SYMPOSIUM FEBRUARY 2, 1979

CELEBRATING THE
THIRTY-FIFTH ANNIVERSARY
OF THE PUBLICATION OF

"STUDIES ON THE CHEMICAL
NATURE OF THE SUBSTANCE
INDUCING TRANSFORMATION
OF PNEUMOCOCCAL TYPES"

Induction of Transformation by a Desoxyribonucleic Acid
Fraction Isolated From Pneumococcus Type III

By Oswald T. Avery, M.D., Colin M. MacLeod, M.D., and
Maclyn McCarty,* M.D.

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The Rockefeller University
Caspary Auditorium

INTRODUCTIONS

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Introduction by Dr. Joshua Lederberg

It isn't very often that a paper deserves to be reprinted 35 years after the original publication; but the paper we are talking about today is no ordinary publication: since in many respects it can be said to be the pivot on which the larger part of contemporary chemical biology absolutely rests. I am particularly delighted at the considerable number of people who have come from outside, for this celebrating occasion and especially for those who. were able to share with me my own sense of excitement and exhilaration at the time that this report first appeared, and to whom the text will appear very, very familiar on a re-reading. I discovered that I had already written my introduction to today's proceedings many years ago, in fact, very nearly 35 years ago and so rather than repeat I'll exhibit it for your own critical examination. I don't know how well this will come out in handwriting which was a little bit better in those days than it is today: but they were my own notes on first reading "The Ode to DNA" together with a small misrepresentation of the date, it was 1944 and not 1943. Having satisfied you of the authenticity of the handwriting I'll give you a more legible version of it with the appropriate documentation (and documentation above all is what I am seeking at the present time). I know I have many of my former colleagues here who can testify to the enormous

excitement that went through the halls of Schermerhorn Hall at Columbia University in 1944 and when I first encountered it in early 1945, and with the real sense that this was indeed the dawn of a new age. A great deal has been written about the implications of this discovery not to mention of course the enormous output of scientific publication that has depended directly on this first demonstration that the genetic substance was DNA, connected with the existence and validation of the first assay system for the biological function of DNA that became available to us. What I hope we might hear more of today is how this discovery came about, and particularly the sense of the way in which there was a dawning in the minds of the investigators that they were dealing with DNA; and that they indeed were on the verge of having to make what would be a revolutionary claim. About such matters much less is published; and as you well know you can search between many of the lines of the text that has been reproduced for you, and find very little trace of the details of the development of ideas that in fact led to those particular circumstances. For an introduction to the scene I will be calling shortly on Professor Dubos. We will hope to hear in greatest detail from Professor McCarty himself and then I am delighted to be able to tell you that Professor Hotchkiss found it possible to return from Paris specifically for this occasion and will also be able to participate towards the end of our discussion. So without further ado Professor Dubos.

1945 2100. Satday.

I had the evening all to myself, and particularly the excruciating pleasure of reading Avery '43 on the desoxyribose nucleic acid responsible for type transformation in Pneumococcus. Terrific + unlimited in its implications. Viruses are gene-type compounds, but they cannot grow on synthetic or even dead media, and their capacity for production is limited to reproduction. The TF of Pneumococcus has every characteristic of a mutation. The obvious questions still to be considered: the fraction of serum that is involved in the reaction system; the induction of mutation in the TF by the use of X-Ray and more controllable methods; the [illegible] of its antigenic specificity + relations to the specific polysaccharide whose manufacture it regulates or initiates. Also the possibility of activity of TF in vitro or in killed systems must be investigated, though the presence of phosphatases + desoxyribonuclease present a difficult problem. I can see real cause for excitement in this stuff though.

I read a paper by Lester, E. today on observations on young of females treated c nephrotoxin which was stimulating by its limitations. The genetic studies were bungled + no comprehension of the significance of the work noted. But I suppose that lacking the stimulus of more modern work I would be as dumb. Read Guyer + Smith's old paper ('20) seem to have gotten the point even then. What a pity more careful studies weren't made then. But microorganisms are obviously superior.

Demonstration of the [illegible]lication of TF as its polysaccharide [illegible]cts!!

[illegible] synthetic enzyme for [illegible] may be TF itself.
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20 Jan. 1945 2100. Satday

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Direct demonstration of the multiplication of TF as well as its polysaccharide products!

or the synthetic enzyme for it c̄H maybe TF itself. Dual function - reproduction/production. Is the spatial specificity different???

Professor Rene Dubos:

I should mention that I have been on this campus now for 52 years and therefore I cannot avoid looking at anything that happens here or outside from a historical point of view. Perhaps the most useful contribution I could make today on this wonderful occasion is to try to create for you the historical perspective in which one must evaluate the occasion that we are celebrating. And if you don't mind I'll go back exactly 100 years ago. Now believe it or not, 100 years ago, there was published in Germany a book by one of the most famous botanists of the time, Karl Nägeli, in which he demonstrated to his satisfaction that there were no such things as bacteria. Bacteria were considered only very primitive forms of fungi which changed shape and physiological activities as they attempted to adapt themselves to different environmental conditions. He was convinced of that and so was the rest of the world. Just to illustrate that this was not peculiar to him, I have traced five or six of the most famous publications of that time that accepted this point of view. Let me just mention two names that I select only because they are so famous for other contributions: namely Dr. Thomas Huxley, who in 1872 published a paper in which he stated that there was no such thing as a bacterium: but those bacteria were "undifferentiated forms of protoplasm" that could change one into the other and adopt all sorts of different morphologies and physiological activities. The other person I want to mention is Lister; the most famous surgeon of the time who introduced antiseptic techniques in

surgery and who also held the same point of view. So throughout the 19th century, until 1877, there prevailed a theory that was called polymorphism, according to which those low forms of life could change one into the other under certain conditions. The first person to take a stand against this view was Pasteur who in 1859 introduced the concept of specificity not so much of morphological specificity but of physiological specificity in the activities of microorganisms. But Pasteur's technique in those early days could not really prove that each organism had a specificity of its own, and could be clearly differentiated from the others. This demonstration occurred only in 1876 and by three different persons. One was Pasteur himself who in 1876 published an enormous book "Studies on Beer" in which he provided the most exquisite evidence of the physiological specificity of microbial action. The other one was by a German botanist F. J. Cohn who published the first book on bacteria in which he clearly described the different morphological structures that we still recognize today. And then finally that very same year there was Robert Koch, also in Germany, who through his studies on anthrax demonstrated in the most exquisite manner that the anthrax bacillus had a very definite shape, a very definite life history, very different from that of other organisms. Well now, let's move to 50 years later the time when I first entered this campus in 1927 to join Dr. Avery's department. It is very difficult for you to understand the atmosphere of the department because you must first realize that we were a hospital

department, a department dedicated to the control of lobar pneumonia. All our effort, whatever we did as bacteriologists, chemists, physicians, was focussed on that grand problem, the control of lobar pneumonia. And then there was something else in the department, the demonstration that had occurred a few years before that lobar pneumonia was not caused by a single type of pneumococcus but by different kinds, many different types of pneumococci. Each one could be clearly differentiated from the others by immunological reactions and more importantly by the possession of a substance that surrounded the bacterium, a polysaccharide, that was specific for each type of pneumococcus. One of the great achievements of Dr. Avery's department was to isolate those capsular polysaccharides that determine the specificity of pneumococci and to demonstrate the structure. As a matter of fact, this was an achievement of such importance that this year the Lasker Prize was given to Michael Heidelberger, who was then a member of Dr. Avery's staff, and who was the first one to demonstrate the chemical characteristics of the capsular polysaccharide. As a matter of fact the Lasker Award was shared this year by Dr. Austrian, who recently confirmed that by using these polysaccharides one can immunize human beings against lobar pneumonia, the immunization being very specific for each particular type. If I mention these facts it is in an attempt to make you sense how committed as a group we were to specificity, and moreover how convinced we were that the approach to the control of pneumonia was through practical application of this

concept of specificity focussed on the capsular polysaccharide. Now in 1928 there occurred in Great Britain a discovery which was terribly upsetting to all of us and especially to Dr. Avery. Namely, an officer of the British Ministry of Health, Fred Griffith, demonstrated to his satisfaction, by very crude experiments in mice, that those pneumococcus types, that all of us were convinced were so stable, in reality could be changed from one to the other in the bodies of mice. These experiments were extremely complicated technically: they were not very convincing, except that Fred Griffith was a person of such technical mastery that one had to take notice of them. Now he took notice of it. We had countless discussions in the laboratory about the possibility that specific pneumococcus types could change one into the other but Dr. Avery could not accept it. It went too much against all that he had taught for 10 or 15 years, and too much against the achievements of his laboratory, so that he did not repeat the experiments of Fred Griffith even though fully aware of them. He left for his summer vacation in 1929 going to Deer Island, Maine where he went every summer; and I stayed in the laboratory as I did in those days(and if I mention myself it's not for my participation in the problem;it is that I was witness to the experiments and participated in the experiments that confirmed Griffith's studies here on the 6th floor of the Hospital). I shared the laboratory with a

Canadian physician who took care of patients suffering from lobar pneumonia, but who also worked in the laboratory as we all did at that time. Henry Dawson was a British Canadian, and he was absolutely convinced that anything done in England had to be right; and I can assure you I am not trying to play with this fact! He decided he would try to repeat Griffith's experiments. I participated in the project with him during the summer and he actually duplicated Griffith's results. If I mention this it is to convey to you that in scientific life, there are all sorts of human elements that are not sufficiently recognized. I think it would not have been confirmed in our laboratory if Henry Dawson had not been a British Canadian. So he did repeat the experiment: and very soon he did what Griffith had not been able to do, he showed that the transformation of pneumococcus types could appear - not only in the mouse through very messy, complicated experiments - but could be done in the test tube. This was an achievement of phenomenal importance because it meant that from then on it could be studied by all sorts of techniques. Dr. Avery came back and accepted the fact. Unfortunately, he became ill and he had to take some time off to be operated on; so that for about 6 months or so he was not in the laboratory. By that time everybody in the laboratory was working on the transformation of types in the test tube. It was as I said first done by Dawson, then

duplicated and extended by others. When Dr. Avery came back then he became completely committed to the problem. Many persons have been surprised that so little was published from our department until 1943-until the paper which we are celebrating today. Let me restate in a few words what I described in many pages in the book where I have reported these events. First, all the people working in the department had a clinical responsibility, namely the control of lobar pneomonia; second, that all of us without the exception were working in one way or another toward developing sera for the treatment of pneumonia; third, that the reproduction of the phenomenon of transformation of types in the laboratory was extremely difficult; not that it could not be done, but most experiments failed until about 1940. The phenomenon was there all the time but could not be reproduced at will.

Two things happened in 1936. One that seems totally unrelated to the topic is that by 1936 the sulfa drugs had been introduced; and sulfapyridine as I recall was found especially to be very effective in the treatment of lobar pneumonia, which in a way relieved the pressure from all of us - the pressure of devoting all of our activities to the development of therapeutic sera. Then also the techniques for the transformation of pneumococcus types began to improve through the skill of Colin MacLeod who was then in the department. A great deal of work went on at that time because they were

methods that were a little more dependable. Dr. Avery at that time was completely involved in the problem even though admittedly he had no picture of what it meant from a very broad point of view, but always emphasized the importance that there was a technique whereby man could change the hereditary characteristics of a bacterium. Then fortunately Maclyn McCarty came who very soon introduced techniques whereby the transformation of types could be achieved without fail, and from then on I will let him tell the story.

Dr. Maclyn McCarty:

Thank you René.

The day to day activities of laboratory research are usually not recorded in the fashion that makes it possible years later to recapture the full flavor of a project or indeed to extract detailed information about the rationale that led to each step along the way. This is surely true of the search for the chemical identity of the pneumococcal transforming substance. Among the questions about the work which I found myself unable to deal with satisfactorily in the past is a central one: when and how did we first become convinced that the active material was almost certainly DNA? There was clearly no flash of sudden revelation, no moment at which we could shout "Eureka." I can recall a long period when the work was carried out in an atmosphere of only half-suppressed excitement as the scent of success became unmistakeable; but my recollections of this period do not include a tidy picture of the set of experimental findings that led to the excitement in the first place. The present occasion seemed like an appropriate time to revisit the old laboratory notes and devote some time and effort to determining whether it is possible to reconstruct the order of events and our interpretation of them. While I can claim to be no more than partially successful in the attempt, certain aspects of the matter were clarified by the exercise. You will even find

I am sure that some of my deductions from the history deviate a little from some of those recollections that René Dubos has just told you about.

The sequence of laboratory notes that I consulted for this purpose begins in October 1940. This may come as a surprise to those who picture the work as progressing continuously through the 1930's after the studies of Alloway in Avery's laboratory on the successful use of cell-free extracts in transformation. The second and last of Alloway's papers appeared in the February 1933 issue of the Journal of Experimental Medicine. It is true that the subject was pursued for the next few years - first by Edward S. Rogers, who was in the laboratory from 1932 to 1934, and then much more extensively by Colin MacLeod when he arrived in 1934 - but there was a hiatus of sorts in the last years of the decade. Colin had tackled many aspects of the problem in an effort to make the phenomenon more consistent and reliable so that it could be subjected to further analysis. This work involved the preparation of active extracts; the definition of susceptible R strains of pneumococcus; the nature of the serum factor required in the system; the occurrence of enzymes in the pneumococcus and other materials that destroy the transforming substance; and so on. Those studies were not published but were described in some detail in the annual spring scientific report from the laboratory in 1935 and again in 1937. However, for the next three years - in the annual scientific reports of 1938 through 1940 - the subject

was not mentioned. As a result of frustrations generated by the vicissitudes and uncertain reproducibility of these experiments, this particular research effort had apparently given way to a considerable degree to more immediately successful enterprises. These included, among others, MacLeod's work on the sulfonamides, on which he published several papers in 1939-41, and the definitive studies with Avery on another important topic, one that had had its origin in the laboratory. This dealt with the substance that appears in human blood during the acute phase of many conditions and came to be known as the C-reactive protein.

Regardless of the details of their research during this period, the sequence of events explains the fact that in the famous letter to his brother Roy, written in May 1943, Avery introduced his description of the ongoing research on transformation by saying: "For the past 2 years, first with MacLeod and now with Dr. McCarty I have been trying to find out what is the chemical nature of the substance in the bacterial extract which induces the specific change". I am suggesting that he shortened the period slightly, since it was a little over 2½ years from the resumption of this research at the time the letter was written. The important point is that he did not say "For the past 10 years". It is my belief that Avery and MacLeod had agreed in the spring of 1940 that after the summer holidays they would together return to the study of the transforming substance and devote full effort to it. The first page in the loose leaf laboratory notebook,

dated October 22, 1940, has the description of an experiment written by MacLeod but bearing a heading on the sheet in Avery's hand. The heading says: "Experiment 1 (T.P.)" [First slide, please (#1)]

I realize that some of these pictures of laboratory notes, of which I will have several, may not be legible all the way to the back of the auditorium. Because they are primarily of historical value though, I thought best to show them in their original and to convey to you as I can what the important information contained on each is. This is the heading that I referred to: "Experiment 1 (T.P.)" which is clearly written in Avery's hand. The T.P. referring to the laboratory shorthand for transforming substance meaning "transforming principle." I think this implies that, at least to me, that this was looked upon as a sort of rebeginning in this study. The experiment itself epitomizes the past and persistent difficulties, because it involves a test of the effect of fluoride in the protection of the transforming principle from destruction during the lysis of pneumococci, an effort being made to improve the production of active extracts.

The nature of the search involved in this case is deceptively simple on paper. It can be illustrated by a kind of rough diagram that was commonly used in the laboratory for informal discussions but never published. I thought to put it on the board but thought it might be more legible if I just sketched one for a slide.

[The next slide, please (#2).]

This is the type of rough sketch. In the model system selected, cell-free extracts were prepared from Type III pneumococci, an organism that produces a capsule of a high molecular weight polysaccharide, which is indicated here by the stippled area around the diplococcus. This disaccharide was at that time of known composition composed of only two sugars, equal amounts of glucuronic acid and glucose, with a disaccharide repeating unit of glucuronido-1 → 3-glucose. When added under appropriate conditions to an unencapsulated rough pneumococcus derived from Type II, in the appropriate medium for the growth of this organism and for transformation to occur, this extract will lead to the emergence of pneumococci that are encapsulated with the Type III capsular polysaccharide. Since the Type II organism originally produced a chemically and serologically quite different polysaccharide, one that has rhamnose as its major constituent, it would appear that the Type II rough strain has been induced to synthesize an entirely new product which is continued to do indefinitely on subculture. The specificity is determined by the extract. An extract from a Type I pneumococcus induces the appearance of Type I pneumococci in the transform cell, a Type VI extract will induce the appearance of a Type VI organism at this point. Thus a specific and heritable change has been induced. The aim of the research was simply to identify the substance in the extracts responsible for this striking bio-

logical effect. The search began, I believe, without preconceived notions as to the probable answer, and was thus prepared to follow any course dictated by the facts that were uncovered. The techniques available for separation and analysis of biological macromolecules were quite primitive by today's standards, and progress toward this well-defined goal was certainly not rapid.

When Avery and MacLeod returned to this task in October 1940 they prepared Type III pneumococcal transforming extracts by a slight modification of the procedure originally described by Alloway. This involved the rapid lysis of a heavy suspension of Type III pneumococci by the addition of bile salt, sodium deoxycholate. In order to minimize destruction of the transforming activity by the autolytic enzymes, ones that were present in the pneumococcus, lysis was carried out at 0° in an ice-bath; and as soon as it appeared to be complete, usually in about 15 to 30 minutes, the lysate was rapidly brought to 65° C., and held there for 30 minutes to inactivate the offending enzyme. This procedure had the merit of releasing essentially all of the soluble components of the pneumococcal cell but the distinct disadvantage of being highly variable and unpredictable in the biological activity of the product in the transforming system. This had been one of the major stumbling blocks, as noted by Dr. Dubos, in the progress of the research in the mid-1930's, and it continued to plague these renewed efforts.

[Next slide, please (#3).]

An example of this is provided by an experiment of November 5, 1940 in which the first 3 extracts prepared that fall were compared for transforming activity. Extracts 1 and 2 were preliminary runs made from 3-liter batches of pneumococci, while Extract 3 used the same procedures scaled up in size to involve organisms from 36 liters of culture. In all cases fluoride had been used in an effort to inhibit inactivation of transforming substance during lysis. These S3's or 3S's referred to the plates;plating out the transforming cultures at the end which one could identify quite readily in terms of the kind of picture you see in the reprinted article: The difference between the R pneumococcus and the transformed Type II cells. These"100%"figures relate to the fact that quite frequently one found no R strain colonies on these plates at all after transformation - despite the fact that it is quite clear that 100% of the R cells were not being transformed. But you will see that this large extract Type III was completely negative even with 1ml. of the undiluted extract used in the test. Parenthetically, it is worth noting that even the active Extracts, 1 and 2, are feeble by comparison with the activity of materials that were obtained later, as you will see; and they must have represented only a minute fraction of the transforming activity originally present in these cells.

During the remainder of the fall and early winter Avery and MacLeod persevered in trying to improve the situation,

focussing primarily on attempts to further limit the enzymatic destruction of the transforming substance. At the same time, they explored a number of different approaches to the fractionation of active extracts, the most successful and revealing of which was the application of the chloroform method of Sevag to deproteinization of the material. Repeated shaking of the extracts with chloroform and amyl alcohol reduced the amount of protein to levels no longer detectable by qualitative tests without affecting transforming activity, and this became a part of the purification process for all future extracts.

On January 28, 1941 in connection with a repeat experiment, on the lack of effect of crystalline ribonuclease on transforming activity, an important step was taken toward getting on the right track. For the first time, the Dische diphenylamine test for deoxyribose was applied to the transforming extracts. There is no indication in the notes as to who called their attention to this test, which had first been described actually a decade earlier.

[The next slide, please (#4).]

In any event, Extract 5 (1940) which was used in this ribonuclease experiment gave a positive reaction. This is the record of the Extract here. This is a controlled preparation of thymus nucleic acid. Colin wrote the following conclusion, which is at this point on the laboratory sheet: "Thus it would appear as though these transforming extracts may contain a little desoxyribonucleic acid in addition to

the large amount of ribosenucleic acid present".

It may seem strange to some that the occurrence of deoxyribonucleic acid in pneumococci, or in any other bacteria, would still be an issue as late as 1941. However, the fact on the distribution of nucleic acids in nature were not yet well defined. Indeed, we were at that point just beginning to emerge from a period when there were thought to be two general classes of nucleic acid in nature: plant nucleic acid, as typified by yeast preparations, and animal nucleic acid, as typified by thymus and fish sperm preparations. The textbooks which had served as my introduction to biochemistry a few years earlier subscribed to this view, and one of these added the following statements which serves to ullustrate the state of knowledge in the 1930's: "It is not known with certainty that the nucleic acids ever occur in the cytoplasm".

While MacLeod expressed some caution in subsequent notes about accepting the diphenylamine reaction as wholly specific for deoxyribose, these doubts seem to have been dispelled and gradually the test became to be applied with some regularity to monitor fractionation experiments.

Another important step was taken in mid-March 1941 when they gave up the Alloway method of making extracts by lysis of pneumococci. The procedure was introduced of heat-killing the pneumococci at 65° C immediately after harvesting,

followed by extracting the heat-killed cells by shaking in saline that contained deoxycholate at higher concentrations than that used in initiating lysis of living cells. This procedure clearly reduced the efficiency of solubilizing the contents of the pneumococci, but this was more than offset by the advantage of inactivating the enzyme that attacked the transforming substance prior to the extraction process. As the technique was perfected, it became possible consistently to obtain crude extracts of higher potency than any that had been made by the old procedure.

The laboratory notes suggest some interesting byplay between Avery and MacLeod in the adoption of this new procedure and even a possibility that there was a difference of opinion about the promise of this approach. While most of the notes during this period were kept by MacLeod, the first experiment on the extraction of heat-killed cells and all of the subsequent data on the preparation and its testing between the 11th and 19th of March were recorded in Avery's hand.

[Next slide (#5).]

This is the full laboratory sheet of the first page of this experiment which is headed by Avery as desoxycholate saline extraction of heat-killed Type III pneumococci. I can't read it myself. This had been a trial run on a 2-liter batch of organisms, and on March 18th a 40-liter lot was grown and the organisms were divided into two equal

portions: Avery extracted his half after heat-killing the cells, keeping the notes himself, and MacLeod handled the other half by the usual deoxycholate lysis followed by heating. The results of this experiment were rendered somewhat less conclusive than they should have been by the usual recurring difficulties with the transforming system in which the extracts were titrated. However, Avery's extract was at least as active as MacLeod's , and at the same time, had much less serologically active material by precipitin test. It must have settled the issue in any event, since one week later MacLeod prepared an extract from a 50-liter batch using the extraction of heat-killed cells, and all subsequent extracts for the next few years used this approach.

The spring and early summer of 1941 were devoted to collecting material by this procedure and using it in attempts to develop effective fractionation procedures, chiefly by means of alcohol and calcium precipitation. It has been observed since Alloway's day that pneumococcal extracts tended to be quite viscous and to yield variable amounts of stringy or fibrous precipitate on the addition of alcohol. The separation of this type of precipitate from the more voluminous flocculant precipitates produced by alcohol naturally became one object of these fractionation experiments. Here a red herring was drawn across the trail in the form of the Type III capsular polysaccharide, a major

constituent of the extracts. It became evident that the polysaccharide was separating out in the form of fibrous strands of precipitate at concentrations of alcohol below 50%, and for a period all material precipitating in this manner appears to have been equated with polysaccharide.

The results of fractionation with alcohol were rendered variable by fluctuations in the concentration of materials in the extracts, and while transforming activity tended to go along with the polysaccharide in some fractionations, there was a considerable degree of separation of the two in certain other experiments. In early July 1941, just as he was departing to assume the chair of microbiology at New York University, MacLeod summarized the current status of the preparative methods and included the final comment: "In this process of purification there has been a great loss of activity. It may be necessary to increase the concentration of Type III polysaccharide to improve the results".

While I am not certain that this comment refers to it, the idea had arisen that the presence of Type III polysaccharide in the transforming system might be necessary to serve as a template or primer for the _de novo_ synthesis of polysaccharide by the transformed pneumococci. This explains the fact that one of the first projects that I was involved in after joining the laboratory in September 1941 was to return to a problem that had been looked at much earlier by both Rogers and MacLeod. This dealt with the effect on transforming extracts of the Dubos enzyme, derived from the

soil bacillus, that specifically hydrolyzes the Type III polysaccharide, yielding as an end-product of complete hydrolysis the disaccharide repeating unit. This project served to acquaint me at first hand with the important store of knowledge of the laboratory on the pneumococcal polysaccharides as well as with the exasperating variability in the behavior of the transforming system.

Because of this variability, it was not until the end of November that I succeeded in completing an unequivocal experiment showing that the enzymatic destruction of Type III polysaccharide had no effect on the transforming activity of an extract.

[Next slide please (#6).]

This depicts the titration of the material in this particular experiment. The material was treated with the enzyme until it was no longer reactive with the Type III antisera that were available, which meant that it had to have less than .2 micrograms per ml of active polysaccharide. Then they were dialyzed to remove split products and tested in the transforming system, the same kind of a setup as before except this happens to have been done in duplicate. This first section is the enzyme treated material which was free now of Type III polysaccharide. This was the same with the heated enzyme as a control and no enzyme, these two retaining their full serological activity with the anti-polysaccharide. And as you can see there was no loss of transforming activity as a result of elimination of the polysac-

charide. As a result of this reconfirmation that the polysaccharide is not needed in transformation, it seemed desirable to devise preparative methods that would provide us with purified extracts devoid of the polysaccharide. The first step was to reduce the amount of polysaccharide initially present in the crude extract so the removal of the residium from preparative lots would not overwhelm our limited supplies of the SIII enzyme. One possible way to achieve this was to eliminate the customary procedure of adding excess glucose to the last two hours of cultivation of the Type III pneumococci. This was done in order to enhance growth and the yield of organisms for extraction. It. was effective for this purpose but also had the effect of greatly enhancing the production of the capsular polysaccharide.

In an experiment stimulated by the results with the SIII enzyme and carried out just a few days before Pearl Harbor, a 50-liter batch of the organisms was grown without the addition of extra glucose and the cells extracted as usual after heat-killing.

[Next slide please (#7).]

This is the titration of that extract which was done in the early part of December. Not only did the extract have less polysaccharide than previous lots, but it turned out by chance to be the most potent extract that had yet been obtained, clearly encouraging further efforts in this direction. As you can see in the sera dilution of the ex-

tract it was active at a level of three-tenths of an ml of 1 to 10,000 dilution of the extract in permitting transformation. This protocol also shows, down at the bottom, (it probably will not be legible to you) that I experienced my own personal Pearl Harbor by breaking the flask and losing all of this potent preparation!

Subsequent experiments revealed that it was advantageous to wash the heat-killed cells repeatedly in saline before extracting the deoxycholate, thus further reducing not only the polysaccharide but also the ribonucleic acid content of the preparation without comparable loss of transforming activity. By the early months of 1942 we were thus able to make preparations that could be rendered free of protein, as far as our ability to detect, as indicated by the biuret test, and can also be rendered essentially free of the Type III polysaccharide with the use of the Dubos enzyme. It then became obvious that these preparations still yielded viscous solutions and contained material that formed fibrous precipitates on the addition of alcohol, establishing that the polysaccharide was not solely responsible for these characteristics.

Some of our experiments indicated that the fibrous alcohol precipitates carried most of the diphenylamine reactive material and also most of the transforming activity of the extract. Separations of this kind were not always as sharp as could be desired. It took a while to learn the

optimum conditions for this precipitation, since the results depended upon a variety of factors, such as the concentration of the material in solution and the manner in which the alcohol was added. One of the earliest successful experiments of this type, carried out on January 27, 1942, indicates that the fractionation of transforming activity by this procedure could be quite sharp.

[Next slide please (#8).]

This is such a fractionation, in which fraction A represents the fibrous precipitate at one volume of alcohol and fraction B is the material that one got by adding additional alcohol and, as you can see in this particular experiment, the activity was highly localized in the first fraction and there were only the scattered positive tubes in the lower dilutions in the other material.

Our interpretation of these findings was assisted by the availability at about this time of authentic preparations of mammalian DNA from Alfred Mirsky, who had his laboratory two floors above us in the Hospital. He had provided us with material isolated from thymus, spleen and sperm by his elegant procedure based on the differential solubility of nucleohistone in salt solutions. These DNA preparations were supplied as fibrous alcohol precipitates that had been dried with ether, and when they were dissolved in saline gave highly viscous solutions, and many of their properties were purely related to the kind of material we were obtaining from the pneumococcus. This

similarity between the active transforming material precipitated by alcohol in fibrous form from our preparations was indeed striking.

Thus by the spring of 1942 our attention had clearly become focused on the possibility that DNA was the substance responsible for transforming activity. At that point we obtained experimental evidence of a quite different sort that provided independent support for this view. Alexandre Rothen was then working with an ultracentrifuge, the equipment filling a sizable room in the basement of what is now called Flexner Hall, with most of the space being taken up by the schlieren optical system. He undertook to examine some of our material analytically using a sector-shaped cell that was divided into two compartments by a perforated partition about two-thirds of the way down the cell.

In the first experiment the material was spun at 50,000 r.p.m. until the fastest moving visible boundary was well down in the lower compartment. The material was then removed from the two compartments separately, precipitated in alcohol for sterilization, reconstituted in saline and titered for transforming activity.

[Next slide please (#9).]

This represents that first experiment. The material in this slide is the material in the upper portion (the upper two-thirds of that sectored cell). This is the

material in the lower third. This is the control on centrifuge material. The results I think are quite clear in indicating that the transforming substance was concentrated in the lower chamber. Although we were dealing with small volumes of material, it was possible to carry out a few qualitative chemical tests showing that this was also true of the DNA. This is the diphenylamine test showing the upper was negative and the lower segment of the material was positive in the diphenylamine test. This evidence that the active material was of high molecular weight was confirmed when several additional analytical runs in which the visible boundary was moved to different levels of the cell.

We then proceeded to use the concentration head of the ultracentrifuge on a pool of transforming extracts that had been prepared in late 1941 and stored in the frozen and dried state. Centrifugation at 30,000 r.p.m. for 4 to 6 hours sufficed to concentrate the transforming activity in the lower 1 ml of the 6 ml in the tubes.

[Next slide please (#10).]

This is simply a titration of one of those runs. The upper material of one tube is here, the lower of that tube here. This is the upper half (on this one page) of another tube; and as you can see the occasional scattered positive results were found in the upper part of the tube, but the 95 or more percent of the material was concentrated in the lower segment.

Indeed at 6 hours centrifugation there was a well defined pellet at the bottom of the tube, gelatinous and translucent in appearance, that could be easily separated from the supernatant fluid. The pellet proved to contain 95-99% of the transforming activity; and on chemical and serological analysis the only known constituent of the extract that was concentrated in the pellet was the diphenylamine reactive material and thus presumably the DNA.

By the summer of 1942 there was thus more than one kind of evidence implicating DNA as the substance responsible for transforming activity. These results stimulated another look at the various enzyme sources that had been tested for their effect on the transforming substance.

[Lights please.]

These included in addition to certain crystalline enzymes, crude preparations of bone, kidney and intestinal phosphatase, a purified preparation of pancreatic lipase, pneumococcal autolysates, and mammalian sera. They were compared under various conditions for their effect on transforming activity and their ability to depolymerize authentic DNA. Here again the results were unequivocal, and only those preparations that clearly acted on DNA were able to inactivate the transforming substance. While only a correlation, the findings provided enough support for what now seemed a most likely possibility to lead us on to

a final major step - that of attempting to prepare highly purified preparations of pneumococcal DNA.

The plan adopted was to first perfect our purification process and then to prepare several lots of purified material that could be subjected to detailed analysis. The latter was to include elementary chemical analysis, semi-quantitative assay by chemical and immunological techniques to detect contaminating substances, enzymatic analysis, ultracentrifugal and electrophoretic studies, and of course quantitative titration of transforming activity. The purification procedure devised was based on the experience gained in preceding years and concluded with several reprecipitations of the material by spinning the fibers on a glass stirring rod during the addition of the minimal amount of alcohol required.

The first of these purified products was available for analysis in October 1942 and in the ensuing months three other preparations, each from organisms from 200-300 liters of culture, were completed and studied in detail. By the time of Avery's letter to his brother in May 1943 most of the work had been done, and the "new batch" to which he refers to get "further evidence of purity and homogeneity by use of ultracentrifuge and electrophoresis" was already in its early stages of preparation. This was the final lot prepared prior to writing up the work. By comparison with products isolated from mammalian sources, our preparations appeared to be good quality DNA, highly active in transformation, and all of the analytical data were consistent with

the conclusion that the primary constituent of the material was responsible for the biological activity.

During this period I am afraid that both Colin and I became increasingly impatient with Avery's caution, even though we were not unaware of the importance of being sure of our ground. We were just young enough to become convinced more readily. Avery expressed his doubts repeatedly in the letter to Roy and they were also obvious on almost a daily basis in the laboratory. The three of us made our pilgrimage to The Rockefeller Institute Laboratories at Princeton at about this time - my unverifiable recollection is that it was in April 1943 - to consult John Northrop and Wendell Stanley. Both of these workers had experienced skeptical responses to their reports attributing biological activity to a crystalline product: Northrop with pepsin and Stanley with tobacco mosaic virus. They of course were not able to provide us with a magic formula for eliminating the possibility that a contaminating substance was responsible for the transforming activity of purified pneumococcal DNA, and their advice was in essence - "you just have to do the best you can."

It was on the train on the way back from Princeton that Colin said to Avery: "What else do you want, Fess? What more evidence do you need?" To the best of my recollection, Avery gave no specific answer, but I think that the "else" he would have liked to have had was a purified DNase to try

on the transforming DNA. This is in keeping with his reliance on other occasions on information obtained by applying enzymatic tools to biological problems. The very existence of the Dubos SIII enzyme, which played so important a role in final purification of the transforming DNA, depended on his earlier conviction that such an enzyme would be of great value in establishing that the type-specific antigens of pneumococcus were indeed polysaccharides rather than some contaminating substance. He had been exposed to this kind of skepticism once before. I think that he knew that we could not wait to publish our results until we had a suitable DNase, since my efforts to prepare the enzyme were just beginning at this time.

I have found on occasion that it is assumed by some, that data on inactivation of the transforming substance by a well-characterized pancreatic DNase, was included in the 1944 paper. It was actually two years later when this information appeared in print, and it was thus somewhat anticlimactic. It did, however, serve as a coup-de-grace to the notion, to which a few had clung, that a trace of protein in the DNA preparations accounted for their biological activity. In addition, work on the enzyme provided indirect evidence of another sort, through the effect that knowledge of the properties of DNase had on the method of preparation of transforming DNA. Pancreatic DNase was found to require magnesium or manganese ion for its activity, and it was then shown that the DNase present in pneumococci also depended on divalent cations.

It thus became possible to return to the procedure of Alloway and to use lysis of living pneumococci for the efficient production of increased yields of transforming DNA by the simple expedient of including chelators of the activating ions in the lytic system. All of the difficulties with this procedure over the years were solved by this one modification, based on learning something of the inactivating enzyme.

I will close by going back for a moment to the actual writing of the first paper, after the collection of data was completed. This began in the summer of 1943, with Avery working on drafts of the introduction and discussion at his customary summer retreat on Deer Isle, Maine, and I collecting protocols and preparing a draft of the experimental part in New York. In the fall we reserved a small, quiet room in the library where we repaired for many long hours to revise and polish the manuscript. Avery applied to our efforts the same stringent criteria for which he was justly so famous in what was called "Fessing" the manuscripts of others. It was a great relief to me when we got the completed version for the Journal of Experimental Medicine, which you have a reprint of in your hands, into the hands of Dr. Rous on November 1st.

Thank you.

Slide 1. Exp. 1 (T.P.)

Difficulty in reading slides of lab notes
Historical intent - use anyway and
try to explain what they show.

Slide 2 Freehand example of what could be put
on blackboard

Slide 3 Titration of Exts 1/40, 2/40 and 3/40.
Explain R and SIII

Slide 4 1st use of diphenylamine test

Slide 5 Extraction of heat-killed cells

Slide 6 SIII Enzyme Exp.
Dialyzed

Slide 7 Prep. from cells s̄ added glucose
Call attention to titer

Slide 8 Alcohol fractionation

Slide 9 Analytical ultracentrifuge

Slide 10 Concentration by ultracentrifuge

Exp. 1 (T.P.)

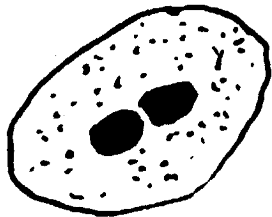
A/66 Lysis Fluoride

Oct 23/40 Effect of Fluoride on Autolysis of Pneumococcus Type III and on preservation of the Transforming Principle.

3 litres of 8 hour culture of A/66 in P.B. $^{\circ}$ 7.54 Taken up in 30 cc Saline. Very heavy [illegible]
15 cc Heated immediately at 65° for 30'
Remainder (about 20 cc) put in an ice bath until ready for manipulations.

Bacteriolytic Enzyme   Prepared Oct 20th - 21st 300 cc 8 hour culture (R[illegible]) taken up in 10.0 cc and permitted to autolyse for 16 hours at 37° all cellular detritus. However, after 24 hours in ice box heavy suspension on blood plate showed fairly numerous R colonies, and organisms grew out in broth. Kept in ice box Oct 20th to 22nd

Type III S

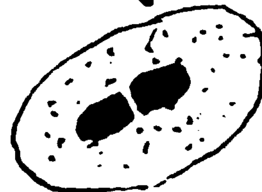


Extracted

Type III
cell-free
extract

+

Type II R



Type III S

Transformed pneumococcus

[illegible] /40.

Titration of activity of Extracts 1/40, 2/40, 3/40.

Extracts 1 & 2 have previously shown high potency. Extract 3 inert.

System: CF Elder. heated at 60° for 15'. 0.5 cc.
Broth. Lac 757.
R/36. – 10^{-5}, 1 drop.

Tube		Extract	Am't Ex	Transformations
1	a	1/40	0.5	III S 100%
	b		0.4	III S 100%
	c		0.3	III S 100%
	d		0.2	R
	e		0.1	R
2	a	2/40	0.5	III S 100%
	b		0.4	III S 100%
	c		0.3	III S 100%
	d		0.2	III S 100%
	e		0.1	R
3	a	3/40	1.0	R
	b		0.5	R
	c		0.1	R
	d		0.05	R
	e		0.01	R

[illegible] Diphenylamine Reagent (desoxypentose) Blue Color
two cc. of " c̄ one of test material

3.	Ex 5/40	+±
4.	Ex 5/40 digested c̄ Ribonuclease in dialysis sac	+
5.	Uranium ppt. of Ex 5/40 digested c̄ R.N.	trace
6.	" Sup. " " "	trace
7.	Yeast nucleic acid 1 mg/cc	—
8.	Thymus " 1 mg/cc (approx)	++++
9.	Type III SSS 1:1000	—
10.	Ribonuclease 0.5 mg/cc	—
11.	Reagents alone (borate buffer)	—

Thus it would appear as though these transforming extracts may contain a little desoxyribosenucleic acid in addition to the large amount of ribosenucleic acid present.

The solutions A, B, C - were dialyzed overnight in the ice box against tap H_2O

Desoxy Cholate — [illegible] [illegible] [illegible]
Type [illegible]

Mar 11 41 5

2 Liters [illegible] [illegible] Type [illegible] [illegible] [illegible] [illegible] 3' — [illegible] first heated [illegible] [illegible] [illegible] [illegible] 20 [illegible] [illegible] [illegible] [illegible] [illegible] 25 cc

[illegible] [illegible] heated 15 [illegible] [illegible] [illegible] [illegible] 0.75 cc 10% [illegible] was added — the [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] 7.2

To the mixture [illegible] [illegible] was added [illegible] [illegible] 1.5 Chloroform — [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] Unfortunately no observation of [illegible] [illegible] [illegible]

After shaking [illegible] [illegible] in continuous [illegible] [illegible] [illegible]

3/12 — Removed [illegible] [illegible] — [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible]

3/13 [illegible] — [illegible] [illegible] [illegible] [illegible] the [illegible] [illegible] of [illegible] —

[illegible] [illegible] up in 10 cc [illegible] — [illegible] into solution completely — immediately — slight yellowish [illegible] — [illegible] — [illegible] [illegible] Reppted in [illegible] [illegible] — [illegible] 1 hr [illegible] [illegible]. [illegible] up in 10 cc [illegible] [illegible] + [illegible]. [illegible] 0.6 + 0.3 [illegible] [illegible] [illegible] [illegible] [illegible] — [illegible] [illegible] [illegible].

Transforming [illegible].

[illegible] 1 [illegible] CA [illegible] CF Bellevue [illegible] 0.5 cc 1.30 [illegible] [illegible] 3'60° — [illegible] [illegible] + R 36 A

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Tube		Extract	Amt.	Transformation
1.	a	Abs. Heated Cell.	0.5	—
	b	Desoxy Abs.	0.3	
	c		0.1	Chocolate [illegible] not satisfactory
2	a	[illegible] [illegible]	0.5	—
	b	ext. of [illegible]	0.3	
	c		0.1	
3	a	Ext. [illegible]	0.5	S, S[illegible]
	b	(1+[illegible])	0.3	R
	c		0.1	R
Control		—	—	R

Repeated 3/14 — [illegible] [illegible] of the [illegible] — [illegible] 0.3 cc Cochran CF (3/10/40) [illegible] [illegible] instead of Bellevue CF as above.

Transformation Tests

Neo #37 + 10% Broth
R36A.. - 10^{-4} 0.05cc inoculum.

Duplicate tubes throughout

Tube		Material		Amt cc.	Turbidity Set 1	Turbidity Set 2	Transformation Set 1	Transformation Set 2
1.	a.	Tube A Active enzyme	Undiluted	0.5	+	+	S III - 80%	S III - 80%
	b			0.3	+	+	S III - 80%	S III - 80%
	c.			0.1	+	+	S III - 80%	S III - 90%
2.	a		1:10	0.5	+	+	S III - 80%	S III - 80%
	b			0.3	+	+	S III - 80%	S III - 80%
	c			0.1	+	+	S III - 80%	S III - 80%
3.	a	Tube B Heated enzyme	Undiluted	0.5	+	+	S III - 50%	S III - 90%
	b			0.3	+	+	S III - 50%	S III - 90%
	c			0.1	+	+	S III - 50%	S III - 70%
4.	a		1:10	0.5	+	+	S III - 50%	S III - 80%
	b.			0.3	+	+	S III - 80%	S III - 60%
	c			0.1	−	−	S III - 2 col.	R
5.	a.	Tube C No enzyme	Undiluted	0.5	+	+	S III - 60%	S III - 50%
	b			0.3	++ (Contam.)	+	R (contam.)	S III - 60%
	c			0.1	+	+	S III - 50%	S III - 60%
6	a.		1:10	0.5	+	+	S III - 80%	S III - 80%
	b			0.3	+	+	S III - 70%	S III - 80%
	c.			0.1	−	+	R	S III - 60%

Comment -

Transforming activity is not reduced appreciably by treatment with the S III B enzyme which removes practically all of the serologically detectable SSS III.

8/4 Ppt. from 20cc. Taken up in 2.0cc M/40 PO_4 buffer. Dilutions made in M/40 buffer.
Broth - Neo 37 + 10% Bile - prepared 8/27
Inoculum - R36A12 - 10^{-4} 0.05cc.
Tubes washed c̄ soap in prep. room.

Tube		Material		Amount cc.	Turbidity	Transformation
1.	a.	Ext.# 24	1:10	0.5	+	S III - 50%
	b			0.3	+	S III - 50%
	c.			0.1	+	S III - 50%
2.	a.		1:100	0.5	+	S III - 40%
	b			0.3	+	S III - 40%
	c.			0.1	+	S III - 40%
3	a		1:1000	0.5	+	S III - 60%
	b			0.3	+	S III - 40%
	c.			0.1	+	S III - 40%
4	a		1:10,000	0.5	+	S III - 50%
	b			0.3	+	S III - 30%
	c			0.1	−	R

Despite the small amount of material obtained, the titer on the basis of equal volumes is greater than that of any previous extract. Its activity is about that of Extract # 20 which was prepared from 66 liters instead of 5 l.

12/16/41 Bulk of this extract lost by breaking of flask. Remaining amount taken up in 10cc distilled H_2O - Frozen & dried -
(See over for test on dried material)

Extract #26 - Attempted Purification (continued)

4/27/42 - Fraction A. precipitate gelatinous and supernatant 5% alcohol cloudy. Due to lack of electrolyte. 1.0 gm. of sodium acetate added with formation of more flocculent and more stringy ppt. Refrigerated 2 hours and centrifuged. Precipitate taken up in 5cc saline + 5cc m/20 PO4 buffer pH 7.6

Fraction B. Flocculent ppt. recovered by centrifuging. Taken up in 5cc saline + 5cc m/20 PO4 buffer pH 7.6

Transforming Tests on Fractions

CA Neo #46 + 10% Broth

Inoculum R36A 10^-4 0.05cc

Tube		Material	Dilution	Amt cc.	Turbidity	Plates
1	a	Fraction A	1:10	0.5	+	S III
	b			0.3	+	S III
	c			0.1	+	S III
2	a	[Ppt after 1.2 volumes alcohol. Taken up in 10cc]	1:100	0.5	+	S III
	b			0.3	+	S III
	c			0.1	+	S III
3	a		1:1000	0.5	+	S III
	b			0.3	+	S III
	c			0.1	+	S III
4	a		1:10,000	0.5	+	S III
	b			0.3	- ?	S III
	c			0.1	-	R
5	a	Fraction B.	1:10	0.5	+	S III
	b			0.3	+	S III
	c			0.1	-	R
6	a	[Ppt @ 4 vol. alcohol. Taken up in 10cc]	1:100	0.5	+	S III
	b			0.3	-	R
	c			0.1	-	R
7	a		1:1000	0.5	-	R
	b			0.3	-	R
	c			0.1	-	R

Transformation Test

Alcohol precipitates from each chamber and the control, dissolved in 1.0cc saline

Dilutions prepared in saline

C.A. Neo #81 + 20% Broth

Inoculum · R36A.s 10⁻⁴ 0.05cc

Duplicate tubes

Tube		Material	Dil^n	Amt cc	Series I Diffuse growth	Series I Plate	Series II Diffuse growth	Series II Plate
1	a.	Material from upper chamber. Taken up in 1.0cc saline	1:10	0.5	−	R	−	R
	b.			0.3	+	S	+	S
	c			0.1	−	R	−	R
2	a.		1:100	0.5	+	S	? −	R
	b.			0.3	−	R	−	R
	c.			0.1	−	R	−	R
3	a.		1:1000	0.5	−	R	−	R
	b			0.3	−	R	−	R
	c.			0.1	−	R	−	R
4	a.	Material from lower chamber. Taken up in 1.0cc saline	1:10	0.5	+	S	+	S
	b.			0.3	+	S	+	S
	c			0.1	+	S	+	S
5	a.		1:100	0.5	+	S	+	S
	b			0.3	+	S	+	S
	c.			0.1	+	S	+	S
6.	a.		1:1000	0.5	+	[illegible]	+	S
	b.			0.3	+	[illegible]	+	S
	c			0.1	+	S	+	S
7	a.	Control extract. 0 bac. · taken up in 1.0cc.	1:10	0.5	+	S	+	S
	b			0.3	+	S	+	S
	c.			0.1	+	S	+	S
8	a.		1:100	0.5	+	S	+	S
	b			0.3	+	S	+	S
	c			0.1	+	S	+	S
9	a.		1:1000	0.5	+	S	+	S
	b			0.3	+	S	+	S
	c			0.1	+	S	+	S

Chemical Test

Undiluted material used (i.e. remainder of 1.0cc after dilutions were prepared)

Bial · 0.2cc + 0.4cc reagent – boil 2 min.

Diphenyl · 0.2cc + 0.4cc reagent boil 10 min. Read after 2 hours

	UPPER	LOWER	CONTROL	SALINE
Bial Test	+	++	+++	0
Diphenylamine	0	++	++	0

... Hypothetical & concentration band I (cont)

Concentration Test of 6 hour material

The material removed from the four tubes which had 6 hours of centrifugation is tested first because the gelatinous pellet in tubes VII – VIII suggests the possibility that the activity may be packed on the bottom.

TP.56 + 27 material
CA. Hoo #122 + 20% Broth - 2.0cc - each tube
Inoculum R86A2 - 0.05cc / 10^{-4} dil

Material taken up in saline (each fraction made up to 6.0cc)
Dilutions in saline

Tube		Material	Dil.	Amt. cc	Diffuse growth	Plate
1.	a.	Tube I	1:10	0.5	–	R
	b.			0.3	–	R
	c.			0.1	–	R
2.	a.	(9)	1:100	0.5	–	R
	b.			0.3	+ (slow)	S III
	c.	Upper 5.2cc		0.1	–	R
3.	a.	(separated mechanically)	1:1000	0.5	–	R
	b.			0.3	–	R
	c.	Brought up to 6.0cc c saline		0.1	–	R
4.	a.	for test	1:10000	0.5	–	R
	b.			0.3	–	R
	c.			0.1	–	R
5.	a.	Tube II	1:10	0.5	+	S III
	b.			0.3	+	S III
	c.			0.1	+	S III
6.	a.	(10)	1:100	0.5	+	S III
	b.			0.3	+	S III
	c.	Lower 0.8cc		0.1	+	S III
7.	a.		1:1000	0.5	+	S III
	b.			0.3	+	S II
	c.	Taken up in 6.0cc saline		0.1	–	R
8.	a.	for test	1:10,000	0.5	–	R
	b.			0.3	–	R
	c.			0.1	–	R
9.	a.	Tube III	1:10	0.5	–	R
	b.			0.3	+	S III
	c.			0.1	+	S III
10.	a.	(11)	1:100	0.5	–	R
	b.			0.3	–	R
	c.	Upper 5.0cc		0.1	–	R
11.	a.	(separated by pipetting smoothly)	1:1000	0.5	–	R
	b.			0.3	–	R
	c.	Taken up in 6.0cc saline		0.1	–	R
12.	a.	for test.	1:10,000	0.5	–	R
	b.			0.3	–	R
	c.			0.1	–	R

Summary for combination of protocol