STUDIES ON THE SEMI-SYNTHETIC MEDIA FOR THE

*Pasteurella pestis*1ST REPORT: ON THE NUTRITIONAL REQUIREMENT
OF *Past. pestis*BY HIDETAKE YAOI*, KAMESABURO, YOSHINO
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In 1939 Rao (1) reported that *Past. pestis* could grow on media composed merely of hydrolysed gelatin, cystine and inorganic salts. Further, he specified, using chemically defined amino-acids media, proline, phenylalanine and cystine as indispensable substances and glycine as growth-promoting substance. In 1940 Rao (2) reported that hematin promoted the growth of *Past. pestis* sown in a chemically defined amino-acids media. Cozymase, nicotinic acid, thiamin also possessed similar but less activity.

As Rao's observation has been depended on a subculture only, to which an amount of carefully washed bacterial cells were sown, we attempted to find out the possible variations in the growth during the repeated subcultures and obtained some newer informations, which will be described in the following lines.

EXPERIMENTAL

Experiment 1. On the variations in the growth-rate following the repeated subcultures.

(a) Experimental methods.

The recipe of the basic media used was as follows:

Hydrolysed casein, prepared after the method of Mueller (3).

KH_2PO_4 0.5 g

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g

NaCl 5.0 g

Na-citrate 4.0 g

Cystine (1% soln. in 1:40 HCl) ...1 cc

made up to 1,000 cc with distilled water.

The amount of hydrolysed casein to be used was regulated so as to make the finished culture-media containing nitrogen in 1.0 mg per cc. The mixtures were adjusted to pH 7.0 with normal NaOH solution, filtered through filter-paper and then dispensed in 2 cc amounts in small test tubes and sterilized at 120°C for 20 minutes.

The reaction of the sterilized media was invariably found to be around pH 6.6, and this pH value was conveniently used as the initial pH of the media for experimental

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use, because it has been confirmed by a preliminary experiment that the optimal pH for the transplantation to be 6.6—7.8, and the media tended to shift to alkaline reaction following the bacterial growth, and moreover, it was found that the bacteria grew most rapidly whenever the media had the initial pH of 6.6.

Bacterial strains: The following strains of plague, from the collection at this Institute, were used in the nutrition experiments:

Virulent strains: Yokohama-strain and Kuma-strain.

Avirulent strains: Tj-strain, M II 40-strain and EV-strain.

Of these strains, two of Yokohama and Tj strains belonged to the southern-type, and 3 of Kuma, M II 40 and EV belonged to the northern-type of strains.

Inoculation: As an inoculum, the growth on the plain agar slant cultivated at 31°C for 18 hours as scraped off with a platinum-loop and, after being washed once with saline solution, was suspended in normal saline in the ratio of 0.2 mg per cc.

This bacterial suspension was inoculated 0.1 cc each into 2 cc amounts media and incubated at 31°C. The cultures were shaken by hand every 24 hours. With each strain, 5 subcultures were made. The transplantation to the next culture media was made every 5—7 days, with one loopful growth each into new media.

Then the observations were made on 7 successive generations.

(b) Experimental results.

Following the growth of bacteria first pellicles appeared on the surface of culture-media and next with the growing of its thickness turbidity in the whole media also increased. The pellicles growing up and down along the wall of test tube, bacterial deposits were formed at the bottom of test tubes. With the formation of clot, the growth became heavier and heavier until at last it gained the state incapable of making homogeneous suspensions upon shaking by hand. After 2 weeks, pellicles were formed no more, turbidity decreased, showing mainly the bacterial deposit at the bottom of the test tube. For all, any bacterial growth was no more seen. The reaction of the culture media at this time was found to be around pH 8.4.

Table 1.

Strain	Yokohama					M II 40				
	1	2	3	4	5	1	2	3	4	5
Day										
Subculture										
1st.	++	+++	+++	+++	+++	++	++	+++	+++	+++
2nd.	+	++	++	++	+++	++	++	+++	+++	+++
3rd.	±	+	++	++	+++	++	++	+++	+++	+++
4th.	±	+	++	+++	+++	++	++	+++	+++	+++
5th.	—	±	+	++	+++	++	++	+++	+++	+++
6th.	—	±	+	++	+++	++	++	+++	+++	+++
7th.	—	±	++	++	+++	++	++	+++	+++	+++

When 5 test tubes for the same test show different growth, for instance ++ & +, it was recorded as ++.

To conclude, appearance of bacterial growth and variation of pH of media, caused by the *Past. pestis* grown in the semi-synthetic media used were on the whole the same with observable in the ordinary broth-cultures.

Observation on the growth-rate: The rate of growth was observed every 24 hours and recorded with the following set of relative values: —, ±, +, ++, ##, ###.

As seen in Table 1, 1) in case of the avirulent strain any lag of the growth-rate was not noticeable through every generations of subculture. On the other hand, 2) in case of the virulent strain a gradual fall in the growth-rate was seen up to the 5th generation at least, becoming rather stationary at the 5th to 7th generations.

Making 5 subcultures for each strains, the above divergent tendencies between 2 different strains were uniformly observed.

(c) Discussion.

Although Rao stated that both virulent and avirulent strains had more or less identical nutritional requirements, we were able to detect a marked difference between 2 different strains.

Namely, though, it may seemed unnecessary for *Past. pestis* any special growth factor as the requisite nutritive element, because both strains are able to grow in the semi-synthetic media composed of amino-acids and inorganic salts, the fact that the virulent strains become to grow very slowly following the number of subcultures, differing from the avirulent strains, seems to indicate the existence of the fundamental difference in the nutritional requirements between 2 different strains.

Experiment 2. On the growth-promoting factors.

When we transferred the organisms from 6th to 7th generation, the effect of some growth factors were tested as follows.

Experimental method.

The identical basic media as used in the previous experiment, were each added with;

- 1) Hematin, prepared from hemoglobin (Takeda) in 1 γ /cc:
- 2) Cozymase, purified from yeast (Sankyo) after the method of Williamson and Green (5), in 0.75 γ /cc.:
- 3) Nicotinic acid, purified at the Chemical Laboratory of the N. I. H., in 1 γ /cc.;
- 4) Thiamin (Sankyo) in 10 γ /cc:
- 5) Pyridoxin (Sonne-Board) in 10 γ /cc: and
- 6) Yeast dialysate, prepared from yeast (Sankyo) after the method of Cohen and Wheeler (4) in 5%.

The above substances were used either in separate or combined, but the doses or concentrations were the same in either cases. As an inoculum, one loopful from the 6th subculture on the 5th day, was first inoculated into 1 cc of the basic media, from which one loopful each was transplanted into the media upon test. Thus the dose of inoculum was reduced to make the efficacy of the above substances more distinctly detectable. Virulent strain only was used in this experiment. Five subcultures for

each test-culture were made. The temperature of incubation and the recording way of result are identical with the previous experiments.

Table 2.

Day	1	2	3	3	5	6
Medium						
Basal medium	—	—	±	+	++	+++
" + Yeast dialysate	—	±	++	+++	+++	+++
" + Hematin	—	—	++	++	+++	+++
" + Cozymase	—	—	++	+++	+++	+++
" + Nicotinic acid	—	+	+	++	+++	+++
" + Thiamin	—	—	++	+++	+++	+++
" + Pyridoxin	—	+	++	+++	+++	+++
" + Hem. + Cozymase	—	—	++	++	+++	+++
" + Hem. + Nic. ac.	—	—	++	+++	+++	+++
" + Hem. + Thiamin	—	+	++	+++	+++	+++
" Hem. + Pyridoxin	—	+	++	++	+++	+++

As seen in Table 2, all substances tested almost equally well accelerated the growth of plague bacillus. Nicotinic acid only was found to be less effective than the others.

Moreover, the substances tested seem though not indispensable, but essential for the luxuriant growth of the virulent strain of *Past. pestis*.

CONCLUSION

1. The nutritional requirements of *Past. pestis* with reference to the virulent and avirulent strains have been studied.

2. *Pasteurella pestis*, both virulent and avirulent strains are capable of growing in a semi-synthetic media composed of amino acids and some inorganic salts, unaided by other special growth factors.

3. Avirulent strains of *Past. pestis* show no retardation in the growing during 7 subcultures, but virulent strains show apparently the gradual lag through 5 generations, at least.

4. Hematin, cozymase, thiamin, piridoxin and yeast-dialysate are almost equally capable of promoting the growth of virulent *Past. pestis*. Nicotinic acid also facilitates the growth but seems to be less effective than the others.

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