VARIATION AND TYPE SPECIFICITY IN THE BACTERIAL SPECIES HEMOPHILUS INFLUENZAE

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During the course of a study of Pfeiffer bacilli, strains were grown on special transparent agar and it was observed that when one particular strain was grown in this way two kinds of colonies developed. The colonies of one kind were like those usually described as typical for this organism while the colonies of the other kind were opaque and were very iridescent when viewed by oblique transmitted light. It was also observed that the organisms forming the colonies of one kind differed in morphology from the organisms forming colonies of the other kind (1). Further study indicated that the phenomenon was undoubtedly an example of variation or bacterial dissociation. During recent years much new and important knowledge concerning variations in bacteria of other species has been obtained (2) and it has seemed important to study in greater detail, in the light of this new knowledge, the variations occurring among the so-called influenza bacilli, or Pfeiffer bacilli.

The bacteria of this group do not form a well characterized bacterial species, and it has long been recognized that individual strains differ from one another in morphology and virulence, in the appearance of the colonies which they form, in their ability to form indole, in power to ferment sugar and to induce hemolysis, in their immunological reactions, and even in their requirements for growth in artificial cultures. The literature relating to the biological characteristics of the bacteria forming this heterogenous group has been recently reviewed by Jordan (3) and Scott (4). In 1920 the Committee on Nomenclature of the American Association of Bacteriologists (5) proposed the name *Hemophilus influenzae* for this group of organisms. Soon after this, more accurate studies of the growth requirements of the organisms of this group were made (6, 7, 8). In the present paper the term *Hemophilus influenzae* is employed to include only those organisms

which are non-hemolytic and which require for growth both of the accessory factors X and V.

The difficulty of identifying and classifying strains of this group is further increased by the fact that many individual strains, when grown under artificial conditions, may show temporary or permanent variations in certain characters. The colony which is generally considered typical for *Hemophilus influenzae* is small, round, discrete, translucent, and finely granular on the surface. Certain writers have, however, described colonies which differ in appearance from those described as typical, and in a few instances the writers have found this atypical colony formation associated with variations in morphology of the individul bacteria and with certain modifications of other biological characters.

A number of writers have stated that the colonies formed by strains derived from cases of meningitis, septicemia, or arthritis are more opaque than those described as typical, (Cohen (9), Henry (10), Taylor (11), Grekowitz (12), and others). Scott (4) has stated that the colonies formed by certain strains derived from the healthy nasopharynx, or from cases of meningitis or arthritis are not only slightly more opaque than the typical colonies but that they "are often distinguishable by a bluish iridescence in oblique light." He stated that the bacilli of these strains are of "abnormal size." He also noticed that these properties were not fixed but, that after growth on artificial media, these strains "may be indistinguishable from some, at least, of the strains of respiratory origin."

Kristensen (13) has mentioned the formation of "coarse" colonies and he states that the coarse appearance of the colonies is undoubtedly associated with a coarse morphology of the individual bacteria, particularly with the occurrence of thread forms.

Certain writers have noted other unusual properties of strains from pathological sources. For instance, Wollstein (14) has observed that the bacteria of strains from the cerebrospinal fluid of cases of meningitis autolyze more readily than the bacteria of strains from the respiratory tract. A number of writers (15, 16, 17, 18, 19, 12) have mentioned that influenza bacilli from pathological sources are pleomorphic. Wollstein (20), however, noted that in young cultures of freshly isolated pathological strains most of the bacteria are short and uniform rods. If growth continues in these cultures for some time the uniformity becomes less evident, and after these strains have been subcultured for a long time even the organisms of the young cultures are pleomorphic (14). Cohen, Ritchie (21), and Henry have also observed that in young cultures of meningitis strains the bacteria appear as uniform rods while in older cultures they are pleomorphic.

Cohen, Henry, Ritchie, Parker and Parker (22), Nabarro and Stallman (18), Taylor, and Wollstein have observed that strains isolated from pathologic sources are usually more pathogenic for animals than are the usual respiratory strains, and some investigators (22, 23, 24) have observed that during subculture these strains lose their pathogenicity. Wollstein, Povitsky and Denny (25), Rivers and Kohn (26) have found a considerable degree of immunological relationship between the strains isolated from cases of meningitis.

In the present study, 155 strains of hemoglobinophilic bacilli were isolated from comparatively widely distributed sources. Only 97 of these strains, however, were non-hemolytic and required both X and V factors for growth. Of these 97 strains 82 have never been observed to form the atypical opaque colonies previously mentioned. Whether under suitable conditions these strains would also produce atypical colonies cannot be stated at present. Fifteen of the strains, however, at the time they were isolated, when grown on transparent agar plates produced opaque iridescent colonies. The sources[1] of these fifteen strains were as follows:

Seven strains were isolated in pure culture from the spinal fluids of meningitis patients. One of these patients suffered from arthritis preceding the onset of the meningitic symptoms. In this case influenza bacilli grew in cultures from the joint as well as from the blood, and these strains were identical with the strain from the meninges, identical not only in their serological reactions but also in biochemical reactions and cultural characteristics. Two strains were obtained in pure culture from the blood of cases of pneumonia. One strain was isolated in pure culture from the purulent exudate in a case of empyema associated with pneumonia. Two strains were isolated from the sputum of patients suffering from atypical pneumonia. In the sputum of both of these patients pneumococci of Group IV were also present; the influenza bacilli, however, predominated. Two strains were isolated from the throats of patients suffering from pharyngitis. In the cultures from the throats of both of these patients at least 90 per cent of the colonies were of influenza bacilli. In these cultures typical influenza bacilli colonies were present as well as opaque iridescent ones. One strain was isolated from the nose of a monkey suffering from tuberculosis.

For determining the appearance of colonies, all strains were grown on Levinthal's transparent medium (27).

In the preparation of this medium the authors' directions were slightly modified in order to avoid the need of sterilization by filtration. 4 per cent sterile nutrient agar was employed, and just before pouring plates, the agar was melted, partially cooled, and mixed with an equal volume of Levinthal's broth (prepared by boiling 10 per cent blood broth for 5 minutes, filtering through paper and then through a Berkefeld candle).

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[1] Certain of these strains have been received from Dr. Martha Wollstein of the Babies Hospital, New York City; Dr. Anne G. Kuttner of the Pediatric Clinic of Johns Hopkins Hospital; Dr. J. D. Trask, Jr., of the Department of Pediatrics of Yale University, and Dr. D. W. Weiss of the Department of Pediatrics of Washington University, St. Louis, to whom the author is gratefully indebted.

When cultures of these 15 strains were grown on plates prepared with this medium, the colonies were large, sometimes 3 mm. in diameter. The surfaces of the colonies were smooth and mucoid and the edges were continuous. If the colonies were close together, they tended to coalesce. They were slightly opaque and in strong light, obliquely transmitted, they were markedly iridescent. This iridescence is quite distinct from the bluish luster sometimes exhibited by other colonies of *Hemophilus influenzae.*

It was frequently observed that as these strains were subcultured there appeared among these opaque colonies other colonies which resembled the "typical" *Hemophilus influenzae* colony. These new colonies which appeared among the iridescent colonies were usually smaller and more discrete; they were translucent and non-iridescent in transmitted light. The surface varied in roughness; some were only slightly granular with the margins slightly indented, others were deeply wrinkled with the contours broken by deep serrations. When cultures were made from these rough colonies, it was found that all the colonies which developed were granular like the mother colony. It was obvious, therefore, that these cultures were variants of the original strains from the patients. Some of the conditions under which this variation or dissociation takes place will be described later.

The phenomenon which has been observed in the case of these organisms so closely resembles the formation of variants of Pneumococcus (28) and other organisms that it has seemed justifiable to adopt a similar terminology and to speak of the 15 strains just described, which form smooth colonies, as S strains, and the strains derived from them forming granular colonies, as R strains. The smooth iridescent colony will hereafter be spoken of as an S colony, and the rough translucent colony will be called an R colony.

The differences in size and opaqueness between colonies of the two kinds are illustrated in Fig. 1. But the most distinguishing characteristic of the S colony, the iridescence, is not shown in the photograph. So marked are these differences that when colonies of different kinds coalesce the S colonies appear "moth-eaten" and the presence of a bacteriophage is suggested. It should be emphasized, however, that these differences only appear striking in the case of cultures grown on Levinthal transparent agar plates. On blood or chocolate agar the

presence of R colonies can only be detected with very great difficulty. Moreover, the differences between the colonies of the two kinds are striking only in young cultures. If growth has continued in the incubator over 24 hours, or if the plates have been allowed to remain at room temperature for several days, the S colonies become as translucent as the R colonies. Even on the old plates, however, the surface of the S colonies remains smooth, provided sufficient moisture is present to prevent drying. When touched with a platinum wire, the S colonies are soft and yielding, while the R colonies are firmer and more tenacious. The bacteria from the S colonies can be readily and uniformly suspended in salt solution, those from the R colonies are suspended in salt solution with greater difficulty and they tend to agglutinate spontaneously.

Morphology of the Bacteria of the S and R Strains

In stained preparations made from a young R colony, the bacteria are of various sizes and lengths, short rods and also long thread forms being seen. In preparations made from a young S colony, on the other hand, practically all of the bacteria appear as short rods, almost uniform in size. In older plate cultures, the bacteria from the S colonies also become pleomorphic and long threads and bizarre forms are seen. This tendency of the S bacteria to become pleomorphic is, however, more marked in broth cultures than in plate cultures. What seems to be of special significance is the fact that the S bacteria, when carefully stained and studied, are found to be surrounded by capsules which, though usually less thick than those of pneumococci, are, nevertheless, perfectly distinct and definite. Welch's (29) and Muir's (30) stains have been found most useful in demonstrating them. The morphology of S and R bacteria is shown in Figs. 2 to 5. That hemoglobinophilic bacteria from cases of meningitis are encapsulated was recently suggested by Grekowitz (12). He observed that the stained bacteria were surrounded with a halo. The bacteria of the S colonies also undergo autolysis more readily than do those of the R colonies. This may explain the irregular staining and the occurrence of shadow forms in old S cultures.

Pathogenicity of S and R Strains

As is well known the bacteria of the *Hemophilus influenzae* group, when inoculated into animals, do not have a marked tendency to invade the blood and tissues. Nevertheless, the inoculations are frequently followed by marked toxic reactions, and death not infrequently results, even though at autopsy the cultures may be sterile. Individual animals also differ markedly in their susceptibility to the action of these organisms and their products, so that it is difficult to determine the minimal lethal dose with great accuracy. In comparing the pathogenicity of different strains, therefore, it is not sufficient to determine the least amounts of the cultures that will cause the death of animals, or even to compare the intervals of time elapsing between the injection of given amounts and the fatal outcome. Other circumstances are also significant. When the injections are made into the peritoneum it has been found to be important to determine whether, at the time of death, living organisms are still present, or whether they have disappeared; and, if they are still present, to observe the morphological changes which they have undergone; also to note the character of the cellular reactions in the exudate, and to establish whether or not invasion of the blood has occurred. In comparing the virulence of S and R strains, observations of all of these circumstances have been made, and the results of many experiments are given in a condensed form in Table I.

In all the experiments 20 hour broth cultures have been employed. By this method the dosage could be more accurately determined than by using suspensions of organisms grown on plates. The medium used was Levinthal's broth (prepared with 2½ per cent blood) and this was introduced into Erlenmeyer flasks in small amounts, so as to give an extensive surface exposure to the air. Rabbits, rats, and mice were used for the experiments. The injections were made intraperitoneally in the case of the mice and rats, and intravenously and intracutaneously in the rabbits.

When fatal doses of influenza bacilli are injected into animals, they usually appear very sick within a few minutes, the breathing becomes labored, they refuse to eat, and frequently diarrhea occurs. Within 2 hours the eyes become watery. In many cases especially in mice the conjunctival exudate later becomes purulent. Death usually occurs within 24 hours. At autopsy there is found hyperemia

and frequently hemorrhage in the tissues. These are especially marked in the case of rabbits, in the thymus, lungs, and mesentery. Microscopic examination of the lungs reveals the presence of edema and hemorrhage.

In comparing cultures of S and R strains, it has been found that in all the animals studied the minimal lethal dose of the S culture has been uniformly smaller than that of the corresponding R culture. Indeed

TABLE I

Results of Inoculations of S and R Strains

Animal	Strain	Route of inoculation	M.L.D.	Organisms in culture from		Stained preparations of peritoneal exudate		
				Peritoneum	Heart's blood	Bacteria	Globoid forms	Leucocytes
	culture		*cc.*					
Mouse	S	Intraperitoneal	0.1–0.5	Many	Many	Many	Rare	Few
"	R	"	1.0–2.0 or avirulent	Few	Few or none	Few	Many	Many
Rat	S	"	0.25–1.0	Many	Many	Many	Rare	Rare
"	R	"	2.0–3.0 or avirulent	Few	Few or none	Few	Many	Many
Rabbit	S	Intravenous	0.5–1.0		Many			
"	R	"	2.0–3.0 or avirulent		Few or none			
	filtrate							
"	S	"	1.0–3.0		—			
"	R	"	3.0–5.0 or avirulent		—			

many R strains have been found to be completely avirulent. In animals dying after intraperitoneal injections, it has been found that, if the strains injected were of the S variety, organisms are still present in the exudate, while if R organisms were injected, few or no bacteria remain. A marked difference between the bacteria of the two kinds as relates to their invasion of the blood has also been noted. If the cultures injected were of the S variety, many organisms grow from the cultures of the blood at autopsy, while if R organisms were injected, the cultures from the blood at autopsy show a very scanty growth, or

they are sterile. When stained preparations of the peritoneal exudate are made, it is found that when R strains have been injected the bacteria which are present frequently do not appear as typical influenza bacilli, but are swollen, at times appearing as almost round, globoid, irregularly staining masses (Fig. 7). These bodies resemble the swollen forms of *Vibrio cholerae* which Pfeiffer (31) described as occurring in the peritoneal exudate of immunized guinea pigs. Similar changes in the bacteria are sometimes found in the exudates of animals inoculated with S strains, but in this case the changes are not of so extreme a degree nor have so many of the bacteria undergone this modification in form (Fig. 6). The exudate in an animal inoculated with an R strain is usually scant and contains many leucocytes, while that from an animal inoculated with an S strain is more viscous and few leucocytes are present. There is also observed a difference in the persistence of the bacteria in the blood, when injections of strains of the two kinds are made intravenously. In the case of organisms of the R variety, few bacteria are present in the blood at autopsy, or they may have entirely disappeared.

It has also been found that when injections of the strains of the two kinds are made into the skin of rabbits, there are even more marked differences in the effects produced than when the inoculations are made intraperitoneally or intravenously. This method of study has the additional advantage in that several inoculations may be made at one time into a single animal, and the effects of different strains may thus be observed in the same animal. In this way the influence of individual differences in susceptibility of different animals may be eliminated.

In all the inoculations, 0.1 cc. of a broth culture was introduced as superficially as possible into the shaven skin. The course of the reaction following the inoculation of an S culture is as follows. The fluid is slowly absorbed and the involved area appears blanched for several hours, after which it becomes erythematous. Within 24 hours the affected area measures 2 to 3 cm. in diameter. In the center there is a small area of necrosis and the skin about this is very red and edematous. At the end of 48 hours the reaction begins to decrease in intensity, and the involved area of the skin becomes smaller so that in a week it measures about 1 cm. in diameter. The skin is now only slightly red, but there is definite induration and only after 10 to 20 days has the reaction entirely disappeared. On the other hand, the skin reaction which follows the injection of a culture of an R strain is less

severe, the erythema is much less marked, and the duration of the reaction is much shorter.

It has been observed also that there is a close correlation between the severity of the skin reaction produced by a given culture and the minimal lethal dose of this strain for laboratory animals. Strains which form very rough colonies, and which are apparently without virulence, as measured by intraperitoneal inoculation, have been found to produce no skin reactions, or very slight ones which fade within 48 hours; while those strains which produce less rough colonies, and which are moderately virulent, induce more marked skin reactions. Dold (32), working with different strains of streptococci, has also noted a close correlation between the severity of the skin reaction produced and the virulence of the culture.

Immunological Reactions of S and R Strains

Many investigators have attempted to determine the immunological relationships between various strains of influenza bacilli. In most of these attempts the method of agglutination with univalent sera has been employed, though studies employing complement fixation have also been made. While certain observers have been able to demonstrate some immunological relationships between certain cultures, it has not been possible to develop any useful or accurate method of grouping these organisms, such as has been arrived at in the case of pneumococci and Friedländer's bacilli.

The observations which have just been reported, especially the fact that S and R forms of influenza bacilli occur, and that the one form is apparently a variant of the other, and furthermore, that the bacteria of the S form are possessed of a capsule, have suggested that the mechanism underlying the immunological relationships of influenza bacilli may not be essentially different from that upon which the immunological relationships of pneumococci depend, and that the difficulties previously encountered in grouping these organisms may depend, to some extent at least, on the facts that the method employed has chiefly been that of agglutination, and that S and R strains were used indiscriminately in the testing. This supposition was supported by the observation that when the serum of an animal immunized against one of the S strains was added to the filtrate of a fluid culture

of the same strain a specific precipitation occurred. The same reaction occurred when washings of a plate culture were added to the serum. The bacteria were washed in salt solution and removed from the fluid by centrifugalization. On the other hand, when the same culture filtrate or bacterial washing fluid was added to an immune serum produced by the injection of R organisms, even though derived from the same S strain, no precipitation occurred. Moreover, no precipitation occurred when filtered R broth cultures, or washings from plate cultures of R strains were added to S immune serum. These observations indicated that in the culture fluids of S strains there exists a soluble substance, which reacts with the homologous immune serum, and that this substance is easily removed from the S bacteria by washing. On the other hand, no such substance in the R strain could be demonstrated, nor did the serum produced by the injection of R strains contain any antibodies effective against this soluble substance.

It was now important to determine whether this soluble substance was specific only as regards the homologous strain, or whether, so far as this reaction is concerned, all S strains are immunologically identical, or whether one soluble substance is specific for a certain number of strains, which thus would form an immunological type. Several groups of rabbits were, therefore, immunized, each one against one particular S strain.

For the purposes of this particular experiment, three groups of rabbits were immunized, one against the Strain 35S, one against 41S, and one against 51S. The best immune sera were obtained by giving repeated intravenous injections of living organisms which, immediately before the inoculation, were scraped from plates and suspended in 20 per cent normal rabbit serum. After the course of immunization had been completed, sera obtained from the rabbits were tested against the soluble substances of each one of the S strains used in immunization.

In making the tests, washings from plate cultures were employed, as they have been found to be not only more active but also more convenient to prepare than are the culture filtrates. The bacteria from a 20 to 24 hour Levinthal agar plate culture were suspended in 3 cc. of physiological salt solution. The mixture was immediately centrifugated at high speed and the clear supernatant fluid was used for the tests. Various dilutions of the fluid from the cultures of each of the three S strains were made. 0.4 cc. of each dilution was placed in a precipitin tube. To each tube was then added an equal quantity of serum, diluted with saline in the

proportion of 2 parts of serum to 3 parts of saline. The tubes were placed in the incubator for 1 hour at 37°C. and then left on ice overnight, and examined on the following morning.

The results of this experiment are shown in Table II. It is seen that precipitin reactions occurred in the mixtures of the soluble substances of 41S and 51S with 41S serum and also in the mixtures of these soluble substances with 51S serum. Moreover, all these reactions were of equal intensity. On the other hand, the soluble substance from Strain 35S reacted only with its homologous serum, no precipitation occurred when the 35S serum was mixed with the soluble substance of either of the other strains. It was evident then that Strains 41S

TABLE II

Precipitation of the Soluble Substance of S Strains in Anti-S Serum

Serum	Antigen	Final dilution of supernatant fluid							Controls
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	
35S	35S	++++	++++	++++	++++	++++	++	+	−
"	41S	−	−	−	−				
"	51S	−	−	−	−				
41S	35S	−	−	−	−				
"	41S	++++	++++	++++	++++	++++	+++	+	−
"	51S	++++	++++	++++	++++	++++	+++	+	−
51S	35S	−	−	−	−				
"	41S	++++	++++	++++	++++	++++	+++	+	−
"	51S	++++	++++	++++	++++	++	++	+	−

and 51S are of one immunological type, so far as could be tested by this method, while 35S is of an entirely different type. Other similar experiments have been carried out to determine the immunological specificity of each one of the 15 S strains so far isolated. It has been found that two of these strains are of the same type as 35S, and this type has been called Type a, while the 12 remaining strains are of the same immunological type as 51S, as tested by the precipitin reaction, and this type has been called Type b. It is of interest that of the three Type a strains, two are those which were isolated from cases of atypical pneumonia, and the third is one of those which were isolated from cases of pharyngitis. The source of the remaining strains, all of

which are of Type b, has been previously mentioned. It is of interest that all of the seven strains[2] from cases of meningitis are of Type b. There is also in the laboratory a specimen of immune serum prepared, before this work was undertaken, by the injection of a rabbit with a strain of *Hemophilus influenzae*, also isolated from a case of meningitis. The strain itself now produces only R colonies but the immune serum has been found to be active against Type b strains.

The immune sera which were prepared by the injection of the S strains, were now used for testing the agglutination reactions of these various S strains. When the tests were carried out at 37°C. for 2 hours and overnight on ice, the same type specificity as revealed by the precipitin tests became evident. The bacteria of 35S, Type a, were agglutinated only in 35S serum, not in that produced by the injection of Strain 51S, Type b, and *vice versa*. This type-specific agglutination at 37° has been confirmed by many other tests of S organisms. The agglutinating powers of the sera produced by the injections of R organisms, and the agglutinability of the R strains in the various sera, however, have not given results which are so easy to interpret. At 37°, S strains are not agglutinated by any of the R sera. So far as tested, however, all R strains are agglutinated by all R sera.

When, however, instead of carrying out the agglutination reactions at 37°, the tests were made at 47°C. for 4 hours and overnight on ice, somewhat different results were obtained. Under these conditions there was observed no specificity of the reactions of the S strains as regards type. The Type b strain, 51S, was now agglutinated in Type a serum and both 35S, Type a, and 51S, Type b, were equally well agglutinated in Type b serum. Moreover, the S strains of both types were now agglutinated by all the sera produced by the injection of R organisms. Also all R strains, at this temperature, are agglutinated in all the sera, whether R or S of Type a or Type b.

There is one difference, however, as regards the character of the agglutination reaction, between the agglutination of the S culture in the type-specific S sera and all the other agglutination reactions. In

[2] Since the completion of this paper two other strains from cases of meningitis have been received. Both strains are of Type b. A precipitin test was also made with the spinal fluid from one of the patients and a positive reaction was obtained in Type b serum.

the former case the agglutinated bacteria form a firm mass or disc at the bottom of the tube and this disc can not easily be broken up by shaking. In all the other agglutination reactions the bacteria form a loose precipitate which, on shaking, becomes easily separated into small granular masses which become distributed throughout the fluid.

In the light of the careful studies of the immunological relationships in another group of organisms, Pneumococcus, by Avery (33) and others, one may venture to offer a probable explanation of the phenomena just described. Influenza bacilli of the S variety undoubtedly produce a soluble substance which is specific for each type. The chemical nature of the soluble specific substance of Type a is now being studied by Dr. Goebel, and it has been found that this substance belongs among the carbohydrates, just as do the soluble specific substances of pneumococci and Friedländer's bacilli. The fact that influenza bacilli of the S form possess capsules, while those of the R form have no capsule and also form no soluble specific substance, suggests that the soluble specific substance is related to this morphological structure, as has been demonstrated to be the case with pneumococci and Friedländer's bacilli (34).

It has been shown that in the case of pneumococci the S forms possess not only a type-specific antigen, but also a species-specific antigen, the latter being the so-called nucleoprotein which forms a large part of the cell. Immunization with R pneumococci gives rise only to antibodies which react with all R pneumococci, no matter from which type they are derived. Immunization with S pneumococci gives rise mainly to the development of type-specific antibodies, but in all cases such sera contain small amounts of species-specific antibodies, the amounts depending upon the method of immunization employed.

In the case of influenza bacilli, it seems probable that immunization with the S forms also gives rise to species-specific antibodies (anti-R) in addition to type-specific antibodies. When agglutination reactions of S organisms are carried out at 37° only the type-specific antibodies in the S sera are effective, but when the reactions are carried out at 47° the species-specific antibodies can also act. It would of course be hazardous, with present knowledge, to offer an explanation of this, though it may be suggested that at this temperature the capsular substance is dissolved or removed from the surface of the bacteria and the body of the bacterium is exposed.

Further studies, which are under way, it is hoped may throw more light on the question of the immunological relationships of this group of microorganisms.

Biochemical Reactions of S and R Strains

Extensive studies have been made to determine whether any differences exist between the S and R strains in relation to certain biochemical reactions. All of the strains, both S and R variants, have been found to produce indole and to reduce nitrates to nitrites.

The ability of the various strains to attack different sugars has been studied.

The sugars employed have been glucose, xylose, galactose, mannite, levulose, maltose, saccharose, lactose, and dextrin. The medium used has been Dunham's peptone solution to which were added the growth accessory factors for influenza bacilli, as in the preparation of Levinthal's broth. The various sugars were added to tubes of this medium, the tubes were inoculated with the strains to be studied, and were left in the incubator for 10 days.

The only sugars fermented by any of the strains were glucose, galactose, and xylose. All of the S strains fermented glucose. The twelve Type b strains also fermented galactose and all but one fermented xylose. On the other hand, only one of the Type a strains fermented galactose and none of them fermented xylose. The R strains fermented the same sugars as did the S strains from which they were derived. With none of the strains, however, was acid production very marked, and the fermentation reactions of a given strain were not entirely constant and regular, when the tests were repeated. This irregularity of fermentation reactions by strains of influenza bacilli has also been noted by others (Stillman and Bourn (35), Rivers and Kohn). While, therefore, there have been some differences noted in the fermentation reactions exhibited by strains of Types a and b, it is not likely that these are distinguishing characteristics of the two types of influenza bacilli.

Several observers have previously noted that influenza bacilli are soluble in bile or in solutions of bile salts (Sellards and Sturm (36), Neufeld and Etinger-Tulcynska (37)), though little attention has been paid to this observation in the literature dealing with this organism. Without knowledge of this phenomenon, it was noted that several S

strains were soluble in bile. Consequently all of the S as well as the R strains were tested as to their solubility. All of the strains were found to be soluble in bile, and no differences in this respect were noted between the S and R strains.

Dissociation of S and R Forms

It has been found that cultures of the newly isolated S form of influenza bacilli are very unstable and that under artificial conditions reversion to the R form frequently occurs quickly and readily.

Transformations may occur when S strains are grown on Levinthal plates or on blood or chocolate agar slants or in Levinthal broth. They may occur when daily transfers are made in broth that promotes luxuriant growth, or when a broth culture is kept in the incubator over a long period of time, or when, after growth has occurred, the broth cultures are kept at 22°C. They may occur when the cultures are kept sealed from the air by vaseline. In S cultures on the surface of blood agar in tubes, transferred every day, R colonies have appeared within a week. Different S strains, however, vary in the readiness and rapidity with which R forms appear. When freshly isolated, the S strains appear more unstable than they do later after they have been repeatedly cultivated. In a relatively stable strain grown through repeated transfers in Levinthal's broth, without replating, transformations do not usually appear until ten or twenty transfers have been made. It has been found, moreover, that the change is delayed in broth cultures in which the conditions are not too favorable for growth, and when the cultures are sealed with vaseline. One such strain has now been kept at 37°C. for 12 months, and subcultures still show that S forms only are present. Other strains, however, kept under the same conditions have not shown the same degree of stability. At 4°C. in Levinthal's broth, under seal, transformations of the S strains have not occurred, but at this temperature the organisms remain alive for comparatively short periods. The longest time such a culture has remained viable has been 15 weeks.

To maintain continuously S strains free of R forms, it has been found necessary to transfer the culture daily on Levinthal agar plates, picking out for each transfer a large typical iridescent colony.

Stryker (38), Griffith, and Reimann (39) have shown that when type-specific strains of pneumococci are grown in type-specific serum transformations may occur. Four type-specific strains of influenza bacilli, two Type a and two Type b strains, have been grown in media containing the two Types of anti-S influenza serum.

The cultures were grown in tubes of Levinthal broth, each containing 10 per cent of one of the various kinds of immune serum, or as controls, of normal serum. Transfers of the cultures in these media were made daily. Inoculations from these tubes were also made daily on Levinthal agar plates, in order that the relative number of R and S colonies might be determined.

TABLE III

Interconvertibility of S and R Strains in Antisera

Strain	Type	Number of transfers	Kind of colony after growth in				
			Anti-S serum Type a	Anti-S serum Type b		Anti-R serum	Normal serum
			+35S	+41S	+51S	+35R	
35S	a	4	*20S:1R	S	S	S	S
		10	R	S	S	S	S
		20		S	S	S	S
60S	a	5	*1S:12R	S	S	S	S
		7	R	S	S	S	S
		20		S	S	S	S
						+41R	
41S	b	2	*50S:1R			S	S
		4	R			S	S
		10				R	*1S:1R
		13				R	R
						+51R	
51S	b	2			S	S	S
		4			R	S	S
		20				S	S
60R		1				R	R
		2				*10S:1R	R
		5				S	R

* The figures indicate the relative number of colonies of the two kinds on plates.
\+ Designation of the particular strain employed in immunization.

From Table III it is seen that when the type-specific strains were grown in media containing the homologous anti-S serum, R colonies appeared on the plate transfers within a short time, much earlier than when growth had occurred in media containing heterologous anti-S serum, anti-R serum, or normal serum.

Transformation in the opposite direction, that is, a change of the R form into the S form, has been more difficult to accomplish, just as others have found to be the case with Pneumococcus.

Dawson and Avery (40) and Dawson (41), however, have shown that when R strains of pneumococci are repeatedly grown in anti-R serum, transformation into the S form may sometimes occur. Four R strains of influenza bacilli have been repeatedly grown in media containing anti-R serum, using the same technique as in the experiments with S strains described above. In the case of only one of the R strains studied, 60R, did any change from the R to the S form occur (Table III). This strain had only recently been derived from an S strain, while the other R strains studied had been under cultivation as R strains for several months. In this instance, S colonies appeared on the plates in the second transfer, and conversion was complete after five transfers. At no time were R colonies present on the plates inoculated from the control tube which contained normal serum. The S culture in this instance was shown to be of Type a, as was the original S strain from which the 60R strain was derived.

It seems certain that in these experiments the conversion was influenced by the presence of antibodies, and that the appearance of S colonies on the plates cannot be ascribed to the presence of S organisms in the original culture. The culture employed, however, was not grown from a single isolated cell which would have been necessary to eliminate completely this possibility.

Attempts to convert R into S forms in animals have so far been successful only occasionally.

Experiments have been made with two R strains; one of these had been derived from an S strain several months previously and the other had been derived from an S strain only a few weeks before. Neither of these R cultures produced any soluble specific substance. Both of these strains were repeatedly passed through mice and rats, and were inoculated intracutaneously into rabbits together with killed Type a organisms. No successful results have been obtained with the older strain. With the R strain recently isolated, however, two reversions to the S form have occurred. This strain, 60R, is the same one which reverted on passage through media containing anti-R serum. One conversion of 60R took place in a mouse, following an intraperitoneal inoculation of the organisms from 2.5 cc. of a broth culture suspended in 0.5 cc. of broth. The mouse died 36 hours after the inoculation, and S and R colonies were present in the peritoneal and heart's blood cultures. The peritoneal washings of this mouse failed to kill another mouse. Fourteen other mice were inoculated with this strain, and the cultures from these mice contained only R organisms, or they were sterile. The other conversion of 60R

occurred in the skin of a rabbit. The rabbit was inoculated with 0.5 cc. of a broth culture, mixed with 1 cc. of vaccine prepared from the Type a strain, 35S. A very marked erythematous lesion, 3.5 cm. x 10 cm., with central necrosis, was produced. Cultures were made daily from the lesion. The first culture contained many R colonies, the second contained S and R colonies, and the third only a few R colonies. All cultures thereafter were sterile. The cultures from the other rabbits of this series gave only R colonies. The S cultures which were obtained from the mouse and the rabbit were both of Type a.

SUMMARY

During the course of a study of different strains of influenza bacilli, fifteen strains have been found to form colonies of a different appearance from that usually considered typical of influenza bacilli. These colonies are smooth, more opaque, and are iridescent in oblique transmitted light. Most of these strains were isolated from patients in whom these organisms seemed to play a pathogenic rôle. When these strains were grown repeatedly on blood agar, other colonies appeared which were smaller, less smooth, less opaque, and not iridescent, and when subcultures were made from these rough colonies, all of the colonies were of this character. Further study of the cultures obtained from these smooth and rough colonies have shown that one is a variant of the other. The strain from the smooth colony has been called an S strain, that from the rough colony an R strain. Certain differences in the morphology of the organisms in the R and S strains have been observed. Of special importance is the fact that the bacteria of the S strains are possessed of capsules. It has also been found that the S strains are somewhat more virulent for laboratory animals than are the R strains.

In the supernatant fluid of broth cultures of S strains, and in the washing fluid of S bacteria grown on agar, there is present a soluble substance which, in the presence of homologous immune serum, gives rise to a precipitate. No reaction of this kind, however, occurs with the cultures of the R strains. By means of cross precipitin reactions, employing antisera against the different S strains, it has been found that the fifteen strains studied may be divided into two distinct immunological groups. Three of these strains belong in one group, Type a, and the remaining twelve in another group, Type b. Seven of the strains studied were isolated from the spinal fluid in cases of menin-

gitis, and all of these strains are of Type b. Agglutination tests performed at 37°C. with these fifteen S strains have revealed the same specific type relationships among the organisms as did the precipitin tests. The R strains on the other hand, exhibit no similar type agglutinations. If the agglutination tests are made at a higher temperature, 47°C., the S strains also fail to show the specific type reactions which occur at 37°C. Certain differences between other biochemical reactions exhibited by the two types of strains have been noted, but it is not believed that they are sufficiently constant to be of great significance.

When S strains are grown on artificial media outside the animal body, they tend to be converted into the R form. The rapidity and the readiness with which this conversion occurs depend on certain conditions, such as the kind of media employed, the temperature at which the cultures are kept, and the atmospheric conditions under which they are cultivated. The rate of conversion is increased when the S strains are grown in media containing anti-S immune serum of the homologous type. On the other hand, conversion of R strains into the S form occurs with much less readiness, and then only if very particular conditions are present. On one occasion conversion occurred when an R strain was grown in a medium containing anti-R immune serum. On two other occasions this same strain changed from the R to the S form during passage through animals. With other R strains it has so far been impossible to bring about this transformation.

These studies indicate that the bacteria belonging in the group *Hemophilus influenzae* exhibit changes in pathogenicity and immunological specificity, which are analogous to those shown by the bacteria of the pneumococcus group. It is important to continue this study, with the technique which has been developed, to include a much larger number of strains. On account of the readiness with which the S strains of influenza bacilli lose their type specificity when grown on artificial culture media, it is important that the organisms be studied as soon as possible after removal from their pathological sources. It is not impossible that many strains lose their specificity immediately after removal from the host, and that the specific immunological differentiation of many strains may, for that reason, be very difficult, if not impossible.

CONCLUSIONS

Strains of influenza bacilli are of two kinds, which have been called S and R. The S strains are distinguished (1) by the appearance of their colonies, smooth surface, large size, opaqueness, and iridescence in oblique transmitted light, (2) by the fact that the individual bacteria are capsulated, and (3) by the fact that they produce a soluble specific substance which is present in culture filtrates and washings of the bacteria. R strains form colonies that are rough and irregular in outline, are less opaque than the S colonies, are of smaller size, and are not iridescent; the individual bacterium possesses no capsules, and these strains produce no soluble specific substance. The S strains are also more pathogenic for animals than are the R strains.

By means of cross precipitation reactions it has been possible to divide the fifteen S strains studied into two distinct immunological types. The same specific types are shown by means of agglutination reactions carried out at a temperature of 37°C. Spontaneous conversion of S strains into the R form occurs in artificial culture media with great readiness. This may be delayed by certain cultural procedures, or may be hastened by growth in media containing type-specific antiserum. Artificial conversion of R strains into the S form has been observed but the changes are carried out only with great difficulty.

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EXPLANATION OF PLATE 19

FIG. 1. Plate culture showing S and R colonies of Strain 35, taken by oblique light, × 7.5. Culture 20 hours old. Note the difference in size and deflection of light.

FIG. 2. Bacilli from an S colony of Strain 35S, × 1000. Gram's stain. Culture 20 hours old. Note the uniformity of the rods and compare it with the pleomorphism of the bacilli from an R colony of the same age, Fig. 3.

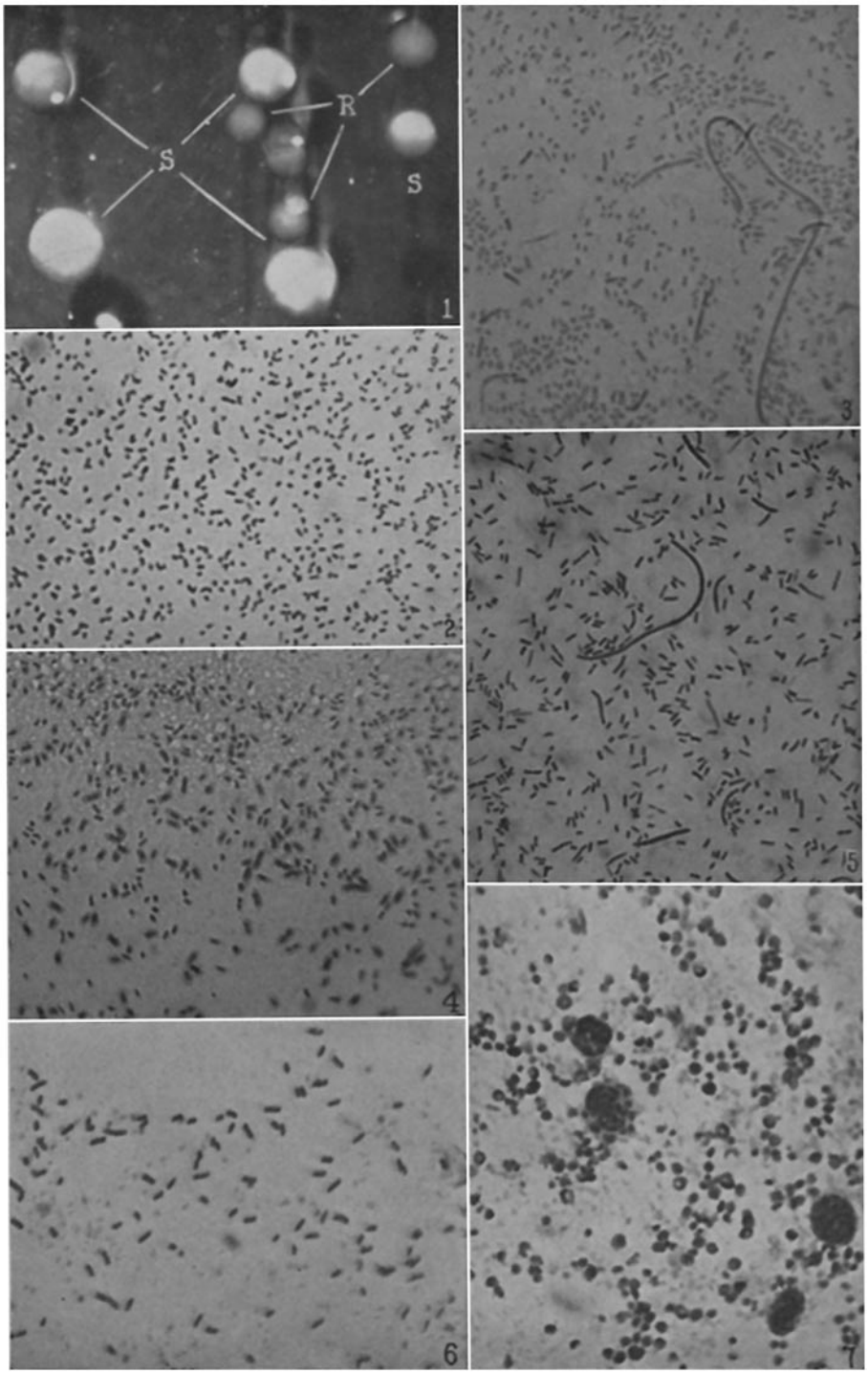
FIG. 3. Bacilli from an R colony of Strain 35R, × 1000. Gram's stain. Culture 20 hours old. Note the variation in the length of the rods, compare with Fig. 2.

FIG. 4. Bacilli from an S colony of Strain 35S, × 1000. Muir's capsule stain.

FIG. 5. Bacilli from an R colony of Strain 35R, × 1000. Muir's capsule stain.

FIG. 6. Smear of the peritoneal exudate of a mouse which died 3½ hours after an inoculation of 35S. Wright's stain, × 1000.

FIG. 7. Smear of the peritoneal exudate of a mouse, which died 32 hours after an inoculation of Strain JR. Gram's stain, × 1000. The bacilli have become shaped like globules.



(Pittman: Type specificity in *Hemophilus influenzae*)