TITRATION OF *CL. WELCHII* EPSILON TOXINS AND ANTITOXIN.

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THE principal toxin produced by *Cl. welchii* Wilsdon Type D was termed epsilon by Glenny, Barr, Jones, Dalling and Ross (1933), who described the toxin as necrotic and lethal. At the time of publication this toxin had not been closely studied: we can now state that ε toxin forms necrotic areas when injected intracutaneously into rabbits and guinea-pigs, is lethal to mice and rabbits injected intravenously, and produces extensive oedema and death when given subcutaneously to guinea-pigs. The phenomenon, reported by Bosworth and Glover (1934–1935), of increase in toxicity of *Cl. welchii* Type D filtrates after a short exposure to trypsin greatly increased the interest of ε toxin. Further interest has been stimulated by the conclusion, reached by Turner and Rodwell (1943), that most Type D filtrates, after a short period of growth, contain, in addition to some toxin, an almost atoxic, relatively thermostable precursor referred to as a proto-toxin, which has the same combining power as toxin, and which can be converted into toxin by various proteolytic enzymes. The total amount of antigen, proto-toxin and toxin produced in a broth culture may almost reach a maximum in 6 to 24 hours; during further incubation much of the proto-toxin is changed to toxin by the action of enzymes produced by the organism, but filtrates from 4 or 5 days' growth still contain some unactivated proto-toxin since further activation can be obtained by the action of trypsin. The observation by Glenny (1936) that toxins increase in toxicity when kept in phosphate buffer solution can be explained by the improvement in the conditions for the action of the bacterial enzyme.

Methods of testing toxins can be divided into two main divisions—tests for toxicity and tests for power of combination with antitoxin. The former cannot be regarded as accurate unless many animals are used, but any form of combining power tests yields results with an accuracy of well within 10 per cent for an expenditure of few animals. Toxicity tests are made without reference to any arbitrary standard, and apart from variation of susceptibility of the animals used and the conditions under which they are kept, results obtained in different laboratories can be compared directly. A measurement of toxicity, however, is of very little practical use, except to determine the extent of change of proto-toxin into toxin during growth or after treatment of a filtrate with trypsin. Combining power tests on the other hand are of special importance in assessing the total amount of antigen present, whether in the form of toxin, or toxoid, and in the case of epsilon toxin of proto-toxin as well. An arbitrarily chosen antitoxin must be used, and so no comparison can be made between the results of different workers using different standards. The unit of antitoxin used in these laboratories was fixed in 1933, but unfortunately we have not yet had an opportunity of comparing our unit with that adopted by Turner and Rodwell (1943).

The flocculation reaction.

Mixtures of unconcentrated antitoxic serum and untreated toxin show several zones of flocculation, and it is difficult to determine which is the specific zone. Antitoxin refined by the pepsin process when mixed with untreated toxin gives a definite zone of flocculation with an optimal point bearing a definite relation to *in vivo* end points, but non-specific flocculation also occurs, and can be recognized by its rapidity (less than 15 minutes under our conditions of test), or by the appearance of flocculation in all mixtures with excess toxin.

Trypsin treated toxins flocculate specifically with unconcentrated or refined antitoxin, and can be used for all preliminary testing of antitoxin.

The Lf dose is determined against an arbitrarily chosen refined antitoxin by mixing a graded series of volumes of toxin with 10 units of antitoxin, heating the mixtures in a water bath at 50° C., and determining the volume of toxin equivalent to one unit in the mixture first to flocculate. It cannot always be assumed that, for any chosen standard antitoxin, the antitoxic value as measured by *in vivo* methods can be satisfactorily taken as equal to that determined by the flocculation method. Unsatisfactory results are obtained if the standard serum originally chosen is non avid, although, when once the *in vitro* unit has been established, subsequent standard sera can be used provided that they are compared with the original standard by the same method of testing. Trypsin activation of epsilon toxin so increases the toxicity that it can be assumed that an Lf mixture should be only just non-toxic to mice, i.e. the L+ dose should be practically equal to the Lf dose. Our original pepsin treated antitoxin fortunately fulfilled these requirements, so it is safe to assume that the antitoxin had a serum ratio of 1·0, and the values assigned to any toxin or antitoxin have a theoretical as well as practical basis.

From the chosen antitoxin the Lf dose of a toxin can be determined, and using this dose the flocculation value of any antitoxin can be measured. Mixtures of activated toxin with refined antitoxin usually flocculate in from 30 to 45 minutes; with natural serum flocculation may appear in one hour, but usually not until several hours have elapsed.

With a satisfactory flocculation reaction at our disposal, other methods of measuring combining power have assumed less importance, because flocculation involves all forms of antigens equally, while the end point reached in any animal test depends upon (1) the total amount of antigen, (2) the amount of active toxin present, (3) the relative affinity for antitoxin of toxin, proto-toxin and toxoid, and (4) the avidity of the standard antitoxin in use. In some circumstances the animal tests for combining power have a qualitative rather than a quantitative value, in that they show the specificity of the flocculation reaction, since local reactions produced in the skin of rabbits or guinea-pigs are usually so typical of epsilon toxin. This point is not so important in the case of trypsin treated filtrates because trypsin destroys most other toxins or reacting antigenic material. The titration of antitoxins to be used clinically should always be made finally by animal experiment to demonstrate that the antitoxin can prevent typical symptoms or death produced by the toxin.

Intravenous injection into mice—lethal dose (L.D. 50).

The dose under test is contained in 0·5 ml. by making use of the appropriate dilution; mice weighing between 18 and 20 g. are used. It is advisable to

keep mice under observation for 96 hours, although with the majority of toxins examined deaths rarely occur after 24 hours. We do not yet know the significance of later deaths which sometimes follow the injection of certain toxins, but the reason for such deaths following the injection of mixtures of toxin and antitoxin is considered later. When accurate results are not needed it is sufficient to use pairs of mice at each dose, and to confirm the result on a second occasion. The end point taken would be the calculated dose that would kill 2 out of 4 mice injected. Mice may die within 3 hours after the injection of 50 L.D. 50 doses. The average filtrate after 24 hours' growth contains about 50 L.D. per ml.; after activation with trypsin this increases to 3000.

Combining power; L+ in mice.

This is the smallest dose of toxin which mixed with one unit of antitoxin kills 50 per cent of mice injected within 72 hours. When trypsin activated toxin is used, very few mice surviving for 24 hours eventually die. The significance of later deaths with unactivated toxins is considered later. It is essential in all combining power tests that the test dose should contain a sufficient number of reacting doses whether L.D. for L+ tests or M.R.D. for Lr tests. It is necessary therefore to use multiples of the L+ dose when tests are made on unactivated toxin. In practice the amount of toxin in the mixture injected intravenously is 10 times the L+ dose of untreated toxin, and 1·0 or 2·5 times when trypsin activated toxin is used. The L+ dose of toxin is used to confirm the readings of a flocculation test upon a toxin or to confirm the antitoxic value of a serum. It must be emphasized that L+ determinations of short growth unactivated toxins are misleading. Upon activation by trypsin no more antigen is produced, but the L+ dose diminishes considerably.

Intracutaneous injection into rabbits.

Considerable individual variations occur among rabbits injected intracutaneously with 0·2 ml. of dilutions of toxin. It is our custom to inject rabbits two days before use with 1 test dose of toxin mixed with 20 per cent less antitoxin than that which is sufficient just to prevent necrosis in the majority of rabbits injected. Only those that show a necrotic reaction are accepted for test purposes. In such chosen rabbits a positive necrotic reaction is produced by the intracutaneous injection of 0·2 ml. of a dilution of about 1 in 500 of a short growth toxin or of a 1 in 50,000 of an activated short growth toxin, or a long growth toxin. After 24 hours the reaction is usually a rather diffuse flat-domed swelling about one inch in diameter, with a vaguely outlined yellowish necrosis in which are scattered small purplish haemorrhages. After 48 hours the centre of the swelling has become slightly concave, and is occupied by a sharply defined circular area of whitish yellow necrosis about 1 inch in diameter. The injection of half this necrotizing dose produces in 48 hours a well-defined mottled purplish area without definite necrosis. It is usually possible to determine within a few hours at which site of injection either of these reactions will occur; reliable readings are made at the end of 2 days.

Some but not all the accepted rabbits react to injections of one hundredth or less of the minimal necrotic dose; this reaction is a rather diffuse slightly raised red area. It is not yet known whether the failure of other rabbits to produce this non-necrotic lesion is due to the range of susceptibility accepted by us. It has

been noticed that toxins have a greater tendency to produce this reaction when tested shortly after preparation, and that much smaller fractions of the minimal necrotic dose of trypsin activated toxin produce this type of reaction ; the range from no reaction of any kind in susceptible rabbits to a definite necrotic reaction is considerably greater when activated toxins are used.

Minimal necrotic dose, M.N.D., is the least quantity of toxin which, when injected intracutaneously, will produce definite necrosis in a rabbit.

The Ln dose in rabbits is the smallest dose of toxin which, mixed with 1 unit of antitoxin, causes a necrotic reaction in 50 per cent of the rabbits injected. This test has the advantage over the mouse lethal test in that specificity of the reaction is at once evident. Once the Ln of a toxin is known it can be used to titrate the antitoxic value of sera. We use the expression M.N.D. and Ln in distinction from M.R.D. and Lr, because we may ultimately use the end point of non-necrotic reaction when more work has been done.

Intracutaneous injection into guinea-pigs.

In guinea-pigs injected intradermally with 0·2 ml. of a 1 in 10 dilution of an average ε toxin or with one hundredth of this amount of activated toxin, the reaction at 24 hours consists usually of one, but sometimes of several sharply cut white necroses, sometimes irregular in shape (occasionally with patches of purplish haemorrhagic mottling), surrounded by a wide flush about one inch in diameter ; the whole reaction is raised as a dome above the general surface of the skin. By 48 hours most of the swelling and the flush have disappeared ; the necrosis alone remains, but may appear slightly smaller than at 24 hours. This reaction is so typical of epsilon toxin that it can be used to give a fairly reliable guide to the presence, but not the absence of this toxin in material of which the quantity available is not sufficient to enable tests to be made by means of neutralization tests with appropriate specific antitoxins.

Combining power tests in guinea-pigs are often unreliable if unactivated toxin is used, possibly because sufficient reacting doses are not available. With activated toxin, however, the test is quite reliable both for the determination of the Lr dose of a toxin and for the titration of antitoxin.

Subcutaneous injection into guinea-pigs.

This method of testing has been used by us occasionally for experimental purposes, especially with a view to finding whether *Cl. welchii* Type D may produce some toxin other than α, ε, θ or K, but we have not yet had any significant results. The lethal dose for guinea-pigs is about 1·0 ml. for a short growth filtrate before activation and 0·02 to 0·05 ml. after activation.

Measures of toxicity and combining power before and after activation with trypsin.

In Table I we record measurements of a 24-hour filtrate before and after treatment with trypsin. The figures quoted for the three methods of determining toxicity are not accurately determined. A large number of tests are needed for accurate measurement, and the result does not justify this expenditure of animals. The three activation ratios here recorded are within 25 per cent of the average, but with many toxins tested by us the three ratios may range from half to double the mean unless many tests have been made. We are inclined to the opinion

TABLE I.—*Showing the Various Measurements for Toxicity and Combining Power of a Short Growth* Cl. welchii *Epsilon Toxin (NX.324) before and after Activation with Trypsin.*

Toxicity.	Volume in ml.		Activation ratio.
	Unactivated.	Activated.	
L.D. in mice	0·034	0·00025	136
M.N.D. in rabbits	0·010	0·00006	167
M.R.D. in guinea-pigs	0·06	0·0006	100
Combining power.			Activation index.
Lf	0·066	0·066	1·00
L+ in mice	0·126	0·068	1·85
Ln in rabbits	0·10	0·072	1·39
Lr in guinea-pigs	..	0·11	..

that the M.N.D. in rabbits is a slightly better measure of activation ratio, because the specific type of reaction produced gives this method of testing toxicity an advantage over methods involving death of mice which might be non-specific, which outweighs the disadvantage of the subjective determination of a positive reaction as opposed to the objective recording of survival or death.

The combining power tests show that the Lf dose has not altered upon activation, thus indicating that the total antigenic content remains unaltered. The L− dose of this unactivated toxin is 1·9 times the Lf dose, indicating the presence of a considerable amount of proto-toxin with less affinity than toxin for antitoxin. After activation the L+ dose is within experimental error equal to the Lf dose. The change in volume of test doses after activation is an indication of the degree of conversion of prototoxin into toxin, and as these test doses can be determined with a high degree of accuracy, we are introducing the term "activation index" for the ratio of the L+, Ln or Lr doses before and after activation. Table I shows that the L+ and Ln doses of the unactivated toxin are not equal but of the same order of magnitude; after activation they were equal for this particular toxin, but the Lr in guinea-pigs was 1·44 times the Ln in rabbits. Considerable difficulty has often been encountered in fixing the Lr dose of unactivated toxin in guinea-pigs; certain factors are involved which have not yet been determined.

An analysis of the results of tests upon 16 short growth toxins before and after activation gave the following results:

1. The L+ dose in mice of unactivated toxins was usually within 20 per cent on either side of 1·6 times the Lf, but may vary from 1·33 to 2·05 times the Lf, indicating that the proportion of prototoxin already converted to toxin may differ considerably in filtrates from a 24-hour growth.

2. The L+ doses in mice of unactivated toxins were mostly about 20 per cent greater than the Ln in rabbits, but this may have been due to the quality of the standard antitoxin used. These two test doses are practically equal for activated toxins.

3. The Lr dose in guinea-pigs was about 1·5 times the Lf, L+ or Ln.

4. Activation ratios varied from approximately 20 to 150 or 200.

5. Activation indices measured by the change in L+ dose varied from 1·33 to 2·1.

6. As a general rule the L+ to Lf ratio of a toxin before activation (the activation index) varied with the magnitude of the activation ratio.

In filtrates from 4-day growths, prototoxin may be changed to toxin to such an extent that the activation ratio after treatment with trypsin is only from 2–5.

Accuracy of titrations.

It is our custom when testing the antitoxic value of any serum to include in the series of tests two sera that are used as substandards in order to determine whether the test toxin has deteriorated, to check the efficiency of the assistants doing the work, and to collect data in relation to the behaviour of two sera differing in quality. In this paper reference is made to three sera, A (RR.2364), a good avid, B (R.6615), slightly non-avid, and C (R.4182), a very non-avid antitoxin. The results of 29 titrations using 2 mice at each level are given in Table II, referring to intravenous injections into mice using an unactivated toxin ; Table III gives the results of 91 tests using an activated toxin. The results in Table II are obtained from the injection of a test dose of unactivated toxin equivalent to 10 units of antitoxin, and in Table III using an activated toxin with a test dose equivalent to 2·5 units of antitoxin. It can be seen that the neutralization curve is neither very steep nor very flat. Any series of results are affected by the accuracy of many measurements, by the character of the toxin and antitoxin under consideration, and by the conditions and degree of uniformity of the test animal. The true characteristic of a toxin in relation to an antitoxin can only be determined by special experiment with all factors carefully controlled. Curves plotted from Tables II and III would represent a characteristic involving not only the toxin and antitoxin concerned, but the general conditions of mice taken from large stocks supplied by different dealers, and also the accuracy and uniformity of a number of assistants making these routine tests. In the series of tests recorded one source of error was the use on several occasions of solutions of toxin several hours after they were made.

An analysis of the detailed results summarized in Table II has been used to

TABLE II.—*Showing the Degree of Accuracy of Titrations, by Intravenous Injection into Mice, of Unactivated* Cl. welchii *Epsilon Toxin* (*NX*.285) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of mice. Dying in (days)— 1	2	3	4	Living.	Percentage dying in— 1 day.	4 days.	Average death time in days.
A	165	4	0	2	1	51	7	12	2.00
	180	18	2	4	1	33	31	43	1·52
	200	45	7	1	2	3	78	95	1·27
B	200	1	0	5	0	52	2	10	2·67
	220	24	6	4	2	22	41	62	1·55
	240	39	6	8	0	5	67	91	1·42

assess the antitoxic titre of one substandard compared with the other ; if tests are made when the toxin solution has deteriorated, a comparison of values can still be made providing the deterioration is not beyond the range of tests. Such an analysis shows that out of 29 tests made at 10 per cent differences using 2 mice at each test, 2 gave no answer ; of the remaining 27, 16 (59 per cent) showed the antitoxic value of B to be 1·15 or 1·20 times that of A, and 24 (89 per cent) to be between 1·10 and 1·25, if the end point adopted was an equal number of survivals

and deaths at the end of 48 hours. If the end point was extended to 96 hours, 19 tests (70 per cent) showed B to be from 1·15 to 1·20 times A, and 24 (89 per cent) between 1·10 and 1·25. Similar figures for the activated toxin (Table III) were : No answer in 13 tests out of 90, in which both sera were used, and of the remaining 77, 43 (56 per cent) as 1·00 or 1·05, and 66 (86 per cent) between 0·95 and 1·10 if 48 hours was taken as the determining limit of time, and 45 (58 per cent) as 1·00 or 1·05, and again 72 (93 per cent) between 0·95 and 1·10 if the later death times were taken.

TABLE III.—*Showing the Degree of Accuracy of Titrations, by Intravenous Injection into Mice, of Activated* Cl. welchii *Epsilon Toxin* (*NX*.316) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of mice. Dying in (days)— 1	2	3	4	Living.	Percentage dying in— 1 day.	4 days.	Average death time in days.
A	165	16	2	1	0	163	9	10	1·21
	180	51	5	7	4	115	28	37	1·46
	200	140	6	5	1	28	78	85	1·12
	220	165	4	2	2	9	91	95	1·09
B	165	15	1	2	0	164	8	10	1·28
	180	38	7	4	2	131	21	28	1·41
	200	111	5	10	9	45	61	75	1·38
	220	165	1	4	1	11	91	94	1·06

In our experience careful selection of mice reduces the number of deaths occurring out of order, use of freshly dissolved toxin avoids the occasional unsatisfactory test, and titrations by expert assistants bring this type of test to a high degree of accuracy.

Tables IV and V give similar results for control tests for intracutaneous injection into rabbits and Table VI into guinea-pigs, using the same substandard sera. The symbols used for recording reactions are — for no reaction, S for a small indefinite reaction which might be non-specific or might be a very small definite reaction, ± for a border line but definite reaction, and + for any fully developed reaction. Tests are made in rabbits on both types of toxin at a 1 unit level and in guinea-pigs at a level of 0·2 unit.

TABLE IV.—*Showing the Degree of Accuracy of Titrations, by Intracutaneous Injection into Rabbits, of Unactivated* Cl. welchii *Epsilon Toxin* (*NX*.285) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of tests showing reactions recorded as— —	S.	±	+	Percentage number of positive reactions.
B	200	32	5	4	1	12
	220	7	8	15	12	64
	240	2	3	2	35	88
	270	0	0	2	40	100

— = No reaction. S. = Small indefinite reaction. ± = Border line but definite reaction. + = Fully developed reaction.

TABLE V.—*Showing the Degree of Accuracy of Titrations, by Intracutaneous Injection into Rabbits, of Activated* Cl. welchii *Epsilon Toxin* (*NX*.316) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of tests showing reactions recorded as—				Percentage number of positive reactions.
		—	S.	±	+	
A	165	18	11	3	4	19
	180	13	6	6	11	47
	200	4	2	1	29	83
	220	1	1	1	33	94
B	180	35	1	0	0	0
	200	22	6	4	4	22
	220	4	5	11	16	75
	240	1	2	1	32	93

TABLE VI.—*Showing the Degree of Accuracy of Titrations, by Intracutaneous Injection into Guinea-pigs, of Activated* Cl. welchii *Epsilon Toxin* (*NX*.316) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of tests showing reactions recorded as—				Percentage number of positive reactions.
		—	S.	±	+	
A	165	37	7	3	5	15
	180	17	15	6	14	38
	200	7	9	8	28	69
	220	2	2	2	46	92
B	180	41	7	1	3	8
	200	24	9	3	16	36
	220	9	6	10	27	71
	240	6	6	5	35	77

These results show that the accuracy of tests using the intracutaneous method in rabbits or guinea-pigs is of the same order as that given by intravenous injection into mice. Table VII records the antitoxic values of the two sera determined

TABLE VII.—*Showing Calculated Antitoxic Value in Units per ml. of Two Standard Sera Tested by Different Methods.*

Method of injection.	Intravenous injection.		Intracutaneous injection.		
Animal.	Mouse.		Rabbit.		Guinea-pig.
Toxin.	Unactivated.	Activated.	Unactivated.	Activated.	Activated.
Serum A	183	185	..	182	188
Serum B	215	189	214	211	216
Number of observations.	58	182	42	36	52

by calculating the amount of each serum which mixed with a test dose of toxin would produce after injection an equal number of survivals and deaths or reactions and no reactions according to the method of testing. The number of mice used at each level tested is sufficient to show that the value of B in relation to A is significantly different according to whether activated or unactivated toxin is

used in the mouse intravenous method. This difference is due to a difference in avidity of the two antitoxic sera. The uniformity of all other values is a confirmation of the accuracy of determination of the test doses of the toxins.

Avidity.

Antitoxic sera differ in relation to the degree of firmness with which they combine with toxin. Non-avid antitoxin combines loosely with toxin and mixtures dissociate upon dilution. Mixtures of toxin and non-avid antitoxin which are over-neutralized when judged by the flocculation test are toxic to animals, and the *in vivo* value assigned to a non-avid antitoxic serum is less than the *in vitro* value. Mixtures may continue to dissociate after injection and toxin is slowly freed, causing late deaths. The difference in the firmness of combination between antitoxin and toxin and that of antitoxin and toxoid is far greater with a non-avid antitoxin; this explains the decreased efficiency of the serum B when tested in mice against an activated toxin. Glenny, Pope and Waddington (1925) showed that mixtures of diphtheria toxin with non-avid antitoxin could be prepared in such proportions that the intracutaneous injection of 0·2 ml. into guinea-pigs causes no reaction, while 0·2 ml. of a 1 in 10 dilution of the mixture would produce large reactions. Glenny and Barr (1932), also referring to diphtheria toxin and antitoxin, stated that "the simplest measure of firmness of combination is the dilution ratio, which is the amount of antitoxin necessary to neutralize the Lr dose of toxin in a total volume of 2 ml., divided by the amount required to neutralize the same dose in 200 ml."

No attempt has yet been made by us to fix the dilution to be used to determine any definite dilution ratio as a measure of avidity of epsilon antitoxin, but a few comparisons have been made of titrations of certain sera at different levels. Table VIII shows a comparison made between titrations of sera A and B at three

TABLE VIII.—*Showing the Effect of Dilution upon Mixtures of Antitoxin and an Activated Toxin (NX.316).*

Serum.	Test for (units per ml.).	Number of deaths in mice out of total injected intravenously with 0·5 ml. of mixture diluted to—		
		2 ml.	10 ml.	100 ml.
A	150	2/22	1/22	..
	165	16/28	11/28	0/21
	180	18/24	18/24	2/21
	200	14/14	16/18	10/26
	220	..	..	13/18
	240	..	..	10/10
B	135	..	0/6	0/8
	150	..	0/4	3/4
	165	1/10	3/10	6/6
	180	2/6	4/6	8/8
	200	7/10	8/8	..
	220	4/4	..	..
		Calculated value.		
A	..	163	170	207
B	..	189	173	160

different levels. Mixtures of 40 L+ doses and appropriate dilutions of serum were made in volumes of 2, 10 and 100 ml., and 0·5 ml. of the diluted mixtures were injected intravenously into mice. More mice died after injection of dilutions of mixtures of toxin and antitoxin B than died after injection of less dilute mixtures, showing that B was not avid. On the other hand, dilutions of mixtures containing A caused fewer deaths than less dilute mixtures because combination between toxin and an avid antitoxin was relatively firm, and dilutions reduced the available free toxin. Thus the relative value of a non-avid antitoxin to an avid one depends upon the amount of toxin used in the test dose.

If A were regarded as a fixed standard with an antitoxic content of 185 units per ml. (Table VII) and test doses were adjusted at each level of testing, then the apparent titre of B would be 215, 189 or 132 units per ml. according to the level at which tests were made. These figures emphasize the point that *in vivo* titrations cannot give an absolute value to a non-avid antitoxin. The flocculation test alone shows the total antitoxic content : *in vivo* tests show how much antitoxin is available to bind toxin under the particular conditions of the experiment.

Table IX gives the results of titrations at two levels of C, a less avid antitoxin

TABLE IX.—*Showing the Effect of Dilution upon Mixtures of Antitoxin and an Activated Toxin* (*NN*.1314).

Serum.	Test for (units per ml.).	Number of deaths in mice out of total injected intravenously with 0·5 ml. of mixture diluted to :—	
		2 ml.	20 ml.
B	200	..	2/6
	220	0/6	4/6
	240	1/6	6/6
	270	6/6	..
C	220	..	0/9
	240	..	2/9
	270	..	8/9
	300	..	8/9
	330	0/9	9/9
	360	3/9	..
	400	6/9	..
		Calculated value.	
B	..	252	210
C	..	380	257

compared with B ; at the strongest level of testing C appears to contain 380 units per ml., but only 257 if one-tenth of the amount of toxin is used for titration. Thus the two Tables VIII and IX show that A is avid, B is less avid, and C still less avid ; that the avid serum appears more effective when less toxin is used unless the fractional test doses are adjusted, but the non-avid sera are less effective against smaller doses of toxin ; consequently the values of non-avid antitoxins in relation to avid ones appear lower if less toxin is used as the basis of comparison. The L+ doses of the toxins used in the experiment recorded in Table IX have not been correctly adjusted ; the object of the experiment was to compare apparent values at different dilutions rather than to determine exact values.

In titrations of avid antitoxin against activated toxin by the mouse intravenous method, a certain number of mice die after the first 24 hours, and less after 48 hours, but the number so dying increases when titrations are made against unactivated toxin, and still further increases when non-avid antitoxin is titrated. Results given in Table III (activated toxin) show that of the mice used for testing serum B, 8·1 per cent of all deaths occurred after 48 hours and in Table II (unactivated toxin) 20·0 per cent; the corresponding figures for the avid serum A were 5·3 per cent and 12·8 per cent. Many more late deaths occurred when the least avid C was tested. Results are given in Tables X and XI, showing that the percentage of late deaths occurring among mice used for testing C reached 89 per cent, compared with 44 per cent with B when an unactivated toxin was used and 30 per cent compared with 9 per cent using an activated toxin.

No single set of figures can express differences in titration curves of one serum compared with another against the same toxin, or of one serum against different toxins; account must be taken of the number of mice dying and of the time of death. It is of advantage to adopt some standard method of expressing a combination of the number of animals dying and the average death time. We suggest the term "lethal index" for the product of the percentage number of animals dying and a factor representing the acceleration in death time. Since in our experiments mice were not observed after the fourth day, those dead on that day died at least one day earlier than those living on that day, so we assume the acceleration in death time to be one day, and for those dying on the first day to be 4 days. Thus factors of 1·00, 0·75, 0·5, 0·25 are allotted for average death times of 1, 2, 3 and 4 days respectively. The formula for the lethal index thus becomes the percentage number dying multiplied by the average death time in days subtracted from 5 and divided by 4. For example the lethal index for the titration in Table X of serum B for 200 units per ml. becomes—

$$\frac{9 \times 100}{48} \times \frac{5 - 2{\cdot}78}{4} = 10{\cdot}4$$

It must be pointed out that a lethal index of this type is only applicable if cause and effect are in a linear relationship. As late deaths are due to slow dissociation this method would appear applicable.

TABLE X.—*Comparing the Number of Late Deaths among Mice Injected Intravenously with Mixtures of an Unactivated Toxin (NX.285) with Varying Amounts of Two Sera of Different Degrees of Non-avidity.*

Serum.	Test for (units per ml.).	Number of mice. Dying in (days)— 1	2	3	4	Living.	Percentage dying in— 1 day.	2 to 4 days.	Average death time in days.
B	200	3	0	2	4	39	6	12	2·8
	220	7	7	6	4	24	15	35	2·3
	240	24	15	6	0	3	50	44	3·6
	270	39	3	0	0	0	93	7	3·1
C	300	0	0	4	7	31	0	26	3·6
	330	0	2	19	5	18	0	59	3·1
	360	1	13	22	6	6	2	85	2·8
	400	3	24	15	4	2	7	89	2·3
	450	7	9	0	0	0	44	56	1·6

Results from Table X can now be compared graphically in Fig. 1, the darkened areas representing the percentage number of mice dead on or before the fourth day; the black area represents the lethal index and the shaded area varies in length with the number and time of late deaths. The absence of any shaded area, as in the fourth column in Fig. 2, shows that all mice died on the first day. The shaded areas in Fig. 1 show that many more late deaths occurred in titrations of C than of B; it can also be seen that the neutralization curve for the very non-avid antitoxin is less steep than that of the slightly non-avid if judged by the proportion of mice dying, and still less steep if judged by the lethal index. Results of the same two sera against an activated toxin (Table XI) shown in Fig. 2 afford a contrast with those shown in Fig. 1.

TABLE XI.—*Comparing the Number of Late Deaths among Mice Injected Intravenously with Mixtures of an Activated Toxin (NX.308) with Varying Amounts of Two Sera of Different Degrees of Non-avidity.*

Serum.	Test for (units per ml.).	Number of mice. Dying in (days)— 1	2	3	4	Living.	Percentage dying in— 1 day.	2 to 4 days.	Average death time in days.
B	165	4	0	0	0	18	18	0	1·0
	180	7	1	1	0	13	32	9	1·3
	200	20	0	2	0	0	91	9	1·2
	220	22	0	0	0	0	100	0	1·0
C	270	2	0	0	0	14	12	0	1·0
	300	9	4	2	0	5	41	30	1·53
	330	13	5	0	0	2	59	25	1·28
	360	6	2	0	0	0	75	25	1·2
	400	4	0	0	0	0	100	0	1·0

Fig. 3, prepared from figures given in Table II, can be compared directly with Fig. 1, as titrations were made against the same unactivated toxin. There is very little difference between the diagram for the avid antitoxin A and that for the slightly non-avid one B. The difference in avidity of these two sera is considerable when judged by the effect of dilution upon toxin antitoxin mixtures (Table VIII). It would appear therefore that many late deaths and flat neutralization curves are properties only of very non-avid antitoxin such as C.

The three sera were tested against a short growth toxin before and after activation; tests were made at the same time under comparable conditions, using 10 mice at each level. As no early deaths followed the injection of the first series of tests up to 360 units on serum C against unactivated toxin, a further series of 10 mice were used on tests from 360 to 550 units. Results are given in Table XII. All tests were made against a test dose equivalent to 10 units. Only 1 mouse out of 40 died after the 2nd day when injected with mixtures of the avid serum A with unactivated toxin, 10 out of 40 injected with mixtures containing B, and 38 out of 90 when C was used. All 20 mice at the 360 unit level died on the 2nd, 3rd or 4th day. In all the tests using the same toxin after activation only 3 mice out of 110 died after the 2nd day and only 4 on that day.

Figs. 4 and 5 show graphically the results given in Table XII. In the diagram for C in Fig. 4 one area for the 360 test is shaded by diagonal lines in two directions

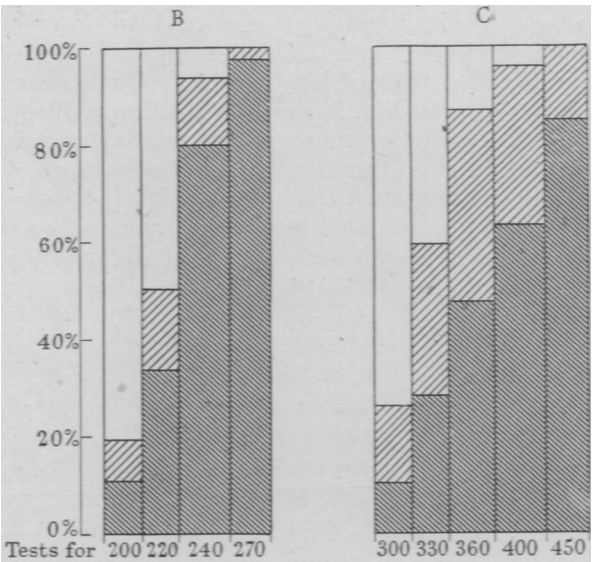

FIG. 1.—Comparing the results of titrations, by intravenous injection into mice, of slightly non-avid serum B with those of the least avid serum C, using an unactivated toxin NX.285 (Table X).

The height of the darkened columns gives the percentage number of mice dying, that of the shaded columns indicates the delay in death time and that of the black column the acceleration in death time between 1 and 5 days.

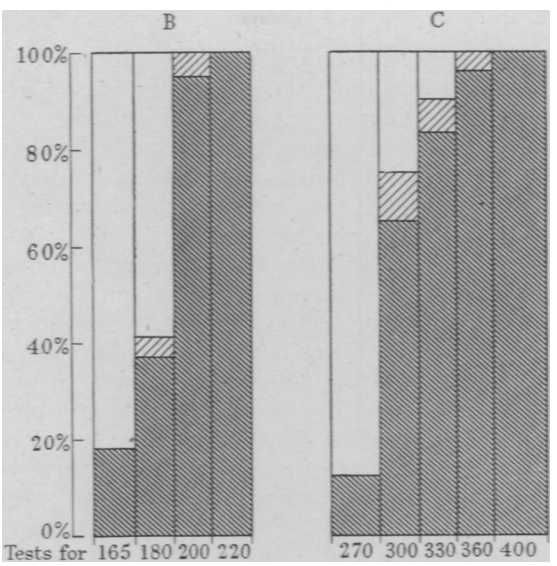

FIG. 2.—Comparing the results of titrations, by intravenous injection into mice, of slightly non-avid serum B with those of the least avid serum C using an activated toxin NX.308 (Table XI).

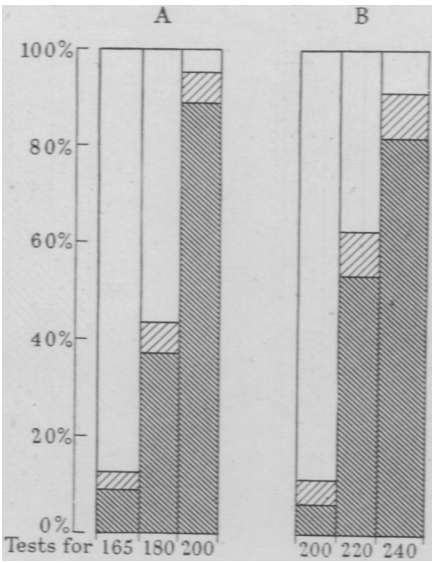

FIG. 3.—Comparing the results of titrations, by intravenous injection into mice, of the avid serum A with those of the slightly non-avid B using an unactivated toxin NX.285 (Table II).

TABLE XII.—*Showing the Titration by Intravenous Injection into Mice of 3 Sera against the Same Toxin (NX.327) Before and After Activation.*

Anti-toxin.	Test for (units per ml.).	Unactivated toxin. Number of mice dying in (days)— 1	2	3	4	Living.	Average death time in days.	Activated toxin. Number of mice dying in (days)— 1	2	3	4	Living.	Average death time in days.
A	165	0	4	0	0	6	2·0	—	—	—	—	—	..
	180	2	3	0	1	4	2·0	0	0	0	0	10	..
	200	5	5	0	0	0	1·5	7	0	1	0	2	1·25
	220	9	1	0	0	0	1·1	10	0	0	0	0	1·00
B	180	0	0	0	0	10	..	0	0	0	0	10	..
	200	0	0	1	3	6	3·8	3	0	1	0	6	1·50
	220	3	2	2	2	1	2·3	10	0	0	0	0	1·00
	240	5	2	1	1	1	1·8	—	—	—	—	—	..
C	270	0	0	1	4	5	3·8	0	0	0	0	10	..
	300	0	0	4	2	4	3·3	1	0	1	0	8	2·0
	330	0	2	2	3	3	3·1	7	3	0	0	0	1·3
	360	0	6	9	5	0	2·9	9	1	0	0	0	1·1
	400	1	2	7	0	0	2·6	10	0	0	0	0	1·0
	450	2	7	1	0	0	1·9	—	—	—	—	—	..
	500	7	2	1	0	0	1·4	—	—	—	—	—	..
	550	9	1	0	0	0	1·1	—	—	—	—	—	..

to indicate the difference between the results on the two occasions when this test was made. The upper limit corresponds to the blackened area for the first series of tests and the lower limit for the second series. The difference in the two sets of results amounted to 0·5 day in the average death time. This can be partially explained by the fact that the mice in the second series were injected 6 hours later in the day than those in the first series, but readings of deaths were taken at the same time on both series, so that a death on the second day in the first series was a death within 46 hours, and in the second series within 40 hours. The

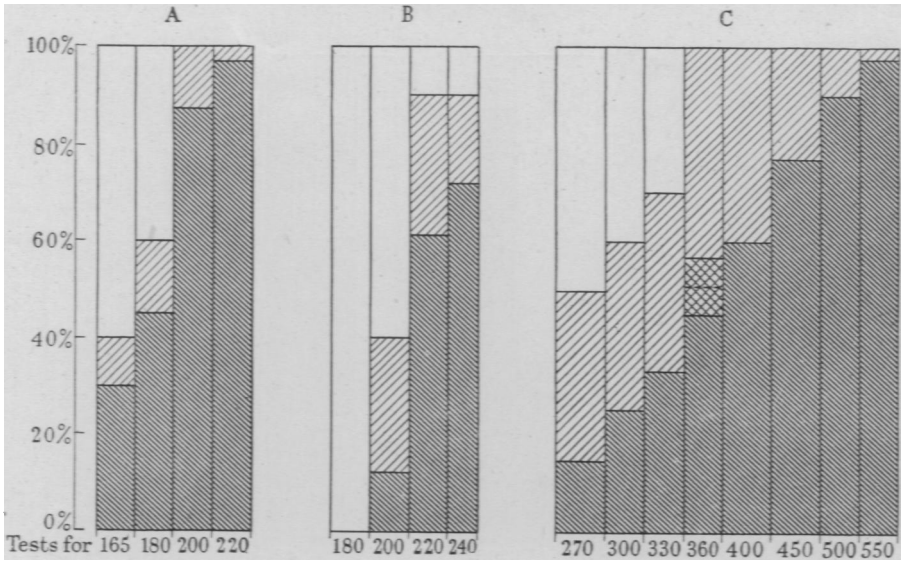

FIG. 4.—Comparing the results of titrations, by intravenous injection into mice, of 3 sera, A, B and C, using an unactivated toxin NX.327 (Table XII).

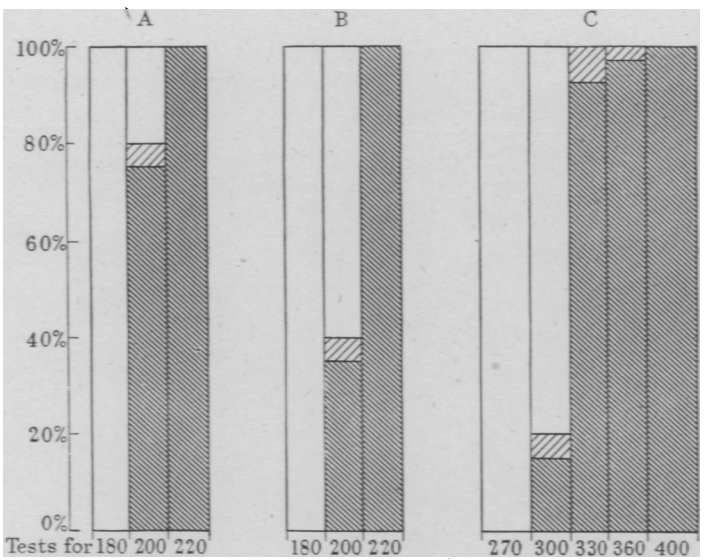

FIG. 5.—Comparing the results of titration, by intravenous injection into mice, of 3 sera, A, B and C, using toxin NX.327 after activation (Table XII).

slope therefore of the curve for lethal index would not be quite so flat as in the diagram.

The results recorded in various tables are summarized in Tables XIII and XIV, showing the ratios of the apparent antitoxic titres of the three sera determined by intravenous injection into mice. More late deaths occur in titrations

TABLE XIII.—*Showing the Comparative Antitoxic Strength of Three Sera Determined by Intravenous Injection into Mice against Activated and Unactivated Toxins, judging the End Point of Titration by 50 per cent Survivals at end of* (a) *1st day*, (b) *2nd day*, (c) *4th day*.

Table.	Toxin No.	Type of toxin.	Amount of toxin used (L+ doses).	Ratio of antitoxic titre $\frac{B}{A}$ (a)	(b)	(c)	Ratio of antitoxic titre $\frac{C}{A}$ (a)	(b)	(c)	Ratio of antitoxic titre $\frac{C}{B}$ (a)	(b)	(c)
III	NX.285	Unactivated	10·0	1·21	1·19	1·19	..			..		
X	NX.285	„	10·0	..			..			>1·88	1·71	1·42
XII	NX.327A	„	2·5	1·20	1·22	1·18	2·40	2·32	1·56	2·00	1·90	1·32
II	NX.316	Activated	2·5	1·03	1·03	1·02	..			..		
XI	NX.308	„	2·5	..			..			1·65	1·57	1·57
XII	NX.327B	„	2·5	1·06	1·06	1·05	1·65	1·61	1·61	1·55	1·52	1·53

TABLE XIV.—*Showing the Comparative Antitoxic Strength of Three Sera Determined by Intravenous Injection into Mice against Multiples and Submultiples of Usual Test Doses of Activated Toxins, judging the End Point of Titration by 50 per cent Survival at end of 4 days.*

Table.	Toxin No.	Amount of toxin injected (L+ dose).	Ratio of antitoxic titre $\frac{B}{A}$	Table.	Toxin No.	Amount of toxin injected (L + dose).	Ratio of antitoxic titre $\frac{C}{B}$
VIII	NX.316	10·0	1·16	11	NX.308	2·5	1·57
XII	NX.327B	2·5	1·05	12	NX327.B	2·5	1·53
II	NX.316	2·5	1·02	9	NN.1314	2·5	1·52
VIII	NX.316	2·0	1·02	9	NN.1314	0·25	1·25
VIII	NX.316	0·2	0·77				

with non-avid than with avid antitoxin; it therefore follows that the relative titres appear to alter according to whether the end point chosen is 50 per cent survival after 24, 48 or 96 hours. Relative values are also affected by the type of toxin used for titrations and the level at which titrations are made relative to the L+ dose.

Of the two unactivated toxins NX.285 was a solution of toxin precipitated by ammonium sulphate and dried. In our experience this process frequently reduces the number of lethal doses in the L+ dose; NX.285, although prepared from a long growth filtrate, only contained 7 mouse lethal doses in the test dose equivalent to 10 units; NX.327A had not been precipitated or dried, and was a filtrate from a 24 hours' growth containing 20 lethal doses in the test dose equivalent to 2·5 units.

Several factors contribute to the size of the ratios of the antitoxic values of the three sera calculated from the results of the various tests. The day on which readings are made to determine 50 per cent survivals has practically no effect upon the relative values of A and B, and there is little difference between the apparent strength of the least avid serum C in relation to the other two, whether readings be made on the first or on the second day, but the ratio of the value of the serum to either A or B falls considerably (from 2·4 to 1·56 or from 2·0 to 1·32) if readings are postponed until the fourth day, when titrations are made with unactivated toxin, but not when activated toxin is used.

The antitoxic values of non-avid sera appear relatively higher when unactivated toxin is used than when the test toxin is activated. The slightly non-avid B

compared with the avid A appears about 15 per cent higher when titrated against the unactivated as compared with the activated toxin. The least avid serum C in relation to A appears 50 per cent higher against the unactivated toxin if end points of death or survival are taken after one or two days, but approximately the same if the readings are made on the fourth day, although the less non-avid B appears about 12 per cent higher with the non-activated toxin when judged by this end point. It follows that the comparison between the least avid C and the less avid B shows a higher ratio against unactivated than against activated toxin, judged by readings after 1 or 2 days and the reverse at the end of 4 days. The concentration of mixtures injected (Table XIV) has some effect (about 10 per cent) on the relative titres of non-avid antitoxin titrated against activated toxin in the range from 10 L+ doses of toxin in the mixture to 2 or 2·5 L+ doses, but a considerably greater effect when the amount of toxin is reduced to 0·2 or 0·25 L− doses.

Most of the tests recorded have been made upon routine toxins prepared by Mr. J. Macsween, and we are specially indebted to him for his co-operation in preparing some special batches of toxin for experimental purposes.

SUMMARY.

1. The flocculation reaction can be used to determine the total antigen content of *Cl. welchii* epsilon toxins both before and after activation by trypsin provided the antitoxin used in the test has been refined by the pepsin process.

2. Antitoxic values of sera can be estimated by the flocculation reaction if the test toxin has been activated by trypsin.

3. Toxicity of filtrates can be determined by intravenous injection into mice and by intracutaneous injection into rabbits and guinea-pigs.

4. Combining power tests can be made by the same methods with one reservation, namely that only trypsin activated filtrates are suitable as test toxins for the titration of antitoxin in the skin of guinea-pigs.

5. The combining power of toxins or the antitoxic value of sera can be measured with a high degree of accuracy by intravenous injection into mice.

6. Titration of non-avid antitoxin by *in vivo* methods gives results that vary considerably with conditions of test.

7. Mice injected intravenously with mixtures of activated toxin and avid antitoxin usually survive for many days if they do not die within 24 hours; if the mixtures contain unactivated toxin and non-avid antitoxin many mice surviving 24 hours die during the following days.

REFERENCES.

BOSWORTH, T. J., AND GLOVER, R. E.—(1934–1935) *Rep. Dir. Inst. An. Path. Camb.*, **4**, 79.

GLENNY, A. T.—(1936) 2nd Internat. Cong. Microbiol. London. Abstract of Communications, 209.

Idem AND BARR, M.—(1932) *J. Path. Bact.*, **35**, 91.

Idem.—JONES, M. LL., DALLING, T., AND ROSS, H.—(1933) *ibid.*, **37**, 53.

Idem, POPE, C. G., AND WADDINGTON, H.—(1925) *ibid.*, **28**, 279.

TURNER, A. W., AND RODWELL, A. W.—(1943) *Aust. J. exp. Biol. med. Sci.*, **21**, 17.