SYNTHESIS OF THE FRACTION I ANTIGENIC PROTEIN BY *PASTEURELLA PESTIS*

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The production of the capsular antigens of *Pasteurella pestis* grown at 37 C and the apparent absence of these antigens in cultures grown at 26 C may be viewed as a temperature-dependent biosynthesis which can be investigated as a problem in protein synthesis and conveniently measured by serological techniques. The capsule, found in virulent and many avirulent strains of *P. pestis* grown at 37 C, has variously been described as the "envelope antigen" (Schütze, 1934), the "capsular substance" (Amies, 1951), or "fraction I" (Baker *et al.*, 1947). Englesberg and Levy (1954) are of the opinion that this antigen (produced only at temperatures near 37 C), whether it be called envelope antigen or capsular substance, is synonymous with fraction I, and Crumpton and Davies (1956) have shown by agar diffusion that at least three temperature-dependent antigens exist. The V and W antigens recently discovered by Burrows and Bacon (1956) and which have been related to virulence in a number of strains of *P. pestis* are also dependent on temperature for their synthesis. These antigens appear to be more intimately associated with the cell wall than are those described above. Although the capsular material has been shown to be a carbohydrate-protein complex, available data indicate that the protein of fraction I is the major component. The present work is concerned solely with the synthesis of fraction I measured serologically with antibody prepared against a purified preparation of this protein which has been demonstrated to be homogeneous by several agar diffusion techniques. These results were introduced in a previous report in which antigen synthesis by *P. pestis* was observed with whole cells and in fragments of these cells disrupted by sonic oscillation (Fox, 1957).

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MATERIALS AND METHODS

An avirulent strain of *P. pestis* (A1122) was used for the majority of experiments described in this paper. A second avirulent strain (Tjiwidej) was also employed in several experiments involving antigen synthesis with cell fragments.

Cultures were maintained on Blood Agar Base (Difco) slants. For daily experiments, growth from two 48 hr slants grown at 26 C was washed off into 250 ml of heart infusion broth (Difco) containing 1 per cent xylose (sterilized separately) in a 3 L Fernbach flask. The cultures were incubated for 24 hr at 26 C on a reciprocating shaker to insure adequate aeration. A drop of sterile olive oil was added as an antifoaming agent. Vitamin free casein hydrolyzate, supplied by Nutritional Biochemical Corp., Cleveland, Ohio, was used as a source of amino acids for the biosynthesis of antigen by resting cells and cell fragments.

Hemagglutination inhibition was employed as a convenient and extremely sensitive method for detecting the soluble antigen when present in solution or on the surface of the bacterial cell wall. Several preparations of fraction I protein were used for the immunological and serological procedures. One antigen preparation was obtained through the courtesy of the Hooper Foundation, California, and used for immunization. The second was prepared in this laboratory by the initial extraction of cells with thiocyanate according to Amies (1951) and final purification by the method of Baker *et al.* (1952). The latter was designated as fraction IB according to its precipitability in ammonium sulfate at 30 to 33 per cent saturation. This material served to sensitize sheep red blood cells in the hemagglutination test. Antisera with high titers were obtained from rabbits with the former antigen administered with Freund's adjuvant according to the method of Cohn (1952). Booster doses without adjuvant were given intramuscularly 30

days after the initial injection. Antisera with the highest titers (assayed by precipitin tests) were collected 10 days following the second injection. When these antisera were pooled and assayed with crude antigens, they reacted as a homogeneous antigen-antibody system as demonstrated by several agar diffusion techniques (Oudin, 1952; Ouchterlony, 1949). Fraction I protein, when assayed with an antiplague serum (Lederle) prepared by immunization with whole cells, revealed only a single precipitin band, demonstrating the homogeneity of the antigen. Hemagglutination assays were performed with sheep red blood cells treated with tannic acid and sensitized with fraction I protein. The technique was essentially that described by Chen and Meyer (1954). For a diluent, horse serum was substituted for normal rabbit serum, thus avoiding the task of adsorbing the normal serum with red cells. Pooled antisera prepared in this laboratory gave strongly positive hemagglutination reactions in dilutions of 1:6400 to 1:12,800 with sensitized sheep red blood cells.

In the general procedure for observing antigen synthesis, *P. pestis* was grown in heart infusion broth for 24 hr at 26 C. The cells were harvested and washed twice in buffered saline (0.85 per cent NaCl in 0.01 M potassium phosphate, pH 7.0), suspended in various media (10 ml of suspension in a 125 ml flask) at a given optical density and incubated at 37 C with vigorous agitation for periods up to 3 hr. At the end of that time the suspensions were neutralized with dilute HCl (as amino acid oxidation proceeded the pH gradually rose to about 8) and an aliquot was taken for assay. The cells were removed by centrifugation and resuspended in diluted antibody (1:50 or 1:100) for 45 min at room temperature with occasional mixing. Upon completion of antibody adsorption, the cells were removed by centrifugation and the residual antibody was titrated by the hemagglutination technique. Diminution of the antibody titer following the adsorption was a measure of antigen synthesis on the cells incubated at 37 C.

Disrupted cell preparations were obtained by exposing heavy suspensions of washed cells in buffered saline to the sonic vibration in a Raytheon 9-kc sonic oscillator at 3 C for 60 min (1 part packed cells to 4 or 5 parts saline). The resulting suspensions were separated into particulate and soluble fractions by centrifugation at 5 C for 30 min under a force of 25,000 × G.

Turbidity measurements were made with a Coleman Nepho-Colorimeter, Model 9, with an arbitrary linear scale of 1 to 100. A value of 100 was standardized so that a suspension of that density in an 18 mm tube contained 2.5×10^9 cells per ml or 0.5 mg per ml dry weight of cellular material.

Respiration measurements were carried out according to standard Warburg techniques. Large cups (125 ml) were used when respiration and antigen synthesis were simultaneously measured over a period of 3 hr.

RESULTS

Antigen synthesis in whole cells. In order to obtain antigen synthesis in the absence of cellular multiplication, *P. pestis* cells grown at 26 C were washed and incubated with rapid agitation at 37 C in a medium of 1 per cent xylose and 2 per cent amino acids supplied as acid-hydrolyzed casein. Optical density measurements were taken initially and at the end of each hour. At no time during this incubation period did the optical densities of the suspensions change significantly. At the end of the third hour cells were removed for antibody adsorption. The results of the hemagglutination inhibition are shown in table 1. The nearly complete inhibition of hemagglutination demonstrated that antigen was synthesized and had the capacity to remove all the antibody from the solution. The cells from a control flask shaken at 26 C had little if any capacity to adsorb antibody; this was also true of cells suspended in saline at 37 C. Although 2 per cent casein hydrolyzate (or even 1 per cent) was adequate for antigen synthesis, when the concentration was decreased to 0.5 per cent the yield of antigen diminished, and when less than 0.5 per cent casein hydrolyzate was supplied, no antigen synthesis was detected.

A requirement for oxygen for antigen synthesis was demonstrated in the experiment in which a flask was allowed to remain stationary; here only small amounts of antigen were synthesized. In an anaerobic experiment (not shown in table 1), in which flasks were flushed with N_2, sealed, and shaken at 37 C, no antigen was synthesized. It may be noted that in the control cells incubated at 26 C, a small amount of antigen was detected, i. e., the last strongly positive dilution was 1:3200 instead of 1:6400 as in the series with unadsorbed antiserum. This trace amount of

TABLE 1

Antigen synthesis by resting cells of Pasteurella pestis assayed by hemagglutination inhibition

Antiserum Dilution	Hemagglutination* by Antisera after Adsorption by Antigen Produced on Cells Suspended in:					Unadsorbed Antiserum
	2% Amino acids,† 1% xylose, shaken, 37 C	2% Amino acids, 1% xylose, shaken, 26 C	Buffered saline, shaken, 37 C	2% Amino acids, 1% xylose, stationary, 37 C	0.5% Amino acids, 1% xylose, shaken, 37 C	
1:50	±	++	++	++	++	++
1:100	−	++	++	++	++	++
1:200	−	++	++	++	++	++
1:400	−	++	++	++	++	++
1:800	−	++	++	++	+	++
1:1,600	−	++	++	+	±	++
1:3,200	−	++	++	±	−	++
1:6,400	−	+	±	−	−	++
1:12,800	−	±	−	−	−	±
1:25,600	−	−	−	−	−	−
Saline	−	−	−	−	−	−

* Strongly positive = ++; weakly positive = +; doubtful = ±; negative = −.

† Acid-hydrolyzed casein, total solids in 0.04 M potassium phosphate buffer pH 7.0.

Cells grown at 26 C washed twice in buffered saline, suspended in above media (25 ml per 500 ml flask) for 3 hr. Optical density of suspensions = 30 when diluted 1:10. After 3 hr incubation, 10 ml of suspension removed, centrifuged, and cells suspended in 3 ml saline containing 0.06 ml (1:50) antiserum and incubated room temperature 45 min. Cells removed and antibody in supernatant liquid titrated by hemagglutination.

fraction I was always present on cells grown at 26 C. It could be liberated into the soluble fraction of sonically disrupted cells and tended to mask some of the results with these latter preparations. The amount of the initial antigen could be decreased considerably when cultures were originally grown at a slightly lower temperature, that is, between 22 and 24 C, although at these temperatures the rate of growth was slightly slower than at 26 C.

To insure that all the antigen was being measured, acetone powders were prepared from cells after the 3 hr incubation period at 37 C. These powders were extracted over night with saline and antibody was added to a final dilution of 1:50. Inhibition titers obtained by these methods were no less than before, indicating in all probability that the antigen formed was on the surface of the cells and that no intracellular antigen was liberated in the preparation of the acetone powder. As an additional control to insure the immunological specificity of the antibody adsorption, rabbit antiovalbumin was mixed with *P. pestis* cells under the standard conditions described above and then titrated by hemagglutination with sheep red blood cells sensitized with ovalbumin. No diminution of the antiovalbumin titer was observed after adsorption by *P. pestis* cells, demonstrating that the adsorption of fraction I antibody was a serologically specific reaction with the newly formed antigen protein. In spite of the fact that xylose or glucose could be fermented anaerobically, antigen was not synthesized without oxygen in the presence of these compounds. Carbohyhydrates such as xylose or glucose were not necessary for antigen synthesis if a sufficient concentration of amino acids (1 per cent or more) supplied as casein hydrolyzate was present and aeration was maintained. A synthetic mixture of 11 amino acids previously demonstrated by Silverman *et al.* (1954) to be sufficient for growth of *P. pestis* in a synthetic medium was not as efficient as the 1 per cent casein hydrolyzate for antigen synthesis.

In a medium of 2 per cent casein hydrolyzate in 0.04 M phosphate buffer, antigen synthesis could be detected after 1 hr and increased exponentially for several hours. This is demonstrated in table 2. The absolute rate of antigen synthesis was determined with purified fraction I protein as a standard and it was calculated that 5 mg of cells, dry weight, synthesized approximately 2 μg of fraction I protein in 3 hr. During this time no antigen was detected in solution in the suspending medium (although in growth experiments of 24 hr or more fraction I was solubilized).

Amino acid oxidation. During synthesis of fraction I antigen by *P. pestis* cells in a buffered, 2 per cent, casein hydrolyzate solution without carbohydrate, oxygen was consumed at a rapid rate as measured by the Warburg manometric technique. Paper chromatography of this medium before and after antigen synthesis showed a definite decrease of amino acids (serine, proline, alanine, glycine, and aspartic acid) and a slight decrease in the concentrations of several others. It was difficult to estimate the extent of utilization of these amino acids due to the problem of

TABLE 2

Hemagglutination inhibition assay of antigen synthesis by resting cells of Pasteurella pestis after varying periods of incubation in amino acids

Antiserum Dilution	Hemagglutination by Antisera after Adsorption by Antigen Produced on Cells Incubated Aerobically in Casein Hydrolyzate* for:					Unadsorbed Antiserum
	0 Hr, shaken, 37 C	1 Hr, shaken, 37 C	2 Hr, shaken, 37 C	3 Hr, shaken, 37 C	3 Hr, shaken, 26 C	
1:50	++	++	++	±	++	++
1:100	++	++	++	−	++	++
1:200	++	++	+	−	++	++
1:400	++	++	±	−	++	++
1:800	++	++	−	−	++	++
1:1,600	++	±	−	−	++	++
1:3,200	++	−	−	−	++	++
1:6,400	+	−	−	−	+	++
1:12,800	±	−	−	−	−	+
1:25,600	−	−	−	−	−	−
Saline	−	−	−	−	−	−

* 2 Per cent casein hydrolyzate in 0.04 M potassium phosphate, pH 7.0. Conditions same as described in table 1.

TABLE 3

*Hemagglutination inhibition assay of antigen synthesis by resting cells of Pasteurella pestis in media of limited amino acid concentrations**

Antiserum Dilution	Hemagglutination by Antisera after Adsorption by Antigen Produced on Cells Suspended in Phosphate Buffer Containing:					Unadsorbed Antiserum
	1% Amino acids	0.1% Amino acids	0.1% Amino acids and 15 mg/ml DL-serine	15 mg/ml DL-serine	1% Amino acids and chloramphenicol 0.5 μg/ml	
1:100	+	++	++	++	++	++
1:200	±	++	++	++	++	++
1:400	−	++	++	++	++	++
1:800	−	++	++	++	++	++
1:1,600	−	++	++	++	++	++
1:3,200	−	++	++	++	++	++
1:6,400	−	+	±	+	++	++
1:12,800	−	±	−	+	−	+
1:25,600	−	−	−	−	−	±
Saline	−	−	−	−	−	−

* Conditions same as described in table 1.

separating all the amino acids of the casein hydrolyzate by 2-dimensional paper chromatography.

When the concentration of casein hydrolyzate in the buffer was limited to 0.1 per cent and the medium was supplemented with an amino acid such as serine (which is rapidly oxidized by *P. pestis*), at an adequate concentration to insure rapid oxygen consumption throughout the 3 hr incubation period, antigen synthesis was negligible. These data are shown in table 3 and figure 1. If the antigen synthesis alone were to account for the utilization of the amino acids, 0.1 per cent casein hydrolyzate would probably have been sufficient, but the situation was complicated by the accompanying oxidation of some of the essential amino acids. Consequently, a limited concentration of casein hydrolyzate even in the presence of a rapidly oxidized substrate (serine) was inadequate for antigen formation. Neither glucose or xylose were stimulatory under these conditions. An alternative explanation may be that the energy obtained by the oxidation of serine or xylose was unavailable for the protein synthesizing mechanism.

The effect of a number of inhibitors of assimilation and protein synthesis was tested with the *P. pestis* antigen system. Diazouracil or 2,4-dinitrophenol (10^{-4} M) only partially blocked antigen synthesis and had no effect on respiration. Chloramphenicol in a concentration as low as 0.5 μg per ml completely inhibited antigen synthesis, whereas, the rate of oxygen consumption was only slightly retarded. The data obtained with chloramphenicol are shown in table 3 and figure 1.

Antigen synthesis in disrupted cells. A suspension prepared from 1 part washed cells, and 4 parts buffered saline was treated in the sonic oscillator for 60 min at 3 C. The cellular debris and extract were then separated by high-speed centrifugation under a force of 25,000 × G for 30 min at 0 C. The resulting supernatant liquid was viscous and opalescent. Plate counts of the material obtained after sonic treatment revealed that from 2 to 4 per cent viable cells remained in the debris. In table 4, it may be seen that both the supernatant liquid and the debris had to be reconstituted in order to initiate antigen synthesis. In the experiments where extract or cellular debris were used alone, no antigen was evolved. The latter experiment also proved that the small percentage of viable cells remaining in the debris could not account for the antigen

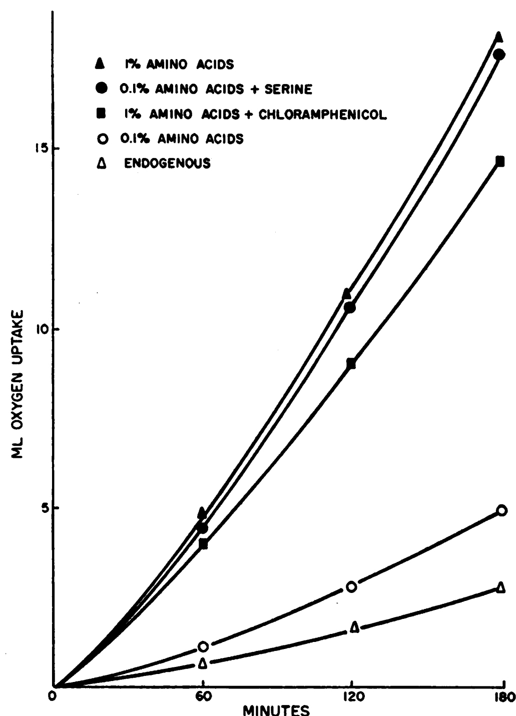


Figure 1. Rates of amino acid oxidation by *Pasteurella pestis*. Warburg cups (125 ml) containing 2 ml 30 per cent cell suspension in sidearm. Cells tipped into 17 ml 0.04 M phosphate buffer containing the above substrates. Center well contained 1 ml 20 per cent NaOH, atmosphere was 100 per cent oxygen, 38 C.

TABLE 4

*Hemagglutination inhibition assay of antigen synthesis in preparations of disrupted cells of Pasteurella pestis**

Antiserum Dilution	Hemagglutination by Antisera after Adsorption by Antigen Produced on Fractions of Disrupted Cells in Casein Hydrolyzate				Unadsorbed Antiserum
	Extract and debris, 37 C	Saline and debris, 37 C	Saline and extract, 37 C	Extract and debris, 26 C	
1:100	++	++	++	++	++
1:200	±	++	++	++	++
1:400	−	++	++	++	++
1:800	−	++	++	++	++
1:1,600	−	++	++	++	++
1:3,200	−	++	++	++	++
1:6,400	−	+	+	+	++
1:12,800	−	±	−	±	±
1:25,600	−	−	−	−	−
Saline	−	−	−	−	−

* Each 125 ml flask contained 4 ml extract and 1 ml debris (or an equivalent volume of saline), 1 ml 10% casein hydrolyzate, and 1 ml 0.2 M potassium phosphate, pH 7.0. Optical density was 130 when suspension was diluted 1:10. Each flask incubated 3 hr with rapid agitation. At end of 3 hr, 3 ml suspension mixed with 0.03 ml antiserum 45 min at room temperature, the debris removed by centrifugation, and supernatant liquid titrated in the above hemagglutination test.

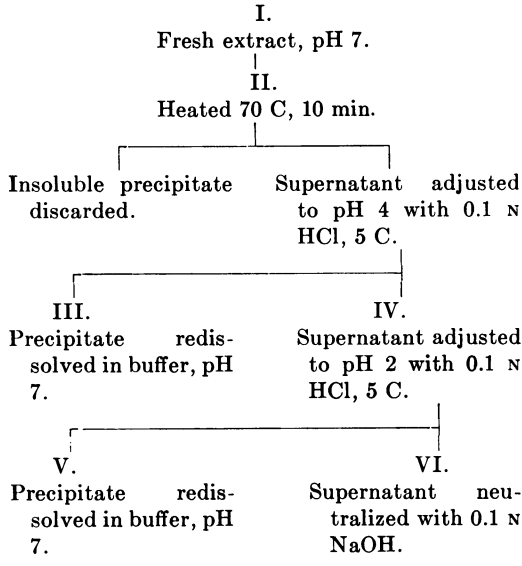
synthesis that resulted when debris and supernatant were reconstituted. The cultures of *P. pestis* used for the experiments with disrupted cells were grown at a slightly lower temperature (22 to 24 C) than those cultures used for whole cell experiments in order to minimize the amount of antigen already present. However, traces of antigen were detectable in extracts of these cultures.

An attempt was made to characterize the material in the supernatant responsible for the "activation" of the particulate fraction. Table 5 presents the protocol in which the active soluble fraction was separated from the bulk of extraneous cellular extract. The initial heating treatment in a 70 C water bath for 10 min precipitated most of the protein and nucleic acid, leaving after centrifugation, a clear light yellow liquid with low viscosity. Table 5 also lists the hemagglutination inhibition titers obtained with various fractions of the supernatant when added back to the particulate material and assayed as outlined in table 4. The active soluble material was precipitated at pH 2 with dilute HCl but not at pH 4. The bulk of material precipitated at pH 2 could be redissolved in buffered saline by the addition of dilute NaOH to neutralize the acidic material of the precipitate. Titers shown in table 5 indicate that the soluble component was not completely stable under the conditions described, and in fact, a number of preparations lost a large percentage of activity after the heating treatment and the acid precipitation. The active material from step II was subjected to a number of enzymes for 12 hr at 37 C. Neither ribonuclease, desoxyribonuclease, nor trypsin appreciably diminished the activity of the soluble fraction. The active material was nondialyzable before and after the enzymatic treatment.

When extract and cellular debris were recombined to initiate antigen synthesis, all the antigen

TABLE 5

*Fractionation and assay of the cellular extract**



Extract Fraction	Last ++ in Hemagglutination Reaction†
I.	1:100
II.	1:200
III.	1:3200
IV.	1:200
V.	1:400
VI.	1:3200

* Fresh extract prepared in sonic apparatus as described in text. Each fraction was neutralized and an amount equal to 4 ml of original extract was used for antigen synthesis with cellular particles.

† Antigen synthesis experiments, antibody, adsorption, and hemagglutination performed as described in table 4.

formed was associated with the sedimented material. Antibody was adsorbed only by the debris resuspended in saline; no additional antibody could be adsorbed in the soluble fraction.

As in the case with whole cells during antigen synthesis, oxygen was necessary and was rapidly consumed by the disrupted cell preparations. Manometric measurements demonstrated that the oxidative activity in casein hydrolyzate (or xylose) was wholly associated with the particulate fraction and was not stimulated by the extract which had no oxidative property alone. Chloramphenicol (1 μg per ml) completely inhibited antigen synthesis in the cell fragment experiments; the rate of oxygen consumption, as with whole cells, was only slightly decreased.

DISCUSSION

In the course of this text the term "antigen formation" was used synonymously with "protein synthesis" in discussing the synthetic processes that occurred when *P. pestis* cells or fragments were incubated at 37 C. It is assumed that the phenomenon described may be called *de novo* protein synthesis, in view of the singular nutritional requirements in the form of amino acids for the production of the fraction I antigen and the extreme sensitivity of the system to the inhibitory action of chloramphenicol. Moreover, fraction I protein was the serological reagent for the production of antisera and the sensitizing agent in the hemagglutination test. If a protein precursor existed in cells grown at 26 C, it is probable that more than a trace amount of antibody would have been adsorbed by these cells or cellular extracts before incubation at 37 C.

Experiments thus far have not revealed any clues as to the site or metabolic level where the increase in temperature affects the antigen synthesis. Antigen synthesis does not appear to be solely dependent upon some "activation" process (e.g., formation of specific nucleic acids serving as templates) which is initiated by an increase in temperature. For example, cells which were grown at 26 C and resuspended in casein hydrolyzate medium for 2 hr at 37 C and then reincubated at 26 C for several hours longer, produced antigen only during the initial incubation at 37 C. Although ribonucleic acid was rapidly synthesized and the process of protein synthesis had proceeded for 2 hr, antigen production did not continue at the lower temperature, though the necessary biochemical prerequisites presumably were still present.

Amino acids supplied as 1 per cent casein hydrolyzate were an adequate source of energy and precursors for protein synthesis. The absence of antigen formation when the amino acid concentration was decreased to 0.1 per cent even though the medium was supplemented with an excess of serine or other oxidizable amino acids, may be attributed to the exhaustion of certain essential amino acids which were present in the hydrolyzate at low concentrations. Polypeptides

from the nondialyzable portion of a pancreatic digest of casein failed to stimulate antigen formation with whole cells or in disrupted cell preparations of *P. pestis* although these polypeptides were shown by Fox and Krampitz (1956) to enhance markedly the synthesis of the M-protein of nonproliferating, group A, hemolytic streptococci.

Attempts to obtain antigen synthesis were unsuccessful with cell fragments prepared in the Mickle apparatus or with the Hughes press (Hughes, 1951). In the Raytheon sonic oscillator cell suspensions exposed longer than 60 min lost much of the antigen synthesizing capacity. Cell suspensions exposed less than 60 min retained a proportionally higher viability than the 2 to 4 per cent found in the 60 min preparations. Antigen formed by these viable cells interfered with the results obtained with the extract and cell fragment preparations.

The relative instability of the soluble fraction from the disrupted cells made it difficult to separate the active component from extraneous material. The active material precipitated at pH 2 exhibited a strong ultraviolet adsorption maximum in the 260 mμ range, but no conclusions can be made at the present stage of analysis concerning the nature of the soluble factor. Yeast and thymus nucleic acids or the hydrolysis products of these materials would not substitute for the soluble fraction of the disrupted cell system.

Other investigators recently have reported protein synthesis in disrupted bacteria. Gale (1956) obtained enzyme synthesis in fractions of staphylococci in which the formation of enzymatic activity was dependent upon the addition of soluble ribonucleic acid extracted from the cells. Aronson and Spiegelman (1957), using ruptured protoplasts of *Bacillus megaterium* have demonstrated a mutual dependency of ribonucleic acid and protein synthesis; neither proceeding without the presence of nucleotides, amino acids, and glucose.

Hoagland and Zamecnik (1957) and Zamecnik *et al.* (1957) in their study of amino acid activation have shown that ribonucleic acid in a rat liver enzyme preparation can couple with amino acids and subsequently transfer those amino acids into the protein of microsomes and at the same time incorporate mononucleotides into the ribonucleic acid fraction.

It would appear that the soluble fraction necessary for antigen synthesis in disrupted cells of *P. pestis* may be similar to these various soluble systems of protein synthesis.

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SUMMARY

The temperature dependent synthesis of the fraction I antigen of *Pasteurella pestis* has been studied in nonproliferating, washed cell suspensions incubated at 37 C but initially grown at a lower temperature (26 C) to suppress prior antigen formation. A sensitive and serologically specific hemagglutination technique was used to measure the fraction I protein synthesis. In the presence of phosphate buffer and adequate aeration only casein hydrolyzate was required for protein synthesis. Neither carbohydrates nor peptide fractions were stimulatory.

Chloramphenicol (1 μg per ml) completely inhibited antigen formation without appreciably reducing oxygen uptake by the system. Dinitrophenol and diazouracil only partially inhibited antigen synthesis.

Sonically disrupted cells were capable of antigen synthesis only if the supernatant as well as the debris (separated by high-speed centrifugation) were recombined. The active constituent in the soluble fraction was nondialyzable and resistant to trypsin, ribonuclease and desoxyribonuclease. The site of antigen synthesis was associated with the debris; no fraction I antigen was detectable in solution.

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