THE RELATION OF THE TOXICITY OF DIPHTHERIA TOXIN TO ITS NEUTRALIZING VALUE UPON ANTITOXIN AT DIFFERENT STAGES IN THE GROWTH OF CULTURE.

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Until within a fairly recent time the filtered or sterilized bouillon in which the diphtheria bacillus had grown and produced its "toxin" was supposed to require for its neutralization an amount of antitoxin directly proportional to its toxicity as tested in guinea-pigs. Thus, if from one bouillon culture 10 fatal doses of "toxin" were required to neutralize a certain quantity of antitoxin, it was believed that 10 fatal doses from every culture, without regard to the way in which it had been produced or preserved, would also neutralize the same amount of antitoxin. Upon this belief was founded the Behring-Ehrlich definition of an antitoxin unit.

The results of tests by different experimenters with the same antitoxic serum but with different toxins proved this opinion to be incorrect. Ehrlich * deserves the credit for first clearly perceiving and publishing this. He obtained from various sources twelve toxins and compared their neutralizing value upon antitoxin; these tests gave most interesting and important information. The results in six toxins, which are representative of the twelve, are as follows: (See Table I.)

From the facts set forth in the tables Ehrlich has derived interesting theories, which, if true, would add greatly to our knowledge of toxins and would also have a very direct influence upon the present methods of standardizing antitoxin. He believes that the diphtheria bacilli in

* Ehrlich, Die Wertbemessung des Diphtherieheilserums und deren theoretische Grundlagen, *Klinisches Jahrbuch*, vi (1897).

TABLE I.—(FROM EHRLICH.)

Toxin specimen number of Ehrlich.	Estimated "minimal" fatal dose for 250-grm. guinea-pigs.	Smallest number of fatal doses of toxic bouillon required to kill a 250-grm guinea-pig within 5 days when mixed with 1 antitoxin unit. "L_+" Ehrlich.	Fatal doses required to "completely neutralize 1 antitoxin unit" as determined by the health of the guinea-pig remaining unaffected. "L_0" Ehrlich.	$L_+ - L_0$	Data upon "toxin" specimen given by Ehrlich.
2.	0.03	42	32	10	*Preserved 2 years.
4.	0.009	39.4	33.4	6	Old, deteriorated from .003 to .009.
7.	0.0165	76.3	54.4	22	Fresh toxin, preserved with tri-kresol.
9.	0.039	123	108	15	A number of fresh cultures grown at 37° C. 4 and 8 days.
10.	0.001	29.2	27.5	1.7	Precipitated from greatly deteriorated "toxin."
12.	0.0025	100	50	50	Tested immediately after its withdrawal.

their growth produce toxin which, so long as it remains chemically unaltered, has a definite poisonous strength with a definite value in neutralizing antitoxin. This neutralization he believes to be a chemical union, in which 200 fatal doses of toxin for a 250-gramme guinea-pig combine with one unit of antitoxin. The toxin is, however, an unstable compound and begins to change almost immediately into substances which are not, at least acutely, poisonous, but which retain their full power to neutralize antitoxin. These substances, according to Ehrlich, fall into three groups. The first has more affinity for combining with the antitoxin than the toxin itself (protoxoids). The second has the same affinity (syntoxoids). The third has less affinity (epitoxoids).

* For number 9 only does Ehrlich give the duration of the culture growth in the incubator by which the toxin was produced.

According to him, if a mixture of toxoids and toxin is added to antitoxin, the protoxoids first combine with the antitoxin, then the syntoxoids and the toxin combine in equal proportions, so long as the supply lasts, with the amount of antitoxin remaining, or if there is a surplus, with enough to satisfy them; finally, if any antitoxin remains, the epitoxoids unite with it.

If to a mixture, in which all three toxoids as well as toxin have united with antitoxin, some additional toxic culture bouillon be added, the new protoxoids displace first the epitoxoids and then, if free protoxoids remain, the toxin and the syntoxoids from their antitoxin, and thus liberate as well as add free toxin to the solution.

Ehrlich states as an important law that toxin molecules change into toxoid molecules either by three toxin molecules becoming two toxoid and one toxin molecules, or that two toxin molecules change into one toxoid and one toxin molecule.

The practical conclusion which he draws is that from the absolute amount of poison in a given quantity of bouillon alone, nothing can be determined as to its neutralizing value for antitoxin, and that, therefore, the old Behring-Ehrlich method must be greatly modified; namely, that in any bouillon we must detect not only the amount of toxin but also of toxoids before we can judge of its neutralizing value. This he believes he can accurately accomplish.

He gives an interesting theory to explain the production of antitoxin in the blood. This he does upon the supposition that, when absorbed, the toxin combines with a portion of certain selected cells, and that this portion by its union with toxin becomes, at least physiologically, dead. The cell replaces this dead matter with new and similar substance; after several repeated losses and replacements of this substance the cells produce it in excess. This substance, whether originally in the normal cell or reproduced there, and whether remaining in the cell or thrown out into the circulation, is antitoxin.

Ehrlich attempts to separate into its constituent toxoids and toxin each culture bouillon by a formula worked out by him. He believes that his investigations show that the total amount of pure toxin which neutralizes one unit of antitoxin is 200 minimal fatal doses, and that in ordinary culture fluids, which are mixtures of toxoids and toxin, an antitoxin unit neutralizes 200 fatal doses of toxin minus the number of toxin molecules changed into toxoid molecules. Letting A = number of toxin molecules and Z = epitoxoids, then one unit of antitoxin fully neutralized equals (200 — A — Z) toxoid-antitoxin + A toxin-antitoxin + Z epitoxoid-antitoxin. By toxoids are designated both protoxoids and syntoxoids.

To find epitoxoids his first step is to add as much toxin-toxoid mixture to the quantity of the toxic fluid which just neutralized one unit of antitoxin, as shown by the complete health after its injection of the guinea-pig, as will suffice to produce death on the fourth day. This amount will always be found to be above three fatal doses. This apparently excessive amount he believes to be explained by the fact that part of the almost harmless epitoxoids has been displaced by toxin. Ehrlich considers death on the fourth day to be caused by one free minimal fatal dose of toxin.

Let us, as Ehrlich, lump syntoxoids and protoxoids together under the name " toxoids," then suppose we have a toxin composed of 50 toxin molecules + 50 (syntoxoids and protoxoids) toxoids and 100 epitoxoids. To satisfy these 200 molecules of toxin, toxoids and epitoxoids we add one unit of antitoxin. After chemical combination we have 50 toxin-antitoxin and 50 toxoid-antitoxin and 100 epitoxoid-antitoxin. To displace the epitoxoids from the antitoxin we would have to add enough fatal doses to contain 100 molecules of toxin and toxoids, this would be then 50 of toxin and 50 of toxoid. A half molecule more of each would leave a pure toxin molecule, which would, according to Ehrlich, cause the guinea-pig's death in four days.

Ehrlich's formula to find the epitoxoids is:

$$\text{Epitoxoids} = \frac{200^{*}\,(L_{+}\dagger - L_{0}\ddagger - 1\S)}{L_{0} + (L_{+} - L_{0} - 1\S)} \text{ which with the above toxin would be } = \frac{200 \times 50}{50 + 50} = 100\,.$$

* 200 = number of molecules of toxin or toxoid required according to Ehrlich to neutralize one unit of antitoxin.

† L_{+} = fatal doses of toxin required to kill a guinea-pig in four days after having been mixed with one unit of antitoxin.

‡ L_{0} = fatal doses of toxin required to fully neutralize one unit of antitoxin.

§ 1, though given by Ehrlich, appears to us to be incorrect, for by his own statement one molecule of toxin and toxoid would be added, not 1 of toxin (1 fatal dose) alone. The amount to be subtracted from the difference between L_{+} and L_{0} would therefore be, not one, but a fraction of one.

The above summary merely gives an outline of Ehrlich's most interesting article. To become fully acquainted with the reasons for his theories the article itself must be carefully read. He concludes with the statement that his investigations have cleared up the difficulties surrounding the estimation of the amount of antitoxin in any given quantity of serum, and that the formula resulting from his investigations enables us to determine in any selected toxic diphtheria culture fluid the amounts of toxin and toxoid present, so that a unit of antitoxin is no longer a varying quantity, but of an exact and determinable size and one which can at any time be reproduced.

Interest in both his theoretical reasoning and in his practical conclusions led us to subject both to a series of tests which have, we believe, added some interesting facts to those already published by Ehrlich as well as cast doubts on some of his conclusions.

ORIGINAL INVESTIGATIONS.

In the following pages are given the results obtained by testing the toxicity in guinea-pigs and the neutralizing value upon antitoxin of fluid removed from bouillon cultures of different diphtheria bacilli after varying periods of growth.

Five cultures of diphtheria bacilli were used. Two of these were employed for the most important of the experiments and a short description of these may be of value.

One of them, No. 8, was obtained three years ago from a case of mild tonsillar diphtheria. For these three years this special culture has never been passed through animals, but has been transplanted to fresh bouillon every two to three days. It has kept almost intact its original virulence. It kills 250-grm. guinea-pigs in doses of about .005 cc. of a 48-hour bouillon culture in from four to five days, and produces a toxin in from two to five days of such a strength that usually .005 cc. of the filtered bouillon culture kills within five days most guinea-pigs weighing 250 grammes. The toxin produced by this bacillus has been constantly employed, with apparently uniform results in testing antitoxin.

The other culture, "Greenwood," was obtained six months ago from a case of diphtheria occurring in a localized epidemic in a town situated in an isolated mountainous district. This epidemic was remarkable for the frequency with which paralysis appeared in rather mild cases. This peculiarity is noticed in its effects in animals. The other three cultures were obtained one year ago from cases of diphtheria occurring in New York City. These four cultures are of only moderate virulence and have been transplanted to fresh bouillon every few weeks.

The bouillon used for the development of toxin for these experiments, after being prepared from meat in the usual way, was first poured into a single vessel and made neutral to litmus. After adding to each litre of this common stock of neutral bouillon seven cubic centimetres of normal soda solution, the whole was boiled, filtered, poured into 600 ccm. flasks, stoppered with cotton and covered with filter-paper caps. The bouillon contained two per cent of Witte's peptone and filled the flasks one-half full. The bouillon, after thorough sterilization in the flasks, was inoculated from the pellicle of cultures made 48 hours previously. The growth of all cultures was abundant. After the period of growth at 35° C. had lasted the desired time, a measured quantity was withdrawn from each flask by means of sterilized pipettes. The amounts of fluid withdrawn were mingled together and, after the addition of 0.5 per cent of carbolic acid, stored in small sterilized glass bottles, stoppered and placed in the ice chest and there kept until used. As a rule a fresh vial of "toxin" was used for each experiment, as the exposure to the air of the toxin left in the bottle is apt to cause a deterioration in its strength.

The following tables give the actual and comparative toxicity and neutralizing value of "toxin" removed from bouillon cultures of diphtheria bacilli between the 6th hour and 49th day of their growth. Each table gives the details of a single experiment. It was found impossible to rely upon the L_+ and L_0 values unless all the guinea-pigs were inoculated at the same time. Otherwise slight changes in the condition of the animals and of the toxin were found to make the results somewhat inaccurate or at least subject to suspicion.

TABLE II.

GIVING RESULTS OF TEST OF TOXIN PRODUCED BY BACILLUS 8 AT END OF 6 HOURS' GROWTH IN SLIGHTLY ALKALINE 2% PEPTONE BOUILLON.

Toxin tested with one-tenth of an antitoxin unit on account of the slight toxicity of the fluid.

	Weight of guinea-pig.	Amount of toxic bouillon in cc.	Amount of antitoxic serum in cc.	Result in guinea-pig.	
1	278	3.00	*.00012	No deleterious effects.	
2	234	3.20	.00012	No deleterious effects.	
3	237	3.40	.00012	Slight temporary decrease in weight, no induration.	
4	245	3.60	.00012	Slight temporary decrease in weight, no induration.	
5	242	3.80	.00012	Slight temporary decrease in weight, slight induration.	
6	250	4.00	.00012	Considerable induration. Death 8th day.	
7	245	4.40	.00012	Died 6½ days,	
8	248	.3769	†.38	Died 7½ days,	Control tests of toxin to establish the minimal fatal dose for 250-gramme guinea-pigs.
9	265	.3816	.36	Died 6½ days,	
10	249	.44	.44	Died 5½ days,	
11	221	.40	.45	Died 4½ days,	
12	230	.45	.487	Died 3½ days,	
13	240	.5	.51	Died 3½ days,	

The minimal surely fatal dose for every 250-grm. guinea-pig, estimated from results of above tests, equals 0.45 cc. One-tenth of a unit, 0.00012 cc., of serum neutralized 3.40 cc., or 7.55 fatal doses, of toxin sufficiently to protect the health of the guinea-pig.

The "minimal fatal dose" is here defined, so as to be in accord with Ehrlich, as the amount which will kill most guinea-pigs of 250 grms. weight on the fourth day and the remainder that live longer certainly on the fifth day. The amount of toxin given is calculated according to the gramme weight of the pig, since experience has taught that on the average guinea-pigs weighing between 225 and 275 grammes are susceptible to toxin directly proportional to their weight.

* .00012 cc. of this serum equals one-tenth of an antitoxin unit as standardized by Ehrlich's test serum.

† Equivalent amount of toxin for a 250-grm. guinea-pig.

TABLE III.

GIVING RESULTS OF TEST OF TOXIN PRODUCED BY BACILLUS 8 AT END OF 22 HOURS' GROWTH IN SAME CULTURE AS TESTED AT 6 HOURS.

	Weight of guinea-pig.	Amount toxic bouillon in cc.	Amount serum in cc.	Result in animal.		
				Weight on 5th day.	Amount induration on 5th day.	General condition on 5th day, if alive.
1	260	2.00	.0012	265	None.	Good.
2	257	2.10	.0012	242	Slight.	Good.
3	250	2.16	.0012	237	Considerable.	Good.
4	252	2.20	.0012	215	Considerable.	Sick.
5	265	2.36	.0012	225	Considerable.	Dying.
6	252	2.38	.0012		Marked.	Dead on 5th day.
7	250	2.40	.0012			Dead on 3rd day.
8	248	.016	*.016	Died 6th day.	Control tests of toxin to establish the minimal fatal dose for a 250-grm. guinea-pig.	
9	248	.018	.018	Died 4th day.		
10	232	.0168	.018	Died 6th day.		
11	238	.020	.021	Died 3rd day.		

Minimal fatal dose estimated to be about .019 cc.
$L_0 = 2.06 = 108.4$ minimal fatal doses.
$L_+ = 2.39 = 125.8$ minimal fatal doses.
$L_+ - L_0 = .33 = 17.4$ fatal doses.

Epitoxoids by Ehrlich's formula $\frac{200 \times 16.4}{108.4 + 16.4} = 27.8$.

.0012 cc. of this serum equals one unit of Ehrlich's test serum.

TABLE IV.

GIVING RESULTS OF TEST OF "TOXIN" PRODUCED BY BACILLUS 8 AT END OF 48 HOURS' GROWTH. TESTED vii-6-'98.

	Weight of guinea-pig.	Amount of toxin in cc.	Amount serum in cc.	Result in animal.		
				Weight on 5th day.	Amount of induration on 5th day.	General condition on 5th day.
1	220	.500	.0012	239	Pin-point.	Good.
2	224	.512	.0012	222	Bean-sized.	Good.
3	288	.524	.0012	280	Slight.	Good.
4	257	.536	.0012	248	Moderate.	Good.
5	270	.548	.0012	250	Considerable.	Good.
6	262	.560	.0012	238	Marked.	Good.
7	235	.582	.0012	176	Marked.	Sick (died vii-13).
8	265	.594	.0012	217	Marked.	Sick (died vii-14).
9	250	.604	.0012	192	Marked.	Very sick (died vii-12).
10	280	.616	.0012			Died vii-10 (4th day).
11	264	.628	.0012			Died vii-9 (3rd day).
12	260	.0062	†.0060	Died vii-12 (6th day).		Control tests of toxin to establish the four-day or "minimal fatal" dose for 250-grm. guinea-pig.
13	259	.0067	.0065	Died vii-12 (6th day).		
14	272	.0076	.0070	Died vii-12 (6th day).		
15	246	.0073	.0075	Died vii-10 (4th day).		
16	246	.0083	.0085	Died vii-12 (6th day).		
17	248	.0089	.0090	Died vii-9 (3rd day).		

.0075 estimated minimal fatal dose for 250-grm. guinea-pig.
$L_+ = .610 = 81.33$ fatal doses.
$L_0 = .512 = 68.26$ fatal doses.
$L_+ - L_0 = .098 = 13.07$ fatal doses.
Epitoxoids by Ehrlich's formula 30.'

* Equivalent of toxin dose for a 250-grm. guinea-pig.
† Equivalent amount for a 250-grm. guinea-pig.

TABLE V.

GIVING RESULTS OF TEST OF TOXIN PRODUCED BY BACILLUS 8 AT END OF 6 DAYS.

Weight of guinea-pig.	Amount of toxin in cc.	Amount of antitoxin in cc.	Result in animal.
200	.48	.0012	Remained full weight and alive.
210	.48	.0012	Died with paralysis after 20 days; on 11th well and full weight.
214	.51	.0012	Died in 3½ days.
217	.53	.0012	Died in 4½ days.
212	.0047	*.0055	Died 4th day. } Control tests to establish four-day fatal dose for guinea-pigs.
254	.0056	.0055	Died 7th day.
208	.0050	.0058	Died 7th day.
208	.0052	.0062	Died 4th day.

Estimated minimal fatal dose, .0062.

L_+=.535=86.3 fatal doses.

L_0=.475=76.6 fatal doses.

$L_+ - L_0$=.06=9.7 fatal doses.

Epitoxoids by Ehrlich's formula=20.16.

The toxin from the Greenwood culture could not be tested before 3 days, since up to 48 hours the culture fluid was not toxic. Minimal fatal dose 48 hours = 5 cc.

TABLE VI.

SHOWING RESULTS OF TESTING TOXIN REMOVED AT END OF 72 HOURS' GROWTH FROM GREENWOOD CULTURE.

Weight of guinea-pig.	Amount of toxin in cc.	Amount of serum in cc.	Result in animal.
220	5.90	.0012	Unaffected.
260	6.00	.0012	Slight induration.
248	6.80	.0012	Died 7th day.
225	7.30	.0012	Died 2nd day.
228	.050	†.055	Died in 6 days. } Control tests of toxin for minimal fatal dose.
238	.056	.060	Died in 3½ days.
210	.0546	.065	Died in 3½ days.

Four-day or minimal fatal dose of toxin estimated at .058.

L_0=about 5.95=102.6 fatal doses.

L_+=about 7.00=120.7 fatal doses.

$L_+ - L_0$=about 1.10=18.1 fatal doses.

Epitoxoids by Ehrlich's formula=29.

* This culture was developed in a different flask of bouillon from that used for the other tests. The bouillon was from different meat, but was otherwise similar.

† Equivalent for 250-grm. guinea-pig.

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TABLE VII.

SHOWING RESULTS OF TESTING TOXIN REMOVED AT END OF 6 DAYS' GROWTH FROM GREENWOOD CULTURE.

Weight of guinea-pig.	Amount of toxin in cc.	Amount of serum in cc.	Result in animal.	
229	.4172	.00012	On 2nd day lost 13 grms., but on 4th day regained 7 grms. and remained well.	
193	.4323	.00012	Died in 50 hours.	
195	.0312	.00400	Died in 2½ days.	Control tests for minimal fatal dose of toxin for 250-grm. guinea-pig.
209	.038	.00452	Died in 3½ days.	

Estimated minimal fatal dose about .038.

One-tenth unit .00012 completely protected guinea-pig from .4172 cc. of toxin = 10.9 fatal doses.

L_+ would equal about 104 fatal doses.

TABLE VIII.

SHOWING RESULTS OF TESTING TOXIN REMOVED FROM SAME FLASK OF GREENWOOD CULTURE AT END OF 7 WEEKS.

Weight of guinea-pig.	Amount of toxin in cc.	Amount of serum in cc.	Result.	
220	2	.0012	Unaffected.	
250	3	.0012	Slight induration, temporary loss in weight.	
217	4	.0012	Temporary induration and loss in weight. Paralysis 20th day. Death 25th day.	
210	5	.0012	Death on 5th day.	
215	6	.0012	Death on 2nd day.	
245	.10	*.1020	Lived, loss 30 grams in weight.	Control tests of toxin to establish the four-day "minimal" fatal dose for a 250-grm. guinea-pig.
270	.12	.1110	Lived.	
237	.13	.1390	Lived after recovering from paralysis.	
234	.14	.1495	Died 22nd day, paralysis.	
227	.16	.1760	Died 24th day, paralysis.	
250	.18	.1800	Lived after recovering from paralysis.	
262	.20	.1907	Died 2nd day.	
234	.19	.2030	Died 3rd day.	

Minimal or four-day fatal dose estimated from above tests at .19 cc.

L_+ = about 5.10 = 26.8 fatal doses.

L_0 = " 3.00 = 15.8 " "

$L_+ - L_0$ = about 2.10 = 11.0 fatal doses.

Epitoxoids by Ehrlich's formula, $\frac{200 \times 10}{15.8 + 10} = \frac{2000}{25.8} = 77.5.$

The unusual amount of paralysis in the guinea-pigs receiving much less than the four-day fatal dose is very striking in the above series. Toxin from this culture of shorter duration of growth had this same characteristic, but to a less extent.

* This column of figures shows equivalent toxin for a 250-grm. guinea-pig.

TABLE IX.

GIVING MINIMAL FATAL DOSE; NUMBER OF FATAL DOSES OF TOXIN NEUTRALIZED BY ONE UNIT OF ANTITOXIN SUFFICIENTLY TO PROTECT LIFE OF ANIMAL FOR 4 DAYS, L_+; TO PROTECT THE HEALTH OF THE ANIMAL, L_0; THE DIFFERENCE BETWEEN L_+ AND L_0; AND THE AMOUNT OF EPITOXOIDS AS DETERMINED BY EHRLICH'S FORMULA.

Culture 8 development.	Minimal fatal dose in cc.	L_+ in minimal fatal doses.	L_0 in minimal fatal doses.	$L_+ - L_0$ in minimal fatal doses.	Epitoxoids (Ehrlich). Number of molecules in amount of "toxin" required to neutralize one unit.
6 hours	.45	*72			
22 hours	.019	125.8	108.4	17.4	27.8
‡28 hours	.018	91.6	77.7	13.9	28
48 hours	.0075	81.33	68.26	13.07	30
†48 hours	.016	81.2	73.4	7.8	17
† 6 days	.0062	86.3	76.6	9.7	20.16
‡ 3 weeks	.0095	42.3	34.2	8.1	34.3
‡ 8 weeks	.038	31.6	21	10.6	46.8
Culture Greenwood.					
3 days	.058	120.7	102.6	18.1	29
6 days	.038	*104			
7 weeks	.19	26.8	15.8	11	77.5

The above tabulated results obtained from testing the absolute and comparative toxicity and neutralizing value upon antitoxin of fluid removed at different periods in the growth of two cultures are certainly striking, and fully substantiate Ehrlich's claim that the original Behring-Ehrlich definition of an antitoxin unit is very insufficient. The small number of fatal doses of 6-hour "toxin" which .1 unit of

* Estimated from the number of fatal doses sufficiently neutralized by one-tenth of a unit to protect the health of the guinea-pig.

† The bouillon in which this toxin was formed was different from that in which the others developed.

‡ Tests completed too late to insert tables. Details carried out as in those where full tables have been given.

antitoxin neutralized—7.5, which is the equivalent of about 72 for one unit—surprised us, for, according to Ehrlich's theories, we should expect the "toxin" to be nearer purity then than at 22 hours. It is probable that changes produced in the culture bouillon account for this and later unexpected results. A second peculiar result of the test was the rapid decrease (from 126 to 91) in the number of fatal doses required to neutralize one unit between the 22nd and the 28th hour, to be followed by slight irregular changes (81 at 48 hours, 86 at 6 days) before the final apparently permanent tendency to decrease (42 at 3 weeks, 31 at 6 weeks).

"Toxin" from culture Greenwood in its test at 3, 6 and 49 days shows also very marked variations in the actual and in the comparative toxicity and neutralizing strength of the culture bouillon. We note that at 3 days the culture fluid required 120 fatal doses to neutralize one unit, about the same amount as culture 8 required at 22 hours. The variation in these two cultures is more apparent than real in that, although the neutralizing value of a fatal dose was different at similar periods of growth, nevertheless when we consider that culture 8 began to produce toxin immediately and culture Greenwood only at the end of 48 hours, we see that at about the same period in their toxin production the neutralizing value of a minimal fatal dose was about the same in both.

Before discussing further these tests, let us examine the neutralizing value of a fatal dose in different cultures, grown in similar bouillon, at the same period of growth and of the same culture in bouillon from different sources.

TABLE X.

THE ACTUAL AND COMPARATIVE TOXICITY AND NEUTRALIZING VALUE OF THE FLUID FROM FIVE DIFFERENT CULTURES AFTER SIX DAYS OF GROWTH.

Culture.	Minimal fatal dose for 250-grm. guinea-pig.	Number of fatal doses neutralized by one-tenth of an antitoxin unit.
8	.006	9
213	.085	9.76
236	.030	13.9
238	.043	9.07
Greenwood	.038	10.9

The results given above from the tests of the neutralizing value of the toxin removed from five cultures after 6 days' growth at 36° C., the time when toxin is usually withdrawn by us, reveals a great lack in uniformity in the neutralizing value of a fatal dose, although no such difference exists as in the case of the same culture fluid at widely different periods of development.

The actual and comparative toxicity and neutralizing value of the "toxin" produced by the same bacillus in the fluid removed from two flasks of bouillon derived from different meats but of equal alkalinity is shown by comparison of Tables IV and XI.

The data upon the tests of one culture bouillon removed 48 hours after inoculation with bacillus 8 have been given already in Table IV.

The minimal fatal dose was .0075.

L_+ = .610 or 81.33 fatal doses.

L_0 = .512 or 68.26 fatal doses.

The data of the test of the "toxin" developed by the same bacillus 8 in a different bouillon is given fully in Table XI, since a comparison of the two is important, in that the uniformity of the two toxins indicates the possibility of the same bacillus under suitable conditions furnishing a toxin of always about the same relative toxicity and neutralizing power.

TABLE XI.

TEST OF TOXIN PRODUCED BY BACILLUS 8 AT THE END OF 48 HOURS IN A BOUILLON DIFFERENT FROM THAT RECORDED IN TABLE IV.

	Weight of guinea-pig.	Amount of toxic bouillon in cc.	Amount of serum in cc.	Result in animal.	
1.	265	1.125	.0012	Unaffected.	
2.	263	1.15	.0012	Slight skin necrosis. No loss in weight.	
3.	270	1.175	.0012	Slight induration. No loss in weight.	
4.	277	1.20	.0012	Death 7th day.	
5.	257	1.30	.0012	Death 4th day.	
6.	250	.011	*.011	Died 6th day.	Control tests of toxin to establish four-day fatal dose in guinea-pigs.
7.	235	.0112	.012	Died 21st day after paralysis.	
8.	250	.0106	.013	Died 6th day.	
9.	205	.0118	.014	Died 6th day.	
10.	264	.0158	.015	Died 5th day.	
11.	240	.0153	.016	Died 4th day.	

Minimal or four-day fatal dose estimated at .016 cc.
L_+=1.30=81.2.
L_0=1.175=73.4.
$L_+ - L_0$=125=7.8.
Epitoxoids "Ehrlich"=17.

* The equivalent in a 250-grm. guinea-pig of the amount of toxin given in the actual animal.

Certainly the toxins produced by bacillus 8 in these two different bouillons as given in the Tables IV and XI are remarkably similar in the neutralizing value of a fatal dose, although the toxicity of one toxin as shown in Table XI is only one-half of the other as shown in Table IV. Six toxins developed during the six-days' growth of cultures of No. 8 in slightly alkaline bouillon have at different times in the past three years been used in antitoxin tests, and all have had nearly the same antitoxic neutralizing value for a fatal dose.

These results have encouraged us to hope that, with a closer study of the relations existing between the changes taking place in the bouillon during the different stages of the growth of the bacilli, and of both the development of the toxin and its neutralizing value, we may be able to obtain from any culture of known characteristics at a certain period of its toxin production a toxin of always about the same neutralizing power. A comparison between different laboratories of the neutralizing value of the toxins produced by their bacilli would help to obtain this information.

Later it would be a very simple matter to select one of the cultures which proved most constant in its characteristics and distribute this to all the testing laboratories of a country. In this way the relative toxicity and neutralizing value of the culture fluid grown under the most varying conditions would soon become known.

When we examine the results of the above tests they do not appear to us to reveal the presence of a substance of such a nature as that of the epitoxoids, as defined by Ehrlich, nor do they seem to indicate the possibility of detecting, except in so far as comparative tests suggest, the purity or constituents of a fatal dose of diphtheria culture fluid or "toxin." If epitoxoids have the properties described by Ehrlich, then they should in proportion to their presence increase the gap between L_+ and L_0, but we find no evidence of this in the tables for the fluids having by Ehrlich's formula and theory far the greatest amount of epitoxoids; the deteriorated seven and eight-weeks' toxins have almost the smallest gap between L_+ and L_0. Again we find decidedly more epitoxoids at the end of 22 and 48 hours' growth of the culture 8 than at 6 days and 3 weeks. This certainly seems unlikely, for with the increased amount of deteriorated toxin present in the culture of longer growth in the incubator, we should expect an increase, not a decrease of epitoxoids, if such exist. Again the remarkable variations in number of fatal doses required to neutralize one unit between the 6, 22, 28 and 48-hour toxin are difficult to understand on Ehrlich's theory.

There is indeed, with the use of increased quantities of antitoxin, a larger and larger gap between the amount of toxin which just suffices to produce a slight temporary induration or effect upon the health of the test guinea-pig and the amount which kills upon the fourth day. This difference is about three fatal doses when one-tenth of a unit is neutralized and about eight to fourteen when one unit is employed. In the above tables it will be seen that there is an average of about 8 fatal doses between the amount of toxin which kills a guinea-pig on the fourth day and the amount which kills at a later period from paralysis or increased weakness. This amount is not greater, however, in the old deteriorated toxin than in the fresh, and shows great variations.

This inhibition, through antitoxin, of the otherwise quickly fatal effect of such an excess as six to ten fatal doses of toxin, suggests a difficulty in accepting as proven the chemical theory of the neutralizing effect of antitoxin as being due to its direct combination with the toxin.

Ehrlich not only forms a theory to explain the varying neutralizing value of a fatal dose of toxin, but through a formula, as already stated in the earlier part of this article, he attempts, and, as he believes, attempts successfully, to separate a fatal dose of "toxin" into its constituents and thus detect with accuracy what amount of antitoxin it should neutralize. He claims that by such separation only can a toxin and therefore an antitoxin be properly standardized. Neither his theoretical reasoning nor the results of the working of his formula seem to us convincing of the accuracy of these claims. Not only the weight of Ehrlich's authority, but the practical importance to all those who test antitoxin of his conclusions, demand that we discuss his views.

Upon the four-day limit of life for a guinea-pig which has received a fatal dose of toxin he obtains the values of L_+, L_0, and therefrom the epitoxoids. A dose of toxin which kills most 250-grm. guinea-pigs in four days is for practical purposes called the minimal surely fatal dose for all pigs of that weight. It is, however, an arbitrary standard, and is in fact much more than the minimal fatal dose for most guinea-pigs. A glance at the control pigs in any table—Table IV is a good example—will show how much beyond the actual fatal dose for guinea-pigs is the amount which kills on the fourth day. A minimal fatal dose for a single pig will kill at the end of a week or ten days, or later still by paralysis in the 2nd or 3rd week. This seems so apparent as hardly to need stating.

If, however, Ehrlich should choose such a real limit, or even put it at the sixth or seventh day, it would entirely alter the values of L_+ and L_0, and therefore the results of his formula. The value of L_+ can be fairly accurately determined, but not that of L_0, since slight induration or loss of weight may or may not occur in different animals from the same amounts. His assumption that one unit of antitoxin neutralizes 200 fatal doses of pure toxin or of its derivatives seems to us purely theoretical. Even if the toxin molecules be divided as he believes, still in any given bouillon there is actual destruction, as well as production, going on all the time of all substances, not only those toxic to guinea-pigs, but also of those which, though not toxic, still neutralize antitoxin.

If Ehrlich's formula really revealed the nature of a "toxin," it should answer when employed with larger or smaller amounts of antitoxin than one unit. The L_+, L_0, and the minimal fatal dose should have the same relative value when .1, 1 or 10 units of antitoxin are mixed with varying amounts of toxin. The 200 would merely become 20 or 2000. If, however, we test the formula by solving the same toxin when different known quantities of antitoxin are employed, the amount of epitoxoids will vary with every test; thus toxin from culture Greenwood of three days' growth tested against 1 unit shows:

TABLE XII.

GREENWOOD TOXIN OF THREE DAYS' GROWTH, THE FLUID BEING THE SAME AS TESTED IN TABLE VI AND TESTED AT THE SAME TIME.

	Weight of guinea-pig.	Amount of toxic bouillon in cc.	Amount of serum in cc.	Result in animal.
1.	245	.80	.00012 = .1 unit	Unaffected.
2.	247	.84	.00012 = .1 unit	Died 8th day.
3.	222	..86	.00012 = .1 unit	Died 8th day.
4.	247	.88	.00012 = .1 unit	Died 4th day.

Minimal fatal dose .058 (see Table VI).

$L_+ = .88 = 15.2$ fatal doses.

$L_0 = .80 + = 13.9$ fatal doses.

$L_+ - L_0 = .08 + = 1.3$ fatal doses.

Epitoxoids in .1 unit $= \frac{20 \times (L_+ - L_0 - 1)}{13.9 + (L_+ - L_0 - 1)} = \frac{20 \times .3}{13.9 \text{ plus } .3} = .42$

Epitoxoids in 1 unit $= 4.2$.

Epitoxoids in the same toxin tested by formula when 1 unit of antitoxin is employed = 29.

This table illustrates also that the new method of grading the value on the L_+ amount with one unit is somewhat more severe than the old method of grading upon the L_0 with one-tenth of an antitoxin unit.

SUMMARY AND CONCLUSIONS.

Until recently diphtheria " toxin " was supposed to be a single definite substance and to have a definite toxicity in animals and neutralizing power for antitoxin. A fatal dose of toxin, without regard to the conditions under which it was produced or preserved, was supposed to require always the same quantity of antitoxin to neutralize it. Ehrlich's researches have completely done away with this theory, and have substituted for it one which assumes the toxin to be only at its origin a single definite chemical compound with definite physiological and antitoxic properties. According to Ehrlich the toxin is an unstable substance which readily loses its toxicity, while at the same time its affinity for antitoxin may be either increased or decreased. Its neutralization by antitoxin he considers to be due to a chemical union between the toxin and the antitoxin.

The results of our experiments as detailed in this paper are fully in accord with those published by Ehrlich, as to the varying neutralizing value of a minimal fatal dose of "toxin"; they, however, go further and indicate roughly a general law in accordance with which these changes occur.

The neutralizing value of a fatal dose of toxin is at its lowest in the culture fluid when the first considerable amounts of toxin have been produced. After a short period, during which the quantity of toxin in the fluid is increasing, the neutralizing value of the fatal dose begins to increase, at first rapidly, then more slowly.

While the culture is still in vigorous growth and new toxin is being produced, the neutralizing value of the fatal dose fluctuates somewhat, but with a generally upward tendency. After the cessation of toxin production the neutralizing value of the fatal dose increases steadily until it becomes five to ten times its original amount.

In our experiments the greatest value for L_+ was 126, the least 27. As at 6 hours L_+ was only 72 and at 28 hours only 91, we doubt whether L_+ ever reaches above 150, and therefore hardly expect Ehrlich's figures of 200 to be realized. When we seek to analyze the above-described process, we find certain facts which seem partly to explain it. Experiments have shown that filtered toxin, preserved for any length of time in conditions under which access of air occurs, gradually loses in both its toxicity and neutralizing power, and that it loses more rapidly in the former property than in the later. Thus, while the fatal dose of a toxin preserved for one year rose from .01 cc. to .55 cc., it lost only half as much in neutralizing value, one unit neutralizing at first 1 cc., at the end of the year 25 cc. These processes take place more rapidly at room temperature than in the ice chest, and in the incubator than in the room.

In the fluid holding the living bacilli we have, therefore, after the first few hours of toxin formation, a double process going on, one of deterioration in the toxin already accumulated, which tends to increase the neutralizing value of the fatal dose, the other of new toxin formation, which probably tends to diminish the neutralizing value. The chemical changes produced by the growth of the bacilli in the bouillon tend to aid one or the other of these processes and so to make from hour to hour slight changes in the value of the fatal dose. Later, with the period of cessation of toxin production, the gradual deterioration of the toxicity alone continues, and the fatal dose gradually and steadily increases in its neutralizing value.

Ehrlich's theories, as to the splitting up of "toxin" into toxoids having little or no toxicity but on the average full neutralizing power for antitoxin, have not in our opinion been substantiated by the results of these experiments. The difference between the amount of toxin mixed with a unit of antitoxin which causes the first symptoms and that causing death upon the fourth day would be, it is true, explained by his theory, but the failure of this difference to be greater where, by his theories, epitoxoids should be in great abundance prevents our acceptance of his views. The fact of the greater neutralization value of a fatal dose of a deteriorated toxin would be accounted for on his protoxoid theory. This, however, is not proof of its correctness, as other theories, such as the production by the diphtheria bacillus of two or more closely allied toxins, similar to the allied alkaloids produced by plants, would equally account for it, if we supposed the one which had the greater neutralization value was more resistant to destruction than the other. We only advance this theory to call attention to the fact that many theories can on paper explain a process without necessarily being thereby established.

Even if his theories prove partially correct, we feel certain that his formula for standardizing toxins is founded upon error and cannot be employed for the purpose intended by him.

While we do not believe, therefore, that he has changed the principles of testing antitoxin, yet we believe he has contributed greatly to uniformity in results by calling attention to the necessity of selecting a suitable toxin and by employing and distributing an antitoxin as a standard to test toxins by. In this way smaller testing stations can make their results correspond with those of the central station.

In spite of the great variations in the neutralizing value of a fatal dose in different toxins, we do not believe there has been any such great difference in the toxins used by the different stations for testing purposes. Most laboratories have taken the culture fluid at about the time of its greatest toxicity, and the neutralizing value of a fatal dose of this toxin would seldom vary more than 10 per cent above or below the standard now adopted in Germany by the government testing station, this latter being presumably as close as possible to that used to establish the original Behring-Ehrlich unit.

Where error has been made, it has usually been by taking too old culture fluids, which would cause the antitoxin strength of samples tested to be estimated below and not above its real value. Culture 8, which is used not only by us but by many other laboratories in the United States and Europe, fortunately produces on the 6th day, the time at which the culture is usually removed, a toxin which grades Ehrlich's antitoxin within five per cent of the strength given by him.

We believe that by using such a bacillus, we can, after gaining a fuller knowledge of its characteristics, obtain a toxin of a known and suitable neutralizing value, and thus always correctly standardize an antitoxic serum. Meanwhile a fairly permanent antitoxin, such as Ehrlich provides, is of immense value in insuring a uniform though not necessarily correct standard among the different testing stations and in allowing of comparison between them.