HYBRID FORMATION BETWEEN BACTERIAL ENDOTOXINS

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During the past several years, work from this laboratory has been concentrated on the physical structure of lipopolysaccharide endotoxins from Gram-negative bacteria and on the relationship between physical structure and biological activity. It was hypothesized that a minimum molecular size and a critical tertiary or quaternary structure were necessary for endotoxins to elicit their host-reactive properties (1, 2). Recently, we presented evidence in support of this hypothesis by demonstrating that endotoxins appeared to dissociate reversibly in the presence of bile salt (3, 4). In the dissociated state, pyrogenicity, antigenicity, and other biological properties of endotoxin were greatly diminished;[1] the pyrogenicity, for example, was reduced over one hundred fold (4). On the basis of these and other data, a working model was developed wherein the endotoxin was diagrammed as a micellar bundle of subunit chains held together by secondary or tertiary forces (2, 4).

Certain pure polysaccharides, such as the celluloses, are known to be composed of parallel polyglucose subunit chains (5). Nucleic acids and some proteins also possess multichain structures (see reference 6). When paired strands of nucleic acid are separated and mixed with the separated nucleic acid strands from another species or variety of organism before annealing, some of the duplexes formed contain one strand from each of the original nucleic acids (7). This hybrid formation between pairs of nucleic acids has been used to measure the homologies between the nucleotide sequences in nucleic acids of different origins (7–9). In proteins composed of subunit chains, notably the immunoglobulins, hybrid formation has been shown to occur between the subunit chains from two types of antibody, between antibody and normal globulin, and and between antibodies from two species of animals (10, 11).

The present report describes the formation of hybrids between the lipopolysaccharides of different Gram-negative bacteria. Hybridization was readily detected by immunological procedures because of the antigenic specificity of these

[1] Tarmina, D. F., et al. In preparation.

molecules. It is believed that the demonstration of hybrid formation between endotoxins and the ultimate elucidation of the mechanism of hybridization will contribute to the formation of a realistic structural model for these molecules.

Materials and Methods

Endotoxins.—Extracts prepared from several strains and species of bacteria were used in these experiments. The source of endotoxin used in any particular procedure will be indicated in the text; however, a more complete description of the preparations follows:

1. Strain S 795 of *Salmonella enteritidis* was grown in a synthetic medium (see reference 4) containing inorganic salts and 0.5% glucose as the sole carbon source. Freshly harvested and washed cells were extracted with water saturated with diethyl ether as described previously (12, 13). A phenol-water extract of fresh whole *S. enteritidis* cells was prepared by the method of Westphal et al. (14). In one case an endotoxin was extracted by the aqueous ether method (12) from *S. enteritidis* which had been grown in the synthetic medium containing randomly labeled glucose-^{14}C as the carbon source. This endotoxin had a specific activity of 11.2 μc/mg and is designated Se ^{14}C endotoxin.

2. *Escherichia coli* strains 0113 (Braude) and 0111:B4 (Difco) were grown in the synthetic medium referred to above. Washed suspensions of bacteria were disrupted in a refrigerated pressure cell (Sorvall, Ivan, Inc., Norwalk, Conn.) and separated into cell wall and protoplasm as described previously (15). Endotoxins were extracted from the cell walls by the phenol-water procedure; native protoplasmic polysaccharides (NPP) (formerly called native haptens) were extracted from the protoplasm with trichloroacetic acid (16).

3. *Salmonella typhi* 0-901 was grown in trypticase soy broth. Endotoxin was extracted from fresh whole cells by the phenol-water procedure.

4. *Proteus mirabilis* strain VI (17) was grown in Brucella broth (Albimi Laboratories). The cells were disrupted in a refrigerated pressure cell, and endotoxin was extracted from the separated, washed cell walls with phenol-water. We are indebted to Dr. Claes Weibull for this preparation.

5. *Serratia marcescens* (Havas strain) was grown in the synthetic medium with 0.5% glucose. Freshly washed cells were extracted with cold trichloracetic acid by the procedure described previously (12).

6. For preparation of the acid hapten, a phenol-water endotoxin extracted from cell walls of *E. coli* 0113 (Braude) was hydrolyzed with 0.1 N acetic acid in a boiling water bath for 180 min (18). After neutralization, the preparation was centrifuged at 60,000 *g* for 4 hr, and the supernatant fluid was dialyzed against distilled water and lyophilized.

Method of Hybridization.—Endotoxin, acid hapten, and/or native protoplasmic polysaccharide were dissolved in water to 2 mg/ml concentration. Equal amounts of the solutions were mixed, and to this was added 1 volume of 4% sodium deoxycholate (NaD) in 0.2 M Tris (hydroxymethyl)aminomethane buffer, pH 8.0 (Tris). This final mixture, containing 1 mg/ml total polysaccharide (or 500 μg/ml of each component) in 2% NaD in 0.1 M Tris, was incubated for 15 min at room temperature (22–24°C). Then the mixture was precipitated with 6 volumes of absolute ethanol for 30 min at room temperature and centrifuged at 1000 *g* for 30 min. The precipitate was suspended in 6 volumes of absolute ethanol and recentrifuged. The final washed precipitate was dissolved in a volume of water equal to that of the 2% NaD-polysaccharide solution and dialyzed overnight at 4°C against distilled water (hybrid or HYB.). In some cases, indicated in the text, NaD was removed from the samples by dialysis against daily changes of distilled water (adjusted to pH 7–8 with a small amount of NaOH) for 5 days at 4°C.

Controls for the hybridization included treating the individual components of the hybrid

pair with 2% NaD and removal of the NaD by ethanol extraction before the samples were mixed and dialyzed (MIX.). In addition, samples were mixed in Tris and the mixture precipitated with ethanol and dialyzed (ALC. MIX.).

Immunological Procedures.—The presumptive hybrids and control mixtures were compared by the immunodiffusion technique of Ouchterlony (19). Precipitation patterns were recorded

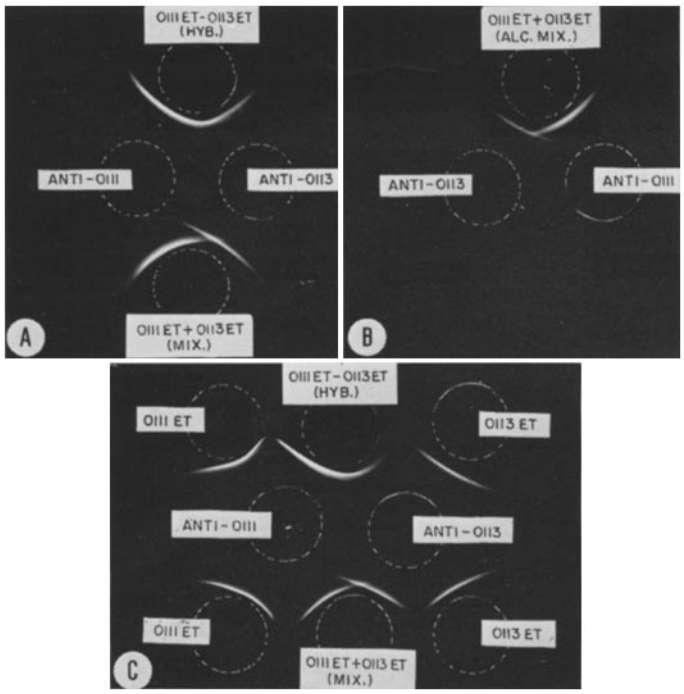
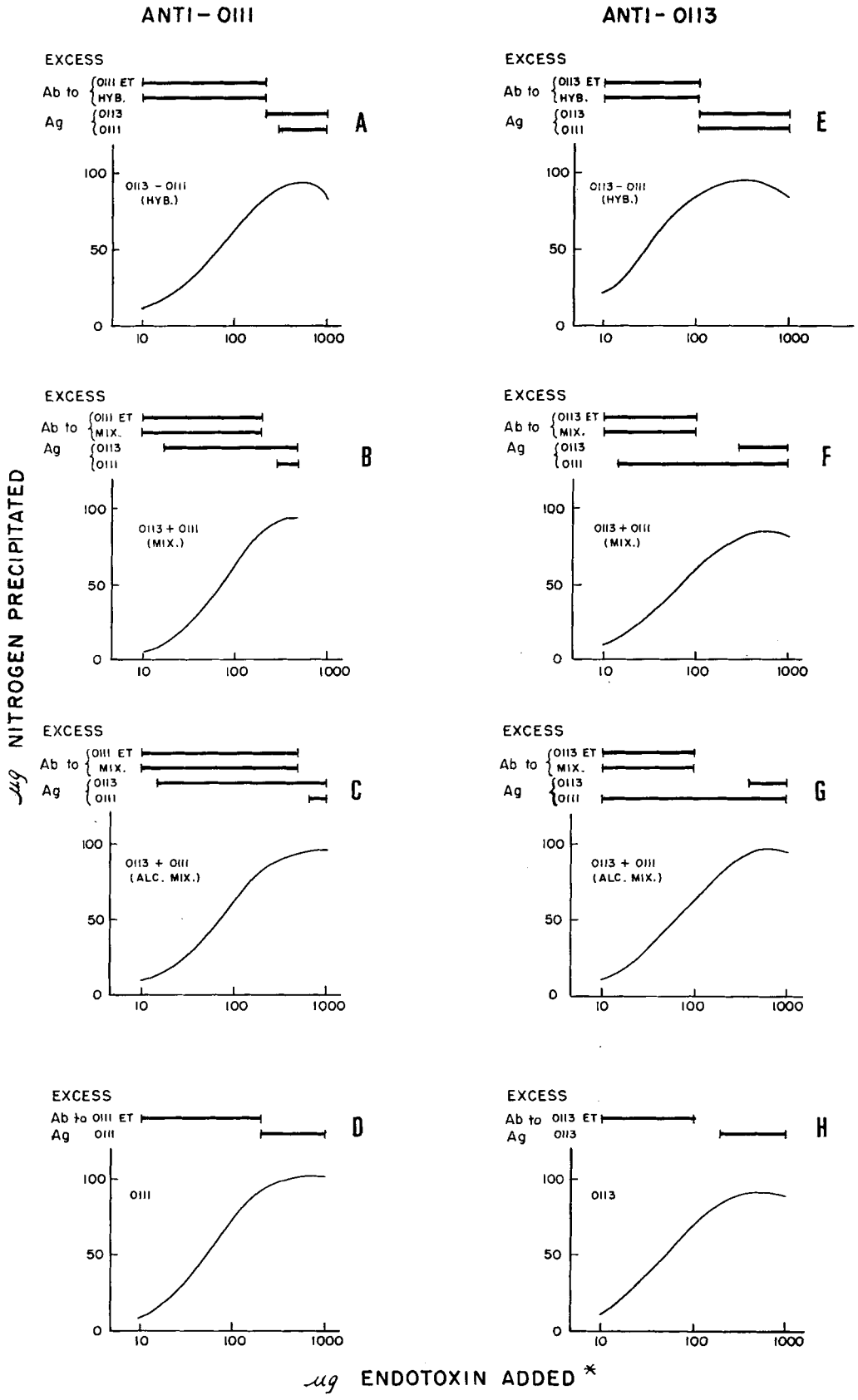


FIG. 1. Hybrid formation between *E. coli* 0111 and *E. coli* 0113 endotoxins (ET). (A) Comparison of the immunodiffusion pattern of the hybrid versus the pattern of endotoxins which were treated separately with NaD, precipitated with ethanol, and then mixed; (B) immunodiffusion pattern of the mixture of endotoxins which was only precipitated with ethanol; (C) relationship of the precipitation patterns of the hybrid, the mixture, and those of the parent endotoxins. Total endotoxin concentration was 1 mg/ml, i.e., 500 μg of each endotoxin per milliliter.

photographically while the plates were illuminated with oblique light. After being photographed, those plates containing ^{14}C-labeled endotoxin were washed, dried under filter paper, and taped to high contrast Kodak projector plates, agar to emulsion, for 3 wk. After development of the projector plates by standard procedures, prints were made using the projector plates as negatives. Antigen and antiserum wells of the plates pictured in this paper have been outlined with white ink for better contrast.

Quantitative precipitation tests were performed by adding varying amounts of the endotoxin or polysaccharide materials to a constant volume (0.5 ml) of rabbit antiserum. Total volumes were then adjusted to 1.5 ml with phosphate buffered saline (0.15 M NaCl; 0.0033 M



* The amount of endotoxin added is expressed as the total amount; each serotype would provide half the amount indicated. The curves in D and H were obtained with half the amount of endotoxin indicated because only one endotoxin was contained in each sample.

PO_4; pH 7.4) (PBS), and the samples were incubated for 2 hr at 37°C and for 18 hr at 4°C. After two washings of the precipitates with ice-cold PBS, the total nitrogen precipitated was determined colorimetrically by a modification of the Nessler reaction (20) with ammonium sulfate as the standard. Supernatant fluid analyses for antigen and antibody excess were carried out in the usual manner (21).

Antisera were obtained from rabbits after multiple subcutaneous and intravenous injections of heat-killed whole bacteria.

Bioassays.—Assays for pyrogenicity in rabbits and lethality for chick embryos were performed as described previously (22).

RESULTS

Hybrid Formation between E. coli Endotoxins.—Previous investigations have shown that a 2% concentration of NaD was sufficient to dissociate endotoxins completely, as demonstrated by the absence of a fast peak in the analytical ultracentrifuge (4). This concentration of bile salt, therefore, was used throughout the present experiments.

Fig. 1 shows precipitation patterns obtained in immunodiffusion plates with the hybrid formed between endotoxins of *E. coli* serotypes 0111 and 0113 together with the precipitation patterns given by control solutions. The junction of continuity in the hybrid pair and the crossing of the precipitation arcs in the mixtures of endotoxins indicate, qualitatively, that the antigenic groups, which previously were unique to each variety of endotoxin, had combined to form a single molecular species and that hybridization was mediated through NaD treatment. Quantitative evidence for hybrid formation is presented in Fig. 2. Of particular importance are the supernatant fluid analyses. When the hybrid was precipitated by either antiserum, the antigens particular to both varieties of endotoxin were precipitated and thus were removed from the supernatant fluid at the same rate (Fig. 2, A and E). However, the mixtures of NaD-treated endotoxins (Fig. 2, B and F) or the alcohol-precipitated mixtures (Fig. 2, C and G) showed no evidence that the antigenic groups of the two endotoxins were on the same molecule. In these latter instances, the endotoxin heterologous to the antiserum remained in the supernatant fluid while the homologous endotoxin was precipitated.

Hybrid Formation between E. coli and S. enteritidis Endotoxins.—Results similar to those above were obtained when an *E. coli* 0111 endotoxin and a phenol-water-extracted endotoxin of *S. enteritidis* were treated with 2% NaD under conditions conducive to hybrid formation (Figs. 3 and 4). The control

FIG. 2. Hybrid formation between *E. coli* 0111 and *E. coli* 0113 endotoxins (ET). (A and E) Quantitative precipitation curves of the hybrid; (B and F) endotoxins which were treated separately with NaD, alcohol precipitated, and mixed; (C and G) the mixture of endotoxins which was only precipitated with ethanol; (D and H) and the NaD-treated, alcohol-precipitated endotoxins alone. The zones of excess antigen (Ag) and antibody (Ab) in the supernatant fluids are indicated by the bars above the curves.

mixtures again indicated that the hybridization was mediated through the NaD treatment and not by the alcohol precipitation or the dialysis procedures.

Hybridization between *S. enteritidis* and *E. coli* 0113 endotoxins was demonstrated by a different method. A biosynthetically ^{14}C-labeled endotoxin from *S. enteritidis* was mixed with endotoxin from *E. coli* 0113. The mixture was treated with 2% NaD and dialyzed to remove the NaD. The control consisted of a mixture of the endotoxins which was dialyzed without prior NaD treatment. Immunodiffusion patterns of these preparations are shown on the left side of Fig. 5 (A, C, and E). Radioautographs of these same plates are on the right side of the figure (B, D, and F). Of particular interest are the bottom pair of

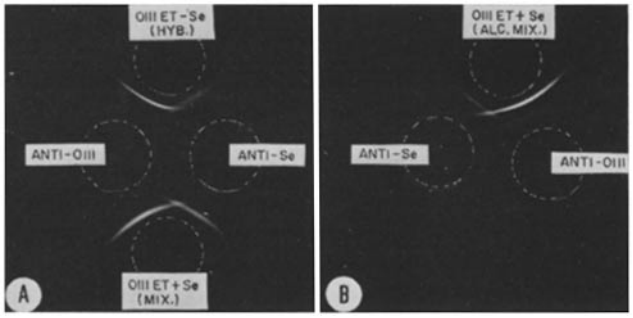


FIG. 3. Hybrid formation between *E. coli* 0111 and *S. enteritidis* (Se) endotoxins (ET). (A) Comparison of the immunodiffusion patterns of the hybrid versus the pattern of endotoxins which were treated separately with NaD, precipitated with alcohol, and mixed; (B) immunodiffusion pattern of the mixture of endotoxins which was only precipitated with alcohol. Total endotoxin concentration was 1 mg/ml, i.e., 500 μg of each endotoxin per milliliter.

plates (E and F). In these the junction of continuity of the hybrid preparation is best demonstrated. It is also apparent that the anti-*E. coli* serum and the anti-*S. enteritidis* serum did not contain antibodies to cross-reacting antigens. Despite the lack of cross-reactions between the systems, however, the radioautograph of the control mixture of endotoxins (Fig. 5, F) indicates that radioactive material was precipitated by anti-*E. coli* serum. An explanation for this observation will be offered in the discussion of possible mechanisms of hybrid formation.

Attempts to Form Hybrids between Endotoxins and Other O-Antigenic Materials.—With the same procedure employed for hybridizing endotoxins, attempts were made to induce hybridization between native protoplasmic polysaccharides of *E. coli* and heterologous endotoxins. Fig. 6 (A and B) shows that the presumptive hybrids exhibited immunodiffusion patterns which indicated no evidence of dual antigenic specificities on one molecule. That hybridization did

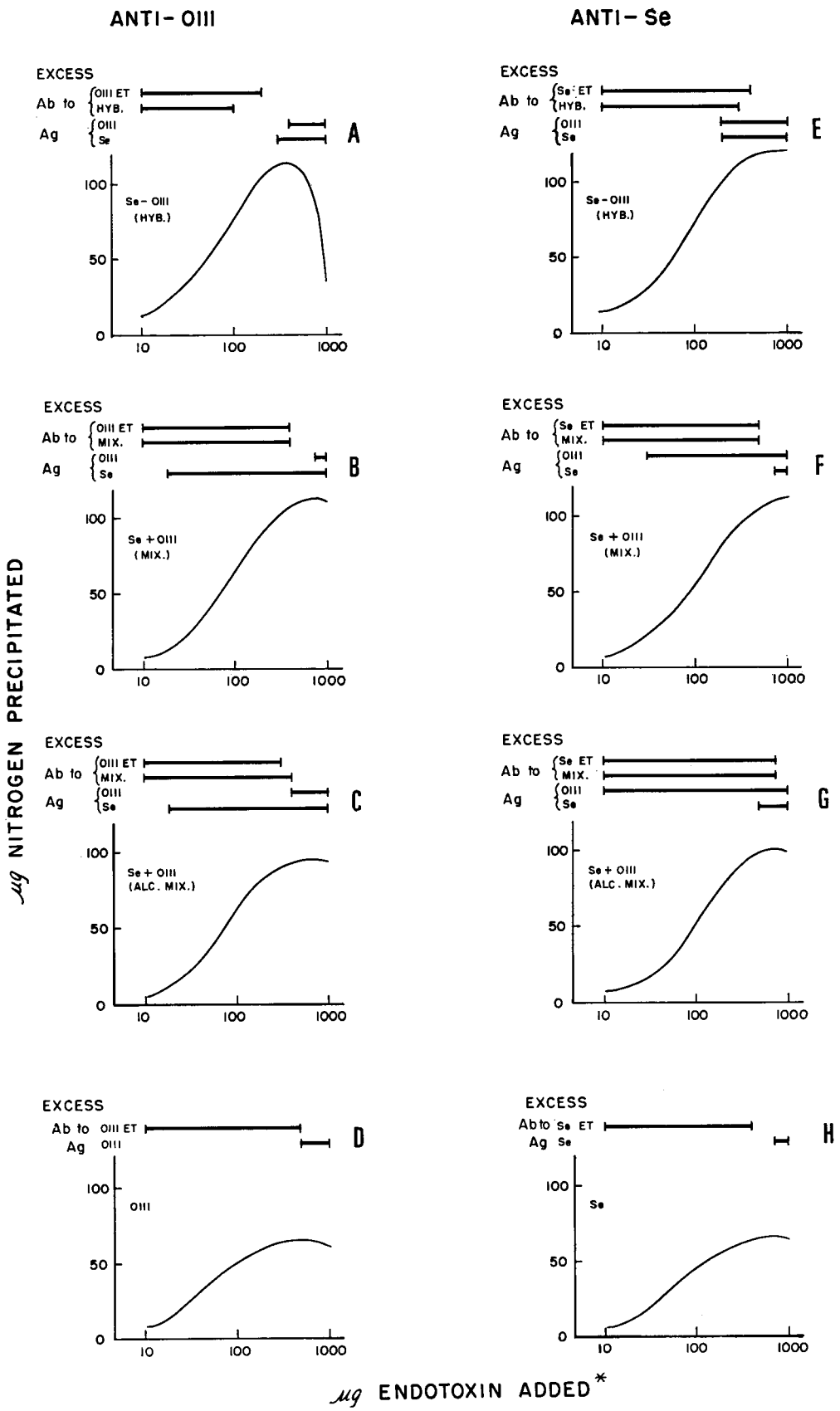


FIG. 4. Hybrid formation between *E. coli* 0111 and *S. enteritidis* (Se) endotoxins (ET).

*See legend and footnote for Fig. 2.

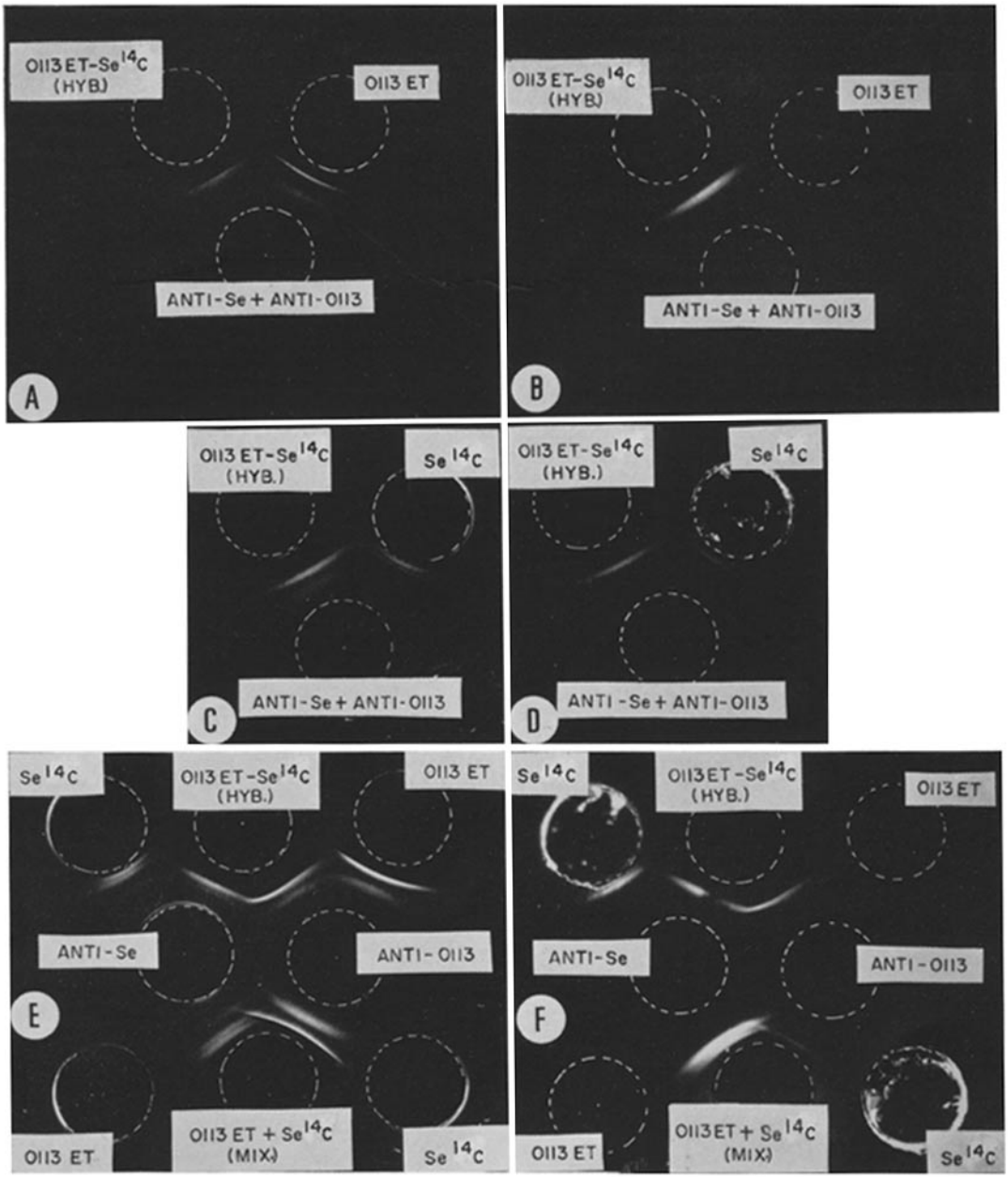


FIG. 5. (A, C, and E) Immunodiffusion patterns and (B, D, and F) corresponding radioautographs of *E. coli* 0113 endotoxin (ET) and ^{14}C-labeled endotoxin from *S. enteritidis* ($Se^{14}C$): Comparison of hybrid, control mixtures, and individual endotoxins. Total endotoxin concentration for the hybrid and the mixture was 1 mg/ml, i.e., 500 μg of each endotoxin per milliliter.

not occur was confirmed by demonstrating that quantitative precipitation of these presumptive hybrids with an antiserum of one specificity did not precipitate both antigenic specificities concomitantly (Fig. 7). In Fig. 6, C, it is also

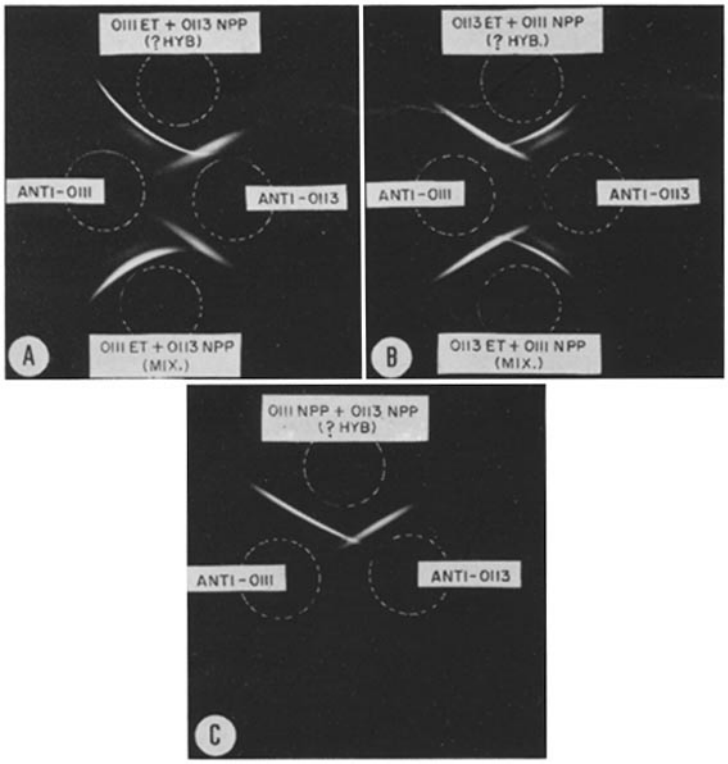
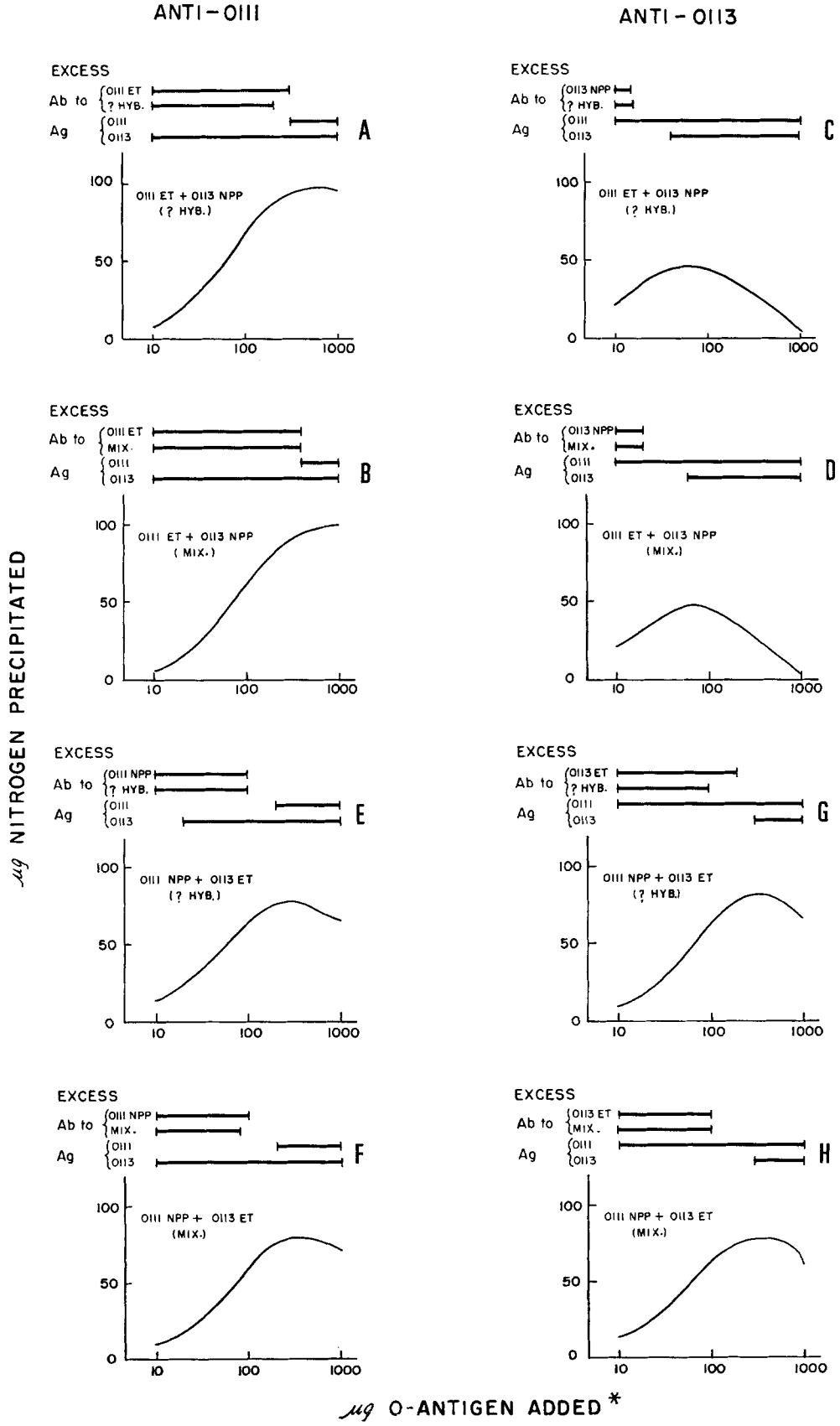


FIG. 6. Attempt to form hybrids between *E. coli* endotoxins (ET) and native protoplasmic polysaccharides (NPP). (A) Immunodiffusion patterns of the attempted hybrid between *E. coli* 0111 ET and *E. coli* 0113 NPP compared with the patterns of the components treated separately with NaD and precipitated with ethanol before mixing; (B) immunodiffusion patterns of the attempted hybrid and control mixture of *E. coli* 0113 endotoxin and *E. coli* 0111 NPP; (C) immunodiffusion pattern of the attempted hybrid of two NPP. The total 0-antigen concentration was 1 mg/ml, i.e., the endotoxin and NPP each provided 500 μg/ml.

seen that the two native protoplasmic polysaccharides would not hybridize with each other.

The polysaccharide hapten produced by acid degradation of endotoxin could not be induced to hybridize with the complete endotoxin from another strain of *E. coli*, as evinced in Figs. 8 and 9. Thus it appeared that only endotoxins could form hybrids with endotoxins and that other materials, although antigenically



* See footnote, Fig. 2.

related to endotoxins, lacked some essential component necessary for hybrid formation.

Spectrum of Endotoxins Participating in Hybrid Formation.—As of now, only endotoxins from bacteria of the family Enterobacteriaceae have been tested for capacity to form hybrids with each other, but hybridization appears to have been achieved between representatives of several genera in this family (Fig. 10). In preparation of these hybrids, the NaD was removed by dialysis rather than by extraction with ethanol; the controls consisted of mixtures of endotoxins that were dialyzed for a similar length of time.

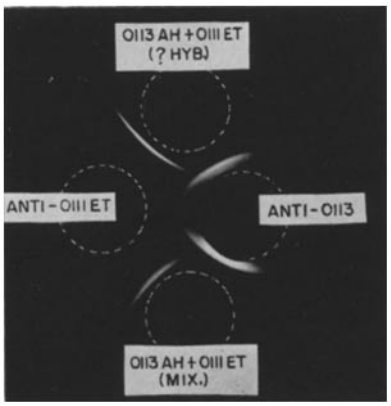


FIG. 8. Attempt to form a hybrid between *E. coli* 0111 endotoxin (ET) and the acid hapten (AH) from *E. coli* 0113 endotoxin. Immunodiffusion patterns of the attempted hybrid and the control mixture. The total O-antigen concentration was 1 mg/ml, i.e., the ET and the AH each provided 500 μg/ml.

Biological Activity of Hybrid Endotoxins.—Complete biological assays were not performed on the hybrid endotoxins and their controls because spot checks on selected preparations showed that the pyrogenicity for rabbits and lethality for chick embryos of the hybrids were not significantly different from values which might be expected in mixtures of the two endotoxins. Examples of the values obtained are presented in Table I.

DISCUSSION

Not all the implications of hybrid formation between bacterial lipopolysaccharides can be clearly perceived at this time. A homologous or comple-

FIG. 7. Attempt to form hybrids between *E. coli* ET and *E. coli* NPP. (A, C, E, and G) Quantitative precipitation curves of the attempted hybrids and (B, D, F, and H) the curves of the control mixtures. The zones of excess antigen (Ag) and antibody (Ab) in the supernatant fluids are indicated by the bars above the curves.

mentary structure among the various endotoxins that have been shown to hybridize is suggested by analogy with hybrid formation between nucleic acids

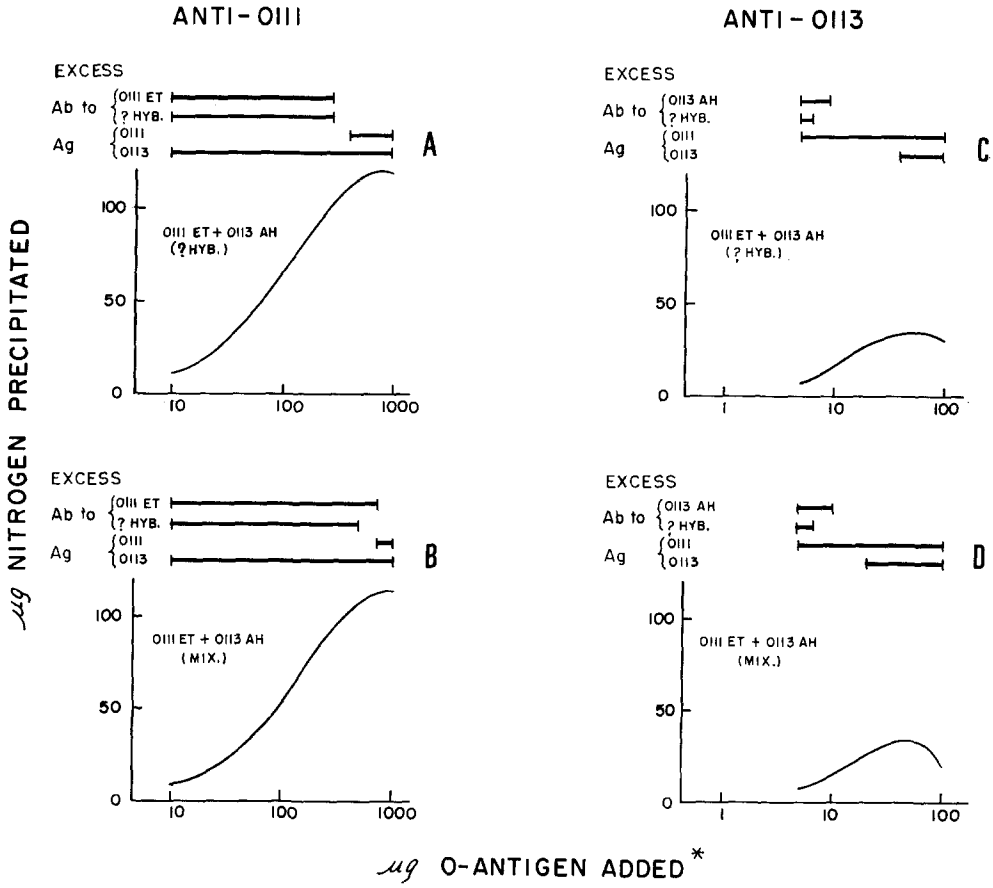


FIG. 9. Attempt to form a hybrid between *E. coli* 0111 endotoxin (ET) and the acid hapten (AH) from *E. coli* 0113 endotoxin. (A and C) Quantitative precipitation curves of the attempted hybrid and (B and D) the curves of the control mixture. The zones of excess antigen (Ag) and antibody (Ab) in the supernatant fluids are indicated by the bars above the curves.

*See footnote, Fig. 2.

wherein the nucleotide sequence and base-pairing are the factors that determine the success of the reaction (7, 9). Although the mechanism for the hybridization of endotoxins remains unknown, we can suggest three possible structures which could account for the properties of the hybrid molecules (Fig. 11).

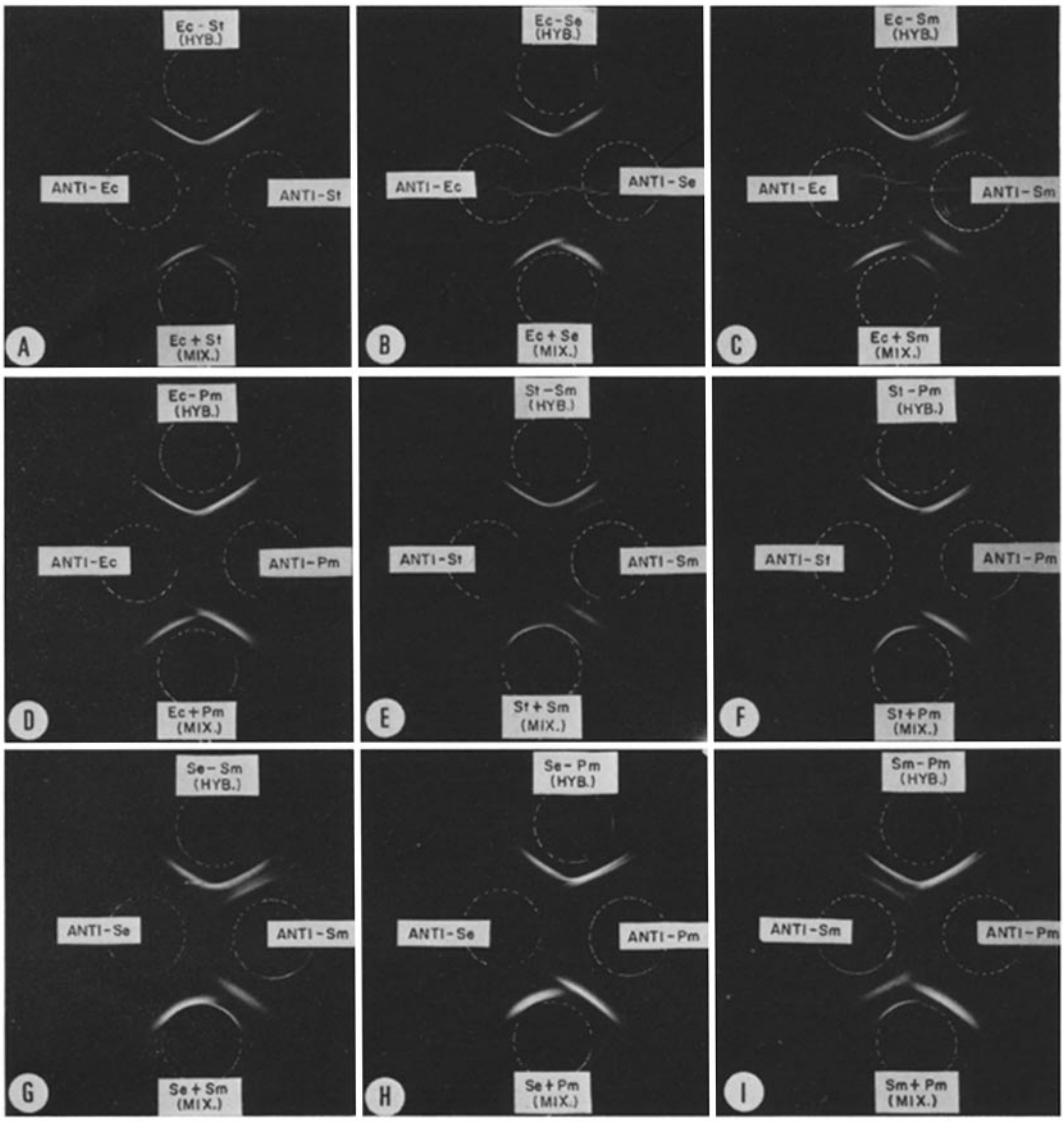


FIG. 10. Hybrid formation among several endotoxins. In this case the NaD was removed from the hybrids by dialysis. Controls are mixtures of the endotoxins which were only dialyzed. The abbreviations for the endotoxins are as follows: *E. coli* 0111 (Ec); *S. typhi* (St); *S. enteritidis* (Se); *Serratia marcescens* (Sm); *Proteus mirabilis* (Pm). Endotoxin concentration was 1 mg/ml; each endotoxin contributed 500 μg/ml.

Free subunit chains, upon dilution or removal of bile salt, might reaggregate homogeneously into the basic endotoxic elements which could then concatenate randomly into mixed chains, as in Fig. 11, A. However, solubility behavior and electron micrographs do not indicate a chain of great length in the freshly

TABLE I

Biological Activity of Hybrid and Parent Endotoxins

Endotoxins	Pyrogenicity FI_{40}*	Toxicity $CELD_{50}$‡
	μg	μg
E. coli 0111	0.95	0.0045
E. coli 0113	0.15	0.0095
S. enteritidis	0.47	0.0065
0111-Se (HYB.)	0.18	0.0035
0111-0113 (HYB.)	0.24	0.0040

* The FI_{40} (fever index 40) represents the amount of endotoxin which will produce a febrile response in rabbits such that the area under the fever curve, plotted on a standardized graph paper, will be 40 cm^2 (22).

‡ $CELD_{50}$ represents the median lethal dose of endotoxin injected intravenously into 11-day-old chick embryos.

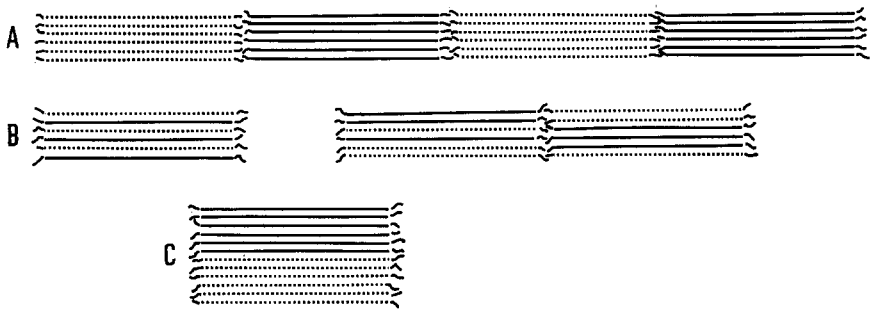


FIG. 11. Schematic diagram of three possible mechanisms to explain the hybridization of endotoxins.

isolated hybrids. Alternatively, and much more attractively from a mechanistic point of view, the free subunit chains from two endotoxins could combine with each other randomly to form heterogeneous endotoxic elements (Fig. 11, B). These elements would then have the same potential for end-to-end polymerization, especially after exposure to phenol, that has been demonstrated in other endotoxins. Least likely, apparently, is the side-to-side association of homogeneous endotoxic elements in mixed pairs that is diagrammed in Fig. 11, C.

Electron micrographs of hybrid endotoxins (Rudbach, unpublished data) show that hybrid molecules have essentially the same diameter as normal endotoxins (3, 4). It should be possible to discriminate among the hypothetical models by proper application of immunological and biophysical techniques.

As reported here, neither native protoplasmic polysaccharide nor acid hapten could be hybridized with endotoxin. If continuing tests of various chemically-altered endotoxins and of endotoxin-related microbial products show that only endotoxic lipopolysaccharides are able to form hybrids with reference endotoxins, hybridization could be used as an in vitro test for endotoxins. It will be noted that, even when evidence of hybridization is obtained, there may be certain antigenic materials in a preparation of endotoxin which do not participate in hybrid formation (cf. *S. marcescens* preparation, Fig. 10, C, E, G, I). Such a finding suggests contamination of the original extract by nonendotoxic materials. In the example just cited, acid polysaccharide and/or haptenic substances serologically related to the endotoxin are likely to have been present.

It is probable that endotoxins, like other molecules, are in a state of equilibrium between association and dissociation. With endotoxins this equilibrium might be established among the toxic elements, the subunits, or both. Indeed, if this is the case, then one would expect some spontaneous hybridization to occur when endotoxins are mixed, even in the absence of a dissociating agent. In the experiment in which radioautography was used to detect hybridization between a ^{14}C-labeled *S. enteritidis* endotoxin and an *E. coli* endotoxin, some radioactivity was precipitated by anti-*E. coli* serum from the mixture of endotoxins which had been dialyzed for 5 days (Fig. 5). The resolution of the radioautograph is insufficient to show whether or not this faint line forms a junction of continuity with the precipitation pattern of the *S. enteritidis* antiserum–endotoxin. If a junction of continuity should be present, then some spontaneous hybridization would be indicated, as suggested above.

Previously we had come to regard the native protoplasmic polysaccharide (NNP) of *E. coli* as a product of abortive lipopolysaccharide biosynthesis rather than as a direct structural precursor of cell wall endotoxin (23). This conclusion was based on the size relationship between the NPP and the smaller endotoxic subunits (24). The present findings support the same opinion. If the endotoxin micelles were simply bundles of NPP chains, or even if some NPP chains were contained in them, one might expect that heterologous NPP could be incorporated into the toxic particles during reversible dissociation of the complexes with NaD. This, however, did not occur. Likewise, heterologous acid hapten failed to complex with endotoxin under the influence of NaD, although this particle is approximately the same size as the "surfactant hapten" of completely dissociated endotoxin (4, 24). Presumably, components that are necessary for complementation or binding in the endotoxin micelle are absent from NPP and have been destroyed during production of the acid hapten. Ingredients

of most endotoxins in which NPP is known to be deficient or lacking include heptose, 2-keto-3-deoxyoctulosonic acid, long chain fatty acids, and phosphorus, but not all of these are missing from acid hapten. The problem of identifying the active form and mode of attachment of the essential components remains formidable, but a solution might provide a chemical basis for the biological activity of endotoxins.

SUMMARY

When endotoxins extracted from enteric bacteria were mixed in the presence of sodium deoxycholate, and the bile salt was subsequently removed by dialysis or by extraction with ethanol, a new type of endotoxin was formed. The latter material was as biologically active as the original endotoxins and possessed a combination of antigenic determinants that were previously unique to each of the individual endotoxins in the mixture. This hybrid formation between endotoxins was detected by immunodiffusion and radioautography and by quantitative precipitation procedures.

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