INVESTIGATIONS CONCERNING THE COMMON ANTIGENIC COMPONENT IN SHIGELLA PARADYSENTERIAE, FLEXNER AND ESCHERICHIA STRAINS

by

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INTRODUCTION

This thesis is presented as an effort to carry forward work done by Swanson on the antigenic relationships of Shigella paradysenteriae Flexner and Escherichia strains. (Unpublished thesis presented to the University of Oregon Medical School - June 1940). On the basis of her work, Swanson suggests that the dysentery bacillus possesses a whole antigen, the haptene component of which is shared by certain Escherichia strains.

Certain experiments performed by another worker in the same laboratory (Brandon - unpublished thesis, 1940) differ in results from Swanson's work and suggest that the common antigenic fraction may be present in Bact. coli as a surface antigen, and present in certain pathogenic organisms of the entire group as a deep somatic antigen.

This thesis consists of two parts. Part one is a discussion of work done repeating Swanson's experiments and further experiments carried out along similiar lines in an effort to determine whether further studies should be along the line of the haptene nature of the common antigenic fraction, or should investigate the possibility of deep and surface antigens. Part two is devoted to investigations concerning the nature of the possible haptene contained in Escherichia coli, as experimental work in part one confirmed Swanson's finding and indicated this course.

PART I

ANTIGENIC RELATIONSHIPS OF SHIGELLA PARADYSENTERIAE FLEXNER, AND ESCHERICHIA STRAINS

History

The observation first made by Bordet in 1899 and later by Gruber, Durham and others, that high titer agglutinating sera would agglutinate not only the homologous organism but also related, and in some cases unrelated organisms, though to a lesser extent, had far-reaching implications in the field of immunology. The relation between immunizing bacteria and antibodies was not always a simple one, that is, immune sera could be produced with one bacterial strain containing antibodies for unrelated organisms, and agglutination was observed with some strains in sera though no known history of immunisation with the homologous or related organisms could be obtained.

In the field of enteric organisms Hiss in 1904 reported a serological relationship between Shigella paradysenteriae Flexner strains and the typhoid bacillus. A serological relationship has been observed by many workers between Shigella paradysenteriae Flexner and Escherichia coli. Park (1904) found sera prepared against certain coli strains to contain agglutinins for Flexner, and sera prepared against Flexner to contain agglutinins for coli. Later (1937) Ingalls reported that a high titer rabbit serum for a Shiga dysentery organism agglutinated an apparently unrelated non-pathogenic organism from the intestinal flora of a rabbit, suggesting a common antigenic factor. Severs (1937) found that a number of Flexner cultures, agglutinating in five Bact. coli antisera, failed to remove coli agglutinins in absorption experiments. Also, that Bact. coli antigens failed to remove Flexner agglutinins from Flexner antisera. Mackie (1939) on the other hand, demonstrated

partial removal of coli agglutinins by Flexner antigens, and also of Flexner agglutinins by coli antigens and stated that this heterologous absorption was strong evidence of a fundamental antigenic relationship between strains of Shigella Flexner and Bact. coli.

Both the Flexner dysentery and Esch. coli groups of bacteria are extremely heterogenous in their antigenic composition. The most complete and satisfactory serological classification of the Flexner group was published by Andrewes and Inman in 1919. On the basis of agglutination and absorption experiments, they reported the existence of at least five distinct types, designated as V, W, X, Y and Z, and two subraces Vs and Wx, essentially members of the V and W races respectively but containing so large a proportion of the secondary components as to modify their serological behavior. This classification appears to include the majority of the Flexner strains, but there are occasional types which appear to be antigenically distinct.

The Escherichia group (including Esch. coli and Esch coli mutabile) appears to be even more heterogenous and difficult to classify, than the Flexner group, and includes a wide variety of organisms distributed throughout nature as intestinal parasites, and water and soil inhabitants. Many attempts have been made to classify coli strains into specific groups but with no success. Lepper (1921) reported that each strain of Bact. coli is practically specific, coli antisera agglutinating only the homologous organism to high titer. Bredenbroke (1937) arrived at the same conclusion. And Sievers (1937) concluded that it is impossible to classify coli strains into specific groups. Bact. coli mutabile strains appear to form a more homogenous serological group than do the Bact. coli organisms.

Miss Swanson, whose work served as a basis for this thesis found that:

1. Normal rabbit sera have the capacity to agglutinate a wide variety of gram negative intestinal bacilli including strains of Shigella paradysenteriae Flexner and Bact. coli. The titer, however, was low, seldom exceeding 1:80.

2. Sera of animals immunized with Flexner strains show increased agglutinins for both coli and Flexner. Homologous absorption removes agglutinins for both Bact. coli and Shigella flexner. Absorption of Flexner antisera with the coli, however, removes coli agglutinins but not Flexner agglutinins.

3. Sera of animals immunized with coli show increased agglutinins for coli, but no rise in titer for Flexner organisms. Absorption with Flexner causes no decrease in coli titer.

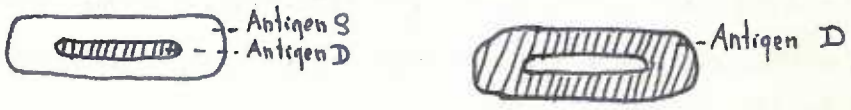
The following explanations of these phenomena were considered by Miss Swanson:

1. Simple antigenic relationship between the two groups on the basis of a shared whole antigen. This thesis was rejected for though Flexner immune sera agglutinated Bact. coli, Bact. coli immune sera did not agglutinate Flexner organisms, nor was Bact. coli capable of absorbing Flexner agglutinins from antiflexner sera, though Flexner removed coli agglutinins from antiflexner sera.

2. The relationship could be ascribed to an amnestic reaction, that is, the production in response to a heterologous antigen of an antibody that has been produced in the tissues on some previous occasion. There appeared, however, no reason why Flexner agglutinins should not be stimulated by coli immunization in the same way that

coli agglutinins were stimulated by Flexner immunization, if this were the mechanism, as normal titers for Flexner (proof of antibody producing mechanism) are as high as some of the normal titers for coli. Also the rise in Bact. coli titer as a result of Flexner immunization was higher than that gererally due to amnestic reaction. This hypothesis, therefore, could not be offered as an explanation.

3. That an actual antigenic relationship, though not a simple one, exists between Shigella flexneri and Esch. coli seems indicated. And the third possibility considered was that Shigella flexner might be thought of as carrying a deep antigen which is serologically similiar to a surface antigen belonging to certain Escherichia strains.



Flexner bacillus Escherichia bacillus

This hypothesis explains the occurence in Flexner antisera of D and S agglutinins for both Flexner and coli, due to D and S antigenic factors in the bacterial coli. It also explains the absorption of coli agglutinins, but not Flexner agglutinins from Flexner antisera by related coli organisms. But it does not explain the absorption of coli agglutinins from Flexner antisera by Flexner organisms, as the Flexner organism, according to this hypothesis, contains no D antigen on the surface of the coli and in a position to absorb D coli aglutinins.

4. Therefore a fourth possibility, that the relationship might be due to the sharing of a common haptene component, was considered. If the dysentery bacillus may be thought of as containing a whole

antigen, a haptene component of which is shared by certain coli organisms, this hypothesis appears to explain adequately all the experimental data. It explains:

a. The occurence in Flexner antisera of agglutinins to high titer for both Flexner and coli organisms, the whole antigen being agglutinogenic for itself and therefore for any part of itself

b. The failure of Bach. coli to cause production of agglutinins for Flexner, as it contains in common with dysentery only the haptene fraction which is not agglutinogenic.

c. Absorption of coli agglutinins in Flexner antisera by both coli and Flexner due to common antigenic fraction.

d. Failure of absorption of Flexner agglutinins in Flexner antisera by coli on the basis that the coli organism can absorb only those agglutinins with which they can react, that is, those that were produced by a common haptene fraction of the Flexner bacterial cell. The agglutinins produced by the remainder of the bacterial cell are untouched and are in a sufficient majority to prevent a noticeable reduction in titer.

Thus the haptene hypothesis appears to provide an adequate explanation. However, one apparent conflict arose. In the course of Miss Swanson's work, Mr. Brandon in the same laboratory performed certain experiments with antityphoid sera in which the agglutinin titer for coli had also increased, and found nonreduction of coli agglutinins on absorption with the homologous organism. These results represent evidence favoring the hypothesis of a surface antigen in coli, present as a deep antigen in certain pathogenic organisms, and are not in line with Swanson's work, in which she demonstrated a reduction of agglutinins

for Bact. coli in Flexner antisera which had been homologously absorbed.

It was thought advisable therefore to investigate this field further before devising experiments to test more directly either of these hypotheses.

EXPERIMENTAL WORK

Part A

Materials and Methods

Bacterial strains used in the preparation of antigens and antisera were those used by Swenson. Her cultures were obtained from the Bacteriology Department of the University of Oregon Medical School. The three Flexner strains used were 362, 362 A and Warden belonging to the strains Z, W, W respectively. Six coli cultures having a high agglutination titer in the Flexner antisera for 362, 362 A and Warden, as determined by Swenson, were pickedfor the experiment.

The cultural reactions were first checked. The Flexner organisms were inoculated into tubes of lactose, mannitol, sucrose and maltose. Results were similiar to those obtained by Swenson, and are shown in Table I.

Six coli strains were streaked on endo plates, and inoculated into tubes containing lactose, sucrose, gelatin, tryptophane to test for production of indol, glucose phosphate for the methyl red and Voges-Proskauer tests. Cultures were also tested for growth in citrate, and motility by examining hanging drop preparations of 18 hour tryptophane broth cultures. Origin and cultural reactions of these strains are shown in Table II.

In the preparation of antigen, care was taken to use smooth strains. 20 hour infusion agar cultures suspended in 0.85% soln. of NaCl containing 0.5% phenol were used in agglutination tests. The suspensions were standardized to the density of the $BaSO_4$ nepholometer tube No. 5 containing approximately 900,000,000 bacilli per cc.

TABLE I

CULTURAL CHARACTERISTICS OF STRAINS OF SHIG. PARADYSENTERIAE FLEXNER

Strain	Lactose	Maltose	Glucose	Sucrose	Mannitol	Gelatine liquifaction	Indol	M.R.	V.P.	Colonies on endo
352A	—	A	A	—	A	—	-	-	-	colorless
352	—	A	A	—	A	—	-	-	-	"
Warden	—	A	A	—	A	—	-	-	-	"

TABLE II

CULTURAL CHARACTERISTICS AND ORIGIN OF 6 STRAINS OF BACT. COLI

Strain	Origin	Lactose	Sucrose	Gelatin	Indol	M.R.	V.P.	Citrate	Motility
Bact. coli	Fecal								
R.A.	Fecal	AG	—	—	+	+	—	—	+
Mayfield	Urine	AG	—	—	+	+	—	—	+ +
Cruse	Urine	AG	—	—	+	+	—	—	+
Hogan	Fecal	AG	—	—	+	+	—	—	+
Bact. coli mut.									
Sparks	Unknown	AG(late)	—	—	+	+	—	—	+
199	Unkown	AG *	—	—	+	+	—	—	+

The sera used were those monovalent high titer rabbit sera prepared by Swanson for the Flexner dysentery strains 352, 352 A, Wardens and for Bact. coli strains Mayfield, RA, 199. Normal serum taken from each rabbit before immunization was use as a control.

Absorption experiments were performed with living antigens. An 0.85% saline suspension of the combined 24 hr. growth of four blake bottles was centrifuged and freed from media by again washing and centrifuging. 10 cc of a 1:10 dilution of the serum to be absorbed was then added to the cells. The suspension held in a water bath at 37 C for two hours, and placed in the ice-box overnight. Serum and bacteria were then separated by centrifugation. The supernatant serum was tested in serial dilution with phenolized antigen for completeness of absorption. If the titer for the absorbing strain was positive in a final dilution of greater than 1:20 the serum was reabsorbed until the titer fell to 1:20. Two absorptions sufficed, in all cases, to reduce the titer. Controls consisting of 1:10 dilution of heated unabsorbed serum were run in all cases. These showed no reduction of titer when tested with the homologous organism.

AGGLUTINATION TESTS PERFORMED WITH ESCHERICHIA STRAINS

The following agglutination tests were carried out.

1. Agglutination of Bact. coli antigens in normal sera - Table III
2. Agglutination of Bact. coli antigens in coli immune sera - Table IV
3. Agglutination of Bact. coli antigens in Flexner antisera - Table V

Agglutination tests with Bact. coli in the normal serum of animals immunized to Shig. flexneri were impossible as normal sera were not available.

The results of these experiments are shown, compared to those obtained by Swanson, in tables III, IV and V respectively. They check closely, first in the presence of agglutinins for Bact. coli in normal serum, though in low titer, second, in the titer for the homologous organism in coli immune sera; and third, and most significant, in the high titer for coli organisms in Flexner immune sera.

Also, in agreement with Swanson, there seems to be no correlation between Flexner type antisera and coli agglutinin titer, which suggests that the shared antigenic components are of a group nature rather than related to type specificity.

As might be expected, the Bact. coli titer in Flexner antisera (Ave titer 1:1280) is not as high as in the homologous serum. (Ave 1:30,000) or as high as the titer for the Flexner organisms in its homologous serum where agglutination occured in dilutions as high as 1:81,920. However, in the agglutinin test with strains of Bact. coli in the three types of Flexner antisera, the average titer of 1:1280 showed a marked increase over the coli agglutinin level in the corres-

ponding normal serum in which the titer did not exceed 1:40 in any case.

It might be pointed out here that, in comparing Swanson's work with our own, a difference in titer of from one fourth to four times the dilution figure was occasionally observed. As a change in the titer of this degree simply means a difference of one to two tubes in the serial dilution and as our antigens were prepared over a year later than Swanson's and slight alteration of strain might have occured, we considered the variation no greater than that which might be expected.

The three Shig. flexner antisera for strains 352, 352 A and Warden, were homologously absorbed and tested for aglutinins for the six Escherichia antigens used previously in the agglutination tests. The results are shown in Table VI. In all cases, in agreement with Swanson's work, absorption with the homologous Flexner organism caused a striking reduction or a complete removal of the coli agglutinins as well as a complete removal of the homologous agglutinins. In those cases in which removal of coli agglutinins was not complete the residual titers was no higher than those that might be expected from normal rabbit sera as is seen in Table III.

TABLE III

AGGLUTININ TITER FOR 5 BACT. COLI STRAINS IN 3 NORMAL RABBIT SERA

NORMAL SERA	199	R.A.	Mayfield
Strain			
199	1:20	1:40	1:40
R.A.	1:40	1:40	1:40
Mayfield	1:40	1:20	1:20

AGGLUTININ TITER OBTAINED BY SWANSON WITH THE SAME SERA

NORMAL SERA	199	R.A.	Mayfield
Strain			
199	1:40	1:40	___
R.A.	1:20	1:40	1:10
Mayfield	1:40	1:10	1:20

TABLE IV

AGGLUTININ TITER FOR 3 COLI STRAINS IN 3 BACT. COLI ANTISERA PREPARED FROM RABBITS WHOSE NORMAL SERA WAS PREVIOUSLY TESTED

Immune sera / Strain	R.A.	199	Mayfield
R.A.	1:10240	1:81920	1:40960
199	1:20480	1:40960	1:20480
Mayfield	1:5120	1:10240	1:40960

RESULTS OBTAINED BY SWANSON WITH THE SAME SERA

Immune sera / Strain	R.A.	199	Mayfield
R.A.	1:10240	1:81920	1:40960
199	1:10240	1:300000	1:20480
Mayfield	1:5120	1:81920	1:20480

TABLE V

AGGLUTININ TITER FOR 6 COLI STRAINS IN ANTISERA FOR 3 FLEXNER STRAINS

Immune sera	352	352A	Warden
Strain			
Homologous org.	1:81920	1:10240	1:40960
Bact. coli			
Mayfield	1:1280	1:1280	1:640
R.A.	1:2560	1:1280	1:1280
Hogan	1:160	1:1280	1:2560
Cruse	1:1280	1:1280	1:1280
Bact. coli mutabile			
199	1:2560	1:2560	1:2560
Sparks	1:2560	1:2560	1:640

AGGLUTININ TITERS OBTAINED BY SWANSON WITH THE SAME ANTISERA

Immune sera	352	352A	Warden
Strain			
Homologous org.	1:81920	1:10240	1:40960
Bact. coli			
Mayfield	1:1280	1:1280	1:640
R.A.	1:1280	1:2560	1:1280
Hogan	1:80	1:160	1:5120
Cruse	1:320	1:1280	1:1280
Bact. coli mutabile			
199	1:2560	1:1280	1:1280
Sparks	1:1280	1:2560	1:640

TABLE VI

COLI AGGLUTININ TITERS FOR HOMOLOGOUSLY ABSORBED FLEXNER ANTISERA

Immune sera	362		362A		Wardem	
	immune	absorbed	immune	absorbed	immune	absorbed
Bact. coli strain						
Mayfield	1:1280	0	1:1280	0	1:640	0
R.A.	1:2560	0	1:1280	1:20	1:1280	1:20
Hogan	1:160	0	1:1280	0	1:2560	0
Cruse	1:1280	0	1:1280	0	1:1280	0
Bact. coli mut.						
199	1:2560	1:20	1:2560	0	1:2560	1:20
Sparks	1:2560	0	1:2560	0	1:640	0

RESULTS OBTAINED BY SWANSON IN A SIMILAR ABSORPTION EXPERIMENT

Immune sera	362		362A		Wardem	
	immune	absorbed	immune	absorbed	immune	absorbed
Bact. coli strain						
Mayfield	1:1280	0	1:1280	0	1:640	1:20
R.A.	1:1280	0	1:2560	0	1:1280	0
Hogan	1:80	-	1:160	-	1:5120	-
Cruse	1:320	-	1:1280	-	1:1280	-
Bact. coli mut.						
199	1:2560	0	1:1280	1:20	1:1280	1:20
Sparks	1:1280	-	1:2560	-	1:640	-

SUMMARY

The results of these experiments substantiate Swanson's findings 1. that normal sera may contain agglutinins for Esch. coli but only in low titer. 2. that immunization with Shig. flexneri increases the titer for each Esch. coli. 3. that absorption of Flexner sera homologously removes coli agglutinins.

EXPERIMENTAL WORK

Part B

STUDIES ON NEW STRAINS AND ANTISERA

It seemed advisable after confirming the work with Swanson's strains to go over the same ground with freshly collected coli strains and freshly prepared antisera, both to further check results and, in case of agreement, to increase the body of evidence in favor of the haptene hypothesis.

Materials and Methods

52 Bact. coli strains were collected largly from fecal and urine specimens from patients at the Multnomah Hospital. These strains are listed on page 20 and the origin, where known, is given.

The cultural characteristics of the 52 coli strains were studied for the production of acid and gas in broth tubes containing lactose and sucrose, for liquifaction of gelatin, production of indol from tryptophane, growth in citrate, methyl red and Voges-Proskauer reactions in glucose phosphate broth. The cultural reactions are shown in Table VII. These strains giving reactions typical of Escherichia coli communis or communior, 50 in all, were used in the preparation of phenolized antigen as described earlier in this thesis.

BACT. COLI STRAIN HISTORY

Bact. coli strains

Fecal strains	Urine strains	Blood strains	Unknown
Murry	Urine 1	Lewis	Greble
Isaacson	Urine 2	Chuinard	Johnson
Peterson	Urine 3		Lanier
34B	Gordon		Probst
32A	Wong		Dolty
Hillman	Findley		Goodwin
Wong	Bennett		Huntley
Travis	Johnson		Lauter
Hackett 1	Reider		Gillman
Hackett 2	Merriman u1		5-2
Cherry	Lee		2691
Emerson	Merriman u2		Olive M
Merriman s	Bullard		Holstrom
	Dant		70 MR
	Bolt		251a
			269R
			269Lv
			139b

Bact. coli strains (mutabile)

Fecal strains	Unknown
Borden	153
	78A

TABLE VII

CULTURAL CHARACTERISTICS OF 50 STRAINS OF BACT. COLI

Strain Bact. coli	Lactose	Sucrose	Gelatin liquifaction	Indol	M.R.	V.P.	Citrate	Motility	Colonies on endos
Murry	AG	AG	—	+	+	-	-	+	colored
Isaacson	AG	AG	—	+	+	-	-	+	+
Peterson	A G	—	—	-	+	-	-	-	+
34B	AG	—	—	+	+	-	+	+ +	+
32A	AG	AG	—	+	+	-	-	+	+
Hillman	AG	—	—	+	+	-	-	+	+
Wong u	AG	AG	—	+	+	-	-	-	+
Travis	AG	—	—	+	+	-	-	+	+
Hackett 1	AG	—	—	+	+	-	-	+	+
Hackett 2	AG	—	—	+	+	-	-	+	+
Cherry	AG	—	—	+	+	-	-	+	+
Emerson	AG	AG	—	+	+	-	-	-	+
Merriman S	AG	—	—	+	+	-	-	+	+
Lewis	AG	AG	—	-	-	-	+	-	+

TABLE VII

(CONTINUED)

Strain	Lactose	Sucrose	Gelatine liquifaction	Indol	M.R.	V. P.	Motility	Citrate	Colonies on endo
Lanier	AG	—	-	+	+	-	-	-	colored
Probst	AG	—	-	+	+	-	-	-	"
Dant	AG	AG	-	+	-	-	-	+	"
Bolt	AG	—	-	+	+	-	-	-	"
Dolty	AG	—	-	+	+	-	-	-	"
Goodwin	AG	—	-	+	+	-	+	-	"
Huntley	AG	—	-	+	+	-	-	-	"
Lauter	AG	—	-	+	+	-	-	-	"
Gillmon	AG	AG	-	+	+	-	-	-	"
5-2	AG	—	-	+	+	-	+	-	"
269L	AG	—	-	+	+	-	-	-	"
Olive H	AG	—	-	+	+	-	+	-	"
70-M-R	—	—	-	+	+	-	+	-	"
251a	AG	—	-	+	+	-	+	-	"
269R	AG	—	-	+	+	-	+	-	"
269Lv	AG	—	-	+	+	-	-	-	"
139b	AG	—	-	+	+	-	-	-	"
Borden	—	—	-	+	+	-	-	-	"

TABLE VII

(CONTINUED)

Strain	Lactose	Sucrose	Gelatine liquifaction	Indol	M.R.	V.P.	Citrate	Motility	Colonies on endo
Chuinard	AG	—	—	+	+	-	-	+	colored
Urine 1	AG	—	—	+	+	-	-	-	"
Urine 2	AG	—	—	+	+	-	-	-	"
U rine3	AG	—	—	+	+	-	-	-	"
Gordon	AG	—	—	+	+	-	-	+	"
Wong u	AG	AG	—	+	+	-	-	-	"
Findley	AG	—	—	+	+	-	-	+	"
Bennett	AG	AG	—	+	+	-	-	+	"
Johnson	—	—	—	+	+	-	-	+	"
Reider	AG	AG	—	+	+	-	-	+	"
Merriman U1	AG	—	—	+	+	-	-	+	"
Merriman U2	AG	—	—	+	+	-	-	+	"
Lee	AG	AG	—	+	+	-	-	+	"
Ballard	A G	AG	—	+	+	-	-	+	"
Greble	AG	—	—	+	+	-	-	+	"
Johnan	AG	AG	—	+	-	-	+	-	"

AGGLUTINATION OF FRESHLY ISOLATED ESCHERICHIA STRAINS IN HIGH TITER FLEXNER ANTISERA

The agglutinin titers of 50 Bact. coli antigens were determined in each of three Flexner antisera 352, 352 A and Wardon. Serum dilutions up to 1:160 were made routinely. In those cases in which agglutination occured in this dilution, tests were run to titer. Table VIII summarizes the results. The range of agglutination titer for the coli strains varied from 0 to 1:10240, fifteen out of 50 organisms agglutinating markedly above the normal level.

AGGLUTININ TITER OF 6 COLI STRAINS IN HOMOLOGOUSLY ABSORBED HIGH TITER FLEXNER ANTISERA

Six coli strains having a markedly high agglutinin titer in all three Flexner antisera were chosen for agglutinin absorption experiments. The coli agglutinin titer of the three Flexner antisera homologously absorbed was tested with each of the six high agglutinating strains. The results (Table IX) show marked absorption, the titer being reduced to 0 (1:20 dilution) in most cases.

TABLE VIII

AGGLUTININ TITER FOR 50 COLI STRAINS IN 3 HIGH TITER FLEXNER ANTISERA

Immune sera	352	352A	Warden
Bact. coli strain			
Reider	1:2560	1:10240	1:80
Merriman u1	1:10240	1:2560	1:640
Merriman u2	1:2560	1:5120	1:640
Bullard	1:20	1:20	0
Findley	1:20	1:80	1:20
Chuinard	1:80	1:20	0
Bennett	1:80	1:640	1:640
Wong u	1:40	1:40	1:320
Emerson	0	0	1:20
Lee	1:80	1:640	1:640
Johnson	1:20	0	1:20
Travis	1:40	0	0
Cherry	0	1:40	0
Hackett 1	0	0	1:40
Hackett 2	0	0	1:40
269Lv	1:40	1:80	0
269R	1:20	1:20	1:40
251b	1:80	0	0
70MR	0	0	0
Merriman s	1:2560	1:1280	1:1280
Johnsn	1:40	1:40	-
Isaacson	0	1:80	-

TABLE VIII

(CONTINUED)

Immune sera	352	352A	Wardem
Bact. coli strain			
139b	1:80	1:160	1:40
Greble	1:320	1:320	1:40
Dolty	1:640	1:160	1:1280
5-2	1:1280	1:640	1:160
Bolt	1:320	1:1280	1:160
Lauter	1:80	1:20	1:80
Murry	1:160	1:320	1:160
Peterson	0	1:20	-**
Lanier	1:20	1:80	-
Gordon	1:20	1:40	-
Probst	1:20	1:40	-
Dent	1:20	0	-
34B	1:40	1:40	-
Goodwin	0	1:320	-
Huntley	0	0	-
32A	1:40	0	-
Urine 1	0	0	-
Urine 8	0	1:40	-
269L	1:20	0	-
Olive M	1:20	0	-
Holstrom	1:20	1:80	-
6	1:40	0	-
Holbrook	0	0	-

** There was not sufficient antiserum to run tests on all strains

TABLE VIII

(CONTINUED)

Immune sera	362	362A	Warden
Bact. coli mut. strain			
163	0	1:20	-
73A	1:20	1:80	-
Borden	1:20	1:20	-

TABLE IX

AGGLUTININ TITER OF 6 BACT. COLI STRAINS IN HOMOLOGOUSLY ABSORBED HIGH TITER FLEXNER ANTISERA

Immune sera	352		352A		Warden	
	immune	absorbed	immune	absorbed	immune	absorbed
Bact. coli strain						
Greble	1:320	0	1:320	0	1:40	0
Dolty	1:640	0	1:160	1:40	1:1280	0
6-2	1:1280	0	1:640	1:20	1:160	0
Bolt	1:320	0	1:1280	0	1:160	0
Lauter	1: 80	0	1:20	0	1:80	0
Murry	1:160	1:40	1:320	0	1:160	0

AGGLUTININ TITERS OF BACT. COLI STRAINS IN THREE NORMAL SERA LATER IMMUNIZED TO FLEXNER

As only a small amount of normal sera was available the coli strains were not run routinely with the normal serum. It seemed advisable at this point, however, to determine the coli agglutinin titer for as many strains as possible in normal serum of each of the rabbits which had later been immunized with the Flexner strains 352, 352 A and Worden, and to determine what fraction, if any, of the normal agglutinins to coli could be removed by absorption of the serum with the Flexner organism.

Agglutination tests were done with 21 coli strains in each of the three Flexner antisera 352, 352 A and Worden. Table X shows the results of these agglutination tests compared with those run with the same organism on the corresponding immune serum. In very few cases did agglutination occur above a dilution of 1:40in normal sera. With eight out of 21 strains a marked increase in coli agglutinins was noted in the dysentery antisera. It is an interesting observation that those strains having a high titer in the immune sera were usually those having a high titer in the normal sera.

Here again the increase in the coli titer in immune serum did not appear associated with Flexner type, indicating the basic rather than race specific nature of the relationship.

TABLE X

AGGLUTININ TITERS OF BACT. COLI STRAINS IN 3 NORMAL SERA AND IN CORRESPONDING FLEXNER IMMUNE SERA

Serum	352		352A		Warden	
Strain	normal	immune	normal	immune	normal	immune
Reider	1:160	1:2560	0	1:10240	0	1:80
Merriman u1	1:160	1:10240	1:20	1:2560	1:40	1:640
Merriman u2	1:40	1:2560	1:20	1:5120	1:40	1:640
Merriman u3	1:40	1:2560	1:20	1:1280	1:40	1:1280
Bullard	1:20	1:20	1:20	1:20	1:20	1:20
Findley	1:20	1:20	0	1:160	1:20	1:20
Chuinard	1:20	1:80	1:20	1:20	1:20	1:20
Bennett	1:20	1:80	1:20	1:640	1:40	1:640
Wong u	1:40	1:40	1:80	—	0	1:160
Emerson	0	0	0	0	0	0
Lee	1:40	1:80	1:40	1:640	1:40	1:640
Johnson	1:20	1:20	0	0	0	0
Travis	1:40	1:40	0	0	1:20	1:20
Cherry	1:40	1:40	0	0	1:20	1:20
Hackett 1	0	0	0	0	1:40	1:40
Hackett 2	0	0	0	0	1:40	1:40
269Lv	0	1:40	0	1:80	0	0
269 R	0	1:20	1:20	0	1:40	1:40
251 a	0	1:80	1:20	1:20	1 :40	1:20
139 b	1:20	1:80	0	1:160	0	1:80
70MR	0	0	0	0	1:20	1:20

EFFECTS OF ABSORPTION WITH FLEXNER STRAINS ON THE O LI AGGLUTININ LEVEL IN NORMAL SERA

For the absorption experiment 10 coli strains were chosen in so far as was possible, had normal agglutinin titers between 1:30 and 1:40 in the normal sera. In order to determine whether absorption with Flexner organisms had any effect on the normal agglutinins for coli and whether the amount of removal of agglutinins, should any occur, could be correlated with Flexner race, the normal serum, later immunized to Shig. paradysenteriae Flexner Wardem, was absorbed with the Wardem, 352, 352 A strains of Flexner respectively. Strains 352 A and Wardem belong to the "W" race and strain 352 to the "Z" race. Therefore, if any race specific factor were involved in the relationship to the coli organism the effect of absorption of the normal serum with 352 A and Wardem should be more alike than the effect of the absorption with 352. The results are indicated in Table XI and show a definite lowering of titer though no correlation between Flexner race and removal of agglutinins is evidenced. This provides additional evidence in favor of the existance of a basic non-race specific antigenic constituent common to Shig. paradysenteriae Flexner and certain strains of Bact. coli.

TABLE XI

COLI AGGLUTININ TITER IN NORMAL SERUM ABSORBED WITH FLEXNER STRAINS

Serum	Normal	Absorbed with 352	Absorbed with 352A	Absorbed with Warden
Bact. coli str.				
Hackett 1	1:40	0	0	0
Hackett 2	1:40	0	0	0
Lee	1:40	1:20	0	0
2092	1:40	1:20	0	1:20
Merriman u1	1:40	0	1:20	0
Merriman u2	1:40	1:20	1:20	1:20
Merriman s	1:40	0	0	0
Wong u	1:40	0	0	0
Findley	1:20	0	1:20	0
Bennett	1:40	0	1:20	1:20

SUMMARY

The results of these experiments carried out with 50 freshly isolated Bact. coli organisms and freshly prepared Flexner antisera for strains 352, 352 A and Warden substantiate the results of Part A, namely, that (1) normal serum may contain agglutinins for Esch. coli but only in low titer, (2) immunization with Shig. Flexner increases the titer for Esch. coli, (3) absorption of Flexner antisera homologously removes the coli agglutinins.

In addition it was shown that absorption of normal serum with Flexner strains caused a reduction of the Bact. coli titer below the normal level, which is additional evidence in favor of an antigenic factor in the dysentery bacillus having a specific relationship to a component of certain Bact. coli strains. This relationship did not appear to be related to the Flexner type.

DISCUSSION AND CONCLUSION

The results of these experiments performed both with Swanson's material and with freshly isolated strains and freshly prepared immune sera, agree with Swanson's findings that:

(1) Normal serum may contain agglutinins, but only in low titer, for both Shig. flexneri and Bact. coli.

(2) Immunization with Shig. flexneri significantly increases the titer for Bact. coli.

(3) Absorption of Flexner antisera, homologously, removes coli agglutinins even below the normal level.

(4) Removal of coli agglutinins both from normal and from Flexner immune serum, by members of the various races, shows no correlation between amount of removal and race.

This evidence suggest the existence of a basic non-race specific antigenic constituent common to Shig. paradysenteriae Flexner and certain strains of Bact. coli. That this common antigenic factor exists as a whole antigen in Shig. flexneri and as a haptene in Bact. coli, is the hypothesis suggested by Swanson. The experimental work in this paper confirms the work done by Swanson and points to the haptene hypothesis as the only explanation adequate to account for the experimental data.

First, the occurence in Flexner antisera of agglutinins for both Shig. flexneri and Bact. coli may be explained in that the whole antigen in the dysentery bacillus is agglutinogenic for itself or any part of itself.

Second, it explains the failure of Bact. coli to cause the production of agglutinins for Shig. flexneri as it contains, in common with the Flexner organism, only a haptene fraction which is non-antigenic.

Third, the absorption of coli agglutinins from Flexner antisera

by both Shig. flexneri and Bact. coli may be accounted for in that both organisms contain a common antigenic fraction with a grouping specific for the coli agglutinins.

Fourth, the failure of absorption of Flexner agglutinins from Flexner antisera by Bact. coli may be explained on the basis that the coli organism can absorb only those agglutinins with which they can react, that is, those that were produced by a common haptene fraction of the Flexner bacterial cell, whereas the agglutinins produced by the remainder of the Flexner bacterial cell are untouched and sufficiently in the majority that the Flexner titer may remain unchanged.

This hypothesis, therefore, seems adequate to explain the experimental data.

Regarding the work of Brandon on the basis of which he suggested the existence of a common antigenic factor present in Shig. flexneri as a deep antigen and in certain Escherichia strains as a surface antigen, the conflict between his experimental work and that of Swanson and our own become less significant on further examination of his data. Concerning the normal serum agglutinin level for the Flexner and coli organisms, concerning the increase in coli agglutinins caused by immunization with pathogenic enteric organisms, and concerning the lack of increase of agglutinins for these pathogenic organisms in serum of rabbits immunized to coli, the results obtained by Brandon and Swanson are in complete agreement. The point of difference in experimental data, and the point on which Brandon based his hypothesis was the failure of the typhoid organism to reduce markedly the coli agglutinin titer on absorption of the homologous serum.

Brandon carried out experiments both with high titer anti typhoid serum prepared from rabbits, homologously absorbed and agglutinated with Bact. coli, and with human typhoid immune serum from

cases, also homologously absorbed and agglutinated with coli strains. The results are shown on p. 37 and 38

Agglutination absorption tests with anti typhoid rabbit sera show a variation in the reduction of coli agglutinins on homologous absorption. In several cases the reduction occured only to the next serial dilution or from 1:250 to 1:1280 as with the Hillman organism in Bact. paratyphosum B serum homologously absorbed. However, in the majority of cases, the reduction was more, the greatest lowering being from a titer of 1:2560 to a titer of 1:160. The lack of a significant reduction of agglutinins in the case of the Hillman organism is not in agreement with our results, but is the only instance in which lack of reduction is striking and seems insufficient evidence on which to base any definite conclusions.

The experiments performed with human typhoid antisera homologously absorbed and agglutinated with coli and paratyphoid organisms, show interesting results in that the coli agglutinin titer is not lowered significantly while the paratyphoid agglutinin titer is lowered markedly. However, it seems questionable whether the results obtained in this experiment are comparable to the rest of the experimental data, which is based on results obtained with controlled and artificially immunized rabbit sera. In this case the source of the coli agglutinins is unknown. Adequate controls on the normal sera, of course, could not be run. And it is quite impossible to determine whether or not the coli agglutinins to high titer resulted from immunizing action of the typhoid organism. It might be possible during typhoid fever, under circumstances of intestinal ulceration and irritation, for the coli organism to reach the blood and give rise independently to agglutinins which might or might not have any antigenic relationship to the invading disease organism.

Therefore, since in the first experiment, failure of the homolo-

AGGLUTININ TITER FOR ESCH. COLI IN HOMOLOGOUSLY ABSORBED TYPHOID AND PARATYPHOID RABBIT ANTISERA (FROM BRANDON)

Esch. coli strain	Bact. paratyphosum B homologously abs.	Bact. typhosum 0901 homologously abs.
Flynn	0	0
Control	0	0
Baldwin	0	0
Control	0	0
No. 5	1:320	0
Control	1:2560	0
Grady	0	0
Control	0	1:80
Swanson	1:80	1:160
Control	1:160	1:320
No. 271	0	0
Control	0	0
Miller	1:80	1:80
Control	1:640	1:160
Hillmen	1:1280	1:160
Control	1:2560	1:2560

AGGLUTININ TITER FOR ESCH. COLI IN HOMOLOGOUSLY ABSORBED TYPHOID ANTISERA OBTAINED FROM HUMAN CASES (FROM BRANDON)

Antigens	Serum dilutions 1:40	1:80	1:160	1:320	1:640	1:1280
Bact. typhosum 0901	0	0	0	0	0	0
Control	+4	+4	+4	+4	1L	0
Esch. coli Hiller	+3	+3	+3	+1	0	0
Control	+3	+3	+3	+3	0	0
Esch. coli Hilman	+4	+3	+3	+2	1L	0
Control	+4	+4	+4	+4	1L	0
Esch. coli Grady	+4	+4	+4	+4	+1	1L
Control	+3	+3	+3	+3	+2	+2
Esch. coli 271	+4	+4	+4	+4	+4	+3
Control	+4	+4	+4	+4	+4	+4
Esch. coli Swanson	+3	+3	+3	1L	0	0
Control	+4	+4	+4	+3	0	0
Bact. aberdeen	0	0	0	0	0	0
Control	0	0	0	0	0	0
Bact. paratyphosum B 8006	0	0	0	0	0	0
Control	+3	1L	0	0	0	0
Bact. paratyphosum B 289b	0	0	0	0	0	0
Control	+3	1L	0	0	0	0

gously absorbed rabbit anti typhoid serum to show marked reduction of coli agglutinins is not the rule, and since it seems doubtful that the results of the second experiment are strictly comparable to the rest of the experimental data, it seems to us that Brandon's conclusion of an antigenic relationship between Bach. coli and Shig. flexneri through a deep antigen in the dysentery basillus shared as a surface antigen in Bact. coli is not supported by sufficient experimental data.

As our own experimental work supported Swanson's work showing a removal of coli agglutinins by absorption of anti dysentery serum with the homologous organism, and thus favors the haptene theory, and as we cannot agree that Brandon's work contradicts this hypothesis, we feel that a direct search for a haptene is justified.

PART II

INVESTIGATIONS CONCERNING A HAPTENE IN BACT. COLI

Review of the Literature on Haptenes

Before proceeding with work on the isolation of a haptene from Bact. coli it seemed advisable to review the literature on the chemical nature and immunological significance of haptenes in general, in order to gain a background for the work involved.

History. Fundamental to the understanding of haptenes is a background in the general problem of antigen-antibody reactions, and in the antigenic or chemical composition of bacteria. The study of partial antigens, or haptenes, was a logical outgrowth of attempts to determine the relationships between different groups of bacteria as suggested by serological reactions.

The term antigen applies to any substance which (1) causes the development of specific antibodies in animals into which it is injected parenterally and (2) reacts specifically with the antibodies produced. A haptene possesses only the second of these properties.

The development of our knowledge of the various reactions which may occur when the blood or serum of an animal is mixed with various bacteria, bacterial products, foreign cells, or foreign protein began in 1888 when Nuttall demonstrated that defibrinated blood had the power of killing bacteria. In 1890 Von Behring and Kitasato showed that the serum of animals which had received repeated injections of sub-lethal doses of tetanus toxin acquired the property of specifically neutralizing these toxins. In 1893 Pfeiffer recorded the occurence of bacteriolysms, and Bordet clarified the

reaction by showing the participation of two factors in the lysing of bacteria, one present in normal serum and heat labile, and the other a specific, relatively heat stable factor produced under the influence of an antigen. Gruber and Durham in 1896 published the first detailed studies on agglutination of bacteria. In 1897, Kraus observed the precipitin reaction with filtrates of bacterial cultures and the homologous sera. The complement fixation test first was developed by Bordet and Gengou(1901) who showed that antigen, sensitized by specific antibody, fixes complement. Neufeld(1904) pointed out the antigen-antibody nature of specific phagocytosis.

That antigenic capicity was not limited to bacterial cells was shown by Bordet who described theappearance of lytic antibodies in the sera of animals injected with red blood cells of an animal of some other species and showed that this lysis depended on the interaction of two distinct substances as in the case of bacteria.

The description of the general types of immune reactions, aside from the practical applications in medicine, was the point of departure for a vast amount of research into the nature of the mechanisms involved. The essential feature of these antigen-antibody reactions is specificity, a phenomenon common toall biological processes from the fertilization of the ovum by the specific spermatazoa, on through the processes which lead to the formation of specific structures characteristic of the species, to the specific reactions to injury which lead to repair by the proper tissues elements and to the specific methods of defense against invading organisms. The study of bacteria in which there are so few possible points of structural differentiation, which are yet specific, and which are endowed with the capacity of indefinite reproduction of pure strains, was a great step

forward towards bringing the broad biological phenomena to its simplest terms. And further, fractionation of bacteria into various antigenic or chemical components and use of these and of artificially conjugated antigens in serological studies, have permitted subjection of the problem to chemical analysis, which must provide the basis for an understanding of specificity.

The precipitin reaction provided a method for differentiating proteins, much more delicate than chemical means, and showed that proteins have a twofold specificity, that of the particular protein and that of species, and that each protein contains a number of serologically distinct components. It was found that with relatively slight immunization, an antisera would be produced which would react almost solely with the same or homologous antigen, but if the serum were raised to a high titer, the range of reactivity would broaden until distinct reactions could be obtained with less closely related antigens, the non-specific reactions being more marked with the more closely related proteins. On the other hand the power of producing antibodies is greater the less closely related is the antigen to the serum protein of the animal being immunized. Serological relationships have been found to correspond closely with zoological classifications bases on morphology, and have provided a means of classifying many bacteria. Thus, immunological studies show degree of relationship in the plant and animal world as well as specificity.

Chemistry. Of the Basic carbohydrates, lipoid, and protein material, the property of antigenicity has been associated until recently exclusivly with the latter. The first immunological

studies were made with bacteria or blood but it was soon found that all soluble proteins are antigenic if the term protein is limited to "those colloidal amino acid aggregates which contain the full quota of amino acids found in complete protein."**

Antigenic capicity seems related to the aromatic radicals in the protein. Gelatin which contains mostly diamino acids and is lacking in the aromatic amono adids, tyrosine, phenylalanine, and is low in tryptophane, has no antigenic power. Other proteins possessing the full complement of aromatic radicals but deficient in some other amino acids are actively antigenic--examples being gliadin which lacks lysine, egg albumin which lacks glycine, casein which lacks cystine and glycine. Proteins which consist chiefly of diamino acids with but small amount of mono-amino acids are lacking in antigenic properties.

The antigenic capacity of proteins also appears to be related to molecular size, or properties related to molecular size as, under circumstances in which the protein molecule is broken down, it loses its antigenic capacity. In this connection Zinsser (1923) suggests that the antigens must be non-diffusable colloids, therefore, which cannot enter the cells to be destroyed there, necessitating the formation of soluble extracellular antibodies which may react in some way with the foreign protein to cause its destruction, may be antigenic. It would deem to follow that when the foreign protein molecule is small enough to be taken into the cell by diffusion, antibody formation becomes unnecessary for its disintegration and consequently we have no antigens that are not colloidal. In support of this conception it was shown that the cleavage product of a protein even when injected

** Wells-Chemical Aspects of Immunity p. 25

together, lack antigenic power and when artificially united as plasteins, the antigenic power of the original material was restored. (Gay and Roberton, 1912)

Also antigenic activity varies with different proteins. This may be related to some extent to the solubility but this is not the only factor as serum albumin is much less strongly antigenic than serum globulin from the same serum (Dale and Hartley, 1922). Also the antigenic activity of a protein varies with the different animals (even of same species) into which it is injected. (Wells page 28) The $[H^+]$ of the protein injected may also have some influence on antibody formation, acid being somewhat more effective than alkali in some cases. (Falk, 1923)

The best approach to the systematic investigation of serum reactions along chemical lines is the study of the effects of altered, artificially conjugated, antigens.

Immunological properties of proteins are effected in different ways by chemical alteration. Treatment with digestive enzymes, alkalis, acids, decreases or destroys antigenic activity, alkalis being more effective than acids. The antigenic properties altered by alkali are restored in part by nitration and to a less degree by iodization. (Johnson and Wormall, 1932)

More profitable for study are alterations in the molecule not destroying immunizing power and ability to react with antibodies. Obermeyer and Pick (1906) found that in certain reactions such as coupling with diazobenzene or oxydizing with permanganate, species specificity is preserved to a large extent. For example precipitins for the oxyprosulfonic acids, formed upon oxidation of proteins by are

permanganate, react with the antigen but not with the original protein or with other oxyprotsulfonic acids. However, greater changes in the serological properties of proteins are produced by the action of nitric, nitrous acid, and iodine. (Landsteiner)** Serum proteins treated in this way lose their original species specificity but gain a new specificity, associated with the newly introduced group, and shared by normally unrelated serum proteins that are chemically altered by the same procedure. Thus an anti-serum prepared against the nitrated serum of a particular animal species failed to react to any extent with the unaltered serum of that species but reacted with a wide range of nitrated sera of unrelated species.

Since the nitro group and halogen elements were known to enter the benzene ring and since such chemical alterations were found to profoundly effect the specificity of such proteins it appers that the aromatice nucleus, especially the tyrosing grouping, plays a part of peculiar importance in determining specificity. It appears that the changed activity of iodo-proteins and bromo-proteins is not due to iodine or bromine in themselves but to the tyrosine radical disubstituted by halogen in the 3,5 positions with a hydroxyl vicinal to the halogen.

Mutsaars (1939-by means of the method of specific inhibition) has clearly demonstrated that the serological specificity of xantho-proteins is due to the nitrotyrosine grouping. Using numerous related compounds the author found that the reactivity depends on the presence of nitro and hydroxyl substituents in the benzene ring, and a carboxyl group, free or esterified. Nitration of gelatin did not confer antigenic propeties upon this protein and nitro-gelatin

** 1935--The Specificity of Serologica logReactions page 25

was not precipitated by immune sera to other nitro-proteins.

~~In this connection~~ It is of interest that by iodization and nitration of different proteins are made serologically alike. Although their original chemical differences cannot have been completely done away with, it would appear that the predominance of altered structures masks the basic chemical differences.

Again there are reactions which effect aromatic groups without changing species specificity. Azo compounds formed by coupling proteins with diazobenzene still possess species specificity, although azo groups are undoubtedly linked to tyrosine. On the other hand species specificity can be changed without substituting aromatice groups. In fact some decrease in specificity occurs when natural proteins are altered, as by heat, formaldehyde, etc.

Landsteiner has shown that the salt forming groups of the amino acids, the carboxyl, hydroxyl, and amino groups, also play a significant part in the determination of specificity. By esterification, methylation, and acetylation, he has shown alteration of the immunological specificity. For example in the esterification of acid radicals by means of acid in alcoholic solutinn(1916-Landsteiner) or an intense treatment with diazomethane, ~~in addition to esterification.~~ the changed proteins behaved Similarly to the nitrated and iodized proteins in that species specificity is to a large extent lost and a serological relationship appears between similarly treated proteins of different species. If one visualizes the protein molecule as a spherical or eliptical coiling of peptide chains with carboxyl groups at the periphery of the molecule, the pronounced effect of esterification of these groups suggests that the groupings of the periphery, oriented toward the solvent, play a prominent part.

Thus changes in specificity are determined from the character

and position of the substituting groups and also from the changes in the structure of the molecule as a whole which occur during the of substitution.

More recently Landsteiner has approached the problem from another angle starting with substances known and of relatively simple composition and building up synthetic antigens with known chemical groupings, and studying the influence of these groupings on specificity. The chemical grouping of the substance to be tested is linked by means of the diazo reaction to some convenient protein molecule:

$$C_6H_5OH + C_6H_5N_2Cl \longrightarrow C_6H_5{-}N{=}N{-}C_6H_4OH$$

phenol — benzene diazonium chloride — p-hydroxy azobenzene

The colored products formed by coupling with diazonium compounds, designated as azoproteins will give but weak reactions with immune serum for the unchanged protein and will elicit the formation of antibodies even when prepared from the serum of the same species. If the grouping is joined with two immunologically distinct proteins and one combination is used for producing antisera and the other for testing reactions with the antisera in vitro, the reactions which occur must be due to the attached groupings. In this way it has been possible to prepare antisera that give specific precipitation with synthetic antigens in which the active grouping consists of such substances as metanilic acid, para-arsenilic acid, laevo, dextro and meso-tartaric acid, glucosides and galactosides.(Landsteiner and Lamp)

These experiments have shown immunological specifity dependent on differences in chemical structure of the kind that determine the ordinary interactions between organic compounds.

The relation between structure and specificity has been considered on the basis of molecular field theory.(Erhlenmeyer and Bergen, 1932) Because of the close serological relationshop existing between

p PhO H NH , PhNHC H NH , and PhCH C H NH , when coupled by the diazo reaction with horse serum it was concluded that haptenes differing only in groupings whose fields of force, are equal are indistinguishable by immunological means.

The active grouping alone was not sufficient, in most cases, to cause precipitation with antisera prepared against it, by injections of the grouping, but a complete synthetic antigen was necessary. However, the simple group alone would combine with or tie up the corresponding bodies in the antisera, preventing subsequent precipitation by the complex antigen. Marrack and Smith (1932) showed by colorimetric technique. The direct union between certain azo dyes and the antibodies produced against them in combination with protein as a whole antigen.

As a result of these works Landsteiner formulated the conception of the partial antigen or haptene, that part of the antigenic complex that carries the specific reacting group, which alone is unable to stimulate antibody production, but which will combine specifically with the antibodies that are produced in response to the injection of the complete antigen. The view that the two defining properties of antigens, their capacity to immunize and to combine with antibodies, were inseperable and exclusively associated with protein became no longer tenable with the isolation of protein free materials from bacterial cells, of a CH and lipid nature, which, though unable to cause production of antibodies, proved capable of specifically precipitating sera prepared from whole bacteria. Subsequent work with haptene-like substances, particularly many of the polysaccharides separated from bacterial cells, has shown them to differ from Landsteiner's original concept in being capable of causing a precipitate with antisera pre-

pared from the whole antigen of which they were a part. It seems probable that this precipitating capacity is a function of the molecular size and complexity. Landsteiner and van der Scheer, (1932) have shown that azo dyes prepared from p-aminosuccinamic acid, p-aminoadipanitic, and p-aminosuberanitic acids will give a precipitate with the corresponding antisera (prepared from these compounds coupled with protein) without previous linkage to protein and that the intensity of the reaction with these compounds appears to run parallel to the length of the aliphatic chain.

POLYSACCHARIDE HAPTENES

As early as 1904 Pick found in young typhoid cultures a material that did not give the ordinary protein reactions, which was resistant to heat and proteolytic enzymes, and was soluble in alcohol. It possessed the power of giving a specific precipitin reaction with homologous immune serum but lacked the capacity of inciting antibodies. In 1917, Dochez and Avery observed that the culture filtrates of the three main types of pneumococcus contain a soluble substance which gives a specific reaction with the homologous immune serum. Sinsser and Parker (1923) isolated from the pneumococcus, influenza baccillus, and staphylococcus a similar substance free from gross amounts of protein, precipitated by alcohol, and thermostabile which they called residue antigen. In 1923 Heidleberger and Averypublished the first of a series of papers dealing with a chemical study of the antigenice constituents of the pneumococcus capsule. From the filtrate of eight day broth cultures, a material was precipitated by alcohol which was found to have the chemical structure of a complex polysaccharide. It

failed to stimulate antibody production in vivo but gave specific precipitation at dilutions of 1:300,000 when mixed with antisera prepared by the inoculation of rabbits with the corresponding strain of pneumococcus. The activity was not destroyed by boiling or by the action of the proteolytic enzymes. In 1925 Laidlaw and Dudley isolated an immunologically active material from the tubercle bacillus. Early work was done on B. pneumoniae Friedlander, and encapsulated bacillus. (1927)

Since then serologically active carbohydrates have been found in many bacteria. A summary is give in the following table, page 50 51

Methods used in the separation of these carbohydrate fractions include the alcoholic precipitation of material from broth culture filtrate, alkaline or acid hydrolysis of the bacterial cells and alcoholic precipatation of the carbohydrate from the supernatant. In 1931 Morgan isolated carbohydrate material from Shig. shiga. The method used consisted, essentially, of acid hydrolysis of cells, precipitation of supernatant as a basic lead complex which was resuspended in water and decomposed by saturation of the suspension with CO . A rapid means of extracting group specific polysaccharide from the hemolytic streptococcus was devised by Fuller (1938) and consists of dissolving the bacteria in formamide at 150° C and which serves to release the polysaccharide from combination with the other substances. Tubercle bacillus polysaccharides were separated cataphoretically by Siebert (1938). Boivin (1936) has separated specific polysaccharide from the rest of the bacterial cell by dialysis, prolonged and through a collodion membrane of high permeability. (In 1931 Morgan isolated carbohydrate material from Shig. shiga. The method used consisted essentially

SOME ORGANISMS FROM WHICH ACTIVE CARBOHYDRATES HAVE BEEN ISOLATED

Streptococci	Lancefield 1925
B. proteus	Przesmycki and Szczuka 1927
B. anthracis	Przesmycki 1927
B. lactis aerogenes	Tomcsik 1927
Salmonella group	Heidelberger 1928
Yeasts and Fungi	Tomcsik 1930
B. dysenteriae	Morgan 1931
Meningococci	Rake and Scharp 1937
V. cholera	Linton and Shivastava
Gonococci	Casper 1934
Members of the phytomonas and pasteurella group	Dingle 1934
Brucella group	Pavilli and Piemontesi 1934
Spirochaetes	Mandle and Bruce White 1934
Vaccinia virus	Ch'en 1934
Rickettsiae	Castaneda 1934
B. influenzae	Pitman and Goodner 1935
Staphylococci	Julianelle 1936

of acid hydrolysis of cells, precipitation of supernatant as a basic lead complex which was resuspended in water and decomposed by saturation of the suspension with CO_2.)

Since the discovery of the prominent role played by polysaccharides in determining the specificity of serological reactions these substances have been subjected to much chemical study in an effort to understand their significance.

Polysaccharide materials, in general, are resistant to pepsin and trypsin and on treatment with acid and alkali their activity remains undiminished until tests for reducing sugars indicate hydrolytic cleavage of the molecule. They are not destroyed by heat, vary in solubility in water, are precipitated by alcohol, acetone, ether, colloidal iron, do not dialyse.

Most work has been done on the specific soluble substance of the pneumococcus capsule which has been found to determine type specificity. Chemical analysis has shown that the carbohydrate materials isolated from different types are differentiated as sharply by their chemical composition as by their serological reactions.

The carbohydrate of type III pneumococcus (yield = 2gms/10 litres glucose broth culture, Goebel 1930) is a colloidal, strongly acid polysaccharide with a molecular weight between 1000 and 5000. According to Heidelberger (1932) it is built up of aldobionic acid units which consist of one molecule each of glucose and glucuronic acid combined in glucosidic union by means of the aldehyde group of glucuronic acid. The nitrogen content is approximately 1%. Pneumococcus type II yields a weakly acid carbohydrate which gives glucose on hydrolysis, while type I gives a carbohydrate occuring in the acetylated form, having a

positive naphtheresorcinol test for uronic acid. Upon oxidation mucid acid is formed, indicating the presence of galactose. It appears to contain 6% nitrogen, ⅓ of which can be liberated by treatment with nitrous acid indicating free amino nitrogen. This portion of the nitrogen, at least, according to Landsteiner, is part of the specific substance and belongs to an amino sugar.

Work on the specific carbohydrated material from the tubercle bacillus has shown arabinose, mannose, galactose, glucose, trehalose, inosite and acids to be among the hydrolytic products (Laidlaw et al 1926).

Polysaccharides obtained from the three types of Friedlander's bacillus (Klopstock and Mitchsky 1927) give glucose and glucuronic acid on hydrolysis. Type A, the most studied, is probably built up of units consisting of one molecule of glucose, one aldobionic acid and a second unidentified sugar. The aldobionic acid contains glucose, glucuronic acid and is isomeric with the acid of Pneumococcus type III, probably having a different position of the linkages between the components.

The carbohydrates obtained from the cholera vibrio have been shown to contain galactose, arabinose and aldobionic acid (Linton and Shrivastava 1934).

In the Salmonella group differences were found between the carbohydrates of the "S" and "R" forms.

Morgan (1939) described the specific carbohydrate obtained from Shig. shiga as a weak acid containing d-galactose, hexosamine, l-rhamnose. It contained 1.8% N and had a specific reaction capacity in dilutions of 1:12,000,000.

Until recently these protein-free carbohydrates or haptenes have been considered universally non-antigenic. That any large colloidal molecule other than the protein could serve as an antigen has been doubted. However, Francis and Tillet (1930) and Zozaya and Clark (1932) in studying the polysaccharide fractions that have been separated from various types of pneumococci, showed that when these substances are tested by the ordinary methods of inoculation into laboratory animals, they have no antigenic action. But when these haptenes are injected into the skin of human subjects in very small doses, they may lead to the formation of specific antibodies.

The antigenic power of polysaccharides in rabbits, by means of complement fixation tests has been demonstrated by Chow (1937) for material obtained from pneumococcus type I, and by Nishimura (1929) for inulin, soluble starch and dextrin. The precipitin tests in these instances were negative. Wong (1938) found that the polysaccharide fractions prepared from B. rhinoscleromatis by acid hydrolysis, but not those prepared by alkaline hydrolysis, antigenic. This suggests the significance of the method used for preparation in determining antigenicity.

It appears that the acetyl group is not responsible, as was previously thought, for antigenic activity of polysaccharides. Wong (1939) working with polysaccharides prepared from Bach. coli and B. rhinoscleromatis came to the conclusion that the acetyl group probably is not responsible for antigenic activities of the polysaccharides prepared by acetic acid hydrolysis. Felton (1939) claims that the presence or absence of acetyl groups in type I pneumococcus polysaccharide is of no significance for its antigenicity in mice.

It is possible that polysaccharides owe such antigenic power as they possess to the same factors that apparently determine their capacity for precipitating antibody in vitro, that is, their structural complexity and high molecular weight. The state of dispersion of an antigenic material may have some relation to antibody stimulation in tissues. Zozaya and Clark (1933) have reported that the injection into animals of polysaccharides adsorbed on collodion particles, charcoal, aluminum hydroxide or casein, may be followed by the appearance of specific antibodies in the blood.

The importance of carbohydrates in determining the specificity of serological reactions is assuming a place on a par with that of proteins. The multiplicity of the compounds which may result from the asymmetry of carbon atoms in sugars and sugar acids, the position of the oxygen bridges, the α and β glucosidic unions indicate the possibilities for variation.

Methods used to study the effect of the chemical constitution of polysaccharides on specificity include the use of synthetic antigens, particularly those prepared from stereo-isomeric tartaric acids, and glucosides.

In this connection, in work on conjugated carbohydrate-proteins, (Goebel, Babers and Avery, 1932), have synthesized the α and β p-amino-phenal glucosides and by studying their immunological reactions, coupled with protein, have shown an immunological difference between them which closely parallels the distinction between type II pneumococcus and type B Friedlander's bacillus.

Landsteiner and Van der Scheer (1932) have shown the serological difference between steric isomers. These workers have also prepared p-amino benzoyl-glycylglycine and the similarly acylated glycyl-leucine, leucyl-glycine, and leucyl-leucine and have coupled them with proteins.

Each possessed a characteristic specificity although there was some crossing between glycyl-leucine and leucyl-leucine, indicating that the specificity depended more on the structure of the terminal amino acid carrying the free COOH group than on the other components of the dipeptide.

Polysaccharide derivatives produced by methylation, esterification etc. have been studied. Landsteiner ascribes special significance to sugar acids because of the influence on specificity of acid groups in general. Esterification of the polysaccharide of type I pneumococcus, for instance, has been shown to abolish reactivity (Chow and Goebel, 1936). Examination of split polysaccharide products in experiments of Heidelberger and Kendall (1933) show, through precipitation or inhibition, that substances built up of aldobionic acid units are of significance in the specific reactions of the carbohydrate of type III pneumococcus.

Goebel and Hotchkiss (1937) have synthesized azoprotein antigens containing respectively glucuronic and galacturonic acids. These are serologically sharply differentiated from each other and from antigens containing glucose or galactose.

Other evidence suggesting the importance of uronic acids is presented by Goebel (1936). In attempting to find a chemical basis for the immunological cross reactions that are displayed by the specific polysaccharides of pneumococcus types III and IV he found that the polysaccharides of these strains contain an identical uronic acid component and feels that this molecule is probably the common structure within each of the polysaccharides which gives rise to overlapping specificity.

Not all polysaccharides are as clearly distinguishable chemically, as well as serologically, as are those of the capsular material of the pneumococcus. Though serologically distinct, the carbohydrates of the

Salmonella group cannot be clearly differentiated chemically. This is also true of the carbohydrate obtained from the three serological types of Friedlander's bacillus. With the exception of solubility they are very much alike chemically though entirely different in their immunological reactions. Goebel and Avery (1927) attribute the serological dissimilarity to a different type of linkage between sugars and sugar acids.

On the other hand it appears that chemically different carbohydrate fractions obtained from varied sources can account for heterogeneous reactions, due to common groupings in the polysaccharide molecule (Landsteiner). Thus the reaction of gumarabic with immune serum for pneumococcus type II and III, of type II pneumococcus carbohydrate with immune sera for a strain of B. lepisepticus, of acetylated polysaccharide of pneumococcus type I with antibodies against human "A" blood, of polysaccharides of the gonococcus and meningococcus with anti-pneumococcus serum type III.

Also Zozaya (1931) reported immunological relationship between the polysaccharides of B. anthracis, B. subtilis, B. proteus and B.mesentericus.

The specific carbohydrate of pneumococcus type XIV is of special interest as horse serum immunized with this organism contains agglutinins for human erythrocytes (Finland and Curnen, 1938).

Thus it seems probable that the study of polysaccharides will provide information on the apparent mosaic structure of cell antigens.

LIPOID HAPTENES

Evidence in support of lipoids as specific substances in serological reactions is increasing. Separation of specific lipoids in unquestionably pure state, and their accurate chemical characterisation, has not been accomplished (Landsteiner 159). Solubility in organic solvents, lack in antigenic activity and resistance to treatment with alkali as in the case of the Forssman haptene, have been the main criteria for grouping as lipoids.

Such material is the active substance in the complement-fixation, and flocculation reactions of syphilitic sera with extracts of normal organs. Also the inactivation of toxins and hemolysins, such as the neutralisation of tetanolysin by very small amounts of cholesterol point to the significance of lipoids in immune reactions.

In experiments on the production of antibodies to sterols by the injection of these substances along with serum proteins, Weil and Besser obtained antibodies with cholesterol, hydroxycholesterol and dihydrocholesterol, while no immune response was caused by cholesterol oxide, dibromides, or esters of cholesterol. Antisera for cholesterol and dihydrocholesterol differentiated these two compounds and did not give reactions with the non-reactive sterols.

The role of the lecithin and cholesterol from organ extracts in non-specific reactions must be considered. Cerebrosides and phosphatides are present in rather large amounts in all tissue and specific reactions would appear not to be due to these but to unknown substances in small amounts. Fischer (1933), in experiments using adsorption and elution methods for purification found adsorption with aluminum hydroxide removed from the extracts of heart muscle, the substance which reacts with syphilitic sera. The active substance adsorbed on aluminum hydroxide and eluted with benzene yielded on hydrolysis more fatty acid

and reducing sugars and less phosphorus and nitrogen than the original extract.

Serologically active phosphatide fractions were separated by K. Meyer (1921) from alcoholic extracts of tape worms and tubercle bacilli. Machebocuf (1937) found the haptenic, lipid fraction from the tubercle bacillus to be a mixture (chiefly in the form of magnesium salts) consisting of high molecular fatty acids combined by ester linkages with either glycero-phosphonic acid or inositol-monophosphoric acid. The preparation lost its serological activity progressively as the fatty acids were liberated with dilute mineral acids. Which of the two sorts of complex acids carried the haptenic property was undetermined.

Boivin and Mesrobeanu (1933) discovered carbohydrate lipid complexes in the "smooth" forms of gram negative bacteria, which were highly antigenic, labile substances, inciting the production of agglutinins of the granular "O" type, and which are endotoxins. Such materials have been isolated from the dysentery bacillus by Morgan (1937), from the pasteurella group by Pirosky (1938), from B. pyocyaneus by Boivin (1937), from B. anthracis by Ionesco Mihaiesli (1937), from E. typhosa by Topley and Raistrick (1937). In the case of Proteus X19, the carbohydrate-lipid antigen appears to represent the material responsible for the Weil-Felix reaction (Cruesa 1938). Morgan, in his work with the Shiga bacillus extracted the dry cells with diethylene glycol (1937). The material obtained gives both anti-bacterial and heterophil antibodies and has the properties of Shiga endotoxin. It consists of a combination of polysaccharides with fatty acids and also with acetic and phosphoric acids. The heterophil activity of the complex appears to be less than that of the polysaccharide.

The presence of serologically related substances of a non-protein lipid nature in cells of animals and in various bacteria, entirely

unrelated, was first made clear by Forssman (1911). This particular type of non-protein hapten is found in the organs and corpuscles of of a wide variety of animals including guinea pigs, horses, chickens, the cat and dog family and when injected into a rabbit causes the production of Forssman or heterophil antibodies. These antigens are absent from the organs of man, rabbit, ox, sheep and rat. It is of interest that the organs of the sheep, do not stimulate the production of Forssman antibody while the sheep red corpuscles are a notably rich source of heterophile antigen. It appears to be a general rule that those species that contain heterophile antigen in their red corpuscles, do not contain it in their tissues, at least in the full and effective antigenic form. Also only animals of the rabbit type, those not containing heterophile antigen in their tissues, are capable of producing heterophile antibody in response to injection of suitable material.

Considerable interest has been shown in the Forssman antigens of bacteria. The first description of heterospecificity among unicellular organisms was presented in 1904 by Ballner and von Sagasser who found that the serum of rabbits immunized to red yeast agglutinated the typhoid and colon bacillus and varieties of the dysentery organism.

Organisms found to contain Forssman antigen are the Shiga bacillus (Braim and Schiff - 1930); the pneumococcus (Bailey and Short - 1931); less frequently, strains of the hemorrhagic septicemia group (Buchbinder 1934); the paratyphoid bacillus; anthrax bacillus (Combiesco - 1930); streptococcus (Bailey and Short - 1931). Bailey and Short (1934) found the following species, in addition to the above, to contain Forssman antigen: Alcaligenes bookeri, Alcaligenes faecalis, Bacillus cereus, Bacillus megatherium, Bacillus petasites, Clostridium oedematiens,

Clostridium welchii, Lacto bacillus acidophilus, Lactobacillus bulgaricus, Neisseria catarrhalis, Neisseria gonorrhoeae and Sarcina aurantiaca. There appears to be no evidence of the occurrence of the antigen in Shig. flexneri or in Bact. coli.

Forssman antigen is soluble in common lipoid solvents - alcohol, ether, chloroform - but not in acetone, and therefore seems to be of lipoid nature. It is heat stable. Alcohol, ether or chloroform extracts, when injected into suitable animals are non-antigenic, but if such material is mixed with protein, as with serum, specific antibodies are produced (Landsteiner 1921). This is not the case with carbohydrate haptenes. Doerr and Hallauer (1925) suggest that the proteins act merely as physical carriers, kaolin or collodion particles and the like being employed to the same end. Landsteiner (1932) feels that the protein forms a new compound of loose construction which functions as a whole antigen.

Though the existence of the Forssman haptene in lipoidal form in animal cells would seem probable, various evidence points to the carbohydrate nature of the Forssman antigen in bacteria. Certain bacterial carbohydrates prepared by Landsteiner and Lewing (1932) combine specifically with Forssman lysins. Further fractination of the Forssman substances with characteristic properties, yields materials soluble in water and dilute alkali, but very slightly soluble in most organic solvents, containing a greater quantity of reducing sugars on hydrolysis along with considerable amounts of fatty acids (Landsteiner 1932). This suggests that carbohydrate may be involved in the specific grouping. That the carbohydrate is linked to the fatty substance rather than being mixed with it is indicated by the solubility of the original material in alcohol, and by the diffulty of separating the carbohydrate material. The Forssman antigen of the anthrax bacillus (Combiesco 1930)

appears to be related to the SSS of that bacillus. Buchbinder states (1935) that bacterial Forssman antigens are probably all of carbohydrate nature or at least associated with sugar.

The study of bacteria containing the Forssman antigen has revealed the existence of distinct immunologic varieties (Zinsser 1931). In addition, evidence has accumulated in the past few years that other heterophile relationships between bacteria and animal tissues, similar in nature to that described by Forssman, but clearly distinct from it, also exist, and that the Forssman antigen is not unique, but is merely one of many substances of a similar nature.

A new type of haptene of polypeptide nature was isolated from the capsular substance of B. anthracis and B. mesentericus by Ivanovics and Bruckner (1937). It was found to be an acid yielding, on acid hydrolysis, the optical isomer of l (+) glutamic acid. The substance is considered to be a high molecular polypeptide containing only the one amino acid with 40-80 acid units to the molecule. It is suggested that the capsular material makes for virulence of the organism since it is built up of "unnatural" d(-) acid and is therefore resistant to digestion by enzymes.

In considering the literature on bacterial haptenes, it appears that carbohydrate materials play the most significant role. The status of the lipoid material is less definite. The specificity of Forssman and similar intigens, distributed widely throughout the plant and animal kingdoms associated with lipoid material and extracted by organic solvents, has been shown in many cases to be determined by carbohydrate groupings in the complex.

EXPERIMENTAL WORK

TESTS FOR THE PRESENCE OF FORSSMAN ANTIGEN

Instarting investigations on the nature of a possible haptene in Bact. coli it seemed to us that an attempt to isolate a carbohydrate material having specific reacting properties would be the best approach. However, due to the wide distribution of the Forssman antigen in bacteria, before starting this work we decided to carry out certain simple experiments to gain information of the presence or absence of this factor in Shig flexneri and Esch. coli.

High titer anti sheep cell sera (rabbit) containing a high content of Forssman agglutinins was used to test for Forssman antigen in the three Flexner strains, 352, 352 A and Warden, and 9 Bact. coli strains having a high titer in Flexner antisera. The results are shown in Table XII.

In no case does the agglutination titer exceed that which might be expected in normal sera.

As a check, antisera for the above strains were tested with a suspension of sheep cells. No lysis of the cells occured. This evidence appears to exclude the presence of Forssman antigen in the strains of Bact. coli and Shig. flexneri tested.

These findings, together with the complete lack of evidence in the literature of the existence of Forssman antigen in strains of Shig. flexneri and Bact. coli, convinced us that we should search for a haptene of carbohydrate nature.

TABLE XXI

AGGLUTINATION TESTS IN HIGH TITER ANTI-SHEEP CELL SERA WITH SFLEXNER AND 9 COLI STRAINS

Serum dilution	1:20	1:40	1:80	1:160
Bacterial strain				
Flexner strains				
552	+	0	0	0
302A	+	0	0	0
Harden	+	0	0	0
Coli strains				
Morrimen u1	+	0	0	0
Mayfield	+	0	0	0
Sparks	+	+	0	0
Cruse	+	+	0	0
R.A.	+	+	0	0
199	0	0	0	0
Morrimen s	+	+	0	0
Bolt	+	+	+	0
Reider	+	+	0	0

PREVIOUS WORK DONE ON THE ISOLATION OF CARBOHYDRATE FRACTIONS FROM BACT. COLI AND DESCRIPTION OF THE METHODS USED

In 1900 Emmerling and Schmadinger attempted to isolate carbohydrate material from the filtrate of broth cultures of Bact. coli. Even after 80 days incubation the bulk of the substance was associated with the bacterial bodies.

Isolation of a specific carbohydrate material from Bact. coli was accomplished in 1927 by Dorothea Smith. She also found that the specific soluble substance isolated from an encapsulated strain remained in the capsule and did not diffuse into the medium, as there was no trace of a precipitin reaction with 24-72 hr. broth culture filtrates at a dilution of 1:4. Undiluted filtrates gave a good but not a heavy reaction. She concluded that practically no carbohydrate was diffusable.

In the preparation of polysaccharide, Smith used tin pie plates covered with tin plates of larger size which contained veal infusion agar plus 0.1% dextrose. Two-day cultures were emulsified in water and hydrolysed with alkali. The bacterial sediment obtained on centrifugation was discarded and supernatant fluid was precipitated by addition of 1.2 volumes of 95% alcohol. Purification of the material was carried out by repeated solution in water and precipitation with alcohol.

The final product gave a positive precipitin test with serum prepared from the homologous organism in a dilution of 1:2,000,000. It was a white powder, soluble in hot water, giving an opalescence even in 1% solution. It was hydrolysed by boiling with acid and the hydrolysate reduced Fehling's solution. The amount of reducing substance calculated on the basis of glucose was found to be 80%. The material gave a slight naphthoresorcinal test for glucuronic acid, and a slight test

test for orcinol, indicating the presence of glucuronic acid, but too slight for a pentose. The nitrogen content (microkjeldahl) was found to be 0.6%. Further analysis showed a carbon content of 43.56% and a hydrogen content of 6.41%. For $(C_6H_{10}O_5)$ the percentage of carbon is 44.4% and of hydrogen 6.2%. The carbohydrate nature of the material was therefore evident.

Concerning the relative amounts of specific carbohydrate in capsulated and non-capsulated strains, Smith found the non-capsulated strains to contain only 2/3 as much polysaccharide by weight, per unit culture area, as the capsulated strains, with 100 times less activity. Mueller (1924) and Heidleberger (1925) suggest that capsular substance and specific soluble substance are identical. In the case of a morphological capsule, the specific substance is produced in much larger quantity and located peripherally. Other substances such as bacterial mucins may also take part in capsule formation. In the case of capsular Bact. coli strains there was no indication of more than a trace of mucin, though in the preparation of specific carbohydrate from a viscid strain of Friedlander's bacillus, there was obviously a very large mixture of an impurity that was probably a mucin.

Tomcisk (1927) extracted a specific substance from the capsules of a number of Bact. coli strains. 48 hour meat infusion agar cultures from Kolle flasks were suspended in water, KOH was added to 10%, and the suspension was placed in the incubator at 37°C until the capsules disappeared, which was usually at the end of 3-4 hours. After centrifugation and addition of acetic acid to the supernatant fluid to precipitate nucleoprotein, the liquid was filtered. The specific substance was precipitated from the filtrate by the addition of three times the volume of 95% alcohol. Purification was carried out by

reprecipitation from water solution by alcohol. The final product was a white powder soluble in water with a nitrogen content of 1.4% and specific activity in a dilution of 1:200,000 in the homologous sera. Protein tests were negative. After hydrolysis for 4-5 hours at 100°C in 1 N H_2SO_4 the amount of reducing substance was 60% calculated on the basis of glucose.

Dernce and Wight (1935) extracted a soluble substance from an encapsulated strain of Bact. coli which reacted with a pneumococcus type I horse serum, and not in normal horse serum, or horse serum immunized to types II or III, or other strains.

EXPERIMENTAL WORK

ISOLATION OF A CARBOHYDRATE MATERIAL FROM BACT. COLI

METHODS

On the basis of the work done previously on the isolation of carbohydrate material from Bact. coli, and the materials available in the laboratory the following proceedures were worked out.

Alkaline Hydrolysis 66 blake bottles containing beef infusion agar were inoculated with a saline emulsion of 24 hr. agar slant cultures. The blake bottles were incubated 48 hrs.

The organisms were washed from each bottle with 4cc of 95% ethyl alcohol and placed in the icebox overnight, to aid sedimentation. The next day, as much of the supernatant as possible was removed and the cells were collected from the remaining fluid by centrifugation. The organisms were then suspended in 200cc of distilled water, centrifuged to free from media constituents, and dried in a desiccator over $CaCl_2$.

The dried bacteria (wt-2.36 gm) were resuspended in water in the proportion of one part bacilli to 150 parts of water, mixed, and placed in the icebox over night to permit hydration.

3.6 cc of glacial acetic acid was added to make a 1% solution and hydrolysis was carried out for 30 minutes over a boiling water bath. The mixture was then cooled and centrifuged. The sediment was discarded and four times the volume of 95% ethyl alcohol, or 1340cc was added to the supernatant fluid. The liquid became opalescent and later a fine white precipitate settled out. The mixture was placed in liter graduated flasks to provide a high column for sedimentation of the precipitate. The graduates were placed in the icebox overnight to allow

time for precipitation and the following day as much of the supernatant as possible was siphoned off. The rest of the material was centrifuged and the precipitate collected. In order to further purify the material it was resuspended in a small amount of distilled water (150cc) and the mixture was placed in the incubator several hours to aid solution. Centrifugation showed that little of the material had gone into solution so the precipitate was reemulsified and the mixture was heated in a water bath at 75°C for 2 hrs. This time on centrifuging less precipitate was collected and the supernatant was distinctly opalescent. Precipitation of the supernatant was repeated with 4 volumes of 95% ethyl alcohol. And the process of solution and precipitation was repeated once more. ~~This method is represented diagrammatically on the following page.~~ (70)

The final product was dried in a desiccator and weighed. From 2.36 gms of dried bacteria 0.11 gm of the material was obtained, or a yield of 4.6%. In appearance it was a white amorphous powder, soluble with difficulty in hot water and giving an opalescent solution.

EXPERIMENTS PERFORMED WITH THE MATERIAL OBTAINED FROM ACID HYDROLYSIS OF BACT. COLI

Serological tests Solutions of the material in approximately 1:5000 and 1:10,000 dilution were tested with the homologous antisera (R. A.) and antiflexner sera in the ring precipitation tests. Normal sera was used as a control.

½ of a cc of saline and ½ of a cc of test solution were placed in each tube (serum tubes were used) and 2 cc of serum was carefully underlayered. The test tubes were allowed to stand ½ hr. The results are shown on page 71.

TABLE XIII

PRECIPITIN TESTS WITH THE FRACTION PREPARED BY ACID HYDROLYSIS OF BACT. COLI R.A. IN NORMAL, ANTI-COLI AND ANTI-DYSENTERY SERA

Antigen dilution	1:10,000	1:20,000
Serum		
Normal	no ring	no ring
Anticoli	definite ring	definite ring
Antidysentery	slight ring	no ring

The final dilution in tubes "A" was approximately 1:10,000 and in tubes "B" approximately 1:20,000. The controls containing normal sera showed no ring. Both tubes containing the homologous coli antisera showed definite rings. The tubes containing Flexner antisera showed faint rings which disappeared on standing.

The value of these results is limited by the small number of tests performed. However, the definite ring formed in the case of the anti coli sera is evidence for the presence of a specific reaction substance. The slight ring formed with the antiflexner sera suggests that the isolated fraction may contain a serologically active component common to both Bact. coli and Shig. flexneri.

Contamination of the test solution with a gram negative bacillus prevented the use of the material for further tests.

Chemical tests As the precipitin tests indicated that the material isolated contained a specific reactive substance the following chemical tests were performed to gain evidence of its nature.

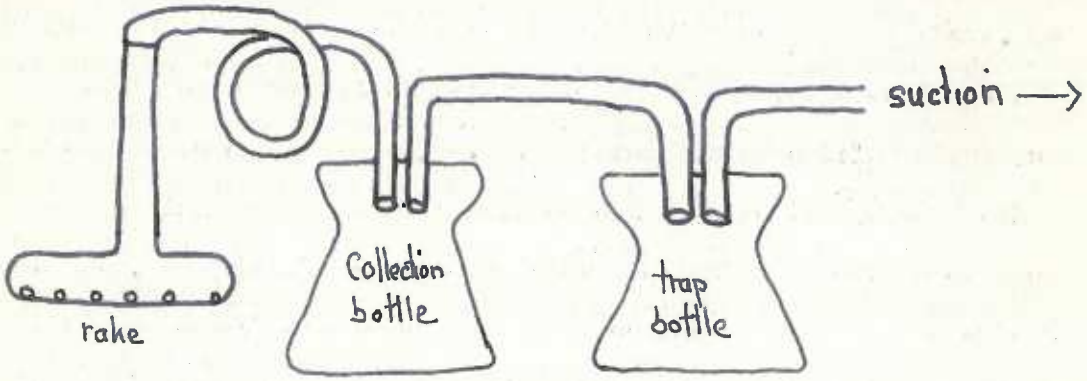
The nitrogen content as determined by the microkjeldahl method was found to be approximately 8.2%. Hydrolysis of a 1:150 dilution of the material in $1N H_2SO_4$ was carried out in a boiling water bath for 2½ hrs. A Schaeffer-Hartmann determination on a portion of the material indicated that reducing substance was present to the extent of 88% (calculated on the basis of glucose). Further evidence of the carbohydrate nature of the material was the strongly positive Molisch test. The material gave no color with iodine. Protein tests (Millon's, sulphosalicylic acid) were negative.

The experiments performed indicate that the fraction isolated from Bact. coli by acid hydrolysis is carbohydrate in nature and possesses specific reactive properties.

ALKALINE HYDROLYSIS OF BACT. COLI

As most of the work on the isolation of a polysaccharide fraction from Bact. coli has been done by alkaline hydrolysis of the bacterial cell, it seemed advisable to prepare a second fraction by this method and compare the materials obtained.

As in the previous extraction the handling of a large number of blake bottles had proven inconvenient, a new method was worked out for the harvesting of bacteria. This time cultures were grown on 250 petri dishes which were found to have approximately the same surface area as blake bottles and were easier to handle. Cultures were incubated 48 hrs. About 4cc of distilled water was added to each plate by means by means of a sterile pipette. For collection of bacteria from the surface of the plate a glass suction rake was improvised. The rake consisted of glass tubing, having a two inch cross piece with small holes along one side, and 6 inch handle which was attached to suction.



The rake was swept rapidely over the surface of the plates and the bacteria were sucked through the handle into the collection bottle. This method greatly facilitated rapid collection of bacteria.

1.4 cc of 1 N NaOH was then added for each plate used, and the mixture was heated in a water bath at 70° for ½ hr. The mixture was the made slightly acid with HCl. One crystal of NaAc and 1cc of 95% alcohol was added per plate and the flask allowed to stand several hours.

for precipitation of extraneous material. The material was then centrifuged and the deposit was discarded. The supernatant was made neutral by the addition of a few drops of weak alkali and twice the volume of 95% alcohol was added. The mixture was placed in the icebox overnight in graduated titer cylinders to provide a tall column for sedimentation. As much of the supernatant was siphoned off as was possible without disturbing the precipitate. The remaining material was centrifuged, the fluid discarded and the deposit dissolved in hot water. The process of precipitation was repeated twice. The final material was white and powdery and gave a straight opalescent solution.

EXPERIMENTS PERFORMED WITH THE MATERIAL OBTAINED FROM ACID HYDROLYSIS OF BACT. COLI

Serological tests: Solutions of the material in approximately 1:5000 and 1:10,000 dilution were tested with the homologous anti-sera (B.A.) and antiflexner sera in the ring precipitin test. Normal sera was used as a control. Tests were carried out as described previously, and are shown on page 75. Positive tests occured only in the case of the homologous antisera.

Chemical tests: The nitrogen content as determined by the micro-kjeldahl method was found to be 8.5% as compared to 8.2% for the fraction prepared by acid hydrolysis. A portion of the material in 1 N H Cl was hydrolysed by heating for 2½ hrs. in a boiling water bath. A Schaeffer-Hartmann determination on a portion of the material indicated that reducing substance was present. The Molisch test was positive but not as strongly so as in the case of the other fraction. Protein tests (Millon's, sulphosalicylic acid) were negative.

These tests indicate that this fraction is carbohydrate in nature

TABLE XIV

PRECIPITIN TESTS WITH THE FRACTION PREPARED BY ACID HYDROLYSIS OF BACT. COLI IN NORMAL, ANTI-COLI AND ANTI-DYSENTERY SERA

Antigen dilution	1:10,000	1:20,000
Serum		
Normal	no ring	no ring
Anticoli	ring	ring
Antidysentery	no ring	no ring

and possesses specific reactive properties. Neither the chemical nor the serological tests were as conclusive as in the case of the fraction obtained by acid hydrolysis.

SUMMARY

The experimental work on the fractions obtained from the E. A. strain of Bact. coli by alcoholic precipitation of the supernatant from both alkaline and acid hydrolysate showed, (1) a positive precipitin test with both fractions in the homologous antisera, (2) the slightly positive reaction of the fraction obtained on acid hydrolysis in antidysentery sera, indicate the presence of a specific carbohydrate substance which is common to both organisms.

That this specific substance was carbohydrate in nature was indicated by, (1) the method used in its preparation, (2) its similarity in appearance to other carbohydrate fractions prepared from various bacteria - being a white powder, soluble (with difficulty) in H_2O giving an opalescent solution, and precipitated as a white flakey material on addition of alcohol, (3) the presence of reducing sugar on hydrolysis and positive Molisch reactions, (4) Protein tests were negative, (5) The total nitrogen content (3.2 - 3.8%) was within the range found in other carbohydrate materials isolated from bacteria. Further serological experiments and chemical tests on more completely purified material are necessary for an understanding of the significance of this specific carbohydrate substance. The methods used are given rather completely in the hope that they will be of use in a continuation of this work.

GENERAL SUMMARY

Agglutination and absorption experiments investigating the antigenic relationship of the Shigella paradysenteriae Flexner and Escherichia strains indicated that this relationship was due to a haptene component in Bact. coli contained as a whole antigen in the dysentery bacillus. These results confirmed those obtained in similiar work by Swanson in the same laboratory. Further analysis of an apparent conflict with Brandon, working on similiar experiments, showed that his experimental data was not sufficient to contradict our hypothesis.

We decided therefore that we would directly investigate this hypothesis by attempting to isolate a haptene.

In considering the literature on bacterial haptenes it appeared that carbohydrate materials play the most significant role and that work on isolation of a carbohydrate material from Bact. coli would be the best approach to the problem. However, due to the wide distribution of Forssman antigen in bacteria associated with lipoid material we decided to carry out certain simple experiments to gain information concerning the presence or absence of this factor in Shig. flexneri and Esch. coli. Results gave no indication of the presence of this factor and convinced us that we should search for a haptene of carbohydrate nature.

A review of the literature showed that carbohydrate fractions had been isolated from Bact. coli by alcoholic precipitation of supernatant fluid obtained by both alkaline and acid hydrolysis of the bacterial cell.

In our work a method employing acid hydrolysis similar to that used by Wong (1938) and one involving alkaline hydrolysis as employed by Lovell (1937) were used.

The material obtained in both cases appeared to be carbohydrate in nature.

Experiments with broth culture filtrates in which positive precipitin tests were obtained in the undiluted filtrate indicated that the specific substance diffused to some extent.

Insufficient work has been done on these carbohydrate materials to merit any definite conclusions. However, the positive precipitin tests given with the homologous antisera and in one case with anti-dysentery sera suggest that further work along this line would be profitable. To this end the literature reviewed and methods used have been rather completely included in this thesis.

BIBLIOGRAPHY

Andrewes, F.W., and Inman, A., "Study of the serological races of the Flexner bacilli," Med. Res. Council Report, 1913 p.41

Bloor, F., "Lipids of certain enteric bacilli," J. Bact., 37:301 1939

Boivin, A., "Possible coexistence of a complete somatic "O" antigen and corresponding polysaccharide hapteno in certain bacteria," Comp. Rend. Acad. de Science, 203:694, 1936

Brandon, G., "Studies on the "O" antigens of typhoid, paratyphoid and colon bacilli," Unpublished thesis presented to the University of Oregon Medical School, June 1940

Buchanan, R.E., and Fulmer, E.I., "Physiology and Biochemistry of Bacteria," Baltimore: Williams and Wilkins, 1930 p.87, 91

Buchbinder, L., "Heterophile phenomena in immunology", Arch. Path., 19:841, 1936

Bull, C.G., "A Manual Knowledge of Bacteriology and Immunology", Chicago: University of Chicago Press, 1928 p.733

Chase, M.W., and Landsteiner, K., "Immunochemistry", Ann. Rev. Bioch., 1939, p.595

Dingle, J.H., and Fothergill, L.D., "The isolation and properties of the specific polysaccharide of Type B. Hemophilus influenzae.

Felton, L., Houffmann, C., and Stahl, H., "The precipitation of bacterial polysaccharides with calcium phosphate", J. Bact., 29:149, 1935

Fuller, "Formamide method for the extraction of polysaccharide from hemolytic streptococci," Brit. J. Exp. Path., 19:130, 1938

Goebel, W.F., "Chemo-immunological studied on soluble specific substances of the pneumococcus", J. Biol. Chem., 110:591, 1935

Goebel, W.F., Babers, F.A., and Avery, O.T., "Conjugated carbohydrate-proteins" J. Exper. Med., 55:761, 1932

Hawk, P.H., and Bergeim, O., "Practical Physiological Chemistry", Philadelphia: Blakiston's, 1931 Chap. II

Heidleberger, M., and Avery, O.T., "Isolation of carbohydrate material from the pneumococcus," J. Exper. Med., 38:73, 1923

Heidleberger, M., and Avery, O.T., and Goebel, W.F., "Relation between polysac polysaccharides from a strain of B. friedlandori and pneumococcus type II," J. Exper. Med., 42:701, 1925

Heidelberger, M., "Immuno-chemistry", Ann. Rev. Bioch., 1932, p.491

Heidelberger, M., "Immunochhemistry," Ann. Rev. Bioch., 1933, p.503

Hiss, P.H., "Agglutinative characters of bacilli, J. Med. Res., 1904, vol.13

Ingalls, M.S., " A contribution to the nature and origin of natural agglutinins," J. Immun., 33:123, 1937

Jordan, E.O., and Falk, I.S., "The Newer Knowledge of Bacteriology and Immunology," Chicago: The University of Chicago Press, 1928, chap. 2,52,

Juliamello, L.A., "Immunological specificity of Bact. aerogenes and its antigenic relation to pneumococcus type II and Freidlander's Bacillus," J. Immunol., 32:21, 1937

Jungebint and Rees., "On the presence of Heterphile antigen in bacteria of the paratyphoid-dysentery group", J. Immun., 20:117, 1931

Landsteiner, K., "Coli antigens and individual specificity," J. Immun., 15:589, 1928

Landsteiner, K., "Specificity of Serological Reactions," Springfield: Thomas, 1936

Lovell, R., "Classification of Bact. coli from diseased calves," J. Exper. Med., 44:125, 1937

Mackie, T.J., "Agglutination absorption Reactions between Shig. dysenteriae and Bach. coli," J. Bact., 37:27, 1939

Mackie, T.J., "The Immunity reactions of the coli group," J. Bact. and Path., 18:137, 1913

Morgan, W., "Immunochemistry: preparation and properties of a specific polysaccharide from Shig. paradysenteriae Shiga," Biochem. J., 1930, 30:909

Morgan, W., "Isolation of a polysaccharide from Shig. shiga," Brit. J. Exper. Path., 13:342, 1932

Neuberger, A., and Quill, M.E., "Possible relationships of carbohydrate components of proteins to their antigenic properties," Biochem.J., 34:109, 1940

Smith, D., "Studies on Pathogenic B. coli from bovine sources" J. Exper. Med., 46:165, 1927

Swanson, P., "The antigenic relationships of Shig. paradysenteriae Flexner and Escherichia strains," Unpublished thesis presented to the University of Oregon Medical School, June, 1940

Zomsisk, J., "On the Carbohydrate-like specific substance in the Colon-aerogenes group," Proc. Soc. Exper. Biol. and Med., 24:810, 1927

Topley, W.W.C., "An outline of Immunity," Baltimore: William Wood, 1938 chaps. Sand5

Topley, W.W.C., and Wilson, G.S., "Textbook on the Principles of Bacteriology and Immunology," Balti ore: William Wood and Co., 1939

Wells, H.G., "The Chemical Aspects of Immunity," New York: Chemical Catalog Co., 1925

Wight, E.C., and Barnes, L.A., "Serological relationship between Pneumococcus type I and an encapsulated strain of Esch. coli," J. Exper. Med., 62:281, 1935

Wong, S.C., "Immunological studies of polysaccharides of an encapsulated bacillus," Proc. Soc. Exper. Biol. and Med., 38:107, 1938

Wong, S.C., "Immunological properties of a bacterial polysaccharide prepared by acetic acid hydrolysis," Chinese M. J. Supp. 2 p.458

Wong, S.C., "Significance of the acetyl group for the antigenic activity of bacterial polysaccharides," Proc. Soc. Exper. Biol. and Med., 40:357, 1939

Zinsser, H., and Bayne-Jones, "Textbook of Bacteriology," New York: D. Appleton-Century Co., 1934

Zozaya, J., "Serological studies of the polysaccaride of the meningococcus, B. anthracis, B. proteus, B. subtilis, B. Mesentericus," J. Exper. Med., 54:725, 1931