THE PRINCIPLES OF IMMUNITY APPLIED TO PROTECTIVE INOCULATION AGAINST DIPHTHERIA[1].

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(With 7 Charts.)

When diphtheria toxin is injected in a suitable form and in sufficient quantity into an animal, antitoxin will presently appear in the blood. If the injection be made into an animal that has not previously received a stimulus there is a latent period of about three weeks before any antitoxin can be detected. The amount present in the blood gradually increases, reaching a maximum about eight weeks after the injection, and then a gradual fall in level of antitoxic content is seen. If, however, the same amount of the same antigen be injected into a previously treated animal, antitoxin appears in the circulation at a far greater rate. The latent period is only three days, and the maximum antitoxin level is reached in about eight days. The maximum level reached is from 10 to 100 times that reached after an injection into a normal animal. The two types of response are illustrated in Charts 1 and 2 and also in Chart 3, which shows the antitoxic content of a horse after two separate injections of a toxin antitoxin mixture. There is a great contrast between the extent and rapidity of the antitoxic response on the two occasions.

We distinguish between the two effects by saying that the injection of an antigen into a non-immune animal acts as a primary stimulus; the injection into an immune animal acts as a secondary stimulus. Thus we say that an immune animal is one that has been so altered by a previous stimulus that it is able to respond to a fresh stimulus more rapidly and to a greater extent than a non-immune animal can.

These two types of antigenic response are of fundamental importance. The differences in rate of response by non-immune and immune animals injected with diphtheria toxin have been established by Dr Sudmersen and myself in a number of different animals, guinea-pigs, rabbits, sheep, goats, cows, horses and men. With another colleague, Miss Allen, I was able some years ago to establish the same difference in response to four other toxins, those of tetanus and of three gas gangrene organisms, *B. welchii*, vibrion septique and *B. oedematiens*. Even the formation of precipitin to foreign protein is more rapid and extensive in a sensitive than in an untreated animal.

This simple principle of increased power to respond has been established by us in many animals for these five different toxins, and results of other

[1] A Lecture given at St Mary's Hospital, 14th May, 1925.

observers with still other toxins do not appear at variance with our results. One is encouraged therefore to believe this principle to be of universal application to all soluble toxins. It follows that the work of so many observers for the past 30 years or more, on diphtheria immunity, resulting at last in extensive

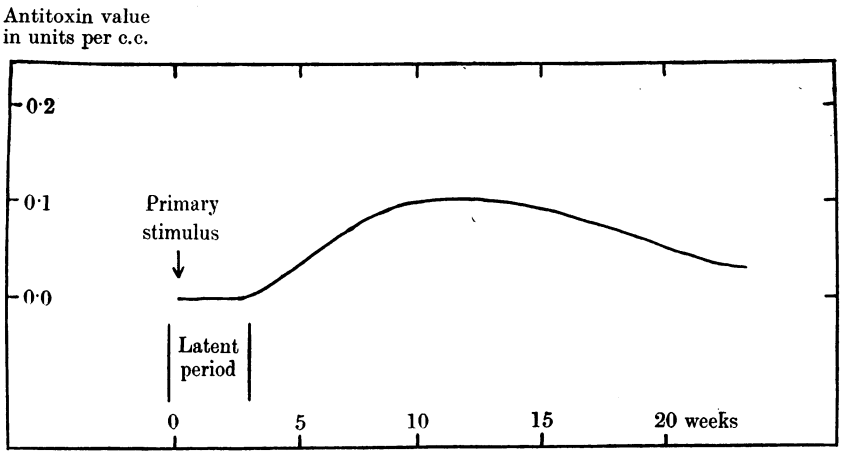

Chart 1. Showing the course of antitoxin production after a single injection in a non-immune animal.

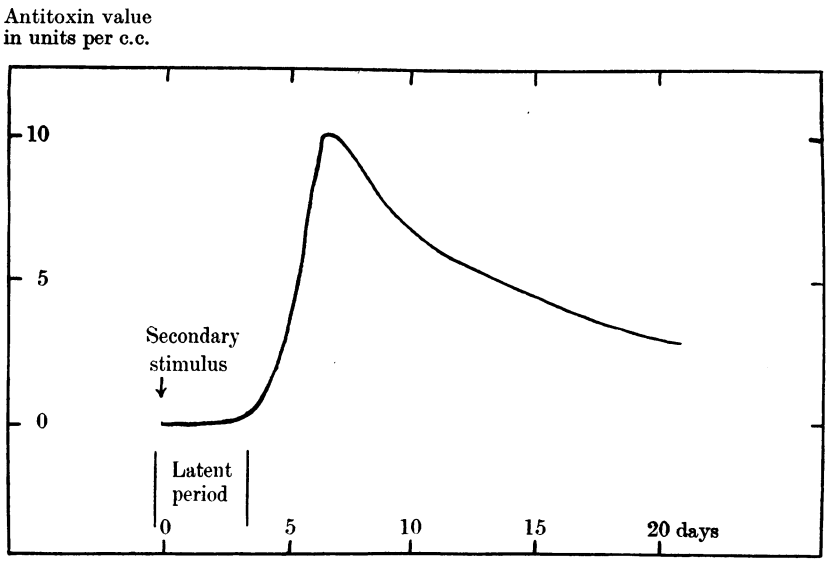

Chart 2. Showing the course of antitoxin production after an injection in an *immune* animal.

preventive inoculation, has a very distinct bearing upon protection against many other diseases and that such a discovery as that of scarlet fever toxin is being immediately followed by equally extensive protective measures based upon knowledge gained from work on diphtheria.

Given the toxin produced by the casual organisms of any disease, preventive measures are in sight. We know that it needs very little work to produce some form of tetanus and gas gangrene prophylactic that would protect the soldiers of a future war from the danger of tetanus and gas gangrene. In the last war protective vaccination against typhoid saved countless lives; the injection of tetanus antitoxic serum into the wounded also saved many lives, but this end was gained by a method vastly more cumbrous and less efficient than simple preventive inoculation to produce active immunity which could be given like the bacterial vaccines during the relative peace of training.

The injection of toxin into an animal not only acts as a stimulus to antitoxin production but also increases the power of an animal to produce anti-

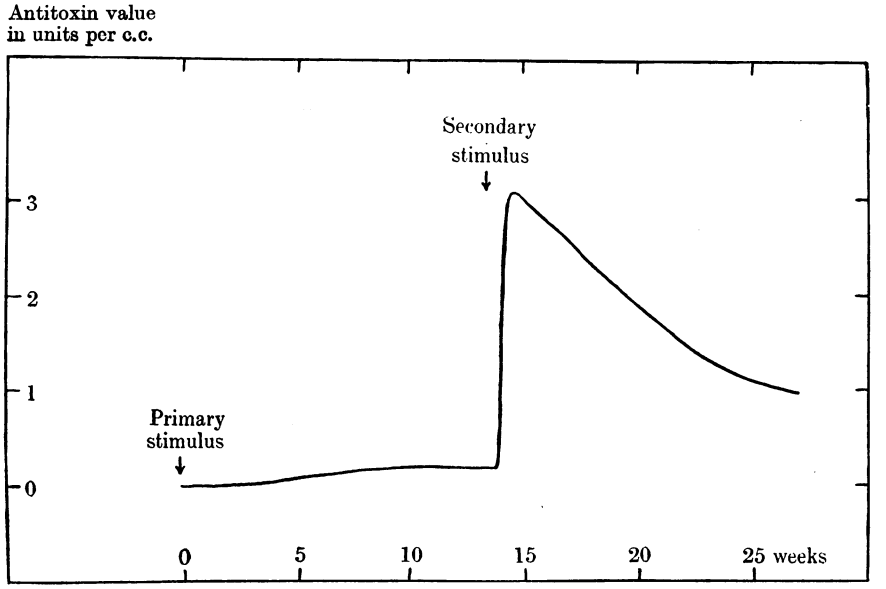

Chart 3. Showing the course of antitoxin production after both a Primary and a Secondary Stimulus consisting of the same amount of the same toxin antitoxin mixture.

toxin. This increased power remains although all antitoxin may gradually be lost; the antitoxin content may fall but potential immunity remains. So far as we know this potential immunity continues throughout life. A small primary stimulus may result in no appreciable production of antitoxin, but a second injection may act as a secondary stimulus showing increase in power of production. Thus we see that the power of production is independent of antitoxin content.

We believe that men and horses, and possibly other animals, are repeatedly infected to a slight extent with diphtheria, and that these small transient infections result in the absorption of minute doses of toxin. It is important, therefore, to trace the effect upon an animal of repeated small doses of toxin.

If a small dose of toxin, for example $\frac{1}{50}$th of a guinea-pig fatal dose, be

injected into a non-immune guinea-pig no antitoxin is produced and no increased power of production can be detected. After several such small injections a slight increase in power of production may be evident, but at least twenty and often thirty or even fifty weekly injections are needed before any antitoxin content is evident and the power to respond has so increased that

Table I.

Showing the different animals and toxins upon which the Primary and Secondary Stimulus phenomena have been established.

Toxin	Animals
Diphtheria	Guinea-pigs, Rabbits, Sheep, Goats, Cows, Horses, Men
Tetanus	Rabbits, Horses
Gas Gangrene	Rabbits, Horses
B. welchii	Rabbits, Horses
Vibrion septique	Rabbits, Horses
B. oedematiens	Rabbits, Horses

the animal is able to make a secondary response to an injection. During the early stages of such a course of injections the animal's power to respond slowly increases until a stage is reached when it is able to respond rapidly to a large stimulus although it has not yet responded to the small injections. After a few more small injections a little antitoxin is detectable in the blood of the animal and soon full secondary responses occur and a high level of content is reached.

We suggest that the natural immunity in man and in animals is produced by similar repeated stimulation. We have been able to show that the antitoxic value of normal horses changes and that when antitoxin first appears in the blood the content is extremely low and may soon disappear. We also know that horses without normal antitoxin, most of which must be regarded as being without power to respond, are of little use for the production of high value antitoxin. Such antitoxin can only be produced from horses educated to increased response.

I have traced the antitoxic content of three horses at weekly intervals over a period of three years. These three horses during this time have been used for farm work. During the whole period no antitoxin has been detected in one horse; its antitoxic value has remained at below $\frac{1}{2000}$th of a unit per c.c. The second horse had no antitoxin in its blood for over a year and a half from the time it was first observed and then, as will be seen from Chart 4, the titre rose to $\frac{1}{100}$th of a unit for a number of weeks and gradually fell back to less than $\frac{1}{2000}$th. Thus apparently we were able to demonstrate the presence of antitoxin when first it appeared in the blood. Some months later a larger rise occurred and later a still larger rise.

Each succeeding stimulus caused a greater response. A third horse showed

far bigger fluctuation; this horse has been immune for a number of years and so was able to respond to stimuli to a greater extent than less immune horses; on at least two occasions the value rose from a small fraction to well over one unit of antitoxin.

These three horses have had, as far as we know, equal chances of infection and it is suggested that our first horse possesses some other line of defence, some anti-bacterial protection against diphtheria and no small infection gains sufficient hold to produce any toxin. Our second horse has gradually lost its

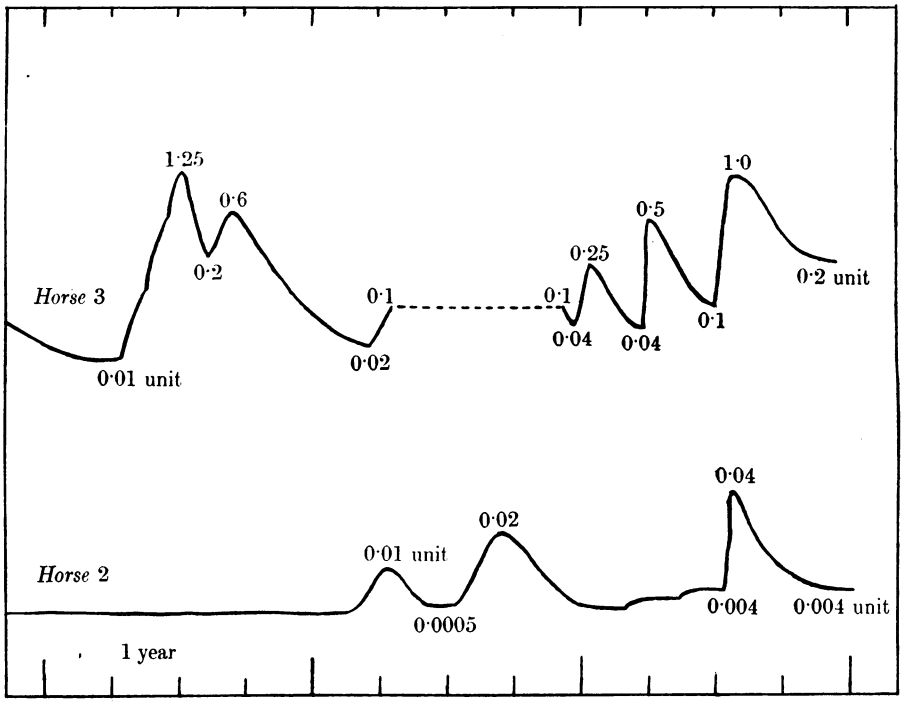

Chart 4. Showing the fluctuations in antitoxic titre of normal horses tested weekly over a period of three years.

defence and became open to attack and so received small stimulation with toxin.

If natural immunity is the result of definite infections we must concede that any conditions likely to increase the number of such small infections must also increase the chances of immunity. A consideration of the conditions influencing natural immunity in man and in horses supports this idea. Horses provide more data than other animals because they have been used on a large scale for the production of diphtheria antitoxin. It is obvious from what we have already seen that considerable time can be saved if we choose for immunisation horses that have already responded to a natural stimulation and which possess a well-developed power of response. Many years ago we

tested the suitability of horses intended for antitoxin production by measuring their antitoxic response to a single injection of toxin. Table II shows that in

Table II.

Showing the increase in antitoxic value after the injection of toxin into 103 *horses.*

Increase of antitoxic value within 10 days	Number of horses	
Less than 0·5 unit per c.c.	23	30 rejected
Between 0·5 and 1·0 unit per c.c.	7	
Between 1·0 and 5·0 units per c.c.	55	73 accepted
Over 5·0 units per c.c.	18	

a group of 103 horses tested, 73 gave a good response and 30 gave a poor response and were rejected as unsuitable. We found that almost all the horses giving a good response also had a good antitoxic content and that it was hardly worth specially testing all horses to discover the few which possessed potential immunity without antitoxic content. Choice was therefore made according to the natural antitoxic value of horses. We established a rapid test to decide which incoming horses possessed the required level of antitoxin. The subcutaneous injection of toxin produces an oedema almost entirely suppressed by antitoxin. Table III shows the connection between the size of reaction produced and the

Table III.

Showing the connection between the normal antitoxic value of horses and the area of swelling caused by a subcutaneous injection of 1 *c.c. of a certain weak toxin.*

Normal antitoxic value in units per c.c.	Number of horses showing swelling of: Less than 10 square inches	10–50 square inches	50 square inches	Average size of swelling in square inches
Less than 0·05	1	7	3	22·8
Between 0·05 and 0·2	21	9	2	11·5
Over 0·2	35	3	0	5·0

antitoxic level of the horse. The suitability of horses for antitoxin production depending to a great extent upon their normal antitoxin content was therefore judged by the size of the local reaction occurring after the subcutaneous injection of a given amount of toxin. In man there is now a much neater test, the Schick test for immunity. Toxin injected even in such small amounts as one-millionth of a c.c. or less of strong toxin will produce a small inflammatory area. The strongest toxin that I have tested was made by my colleague, Dr Watson. A reaction was produced by less than one two-millionth of a c.c. (see Table IV). This reaction is preventable by antitoxin either injected at the same time or already present in the blood and thus forms a convenient measure of antitoxin content. Presence or absence of a reaction following the injection of a given dose of toxin determines the absence or presence of a fixed level of antitoxin content. The dose must be fixed by the level of content to be established.

Schick fixed the amount of toxin at $\frac{1}{50}$th of a guinea-pig fatal dose. By the intracutaneous injection of this dose—the Schick dose of toxin—it is relatively easy to demonstrate by the absence of reaction those individuals with sufficient antitoxin—about $\frac{1}{30}$th of a unit per c.c. of blood—to protect them against any reasonable danger of infection. We speak frequently therefore of the Schick level of immunity, and a Schick negative reactor is one who has more antitoxin than that indicated by this level, and the Schick positive reactor contains less. We have suggested that the Schick dose of toxin should be determined as the equivalent of $\frac{1}{1000}$th of a unit of antitoxin rather than $\frac{1}{50}$th of a fatal dose because a more uniform level would be so established by basing the dose injected upon its power of combining with antitoxin. Table IV has been prepared to show the connection between different measurements of toxin and antitoxin to which reference may be made.

Table IV.

Showing the connection between different measurements of an average toxin and antitoxin.

Measurement of	Antitoxin	Toxin	Volume (typical fresh toxin) c.c.	
Combining power	1 unit	1 L+ dose	0·21	Kills guinea-pigs in 4 to 5 days
	,,	1 Lo ,,	0·18	Causes minimal oedema in guinea-pigs
	,,	1 Lr ,,	0·175	Causes minimal intradermic reaction
	,,	1 Lf ,,	0·155	Flocculates most rapidly
	$\frac{1}{1000}$th unit	1 Schick dose	—	Causes minimal intradermic reaction
Toxicity	None	1 M.L.D.	0·002	Kills guinea-pigs in 4 to 5 days
	None	1 M.R.D.	0·000002	Causes skin reactions

Antitoxin

Least detectable, $\frac{1}{2000}$th unit per c.c.
Average normal value of old horses, $\frac{1}{25}$th unit
Average value of immunised horses, 600–800 units
Highest value produced, 2500–3000 units

Approximate relations in a typical toxin

Fresh toxin, 1 Lo dose contains 100 M.L.D.'s.
Old toxin, 1 Lo dose contains 20–80 M.L.D.'s.
1 M.L.D. = 50 Schick doses
1 Schick dose = 20 M.R.D.'s.
Average toxin 10–15 Lf doses per c.c., 600–1000 M.L.D.'s per c.c.
Strongest toxin 28 Lf doses per c.c., 2000 M.L.D.'s per c.c., 2,500,000 M.R.D.'s per c.c.

We will now consider the conditions influencing the immunity rate in man and in horses. In a given population more Schick positive reactors are found among children than among adults. The figures given in Table V showing the connection between age and immunity rate were obtained by Park (1921) in New York, whose observations now cover several hundred thousand children. Corresponding figures on a large scale were obtained by Groer and Kassowitz in Vienna. Park showed that while only 40 per cent. of children from 6 months to 3 years old were Schick negative, this proportion rose to 60 per cent. among those between 3 and 5 years old and with increased age rose to 70 per cent., 80 per cent. and 88 per cent. among adults. Very young children are immune or not according to what their mothers are; this passively conferred immunity is lost at the end of about six months.

Different groups of individuals also vary. Zingher, in 1921, stated that in schools located in the better sections of the city and attended very largely by children of the well-to-do, positive reactors ranged as high as from 50–70 per cent., that is, less than half of the children were immune, while in a school located in the more crowded sections only from 16–25 per cent. gave a positive Schick reaction, that is, over 75 per cent. of them were immune.

Dudley, in 1922, quoted the figures from a school that had had two distinct outbreaks of diphtheria. Of the boys who had lived in the school long enough to have been in contact with both epidemics 95 per cent. were Schick negative, those that had been in contact with one epidemic showed 80 per cent. negative reactors, while of the newer boys who had entered the school after the last

Table V.

Showing the factors influencing natural immunity.

Men							
	Proportion Schick negative						
Age	0–6 months	½–3 years	3–5 years	5–10 years	10–20 years	Over 20 years	
	85% (passive)	40%	60%	70%	80%	88%	Park
Density of population	Isolated 30–50%				Crowded 75–84%		Zingher
Contact	No exposure 60%		One epidemic 80%		Two epidemics 95%		Dudley
Horses							
	Proportion containing antitoxin						
Age	0–9 months	1–2½ years	3–5 years	10–17 years			
	65% (passive)	0%	0%	100%			Sordelli
Density of population	After 1917				Before 1917		
	($\frac{1}{10}$th unit or more)						
	35%				70%		
Contact	On arrival in camp				After 9 days in camp		
	($\frac{1}{25}$th unit or more)						
	37%				100%		

outbreak only 60 per cent. were immune. Similar figures were obtained by O'Brien (1923) who found 86 per cent. immune among boys of a training-ship in which diphtheria had been prevalent; among incoming boys only 57 per cent. were immune.

The first two conditions, age and density of population, affecting the immune rate agree with our assumption of many small infections. Dudley's figures deal with exposure to bigger infection. This results in speeding up the production of immunity amongst those who may be regarded as partially immune, while those in less advanced stages develop the disease. It must be accepted that the infection in which the bacilli are present in sufficient numbers and for a sufficient length of time that they are easily detected bacteriologically will usually result in an attack of the disease in the case of the non-immune. This difficulty of detection of infection is even greater in horses. No observations have been made on the diphtheria bacillus in horses beyond those of

Cobbett in 1899 and Minnett in 1922, and quite recently those of Kliewe and Westhues (1925).

Table V also shows how these three factors—age, density of population and known contact—can be shown to have a similar bearing upon the proportion of horses containing antitoxin in their blood.

Sordelli (1920) tested a very small group of horses and found none between 1 and 5 years old to contain over $\frac{1}{50}$th of a unit of antitoxin, while all tested over 10 years old contained this amount. Very young horses were passively immune. These observations are supported by the general experience of most workers, that old horses are more useful for antitoxin production. My own

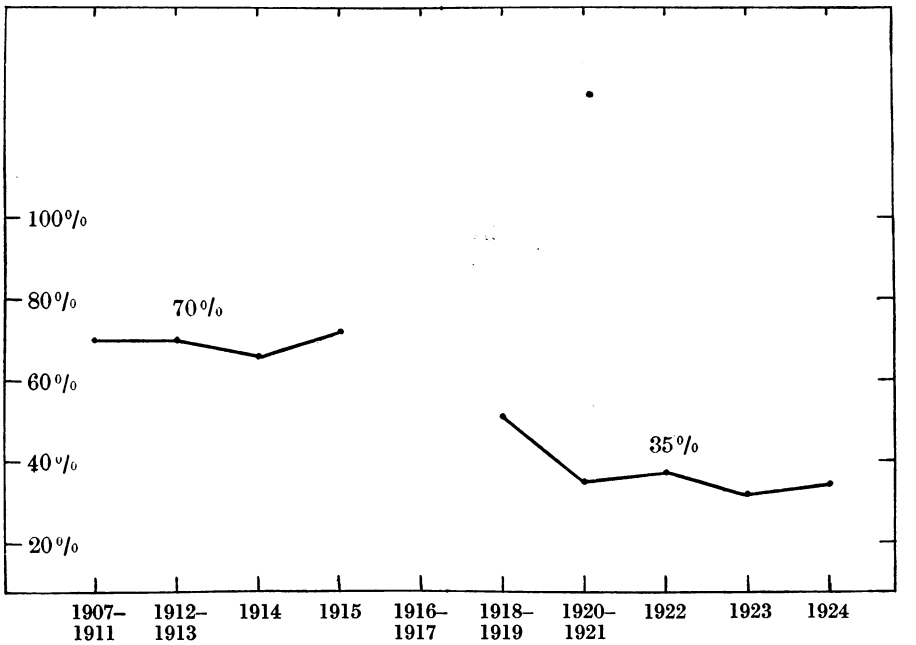

Chart 5. Showing the percentage number of horses containing 0·1 unit or more per c.c. of blood.

observations have been limited entirely to horses over 8 years old. Chart 5 gives some results that I have collected from 1400 horses. It will be seen that it is possible to divide these horses into two groups. Among horses tested before the war and until 1917 about 70 per cent. contained over $\frac{1}{10}$th of a unit per c.c. of blood. If the level had been placed at $\frac{1}{30}$th of a unit corresponding with Schick negative persons the proportion would have been slightly higher and curiously near the figure obtained for adult men. More recently, the horses tested have shown a much lower degree of natural immunity, and in the past five years the number of horses containing over $\frac{1}{10}$th of a unit of antitoxin per c.c. of blood has averaged 35 per cent. It is suggested that the difference between the two groups of horses is probably connected with the gradual decrease of the number of horses used in towns, owing to the increasing

motor traffic, and that this decrease of the number of town horses was greatly accelerated by the high mortality of horses during the war. All the horses tested were old animals purchased for antitoxin production at the Wellcome Physiological Research Laboratories, and the majority of the horses in the first group were of the type used in tradesmen's carts and cabs. Few horses in the second group could have had the same early history, but many have had a more isolated existence in country districts.

Here we have an analogy again, men and horses showing differences in immunity rate according to the type of population. It would be of great interest to obtain figures for the antitoxic content of horses in different parts of the world, comparing those in close contact with men and other horses with those living relatively isolated existences, and to compare their immunity rate with that of man in the same district.

We have no figures for increased immunity rate for horses in contact with known carriers. This is only to be expected when we consider that only two observers have detected the diphtheria bacillus in horses during a period of 20 years. But we can show that horses coming in contact with large groups of horses and their attendants frequently show an increased degree of immunity. We have often found horses that arrive at the stables possess a certain level of antitoxin and when tested a week or ten days later show a much higher level. The most marked case was that of a horse that rose from $\frac{1}{10}$th of a unit to 10 units in 9 days, and to over 20 units in 14 days, although he had not received any injections of diphtheria toxin. Of the last hundred horses that I have tested 26, when re-tested between one and two weeks later, without having received any injection of toxin, showed a definite increase in antitoxic content. This increase was probably caused by some stimulus received due to the increased chances of infection when the horses arrived among a large group of animals and their attendants, such as would occur during their short time in the London sale yards and also on their arrival at our stables. Thus we see that one horse in every four receives a stimulus if they remain for two weeks in contact with many other horses or roughly speaking some stimulation occurs on an average once every two months.

Another interesting example of this increased immunity when horses come into contact with others occurred quite recently. I was anxious to obtain samples of serum from horses of different types of population and to see what fluctuations in antitoxic content occurred. Last autumn my colleague, Captain Dalling, was serving as a veterinary officer in a military camp in Scotland, and obtained some serum from eight horses which were sent to hospital soon after their arrival in camp, suffering from slight abrasions. We see from Table VI that five of the eight horses contained no antitoxin that we could detect. The samples taken nine days later from these horses all showed definite antitoxin.

Little is known about naturally occurring diphtheria antitoxin in other animals. Many years ago Dr Sudmersen and I tested the blood of some half-

dozen cats, and found antitoxin in five out of the first six that we tested; since then we have tested some 30 other laboratory cats and have not detected a single other instance of antitoxin. It is generally accepted that rabbits and guinea-pigs do not acquire natural immunity to diphtheria, and for this reason guinea-pigs are so useful in the constancy of their reactions to diphtheria toxin. It must be remembered, however, that the age at which guinea-pigs are used in the laboratory averages from two to three months; at this age they would have had little opportunity of acquiring any degree of immunity, nor are they

Table VI.

Showing the alteration in natural antitoxin of 8 *horses.*

Horse number	Antitoxic value on arrival in Yeomanry camp	Antitoxic value 9 days later
1	Less than $\frac{1}{2000}$th unit per c.c.	$\frac{1}{25}$ unit per c.c.
2	,, ,, ,,	$\frac{1}{25}$,, ,,
3	,, ,, ,,	$\frac{1}{25}$,, ,,
4	,, ,, ,,	$\frac{1}{2}$,, ,,
5	,, ,, ,,	8 units ,,
6	$\frac{1}{25}$th unit per c.c.	$\frac{1}{25}$ unit ,,
7	$\frac{1}{10}$,, ,,	$1\frac{1}{2}$ units ,,
8	$\frac{2}{5}$,, ,,	$\frac{3}{5}$ unit ,,

likely to possess antitoxin conferred by maternal transmission, because the age of guinea-pigs that are used for breeding is usually between 1 to 3 years old, and at this age neither children nor horses have received many stimulating infections. We have recently, however, had definite evidence that guinea-pigs over a year old are slightly more easy to immunise against diphtheria toxin than younger guinea-pigs. They reach a Schick negative level after half the number of weekly injections of a Schick dose of toxin required by young guinea-pigs. This would suggest that some slight stimuli had been received unless activity of response is a function of age apart from stimulated activity. It is possible that stray guinea-pigs or rabbits have become highly immune naturally, but such instances must be so infrequent that any disturbance of test they might cause has in the past been written off as some unaccountable mistake. It would be extremely interesting to test a number of guinea-pigs or rabbits which had been in close contact with diphtheria cases or carriers. The same applies also to cats and other domestic animals. It is possible that the chance of infection having been carried by animals or of animals being a centre of infection could be best studied by testing the natural antitoxin of a large number of such animals rather than by bacteriological means. We know from our experience in horses that the frequent failure to find the diphtheria bacillus cannot be taken as evidence of its continued absence.

We will now consider and classify the various types of individuals recorded in Table VII, their need of immunisation, and the type of response to be expected to such immunisation. First, we have the Schick negative individuals who need no further treatment. They possess enough antitoxin to neutralise any toxin that might suddenly be formed after a heavy infection. Their power

of rapid response will enable them to replenish antitoxin lost in neutralising such toxin. Amongst the Schick positive reactors there are those who possess some small amount of antitoxin, not sufficient to suppress the Schick reaction; such persons will respond readily to the injection of toxin antitoxin mixtures or other diphtheria antigens and become rapidly immune. Their antitoxic content is low, but the presence of some antitoxin shows that they have reacted previously to a stimulus and their power of response is high.

We have shown in different animals that a small amount of diphtheria toxin used in the Schick test is enough to act as a secondary stimulus and causes a definite increase of antitoxic content of immune animals. My colleagues, Dr O'Brien and Dr Okell, tell me that they have fully confirmed on children the previous animal experiments on this point. Thus we have Schick

Table VII.

Showing the degrees of immunity in a normal population.

	Schick test		Circulating antitoxin	Secondary response	Increased response
	Group 1 ↓	2 ↓	3 ↓	4 ↓	5
Schick test	Negative	Positive	Positive	Positive	Positive
Antitoxin in the blood	Over $\frac{1}{30}$th unit per c.c.	Under $\frac{1}{30}$th unit	None	None	None
Response to injection	Secondary	Secondary	Secondary	Intermediate	Primary
Earlier stimulation	Many	Many	Many	Few	None
Response to immunisation	—	Rapid	Rapid	Slower	Slowest
Description	Schick negative immune	Schick positive immune	Potentially immune	Sub-immune	Non-immune

positive individuals who are sufficiently immune that a second Schick test given one or two weeks after the first test will give a negative result, although the first test was positive. For this reason observations connecting Schick reactions and antitoxic level must be accepted with reservation. Such observations have sometimes been made after the Schick test had been done. Determinations made upon blood taken a week or two after the individual was tested may show a higher titre than that which determined whether the individual would react or not.

We know that an animal may be potentially immune and yet have no detectable antitoxin in its blood. We have shown this in guinea-pigs, rabbits and horses; it probably occurs in man. Thus we may have a third group of persons capable of rapid response to injection of diphtheria antigen even though their blood has no antitoxin and this rapid response is the only way of telling that they have received in the past sufficient stimulation to become immune. They have no antitoxin content but are potentially immune.

People falling into any of our first three groups are potentially immune to a marked degree, and, as we have already shown, immunity is not necessarily correlated with the degree of circulating antitoxin or content, so that a person

falling into our third group may have as great a power of response or greater than one in the first group.

We have already mentioned that a few small stimuli may slightly increase an animal's power of response, and so among normal individuals we will have some with a slightly increased power rendering them more easily immunised than those who have not received such slight stimulation. This fourth group consists of persons with varying degrees of a slightly increased power of response, but none is sufficiently immune to give a secondary response to injection.

Our fifth group of the population is of interest. Children are in this group through lack of opportunity to rise higher, but few adults can have been without many opportunities for infection. As we have already suggested for horses, it is possible that Schick positive adults have some other defence, some antibacterial protection so that no infection has persisted long enough for toxin to have been formed. No antitoxin stimulation ever occurs and such adults remain absolutely non-immune and therefore difficult to immunise. Probably few adults are in our fourth group and most of the Schick positives are in the fifth group.

If we are right in assuming that natural immunity in man and in animals is caused by repeated stimulation with small doses of toxin produced by small transient infections, then in any chosen group of children subject to the same general conditions, the number who are already Schick negative must be an index of the number and extent of infection and stimulation. It follows, therefore, that if in any group there are many people falling into our first division, there will be a corresponding large number of the Schick positives in the second and third division, all of whom will respond to artificial immunisation so rapidly that they will become Schick negative in a few weeks, and of those who are apparently non-immune more will be in our fourth than in our fifth group. There is thus a close connection between the Schick negative rate of the population before artificial immunisation and the ease with which they will become immunised. This has been shown by Zingher (1922), who compared the immunising effect of certain toxin antitoxin mixtures upon the children of different schools. Chart 6 shows that the schools with a high natural immunity rate were more easily immunised than those with a low initial rate. The chart also serves to show that one toxin antitoxin mixture used was distinctly weaker than another, but the worse mixture tested on certain schools, F and G and to a less extent on H and K, gave better results than the better mixture on schools D and E. Thus it is evident that the natural immunity rate must always be considered when the efficiency of any protective measure is being judged. Zingher's results were confirmed by O'Brien and colleagues (1923) in England with far fewer numbers, but this work was done with antigens already standardised upon guinea-pigs.

This difference in ease of immunisation of the positive reactors in a naturally highly immune population can be compared to a certain extent with our experi-

ence in immunising horses for the production of diphtheria antitoxin. As a general rule only those horses possessing a reasonable degree of antitoxin (over $\frac{1}{100}$th at least and usually over $\frac{1}{10}$th of a unit per c.c.) are used for immunisation. If our general theory is right, then among pre-war horses with a high antitoxic rate the number of stimuli received and therefore the power of response of the already immune horses must be greater than that of the horses since 1917. In our experience horses now are more difficult to immunise than they were twelve years ago; this may not be the experience of other

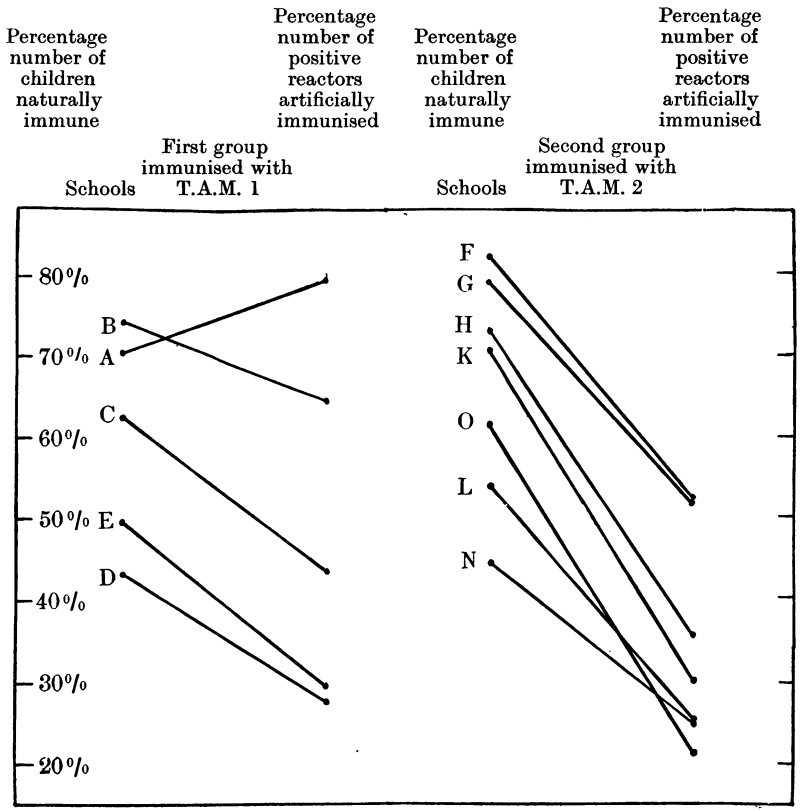

Chart 6. Showing the correlation between immunity rate and rapidity of artificial immunisation in different schools (from figures given by Zingher, 1922).

observers because they may not have met with the corresponding decrease in antitoxic content. Sordelli, for example, uses in the Argentine only horses that are over 12 years old and that have been cast out from the army or police; such horses should have a very high immune rate and should readily respond to immunisation.

We have considered the different stages of immunity in man and in animals; we must now consider methods of immunisation. Animals that are already immune and possess some circulating antitoxin can tolerate the injection of unaltered toxin. Guinea-pigs that are Schick negative can stand 50–100 fatal

doses of toxin; horses with $\frac{1}{5}$th of a unit of antitoxin in their blood can be injected safely with 10 c.c. of strong toxin, $\frac{1}{100}$th of a c.c. of which would seriously affect or even kill other horses without any natural immunity. If the toxin be rendered inert by the action of formaldehyde animals tolerate unlimited quantities. Formalin so modifies the toxin that all specifically harmful effects are destroyed, but the toxoid so formed can still combine with antitoxin and will stimulate the production of antitoxin. The origin of formalinised toxin is difficult to trace. Twenty-five years ago at least the action of formalinised toxin was recognised at these laboratories and elsewhere as a well-known fact; there appears to have been no publication on the subject and I do not know from whom or from what other laboratory we obtained our information. Loewenstein in 1908 first published an account of the action of formalin upon tetanus toxin although he had used such formalinised toxin in 1904 at a time when we also were using formalinised diphtheria toxin on a large scale upon horses. The introduction of preventive inoculation revived interest in toxoid and shortly after the end of the war we produced various batches of toxoid and we eventually replaced the toxin in neutral mixtures by partially modified toxoid. The flocculation test established by Ramon further accelerated the work upon toxoid as this test gave a simple method for testing the strength of such modified toxin which was otherwise extremely difficult to do by animal means.

We find that toxin and toxoid have equal antigenic properties when injected into animals *already immune*, and, further, that the degree of response in the animal is dependent to a great extent upon the amount injected. If, therefore, our problem was concerned only with the immunisation of those people already possessing an increased power of response, then the best antigen would be the most powerful toxoid which can be concentrated and purified by such a method as that published for toxin by Watson and Wallace (1924). A single injection of such material would render anyone in our first three groups Schick negative within a week or two. The chief problem, however, is the immunisation of the non-immune. It is necessary to introduce diphtheria antigen, whether toxin or toxoid, in some form or other in order to produce immunity. Toxin alone, when given in sufficiently small doses to produce no ill-effects, fails as an antigen unless frequent injections are made. For example, it is not possible to inject into a guinea-pig more than twenty or thirty times the Schick dose at one time without seriously affecting the condition of the animal. With such an injection we have never succeeded in rendering a guinea-pig Schick negative. Guinea-pigs have, however, become Schick negative in 25 days from the commencement of the experiment after injecting a total of only fourteen times the Schick dose spread over many days. Other guinea-pigs have become immune within three weeks of the first injection of a daily series totalling less than $\frac{1}{10}$th of the minimal quantity of toxoid that is required as a single injection to bring about the same result. Thus in non-immune animals the time spacing of the injection is of considerable importance.

Theobald Smith (1907) first showed that neutral mixtures of toxin and antitoxin would produce immunity. Park (1913) first used such preparations of a large scale and used what we term 3 L + mixtures, roughly speaking, undilute toxin with sufficient antitoxin added that the mixture causes no serious harm in unprotected children; relatively severe reactions, however, were obtained. A single injection of such a mixture produces reasonable immunity in a guinea-pig. A bigger stimulus, however, is needed for human beings, and it was found that three injections were necessary in order to produce the required effect. Later, Park, Schroder and Zingher (1923) found that $\frac{1}{10}$th L + mixtures were equally successful; that is, they found that if they used only $\frac{1}{30}$th of the amount of total toxin previously used, again partially neutralised, equally good results were obtained.

When we consider the total amount of toxin in the mixture used by Park and Zingher it would be thought that if, instead of giving a small amount of toxin partially neutralised so that only a portion of the toxin would be available for stimulation, we give undiluted and harmless toxoid containing at least 100 times as much antigenic material, high immunity should result. Again, this toxoid can be concentrated at least forty-fold. We have thus a preparation containing many thousand times as much specific antigen than that in mixtures shown to be successful in the past, and yet the results have been disappointing. Certainly the most rapid production of immunity that we have achieved in animals has been with the use of concentrated toxoid; the latent period following a primary stimulus in a guinea-pig which we have already given as about three weeks has been reduced to half that time, and we have been able to detect antitoxin in the blood of a rabbit nine days after a single dose of harmless concentrated toxoid. Improvement in antigenic efficiency has only been slight in spite of great concentration of antigens.

It thus appears that the immunity produced by the injection of an antigen into a non-immune animal does not depend only upon the total amount of antigen injected, the time spacing of the stimulus or the continuance of the stimulus is of great importance. If the material is too rapidly absorbed and eliminated, for example, when unneutralised toxoid is injected intravenously, only slight stimulation may occur, however big the dose injected. The efficiency of toxin antitoxin mixtures depends upon the slow dissociation between the toxin and antitoxin, so that the animal injected is constantly stimulated. Evidence of dissociation can be briefly given in Table VIII. It is possible to produce a mixture of toxin and antitoxin causing no reaction when injected undilute into the skin of an animal; when diluted, $\frac{1}{100}$th of the dose will produce a marked intracutaneous reaction. Similar mixtures cause no oedema when injected subcutaneously into guinea-pigs, but after absorption from the site of injection sufficient toxin becomes free to kill the animal in five days. Most ordinary mixtures of toxin and antitoxin in which the toxin has been sufficiently neutralised to prevent the appearance of any oedema when injected subcutaneously into an animal, will cause late paralysis. If a mixture of toxin and

antitoxin containing phenol is frozen or if more phenol be added to a mixture, toxin can be recovered from combination. If the precipitate that occurs when toxin and antitoxin are mixed be heated, antitoxin can be recovered. This precipitate is equally antigenic in widely different doses; the decrease in the amount of antigen injected is balanced by the increase in dissociation upon dilution. Other points bearing out the same idea of slow stimulation by dissociation of toxin appear to be the efficiency of even highly diluted mixtures; that toxoid partially neutralised with antitoxin is as efficient as unneutralised toxoid when injected subcutaneously, and partially neutralised toxoid is

Table VIII.

Showing evidence of dissociation between toxin and antitoxin.

Injection into guinea-pigs.

Toxin + Modified antitoxin	Injected intracutaneously	0·2 c.c. undilute—no reaction 0·2 c.c. of 1 in 100 dilution—large reaction
Toxin + Modified antitoxin	Injected subcutaneously	No local oedema, death 5th day
Toxin + Antitoxin	Injected subcutaneously	No local oedema, late paralysis

Action of chemical and physical agents.

Toxin + Antitoxin	5% phenol added (or frozen)—free toxin
Toxin antitoxin precipitate	Heated—free antitoxin

Antigenic values.

Lo mixtures are antigenic
Emulsion of toxin antitoxin precipitate equally antigenic in doses of 0·1 c.c., 0·01 c.c., and 0·001 c.c.
Addition of antitoxin to toxoid makes little difference

better than unneutralised toxoid given intravenously. It is suggested that the presence of antitoxin spaces out the stimulation. This shows that one of the aims of our work must be to determine the best method in which to present such a potent antigen as concentrated toxoid in such a way that the maximum stimulation occurs.

. There is another serious complicating factor, and that is the presence of impurities in toxin. We see from Table IX that the usual broth filtrate contains over 99 per cent. of material that we do not want; this material varies in every brew of toxin and has a great bearing upon the ordinary physical and chemical reactions of toxin. Very little that has ever been written about such reactions is true for every kind or batch of diphtheria toxin. It is even possible that when pure toxin is obtained it will be found to be heat stable. Certainly, different batches of toxin vary considerably in their stability to heat, and when we endeavour to modify different batches of toxin by the action of formalin we find that not only, as would be expected, the amount of formalin necessary depends upon the amino acid content of the toxin but with many batches it is extremely difficult or impossible completely to modify the toxin without considerable destruction of total antigenic material taking place. The same applies equally to precipitation with acid. With one batch of toxin it is possible to precipitate with acid and to recover all the toxin from the precipitate. With another batch of toxin the addition of enough acid to

Table IX.

Composition of diphtheria toxin.

Toxin (broth) filtrate)	99% = non-specific material	Antigenic material	Interference in immunity response
		Pseudo-constituent (bacterial protein)	Severe reactions in certain individuals (mostly adults)
		General broth material	Harmful
	1% = specific antigen	Toxin	Antigenic; combines with antitoxin; causes oedema and death
		Present in approximately equal parts in fresh filtrates; on keeping toxin changes to toxoid or can be completely changed to toxoid by the action of formaldehyde and certain other agents	
		Toxoid	Antigenic; combines with antitoxin; causes no ill effects

produce a precipitate will cause destruction of almost all the toxin and only 5 or 10 per cent. can be recovered from either the precipitate or the supernatant liquid.

It must be borne in mind that much of the earlier work upon destruction of toxin by chemical and physical means did not take into account sufficiently the possible modification of toxin into toxoid and if the fatal dose of a toxin had altered it was thought that toxin had been destroyed, whereas frequently toxin might have been changed only into toxoid. It was not until the introduction of the flocculation test by Ramon that it was possible to do any work on the subject on a very large scale. The animal methods for detecting the total toxin and toxoid content are extremely difficult if most of the toxin has been changed into toxoid.

The importance of the non-specific material in toxin cannot be exaggerated. Anyone who has immunised horses on a large scale knows perfectly well that a large volume of weak toxin is not so useful as small volumes of strong toxin and, further, that certain batches of toxin are of far less utility than others of the same strength. Much of the difficulty of immunisation depends upon the non-specific injurious effects of toxins upon horses; any substances tending to lower the condition of an animal reduce its response.

Adults are susceptible to certain constituents of diphtheria toxin other than the specific toxin or toxoid. The severe reactions occasionally reported after the use of toxin antitoxin mixtures or of toxoid are due to the non-specific constituents rather than to the presence of specific toxin. The latter can be detected if present even in minute quantities by means of animal tests, but non-specific material cannot be so detected. The mixture issued from the Wellcome Laboratories for the past year or two has been toxoid diluted tenfold and partially neutralised with antitoxin. The use of toxoid in the place of toxin makes the preparation harmless even without the antitoxin and is a safeguard against such dangers as freezing. The antitoxin delays absorption

and by slow dissociation causes a steady and more continued stimulation. Dilution greatly lessens the non-specific effects.

Again, if any of the non-specific substances present are themselves antigens the response to diphtheria toxin will be decreased. We have shown by repeated injections of toxin antitoxin mixtures or of toxoid into rabbits that the response in antitoxin production is lessened if at the time of stimulation the animal is

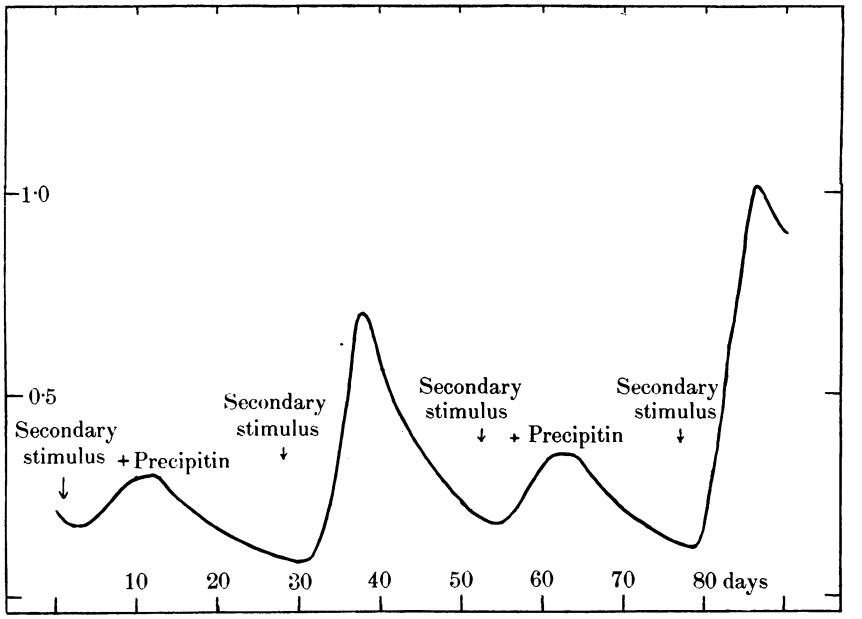

Chart 7. Showing the reduction of antitoxin response owing to precipitin formation. Antitoxic value in units per c.c.

Table X.

Showing the highest titre of serum obtained from horses injected with one, two or three different toxins.

Horse	A	B	C	D	E	F	G	H	K	L	M	N
Tetanus	1,000	—	—	300	600	500	—	—	400	300	200	200
Vibrion septique	—	10,000	—	1,000	—	—	2,000	10,000	300	300	1,000	3,000
B. welchii	—	—	5,000	—	1,000	2,000	4,000	3,000	500	1,000	1,000	100

Vertical columns represent single bleedings of a horse. Figures represent units per c.c.

1 unit tetanus = 10 times the amount protecting against 100 M.L.D. (for guinea-pigs) of a certain toxin.
1 unit vibrion septique = the amount protecting against 10 minimal oedema causing doses (in mice) of a certain toxin.
1 unit B. welchii = twice the amount protecting against 2 M.L.D. (for mice) of a certain toxin.

producing precipitin to horse serum. This may be seen in Chart 7. We had instances during the war of a similar lowering of antitoxic response when an animal is called upon to produce several antibodies at once. Table X shows that the highest value of tetanus antitoxin was 1000 units per c.c., when the horses were not injected with other toxins, 300 when producing either *B.*

welchii or vibrion septique antitoxin as well, and 200 to 400 when producing both these antibodies. Similar lessened values were obtained for *B. welchii* or vibrion septique serum when obtained associated with the other antibodies.

We may briefly summarise our main points by saying that any antigenic response depends upon the immunity state of the individual or animal as well as upon the type of antigen. Such a statement as "a single injection of a given antigen has rendered a man Schick negative in 6 days" is to be put to the credit of the man probably more than to that of the antigen. The true test of any type of antigen would be the rate at which it would immunise the Schick reactors in a population with a very high positive rate. We have also seen that there is little point at present in considering the relative merits of toxoid and of toxin antitoxin mixtures; we must rather consider the relative merits of toxoid prepared in different ways, and of toxin antitoxin mixtures prepared from different types of toxin.

Our chief line of work for the future lies in the investigation which Dr Watson and I, with other colleagues, are now carrying out upon the factors affecting the production, modification, precipitation and destruction of toxin in the hope that we may be able to trace the influence of some of the complicating non-specific materials upon the antigenic values of toxin.

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