

THE BASIS OF VIRULENCE IN *PASTEURELLA PESTIS*:
THE DEVELOPMENT OF RESISTANCE TO PHAGOCYTOSIS
IN VITRO

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Received for publication March 12, 1956

STUDIES of the comparative behaviour of virulent and avirulent strains of *Pasteurella pestis*, following their intraperitoneal injection into mice, brought to light an important difference between the two types which was not evident from comparative studies *in vitro*. After a short period *in vivo* virulent organisms acquired the ability to resist phagocytosis by mouse polymorphonuclear cells whereas avirulent organisms did not (Burrows and Bacon, 1954*b*). Observations on the behaviour of virulent cells *in vivo* were interpreted to indicate that virulent organisms acquired resistance to phagocytosis through modification of their surface properties and that they exerted an inhibitory action on the polymorphs. Whether or not these two phenomena were determined by a single agency, and whether or not the interpretation of the observations made *in vivo* were substantially correct, demanded a study of the development of resistance to phagocytosis under controlled conditions *in vitro* for satisfactory answer.

In this paper we report attempts to obtain phagocytosis-resistant virulent organisms by treatments *in vitro* and to investigate factors which influence the change from sensitivity to resistance to phagocytosis. Throughout, to avoid monotonous repetition, the phrase "to phagocytosis by mouse polymorphonuclear leucocytes" is to be understood whenever the terms sensitive, sensitivity, resistant and resistance are used.

Some observations concerning the production of a phagocyte-inhibiting factor *in vitro* are included.

MATERIALS AND METHODS

Strains and suspensions

As in former studies, the strains mostly used were the avirulent Tjiwideoj smooth, referred to throughout as TS, and the fully virulent MP6 (Burrows and Bacon, 1954*a*). They were maintained in the dried state. Stock suspensions were prepared from dried material by inoculation into flasks of a semi-synthetic medium, developed by Ross, Hakes and Herbert (1956), which were kept well shaken throughout incubation for 48 hr. at 28°. Population densities of $1-2 \times 10^{10}$ organisms per ml. were routinely achieved. These stock suspensions were kept at 4° where they retained full viability for at least 6 weeks. Slopes containing 5 ml. of tryptic meat digest agar (TMA) were inoculated with 2 drops of the stock suspension, incubated for 17 hr. at 28°, unless otherwise stated, and the resulting growth suspended in phosphate buffer pH 7.0 to the density required for use. The use of stock suspensions was a deviation from former procedure designed to ensure uniform inoculation of slopes in an endeavour to obtain more uniform suspensions. The stock suspensions were replaced at 4-week intervals.

Suspensions of mouse polymorphonuclear leucocytes

White mice (Porton strain weighing 30–35 g.) were injected intraperitoneally each with 1.5 ml. of 2 per cent wheat starch in physiological saline. After 16 hr. 1 ml. of a solution containing KH_2PO_4 3.6 g., NaCl 3.6 g., sodium acetate 1.6 g., heparin 0.15 g., glucose 4.0 g. and gelatin 10 g. per l., pH 7.0, was injected i.p. into each mouse. This solution was based in part on the recommendations of Tullis (1952) and is referred to as PGA. The animals were killed 5 min. later, by cervical dislocation, and the fluid in the peritoneal cavity containing polymorphs in suspension gently transferred to a paraffin waxed tube, using a waxed pipette. In most experiments the peritoneal fluids derived from ten mice were pooled for use. To this volume of pooled fluid 1 ml. of freshly drawn mouse heart blood was added, the waxed tube sealed with a waxed bung and placed at 4° until required. The presence of erythrocytes in the polymorph suspension facilitated subsequent microscopic examination of stained films by serving to delineate the extent of the spread material so that the edges, where polymorphs accumulated, could easily be found.

From 10 mice *ca.* 7.5 ml. of suspension could be recovered, each 1 ml. containing *ca.* 15 million polymorphs. When necessary the suspension was concentrated by centrifuging at 500 r.p.m. for 5 min. and removing the appropriate volume of supernatant. After harvesting from the mouse or after centrifuging or storage at 4° polymorph suspensions were incubated for 30 min. at 37° to permit recovery from any adverse effects prior to use in a phagocytic system. In our experience mouse polymorphs *in vitro* required the gentlest treatment to avoid their disruption and the composition of the medium for harvesting was critical. The addition of gelatin greatly assisted the preservation of normal morphology and ingestive capacity. Fragility of polymorphs *in vitro* did not appear to result from ascorbic acid deficiency (Nungester and Ames, 1948) or from incompatibility of different pooled peritoneal fluids. Heparin proved a more satisfactory anti-coagulant than sodium citrate or ethylenediamine tetra-acetic acid.

In practically all experiments polymorphs were used within 2 hr. of collection from mice.

Phagocytosis

To compare the abilities of different media to permit the change from sensitivity to resistance to phagocytosis 1 ml. volumes of the media under test were inoculated with 0.1 ml. of suspensions in buffer containing 2.5×10^9 organisms per ml. The tubes containing the inoculated media were then rotated at 55 r.p.m., with their long axes inclined at 30° to the horizontal, for the required time and at the required temperature. The tubes were then centrifuged at 3000 r.p.m. for 20 min. and supernatants completely removed. To the deposited organisms 0.8 ml. of polymorph suspension was added, the organisms re-suspended therein and 0.2 ml. volumes transferred to each of 3 waxed tubes. These were rotated at 55 r.p.m. at 37° (Robertson and Sia, 1924) for 30 min., then plunged in iced water. Films of material from each tube were prepared, on slides kept at *ca.* 40° on a warm surface, using the normal method for preparing blood films. After fixing and staining by Leishman's method, phagocytosis was estimated in the customary manner by counting the numbers of ingested organisms contained in a random sample of 100 polymorphs and the percentage of polymorphs containing ingested bacteria, for each film. The means of the triplicate determinations of phagocytosis are recorded in the Tables by the two numbers representing these two values.

The following procedure was used to determine the ability of strains to excrete factors having an inhibitory action on mouse polymorphs. Organisms were incubated in particular environments for the desired period then centrifuged at 10,000 r.p.m. for 1 hr. Of the supernatants 1 ml. was used to re-suspend 20 million polymorphs, previously deposited by light centrifugation in waxed tubes, and the mixture incubated at 37° for the prescribed time. The polymorphs were again deposited, the supernatants completely removed, and the polymorphs re-suspended in 1 ml. of PGA. Of these suspensions 0.2 ml. volumes were transferred to waxed tubes, 0.05 ml. of a suspension of the test organism (usually TS) at a density of 5×10^9 organisms per ml. was added to each tube and the tubes rotated and films prepared as above. Fresh suspensions of organisms and of polymorphs were employed for each experiment.

An electrically operated device, which incorporated three Post Office telephone meters, greatly reduced the tedium of phagocytosis estimations.

RESULTS

In experiments *in vitro* the sensitivity to phagocytosis of virulent organisms harvested from TMA slopes which had been incubated at 37° varied very considerably from experiment to experiment. Frequently this sensitivity was lower than was desirable in experiments aiming to determine factors controlling the development of resistance. From slopes which had been incubated at 28° organisms consistently showed high sensitivity and were therefore more satisfactory for

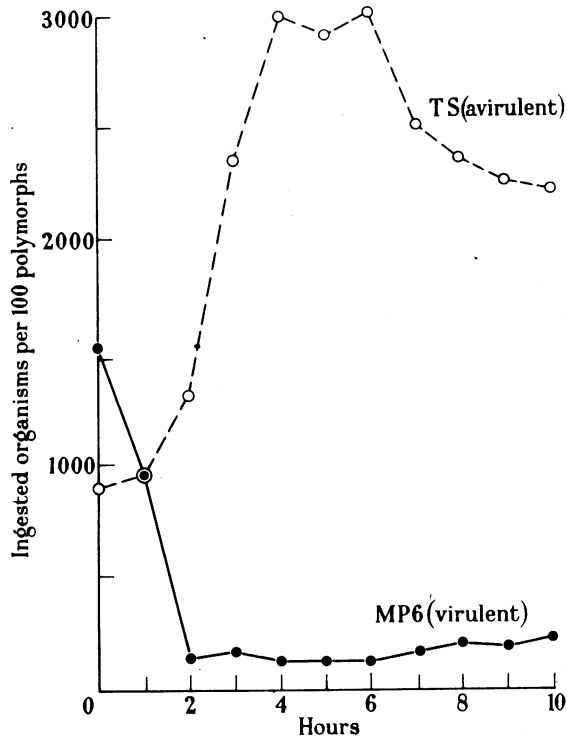


FIG. 1.—Effect of time of incubation on development of resistance to phagocytosis—1 ml. volumes of tryptic digest meat broth pH 7.0 containing 2.5×10^8 virulent or avirulent organisms incubated with rotation at 37° and phagocytosis of resulting organisms determined at hourly intervals.

our purpose. In view of the known temperature dependence of the metabolic processes leading to the accumulation of capsular (or "envelope") material in *P. pestis* (Schütze, 1932), and of the correlation between capsulation and resistance to phagocytosis in other organisms, observations were made of the extent of capsulation of organisms following various treatments leading to resistance. However, no correlation was found between sensitivity to phagocytosis and absence of a visible degree of capsulation with our virulent strain, or between resistance and visible capsulation with our avirulent strain, under our conditions. Although we did not observe visibly capsulated virulent organisms to show high sensitivity, non-capsulated virulent organisms frequently showed a

high level of resistance. These observations suggested that resistance was independent of capsular material.

Preliminary trials clearly showed that sensitive virulent MP6 organisms became resistant to phagocytosis when incubated for 2 hr. at 37° in normal mouse, rabbit or horse serum, and that avirulent TS organisms failed to do so. Similar incubation of these two representative strains in certain batches of tryptic meat broth (TMB) also resulted in the development of resistance by MP6 and in the retention of sensitivity by TS. Our earlier experiments were devoted to

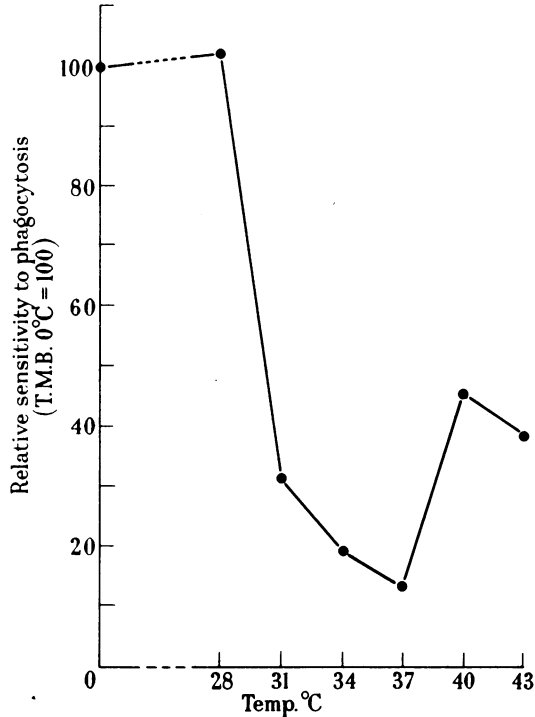


FIG. 2.—Effect of temperature on development of resistance to phagocytosis—1 ml. volumes of tryptic digest meat broth pH 7.0 containing 2.5×10^8 virulent organisms incubated for 3 hr., with rotation, at temperatures shown and phagocytosis of resulting organisms determined.

establishing, so far as possible, the optimum conditions for the development of resistance by virulent organisms in this readily available medium. Maximum resistance was found to result within 3 hr. of incubation, with rotation, at 37° in TMB having an initial pH within the range 6.2 to 7.4. Resistance did not develop within this period in static culture, and developed less in shaken culture than when gentle rotation was employed. Fig. 1, 2 and 3 illustrate the effects of varying the time and temperature of incubation and the initial pH of TMB individually while keeping the other important factors at optimal values.

Complete resistance to phagocytosis was never achieved in these experiments. We think it probable that organisms remaining sensitive after the bulk of the bacterial population has developed resistance constitute the non-viable fraction

of the population. It can be seen from Fig. 1 that even following prolonged periods of incubation in TMB this residuum of sensitive organisms remains.

The very different behaviour of avirulent TS organisms under the conditions which permit virulent MP6 organisms to develop maximum resistance to phagocytosis is illustrated in Fig. 1. As judged by the method employing India ink for capsule demonstration (Rowland, 1914), neither strain showed visible capsulation of organisms over the period 0–3 hr. in the experiment from which the results given in Fig. 1 were taken. Visible capsulation was first evident with both strains

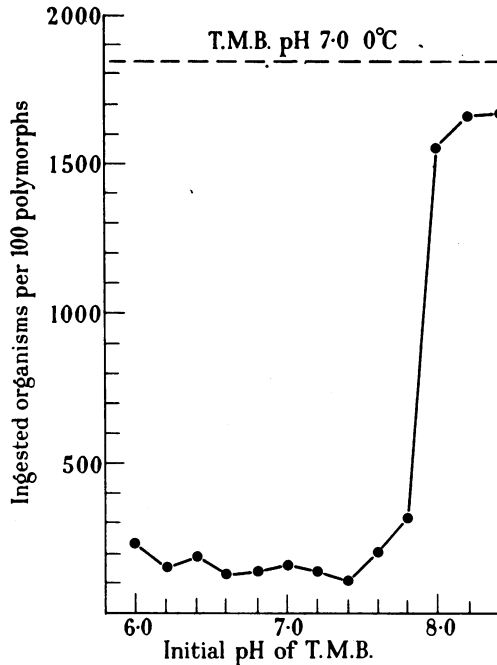


FIG. 3.—Effect of initial pH of medium on development of resistance to phagocytosis—1 ml. volumes of tryptic meat digest broth, previously adjusted to the pH values shown, inoculated with 2.5×10^8 sensitive virulent organisms and incubated with rotation for 3 hr. at 37° . Phagocytosis of resulting organisms then determined.

at 5 hr., that is at a time well after maximum resistance of virulent organisms had been reached, and was very evident in both strains by the 10th hr. In this well capsulated state TS retained high sensitivity to phagocytosis. We concluded from these observations that resistance to phagocytosis was not determined exclusively by the presence of a degree of capsulation observable by the India ink method.

Both virulent and avirulent organisms multiplied slowly when incubated with rotation in TMB pH 7.0 at 37° . With 3 hr. incubation both types increased their viable count by a factor of *ca.* 1.8 only.

The test for ability to develop resistance, or failure to do so, when incubated under precise conditions in TMB readily allowed us to differentiate our virulent and avirulent representative strains MP6 and TS. This test was applied to a

random selection of laboratory strains of *P. pestis* to confirm the generality, or otherwise, of this distinction between virulent and avirulent types. Organisms derived from the same suspensions as those used for the estimations of sensitivity to phagocytosis were injected intraperitoneally into mice, at levels of 10^4 organisms per mouse, a dose which was known from experience to differentiate clearly virulent from avirulent strains. The results are given in Table I.

TABLE I.—*Differentiation of Virulent from Avirulent Strains of Pasteurella pestis by Phagocytosis In Vitro.*

Experiment.	Strain.	Phagocytosis.*	Assessment by phagocytosis <i>in vitro.</i>	Virulence† for mice.
1	MP6	30 : 20	V	10
	TS	1522 : 99	AV	0
	A1122	1567 : 97	AV	0
	5341	1159 : 98	AV	0
	14	1842 : 98	AV	0
	I-72	119 : 52	V	8
	F9581	55 : 25	V	10
	L27	40 : 23	V	10
L36	47 : 20	V	10	
2	MP6	41 : 20	V	10
	TS	1541 : 94	AV	0
	Java	1883 : 100	AV	0
	Soemedang	1422 : 100	AV	0
	A2256 Ppl	1203 : 98	AV	0
	H327	49 : 21	V	10
	139L	43 : 19	V	10
	Yokohama	22 : 13	V	10
M1 (purine dependent)	51 : 22	V	0	

Organisms harvested from 18-hr. growths on tryptic digest meat agar at 28°, incubated with rotation for 3 hr. at 37° in tryptic digest meat broth pH 7.0 (2.5×10^8 organisms/ml.) and tested for sensitivity to phagocytosis *in vitro*.

* Phagocytosis recorded by numbers of organisms ingested by 100 polymorphs, followed by percentage of polymorphs containing ingested organisms.

† Virulence recorded by deaths out of 10 mice injected intraperitoneally with 10^4 organisms suspended in 0.2 ml. buffer.

V = virulent. AV = avirulent.

With the exception of strain M1, the assessments of virulence or of avirulence on the basis of this test *in vitro* agreed completely with the assessments based on animal assay. Strain M1 was derived from MP6 and is characterised by dependence on exogenously supplied purine for growth (Burrows and Bacon, 1954b). Its avirulence reflects the non-availability of purines in the mouse. In the presence of purines, *e.g.*, in TMB, this mutant shows all the properties of its virulent parent, including the ability to develop resistance to phagocytosis.

From time to time different batches of TMB either failed to permit virulent organisms to develop resistance or permitted the development of moderate resistance only. Certain of these batches were improved by the addition of glucose and/or cystine but others were not. These failures stimulated an attempt to define the minimal nutritional conditions necessary to permit the development of resistance by virulent organisms in synthetic media. Despite exhaustive trials no entirely satisfactory chemically defined medium was evolved which would

regularly permit MP6, previously grown at 28°, to develop a level of resistance comparable with that achieved in effective batches of TMB. A medium containing phosphate buffer, glucose, acetate, asparagine, cystine, methionine, phenylalanine, valine, leucine, isoleucine, proline, glutamic acid, spermine, haemin, biotin, pantothenate and magnesium salts, pH 7.0, at times permitted an impressive development of resistance, yet at other times, under what we believed to be identical conditions, failed to be effective.

As mentioned earlier, virulent organisms derived from TMA slopes incubated for 17 hr. at 37° showed widely varying degrees of resistance from day to day. This variation ranged from the high sensitivity of organisms recovered from growths at 28° to the high resistance achieved by such organisms following 3 hr. incubation in TMB at 37°. Organisms recovered in a sensitive condition from slopes grown at 37° rapidly became resistant on incubation in TMB at 37° and frequently, but not invariably, on incubation in phosphate buffer supplemented with ammonium salts, glucose and cystine pH 7.0 at 37°. The high sensitivity of organisms recovered from slopes incubated at 37° apparently resulted from previous exhaustion of glucose, or its equivalent, during the 17 hr. incubation period. Supplementation of these exhausted slopes with buffer plus glucose, followed by a further 2 hr. incubation at 37°, yielded organisms with considerably increased resistance. Supplementation with buffer alone was ineffective.

Growth of virulent organisms in TMB at 37° to population densities in excess of *ca.* 2×10^9 organisms per ml. yielded organisms with low resistance to phagocytosis. Supplementation of TMB with galactose (a sugar whose metabolism by *P. pestis*, unlike that of glucose, did not result in gross alteration of the pH of the medium) permitted the recovery of larger populations showing a high level of resistance. Fig. 4 illustrates this effect.

Virulent organisms did not achieve high resistance following incubation in CCY medium (Gladstone and Fildes, 1940) at pH 7.0 for 3 hr. at 37°. The efficiency of this medium was however considerably improved by the addition of glucose as illustrated in Table II.

TABLE II.—*Effect of Added Glucose on Development of Resistance to Phagocytosis by Virulent Organisms Incubated in CCY Medium for 3 hr.*

Medium.	Addition.	Temperature.	Phagocytosis.*
CCY	—	0°	1365 : 87
"	—	37°	589 : 82
"	Glucose 0.1%	"	181 : 58
"	" 0.5%	"	184 : 62
"	" 1.0%	"	127 : 50
"	" 1.0%	"	103 : 47
"	{ + cystine 200 μM	"	"
"	Cystine 200 μM	"	895 : 92
TMB	—	"	76 : 33

* Phagocytosis recorded by numbers of organisms ingested per 100 polymorphs, followed by per cent of polymorphs containing ingested organisms.

These observations are consistent with the view that the development of resistance is in part dependent on the free availability of a utilisable carbohydrate and that metabolism in the absence of free carbohydrate results in failure to develop resistance, or in loss of resistance previously acquired.

Persistence of the resistant state

As shown in Fig. 1, virulent organisms after acquiring resistance retain the property for at least a period of 7 hr. of continued incubation in TMB at 37°. When rendered resistant by incubation in TMB and stored in the refrigerator they retained resistance for 4 to 5 hr. and then rapidly became sensitive. Low

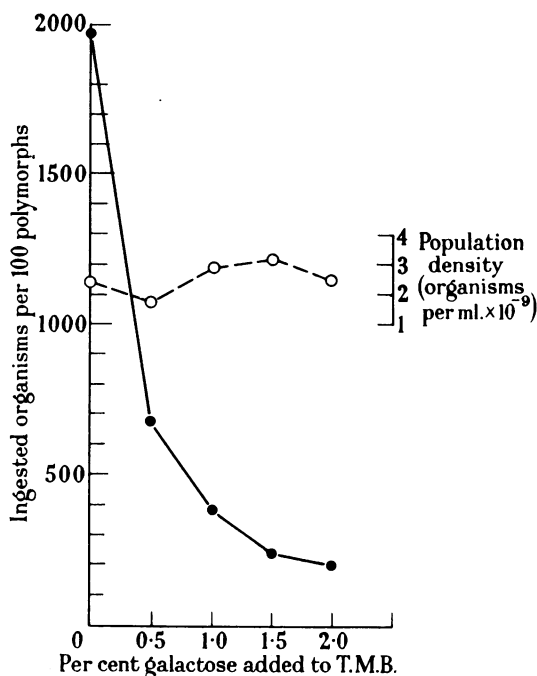


FIG. 4.—Effect of galactose on resistance to phagocytosis of large populations of virulent organisms. Tryptic digest meat broth pH 7.0 supplemented with galactose to the concentrations shown and inoculated with sensitive virulent organisms to a density of 2×10^8 organisms per ml. Incubated static for 11 hr. at 20°, followed by 6 hr. at 37° rotated. Volumes containing equal numbers of organisms centrifuged and organisms tested for resistance to phagocytosis.

concentrations of streptomycin (10 μ g./ml. final conc.) added to TMB completely suppressed the development of resistance by sensitive virulent organisms. However high levels of streptomycin (400 μ g./ml. final conc.) acting on resistant organisms for 1 hr. at 37° did not render them sensitive to phagocytosis. Similarly, treatment for 30 min. at 37° in M/10 KCNS—a reagent whose action results in solution of capsular material (Amies, 1951)—or with formalin or tannic acid, both at 0.5 per cent final conc., followed by two washings in saline, did not materially affect the resistance of virulent organisms. Treatments in saline followed by two washings in saline were used as controls. Thus neither agents acting as metabolism inhibitors, nor those which would be expected to react non-specifically with surface proteins, materially affected the resistance of virulent organisms once they had achieved a resistant state.

Production of polymorph-inhibiting factor(s) in vitro

Concurrently with the development of resistance by virulent organisms *in vivo* there is evidence that the phagocytic activity of polymorphs in infected mice becomes seriously impaired, rendering them incapable of ingesting a secondary infection with phagocytosis-sensitive avirulent organisms (Burrows and Bacon, 1954b). However *in vitro* the development of resistance by virulent organisms in TMB was not accompanied by the excretion into the medium of detectable amounts of a factor, or factors, which inhibited the ingestive capacity of mouse polymorphs. Similarly we failed to demonstrate the production of a free polymorph-inhibiting factor during the development of resistance by virulent organisms incubated in horse serum, under a number of conditions. These conditions included variation of O₂ and CO₂ tensions.

Successful demonstrations of the production of a polymorph-inhibiting factor *in vitro* have, to date, been confined to experiments in which mouse peritoneal

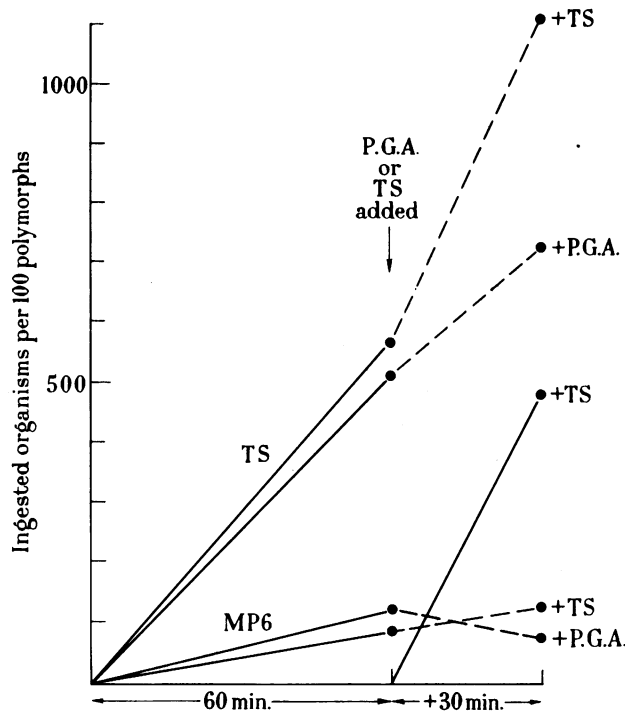


FIG. 5.—Inhibition of the phagocytic activity of mouse polymorphonuclear leucocytes following incubation with virulent organisms. Virulent MP6 and avirulent TS organisms grown at 28° then incubated with rotation 3 hr. at 37° in tryptic digest meat broth pH 7.0, sedimented and re-suspended in PGA to 1×10^9 organisms per ml. Polymorph suspension in PGA plus mouse peritoneal fluid distributed in 0.2 ml. volumes to 15 waxed tubes. To 3 was added 0.025 ml. PGA, to six 0.025 ml. TS suspension and to remaining six 0.025 ml. MP6 suspension. After rotation at 37° for 1 hr. and preparing films from each tube 0.025 ml. TS suspension was added to the three tubes originally receiving PGA, and to three of each of six tubes originally receiving TS or MP6 suspension. The remaining 3 of each series of 6 received 0.025 ml. PGA. All tubes then rotated at 37° for further 30 min. and films prepared from each and phagocytosis estimated.

fluid or heparinised normal mouse whole blood have been present in media in which virulent organisms were incubated at 37°.

An indirect demonstration of the production of inhibition by virulent organisms, and not by identically treated avirulent organisms, is given in Fig. 5. The experiment compared the ability to ingest TS organisms of polymorphs exposed to the products of virulent or of avirulent organisms, in a phagocytic system containing mouse peritoneal fluid.

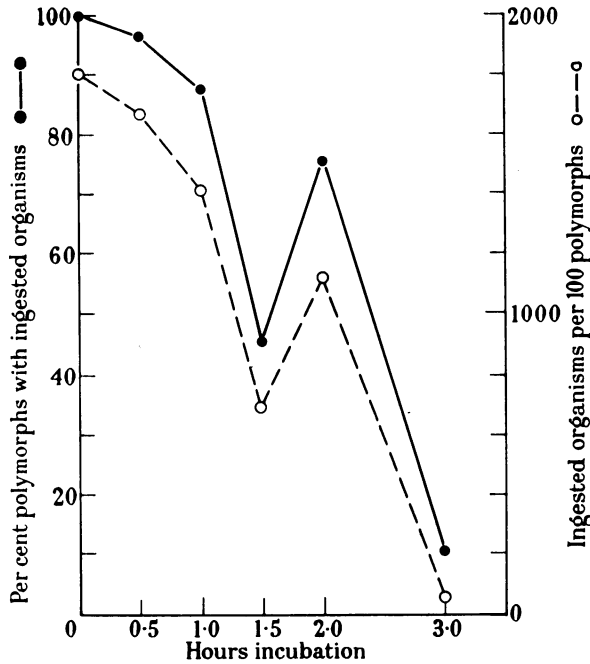


FIG. 6.—Production of polymorph-inhibiting factor(s) by virulent organisms incubated in mouse whole blood. Heparinised (1/6500 heparin final conc.) normal mouse whole blood inoculated with virulent organisms to 1×10^8 per ml. and incubated at 37° with rotation for times shown. Cultures centrifuged and 0.8 ml. supernatants added to 0.2 ml. of a suspension containing 1×10^8 polymorphs per ml. in PGA in waxed tubes. Tubes were then rotated for 2 hr. at 37°. From each tube three 0.2 ml. volumes transferred to waxed tubes, 0.05 ml. of a suspension of TS (5×10^9 /ml.) added to each; rotated 30 min. 37° films prepared and phagocytosis estimated.

It is evident that polymorphs incubated with resistant virulent organisms have their ability subsequently to ingest sensitive organisms very greatly reduced.

Fig. 6 gives the results of an experiment to estimate the effect of time of incubation on the production of a polymorph-inhibiting factor by virulent organisms incubated at 37° in heparinised whole mouse blood. The magnitude of this production varied in three experiments of this type but the peculiar inflection of the curve at the two-hour plot was a constant feature. Its significance is not understood.

We have no evidence that avirulent organisms at any time excrete a polymorph-inhibiting factor *in vivo* or *in vitro*. In contrast to peritoneal smears

derived from mice moribund from virulent infection, those from mice moribund from infection with avirulent organisms (2×10^8 per mouse) show the majority of polymorphs to contain large numbers of ingested organisms. It is unlikely that such ingestion would be observed if the large numbers of uningested organisms present were excreting, or had excreted, effective amounts of a polymorph-inhibiting factor.

Destruction of P. pestis following phagocytosis

Information as to whether or not phagocytosis of virulent organisms leads to their destruction was considered of importance in this study. Numerous observations of stained preparations during estimations of phagocytosis allow us to state that both ingested virulent and avirulent organisms stained less densely than did uningested organisms and, with further time, became rounded and swollen and eventually unrecognisable. Again, in experiments *in vivo*, the high ingestion of sensitive virulent organisms observed immediately following injection progressively declined to become practically zero within a 5-hr. period (Burrows and Bacon, 1954*b*). If ingested organisms remained stainable and recognisable this decline would not have been observed, unless, during this period, a complete replacement of the polymorphs originally present by fresh elements had occurred. In closed phagocytic systems *in vitro*, where such replacement cannot occur, a decline in phagocytosis with time was repeatedly observed. Indirect evidence therefore is in support of the destruction of organisms following phagocytosis.

As a direct approach to this problem the following experiments were conducted. Sensitive virulent organisms were added to a suspension containing known numbers of polymorphs and phagocytosis allowed to proceed. The polymorphs were sedimented and washed twice with PGA and numbers of ingested organisms determined on a small sample. The remainder were re-suspended in PGA to known concentration and divided into two parts. To one was added normal rabbit serum, to the other an equal volume of an antiserum prepared in rabbits against mouse polymorphs, and both incubated 1 hr. at 37°. This antiserum caused the lysis of mouse polymorphs with the liberation of their contents but did not affect the viability of virulent organisms.

Equal volumes of the two suspensions were then plated on TMA containing sodium metabisulphite at 0.075 per cent final conc. and incubated for 48 hr. at 37°. This medium was effective for the growth of *P. pestis* to colony size from single organisms. If organisms remained viable after ingestion each polymorph containing one or more ingested organisms should have given rise to one colony (normal serum treated) and each ingested organism liberated by antiserum treatment similarly should have produced one colony. The results are given in Table III.

In other experiments the lethal effects on mice of known numbers of virulent organisms, contained intracellularly in mouse polymorphs, were compared with those of extracellular organisms. To sterilise organisms adhering to polymorph surfaces the polymorphs containing ingested organisms were incubated with streptomycin, an antibiotic to which the polymorph cell membrane apparently is impermeable (Schaffer, Kucera and Spink, 1953). The results indicated that for lethal effects equivalent to those of 150 extracellular organisms, or to 150 extracellular organisms surviving streptomycin treatment, some 26,000 ingested

TABLE III.—*Destruction of Virulent Organisms by Phagocytosis*

		Expt. 1.*	Expt. 2.†
Normal serum treated	{ Number of colonies expected if each polymorph with ingested organisms contains at least one viable organism	114	152
	{ Colonies obtained	58	92
Lytic serum treated	{ Number of colonies expected if all ingested organisms were liberated and viable	1631	3488
	{ Colonies obtained	77	52

* Expt. 1.—983 organisms ingested per 100 polymorphs ; 69 per cent polymorphs contained ingested organisms.

† Expt. 2.—2089 organisms ingested per 100 polymorphs ; 91 per cent polymorphs contained ingested organisms.

Polymorph suspensions diluted after treatment so that plated volumes contained 166 whole or disrupted polymorphs.

virulent cells were necessary. These results support the conclusion that phagocytosed virulent organisms are rendered non-viable.

DISCUSSION

The progress of this work has been hampered by the difficulty of obtaining reproducible experimental results from day to day. In addition, experiments which in the past were readily confirmed, have, when repeated many months later, failed to yield results in accordance with earlier findings. As a result much experimental work has had to be omitted from this paper. The behaviour of *P. pestis in vitro* appears to be greatly influenced by subtle changes in the environment which at present we cannot completely define. As an example, when the work was started we were able consistently to convert sensitive virulent organisms, taken from growths at 28°, to the resistant state by incubation at 37° in a medium containing phosphate, ammonium salts, glucose and cystine. This effect was sufficiently reproducible to permit titration of optimal levels of cystine for this development. Maximum resistance developed in 3 hr. over the range 25–400 μM cystine ; levels between 400–1600 μM were less effective, and above 1600 μM was inhibitory. The same experiments repeated a year later failed to yield resistant organisms at any cystine level. The possibility of genetic variation of our organisms maintained in the dried state cannot be excluded, neither can the possible selection of different substrains during the growth of stock suspensions. These latter however were all routinely checked for virulence for mice, and for other properties before being brought into use, but variations in virulence or other properties were not detected.

The ability of virulent organisms to develop resistance to phagocytosis under defined conditions *in vitro*, and under the conditions existing *in vivo* clearly distinguishes them from avirulent laboratory strains of *P. pestis*. The suppression of the development of resistance by streptomycin indicates that active metabolism of the virulent organism is essential for this process. This supports the conclusions from earlier studies on the behaviour of the purine-dependent mutant M1 *in vivo*. Further, metabolism in the presence of an available carbo-

hydrate at temperatures close to 37° are necessary conditions for the expression of the potentiality for the development of resistance.

Present evidence does not permit us to decide whether or not the factor determining resistance to phagocytosis is identical with that causing impairment of the phagocytic activity of mouse polymorphonuclear cells. While resistance can be achieved in a variety of media, a free phagocytosis-inhibiting factor has only been demonstrated in media containing mouse fluids. This suggests that resistance and inhibition are independent phenomena. However, if we assume that an excreted factor protects resistant organisms from phagocytosis, and if in sufficient quantity can impair the ability of polymorphs to phagocytose sensitive organisms, our findings could be explained on the basis of the differing quantitative production of this factor in media with and without added mouse fluids; or on equal production in both but with instability in the absence of the latter. Provided a sufficiency of the factor was produced to give maximum resistance, amounts of the factor in excess would make no contribution in our tests for resistance, but would be detectable in a test for inhibition of phagocytosis. We are now developing new techniques which may permit a decision to be made as to the identity or otherwise of resistance factor(s) and polymorph-inhibiting factor(s).

Our experiments similarly do not give unequivocal evidence supporting the view that virulent organisms in the sensitive and resistant conditions differ in their surface properties rather than in their excreted soluble metabolic products. Neither do they tell us whether the important differences between resistant virulent and avirulent organisms, which can be detected by the tests described in this paper, are associated with antigenic differences hitherto not recognised. Studies which indicate that virulent organisms in the resistant condition do in fact possess an antigen not evident in sensitive virulent, or avirulent, organisms will be reported later.

SUMMARY

Virulent strains of *Pasteurella pestis* can be differentiated from protective avirulent strains by phagocytic tests *in vitro*. When grown on agar slopes at 28° both strains are highly sensitive to phagocytosis by mouse polymorphonuclear leucocytes. Virulent organisms grown at 28° and then incubated for 3 hr. in tryptic digest meat broth at 37° change to a condition in which they are highly resistant to phagocytosis. Avirulent organisms treated identically retain their high sensitivity to phagocytosis. The degree of aeration, pH of the medium, time and temperature of incubation and availability of a carbohydrate source are factors determining the suitability of media to permit the change of virulent organisms from the sensitive to the resistant state. In media containing mouse peritoneal fluid or mouse whole blood virulent organisms produce a factor(s) which inhibits the phagocytic activity of mouse polymorphonuclear leucocytes. Evidence is given that phagocytosed virulent organisms are rendered non-viable.

We gratefully acknowledge the interest and advice of Dr. D. W. Henderson throughout this work. We also wish to express our appreciation of the excellent technical assistance rendered by Miss Barbara Alkins, Mrs. Corinne Ness and Miss Dorothy Burrell.

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