Report of Dr. Avery with Drs. Stillman, Goebel, Dubos, Francis, Babers, Goodner, Alloway and Terrell.

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VI. Publications.

I. The decomposition of the capsular polysaccharide of Type III Pneumococcus by a specific bacterial enzyme. (Drs. Avery, Dubos, Goodner and Francis.)

1. Methods of purification and concentration of enzyme. In previous reports we have described the isolation of a microorganism capable of decomposing the capsular polysaccharide of Pneumococcus Type III, and the extraction from cultures of this bacillus of the active enzyme responsible for this action on the polysaccharide. This enzyme is not only active *in vitro* but retains its specific activity *in vivo* as demonstrated by the protective action afforded mice infected with Pneumococcus Type III.

The work of this year has been concerned chiefly with a study of the action of the enzyme on the course of an experimental disease induced by infecting rabbits intradermally with a rabbit virulent strain of Pneumococcus Type III.

However, before describing this study, modifications in the technique of preparation of the enzyme will be given. It will be recalled that the yields of enzyme were much increased by the addition of small amounts of yeast extract to the synthetic mineral medium originally used. Unfortunately, the preparations thus obtained exhibited a definite primary toxicity expressing itself by a sharp rise in temperature of the injected animals and at times death within a few minutes after injection. The production of non-toxic preparations has been attempted along three

different lines: (a) the amount of yeast extract in the medium has been reduced to the minimum concentration compatible with good yields of enzyme (.03 per cent); (b) toluene, which had been added to some cultures in order to achieve a more rapid and more complete extraction of the enzyme, has been completely eliminated from the operation; (c) the autolysate is now purified by a process of differential adsorption on aluminum hydroxide; this process is such that, under well defined conditions, the toxic principle is adsorbed on the aluminum gel while the enzyme remains in solution.

Concentration of the enzyme preparations has been achieved by two different techniques: (a) distillation under reduced pressures at temperatures not exceeding 35°C; (b) ultrafiltration through a cellulose acetate ultrafilter; in this case the enzyme remains in the filter while most of the irrelevant material is eliminated with the filtrate.

Throughout the steps of preparation, purification and concentration of the enzyme, the operations have been controlled by quantitative determinations of activity by the titration method described in the previous report. We have found it convenient to express the potency of the preparations in terms of units. The unit is defined as 100 times the minimal amount of enzyme required for the decomposition of .01 mg. of chemically purified capsular polysaccharide in 18 hours at 37° C.

The purified, concentrated enzyme preparations exhibit little if any toxicity for normal rabbits when injected intravenously. Normal monkeys which received very

large amounts of these preparations by the intravenous route exhibited no reaction recognizable by change in the temperature or behavior of the animal. However, the study of the blood picture revealed a sharp rise in the white cell count for a few hours following injection. Whatever the nature of the leucocytic reaction, we are inclined to consider it as beneficial in view of the essential part played by phagocytosis in the course of events following enzyme treatment of infected animals.

2. The protective action of the specific enzyme in dermal infection of rabbits with Type III Pneumococcus. In a previous report it has been shown that the enzyme which specifically decomposes the capsular polysaccharide of Type III Pneumococcus has a marked protective action in mice infected intraperitoneally with this organism and is capable of bringing about the recovery of these animals when given even after the infection is already established. The intraperitoneal injection of mice with pneumococci leads quickly to a generalized infection. On the other hand, the dermal infection of the rabbit, previously studied by one of us in detail, gives rise to a disease which is primarily localized and in which the infecting organisms in the local lesion are less accessible to the action of an agent administered intravenously. However, the specific enzyme has a marked curative action in the disease brought about by infecting rabbits intradermally with a highly virulent strain of Type III Pneumococcus. The use of this enzyme in suitable amounts in the infected animal promptly frees the blood stream of bacteria, leads to a

sterilization of the focal area of infection, and a consequent complete cessation of the disease process. A mortality rate of 5 per cent in treated animals is in sharp contrast to a mortality rate of 95 per cent in untreated rabbits. The curative action of the enzyme is type-specific. Its activity is destroyed by heat. The results of this study yield further evidence that the capsular substance is an important conditioning factor in pneumococcal infection, since, in so far as known, the only action of which the specific enzyme is capable is that of decomposing the capsular polysaccharide.

Animals which have recovered following enzyme injections within 24 to 48 hours after infective inoculation show no active immunity against subsequent massive reinfection. On the other hand, animals which were treated repeatedly over a period of days with small amounts of enzyme and in which the disease was not arrested until the fifth to ninth days, show a high degree of active immunity against reinfection.

An elaboration of one phase of these studies has dealt with the question of the minimal amounts of enzyme necessary to bring about recovery of the infected rabbit and with the correlation of certain factors involved in these quantitative relationships.

Drs. Dubos and Avery have shown, by <u>in vitro</u> experiments, that, after a definite incubation period, the total amount of specific substrate decomposed bears a quantitative relationship to the concentration and activity of the enzyme preparation used. If it were possible to apply

this finding to the experimental infection, the minimal amount of enzyme necessary to bring about recovery should have a quantitative relationship to the amount of specific capsular polysaccharide in the animal body. This substance is of course associated with the pneumococci but may also be present in soluble form entirely free from the microorganisms. It is technically impossible to determine the total amount of the specific polysaccharide in the infected animal or even to estimate the total number of pneumococci. The nearest approximation is the determination of the number of viable pneumococci in the circulating blood. Other factors the same, this index should, on theoretical grounds, bear some quantitative relationship with the amount of enzyme necessary to bring about recovery.

A series of 85 rabbits has given definite evidence that this is actually the case. For example, 2.5 units of enzyme were sufficient to bring about recovery when the bacteriemia amounted to 3 pneumococci per cc. of blood, 6.6 units sufficed with 15 organisms per cc., 20 units were required with bacteriemias between 100 and 1,000, 50 units sufficed with bacteriemias between 1,000 and 5,000. However, 100 units failed to bring about recovery when the number of pneumococci per cc. of blood exceeded 20,000.

The results indicate that as the bacteremia increases the amount of enzyme required to bring about recovery is greater although the two ratio between the two factors is not proportional.

Studies of the specific action of the enzymes on

Type III infections in mice and rabbits have furnished convincing evidence that this agent exerts a favorable influence on the course of the disease in these animals. Interesting as these results are, it is at once apparent, however, that the experimental conditions under which the enzyme has thus far been tested are not entirely comparable to those prevailing in pneumococcus pneumonia. A crucial experiment has therefore been undertaken to determine whether with this particular type of Pneumococcus an experimental pneumonia can be produced in monkeys which in clinical course and pathology more closely resembles the spontaneous disease in man; and finally, whether the enzyme would be effective under the more complicated conditions of an actual consolidation of the lung.

For this study, the Java (M. Cynomologos) monkey was selected; since other workers in attempting to produce experimental pneumonia, have found the more common M. Rhesus and the Cebus species rather unsatisfactory. In addition, the Java monkey has proven to be somewhat hardier and rarely infected with tuberculosis. A strain of Type III Pneumococcus, virulent for rabbits, was chosen for the original experiment; and this strain as recovered from an infected monkey has been reinoculated serially into the succeeding animals. The use of morphine, suggested by Robertson and his co-workers in the production of experimental pneumonia in dogs, has been adopted, for it was thought that morphine would abolish coughing, perhaps reduce the resistance of the animal temporarily and hence allow the organisms to gain a foothold in the lung. The drug is administered 2-3 hours before inocula-

tion. Roentgenograms of the chest are taken before inoculation as control, and at least once daily thereafter.

The technique of the inoculation is as follows: The animal is anaesthetized with ether. While the jaws are held widely open, the tongue is drawn forward with lingual forceps, the epiglottis grasped with plain forceps, and the larynx thus made clearly visible. A hard rubber catheter is passed into the trachea until resistance is encountered. The organisms centrifugated from a fresh culture and resuspended in 0.5 cc. of soluble starch solution are introduced from a syringe fitted into an adaptor in the end of the catheter. The material is allowed to flow into the trachea while the animal is held upright. An equal volume of air is then slowly injected to ensure the emptying of the catheter. Care is taken at all times to avoid contamination with material from the mouth.

Following the inoculation, daily blood cultures, blood counts, and temperature records are made in addition to the x-rays.

By this method it has been possible to produce a pneumonia which is distinctly lobar in distribution and tends progressively to involve more than one lobe. The spread of the lesion has been clearly followed by means of the roentgenograms. At autopsy the gross pathological findings are quite comparable to the typical lobar pneumonia in man. This similarity has been further confirmed by the microscopic examination of histological sections.

Although the demonstration of lobar pneumonia in monkeys has been successfully accomplished, certain obstacles

have been encountered which have delayed the study of the therapeutic capacity of the enzyme. These are primarily the variations in the resistance of the individual monkeys, and, consequently, the difficulty in establishing a predictable course of disease with a minimal standard dose of micro-organisms. Within a narrow range of dosage, 0.2 - 0.4 cc., one animal may develop pneumonia and die in 48 hours, with massive pulmonary involvement and overwhelming septicemia, whereas another may survive a well-developed, progressing pneumonia, with or without septicemia, after 7 - 8 days. With the repeated passages of the culture and the use of morphine, however, it is possible to produce a fatal pneumonia in practically all cases.

Since the purpose of the experiments is to attempt an evaluation of the effect of enzyme treatment on the pneumonia, it is highly desirable to be able to induce infection which will run a uniform course of 4 to 5 days, terminating fatally when untreated. This would enable one to have fairly accurate information of the animal's condition before any therapeutic interference was attempted. At present, the effort is directed toward balancing the host factors and those of the invading organism to this end. It is thought that these factors can be fairly well controlled and that the study may soon be carried to a definite conclusion.

II. Isolation of microorganisms decomposing the capsular polysaccharide of different types of Pneumococcus. (Drs. Dubos and Goodner.) The search for other microorganisms decomposing the specific polysaccharides of Pneumococcus Types I and II is being continued.

In principle, the method remains the same as that elaborated two years ago for the isolation of the bacillus capable of decomposing the Type III capsular polysaccharide. The method consists in the use of very specific media containing the substance to be decomposed as sole source of energy; these media are incubated under a great variety of conditions so as to bring out as many as possible of the organisms potentially capable of decomposing the substance in question.

In fact, several cultures (at least 5) have been obtained thus far which are capable of decomposing the Type II specific substance, but none of them has been sufficiently purified to warrant a statement as to the nature of the active organism. The work has been rendered difficult by the fact that all these cultures decompose the polysaccharide only very slowly in liquid media, although some of them are quite active in sand and soil media. An interesting lead has been the successful preparation during the past few weeks of catalysts which, when added in small amounts to the liquid medium, greatly increase the rate of decomposition. Concerning the nature of these catalysts, we have no real knowledge, but there is some suggestion that they may be soluble organic compounds of iron.

III. Chemo-immunological studies on conjugated carbohydrate-proteins. (Drs. Avery, Goebel and Babers.)

1. The specificity of antigens prepared by combining α and β glucoside of glucose with protein. The study of antigens prepared by chemically combining sugar derivatives with protein has revealed the fact that carbohydrates exert a determining influence on the immunological specificity of compounds of which they form a part. In preceding studies evidence has been acquired that mere differences in the structural configuration of relatively small groups in the hexose molecule may determine the serological specificity of two sugar derivatives identical save in this one respect. As previously shown, the β-glucosides of glucose and galactose differing from each other only in the interchange of the H and OH groups on a single carbon atom, exhibit an individual specificity irrespective of the protein to which they are attached.

These earlier studies concerned themselves with the specificity of similar glucosides of two different hexoses, glucose and galactose. A paper now in press deals with the specificity of different glucosides of the same sugar, namely the α and β forms of glucose coupled to globulin. In both the "α and β antigens" thus formed the glucosidic linkages to the protein differ from each other only in their spatial relations to the rest of the antigenic molecule. Since the ultimate composition of both antigens is the same, the differences observed in their immunological specificity are referable only to known differences in the molecular structure of each. The results show that the mere change in the spatial

arrangement of the groups on the first carbon atom in these two derivatives of glucose suffice to confer on each antigen a marked degree of differential specificity. These structural changes are so sharply reflected in serological specificity that it is possible by means of immune serum to differentiate selectively between the two isomeric glucosides of the same sugar. However, the antibodies present in both α and β antisera cross react with the heterologous test antigen. Recognizing the difference in the chemical structure of the two glucosides it becomes necessary to account for the overlapping specificity of the two substances. The chemical basis for the immunological crossing appears to lie in the fact that the spatial arrangement of the polar groups on the remaining five carbon atoms is identical in both glucosides. This partial similarity of molecular groupings might then account for the degree of immunological likeness exhibited by the two substances. Although in a portion of the molecule of both glucosides the structural relationship is identical, nevertheless they behave chemically as separate chemical entities and possess serologically a separate and distinct specificity. For, if complete reciprocal inhibition of precipitins is accepted as the criterion of serological identity, then the failure of both glucosides to exhibit this capacity may be taken as further evidence of differences in the immunological specificity of each.

The lack of reciprocal absorption of agglutinins by two organisms mutually agglutinable in the immune serum of

each is generally conceded to indicate a lack of immunological identity. Relationships of this order are known to exist between Pneumococcus Type II and Friedländer bacillus Type B. In both instances the reactive substance has been identified chemically as the specific polysaccharide peculiar to the capsule of each organism. While the structural constitution of these complex sugars is not as yet fully known, considerable knowledge has been gained concerning their chemical properties. For example, it is known that the Type II polysaccharide is built up of glucose units and that chemically it bears a close resemblance to the polysaccharide recovered from the Type B Friedländer bacillus. However, the two substances are not chemically identical, although the similarity between them is sufficient to result in a certain likeness in immunological specificity. In the absence of precise knowledge of the structural relations of the two polysaccharides, it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This similarity of molecular grouping might then account for the immunological similarity of the two substances.

Considerable evidence for this point of view is found in the results of the present study concerning the specificity of the α and β glucosides of glucose. A comparison of the serological relationships between the two isomeric derivatives of glucose and the capsular polysaccharides of the two organisms in question is presented in the following Table.

Comparison of the Serological Relationship between Isomeric Derivatives of Glucose and the Capsular Polysaccharide of Two Unrelated Species of Bacteria.

α Gluco-globulin antiserum			Antipneumococcus serum Type II		
	Precipitans are			Agglutinins are	
By addition of	inhibited for	not inhibited for	Absorbed with	removed for	not removed for
α glucoside	α and β test antigens✓		Pneumococcus Type II	Pneumococcus Type II, B. Friedländer Type B	
β glucoside	β test antigen	α test antigen	B. Friedländer Type B	B. Friedländer Type B	Pneumococcus Type II

β Gluco-globulin antiserum			Anti-Friedländer serum Type B		
	Precipitins are			Agglutinins are	
By addition of	inhibited for	not inhibited for	Absorbed with	removed for	not removed for
β glucoside	α and β test antigens		B. Friedländer Type B	Pneumococcus Type II, B. Friedländer Type B	
α glucoside	α test antigen	β test antigen	Pneumococcus Type II	Pneumococcus Type II	Friedländer Type B

✓The test antigens in all instances were prepared by combining the respective glucoside with a protein biologically unrelated to that in the immunizing antigen.

Analogous relationships extending even to the cross immunity reactions and the lack of reciprocal absorption and inhibition of antibodies are evident in both the groups of simple and complex carbohydrates. While the comparison is instructive it is not necessarily valid in the case of the capsular polysaccharides, for the final interpretation of these relationships must await further knowledge of the structural relations of the more complex bacterial sugars.

A possible explanation of the cross relationships between the Type II Pneumococcus and the Type B Friedländer bacillus has been advanced by Enders in his recent study on the presence in Pneumococcus of a type-specific agglutinogen unrelated to the specific carbohydrate.

In the case of the synthetic antigens containing α and β compounds of glucose alone, the evidence indicates that the immunological specificity of the reactive glucosides is determined by known variations in chemical constitution and is independent of the protein to which they are attached. In view of these findings it seems not unlikely that in the case of the polysaccharides because of their more complicated structure and the greater possibility for variation in molecular configuration, there may be found many instances of a similar overlapping specificity among carbohydrates of unrelated origin.

In attempting to interpret the relationship between the chemical constitution and the serological specificity of carbohydrates, especially those of bacterial origin, it is of obvious advantage to begin with a study of the more simple

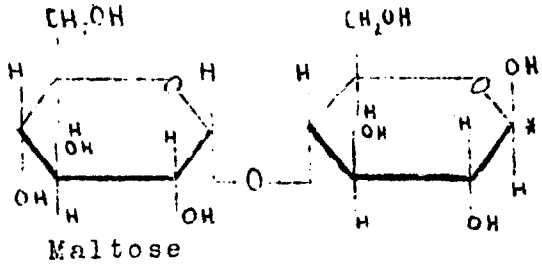
mono and disaccharides. In these sugars both the chemical composition and the spatial arrangement of the groups within the molecule are definitely known. With this end in view the following study of disaccharides is in progress.

2. The synthesis of glucosides of the disaccharides, cellobiose and maltose. (Drs. Goebel and Babers.) The opportunity for inter-molecular differences in structure is almost infinite in complex polysaccharides such as the type-specific capsular polysaccharide of pneumococci. These polysaccharides are made up of disaccharide units which are in turn composed of a simple hexose and a hexose uronic acid. Very little is known concerning the manner of linkage of these disaccharides or the units composing them. Still less is known of the effect which internal differences in linkage may have on the immunological specificity of the polysaccharide.

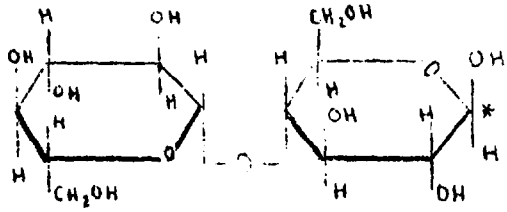
It seems, therefore, quite important to determine what effect differences in linkage of the hexose units would have on the immunological relationships of two disaccharides of known structure.

Maltose, the disaccharide obtained by the partial hydrolysis of starch, and cellobiose, that obtained in a similar manner from cellulose, have been shown to be composed of two glucose units, the aldehyde group of one being linked to the fourth carbon atom of the other hexose. Thus maltose and cellobiose are each glucosides of glucose linked through the same carbon atom. These disaccharides differ, however, in that maltose is an alpha glucoside of glucose while

cellobiose is a beta glucoside. This difference is best shown by the following structural formulae:



Maltose



Cellobiose

The free reducing group of each disaccharide is indicated ny an asterisk*.

The para aminophenol glucosides of maltose and cellobiose havobeen prepared, diazotized and coupled to protein and the resulting antigens used to immunize rabbits. The immunological work is now being carried out.

IV. Studies on the interconvertibility of the specific types of Pneumococcus. (Dr. Alloway.) Studies have been made during the past few months on the nature of the phenomenon first described by Griffith in 1928, and subsequently investigated by Neufeld, Dawson, and Sia, dealing with the transformation of R organisms derived from one type of Pneumococcus into S forms of a different type. These investigators induced the change through the use of intact bodies of heat-killed S pneumococci either injected subcutaneously into mice, together with a small inoculum of living R organisms, or used in vitro together with anti-R serum in a medium inoculated with R forms. The R cells assumed the properties of the heat-killed S organism used in the culture medium.

The phenomenon is of unusual biological significance, for it is a unique example of a complete transformation of the biological characters of a microorganism by means of a specific stimulus. An organism thus transformed not only acquires a type-specificity not present in the R cell, but it undergoes definite morphological changes, develops a high degree of virulence, and possesses antigenic properties which differ markedly from those present in the original bacterium. These properties, once acquired, are retained by the organism indefinitely under suitable conditions of growth. The acquisition of these new characteristics is always associated with the regaining of the function of producing the specific capsular polysaccharide.

It seemed worth while to attempt an analysis of the substance present in the heat-killed organism essential in the reaction, and to learn more of its general nature. Attention has been directed, therefore, towards the separation in a filtrable, cell-free state, of the substance active in effecting transformation. Studies already published have established several facts. Cell-free extracts which are active in causing transformation in type may be obtained from S pneumococci disrupted by repeated freezing and thawing. Such extracts under suitable conditions may be filtered through Berkefeld candles, and thus separated from all intact organisms. Avirulent R pneumococci derived from S forms of a specific type may be changed by growth in broth containing anti-R serum and the filtered extract of S pneumococci of a

different type into virulent S organisms identical in type with that of the bacteria extracted. The constituents of the extract supply an activating stimulus of a specific nature in that the R pneumococci acquire the capacity of elaborating the capsular material peculiar to the organisms extracted.

Subsequent studies have shown that still further purification can be accomplished by the adsorption of pneumococcus extracts on aluminium gels. Most of the protein and lipoid constituents in the extract are carried down with the aluminium, the active substance remaining in the supernatant fluid. This supernatant, a water-clear fluid, is specifically as effective in inducing transformation in type as are the suspensions of whole, heat-killed organisms.

It is hoped that by further procedures such as selective adsorption, selective dialysis, and chemical analyses, more exact knowledge of the active substance may be acquired, and an explanation provided for a phenomenon which possesses wide biological significance.

V. The Degree and Duration of Active and Passive Immunity to Pneumococcus in Rabbits. (Dr. Ernest Stillman.) The experiments, previously reported, on the persistence of pneumococcus antibodies in the sera of rabbits following various immunization procedures is still in progress. The trend of this work is illustrated by the fact that the serum of one rabbit, last exposed to inhalation of virulent Type I pneumococci 6 years ago, still contains sufficient antibodies to protect mice against large doses of pneumococci.

In colaboration with Dr. Goodner, a large series of rabbits have been given identical courses of injections of a suspension of heat-killed pneumococci, containing a mixture of equal amounts of Pneumococcus Types I, II and III. At monthly intervals groups of these animals are being examined as regards the agglutinin titer and mouse protective value of their serum, and as to actual active immunity or susceptibility to infection by the dermal route. The dermal infection permits of a thorough evaluation of the active immunity since the results do not depend so much on survival or death as they do on the clinical findings.

This investigation has now been under way for four months. While it is yet too early to predict the duration of immunity it has become apparent that it persists beyond the time of the disappearance of circulating antibodies. Furthermore, it has been found that the immunity against the Type III Pneumococcus is the first to disappear, and the indications are that the immunity to Type I will have the longest duration.

Production of experimental pneumonia in animals.

The development of a technic for the production of experimental lobar pneumonia in laboratory animals is still being attempted. Although by the inhalation method pneumonia may occasionally be produced in partially immunized mice under the influence of alcohol, these animals are too small for detailed study. Experiments have shown that partially immunized rabbits, whether infected by inhalation or by nasal inoculation, fail to localize the infection in the lungs. Although guinea pigs are subject to spontaneous epidemics of pneumococcus lobar pneu-

monia, they are highly resistant to experimental infection with this organism. Numerous attempts to raise the virulence of pneumococci for guinea pigs have failed. Various methods for lowering the natural resistance of guinea pigs are now being employed with the hope that these animals may become more susceptible to pneumococcus infection.

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