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THE significance of the presence of natural diphtheria antitoxin in horses does not appear to have been as fully recognised as its importance warrants. Kolmer for example, writes as follows: "There is absolutely no way of judging which horses will produce the highest grades of antitoxin. Roughly estimated, those horses that are extremely sensitive and those that react feebly are the poorest, but there are exceptions even in these cases. The only reliable method, therefore, is to bleed the horses at the end of six weeks or two months and test their serum. As shown by Park and Zingher, persons yielding negative Schick tests respond to toxin-antitoxin injections with the production of antitoxin more readily than persons who react in a positive manner. Taking advantage of these data, Hitchens and Tingley have injected 0·2 c.c. diphtheria toxin, equal to 3 M.L.D. for 250-grm. pigs, into the conjunctiva of one eye of horses under examination for the purposes of immunisation, and found that many yielded negative tests read at the end of forty-eight hours, indicating the presence of natural diphtheria antitoxin in the blood of normal horses; these animals are probably to be preferred in the production of antitoxin, as they are likely to yield highly potent sera." Again, the bacteriological committee of the Medical Research Council (*Diphtheria* 1923, page 129) say: "It has been found (Bolton, 1896) that the serum of some normal untreated horses shows a certain degree of antitoxic power. Do such horses prove to be the best antitoxin producers? Apparently not. Bolton found that those horses whose serum was normally antitoxic tolerated toxin unusually well, but produced no more antitoxin than the others." Cobbett (1899) stated that his own experience on the point was not sufficiently advanced to win any expression of opinion. Sordelli on the other hand points out (1921) that old horses have more natural antitoxin than young ones. Since horses with natural antitoxin can be given much larger initial doses of toxin than those without natural antitoxin, Sordelli expresses his preference for horses more than twelve years old.

Our experience with many hundreds of horses within the last fifteen years leaves us with no room for doubt that horses containing natural antitoxin are much more readily immunised and give serum of higher titre within reasonable time limits than those with no natural antitoxin.

We would here point out that such a phrase as "without normal antitoxin" is frequently used in the literature without a clear definition of its meaning. The phrase must always mean "with either no antitoxin or at least no detectable antitoxin." In the early history of immunology, the smallest detectable amount was probably about 0·1 unit per c.c. As the methods of titration improved, the

minimal amount detectable became smaller; to-day when we use the phrase we mean "with no detectable antitoxin, *i.e.* less than 0·0005 unit per c.c.," for this quantity is the least we can with certainty detect by the Römer technique.

Many horses before treatment contain sufficient antitoxin to enable them easily to tolerate from 1 to 10 c.c. of strong diphtheria toxin while other horses containing no normal antitoxin would be seriously affected or even killed by 0·01 c.c. of this toxin. Obviously the two classes of animals need different treatment. The antitoxic value of normal horses has been shown by Glenny and Sudmersen (1921) to represent active immunity.

Horses with some antitoxin in their blood rapidly respond to an injection of toxin and Glenny and Sudmersen (1921) have shown that 73 out of 103 such horses produced over 1 unit of antitoxin per c.c. within 10 days of an initial injection of 1 c.c. of a weak toxin. In our experience, horses with little normal antitoxin are seldom worth including in a stud intended to produce diphtheria antitoxin; all horses with less than 0·01 unit per c.c. and when practicable those with less than 0·1 unit per c.c. are discarded or used for other purposes. In one small group of horses whose ultimate ability to produce antitoxin was compared with the normal value, it was found that 11 out of the 15 horses with less than 0·01 unit per c.c. failed to produce usable antitoxin while only 4 out of 34 containing more than 0·01 unit failed.

It is also our opinion that the ability to respond to hyper-immunisation depends upon continued activity of response in the past to natural infection and that this practised activity is not represented by the antitoxic level of the blood at any given time. It is our experience that although a horse without normal antitoxin may be stimulated by the injection of toxoid or toxin-antitoxin mixtures to produce small amounts of antitoxin yet such a horse does not reach the same degree of utility for production as the majority of horses found naturally to possess the same level of antitoxic content. There are thus degrees of immunity depending on the one hand upon experience and on the other upon the antitoxic level reached. The failure of certain horses possessing natural antitoxin to produce high grade antitoxin may be because the antitoxic titre of their blood by which their capacity for production is judged is not always a correct indication of their activity of immunity. A horse with 0·1 unit per c.c. who first developed natural immunity many years ago has probably become a better antitoxin producer than one with the same amount of antitoxin who first developed natural immunity only a few months ago.

Much time can be saved by determining the initial dose of toxin that can be tolerated by each individual horse and so it has been our custom since 1907 at these laboratories to test the normal antitoxic value of all horses intended for routine production of diphtheria

antitoxin and to decide upon the initial dose of toxin to be given according to the results of such tests. At first it was hoped to obtain a rapid decision by injecting all horses with 1 c.c. of very weak toxin and judging the immunity of the horses by the size of the local reaction 24 hours later. This afforded a convenient guide; out of 25 horses with less than 0·1 c.c. 73 per cent., while out of 55 horses with more than 0·1 of a unit only 9 per cent., gave large reactions.

Since 1907 we have tested the antitoxic content of the blood of about 1350 normal horses at the time of their arrival at these laboratories. Our methods of testing during the period covered by these statistics have varied, and the levels at which we have tested for antitoxin have also varied. During the greater part of the time it has been possible to say whether any horse was over or under 0·2 unit and also whether it was over or under 0·1 of a unit, but during 1916 or 1917 horses were only tested at the 0·2 unit level and figures for the 0·1 unit level are missing, while during 1918 to 1919 the reverse was the case. Table I. records the number of horses

TABLE I.

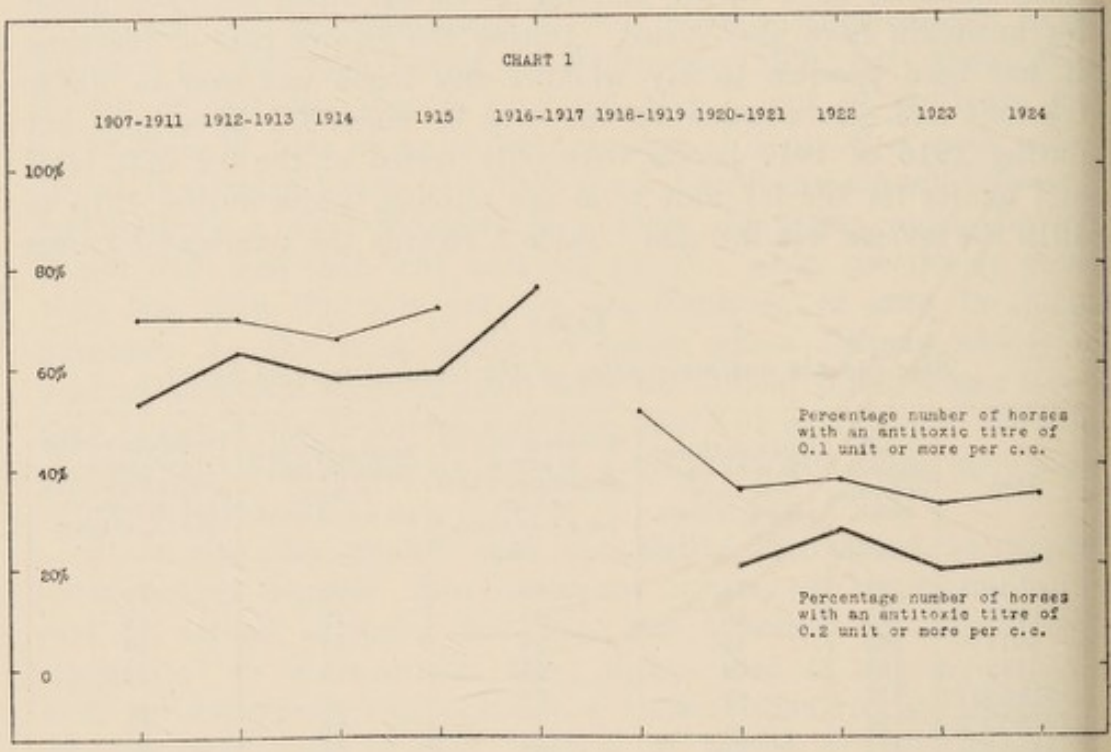
Showing the antitoxic value of the blood of normal horses.

Year.	Total number of horses tested.	Number of horses with 0·2 unit or more per c.c. of blood.	Percentage number of horses containing 0·2 unit or more per c.c. of blood.	Number of horses with 0·1 unit or more. per c.c. of blood.	Percentage number of horses containing 0·1 unit or more per c.c. of blood.
1907-1911	98	52	53	69	70
1912-1913	103	65	63	72	70
1914	103	60	58	68	66
1915	184	109	59	132	72
1916-1917	117	89	76	...	...
1918-1919	126	...	...	64	51
1920-1921	182	37	20	64	35
1922	168	46	27	63	37
1923	120	23	19	38	32
1924	150	32	21	51	34

tested and percentages above or below these two levels. The years 1916 to 1919 form a slight break in the continuity of the records. The table has been prepared by grouping together the figures for succeeding years until results from 100 or more horses were available. It will be seen from the figures for 1907 to 1915 that the percentage number of horses containing 0·2 or 0·1 unit respectively remained constant during this period. Of 488 horses tested 58 per cent. contained 0·2 unit or more per c.c. of blood and 70 per cent. contained 0·1 unit or more.

From 1920 however far fewer normal horses were immune: of 620 tested 22·3 per cent. contained 0·2 or more and 34·8 per cent. contained 0·1 unit or more. These differences would appear truly

significant. It must be admitted that the methods of testing for antitoxin for the periods were different. In the earlier period each titration was made by subcutaneous injection into single guinea-pigs, in the later period titrations were made by intracutaneous injection; but direct comparisons of the subcutaneous and intracutaneous methods have never shown any discrepancy and we are confident that the change of method does not invalidate our figures. The percentage of horses during 1918 to 1919 showing over 0·1 unit, *i.e.* 51 per cent., is intermediate between the 70 per cent. of the earlier period and 34·8 per cent. of the later period and this suggests that the reduction in the number of immune horses was gradual. This figure was



arrived at by the intracutaneous method so that we have by the one method of testing the figures of 51 per cent. for 1918 to 1919 compared with 32 to 37 per cent. in subsequent years.

The difference in the degree of immunity of normal horses during the period 1907 to 1917 as compared with 1918 to 1923 is also shown graphically in chart 1. The explanation is probably connected with the gradual decrease in the number of horses used in the towns owing to rapidly increasing motor traffic; this decrease was greatly accelerated during the war. The majority of our horses in the early period under review were of the type used in tradesmen's carts and in cabs. It is probable that fewer horses which we bought in the later period had the same early history but many probably had had a more isolated existence in country districts. It might be mentioned here that the great majority of horses tested were over seven years old.

Since 1920 the serum of each horse has been tested out at definite levels shown in table II. At present the lowest level for

TABLE II.

Showing classification of horses according to antitoxic value of blood.

Antitoxic value in units per c.c. of blood.	1920.	1921.	1922.	1923.	1924.	Total.
Less than 0·0005 . . .	...	18	19	29	28	94
Between 0·0005 and 0·001 .	...	3	6	9	8	26
Less than 0·001 . . .	...	5	12	2	...	19
Between 0·001 and 0·002 . .	...	1	5	3	6	15
Less than 0·002 . . .	15	2	2	...	...	19
Between 0·002 and 0·004 . .	6	3	2	3	5	19
Less than 0·004 . . .	3	1	...	...	...	4
Between 0·004 and 0·01 . .	7	4	2	8	8	29
,, 0·01 ,, 0·02 . .	7	7	8	2	11	35
,, 0·02 ,, 0·04 . .	2	7	28	10	15	62
,, 0·04 ,, 0·1 . .	12	15	21	16	18	82
,, 0·1 ,, 0·2 . .	14	13	17	15	19	78
,, 0·2 ,, 0·5 . .	14	8	31	13	22	88
,, 0·5 ,, 1·0 . .	6	3	6	5	5	25
,, 1·0 ,, 2·0 . .	3	1	4	1	3	12
,, 2·0 ,, 5·0 . .	1	...	5	3	0	9
,, 5·0 ,, 10·0 . .	1	...	...	...	2	3
Over 10	...	...	...	1	...	1
Total . . .	91	91	168	120	150	620

which we test is 0·0005 unit. The existence of very small quantities of antitoxin between, say 0·0005 and 0·001 unit per c.c., needs several carefully controlled tests to establish with any certainty. If an intracutaneous reaction is obtained when testing at either the 0·0005 or 0·001 level it is easy to say that the serum contained less than this amount, but the dilution of toxin used is so great that guinea-pigs may fail to respond through a variety of causes, and unless a negative reaction at the 0·0005 level, indicating the presence of small traces of antitoxin, be repeated it is difficult to determine with certainty whether the failure of guinea-pigs to react is significant. It has also happened several times in the last few years that the test toxin has weakened slightly in specific toxicity although the combining power has remained unchanged. This toxin has been of little use for titration below a 0·002 or 0·001 level. For this reason it has not always been possible to test sera to the same extent and in the classification of horses given in table II. it will be found that in addition to such levels as "under 0·0005" "between 0·0005 and 0·001," "between 0·001 and 0·002," etc., we have had to include three additional groups "under 0·001" and "under 0·002" and also "under 0·004" when the toxin was untrustworthy in tests at lower levels. With the exception of these few horses whose titre was not accurately fixed, the serum of each horse of the 620 tested during this period was tested several times until a

definite reaction had occurred at one level and no reaction was found at our next level of testing. The figures given in table II. show that approximately equal numbers of horses contained less than and more than 0·04 unit per c.c. Because of the frequent error involved in testing for less than 1/1000 of a unit (particularly in hot weather when toxin dilutions may deteriorate in a few minutes) it is probable that the majority of horses recorded as containing between 0·001 and 0·0005 unit per c.c. really contained less than 0·0005. It was only when for various purposes it was essential to determine with certainty the absence of all detectable antitoxin that confirmatory tests at this level were made.

Fluctuations in antitoxic value.

It occasionally happened that horses were tested on more than one occasion without having received an injection of diphtheria toxin. A few were tested repeatedly at short intervals.

The best examples to record are those of certain normal horses that were injected with diphtheria antitoxin obtained from other horses and the rate of loss of the passive immunity so conferred was determined by tests upon samples of blood taken at weekly intervals from these horses. Some of these results have already been published by Glenny and Hopkins (1923) who showed that the course of loss of passive immunity is such that the logarithms of the antitoxic titre of the horse fall upon a straight line such as that, 980 (1), shown on chart 2 for the first injection of horse 980. This horse before its injection with antitoxic serum contained less than 0·0005 unit per c.c.; at the end of about twelve months the antitoxic titre had again fallen below this level. A similar curve, 981 (1), was also published and is shown on chart 2 for the first injection of horse 981. Horse 982 however shows a most irregular feature.

> It was pointed out (Glenny and Hopkins, 1923) that horse 982 "still showed a high antitoxic content over 40 weeks after injection and little or no loss occurred from the 29th to the 40th weeks and then a marked increase in antitoxic value was seen. The various readings of the antitoxic content plotted against time do not fall upon a straight line. It is, however, possible to draw, as we have done in curve 59, a series of short sections parallel to curve 58; these sections are linked with lines roughly parallel to the base line or showing a tendency to rise. Each such interrupted section represents an active immunity response. At the time of injection of diphtheria antitoxin this horse already possessed a normal antitoxin value of 0·008 of a unit per c.c.: the other two horses, 980 and 981, both contained less than 0·0005 of a unit. Horse 982, already actively immune, could respond to any accidental stimulus by a rapid production of antitoxin, thus showing an irregular curve made up of constantly diminishing passive immunity and frequently varying active immunity."

Since the above was written many other titrations have been made upon weekly samples of blood from horse 982, and horses 980 and 981 have each received another injection of antitoxic serum. The antitoxic titres of all the samples of blood tested are shown graphically on chart 2.

Horse 982 affords a good illustration of the course of production of active immunity naturally acquired by an animal. At first the responses are small, and the rate of loss of apparent immunity is delayed only two weeks, but later

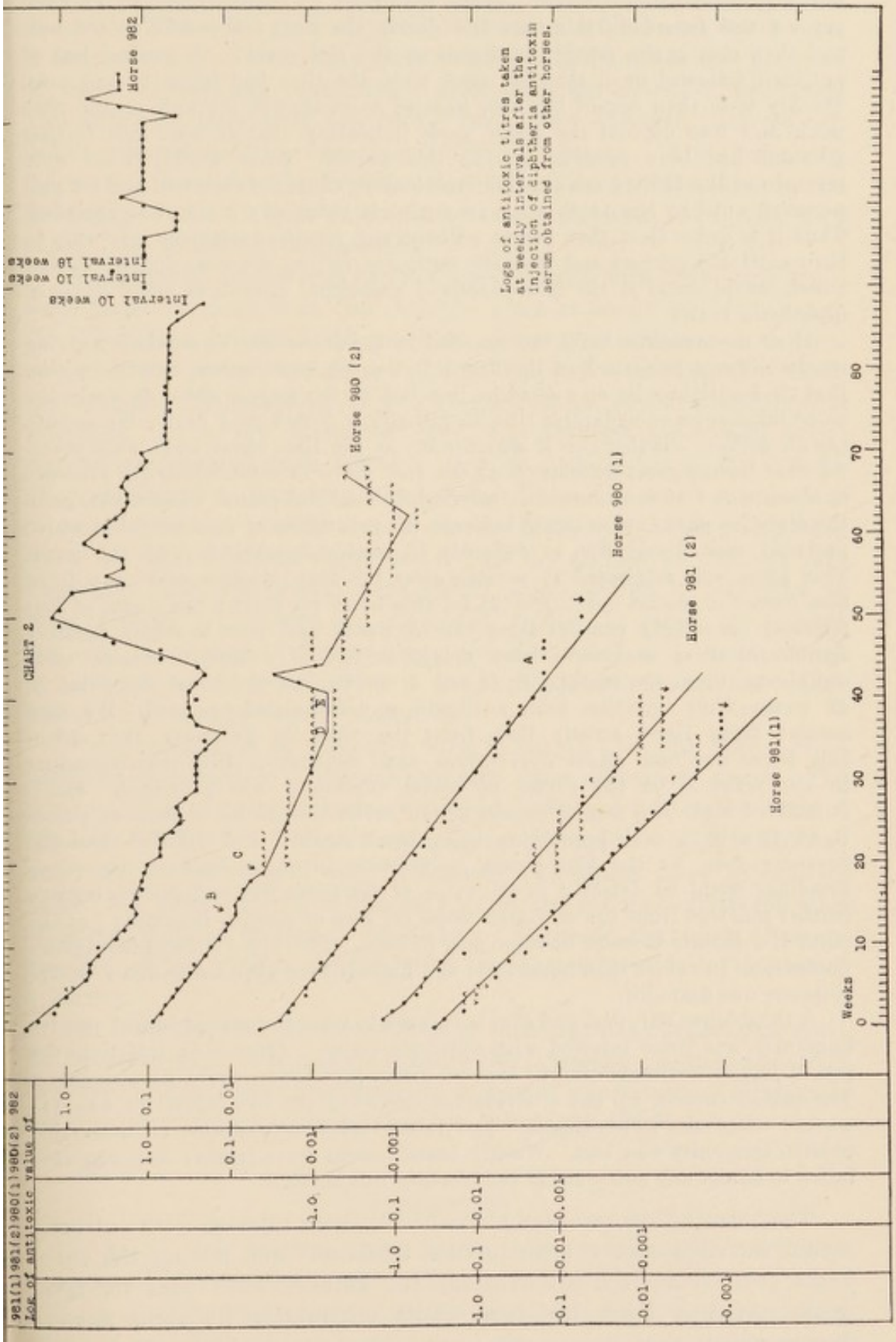


CHART 2
Logs of antitoxic titres taken at weekly intervals after the injection of diphtheria antitoxin serum obtained from other horses.
Horse 982
Interval 18 weeks
Interval 10 weeks
Interval 10 weeks
Horse 980 (2)
Horse 980 (1)
Horse 981 (2)
Horse 981(1)
A
B
C
D
E
Weeks
0
10
20
30
40
50
60
70
80
981(1)981(2)980(1)980(2) 982
for of antitoxic value of
1.0
0.1
0.01
0.001

the responses increase until eventually an increase in antitoxin content of one unit may occur within a week. At the 50th week an antitoxic titre of 1·5 units per c.c. was recorded; this titre fell during the next few weeks to 0·2 unit and then rose again, reaching 0·6 unit at the 59th week. A gradual loss of antitoxin followed until the 88th week when the titre had fallen to 0·02 unit. Weekly tests then ceased but two isolated tests showed 0·1 unit at the 98th week and 0·25 unit at the 108th week indicating that at least one further stimulus had been received during this period. When weekly tests were resumed at the 126th week small fluctuations in value between 0·04 and 0·2 unit occurred until at the 145th week an antitoxic value of 0·5 unit was recorded. Thus it is quite clear that horses without any specific treatment may vary in their antitoxic content and that the antitoxin follows a course similar to that which would occur if the horse received occasional stimuli of small doses of diphtheria toxin.

If we now consider horse 980 recorded by the curve, 980 (1), on chart 2 giving results already published of the first injection of horse serum, it will be seen that the logarithms lie on a straight line but at the end of about 65 weeks the value takes some considerable time to fall below 0·0015 of a unit. We cannot say for certain whether this is significant; at the time these tests were made we were having great difficulty with our test toxin in establishing the presence or absence of 1/1000 of a unit. This slightly doubtful period when a change in the direction of the curve might indicate the production of immunity but which probably was merely due to difficulty in testing is marked A on the curve. This horse was reinjected 17 months after the first injection and as will be seen from the second curve, 980 (2), for this horse on chart 2 the course of loss followed on exactly parallel lines for 14 weeks and then a slight, possibly significant, delay occurred; later delays in loss of antitoxin became more significant (these are marked B, C and D on the curve) but at the point E, 42 weeks after injection with antitoxin, a definite rise occurred. We may assume from these results that from the time in February 1922 when this horse was first under observation until September 1923 (corresponding to the point B on the curve) no active immunity was produced. Small stimuli of unknown origin caused active production to be evident at points B, C, D and E, each production being more marked and definite than the previous one. At the first points no increase in value between successive bleedings could be detected in the slope of the curve but each section became further removed from the standard slope for loss of passive immunity; at the point E a definite increase in value may be seen. This horse showed far smaller fluctuations in values than horse 982; the highest titre apparently due to active response was 0·03 unit.

A third horse 981, that had also been used to trace the rate of loss of passive immunity, was twice injected with antitoxic serum. After both injections the rate of loss of antitoxin showed that no active production could have occurred. The period covered by the observations following the two injections was 114 weeks; after the antitoxic titre had fallen below 0·0005 unit all detectable passive immunity was lost. Weekly examinations for a further 30 weeks have failed to detect any antitoxin in the blood of this horse.

The three horses recorded above had all been injected with antitoxic serum but had received no further treatment and during the three years covered by the observations the animals were used for farm work. Another horse has been under observation for eight months during which time it received no injection of any kind. This horse was not used for a passive immunity experiment but was frequently

bled to supply horse serum. When the horse first arrived at the stables a small amount of antitoxin was detectable, 0·004 unit per c.c. of blood. The titre gradually fell to between 0·0005 and 0·001 unit. A large number of tests were made establishing definitely the presence of traces of antitoxin until five months after the arrival of the horse. The antitoxic titre then fell below the lowest strength that we can detect with certainty, *i.e.* less than 0·0005 unit per c.c. After remaining below this level for three weeks the value rose to 0·004 and in the following week to 0·02 unit and remained between 0·01 and 0·02 during the following two months.

A number of horses that had not been injected with diphtheria toxin were tested on more than one occasion after at least a week's interval. Of 131 horses so tested, 26 per cent. showed an increase in normal value on the second occasion. It would appear therefore that there is a tendency for horses arriving at a large stud to receive a diphtheritic stimulus. In a few instances very big increases have been seen; one horse increased from 0·1 of a unit to over 20 units in 14 days. There appeared to be a tendency for definite changes in normal value to occur at certain periods or with certain batches of horses purchased; thus, out of 16 horses which arrived at the stables in January and February 1923, and which were tested again within a fortnight of their arrival, 10 showed an increased value. It must be pointed out that a number of horses may have fluctuated to a slight extent in antitoxic content but not to a degree detectable within the wider limits that we have usually tested for.

These variations in antitoxic titre indicated that the horses were periodically receiving a specific stimulus from diphtheria toxin. The source of this stimulus remains untraced. We know from the work of Minnett (1920) that *C. diphtheriæ* may be present in wound infections, but our bacteriological and veterinary colleagues have repeatedly looked for wound infection and have examined the mucous membranes of horses without finding *C. diphtheriæ*. Repeated swabbings of every member of the staff in contact with the horses have failed to detect a carrier.

It is evident that the possibility of these normal fluctuations must be borne in mind when investigating the influence on antitoxic titre of horses of injections of non-specific agents, of small doses of diphtheria toxin or of avirulent *C. diphtheriæ* (*cf.* Arkwright, 1909).

Conclusions.

1. As is well known, many horses never subjected to artificial immunisation have diphtheria antitoxin in their blood. Thus, while 0·01 c.c. of an ordinary brew of strong diphtheria toxin would seriously affect or even kill many normal horses, others contain so much normal antitoxin that 10 c.c. of the same toxin would not harm them.

2. Horses possessing natural antitoxin in their blood produce antitoxin rapidly; those with no normal immunity do not respond rapidly to immunisation.

3. The source of natural immunity possessed by some horses has never been satisfactorily identified.

4. Of 1350 horses tested on admission during 17 years about half contained 0·1 unit or more antitoxin per c.c.

5. The percentage of horses possessing normal immunity has greatly decreased in recent years.

6. Horses possessing normal immunity showed considerable fluctuations of antitoxic titre while under observation though no injection of diphtheria toxin had been given. The source of the stimulus was not found.

REFERENCES.

ARKWRIGHT, J. A. . . . . *Journ. Hyg.*, 1909, vol. ix. p. 409.

BOLTON, B. M. . . . . *Journ. Exper. Med.*, 1896, vol. i. p. 543.

COBBETT, L. . . . . *Lancet*, 1899, vol. ii. p. 332.

GLENNY, A. T., AND HOPKINS, B. E. *Journ. Hyg.*, 1923, vol. xxii. p. 37.

GLENNY, A. T., AND SUDMERSEN, H. J. *Journ. Hyg.*, 1921, vol. xx. p. 176.

KOLMER, J. A. . . . . Infection, Immunity and Biologic Therapy, 1923, ed. 3, p. 213.

MINNETT, F. C. . . . . *Journ. Comp. Path. and Therapy*, 1922, vol. xxxv. p. 291.

SORDELLI, A. . . . . *Revista de la Asociacion Medica Argentina*, 1921, vol. xxxiv., No. 199.

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