STUDIES ON THE TUBERCULIN REACTION AND ON SPECIFIC HYPERSENSITIVENESS IN BACTERIAL INFECTION.

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For a number of years we have been interested in defining, if possible, the rôle which anaphylactic sensitization to bacterial cell constituents might play in the symptomatology and pathology of infectious diseases. We have studied this problem from a number of different points of view and some years ago published, with Parker (1), a series of observations on the sensitization of guinea pigs with typhoid bacilli in which we made use of the isolated uterus method of Dale.

It has been quite apparent to all workers in bacterial anaphylaxis that the sensitization of animals to the cell materials of bacteria is much more difficult than is analogous sensitization with sera, egg albumin, etc., and it has usually been assumed, and we believe correctly, that this was due to the relatively small amount of coagulable protein in the bacterial cell.

In thinking about the problems of bacterial hypersensitiveness since that time, it has seemed to us of fundamental importance to obtain, if possible, a clear understanding of skin reactions such as the tuberculin, typhoidin, mallein, etc., tests, which represent more or less specific forms of hypersensitiveness in infected animals and man but, at the same time, differ in a number of important and perhaps fundamental aspects from phenomena generally associated with true anaphylaxis.[1]

[1] By true anaphylaxis we mean the reaction of protein hypersensitiveness, in which it is now generally agreed that an antigen-antibody union is involved in which the predominant rôle is played by the sessile antibodies, and which can be

I.

The Two Different Types of Skin Reaction.

A basic fact in regard to skin reactiveness is the occurrence, in man and in guinea pigs, of two fundamentally different types of such reactions, both of them to a considerable degree specific, yet differing both in the nature of the completed reactions and in the time factors concerned in their development. We specify guinea pigs and man because, in rabbits, the distinction is not so clear, a matter to which further reference will be made below. The two forms to which we refer are as follows:

1. The intradermal reaction, which appears in from 2 to 3 to 15 minutes after injection of the antigen. It expresses itself in the development of a growing wheal, often surrounded by a red areola of variable size. This reaction may last from ½ hour to 1 or 2 hours, and fades again, usually without leaving any profound injury of the tissues. This is the reaction obtained with such substances as horse serum and has been extensively used in man to determine whether or not the particular individual was sensitive to horse serum when therapeutic injections were intended.

2. The other type of skin reaction is one in which there is no immediate effect, but in which within 4, 5, or more hours, a swelling becomes apparent which in the course of 12 to 24 hours results in a swollen, edematous area of varying intensity, often with a central necrotic spot and, occasionally, hemorrhage. This reaction may not reach its highest development until about 48 hours after the injection, and is accompanied by distinct signs of inflammation and some cell death.

passively and specifically transferred to normal animals with the serum of other animals as long as this serum contains antibodies. We do not wish to take up space with this definition, but refer the reader to the recent and excellent summaries of Doerr (Doerr, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 947), Coca (Coca, A. F., in Tice, F., Practice of medicine, New York, 1920, i, 107), and Wells (Wells, H. G., *Physiol. Rev.*, 1921, i, 44). Although differing somewhat in nomenclature and in classification, the three summaries lay down fundamental principles which are essentially alike and with which we agree.

The classical examples of such skin reactions are the tuberculin intradermal test, the typhoidin reaction, the mallein reaction, and the reactions studied by Fleischner, Meyer, and Shaw (2) with *Bacillus abortus* and *Bacillus melitensis* in guinea pigs.

As we shall see, the two reactions may occur in the same animal, after one and the same injection, but, in guinea pigs, can usually be differentiated, since one begins to fade before the other appears.

It seemed that our first task, then, was to determine whether either one of these varieties of skin reactions or perhaps both of them could be correlated with true anaphylaxis.

Relationship of the "Immediate" Skin Reaction to Anaphylaxis.

The immediate skin reaction has been used for some years as an index of safety before the injection of therapeutic horse serum in the specific treatment of pneumonia and other diseases. Its application in this connection was based primarily upon the publications of Moss (3), of Knox, Moss, and Brown (4), of Longcope and Rackemann (5), of Mackenzie and Leake (6), and of others. It should be noted, however, that the work of Moss and his collaborators, on which the assumption that the skin reaction was a reliable index of general hypersensitiveness is based, was done with rabbits, and in rabbits the skin manifestations of anaphylaxis, both intracutaneous and subcutaneous (Arthus phenomenon) are always in the form of delayed reactions, for reasons that we believe depend upon peculiarities of the rabbit, rather than upon fundamental differences in the nature of the reaction. This peculiarity in rabbits, however, is being investigated in our laboratory at the present time in a study on the Arthus phenomenon and will be reported upon subsequently. Meanwhile, it seemed to us necessary, since we were working with guinea pigs, to determine whether in these animals an immediate skin reaction to a protein antigen such as horse serum, egg albumin, etc., could be elicited at all, and, if so, whether its development was parallel to that of general protein hypersensitiveness.

In consequence, we carried out a number of experiments in this direction.[2]

[2] These experiments were done with the assistance of Dr. S. T. Woo and Mrs. J. T. Parker, who was carrying out skin reactions in guinea pigs for another purpose at the time.

Series of guinea pigs were given from 1 to 2 cc. of horse serum intraperitoneally. At the end of from 2 to 3 weeks, they were given intracutaneous injections of horse serum in dilutions ranging from 1 : 5 to 1 : 20. Normal controls were always injected at the same time and intravenous injections of varying quantities of horse serum were made on guinea pigs similarly sensitized in order to determine general anaphylaxis. We omit protocols since the experiments were extremely simple and we do not wish needlessly to prolong this paper.

Our results may be stated briefly as follows: Guinea pigs in the sensitized condition, that is 2 to 3 weeks after the intraperitoneal injection of 1 cc. of horse serum, will generally, though not always, show an immediate reaction to the intracutaneous injection of 0.1 cc. of a 1:10 or 1:20 dilution of horse serum.

There is a considerable increase in the size of the wheal formed at the point of injection, the increase beginning usually within 5 or 10 minutes, and increasing up to from ½ to 2 hours, then gradually fading. It was usually completely gone in from 2 to 4 hours and in most cases was accompanied by a red areola quite comparable to the reaction observed in human beings under similar conditions. These reactions never caused any serious or lasting injury to the tissues.

This reaction was roughly parallel to the sensitiveness of the guinea pigs to intravenous horse serum injections.

It can be produced in guinea pigs by passive sensitization, appearing on the 2nd day after the injection of the anti-horse rabbit serum almost as well as in the actively sensitized guinea pigs.

Guinea pigs that have been shocked and become antianaphylactic are desensitized to skin reactions made on the following day.

Relationship of the Delayed Tuberculin Type of Skin Reaction to Anaphylaxis.

Having thus determined that immediate skin reactions to horse serum in guinea pigs might be regarded, with reasonable certainty, as one of the manifestations of general hypersensitiveness, we next proceeded to investigate the same point in connection with the second type of reaction; namely, that represented by tuberculin, mallein reactions, etc. We selected tuberculosis and the tuberculin reaction as the first type to be studied because with tubercle bacilli it is relatively easy to produce, at will, infections of varying intensities and different speeds of progression, and since the volume of clinical

data and the careful studies of tuberculin skin reactions in guinea pigs by Baldwin, Krause, and others had cleared the way for a free approach to the basic problems. The reaction, moreover, is essentially the same type of occurrence as that following the injection of typhoidin, mallein, or abortin preparations in animals respectively infected with the organisms from which the antigens have been produced.

It is quite well known that in an interpretation of reactions such as the tuberculin reaction, the field is fairly divided between those that hold these phenomena to be manifestations of true anaphylaxis and those who deny this.

Calmette (7), who separates the tuberculin reactions distinctly from anaphylaxis, nevertheless states that tuberculous animals or man develop a lytic principle, presumably in the nature of a bacteriolysin, which goes into reaction with the specific antigen in the tuberculin preparations. This fundamentally is, in a sense, an anaphylactic conception. Friedberger has frankly regarded the phenomenon as an anaphylactic one, developing his ideas along his theories of anaphylatoxin formation.

Study of the reactions from a theoretical point of view was begun by Römer (8) in 1909, and by Baldwin (9) in 1910. Baldwin's work is fundamental, in showing that, in spite of previous assertions, guinea pigs could not be rendered skin-sensitive by implantation of porous filter capsules or celloidin capsules containing tuberculoprotein, or living tubercle bacilli. He showed that skin sensitiveness could never be produced without actual infection with living organisms. Animals treated with tuberculoprotein, however, often showed reactions to intravenous inoculation of the homologous preparation which could be recognized as anaphylactic. His conclusions can be summarized as follows:

Tuberculous animals become sensitive to anaphylactic test, but not uniformly so. There is no absolute relation between the degree of sensitiveness and the stage of the disease. Injections of the tuberculoprotein may sensitize normal guinea pigs. Sensitized guinea pigs, however, do not react to the ordinary tuberculin test, though some respond slightly to the intradermal test. He adds: "This difference between anaphylactic sensitization and tuberculin reactivity need not be fundamental, as it is possibly due to another factor as yet undetermined." His experiments on the transfer of passive anaphylaxis to tuberculoprotein were inconclusive, but it has been shown since then, by Austrian (10) and others, that passive sensitization can be attained. From a theoretical point of view the most important observation of Baldwin is the fact that there seemed to be a discrepancy between skin sensitiveness and general anaphylaxis. Krause (11), following out the work of Baldwin, confirmed and extended the above observations and established an interesting relationship between skin sensitiveness and the progress

of infection. He asserts that skin sensitiveness develops simultaneously with the development of the initial focus, increases progressively with the lesions, varies directly with the extent and intensity of the infection, and diminishes with healing. It is blunted by a general tuberculin reaction which suggests analogy to saturation, such as that which occurs in connection with anaphylaxis.

In dealing theoretically with the tuberculin reaction, especially as regards the possibility of its being an anaphylactic phenomenon, it is of great importance to determine whether or not the reaction can be transmitted passively. The evidence on this is contradictory. Only a few writers have reported positively in this connection. In 1909, Bail (12) injected finely divided tissue mash of tuberculous organs of guinea pigs into normal guinea pigs, and 24 hours later gave the animals so treated 0.5 cc. of old tuberculin, or a preparation which in this quantity had practically no effect upon normal animals. The animals prepared with the tuberculous tissue died in some cases, while controls treated with normal tissue suspensions showed no symptoms.

Helmholz (13) in the same year reported positive skin reactions in normal guinea pigs 2 to 6 days after he had injected them intraperitoneally with the defibrinated blood of tuberculous guinea pigs. Both of these observations would be of fundamental importance if they could be confirmed.

In considering the mechanism of the tuberculin reaction, it will be well to examine also the work that has been done on the typhoidin reaction. Gay and Claypole (14) believed that positive skin reactions in rabbits were parallel with the degree of immunity of the animal. They succeeded in transferring the susceptibility to typhoidin from an immune to a normal animal by inoculation of 20 cc. of typhoid-immune serum, testing 24 hours later. These experiments were repeated and confirmed by Meyer and Christiansen (15); and in their first work with rabbits, these last observers, using what they called a typhoid autolysate (by which they mean an alcohol precipitate of a heated distilled water suspension of a 48 hour agar culture), concluded that "the typhoidin and similar reactions in rabbits are anaphylactic in nature and the result of an interaction of antigen and antibody." They stated that "the logical assumption from these facts is than cutaneous hypersensitiveness is the result of bacterial protein sensitization." Later Meyer (16) found that injected rabbits react with typhoidin more intensively than do immunized rabbits, and drew the conclusion that cutaneous hypersensitiveness does not indicate that the rabbit is particularly immune, and that no definite relationship existed between agglutinins and complement-fixing antibodies and skin sensitiveness. From these first two papers of Meyer's we gather that he believed that in rabbits skin sensitiveness to typhoidin is a sign of infection, rather than of immunity, but that as stated in his own words "cutaneous hypersensitiveness of rabbits . . . . . is, in all probability, the result of sensitization with typho- or similar bacterial proteins." Nichols (17) also considered the typhoidin reaction as he observed it in human beings as a protein sensitization.

The apparent discrepancies between the results of Baldwin with the tuberculin reaction and those of the workers just mentioned with the analogous typhoidin reaction, probably depend upon the fact that Baldwin used guinea pigs and the other observers used rabbits. When, subsequently, Fleischner, Meyer, and Shaw (2) studied cutaneous hypersensitiveness in guinea pigs treated with repeated intraperitoneal injections of *B. abortus*, *B. typhosus*, and old tuberculin, and carried out parallel experiments with animals infected with living organisms, the conclusions that they reached coincided with those of Baldwin in the case of the tuberculin reaction. They found, in other words, that guinea pigs treated with dead bacterial proteins might become anaphylactic, but did not give skin reactions.

It will be seen from this brief review of the literature that, in spite of a great deal of careful work by experienced observers, there is still a considerable degree of confusion. We considered it best, therefore, in studying the tuberculin reaction to begin at the bottom and reexamine the conditions in all their details, substituting reactions with the isolated guinea pig uterus for the intravenous test for anaphylaxis. This point is particularly important since the intravenous injection of bacterial extracts into guinea pigs is apt to give rise to confusing symptoms; and in carrying out such experiments we have again and again felt uncertain as to whether or not an individual, mild reaction should be interpreted as a feeble manifestation of anaphylaxis, as due to toxicity of the bacterial preparation, or perhaps even as the result of the injection of finely divided colloidal particles.

The first stages of our work, then, concern themselves with a study of tuberculous guinea pigs and guinea pigs treated with various tubercle bacillus extracts, determining in both cases the relationship of intracutaneous tuberculin reactions with anaphylaxis as manifested by tests with the isolated uterus. We summarize the final procedures in order to avoid a needless account of much preliminary groping for a suitable technique.

Technique.—The tubercle bacillus used throughout was a human type of moderate virulence originally isolated, we believe, at Saranac. Many different methods of extraction were used, but the preparation with which most of the work was done was made as follows:

About 100 mg. of ground and powdered tubercle bacilli were shaken up in a shaking machine for 3 to 4 hours, with 200 cc. of salt solution to which had been added enough normal sodium hydroxide to give a final concentration of 0.2 per cent of the alkali. When the powder was added to this, the buffers reduced the alkalinity, which finally ranged somewhere about pH 8. After shaking, the bottles

were preserved in the ice chest, and sometimes reshaken on subsequent days. The gross particles were removed before this was used, either by filtration through a Berkefeld filter, or by high speed centrifugation, and the slightly opalescent fluid was the basic preparation finally employed in most of the experiments, and fractionated subsequently for special purposes. In sensitizing guinea pigs it was very often the unfiltered extract which was intraperitoneally injected without centrifugation.

The following method was found to be the most suitable for the sensitization of normal guinea pigs with the tubercle bacillus extracts. Young female guinea pigs of about 150 to 200 gm. weight received from 1 to 2 cc. intraperitoneally, at first on alternate but in later experiments on successive days, until ten to twelve injections had been given. It was found best to test these guinea pigs not earlier than 20 days and usually not later than 28 days after the last injection.

Skin reactions were invariably done intracutaneously with a No. 26 gauge needle on a tuberculin syringe, and it was attempted to inject about 0.05 cc., but it was found that the concentration rather than the actual amount was the thing that seemed to count most. We gauged the skin reactions largely on the size of the wheal produced, attempting to produce the same sized wheal in comparative tests. It is next to impossible in guinea pigs to inject absolutely accurate quantities in separate tests, but, as a matter of fact, the variations in quantity injected could never have been very great or significant.

The uterine tests were all done in the same way, in a bath of 200 cc. of Ringer's solution with 0.5 to 1 per cent glucose and oxygen bubbling through the bath.

Studies with Tuberculous Guinea Pigs.

Guinea pigs were intraperitoneally injected with relatively large doses of tubercle bacilli, without any attempt to equalize the doses or to grade them, since we wanted to perform the test on guinea pigs with varying degrees of tuberculous involvement and to check up by autopsy after the results had been obtained.

Skin reactions were done on these animals with the undiluted but filtered extracts described above, and with old tuberculin in dilutions usually of 1 : 5 or 1 : 10, at varying intervals from the 1st day after infection. When definite skin reactions appeared, some of the guinea pigs were killed and the uteri put into the Dale apparatus, the extract mentioned above being used in most of these experiments, since, in working with old tuberculin, the margin between the nonspecific action on the normal uterus and the specific action on the sensitized uterus is a relatively narrow one, a thing also found by Weil (18), who is, as far as we know, the only one who has applied this method to tuberculosis, his report dealing with some isolated experiments carried out in 1917.[3] A great many animals

[3] Weil obtained uterine reactions with old tuberculin 9 weeks after injection with 0.001 cc. of old tuberculin, while the normal uterus did not react to less than 0.3 cc. The capacity of the bath is not given. He states that the reaction is an irregular one and occurs "at some stage of the disease."

were tested in this way, and the results were perfectly consistent, so that it is unnecessary to go into detailed study of the protocols. Whenever uterine tests were done, the tubercle bacillus extract used was titrated on the same day against normal uteri to make sure that we did not approach the non-specific limit, which varied considerably in these extracts. Also, no skin reactions were ever carried out without being accompanied by tests, with the same material in the same quantities, on normal guinea pigs, since, in working with alcohol precipitates and other chemically manipulated substances, mild non-specific reactions will occasionally appear.

The question to be answered was the following. In the course of a tuberculous infection does true anaphylaxis to tubercle bacillus products develop, and, if so, is it parallel in time and in intensity with the intradermal tuberculin reaction?

Figs. 1 to 3 illustrate the results obtained. Positive skin reactions were usually obtained within 9 or 10 days after inoculation, the earliest, observed by us, being between the 6th and 7th days. At this time the animals were never anaphylactic. Fig. 1 shows complete lack of anaphylactic reaction in the uterus in an animal 10 days after inoculation, the day after a positive skin test had been obtained. Fig. 2 shows two uteri simultaneously put into the bath, both of them 9 days after inoculation with tubercle bacilli, one giving an entirely negative skin reaction, the lower one giving a very marked $++++$ reaction. Neither showed a sensitive uterus.

Fig. 3 shows a different state of affairs; namely, an animal inoculated subcutaneously with tubercle bacilli and tested 3 weeks after inoculation. This animal showed a powerful anaphylactic, as well as skin reaction. These records and a considerable number of similar ones indicate: (1) that guinea pigs suffering from infection with tubercle bacilli may become both skin-reactive and anaphylactic; (2) that the skin reaction develops early and may exist without any detectable signs of general anaphylaxis as evidenced by the uterine reaction; (3) that within 3 weeks or later, after the animals are still in fairly good condition, the skin reaction and the uterine reaction may coexist.

It is during this period that a simulated parallelism may have led others, working with a less delicate anaphylactic method, into confusion. Towards the end when the guinea pigs are moribund, both the skin reaction and general anaphylaxis may fade, another feature

which simulates parallelism; but many reactions like those above have convinced us that there is no question about the correctness of Baldwin's original contention, that the two conditions, skin reactiveness (and probably, therefore, the other forms of tuberculin hypersensitiveness) and anaphylaxis do not necessarily coincide.

Studies on Guinea Pigs Sensitized with Materials from Killed Bacteria.

The next question to be answered was that concerning the conditions existing when guinea pigs were not infected, but when they were treated with products of dead tubercle bacilli. Young female guinea pigs were prepared by the method of sensitization outlined above. They were run through in sets of six or more. Between July, 1920, and January, 1921, five sets of such guinea pigs were run through, all of them treated with ten or more injections of extract. Skin reactions were done on these animals, both during the process of sensitization, that is in the course of the 10 to 20 days during which they were being treated, and at varying times thereafter, up to the period of 3 weeks after the last injection when they were tested for uterine sensitiveness.

Practically none of these guinea pigs showed typical delayed skin reactions comparable to the true tuberculin reaction. In only two cases did we see reactions which might have been regarded as moderate tuberculin reactions. It may be noted that Baldwin also observed a few exceptions to his other negative results. An example of one set in which these exceptions occurred may be worth recording since it will typify our general procedure.

Experiments on the Sensitization of Guinea Pigs with Tuberculoprotein.—Six guinea pigs (Nos. 1 to 6), were injected intraperitoneally with 1 cc. of unfiltered tubercle bacillus extract as follows: September 1, 3, 7, 9, 11, 13, 20, 28, 1920. The first skin reactions were done on four of the guinea pigs with two preparations of old tuberculin diluted 1 : 5, and undiluted extract similar to that injected on September 28. Negative results.

October 2. All the guinea pigs were reinjected with 2 cc. of the extract; skin reactions on this day were negative.

October 7. Skin reactions done on three of the guinea pigs at the same time with four normal controls and one tuberculous control. When these reactions

were read on October 8, Guinea Pig 2 showed what ordinarily we should have called a +++ reaction. It should be mentioned that this guinea pig was very much emaciated and we were suspicious of its having been spontaneously infected with tuberculosis. It was killed and autopsied, and a few small yellowish spots on the surface of the liver about 2 mm. in size at the base were taken for section and examined,[4] but could not be identified as tuberculous (we may mention that this occasional appearance of small knob-like lesions on the liver of extract-treated guinea pigs has not been uncommon). The uterus of this animal was found to be moderately sensitive. Subsequent tests of the rest of these guinea pigs showed only one other suspicious skin reaction, less marked than that on No. 2.

The isolated uteri of these extract-treated guinea pigs were, with very few exceptions, found to be sensitive to extract anywhere from 20 to 30 days after the last injection. Fig. 4 shows two guinea pigs in which positive, typical reactions were obtained with the uteri with 4 cc. of a rather weak extract which in these quantities had no effect upon normal uteri. In both of these animals, the skin reactions had been consistently negative.

It may be noted in passing, however, that although these extract-treated guinea pigs did not show the typical tuberculin reaction with well defined swollen areolæ, etc., after 24 hours, they did occasionally show an immediate reaction not incomparable to the immediate reaction described above for horse serum. Whenever such reactions were observed, they occurred at about the time when the guinea pigs showed anaphylactic reaction to uterine test. This is mentioned in passing as showing that even with the products of the tubercle bacillus the two kinds of skin reaction can be elicited.

Moreover, the two graphic records shown in Fig. 4 are only two of very many in which typical uterine reactions were elicited in guinea pigs similarly prepared with considerably smaller quantities of various kinds of tubercle bacillus preparations. It will be seen in the lower record that a repetition of the extract showed the uterus to be desensitized, a matter which has been noted many times.

Therefore, from a considerable number of experiments similar to those described in perfect accord with the results obtained by Baldwin by other methods, we may conclude that delayed reactions, of the tuberculin reaction type, may develop independently of generalized anaphylaxis in the ordinary sense in which this word is used, may be

[4] The examination was made by Dr. Frederick Parker.

present in tuberculous guinea pigs before anaphylaxis to tuberculoprotein has developed, and are with very few exceptions entirely lacking in guinea pigs rendered anaphylactic by sensitization with tubercle bacillus products.

These facts, then, definitely confirm the opinion, first clearly advanced by Baldwin, that tuberculin hypersensitiveness may develop independently of general tuberculoprotein anaphylaxis, and that the former type of hypersensitiveness is associated particularly, perhaps solely, with the existence of an infection. This, too, is in complete keeping with the experience of Fleischner, Meyer, and Shaw who found that intradermal tests were positive only in guinea pigs infected with the bacillus of bovine abortion, but consistently negative in these animals intensively immunized by intraperitoneal injections of dead organisms, or with extracts of organisms.

II.

The fundamental facts may then be stated as follows: Under the influence of contact with living tubercle bacilli, and probably other bacteria, two distinct varieties of hypersensitiveness develop in guinea pigs. One of them, true anaphylactic hypersensitiveness, develops late and can be readily induced by appropriate treatment with dead bacterial materials, extracts, etc. The other, the typical tuberculin reaction (and probably the mallein, typhoidin, and abortin reactions) may be regarded as infection phenomena, for, while we may eventually succeed in inducing these reactions by modified methods of sensitization,[5] they are most easily and characteristically elicited as soon as an infection is established, while, to date, they have but rarely and atypically followed upon any form of artificial sensitization. And yet, in spite of the apparent mutual independence of these two forms of hypersensitiveness, both reactions, or either one of them alone, may be elicited with one and the same bacterial extract in appropriately prepared animals.

This forces us to inquire whether a single constituent of such bacterial extracts is responsible for both types of reactions, or whether it is possible to separate, from such preparations, two functionally

[5] See below.

distinct fractions, one capable of eliciting the typical anaphylactic response, the other active in the tuberculin sense.

The latter opinion is rendered likely by the generally accepted view that the true anaphylactinogens are proteins, whereas most of the work done with tuberculin seems to indicate that these substances are of a simpler structure.

Löwenstein and Pick (19) among others conclude that the active substance in tuberculin is a protein-free, alcohol-precipitable substance which is not coagulated by boiling either in neutral or acid reaction, cannot be precipitated with ammonium sulfate, gives a negative biuret reaction, but is precipitated with tannic acid. We will not go into the details of analysis, but their final conclusion is that the active substance is not a protein, but belongs in the class of protein split products which Fischer has spoken of as "polypeptides." They claim that dialysis of the concentrated tuberculin solution weakens it after a short time, and that after prolonged dialysis its activity is entirely lost; they do not, however, state the nature of the membranes used, nor the particular conditions under which dialysis was performed. The active tuberculin substance, moreover, which Calmette uses for his ophthalmoreaction is the white, flocculent, and highly soluble substance which is precipitated out of tuberculin solutions upon the addition of ten or twenty volumes of absolute alcohol.

Along the lines of these investigations we proceeded to attempt separation of the various substances which might be obtained from tubercle bacillus extracts.

Attempts at Chemical Separation of the Tuberculin from the Anaphylactinogen.

From powdered tubercle bacilli of the human type[6] we produced extracts by shaking in a 0.02 per cent sodium hydroxide solution in physiological salt solution. 3 or 4 hours shaking and perhaps a day or so in the ice chest sufficed to bring a considerable amount of the material into solution. This extract was centrifugalized until all the particles had been removed and a moderately opalescent supernatant fluid was decanted.

This material, upon being acidified to approximately pH 5 to 6, with 2 per cent acetic acid in the cold, became turbid and soon precipitated in large flakes. Further acidification up to pH 4 did not redissolve these flakes. The precipitate represented the bulk of the dissolved substance in the extracts. The precipitate could be redissolved in a slightly alkaline salt solution and reprecipitated with

[6] These bacilli were kindly furnished by Dr. S. A. Petroff of Saranac.

acid. Because of their precipitability by acetic acid in the cold, we designated these substances as nucleoproteins or phosphoproteins, although we do not wish to commit ourselves, chemically, since we are aware of the indefiniteness of these biochemical terms and realize fully our incompetence to deal authoritatively with a chemical problem of such difficulty, without further intensive study.

After removal of these acid-precipitable substances by centrifugation and filtration through Berkefeld candles, the fluid was brought to a boil in the acid condition, and sometimes a very faint turbidity developed which was taken to represent the presence of coagulable protein, albumin or globulin, or both. These precipitates were so fine and slight that only Berkefeld filtration would remove them, and even this was not always completely successful.

The fluid was then neutralized; that is, brought to an approximate pH of 7.

When this neutral fluid was filtered hot, we observed on numerous occasions that a slight precipitate developed over night in the ice chest, and that this precipitate redissolved on heating, a point which indicated the possibility that the tubercle bacilli may contain Bence-Jones protein. This point, however, will need further chemical analysis, a task which we have not yet had time to undertake. The water-clear fluid which was left after removal of all these substances gave in all cases a very definite precipitate with alcohol. The precipitate could be thrown down, collected, and redissolved in water with salt solution, and like the similar precipitate obtained from crude tuberculin was astonishingly soluble.

This final water-clear material gave no biuret reaction, and usually gave no sulfosalicylic acid reaction, though occasionally this was very faint; in most cases it gave no Millon reaction, was not clouded on boiling with acid, and usually gave no xanthoprotein reaction, though in some cases a slightly yellowish color appeared when the ammonia was added in the second part of the test. This material, for want of a better name, we speak of as the "proteose" residue.

Having thus fractionated the original tubercle bacillus extract, we proceeded to carry out biological reactions with various parts, comparing chiefly the whole extracts with the dissolved substances which precipitated with acid in the cold (nucleoproteins) and with the final solutions from which the acid-precipitable, acid- and heat-coagulable substances and the material suspected of being Bence-Jones protein had been removed (proteose residue).

We have done a large number of skin reactions on tuberculous guinea pigs with the apparently protein-free proteose residue, always controlling with tests on normal guinea pigs. This precaution is absolutely necessary, since we have found (as we were warned to expect by Baldwin) that repeated alcohol precipitation or prolonged boiling in an excessively acid reaction (pH 4 or below) may eventually

lead to non-specific toxicity, both for the skin test and for the isolated uterus.

Invariably this final proteose material gave skin reactions, often quite as powerful, and never more than slightly less than the original whole material. And, indeed, it was to be expected that a certain amount of the tuberculin active substance might be carried down in the flocculent precipitate formed when the acid is first added, so that the solution is thus deprived of a certain percentage of its activity. The protocol presented in Table I is an example of experiments of this kind.

TABLE I.

Skin Reactions Read after 24 Hours.

No. of tuberculous guinea pigs.	Whole extract 1:2.	Proteose 1:2.	Whole extract 1:5.	Proteose 1:5.
7	++++	++++	+++	++
8	+++	++	+++	+++
9	+++	++	+++	++
	Whole extract undiluted.	Proteose undiluted.		
10	++++	+++		
11	+++	+++		

Two normal controls show no non-specific reactions.

++++ indicates a very marked reaction; +++ a large reaction, slightly less than the preceding; ++ a moderate reaction; + a definite but faint reaction; ± doubtful; − negative.

Reactions of this nature, often repeated, indicate definitely that in removing the acid-precipitable and the coagulable protein substances from the tubercle bacillus extracts, we did not remove any considerable part of the skin-reactive substance. Moreover, they justify the assumption that, in the tuberculin skin reactions, the antigen is not necessarily a protein substance, but probably belongs in the category of the so called proteoses or perhaps polypeptides.

It must be stated, however, that the nucleoprotein fraction, precipitated from the original extracts with acid in the cold, always retained tuberculin activity. In spite of repeated reprecipitation and resolution, we have never succeeded in entirely removing the capacity

of inducing skin reactions of the tuberculin type from these acid-precipitable substances. This may be due to the adsorption of the proteose material by the heavy flakes of the precipitate. On the other hand, subsequent experiments suggested the possibility that these nucleoproteins (?) which constitute the bulk of the soluble material of the bacterial cell might represent the mother substance from which the proteose materials are derived.

However this may be, the proteoses alone are fully capable of giving rise to the typical tuberculin reaction in the infected guinea pigs.

This being the case, what is the effect of this proteose fraction upon the isolated uterus of the extract-sensitized or anaphylactic guinea pig in which no tuberculin skin reaction can be elicited?

In these experiments our results were often not so clean-cut as they might have been. Nevertheless, in a considerable number of tests there was indication that the proteose fraction, powerfully skin-reactive, had little or no effect upon the uteri of extract-sensitized animals, which contracted powerfully when brought in contact with the whole extracts or the acid-precipitable fractions in amounts that had no effect upon the normal uterus.

Fig. 5 shows the uterus of a guinea pig, sensitized with extract, which was unaffected by 2 and 2.5 cc. of the proteose fraction, but contracted powerfully and went into a spasm when 1.5 cc. of the whole extract were added to the bath.

Fig. 6 shows a similar experiment in which 2.5 cc. of the proteose fraction had no effect, whereas as little as 1 cc. of the acid-precipitable fraction produced a strong contraction and spasm.

A number of records (six) as sharp and unambiguous as the above were obtained. But in addition, we must state that on three occasions the proteose material contracted the uteri of highly sensitized and of tuberculous guinea pigs when relatively large amounts of proteose were used. Whether this means that we had not freed the proteose completely of protein, or whether it signifies that, with sufficiently energetic treatment, the proteoses, too, may form antibodies, is a question that cannot be definitely answered. We incline to the latter view for reasons that will be discussed below.

Notes on the Dialysis of Tuberculin.

It will be remembered that the chief clinical difference between the immediate skin reaction of the anaphylactic type and the tuberculin type of reaction is the fact that the former consists in a rapidly developing urticaria-like swelling which disappears in a relatively short time, and leaves little or no injury behind. The latter, however, comes on slowly, and when once developed is accompanied by manifestations of inflammation and, eventually, profound injury of cells, often with cell death, a state of affairs which at least suggests the penetration of the injurious substances into the cells themselves. This, taken together with our knowledge of the relatively simpler structure of the tuberculin substances and Löwenstein and Pick's claims concerning their diffusibility, suggests a number of interesting lines of reasoning.

Is it not possible that the diffusibility of antigenic substances is a very important factor not only in the reaction of these diffusible substances with the cells, but also in the formation of antibodies? Is it not likely that substances which are practically non-diffusible should be excluded from direct reaction with the tissue cell and that the mechanism of antibody formation is a device for a reaction with non-diffusible materials? And, if this is so, is it reasonable to suppose that the reaction of the body to substances which are relatively more diffusible should become increasingly intracellular, as diffusibility increases, and that antibody formation, in the usual sense of the word, should, therefore, become less and less essential as relative diffusibility increases? Such a view would explain at the same time the apparent intracellular nature of such reactions as the tuberculin reaction and the difficulties encountered in attempts at passive transfer of such sensitiveness. Accordingly, we thought it worth while to attempt to separate by dialysis the substance which produced the tuberculin skin reaction from that which caused typical anaphylaxis.

In planning experiments upon the diffusion of antigenic substances through semipermeable membranes, we are quite aware of the many pitfalls which entrance into such a field on the part of the biologist implies. We know that it is by no means proven that the living cell membrane is at all permeable to substances like the proteoses and that experiments with dialysis *in vitro* may lack some of the essential criteria that govern dialysis in the animal body. Nevertheless, it seemed worth while to investigate the diffusibility of the tuberculin active substances. We at first tried to work with membranes graded by the methods of Brown (20) and of Gates (21). But since the differences in diffusibility of proteins and the proteose-like substances with which we are concerned can be very slight ones only, we abandoned this and worked empirically, attempting, in many tests, to find membranes which would let through the tuberculin active substances, but hold back the anaphylactinogenic ones.

The general procedure consisted in taking various tubercle bacillus preparations, the skin reactiveness of which had been determined on tuberculous guinea pigs, and subjecting them to dialysis either in celloidin or in fish bladder bags, in

a closed system, testing the material outside the bag from time to time on tuberculous and normal guinea pigs. As soon as the material outside gave indication of containing the skin-reactive substance, the same material was used upon a sensitized and a normal uterus. In a few experiments we obtained results which at least indicate that the tuberculin active substance is more readily diffusible than the anaphylactinogens.

Experiment 1.—A glycerol salt solution extract of powdered tubercle bacilli was started on February 7, 1921. 1 gm. of powdered tubercle bacilli was infused in 500 cc. of a 5 per cent glycerol salt solution, heated to 70°C. for 1 hour, and placed in the incubator over night. It was then shaken for several hours on consecutive days. This material was simmered down to 150 cc. at a temperature of 75 – 80°, never quite being allowed to come to 80°, and was then filtered through paper. It gave a strong sulfosalicylic acid reaction. February 15. 40 cc. of this material

TABLE II.

Readings on Skin Reactions Done February 17.

Guinea pig No.	Skin reactions.	
	With material outside bag (B_2).	With material inside bag (B_2).
12	++	++++
13	++	++++
14	++	++++
15	−	++++
16	±	++
17	±	++
18	+	+++
Normal control.	−	±
" "	−	±

were placed in a fish bladder bag in a closed system with 40 cc. of salt solution outside. February 17. Skin reactions were done with the material inside and outside the bag, and it was found that a very definite though mild skin reaction resulted from the material outside the bag. The relative strength of the skin reaction with material inside and outside the bag are given in Table II.

February 18. This material was tested upon normal uteri and it was found that 2.5 cc. of the original material and the material outside the bag, added to a 200 cc. Ringer's bath in which a normal uterus was rhythmically contracting, produced no marked changes in the rhythm. The material was then tested on the uterus of a guinea pig sensitized as above described with tubercle bacillus extract. As will be seen in Fig. 7, 4 and 6 cc. of the material outside the bag, which gave a skin reaction, produced no spasm of the sensitized uterus, whereas 2 cc. from inside the bag gave a typical and marked reaction. We realize that this may have been a quantitative result, due to a greater sensitiveness of the skin than of the uterus.

This does not seem likely under the circumstances, but, nevertheless, must be considered.

We have noted above that Löwenstein and Pick claimed that they could completely free old tuberculin of its activity by prolonged dialysis against running water. For this reason, we determined to try the reverse experiment; that is, attempting by prolonged dialysis to remove the skin-reactive substance entirely from the inside of a dialyzing bag, leaving the anaphylactinogenic substance behind. We tried this with various preparations, but never succeeded even with prolonged dialysis in freeing the materials on the inside of the bag entirely of a skin-reactive substance, although such a result had been claimed by Löwenstein and Pick. In one case only did we obtain a result worth reporting. We placed 25 cc. of concentrated old tuberculin from the Department of Health of the City of New York in a fish bladder dialyzing bag, and dialyzed this against running water. The old tuberculin used still gave a powerful sulfosalicylic acid reaction for protein, and gave a definite precipitate on being acidified with acetic acid in the cold after dilution to about 1 : 20. The precipitate did not redissolve upon heating, but was rather intensified. With this preparation we had what we must now consider a rather fortunate accident. Because we were dialyzing with running water, we could not carry out the procedure in the refrigerator, and bacteria began to grow in the dialyzing bag. In the course of 5 days, the material from the inside of the bag gave practically no skin reaction on tuberculous guinea pigs as compared with old tuberculin diluted 1 : 3, which corresponded to approximately the dilution which had been attained by the substance in the course of dialysis. This material which no longer gave skin reactions was tested against normal and sensitized uteri, and it was found that, although 3 cc. of the material gave no reaction on the normal uterus, powerful reactions with sensitized uteri were obtained in quantities as low as 0.5 cc. and a weaker but still noticeable reaction obtained with 0.2 cc. The possibility that substances like histamine were formed by the bacteria and accounted for the uterine reaction can be excluded by the failure of this material to affect the normal uterus. Whatever may have been the reason for this result, whether dialysis had anything to do with it or whether it means simply that the bacterial growth had destroyed the tuberculin substances while leaving the anaphylactinogens intact, it seems a definite and sharp separation of the two functions.

III.

Difference between Infected Animals and Those Treated with Dead Bacterial Substances.

Neither chemical fractionations nor the diffusion experiments furnish absolute proof that the anaphylactinogen can be completely separated from the tuberculin active substances. Nevertheless,

both series of experiments point in this direction, and, taken together with the mutual independence of the two types of reaction in guinea pigs, render such a conception an extremely likely one.

However this may eventually prove to be, one fundamental fact remains definite; namely, that the proteose fraction alone can elicit the particular form of hypersensitiveness which we speak of as the tuberculin reaction; and that the potency of the proteose residue in this respect is but slightly less then that of the whole extract, in spite of the fact that proteins have been removed as completely as is possible by boiling with acid.

Assuming, then, that the tuberculin type of reaction is a response to a proteose antigen, while the anaphylactic reaction is associated with the proteins, we are still confronted with the puzzling fact that the proteose reaction occurs only in infected animals and cannot ordinarily be induced in animals treated and rendered anaphylactic by injections of dead bacterial substances.

It might be assumed that the living tubercle bacillus in contact with the animal tissues produces a sensitizing substance which is not present in culture fluids or in dead tubercle bacilli. This idea is rendered improbable by the consideration that, although we almost uniformly fail in sensitizing guinea pigs to the cutaneous test by prolonged treatment with concentrated culture fluids or with bacillus extracts, nevertheless, skin reactions can be elicited in tuberculous animals by the proper application of such substances.

Another possibility, however, is that the substance which sensitizes to the tuberculin reactions is actually represented in the various tuberculin preparations and tubercle bacillus extracts, but that the intermittent method of injection, which must necessarily be used in the preparation of guinea pigs or other animals, does not simulate the manner in which these substances are being constantly diffused out into the animal tissues from growing foci. The supposition that the difference may lie in the manner of sensitization, both as to the time factor and in regard to the quantitative relations, is suggested by such experiments as those of Krause who has shown that cutaneous hypersensitiveness coincides with the establishment of a focus, diminishes with the healing of the focus, and varies directly with the intensity of the disease. It is suggested that in specific phenomena

of hypersensitiveness, of the tuberculin, typhoidin, mallein types, etc., we may be dealing with antigens that are subject to laws of sensitization and antibody formation, quite different from those governing the phenomena of protein anaphylaxis; in which the hypersensitiveness is elicited only by an intensive and concentrated contact with the antigenic substances; and in which, soon after the stimulus is removed, the hypersensitiveness diminishes. This, at least, seems a logical conclusion in regard to guinea pigs.

It should also be borne in mind, as has been suggested by Krause, that a general tuberculin reaction blunts skin hypersensitiveness considerably, and that a similar blunting of both the anaphylactic and the skin hypersensitiveness has been noted by others, as well as by ourselves, in the late stages of a fatal tuberculosis in guinea pigs, observations which indicate a reaction at least analogous to antigen-antibody reactions in general.

These considerations suggested to us the possibility that, although we might be giving the animals which we had treated with bacterial extracts some of the material which sensitizes them in the course of infection, we were not, perhaps, administering to them a sufficient amount of this proteose-like substance and were not giving it with the continuity with which it passes into the circulation of an animal suffering from an active process.

In order to obtain some light upon this, we carried out a number of experiments as follows:

Tubercle bacillus cultures were grown on 5 per cent glycerol peptone broth. When the growth had reached the size of a silver dollar or slightly more, but one-quarter of this growth was carefully lifted into another flask containing 100 cc. of similar glycerol broth, and the remaining three-quarters was killed at 80°C. for ½ hour. This dead growth, at least three-quarters of the original growth, was now washed several times with salt solution and infused in a flask containing 100 cc. of broth of the same lot as that on which the living one-quarter had been inoculated. The two flasks were then put into the incubator, and after the 2nd day skin reactions were done every day with the fluids from these flasks on tuberculous guinea pigs.

Fig. 8 shows the results. The reaction marked 1 on this figure is the reaction obtained with 0.1 cc., intracutaneously injected, of a 1:3 dilution of the 4 day growth in a highly sensitive guinea pig. The

reaction marked 2 shows the corresponding reaction carried out with the broth in which the dead tubercle bacilli, three times greater in bulk than the living ones, had been infused for the same length of time and used in the same dilution.

In order to eliminate any obstruction to diffusion which might have been brought about in the dead culture by the fact that heat was used in the killing, we did other experiments in which 0.5 per cent carbolic acid was added to the culture material to kill it.

Such an experiment is the following one. In this case a living growth about 1.2 cc. in diameter was floated on a flask of 100 cc. of glycerol broth and the remainder, about four times this amount, was submerged by shaking in 100 cc. of a similar broth flask, and 0.5 per cent carbolic acid added. On the 6th day reactions were done on tuberculous guinea pigs and on a control with the results shown in Table III.

TABLE III.

Skin Reactions Obtained with Material Killed with Carbolic Acid.

Broth.	Dilution.	Results with tuberculous guinea pigs.			Results with normal control.
		No. 19.	No. 20.	No. 21.	
From living bacilli.	1:10	+	++	++	−
	1:20	+	+	±	−
From killed bacilli.	1:10	+	+	−	−
	1:20	±	±	−	−

Reactions of this type would indicate that the materials which caused skin reactions of the tuberculin type were being constantly diffused out from the growing cultures, while a limited amount only could be extracted from dead tubercle bacilli.

It is not impossible also that such a conception might indicate that these proteose-like materials were constantly being produced from the more complex material which we have spoken of tentatively as nucleoproteins, which represent the bulk of the soluble bacterial constituents and that this is the reason why we have never been able to free these acid-precipitable substances of their skin reaction capacity.

With this in mind we have recently treated four guinea pigs with large amounts of the so called nucleoproteins, giving each animal

four intraperitoneal injections, each of which represented at least 50 cc. of original extract. And in these animals, 14 days after the last injection, we saw the first hopeful indications of positive skin reactions of the tuberculin type, artificially induced. These experiments will be continued.

IV.

DISCUSSION AND CONCLUSIONS.

The work reported in the preceding sections justifies, we think, a number of definite conclusions. In addition to this, some of the experiments indicate a line of thought which may lead to considerable alteration in our conceptions, both of phenomena of bacterial hypersensitiveness and of infection.

1. In guinea pigs two fundamentally different types of intradermal reactions may be observed. One of these is the immediate, transitory reaction which develops in animals sensitized against proteins (horse serum, etc.) and may be regarded as one of the manifestations of general protein hypersensitiveness, or anaphylaxis; the other is the tuberculin type of skin reaction which develops more slowly, leads to a more profound injury of the tissues and is independent of anaphylaxis as ordinarily conceived.

2. The tuberculin type of hypersensitiveness (as well as probably the typhoidin, mallein, abortin reactions, etc.) does not develop at all in guinea pigs sensitized with proteins, like horse serum, etc. While this form of hypersensitiveness may eventually be induced with materials not bacterial in origin, it has been observed up to date only as a reaction of bacterial infection.

3. Methods of treatment with protein material from bacterial cultures which sensitize guinea pigs to anaphylactic reactions with the bacterial extracts, do not sensitize them to the tuberculin type of reaction. Such sensitization is easily accomplished only by infecting the animals with living organisms. No reliable method of sensitizing guinea pigs to such reactions with dead bacterial material has as yet been worked out, though a few hopeful experiments have been obtained with massive injections of large amounts of the acid-precipitable substances (nucleoproteins?) from bacterial extracts.

4. In animals made hypersensitive to the tuberculin type of reaction by infection with living bacteria, the reaction may be elicited by intradermal injections of bacterial extracts from which all coagulable proteins, nucleoproteins, and Bence-Jones proteins have been removed, as well as this can be done by boiling with acid, etc. This proteose residue alone suffices to elicit such reactions. The exact chemical nature of the so called proteose residue must be further studied and analyzed when we have had opportunity to produce bacterial extracts in large quantity.

These points seem incontrovertible on the basis of our own experiments, as well as those of other workers.

There thus seem to develop two definite forms of hypersensitiveness in guinea pigs infected with bacteria, typical anaphylaxis in which the protein material of the bacterial cells is concerned, which develops late and which can be induced by repeated injections of dead bacterial material, and a hypersensitiveness to non-protein constituents which differs from the former, both in the laws that govern sensitization and in the manifestations which follow injections into the sensitized animals.

While there is virtual agreement among immunologists concerning the essential mechanism of protein anaphylaxis, its dependence upon an antigen-antibody reaction, and the dominating rôle played by the sessile antibodies, the mechanism of hypersensitiveness to tuberculin and similar bacterial substances is still a problem of much uncertainty.

The most striking difference between the two phenomena lies, as we have seen, in the criteria of sensitization, in that hypersensitiveness to the tuberculin type of reaction can hardly ever be induced by any of the ordinary methods of preparation with the constituents of dead bacteria, but develops promptly (7 to 10 days) in the course of actual infection with living organisms.

The considerable specificity of such reactions forces the conclusion that the sensitizing substance must, in some way, be derived from the infecting microorganisms.

The idea that the failure of sensitization with dead culture materials is perhaps due to the elaboration in the body of infected animals of bacterial products not represented in extracts of test-tube cultures is rendered unlikely by the fact that in the tuberculin-sensitive,

infected animals, we can produce the reactions by the application of such dead extracts. It is neither logical nor in keeping with biological experience to assume that one substance will sensitize to reaction with another. This mistake was made early in the study of anaphylaxis in another connection and caused considerable delay of progress.

Krause has shown that tuberculin sensitiveness may be blunted in infected animals by massive, but sublethal injections of tuberculin, and we have obtained some indications of the same thing. Moreover, others as well as ourselves have seen tuberculin reactivity decline in guinea pigs and in man in the stages of very severe infection. These facts would eliminate any assumption of mere cumulative injury as explaining this type of reaction, and stamp it as a mechanism at least analogous to ordinary anaphylaxis.[7]

The only remaining possibility to explain the difference between infected animals and those treated with dead bacterial constituents would be to assume that the difference must lie in the manner in which the sensitizing substance is administered to the animals, and that sensitization with the proteose residue materials depends upon criteria of sensitization differing in regard to the time and quantity factors from those governing protein sensitization. If one considers the relatively simpler chemical structure and perhaps physically greater diffusibility of the materials concerned in this reaction, one might readily expect such differences in the methods needed for sensitization.

In keeping with such a line of reasoning our experiments have shown that the tuberculin active materials are constantly and rapidly being diffused out into the culture fluid from growing organisms, in quantities greater than can be extracted from similar amounts of the dead bacteria. It seems reasonable to assume from this that the same thing may happen in the animal body harboring a growing focus. And it would seem quite likely that the association of the tuberculin type of reaction with actual infection may depend upon the fact that sensitization to these non-protein substances depends upon a constant steady absorption of large amounts of the material.

Moreover, the only hopeful experiments on the artificial production of tuberculin sensitiveness in guinea pigs obtained by us were those

[7] Direct attempts to show such cumulative toxic action have failed in our hands.

in which massive doses of the nucleoprotein material injected into guinea pigs gave rise to a moderate skin sensitiveness.

Does the so called proteose residue form antibodies, and, if so, are substances analogous to antibodies involved in the tuberculin type of hypersensitiveness?

The failure to transfer passively this form of hypersensitiveness to normal animals with the blood and tissues of tuberculin-sensitive ones would suggest that no antibodies are involved. But this is not conclusive on the basis of available experimental facts. We are inclined to believe that antibodies of a sort are involved, for the following reasons: (*a*) In our experiments with the uteri of highly sensitive extract-treated guinea pigs and of tuberculous guinea pigs, we have occasionally had positive reactions when the proteose residue alone was used. (*b*) We believe that these proteose substances are entirely analogous to the substances studied by Avery and Dochez (22) in the urine and blood of typhoid and pneumonia patients. They obtained precipitin reactions against homologous immune sera with the urine of infected cases concentrated by evaporation after boiling with acetic acid to remove coagulable proteins. (*c*) Petroff, with whom we discussed this proteose residue early in our work, has produced it, and tells us that he has obtained precipitin reactions with it by titrating it against the serum of a sheep treated for a long time with tubercle bacillus products.

In suggesting an antibody response to a non-protein antigen we are aware that we are opposing what has been regarded as a well established doctrine in immunity; this is justified, or at least mitigated, we believe, by the consideration that reactions of the antigen-antibody type are the only explanation of specificity; and tuberculin, mallein, and typhoidin reactions are to a considerable degree specific. If such reaction bodies cannot be produced by precisely the same methods of administration as to time and quantity which are successful in calling forth protein antibodies, this should not astonish us, since, after all, the substances that we are dealing with are simpler in chemical structure than are the proteins, and physically are probably of relatively greater diffusibility. It may be that the greater diffusibility of the proteose-like substances transfers much of the actual reaction phenomena to an intracellular location, and that this

to some extent influences the presence of circulating antibodies. It may also be that these more diffusible non-protein antigens are more rapidly eliminated from the animal body than are the proteins. Indeed, the above mentioned observations of Avery and Dochez, and the recent work of Wildbolz (23), Lanz (24), Imhof (25), and Gibson and Carroll (26), who demonstrated tuberculin active antigens in the urine of active cases, would corroborate such a view. The evidence available at the present time, however, concerning antibody formation to these non-protein substances is, we recognize, largely indirect, at least as far as our own work is concerned, and we present it in the present connection purely as a working hypothesis.

Finally, perhaps the most important theoretical consideration indicated by our experiments is the following. We have in the tuberculin reaction a form of hypersensitiveness which seems to be (in guinea pigs, at least) analogous entirely to the typhoidin reaction, the mallein reaction, and the abortin reaction. Whenever reactions of this type have been carefully studied, whatever the bacteria involved, they have been associated with infection as in tuberculosis, and have been followed by analogous clinical manifestations. It would seem perhaps that we are dealing with a law applicable to bacterial infection in general.

It would appear that certain non-coagulable substances of uncertain chemical constitution are being constantly elaborated in the course of bacterial growth, and passed into the circulation of infected animals. As a result of this, infected animals become sensitized to these heat- and acid-resistant materials, in tuberculosis in the course of 1 to 2 weeks, in the case of more rapidly growing bacteria perhaps sooner. Early in the course of infection, the animal becomes sensitized and subsequently the further elaboration and distribution of these materials from the bacterial focus plays a fundamental part in the injury of the animal. These proteose-like substances, like tuberculin, possessing but slight toxicity for the normal animal, become highly toxic to the sensitized one. Thus, these substances, while not being true exotoxins in the ordinary sense, would still represent a highly toxic bacterial product comparable in its injurious effect to toxins when produced in the body of an animal thus sensitized.

If there is any value in these deductions the attention of bacteriologists should be turned to the non-protein constituents of bac-

terial cells in their further immunological studies, as well as to the protein materials.

It is obvious that the next step in our investigations must consist in producing the non-coagulable material from bacterial extracts in considerable quantity, to determine their antibody-forming properties in detail, and elucidate, if possible, the laws which govern sensitization with them. This work has been begun, but it has seemed advisable to publish this as far as we have gone because it will take a long time before it can be completed.

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EXPLANATION OF PLATES.

PLATE 40.

FIG. 1. Guinea Pig 22. Another instance of positive skin reaction after 9 days, with negative uterine test. This animal was of the same lot as Guinea Pig 23.

FIG. 2. This record is a simultaneous record of two guinea pigs inoculated on November 21, 1920, with large amounts (2 cc.) of a suspension of a young tubercle bacillus culture (human). No. 23 (upper curve) inoculated intraperitoneally, and No. 24 (lower curve) subcutaneously. On December 1, 1920, skin reactions were done with two types of extract, one a sodium carbonate extract and the other a sodium hydroxide extract. No. 23, which was apparently weaker and emaciated, showed practically no reaction in either place, whereas No. 24, to our surprise, showed a definite reaction at both points. In spite of the differences in skin reactions, it will be seen that the uterine reactions in both guinea pigs were negative. The two uteri were put into the same bath simultaneously in order to subject them to exactly the same conditions. The negative reaction of the uterus was confirmed with the other horns of both guinea pigs. The surprising thing is the earliness with which the skin reaction appeared, which tends to confirm one of a series of observations that the skin reaction appears much earlier than the uterine reaction.

FIG. 3. Guinea Pig 25. Inoculated subcutaneously with tubercle bacilli on November 22, 1920. Showed that powerful anaphylactic reaction may develop within 3 weeks after a heavy inoculation with tubercle bacilli. This, however, is unusually early. Skin reaction in this guinea pig was positive.

PLATE 41

FIG. 4, *a* and *b*. (*a*) Guinea Pig 26. (*b*) Guinea Pig 27. Two guinea pigs of a series injected every other day intraperitoneally with unfiltered alkaline extract of powdered tubercle bacilli. Last injection given November 29, 1920. No. 26 gave a negative skin reaction on December 6, but a positive uterine reaction on December 7. A similar uterine test with another guinea pig (No. 28) showed a very moderate uterine reaction, and this, with other experiments, indicates that the uterine or anaphylactic reaction does not develop until at least 8 or 9 days after the last preparatory injection. Guinea Pig 27 in the second record was treated exactly like the preceding animal, but here the tests were not done until December 21, slightly over 3 weeks since the last extract injection. Here again the skin reaction was entirely negative and the uterine test very much more powerful. The uterine reaction does not develop to its fullest extent until about 2 or 3 weeks after the last injection.

FIG. 5, *a* to *c*. (*a*) Normal guinea pig. (*b*) Guinea Pig 29. (*c*) Guinea Pig 30. The records show a failure of reaction of the uteri of two different sensitized guinea pigs (Nos. 29 and 30) after the addition of the proteose residues alone, but

powerful reactions when, following this, whole extracts were introduced into the bath. The record at the top is a normal control with the same extracts, showing that the reactions below are specific.

PLATE 42.

FIG. 6, *a* and *b*. This is one of two preparations in which the materials used consisted of two fractions obtained from the same 50 cc. of a 0.02 per cent alkaline extract of powdered tubercle bacilli. The extract was centrifuged until clear, the supernatant fluid acidified to pH 4.5, which was the point at which the flocculent precipitate did not increase. This was centrifuged away and spoken of as nucleoprotein. This nucleoprotein was twice redissolved in more than 50 cc. of alkaline salt solution and reprecipitated with acid, the material being centrifuged in each case in order to purify as much as possible. The supernatant fluid was then boiled in the acid condition to remove coagulable proteins and twice filtered through a Berkefeld filter. It will be noted that in looking at these two preparations together after testing against different strips of the same sensitized guinea pig uterus (No. 31), the total amounts of the two fractions gave the same results, though in different proportions, an additional proof that cumulative effects had nothing to do with the results.

FIG. 7. February 18. The sensitized guinea pig treated with Material B_2 which, as stated before, gave definite though somewhat weakened skin reactions. Here, 6 cc. on the outside of the bag, which still gave skin reactions, gave no anaphylactic reaction. The high rise is probably due to the fact that this uterus was very irritable. That the rise was not an anaphylactic reaction is apparent from the immediate return to the base-line, and the fact that there was no apparent desensitization to the subsequent addition of 2 cc. from the inside of the bag. Dialysate was positive on skin and negative on uterus.

PLATE 43.

FIG. 8. Drawing of skin reactions on a tuberculous guinea pig, carried out with broth from a culture of living tubercle bacilli 4 days old, 1:3 dilution, at the point marked 1, and a similar reaction with a similar amount of broth from a killed culture, 1:3 dilution, at the point marked 2.

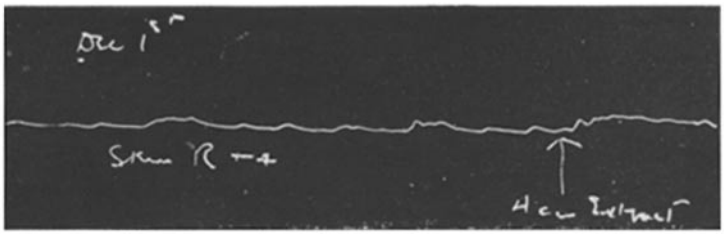

FIG. 1.

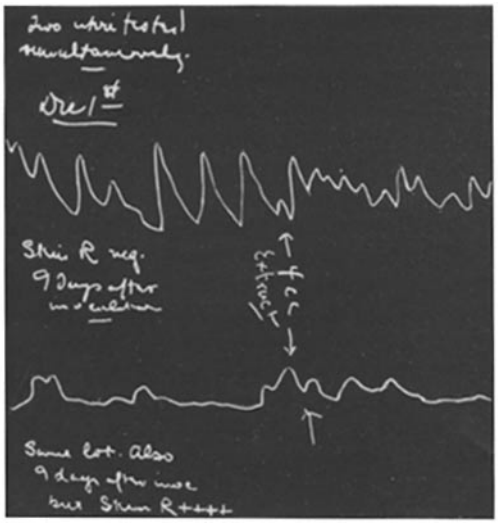

FIG. 2.

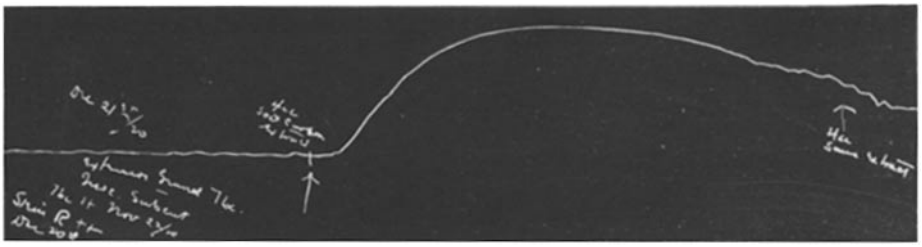

FIG. 3.

(Zinsser: Tuberculin reaction.)

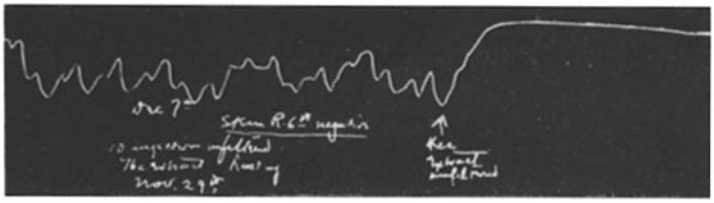

FIG. 4, *a*.

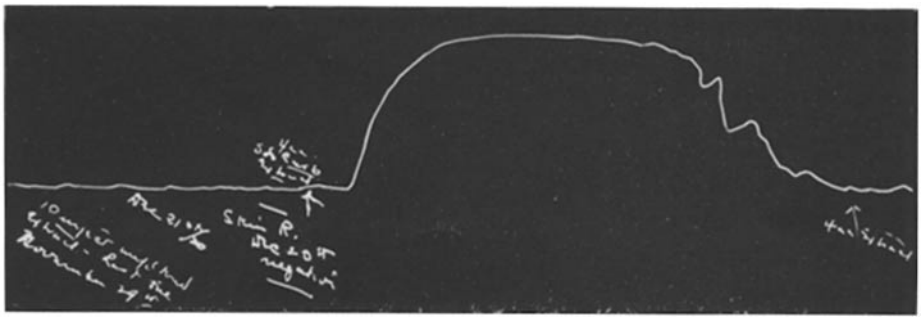

FIG. 4, *b*.

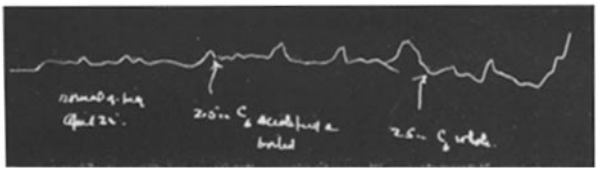

FIG. 5, *a*.

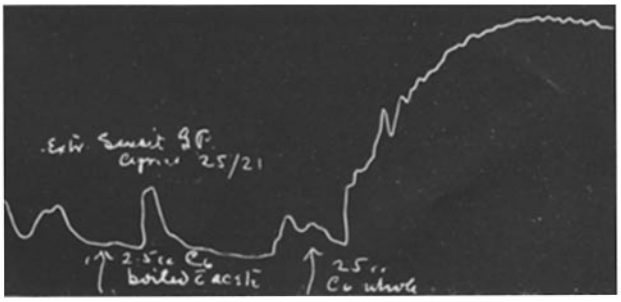

FIG. 5, *b*.

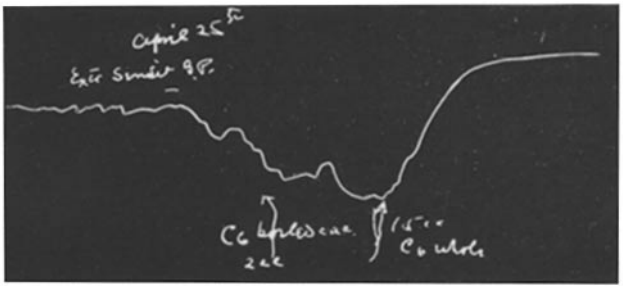

FIG. 5, *c*.

(Zinsser: Tuberculin reaction.)

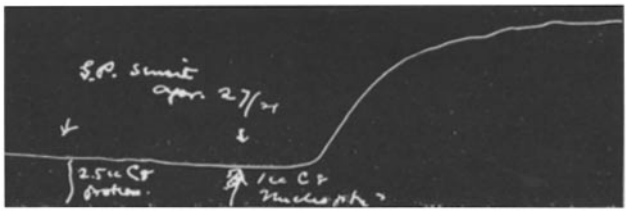

FIG. 6, *a*.

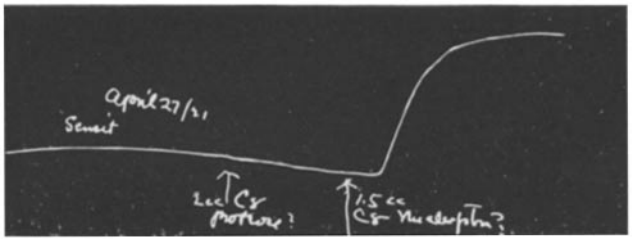

FIG. 6, *b*.

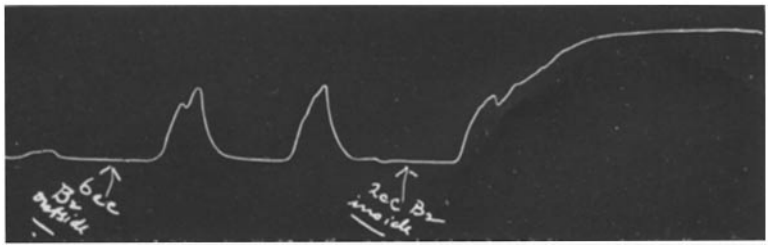

FIG. 7.

(Zinsser: Tuberculin reaction.)

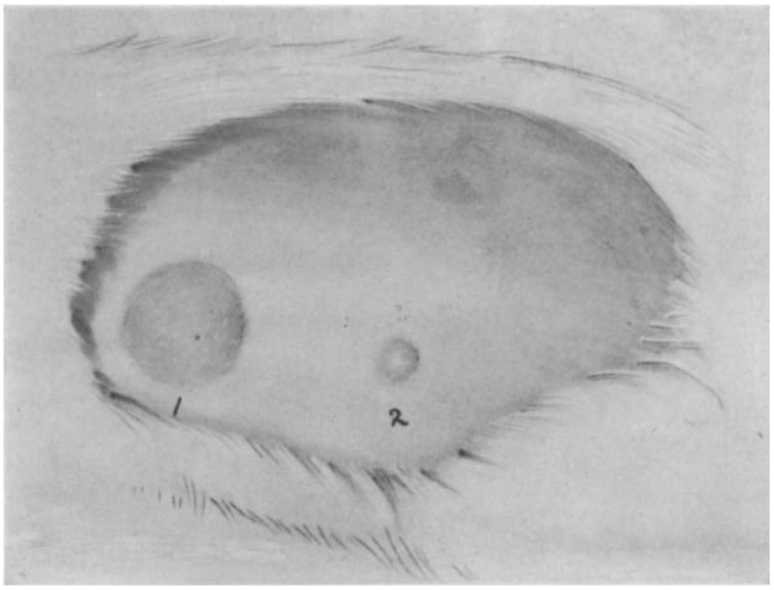

FIG. 8.

(Zinsser: Tuberculin reaction.)