THE COMBINATION OF ANTITOXIN
WITH TOXIN AND TOXOID

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(With 4 Figures in the Text)

There are a number of factors concerned in the combination of antitoxin with crude toxin and toxoid contained in toxic culture filtrates. These factors must be considered before theories are advanced to account for any observed phenomena.

The object of this paper is to catalogue some of these factors, to give such experimental evidence as we have accumulated and to give a few instances from the literature where these factors have been ignored. The chief factors are as follows.

The number of M.L.D. (or LD_{50}) contained in 1 Lf of a freshly harvested toxin varies owing to the presence of natural toxoid formed during the growth of the organism; as toxin ages more is changed into toxoid and the number of M.L.D. per Lf decreases.

Antitoxin has a greater affinity for toxin than for toxoid either naturally produced or formed by the action of formaldehyde on toxin.

Combination between antitoxin and toxin or toxoid becomes firmer with time, this effect is more pronounced with toxoid or formol toxoid than with toxin.

The relative affinity of formol toxoid for antitoxin compared with that of toxin varies with different preparations and can be correlated to some extent with immunizing efficiency. Barr & Glenny (1949) described a method for measuring affinity of toxoid for antitoxin in terms of an 'affinity coefficient'.

The 'differential region' of Ehrlich, which is the difference between Lo and L+ doses, varies in extent depending upon the relative amounts of toxoid and toxin in a toxic filtrate and can be correlated with the number of LD_{50} per Lo or Lf doses. Diphtheria antitoxin or toxin is seldom tested now by subcutaneous injection into guinea-pigs but another form of differential region, the 'length of range', is of considerable importance in the choice of a suitable toxin for use in antitoxin titrations by guinea-pig or rabbit intracutaneous methods. Because, in our experience, results are more consistent and obtained sooner, we greatly prefer guinea-pigs to rabbits for most titrations, and only have recourse to rabbits for the detection of very small concentrations of toxin or antitoxin; such tests are seldom needed. The length of range in this type of testing is measured by the percentage difference in the amounts of antitoxin, or of culture filtrate, in two mixtures one of which invariably fails to cause any reaction and the other always causes some well-defined reaction. In tests, such as tetanus toxin-antitoxin titrations, the results of which depend upon death or survival, the length of range is shown by the difference in times of death caused by the injection of a series of mixtures of different constitution. For accurate titration of antitoxins, test toxins must be carefully chosen to ensure sharpness of

end-point. The difference between the Lf and Lr doses of a toxin may be regarded as another form of differential region, which in certain circumstances increases in extent during the ageing of culture filtrates when some of the toxin becomes modified into toxoid.

Antitoxins differ in avidity, that is in the firmness with which they combine with toxin or toxoid. Antitoxic fractions differing widely in avidity can be salted out from a single serum (Barr & Glenny 1931 a, b). These authors describe three types of antitoxin, flocculating avid, non-flocculating avid and non-avid. The proportions in which these types are present in any single serum would determine the average avidity of the preparation, but there may be a continuous range of qualitative differences in single preparations. Barr (1951) gives evidence of 'heterogeneity among antigens'; she suggested (Barr, 1949) that 'toxins may consist of molecules possessing a range of affinities for antitoxin', and that goodness of fit would occur between a toxin and serum composed of components of maximum mutual affinity present in optimal proportions.

The looseness of combination between non-avid antitoxin and toxin does not influence the flocculation test beyond slowing down the reaction and producing visibly sparser floccules than would be obtained from the same toxin reacting with an avid antitoxin. *In vivo* titrations do not measure the full amount of non-avid antitoxin in a serum owing to the dissociation of the toxin-antitoxin complex which occurs on increasing the dilution, so that the serum ratio and the dilution ratio of such sera are both less than 1·0. The serum ratio is the *in vivo* titre measured at a fixed level of testing divided by the *in vitro* titre. The dilution ratio is 'the amount of antitoxin necessary to neutralize the Lr dose of a toxin in a total volume of 2 ml. divided by the amount required to neutralize the same dose in 200 ml.' (Glenny & Barr, 1932). Barr (1949) showed that the values of the dilution ratio differed according to the toxin used in the test, but consistent results could be obtained for the majority of sera if the ratio was referred to that of a suitable standard serum tested against the same toxin.

If non-avid antitoxin is used for the titration of toxin the differences between Lf and Lr and between Lo and L+ no longer depend entirely on the amount of toxoid present. Three out of four of these measurements have little meaning if made with non-avid antitoxin without specifying the conditions under which the tests were made. It is essential to consider the level at which Lr tests are made and to take into account the dilution ratio of the serum. When the subcutaneous method is used the presence or absence of oedema depends upon the presence or absence of free toxin in the mixture before it is absorbed and further diluted in tissue fluids: dissociation caused by this dilution may render lethal a mixture that contained no free toxin when injected. Thus the L+ dose may be in effect smaller than the Lo, and an injected guinea-pig may die on the fourth day without exhibiting any local swelling. Instances of this were reported by Glenny (1913) who did not at that time know the reason for this effect.

The work to be recorded in this paper was mostly done during the years 1935–8; and much still remained to be done when we were obliged to cease because of the need for large-scale production of tetanus and gas-gangrene antitoxins. The results

obtained may be of value in the consideration of the significance of recently published findings of other workers.

The differential region

Cohn & Pappenheimer (1949) compared the extent of the differential region of a diphtheria toxin when tests were made against antitoxin prepared in various species. They concluded that the long range between Lo and L+ was peculiar to the use of horse antitoxin and was not found in tests against human, monkey, rabbit and guinea-pig antitoxins. The evidence submitted does not justify the conclusions drawn. A relatively wide differential region is to be expected when toxins are tested against avid antitoxins owing to the presence of toxoid in every culture filtrate. This was true whether the serum was obtained from a horse or from any other animal. Serum prepared from a hyperimmunized horse by suitable methods is almost always avid but antitoxin produced in any animal, whatever the species, by methods of immunization such as Cohn & Pappenheimer describe would frequently be non-avid and would not exhibit the true differential region. Barr (1951) reported on the formation of abnormal (non-avid) diphtheria antitoxin in the early stages of immunization and also suggested that the production of non-avid antitoxin in hyper-immunization might be due to the 'forcing of an incompletely developed antitoxin-producing mechanism'. A small differential region is not typical of human serum titrations nor of those of guinea-pigs. The following groups of results, which are representative of many thousands of such subcutaneous tests, substantiate this statement.

During the first 6 months of 1912, fifty-two naturally immune persons were tested at fairly wide range at these laboratories; many bleedings from actively immunized guinea-pigs were also tested, some at closer ranges. The results of the first 100 tests in guinea-pigs used in 1912 to titrate human blood and of the first 200 used for testing guinea-pig blood are given in Table 1.

Table 1. *The results of diphtheria antitoxin titrations on samples of human and guinea-pig sera, performed by the guinea-pig subcutaneous method*

	No. of guinea-pigs in tests on	
Result	Human sera	Guinea-pig sera
No swelling, survived	35	44
Slight swelling, survived	6 ⎫	12 ⎫ 82 in
Large swelling, survived	16 ⎬ 36	35 ⎬ differential
Large swelling, died on 4th, 5th or 6th day	14 ⎭	35 ⎭ region
Large swelling, died earlier than 4th day	29	74

If the generalizations made by Cohn and Pappenheimer on their very limited evidence were correct, a random selection of results of titrations would yield very few records of reactions within the differential region, but our results show that 36 % of tests on human blood and 41 % of tests on guinea-pig blood give results within this range. Evidence is also given by the results of ten tests made upon human blood taken from one individual during February and March 1912. Mixtures were made containing the same volume of blood and varying amounts of

toxin (Table 2). In these tests the differential region, in relation to the amount of antitoxin present in the fixed volume of blood, extended from under 0·01 to over 0·015 ml. of test toxin.

Table 2. *The results of diptheria antitoxin titrations on human serum from one individual, performed by the guinea-pig subcutaneous method*

No. of test mixtures	Volume of test toxin (ml.)	Result of subcutaneous injection
2	0·01	Moderately large swellings, both guinea-pigs survived
4	0·015	Medium or large swellings, three died in 8, 9 or 20 days, one survived
4	0·0175	Large swellings, all died in 3 days

Titration of tetanus antitoxins against toxins of different constitution

In the past the titration of tetanus antitoxin afforded a most striking example of the influence of avidity of antitoxin and of toxoid content of test toxins on the result of titration; Glenny (1936) stated that 'the titration of tetanus antitoxin varies with the toxin used because official standard sera are non-avid'. Barr & Glenny (1945) showed that non-avid tetanus antitoxin was produced if hyperimmunization of horses was started before basal immunity was well established. The method of resting horses after a few preliminary injections was first reported by Glenny, Pope, Waddington & Wallace (1925), but it was not universally adopted for many years. Thus it followed that not only international standards, but also laboratory standards were non-avid so that different laboratories using different preparations of test toxin could not agree upon the titres of sera upon which comparative tests were made.

The following experiment was carried out in order to determine the effects upon standardization of both the avidity of antitoxin and the toxoid content of test toxins. Table 3 describes in detail the eight different toxins used. Very rough toxicity

Table 3. *Description of tetanus toxins used for titration*

Batch	Description	Length of growth in days	Approximate number of M.L.D. in test dose	Test dose equivalent to 0·1 unit of avid standard serum
T1	Liquid	2	5000	0·094 ml.
T2	Solid*	2	1000	3·07 mg.
T3	Liquid	6	2000	0·013 ml.
T4	Liquid	7	2000	0·013 ml.
T5	Solid*	8	500	0·55 mg.
T6	Solid*	9	400	0·27 mg.
T7	1 part liquid toxin T1, 9 parts liquid formol toxoid	—	100	0·39 ml.
T8	Liquid	35	50	0·142 ml.

* Precipitated with ammonium sulphate and dried.

tests were made to indicate the proportions of toxin and toxoid in each preparation. Toxins 5 and 6 are of the type usually used for routine standardization; 6 was in use at these laboratories for many years.

Table 4 records the results of titrations of five sera with serum ratios of 1·00 or more. The ratios are based on the flocculation titre of an *in vitro* standard serum chosen by the authors in 1937. The test doses of the eight toxins were fixed against antitoxin A, the serum with the highest ratio. This serum was our laboratory standard, chosen as a known avid serum to which we had allotted a value of 500 U.S.A. units; this round figure, chosen for convenience, was within 1 % of the lowest titre which could be assigned to it when compared with the British standard J against any routine test toxin. Serum J was non-avid like all other official standard sera in use at that time; D was an avid serum specially produced as suitable for the preparation of a new dried standard to replace J. D came from a horse which had been given a few injections of toxin a year or more after its initial prophylactic injections.

Table 4. *The uniformity in titration of avid tetanus antitoxic sera against test toxins standardized against an avid serum*

Antitoxin	A	B	C	D	E
Serum ratio	1·44	1·38	1·35	1·34	1·00
Antitoxin titre unit/ml. mean value from eight toxins ...	500	322	611	26·3	807

Toxin used for titration	Percentage differences between mean value and those determined against each toxin				
T 1	—	− 2·6	+ 1·8	− 4·9	+ 1·0
T 2	—	+ 0·6	+ 1·6	− 3·8	− 6·5
T 3	—	0·0	+ 2·1	+ 3·0	− 4·2
T 4	—	− 0·6	+ 2·4	+ 1·9	− 4·7
T 5	—	− 3·5	− 2·1	+ 2·1	+ 2·1
T 6	—	+ 0·3	− 16·1	+ 0·7	− 5·3
T 7	—	+ 0·3	+ 7·7	− 4·2	+ 12·1
T 8	—	+ 5·8	+ 2·8	+ 5·3	+ 5·6

Results in Table 4 show that with few exceptions all the widely different toxins gave consistent results except for T 6 against C and T 7 against E; these results appear to be outside experimental error. Very different results were obtained when sera with lower serum ratios were titrated against the same toxins. In Table 5 the apparent titres of six sera against toxins 2 to 8 are compared with those against toxin 1. With certain exceptions, far outside experimental error, the figures in this table fall into a regular pattern showing that with non-avid sera higher titres were found when they were tested against toxic filtrates containing the most toxoid in the test dose and the less avid the serum the more marked was the difference. The average of the ratios of the titres of the seven sera against toxins 1 to 4, with 1000 or more M.L.D. per test dose, is 1·07, against toxins 5 and 6, with 400–500 M.L.D. per test dose, it is 1·45, while against T 7 and T 8, which contained more toxoid, and had only 50–100 M.L.D. per test dose, it is 1·86. When the results are compared according to the serum ratios of the sera, the average of the ratios of the titres against all toxins compared with the titres determined against toxin 1 is 1·28 for F, G and H with serum ratios between 0·75 and 0·92, and 1·54 for the far less avid

sera K and L. The titres of these two sera against toxins 7 and 8, containing a great excess of toxoid, range from 2·18 to 2·87, averaging 2·41 times the titres found for them when tested against T 1, the toxin with the least toxoid. Thus very non-avid sera appear to have very little affinity for toxoid.

Table 5. *The variation in the apparent antitoxin titres of non-avid tetanus antitoxins titrated against test toxins standardized against avid serum A.*

Antitoxin	F	G	H	J	K	L
Serum ratio	0·92	0·87	0·75	—	0·37	0·32
Antitoxin titre unit/ml. determined against toxin T 1	78	475	1300	3·45	245	805

Ratio of apparent titres to those obtained against Toxin T 1

Toxin used for titration							Mean ratio
T 1	1·00	1·00	1·00	1·00	1·00	1·00	1·00 ⎫
T 2	1·09	1·08	1·05	1·02	1·06	1·04	1·06 ⎪
T 3	0·97	1·18	1·08	1·07	1·13	1·02	1·08 ⎬ 1·07
T 4	1·23	1·19	1·08	1·11	1·17	1·13	1·15 ⎭
T 5	1·45	1·32	1·18	1·46	1·71	1·96	1·51 ⎫ 1·45
T 6	1·09	1·87	1·49	1·14	1·25	1·49	1·39 ⎭
T 7	1·74	1·38	1·34	1·45	2·18	2·32	1·74 ⎫ 1·86
T 8	1·65	1·70	1·53	1·85	2·28	2·87	1·98 ⎭
Mean ratio	1·28	1·34	1·22	1·26	1·47	1·61	1·36

1·28   1·54

Titrations against toxins 5 and 6 gave evidence of variations in 'goodness of fit'; four sera gave higher titres and two gave lower titres against toxin 5 than against toxin 6. In order to establish this individual goodness of fit beyond all doubt, sera G and L were tested many times against the two toxins using generally only one but sometimes two mice for each test mixture. Table 6 gives the results of all the titrations made on these two sera and the conclusions drawn from them. With so many tests fairly accurate assessments of titre can be made by simple inspection of the results. More accurate estimates can be made by calculating the titre at which tests made would result in the number of early deaths balancing that of survivals. The comparisons between the allotted titres show that serum G appeared 1·39 times stronger against toxin 6 than against toxin 5, while L against T 6 was only 0·76 time that against T 5. Also L appeared 2·49 times the strength of G when T 5 was used for testing but only 1·37 times when T 6 was used.

The results in Table 5 of titrations of non-avid antitoxins against T 7 and T 8 have shown that there was no great difference between natural and formol toxoids with regard to their affinities for antitoxin. In the next section of this work formol toxoid has been used to show the differences in affinity of diphtheria toxin and toxoid for antitoxin. This has been done by direct titration of mixtures of the two components and by comparing the extent of the Danysz phenomenon when the first addition was either toxoid or toxin and the second was toxin.

Table 6. *Summarizing the results of titrations in mice of two tetanus antitoxic sera against two toxins to establish evidence of differences in 'goodness of fit'*

Antitoxin	Test for (units/ml.)	Toxin T5, no. of mice*			Toxin T6, no. of mice		
		a	*b*	*c*	*a*	*b*	*c*
G	600	2	5	11	—	—	—
	650	8	10	1	—	—	—
	700	15	3	0	0	0	10
	800	—	—	—	0	4	14
	900	—	—	—	6	6	5
	1000	—	—	—	14	1	0
L	1000	—	—	—	0	3	7
	1100	—	—	—	1	7	7
	1200	—	—	—	2	9	3
	1350	0	2	6	9	5	0
	1500	2	7	6	7	0	0
	1650	3	7	1	—	—	—
	1800	10	3	0	—	—	—

Titre by inspection (G)	600–650	900
Titre by calculation (G)	632	880
Titre by inspection (L)	1500–1650	1200
Titre by calculation (L)	1575	1203
Ratio, G : L	1 : 2·49	1 : 1·37

* *a*, Dying before; *b*, dying on; and *c*, surviving beyond the 4th day.

The relative affinities of diphtheria toxin and toxoid for antitoxins of different quality

The different preparations of antitoxin used in this work are described in Table 7. Figures for dilution ratios, Glenny & Barr (1932), are not recorded because it has been shown by Barr (1949) that the figures allotted depend upon the toxin used. Similarly, Barr & Glenny (1938) found that sera obtained from one horse with a high serum ratio, i.e. with *in vivo* titre greater than *in vitro* titre, gave different flocculation titres when tested against different batches of toxoid. For the purpose of these experiments it appears sufficient to record in general terms the relative qualities of the antitoxin used. All antitoxins from G to M had abnormal serum ratios, the lowest being 2·2 for G. Serum H has been reported in considerable detail by Barr & Glenny (1938) under its laboratory number EX. 379. Preparation M was prepared from serum G by fractional precipitation with ammonium sulphate and had an *in vivo* titre of 7000 units and an *in vitro* titre of 370 as already reported in the same paper. One other preparation, N, needs special mention; it was obtained by distilled water dialysis of an early ammonium sulphate fraction of a mixture of many antitoxins. When freshly prepared there were many zones of flocculation but all flocculating power was soon lost: *in vivo* titrations were difficult, which suggests that the antitoxin was very non-avid.

Direct titrations for combining power were made upon mixtures containing in a total of 10 ml., 1 Lr of a test toxin and 9 Lf of seventeen different batches of toxoids: the Lr dose was determined against three preparations of antitoxin C, F and H. The results showed that the amounts of toxoid in combination with antitoxin when

only a residual trace of toxin remained free were between 8·0 and 39·4 % (average 24·1 %) when slightly non-avid antitoxin was used, from 10·3 to 47·4 % (average 28·5 %) with antitoxin of average avidity and from 13·0 to 58·3 % (average 40·1 %) when an avid antitoxin was used. Of the seventeen batches tested against each of the three sera, all combined better with the avid than with the average antitoxin, fourteen combined better with the average than with the slightly non-avid anti-toxin and three gave the same results with both these antitoxins. Results from seven of these batches of toxoid have already been published by Barr & Glenny (1949). Of the formol toxoids used in the present work FT 1 to 4 had the lowest affinity coefficients of any examined by Barr & Glenny (1949); the remaining toxoids 5 to 8 were not abnormal in any way. The toxins 9 to 13 had been prepared from four different types of media and two, 10 and 12, had been made some years before the experiment and had been used for some time as routine test toxins.

Table 7. *Description of preparations of diphtheria antitoxin lettered in order of degree of avidity*

Quality	Type of preparation	Batch letters
Very non-avid	*Early ammonium sulphate fractions	A, B
Slightly non-avid	Natural serum	C
	Concentrated by ammonium sulphate precipitation	D, E
Average avidity	Concentrated by ammonium sulphate precipitation	F
High serum ratio:	Natural serum	G, H
$\dfrac{in\ vivo\ \text{value}}{in\ vitro\ \text{value}}$	*Early ammonium sulphate fraction	J, K, L, M
Unknown	Water insoluble globulins	N

* Precipitated by minimal amounts.

Other direct tests for combining power were made by titrating mixtures contain-ing 1 Lf of toxoid and 1 Lr of toxin. All batches of toxoid were treated with sodium metabisulphite to remove any remaining formaldehyde. All mixtures were made up to a total volume of 20 ml. with borate buffer diluent and 0·2 ml. injected intra-cutaneously into guinea-pigs. The end-point chosen was the production of a minimal reaction. Results including those of control tests with toxin are given in Table 8. The amount of antitoxin needed to neutralize the double amount of antigen was always below 2 units because 1 Lf of these toxins was considerably less than 1 Lr. The figures given in the table are the average figures from several titrations. Suffi-cient tests were made to ensure an accuracy of at least 5 %, possibly of 2 %. Union between antigen and antibody was almost as complete in 30 min. as in 24 hr., and there was little decrease in the amount of antitoxin needed if the mixtures were allowed to stand, except in the second series when mixtures contained toxoids with high affinity coefficients and non-avid antitoxin.

At the end of 24 hr. only 0·22 unit of slightly non-avid antitoxin C had com-bined with part of 1 Lf of toxoids with low affinity coefficients. This figure increased to 0·41 unit of non-avid or 0·61 unit of avid antitoxin combined with toxoids with higher affinity coefficients. If the toxoid in the mixture was replaced by 1 Lf of toxin the figure was increased to 0·76 unit of either avid or slightly non-avid anti-

toxin. It follows that (1) toxoid has less affinity than toxin for antitoxin, (2) this difference is greater if the toxoid has a low affinity coefficient, and (3) the difference is further accentuated if non-avid antitoxin is used.

Table 8. *The number of units of diphtheria antitoxin needed to neutralize a mixture of either* 1 *Lf of toxoid or of toxin and* 1 *Lr of toxin*

Antitoxin	Antigen		Affinity coefficient of toxoid*	Time between mixing and injection		
				30 min.	2 hr.	24 hr.
C	Toxoid FT	1	Poor	1·25	1·20	1·20
C		2	Poor	1·25	1·25	1·20
C		3	Poor	1·25	1·25	1·18
C		4	Poor	1·30	1·30	1·30
	Mean			1·26	1·25	1·22
A	Toxoid FT	5	Good	1·55	1·48	1·35
B		5	Good	1·67	1·67	1·43
E		5	Good	1·60	1·55	1·45
E		6	Good	1·45	1·45	1·42
	Mean			1·57	1·54	1·41
K	Toxoid FT	5	Good	1·59	1·59	1·52
K		6	Good	1·65	1·65	1·62
L		5	Good	1·70	1·70	1·70
	Mean			1·65	1·65	1·61
C	Toxin T	9	—	1·80	1·75	1·72
E		10	—	1·95	1·85	1·75
F		9	—	1·85	1·80	1·72
H		9	—	1·85	1·80	1·80
	Mean			1·86	1·80	1·76

* See Barr & Glenny (1949).

Antitoxins A to E were of less and G to M of more than average avidity.

Danysz effect

Further experiments were made by adding the antigen in two fractions to the antitoxin. Mixtures were made of 1 Lf of toxoid (Table 9) or 1 Lf of toxin (Table 10) with varying amounts of antitoxin in a total volume of 20 ml.; a second addition consisting of 1 Lr of toxin was made either 30 min., 24 hr. or 3 days later. Intracutaneous injections of 0·2 ml. of the final mixtures were made into guinea-pigs 5 min., 2 hr. or 24 hr. after the second addition.

Comparison of the results given in Tables 8 and 9 for the slightly non-avid antitoxin C and the poor quality toxoids 1–4 show that if the toxoid was allowed to react with antitoxin before the addition of toxin it combined with larger quantities. Thus when the toxoid and toxin were mixed before adding the antitoxin the toxoid had combined with only 0·22 unit of antitoxin at the end of 24 hr. (Table 8). Table 9 shows, however, that if the toxoid was allowed to react for 30 min. with the antitoxin unhindered by the superior combining toxin, and injections made at the same interval of 24 hr. after the addition of the toxin, the amount of antitoxin combining with toxoid was only increased to 0·29 unit; when the toxoid was allowed a start of 24 hr. ahead of the toxin, this figure was increased to 0·36, and if 3 days

25-2

were allowed it became 0·49. Thus only half of the poor quality toxoids left in contact with excess of slightly non-avid antitoxin for 3 days remained in firm combination when toxin was allowed 24 hr. to displace some of the toxoid. If, however, toxin had only 2 hr. in which to displace toxoid the amounts of antitoxin in combination with toxoid were 0·32, 0·52 and 0·64 unit according to whether the toxoid had a start of 30 min., 2 hr. or 24 hr.

Table 9. *The number of units of diphtheria antitoxin needed to neutralize two additions of antigen, the first 1 Lf of toxoid, the second 1 Lr of toxin*

Time elapsing between two additions		30 min.			24 hr.			3 days		
Time between second addition and injection		5 min.	2 hr.	24 hr.	5 min.	2 hr.	24 hr.	5 min.	2 hr.	24 hr.
Antitoxin	First addition (toxoid FT)									
C	1	1·35	1·27	1·25	1·55	1·45	1·30	1·70	1·65	1·42
	2	1·40	1·27	1·27	1·60	1·55	1·40	1·70	1·55	1·50
	3	1·40	1·35	1·27	1·65	1·42	1·40	1·70	1·65	1·45
	4	1·50	1·40	1·35	1·80	1·65	1·35	1·80	1·70	1·60
	Mean	1·41	1·32	1·29	1·65	1·52	1·36	1·73	1·64	1·49
F	1	1·50	1·42	1·35	2·00	1·70	1·57	2·00	1·80	1·70
	2	1·70	1·60	1·50	2·00	1·90	1·75	2·15	2·00	1·72
	3	1·65	1·50	1·42	1·90	1·80	1·65	2·15	1·95	1·72
	4	1·80	1·70	1·60	2·25	2·00	1·80	2·40	2·20	1·85
	Mean	1·66	1·56	1·47	2·01	1·85	1·69	2·18	1·99	1·75
H	1	1·60	1·50	1·40	2·00	1·72	1·70	2·10	1·90	1·72
	2	1·90	1·72	1·50	2·10	2·00	1·90	2·30	2·00	1·80
	3	1·60	1·50	1·50	2·10	1·90	1·72	2·20	2·00	1·80
	4	1·85	1·75	1·60	2·20	2·10	1·90	2·20	2·00	1·80
	Mean	1·74	1·62	1·50	2·10	1·93	1·81	2·20	1·98	1·78
E	5	1·70	1·65	1·65	2·25	2·15	1·95	2·30	2·25	2·05
	6	1·70	1·65	1·55	2·20	2·00	1·80	2·50	2·25	2·00
	Mean	1·70	1·65	1·60	2·23	2·08	1·88	2·40	2·25	2·03
K	5	1·75	1·75	1·75	1·95	1·95	1·90	2·00	2·00	1·95
	6	1·90	1·90	1·80	2·15	2·10	2·05	2·25	2·10	2·10
	Mean	1·83	1·83	1·78	2·05	2·03	1·98	2·13	2·05	2·03

When another slightly non-avid antitoxin, E, was added to 1 Lf of better quality toxoids, 5 or 6, mixed with 1 Lr of the test toxin, more antitoxin was needed for neutralization by either method of titration. If the antitoxin was added to the total antigen (Table 8) and allowed to act for 24 hr. before injection, an average of 0·435 unit combined with the toxoid, but when the toxoid alone was mixed with the antitoxin and the toxin was added later (Table 9) the amount of antitoxin combined with the toxoid ranged from 0·60 to 1·40 units; the smaller of these amounts was enough if the toxoid acted alone for only 30 min. and the added toxin was allowed 24 hr. to displace some toxoid from combination; the larger amount was needed when the toxoid had a start of 3 days and the toxin was given only 5 min. for displacement.

An antitoxin of average avidity and one with a high serum ratio combined more

firmly with toxoids of either quality; the figures in Table 9 for the toxoids with low affinity coefficients ranged from 0·47 to 1·18 units of antitoxin F (of average avidity) and from 0·50 to 1·20 of the high ratio antitoxin H. More antitoxin was needed for neutralization if the better toxoids 5 and 6 were used.

Glenny (1931) suggested that the presence of toxoid in culture filtrates might account in part for the Danysz effect: some confirmation is afforded by consideration of the results when avid antitoxin and toxoid of high affinity are used. When a single addition of antigen was made to antitoxin (Table 8) an average of 1·61 units of K or L neutralized the mixture; if two additions were made separated by an interval of 24 hr., an average of 1·98 units of K was needed. When no formol toxoid was present 1·72 units of F or 1·80 of H neutralized the mixture of 1 Lf plus 1 Lr of toxin (Table 8); when two separate additions were made (Table 10), between 2·20 and 2·25 units of F, or H were needed.

Table 10. *The number of units of diphtheria antitoxin needed to neutralize two additions of toxin, the first 1 Lf, the second 1 Lr*

Time elapsing between two additions		30 min.			24 hr.			3 days		
Time between second addition and injection		5 min.	2 hr.	24 hr.	5 min.	2 hr.	24 hr.	5 min.	2 hr.	24 hr.
Antitoxin	Toxin									
C	9	2·00	1·90	1·85	2·20	2·10	1·90	2·40	2·30	2·05
	11	1·90	1·75	1·75	2·05	2·00	1·80	2·40	2·20	1·90
	Mean	1·95	1·83	1·80	2·13	2·05	1·85	2·40	2·25	1·98
F	9	2·25	2·20	1·90	2·75	2·50	2·20	2·80	2·75	2·30
	11	1·95	1·80	1·72	2·50	2·25	2·25	2·70	2·50	2·10
	Mean	2·10	2·00	1·81	2·63	2·38	2·23	2·75	2·63	2·20
H	9	2·25	2·05	1·95	2·40	2·25	2·20	2·40	2·25	2·05
	11	2·40	2·10	2·00	2·25	2·25	2·25	2·70	2·50	2·40
	Mean	2·33	2·08	1·98	2·33	2·25	2·23	2·55	2·38	2·23
E	10	2·00	2·00	1·85	2·80	2·60	2·25	2·80	2·70	2·40
K	10	1·95	1·95	1·90	2·10	2·10	2·00	2·10	2·10	2·10

The other results in Tables 9 and 10 show that the Danysz effect may be very small if non-avid antitoxin is used. A single addition of toxin 9 to antitoxin C (Table 8) needed 1·72 units; when the toxin was added in two fractions separated by 24 hr. (Table 10) 1·90 units was sufficient. Kuhns & Pappenheimer (1952) have unwittingly confirmed this. From the figures given by them it would appear that examples of what they term 'non-precipitating serum' which failed to produce a Danysz effect were non-avid antitoxins. The relatively poor affinity between toxoid and non-avid antitoxin would account for this reduced effect.

Presence of toxoid does not appear to be the only cause of the Danysz phenomenon. It is also possible that multiple combination reported by Healey & Pinfield (1935) might be a factor of considerable importance; the Danysz effect was therefore studied from a different angle.

Combination of three or more units of antitoxin with 1 *Lf of toxin or toxoid*

Excess diphtheria antitoxin was added to toxin or to formol toxoid and the mixtures, after standing for some days, were tested for free antitoxin by the guinea-pig intracutaneous method at the Lr/100 level. The lengths of time elapsing between the first addition of toxin or toxoid and the subsequent addition of test toxin, in addition to the dilution of the original mixture before the second addition differ from the experiments of Danysz.

The preparations of antitoxins used in this experiment were chosen for their abnormal serum or dilution ratios, in the unfulfilled hope that results would shed some light upon the cause of these abnormalities. Although only five antitoxins were used three types of reactions were found: in the presence of excess antitoxin, three formed combinations of 3 units of antitoxin to each Lf of toxin (TA3), one formed a 4-unit compound (TA4) and one formed higher multiples.
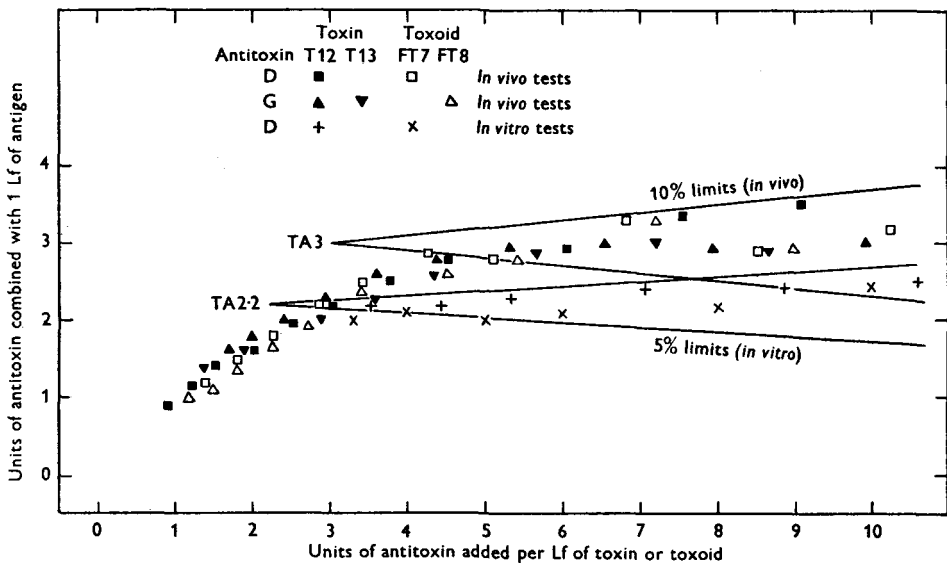
Fig. 1. Multiple combination of diphtheria antitoxins with toxins or toxoids measured by *in vivo* and *in vitro* methods. In Figs. 1–4 the diverging lines show the effect, on the calculated amount of antitoxin combined, of error in estimation of antitoxin free.

Text-fig. 1 depicts the number of units of antitoxin D combined with 1 Lf of toxin 12 and with 1 Lf of toxoid 7, and similar results for antitoxin G combined with toxins 12 and 13 and toxoid 8. The number of units in combination were arrived at by subtracting the antitoxin found from that added; the limits of error of any observations must therefore be determined by the limits of error of the tests for antitoxin uncombined. These limits are probably not much more than 5 % on either side, but it appears reasonable to accept 10 % to allow for additional small errors in composition of the various mixtures. The diagram shows that the amount of antitoxin in combination with 1 Lf of antigen, whether toxin or toxoid, was at least 2 units if 3 units had been added per Lf. The amount in combination gradually increased with increasing amounts added to the antigen. When between 5 and 6 units

had been added two mixtures containing toxin had formed TA 3, but two containing toxoid had not quite reached this stage. Thirteen mixtures were made containing from 6·0 to 10·2 units of antitoxin per Lf of toxin or toxoid and all formed TA 3.

One remarkable feature of Fig. 1 is that it shows that a slightly non-avid antitoxin behaves similarly to one with a high serum ratio and that formol toxoid behaves very similarly to toxin. Of special interest, however, is the difference in the results obtained according to whether tests were made *in vivo* or *in vitro*. Flocculation tests were made on mixtures containing antitoxin D and toxin 12 or toxoid 7. The results did not confirm those found by *in vivo* tests; the compounds formed were slightly higher than TA 2 and averaged 2·2 units of antitoxin in combination with 1 Lf of antigen. Slightly more antitoxin was fixed by the toxin than by the toxoid, 2·3 units combined with toxin and 2·1 with toxoid. In the diagram the scales of antitoxin added or in combination refer to *in vivo* or *in vitro* units according to which method of titration was used. The two points representing results of two types of tests on any one mixture are thus not in the same vertical line. For example, one mixture of antitoxin D and toxin 12 contained 6·0 *in vivo* units of which 2·92 appeared bound, and 7·1 *in vitro* units with 2·4 bound.

Table 11. *The serum ratios of the antitoxin free or combined in mixtures containing excess of antitoxin*

| Antitoxin | Antigen | Antitoxin added per Lf | | Serum ratio of antitoxin | | |
		In vitro	*In vivo*	Original	Free	Combined
D	T 12	3·54	3·02	0·853	0·63	0·98
		4·43	3·77		0·55	1·16
		5·31	4·53		0·57	1·23
		7·08	6·04		0·67	1·21
		8·85	7·54		0·65	1·41
		10·6	9·06		0·69	1·37
D	FT 7	3·34	2·85	0·853	0·51	1·08
		4·0	3·41		0·50	1·17
		5·0	4·26		0·48	1·38
		6·0	5·11		0·60	1·32
		8·0	6·82		0·60	1·55
		10·0	8·52		0·75	1·16
		12·0	10·23		0·70	1·69
G	T 12	4·68	9·89	2·11	2·89	1·31
H	T 12	4·62	18·13	3·92	5·80	2·00
		6·16	24·17		4·59	2·65
		7·71	30·21		4·62	2·27
H	T 13	2·65	10·97	4·14	11·8	2·02
		3·18	13·18		8·86	1·79
		4·24	17·57		6·09	2·25

The different results obtained by the two methods of testing show that the serum ratio of the antitoxin, apparently free in any one mixture, was not the same as that combined. Table 11 shows that in general the abnormality of the ratio of

the original antitoxin was exaggerated in the antitoxin uncombined so that the antitoxin fixed was less abnormal than the original.

One other antitoxin, N, produced TA3 when combined with toxins 12 and 13. This was a preparation of globulin prepared by distilled water dialysis. The *in vitro* titre was unknown because when first prepared flocculation tests could only be made by blending with known sera; such blends gave several zones but all flocculating power was soon lost. The results shown in Fig. 2 suggest some degree of goodness of fit; toxin 13 combined more readily with the antitoxin than did T 12. TA3 was not formed with T 12 unless over 8 units were added, while T 13 produced TA3 with the addition of 5·75 units to each Lf dose of toxin. There was a tendency for higher compounds to be formed with this serum particularly in combination with T 13.
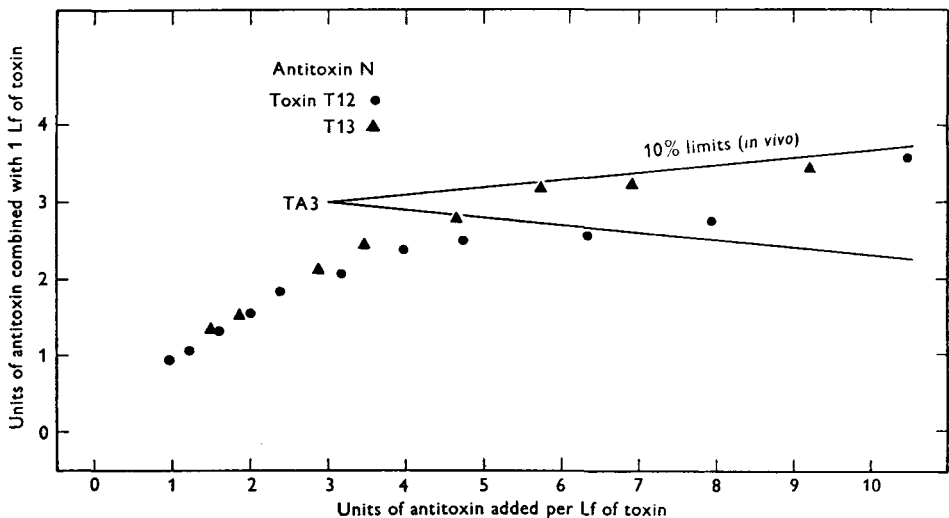
Fig. 2. Multiple combination of diphtheria antitoxin with toxin measured by *in vivo* methods.

The fourth antitoxin used (H) was a natural serum with a serum ratio of about 4. Fig. 3 shows that TA4 was formed in combination with toxins 12 and 13 and with toxoid 8 judged by *in vivo* tests. The flocculating antitoxin, however, formed only TA2 or slightly above. Although the diagram shows the formation of TA2 in mixtures containing from 2·65 to 7·7 units per Lf of toxin the *in vivo* titres of the antitoxin in the same mixture were between 11·0 and 30·2 units.

The fifth antitoxin, M, used in this experiment had a serum ratio of about 20; it was a small fraction precipitated from a high ratio natural serum by minimal amounts of ammonium sulphate. An exact *in vivo* antitoxin titre was difficult to allot to any mixture owing to the long range of reactions exhibited in the tests. The reason for this is not apparent. This preparation behaved in a different manner from the others, or at least its behaviour was greatly exaggerated. High multiple combinations were formed (Fig. 4). In the presence of 3 units of antitoxin, 2 units were fixed by each Lf of toxin, for every 5 units in excess of 3 a further 2 units were fixed so that the formula for the amount of antitoxin combined with each Lf
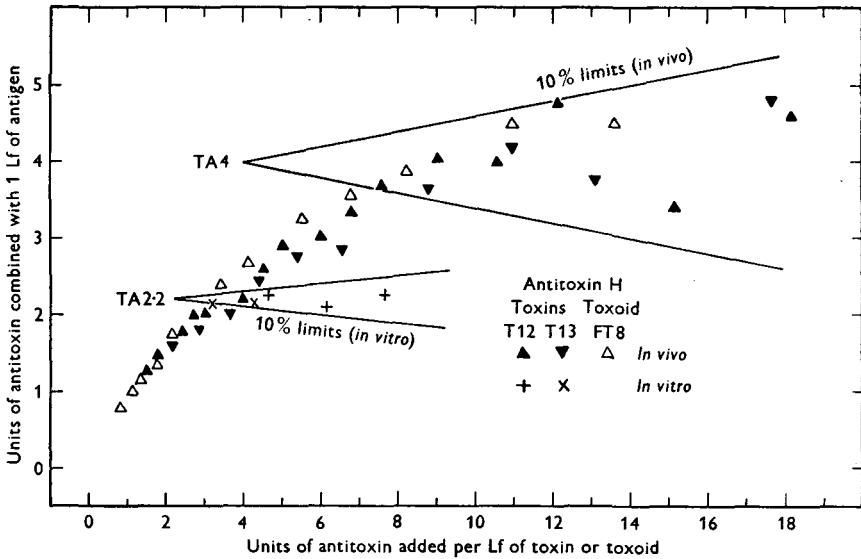
Fig. 3. Multiple combination of diphtheria antitoxin with toxins or toxoid, measured by *in vivo* and *in vitro* methods
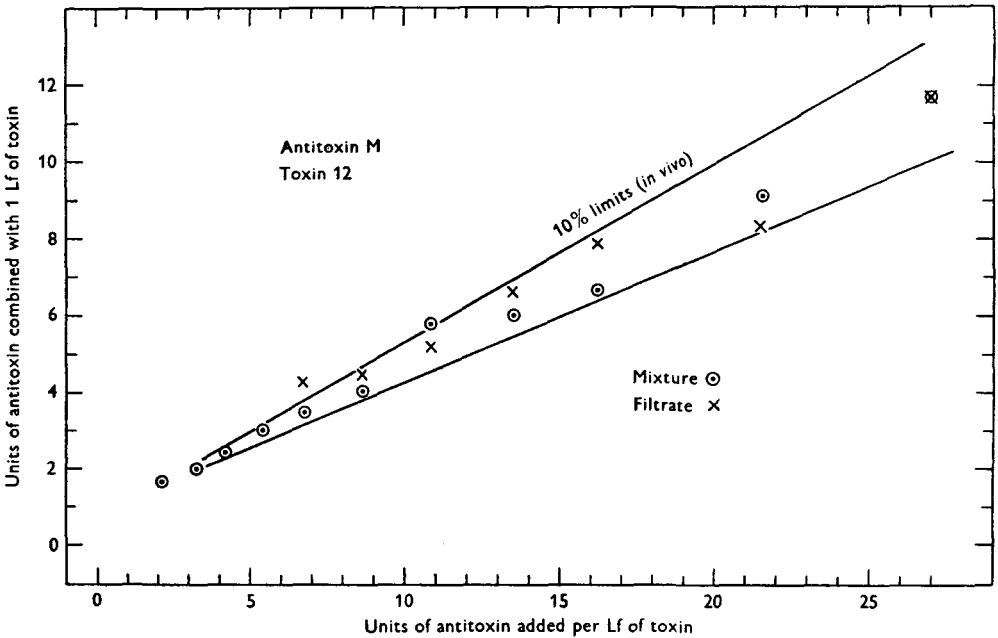

Fig. 4. Multiple combination of diphtheria antitoxin with toxin, measured by *in vivo* methods

of toxin is $2 + \frac{2}{5}(n - 3)$. Applying this formula over the range of n (the number of units added per Lf) 3·25–27·08, it is found that the calculated amount in combination is within 5 % of the amount found in 8 times out of 10 and in only one instance is the discrepancy greater than 10 %.

DISCUSSION

If it is accepted that toxic filtrates contain varying proportions of toxin and toxoid, that both combine with antitoxin although they may differ somewhat in affinity, and that both are antigenic, any measure of toxicity, whether M.L.D. or M.R.D., must be regarded as of little use alone, either as a basis upon which to judge the amount of antigen present in a filtrate or product thereof or to measure the result of immunization in any form of tolerance test. Glenny & Walpole (1915) stated that 'a batch of diphtheria toxin is appraised by the immunologist by its binding-unit content per c.c., a quantity which for all purposes is most readily measured as the L+ dose'. Nevertheless, almost all workers made use of tests for toxin content, usually a measurement of M.L.D., until the flocculation test was established. It is not uncommon to see statements that batches of tetanus *toxoid* contained a certain number of M.L.D. per ml., presumably meaning that that was the lethal capacity of the toxin of origin. The popularity of the M.L.D. test is diffi-cult to understand because, in addition to its use being theoretically unsound, it is in practice more difficult to measure with any degree of accuracy than a combining power test.

The final test of the efficiency of any antigen is the measurement of the degree of immunity produced by its use. The immunity produced by the injection of diphtheria prophylactic into children is usually judged by means of the Schick conversion rate. As this is a measure of the antitoxin content of the blood it must be standardized by means of a combining power test. Glenny (1925) stated 'the Schick dose of toxin should be determined as the equivalent of 1/1000th of a unit of antitoxin rather than 1/50th of a fatal dose'. The latter definition still remains in many countries. Similarly, various prophylactics may be tested in animals by determining their tolerance for specific toxin after injection. As this depends upon the neutralizing power of circulating antitoxin the test injection should be fixed in terms of combining power as well as toxicity. Official regulations in certain countries stipulate the number of M.L.D. that must be tolerated by the test animals without defining the relation between toxicity and combining power of the challenge toxin.

The results recorded in this paper afford further evidence of the greater affinity of antitoxin for toxin than for toxoid, and of the fact that this difference in affinity is greater when the antitoxin is non-avid. The poor avidity of some sera suggests the presence of misshapen or distorted molecules of antitoxin, and the variation in goodness of fit between two non-avid tetanus antitoxins and two toxins, shown in Table 6, provides evidence of corresponding distortion of toxin or toxoid molecules. The existence of goodness of fit suggests that degrees of non-avidity are due not to the presence of varying proportions of a single pattern of distorted antitoxin, but to various types or degrees of distortion which may have their counterpart in the antigen. This idea is supported by the work of Petrie (1942–4), who found that there was a better fit between tetanus antitoxin and the toxin used to immunize the horse from which it came.

There is, however, evidence that only a single form of distortion exists. The affinity coefficients of various batches of diphtheria toxoid followed the same general

sequence whether titrated against slightly non-avid, average or avid antitoxin, although the absolute values of this coefficient depended on the avidity of the antitoxin used (Barr & Glenny, 1949). If toxoids contained several different forms of distorted molecules, differences in goodness of fit between toxins and antitoxins would be expected to be found more frequently. The antigenic efficiency of a toxoid, in relation to its potency as measured by the flocculation test, might be expected to depend to some extent on the proportion of well-formed antigen that it contained, and the avidity of antitoxin produced by injections of a toxoid might depend on the same condition. Evidence in support of this is lacking. Barr & Glenny (1945) reported that non-avid tetanus antitoxin is almost invariably formed in horses given a course of injections before good basal immunity has been established: these authors have supervised the hyperimmunization of many thousand horses, but have no evidence that any particular batches of toxin or toxoid induced the formation of non-avid antitoxin. This suggests that distorted antigen is not antigenically different from the normal one. The most feasible explanation would appear to be that 'different molecules of an antigen might have a common basic structure with slight steric differences which assisted or hindered firm combination' and that 'a similar state of affairs might exist among antitoxin molecules' (Barr, 1951).

Evidence has been given that the combination between toxoid and antitoxin of normal avidity becomes firmer with time, but that such improvement was much less marked with antitoxin of poor avidity. Such conditions must have a considerable effect on the Danysz phenomenon. An additional cause of this phenomenon is the formation of multiple complexes TA3, TA4, etc., as determined by *in vivo* tests, in mixtures containing an excess of antitoxin. Multiple combination occurred whatever the avidity of the antitoxin used.

SUMMARY

1. Non-avid tetanus antitoxin appears to have a higher titre in relation to an avid standard if titrations are made against test toxins with a high toxoid content. The least avid serum tested appeared to be 2·87 times as potent when titrations were made against a toxin grown for 35 days than when a 2-day growth was used. Using the same test doses avid sera did not show this discrepancy.

2. A mixture of short-growth toxin and formol toxoid reacted similarly to the long-growth toxin.

3. Some non-avid tetanus antitoxic sera showed evidence of difference in goodness of fit with two test toxins; one serum was 2·49 times the titre of another when tested against one toxin and only 1·37 times when tested against another toxin.

4. Diphtheria toxoid has less affinity than toxin for antitoxin; this difference is greater with formol toxoids of poor immunizing efficiency; the difference is further accentuated if non-avid antitoxin is used.

5. The Danysz effect may be very small if non-avid antitoxin is used.

6. In the presence of excess antitoxin, toxin or formol toxoid can combine with 3, 4 or more times its equivalent as judged by *in vivo* titrations for free antitoxin.

7. The serum ratio, i.e. *in vivo* titre/*in vitro* titre of high or low ratio antitoxin in multiple combinations is nearer unity than that of the original antitoxin.

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