

## STUDIES IN THE ENZYME MAKE UP OF *PASTEURELLA PESTIS*.

### Part II.

#### PHOSPHATASE ACTIVITY OF VIRULENT AND AVIRULENT STRAINS.

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BARBER AND KAUPER (1951) demonstrated a correlation between the phosphatase activity and virulence in *Staphylococcus pyogenes* where the avirulent strains, as distinguished from virulent ones, were coagulase (Chapman *et al.*, 1934) and phosphatase negative. Phosphatase activity in relation to antigenicity in *Corynebacterium diphtheriae* and other diphtheroids was studied by Bray (1944). Recently, Shrivastava *et al.*, (1954) have reported the phosphatase activity in relation to antigenicity and virulence in *Salmonella typhosa*, using sodium  $\beta$ -glycerophosphate as the substrate. They have shown that with the enhancement of virulence after mouse passage, according to the method of Perry *et al.* (1933), the phosphatase activity increases.

In view of these reports, it was thought of interest to study this enzyme in a few virulent and avirulent strains of *P. pestis* under closely comparable conditions. The results obtained are reported in the present communication.

#### METHODS AND MATERIALS.

The strains were the same as described earlier (Sagar *et al.*, 1956). The cell suspension was obtained by harvesting the 48 hour growth on papain agar at

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28°C. For inoculum, the cultures were initially grown for 24 hours on the same medium. The cells were washed once with normal saline and re-suspended to a turbidity of 25 per cent transmission, in a solution containing equal volumes of saline and veronal buffer (Michaelis, 1931), in a Lumetron photo-electric colorimeter Model A, using red filter, 650 m $\mu$ .

The technique for the enzyme assay was that of Bodansky (1932, 1933) with modifications permitting the use of Fiske and Subbarow (1925) reagents. The reaction mixture consisted of 1 c.c. of the standard cell suspension and 2.5 c.c. of the buffered substrate prepared by diluting 1 per cent sodium  $\beta$ -glycerophosphate (BDH) with an equal volume of veronal buffer, pH 8.0, unless otherwise mentioned. After incubation the reaction was stopped by the addition of 0.5 c.c. of 30 per cent trichlor-acetic acid. The precipitated proteins were centrifuged off and 2 c.c. aliquot from the supernatant was used to develop the colour, which was read in the Klett Summerson photo-electric colorimeter, using red filter (660 m $\mu$ ). A control, in which the reaction was stopped immediately after the addition of the enzyme, was run simultaneously. The phosphatase activity was finally expressed in terms of  $\mu$ g. of inorganic phosphorus released in the total quantity (4 c.c.) of the reaction mixture.

#### RESULTS.

Since, in the recent studies on the deaminases of *P. pestis* (Sagar *et al.*, *loc. cit.*), the medium was found to have a marked effect on the enzyme production, a similar experiment was carried out to compare the phosphatase activities of the cells harvested from three different agar media and their respective broths. The results are reported in Table I for one each of virulent and avirulent strains. The data indicate that the best phosphatase activity was shown by the cells grown on either papain or tryptic casein digest agar. The cells harvested from heart infusion agar were significantly poor in phosphatase. Further, in contrast to the deaminase activity, the cells grown on the solid medium were consistently found to have more

TABLE I.

*Effect of medium on the phosphatase activity of Pasteurella pestis.*

Media.	$\mu$ G. OF INORGANIC PHOSPHORUS RELEASED.	
	Virulent 5/P.	Avirulent 53/H.
Tryptic casein digest agar ...	6.0	5.7
Tryptic casein digest broth ...	5.1	5.1
Beef heart infusion agar ...	3.1	2.8
Beef heart infusion broth ...	3.1	2.4
Meat papain agar ...	6.3	6.0
Meat papain broth ...	5.4	5.4

phosphatase than those grown in the liquid medium. Papain agar was, therefore, selected for the growth of the cultures.

*Effect of pH and temperature on the phosphatase activity.*—For a study of the effect of pH, 1 c.c. of the cell suspension was added to 2.5 c.c. of the buffered substrate of appropriate pH, ranging from 5.0 to 9.5, and the amount of inorganic phosphorus released in 2 hours was estimated as usual. The values for 2 strains, virulent 124/P and avirulent 53/H, have been plotted in Graph 1A. These curves show a gradual rise in the phosphatase activity up to pH 8.0 and a subsequent steep fall indicating that the optimum pH for this enzyme was at 8.0 for both the strains. Another experiment, in which the pH was kept at 8.0, and the incubation temperature varied from 27°C. to 47°C., was carried out to determine the effect of temperature. From the results in Graph 1B, it would be seen that the optimum temperature for this enzyme was at 37.5°C., which was identical with that observed for deaminase (Sagar *et al.*, *loc. cit.*). The fall in activity beyond that temperature was, however, not so abrupt.

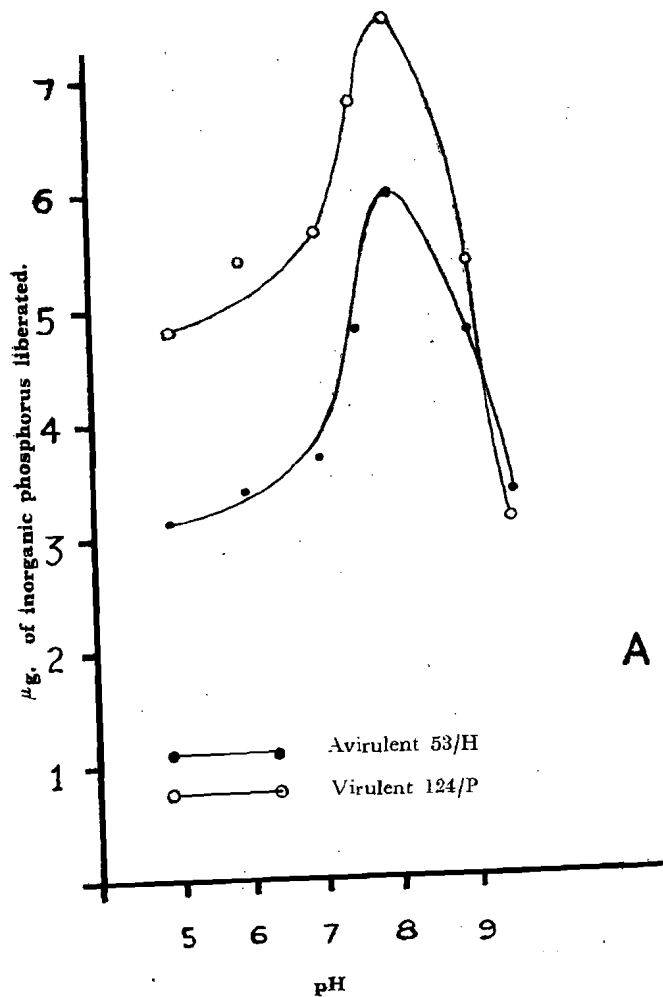
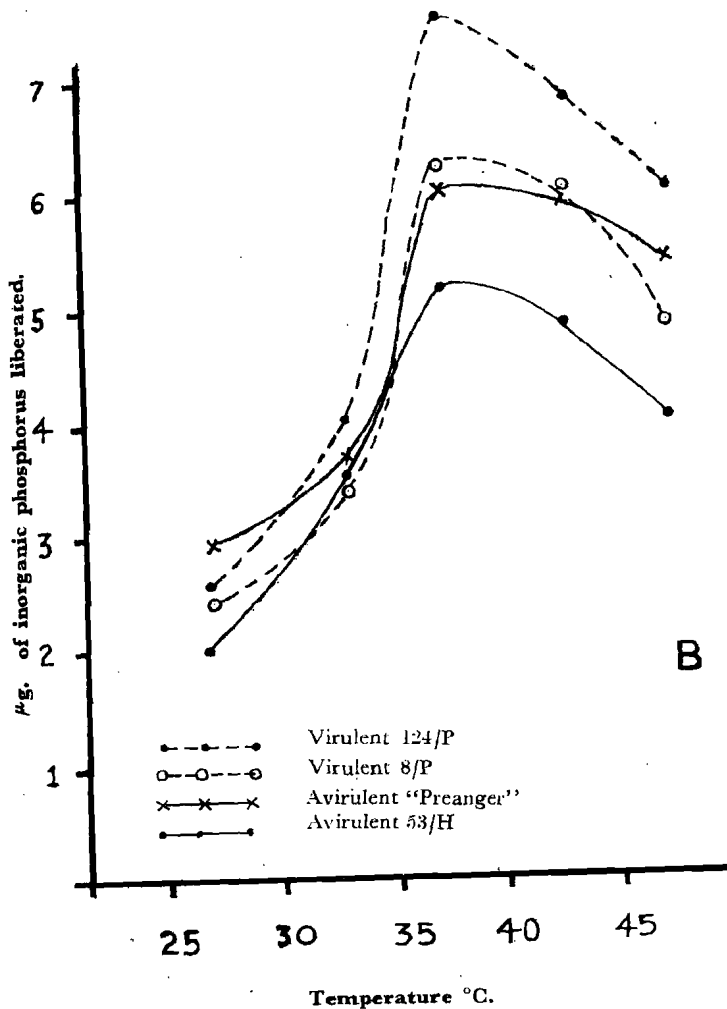
*Phosphatase activity in virulent and avirulent strains of P. pestis.*—The amount of inorganic phosphorus liberated by 9 virulent and 8 avirulent strains of *P. pestis* in 2 hours is shown in Table II. It would be clear that all the strains were capable of dephosphorylating  $\beta$ -glycerophosphate and the activity of the enzyme, as represented by the amount of inorganic phosphorus released, varied from 2.8  $\mu$ g. to 7.5  $\mu$ g. Exception was noted in one of the strains, avirulent I, which gave an abnormally high phosphatase activity; the inorganic phosphorus released being 72.0  $\mu$ g. These data do not, however, reveal any general relationship between virulence and phosphatase activity.

TABLE II.

*Alkaline phosphatase activity of certain virulent and avirulent strains of Pasteurella pestis.*

Cultures (virulent).	$\mu$ g. of inorganic phosphorus released.	Cultures (avirulent).	$\mu$ g. of inorganic phosphorus released.
Fukeim China ...	4.8	53/H	5.4
Q ...	3.4	Massland	5.7
327/L ...	5.4	P	2.8
120/5H ...	7.5	P.M.	2.8
138/P .....	7.2	Tjiwidej rough	6.4
Vimla Bai ...	4.4	Preanger	6.8
8/P ...	5.7	Somadang	4.0
124/P ...	6.8	i	72.0
195/P ...	7.1	...	...

## GRAPH.

A. Effect of pH on the phosphatase activity of *P. pestis*.B. Effect of temperature on the phosphatase activity of *P. pestis*.

The progress of hydrolysis of  $\beta$ -glycerophosphate by *P. pestis*.—With a view to study the difference, if any, between the rate of hydrolysis of the  $\beta$ -glycerophosphate by the virulent and avirulent strains of *P. pestis*, the phosphatase activity was followed up to a period of 5.5 hours by 4 virulent and 4 avirulent strains and the results are presented in Table III. Adequate volumes of the reaction mixture (pH 8.0) were prepared in 250 c.c. flasks in the same proportion as mentioned earlier in this paper. Aliquots of 3.5 c.c. were pipetted out at different intervals in 0.5 c.c. of 30 per cent trichloro-acetic acid. The data in Table III show that the amount of inorganic phosphorus increased up to 4½ hours and remained almost steady thereafter. Here again, no correlation between virulence and dephosphorylation was noticeable.

TABLE III.

Progress of hydrolysis of  $\beta$ -glycerophosphate by virulent and avirulent strains of *Pasteurella pestis*.

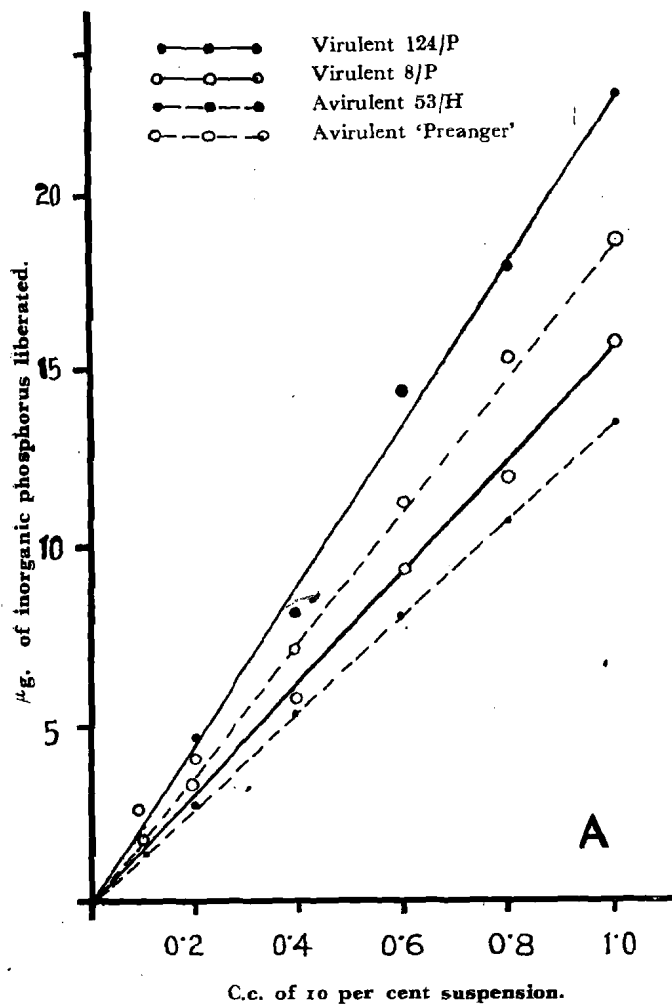
Strains.	µ C. OF INORGANIC PHOSPHORUS RELEASED:								
	½ hr.	1 hr.	2 hrs.	3 hrs.	3½ hrs.	4 hrs.	4½ hrs.	5 hrs.	5½ hrs.
Virulent 124/P ...	1.7	3.7	7.5	10.4	12.4	14.0	14.7	15.4	15.7
Virulent 8/P ...	2.0	3.4	6.8	8.6	10.8	12.0	13.7	14.4	14.4
Virulent 138/P ...	1.0	3.7	7.2	9.4	11.1	12.0	12.8	12.8	12.7
Virulent 327/L ...	1.0	3.1	5.7	8.6	10.8	11.7	12.6	12.7	13.0
Avirulent 53/H ...	0.70	3.4	5.1	7.0	8.3	8.9	9.7	10.0	10.0
Avirulent Preanger	1.4	2.8	6.4	8.9	10.4	12.0	13.4	14.8	14.8
Avirulent Massland	1.4	3.4	6.8	8.9	10.0	10.8	11.7	12.4	12.4
Avirulent Tjiwidej rough	1.4	2.8	6.0	8.6	10.2	11.1	12.9	12.9	13.1

Effect of the concentration of the enzymes and the substrate on the hydrolysis of sodium  $\beta$ -glycerophosphate by *P. pestis*.—A study of the enzyme concentration and phosphatase activity was made by adjusting the turbidity of the cell suspension to 10 per cent transmission and adding different quantities of this suspension (0.1 c.c. to 1.0 c.c.) to 2.5 c.c. of the buffered substrate, the final volume in each case being made to 3.5 c.c. with buffered saline. The phosphatase activity of two virulent and two avirulent strains in 2 hour has been plotted in Graph 2A against the amount of cell suspension. It would be seen that under the conditions of the experiment and the range of the enzyme concentration, the quantity of inorganic phosphorus released was directly proportional to the enzyme concentration.

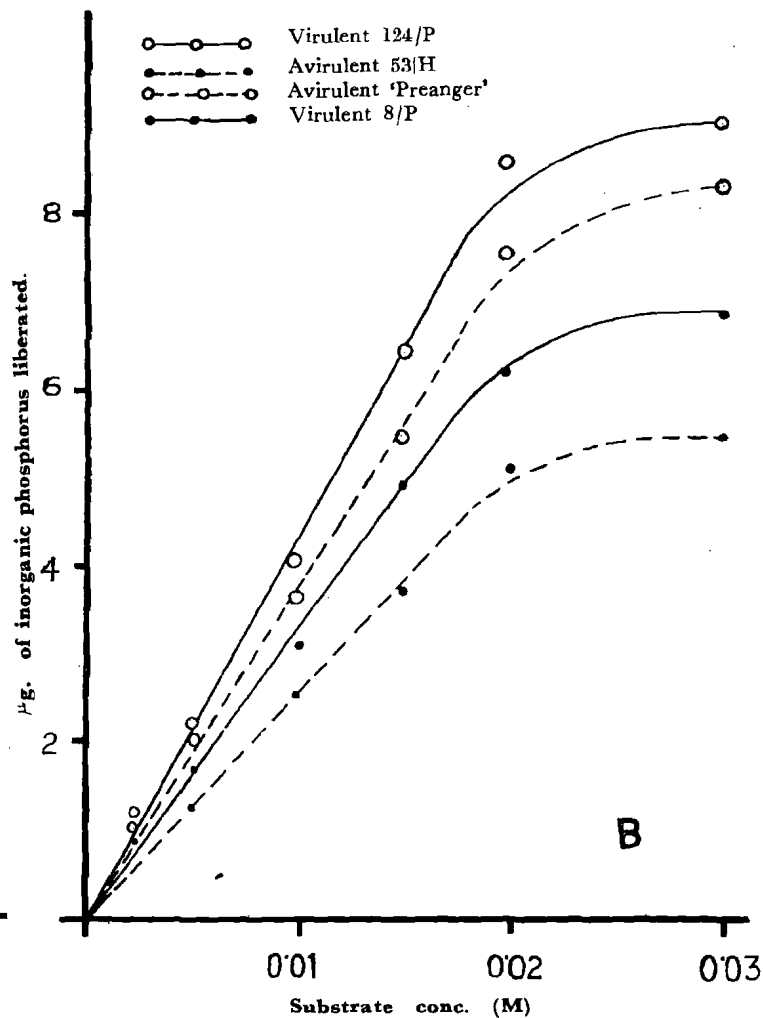
The phosphatase activity with respect to the substrate concentration was studied by incubating 2.5 c.c. of the buffered substrate of different molarity with 1 c.c. of the standard cell suspension and estimating the amount of inorganic phosphorus released in 2 hours as usual. The results for 4 cultures (2 virulent and 2 avirulent) are plotted in Graph 2B. It may be seen that the phosphatase activity

GRAPH 2.

A. Effect of the enzyme concentration on the phosphatase activity of *P. pestis*.



B. Effect of the substrate concentration on the phosphatase activity of *P. pestis*.



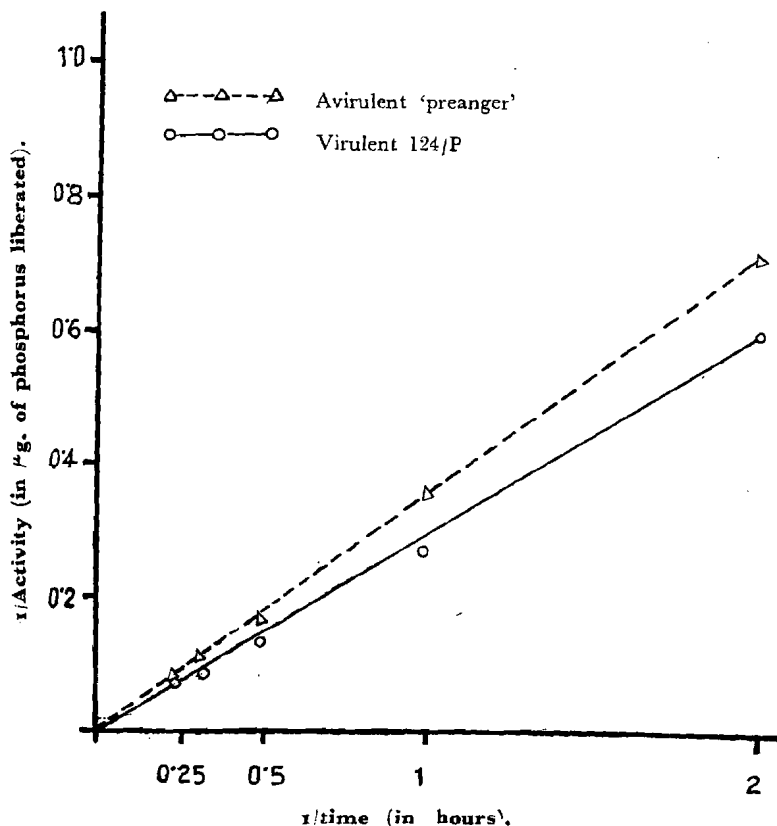
is directly proportional to substrate concentration up to 0.02 mols. after which it attains a constant value and becomes more or less independent of the substrate molarity.

## DISCUSSION.

A comparative study of the phosphatase activity of 9 virulent and 8 avirulent strains of *P. pestis* showed that all the cultures were capable of dephosphorylating  $\beta$ -glycerophosphate to an appreciable extent and that the amount of inorganic phosphorus released in 2 hours varied between 2.8  $\mu$ g. and 7.5  $\mu$ g. except for one of the strain, avirulent I, which gave an abnormally high phosphatase activity. The purity of this culture was confirmed by Gore's method, described by Taylor (1933). It is difficult to account for such an abnormal behaviour, but it may be mentioned that as compared to other strains, this one showed a much higher deaminase activity with serine (Sagar *et al.*, *loc. cit.*).

GRAPH 3.

Verification of inverse law for bimolecular reaction.



The optimum temperature and pH for this enzyme were found to be 37.5°C. and 8.0, respectively. Since there was no tendency in the hydrolysis to rise at pH below 7, the acid phosphatases are presumably between this organism.

Application of the rate equations to the velocity of  $\beta$ -glycerophosphate hydrolysis showed that the reciprocals of time when plotted against the reciprocals of activity (Graph 3), gave a straight line. This would indicate the reaction to be of the second order.

Taking into account the various factors and the kinetics of the enzyme, it may be concluded that there is no general correlation between the phosphatase activity and virulence in *P. pestis*.

#### SUMMARY.

1. The medium for the growth of the cells had a significant effect on the enzyme production and the best activity was observed when *P. pestis* was grown on either meat papain or casein tryptic digest agar.
2. Screening of phosphatase activity, using  $\beta$ -glycerophosphate as the substrate, with 9 virulent and 8 avirulent strains of *P. pestis* showed no correlation between the enzymic activity and virulence of the organism.
3. The optimum pH and temperature were found to be 8.0 and 37.5 °C., respectively, for both virulent and avirulent strains.
4. The hydrolysis of  $\beta$ -glycerophosphate was found to be bimolecular.

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