the "mucocomplex" characteristic of bacterial cell walls. The marked resistance of *Mycoplasma* to cycloserine and penicillin therefore adds evidence to the concept that these antibiotics act by affecting bacterial pathways which synthesize the cell wall.

Summary. L phase growth of bacteria was produced by exposing growing organisms to high concentrations of penicillin and D-cycloserine. Of 96 bacterial strains examined, 16 produced L growth which could be maintained in serial subculture. Exposure of bacteria to cycloserine gave L phase growth only in strains which also produced L growth on exposure to penicillin. The penicillin and cycloserine sensitivities of the bacteria from which the L forms were isolated were compared with the sensitivities of the L forms. The concentrations of penicillin which inhibited L growth ranged from 2 to 1000 times the concentrations required for inhibition of bacterial growth. The concentrations of cycloserine required for inhibition of L phase growth were 4 to 60 times greater than those required for inhibition of bacterial growth. *Mycoplasma* exhibited marked resistance to both agents. The resistance of the L phase growth and *Mycoplasma* to penicillin and cycloserine is evidence to substantiate the role of these antibiotics in affecting bacterial pathways which synthesize the cell wall.

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Evidence for a Common Hapten Associated with Endotoxin Fractions of *E. coli* and other *Enterobacteriaceae.** (27734)

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E. coli may be serologically characterized by 3 major groups of antigens; the 0, or heat stable, antigens located in the endotoxin moiety; the H or flagellar antigens; and the K or envelope antigens. Kauffmann(1) and Ewing *et al.*(2) have characterized the antigenic relationships among the 145 known *E. coli* 0 antigen groups by means of the bacterial agglutination test, and have demonstrated a number of cross reactions between various groups. Serologic cross reactions have also

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been described among 0 antigens of members of the family *Enterobacteriaceae,* particularly between *Salmonella* and *Arizona* groups and *Shigella* and *E. coli,* but such reactions appear to be limited in number(3). Brodhage(4) has recently described a C, or common, antigen extracted with urea which cross reacts with various gram negative bacteria in the hemagglutination test; he distinguishes this from the 0, H and rough antigens. In addition, fimbrial antigens, which have the capacity to agglutinate erythrocytes, have been described by Duguid and his associates(5).

During studies reported elsewhere(6,7) which employed the hemagglutination test to characterize *E. coli* from urinary tract infections and to determine the distribution of antibodies in human serum and colostrum, it became evident that further information was needed concerning the serologic cross reactions among the various 0 groups which might be detected by this test. Accordingly, 137 of the 145 prototype 0 group strains and almost all of their corresponding rabbit antisera‡ were examined by means of the hemagglutination test described by Neter *et al.*(8) for reactions with homologous and heterologous antigens and antisera. All but a few antigens coated on erythrocytes reacted with their corresponding sera, and a small number of cross reactions were observed according to the general pattern described previously by others employing the bacterial agglutination test. Rabbit antiserum prepared against *E. coli* 014, however, was found to agglutinate erythrocytes coated with almost all of the 137 *E. coli* crude 0 antigen preparations tested. The nature of the nonreciprocal cross reaction observed with *E. coli* 014 antiserum in the hemagglutination test with fractions from numerous *E. coli* 0 groups, as well as with similar material from *Salmonella, Shigella, Proteus* and other *Enterobacteriaceae,* is the subject of the present report.

Materials and methods. Antigens were prepared from protype *E. coli* strains obtained from the Communicable Disease Center (CDC), and other bacteria in the collection of the Dept. of Microbiology, University of Virginia, by serial cultivation of organisms several times on heart infusion agar slants. Saline (0.9% NaCl) suspensions of these cultures were inoculated on the surface of heart infusion agar in petrie dishes and incubated overnight at 37°C. Cultures were harvested into 10 ml of saline and boiled for 2 hours, followed by centrifugation at 5,000 × *g* for 15 minutes at 0°C, and final addition of 1 ml of 95% ethyl alcohol to supernates. Supernates were stored at 4°C until used as antigen; sediments were discarded. Cultures were also prepared in chemically defined minimal liquid medium(9) supplemented with vitamins and amino acids, and treated as above to make antigens. Lipopolysaccharide endotoxin of *E. coli* 014 was prepared by a modification of the cold trichloracetic acid method of Boivin(10) after growing the organisms in chemically defined medium. Other lipopolysaccharides obtained commercially (Difco) had been prepared by the phenol extraction method for *E. coli* 055:B5 and 0127:B8 or by a modified Boivin method. (*S. typhosa* 0901, *S. marcescens, Sh. flexneri* and *Brucella abortus.*)

A 1:10 dilution of crude antigen, or 100 μg per ml of a lipopolysaccharide in Hanks' balanced salt solution (BSS) were incubated for 1 hour at 37°C in a water bath shaker with a 2½% suspension of human 0 erythrocytes; erythrocytes were obtained fresh and washed 3 times in BSS. The sensitized erythrocytes were then washed 3 times in BSS and adjusted to a final 2½% suspension in this medium. Antigen coated cells were employed in the hemagglutination test immediately after preparation. All sera were diluted serially in BSS. The hemagglutination test was conducted with 0.25 ml aliquots of cell suspensions and serum in plastic trays,§ incubated for 1 hour at 37°C, and read grossly by pattern and after shaking for agglutination.

Bacterial agglutination tests were performed by the method of Edwards and Ewing (3); agar gel precipitin tests were performed

‡ We are indebted to Dr. John Winn of the Laboratory Branch, Communicable Disease Center, Dept. of H.E.W., Atlanta, Ga., for supplying these reagents.

§ Obtained from Linbro Manufacturing Co., New Haven, Conn., as disposo-trays.

TABLE I. Inhibition of Hemagglutination Cross Reactivity of *E. coli* 014 Antiserum by Various Crude *E. coli* 0 Antigen Preparations and *S. typhosa* Lipopolysaccharide.*

Coated erythrocytes	014 antiserum preincubated with various antigens								Homologous antiserum preincubated with	
	BSS control	014	034	035	036	037	0144	*S. typhosa*	BSS control	014† antigen
E. coli 014	+	0	+	+	+	+	+	+	+	0
" 034	+	0	0	0	0	0	0	0	+	+
" 035	+	0	0	0	0	0	0	0	+	+
" 036	+	0	0	0	0	0	0	0	+	+
" 037	+	0	0	0	0	0	0	0	+	+
" 038	+	0	0	0	0	0	0	0	+	+
" 0144	+	0	0	0	0	0	0	0	+	+
S. typhosa	+	0	0	0	0	0	0	0	+	+

+ = hemagglutination; 0 = no hemagglutination.

* All antiserums tested at a dilution of 1:400, crude antigens diluted 1:10, lipopolysaccharide used at 100 μg.

† Separate studies with crude and Boivin preparation of 014 antigens.

by a modification of the Ouchterlony method (11).

Rabbits were immunized by 5 serial intravenous injections given 4 days apart of a boiled 0.3% formalin treated culture derived from a single, serologically confirmed, colony of *E. coli* 014 grown in brain-heart infusion broth. Rabbits were also immunized with a single intravenous injection of 20 μg of purified 014 Boivin type lipopolysaccharide, and bled at 6 days. *Salmonella* and *Shigella* 0 antigen groups used in hemagglutination tests were tested with commercial grouping antisera (Lederle).

Sera were adsorbed by incubating 10 ml of a 1:400 dilution of a serum with 3 successive 1-ml amounts of packed, antigen coated erythrocytes, each for 30 minutes at 37°C. Hemagglutination-inhibition or blocking tests were performed by preincubating a 1:400 dilution of rabbit immune serum with a 1:10 dilution of crude antigen for 30 minutes at 37°C. Coated erythrocytes were then added to the reaction mixture and read for hemagglutination as described above.

Results. Cross reactions among E. coli *0 antigen preparations and antisera.* All but 9 of the 145 known *E. coli* 0 antigens and antisera were available for study; missing were 29, 31, 47, 67, 68, 72, 87, 94, and 107.‖ A 1:400 dilution of all of the available antiserums, except 012 and 092, gave positive hemagglutination tests with the homologous antigens. A small number of cross reactions were observed. A 1:400 dilution of CDC lot #1 of 014 rabbit antiserum, however, reacted not only with its homologous antigen, but also with erythrocytes coated with all other antigens with the exception of 010, 11, 12, 18ac, 22, 25, 60, 92, 115, 120 and 130; many of the latter reacted with lower dilutions of 014 antiserum. Antigen 014 coated cells reacted only with 014 antiserum and with no other enteric antiserum. Similar, but much less marked, broad cross reactions were noted with antisera to *E. coli* 056 and 0144. Erythrocytes coated with antigens 056 and 0144, however, reacted only with homologous and 014 antisera. Serially diluted 014 antiserum reacted with its homologous antigen at a titer of 1:1600 and with 9 other *E. coli* 0 antigen preparations at titers ranging from >1:400 to 1:1600; the cross reactions were not inhibited by heating the serum at 56°C for 30 minutes. *Hemagglutination-inhibition tests.* A 1:400 dilution of 014 antiserum was incubated with an equal volume of a 1:10 dilution of the 137 available *E. coli* crude 0 antigen preparations, and then tested for hemagglutination with erythrocytes coated with *E. coli* 01. Hemagglutination of 01 coated erythrocytes by 014 serum was completely inhibited by any one of these antigens except 062 and 092; erythrocytes coated with *E. coli*

‖ Five of these are no longer included as *E. coli* 0 antigens(2).

TABLE II. Hemagglutination Absorption Tests with Various *E. coli* 0 Antigens.

Coated erythrocytes	1:400 dilution of serum							
	014	014–01	014–04	014–0750	14–014	01	04	075
01	+	0	0	0	0	+	0	0
04	+	0	0	0	0	0	+	0
075	+	0	0	0	0	0	0	+
014	+	+	+	+	0	0	0	0
BSS	0	0	0	0	0	0	0	0

+ = hemagglutination, 0 = no hemagglutination, — = serum absorbed with antigen –.

01 antigen reacted strongly with 014 antiserum preincubated only with BSS.

In a second study rabbit 014 antiserum was tested with crude 0 antigens prepared from *E. coli* 014, 6 other *E. coli* 0 groups, and *S. typhosa* lipopolysaccharide (Table I). The antigen coated cells reacted strongly with homologous antisera and all were agglutinated by 014 antiserum; no other cross reactions were observed. Following preincubation with any of the 7 other antigens, the 014 antiserum failed to agglutinate any of the treated cells except those coated with 014 antigen. Preincubation with 014 antigen blocked the cross reaction of 014 antiserum with all the treated cells, including those coated with 014 antigen. Incubation of diluted heterologous *E. coli* or *Salmonella* group D antisera with a 1:10 dilution of *E. coli* 014 crude antigen or 100 μg of 014 lipopolysaccharide did not inhibit their ability to agglutinate cells coated with homologous antigen; only the 014 reaction with its own serum was blocked. It appeared from these experiments that 014 antiserum was reacting with an antigen common to almost all of the *E. coli* 0 groups and *S. typhosa* lipopolysaccharide, but that this common antigen was different from the previously well defined 0 antigens of 014 or other *E. coli* groups and *S. typhosa*. *Hemagglutination absorption tests.* Further evidence for the above hypothesis was provided by studying absorption of the 014 antiserum by erythrocytes coated with various *E. coli* crude 0 antigen preparations (Table II). Absorption of 014 serum with antigens 01, 04, and 075 removed all cross reactivity without affecting hemagglutination of 014 coated erythrocytes. The 014 antigen, however, removed both homologous and heterologous activity from the 014 serum. *Precipitin studies.* The 014 rabbit antiserum used in the above tests was lot #1 provided by the Communicable Disease Center. This serum was tested undiluted in Ouchterlony gel diffusion plates for formation of precipitin lines against undiluted crude 0 antigens of *E. coli* 01, 2, 3, 4, 5, 6, 7, 9, 14, 60, 62 and purified lipopolysaccharides (1 mg/ml) of *E. coli* 014, 055, 0127, *S. typhosa, S. marcescens, Sh. flexneri, B. abortus* and *Staphylococcus A*. Plates were held for 3 weeks at 4°C in a moist chamber before discard. Precipitin lines were observed only with 014 crude and liposaccharide antigens; this was a single band which became visible within 2 to 3 days. Precipitin lines were clearly observed when many of the above antigens were tested against homologous antisera; none of the latter, however, formed precipitin lines with Boivin type 014 antigen prepared in this laboratory. *Role of growth media.* The possibility arose that the cross reactions observed with 014 antiserum may have been due to a factor in the growth medium such as polypeptides or agar. Accordingly, 12 different *E. coli* 0 groups, including 014 were grown in a defined liquid synthetic medium free of these ingredients. Antigens prepared with this medium and coated on erythrocytes exhibited the same cross reactions with the 014 serum as previously observed. Furthermore, purified *E. coli* 055 and 0127 lipopolysaccharides (and similar preparations from other *Enterobacteriaceae, vide infra*) coated on erythrocytes strongly reacted with the 014 serum, suggesting that some component related to the endotoxin moiety, perhaps a hapten was responsible for the cross reactions observed. *Inhibition tests with various sugars.* A 1:400 dilution of 014 antiserum was incubated for 30 minutes with an equal volume of a 10% solution of the fol-

TABLE III. Agglutination of Erythrocytes Coated with Various Bacterial Antigens by Rabbit Antisera Prepared against *E. coli* 014 at Different Laboratories.*

Organism	Preparation	C. D. C. lots			U. Va. vaccine‡	
		#1	#555	#649	Formalin	Boivin
E. coli 014	liposacch.†	>400			40	80
" 014	crude	1600	400	1600	640	80
" 055	liposacch.	800	>1600	800	640	320
" 0127	"	800	1600	800	640	80
E. carotovora	crude	800			640	320
P. vulgaris	"	400	>1600	400	320	320
Shigella flexneri	liposacch.	200	400	200	80	160
" *dysenteriae*	crude	400	800	800	10	80
" *schmitzii*	"	>400	800	800	320	80
" *paradysenteriae*	"	400	1600	800	>640	40
Sal. typhosa	liposacch.	800	>1600	800	640	320
" *paratyphi* A.	crude	200	1600	800		40
" *hirschfeldii*	"	>400	1600	800	320	80
" *enteritidis*	"	>400	1600	400	160	80
" *pullorum*	"	>400	1600	400		80
" *schottmulleri*	"	>400	1600	400	320	80
Kl. pneumoniae	"	100	400	100		20
A. aerogenes	"	100	200	400		10

* Titers are reciprocal of highest positive dilution.
† Purified lipopolysaccharide prepared by modified Boivin or Westphal methods.
‡ Titer of all preimmunization sera <1:10, formalinized vaccine given in 5 injections 4 days apart; Boivin vaccine given as single intrav. inj. of 20 μg, bled at 6 days.

lowing sugars: d glucose, d+ galactose, d+ mannose, l-rhamnose, lactose, sucrose, l arabinose, maltose, d levulose, and d glucosamine; the mixture was tested for reactivity with erythrocytes coated with crude 0 antigen of a coliform (*Erwinia carotovora*) which reacted strongly with this serum. No inhibition of hemagglutination was observed by the sugars tested. *Relation to type of erythrocytes.* Rabbit sera prepared against *E. coli* 014 did not agglutinate untreated washed human erythrocytes of types A, B, or 0, (RH+) or chicken or sheep red cells. Absorption of 014 antiserum 3 × with these erythrocytes did not alter reactivity with erythrocytes coated with various *E. coli* 0 antigens. Sheep cells served equally well as human 0 cells for hemagglutination tests and cross reaction studies. *Cross hemagglutination reactions with other organisms.* Cross reactions observed with various rabbit sera prepared against *E. coli* 014 are summarized in Table III. Cross reactions, frequently at higher titers than with 014 antigens, were observed with a large variety of groups belonging to the *Enterobacteriaceae* family. An exception was *S. marcescens.* All other gram negative organisms tested by the hemagglutination test, including *Brucella abortus,* (lipopolysaccharide), and crude preparations of *Pseu. aeruginosa,* and *B. pertussis,* were unreactive as were the following gram positive organisms: *Staph. aureus, Strep. fecalis, B. subtilis, B. megatherium, S. lutea,* and *D. pneumoniae. Results with various lots of 014 rabbit antisera, normal rabbit serum and γ & β globulins.* Two further lots of independently prepared rabbit 014, 056 and 0144 antisera were obtained from the Communicable Disease Center.¶ Results with the 2 lots of 014 antiserum, both of which were highly active, are shown in Table III. One lot of 056 antiserum was weakly active (at a dilution of 1:50) against a few *E. coli* antigens and *S. typhosa* lipopolysaccharide, but the 2 lots of 0144 sera reacted only with the homologous antigen. Three lots of rabbit 014 serum were obtained from the laboratory of Department of Health of Ontario, Canada;** all of these displayed the cross reactions with *E. coli, Sh. flexneri* and *S. typhosa* described above. Positive re-

¶ We are indebted to Dr. W. H. Ewing for supplying these sera, which had been shown to agglutinate bacteria only of *E. coli* group 014.

** We are indebted to M. Finlayson for supplying these sera.

sults with rabbits immunized with multiple injections of formalinized 014 vaccine†† or a single injection of 20 micrograms of 014 Boivin type antigen prepared in this laboratory are summarized also in Table III. A large number of sera obtained from healthy nonimmunized rabbits used at a dilution of 1:10 and 2% rabbit pooled γ and β globulin‡‡ failed to agglutinate erythrocytes coated with *E. coli* 014 or other 0 antigens. *Independent confirmation of results.* A sample of 014 antiserum prepared with formalinized vaccine in this laboratory was kindly studied by Dr. Erwin Neter, Children's Hospital, Buffalo, N. Y. He was able to corroborate its capacity to agglutinate erythrocytes coated with crude boiled 0 antigen preparations of *E. coli* 055 and 0111, *S. sonnei* lipopolysaccharide (Westphal[12]) and crude antigens of *Salmonella* B, C, D, E, at titers of 1:400 to 1600, but not against cells coated with Vi (Ballerup, purified), Staphylococcus (heterogenic, crude) antigens, or Westphal prepared 0 antigens of *Salmonella* B, C, and D or *E. coli* 0111.

Discussion. These studies strongly suggest that when boiled, formalinized cultures or Boivin type 0 antigens (endotoxin, lipopolysaccharides) of *E. coli* 014 are injected into rabbits at least 2 types of antibodies are produced. One of these is the well known antibody responsible for highly specific bacterial agglutination, hemagglutination and precipitin formation with homologous antigen. The other antibody reacts only in the hemagglutination test with crude or Boivin type 0 antigen preparations of more than 100 of the 145 *E. coli* 0 groups and with supernates of boiled cultures prepared from many other *Enterobacteriaceae,* including many serologic types of *Salmonella* and *Shigella.* It is not known as yet whether the latter antibody will fix complement, react in the passive cutaneous anaphylaxis test or possess bactericidal activity against heterologous *Enterobacteriaceae.* These studies are in progress. The data suggest that the lipopolysaccharide endotoxin fractions (particularly the Boivin type) of *E. coli* and other *Enterobacteriaceae* possess 2 types of antigenic determinants. One is the classical, specific 0 antigen; the other is a hapten common to almost all members of this family. The 014 lipopolysaccharide appears to differ from others in that the common hapten is situated or structured in a manner such that it can behave as a complete antigen and induce antibody formation. The nature of this reactive site has not been established; inhibition studies with a limited number of sugars have been unrevealing. It is doubtful that the dideoxy sugars, such as tyvulose, abequose, paratose or colitose are involved because the heterospecificity of the 014 antiserum includes many different *Salmonella* 0 groups which are characterized by only one or another of these sugars(10). It is hoped that the determinant group of the postulated hapten can be revealed by study of hydrolysates of various *Enterobacteriaceae* endotoxins and search for inhibition by other sugars. The experiments reported here indicate that the postulated common hapten among *Enterobacteriaceae* is unrelated to Forsmann antigen, to the human blood group substances, to the Rantz factor(13) described with various gram positive organisms, to the C. antigen of Brodhage(4), or to the hemagglutinating fimbrial antigens(5). The hapten appears to be closely related to the endotoxin moiety (as prepared by the Boivin method or commercially) but may not be identical with it since Neter§§ has found that certain highly purified preparations such as *S. enteritidis* endotoxin of Ribi *et al.*(14) and certain of those prepared by Westphal (*vide supra*) do not cross react with 014 antiserum.

An alternate explanation of the results obtained in these experiments is that *E. coli* 014 antigen stimulates a non-specific anamnestic type of response in rabbits which recalls a host of antibodies to many *Enterobacteriaceae* with which they have had previous contact. This alternate hypothesis is questionable for 4 reasons: 1) it would presume that the rabbits had a previous experience with a very large number of many different *E. coli, Sal-*

†† This serum agglutinated *E. coli* 014 bacteria at a dilution $>1:160$, but failed in the bacterial agglutination test to react with *E. coli* 01 or 055 at a titer of $<1:10$.

‡‡ Obtained from Pentex, Inc., Kankakee, Ill.

§§ Personal communication.

monella, Shigella and other *Enterobacteriaceae,* some of which have not been previously isolated from rabbits, and that this anamnestic rise frequently gives titers higher than those achieved with *E. coli* 014; 2) *E. coli* 014, and perhaps 056 and 0144, were the only antigens to induce such a phenomenon, while other equally active 0 antigens failed to do so; 3) inhibition and absorption studies indicate that any one of a large number of unrelated *Enterobacteriaceae* antigens can block the 014 antiserum cross reactivity, but only the 014 antigen can block its reaction with 014 serum; 014 antigen does not block combination of heterologous 0 antigens with their corresponding sera suggesting that the antibody produced is quite different than those classically associated with 0 antigens; 4) only the hemagglutination test is involved in the 014 phenomenon; no response of agglutinating or precipitating antibodies was observed as might be expected if the alternate hypothesis were correct or if an antigen other than a hapten were involved.

Summary. Sera from rabbits immunized at 3 different laboratories with *E. coli* 014 antigens prepared from boiled whole cultures or with Boivin type extracts agglutinate human and sheep erythrocytes coated with lipopolysaccharides or crude 0 antigen type preparations from a wide variety of *E. coli* and other *Enterobacteriaceae* but do not produce heterologous bacterial agglutination or precipitin formation. Inhibition and absorption studies suggest that the observed phenomena are due to a hapten associated, but not necessarily identical, with the crude endotoxin moiety of *Enterobacteriaceae* which is so structured in *E. coli* 014 as to be able to induce antibody formation.

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Observations on Some Little-Known Adenoviruses. (27735)

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Considerable information has been published on the natural history of certain adenoviruses, *viz.,* types 1, 2, 3, 4, 5, and 7(1). Relatively little data are available on the other serotypes. This report summarizes observations made on some of the little-known

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