THE PROTECTIVE ACTION OF TYPE I ANTIPNEUMOCOCCUS SERUM IN MICE

I. The Quantitative Aspects of the Mouse Protection Test

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(Received for publication, June 6, 1935)

The demonstration of the protective or curative action of an antibacterial serum such as antipneumococcus serum is conditioned not alone by the antibody content but in a large measure by as yet ill defined characteristics of the animal. It is well recognized that the action of the specific antibody alters certain qualities of the bacterium or its products. The eventual outcome, however, is in large measure dependent upon the various non-specific factors of the animal body. The evaluation of the latter is a particularly complex and difficult problem. For example, it is known that unidentified characteristics of various animal species are of great importance. Then, too, the rôle of genetic factors within a species is clearly appreciated. Moreover, within the same genetic strains, certain physiological variables appear to be of the greatest significance. When the latter can be correlated with the constitutional response they are termed "individual host factors."

Previous communications (1, 2) have dealt with the nature and influence of the individual host factors of the rabbit with reference to the curative and protective actions of specific antipneumococcus serum. Under relatively controlled genetic conditions the end-result appeared in any given instance to be conditioned by the quantitative interrelationships of four variables: (*a*) the number of infecting organisms, (*b*) the amount of antiserum (in effect the amount of antibody), (*c*) the weight of the animal, and (*d*) the number of circulating white blood cells. No evidence was obtained to show that either the weight or the number of cells is other than a reflection of a more complex physiological system. They serve, however, as indices, and, as such, they express the quantitative aspects of non-specific host resistance in the rabbit.

The present series of papers deals with the problem of the host factors which are concerned in the protective action of antipneumococcus serum in white mice. As in any study of this kind, it was first necessary to determine the general quantitative characteristics of the mouse protection test in order to learn what combinations of serum and culture might be used advantageously in the evaluation of the rôles played by the various host factors.

The mouse protection test for the evaluation of the potency of specific antipneumococcus serum has long been used and has for many years been the subject of controversy, particularly since it is a method for standardization. The earlier methods, such as those of Neufeld (3), and of Cole and his associates (4), were used to establish minimal requirements for the protective capacity of a serum. The latter method, as later adopted by the United States Hygienic Laboratory (5), was based on the simultaneous injection of culture and serum, and required that 0.2 cc. of serum should protect two of three mice when injected together with 0.1 cc. of a culture of a virulence such that 0.000,001 cc. would bring about the death of normal controls. It was recognized that a considerable individual variation among mice did not permit the more stringent requirement of 100 per cent protection.

Subsequent improvements in culture media and the knowledge of bacterial dissociation have made it possible to obtain cultures of Pneumococcus containing at least 100 times as many virulent organisms as did cultures at the time these protective tests were first described. Since the earlier requirements were based on the amount of culture rather than on the number of minimal fatal infective doses, the result has been to increase the severity of the test.

A second development within the past few years has been the introduction of various forms of concentrated sera. As will be shown in the text, these concentrated preparations, although high in antibody content, give misleading results with the original minimal potency tests.

In view of these facts, Felton (6) has developed a second type of protection test designed to give a quantitative estimation of serum potency. This test consists essentially in the simultaneous injection of varying amounts of antiserum together with a fixed quantity of culture, usually 100,000 minimal fatal infective doses. The end-point or point of quantitative evaluation is taken as the smallest quantity of serum which under these conditions will protect two-thirds of the mice. This last requirement again recognizes the variation among individual mice. Although this system of titration is now widely used, the results are not as quantitative as might be desired, since it is often difficult for two or more workers to arrive at the same estimation of potency on a given sample of serum except by the use of very large numbers of mice.

EXPERIMENTAL

Mice.—Female white mice of the Rockefeller Institute strain were used throughout. These mice were maintained on a diet of bread and milk. This strain, while highly inbred over a long period of years, is fairly representative of the type of mice in general use for protection tests.

Culture.—The original Neufeld strain of Type I Pneumococcus was used in all of these experiments. Cultures were maintained in rabbit blood broth and possessed a virulence such that 0.000,000,01 cc., given intraperitoneally, invariably produced fatal infection.

Sera.—Type I antipneumococcus horse sera were used in these experiments.[1]

Injection.—The exact details of each experiment are described in the respective protocols. The general method was to dilute the serum and culture so that the desired quantity of each would be contained in 0.5 cc.; the serum was diluted in saline, the culture in broth. Serum and culture were mixed in the same syringe before injection. The minimal period of observation of the test mice was 6 days.

The Protective Action of Antipneumococcus Serum When Injected Simultaneously with Culture

The first experiment deals with the comparative protective actions of Type I antipneumococcus horse and rabbit sera when injected intraperitoneally with various amounts of Type I pneumococcus broth culture. These tests are essentially elaborations of the test originally employed in this laboratory. The results are shown in Text-fig. 1.

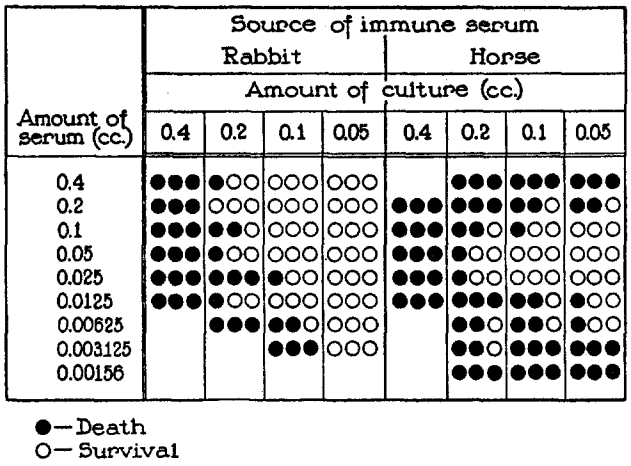
Dilutions of an 18 hour blood broth culture of Type I Pneumococcus were made in broth so that designated amounts ranging from 0.05 cc. to 0.4 cc. were contained in 0.5 cc. Dilutions of the antipneumococcus sera were made in saline so that amounts ranging from 0.001,562,5 cc. to 0.4 cc. were contained in 0.5 cc. Both horse and rabbit antipneumococcus sera had comparable agglutinin titers. The mice weighed from 18.5 to 21 gm.

This particular lot of antipneumococcus *rabbit* serum, within the range of amounts used, protected all mice against 0.05 cc. of culture. Against 0.1 cc. of culture complete protection was obtained with 0.05 cc. of serum and with larger amounts. With smaller amounts of serum the results were irregular, until with 0.003,125 cc. all mice died. Against 0.2 cc. of culture, protection was irregular throughout, and when 0.4 cc. of culture was used no protection was demonstrated.

With this lot of antipneumococcus *horse* serum all mice were protected against 0.05 cc. of culture by 0.1 cc., 0.05 cc., and 0.025 cc. quantities. With amounts of serum greater or smaller than these, at first irregular, and finally completely

[1] These sera were furnished through the courtesy of Dr. Augustus Wadsworth of the Division of Laboratories of the New York State Department of Health.

negative, results were observed. Similar results were obtained with varying amounts of serum against 0.1 cc. of culture, although here complete protection was obtained only with 0.05 cc. and 0.025 cc. amounts of serum. With 0.2 cc. of culture the results were irregular throughout except that with the larger amounts of serum no protection was afforded. No protection was obtained with any amount of serum when 0.4 cc. of culture was used.



TEXT-FIG. 1. Protection tests with Type I antipneumococcus horse and rabbit sera. Serum and culture administered simultaneously. Type I pneumococcus culture possessed a virulence such that 0.000,000,01 cc. produced fatal infection in controls.

These results, entirely typical in our experience, clearly point to certain well defined characteristics of the protection test. These may be listed as follows:

1. The results show a considerable amount of irregularity. Thus, in many instances in which three mice received exactly the same amounts of serum and culture, some died whereas others survived. This irregularity is ascribed to variations in the capacities of the individual animals to utilize the protective qualities of the serum.

2. With very small amounts of serum no protection is obtained against relatively large amounts of culture. Here the amount of antibody is obviously inadequate in proportion to the number of infective organisms. The minimal amount of serum below which no

protection can be demonstrated with a given amount of culture is designated as the "limiting titer zone." A complete description of this phase will be presented later in this paper.

3. There appears to be a limiting maximum amount of culture against which protection can be demonstrated. Thus, with both horse and rabbit immune sera, some protection was obtained against 0.2 cc. of culture, but all mice died when 0.4 cc. of culture was injected. Neufeld has designated the upper limit of culture against which protection can be obtained with a given amount of immune serum as the *Schwellenwert* (7). In the present paper the term will be used in a more general sense as referring to the maximum amount of culture against which protection can be demonstrated irrespective of the amount of serum.

4. With the antipneumococcus rabbit serum, the protection with 0.4 cc. of serum was as good as that with 0.05 cc. On the other hand, with 0.4 cc. of antipneumococcus horse serum, no protection was obtained. This characteristic failure of large amounts of antipneumococcus horse serum to afford protection is termed the "prozone." Evidence of its existence is to be found in the protocols of many workers (*cf.* Yosioka (8), Felton (6), Sobotka and Friedländer (9)).

5. These limiting quantitative characteristics serve to define a zone of optimal protection.

Comparative Protection Tests with Various Lots of Antipneumococcus Horse Sera

Protection tests have been carried out with several lots of Type I antipneumococcus horse serum. For purposes of comparison, protection tests on three of these lots are shown in Table I. The results of agglutinin titrations with the same sera are shown in Table II.

It will be noted that serum Lot A gave definite agglutination in a dilution as high as 1–64; Lot B, 1–256; and Lot C, a concentrated preparation, in a final dilution of 1–512. Assuming that the antibody content of these sera actually range in this order, it is of interest to examine the results of the protection tests presented in Table I. Each of the three sera show the characteristic prozone of non-protection, the protective zone, and a limiting titer zone. For each serum there is a particular optimal amount which appears to give the highest

degree of protection. Thus, Lot A gave maximum protection with 0.2 cc., Lot B with 0.05 cc., and Lot C with 0.025–0.0125 cc. It will be observed that these optimal amounts are inversely proportional

TABLE I

Comparison of Protection Tests with Three Lots of Antipneumococcus Horse Sera (Type I)

Serum and culture injected simultaneously. Each animal received the equivalent of 0.1 cc. of an 18 hour blood broth culture of Type I Pneumococcus.

Amount of serum	Type I antipneumococcus horse serum		
	Lot A	Lot B	Lot C
cc.			
0.4	D D S S	D D D	D D D
0.2	D S S S	D D S	D D D
0.1	D D S S	D S S	D D D
0.05	D D S S	S S S	D D S
0.025	D D D S	D S S	S S S
0.0125	D D D S	D D S	S S S
0.00625	D D D D	D D S	D S S
0.003125		D D D	D D D
0.0015625		D D D	D D S

D = death.
S = survival.

TABLE II

Agglutinin Titrations of Three Lots of Type I Antipneumococcus Serum

Titrations against a washed heat-killed suspension of Type I pneumococci.

Sera	Final dilution of serum						
	1–16	1–32	1–64	1–128	1–256	1–512	1–1024
Lot A	++++	++++	++±	±	−	−	−
Lot B	++++	++++	++++	++++	++	−	−
Lot C	++++	++++	+++	+++	++±	+±	±

to the agglutination titers, in that the higher the titer the smaller is the amount of serum which gives optimal protection. Likewise, the prozone becomes more marked the higher the agglutination titer of the serum. Repetitions of these tests have shown that the "optimal

protective amount" is a definite characteristic of each lot of antipneumococcus horse serum.

Each of these lots of immune serum has been used clinically in the treatment of Type I lobar pneumonia. Lots B and C gave excellent results, but Lot A proved less efficient therapeutically. It may be pointed out that of these three lots of antipneumococcus horse sera the only one which would pass the original minimal potency requirement is Lot A.

Results Obtained by the Injection of Immune Serum 18 Hours before Infection

Neufeld (3) has regularly followed the practice of injecting the immune serum several hours previous to the time of infection. It is of course well known that in the case of certain infective organisms, as, for example, the hemolytic streptococci of Group A (Lancefield), this method must be used in order to demonstrate the protective action of immune sera. A series of experiments was therefore undertaken to learn if the earlier administration of immune serum might exalt its protective qualities.

Mice were injected with varying amounts of antipneumococcus horse serum and 18 hours thereafter were infected with varying numbers of pneumococci. The results of this experiment are shown in Table III together with comparable data on a series in which serum and culture were injected simultaneously.

From these results it will be noted that with the "delayed" method of infection the prozone is entirely eliminated. In other experiments, not shown in the table, as much as 0.8 cc. of serum was administered several hours prior to infection and yet no prozone was demonstrable. Although this procedure abolished the prozone, the limiting titer zone was altered so that the amount of serum required to give protection was larger.

The optimal protective amount of serum also differs with different methods of conducting the protection test. With 0.1 cc. of culture all mice were protected with 0.2 cc. of serum by the delayed method, whereas four of six died by the simultaneous method. On the other hand, with 0.025 cc. of serum and 0.1 cc. of culture, three of four mice died with delayed infection whereas all survived by the simultaneous

method. Thus, the optimal protective amount is smaller with the simultaneous method than with the delayed method, and in a certain sense it may be said that the efficiency of the serum appears to be greater with the former method.

A shift in *Schwellenwert* was also produced, for it may be noted that some mice were protected against infection with 0.4 cc. of culture by the delayed method, whereas all died by the simultaneous method.

On the basis of the data presented, it is not possible to correlate the results obtained by the two methods. However, certain inferences may be drawn. It seems altogether likely that 18 hours after serum

TABLE III

Results Obtained by Simultaneous Injection of Serum and Culture as Compared with Those Obtained by the Administration of Serum 18 Hours Previous to Infection

	Serum injected 18 hrs. in advance of culture				Serum and culture injected simultaneously			
Culture	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.
Serum cc.								
0.2	DD SS	D SSS	SSSS	SSSS	DDD	DDDDD S	DDDD SS	DDD SSS
0.1	DDD S	D SSS	D SSS	SSSS	DDD	DDDD SS	DDD SSS	SSSSSS
0.05	DDDD	DDD S	D SSS	SSSS	DDD	DD SSSS	SSSSSS	SSSSSS
0.025	DDDD	DDD S	DDD S	SSSS	DDD	DDD SSS	SSSSSS	SSSSSS

D = death.
S = survival.

injection a large proportion of the antibody is no longer present in effective form in the peritoneum, and that this may account for the shift in the optimal amount of serum. Reasoning on this basis, it would appear that about seven-eighths of the effective antibody has been lost during the interval. Entirely similar findings resulted from comparable titrations by the Felton technic (6). On the basis of this conception, it would seem that protective action of serum administered with the simultaneous method is much more efficient in that smaller amounts of antibody suffice. On the other hand, this interpretation does not account for the increased protection against larger amounts of culture when serum is administered in advance, that is to say, for

the increased value of the *Schwellenwert.* If, however, one reasons from other experiences, this result might be expected in view of the fact that foreign protein in the peritoneum of the mouse acts as a stimulus to the cellular system.

Protection Test in Mice Which Had Received a Previous Injection of a Non-Specific Agent

In order to test the foregoing hypothesis, a series of protection tests was carried out in which serum and culture were injected simultaneously into animals which had received a previous injection of a non-specific irritant. The results of one experiment of this order are shown in Table IV.

TABLE IV

The Protective Action of Type I Antipneumococcus Horse Serum in Normal as Compared with Nucleinate Prepared Mice

Serum and culture injected simultaneously. Each animal received the equivalent of 0.1 cc. of an 18 hour blood broth culture of Type I Pneumococcus. Each animal in the column marked Prepared had received an intraperitoneal injection of 0.5 cc. of 5 per cent sodium nucleinate 18 hours prior to the injection of serum and culture.

Amount of serum	Normal mice	Prepared mice
cc.		
0.4	D D D	S S S S S
0.2	D D S	S S S S S
0.1	D S S	S S S S S
0.05	S S S	S S S S S
0.025	D S S	S S S S S
0.0125	D D S	S S S S S
0.00625	D D S	D D D D D

D = death.
S = survival.

The irritant used in this experiment was sodium nucleinate prepared from yeast nucleic acid. It had been determined that this agent brings about a rapid increase in the number of cells in the peritoneum beginning at 4 to 6 hours after injection. The cells were found to persist in increased numbers for 2 to 3 days. In the experiment cited each animal received an intraperitoneal injection of 0.5 cc. of a 5 per cent solution of sodium nucleinate. 18 hours thereafter the animals of

this series together with normal control mice received injections of varying amounts of antipneumococcus horse serum together with 0.1 cc. of culture.

The results with the series of normal mice show the characteristic prozone in which larger amounts of serum failed to afford protection. With "prepared" mice, however, the prozone was completely eliminated in that all animals receiving 0.0125 cc. or more of serum survived. In other protection tests of this type the limiting titer zone has not been so sharp as in the experiment recorded, although as a rule the prozone has been greatly reduced or completely eliminated. With certain lots of high-titered concentrated sera it has not been possible to abolish entirely the prozone phenomenon by preliminary injection of sodium nucleinate.

In other experiments it was found that the previous preparation of mice also altered the value of the *Schwellenwert*. For example, prepared mice were regularly protected against 0.4 cc. of culture although in normal mice this result was rarely achieved.

These results indicate that both the prozone and the *Schwellenwert* can be modified or shifted as a result of the previous administration of a non-specific agent. It is probable that changes in the number and character of the cells in the peritoneum are responsible for these results.

The Quantitative Aspects of the Mouse Protection Test

An extensive study has been made of certain quantitative aspects of the mouse protection test with antipneumococcus horse serum. For the present purposes the results with one lot of serum have been divided into two sections. The first deals with a series of graded combinations of serum and culture involving large amounts of the latter, the second with the extension of the limiting titer zone in a range involving progressively diminishing amounts of both serum and culture. The results in the first section are shown in Table V.

Amounts of Type I Pneumococcus broth culture varying from 0.05 cc. to 0.4 cc. were injected intraperitoneally together with amounts of immune serum ranging from 0.001,562,5 cc. to 0.4 cc. The results are shown in terms of percentage survival. The number of mice averaged seventeen for each combination of serum and culture.

It will be noted that with any given amount of culture the highest survival rate occurred with 0.025–0.05 cc. of serum. With smaller or greater amounts of serum progressively fewer mice survived. Furthermore, it will be noted that with any particular amount of serum the survival rates progressively increase as the amount of culture is decreased. Thus, the results form a definite fan-like pattern with a pivotal point of complete protection and radiating therefrom many axial lines of progressively diminishing protection.

These results serve to emphasize the four general characteristics of the mouse protection test which have been previously pointed out;

TABLE V

Survival Rates with Various Combinations of Antipneumococcus Horse Serum and Culture

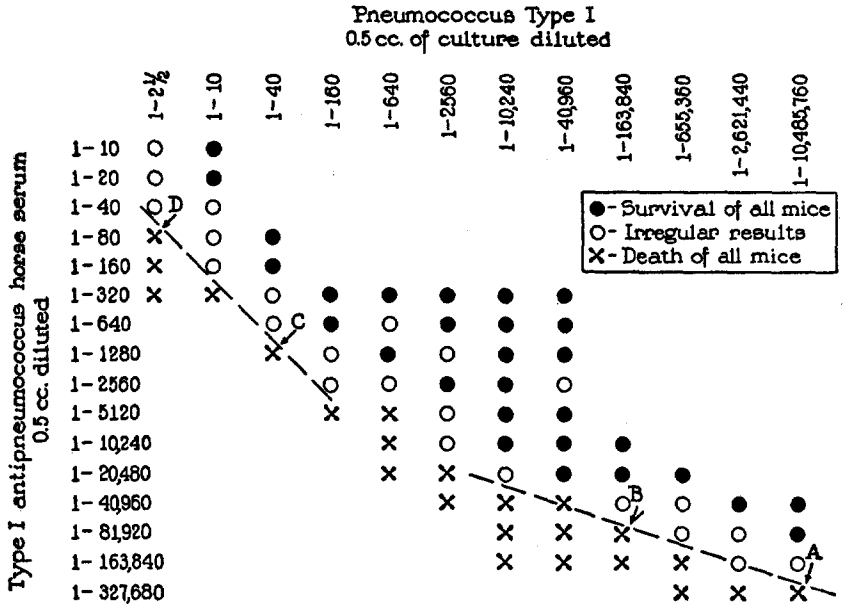
Amounts of immune serum (Lot D)	Amounts of culture				
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	
cc.					
0.4		0	5	0	Per cent survival
0.2	0	14	26	60	
0.1	0	13	41	80	
0.05	9	33	73	100	
0.025	0	45	64	100	
0.0125	0	17	48	83	
0.00625	0	0	30	33	
0.003125		0	0	17	
0.0015625		0	0	0	

viz., the prozone, the *Schwellenwert*, the limiting titer zone, and the optimal protective amount of serum. The irregularity in each group of mice can only be ascribed to variations in individual host factors.

The second part of this experimental analysis deals with the results of mouse protection tests involving progressively diminishing amounts of both culture and serum, and particularly concerns the limiting titer zone, previously defined as the smallest amount of serum which will protect mice against a given amount of culture. This end-point is obviously not solely dependent upon amounts of either serum or culture, but rather upon the quantitative relationships of one to the other. It might be expected that if protection is predicated by a

proportional equation between antibody molecules and infective organisms, it should be possible to demonstrate a linear interdependence between them.

In order to study this question, a large series of mice was injected with varying amounts of serum together with varying amounts of culture; the serum was progressively diluted by multiples of two, the culture by multiples of four. The results are shown graphically in Text-fig. 2.



TEXT-FIG. 2. Schematic representation of results of mouse protection test involving progressively diminishing amounts of both serum and culture.

For each of these combinations of serum and culture, a minimum of three mice was used. It is realized that the number of mice used to test each combination is not great enough to permit of rigid quantitative analysis. This deficiency, however, is to some extent compensated by the supporting results in adjacent series.

When graphically plotted as in Text-fig. 2, the results tend to arrange themselves in three distinct zones: a zone of complete protection, a

second zone of irregular protection, and a third of complete lack of protection.

Two broken lines have been inserted in the chart to show the general position of the boundary between the zones of protection and non-protection. Attention may be drawn to the slopes of these lines. Line AB indicates that in this range a fourfold increase in the amount of serum permits of a 64-fold increase in the amount of culture. On the other hand, line CD indicates that with a fourfold increase in the amount of serum the amount of culture can only be increased by the same multiple.

Any attempt to explain these results must take into account the fact that the organisms against which it is desired to protect the animal are living and multiplying biological units. It is known that when large numbers of pneumococci are injected, multiplication begins within 30 minutes. On the other hand, complete phagocytosis and bacterial destruction are not accomplished for 4 hours or longer. Therefore, when large numbers of pneumococci are injected their number has been many times augmented before bacterial destruction has been completed. Hence, the animal to be protected must overcome and destroy not merely the number of bacteria originally introduced, but their numerous progeny as well. From the evidence now available, it would seem likely that when extremely few pneumococci are injected the lag phase preceding bacterial multiplication is prolonged, and if this be of sufficient length, bacterial destruction may supervene before any cell division occurs. When amounts of serum and culture are properly chosen in order to illustrate this point the animal is confronted only with the number of pneumococci originally injected. It is in exactly this range that the maximum efficiency of the immune serum is demonstrable.

DISCUSSION

The present paper deals with the quantitative aspects of the mouse protection test as applied to antipneumococcus horse and rabbit sera. The test has been subjected to quantitative analysis and both the dosage of protective serum and the number of infecting organisms have been varied through wide limits.

The quantitative characteristics of the mouse protection test for

the evaluation of antipneumococcus sera may be summarized as follows:

1. The Limiting Titer Zone.—The smallest amount of immune serum which will protect a mouse against a given quantity of culture is apparently related both to the number of virulent organisms and to the antibody content of the serum. The evidence presented tends to show that this relationship is not strictly one of multiple proportions. In this connection it is a matter of considerable interest that specific protection can be obtained with an amount of serum as small as 0.000,006 cc., although *in vitro* reactions of agglutination, precipitation, and complement fixation fail with amounts below 0.002 cc.

2. The Schwellenwert.—The maximum amount of culture against which protection can be demonstrated with any amount of serum is termed the *Schwellenwert.* Actually, this threshold is far from exact, since a great many irregular results are obtained. This characteristic is obviously to some extent dependent upon the antibody content of the serum, but there appears to be a final maximum of culture beyond which protection is not obtained in the normal mouse, no matter what the potency of the serum may be. In other words, the *Schwellenwert* is largely determined by the relation between the number of organisms and some host factor or factors. It has been shown that, by increasing the number of cells in the peritoneum, protection can be demonstrated against larger numbers of bacteria. This result suggests that the number of cells may be of considerable significance in fixing the value of the *Schwellenwert.*

3. The Prozone.—For each lot of antipneumococcus horse serum there appears to be a particular optimal quantity which gives maximum protection against large numbers of pneumococci. With progressively larger amounts of serum fewer animals survive. This in many respects corresponds to the well known prozone in specific agglutinin and precipitin tests. Contrary to this viewpoint, however, is the fact that antipneumococcus rabbit sera, although possessing antibody titers equivalent to those of horse antisera fail to exhibit this prozone effect. Experiments now in progress indicate that the addition of various heterologous substances may produce an artificial or pseudo-prozone.

The chief purpose of this work has been not only to define the

quantitative characteristics of the protection test *per se*, but also to determine the level of dosage at which maximum variation in the results occurs. In short, if one is to acquire knowledge of the physiological variations or host factors which are so intimately associated with the end-results of any animal test, it is first essential to determine the experimental conditions under which their effects are most evident. In the case of the mouse protection test with antipneumococcus serum, such a fortuitous combination of circumstances occurs in that range of serum and culture relations which gives the most irregular results. It is at this point that inherent physiological differences between various mice, a series of factors which until now has not been controlled, exert their major effect and cause the results in any individual mouse to be unpredictable even though the test be done under so called standard conditions. In subsequent papers an attempt will be made to analyze and evaluate these modifying host factors.

Biological evaluations can be termed quantitatively accurate only if all variables are recognized and controlled. Since the determination of the antibacterial potency of an immune serum is based upon its action in protecting animals against fatal infection, the number of variables is large, and the system, unlike that for the *in vivo* titration of toxin or antitoxin, is in a constant state of flux.

SUMMARY

The mouse protection test for the evaluation of type specific antipneumococcus serum has been studied with reference to the quantitative relationships between the amount of antibody and the number of injected bacteria. By varying both these factors through wide limits certain definite characteristics of the protection test have been defined. These are the *Schwellenwert*, the prozone, and the limiting titer zone. The modifications produced in the manifestations of these characteristics by changes in the technic of performing the test and also by certain non-specific reagents are described. The dependence of the outcome of the test upon variable factors intrinsic to the host is discussed.

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