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The International Reference Preparation of Influenza Virus Haemagglutinin (Type A)

P. KRAG [1] & M. WEIS BENTZON [2]

This paper describes the international collaborative assay that led to the establishment in 1967 of the International Reference Preparation of Influenza Virus Haemagglutinin (Type A) and the studies completed during the following years on the use of the preparation for evaluating the haemagglutinin content of 46 influenza virus vaccines in terms of international units. The WHO Expert Committee on Biological Standardization (1967) defined the International Unit as 0,09361 mg of the International Reference Preparation.

Altogether 14 laboratories in 12 countries took part in one or both studies, using a total of 24 methods (HA titrations and, in a few cases CCA titrations). Major differences in the HA titres were found between laboratories, while the potencies (the haemagglutinin content values) relative to the International Reference Preparation were free from most of these differences. Haemagglutination titres varied over a range factor up to 50, while the corresponding relative "potencies" varied with a factor of only 2. The CCA method used in a few laboratories gave results close to the lowest haemagglutination titres and showed relatively small variations between laboratories. The analyses of variance disclosed differences in the variation within laboratories, but for the majority of the laboratories the variation allowed an overall estimate of a standard error.

The calculation of haemagglutinin content (in IU) from relative potencies is described. Advice is given on the selection, preparation, and titration of a local reference vaccine *with a view to expressing its haemagglutinin content in international units.*

The test results with 46 local vaccines are also given. The deviations of the relative potencies from the average per vaccine showed a distribution with eight major discrepancies instead of the expected one. The background for these cases is discussed.

Following requests from the fifteenth and twentieth WHO Expert Committees on Biological Standardization (1963, 1968), the International Laboratory for Biological Standards in the Statens Seruminstitut, Copenhagen, arranged two series of collaborative assays on a number of influenza virus vaccines, including a preparation proposed as an international reference preparation that might be useful in the influenza haemagglutination test. This paper describes studies on this material that was established as the International Reference Preparation of Influenza Virus Haemagglutinin (Type A) by the WHO Expert Committee on Biological Standardization (1968).

[1] Director, Department of Biological Standardization Statens Seruminstitut, Copenhagen, Denmark.

[2] Actuary, Department of Biostatistics, Statens Seruminstitut, Copenhagen, Denmark.

THE INTERNATIONAL REFERENCE PREPARATION (IRP)

The material was obtained through the collaboration of certain laboratories in the United Kingdom. The World Influenza Centre provided the 1/57 strain of Type A influenza virus, No. 30338. The Microbiological Research Establishment, Salisbury, prepared from this strain 300 ml of highly concentrated influenza virus; the strain was grown in the allantoic cavity of 11-day-old chick embryos for 48 hours at 35.3°C. After being harvested, the allantoic fluids were pooled and formol was added to a final concentration of 1 : 10 000. The virus was then concentrated by centrifugation. The resuspended deposit was held at 4°C and sent to the Division of Biological Standards, National Institute for Medical Research, Mill Hill, London, England, on 3 May 1966.

A trial batch, diluted 1 : 30 in M/100 phosphate buffer with 1% bovine serum albumin fraction V

(Armour), could be filtered through a Millipore membrane of average pore diameter 650 nm, but not through one of 450 nm pore diameter without loss of titre.

The bulk of the material was diluted 1 : 24 in M/100 phosphate buffered saline with bovine serum albumin, filtered through a 650-nm membrane and filled into 1 ml ampoules on 5 May 1966 and freeze-dried immediately afterwards according to the procedure normally used in the Division of Biological Standards, and transferred to the Statens Seruminstitut, Copenhagen.

The thermostability was found acceptable as no reduction in titre was found after 2 months' storage at 37°C, while a reduction of the log titre from 3.54 to 3.40 was seen after the same storage time at 56°C.

The dry weight of the contents of the ampoules of the final preparation of influenza virus type A haemagglutinin was estimated from 18 ampoules; it varied within a range of 1.29 mg about an average value of 18.73 mg (coefficient of variation $\pm 1.8\%$).

THE ASSAYS

A total of 14 laboratories (I-XIV)[1] participated in these studies; the two series were designated assay *x* and assay *y*. Six of the laboratories participated in both assays with a total of 8 methods (one of the six laboratories used two different methods in assay *x*). The remaining 8 laboratories (3 in assay *x* and 5 in assay *y*) used a total of 14 different methods (1–4 per laboratory); the different methods per laboratory are indicated in this report by an index letter (a–d). The numbers I–XIV were allocated to the laboratories according to the level of haemagglutinin content determined for the IRP (the laboratory sensitivity level).

In the first series (assay *x*, in 9 laboratories) the proposed international reference preparation of influenza virus haemagglutinin (type A) and 6 distributed vaccines were included (A_{1-4} and B_{1-2}; the results from this study were the basis for the establishment in 1967 by the WHO Expert Committee on Biological Standardization (1968) of the International Reference Preparation of Influenza Virus Haemagglutinin (Type A). The WHO Expert Committee also defined the International Unit as the activity contained in 0.09361 mg of the International Reference Preparation. For practical purposes, and since it is recommended to reconstitute the total content of each ampoule with 1 ml of saline, it can be accepted that such a suspension contains 200 International Units per ml.

The second series (assay *y*, in 11 laboratories) included, firstly, a comparison between the IRP and one distributed vaccine, B_3, and also the testing of some 40 local vaccines to examine the usefulness of the IRP as a reference for type A vaccine as well as for type B vaccine, and to classify, in international units, a large number of vaccines in production in 10 different areas. The distributed vaccines were obtained from some of the participating laboratories.

The average results per method for all the distributed vaccines are shown in Table 1 (the detailed results can be obtained from Copenhagen on request). Each vaccine was tested under a code number on three different testing days; as a rule, log titres were estimated corresponding to 50% agglutination, mostly according to the Kärber principle; the majority of the laboratories used the haemagglutination (HA) test and only four laboratories used the chicken red cell agglutination (CCA) test (IVb, XIb, XIIIc, and XIVc & d).

RESULTS

The average log titres obtained by the HA and CCA methods for the International Reference Preparation are given in Table 1; the HA values for the IRP vary over a range of 1.67 (assay *x*) and 0.76 (assay *y*); the few CCA values are more in agreement with a range of about 0.20 in each of the assays.

These systematic differences, which in ordinary titres correspond to range factors of 47 or 6 for HA and of 1.6 for CCA, show the need for an international reference preparation and an international unit, as such differences have made international comparison of influenza virus vaccines very difficult.

The number of HA units, the haemagglutination titre referred to 1 ml of virus suspension, has been calculated for the 12 laboratories concerned with assay *x* using the average results for the IRP; the range of their logarithms is 1.71 corresponding to a range factor of 51, practically the same as that for the ordinary titres; it should, however, be noted that 4 of the 12 laboratories are responsible for this high range.

The ranges for some of the distributed vaccines in assay *x* were 1.33 for vaccine A_1, and in assay *y*

[1] The participating laboratories are listed on p. 486. In the reports of the original assays (assay *x* and assay *y*) the methods used were identified by a different series of code numbers (1–16) and these numbers are shown in the tables and text of the present article (often in parentheses) with superscripts to identify the assay series (e.g., 15^x).

Table 1. Haemagglutinin content of 8 distributed influenza virus vaccines

Method & laboratory codes		Log titre IRP	Log relative "potency"							Residual variance × 10^4	f [b]
			Type A vaccines [a]				Type B vaccines [a]				
			A_1	A_2	A_3	A_4	B_1	B_2	B_3		
Haemagglutination test											
I	8 [x]	3.75	−0.05	0.21	0.24	0.12	0.31	0.00		128 [c]	4
	11 [y]	3.21							−0.30 ***	76 [d]	6
II	12 [x]	3.40	−0.05 ††	⩾0.34	0.00 *	0.23	⩾0.33	−0.68 ***		868 [d]	4
III	11 [x]	3.25	−0.15 †	0.24	0.15	0.11	0.16	−0.18		109 [c]	4
IVa	6 [x]	3.18	0.05 †	0.00 **	0.23	0.00 †	−0.50 ***	−0.08		177 [e]	4
	1 [y]	3.28							0.00	114	18
Va	12 [y]	3.17							−0.01	19	8
VIa	2 [y]	3.13							0.02	111	8
VIb	3 [y]	3.13							−0.05	61	8
Vb	13 [y]	3.11							−0.06 †	55	8
VII	14 [y]	3.05							−0.07 *	47	14
VIII	1 [x]	3.12	−0.15 †	−0.41 ***	0.17	0.25	0.23	0.00		104 [e]	4
	4 [y]	3.00							0.06	95	8
IX	2 [x]	2.93	−0.01	0.19	0.36	0.13	0.24	−0.03		47 [c]	4
	5 [y]	2.88							0.18 *	56	12
Xa	10 [x]	2.77	−0.10	0.38	0.11	0.34	0.38	0.14		63 [c]	4
	6 [y]	2.82							0.23 **	60	10
XIa	15 [y]	2.77							0.10	13	8
XII	7 [y]	2.75							0.02	30	10
XIIIa	3 [x]	2.68	0.04	0.43 †	0.11	0.25	0.48	0.03		69 [c]	4
Xb	9 [x]	2.54	−0.08	0.40	0.45	0.30	0.45	0.08		63 [c]	4
	8 [y]	2.52							0.20 *	149 [d]	10
XIVa	13 [x]	2.33	−0.02	0.45 ***	0.64 ***	−0.08 ***	−0.63 ***	0.56 ***		98	16
XIVb	14 [x]	2.26	−0.16 *	−0.14 ***	0.19 †††	0.26	0.19 *	0.20 ***		247	16
XIIIb	4 [x]	2.08	0.13 †	0.41 †	0.57 **	0.13	0.47 †	0.00		229 [c]	4
Chicken cell agglutination test											
XIIIc	7 [x]	2.19	0.03	0.19	0.27	0.13	0.28	−0.16		37 [c]	4
	9 [y]	2.17							−0.15 **	10	12
IVb	5 [x]	2.02	−0.05	0.06 *	0.12	0.04	−0.06 *	−0.25		250 [c]	4
	10 [y]	2.18							−0.37 ***	8	18
XIVc	15 [x]	2.01	−0.07	0.31	0.58 **	−0.12	⩽−1.28 ***	0.13		62	4
XIVd	16 [x]	2.00	−0.05	0.36	0.45	0.34 †	0.46	−0.01		15	4
XIb	16 [y]	1.99							−0.16 **	11	8

[a] The type A vaccines used in assay *x*—A_1, A_2, A_3, and A_4—were designated vaccines B, U, PA, and BA, respectively, in an earlier report. The type B vaccines used in assay *x*—B_1 and B_2—were designated BBA and PB, respectively, in an earlier report. The type B vaccine used in assay *y* was designated JB in an earlier report. Vaccines A_1, A_4, B_2, and B_3 were freeze-dried vaccines and vaccines A_1, A_3, and B_2 were fluid vaccines.

[b] f = the number of degrees of freedom.

[c] Values used to calculated the overall average in assay *x*.

[d] These data were excluded from Tables 5, 6 and 9.

[e] Values used, together with those referred to in footnote "c", to study the variation.

†, ††, ††† indicate that the average (although it does not deviate significantly from the overall average value) contains 1, 2, or 3 single values that do deviate significantly from the overall average value (see Table 5).

*, **, *** indicate deviations of log relative potencies frcm their average that exceed the 5%, 1 % and 0.1 % levels of significance.

Table 2. Distribution of the residual variances in the assays (Methods I - XIVd)

Residual variance	Assay x [a]	Assay y [a]	No. of methods HA	No. of methods CCA
0.0008	(XIVd) [b]	(IVb) XIa (XIb) (XIIIc)	1	4
0.0016		Va XII	2	
0.0032	IX Xa Xb (XIIIc) (XIVc) [b]	Vb VIb VII IX Xa	8	2
0.0064	I III VIII [b] XIIIa XIVa [b]	I [b] IVa VIa VIII	9	
0.0128	IVa [b] (IVb) XIIIb XIVb [b]	Xb	4	1
0.0256				
0.0512	II [b]		1	
0.1024				
weighted average	HA & CCA: 0.0116	HA: 0.0058 CCA: 0.0011		

[a] Method numbers for CCA results are placed in parentheses. The 8 methods used in assay x as well as in assay y are underlined.

[b] The residual variances for these methods were not included in the weighted average.

were 0.56 for vaccine B_3; the log relative potencies [1] (the log relative haemagglutinin contents) for these two vaccines had ranges of 0.28 and 0.30. This shows very clearly the considerable reduction in the laboratory variation by replacing titres with relative potencies; the reduction factor was about 11 in assay x and about 2 in assay y. It is therefore not practicable to translate HA results to CCA results by using the factor 20 mentioned by the WHO Expert Committee on Biological Standardization (1968) in the requirements for inactivated influenza vaccine. It is, however, possible to obtain uniformity of results by expressing the results in international units calculated from log relative potency based on the IRP titres. The relative potencies show smaller ranges than that for the IRP, and the trend of the IRP values is not evident in the log relative potencies.

In addition to reducing the variations between laboratories and between methods, the variations from day to day for each method can be reduced by using a reference vaccine. The reduction may depend on the type of vaccine. In the following section, an analysis of the different types of variation is given.

[1] The term " relative potency ", as used in this paper, refers to the ratio between the haemagglutinin content of the test vaccine and that of the reference vaccine.

Reproducibility of the method

Experimental errors. Analyses of variances were carried out for both assay series on the results from 3 days—in assay x on the results with 3 distributed vaccines of the same origin, in assay y on 2 distributed vaccines of different origin, and on at least 3 local vaccines, which were often samples taken from current production. In these computations the titres were corrected for systematic variations due to vaccine and for variations from day to day. The mean squares of these corrected titres (residuals) were used for estimating the variance due to experimental errors (the residual variance). These estimates are given in Table 1 (last column). Table 2 shows the distribution of the estimates and the average values used for the calculation of standard errors.

Variances estimated for each laboratory for the HA method varied from 0.0013 to 0.0868 with 17 out of 25 values between 0.0032 and 0.0128; for the CCA method 6 of the 7 variances were between 0.0008 and 0.0062 and one was 0.0250; this is less than the distribution for the HA test. The distribution of the residual variances is broader than the expected distribution and somewhat skew, with many relatively low values. The high values for the residual variances were found in the 6 methods quoted below:

Method	*Residual variance*
Assay x	
II	0.0868
IVa	0.0177
IVb	0.0250 (CCA method)
XIIIb	0.0229
XIVb	0.0247
Assay y	
Xb	0.0149

For all the above HA methods (5) the usual 2 two-fold dilution series were used, and no connexion was seen between the number of categories of haemagglutination used in reading the results and the high variance. The residual variances were generally lower in assay *y*.

All the results for laboratory II (12^x) were excluded from further evaluation, because of the degree of variation and also because of the occurrence of two values that could not be properly defined. For information, Table 2 shows the variance for laboratory II. Among the results in assay *y*, those from laboratory I (11^y) for vaccine B_3 were excluded from Tables 5 and 6, and those from laboratory Xb (8^y) for local vaccines x, y, z, and w were excluded from Table 10; the reasons for these exclusions were that in laboratory I, the log relative potency for vaccine B_3 (−0.30) was outside the normal distribution; in laboratory Xb, among the results with vaccines x, y, z, and w there were 3 major deviations among the 12 log relative potencies ($s_R^2 = 0.0149$; see also Table 10).

In assay *x* the difference between the HA and CCA test results was moderate. The values of s_R^2 were 0.0180 with f = 72 (the value was 0.0139 with f = 68 if laboratory II (12^x) was excluded), and 0.0091 with f = 16, respectively; the value $s_R^2 = 0.0116$ with f = 44 was used for the whole assay, corresponding to s_R^2 values from laboratories I, III, IVa, IVb, VIII, IX, Xa, Xb, XIIIa, XIIIb, XIIIc (1^x–11^x). In assay *y* it was found necessary to remove the variance component originating from the contrast between the distributed vaccines and the local vaccines (see below and Table 3); the s_R^2 distribution noted in Table 2 remains, however, almost unchanged with the following exceptions: the value for laboratory VIa is reduced from 0.0111 to 0.0010, and that for laboratory VIII is reduced from 0.0095 to 0.0037. The following s_R^2 values were based on the reduced s_R^2 values: HA = 0.0058; f = 98; CCA = 0.0011; f = 32. Because of the clear difference between HA and CCA methods in assay *y* the following values of the standard error were used:

assay *x*,	HA & CCA	0.108
assay *y*,	HA	0.076
assay *y*,	CCA	0.033

The footnotes to Table 4 indicate how these SE values should be used to compare single log relative potencies, average values per vaccine and per laboratory, and the overall averages.

The overall reduction in the variance from assay *x* to assay *y* is contributed to by the occurrence of low residual variances in some laboratories and partly by a reduction in the variation in some of the laboratories from assay *x* to assay *y*. In one laboratory, two different HA methods, Xa and Xb, were used in both assay *x* and assay *y*; three of these s_R^2 values are equal to 0.0060, while one (Xb, assay *y*) is 0.0149.

Day-to-day variations. The variances due to day-to-day variations have been compared with the average residual variance. Cases in which a pronounced variation from day to day is observed have been used for testing whether a reduction could be obtained by means of a reference vaccine. Such cases are listed in Table 3. In the table the vaccines are divided into two groups, the distributed vaccines and the local vaccines. The variations from day to day in the average titres for the two groups are compared and a mean square of the differences between these averages (called " contrast ") has been calculated. It will be seen from the table that strongly significant day-to-day variations are observed for 12 methods. In these cases the day-to-day variation may be eliminated by using one of the vaccines in the group as reference. Thus the IRP should be useful as a reference for the distributed vaccines.

If the day-to-day variations for the two groups of vaccines were the same, a significant variance ratio should be obtained for both the distributed vaccines and the local vaccines. It appears from Table 3 that this happens only in one case, i.e., assay *y*, laboratory XIb. This indicates that the reference vaccine is not useful for the elimination of day-to-day variations for the local vaccines. By the direct comparison of the averages, significant contrasts are, however, found in two cases only. Therefore a detailed study of the haemagglutinin content of the different vaccines in relation to the reference has been carried out.

Deviations of relative potencies

The variation in each assay is described by the distribution of the deviations of the log relative potencies from their overall averages. Table 4 shows these deviations for the average log relative potencies

Table 3. Survey of results of the methods for which a strongly significant day-to-day variation was observed for distributed vaccines and/or for local vaccines

Assay	Laboratory	Distributed vaccines [a]			Local vaccines [a]			Contrast [a,b]	
		No. of vaccines	$s^2 \times 10^4$	v^2 [c]	No. of vaccines	$s^2 \times 10^4$	v^2 [c]	$s^2 \times 10^4$	v^2 [c]
x	Xa (10)	3	648	5.6 **	1	203	1.8	56	0.5
	XIVa (13)	3	2162	18.6 ***	0	—	—	—	—
	XIVb (14)	3	2186	18.8 ***	0	—	—	—	—
	I (11)	2	604	10.4 ***	2	150	2.6	76	1.3
	IVa (1)	2	152	2.6	8	2050	35.3 ***	92	1.6
	VIa [d] (2)	2	2	0.03	3	1141	19.7 ***	414	7.1 **
	VIb (3)	2	179	3.1	3	310	5.4 **	110	1.9
	VII (14)	2	3	0.05	6	442	7.6 ***	127	2.2
	VIII [d] (4)	2	21	0.4	3	421	7.3 **	271	4.7 *
	Xa (6)	2	28	0.5	4	360	6.2 **	44	0.7
	Xb (8)	2	152	2.6	4	1266	21.8 ***	119	2.1
	XIb [e] (16)	2	160	14.6 ***	3	226	20.5 ***	1	0.1

[a] *, **, and *** indicate the variance components that pass the 5, 1, and 0.1 % limits of significance, measured by the variance ratio, using the mean s_R^2 values quoted below.

[b] Contrast = the mean square of the differences between the average titres for the two groups of vaccines.

[c] v^2 = the ratio between the variance due to day-to-day variations and the average residual variance:

assay x: $s_R^2 \times 10^4 = 116$

assay y, HA: $s_R^2 \times 10^4 = 58$

CCA: $s_R^2 \times 10^4 = 11$

[d] The s_R^2 from Table 1 was remarkably reduced by removing the large contrast component: that for VIa was reduced from 111 to 10, and that for VIII from 95 to 37.

[e] CCA test.

for each vaccine by laboratory; between zero and four values per laboratory pass the 1% limit of significance.

Table 5 shows, for both series of assays, the relation between the deviations of the log relative potencies from their overall averages and the occurrence of significant deviations of single log relative potencies. It should be noted that 11 significant deviations of single relative potencies are found for combinations of method and vaccine, where the average value had a deviation within the 5% limit.

Table 6 shows for assay x the occurrence of deviations of single relative potencies in three groups of laboratories for two groups of vaccines. With vaccines A_1, A_3 and A_4, for which there were few deviations, the methods XIVa, XIVb, XIVc, and XIVd resulted in 16.1% of the deviations passing the 1% limit of significance against 0% and 1.6% for the other methods. With vaccines A_2, B_1, and B_2, methods IVa and VIII and especially methods XIVa, XIVb, XIVc, and XIVd gave rise to a large number of pronounced deviations, many of which are negative.

For each of the six groups of results given in Table 6 additional information is given in Table 7 regarding the ranges of the log relative potency per group and their averages. The weighted residual variance per group of methods is also given. The ranges are in the region of 0.74, except for the values for vaccines A_1, A_3, and A_4 with methods IVa and VIII, and for vaccines A_2, B_1, and B_2 with methods XIVa, XIVb, XIVc, and XIVd.

For methods IVa and VIII the average log relative potency for vaccines A_2, B_1, and B_2 was -0.13 compared with 0.17 and 0.18 by the other methods,

Table 4. Average values per vaccine of log relative potencies, and average residual variances per method group, together with a summary of significant deviations of average log relative potencies from the overall average values †

Methods	Assay x [a]							Assay y [a]		
	Type A vaccines				Type B vaccines		$s_R^2 \times 10^4$	Methods	Vaccine B_3	$s_R^2 \times 10^4$
	A_1	A_2	A_3	A_4	B_1	B_2	(f)			(f)
Selected methods (HA and CCA) No. 2-5 & 7-11	− 0.03	0.28	0.27	0.17	0.31	− 0.04	116 [b] (44)			
HA methods No. 1-4,6, & 8-14	− 0.05	0.21	0.27	0.17	0.18	0.00	180 (72)	1–8 and 12–15	0.05 [c]	58 (98)
CCA methods No. 5,7,15, & 16	− 0.04	0.23	0.36	0.10	0.23 [d]	− 0.07	91 (16)	9,10, 16	− 0.23	11 (32)

Methods	Statistical significance [e]	Deviations of the average relative log potencies									
No. 1–11	*	1	1		—	2	—	—	1-10	3	—
&	**	—	1	2	—	—	—	—	&	3	—
13–16	***	—	3	1	1	2 [d]	2	—	12-16	1	—

† The overall averages of log relative potencies in assay x were calculated using only the values indicated in Table 1; this may explain some of the deviations from the above averages. The SE was based on the residual variance for IRP and the two other batches with the same origin (also indicated in Table 1).

In assay y the SE was based on the residual variance for 4–9 vaccines per method.

In assay x the SE for the deviation of a single log relative potency from the overall average log relative " potencies " (for the 9 selected laboratories) is 0.153, with the following exceptions: the magnitude of the SE applicable for the actual deviations depends on the number of testings per day and number of testing days involved in the comparison; with two or three testing days the standard error is 0.108 and 0.088 respectively. For laboratories XIVa and XIVb, using 1, 2, or 3 testings per day, the SE for the averages ranges from 0.062 to 0.051; the SE for single relative " potencies " ranges from 0.125 to 0.088.

The SE values for assay y depend on the method as follows:

	Deviations of averages		Deviations of single values	
	HA	CCA	HA	CCA
Deviations from overall averages	0.062	0.027	0.107	0.046
Deviations from average per 3 days	—	—	0.088	0.038

[a] s_R^2 = the average residual variance, and
(f) = the number of degrees of freedom (figures in parentheses).

[b] This value for the average residual variance is for laboratories no. 1–11.

[c] The result for vaccine B_3, from laboratory I (11^y), was excluded as it was outside the range of the other values.

[d] The result for vaccine B_1, by method XIVc (15^x) was excluded as it was outside the range of the other values.

[e] *, **, *** indicate deviations of the relative log potencies from their average that exceed the 5 %, 1 %, and 0.1 % levels of significance.

Table 5. Relation between significant deviations of the single log relative potencies and of their averages †

No. of deviations of single log relative potencies exceeding the indicated level of probability		Significance level of the deviations of the average log relative potency				Total
*** + **	*	***	**	*	none	
⩾2		5				5
1	⩾1	3 (1)			1	4 (1)
1		2	2 (1)	4 (1)	4	12 (2)
	2		2 (2)	1 (1)		3 (3)
	⩾1		2	2 (1)	6 (1)	10 (2)
none					70 (7)	70 (7)
		10 (1)	6 (3)	7 (3)	81 (8)	104 (15)

† The figures 1 and 2 in the two columns at the left-hand side of the table indicate that one or two of the three single log relative potencies for a vaccine pass the indicated limit; ⩾1 and ⩾2 are used, when no result for day 3 is available. The figures in parentheses in the main table indicate the deviations found in assay *y*.

When 2 or 3 days gave results that deviated significantly from the average these deviations agreed in direction in 14 cases, but for method XIVb with vaccine A_3 the deviations were as follows (standard error is shown in parentheses):

day 1	*day 2*	*day 3*
− 0.26 *	− 0.23 *	+ 0.25 **
(0.099)	(0.088)	(0.088)

*, **, and *** indicate the 5, 1, and 0.1 % levels of significance.

while no such discrepancy is seen for vaccines A_1, A_3, and A_4 (values 0.09–0.16); the residual variances for the three method groups are almost the same (0.0116, 0.0141, and 0.0146, respectively).

In assay *y*, the distribution of the deviations for vaccine B_3, estimated by 15 laboratory methods (see Table 6), was similar to that for vaccines A_1, A_3, and A_4; the results for one laboratory were excluded because they were not acceptable (laboratory I : 3 major negative deviations, large dilution steps, and less well defined readings).

In assay *x*, as well as in assay *y*, it can be seen that the between laboratory variations for the type B vaccines follow those of the type A vaccines, and that the log relative potencies based upon the IRP (type A) show the same variation as the log relative potencies based on a type B vaccine.

Comparison of vaccines

The review of the variation within laboratories in assay *y* showed that the laboratories using the CCA test had a rather low s^2 value, while the results from those laboratories that used the haemagglutination test could be arranged into three groups, each with a characteristic level of s^2 values. This grouping is shown in Table 8, in which each group is characterized by the weighted average of s^2.

To illustrate the practical implications of these differences in standard error a calculation has been made following the same principles as those used for smallpox tests (Bentzon & Krag, 1963). The number of testing days required to give a probability greater than 95% of obtaining a significant difference between the average log titres for two vaccines having a true "potency" ratio of 2, 1.6, and 1.25 was calculated for each of the four groups; if the desired ratio is 2, only the CCA test and the best HA tests will give sufficient evidence from a single testing day; if the ratio is taken as 1.6, 2 testing days will be required for the best HA tests, 4 testing days for the medium tests, and 7 testing days for

Table 6. The total number of test results and the number of deviations of single log relative potency values from their average that exceeded the given level of significance †

Methods	Level of significance[a]	Assay *x*		Assay *y*
		Vaccines A_1, A_3, and A_4	Vaccines A_2, B_1, and B_2	Vaccine B_3
I, III, IVb, IX, Xa, Xb, XIIIa, XIIIb, XIIIc (2–5, 7–11)	total	63	60	
	*	2	2	
	**	1	3(1)	
	***	0	0	
	percentage ≥ **	1.6	5.0 (1.7)	
IVa, VIII (1 & 6)	total	14	14	
	*	3	0	
	**	0	0	
	***	0	4(4)	
	percentage ≥ **	0	28.6 (28.6)	
XIVa, XIVb, XIVc, XIVd (13–16)	total	31	31	
	*	5	0	
	**	3(1)	1	
	***	2(1)	13(8)	
	percentage ≥ **	16.1 (6.5)	45.2 (25.8)	
all	total	108	105	45
	*	10	2	10
	**	4(1)	4(1)	2
	***	2(1)	17(12)	1(1)
	percentage ≥ **	5.5 (1.8)	20.0 (12.4)	6.7(2.2)

† The figures in parentheses represent negative deviations. For assay *x* the deviations exceeding the 1 % level of significance are distributed between vaccines as follows:

	A_1	A_3	A_4	A_2	B_1	B_2
Deviations exceeding the 1 % level of significance	2 (1)	2	2 (1)	8 (6)	8 (7)	5

[a] *, **, and *** indicate the 5, 1, and 0.1 % levels of significance.

the worst. These remarks are, of course, of value only if the laboratories maintain the same within laboratory variation as found in the experiments used as the basis for this study.

Tests used for describing the strength of smallpox and polio vaccines were studied by Bentzon & Krag (1963) and Krag (1963) regarding the relation between the number of repetitions needed to demonstrate a certain ratio between the strength of two vaccines: the methods examined were:

Table 7. Range of log relative potencies and the average per method group

Methods	No. of results included in the average	Vaccines A_1, A_3, and A_4: log relative potency		Vaccines A_2, B_1, and B_2: log relative potency		Residual variance[a]
		Average	Range	Average	Range	
I, III, IVb, IX, Xa, Xb, XIIIa, XIIIb, and XIIIc (No. 2–5 and 7–11)	27	0.14	0.72	0.18	0.71	0.0116 (44)
IVa, VIII (No. 1–6)	6	0.09	0.40	− 0.13	0.73	0.0141 (8)
XIVa, XIVb, XIVc, and XIVd (No. 13–16)	12	0.16	0.80	0.17	1.19	0.0146 (40)

[a] The figures in parentheses are the number of degrees of freedom.

Smallpox

Scarification in rabbit skin; 2-fold dilutions — 2 rabbits

Pock-count in eggs; 6 eggs for each of five 3-fold dilutions — 30 eggs

Polio

Immunization of chicks / Immunization of guinea-pigs — 10 animals for each of five 5-fold dilutions — 50 animals

Influenza

Haemagglutination; two 2-fold dilution series — no animals

Tests as above on two days allowed the following distinctions to be made between vaccines:

Pock count	2-fold
Scarification	16-fold
Polio vaccine	5–16-fold (or more in some laboratories)
Influenza, CCA and some HA tests	1.4-fold, but other HA tests 2-fold or more (in some laboratories 3 testing days were needed for a 2-fold distinction)

Local vaccines

It has been the practice in some countries, for control purposes, for acceptance limits of haemagglutinin content to be specified for vaccine—e.g., 10 000 –15 000 HA units or 500–750 CCA units (Miller & Stanley, 1944). As the variation between CCA results is relatively small, it is possible to express the above limits in international units. The average log value for the CCA results for the International Reference Preparation in assay x was 2.060. As the content of one ampoule of the International Reference Preparation is 200 International Units, the ratio IU/CCA units is 200/antilog 2.060 = 200/114.8 = 1.74.

The above limits expressed in CCA units are therefore equivalent to 870–1 305 IU, or in practice 1 100 IU $\pm$20 %, when expressed in terms of the International Reference Preparation. In the present study, a number of influenza virus vaccines of different origin were obtained and their haemagglutinin content was evaluated in terms of the International Reference Preparation.

In assay x, 8 local vaccines were tested. It is not known whether these local vaccines were represen-

Table 8. Number of testing days required to establish at the 5 % level of significance an n-fold difference in strength between two vaccines with a probability greater than 95 %

Method	Laboratories	Weighted average of residual variance	"n" values		
			2.00	1.60	1.25
CCA	IVb, XIb, XIIIc	0.0009	0.3	0.6	2.1
HA	Va, XIa, XII	0.0021	0.6	1.4	5.6
HA	Vb, VIb, VII, IX, Xa	0.0053	1.6	3.7	14.6
HA	I, IVa, VIa, VIII	0.0104	3.1	6.9	27.6

Table 9. Distribution of deviations of single relative potency values from their averages

	HA		CCA		HA & CCA[a]		Expected percentage distribution
	No.	% of total	No.	% of total	No.	% of total	
3.29 × SE	1						0.1
2.58 × SE	6	13	1	6	5	10	0.4 5.0
	12		2		13		4.5
1.96× SE	33	23	11	20	40	21	28.3
1.00 × SE	92	64	40	74	128	69	66.7
total	144		54		186		100.0

[a] Laboratory Xb (8 *y*) was excluded.

tative of local reference preparations or whether they were samples of vaccines produced for clinical use, but only 3 vaccines contained more than 870 International Units—the lowest acceptance limit according to the criteria cited above; only 1 passed the upper limit of 1 305 IU. One vaccine was close to the limit, with a haemagglutinin content of 718 IU; 3 vaccines contained only about 400 IU, and 1 only 200 IU.

In assay *y*, the participants tested 3–7 local vaccines together with the 2 distributed vaccines, giving a total 198 log relative potency values. The within laboratory variation has been determined by computing the deviations between single relative " potencies " and the average per laboratory and per vaccine. The magnitude of these deviations has been estimated on the basis of the standard error described for the whole of assay *y*. The distribution of these deviations is given in Table 9 together with the expected distribution. It is seen that all three classes of significant deviations appear more often than expected while the group of deviations corresponding to 1.00–1.96 SE is poorly represented. It is not possible to normalize the distribution by introducing a standard error factor.

The CCA results were nearest to the expected distribution; the exclusion of results for 4 vaccines from laboratory Xb (8*y*) improves the distribution slightly. Six of the 8 highly significant values ($P < 1.0$) are found among the results from only 3 of the 16 laboratories where they appear in connexion with four deviations exceeding the 5% level of significance (Table 10).

In the analysis mentioned earlier these three laboratories had maximum residual variances of 0.0114, 0.0111, and 0.0149, respectively (average 0.058); their day-to-day variation for local vaccines was high (Table 3). In two of the laboratories (VIa and Xb) the IRP showed no differences from day to day, while all the local vaccines showed a relatively high day-to-day variation.

Comparison between methods within laboratories

A total of 20 vaccines were tested by two methods (5 laboratories each using two methods). When the differences between these pairs of log relative poten-

Table 10. Log relative potencies by days

Method	Vaccine	Day 1	Day 2	Day 3	Average	Residual variance	Range of IRP
IVa (1 *y*)	6	0.68 **	0.15 **	0.38	0.40	0.0114	0.15
VIa (2 *y*)	x	0.53	0.68 *	0.30 *	0.50	0.0111	0.00
	y	0.44	0.72 **	0.30 *	0.48		
Xb (8 *y*)	x	0.68 **	1.05	1.05	0.93		
	y	0.45 ***	0.93	0.90	0.75	0.0149	0.00
	z	0.60 **	1.06 *	0.90	0.85		

Table 11. The distribution of results from assay y for 46 local vaccines by haemagglutin content (in IU), with notes on the standard error per laboratory, and results for vaccine B3 and the IRP in IU and log titres respectively *

Haemagglutinin content (IU)	Laboratory											
	I [a]	IV [b]	V [c]	VI [c]	VII [d]	VIII	IX	X [c]	XI [c]	XII [e]	XIII	Total no.
6 400										1		1
3 200								2				2
1 600						1	1	2				4
800	1	7	1	3		1	3		3		2	21
400	1		2			1	1			1	3	9
200					5					2		7
100	1				1							2
50												
Total no.	3	7	3	3	6	3	5	4	3	4	5	46
SE × 10^4	76	(114) 8	19 55	111 61	47	95	56	60 149	13 11	30	10	
Vaccine B3 (IU)	100	200 85	191	189	170	231	304	331	183	208	142	
IRP (log titre)	3.2	3.3 2.2	3.2 3.1	3.1 3.1	3.0	3.0	2.9	2.8 2.5	2.8 2.0	2.7	2.2	

* The underlined figures are the SE values and titration results corresponding to the CCA test.

[a] These relatively weak vaccine preparations were not intended for human use and were prepared specially for the study: the results were not included in Tables 3, 4 & 6.

[b] The HA results with the larger SE were not used in calculation of the average (these HA results were larger than CCA results).

[c] Weighted average calculated on the HA and the CCA results, or on 2 HA results.

[d] Three of the vaccines were live vaccines containing 90, 125, and 160 IU; the effect of these may not be comparable with the effects of other types of vaccines.

[e] One vaccine containing 140 IU was used in protection experiment in humans. Only 13 % of the vaccinated had a moderate influenza attack, after challenge compared with 55 % of the controls.

cies were examined the variation was found to be random. Among the 60 differences, 6 were outside the 1 % level of significance. The 20 pairs of average vaccine results showed differences corresponding to the expectation (based on the s^2 values per laboratory, noted in Table 1), except for the values found in laboratory IV where vaccines 2 and 6 showed method differences exceeding the 0.1 % level of significance; major deviations occurred on days 2 and 3 and on days 1 and 2, respectively, in the HA results.

Classification of local vaccines

Table 11 summarizes the average results for the 46 different vaccines tested. When two methods were used a weighted average has been taken to express the haemagglutinin content of the vaccines, all of which are expressed in international units. It is clear that the variation among the results is considerable and that the majority (21) lie between 400 and 800 IU; the two values between 50 and 100 IU, and two of the seven values between 100 and 200 IU, are results with vaccines not intended for human use.

Only 7 of the 46 vaccines had a haemagglutinin content of 800 IU or higher. It is known that vaccine *y* from laboratory XII (7*y*), containing 140 IU, was identical with a vaccine that had given protection to chicks and humans (Beare et al., 1968). Regarding the two vaccines containing 415 and 125 IU, it is

known from laboratories XIII (9v) and VII (14v) that vaccines produced from the same strains and by the same procedures as the assay vaccines have shown a clinical vaccination effect:

Laboratory	*% of persons infected/ no. of persons vaccinated*	*% of persons infected/ no. of nonvaccinated persons observed*
XIII (9v)	4/800	12/3 000
	4/400	21/1 300
	15/900	20/900
VII (14v)	5/30 000	19/22 000

If precise specifications are to be made for haemagglutinin content, in terms of the International Reference Preparation, the limits to be imposed will have to be agreed upon. At present, national control authorities would have to decide for themselves the acceptable limits, but it would be useful if the World Health Organization requirements could be formulated to give guidance on these specifications. It should, of course, be realized that the haemagglutinin content (in IU) expresses only the haemagglutinating effect of a vaccine. Results from protection tests are the ultimate basis on which to determine the criteria of potency.

THE USE OF THE INTERNATIONAL REFERENCE PREPARATION FOR CALIBRATING A LOCAL REFERENCE PREPARATION (LRP)

An influenza virus suspension with an HA titre between 400 and 2 000 (2.60–3.30 in log titre) should be selected or prepared by dilution from more concentrated material. The IRP and the LRP should then be titrated on a series of testing days—3–6 days depending on the magnitude of the local within laboratory variation. It is preferable to use two 2-fold dilution series for which the basis of dilution differs by a factor of $\sqrt{2}$ as in the example below:

Series a	*Series b*	*LRP*		*IRP*	
1/128		4		4	
	1/181		4		4
1/256		4		4	
	1/362		4		4
1/512		4		4	
	1/724		3		2
1/1024		2		3	
	1/1448		0		0
1/2048		0		0	
	1/2896		0		0
Log titre		3.010	2.930	3.086	2.855
Ave. log titre		2.970		2.971	

Assuming that the average log titres for five days are, IRP, 2.996 : LRP, 2.989, then the average log relative potency is −0.007. The IRP contains 200 IU per ampoule (per ml). Therefore the haemagglutinin content of LRP is the antilogarithm of (2.301 −0.007) = 2.294; that is, 196.8 IU. If we further assume that the LRP was distributed in ampoules each containing 1 ml, and that it was freeze-dried before testing, and the average content per ampoule was 17.65 mg, then 1 IU will be 0.0897 mg of the dry material in the LRP.

RÉSUMÉ

PRÉPARATION INTERNATIONALE DE RÉFÉRENCE D'HÉMAGGLUTININE DU VIRUS GRIPPAL (TYPE A)

Le présent article décrit l'étude collective internationale qui a conduit à constituer, en 1967, la préparation internationale de référence d'hémagglutinine du virus grippal (type A) ainsi que les études relatives à l'emploi de cette préparation pour évaluer le pouvoir immunisant de 46 vaccins antigrippaux en termes d'unités internationales. Le Comité OMS d'experts de la Standardisation biologique (1967) a défini l'unité internationale d'hémagglutinine du virus grippal (type A) comme l'activité de 0,09361 mg de la préparation internationale de référence.

Quatorze laboratoires, dans 12 pays, ont participé à l'une de ces études, ou aux deux, en utilisant au total 24 méthodes de titrage — épreuves d'hémagglutination (HA) et, dans quelques cas, épreuves d'agglutination des hématies de poulet (CCA). On a relevé de fortes différences entre les titres HA obtenus par les divers laboratoires (facteur de variation: 50); en revanche, les divergences ont été beaucoup moins nombreuses en ce qui concerne la mesure de l'activité des vaccins par rapport à la préparation internationale de référence (facteur de variation: 2). La méthode CCA utilisée dans un petit nombre de laboratoires a fourni des résultats voisins des titres HA les plus faibles et relativement peu variables d'un laboratoire à l'autre. L'analyse de la variance a révélé des différences dans les variations propres à chaque laboratoire, mais, dans la majorité des cas, ces variations ont permis l'estimation globale d'une erreur type.

On décrit le mode de calcul utilisé pour convertir les titres en activités relatives puis en unités internationales.

Des recommandations sont formulées concernant le choix, la préparation et le titrage d'un vaccin de référence local dont l'activité serait exprimée en unités internationales.

L'analyse des résultats des épreuves pratiquées sur 46 vaccins locaux et des écarts des activités relatives par rapport à la moyenne, par vaccin, a fait ressortir une distribution comportant huit divergences majeures au lieu de une, comme on l'escomptait. De nombreux vaccins (21) possédaient une activité comprise entre 400 et 800 unités internationales, nettement inférieure à la limite adoptée auparavant, soit 1100 unités internationales; 3-4 vaccins seulement faisaient preuve d'une activité supérieure à cette limite.

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Annex

LIST OF PARTICIPATING LABORATORIES

AUSTRALIA

Commonwealth Serum Laboratories
Parkville

CANADA

Laboratory of Hygiene
Ottawa

FRANCE

Institut Pasteur
Centre National de la Grippe
Paris

HUNGARY

State Institute of Hygiene
Budapest

INDIA

Pasteur Institute of Southern India
Coonoor (Nilgiris)

JAPAN

National Institute of Health
Japanese Influenza Centre
Tokyo

NETHERLANDS

N. V. Philips-Duphar
Weesp

NORWAY

Statens Institutt for Folkehelse
Virusavd. B
Oslo

SWITZERLAND

Swiss Serum and Vaccine Institute
Research Department
Berne

UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND

Evans Medical Ltd.
Liverpool

Medical Research Council Laboratories
Holly Hill
London

Medical Research Council Laboratories
Mill Hill
London

UNITED STATES OF AMERICA

National Institutes of Health
Division of Biologics Standards
Bethesda
Maryland

Park, Davis and Company
Detroit

YUGOSLAVIA

Institute of Immunology
Zagreb