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BIOCHEMISTRY OF THE PLAGUE BACILLUS
(Pasteurella pestis)
- USSR -

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by Ye. M. Gubarev and M.N. Ivanovskiy

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BIOCHEMISTRY OF THE PLAGUE BACILLUS
(PASTEURELLA PESTIS)

- USSR -

By

Ye. M. Gubarev and N. N. Ivanovskiy

(Biokhimiya Chumnyh Mikroba (Pasteurella Pestis) (English version above), Medgiz, Moscow, 1958, pages 1-115)
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AUTHOR'S NOTE

The contents of the book by Ye. M. Gubarev and N. N. Ivanovskiy, *Biochemistry of the Plague Bacillus*, ranges far beyond the frame of reference set by the title: in addition to summarizing the literature material and the authors' own investigations on the chemical composition and the biochemical activity of the plague and other similar bacilli, the book contains much general information on the biology and evolution of the plague causative, its pathogenesis, and the clinical aspect of plague infection. Although this information is presented in general form, the book can serve as an introduction to a more detailed study of the problem in the original sources. In this regard the extensive and carefully compiled literature list included in the book will be of great benefit. These qualities make the book profitable and interesting not only for a narrow circle of specialists, but also for many lovers of natural science and medicine.
INTRODUCTION

Plague has been a monstrous menace to mankind both in antiquity as well as not very long ago.

The Asiatic-European epidemic of the 14th Century took more than 65 million people. During the course of this epidemic about one-fourth of the entire population of Europe perished. It has been only in the 20th Century through gradually developed anti-epidemic measures that it has been possible to sharply cut down the number of fatalities from the still numerous epidemic outbreaks of plague infection. Mortality from the plague, amounting to 25-50% for the bubonic form and 100% for the pneumonic form, has been greatly reduced only during the last 15 years.

At present, beginning with the successes of chemotherapy, and subsequently owing to the use of antibiotics mortality from the plague can be reduced to practically zero when early and proper treatment is carried out. It is difficult to point to any other such examples of success in the struggle of man with the hostile forces of nature.

Beginning from 1938, when sulfanilamide preparations began to be used in the treatment of the plague, an essential turning point in the treatment of this disease was recorded. But even such powerful agents, as sulfathiazole, sulfadiazine, and sulfamerazine, have afforded favorable results only for the treatment of the bubonic form of plague. The introduction of antibiotics in the postwar years into therapeutic practice has made possible an alleviation of the severity of prognosis in plague victims. At the present the high effectiveness of streptomycin for primarily pneumonic plague and for septicaemic plague has been conclusively established.
Other antibiotics — chloramphenicol (chloromyceitin) and terramycin (oxytetracycline) — have shown approximately the same effectiveness.

However, in spite of many reports of cures of almost all victims of any form of the plague in individual epidemic outbreaks, we must never wholly remain content with the successes achieved and with the availability of antibiotics. Various microorganisms, including also the plague bacillus, have the ability of forming varieties which are resistant to streptomycin and to other antibiotics. This does not exclude the possibility of forming, for example, streptomycin-resistant plague bacilli not only artificially, but also under natural conditions, the possibility of infection of man by streptomycin-resistant strains of the plague bacillus, although the emergence of strains simultaneously resistant to many antibiotics appears improbable. Therefore, the most important problems to be dealt with are as follows: discovery of the mechanism of action of antibiotics on plague; the mechanism of the formation of drug-resistant strains of the plague bacillus; the development of methods of suppressing or eliminating this resistance; and research into new chemotherapeutic agents. It is unnecessary to state that the solution of such problems is possible only on the basis of profound understanding of the biochemistry of the plague bacillus.

There are also other not less important problems that must be faced in combating plague infection, the solution of which is also inconceivable without careful research into the biochemistry of the plague bacillus. Thus, the experience of many specialists armed with modern methods of treating plague includes cases of patient mortality even after the complete annihilation of the bacilli in the blood and the tissues of the affected organs. In these instances, usually occurring as a result of delayed initial treatment, the patient dies from intoxication caused by the plague bacillus. This intoxication occurs in attacks by any form of the plague. Consequently, controlling intoxication in all, and not only in the instances of the disease that are dangerous to life is an urgent modern problem of plague treatment. Toxins of the plague bacillus, both those exhibiting antigenic properties as well as those which are nonspecific toxic substances, formed by this microbe, require specially careful biochemical research, since this problem has up to now not been satisfactorily illuminated.
Based on the above stated facts we have been convinced that now more than ever it is necessary to sum up the results of biochemical knowledge of the plague bacillus in order to concentrate efforts in solving the problems lying ahead. Without fear of overstatement, it can be said that the complete solution of the main problems related to the modern successful treatment of the plague lies along the approaches of the most extensive use of methods of biochemical research which have successfully been mastered by microbiologists.

Considering the special character of this study, we recommend that the reader examine several monographs on the general biochemistry of bacteria, published in recent years in the Russian language: V. S. Gostev, Biochemical Foundations of Medical Bacteriology (published by the Academy of Medical Sciences, USSR, 1951); Ye. N. Gabarev, Bacteriochemistry (Medgiz Ukrainian SSR, 1952); M. S. Stefenson, Metabolism of Bacteria (Foreign Literature Publishing House, Moscow, 1951); Physiology of Bacteria (Foreign Literature Publishing House, Moscow, 1954). The reader's familiarity with even one of the suggested books will considerably facilitate his understanding of the present monograph.
CHARACTERISTICS OF THE DEVELOPMENT OF
RESEARCH OF THE PLAGUE BACILLUS

From the moment that Yersin isolated the causative organism
of the plague (1894) and during the course of the subsequent years
researchers, cultivating the plague bacillus under various condi-
tions and in various artificial nutritive media, observed changes
in the form both of individually isolated bacterial cells, as well
as of their colonies. In this connection, simultaneously with
the morphological changes several features characteristic of the
plague bacillus -- its tinctorial properties, virulency, anti-
genicity, and many others -- also changed to a greater or lesser
extent. Part of these bacillus signs were lost, for example,
virulency, while part on the other hand were acquired, for example,
the ability to multiply from small culture batches, etc. All
these diverse appear for the plague bacillus during its cultiva-
tion in artificial nutritive media, that is, in a medium habitat
that is new for the microbes.

Much material of the nature indicated exists in studies
from the end of the 19th and the beginning of the 20th centuries,
dedicated to problems related to the study of plague infection.
Thus, for example, N. N. Vesternek (1), growing the plague bacillus
under anaerobic and aerobic conditions, described in detail the
changes occurring in the culture.

Many authors have directed attention to the formation of
the adult forms of the plague bacillus when it is cultivated in
a nutritive medium with high sodium chloride content.

Investigations of this period are factorially descriptive
in character and are presented in a non-systematic crude form.
The large amount of accumulated experimental material on the mutability of the plague bacillus was verified and considerably enlarged upon by the later observations of M. P. Pokrovskaya, A. A. Bezsonovaya, Ye. I. Korobkovaya, N. N. Zhukov - Verezhnikov, G. N. Lenskaya, V. M. Tumanaskiy, and others. These authors described the enormous number of plague bacillus forms, which they were able to obtain as a result of repeated subculturing in artificial nutritive media, and also through the action of bacteriophage. A survey of the factual data on this problem is available in a monograph by V. M. Tumanaskiy, (2).

These studies served to establish the so-called process of dissociation of the plague bacillus culture into coarse and smooth varieties. From these the authors isolated many transitional forms, and countered on the transformation route of the typical natural virulent coarse form into the atypical, avirulent smooth form. The same process of formation of smooth form from the coarse was regarded as a consequence of unfavorable conditions in which the plague bacillus existed. Finally, the problem of the profoundly occurring mutability of the plague bacillus beyond the limits of dissociation was formulated. This mutability is observed in cultures of old museum strains, subjected to frequent subculturing in artificial nutritive media over a long period. These changes of the plague bacillus proceed along the path of similarity with the properties of the pseudotubercular bacillus and with the passage of time advance so far that the problem of the possible transition of the plague bacillus into the pseudotubercular arose.

During the second period of the progress in understanding the mutability of the plague bacillus first and foremost were chiefly the morphological properties of the variety; the biochemical characteristics of the newly obtained forms reduced to the constancy that the R-forms in the biological sense were generally less active than the S-forms. The morphological properties of numerous forms of the plague bacillus were described in the utmost detail. Many observations of this period (for example, the pigment-containing cultures of the plague bacillus) were regarded as rare instances, which the authors were not able to reproduce. Researchers explained them by the "spontaneous" change of the culture, occurring as a result of its prolonged storage without subculturing, partial subculturing, or under the action of a bacteriophage.
Swung up the results of the successes in descriptive morphology of this period of progress in elucidating the mutability of the plague bacillus, G. N. Lanskaya (3) in his monograph presented a detailed treatment of the existing material on this problem.

The problem of the mutability of the plague bacillus, appearing initially basically as purely morphological, has been given subsequentially a new biochemical direction.

A third period in the study of the plague bacillus set in – the functional-morphological period.

The unity of biochemical processes in the cell frequently is noted in the literature and now is a widely known position.

The processes of intracellular metabolism involved the participation of substances, in common for all cells of plant and animal origin, however, not all of these substances are capable of being independently synthesized by the cells. The nutrient substances of the cell are obtained from without and in a ready form or in the form of by-products from which the usable substances can be more easily obtained.

The above considerably eases the task of studying the bio-chemistry of the plague bacillus.

At present the biochemical processes in the plague bacillus cannot be investigated without taking into account its substantial and sharply pronounced variability in morphological features. The range of intraspecies mutability is so great that even experimental microbiologists frequently experience difficulties in diagnosis, when they are dealing with old laboratory strains of the plague bacillus.

Neither data relating to the chemical composition of the plague bacillus, nor its so-called biochemical properties, that is, the conventional indices of metabolism can be regarded independently of the varieties with which the author must deal. Underestimating the variability of the plague bacillus is an explanation for the many apparent contradictions in its properties, which researchers have indicated. Actually the contradictions often do not exist,
since the author frequently study strains of the plague bacillus which sharply differ in their morphological features and biochemical properties.

In the present monograph, in addition to other, no less important problems, an attempt will be made to summarize the data dealing with the biochemical properties of the plague bacillus and to undertake a biochemical analysis of its numerous varieties. Some material in the present work is being published here for the first time.
CHEMICAL COMPOSITION OF THE PLAGUE BACILLUS AND THE
CHARACTERISTICS OF ITS ANTIGENS

Systematic and complete information on the chemical composition of the cells of the Pasteurella pestis have not been published in our own or in the foreign literature. Investigations into this problem are fragmentary in the sense that they deal with the study of not all but only several of the cell components. The first efforts in studying the composition of the P. pestis cells were associated with the efforts of researchers to obtain from a given bacillus those of its constituents which exhibited the properties of antigens, which could be used for immunization.

In a study by Lustig and Galeotti (4) it was first noticed that plague bacilli readily and completely dissolved upon adding potassium hydroxide up to a concentration of 1%. Upon acidifying these products of cell decomposition with acetic acid, a precipitate separated from the solution, called nucleoprotein, exhibiting antigenic and toxic properties. The nucleoprotein of Lustig and Galeotti were suggested by them in obtaining antiplague horse serum, and for a long time was used for this purpose in various anti-plague institutions. The chemical composition of this nucleoprotein has remained thus far unknown.

From the cells of P. pestis, killed each chloroform, S. Rowland (5) extracted using a 5% sodium sulfate solution a toxic protein substance, discovered to include adenine and guanine, which led the author to propose the nucleoproteinic nature of the protein produced. The Rowland nucleoprotein exhibited immunizing properties.

B. L. Gyurazi (6) obtained from a 4-day culture on meat-paste agar a dry preparation of plague bacilli, in which 6.8% bitol, extracted by ether and two protein fractions were found.
Ye. M. Gubarav and N. M. Skomarovsky (7) found 5.5% ether-extracted lipid content in the cells of P. pestis, produced from a seven-day culture in Marten's broth.

The first efforts to systematically study the chemical composition of the plague bacillus was a study of A. Bystremin (8). According to his data, the plague bacillus, on a dry weight basis, consists of 84.1% protein (based on nitrogen), 3.7% lipids, 3.6% ash, and 8.6% unknown substances.

The protein content calculated by A. Bystremin in the plague bacillus undoubtedly is exaggerated, since not all the bacilli is completely deprived of the ability to effect an increase in the opsonic index in vaccination. Shrivastava (14) isolated this nucleoprotein from the dissolved portion of the Haffkine anti-plague vaccine. This substance reacts strongly with antiplague serum, but it is difficult to isolate from the nonspecific proteins present in the nutrient medium. In order to facilitate this separation, Rao (15) used a non-protein gelatine hydrolysate for the cultivation of P. pestis. Later Miller and Johnson (16) used a casein hydrolysate medium for this same purpose.

As the result of the above indicated research it was established that one of the protein of P. pestis is very unstably associated with the cells, and easily enters the nutrient medium during the course of a three-day cultivation. This protein was shown to be serologically active.

Baker and his colleagues (17) isolated two protein fractions from the P. pestis cells, grown in three days on an agar medium.

Both fractions were obtained from a suspension of the bacilli that had been washed free of agar with a physiological solution of sodium chlorides. One of the fractions was soluble the other -- insoluble in water. The same authors (18) described the isolation and properties of two antigenic fractions from the water-soluble portion of the plague bacilli. The cultures were killed with acetone and then the antigens were extracted from them by a neutral salt solution. From the water-salt extract a fraction 1-A containing a carbohydrate associated with a specific protein was isolated by ammonium sulfate. This fraction is a viscous noncrystallizing substance. Through purification it was possible to liberate it from the carbohydrate and the isolated protein substance was called fraction 1-B, which was obtained in the form of a crystalline preparation. Both fractions exhibited similar immunizing properties,
serving as powerful antigens in the immunisation of mice and rats, but for guinea pigs the immunity from either fraction proved to be weak. Both fractions did not show toxicity. In addition to the above-mentioned, a toxic fraction was isolated from the water-soluble portion of the culture. Finally, it was established that the water-soluble precipitate of the bacilli contains antigens capable of inducing a relatively high degree of immunity in guinea pigs.

Seal (19) obtained soluble specific proteins from virulent and avirulent strains of P. pestis, and also from the cells of P. pseudotuberculosis. All the strains were cultivated over a 21-day period at 28°C in a medium containing casein hydrolysate. The proteins were obtained from cultured noncellular filtrates by the method of salting out with sodium sulfate and ammonium sulfate or by acetic acid precipitation. In the latter case protein were obtained which were serologically less active in comparison with the salted-out proteins.

From each filtrate in the salting with sodium sulfate high fractions were obtained. Salt was added to the bacterial filtrate until completely saturated, but the precipitate formed was dissolved in distilled water and precipitated by 50% sodium sulfate (the fraction P-1/2). The filtrate from this precipitation was saturated by the same salt and the fraction P-1-1/2 was isolated. Part nitrogen can be regarded as proteinic. According to the data of this same author, the lipids of P. pestis are deep yellow in color and have a sharply unpleasant odor. The physiochemical properties of the lipids of the plague bacillus are as follows: melting point - 40°C, iodine number - 27.7-29.6, and saponification number - 169-188.

Bystrochin computed the average molecular weight of the fatty acids included in the composition of the plague bacillus lipids. It proved to be equal to 302. The author also stated that the saponification number of the plague bacillus lipids coincides almost precisely with the saponification number of the triglycerides of stearic and arachidic acids. The low iodine number of the P. pestis lipids points to their low content of unsaturated fatty acids. However, due to the fact that the author dried the microbes at 100-105°C, a portion of the unsaturated fatty acids were possibly oxidized.
N. N. Ivanovsky (9) investigated in more detail, using fractions, the nitrogen and phosphorus-containing substances of the plague bacillus (the strain grown on the Chottinger agar at pH = 7.1 and 28°C, a two-day culture).

Presented below is the composition of the air-dried defatted preparation: total nitrogen -- 13.3%, nitrogen of the humous substances -- 0.73%, ammonia -- 0.83%, monoaminoacid -- 7.10%, diaminoacid -- 2%, purine and pyrimidine bases -- 1.68%, total phosphorous -- 1.1%, lipid phosphorous -- 0.18% (in the non-defatted preparation). Inorganic phosphorous was absent. According to the calculation of N. N. Ivanovsky, the plague bacillus contains, consequently, 72.15% protein and 11.1% nucleic acids.

Thus, the plague bacillus proteins contain considerably more nitrogen in monoaminoacids than in diaminoacids (ratio -- 1:3.7).

The greatest number of studies, beginning with the above-mentioned investigation of Lustig and Galetti up to the present, have been concerned with isolating the constituents of P. pestis as isolated antigens of this microbe.

One of the most widely used vaccines for antiplague immunisation is the Haffkine vaccine, developed more than 50 years ago. Its preparation is simple. A culture of any virulent strain of P. pestis, grown on a meat-peptone broth at 28°C over three to four weeks is killed with a 15-minute heating at 54°C and is preserved upon adding 0.5% carbolic acid. As an effective immunizing agent, this vaccine has frequently been investigated to discover its active principle. Thus, even Balfour-Stewart (10) and later Sokhey (11, 12) indicated that the antigen of the Haffkine vaccine, protecting against subsequent infection by the plague, when in solution perhaps could be isolated from the vaccine by centrifuging and was a protein.

Brooks (13) showed that the substance increasing the opsonic index during the immunization process and in producing the antiplague serum is a nucleoprotein, which can so easily and completely be isolated from the P. pestis cells that the water used to wash the of the P-1/2 fraction was dissolved in water and precipitated upon the 1/3 saturation with sodium sulfate, obtaining the fraction P-1/3.
In the filtrate of the last fraction on concentration of the salt increased to 50% saturation; the precipitate forming was designated as the P-1/2-1/3 fraction. The fifth fraction, not precipitating upon complete saturation by salt, was called the residual. The protein fractions were purified by reprecipitation, and by careful dialysis against distilled water, and were stored in dry form.

**SCHEME OF FRACTIONATION OF FILTRATE PROTEINS OF THE PIAGUE BACILLUS CULTURE ACCORDING TO SEAL (19)**

Bacterial Filtrate

```
Saturation ↓ of Na2SO4

Precipitate (contains 4 fractions) Filtrate (contains the fifth fraction)

1/2 saturation ↓ Na2SO4

Precipitate (fraction 1/2) soluble in water Filtrate

1/3 saturation of Na2SO4

Precipitate: fraction P-1/3 soluble in water Filtrate

1/2 saturation Na2SO4 fraction P-1/2 clear

Precipitate: fraction P-1/2-1/3 soluble

(Antigen A) (Antigen B)
```

The P-1/2 fraction represents 75% of all the salted-out proteins and exhibited serological activity. About 90% of this fraction consists of the P-1/3 fraction proteins, also serologically active. The other fractions, with the exception of the residual, also proved to be serologically active.

The author obtained similar active protein fractions from the water-soluble extracts of virulent plague bacilli, grown in agar containing casein hydrolysate at 37°C for 72 hours, since virulent strains usually grow poorly in [purely] agar media. But in the given instance the P-1/3 fraction represents 80-90% of all the salted-out proteins, and it yielded a precipitate with the antiplague serum in a dilution of 1: 1,280,000. But this fraction
is practically absent in the avirulent and nonprotective strains of  
_ _ F. pestis_ and is wholly absent in all the tested strains of  
_ _ F. pseudotuberculosis._

The chemical characteristics of the protein fractions are  
limited by the determination of the amount of nitrogen which varies  
within the limits of 14.9-15.6%, and by the qualitative reactions  
toward sulfur, phosphorous, tryptophan, tyrosine, and the Mohr  
and biuret tests. These reactions proved to be positive almost in  
all instances. On the P-1/2 fraction, obtained from the protective  
strain of _F. pestis_, grown on casein hydrolysate-containing agar,  
yields a negative reaction toward tryptophan. Tryptophan has not  
been discovered in the P-1/2 fraction from a single strain of _F.  
pseudotuberculosis_. The water solubility of all the protein  
fractions obtained was low and usually did not exceed 1:500. This  
solubility was increased at pH = 7.8-8.0 and was good in a N/80  
solution of NaOH.

It is practically impossible to investigate the rotation of  
polarized light using protein fraction solutions owing to the strong  
opalescence of the solutions. In spectrophotometry of 0.01% solu-

The P-1/3 fraction from virulent and avirulent, but protec-  
tive (inducing immunity) strains of _F. pestis_ designated as antigen  
A, is serologically distinct (does not yield overlapping precipita-
tion) from the P-1/2-1/3 fraction of the same origin. The latter  
has been called antigen B. In addition, antigen A differs serologi-
cally from the same fractions of P-1/3 obtained from nonprotective  
avirulent strains of _F. pestis_ and from strains of _F. pseudotuber-
culosis_, which are almost serologically identical to each other  
and yield overlapping reactions with antiseraums against antigen  
B and against virulent cells of _F. pestis_ inactivated through  
heating.

Consequently, antigen A is a specific antigen of virulent  
plague bacilli and corresponds to the membrane antigen described  
earlier by Schutze (21), since antigen B is a total somatic linking  
the plague and the pseudotubercular bacilli. Both antigens A and  
B can be isolated from the filtrates of broth cultures, but only  
antigen A can be obtained from water-soluble extracts of agar cultures  
of virulent _F. pestis_, which antigen also must be considered as a  
specific soluble antigen of virulent bacilli of the plague. An
antiserum prepared to act against this antigen agglutinates only the protective strains of P. pestis and absolutely does not agglutinate the strains of P. pseudotuberculosis.

Later, Seal (19) established that the virulent strains of P. pestis as a result of many passages through nutrient media gradually lose the antigen A until it disappears completely. To obtain the latter, the cells must be cultivated in a fully usable nutrient medium, enriched with casein hydrolysate.

Amies (22) has asserted that the widely accepted representation of the membrane of the P. pestis incorrectly reflects the essence of the problem. This membrane, in his opinion, is only a well developed capsule, visible in the dark field in preparations treated with India ink. The capsule substance, in the opinion of Amies, separates from the surface of the cell through a definite chemical reaction resulting from the use of a water solution of potassium thiocyanate. The substance obtained as a result of this extraction following purification and concentration, is a bacteria exhibiting the properties of the most important antigen of the P. pestis cells. Several milligrams of this antigen constitutes a dose, sufficient to produce powerful immunity in experimental animals. The chemical characteristics of this antigen are insufficiently clear (there is no certainty that it contains polysaccharide), but the author by analogy proceeds from the fact that the capsules of pneumococci and Klebsiella pneumoniae, consisting of polysaccharides, are not extracted by solutions of potassium thiocyanate, since the polypeptide capsule of B. myxoides is soluble under these conditions.

Englesberg and Levy (23) have presented various evidence, including experimental, that refutes the point of view of Amies. These authors used the A-112 avirulent strain of P. pestis, in the cultivation of which it was possible under selected conditions to obtain yield of four billion to five billion cells per one ml of medium with an exceptionally high formation of antigens. The medium contained: 1% casein hydrolysate, 0.2% glucose, 0.1% MgSO4, 7H2O, 2 x 10-4% FeCl3·6H2O, 1 x 10-3% CaCl2, 0.1% NH4Cl = NH4Cl, Na2HPO4, and N/20 phosphate buffer at pH = 7.0. Under aeration conditions the maximum number of cells was obtained on the third day of growth, after which their massive lysis set in, terminating on the sixth day. During the course of these six days not fewer
than three different antigens developed in the medium: fraction 1 (corresponding to antigen A of S. aureus), somatic antigen (probably, corresponding to antigen B), and a toxic fraction.

The largest appearance in the medium of fraction 1, relatively free from the somatic antigen, was noted in the three-day culture. The somatic antigen fraction appeared in the medium during the lysis period, that is, by the fourth to sixth day of cultivation. The somatic antigen yield was determined from the difference between the total amount of soluble antigens in fraction 1, based on turbidity using a specific antiserum. Of course, the presence of a plague toxin fraction, yielding a precipitate from the given serum, altered the preciseness of these determinations, but the quantity of this precipitate was very substantial and could not strongly distort these results. Small amounts of toxin, determined by tests in vivo, appeared in the medium both during the course of the growth phase as well as during lysis.

These authors showed that in a three-day culture there is contained about 50% of all the soluble antigens, hence, they separate by themselves in the medium; the remaining 50% can be obtained by salt solution extraction of the cells, followed by drying of the cells with acetone and an additional extraction. As far as fraction 1 is concerned, it is distributed thusly: 86% appears in the medium, a salt solution extracts an additional 13%, and the remaining 13% can be extracted by the same salt solution, but from cells previously dried with acetone. The quantity of fraction 1, remaining combined with the cells, is a constant value. Only the quantity of this fraction entering into the medium is subjected to fluctuations.

The facility with which fraction 1 enters into the medium during the active growth phase from P. pestis cells is similar to that which has been observed by Dochze and Avery (24) for pneumococci yielding a soluble antigen in the nutrient medium. This fact testifies in favor of the proposition that fraction 1 in the bacillus of the plague is a mycoid substance not of a capsule but of a membrane. The latter differs from a capsule in that it is an exudate, enveloping the body of the cell as a continuous mass of mucin (51). The membrane changes in outline and thickness and is not sharply bounded off from the surrounding environment. Evidence of various kinds supports the proposition that this membrane is a membranous substance of the plague bacillus or at least is a constituent part of the membrane. Fraction 1 and the membrane are formed simultaneously.
in a greater number of cells grown at 37° than those cultivated at a lower temperature. All the virulent and protective (immunogenic) strains of P. pestis exhibited a well-developed membrane and form correspondingly substantial amounts of fraction 1, since all avirulent strains of the membrane have none at all or exhibit only very weakly developed membranes and, corresponding to this, form very little fraction 1.

The immunity of laboratory animals to plague is directly related to the amount of antimembrane or anti-fraction 1 antibody, forming in the serum of these animals. Rabbit or monkey antisera, produced through immunization by fraction 1, is capable of changing the membrane of the virulent plague bacilli forming precipitates in it. But these immune sera, after the antibodies acting against fraction 1 are removed from the sera through specific absorption, lose the ability to form precipitates in the cellular membranes.

Opposing the theory of Amies, testifying to the absence of any myxoidal membrane and to the presence of an actual capsule in the plague bacillus are also the following considerations. To rupture the capsule, yielding a powerful antigen, requires rapid chemical or physical reactions. Amies "successfully" used a potassium thiocyanate solution in this capacity. But this author in his experiments did not consider the ready solubility of the membrane substance surrounding the P. pestis cells. Englesberg and Levy repeated the Amies experiment, but the suspensions of cells in distinction to the Amies experiment were extracted beforehand with distilled water. This experiment showed that 96% of fraction 1 and 8.9% of all the extracted soluble antigens were collected immediately through use of distilled water even before the beginning of extraction with the strong-acting potassium thiocyanate. Consequently, only 1% of fraction 1 was liberated additionally from the cells from infusion in water at pH = 7.8, or in the potassium thiocyanate solution. As far as the other antigens were concerned, the additional infusion of the cells in water at a pH = 7.8 additionally liberated 11% of all the soluble antigens, since the additional infusion in the potassium thiocyanate solution liberated additionally only 6% of the soluble antigens.

Preparations of the cellular suspensions in the India ink medium revealed a clear corona surrounding the bacilli not washed with water. This corona distinctly decreased upon standing in water. Similar preparations of cells, taken directly from the
surface of the agar, did not reveal clearly pronounced capsules in
the presence of the mucin Lsliz' mass in the various irregularly
formed inclusions in many cells.

Thus, the P. pestis cells exhibited readily soluble jelly-
like membranes surrounding them. This fact has been confirmed by
electron-microscopic observations. The membrane dissolves readily,
leaving behind a small amount of material which under microscopic
investigation appears to be similar to the typical bacterial capsule.
The membrane residue enters solution with difficulty, probably as
a consequence of its intimate bond with the bacterial cellular wall.
In this instance the question arose as to whether or not a difference
exists between the readily soluble jelly-like membrane substance
(fraction 1) and the "capsule" substance, strongly bound with the
cells. Inasmuch as fraction 1 readily enters into solution, it
can be assumed that part of it remaining on the cells is an impurity
to the pure capsule substance. The antigen, extracted by the
potassium thiocyanate solution, is most probably not capsule
material, since the solution of the capsule occurs by another
method. The fact that following solution of the membrane a certain
amount of antigen is additionally extracted can be treated as the
solution of only that portion of fraction 1 which is more stably
bound with the cells. However, here in addition to antigen 1 of
the fraction other antigens differing from this one are extracted.

Consequently, the problem of the existence in addition to
the membrane of the capsule and special capsule antigens in the
P. pestis cells awaits further elucidation.

Supplementing the clearly inadequate chemical characteristics
known thus far of the plague bacillus antigens, Ye. M. Gubarev,
S. I. Zaplatina, A. M. Konova (25) investigated several fractions
of these cells. In the present instance the avirulent vaccine
strain EV of the P. pestis and the No 177 virulent strain were
grown on solid agar medium, consisting of aminoacids obtained
through the complete hydrolysis of casein with the addition of
glucose, mineral salts, and a very small amount of yeast autolysate.
The cells obtained after a three-day growth were destroyed by
repeated chilling in liquid air and thawing out at a temperature
of 40°.

The products of cell decomposition were fractionated by
means of salting-out ammonium sulfate. In this way, the following
fractions were obtained: A - at 33% saturation, B - between 33
and 50%, C - between 50 and 75%, and D - at 100%.

The toxicity of the fractions for white mice is not identical. The most toxic are fractions C (at 1%N/0.001 mg of nitrogen) and B (at 1 DIM/0.005 mg of nitrogen), since fractions A and D are considerably less toxic. The toxic properties of all fractions disappear after a minute's heating up to 100° or following a three-day treatment with 0.3% formalin solution at 37°.

The most interesting results were obtained in a comparative investigation of the aminoacid constitution of the hydrolyzates of the three first fractions by the paper chromatography method. The identically obtained fractions of the virulent and avirulent strains exhibited a characteristic difference.

Thus, fraction A from the virulent strain No 177 contains the following aminoacids: glutamic, aspartic, serine, glycine, alanine, norleucine, valine, lysine, arginine, leucine, L-aminobutyric, isoleucine, ornithine, methionine, proline, and one unidentified aminoacid (16 in all) of the number of those identified only the first of ten aminoacids were found in fraction A from the avirulent EV strain, in addition, tryptophan and threonine were found, hence, a total of 12 aminoacids.

The B fractions from both strains also contain a group of ten aminoacids in common: glycine, alanine, norleucine, aspartic, glutamic, serine, tyrosine, arginine and valine. But fraction B from the virulent strain No 177 in addition contains ornithine, phenylalanine, L-aminobutyric acid and tryptophan (a total of 14) since the analogous fraction from the avirulent strain, in addition to the 10 in common, contains two other aminoacids, leucine and isoleucine (a total of 12).

There is also a difference in the C fractions, although the total number of aminoacids here is the same. Nine aminoacids were found in common here: glutamic, glycine, serine, alanine, arginine, tyrosine, leucine, isoleucine, and methionine. But fraction C of strain No 177 contains in addition: ornithine, cysteine, and valine, while the same fraction from the EV strain, in addition to the nine in common contains still other aminoacids: aspartic, histidine, and threonine.

Thus, the studies cited below of Chan and Mayer (31), in which a link was shown between the virulence of the strains and
the amount of protein in the cell membrane, is supplemented by the fact that the virulence of the plague bacillus strains can depend also on the aminoacid constitution of the proteins.

Seal (26) isolated a specific polysaccharide from specific soluble and nonfractionated substances of the plague bacillus. This polysaccharide was obtained also from substances contained in the filtrates of P. pestis cultures, raised on casein hydrolysate broth. In the hydrolysis products of the isolated polysaccharide preparations arabinose was found, identified in the form of the crystalline osazone.

Up till the present an explanation has not been forthcoming as to whether polysaccharides exist in the composition of P. pestis cells in the free state. As a result of successes multiplying from year to year, it has become increasingly clear in the study of the chemical composition of bacteria that all the constituents of the cells to a certain extent are bound up with proteins forming complex compounds. But the degree of attachment of these bonds for various substances is dissimilar. Obtaining polysaccharide fractions from the plague bacillus, as a rule, has been stimulated by investigations studying the total somatic antigen. For this purpose the methods of Ruavena-Meazravenau (extraction of the antigen by trichloroacetic acid) and of Raystricka-Topli (trypsin hydrolysis of cells with subsequent precipitation of the antigen by alcohol), in various modification, are usually applied.

Ye. I. Korobkova, V. Kuznetsova, Ye. Bakhraka, A. Shalayeva (27) obtained such a complex antigen in the unpurified form, using the Raystricka-Topli method. The substance obtained yielded qualitative reactions for proteins and carbohydrates. Boiling a solution of the given substance in acetic acid permitted a separation of the protein fraction, forming a precipitate, and from the soluble portion of this hydrolysate most probably a polysaccharide was precipitated by alcohol. The polysaccharide in the dry form is a white powder, readily soluble in water, not precipitable by trichloroacetic acid and yielding a strongly positive Podobedova-Molish test. Upon hydrolysis of the polysaccharide with sulfuric acid, reducing substances were formed. The polysaccharide was precipitated by antibacterial antiplague serum in a dilution of 1:10,000, and with the same dilution resulted in a precipitation with rabbit antiserum, obtained through immunization by the trypsin hydrolysate of the P. pestis cells. Based on the fact that the P. pestis cells grown on an agar medium produced a two-fold yield of polysaccharide in comparison
with cells grown in broth, the authors proposed the existence of two polysaccharides — one in the capsule substance, and the other in the cell plasma.

S. I. Zaplatina and A. M. Kornova (28), using various methods, obtained polysaccharide preparations from two strains of P. pestis: one virulent and another avirulent vaccine EV and, in addition, from a P. pseudotubercolosis strain. The polysaccharides obtained by these authors were water-soluble substances, yielded a strongly positive Podobedova-Molish test, were not precipitated by trichloroacetic and sulfosalicylic acids, and did not show a biuret reaction. Not one of the polysaccharide preparations obtained revealed toxicity for guinea pigs and white mice. The polysaccharides were precipitated by antiplague serum in dilutions not exceeding 1:1,000 -1:2,000.

Boyden (29) showed that sheep erythrocytes, treated with a dilute tannin solution (1:15,000, 1:20,000), were sensitised upon being treated with soluble protein antigens of P. pestis, after which they are agglutinated by specific antiplague antiserum. But the same protein antigens do not react with normal erythrocytes. However, the polysaccharide antigen from P. pestis is adsorbed by normal erythrocytes.

Evidently, a similar polysaccharide antigen from the plague bacillus was investigated by Chan (30), establishing the ability of his antigen to be adsorbed by normal erythrocytes of sheep, chicks, and guinea pigs, and to yield hemagglutination in the presence of a specific antiserum. This antigen was found in old P. pestis cultures, in extracts from acetone killed dried plague bacilli, and in alcohol precipitated membrane antigens. But this antigen was not found in the purified protein fraction, isolated through salting-out with 30% saturated ammonium sulfate from the water-soluble extracts of the plague bacillus. The antigen tested contained little nitrogen and was suggested to be included among the polysaccharides. It did not increase the resistance of mice to plague infection, but proved to be useful in the serological investigation and identification of fractions isolated from P. pestis cells.

The attempts to sensitise normal erythrocytes with highly purified protein fractions 1-A from P. pestis proved to be unsuccessful. Normal erythrocytes, sensitized by polysaccharide antigen, did not yield hemagglutination with rabbit antiserum against fraction 1-A (protein). During additional tests of the
serum eight rabbits, hyperimmunized by protein fraction 1-B from
P. pestis, with an agglutin titer of 1:32-1:128, and with a comple-
ment fixing titer of 1:8-1:256 did not agglutinize erythrocytes
that had been sensitivized by the polysaccharide antigen of the
plague bacillus. In this way, hemagglutination of erythrocytes
sensitivized by the plague bacillus polysaccharide could not be
used for diagnosis, since the polysaccharide could not be used for
diagnosis, since the polysaccharide antigen itself is not suitable for protective
immunization against plague infection.

Chen and Mleyer (31) studied the hemagglutination of the tannin-
treated and protein antigen 1-B-sensitivized sheep erythrocytes.
The antigen was prepared by salting-out of the water extract from
the P. pestis cells with ammonium sulfate at 33% saturation of the
salt. Following frequent purification through reprecipitation
the antigen 1-B was obtained as a protein preparation, completely
free of carbohydrates. The agglutinating serums were prepared by
immunization of rabbits with the antigen 1-B. Through comparative
studies the advantages of the given hemagglutination over the
reactions of complement fixation and the bacteria agglutinations
were established. Hemagglutination is extremely more sensitive
than the above-mentioned reactions and permits the detection of
high titres of antiplague antibodies both in animals which have
had the plague as well as for people immunized by the antigen 1-B
in those cases when these antibodies have not been revealed at all
by the complement fixation reaction. Among the serums of animals
immunized by the various live and killed virulent vaccines, the
hemagglutination titer as a rule proved to be higher than the titer
of the complement fixation or the plague bacteria agglutination.
An additional advantage of the hemagglutination tests for diagnosis
consists in the possibility of determining the presence of plague
antibodies in the anti-complementary serums such as, the anti-plague
Haffkine horse, which cannot be tested by the complement fixation
method.

Protein hemagglutination is especially useful in conjunction
with the polysaccharide hemagglutination reaction. Inasmuch as
both reactions are strictly specific either for the protein or the
polysaccharide component and the corresponding antibodies, the
various plague antigens can be subjected to direct or indirect
analysis by serological methods. Direct analysis of the antigens
is preferable in the determination of the relative purity of such
preparations as fraction 1-B or the polysaccharide fraction in-
soluble in carbolic acid.
The results of similar investigations have shown that the amount of protein contained in the plague bacilli have a direct relationship to the virulence and avirulence of a strain. These bacilli are surrounded by a protein membrane having a different thickness for different strains. It is suggested that the polysaccharides either form a layer located under the protein layer or that they are found within the cells and can be liberated during lysis caused by natural or artificial factors. Immunological research of various P. pestis antigens has permitted a judgment of the link between the composition of these antigens and the virulence of the cells. As a rule, the serum of rabbits immunized by live avirulent vaccines contain a negligible amount of almost no antibody against membrane protein fractions, while at the same time abound in antibodies acting against the polysaccharides. This fact indicates that in avirulent strains, the amount of protein antigens is reduced, and perhaps even the total amount of protein, with a simultaneous increase in the amount of polysaccharide antigens. The sera of animals immunized by live virulent strains of P. pestis contain, on the other hand, a large amount of antibodies against proteins and react weakly or not at all with the polysaccharide fraction of the plague bacillus. This points to an increase of protein antigens in virulent strains. Finally, the sera of the rabbits immunized by boiled and dried extracts of virulent plague bacilli contain considerable amounts both of anti-protein as well as of anti-polysaccharide antibodies. The latter indicates that virulent microbes, as a result of the extraction, yield greater amounts both of protein as well as of polysaccharide antigens.

The above indicated results of research on antigen properties of various strains using serological methods have filled the gap in chemical research on this problem. The conclusion can be drawn that live avirulent plague cultures are readily distinguished from polysaccharide cells because the protein membrane of these strains is very insignificant, thin, and is readily removed. In their turn, virulent cultures yielding few polysaccharide and many protein antigens do not exhibit difficulty accessible polysaccharides, which in these strains are surrounded by a massive protein membrane. It can also be assumed that the quantitative content of polysaccharides is approximately the same for virulent and avirulent strains, since the killed virulent cells yield considerable amounts of protein and polysaccharide antigens due to the fact that here the massive protein membranes have been substantially damaged and dissolved by
boiling, and the polysaccharides are liberated without difficulty. Consequently, the most essential difference between the P. pestis avirulent and virulent strains, consists in that the latter have greater quantities of protein in the membrane.

The result of research into the various strains of P. pestis using serological methods, allows us to state important conclusions on the different behavior of avirulent and virulent strains in the infection of the animal organism. The difference in the membrane composition for virulent and for avirulent strains, cannot be without significance for the phagocytosis at one or the other. Even Meyer (32) showed that phagocytosis of plague bacilli is suppressed by an unchanged membrane substance containing more than 10% fraction 1 protein, whereupon the addition to the culture by the 1-B protein fraction. Inasmuch as the avirulent and virulent strains, as a rule, form toxin, in the unlimited multiplication of the cells, an identical toxemia from infection by virulent and avirulent cultures must be anticipated. However, following infection by an avirulent strain containing a quantity of fraction 1-B that is inadequate to suppress phagocytosis, cell multiplication proves to be limited to such a degree that neither toxemia nor affection sets in. In addition, following infection by a virulent strain of the plague possessing a thick protein membrane containing more than 10% of fraction 1-B protein, the latter sharply suppresses phagocytosis, which produces favorable conditions for rapid multiplication of cells and rapid liberation of the toxins by the aging cells. Here, consequently, infection has all the possibilities for rapid propagation in the organism with all the resultant consequences. Thus, the virulence or avirulence of a given P. pestis strain depends correspondingly on the ability or inability to suppress phagocytosis, which in its turn depends on the amount of fraction 1-B proteins in the cell membranes.

A substance located under the upper protein layer of the membrane, and immediately enveloping the P. pestis cells has been identified as a polysaccharide antigen, incapable of protecting mice from infection. However, cells breed of the readily soluble protein membrane substance exhibit protective properties for guinea pigs. From these considerations a study of the immunological properties of the polysaccharide isolated from P. pestis was undertaken. To obtain the latter, the cells were treated with carbolic acid, used to remove the proteins. The carbolic acid-insoluble fraction extracted with water contained a large amount of carbohydrates with a negligible nitrogen content. The polysaccharide obtained in the
pure form after addition to normal erythrocytes provided a sharply
pronounced hemagglutination with anti-plague serum. However, immu-
nization of guinea pigs with the polysaccharide did not increase their
resistance to plague infection, the immunized animals perishing
identically as the control. Hence, the protective effect of
antigens of plague bacilli from which the membrane protein has been
removed must involve a certain amount of protein antigen remaining
after treatment of the cells, but not through the agency of the
polysaccharide.

In a study by V. G. Aldinenko (33) several general indices
of the chemical composition of two P. pestis strains were investi-
gated: a virulent (No 291) and an avirulent (EV). The author
cultivated the strains tested under wholly identical conditions on
agar containing Harten's broth for two days at 28°C. Following this,
the culture was washed free of the agar surface with a physiological
solution of sodium chloride, and the cells were repeatedly washed
by the same solution in centrifuging and were dried at 56°C.

Several comparative indices of the chemical composition of
both strains were shown to be identical or similar (the amount of
ash, the total nitrogen content, the nitrogen content in the pro-
teins extracted with diluted KOH, and the amount of tryptophan and
tyrosine in these proteins). However, very many composition indices
for the strains studied proved to be different. Thus, 6.75% lipoids (ether extract) were found in the EV strain, and 6.07% in
the No 291 strain. The carbohydrate fraction was found to be
5.33% for the EV strain and 6.33% for the No 291. And even greater
difference was established for the total phosphorus content — 1.13%
(EV) and 0.62% (No 291) — and for the phosphorus of the lipid
fractions — 0.07% (EV) and 0.04% (No 291). In addition, it was
shown that the protein fractions obtained through precipititation
at the isoelectric point are acidic proteins. Thus, their content
of nitrogen of the dicarboxylic fraction of the aminoacids (in
terms of all the nitrogen of these proteins) was as follows: 31%
(No 291) and 26.8% (EV), since the nitrogen of the diaminocid
fraction equalled 17.33% and 18.1%, respectively.

The difference in chemical composition found for both strains
does not explain, of course, their widely diverse virulence. Such
a difference, possibly, could be detected also for two strains of
the same virulence, however, the very fact of the clear distinction
shown by these two strains in several basic chemical composition
indices is of great interest as evidence of the essential mutability of the given bacillus. It cannot be doubted that basically the extensive mutability of the P. pestis in the formation of strains of this bacillus having very diverse biological properties consists in the variability both qualitatively and quantitatively of the chemical composition of the cells.
The plague bacillus is a typical gram-negative cell with biochemical properties inherent to this group of microorganisms.

Being not strictly aerobic, this bacillus, however, grows considerably more favorably under conditions of adequate access to air oxygen than would appear, evidently, to explain the favorable influence on the growth of the plague culture when oxyhemoglobin being present in the nutrient medium in quantities surpassing by many times those which usually characterize it as a growth factor. And in general it has not been established that oxyhemoglobin is a necessary growth substance for these cells.

Under relatively anaerobic conditions (under a paraffin oil layer) the plague culture develops poorly. Here it must be emphasized that if under aerobic conditions the plague bacillus is somewhat capricious toward composition of the nutrient medium, then in the absence of molecular oxygen satisfactory growth can be obtained only in media containing carbohydrate brewed in the media. It is widely known that carbohydrates serve as the basic substances supplying energy under conditions of anaerobiosis.

The most favorable temperature for the growth of the plague microbe is 25-30°C, the optimal reaction for the nutrient medium is found in the interval of pH = 6.9-7.1.

The isoelectric point of the plague bacillus lies within the limits of pH = 4.0-4.4, typical of gram-negative microbes (N. N. Ivanovskiy, V. M. Tumanskiy, and V. A. Knyazeza, (34)).

As is widely known, one of the species characteristics of the plague bacillus is its ability to grow from artificial nutrient
media only from a culture of a considerable number of microbe bodies (not less than 10,000).

In the growth of the plague bacillus in ordinary solid or liquid nutrient media (the broths of Chottinger and Marten) a decrease in the oxidation-reduction potential of the medium occurs as a consequence of accumulation in the medium of reduction compounds. The nature of the these compounds is unknown. With an electrometric method it has been established that the optimal oxidation-reduction condition of the nutrient medium for the growth of plague culture lies within the limits of 100-150 mv (Ye. E. Bakhrakh (35)). The artificial decrease in the oxidation-reduction potential of the medium by means of addition of sodium bisulfite makes possible the growth of the plague bacillus from individual cells. Analogously, an extract of Sarcina and live Sarcina (the phenomenon of "kemwila" /small-scale culturing/). Thus, the value of the initial culture number of the same strain depends to a great extent on the properties of the medium in which its cultivation is carried out.

The initial culture number of the plague bacillus varies also as a function of the strain. In general strains oxidizing glycerine ("continental"), have a smaller initial culture number than strains which do not oxidize it ("oceanic").

The assertion that the initial culture number of the plague bacillus is almost independent of the medium pH and the cultivation temperature (Ye. I. Korobkova and Ye. A. Mitina (36)) has occasioned great doubt. The reliability of this suggestion appears doubtful in connection with the fact that the functional tie between the value of the oxidation-reduction potential and the medium reaction is well known:

\[
\text{pH} = \frac{\text{Eh}}{0.059} - 2\text{pH}
\]

Also widely known is the effect that temperature has on the value of the oxidation-reduction potential.

As a gram-negative microorganism the plague bacillus exhibits many properties inherent in this group. It is readily decomposed by trypsin, alkalis, easily forms of mucilaginous mass under the action of diluted alkalis, etc.

The hydrolytic property of the plague bacillus is manifested weakly and has been established only in regard to a few substances.
Thus, it has been shown that for the growth of the plague bacillus products of the profound degradation of proteins are necessary -- aminoacids and peptones (A. I. Bysterenin et al (37)). The plague bacillus, evidently, does not decompose proteins, at least so far as blood proteins are concerned (A. A. Trifonova (38)) and gelatine. It in general does not display the ability to hydrolyze urea in nutrient media (N. N. Ivanovskiy and G. N. Lenskaya (39)).

The hydrolytic capacity of the plague bacillus is also very restricted in regard to carbohydrate. The colloidal polysaccharides -- starch, dextrin, and imulin -- are not utilized by the plague bacillus. Evidently, this also is the case with glycogen, although several authors have stated the reverse; it does not hydrolyze, with the exception of several laboratory strains, saccharose, raffinose, and lactose (very weakly). Trehalose and maltose are utilized by the plague bacillus quite intensively.

Accepting the weak hydrolytic activity of the plague bacillus is to a considerable degree based on indirect data. This reservation must be made in connection with the fact that data still is not available on the existence in the plague bacillus of phosphorylase. Therefore, in several cases it is difficult to state along which pathway the substrate is utilized -- through hydrolysis or through the use of phosphorylase.

The plague bacillus exhibits sharply pronounced hyaluronidasie activity, which in virulent strains is considerably higher than in avirulent. The high invasion capacity of the plague bacillus is related to its ability to cleave the hyaluronic acid of the tissues of the animal suffering from plague (Ye. I. Korobkova (40)).

The plague bacillus oxidizes a sizable number of organic compounds -- monosaccharides, polyatomic alcohols, fatty acids, aminoacids, etc. utilizing these as energy sources and as plastic material. It oxidizes hexoses (glucose, fructose, mannose, and others), most pentoses, but also, as might be expected, the decomposition of the latter is accompanied by considerably more difficulty than in the other instances.

Monatomic alcohols (methyl, ethyl, propylens) and diatomic alcohols (ethyleneeglycol) are not oxidized by the plague bacillus; triatomic alcohols -- glycerine -- are not oxidized by all strains;
quadratomic (erythritol) and pentatomic alcohols (arabite, xylite) are not oxidized. Of the hexatomic alcohols the plague bacillus oxidizes manmite, sorbite, and does not oxidize dulcitol.

The products of plague bacillus oxidation of carbohydrates and polyatomic alcohols have remained almost completely uninvestigated.

The plague bacillus ferments glucose with the formation of involatile (90%) and volatile (10%) acids. Of the involatile acids succinic, malic, and citric acids have been identified (N. N. Ivanovskiy, V. S. Basheev [41]) have been identified as well as lactic acid.

Most of the aminoacids in the fatty series are intensively oxidized by the plague bacillus. The well-known generalization that gram-negative microorganisms do not utilize arginine is wholly confirmed also in regard to the plague bacillus — it does not oxidize this acid. Of the aromatic aminoacids the rule of thumb applied only in regard to tyrosine, which is oxidized by the plague bacillus very weakly. Heterocyclic aminoacids (tryptophan and histidine) are not oxidized.

Of the enzymes associated with oxygen respiration, catalase peroxidase (M. N. Dzhaparidze [42]), and cytochromoxidase have been discovered in the plague bacillus.

The dehydrases of the plague bacillus have been studies most inadequately. The existing material on this problem relates only to the ability of the enzyme to dehydrate various substrates under anaerobic conditions, in the presence of methylene blue.

Woodward [42] showed that live plague bacilli and cells killed with phenylmercuricnitrare, and also noncellular preparations decompose yeast ribonucleic acid (ribonucleic ability). Primarily, its action consists in depolymerizing the nucleic acid. In the analysis of its decomposition products hydrochloric acid precipitation and uranyl fractionating are employed. Based on the data obtained it has been concluded that only part of the nucleic acid is hydrolyzed to mononucleotides, for the bulk the process is limited in its depolymerization.

Even in studies of authors of some time ago it was noted that the plague bacillus grows considerably better on a nutrient
medium which has had blood or hemin added to it. These media were suggested for isolating the plague bacillus from weakly infected material. It was shown that hematin, cosinase, thiamine, and nicotinic acid was to be considered as weak plague bacillus growth stimulators. Moreover, it must be noted that in the action of the indicated substances there is no parallelism between growth intensity and respiration intensity. The most stimulating action on respiration is exhibited by nicotinic acid, the least — by hematin.

Rao (14) also undertook an attempt to establish the aminoacid requirements of the plague bacillus. This author showed that three aminoacids are required for the growth of the plague bacillus: proline, phenylalanine, and cystine. However, the author introduced into the composition of the synthetic nutrient medium for cultivating the plague bacillus a considerably larger number of aminoacids (15). Research done in the 1953 period has shown that for the growth of the plague bacillus a small number of aminoacids are sufficient, including the nonobligatory presence of most of their cyclic representatives. Moreover, it is important to emphasize that the aminoacid requirements for the plague bacillus vary as a function of the strain origin.

These data permit the conclusion that the aminoacid and growth substance requirements for gram-negative cells are not large, since they can be synthesized by the cells themselves.

The plague bacillus does not contain an essential number of free aminoacids in view of the fact that gram-negative cells do not require them to be accumulated in protoplasm.

Plague bacillus strains of various origins respond differently to ammonia, nitrites, and nitrates. Some of these oxidize ammonia to nitrous acid, others have the capacity to oxidize it to nitric acid, but the capacity of many plague bacillus strains to reduce nitrates to nitrites is also known (S. F. Konovalova (16), N. V. Uryupina (17)).

Some data found only in the study of Ye. M. Gubarev and T. I. Lipatovaya (18) deal with the mineral nutrition of the plague bacillus. Special attention in this study is attracted to the favorable effect of ammonium and manganese ions for the growth of the plague bacillus, which ions when present at a concentration of 8.3 millimoles in the medium in the form of hydrochloric salts significantly increase the development of the plague bacillus (ammonium ion by
47.7% and manganese ion by 70.7%); a sharp suppressing action is exerted on the process of bacillus multiplication by the iodide ion, but sodium sulfate strengthens the growth of the plague bacillus.

Several strains from the "continental" group exhibit the capacity to form hydrogen sulfide during growth in media containing cystine, cysteine, and methionine. The division of microorganisms into two different groups -- autotrophes and heterotrophes, as is known, is not strict. The plague bacillus -- a "typical" heterotroph -- exhibits the capacity to form nitrites from ammonia, and, probably, uses carbon dioxide (see below for more detail).
The empirically discovered method of staining bacteria, that was also suggested by H. Gram, has over the course of the years after its discovery attracted the attention of numerous investigators due to the fact that it affords a convenient test in the classification of microorganisms.

The relationship of various bacteria to the Gram staining has at present acquired the significance of an important systematic feature of the bacteria, according to which all microorganisms are divided into two groups: gram-negative and gram-positive. This division reflects profound differences in the structure of cells, expressed in the most diverse ways. The properties of bacteria: tinctorial, reaction to acids and alkalis, penetrability of their protoplasm, resistance to poisonous substances, antigenic structure, character of toxins, etc. — differ strongly for gram-negative and for gram-positive microorganisms. However, it must be kept in view that the distinction between gram-negative and gram-positive bacteria is not absolute. The indicated groups are related to each other by gram-unstable microorganisms occupying an intermediate position.

To understand the essence of the structural difference between gram-negative and gram-positive microbes it is necessary to know the chemistry of staining that employs this method. Thusfar there are not to be found adequately complete data explaining in detail this complex staining process. It has been possible only to establish that gram staining of bacteria is associated with the structure of their nucleic acids, entering into the composition of nucleoproteids or partially found in the cell in a free state.

As is widely known, two types of nucleic acids are contained in the composition of each bacterial cell: ribonucleic (cytoplasmatic...
and deoxyribonucleic (nuclear). Bacteria stained according to Gram are characterized by a high content of ribonucleic acid and a relatively low content of deoxyribonucleic acids. The ratio of these acids in gram-positive bacteria is approximately 8:1, and for gram-negative the same ratio is 1.3:1. Thus, gram-negative bacteria contain both kinds of nucleic acids in quantities much closer to each other than exists in gram-positive bacteria.

When staining according to Gram, the chemical composition and condition of the surface layer of the bacteria is especially important (and decisive). For gram-negative microbes the surface layer of the cell contains a protein-ribonucleic complex.

It has also become clear that the isoelectric points of gram-negative bacteria are grouped around pH = 5 but the isoelectric points of gram-positive bacteria—about pH = 2. In other words, the protoplasm of bacteria stained positively according to Gram have a more acid reaction than for bacteria not stained according to this method. The plague microbe is gram-negative. However, cells from young cultures are frequently weakly stained according to the Gram method.

As is known, in the growth process of the bacterial cell the value of its negative charge changes; young cells are more electronegative than adult, and the isoelectric point of the protoplasm of young cells shifts towards the acid side in comparison with old cells. The high content of nucleic acids characteristic of young growing cells probably explains their high capacity to fix with basic stains, that is, their increased basophilicity.

The often observed phenomenon that individual undamaged cells of a plague culture are stained weakly positively according to the Gram method wholly corresponds to the considerations presented above. Staining of individual specimens to a certain extent characterizes the plague microbe as a gram-positive cell. The relative gram-instability of the plague bacillus is confirmed also by the determination of its isoelectric point which lies in the interval of pH = 4.4, that is, in a region adjoining the transitional zone, but closer to the group of gram-negative microbes.

The presence of nucleic acids of the protoplasmic type in the plague bacillus was discovered by way of the Feilgin reaction by Ye. I. Korobkovaya (49), L. V. Lugovaya and Ye. A. Lebedevaya,
V. G. Chernobayev (51), and others. Later, N. N. Ivanovsky (52) carried out a quantitative determination of the nucleic acids (based on phosphorus) in the dried and defatted bacterial bodies. According to his data, the plague bacillus contains approximately 11% nucleic acids on a dry weight basis of the bacterial mass.

To establish the ratio of ribonucleic and deoxyribonucleic acids in the plague bacillus composition N. N. Ivanovsky used the method suggested by Robineau. This method made possible a roughly quantitative distinction in the protoplasm of two types of nucleic acids, which is important in solving several physiological problems such as, for example, the nature of the growth and the development of the cells, the influence of the nutrient medium, composition and other phenomena related to the ratio of both kinds of nucleic acids. The Robineau method applicable to the plague bacillus yielded satisfactory results. The plague bacillus stains readily with various basic aniline stains, in which its preliminary tarmin treatment does not reflect on its ability to receive the stain, a characteristic for gram-negative bacilli.

In the specialized literature on the biochemistry of microbes it is frequently noted that gram-negative bacteria differ in their chemical composition from gram-positive.

As has been noted above, gram-negative microbes contain ribonucleic and deoxyribonucleic acids in amounts closer to each other than observed for gram-negative bacilli. Evidently, gram-negative bacteria include lipids of unsaturated fatty acids in lesser amount than those compounds are to be found in gram-positive microbes.

Considerable interest lies in oxystethylamine (colamine) as a constituent of lipids. It is absent in gram-positive and was discovered in an ether extract of gram-negative bacteria.

The plague bacilli has been almost completely uninvestigated in this area, but enough has been done to afford a basis for asserting its inclusion among gram-negative organisms. Thus, the study of A. I. Bystrenin (53) has shown that "crude" fat, taken from the dried bacterial mass of the plague bacillus, contains fatty acids with low iodine number.

The different species of microorganisms exhibit dissimilar resistance to alkalis and proteolytic enzymes. Analysis of data
relating to this problem shows that gram-positive microbes are considerably more resistant to these agents than gram-negative. The latter are easily and readily subjected to decomposition both by alkalis as well as by proteolytic enzymes.

Even Lustig and Galeotti observed that a culture of plague bacillus is easily decomposed by a 1% potassium hydroxide solution. The decomposition at room temperature and is almost complete. Actually, the alkali concentration can be substantially reduced to 0.2-0.4% and even when cold effect a very intensive lysis of the culture.

Practice shows that digestion of plague bacillus by trypsin to produce a complete antigen according to the Raistrick-Topli method speedily leads to the decomposition of the cells.
The conditions for the existence of pathogenic microorganisms can vary within wide limits. In the organism of an animal susceptible to a given infection the causative microorganisms have conditions of life differing sharply from those which are created upon cultivating the organisms in artificial nutrient media. In the latter bacteria are subjected to the influence of everchanging external conditions. The qualitative and quantitative composition of the medium, the temperature, the pressure of oxygen and of carbon dioxide change, the active reaction of the medium varies, as well as the oxidation-reduction status of the nutrient medium, etc. Existing under the continuous influence of diverse altering physical and chemical factors of the external environment, the microbial cell rapidly responds with adaptation reactions. The adaptation of the microbial cell to the new conditions of existence occurs primarily through the rearrangement of its enzyme systems.

The flexibility and high mobility of the enzyme apparatus of bacteria assured it of the possibility of living in medium which is new to it.

Many published studies on various phases of plague microbiology testify to the capacity of the plague bacillus to alter its metabolism within wide limits. However, discussion of these studies has met with difficulties not only those related to the widely known complexity of the plasticity problem of microorganisms but especially due to the deficiency of experimental material which would yield to systemization and permit on this basis the drawing of generalizing conclusions.

According to the concepts presented it would appear profitable to devise a first experiment of the biochemical analysis of
plague bacillus variability in terms of the broadest cell metabolism factors. Here, experimental data would chiefly be used which summary reflects the reaction of the cell to be changing conditions of life without a special analysis of the possible deviations in individual processes of cell metabolism with which this reaction is associated. Included among the general reactions toward the external environment of the microorganism is primarily the change in the character of its oxidation-reduction processes. The plague bacillus, as a function of the conditions of its cultivation, employs a sometimes more and sometimes less pronounced anaerobic type of metabolism.

As is known, anaerobiosis in aerobic bacteria is a widespread phenomenon, with which microbiologists (facultative aerobes and anaerobes) have often dealt.

Aerobe-microorganisms obtain energy and construct their protoplasm as a result of complex processes of oxidizing carbohydrates and various nitrogen-containing compounds with the participation of molecular hydrogen. These substances can within certain limits substitute for each other in the processes indicated.

When oxygen is in short supply such a diversity of compounds participating in the cell metabolism is not observed. In anoxobiosis, as a general rule, carbohydrate metabolism prevails strongly over all the other forms of metabolism. Nitrogen metabolism is reduced considerably, and oxidation of fat is noted only as an exception.

The shift in aerobic metabolism toward the side of greater anaerobiosis in bacteria has been observed in those cases when the oxygen employed due to various reasons was reduced, but the energy requirements for the metabolism remained as before. Most commonly the factor for decreased use of oxygen by the microorganisms is its low concentration in the medium. However, even with a sufficiently high oxygen content in the medium surrounding the cell, hypoxia and even anoxia can occur in the cell owing to disturbance of the aerobic oxidation mechanisms. This has been observed, for example, in the action of poisons (carbon monoxide, cyanide, salts, etc.), when the salt concentration in the medium is change, etc.

Interesting data is available on the problem of the use of oxygen by bacteria during starvation and at various cultivation temperatures of the bacteria. It has been shown that well-nourished cells require oxygen at a considerably higher level than do cells experiencing deficiencies in nutrient.
Thus, Cahn and Bronner (54) indicated that the poorer the medium is in nutrients, the more profound growth is noted for an intestinal bacillus, that is, in other words, metabolism in the intestinal bacillus becomes more anaerobic. This regularity has been observed for many microbes, including also pathogenic.

Changing the partial pressure of oxygen in the gaseous mixture over the nutrient medium can sharply alter the extent of profound growth of the microbe. Upon reducing the partial pressure of oxygen growth becomes more superficial and, on the other hand, upon increasing the partial pressure of oxygen growth becomes more profound.

The same authors believe that an increase in oxygen sensitivity arises in microorganisms as a result of a carbon deficiency in the source. In general the smaller amount of carbon source in the medium, the more vigorously does the inhibiting action of oxygen on the growth of the microbial cell appear.

Location of the zone of microbe growth in a liquid nutrient medium depends also on temperature.

Upon reducing temperature the growth zone subsides (a more anaerobic process), moreover, this zone subsides also when the temperature lies above the optimal level for the development of the given microorganism. Attempts to explain the phenomena described by a constant value for oxygen diffusion in the cell and, consequently, to establish a relationship between the entrance of oxygen and the size of the cell surface (during starvation the cell's surface is reduced) have proven to be unworkable.

The intensity of metabolism, the medium pH, the carbon dioxide pressure, and, finally, the temperature at which the culture is grown all exert an influence on the process of oxygen use in the cell.

The oxygen requirement is reduced to a minimum if the cell resides in a liquid medium containing a very low salt concentration, that is, here partial anaerobiosis appears. This position, evidently, is wholly substantiated. Finally, it has been shown that vitamins play an exceptionally great role in the life of bacteria.

By means of vitamins the metabolism of the cell can be altered, regulating the synthesis and storage of fat by the cells,
as well as of glycogen, nucleic compounds, altering the development of cellular structures, forming specific enzymes, etc. Thus, for example, an excess of $B_1$ in the medium (in the absence of antagonistically acting vitamins) causes in the metabolism of yeast, organisms even under aerobic conditions a sharp displacement toward the side of intensified fermentation without a noticeable decrease in respiration. The rearrangement of the structure and chemistry of the cell occurring in this instance is very reminiscent of what has been observed in its development under anaerobic conditions.

As evidence favoring the partial transition of the bacterial cell to anaerobic metabolism, we have the following:

1) accumulation in the medium of end products of the incomplete oxidation of organic substances, for example, organic acids,

2) increase in the quantity of required carbohydrates; and

3) increase in the value of the respiration coefficient.

The plague bacillus is not capricious towards the composition of the nutrient media in which it is cultivated, in that it readily and in great quantity yields morphologically altered forms. The polymorphism of cells, as is known, is one of the typical features of plague culture.

The capacity of the plague bacillus to grow in nutrient media of diverse chemical composition and its polymorphism evidence the high adaptative activity of the plague causative to new conditions of life created for it.

The variablility of the plague bacillus metabolism appears in the morphological features of its colonies ($S-, R-, O-forms$, etc.) and individually examined cells in biological (virulency) and biochemical activity.

It is unquestioned that the presence of shifts in the metabolism of the plague bacillus can be most easily perceived from morphological changes, for example, from the phenomenon of culture dissociation.

Dissociation of the cultures of microorganisms is an often-observed effect. It frequently appears in bacteria when they have
aged and as a result of unfavorable conditions of existence. Most authors have relegated this phenomenon of bacteriophagia to a special position.

In distinction to almost all gram-negative bacteria the plague bacillus under natural conditions exists in a rough form, adapting itself to conditions of parasitism. This feature explains many characteristics in the course of plague infection. Only a few authors have noted that they have been successful in isolating from wild rodents the smooth variety of the plague bacillus.

The plague bacillus is included among those species of bacteria which dissociate with difficulty into stable varieties.

The indicated ability explains its discovery under natural conditions in the rough form. A long series of transitional forms (O-forms) link the typical parasitic coarse form with the avirulent relatively stable smooth form. Various intermediary forms are extremely diverse in their morphology and are very unstable. This refers both to the transitional coarse variety, as well as especially to the transitional smooth.

Apparently, the stability of the smooth varieties increases as they approach in biochemical properties the pseudo-tubercular microbe, which under natural conditions is found in the smooth form.

It must be emphasized that cultures of various strains of plague bacillus undergo dissociation with dissimilar facility. In general the "continental" strains dissociate considerably more readily than do the "oceanic".

The membranes of the microbial cell which reflects changes in the metabolism occurring in the cell plays an undoubtedly visible role in the question of plague bacillus dissociation. The cellular membrane in the rough and in the smooth forms differs. It develops poorly in the smooth forms, but in the rough it is well pronounced.

Recently methods have been proposed with which the distinctions between the rough and the smooth varieties of the plague bacillus can be considerably more easily noted. Levine and Jarber (55) were able to readily identify the coarse and smooth colonies of P. pestis on tryptose (Difco) agar containing triphenyltetrazoliumchloride. For many other media such differentiation was very difficult.
A 1% aqueous solution of tetrazol was added to molten tryptose agar, this resulting solution was sterilized by auto-claving up to a concentration of 0.005%. About 20 ml of this medium were placed into a Petri dish and dried for 24 hours at 37°C. The dish was seeded with the coarse and the smooth forms of virulent and avirulent strains of P. pestis and incubated for four days at 30°C. At the end of this period the typically smooth colonies were circular in form and two mm in diameter.

GENERAL CHARACTERISTICS OF COARSE AND SMOOTH FORMS OF PLAGUE BACILLUS

<table>
<thead>
<tr>
<th>Coarse Form</th>
<th>Smooth Form</th>
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</thead>
<tbody>
<tr>
<td>2. Virulent.</td>
<td>2. Avirulent.</td>
</tr>
<tr>
<td>3. Immuneogenic.</td>
<td>3. Exhibits poor immunogenic properties.</td>
</tr>
<tr>
<td>4. Colonies are colored.</td>
<td>4. Colonies more transparent, aehromogenic, with blue shading when viewed</td>
</tr>
<tr>
<td>5. Membranes are well defined (cellular membrane is thicker).</td>
<td>against light.</td>
</tr>
<tr>
<td>6. Serologically less specific.</td>
<td>5. Membranes considerably less defined.</td>
</tr>
<tr>
<td>8. Grow more vigorously.</td>
<td>7. Biochemically less active (decomposition of carbohydrates).</td>
</tr>
<tr>
<td>9. Reduce dyes more vigorously.</td>
<td>8. Grow less vigorously.</td>
</tr>
<tr>
<td>10. Stable form.</td>
<td>9. Reduce dyes less vigorously.</td>
</tr>
<tr>
<td>11. More sensitive to bacteriophage.</td>
<td>10. As a rule, transient.</td>
</tr>
<tr>
<td></td>
<td>11. Less sensitive to bacteriophage.</td>
</tr>
</tbody>
</table>

they exhibited a sharply bounded carmine center, while the coarse yielded colonies irregular in form and were diffusely colored pink. Differentiation was easily possible with the naked eye, but even somewhat better through the microscope. The intermediate forms of the colonies also were visible in this medium, but they were extremely rare, and developed into a subculture as the smooth or coarse forms.
Garber, Wlochow, and Smith (56) used a procedure described by Brown in 1946 in studying the dissociation of brucelli cultures with a purpose analogous to that used for the plague bacillus. A solid nutritive medium was prepared containing the following: tryptone (Difco) -- 2%; glucose -- 0.2%; yeast extract (Difco) -- 0.8%; and an agar -- 2%. 20 ml of the medium was placed in a Petri dish and implanted with infection. After 48 hours of growth at 30° the cultures were observed by the Brown method using a blue light filter. The S-colonies were creamy white, opalescent, with smooth gleaming convex surface and regular edges. The R-colonies were light blue, translucent through a coarse wrinkled even surface and with irregular borders. The colonies differing one from the other displayed their morphological character following subculturing. For example, the R-colonies obtained from the strongly virulent strain of P. pestis displayed its stability after reculturing, but the avirulence was confirmed by bio-tests (infection of mice). Satisfactory differentiation of the colonies was obtained upon reculturing in a medium held for the required 14 days in a refrigerator.

Devignaut (57) obtained data on the chromogenic dissociation of the plague bacillus. In preparing vaccine from the EV strain following the 101th passage on the third day of growth at 26° in Fl bottles he noted an unusually retarded growth. The culture was checked microscopically, and from it a subculture was obtained in a glycerine-lactose and glucose agar. The isolated colonies were shown to be typically S- and R-forms in which the one or the other form of the colonies had a yellowish coloration with the subsequent preservation of its chromogenic character. Inspection of the morphological, cultural, biochemical, and biological properties of this new strain led to the conclusion of the presence of "chromogenic dissociation" and perhaps, also mutation. The newly formed strain was obtained from P. pestis, but since systematic classification of the strains of the plague bacillus and of the P. pseudotuberculosis similar to it was difficult, the problem of the class membership of the strain obtained has remained unclear.
CHANGES IN THE METABOLISM OF THE PLAGUE BACILLUS
IN THE ORGANISM OF AN ANIMAL SUSCEPTIBLE TO THE PLAGUE

Pages 43-48

All forms of the mutability of the plague bacillus are encountered very rarely under natural conditions, and, therefore, in isolating from the organism, as a rule, typical cultures are grown.

Here we can deal with only two distinct forms of the plague bacillus encountered under natural conditions — the glycerine-negative and glycerine-positive, since the smooth form, in the opinion of most researchers, is not isolated from animals. Only a few authors have been successful in isolating it under natural conditions from wild rodents.

As is generally known, under natural conditions the glycerine-negative strains of the plague bacillus generally pass through the organism of grey rats, while the glycerine-positive forms pass through hibernating rodents, among which the marmots and Transbaikalian marmot (Arctomys sibiricus) rank first in importance.

The different relationship of the plague bacillus isolated from the grey rat (the "oceanic" strain) and from the hibernating rodent ("continental" strain) toward glycerine must be explained based on the characteristics of not so much the chemical composition of the tissues of the animals indicated, for example, the fat content (V. M. Tumansky (58)), as much as their metabolism (N. N. Ivanovskiy (59)).

We regard the suggestion of V. M. Tumansky as untenable because fat is a necessary constituent of the organism both of the rat as well as of the marmot. The amount of fat in either
instance is completely adequate for the nutrition of the bacterial cells, if they require fat.

It is reliably known that the synthetic nutrient media assuring a full measure of growth for the P. pestis do not contain fatty acids. As far as glycerine is concerned, if the P. pestis cells require it, then this substance is contained identically in all fats. The difference in the quantitative amount of glycerine in various animals cannot be of significance. Therefore, the assumption of V. M. Tumansky of the possible adaptation of the P. pestis cells toward glycerine specifically in the marmot organism but not in the organism of the rat cannot be grounded on any considerations of the biochemical properties of these bacteria.

The grey rat is in the biological sense a more active animal which during the course of its entire life remains unchangeably most active. The hibernating rodents -- the marmot and especially the Transbaikalian marmot -- spend a considerable portion of their life in the sleeping state; during the course of the year the marmot is found in a sleeping condition for around six months and the Transbaikalian marmot -- for about nine months.

Without dealing with the entire complexity of the problem of the metabolism of hibernating rodents, we note only that the hibernation of the animal leads to a sharp decrease in its oxidative processes occurring with the participation of air oxygen. In this connection, a considerable decrease in the oxygen content sets in as well as an accumulation of carbon dioxide in its tissues.

All this represents a foundation to characterize the metabolism of an animal found in the sleeping state as approaching anaerobiosis (according to respiration type).

In opposition to what has been said of the grey rat, leading a very agile kind of life, according to its metabolism it can be studied in comparison with the hibernating rodents as an animal with pronounced oxybiotic processes with intensive respiration of the tissues.

The differing response of "oceanic" and "continental" strains of the yersinia pestis toward glycerine must constitute a link with the type of metabolism of the basic carriers of the infection.

The brought forth on the problem of the genesis of P. pestis positive and glycerine-negative strains lead us to the
following single supposition. The differing response of "oceanic" and "continental" strains of the plague bacillus toward glycerine is a consequence of their dissimilar capacity for glycolysis emerging as a result of prolonged passage in one instance through the grey rat ("aerobic" metabolism), and in the other -- through the organism of the hibernating rodent ("anaerobic" metabolism).

Our supposition is confirmed by experiments carried out in vitro, in which the possibility was demonstrated of transforming the glycerine-negative strains into glycerine-positive and their reverse transition. These experiments can serve as a model of what is slowly accomplished under natural conditions in the organisms of wild rodents. In practice these changes take place considerably more rapidly owing to the rigid conditions in which the plague cultures exist.

The low oxygen content in the tissues that was observed in hibernating rodents during the sleeping period with the simultaneous considerable accumulation of carbon dioxide in the same tissues is a leading factor which determines basically the high leavening activity of the plague bacillus strains isolated from them. Opposing processes occur upon the emergence in nature of glycerine-negative strains. The high oxygen content in the tissues of the grey rat suppress the leavening capacity of the plague bacillus which leads to the formation of these strains.

Several foreign authors have emphasized that the ability of the plague bacillus strains isolated from wild rodents to oxidize glycerine is not constant and varies strongly. In addition, it has been shown experimentally that prolonged passage in a marmot of a virulent strain of the plague bacillus of "oceanic" origin leads to the emergence in its tissues of the glycerine-positive form, but upon passage of the virulent "continental" strain on the noontide sandstone /polutennaya peschanka/ leads to the formation of the glycerine-negative variety. These investigations have undoubtedly demonstrated the possibility of transition of one variety of the plague bacillus into another under natural conditions.

The variability of the plague bacillus metabolism, as has been made clear, depends on many factors of the external environment. Of these factors those have been studied basically which affect the respiration of the plague bacillus. The rearrangement
in the enzymatic respiratory system of the cell radically alters its entire metabolism. Of course, all forms of the variability of the plague bacillus can never be reduced only to changes in its respiration type. Nonetheless, based on the point of view presented concerning the natural variability of the plague bacillus, the geographical distribution of the different varieties of plague causative organisms can be easily carried out. The origin of the varieties of plague bacillus is related to the species of wild rodents, in which their passage takes place.

Numerous studies confirmed the correctness of this point of view. Thus, Matumoto (60), in studying the properties of 69 strains of P. pestis, obtained results confirming the former data that showed an intimate relationship between the decomposition of glycerine by a plague causative and its geographical distribution. All the strains stemming from the "third constant endemic focus" (Southeast Russia, Central Asia, Mongolia, and Transbaykal'ye) were glycerine-positive, and all the strains emanating from the first "endemic focus" (the Eastern Himalayas) — glycerine-negative. The author concluded that in response of any freshly isolated strain toward glycerine can serve as an indication of the geographical origin of the given epidemic.

Devignaut and Boivin (61) summarized the results of 26 biological tests carried out on liquid cultures of 42 P. pestis strains isolated from rats and from affected persons in the region of Lake Albert (Belgian Congo). The most characteristic feature of the strains was their inclusion in the glycerine-positive group, which was shown by a colorimetric test with bromthymol blue as an indicator. The authors proposed that the designation of "ancient variety of plague" may be given to glycerine-positive strains. The aim of their research was to promote the understanding of the prevalence in various parts of the earth of glycerine-negative and glycerine-positive strains of plague bacilli.

Devignaut, dealing with the relationship between the biochemical variability of the P. pestis strains and their geographical distribution, classifies them according to a combination of two characteristics: the ability to decompose glycerine and to form nitrous acid in the usual broth culture (Table 1).
TABLE 1

<table>
<thead>
<tr>
<th>Variety</th>
<th>Decomposition of Glycerin</th>
<th>Formation of HNO₂</th>
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<tbody>
<tr>
<td>Variety I</td>
<td>P. pestis orientalis</td>
<td>-</td>
</tr>
<tr>
<td>Variety II</td>
<td>P. pestis antiqua</td>
<td>/</td>
</tr>
<tr>
<td>Variety III</td>
<td>P. pestis mediavallis</td>
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The possible permutation is recognized, but mainly the hypothesis is based on the assumption that each variety reserves its special biochemical characteristic for hundreds and in one instance for thousands of years. Variety I emanates from India, Burma, and South China. Carried from Hong Kong by sea, it resulted in the pandemic of 1894 and the following years. Variety II exists in Central Asia and was carried toward the West by eastern conquerors. The variety has been preserved and carried into Libya, Egypt, and Syria, where it has caused epidemics in the last centuries before Christ. During the subsequent period it was propagated by water route into Central Africa, where it exists even at the present time. It was the basis for the great "justianian" pandemic of the Sixth Century. Variety III was formed by gradual transmutations from the second. Supposedly it caused the "black death", taking 25 million persons in Europe in the middle of the 14th Century. It spread into the southeast area of Russia, Mongolia, and Manchuria, where it has existed up to the present.

Baltazar and Aslani (62) have presented the biochemical characteristics of strains of "wild" plague in Kurdistan. 73 strains of P. pestis are described in their report, isolated during the course of epidemic examinations carried out in Kurdistan over four years. Four strains were isolated from sick persons; three -- from the fleas of rodents; 61 -- from three species of marmot; three -- from marmot badgers; and the remaining two -- from other rodents. All the strains revealed similar features: they ferment glycerine, do not decompose ramose and do not reduce nitrites to nitrites. The strains possessing these characteristics have up till the present been described in the USSR, in North Caspian, where the marmots are also the chief carrier of infection. The authors left open the question as to whether related strains emanate from different regions than those of several precursors of the microbes or whether this similarity is explained by the special resistance of marmots to the microbes.
Golem and Zsan (63) dealt with the biochemical distinctions among four Turkish strains of P. pestis. Three strains derive from the coastal towns of Turkey and yield biochemical reactions of the first variety of P. pestis orientalis according to the Devignaut classification. The fourth strain is obtained from a village located five km north of the Turkish-Syrian border, where there was an outbreak, probably of the forest type, in March-April, 1947. This strain is included according to the Devignaut classification among the third variety of P. pestis mediterranea, which ferments glycerine and does not reduce nitrates to nitrites. The same type was isolated by Baltazar and Aslan in Kurdistan close to the Turkish border.
A. A. Bezsonova (64) first pointed to the different response of plague bacillus strains toward glycerine. A. L. Berlin and A. K. Borzenkov (65), in analyzing the literature data and their own observations on the response of various strains of the plague bacillus toward glycerine, discovered an interesting regularity, which was later shown also to be valuable in practice. The so-called "continental" strains of the plague bacillus exhibit a sharply pronounced capacity to oxidise glycerine with the formation of acids, while at the same time the "oceanic" strains do not oxidise it.

The observation indicated later found confirmation by many researchers, and at present the existence of an intimate relationship between the decomposition of glycerine by the plague bacillus and its geographical origin is wholly indisputable.

The dissimilar response toward glycerine of plague bacillus strains from various geographical origins has still not been given an entirely acceptable explanation. The process of glycerine fermentation is characterized by the following important features distinguishing it from the fermentation of glucose:

1) the process is arrested long before the complete decomposition of glycerine; and

2) adding peptone to yeast water leads to a fuller utilization of the glycerine, however, not to its depletion -- instead of peptones aspartic acid or methylene blue can be added, functioning as hydrogen acceptors.

It is clear that the initial phase of the oxidation of glycerine into a carboxyl-containing compound is associated with the
existence of an alien hydrogen acceptor. Subsequently, its functions can be fulfilled by the intermediate products of the fermentation of the glycerine itself.

The products of the oxidation of glycerine are the same as for glucose. However, the amount of oxidation products is less when compared with the amount of reduction products.

In regard to the elucidation of the question of the genesis of glycerine-positive and glycerine-negative strains of the plague bacillus, the experimental data of various authors is of considerable interest, indicating that the strains of "oceanic" origin following prolonged storage in a museum or by means of their adaptation to glycerine acquire the capacity to oxidize it in liquid and solid nutrient media with the formation of volatile and involatile fatty acids (Ye. I. Smirnova) (66). Through qualitative tests it was established that citric acid is amon the involatile acids, and that the involatile fatty acid fraction predominates considerably over the volatile fatty acid fraction. In addition, it was discovered that the museum strain EV also forms volatile and involatile fatty acids, but in insignificant amounts.

The studies referred to established the commonness of the products of the plague bacillus-oxidation of glucose and glycerine. Moreover it was noted that several "oceanic" strains of the plague bacillus during the process of their adaptation to glycerine acquire the capacity to vigorously reduce litmus — decolorizing it, which speaks in favor of the formation by these strains, using glycerine, of sizable amounts of reduction compounds.

With the purpose of explaining the mechanism of the transition phenomenon fo the glycerine-negative strain into the glycerine-positive, S. E. Ivanovskiy (67) made the suggestion that the ability of the plague bacillus to oxidize glycerine is determined by the intensity of its carbohydrate metabolism. According to his reasoning, strains of bacillus strains with a high fermenting activity also exhibit the ability of oxidizing glycerine.

A suggestion was confirmed in the observations of N. V. Shcherbakov. She showed that the EV strain following its cultivation on a layer of vaseline oil on a liquid medium containing glycerine could redy the capacity to decompose glycerine.
Analogous experiments carried out with other museum "oceanic" strains of the plague bacillus showed that under the conditions indicated these strains also acquired the activity of decomposing glycerine in a liquid medium.

The acquisition by the plague bacillus of the capacity to decompose glycerine occurs, apparently, more intensively if the cultivation of the culture takes place in the presence of carbon dioxide. This observation agrees well with the general rule that all heterotrophs employ carbon dioxide in some amount, and wholly corresponds to the well-known theory of Wood and Workman on its participation in carbohydrate metabolism (referred to in Stefenson (68)). Moreover, Wood and Workman found that in the dissimilation of glycerine by the bacteria of the Propionibacterium variety fixation of the carbon dioxide occurs.

Unquestionably, the opposing direction in the intraspecies variability of the plague bacillus is also possible -- the decreased intensity of its carbohydrate metabolism. In other words, not only can the "oceanic" strains of the plague bacillus make the transition to the "continental", but the reverse transition of "continental" strains into "oceanic" can also be realized.

Based on the general considerations stated dealing with the question of the fermenting activity of microorganisms, N. V. Uryupina (47) recorded observations on the intensity of glycerine decomposition by the No 476 strain during aeration in a liquid noncarbohydrate medium. The experiments demonstrated that a small number of passages under these conditions leads to a stable loss of the indicated strain's capacity to decompose glycerine. In the same way under laboratory conditions the transition of the "continental" strain of the plague bacillus into the "oceanic" was carried out. It must, however, be noted that the rearrangement of the enzymatic apparatus of the glycerine-positive strains in their transition into glycerine-negative, apparently, occurs with considerably more difficulty than the restructuring in the opposing direction.

In this way, from the metabolic point of view, the "continental" strains must be characterized as intensively fermenting, whereas the "oceanic" must be considered as strains with a reduced fermenting activity.
According to the data of V. I. Kuznetsova (69), glycerine is oxidized by almost all smooth varieties of the plague bacillus, but more slowly than by the coarse. In this connection attention must be given to the fact that the growth of the smooth forms of the plague bacillus is substantially retarded in comparison with their coarse counterparts.

In view of the special significance of the assimilation of glycerine by the P. pestis cells, in conclusion we deem it useful to present some data on the splitting of this substance by other forms of bacteria. Of the studies cited in the monograph by Braak (70), at least 20 various products are known which form from glycerine under the influence of microbes: hydrogen, carbon dioxide, ethyl, propyl, butyl, and hexyl alcohols, 2, 3-butyleneglycol, trimethyleneglycol, acetaldehyde, acrolein, 1, 3-propanaldehyde, formaldehyde, acetic, propionic, butyric, caproic, lactic, succinic, and acrylic acids. In reality this list far from exhausts the products formed by microbes out of glycerine.

Braak established that B. aerogenes and B. coli yield qualitatively similar but quantitatively differing products of glycerine fermentation. Table 2 presents a picture of the products forming, whose quantity is expressed in percentages of the total amount assimilated glycerine.

The species of microorganisms which more remotely differ from each other form the same products of glycerine fermentation as those characterized by a sharper qualitative distinction.

Of the several species of bacteria various strains oxidize glycerine by dissimilar routes. This was discovered for the strains of Aerobacter aerogenes Magasanik (71). For the capsule strain of this species oxidation of glycerine occurs through glycerine aldehyde and the acetone with the subsequent formation of pyruvic acid, since the capsule strain in oxidizing glycerine forms an intermediate substance β-glycerophosphate, which is also transformed into pyruvic acid.

Certain microorganisms, for example Aerobacter suboxidans, glycerine one of a few substances capable of assuring the growth
of the culture in the capacity of a sole carbon source (King et al.

<table>
<thead>
<tr>
<th>Species of bacteria</th>
<th>CO₂</th>
<th>H₂</th>
<th>Formic acid</th>
<th>Acetic acid</th>
<th>Lactic acid</th>
<th>Succinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. aerogenes</td>
<td>30.15</td>
<td>1.5</td>
<td>6.64</td>
<td>5.05</td>
<td>3.26</td>
<td>8.51</td>
</tr>
<tr>
<td>E. coli</td>
<td>23.31</td>
<td>1.00</td>
<td>11.76</td>
<td>5.12</td>
<td>10.96</td>
<td>7.19</td>
</tr>
</tbody>
</table>

\*1 Detected qualitatively.

<table>
<thead>
<tr>
<th>Species of bacteria</th>
<th>Ethanol acid</th>
<th>Acetic acid</th>
<th>Trime-thylene</th>
<th>Butylene glycol</th>
<th>Glycol glycol</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. aerogenes</td>
<td>48.79</td>
<td>0</td>
<td>2.3</td>
<td>2</td>
<td>3</td>
<td>99.9</td>
</tr>
<tr>
<td>E. coli</td>
<td>37.26</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>97.9</td>
</tr>
</tbody>
</table>

\*1 Detected qualitatively.

In the given instance it has been established that even dioxyacetone, as an intermediate product of glycerine oxidation, cannot assure the growth of the culture as a sole carbon source.

Glycerine is the most important substance in the so-called synthetic media for cultivating mycobacteria. Extracts of the latter (Hunter (73)), containing the dehydrase of glycerophosphate, accumulate pyruvic acid by way of glycerine in the presence of arsenous acid. Consequently, the transition of glycerine occurs here along the pathway of Embden-Meyerhoff reaction scheme through triose.

Asnis and Brodie (74) obtained a partially purified glycerine dehydrase from the Escherichia coli cells. The co-enzyme of this enzyme is diphosphopyridinenucleotide, and the optimum action of the enzyme is at pH = 10.0. The phosphorus is entirely unnecessary for catalyzing the reaction glycerine → dioxyacetone. Cultures of the S₁ strain of B. subtilis grow well on a nutrient medium containing only two organic substances: aspartic acid and glycerine, since the H₂ strain of the same species under these conditions grows slowly (Viame and Bourgeois (75)). According to
more recent data (76) the difference between M2 and the S1 strains reduces to the fact that only the latter contains kinase, phosphorylating dihydroacetone. This enzyme is absent in the M2 strain.

The enzymes of the plague bacillus oxidizing glycerine have not been objects of special research. But there is every reason to assume for the glycerine-positive strains of P. pestis the existence of a dehydrase of liberated or phosphorylated glycerine, forming trioses. (See Note.) This proposal is reenforced by the capacity of the P. pestis cells to assimilate glucose and sugars close to it according to the reaction scheme of Embden-Meyerhoff, in which the trioses are obligatory intermediate products.

[NOTE] We have found confirmation of this viewpoint in a study by G. F. Shamanova and V. A. Blagoveshchenskiy (Biokhimiya (Biochemistry), 1956, 21, No 6, page 729), who have isolated glycerolase from the Clostridium cedenatensis cells. This enzyme in the presence of ATP phosphorylates glycerine and thus includes it in the phosphotriose pathway of carbohydrate transformation both along the route of obtaining the end products of fermentation, as well as in the direction of synthesizing new carbohydrates. Analogously, P. pestis in the Cl. cedenatiens group also has two types: A. - glycerine-positive and B. - glycerine-negative, -- Ed.)
Carbohydrates in the metabolism of the bacterial cell play the role of a chief energy source, expended for various purposes -- to synthesize new compounds (cell growth), for mobility, to sustain the temperature of the cell protoplasm, etc. The syntheses proceed mainly by way of carbonyl-containing intermediate products of decomposition, of various substances and primarily of carbohydrates (pyruvic acid, acetaldehyde, etc.). From the energetic aspect carbohydrates are most completely utilized under aerobic conditions; under anaerobic conditions energy is liberated in considerably lesser amounts. The ratio of the liberated energy differs, approximately in the order of 1:7.

The plague bacilli utilize carbohydrates as building material and as an energy source. In addition to part of the carbohydrates which is involved in the formation of fatty acids, amino acids, and other noncarbohydrate compounds, the P. pestis cells synthesize from carbohydrates their specific polysaccharides.

In studying the chemical composition of the plague bacilli and its antigens, many authors discovered polysaccharides. As of now only polysaccharides associated with proteins have been found in the composition P. pestis cells. V. G. Akimenko (33) obtained a polysaccharide preparation by means of decomposing a cell with trichloroacetic acid when heated and followed by the acetone precipitation of the polysaccharide. Using qualitative tests this author established the presence in the polysaccharide of hexoses, pentoses, amino-sugars, and phosphoric acid.

The synthetic pathways for complex carbohydrates used by the P. pestis cells have not been uncovered, and we can only deal here with the widespread bacterial synthesis of any complex carbohydrate involving participation of enzymes which carry whole fragments of nonosaccharides and have been called transglucosidases.
An example of such is the synthetic activity of E. coli. These bacilli, employing maltose as a substrate, are capable of synthesizing maltotriose and maltotetrose (77). Transglucosidases, apparently, are capable of converting some polysaccharides into others, as was shown for the cells of Acetobacter capsulatum (78).

Many authors have with the purpose of differential diagnosis studied the response of the plague bacillus toward various carbohydrates. Much factual material has been gathered on this problem, indicating that the capacity to decompose carbohydrates changes within substantial limits in the plague bacillus. Thus, for example, freshly isolated strains of the plague bacillus from wild rodents do not oxidize ramose. However, following their prolonged storage in a museum over the course of many years without subculturing they acquire this capacity, some more readily than others.

For various strains of the plague bacillus, especially for the museum strains, the capacity to decompose carbohydrates undoubtedly differs sharply. This is first of all explained by the contradiction of several literature data dealing with the relationship of the plague bacillus toward mono- and polysaccharides, the authors studying strains differing from each other in their fermenting capacity. The capacity of different plague bacillus strains to oxidize carbohydrates, varying within very broad limits, must be explained by their dissimilar fermenting activity, which varies in these strains as a result of being cultivated in media with differing amounts of oxygen.

To confirm the correctness of this thought observations on the "oceanic" strain EV of the plague can be employed, which strain was previously partly adapted to anaerobic conditions by being cultivated in a peptone medium with glucose under a layer of vaseline oil. Experiments indicated that the adapted strain acquired the capacity to more intensely oxidize glucose than the original strain, both under anaerobic conditions, as well as when the culture was aerated (Table 3).
The general direction in altered fermenting capacity of the museum strains of the plague bacillus, preserved for a long period in liquid and solid nutrient media in sealed test tubes, is determined primarily by the relative oxygen deficiency in the surrounding environment which sets in over the course of time. Prolonged residence of the culture under these conditions leads to substantial changes in their carbohydrate metabolism. The plague bacillus evermore profoundly reorganizes its aerobic type of respiration into a more anaerobic type, in this way adapting to the new external conditions which are unfavorable for its existence.

The intensity of carbohydrate fermentation depends not only on the oxygen content in the medium, but also on the geographical origin of the strain and its existence in the coarse or the smooth form.

From numerous, but diversely oriented and nonsystematic, observations the unquestioned conclusion, however, must be drawn that "continental" strains more energetically ferment carbohydrates than do "oceanic". Thus, for example, A. N. Kraynov (76) noted that she could not even once obtain from "oceanic" strains the gram-positive form, whereas from the "continental" strains this could be done relatively easily.

In regard to the intensity of decomposition of sugars by the coarse and by the smooth forms of the plague bacillus the conviction is widespread that the latter are less active in this regard. In addition, however, it is emphasized that the smooth form oxidizes ramnose, which the coarse variety does not oxidize. Considering the differing

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**TABLE 3**

Intensity of glucose oxidation in arbitrary units

<table>
<thead>
<tr>
<th>Strains</th>
<th>When culture under aerobic conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original EV strain</td>
<td>18</td>
</tr>
<tr>
<td>EV strain, adapted to anaerobic conditions</td>
<td>24</td>
</tr>
</tbody>
</table>

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- 57 -
intensity of the growth of the coarse and of the smooth forms of the plague bacillus, and also the absence of data directly defining its fermenting ability, such a conclusion would be premature.

In evaluating the biochemical activity of the smooth forms of the plague bacillus it must always be kept in view that they grow considerably more slowly than do the coarse. This circumstance easily explains many properties of the indicated varieties of the plague bacillus.

In the overwhelming majority of instances two transformation pathways for carbohydrates (80, 81) have been observed in the cell. The first pathway — anaerobic degradation — begins with the phosphorylation of the carbohydrate and concludes with the formation of pyruvic acid, which is subsequently either reduced to lactic acid (I) or is decarboxylated into acetaldehyde with its subsequent reduction to ethyl alcohol (II).

The enzymes of the first phase of carbohydrate metabolism in the P. pestis cells have not been subjected to systematic study, but the existence of several was shown with a certain degree of probability. Thus, the phosphorylating enzymes as initiators of fermentation undoubtedly are present in the plague bacillus, which is demonstrated by the existence of a more or less fermenting capacity in all these strains of this species. In addition to the indicated enzymes, the enzymes known as isomerases are of much significance on the same pathway of carbohydrates metabolism. There is no question that the plague bacillus possesses isomerases catalyzing the transformation of several phosphoric esters of the

\[
\begin{align*}
\text{Pyruvic Acid} & \rightarrow \text{Lactic Acid} \\
\text{CH}_3 - CO - \text{H}_2 - \text{COOH} & \rightarrow \text{CH}_3 - \text{CHOH} - \text{COOH} \\
\text{CH}_3 - CO & \rightarrow \text{CH}_2 - \text{OCH} - \text{CH}_2 - \text{OH}
\end{align*}
\]

monomeric carboxylic acids into others, without which fermentation would also prove impossible.
In the *P. pestis* cells of the avirulent strain A1122 the xylulose-isomerase (62) has been found. This enzyme catalyzes the mutual transformation of three, unphosphorylated pentoses of xylose (aldose) and of xylulose (ketose). Xylose-isomerase was discovered earlier in extracts from the cells of *P. pseudomonas hydrophila* (63), but Mitubashi and Lampen (64) discovered this same enzyme in extracts from *Lactobacillus pentosus*.

Xylose-isomerase has been obtained from the noncellular extract of *P. pestis*, raised in the presence of α-xylose. The isomerase of the plague bacillus belongs to the thiol enzymes, since its activity is doubled or tripled in the presence of cysteine. Also having an activating effect on the enzyme are manganese ions at a concentration of $10^{-2}$ M and magnesium ions at a concentration of $10^{-3}$ M. The optimum action is at pH 8.0. Equilibrium in the reaction catalyzed by this enzyme, in the absence of borate occurs in the presence of 16% xylulose in the reacting mixture, but the addition of borate shifts the equilibrium to 60-65% of the same sugar. Isomerase is suppressed by the addition of trimoxymethyl-aminomethane. At a d-xylose concentration of $3 \times 10^{-3}$ M at pH 7.0 and 30° the enzyme exhibits half of its maximum activity. Unpuri-ified extracts from *P. pestis* cells, grown in the presence of d-xylose, were found to contain xylulokinase, capable of phosphorylating d-xylulose in the presence of adenosinetriphosphate but not capable of phosphorylating d-xylose.

The mechanism of the transformation of the pentoses assimilated by the *P. pestis* cells has not been studied, nor has this process been much examined for other bacteria. It has been shown (Gest and Lampen (85)) that d-xylose tagged (C14) at the first carbon atom is converted by the nonmultiplying cells of *Lactobacillus pentosus* into equimolecular quantities of acetic and lactic acids. In this connection, C14 is detected only in the methyl group of acetone. Hence, the methyl group of the acetic acid is formed using the C1 of xylose and splitting of the pentose occurs through rupture of the bond between C2 and C3. Supplementing this fact by indirect evidence and by several assumptions the authors (85) presented the following scheme for the splitting up of xylose:
Whether the mechanism of the assimilation of d-xylose by the P. pestis cells is similar to the above presented scheme it is difficult to say in view of the absence of experimental data. However, the presence in the plague bacillus of xyloso-isomerase, transforming d-xylose into d-xylulose, can favor the hypothesis of the authors.

Another pathway for the transformation of carbohydrates — the aerobic — is related to the oxidation of pyruvic acid or of acetaldehyde. Acetaldehyde is oxidized into acetic acid, which in its turn serves as a source for the formation of several other acids.

The approach of oxidative degradation of pyruvic acid into another proceeds along a tricarboxylic cycle, called the Krebs cycle.

The existence of mechanisms of the tricarboxylic cycle in the P. pestis cells has been demonstrated by direct studies (86). The nonmultiplying bacilli of the plague accumulate noticeable quantities of intermediate products of the tricarboxylic cycle. Such cells oxidize any substance entering into the cycle of tricarboxylic acids with the formation of end products — carbon.
Acetoldehyde

\[ CH_3 \rightarrow COOH \]

Acetic Acid

\[ COOH \rightarrow CH_2COOH \]

Succinic Acid

\[ CH_2COOH \rightarrow CH-00H \]

Fumaric Acid

Malic Acid

\[ CH\cdot COOH \rightarrow CO \cdot COOH \]

Oxalacetic Acid

\[ COOH \rightarrow CO \]

Pyrurvnic Acid

\[ CH_3 \rightarrow \]

Acetolddehyde

dioxide and water. It has been shown that the acetate also is included in the tricarboxylic cycle by these cells. Thus, the addition to a suspension of cells of labelled (C14) acetate makes possible the subsequent detection of the isotope in the intermediate products of oxidation. Several enzymes of the tricarboxylic cycle have also been found in the plague bacilli -- isocitrate dehydrase and aconitase.

The chain of biochemical reactions called the tricarboxylic cycle is a most important source of energy in the aerobic oxidation of the main bio-organic compounds. It has been shown that such substances as acetic and pyruvic acids can be included in the tricarboxylic cycle, consequently, the products of the degradation of carbohydrates, glycerine, fatty acids, and several aminoacids are used as energy sources through a chain of the cycle reactions.

The process of transforming pyruvic acid into acetic acid and including the latter in the tricarboxylic cycle is very complex and has as yet not been explained in several details. Pyruvic acid with the participation of cocarboxylase, coenzyme A (CoA) and diphosphopyridine nucleotide forms through the agency of the acetyl-coenzyme A acetate which combines with oxalacetic acid and thus is included in the tricarboxylic cycle.

Inasmuch as direct evidence has been presented of the existence in the P. pestis cells of the tricarboxylic cycle and the inclusion of this cycle of acetic acid, the presence in plague bacilli of all the most important enzyme and coenzyme systems supporting these reactions must be regarded as established.
Intermediate and end products of the degradation of carbohydrates occurring in the plague bacillus have been inadequately studied, but they undoubtedly differ as a function of the conditions in which the oxidation of the carbohydrates occurs, and especially as a function of the molecular oxygen content in the surrounding environment. In this connection, evidently, there are no special characteristics in the carbohydrate metabolism of the plague bacillus. Thus, for example, N. V. Uryupina (47) has shown that sodium fluoride -- a typical inhibitor of the tissue metabolism of carbohydrates -- exerts a suppressing effect both on the growth of the culture as well as on the intensity of the glucose use by the plague bacillus.

The nonmultiplying cells of P. pestis have revealed an energetic internal metabolism. This intensive endogenic respiration exists in response to the high intracellular accumulation of intermediate substance of the tricarboxylic cycle. Using labelled cells, it can be shown that endogenic respiration is not associated with the oxidation of exogenic substrates. Whole nonmultiplying cells oxidize any substance entering into the cycle of tricarboxylic acids. The fraction of acids utilized is oxidized to CO₂ and water. Oxidation of the acetate is included in the cycle of tricarboxylic acids, as shown by the determination of the radioactivity of intermediate intracellular substances in the oxidation of tagged (¹⁴C) acetate. The plague bacillus contains isocitrate dehydrogenase and aconitase, which acting jointly convert the citrate into the keto-glutarate. This reaction is reversible.

Englesberg, Levy, and Gobor (87), working with the All22 of P. pestis, established that a profound difference exists between anaerobic and aerobic cells. Anaerobic cells are capable of restrictedly oxidizing glucose with the accumulating of large amounts of pyruvate and other end products, which either are not oxidized further or are oxidized very slightly. On the other hand, aerobic cells yield "complete" oxidation of glucose. There are other differences in the metabolism: anaerobic cells do not exhibit the capacity to adequately oxidize pyruvate, acetate, and C₄-dicarboxylic acids, whereas all these substances are rapidly oxidized by aerobic cells. Anaerobic cells can be transformed into aerobic by aerating the culture in a casein hydrolysate glucose medium for many hours with a negligible increase in the number of cells. This transformation is blocked by ultraviolet rays. Oxygen, consequently, acts here as an excitant for the formation of respiratory enzymes, as has been observed in the East.
Santer and Aji (88) showed that the growth of the plague bacillus in the presence or absence of oxygen profoundly affects its physiology: anaerobic cells do not oxidize acetate, and the oxidation of pyruvate, succinate, fumarate, and malonate proceeds weakly, glucose is oxidized incompletely, with the accumulation of pyruvate and other organic end products; moreover, aerobic cells oxidize acetate, pyruvate, and four-carbon components vigorously and oxidize glucose rapidly and completely.

Anaerobic cells adapted to complete oxidation of glucose are at the same time adapted also to complete oxidation of pyruvate, acetate, O₂-components. Citrate and L-ketoglutarate are oxidized by not a single type of plague bacillus cells. Based on experiments with cell extracts it is possible to partially explain this phenomenon. First of all, it must be assumed that the cycle of the tricarboxylic acids functions in anaerobic cells as a terminal mechanism. In spite of the fact that whole non-multiplying anaerobic cells are not capable of oxidizing L-ketoglutarate and nitrate, these substances are oxidized by cellular extracts more rapidly than other components of the cycle of tricarboxylic acids. This characteristic of cellular extracts to oxidize substances not oxidizable by whole cells is explained by the difficulty experience by the tricarboxylic cycle substances in penetrating the cells. This has been noted earlier by many authors also for other species of bacteria. The fact that many species of microbes can oxidize compounds of the tricarboxylic acid cycle independently of aerobic growth and the substrate employed does not yet show that the enzymes participating in this process are "constitutive", but suggest only that the cycle of tricarboxylic acids is a general terminal pathway of respiration.

For P. pestis the transition to anaerobic existence consists in the loss of the ability to oxidize the substances of the tricarboxylic acid cycle. Enzyme analysis has shown that anaerobic cells of the plague bacillus do not contain at least isocitrate-dehydrase, aconitase, fumarase, and cytochrom. The weak activity in regard to citrate supports particularly the position that the tricarboxylic acid cycle is not operative in anaerobic cells. Aeration of the culture in the casein-hydrolysate glucose medium promotes the synthesis of these enzymes. It is possible to assume that other enzymes participating in this metabolic process also respond to aeration in a similar manner.
Cytochrome, present in aerobic cells, exhibits maximum absorption at 560 millimicrons, it is thermally labile, sensitive toward 
HCl, and is closely associated with the insoluble portion of cells. The adaptive response in P. pestis to O₂ is similar to that found 
in Saccharomyces cerevisiae. However, there is an essential differ-
ence. For P. pestis the initial extent of glucose oxidation 
to pyruvate is constitutive, which demonstrates the existence of 
two mechanisms for glucose degradation. For S. cerevisiae adapta-
tion to anaerobic conditions is expressed in the complete loss of 
the ability to oxidize glucose. Both microorganisms adaptively 
form cytochrome in response to the presence of O₂, but in S. cere-
visiae cytochromes a, b, and c, and cytochrome oxidase, present in 
aerobic cells, disappear when the cells are grown under anaerobic 
conditions and two new components appear — the cytochromes a₁ and 
b₁, whereas in the P. pestis only one cytochrom is found in aerobic 
cells, but in cells growing anaerobically, there is no cytochrom at 
all and only traces of intracellular haematin.

The mechanism of carbohydrate metabolism in the P. pestis 
cells should not be regarded as wholly explained. However, several 
of the most important aspects of this problem have been studied 
adequately. Fermentation of L-[[C²⁰]glucose of a non-multiplying 
cellular suspension of P. pestis proceeds almost exclusively along 
the glycolysis pathway. By determining the carbon dioxide form-
ing in the Warburg apparatus and by analyzing the fermentation 
products, an almost complete absence of glucose transformation 
through glucose-6-phosphomonoester and gluconic acid was shown; only 
0.7% of the total radioactivity of the glucose was detected in 
CO₂.

The same glycolytic pathway has been confirmed by a study 
of the glucose degradation products, since the radioactivity was 
found in succinate and other products. In the presence of the hexo-
somonoester pathway not a single product containing the isotope 
would be formed from L-[C²⁰]glucose. In reality all the products, 
with the exception of formic acid, contained C²⁰, and the greater 
part of the isotope is present in them.

Quantitative analysis of the products of the breakdown of 
pyruvic acid has shown that this process proceeds along two path-
ways: disproportionation, in which two moles of pyruvic acid are 
converted into lactate, acetate, and CO₂; and through "phosphoro-
clastic splitting" in which one mole of pyruvic acid forms acetic
and formic acids. Formation of carbon dioxide in glucose fermentation proceeds, apparently, through the disproportionation of pyruvic acid. Since the P. pestis cells do not contain the hydrogenase of formic acid, this in its turn confirms the supposition of the formation of the main amount of CO$_2$ through the C$_3$ and C$_4$ of glucose. Still another poorly explained fact must be noted. When CO$_2$ no longer is evolved, the glucose requirement continues. The lactate forming here begins to be broken down with the formation of acetic and formic acids.

The above-referred-to investigations characterize the metabolism of glucose by nonmultiplying cells of P. pestis under anaerobic conditions. However, under aerobic conditions dried cells of P. pestis assimilate gluconic acid, and noncellular extracts are capable of converting glucosophosphate into pentosphosphate.

Cohen (89) discovered a hexosomonophosphate pathway in growing cultures of E. coli and suggested that this pathway is the most important as a mode of converting hexosomonophosphate into pentosomonophosphate, utilized for synthetic reactions.

The studies of Santer and Ajl (90) were devoted to studying the hexosomonophosphate approach in noncellular extracts and multiplying cells of P. pestis. These authors set up experiments on growing cells in the Warburg apparatus, where the multiplying culture was placed together with the nutrient medium and radioactive glucose tagged at the C$_1$ was added. As a result of these studies enzymes were discovered which catalyze the conversion of hexosomonophosphate into pentosomonophosphate. Using L-C$^{14}$-glucose as a substrate, the authors showed that the carbon dioxide evolved by growing cells had at least a 300% greater radioactivity than in the experiment with nonmultiplying cells. This fact demonstrates that during the period of growth of the culture the C$_1$ of the radioactive glucose is converted primarily into carbon dioxide with the formation of pentosophosphates. It must here be emphasized that these determinations refer to the period of maximum multiplication of the P. pestis cells. Precisely during this period the radioactivity of the evolved CO$_2$ is the maximum. If the observations of growing cells were continued further, then an increase in the oxygen requirement would not be recorded, and along with this the specific radioactivity of the evolved carbon dioxide would fall off. The latter change in the metabolism of glucose is an indicator of a shift occurring in connection with the transition of cells from a state of multiplication to a resting state.
The basis for the extensive adaptability of microorganisms for changes in conditions of nutrition is the formation of new enzymes in the cells. Such enzymes have been called adaptive. The plague bacillus can serve as an especially graphic example of the formation of the various adaptive enzymes. Several conditions of the formation of glycerine-positive strains of P. pestis have been examined above. The appearance of adaptive enzymes in the process of forming strains which oxidize rhamose can also be noted, as well as the multitude of other similar examples of the same adaptation of the plague bacillus. But, turning primarily to the adaptive enzyme of the plague bacillus we deem it pertinent to note the most intensive studies of recent times illuminating this important biological problem, studied for many species of microorganisms.

Several researchers believe that the appearance of adaptive enzymes in microbes consist simply of an increase in the number of enzymes already existing in cells. Thus, Lederberg (91) has established that lactase, easily detectable in the Escherichia coli cells adapted toward lactose is not found by the usual methods in cells that have not been previously adapted to lactose. However, using ortho-nitrophenol-β-d-galactoside as a substrate, this author found lactase also in the cells of E. coli which had not been adapted to lactose. The truth is that for this purpose the hydrolysis products must be determined by an especially sensitive colorimetric method, since hydrolysis occurs to a very small extent. The authors' conclusion, based on these investigations, reduces to this point in adaptation no new enzymes are formed. This scarcely corresponds to reality, or for such a theory excludes the possibility of adaptive variability of the bacilli which has been observed so often in the most diverse species.
Rickenberg, Yanofsky, and Bonner (92) investigated the mechanism of the loss (disadaptation) of the same enzyme β-α-p-glactoside (lactase) for two strains of E. coli adapted over a long period to lactose. In this connection, the strain W 1185 did not require any growth factors in a medium containing lactose, but the strain W 253 required biotin and methionine. The author studied the loss of the lactose activity when these strains were cultivated in a medium containing glucose and xylose. It was established that for the W 1185 strain not only did the loss of galactosidase not occur under these conditions, but not even its decrease either in whole cells or in noncellular extracts has been noted. For the W 253 strain the synthesis of the same enzyme is wholly dependent on the presence in the medium of 1-methionine. Cultivating the W 253 strain in the absence of methionine even in a medium containing galactose did not assure the synthesis of β-galactosidase. Analogous results were obtained also with other strains of E. coli which required histidine, threonine, or leucine, that is, these strains also proved to be incapable of forming lactose in the absence of the amino acid necessary for their growth.

Based on the fact that the bacteriophage of Escherichia coli multiplies and exerts a lytic action only in those cells in which intensive metabolism and formation of energy do to an added substrate occurs, Benzer (93) showed that the adaptive elaboration of β-galactosidase occurs with approximately the same velocity in almost all cells of the culture. Thus, a S-shaped curve characterizing the process of individual formation of the enzyme does not at all reflect the selection of the corresponding cells. It was shown that the S-shaped form of the curve depends upon the effect of several factors limiting the synthesis of the adaptive enzymes (94). These factors include, as has been shown above, the sources of nitrogen nutrition, the concentration of the substance provoking the synthesis of the enzyme, the duration of the assimilation phase, the amount of oxygen, and the temperature. Upon removal of the factors restricting the adaptation, the process of induced formation of galactosidase no longer is S-shaped but linear.

Monod and Wollman (94) indicated the complete suppression of the synthesis of the adaptive lactase by a phage for those strains of E. coli which are susceptible to the action of a bacteriophage. However, Siminovitch and Jacob (96) showed that for the lysogenic strain of E. coli K 12 the adaptive formation of β-galactosidase...
occurs with the usual velocity coinciding with the multiplication of the bacteriophage. In precisely the same way Jacobs (97) showed that for lysogenic strains of Pseudomonas aeruginosa synthesis of the adaptive enzymes occurs simultaneously with the multiplication of the bacteriophage.

A survey of studies completed in recent years dealing with the mechanism of the formation of adaptive enzymes (98) has shown that the study of this problem has proceeded in various directions.

Cohn and Torriani (99, 100) also investigated the induced formation \( \beta \)-galactosidase in E. coli cells. These authors showed that simultaneously with the appearance of the adaptive enzyme in the tissues a synthesis of a new protein occurs which was detected by immunological methods. The new protein was identified by enzyme as lactose. Extracts from cells not synthesizing the adaptive enzymes also did not contain this protein, but they contained another which energetically reacted in a crosswise fashion with antilactase serum. It was further shown that in the growing culture the synthesis of lactase is accompanied by the simultaneous decrease of this second protein. During nitrogen starvation of E. coli cells in them the synthesis of lactase was never induced, since the amount of the second protein even during the course of a twenty-four hour nitrogen starvation remained constant.

Consequently, synthetic reactions using nitrogen substances are necessary in order to convert the second protein into lactase and this second protein, whose synthesis is not induced, must be regarded as precursor of lactase. In addition, it was established that those species of Enterobacteriaceae, which contained the second protein are capable of forming lactase also. The species not exhibiting the second protein do not have the capacity to synthesize this enzyme.

It was established (101) that the formation of lactase by the E. coli cells can occur only under conditions occurring in the synthesis of new proteins. The strains incapable of synthesizing several amino acids, in their absence, cannot form \( \beta \)-galactosidase, but addition of these indispensable amino acids creates the conditions for synthesis of the adaptive enzymes. Consequently, for the synthesis of lactase, in addition to the second protein, specific amino acids are necessary.
It was shown that the adaptive formation of β-galactosidase can be induced by substances containing whole galactoside radicals. However, such substances as methyl-d-galactoside or alkyl- and aryl-d-galactoside although not hydrolysed by β-galactosidase are capable of inducing the formation of the enzyme. The capacity to induce, generally speaking, has been shown to be independent of the kinship to the enzyme. Such substances as phenyl-β-thiogalactoside, having a strong affinity (relationship) to the enzyme, nonetheless does not induce its formation.

The facts obtained as a result of the study of the mechanism of the adaptive formation of β-galactosidase by the E. coli cells compels us to assume that all substances inducing the synthesis of this enzyme participate in the processes of the general metabolism in this cell. It can be assumed that the inducing substances form intermediate products combining with several cellular components which of themselves do not constitute the enzyme.

The investigations of Halvorson and Spiegelman, (102) showed that analogues of amino acids obstructing the assimilation and inclusion in the protein of individual amino acids of their mixtures suppress the induced synthesis of maltase by yeast cells. Thus, para-fluorphenylalanine almost completely suspends the growth of Saccharomyces cerevisiae, but the adaptive formation of maltase under these conditions is decreased to approximately one-fourth in comparison with the normal. This suppression is specifically and entirely relieved upon the addition of the required quantity of homologous amino acid. The results of these experiments have led to the conclusion that in the formation of adaptive enzymes in non-multiplying cells free intracellular amino acids participate.

An analogous conclusion derives also from the observations of Pinsky and Stoelz (103) who found that for the adaptive formation of hydrogenase in non-multiplying E. coli cells the presence of arginine, aspartic and glutamic acids are necessary in the medium.

A strict relationship between the ability to synthesize and the enzyme and the suitability of the existing free amino acids within the cell for protein synthesis was discovered, consequently, three intracellular amino acids are a determining source of nitrogen for the formation of the new enzyme molecules. Halvorson and Spiegelman believed (104) that there is no data supporting the
theory of the conversion of a previously existing protein complex, which independently of amino acids would be transformed into an active enzyme. The same authors (105), using methyl-glucoside, the unassimilated analogue of maltose, for the induction of maltase formation in the cells of Saccharomyces cerevisiae, found in these cells a quantity of free amino acids expended in synthesizing the given adaptive ferment. Table 4 presents the results of this investigation.

TABLE 4

Millimicrons per 100 mg of dry cells

<table>
<thead>
<tr>
<th>Free Intracellular amino acids</th>
<th>Control cells</th>
<th>Induced cells</th>
<th>Percentage of consumed amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>1.12</td>
<td>0.90</td>
<td>0.94</td>
</tr>
<tr>
<td>Valine</td>
<td>3.06</td>
<td>1.55</td>
<td>1.46</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.69</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.088</td>
<td>0.011</td>
<td>0.018</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>19.7</td>
<td>11.5</td>
<td>10.3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.72</td>
<td>1.23</td>
<td>1.27</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.40</td>
<td>4.90</td>
<td>3.45</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.17</td>
<td>0.48</td>
<td>0.48</td>
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<tr>
<td>Proline</td>
<td>1.00</td>
<td>0.67</td>
<td>0.65</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.47</td>
<td>0.24</td>
<td>0.19</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.26</td>
<td>0.18</td>
<td>0.16</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.87</td>
<td>1.47</td>
<td>1.50</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.68</td>
<td>1.60</td>
<td>1.59</td>
</tr>
</tbody>
</table>

Note: Numbers in columns A and, also B and, pertain to parallel experiments.

In this way, a direct increase in the assimilation of intracellular free amino acids was shown as a result of the synthesis of the adaptive enzyme maltase by suspensions of nonmultiplying yeast cells in a non-nitrogen medium. The amount of amino acids expended in synthesizing maltase was dissimilar: from the existing reserve of threonine only 21% was consumed, whereas isoleucine came to a 56% expenditure.

The mechanism also of the reduced synthesis of pencillinase differs clearly from the above described maltase synthesis.
The adaptive formation of penicillinase proceeds at a constant velocity in the presence of traces of penicillin. Such a quantity of the latter, of course, is destroyed rapidly, on the basis of which Pollock (106) drew the conclusion that penicillin reacts with any component of the cell owing to which a complex in created, still not vulnerable to penicillinase but capable of catalyzing the further formation of this enzyme. The author considered that if each molecule of fixed penicillin induces the formation of a complex through combination with one molecule of the enzyme precursor, then the penicillinase forming must have an activity fifty times greater than catalase in order to assure the degradation of the amount of penicillin which exists in reality. Taking such an activity of penicillinase as being of low probability, the author suggested that the penicillin complex only catalyzes the synthesis of the enzyme, but in itself is not an enzyme. Based on the results of his investigations Pollock proposed the following scheme for the formation of the adaptive ferment.

\[
\text{Penicillin (inducer) } \xrightarrow{\text{receptor}} \\xrightarrow{\text{organizer}} \text{aminoacids } \rightarrow \text{type I precursor } \rightarrow \text{type II precursor } \rightarrow \rightarrow \text{enzyme (penicillinase)}
\]

Cohn and Monod (107) suggested a scheme for the synthesis of the adaptive ferment which was analogous in essence. Shayer (108) discovered in several microbacteria the formation of adaptive enzymes for oxidizing benzoic acids. Berheim (109) studied similar ferments in the Mycobacterium tuberculosis (the BCG 8420 strain).

He showed that the ortho-isomers of amino-, nitro-, fluoro-, chloro-, and bromobenzoic acid stimulate the formation of the adaptive enzymes oxidizing benzoic acid and pyrocatechin. However, the ortho-xybenzoic acid is not active in this regard, but cyclohexane-carboxylic acid also stimulates a synthesis of adaptive enzymes. But the stimulators themselves are not oxidized. A stimulating effect was discovered also in para-fluorobenzoic acid, but the para- and meta-isomers of amino-, chloro-, and bromobenzoic acid not only do not stimulate, but suppressed the synthesis of adaptive enzymes.

The great interest shown the adapted enzymes is explained not only by the fact that they are the basis for the changeability of the microbe and their adaptability to changing conditions of
existence; the problem of adaptation is also associated with the acquisition by the bacilli of resistance to antibiotic and to chemotherapeutic agents. Therefore, the problem of adaptive enzymes will again return to focus on studying the treatment of the plague.

Study of the adaptive enzyme system of the All22 strain of P. pestis cultivated under anaerobic conditions, during the transition of the cells to conditions of aerobiosis has shown that in this transition the adaptive synthesis of dehydrase by isocitric acid, aconitase, fumarase, and cytochrome is carried out.

Englesberg and Levi (110), using the same P. pestis strain, also studied other adaptive enzymes of the tricarboxylic cycle, oxidizing acetate and the system providing the complete oxidation of glucose both by whole cells as well as by noncellular extract. The latter were produced both from aerobically growing cells (aerobic extract) as well as from anaerobically cultivated (anaerobic extract). In addition, the extract obtained from anaerobically growing cells following their adaptation to aerobiosis (aerobically adapted extracts) were tested.

Suspensions of nonmultiplying cells of P. pestis, grown under aerobiosis in a mineral-glucose medium containing a casein hydrolysate oxidize at quite a high velocity pyruvic, acetic, and C4-dicarboxylic acids. Glucose in this instance is oxidized also rapidly and completely. In opposition to this, anaerobically grown cells of P. pestis do not oxidize acetate, but glucose is oxidized slowly and incompletely with the formation of pyruvic acid and other end products of fermentation. Such an incomplete oxidation of glucose and the inability to oxidate acetate is accompanied by a large decrease in the oxidation rate of pyruvic and C4-dicarboxylic acids. Aeration of acids anaerobically grown in a glucose-mineral medium containing casein hydrolysate causes a transformation of the character of the metabolism of these cells from an anaerobic to an aerobic type. As shown in the studies of Englesberg and his colleagues referred to above, the transition from anaerobiosis to aerobiosis is accompanied by the adaptive formation of several enzymes: the dehydrase is isocitric acid, aconitase, fumarase, cytochrome, phosphotransacetylase, condensing enzyme, malonatedehydrase, and enzymes participating in the oxidative decarboxylation of pyruvic -keto glutaric acids.

The best method of synthesizing the designated adaptive enzymes is illustrated in Table 5, in which the enzyme properties at extract from cells grown under aerobic and anaerobic conditions are compared. In addition, for comparison extract has been taken from the bacilli cultivated anaerobically, but subsequently aerated.
(aerobic-adaptive extract). The fermenting activity of extract is presented in Table 5 in micromoles of the converted substrates or in micromoles of synthesized products per one hour per one mg of nitrogen contained in the extract.

The fact that the appearance of enzymes in P. pestis is not a result of the liberation of the aerobic culture from hypothetical inhibitors forming previously in the anaerobically cultivated cells points to the inability of extracts from anaerobically grown cells to suppress the isocitrate dehydrase.

This is evidenced by the inability of anaerobic extract to retard enzyme systems of aerobic extracts, catalyzing the oxidation of nitrate into acetate.

The result presented in Table 5 shows the genuinely adaptive character of the transition from anaerobiosis to aerobiosis, since in this transition we are not limited only by the sharp increase in the activity of the enzymes indicated in Table 5 from No 1 to No 9 inclusive. At the same time a two-fold decrease in the activity of the most important enzymes of glycolysis occurs: phosphate-fructose-kinase, the dehydrase of glycerinaldehydephosphate, whereas acetokinase and decarboxyrase of an oxalacetic culture remains without essential changes. The biosynthesis of adaptive enzymes in P. pestis is in accord with the metabolic changes. Thus, in anaerobiosis the capacity to oxidize acetate is absent, glucose is oxidized incompletely, and pyruvate and C4-dicarboxylic acids are oxidized slowly and weakly. The transition to aerobiosis is marked by a rapid oxidation of acetate, pyruvate, C4-dicarboxylic acids, and also by the rapid and complete oxidation of glucose. In all of the latter processes the designated (No's 1-9) adaptively synthesizing enzymes participate, as well as the enzymes of the tricarboxylic cycle, assuring the complete oxidation of glucose and the assimilation of acetate. Although in this connection a sharp adaptive increase of succinate dehydrazase is not observed, it is difficult to question that it occurs, since the succinate dehydrazase activity of P. pestis extracts is exceptionally high.

The last fact represents additional evidence of the action of the tricarboxylic cycle in the aerobiosis of P. pestis. But there are also other indications of the presence of this cycle in the plague bacillus — the necessity of the presence of precisely
those identified products which participate in the tricarboxylic cycle. Moreover, the synthesis of citrate from acetate and oxaloacetate, and also from pyruvate and oxalacetate, and the rate of these processes evidence the existence of the tricarboxylic cycle in the P. pestis.

The high phosphotransacetylase activity of aerobically grown cells assimilating glucose as carbohydrate source indicates that the free acetate, most probably, is a normal product of the oxidation of glucose. This is confirmed further by the relatively high actinidase content in P. pestis.

Extracts from P. pestis, strain A-1122, obtained both from aerobically, as well as from anaerobically grown cells, contained a very large amount of the dehydrase of glycerinealdehydophosphate and fructose-phosphate-kinase. This evidence the essential significance of the Embden-Meyerhoff series of reactions in the metabolism of glucose in aerobic and anaerobic conditions. There is no grounds to consider this pathway as the only means of converting glucose in anaerobically growing cells. It has been stated above that anaerobically grown cells of P. pestis, taken in the form of a nonmultiply-

ing suspension supplied with glucose in the presence of oxygen, oxidize this sugar incompletely, with profuse release of CO₂ and the simultaneous formation of pyruvic acid. The amount of carbon dioxide forming from glucose falls off with time down to a low level, characteristic for the splitting of the pyruvate by P. pestis cells,

The above described adaptive synthesis of several of the most important enzymes is an example of a well-pronounced control over this synthesis on the part of external conditions. Similar situations on the mechanism of controlling induced synthesis of enzymes have been expressed by Stanier (111).

However, the decisive factor in the change of enzyme systems in the tricarboxylic cycle and other enzymes of the plague bacillus is the presence or absence of oxygen, nonetheless the mechanism of these changes is not clear. Oxygen, of course, can fulfill the function of a substrate, inducing the formation of cytochrome, participating in the final oxidation. This same cytochrome in its turn can be substrate, inducing the synthesis of enzymes of the chain that provides for the transfer of electrons. But here one must not explain the effect of oxygen on the synthesis of such enzymes as phosphotransacetylase, condensing enzyme, fumarase, succinyl-CoA hydratase, and other enzymes of the tricarboxylic cycle in the cell.
Inasmuch as anaerobic cells accumulate acetate as a product of glucose fermentation, here an adequate amount of acetylphosphate is necessary to induce the synthesis of phosphotransacetylase, however the level of acetylphosphate remains as low as in aerobic cells.

In the same way the content of fumarase can be evaluated. Aerobic and anaerobic cells form succinic acid, these cells possess approximately the identical amount of dehydrase of this acid, but the amount of fumarase in aerobic extracts is 5-10 times higher than anaerobic.

The results of research presented do not permit us to indicate any special products of metabolism present even in low, but adequate amounts to induce the synthesis of enzymes. It remains to be assumed that the presence of oxygen is an adequate factor in order to form these substances, exciting the synthesis of adaptive enzymes in the \textit{r. pestis} cells. In its turn the absence of oxygen, apparently, excludes the possibility of an adequate accumulation of substances inducing the synthesis of adaptive enzymes in the aerobic tricarboxylic cycle.

\begin{table}
\[\text{Comparative Enzymatic Activity of Aerobic, Anaerobic, and Aerobically Adapted Noncellular Extracts of the A 1122 Strain of } \textit{P. pestis}\]
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{No. Enzymatic Activity} & \textbf{Aerob. Extr.} & \textbf{Anaerob. Extr.} & \textbf{Aerobically Adapted Extr.} & \textbf{C} \\
\hline
1. Oxidative decarboxylation of pyruvate & 21.9 & 3.7 & 6.9 & 25.4 & 6.9 \\
2. Phosphotransacetylase & 23.5 & 6.1 & 3.9 & 12.4 & 7.0 \\
3. Condensing enzyme & 82.3 & 7.9 & 10.4 & 90.3 & 11.4 \\
4. Isocitrate-dehydrase & 73.6 & 1.2 & 61.2 & 66.4 & 18.9 \\
5. Oxidative decarboxylation of \textit{\alpha}-ketoglutarate & 3.0 & 0.3 & 10 & 4.0 & 1.2 & 4.4 & 4.5 & 3.8 \\
\hline
\end{tabular}
\end{table}
<table>
<thead>
<tr>
<th></th>
<th>Fumarase</th>
<th></th>
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<tr>
<td>6</td>
<td>50.3</td>
<td>11.1</td>
<td>4.5</td>
<td>16.9</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>Malonate-dehydrogenase</td>
<td>13.4</td>
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<td>2.7</td>
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<td>14.3</td>
<td>3.9</td>
<td></td>
<td>3.7</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>Cytochrome (560 m\text{\mu})</td>
<td>#</td>
<td></td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>8</td>
<td>Acetitase</td>
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<td>0.3</td>
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<td>Succinate-dehydrogenase</td>
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<td>555</td>
<td>1.9</td>
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<td>1460</td>
<td></td>
<td>0.9</td>
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<td>Acetokinase</td>
<td>23.4</td>
<td>16.3</td>
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<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>25.2</td>
<td>30.3</td>
<td>0.8</td>
<td>32.5</td>
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<tr>
<td>11</td>
<td>17.5</td>
<td>20.0</td>
<td>0.9</td>
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<td></td>
<td>Oxalacetate-decarboxylase</td>
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<td>1.5</td>
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<tr>
<td>12</td>
<td>Glycerinaldehyde-phosphate-dehydrogenase</td>
<td>48.1</td>
<td>113.6</td>
<td>0.4</td>
<td>61.4</td>
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<tr>
<td>13</td>
<td>Fructoso-phosphate-kinase</td>
<td>22.9</td>
<td>45.6</td>
<td>0.5</td>
<td>28.1</td>
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AMINOACID REQUIREMENTS AND THEIR SIGNIFICANCE
IN THE METABOLIC PROCESSES OF THE PLAGUE BACILLUS

Cultivating the plague bacillus in complex and chemically unknown compositions of nutrient media (meat-peptone broths), usually employed in bacteriology, has satisfied and even now in most instances satisfies bacteriologists. Such media afford good growth under relatively small inoculation doses, but the main cultural and biochemical properties in freshly isolated strains vary only after prolonged storage and many subculturings.

However, in studying several problems of the metabolism, and in particular, to discover the nutrition requirements, the cultivation of the bacilli on nonprotein media of a precisely known composition assuring good growth and preservation of biological properties is of a specially high significance. Such a variety of "synthetic" media, of course, must not contain substances nonuseful and unnecessary in cultivating. The carbohydrate and mineral substance requirements are more or less the same for a considerable number of pathogenic bacteria leading a parasitic form of life. Consequently, the study of the nutritive requirements of these bacteria reduces mainly to discovering what aminoacids and vitamins they require.

Recently, several studies have been published devoted to studying the requirements of P. pestis for various nutrients. Unfortunately, in most of these studies the requirement of the microbe for a given constituent of the medium was not adequately considered and the broad adaptability of strains of this bacillus to unfavorable conditions of nutrition was not always realized.

All aminoacids and growth factors of microbes can be included among the following groups: 1) indispensable, in the absence of

- 77 -
which growth of the species in question is completely impossible; 2) supplementary, whose presence stimulates growth, but whose absence does not suspend growth; 3) unnecessary vitamins and amino acids whose absence does not affect the growth of the culture.

In a study Rao (44) tested the growth of P. pestis on a mixture of amino acids obtained after hydrolysis of gelatine at a concentration corresponding to 0.5% of the original protein, and upon addition of a selection of mineral salts. It was discovered that such a mixture of amino acids does not assure the growth of the plague bacillus. Upon the addition of cystine to the hydrolysate satisfactory growth was obtained. The resultant medium was so fully valuable that adding to it yeast extract or meat broth or rabbit blood or horse urine did not stimulate growth. Of the six virulent and four avirulent of P. pestis tested, only one virulent strain grew satisfactorily in the gelatine hydrolysate without cystine. For the remaining nine strains one gelatine hydrolysate resulted in barely perceptible growth, which was sharply intensified upon the addition of cystine. In as much as the above named biological liquids do not exert an effect on growth in the presence of the hydrolysate containing cystine, this offers to the conclusion that P. pestis does not require any of such growth factors, and that the array of amino acids in the gelatine hydrolysate upon the addition of cystine satisfies the basic requirement.

It must be admitted that the available quantity of cystine in gelatine (0.2%) is clearly inadequate for successful growth. However, on the basis of these data it cannot be learned which amino acids are necessary, which are supplementary, and which are unnecessary. The author's conclusion on the nonusefulness of any vitamins for the growth of P. pestis is interesting. However, it must be kept in view that the hydrolysate may have contained several vitamins of the original protein.

Moreover, Rao (45) demonstrated the possibility of cultivating the plague bacillus in a medium simpler than gelatine hydrolysate. This medium, in addition to glucose and mineral salts, contained dl-amino acids: alanine, leucine, proline, phenylalanine, cystine, l-argine, and l-lysine. The two last amino acids, as shown experimentally, can be removed from the medium without affecting growth. In this way, it was established that a medium consisting only of five
amino acids along with glucose and mineral salts supports satisfactory growth both of the virulent and the avirulent strains of P. pestis. In addition, it was shown that removing one of the three amino acids — proline, phenylalanine, or cystine — from this medium excludes the possibility of growth.

The author drew the conclusion that the last three amino acids are indispensable. Of course, glycine in accordance with those studies, must be regarded as a supplementary amino acid, since its addition to the medium markedly intensifies growth.

A nutrient medium tested by Berkman (112) in cultivating many species of Pasteurella, contained as complete a gelatin hydrolysate as did the medium of Rao. In addition, Berkman added glucose, mineral salts, and l-amino acids: tyrosine, tryptophan, histidine and threonine, dl-valine, and dl-serine. Tryptophan, tyrosine, valine, and methionine, as it is known, are absent in gelatine. The remaining supplementary amino acids are found in gelatine in very small amounts.

M. Doudoroff (113) tried to cultivate P. pestis on a medium of the following composition: glucose — 0.2%, NH₄Cl — 0.1%, MgSO₄ • 7H₂O — 0.05%, FeCl₃ — 0.005%, CaCl₂ — 0.002%, and a M/30 buffer mixture of KH₂PO₄ / Na₂HPO₄ at pH = 7.0. But not one of the P. pestis strains displayed growth in this glucose-mineral medium. A very retarded growth was obtained upon adding to this medium cystine and phenylalanine at 0.002%. Upon subculturing, as a result of habituation a weak growth was obtained even without amino acids. Adding to the medium 0.002% proline intensified the growth of two strains, but had no effect upon the growth of the other strains of P. pestis.

The addition to a glucose-mineral medium containing cystine and phenylalanine of only one of the following growth factors — biotin, pantothenic acid, thiamine, or hematin — does not affect growth. In the same medium (without growth factors) the authors demonstrated the possibility of replacing cystine with thiosulfate, with sulfite, with thioglycolate, with homocystine, but not with methionine. We note that in the animal organism it is possible to replace cystine and cysteine with methionine, but methionine can never be substituted by cysteine or by cystine. Phenylalanine, as one of the two amino acids in the medium must be included among the supplementary substances, since it stimulates growth, which proceeds with the same intensity as it has without it, but after previous adaptation. Tryptophan, tyrosine, or glycine, and also
Phenylacetic and indolacetic acids can never be included among the supplementary amino acids, since these substances do not exert a substantial effect on growth.

M. Doudoroff concluded that all the amino acids tested have no greater importance for the growth of P. pestis than do ammonium salts. It also was noted that several amino acids added to a medium not containing any amino acids caused a certain suppression of growth. Thus, strain adapted to a glucose-mineral medium revealed a retardation of growth in the presence of 0.002% of L- or Dl-leucine when added jointly with cystine or thiosulfate. Much of the data cited above has been subjected to complete or partial reexamination. Herbert (114) also studied the growth of two avirulent and three virulent strains of P. pestis in media of precise chemical composition. The above described nutrient medium of Berkman, containing glucose, mineral salts, and a collection of twenty different natural amino acids, in the opinion of Herbert, also cannot assure the growth of all the strains tested. The last medium is capable only of providing for the growth of a small number of cells after adaptation.

The nutrient medium of Herbert contained glucose, mineral salts, and the following amino acids: L-glutamic acid at M/200, M/500 glycine, and the following acids at concentrations of M/1000: L-arginine, L-histidine, L-oxyproline, Dl-alanine, Dl-valine, Dl-leucine, Dl-isoleucine, dl-norleucine, Dl-aspartic acid, Dl-lysine, and Dl-proline. The following were contained at concentrations of M/2000: Dl-serine, Dl-tyrosine, Dl-histidine, and Dl-methionine. The racemate of tryptophan was added at a concentration of M/5000. In addition, a mixture of seventeen different growth factors participated in this same medium, but since they did not affect growth, we will not list them here. The sole effective growth factor of these seventeen was shown to be hemin, which also was part of the nutrient medium. Thus, this medium contained glucose, mineral salts, hemin, and a selection of eighteen natural amino acids, differing from the Berkman media only by the presence of hemin and probably, by a rational concentration of amino acids. Such a nutrient medium provides for the growth of all five tested strains upon implanting with only ten cells.

In order to explain the significance of each aminoacid, the authors excluded each of them separately from the composition of the nutrient medium. In this way it was possible to divide all the aminoacids in to four groups: 1) aminoacids whose exclusion does
not effect the growth (glycine, alanine, norleucine, aspartic, glutamic acids, lysine, proline, oxyproline, pyrosine, and tryptophan); 2) amino acids, whose exclusion resulted in a slight inhibition of growth (serine, methionine, and arginine); 3) amino acids, whose exclusion strongly retards growth (threonine and histidine); 4) amino acids, in whose absence growth is impossible (valine, leucine, isoleucine, cystine, and phenylalanine).

However, a medium containing only indispensable amino acids (the fourth group), hemin, glucose, and mineral salt does not result in growth. But growth is absent even upon the addition to the medium of amino acids of the third group. Further complication of the medium by adding various amino acids improves growth, which becomes optimal with the presence of all eighteen amino acids.

Herbert established, moreover, the possibility of replacing hemin by thioacetic acid and even by animal charcoal. The author concluded that hemin is necessary for P. pestis cells in the synthesis of catalase, which protects the cells from the harmful accumulation of hydrogen peroxide. Animal carbon, apparently, is capable of replacing catalase owing to its capacity also to decompose hydrogen carbide. The action of thioacetic acid was explained by the author as due either to the capacity to split the peroxide or to suppress the formation of this peroxide.

Englesberg (115) studied the effect of the sulfur-containing amino acids on the growth of an avirulent strain of P. pestis. The Englesberg medium contained glucose, mineral salts, hyposulfite, phenylalanine, valine, isoleucine, and methionine. It was established that removing any of the existing amino acids and hyposulfites prevents the growth of the culture. Hyposulfite can be replaced by cystine, sulfite, or sulfide, but in the presence of methionine. When hyposulfite is present, methionine can replace cystothionine or homocysteine. Consequently, the P. pestis cells are capable of methylating if they synthesize methionine from such substances as cystothionine and homocysteine.

Rackenmacher and James (116), in verifying the nutrient media of Rao, Eoudoroff, Berkman, and Herbert, found that all of the media in question did not assure the maximum growth of the P. pestis cells, representing 27 different strains. They used a medium called "synthetic casein" and containing, in addition to the mineral salts and glucose, a mixture of pure amino acids, qualitatively and quantitatively corresponding to the casein hydrolysate. In
addition, these authors tested the addition to the medium of fourteen different vitamins, but not one of these growth factors had an effect on the growth of the plague bacillus.

The Rockenmacher–James medium made possible the cultivation of all 27 strains, just as well as in the meat broths, under the condition that it was used in the liquid form, but this medium containing agar requires hemin for successful cultivation.

Ye. M. Gubarev, S. I. Zaplatina, and A. M. Konnova (25) studied the aminoacid requirements of P. pestis cells in a somewhat different manner than had been used by the previous authors. Two strains of this species — the avirulent W and the virulent No 177 — were cultivated in a mineral-glucose medium. The medium was prepared in this way: the following salts were added to a phosphate buffer containing K/15 Na₂HPO₄ and KH₂PO₄ at pH = 7.3: calcium chloride — 1 mg%; magnesium sulfate — 1 mg%; ammonium chloride — 100 mg%; iron oxide sulfate — 20 mg%; manganese sulfate — 2 mg%; sodium chloride — 2 mg%; glucose — 200 mg%; and the aminoacid being tested — 160 mg%. The following synthetic aminoacids were tested: methionine, tryptophan, phenylalanine, valine, glutamic acid, glycine, alanine, tyrosine, cysteine, cystine, norleucine, and asparagine.

When a large amount of inoculation material (one billion washed cells in five ml of medium) at 28°C, over a period of five days a very weak growth of both strains for all aminoacids was detected, with the exception of tyrosine and tryptophan. The growth was noted from the turbidity of the transparent nutrient media. The vitality of cells in such media lasted long; thus, implantations in agar dishes containing the Gottinger medium after a seven-month residence in synthetic media yielded normal growth for colonies typical of P. pestis.

In all media containing one aminoacid, an attenuation of the vitality of the bacilli could be noted: upon subculturing in the same media not only was there no adaptation to it, but on the contrary, with each subculturing the growth became weaker and on an average after four subculturings ceased entirely. The cause for this lies, probably, in that a very high implantation number is required for growth. In solid glucose-mineral media (1.5% Difko agar) growth ceased for one aminoacid in most of the cases. It
was detected only to a weak extent on the seventh day in media containing glutamic acid, glycine, phenylalanine, norleucine, or cysteine. An agar medium containing cysteine provided growth for two strains -- the virulent No 177 and the avirulent No 154 -- if the inoculation amounted to 100 million cells. Here the growth was discovered in the form of large colonies after six days, and from 100 million cells not more than 50 colonies developed. Such an insignificant number of cells was adopted quite well to this medium.

Upon prolonged storage of agar dishes (more than seven days) containing media which had one aminoacid, their contamination by air microflora was noted. A growth of typical colonies of P. pestis was discovered around some colonies contaminating the culturing. Foreign microorganisms, proving to be "small-scale feed" of the plague bacillus were not identified by the authors, but supposedly are among the Sarcina or actinomycetes. Individual tests of these "small-scale feedings" that is, their artificial implantation in a Petry dish which had not displayed the growth of P. pestis, stimulated the appearance of typical colonies of the plague bacillus. However, the "small-scale feedings" stimulated the growth of the plague bacillus only in media containing one of the following aminoacids: glycine, norleucine, phenylalanine, or glutamic acid.

The authors did not investigate in more detail the nature of the "small scale feed" and the P. pestis growth stimulator contained therein, since such a stimulator obtained from Sarcina had been studied by Karpuzidi and colleagues in studies which will be examined later.

Ye. M. Gubarev, S. I. Zaplatina, and A. M. Konnova (25) tested many agar media containing, in addition to glucose and the above indicated mineral salts, the most diverse combinations of two or more (up to seven) amino acids, although in these combinations amino acids showing the most favorable effect on the growth of plague bacilli in liquid media were used, in agar media no growth was forthcoming.

Guided by the indications of Rockenmacher and James on the positive effect on the growth of P. pestis that was shown by the amino acid accomodation approximately corresponds to the composition of the casein hydrolysate, Ye. M. Gubarev and his colleagues obtained an agar medium assuring satisfactory growth for almost all strains tested. The composition of this medium is as follows: alanine -- 23.3%, asparagine 12 mg%, cysteine -- 1.4 mg%, glutamic acid -- 54.2 mg%, methionine -- 15.6 mg%, phenylalanine -- 6.6 mg%, glycine -- 21 mg%, tryptophan -- 23.2 mg% tyrosine -- 11 mg%, valine
-- 16 mg%, norleucine -- 10 mg%, magnesium sulfate -- 4 mg%, iron sulfate -- 2 mg%, manganese sulfate -- 2 mg%, sodium chloride -- 2 mg%, and glucose -- 100 mg%, in addition, the medium contained a M/15 buffer of KH₂PO₄ / Na₂HPO₄ at pH = 7.3 and 1.5% Difko agar. Glucose, cysteine and the salts were added steriley to the remaining components of the autoclaved medium.

Of two strains of P. pestis -- EV and No 177 -- only the latter displayed satisfactory growth in this medium at 28°C, forming a continuous deposit of blending colonies in 18-24 hours for implantation doses of from 100 to one million cells. However, an additional testing of seven more strains, of which six were found in the R-form and only AMP strain in the S-form revealed in all cases growth as satisfactory as that observed for strain No 177. Thus, various authors working with the most diversified strains of the plague bacillus demonstrated the extensive adaptability of these cells to the conditions of the nutrient medium. The cells P. pestis do not require vitamins which are indispensible for their growth. In the presence of the appropriate mineral salts and glucose, several strains are capable of being cultivated on one amino acid. However, growth under such conditions can be obtained with a very high culturing number [See Note]. Hence, the indispensible amino acids that are required for the growth of plague bacillus, without which growth is entirely impossible, can not be easily specified. It is much easier to designate the supplementary amino acids, stimulating growth, although to varying degrees. This category includes, apparently, all the eighteen natural amino acids. However, among the supplementary amino acids there are several, such as, phenylalanine, cysteine, glutamic acid, and valine which are more important than the others.

([NOTE] Experiments with higher inoculating numbers are not convincing, since this makes estimate of nutrients difficult. -- Ed.)

Hills and Spurr (117) established the effect that temperature has on the nutrition requirements of the plague bacillus. Studying the nutrition of three avirulent strains of P. pestis at temperatures within the limits of 23-37 in a glucose-mineral medium with the addition of amino acids, these authors found that for temperatures in the limits of 32-33°C, the optimal medium must contain, in addition to glucose and mineral substances, the following: phenylalanine, valine, isoleucine, cysteine, methionine, and hemin.
Five of the six strains tested used leucine instead of valine, although here the growth attained was not equal to the maximum. Growth at 36°C was at an optimum for the case in which alanine, leucine, serine, threonine, biotin, and pantothenic acid were added to the above-mentioned five amino acids were added to the above-mentioned five amino acids and hemin. The absence of alanine or leucine noticeably retarded growth. In the absence of biotin and pantothenic acid the strains tested showed good growth only in a medium containing a selection of twenty amino acids.

Hemin must be included among the supplementary growth factors. Although the latter does not result in a sharply pronounced stimulation, it does favorably affect growth.

Apparently also several vitamins have a noticeable stimulating action on the growth of P. pestis, although their absence in nutrient media does not exclude normal growth. Thus, Sokhey (118), pointing to the necessary requirement of phenylalanine, proline, and cystine for the growth of P. pestis found that thiamine, nicotinic acid, and other vitamins none the less do stimulate the growth of this microbe.

A distinctive growth stimulator of the plague bacillus is a substance (or substances) contained in many species of non-pathogenic micro-organisms, especially in Sarcinas. Most detailed information dealing with this stimulator is available in the reports of K. S. Karpuzidi et al. K. S. Karpuzidi and A. M. Khokhlova (119) showed that the lysate of the microbe—"small-scale feed", added to the usual liquid and solid nutrient media (Martens' broth or the same with agar), sharply reduced the implantation dose, down to one cell of P. pestis. The rates of culture growth are also accelerated. These results were obtained with three strains, of which two — No 112 and No 291 — were virulent and one — vaccine EV — avirulent. The stimulator tested both during the cultivation process as well as upon long storage (up to twelve months) in media did not change the main biological properties of the strains. Unchanged in the strains were the following: vitality, virulence, response toward bacteriophage, agglutinability, and biochemical activity. In a report by K. S. Karpuzidi and L. N. Makarovskyaya (120) it was stated that this same lysate from "small scale feed" essentially shortens the initial stationary phase of growth and the phase of negative acceleration of the multiplication of the strain EV cells of P. pestis.
A. M. Krichevskaya and K. S. Karpuzidi (121) produced the growth stimulator from the lysates of the microbe-"small scale feed" by means of salting-out the lysates with a saturated solution of ammonium sulfate. It was established that all the proteins of the lysates are salted-out only for 100% saturation of this salt, and the filtrate following removal of the salted-out proteins no longer has the ability to stimulate the growth of P. pestis. Of the number of salted-out proteins the protein fraction in which all the stimulator was contained was dissolved in a phosphate buffer at pH = 8.6-9.2. The remaining proteins were inactive. The active fraction was soluble in water and in a neutral reaction was capable of being precipitated from solution by acetone without changing its stimulating properties.

In this way the soluble protein fraction obtained following washing and acetone drying proved to be a powder, which stimulated the growth of P. pestis cells in a concentration of 1:1,000,000. The P. pestis growth factor has been applied, in the form of the preparation described without subsequent purification, in isolating cultures from weekly infected material, from which growth is difficult to induce with the usual nutrient media.

Growing P. pestis cultures in glucose-mineral media with the addition of a given amino acid mixture, as is clear from all that has been said above, is not free of certain difficulties. These difficulties occur since researchers must deal, as a rule, with strains already adapted to this or that nutrient medium. But a richer growth of any strain without previous adaptation can probably be obtained when all or almost all of the array of amino acids are present in the medium. However, in several cases a medium proved wholly satisfactory although it was not "synthetic", but rather deliberately reduced in proteins. Thus, the hydrolysates referred to above of several proteins yield amino acid mixtures providing a rich growth of the most diverse strains of P. pestis. Of such hydrolysates the most widespread in use is the casein hydrolysate containing glucose and a mixture of mineral substances. Being non-gastric in nature mixture in providing good growth for all strains of P. pestis is convenient for the study of the plague bacillus proteins freed from protein impurities of the nutrient medium.

Hulst and Hulstergie (122) have suggested that nutrient medium was prepared as P. pestis from commercial casein hydrolyzed for
seventy-two hours by sulfuric acid with an admixture of hydrochloric acid, followed by the neutralization of the acids by sodium hydroxide and barium carbonate. The barium sulfate formed is removed by filtering. Several salts of magnesium and iron are added to the hydrolysate in addition to the alcohol extract of the liver. The growth of P. pestis cultures in such a medium does not exceed the growth in meat broths. In filtrates of the culture an active and specific protein antigen has been discovered. The vaccine obtained from such a medium according to the Haffkine method proves to be one-fourth as toxic and four times more active than the original Haffkine vaccine. Finally, the suggested medium is cheaper than meat broth.

For this purpose of reducing production costs of vaccines and more easily standardizing them, Seal (123) proposed the above described casein hydrolysate for a solid nutrient medium. A P. pestis culture in a solid medium produces a cell yield that is 150% greater than that from the same volume of liquid casein hydrolysate medium. In comparison with an agar medium in meat broth the Seal produces a ten-fold cell yield.

Ye. M. Gubarev, S. I. Zaplatina, and A. M. Konnova (op. cit.) can only confirm the advantages presented of the medium containing casein hydrolysate. But these authors join in finding it more advisable to use in the hydrolysis of casein some sulfuric acid, which would assure quite a complete removal in the form of the barium salt.

The above-mentioned studies on the nutrition requirements of P. pestis in terms of amino acids show that for this cell species several amino acids have particular value. Individual studies conducted in this area are of great interest.

Thus, a series of investigations (123, 124) has established that lysis of several bacteria, including E. coli, occurs in glycine solutions. Levine et al. (126) used glycine as an agent lysing P. pestis cells. A suspension of cells in a molar solution of glycine was subjected to lysis. Whereas whole cells assimilate pyruvic and acetic acids, preparations made from cells lysed by glycine, have lost this ability. It is probable that under such conditions damage or degradation of enzymes of the tricarboxylic cycle takes place. But if exposure of cells in a glycine solution is reduced to the extent that only part of the cells are lysed, then the remaining whole bacilli retain the ability to oxidize...
pyruvic acid along with the complete loss of the ability to oxidize acetate. In the latter case the cells acquire the property of accumulating acetic acid through oxidation of pyruvic acid which is here subjected to oxidative decarboxylation.

In the study of Rockermacher and James referred to above (116) it is shown that serine is among the essential amino acids for the growth of P. pestis. This is shown in the cultivation of twenty-seven different strains in media from which individual amino acids were excluded one at a time. However, in the studies of Rao (114, 115) it was shown yet earlier that the strain No 120/H of P. pestis oxidizes serine more rapidly than do fifteen other natural amino acids tested. The observations of Rao have been confirmed by Levine et al (126) in experiments on the avirulent strain of P. pestis A-1122.

In the last case the complete oxidation of serine with the formation of pyruvic and acetic acids as intermediate products was shown. These same authors established that the oxidation of serine by nonmultiplying P. pestis cells is specific for the L-isomer, since for five moles of L-serine as much oxygen is consumed as for ten moles of DL-serine. In this connection, d-serine does not have an affect on the oxidation of the L-isomer. Oxidation occurs also in the presence of the 2, 4-dinitrophenol, which at such concentrations is a powerful inhibitor of the oxidation of several substrates for several species of bacteria. The fact that the P. pestis cells treated with glycine under the above-mentioned conditions is capable of accumulating acetate by way of pyruvate, is used to explain the intermediate stages of serine oxidation. The results of the investigations permit the suggestion that serine oxidation occurs in the following manner /See Note/:

\[
\text{CH}_2 \quad \text{CH} \quad \text{COOH} \\
\text{CH} \quad \text{NH}_2 \quad \text{serine} \\
\downarrow \\
\text{CH}_3 \quad \text{CO} \quad \text{COOH} \quad \text{NH}_3 \\
\text{Pyruvic Acid} \\
\downarrow \\
\text{CH}_3 \quad \text{COOH} \quad \text{CO}_2 \\
\text{ACETIC ACID}
\]
Chargaf and Sprinon (1943) demonstrated an analogous path for Escherichia coli in the conversion of serine into pyruvic acid, an intermediate stage of which is the formation of an aminoacid under the action of the enzyme serinedehydrase (Workman, Wilson, *Physiology of Bacteria*, Moscow, 1954, pages 342). - Eds.
The nutritive requirements of *P. pestis* in regard to amino acids and other organic and mineral substances containing sulfur are of a special importance.

Hills and Spurr (117), Rockenmacher (127) have established virulent and avirulent strains of *P. pestis* requires cystine and methionine. Thus, there exist a requirement for two sources of sulfur in different strains of *P. pestis*. A more detailed study of the sulfur metabolism of the A 1122 strain of *P. pestis* given in the study of Englesberg (115) has shown the following. The growth of this strain in a glucose-mineral medium is satisfactory in the presence of DL-amino acids: phenylalanine, valine, isoleucine, two necessary sources of sulfur - methionine, and hyposulfite - at 0.4 mg/l of each of these compounds. Any of the amino acids containing sulfur, or any of the mineral sources of sulfur alone cannot replace the mixture of hyposulfite with methionine. But in the presence of hyposulfite DL-homocystine or L-cystathionine entirely replaces methionine.

In the presence of DL-methionine hyposulfite can be replaced only by sulfite, sulfide, or L-cysteine, whereas sulfate or DL-homocysteine in this instance entirely excludes the possibility of growth, and L-cystathionine assures only a barely perceptible growth.

In addition, the author succeeded in obtaining a mutant of *P. pestis* -- a strain called M-1, which sharply differs in its sulfur source needs from the previous strain. The M-1 mutant shows satisfactory growth in the same nutrient medium when one of the following substances are used as the only sulfur source: Na₂S₂O₃ 5H₂O, Na₂S 9H₂O, Na₂SO₃, or L-cysteine. But if the sole source is sulfate, DL-homocysteine, or DL-methionine, then the growth is entirely absent and is barely perceptible for the case of L-cystathionine. Weak growth in the last case was explained by the
author as due to contamination of the preparation by some other sulfur compound. Methionine does not assure the growth of the M-1 strain, nor of the A-1122.

Although Doudoroff (113) also found that the A-1122 strain of P. pestis is capable of growing on a glucose-mineral medium upon addition only of phenylalanine and cystine, but growth proceeds under such conditions as would never exclude the selection of the corresponding cells, this possibility being indicated by the author himself: a large amount of implantation material, very slow growth of the culture under a relatively rapid subsequent growth in subcultures. That selection or mutation in this instance is possible has been shown by obtaining the above-described M-1 mutant.

In as much as methionine and cysteine usually are present jointly in proteins or in natural mixtures of amino acids, for example in blood and other tissues, there is nothing surprising in the fact that the requirement for both of these sulfur sources has been elaborated in P. pestis cells under natural conditions of habitation.
Examination of the data obtained in studying the properties of museum strains of the plague bacillus, stored over a long period in artificial nutrient media, and of strains recently isolated from various natural foci of plague propagation demonstrate the existence of a wide range of their intraspecies variability.

The division of plague bacillus strains according to their reaction with glycerin into two groups has been given the widest recognition in the USSR — oxidizing glycerin ("glycerin-positive", "continental", "maternal") and not oxidizing it ("oceanic", "glycerin-negative"). The representatives of each of the indicated groups can be sharply distinguished from each other by their virulence, toxicity, antigenicity, and by other properties. It is entirely obvious that the systematics of the strains presented is artificial and to a certain extent random in its selection of features which constitute its foundation, but it has proven convenient in practical use.

Politzek (128) presented a critical survey of the literature dealing with the classification of varieties of P. pestis, including also the classification of P. pestis proposed by Devignaut.

Three varieties are differentiated in the latter (Table 6).

A rational systematics of strains can be carried out only on the basis of data characterizing in a sufficiently complete manner the metabolism of each of these.
Special difficulties in the classification of strains are presented by those which have been stored for a long period in artificial nutrient media, that is, under conditions differing sharply from the natural. These strains, frequently avirulent, are so decidedly remote from the "wild" strains of the plague bacillus that they are not even always regarded as being of plague origin.

In regard to carbohydrates metabolism of the plague bacillus more is known than of the other forms of metabolism. Therefore it must be used primarily for the systematics of the strains. Carbohydrate metabolism differs sharply in its intensity for strains of differing origins.

Strains isolated from Transbaykalian marmots and common marmots, more energetically oxidize carbohydrates with the formation of acids then do strains obtained from rats and other non-hibernating rodents. Among the museum strains isolated from hibernating rodents, one meets most often forms decomposing ramnose then among "rat" strains, although "wild" strains of the plague bacillus do not decompose it. All museum strains of the plague bacillus change continually, acquiring a progressive capacity to oxidize various carbohydrates with the formation of acids. In addition to this, the circle of carbohydrates that they utilize also expand (ramnose and saccharose).

Thus, the intensity of the formation of acids from carbohydrates increases as one goes from freshly isolated "wrapped" strains to strains of the "Transbaykalian marmot", from "young" museum strains to "old", stored for many years in liquid or solid artificial nutrient media.

In the process of cultivating the plague bacillus in media an increase in the intensity of carbohydrates metabolism occurs more rapidly for "continental" strains then for "oceanic", and in each of these groups the old museum strains are more active then the freshly isolated.

It must be kept in view that all the data presented characterizing "oceanic" and "continental" strains of the plague bacillus
pertain only to those of these groups which have passed for a long period through the same animal, for example in the Transbaykalian marmot, the common marmot, rat, etc. In those cases when passage of the strains occurs interruptedly, now in hibernating rodents, and now in animals not sleeping through the winter, forms allies with intermediate properties.

The interrelationship between virulence, antigenicity, morphological and cultural properties of the plague bacillus and its carbohydrate metabolism is unquestioned. As is generally known, the most virulent strains causing primarily pneumonic plague, are isolated from the Transbaykalian marmots. These strains are typically "continental", with a sharply pronounced capacity to decompose carbohydrates, to form a well-defined capsule, to easily dissociate, and to have a relatively small implantation number. Their antigenic capacity is extremely unstable. Upon loss of virulency the strain rapidly loses its immunogenicity.

The strains isolated from rats are considerably less virulent. The "rat" strains are typical, "oceanic", causing the bubonic form of the plague. Their carbohydrate metabolism is less intensive than for the "continental". Upon loss of virulence the antigenic properties are preserved. In general "oceanic" strains are stable and have a high implantation number.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Fermentation of glycerin</th>
<th>Formation of nitrous acid</th>
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<tbody>
<tr>
<td>P. pestis var. orientalis</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>P. pestis var. antiqua</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>P. pestis var. medievalis</td>
<td>/</td>
<td>-</td>
</tr>
</tbody>
</table>

Remark. - signifies absence, and / signifies the presence of fermentation.
The diagnosis of the plague (P. pestis) and the pseudo-tubercular (P. pseudotuberculosis) bacilli in artificial nutrient media has not presented special difficulties. However, this refers only to freshly isolated strains from focuses of their propagation. The task of differentiating museum strains of the indicated species of microorganisms sometimes is greatly complicated.

Numerous differential-diagnostic media, suggested to distinguish the plague bacillus from the pseudo-tubercular, have as their basis the practical use of the characteristics of their metabolism.

For the great diversity of the media proposed in the differential diagnosis of the plague and the pseudo-tubercular bacilli, they can be combined into a relatively small number of groups. For this summing up the features of the metabolism of the microorganisms which have found in each individual case diagnostic value are used.

The ability of microorganisms to adapt themselves to new and sometimes unfavorable conditions of the external environment has found widespread use in diagnosis. In these cases, the adaptive capacity is determined visually -- there is growth or there is not growth, and its intensity in the given medium being tested.

Of this group of diagnostic tests the use of a medium linking in nutrients both in regard to their concentration as well as to the diversity of organic and inorganic compounds has found amplification.

A. A. Vezhnov (129) has proposed an acid-poor agar for the differential diagnosis of the plague and the pseudo-tubercular
bacilli, based on the fact that the pseudo-tubercular bacillus is less exacting toward the composition of the nutrient medium, than is the plague bacillus. Unfavorable conditions for the growth of bacteria in this medium were compensated by the author by reducing the medium pH down to 5.9-6.1. In acid-poor agar the plague bacillus does not grow, and the pseudo-tubercular bacillus grows weakly.

It is apparent that under rigid conditions of cultivation existing in the Bezsonova medium, only the microbe with a strongly pronounced adaptative capacity can develop. Such a microbe is the pseudo-tubercular.

The same reasoning lies at the bases of the method used by Kurocha for differentiating the plague from the pseudo-tubercular bacilli. This author suggested cultivating both species of bacteria in the Mayed medium, in with asparagine and sodium lactate are used as sources of nitrogen and carbon, respectively. The confirmatory studies of the suitability of this medium showed that most plague strains can grow in it within the limits of 7-21 days, and the pseudo-tubercular strains, with rare exceptions, grow profusely over a period of 1-4 days.

A. A. Bezsonova and A. Yegorova (130) suggested that the growth intensity also of the plague and the pseudo-tubercular bacilli from different implantation amounts in an agar jelly containing meat broth at pH 6.7-7.8 be employed for differential diagnosis. This method, based on the difference in the implantation number, gives good diagnostic results, since the pseudo-tubercular microbe grows from implantation dozes one million or ten million as much as do the plague bacilli.

In cultivating a given microbe in this medium over the course of the first several days a moderate acidification of the latter occurs as a consequence of the complete fermentation of the glucose present in the medium, but subsequently the acids formed are subjected to neutralization by way of the nitrogen basic products of the metabolism, including also ammonia. Since these bases more rapidly and in large amount form in the culture of the pseudo-tubercular bacillus, than the increase of pH in this medium has a more pronounced character. Thus, according to the form of the curve of the medium reaction in these cultures we can differentiate both species of microbes.
It is entirely obvious that in carrying out the research on the medium Otten had to keep in view the buffer capacity of the peptone water. The higher it was, the less "sensitive" was the medium for the purpose described. This was explained by the "uncertainty" of the results and by the "noncoinciding data" in the studies of various researches.

According to the suggestion of A. A. Bezsonova (129), peptone water containing ramnose has found wide application. Freshly isolated strains from focuses of plague propagation do not oxidize the indicated methylpentose, but strains of pseudo-tuberculosis oxidizes readily with the formation of acids. Nonetheless the oxidation of ramnose cannot serve as a reliable indicator for the distinguishing of the pseudo-tubercular bacillus from the plague, since the latter also oxidizes it with the formation of acids, but in considerably lesser quantities.

In the differential diagnosis of the plague and pseudo-tubercular bacilli the use of their characteristics of carbohydrate in the nitrogen metabolism have been widely employed, and precisely the high capacity of the pseudo-tubercular bacillus to ferment carbohydrates and to form ammonia more intensively from the products of protein degradation in comparison with the plague bacillus. This is the basis for the use of the media by Otten and Himelfarb, and others.

In 1926 Otten suggested that peptone water with a low glucose content (0.5%) be used to distinguish the plague bacillus from the tubercular. The initial pH of the medium was set at 7.0-7.2.

N. Ipanovskiy and G. N. Lenskaya (39) in 1944 suggested that the high urease activity of the pseudo-tubercular bacillus be used for the purpose of distinguishing it from plague strains. The "oceanic" strains of the plague bacillus do not decompose urea, however the "continental" strains split it, to a greater or lesser extent. Later, Fokonne (1950) also recommended the use of urea to distinguish the plague strain from the pseudo-tubercular.

An expert committee on the Plague under the Chairmanship of Sokhey (Second Report, Bombay) (31) expressed the belief that the P. pseudo-tuberculosis is easily distinguished from P. pestis. The former is characterized by its mobility, by its nonpathogenicity for rats, by the formation of urease, and the always present ferment-
tation of glycerine and ramnose. In doubtful cases it is recommended to use agglutination, precipitation, and the phage tests.

At the present, the above described basic features of distinction are most often employed in the differential diagnosis of the causatives of the plague and pseudotuberculosis of rodents. Thus, Girard (132) studied the properties of 98 P. pestis strains, most of which were of the "oceanic" type, 29 — of the mediavallis type, and nine — of the antiqua type. He also investigated 40 strains of P. pseudotuberculosis, of which three were isolated from human beings, and the remainder — from various rodents and birds, with the exception of one, which was isolated from a young pig. The author presents a standard plan for differentiating P. pestis from P. pseudotuberculosis, based on the following:

1) test of mobility; 2) test for urease; 3) test for glycerin fermentation; 4) ramnose fermentation.

All the indicated tests are usually negative for P. pestis (this refers to "oceanic" strains).

Devignaut and Bolvin (133) described two complex media, which they had developed with the purpose of facilitating a rapid orientational diagnosis of three varieties of P. pestis and to differentiate these varieties from P. pseudotuberculosis. The first medium was an agar type, containing glycerine, ramnose, and potassium nitrate, and proved in most cases to be adequate for distinguishing between the above indicated types. In doubtful cases a second medium was also used. It is semiliquid medium, containing urea and inulin, and is used for various cultures which are found to exhibit a clear mobility. This medium makes possible the differentiation of all strains of the pseudotuberculosis bacillus from P. pestis. In it mobile cells develop outside a limited zone in the form of dispersed foci, whereas the immobile P. pestis grow in a zone bounded by a sharp line, but do not grow beyond this limit.

To differentiate the plague bacillus strains from the pseudotuberculosis bacillus strains a specific bacteriophage is also employed. Gunisson, Larson, and Lazarus (132) described a bacteriophage which lysed 35 virulent and 37 avirulent strains of P. pestis at 20°, while at the same time not a single one of 45 strains of P. pseudotuberculosis were lysed under those same conditions.
Devignaut and Bolven (135) have presented biological and biochemical characteristics of the plague and the pseudotubercular bacilli for the purpose of differentiating between them. These authors have described methods and results of a comparative study of these strains of three varieties of P. pestis and P. pseudotuberculosis. They tested 14 strains of P. pestis orientalis (variety O); three strains of P. pestis mediovalis (variety M); seven strains of P. pestis antiqua (variety A); five strains of P. pseudotuberculosis, and one strain of a doubtful origin. Two strains of variety O and two strains of variety A were avirulent, all the other strains of P. pestis were avirulent. Variety O did not ferment glycerin, but reduced nitrates to nitrites and formed nitric acid in a broth not containing nitric acid. Two strains of this variety exhibited an exceptionally pronounced property of fermenting ramnose. Variety A differs from variety O by its ability to ferment glycerin, variety M from the others (O and A) by its inability to reduce nitrates or to form NO₃ in a medium free of NO₃.

The pseudotubercular bacillus ferments glycerine, reduces NO₃ into NO₂; it facultatively forms NO₃ in the ordinary broth, ferments ramnose, and forms urease, more actively at room temperature.

Both varieties of P. pestis and P. pseudotuberculosis are sensitive to a phage; the latter has been given the name of Phage yersinianum (136).

The variety mediovalis, found in the southeast USSR, in Kurdistan, and Turkey, differs from two other varieties of P. pestis and from P. pseudotuberculosis by more rapid propagation in cultures, by insensitivity to penicillin, and by a tendency to form pneumonia in mice.

Matumoto (137) studied the strains of P. pestis, carrying out a special survey of enzymatic reactions of the plague bacillus. He investigated the biochemical reactions of 69 P. pestis strains and showed that all of these do not form indole and H₂S; they reduced nitrates to nitrites; they do not alter or easily acidify milk to coagulation; they do not ferment amygdalin, dulcite, erythrite, inosite, imlin, raffinose, saccharose, and sorbite; they ferment glucose, galactose, levulose, mannite, and mannose, and also in most cases arabinose, maltose, xyllose; glycerin and ramnose are
fermented by some strains, and not by others; lactose is not fermented at all or very slightly.

Inconstant results were obtained with adonite, dextrin, glycogen, salicin, starch, and trehalose.

The author believes that, since several strains of *P. pestis* ferment glycerin, and others do not ferment it, the test for glycerin fermentation to differentiate *P. pestis* from *P. pseudotuberculosis* is unsuitable. The observations of the author confirm the earlier discovered relationship between glycerin fermentation and the geographical distribution of the *P. pestis* strains.

Chen (138) in studying 53 *P. pestis* strains of a collection of the University of California found that three of these form acid in a culture medium containing glycerin.

Analysis of the literature data has established that glycerin-negative and glycerin-positive strains are rarely encountered in the same region. All strains without exception obtained from Manchuria, Mongolia, Southeast Russia, Turkestan, and South Russia proved to be glycerin-positive, whereas all strains obtained from other parts of the world were glycerin-negative. The author also notes that the several investigators have demonstrated the conversion of glycerin-positive strains into glycerin-negative upon their prolonged storage in a museum of live cultures or after subculturing.
The virulency of the plague bacillus strains falls off when they are stored in artificial nutrient media. Not all strains under identical conditions of cultivation (temperature, composition of nutrient medium, etc.) lose their virulence equally rapidly. In general, the glycerin-positive apparently lose it more slowly than the glycerin-negative strains -- more readily. Among the glycerin-negative strains there is a great diversity in the rate of decrease in virulence. Thus, several strains retain it tenaciously in spite of prolonged residence in a museum.

Even N. N. Vasternrik (1) attracted attention to the fact that growing the plague bacillus with restricted access to oxygen lead to a decrease in its virulence. This observation can be viewed as a phenomenon related to a change in the type of respiration of the plague bacillus. Alteration in the carbohydrate metabolism, appearing as a result of oxygen deficiency is reflected, undoubtedly, on the nitrogen metabolism of the plague bacillus. The nature of these changes has remained unknown, but they lead to a decrease in the virulence of the strain.

Rockenmacher (139), based on determinations of the catalase activity in 11 virulent and 11 avirulent strains of P. pestis, indicated that the catalase content is considerably higher in virulent strains, and concluded that there is a possibility of using the catalase activity index as a preliminary test in determining the virulence of plague bacillus strains.

S. I. Zaplatina and O. Borodina (140), although they did not find differences in the catalase content in museum virulent and avirulent strains of P. pestis, noted however that the passage of virulent strains through an animal organism increases simultaneously the catalase content and the virulence.
The authors believe that in addition to the ability of the virulent strain to exhibit a considerably higher catalase activity than the avirulent strain, the criteria for differentiating them in vitro as described by Otten do not exist. The authors repeatedly purified the avirulent strain Tjvidey by growing it from a single cell, and then cultivated it in a nutrient medium irradiated by X-ray or ultraviolet rays. The testing of the culture was carried out by intraperitoneal injection in groups of 20-50 mice with doses of $1 \times 10^7$ cells. The mice were observed for a minimum of seven days. Those animals which had perished by this time were dissected, carefully examined, and when a P. pestis culture was obtained from them it was introduced at the same doses into groups of 20 mice. The survival of the animals was taken as evidence of the avirulence of the mutant.

Among 237 strains tested only one virulent strain was found. The question remains unanswered: could not the strain in question randomly change over from a virulent culture? However, such a possibility is considered improbable, since not a single virulent strain was discovered among the remaining 40 individual colonies obtained from an implantation of the original suspension used in the experiment for infecting 50 mice.

This observation allows us to conclude that the ratio of virulent to avirulent cells in the culture was less than 1:40, and from other experiments described in the same study, it appears probable that this ratio is of the order of 1:1000 - 1:10,000.

In view of the unsuccessfulness of efforts to discover the mechanism determining virulence in P. pestis, Barrows and Bacon (142) compared the behaviour of virulent and avirulent strains in vivo and in particular dealt with the degree of resistance of the microbes to phagocytosis by polymophonuclear leucocytes in the abdominal cavity of live mice. In setting up this experiment, before injection of the microbes under examination, leucocytes were mobilized beforehand by intraperitoneal injection of the mice by wheat starch. Initially, the degree of phagocytosis of the virulent and the avirulent strains was identical, but soon the virulent strains exhibited a progressive resistance to phagocytosis and by the end of the fifth hour they ceased entirely to be enveloped by the leucocytes. The microbes manifested here a rapid multiplication in exudate. The avirulent cells continued to be enveloped by the leucocytes, and the number of these cells in the exudated amount to a low level. It was noted that the enveloped bacilli, both virulent and avirulent, became turbid, were pale in color, and no longer could be identified.
as plague bacilli. The virulent bacilli, isolated from the mice after a five-hour contact with the peritoneal exudate in vivo were introduced again intraperitoneally into normal mice. In this instance the bacteria immediately after their administration were not swallowed up by the leucocytes, but, upon freeing the bacteria from peritoneal exudate by centrifuging and again suspending them in buffer solution, the authors did not discover differences in resistance to phagocytosis. This circumstance can stem either from changes in the structure of the bacteria or from the removal of the anti-opsonic or anti-phagocytic factors during the period of the treatment in question; it was established that a six-fold washing of the virulent cells with the phosphate buffer also reduced the force of their resistance to phagocytosis. When the mice were given a second dose of virulent cells, isolated from agar four hours after injection of large doses of these virulent bacilli, noticeable phagocytosis was absent. This compelled the conclusion that the phagocytic strength of the leucocytes becomes extinguished.

In this way, it has been possible only to establish that the more virulent cells are more resistant to phagocytosis, but the mechanism determining the virulence has remained unexplained.

A much greater clarity in understanding the mechanism of virulence has been possible to achieve through serological and chemical investigations of the composition of the membrane protein antigens in virulent and avirulent strains of P. pestis (cf chapter "Chemical Composition of the Plague Bacilli and the Characteristics of Their Antigens").

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Plague infection is a bacteremia with clearly pronounced intoxication, increasing simultaneously with the multiplication of bacilli in the organism.

The existence of toxin in the plague bacillus has been confirmed by clinical, pathologo-anatomical, and experimental data.

The resistance of laboratory animals to plague bacillus toxins is dissimilar. White mice and rats are most sensitive to it; guinea pigs and rabbits are slightly sensitive to these toxins. When cultivated the artificial nutrient media the virulence of the plague bacillus varies to a lesser extent than does its ability to form toxins. A plague culture which has been freed of its toxic properties acquires them again upon passing through the organism of a susceptible animal.

Ferments of young broth culturings of the plague bacillus are less toxic than old. The maximum toxin-formation occurs by the middle to eighth week of culture growth, and then slowly falls off.

Hot and toxins of the plague bacillus are thermally labile; boiling, and in some cases warming up to 37°, reduces their activity. The formation of toxins by the plague bacillus is sharply intensified with the culture is aerated. In this connection, the role of oxygen apparently, to a certain extent consist of increasing the intensity of metabolism of the plague bacillus and to increasing its biomasses.

Thus, the fix has not been possible to obtain any plague bacillus culture pure by the cells in a medium in the form in which it was to be the diptherial or the tetanus bacilli. In this connection, it is often been made that plague toxins are in
stronger combination with intracellular substances. The main argument in favor of adopting this point of view consist of the observations that toxic properties in the culture appear only after destruction of the cells.

We oppose the division of toxins into exo- and endo-toxins in the previous sense of this word.

This does not mean that there is no difference in how strongly various toxins are bound up with cellular substances. Such difference exist in toxins as well as in various enzymes.

At present enough facts have been gathered that support the viewpoint that the strongest toxins of pathogenic bacteria must be considered as enzymes catalyzing degradation reactions, not characteristic of the animal organism, with the formation of poisonous substances. Only under this condition is it possible to explain the high activity of bacterial toxins, exceeding by a thousandfold the toxicity of the most powerful chemical poisons.

Of the bacterial toxins produced in a sufficiently pure form the most active are crystalline preparations of the botulism toxin (200 million fatal mouse doses per one mg of nitrogen) and tetanus toxin (about 75 million fatal doses for mice per one mg of nitrogen) (143). Only the catalytic action of these toxins can provide, in our opinion, for the formation of a sufficient amount of the poisonous products of degradation at the expense of the animal organism.

However, it has not thus far been established what specific processes catalyze these most powerful toxins. We find more fruitful results in the explanation of the catalytic action mechanism of other bacterial toxins, although not available in pure form. Thus, among the B. perfringens toxins, the most active hemolytic poison is lecitinase C, energetically splitting lecitin with the formation of phosphocholin and diglyceride. As a result of this reaction intensive destruction of the cellular membranes of the erythrocytes and hemolysis occurs.

Watanabe (144) in studying the appearance of 60 strains of dysentery bacilli belonging to Schigella flexneri, established that
almost all strains contained the decarboxylase of the following L-
amino acids: histidine, arginine, lysine, ornithine, and glutamic
acid. Using the paper chromatography method this author demonstrated
the formation by the cells in question of the corresponding amines:
histamine, agmatine, cadaverine, putrescine, and L-amino-butyric
acid.

Consequently, the toxins of dysenteric bacteria if not comple-
tely, then to a substantial extent are amino acid decarboxylases,
catalyzing in the organism the formation of such powerful poisons
as histamine, putrescine, cadaverine, etc.

As far as the nature of the pathogenic factors of the plague
bacillus, it should be noted that along with the toxins (or toxin)
of complex structure, characteristic of enzymes, this bacillus also
forms simply alien nonspecific poison substances. These include
many products of degradation of the plague bacillus.

The nature of the nonspecific poisonous substances of the
plague bacillus evidently is varied, but they are all characterized
by the absence of the ability to be neutralized by a specific serum.

During its growth the plague bacillus brings about a sharp
increase in the pH of the medium. The maximum accumulation of alkali,
formed by the plague bacillus, lies within the sixth and eighth
week. This shift in the medium reaction is caused mainly by the
accumulation in it of ammonia and, probably, amines, as established
by K. I. Cherkasova (145). Ammonia was detected not only in broth
media but also in such toxic products as the solution of both "membrane
antigen". Analysis of the latter showed that in individual cases up
to 30% of all the nitrogen is found in the ammonia portion.

Simultaneously with an increase in the pH of the medium for
several strains of the plague bacillus the appearance of a nitrifying
ability whose intensity depends apparently on the medium com-
position, on its oxygen content, and on several other factors.

Formation of ammonia by the plague bacillus takes place not
only in vitro, but also in vivo. Indications to this effect are
found in the studies of N. N. Ivanovskiy, Ye. M. Gubarev, and D. A.
Golov (146). In their study of experimental plague in guinea pigs
they established that the residual nitrogen in the blood is increased, occurring simultaneously with the severity of the affection and of importance for the prognosis of the disease. This increase stems from the urea fraction, determined along with ammonia (147).

Ammonia, and also products genetically related to it — nitric acid and hydroxyls — belong among the physiologically active substances. Animal organisms are more sensitive to ammonia than are plant organisms. The nervous system is especially sensitive; ammonia acts on it as a powerful exciting poison. Ammonia possesses hemolyzing properties.

N. N. Ivanovsky suggested that the main pathogenetic role in plague belongs to ammonia which is formed by the causative agent in the organisms of animals susceptible to this affection. The main source of ammonia is the fermentive decomposition of urea and partly of glutamine and asparagine.

In the organisms of animals the plague bacillus is lysed, releasing into the surrounding environment, in addition to toxins of unknown chemical nature, several enzymes including urease, which in vitro is easily detected in several strains of P. pestis, and in others it is probably adaptively formed in vivo.

With the participation of the enzyme mentioned a large quantity of ammonia is formed.

Urease is a most powerful toxin for mammals. For rabbits a toxic action has been recorded for injections of doses containing 0.03-0.05 mg of crystalline urease, used for immunization aimed at obtaining the anti-enzyme (148). Higher doses can cause fatal poisoning of these animals. It is interesting that after a 60-day immunization rabbits can endure more than 1,000 fatal doses of crystalline urease.

It is possible that there is a certain relationship between sensitivity to urease in various animal species and their susceptibility to the plague.

Actually, tests of unpurified urease preparation (extract from soy beans) on animals susceptible to the plague showed that the most sensitive to urease is the yellow mongoose, Guinea pigs.
are more resistant to urease. The small marmot and the burrowing sandwort [grobenshchikovaya peschanka] is characterized by approximately the same sensitivity to urease, but in general less than the noontime sandwort.

The pathologo-anatomical picture of all rodents succumbing from vegetative urease corresponds in the main features to plague intoxication. The common features include numerous hemorrhages, mucous discharge, swelling of liver and spleen, injections of subcutaneous vessels, and the character of the dystrophic changes in the internal organs. Necrotic nodules in the liver and the spleen are absent.

In a histological investigation of changes in the internal organs of white mice poisoned by ammonia and by "plague toxin" (membrane antigen), substantial features of similarity were discovered. Thus, in the lungs a thickening of the interalveolar partitions due to the multiplication of cellular elements was discovered, blood was contained in the clearances of the alveoles (blood discharge); in the liver -- the parenchymatous elements were greatly swollen, the boundaries between them could not be distinguished, and the centers were pale, and frequently swollen. In several instances a rough basophilic graininess was discovered. Frequently binuclear liver cells were found. The inner-lobular capillaries were filled with blood.

When animals are poisoned by ammonia and by the plague bacillus toxin sometimes in the myocardia and other times in the kidneys dystrophic changes have been observed in the cellular elements: graininess of the kidney epithelium protoplasm, disappearance of the transverse striation in the muscular fibers of the heart, presence of hemorrhages in the kidneys and in the myocardia. Hyperplasia of the follicular apparatus has been noted in the spleen with great constancy, manifested to differing degrees.

In the action of the plague toxin in particular instances hemorrhaging has struck the follicles themselves.

Ammonia and the toxins of the plague bacillus affect to varying degrees the parenchymatous elements of the internal organs, causing dystrophic changes in them, and on the vascular wall, owing to which hemorrhages form in several organs.
The similarity of the toxin action of ammonia and of the plague toxin has been confirmed also by the presence in both instances of binuclear liver cells and the phenomenon of hyperplasia of the follicular apparatus of the spleen.

That urease of pathogenic microbes leading to the accumulation of enormous quantities of ammonia in the blood and in the tissues is of essential importance during the course of the disease is shown by the following facts, pertaining to another affection — brucellosis.

In their experiments Bonaduce and Orlandella (1979) established that all types of brucelli contain significant quantities of urease, degrading urea when these microbes are cultivated in nutrient media.

Ye. M. Gubarev, Grabenko, Gralayev, and Kobzar (147) established that for persons suffering from brucellosis a sharp increase in the amount of ammonia in the blood corresponding to the severity of the clinical manifestations of the disease has been observed. But even in instances of the sluggish progress of the infection the amount of ammonia in the blood is increased several times above the normal level.

The authors, taking into account the data of N. N. Ivanovskiy on the significance of ammonia intoxication for experimental plague of guinea pigs, used subcutaneous injections of 5% aqueous solutions of glutamic acids (5-10 ml per day) with the simultaneous administration of 1% ATP solution (1 ml per day, to bolster the synthesis glutamine) aimed at treating brucellosis. As a result of a ten-day treatment a decrease, sometimes down to the normal, of the amount of ammonia in the blood was observed in the patients with a parallel decrease or disappearance of the clinical symptoms of the affection; high temperature, pains, tendency to perspire, etc.

A. I. Zheltenkov (150) emphasized that the biological activity of "membrane antigen") decreases sharply under the influence of high temperatures, calcium chloride, alums, alcohol, and formaldehyde. Based on this he proposed the protein character of the toxic substance of "membrane antigen." The chemical analysis of the antigen confirmed the presence of protein in amounts not exceeding approximately 0.3-0.4%. At the same time individual batches of "membrane antigen" exhibited a clearly pronounced ability to decompose urea with the formation of ammonia.
Investigations carried out on white mice showed that treating the membrane antigen with substances inactivating urease (iodine, formaldehyde, protargol) was accompanied by the loss or a decrease in toxic properties.

The investigation of Englesberg and Levy (151) was devoted to studying the formation of the plague bacillus toxin. They noted that the growth of the EZ-76 strain in a glucose-mineral medium containing casein hydrolysate at 30°C is characterized by the maximum yield of cells (5.6 x 10⁹ per 1 ml after two days of cultivation). Then a massive lysis of the cells took place. The plague toxin entered the medium during the lysis and subsequently, reaching a maximum on the seventh day of culture growth. This entry of the toxin into a nonprotein medium during its growth is the simplest method, previously underestimated, for obtaining a relatively pure toxin. The latter was obtained from the centrifugate (in the cold) of a seven-day culture precipitate at 100°C saturation of ammonium sulfate, dialysis at 30°C, and by lyophilization. The toxin contains one mouse LD₅₀ per 1.4-1.8 g of substance with a yield of 0.85 g per one l of medium.

It withstands storage without loss of toxicity for many months in the cold in a vacuum-desiccator. The powder is readily soluble in a physiological solution, yielding a solution amber in color. It contains 10.8-11.6% nitrogen, decomposing when heated, and rendered harmless with formaldehyde. Of the two strains EV 76 and A-1122 compared (both avirulent) the latter yielded the toxin one-fourth and one-eighth times as much, respectively, at 30 and 37°C when grown in the above indicated medium. The strain EV 76 initially did not grow at 37°C, but a mutant of this strain was obtained, which grew well at 37°C, using the same yields of cells and toxin as the original strain.

Hoyer and Robbins (152) presented unpublished data of Hoyer, Hoyer, and Pillemer on obtaining from P. pestis cells a purified toxin of exceptionally high activity. The toxin was obtained in the following manner: a strongly toxicogenic, avirulent strain of plague bacillus was grown in hormonal agar at 28°C for seventy two hours. The cells, washed free of agar with a physiological solution, were precipitated from a suspension by two volumes of acetone at 30°C, washed with acetone at low temperature, and dried...
vacuum. This powder was twice extracted with a 2.5% neutral solution of sodium chloride at 40°C centrifuged, and from the resulting extract the toxin was isolated by means of fractionation.

Purification of the toxin consisted in precipitating it from its water-salt extract with a 40% solution of methanol temperature of 50°C, pH = 5.5, and an ion concentration of 0.23. Impurities contaminating the toxin were removed with a subsequent precipitation at a temperature of 0°C, pH = 5.0, and ion concentration equal to 0.04. The toxin was reprecipitated at a temperature of -50°C, ion concentration = 0.04, and pH = 4.7 in 40% methanol. Then the impurities were removed again at 0°C, ion concentration = 0.04, and pH = 4.0. The last reprecipitation of the toxin was carried out at 0°C, ion concentration = 0.21, pH = 4.0. As a result of dialysis against distilled water the last precipitate was freed of about 30% nitrogen substances.

The dialyzed solution of the toxin contained 0.5% protein and 70,000 mouse DL50 per one ml or 66,000 DL50 per one mg of nitrogen. This is the most powerful toxin that has been obtained anywhere from gram-negative bacteria. Analysis of the purified toxin showed a 13.4% nitrogen content, 19% carbohydrates, 2.5% phosphorus, 1.5% sulfur, but the electrophoretic method established that the toxin was not uniform.

It is possible that the success in obtaining such an active toxin depended on the selection of the appropriate avirulent P. pestis strain with high toxigenicity.

The studies published by various authors on the P. pestis toxin are characterized by a description of extremely dissimilar aspects of this poison's action. Under such conditions we are forced to conclude that the substances involved are not the same, that is, the plague bacillus forms several toxins, in addition to several toxic substances not exhibiting antigenic properties.

Goodner established the interesting fact (153) that the toxicity of filtrates of plague culture in a certain sense differ sharply from the filtrates of other pathogenic bacteria. The action of surface-active substances on bacterial toxins, as is known, either causes destruction of partial inactivation of the toxin. As examples we can cite the detoxifying action of alkali salts of the higher
fatty acids on the diphtheria toxin. Free bile acids (cholic, desoxy-
cholic, and lithochoic), but not their binary compounds with glycocol
and taurine, are powerful detoxinizing agents. The effect of these
acids is increased upon the reducing the number of hydroxyl groups
in the bile acid molecule. Thus, desoxycholic acid is fifteen times,
more powerful in detoxifying diphtheria toxin than is cholic acid.

Completely contradictory, that is, not inactivating but
fortifying, action has been discovered for desoxycholic acid on
the toxic filtrate of plague culture. This fact is at present
the only example of the activating effect of bile acids on bacteri-
toxins.

Pannel (151) tested the action of several surface-active
substances on noncellular filtrates of the avirulent P. pestis strain
EP76. It was shown that of the substances tested all the anionic --
for example, desoxycholate, laurylsulfate -- intensify the toxicity
of the filtrates for white mice.

Of the cationic substances several -- for example, cetyl-
trimethylammonium chloride -- also increase the toxicity of fil-
trates, while others -- for example, lauryldimethyl-benzyl-ammonium
chloride -- did not have an effect on toxicity. Finally, non-
ionizing substances, such as the oleate ester of polymerized
polyethylene glycol, do not affect toxicity.

A more detailed study of the effects of desoxycholic acid
on the toxins of the avirulent P. pestis EV 76 strain has revealed
the following (155). Frequent of these microbes with sodium des-
 oxycholate caused a lysis of the cells with the release of high
toxicity substances. Taking as a unit the amount of toxin con-
tained in one billion cells, it was established that after a
15-hour treatment at 4° and a desoxycholate concentration of 0.5
and 2.5% under the same conditions. Toxicity was increased by a, re-
spectively, by 33 and by 70 times. But if the increase of toxicity
here can derive from the intensified lysis, which develops more
slowly in cells introduced into mice without the preliminary des-
oxocholate treatment, then moreover analogous of the bile acid on
the filtrates of a culture obtained through the Berkedfield candle
has been seen. Such a filtrate, containing before the desoxycholate
treatment 2 units of toxin per one billion cells, following an
hour's treatment with the equal volume of a 1.5% sodium desoxycholate
solution at room temperature was found to contain 16 units of
toxin.
Heating the filtrate of the *P. pestis* culture for 30 minutes at a temperature of 67° leads to a complete destruction of two antigen-specific toxins present in solution. But the same warmed filtrate following the desoxycholate treatment is found to exhibit high toxicity. Gunder, Panner, Bartel, and Rothstein, (155) suggested that in addition to formed toxins, culture filtrates contain also a firmly stable protoxin, activated by bile-acid salts.

Of unquestioned interest is a comparison of several toxins of *P. pestis* and *P. pseudotuberculosis*. Schar and Thal (156) obtained plague toxin from the avirulent EV 76 strain following seven-day cultivation in a glucose-mineral medium containing casein hydrolysate at 20°. The culture liquid following this was salted out at 100° saturation of ammonium sulfate, the precipitate was dissolved at 35° saturation by this salt and the toxin was again precipitated at 70° saturation. The preparation of plague toxin as shown by electrophoresis, is a nonuniform protein.

Pseudotubercular toxin was isolated from the toxin strain 105/3 and also from two nontoxic, but virulent strains 14/1 and 16/2. All three strains were grown for two days in the hormonal agar of Hantun at 37°. *P. pseudotuberculosis* cells, killed with toluene, were washed with an 0.9% solution of sodium chloride and extracted by a mixture of phosphate buffer, with a pH of 7.5 and 1% bicarbonate of soda.

Pseudotubercular and plague toxins are inactivated by a 60-minute heating at 60° and are readily converted into anatoxins with formaldehyde. All the toxins tested exhibited high toxicity for mice and rats, but only the pseudotubercular toxins proved to be active for guinea pigs and rabbits. For the plague bacillus toxin rabbits and guinea pigs exhibited high tolerance.

The nature of the action of the toxins of the plague and the pseudotubercular bacilli differs. Under the influence of the *P. pestis* toxin clotting of the blood and a drop in arterial pressure was observed in rats. These symptoms are not produced in rats by the toxins of *P. pseudotuberculosis*. The specific antitoxic serum in proportional amounts completely neutralized the pseudotubercular bacillus toxins in vitro and in vivo. But the *P. pestis* toxin only partially is neutralized by its specific antitoxic serum. The overlapping neutralization of the toxins of *P. pestis* and *P. pseudotuberculosis* by their antitoxic serums has not been observed [See Note].

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(NOTE) Adzel, Ryadal, Darram, Warren, and Wally (J. Bacteriol., 1955, 70, No 2, 158-176) described in detail a method for obtaining and purifying the toxin of plague bacteria, both virulent and avirulent. Starting with the salt extract of the acetone preparation of the bacteria, the authors successively used the following methods for fractionating the protein mixtures: salting out at increasing stages of saturation by (NH₄)₂SO₄, repeated isoelectric precipitation, removal of nucleoproteins by using MnCl₂, precipitation by increasing quantities of methanol adsorption on a calcium phosphate gel with subsequent elution, precipitation by ammonium sulfate at the isoelectric point and the preparative electrophoresis with convection; the resultant toxin was electrophoretically uniform. The following were determined: solubility, electrophoretic mobility (7 x 10⁻⁵ cm²/sec) and the following constants: diffusion (3.43 x 10⁻⁷ cm²/sec), sedimentation (S₀ = 2.73 x 10⁻¹⁳), molecular weight about 70,000; N = 11.1%; P = 0.2%; S = 1.9%. Chromatographic analysis revealed twelve known and three unknown amino acids. Toxicity for mice is as follows: ID₅₀ (vivo) = one millimon. A good antigen, it is distinctive in its specificity both from the capsule as well as from the somatic. — ED.)
HEMOLYSIN OF THE PLAGUE BACILLUS

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The widely known ability of the plague bacillus to cause hemolysis is of considerable interest from the point of view of the analysis of its pathogenetic factors, as shown by Ye. I. Korobkova (157).

Hemolysins are very widespread among pathogenic microorganisms. Several animals also are capable of elaborating hemolytic toxins with very high activity. Included among these animals are many species of snakes, scorpions, bees, and others. However, the study of the character of animal and bacterial hemolysins has uncovered an essential difference between them.

The hemolytic poisons of animal origin possess the activity of lecitinase A, cleaving only a group of an unsaturated fatty acid from the molecules of the lecithins and cephalins with the formation of lysolecithins and lysocephalins. The latter are essentially hemolysins.

Bacterial hemolysins in several cases are included in the lecitinase C group. These enzymes cleave lecithins with the formation of phosphocholin and diglycerides of fatty acids. Both the cleavage products mentioned do not exhibit homolytic acid. Hemolysis here occurs due to the degradation of the lecithins of erythrocytes, in which they are structurally important. Included among the lecitinase C is alpha-toxin B. perfringens hemolyzing only in the presence of calcium ions. This hemolysin attacks erythrocytes of many animals, with the exception of goats and horses (158). However, for most hemolysins of bacteria not included among the lecitinase C group, the mechanism of hemolyzing action has not been established. Thus, the delta-toxin of the same cells of B. perfringens is a hemolysin.
only for erythrocytes of artiodactyla animals. This enzyme is not
activated by Ca\(^{2+}\) ion and the mechanism of its action has not been
explained.

Also inadequately studied is the mechanism of the action
of pneumococcal, staphylococcal, and other bacterial hemolysins.

The plague bacillus exhibits hemolytic properties, but
they are considerably weaker than in other bacteria species. Hemolysin
is discovered in the filtrate of a broth culture and in the autolysates
of agar cultures.

Hemolysin forms slowly, during the growth of a P. pestis
culture and readily diffuses into the surrounding environment. The
hemolysin of the plague bacillus is comparatively resistant to heat
and is destroyed only at 100\(^\circ\).

It is probably that is is a low-molecular substance, rapidly
diffusing into a gel and losing its properties when heated to 100\(^\circ\).
Apparently, the hemolytic properties of the plague bacillus to a
considerable extent depend on volatile bases, which are formed
during the process of the life activity of the microorganism or
during its autolysis. Of the bases formed by the plague bacillus,
one must keep in mind first of all ammonia, whose presence in the
culture has been demonstrated by studies of various authors, and
volatile amines, which also, probably, are formed during the growth
of P. pestis. Ammonia and amines exhibit sharply pronounced ability
to effect hemolysis, in which the intensity of their hemolytic action
depends on the extent of dissociation of the bases. The maximum
hemolytic effect occurs when the medium shows an alkaline reaction,
in which the bases are found to a large extent in an undissociated
state. The substances indicated, as a consequence of their ready
solubility in lipoids, easily penetrate into erythrocytes, causing
by this action the destruction of the stromes and the transfer of
hemoglobin into the surrounding environment. It must, however, be
noted that ammonia and amines destroy not only red blood cells, but
are also a poison for all cells and other tissues of the organism
including, of course, also for the central nervous system. Apparently,
specifically in the attack on the central nervous system by the
volatile bases -- products of the life activity of the plague bacillus
-- must be sought an explanation for blindness and the rapid death
of rats, noted in the experiments of Ye. I. Korobkova (157) when
she tested the toxicity of the filtrates of broth cultures exhibiting
hemolyzing properties.
These observations, favoring the nonspecific nature of the hemolysin of the plague bacillus, must be supplemented by comparative biochemical data. As is well known, the P. pseudotuberculosis analogously with the plague bacilli also exhibits hemolytic properties. However, destruction of erythrocytes by products of the life activity of the latter species of microorganism, at least when grown in a liver agar of the Boyliye type, is noted even by the second day, whereas for the plague bacillus the initial phase of hemolysis can be detected not sooner than the fourth or fifth day of the culture growth. This phenomenon becomes understandable if it is remembered that P. pseudotuberculosis alkalinizes the medium more rapidly than does P. pestis. Vacuum distillation of the volatile substances from a broth culture of the plague bacillus that has been alkalinized by sodium hydroxide can free it from "hemolysins", which are driven off in the distillate. From the distillate ammonia can be separated and identified. A broth culture, concentrated under vacuum, freed in this way of its hemolytic properties, partly loses its toxicity for animals. Thus, there is a certain parallelism between the hemolytic ability of the plague bacillus and the toxicity exhibited.
In spite of the fact that the bacteriophage of the P. pestis, described by d’Erlon 25 years ago and subsequently by N. P. Polkovska, has been shown to be a very powerful factor in the variability of the plague bacillus, thus far sizable successes have not been obtained in the study of this phage. Nonetheless the established fact to the variability of several strains under the influence of the phage has served as a foundation for a conclusion by N. N. Zhukov-Varezhnikov et al (159) on the conversion of the plague bacillus into another species of bacteria.

The action of bacteriophage is often characterized by an incomplete destruction of cells. Lyzed cultures after some time yield "secondary growth". This situation destroyed to a considerable degree the hopes of the first researchers on the bacteriophage of using phages as powerful specific agents in the treatment of infective diseases.

The reaction of the plague bacteriophage causing profound changes in the properties of the bacillus detected in the secondarily growing cultures has also served as grounds for a hypothesis on the conversion of P. pestis into another already existing species that is similar to it — P. pseudotuberculosis.

We will dwell briefly on the investigations which have illuminated the main problems of the biochemistry of the bacteriophage.

We recommend the monograph (160) and the article (161) of Evans on surveys of the biochemistry of phages.

Electron-microscopic study of the coli-phages of the types T₁ - T₉ has revealed differences in their form and size. At present no form has been established for all these types, similar
to spermatozoids. The diameter of the head ranges within the limits of from 45 millimicrons (T3) to 90 millimicrons (T5), and the tail dimensions — 120 x 10 millimicrons (T1) and 170 x 15 millimicrons (T5). The molecular weight of the particles is very high; thus, for the nucleoproteids is equal to: $2.46 \times 10^6 - 5.84 \times 10^6$.

Coliphages constitute four different immunological groups. For the T2 phage the presence of two different (head and tail) antigens was shown. The chemical composition of coliphages are characterized by a predominant nucleoprotein content with a small amount of lipids and carbohydrates. Deoxyribonucleic acid (DNA), of the nucleic acids, is chiefly in evidence — about 90% — but ribonucleic acid (RNA) is also present in small quantities, although several researchers have suggested that the latter is an impurity entering the preparations from bacteria. The aminoacid composition of the virus proteins is similar to that of the bacterial cell-host. In bacteria RNA clearly predominates, representing around 50% of the mass of the entire cell, whereas DNA is contained in cells in quantities equal to 11-22%.

Three phases are distinguished in the action of a phage on bacteria:

1) adsorption of the virus by the bacterial cell; 2) its introduction into the cell; 3) lysis of the bacterial cell with the liberation of newly formed virus particles.

In the first phase of the adsorptiunal union of the phage to the cell, break down of the virus particles and liberation of DNA occurs, proceeding evidently without the participation of the enzyme systems of the cell, inasmuch as this process can occur in killed cells.

A second phase of the action of the phage commences, when, according to the data of Hershey and Chase (162), DNA liberated by the phage particles is enclosed in the bacterial cells, while the protein membrane of the phage remains bound outside the cell and can be liberated by simple agitation of the infected material without affecting its later stages of virus reproduction.

Of the greatest interest for the characteristics of the third phase of phage action (multiplication of the phage and lysis of the cell) are the investigations of Putman and Kozloff (163) on the E. coli cells, infected with the T6 bacteriophage tagged with P32.
They showed that the greater fraction of the isotope was detected in the soluble fractions not containing the virus. But only 1/3 of all the isotope was found in the subsequent generations of the virus.

In one of his studies Kozloff (164, 165) carried out a special investigation of the mechanism with which \( ^{15}N \) and \( ^{32}P \) of the original tagged T6 bacteriophage are utilized for the synthesis of new phage particles. The greater fraction of the original virus material discovered in new generations was formed through the use of the degradation products of the original virus for the synthesis of new viral nucleoproteids.

A considerable quantity of nucleic acid of the bacterial cell is also used for the synthesis of the DNA of phages, which was shown primarily by the conversion of the cellular \( ^{32}P \) into the viral \( ^{32}P \), detected in the DNA of the bacteriophage. This was confirmed by using tagged purines and pyrimidines, since the latter move from the cells into the nucleic acid phages.

In lysogenic cultures the phage particles were converted along with these the absorbed viral particles also were propagated, such that each new generation of cells formed with phage particles adsorbed on them. In the given instance conversion of the prophage into the phage lyzing cells with the release of viral particles into the medium was often observed.

The action of viral particles on cells is capable of inducing profound changes in these cells, observable by objective methods. A rare example of change in the biological properties of bacterial cells under the influence of a phage can be seen in the conversion of the nontoxigenic strain Corynabacterium diphtheriae into a toxigenic. The reverse transformation has also proven to be possible, regularly occurring in the loss by the cells of the lysogenic phage. Thus, Ye. M. Gabarcev, S. A. Kacherova, and G. M. Frenkel' (166) observed the sudden loss of toxin-formation for the PW-8 strain, used in the production of diphtheria toxin, and established that in the present instance formation of a new variety occurred with several altered properties. The appearance of a lemon-yellow pigment was noted in this variety, as well as small changes in the character of growth (morphology of films and colonies), and changes in the enzymatic activity and several characteristics of the chemical composition of the cells.

In the reports of Freeman (167), Freeman and Mores (168) a relationship was established between the action of the bacterial phage and the toxigenicity of diphtheria bacteria. In his study Grossen (169) reported on results of extended observations of this
phenomenon. The author indicated that the conversion of the non-
toxigenic C-4 Corynebacterium diphtheriae strain into the toxigenic
variety (C-4 (P)) is caused by the action of the betaphage. The
reverse conversion of the lysogenic, toxin-forming variety D-4(P)
into the non-lysogenic state C-4 is accompanied by the obligate loss
of toxin-formation. The author succeeded, in addition, in isolating
a new phage capable of converting the C-4 strain into the lysogenic
without being converted simultaneously into a toxigenic.

Inasmuch as phages are parasitizing nucleoproteids in the
bacterial cells, they must be regarded as possessing the property
of causing a strongly pronounced variability of bacteria which is
passed on through heredity. The formation of varieties of bacteria
with sharp differences from the original strain under the influence
of phages has afforded several authors adequate grounds even to
assert that the phages are capable of converting one species of
bacteria into another known species. Thus, G. N. Lenskaya (170)
 wrote: "... the conversion of the plague bacillus into the pseudo-
tubercular takes place along the pathway of qualitative transfor-
mation in the absence of intermediate forms. Only one conclusion
is possible, that the addition of a bacterial phage undoubtedly
accelerates the process of species-formation; we are dealing here
either with a restructuring of the noncellular living substance and
the type of metabolism by means of this factor, or we are dealing
with the acceleration of qualitative changes in the composition of
the nutrient medium ..."

"And thus, on the basis of the factual material presented
we assert that the plague bacillus is capable of being converted
into the pseudotubercular."

Demonstrating that the plague bacillus before its conversion
into the pseudotubercular differs markedly from the latter in diverse
particulars, including also response to the bacterial phage, G. N.
Lenskaya wrote: "To the extent that difference in metabolism exists,
it also exists in response to the bacterial phage. It is enough to
state that the plague bacterial phage does not cause lysis of the
pseudotubercular microbe. Unfortunately, in many studies concerned
with this question, the term 'pseudotubercular' bacterial phage has
been designated as being of a little studied nature, which lyzes
both the plague and the pseudotubercular bacilli."

However, Gunnison and Lazarus (171) showed that the strain
of the P. pestis phage tested by them caused lysis not only of its
host but also lysed 19 of 27 strains of P. pseudotuberculosis selected and six of 37 strains of Schigella dysenteriae. More recently these same authors (172) showed that the plague bacillus phage selected by them for testing lysed 31 of 40 P. pseudotuberculosis strains. All the remaining nine strains also could be lysed by the phage tested, but after its adaptation even to one of the nine initially unlyzable strains. The phage adapted in this manner following transferral into the Schigella dysenteriae cultures reverted to its original properties and ceased to lyse the above indicated nine strains. A new transferral of the phage into the culture of the already utilized strain of the nine strains in question again activated the phage, and it began anew to lyse all the 40 P. pseudotuberculosis strains.

With such a variability in the specific antiplague phages, sometimes without even adaptation, lysing most of the pseudotubercular bacillus strains, the suggestion of G. N. Lenskaya to use a related phage as a criterion for deciding whether the P. pestis has made a transition to the P. pseudotuberculosis can hardly be regarded as well substantiated.

In 1955 N. N. Zhykov-Verznikov (159) turned to the problem of the conversion of the plague bacillus into the pseudotubercular. He wrote: "in this instance the modification occurs under the influence of a bacterial phage introduced into the nutrient medium." As shown by N. N. Zhykov-Verznikov et al, in testing the S-form of one of the P. pestis strains under the influence of high phage concentration was entirely lysed, but the secondary growth appearing after two days yielded a culture containing not the plague, but the pseudotubercular bacillus. In the above referred to article (159) the following is stated: "detailed investigation has shown that the culture obtained in this way differs from the original by more than twenty features. Most of these characteristics coincide with the features of the pseudotubercular bacillus, which, as is know, is close to the plague bacillus, but is well defined independent species."

Assuming that even twenty characteristics distinguishing the new strain from the original coincide with the properties of the P. pseudotuberculosis, this can never be considered as objective evidence of the conversion of one species into another. With little trouble a number of changes more than this can be shown as representing the
characteristics in which this original *P. pestis* strain before its conversion was similar to *P. pseudotuberculosis* strain.

However, the method itself of obtaining new varieties of the plague bacillus by the action of the bacterial phage deserves attention and was thoroughly evaluated and used in practice both before, as well as after the investigations of N. N. Zhukov-Verezhnikov et al.

M. P. Pokrovskaya in 1931 obtained an avirulent mutant called the AMP strain through the action of a specific bacterial phage from one *P. pestis* strain, which mutant exists primarily in the S-form. More recently the same author (173, 174) published the results of many years of study of the AMP plague bacillus strain. Through these observations it was established that the AMP variety retains stability almost complete avirulence and other newly acquired. Its avirulence was tested not only on animals, but also on human beings during the same period in which the effective agents for plague treatment now available to medicine did not exist. M. P. Pokrovskaya first inoculated herself, and then several volunteers with a live culture of a AMP strain and suggested its use as a live vaccine against plague. However, she also did not deal with the problem of the conversion of *P. pestis* into another species under the action of a bacterial phage.

V. M. Tumansky in 1937 (2) treating nineteen different *P. pestis* strains with an antiplague bacterial phage, obtained from one of these a stable mutation. The Tumansky strain proved to be similar, in several cultural and immunobiological properties, with typical *P. pseudotuberculosis* strain. The author was more inclined to study here the conversion of one species into another, but made the following reservation: "it must be assumed that our strain as a mutant of the plague bacillus must be distinguished from the *pseudotubercular bacillus*, but we cannot yet explain this difference."
Conversion of the plague bacillus of pseudotuberculosis of rodents, beginning in 1929, has been frequently noted by co-workers of the Saratov Antiplague Institute. These transformations have been described for old laboratory strains of the plague bacillus and have been related with substantial changes in their metabolism.

Several properties of newly formed varieties of continental strains of the plague bacillus are identical with natural strains of the causative of the pseudotuberculosis of rodents. The observations referred to have also provided grounds for drawing a conclusion of the conversion of the plague bacillus into the bacillus of pseudotuberculosis of rodents. However, the difficulties in distinguishing old laboratory strains of the plague bacillus from pseudotubercular must always be kept in view. We are not at all inclined to evaluate these changes as transformation of one species into another existing species, but consider it useful to examine the facts that are of much importance in the investigation of widely distributed processes of far-ranging variability characteristic of many microorganisms.

**Shifts in Carbohydrate Metabolism**

The general direction in altered carbohydrate metabolism of plague bacillus strains found in live museum cultures amounts to an increase in their fermenting ability. A typical freshly isolated strain of the plague bacillus has an ability of fermenting carbohydrates that is many times less pronounced than old laboratory strains.

All researchers agree that the pseudotubercular bacillus oxidizes carbohydrates more energetically than does the plague.
bacillus. In this connection, the range of sugars fermentable by the pseudotubercular bacillus is considerably broader. Change in the properties of the plague bacillus which lie in the direction of making it similar with the bacillus of pseudotuberculosis of rodents is associated with an increase in fermenting activity of P. pestis. This is clear from data in Table 7 and has been confirmed by quantitative determinations carried out in a study of the intensity of ramose decomposition by strains of P. pestis and P. pseudotuberculosis (N. N. Ivanovskiy and V. S. Basheva) (176).

### TABLE 7

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Response Toward Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. pestis</td>
</tr>
<tr>
<td>Arabinose</td>
<td>/ (-)</td>
</tr>
<tr>
<td>Xylose</td>
<td>/</td>
</tr>
<tr>
<td>Ramose</td>
<td>- (())</td>
</tr>
<tr>
<td>Sorbose</td>
<td>- (())</td>
</tr>
</tbody>
</table>

Symbols: / ferments; - does not ferment; / (-) most strains ferment, a minority do not; - (\(\)) most strains do not ferment, a minority do; / some strains ferment, others do not.

### Shifts in the Ability to Oxidize Polyatomic Alcohols

Shifts in the ability to oxidize polyatomic alcohols in the course of the variability of the plague bacillus are unquestionable and consist principally in changes in its carbohydrate metabolism. Polyatomic alcohols are oxidized considerably more readily and in greater number by the pseudotubercular bacillus than by the plague bacillus. This can be seen from data given in Table 8.

In this way, it is apparent that the variability of the plague bacillus along the line of making it similar with the pseudotubercular is caused by a considerable intensification of use of polyatomic alcohols by P. pestis cells. From this point of view it is completely understandable that all "newly formed" cultures of the pseudotubercular bacillus are obtained only from one variety of the plague bacillus, which intensively decomposes glycerin. It is probable that in the future a microbe similar to P. pseudotuberculosis will obtained also from the "oceanic" strains, although it can be said in advance that
this approach will be longer, than the path necessary for obtaining
this variety from the "continental" strains. It will lie along
a previous formation from a glycerin-negative strain of a glycerin-
positive, and only after this will there be a possibility of its
further approaching the P. pseudotuberculosis.

In conclusion it must be emphasized that in general poly-
atomic alcohols are oxidized with considerably more difficulty
than are monosaccharides, by both species of bacilli.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Response to Polyatomic Alcohols</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plague</td>
</tr>
<tr>
<td>Glycerin</td>
<td></td>
</tr>
<tr>
<td>Erythritol</td>
<td></td>
</tr>
<tr>
<td>Dulcite</td>
<td></td>
</tr>
</tbody>
</table>

Symbols: / ferment; - does not ferment; - (f) most
strains do not ferment, a minority ferment;
£ most strains ferment, others do not.

There is considerable contradictory data in the literature
on the problem of the oxidation of alcohols by the plague and by
the pseudotubercular bacilli. This is due to the fact that the
authors have dealt with cultures found in different stages of vari-
ability.

Shifts in the Nitrogen Metabolism

Several authors have presented information on the ability of
various strains of P. pestis and several strains of P. pseudotuber-
culus to form nitrites when they are cultivated in liquid nutrient
media. In attempts to analyze this ability from the aspect of geo-
graphical origin of the strains, it has not been possible to establish
relationships analogous to their response to glycerin.

Ammonia is a source of nitrites in the cultures of plague and
pseudotubercular bacilli. As is widely know, oxidation of ammonia
into nitric acid is an aerobic process. The plague bacillus is
more aerobic than the pseudotubercular, and therefore the strains of the plague bacillus form nitrates in greater amounts than the strains of the causative of rodent pseudotuberculosis (Table 9).

In addition to this, it is important to emphasize also the evolution of this process in the plague bacillus strain.

The ability to oxidize ammonia into nitrous acid decreases for plague bacillus cultures as we proceed from strains of "oceanic" origin to strains of "continental" origin, and further to strains of rodent pseudotuberculosis, where it is expressed either weakly or not at all. Urease activity in plague bacillus strains appears extremely weakly, and with the usual methods employed in microbiology often is not detected. To the extent of changes appearing in the metabolism of the plague bacillus in the general direction of making it similar to the metabolism of the pseudotubercular microbe, urease activity increases.

**TABLE 9**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intensity of Nitrite Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pestis</em> (&quot;oceanic&quot;)</td>
<td>###</td>
</tr>
<tr>
<td><em>P. pestis</em> No 66 (&quot;oceanic&quot;)</td>
<td>###</td>
</tr>
<tr>
<td><em>P. pestis</em> No 708 (&quot;Oceanic&quot;)</td>
<td>#</td>
</tr>
<tr>
<td><em>P. pestis</em> No 135 (&quot;continental&quot;)</td>
<td></td>
</tr>
<tr>
<td><em>P. pseudotuberculosis</em> No 1</td>
<td>-</td>
</tr>
<tr>
<td><em>P. pseudotuberculosis</em> No 6</td>
<td>-</td>
</tr>
<tr>
<td><em>P. pseudotuberculosis</em> No 31</td>
<td>-</td>
</tr>
<tr>
<td><em>P. pseudotuberculosis</em> No 65</td>
<td>-</td>
</tr>
<tr>
<td><em>P. pseudotuberculosis</em> No 67</td>
<td>-</td>
</tr>
</tbody>
</table>

Symbols: / ferment; - does not ferment; / some strains ferment, others do not.

For the causative of rodent pseudotuberculosis urease activity is pronounced strongly (Table 10).

**TABLE 10**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity of Urease in Arbitrary Units</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pestis</em> EV</td>
<td>0</td>
</tr>
<tr>
<td><em>P. pestis</em> No 476</td>
<td>0</td>
</tr>
<tr>
<td><em>P. pestis</em> No 74</td>
<td>20.5</td>
</tr>
<tr>
<td><em>P. pestis</em> ZHVR</td>
<td>62</td>
</tr>
<tr>
<td><em>P. pseudotuberculosis</em> No 31</td>
<td>174</td>
</tr>
</tbody>
</table>

- 127 -
In connection with the investigations referred to, the evolution of the plague bacillus as it becomes similar to the pseudotubercular is sketched in the following way. "Oceanic" strains exhibit a decreased fermenting ability, they do not decompose glycerin and contain neither urease nor the Pt antigen (cf chapter on P. pestis antigens). On the other hand, "continental" strains emerge from "oceanic" strains, with higher fermenting ability, which appears in the new strains as glycerin oxidation. "Continental" strains to a greater or lesser extent are urease-positive and possess an additional antigen.

These properties are still more manifest in the strains of the pseudotubercular bacillus.

In this way, increase in urease activity in the plague bacillus strains represents a shift in their metabolism in the direction of forming a new stable variety similar in certain respects with P. pseudotuberculosis.

In the opinion of V. M. Tumasnicky (2) P. pestis cells and P. pseudotuberculosis cells have general antigenic properties, in which the antigen of the pseudotubercular bacillus is more active than the antigen of the plague bacillus. This position has been wholly confirmed by experiments on urease obtained from the pseudotubercular and from the plague bacilli.

Urease is a globulin with well-defined antigenic properties. For several animals urease is a powerful toxin. At least one of the general antigens of the plague and the pseudotubercular bacilli is urease. The plague bacillus possesses low urease activity and in addition it is a poor antigen. The pseudotubercular bacillus, on the other hand, exhibits high urease activity, making it in this respect one of the leading pathogenic microbes, and it also has good antigenic activity. The parallelism between the antigenic activity of these microorganisms and their urease ability is apparent.

From this comparison we can draw the supposition that to the extent of increased urease content in the plague bacillus strain, the antigen shared in common with the pseudotubercular bacillus also increases in quantity in the plague strains.

... recently and T. D. Sasykina (177) recorded the observation of 60 plague bacillus strains only three had aldehyde activity weakly manifested. At the same time all of the 60 P. pseudotuberculosis examined by them exhibited a sharp increased aldehyde activity.
Aldehydease, included in the group of yellow oxidase enzymes, is of great interest in connection with the variability of the plague bacillus in the direction of making it similar with the pseudotubercular.

A sharp increase in the aldehydease activity when plague bacillus strains are stored in the laboratory must be regarded as a process of radical rearrangement of the plague bacillus respiration type into a more anaerobic type.

We can note the shifts in the peroxidase and catalase activity from the data in Table 11.

Analysis of the observations described provides grounds for supposing that the oxybiotic processes decrease in the plague bacillus going from the "oceanic" strains to the "continental" and reach the lowest value in the varieties akin to the pseudotubercular bacillus.

The plague bacillus during its existence under various conditions of the external environment exhibits a pronounced capacity for variability. The newly formed varieties of P. pestis appear related in their biochemical properties with several strains of P. pseudotuberculosis. Analysis of the available material on the biochemical activity of this bacillus leads to the conclusion that the plague bacillus is an aerobe to a greater extent than the pseudotubercular. In favor of such a conclusion is the evidence of an increase in the fermentative activity in the transition from plague to pseudotubercular bacillus. The latter shows a high fermentative.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>plague pseudotuberculosis</th>
<th>bacillus bacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td>5.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Catalase</td>
<td>8.0</td>
<td>6.3</td>
</tr>
</tbody>
</table>
and aldehyde ability and reduced levels of catalase and peroxidase. The plague bacillus is characterized by contradictory indices in its enzymatic activity.

In this way, the basic factor determining shifts in the metabolism of P. pestis in the formation of varieties similar to P. pseudotuberculosis is change in the metabolism type, which consists primarily in an increased fermenting activity of the plague bacillus.

It is probable that in connection with change in the metabolism type observable shifts in the inoculation number of various Pasteurella species exist, which has been indicated by Ye. I. Korobkova (178) and by Ye. E. Bakhmakh (179).
The great variability in the plague bacillus has created a considerable difficulty in distinguishing it from the causative of rodent pseudotuberculosis.

For typical cases conducting a differential diagnosis between the plague bacillus and the pseudotuberculosis bacillus is not less with difficulties.

Distinguishing the plague bacillus from the rodent pseudotubercular bacillus is most convincingly demonstrated not in various artificial nutrient media but in animals susceptible to the causatives of these diseases, whose tissue is a medium for the habitation of the bacilli in question.

The plague and the pseudotubercular bacilli are two different species. They have no common circle of animal-carriers. Many observations of various authors have quite precisely established the circle of animals which suffer from plague and pseudotuberculosis of rodents. Undoubtedly, the metabolism of the animals has an effect on the vitality of the microorganism introduced; in some species of animals microbes perish, in others they develop and multiply.

It is necessary to emphasize the leading role of protein (nitrogen) metabolism and of one of its end products. The most diverse organisms — single-celled and poly-celled — cells of plant origin and from the animal world form ammonia during the process of their intracellular metabolism. Ammonium is a protoplasmatic poison for all cells. The difference lies only in the
fact that some are more resistant to it and others are more sensitive. D. N. Pryanishnikov has established that ammonia in plant organisms is deposited in the form of the amide of aminosuccinic acid (asparagin). It has been shown that in animal organisms as well this type of ammonia fixation (glutamine) (180) is widespread.

Other forms of fixation of ammonia in animals are represented by urea and uric acid. It is important to note that only those animals in which the main mass of ammonia is excreted in the form of urea suffer from plague. Animals excreting ammonia either as such, or primarily in the form of uric acid, are nonsusceptible to plague. However, not all animals of the indicated type suffer from plague. For example, dogs and to a certain extent cats are not susceptible to it.

From the epidemiological point of view, the ammonia response of small animals included in the rodent group which are carriers of plague infection in nature is of special interest. It has been established that the susceptibility of animals of this group to plague and their resistance to ammonia to a certain extent proceeds in parallel to each other -- the animals least resistant to plague are the least resistant to ammonia (Table 12).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Smallest fatal dose of ammonia per 100 g of body weight in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small marmot</td>
<td>255</td>
</tr>
<tr>
<td>House mice</td>
<td>219</td>
</tr>
<tr>
<td>Noontime sandwort</td>
<td>140</td>
</tr>
<tr>
<td>Burrowing sandwort</td>
<td>124</td>
</tr>
</tbody>
</table>

Here it must also be emphasized that the resistance of animals to ammonia depends on their physiological condition: sleep, the character of nutrition, etc., and, therefore, several deviations from the general rule are known.

The role of glutamine in ammonium metabolism in the organism is widely known, which amounts to the removal of ammonia from the tissues. Variations in the glutamine in tissues constitutes one of the major aspects of nitrogen metabolism. As can be seen from
the data of Ferdmgn (180), hibernation reflects sharply on the glutamine content in tissues.

The circle of animals suffering from pseudotuberculosis of rodents is considerably broader than that affected by plague. Most animals excreting ammonia primarily in the form of urea are apparently subject to this infection, as well as representatives of animals excreting ammonia chiefly in the form of uric acid (birds).

The intensity of their metabolism probably plays a large role in the susceptibility of animals to plague. In general it can be said that plague affects animals only with an intensive metabolism.

Convincing evidence of the presence of a relationship between metabolism intensity and plague susceptibility of the organism is represented by the marmot — one of the main carriers of plague infection in nature. The marmot is susceptible to plague only during the period of its active life. In its sleeping period, when its metabolism drops to a minimum and protein metabolism falls off especially, the rodent becomes insusceptible to this infection. When contaminated with plague during its sleep period the rodent is not affected by it, but as soon as such an infected marmot awakens its metabolism increases sharply and after several days following incubation it becomes diseased by the plague. In every case it must be emphasized that the generalization of plague infection in the sleeping marmot must be regarded as a rare exception, and not as a rule.

TABLE 13

<table>
<thead>
<tr>
<th>Condition of marmots</th>
<th>Glutamine content in the muscles of the heart in mg-%</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hibernating</td>
<td>225</td>
<td>Artificially excited marmots</td>
</tr>
<tr>
<td>Artificial excitation</td>
<td>180</td>
<td>perish immediately</td>
</tr>
<tr>
<td>Summer Awakening</td>
<td>130</td>
<td></td>
</tr>
</tbody>
</table>

Glutamine Content in the Cardiac Muscle of Marmots
Thus far there has not been available adequately complete data on the relative susceptibility of various species of rodents to the plague, but this susceptibility varies among rodents and changes as a function of the animal's age and the time of year. This has been established for the small marmot, for the Transbaykalian, for the noontime sandwort, and for other animals. The points indicated undoubtedly serve to explain the divergence in the data of the various researchers on the susceptibility of animals to plague infection.
In the human disease of plague and of several species of animals they experience, over a course of three to four days a sharply pronounced septicemia, thus, according to the data of British Anti-plague Commission (1), for sixteen persons suffering with the plague the content of the microbial cells in one ml of blood before death amounted to one million.

The organs of the human and animal cadavers, perishing from plague infection, contain enormous quantities of the causative. The amount is so profuse that frequently the liver, the lungs, the spleen, and the marrow, under microscopic examination consist of tissues solidly packed with microbial bodies.

The sharply pronounced invasiveness of the plague bacillus, its rapid ability to multiply in the tissues of animals susceptible to plague also leads to the formation of a great quantity of microbial bodies. Through the intensively increasing mass of the plague bacillus alone does the death of the animal result. This reliable and well known observation is extremely important in understanding the pathogenesis of plague.

It can be said with complete substantiation that the most threatened part of the body on terms of the pathogenesis of the plague is the cardiovascular system (G. P. Rudnev (181)). Undoubtedly, a great role is also played in the pathogenesis of plague by nonspecific products of metabolism, forming due to micro- and macro-organisms.

Being a poor antigen, the plague bacillus does not induce stable and sustained immunity, and therefore the protective process of the macro organism need not be recapitulated since this has been done by N. N. Zhukov-Jereshnikov and T. N. Mayskiy (108).
From our point of view, for plague the factors of immunity
do not constitute the primary significance, but only secondary, since
even the best antiplague vaccines reduce the fatality of persons re-
ceiving inoculations from 53.4 to 36.5%. In addition, of course we
can never negate the value of vaccinating population in plague foci,
since vaccination undoubtedly promotes a decrease in the susceptibility
of persons.

Vaccination of population is carried out again after several
months. If the vaccine also creates comparative immunity in man
against bubonic plague, it has been proven to be completely in-
effective in regard to its pulmonary form.

In plague prevention various kinds of vaccines are used --
killed and live; the latter have been the preferred. The live anti-
plague vaccine prepared from the EV strain has received much fame;
in the Soviet Union in addition to the latter the live divaccine
1-17 is employed.

Serotherapy has found extensive use as a treatment and pre-
ventive agent against plague, although this form of therapy has always
and especially at present aroused disagreement in the estimates of
its effectiveness. The basic cause of disagreement is that the plague
bacillus is a poor antigen, and therefore cannot be used successfully
in obtaining an antiserum with a high titer. It is sufficient to
state that the antiplague serums produced are not titrated. In use
are antiplague serums from the blood of horses, and also of other
animals immunized by various means -- by live virulent and avirulent
strains of the plague bacillus and by various preparations obtained
from their cultures (nucleoproteids, polysaccharides, filtrates, etc.).

Antiplague serum is administered to the patient repeatedly
and at high doses -- single applications of 200 ml, and the total
amount of serum administered to the plague victim often reaches 1,500
ml. The mechanism of the action of antiplague serum is unclear and
the point of view of various authors on this question diverges widely.
They, Zolotov and Tagil (183) even have said that sulfathiazol + serum
acts best without serum.

At present efforts are underway to use purified and concen-
trated fraction in treating plague. We are speaking here of the use
of nucleoproteins, isolated from antiplague serum.
using antiserum in treating plague in addition to the new antibi-otic, especially in the early stages of the disease, when the toxic factor often is prevalent over the bacterial. Aureomycin is the only anti-biotic which has revealed antitoxic action under experimental condi-tions, other antibiotics have shown only antibacterial action. According to the data of Gerard, gamma-globulin was tested on monkeys infected with plague, with encouraging results.

Also finding use in the treatment of plague is the specific bacteriophage. However, the initially favorable and encouraging results obtained have not been confirmed.

It can be supposed that the authors have not always taken into account the diversity of the bacteriophage strains, the decrease in titer when it is stored, and the stage of the disease at which it is administered. Nonetheless, G. P. Rudnev (161) noted that specific bacteriophage therapy has in general shown a favorable effect on the course of plague infection -- a distinct improvement in the patient's condition develops.

According to the data of M. P. Pokrovskaya, the plague bacterio- phage administered intravenously into the animal organism circulates in the blood system for about twenty hours, while injected intramuscularly it can be detected in the blood even after seventy hours (185).

M. P. Pokrovskaya has indicated that massive doses of plague bacteriophage in vivo have shown the ability of completely lysing the plague bacillus. The latter cannot be detected neither in the tissues of the animal organism nor in its blood smears. However, the animal perishes as a result of the sharply pronounced intoxication by the plague bacillus toxin.

In this way, in treating plague with the bacteriophage the phenomena of intoxication of the affected animal is distinctly manifest and frequently its death occurs even in the complete absence of live cells of the plague bacillus in the organism's tissues.

From what has been said it follows that the specific treat-ment of plague victims can be broken down into two basic stages: controlling the septicemia increasing as the disease develops, and controlling the simultaneously ongoing intensive intoxication of the patient by the products of decay and of the life activity of the plague bacillus.
The first stage in the treatment of plague — eliminating septicemia — has been dealt with through the use of the most diverse substances. For this purpose numerous salves (gray mercury) and injections of a large number of medicinal substances have been employed: collargol, iodine, and corrosive sublimate; carbolic acid, quinine, zinc chloride, Lugol solution, and many others, and even surgical intervention has been resorted to — lancing of the buboes.

G. P. Rudnev has especially recommended gray mercury salve in treating the bubonic form of the plague. This salve, according to his assertion, provides quite good results. The comment of G. P. Rudnev referred to on the good therapeutic effect of mercury salves deserves special attention. In addition to the observations of Ye. K. Gubarev and T. I. Lipatova (18) on the high bactericidal activity of potassium iodide and iodine, which appears even at low concentrations in regard to the plague bacillus, this constitutes a confirmation of the viewpoint that ascribes a substantial role to urease in the "continental" strains of the plague bacillus, the widely recognized most virulent in the pathogenesis of plague.

As is known, urease is a catalytic active protein with a large number of sulfhydryl groups, which are combined with mercury salts and are easily inactivated by iodine ions. Urease is extremely sensitive to ions of mercury and iodine, of which the literature contains many reports. The favorable effects of ions of mercury and iodine on the course of plague infection can be attributed to their inhibitor action on the urease of the plague bacillus.

The substances indicated and the methods of treating plague victims that involve these substances have proven to be in general of low effectiveness and have been abandoned. For a long time the efforts of many researchers to obtain a medicinal preparation for treating plague have been unsuccessful and only fairly recently have very effective compounds been found among the antibiotics. This approach involves the use of sulfanalamide preparations for the purposes indicated.

The synthesis of numerous derivatives of the amide of paraamino sulfonic acid and their extensive use in treating various diseases has stimulated researchers to attempt to treat plague with sulfanalamide preparations. Sulfidino-therapy of human victims of the plague has been employed abroad since 1938 (186). These efforts have yielded encouraging results, but insufficient gains, in
particular, in treating the primary pulmonary form. However, the combined treatment of patients suffering from the primarily pulmonary form of plague with sulfidine and methylene blue has yielded therapeutic effects. In this method of treating the patient a large quantity of sodium salts of sulfidine is introduced intravenously or intramuscularly, as well as considerable quantities of methylene blue (187).

At present the use of antibiotics in the treatment of plague has reached a high level. Of the known antibiotics the most active in this connection is streptomycin, which has firmly been established in the treatment of the plague (188). However, it has several shortcomings. These pertain primarily to its ability to induce in bacteria streptomycin-resistant forms, frequently preserving their virulent properties in this transformation.

However, the studies of Garber, Nolla, and Caruso (189) have not given reason to fear the adaptation of the plague bacillus to streptomycin in the organism of the infected animal. The authors reported on the frequency of the appearance of streptomycin-resistant P. pestis cells, detected in cultures grown in media containing streptomycin from 2 to 128 ED/ml. The resistant forms are regarded by the authors as mutants of normal cells. However, their number, according to the authors' assertion, is so low that in the streptomycin treatment of animals infected with virulent strains of P. pestis only a few cells of resistant mutants survived.

Prolonged streptomycin treatment of plague victims involving the use of large doses leads to side effects that are extremely undesirable in view of the toxic properties of this antibiotic. Streptomycin has a considerable antibacterial activity for the plague bacillus, which appears at an antibiotic concentration of 1:1,428,000.

Streptomycin was introduced into the practice of treating the bubonic and especially the pulmonary forms of the plague in 1946 by Gerard. Systematic injection of streptomycin into the plague victim involving fractional doses every three hours initially at the rate of 4.3 g, and then 1 g per day has yielded very good therapeutic effects. For the course of treatment lasting 164 hours, about 25 g of streptomycin required. When this treatment is commenced early enough it is possible to save almost all persons suffering from plague.

The mechanism of the antibacterial effect of streptomycin and the formation of microbial cells resistant to it cannot be considered as sufficiently elucidated, but the existing fragmentary
facts deserve attention and to some extent are capable of illuminating its curative action for plague.

The resistance to antibiotics and chemotherapeutic agents has been evaluated by many authors from the genetic point of view (190). According to this theory, a certain number of cells (mutants) resistant to the medicinal are spontaneously formed in the populations of microbes.

Supporters of a second theory can be found in Sevag and Rosanof (191) who were not able to demonstrate the existence of even a single streptomycin-resistant cell among enormous populations of sensitive cells of Micrococcus pyogenes var aureus. These authors unsuccessfully tried to detect streptomycin-resistance in cells growing without glucose with a complete array of amino acids, and also when cells cultivated in the presence of glucose, but with a deficiency of aspartic acid or phenylalanine. However, the formation of streptomycin-resistant cells was observed in a medium containing a complete array of amino acids and glucose under aerobic conditions and in contact with the antibiotic. After the cells acquired resistance they were capable of multiplying in the deficient media referred to above.

The antiplague action of streptomycin is similar to the antibacterial action of antibiotics for other bacteria, but in connection with the fact that in the battle of the animal organism with plague infection phagocytosis of bacilli is of not small significance, the stimulating action of streptomycin on the phagocytosis of several pathogenic bacteria in vivo must be noted, thus, for guinea pigs infected with Mycobacterium tuberculosis cultures, under the influence of streptomycin phagocytosis increases strongly, most probably due to the action of the antibiotic on the bacilli (192).

In the treatment of plague other antibiotics are also used, but they have been only slightly investigated. Blyomycin was used without success by L. N. Makarovskaya (193). Aureomycin has yielded a good therapeutic effect, but it apparently is less effective than streptomycin (194) although it exhibits an unquestioned ability to neutralize the action of the plague bacillus toxin in experiments on white mice -- animals which are most sensitive to it (195).

Chloromycetin has yielded positive results in experiments on white mice and monkeys. However, Mercier (196), in treating human victims of the primary pulmonary plague was compelled to abandon it.
rapidly and turn to streptomycin treatments, which led to the recovery of the victims. In a second study the same author (197) and also Kram reported on the favorable outcome chloromycetic treatment used for patients suffering from the pulmonary form of the plague.

According to the data of Quan, Che, and Meyer (198), chloromycetic has the property of fixtating the plague bacillus toxin. Bismarck, levomycetin, and sintomycin apparently are not of interest as therapeutic agents either singly or in combination with streptomycin.

The treatment of guinea pigs infected with plague by streptomycin in combination alamin has afforded a better effect than treatment with streptomycin alone. However, the dosage of streptomycin, the mode of its administration into the organism of the patient, and also the time of commencement and duration of the antibiotic treatment have a substantial effect on the outcome of the disease. It must be emphasized that there are a considerable number of cases in which cultures could not be isolated from the cadavers of the plague victim who had been treated with antibiotics.

In addition, it must be noted that there is still not available the necessary clarity in the evaluation of the results of the use of various antibiotics in plague treatment. It is enough to recall thatGerard (199) evaluated as identical the therapeutic effect of streptomycin, chloromycetic, and terramycin in plague therapy. He reported results obtained from Madagascar in the treatment of patients suffering from the primary pulmonary plague with streptomycin, chloromycetic (chloramphenicol), and terramycin in the following form.

Of ten cases in which patients were treated with streptomycin with a total dose of 12-20 g administered intramuscularly one case of fatality a month after the beginning affection from secondary infection, not yielding to penicillin and other antibiotics, was recorded. In all the other instances rapid disappearance of plague bacillus from the sputum took place. Of eight cases in which another treatment was used during the period of the same outbreak, three patients perished, since the treatment was commenced only on the third day of the disease and death occurred after a few hours following commencement of the treatment.
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TRANS/ATOR'S SUMMARY

This book presents a good deal of general information on the biology and evolution of the plague causative, its pathogenesis, and the clinical aspects of plague infection. It opens with a survey of research done on the plague, emphasizing its unsystematic character and its general neglect of the variability of the plague. This has given rise to apparently contradictory data in the literature. The data on the chemical composition of the plague bacillus is stressed in the book as being fragmentary in not dealing with all of the cell constituents. The membrane and capsule origins of antigens were differentiated. Aminoacid composition was emphasized. Differences in virulence were tentatively traced down to chemical origins.

The Gram properties of various plague strains were related with polysaccharide composition. Various nutrients employed by the several plague strains were delineated. The Gram staining method was used to classify plague bacillus strains.

An outline of variability in plague bacillus metabolism was presented. The plague host was described in relation to metabolic changes of the plague bacillus. Glycerin oxidizing ability was used to further differentiate between plague bacillus strains. This ability was correlated with participation in carbohydrate metabolism. Various culture environments were associated with differences in carbohydrate metabolism.

The basis for plague bacillus adaptability was traced to the formation of adaptive enzymes; these were briefly enumerated and described. The differing responses of plague strains to anaerobic and aerobic culture conditions were delineated.

Anaerobic requirements were correlated with the metabolic process of the plague bacillus. Lysis was associated with varying amino acid .

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Sulfur metabolism was briefly correlated with various strains.

The different responses of old and fresh laboratory strains to various chemical reactions were correlated with virulence and antigenicity.

Metabolic differences of the plague and the pseudotubercular bacilli were correlated with the problem of distinguishing them. Various culture environments were further used in this differentiation. Plague bacillus virulence was associated with duration of storage. Metabolic shifts in the plague bacillus making it similar to the pseudotubercular bacillus were described. The bacteriophages of the plague bacillus and their possible use in therapy were enumerated. Toxins and the hemolysin of the plague bacillus were correlated with various approaches toward therapy.

Differential diagnosis of the plague and the pseudotubercular bacillus was expounded upon briefly. Treatment of the plague was held to involve treatment of intoxication of the organism as well as antibacterial action of serums administered. The varying success of antibiotic use was briefly traced in time.