The Serology of Fifty Strains of *Proteus vulgaris*

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SUMMARY: Of fifty strains of *Proteus vulgaris* from human infections, seven of the 'vulgaris' and forty-three of the 'mirabilis' fermentative type, forty-four were brought into Belyavin's phase A, which represents the modal S form of recently-isolated strains, and analysed serologically by their H and O antigens. Three of the six remaining strains were irreversibly R, and three were too unstable antigenically for the routine analysis.

The antigens determining the somatic O types were resistant to heating at 95°, to ethanol and to dilute HCl, treatments that destroyed the H antigens. Serologically similar O antigens were extracted from the bacilli by hot formamide and by ethanol precipitation of tryptic digests.

As in other recent surveys of *Proteus* serology, the classification of strains by major O antigens was more clear-cut than by H antigens, though some minor O antigens were distributed differently from the major. The forty-four phase-A strains fell into eighteen O-antigenic types, three of these being the classical OX2, OX19 and OXK types, strains of which were included in the survey. The H antigens of each strain were often apparently multiple, but grouping by major H antigens was broader than by O antigens; moreover, H antigens were often common to strains with dissimilar O antigens.

Apart from the antigenic changes accompanying variation from phase A to phases B or C, there were suggestions of other phasic variations in both H and O antigens, which, if substantiated, may necessitate a reconsideration of serological classifications of the group. The three unstable strains in the collection could not be definitely assigned to any O-antigenic type.

The serological study of *Proteus vulgaris* and closely allied microbes covers a period of fifty years. The earlier work was directed, among other things, mainly to finding a species antigen by which *P. vulgaris* could be identified (Wolf, 1899; Klieneberger, 1908; Cantu, 1911; van Loghem & van Loghem-Pouw, 1912; Horowitz, 1916; Bengston, 1919; Wenner & Rettger, 1919; Taylor, 1928). As a result of these investigations, it was generally conceded that *P. vulgaris* was antigenically heterogeneous. One type of heterogeneity in *Proteus* spp., the distinction of flagellar and somatic antigens, was discovered by Weil & Felix who also discovered differences in the O antigens of the classical strains, X19, X2 (Weil & Felix, 1917; Weil, 1920, 1923; Felix, 1923). With the description of the XK strain (Fletcher & Lesslar, 1926) a third O-antigen complex was recognized.

Work on the flagellar and somatic antigens of the *Proteus* group during the past twenty years has defined the heterogeneity of the species antigens more fully (Moltke, 1927; Jacob, 1932; Varela, Zozaya & Imaz, 1944; Winkle, 1943, 1944; Rustigian & Stuart, 1945; Perch, 1948) with regard to both H and O antigens. In particular, Winkle (1944) and Perch (1948) have made a detailed receptor analysis of their strains and have numbered or lettered the determinant groups identified, thus allotting an antigenic 'formula' to each strain and group.

In the course of her investigations, Perch tested Winkle's type strains with her own sera.

Our serological investigation started in 1936, with the collection of 21 strains from human infections (see Miles, 1951) and of authentic stock strains for comparison. The serological analysis was interrupted by the 1939-45 war, during which most of the strains were maintained in stock nutrient agar slabs. A number of *Proteus* strains from infected burns were collected in 1943. On the resumption of serological work in 1946, many of the somatic serological characters previously found in the pre-war strains had disappeared or were difficult to elicit; and those even of the more recent burn strains were variable on retest. For this reason, the status of each stock strain was re-investigated (Belyavin, 1951), with the discovery of a modal phase A, equivalent to the fully Smooth form of other Gram-negative enterobacteria, and two other phases, B and C, of which B corresponds to a partially Rough form, with loss of the type O-antigen, saline-stability and virulence for mice. Since the variations A→B and A→C were reversible, it proved possible to coax most of the degraded stock strains into phase A, and we report below an analysis of the O and H antigens of *P. vulgaris* in Belyavin's phase A.

Symbols. We have used the sign PR, followed by a number, to designate our strains, except for those which were already named (X2, X19, etc.). Suspensions treated to destroy flagellar antigens, and the corresponding antisera, are labelled OPR1, OPR3, etc.; and suspensions of flagellated forms and antisera to them, HPR1, HPR3, etc. A collection of strains having the same phase A O-antigen constitute a 'type', and those with the same main H antigen, a 'group'; but we intend these terms to be only temporary descriptions pending an agreed designation for the *Proteus* antigens.

Character and source of the strains

The strains were isolated from human urinary, wound, pleural and burn infections (where they were either the sole or the predominant microbe as judged by examination of morbid material and direct plate cultures), with the exception of six strains from collections, and PR37 and 39, from laboratory mice dying of an intercurrent spontaneous *Proteus* infection during toxicity tests with *Brucella* endotoxins.

All strains were typical *P. vulgaris*, swarming on 2 % nutrient agar, liquefying gelatin, producing H₂S from peptone water and splitting urea with the production of NH₃. Of the fifty strains, seven (PR5, X2, XL, X19, PR29, 37, 39) were '*P. vulgaris* Hauser' (Bergey, 1948), in that they readily fermented maltose and sucrose, and readily produced indole. One (PR36) readily though feebly fermented maltose and sucrose, and was indole-negative; and the remaining forty-two were '*P. mirabilis* Hauser' (Bergey, 1948), being indole-negative, and failing to ferment maltose or sucrose within 5 days. Both the '*vulgaris*' and '*mirabilis*' varieties are called *P. vulgaris* throughout this paper.

Pus strains. PR5, 7, 9, 10, 13, 19, 21.

Urinary strains. PR1, 3, 4, 11, 12, 15, 16, 17, 18, 20, 22, 23, 31, 32, 33, 35, 36.

Mouse strains. PR37 and 39.

Burn strains. PR 41–52, isolated in 1943 by Dr L. Colebrooke from infected burns in ten patients during a ward outbreak of *Proteus* infection; and PR 53–58, from five patients during a second outbreak in a burns ward.

Stock strains. *Proteus* X2; *Proteus* X19; *Proteus* XK; *Proteus* XL (all from Col. H. J. Bensted); PR 29, *P. vulgaris* originally from Prof. Rettger (Wenner & Rettger, 1919); PR 30, N.C.T.C. 3156, selected because it was reported to react serologically with *Brucella* antisera. We also received from Dr F. Kauffmann seventy-one of the strains used by Dr Perch in her analysis, representing forty-nine of the somatic groups described in the simplified antigenic schema of Kauffmann & Perch (Perch, 1948). Some of these strains were tested against sera representing seventeen of our somatic types, with a view to identifying receptors common to the two classifications.

Up to 1945, the strains were maintained in soft agar stabs, subcultured at 6-weekly or 2-monthly intervals; in 1946 and after, they were kept in the dry state by Stamp's (1947) method.

METHODS

Preparation of phase A strains

Stability of suspensions. The strains were tested for thermal, saline and acriflavine stability. Thick suspensions in distilled water were killed by heating for 1 hr. at 56°, and diluted to 2×10^9 cells/ml. for the saline, and to 8×10^9 /ml. for the thermal tests (Pandit & Wilson, 1932), the density being estimated in a simple visual comparator against a counted suspension of 2×10^9 *Salmonella typhi*/ml. The 8×10^9 /ml. suspensions were heated in a 95° bath for 60 min. and examined during the heating at 15 min. intervals; and the 2×10^9 /ml. suspensions mixed with equal volumes of 5, 4, 3, 2, 1 and 0 % solution of NaCl, and read after 18 hr. incubation at 50°.

The acriflavine test, described by Pampana (1933), was made on a number of single colonies on MacConkey agar cultures of each strain by suspending them in a drop of 0.85 % saline on a slide, and mixing with an equal volume of 1/500 acriflavine in 0.85 % saline. Agglutination of the cells, readily visible with a $\times 3$ lens within 2 min., was recorded as positive. It is clear from Table 1 that acriflavine stability is not associated with saline and thermal stability. Heat-stable suspensions give rise as often as heat-labile suspensions to acriflavine unstable colonies and in comparable proportions; and in only one instance did the selection of an acriflavine-stable colony affect the heat stability of the strain. Comparison of the acriflavine and the saline tests is less valid because saline stability was at first tested with flagellated suspensions. Later work proved that flagellated suspensions were often saline-stable when the derived O suspensions were unstable. In any series of colonies from a strain quite stable to both heat and saline, acriflavine-stable colonies may nevertheless be exceedingly rare. We finally abandoned the acriflavine test as a method of selecting smooth strains. The decision was also justified by the fact that many variants, which later proved to be antigenically degenerate and to have lost virulence, were as acriflavine-stable as the parent strain.

Table 1. *The relation of thermal, saline and acriflavine stability of flagellated strains of Proteus vulgaris*

Strain	Source	Agglutination by		Acriflavine test*
		Saline	Heat (95°)	
PR 1	(a) From stock	+	0	3/20
	(b) Acriflavine-stable, from (a)	0	0	6/10
	(c) After mouse-passage	0	0	0/5
PR 3	(a) From stock	0	0	2/20
	(b) Acriflavine-stable, from (b)	0	0	3/9
	(c) After mouse-passage	0	0	0/5
PR 23	(a) From stock	+	+	2/20
	(b) Acriflavine-stable, from (a)	tr.	0	2/6
	(c) After mouse-passage	0	0	0/5
PR 4	From stock	0	0	0/7

* Number of colonies positive/number tested.

Selection of colony types for preparation of suspensions. All the parent strains and variant forms discovered during the investigation could be assigned to one of the three phases described by Belyavin (1951). The colonial types found could be divided into a modal phase A, which always gave rise to saline-stable O suspensions, and at least two other phases of which one (B) had an antigenic constitution of different specificity when tested against a series of fifteen somatic antisera, and tended to be unstable in saline suspensions. Whereas the phase A antigens were type specific, the antigenic complex of the B variant had two new antigens, one of broader specificity, a cosmopolitan antigen, the other narrower—indeed, strain specific. These components were present on the surface in varying amounts as judged by agglutination and absorption tests, together with small amounts of the type-specific antigen; moreover, even predominantly type-specific strains contained small amounts of the B phase-antigenic complex. These antigenic components could be detected in suspensions by the use of appropriate antisera, and serological tests of suitability eventually replaced those for saline and thermal stability.

Flagellar (H) suspensions. The dry stock cultures were seeded into CCY broth (Gladstone & Fildes, 1940) incubated for 5–6 hr. at 37° and plated on to MacConkey's agar. The next day, single colonies were seeded on to nutrient agar, the 20 hr. growth washed off with distilled water, centrifuged once, and resuspended in distilled water. The suspension was killed by heating at 56° for 1 hr. and preserved by 0.25–0.4 % formalin. Such suspensions were adequate for absorbing flagellar antibody, but in many instances were inferior to liquid cultures as agglutinating suspensions. Liquid cultures were prepared by inoculating single colonies into 40 ml. of 1 % peptone water, incubating for 3–5 hr. at 37° and then at 22–25° for 3 days. The cultures were killed by heat and formalized without washing.

On the whole, slightly higher titres were obtained with the formalized peptone cultures, and the agglutination was more floccular. However, the flagellar agglutination of a strain was sometimes variable, and occasional subculture by swarming over a 2 % agar surface, or through 0.5 % nutrient agar in U tubes

was necessary to produce a strongly agglutinating flagella suspension. With some strains even this treatment failed.

Somatic (O) suspensions. Since non-motile, non-swarming strains may be flagellated, though poorly (Colquhoun & Kirkpatrick, 1932) we did not attempt to induce non-motile variant strains for making O suspensions, but relied on the destruction of H antigens by ethanol, and by acid (Duncan, 1935). One volume of 0.01N-HCl at 37° was added to an equal volume of distilled water suspension in a water-bath. After 5 min. a one-tenth volume of 0.1N-NaOH was added to stop the reaction. The suspension was centrifuged once, resuspended in distilled water and heated for 1 hr. at 56°; preservative was not added. The rate of destruction of flagella at a given temperature and acidity is a function of bacterial density (Duncan, 1935). We confirmed that this treatment completely destroyed all flagellar antigen in suspensions containing 100×10^9 cells or less/ml.; all suspensions for acid treatment were diluted first to this concentration. The acid method is quick and simple, but suspensions of heat- and saline-agglutinable strains became much more so during acid treatment and were unsuitable for serological tests. Ethanol treatment, on the other hand, made these unstable forms more stable (Belyavin, 1951), and because we wished to analyse all forms serologically, it was adopted as a routine. Ethanolized type-specific suspensions induced good antibody formation in rabbits, and agglutinated more strongly than type-specific, acid-treated suspensions, although the latter gave slightly more consistent end-points in repeated titrations. We must emphasize that modal phase A (type-specific) strains yielded equally stable suspensions whether they were treated with acid or ethanol and gave precisely the same serological results.

The suspensions were prepared by washing off a nutrient agar culture in 96% methylated spirit and heating for $1\frac{1}{2}$ hr. at 56°. The suspension was spun down, washed twice in distilled water, finally resuspended in distilled water and stored at +5°. No preservative was added. We did not use as a routine O suspensions prepared by heating to 100° for 30 or 60 min., as recommended by Perch (1948). With phase A strains the results with ethanol, HCl and heating to 100° were identical, but phase B and phase C strains, initially unstable, were often more so by this treatment, and it was convenient to use ethanol, which was suitable for all three phases. Moreover, the results of HCl treatment were useful in detecting phase B strains (Belyavin, 1951).

Preparation of antisera. Albino rabbits, 2–3.5 kg. in weight, were immunized by the bi-weekly intravenous injection of bacterial suspension. The initial dose was 10^8 organisms and the dose was doubled at each injection, six to seven doses being given. Usually 'first-course' bleedings were used; a second course of injections sometimes induced a broadening of specificity in the resulting antisera, and 'second-course' bleedings were seldom used.

Serological methods

Agglutination tests. Twofold dilutions of antisera were mixed with an equal volume (0.3 ml.) of suspensions containing 2×10^9 cells/ml. and held at 50°. H agglutination was read at 4 and 18 hr. and O agglutination after 18 hr. The

end-point of the titrations was the reciprocal of the serum dilution producing agglutination barely perceptible to the naked eye, but easily visible with a $\times 3$ hand-lens when viewed by oblique illumination against a dark background ('standard' agglutination). For intermediate values in the two-fold series, interpolation factors were calculated by the method of Dreyer & Inman (1917). As in the *Salmonellae*, H and O agglutination respectively were floccular and granular.

Slide agglutinations were used in screening tests of O suspensions against type sera. Two loopsful (2 mm. loop) of neat serum were mixed with *c.* 0.05 ml. of ethanolized O suspension containing about 50×10^9 cells/ml., i.e. a final serum dilution of 1/3–1/5. The test was read in oblique transmitted light after rocking the slide for 1–2 min. The agglutinations were quite clear-cut and easy to read. Slide agglutination tests are liable to be falsely negative when compared with tube titrations, unless highly diluted sera are used. To establish the reliability of the slide test, ninety titrations were made in parallel (Table 2).

Table 2. *The relation between slide and tube agglutination tests of O suspensions of Proteus vulgaris*

Serum no.	Slide negative			Slide positive			Totals
	Tube negative	Tube positive		Tube negative	Tube positive		
		Up to 20	>20		Up to 20	>20	
1	13	0	0	0	0	2	15
2	15	0	0	0	0	0	15
3	12	1	0	0	0	2	15
4	14	1	0	0	0	0	15
5	13	2	0	0	0	0	15
6	11	0	0	0	2	2	15
Totals	78	4	0	0	2	6	90

In ninety tests, four were slide-negative but tube-positive. All four, however, gave tube titres, which, being less than $\frac{1}{100}$ th part of the homologous titre, were not significant in type identification. A similar series of thirty tests with four other sera, but containing a higher proportion of slide-positive suspensions gave similar results. It was not uncommon to find that slide-positive suspensions were agglutinated only to low titres in tubes. The slide test then is reasonably reliable for 'screening' ethanolized O suspensions.

Absorption tests. Two methods of absorption were used. In one, the serum at a fixed dilution (1/10 or 1/20) was absorbed by an equal volume of falling concentrations of antigen, and after centrifugation the supernatant from each tube tested directly against one or more suspensions. In the other, after absorption of the serum with a single concentration of antigen, the residual antibody was titrated in the usual manner. In both cases the absorption was carried out for 2 hr. at 37°. Both tests were used in parallel, one acting as a check on the other, when small numbers of strains and sera were involved. When more than three strains and three sera were tested the second method was used as being more economical of time and materials. Table 3 exemplifies

both methods in determining the antigenic relationship of two strains falling within the same somatic type. When a serum was absorbed, particularly by a heterologous strain, the agglutinins were usually more completely removed by low concentrations of absorbing antigen than with high, as is evident in Table 3*a*. For absorption of sera in bulk, therefore, a low multiple of the minimal absorbing dose was always used. For a large number of suspensions, the absorbed sera were first tested by the slide method to eliminate non-reacting strains.

Table 3. 'Mirror' cross-absorption tests in which a given serum dilution is (a) absorbed by graded doses of antigen, and (b) by a single dose of antigen (5×10^9 cells/ml.) with subsequent titration of residual antibody

Serum (1/10)			(a)					
			Initial conc. of absorbing suspension (10^9 /ml.)					
			80	40	20	10	5	0
Against	Absorbed by	Tested against	Agglutination result					
PR 7	OPR 7	OPR 7	S	S	tr.	tr. +	T-	T-
		OPR 10	0	0	0	0	0	T-
	OPR 10	OPR 7	T-	S+	T-	S+	T-	nt
		OPR 10	tr.	tr.	tr.	tr. +	tr.	nt
PR 10	OPR 7	OPR 7	tr.	tr. +	tr. +	tr. +	tr. +	T-
		OPR 10	T-	T-	T-	T-	T-	T-
	OPR 10	OPR 7	0	0	0	0	0	nt
		OPR 10	S	S+	S+	0	0	nt

tr., tr. +, S, S+, T-, T=degrees of trace, 'standard' and total agglutination; nt= no test; 0=no agglutination.

Serum (1/10)		Tested against suspension	(b)		
			Titre after absorption with		
			Nil	OPR 7	OPR 10
OPR 7		OPR 7	1280	80	640
		OPR 10	320	<20	20
OPR 10		OPR 7	320	20	<20
		OPR 10	1280	640	<20

RESULTS

The phase A somatic antigens

Table 4 shows the cross-reactions of HCl-treated suspensions from strains with their corresponding sera. It is arranged to display the main agglutinating types. A few are singleton strains, that is, they react only with their own sera, and their sera do not agglutinate any other strain in the collection. A summary of all types with their component strains is given in Tables 5*a* and *b*. Eighteen serological types emerge from forty-four strains, of which ten are singletons. Three strains, PR 5, 20 and 41, were irreversibly rough when tested in 1948. It was not possible to classify PR 19, 21, and 22 by agglutination reactions, because the strains were unstable, with a variable antigenic type. Both PR 19 and PR 21 at times exhibited affinities with the X2 type and PR 19 and PR 22

Table 4. The O-agglutination reactions of thirty-one sera and thirty-four phase A strains of Proteus vulgaris

O-suspension of (PR)	O-Antisera to (PR)																																		
	4	9	31	3	13	15	23	XK	30	42	11	17	45	7	10	X19	XL	X2	44	46	47	50	52	51	53	1	12	43	32	37	39				
4	1280	1000	5120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	0	0	0	0	0	0	0	40	0	0	40	0	0			
9	1280	1280	5120	20	0	0	0	0	0	0	0	120	0	0	0	20	120	40	0	0	0	0	0	0	0	0	0	0	0	20	0	0			
31	640	1280	5120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0	0	0	30	0	0				
3	0	0	0	640	2560	1000	160	2560	0	0	0	0	0	20	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0			
13	0	0	0	320	2560	2560	640	5120	0	20	0	0	0	0	0	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
15	0	0	0	640	1280	5120	1280	5120	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
23	0	0	0	320	1280	640	1920	10240	20	480	0	60	0	40	20	0	0	20	0	0	0	10	20	0	10	10	0	0	0	0	0	0			
XK	20	0	0	60	240	320	640	10240	20	1000	0	20	0	40	20	20	20	20	10	0	0	0	0	0	10	10	60	0	20	0	0	0			
30	20	20	0	20	160	320	480	640	480	40	640	1280	40	40	20	20	20	20	10	0	0	10	10	10	10	10	0	10	0	0	0	0			
42	0	0	0	0	40	60	20	80	160	2560	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
11	0	0	0	0	0	0	0	0	320	0	1280	1280	1000	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0			
17	0	0	0	0	0	0	0	0	160	0	1000	1000	2560	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
49	0	0	0	0	0	0	0	20	0	0	640	1000	1280	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
45	0	0	40	0	0	0	0	0	0	0	480	1000	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
7	0	0	0	0	0	160	0	0	0	0	0	0	0	5120	1280	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
10	0	0	0	0	0	40	0	0	0	0	0	0	0	1280	5120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
X19	20	0	0	30	0	0	20	0	0	0	0	0	0	0	0	5120	5120	60	0	0	0	0	0	0	0	30	0	0	0	0	0	0	0		
XL	0	0	0	80	0	0	0	0	0	0	0	0	0	0	0	5120	5120	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
X2	0	0	0	40	0	0	20	0	0	0	0	0	0	0	0	0	0	2560	0	0	0	0	0	0	0	60	0	0	0	0	0	0	0		
44	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5120	5120	2560	1280	1280	0	0	0	0	0	0	0	0	0	0		
46	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0	0	0		
47	0	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	5120	2560	1280	1280	0	0	0	0	0	0	0	0	0	0		
48	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0	0	0		
50	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0	0	0	0	
52	0	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0	0	0	0	
51	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	640	0	0	0	0	0	0	0	0	0	
53	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1280	0	0	0	0	0	0	0	0	0	
1	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	1280	0	0	0	0	0	0	0		
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1280	1280	0	0	0	0	0	0	0		
43	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	0	0	0	
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	0	0	
37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	3800	0	0
39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	2560	0	0

with type 10. The lability of these strains is well exemplified by the behaviour of PR19. The stock strain was at first Rough, but the apparently stable phase A selected from it reacted moderately with an X2 antiserum. Tested 4 days later, it had lost its X2 reactivity, and agglutinated to low titre with a type 10 serum; when tested a week later, it would react with none of the phase A sera. The other two varied analogously but both finally reached a state when they reacted to a very low titre with over half a dozen phase A sera, as though they were now characterized by a minor 'cosmopolitan' antigen. No serum was prepared against PR58, the fiftieth strain, but since it failed to agglutinate with any of the type O-antisera, it is presumably a singleton.

Table 5a. *The O antigenic types of forty-four phase A strains of Proteus vulgaris, including the four classical OX strains*

Somatic type	PR strains	Total
1	1, 12	2
2	4, 9, 31	3
3	11, 17, 18*, (30), 35, 45, 49, 56	8
4	7, 10	2
5	44, 46, 47, 48, 50, 52, 54, 55, 57	9
6	51	1
7	53	1
8	29	1
9	43	1
10	32	1
11	36	1
12	33	1
13	37, 39	2
14	16	1
15	58	1
X 2	X 2	1
X 19	X19, XL	2
XXa	23, (42), XK }	6
b	3, 13, 15 }	
Total		44

() = Strains carrying additional type antigens.

* See text (p. 187).

Table 5b. *H antigenic groups of twenty phase A strains of Proteus vulgaris, including the four classical X strains*

Flagellar group	PR strains	Total
A	4, 9	2
B	1, 3, 10, 12, 13, 15, 23	7
C	7, 11, 17, 19, 22, X 2, X 19, XK, XL	9
D	16	1
E	21	1

In both tables, numbers in **heavy type** indicate strains against which sera were prepared.

Types containing more than one strain are discussed below, especially those that are heterogeneous with respect to their minor antigens.

Somatic types 1, 2 and 3. These three types, containing two, three and eight

strains respectively, are each antigenically homogeneous; each component strain will remove all the O antibody from the type sera. This, of course, does not preclude the possibility of strains belonging to these types, with heterogeneous minor antigens. Strain PR 18 is not classed as type 3 by agglutination or absorption tests; the strain is rough, although strain-specific receptors are present. However, it contains a trace of the type 3 antigen, because it induces type 3 antibody in the rabbit.

Type XK. This type certainly includes five strains: PR 3, 13, 15, 23 and XK and possibly PR 42. Strains PR 3, 13 and 15 agglutinate to a lower titre than strains PR 23, and XK with OXK antiserum. Conversely, strains XK and PR 23 agglutinated to low titres with PR 3 and 15 antisera. The cross-adsorption tests with ethanolized suspensions (Table 6) subdivide the type into two, *a* and *b*, the classical XK strain falling in type *XKa*. All these strains are antigenically distinct from the other X strains, X 2, X 19 and XL.

Table 6. *Cross-absorption of tests of type OXK strains*

Serum	Tested against suspension	Titre after absorption by					
		Nil	OPR 3	OPR 13	OPR 15	OPR 23	OXK
OPR 3	OPR 3	320	0	0	80	160	160
	OPR 13	160	0	0	40	80	80
	OPR 15	80	0	0	<20	<20	<20
	OPR 23	40	nt	nt	nt	nt	nt
	OXK	40	nt	nt	nt	nt	nt
OPR 15	OPR 3	320	0	0	0	80	80
	OPR 13	320	0	0	0	0	0
	OPR 15	1280	0	0	0	0	0
	OPR 23	100	0	0	0	0	0
	OXK	80	0	0	0	0	0
OXK	OPR 3	640	160	120	40	0	0
	OPR 13	1280	240	160	80	0	0
	OPR 15	1280	240	240	160	0	0
	OPR 23	2560	480	480	480	0	0
	OXK	2560	1280	1280	1280	0	0

nt=no test because unabsorbed serum too weak.

Difficulties in classification are raised by the behaviour of strains PR 30 and PR 42. PR 30 reacts strongly with sera for subtypes *a* and *b*, but PR 42 does not react with the subtype *a* sera, and only agglutinates to 5 % of full titre with subtype *b*. Nevertheless, its own serum agglutinates OXK to at least 50 % of full titre; moreover, PR 30 carries an antigen reacting to full titre with the sera of the otherwise quite distinct type 3, and PR 42 has an antigen not related to any other in the collection. It is not clear, therefore, whether these strains should, in fact, be referred to the XK type. Two of Dr Perch's strains behaved similarly, i.e. as though possessing an XK antigen in addition to that characterizing a different somatic type. We have no evidence that such an antigenic constitution is quantitatively variable, with one or other type antigen predominating at the surface in different phases. If phase variation of this

kind were proved, the validity of the XK type antigen as a constant feature characterizing XK strains would need reconsideration.

Type 4. This consists of two strains, PR 7 and PR 10. They cross-agglutinated, but not to reciprocally equal titres. The two strains are not antigenically homogeneous (Table 3a).

Type 13. This consists of two antigenically homogeneous strains, PR 37 and 39.

Type X 2 and X 19. These types, although antigenically distinct, may be considered together, as they are represented in the collection by only three strains, viz. X 2, X 19, and XL. Strain X 2 is a singleton. On the other hand strains X 19 and XL as originally described are closely related although not homogeneous (Table 7). Whereas XL removes agglutinins for both itself and

Table 7. *Cross-absorption tests of Proteus OX 19 and OXL*

Serum (1/20)			Initial conc. of absorbing suspension (10 ⁹ /ml.)					
Against	Absorbed by	Tested against	80	40	20	10	5	0
OXL	OX 19	OX 19	0	0	0	S—	T	T
		OXL	S+	T—	T—	T	T	T
	OXL	OX 19	0	0	0	0	S	nt
		OXL	0	0	0	S+	T—	nt
OX 19	OX 19	OX 19	0	0	0	T—	T	T
		OXL	0	0	0	T—	T	T
	OXL	OX 19	S+	S+	tr. +	T—	T—	nt
		OXL	S+	S+	tr.	tr. +	T	nt

Symbols as in Table 3.

X 19 from anti-XL serum, X 19 even in high concentration, removes only its own antibody. Conversely, XL does not remove all antibody from the X 19 antiserum, although it obviously removes more of its own agglutinin. The relationship of these two strains is of considerable interest. The results described suggest that they differ in their somatic antigens only by the presence of an additional receptor in strain XL, and in this we agree with Kauffmann & Perch (1947). On the other hand, Winkle (1944) allots the additional antigen to strain X 19 and identifies it also in strain X 2. The ethanolized OXL suspension used gave a well-marked though partial agglutination to a titre of 640 with a strong phase B serum (58 α) which did not occur with OX 19. The antigen that distinguishes XL from X 19 may therefore be a phase B antigen. The two strains may be even more complex. A bacteriophage from the Pasteur Institute reported to act on the *Proteus* strain OXL, when tested against subcultures of X strains obtained from the National Collection of Type Cultures, indeed attacked the strain XL but not X 19. In the case of the X strains we received from Col. Bensted, however, OX 19 was attacked and not OXL. This discrepancy is unexplained.

Types 6, 7, 8, 9, 10, 11, 12, 14 and 15. These types are all represented by single strains. It is possible that some of these, notably PR 16, are in a 'strain-specific' phase. Type 9 contains PR 36. This strain has a 'rough' colonial

morphology and tends to be unstable in high concentrations of saline. The creation of an O type to accommodate this strain must be regarded as tentative.

Somatic antigen-analysis by precipitin reaction. Two kinds of soluble extract were tested against O-antisera, and gave identical results. The first was obtained by ethanol precipitation of tryptic digests of acetone-extracted cells (Miles, 1951) and the second, more simply by treating the centrifuged deposit from 2 ml. of a 5 or 20 hr. culture in CCY broth with hot formamide, by the method of Fuller (1938). The extract was layered on undiluted serum and precipitation at the interface occurred within 10 min. at room temperature. Eighteen strains were tested against sixteen sera (Table 8). The reactions confirm the results with cross-agglutination tests (Table 4), but strains belonging to antigenically heterogeneous types are sharply separated into subtypes without cross-precipitation; e.g. the reactions between PR3, 13, 15, 23 and XK, and of PR7 and 10. The effect might be due to selective extraction of different fractions, or to a concentration effect; the same sera were used for agglutination and precipitation, and, being induced by one or at most two courses of injections, perhaps contained insufficient antibody for a visible heterologous precipitin reaction. The formamide method which can be applied to 2 ml. of a 5 hr. culture would clearly be useful for rapid O typing of *Proteus* strains.

Table 8. *The cross-precipitation reactions of sixteen antisera and formamide extracts of eighteen phase A strains of Proteus vulgaris*

Extracts from (PR)	O-Antisera to (PR)															
	4	9	3	23	XK	30	11	17	7	10	X19	XL	X2	1	21	32
4	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
13	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
23	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—
XK	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—
30	—	—	—	—	—	±	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—
17	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—
X19	—	—	—	—	—	—	—	—	—	—	+	±	—	—	—	—
XL	—	—	—	—	—	—	—	—	—	—	±	+	—	—	—	—
X2	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—
32	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+

The flagellar antigens

Table 9 shows the flagellar cross-agglutination titres for nineteen of the strains in the collection; the analysis was not carried beyond this point. Eighteen fall into five groups, containing respectively one, one, two, seven and nine

strains and comprising strains belonging to nine O types. The word 'group' is not used here in the strict sense of a serological class, but merely implies a collection of strains, and these we designate A, B, C, D and E (Table 5*b*).

They are not as clearly defined as the O types by the cross-agglutination reactions, and cross-absorption tests reveal that all are antigenically heterogeneous.

Table 9. *The H-agglutination reactions of fifteen sera and nineteen phase A strains of Proteus vulgaris*

H-suspension of (HPR)	H-antisera to (HPR)														
	9	4	1	3	13	23	15	XK	19	17	11	X19	X2	XL	16
9	2560	7600	60	0	0	0	0	0	0	0	80	0	80	0	0
4	3800	10240	60	0	0	0	0	0	0	0	80	0	160	0	0
12	0	0	1280	5120	0	120	0	0	0	0	0	0	0	0	0
1	0	0	1280	5120	3800	>10240	30	2560	0	0	0	0	0	0	0
3	0	0	2000	20000	250	160	3800	640	0	0	0	0	0	0	0
13	320	0	480	1280	5120	2560	480	480	0	0	0	0	80	0	0
23	0	0	1000	120	1280	>20480	640	15360	0	0	0	0	60	0	0
15	320	160	1280	7600	320	80	5120	120	0	0	0	0	80	0	0
10	0	0	750	5000	3000	160	<40	10000	5000	5120	160	nt	0	0	0
7	0	0	0	0	160	0	80	15360	160	160	3800	3800	nt	750	0
XK	0	0	0	160	0	160	40	15360	240	2560	5120	2560	1280	640	0
19	0	0	0	0	0	0	0	640	3800	3800	0	0	0	0	0
17	0	0	0	0	0	0	0	160	nt	5120	80	<40	<40	<40	0
X19	0	0	0	0	0	0	0	20480	160	640	1280	5120	1280	1280	0
X2	0	0	0	160	0	0	0	10240	120	5120	3800	2600	1280	1000	0
XL	0	0	0	0	0	0	0	15360	1000	5120	3800	1280	1280	1280	0
11	0	0	0	60	0	0	0	15360	0	1280	5120	2560	2000	1280	0
22	0	0	0	0	0	0	0	15360	0	160	1280	3750	0	160	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7620

nt=no test.

A, D and E groups. PR 4 and 9 comprise the A group. Their flagellar antigens are not entirely homogeneous, and they differ in at least one receptor. D group is represented by PR 16, and E by PR 21. Both have an antigenic flagellar constitution distinct from that of the other strains examined.

B group. Strains PR 12, 1, 3, 13, 23, 15 and 10 constitute B group. Cross-absorption of three anti-sera HPR 3, HPR 23 and HXK by four of the strains (Table 10) show at least three distinct antigens and from the cross-agglutination tests of PR 12 and XK, two others may be inferred. One receptor carried by HXK is common to the whole group.

C group. This is formed by the strains PR 7, PR 19, PR 17, X 19, X 2, XL, PR 11 and PR 22. XK can also be considered as falling mainly in this group, but its exact relationship is discussed below. The cross-absorptions with three of the main sera (Table 11) reveal the distribution of four antigens. The strains 19 and 17, as they agglutinate only with their own sera and with that of XK, can be considered as forming a minor subdivision and might with justification be taken out of the major group of strains. They are retained, however, on the basis of their antigenic relationship with strain XK.

The flagellar antigens of PR 7, 11, 22 and strains X 19, X 2, XL and XK have something in common. It is noteworthy that XK has wide antigenic relationships, and it is of particular interest that although the antiserum against XK agglutinates both group B (PR 1, 3, 12, 13, 15, 23, 10) and group C (PR 7, 19, 11, 22 and strains X 19, X 2, XL) to a high titre, it was itself agglutinated only by the sera of group C (Table 9).

Table 10. *Cross-absorption tests of strains in flagellar antigen, group B*

Serum	Tested against suspension (HPR)	Titre after absorption by				
		Nil	(HPR)			
			1	3	23	XK
HPR 3	1	5120	< 80	< 80	80	320
	3	20000	5120	1920	5120	3800
	13	1280	< 80	< 80	< 80	< 80
	15	7600	5120	1280	5120	5120
	23	120	< 80	< 80	< 80	< 80
HPR 23	1	> 10240	80	5120	80	3800
	3	160	< 80	< 80	< 80	< 80
	13	2560	< 80	2560	< 80	2560
	15	80	nt	nt	nt	nt
	23	> 20480	80	1920	< 80	2560
HXK	1	2560	< 80	< 80	< 80	< 80
	3	640	< 80	< 80	< 80	< 80
	13	480	< 80	< 80	< 80	< 80
	15	120	< 80	< 80	< 80	< 80
	23	15360	< 80	< 80	< 80	< 80

Table 11. *Cross-absorption tests of strains in flagellar antigen, group C*

Serum	Tested against H suspension	Titre after absorption by				
		Nil	(H)			
			XL	X 2	X 19	XK
HXK	XK	15360	10240	10240	10240	80
	PR 19	640	< 80	< 80	< 80	< 80
	PR 17	160	< 80	< 80	< 80	< 80
	PR 11	15360	160	80	160	5120
	XL	15360	2000	1000	160	10240
	X 2	10240	2000	160	160	10240
	X 19	20480	2000	160	640	15360
HX 19	XK	2560	< 80	< 80	< 80	< 80
	PR 19	< 40	nt	nt	nt	nt
	PR 17	< 40	nt	nt	nt	nt
	PR 11	2560	< 80	< 80	< 80	< 80
	XL	1280	< 80	< 80	< 80	640
	X 2	2600	< 80	< 80	< 80	1280
	X 19	5120	< 80	< 80	< 80	1000
HPR 11	XK	5120	< 80	< 80	< 80	< 80
	PR 19	< 40	nt	nt	nt	nt
	PR 17	80	nt	nt	nt	nt
	PR 11	5120	< 80	< 80	< 80	< 80
	XL	3800	< 80	< 80	< 80	< 80
	X 2	3800	< 80	< 80	< 80	160
	X 19	1280	< 80	< 80	< 80	160

nt = no test, because unabsorbed serum too weak.

The anomaly may have its basis in a diphasic variation. We attempted to discover such a variation by absorbing two portions of the XK antiserum with HX 19 and HXK respectively, and diluting the absorbed sera so that they were monospecific for HXK and HX 19 respectively. The monospecific titres were about 1/30. Single colonies on MacConkey agar plated with the N.C.T.C. HXK

and with Col. Bensted's strain of HXK were tested against both sera by the slide method. All colonies from the first strain were agglutinated equally well by both sera, whereas those from the Bensted strain went feebly or not at all with the monospecific 'HXK' serum, and in very different degrees with the monospecific 'HX19' serum. The matter was not pursued, but the results, though providing no evidence for diphasic variation, suggest that the content, or the development of the flagellar antigenic components in the same strain, may be variable. Further work on these lines is necessary.

Comparison of the antigenic complexes with those in Dr Perch's strains

Forty-four of our strains were allotted to eighteen somatic types, of which three are the classical X types, X2, X19 and XK. We examined thirty-eight of Dr Perch's seventy-one strains, representing the thirty O types fully described by Dr Perch in her extensive classification of 388 strains. We did not investigate the remaining nineteen types recorded in the simplified scheme of Kaufmann & Perch (Perch, 1948) since the details of the minor antigen are not recorded. Excluding the necessary identities of the X strains common to both series, the correspondences were:

Perch		Present paper
'O' group 30	=	'O' type 2
group 23	=	type 4
group 4	=	type 10
group 10	=	type 3

In addition, the strain F10 (Perch O group 29) agglutinated with our O type 2 antiserum to approximately 10 % of full titre, and U509 (Perch O group 28) to nearly 50 % of full titre against our OPR42 serum. Since U509 did not react with type XK sera it is provisionally assumed to share a minor antigen with strain PR42.

It cannot be asserted that the antigenic constitutions of the identified types are identical in every case, but they are clearly closely related. The lack of correspondence between the type composition of the two samples of organisms is of some interest. Perch was able to identify every one of Winkle's eight somatic types (excluding the X types) with one of her own (Perch, 1948). Some of the organisms in the two collections were isolated from geographically continuous regions, i.e. Denmark and Germany, although some of Winkle's strains came from Poland or the Polish-German frontier region. Our strains were collected in Britain (London and Birmingham). In all these collections the strains were of human origin. Perch's O group 10 (our O type 3) is particularly interesting. The strains of this group form 14.4 % of the 388 strains represented in Perch's extended scheme, and 18.2 % of the typable strains in our collection; that is, in both cases, the type is one of the most common, if not the most common, somatic antigen. It is completely absent from Winkle's strains; and since these numbered over 1000 it seems unlikely that its absence from his collection is due merely to errors of random sampling. It is conceivable that the differences in the distribution of O antigens in these collections is due

to the limited geographical distribution of the various O antigenic types. Alternatively, the apparent dissimilarity in somatic antigen constitution between our collection of strains and that of Perch, may in our own series be the result of the selection only of strains that were in phase A or could be induced to revert to it, with the consequent elimination of certain antigens found widely distributed in degenerate strains which are characterized by the more cosmopolitan phase B and C antigens.

Applications of Proteus typing

Much information of epidemiological and clinical interest may emerge from typing *Proteus* strains. In this connexion it is worth recording the O types of strains PR 41-58 isolated on two occasions from infected patients in a burns ward (Table 12). The result, though not conclusive, suggests that the hospital infection of wounds and burns, recognized with staphylococci and streptococci, might also be demonstrated unequivocally with typed *P. vulgaris*.

On the first occasion, type 5 was found in six of ten infected patients; and patients nos. I and IV swabbed at an interval of a few days had either a mixed infection or a replacement of type by another (untypable → XK, 3 → 5). On the second occasion, investigated a few weeks later, types 5 and 3 are still prevalent.

Table 12. *O*-antigenic types isolated during two outbreaks of *Proteus* infection in burns

Patient	Type of <i>P. vulgaris</i>	Patient	Type of <i>P. vulgaris</i>
I	{ (not typable) XK	VIII	5
II	9	IX	6
III	5	X	5
		XI	7
IV	{ 3 5	XII	{ 5 5
V	5	XIII	3
VI	5	XIV	5
VII	3	XV	15

The strains isolated from patients I, IV and XII were in each case obtained within a few days.

DISCUSSION

Our analysis of the fifty strains is incomplete in two senses: (a) we have not made antisera to all the H strains, and so do not know the entire cross-reactivity of the collection; and (b) we have based our classification on the orthodox O and H antigens. With regard to (a), we felt it sufficient to demonstrate, as others have recently done, the antigenic heterogeneity of the *P. vulgaris*; the fact that H antigens are shared by strains with quite different O antigens; the occurrence of OX strains (in our limited collection only type OXK) in situations remote from typhus infections; and a concordance of some of our types with those in other collections. We do not wish necessarily to perpetuate our numbered typing of O antigens, nor the lettered grouping of H antigens. The proper

naming of the types and groups must follow concerted action by workers in the field, whose strains are extant.

With regard to (b), our O antigens are those which, as bacterial suspensions, withstand heating at 95°, and treatment with ethanol or dilute mineral acid; and which, as bacterial extracts, withstood tryptic digestion followed by ethanol precipitation or exposure to formamide at 150°. Their serology was similar, whichever of the five treatments was used. But it is quite possible that other important surface antigens are destroyed by these processes and do not appear in the analysis. Moreover, the analysis was made only upon strains in what we regard as the modal S form of recently isolated strains—the phase A of Belyavin—and it is possible, by extending the analysis to antigens that predominate on the surface of phase B and phase C strains, that significant grouping might appear. The existence of phasic changes is suggested both by our own work and by Perch (1948) who described the induction of new flagellar antigens in three strains, two of them with identical somatic antigens. She reported also ‘incomplete antigens’ which appeared erratically in successive cultures of a given strain, but does not appear to use them in her antigenic scheme. In any event, the serological qualities of the various phases, of which only three are yet known, necessitate a careful serological study of the antigenic configuration of common variants of *P. vulgaris*, including its Rough forms, as a preliminary to a definitive classification.

Some interest attaches to the precise status to be accorded to the somatic XK type. Antigens of this type were found in two strains in our collection (PR 30 and PR 42) which also carried antigens (type 3) of other types. Two such strains (F 58 and F 151) were also detected in the seventy-one strains received from Dr Kauffmann. Winkle (1944), in his somatic antigenic table, lists two other somatic groups which carry one antigen common to the OXK group, in addition to antigens characteristic of their own. Hemmes (1948), in a survey of ninety strains of *P. vulgaris*, tested serologically against eight scrub-typhus sera agglutinating the OXK strain to a high titre, reports the wide distribution of XK antigen, which she identified in a very large proportion of her strains both by agglutination and absorption tests. Only five rabbit anti-proteus sera were used. It is noteworthy that thirty-four of her ninety strains were auto-agglutinable.

The sharing of antigens from groups other than OXK is not found in any strain in our series, which however is limited in number. In the 388 strains analysed in the extended schema of Perch, however, only one other strain is listed as sharing a somatic antigen from a group other than OXK.

Winkle gives three somatic groups with one antigen in common, distinct from any found in the OXK group. According to Perch, however, these three somatic groups form three subgroups of her own somatic group 23, and may therefore merely represent the members of a heterogeneous somatic group. The evidence therefore points to the XK group as being the outstanding possessor of this peculiarity. The question therefore arises as to whether some of the OXK antigens concerned are not characteristic of a distinct phase in an antigenic variation.

With regard to the relation of the 'mirabilis' and 'vulgaris' varieties, it is to be noted that there we found no O antigens shared by strains of the two types; but the number of 'vulgaris' varieties in our collection was too small to give this observation much weight in determining whether the two fermentative types deserve specific rank.

We are indebted to Lt.-Col. H. J. Bensted and Dr L. Colebrook for strains, and particularly to Dr F. Kauffmann and Dr B. Perch of the State Serum Institute, Copenhagen, who generously put the key strains of their collection at our disposal.

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