CELLULAR REACTIONS TO FRACTIONS ISOLATED FROM TUBERCLE BACILLI

F. R. SABIN

Laboratories of The Rockefeller Institute for Medical Research, New York

The cellular reactions in tuberculosis form one of the striking features of the disease and it is important to note that they can be produced by the dead bacilli. In 1890, Maffucci (1) studied the effect of subcutaneous injections of dead organisms; he recorded the formation of abscesses and the subsequent death of the animals from marasmus. The next year, Koch (2), in the well-known article describing the so-called Koch phenomenon, tested the reaction to subcutaneous injections of dead, as well as living tubercle bacilli, in normal and tuberculous guinea pigs. In normal animals he also produced local abscesses with dead organisms. However, in 1890, Wyssokowitsch (3) had also studied the effects of dead tubercle bacilli, using both subcutaneous and intraperitoneal injections in rats. He found nodules of epithelioid cells and giant cells and infiltrations with neutrophilic leucocytes; that is, the production of tubercles. These observations were repeated and extended by Prudden and Hodenpyl (1891) (4) and by Prudden (1891) (5), and were then repeatedly confirmed (6–11).

The fact that dead bacilli will produce the lesions of tuberculosis is of great significance and sets the disease apart from other infections. It demonstrates that, in the actual infection, dead bacilli, as well as living, may play a rôle and gives an especial interest to the study of the tissue response to chemical fractions isolated from the organism. Proteins, carbohydrates, and lipoids all play a part in these reactions, but the lipoids alone produce tubercles. These substances have the power to stimulate the formation of the epitheloid cell and its multinuclear form, the Langhans giant cell, which together are the essential structural units of the tubercle.

While chemical analyses of the organisms give only the vaguest clues

141

to the complex compounds through which tubercle bacilli disintegrate in the body, it is, nevertheless, remarkable how closely the cellular reactions to certain chemical fractions reproduce the tubercle and how many of the various accessory cellular responses can be elicited by other fractions.

In the reactions to the fractions of the earliest analyses, however, it is not possible to make a sharp discrimination between the effects of proteins, carbohydrates, and lipoids. The earliest analyses of tubercle bacilli showed a high content of phosphorus and of lipoids. Hammerschlag (12) isolated material soluble in alcohol and ether and found that it made up about 27 per cent of the weight of the organisms. This material, on analysis, yielded lecithin, fat, and a certain toxic substance. On injecting the material soluble in alcohol and ether, and thus containing lipoids, the animals died with the symptoms which have since been definitely related to proteins and carbohydrates. This indicates that his material was a mixture. Hammerschlag also isolated a protein from the bacterial residue. De Schweinitz and Dorset (13) found even more lipoidal material, namely, 37 per cent.

The earliest report of a study of cellular reactions to materials from tubercle bacilli is that of Weyl (14), in 1891, who extracted the bacilli with caustic soda and obtained material which caused necrosis. Auclair (15) and Auclair and Paris (16) made a series of interesting biological studies with fractions from the tubercle bacillus. They killed the organisms with sunlight. Auclair (1897) collected the volatile substances from the organisms, responsible for the characteristic odor of the cultures, and found them toxic. With small doses there was congestion and hemorrhage in the lungs and liver; in larger doses, death. In tuberculous animals there was a rise in temperature and a rapid loss of weight. In 1898 Auclair described the serious illness of eight people from the volatile products set free while the ether extract was being filtered. Koch (1897) (2) had already called attention to the danger of grinding tubercle bacilli in open mortars. The effects of these volatile products have been repeatedly experienced by those who have worked with this organism. In the experience of the H. K Mulford Company which has handled enormous quantities of these organisms, the bovine organism liberates more of the volatile material than the other strains.

Auclair suspended the bacilli in ether and noted that two types of organisms could be demonstrated; cocci which floated in the ether and made it milky, and bacilli which sank to the bottom on standing. The

cocci, inoculated subcutaneously, gave small nodules which caseated. The bacilli, inoculated in the same way, gave lesions which were resorbed; abscesses were rare but the animals showed loss in weight. He found that ether extracted a lipoid analogous to cholesterin; alcohol, a lecithin; and chloroform, a wax. The extracts obtained from ether and chloroform were both acid-fast as were the organisms after their removal. He found that all of the lipoidal substances gave cellular reactions comparable to those produced by the bacillus. Injected under the skin, the ether-soluble material gave abscesses which caseated. With intratracheal injections, it produced pneumonia with marked caseation. The chloroform-soluble material was slower in reaction; subcutaneously it caused sclerosis, and intratracheally a reaction like tuberculous pneumonia. From these results Auclair emphasized caseation as due to lipoids from the organisms. These biological reactions to the materials extracted by Auclair were then tested in different parts of the body (17-20). Dominici and Ostrovsky (21) extracted heat-killed tubercle bacilli with water and obtained substances which, injected subcutaneously, gave tubercular tissue in the organs. They concluded that lipoids were not essential for the production of tubercles, but inasmuch as the tuberculo-phosphatide separated by Anderson, to be described later, is soluble in water, it is possible that this aqueous extract contained not only protein and polysaccharide but also some phosphatide. With this aqueous extract they also produced a general stimulation of lymphoid tissues and showed a remarkable increase in lymphoblasts (see their plate XXXIII) in the lymph follicles.

The lipoidal material which Levene (22) isolated from the tubercle bacillus was tested at the Trudeau Sanatorium and produced sterile abscesses under the skin. Morse and Stott (23) and Ray and Shipman (24) isolated lipoids from tubercle bacilli for the purpose of studying the cellular reactions to them. Morse and Stott used first ether and then boiling alcohol for the extractions. Ray and Shipman retested these methods and used also the more effective methods of Long, namely, alcohol followed by hot toluol. These lipoids were then extracted by ether and by chloroform. With the lipoids extracted by these means, both groups obtained tubercular tissue consisting of epithelioid cells, giant cells, and fibroblasts, and recognized that the formation of the tubercle may be considered as a foreign body reaction toward tuberculo-lipoids. Although these are important steps, it is not possible to recognize the nature of the lipoidal material extracted and their reports of the biological testing are not conclusive because they used diluting

menstrua for the tuberculo-lipoids, olive oil in the one case and sodium stearate in the other, without adequate control of their reaction.

Acid-fastness in tubercle and paratubercle bacilli was from the very beginning a factor of great interest in connection with the chemistry of the lipoids of these organisms. That certain organisms possessed the property of resistance to decolorizing both with acids and with alkalies was first noted by Neisser; this property played an important rôle in the work of Koch, and was further analyzed by Ehrlich. Koch (2) (1897) noted that if tubercle bacilli were ground in a mortar they lost this property entirely. Benians (25) and Sherman (26) confirmed this and showed that acid-fastness could be made to disappear from tubercle bacilli by simply rubbing them between two slides. These observations suggest that if the property is due to a specific substance, a simple dispersion of the substance into sufficiently fine particles is sufficient to remove the reaction. As early as 1886, Bienstock (27) and Gottstein (28) both showed that the property of acid-fastness was associated with lipoids. Auclair found that all of the lipoidal substances, as he extracted them, were acid-fast; and that, after their removal, this property was still retained by the bacilli. It is now clear that Auclair's separation of the lipoids was partial and that the complete defatting of the organisms by lipoidal solvents is extremely difficult, if not impossible without the breaking up of the bacilli. Aronson (29) found that the wax from the organism is acid-fast; this was confirmed by Borrel (30) and by Bulloch and Macleod (31). Bulloch and Macleod showed that the substance which had this property was extremely resistant to saponification and was an alcohol. In 1910, Aronson showed that saponification of the entire organism with strong alcoholic caustic potash gave an unsaponifiable material which was acid-fast. He found also that it was a higher alcohol. This has now been shown in the more recent analyses of Tamura (32), Goris (33), and Anderson and his associates (34-42). In Anderson's analysis, it has proved that the material soluble in alcohol-ether entirely lacks the property of acid-fastness after the traces of unsaponifiable material have been removed from it.

Early in the study of tubercle bacilli (1883 and 1884), Malassez and Vignal (43) suggested that there is a non-acid-fast phase of Koch's organism. They found a non-acid-fast organism in a nodule from the skin of a child who had died of tuberculous meningitis, inoculated the material into guinea pigs and recovered acid-fast bacilli. Ferran (1897) (44) then showed that non-acid-fast forms could be developed in cultures of tubercle bacilli by reducing the amount of peptone and glycerin

in the media in each succeeding transplant. The cultures which he obtained in this way contained some acid-fast organisms and were of low virulence. In 1903, Auclair (15) repeated this experiment, but by omitting the glycerine altogether, he degraded a culture into forms which were wholly non-acid-fast and avirulent. Moreover, he was unable to cause these organisms to recover their virulence. He analyzed this culture by the same methods he had been using for the acid-fast forms, obtained the same type of acid-fast lipoids as from the virulent cultures, but in less amount. Moreover, these lipoids gave the same biological reactions as those from virulent tubercle bacilli. The occurrence of non-acid-fast forms of tubercle bacilli has now been repeatedly reported (45 to 57) since their first discovery by Malassez and Vignal and Ferran. Moreover, Kahn's observations (56, 57), in which he has isolated a single acid-fast bacillus from a culture of tubercle bacilli, watched it fragment into cocci and then still further subdivide into colonies of tiny, non-acid-fast organisms, and then regenerate the original form, make conclusive proof of a non-acid-fast cycle in the life of the tubercle bacillus. This fact enhances the interest in the early observation of Auclair that the non-acid-fast form contains an acid-fast lipoid which can be demonstrated chemically.

In comparing the biological reactions to fractions from different chemical analyses, it is of the utmost importance to know the nature of the material used. In a recent extensive review, Albert-Weil (58) has made tables of many of the chemical analyses which elucidate the comparisons where possible (13–16, 22–24, 29–33, 59–68). He has presented charts which analyze the sequence of the procedures of Goris (38) and of Anderson (37–42) who have made the most extensive studies of the tuberculo-lipoids. Many factors make the comparisons of these analyses difficult: variations in the strains of the bacteria used, in the media on which they were cultivated, the age and the condition of the organisms when the analyses were made, the solvents used, and finally, as Albert-Weil has pointed out, the order in which they were applied. Perhaps the most important factor contributing to the difficulties in comparing different biological studies of the lipoids from different extractions has been brought out by Anderson, namely, that no one solvent makes a complete separation of one lipoid from the others. For example, he found that a little of the unsaponifiable acid-fast material which is soluble in chloroform came down with the entirely non-acid-fast material soluble in alcohol and ether. This means that some of the materials of the earlier analyses were probably mixtures of the lipoids

separated by Anderson. No claim of purity, in the chemical sense, is
made for the products of the recent analyses, but the products have been
submitted to biological testing in succeeding stages of purification. In
the case of some of these products, a relatively simple and entirely con-
stant biological reaction has been observed.

Chemical analysis of the bacteria depended from the start on devising
suitable media for the growth of the organisms. This was clear to
Hammerschlag (12) who made the first analysis. He said that his
analysis, made not long after the discovery of the organism (1882) (2)
had to await the introduction of glycerin (Nocard and Roux, 69) into
the media, which made possible an adequate growth of the bacilli.
Hammerschlag used glycerinated bouillon and glycerin-peptone agar.
In 1892, Kühne (70) showed that tubercle bacilli could be grown on
simple media without protein, and in 1894 Proskauer and Beck (71)
developed what is still regarded as the simplest formula on which growth
is possible, namely, ammonium carbonate, mono-potassium phosphate,
magnesium sulphate, and glycerol. Luxuriant growth is necessary for
chemical analysis. Armand-Delille, Mayer, Schaeffer and Terroine
(72) studied the nitrogenous compounds in the media and found that
the monoamino acids were the indispensable part of the peptone, while
in the bouillon certain extractives (carnosin, creatin, and sarcosin) and
diamino acids (argainine and histidine) were important. Kendal, Day
and Walker (73) then showed that in albumen-free media containing
asparagin, glycerin, and 1 per cent of either mannite or glucose, the
organisms could synthesize their nitrogenous compounds from the
asparagin and their fats and waxes from the glycerin and sugar. The
early work of De Schweinitz and Dorset (13) had already shown that
phosphorus pentoxide made up 55 per cent of the mineral content of
tubercle bacilli and Baudran (74) found that tubercle bacilli use the phos-
phates in the media. These studies were followed by more extensive
research on the metabolism of the organism and the various substances
necessary for their best growth (75–80). The facts disclosed by these
investigations have led to all of the synthetic media, of which the Pros-
kauer and Beck (71), the Sauton (81), and the Long (75, 76) are ex-
amples. Only by the use of such media can one be sure that all of the
complex carbohydrates, lipoids, and proteins found in the bacilli and
in the fluid media on which they have been grown have been synthe-
sized by the organisms.

A recognition of the difficulties in comparing the results of previous
analyses and of the fundamental importance of using standard strains

of organisms and synthetic media without protein led to a coöperative plan by the Research Committee of the National Tuberculosis Association, William Charles White, United States Public Health Service, Chairman. This plan was to select standard strains of organisms, have them grown in large quantities on synthetic media, analyzed chemically, and have the various products tested biologically to determine how many of the factors of the disease could be reproduced. Certain standard strains of tubercle bacilli, human, bovine, and avian, together with a strain of the timothy grass bacillus and a strain cultivated from a leprous lesion, were chosen. The strains used are shown in Anderson's tables (1932). The first work was done with the human strain H-37, and then fractionation by the same procedures was made of the others. The organisms were grown by the H. K. Mulford Company on Long's synthetic media made entirely from chemicals of the same standard lot, purchased at one time. All of the cultures have been grown in pyrex glass. No pains have been spared to make the cultural conditions as uniform as possible. Moreover, the organisms have been furnished to the chemists in quantities adequate for analysis. The analyses of the lipoids and carbohydrates related to them have been made by Dr. R. J. Anderson, with the aid of Drs. E. G. Roberts, E. Chargaff, M. L. Burt, M. C. Pangborn, and N. Uyei, Sterling Chemical Laboratory, Yale University. The proteins and carbohydrates of the bacilli have been extracted by Prof. T. B. Johnson, of Yale University, with the aid of Drs. R. D. Coghill, E. B. Brown, and A. G. Renfrew. Dr. M. Heidelberger, of Columbia University, has studied the several carbohydrates from the organisms. The bacillary proteins and carbohydrates found in the media have been isolated and tested biologically by Drs. E. R. Long and F. B. Seibert, of the University of Chicago. It has been our privilege to receive some of each of these fractions for biological study. A complete bibliography of this work up to 1929 has been published (82).

Proteins from tubercle bacilli are responsible for the reactions that give the skin test (83, 84). Injected intravenously they produce the changes in the cells of the cirulating blood common to all other proteins, and give a rise of temperature in the normal animal. The tuberculous animal is remarkably sensitive to them; they give a rise in temperature or, after sufficient dosage, a rapid fall in temperature, followed by death. The cellular reaction to the proteins is mainly a proliferation of plasma cells (85). There is also damage to the endothelium of the vessels, causing hemorrhage (86). Injections of the tuberculo-polysaccharides may cause death in the tuberculous animal, and in the tissues of the

normal animal they are chemotactic and toxic to the neutrophilic leuco-
cytes (86–89). The rise in temperature which follows the injection of
polysaccharide is probably produced by an accompanying nitrogenous
compound. The lipoids, on the other hand, produce a great variety
of cellular reactions, reproducing many of the lesions characteristic of
the disease (86–89).

CELLULAR REACTIONS TO TUBERCULO-LIPOIDS. With the abundant
material furnished by the H. K. Mulford Company, Doctor Anderson
(34–42) separated the lipoids of tubercle bacilli into a phosphatide, an
acetone-soluble fat, and a wax. Each of these three substances gives
rise to cellular reactions which are so constant and so characteristic
that the material injected can be determined from the lesions.

I. *Cellular Reactions to Tuberculo-Phosphatide and Phthioic Acid.*
Phosphatide. An alcohol and ether extract was first made from the
bacilli. This material was then extracted with acetone, and a phos-
phatide which was insoluble in acetone and a fat which was soluble,
were obtained. Traces of wax and fat were removed from the phospha-
tide and then it was further purified. The first preparation which we
received from Doctor Anderson contained a few acid-fast bacilli in small
clumps. After this lot, Doctor Anderson filtered the material through
porcelain candles, a procedure which completely removed all of the acid-
fast débris. The material is stable as far as can be judged from its
biological reaction which has not changed in the material which was
prepared in 1926. The phosphatide is a soft, white, granular substance.
It has been examined for us under crossed Nicol prisms by Dr. R. W. G.
Wyckoff of The Rockefeller Institute, who reported it to be predominantly
crystalline. This, of course, does not mean chemical purity, which
Doctor Anderson has not yet obtained. He found that the phosphatide
from the human strain of tubercle bacilli, H-37, contained 0.36 per cent
nitrogen, the state of which he has not yet determined. The phospha-
tides from the other acid-fast organisms contain different percentages
of nitrogen, as shown in Anderson's table II (1932, 34). In 1920,
Linossier (65) extracted phosphatides from tubercle bacilli which he
said were sticky or crystallizable with difficulty. It may thus be con-
cluded that the tuberculo-phosphatide is a material which can crystallize.

Another property of no less importance is that when distilled water
is first applied to the phosphatide, it breaks into typical myelin figures,
looking much like the material in the sheaths of fresh, medullated nerve
fibers, except that it is less refractive. This property is of great interest
in the biological tests, because it enables one to identify the material

after it has been phagocytized by cells.   The phagocytic cells of the connective tissues have been extensively studied both in their reactions toward other damaged cells, such as leucocytes in the tissues and free red blood cells, and toward insoluble particles, such as carbon, trypan blue, and carmine.   These particles, which the cells have no power to disintegrate, have been used experimentally because they can be identified.   To such experimental materials may now be added the tuberculophosphatide, which can be identified by the myelin figures, if examined soon after it has been phagocytized.   Moreover, one may follow to some extent the signs of the breaking up of this material within the cells, and so study the processes by which the epithelioid cell of tuberculous infections is produced.

In the fresh state, this myelin-like material stains faintly pink in neutral red; after it has been taken into cells, this characteristic stain is retained for a time.   In rabbit tissues the phagocytic cells soon secrete a fluid around the myelin-like masses making them stain red in this dye and obscuring the lipoidal material.   In the guinea pig, on the other hand, the cells can be found engorged and distended with entirely unchanged myelin-like masses for at least twenty-four hours after the injection of the material.   The description of the processes through which the cells which have phagocytized masses of phosphatide become typical epithelioid cells, will follow.   These observations, based on the identification of the phosphatide within a phagocytic cell, establish the point that the epithelioid cell represents a foreign body reaction; thus, the epithelioid cell is the final stage of a cell which has taken in, and in part disintegrated, a foreign lipoid.

The next step in these biological studies was to determine which type of cell of the connective tissues phagocytizes this material and thus gives rise to epithelioid cells.   For this purpose the omentum offers great advantages.   It can be separated into its two layers and spread as a film on a slide, allowing the study of the cellular changes in the living state without any distortion of the position of the cells.   In no other place in the body are the cells of the connective tissues to be seen in simpler form and arrangement.   In the normal omentum there are small masses of cells closely packed together, forming the so-called milk spots.   The milk spots are composed of monocytes and of young connective tissue cells, somewhat less differentiated.   They have basophilic cytoplasm and contain a few vacuoles and many mitochondria. There may also be the simplest type of connective tissue cell, the so-called reticular cell, in which no differentiation of granules or vacuoles

in the cytoplasm can be detected. At times there are some lymphocytes and an occasional branched clasmatocyte. Similar small collections of young connective tissue cells, true milk spots, are to be found everywhere in the connective tissues, as was shown by Möllendorff and Möllendorff (90). In the interspaces between the milk spots of the omentum are scattered branched cells of the macrophage or clasmatocyte type. They always contain some debris in their vacuoles, for they are constantly functioning with reference to substances which are passing through the omentum. These clasmatocytes of the interspaces are the cells which engorge themselves with any of the particulate materials used experimentally for the identification of these cells. They also engulf in large numbers any dead leucocytes and red cells that get free into the tissues. The mass of material which these cells will take up is important in determining their functional nature as macrophages or "big eaters," since a monocyte or even such cells as the secretory cells of the liver will take in a small amount of any of the substances with which the clasmatocyte will engorge itself.

The cells of the milk spots have more deeply basophilic cytoplasm than the clasmatocytes around them and they also have massive amounts of mitochondria, signs which are recognized as characteristic of young cells in the connective tissues. The experiments with the tuberculophosphatide show that it is these young cells of the milk spots which phagocytize this material in massive amounts rather than the mature clasmatocytes adjacent to them.

The tuberculo-phosphatide, injected into the peritoneal cavity as a suspension in distilled water, is taken in by the clasmatocytes, as seen in the omentum, only in small amounts, so that there is only a slight change in their activity and no change in their shape; but it is phagocytized in large amounts by the cells of the milk spots. Lymphocytes, if present, are not involved. Within three days after the injection, the young connective tissue cells and monocytes become greatly increased in size and to some extent in numbers, so that the milk spots appear swollen to the unaided eye. When these cells are stained with neutral red, it appears that the lipoidal material has been segregated within a few vacuoles in the cytoplasm. Some of these cells with highly vacuolated cytoplasm wander into the peritoneal fluid, showing that they are motile; they may also divide. In this state these cells look like clasmatocytes, but it can be shown that they were derived from the monocytes of the milk spots and not from cells which were functioning as clasmatocytes before the experiment began. They represent a stage in the transformation of monocyte into epithelioid cells.

The cells which have engorged the material appear to break it into finer and finer particles and these gradual processes we have arbitrarily described in three stages. The first stage has the largest vacuoles filled with the phosphatide, either intact or slightly changed. These vacuoles are of irregular size and shape. For convenience we shall call this stage the "phosphatide cell." In the rabbit treated with the phosphatide from the human organism H-37, this first stage lasts three or four days.

The second stage shows the cytoplasm filled with a rosette of many coarse vacuoles, uniform in size, making the fixed cell appear to have a foamy cytoplasm. We speak of this stage as the course-vacuoled epithelioid cell; it is reached in five to seven days. The typical epithelioid cell is the third stage and has a rosette of such tiny vacuoles that the cytoplasm in the fixed cell appears dense and nearly uniform in structure. This stage is reached in the second week. The rabbit cells break down the phosphatide from H-37 in a uniform manner, the vacuoles being of approximately the same size in each cell at any given time. In this regard there are marked variations with the phosphatides from the other acid-fast strains.

These different stages in the development of epithelioid cells after the injection of tuberculo-phosphatide have been illustrated in part by Sabin, Doan, and Forkner (89). They will be shown further in a forthcoming paper by Smithburn and Sabin (91) from a study of the reactions of cells to the phosphatides from the various strains of acid-fast bacteria.

With repeated intraperitoneal injections of the phosphatide there is a marked maturation of monocytes and the production of epithelioid cells in such numbers that the outlines of the milk spots become obscured. That the phosphatide from the human strain is not greatly irritating to the cells of the rabbit is shown by the fact that leucocytes are called from the vessels only after the first injection; emigration of leucocytes does not occur after succeeding injections, and those present after the first injection are quickly phagocytized by clasmatocytes and destroyed. There is an increase in undifferentiated connective tissue cells in the omentum with repeated injections of the phosphatide, but it is minimal in the rabbit. In the guinea pig this reaction is much more marked. After from ten to fourteen daily doses of the phosphatide, epithelioid cells are to be found in large masses, in circumscribed tubercles, and in tuberbular granulation tissue. Injection of phosphatide into the pleura or directly into the lung in the rabbit brings out with

especial clearness the fact that the epithelioid cell is the characteristic response to this material.

The response to the tuberculo-phosphatide is clearly the phagocytosis of this material. Other immediate reactions are minimal and consist only in a transient emigration of leucocytes from the tissues. However, important cellular reactions always develop during the second week after the introduction of this material into the tissues; the occurrence of large numbers of Langhans giant cells, the local stimulation of lymphocytes and of plasma cells, and the process of caseation.

The Langhans giant cell, as seen in supravital reaction, has the same type of rosette as the typical epithelioid cell of the third stage. Forkner (92—see also 89) has shown that this type of giant cell is a multinuclear epithelioid type, while the so-called foreign body type is produced in the reaction to foreign material by the fusion of cells, usually monocytes. In the early reaction to the phosphatide, there is a marked tendency for epithelioid cells with two nuclei to occur and by the second week there may be great numbers of Langhans giant cells; in certain animals some of the lesions of the omentum may be predominantly of epithelioid giant cells and frequently the reaction in the retrosternal lymph nodes is made up entirely of these types. The epithelioid giant cell never shows the first stage of the phagocytosis of the lipoid and almost never the stage of the coarse vacuoles. It is a typical epithelioid cell which has become multinucleated. These giant cells show no especial relationship to caseation.

In every experiment in which as much as two weeks have elapsed after the injection of the phosphatide, the tissues have been found with considerable infiltrations of lymphocytes. In some cases the tubercles have had complete capsules of lymphocytes like those to be seen in the actual infection. The areas in which giant cells predominate are likely to show many lymphocytes as well. At present we are unable to analyze this late association of lymphocytes with epithelioid cells in the reaction to the phosphatide, but we have seen no evidence that it is an immediate and direct effect of the phosphatide. In this connection the observations of Dominici and Ostrovsky must be recalled (21).

The epithelioid cell may remain for long periods of time; some have been found six and one-half months after the injection of the lipoid, but in every experiment with any of the phosphatides from the acid-fast bacilli, we have found considerable amounts of caseation during the second week. We have not designated any nodule caseous unless it showed clearly a border of intact epithelioid cells around a center of

necrotic tissue; indeed, in many of the caseous nodules the outlines of the dead epithelioid cells are still present in the center, surrounded by leucocytes. It is our view that caseation is the end stage of the epithelioid cell, the infiltration of the leucocytes being secondary to the death of these cells. Thus the so-called "caseation fraction" of Auclair would be interpreted by us as a material which had produced epithelioid cells. Whether or not the death of epithelioid cells is hastened by tuberculo-protein is being investigated. Caseation is more extensive in the tissues of the guinea pig than in those of the rabbit, after the phosphatide from H-37.

In general the reactions to the phosphatide from the bovine and avian strains of tubercle bacilli, and the timothy grass bacillus and the lepra strain are like, in kind, to those from the human tubercle bacillus but differ in the time which the cells require to break down the lipoid. The reaction to the avian phosphatide is most like that to the human. It differs in that the intracellular dispersion of the material is a little slower, so that at the end of ten days, the epithelioid cells, which are present in massive numbers, are largely in the second stage rather than in the third, that is to say, they are epithelioid cells with coarse vacuoles. There may even be a few cells still in the first stage. There may be only a few giant cells, or, in other animals, they may occur in massive numbers. There is a marked tendency for these giant cells to be of complex type. Instead of being typical epithelioid giant cells of moderate size and with peripheral arrangement of nuclei, these cells are large, have one or more wide cytoplasmic areas corresponding to the rosette of the epithelioid cell, and great masses of nuclei closely packed together either in the center or at one end. They may have resulted from the fusion of several Langhans giant cells. In the reaction to this phosphatide there are quite extensive masses of plasma cells and the same infiltration with lymphocytes as with the material from the human tubercle bacillus. Caseation is marked in the areas of epithelioid cells, whether in the subcutaneous nodules or in the omentum, as well as in the lymph nodes draining these areas.

The reactions of the cells of the rabbit toward the phosphatide from the bovine organism and from the timothy grass bacillus are much alike; these materials are broken down more slowly than the phosphatide from H-37 and from the avian bacillus. In the lesions which have been produced by the bovine phosphatide, there are marked variations in the epithelioid cells, even after two weeks; in some areas these cells are of the third stage with enormous numbers of giant cells; in other areas

the epithelioid cells are in all three stages, showing an irregular breaking down of the phosphatide. There may be an increase in undifferentiated connective tissue cells, and more infiltration with leucocytes, lymphocytes, and plasma cells than after the phosphatide from H-37.

Two weeks after the injection of the phosphatide from the timothy grass bacillus there seems to be less formation of epithelioid cells but this is only because all of the large nodules have become caseous; the diffuse reaction of intact epithelioid cells shows the same mixed types as after the bovine phosphatide. After ten doses of the former, many of the epithelioid cells are still in the second stage and some even in the first. The material seems to be more irritating than any of the other phosphatides, for even after one intraperitoneal injection there are several abscesses in the omentum; and in the later reaction there are many leucocytes scattered in the tissues and phagocytized by clasmatocytes. There are also many plasma cells.

The study of the phosphatide from the lepra organism is incomplete since only small amounts have been available. The material is more irritating and the tissues show more of an increase in connective tissue cells. Also there has been much more variation in the reaction of different animals. In one experiment there was a considerable diffuse reaction of very complex epithelioid cells, a few of them being in small clumps or tubercles. In the regional lymph nodes there was, however, a massive reaction of epithelioid giant cells, a few of them being the compound types. There was also some caseation and a general infiltration of the tissues with leucocytes and many clumps of plasma cells. In another animal the reaction was almost wholly of leucocytes, enormous numbers of them having been called from the vessels and phagocytized by clasmatocytes.

From this study with the phosphatides of the acid-fast organisms, we have designated the reaction of the material toward the production of epithelioid cells and their multinuclear form, the Langhans giant cells, as the specific reaction toward tuberculo-lipoid. By this use of the term "specific reaction" is meant to imply that emphasis should be put on the epithelioid cell, either in mono- or multinucleated form, as the one structure which is essential in tubercles or in tubercular infiltrations or in tubercular granulation tissue. The phosphatides from all of the acid-fast organisms included in this study produce epithelioid cells and Langhans giant cells which go on to caseation. Caseation is, therefore, a part of the specific reaction.

All other reactions, such as the calling of leucocytes from the blood

vessels, their phagocytosis and destruction by clasmatocytes, the development of plasma cells, the increase in lymphocytes, in young connective tissue cells, in fibroblasts, and the development of new blood vessels, we have termed non-specific. On this basis, it is clear that the phosphatide from the human strain of tubercle bacilli gives the more specific response, while all of the other phosphatides give more non-specific reaction as well, the reactions to the phosphatide from the timothy grass bacillus and the lepra strain being the more mixed.

Of the various controls only one material so far tested acts just like the tuberculo-phosphatide, namely, lecithin. A quantity of this substance was prepared for us from the brain, by Dr. P. A. Levene. The lecithin is phagocytized by the same mononuclear cells as the tuberculo-phosphatide and produces epithelioid cells and giant cells in massive tubercles. In the original report (89) it was stated that only a small reaction was found in the omentum. Subsequent studies of sections from many places in the peritoneal cavity have shown an extensive reaction elsewhere. A dilauryl acetic acid containing the same number of carbon atoms as the phthioic acid, namely 26, synthetized for us by Prof. Roger Adams, Department of Chemistry, University of Illinois, is also phagocytized by the cells in the milk spots rather than by clasmatocytes, but this material does not change these cells into epithelioid types in the same period of time.

An important property of the tuberculo-phosphatide is that it acts as an antigen. This reaction was first described for alcoholic extracts of tubercle bacilli by Meyer (93); it has been studied for methyl extracts by Boquet and Nègre (94). Pinner (95) showed the phosphatide by Anderson to be antigenic, and further studies of this reaction have been made by Doan (96), and Doan and Moore (97).

Phthioic acid. All of the primary divisions of the lipoids, the phosphatide, the waxes, and the acetone-soluble material yielded on analysis certain hitherto unknown, saturated fatty acids of high molecular weight. A fatty acid designated Fatty Acid I, from the phosphatide A-3, of the human strain of organisms, was tested first in the rabbit. All of the tendency of the phosphatide toward the production of epithelioid cells was carried over into this acid; and this acid produced as great an amount of epithelioid cells as the original phosphatide (see table 1, in 88). Tubercles surrounded by lymphocytes were characteristic of the reaction. It was, however, more irritating than the phosphatide, there was a greater increase in connective tissue cells, and there were large abscesses. Typical caseation, in the sense we have defined

it, was present only in small foci. The cellular reactions simulated those of the disease more closely than those after the phosphatide. On further analysis of Fatty Acid I, Doctor Anderson obtained a hitherto unknown fatty acid of high molecular weight, which has been found in the biological test to be the only substance from the original acid producing a response of tubercular tissue. Doctor Anderson therefore named it phthioic acid. The empirical formula of this acid is $C_{26}H_{52}O_2$. The phosphatide also yielded glycerophosphoric, oleic, and palmitic acids. Each of these was tested by intraperitoneal injections and gave leucocytes both free and in clasmatocytes, some increase in blood vessels, and a non-specific thickening of the omentum in varying degrees.

The phthioic acid was a heavy oil and proved much too irritating to be used undiluted; it caused necrosis and gave very complex cellular responses. In spite of this complexity of these reactions, it could be made out that there was a marked maturation of monocytes. It was necessary, therefore, to find a bland oil for a diluting menstruum; olive oil was tried and rejected on account of its extreme irritation of the connective tissues. Mineral oil was chosen because, though not inert, it gave only a moderate increase in fibroblasts and a minimal production of epithelioid cells. Diluted in mineral oil, the phthioic acid from the phosphatide A-3 gave a diffuse reaction of epithelioids as well as typical small tubercles, like those from the phosphatide and closely simulating those of the disease. None of the highly vacuolated types of cells produced by the phagocytosis of the complex lipoid appeared with the fatty acid; the epithelioid cells were all of the typical third stage. These results may be interpreted as indicating that the original phosphatide in these experiments was phagocytized by cells and reduced to some state comparable to the fatty acid. In the one case the degradation of the material was done within the cell and in the other in the test tube. However, it is probable that in the disintegration of the bacilli that occurs in the actual infection, the phagocytosis of the material by cells takes place when the substances are in the more complex molecule. Interesting evidence bearing on this point is to be found in the work of Bickford (98) who has shown that there is a specific type of early epithelioid cell in the meninges which is produced by the injection of living or dead tubercle bacilli or tuberculo-phosphatide.

The fatty acids from the purified and soft waxes also produced typical tubercles in varying amounts, but not as massive reactions as that from the fatty acid from the phosphatides. The acetone-soluble material contained more of these fatty acids than the other lipoids. Doctor

Anderson separated these fatty acids and found an isomer of stearic acid, which he called tuberculo-stearic acid, and phthioic acid. The former was irritating but did not produce tubercular tissue.

The acetone-soluble material yielded enough phthioic acid for fractionation and Doctor Anderson separated the acid from this source into a dextro- and a levorotatory form. The specific activity toward the production of epithelioid cells was carried only in the dextrorotatory acid. The response to both of these acids is complex and the actual increase in new tissue is fully as great with the levorotatory acid. Introduced intraperitoneally in mineral oil, the levorotatory form gives a marked increase in thickness of the omentum and subperitoneal tissues. There is an increase in young connective tissue cells, an infiltration of the tissues with leucocytes, much phagocytosis of them by clasmatocytes, an increase in lymphocytes and plasma cells, together with the development of many monocytes but practically no epithelioid cells. After the dextrorotatory acid, on the other hand, there is also a marked production of epithelioid cells, and a few epithelioid giant cells; there is relatively little tendency toward the production of these cells in clumps or tubercles and we have not found these cells surrounded with lymphocytes. There is the same complex non-specific reaction toward leucocytes and plasma cells with the dextrorotatory acid as with the levorotatory, but the presence of the epithelioids in the one case makes such a difference in the appearance of the tissues that the type of acid used can be determined from the tissues without reference to records.

The question has been raised by Boissevain and Ryder (99) as to whether the alcohol-ether soluble material isolated by Anderson is really a phosphatide or merely debris of bacilli, and therefore whether the biological reactions which we have recorded with this material are necessarily associated with lipoids. The fact that dead tubercle bacilli also produce tubercles makes this question pertinent. Acid-fast debris has not been present in any of the phosphatides prepared by Anderson after the first lot; the filtering through candles removed all demonstrable acid-fast masses. Therefore the question at issue is the presence of non-acid-fast debris. It is possible that this question cannot be wholly settled until the nature of the nitrogen present in the phosphatide has been determined and until complete purification of the material has been accomplished, but the evidence up to the present time in favor of the hypothesis that the epithelioid cell is a foreign body reaction to tuberculo-lipoid is as follows. Epithelioid cells have been produced only by extracts which have been obtained by lipoidal solvents, not by

tuberculo-proteins or polysaccharides; thus if the epithelioid cell can be produced chemically at all, it must be by lipoids. The predominantly crystalline nature of the dry phosphatide and its transformation immediately on wetting into myelin figures are against the view that this material is amorphous debris. The identification of the phagocytosis of this myelin-like material by cells in a definite location, namely, in the milk spots of the omentum, so that the reaction can be followed into typical epithelioid cells, gives evidence that the epithelioid cell represents a foreign body reaction toward a lipoid. The production of the epithelioid cell in the same location by pure lecithin also suggests that the epithelioid type may be a form of reaction toward ingested lipoids.

Doctor Boissevain has also brought up the interesting question of the discrepancy between the amount of epithelioid tissue produced by dead tubercle bacilli and the corresponding amount of phosphatide on the basis of its being 6 per cent by weight of the organisms. These two quantitative reactions cannot be compared for the phosphatide contains only a part of the specifically active lipoid. Doctor Anderson has shown that all of the lipoids extracted by solvents, the alcohol-soluble, chloroform-soluble, and the acetone-soluble material, contain phthioic acid; moreover, lipoidal solvents do not remove all of these substances, for the so-called "defatted bacilli" are still acid-fast and produce epithelioid cells, in less amount, however, than intact dead bacilli. The total lipoid can only be extracted by methods which completely disintegrate the bacilli. If the total lipoidal material were available, comparative tests between the amount of the epithelioid reaction to this material and dead bacilli could be made. For such tests, however, the intraperitoneal route is not as good as the subcutaneous on account of the wide dispersion of the reaction in the peritoneal cavity. In this area the reaction is produced not only in the omentum but under the serosal lining of the body-wall, the wall of the intestine, in the mesentery, the diaphragm, in the capsules of the liver and spleen, and around the reproductive organs. The cellular reactions are also reflected by the cells free in the peritoneal fluid and always appear in the retrosternal lymph nodes which drain the peritoneal cavity. They are not found in the mesenteric lymph nodes unless the bowel wall has been involved. The value of the intraperitoneal route is the opportunity to follow the cellular reactions in living tissue without any distortion in the arrangement of the cells. In subcutaneous tests, however, all of the cellular reactions are in a restricted area except that which is to be found in the regional lymph nodes.

There are, however, certain quantitative discrepancies in our results which cannot be explained at present.   In all of the tests with the more purified forms of the phthioic acid there has been a marked loss in the amount of specific activity from the reaction of the corresponding amount of the original phosphatide and the mixture of fatty acids extracted from the phosphatide.   Moreover, the acetone-soluble material which contained more of the phthioic acid than the phosphatide itself shows little tendency for the epithelioid cells to be in large masses or tubercles. The question as to whether these phenomena are due to any accessory factors cannot be answered at the present time.

II. *Cellular Reactions to the Unsaponifiable Wax.*   Doctor Anderson considers the so-called waxes to be very complex phosphatides.   From them he isolated an unsaponifiable material having the property of acid-fastness which discriminates the group of the tubercle and para-tubercle bacilli.   Doctor Anderson has found this material to be a higher alcohol with the formula of $C_{94}H_{188}O_4$ and has discussed the relationship of this material to the higher alcohols of other analyses.   We have had the privilege of studying also a similar acid-fast material prepared from tubercle bacilli by Dr. P. A. Levene.   Both specimens were completely insoluble in water and had therefore to be suspended in mineral oil.   In this menstruum they produced identical reactions, namely, a marked production of young connective tissue cells, both diffusely and in small clumps.   There has been no sign that this material is phagocytized by any of the cells.   The new cells which appear after the injection of this material are round or oval and of about the size of monocytes.   Little differentiation can be made out in the cytoplasm which is slightly basophilic.   However, the cytoplasm is so delicate that it cannot be made out at all in sections, so that the clumps or pseudo-tubercles of them look like masses of large nuclei without cytoplasmic outlines around them.   There are considerable numbers of leucocytes scattered diffusely and infiltrating the pseudo-tubercles. Similar clumps of cells characterize the reaction to the levorotatory phthioic acid.   Thus the cellular reaction to the unsaponifiable alcohol is a double one: undifferentiated connective tissue cells and leucocytes.

III. *Cellular Reactions to the Acetone-Soluble Fat.*   By far the most complex cellular reactions are produced by the acetone-soluble fat. We have tested this material from the human, bovine, and avian tubercle bacilli and from the timothy grass bacillus.   The supravital studies show that every type of connective tissue cell has been stimulated.   The analysis of this material by Doctor Anderson shows that it is a complex

mixture of fatty acids, butyric, palmitic, stearic, cerotic, linoleic, linolenic, tuberculo-stearic, and phthioic acids. Corresponding to the phthioic acid there is a diffuse reaction of epithelioid cells; besides this, there is a general infiltration of the tissues with leucocytes, and many of them are in clasmatocytes; there is also a great increase in undifferentiated connective tissue cells, fibroblasts, lymphocytes, plasma cells, and a marked increase in new blood vessels with hemorrhage. The diffuse character of the epithelioid reaction may be associated with the intensity of these non-specific reactions. Further studies with the material from the various acid-fast strains of organisms are necessary in order to analyze the complexity of these reactions, which may be due in part to the high acidity of the material. As a control for this part of the lipoid, we have tested the acetone-soluble lipoid prepared from the streptococcus by Doctor Heidelberger. It appears to be the only type of lipoid in this organism; it is present only in small amounts in these organisms but gives very complex cellular responses with, however, no epithelioid cells.

From these studies of cellular reactions toward tuberculo-lipoids, the mass of evidence seems to indicate that they are remarkable stimulants of the cells of the connective tissues. Some of this material, such as the phosphatide, seems to act as a foreign body and produces effects through being phagocytized by cells; other materials, such as the unsaponifiable higher alcohol, act as a stimulant without being phagocytized; and the third lipoid, the acetone-soluble fat, is an extremely complex irritant. Reason for the cellular lesions in tuberculosis can be found in all these reactions. The view that the epithelioid cell is the result of the phagocytosis of a tuberculo-lipoid by monocytes and young connective tissue cells is in agreement with the view that the tubercle in the disease arises locally from fixed connective tissue cells.

CELLULAR REACTIONS TO POLYSACCHARIDES. Polysaccharides obtained from the lipoids by Doctor Anderson and from the whole bacillus by Doctor Heidelberger have been tested on the cells of the connective tissues. All of these materials give the same effect, namely, the calling of leucocytes from vessels and the damaging of them so that they are quickly phagocytized by clasmatocytes. This is a constant reaction and occurs regardless of the number of injections. There is no substance tested from the tubercle bacillus that did not call leucocytes from the vessels and in some instances this has been an extreme reaction in response to some of the lipoidal fractions, as for example, the acetone-soluble fat. However, this reaction is so consistently found with all

of the polysaccharides as to raise the issue as to whether the material in the other fractions which so damages the leucocytes that they are quickly taken in by clasmatocytes may not be the content of carbohydrate.

CELLULAR REACTIONS TO PROTEIN.   It was shown by Doctor Miller (85) that the various preparations of protein obtained from the tubercle bacilli all have a remarkable power toward the production of plasma cells.   With repeated injections of the protein he could follow the complete life cycle of these cells, producing its youngest stages, the well-known mature, Marschalkow type, and the final stage, the so-called Russell body cell.   Of all of the materials from the tubercle bacillus, the tuberculo-protein gives plasma cells in the most massive amounts. They are found, however, in considerable numbers after repeated injections of the polysaccharide; and, as has been stated, they are increased over normal numbers after the phosphatide, especially from the organisms other than the human.   It is thus interesting to speculate as to whether a response of plasma cells to polysaccharide or lipoid may be a biological test of the presence of some nitrogenous compound.

SUMMARY

In these studies on the cellular reactions to chemical fractions from the tubercle bacillus, it has been shown that there are three different types of complex lipoids in the organism which can be discriminated by the cellular reactions they produce.

The phosphatide reproduces the tubercle; it is phagocytized by certain cells of the connective tissues, namely, by monocytes, and partially degraded within them, thereby forming the epithelioid cell.   The fact that this material is phagocytized, and good evidence of this is obtained by seeing the characteristic myelin figures of the original material within the living cell, goes far to indicate that it is the substance itself and not some contaminating impurity which is responsible for this action.

The only constituent of this phosphatide which can produce this reaction is a saturated fatty acid of high molecular weight, phthioic acid, of the formula $C_{26}H_{52}O_2$.   All of the other lipoids of the original fractionation, the wax and the acetone-soluble fat, contain also some of the phthioic acid, and therefore possess varying degrees of specific biological activity.

The waxes contain phthioic acid and an unsaponifiable residue which

is a higher alcohol, $C_{94}H_{188}O_4$. This unsaponifiable base of the wax does not seem to be phagocytized by cells, but in spite of its insolubility in water, when injected in an oil, acts as a remarkable stimulant toward the production of undifferentiated connective tissue cells. It is always irritating and calls leucocytes from the vessels. These two exceedingly complex phosphatides, the tuberculo-phosphatide and the waxes, then, may be considered as the types of lipoidal substances especially characteristic of the acid-fast strains of the organism, the phosphatide and the phthioic acid being responsible for the epithelioid cell—so prominent a factor in the cellular reactions of the disease, while the unsaponifiable material is responsible for the acid-fastness of the bacillus.

The especial interest of the acetone-soluble material is that it may be more like the lipoids of other strains of organisms; the extremely varied cellular reactions which it produces may be due to the fact that it is a complex mixture of fatty acids.

The characteristic cellular response to tuberculo-protein is the plasma cell. The tuberculo-polysaccharides are chemotactic and toxic to neutrophilic leucocytes.

BIBLIOGRAPHY

(1) MAFFUCCI, A. Centralbl. f. allg. Path. u. path. Anat., 1890, i, 825.
(2) KOCH, R. Berl. klin. Wochenschr., 1882, xix, 221.
    Deutsch. med. Wochenschr., 1891, xvii, 101.
    Deutsch. med. Wochenschr., 1897, xxiii, 209.
(3) WYSSOKOWITSCH, W. Zeitschr. Hyg. u. Infektionskrank., 1886, i, 3.
(4) PRUDDEN, T. M. AND E. HODENPYL. New York Med. Journ., 1891, liii, 637, 697.
(5) PRUDDEN, T. M. New York Med. Journ., 1891, liv, 617.
(6) STRAUS, I. AND N. GAMELEIA. Arch. méd. expér. et d'anat. path., 1891, iii, 705.
(7) GRANCHER, J. AND LEDOUX-LEBARD. Arch. méd. expér. et d'anat. path., 1892, iv, 1.
(8) VISSMAN, W. Arch. path. Anat. u. Physiol., 1892, cxxix, 163.
(9) ABEL. Deutsch. med. Wochenschr., 1892, xviii, 482.
(10) KOSTENITSCH, J. Arch. méd. expér. et d'anat. path., 1893, v, 1.
(11) MASUR, A. Beitr. z. path. Anat., 1894, xvi, 256.
(12) HAMMERSCHLAG, A. Centralbl. f. klin. Med., 1891, xii, 9.
(13) DE SCHWEINITZ, E. A. AND M. DORSET. Journ. Amer. Chem. Soc., 1895, xvii. 605.
    Centralbl. f. Bakt., Abt. i, 1896, xix, 707; Abt. i, 1897, xxii, 209.
    Journ. Amer. Chem. Soc., 1898, xx, 618.
    Centralbl. f. Bakt., Abt. i, 1898, xxiii, 993.
    Centralbl. f. Bakt., Abt. i, Orig., 1902, xxxii, 186.
(14) WEYL, Th. Deutsch. med. Wochenschr., 1891, xvii, 256.

(15) AUCLAIR, J. Étude Expérimentale sur les Poisons du Bacille Tuberculeux humain. (Essais de vaccination et de traitement.) Thèse de Paris. 1897, G. Steinheil.
Rev. de la Tuberculose, 1898, vi, 97.
Arch. méd. expér. et d'anat. path., 1903, xv, 469.

(16) AUCLAIR, J. AND L. PARIS. Arch. méd. expér. et d'anat. path., 1907, xix, 129.
Arch. méd. expér. et d'anat. path., 1908, xx, 737.

(17) ARMAND-DELILLE, M. P. Compt. rend. Soc. biol., 1901, liii, 885, 1127.

(18) BERNARD, L. AND M. SALOMON. Compt. rend. Soc. biol., 1903, lv, 1233.

(19) OPPENHEIM, R. AND M. LOEPER. Compt. rend. Soc. biol., 1903, lv, 330.

(20) COURCOUX, A. AND L. RIBADEAU-DUMAS. Compt. rend. Soc. biol., 1904, lvii, 633.

(21) DOMINICI, H. AND E. OSTROVSKY. Recherches sur les Poisons du Bacille de la Tuberculose. Masson et Cie., 1914.

(22) LEVENE, P. A. Journ. Med. Res., 1901, vi, 135.
Journ. Med. Res., 1904, xii, 251.

(23) MORSE, P. F. AND E. STOTT. Journ. Lab. Clin. Med., 1916–17, ii, 159.

(24) RAY, L. W. AND J. S. SHIPMAN. Amer. Rev. Tuberc., 1923, vii, 88.

(25) BENIANS, T. H. C. Journ. Path. and Bact., 1912–13, xvii, 199.

(26) SHERMAN, H. Journ. Inf. Dis., 1913, xii, 249.

(27) BIENSTOCK, B. Fortschr. d. Med., 1886, iv, 193.

(28) GOTTSTEIN, A. Fortschr. d. Med., 1886, iv, 252.

(29) ARONSON, H. Berl. klin. Wochenschr., 1898, xxxv, 484.
Berl. klin. Wochenschr., 1910, xlvii, 1617.

(30) BORREL, A. Bull. Inst. Pasteur, 1904, ii, 409.

(31) BULLOCH, E. AND J. J. R. MACLEOD. Journ. Hyg., 1904, iv, 1.

(32) TAMURA, S. Zeitschr. Physiol. Chem., 1913, lxxxvii, 85.

(33) GORIS, A. Ann. Inst. Pasteur, 1920, xxxiv, 497.

(34) ANDERSON, R. J. Journ. Biol. Chem., 1927, lxxiv, 525, 537.
Proc. Nat. Acad. Sci., 1929, xv, 628.
Journ. Biol. Chem., 1929, lxxxiii, 169, 505.
Journ. Biol. Chem., 1929, lxxxv, 327, 339, 351.
Journ. Amer. Chem. Soc., 1930, lii, 1607.
Physiol. Rev., 1932, xii, 166.

(35) ANDERSON, R. J. AND E. G. ROBERTS. Journ. Biol. Chem., 1929–30, lxxxv, 509, 519, 529.
Journ. Biol. Chem., 1930, lxxxix, 599, 611.
Amer. Rev. Tuberc., 1930, xxii, 664.
Journ. Amer. Chem. Soc., 1930, lii, 5023.

(36) ROBERTS, E. G. AND R. J. ANDERSON. Journ. Biol. Chem., 1931, xc, 33.

(37) ANDERSON, R. J. AND E. CHARGAFF. Journ. Biol. Chem., 1929, lxxxiv, 703.
Journ. Biol. Chem., 1929–30, lxxxv, 77.
Zeitschr. Physiol. Chem., 1930, cxci, 157, 166.

(38) ANDERSON, R. J., E. G. ROBERTS AND E. CHARGAFF. Trans. Nat. Tuberc. Assoc., 1929, 25th Annual Meeting, p. 206.

(39) ANDERSON, R. J. AND A. G. RENFREW. Journ. Amer. Chem. Soc., 1930, lii, 1252.

(40) ANDERSON, R. J., E. G. ROBERTS AND A. G. RENFREW. Proc. Soc. Exp. Biol. and Med., 1929–30, xxvii, 387.

(41) CHARGAFF, E. AND R. J. ANDERSON. Zeitschr. Physiol. Chem., 1930, cxci, 172.

(42) CHARGAFF, E., M. C. PANGBORN AND R. J. ANDERSON. Journ. Biol. Chem., 1931, xc, 45.

(43) MALASSEZ, L. AND W. VIGNAL. Arch. d. Physiol. norm. et path., 1883, Series iii, ii, 369.
Arch. d. Physiol. norm. et path., 1884, Series iii, iv, 81.

(44) FERRAN, J. Compt. rend. Acad. Sci., 1897, cxxv, 515.

(45) MARMOREK, A. Zeitschr. f. Tuberc. u. Heilstättenwesen, 1900-01, i, 444.

(46) MUCH, H. Beitr. z. Klin. d. Tuberk., 1907, viii, 85, 357.

(47) MAHER, S. J. The relation of acid-fast tubercle bacillus to other forms of bacterial life. Report of Ninth Internat. Conference on Tuberculosis, Brussels, 1910. Berlin, 1911, p. 350.
Amer. Rev. Tuberc., 1925, xii, 365.
Beitr. z. Klin. d. Tuberk., 1931, lxxvii, 40.

(48) FONTES, A. Mem. Inst. Oswaldo Cruz, 1910, ii, 186.

(49) MELLON, R. R. AND E. JOST. Proc. Soc. Exp. Biol. and Med., 1926-27, xxiv, 743.
Amer. Rev. Tuberc., 1929, xix, 483.

(50) MELLON, R. R. Proc. Soc. Exp. Biol. and Med., 1931, xxix, 206.
Tubercle, 1931, xiii, 10.

(51) SUYENAGA, B. Amer. Rev. Tuberc., 1925, xii, 260.

(52) DUFFY, F. M. Amer. Rev. Tuberc., 1927, xvi, 330.

(53) NÈGRE, L., A. BOQUET AND J. VALTIS. Compt. rend. Soc. biol., 1928, xcix, 45.
Ann. Inst. Pasteur, 1930, xliv, 247.

(54) NÈGRE, L., J. VALTIS AND A. SAENZ. Compt. rend. Soc. biol., 1931, cvii, 942.

(55) MILLER, F. R. Science, 1931, lxxiv, 343.

(56) KAHN, M. C. Ann. Inst. Pasteur, 1930, xliv, 259.

(57) KAHN, M. C. AND J. C. TORREY. Amer. Rev. Tuberc., 1928, xviii, 815.

(58) ALBERT-WEIL, J. Les Poisons du Bacille Tuberculeux et les Réactions Cellulaires et Humorales dans la Tuberculose. Paris, J. B. Baillière et Fils, 1931.

(59) KLEBS, E. Centralbl. f. Bakt., Abt. i, 1896, xx, 488.

(60) RUPPEL, W. G. Zeitschr. Physiol. Chem., 1898-99, xxvi, 218.

(61) KRESLING, K. Centralbl. f. Bakt., Abt. i, 1901, xxx, 897.

(62) MAYER, A. AND E. F. TERROINE. Compt. rend. Soc. biol., 1907, lxii, 398.

(63) DORSET, M. AND J. H. EMERY. Centralbl. f. Bakt., Abt. i, Ref., 1906, xxxvii, 363 (Abstract).

(64) AGULHON, H. AND A. FROUIN. Bull. Soc. Chim. biol., 1914-1920, i-ii, 176.

(65) LINOSSIER, G. Les Lipoïdes dans l'Infection et dans l'Immunité. Paris, 1920. Libraire J. B. Baillière et Fils.

(66) BOQUET, A. AND L. NÈGRE. Compt. rend. Soc. biol., 1920, lxxxiii, 922.
Compt. rend. Soc. biol., 1922, lxxxvi, 581.
Ann. Inst. Pasteur, 1923, xxxvii, 787.

(67) NÈGRE, L. AND A. BOQUET. Compt. rend. Soc. biol., 1921, lxxxiv, 76.

(68) FROUIN, A. AND M. GUILLAUMIE. Compt. rend. Soc. biol., 1923, lxxxix, 319.

(69) NOCARD, E. AND E. ROUX. Ann. Inst. Pasteur. 1888, i, 19.

(70) KÜHNE, W. Zeitschr. f. Biol., 1892, xxx, 221.

(71) PROSKAUER, B. AND M. BECK. Zeitschr. f. Hyg., 1894, xviii, 128.

(72) ARMAND-DELILLE, P., A. MAYER, G. SCHAEFFER AND E. TERROINE. Compt. rend. Acad. Sci., 1912, cliv, 537.
    Journ. Physiol. et de Path. Gén., 1913, xv, 797.
    Compt. rend. Soc. biol., 1913, lxxiv, 272.

(73) KENDAL, A. I., A. A. DAY AND A. W. WALKER. Journ. Infect. Dis., 1914, xv, 417.

(74) BAUDRAN, G. Compt. rend. Acad. Sci., 1910, cl, 1200.

(75) LONG, E. R. Amer. Rev. Tuberc., 1919-20, iii, 86.
    Trans. Nat. Tuberc. Assoc., 16th Annual Meeting, 1920, p. 332.
    Amer. Rev. Tuberc., 1921-22, v, 857.
    Tubercle, 1924-25, vi, 128.

(76) LONG, E. R. AND F. B. SEIBERT Amer. Rev. Tuberc., 1926, xiii, 393.

(77) MAYER, A. AND G. SCHAEFFER. Compt. rend. Soc. biol., 1919, lxxxii, 113.

(78) FROUIN, A. Compt. rend. Soc. biol., 1921, lxxxiv, 606.

(79) LONG, E. R. AND L. K. CAMPBELL. Amer. Rev. Tuberc., 1922-23, vi, 636.

(80) LONG, E. R. AND L. L. FINNER. Amer. Rev. Tuberc., 1927, xvi, 523.

(81) SAUTON, B. Compt. rend. Acad. Sci., 1912, clv, 860.

(82) WHITE, D. C. A national research program in tuberculosis. Nat. Tuberc. Assn., Technical Series no. 9, New York, 1929.

(83) SEIBERT, F. B. Amer. Rev. Tuberc. 1928, xvii, 402.
    Journ. Biol. Chem., 1928, lxxviii, 345.
    Amer. Rev. Tuberc., 1928, xvii, 394.

(84) SEIBERT, F. B. AND B. MUNDAY. Amer. Rev. Tuberc., 1931, xxiii, 23.
    Tran. Nat. Tuberc. Assoc., 26th Annual Meeting, 1930, p. 234.

(85) MILLER, F. R. Journ. Exp. Med., 1931, liv, 333.

(86) SABIN, F. R. AND C. A. DOAN. Journ. Exp. Med., 1927, xlvi, 645.

(87) WHITE, W. C. Trans. Assoc. Amer. Phys., 1928, xliii, 311.

(88) SABIN, F. R., F. R. MILLER, C. A. DOAN AND B. K. WISEMAN. Journ. Exp. Med., 1931, liii, 51.

(89) SABIN, F. R., C. A. DOAN AND C. E. FORKNER. Journ. Exp. Med., 1930, lii, Supplement no. 3, 1.

(90) MÖLLENDORFF, W. AND M. MÖLLENDORFF. Zeitschr. f. Wissensch. Biol., Abt. B, 1925-26, iii, 503.

(91) SMITHBURN, K. C. AND F. R. SABIN (to be published later).

(92) FORKNER, C. E. Journ. Exp. Med., 1930, lii, 279.

(93) MEYER, K. Zeitschr. f. Immunitäts., 1912, xiv, 355, 359.
    Zeitschr. f. Immunitäts., 1912, xv, 245.
    Zeitschr. f. Immunitäts., 1914, xxi, 654.

(94) NÈGRE, L. AND A. BOQUET. Antigénothérapie de la Tuberculose. Masson et Cie., 1927.

(95) PINNER, M. Amer. Rev. Tuberc., 1925, xii, 142.
    Amer. Rev. Tuberc., 1927, xv, 714.

(96) DOAN, C. A. Proc. Soc. Exper. Biol. and Med., 1928-29, xxvi, 672.
    Trans. Nat. Tuberc. Assoc., 26th Annual Meeting, 1930, p. 188.

(97) DOAN, C. A. AND D. M. MOORE. Amer. Rev. Tuberc., 1931, xxiii, 409.

(98) BICKFORD, J. V. (to be published later).

(99) BOISSEVAIN, C. H. AND C. T. RYDER. Amer. Rev. Tuberc., 1931, xxiv, 751,