the terminal glucosamine present in most other types of cores) is required; furthermore, this core must be complete but unsubstituted by O side chains. The linkage region has not yet been isolated to prove that a covalent linkage exists. However, suggestive evidence is provided by the fact that this linkage requires the function of the *rfaL* gene, which determines a translocase needed to link the O side chain to core.

Recently, several kinds of mutants blocked in the biosynthesis of ECA have been found. They indicate connections between the biosynthesis

of ECA and LPS. The *rfe* genes (close to *ilv*) participating in the synthesis of the O side chain of several (but not all) *Salmonella* and *E. coli* serogroups are required for the synthesis of ECA in all groups. It seems possible that the common step is the synthesis or modification of a carrier molecule for the synthesis of both these polysaccharides. Other, *rff*, genes, also close to *ilv*, are required for the synthesis of ECA only; they may be responsible for steps specific to ECA, for instance in the synthesis and transfer of ManNUA. Further, the *rfb* gene cluster of *Salmonella typhimurium* is also involved in determining the presence of ECA. Some ECA-negative mutants are very sensitive to detergents, suggesting an altered (outer) membrane structure. Further work with these mutants can be expected to give information of the biosynthesis of ECA as well as of its function in the bacteria and in relation to disease caused by these bacteria.

Only a few kinds of whole heat-killed bacteria – those in which ECA is linked to LPS, provisionally called "immunogenic strains" – elicit the production of anti-ECA antibodies. The LPS-linked ECA is a fairly good immunogen in rabbits and poor in mice. The non-LPS-linked ECA can also immunize rabbits when presented in a suitable form; a large aggregate size is a definite requirement. LPS and some lipids and detergents prevent this immunization; the injection of such mixtures does, however, not lead to true tolerance, as the animals become primed to a subsequent injection of an immunogenic form of ECA. The nature of this phenomenon urgently needs clarification.

ECA is probably present on the outer surface of the bacteria and can therefore be expected to be involved in reactions of the bacteria with their animal hosts. Anti-ECA antibodies are elevated in severe or chronic infections caused by enteric bacteria; their diagnostic potentialities would still require more study. ECA-negative mutants have a slightly reduced virulence. A protective function of anti-ECA antibodies has been suggested in experimental infection, but a correlation with presence or prognosis of human disease has so far not been shown. Further studies are, however, indicated, especially if better immunizing methods can be developed.

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