OBSERVATIONS ON THE ANTIGENIC STRUCTURE OF *PSEUDOMONAS AERUGINOSA*

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(With 1 Figure in the Text)

Our interest in *Pseudomonas aeruginosa* arose from a desire to study the phages carried by this organism and to find a method of typing which could be utilized in studies of hospital infection.

Both these aims demanded a more accurate knowledge of the antigenic structure of *Ps. aeruginosa* than is yet available. Studies on the antigenic structure of this species have been undertaken by many investigators: Aoki (1926), Christie (1948), Gaby (1946), Mayr-Harting (1948), Munoz, Scherago & Weaver (1945) and others. Christie was able to subdivide 138 strains into thirteen groups on the basis of O agglutination. In addition, he recognized several H antigens. Other investigators were either unable to differentiate between O and H agglutination or failed to describe the differentiation in their publications.

In attempting to use the usual agglutination methods in antigenic analysis we encountered some of the difficulties described by Mayr-Harting in the preparation of suspensions for O agglutination by alcohol treatment. We resorted therefore in the first place to precipitation tests with extracts (Boivin & Mesrobeanu, 1937), presumably containing only O antigens of the organisms, and were able to classify 90% of 116 strains into six serological groups. These results could later be confirmed by agglutination as well as by bacteriophage sensitivity tests.

The difficulties encountered in agglutination tests with *Ps. aeruginosa* have apparently not been encountered in the study of the related genus *Vibrio*. White (1938) has, however, described a mucoid substance produced by rugose variants of *V. cholera* which interferes with O agglutination of this organism. Although none of our *Pseudomonas aeruginosa* strains could be described as rugose, many of them produced mucoid growth particularly on solid media. The nature of the mucoid substance has been investigated in the hope that a knowledge of its composition may prove of assistance in the preparation of agglutinable O suspensions.

MATERIAL AND METHODS

Stocks of bacteria were maintained in the dry state (Stamp, 1947). For each experiment fresh subcultures were made in Difco nutrient broth (D.N.B.). Thirty-six strains (A to AQ) which have been used in bacteriophage studies were investigated serologically; with an additional eighty strains, all of human origin, only precipitation tests have been carried out. For the latter only those strains

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were included which possess the biochemical properties of *Ps. aeruginosa*, produce yellow, green or red pigments and grow on nutrient agar containing 1 % Cetavlon.

Preparation of suspensions for the immunization of rabbits

H suspensions. The growth from nutrient agar plate cultures was washed off in 0·35 % formol saline and diluted to an opacity equivalent to approximately 3000×10^6 organisms per ml.

O suspensions. The growth from nutrient agar plate cultures was washed off in absolute alcohol using 50 ml. of alcohol for each 6 in. agar plate. The suspension in alcohol was heated to 56° C. for ½ hr. and then centrifuged. The bacteria were washed twice in distilled water and finally resuspended in saline. The suspension was diluted to an opacity equivalent to approximately 3000×10^6 organisms per ml.

Immunization of rabbits

Each rabbit received eleven injections at 2–3-day intervals, the first five being given subcutaneously and the remainder intravenously. Because of the toxicity of some of the suspensions it was necessary to give small initial doses (0·1–0·25 ml.) and to increase gradually with succeeding doses to a maximum of 0·5–0·75 ml.

The rabbits were bled 7 days and again 10 days after the last injection, and each pair of sera pooled. Most of the rabbits received a second similar course of immunizing injections 3–4 months after the first course.

Agglutination titrations

Suspensions for H agglutination tests were prepared from broth cultures which had been aerated to prevent pellicle formation, by the addition of formalin to a concentration of 0·35 % and dilution of the culture to the required opacity.

For our initial experiments suspensions for O agglutination tests were prepared from nutrient agar plate cultures by a method similar to that used in the preparation of immunizing suspensions. They were, however, subjected to heating in alcohol for 30 min. at 37° C. instead of 56° C., and thereafter kept in the refrigerator for 24–36 hr. before the removal of the alcohol.

In later experiments on the preparation of O suspensions the effects of dilution of the bacterial suspension before the addition of alcohol and of variation of the final concentration of alcohol were investigated.

In the method which was finally adopted the growth from one 4 in. agar plate was suspended in 40–50 ml. of saline. Nine volumes of absolute alcohol were then added, the mixture kept for 30 min. at 37° C. and overnight in a refrigerator. The alcohol was removed by centrifugation, the bacteria washed once in saline, resuspended in saline and diluted to an opacity equivalent to approximately 2000×10^6 per ml. Attempts at the preparation of O suspensions by heat or acid treatment (Duncan, 1935) proved unsuccessful.

To 0·25 or 0·5 ml. amounts of serial dilutions of serum in 1×8 cm. round-bottomed tubes were added equal volumes of bacterial suspension with a density of approximately 2000×10^6 per ml. The tubes were incubated at 37° C. for 2 hr. and left at room temperature for a further 12–18 hr. before reading. The amount of

agglutination was assessed according to the clearing of the suspension and the pattern of sediment on the bottom of the tubes. We attributed the lack of clear differentiation between O and H agglutination to the fact that the organisms are monotrichate. In most cases both with alcohol- and formalin-treated suspensions agglutination was granular. With a few strains which throughout gave anomalous results agglutination appeared to be atypical, and uniform resuspension resulted from gentle shaking even of apparently completely agglutinated bacteria.

Preparation of extracts for precipitation tests

The method is based on that of Boivin & Mesrobeanu (1937). A 50 ml. amount of an overnight D.N.B. culture is centrifuged and the supernatant fluid discarded. The deposited bacteria are resuspended in 0·5 ml. of distilled water, and 0·5 ml. of N/2-trichloracetic acid added. The mixture is allowed to stand in the refrigerator for 3 hr., after which it is centrifuged until the supernatant fluid is clear. The supernatant is removed and neutralized with N/4-NaOH using brom-thymol blue as indicator. The neutral extracts are stored in the refrigerator until tested.

Extracts made by the method employed by Lancefield (1933) in the serological grouping of streptococci have given less satisfactory results than trichloracetic acid extracts.

Precipitin tests

The extracts are carefully layered on small amounts of neat serum in narrow test-tubes. The tubes are examined within a few minutes for rings of precipitates. They are then allowed to stand at room temperature overnight, after which they are examined for precipitates which have settled out on the bottom or sides of the tubes. Dreyer tubes have been found satisfactory, particularly for observation of the precipitates which settle out overnight.

RESULTS

Antisera were initially prepared against only six strains of *Ps. aeruginosa*. These were tested in precipitation tests against extracts of each of the thirty-six strains available. Eighteen of the extracts failed to precipitate with any of the sera. Immune sera were therefore prepared against formolized whole organisms (H) and alcohol-treated (O) suspensions of each of these eighteen strains.

Our antigenic analysis was based on a study of the twenty-four strains for which antisera were prepared.

The results of precipitation tests with sera prepared against formolized whole organisms are recorded in Table 1. They show that of the twenty-four strains twenty could be placed into six groups, whereas extracts of four strains reacted either with many sera (N) or with none significantly (P, X, AD). The biggest group consists of nine strains (K, L, R, S, V, AC, AG, AL, AP). That these are not identical is shown by differences in precipitin tests with the corresponding nine sera. We have also obtained evidence in agglutination tests of differences between individual strains of this group. There appears, however, to be sufficient overlap to group them together. The remaining five groups contain 1, 2, 4, 2 and 2 strains respectively. Almost identical results have been obtained in precipitation tests with sera

prepared against the alcoholized (O) suspensions. Of the remaining twelve strains in this first series two fall into group I, five into group II, three into group IV and two into group V.

Table 1. *Precipitation tests with trichloracetic acid extracts and antisera against formolized whole organisms*

	Groups and Extracts																							
	I	II		III				IV									V		VI					
Sera	A	B	D	G	O	Q	AH	K	L	R	S	V	AC	AG	AL	AP	Z	AB	AK	AN	N	P	X	Al
A	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−
B	−	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
D	−	±	+	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
G	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
O	−	−	−	+	+	±	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Q	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−
AH	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
K	−	−	−	−	−	−	−	+	⊕	±	±	±	+	⊕	+	+	−	−	−	−	±	−	−	−
L	−	−	−	−	−	−	−	⊕	±	⊕	⊕	±	⊕	±	±	−	−	−	−	−	±	−	−	−
R	⊕	−	−	−	⊕	−	−	⊕	⊕	+	+	±	±	+	±	⊕	−	−	−	−	+	−	−	−
S	−	−	−	−	−	−	−	tr	⊕	+	+	⊕	−	+	⊕	⊕	−	−	−	−	±	−	−	−
V	−	−	−	−	−	−	−	⊕	⊕	±	±	+	+	⊕	±	±	−	−	−	−	±	−	−	−
AC	−	−	−	−	−	−	−	±	⊕	±	±	±	+	±	±	+	−	−	−	−	±	−	−	−
AG	−	−	−	−	−	−	−	−	−	±	±	⊕	⊕	+	⊕	−	−	−	−	−	⊕	−	−	−
AL	−	−	−	−	−	−	−	+	±	±	−	+	+	±	±	±	−	−	−	−	−	−	−	−
AP	−	−	−	−	−	−	−	⊕	−	−	−	±	±	−	±	+	−	−	−	−	−	−	−	−
Z	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−	⊕	−	−	−
AB	−	−	−	=	−	−	−	⊕	−	−	−	−	−	−	−	−	±	+	−	−	±	−	−	−
AK	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	⊕	±	−	−	−	−
AN	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−
N	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	⊕	−
P	−	−	−	−	−	−	−	⊕	−	−	−	−	⊕	−	−	−	−	−	−	−	⊕	−	−	−
X	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	tr	−
AD	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+ = strong reaction.
± = slight reaction.
⊕ = slight reaction usually visible only after standing overnight, and variable in repeated tests.
tr = trace of precipitation.

A summary of the results obtained in agglutination tests with the same sera used in precipitation tests and formolized (H) suspensions of each strain is recorded in Table 2. Each serum was tested in only three dilutions, 1/10, 1/100 and 1/1000, and the agglutination for each strain compared to that for the homologous organism. The results show that organisms which by precipitation tests had been found to be related, also show the closest relationship in agglutination tests. There are, however, many additional cross-agglutinations which are probably due to the sharing of common flagellar antigens.

We anticipated that agglutination tests with O suspensions and antisera prepared against alcoholized organisms would show cross-reactions corresponding more closely to those encountered in precipitation tests.

In view of the suggestion of Mayr-Harting that alcohol treatment affects agglutinability of the somatic antigens, preliminary experiments were carried out to determine the most satisfactory method of preparing O suspensions of our strains. There appear to be marked differences in the details of methods which have been

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successfully employed for the preparation with alcohol of O suspensions of other organisms. Thus Weil & Felix (1920) added alcohol to a final concentration of 96 % to spun deposits of bacteria. Craigie (1931) states that alcohol in a concentration of 30 % or more is sufficient to destroy H antigen.

Table 2. *Agglutination tests with H suspensions and antisera prepared against formolized whole organisms (H)*

	Groups and Antigens																							
I	II		III				IV									V		VI						
A	B	D	G	O	Q	AH	K	L	R	S	V	AC	AG	AL	AP	Z	AB	AK	AN	N	P	X	AD
+	−	−	+	+	+	+	±	+	±	±	±	tr	±	±	−	−	−	−	−	±	tr	±	±
−	+	+	tr	−	−	±	−	±	tr	−	±	±	−	tr	−	+	+	−	+	±	−	tr	tr
−	+	+	tr	−	±	tr	tr	±	±	tr	±	tr	tr	tr	−	+	+	tr	+	±	tr	tr	±
±	−	−	+	+	+	+	±	+	+	±	±	−	tr	±	±	−	tr	−	tr	+	+	+	+
+	tr	−	+	+	+	+	−	±	tr	−	tr	−	−	−	−	−	−	−	tr	tr	−	tr	tr
±	−	−	+	+	+	+	±	±	±	tr	±	±	tr	±	±	−	−	−	±	±	±	±	±
tr	−	−	±	+	+	+	tr	+	±	tr	±	tr	tr	±	−	−	±	−	−	±	tr	tr	±
−	−	−	−	−	−	−	+	+	+	+	+	±	±	±	±	−	−	±	tr	+	±	±	+
+	−	−	+	±	+	+	+	+	+	±	+	±	+	+	±	−	tr	−	−	+	±	+	+
tr	−	−	±	−	±	tr	+	+	+	+	+	±	±	+	+	−	±	±	tr	+	+	+	+
tr	−	−	−	tr	−	−	+	±	+	+	+	±	±	±	+	−	−	tr	tr	+	+	+	+
−	−	−	−	−	−	−	+	+	+	±	+	±	+	+	+	−	tr	−	−	+	±	±	+
−	−	−	−	−	−	±	+	+	+	+	+	+	+	+	±	−	±	tr	±	+	+	±	+
−	−	−	−	−	−	−	±	±	±	±	±	±	+	±	±	−	−	−	−	±	±	±	±
−	tr	−	tr	−	tr	tr	+	±	+	±	+	±	+	±	+	−	±	−	tr	+	+	±	+
tr	−	−	tr	tr	tr	−	±	±	±	+	±	±	±	±	+	−	−	−	−	+	±	±	+
−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	tr	−	−	−	tr
±	+	+	±	±	±	±	tr	±	±	−	±	tr	−	tr	−	+	+	−	+	tr	tr	−	tr
−	+	+	−	−	−	−	tr	−	−	−	tr	−	−	−	±	±	+	+	+	−	tr	−	±
tr	±	±	tr	−	tr	−	tr	tr	±	±	±	−	tr	tr	±	−	±	±	+	tr	tr	−	tr
−	−	−	−	−	tr	±	+	±	±	+	±	±	±	±	±	−	−	−	−	+	±	+	+
±	−	−	±	+	+	+	+	+	+	+	+	±	+	+	+	−	−	−	tr	+	+	+	+
±	−	−	±	±	±	tr	+	+	±	±	±	tr	±	±	+	−	−	−	−	+	±	+	+
tr	−	−	tr	tr	tr	tr	+	±	+	±	±	tr	±	+	±	−	−	−	−	+	+	+	+

\+ = agglutination equivalent to that with homologous organisms.
± = agglutination approximately one-tenth that with homologous organisms.
tr = trace of agglutination.
The squares are drawn in on the basis of the results of precipitation tests (Table 1).

Organisms were submitted to a variety of treatments and examined for agglutinability by homologous sera prepared against formolized whole bacteria (H) and alcohol-treated bacteria (O). Parallel experiments were made in this way with a member of each serological group. Heating to 56° C. or higher markedly reduced agglutinability by both O and H antisera. The agglutinability of the O antigen was also reduced when concentrated suspensions were treated with 90 % alcohol in the usual manner. This effect was specially marked with mucoid cultures. If, however, the suspensions were diluted before the addition of alcohol to a final concentration of 90 %, then agglutinability by homologous H antisera was reduced moderately, leaving their agglutinability by O antisera but slightly affected. Typical results are recorded in Table 3.

Mucoid cultures were extremely difficult to emulsify after centrifugation, and

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the emulsification became no easier even after repeated washing in saline or water. Mucoid material could be separated from thick suspensions by centrifugation at 10,000 r.p.m. for 1 hr. in a Spinco Model L centrifuge. It appears to contain at least two fractions which can be separated by treatment with trichloracetic acid.

Table 3. *Effect of dilution of bacterial suspension before addition of alcohol.*

Dilution of suspension before addition of alcohol	Dilution of antiserum AN O								
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	C
1/64	4	4	4	4	2	tr	0	0	0
1/32	4	4	4	4	3	1	0	0	0
1/16	4	4	4	4	3	1	0	0	0
1/8	4	3	3	2	2	1	0	0	0
1/4	4	4	4	2	2	1	0	0	0
1/2	2	2	2	2	1	0	0	0	0
1/1	tr	1	1	1	tr	tr	0	0	0

Initial suspension: growth from one 6 in. agar plate suspended in 5 ml. saline.
4 = complete agglutination.
3 = almost complete agglutination.
2 = marked agglutination easily visible to the naked eye.
1 = scanty agglutination producing fine sediment or agglutinates visible with the aid of a lens.
tr = trace of agglutination.

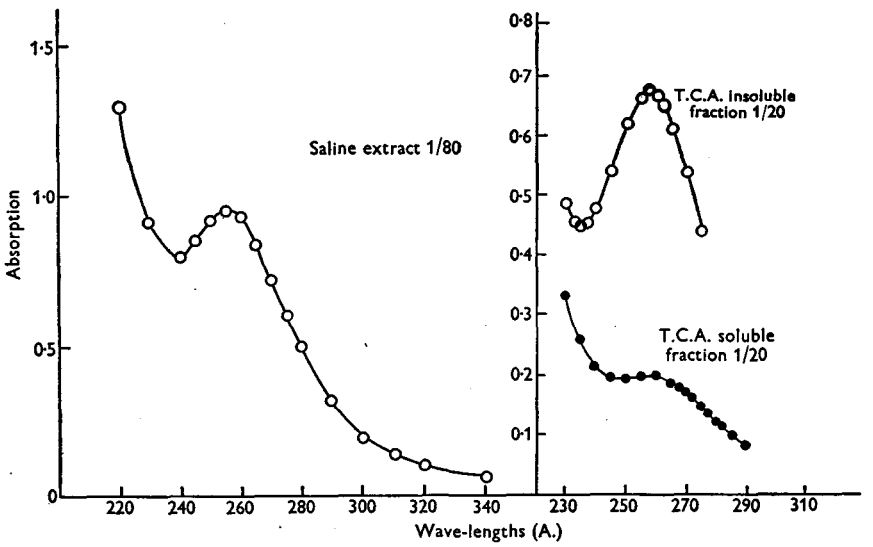

Fig. 1. U.V.L. absorption of saline (0·9 % NaCl) extract of nutrient agar culture of Strain AP, and of fractions obtained from it by trichloracetic acid precipitation.

The T.C.A.-insoluble fraction contains nucleic acid, is easily precipitable by alcohol, and reacts poorly in precipitin tests even with homologous antisera. The T.C.A. soluble fraction forms a less bulky precipitate in alcohol, and reacts strongly in precipitin tests with homologous antisera (see Fig. 1).

We concluded that the development of the mucoid consistency probably depends at least in part on autolysis with release of nucleoprotein. Treatment with alcohol

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may precipitate the nucleoprotein which appears to be a relatively inert antigen on the surface of the bacteria, thereby masking specific O agglutination. We were, however, unable to prepare more satisfactory suspensions by preliminary washing of bacterial suspensions with water, physiological saline or 6 % salt solution.

Table 4. *Agglutination tests with alcoholized (O) suspensions, and antisera prepared against alcohol-treated organisms*

O antisera	Groups and Antigens: I	II		III				IV									V		VI				
	A	B	D	G	O	Q	AH	K	L	R	S	V	AC	AG	AL	AP	Z	AB	AK	AN	N	P	X
A	+	–	–	–	–	–	tr	–	–	–	–	–	–	tr	–	–	–	–	tr	–	±	.	±
B	–	+	+	–	–	–	tr	tr	tr	–	–	–	–	–	tr	–	–	–	tr	–	tr	.	tr
D	–	±	+	±	±	±	±	tr	tr	–	–	–	–	tr	–	–	–	tr	tr	tr	tr	.	tr
G	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	tr	–	±	.	±
O	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	tr	–	–	–	–	±	.	±
Q	–	–	tr	+	+	+	+	tr	tr	–	–	–	tr	tr	tr	–	–	tr	–	tr	±	.	tr
AH	–	–	–	+	+	+	+	tr	–	–	–	–	tr	tr	–	–	–	tr	tr	tr	±	.	tr
K	–	–	–	–	–	–	tr	+	+	+	+	+	+	±	+	+	–	–	–	±	±	.	±
L	–	–	–	–	–	–	–	+	+	+	+	+	+	±	+	±	–	–	±	±	±	.	±
R	–	–	–	–	–	–	–	±	±	+	+	±	±	±	±	tr	–	–	–	tr	±	.	±
S	–	–	–	–	–	–	–	tr	±	+	+	±	±	±	±	tr	–	–	–	–	±	.	±
V	–	–	tr	tr	–	tr	±	+	+	+	+	+	±	+	+	+	–	±	tr	tr	±	.	±
AC	–	–	tr	–	–	–	±	±	±	±	±	+	+	±	+	+	–	±	tr	±	±	.	±
AG	–	–	–	–	–	–	±	±	+	+	+	+	±	+	±	tr	–	–	tr	–	±	.	±
AL	–	–	–	–	–	–	±	+	±	+	+	+	+	±	+	±	–	–	tr	–	+	.	+
AP	–	–	–	–	–	–	±	+	±	+	+	+	+	±	+	+	–	–	–	–	±	.	±
Z	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	+	±	tr	–	.	–
AK	–	–	–	–	–	–	–	tr	tr	–	tr	–	tr	–	tr	tr	–	–	+	+	tr	.	tr
AN	–	–	–	–	–	–	–	–	tr	–	tr	–	tr	–	–	–	–	–	+	+	±	.	tr
N	–	–	–	–	–	±	±	±	+	–	±	±	±	±	±	–	–	±	–	±	+	.	+
P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
X	–	–	–	±	+	±	±	tr	+	±	+	±	±	±	±	±	–	–	–	–	+	.	+
AD	–	–	–	–	–	–	tr	±	±	–	+	+	tr	–	+	±	–	–	tr	–	+	.	+

+ = agglutination equivalent to that with homologous organisms.
± = agglutination approximately one-tenth that with homologous organisms.
tr = trace of agglutination.
O suspensions of strain P were inagglutinable even by homologous serum.
Z O serum was not available.
The squares are drawn in on the basis of the results of precipitation tests (Table 1).

The most constant O agglutination was obtained with suspensions which were diluted in saline before addition of alcohol to a final concentration of 90 %. It is remarkable that 30 or 60 % alcohol, though destroying H antigen, gave less satisfactory results than 90 % alcohol.

That the agglutination obtained with suspensions which were diluted and then treated with 90 % alcohol at 37° C. is in fact O agglutination is confirmed by the demonstration that an antiserum to the isolated and purified somatic antigen of one of the strains (L) agglutinates alcohol-treated suspensions as well as untreated and formolized suspensions of the same organism. The serum furthermore agglutinates suspensions only of those strains which by precipitation tests can be grouped with it (K, L, R).

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The results of cross-agglutination tests with O suspensions treated with 90% alcohol as described above, and antisera against alcohol-treated (O) suspensions, are recorded in Table 4. They are in striking agreement with those obtained in precipitation tests. As in precipitation tests, four of the strains (P, N, X, AD) could not with certainty be placed into any of the six main serological groups. None of the four strains gave typical O agglutination. In fact, the P O suspension was almost completely inagglutinable even by homologous serum, whilst incomplete fine agglutination, easily broken up by shaking, was produced by the other three strains (N, X, AD). It is probable that the precipitin and agglutination tests agree because the organisms possess one main type-specific somatic antigen which

Table 5. *Susceptibility of* Pseudomonas aeruginosa *strains to bacteriophages*

	Groups and Strains of *Ps. aeruginosa*																							
	I	II		III				IV										V		VI				
hages	A	B	D	G	O	Q	AH	K	L	R	S	V	AC	AG	AL	AP	Z	AB	AK	AN	N	P	X	AI
A	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D	−	−	+	−	−	−	−	±	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Q	+	−	−	+	+	+	+	−	−	−	−	−	+	−	−	−	±	−	−	−	−	−	−	−
G	−	−	−	+	+	+	−	+	−	+	+	+	−	+	+	+	−	−	±	−	+	+	+	−
O	−	−	−	±	±	±	±	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
AD	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+
K	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	±	−	−	−	−	−	−	−	−
Z	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
AB	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−

\+ = confluent lysis with concentration of phages just producing confluent lysis on strain used for its propagation (test dose).

± = isolated plaques only with test dose of phage.

The squares group together strains of organisms which are similar in precipitation tests, and the phages used for differentiation within the group.

can be extracted in T.C.A., and that this antigen is absent from the strains N, X and AD. Agglutination tests, however, show some similarity between these strains and groups II and III, which may depend on the presence of a second specific somatic antigen which is not extracted by T.C.A. treatment. The presence of such an additional antigen in the strain D would also explain the reaction in O agglutination tests of members belonging to serological group III with 'anti-D' serum.

Although it has been possible to differentiate between strains by O agglutination, the method has not given sufficiently reproducible results for accurate quantitative studies, and we have been unable so far to prepare by absorption with alcohol-treated suspensions antisera which could be used for analysis of flagellar antigens. Preliminary results, however, suggest that a multiplicity of flagellar antigens exist, and that a study of their distribution will indicate further subdivisions within the groups sharing common somatic antigens.

Precipitin tests have been carried out with sera representative of each of the serological groups, and T.C.A. extracts from eighty additional strains of *Ps. aeruginosa* isolated from material submitted for bacteriological examination. On the basis of the results, seventy-two of the eighty strains could be placed in one

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or other of the six serological groups defined. Only 15 % of the eighty strains fell into group II, 2·5 % into group III and none into groups I and V. The majority fell into groups IV (42 %) and VI (30 %), and it is therefore unlikely that precipitin tests alone will be sufficient identification for studies of hospital infection.

Striking differences in bacteriophage sensitivity, however, make it possible to differentiate between members of the individual groups. The results recorded in Table 5 were obtained with nine phages selected from fifteen which had been isolated from lysogenic strains of *Ps. aeruginosa*. The phages were all used at concentrations just sufficient to produce confluent lysis on the hosts used for their propagation. Within the most complex group (IV) four strains, R, S, V, AG, appear to be identical in phage sensitivity, but absorption tests indicate minor serological differences between them.

The strain L which has been used in many of our investigations has been found antigenically identical to another, W, which has not been subjected to such a detailed study. The strains L and W are also identical in phage sensitivity and are the only ones encountered which produce a red pigment.

DISCUSSION

The reduced agglutinability of some strains of *Ps. aeruginosa* which results from treatment by methods usually employed for the preparation of O suspensions of motile bacteria has presented an obstacle to the antigenic analysis of this species which has not yet been completely overcome.

Alcohol does not appear to affect the power of the somatic antigen to stimulate antibody formation in the rabbit. It does, however, reduce agglutinability of some strains, particularly if the suspensions used are highly concentrated. Our results suggest that this depends on the precipitation by alcohol of material which is antigenically inert, and which may have its origin in autolysis of bacteria.

Precipitation tests can, however, be conveniently employed as a first step in the analysis. Subdivision within each group can be achieved by the determination of phage sensitivity. Further differentiation will become possible with knowledge of the distribution of flagellar antigens.

The antigenic differences observed in precipitation tests probably depend on qualitative differences in a single polysaccharide-containing antigen rather than on the presence of entirely distinct antigens in the different strains. Only the major differences are detectable by the precipitation methods we employed. That many minor differences occur within each group is suggested by the slight variations in precipitation tests obtained with extracts within individual groups, and by differences in phage sensitivity. We have not yet made a detailed study of the antigenic variations which may occur in individual strains. Rough variants devoid of the group-specific antigen have, however, been obtained from several, and from one strain (L) variants have been obtained which yield T.C.A. extracts precipitating with heterologous antiserum but not with antiserum prepared against the parent organism.

The specific somatic antigens extracted from several of the strains have already been submitted to a more detailed study by Mead (1951) in his investigations on

their bacteriophage-inactivating properties. A more complete serological study will also be undertaken.

We have confirmed Boivin & Mesrobeanu's findings that trichloracetic acid extracts are antigenic. Antisera so far prepared against the purified antigens will agglutinate formolized or alcoholized suspensions only of those strains which on the basis of precipitation tests have been grouped together. Agglutination tests with sera prepared in this way may well prove to be more convenient than precipitation tests as a first step in the identification of strains. Until such sera are available it is intended to use precipitation tests in the study of hospital infection with this organism.

SUMMARY

Twenty-four selected strains of *Pseudomonas aeruginosa* have been classified on the results of precipitin tests with trichloracetic acid extracts into six serological groups.

This differentiation was confirmed by O agglutination.

Four of the six serological groups can be further subdivided according to their sensitivity to one or more of nine phages.

Alcohol treatment reduces agglutinability of some strains, particularly if alcohol is added to very concentrated suspensions of bacteria in the preparation of O suspensions.

The precipitation method has been applied to eighty successive strains of *Ps. aeruginosa* isolated, and with the available sera seventy-two could be placed into one or other of the six serological groups.

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