

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

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I. The production and treatment of Type III pneumococcus pneumonia in monkeys. (Drs. Francis and Terrell.)

In their first papers, describing the action of an enzyme of bacterial origin upon the capsular polysaccharide of Type III Pneumococcus, Avery and Dubos also presented convincing evidence that this enzyme is capable of decomposing the same substance when encountered in the animal body. Mice were infected with many lethal doses of Type III pneumococci. It was found that as late as 12 to 18 hours after the infection was established intraperitoneally, the administration of the enzyme brought about recovery in most instances. Furthermore, when the enzyme was given 24 to 48 hours preceding the injection of many lethal doses of Type III organisms into mice, it was found to have a marked protective action resulting in recovery of most of the animals. The mechanism of the therapeutic effect was shown to be due to its action on the capsular polysaccharide, decomposing it, and thereby rendering the bacterial cells vulnerable to attack by the phagocytic cells of the animal body. This study was limited to effect of the enzyme

on Type III Pneumococcus in an experimental infection primarily septicemic in nature.

Later, Goodner, Dubos and Avery studied the action of the enzyme upon the localized infection of the skin of rabbits (termed by Goodner "dermal pneumonia") produced by a virulent strain of Type III Pneumococcus. In this form of infection, the lesion is primarily localized and is accompanied by septicemia of varying degree in different animals. Here again, a definite therapeutic effect of the enzyme was demonstrable as shown by prompt subsidence of the cutaneous lesion, and sterilization of the blood. However, with the amounts of enzyme used in the studies, there was found to be a degree of septicemia above which the enzyme was unable to control the infection, although the duration of life of the rabbits might be prolonged over that of control animals. Since excellent results had been obtained in the treatment of septicemic and local infections of the smaller animals, it seemed advisable to study the effect of the enzyme under conditions in which the disease process more closely resembled that in the human patient suffering from pneumococcus lobar pneumonia. In this disease, the organ involved is highly vital, the course is comparatively constant, the pathology typical and recovery problematic. Consequently, an attempt was made to produce in monkeys a disease resembling lobar pneumonia in man, which could then be subjected to treatment with the specific enzyme under conditions more closely approaching those encountered with patients in the hospital wards.

The Java monkey (*M. cynomolgus*) was selected for the purpose, and this choice has proved to be a fortunate one, since

the *M. rhesus* is much more resistant to the strain of Type III Pneumococcus employed. In the first part of the study the animals were given morphine and then under ether anesthesia a small catheter was inserted into the trachea; the inoculum, consisting of organisms centrifuged from a given amount of culture and resuspended in 0.5 cc. of soluble starch was allowed to flow into the lungs. During the procedure the animal was tipped to the right with the hope of localizing the material in the right lower lobe. This technic was, in the majority of instances, successful. However, the location of the infection was not always predictable, since the exact site of the catheter at the time of injection was not known. At times, too, during the recovery from ether anesthesia, the monkey often went through a stage of excitement, with hyperventilation and exaggerated physical activity, or the reverse condition with cessation of breathing which required artificial respiration. Such incidents apparently resulted in pneumonia scattered diffusely through the lungs and accompanied by a high septicemia. In spite of these variable factors, localized lobar pneumonia was produced in a high proportion of the animals.

In the latter part of the study, a more accurate method has been employed using the technic described by Terrell and Robertson. With this method, the animal is given a dose of morphine sufficient to make it quiet. The animal is placed in the supine position, and a small radio-opaque catheter is inserted into the trachea. With the aid of the fluoroscope the catheter is then guided with direct visual control into the

bronchioles of the lobe or lobules, in which the infection is desired. When the catheter is so placed, the organisms suspended in 0.5 cc. of 5 per cent cornstarch emulsion are injected. The method has several advantages: First, it eliminates general anesthesia; second, the site of injection is known accurately; third, with better localization the number of organisms required for infection is somewhat smaller and the septicemia consequently tends to increase more gradually.

Throughout the study, temperatures were recorded 3 or 4 times daily and the course of the pneumonia was followed by daily roentgenograms. Daily blood counts and blood cultures were made. In general, within 18 hours after infection, a definite well localized pneumonic consolidation can be seen in the X-ray. Concomitantly, a gradually rising fever is noted. The white blood cells may be increased in number, or, if septicemia is present, they may have decreased. The lesion continues to spread so as to involve the entire lobe and may extend to other lobes. The temperature remains elevated, the septicemia increases, and the leukocyte count tends to fall progressively. Death may follow in 4 to 7 days in the typical case. On the other hand, in a certain number of instances associated at times with either a diffuse infection of the lungs or with the well localized lesion, there is an immediate fall in leukocytes, a heavy septicemia and a tendency to subnormal temperature with a rapidly spreading lesion and an early fatal termination.

There is also a group of monkeys in which recovery takes place spontaneously after 3 to 7 days. In these, septicemia

may be absent or slight in degree, although in two instances the bacteremia has been comparatively high. There is a tendency of this group to respond with definite fever, leukocytosis and a well localized lesion, but with relatively less spread of the pneumonic process. The spontaneous recoveries occurring in this group make it difficult to speak of control animals in the usual sense, since the factor of individual variation, so far as we have been able to discover, cannot be eliminated or allowed for. This is especially true when the purpose of the experiment makes a comparatively long, but gradually progressive and fatal course the most desirable. From the point of view of the production of pneumonia, of course, it does not matter, but when an attempt to evaluate a therapeutic agent is made, the ideal is to approach absolute comparisons of treated and untreated animals. This has not been possible.

The studies to date include the results in 82 monkeys with definite pneumonic lobar consolidation of various degrees. Of these, 20 animals, or 24.4 per cent, had no septicemia and all recovered. Twenty-three animals, 28 per cent, had rapidly mounting septicemia greater than 5,000 colonies per 1 cc. of blood in the first three days. All of these animals died. The remaining animals were subdivided into two groups on the basis of the degree of septicemia present: (1) those in which septicemia was present but not greater than 250 colonies per 1 cc. of blood in the first 3 days; (2) those in which the septicemia ranged between 250 and 2,000 colonies in the first 3 days. The first of these two groups comprises 26 animals, or 31.9 per cent, and the second includes 12 animals, or 14.6 per cent. It is in these

two groups that the conditions seem most favorable to study the possible effect of the enzyme upon the course of the experimental disease. The results obtained by treatment are tabulated below, comparing the mortality rate in a series of untreated animals with that of similar animals receiving treatment with the Type III polysaccharide-splitting enzyme. The reason for dividing the groups on the basis of the first 3 days is that all of the animals treated, received the enzyme in the first 3 days after infection.

Animals with pneumonia and septicemia less than 250 col./cc. in first 3 days.

No. of animals		Recovered	Died	Mortality per cent
Untreated	Treated			
15		7	9	55.3
	10	10	0	0
Animals with pneumonia and septicemia from 250 to 2000 col./cc. in first 3 days.				
No. of animals		Recovered	Died	Mortality per cent
Untreated	Treated			
8		2	6	75
	4	4	0	0
Summary of both groups.				
No. of animals		Recovered	Died	Mortality per cent
Untreated	Treated			
24		9	15	53.1
	14	14	0	0

The results on the basis of comparative mortality figures are striking. In the animals of these groups which received treatment there have been no fatalities, whereas, well over 50 per cent of the untreated animals have succumbed. Five more animals were treated on the first day and one on the second day after infection. In all of these a definite consolidation was noted, in several the fever had reached 105° to 106°, and they

seemed on a clinical basis to be suitable cases for the therapeutic test; however, at the time of treatment their blood cultures were sterile and remained so. They all recovered. Six others with very high initial septicemias were treated and although they all died, one treated on the first day with a septicemia of 18,000 colonies per cc. at that time did not die until the fifth day, and then with 2,000 colonies of Type III Pneumococcus per cc. Another with 25,000 colonies per cc. on the second day of disease lived until the fourth day, and at the time of death the septicemia had been reduced to 3,700 colonies per cc. This marked reduction of the number of organisms in the circulating blood has been noted in others of the treated group in which death occurred early.

In addition to the effect of the enzyme on the septicemia, there is usually seen in the X-rays a definite prompt effect upon the pulmonic consolidation. The spread of the lesion is limited and the advancing margin begins to clear. At the same time, the fever tends to subside and the animal is alert and active, and takes interest in his food and surroundings. The immediate constitutional effect of the enzyme has varied with different preparations and with the severity of the disease. Some preparations have caused a fall in temperature and white blood count and have made the animals appear sicker. This is especially noticed when the monkey is already extremely sick. In animals moderately sick there is a tendency to a febrile rise following treatment. It may be that the fall of the blood count is partially due to an increased mobilization of the leukocytes at the site of infection.

The treatment has usually consisted in the intravenous administration of 10 cc. of enzyme, varying from 2.5 to 20 units per cc. Following the original treatment, blood cultures and white counts are made and further treatment has been based on the results of these studies in conjunction with the general clinical appearance and the X-ray evidence. In some instances three treatments have been given in a period of 24 hours. In the later treatments the intraperitoneal route has sometimes been employed with satisfactory results, and perhaps some reduction in the amount of general reaction.

Although the study must be carried further, the results so far appear promising. Recently, it has been our privilege to move the monkey clinic to quarters which combine many features that tend to expedite the actual work. It is hoped that with more experimental animals, and with further improvement in technical procedures of concentration and purification of the enzyme, more potent and highly purified preparations may be available and that unequivocal results of its therapeutic effect may be obtained.

Pathology: As a side-product of the primary clinical study of experimental pneumonia in monkeys, an excellent opportunity has been presented for the study of the pathology of the experimental disease in animals dying at different times with various degrees and stages of involvement. The microscopic preparations have been made with the kind cooperation of Dr. Rhoads and Mrs. Whitehouse. The description of the microscopic lesions is limited to sections obtained from the lungs of monkeys infected by allowing the inoculum to flow by gravity to a given site.

In the animals dying early in the disease there has been an accompanying heavy septicemia. As early as 24 hours an entire lobe may be involved. Its color at this time is a dark bluish-red. It is large, firm and airless, except, perhaps, at the extreme margins. There is usually a moderate degree of fibrinous pleurisy, sometimes a small amount of serous fluid. The septum between it and adjacent lobes is obliterated by adhesions. On cut section, the involved lobe is comparatively dry, but a viscous fluid, typical of Type III pneumococcus pneumonia, may be scraped off. Microscopically, the most advanced process may be seen in the portion nearest the root of the lobe, while extending peripherally different stages may be encountered. In the region of the main vessels and bronchi there may already be seen a marked cellular exudate primarily composed of leukocytes. Fibrin has appeared in different parts. The alveolar walls are moderately swollen and their capillaries are engorged. Ordinarily, the number of red blood cells in the alveolar exudate is not striking. The lymphatics of the larger bronchi and bronchioles, although dilated, show little evidence of infection. The walls of the bronchioles show little infiltration, although much exudate is present in their lumina. The walls of the blood vessels do not appear to be involved. In this area comparatively few organisms are seen.

Extending peripherally in a gradual transition from the preceding phase, there is seen marked engorgement of the alveolar capillaries with swelling and desquamation of the alveolar epithelium. The alveoli are filled with exudate composed of edema fluid, moderate numbers of polymorphonuclear leukocytes and erythrocytes usually in small numbers. There may

be a few deposits of fibrin spicules and organisms are numerous in the exudate, but not notable in the walls of the alveoli. At the margins of the lesion the predominant features are the filling of the respiratory lobules with edema fluid containing great numbers of bacteria and comparatively few cells. There may be extreme congestion of the alveolar capillaries so that the walls are outlined by erythrocytes filling the capillaries. The alveolar walls are swollen, but the outstanding feature is that, in spite of the number of organisms in the material filling the alveoli, they are infrequent in the walls of the blood vessels, the bronchioles and alveoli.

In animals dying on the fourth to seventh days, one may find several lobes completely involved, and the whole lung covered with fibrous pleurisy and adherent to the thoracic wall. The lobe which was the site of the original pneumonic consolidation is a slate grey in appearance, or bluish grey. It is large, firm and airless. At times a local area, red in appearance, may be seen. The lung on cross section is reddish grey and very dry with rough, granular surface. Microscopically, most of the lobe will be seen to be in an advanced stage of the pneumonic process. The alveolar walls are poorly distinguished because of the dense character of the exudate and the large amounts of fibrin. The alveolar capillaries contain erythrocytes but are not prominent. In many phases there is a shrinkage of the exudate away from the walls of the alveoli, but strands of fibrin can be seen extending from one alveolus to another. In the terminal bronchioles the process is quite marked. In the areas of dense involvement, especially where fibrin is abundant,

few organisms are seen and the number of leukocytes is less. The fibrin appears to be most marked in areas adjacent to the blood vessels. There is practically no evidence to suggest vascular thrombi, and the walls of the vessels are surprisingly free of evidences of infection. In the larger bronchi, much exudate may be seen in the lumen, but there is little infiltration of the walls even adjacent to areas of marked pneumonia. The lymphatics do not appear to be infected. In certain areas, perhaps most frequently in the peripheral portions of the lobe so involved, one notes the appearance of large yellowish cells in the alveoli. Where they are present the fibrin is disappearing and the polymorphonuclear leukocytes are much diminished. These cells are the typical forms seen in resolution, and are apparently actively phagocytic.

In the other lobes, depending upon the duration of involvement, the type of lesion appears to vary with the age. In some instances there seems to be an area of direct extension from lobe to lobe, but usually the oldest lesion is seen about the hilus, while in the lobe in which the process is most recent the peripheral portions may show engorgement of the alveolar capillaries with much edema fluid and many organisms in the alveoli typifying the advancing margin of the spreading infection.

In animals sacrificed after spontaneous recovery has begun, there is frequently a persistence of fibrous pleural adhesions. The involved lobe is smaller than normal, has a rubbery consistency, and its color is a rather pale greenish yellow. The surface may be wrinkled. On cutting the lung, it is rather firm,

dry, but flabby. Very little air is present. In the microscopic section the striking feature is the predominance of the large yellowish phagocytic cells. Certain alveoli contain large numbers of them with few polymorphonuclear leukocytes and less fibrin.

Elsewhere the alveoli are clear but for a small number of these cells; the alveolar walls are becoming less swollen; the epithelial cells are being replaced. The capillaries are perhaps more dilated than in the stage of dense consolidation. Scattered throughout the section are portions in which fibrous tissue is making its appearance and beginning organization. At times the plugs of exudate are coated with a layer of epithelium and almost suggest the organization of a thrombus with small new vascular channels making their appearance. The complete clearing of the infection is comparatively rapid, although just as the laying down of fibrin is marked in the acute process, so is the amount of fibrous tissue in the reparative process.

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II. Nature of Lysis of Pneumococcus by Autolysis, Bacteriolytic Enzyme and Bile. (Dr. Dubos.)

Among the interesting properties of the Pneumococcus cell is the ease with which it undergoes autolysis and the readiness with which it is dissolved by bile and bile salts. These two properties serve to differentiate pneumococci from other Gram-positive cocci; they are also of some interest in the study of bacterial variation in the Pneumococcus group, since the R variants are more resistant both to autolysis and bile lysis

than the S forms. Finally, these two processes result in the loss of the characteristic antigenic property of the S cell, i. e., its power to stimulate the production of the type-specific capsular antibodies.

What is the nature of the phenomena which cause such striking modifications of the cell? It has long been realized that autolysis is the result of the breakdown of some cell constituents by the so-called autolytic enzymes. Cell-free preparations of these enzymes have the property of changing heat-killed pneumococci into a mass of Gram-negative debris; this action is fairly limited to the Pneumococcus group, but, within this group, both R and S forms, irrespective of type, are equally affected.

A very satisfactory method of preparation of the bacteriolytic enzyme is as follows: A chilled suspension of pneumococcus cells is thrown into 15 volumes of chilled acetone, the precipitate separated by centrifugation and dried as a grey powder. When resuspended in a medium in which the cellular enzymes cannot function, this material may be shown to consist of apparently normal Gram-positive cells. If, however, the cells are resuspended in saline they undergo lysis instantaneously, leaving only a mass of Gram-negative debris. Outside of the interest attached to this phenomenon of flash lysis, the technique provides a rapid method of obtaining a solution which in turn is very active in causing lysis of heat-killed pneumococci.

Although autolysis was soon recognized as an enzymatic phenomenon, most investigators have felt that bile lysis was of a more physical nature, particularly because of its amazing

rapidity, even at ice box temperature. It was also felt that the property of undergoing lysis in bile was in some way associated with the life and death of the cell, since any procedure which killed the organism would also render it bile insoluble. However, the following experiments, very briefly outlined, seem to indicate that bile lysis is only an activation of the autolytic enzymes, and that it does not occur when these enzymes are inhibited or destroyed.

When pneumococcus cells are suspended in citric acid, for instance, they slowly die but remain Gram-positive and retain their morphology. If these dead cells are then suspended in a buffer solution at pH 7.0, they undergo lysis at a normal rate, they are found to be bile soluble, and when subjected to freezing and thawing give an active preparation of bacteriolytic enzyme. This experiment shows that dead cells remain bile soluble when their enzymes have not been destroyed, but have been only placed under conditions where they cannot function. When, however, the cells are killed by agents such as heat, mineral acids, salts of heavy metals, which not only inhibit but also destroy the bacteriolytic enzymes, they are no longer bile soluble.

A direct evidence of the activating effect of bile on lysis by the bacteriolytic enzyme is supplied by the fact that the rate of breakdown of heat-killed cells by the enzyme is very much accelerated in the presence of bile or bile salts.

The enzymatic nature of bile lysis is also indicated by the influence of temperature on the phenomenon. The rate of bile lysis was measured at 0° C., 5° C., 20° C., 37° C., 45° C.,

53° C. and 60° C. Between 0° C. and 45° C. the phenomenon was found to have a temperature coefficient of 2 to 3. At 53° C. lysis began very rapidly but soon stopped and never was completed, suggesting that the enzyme itself had become inactivated. At 60° C., no lytic action could be detected.

All these facts seem to indicate the enzymatic nature of bile lysis. The following observations also tend to prove that autolysis, bile lysis and lysis of heat-killed cells by sterile preparations of bacteriolytic enzymes are fundamentally the same phenomenon; all 3 processes have a temperature optimum at 45° C. and are inhibited at temperatures greater than 52° C.; the range of pH lies between 5.5 and 8.2 with an optimum near the neutral point; the rate of all three processes becomes exceedingly slow in distilled water, but increases with salt concentration, at least up to 5 per cent NaCl; finally, in all three cases, lysis does not occur in the presence of agents known to inhibit enzymes.

It seems that the foundations have now been placed on which to build a study of the mechanism of lysis. Four observations are being studied as possible leads towards an understanding of this mechanism. 1. The fact already referred to that cells precipitated with acetone exhibit flash lysis. 2. Activation of lysis by bile and bile salts. 3. The rate of autolysis and of lysis of heat-killed cells by bacteriolytic enzymes is very much activated by small amounts of iodine. This activation, however, can be recognized only within a narrow range of iodine concentration. Above a certain concentration the enzymes are completely inhibited by iodine, the cells remain permanently Gram-positive and are no longer soluble in bile. It may be

mentioned here that cells thus treated with an excess and then washed free of iodine are still susceptible to lysis by the bacteriolytic enzymes.

It has been mentioned early in this discussion that all heat-killed pneumococci, irrespective of types, are susceptible to lysis by the bacteriolytic enzyme. It should be added here, however, that from this point of view the Type I cells are by far the most resistant, and the Type III the least resistant. It is perhaps more than a coincidence that the Type I contains also the most stable type-specific antigenic complex, and Type III the least stable.

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III. Study of the Antigenic Complex of Type-specific Pneumococci. (Dr. Avery and Dr. Dubos.)

When virulent, encapsulated pneumococci are subjected to any form of lysis, they lose their property of stimulating type-specific antigenic response in rabbits. Such preparations of lysed cells, however, are still capable of immunizing mice against infection with virulent pneumococci of the homologous type. This observation suggests the existence in the intact virulent Pneumococcus cell of two distinct antigenic functions, both of which are type-specific.

1. Capsular polysaccharide antigen. This antigen is concerned with the production in the immunized animal of a type-specific antibody directed against the polysaccharide constituent of the Pneumococcus capsule. Practically all rabbits immunized with suspensions of intact heat-killed Type I pneumococci respond with the production of type-specific precipitins,

agglutinins and protective antibodies. If, however, these same cells are first allowed to undergo autolysis, or are dissolved by bile, or their heat-killed bodies are digested by the addition of bacteriolytic enzymes, and these preparations are injected into rabbits, the animals fail to exhibit type-specific response. As shown earlier in this report, these three forms of lysis are only different expressions of the activity of the same lytic enzymes. We may say, therefore, that the capsular polysaccharide antigen is rendered ineffective by the action of the cells' own enzymes.

When rabbits are immunized with a suspension of heat-killed Type III organisms, only a few animals respond with the production of type-specific antibodies. When, however, iodized cells are used for immunization, practically all animals exhibit the formation of type-specific precipitins, agglutinins and protective antibodies. As has been stated earlier, iodized cells are easily attacked by the bacteriolytic enzymes and thus changed to a mass of Gram-negative debris. Now it has been found that treatment with the bacteriolytic enzyme does not seem to modify the type-specific antigen of the iodized cells. In fact, rabbits immunized with the Gram-negative debris obtained by digesting iodized cells with the bacteriolytic enzyme gave as good type-specific precipitins and protective antibodies as animals treated with undigested iodized cells. It is hoped that this observation may be made use of in the study of the chemistry of the capsular polysaccharide antigen.

2. Secondary type-specific antigen. As stated before, mice immunized with the Gram-negative material obtained by digesting

heated Type I cells with the bacteriolytic enzyme exhibit marked resistance against infection with the homologous organism, but no resistance against Type II. The same degree of active immunity can be induced in mice with autolyzed cells or cells dissolved in bile. Besides being resistant to the Pneumococcus enzymes, this antigen exhibits certain remarkable properties which have made possible some progress in its purification; it is insoluble in 95 per cent alcohol, but soluble in 70 per cent alcohol; it is soluble in acetic acid; it is not inactivated by crystalline trypsin; it goes through Berkefeld filters. Antigenic material thus purified reacts specifically in sera which have been freed of antibodies against the type-specific polysaccharide.

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IV. Methods of Preparation of the Enzyme Decomposing the Capsular Polysaccharide of Type III Pneumococcus. (Dr. Dubos.)

The methods of preparation, concentration and purification of the bacterial enzyme capable of decomposing the capsular polysaccharide of Type III Pneumococcus have been described in previous reports. These methods have been simplified in some details. In particular, the amount of yeast extract in the culture medium used for growing the SIII bacillus has been reduced to .005 per cent; this has somewhat decreased the yield of enzyme, but has also reduced the toxicity of the preparations. (It will be recalled that the toxicity has been found to be due partly to the action of the SIII bacillus on yeast extract.) As a result, it has been found possible to eliminate one step in the purification process, i.e., the adsorption on

aluminum gel. We have also modified the composition of the ultrafilters used for concentration, employing for the membrane a solution of 3.5 per cent cellulose acetate instead of 5½ per cent as previously used. This has resulted in the elimination by filtration of a greater part of irrelevant substances, and also in a more rapid concentration.

At the present time, the main difficulty seems to be in the great losses which occur at the time of concentration, probably by adsorption on the ultrafilter membranes. Precipitation of the enzyme with acetone or alcohol has given some successful but irregular results. It is obvious that the handling of the enzyme at this stage of concentration and purification will require much more study before the methods can be completely standardized.

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V. Isolation of Pure Cultures of Organisms Decomposing the Capsular Polysaccharide of Type II Pneumococcus. (Dr. Dubos and Dr. Goodner.)

The work on cultures capable of decomposing the capsular polysaccharide of Type II Pneumococcus was continued during the spring and the summer. Several cultures have been obtained pure, some of them in a very active form and soluble enzymes extracted from them in a few cases. Unfortunately, during the fall, the cultures began to lose their activity and at the present time we have only two left which are capable of decomposing the Type II polysaccharide. Furthermore, the activity of these two cultures has become so attenuated that it is now impossible to obtain from them active enzyme preparations. Time permitting it is planned to resume the work this spring.

VI. Pneumococcus Dermal Infection in Rabbits. (Dr. Goodner.)

In the symptom-complex which follows the intradermal injection of pneumococci in rabbits, the number of organisms in the blood stream at a given time seems to be an accurate index of the relative severity of the disease. The data which have accumulated during the past seven years are being analysed to learn if this index may be correlated with any determinable constitutional factors. So far, this has not been possible, but it is apparent that the white cell count at the time of infective inoculation is significant. In those instances in which the initial white counts deviate most greatly from the average, the intensity of the bacteriemia 24 hours after infective inoculation is the greatest. This is especially true in rabbits which have high initial white counts, for these invariably show a massive blood invasion---a rather paradoxical finding.

When so-called "sub-effective" amounts of specific anti-serum are injected intravenously 24 hours after infective inoculation with Type I pneumococci, some animals survive while others die. If a definite "sub-effective" amount of the same serum is used in a large series of rabbits there is apparently no single indicator of what result may be expected. Some animals with a very high grade of bacteriemia survive, while others with a low degree of bacteriemia die. However, if the bacteriemia at the time of treatment is plotted against the white cell count at that time with respect to the end result it is found that animals which survive occupy one section of the figure while those which die are oppositely placed. With a constant amount of serum the number of white cells must be greater in proportion to the degree

of bacteriemia if the animal is to survive. Increasing amounts of antiserum appear somewhat to compensate for a lack of white blood cells.

The Determination of Active Immunity by Dermal Infection.

A project is being carried out (with Dr. Stillman) to ascertain the duration of active immunity as determined by intracutaneous infection of rabbits which have previously been immunized by intravenous injections of heat-killed pneumococci, Types I, II and III. Each succeeding month animals, not previously tested, are given intradermal injections of living virulent pneumococci of the various types, and the reactions to these injections studied. At the time of infection, determinations are also made of the presence of circulating antibodies. This study has now reached the tenth month, and will be concluded shortly. It may be tentatively stated that there appears to be no close correlation between the degree of active immunity and the presence and titer of circulating specific antibodies.

An analysis of an extensive series of dermal infections carried out to determine if rabbits possess active immunity, has made it possible to devise a significant scale by which the degree of active immunity may be judged. It is believed that the commonly accepted end-point of immunity, viz., survival and death, does not give a very accurate picture of immunity, for careful study reveals that there are differences in the rate with which animals die, and certainly even greater differences in response to infection among those which survive. For example, an animal's immunity may be of such a character as to bring about recovery, but the animal may nevertheless suffer from a

severe and protracted infection differing in no essential respect from that occurring in one which may succumb. On the other hand, the degree of resistance may be such that the animal may develop only a slight localized area of infection and show no temperature elevation. Thus, while it is possible arbitrarily to state that animals which die are non-immune, and those which survive are immune, it would seem more reasonable to hold that survival and death merely divide the gradient of response to infection into two phases, in each of which considerable differences may be encountered.

The Effect of Pneumococcus Autolysates upon Pneumococcus Dermal Infection in the Rabbit. It has previously been reported that the edema fluid removed from the lesion during the earlier phases of the dermal infection in rabbits possesses the property of inhibiting the coagulation of normal rabbit blood. Pneumococcus autolysates possess the same property. It has been suggested that, in the course of the infective process, some of the less resistant microorganisms may autolyse and thus, through the action of their end-products, pave the way for the invasion of the few which survive. Experiments have been undertaken to determine if bacterial autolysates might enhance the virulence of Pneumococcus.

Sterile autolysates of virulent or non-virulent strains do enhance the invasiveness of pneumococci. Thus, a strain of Type III Pneumococcus (PH) which is capable of bringing about the death of a rabbit when given intradermally in certain amounts becomes capable, when autolysate is added, of producing the same result in much smaller amounts. On the other hand,

another Type III Pneumococcus (A 65) which does not inherently possess a lethal capacity does not acquire this property from the autolysate, but does seem capable of greater invasiveness.

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VII. Study of Pneumococcus Infection and Immunity in Rabbits by the Inhalation Method. (Dr. Stillman.)

Pulmonary lesions produced by inhalation method. The problem of the production of experimental Pneumococcus lobar pneumonia in small laboratory animals is still under study. All of the "successful results" of the experimental production of the disease reported in rabbits have been obtained by the intrathoracic or intratracheal inoculation. As lobar pneumonia may frequently be produced in mice by the artificial inhalation method, this method has been applied to partially immunized rabbits. Unfortunately, however, rabbits failed to show any evidence of pulmonary localization of the infection. This failure may be due to the arrangement of the lymphatics in the rabbit's lung. The infection early reaches the pleura and the pericardium and as a result the animals succumb before lesions develop in the lungs. A study has also been made of the pulmonary reactions of rabbits following intranasal instillation of pneumococci. It was found that the mortality of rabbits so infected varied in direct proportion to the number of pneumococci instilled into the nose. Although no lesions of pneumonia were produced, characteristic vascular and lymphatic inflammatory changes were found in the lungs of rabbits infected by the nasal route.

Duration of demonstrable antibodies in serum of rabbits following inhalation of pneumococci. The duration of type-specific agglutinins and protective antibodies in the serum of rabbits following inhalation of living pneumococci has been studied. Type-specific agglutinins could be demonstrated only for a short period after the course of exposures was terminated. However, Type I protective antibodies were still detectable in the sera of these animals years after their final exposure to pneumococci. The protective antibodies developing after exposure to inhalations of Type II pneumococci, on the other hand, remained demonstrable in their serum for only a relatively short period.

Active and passive immunity in rabbits. The duration of type-specific antibodies in rabbits following inoculation of suspensions of heat-killed pneumococci is still under investigation. In conjunction with Dr. Goodner, the active immunity of these rabbits as determined by dermal infection and the correlation of this with the presence of type-specific antibodies in the serum is being studied.

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VIII. Interconvertibility of Specific Types of Pneumococcus.

(Dr. Rogers.)

It is an established although poorly understood fact that rough (R) pneumococci may be experimentally restored to smooth forms of the same type as that from which they were derived, and that they may be "transformed" into smooth pneumococci of a serological type different from that of their origin. Since the initial work of Griffith subsequent studies by Neufeld and

Levinthal, Dawson and Sia, and more recently by Alloway have not only confirmed the occurrence of this phenomenon, but have added much to our knowledge and technical control of it.

The transformation of pneumococci from one type to another, which at first could only be accomplished by an in vivo technique employing pneumococcus "vaccines" as the transforming agent, can now be carried out in vitro using sterile, cell-free pneumococcus extracts in the place of vaccines. It has, furthermore, been shown that the organisms thus converted from one Type into another undergo a real and permanent change, acquiring all the biological characteristics of the new type, i.e., virulence, type-specificity, type-stability upon repeated subculture, and specific antigenicity.

Alloway was the first to succeed in using filtered cell-free extracts of pneumococci as the transforming agent. These extracts were obtained by dissolving virulent cultures with sodium desoxycholate, precipitating the bacterial solutions in absolute alcohol, extracting the precipitate with salt solution and further purifying by charcoal adsorption and Berkefeld filtration. (J. Exp. Med., 1932, 55, 91; 1933, 57, 265.) The extract of type-specific pneumococci so prepared, containing the so-called "transforming factor," was added to small quantities of broth enriched by the addition of 25 per cent ascitic or chest fluid, and to the whole was added a very small inoculum of living "R" cells to be transformed. Transformation usually occurred after from one to four subcultures had been made.

Alloway found that although these extracts are rich in specific soluble substance the degree of transforming activity

of an extract is not necessarily correlated with the amount of the specific capsular polysaccharide present. Moreover, the chemically isolated and purified specific soluble substance possessed no transforming qualities. It has also been shown that the transforming factor lacks the thermostability possessed by the purified carbohydrate. So far, it appears that the transforming factor remains closely associated with the specific soluble substance, though they are not identical in their physical properties in so far as we have been able to test them. The work of Alloway has been confirmed in our recent studies, and further efforts at purification of the transforming factor are being made at the present time.

It has been found that by careful adjustment of the final pH of the media employed in obtaining transformations, sufficient growth occurred to permit as many as three transfers within a twenty-four hour period. This not only adds greatly to the rapidity with which transformations may be obtained, but also seems to produce more constant results. A few experiments upon the so-called "serum factor," which is an essential adjunct in the cultural technique, would seem to indicate that the importance of this substance is dependent upon a certain minimal content of anti-R pneumococcus antibodies, perhaps even more than upon its nutrient value. Active extracts, in addition to being thermolabile, lose their activity upon ageing for even so short a time as three or four weeks in the ice box. The nature of this degradation is being investigated.

Still further purification is being attempted employing various enzymes, adsorbents, immunological and chemical methods,

but as yet the data are insufficient to warrant conclusions.

It is desired, by the methods outlined, to reduce the transforming factor to its simplest and most active form in order to acquire knowledge of its biological and chemical properties, and so to arrive at a better understanding of this most unusual and significant phenomenon.

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IX. Study of the Chemical Nature of Antibodies in Antipneumococcus Serum. (Drs. Goebel and Avery.)

The investigations of Northrup concerning the nature of pepsin and trypsin have led to the isolation of crystalline proteins possessing unique chemical properties and biological activity. The success of these investigations, and the fact that these crystalline proteins have properties which differentiate them sharply from other biologically inert proteins, have led us to believe that the immune protein of antipneumococcus horse serum might, by means of some unusual property, likewise be separated and differentiated from the accompanying inert serum proteins. As a starting material for this investigation we chose that fraction of antipneumococcus serum (Type I) which precipitates from whole serum on the addition of large quantities of water. This fraction, which is insoluble in water, contains practically all of the antibodies present in the original serum. It was found that this material could be further separated, into an inert water insoluble englobulin, an inert water soluble pseudo-globulin, and a second water soluble pseudo-globulin which contained all of the antipneumococcus antibodies. This biological specific protein possesses certain

unusual chemical properties which permit its separation, as well as its differentiation, from the other inert serum proteins. This substance has a lower total nitrogen content, and a much higher amino nitrogen content than the proteins of normal serum. A chemical analysis by the Van Slyke method reveals that the distribution of basic amino acids is different from that of normal horse serum proteins. An electrometric titration of this unusual protein has shown that its isoelectric point lies at pH 7.6. The protein is soluble in distilled water on either side of its isoelectric point, but is precipitated from water at pH 7.6. The protein is extremely active biologically; a solution containing as little nitrogen as 0.007 mgs. per cc. causes specific agglutination of pneumococci. The protein contains all of the various antipneumococcus antibodies, i.e., it contains the type-specific and species-specific antibodies. Attempts have been made to separate these antibodies, but their chemical properties are so closely related, despite the fact that their immunological properties are sharply differentiated, that we have not as yet succeeded in accomplishing this. We have also attempted to crystallize the biologically active protein, but thus far without success. Studies on the protective power of this protein against pneumococcus infection are at present being carried out.

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X. Derivatives of Glucuronic Acid and Glucuron. (Drs. Goebel and Babers.)

The presence of glucuronic acid in the specific carbohydrates of Types II and III Pneumococcus, in those of Types

A, B and C Friedländer bacillus, and in the specifically reacting carbohydrate derived from gum acacia, has led to the belief that this sugar acid has an important function in directing the specific immunological properties of the polysaccharides of which it forms a part. A study of the chemistry of glucuronic acid, and its lactone, glucuron, is in progress. The ultimate objective of these studies is to prepare a glycoside of glucuronic acid which may be chemically combined with protein. The immunological behavior of the resulting complex may then be directly compared with the responses elicited by the hexose and disaccharide antigens which have already been studied.

An excellent source of glucuronic acid is the urine of dogs fed on a diet of d-borneol. Glucuronic acid is isolated directly and easily from the urine as the zinc salt of bornyl glucuronide. The latter compound yields a mixture of glucuron and glucuronic acid on hydrolysis. It is not possible to separate glucuron from glucuronic acid by fractional crystallization. It has, however, been found possible to convert this mixture quantitatively into glucuron, as well as to reconvert glucuron quantitatively into pure glucuronic acid by mild hydrolysis with barium hydroxide.

The comparative reducing values of glucuronic acid and glucuron have for the first time been quantitatively determined. A report of this study is now in press.

In a previous report (1931) there was described the preparation of α and β triacetyl glucuron. These derivatives have now been secured in optically pure forms. They show a difference in molecular rotation of 36,400, a value which is

in excellent agreement with the known differences in molecular rotations of the pentacetyl hexoses. A crystallographic study of these two derivatives is being made by Dr. Ralph Wykoff.

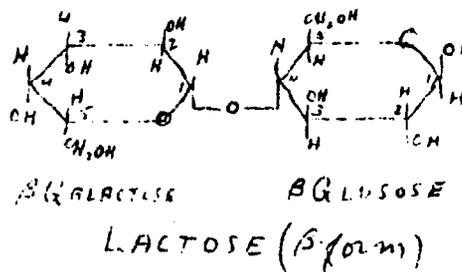
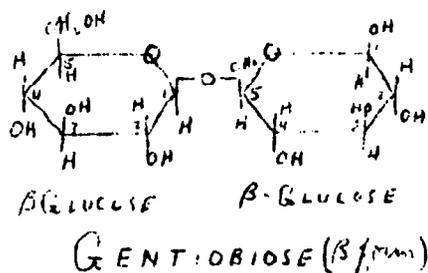
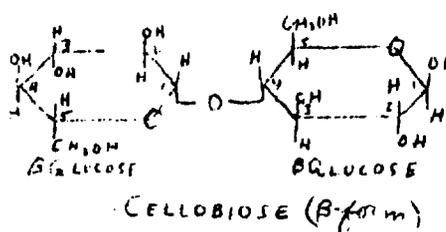
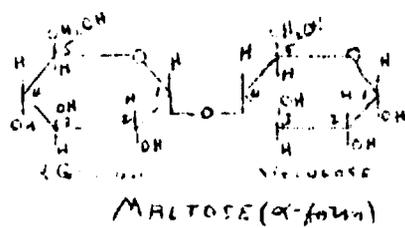
When either α or β triacetyl glucuron is treated with acetyl chloride and hydrogen chloride, an aceto chloro derivative is formed which is probably a mixture of α and β . 1-chlor 2, 4 diacetyl glucuron. This compound serves as the source material for the synthesis of glucuronides. When aceto chloro glucuron is dissolved in moist ether and shaken with silver carbonate, the chlorine atom is replaced by hydroxyl yielding a crystalline derivative which appears to be 1 hydroxyl, 2, 4 diacetyl glucuron. We have prepared a non-crystalline methyl diacetyl glucuronide from diacetyl chloro glucuron, and we have succeeded in synthesizing a small quantity of a crystalline diacetyl p-nitrobenzyl glucuronide. The condensation of diacetyl chloro glucuron with phenolic derivatives is very difficult to affect. It is necessary to overcome the difficulties before the synthesis of glucuronides can be profitably attempted, and we are still making serious efforts to do so.

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XI. The synthesis of p-aminophenol glucosides of lactose and gentiobiose.

The result of the studies of the immunological behavior of synthetic carbohydrate protein antigens has proven to be so instructive that we have extended this investigation to the disaccharides. The factors which control the immunological specificity of carbohydrates appear to be the position of intermolecular linkages and differences in intra-molecular

stereochemical arrangements. The disaccharides, whose chemical constitutions have been well established, are ideal substances for investigating the effect of such changes on immunological response. The four disaccharides which are being studied are represented graphically below. The differences in spatial constitution and inter-molecular linkage are readily discernable from these graphic formulae.



In order to combine chemically a sugar with a protein it is necessary to synthesize the p-aminophenol glucoside. These derivatives are then combined by diazotization of the amino group, and coupling the diazonium glycoside with protein in alkaline medium. We have synthesized the heptacetyl *p*-nitrophenol, the *p*-nitrophenol and the *p*-aminophenol glycosides of maltose, lactose, cellobiose, and gentiobiose. These glycosides have been combined with protein and a study of their antigenic properties is nearing completion.

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