STUDIES ON PNEUMOCOCCUS VARIATION

I. VARIANTS CHARACTERIZED BY RAPID LYSIS AND ABSENCE OF NORMAL GROWTH UNDER THE ROUTINE METHOD OF CULTIVATION

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INTRODUCTION

The pneumococcus variants to be described in this paper were first observed during a series of studies on the smooth to rough transformation. These new variants are characterized by a tendency to undergo a rapid spontaneous lysis under certain cultural conditions at 37°, and some of them grow much more slowly than do the ordinary strains of pneumococcus. This characteristic behavior is best detected by growing these variants on blood agar at 37°. Under these conditions the effects of the lytic process and speed of growth on the colony form are most evident. With some strains the lytic process commences a few hours after cell division has started, the result being that the pneumococcus cells are completely lysed and apparently non-viable before the colonies attain a size visible under the colony microscope, and there is no appreciable growth at twenty-four hours. In other strains the lytic process begins later so that colonies varying in size from 0.1 mm. to 1 mm. are produced during a growth period of between eight and twenty-four hours, and these colonies subsequently undergo a rapid lysis thus acquiring a transparent phantom-like appearance at the end of twenty-four hours.

For the sake of brevity the letters P-C will be used to designate all of the pneumococcus variants which show this tendency to lysis and consequently form small phantom colonies or fail to grow appreciably on blood agar at 37°.

The P-C variants not only have been produced by artificial methods in the laboratory but have also been isolated directly from cases of pneumococcus infection in man. They appear to result from a dissociative process other than that which produces the ordinary rough to smooth transformation, both virulent and avirulent variants exhibiting lytic properties. However, there are probably important connections between these two dissociative processes. The resemblance of the colonies of the P-C variants, under certain conditions, to G colonies of Hadley (1931) is rather striking. This aspect of dissociative phenomena will be discussed at the end of this paper.

Phantom colony variants of the anthrax bacillus which produced numerous atypical forms after twenty-four hours incubation were described by Nungester (1929). It seems likely that certain of the lytic variants of the Shiga dysentery bacillus described by Twort (1920), Arkwright (1921) and others were not cultures containing a bacteriophage but were closely similar to the type of variant to be described in this paper. Hadley (1927) mentions certain lytic variants which are apparently not due to the direct action of the bacteriophage to which he applies the term suicide cultures. Numerous other examples of what appears to be exaggerated autolysis may be found in the literature.

Dawson (1928) has reported small and lytic colony forms of the pneumococcus among what he considered to be intermediate variants. More recently this same author (Dawson, 1933) has reported a new type of rough variant of the pneumococcus which exhibits extreme pleomorphism. Rakieten (1930) obtained from a virulent type II culture a variant which grew better at 20°C. than at 37°C., and also a variant showing very atypical morphology and forming microscopic colonies. Blake and Trask (1933) also report a small colony form intermediate between rough and smooth.

The present work is concerned with methods of producing the P-C variants, with the effect of the characteristic lytic process on their colony form and cell morphology, and with the effects of simple chemical and physical changes such as acidity, oxygen

tension, carbon dioxide tension, and temperature in hastening or preventing the lytic process. The latter part of the paper is concerned with methods of causing the P-C variants to revert to normal strains, and with studies on the antigenic composition and virulence of the P-C variants.

The effect of temperature on the lytic process must be mentioned at this time because it has an important bearing on the methods of isolation, cultivation, and artificial production of the P-C variants. Lowering the temperature inhibits the lysis of the P-C variants so that although they grow scantily or not at all at 37° these P-C variants give a type of growth indistinguishable from that of normal strains when cultivated on blood agar at 25°C. for three days. This observation made it possible, simply by cultivation at 25°, to carry the variants in a relatively stable form in stock culture, to isolate phantom colony forms from cases of human infection, and to devise an effective method for the artificial production of P-C variants.

METHODS

The media used in the study of the P-C variants were as follows:

(1) Five per cent horse blood infusion agar.
(2) One per cent glucose infusion agar.
(3) Infusion broth containing one drop of rabbit whole blood to each 5 cc.
(4) One per cent glucose infusion broth.

In cultivating P-C variants and studying the effects of temperature, two incubators, one maintaining a temperature of 25°C., and one a temperature of 37°C., were used. In all cases unless otherwise specifically noted the pH of the media was 7.6 and the conditions of O_2 and CO_2 tensions those of the laboratory air.

The process of lysis and the accompanying changes in morphology in the P-C variants were observed by growing the organisms on blood or serum agar on a cover slip inverted over a hollow ground microscope slide, and watching the development of the colonies under the microscope.

Studies of the effect of oxygen and carbon dioxide tensions

were conducted by placing the cultures in closed jars and pumping out the air to the desired tension, then replacing with nitrogen, or carbon dioxide, or both. The pH was varied by adding lactic acid or sodium hydroxide to the media and checking on a colorimetric scale.

The cultures used in the study of antigenic properties and virulence were grown in rabbit-blood broth at 37°, or on blood agar at 25° and then emulsified in broth. Autolysates were made by adding 0.5 per cent of phenol to the forty-eight hour blood broth cultures and allowing them to stand for three days. The autolysates were then boiled successively at pH 8.5 and 4.0 to remove antigenic constituents which might interfere with the precipitin titration for specific soluble substance. The pH was then brought back to 7.0, the precipitated protein centrifuged off, and the supernatant used for the precipitin tests.

PRODUCTION OF P-C VARIANTS BY DISSOCIATION OF STOCK CULTURES AT 37° AND 25°

P-C variants of the pneumococcus exhibiting the characteristic lytic phenomenon were discovered during attempts to convert an unstable type I, rough strain into a smooth by growth in a special potato extract medium. After incubation for twenty-four hours in the potato medium, the R culture gave, on plating to blood agar, a mixture of colony types.

Selection and subculture of some of the colonies yielded a strain which showed distinct lysis in twelve hours on blood agar at 37°. This strain was moderately virulent for mice, being recovered unchanged from the heart blood.

Similar P-C variants were obtained using the following methods to produce the dissociation.

1. From type I S and R strains by growth in human whole blood for ten to twenty generations at 37°.

2. From Type I S by growth in 10 per cent type I antiserum broth for five generations at 37°.

3. From type I S by growth on an agar medium containing 1 per cent peptone, 1 per cent laked horse red corpuscles, and 0.5 per cent glucose at 37° for three generations.

4. From the type I S stock culture by a dissociation of unknown cause which occurred during the routine procedure used to maintain virulence, e.g.: daily transplants in rabbit-blood broth and mouse passage once a week. The P-C variants later disappeared from the stock culture.

5. From types I, II and several strains of group IV by the daughter colony dissociation which occurs during cultivation on blood agar at 25°.

In all cases the P-C variants were isolated by subcultures on 5 per cent horse-blood agar and colony selection.

The fifth method mentioned above, namely, the daughter colony dissociation at 25° was found to be the most satisfactory method for the artificial production of P-C variants from smooth cultures. The culture is plated on blood agar so that well separated colonies are formed, and is then allowed to grow at 25° for three to eight days. At varying times during this period raised patches or papillae appear on the flattened colonies. The appearance of these papillae indicates the beginning of dissociation and if the colony is subcultured at this time or later a few dissociant colony forms are obtained, all or part of which may be P-C variants. Farago (1932), describes a similar dissociation of the pneumococcus at 37° but this author did not succeed in isolating any dissociants.

MORPHOLOGY OF THE COLONIES OF P-C VARIANTS WHEN CULTIVATED ON BLOOD AGAR AT 25°C.

1. Colony characteristics of type I

As indicated in the introduction, most of the P-C variants will produce colonies 1.0 mm. to 3.0 mm. in diameter which are similar to those of normal smooth strains when grown on the blood agar at 25° for three to five days. When cultures which have formed daughter colonies are subcultured on blood agar at 25° several dissociant colony types are obtained. Among these the three that occur with greatest frequency are as follows: (1) Large, smooth, shining, convex opaque; (2) large, smooth, dull, flat, translucent; and (3) small, smooth, slow growing, shining convex.

Of these three colony forms at 25° one, two, or all three may exhibit the lytic properties characteristic of P-C variants when cultivated at 37°. Studies on the dissociation of several strains of type I obtained from different sources showed that the lytic properties are not all equally marked in dissociants from different strains.

The production of dissociants giving the translucent and opaque colony types at 25° takes place with great regularity in the daughter colony dissociation of practically all pneumococcus cultures. Similar translucent and opaque colony forms have been observed in normal smooth strains when grown at 37°. In all cases these properties are permanent and probably represent a third type of variation in addition to the P-C variation and the S to R variation.

For the purposes of the present discussion no important differences exist between the P-C variants which give convex opaque colonies at 25° and those which give flat translucent colonies at 25°. Both forms show all of the typical P-C characteristics. The fact that the appearance of the lytic properties characterizing the P-C variation coincides with the appearance of the opaque and translucent variants undoubtedly has some, as yet undetermined, significance. Further work on the translucent and opaque colony forms is now in progress.

The small colony P-C variants (third colony form listed above) which are obtained less regularly than the larger colony forms seem to differ most from the normal smooth form. They show marked departure from the latter in cultural characteristics, grow slowly, and are usually of low virulence. When kept on blood agar at room temperature they are very stable.

2. Colony characteristics of types II, III and IV P-C variants

The P-C variants obtained from type II by daughter colony dissociation at 25° are in general very similar to those obtained from type I except that slow-growing small-colony variants occur relatively more frequently in the type II dissociant cultures. Variants corresponding closely to those described by Rakieten[1] (1930) were obtained in some cases.

[1] See Introduction.

No P-C variants could be obtained from the stock type III cultures. However, a typical P-C strain of type III giving no growth or phantom colonies on blood agar at 37° and normal smooth transparent colonies at 25° has been isolated from the sputum of a case of type III lobar pneumonia.

DIRECT ISOLATION OF PNEUMOCOCCUS P-C STRAINS FROM CASES OF HUMAN INFECTION BY CULTIVATION AT 25°

It has just been indicated that P-C variants of the pneumococcus have been obtained not only from stock laboratory cultures but also directly from material derived from pneumococcus infection in man. Samples of sputum from cases of lobar pneumonia were washed and plated directly on blood agar, and incubated at 25°. After two or three days colonies that appeared to be pneumococcus were transferred to fresh blood-agar and one set of these was kept at 25°, the other at 37°. Blood cultures were taken in the usual way in flasks containing 100 cc. of broth and incubated for twenty-four hours at 37°. The culture was then plated on blood agar at 25° and 37°. The usual methods of identifying the organisms were used and all were tested for virulence by mouse injection.

The results from eleven cases are presented in table 1. One of the blood cultures, D, was taken from a case of pneumococcus meningitis, the rest of the samples were obtained from pneumonia patients. The identification of the P-C strains was made simply by noting the character of growth at 37° as shown in the last column of the table.

Of the eleven strains isolated, six showed definite lytic properties on blood agar at 37° as may be seen in the last column; and five grew normally without lysis at 37° as indicated at the bottom of the table. All of the strains gave large colonies of normal appearance at 25° as shown in the next to the last column. The number of cases so far studied is not large but these results serve to show that P-C variants occur in about 50 per cent of cases.

All of the P-C strains isolated from human sources were virulent for mice and all were recovered as P-C strains from the heart blood of the mice after death.

Besides the strains reported in the table, type I phantom colony variants were isolated at autopsy from a case of lobar pneumonia which had been shown by previous typing to be caused by a type I organism. Plating of the material from the pneumonic lung on blood agar at 37° gave no growth, but at 25° a scant growth which included three distinct colony types was obtained.

TABLE 1

Lytic characteristics of pneumococcus strains isolated from human cases of pneumococcus infection by cultivation at 25°C.

SOURCE	TYPE	CHARACTER OF GROWTH ON BLOOD AGAR AT 25°C. IN 3 DAYS	CHAPACTER OF GROWTH ON BLOOD AGAR AT 37°C. IN 36 HOURS
Blood A	IV	++ Large colonies, no lysis	− Complete lysis
Blood D	XII	++ Large colonies, no lysis	− Complete lysis
Blood G	?	++ Large colonies, no lysis	− Complete lysis
Sputum 1	V	++ Large colonies, no lysis	+ Colonies with irregular edges. Slight lysis
Sputum 4	?	++ Large colonies, no lysis	− Complete lysis
Sputum 5	III	++ Large colonies, no lysis	± Phantom colonies, marked lysis
Blood C	?	The last five strains here tabulated all gave a vigorous massive growth with no signs of lysis both at 25° and 37°	
Blood E	?		
Blood F	II		
Blood H	II		
Sputum 2	?		

− = no appreciable growth.
± = 50 to 200 colonies.
\+ = over 200 distinct colonies
++ = massive growth.

Two of these grew normally at 25° but not at 37°. The third strain was extraordinary, in that it showed distinct lysis and formed phantom colonies even at 25°.

A few other such variants which show the lytic phenomena at 25° have been isolated but because of the methods used in isolation of the P-C variants, most of those so far studied grow

normally at 25°. Many of the freshly isolated cultures probably contain at least a few elements that grow normally at 37° and continued cultivation at this temperature selects the organisms which grow best and show the least tendency to lysis; thus, finally, a culture growing normally at 37° is produced.

MORPHOLOGICAL CHANGES PRODUCED BY THE ACTION OF THE LYTIC PROCESS ON THE CELLS OF THE P-C VARIANTS

Morphological changes in the P-C variants grown on blood or serum agar have been observed by means of "klatch" preparations and by growing the organisms on agar in a hollow ground microscope slide and watching the development of the colonies, as described in the section on methods. Growth occurs normally until a few chains or a small colony is produced. Then a swelling of certain of the members of the chains occurs and this proceeds until part or all of the colony consists of swollen organisms. The swelling is followed by rupture and lysis of the cells so that finally a mass of gram-negative detritus is formed. Usually a nucleus of intact organisms, or at least one or two cells, will remain. These will develop to a normal colony if the culture is left at 25°, or may, after a long delay, grow up at incubator temperature to form a shining daughter colony on the dull surface of the original lysed colony.

The size and nature of the colonies formed depend on the speed of growth, the age of the culture at which the lytic process begins, and the rapidity with which it proceeds. Sometimes the organisms swell and break up soon after the culture is placed in the incubator. With other strains growth proceeds along with the process of lysis so that colonies showing extreme pleomorphism with balloon forms, bacillary forms, granular material, and only a few normal organisms result. These changes also occur to a less extent in some of the P-C strains when grown at 25°. In aerobic broth cultures grown at 37° a similar pleomorphism often occurs.

This process might be attributed either to autolysis or the action of the bacteriophage were it not for the fact, as shown later, that the lysis may be completely prevented by growing the cul-

ture under reduced oxygen tension or in the presence of carbon dioxide. There is no evidence that the granular material in the lysed colonies is viable. These colonies are often transplantable, but, when this is found to be the case, the presence of intact viable organisms cannot be ruled out. No attempts at filtration of these lysed cultures have been made.

COMPARISON OF P-C VARIANTS AND NORMAL STRAINS WITH REGARD TO BIOCHEMICAL CHARACTERISTICS

1. Growth on agar media under varying conditions of hydrogen ion concentration, carbon dioxide tension, and oxygen tension

The only cultural condition so far discussed that affects the lytic process in the P-C variants is temperature, but, as previously mentioned, pH, oxygen tension, and carbon dioxide tension have a marked effect on the lytic phenomenon and consequently determine whether a given P-C strain grows normally, forms small phantom colonies, or does not grow at all.

The effect of these factors and a comparison of the amount and type of growth obtained with normal and P-C strains under cultivation on blood agar at 37°, and glucose agar at 37° is presented in table 2. In the first column is noted the presence or absence of growth of normal and P-C strains at 37°C. under the standard conditions on blood agar of pH, 7.6 atmosphere tensions of oxygen (150 mm. of mercury) and carbon dioxide (less than 0.5 mm. of mercury). It will be seen that under these conditions only one of the P-C variants grows at all and this gives phantom colonies. Raising the tension of carbon dioxide to 40 mm. produces, as shown in the second column, a normal, vigorous growth of all the P-C variants except one. Tensions of carbon dioxide as low as 2 mm. of mercury will produce a considerable inhibition of lysis and consequent stimulation of growth of the P-C variants. For this reason the carbon dioxide produced by normal cultures growing in a closed space with the P-C variants is often sufficient to stimulate the growth of the latter and produce colonies of normal appearance. Valley and Rettger (1926, 1927) found that carbon dioxide in small amounts stimulates the

growth of a great many bacterial species on solid media and these observations have been confirmed by Walker (1932) for liquid media. In most cases the removal of carbon dioxide seems merely to prolong the lag phase, but, as has already been pointed out, the P-C variants of the pneumococcus when grown in the pres-

TABLE 2

Effect of oxygen, carbon dioxide and pH on growth and lysis, of P-C variants and normal strains on agar media at 37°C.

	BLOOD AGAR AT 37°C.								GLUCOSE AGAR AT 37°C.			
pH →	7.6	7.6	7.6	7.6	7.6	7.1	6.9	6.4	7.6	7.6	7.6	7.6
CO_2 tension → mm. Hg.	<0.5	40	<0.1	<0.1	<0.2	<0.5	<0.5	<0.5	0.5	140	<0.5	30
O_2 tension → mm. Hg.	150	110	0.5	8	40	150	150	150	150	110	5	5
Normal I s	++	++	−	++	++	++	++	++	±	−	++	+
I s 71	±P-C	++	−	++	−	−	−	−	−	±	−	+
I s 72	−	++	−	−	−	−	−	−	−	−	−	++
I s a 112	−	++	−	±P-C	−	−	−	−	−	−	−	−
I s a 134	−	++				−	−	−				
II s Normal	++	++		++	++	++	++	++	±	±	++	++
II s 21	−	++	−	−	−	−	+P-C	+	−	±	−	± or −
II s 241	−	−	±P-C	±	−	−	+P-C	−	−	−	−	−
IV s P-C	−	++	−	+P-C	−	−	+	−	−		−	±P-C

++ = Normal growth, no lysis.
± = Scant growth.
− = No growth, complete lysis.
P-C = Phantom colonies showing definite lysis.

Origin and description of P-C strains as follows: I s 71, stock culture, virulent, rapid growing 25°; I s 72, stock culture, avirulent, slow growing 25°; I sa 112, direct from human autopsy, grows normally 25°; I sa 134, direct from human autopsy, phantom colonies at 25°; II s 21, stock culture, virulent, rapid growing 25°; II s 241, stock culture, avirulent, slow growing 25°; IV s P-C, direct from human blood grows normally 25°.

ence of the amounts of carbon dioxide ordinarily found in the atmosphere do not show any prolonged lag but undergo lysis and cease to grow after cell division has actually started. It was found that if the carbon dioxide is removed, by means of soda lime, as rapidly as it is formed no strain of pneumococcus will grow either at 25° or 37°.

The effect of reducing the oxygen tension on growth of the P-C variants on blood agar at 37° is shown in columns 3, 4, and 5 of table 2. One P-C strain grows normally at 8 mm. partial pressure of oxygen and less than 0.5 mm. of carbon dioxide, and in several others the lysis is somewhat inhibited so that instead of no visible growth, phantom colonies are produced. With the exception of one type II variant both normal and P-C strains fail to growth appreciably under conditions approaching a complete removal of oxygen as may be seen in the third column.

Although lowering the pH, as is indicated by the data recorded in the sixth, seventh and eighth columns of table 2, does produce some increase in the growth of the P-C variants, especially those of type II, the stimulation is much less than that produced by quantities of carbon dioxide too small to affect the pH of the medium appreciably. With large amounts of carbon dioxide the effect may be in part due to pH but in general the carbon dioxide seems to have a specific stimulating action on the growth of the P-C variants.

A comparison of the normal and P-C strains on glucose agar at 37° under atmospheric conditions, and with low oxygen tension, increased carbon dioxide tension, and both increased carbon dioxide and lowered oxygen is given in the last four columns of table 2.

None of the P-C variants will grow on 1 per cent glucose infusion agar at normal oxygen tension and 37°. The normal smooth cultures of types I and II do, however, grow moderately well on aerobic glucose agar, and the growth is greatly improved by lowering the oxygen tension to 5 mm.

It will be noted that carbon dioxide produces a slight improvement in the growth of several of the P-C variants on glucose agar but this improvement is not nearly as striking as that produced by a corresponding increase in carbon dioxide on the growth of P-C variants on blood agar. Lowering the oxygen tension does not by itself produce any improvement of the growth of the P-C variants on glucose agar but on blood agar a definite improvement in growth due to this change in cultural conditions was observed with three strains.

2. Effects of varying the constituents of blood agar media

The effect of varying the amount of blood cells and serum in infusion agar has been studied. The addition of more serum to the medium slightly improves the growth of some of the P-C variants. On a medium containing 1 per cent of laked cells but no serum a vigorous growth of the normal strains occurs. Many P-C strains on the other hand grow poorly or not at all on this medium at 37°. The use of hormone blood agar in place of infusion blood agar makes no difference in the growth of the P-C variants. Growth on chocolate agar, or Mueller's meatless medium, which contains 1 per cent peptone and 10 per cent of horse blood, is generally somewhat poorer than on blood agar.

3. Cultural conditions in broth media. Effect of pH and oxygen tension

Certain of the P-C variants, especially those that give visible colonies on blood agar at 37°, will grow normally in blood broth or horse-serum broth at 37° and pH 7.6, the reaction generally used in cultivation of the pneumococcus. On the other hand, a great many strains have been produced artificially and also isolated from cases of pneumococcus infection that grow slowly in broth or not at all under these conditions of temperature and pH. These also fail to grow on blood agar at 37°. Thus, if routine methods of isolation were employed their cultivation would be very difficult, or impossible. Studies of growth in broth at various degrees of acidity show that the P-C variants have a pH growth range different from the normal forms. The results of these experiments are summarized in table 3. None of the P-C variants will grow on blood broth above pH 7.8 but all normal-growing strains grow just as well at pH 8.2 as they do at 7.6. The acid death point seems to be about the same in all cases.

Sealing the tubes of blood broth with vaseline before incubation makes no difference in the growth of the P-C variants but with glucose broth sealing the tubes makes a great difference in the amount of growth obtained. None of the P-C variants will grow aerobically on old glucose broth but some give a very slight growth on freshly prepared or recently boiled aerobic broth after

forty-eight hours at 37°. Lowering the pH to 7.0 also improves the growth of some of the strains.

Most of the P-C variants grow well in recently boiled 1 per cent glucose broth sealed with vaseline; but a longer period of incubation at 37° is required than is necessary with normal strains. Under these conditions the pH growth-range is similar to that on blood broth but is somewhat narrower as may be seen in the last two columns of table 3.

TABLE 3

Growth pH range in aerobic and vaseline sealed blood broth and glucose broth

STRAIN*	pH RANGE OF GROWTH AT 48 HOURS			
	Blood broth aerobic	Blood broth sealed	Glucose broth aerobic	Glucose broth sealed
I S normal	8.4–6.1	8.4–6.1	8.4–7.0	8.4–6.6
I S 71	7.8–6.1	7.8–6.1	7.0 irregular	7.8–6.8
I S 72	7.2–6.1	7.2–6.1	No growth	7.8–7.4
I Sa 111	6.8–6.1	6.8–6.1	No growth	
II S normal	8.4–6.1	8.4–6.1	8.4–6.6	8.4–6.6
II S 21	7.6–6.1	7.6–6.1	7.4–7.0 irregular	7.8–6.6
II S 241	No growth	No growth	No growth	No growth

* Origin and description of PC strains as in table 2.

I S 71, stock culture, virulent, rapid growing 25°.

I S 72, stock culture avirulent slow growing 25°.

I Sa 111, avirulent dissociant of strain from human autopsy, grows normally 25°.

II S 21, stock culture, virulent, rapid growing 25°.

II S 241, stock culture avirulent, slow growing 25°.

REVERSION OF THE P-C VARIANTS TO NORMAL FORMS

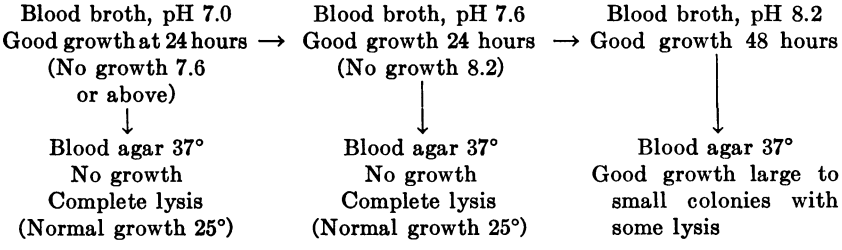
The P-C variants show great differences in stability. Strains that give some growth on blood agar at 37° may be considered to contain a few normal-growing elements and these may be changed back to normal-growing cultures after several generations at incubator temperature and selection of the largest colonies. If the culture contains many normal forms along with the P-C, the normal forms stimulate the P-C variants by the carbon dioxide they produce, when grown on solid media at 37° in a closed space. In liquid media at 37° the normal forms if originally present in

the culture tend to overgrow the P-C variants, especially in broth above pH of 7.6.

P-C variants that grow at 37° in blood broth at pH 7.6 but not on blood agar may be carried unchanged in blood broth for many generations before any sign of reversion appears. If broth of pH 7.0 is used the rate of reversion is still slower. In general the more alkaline the medium the sooner the appearance of normal growing forms in the culture.

With some strains a rapid reversion to normal can be brought about by growing the P-C variant first in blood broth at pH 7.0 and then transferring a drop of this broth culture at twenty-four hours to blood broth at pH 8.2. Growth may be delayed in the alkaline blood broth but when it occurs many normal forms are obtained on plating to blood agar. The more stable P-C strains cannot be caused to revert in this manner in two generations but the same thing may be accomplished by several transfers in broth of gradually increasing pH until growth in the alkaline broth occurs.

An outline of this procedure is shown in the diagram given below.



The various colony forms which grow out of the reverted strains seem to represent definite stages in the reversion process and in certain cases stable strains each representing one of these stages have been isolated. The colony forms on blood agar at 37° observed in these various stages of reversion are:

(1) Small phantom colonies; marked lysis.
(2) Medium irregular colonies; slight lysis.
(3) Large normal growing colonies; no lysis.

The first two types revert rather easily to the forms which give

no appreciable growth on blood agar at 37°. The last type represents the stable, vigorously-growing form ordinarily found in stock cultures.

Type II P-C strains are exceptional in that they revert to normal growing forms on mouse passage and also in blood broth even in a single generation without change in pH. This abrupt reversion has not been noted in the case of pneumococcus P-C strains of types I, III or IV.

Another type of reversion occurred in the type III P-C variant isolated from pneumonia sputum (see table 1). After several generations on blood agar at 25° this strain began to form opaque patches in the transparent colonies. These opaque patches were found to contain a dissociant which grew normally at 37°, and was very similar to the stock culture of type III. The same type of reversion in normal-growing forms (as observed in the case of type III, by daughter colony dissociation at 25°) was also found to occur in another strain of unknown type isolated from pneumonia sputum.

In general the reverted strains are similar in virulence and antigenic composition to the P-C strains from which they are derived. It is evident, therefore, that the change from normal to P-C and back again to normal may be accomplished without any appreciable alterations in the other characteristics of the pneumococcus. As with the transformation of normal to P-C so also with the reversion of P-C to normal, methods which involve prolonged cultivation, aging, or dissociation of the cultures will produce changes in virulence and antigenic composition in addition to causing the appearance or disappearance of lytic properties.

COMPARISON OF P-C AND NORMAL STRAINS WITH REGARD TO VIRULENCE AND ANTIGENIC COMPOSITION

As stated in the previous section, stimuli which cause S to R dissociation, such as prolonged cultivation without animal passage, or growth in antiserum, will cause a reduction in virulence or the appearance of R forms in the P-C variants just as they do in the normal-growing forms. P-C variants showing all degrees of virulence have been isolated, so that it is evidently meaningless

to speak of the virulence or avirulence of P-C variants as a general class.

P-C variants derived by the daughter colony dissociation from a smooth strain behave exactly like the normal smooth forms in that their virulence may be raised by mouse passage or lowered by prolonged cultivation on blood agar. Rough P-C variants can-

TABLE 4

Virulence and antigenic composition of P-C variants

STRAIN*	MAXIMUM DILUTION WHICH KILLS MICE 0.2 CC. 24 HOUR BLOOD BROTH CULTURES	TIME OF DEATH OF MICE (AVERAGE) WITH MAXIMUM DILUTIONS	MAXIMUM DILUTION† OF AUTOLYSATE WHICH GIVES PRECIPITATE WITH HOMOLOGOUS ANTISERUM	TYPE SPECIFICITY AS DETERMINED BY AGGLUTINATION
I s normal	10^{-6}	1–2 days	1:160	Type I
I s 71	10^{-6}	1–2 days	1:120	Type I
I s 712	Undiluted	1 day	1:160	Type I
I s 72	—	Survived	1:160	Type I
I s 732	—	Survived		None, R
II s normal	10^{-6}	1 day	Not done	Type II
II s 122	10^{-4}	6–12 days	Not done	Type II Slight agglutination I and III
II 241	—	Survived	0	None, R
III s normal	10^{-6}	1 day	1:100	Type III
III s P-C	10^{-6}	1 day	1:100	Type III

* All strains designated by numbers are P-C variants.

I s 71, 72, II s 241 are the same strains as those shown in table 3.

I s 712; a dissociant of I s 71.

I s 732; a rough P-C variant from the strain I s 71.

II s 122; a smooth dissociant from stock type II culture.

† The autolysates were boiled successively at pH 3.5 and 8.5 and the coagulum removed (see section on methods). The precipitates obtained with the antisera were considered to be due to the specific soluble substance.

not be obtained directly from normal growing rough cultures and must therefore be produced from the smooth P-C strains.

In table 4 are presented the results of comparative studies on the virulence and antigenic composition of normal-growing smooth strains and P-C variants of various degrees of virulence for mice. The first column gives the results of virulence titra-

tions in mice using 0.2 cc. of various dilutions of twenty-four-hour blood broth cultures. Studies on the antigenic composition were confined to tests for the specific soluble substance by means of precipitin titrations on broth culture autolysates (column 2), prepared as described in the section on methods, and by means of agglutination tests on broth suspensions of pneumococcus with homologous and heterologous sera (column 3).

These results show that the P-C variants can equal the normal-growing strains in virulence. Some of the P-C strains show a decreased virulence with a retention of the other smooth characteristics. This diminished virulence is evident in two ways; first, by a decreased titre for mice, as with strain I S 712; second, by an increase in the length of the time required to kill mice as is strikingly shown with the strain II S 122.

From the results presented in the last two columns of table 4 it may be seen that the virulent, and certain of the avirulent, P-C variants of type I form as much specific soluble substance as do the normal-growing forms. Strain I S 712 has all of the typical smooth characteristics except high virulence for mice and strain I S 72 is smooth by antigenic tests but avirulent by the mouse test.

The virulent P-C variant of type III mentioned in table 4 was found by precipitin tests with the autolysate to form the same amount of specific carbohydrate as the normal-growing strain. No avirulent P-C variants of type III have as yet been isolated. Further studies on the relations between the antigenic composition, the virulence, and the colony form of normal and P-C variants are now in progress.

DISCUSSION

Definite evidence has been presented in this paper that the P-C variation as characterized by the tendency of the P-C strains to lyse at 37°C., represents a change that is entirely independent of the alterations in antigenic composition which occur in the smooth-to-rough transformation. Additional examples of independent variations in the properties of pneumococcus cultures have been observed during the course of the work and will

be reported later. Nungester (1933) has recently called attention to this independent variation of the properties in other bacterial species. On the basis of the evidence at hand we believe that the conception of a single change from rough to smooth or smooth to rough with intermediate forms is too limited and is not in accord with experimental observations on the variations brought about by daughter colony dissociation. This method as employed in the course of this work did not produce a smooth to rough transformation.

The P-C variants when grown under certain conditions resemble both in colony form and morphology the G forms of Hadley (1931), who considered these G forms to represent a filterable stage in a life cycle. Attempts to show whether or not the lysed P-C variants represent a filterable stage in a life cycle by filtration experiments or serial plate washings, as described by Hadley, have not been made. In the case of the P-C variants a further study of the nature of the lytic process itself is in our opinion more important than attempts at filtration. As shown in the present work the colony form and morphology of the P-C variants is due to the operation of a lytic process which may be inhibited by appropriate alterations in temperature, pH, and oxygen and carbon dioxide tensions; under the changed environmental conditions the P-C strains produce colonies identical with those of normally growing pneumococcus strains. A further elucidation of the chemical nature of lytic processes should lead to a definite conclusion as to whether they represent changes from non-filterable to filterable forms in a life cycle, or an irreversible destructive chemical process similar to the autolysis of dead cells.

A much more detailed knowledge of the metabolism of living cells will be required before any conclusion may be drawn as to the chemical nature of the lytic processes which occur in the P-C variants of the pneumococcus on blood agar at 37°. The effect of carbon dioxide in preventing the lysis is very striking but as yet no hint as to its mode of action has been obtained. The fact that the P-C variants grow well at 25° but not at 37° indicates that some destructive chemical reaction in the P-C cells is speeded up by raising the temperature, and this reaction is inhibited by carbon dioxide and to a less extent by lowering the pH.

In some cases the reduction of oxygen tension or the production of more strongly reducing conditions in the medium inhibits the lytic process. This latter observation indicates that the lytic process is connected with an oxidative mechanism in the cell. No transmissible lytic agent has been demonstrated in the filtrates of P-C variant cultures. The lytic action of the bacteriophage is not readily affected by changes in temperature, pH, and CO_2 tension as is the lytic process in P-C variants.

P-C variants have been frequently isolated from blood, sputa, and the lungs of pneumonia patients. This is not surprising in view of the fact that conditions in the body are nearly optimal for the production and growth of these variants. In the living body the low oxygen tension, high CO_2 tension and pH near 7.3 favor the appearance of the P-C variation whereas the high oxygen tension, minimal CO_2 tension, and pH of 7.6 or above occurring under conditions of artificial cultivation lead to the disappearance of the P-C variants and the maintenance of those organisms which exhibit the cultural characteristics of the typical pneumococcus. At present nothing is known concerning the significance of the demonstration of P-C variants in cases of pneumonia. It is possible that they may be derived in the body from the so-called normal strains and therefore be of minor importance. On the other hand they may represent the primary infective agent in pneumonia. The unusual cultural properties of the P-C variants and the attendant difficulties in isolating and cultivating them by routine methods might lead to errors and misconceptions in the experimental study of pneumococcus infection. For this reason a further study of the rôle of P-C variants in disease is indicated.

SUMMARY

Methods are described for the isolation from human sources and the artificial production of stable strains of the pneumococcus which undergo rapid lysis or fail to grow under the ordinary conditions at 37°. Strains showing such characteristics have been termed phantom colony variants or P-C variants.

The effects of cultivation at 25°, carbon dioxide, pH, and oxygen tension on the growth and lysis of P-C variants, are described.

The P-C variant strains are compared with normal forms as regards growth requirements, virulence and antigenic composition.

Evidence is adduced that the P-C variation is a change independent of the ordinary smooth-to-rough variation.

Methods are described for causing reversion of the phantom colony variants to normal-growing smooth forms by cultivation in alkaline media, and under certain other conditions.

The direct isolation of the phantom colony variants from cases of human infection indicates further study of their possible rôle in disease.

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