that for pregnancy or non-pregnancy. 3. Threonine, histidine, lysine and tryptophan showed the greatest changes from one phase of the reproductive cycle to another. 4. While trends of amino acid excretion were similar from one pregnancy to another, the quantities excreted in relation to duration of gestation were different.

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Production of Protective Antigens by *Pasteurella pestis* in a Synthetic Medium. (21024)

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Wright *et al.*(1) have reported the production of immunogenic substances by *Bacillus anthracis* in a synthetic medium. It is the purpose of this note to report preliminary observations of a similar nature with *Pasteurella pestis*.

Materials and methods. The avirulent strain A-12 of *P. pestis*, a single colony isolate of strain A-1122 of Jawetz and Meyer(2), was cultivated in a synthetic medium based on that developed by one of us (K.H.). The formula for the medium is given in Table I. The amino acids were dissolved in the salt solution by heating and, after cooling, the mixture was adjusted to pH 7.5 with 10 N NaOH. The medium was distributed in 234 ml amounts into 2.8 l Fernbach flasks and

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TABLE I. Formula for Synthetic Medium Used for Production of Immunogenic Substances by *Pasteurella pestis*, Strain A-12.

Ingredient	g/liter	Ingredient	g/liter
l glutamic acid	12.0	K_2HPO_4	2.2
dl phenylalanine	.8	KH_2PO_4	1.7
dl methionine	.48	$MgSO_4 \cdot 7\ H_2O$	4.9
dl valine	1.60	Na gluconate	2.2
dl leucine	.4	$FeSO_4 \cdot 7\ H_2O$	.0028
dl lysine HCl	.4	$MnSO_4 \cdot H_2O$	.0017
l proline	.8	$NaHCO_3$†	2.5
dl threonine	.32	thiamine HCl‡	.001
l cysteine HCl	.1	Ca pantothenate‡	.001
glycine	.1	biotin‡	.0005
dl isoleucine	.62	distilled water	1 liter
xylose*	5.		

* Prepared as a 25% solution and sterilized at 121°C for 15 min. Added to the medium before inoculation.

† Prepared as a 6.25% solution and sterilized by filtration through a sintered glass filter. Added to the medium before inoculation.

‡ Prepared as a stock solution containing 0.25 mg/ml each of thiamine HCl and Ca pantothenate and 0.125 mg/ml of biotin. Sterilized by filtration through a sintered glass filter. Added to the medium before use.

autoclaved at 121°C for 15 minutes. Immediately before use xylose, the solution of vitamins, and sodium bicarbonate were added aseptically. A 24-hour culture of the strain A-12 of *P. pestis* grown on Bacto blood agar base at 26°C was suspended in 0.06 M phosphate buffer (pH 7.2). The synthetic medium inoculated with 2.5 ml of this suspension which contained approximately 1 x 10^9 cells per ml, was incubated at 37°C on a reciprocating shaker for 24 to 96 hours. During growth the pH was adjusted as required to 7.0-7.2 by the addition of either 2 N HCl or 2 N NaOH. Five ml of 25% xylose added twice daily also served to control the reaction. Initially the pH of the culture rose to 8.2-8.5 but after the first adjustment it generally remained near neutrality for at least 96 hours. After incubation the cultures were centrifuged at about 15,000 G for 30 minutes in a Servall SS-2 centrifuge at approximately 10°C. The supernatant was filtered through a sintered glass candle of UF porosity and to 50 parts of filtrate was added one part of sterile 5.0% potassium aluminum sulfate. The mixture, adjusted to pH 6.0 to facilitate precipitation, was left at 4°C overnight and the precipitate collected by centrifugation was washed 3 times with sterile 0.85% saline solution and finally resuspended in a volume of saline (containing 1:5000 merthiolate) equal to 1/50 or 1/25 the original amount of filtrate. To test the immunogenicity of the alum adsorbed antigen, various amounts of the material were inoculated into each of a group of 10 mice; each animal of a group received only one dose. One week after immunization the mice were challenged intraperitoneally with approximately 500 cells (100 LD_{50}) of

TABLE II. Protective Effect of Filtrates Obtained from Cultures of *Pasteurella pestis* Strain A-12, Grown in a Synthetic Medium when Injected into Mice (Namru strain).

Culture No.	Culture Hr incubation	ml inj.*	Survival out of 10	ED_{50},† ml
1.1	48, aerated	10.3	9§	
1.2	48, static	9	0	
2.0	24, aerated	10	1	
		2	1	
		.4	0	
3.2	88, "	10	10	
		2	8	
		.4	9	
3.1	72, "	10	10	
3.3	48, "	10	6	5.0
		2	2	
		.4	3	
4.1	92, aerated, containing .01 M $MgSO_4$ and .01 M Na-citrate‡	10	8‖	
		2	9	
		.4	9	
4.3	88 (*idem*)	7	10	
		1.4	10	
		.28	8	
4.2	42 (*idem*)	10	10	.4
		2	9	
		.4	5	
5.1	94, .02 M $MgSO_4$	5	8§	
		1	8	
		.2	7	
5.2	94, .01 "	5	6§	
		1	7	
		.2	9	
5.3	94, .005 "	5	8	
		1	7	
		.2	8	
5.4	94, .0025 "	5	9	.2
		1	8	
		.2	5	

Except where noted, filtrates were treated with 5% alum.

* Alum or phosphate adsorbed antigen expressed as ml of whole filtrates.

† Determined by method of Litchfield and Wilcoxon (3).

‡ Filtrate treated with 10% $CaCl_2$ and 10% $Na_2HPO_4 \cdot 12H_2O$.

§ Survival out of 9 mice tested.

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a virulent strain of *P. pestis* suspended in 0.1 ml phosphate buffer. Untreated mice were infected simultaneously with the test animals and 90.0 to 100% of these control animals succumbed to the infection. The mice were observed for 2 weeks following challenge and a portion of those that died were autopsied and cultures prepared from the spleen.

Results. The results shown in Table II indicate that with the exception noted below, all of the preparations were able to confer protection. It appeared that in static cultures insufficient antigen was produced within 48 hours to be detected by mouse assay. Similarly, preparations from 24-hour shaken cultures did not protect mice with amounts used. By 42 hours, however, sufficient antigen was produced in shaken cultures so that in one case (filtrate 4.2) the ED_{50} as determined by the method of Litchfield and Wilcoxon(3), was 0.4 ml of filtrate. With another preparation (filtrate 3.3) obtained from a 48-hour shaken culture the ED_{50} was 5.0 ml while with a third culture filtrate, 4.1, the ED_{50} was 0.2 ml after 92 hours incubation. In the remaining tests the survival ratio (survivors/total number tested) was too high to furnish data appropriate for the computation of the ED_{50}.

The medium described in Table I was extremely turbid as a result of the formation of insoluble magnesium phosphate. By decreasing the concentration of $MgSO_4$ from 0.02 M to 0.005 M the turbidity was reduced considerably with no apparent effect on the production of protective antigen (filtrate 5.1, 5.2, and 5.3, Table II). The precipitation of phosphates could also be eliminated by the incorporation of 0.01 M sodium citrate in the medium, however, the citrate inhibited the precipitation of alum. An attempt was made to concentrate the protective substances by adsorption onto calcium phosphate formed by the addition of 10.0% $CaCl_2$ and 10.0% $Na_2HPO_4 \cdot 12\ H_2O$ to the filtrate. Preparations 4.1, 4.2, and 4.3 listed in Table II were treated in this manner and each, on assay, induced a degree of protection of the same order as the alum adsorbed filtrates.

Injection into mice of the supernatants obtained following adsorption of the protective substances indicated that the immunizing antigens were removed incompletely with alum or calcium phosphate. These supernatants were toxic for mice when injected subcutaneously and the majority of the animals died within one to 3 days. Adsorption on these salts may serve to separate toxic components from the immunogenic substances.

Serological studies of the filtrates obtained from the cultures of *P. pestis* showed the presence of Fraction I of Baker *et al.*(4). Whether Fraction I antigen is solely responsible for the protection afforded mice cannot be assumed at present since other antigens were also detected.

Summary. These studies show that the soluble substances produced by *P. pestis* when grown at 37°C in a completely defined medium are capable of producing a high degree of protection when injected into mice. Further studies to determine the optimal conditions for growth and antigen production are necessary and are in progress.

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