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United States Standard Diphtheria Toxin for the Schick Text and the Erythema Potency Assay for the Schick Text Dose

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A diphtheria toxin shown to have high toxicity and avidity and to combine with antitoxin in multiple proportions was selected for the U.S. standard diphtheria toxin for the Schick test and freeze-dried. Assayed values per vial were 1.09 L_f, 1.09 L_r, 1,090 Schick test doses (STD), 33 LD_{80}, 38 LD_{50}, and 43,000 minimum skin reactive doses. One STD (L_r/1000) is slightly more toxic than one unit of the international standard (L_f/1,000). Experiments showed that the potency assay of Schick test toxin by guinea pig erythema toxicity, determined relative to the toxicity of the standard, was highly reproducible and significantly more reproducible than the lethal (minimum lethal dose) test and that the STD, defined as one L_r/1000, was equivalent to approximately 1/50 minimum lethal dose. The erythema potency assay was prescribed in the U.S. standards for Schick test toxin effective in 1969.

Revised United States requirements for diphtheria toxin for the Schick test were effective from January 1969 (19). This paper describes the experimental work which supported the change from the potency requirement that the Schick test dose (STD) shall contain 1/50 minimum lethal dose (MLD) to the requirement that the STD shall be equal in guinea pig intradermal erythematous reactivity to that of the STD of the U.S. standard diphtheria toxin for the Schick test. The STD is defined as that amount of the standard toxin which, when mixed with 0.001 unit of the U.S. standard diphtheria antitoxin and injected intradermally in a guinea pig, will induce an erythematous reaction 10 mm in diameter.

The Schick test (16) was developed to determine if an individual is susceptible or immune to diphtheria, and the dose is defined as 1/50 MLD for the guinea pig. Because of variation in the susceptibility of guinea pigs to the lethal activity of diphtheria toxin, the STD was difficult to standardize (18). In 1925, Glenny (4) proposed that the STD be standardized as that amount of toxin which is neutralized by 0.001 unit of a standard antitoxin. In 1931, the Permanent Commission on Biological Standardization of the League of Nations (14) accepted the use of two tests, erythema potency and antitoxin-combining power, as alternatives to the guinea pig lethal test for standardization of diphtheria toxin for the Schick test.

In 1957, Gerwing et al. (3) questioned the inclusion of the combining-power test in the routine assay of the Schick toxin product and recommended that the STD of the toxin be standardized to the equivalence of the STD of the international Schick toxin (diphtheria) by use of an animal erythema potency test. The U.S. newly prescribed potency test (19) is similar to the test described by Gerwing et al. The U.S. specification for the parent toxin tends to preclude the need for a combining-power test.

This paper describes the selection of the parent toxin for the U.S. standard, the studies performed in the development of the dermal erythema potency test, the preparation of the standard, the properties of the standard in comparison with the properties of the international Schick toxin, and the relationship of the activity of commercial Schick test toxins assayed by the dermal toxicity test and the lethal test.

MATERIALS AND METHODS

Animals. Guinea pigs and rabbits were obtained from the Rodent and Rabbit Production Section, Division of Research Services, National Institutes of Health. The guinea pigs weighed 230 to 300 g for lethal tests, 500 to 800 g for skin tests, and 300 to 500 g for the L_+ test. Rabbits used for skin tests weighed 1,500 to 2,000 g.

Toxins and antitoxin. Three samples of bulk parent diphtheria toxin were obtained from U.S. manu-

facturers. Subsequently, one of these lots was procured for the U.S. standard diphtheria toxin for the Schick test. The international Schick toxin was studied for a relationship with the U.S. standard, and 18 commercial lots of Schick toxin were assayed to determine suitability of the erythema assay.

The U.S. standard diphtheria antitoxin, filling lots A15, A16, and A17, was used. The unit of the U.S. standard [antitoxin unit (AU)] is equivalent to that of the international standard [international unit (IU)].

Diluent. Phosphate-buffered saline (PBS; 0.066 M, *p*H 7.4; reference 17) containing 0.2% gelatin (PBSG; reference 13) was used for preparing all dilutions. The *p*H and gelatin were critical to prevent deterioration of the toxin. At *p*H 7.0, there was about a fourfold decrease in skin reactivity.

MLD. Minimum requirements for diphtheria toxin for the Schick test (October 15, 1948) defined the MLD as the smallest amount of toxin which, when injected subcutaneously into 250 g ± 10% guinea pigs, will cause death of all animals injected with at least 75% of deaths taking place between 72 and 96 hr (2, 5). Experience indicated that the death interval requirement of 72 to 96 hr is difficult to meet. In practice, a medium death time between 60 and 120 hr with death of all animals injected was accepted as satisfactory. The lethal action of the standard was titrated by using nine doses ranging from $\frac{1}{30}$ to $\frac{1}{42}$ vial content and 8 to 20 guinea pigs per dilution in 10 experiments. No experiment included the full range of doses. Results per dose were combined for estimations of the lethal dose levels.

Minimum skin reactive dose. In our study, the minimum skin reactive dose (MRD) was defined as the smallest amount of toxin in a volume of 0.1 ml which induced an erythematous reaction 10 mm in diameter in guinea pigs or rabbits 40 to 48 hr after intradermal injection (6, 15). The optimum volume of the injection (0.1 ml) and optimum diluent, PBSG, were determined in preliminary tests. The nature of the reactions was observed up to 100 hr. Injection of Evans Blue dye used in early tests did not increase the precision of reading of the reactions and was discontinued.

For each test, eight guinea pigs and eight twofold dilutions of each of two toxins were used. Hair was removed from the flanks and back of the animals with an electric clipper followed by application of a depilatory agent (Nair; Carter Products, Inc., New York, N.Y.). The denuded skin was divided into 16 areas (Fig. 1). The test volume of 0.1 ml was injected by using a 27-gauge needle and a 1.0-ml tuberculin syringe. Each of the eight dilutions of each toxin was injected into each of the eight guinea pigs in the randomized manner as shown in Table 1. Between 40 and 48 hr, the reaction induced by each dilution per animal was recorded as the average of the long and short diameters. Then the average diameter of the eight reactions per dilution of toxin was calculated.

Toxin-antitoxin tests: the L_r dose. We determined the smallest amounts of toxin (L_r doses) which, when mixed with 0.1, 0.01, and 0.001 AU of antitoxin, induced an erythematous reaction 10 mm in diameter (3, 9). The guinea pigs were prepared as for the MRD test. For the $L_r/10$ test, eight small-increment (about

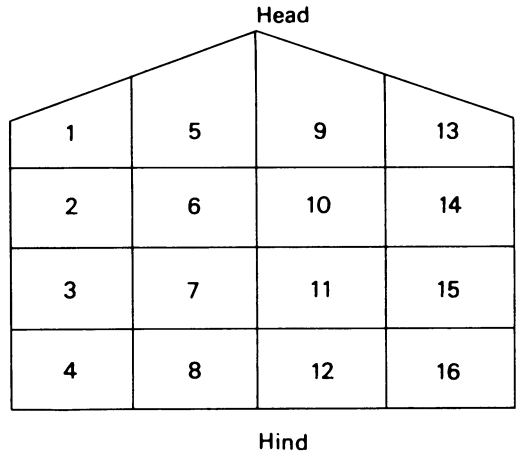

FIG. 1. *Location of the 16 intradermal sites on a guinea pig. See Table 1 for randomization scheme for injection.*

TABLE 1. *Randomization scheme for the injection of eight dilutions of two toxins on each of eight guinea pigs*

Diphtheria toxin		Injection sites							
Lot	Dilution	1[a]	2	3	4	5	6	7	8
A	A1	3	10	14	1	12	7	16	5
	A2	5	3	12	16	10	1	7	14
	A3	11	2	6	9	4	15	8	13
	A4	13	11	4	8	2	9	15	6
	A5	4	8	15	2	13	6	9	11
	A6	15	9	11	6	8	13	2	4
	A7	12	16	7	10	5	14	1	3
	A8	7	1	3	14	16	5	10	12
B	B1	14	7	1	5	3	16	12	10
	B2	16	12	5	3	7	10	14	1
	B3	6	15	9	13	11	8	4	2
	B4	8	4	13	11	15	2	6	9
	B5	9	13	2	15	6	4	11	8
	B6	2	6	8	4	9	11	13	15
	B7	1	5	10	7	14	12	3	16
	B8	10	14	16	12	1	3	5	7

[a] Number of animals.

10%) dilutions of each toxin were mixed, respectively, with a constant amount of standard antitoxin that provided 0.1 AU per 0.1-ml test dose. The 16 mixtures (two toxins) were incubated at room temperature (22 ± 1 C) for 1 hr and injected intradermally into the eight guinea pigs in the randomized manner, and results were read in the same manner as for the MRD test. The $L_r/100$ and $L_r/1{,}000$ values were determined in a similar manner.

The L_+ dose. The L_+, $L_+/10$, $L_+/100$, and $L_+/1{,}000$ doses of a toxin were determined as the smallest amounts of toxin which, when mixed with 1, 0.1, 0.01,

TABLE 2. *Relative toxicity (MLD and MRD)[a] of toxins A and B*

Toxin	MLD (μliters) (guinea pig)	MRD (μliters)	
		Guinea pig	Rabbit
A	0.253 (3,953)[b]	3.72×10^{-4} (2.7×10^{6})	2.33×10^{-4} (4.3×10^{6})
B	0.758 (1,319)	10.46×10^{-4} (0.96×10^{6})	6.18×10^{-4} (1.6×10^{6})
Relative toxicity	3.0	2.8	2.7

[a] MLD, minimum lethal dose; MRD, minimum skin reactive dose.
[b] Values in parentheses denote the number of MLD or MRD doses per milliliter.

TABLE 3. *Combining power (L_r and L_+ doses) of toxins A and B*

Diphtheria antitoxin unit	Amt of toxin (μliters)					
	L_r dose			L_+ dose		
	Toxin A	Toxin B	Relative toxicity	Toxin A	Toxin B	Relative toxicity
1.0				14.550	37.040	2.5
0.1	1.250	3.350	2.7	1.790	4.868	2.7
0.01	0.108	0.289	2.7	0.318	1.050	3.3
0.001	0.012	0.032	2.7	0.314	1.200	3.8

and 0.001 AU antitoxin, respectively, and injected subcutaneously in three or more guinea pigs caused death of all animals within 120 hr. For each titration, six small-increment dilutions of each toxin were mixed with a constant amount of standard antitoxin that provided the appropriate unitage per 3-ml test dose. The mixtures were incubated for 1 hr at 22 ± 1 C.

Antitoxin-combining avidity velocity. Antitoxin-combining avidity velocity (10) of the toxins was determined at the $L_r/100$ level. Toxin-antitoxin mixtures held at 22 ± 1 C were tested at 0, 0.5, 1, 3, and 24 hr to determine that amount of noncombined toxin that would induce a 10-mm reaction in guinea pigs.

Toxin-combining avidity at different antitoxin concentrations. Toxin-combining avidity (3, 7) was determined with the $L_r/10$, $L_r/100$, and $L_r/1{,}000$ and the L_+, $L_+/10$, $L_+/100$, and $L_+/1{,}000$ tests.

RESULTS

Selection of the parent diphtheria toxin for the standard. Preliminary comparisons of the three samples of toxin, by using the guinea pig MLD and the chick mean lethal dose (1, 11) tests, showed that one toxin was much lower in toxicity than the other two. This toxin was eliminated from further study. The results also pointed up that the two lethal test systems measured different dose-response characteristics of the toxins and that a correlation could not be made in absolute terms. The impression, not proven, was that the correlation was poor, and work with the chick test was discontinued. The other two toxins, coded toxins A and B, differed about threefold in toxin activity with toxin A being the more toxic. As measured by the various tests, the relative toxicity values did not differ significantly. These values were 3.0 in the MLD test and 2.8 (guinea pig) and 2.7 (rabbit) in the MRD tests (Table 2); 2.7 in the L_r test with 0.1, 0.01, or 0.001 AU; and 2.5, 2.7, 3.3, and 3.8 with the L_+ test at four antitoxin levels ranging from 1.0 to 0.001 AU (Table 3).

Figure 2 shows that the two toxins combined in multiple proportions in the L_+ and the L_r tests. In the L_+ curves, the deviation from the straight line for toxin A at 0.001 AU and for toxin B at 0.01 AU is due to the limit of the sensitivity of the test for the particular toxin. The avidity of the two toxins was comparable as shown by the parallel lines of the L_r and L_+ combining values at different antitoxin concentrations (Fig. 2) and by the velocity of antitoxin combination at $L_r/100$ which was immediate and stable (Table 4).

The results of the above tests indicated that toxin A had properties recommended for Schick test toxin (3, 5) and would be suitable for a standard preparation. The results also indicated that an erythema potency test for Schick test toxin would be satisfactory.

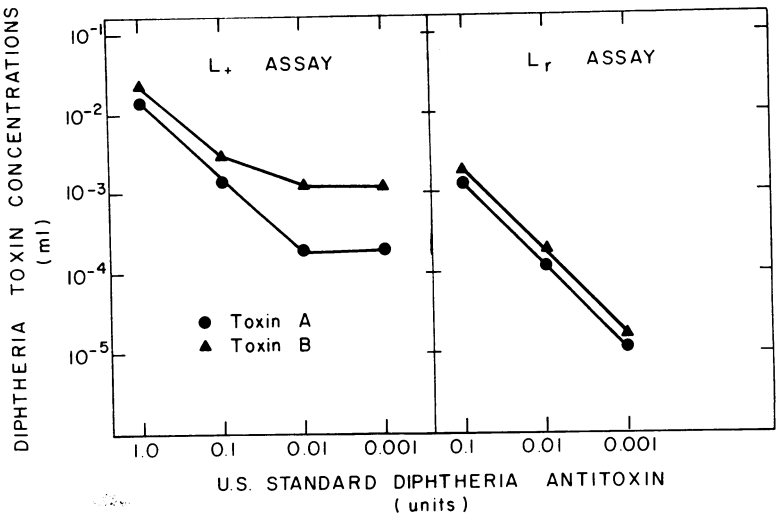

FIG. 2. *L_+ and L_r doses of toxins A and B titrated at 10-fold levels of antitoxin with guinea pigs.*

TABLE 4. *Avidity of combination of toxins A and B with antitoxin at the L_r/100 level*

Incubation time (hr)	Diphtheria antitoxin unit	Toxin in L_r/100 dose (μliters)		
		Toxin A	Toxin B	Relative toxicity
0	0.01	0.105	0.3050	2.9
0.5	0.01	0.105	0.3025	2.9
1.0	0.01	0.105	0.3025	2.9
3.0	0.01	0.1075	0.3025	2.8
24.0	0.01	0.1125	0.3100	2.8

Nature of the skin reaction. The erythematous, edematous, and necrotic manifestations of the skin reactions, within certain dose limits, were linear (Fig. 3). The erythema and edema reactions showed a closer relationship with each other than with the necrosis reactions. However, the erythema reactions with average diameters from 10 to 20 mm provided a straight line over the widest range of test doses which was 64-fold and they remained constant in size from 40 to 72 hr (Fig. 4). Necrosis persisted for a longer period, but the larger doses and the narrower range of doses required for linearity made this measurement less desirable than erythema. Erythema and measurement between 40 and 48 hr were selected as criteria for evaluation of reactions.

Skin reactivity of guinea pig and rabbit. Linear and parallel erythema dose responses to toxin A were obtained in the rabbit as well as in the guinea pig (Fig. 5). A comparison of Fig. 5 with Fig. 3 shows the reproducibility of the size of the reactions induced in the guinea pig with a given amount of toxin. Although the rabbit was somewhat more reactive (Table 2), the guinea pig was selected as it gave more reliable results. Some rabbits had severe inflammatory reactions to the depilatory agent and others developed an undergrowth of coarse hair which obliterated the reactions.

Preparation of the U.S. standard diphtheria toxin for the Schick test. From the data in Tables 3 and 4, it was calculated that toxin A contained 85.52 L_r per ml. Parent toxin A was diluted in 0.066 M PBS (*p*H 7.4) containing 0.1% human albumin to provide, per ml, 1 L_r or 1,000 STD. Each milliliter contained 0.01169 ml of the parent toxin. Ten thousand glass vials were filled with 1.0 ml of the diluted toxin with an accuracy of ±0.01 ml, and the contents were freeze-dried. The vacuum in the vials was replaced with argon, and the vials were flame-sealed. The moisture content was <1.0%. (In an earlier preparation, nitrogen replaced the vacuum and the moisture content was >1.0%. This preparation was discarded.) The drying was performed by a U.S. licensed manufacturer. The preparation satisfactorily passed the general requirements for a product for human administration. It was designated lot 2.

Properties of the U.S. standard and comparison with the international standard. Assays of the dried U.S. toxin showed that drying had caused no demonstrable deterioration of the toxicity and combining power. Table 5 gives the average values per vial which were 1.09 L_f, 1.09 L_r, 43,000 MRD, and 38 LD_{50} (96 hr) or 33 LD_{80} (96 hr). Titrations of the lethal action are given in Table 8.

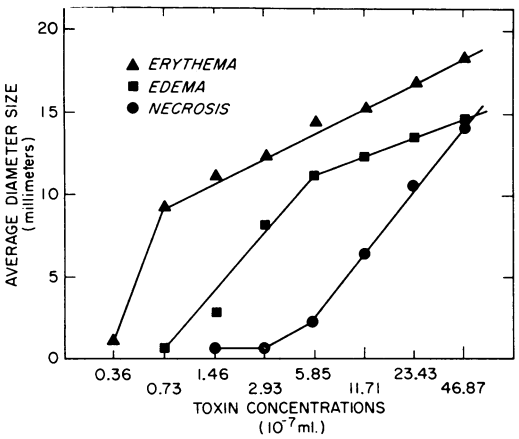

FIG. 3. *Size of erythematous, edematous, and necrotic dermal response of guinea pigs to graded doses of toxin A at 44 hr after injection.*

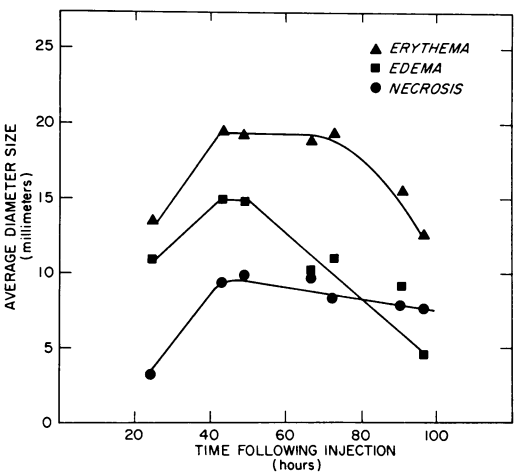

FIG. 4. *Time of appearance and duration of maximum dermal erythema, edema, and necrosis induced by toxin A.*

Table 5 also gives the values (averages) of the international standard which were obtained in a World Health Organization collaborative study (working document WHO/BS/274, 1954). WHO accepted this and other working reports and assigned 900 IU per vial (20).

In the preparation of each of the two standards, the aim was to provide 1,000 STD per vial. Based on subsequent assays, the assigned STD values were 900 IU for the international standard and 1,090 (L_r/1,000) for the U.S. standard. The respective L_f contents per vial were 0.9 and 1.09. The 900 IU did not conflict with the British or the Canadian requirements which were in effect at that time (WHO/BS/274, 1954).

The physical preparation of the U.S. standard differed from that of the international standard in that the toxin was less purified, human albumin was used, and argon replaced the vacuum in the vials, whereas the international preparation contained purified toxin and bovine albumin and vials were sealed under vacuum (Table 5).

The STD of the U.S. standard is slightly more reactive than 1 unit of the international standard. With our assayed guinea pig MRD values, the international standard had 21 MRD per IU (19,000/900), whereas the U.S. standard had 39 MRD per STD (43,000/1,090). In another experiment, each toxin was diluted to provide 1 L_r/1,000 per 0.1 ml. Then the MRD values were titrated simultaneously on guinea pigs by using serial dilutions. The log doses were plotted against the diameter of the reactions. The dilution of the U.S. standard that induced a 10-mm reaction was 38, whereas that of the international standard was 28. This suggests that the international standard may contain some toxoid. The presence of toxoid is also suggested by the ratio of 0.9 L_f to 0.74 L_r per vial which was 0.82. With the U.S. standard, the ratio was 1.0. These values indicate that 1 STD (L_r/1,000) of the U.S. standard is slightly more avid than 1 IU of the international standard.

Stability of the U.S. standard. The stability of the toxicity of the standard was determined as prescribed for Schick test toxins (19). Table 6 shows that heating of the reconstituted toxin at 37 C for 24 hr caused no loss in toxicity. The table also shows that the MRD test was reproducible within and between laboratories. The dried standard which has now been stored at

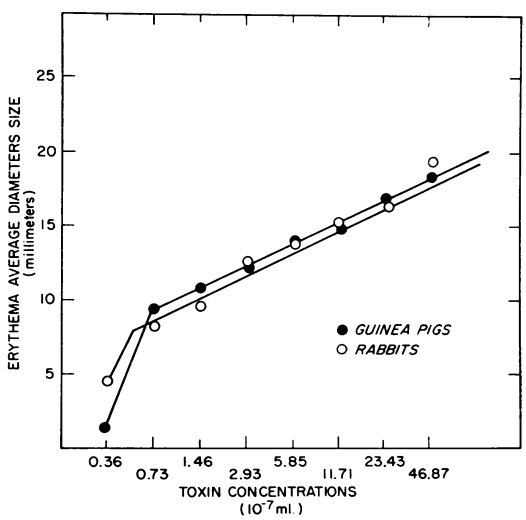

FIG. 5. *Comparison of the dermal erythematous dose response of guinea pigs and rabbits to toxin A.*

TABLE 5. *Comparison of the U.S. standard Schick toxin with the international Schick toxin*

Property	International standard per vial[a]	U.S. standard per vial[b]
Total weight	3.8 mg	5.36 mg
Albumin	Bovine, 1.0 mg	Human, 1.0 mg
Na_2HPO_4	$\cdot 2H_2O$, 2.37 mg	$\cdot 7H_2O$, 3.57 mg
KH_2PO_4	0.36 mg	0.36 mg
Gas	Vacuum	Argon
Moisture		<1.0%
Diphtheria toxin		
Total solids	Purified, 5.0 μg	Not purified, 425.0 μg[c]
Total N		31.0 μg[c]
Protein N	0.40 μg[c]	0.87 μg[c]
Toxicity		
Lethal dose, guinea pig	20 LD_{50} (ca. 15 LD_{75})	38 LD_{50}, 33 LD_{80}[d]
Minimum skin reactive dose		
Rabbit	67,500	54,300[e]
Guinea pig	27,700, 19,000[b]	43,000
Combining power		
L_f	0.9	1.09[f]
L_r guinea pig	0.73, 0.75[b]	1.09
Schick test dose	900	1,090
L_f/1,000	900	1,090
L_r/1,000	730, 750[b]	1,090

[a] Data from working document WHO/BS/274, 1954, except as indicated otherwise. Values reported by different participants were averaged. International standard for diphtheria antitoxin was used for combining-power tests.

[b] Determinations made by the authors. U.S. standard diphtheria antitoxin was used for combining-power tests.

[c] Calculated from assays of parent toxin.

[d] From Table 9 with deaths at 96 hr.

[e] Calculated from Table 2.

[f] Methods: H. R. Dean and R. A. Webb, J. Pathol. Bacteriol., **29**:473, 1926. G. Ramon, C. R. Soc. Biol. **86**:661, 711, 813, 1922.

TABLE 6. *Heat stability of the U.S. standard diphtheria toxin for Schick test*

No. of test	Avg diameter of erythema reaction (mm)							
	Laboratory no. 1				Laboratory no. 2			
	Unheated[a]		Heated[b]		Unheated[a]		Heated[b]	
	1/25[c]	1/60[c]	1/25	1/60	1/25	1/60	1/25	1/60
1	12.1	8.7	11.2	8.5	12.2	9.2	12.2	9.0
2	12.0	9.1	11.8	9.2	12.0	8.8	12.2	8.8
3	12.0	8.9	10.9	9.4	12.3	9.4	12.3	9.5
4	11.9	9.1	11.7	9.5	12.3	9.6	12.0	9.1
Avg	12.0	9.0	11.4	9.2	12.2	9.3	12.2	9.1

[a] Standard reconstituted on day of test.

[b] Vial of standard was reconstituted in 1.0 ml of water and heated at 37 C for 24 hr.

[c] Dilution of 1:109 dilution of the reconstituted toxin (approximately 1 STD per 0.1-ml injection).

4 C for more than 9 yr has shown no change in toxicity (Table 7).

Potency assay of diphtheria toxin for the Schick test. The suitability of the erythema potency assay was shown in experiments that provided information on reproducibility of assay results, relationship between toxicity of the STD as measured by the former MLD test and the new erythema toxicity test reative to the standard, and the greater precision of the latter test.

TABLE 7. *Stability of U.S. standard diphtheria toxin for Schick test during 9 years of use*

Year	No. of tests	Dilution of 1 STD[a]	
		1/8	1/40
1963	6	14.4[b, c]	10.6[c]
1964	11	13.8	10.5
1965	7	14.4	10.7
1966	11	14.4	10.8
1967	7	13.6	10.4
1968	4	14.2	10.7
1969	6	14.0	11.1
1970	3	14.4	10.6
1971	2	14.2	10.9
Totals and mean values	57	14.2	10.7
Individual range		12.9–15.6	9.7–12.2

[a] New vial of standard toxin was used for each of the 57 potency assays. STD, Schick test dose.
[b] Mean erythema reaction of potency assays performed per year.
[c] Values are expressed as millimeters.

In the experiment on reproducibility, 10 replicate potency tests of a commercial lot of Schick toxin were performed independently in each of two laboratories with different vials of the standard for each test. The toxin in one vial of the standard was diluted 1:109 to provide 1 STD per 0.1 ml and further diluted 1:25 and 1:60. The lot under test was also diluted 1:25 and 1:60. Each dilution in duplicate was injected intradermally into each of four guinea pigs in the predescribed random scheme. The diameters of the erythema induced by each dilution were read at 44 hr. Then the average reaction per dilution and ratio of the reactions of the test toxin and standard at each dilution were calculated. Table 8 shows that there was good reproducibility within and between laboratories and also that there was consistency in the toxic content between vials of the standard. The diameters of the reactions induced by the two dilutions were slightly greater than 11 and 8 mm, respectively. The latter was at the lower limit of the linear dose-response curve (Fig. 3 and 5). Dilutions of 1:8 and 1:40 of the STD were used in the tests reported in Table 10 and are currently used for routine potency assays of Schick test toxin.

Lethal reactivity of the standard. Table 9 shows the results of the titrations of the lethal activity per vial of the standard with 295 guinea pigs. The animals were observed for 10 days. Deaths per toxin dilution were accumulated at 3, 4, 5, and >5 to 10 days, and survivals at 10 days were recorded. Considerable variation in death time occurred within a single dose and was not necessarily dose-related. The range for a single dose was as wide as 18 hr to survival at 10 days. Overall, however, the death time was dose-related between the 1/30 to 1/39 doses. With the accumulated deaths and survivals at 96 hr, it was calculated by the Reed-Muench method that a vial contained 37.3 LD_{50} and 34 LD_{80}. Inspection of the table shows that at 96 hr 50% of the animals died at dose 1/38 and 79% died at dose 1/33 (88% died at the end of 120 hr). In 18 subsequent tests of the 1/33 vial with 144 guinea pigs (Table 10), 79.9% died by the end of 96 hr (93% died by the end of 120 hr). Based on 1,090 STD per vial, 1 STD would be equal to 1/33 LD_{80} and 1/28.7 LD_{50}. The MLD could not be determined directly from the table, but it was calculated that each vial contained 24.8 MLD. With this value of 24.8 MLD and 1,090 STD per vial, there would be 44 STD per MLD. In other words, 1 STD would contain about 1/50 MLD.

Table 10 compares the lethal and the erythema tests of 18 lots of Schick toxin submitted to the Division of Biologics Standards for release. These lots had been evaluated by the lethal test by the manufacturers. In the lethal test, eight guinea pigs each were injected subcutaneously with 5 ml of each toxin under test (50 STD) and eight other guinea pigs were injected with 5 ml of 1/165 dilution of the standard. The latter was estimated to provide 1 MLD. Later, results given in Table 8 indicated that this dose contained 1 LD_{80}. Variability of death time was observed as previously reported. Only with lot I-1 did 75% of the deaths occur between the specified time of 72 and 96 hr. Differences between lots were not significant. Combined, 85% of the 144 guinea pigs injected with the 18 toxins died within 96 hr, 96% died within 120 hr, and 4% died between 124 and 209 hr. With the standard, 80% died within 96 hr, 93% died within 120 hr, 5% died between 125 and 156 hr, and 2% survived. The combined results indicated that 50 STD of the toxins under test were near 1 MLD and that lethality was slightly greater than 1 LD_{80} of the standard. It seems reasonable to assume that 50 STD of the standard would be approximately 1 MLD.

By the erythema test, no significant differences between the 18 toxins were shown and there was consistency between the reactions of the standard between tests. The ratio of the potency of each lot to the standard fell well within the accepted range of 0.77 and 1.30. In fact, the smallest and largest ratios were 0.88 (lot C-1) and 1.06 (lot A-1), respectively. The largest difference between the ratios of the two dilutions of a single lot was only 0.09 (lot H-3), whereas the difference of the mean of the lots was 0.02. An analysis of vari-

TABLE 8. *Reproducibility of the erythema potency assay within and between laboratories*

No. of test	Dilutions of the STD[a] of the toxin under test and the standard avg diameter of the reaction of erythema (mm)											
	Laboratory no. 1						Laboratory no. 2					
	Test toxin		Standard toxin		Ratio of test to standard		Test toxin		Standard toxin		Ratio of test to standard	
	1/25	1/60	1/25	1/60	1/25	1/60	1/25	1/60	1/25	1/60	1/25	1/60
1	11.8	8.3	12.0	8.9	0.98	0.93	11.4	7.9	10.8	8.8	1.06	0.90
2	11.5	8.3	11.4	8.7	1.01	0.95	11.6	8.3	11.8	9.7	0.98	0.86
3	11.8	8.0	11.5	8.5	1.03	0.94	11.0	7.9	11.1	8.6	0.99	0.92
4	11.7	8.5	11.7	7.4	1.00	1.15	11.8	7.8	11.2	9.0	1.05	0.87
5	11.2	7.5	11.5	8.2	0.97	0.91	10.7	8.2	11.0	8.2	0.97	1.00
6	11.0	7.5	11.2	7.7	0.98	0.97	10.6	8.1	11.5	9.1	0.92	0.89
7	12.6	8.4	12.2	9.1	1.03	0.92	11.0	7.9	11.3	9.1	0.97	0.87
8	11.6	7.9	11.6	7.7	1.00	1.03	10.9	7.7	11.3	9.0	0.96	0.86
9	11.7	8.5	11.7	8.3	1.00	1.02	11.0	8.0	10.9	8.6	1.01	0.93
10	11.7	8.2	12.0	8.4	0.98	0.98	11.0	8.5	10.8	8.2	1.02	1.04
Mean	11.66	8.11	11.68	8.29	0.998	0.981	11.1	8.03	11.17	8.83	0.993	0.914
Variance	0.1777	0.1411	0.0955	0.3020	0.00044	0.00517	0.1466	0.1600	0.1033	0.2077	0.00182	0.00378
Standard deviation	0.4215	0.3756	0.3900	0.5497	0.0203	0.0703	0.3829	0.2449	0.3210	0.4557	0.0412	0.0621
Standard error of mean	0.1333	0.1188	0.0977	0.1738	0.00663	0.02273	0.1211	0.0775	0.1016	0.1441	0.01349	0.0194

[a] Schick test dose.

TABLE 9. *Titration of lethal action of the U.S. standard Schick toxin with guinea pigs*

Portion of vial	Dilution[a] (5 ml)	No. of tests	Total no. of guinea pigs	Accumulated deaths				Survival (10 days)	Per cent dead				Survival (10 days)
				3 days[b]	4 days	5 days	>5 days		3 days	4 days	5 days	>5 days	
1/30	1/150	4	46	34	41	44	46	0	74	89	96	100	0
1/33	1/165	5	61	33	48	54	60	1	54	**79**[c]	88	98	2
1/35	1/175	4	53	29	41	47	53	0	55	77	89	100	0
1/37	1/185	3	35	12	19	27	33	2	34	54	77	94	6
1/38	1/190	2	20	8	10	14	18	2	40	**50**	70	90	10
1/39	1/195	2	20	3	5	7	15	5	15	25	35	75	25
1/40	1/200	2	20	2	5	9	16	4	10	25	45	80	20
1/41	1/205	2	20	5	7	10	15	5	25	35	50	75	25
1/42	1/210	2	20	7	7	9	17	3	35	35	45	85	15

[a] Vial of standard toxin (1,090 Schick test doses) was reconstituted in 1 ml of water and diluted in phosphate-buffered saline with 0.2% gelatin. Guinea pigs were injected subcutaneously in groups of 8 to 20 per dilution.

[b] Accumulated results from 10 separate experiments.

[c] Numbers in boldface indicate the approximate LD_{80} and the LD_{50} doses.

ance showed that the difference in precision of the lethal and erythema assays was highly significant. The ratio of the skin test variances suggested that one skin test provided as much precision as 50 death-time tests.

DISCUSSION

The method for evaluating the potency of the STD of diphtheria toxin has seen changes since the original definition of 1/50 MLD for the guinea pig (16). Nevertheless, the toxic activity of an STD has remained quite constant worldwide through the years. Variation in guinea pig response prompted the use of an animal erythema skin test assay (5). Later, Glenny defined the STD as that amount of toxin neutralized by 0.001 unit of diphtheria antitoxin. The Permanent Commission on Biological Standardization of the League of Nations (14) accepted both the erythema and the combining-power potency tests and defined the STD as "(a) that amount of toxin which when diluted 1/25 produces a positive and when diluted 1/50 produces a negative intracutaneous reaction in guinea pigs provided that (b) when the STD is mixed with 1/750th part or more of an International Unit (IU) of diphtheria antitoxin and injected intracutaneously causes no local reaction and when mixed with 1/1250th part or less of an IU, similarly injected, causes a marked positive Schick reaction." The latest international action was to adopt a standard for the Schick test toxin (20). The IU was defined as the activity of 1 L_f/1,000 of the standard. Latest available information shows that the toxic activity of the STD in various countries is equal to the combining power of either 1 L_f/1,000 or 1 L_r/1,000. For the potency assay, Great Britain (*British Pharmacopoeia*, 1958 and 1968, The Pharmaceutical Press, London) has changed from use of the combining power test as defined by the League of Nations to the erythema test with potency expressed relative to the international standard. Canada (Food and Drug Act and Regulations with Amendments to 12 May 1970, C.04.140-C.04.147) has retained the two tests, and Japan (8) uses the MLD, the MRD, and the L_r/1,000 combining power tests. The U.S. retained the potency requirement of 1/50 MLD until recently, when the STD of the U.S. standard was defined as 1 L_r/1,000 and an erythema potency requirement relative to the standard was prescribed.

This paper has shown that the erythema activities of an STD of the U.S. standard and of 18 commercial lots of Schick toxin, assayed by the lethal test, are equivalent. Therefore, the amount of diphtheria toxin in an STD currently standardized on the basis of an L_r/1,000 is the same as that amount of toxin in an STD previously standardized on the basis of 1/50 MLD. The specification that the potency of 1 STD be equivalent to 1 L_r/1,000 of the U.S. standard conforms with the recommendation of earlier workers (5). The U.S. STD, however, is slightly more toxic than the IU of the international standard for Schick toxin. The vial of the international standard has an assigned unitage of 900. From the values obtained in a collaborative study (WHO/BS/274, 1954), we have estimated that 1 IU has 1 L_f/1,000, 0.81 L_r/1,000, and 31 MRD. Our own assayed values were 0.83 L_r/1,000 and 28 MRD. It is

TABLE 10. *Comparison of the potency test results of 18 lots of Schick test toxin assayed by the lethal (MLD) and the erythema (MRD) toxicity tests*[a]

Test toxin lot no.	Time of death (hr) in MLD test (50 STD)										Avg diameter of the reaction of erythema (mm) in erythema toxicity test (dilutions of an STD)					
	Test toxin, 50 STD				Median death	Standard toxin, 1/33 vial content				Median death	Test toxin		Standard toxin		Ratio of test to standard	
											1/8	1/40	1/8	1/40	1/8	1/40
A-1	42	53	63	66	71.5	53	63	77	77	77	13.39	10.73	13.60	10.17	0.98	1.06
	77	84	132	173		77	87	109	109							
A-2	58	60	62	65	65	55	62	66	72	79	13.61	10.30	13.20	10.21	1.03	1.01
	65	65	65	79		86	107	162	S[b]							
B-1	59	71	71	77	86	53	59	71	73	75	13.97	9.88	14.97	10.41	0.93	0.95
	95	95	95	95		77	95	112	130							
B-2	53	53	53	59	59.5	53	53	56	56	57.5	13.77	10.04	13.57	10.33	1.01	0.97
	60	63	63	66		59	63	66	77							
B-3	53	63	66	66	66	59	60	63	83	66	14.27	9.92	14.70	10.81	0.97	0.92
	66	98	101	128		87	90	101	128							
C-1	66	66	70	85	85	44	48	56	77	80	14.13	9.50	15.57	10.78	0.91	0.88
	85	90	101	111		83	107	115	131							
C-2	56	56	63	63	69	51	51	56	56	56	13.81	10.47	14.01	10.46	0.99	1.00
	75	75	75	80		56	59	80	117							
D-1	55	62	62	65	65	52	58	65	82	82.5	14.16	10.54	14.49	11.00	0.98	0.96
	65	76	86	124		83	83	110	115							
E-1	71	79	86	86	87.5	48	52	58	66	67	14.05	10.82	13.59	10.54	1.03	1.03
	89	110	151	209		68	86	96	134							
E-2	56	77	80	87	90	68	69	80	87	90	14.30	10.78	14.80	11.58	0.97	0.93
	93	93	104	111		93	108	156	S							
F-1	56	57	57	59	62.5	56	57	77	80	85.5	14.75	10.97	14.94	11.03	0.99	0.99
	66	66	73	96		91	104	111	125							
F-2	56	60	63	66	71.5	48	56	56	60	63.0	13.64	10.50	13.30	10.08	1.03	1.04
	77	77	77	78		66	66	77	92							
F-3	55	59	59	71	71	55	62	62	62	74	14.44	9.86	13.67	10.23	1.05	0.96
	71	72	79	79		86	95	103	110							
G-1	63	66	66	101	101	39	47	50	53	54.5	13.97	9.90	13.85	10.27	1.01	0.96
	101	104	104	104		56	66	77	101							
H-1	52	58	59	63	63	52	52	60	62	62	12.63	9.75	13.53	10.74	0.93	0.91
	63	74	94	114		62	88	117	S							

TABLE 10. *Continued*

I-1	63	73	80	80	83.5	49	53	73	77	83.5	13.47	10.48	13.68	10.58	0.98	0.99
	87	87	95	111		90	90	91	104							
I-2	47	53	53	56	59	39	47	50	53	54.5	13.55	9.99	13.59	10.14	1.00	0.98
	63	63	77	101		56	66	77	101							
I-3	50	61	63	77		48	56	56	60							
	80	87	87	104	78.5	66	66	77	92	63.0	13.64	10.13	13.81	10.58	0.99	0.96
Mean			74.19		74.14			74.08		70.56	13.86	10.25	14.05	10.55	0.988	0.9722
Variance			392.44		147.26			843.32		133.17	0.2274	0.1802	0.4658	0.1505	0.0014	0.00228
Standard deviation			19.81		12.135			29.04		11.54	0.4769	0.4245	0.6825	0.3879	0.0142	0.0477
Standard error of mean			1.41		2.860			2.074		2.720	0.1124	0.1000	0.1609	0.0914	0.0088	0.01127

[a] MLD, minimum lethal dose; MRD, minimum skin reactive dose; STD, Schick test dose.
[b] Survived 10 days.

hoped that other laboratories will make comparative tests of the two standards.

Our experimental observations with the erythema potency assay are in agreement with other investigations (3, 15). The test is accurate and reproducible. The design of the U.S. assay is similar to the one recommended by Gerwing et al. (3). In contrast to the limitations of the lethal assay (2, 4, 12, 18), which lacks precision due to the variability of death-time of guinea pigs, the erythema assay provides a sensitive quantitative measurement of toxin. The size of the reaction is directly proportional to the log of the toxin concentration. Besides being significantly more precise, the assay is cheaper. It provides for replicate testing and simultaneous testing of several toxins on the same animals.

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LITERATURE CITED

1. Branham, S. E., and M. F. Wormald. 1954. The use of the chick in titration of diphtheria antitoxin. J. Immunol. 72: 478–484.
2. Ehrlich, P. 1897. Die Wertbemessung des Diphtherie-heilserum und theoretische Grundlagen. Klin. Jahrb. 6:299–305.
3. Gerwing, J., D. A. Long, and M. V. Mussett. 1957. The assay of diphtheria toxin. Bull. W.H.O. 17:537–551.
4. Glenny, A. T. 1925. The principles of immunity applied to protective inoculation against diphtheria. J. Hyg. 24:301–320.
5. Glenny, A. T. 1931. System of bacteriology in relation to medicine. London Med. Res. Council 6:106.
6. Glenny, A. T., and K. Allen. 1921. The testing of diphtheria toxin and antitoxin by intracutaneous injection into guinea pigs. J. Pathol. Bacteriol. 24:61–63.
7. Glenny, A. T., and M. Barr. 1932. The "dilution ratio" of diphtheria antitoxin as a measure of avidity. J. Pathol. Bacteriol. 35:91–96.
8. Japanese Minimum Requirements of Biologic Products. 1968. Schick toxin (diphtheria toxin for diagnosis), p. 203–288. Ministry of Health and Welfare, Tokyo, Japan.
9. Jensen, C. 1933. Die intrakutane Kaninchen methode zur auswertung von Diphtherie-Toxin und Antitoxin. Acta Pathol. Microbiol. Scand., Suppl. XIV.
10. Jerne, N. K. 1951. Study of avidity based on rabbit skin responses to diphtheria toxin-antitoxin mixtures. Acta Pathol. Microbiol. Scand. Suppl. 87:3–183.
11. Kolb, R. W., S. E. Branham, and D. B. Riggs. 1955. Comparison of the guinea pig and chick in evaluation of diphtheria toxin for the Schick test. Appl. Microbiol. 3:242–247.
12. Miles, A. A. 1951. Biological standards and the measurement of therapeutic activity. Brit. Med. Bull. 7:283–291.
13. Moloney, P. J., and E. M. Taylor. 1931. Stabilization of diluted diphtheria toxin Trans. Roy. Soc. Canada, Sect. 52 5: 149-151.
14. Permanent Commission on Biological Standardization of the Health Organization of the League of Nations. 1931. Report C. H. 1056 (1) annex II series L. of N. Publications, III Health III, 10.
15. Romer, P. H. 1909. Ueber den Nachweis sehr Kleiner Mengen des Diphtherie-giftes. Z. Immunitaetsforsch. 3:208-216.
16. Schick, B. 1913. Die Diphtherietoxin-Hautreaktion des

Menschen als Vorprobe der prophylaktischen Diphtherieheilseruminjection. Muenchen Med. Wchenschr. **60**:2608–2610.

17. Seibert, F. B., and E. H. Dufour. 1954. Comparison between the international standard tuberculins. PPD-S and old tuberculin. Amer. Rev. Tuberc. **69**:585–594.
18. Trevan, J. W. 1927. The error of determination of toxicity. Proc. Roy. Soc. Ser. B **101**:483–514.
19. U.S. Public Health Service Regulations (42 C.F.R. Part 73:2000–73:2006). Revised September 2, 1970.
20. World Health Organization. 1955. Diphtheria toxoid and Schick test toxin. Techn. Rep. Ser. **96**:7–8.