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CONTRIBUTION TO THE STUDY OF THE BEHAVIOR OF PATHOGENIC  
MICROBES IN HEMATOPHAGOUS INSECTS FIRST REPORT

[Following is a translation of an article by Georges Blanc and Marcel Baltazard, in the French-language periodical Archives de l'Institut Pasteur du Maroc (Archives of the Pasteur Institute of Morocco), Vol III, No 2, 1944, pages 21-49.]

As a result of our research on the behavior of typhic viruses in the rat flea, we were led to enlarge the field of our observations and of our experiments and to study the behavior of various pathogenic microbes in this hematophagous insect.

From studies on the plague bacillus (G. Blanc and M. Baltazard, C. R. Soc. Biol., 136, 1942, p 646), we passed on to the study of Whitmore's bacillus (G. Blanc and M. Baltazard, Annales Inst. Pasteur, 60, 1942, p 281) and Gessard's bacillus (G. Blanc and M. Baltazard, C. R. Acad. Sc., 215, 1942, p 43). The perfect adaptation of these germs to life and to multiplication in the digestive tube of fleas seemed to constitute for us an interesting biological characteristic that could even, at least in certain cases, have a systematic value utilizable in classification and that would permit the separation or the bringing together of germs in the same genus or the same family.

Thus we were able to observe that other very virulent germs, that gave an intense septicemia to laboratory animals, were not fit for supporting themselves in the digestive tube of fleas; for example, the carbon bacillus and various Pasteurellas (avian strain, rabbit strain, porcine strain) (G. Blanc and M. Baltazard, Report on the Operation of the Pasteur Institute of Morocco, 1942, p 11). Now, most bacteriologists classify Yersin's bacillus in the genus Pasteurella. The difference in behavior of the plague bacillus and the Pasteurellas in the flea moves us to separate these germs and to classify them in different genera. Although the plague bacillus cannot be retained in the genus Pasteurella, it seems that it should be placed in a common genus with Malassez's bacillus which displays so many characteristics that are common with it.

But there is a biological characteristic, not yet studied, on which we have believed it indispensable to throw light, before considering this relationship as completely established: it is the characteristic of the behavior of this bacillus in Xenopsylla cheopis. It seemed likely, a priori, that this behavior was probably the same behavior as found in the bacilli that give laboratory animals an infection of the pseudotuberculous type, such as the plague bacillus, Whitmore's bacillus and the pyocyanic bacillus. Experiments have confirmed this supposition. We also wanted to extend our research to pathogenic germs of other groups and we began to study the Salmonellas. The results that we set forth here are the first ones obtained with these different germs.

### General Techniques

Fleas (Xenopsylla cheopis) are infected by biting an animal that is also infected with the germ to be studied. Sometimes the animal, most frequently a guinea-pig, is placed directly in a growing vat of young fleas containing thousands of fleas. Since this technique of flea growing has previously been given very much in detail, we shall not repeat it here (G. Blanc and M. Baltazard, Archives de l'Institut Pasteur du Maroc, 2, 1941, p 465). Other times the animal is bitten by fleas placed in a tube with a wide opening, a type of Borrel tube, closed at both ends with a sifting silk cloth.

Once or twice a day, the fleas are gorged. For that purpose, one end of the tube is applied to the ventral wall of the infected guinea-pig and kept there a length of time sufficient for allowing all the fleas to take their bloody meal, that is, about one half hour.

It is possible to ascertain whether they are infected by removing some fleas, either from the vat or from the tube. The insects are crushed carefully in a rough-bottomed glass, the trituration is diluted in a few cubic centimeters of physiological water or of bouillon, seeded on culture media and inoculated in a susceptible animal.

In order to ascertain if the fleas are able to transmit the infection by biting, it suffices, after having observed that they are properly infected, either to put a fresh guinea-pig in the vat, or to make the fleas contained in the Borrel tube bite a fresh guinea-pig. The illness of the animal bitten in this way provides the desired proof.

In every case we used concurrently the two techniques that have just been outlined. It happens that they are insufficient. In order to infect the fleas effectively, it is necessary to make them feed on an animal whose septicemia is intense; it is necessary to be sure that the insect has in fact ingested a considerable amount of the germs being studied to learn what happens to them in their digestive tube. Now, some

pathogenic microbes do not cause a real septicemia in laboratory animals, but merely a more or less slight bacteremia that is more less transitory. In these cases, we were forced to use a special procedure. We deliberately rejected Hindle and Duncan's technique (E. Hindle and J. Duncan, T. Parasitology, 17, 1925, p 434) used by them to study the behavior of various microbes in the digestive tube of Argas persicus and which consists of making the tick feed through an animal membrane: for example, guinea-pig mesentery, covering a cupula containing a mixture of inactivated serum and of microbial culture, Haller's organ having been previously removed. Since this very delicate method seemed to us to be inapplicable to fleas, we used, to infect them, their property of gorging themselves greedily on a rabbit, and we put them to feed on an animal in which we had produced experimentally an intensive bacteremia. The following is the method: a rabbit is fastened down on its back and the skin of its belly very carefully depilated. It receives three to four cc. of a microbial emulsion, titrated at three billion, by means of an injection in a marginal vein of one ear. If we admit that the total volume of blood circulating attains a maximum of 100 cc., there must be a transitory bacteremia of about 120 to 160 million germs per cubic centimeter, 120,000 to 160,000 per cubic millimeter, or 6,000 to 8,000 in one-twentieth of a cubic millimeter, the volume of blood that authorities admit as the amount absorbed by a flea during one feeding. All this mathematical reasoning has only a theoretical value, since the distribution of microbes in circulating blood is not uniform, but, in fact, under the indicated experimental conditions, by using the germs studied here on intensive bacteremia is observed which allows us to state that the fleas that are put on to feed must be infected in their majority.

Five minutes after the rabbit has received the intravenous injection of the microbial dilution, the fleas are placed on it to feed in the microbial dilution, the fleas are placed on it to feed in the following manner: several hundred fleas, recently hatched and never having bitten, are put in a wide tube of the Borrel type, closed at both ends with a piece of sifting silk No. 8. One of the ends of this tube is applied to the ventral wall, carefully depilated, of the rabbit and kept there sufficient time for the fleas to gorge themselves, or usually one half hour. Examinations under a binocular magnifier allow us to make certain that all the fleas are well distended.

During the operation, a drop of blood taken from the marginal vein of the ear opposite the one that was used as an inoculation point, is seeded on usual media. This enables us to verify the intensity of the induced bacteremia. As soon as the fleas are distended, ten of them are crushed in several drops of bouillon and the dilution is cultivated, which allows us to verify that the fleas have really ingested microbes during their bloody feast.

Once the fleas are properly infected, they are then put to feed

on fresh guinea-pigs that are changed as often as seems necessary to avoid superinfections. At fixed time intervals, generally every four days, some fleas are removed from the Borrel tube, are crushed and diluted in several drops of bouillon or physiological water. The dilution is cultivated and inoculated in susceptible animals, in order to judge the condition of infection of these fleas. The excrements are collected from the Borrel tube at several different times and are cultivated and inoculated, which enables us to determine if the germs ingested by the fleas are regularly eliminated and, consequently, are multiplying in the digestive tube. Finally, some excrements are preserved dry and, by means of repeated cultivation, it is possible to determine if the microbes that they contain are susceptible to being preserved in this state of anhydrobiosis for a more or less long period of time.

#### BACILLUS OF PSEUDOTUBERCULOSIS OF RODENTS

We used two strains for our experiments, one brought from Paris, isolated from a guinea-pig that came from Doctor Boquet's laboratory, the other one isolated at Casablanca from a rabbit that had died of pseudotuberculosis. Both were carefully examined from the point of view of their biochemical characteristics and their pathogenic properties. An agglutinant serum that was prepared in a rabbit with types S and R isolated from the Paris strain, enables us to add an agglutination examination to the study of these characteristics.

#### Infection Experiments on Breeding Fleas Kept in a Vat

The Paris strain is the one used, freshly isolated and strongly virulent; it kills the guinea-pig in seven to twelve days after intramuscular injection in the thigh (Figs. I and II) [all figures are appended at the end]. The transfer guinea-pigs, also inoculated in the thigh with one cc. of spleen dilution in 10 cc. of physiological water, die in eight to twelve days.

On 27 June 1943, a guinea-pig that had been inoculated on the 24th in the left thigh, with one cubic centimeter of the dilution in 10 cc. of physiological water of a 24-hour culture of the pseudo strain, is put in a vat containing several thousand fleas, Xenopsylla cheopis, that had been raised without ever having bitten. On the following morning, the guinea-pig is found dead. In the autopsy, the signs of an acute infection are found. At the level of the injection, in the left thigh, there is bloody suffusion without abscess, the left inguinal ganglions have increased in volume and are hemorrhagic but not purulent. The left sub-lumbar ganglion has increased in volume and is purulent. The spleen is very swollen, black, soft, friable, without abscess. The lungs display numerous hemorrhagic spots, the liver is congested and marbled. Bacilli,

most frequently grouped in a mass according to the usual type of pseudotuberculosis bacillus, are found on spleen and liver smears.

Spleen and blood cultures yield numerous colonies. One drop of blood produces 530 colonies, which represents about 16,000 germs per cubic centimeter and less than 20 germs per cubic millimeter, therefore, less than one germ per twentieth of a cubic millimeter, the normal stomach capacity of the flea, according to Dujardin-Baumetz.

On the same day, a fresh guinea-pig is put in the vat and does not become infected. At various times, batches of fleas are taken from the vat for inoculation in guinea-pigs.

On 2 July, or four days after feeding on an infected guinea-pig, one hundred fleas are crushed and diluted in 3 cc. of physiological water. Half the suspension is inoculated in a guinea-pig peritoneally. It dies five days later, with pseudotuberculosis lesions. The spleen and blood yield positive cultures of Malassez's bacillus.

Ten days after the infecting feeding, thirty-five fleas are crushed as before. Two guinea-pigs are inoculated, one in the peritoneum, the other in the thigh. The first one will die seven days later; the second one with internal lesions of the infection with a slow evolution. Finally, twenty-two days after the infecting feeding, five fleas are taken from the vat and inoculated in a guinea-pig intraperitoneally. This guinea-pig dies of pseudotuberculosis nine days later. The guinea-pig left in the vat is not infected. Therefore, the fleas have been infected by a single feeding on a pseudotuberculous guinea-pig. They remained infected at least for twenty-two days, but did not transmit the infection by biting.

Another experiment made under the same conditions enabled us to ascertain that one single feeding on an infected guinea-pig was sufficient to infect the fleas. This time, twenty-four hours after the infecting feeding, twelve fleas that were picked out of the vat infected a guinea-pig peritoneally. Three fresh guinea-pigs that were placed in the same vat and that were subjected successively to bites during these twenty-four days, did not become infected.

Finally, a third experiment was performed with three hundred fleas taken from the vat, placed in a Borrel tube, and fed twice a day on the same guinea-pig for eighteen days. The guinea-pig was not infected, but the two hundred fleas, approximately, that remained on the eighteenth day infected the two guinea-pigs in which they were inoculated. The result of this first series of experiments is that the Xenopsylla cheopis fleas are infected on the guinea-pig stricken with pseudotuberculosis, although the septicemia is not very intense and represents less than twenty germs per cubic millimeter.

### Infection Experiments on Breeding Fleas Placed in a Tube

Since it was a fact that septicemia in guinea-pigs is always quite weak and slow in appearing, we attempted to infect the fleas more intensely by making them gorge on a rabbit in a state of strong bacteremia as a result of intravenous inoculation in accordance with the method that we indicated above. Four experiments enabled us to observe the infection of the fleas fourteen, twenty, twenty-four and thirty-five days after the single infecting feeding.

The following is, by way of example, the report of one experiment:

7 February 1944: At 1040, rabbit 28/24 receives, in the marginal vein of the right ear, 3 cc. of a suspension of pseudotuberculosis bacilli from a 24-hour culture of strain 195. The suspension has approximately 4 billion germs per cubic centimeter. At 1045, 200 Xenopsylla cheopis, fresh and never having bitten, are put to feed on the rabbit. These fleas are enclosed in a Borrel tube, closed at both ends with a piece of sifting silk. They are kept in place for one half hour.

At 1125, a few drops of blood, taken from the rabbit's left ear, are cultivated on an agar tube. A hemoculture is also made of 2.5 cc. of blood taken by cardiac puncture. At 1130, finally, ten of the fleas that have just fed are crushed and 20 drops of bouillon are added. A loopful of the dilution is seeded in an agar tube and the rest in another tube.

The following is the result of these operations. Hemocultures: heart blood, very many colonies; ear blood, very many colonies. The first psyiloculture is positive, fifteen colonies are counted in the tube seeded with a loopful of dilution and more than one hundred in the other tube; the fleas that fed on the rabbit have, in fact, ingested Malasses's bacilli.

8 February: The fleas are fasting.

9, 10 and 11 February: The fleas bite a guinea-pig.

On 11 February, another psyiloculture is made by crushing ten fleas, seeding is made in two tubes as before. The two tubes yield very many quasi-confluent colonies of pseudotuberculosis bacilli.

12 February: The fleas are fasting.

13, 14 and 15 February: The fleas bite a fresh guinea-pig.

On 15 February, a third psylloculture that yields very numerous colonies.

16 February: The fleas are fasting.

17, 18 and 19 February: The fleas bite another guinea-pig. On 19 February, fourth psylloculture, yielding the same result as the preceding one.

20 February: The fleas are fasting.

21, 22 and 23 February: The fleas bite another guinea-pig. On 23 February, fifth psylloculture. This time the colonies of pseudotuberculosis bacilli are much less numerous in both tubes.

24 February: The fleas are fasting.

25, 26 and 27 February: The fleas bite another guinea-pig. On 27 February, sixth psylloculture that is practically negative. There are only three colonies in the tube seeded with 20 drops of the dilution.

28 February: The fleas are fasting.

29 February, 1 and 2 March: The fleas bite another guinea-pig. On 2 March, seventh psylloculture that yields twelve colonies in the tube seeded with a loopful of the dilution and 50 in the one seeded with the 20 drops.

3 March: The fleas are fasting.

4, 5 and 6 March: The fleas bite a fresh guinea-pig. On 6 March, eighth psylloculture that yields the same results as the preceding one. The experiment is in its twenty-ninth day and since the number of fleas left alive in the tube is still considerable, it is continued.

7 March: The fleas are fasting.

8, 9 and 10 March: The fleas bite a fresh guinea-pig. On 10 March, ninth psylloculture. It is negative.

11 and 12 March: The fleas are fasting.

13 March: The experiment is stopped on the thirty-fifth day. Two hundred fifty fleas remain alive and are crushed in several drops of bouillon. A tenth psylloculture will produce a positive culture with numerous colonies of pseudotuberculosis bacilli.

Two cubic centimeters are added to the suspension of the crushed fleas and the whole is inoculated in guinea-pig 95/51, peritoneally. This guinea-pig is sacrificed on the sixth day. The spleen is very swollen, is covered with a slight exudate and displays some small abscesses. There are some abscesses on the omentum; the liver is swollen but without abscesses. The same applies to the lungs. Liver and spleen smears show the characteristic masses of bacilli in a small number. These bacilli are abundant on the smears of epiploic abscesses.

Seedings of heart blood and spleen produce cultures of Malassez's bacillus. On the thirty-fifth day of the experiment, the fleas, after a single infecting feeding, were still infected.

### Experimental Disease in Guinea-Pigs

The guinea-pigs that were inoculated with fleas coming from the vats or the tubes die of pseudotuberculosis in a few days, usually six to seven (Figs. III and IV). The spleen is swollen, but without abscess or with only a small number; the liver is swollen and it is not rare for it to present many small abscesses. Very few bacilli are found on spleen or liver smears, usually in small intra or extracellular clusters, like the ones usually observed in pseudotuberculosis. Cultures of the spleen and of the blood are positive. This clinical and anatomopathologic picture is one of an acute infection caused by a virulent microbe. As we have already said, the guinea-pigs bitten by the infected fleas never incur pseudotuberculosis, either acute or chronic. It is not possible to state that they do not acquire any infection without symptoms. The virulence of Malassez's bacillus is relatively very weak and it is not possible to conclude from the fact that infection and immunity is absent in the bitten guinea-pigs that the bacillus cannot be inoculated by biting.

As we have shown for the pyocyanic bacillus (G. Blanc and M. Baltazard, *C. R. Acad. Sc.*, 215, 1942, p 43), it is possible that this transmission is accomplished without producing either infection or immunity.

In the course of our experiments, we observed a fact with a doubtful explanation: the appearance of an infection by Yersin's bacillus in two guinea-pigs, one of which had been bitten by fleas infected with pseudotuberculosis bacilli and the other inoculated with these same fleas. We believe it to be interesting to publish the observation with all its details.

On 27 November 1943, rabbit 10/80 receives, in the marginal vein of the left ear, 2 cubic centimeters of a dilution of pseudotuberculosis bacillus culture, representing about ten billion germs. The strain was

isolated in Casablanca from a rabbit that displayed typhic lesions of pseudotuberculosis, has all the biochemical characteristics of Malassez's bacillus and causes a typical pseudotuberculosis infection in guinea-pigs. Let us add that this same strain was used in other infection experiments by fleas and that its specific characteristics have remained unchanged for the year that we have been preserving it.

Rabbit 10/80 received the ten billion germs at ten o'clock. A quarter of an hour later, two hundred *X. cheopis* breeding fleas that have never bitten are put on the rabbit to gorge. In accordance with the method that we have presented, the fleas bite through the piece of sifting silk that closes the Borrel tube in which they have been placed. The operation lasts one half hour, after which it is easy to observe with a binocular magnifier that the fleas are all well distended. Then a hemoculture is performed by drawing heart blood and another one by seeding one drop of blood taken from the rabbit's right ear, the ear opposite the one that received the culture.

Both hemocultures are positive and yield many colonies on the surface of the agar. Finally, ten fleas that have just bitten are crushed in several drops of bouillon. Seeding the dilution on slant agar yields about one hundred colonies of a germ identified as the pseudotuberculosis bacillus. Therefore, circulation of the inoculated germs and passage in the digestive tube of the fleas have occurred. These fleas are kept fasting for 24 hours, and are then put to feed on a fresh guinea-pig, which does not become infected. The last day, that is, 1 December, ten fleas are taken from the tube and crushed in 20 drops of bouillon. One tube is seeded with a loop and another one with the rest of the dilution. This second tube yields a surface culture, the first one produces about five hundred colonies. The microbe identified is the pseudotuberculosis bacillus. Blood is drawn four times during four days by heart puncture from the bitten guinea-pig; the culture is negative. After a day of fasting, the fleas are put back to feed on another guinea-pig for four consecutive days. On the last day, hemoculture of the guinea-pig and culture (pylloculture) of ten crushed fleas, as before. This time still the same result: numerous colonies of pseudotuberculosis bacilli. Negative hemoculture. Again, one day of fast followed by three days of feeding, on a fresh guinea-pig. Same operations as usual. Negative hemoculture, positive pylloculture. Once again the same operations are performed: pylloculture made on 18 December yields a bacillus that is confirmed as having all the characteristics of Malassez's bacillus and which, when it is inoculated in the guinea-pig, gives it a typical pseudotuberculosis infection. But a new fact appears: the guinea-pig (95/47) is bitten on 16, 17 and 18 December, that is, on the 20th, 21st and 22nd days of the experiment, and becomes infected. Its observation will be given below.

When the fever appears, the hundred surviving fleas are crushed

and inoculated in a fresh guinea-pig (10/95), which becomes infected like the preceding one. A culture of the inoculated product yields a pseudo type bacillus, but, since the colonies are not pure, a precise identification, unfortunately, is not made.

In order to isolate the germs responsible for infecting the two guinea-pigs, under the best possible conditions, in order to avoid enabling the microbes to pass into the blood during the agonal period, the two guinea-pigs are sacrificed before the final period of the disease. The following are their observations: guinea-pig 95/47, bitten on 16, 17 and 18 December by the fleas in tube 195, that is, on the 20th, 21st and 22nd days of the experiment. After three days of incubation without fever, the temperature rises, on the following days, to 40.4 C., 40.7 C., 41.3 C., drops back to 40 C, then to 39.6 C. on the tenth day (Fig V), the date on which the animals is sacrificed. On autopsy, the presence of a very large inguinal ganglion, the size of a small nut, located to the left, that is to say, on the side where the tube containing the fleas was applied on the central [Sic; obviously should read "Ventral"] surface, is observed. When it is sectioned, the ganglion shows an alveolar structure; there is a little pus in the crypts. The spleen is very swollen, weighs 3.50 g., is filled with abscesses. The liver is interspersed with very many small abscesses. Lungs without notable lesions. Kidneys swollen and white, suprarenals swollen and pale. The left lumbar ganglion, the size of a bean, is purulent. The spleen smears show numerous short bacilli with bipolar staining; ganglion smears display countless bacilli of the same type. Cultures of the heart blood, of the spleen and of the inguinal ganglion are positive. The isolated germ has all the characteristics of the plague bacillus, including agglutinability by an antiplague serum. When it is inoculated in a guinea-pig, it kills it with all the signs of the plague. It is pathogenic for the rat (refractory to the pseudotuberculosis bacillus).

Guinea-pig 10/95, inoculated intraperitoneally with the suspension of the hundred Xenopsylla cheopis, comes down with an illness that has a more rapid evolution. Inoculated on 20 December, it has 40.4 C. on the 22nd, 41 C. on the 23rd and the 24th, the fifth day, or 25 December, its temperature is 40 C. (Fig. VI). A very swollen inguinal ganglion can be felt on the left. The animal is sacrificed that same day. At the autopsy, the presence is noted of a subcutaneous abscess at the level of the inoculation and a swollen left inguinal ganglion, abscessed, the size of a walnut. The left lumbar ganglion is swollen, the size of a bean and is purulent. No pus in the peritoneum, no abscess. The spleen is a bit swollen and granulous, without coating; the liver, the lungs, the kidneys appear to be normal.

Very numerous short bacilli with bipolar staining are seen on a smear of the spleen, the inguinal ganglion and of the lumbar ganglion.

Blood and spleen cultures yield, in a state of purity, in very numerous colonies, an immobile bacillus identified as Yersin's bacillus. Inoculation of the guinea-pig causes an experimental plague.

Strain 95/47 was transferred to numerous guinea-pigs. It served to infect fleas (*X. cheopis*) on which the strain is kept. The bite of these fleas actually kills the guinea-pigs with plague, within sixty hours on an average.

On the other hand, strain 195, isolated on 18 December from fleas after their last feeding on guinea-pig 95/47, behaved after its isolation like a pseudotuberculosis bacillus, both because of its biochemical characteristics and its pathogenic ability.

When it was used for a flea infection experiment, using the rabbit method, it was followed for 37 days. None of the guinea-pigs bitten was infected. The psyllocultures always showed Malassez's bacillus. Finally, on the thirty-seventh day of the experiment, 250 live fleas and 20 dead fleas are crushed and all put in suspension in 2 cc. of physiological water and injected, intraperitoneally, in a guinea-pig that dies of pseudotuberculosis five days later. Culture of the organs yields a pseudotuberculosis bacillus.

How are these facts explained?

Let us reject immediately an explanation that comes to mind: that the pseudotuberculosis strain isolated from the rabbit and that was used in the experiment with tube 195 was, in reality, a more or less attenuated plague strain. That is not at all the case. When this strain, still preserved in our laboratory, was transferred several times by guinea-pigs, it showed itself to be virulent for these animals to which it gives a disease that has all the characteristics of pseudotuberculosis, including the completely typical aspect offered by the germs on spleen or liver smears and it retains all the bacteriological characteristics of a pseudotuberculous bacillus.

We have left a choice between two other explanations.

Summing up the facts, it is seen that everything occurred as if Malassez's bacillus had undergone a mutation in the digestive tube of the fleas, transforming it into Yersin's bacillus. Aside from the fact that such a mutation appears difficult to accept a priori and that in fact it has never been observed until now [See Note], it probably would not explain the case of guinea-pig 95/47, if this hypothesis were accepted. This guinea-pig is bitten on 16, 17 and 18 December by fleas recognized as infected with pseudotuberculous bacilli. On 25 December, it dies of plague, but the psylloculture made on 18 December, after the three biting sessions, still gives Malassez's bacillus; therefore, the mutation probably occurred in the guinea-pig and not in the flea and these bacilli,

transformed into plague bacilli, were probably the ones that infected the fleas which, in their turn, gave the plague to guinea-pig 10/95 in which they were inoculated. It would be vain to make such a hypothesis, because no fact of the same order has come up in support of it. And this second explanation must be given up, just like the first one. One final explanation remains: the last guinea-pig bitten by the fleas, guinea-pig 95/47, was in a condition of latent infection with plague. This explanation, although it is not very satisfactory, must still be accepted, for at the time when we were performing our experiments on pseudotuberculosis, we did not have any experiment on plague under way, nor did we have any infected fleas, guinea-pigs or rats.

([Note:] The only mutations pointed out, as far as we know, between the two bacilli were done so by A. Bessonova, G. Leriskaya, P. Molodtsova and O. Mossolova: "Some Cases of Spontaneous Transmutation of B. pestis into B. pseudotuberculosis rodentium," J. Parasit. Epid. Microb., 15, 1936, p 151. These authors observed, out of 214 strains of plague bacilli, one mutation into pseudotuberculosis in five of them, three of human origin and two from rodents.)

It must indeed be admitted, nevertheless, since the appearance of Yersin's bacillus occurred in a sealed tube on fleas that had bitten only non-inoculated guinea-pigs on which they had fed, that one of the guinea-pigs infected them, in this case the last guinea-pig, 95/47.

It must be admitted that this guinea-pig was infected with plague in a latent form at the time when it was bitten by fleas, that the bites transformed this latent infection into acute infection starting at the region injured by the bites, hence the appearance of the left inguinal bubo. It must be admitted that, in the course of this evolution, a certain number of fleas became infected and that these are the ones that gave the plague to guinea-pig 10/95 when they were inoculated in it. For the moment, we are obliged to retain this explanation as the only one admissible, even though it is not very satisfactory. It is rather curious that a similar spontaneous infection of a guinea-pig has never been observed in our laboratory and that chance made this first spontaneous infection appear during experiments performed with the pseudotuberculosis bacillus.

#### SALMONELLOSIS BACILLI

A small number of observations can be picked up in medical bibliography concerning the presence of Salmonella in hematophagous arthropods. In 1937, C. H. Huang, H. C. Chang and V. T. Lien (Chinese Med. Journ., 52, 1937, p 345) observed a small epidemic with Salmonella enteritidis in Peiping. Several of their patients were also stricken with recurrent fever. The authors isolated Salmonella enteritidis by examining lice taken from these patients. In 1940, R. R. Parker and E. A. Steinhaus

(Public Health Reports, 58, 1943, p 1010) observed an infection with S. enteritidis in some guinea-pigs used for transferring spotted fever. They ascertained that the Dermapteron andersoni that bit these guinea-pigs became infected and were able to preserve the Salmonella for at least 35 days. There could have been infection of the eggs and the presence of bacilli in the excrements. In 1942, A. Messerlin and G. Couzi (Bull. Inst. Hyg. Maroc, 2, 1942, p 15) observed an infantile epidemic with Salmonella suispestifer in the douar of the Oulad ben Azzouz (Gharb, Morocco) and, under the supposition that the infection came perhaps from a breeding center where there was an epizootic of hog cholera by means of biting insects, they conducted a series of experiments that enables them to observe that anopheles, human fleas, swine lice, may be infected. After several months, the authors were able to find the bacillus on swine lice in the cadavers that were preserved in the laboratory without special precautions. Finally, Van Oye (according to Presse Medicale, 25 September 1943) succeeded in infecting bugs by making them bite mice infected with an avian Salmonella. These bugs remained infected for about one month and the bacilli were found in the excrements. Transmission to mice by biting is possible during the two weeks that follow the infecting feeding.

Our experimental research bore on two species of Salmonella: S. suispestifer and S. schottmulleri, the two strains that were isolated from man. The precise determination of the two species was made by studying the biochemical characteristics and by means of the cross agglutination method accomplished with agglutinant serums prepared in our laboratory and with others coming from official laboratories. We completed the diagnoses by means of a comparative study of the glucidolipidic antigens (G. Blanc and B. Delage, Maroc Medical, 243, 1944, p 71).

#### SALMONELLA SUIPESTIFER

The strain that we utilized was isolated by Couzi from the blood of a child during the epidemic of which we spoke above.

The experiments bore primarily on the infection of fleas. Some attempts to infect Aedes aegypti gave a positive result.

#### Infection of Fleas

One or several infected guinea-pigs are placed in succession in a vat containing thousands of fresh fleas that have never bitten, after which fresh guinea-pigs are put in and taken out before the appearance of fever and are observed after having taken care to free them completely of any fleas that might have remained on them. Another method consists of putting a certain number of fresh fleas in a Borrel tube whose

cnds are closed with a piece of sifting silk. The fleas contained in the tube are applied to bite through the sifting silk first infected guinea-pigs, then fresh guinea-pigs. In the interval between biting sessions, the tube is placed on a Borrel tube cover where it is easy to collect the fleas' excrements. In order to collect a large amount of excrements, a white rat is placed in a vat containing infected fleas and the rat is sacrificed, dying as a result of bites after 24 hours. This rat, whose fur has become black filled with excrements, provides a considerable amount of excrements (about 1 gr.). In order to collect these excrements, the hairs are pulled out, dried in a vacuum, sifted and the powder that is collected is kept in phials sealed under vacuum.

#### Infection of Stegomyias

About one hundred Aedes aegypti are put in a cage, in the dark, in a very humid atmosphere.

An earthenware container, filled with water and closed with hydrophilous cotton, is suspended inside the cage. The rough, damp surface of the container permits Aedes to be layed and the constant evaporation of the water ensures a suitable hygrometric condition. Several infected guinea-pigs, secured on their back and their ventral wall depilated, are placed successively in each cage. Each biting session lasts about one hour; there is one in the morning and one in the afternoon. Fresh guinea-pigs follow the infected guinea-pigs under the same experimental conditions.

#### Results Obtained

The fleas, Xenopsylla cheopis, become infected on the guinea-pig. They remain infected for at least forty days. The Salmonella can be isolated both from the fleas and from their excrements.

The fresh guinea-pigs bitten by these fleas may become infected.

Some experiments made with Stegomyias enabled us, in one case, to find S. suispestifer in an insect ten days after the last infecting feeding and to produce once infection of the guinea-pig by biting.

The following are, by way of example, the record of two experiments:

1. Vat with Xenopsylla. Vat 185:

2 September 1943: Several thousand fleas (X. cheopis) that have just hatched and that have never bitten are put in a vat.

On the same day, a guinea-pig, 10/11, that has just been inoculated is put in the vat. It received intraperitoneally 4 cc. of a suspension of the spleen of a guinea-pig infected with S. suispestifer (Couzi strain). Guinea-pig 10/11 dies on 8 September. The hemoculture is positive.

- 9 September: Another guinea-pig, 10/21, inoculated intramuscularly with a culture of the same strain of S. suispestifer, is put in vat 185. It is found dead on 13 September. The hemoculture is positive.
- 13 September: A first sounding is taken to ascertain if the fleas that have bitten guinea-pigs 10/11 and 10/21 have become infected. One hundred fifty are withdrawn from the vat and crushed, put in suspension in four cubic centimeters of physiological water and inoculated intraperitoneally in guinea-pig 10/24. This one dies on 21 September. The spleen is enormous, friable, black with a thick coating. The hemoculture and the splenoculture are positive.
- 15 September: Another guinea-pig, 10/25, inoculated with the suspension of spleen from guinea-pig 10/21, is put in the vat. It dies on 21 September. The hemoculture is positive.
- 21 September: A fourth guinea-pig, 10/14, inoculated the evening before intramuscularly with a culture of S. suispestifer, is put in vat 185. It remains there until the next day. Found dead on 22 September, the hemoculture is positive.
- 23 September: One hundred fleas are taken from the vat, crushed, put in suspension in 3 cc. of physiological water. It is inoculated intraperitoneally in guinea-pig 15/16. The animal dies six days later, on 29 September. In the autopsy it is noted that the spleen is very swollen, black, covered with a coating. The left lung is the seat of an intense congestion. There are some infarcts. The suprarenal capsules are very swollen and are purplish in color. The blood and spleen clutures yield S. suispestifer. When the fleas in the vat have been duly infected, fresh guinea-pigs are put in to be left only a few days.
- 24 September: Guinea-pig 95/16 is put in the vat until 2 December. It will become infected but will survive infection that is revealed by fever and by a positive hemoculture with S. suispestifer (Fig. VIII).
- 2 October: Another fresh guinea-pig, 95/20, replaces it. It is left in the vat for seventeen days without showing the slightest febrile reaction. When it is removed and punctured for a hemoculture, it immediately shows a rise in temperature above 40.2 C. It will

die on 29 December. The spleen is slightly increased in volume. A pleuropericarditis with intense pulmonary congestion is observed. The blood and spleen cultures give S. suispestifer. The acute infection seems to have been touched off by the traumatism of the heart puncture (curve VII).

12 October: One hundred fleas are washed, crushed in 6 cc. of physiological water. The whole is inoculated in the peritoneal cavity of guinea-pig 95/24 which will die on 18 October. Twenty other fleas are inoculated in the same way in another guinea-pig, 95/25, which will also die of S. suispestifer infection on 20 October (curve XI).

19 October: Another guinea-pig is put in the vat where it is left until 2 November. It incurs a febrile infection and dies on 9 November. Death is probably due to the heart puncture. The macroscopic lesions are little pronounced. The spleen has increased in volume, without coating; examination of the smears does not show any bacilli. The blood and spleen cultures yield S. suispestifer.

2 November: The experiment is stopped. The hundred fleas remaining in the vat are crushed, 3 cc. of physiological water added; the whole is inoculated in the peritoneal cavity of guinea-pig 10/64 after having seeded a few drops of the suspension. This culture yields numerous colonies of S. suispestifer. The guinea-pig dies on 10 November (Fig. X). In the autopsy a very swollen, black colored spleen with coating is found; the liver appears normal. The adrenal glands are swollen and purplish. An intense congestion of the upper, median and lower lobes of the right lung.

The blood culture and the spleen culture are positive. S. suispestifer is isolated.

It may be concluded from this experiment that lasted two months that X. cheopis fleas become infected by biting guinea-pigs themselves infected with S. suispestifer. The infection was maintained from 23 September until 2 November, or forty days. Fresh guinea-pigs bitten by fleas became infected. S. suispestifer was isolated from the guinea-pigs, the fleas and their excrements.

## 2. Case with Steromyias.

19 July: An infected guinea-pig is put in a cage containing about three hundred A. aegypti, captured in nature.

20 July: Another exposure to bites in the cage. The guinea-pig is dying.

22 July: Another infected guinea-pig is put in the cage.

23 and 24 July: The same guinea-pig is subjected to *Stegomyias* bites twice a day.

26 July: A fresh guinea-pig, A.69, is put in the cage in the morning and then isolated in the afternoon. Its temperature is taken every day. No fever appears at any time. It dies on 30 August. In the autopsy, few signs; and inguinal ganglions, the size of a grain of wheat, are menorrhagic. The spleen is swollen, dark in color; there is little peritoneal liquid. The hemoculture and spleno-culture yield *S. suipestifer*. A liver fragment is crushed in 10 cc. of physiological water; 3 cc. of this suspension are inoculated intraperitoneally in a guinea-pig that will die of infection by *S. suipestifer*. Other transfers, also positive, are continued.

From 27 to 30 July: Another guinea-pig, A.70, is put in the cage. Then guinea-pig A.80 replaces it from 2 to 4 October. Neither one will become infected.

29 July: Ten crushed *Stegomyias* are inoculated in a guinea-pig that does not become infected.

5 August: Ten live *Stegomyias* remain in the cage. They are crushed in 1 cc. of physiological water and inoculated in guinea-pig A.83. This one will not become infected.

The point to remember from this experiment is the possibility for *Stegomyias* to become infected and to transmit the infection by biting. It seems that the infection is less strong than the flea infection and that few insects become infected, because, in this experiment, we did not find *S. suipestifer* in the crushed *Stegomyias*. On the other hand, in another experiment, it was possible to isolate the *S. suipestifer* strain in *Stegomyias* ten days after their last infecting feeding.

#### Experimental Disease in Guinea-Pigs

Guinea-pigs that were inoculated in the peritoneum with crushed fleas incur a short-lasting and always mortal infection. Fever appears from the 3rd to the 5th day, the temperature rises above 41 C., remains on a plateau, and defervescence, most frequently very abrupt, announces death which occurs from five to ten days after inoculation. The temperature curve is comparable to the one with the transfer animals (Figs. II, X, XI). The lesions observed in the autopsy are acute infection, black, very swollen spleen, swollen, purplish adrenal glands. Hemoculture and splenoculture are always positive, but the bacilli on spleen smears are relatively few in number, sometimes very sparse. The pullulation of germs that is so pronounced in plague is never observed.

The guinea-pigs bitten by the infected fleas react differently. The illness is of the subacute type. Incubation for about ten days, apparent incubation, period preceding the fever, but, well before its appearance, it is possible to isolate S. suispestifer from the organs and even from the blood. The fever curve, in case of death of the animal, does not differ from the one described for animals infected by inoculation of crushed fleas and the lesions found at the autopsy are identical. Therefore, there is no reason for speaking properly of a clinical form, chronic in type, but merely an extension of incubation. The illness breaks out later but it is as acute. Sometimes the animal recovers from its infection. We observed a guinea-pig, put in a vat to be bitten for eight days, that incurred a long pyretic form, more than ten days, and that, nevertheless, survived. A heart puncture made on the fifth day of the fever enables us to isolate S. suispestifer.

Finally, there is another form that seems indeed, to be found: the inapparent form. A real blood infection without symptoms and especially without fever. But the animal that is infected in this way is in a state of unstable physiological equilibrium, and even an unimportant traumatism immediately transforms this inapparent infection into febrile, acute, mortal illness. In one case, for example, we have a guinea-pig that is kept in a vat of hyperinfected fleas for seventeen days. Its temperature is taken regularly and is completely normal and the animal, in spite of the anemizing bites, is in perfect condition. It is taken out of the vat, freed of its fleas. Its temperature is 39.2 C., heart puncture and hemoculture is positive but very poor. Only one tube of two produced a few colonies of S. suispestifer. In the evening, the temperature is 40 C. It remains on a plateau between 40 C. and 41 C. for nine days. The animal dies on the tenth day. The spleen has increased little in volume. Bacilli are found there only with culture but not with direct examination. There is an intense pulmonary congestion with pericarditis, lesions caused by the puncture and point of departure for generalization of the infection.

In another case, a guinea-pig, taken from the vat under the same conditions, after twelve days, without having displayed the slightest sign of infection, is bitten by non-infected fleas for the purpose of isolating S. suispestifer, by the very sensitive method of xenodiagnosis; the animal is kept twice a day for twenty minutes spread out on its back with a tube containing about three hundred fleas applied to its abdominal wall. Shortly after this ordeal, its temperature rises and the animal dies from the results of a heart puncture. Discrete lesions in the autopsy. No bacilli on direct examination of spleen smears. Blood and spleen cultures positive.

#### Virulence of Flea Excrements

The Salmonella suispestifer ingested by the fleas are not only

preserved in the digestive tube of the insect but they multiply there and are eliminated with the feces in great quantity. The culture of a small amount of excrements yields numerous colonies on solid media. The preservation of the S. suipestifer in the dry excrements is determined to have a considerable duration with maintenance of virulence. At present, after exactly one year, this preservation seems to be undiminished, since cultures of excrements still yield a considerable number of quasi-confluent colonies, even with one twentieth of a milligram.

#### Summary

The following facts may be retained from these first studies on the behavior of S. suipestifer in biting insects, and especially in a rat flea, Xenopsylla cheopis:

1. A flea that bites a guinea-pig infected with S. suipestifer becomes infected itself. The ingested bacilli multiply in the digestive tube of the insect, passing into the excrements where they can be preserved dry for a considerable time.

2. An infected flea that bites a healthy guinea-pig may give it the infection.

3. The guinea-pigs infected by flea bites come down with a febrile illness, usually mortal. They may also incur an infection without symptoms and particularly without febrile reaction. This infection is accompanied by septicemia. Some animals stricken by this inapparent salmonellosis are in an unstable physiological equilibrium, and even a slight traumatism may be sufficient to transform this inapparent infection into an acute type of infection.

It seems possible to draw some suggestions from these facts:

A. Infections with S. suipestifer, in particular hog cholera, may probably be propagated by hematophagous insects.

B. The very long preservation in the virulent state of S. suipestifer in the excrements of hematophagous insects may probably constitute an important factor in the preservation and the transmission of this salmonellosis.

C. The existence of inapparent and fragile forces of salmonellosis in laboratory animals is perhaps not peculiar to them and their existence in domestic animals and even in man must be considered.

#### SALMONELLA SCHOTTMULLERI (Paratyphi B)

The results obtained with the Salmonella of hog cholera led us to

study the behavior of the paratyphic B bacillus in man, *S. schottmulleri*, in the same hematophagous insect, the rat flea *Xenopsylla cheopis*. The precise determination of the strain used (strain isolated from human blood by Doctor Cousi in Rabat) was made as before by examining its biochemical characteristics on culture media, by agglutination with various serums and by determining the presence of the specific glucidolipidic antigen (G. Blanc and B. Delage, *loc. cit.*). The paratyphoid B strain, that is very little pathogenic for guinea-pigs, did not enable us to use the rat method utilized for studying *S. guineastifer*. We used the method, also described here, of the transitory bacteremia of the rabbit that makes it possible for fleas to become infected easily. An experiment report follows;

On 16 December 1943, a rabbit receives 4 cc. of a suspension of paratyphic B bacilli, 3 billion germs per cubic centimeter, in the marginal vein of the right ear. The inoculation is administered at 10 o'clock. At 1030, a Borrel tube containing 240 *Xenopsylla cheopis* is applied on the depilated ventral surface of the animal which is secured, as previously, in a dorsal position. The fleas have hatched recently and have never bitten. They gorge themselves greedily. At 1030 [sic], the tube is removed. Examination with a binocular magnifier enables us to verify that most of the fleas have bitten. Five minutes later, at 1035 [sic], culture of several drops of blood drawn from the rabbit's left ear, and hemoculture of 2 cc. of blood drawn by heart puncture; at the same time, ten fleas are crushed in twenty drops of bouillon. Both hemocultures will be positive, and the few drops of blood will yield confluent colonies on slant agar. The psyiloculture will also be positive. A loopful of the suspension seeded on agar yields 13 colonies identified, just like the ones originating in the hemocultures, as being of a paratyphic B bacillus. 17 December: The fleas are put to bite on a fresh guinea-pig, then ten are crushed, with the addition of 20 drops of bouillon. This second psyiloculture is positive; one loopful of the suspension yields five colonies.

18 December: The fleas are left fasting.

19, 20 and 21 December: They bite the guinea-pig.

21 December: Third psyiloculture, made as before with ten fleas. One loopful yields ten colonies of paratyphic B.

22 December: Fleas fasting.

23, 24 and 25 December: Biting the guinea-pig.

25 December: Fourth psyiloculture, producing 20 colonies per loopful.

26 December: Fleas fasting.

- 27, 28 and 29 December: Biting the guinea-pig.
- 29 December: Fifth psyiloculture, yielding one single colony per loopful.
- 30 December: Fleas fasting.
- 31 December, 1 and 2 January: Biting the guinea-pig.
- 2 January: Sixth psyiloculture, producing six colonies per loopful. One hemoculture of the guinea-pig that has been bitten since 17 December is negative.
- 3 January: Fleas fasting.
- 4 January: One milligram of excrement from the fleas is taken from the Borrel tube and diluted in one cc. of bouillon. A seeding is made in an tube of agar with one loopful of the dilution. The culture yields a great number of colonies of paratyphic B bacillus.
- 4, 5 and 6 January: Biting on a fresh guinea-pig. Seventh psyiloculture. Also positive, but this time the tube seeded with one loopful of the dilution produces a great number of colonies.
- 7 January: Fleas fasting.
- 8, 9 and 10 January: Biting the guinea-pig.
- 10 January: Eighth psyiloculture, yielding very many colonies, like the preceding one.
- 11 January: Fleas fasting.
- 12, 13 and 14 January: Biting the guinea-pig.
- 14 January: Ninth psyiloculture, yielding, like the two preceding ones, a great number of colonies.
- 15 January: Fleas fasting.
- 16, 17, 18 and 19 January: Biting the guinea-pig.
- 19 January: Tenth psyiloculture. Still very rich.
- 20 January: Fleas fasting.
- 21 to 27 January: Biting the guinea-pig, interrupted by one day of fasting.

27 January: Eleventh psyilloculture, producing only a few colonies.

28 January: Fleas fasting.

29, 30 and 31 January: Biting the guinea-pig.

31 January: Twelfth psyilloculture, as poor as the preceding one; there are only a few colonies, still of paratyphic B.

Starting with 31 January, the forty-seventh day of the experiment, four more psyillocultures, all negative, are made, the last one with forty fleas still alive (there are fifty dead fleas), made on 16 February.

#### Summary

The following facts can be retained for the present from these first studies on the behavior of S. schottmulleri (paratyphic B bacillus) on the rat flea Xenopsylla cheopis:

1. A flea that bites an animal (rabbit) in a state of intense bacteremia becomes infected.
2. The infected flea may be host to bacilli for at least forty-seven days.
3. The bacilli ingested by the flea multiply in its digestive tube and pass out in the excrements.

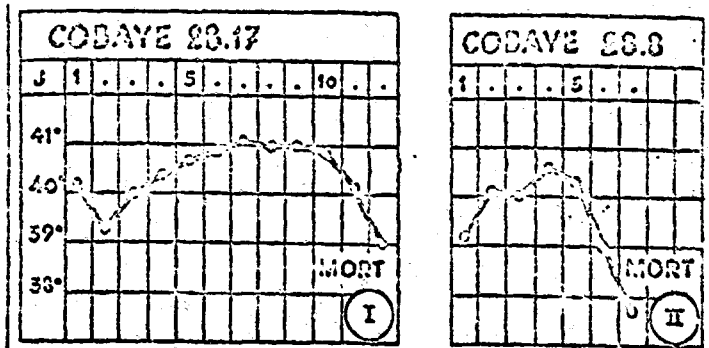
Other facts require additional research for their interpretation. Everything seems to take place as if, at a given moment (psyillocultures 7, 8, 9 and 10), there was an intense multiplication of the bacilli followed by a cessation and by the disappearance of the germs. The objection to this is that, as the experiment progressed, there is a decrease in the number of fleas and that if they have not all been infected the number of fleas carrying germs is decreasing. A conclusion can be drawn only by repeating the experiments.

The guinea-pigs bitten by the fleas were not infected and the hemoculture remained negative. No interpretation can be given to this either. The paratyphic B bacillus is too little virulent to infect the guinea-pig, even though it could possibly be transmitted by biting. We have demonstrated elsewhere, in connection with the pyocyanic bacillus, that although the flea bite may never be infectious it was possible to find the germ in the skin of the bitten guinea-pig; perhaps the same is true of the paratyphic B bacillus.

#### CONCLUSION

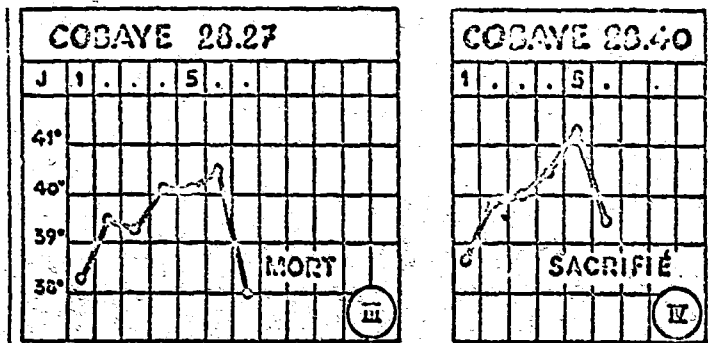
The results obtained with two species of Salmonella, one of them primarily of animal infection, the other one exclusively of human infection, appear to be completely comparable. If they are compared with the facts observed by Parker with S. enteritidis, we are led to extend to the entire group of Salmonella (paratyphic B) the characteristic of being able to develop in the digestive tube of hematophagous arthropods, to pass out in the excrements and to be preserved there for a considerable period of time. These facts will perhaps enable us to consider a active role for hematophagous insects in the transmission and preservation of bacilli of the genus Salmonella.

Pseudotuberculosis by Inoculating Cultures



LEGEND: COBAYE = guinea-pig; J = day of the experiment; MORT = dead;

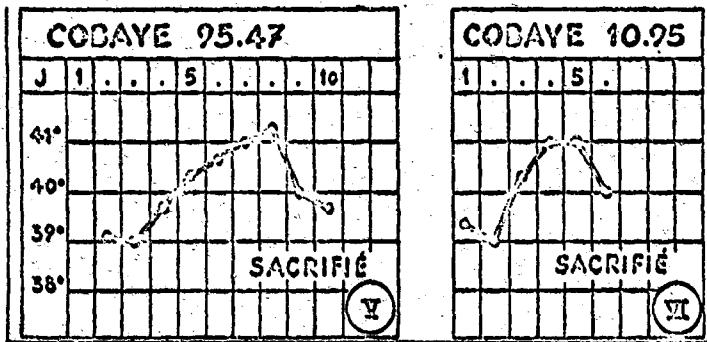
Pseudotuberculosis by Inoculation of Crushed Fleas



LEGEND: COBAYE = guinea-pig; J = day of the experiment; MORT = dead; SACRIFIÉ = sacrificed

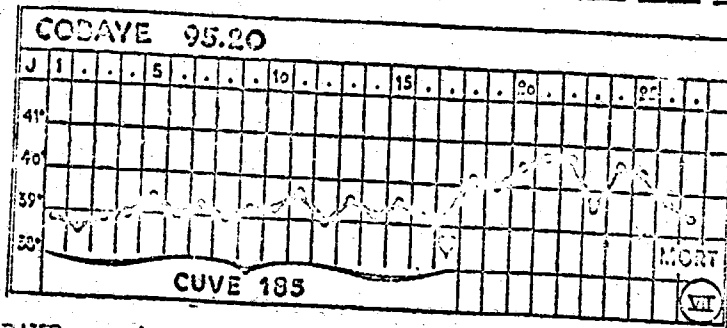
Plague by Flea Bites  
(Tube 195)

Plague by Inoculation  
of Fleas (Tube 195)



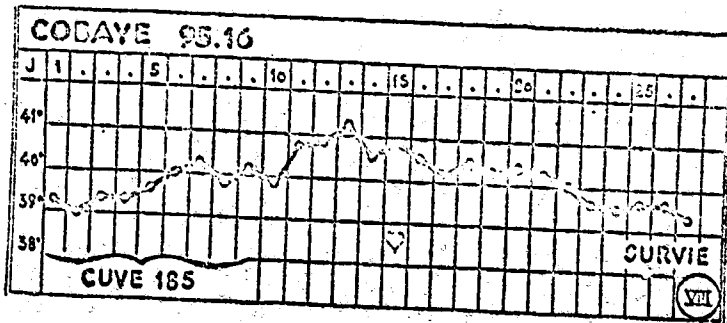
LEGEND: COBAYE = guinea-pig; J = day of the experiment; SACRIFIÉ = sacrificed

Salmonellosis Due to S. pestifer by Infected Flea Bites



LEGEND: COBAYE = guinea-pig; J = day of the experiment; CUVE = vat; MORT = dead

Salmonellosis Due to S. pestifer by Infected Flea Bites

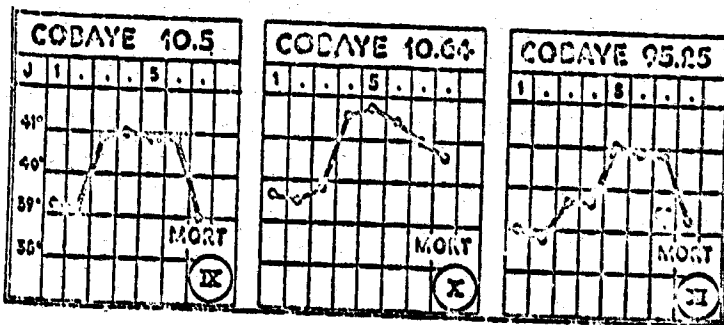


LEGEND: COBAYE = guinea-pig; J = day of the experiment; CUVE = vat; SURVIE = survives

Salmonellosis  
Due to S. p.,  
Spleen Trans-  
fer, Peritoneum

Salmonellosis  
Due to S. p.,  
Inoculation  
100 Fleas,  
Peritoneum

Salmonellosis  
Due to S. p.,  
Inoculation  
20 Fleas, Pe-  
ritoneum



LEGEND: COBAYE = guinea-pig; J = day of the experiment; MORT = dead

- END -

- 25 -