A STUDY OF BACTERIAL CARBOHYDRATES WITH SPECIAL REFERENCE TO THE TUBERCLE BACILLUS

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Bacterial carbohydrates.

The numerous studies of bacterial antigens have persuaded many investigators that the rôle which different carbohydrates play in the phenomena of immunity is a considerable one. Bunji Imai has reported that he obtained immune-sera, which gave precipitation with a certain kind of inulin. Ken Nodzu and B. Imai have demonstrated the antigenic power of soluble starch by the complement fixation test. After heating the immune-sera at 60° C for 1 hour, no changes in the complement fixation occurred. The antibodies derived from starch are strictly specific. Ken Nodzu and Y. Masuda insist that they can immunologically differentiate starches, which it is very difficult to do by morphological features. S. Nishimura experimented with inulin, soluble starch and dextrine and obtained antisera against all of them. All the sera of rabbits immunized with these polysaccharides contained specific antibodies and gave a clearly positive complement-fixing test. The precipitation test was negative with all of them. The three kinds of polysaccharides named have the power to produce antibodies without any vehicle. The polysaccharides employed gave a negative protein colour reaction, but they contained nitrogen. The nitrogen found in the soluble starch solutions was obviously protein. The author states that the sera lost their strength rapidly in the period following the immunization.

The function of carbohydrates as determinative substances in bacterial specificity has only recently been disclosed, despite the fact that the presence of these substances in bacteria and yeast has long been known. There is, already, much literature dealing with the antigenic nature and chemical properties of the bacterial carbohydrates, and we attempt to give here a summary of the principal views on this subject.

Schreibler (1874) studied a polysaccharide isolated from the gum of Streptococcus (Leuconostoc) mesenterioides.

Dochez and Avery (1917) observed that the urine and blood of pneumonia patients and the filtrates of pneumococcus cultures contained a specifically reacting "soluble" substance, which reacted with antipneumococcus serum of the homologous type. This observation led to the isolation and identification of these specifically reacting substances from very different bacteria. Little attention was given to the carbohydrates of pathogenic bacteria until Toenniessen's work on Friedländer's bacillus. A polysaccharide galactane that yields galactose upon hydrolysis was found in the capsules of these bacilli. Zinsser (1921) and Zinsser and Parker (1923) prepared from several organisms (pneumococcus, staphylococcus and typhoid, colon, and influenza bacilli) residue antigens which were almost protein-free, reacted by precipitation with homologous immune-sera, but failed to elicit antibody production in animals. Kramár (1922) obtained from B. radicicola a polysaccharide dextrane and from B. anthracis a substance of a glucoprotein nature. Heidelberger and Avery (1923 and 1924) carried out a fruitful investigation of the carbohydrates of pneumococcus. They isolated from Types II and III a soluble specific substance which bore close resemblance to a polysaccharide. It was nitrogen-free, non-antigenic in rabbits, but precipitated homologous antisera. Mueller and Tomcsik (1924) carried out special studies with yeast and Mueller, Smith and Litarczek (1924) with bacilli of the Friedländer group. The results are in certain fundamental aspects, in harmony with the studies carried out by Heidelberger and Avery. Heidelberger, Goebel and Avery (1925) stated that the soluble specific substance of Type I pneumococcus differs sharply from the corresponding substances of the other two types, each of which, in turn, differs from the other. The evidence is believed to favour the view that the polysaccharides isolated are the actual specific substances of pneumococcus. Landsteiner and Furth (1927) have described precipitable substances, probably of a carbohydrate nature, isolated from B. typhosus, paratyphosus B, and enteritidis. Heidelberger, Schwartzman and Cohn (1928) studied a carbohydrate fraction of B. typhosus. Przemycki (1925) and Furth and Landsteiner (1928) obtained a specific carbohydrate substance from the B. proteus. Combiesco, Soru and Stamatesco (1929) have described a specific

carbohydrate obtained from B. anthracis. From a rough strain of pneumococcus Tillett and Francis (1930) obtained a non-type-specific polysaccharide, designated the C fraction. Wadsworth and Brown (1931) have reported the isolation of a type-specific substance of a carbohydrate nature from pneumococcus which they regard as different from the polysaccharides of Heidelberger and Avery and also from the C fraction of Tillett and Francis. Heidelberger and Kendall (1931) isolated three nitrogen-containing polysaccharides from autolyzed cultures of Type IV pneumococcus, a type-specific carbohydrate differing markedly from those of Types I, II and III. Zozaya (1931) studied the serological specificity of the polysaccharides of meningococcus, B. anthracis, B. proteus, B. subtilis and B. mesentericus. Casper and Miller and Boor (1933) have reported the isolation of carbohydrate fractions from gonococcus and, for comparison, carbohydrates were prepared also from Micrococcus catarrhalis, Streptococcus hemolyticus, Staphylococcus aureus, and a rough strain of pneumococcus. Zozaya and Wood, Webster and Rake and Miller and Boor carried out an investigation of the carbohydrates of meningococcus. Meisel and Mikulaszek (1933) have isolated polysaccharides from the Proteus x (H, O, R), and from Typhus-Paratyphus group. Boivin and Mesrobeanu and Boivin, J. Mesrobeanu, L. Mesrobeanu and Nestorescu (1934) studied the carbohydrates from B. Aertrycke, B. Gärtner, B. Eberth, B. paratyph. A and B, B. coli, B. Shiga, Vibrio choler., B. anthracis, Staphylococcus aureus, 5 types of Proteus, B. pyocyaneus, B. prodigiosus, B. subtilis and B. tuberculosus. The carbohydrates were extracted by a special method with trichloracetic acid and afterwards precipitated with acetone. The quantity of the specific polysaccharide obtained from dried B. Aertrycke varied from 4,2 to 7,4 per cent, and the quantity of glucose liberated from the polysaccharide by hydrolysis varied from 38 to 41 per cent. The maximum supply of polysaccharide, 14 per cent, was obtained from a type of Proteus (Boivin, Mesrobeanu and Nestorescu).

Numerous studies carried out within the past decade have demonstrated that the presence of specific carbohydrates was found not only in the encapsulated organisms, but also in a number of other microorganisms, including the tubercle bacil-

lus, about which we will speak in the following chapter of our present study.

The investigations of the physical and chemical properties of bacterial carbohydrates showed that they were amorphous, white or light yellow in colour and entirely soluble in distilled water and in 0,85 per cent sodium chloride solution. They are insoluble in ethyl alcohol, ether, chloroform and acetone. Miller and Boor found in the case of gonococcus and meningococcus an alcohol-soluble carbohydrate. When about 1,5 volumes of alcohol or 3 volumes of acetone are added to an aqueous solution of the bacterial carbohydrates, the soluble specific substances are entirely precipitated. The bacterial carbohydrates are often ash-free, but some preparations contain a little ash. They seem to be resistant to the action of weak acids and alkalis. They are only partially dialysable after 10—15 days' dialysis. When boiled for 4—12 hours with a sufficient excess of mineral acid (H_2SO_4, HCl) the carbohydrate is slowly hydrolysed with formation of reducing sugar, which it has not yet been possible to identify fully.

The tests for protein, e. g. biuret and xanthoproteic, are negative. Negative ninhydrin reaction indicates the absence of an amino-acid impurity in appreciable quantity. The polysaccharides also fail to form precipitates when treated with solutions of silver nitrate, copper sulphate, phospho-tungstic acid, tannic acid, picric acid and sulphosalicylic acid. Boivin and Mesrobeanu have found that the polysaccharide of B. Aertrycke was precipitated by phospho-tungstic acid if the solution was rendered strongly acid by Hl. The Millon and Hopkins tests are also negative. With Millon's reagent a jelly is sometimes formed in the cold, but the precipitate redissolves on heating and no colour is developed. The majority of bacterial carbohydrates is precipitable by solutions of uranium nitrate and basic lead acetate; the Friedländer polysaccharides are precipitated also by barium hydroxide and neutral lead acetate. Negative orcinol, phloroglucinol and resorcinol reactions indicate the absence of pentose and ketose radicals (Miller and Book). There is no colour developed with iodine-potassium iodide solution, or there may be developed a slight colour-reaction of glycogen. The Molisch test is strongly positive, which is considered as a carbohydrate reaction. Miller and

Boor (1934) have found that certain bacterial "nucleoproteins" gave strongly positive Molisch reactions, which indicates the presence of a carbohydrate radical. The unhydrolysed bacterial polysaccharides does not show reduction with Fehling's and Benedict's solution. However, this test is positive with hydrolysates obtained by boiling the carbohydrates with 2 per cent hydrochloric acid or N. H_2SO_4 for several hours. Potassium permanganate is not immediately reduced by the bacterial polysaccharides. The nitrogen content found by Kjeldahl microdetermination was 1—2,5 per cent for Proteus X polysaccharides, 3,7 per cent for meningococcal, 4, 2 per cent for gonococcal and 1,5—3 per cent for Typhus-Paratyphus polysaccharides. The carbohydrate preparations isolated by Furth and Landsteiner (1929) from the Salmonella group gave on analysis figures of 0,5 to 1,4 per cent for nitrogen; they showed weak or negative reactions for proteins. The polysaccharides of Friedländer's bacillus have acidic properties. They do not give a glucuronic acid test like the specific substance of pneumococcus. The carbohydrates prepared from the main serological types of the typhoid-paratyphoid groups do not exhibit very pronounced chemical differences in spite of serological dissimilarity (Furth and Landsteiner). In this respect the results differ from those observed with the polysaccharides of pneumococci. The polysaccharides of Friedländer's bacillus are optically active and rotate the plane of polarized light to the right. An aqueous solution of the gonococcal polysaccharide was optically inactive. The bacterial polysaccharides are generally considered as relatively heat-stable substances, but according to Boivin and Mesrobeanu certain polysaccharides may be denatured by heat. The differences in resistance to the action of acid and alkali were found to be characteristic for various specific carbohydrates. The polysaccharides may be absorbed by kaolin, tricalcic phosphate, coal, etc. According to the precise chemical studies made by Heidelberger and Avery, and Mueller the structure of bacterial carbohydrates is that of a complex carbohydrate. It was originally assumed that the ability of specific polysaccharides to precipitate homologous antibodies was a function of a relatively high molecular weight. Heidelberger and Kendall (1932) have shown that the formula weights of the specific carbohydrates are probably less than 10.000. In a further communication

Heidelberger and Kendall (1933) described a series of carbohydrates ranging from 550—1.800 in formula weight. Avery and Goebel (1933) showed that the carbohydrate present in the intact bacterial cells (Pneumococcus I) and in filtrates of autolysed broth cultures has been chemically identified as an acetyl polysaccharide. Owing to the marked instability of the acetyl groups, the specific polysaccharides which are originally isolated by treatment with alkali represent the deacetylated polysaccharides. They still retain the dominant type-specificity of the native substance, but have through the loss of their acetyl groups suffered a corresponding loss of properties possessed by the acetyl polysaccharides.

Dubos and Avery (1931) isolated an organism from peat soil which decomposes the specific capsular polysaccharide of Type III pneumococcus. The endocellular enzyme of this organism is specific. It does not attack the polysaccharides of Type I and Type II pneumococcus, nor any of the other bacterial polysaccharides thus far tested. Dubos (1932) described a method for the preparation, concentration and purification of this bacterial enzyme.

One of the striking characteristics of the bacterial carbohydrates is their failure to produce antibodies when injected into the animal organism in the form in which they are obtained separately from the bacteria. In the state in which they occur in the bacterial cell they are not only type-specific, but are also antigenic as well. Specific antibodies are formed when the undisrupted bacterial cell substances are present in the immunizing material (Zinsser and Tamiya). A great deal of contradictory evidence has been reported in the study of the bacterial polysaccharides as antigens. Avery and Morgan (1925) and Avery and Heidelberger failed to induce antibody formation in animals injected whith the purified polysaccharides of the pneumococcus. Schieman and his coworkers, Saito and Ulrich, Enders and Wadsworth and Brown were able to produce active type-specific immunity in animals with the carbohydrates from the pneumococcus cells, which were prepared by a method different from that used by Heidelberger and Avery. It was supposed, however, that in the few successful cases the carbohydrate used for the immunization usually has been of doubtful purity.

Zozaya (1932) and Zozaya and Clark studied the antigenic properties of various bacterial polysaccharides (Pneumococcus, Meningococcus, B. anthracis, Streptococcus viridans, B. proteus, B. dysenteriae) and dextrane adsorbed on colloids, especially on collodion and carbon. The bacterial polysaccharides were not free of nitrogen, but the dextrane was free of nitrogen and ash. Evidence was given that the bacterial polysaccharides and the dextrane can be rendered antigenic by haptogenic adsorption upon a colloid carrier. Dextrane reacted immunologically with antisera from pneumococci, some of the Salmonella and some of the types of Streptococcus viridans. All the bacterial carbohydrates were non-antigenic alone. The experiments with the antisera thus obtained demonstrated that the carbohydrate antibodies are specific. The experiments also suggest that some of the bacterial polysaccharides may contain several active antigenic groups in their molecular structure.

An attempt was made by Miller and Boor in which rabbits were injected intravenously with gonococcal carbohydrate mixed with pig serum. The injected animals developed high titers of anti-pig serum precipitins, but not at all for the carbohydrate. The bacterial polysaccharides must be combined with another cellular constituent, possibly a protein, to form a complex and easily dissociable antigen. Boivin, J. Mesrobeanu, L. Mesrobeanu and Nestorescu (1934) found that the complexes obtained from B. Aertrycke, B. coli and Proteus possess antigenic properties. If boiled a long time with diluted acetic acid, these complexes were cleft into a water-soluble specific polysaccharide and an insoluble couple.

Many recent studies show that complex antigens containing non-protein fraction may occur in nature. It is evident that the antigenic specificity of the complex antigens of certain bacterial cells is determined by the presence of specific polysaccharides. Goebel and Avery (1929) prepared by chemical methods two synthetic sugar-protein complexes with different optical properties and immunized animals with them. The immune-sera prepared with synthetic glucoprotein and galactoprotein contained two separate kinds of antibodies: one variety stimulated by the conjugative sugar-protein, and the other evoked by the protein itself. It was shown that each variety of antibody was specifically related to the corresponding

component of the antigen. It was also evident that the chemical constitution of the sugar radical, regardless of the nature of the protein to which it was attached, determines the serological specificity of the conjugated antigen. It occurred to Goebel and Avery (1931) to prepare a conjugated carbohydrate-protein from the specific polysaccharide of Type III pneumococcus and serum globulin. The synthetic antigen elicits in rabbits a type-specific antipneumococcus response, which neither one of its constituents alone is capable of inciting when injected singly into these animals. Rabbits immunized with this antigen acquired active immunity against infection with virulent Type III pneumococcus. The sera of these rabbits contain type-specific antibodies which precipitate the Type III pneumococcus polysaccharide, agglutinate Type III pneumococci and protect mice against Type III infection. Goebel, Babers and Avery (1932) prepared the alpha-p-aminophenol and beta-p-aminophenol glucosides of the glucose and coupled these to the globulin of the horse serum. The α-glucoside-protein and β-glucoside-protein differed from one another only in the alpha and beta linkage of the glucoside to the protein molecule. These synthetic glucoproteins were studied immunologically and it was shown that the structural changes of the α- and β-glucosides of glucose are so sharply reflected in serological specificity that it was possible by means of immune-sera to differentiate selectively between the two isomeric glucosides of the same sugar. The results of the present study support the view that the immunological specificity of carbohydrates is determined by their chemical constitution.

These works revealed the important fact that the diazotized glucose and galactose, bound to protein, which differ one from the other chemically only in specific rotation and in the spatial configuration (molecular configuration) of a single carbon atom, exhibit distinct immunological specificity. For the first time, it has been shown by direct experimental evidence that asymmetry of the carbon atoms in the sugar radical alone suffices to determine differences in the specifity of sugar-protein antigens. It was apparent that the simple sugar derivatives (glucoside and galactoside) by themselves are not precipitated in the presence of immune-sera which were prepared with protein containing the homologous diazotized compounds.

The lack of specific precipitation may be referable to the fact that the glucosides are crystalloids and of relatively small molecular size when compared with the colloidal and highly complex carbohydrates of certain bacteria which react readily in precipitin tests with specific antibacterial sera. The immunological specificity of bacterial polysaccharides has thus a close analogue in the serological specificity exhibited by glucoprotein and galactoprotein.

The cutaneous reactions with bacterial carbohydrates have been studied by many investigators. Tillett and Francis (1929) tested pneumonia patients intracutaneously with purified, protein-free carbohydrates of Types I, II and III pneumococci. The material obtained according to the method employed by Heidelberger and Avery was injected in 0,1 cc amounts into the skin on the flexor surface of the forearm. It has been shown that in patients convalescent from pneumococcus lobar pneumonia the intradermal injection of type-specific capsular polysaccharide elicited a typical skin reaction, which took the form of an immediate wheal and erythema which reached its height within 15 to 30 minutes. It can first be elicited at or about the time of recovery. In testing the sera of the patients, it was found that in all instances in which a positive skin reaction was elicited with the specific carbohydrates, agglutinins for the homologous type of pneumococcus and precipitins for the reacting polysaccharides were present. Francis and Tillett (1931) found that the injection of the type-specific capsular polysaccharides of Types I, II and III pneumococci into the skin of rabbits actively or passively immunized to one of these types of Pneumococcus, elicits a type-specific cutaneous reaction. Francis (1933) stated that the skin test has proved to be an extremely valuable guide to serum therapy, and a definite prognostic aid. When positive, it denotes that recovery has begun, when negative, it indicates further serum therapy. Miller and Boor (1934) obtained the cutaneous reaction by the intracutaneous injection of 0,1 cc of a 1:1000 solution of gonococcal carbohydrate rendered hypersensitive by the gonococci. Undetermined as the problem at the present may be, it is yet an interesting fact that the protein-free bacterial polysaccharides are capable of producing a reaction in the skin, which is urticarial-like in appearance and runs its course in 1 to 2 hours.

The mechanism of the positive skin test is apparently the resultant of antibody and tissue activity (Francis).

The aggressin-like action of large doses of polysaccharides prepared from Types I, II and III pneumococci has been established. Sia (1926) and Ward (1930) studied the pneumococcicidal power of serum-leucocytes mixtures and of defibrinated human blood and found that the specific carbohydrate of the pneumococcus exerts a strong and type specific antibactericidal action on such systems. The authors suggest that the specific carbohydrate inhibits the bactericidal properties by uniting with its corresponding antibody and thus completely preventing the opsonization and subsequent phagocytosis of the organisms. Ward (1932) studied the neutralization of this action of the bacterial carbohydrates by the corresponding antisera. Antipneumococcus serum, after absorption with the specific carbohydrate, no longer forms a precipitate with carbohydrate, but still has a definite, though diminished bactericidal action on virulent pneumococci in a bactericidal test, and retains also some of its power to neutralize the antibactericidal effect of the specific carbohydrate. Accordingly it is unjustifiable to assume that an animal or human being has no type-specific immunity against a type pneumococcus because no specific precipitins can be demonstrated in the serum. Ward and Enders (1933) have found that the specific carbohydrate which will suppress the bactericidal action of defibrinated human blood does not prevent, under certain conditions, the phagocytosis of virulent pneumococci. The authors suppose that the anticarbohydrate antibody is the only antibody in immune-serum which can induce the phagocytosis.

The rôle of bacterial carbohydrates in anaphylaxis has been a subject of recent experimental investigation. Tomcsik (1927) and Tomcsik and Kurotchkin (1928) isolated carbohydrates from different bacilli (B. lactis aerogenes, pneumobacillus and a yeast) which produced anaphylactic shock in guinea-pigs passively sensitized with homologous immune-sera. Lancefield (1928) working by the same method, obtained anaphylactic shock with the carbohydrate from streptococci. The author doubts whether the carbohydrate alone was responsible for the shock because the products used contained small amounts of nitrogen. Avery and Tillett (1929) working

with the highly purified polysaccharide of the type-specific pneumococci, obtained anaphylactic shock in guinea-pigs passively sensitized with homologous anti-pneumococcus serum from rabbits. Guinea-pigs could not, however, be actively sensitized with the purified polysaccharides alone. Since the materials used were protein-free and many of them also nitrogen-free, the results conclusively demonstrate the capacity of complex sugar to induce anaphylactic shock in animals passively sensitized with antibacterial sera.

Tillett and Avery (1929) demonstrated the capacity of artificially prepared sugar-proteins to produce both active and passive anaphylaxis. The anaphylactic reactions were specific and depended for their specificity on the carbohydrate component and not on the protein fraction of the synthesized sugar-protein. The unconjugated glucosides themselves were not capable of inducing anaphylactic shock.

Biologically the bacterial polysaccharides belong to the group of Landsteiner's haptens; they are precipitable, give a complement fixing test with homologous antibodies and are specific. Of all the reactions of immunity the precipitin test is perhaps the most striking. It is the most specific and least subject to error and technical difficulties. The most striking evidence of the specificity of bacterial polysaccharides has been demonstrated with substances prepared by different methods from the encapsulated organisms (pneumococcus and Friedländer's bacilli). It seems to be less striking with the carbohydrates regarded as somatic in origin and isolated from bacteria in which it has been impossible to demonstrate capsules. Several hetero-antigenic relationships among bacteria have been reported. Avery, Heidelberger and Goebel find it between strain E of Friedländer's bacillus and Type II pneumococcus, and Zozaya among many other bacteria. Miller and Boor showed with the cross-reactions between the carbohydrates of gonococcus and meningococcus and antisera to those two organisms the presence of a biological similarity of these carbohydrates. So were the reactions of B. catarrhalis carbohydrates, which were positive with the antigonococcus and negative with the antimeningococcus serum, and likewise the failure of anti-catarrhalis serum to react with the carbohydrates of the gonococcus strains. The precipitin reactions of antipneumococcus

serum Type III with the carbohydrates of gonococcus and meningoccus also were observed by Miller and Boor. Several authors have considered the possibility that bacteria grown on solid media may adsorb traces of agar or egg white which cause the hetero-reactions (Furth and Landsteiner 1929, Zozaya and Medina 1933). Miller and Boor made the corresponding control tests with agar and egg white, but they were all negative. The carbohydrates prepared from gonococci and meningococci grown in liquid media gave the same non-specific cross-reactions as those prepared from organisms grown on agar medium. It seems improbable, therefore, that the non-specific reactions are due to the substances adsorbed by the bacteria during their cultivation.

Several recent investigators have studied the changes produced by the dissociation process in the antigenic properties of bacteria. Arkwright (1921) found that the structure of the heat-stable "O" agglutinogens was entirely changed during the course of the dissociation process, but there was found no change of the heat-labile "H" agglutinogens. Friedländer (1922) found that the "glatt" forms of paratyphoid bacilli showed a higher water content (91,3 per cent) than did the "rauh" variants (85,8 per cent). Reimann and Julianelle (1926) described the differential distribution of the polysaccharide in the case of smooth and rough variants of the pneumococcus and pneumobacillus. One of the important differences between the two variants of the pneumococcus, the so called S and R forms, is the presence round the S forms of a capsule which has been shown to contain a complex polysaccharide. The type-specificity of the pneumococcus and the virulence of the S cells are associated with the presence of this capsular polysaccharide. The chemical structure of the polysaccharides has been shown to vary from one type of the pneumococcus to another. White (1928 and 1931) studied the carbohydrates obtained from the smooth and rough variants of Salmonella strains. It was shown that smooth forms differ from their rough variants in possessing a specific carbohydrate which is not damaged by boiling in neutral solution and non-antigenic on animal inoculation. It reacts precipitatively in a manner exactly correlated to the agglutinative reaction of the bacterial soma from which it is derived. The alkaline lysates of smooth bacterial bodies

contain substances which are antigenically identical with the body substance of the rough organism. The lysates from the smooth strains yielded on injection into animals purely rough antisera which acted equally on all rough Salmonella variants and on rough variants alone. A serum prepared against a similar lysate of smooth B. suipestifer gave a mixed smooth and rough serum. The presence of the rough somatic antigen in the smooth antigenic complex was thus established. Furth and Landsteiner (1929) and White concluded from their studies that the carbohydrates of the smooth variants of the typhoid-paratyphoid group present the essential fraction of the "O" agglutinogen. Furth and Landsteiner have shown that the rough Salmonella possesses a carbohydrate-containing factor active in precipitation tests. It was found that S sera react serologically only with S carbohydrates, but R sera, on the contrary, react with R and S carbohydrates. Meyer (1930) obtained a carbohydrate from the rough variant of B. dysenteriae Shiga. Meisel and Mikulaszek demonstrated with the aid of complement fixing text the presence of the serologically active polysaccharides in the S, R and O (smooth not flagellated variant) variants of the typhoid-paratyphoid group. They were found group-specific, but not type-specific. White thinks that roughening involves loss of a specific carbohydrate containing a substance which in the smooth organisms dominates the physical and chemical nature of the bacterial surface.

The toxicity of different bacterial carbohydrates on laboratory animals has been but little investigated and has been left an open question for the present. Ecker and Rimington (1927) report obtaining from r. B. Aertrycke a carbohydrate which contains material possessing toxic properties. Boivin and his co-workers obtained toxic polysaccharides from B. Aertrycke, B. coli and Proteus. The rabbits injected with these substances died within 24 hours. No remarkable manifestation preceded death. Miller and Boor have found that both the gonococcal and meningococcal carbohydrates were non-toxic for rabbits and mice.

It is probable that the cells of every species of microbes contain a toxic and antigenic specific complex of carbohydrate nature. The differences between the results obtained by the

investigations of bacterial polysaccharides appear to be due to the variety of methods by means of which the specific polysaccharides were obtained from bacterial cells and from fluid cultures. Undoubtedly, with certain methods denatured carbohydrates (absence of acetyl group, etc.) were obtained.

Carbohydrates of the tubercle bacillus.

Many of the investigators of the chemistry of tubercle bacilli have reported the presence of substances which reduce cupric oxide in an alkaline medium and are, therefore, considered as carbohydrates. All the conflicting reports which have appeared in the earliest literature need not here be reviewed in detail. Only the more important and recent studies will be considered in our present paper. The problem is complicated by the fact that the carbohydrates of the bacilli may be free or in chemical combination with the lipins (glucolipins) and proteins (glucoproteins). Furthermore, it is known that sugar may be an invariable constituent of nucleic acids.

Hammerschlag (1891), in studying the chemical composition of the tubercle bacillus, found that the residue left after the removal of the lipins and proteins was cellulose. Freund (1886), Lange and Dreyfuss (1894) detected the presence of cellulose in tuberculous organs, both in man and cattle, and believed it was present in the bodies of tubercle bacilli.

Ruppel (1898) extracted from the tubercle bacilli a material, which on hydrolysis with hydrochloric acid liberated substances reducing cupric oxide. It was considered a proteinoid substance containing carbohydrate, and analogous to keratin or chitin. Benedix (1901) secured from the reducing substance on treatment with phenyl hydrazin an osazone melting at 153 to 155°, which was considered characteristic for pentosazones. He was convinced that pentose was present in tubercle bacilli and especially in their nucleoprotein portions.

Levene (1904) found a glycogen-like body in tubercle bacilli cultivated on mannite media or on ordinary glycerol broth. It could be precipitated with alcohol and basic lead acetate. On the addition of iodine the material gave a colour test similar to that with glycogen. It contained traces only of nitrogen and phosphorus.

Baudran (1906) macerated tubercle bacilli 8—10 days with 1 per cent hydrochloric acid, and obtained a residue containing much waxy material and also a substance which was considered to be cellulose.

Panzer (1912) extracted tubercle bacilli with hot water and obtained a substance which in concentrated solution gelatinized on cooling. It resembled gum arabic and contained no nitrogen, phosphorus or sulphur. It was precipitated by alcohol and by lead acetate, and reduced Fehling's solution after hydrolysis by hydrochloric acid. The author concluded that the extracted jelly was a pectin which was not galactane.

Kozniewsky (1912) noted that mineral acids in a concentration of 3 to 5 per cent set free from tubercle bacilli an inactive reducing sugar, the source of which seemed to be polysaccharides.

Laidlaw and Duley (1915) obtained from 1000 gr of tubercle bacilli 0,9774 gr of a substance which showed all the characteristics of glycogen and a hydrate complex of carbon of a gummy nature, precipitable by a specific method with the antituberculous sera.

Aguehon and Frouin (1919) obtained from tubercle bacilli a gummy polysaccharide from which glucose split off on hydrolysis.

Zinsser (1921) and Zinsser and Parker (1923) noted that a slightly alkaline extract of tubercle bacilli freed as far as possible from protein by acid precipitation and heat coagulation, contained a substance precipitable by alcohol, which produced typical skin reaction in tuberculous guinea-pigs. A specific precipitin reaction was also given by this material with the serum of an animal injected repeatedly with dead tubercle bacilli. It was shown that the substance separated by slight acidification of the alkaline extracts gave a strongly positive skin test in tuberculous guinea-pigs. Similar extracts for which the term "residue antigen" has been suggested by Zinsser and "specific soluble substance" by Heidelberger and Avery have been prepared from many types of bacteria and yeasts. These extracts contain non-protein substances of the nature of polysaccharides, which appear to be responsible in large measure for the specific precipitating, agglutinating and complement-fixing properties of bacteria.

Mueller (1926) attempted to prepare the "specific soluble substance" from old tuberculin made from synthetic broth by the following formula: KH_2PO_4 5,0 gm, $MgSO_4$ 0,6 gm, Magnesium citr. 2,6 gm, Asparagin 5,0 gm, Glycerol 20 cc, Water 1000 cc, pH 7,0. In testing different functions of the material obtained, the author was forced to the conclusion that this substance was quite separate and distinct from that producing a skin reaction. A specific ring precipitin test was given by this substance up to a dilution of 1:2.000.000. The cause of the precipitin reaction is a non-protein gum which contains 0,3 per cent of nitrogen and a very slight trace of phosphorus. It does not reduce Benedict's solution, but gives a very strong alpha-naphthol test for carbohydrate. It gives no precipitate with picric, tannic, uranium nitrate, silver nitrate, $HgCl_2$, nor with safranin. The optical rotation was determined by a 3 per cent aqueous solution in a 1 cm tube $[\alpha]_D = +17{,}3^0$. The non-protein gum fixes the complement and precipitates in the presence of homologous immune-serum, but fails to give a skin test in tuberculous animals. The method of preparing this material is fully described by the author. Obviously this gum corresponds very closely to that described by Laidlow and Duley, which was prepared from whole tubercle bacilli themselves.

The biological properties of the carbohydrates obtained by very different methods from tubercle bacilli have been studied by many recent investigators. White (1928) reported that a polysaccharide isolated by Dr. Anderson from the human bacilli was toxic for tuberculous guinea-pigs. With doses of 10 mg the animal either died within a few hours, in which case there was a precipitous fall in temperature; or it survived and showed only a temporary fall in temperature with subsequent rise above the normal level. The change in the blood cells was characterized by a rise in the neutrophilic leucocytes and a fall in lymphocytes. These phenomena were confirmed by Sabin, Doan and Forkner (1930). The carbohydrate isolated by Dr. Anderson from the human tubercle bacilli does not include the entire polysaccharide content of the organism. In studying the action of this particular polysaccharide in normal tuberculous animals, the authors have found that it is non-toxic when introduced intravenously into the normal

animal. Introduced intraperitoneally it is irritative, and each succeeding dose continues to elicit a fresh emigration of leucocytes from the vessels. These leucocytes appear to be damaged, for they are actively engulfed by clasmatocytes. Guinea-pigs with extensive tuberculosis may die soon after subcutaneous or intraperitoneal injections of the polysaccharide. The animals showed a progressive fall in blood pressure and body temperature from the time of injection to death.

Sabin, Miller, Doan and Wiseman (1930) tested four preparations of tuberculo-polysaccharides: 1) a sugar isolated by Anderson from human tubercle bacilli, 2) an analogous sugar from the bovine strain, 3) a sugar prepared by Heidelberg, and 4) similar preparations made by the H. K. Mulford Company. These four polysaccharides did not show such a marked killing power for guinea-pigs, as was found in the original studies of Sabin, Doan and Forkner. They may have some killing power under certain conditions, but this is not so constantly related to dosage as in the case of proteins. The temperature reaction in tuberculous and normal animals is elicited by the tuberculo-protein and not at all by the polysaccharides. The polysaccharides used in all the experiments contained probable enough protein, or its degradation products, to account for the temperature reaction. Both proteins and polysaccharides cause a change in the white blood cells when introduced by any route.

J. van Allen Bickford (1932) concluded from his studies that normal rabbits which were inoculated with the tuberculo-polysaccharide prepared by the H. K. Mulford Company from the media in which the tubercle bacilli had been grown, showed a rise in temperature similar to that occurring after the injections of tuberculo-protein. The polysaccharide contained a small percentage of nitrogen to which the rise in temperature may be due.

Pels Lensden (1933) boiled tubercle bacilli for 30 minutes at 100° C with a solution containing 0,7 per cent NaCl and 0,05 per cent $NaHCO_3$. The precipitate of the bacteria was removed by centrifugation and the supernatant liquid, named by the author "coctigen", was used for different studies. The liquid thus obtained was free from protein but showed reactions of polysaccharides. It contained a substance precipi-

table by alcohol. Immunologically the coctigen belongs to that group of specifically reactive substances which Landsteiner has named "haptens". A specific precipitin reaction was given by the coctigens prepared from human tubercle bacilli with the serum of rabbits injected repeatedly with a suspension of boiled human tubercle bacilli. The precipitin reaction failed with anti- "bovine coctigen" serum and bovine coctigen. The author supposed that this serological reaction might be used for the differentiation of human and bovine tubercle bacilli. There was also found a certain difference in the toxicity of the human and bovine coctigen for rabbits. The coctigens were active in the specific complement-fixing reaction. The presence of the tuberculous antibodies was demonstrated by means of coctigen only in the serum of tuberculous guinea-pigs and not in the serum of tuberculous men. Anderson and Newman (1933) isolated from the acetone-soluble fat of the human tubercle bacillus a crystallizable disaccharide trehalose. This water-soluble substance is set free after the saponification of the acetone-soluble fat. Pangborn and Anderson isolated the carbohydrate trehalose from the lipoids of the Timothy-grass bacillus.

In summarizing the review, we see that much work has been done in the course of the past decade in the studies of the bacterial carbohydrates and many important data have been presented showing that the tubercle bacilli contain also soluble substances of a carbohydrate nature. In our present study we will consider not only the polysaccharide fractions from different cultures of tubercle bacilli, but also the varieties of the polysaccharides prepared from different types of the tubercle bacillus and its smooth and rough variants. It began therefore with the preparation of carbohydrate fractions from these bacilli.

Outline of Methods and Experimental Observations.

It was evident from the very first that the isolation of the natural specific polysaccharides from tubercle bacilli would be a difficult matter and, indeed, it was satisfactorily accomplished only after the sacrifice of much material in preliminary experimentation. We were convinced that by means of the complicated chemical procedures used by several previous authors only denatured polysaccharides could be obtained from bacterial

cells. The changes of the chemical structure of the polysaccharides obtained depended upon the procedure employed in their isolation. It is evident that only such a procedure, which excludes the use of high temperature and of brutal chemical reagents, could be considered as satisfactory for the isolation of the natural bacterial polysaccharides. The method finally adopted in our present investigations differs from those of many other experimenters principally in the avoidance of the use of heat and of an excess of alkali and acid. Our method is based on the fact, studied exactly by Boivin and his co-workers, that under appropriate conditions the aqueous extract of specific carbohydrates is almost completely freed from the accompanying proteins by the addition of trichloracetic acid.

Method: The organisms grown on different solid and liquid culture media generally used for the cultivation of tubercle bacilli were gathered on filter paper where the bacterial mass was freed from excess water. The material obtained was then triturated in the mortar and then emulsified with N/4 trichloracetic acid solution in the proportion of 10 gr of the material to 100,0 cc of the solution. The emulsion was allowed to stand overnight in the cold, shaking it 3 times for 2—3 minutes. The precipitate of the coagulated proteins and bacterial cells was removed by centrifugation and filtration through the paper filter. The liquid obtained was neutralized (pH 7,2) with sodium hydroxide, and the slight precipitate was removed by centrifugation. The yellow supernatant fluid which contains the specific polysaccharides is by no means free from all the accompanying contaminations, but it is, nevertheless, suitable for many purposes (precipitation test etc.) without further purification. It is known that a pure product of the bacterial polysaccharide is still difficult to obtain, but the preparing of crude preparations is based on the fact that the specific polysaccharide is almost completely precipitated from aqueous solution by 2 volumes of 96 per cent alcohol or by 3 volumes of acetone. Ward and Smith, Miller and Boor stated that mere repetitive precipitation suffices to eliminate most of the accompanying contaminations. Our method of purification was as follows: To the obtained filtrate, slightly acidified by HCl, were added 2 vol. of 96 per cent ethyl alcohol or 3 vol. of acetone with constant stirring. After standing overnight in the cold

the precipitate, containing the specific polysaccharides, was separated by centrifugation; the alcoholic or acetonic supernatant liquid was siphoned off and discarded. It must be noted that the residue from the supernatant liquid obtained by evaporation to dryness was found to be protein-free by all the protein tests to which it was subjected, but it contained a fraction of carbohydrate which does not concern us here. The precipitate was drained free from excess liquid and while still damp it was dissolved in the minimum amount of hot water. The small amount of insoluble precipitate was removed in the centrifuge and after being washed with about 5 cc of water was discarded. The polysaccharide was precipitated from the slightly acidulated supernatant and washing solution by the addition of 2 volumes of 96 per cent alcohol or 3 volumes of acetone. The precipitate was collected in the centrifuge and dissolved again in hot water. The solution of polysaccharide at this point was clear and colourless. It was rendered slightly acid by the addition of a trace of HCl and the polysaccharide was precipitated again by alcohol or acetone. The precipitate of the specific polysaccharide was now collected and washed in the centrifuge successfully with absolute alcohol and ether. It was then dried over H_2SO_4 in vacuo in the cold. The final product was a yellow powder which was completely soluble in water containing a trace of sodium hydroxide. The purification was more complete when the aqueous solution of polysaccharide, slightly acidified by the addition of HCl, was dialysed in a cellophane bag against distilled water until no chlorine ion was detectable in the dialysate. It should be noted, however, that by the repeated precipitations and dialysis there results a considerable loss in carbohydrate.

In preparing the specific polysaccharides from several species of non-acid resistant bacteria, Boivin and Mesrobeanu found that from the different reagents (mercuric chloride, sulphosalicylic acid, Esbach's reagent etc.) employed for the precipitation of the proteins trichloracetic acid has given the best results. In their cases extraction continued for 3 hours was found sufficient, but we have seen that from tubercle bacilli a satisfactory extraction of the specific polysaccharides can be obtained only after about 24 hours of contact. It is possible that a certain part of the carbohydrate still remains attached to the surface of the precipitated proteins, and the trichloracetic acid thus

does not assure a complete liberation of the bacterial polysaccharides. The supply of polysaccharides was not markedly better if the tubercle bacilli were first extracted with acetone and ether to remove the cell lipoids. In our experiments the quantity of the specific polysaccharide obtained from the extracts by the first precipitation with alcohol or acetone varied from 4,6 to 6,9 per cent. After the final purification of the polysaccharide its quantity was found from 1,5 to 2,5 per cent of the weight of the dried tubercle bacilli. Boivin and his co-workers have found the supply for the tubercle bacilli about 0,5 to 1,0 per cent. We must agree that the method used in this paper for the extraction of the specific polysaccharides from tubercle bacilli can not yet be considered a perfect one, and it is possible that the contact with trichloracetic acid is also susceptible to denature slightly the specific polysaccharides. Boivin and Mesrobeanu (1934) found that the specific complexes obtained from different microbes are not equally sensible towards trichloracetic acid and many other factors.

Physical and Chemical Properties.

Our present study began with the preparation and investigation of carbohydrate fractions from Bacillus tuberculosis hominis and Bacillus tuberculosis bovis, the two organisms of special interest to us. Subsequently Bacillus tuberculosis bovis Calmette-Guérin (abbrev. B. C. G.), Bacillus tuberculosis avium and Turtle bacillus (Friedmann) were added for purposes of comparison. The organisms were grown on ordinary glycerin broth, glycerin agar, glycerin potato and in the synthetic broth of Sauton.

Precipitation with acetone generally showed a greater supply of the specific polysaccharide than precipitation with alcohol. The addition of alcohol to the extract produced a flocky precipitate; that obtained with acetone consisted of small grains. The investigations of the specific carbohydrates prepared from all the organisms named showed that they were amorphous, light yellow in colour in the dry state, and entirely soluble in hot destilled water and in 0,85 per cent sodium chloride solution. They were insoluble in ethyl alcohol, ether, chloroform and acetone. The purified carbohydrates were ash-free; they

gave negative colour tests (biuret and xanthoproteic) for proteins. The solutions of polysaccharides also failed to form precipitates when treated with excellent precipitants of the protein materials: copper sulphate, silver nitrate, phospho-tungstic acid, picric acid, sulphosalicylic acid, tannic acid and mercuric chloride. The ninhydrin, Millons and Hopkins-Cole tests for nitrogenous compounds were also negative. The nitrogen content of the purified polysccharides found by the Kjeldahl micro-determination varied from 0,54 to 1,2 per cent. They contained but slight traces of phosphorus.

The unhydrolysed polysaccharides did not reduce Benedict's and Fehling's solutions. Potassium permanganate was not immediately reduced by them. There developed a slight colour-reaction of glycogen with iodine-potassium iodide solution. Negative orcinol, phloroglycinol and resorcinol reactions indicate the absence of pentose and ketose radicals in the tuberculo-polysaccharides. The Molisch test for dextrose was strongly positive with purified polysaccharides prepared from all types of tubercle bacilli. They were precipitated by the solution of uranium nitrate, basic lead acetate, neutral lead acetate and barium hydroxide. A noticeable difference, however, in the strength of these reactions was seen. The summary of reactions made with the solutions, which contained nearly the same amounts of the polysaccharides, is given in Table I.

Table I.

Reactions with Carbohydrate Preparations.

Reagentia	T. humanus	T. bovinus	B. C. G.	T. avium	Friedmann's b.
Uranium nitr.	++	+++	+	±	++
Basic lead acet.	++	+++	+	+	++
Neutr. lead acet.	++	+++	±	±	+
Barium hydrox.	±	++	±	±	+

It is evident that Table I gives but roughly comparative data of the specific polysaccharides prepared from different types of mycobacteria.

The purified specific polysaccharides obtained from tubercle bacilli were in aqueous solution optically active and rotated the plane of polarized light to the right. For hydrolysis tests of the specific polysaccharides 0,25 gm of dried substance was

dissolved in 25 cc of acid, and boiled under a reflux for several hours. When boiled for 3—6 hours with normal sulphuric acid or 2 per cent hydrochloric acid the polysaccharides were slowly hydrolyzed with formation of reducing sugar, which rotated the plane of polarized light to the right. The reducing sugars, calculated by the Baudouin-Lewin [1]) micromethod as glucose, rose to 23,4 per cent. It seemed that the reducing sugars prepared from different types of tubercle bacilli did not exhibit pronounced chemical differences.

Toxicity for Laboratory Animals.

The toxicity tests were accomplished with the carbohydrates which were not carried through the final step of repeated precipitation. The aqueous solutions used for the injections contained from 0,5 to 1,0 gm per 1000 cc of specific carbohydrates. Rabbits injected intravenously with 1,0 to 2,0 cc of solutions prepared from the human and bovine polysaccharides showed, generally, at first a more or less noticeable loss in weight, which was restored afterwards. Of 12 rabbits injected, two died with evidence of convulsions. One died 3 hours, and the other 24 hours after the injection. The injection of the carbohydrates obtained from B. C. G., typus avium and Friedmann's bacillus was borne by the rabbits without evidence of deleterious effect. The purified preparations of tuberculo-polysaccharides did not show a remarkable toxic effect when repeatedly introduced subcutaneously into normal guinea-pigs. When introduced intraperitoneally, they were irritative, and caused an emigration of leucocytes from the vessels.

As noted in the preceding review of literature, the specific polysaccharides generally cause a remarkable change in the white blood cells when introduced by any route. In the course of our present work, the changes in the white blood cells were studied on rabbits injected intravenously with 1,0 cc of the solutions of specific carbohydrates obtained from various types of tubercle bacilli. The number of leucocytes per cmm was repeatedly determined at several intervals after the injection of polysaccharides. Table II gives comparative data for the changes of the white blood cells.

1) Bullet. Soc. Chim. Biolog. 1927, 9, 280.

Table II.

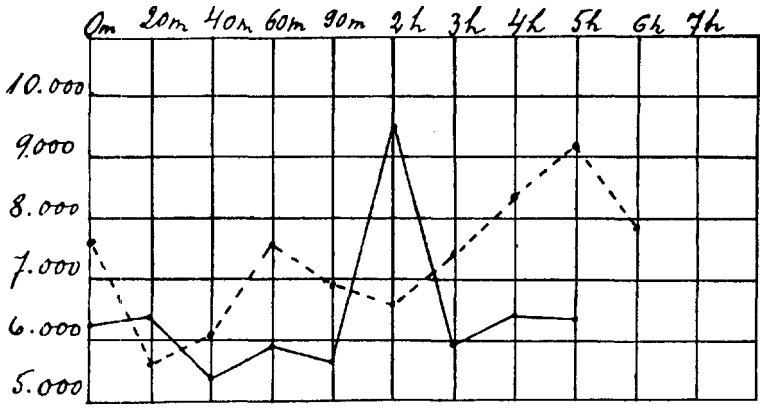
Effects of Tuberculo-Polysaccharides on Normal Rabbits.

Blood count	Rabbits injected with: 1. T. human.	2. T. human.	3. T. bovin.	4. T. bovin.	5. B.C.G.	6. B.C.G.	7. T. avium	8. Friedmann
B. i. [1])	6250	7560	9460	10810	13530	5340	14370	8370
20 m. a.i.[2])	6340	5560	3530	4410	6250	7870	18100	8030
40 „ „	5340	6030	4560	5090	12930	8590	20250	8590
60 „ „	5900	7550	2310	3530	18910	14560	37150	11340
90 „ „	5650	6940	3030	3260	28530	9930	16530	7710
2 hours „	9500	6650	1430	2910	26340	16750	18280	8750
3 „ „	5960	7450	1560	2420	13620	11680	12370	6870
4 „ „	6430	8340	2620	3120	15180	6250	14430	7950
5 „ „	6340	9120	4810	4910	13560	5840	16100	8240
6 „ „	—	7860	6680	10960	—	—	10000	7360
7 „ „	—	—	6620	10640	—	—	—	—

Our diagrams 1, 2, 3 and 4 illustrate very well the oscillations of the number of leucocytes in 7 hours after the injections. We are inclined to believe that the changes in the blood count

Diagram 1.

Typus humanus. —— Rabbit 1. - - - - - - Rabbit 2.



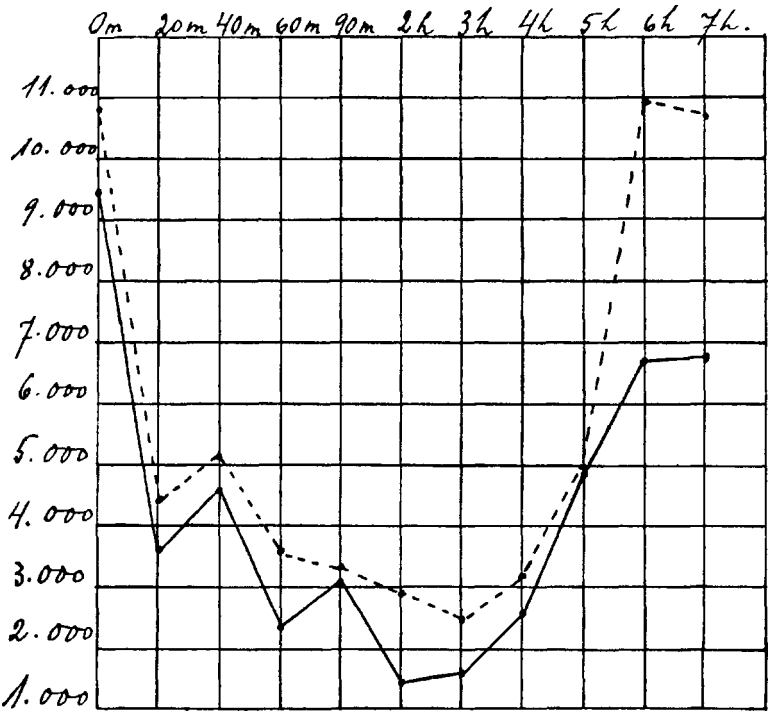
were elicited by the polysaccharides and not by the tuberculo-proteins, because the solutions of polysaccharide used in all these experiments were protein-free and contained but hardly detectable traces of nitrogen. It should be pointed out that the products of hydrolysed polysaccharides did not show a marked effect on normal rabbits.

1) B. i. = before injection, 2) a. i. = after injection.

The rabbits which received the polysaccharides of human type tubercle bacilli intravenously showed a temporary fall of the number of the white blood cells with subsequent rise above the normal level. Similar phenomena followed the administration of the B. C. G. polysaccharides. After the injection of the polysaccharides prepared from the avian type and from Friedmann's turtle bacillus

Diagram 2.

Typus bovinus. — Rabbit 3. - - - - - - Rabbit 4.

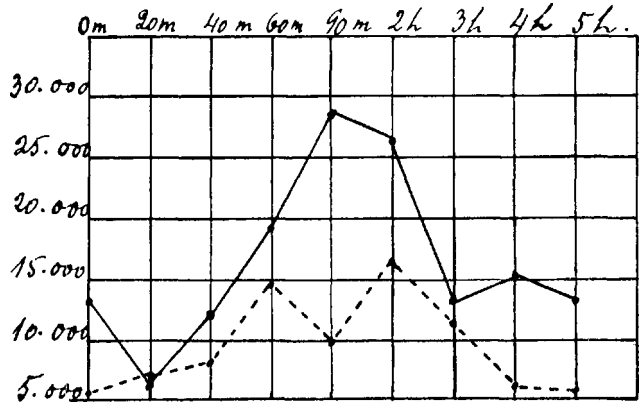


a leucocytosis generally was found which reached its height in 1 hour. The most striking and typical reactions were found after the injection of the polysaccharides obtained from two strains of the bovine type tubercle bacilli. The specific type of curve is shown in diagram 2. The reaction involved a preliminary fall of the number of leucocytes which occurred almost at once after the injection and lasted 4 hours. This fall was followed by a gradual rise and return to the original level in approximately 7 hours. It will be noted that there was also a

marked change in the proportions of the white blood cells, which was characterized by the fall of the percentage of lymphocytes and by a rise of the percentage of neutrophilic leucocytes 20 min. after the injection of the polysaccharides. From 40 min. to 1 hour after the injection a reverse relation of the percentages of these blood cells was found in every instance. Later on, however, the normal relations of the white blood cells were established, while the fall of the total numbers of lympho-

Diagram 3.

B. C. G. — Rabbit 5. - - - - - - Rabbit 6.



cytes and neutrophilic leucocytes, which had begun at once after the injection of the polysaccharides, lasted about 4 hours.

Our experiments furnish additional evidence that the polysaccharides isolated from different types of Mycobacterium tuberculosis are toxic for normal rabbits. The polysaccharide reaction is evidently type-specific and the degree of the reaction to the polysaccharides is governed not only by the amount of the polysaccharide injected, but also by its biochemical properties. The results of our present experiments on rabbits compel us to presume that the striking difference in the changes of the white blood cells obtainable with the purified tuberculopolysaccharides may be used as a valuable aid in the differentiation of human and bovine tubercle bacilli. Further investigation in this direction is now in progress. It should be mentioned that these experiments postulate great caution, because

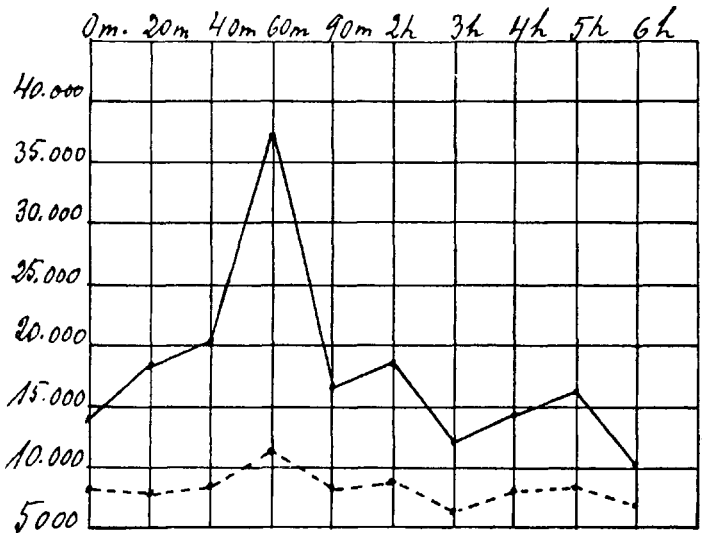
quite similar effects on the white blood cells of rabbits may be elicited by very different products, salts, proteins, carbohydrates, etc. (Hussey, Beard and Beard, etc.).

Antigenic properties of the tuberculo-polysaccharides.

Many contradictory views have been reported in the study of the bacterial carbohydrates as antigens. In summarizing the results hitherto obtained, we may take it for granted that the

Diagram 4.

— Typus avium. - - - - - - Friedmann's bacillus.



majority of authors have failed to induce antibody formation in animals injected with purified bacterial polysaccharides. In our experiments, here reported, all the sera of rabbits and guinea-pigs, which had been repeatedly injected intravenously, subcutaneously and intraperitoneally with purified polysaccharides prepared from various types of tubercle bacilli, contained no antibodies demonstrable by the precipitin reaction and the complement-fixing test. Attempts to immunize animals with specific polysaccharides mixed with proteins were not made.

From these observations it appears that tuberculo-polysaccharides are non-antigenic alone. In the state in which they occur in the bacterial cell, they seem to be antigenic and

produce antibodies in the actively immunized organisms. The presence of the antibodies in blood sera may be demonstrated by those immunological reactions, which are considered the most sensitive. As has been mentioned in the review of literature, of all the reactions for immunity the precipitin test is perhaps the most specific. In this paper we will but shortly report the results of a series of precipitin tests on polysaccharide preparations from human and bovine tubercle bacilli with immune-sera obtained from tuberculous cattle and men.

Precipitin test. The tests were made in small vials which had been thoroughly cleansed with hot chromic acid solution and many applications of distilled water. 0,2 cc of serum was diluted with 0,3 cc of 0,85 per cent sodium chloride solution and then mixed with 0,5 cc of varying dilutions of the polysaccharide in 0,85 per cent sodium chloride solution adjusted to pH 7,1—7,2. The tubes were incubated for 4 hours at 37° C. The test was then examined for the presence of precipitate and the tubes were placed in the ice-box overnight when a second reading was made. The reaction was designated positive, when a definite clouding or precipitate was seen when examined in bright illumination against a black background. The tests were also controlled by the agglutinoscope.

Table III presents the results of precipitin reactions in which polysaccharides from the human type and the bovine type served as antigens. The antigenic solutions used for these tests contained from 1:2000 to 1:3000 of specific polysaccharides, and were adjusted to pH 7,2. The precipitating sera were obtained from cattle in which tuberculosis had been recognized long before, or in which the symptoms of tuberculosis were not detected by physical examinations. For purposes of comparison all these animals were tested with tuberculin (ophthalmo-reaction) [1]), and the results of the reactions are given in Table III.

From Table III it will be seen that in 22 per cent of all 50 sera examined the precipitin reaction was not in harmony with the tuberculin reaction. In 10 per cent of the sera positive results were obtained with the precipitin tests, but the animals from which these sera were received showed negative results with the tuberculin test. On the other hand, 12 per cent

1) We are indebted to Dr. F. Laja, Professor of Veterinary Medicine, University of Tartu, Estonia, for making the tuberculin tests.

Table III.

Precipitin and Tuberculin Tests.

Sera	Precipitinogen		Tuberculin
	Typus humanus	Typus bovinus	Ophthalmoreaction
3	—	+	+
26	—	+++	—
27	—	—	—
34	—	—	—
35	—	++	—
82	—	++	+
86	±	++	+
131	±	+	++
136	—	+++	+++
137	—	±	+
152	++	+++	+
153	+	++	++
157	—	+++	+++
161	—	++	+
162	—	+	±
165	—	+	+
167	+	+++	+++
171	++	+++	++
173	+	++	++
179	—	—	+++
183	—	—	++
185	—	—	—
194	—	++	++
195	—	—	+
199	—	+	++
205	—	+	++
209	±	+++	+++
213	∓	+++	+++
214	—	+++	+++
219	—	+	++
221	—	+	+
222	—	—	—
223	—	+	++
224	—	+	++
226	—	+	++
227	—	++	++
228	±	+++	±
230	—	++	—
231	+	++	—
232	—	—	+
234	—	+	—
237	±	+++	++
284	—	±	±
306	—	+	±
314	++	—	+
404	—	—	+
415	—	+	++
417	±	++	—
480	—	+	++
959	—	—	++

of the sera failed to react with the tuberculo-polysaccharides, although they were obtained from animals in which the tuberculin produced an appreciable ophthalmic reaction. The most probable explanation of this discrepancy is that 1) some of the tuberculous animals failed to react with tuberculin instilled into the eye, and 2) some of the sera obtained from tuberculous animals did not contain workable titers of specific precipitin. It will be noted that the positive precipitin reactions were observed especially with the polysaccharides prepared from bovine tubercle bacilli. With 13 sera we also observed a positive reaction with the polysaccharides of human types, but these reactions were by far less striking than those with the polysaccharides prepared from bovine types. This very striking difference suggests that the tuberculosis of the cattle was caused by Mycobacterium tuberculosis bovis. From 50 sera examined one serum (314) reacted with the polysaccharides from the human type and failed to react with the polysaccharides of the bovine type. The animal from which this serum was obtained showed a positive ophthalmoreaction with tuberculin. Thus, it may be supposed in this case, that the animal was infected with human tubercle bacilli. The above results of our experiments support the view that precipitin tests with the specific polysaccharides may serve as a valuable aid in the differentiation of human and bovine tubercle bacilli. It will be noted that the sera obtained from normal cattle, rabbits and guinea-pigs always failed to react with the polysaccharides of either of those organisms. An inconsistency is to be noted in the case of human sera. From our experiments it was evident that the carbohydrates prepared from human and bovine tubercle bacilli only seldom were precipitated by the sera obtained from tuberculous men. Of 12 sera taken from patients suffering from 3 to 5 years with open pulmonary tuberculosis two sera reacted with the human polysaccharide, and failed to react with the bovine polysaccharide. On the other hand, 8 sera obtained from persons ill with tuberculosis in the bones and joints failed to react with either of these polysaccharides. Of 100 sera sent into our laboratory for the purpose of serological reactions only 6 reacted slightly with the polysaccharides prepared from human tubercle bacilli, although many of the persons with negative serum were infected with tuberculosis.

It was also found that precipitin tests repeatedly made with the serum from a dog, which was supposed to be ill with tuberculosis, were strikingly positive with the carbohydrate fractions prepared from human tubercle bacilli and negative with those obtained from the bovine type. The precipitin tests carried out for purposes of comparison showed that the polysaccharides isolated from living tubercle bacilli react far better than the polysaccharides prepared from tubercle bacilli previously killed by heating for 30 min. at 115° C. This may be explained by the fact that the heating has denatured the specific polysaccharides.

We have been unable to demonstrate any precipitin in the sera of rabbits immunized with purified tuberculo-polysaccharides. None of the rabbits which had been repeatedly injected intravenously with polysaccharides prepared from human and bovine tubercle bacilli developed antibodies demonstrable by the precipitin test.

It seemed important to investigate whether experimental infection in guinea-pigs would produce precipitin for the specific tuberculo-polysaccharides. Two series of guinea-pigs were infected by the subcutaneous injection of virulent tubercle bacilli, one series with the human type, the other with the bovine type. The first examination of the sera obtained from these animals was made 30 days after the inoculation with tubercle bacilli. All the guinea-pigs showed a positive tuberculin reaction and marked symptoms of tuberculosis were found in them. In both instances, however, the precipitin reactions were practically negative. The second series of precipitin tests was made 60 days after the inoculation. In these experiments some differences in precipitation were found. Of 6 sera obtained from guinea-pigs inoculated with the bovine type only 3 sera reacted with the polysaccharides obtained from the bovine strain of tubercle bacilli, and not one of them reacted with the polysaccharides from the human tubercle bacilli. On the other hand, of 6 sera obtained from guinea-pigs infected with the human type 2 sera showed a precipitin reaction (which might be considered practically positive) with the polysaccharides from the human strain of tubercle bacilli, and one serum showed but a slightly positive precipitin reaction with the polysaccharides from the bovine type of tubercle bacilli. Concer-

ning these irregular precipitin reactions it should be noted that the tuberculous infection of our guinea-pigs was too intensive and the specific precipitins could not develop during the short course of the lethal disease.

Complement fixation. In order to decide whether or not antigenic differences of the tuberculo-polysaccharides of the human and bovine types exist, the complement-fixing tests were made with the sera obtained from tuberculous men, cattle and guinea-pigs. In summarizing the results of our experiments, it must be noted that the purified tuberculo-polysaccharides reacted but slightly with some of our sera. The inconsistency was, however, so evident that we are yet obliged to abstain from a definite conclusion. Hardly detectable complement fixation was seen with some sera of tuberculous cattle, less with the sera of tuberculous guinea-pigs and none with the sera of tuberculous men. The explanation of this discrepancy is difficult to give, but we hope that further studies, which are now in progress, may throw more light on this question.

Cutaneous reaction. Of the phenomena manifesting hypersensitiveness to bacteria or their products, the skin test has attracted the greatest attention of the investigators, and the results hitherto obtained have proved to be an extremely valuable aid in the study of different infections. In our present study tuberculous guinea-pigs were tested with tuberculo-polysaccharides. The animals used in these experiments were all tested with tuberculin, and the tests were negative before the inoculation with tubercle bacilli. The skin tests made with the tuberculo-polysaccharides were also negative before the inoculation. Two series of guinea-pigs were inoculated with tubercle bacilli. The first series received subcutaneously $^1/_{50}$ mg of a 15-day culture of typus humanus strain and the second series a similar quantity of typus bovinus strain. The skin tests with the purified tuberculo-polysaccharides were begun about 4 weeks after inoculation, and were continued for 3 months. The skin test was positive to tuberculin in each instance before the injection of tuberculo-polysaccharides. For the skin tests about 0,001 mg of purified polysaccharide dissolved in 0,1 cc of 0,85 per cent sodium chloride solution was given intracutaneously. The polysaccharides used in these experiments were prepared from virulent strains of human and

bovine tubercle bacilli, from B. C. G. and Friedmann's turtle bacilli. The results of our experiments are shown in Table IV.

Table IV.

Skin Tests with Polysaccharides.

Guinea-pig infected	After 4 weeks				After 8 weeks				After 12 weeks			
	Type of polysaccharide injected											
	hum.	bov.	B. C. G.	Friedm.	hum.	bov.	B. C. G.	Friedm.	hum.	bov.	B. C. G.	Friedm.
52. humanus	—	—	—	—	±	—	—	—	+	—	—	—
53. „	—	—	—	—	—	—	—	—	±	—	—	—
54. „	—	—	—	—	±	—	—	—	+	—	—	—
55. „	—	—	—	—	+	—	—	—	++	—	—	—
56. „	—	—	—	—	±	—	—	—	+	—	—	—
57. „	—	—	—	—	—	—	—	—	±	—	—	—
65. bovinus	—	±	—	—	—	+	±	—	—	+	±	—
66. „	—	+	—	—	—	++	±	—	±	++	+	—
67. „	—	±	—	—	—	+	—	—	—	++	±	—
68. „	—	—	—	—	—	±	—	—	—	+	—	—
69. „	—	±	—	—	—	+	±	—	—	+	±	—
70. „	—	±	—	—	—	+	±	—	—	++	+	—

A study of Table IV shows that in guinea-pigs previously infected with various types of tubercle bacilli the intradermal injection of type-specific purified polysaccharide elicits a skin reaction, which reaches its height within 24 hours. The skin reaction generally begins with erythema and edematous swelling, but afterwards a nodular infiltration develops in the upper layer of the skin. The hypersensitiveness to the specific polysaccharides develops but slowly, and it can be demonstrated by the skin test only after a certain period of infection, where the guinea-pigs already show a strongly positive tuberculin reaction. The hypersensitization of the cells to the specific polysaccharides seems to be always of the delayed type of reactions, which takes place in the organism of tuberculous animals. We might conclude from our few preliminary experiments that some difference is to be found in the sensitizing activity of the human and bovine tubercle bacilli. It seems that hypersensitiveness to the type-specific polysaccharides develops sooner and is more evident in guinea-pigs inoculated with bovine tubercle bacilli than in guinea-pigs inoculated with

human tubercle bacilli. It appears from Table IV that the tuberculo-polysaccharides used in our present experiments were strongly type-specific. Our observations also permit the conclusion that there exists an appreciable difference between the carbohydrates prepared from B. C. G. and virulent bovine tubercle bacilli, although a certain degree of relationship between them was demonstrated by the cutaneous reactions.

A series of experiments was performed to compare the sensitizing power of the tuberculo-polysaccharides in guinea-pigs if they are injected alone. Guinea-pigs were rendered hypersensitive to the polysaccharides by repeated subcutaneous injections of 1,0 cc of a 0,5 : 1000 solution of carbohydrate. 4 guinea-pigs of the first series received 5 days successively an injection of the polysaccharide prepared from the human type of tubercle bacilli, and 4 guinea-pigs of the second series received similar injections of the polysaccharide obtained from the bovine type of tubercle bacilli. The cutaneous reactions were made 4 weeks after the last subcutaneous injection. The specific polysaccharides used for the skin tests were prepared from the virulent strains of human and bovine tubercle bacilli, from B. C. G. and Friedmann's turtle bacilli. The results of these tests are shown in Table V:

Table V.

Skin Tests in Sensitized Guinea-Pigs.

Guinea-pig sensitized	Type of polysaccharide			
	Humanus	Bovinus	B. C. G.	Friedmann
80. humanus	+ +	±	−	−
81. „	+	−	−	−
82. „	+ +	±	±	−
83. „	+	−	±	−
84. bovinus	−	+ +	+	−
85. „	−	+ +	±	−
86. „	−	+	+	−
87. „	−	+ +	+	−

The results shown in Table V were found 24 hours after the intracutaneous injection of 0,1 cc of a 1,0 : 1000 solution of carbohydrate. It appears from Tables IV and V that no striking difference was detected in the specificity of the hypersensitiveness evolved by the specific polysaccharides and by the living organisms themselves. A comparison of the skin tests

shows that the reactions made with polysaccharide were type-specific. It should be mentioned that guinea-pigs rendered sensitive to the specific polysaccharide prepared from the bovine type of tubercle bacilli were found to be also allergic to the polysaccharide obtained from B. C. G.

Anaphylaxis. It has been reported that carbohydrates isolated from different bacilli are capable of producing anaphylactic shock in guinea-pigs passively sensitized with homologous immune-sera. In the present investigation we attempted to answer the important question whether this method is practically available for the differentiation of human and bovine tubercle bacilli. For this purpose four rabbits were passively sensitized by the intravenous injection of 2 cc of the serum obtained from tuberculous cattle. The serum showed a strongly positive precipitin reaction with the polysaccharide prepared from bovine tubercle bacilli and no precipitation with the polysaccharide obtained from human tubercle bacilli. 24 hours after the injection of the serum two rabbits received intravenously 2 cc of a 1 : 1000 solution of bovine polysaccharide and the other two rabbits an injection of the human polysaccharide. No evident signs of an anaphylactic shock followed in any of our rabbits. It should be mentioned that the inconsiderable fall of temperature and the type of the changes in the blood cells (striking leukopenia), which immediately followed the injection of the homologous type-specific polysaccharide, might be considered with a certain reservation as the symptoms of an anaphylactic shock. The experiments performed by the same method on guinea-pigs showed not much better results. It is possible that the serum obtained from tuberculous cattle did not contain enough antibodies, which are necessary for the reactions of an evident anaphylactic shock. Further studies with the specific sera obtained from artificially immunized animals are now in progress.

Dissociation and changes in the carbohydrates of tubercle bacilli.

During the past several years the dissociation of bacteria has aroused increasing attention. Several investigators have reported that the dissociation process produces profound changes in the chemical nature of bacterial cells. Some differences have

also been found in the chemical structure of the specific polysaccharides obtained from smooth (S) and rough (R) variants. It was reported that S sera react only with S carbohydrates, but R sera, on the contrary, react with R and S carbohydrates. As is well known, the smooth virulent avian strain of tubercle bacilli, when cultivated on the glycerin-potato medium, takes at last a rough and dry aspect and gradually diminishes its virulence. The probable explanation of this phenomenon is difficult to give. Winn and Petroff, Sabin, Miller, Doan and Wiseman, Birkhang[1]), Saenz and Costil studied the effects of the dissociation of the avian strains and found that the two dissociated avian strains S and R produce two different combinations of the manifestations of the disease. The initial variant S strain, which always predominates in cultures newly isolated from the tuberculous organs, is extremely virulent to poultry and rabbits. This strain conserves its virulence, when cultivated on Loewenstein's egg-medium. The avian R strains were found to be strikingly less virulent or avirulent to animals. It was shown that smooth forms differ from their rough variants also in the contents of pigment, lipides and in antigenic properties. Reed, Rice and Birkhang have found that the human and bovine S strains are agglutinated by a special solution of citric acid of pH2,4—3,0, R strains by pH4,2—4,8. The avian S strains are agglutinated by pH3,6—4,0, R strains by pH6,8—7,2.

In our present study a preliminary attempt was made to differentiate the carbohydrates prepared from the smooth and rough variants of the avian strains of tubercle bacilli. The avian type seems to be especially available for this purpose, because the dissociation process of this organism is relatively easy to carry out, while the dissociation of bovine strains and particularly of human strains is known to be a very laborious task. Our present experiments have been made with carbohydrate preparations isolated from smooth and rough strains cultivated 15 days on an asparagin-egg-agar medium, which is a certain modification of Loewenstein's medium.

We will but shortly summarize here the results hitherto obtained. It was found that smooth forms differ from their rough variants in possessing a larger percentage of specific carbo-

1) Birkhang, C. R. Soc. Biol. 1933, 113, 814.

hydrate. The quantity of purified polysaccharide extracted from S strain was found to be 2,5 per cent of the weight of the dried bacilli, the quantity obtained from R strains rose only to 1,2 per cent. It seems to be possible that roughening of the avian tubercle bacilli involves loss of a specific carbohydrate. Such a striking difference in carbohydrate content suggests that the pathogenicity of the avian tubercle bacilli depends to a certain degree on the specific carbohydrates present in the bacterial cells. On the other hand, the nitrogen content was found greater in the purified R polysaccharide. It varied from 0,8 to 1,2 per cent in R polysaccharides and from 0,6 to 0,9 per cent in S polysaccharides. The explanation of this phenomenon is yet difficult to give. Furthermore, hydrolysis tests were made. The purified polysaccharides obtained from the avian S and R strains were boiled for 6 hours with 2 per cent hydrochloric acid and then the quantity of the reducing sugar was determined by the micromethod. The reducing sugars liberated from S polysaccharide and calculated as glucose rose to 23,0 per cent, those liberated from R polysaccharide rose to 15,5 per cent. The reducing sugars prepared from S and R polysaccharides did not exhibit evident chemical differences.

Investigation of the toxicity as well as of the immunological behaviour of the S and R polysaccharides isolated from avian strains is now in progress. The skin tests made with them in animals, which were inoculated with S and R variants of avian tubercle bacilli, suggest, however, the different natures of S and R polysaccharides. It should be mentioned that the differences in reaction to bacterial dissociation demonstrate the significance of this method in the study of tuberculosis.

Discussion.

The understanding of the nature of antigenic specificity is one of the most important goals of modern bacteriology. It is now generally acknowledged that the type-specificity of bacteria depends not only upon the different kinds of proteins present in the bacterial cells, but also upon the lipoids and carbohydrates. The results hitherto obtained have persuaded many investigators that bacterial carbohydrates may be considered as determinative substances in bacterial specificity, but they have only recently

attracted the attention that they deserve. Undoubtedly, the rôle which bacterial carbohydrates play in the phenomena of immunity is a considerable one.

Our present report concerns certain properties of the polysaccharides obtained from different types of tubercle bacilli: human, bovine and avian types, B. C. G. and Friedmann's turtle bacillus. It is evident that the use of high temperature and of brutal chemical reagents in the isolation of bacterial polysaccharides causes changes in their chemical structure, and with such methods only denatured preparations of carbohydrates can be obtained. The method finally adopted in our present investigations is a modification of the procedure used by Boivin and his collaborators. This method differs from those of many other investigators in the avoidance of undesirable factors such as heat and excess of alkali and acid. It is quite possible that extraction by means of trichloracetic acid does not assure a complete liberation of the carbohydrates from tubercle bacilli, and a certain part of tuberculo-polysaccharides was lost in our isolation process. The quantity of the purified specific polysaccharides obtained from tubercle bacilli varied from 1,5 to 2,5 per cent of the weight of the dried tubercle bacilli. Boivin and his collaborators have found the supply about 0,5 to 1,0 per cent. We consider the possibility that our method is not yet perfect, as well as the possibility that contact with trichloracetic acid is liable slightly to change the chemical nature of the bacterial carbohydrates.

It should be noted that the tuberculo-polysaccharides described in our present paper might be regarded as somatic in origin, because it has been impossible to demonstrate capsules on tubercle bacilli. Undoubtedly, the tuberculo-polysaccharides in bacterial cells are combined with another cellular constituent, possibly protein (glucoprotein), and form a highly complex antigen. This specific complex antigen is evidently easily dissociable, and its antigenic specificity depends upon components of relatively simple chemical composition, among which the specific polysaccharide should be noted.

The physical and partly also the chemical properties of the tuberculo-polysaccharides were found to be nearly similar to the properties of other bacterial carbohydrates. The purified tuberculo-polysaccharides were protein- and ash-free. Their

nitrogen content varied from 0,54 to 1,2 per cent. They contained but traces of phosphorus. When precipitated by the solution of uranium nitrate, basic lead acetate, neutral lead acetate and barium hydroxide, a noticeable difference in the strength of the reaction was found with the polysaccharides isolated from different types of tubercle bacilli. The polysaccharides of all types rotated the plane of polarized light to the right. When boiled for 3—6 hours with a sufficient excess of mineral acid a reducing sugar was formed, which rotated the plane of polarized light to the right.

The toxicity and killing power of the human and bovine tuberculo-polysaccharides, as determined in normal rabbits and guinea-pigs, were found to be insignificant, but in any case higher than those of the polysaccharides isolated from B. C. G., avian type and Friedmann's bacillus.

The changes in the number and proportions of leucocytes studied on rabbits, which intravenously received 1,0 cc of the solution of tuberculo-polysaccharides, may be used as a valuable aid in the differentiation of human and bovine tubercle bacilli. The injection of the bovine polysaccharide involved a striking preliminary fall of the number of leucocytes, which lasted about 4 hours. This fall was followed by a gradual rise and return to the original level in 6—7 hours. The polysaccharide reaction is evidently type-specific and depends not only upon the amounts of the polysaccharide injected, but also upon its biochemical properties.

We failed to produce demonstrable antibodies in rabbits and guinea-pigs repeatedly injected with purified tuberculo-polysaccharides. Nevertheless, we are inclined to believe that purified tuberculo-polysaccharides are not entirely non-antigenic. It appears that after the injection of bacterial carbohydrates the formation of antibodies is remarkably slower, and the evolution of immunizing reactions is more delicate than is generally found after the injection of bacterial proteins. The presence of the specific antibodies, however, may be demonstrated by precipitin tests in blood sera of tuberculous cattle, especially in those cases, in which the tuberculous infection has lasted a long period. In 22 per cent of all 50 sera examined the precipitin reaction made with specific tuberculo-polysaccharides was not in harmony with the tuberculin reaction made for purposes of comparison.

In 10 per cent of the sera positive results were obtained with the precipitin test, and negative ones with the tuberculin test. In 12 per cent of the sera a reverse result was obtained. The most probable explanation of this discrepancy is that the ophthalmoreaction with tuberculin cannot be considered as an absolute indicator of the tuberculous infection of cattle and it postulates great caution in performance. We may conjecture that precipitin tests with the specific tuberculo-polysaccharides may serve as an aid in the differentiation of tubercle bacilli. It will be noted that the tuberculo-polysaccharides only rarely were slightly precipitated by the sera obtained from tuberculous men.

Our experiments on guinea-pigs infected by the inoculation of virulent tubercle bacilli showed that type-specific precipitins, demonstrable by the precipitin test with tuberculo-polysaccharides, appeared in the blood only at a certain period after the tuberculin reaction had been already rendered positive. It is possible that the tuberculous infection of our guinea-pigs was too acute and its course too short in order to enable the production of specific precipitins. The complement fixation tests were found to be negative with all the sera of tuberculous guinea-pigs examined in our present investigation.

The hypersensitization of the cells to the specific tuberculo-polysaccharides also was found to be of the delayed type of reaction. The hypersensitiveness was detected by the skin test only when the tuberculous infection of guinea-pigs was already perfectly developed. The preparations of tuberculo-polysaccharides used for our skin tests were found to be strongly type-specific. It is interesting that an appreciable difference between the carbohydrate isolated from B. C. G. and virulent bovine tubercle bacilli was found, although there existed a certain degree of relationship between them. Guinea-pigs rendered hypersensitive to the tuberculo-polysaccharides by the repeated injections of these substances showed no striking difference in the specificity of the hypersensitiveness evolved by the specific polysaccharides and by the living tubercle bacilli themselves. Rabbits and guinea-pigs passively sensitized by the intravenous injection of blood sera obtained from tuberculous cattle showed no evident signs of an anaphylactic shock after the intravenous injection of the type-specific polysaccharide.

In studying the carbohydrates isolated from the smooth (S)

and rough (R) variants of the avian tubercle bacillus, we found that smooth forms differ from their rough variants in possessing a larger percentage of specific carbohydrate. The nitrogen content was greater in the R polysaccharide, but the quantity of the reducing sugars liberated on the hydrolysis process was greater in the S polysaccharide. The skin tests made in animals inoculated with S and R variants of the avian tubercle bacillus suggest the different natures of S and R polysaccharides.

We have to-day only insufficient knowledge of the reactions caused by the specific tuberculo-polysaccharides in the different tissues of infected organisms. In tuberculous animals this complex chemical factor is probably liberated from the bodies of dead tubercle bacilli. The amount of the reaction to the bacterial carbohydrates is evidently governed by the quantity of the liberated factor and by the amount of anticarbohydrate produced in the organisms of the infected animals. Our experiments described above showed that the specific tuberculo-polysaccharides are toxic for normal rabbits and guinea-pigs. Furthermore, immunization with these substances is possible only in appropriate conditions and its development evidently demands a very long period. Thus it may be supposed that the specific tuberculo-polysaccharides in tuberculous organism develop reactions which are undoubtedly injurious to health.

Conclusions.

A summary review of the literature concerning bacterial carbohydrates is given.

The method of isolation and purification of the tuberculo-polysaccharides is described and discussed.

The properties of the specific polysaccharides isolated from human, bovine and avian types, B. C. G. and Friedmann's turtle bacillus were investigated.

The purified tuberculo-polysaccharides were found to contain from 0,54 to 1,2 per cent of nitrogen, to be protein-free by chemical test, and to reduce the Fehling-Benedict solution only after hydrolysis.

They were but slightly toxic for normal rabbits and guinea-pigs and caused a remarkable change in the number and proportions of the leucocytes.

They failed to engender antibodies in rabbits and guinea-pigs if injected alone. Precipitins were demonstrated in the sera of tuberculous animals and men.

The tuberculo-polysaccharides caused typical cutaneous reaction in tuberculous guinea-pigs and in guinea-pigs rendered hypersensitive by the injection of bacterial carbohydrates.

An attempt was made to differentiate the carbohydrates isolated from the smooth (S) and rough (R) variants of the avian strains of tubercle bacilli.

Attention is called to the fact that the specific tuberculo-polysaccharides cause reactions injurious to health.

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