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Assay of Potency of the Proposed Fifth International Standard for Gas-Gangrene Antitoxin (Perfringens)

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As part of a collaborative assay of the proposed Fifth International Standard for Gas-Gangrene Antitoxin (Perfringens), five ampoules of the proposed replacement material were assayed in the authors' laboratory against the then current Fourth International Standard. Both in vitro *and* in vivo *methods were used. This paper presents the results and their statistical analysis.*

The two methods yielded different results which were not likely to have been due to chance, but exact statistical comparison is not possible. It is thought, however, that the differences may be due, at least in part, to differences in the relative proportions of zeta-antitoxin and alpha-antitoxin in the Fourth and Fifth International Standards and the consequent different reactions with the test toxin that was used for titration.

A collaborative assay was arranged by the National Institute for Medical Research (Department of Biological Standards and Division of Immunological Products Control), London, England, in order to assign a potency to the proposed Fifth International Standard for Gas-Gangrene Antitoxin (Perfringens) (*Clostridium welchii* Type A Antitoxin) (Evans & Perkins, 1963).[4] As collaborators in this assay we received six ampoules of the proposed standard. We compared five of them with the Fourth International Standard Antitoxin, then current. The proposed replacement material has since been established as the Fifth International Standard, and will be referred to as such hereafter.

RECONSTITUTION OF SERUM

Each of the five ampoules was weighed, and the freeze-dried contents of each ampoule were dissolved in 1.6 ml of saline. A 3.4-ml aliquot of glycerol was added to the solution which was then thoroughly mixed. The mixture was decanted into tubes closed by ground-glass stoppers. The empty ampoules were carefully cleaned and then weighed to determine the weights of their freeze-dried contents.

For the calculation of potency we assumed that the entire contents of each ampoule were contained in 5 ml of the solution prepared in the above manner. Thus we did not take into account the following facts: (*a*) that the volume of reconstituted serum is slightly greater than the volume of solvent added; (*b*) that mixing 1.6 ml of saline and 3.4 ml of glycerol does not yield exactly 5.0 ml of glycerol-saline; and (*c*) that there remained a barely visible film of dried serum in the cut-off tops of the ampoules.

METHODS OF TITRATION AND RESULTS

Following the suggestions of the National Institute for Medical Research, we titrated the five ampoules of the Fifth International Standard by an *in vivo* as well as by an *in vitro* method.

In vivo *method*

White mice weighing 16-18 g were inoculated with mixtures containing a fixed dose of test toxin and varying doses of the six sera (Fourth International Standard and the five ampoules of the Fifth International Standard); each mixture was inoculated into six mice. Death of the animals within 48 hours after the inoculation was taken to indicate the presence of an effective dose of free toxin. Later deaths were not recorded; but in all the experiments only two mice in fact died more than 48 hours after inoculation. The doses of serum formed an approximately geometric series with the ratio

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$f = \sqrt[20]{10}$; e.g., the following doses of serum were used:

$$f^3 \times 0.2 \times \frac{1}{50} \approx 0.28 \times \frac{1}{50}\text{ml}$$

$$f^2 \times 0.2 \times \frac{1}{50} \approx 0.25 \times \frac{1}{50}\text{ml}$$

$$f^1 \times 0.2 \times \frac{1}{50} \approx 0.22 \times \frac{1}{50}\text{ml}$$

$$f^0 \times 0.2 \times \frac{1}{50} \approx 0.20 \times \frac{1}{50}\text{ml}$$

$$f^{-1} \times 0.2 \times \frac{1}{50} \approx 0.18 \times \frac{1}{50}\text{ml}$$

$$f^{-2} \times 0.2 \times \frac{1}{50} \approx 0.16 \times \frac{1}{50}\text{ml}$$

$$f^{-3} \times 0.2 \times \frac{1}{50} \approx 0.14 \times \frac{1}{50}\text{ml}$$

As test toxin the preparation Prg 707 was used at a dose of 0.95 mg; this dose is also used in the national control of perfringens sera in Germany and represents the test dose of toxin against 0.2 IU of antitoxin (mouse L+/5). The serum-toxin mixtures were held for one hour at room temperature and then inoculated in a volume of 0.5 ml per mouse.

The results of the animal experiments (i.e., the survival rates corresponding to the individual serum doses) were analysed by the so-called area method (Spearman, 1908; Kärber, 1931; van der Waerden, 1940). For each of the five ampoules, this method yielded estimates for the mean protective doses of the Fourth and Fifth International Standards; hence we obtained an estimate of the potency of each ampoule of the Fifth International Standard relative to the Fourth. Since the potency of the Fourth International Standard is 20 IU/ml this gave an estimate of the absolute potency of each ampoule. The results, together with the dry weights of contents of the ampoules, are given in Table 1.

The geometric mean of the potencies of the individual ampoules is 268 IU/ampoule, so that 1 ml of the glycerol-saline solution of the Fifth International Standard, prepared by dissolving the contents of one ampoule in 5 ml of glycerol-saline, contains 53.6 IU on the average.

In vitro *method*

The lecithin (or Nagler-Oakley) method was used, in which sterile egg-yolk emulsion serves instead of mice as indicator for the excess toxin. Again volumes of 0.5 ml of toxin-antitoxin mixtures were used; after the mixtures had been held for one hour at room temperature, 0.5 ml of egg-yolk emulsion (1 egg-yolk in 250 ml of saline) was added to each mixture. For the *in vitro* experiments as well Prg 707 was used as test toxin, but now in a dose of 0.64 mg. This dose was chosen as it had yielded a visual extinction value of ++ in a preliminary test with a fixed serum dose (0.2 IU) and graduated toxin doses; the visual extinction value ++ corresponds to a photometrically determined extinction of about 0.3. Again serum doses were used which were graduated according to a nearly geometric series with ratio $\sqrt[20]{10}$. The extinctions of the serum-toxin-yolk mixtures were determined photometrically 20 hours after adding the egg-yolk emulsion to the toxin-antitoxin mixtures. Two *in vitro* experiments were done; in the first only one tube was used for each serum dose, in the second two.

EVALUATION OF RESULTS OF *IN VITRO* EXPERIMENTS

The extinction values of the serum-toxin-yolk mixtures were plotted against the logarithms of the serum doses; in the second experiment, the arithmetic means of the two parallel measurements were used as extinction values. Such a dose-response curve is likely to be sigmoid, but one may expect its middle part to be approximated by a straight line. This was in fact observed in both experiments. (The accompanying figure shows the results of the second experiment.) Therefore, to simplify the evaluation, we used only the points in the middle parts of the response curves, corresponding to four successive serum doses (Table 2), and accordingly took as basis the following model for the relation

TABLE 1

RESULTS OF *IN VIVO* TITRATIONS OF FIFTH INTERNATIONAL STANDARD FOR GAS-GANGRENE ANTITOXIN (PERFRINGENS)

Ampoule	Dry weight of contents (mg)	IU/ampoule	IU/mg
1	89.1	280.5	3.14
2	90.3	276.5	3.06
3	89.2	250.0	2.80
4	89.4	270.0	3.02
5	90.5	265.0	2.93

RESULTS OF SECOND *IN VITRO* EXPERIMENT WITH SERUM-TOXIN-YOLK MIXTURE

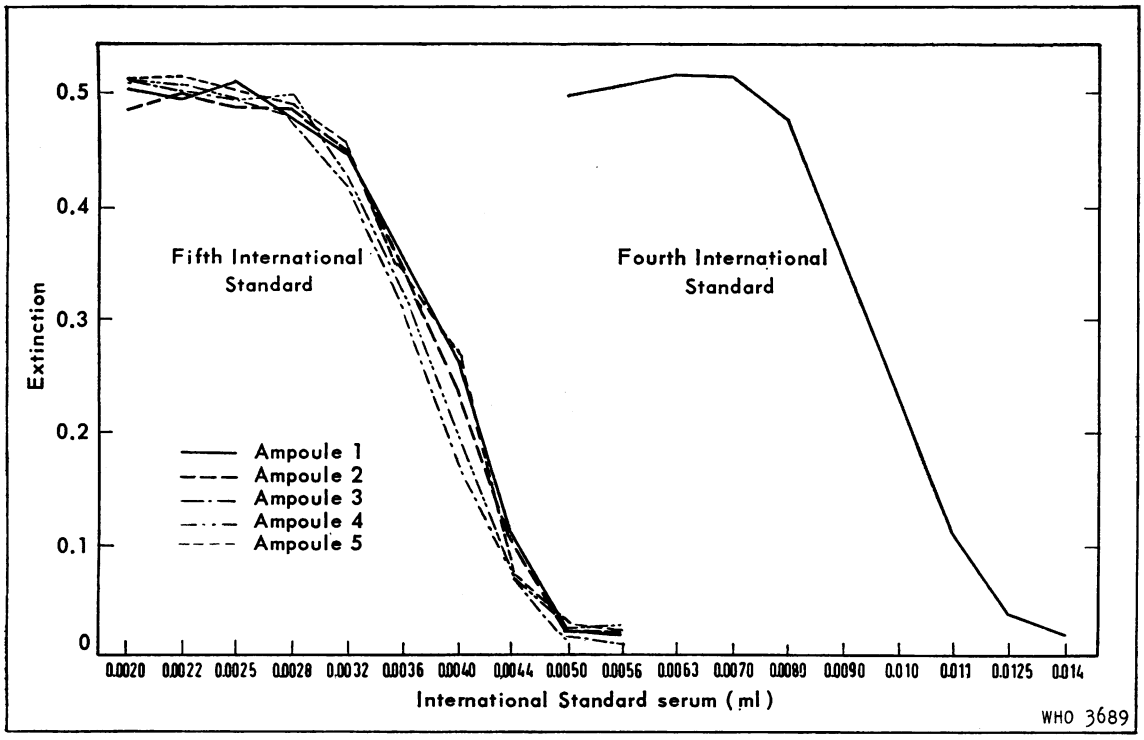

TABLE 2
RESULTS OF *IN VITRO* EXPERIMENTS WITH SERUM-TOXIN-YOLK MIXTURE

Fourth International Standard		Fifth International Standard					
Dose (ml)	Extinction value	Dose (ml)	Extinction value				
			Amp. 1	Amp. 2	Amp. 3	Amp. 4	Amp. 5
			First experiment				
0.0090	0.44	0.0036	0.40	0.41	0.42	0.43	0.34
0.0100	0.31	0.0040	0.31	0.34	0.34	0.30	0.30
0.0110	0.20	0.0044	0.20	0.19	0.20	0.16	0.17
0.0125	0.08	0.0050	0.08	0.07	0.06	0.045	0.045
			Second experiment				
0.0080	0.478	0.0032	0.445	0.448	0.420	0.435	0.455
0.0090	0.360	0.0036	0.355	0.352	0.318	0.340	0.348
0.0100	0.228	0.0040	0.258	0.233	0.175	0.205	0.268
0.0110	0.105	0.0044	0.106	0.099	0.065	0.065	0.068

between the doses of the sera and the extinction values:

$$(1) \qquad y_{ik} = \mu_i + \beta_i (x_{ik} - \bar{x}_i) + e_{ik}.$$

Here x_{ik} is the logarithm of the k^{th} dose ($k = 1, 2, 3, 4$) of the i^{th} serum ($i = 0$: Fourth International Standard; $i = 1, 2, \ldots 5$: i^{th} ampoule of the Fifth International Standard), y_{ik} the corresponding observed extinction value, and $\bar{x}_i$ the arithmetic mean of the logarithms of the four doses of the i^{th} serum. The μ_i and β_i ($i = 0, 1, \ldots 5$) are certain constants (parameters). Lastly, the e_{ik} are terms ("experimental errors") which form random variables with regard to the experiment being repeated infinitely under the same conditions; these random variables were supposed to be normally and independently distributed with mean 0 and a standard deviation σ which depends on neither i nor k. In each of our experiments the logarithms of the serum doses satisfied the equations

$$x_{0k} - \log 2.5 = x_{1k} = x_{2k} = \ldots = x_{5k} \quad (k = 1, 2, 3, 4)$$

and consequently also the equation

$$\bar{x}_0 - \log 2.5 = \bar{x}_1 = \bar{x}_2 = \ldots = \bar{x}_5,$$

if 1 ml of the original solution of the serum in question (which as regards the Fifth International Standard contained the contents of the ampoule in 5 ml of glycerol-saline) was taken as unit of dosage.

By means of the usual method of regression analysis (see, for instance, Finney, 1952), maximum likelihood estimates $\hat{\mu}_i$, $\hat{\beta}_i$ ($i = 0, 1, \ldots 5$) for the unknown parameters μ_i and β_i are computed:

$$(2) \quad \hat{\mu}_i = \bar{y}_i = \frac{1}{4} \sum_{k=1}^{4} y_{ik}, \quad \hat{\beta}_i = b_i = S_{xy}^{(i)} / S_{xx}^{(i)},$$

where

$$S_{xx}^{(i)} = \sum_{k=1}^{4} (x_{ik} - \bar{x}_i)^2, \quad S_{xy}^{(i)} = \sum_{k=1}^{4} (x_{ik} - \bar{x}_i)(y_{ik} - \bar{y}_i).$$

In a subsequent analysis of variance one tests the hypothesis that the "true" regression coefficients β_i ($i = 0, 1, \ldots 5$) are equal and thus the response lines of the individual sera are parallel; this parallelism may be expected and is the assumed basis for referring to the potency of one serum relative to another, i.e., the ratio of equally effective doses of the two sera. Further it is proved whether there is any difference between the "true" extinction means μ_i.

This variance-analysis is shown for the first experiment in Table 3 and for the second in Table 4.

By means of the variance-ratio test it follows from Tables 3 and 4 that in both experiments the sum of squares for "regression" is highly significant, indicating the close dependence of the extinction values on the serum dose. On the other hand, the sum of squares for "parallelism" and the sum of squares "between sera" do not prove significant in either of the two experiments (the significance point of F with 5 and 12 degrees of freedom is 3.11 for the significance level P = 0.05). We may therefore conclude that the differences between the estimated

TABLE 3
ANALYSIS OF VARIANCE FOR FIRST *IN VITRO* EXPERIMENT

Nature of variation	Degrees of freedom	Sum of squares	Mean square [a]	F [b]
Between sera	5	0.0056	0.0011	2.3
Regression	1	0.4032	0.4032	840
Parallelism	5	0.0024	0.00048	1.0
Error	12	0.0057	$0.00048 = s^2$	
Total	23	0.4169		

[a] Mean square = sum of squares/number of degrees of freedom.
[b] F = mean square/s^2.

TABLE 4
ANALYSIS OF VARIANCE FOR SECOND *IN VITRO* EXPERIMENT

Nature of variation	Degrees of freedom	Sum of squares	Mean square [a]	F [b]
Between sera	5	0.00737	0.00147	1.82
Regression	1	0.43124	0.43124	532
Parallelism	5	0.00075	0.00015	0.19
Error	12	0.00973	$0.00081 = s^2$	
Total	23	0.44909		

[a] Mean square = sum of squares/number of degrees of freedom.
[b] F = mean square/s^2.

regression coefficients b_i ($i = 0, 1, \ldots 5$) as well as the differences between the observed extinctions means $\bar{y}_i$ for the individual sera are due to experimental error only.

After it has been ascertained that there is no reason to doubt the parallelism of the (true) response lines of the six sera, we obtain an estimate for the true common regression coefficient β, the slope of the parallel response lines of the sera, with the weighted mean

$$(3) \qquad b = \sum_{i=0}^{5} S_{xx}^{(i)} b_i \Big/ \sum_{i=0}^{5} S_{xx}^{(i)}$$

of the individually estimated regression coefficients b_i. Then

$$(4) \qquad M_i = \bar{x}_i - \bar{x}_0 + \frac{\bar{y}_i - \bar{y}_0}{b} = \log 2.5 + \frac{\bar{y}_i - \bar{y}_0}{b} \qquad (i = 1, \ldots 5)$$

forms an estimate for the logarithm of the potency of the i^{th} ampoule of the Fifth International Standard relative to the Fourth, and the (estimated) variance of M_i is

$$(5) \quad V(M_i) = \frac{1}{b^2}\left[V(\bar{y}_i - \bar{y}_0) + (M_i - \bar{x}_i + \bar{x}_0)^2 V(b)\right] = \frac{s^2}{b^2}\left[\frac{1}{2} + \frac{(M_i - \log 2.5)^2)}{\sum_i S_{xx}^{(i)}}\right],$$

s^2 being the mean square for " error " in the analysis of variance. In both experiments, the quotient of s^2 and $b^2 \sum_i S_{xx}^{(i)}$ has a value small enough to allow us to determine 95% confidence limits for the true value of the logarithm of potency of the i^{th} ampoule of the Fifth International Standard relative to the Fourth (i.e., limits for which the assertion that they include the true value of log-potency will be correct, on an average, in 95 out of 100 cases when the assertion is made) in the form of

$$(6) \qquad M_i - t_{0.05}\sqrt{V(M_i)}\,,\; M_i + t_{0.05}\sqrt{V(M_i)}$$

(see Finney 1952, p. 115). Here $t_{0.05}$ means the significance point of Student's t-distribution with 12 degrees of freedom (the number of degrees of freedom due to s^2) for the significance level P = 0.05. The antilogarithms of the limits (6) provide corresponding confidence limits for the true value of the (relative) potency itself. The values of the estimates M_i for the log-potencies and of their standard deviations $\sqrt{V(M_i)}$, obtained according to (4) and (5), and the values of the estimates and 95% confidence limits for the potencies themselves are shown in Table 5.

TABLE 5

LOG-POTENCY ESTIMATES FOR FIFTH INTERNATIONAL STANDARD IN *IN VITRO* EXPERIMENTS

Exp. No.	Amp. No. (*i*)	M_i	$\sqrt{V(M_i)}$	Relative potency	95 % confidence limits
1	1	0.4020	0.00628	2.523	2.445 - 2.604
	2	0.3999	0.00628	2.511	2.433 - 2.592
	3	0.3989	0.00628	2.506	2.428 - 2.586
	4	0.4075	0.00629	2.556	2.476 - 2.638
	5	0.4156	0.00632	2.604	2.522 - 2.688
2	1	0.3986	0.00774	2.504	2.408 - 2.603
	2	0.4016	0.00774	2.521	2.426 - 2.621
	3	0.4166	0.00778	2.609	2.510 - 2.713
	4	0.4100	0.00776	2.570	2.472 - 2.672
	5	0.4011	0.00774	2.518	2.422 - 2.618

For each ampoule of the Fifth International Standard we now test whether there is a significant difference between the values obtained for the potency relative to the Fourth Standard in the two experiments; for this we make use of the corresponding values of $\sqrt{V(M_i)}$ set out in Table 5. As is to be expected, there is no such difference for any of the five ampoules; this fact is also expressed in Table 5 by the considerable overlapping of the two confidence intervals for the true relative potency of each ampoule. For this reason a mean value is now calculated from the two values for the potency of each ampoule of the Fifth Standard relative to the Fourth, by forming a weighted arithmetic mean from the corresponding logarithms, with the reciprocals of the variances of the individual values as weights. These mean values, together with corresponding 95% confidence limits for the true relative potencies, are given in Table 6.

Table 6 does not allow of any exact statistical statement about the differences in the relative potencies of the individual ampoules of the Fifth International Standard, even though the considerable overlapping of the confidence intervals suggests that there are no true differences between the potencies of the five ampoules relative to the Fourth Standard. But the verification of this suggestion follows in association with formula (4)

TABLE 6
MEAN POTENCY VALUES FOR FIFTH INTERNATIONAL STANDARD IN *IN VITRO* EXPERIMENTS

Ampoule No.	Relative potency	95 % confidence limits
1	2.515	2.458 - 2.575
2	2.515	2.458 - 2.575
3	2.546	2.488 - 2.606
4	2.562	2.503 - 2.622
5	2.569	2.510 - 2.630

from the above-mentioned fact that in neither of the two experiments are there significant differences between the extinction means $\bar{y}_i$ $(i = 0, 1, \ldots 5)$ for the individual sera.

The (now permitted) averaging of the relative potencies for the five ampoules is, however, not done with the mean values from the two experiments included in Table 6. Instead, we first calculate an estimate $\overline{M}$ for the logarithm of the potency of the five ampoules of the Fifth Standard as a group relative to the Fourth, separately for each of the two experiments, according to the formula:

$$\text{(4a)} \qquad \overline{M} = \log 2.5 + \frac{\bar{y}' - \bar{y}_0}{b},$$

which is analogous to (4); here $\bar{y}' = \frac{1}{5}(\bar{y}_1 + \bar{y}_2 + \ldots + \bar{y}_5)$ is the mean of the extinction values for *all* doses of the Fifth International Standard. Corresponding to (5) we obtain as (estimated) variance of $\overline{M}$

$$\text{(5a)} \qquad V(\overline{M}) = \frac{s^2}{b^2}\left[\frac{6}{20} + \frac{(\overline{M} - \log 2.5)^2}{\sum_i S_{xx}^{(i)}}\right]$$

The following are the numerical results for $\overline{M}$ and $V(\overline{M})$:

Experiment No.	$\overline{M}$	$V(\overline{M})$
1	0.4048	0.238×10^{-4}
2	0.4056	0.361×10^{-4}

Now we take a weighted mean $\overline{\overline{M}}$ from these two values for $\overline{M}$, as has been done above with the two values obtained for the logarithm of the potency of each individual ampoule relative to the Fourth Standard, and get

$$\overline{\overline{M}} = 0.4051$$

with the variance

$$V(\overline{\overline{M}}) = 0.143 \times 10^{-4}.$$

According to this the potency of the Fifth International Standard relative to the Fourth estimated from the two experiments is 2.542, with 2.496 and 2.588 as 95% confidence limits for the true value of potency.

If we take the geometric mean of the values in Table 6 for the relative potencies of the five ampoules (i.e., the antilogarithm of the arithmetic mean of their logarithms), we get a value for the potency of the Fifth Standard relative to the Fourth which agrees essentially with the above, but we cannot obtain in this way any valid confidence interval for the true value of relative potency.

As the absolute potency of the Fourth International Standard is 20 IU per ml, it follows from the above value for the relative potency of the Fifth Standard that the latter standard dissolved in the described manner (the contents of one ampoule being contained in 5 ml of solution), has an absolute potency of 50.8 IU per ml, with 95% confidence limits of 49.9 and 51.8 IU per ml for the true value.

DISCUSSION

The value obtained for the absolute potency of the Fifth International Standard by means of the lecithin (or Nagler-Oakley) *in vitro* method differs from the value for this potency obtained from the animal test by evaluation with the area method. The latter was 53.6 IU per ml. This figure falls considerably outside the confidence limits for the true potency, which were calculated from the results of the *in vitro* experiments. Therefore it is hard to believe that the difference is due to chance. An exact statistical comparison of the two values is not possible. It is true that, by means of the error formula given by van der Waerden (1940), estimated variances can be calculated for the logarithms of the estimates obtained by the area method for the potencies of the individual ampoules, and hence also a variance for the logarithm of their geometric mean; it amounts to 0.78×10^{-4}. But, as opposed to the evaluation of the *in vitro* experiments, the distribution law relating this estimated variance to its true value is unknown. As an approximation, however, we may regard the variances we have found for the logarithms of the two values for the potency

we are comparing (50.8 and 53.6 IU per ml) as true variances. For testing the significance of the difference we then have to judge the quotient

$$\frac{\log 53.6 - \log 50.8}{\sqrt{(0.143 \times 10^{-4}) + (0.78 \times 10^{-4})}} = 2.42$$

by a table of the normal distribution (with mean=0 and standard deviation = 1). According to this, a value of the quotient which is absolutely (i.e., without regard to sign) equal to or greater than 2.42 has a probability less than 0.02. Therefore we may say that at least there is a strong suspicion that the difference between the values obtained for the potency of the Fifth International Standard by the two methods is not due to chance.

If the difference between the two values is real, the following explanation may be offered for it. The toxin preparation Prg 707 used for the titration is rich in zeta-toxin, but also contains a quantity of alpha-toxin. Whereas the Nagler-Oakley method detects only the zeta-toxin, it is likely that the values obtained in the animal experiments are due to two different systems, the zeta-toxin/zeta-antitoxin system and the alpha-toxin/alpha-antitoxin system. One of us (Prigge) pointed out in 1937 that alpha-toxin appears to be not only haemolytic but also, when given in a high dose, lethal. The relative proportion of zeta-antitoxin and alpha-antitoxin in the Fifth International Standard may be different from that in the Fourth International Standard, so that different results may be obtained by the use of different toxin preparations (see Prigge, 1936, p. 482, Table III) or of different indicators—i.e., of egg-yolk, which is sensitive only to zeta-toxin, or of mouse, which is sensitive to both zeta-toxin and alpha-toxin. However, if a laboratory uses a toxin preparation which is monovalent (e.g., merely contains zeta-toxin), the choice of the indicator—mouse or egg-yolk—is irrelevant.

RÉSUMÉ

Dans le cadre des essais en collaboration visant à établir le cinquième étalon international de Sérum Anti-gangrène gazeuse perfringens (*Clostridium welchii*, type A), on a comparé 5 ampoules de ce sérum avec le quatrième étalon international actuel.

Conformément aux recommandations du National Institute for Medical Research, les essais ont été pratiqués *in vitro* et *in vivo*. Pour les premiers, on a utilisé l'émulsion de jaune d'œuf (méthode Nagler-Oakley), et pour les seconds la souris.

On a constaté que l'activité du cinquième étalon était, *in vitro*, de 50,8 unités internationales par millilitre et, *in vivo*, de 53,6 UI/ml. D'après l'analyse statistique des résultats, on semble fondé à admettre que la différence entre ces deux valeurs n'est pas uniquement imputable aux erreurs expérimentales.

Cette différence s'expliquerait comme suit:

La préparation de toxine (Prg 707) employée pour les titrages est riche en toxine zêta, mais contient aussi une certaine quantité de toxine alpha. Alors que la méthode Nagler-Oakley n'entraîne qu'une réaction à la toxine zêta, les valeurs obtenues dans l'essai sur l'animal reposent vraisemblablement sur deux mécanismes différents: le système toxine zêta/antitoxine zêta et le système toxine alpha/antitoxine alpha. On sait en effet depuis 1937 que la toxine alpha a non seulement des effets hémolysants, mais encore — à forte dose — une action létale. Si donc le rapport entre l'antitoxine zêta et l'antitoxine alpha n'est pas le même dans le cinquième étalon de sérum que dans le quatrième, on peut obtenir des résultats différents selon que l'on emploie le jaune d'œuf ou la souris. Cela ne vaut naturellement que dans le cas où la toxine d'essai contient plus d'une toxine partielle; si un laboratoire utilise une toxine monovalente, la nature de l'indicateur est sans effet sur le résultat.

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