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METHODICAL BASES FOR THE STUDY OF THE BIOLOGICAL PROPERTIES OF THE PLAGUE CAUSATIVE AGENT

[Following is the translation of an article by M. I. Levi, Published in the Russian-language periodical Sbornik Nauchnykh Rabot Elistinskoy Protivochumnoy Stantsii (Collection of Scientific Works of the Elistinskaya Antiplague Station), No 2, 1961, pages 9--35. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

Some investigators propose that the virulence of the plague causative agent is provided for by a specific antigen which is included in the composition of the bacterial cell, and others consider that this property is manifested thanks to a specific combination of several antigens, each of which separately does not guarantee it. The problems of studying the virulence of the plague causative agent take on special importance for our concepts concerning the epizootic process and the mechanism of preservation of the microbe during the so-called interepizootic period, when the existing methods and means are not sufficient for detecting the causative agent

Just recently proposals were made that findings of weakly virulent strains are special laboratory cases, the rapid lowering of the virulence of natural strains following passages on artificial nutrient media were falsely interpreted as the detection of weakly virulent strains in nature. Expressly, therefore, a number of investigators, and mainly Ye. Ye. Punskiy (1956) and L. A. Timofeyeva (1960), insist on the prompt study of the virulence of strains immediately following isolation and identification.

At the present time it can be considered substantiated that weakly virulent and avirulent strains of the plague causative agent are encountered in nature.

It is known, however, that virulent strains of the plague causative agent are heterogeneous. In accordance with the classification of D. Tumanskiy, three varieties of the plague microbe are distinguised: Rat, marmot and suslik. A study of the varieties of the plague microbe has a direct relation to the main regularities of the natural focalness of plague.

Our personal experience in studying the virulence of the plague microbe and published information made it possible to develop a method and arrangement for studying the biological properties of strains of the plague microbe. These are presented below.

Cultural Properties

An isolated study of virulence cannot be utilized for any constructive conclusions without a comprehensive study of the cultural, biochemical and antigenic properties of the strain. In connection with this in our test we studied the growth of strains on solid and liquid nutrient media, both ordinary and special (blood agar, glycerin agar, peptone deficient agar), capsule formation, behavior on colored media, and particularly the fermentation of glycerin, nitrates, rhamnose, urea, and also motility.

The history of the isolation of each strain and the history of the study of its properties were taken into consideration. As far as possible the history of the passages of the strain on artifical nutrient media and laboratory animals was reestablished. For these purposes we used the following conditional designations: B - broth, A - agar, M - white mice, S - guinea pig. The incubation temperature was indicated over the letter and the subscript on the right stood for the number of passages. For example, since the time of isolation, 24 Jul 46, strain No went through

$C_1 - A^{37}_{12} - B^{37}_{1} - M_1 - A^{28}_{14} - A^{37}_{1} - A^{28}_{107} - C_1 - A^{28}_{1}$ passages.

Virulence

As a rule, strains which were highly virulent for guinea pigs turned out to be just as highly virulent for white mice. The work by R. V. Kovaleva (1958) presents the observations on the comparative virulence for white mice and guinea pigs of strains which were isolated during as epizootic on Brandt's voles in the Mongolian People's Republic. It turned out that these strains caused the death of white mice in small doses, while the guinea pigs died only following the administration of very large doses. Similar properties were detected by A. M. Shamova (1959) in strains which were isolated during a plague epizootic in flat-skulled voles. The observations of R. V. Kovaleva and A. M. Shamova were supported by L. A. Timofeyeva (1960). M. I. Levi (1960) reported that strains of the plague bacteria which were isolated during an epizootic in common and social voles in the Armenian SSR also possessed a high virulence for white mice and a weak virulence for guinea pigs. Thus, for the first time in literature information appeared concerning the dissociation of virulence for the above mentioned two species of laboratory rodents.

M. I. Levi, B. G. Valkov, A. I. Shtelman and Yu. V. Kanatov (1959) noted the dissociation of virulence in strains of the plague causative agent not only for white mice and guinea pigs, but also for wild rodents. It turned out that strains which possessed a similar virulence for some rodents displayed expressed differences in their virulence for other rodents. Moreover, it became apparent that virulence should be tested not only on laboratory animals, but also on wild rodents. A subsequent study of the virulence of a number of strains showed that for relating them to varieties it is desirable to test this feature on those species of wild animals which are suspected of or known to be the main carriers of plague in the natural foci.

Unconditionally this greatly complicates the testing of the virulence of strains. In our tests we decided to limit the collection of animals to a minimum (white mice, guinea pigs, little susliks -- the main carriers in the focus of the Severo-Zapadnyy [northwest] Pricaspian area, and midday gerbils-- the main carriers in Volzhskiy-Uralskiy sand plains). This collection guaranteed, as will be shown below, the most accurate demarcation of the suslik and gerbil varieties.

M. I. Levi, B. G. Valkov, A. I. Shtelman and Yu. V. Kanatov (1959) demonstrated that the midday gerbils from the left bank of the Volga were different from the right bank gerbils in infectious sensitivity to plague infection. Thus, the left bank animals turned out to be approximately 50,000 times more resistant than right bank animals. It is not excluded that similar intraspecies differences are also inherent to certain other rodents. For eliminating the influence of this factor on the results of studying the virulence of strains, in all of our tests we used small susliks, captured in the eastern sector of Rostovskaya Oblast (village of Zavetnoye), and left bank midday gerbils, captured at Dosang village.

THUS, THE VIRULENCE OF THE PLAGUE MICROBE IS A RELATIVE CONCEPT AND DEPENDS ON THE SPECIES OF ANIMAL ON WHICH IT IS TESTED.

Thereby it is acknowledged that virulence has not only a quantitative, but also a qualitative nature.

Every titration of virulence on animals is usually accompanied by the statistical processing of the material obtained. This will be the calculation of the minimum, unconditional or 50% lethal dose or the standard deviation from the values obtained.

In connection with this it is desirable that the number of animals and the selection of infecting doses facilitate the processing of the resulting data with the help of methods of variation statistics. Therefore, for the study of the virulence of the strains we selected 4 animals (white mice - 6) for each dose, and the following dose exceeded the preceding dose by 10 times. Without a doubt the selection of the range of doses was influenced by the state of virulence of the strain at the moment of study and the degree of resistance of the species of animals taking part in the test. Each time the state of virulence of the strain was studied in a preliminary test on white mice. In this test we used 4 animals (2 males and 2 females) for each dose (1, 10, 100, 1000, 10 000, & 100 000 microbial bodies). In all the tests we used only the subcutaneous administration of a culture of the plague microbe in a volume of 0.2 ml in the region of the haunch of the right rear limb. After determining the virulence of the strain for white mice the animals for the main test were infected. In both cases the culture of the causative agent was passed 2--3 times through the organism of a white mouse, after which it was incubated for 48 hours on agar at 37°. A check of the inoculation doses before the test and following infection of all the animals did not expose any significant differences. Considering the degree of resistance of the animals, the white mice were infected with 7 doses from 0.1 up to 100,000 m.b.[microbial bodies]; guinea pigs -- 7 doses from 0.1 up to 100,000 m.b.; small susliks -- 8 doses from 0.1 up to 1,000,000 m.b.; midday gerbils -- 10 doses from 0.1

up to 100,000,000 m.b. The doses were verified by seeding on nutrient agar (3 dishes for each dose). Animals of approximately the same weight were selected from each species.

Both in the preliminary and in the main tests, animals which died following infection were autopsied. Seedings from the site of administration, the lymph nodes, blood, spleen, liver, and lungs were made on nutrient agar. Inoculation in broth turned out to be a valuable assistance, since it made it possible to expose additionally from 5 to 8% of the total amount of positive seedings following the calculation of the data from inoculation on agar alone. The summarized data on the effectiveness of inoculations from the internal organs from infected animals which died and which were sacrificed are presented in table 1.

In previous works (M.I. Levi, B. G. Valkov, A. I. Shtelman, Yu. V. Kanatov, 1959; M.I. Levi, B. G. Valkov, Ye. I. Novikova, G. B. Minkov, 1959) it was pointed out that sometimes the growth of the plague microbe was not detected in the seedings from the internal organs, in spite of the fact that shortly before death the microbes were detected in seedings of blood on agar. After the employment of parallel seedings on agar and in broth the number of similar cases was reduced to a minimum.

The calculation of the growth of colonies on agar plates was performed twice - after 3 and 6 days. The surviving animals were sacrificed in one and a half months following infection. The maximum amount of blood was collected from the sacrificed animals and subsequently the serum was removed for investigation in the passive hemagglutination reaction. Seedings from the internal organs of the sacrificed animals were made on agar and in broth. The pathologoanatomical picture was considered in all the dead animals and in the surviving ones which were sacrificed after a month and a half. The internal organs from some of the dead and sacrificed animals were placed in formalin for a subsequent histological investigation. In those cases when the plague microbe was isolated from rodents after the 20th day from the moment of infection, its virulence was checked on white mice for a comparison with that which was determined in the preliminary and the main tests. The arrangement for studying the virulence of those cultures which were isolated in later periods corresponded fully with the arrangement for titrating the virulence of the strain in the preliminary test. It should be mentioned that in all the work on studying the biological properties of strains of the plague causative agent we used highly nutritious agar, on which colonies grew even when individual microbial bodies were seeded.

For a preliminary checking of the agar we placed doses of 1, 10, 100 and 1000 m.b. on it. These were from 3 strains --EV 76, 17, and 297, which represent the varieties of the plague microbe which are known in literature. In a number of cases the agar was additionally tested with the strain being studied. Only those series of agar were considered suitable on which colonies grew with just a seeding of 10 m.b., and if following seeding of 100 m.b. from 20 up to 50 colonies were noted.

Usually the results of the tests for virulence were placed in a table, where a calculation was made of the number of infected, dead, and surviving animals, and also the average life duration of the animals.

From figure 1 it is possible to obtain a more complete judgment concerning the results of the test (test 3 from the work by M. I. Levi, B. G. Valkova, A. I. Shtelman and Yu. V. Kanatova, 1959).

When calculating the LD_{50}, consideration was given to only those animals from which the plague bacteria were isolated. If the animals died without the isolation of a culture prior to the 11th day, then they were excluded from the count, and if the culture was not isolated from animals which died after the 10th day, they were considered as surviving.

We were not confined to the determination of the LD_{50}, having supplemented it with the standard deviation. We will cite an example of the calculation of the LD_{50} according to the method of Reed and Mench and the standard deviation according to the formula of Pizz, taken from the data which is contained in figure 1 (right bank midday gerbils, from the "Chernozemelskiy" state farm).

The dose, equaling the LD_{50}, in this case is found between 60% and 0, and the logarithm of the LD_{50} -- between 2 and 3. The mantissa to the whole logarithm is calculated by the formula $x = \frac{60 - 50}{60 - 0} = 0.1667$. Thus the $LgLD_{50} = 2.1667$. The antilogarithm correspondingly equals 147 m.b. The standard deviation is calculated according to the formula of Pizz, where

$$CO = \sqrt{\frac{0.79 \cdot h \cdot R}{n}} \; ; *$$

h -- the logarithmic difference between the two nearest doses, equaling 1; n -- the number of animals for each dose; in this case -- 4; $R = lgLD_{75} - lgLD_{25}$. The doses which correspond to the LD_{75} and the LD_{25} are determined according to a formula which is similar to the one for determining the LD_{50}. Thus, the mantissa of

$$lgLD_{75} = \frac{60 - 25}{60 - 0} = 0.5833; \; lgLD_{75} = 2.5833;$$

(instead of the LD_{75}, the dose was determined which corresponded to 25% of survival, since these values are identical).

$$lgLD_{25} = 1.4231; \; R = 2.5833 - 1.4231 = 1.1602;$$

$$lgCO = \sqrt{\frac{0.79 \cdot 1 \cdot 1.1602}{4}} = \sqrt{0.2291} = \pm 0.48.$$

* CO - [standartnoye otkloneniye, standard deviation]

In the work by M. I. Levi, B. G. Valkov, A. I. Shtelman and Yu. V. Kanatov (1959) it was pointed out that the difference in the two determinations of the $lgLD_{50}$ will be reliable only in the case that the sum of the positive values of the logarithms of the standard deviations is less than the difference of the logarithms of the LD_{50}, that is if

$$(lgLD^1_{50} - lgLD^2_{50}) > (lgCO_1 + lgCO_2).$$

However, the stated hypothesis was also subjected by us jointly with Ye. S. Biryukova to an experimental verification on white mice in 1957--1958. We selected 3 strains for this purpose (1 - highly virulent, and 2 - less virulent for white mice). The arrangement for each test was as follows. A 48-hour culture, incubated at 37°, was cultivated into five lines based on the optical standard. Virtually five similar titrations of virulence of the strain on white mice were produced. In each line at the same time all the doses were seeded on nutrient agar -- three dishes for each infecting dose. The results obtained are summarized in tables 2 and 3. The results of the infection of the animal are presented in two variants. Variant A: When calculating the LD_{50} and the CO we took into consideration the loss of animals whether or not a culture was isolated from the dead animals. Variant B: We considered only those animals from which a specific culture was isolated. In those cases when the culture was not isolated, a decision was made depending on the periods of death of the animals.

For clarity we presented the results of the test for virulence in the five lines of each strain in figure 2 (the principle of describing the data is the same as in figure 1 from the work of M. I. Levi, B. G. Valkov, Ye. I. Novikova and G. B. Minkov, 1959). Variations of the lgCO are given according to both sides for the value of the $lgLD_{50}$.

It is necessary to note that differences in the $lgLD_{50}$ in a number of cases exceeded the sum of the positive values of the CO. Here variant B is distinguished by greater differences than variant A.

In variant A the difference in the $lgLD_{50}$ for strains 133 and 261 did not exceed the limits of the sum of positive values for lgCO, while for strain 142 in certain cases these differences exceeded the sum of the values of the lgCO.

In variant B for each strain it was possible to select two such lines at which the sum of the lgCO turned out to be less than the difference of the $lgLD_{50}$. Thus, we are obliged to state that the sum of the positive values of the lgCO cannot serve as an absolute criterion for evaluating the reliability of the differences of the LD_{50}. It should be pointed out that the results of seeding the doses on nutrient agar were sufficiently uniform (figure 3).

Figure 3 depicts the average indices of the seedings of infecting doses of two strains. The role of the sum of the values of the lgCO for evaluating the reliability of the differences of the $lgLD_{50}$ will be given

a special investigation. However, here we will only point out that the insignificant excess of the difference in the LD_{50} over the sum of the CO indicates the reliability of the differences in this test, a tendency, but it cannot be sufficient for the recognition of the reliability of the differences in the LD_{50} for all analogous tests, while a 2--3 fold excess of the sum of the lgCO may be considered reliable for recognizing the absolute reliability of these differences.

Bacteremia

It was shown in previous works (M. I. Levi et al., 1959; M. I. Levi and Yu. M. Rall, 1960) that for a knowledge of the biological differences of strains of plague bacteria, a study of the nature of bacteremia in infected animals has an unconditionally greater importance than the study of virulence. Thanks to a comparative study of bacteremia in strains 1228 (297) and 1256 (403) it was possible to isolate the gerbil and suslik varieties (M.I. Levi, 1959; M. I. Levi and Yu. M. Rall, 1960; M.I. Levi, 1960). It was shown that the transmissive [vector ?] mechanism of transmission in plague conditions a number of specialized adaptations of the plague microbe which may explain the peculiarities in the nature of bacteremia in various animals. A theory for the mechanism of transmission in infectious diseases has been worked out in a general form by L. V. Gromashevskiy (1958, 1959). Though the present article is devoted to the methodical bases for studying the biological properties of the plague microbe, it is necessary to briefly characterize its theoretical grounds.

At the basis of our method for studying the biological properties of the plague microorganism lies a number of theoretical prerequisites of the modern epizootology of plague, which may be formulated as the THEORY OF ADAPTATION MUTABILITY of the causative agent.

The THEORY OF MULTIPERSISTENCE presumes that at the present time in each natural focus there is one main carrier, several secondary carriers which are close to the main carrier based on systematic features, and several accidental carriers, which for some reason or other have been drawn into the epizootic process. It should be pointed out that even earlier for several natural foci, especially the suslik and marmot foci, a number of investigators acknowledged one main carrier. However, multipersistence as a general regularity of the epizootology of plague which is characteristic for all natural foci, consequently the theory of multipersistence, was only formulated very recently in the works by Yu. M. Rall, V. N. Fedorov, M. I. Levi, et al.

The THEORY OF ADAPTATION MUTABILITY of the plague causative agent as an epizootological regularity was developed in our last works (M.I. Levi, 1959; M.I. Levi, 1959; M. I. Levi, 1960, M. I. Levi and Yu. M. Rall, 1960). This regularity can be expressed in a general form by

stating that each natural focus is characterized by its own variety or race of causative agent, displaying the features of adaptation to the main carrier (leading host).

Both of the mentioned theories are enlisted for an explanation of the main regularities of the epizootic process in plague only from historical positions.

In previous investigations a method was developed for the quantitative study of bacteremia. This made it possible to establish a series of new facts in favor of the theory of adaptation mutability of the plague causative agent.

Blood for the investigation of bacteremia was collected from a cut on the tail. A small suslik, a midday gerbil, or a white mouse was secured with the help of two dressing forceps. One was used to fasten down the withers and the other -- the skin in the area of the tail. For susliks and midday gerbils scissors were used to remove the hair covering of the tail. With sterile scissors or a razor (safety or straight) we cut off the tip of the tail and the blood which entered the cut was gathered with a loop for seeding. If the blood did not appear, then the tail was massaged lightly. Sometimes it is more expedient to cut a little hole in the tail. The blood would drain into this. For each subsequent taking of blood it is possible to remove the scab which has formed at the site of the cut, however it is better to make a new cut. The blood seedings from all the animals are made with the same bacteriological loop on nutrient agar, to which gentian violet in a dilution of 1:400 000 may be added.

Our experience testified to the fact that it is most convenient to use a bacteriological loop which withdraws 1 mm^3 of blood from the rodent. The volume of the blood which is withdrawn by the loop may be determined by three methods: 1) by a comparison of the amount of erythrocytes in 1 mm^3 of the rodent's blood with the amount of erythrocytes in one drop of blood, the loop is rinsed with 1 ml of a physiological solution; 2) by the direct measurement of the amount of blood in a loop with the help of a micropippete; 3) by numerous transfers of a measured amount of blood, here the quotient from the division of the entire transferred volume by the number of transfers represents the volume of the loop. Of these methods the most reliable is the first, according to which the volume of the bacteriological loop=

$$\frac{\text{amount of erythrocytes in 1 mm}^3\text{ of physiological solution x 1000}}{\text{amount of erythrocytes in 1 mm}^3\text{ of rodent's blood}}.$$

It is necessary to perform the measurement of the so-called "average loop," since it is not always possible to collect a full loop of blood. The amount of erythrocytes is calculated in a counting chamber by the usual hematological method.

The blood for the study of bacteremia is collected from the cut on the

tail after every 8 hours from the moment of infection for a period of 10 days. Special observation showed that in later periods bacteria are encountered very rarely in the blood. The sense of the method of the quantitative study of bacteremia lies in the determination of the degree of insemination of the blood by the causative agent throughout the course of the acute period of the infection.

The selection of the preferred volume of the bacteriological loop is determined by that amount of blood which is taken up by the most widely distributed species of fleas.

We accepted that on the average a fully saturated flea takes in 0.2 mm^3 of blood from the rodent.

WE DESIGNATE AS EPIZOOTOLOGICALLY SIGNIFICANT THAT BACTEREMIA AT WHICH IN 1 mm^3 OF THE RODENTS'S BLOOD THERE ARE 5--10 MICROBES. IN THIS CASE EACH FULLY SATURATED FLEA RECEIVES AN AVERAGE OF 1--2 MICROBIAL CELLS. Epizootologically a significant bacteremia is a necessary condition for the epizootic process. The more prolonged the bacteremia and the higher the concentration of bacteria in the blood, then the more frequently will blood-sucking insects be infected. In this manner the method of the quantitative study of bacteremia may be used for evaluating the role of this or that rodent in the epizootic process.

In our investigations the method of the quantitative study of bacteremia was used mainly for the study of the biological properties of strains of the plague causative agent which were isolated in various natural foci of the USSR. We propose becoming familiar with those methods of depicting the results of the study of bacteremia which were used by us for the first time.

In the investigations by P. N. Stupintskiy (1939) and other authors, the results of bacteremia were depicted in the form of a broken curve which connected points reflecting the quantitative aspect of bacteremia. The time was set off along the axis of abscissas, and along the axis of ordinates -- the amount of microbial cells in the investigated material following titration on nutrient broth. M. I. Levi, B. G. Valkov, A. I. Shtelman and Yu. V. Kanatov (1959) calculated the amount of colonies which grew in seedings of blood on solid nutrient media. However, such a method did not permit a comparison of the data from a study of bacteremia in many rodents on one drawing. In connection with this we made the suggestion to depict the data of bacteremia in the form of a graph which would make it possible to reflect the results of the investigation of many rodents. This form of representation was published for the first time in the work by M. I. Levi, B. G. Valkov, Ye. I. Novikova and B. G. Minkov (1959). For each animal which was investigated a special series of squares was set aside. Each of these should have reflected the results of a seeding of blood during a certain period of the investigation. The intensity of bacteremia was denoted by means of crossing out a various portion of the square. With 1--10 colonies in a seeding one-fourth of the square was crossed out, with 11--50 - half of the square, with

51--100 - three-fourths of the square, and finally, if the amount of colonies exceeded 100 the entire square was crossed out. It is easy to be convinced of the advantage of such a method of depicting the results of a bacteremia investigation by comparing it (see the work by M. I. Levi, 1960) with the curves in figure 2 in the article by M. I. Levi, B. G. Valkov, A.I. Shtelman and Yu. V. Kanatov (1959). It is possible to compare the results obtained because the animals are infected with similar doses and in the same periods, and the bacteremia investigations are carried out in the same periods and with the same loop.

With the help of graphs it is possible to obtain a satisfactory impression concerning bacteremia in a large number of rodents, however, for a comparative evaluation in a number of cases it is necessary to correlate the data of the graphs with the help of relative values, which we called the bacteremia indicator and the bacteremia index. Squares which were not crossed out were evaluated as 0, blacking out of one-fourth of the square is denoted by the figure 5, one-half of the square -- by the figure 6, three-fourths of the square -- by the figure 7, and if the square is completely crossed out - the figure 8. A similar evaluation is connected with the role of the duration and intensity of bacteremia for the infection of blood-sucking arthropods. THE BACTEREMIA INDICATOR IS THE SUM OF ALL THE VALUES-THE FIGURES DURING THE INVESTIGATION OF BACTEREMIA IN A SPECIFIC ANIMAL. The bacteremia indicator makes it possible to evaluate the epizootological significance of a specific infected animal.

THE BACTEREMIA INDEX IS THE AVERAGE BACTEREMIA INDICATOR FOR ANIMALS WHICH ARE INFECTED WITH THE SAME DOSE OF THE CAUSATIVE AGENT. For the computation of the bacteremia index, the bacteremia indicators for all the animals which were infected with the same dose are added up and divided by the number of animals. A combination of the bacteremia indices for various doses makes it possible to evaluate relations of the animals to the administration of the plague microbe. As an example, here are the bacteremia indices for the left bank midday gerbils, infected with strains 1228 (297) and 1256 (403)*), compiled on the basis of the graphs which were presented in the work by M. I. Levi (1960). See figures 4 and 5.

In the previous investigations by P. N. Stupnitskiy (1939) and other authors, the so-called agonal septicemia was detected. The intensity of this depended on the multiplication of the causative agent in the internal organs of the animal. In experimental animals, several authors noted primary bacteremia which set in after certain periods following the administration of the bacteria and depended on the infiltration of the causative agent from the site of administration and its dissemination in the organism of the animal.

*Here and subsequently the number of the strains corresponds to the reference journal of the Rostov/Don Antiplague Institute. The figure in parentheses indicates the number under which the strain is encountered in the literature.

A systematic study of bacteremia in various species of rodents, infected with various strains of the plague causative agent, made it possible to isolate an infectious bacteremia along with the primary bacteremia and agonal septicemia. This set in during the climax of the infectious process and lasted considerably longer than agonal septicemia. As it was cleared up in previous investigations, the nature of bacteremia depends on the virulence of the causative agent and its origin, and also on the species of rodent infected. Infectious bacteremia is encountered particularly often in those cases when the study of this indicator was carried out on animals which played the role of the leading host (main carrier) in the natural focus in which the strain was isolated. On the other hand, in the event of a disparity between the strain of the causative agent and the rodent on which bacteremia is studied, agonal septicemia is encountered more often than infectious bacteremia, and from time to time the infiltration of bacteria into the blood is not noted (especially with moderate infecting doses).

For the experimental study of bacteremia there is real significance in the selection of the infecting doses and the degree of resistance of the animals. While for the study of bacteremia in resistant species it is possible to use infecting doses with a tenfold index, highly sensitive species should be infected with small doses with an index of 2.

Bacteremia is one of the most important indicators of the susceptibility of a rodent to plague. WHILE EARLIER THE STUDY OF SUSCEPTIBILITY AND INFECTIOUS SENSITIVITY WAS PRACTICED ONLY FOR STUDYING THE RELATIONSHIP OF THIS OR THAT SPECIES OF ANIMAL TO A MICROBE (N. G. OLSUFYEV(1960), T. N. DUNAYEVA (1955)), WE SUGGESTED THE USE OF INFECTIOUS SENSITIVITY AND BACTEREMIA INDICATORS FOR CHARACTERIZING THE VERY CAUSATIVE AGENT, FOR RESOLVING THE PROBLEM CONCERNING THE ORIGIN OF THIS OR THAT STRAIN.

In connection with the fact that the difference in the virulence for certain species of animals is less significant than the difference in the nature of bacteremia, the latter indicator was used in our investigations for clearing up a number of important properties of the strains. This made it possible to essentially supplement the classification of varieties of the plague microbe.

The study of bacteremia, just as the investigation of virulence, should be carried out simultaneously on various species of rodents with an obligatory consideration of the sex and weight of the animal. This does away with the additional possibilities of errors and reduces the number of controls. As a rule, when studying the strains from various natural foci of the USSR, we used the same dilutions of a culture for the simultaneous infection of 80 midday gerbils (40 - for studying virulence and 40 - for studying bacteremia), 64 small susliks (32 - for studying virulence and 32 - for studying bacteremia), 28 guinea pigs and 42 white mice (of these, 28 animals were for the study of bacteremia). For each dose 4 animals (2 males and 2 females) were taken for the study of virulence. For the study

of bacteremia the animals were infected with the same doses as for the study of virulence, and 2 males and 2 females were infected with each dose. We did not notice significant or regular differences in the sensitivity to plague on the part of laboratory and wild rodents depending on their sex.

The investigations of bacteremia in experimentally infected animals over a number of years led us to a certain unexpected conclusion concerning the harmlessness of the method of taking blood from the animals. It turned out the LD_{50} for rodents in which bacteremia was not studied was by far higher than for those in which bacteremia was studied. Since both groups of animals were infected simultaneously with the same dilutions of a culture, and the resulting differences by far exceeded the probable error, then it must be recognized that the taking of blood from the tails of infected animals raised the percentage of their death.

For technical reasons our investigations were carried out mainly in the winter period, however for a comparison the investigations on two strains (1252 and 1230) were repeated in the spring and summer. No principle differences were noted in the nature of bacteremia, however, in the spring and summer the bacteremia turned out to be much more intensive in small susliks, somewhat more intensive in midday gerbils, and for the white mice it turned out to be almost the same as in the winter (see figures 6, 7, 8, and 9).

All that has been said makes it possible to appraise the data of bacteremia which are obtained in the winter period as fully reliable for attributing a strain under study to this or that variety of the plague microbe. It should be added that the carrying out of similar tests in the spring or summer is more preferable, since bacteremia is expressed more sharply, and the difference in the death of traumatized and non-traumatized animals is less.

Ye. Ye. Punskiy (1959), M. A. Timofeyeva (1960) and other authors think that the testing of the virulence of strains of the plague microbe should be conducted immediately following isolation. We consider that this proposal remains true for establishing the virulence of strains which are circulating under natural conditions, however, for studying the nature of bacteremia it is possible to use strains which have been stored for some time under laboratory conditions. Thus, strain 1228 (297), which was stored for 5 years on nutrient media under laboratory conditions, caused, though in a somewhat weakened form, bacteremia which is characteristic for strains of the suslik variety. It stands to reason that for the study of bacteremia the strain should possess a relatively high virulence for laboratory animals.

Earlier it was pointed out that all the surviving animals were sacrificed in one and a half months from the moment of infection. The internal organs of the animals were subjected to a bacteriological investigation and the blood serum was investigated for the content of antibodies to the plague causative agent.

The content of antibodies in the serum of sacrificed animals is

investigated with the help of the passive hemagglutination reaction (the erthrocytes were sensitized with fraction IA of the plague microbe). M. I. Levi (1960) reported that in left bank midday gerbils the gerbil strain 1256 (403) caused the formation of antibodies much more often and in a higher titer than the suslik strain 1228 (297). However, in small susliks both strains caused the production of antibodies in approximately the same titer and with the same frequency. Thus, left bank midday gerbils can be considered as DETECTOR ANIMALS. It is possible to use them to distinguish the suslik strain from the gerbil. It seems to us that the formation of antibodies in the organism of animals is connected with the infiltration of a certain amount of bacteria into the blood.

It was pointed out earlier that in highly sensitive species of animals, antibodies are formed very rarely in response to the administration of a highly virulent strain, while in the organism of small susliks and left bank midday gerbils antibodies are formed relatively often and in a high titer (M. I. Levi, 1960). In all probability this circumstance is connected with the fact that in sensitive species if infection leads to bacteremia, then most often of all it is the result or the repletion of the internal organs with the causative agent and exudation of the latter into the blood which is connected with this. With sensitive species the infectious process during experimental plague proceeds according to the principle "all or nothing," that is, either the causative agent does not overcome the barriers of the organism and dies, without causing any significant immunological shifts, or it penetrates into the internal organs and multiplies intensively there, and as a result of this antemortem speticemia and the death of the animal sets in. In both cases antibodies are hardly ever formed.

Since the physiological condition of rodents, especially hibernating ones, seriously influences the results of studying virulence (N. I. Kalabukhov) and bacteremia, then it is desirable to have a simultaneous or almost simultaneous setting up of the tests with the strains being compared.

Antigenic Structure

In recent years several authors (Javets and Meyers, 1943; Burrows and Bacon, 1956; Crampton and Davis, 1956; Lawton, Fukui and Surgalla, 1960) have connected the virulence of the plague microbe with the presence of specific antigens -- Vi, V and W. It turned out that the EV 76 strain possesses the V- and W- antigens, but at the same time is devoid of virulence for all the animals on which it has been tested. We propose that the virulence of the plague microbe is connected not with any one antigen, but apparently with a number of properties of the microorganism which guarantee the paralysis of phagocytosis and the tissue mechanisms of defense of the animal -- the penetration of the causative agent through the barriers, and the multiplication of the microbe in the organs and tissues of the animals with the formation of toxic products, causing the death of warm blooded animals. V. D. Timakov and V. G. Petrovskaya (1960) propose that no one of the features of virulence has an absolute significance, since this property has a multitude of determinants. It

should be assumed that the virulence of a strain is manifested only in the event that there are conditions for the manifestation of all of the properties of the microbe. In connection with this we dropped the investigations for seeking an antigen which specifically determined the virulence of the microorganism. Apparently the lowering or loss of virulence in different cases may be caused by various reasons. We decided to limit ourselves to the characteristics of several antigens in a number of strains of the plague microbe which are attributed to various biological varieties. In one of the articles of this collection it will be shown that this problem turned out to be too difficult for us and the problem is still waiting to be resolved.

For studying the antigenic structure of strains of the plague microbe, we obtained the immune rabbit serum to each strain. We used the method of immunization of the rabbits with a killed and then with a live culture of microbes. Since a number of antigens of the plague microbe, for example fraction I, are formed only as a result of incubation of the bacteria at a temperature of 37°, we incubated the strain at this temperature on slopes with blood agar (blood of a sheep) for 72 hours. Then the culture was washed off with a physiological solution and diluted with it to a concentration of 1 billion microbes in 1 ml based on the optical standard. Formalin in a volume of 0.2 ml was added to 100 ml of such a suspension. Then the flask was closed with a rubber cork and left in a dark,cool place for 2 days with frequent periods of vibration. After this a control seeding was made on nutrient agar. If the solution did not contain live bacteria then it could be used for the immunization of rabbits.

The immunization of rabbits is carried out by means of the intravenous administration of the antigen. The killed vaccine was introduced into the ear vein in quantities of 1, 2, and 3 ml with an interval of 3 days. Following the first cycle 2 more analogous cycles are performed with an interval of a week between them. Then 0.5 ml of blood is collected from the ear vein for setting up the agglutination reaction with the homologous strain. After a week following the conclusion of immunization with killed vaccine, immunization with a live culture was undertaken. It was incubated at 37° on blood agar for 72 hours. The live vaccine was introduced in quantities of 10^3, 10^4, 10^5 (1 cycle), 10^5, 10^6, 10^7 (2 cycles), 10^7, 10^8, 10^9 (3 cycles) of microbial bodies with intervals of 3 days between injections and a week between cycles. In a week following the conclusion of immunization with the live culture, 0.5 ml of blood was collected from the ear vein of the animal and investigated in the agglutination reaction with the homologous strain. If the titer of the serum is lower than 1:160, then the last cycle of immunization with a live culture is repeated. Introduction of the plague microbe in amounts exceeding 1 billion microbial bodies has an unfavorable influence on the rabbits: In the first days following the injection their gait was unsteady, sometimes paralysis of the rear extremities was observed, and now and then the rabbits died. The cited arrangement of immunization made it possible to obtain immune serum with a titer of antibodies of 1:160--1:1280 in the agglutination reaction, 1:10--1:20 in the precipitation reaction, 1:10 000 -- 1:40 000 in the passive

hemagglutination reaction, and 1:320--1:1280 in the complement fixation reaction.

For the study of antigenic composition, the virulent strains of the plague microbe were incubated in glass separating flasks on blood agar at 37°. After 72 hours the culture was washed off with a physiological solution, and the bacteria were killed with cooled acetone. The dry powder of killed bacteria was subjected to processing in accordance with the method described by Baker et al. (1952) with insignificant changes. The toxic fraction was titrated on white mice.

Conclusions

The investigations of the biological characteristics of the various strains of the plague causative agent are ~~of the most important~~ significance, ~~for~~ the theory of adaptation mutability. Such works are exceedingly laborious, therefore, V. M. Tumanskiy (1959) recommended that a large number of workers from the antiplague institutes, stations and departments be drawn into them. He also recommended that this be carried out according to a common method and plan. The method ~~which we suggested for~~ studying the biological properties of the plague microbe, consisting of four sections -- cultural properties, virulence, bacteremia, and antigenic structure, ~~was used for the~~ study of 11 strains of the plague causative agent from various natural foci. The works from a group of authors from various antiplague institutions are listed below. It is necessary to state that in our investigations the histological division was provided for the study of pathohistological changes of the internal organs and tissues of laboratory and wild rodents which were infected with various strains. It was carried out under the leadership of V. N. Lobanov, however, these investigations are still not concluded.

It can be thought that the bases of our method for studying the biological properties of the plague causative agent will find application in a number of research establishments. It is necessary to study a large number of strains of the plague microbe, since only such a mass of material makes it possible to make rational theoretical and practical conclusions.

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Table 1

Comparison of results of seeding the organs of rodents on agar and in broth.

No of strain	Results and number of seedings			
	+/+	+/-	-/+	-/-
1252	394	27	33	708
1253	422	87	26	738
1258	395	6	22	847
1229	411	12	20	787
1230	456	43	41	686

Infecting dose in decimal logarithms	Outcome of infection		Cumulative data		% survived
	died	survived	died	survived	
1	1	3	1	6	86
2	1	3	2	3	60
3	4	0	6	0	0
4	4	0	10	0	0
5	4	0	14	0	0
6	4	0	18	0	0

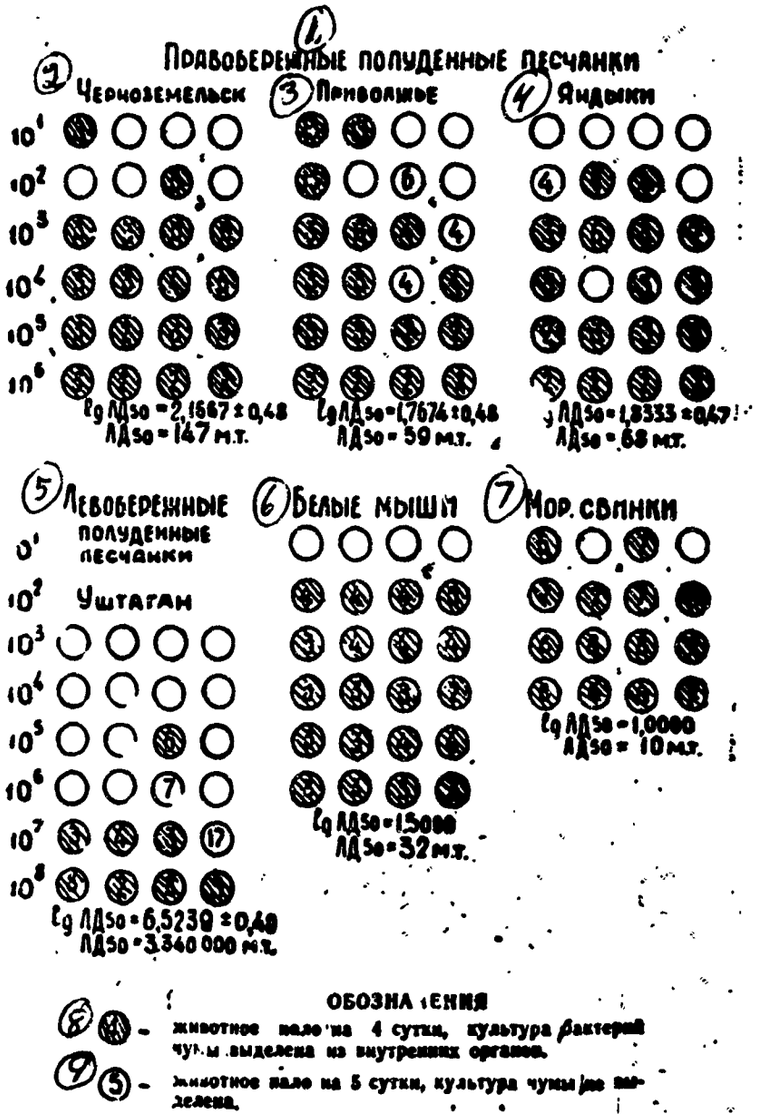

Figure 1. Virulence of strain 1230 (1042) for various animals.
1 - right bank midday gerbils; 2 - Chernozemelsk; 3 - Privolzhye; 4 - Yandyki; 5 - left bank midday gerbils, Ushtagan; 6 - white mice; 7 - guinea pigs; 8 - animal died in 4 days, culture of bacteria isolated from internal organs; 9 - animal died in 5 days, plague culture not isolated. In the explanations under the six different sections of the overall drawing, the letters m.t. stand for microbial bodies, and the Russian letters ЛД stand for LD.

Table 2

Variation of the LD_{50} in one test

Number of strain	Infecting dose	Results of infecting white mice									
		Variant A					Variant B				
		I	II	III	IV	V	I	II	III	IV	V
261	10^{-1}	0/6	1/6	1/6	0/6	0/6	0/6	0/5	0/5	0/6	0/6
	10^{0}	2/6	1/6	1/6	1/6	2/6	2/6	1/6	1/6	1/6	2/6
	10^{1}	1/6	3/6	6/6	6/6	5/6	0/5	3/6	5/5	6/5	4/5
	10^{2}	6/6	6/6	5/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6
	10^{3}	5/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6
	10^{4}	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
$1gLD_{50}$		1.2983 ± 0.40	0.7143 ± 0.40	0.3906 ± 0.32	0.3976 ± 0.11	0.3729 ± 0.35	1.3906 ± 0.32	0.8444 ± 0.39	0.5000 ± 0.30	0.3976 ± 0.34	0.3684 ± 0.30
142	10^{2}	2/6	4/6	4/6	2/6	5/6	1/5	2/4	4/6	1/5	3/4
	10^{3}	5/6	6/6	4/6	3/6	5/6	4/5	4/4	3/5	3/6	5/6
	10^{4}	5/6	6/6	6/6	6/6	6/6	4/5	6/6	5/5	6/6	6/6
	10^{5}	6/6	6/6	6/6	6/6	6/6	5/5	6/6	6/6	5/5	6/6
	10^{6}	6/6	6/6	5/6	6/6	6/6	6/6	6/6	5/6	5/5	6/6
	10^{7}	6/6	6/6	4/6	5/6	6/6	6/6	6/6	4/6	5/6	6/6
$1gLD_{50}$		2.4717 ± 0.35	1.7463 ± 0.34	2.5417 ± 0.54	2.8333 ± 0.43	1.7042 ± 0.36	2.6316 ± 0.37	2.0000 ± 0.36	2.5909 ± 0.56	3.0000 ± 0.41	1.8333 ± 0.42
133	10^{3}	5/6	6/6	6/6	5/6	5/6	4/5	1/1	3/3	3/6	3/4
	10^{4}	5/6	5/6	6/6	4/6	5/6	2/3	3/4	3/3	2/4	3/4
	10^{5}	5/6	5/6	1/6	6/6	6/6	4/5	5/6	1/6	4/4	5/5
	10^{6}	5/6	6/6	5/6	6/6	5/6	4/5	5/5	3/4	6/6	3/4
	10^{7}	5/6	5/6	2/6	5/6	6/6	5/6	5/6	2/6	5/6	6/6
	10^{8}	6/6	5/6	6/6	6/6T	6/6	5/5	4/5	6/6	6/6	5/5
$1gLD_{50}$		3.0000 ± 0.50	2.8333 ± 0.60	3.7222 ± 0.73	3.4444 ± 0.45	2.8929 ± 0.52	3.3333 ± 0.64	4.2333 ± 0.50	5.3462 ± 0.71	3.7693 ± 0.50	3.0000 ± 0.51

Denominator: Number of infected animals; Numerator: Number of animals which died.

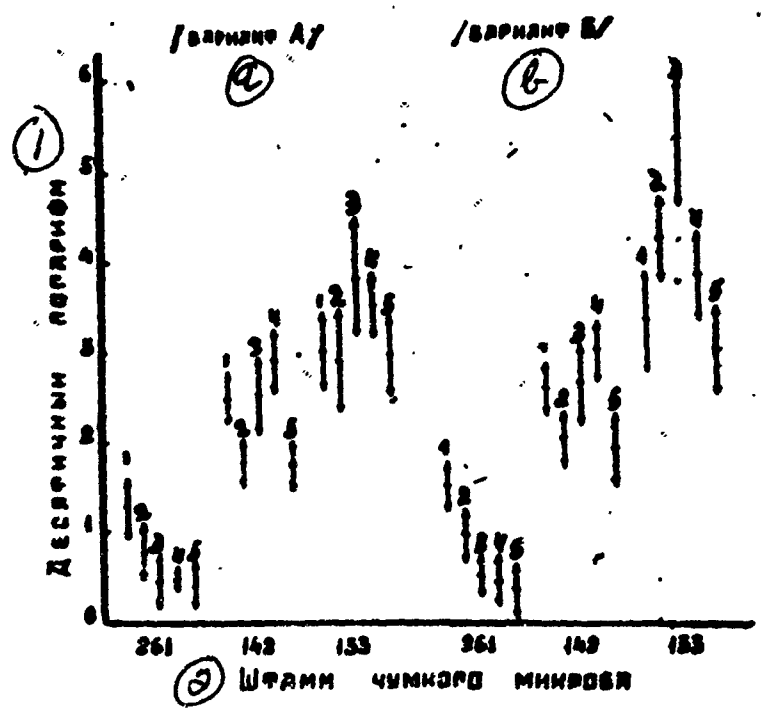

Figure 2. Variation of the LD_{50} in one test.

a - variant A; b - variant B;

1 - decimal logarithm; 2 - strain of plague microbe.

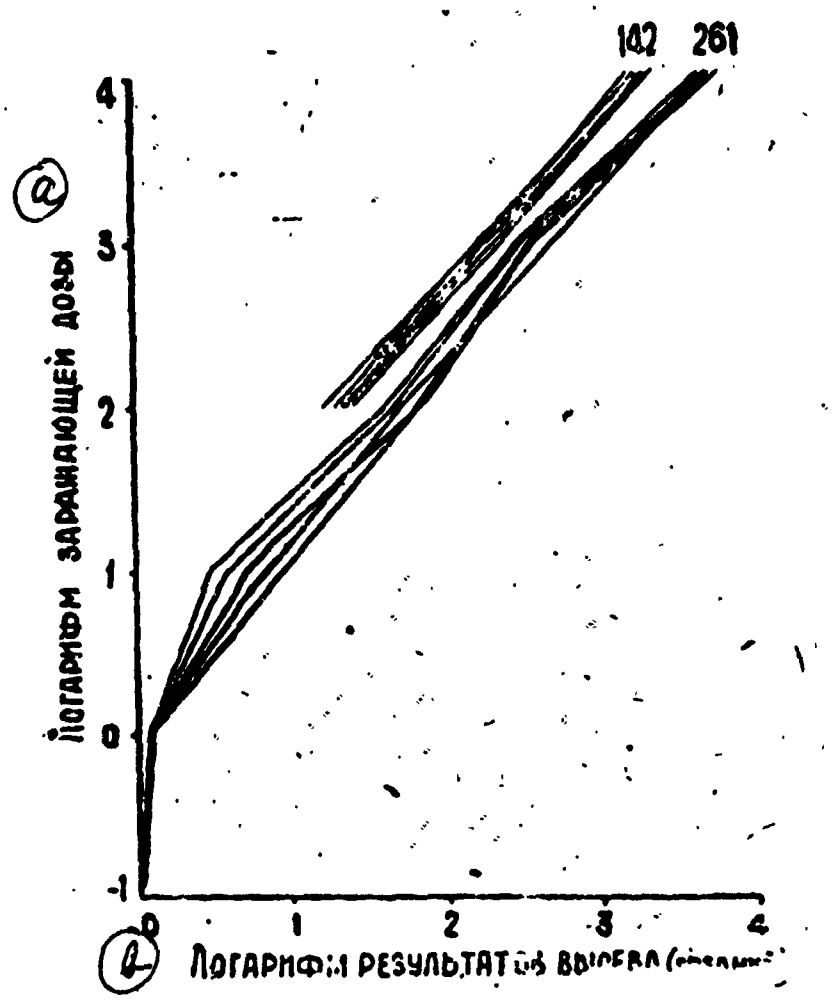

Figure 3. Variations in the results of seeding (average) for strains 261 and 142.

a - logarithm of infecting dose; b - logarithm of the results of seeding (average).

Table 3

Experimental verification of variations of the results of seeding the plague microbe on agar dishes in one test.

No. of strain	Dose based on optical standard	Results of seeding on nutrient agar in lines				
		I	II	III	IV	V
261	10^{-1}	0 0 0 (0)	0 0 0 (0)	0 0 0 (0)	0 0 0 (0)	0 0 0 (0)
	10^{0}	0 0 1 (0.3)	0 0 1 (0.3)	0 0 1 (0.3)	0 0 1 (0.3)	0 0 1 (0.3)
	10^{1}	2 5 2 (3)	7 5 0 (0)	6 15 5 (9)	5 2 8 (5)	8 4 5 (6)
	10^{2}	42 54 40 (45)	73 47 30 (48)	81 70 58 (70)	66 54 64 (61)	37 74 64 (58)
	10^{3}	450 380 475 (435)	501 515 398 (471	450 315 550(438)	478 520 600 (532)	399 439 501 (446)
	10^{4}	6090 4850 3999 (4980)	5014 4900 4850 (4931)	5373 4860 4050 (4761)	3702 4789 7001 (5164)	3837 4800 5039 (4559)
142	10^{2}	31 22 22 (25)	19 23 28 (23)	23 22 20 (22)	21 24 31 (25)	17 29 31 (26)
	10^{3}	280 257 280 (226)	278 300 117(232)	220 247 301(256)	185 217 205 (202)	278 199 217 (231)
	10^{4}	3050 3015 2985 (3017)	2853 2799 3045 (2899)	2555 2400 3015 (2657)	2713 2988 2560 (2754)	2015 2897 3750 (2887)
	10^{5}	profuse growth	profuse growth	profuse growth	profuse growth	profuse growth
	10^{6}	profuse growth	profuse growth	profuse growth	profuse growth	profuse growth
	10^{7}	profuse growth	profuse growth	profuse growth	profuse growth	profuse growth

The average indices are in parentheses

Table 4

Determination of the amount of blood, sucked up by various species of fleas (based on the data of L. V. Bryukhanova, Ye. A. Sardar and M. I. Levi, 1957)

Number	Species of fleas	Average amount of blood, sucked up by the flea (in mg)	
		female	male
1	X. cheopis	0.14	0.14
2	X. conformis	---	0.05
3	Cer. tesquorum	0.26	0.23
4	Cer. consimilis	0.21	0.11
5	Cer. fasiatus	0.24	0.09
6	L. segnis	0.10	0.03
7	N. setosa	0.21	0.18

Figure 4. Bacteremia index for strain 297.

1 - small suslik; 2 - midday gerbil; 3 - logarithm of infecting dose.

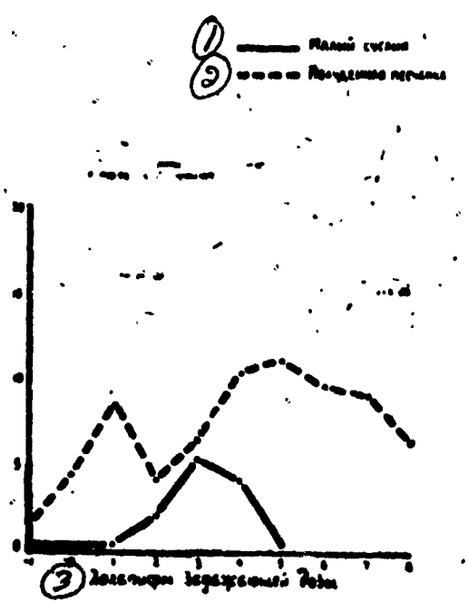

Figure 5. Bacteremia index for strain 403.

1 - small suslik; 2 - midday gerbil; 3 - logarithm of infecting dose.

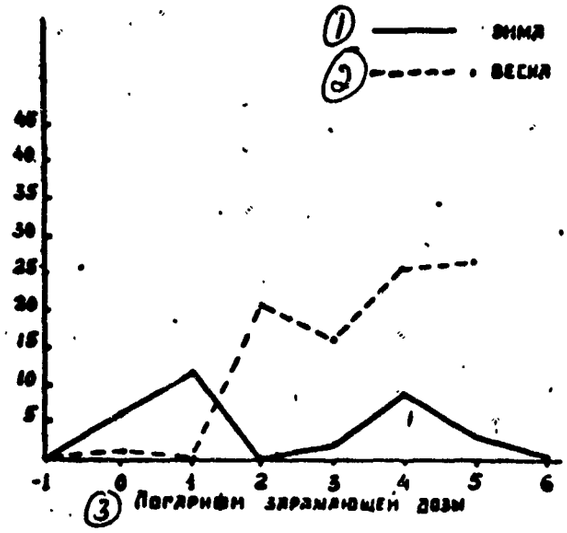

Figure 6. Bacteremia index in small susliks in the winter and spring of 1960. Strain 1252. 1 - winter; 2 - spring; 3 - logarithm of infecting dose.

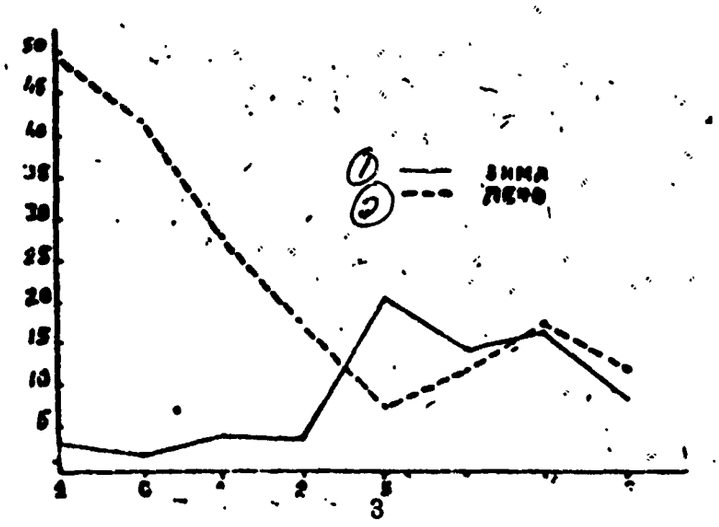

Figure 7. Bacteremia index in small susliks in the winter and summer of 1960. Strain 1230. 1 - winter; 2 - summer; 3 - logarithm of infecting dose.

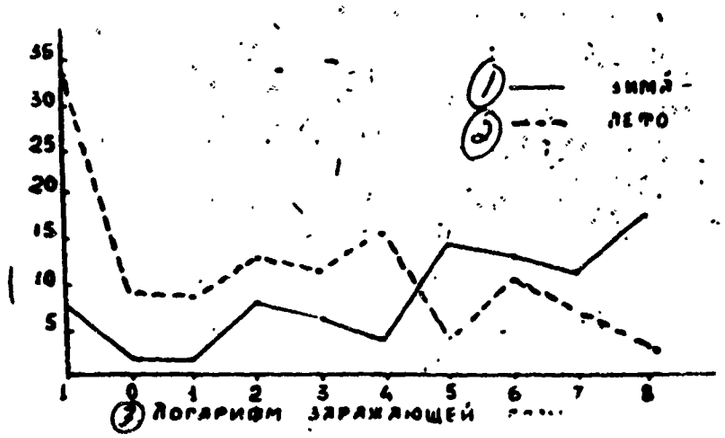

Figure 8. Bacteremia index in midday gerbils in the winter and summer of 1960. Strain 1230. 1 - winter; 2 - summer; 3 - logarithm of infecting dose.

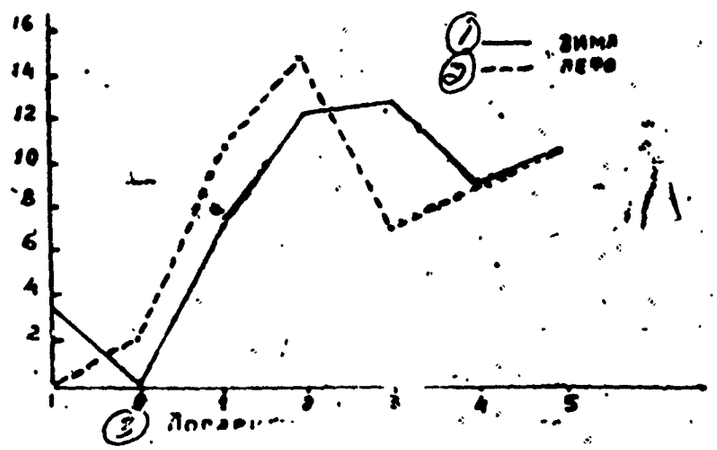

Figure 9. Bacteremia index in white mice in the winter and summer of 1960. Strain 1230. 1 - winter; 2 - summer; 3 - logarithm of infecting dose.