VIRULENCE OF SALMONELLA TYPHIMURIUM

II. Studies of the Polysaccharide Antigens of Virulent and Avirulent Strains

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In a previous communication (Pike and Mackenzie, 1940) we have recorded the results of studies which were part of an attempt to identify and evaluate the factors which determine the virulence of *Salmonella typhimurium* for mice. First, it was shown that quantitative determinations of the virulence of *Salmonella typhimurium*, if they are to have validity, should include careful consideration of the dosage factor and of the degree of resistance of the mice to this infection: with some strains of this micro-organism a small increment in dosage causes a large increase in percentage mortality; measurement of virulence with highly susceptible mice may fail to reveal differences which are clearly demonstrated when adequate numbers of more resistant mice are used. Furthermore, it was shown that invasiveness and resistance to phagocytosis are at most minor determinants of virulence, and that sustained ability to multiply in the tissues of the host is the character which differentiates most unmistakably the virulent from the avirulent strain.

That the immunizing capacity and the toxicity of several members of the typhoid-paratyphoid group of bacteria depend upon polysaccharide antigens has been reported by Boivin, Mesrobeanu, and Mesrobeanu (1933), Raistrick and Topley (1934) and Felton and Wakeman (1937); these studies have not, however, revealed whether or not these antigens are also important as determinants of virulence. Previous studies (Mackenzie, Fitzgerald, and Pike, 1935), in which intact bacilli treated with

alcohol were used, indicated that the virulence of *Salmonella typhimurium* did not depend upon the smooth somatic antigen. In these experiments no attempt was made to isolate the polysaccharide antigens or to determine their significance as determinants of virulence. Even though there is considerable evidence (Boivin, Mesrobeanu, and Mesrobeanu, 1933; Raistrick and Topley, 1934) which seems to indicate that the principal constitutents of the smooth somatic antigen are polysaccharides, it seemed necessary in attempting to analyze the virulence of this species, to study the properties of the isolated and partially purified polysaccharide antigens.

EXPERIMENTAL

Cultures

Two smooth cultures of *Salmonella typhimurium*, BA_2 and TMO, and two rough cultures, V47D and RZ1, which we have used in this work have been described in detail elsewhere (Mackenzie, Fitzgerald, and Pike, 1935; Pike and Mackenzie, 1940). The colonies formed by V47D and RZ1 have grown progressively less rough over a period of 4 years but this change has not been accompanied by changes in any of the other characteristics of rough cultures. Observations on the virulence, the immunizing power, the invasiveness, and the resistance to phagocytosis of these strains have been recorded (Mackenzie, Fitzgerald, and Pike, 1935; Pike and Mackenzie, 1940).

Methods

Mass cultures were grown on beef infusion agar pH 7.6 in 15 cm. petri dishes. The 18- to 20-hour growth was collected by flooding the agar with sterile distilled water and suspending the bacteria by scraping the surface of the agar with a platinum wire. The heavy suspensions thus obtained were filtered through absorbent cotton to remove particles of agar.

Three methods were employed in the preparation of antigenic material:

1. The method of Raistrick and Topley (1934). An equal volume of acetone was added to suspensions of bacteria in distilled

water in large centrifuge tubes. After standing at room temperature for 1 hour the tubes were centrifuged, the supernatant fluid discarded and the sedimented bacteria emulsified in acetone. After standing at 37°C. over night the tubes were again centrifuged, the supernatant fluid discarded, the sediment washed twice with small amounts of acetone and dried. The tryptic digestion and fractionation of the dried bacilli were carried out exactly as described by Raistrick and Topley (1934). At this point it became apparent that the yield of each fraction was relatively small. Since the fractions which Raistrick and Topley precipitated by concentrations of alcohol from 50 to 68 per cent differed so little and since they subsequently used only the 68 per cent alcohol precipitation (Topley *et al*, 1937), we combined the 68/50, 68/60, and 68/68 fractions and reprecipitated this material with 68 per cent alcohol. This material will be referred to as the Raistrick-Topley antigen.

2. The method of Boivin et al. (1933, 1934a and b, 1935a and b). To the tryptic digest, prepared as in the Raistrick-Topley method above, trichloracetic acid was added to a concentration of N/4 and extraction carried out at 4°C. for 3 hours. The mixture was then centrifuged and the opalescent supernatant fluid placed in dialyzing sacs made with a 5 per cent solution of collodion in glacial acetic acid. The material was dialysed for 48 hours against running water and, after acidulation, for 24 hours against distilled water. The contents of the sacs were then removed, acidulated, and precipitated with 5 volumes of alcohol. The precipitate was washed with alcohol and ether and dried. This material constituted the Boivin antigen.

3. Extraction with distilled water at 60°C. This method was essentially that described by Smith (1938) for the preparation of toxic extracts from *Eberthella typhosa*. Similar methods have previously been used for extracting toxic and antigenic substances by Pick (1902, 1912), Weil and Felix (1920), Fukuhara and Ando (1913), Chase (1931), White (1931), and Landsteiner and Levine (1932). Bacterial suspensions were prepared as before except that cold distilled water was used and the suspension was kept in an ice bath. This cold suspension was centrifuged, the super-

natant removed, and the bacteria resuspended in distilled water. After heating in a water bath for 4 hours at 59 to 60°C., the suspension was again centrifuged and the supernatant extract filtered through a Berkfeld N candle. The filtrate was precipitated with 5 volumes of alcohol, the white precipitate washed

TABLE 1

Yield and chemical analysis of antigenic fractions prepared from Salmonella typhimurium

PREPARATION	STRAIN	YIELD	QUALITATIVE CHEMICAL ANALYSIS			QUANTITATIVE CHEMICAL ANALYSIS						
			Protein	Amino acid	Carbohydrate	Glucose	C	H	N	S	P	Residue
		per cent				*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Raistrick-Topley	BA_2, smooth high virulence	8	−	+	+	40.0	37.8	6.3	5.0	0.5	3.5	14.4
	TMO, smooth low virulence	8	−	+	+	39.5	38.5	6.3	6.7	0.4	3.1	11.3
	V47D, rough very low virulence	1	−	+	+	23.5	35.2	6.2	6.4	0.6	4.9	21.0
	RZ1, rough avirulent	1	−	+	+	24.0	20.2	4.7	4.9	0.5	9.6	41.2
Boivin	BA_2	9	−	+	+	45.1	44.5	6.6	5.5	0.5	0.9	7.0
	TMO	8	−	+	+	51.9	39.4	7.5	4.7	0.6	1.2	3.5
	V47D	1	−	+	+	44.1						
Aqueous extract	BA_2	7	−	+	+	43.9						
	TMO	6	−	+	+	37.8						
	V47D	4.5	+	+	+	16.1						
	RZ1	4.5	+	+	+	17.4						

with alcohol and ether and dried. This preparation will be called the aqueous extract.

With one exception, we have applied these methods to the preparation of antigens from all 4 strains and have obtained yields as shown in table 1. The yield from each strain is expressed as a percentage of the weight of acetone-dried bacilli. Since the aqueous extracts were prepared from bacteria which

had never been dried, the weight of dried bacteria in this case was estimated from previously acquired knowledge of the average amount of growth of these strains. The amount of growth varied considerably among different strains, but for any one strain it was fairly constant. Although the yield was much higher from smooth strains (8 to 9 per cent) than from rough strains (1 per cent) by both the Raistrick-Topley and Boivin methods, the 2 methods showed little difference in the yield from any one culture. Precipitation of the aqueous extracts of smooth cultures yielded slightly less material (6 to 7 per cent) than precipitation of material obtained by the other methods; the yield from rough cultures by aqueous extraction was relatively high (4.5 per cent).

Although the solubility and appearance of the dried antigens was dependent to some extent upon the speed of drying, definite differences in solubility in 0.85 per cent NaCl were noted. For use as stock solutions the antigens were usually dissolved in a concentration of 0.4 per cent. In this concentration the aqueous extracts of smooth cultures dissolved readily forming opalescent solutions. The Boivin smooth antigens dissolved more rapidly than the Raistrick-Topley antigens but both formed stable solutions of about the same concentration. The antigens prepared from rough cultures, with the exception of the Boivin antigen from V47D, were less soluble and formed, even in distilled water, much less stable suspensions than the smooth antigens. Neither of the RZ1 antigens formed a stable suspension.

THE CHEMICAL PROPERTIES OF THE ANTIGENS

The results of qualitative and quantitative chemical analyses are shown in table 1.

Exton and Esbach tests for protein were performed on solutions or suspensions containing at least 0.1 per cent of the antigens and were negative on all preparations except the two aqueous extracts of rough cultures. Positive biuret, Millon, and xanthoproteic tests indicated the presence of peptide linkages, phenolic bodies (probably tyrosine), and phenyl groups in all preparations. The presence of carbohydrate groups in all the antigens was shown by positive Molisch tests.

Reducing substances expressed as glucose were determined by the Hagedorn-Jensen method after hydrolysis by boiling for 1 hour in normal HCl. Content of reducing sugars was of the same order for all the preparations from smooth strains, 38 to 52 per cent, but there was a suggestion that antigens with slightly higher reducing sugar content were obtained by the Boivin method. The antigens from rough strains tended to have a lower content of reducing substances than smooth antigens prepared by the same methods.

The elementary analysis of the Raistrick-Topley antigens from strains BA_2 and TMO agreed closely with the results reported by Raistrick and Topley (1934). The low carbon and high phosphorus content of the Raistrick-Topley antigen from the rough strain, RZ1, and the relatively large amount of residue in antigens from rough cultures may be significant; the low phosphorus and low residue in the Boivin antigens were probably results of dialysis.

TOXICITY AND ANTITOXIC IMMUNITY

The toxicity of various preparations was determined by intraperitoneal inoculation of mice weighing 20 to 23 grams. The concentration of antigen in the inoculum was such that the desired dose was contained in 0.5 ml. of 0.85 per cent NaCl. The results are shown in table 2. The Raistrick-Topley antigens from strains BA_2 and TMO were perhaps slightly more toxic than equal weights of whole acetone-dried bacilli.

The capacity of the Boivin preparations from strain BA_2 to induce antitoxic immunity was tested by immunizing mice with 7 injections at 3-day intervals increasing from an initial dose of 0.01 mgm. to a final dose of 0.1 mgm. The test dose was given 1 week after the last immunizing dose. The results, shown in table 3, indicate that the immunizing procedure protected against only about 1 minimal lethal dose. Other observations on the increased resistance of mice surviving toxicity tests were comparable to the results of this experiment.

TABLE 2

Numbers of deaths in mice following the intraperitoneal injection of various amounts of acetone-dried bacilli and polysaccharide antigens

STRAIN	PREPARATION	AMOUNTS OF ACETONE-DRIED BACILLI AND POLYSACCHARIDE ANTIGENS							
		10 mgm.	5 mgm.	2.5 mgm.	2.0 mgm.	1.0 mgm.	0.5 mgm.	0.25 mgm.	0.1 mgm.
BA_2	Acetone-dried bacilli				3/5*	8/25			
	Raistrick-Topley				10/10	27/35	0/10		
	Boivin						9/10	0/10	
	Aqueous extract						5/5	10/10	0/5
TMO	Acetone-dried bacilli				5/5	13/25			
	Raistrick-Topley				10/10	20/35	0/10		
	Aqueous extract						5/5	6/10	
V47D	Acetone-dried bacilli	4/5	5/5		0/10	0/15			
	Raistrick-Topley		2/2	2/2		0/2			
RZ1	Acetone-dried bacilli	5/5	5/5		4/10				
	Raistrick-Topley		0/2						

* In this table the numerator of each fraction records the number of deaths and the denominator the number of mice tested.

TABLE 3

Deaths among normal and immunized mice following the injection of the Boivin antigen prepared from BA_2

DOSE	NUMBER OF MICE	DEATHS	
		Immunised	Controls
mgm.			
2.0	5	5	5
1.0	5	3	5
0.5	5	1	4

PRODUCTION OF IMMUNITY TO INFECTION

In order to determine the most effective doses of the antigenic fractions for the production of active immunity to infection with virulent *Salmonella typhimurium*, groups of 10 mice were vaccinated intraperitoneally. The mice in each group received 2 injections of the same dose with an interval of 7 days between injections. The doses employed were 0.1, 0.01, 0.001, and 0.0001

mgm. The antigens used were the Raistrick-Topley antigens from strains BA_2 and TMO and the Boivin antigen from BA_2. Two weeks after the second immunizing injection, half the mice were injected intraperitoneally with 1,000 and half with 100,000 BA_2. The degree of protection was found to be roughly proportional to the size of the immunizing doses but the dose of 0.1 mgm. showed little advantage over 0.01 mgm. Since 0.1 mgm. approached the lethal dose of the aqueous extract of BA_2, 0.01 mgm. was employed in subsequent comparative immunity tests.

TABLE 4

Immunity of mice vaccinated with polysaccharide antigens of Salmonella typhimurium

VACCINATED WITH		NUMBER OF MICE	SURVIVING 28 DAYS	MEAN TIME TO DEATH	MEAN SURVIVAL TIME	COMPLETELY RESISTANT
Strain	Preparation					
			per cent	*days*	*days*	*per cent*
BA_2	Acetone-dried bacilli	25	40	16.67	21.20	4
	Raistrick-Topley	25	28	14.83	18.12	16
	Boivin	25	56	14.64	22.48	56
	Aqueous extract	25	0	11.68	11.68	0
TMO	Acetone-dried bacilli	25	40	16.07	20.84	40
	Raistrick-Topley	25	48	14.62	21.04	40
	Aqueous extract	25	4	14.04	14.60	4
Unvaccinated controls		100	3	8.02	8.59	0

Table 4 shows the results of immunity tests on mice vaccinated with polysaccharide antigens from BA_2 and TMO. Groups of 25 mice were vaccinated with two doses of 0.01 mgm. a week apart and tested by the intraperitoneal injection one week later of 1,000 live bacilli of strain BA_2. We have recorded the percentage of mice surviving for 28 days, the mean time to death of the mice which succumbed, the mean survival time limited to 28 days, and the percentage of completely resistant mice as indicated by negative cultures from the blood and spleen after 28 days.

Of 100 unvaccinated controls only 3 per cent survived. There was no significant difference in the protection induced by the

acetone-dried bacilli and the Raistrick-Topley antigens of either culture. The Boivin antigen from BA_2 gave a little more protection than the Raistrick-Topley antigen: the difference between 56 and 28 per cent survivors is probably, but not certainly, statistically significant. The aqueous extract antigens conferred practically no protection on the mice although they slightly prolonged the mean time to death.

SEROLOGICAL ANALYSIS

The serological analysis was chiefly concerned with: (a) an analysis of the antigenic properties of the polysaccharide from strains BA_2 and TMO, (b) the similarities and dissimilarities of the polysaccharide antigens from smooth and rough strains, (c) a comparison of polysaccharide antigens prepared by different methods from the same culture, and (d) the relation of these antigens to somatic agglutinogens. In attempting to analyze the antigenic properties of the polysaccharide antigens, we have studied agglutinogenesis, precipitinogenesis, and specific absorptive capacities for agglutinins and precipitins. Although the analysis did not include antigens prepared by all 3 methods from all 4 strains, it did serve as a basis for comparing the Raistrick-Topley antigens from the 4 cultures and the BA_2 antigens prepared by the 3 methods.

Rabbits were immunized with 7 injections at 5 day intervals starting with 0.015 mgm. of the antigens from smooth cultures and 0.15 mgm. of the antigens from rough cultures and increasing the dose to 0.7 mgm. The small initial dose of smooth antigens was necessary because of the toxicity of these antigens for rabbits.

Somatic agglutinins were determined with suspensions of 24-hour agar cultures heated for 4 hours at 56°C. in 95 per cent alcohol. The antigens for detecting flagellar agglutinins were 24-hour broth cultures to which 0.2 per cent formalin was added.

Precipitin tests were performed by stratifying dilutions of antigen in 0.2 ml. amounts on 0.2 ml. of serum dilution, usually 1 to 10. The tubes were placed in a 50°C. water bath, observed for ring formation after 20 minutes, shaken, and allowed to remain at 50°C. for 4 hours. Final readings were made after stand-

ing over night at room temperature. When it was desired to determine the optimal proportions of antigen and antibody, the tests were observed at frequent intervals until the first tube to show flocculation had been recorded. Although rings appeared within a few minutes, flocculation usually did not appear for an hour or more.

In absorbing sera with polysaccharide antigens it was found that one absorption at the optimal ratio, or with an excess of antigen, sufficed to remove detectable homologous precipitins but in no case did one absorption of a low dilution of serum completely remove homologous somatic agglutinins. This apparent complete removal of precipitins was presumably due to the fact that a relatively high concentration of antibody was necessary to cause flocculation. A single absorption lowered the titer of precipitin to a concentration at which the test failed to reveal the presence of precipitin. Precipitation did not take place if the sera were diluted beyond 1 to 40. Complete removal of homologous agglutinins was obtained, however, by repeated absorptions.

Since temperature has been shown to affect the precipitation of antibody by pneumococcus SSS (Heidelberger and Kendall, 1935; Brown, 1935), absorptions were performed at 4°C., at 50°C. and at several intermediate temperatures. It was found that absorption at the low temperatures was no greater than at 50°C. Flocculation took place much less rapidly at low temperature.

A serological analysis of the Raistrick-Topley fractions of all 4 strains is shown in table 5. None of these preparations produced flagellar agglutinins. The antigens from smooth strains gave rise to O agglutinins and to precipitins for the smooth polysaccharide antigen. Reciprocal absorption of agglutinins with the antigens of BA_2 and TMO was complete—further evidence of the serological identity of these 2 strains.

Antigens from smooth strains and those from rough strains were shown to be serologically distinct by the absence of cross agglutination and cross absorption of agglutinins. The study of the rough antigens was limited by the small quantities obtained

and by the insolubility of the RZ1 antigen. A suspension of this antigen which was sufficiently stable to use in precipitin tests was never obtained. Like the smooth antigens, the V47D preparation was shown to be a complete antigen; it gave rise to agglutinins and precipitins and specifically precipitated the antibody. Although the RZ1 antigen failed, after prolonged immunization, to produce agglutinins in a rabbit, it absorbed agglutinins from V47D serum. Whether this antigen is a haptene or whether the failure to produce antibodies was due to the rabbit is not known.

Tests for the inhibition of O agglutination by the polysaccharide antigens were performed by incubating together decreas-

TABLE 5

Antigenic analysis of the polysaccharide antigens prepared by the Raistrick-Topley method from smooth and rough strains of Salmonella typhimurium

STRAIN	SOMATIC AGGLUTININS								PRECIPITINS			
	Titers of agglutinins for				Titers for homologous strains after absorption with polysaccharide antigens from				Smallest amount of antigen giving flocculation with 0.02 ml. serum prepared with			
	BA_2	TMO	V47D	RZ1	BA_2	TMO	V47D	RZ1	BA_2	TMO	V47D	RZ1
									mgm.	*mgm.*	*mgm.*	*mgm.*
BA_2.....	1280	1280	0	0	0	0	1280	1280	0.006	0.006	None	None
TMO...	1280	1280	0	0	0	0	1280	1280	0.006	0.012	None	None
V47D...	0	0	640	320	640	640	0	0	None	None	0.003	
RZ1....	0	0	0	0								

ing amounts of antigen and a constant amount of serum for 4 hours at 50°C. Any precipitate which formed was allowed to settle over night. The supernatant fluid was decanted; to it was added an equal quantity of alcoholic antigen, and agglutination allowed to take place in the usual manner. The amount of serum used was such that the final dilution corresponded to the highest dilution that would give complete agglutination. The results are summarized in table 6.

The data show that the amounts of polysaccharide antigen from strains BA_2 and TMO required completely to inhibit agglutination of either BA_2 or TMO by sera prepared with live bacilli and with polysaccharide antigens of these strains differed

only slightly. The differences recorded were only those represented in each instance by one tube in a series of antigen dilutions. It should also be noted that both the smooth and the rough polysaccharide antigens completely inhibited agglutination by homologous sera produced with live bacilli. This would seem to be additional evidence that the polysaccharide antigen is the somatic antigen upon which O agglutination depends.

TABLE 6

Inhibition of somatic agglutination by polysaccharide antigens prepared by the Raistrick-Topley method

SERUM PRODUCED WITH	ANTIGEN IN AGGLUTINATION TEST	SMALLEST AMOUNT OF ANTIGEN REQUIRED TO INHIBIT AGGLUTINATION COMPLETELY*		
		BA_2	TMO	V47D
		mgm.	*mgm.*	*mgm.*
BA_2—Live culture	BA_2	0.2	0.2	
	TMO	0.2	0.2	
BA_2—Raistrick-Topley	BA_2	0.2	0.4	
	TMO	0.4	0.8	
TMO—Live culture	BA_2	0.4	0.4	
	TMO	0.8	0.8	
TMO—Raistrick-Topley	BA_2	0.2	0.4	
	TMO	0.8	0.8	
V47D—Live culture	V47D			0.2

* Serum dilution in the inhibition tests was the highest dilution of serum giving complete agglutination.

At the outset it was thought that possibly the optimal proportions technic of Dean and Webb (1926) might be used to detect differences in the antigen content of different fractions. The amounts of BA_2 and TMO fractions prepared by all 3 methods which gave optimal flocculation in various sera were determined. It was found that the relationship of fractions as determined in one serum did not necessarily hold for another serum. For example, the aqueous extract antigen of BA_2 gave optimal flocculation in an antiserum for the polysaccharide prepared from BA_2

by the Raistrick-Topley method in one quarter of the optimal amount required for the homologous antigen, while in an antiserum for the polysaccharide prepared from BA_2 by the Boivin method these same fractions gave optimal flocculation in equal amounts. These discrepancies may have been due to differences in the antigenic constitution of the fractions, but the meaning of the results was not sufficiently clear to justify any conclusions.

The serological relations of the three BA_2 fractions to each other were studied by reciprocal absorption of agglutinins. All three completely absorbed somatic agglutinins from antisera prepared with each of the fractions and from sera prepared by the injection of live bacilli.

As in the case of the polysaccharide antigens prepared from BA_2 by the Raistrick-Topley method, the Boivin preparation gave rise to somatic agglutinins only, but the aqueous extract preparation also induced the formation of flagellar agglutinins in high titer.

Since it has been shown (Sordelli and Mayer, 1931; Morgan, 1936) that polysaccharide antigens prepared from bacteria grown on agar media may be contaminated with a non-specific polysaccharide from the agar itself, the sera used in this work were examined for the presence of antibodies for constituents of the medium. The antigen was prepared by precipitating a concentrated aqueous extract of beef infusion agar with acetone, dissolving the precipitate in 0.85 per cent NaCl, and reprecipitating with alcohol. This precipitate dissolved in a small amount of saline was tested against antisera for polysaccharides prepared by the 3 methods. Antisera for the Raistrick-Topley and Boivin polysaccharides gave no reaction with this antigen but an antiserum for the aqueous extract preparation gave a precipitin reaction only with a concentrated solution of the agar antigen. This antigen was evidently present in the polysaccharide prepared by the aqueous extract method in sufficient amount to induce the production of antibodies; but it could not have introduced an error in the results of the serological reactions with this fraction because of the high dilutions at which these reactions took place. The nonspecific antigen was not studied further except to show

that it probably originated from either the beef or the peptone since no acetone-precipitable substances were present in an extract of pure agar.

DISCUSSION

Both Boivin and Mesrobeanu (1935a) and Raistrick and Topley (1934) have indicated their belief that they were dealing with the same substance in the antigenic material which they respectively have prepared. The former (Boivin and Mesrobeanu, 1934c), however, stated that their material contained a higher concentration of toxic and antigenic substance because of the lower nitrogen content and higher toxicity. We have tried both methods on the same strains: although a slight difference was observed in toxicity, the nitrogen content of the two preparations was almost the same; the lower residue and much lower phosphorous, probably inorganic, of the Boivin preparations probably indicate greater purity. We found little difference in the time and labor involved in the two methods.

With regard to the presence of polysaccharide antigens in rough cultures of gram-negative bacilli reports disagree. White (1929) failed to obtain a soluble specific substance from rough Salmonellas by extraction with acetic acid but Furth and Landsteiner (1929) at about the same time reported the extraction of a haptene from rough *Salmonella typhimurium* by means of alkaline hypochlorite. Later, White (1931), using the alkaline method by which Meyer (1930) obtained a haptene from rough *Shigella dysenteriae*, confirmed the work of Furth and Landsteiner. The necessity for using an alkaline extraction was also shown by Meisel and Mikulaszek (1931). Boivin and Mesrobeanu during the course of their work failed in many attempts to obtain a complete antigen from rough strains of *Salmonella typhimurium;* Raistrick and Topley record no observations on rough strains. Using the methods of these last named investigators we have obtained antigenic polysaccharides from rough strains; and, at least one of the preparations, the polysaccharide prepared by the Raistrick-Topley method from V47D, was a complete antigen.

The toxicity of our Boivin and Raistrick-Topley fractions was

slightly less than that reported by others. Boivin *et al.* (1935b) obtained antigens which regularly killed mice in doses of 0.1 mgm. while Martin's (1934) fractions prepared by the Raistrick-Topley method were lethal in doses of 0.5 mgm. Since a dose of 0.5 mgm. in mice would be the equivalent of over 1 gram for man, this cannot be considered more than a low grade of toxicity. Although animals immunized with these toxic preparations show increased resistance to the toxin, the number of minimal lethal doses to which the animals become resistant is small: hence their antitoxinogenic properties are also of a low grade.

The comparison of antigens prepared by the simple method of aqueous extraction at 60°C. with those prepared by the Boivin and Raistrick-Topley methods revealed significant differences. The polysaccharides prepared by the aqueous extract method were the most toxic antigens but, in our hands, they were incapable of inducing active immunity to infection.

Our serological analyses of these antigens are in accord with previous observations of others which have seemed to justify the conclusion that these polysaccharide antigens are identical with, or at least constitute the serologically significant components of, the antigen upon which somatic agglutination depends. Polysaccharides prepared by all 3 methods induced in rabbits O agglutinins and specifically absorbed the O agglutinins from sera prepared with intact acetone-dried bacilli. Thus the results of this study of the properties of the polysaccharide antigen prepared by the aqueous extract method indicate that the toxicity of these preparations and their capacity to induce in mice an active immunity are determined by different components. Furthermore, in view of the frequently demonstrated heat stability of the immunizing antigens of this group of bacteria, it seems improbable that heating (4 hours at 60°C.) destroyed the immunizing capacity of the aqueous extract polysaccharide without destroying its ability to produce somatic agglutinins. These results also cast some doubt on the validity of conclusions which assign to the smooth somatic antigen a predominant rôle in the determination of active immunity.

The presence of protein in the aqueous extracts of rough strains

and its apparent absence from similar preparations from smooth strains is not easily explained. One might postulate either a difference in the solubility of the smooth and rough proteins or some physical difference in the rough and smooth cells which causes the smooth protein to escape extraction by the methods used.

The similarity in all respects of the polysaccharide antigens of the highly virulent strain BA_2 and those of the much less virulent strain TMO confirms our previous contention (Mackenzie, Fitzgerald, and Pike, 1935) in regard to the independence of virulence and the smooth somatic antigen. The polysaccharides of these 2 strains seem to be quantitatively and qualitatively the same. The unavoidable implication is that the virulence of these strains is largely determined by serologically inactive material.

CONCLUSIONS

1. Antigenic polysaccharides prepared from *Salmonella typhimurium* by the method of Raistrick and Topley and by that of Boivin and Mesrobeanu possess minor chemical and antigenic differences.

2. Polysaccharide antigens prepared by different methods may be serologically indistinguishable and yet show significant differences in toxicity and immunizing capacity; hence, these two characters are apparently not determined by the same components of the bacterial cell.

3. Qualitatively and quantitatively identical polysaccharide antigens were obtained from 2 smooth strains of *Salmonella typhimurium* although these strains have manifested a persistent and large difference in virulence for mice.

4. The smooth polysaccharide antigen of *Salmonella typhimurium* is not the major determinant of virulence.

5. Important determinants of virulence are serologically inactive.

6. A polysaccharide which is a complete antigen has been obtained from a rough culture of *Salmonella typhimurium;* it is serologically quite different from the polysaccharide of smooth strains.

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