Report of Drs. Avery and Horsfall (assisted by Drs. Adams, Binkley, Curnen, Goebel, McCarty, Mirick, Perlman, Stillman, and Ziegler).

Study on the chemical nature of the substance inducing transformation of specific types of pneumococcus (Avery and McCarty). Biologists, especially the geneticists, have long attempted by chemical means to induce in higher organisms predictable and specific changes which thereafter could be transmitted in series as hereditary characters. Among microorganisms the most striking and perhaps the only known example of inheritable and specific alterations in cell structure and function that can be experimentally induced and that are reproducible under well defined and adequately controlled conditions is the transformation of specific types of Pneumococcus. This phenomenon was first described by Griffith who succeeded in transforming an attenuated and non-encapsulated (R) variant derived from one specific type into fully encapsulated and virulent (S) cells of a heterologous specific type of Pneumococcus. A typical instance will suffice to illustrate the techniques originally used and serve to indicate the wide variety of transformations that are possible within the bacterial species. For example, Griffith found that mice injected subcutaneously with a small amount of a living R culture derived from Pneumococcus Type II together with a large inoculum of heat killed Type III (S) cells frequently succumbed to infection and that heart's blood of these animals yielded Type III pneumococci in pure culture. The fact that the R strain was avirulent and incapable by itself of causing fatal septicemia and the additional fact that the heated suspension of Type III cells contained no viable organisms brought convincing evidence that the R forms growing under these conditions had newly acquired the capsular structure and biological specificity of Type III pneumococci. Griffith wisely refrained from offering any explanation of the phenomenon

beyond suggesting that the dead bacteria in the inoculum might furnish some specific protein that enables the R forms to manufacture a capsular carbohydrate of the same specific type as that of the S cells which served as initial source of the inducing substance.

Although the experiments in mice were successful, Griffith was unable to reproduce the phenomenon without mouse passage and on the basis of negative results concluded that incubation of the bacterial mixture in vitro failed to induce transformation.

These original observations were later confirmed by Neufeld and Levinthal abroad and by Dawson and Alloway in this laboratory. Subsequently Dawson was the first to succeed in inducing transformation in vitro. This he accomplished by growing R cells in a fluid medium containing anti-R serum and heat killed encapsulated S cells. In the test tube as in the animal body he showed that transformation can be selectively determined depending on the type specificity of the S cells used in the reacting system. Later Alloway was able to cause specific transformation in vitro using sterile extracts of S cells from which all formed elements and cellular debris had been removed by Berkefeld filtration. He thus showed that crude extracts containing the active material in soluble form are effective, inducing the same specific transformations as do the intact cells from which the extracts were prepared.

The present report is concerned with a more detailed analysis of this phenomenon. Our major interest has centered in attempts to isolate the active principle from crude extracts and to identify if possible its chemical nature or at least to characterize it as belonging to a general group of known chemical substances. For purpose of study, the typical example of transformation chosen as a working model was the reacting system with which

we have had most experience and which consequently seemed best suited for further analysis. This particular example represents the transformation of Type II to Type III pneumococcus through the intermediary R form. Each constituent of the reacting system presented problems which required clarification before it was possible to obtain consistent and reproducible results. The various components of the system will be briefly discussed in the following order: 1) Culture medium; 2) The R-strain; 3) Preparation of transforming extracts of Type III cells; 4) Purification of the active material; 5) Chemical nature and biological activity of the purified substance.

Culture medium: The basic medium consists of the standard meat infusion broth routinely used in the cultivation of pneumococci. Earlier studies revealed that in order to obtain transformation it is necessary to add serum to the basic medium. Anti-R rabbit serum was used in the first successful experiments in vitro. It soon became evident, however, that the effectiveness of different lots of serum varied and that the differences observed were not necessarily dependent upon the content of R antibodies, since many sera of high titer were found to be incapable of supporting transformation. This suggested that factors other than R antibodies are involved in the system.

MacLeod first showed that sera from various animal species irrespective of their immune properties, and indeed normal sera of diverse origin contain an enzyme capable of destroying the transforming principle in potent extracts. (The nature of this enzyme and the specific substrate upon which it acts will be referred to later in this report). It was found that this enzyme was inactivated by heating the serum at 60°-65°C. and that sera previously heated at temperatures known to destroy the enzyme were then

effective in the transforming system. Further analysis has shown that certain sera in which R antibodies are present and in which the enzyme has been inactivated may nevertheless fail to support transformation. This fact suggests that still another serum factor is essential. The content of this factor varies in different sera, and at present its identity is unknown.

Recognition of these factors and their role in the transforming system has greatly facilitated the standardization of the medium required for obtaining consistent and reproducible results.

The R strain (R 36 A): The R variant used in present experiments was derived from Pneumococcus Type II. Irrespective of their type derivation the R variants of pneumococcus are characterized by the lack of capsule formation and the consequent loss of type specificity and virulence. The designation of these variants as "R" forms refers merely to the fact that on artificial media the colony surface is "rough" in contrast to "smooth" surface of colonies of encapsulated "S" cells. The R strain referred to above as "R36A" was derived by growing an "S" culture of Pneumococcus Type II in broth containing Type II antiserum for 36 serial passages and isolating the R variant thus induced. The strain (R36A) has lost the type-specific characteristics of the parent culture and consists only of attenuated, non-encapsulated R cells. The change S⟶R is often a reversible one, provided the R cells are not too far "degraded". The reversion of the R form to its original specific type may frequently be accomplished by successive animal passages or by repeated growth in anti-R serum. Under these conditions, however, the R culture invariably reverts to the same specific type as that from which it was derived. Strain R36A has become relatively fixed in the R phase and has never been found to revert spontaneously to the Type II S form.

Conversion of S⟶R and vice versa within the limits of a given type is quite a different matter than is the transformation of organisms of one specific type into those of another specific type. The latter has never been observed to occur spontaneously and indeed can be induced experimentally only by the special techniques outlined earlier in this report. Under these conditions, the enzymatic synthesis of a chemically and immunologically different capsular polysaccharide is specifically oriented and selectively determined depending on the type specificity of the S cells used as the source of the transforming principle.

In the course of the present study it was noted that the stock culture of R36 on serial transfers in blood broth undergoes spontaneous dissociation and gives rise to a number of other R variants which can be distinguished one from another by colony form. The significance of this in the present instance lies in the fact that of 4 different variants isolated from the parent culture only one strain, designated R36A, is susceptible to the transforming action of potent extracts, while the others failed to respond and have since remained inactive in this regard. The knowledge of differences in the responsiveness of different R variants to the same specific stimulus emphasizes the care that must be exercised in the selection of a suitable R variant for use in transformation experiments.

Although the significance of the following fact will become apparent later on, it must be mentioned here that pneumococcal cells possess an enzyme capable of destroying the activity of transforming extracts. Indeed, autolysates of R36A are highly active in this respect. Earlier workers found that it is essential to use a small inoculum of young and actively growing R cultures in transformation tests. This requirement may best be explained on the assumption that when large inocula are used enough enzyme may be released by autolysis to inactivate the transforming principle present in

the system.

Preparation of transforming extracts from Type III pneumococci: The present method of preparation of extracts from pneumococcal cells represents the end results of numerous experimental approaches carried out over a period of years. The procedures used in extraction and purification have been evolved gradually as evidence has been obtained concerning the nature and properties of the active substance.

Type III cells are grown in large quantities (50-75 liters) and collected by centrifugation in a steam driven sharples centrifuge. The packed cells are resuspended in physiological saline and heat killed at 65°C. This temperature is chosen in order to inactivate the enzyme capable of destroying the active substance since Type III pneumococci, as well as strain R36A, possess such an enzyme. The heated cells are recovered by centrifugation and washed three times with physiological saline which results in the removal of much of the type-specific capsular polysaccharide and large quantities of protein and ribonucleic acid (yeast type). The loss of active material incurred in the washing process is slight compared to the purification achieved. The washed cells are then extracted twice by shaking in saline containing 0.5 per cent sodium desoxycholate. Extracts prepared in this manner after removal of the desoxycholate by alcohol contain the major portion of the active principle as measured by their capacity to induce transformation.

Purification of active substance: Reprecipitation of the crude extract with 3 volumes of alcohol precipitates the active material and removes the last traces of desoxycholate which remain soluble in the alcohol. The precipitate is redissolved in saline and the great bulk of protein is removed by the chloroform method of Sevag. The process of deproteinization

is repeated several times. The specific enzyme prepared from a soil bacillus capable of decomposing Type III polysaccharide is then employed to digest the capsular polysaccharide remaining in the extract. After digestion of capsular polysaccharide is completed as determined by loss of reactivity with Type III antibody solution, the extract is again treated by the Sevag method to remove the enzyme protein and any remaining pneumococcal protein. This process is repeated until the supernatant fails to give the biuret reaction. The final step in purification is the repeated precipitation of the extract by the dropwise addition of 0.9 - 1.0 volume of absolute ethyl alcohol with constant stirring. At this critical concentration of alcohol, the active material separates out in long, extremely fine fibers which collect on the stirring rod. Digestion products of the capsular polysaccharide together with the somatic C carbohydrate, ribonucleic acid and remaining traces of protein are left in the residue. After several reprecipitations, the material is finally dissolved in distilled water and dialyzed against distilled water to remove free salts and other diffusible substances.

Chemical nature and biological activity of the purified substance: In the course of chemical fractionation of active material it was repeatedly found that biological activity was always associated with that fraction which gave a strongly positive reaction in the diphenylamine test, suggesting that the active substance might be desoxyribonucleic acid (thymus type). Further evidence for this view was found on elementary analysis of several preparations isolated by the procedures outlined above. In the following table analytical figures on two highly purified preparations are compared with the theoretical values for the sodium salt of desoxyribonucleic acid on the basis of the tetranucleotide structure of the molecule.

	Preparation #37	Preparation #42	Theory for desoxy-ribotetranucleotide
Phosphorous	8.57 per cent	9.04 per cent	9.05 per cent
Nitrogen	14.21 per cent	15.36 per cent	15.32 per cent
N/P ratio	1.66	1.69	1.69
Carbon	34.27 per cent	35.50 per cent	34.20 per cent
Hydrogen	3.89 per cent	3.76 per cent	3.21 per cent

The observed values correspond fairly closely to those calculated from theory. These figures by themselves do not establish that the substance isolated is a pure chemical entity. However, on the basis of the N/P ratio it would appear that little protein or other substances containing nitrogen or phosphorous are present as impurities since if they were this ratio would be considerably altered.

On enzymatic analysis of the active material it was found that crystalline trypsin or chymotrypsin and combinations of both caused no appreciable loss in activity. Moreover, digestion with crystalline ribonuclease does not impair biological activity. Among various crude preparations of enzymes derived from sources such as bone, kidney, pneumococcal cells, pancreatin and sera only those which attack authentic samples of desoxyribonucleic acid have been found to cause any detectable loss in the activity of potent preparations of the transforming principle. This is further evidence of the chemical relationship suggested by the analytical figures above.

Preliminary investigations in collaboration with Dr. Rothen indicate that the substance in highly purified form is probably highly polymerized and has a molecular weight of approximately 0.5 - 1 million. Other studies of the physical chemical properties of the active substance are now in progress.

It should also be pointed out that as the crude extracts are purified, serological activity with Type III antiserum progressively decreases. Solutions of the purified substance itself give only faint trace reactions with high titer Type III antipneumococcus rabbit serum. In view of the fact that the Type III capsular polysaccharide and the somatic C carbohydrate react with homologous antisera in dilutions as high as 1 part in 5 million, this loss of serological activity reflects the almost complete removal of all serologically reactive substances from the final preparations.

The fact that the transforming substance in purified state exhibits little or no immunological reactivity is in striking contrast to its biological function in inducing highly specific changes in living pneumococcal cells. As little as 0.02 μgr., representing a final dilution in the reacting system of 1 part in 100,000,000 has sufficed to bring about the transformation of the R variant (R36A) into encapsulated Type III pneumococci.

Assuming that the sodium desoxyribonucleic acid and the active principle are one and the same substance, then the transformation from R → SIII represents a change that is chemically induced and specifically directed by a known chemical compound. Moreover, this substance selectively determines a differentiation of cellular function and structure corresponding in type to that of the S organisms from which the agent was derived. The interaction between the R cell and the transforming principle initiates a series of complex reactions which culminate in the synthesis of the Type III capsular polysaccharide. Thus, the transforming principle - a nucleic acid - and the end product of the synthesis it evokes - the Type III polysaccharide - are each chemically distinct and both are requisite in the type specific differentiation of the cell of which they form a part. The former has been likened to a gene, the latter to a gene product, the accession of which is mediated through enzymatic synthesis. The genetic interpretation of this

phenomenon is supported by the fact that once transformation is induced, thereafter without further addition of the inciting agent both capsule formation and the gene-like substance are reduplicated in the daughter cells. The changes induced are therefore not transient modifications but are transmitted through innumerable transfers in ordinary culture media.

If the present studies are confirmed and the biologically active substance isolated in highly purified form as the sodium salt of desoxyribonucleic acid actually proves to be the transforming principle, as the available evidence now suggests, then nucleic acids of this type must be regarded not merely as structurally important but as functionally active in determining the biochemical activities and specific characteristics of pneumococcal cells.

Leathes has pointed out that in the chemical make up of protoplasm proteins are not the only essential substances. Among the other constituents he ranks the nucleic acids as preeminent, and referring to their occurrence as major components of nuclear chromosomes he raises the question "whether the virtues of nucleic acids may not rival the amino acid chains in their vital importance."

Studies on primary atypical pneumonia (Horsfall, Curnen, Mirick, and Ziegler). In 1930 it was first recognized clearly that there existed a common clinical form of pneumonia which differed from the usual bacterial pneumonias. In the intervening years this illness, now termed "primary atypical pneumonia", has been encountered with increasing frequency. Interest in the condition stems from the facts that it is, at the present time, about as often seen as is bacterial pneumonia and that the cause or causes of it have not been definitely established.

There is good reason to believe that this clinical syndrome is

not a new disease. It was probably recognized occasionally during the second half of the last century and undoubtedly was observed not infrequently during each of the first three decades of this century.

The recent marked increase in the use of the x-ray in acute respiratory diseases, the establishment of active full-time health units in some schools, colleges and camps and the ineffectiveness of sulfonamide chemotherapy in the illness seem to have been the most important factors in bringing this syndrome into clear relief.

There can be no doubt that this condition, during the past three years, has been increasing in incidence more rapidly than can be accounted for on the basis merely of increased awareness and recognition of it by physicians. In certain Army camps in the continental United States the incidence of the illness has been as high as 1.3 per cent of the total command. It is recognized both by the Army and the Navy as responsible for more man-days lost from duty than almost any other acute infectious disease. The frequency with which the illness occurs among civilians cannot now be estimated due to the fact that, with the exception only of New York City, the disease is not reportable.

Almost all investigators who have studied cases of primary atypical pneumonia think that the illness is not the result of bacterial infection. Some workers have suggested the possibility that the syndrome was caused by a virus and as a result the term "virus pneumonia" has come into common usage.

There is evidence that the syndrome is not a single disease entity. At least three different infectious agents have each been shown to be etiologically related to certain small groups of cases. These are the psittacosis group of viruses, Rickettsia diaporica, and a virus infectious

for the mongoose. Additional infectious agents have been suggested as possessing a causal relationship to other cases although the evidence upon which these suggestions have been based seems insufficient to permit of critical assessment.

A comprehensive study of primary atypical pneumonia was begun in this hospital one year ago. The primary objectives of the study were two-fold; firstly, a detailed study of all the clinical manifestations of the illness, and secondly, an investigation of the nature of the infectious agents responsible for the syndrome. During this year 112 patients were admitted to this hospital with acute respiratory diseases. Of these patients 80 were found to have primary atypical pneumonia. Specimens obtained from these patients constituted the source material for the laboratory studies. In addition, specimens were also obtained from 211 patients with the syndrome in other hospitals, both civilian and military. All specimens have been stored at -70°C. and consequently are constantly available for study. This large library of potentially infectious source material has already proven of great value.

The laboratory studies were facilitated by funds made available through a contract between the Office of Scientific Research and Development and The Rockefeller Institute. Complete laboratories and animal quarters suitable for the study of virus diseases were fitted out and specially equipped for this investigation. Additional technical assistants were engaged.

Clinical studies. As a result of the detailed studies of 80 patients with primary atypical pneumonia it was possible to formulate a fairly accurate clinical picture of the syndrome. The illness differed strikingly from the bacterial pneumonias. The clinical history, physical

examination, chest x-ray, and clinical course had but little in common with those encountered in the bacterial pneumonias. The most common findings were fever, relative bradycardia, cough, headache, slight alteration in the physical signs over the chest, definite x-ray evidence of consolidation, a normal leucocyte count and normal urine. Four characteristic observations were common; (1) the patient seldom appeared as ill as the chart would lead one to expect, (2) the pulse rate was seldom as rapid as the temperature would indicate, (3) the physical examination rarely suggested that consolidation of the extent demonstrable by x-ray was present, and (4) the clinical severity of the illness was often unrelated to the extent of pulmonary involvement.

An analysis of the clinical data revealed the following average findings; fever persisted for 10 days and reached 103.5°F., the pulse rate was 23 beats per minute slower than the temperature indicated, the respiratory rate was 25 per minute, leucocytes were 7,800 per cu. mm. and the erythrocyte sedimentation rate was 38 mm. per hour. In 75 per cent of instances the consolidation occurred in the lower lobes, and more commonly on the left than the right side. In 70 per cent of instances only one lobe was consolidated. Evidence of beginning resolution of the pneumonic process could be discerned by x-ray on the 10th day but complete resolution did not occur until the 17th day. Complications were uncommon, although recrudescence of fever was not infrequent. Either extension of the consolidation or recurrence of it occurred in some cases. Pleuritis associated with small collections of fluid and a mild form of meningismus, or transient encephalitis, each occurred in about 5 per cent of cases. Slight splenomegaly, arthralgia and transient alterations in the electrocardiogram were each found in some cases. This group of unusual phenomena led to the hypothesis that the

syndrome might in certain instances be associated with a generalized infection. Sulfonamide chemotherapy seemed not to alter the clinical course of the illness. The mortality rate was zero.

Biochemical studies on primary atypical pneumonia (Emerson, Hoagland, Curnen, Mirick and Ziegler). Through the cooperation of other services in the hospital numerous biochemical studies were carried out on selected patients. It was found that the icteric indices, prothrombin levels, serum CO_2 and serum chloride levels were within normal limits. Plasma carotene and vitamin A levels were reduced but only to an extent commonly encountered in many other acute infectious diseases as well. Plasma α amino acids were within normal limits and chloride balance studies indicated that there was no striking abnormality in chloride metabolism. These latter findings are noteworthy since they serve additionally to differentiate this syndrome from those associated with the bacterial pneumonias. As is well known, the plasma α amino acid levels are definitely reduced in bacterial pneumonia and chloride metabolism is distinctly abnormal in most cases.

Bacteriological studies on primary atypical pneumonia (Curnen, Mirick, Ziegler, and McCarty). The bacterial flora of the nose, throat and sputum was studied in each case and an effort was made to obtain both qualitative and quantitative data. The sputa usually contained relatively few bacteria and in no instance was it possible to determine the type of pneumococcus by the direct Quellung technique. However, by mouse inoculation one or another type of pneumococcus was obtained from 60 per cent of the sputa. It is noteworthy that neither Type I nor Type II Pneumococcus was isolated. The distribution of pneumococcus types was not dissimilar to that which has been found in the throats of healthy individuals. β hemolytic streptococci, hemolytic staphylococci and H. influenzae, though occasionally

found were not encountered with sufficient frequency or in numbers sufficient to make them seem of etiological significance. None of the strains of H. influenzae isolated were smooth and none could be typed. Numerous other species of bacterial micro-organisms were also isolated from specimens obtained from the respiratory tracts of patients. All of these organisms are known to be present commonly in the upper respiratory tract of healthy persons. In no instance was any evidence obtained which suggested that any of the bacterial species isolated were causally related to the illness. Blood cultures were done on all patients and none showed bacterial growth. Moreover, pleural fluid and cerebrospinal fluid obtained from patients failed to reveal the presence of bacteria on culture.

Serological studies on primary atypical pneumonia (Horsfall, Thomas, Curnen, Mirick and Ziegler). Acute phase and convalescent sera from patients were tested for the presence of antibodies against a number of viruses known to be capable of inciting acute respiratory disease in human beings. The agents selected for these tests were, psittacosis virus, lymphocytic choriomeningitis virus, influenza A virus, influenza B virus and swine influenza virus. In no instance was a significant increase in specific antibodies against any of these viruses demonstrable during convalescence of patients admitted to this hospital.

Acute phase and convalescent sera from patients were also tested for the presence of the so-called C reactive protein. It will be recalled that notably in pneumococcus pneumonia but also in other acute infectious diseases there is present in the serum during the acute phase of the illness an abnormal protein which has the peculiar property of reacting with the C or somatic polysaccharide of pneumococcus to form a precipitate. It was found that this protein was almost invariably present in the acute phase sera of patients with primary atypical pneumonia and that it disappeared

during early convalescence.

In the course of certain serological tests a peculiar and unexpected phenomenon was encountered. It was found that approximately 35 per cent of patients with primary atypical pneumonia developed in their serum during early convalescence the capacity to fix complement with a wide variety of apparently unrelated antigens. The convalescent serum in high dilution of some patients reacted with many of these antigens although the acute phase serum from the same patients did not. Similar reactions were not observed when convalescent sera from other acute infectious diseases, either of bacterial or virus etiology, were tested. The reaction could be demonstrated with antigens prepared from a variety of organs obtained from a number of unrelated species and it is not yet apparent what component common to these antigens may explain the phenomenon. In general, tissues infected with one or another virus provided more active antigens than did normal tissues. This phenomenon seriously complicates the interpretation of complement fixation tests in this syndrome. Moreover, it serves to cast some doubt on the significance of certain studies which have been based wholly or largely upon the results obtained with the complement fixation technique in this illness.

Etiological studies on primary atypical pneumonia (Horsfall, Curnen, Mirick, Thomas, Ziegler). Specimens of sputum, throat washings, plasma, pleural fluid and cerebro-spinal fluid obtained during the acute phase of illness from a number of patients with primary atypical pneumonia were tested for the presence of infectious agents. In numerous instances these specimens were inoculated in mice, guinea-pigs, hamsters, rabbits, cotton rats, white rats, hooded rats and monkeys by a variety of routes including the intranasal, intratracheal, intraperitoneal, intravenous, subcutaneous and intracerebral. Many specimens were also inoculated in chick-embryos of various ages by the following routes; intra-

allantoic, intraamniotic, and on the chorio-allantoic membrane. Serial passages were carried out in all species with the exception of monkeys. In no instance were obvious signs of infection produced which could be reproduced on transmission in series. Indeed in only two of 21 sputa tested was pulmonary consolidation produced repeatedly in cotton rats following primary intranasal inoculation. However, it was not possible in either instance to reproduce this lesion on serial passage in this species despite many variations in experimental procedure.

Although no obvious signs of infection, transmissible in series, were encountered in any of the species tested, it was discovered that animals inoculated with certain specimens or with passage material derived from these specimens subsequently developed in their serum, antibodies which were capable of specifically neutralizing a heterologous virus. This heterologous virus was previously shown to be present, though latent, in the lungs of many apparently healthy mice and has been termed "pneumonia virus of mice". Because of this unexpected observation that animals inoculated with specimens obtained from cases of primary atypical pneumonia produced antibodies which completely neutralized large amounts of a heterologous virus derived from the lungs of mice, the possibility arose that there might be in the agent recovered from patients and in the "pneumonia virus of mice" minor common antigens. Moreover, if such an accidental antigenic relationship actually existed, means would become available of detecting the presence of the human agent even though it were not capable of producing obvious signs of infection in available animal species. This hypothesis has proven fruitful and has made possible the development of experimental procedures, based almost entirely upon specific immunological tests, by means of which certain characteristics of the human agent have been defined.

Evidence has been obtained which indicates that 15 strains of a virus have been recovered from specimens obtained from a total of twenty-two patients. Two strains were recovered from throat-washings, eight from sputa and five from citrated plasma. All 15 strains appeared to possess minor antigenic components also present in the heterologous pneumonia virus of mice, hereinafter referred to as PVM. Although none of these strains was capable of producing obvious signs of infection on serial passage in any animal species tested, nevertheless, immunological evidence has been obtained that the agent could be passed in series in both chick embryos and cotton rats. It could also be passed serially in chick embryo solid tissue culture, was antigenic for rabbits and caused an inapparent infection in mice. The virus was filterable through Berkefeld V candles, did not lose activity after storage at -70°C. for more than eight months, withstood rapid freezing and thawing 10 times, and was inactivated by 56°C. for 30 minutes.

Evidence indicating the presence of this agent in specimens from patients with primary atypical pneumonia has been obtained in a variety of ways. Cotton rats were inoculated and rabbits were injected with sputum, throat-washings, or plasma from patients as well as with animal or tissue culture passage material derived from these specimens. Sera obtained from these animals before and at intervals after inoculation were tested for the presence of neutralizing antibodies against PVM. Mice inoculated with similar specimens were subsequently tested for active immunity against infection by PVM.

One of three filtered throat washings tested contained an agent which following serial passage in chick embryos was capable of stimulating the production of specific neutralizing antibodies against PVM in rabbits. Of 15 acute phase plasma tested 5 were found to contain an agent which was

capable of stimulating the production of neutralizing antibodies against PVM either following intravenous injection of rabbits or intranasal inoculation of cotton rats. Of 18 specimens of sputum and one throat washing tested by the inoculation of cotton rats 8 sputa and the throat washing were found to stimulate the production of neutralizing antibodies against PVM. It should be pointed out that normal rabbit sera and normal cotton rat sera did not contain antibodies against the heterologous mouse virus.

Mice which had been inoculated intranasally with many of the positive specimens were found on subsequent test to have developed active immunity against infection by small but significant quantities of PVM. Mice similarly inoculated with such specimens previously inactivated at 56°C. for 30 minutes or with negative specimens failed to develop resistance against infection with PVM.

When rabbits were injected or cotton rats were inoculated with PVM itself it was found that this virus stimulated the production of neutralizing antibodies against itself much more quickly and in far higher titer than did the human virus. Moreover, mice inoculated with sublethal doses of PVM developed active immunity against far greater quantities of the mouse virus than did other mice which were given the human agent. The quantitative and temporal differences in the manifestations of immunity produced by these two viruses indicated that whereas they possessed antigens in common they were not antigenically identical.

A number of strains of the human virus were passed in series either in cotton rat lungs or in chick embryos as well as on the chorioallantoic membrane or in tissue culture. Evidence for the presence of the agent in passage material was obtained by the demonstration of neutralizing antibodies against PVM in the sera of inoculated cotton rats. The virus was capable of causing contact infection in cotton rats as was shown by the fact

that normal rats placed in the same cage with previously infected rats developed in most instances antibodies against the heterologous mouse virus. This finding was of some interest since it seems probable that the human infection itself is transmissible by contact. Histological sections of infected rat lungs and infected egg membranes stained by the Giemsa method and impression films stained by the Macchiavello or Gram method failed to reveal the presence of elementary bodies, inclusion bodies, Rickettsiae or bacteria.

The fact that the agent recovered from current cases of primary atypical pneumonia appeared to possess antigenic components also present in the pneumonia virus of mice served to confirm unpublished experiments carried out (Horsfall) in the Laboratories of the International Health Division of the Rockefeller Foundation during 1939. At that time it was found that rabbits immunized with chick embryo tissue culture passage material derived from a Berkefeld V filtrate of a throat washing obtained from a patient with primary atypical pneumonia produced antibodies capable of specifically neutralizing PVM. It seems of significance to point out that this particular filtered throat washing, studied 3 years ago, was also the source of the first recovered strain of the mongoose infectious virus. In the light of the present findings it was considered of importance to restudy this latter agent. A suspension of infected mongoose lungs which had been stored at -70°C. for over 2½ years was available. It was found that the agent present in this suspension could be passed in series both in cotton rat lungs and on the chorioallantoic membrane. Moreover, cotton rats inoculated with passage material produced neutralizing antibodies against PVM indicating that the mongoose infectious virus also possessed antigonic components present in the virus of mouse origin.

Although these studies suggested that the agent recovered from current cases was similar to the agent recovered from other cases three years ago it was of importance to determine whether following atypical pneumonia there occurred in human beings a specific antibody response to this agent. Neutralization tests with patients' sera and the human virus were difficult to devise since the virus failed to produce signs of infection on passage in available animals. However, advantage was taken of the fact that one sputum known to contain the agent produced pulmonary consolidation on primary inoculation in cotton rats, though it did not do so on serial passage. When mixtures of this sputum and either acute phase or convalescent serum from patients with atypical pneumonia were inoculated in groups of cotton rats, it was found that an increase in antibodies was demonstrable in the convalescent sera of 11 of a total of 18 patients tested. It seems noteworthy that all 18 convalescent sera completely neutralized the agent.

Convalescent human sera not only neutralized the agent responsible for pulmonary consolidation but also neutralized the agent responsible for the stimulation of antibodies against PVM in cotton rats. Moreover, rats inoculated with the human virus produced antibodies which neutralized not only PVM but also the agent in the sputum responsible for consolidation in rats. This evidence strongly suggested that pulmonary consolidation in the cotton rat and the development of antibodies against PVM were the result of infection by one and the same agent.

Acute phase and convalescent sera from these same patients were tested against many heterologous viruses some of which are known to be capable of inciting acute respiratory diseases in human beings. In no instance was a significant increase against any of these other agents demonstrable. The available evidence suggests that the mongoose infectious virus

which appeared to be etiologically related to certain cases of primary atypical pneumonia studied in 1939 is related antigenically to the heterologous virus of mouse origin, the "pneumonia virus of mice". Since all 15 strains of the agent recovered from current cases of atypical pneumonia appear also to be antigenically related to PVM, it seems reasonable to think that they are either identical with the mongoose infectious virus or are very closely related to it both in antigenic composition and biological characteristics. Moreover, serological studies carried out with current cases indicate that in a large proportion of instances there was a demonstrable increase in antibodies against the human virus during convalescence. This is strong evidence that the agent actually had produced infection in these patients and suggests that it may have been causally related to the illness.

Most surprising perhaps was the fact that in some cases the agent was recovered from the plasma. This indicated that the infection was not confined to the respiratory tract but instead was in certain instances generalized. It is well known that in almost all virus diseases in which a systemic infection occurs, the immunity which follows such illnesses is persistent. By analogy, it also seems possible that in primary atypical pneumonia immunity may be enduring. In this connection it may be of interest that so far as is known, there have not yet been reported instances of two attacks of this syndrome in one individual. If, as seems possible, immunity following this infection does persist in time, the implications in relation to the possibility of developing effective prophylactic measures are obvious. Experiments designed to test the feasibility of actively immunizing susceptible animals using infected chick embryo material as a vaccine are now under way.

Numerous attempts have been made to devise a rapid and reliable

serological test for the presence of antibodies against the human virus in order that an etiological diagnosis could be made more readily. Although some suggestive preliminary results have been obtained by means of a carefully controlled complement fixation technique there are as yet insufficient data to indicate that this procedure will actually serve to be useful in solving the present diagnostic problem.

Studies on the interference phenomenon (Ziegler, Mirick and Horsfall). It is well known that certain viruses are capable of rendering an infected animal insusceptible to infection by a second and sometimes antigenically unrelated virus. This is termed the interference phenomenon. Andrewes has recently reported that a neurotropic strain of influenza A virus and the parent pneumotropic strain from which it was derived were capable of reciprocally interfering with each other when tested in tissue culture. The possibility that influenza A and influenza B viruses, which do not possess common antigenic components, might interfere reciprocally with each other in the chick embryo seemed worth investigating, particularly since it was considered possible that one or the other might be capable of interfering with the virus recovered from current cases of primary atypical pneumonia. Should the latter phenomenon be demonstrable means would become available of testing readily and rapidly not only for the presence of the human virus but also for antibodies against it. It has been found that influenza A virus actually does interfere in chick embryos with subsequent infection by influenza B virus. Similarly, it has been found that influenza B virus in the chick embryo interferes with subsequent infection by influenza A virus. Small quantities of either virus inoculated in chick embryos are capable of rendering such embryos almost if not entirely resistant to subsequent infection with the other virus even though

very large amounts of the latter are given.

So far it has not been possible to demonstrate conclusively that the virus recovered from cases of primary atypical pneumonia is capable of interfering with the infection of chick embryos by either of the two influenza viruses. However, it has been found that the human virus possesses the capacity of interfering in mice with subsequent infection by the pneumonia virus of mice. The available evidence indicates that this is an additional instance of true interference, even though there exists an antigenic relationship between these two viruses, since when they are both inoculated simultaneously the phenomenon still is repeatedly demonstrable.

Antigens of the Flexner group of dysentery bacilli (Goebel, Binkley and Perlman). Epidemic dysentery among members of the armed forces and civilian population is at present a problem of special concern to public health officials and to military authorities. Since the outbreak of the world conflict clinical bacillary dysentery has been controlled largely through the use of the newer sulfonamide drugs. The problem of procuring an efficacious prophylactic agent for the prevention of the disease among closely grouped peoples where the general sanitary conditions cannot be adequately controlled, has not been effectively solved. The reasons for this are manifold. There is no assurance that the immunization of humans with suspensions of dead dysentery organisms will prevent the disease. The use of such vaccines is dangerous due to their great toxicity and the reactions to which they give rise. Furthermore, the number of different types of enteric organisms which can cause the disease is considerable and any effective vaccine must include organisms of all the known types of dysentery.

Dysentery bacilli isolated from the stools of patients in the epidemics studied in this country during the past few years belong for the

most part to the Flexner group although Sonne, Newcastle, and Schmitz infections are frequently encountered. The more virulent Shiga bacillus has been demonstrated only infrequently as the causal agent of dysentery outbreaks here in our temperate zone.

There are some nine well defined types of organisms in the Flexner group. These types are interrelated immunologically and it is currently believed that this relationship can be attributed to a common antigen. The individual specificities exhibited by members of this group are thought to be due to an additional specific antigen peculiar to each serological type.

We have undertaken a study of the antigens of the Flexner paradysentery group with the ultimate objective of using these substances as immunizing agents for the prevention of the disease in man. We have chosen the V strain (Boyd I) for study because of the broad serological crossing which this strain exhibits with other members of the Flexner group.

The antigen prepared with diethylene glycol: a. Preparation of the antigen from the V strain of Shiga paradysenteriae (Flexner). The type specific antigen of the Flexner V strain can be obtained in crude form by extraction of the organisms with diethylene glycol following the technique used by Morgan. Further fractionation by various procedures has yielded a sufficient quantity of a highly purified material to characterize and to learn much of its immunological and chemical properties. We are fortunate in having at our disposal the modern techniques of electrophoresis and the photometric quantitative precipitin titration which have been used very effectively in following the purification of the antigenic fractions. 1.6 gms. of the specific antigen, derived from 300 gms. of dried dysentery organisms, has been obtained in nearly pure form. The following is an account of the chemical and immunological properties of this material.

b. Properties of the antigen. The specific antigen of the V strain of paradysentery bacillus is a carbohydrate-protein-lipid complex containing 4.5 per cent nitrogen, 14 per cent phospholipid, and approximately 60 per cent carbohydrate. When examined by electrophoresis the antigen is essentially pure. The substance is powerfully antigenic in rabbits. Three intravenous injections of 50 micrograms each on alternate days suffices to evoke potent precipitating and agglutinating antisera in rabbits. The intradermal and subcutaneous routes of inoculation are equally effective in giving rise to antibodies.

The purified antigen is highly toxic, about 1000 micrograms suffice to kill both rabbits and mice. On the basis of body weight the rabbit is one hundred times more susceptible than is the mouse to the toxic action of the antigen.

We have accumulated a great deal of evidence which indicates that toxicity is an inherent quality of the antigenic molecule and that its toxic properties cannot be attributed to an accompanying substance which is separable by chemical fractionation. Nor can the toxicity be attributed to the phospholipid constituent for when the latter is removed, neither the precipitability, antigenicity nor toxicity of the resultant carbohydrate-protein complex is altered.

Preliminary attempts to alter or impair the toxicity of the antigen by chemical means have been made, but thus far without success. Deamination of the protein constituent with nitrous acid fails to destroy toxicity nor does treatment with formaldehyde. Substitution in the tyrosin molecule with diazonium salts likewise does not impair the toxic properties of the antigen nor do mild oxidizing agents. The toxic antigen is singularly resistant to chemical manipulation and only drastic procedures appear to

destroy its toxicity and at the same time its capacity to elicit antibodies. Because of the differential between the immunizing and toxic doses in experimental animals we are of the opinion that the antigen as such may be safely used as a prophylactic agent and we are preparing to conduct experiments on human beings with this end in view.

c. Isolation of a type specific polysaccharide. The extraction of the V strain of dysentery bacilli with diethylene glycol yields not only the phospholipid-carbohydrate-protein complex but a second immunologically active constituent as well. The latter has been identified as a polysaccharide and is highly reactive in homologous antiserum. This polysaccharide contains nitrogen probably hexosamine nitrogen and although specifically reactive in high dilution in V antiserum, it fails to evoke antibodies or to exhibit toxic properties when injected into rabbits and mice. It is believed that the polysaccharide is identical with the carbohydrate moiety of the antigenic complex and may occur free and uncombined in the bacterial cell.

Preparations of the antisera by tryptic digestion. The extraction of dysentery bacilli with diethylene glycol is not the most efficient means to procure the type specific antigen. Cells which have been extracted with this solvent yield on tryptic digestion large amounts of an antigenic and serologically reactive product. This material is toxic and evokes specific precipitins and agglutinins when injected into rabbits. Fractionation of the substance yields two major products which are essentially pure when examined by electrophoresis. One fraction is a non-nitrogenous and non-toxic polysaccharide which is immunologically inert. The other is a toxic protein-carbohydrate complex, free of lipid which evokes in rabbits type specific agglutinins and precipitins. The latter substance has both

chemical and immunological properties which lead us to believe that it is very similar to the type specific antigen obtained by diethylene glycol extraction. The tryptic antigen is less toxic than the latter, however, and when injected into rabbits evokes precipitins and agglutinins. The antibodies are not qualitatively nor quantitatively identical with those evoked either by the untreated cells or by the diethylene glycol antigen. It is our opinion that the antigen obtained by the enzymatic procedure represents a degradation product of the natural antigen in which the phospholipid has been removed by the lipase or crude preparation of the trypsin employed and in which the protein moiety has been partially degraded by hydrolysis.

Antigenicity and toxicity of the V strain antigen. As pointed out above, toxicity appears to be an inherent quality of the V strain antigen. The most convincing evidence in support of this view is the fact that a lysogenic (VR) strain of the parent organism obtained by secondary growth from phage lysates fails to synthesize any trace of the type specific toxic antigen. The organisms themselves exhibit no toxic properties when injected into experimental animals. Quantities as great as 30 mg. can be given to mice intraperitoneally without effect, whereas 1 mg. of the parent strain kills.

When the specific antigen derived from the V strain is administered intradermally in rabbits in amounts of 2000 micrograms (two lethal doses by the intravenous route) the animals do not die. However, there is considerable local reaction characterized by edema, hemorrhage and central necrosis. This lesion persists for some ten days. At the end of this period the sera of such animals contain a high concentration of antibodies.

If the animals are reinoculated at the end of the ten day period, the dermal reaction is much more intense than at first. The freshly inocu-

lated area shows far greater induration and necrosis than the original. Furthermore, the site of the first area flares up and the reaction persists for several days.

The effects of subcutaneous inoculation of this antigen in rabbits is less dramatic. When 2000 micrograms are injected, there is no localized reaction. There is a systemic reaction in the form of general malaise and a transient rise in temperature. On subsequent inoculation these phenomena do not manifest themselves. The animals immunized by this route develop in their serum powerful specific agglutinins and precipitins in high titers.

Isolation of a polysaccharide from the lysogenic (VR) strain. The tryptic digestion of the organisms derived from mass cultures of the lysogenic (VR) strain followed by chemical fractionation of the digest fails to reveal any trace of the type specific antigen. Furthermore, the VR organisms do not agglutinate in V antiserum nor do the V organisms react in anti VR serum. From the enzymatic digest of the VR strain a polysaccharide has been isolated which contains neither phosphorous nor nitrogen, and which possesses no immunological activity. The significance of this substance is not known. It appears to be identical with the inert polysaccharide isolated in larger quantities from the tryptic digest of the V strain since both substances have identical specific rotations and yield the same percentage of reducing sugars on hydrolysis.

Immunological relationship of Shiga paradysenteriae. As indicated previously in this report members of the Flexner group are immunologically interrelated and it is believed that their serological crossing can be attributed to at least one common antigen, whereas their type specificities are caused by an additional specific antigenic constituent. Even the most modern work, that of Boyd in India, has not materially modified the hypothe-

sis propounded by Inmans and Andrews over twenty years ago.

During the past few months we have prepared in highly purified form the antigens from Flexner Z and Newcastle strains. These substances are analogous to the antigen derived from the V strain i.e., they are lipocarbohydrate-protein complexes which precipitate in homologous antiserum. The antigens exhibit serological crossing in the sera of rabbits immunized to other Flexner types. When measured quantitatively by the photometric method the extent and degree of crossing is almost identical with that observed by the agglutination reaction of heat killed organisms. Cross absorption experiments with the pure V and Z polysaccharides and their respective antisera show a relationship which is exactly analogous to the cross reaction of Types III and VIII pneumococcus. For example, when V antiserum is absorbed with homologous polysaccharide, both precipitins and agglutinins for the homologous and heterologous antigens are removed. When the absorption is carried out with the heterologous Z polysaccharide, the homologous antibodies remain and only the heterologous agglutinins and precipitins are removed. Since these cell free antigens represent pure or nearly pure chemical entities, it seems not unlikely that their immunological crossing and that of the organisms from which they are derived is not caused by a multiplicity of antigenic constituents but may be attributed to similarities in the chemical constitution of the polysaccharide portion of the respective bacterial antigens. We hope to establish this hypothesis by more extensive work and to explain the immunological relationship of the organisms of the Flexner group strictly on the basis of chemical similarities of the type specific antigens.

Studies on the bacterial flora in acute respiratory diseases (Stillman). In the last pandemic of influenza the high mortality rate was

largely due to the frequent occurrence of highly fatal pneumonias caused by pathogenic microorganisms which the patient had newly acquired or happened to be carrying at the time in the upper respiratory tract. These opportunistic pathogens, notably the various types of influenza bacilli, hemolytic streptococci, and pneumococci, are also frequently found as secondary invaders in the purulent complications associated with the common cold. If influenza and the common cold are in fact due to viruses, it seems not unlikely that these primary agents may so alter the defense reactions of the patient as to permit secondary invasion by microorganisms which can be carried with impunity by the healthy individual for considerable periods of time. If this hypothesis is correct then the bacterial pneumonias and the suppurative lesions described above must be regarded not as primary diseases but as secondary complications of the initial virus infection.

In a study made in 1920-21 on the occurrence of H. influenzae in cases of pneumococcal lobar pneumonia, this organism was isolated from 85 per cent of the patients studied. Although hemophilic bacilli are only rarely present in nasal cultures from healthy individuals, they have been found in similar cultures in 23 per cent of cases of lobar pneumonia. At the time these studies were made the differentiation between the encapsulated (S) and the non-encapsulated (R) forms of this group of organism had not been recognized and no serological classification of specific types had been developed. When it was found that a large number of patients with atypical (virus) pneumonia also harbor in their nasopharynx H. influenzae our interest in this organism was reawakened, particularly since it is now possible by means of immune sera to classify strains into known specific types. Type specific immune sera have been prepared by the immunization of rabbits with strains of H. influenzae of known specific types. A study

of the bacterial flora of patients suffering from various mild upper respiratory diseases is being continued with the hope that the results may serve as a background in the event of an epidemic of respiratory infection of viral origin, complicated by secondary bacterial invasion as occurred in the last pandemic of influenza. Special attention is directed toward the occurrence of type-specific strains of H. influenzae.

Studies are now in progress on experimental pneumonia induced in mice by the method of inhalation which simulates more closely the natural pathway of infection. It has been found that pulmonary consolidation occasionally occurs in mice sprayed with H. influenzae followed by inhalation of pneumococci and that this lesion does not occur when either organism is used alone. Experiments are being carried out to determine whether the combination of these two organisms is the essential factor or whether in association together they serve as a provocative stimulus to the pneumonia virus of mice which is known to be harbored in a latent state by many animals of this species.

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