FACTORS INFLUENCING THE DEVELOPMENT OF POTENTIAL IMMUNITY AND THE CHARACTER OF THE SECONDARY RESPONSE.

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IN 1931 Glenny stated : " Antigens suitable for human use have not yet been produced of sufficient potency to immunize successfully by means of a single injection. The power of an antigen therefore to induce high immunity in a guinea-pig after a single dose is not necessarily an index of its value for human immunization, but is of use in comparing the relative strengths of different batches of the same preparation." After a lapse of twenty years these observations still appear to hold good.

The method used for testing a batch of antigen should depend on the purpose for which the material is intended. Antigens may be used (1) for the hyper-immunization of horses in the production of therapeutic sera, and (2) for the prophylactic immunization of animals and man. Group 2 may be further sub-divided into prophylactics intended to establish basal immunity, and those required to boost antitoxic levels that have fallen with the passage of time. It is possible (Barr and Glenny, 1951) that the ideal course of human immunization should include the use of more than one type of prophylactic.

Carlinfanti (1950) stated that " the two-dose method is totally inadequate to reveal quantitatively the difference between a good and a poor prophylactic." In his work he was comparing different types of diphtheria prophylactic, to which the primary responses could not be expected to be comparable. In contrast to this, Barr and Glenny (1949), in work restricted to the comparison of crude diphtheria formol toxoids, failed to find a satisfactory single dose that would detect differences so well as the two-injection method.

Most workers interested in the prophylactic immunization of man against diphtheria and tetanus would agree with the aims of active immunization suggested by Barr and Glenny (1951). These include the establishment of sound potential immunity, the production of good antitoxic titres, and the maintenance of some antitoxin in the circulation for many years after the course of prophylactic injections. We are of the opinion that at the present time this state of affairs cannot be brought about in man by a single injection of any type of prophylactic. It therefore appears to us that, unless strong evidence to the contrary is forthcoming, Carlinfanti's one-injection method should not be used as a routine test for preparations intended for human immunization. In our experience a single-dose method may be of great value when used for the purpose suggested by Glenny (1931), but it does not measure the degree of potential

immunity established. We have found (unpublished) that a very small dose (0·05 Lf dose) of an adsorbed diphtheria prophylactic may confer excellent potential immunity in guinea-pigs that at no time showed detectable circulating antitoxin after the injection. This potential immunity was demonstrated by the appearance of 1 unit or more of antitoxin per ml. in the circulation of most of the animals 10 days after a second injection given as long as seven months after the first. Individual results were very variable, however, and some animals gave a poor response. This result, together with others we are recording in this paper, suggests that potential immunity had declined in the poor responders during the long interval between the two injections.

While a temporary advantage is gained in prophylactic immunization by antitoxin-production after a single injection of prophylactic, it appears to us that so long as additional injections are needed, the establishment of basal immunity should be regarded as the most important function of the primary stimulus. A good secondary response should follow the second injection, and in order to ensure this the first dose should be relatively large.

Experience in hyperimmunization (Barr and Glenny, 1945) has shown that potential immunity increases long after antitoxin-production has declined following prophylactic injections. This conclusion was drawn from the fact that in the production of tetanus antitoxin, horses given preliminary injections many months before their hyperimmunization course produced antitoxin of better quality and higher value than those given a shorter resting period. (The term " quality " refers to the degree of avidity as measured by the serum ratio *in vivo* value/*in vitro* value : a low value for this ratio indicates loose combination of the antitoxin with toxin, so that dissociation occurs *in vivo*).

The experiments recorded here show that the amount of antitoxin produced from primary stimulation may bear little relation to the degree of potential immunity established by different prophylactics, and that if too long a delay is left between the first and second injections, potential immunity, judged by values 10 days after a second injection, may decrease. This suggests that the spacing of injections and of boosting doses is of great importance in human immunization, and it appears reasonable to suppose that there exists an optimal interval, most suitable for the majority of persons, which would be dependent on the nature of the prophylactic and the dose used in relation to body-weight.

Antitoxin-production in guinea-pigs after primary stimulation with diphtheria prophylactic.

Two groups, each of eighty guinea-pigs, were injected subcutaneously, the one with 1 Lf dose of P.T.A.P., and the other with 1 Lf dose of A.P.T. The P.T.A.P. was a routine matured batch containing 60 Lf doses and 10 mg. $AlPO_4$ per ml. All injections were made in a dose of 1 ml., the material having been suitably diluted in normal saline. The animals were bled at monthly intervals after injection, until they were due for re-injection in the second part of the experiment. All blood samples in this work were titrated individually for antitoxic content at approximately 2-fold differences : the guinea-pig intracutaneous method was used (Glenny and Llewellyn-Jones, 1931), and at least two tests giving the same end-point were made on each sample.

In this particular experiment individual guinea-pigs were not distinguishable. This fact limits the conclusions that we were able to draw, because although the

scatter of values was determined, it is not known whether all guinea-pigs reached the height of their response at the same time. The differences between the values of the P.T.A.P. group and the A.P.T. group were sufficiently great to justify the

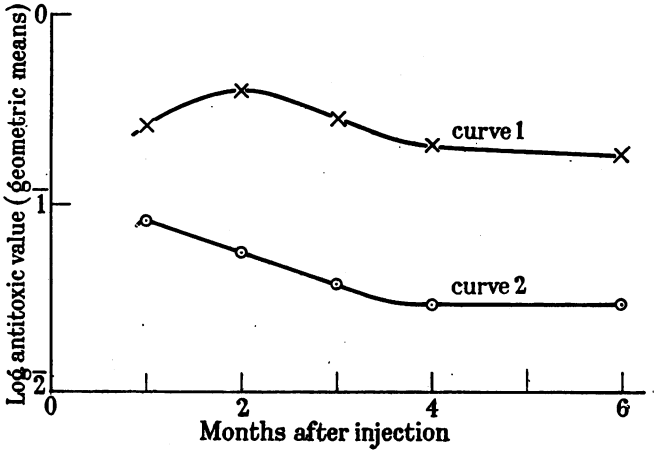

FIG. 1.—The response of guinea-pigs to a single injection of diphtheria prophylactic (geometric means).
Curve 1: 1 Lf dose of P.T.A.P.
„ 2: 1 „ „ A.P.T.

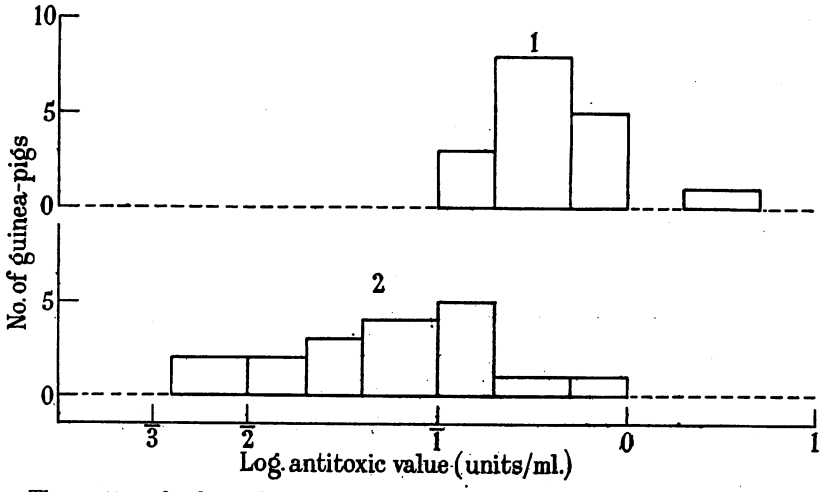

FIG. 2.—The scatter of values of guinea-pig sera 2 months after a single injection of diphtheria prophylactic.
(1) 1 Lf dose of P.T.A.P.
(2) 1 „ „ A.P.T.

comparison of response curves for the two groups. These are shown in Fig. 1, where the logarithms of the geometric means are plotted against time after injection. The peak was reached with P.T.A.P. at two months and that for A.P.T. at one month or earlier, though we are unable to say that all animals reached their peak at these respective times. The values of guinea-pigs injected

with P.T.A.P. were very appreciably higher than those of animals injected with A.P.T., and the scatter of values at two months is shown for both groups in Fig. 2. At this stage the geometric mean for the P.T.A.P. group was at the peak, and that for the A.P.T. group had fallen slightly. It will be seen that the values of the former showed a small scatter, and those of the latter a much larger scatter with a tail below the peak of the distribution. A similar type of scatter was observed at all times when blood samples were titrated. The evidence from Fig. 1 and 2 is in agreement with that presented by Holt (1950*a*) for primary responses to a larger dose of these two prophylactics. It will also be noted in Fig. 1 that a flattening of both curves appeared to be occurring four months after injection.

Holt (1949), apparently using pooled sera, obtained a " baseline " or steady level three months after a single injection of P.T.A.P. in guinea-pigs. In our experience in the tracing of primary response curves in individual but not distinguishable guinea-pigs, falls in value may continue to occur in some animals after four months. This conclusion was drawn from the values obtained from groups in which no deaths occurred in the later stages of the experiment : most of such groups are observed by us for six months after injection. Holt (1949) quotes the observations of Glenny and Sudmersen (1921) in an experiment in which a single dose of toxin-antitoxin mixture was injected subcutaneously into guinea-pigs, the antitoxic titres of which were followed for a period of two years and " for the greater part of the latter period of observation the animals showed a constant titre of circulating antitoxin—the final baseline of immunity." He continues : " Glenny and Barr (1947) have reported a similar phenomenon in horses in which tetanus antitoxin titres were observed over a period of five years following a single injection of tetanus toxoid." Since this observation concerns a matter of some fundamental importance in connection with primary stimulation, we feel it necessary to point out that this work has been misinterpreted by Holt.

Reference to the original paper (Barr and Glenny, 1947) fails to reveal records of any experiments involving single injections of tetanus toxoid, nor were observations made on the antitoxic titres of horses over a period of five years. The experiments recorded by Barr and Glenny (1947) were concerned with the amounts of tetanus antitoxin remaining in the blood of *hyperimmunized* horses about *one year after the last injection of toxin :* one of the horses had been hyperimmunized with tetanus toxin for five and a half years. It is presumably possible that an occasional horse might have detectable antitoxin five years after a single injection of tetanus toxoid, but so far as we are aware, evidence to this effect is lacking.

The effect of a second injection of prophylactic into guinea-pigs.

The groups of guinea-pigs used in the first part of the experiment were each subdivided into four distinguishable groups and re-injected with the same dose of the same prophylactic, but at different times after the first injection. These intervals were 1, 2, 3 and 6 months. The animals were bled 10 days and 1, 2 and 3 months after re-injection and the individual sera titrated for antitoxic content.

The guinea-pigs were not individually distinguishable, but the scatter of values within the groups was small, and response curves have been plotted using the geometric means ; these are shown in Fig. 3.

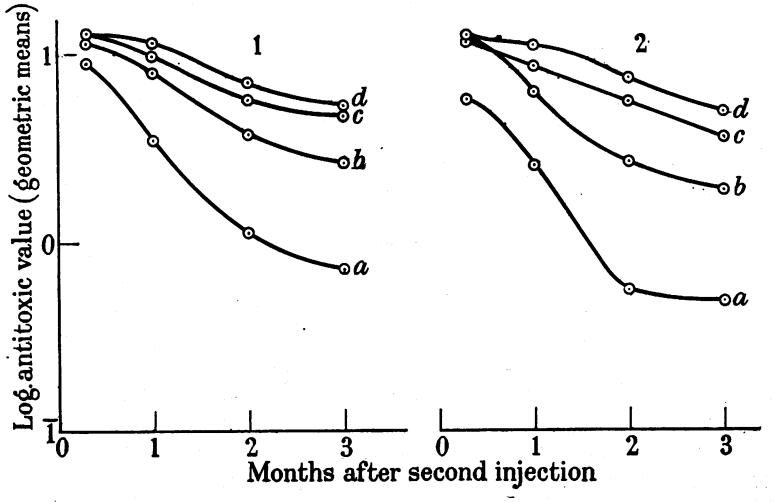

FIG. 3.—The response of guinea-pigs to a second injection of diphtheria prophylactic (geometric means).

(1) Both injections 1 Lf dose of P.T.A.P.
(2) „ „ 1 „ „ A.P.T.

(*a*) 2nd injection 1 month after 1st.
(*b*) „ „ 2 months „
(*c*) „ „ 3 „ „
(*d*) „ „ 6 „ „

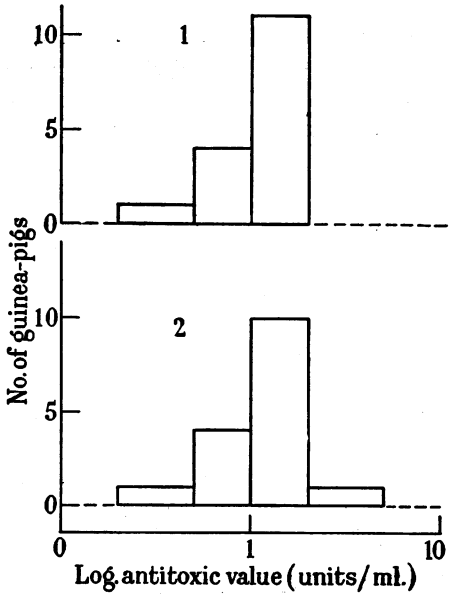

FIG. 4.—The scatter of values of guinea-pig sera 10 days after the second injection of prophylactic given 6 months after the first.

(1) Both injections 1 Lf dose of P.T.A.P.
(2) „ „ 1 „ „ A.P.T.

A remarkable similarity exists between the responses to the second injection of comparable groups injected with the two prophylactics, whatever the interval between injections. It will be noted that an increase in this interval from 1 to 2 months increased the 10-day response: with a further increase in interval this response was not affected within the limits of experimental conditions. The effect of increased interval was slightly greater in the A.P.T. group. A further point of interest is the difference between the rates of fall in value from the height of the response. The rate of loss decreased as the interval between injections was increased.

These observations apply to the responses to the second injection of animals injected with both prophylactics. Whereas considerable differences in response between groups were observed after the first injection, a remarkable similarity was apparent between comparable groups after re-injection with the different preparations. Evidence of this is provided in Fig. 4, which shows the scatter of values in groups of 16 guinea-pigs 10 days after the second injections given 6 months after the first. The results are almost identical.

This experiment shows that potential immunity (judged by the 10-day response to the second injection) may be maintained for a considerable time after the peak of a primary response. It also shows that with a dose of 1 Lf, the potential immunity existing over the period of observation was in general unrelated to the degree of antitoxin-production provoked by the first injection (Fig. 1 and 3). It is probable that potential immunity would eventually decline, but it is not practicable to house and re-inject guinea-pigs over a period of several years. In the next experiment we therefore used P.T.A.P. in smaller doses.

The effect of injection of smaller doses of P.T.A.P.

A group of 100 guinea-pigs, all distinguishable from one another, were injected subcutaneously, each with 0·25 Lf dose of the same batch of P.T.A.P. as that used in the preceding experiment. They were subdivided into five groups according to the interval fixed before the second injection: the intervals chosen were 1, 2, 3, 4 and 6 months after the first injection. All guinea-pigs were bled and the individual sera titrated for antitoxin content immediately before re-injection: three groups had one or more additional blood samples taken. This was done to determine the degree of variation in antitoxic titre and form of primary response curves for different animals treated in the same way.

Fig. 5 shows the antitoxic values of 9 guinea-pigs bled 1, 2 and 3 months after the first injection. One animal failed to produce any detectable antitoxin over the period examined, one reached its highest titre 1 month after injection and the remainder 2 months after injection. The scatter of values at 2 months, when most of the guinea-pigs reached the height of the response, was relatively large, extending from under 0·001 to 0·2 unit per ml. Fig. 6 shows the scatter of antitoxic values among 32 guinea-pigs bled 2 months after the first injection. A comparison of this result with that shown in Fig. 2 (1) clearly illustrates the dependence of primary response on dose. The reduction of the dose to one-quarter greatly increased the scatter of values.

Table I gives the antitoxic values of all guinea-pigs surviving for the duration of the experiment, at the time of receiving the second dose of prophylactic and at fixed chosen times thereafter. The animals are grouped according to the time at

which the second injection was given, and arranged in order according to their antitoxic values 10 days after the second injection.

An excellent degree of uniformity existed among the 10-day values of the guinea-pigs in Group 3. This suggests that there was but little variation in the degree of potential immunity in individuals 3 months after the first injection of 0·25 Lf dose. The scatter of 10-day values was greater in the other groups, and while some of those in Group 4 were higher, others were lower than those of Group 3. This suggests that a further increase in potential immunity occurred in some animals between the third and fourth month after the first injection : in a few there was a decrease. A general decrease appeared to have occurred by the sixth month.

There appears to be a rough correlation between the values produced by individual guinea-pigs after the first and after the second injection. The guinea-pigs in Groups 2, 3 and 4 were all bled two months after the first injection, and a comparison between the values of these samples and those of the same animals 10 days after the second injection is given in Table II. Although the numbers are small, it appears that the majority of animals reaching low values after the first injection did not respond so well to the second injection as did those producing higher values after the first injection.

A striking point emerging from the data in Table I is the variability in the amount of antitoxin lost over the period observed after secondary stimulation. In general the fall in value among good responders, from 10 days to 6 months, decreased as the interval between injections was increased, and was extremely small in some of the animals in Group 5. Owing to the wide limits of our tests rates of loss cannot be calculated, but it is perfectly clear from the figures that many differences in individual rates are well outside the limits of error of the experiment. This is illustrated in Fig. 7, discussed at a later stage : the limits of error in the allocation of values have been shown in plotting these curves.

The loss of antitoxin in the poorer responders cannot be correlated with interval between injections, but appeared to be greater in those animals that had no detectable circulating antitoxin at the time of the second injection. It is of interest to note that the absence of antitoxin at this time does not mean that no antitoxin had ever been detected in these animals after the first injection. All but one had produced antitoxin at an earlier stage. This suggests that the power of continued production is poor in some animals after both the first and the second injection.

Holt (1949) considers that " pure primary response " curves show no peak and that any peaking with time, observed after a single injection, is due to a secondary response effect superimposed on a " pure " primary. He claims to have shown that this peak falls to a base-line value at the same level as that obtained in the " pure " primary response curve. If we accept this hypothesis, then those of our guinea-pigs that had circulating antitoxin 2 months after the first injection and subsequently lost it (4 months after injection) must have produced this antitoxin *entirely* as a result of a secondary response effect. Secondary responses are frequently observed after a second injection of antigen into animals that have produced no detectable antitoxin after the first injection. In this experiment, however, we have obtained what is termed by Holt (1950*b*) a " pure secondary response " following the first injection of a very small single dose of prophylactic into non-immune guinea-pigs.

We agree with Holt's (1949) idea that primary responses are made up of component effects, one of which is similar to the secondary response and is caused by residual antigen. Glenny, Buttle and Stevens (1931) visualized the effect of an injection of antigen into a normal animal as a process of continual stimulation by lessening quantities of antigen, acting on tissues whose power of response is rapidly increasing.

It appears, however, that the terms " pure " primary response and " pure " secondary response are unfortunate. There is no evidence to show that a " pure primary response " as conceived by Holt (1949) is not made up of two factors, one

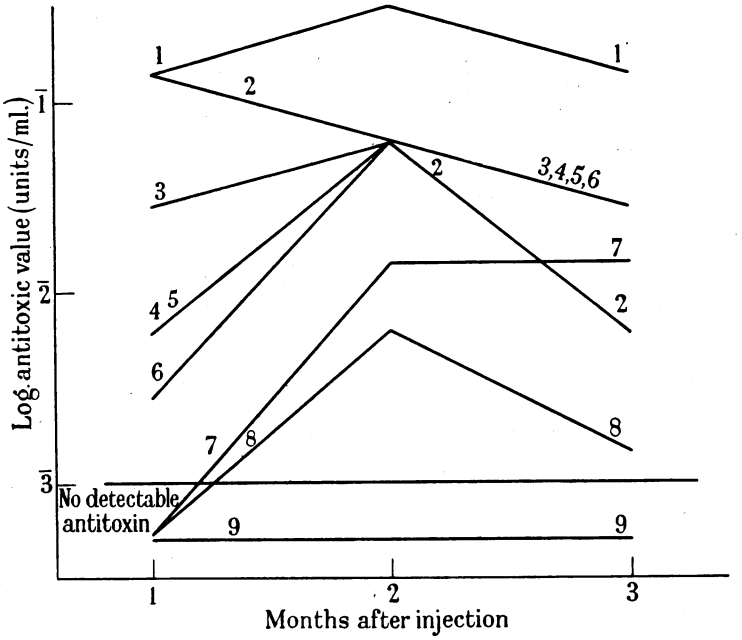

FIG. 5.—The antitoxic values of individual guinea-pigs bled at monthly intervals after a single injection of 0·25 Lf dose of P.T.A.P.

the sensitization of cells or production of potential immunity, and the other the formation of antitoxin. It is departing too far from the essential meaning of the words " primary " and " secondary " to describe the first observable effect after primary stimulation as a " pure secondary response."

The testing of immunity by antitoxin titrations.

In the experiments we have described, individual sera have been tested at approximately 2-fold differences, and confirmatory tests made. Values recorded in Table I, as for example 5 units, are in fact over 5 but under 10 units. In calculating the geometric means of groups and in constructing Fig. 5, the values of individuals were taken as the geometric means of the limits between which they lay ; this means that a value given as 5 units in Table I was taken as $\sqrt{5 \times 10}$ (= 7·07) in the calculation of geometric means.

The volume available for testing the antitoxic value of serum from a guinea-pig is small, and may not suffice for close and accurate testing : the values allotted are not absolute because such sera are frequently non-avid, (Glenny, Barr, Ross and Stevens, 1932 ; Jerne and Maaløe, 1949). The methods used by us have the advantage, however, of revealing individual variations in value and type of response among the animals of a group.

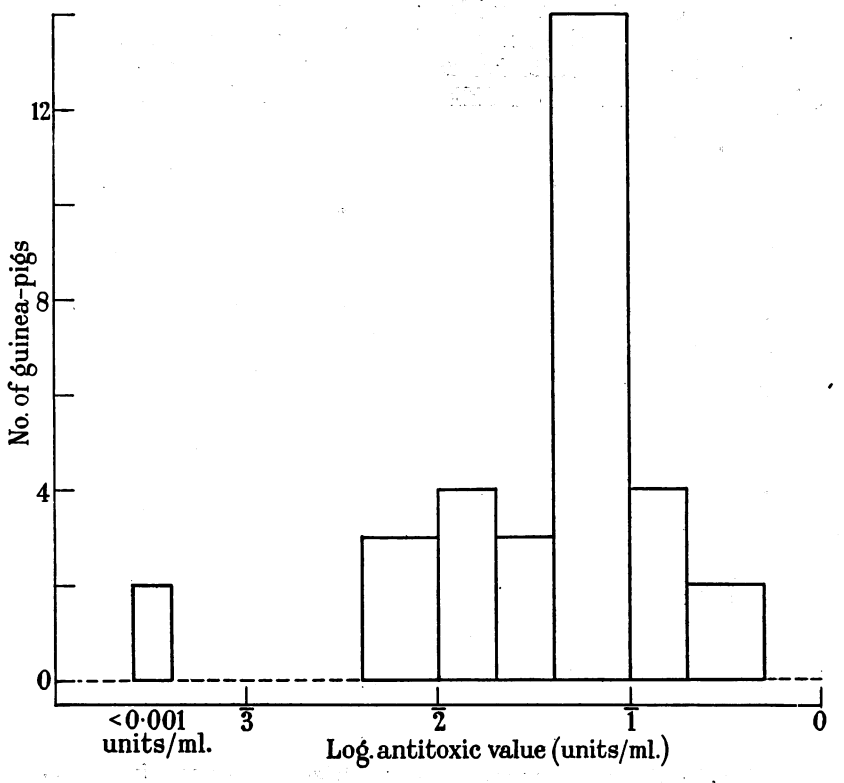

FIG. 6.—The scatter of antitoxic values of sera among 32 guinea-pigs 2 months after a single injection of 0·25 Lf dose of P.T.A.P.

It is unfortunate that conclusions of a fundamental nature have been drawn by some workers on the results of tests on pooled samples. The pooling of samples provides sufficient material for close accurate tests to be made, but individual variation is quite unknown, and essential information is thereby lost ; as a result unjustifiable conclusions may be drawn.

Faragó and Pusztai (1949), using pooled sera from rabbits injected with a combined diphtheria-tetanus-pertussis prophylactic, claimed to have obtained a two-stage secondary response to the tetanus constituent ; the antitoxic value did not rise in the usual uninterrupted manner to the peak of the response. Glenny, in his Sir Almroth Wright Lecture (May, 1950), pointed out that such a two-stage secondary response could apparently be obtained by plotting the arithmetic means of values of the sera of horses that did not reach the peak of the response

at the same time after injection. While it is not denied that a two-stage response might conceivably occur, no such phenomenon has been observed by us in individual animals, and we suggest that the unexplained result of Faragó and Pusztai may have been obtained fortuitously, from the testing of pooled sera from animals that responded in a dissimilar manner.

While the values obtained for pooled sera may represent the average for a group, it is also possible that they do not represent the value of any single member of the group, and one abnormally high value may raise the mean so excessively as to convey an entirely false impression of the whole. More especially is it dangerous to work out quantitative data from response curves constructed from values of pooled serum bled on different occasions. If the animals concerned

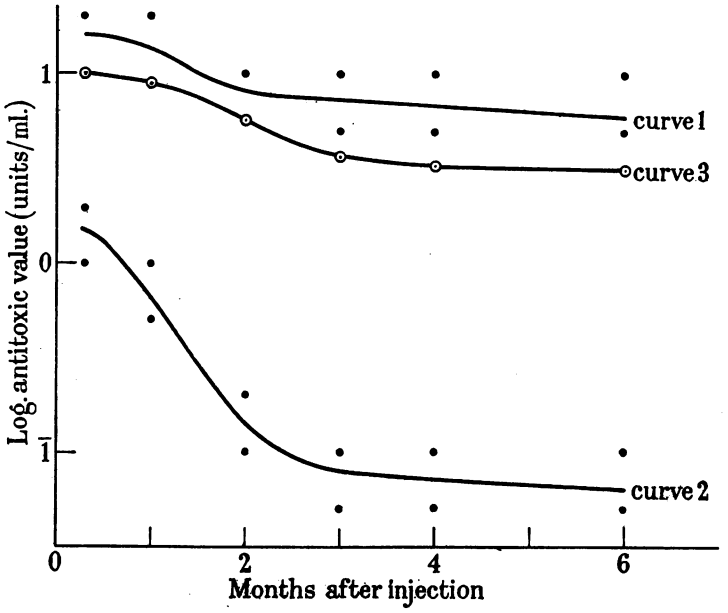

FIG. 7.—Secondary response loss curves taken from Group 5 in Table I.
Curves 1 and 2 responses of individual guinea-pigs.
Curve 3 values of the arithmetic mean for the whole group.
Vertical dots give the limits within which individual observations lie.

reached the peak of their response at different times, and the subsequent rate of loss varied in the group, a curve might be constructed which was truly representative of none of them. Thus a curve constructed from the arithmetic means of a number of individual curves, all showing sharp rises and falls, might show a gradual rise to a peak maintained for a considerable time if the individuals reached their peaks at different times. The information lost in pooling sera in the tracing of response curves includes (1) the scatter of values and (2) the configuration of individual curves: both these are of the utmost importance. Owing to the relatively sharp rise and fall of many secondary response curves, a knowledge of the second factor is usually more important in the tracing of such curves. The chief variable in primary responses is frequently the scatter of values, but the effect of both factors can be seen for primary responses in Fig. 5. Only when

individual variation is known can the limitations of conclusions to be drawn be appreciated.

Fig. 7 shows the fall from the secondary peaks of the first and last guinea-pigs in Group 5, Table I; the figures in Table I show that the first 3 guinea-pigs in this group had the same individual values at the various times of sampling, and that the titres of the last 2 fell in a comparable manner. In constructing curves 1 and 2 in Fig. 7, points were plotted for the limiting levels in the antitoxin titrations (i.e., guinea-pig 1, value recorded as 10 units, points plotted were log 10 and log 20) and curves drawn between the appropriate points. Curve 3 was constructed by plotting the logs of the arithmetic means for the whole group. In calculating arithmetic means, values of over 10 and under 20 were taken as 15 units. This curve would have been obtained if pools consisting of equal volumes of individual sera had been titrated. In the absence of a knowledge of the individual values, this curve might have led us to conclude that a very high degree of immunity existed in all the animals of this group. The mean value 6 months after the second injection was 3·22, and the magnitude of this figure might be taken as indication of a small scatter.

Holt (1950*a*) used pooled sera extensively in some of his experiments. This fact, together with the absence of any mention of procedure, or scatter of values, and the actual figures (e.g., 1·0, 1·5) recorded in tables, renders it practically certain that pooled sera were titrated in at least some of the experiments described in his other publications (1949, 1950*b*); in a few tables the values given are headed "average values."

Holt (1950*b*) considers that the secondary response phenomenon of Glenny and Sudmersen (1921) is the resultant of three separate components, (a) the initial serum titre, (b) the secondary response effect or "leap," and (c) the contribution of the secondary stimulus towards the new base-line level of immunity. In this quotation we have corrected Holt's misleading expression "second" and reverted to the original "secondary" response, because the use of the word "second" seems to imply the response to a second injection. As originally stated by Glenny and Sudmersen (1921), an intermediate response is obtained on injecting an animal that is only partially immune, and could thus follow a second injection of antigen into an animal that had received an extremely small first injection. The term "secondary response" has no bearing on the number of injections given, but is used to describe the *type* of response given after the stimulation of animals in which basal immunity is well established. It would thus be shown after the tenth or fiftieth injection of prophylactic.

Most workers will agree with Holt's general conception of the secondary response, because its magnitude is measured in terms of increased titre (although some workers unfortunately use the word "gain," meaning geometric and not arithmetic). It is probably generally accepted by observers experienced in hyperimmunization that the rate of loss of circulating antitoxin decreases with continued immunization, whereas the actual antitoxic titre may change but little. The conception of the "pure" secondary response is new, and Holt (1950*b*) claims that on occasion a "pure" secondary response can be worked out by subtracting the primary base-line titre and the primary response at corresponding times from the total secondary response: this "pure" secondary response, or "leap," is claimed to show a geometric loss in titre with time, that corresponds well with the rate of loss of globulin molecules. Holt states (on p. 234), however,

that it is only from certain types of secondary response curves that the " pure " secondary response can be derived. It is important to know how often such curves are obtained, and whether they have ever been obtained apart from those in which pooled sera or average values of groups have been used.

Our results, given in Table I, show that in the majority of cases both the primary response and its subsequent base-line are negligible in comparison with the total secondary responses. The latter therefore approximate very closely to Holt's " pure " secondary responses. Consideration of the values in Group 5, Table I, shows that some of the rates of loss among individual animals treated in the same manner are quite different, whether the values adopted as the final " base-line " are taken as those recorded for 3 months or for 6 months after the second injection. This is clear from Fig. 7, and the same differences may be seen among individuals in other groups in Table I.

In drawing the conclusions quoted above, Holt (1950*b*) appears to have plotted points obtained by subtracting the values of two sets of pooled sera (one of these the constant " base-line titre ") from that of a third ; it is possible that average values of individuals were used. In one experiment one of the sets of values subtracted was that of an entirely different group of animals (Table III, Series B, p. 236). Two of the four points finally plotted on a straight line from figures obtained as a result of these calculations (Fig. 3, p. 238) were derived from values originally interpolated from a free-hand curve (Fig. 1 and Table II, pp. 235–6). In another experiment a graph was constructed (Fig. 4, p. 239), the points for which were obtained by subtracting the average values of serum from one group of guinea-pigs from the average values for another group at corresponding times (Table IV, p. 239). These animals had received a single injection of P.T.A.P. and the subcutaneous nodules had been excised 7 days and " 14 days or later " after this injection. The difference between these values was taken to represent a secondary response effect superimposed on a pure primary response (see Holt, 1949, p. 292). It was claimed (Holt, 1950*b*) that a linear relation existed between the logs of these differences and time after injection, but it should be noted that the critical point determining the relation was obtained by subtracting 0·6 from 0·7 (Table IV, p. 239). An error of less than 10 per cent in

TABLE I.—*The Antitoxic Values of Individual Guinea-pigs Injected with* 0·25 *Lf Dose of P.T.A.P. at the Time of a Second Similar Injection and at Fixed Times thereafter.*

Group.	Time of reinjection.	A.T. value at reinjection.	A.T. value after 2nd injection (units/ml.).					
			10 days.	1 month.	2 months.	3 months.	4 months.	6 months
1	1 month	0·10	5	2	1	0·5	0·5	0·5
		0·01	5	1	0·5	0·5	0·2	0·2
		0·02	2	2	2	2	1	1
		0·10	2	2	0·5	0·2	0·2	0·2
		0·04	2	1	0·5	0·2	0·2	0·2
		0·002	2	0·5	0·5	0·5	0·5	0·5
		0·02	2	0·5	0·5	0·2	0·2	0·2
		0·01	2	0·5	0·2	0·5	0·5	0·5
		0·004	1	0·5	0·5	0·5	0·5	0·2
		0·10	1	0·5	0·2	0·2	0·2	0·2
		0·002	0·2	0·2	0·2	0·2	0·2	0·2

TABLE I—(*cont.*).

Group.	Time of reinjection.	A.T. value at reinjection.	A.T. value after 2nd injection (units/ml.).					
			10 days.	1 month.	2 months.	3 months.	4 months.	6 months
2	2 months	0·20	10	10	5	2	1	1
		0·02	10	5	5	2	2	1
		0·10	10	5	2	2	1	1
		0·01	5	5	5	2	2	2
		0·04	5	5	5	2	2	2
		0·04	5	5	2	2	2	1
		0·04	5	5	2	2	1	1
		0·10	5	2	2	2	1	0·5
		0·01	5	2	2	1	1	0·5
		0·004	2	1	0·5	0·2	0·2	0·2
3	3 months	0·02	10	10	10	10	5	5
		0·10	10	10	5	2	2	2
		0·004	10	10	5	5	2	1
		0·01	10	5	2	2	2	0·5
		0·001	5	5	2	2	2	1
		0·02	5	5	2	2	2	1
		0·02	5	5	2	2	2	1
		0·02	5	5	2	2	2	0·5
	Under	0·001	5	2	2	1	1	0·2
4	4 months	0·10	20	20	10	10	5	5
		0·10	20	10	10	5	5	2
		0·01	20	10	5	5	2	2
		0·10	20	10	5	5	2	2
		0·04	10	10	5	5	2	2
		0·02	10	10	5	2	2	2
		0·02	10	5	2	2	2	2
	Under	0·001	10	5	2	2	0·5	0·5
	,,	0·001	10	5	2	2	0·5	0·5
		0·004	5	5	5	2	1	1
		0·04	5	2	2	2	1	1
		0·04	2	1	2	2	1	1
	Under	0·001	2	2	1	0·2	0·2	0·5
5	6 months	0·04	10	10	5	5	5	5
		0·10	10	10	5	5	5	5
		0·04	10	10	5	5	5	5
		0·10	10	10	5	2	2	1
		0·04	10	10	5	2	1	1
		0·04	5	5	5	2	2	2
		0·10	5	5	2	2	1	1
		0·04	5	5	2	1	1	1
		0·004	2	1	0·2	0·2	0·2	0·1
	Under	0·001	1	0·5	0·1	0·04	0·04	0·04

TABLE II.—*The Response of Guinea-pigs to First and the Second Injections, each of 0·25 Lf Dose of P.T.A.P.*

Antitoxic value 2 months after 1st injection.	Value 10 days after 2nd injection.	
	1. 2 units/ml. Number of animals.	5. 10 units/ml. Number of animals.
Under 0·02 unit/ml.	6	3
0·02 to 0·04 unit/ml.	9	8
0·1, 0·2 unit/ml.	1	5

each of these values would have converted the straight line into a definite curve, and the position of the adjacent point in the graph suggests that such a curve would have fitted the points better than the line that was actually drawn.

The suggestion that the secondary response " leap " represents the release of stored antibody is an attractive one. We feel, however, that there is as yet no satisfactory evidence to support it, or to justify Holt's conception of " pure responses " to stimulation. We suggest that a " pure primary response " could more logically be regarded as one that was concerned exclusively with the establishment of good potential immunity ; this can be achieved without any detectable antitoxin appearing in the circulation.

It is difficult to visualize the accumulation and storage of considerable quantities of antitoxin in the body of an animal in whose circulation none has ever been detected before secondary stimulation. In other cases antitoxin may have been lost from the circulation for many years, and yet a typical secondary response may follow an injection of prophylactic. Likewise it is difficult to see why the magnitude of the " leap " from animals primarily stimulated by the same dose of prophylactic should in some circumstances vary according to the type of antigen used as secondary stimulus (our own unpublished observations). Above all, the almost negligible loss of antitoxin from the circulation of some of the guinea-pigs in our experiment, over a period of 6 months, appears to provide strong evidence against the suggestion of the release of stored antibody. This hypothesis cannot account for the considerable differences existing in the rates of loss of antitoxin from the blood of individual animals. Our tests were made at wide intervals, but the individual differences are far beyond the limits of error of the experiment.

The mechanism of antitoxin production in secondary stimulation is obscure, and primary and secondary responses cannot be adequately explained without careful consideration of intermediate response curves. Much work is needed to determine the essential character of potential immunity as established by primary stimulation, and its effect on the subsequent immunological behaviour of animals after further injections.

SUMMARY.

The amount of antitoxin produced following a single injection of prophylactic into guinea-pigs does not necessarily constitute a measure of the degree of potential immunity established. Whereas 1 Lf dose of diphtheria prophylactic P.T.A.P. stimulated greater antitoxin production than the same dose of A.P.T., a remarkable similarity was observed in the immunological behaviour of the guinea-pigs after a second injection of the same prophylactic. This similarity was seen in the heights of the response 10 days after the second injection, and in the rates of loss in

parallel groups that received the second injection 1, 2, 3, or 6 months after the first. Potential immunity (as judged by magnitude of secondary response) increased between the first and second months after primary stimulation and appeared to be maintained thereafter up to 6 months.

When a lower dose (0·25 Lf) of P.T.A.P. was used, potential immunity increased in some but not all animals up to 4 months after the first injection: after 6 months it appeared in general to have declined. The rates of loss of antitoxin in individual guinea-pigs after the second injection showed considerable variation. In general the amount of antitoxin lost by good responders, from 10 days to 6 months after the second injection, decreased as the interval between the injections was increased. Considerable variation was found in the amounts of antitoxin lost by some animals treated in the same manner.

The results obtained using distinguishable guinea-pigs and testing individual sera show the nature of the essential information lost by testing pooled sera. It is suggested that the results obtained by some workers using pooled serum might not have been obtained had individual sera been titrated. Erroneous conclusions concerning fundamental processes may at times have been drawn in consequence.

There is little evidence to support Holt's conception of " pure " primary and " pure " secondary responses, or the suggestion that a secondary response " leap " is due to the release of stored antitoxin.

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