STUDIES ON THE SENSITIZING PROPERTIES OF THE BACTERIOPHAGE.

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INTRODUCTION.

It is surprising that among the numerous investigations on the properties of the bacteriophage no systematic inquiry has been made to determine whether the bacteriophage is capable of sensitizing the tissues of experimental animals, a property which we commonly associate with the antibody-producing function of more complex antigenic substances. The few suggestive observations made do not serve to cast any definite light on this problem. Thus, d'Herelle (1) states, in a brief note, that repeated injections of rabbits and mice with Shiga bacteriophage may sensitize these animals to the Shiga toxin, a condition for which he has coined the term "antiphylaxis." He claims that a form of hypersensitiveness, passive in character, may also be obtained by injecting animals with mixtures of sublethal doses of Shiga toxin and specific antiphage serum. Otto and Munter (2) have not been able to observe the regular occurrence of this phenomenon. Similar experiments by Gratia (3) and by Hauduroy (4) with staphylococcus bacteriophage seem to indicate that prolonged immunization with this phage may not only cause emaciation and premature death of some animals, but that the surviving animals show upon reinoculation an increased susceptibility to the same organisms. Although we have occasionally observed slowly progressing emaciation of rabbits during immunization with staphylococcus bacteriophage, we are inclined to question whether one is justified in interpreting any of the above phenomena as manifestations of true or typical anaphylaxis.

In view of the general trend of more recent studies on the antigenic properties of the bacteriophage (Schultz (5)) which suggest that the

antigenic action of the bacteriophage is strictly limited to a capacity of inducing the formation of specific neutralizing antibodies, we felt that it would be of fundamental importance to determine whether or not the bacteriophage is capable of producing a sensitization effect in suitable animals. Some of the contradictory and confusing results obtained in the past with the complement fixation test in studies on the antigenic properties of the bacteriophage and the ultraviruses, generally, appear to be clearly traceable to unspecific factors, not always fully recognized in the evaluation of the results. The anaphylactic reaction, on the other hand, is characterized not only by an extraordinary degree of specificity but also sensitivity, so that results secured with this procedure would seem to carry definitely more weight than serological reactions carried out *in vitro*.

EXPERIMENTAL WORK.

Preliminary attempts to elicit in guinea pigs, actively sensitized to various bacteriophages, typical symptoms of anaphylactic nature by intravenous reinjection of the same antigen, had to be abandoned, because it appeared definitely impossible to produce either fatal shock or sufficiently pronounced symptoms by this method. We, therefore, decided to make use of the more delicate reactivity of the isolated uterine muscle as it may be measured by the Schultz-Dale method. The procedure of passive anaphylaxis which has proved so successful in recent studies of Tomcsik and Kurotchkin (6) in determining the sensitizing properties of bacterial carbohydrates, was given a trial. The interval between intraperitoneal injection of the antiserum and the testing of the uterine strip varied in these few preliminary experiments from 24 hours to 5 days. The guinea pigs, however, failed to become sufficiently sensitized, although they had been prepared with large doses of potent antiphage rabbit sera. We have, therefore, primarily confined ourselves to a study of active anaphylaxis, using as test objects the isolated uterine horns of *actively* sensitized young, virgin guinea pigs.

A number of modifications of the original Schultz-Dale technic have been described. We have adopted an outfit recommended by Hanzlik and De Eds (7) which in our opinion greatly facilitates the study of surviving organs. As an

immersion fluid in the bath we used freshly prepared Tyrode solution. Other technical details of the actual test do not require any further description since in every other respect we have followed the customary procedures. In the following lines we have given a description of the methods employed in the preparation of the various reagents and of the procedure of sensitizing the animals.

Preparation of Reagents.—Since many of the positive results reported with the complement fixation and precipitin tests on antiphage sera, credited to the bacteriophage itself, are undoubtedly traceable to bacterial residues in the filtrates, we endeavored in our experiments to eliminate the rôle of these concomitant antigens by employing a bacteriophage capable of lysing two distinct bacterial species. By sensitizing, for example, with phage-lysed Flexner bacilli and testing for anaphylaxis with colon bacilli lysed by the same bacteriophage, it became clear that any reaction which might be elicited could only be attributable to a direct antigenic effect of the bacteriophage itself. In order to further obviate any possible interference by extraneous antigenic substances contained in the culture substrate, we have attempted to grow and lyse our organisms in a synthetic medium devoid of protein. While we succeeded in obtaining good growth and also complete bacteriophage lysis of the colon bacillus, cultivated in Uschinsky's medium, it proved impossible for us to adapt the Flexner bacilli to this artificial medium. We have therefore, in the case of Flexner antigens, varied the medium for sensitization and the anaphylactic test by using Martin's broth in preparing the sensitizing lysates and beef infusion broth for the preparation of the lysate used in the bath. As a further check, a small number of guinea pigs were sensitized against Flexner lysates, which had been trypsinized before use, a method that may be employed to free bacteriophage suspensions from residual bacterial and other proteins (Arnold and Weiss (8)) without injuring the bacteriophage. We have moreover on general principles reduced the presence of unlysed bacterial proteins in our lysates to a minimum by employing a bacteriophage of maximum virulence for both the Flexner and colon bacilli in question. In addition, the lysed cultures were always filtered, shortly after complete lysis had been attained, through a Chamberland L 3 candle, well before the onset of any possible secondary growth of resistant bacterial cells.

Method of Sensitization.—Experiments of Zinsser and his associates (9, 10) have shown with sufficient clarity that, in order to obtain positive results with any degree of regularity in the difficult and much contested field of bacterial anaphylaxis, it is necessary to subject the animals undergoing sensitization to an intensive course of treatment. Our guinea pigs therefore were all given a series of seven subcutaneous injections of the respective antigens administered at 3 day intervals, the doses ranging from 0.5 cc. to 3 cc. Some animals were lost during the course of sensitization. The interval between the last sensitizing injection and the sacrificing of the animal for the test varied between 3 and 5 weeks.

We have run two large series, comprising a total of 62 sensitized animals. In addition, a total of twelve normal guinea pigs was included in order to accurately determine the maximum dose of the different lysates and broth cultures, which

failed to cause irritation of the normal uterine horn. This dose varied with the different preparations from 4 to 8 cc., added to a bath of 50 cc. capacity. An amount well below (one-half to one-fourth of the minimum irritating dose) was used in testing the sensitized uterine strips. It should be mentioned in this connection that it was not possible to add even traces of trypsinized material to the bath without provoking maximum contraction of the normal muscle. On the other hand, the Uschinsky medium caused an immediate marked depression in the tonus of the normal muscle. We have, therefore, refrained from employing either one of the media as a vehicle for any reagents used in the final test.

The complete protocols of the two experimental series are as follows:

Series I.—One group of six guinea pigs was sensitized against phage-lysed colon bacilli, grown in Uschinsky's medium (Phage 1,[1] K-13), another group of six animals against the intact, phage-free colon bacilli (K-13), naturally the identical bacterial strain. Six guinea pigs received sensitizing injections of phage-lysed Flexner bacilli, grown in Martin's broth (Phage 1, I-13), and an equal number was sensitized in the same manner against the intact, phage-free Flexner culture (I-13). Four guinea pigs were sensitized with the Flexner lysate, subjected to thorough trypsinization[2] (Phage 1, I-13 trypsinized), while four more animals were injected with uninoculated Martin's broth and Uschinsky's medium, respectively, for purpose of control.

In testing our guinea pigs for acquired hypersensitiveness, we have experimented with various combinations of sensitized uterine horns and test reagents. As previously outlined, animals sensitized to the two phage lysates (Flexner or colon) were tested not only with the homologous bacteriophage suspensions, but also with the same bacteriophage, but propagated on the heterologous organisms. These same animals were also tested for anaphylaxis with autolysates of the corresponding bacteria, obtained by allowing broth cultures in sealed tubes to remain in the incubator for 3 to 4 weeks. The term autolysate as used here and later in the text is meant to designate phage-free broth cultures, partially cleared by spontaneous bacterial autolysis, and bearing no relationship to the bacteriophage phenomenon. As regards animals sensitized to the intact bacterial cells (Flexner or colon), they were subjected to a test not only with the corresponding bacterial autolysates but also with the homologous phage lysates.

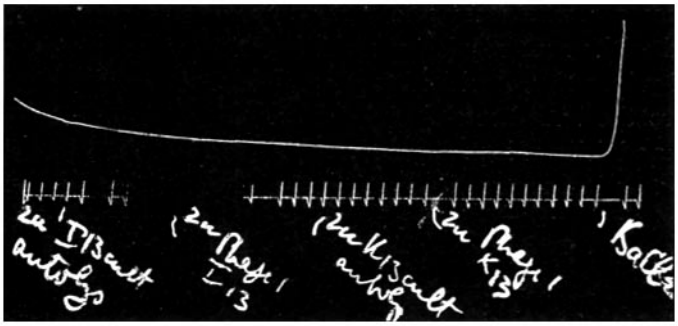
Although not all of the uterine strips reacted in a reliable and clean-cut manner, some failing to respond entirely, we secured uniform and significant tracings with the majority of animals tested. Our

[1] Phage 1 is an antidysentery phage obtained from Dr. d'Herelle and isolated originally by him from the feces of a goose.

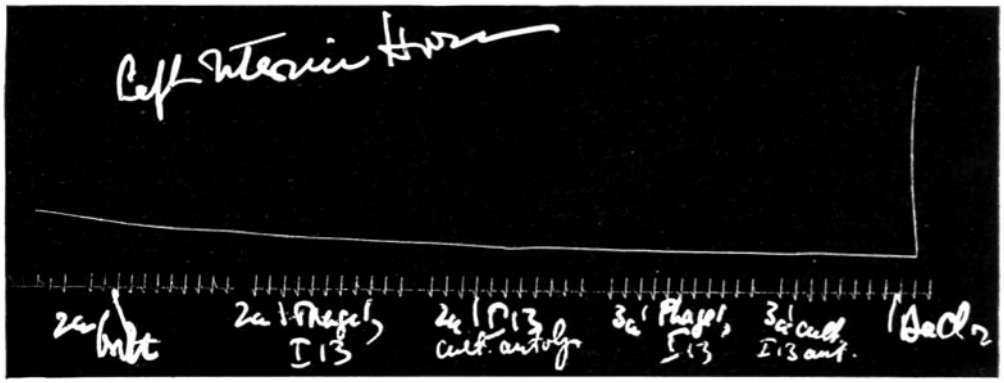
[2] It is common knowledge to students of bacteriophagy that bacteriophages, with certain exceptions (Schultz (11)), resist the action of tryspin.

observations in this series demonstrated very clearly three outstanding phenomena, which may briefly be summarized as follows: First, we obtained with great regularity marked contractions of the uterine strips of guinea pigs, sensitized to phage-lysed bacterial cultures, upon adding to the bath homologous *phage lysates*, while no reaction followed the addition of homologous bacterial *autolysates* to such muscles. Secondly, marked contractions of the uterine horns, taken from animals sensitized to the normal intact bacterial cells, occurred following the addition of the homologous bacterial *autolysates* to the bath, but no such reactions were demonstrable upon adding the corresponding *phage lysates*. These phenomena were equally well marked in both groups, guinea pigs sensitized to the Flexner antigens and those sensitized to the colon antigens. The specificity of these positive reactions is further indicated by the complete and specific desensitization of the sensitized muscle, which in most instances followed immediately after the initial contraction. The third fact, which these experiments brought to light, was the absence of any contraction of phage-sensitized strips, when these were tested against the heterologous phage lysates, *i.e.*, guinea pigs sensitized to Flexner lysates showed no reaction whatsoever with colon lysates, and *vice versa*, Flexner lysates were entirely inactive on strips sensitive to colon lysates, although the bacteriophage in each instance was the same. The control animals, sensitized to the uninoculated Martin and Uschinsky broth, had acquired no demonstrable hypersensitiveness to the respective media. Likewise, strips from animals sensitized to trypsinized Flexner lysates, did not react upon contact with the non-trypsinized Flexner lysates.

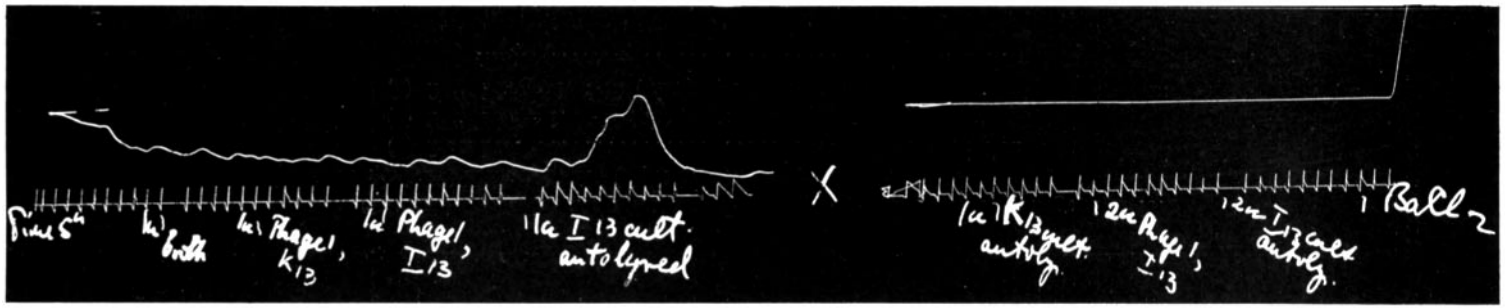
It appears from these experiments that phage-lysed bacteria exhibit antigenic properties quite distinct and different from those belonging to bacteria which have been allowed to undergo disintegration by spontaneous autolysis. In addition, it would seem that the sensitizing effect of a bacteriophage suspension is due exclusively to the dissolved bacterial protein, *altered by phage action*, and not to the bacteriophage itself, because cross-tests, in which the bacteriophage represented the only common antigen, gave consistently negative reactions. It is of interest and seemingly significant that animals, sensitized to trypsinized Flexner lysates, failed to respond when tested



GRAPH 1. Uterine strip from normal guinea pig (right horn).
(*a*) 2 cc. I-13 culture (Flexner autolyzed).....no reaction.
(*b*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli) " "
(*c*) 2 " K-13 culture (*coli* autolyzed)..... " "
(*d*) 2 " Phage 1, K-13 (phage-lysed *coli* bacilli)... " "
(*e*) $BaCl_2$.....maximum contraction.

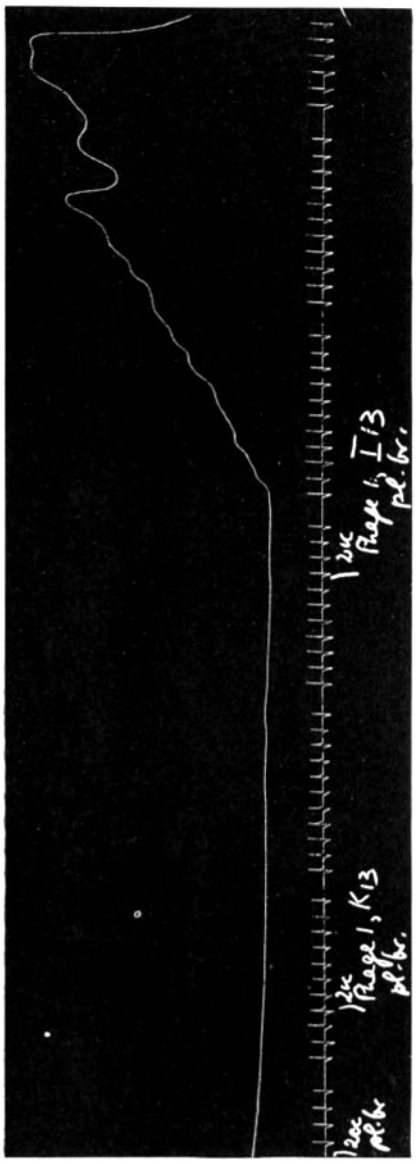


GRAPH 2. Uterine strip from normal guinea pig (left horn).
(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*c*) 2 " I-13 culture (Flexner autolyzed)..... " "
(*d*) 3 " Phage 1, I-13 (phage-lysed Flexner)..... " "
(*e*) 3 " I-13 culture (Flexner autolyzed)..... " "
(*f*) $BaCl_2$.....maximum contraction.



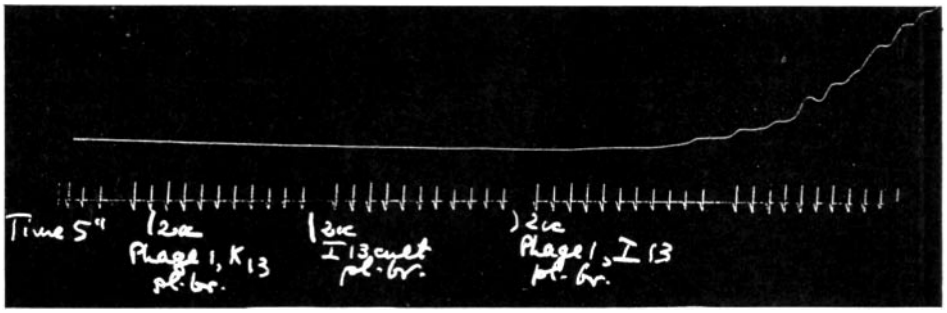
GRAPH 3. Uterine strip from Guinea Pig 16. Sensitized to I-13 culture (Flexner bacilli) (left horn). June 8, 1928.

(*a*) 1 cc. broth.....no reaction.
(*b*) 1 " Phage 1, K-13 (phage-lysed *coli* bacilli).. " "
(*c*) 1 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*d*) 1 " I-13 culture (Flexner, autolyzed).....contraction.
(*e*) Bath changed.
(*f*) 1 cc. K-13 culture (*coli*, autolyzed).....no reaction.
(*g*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*h*) 2 " I-13 culture (Flexner, autolyzed)..... " " *Desensitization.*
(*i*) $BaCl_2$.....maximum contraction.



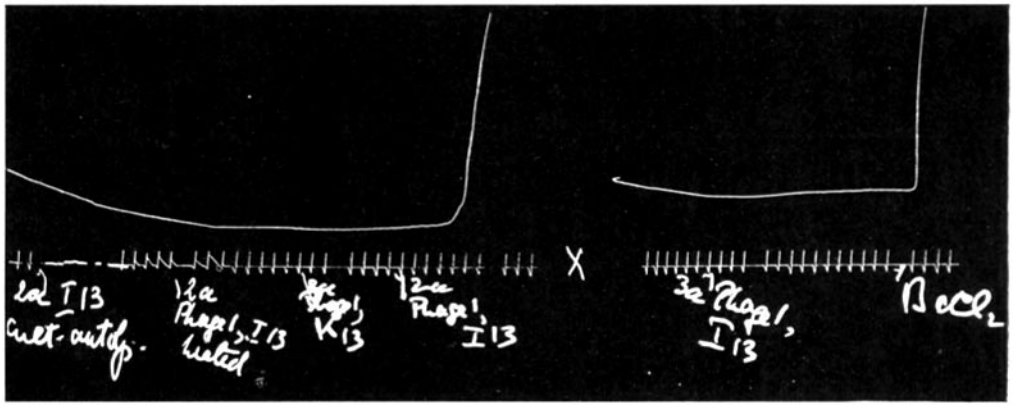
GRAPH 4. Uterine strip from Guinea Pig 8. Sensitized to Phage 1, I-13. Phage-lysed Flexner bacilli (right horn). May 7, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*c*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli).....contraction.



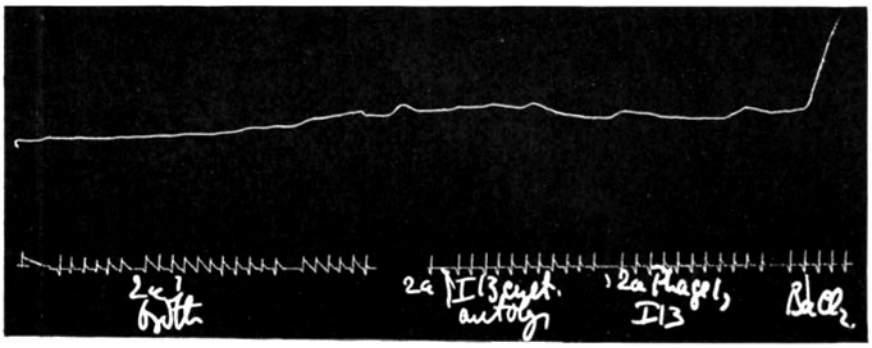
GRAPH 5. Uterine strip from Guinea Pig 8. Sensitized to Phage 1, I-13. Phage-lysed Flexner bacilli (left horn). May 7, 1928.

(*a*) 2 cc. Phage 1, K-13 (phage-lysed colon bacilli).....no reaction.
(*b*) 2 " I-13 culture (Flexner)..... " "
(*c*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli).....contraction.



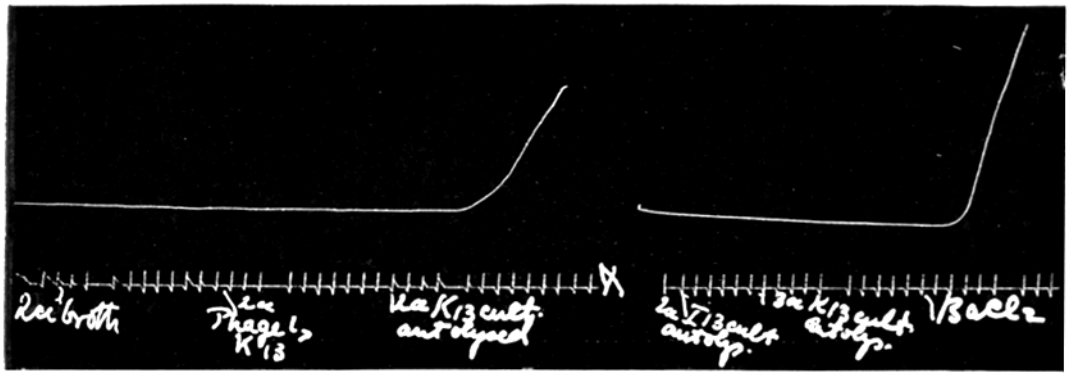
GRAPH. 6. Uterine strip from Guinea Pig 26. Sensitized to Phage 1, I-13. Phage-lysed Flexner bacilli (left horn). July 25, 1928.

(*a*) 2 cc. I-13 culture (Flexner, autolyzed).....no reaction.
(*b*) 2 " Phage 1, I-13 (*heated* phage-lysed Flexner bacilli)..... " "
(*c*) 3 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*d*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli) not heated.....contraction.
(*e*) Bath changed.
(*f*) 3 cc. Phage 1, I-13 (phage-lysed Flexner bacilli).....no reaction. *Desensitization.*
(*g*) $BaCl_2$.....maximum contraction.



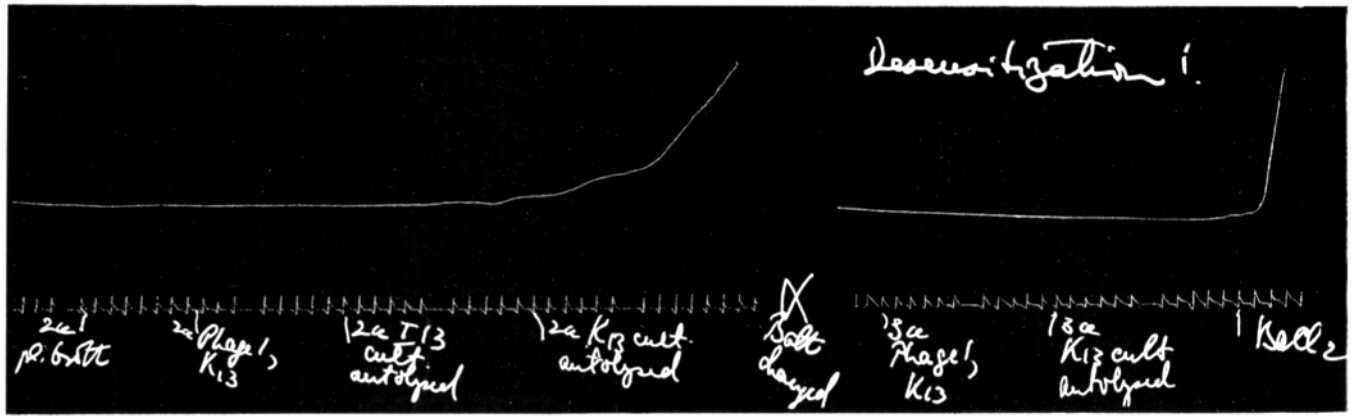
GRAPH 7. Uterine strip from Guinea Pig 34. Sensitized to Phage 1, I-13, trypsinized. Phage-lysed Flexner bacilli, trypsinized (right horn). July 25, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " I-13 culture (Flexner, autolyzed)..... " "
(*c*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*d*) $BaCl_2$.....maximum contraction.



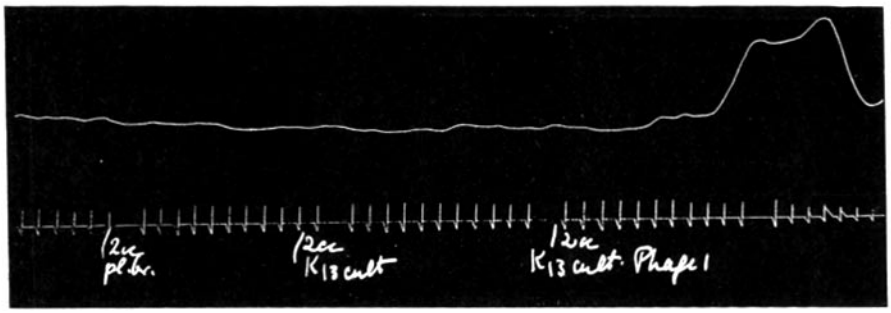
GRAPH 8. Uterine strip from Guinea Pig 29. Sensitized to K-13 culture (colon bacilli) (left horn). August 2, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli).. " "
(*c*) 2 " K-13 culture (colon autolyzed).....contraction.
(*d*) Bath changed.
(*e*) 2 cc. I-13 culture (Flexner autolyzed).....no reaction. *Desensitization.*
(*f*) 3 " K-13 culture (colon, autolyzed)..... " "
(*g*) $BaCl_2$.....maximum contraction.



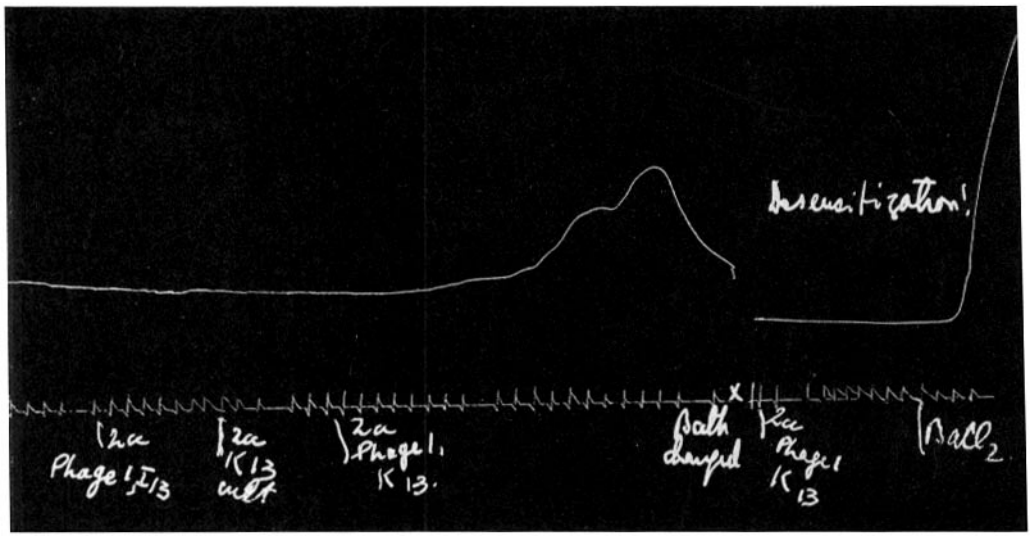
GRAPH 9. Uterine strip from Guinea Pig 30. Sensitized to K-13 culture (colon bacilli) (right horn). August 6, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*c*) 2 " I-13 (Flexner) culture, autolyzed..... " "
(*d*) 2 " K-13 (colon) culture, autolyzed.....contraction.
(*e*) Bath changed.
(*f*) 3 cc. Phage 1, K-13 (phage-lysed colon bacilli). no reaction.
(*g*) 3 " K-13 (colon) culture, autolyzed..... " " *Desensitization.*
(*h*) $BaCl_2$.....maximum contraction.



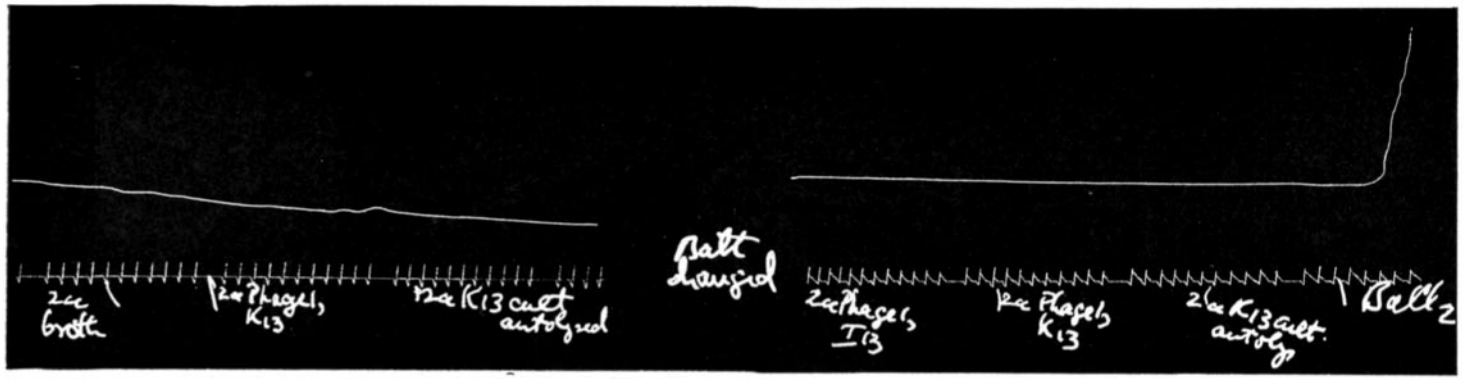
GRAPH 10. Uterine strip from Guinea Pig 10. Sensitized to Phage 1, K-13. Phage-lysed colon bacilli (right horn). May 15, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " K-13 (colon) culture..... " "
(*c*) 2 " Phage 1, K-13 (phage-lysed colon bacilli)..contraction.



GRAPH 11. Uterine strip from Guinea Pig 11. Sensitized to Phage 1, K-13. Uschinsky medium. Phage-lysed colon bacilli (right horn). May 15, 1928.

(*a*) 2 cc. Phage 1, I-13 (phage-lysed Flexner bacilli).....no reaction.
(*b*) 2 " K-13 culture (colon)..... " "
(*c*) 2 " Phage 1, K-13 (phage-lysed colon bacilli)..contraction.
(*d*) Bath changed.
(*e*) 2 cc. Phage 1, K-13 (phage-lysed colon bacilli).....no reaction. *Desensitization.*
(*f*) $BaCl_2$.....maximum contraction.



GRAPH 12. Uterine strip from Guinea Pig 31. Sensitized to Phage 1, K-13, trypsinized. Phage-lysed colon bacilli, trypsinized (left horn). August 7, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli).....practically no reaction.
(*c*) 2 " K-13 (colon) culture, autolyzed.....no reaction.
(*d*) Bath changed.
(*e*) 2 cc. Phage 1, I-13 (phage-lysed Flexner bacilli).....no reaction.
(*f*) 2 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*g*) 2 " K-13 (colon) culture, autolyzed..... " "
(*h*) $BaCl_2$.....maximum contraction.

with the homologous non-trypsinized lysate. This would indicate that the new antigenic complex of phage-lysed bacilli had been destroyed by the action of trypsin in the same manner as the normal bacterial proteins are digested by that ferment.

Series II.—In the attempt to verify the results obtained with the first series of experiments we have run another series under similar experimental conditions, involving virtually the same number of sensitized guinea pigs, the only exception being that in this series the groups of animals, sensitized to the normal Flexner or colon cultures, were injected with spontaneously autolyzed cultures instead of with intact bacterial cells. It was thought desirable to include this modification, in order to approach as closely as possible the physical state and high dispersion of bacterial proteins presumably provided by phage lysis.

The results obtained in this second series were in principle exactly the same as those observed in the former series. We shall therefore omit a detailed description and instead refer the reader to Graphs 1 to 12 which record individual experiments of both series. These graphs also give full particulars as to dosage, time interval, intensity of contraction, desensitization and control measures; they have been selected from a large number of records and are representative of the types of reactions obtained with the various combinations in question.

DISCUSSION.

The observations made in this paper are of interest because they demonstrate two fundamental facts: First, it *would seem that the bacteriophage virus*[3] *per se is not capable of sensitizing guinea pigs to a degree and extent detectable by ordinary experimental methods.* This conclusion is made practically inevitable by the negative outcome of cross-test, in which the bacteriophage constituted the only common antigen. Secondly, it *appears that the bacterial protein, after lysis of the organisms by the bacteriophage, acquires new antigenic properties which differ radically from those of the intact bacteria or bacterial cells dissolved by spontaneous autolysis.*

As to the first conclusion, our results agree with the trend of recent studies on the antigenic properties of the bacteriophage which suggest that antiphage sera, produced with phage antigens, freed from bac-

[3] The bacteriophage is designated a virus here, quite without reference to the disputed question as to its living or non-living nature.

terial proteins by trypsinization, contain only specific neutralizing antibodies and do not possess either complement-fixing or precipitating qualities (Arnold and Weiss). The inability of the bacteriophage in itself to produce either a passive or active sensitization effect would further serve to emphasize the peculiarly restricted antigenic action of this virus, a fact which has been brought out more fully in the recent studies by Schultz, Quigley and Bullock (12) on the antigenic properties of the bacteriophage. The bacteriophage in this respect resembles the ultraviruses and toxins, which, as has been pointed out by Schultz in a recent critical review (5), seem to fall into a special category of antigenic substances which apparently do not engender specific complement-fixing or precipitating antibodies during the immunization of experimental animals.

The fact that phage lysis of bacteria gives rise to new and immunologically distinct antigenic complexes is of fundamental importance, inasmuch as it suggests that the lysis brought about by phage action is not merely a physical phenomenon, but appears to be intimately associated with a profound biochemical change of the bacterial protein. This conclusion seems the most logical interpretation of our results, although it may be possible that some other mechanism, at present not clear to us, underlies the observed phenomena. It is of interest that recent researches on certain filtrable viruses of plants also indicate an alteration of the protoplasma of the host by the pathogenic activities of the implanted viruses. Thus Dvorak (13), basing her conclusions on the precipitin test, has reported a new specificity for plant proteins diseased with the virus of potato mosaic. Purdy's (14) observations on tobacco mosaic virus are of a similar nature. All these contributions strongly suggest that the propagation of ultramicroscopic agents of disease, because of their more intimate contact with the internal mechanism of the cell and because of the peculiar morbid changes which they induce, leads to a more or less complete transformation or to a denaturization of the protoplasm of the cells of the affected host.

It is difficult, *a priori*, to reconcile our observations on the alteration of bacterial protein by phage lysis with the findings of numerous authors secured with the complement fixation, precipitin and agglutination tests, all of which would seem to indicate that antiphage sera

contain a definite quota of specific antibodies which can be shown to react in various ways with the unchanged bacterial antigen. This objection takes for granted that rabbits behave like guinea pigs during immunization with the bacteriophage and, furthermore, that sensitization, as measured by the contraction of the isolated muscle, registers an immunity response identical with the one indicated by the serological reactions *in vitro*. Without discussing for the present the validity of such arguments, there are other factors which would seem to go far towards explaining the discrepancy on a satisfactory basis. It is by no means easy to secure under all conditions a phage lysate, in which all available bacterial protein has been completely transformed into the new antigenic complex by the action of the bacteriophage, since the virulence of many bacteriophages is so limited that, in spite of macroscopic clearing of the culture, an appreciable number of bacterial cells must escape the primary lysis. It has to be considered furthermore that even with bacteriophages of maximum virulence, there is frequently an occurrence of secondary growth of resistant bacterial cells after the acme of lysis is over. These two factors, which depend very much on the composition of the medium, the temperature of incubation and the interval between filtration of the lysate and the completion of lysis, undoubtedly explain the variation in composition and, consequently, in antigenic behavior of different bacteriophage suspensions. We believe that our results with the sensitized muscle have been of such a uniform character and have shown practically no overlapping between the antigenic action of autolysates and phage lysates, because special precautions were taken to obtain as ideal bacteriophage preparations as possible by employing only a lytic principle of maximum virulence for the susceptible organisms and by filtering the lysed cultures immediately after the acme of lysis.

As regards our reactions with strictly bacterial anaphylaxis, we happened to have experienced no difficulty in obtaining maximum contractions of uterine strips, sensitized to normal bacteria, by testing them with autolyzed or even intact homologous bacteria, while Zinsser and his associates stress the necessity of using bacterial extracts, prepared in a laborious way for the anaphylactic test, in order to obtain enough of the reacting proteins in solution.

SUMMARY AND CONCLUSIONS.

1. Marked specific contractions of the uterine horns of guinea pigs, actively sensitized, to phage-lysed Flexner bacilli or to colon bacilli, lysed by the same bacteriophage, occurred on testing either series for anaphylaxis with the homologous phage lysates. These reactions, however, were not due to an antigenic function of the bacteriophage itself, because no reaction whatsoever occurred when the same bacteriophage, propagated on the heterologous organisms, was substituted in the anaphylactic test.

2. Specific uterine reactions of marked intensity were obtained in guinea pigs, actively sensitized to intact or autolyzed Flexner or colon bacilli, respectively, by testing either series for anaphylaxis with homologous, phage-free bacterial antigens.

3. No reaction occurred by testing the uterine strips of animals, sensitized to *intact or autolyzed* bacilli (either Flexner or coli), for anaphylaxis with homologous *phage lysates* and, *vice versa*, there was no contraction of uterine strips sensitized to *phage lysates* upon contact with homologous bacterial *autolysates*.

4. The observations made in this paper suggest that a new and immunologically distinct antigenic complex arises from the bacterial protein after lysis of the organisms by the bacteriophage.

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