Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1970, 42, 535-552

A Research Standard for Human Serum Immunoglobulins IgG, IgA and IgM

D. S. ROWE,[1] S. G. ANDERSON [2] & B. GRAB [3]

A pooled human serum, partly diluted, has been distributed into ampoules and freeze-dried in several batches. The freeze-dried material has been examined in an international collaborative assay and certain properties have also been estimated in individual laboratories.

On the basis of these tests this material was considered to be suitable for use as a standard for the estimation of IgG, IgA and IgM for clinical purposes using the single-radial-diffusion or similar techniques. Greater uniformity of results than is obtained at present should be achieved if this material were in general use.

Estimates of immunoglobulins from different laboratories using this material as a standard showed small but significant variability. This variability was probably related to the heterogeneity of immunoglobulins and of antisera, and it limits the precision of immunoglobulin estimations by techniques at present in use.

Batches of this material have been distributed to various centres. 67/68 has been established as the British research standard for human serum immunoglobulins IgG, IgA and IgM for which the unit of potency is defined as the activity present in 0.8147 mg of dry powder. The average activity per ampoule of 67/86 is 100 units of IgG, IgA and IgM. The average activities of other related preparations have been estimated.

Concentrations of the immunoglobulins IgG, IgA and IgM (*Bull. Wld Hlth Org.*, 1964) in human serum are frequently estimated by immunochemical methods based on the precipitation of antigen–antibody complexes in agar gel. One such technique, that of single radial diffusion, is now widely used. This method was originally described by Mancini, Carbonara & Heremans (1965) and many modifications are at present in use. Solutions of antigen are introduced into small wells cut into agar plates in which antiserum is uniformly distributed. Antigen diffuses from these wells and produces circular areas of precipitate. The amount of diffusing antigen can be related to the area of the precipitate. Techniques such as this could best be quantified by the use of a biological standard for antigen in each titration run. The concentration of antigen under test would then be expressed as a potency in relation to the defined concentration of antigen in the standard.

A proposed standard for human IgG, IgA and IgM has been prepared for use in such tests and has been subjected to international collaborative assays. This paper describes the preparation and tests of this material and records the unitage assigned to it. Preparations related to the proposed standard are now issued by the laboratories listed in Annex 1.

Thirteen laboratories in 8 countries participated in the collaborative study: they are listed in Annex 2 together with the names of the responsible workers. In this report the laboratories will be arbitrarily referred to by a code letter, which is not necessarily related to their order in Annex 2.

THE PROPOSED STANDARD

Material for the standard

A single pool of diluted human serum was prepared as the source of a standard for IgG, IgA and IgM. The pool was distributed into ampoules and freeze-dried. The ampoules were sealed by fusion of the glass and stored at −20°C.

Through the courtesy of the British National Blood Transfusion Service, citrated plasma from a

[1] Director, WHO Reference Centre for Immunoglobulins, Lausanne, Switzerland.

[2] Division of Biological Standards, National Institute for Medical Research, Mill Hill, London, England.

[3] Statistician, Health Statistical Methodology, World Health Organization, Geneva, Switzerland.

number of normal male donors with no history of malaria or hepatitis was made available to the Division of Biological Standards, National Institute for Medical Research, London. Between January and March 1967, a total of 761 donors each provided approximately 200 ml of citrated plasma. Between 400 ml and 450 ml of blood were obtained from each donor, and were mixed with 75 ml of citrate.[1] The citrated blood was held for up to 8 hours at 6°C and then centrifuged at 4600 rev/min at 6°C for 20 min; the plasma was then expressed into a satellite pack. The plasma was frozen in a mixture of alcohol and solid CO_2 for 20 min and then thawed at 8°C for 90 min. The cryoprecipitate was removed by centrifugation at 4600 rev/min for 2 min and the supernatant was stored at 4°C–6°C for up to 6 days. A further 30 donors provided plasma that was not freed of cryoprecipitate but was held frozen at −20°C until pooled with the larger number of samples.

Samples of citrated plasma were received in the Division of Biological Standards either frozen at −20°C or in a liquid state at room temperature, approximately 18°C, and were immediately frozen. Aliquots of samples were thawed and examined for their content of IgD. All samples with a high level of IgD were set aside as source material for a British research standard for IgD, subsequently coded 67/37. On 16 April 1967 and 17 April 1967, samples containing low or normal amounts of IgD were thawed and tested for rheumatoid factor by a latex agglutination test: 35 of these were positive in a spot test that was calibrated just to detect 2 IU of activity per ml. The remaining 465 samples of plasma were pooled and calcium chloride was added to a final concentration of M/90. The bulked samples were stirred at a temperature of approximately 18°C and clotting occurred within 120 min. A sample of the bulk serum was tested for remaining fibrinogen by the addition of thrombin, and none was found. The bulk serum was stored overnight at 4°C from 17 April to 18 April. The serum was then filtered at 4°C through a series of Millipore membranes down to a filter with an average pore diameter of 0.45 μ and divided into 2 lots—one to be distributed into ampoules at the Division of Biological Standards and the second to be distributed into ampoules at the Wellcome Research Laboratories, England.

[1] The citrate solution used was:

Trisodium citrate (dihydric)	2.2 g
Citric acid (monohydric)	0.8 g
Dextrose (monohydric)	1.47 g
Distilled water to	100.0 ml

Each lot was distributed into more than one batch of ampoules. For example in the Division of Biological Standards batch 67/86 was filled on 21 April 1967. The wet weight of contents was estimated on 47 out of 3700 ampoules and was on average 1.021 g ±0.39%. The contents were freeze-dried and then secondarily dried and sealed under pure nitrogen on 7 May 1967. For experimental purposes a small number of ampoules coded 67/139 were sealed on 1 May after only 36 hours of secondary drying, and some of the original ampoules coded 67/138 were sealed and held frozen at −20°C or −70°C without any drying.

A number of batches were filled at the Wellcome Research Laboratories on 20 April and 21 April 1967, including 67/95, 67/97, 67/98 and 67/99. The materials and the ampoules were freeze-dried for 2 days and the ampoules were sealed under nitrogen. There was no secondary drying as this was considered unnecessary in relation to the techniques then in use for primary freeze-drying.

Reconstitution of the standard

In order to examine the dried materials the contents of each ampoule were dissolved in 1 ml of distilled water. From the known weights of wet and dry contents of the ampoules it was calculated that this gave a solution containing 94.4% of the concentration of solids in the source material, and an independent laboratory test confirmed this value. Allowance for this was necessary for the comparison of reconstituted 67/86 with material from the same source which had not been freeze-dried (67/138), and this question is further considered in the later section on the use of the standard (Annex 3).

A series of dilutions of the standard and of serum samples were used in the comparative assays. The diluent commonly used was 0.14 M NaCl. The effects of different diluents are considered in Annex 4.

Stability of the standard

An estimate was made of the stability of IgG, IgA and IgM during the freeze-drying process. Laboratories K and P (see below) used the single-radial-diffusion test to compare material frozen and held at −20°C (67/138) with material freeze-dried and only briefly secondarily dried (67/139) and material freeze-dried and secondarily dried (67/86).

In order to examine the dried materials the contents of each ampoule were dissolved in 1 ml of distilled water. As noted above this gave a solution containing 94.4% of the concentration of solids in

the source material. Allowance for this difference has been made in Table 1, which shows no significant loss of potency during the freeze-drying and secondary drying processes.

TABLE 1

POTENCIES OF FROZEN (67/138) AND FREEZE-DRIED (67/139) MATERIALS RELATIVE TO 67/86 WHICH HAD BEEN FREEZE-DRIED AND SECONDARILY DRIED [a]

Immunoglobulin	67/138	67/139
IgG	1.016 (0.99–1.04)	0.988 (0.92–1.06)
IgA	1.012 (0.98–1.05)	1.020 (0.99–1.05)
IgM	1.045 (0.97–1.13)	1.032 (0.96–1.11)

[a] Each of the values above represents the weighted mean of 4 assays (3 for IgM) provided by 2 laboratories (K and P). The 95 % confidence limits are shown in parentheses.

An estimate was also made of the stability of 67/86 and 67/95 during storage at −20°C. This was done by placing ampoules of these freeze-dried materials at −70°C, −20°C, +4°C, +20°C, +37°C and +56°C for periods of 182 days and 374 days. The samples were then examined by laboratories C and K using several techniques; in general, there was significant degradation of immunoglobulins at +37°C, greater after 12 months than after 6 months. However, there was no significant change in the material held at +4°C or −20°C when compared with the contents of ampoules held at −70°C. Material held at +56°C had been so changed as to be incompletely soluble and was not further examined. The contents of ampoules held at the other temperatures dissolved completely on the addition of 1 ml of distilled water.

Gel-filtration analyses were carried out on the samples of 67/86 which had been stored at the different temperatures for 374 days. A 4-ml quantity of reconstituted serum was applied to a column of Sephadex G-200 equilibrated with 0.2 M NaCl, 0.05 M tris, pH 8.0. The column was eluted at room temperature with the same buffer, the optical densities of the fractions were measured and their immunoglobulin contents estimated by a single-radial-diffusion technique. Materials stored at temperatures from −70°C to +20°C showed similar elution patterns and no evidence of aggregation of immunoglobulins. Materials stored at +37°C showed aggregation of some IgG and IgA (Fig. 1). There was no evidence of fragmentation of immunoglobulins at any temperature, since material reacting with specific antisera was not detected in any fraction eluted later than the intact molecules of each class. The only evidence for protein fragmentation occurred in serum stored at +37°C. This was shown by an increase in the area of the 4th (low molecular weight) optical-density peak.

Analyses by ultracentrifugation showed no differences between 67/86 stored for 374 days at −70°C and −20°C. Compared with these samples, the material stored at +37°C showed an increased proportion of components sedimenting in the 7S region and a reduced proportion of components sedimenting in the 4.5S region.

Immunoelectrophoretic analyses were carried out on 67/86 that had been stored at the various temperatures for 374 days. The following antisera were used: a horse antiserum to human serum proteins, an antiserum reactive with the Fab and Fc fragments of IgG, and antisera specific for IgG, IgA and IgM. Major changes were seen in the materials stored at +37°C. The IgG precipitin line was elongated and extended further towards the anode and less far towards the cathode than did the IgG line of fresh serum or of material stored at lower temperatures (Fig. 2). A similar change occurred in the IgA line. A less striking change occurred in the IgM line; the precipitin line was shortened and did not extend so far towards the cathode. No spurs were present on any of the immunoglobulin precipitin lines, including the line produced by the antiserum reactive with Fab and Fc; thus there was no evidence of splitting of immunoglobulins at any temperature of storage.

Samples of 67/86 held at −70°C, −20°C, +4°C and +37°C for 374 days were examined by single-radial-diffusion tests in laboratories C, K and P, each using slightly different techniques and different specific antisera. In addition, laboratory P examined 67/95. The results are shown in Table 2. Potencies were calculated from the diameters of the precipitates by methods described below. Material stored at 37°C had a lower potency than materials stored at lower temperatures. Possible causes of this loss of potency include loss of antigenic reactivity and changes related to the aggregation of immunoglobulins which was demonstrated by the gel-filtration analyses as shown above. Since the rate of degradation at +4°C was too low to be measurable after 12 months it can be assumed that the rate of degradation at −20°C would be so low as to be negligible for practical purposes. The precise rate of decay of antigen cannot be estimated until samples held at

TABLE 2

POTENCY[a] OF REFERENCE PREPARATIONS AFTER STORAGE AT DIFFERENT TEMPERATURES, RELATIVE TO MATERIAL STORED AT −20°C: PERIOD OF STORAGE 374 DAYS

Laboratory	Immuno-globulin	Temperature of storage		
		−70°C	+4°C	+37°C
		Preparation 67/86		
C	IgG	1.03 (0.98–1.08)	0.98 (0.94–1.02)	0.86 (0.82–0.90)
K	IgG	1.00 (0.96–1.04)	0.95 (0.91–0.99)	0.75 (0.64–0.89)
P	IgG	—	1.02 (0.95–1.08)	0.74 (0.69–0.79)
C	IgA	0.93 (0.90–0.97)	1.01 (0.96–1.06)	0.96 (0.91–1.01)
K	IgA	1.01 (0.98–1.05)	0.97 (0.94–1.00)	0.84 (0.82–0.87)
P	IgA	—	1.04 (0.97–1.11)	0.69 (0.64–0.71)
C	IgM	1.00 (0.97–1.03)	1.04 (1.00–1.08)	1.02 (0.99–1.06)
K	IgM	1.05 (0.94–1.18)	0.96 (0.86–1.08)	1.02 (0.91–1.15)
P	IgM	—	1.01 (0.90–1.13)	0.74 (0.65–0.84)
		Preparation 67/95		
P	IgG	1.01 (0.94–1.08)	0.92 (0.86–0.98)	0.70 (0.65–0.74)
	IgA	1.08 (1.00–1.16)	0.98 (0.91–1.05)	0.71 (0.66–0.76)
	LgM	0.95 (0.85–1.06)	0.97 (0.87–1.08)	0.67 (0.61–0.79)

[a] 95 % confidence limits in parentheses.

+4°C have been stored long enough to show some degradation.

On the basis of these tests it appears that 67/86 and 67/95 stored at −20°C were sufficiently stable for use as standards for the quantitative analyses of IgG, IgA and IgM, using the single-radial-diffusion method. Although the stability of 67/86 and 67/95 was only estimated by the single-radial-diffusion technique, it is anticipated that these preparations would be sufficiently stable for use in other methods.

THE COLLABORATIVE STUDY OF THE PROPOSED STANDARD

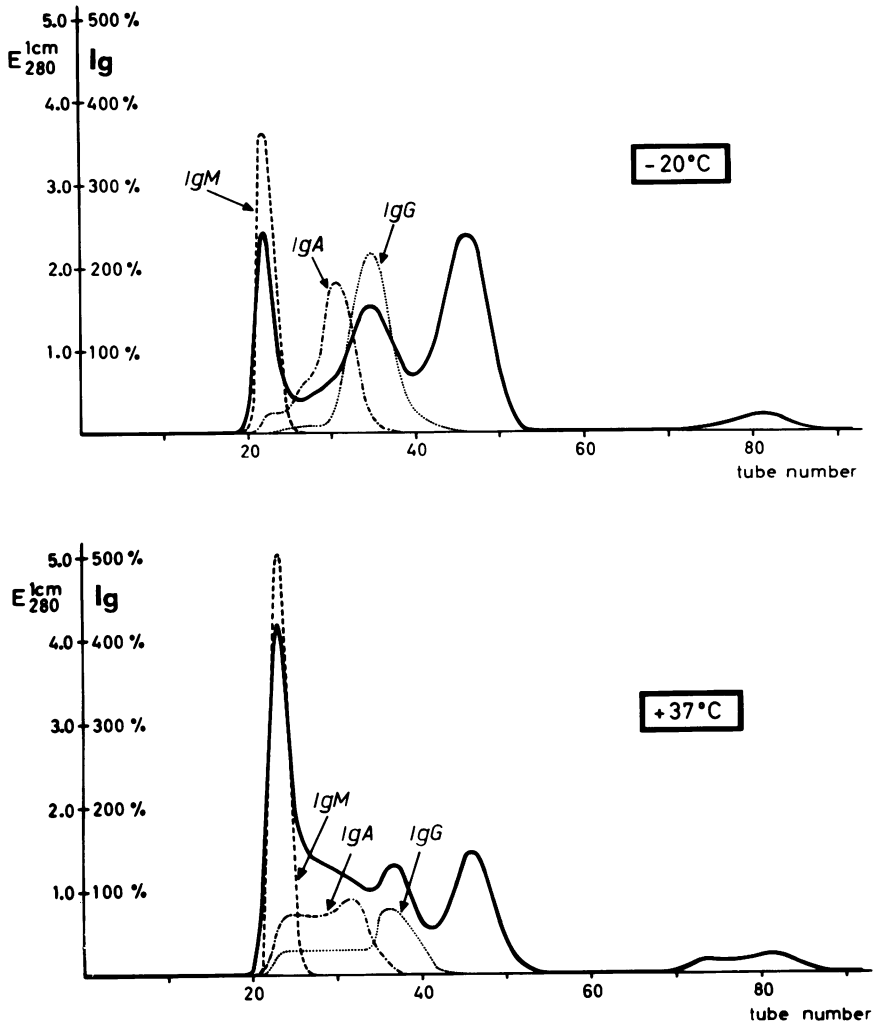
The materials distributed to all participants were the freeze-dried batches 67/86, 67/95 and 67/97, and 6 human sera (frozen) obtained by calcifying citrated plasma that had been obtained from 6 apparently healthy donors. The latter frozen samples were coded 68/160 and 68/162 to 68/166; each ampoule contained 1 ml. In addition, samples of 67/159 were distributed; these consisted of a 1 in 4 dilution of the pool from which 67/86 has been freeze-dried.

The antisera used in different laboratories varied in their characteristics (see Table 3). All participants used a test involving diffusion of antigen through gel. Laboratory N used an Oudin technique (Claman & Merrill, 1964) but all other laboratories used a single-radial-diffusion test. The latter laboratories read the diameter (or 2 orthogonal diameters) of the area of precipitation except for 1 laboratory where the response was expressed in weight of paper. Different laboratories allowed different times for diffusion. The results were read when diffusion was considered to be complete (the precipitates were no longer increasing in size) for IgG in 4 laboratories and for IgA and IgM in 6 laboratories. In the remaining laboratories the results were read at a time when the precipitates were still increasing in size.

Statistical information on the type and the design of the assays preformed in the collaborating laboratories is summarized in Table 4. The number of sera tested simultaneously on the same plate varied

FIG. 1

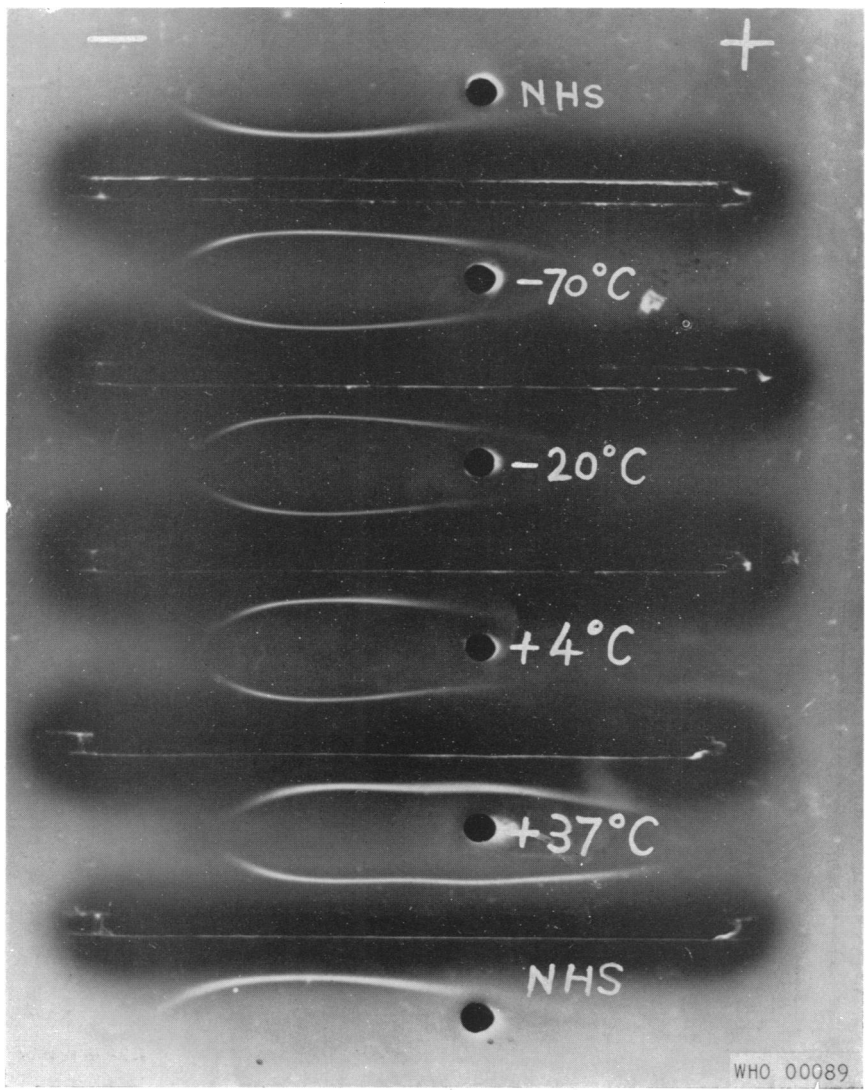
GEL FILTRATION OF 67/86 AFTER STORAGE FOR 374 DAYS AT −20°C AND +37°C [a]



[a] 4 ml of reconstituted serum were applied to a Sephadex G-200 column. Optical densities of eluate fractions are shown by the solid lines, and immunoglobulin contents as a percentage of a arbitrary standard by the broken lines. IgG and IgA show evidence of aggregation after storage at 37°C.

FIG. 2

IMMUNOELECTROPHORESIS OF 67/86 AFTER STORAGE FOR 374 DAYS AT VARIOUS TEMPERATURES [a]



[a] Reconstituted samples and a sample of fresh normal serum were placed in the wells as indicated. After electrophoresis, an antiserum specific to the Fc fragment of IgG was placed in the troughs. Note the elongation of the IgG line towards the anode and its shortening towards the cathode after storage at 37°C. Similar but less marked shortening is also seen after storage at +4°C. NHS is fresh normal human serum.

TABLE 3
SOME CHARACTERISTICS OF THE DIFFERENT ANTISERA USED IN THE COLLABORATIVE ASSAYS [a]

Characteristics	No. of antisera to		
	IgG	IgA	IgM
Species:			
Rabbit	6	4	6
Goat	4	5	3
Sheep	2	2	1
Swine	1	1	1
Horse	0	1	0
Source of immunizing antigen:			
Serum	10	8	8
Colostrum	0	1	0
Milk	0	2	0
Unknown	3	2	3
Type of immunizing antigen:			
Polyclonal	10	6 [b]	3 [b]
Monoclonal	0	5	5
Unknown	3	2	3
Source of antiserum:			
Commercial	3	2	3
Local	10	11	8
Total no. of antisera	13	13	11

[a] No single antiserum was used by more than one laboratory. So far as was known all antisera were absorbed before use and were immunoglobulin class specific when tested by immunoelectrophoresis and other tests. Full details of all the commercial antisera were not available.

[b] Includes 1 antiserum prepared to a mixture of normal and monoclonal proteins.

widely; the number of replicates in each assay was usually 2.[1]

Method of statistical analysis

A small number of assays (or parts of assays) had to be excluded from the statistical analysis because the data were statistically unusable. In most cases the laboratories themselves drew attention to the unsatisfactory results. A total of 292 assays was considered statistically acceptable for inclusion in the present analysis.

The full statistical analysis of the assay results, including the analysis of variance, was carried out at the World Health Organization on an IBM 360/40 computer. As a preliminary step, the average dose–response line for each serum was graphically produced by the computer plotter. The process was repeated with various combinations of different transformations of the dose and response metameters. On the basis of the visual evidence provided by the graphs, a decision was made for each laboratory on the appropriate transformation system to be adopted and the corresponding type of statistical technique to be applied, whether parallel-line or slope-ratio assay (see Table 4).[2] The graphs showed also that in several parallel-line assays the lowest concentrations were responsible for the statistical invalidity of the assay. Such concentrations were excluded from the final analysis.

For each individual assay, the relative potency of the individual sera with respect to the standard 67/86 and its precision (statistical weight) were estimated. The statistical significance of departure from linearity and parallelism [3] of dose–response regression lines was tested by applying the F ratio to the relevant sums of squares of the analysis of variance.

Table 5 shows that 47 out of 292 assays were invalid at the 1% probability level of significance. This is 16.1% of the total number of assays performed. However, a close examination of the situation revealed that the statistical significance of departure from parallelism [3] or from linearity was presumably due to the error variance being smaller than in the other assays which showed no significant departure.

It was also observed that, in general, only 1 or 2 sera were responsible for the formal invalidity of the assays.[4] In several laboratories, almost all the

[1] A set of experiments carried out on one plate was considered to be one assay. When in the analysis of variance the residual error could not be calculated from the between-replicate variation it was taken as the mean square for deviations from linearity.

[2] Laboratory O tested single doses of the various sera against a set of dilutions of the reference serum. The results are reported in Table 6, but they were not included in the statistical analysis, as the standard curve method does not provide information on the validity of the assay and on the precision of the potency estimates, which is statistically comparable with the corresponding information given by the conventional parallel-line or slope-ratio assays.

[3] Tested on the component for intersection in the slope-ratio assays.

[4] In particular, serum 68/159, which was a one-fourth dilution of serum, caused many statistically unsatisfactory results, as some curvature at very low concentrations was often encountered, leading to significant departure from linearity and parallelism and introducing systematic biases in the potency estimates (Finney, 1964).

TABLE 4
BASIC STATISTICAL INFORMATION ON THE ASSAYS PERFORMED IN DIFFERENT LABORATORIES

Laboratory code	Statistical type of assay	Transformation used		No. of assays analysed per antigen				No. of sera tested against the standard per assay	No. of dilutions per serum [a]	No. of replicates per dilution and serum
		Dose	Response	Total	IgG	IgA	IgM			
A	Slope ratio	None	Square	27	9	9	9	2 or 3	3	2
B	Parallel line	Log	Square root	11 [b]	3	4	4	2 to 10	2 to 4	1
C	Slope ratio	None	Square	33	9	9	15	2 or 3	3 or 4	2
D	Slope ratio	None	Square	7	3	3	1	8 or 9	3	2
E	Slope ratio	None	None	16	8	8	—	5 or 6	5	2
F	Slope ratio	None	Square	6	2	2	2	1 to 4	2 to 4	1
I	Slope ratio	None	Square	36	12	12	12	4	3 to 5	1 or 2
J	Slope ratio	None	Square	52	18	18	16	1	3	2
K	Parallel line	Log	Square [c]	53	17	18	18	3	3	2
L	Parallel line	Log	Square	24	8	8	8	2	3 or 5	2
N	Parallel line	Log	None	1	1	—	—	2	3 or 4	3
P	Parallel line	Log	Square [c]	26	9	8	9	3 or 4	3	2

[a] Retained for the analysis.
[b] In addition two assays against NHS (normal human serum) were also performed for each immunoglobulin.
[c] Square root for immunoglobulin IgM.

TABLE 5
DISTRIBUTION OF FORMALLY INVALID [a] ASSAYS, ACCORDING TO LABORATORY AND IMMUNOGLOBULIN

Laboratory code	No. of assays					
	Immunoglobulin G		Immunoglobulin A		Immunoglobulin M	
	Total	Invalid	Total	Invalid	Total	Invalid
A	9	2	9	—	9	4
B	3	—	4	—	4	—
C	9	1	9	—	15	—
D	3	—	3	3	1	1
E	8	3	8	—	—	—
F	2	—	2	—	2	—
I	12	5	12	—	12	1
J	18	1	18	2	16	1
K	17	—	18	6	18	9
L	8	—	8	—	8	—
N	1	1	—	—	—	—
P	9	3	8	—	9	4
Total	99	16	99	11	94	20

[a] Statistically invalid at 1 % probability level of significance. For the 5 % probability level, the total number of invalid assays by immunoglobulin should be increased by 14, 13 and 11, respectively.

individual sera were tested in each assay. As a consequence, unsatisfactory results of a single serum could lead to the apparent invalidity of the majority of the assays. It was therefore considered reasonable, before rejecting any complete set of assay results, to study the degree of heterogeneity between the relative potency estimates of these sera as derived from the results of different assays carried out in the same laboratory.

The homogeneity of the relative potency estimates obtained within each laboratory was studied separately by the χ^2 test (Humphrey, Mussett & Perry, 1953) for each immunoglobulin and each serum.

For the results which did not show heterogeneity, the average logarithmic relative potency was computed by weighting each logarithmic relative potency value with the reciprocal of its variance derived from the internal evidence of the assay. The variance of the average relative potency was then simply the reciprocal of the total of the individual weights.

For the laboratories whose results were found to be heterogeneous, the between-assay variance was computed and the weight of each logarithmic relative potency value was redetermined by taking the reciprocal of the total variance, i.e., including both within- and between-assay variances according to the method described by Bliss (1952). The new

TABLE 6

RELATIVE POTENCY OF EACH SERUM WITH RESPECT TO THE STANDARD SERUM 67/86: WEIGHTED [a] MEAN VALUES FOR EACH LABORATORY

Laboratory code [b]	Immunoglobulin G				Immunoglobulin A				Immunoglobulin M			
	Number of potency estimates	Within-laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate	Number of potency estimates	Within-laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate	Number of potency estimates	Within-laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate
Serum: 67/95												
A	2	0.121	1.002	6 504.5	2	0.026	1.004	3 234.8	2	0.017	1.017	2 332.9
B	1	—	0.959	3 339.5	1	—	0.913	248.2	1	—	0.979	1 602.1
C	2	2.234	0.844	1 245.3	2	0.094	1.036	3 573.6	2	0.072	0.017	6 271.4
D (1)	—	—	—	—	2	0.694	1.012	2 259.2	—	—	—	—
D (2)	—	—	—	—	1	—	0.995	922.6	—	—	—	—
D	3	0.001	1.072	1 262.1	3	0.736	1.009	1 813.7	1	—	1.005	816.2
E (1)	4	0.476	1.000	19 444.1	4	0.384	1.014	8 885.5	—	—	—	—
E (2)	4	1.887	1.023	9 365.9	4	1.263	1.003	7 151.1	—	—	—	—
E	8	4.830	1.008	14 405.0	8	2.012	1.009	8 018.3	—	—	—	—
F	1	—	1.001	1 356.3	1	—	0.984	1 060.6	1	—	1.065	190.0
I	4	3.932	1.027	1 693.2	4	0.452	0.993	2 304.6	4	0.849	0.967	1 340.0
J	2	0.105	0.975	1 287.7	2	0.358	1.019	2 288.1	2	0.917	1.072	1 742.5
K (1)	2	0.074	1.046	4 216.5	3	0.698	0.984	5 459.3	3	3.225	1.028	4 948.3
K (2)	3	0.877	1.038	3 754.8	3	0.979	1.023	3 621.0	3	9.059*	0.995	1 239.7
K	5	1.017	1.041	3 939.5	6	3.511	0.999	4 540.1	6	12.349*	1.016	1 892.1
L	1	—	1.284	454.3	1	—	1.346	92.7	1	—	1.082	50.1
N	3	1.158	1.075	4 125.6	—	—	—	—	—	—	—	—
P	9	11.866	0.972	4 492.5	8	5.985	1.008	1 655.3	9	8.089	1.030	2 117.9
O	1	—	0.962	—	1	—	0.970	—	1	—	1.234	—
Serum: 67/97												
A	2	0.053	1.059	6 130.3	2	0.365	1.010	2 904.9	2	0.503	0.998	2 376.0
B	1	—	1.169	3 172.3	1	—	0.968	244.9	1	—	1.140	1 563.2
C	2	2.601	0.915	1 217.8	2	0.219	1.035	3 576.3	2	0.136	1.008	1 589.2
D (1)	—	—	—	—	2	0.891	0.975	2 344.8	—	—	—	—
D (2)	—	—	—	—	1	—	0.995	922.3	—	—	—	—
D	3	0.059	1.050	1 291.0	3	0.955	0.978	1 870.6	1	—	0.959	854.9
E (1)	4	0.616	0.996	19 526.3	4	3.461	1.010	8 919.4	—	—	—	—
E (2)	4	4.747	1.018	9 414.7	4	0.246	1.006	7 134.1	—	—	—	—
E	8	7.563	1.003	14 470.5	8	3.758	1.008	8 026.7	—	—	—	—
F	1	—	1.006	1 349.0	1	—	0.980	1 064.2	1	—	0.934	258.8
I	4	3.408	1.031	1 689.2	4	4.505	1.014	2 266.5	4	1.230	0.975	1 333.2
J	2	0.050	0.976	2 534.3	2	0.134	1.060	646.4	2	0.100	1.122	645.7
K (1)	2	0.004	1.021	4 219.2	3	1.486	0.988	5 459.0	3	0.124	1.040	4 948.9
K (2)	3	0.275	1.022	3 756.4	3	0.965	1.004	3 621.7	3	1.314	1.001	5 798.0
K	5	0.280	1.022	3 941.6	6	2.796	0.994	4 540.3	6	3.637	1.019	5 373.4
L	1	—	1.127	248.4	1	—	1.172	803.5	1	—	1.161	248.6
N	3	0.177	1.034	3 397.1	—	—	—	—	—	—	—	—
P	3	16 498 **	1.012	375.6	3	27 526 **	1 122	146.4	3	2.528	0.988	1 289.9
O	1	—	1.051	—	1	—	1.000	—	1	—	1.109	—
Serum: 68/159												
A	3	25 710 **	0.264	917.3	3	12.348 **	0.247	1 504.1	3	10.257 **	0.247	704.6
B	3	3.685	0.239	1 777.3	3	3.277	0.267	478.4	1	—	0.238	593.3
C	1	—	0.255	5 492.3	1	—	0.265	5 441.3	2	0.634	0.238	2 919.5
D (1)	—	—	—	—	1	—	0.068	5 344.2	—	—	—	—
D (2)	—	—	—	—	—	—	—	—	—	—	—	—
D	2	0.815	0.158	2 129.4	1	—	0.068	5 344.2	1	—	0.157	1 289.0
E (1)	2	0.325	0.239	24 316.5	2	5.593	0.237	2 026.5	—	—	—	—
E (2)	2	1.361	0.259	14 928.4	2	0.022	0.262	13 376.4	—	—	—	—
E	4	22.585 **	0.249	2 203.7	4	29.051 **	0.247	3 470.1	—	—	—	—
F	1	—	0.275	5 553.6	1	—	0.294	5 578.0	1	—	0.549	232.1
I	4	51.987 **	0.180	152.3	4	9.917 *	0.271	927.2	4	1.043	0.277	1 917.2
J	2	0.048	0.250	9 013.8	2	5.837 *	0.265	4 647.9	—	—	—	—
K (1)	3	0.175	0.268	2 040.7	3	1.780	0.298	3 802.5	3	0.264	0.325	945.8
K (2)	3	0.191	0.259	3 348.9	3	0.240	0.270	4 005.6	3	3.521	0.315	1 377.9
K	6	1.228	0.262	2 694.8	6	13.000 *	0.283	3 192.0	6	4.060	0.319	1 161.9
L	1	—	0.329	125.5	1	—	0.261	288.4	1	—	0.381	17.9
N	—	—	—	—	—	—	—	—	—	—	—	—
P	3	9.976 **	0.251	461.8	3	23.612 **	0.260	106.9	2	1.528	0.273	927.3
O	1	—	0.232	—	1	—	0.228	—	1	—	0.250	—

TABLE 6 (*continued*)

Laboratory code [b]	Immunoglobulin G				Immunoglobulin A				Immunoglobulin M			
	Number of potency estimates	Within laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate	Number of potency estimates	Within laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate	Number of potency estimates	Within laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate
Serum: 68/160												
A	3	0.287	0.979	9 107.1	3	0.397	0.723	9 118.5	3	0.103	1.142	2 650.7
B	3	0.478	1.068	1 768.5	3	2.757	0.794	552.8	3	4.403	1.031	1 109.9
C	3	2.263	1.056	2 610.3	3	0.919	0.750	5 736.0	3	0.046	1.084	6 434.7
D (1)	—	—	—	—	2	0.146	0.713	2 976.2	—	—	—	—
D (2)	—	—	—	—	1	—	0.766	1 141.5	—	—	—	—
D	3	0.164	1.058	1 280.6	3	1.084	0.721	2 364.6	1	—	1.157	699.0
E (1)	2	0.128	0.905	22 770.2	2	1.224	0.773	11 701.2	—	—	—	—
E (2)	2	0.007	0.928	8 738.0	2	0.153	0.718	5 708.0	—	—	—	—
E	4	1.628	0.911	15 754.1	4	9.457 *	0.749	3 757.9	—	—	—	—
F	—	—	—	—	1	—	0.777	2 515.1	1	—	0.818	105.3
I	4	1.062	1.070	2 032.5	4	3.588	0.771	2 497.0	4	0.067	1.192	1 483.7
J	2	0.193	0.981	329.4	2	0.058	0.803	604.5	2	0.095	1.220	902.3
K (1)	3	0.234	1.070	3 417.1	3	0.475	0.736	5 764.5	3	2.856	1.286	1 377.4
K (2)	3	1.561	1.072	5 686.6	3	3.605	0.723	6 393.6	3	3.420	1.319	2 017.6
K	6	1.803	1.071	4 551.8	6	4.638	0.729	6 079.0	6	6.564	1.305	1 697.5
L	—	—	—	—	—	—	—	—	—	—	—	—
N	—	—	—	—	—	—	—	—	—	—	—	—
P	3	38.634 **	1.044	164.5	3	7.003	0.847	289.4	3	16.210 **	1.230	416.4
O	1	—	0.992	—	1	—	0.680	—	1	—	1.172	—
Serum: 68/162												
A	3	0.305	0.964	9 146.9	3	0.928	0.901	8 172.5	3	10.649	0.590	606.3
B	3	0.180	0.985	1 805.3	3	1.724	1.090	521.9	3	5.362	0.527	1 163.7
C	3	3.060	0.986	2 820.0	3	1.582	0.936	4 949.5	3	2.759	0.540	25 522.7
D (1)	—	—	—	—	2	0.999	0.948	2 405.7	—	—	—	—
D (2)	—	—	—	—	1	—	0.954	960.5	—	—	—	—
D	3	0.076	1.007	1 348.3	3	1.004	0.949	1 924.0	1	—	0.640	1 128.
E (1)	2	0.321	0.865	23 612.8	2	0.110	0.942	10 101.0	—	—	—	—
E (2)	2	0.031	0.913	8 862.6	2	0.035	0.910	4 874.8	—	—	—	—
E	4	7.457	0.878	16 237.7	4	1.602	0.932	7 487.9	—	—	—	—
F	—	—	—	—	—	—	—	—	—	—	—	—
I	4	8.330 *	0.866	1 256.8	4	0.959	0.971	2 250.9	4	1.892	0.629	2 014.9
J	2	0.011	0.918	781.1	2	0.370	0.991	579.3	2	0.004	0.685	917.4
K (1)	3	2.689	0.999	3 421.4	3	0.617	1.006	5 976.3	3	0.939	0.601	1 283.2
K (2)	3	0.945	1.030	5 695.8	3	0.095	1.011	6 656.1	3	0.655	0.673	1 960.9
K	6	4.727	1.018	4 558.6	6	0.753	1.008	6 316.2	6	7.227	0.644	1 622.1
L	1	—	1.153	338.7	1	—	1.478	187.3	1	—	0.900	137.9
N	—	—	—	—	—	—	—	—	—	—	—	—
P	3	39.108 **	0.945	171.8	3	11.875 **	0.996	164.1	3	12.177 **	0.640	508.3
O	1	—	0.957	—	1	—	0.832	—	1	—	0.500	—
Serum: 68/163												
A	3	1.491	1.246	2 902.2	3	0.199	1.384	2 182.7	3	1.160	1.936	1 274.0
B	3	4.817	1.387	1 632.4	3	0.689	1.613	314.2	2	0.010	1.979	7 867.7
C	3	24.656 **	1.085	228.8	3	0.103	1.390	2 137.9	4	0.746	1.824	825.8
D (1)	—	—	—	—	2	0.010	1.269	1 736.6	—	—	—	—
D (2)	—	—	—	—	1	—	1.204	744.6	—	—	—	—
D	3	0.265	1.313	983.6	3	0.328	1.257	1 405.9	1	—	1.856	349.0
E (1)	2	0.111	1.099	18 757.6	2	0.364	1.356	6 535.0	—	—	—	—
E (2)	2	0.673	1.166	6 764.6	2	0.143	1.333	3 137.2	—	—	—	—
E	4	7.215	1.117	12 761.1	4	0.736	1.349	4 836.1	—	—	—	—
F	—	—	—	—	—	—	—	—	—	—	—	—
I	4	1.541	1.161	715.4	4	1.356	1.415	967.1	4	0.659	1.972	633.7
J	2	1.374	1.223	1 600.7	2	0.317	1.389	709.7	2	0.011	2.062	1 016.1
K (1)	3	0.971	1.333	3 651.4	3	3.443	1.445	5 092.3	3	0.144	2.414	1 284.4
K (2)	3	0.294	1.307	2 982.8	3	4.638	1.403	4 382.4	3	0.868	1.990	4 795.1
K	6	1.626	1.321	3 317.1	6	9.258	1.426	4 737.3	6	22.418 **	2.152	434.1
L	1	—	1.234	241.1	1	—	1.398	262.4	1	—	2.272	143.8
N	—	—	—	—	—	—	—	—	—	—	—	—
P	3	39.214 **	1.218	160.8	3	10.413 **	1.502	183.6	3	9.996 **	2.131	544.7
O	1	—	1.115	—	1	—	1.294	—	1	—	3.063	—

TABLE 6 (*concluded*)

Laboratory code [b]	Immunoglobulin G				Immunoglobulin A				Immunoglobulin M			
	Number of potency estimates	Within laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate	Number of potency estimates	Within laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate	Number of potency estimates	Within laboratory χ^2 heterogeneity test [c]	Weighted mean relative potency	Average weight per potency estimate
Serum: 68/164												
A	3	0.868	0.955	4 001.3	3	2.950	0.890	3 777.5	3	2.026	1.073	3 585.3
B	3	8.860 *	1.034	238.1	3	1.184	0.893	371.5	3	2.171	1.000	7 028.4
C	3	28.222 **	0.840	223.5	3	1.072	0.862	4 354.4	4	0.897	0.996	5 493.4
D (1)	—	—	—	—	2	0.152	0.876	2 580.8	—	—	—	—
D (2)	—	—	—	—	1	—	0.859	1 051.3	—	—	—	—
D	3	2.999	1.009	1 339.9	3	0.214	0.874	2 070.9	1	—	0.988	830.9
E (1)	2	1.046	0.864	20 599.0	2	5.847 *	0.904	1 586.2	—	—	—	—
E (2)	2	0.470	0.913	12 007.9	2	0.034	0.836	11 491.4	—	—	—	—
E	4	10.170 *	0.887	4 233.4	4	17.739 **	0.873	4 964.0	—	—	—	—
F	—	—	—	—	—	—	—	—	—	—	—	—
I	4	1.620	0.982	815.0	4	0.533	0.913	1 412.3	4	2.655	1.029	1 415.5
J	2	0.060	0.975	1 577.3	2	0.007	0.911	2 229.3	2	0.020	1.138	1 121.1
K (1)	3	0.703	1.011	3 768.9	3	5.637	0.884	5 328.8	3	1.075	1.113	1 665.5
K (2)	3	0.768	1.001	3 065.7	3	1.997	0.878	4 550.4	3	9.846 **	1.045	1 593.7
K	6	1.569	1.006	3 417.3	6	7.697	0.881	4 939.6	6	14.429 *	1.058	3 049.6
L	1	—	0.763	191.8	1	—	1.009	204.4	1	—	1.266	215.7
N	—	—	—	—	—	—	—	—	—	—	—	—
P	3	12.559 **	0.914	209.7	2	14.108 **	0.919	120.4	3	0.127	0.968	1 763.3
O	1	—	0.962	—	1	—	0.787	—	1	—	1.219	—
Serum: 68/165												
A	3	1.146	0.851	4 373.8	3	1.746	1.517	1 842.2	3	9.212 **	0.821	987.3
B	1	—	1.059	3 258.7	3	2.532	1.760	303.4	2	4.862 *	0.938	255.1
C	3	27.552 **	0.762	228.8	3	0.118	1.491	1 949.5	3	3.452	0.731	8 607.6
D (1)	—	—	—	—	2	0.019	1.381	1 546.3	—	—	—	—
D (2)	—	—	—	—	1	—	1.317	662.0	—	—	—	—
D	3	0.045	0.900	1 497.7	3	0.253	1.370	1 251.5	1	—	0.818	977.0
E (1)	1	—	0.756	29 126.9	2	0.597	1.494	5 629.9	—	—	—	—
E (2)	2	0.057	0.742	10 222.3	2	0.086	1.418	2 862.8	—	—	—	—
E	3	0.881	0.750	16 523.8	4	2.644	1.468	4 246.3	—	—	—	—
F	—	—	—	—	—	—	—	—	—	—	—	—
I	4	1.852	0.892	821.2	4	0.271	1.520	882.0	4	2.860	0.804	1 614.8
J	2	0.001	0.792	757.4	2	0.008	1.556	1 410.1	2	0.429	0.903	500.0
K (1)	3	0.885	0.908	3 755.4	3	10.535 **	1.668	730.9	3	1.603	0.944	1 670.4
K (2)	3	6.433 *	0.881	820.7	3	2.813	1.592	4 223.2	3	1.837	0.871	5 638.2
K	6	7.656	0.901	3 400.7	6	14.866 *	1.626	1 263.8	6	8.117	0.887	3 654.3
L	1	—	0.801	170.9	1	—	1.350	424.7	1	—	0.809	276.4
N	—	—	—	—	—	—	—	—	—	—	—	—
P	3	11.415 **	0.791	243.9	2	18.595 **	1.580	84.3	3	2.407	0.800	1 735.8
O	1	—	0.916	—	1	—	1.401	—	1	—	0.828	—
Serum: 68/166												
A	3	0.064	1.254	4 217.7	3	2.709	0.892	4 316.5	3	0.057	1.402	1 514.1
B	3	0.026	1.420	1 618.9	3	2.559	1.012	361.2	2	9.571 **	1.358	129.6
C	3	4.281	1.168	1 632.4	3	0.536	0.881	3 063.3	5	1.330	1.311	3 554.5
D (1)	—	—	—	—	2	0.011	0.887	2 554.7	—	—	—	—
D (2)	—	—	—	—	1	—	0.971	944.6	—	—	—	—
D	3	0.020	1.294	1 003.7	3	1.239	0.900	2 018.0	1	—	1.391	549.5
E (1)	3	400.809 **	0.976	99.1	2	5.201 *	0.925	1 748.2	—	—	—	—
E (2)	2	53.356 **	1.074	167.9	2	0.658	0.911	10 758.5	—	—	—	—
E	5	471.981 **	1.014	141.8	4	6.339	0.918	9 927.7	—	—	—	—
F	1	—	1.326	902.9	1	—	0.832	2 043.6	1	—	1.245	130.5
I	4	1.906	1.203	1 483.6	4	4.677	0.926	2 399.1	4	0.405	1.428	932.4
J	2	0.518	1.229	596.1	2	0.103	0.974	2 622.5	2	3.404	1.637	195.8
K (1)	2	2.019	1.340	4 081.4	3	1.045	0.982	5 458.9	3	4.213	1.477	4 679.4
K (2)	3	0.143	1.285	3 667.0	3	0.078	0.979	3 621.7	3	4.227	1.484	5 463.5
K	5	3.695	1.308	3 832.7	6	1.131	0.981	4 540.3	6	8.476	1.481	5 071.5
L	1	—	1.249	256.1	1	—	1.016	528.1	1	—	1.703	108.2
N	—	—	—	—	—	—	—	—	—	—	—	—
P	3	12.473 **	1.191	258.7	2	29.532 **	0.971	57.1	3	3.210	1.404	1 701.1
O	1	—	1.135	—	1	—	0.863	—	1	—	1.797	—

[a] With weights adjusted when required for heterogeneity between individual laboratory estimates.

[b] For Laboratories D, E and K, results of assays performed with different anti-sera were consolidated separately and entered on lines identified by the suffixes (1) and (2). Data from Laboratory O did not permit the calculation of weighted mean potencies; the results from this laboratory are therefore not included in subsequent tables.

[c] * Significant heterogeneity at 5 % probability level.
** Significant heterogeneity at 1 % probability level.

weights were applied to the individual logarithmic relative potencies in the computation of the average logarithmic relative potency, and its variance was taken as the reciprocal of the sum of the new individual weights.

RESULTS

The results, consolidated by laboratory, are summarized in Table 6. The number of replicates per laboratory was generally between 1 and 3; the χ^2 test showed that within any one laboratory homogeneity was good in the majority of cases, especially for batches 67/95 and 67/97. Heterogeneity between individual estimates of a laboratory was more frequently encountered for 68/159, it occurred in 5 of 36 laboratory means. Significant within-laboratory heterogeneity was frequently observed in laboratory P.

Table 7 shows the number of mean potency estimates that were obtained for each immunoglobulin and each material and the number of cases in which these means were based on heterogeneous individual laboratory potencies. On average, only 13% of the mean potency estimates were affected by within-laboratory heterogeneity, i.e., 42 of 326 values. This proportion was 16% for IgG, 12% for IgA and 11% for IgM.

The over-all combined weighted relative potency was calculated from the various laboratory mean potencies for each immunoglobulin in each sample. The method of estimation was the same as for the calculation of the mean within-laboratory potency. When the between-laboratory heterogeneity was significant at the 5% probability level, new weights were computed on the basis of both the within- and the between-laboratory variances. The final mean relative potencies and their 95% confidence limits are shown in Table 8. Highly significant between-laboratory heterogeneity was the rule, with the notable exception of values for IgA and IgM in samples 67/95 and 67/97, which were homogeneous between laboratories. It should be noted that these materials could be expected to be qualitatively the same as 67/86, since they were derived directly from the same source. Similar between-laboratory homogeneity was not observed for IgG in these 2 samples. This was due to the increased precision of IgG estimates, as proved by the fact that the unweighted between-laboratory variance was not larger for IgG than for IgA.

If Tables 6 and 8 are compared it can be seen that the individual potency estimates of a given laboratory were, in general, not uniformly distributed about the corresponding over-all mean value. As shown in

TABLE 7

TOTAL NUMBER OF LABORATORY MEAN POTENCY ESTIMATES AND THE NUMBER OF ESTIMATES AFFECTED BY WITHIN-LABORATORY HETEROGENEITY

Serum	Independent laboratory mean potency estimates					
	Immunoglobulin G		Immunoglobulin A		Immunoblogulin M	
	Total no.	No. heterogeneous [a]	Total no.	No. heterogeneous [a]	Total no.	No. heterogeneous [a]
67/95	14	0	14	0	11	1
67/97	14	1	14	1	11	0
68/159	13	3	13	5	10	1
68/160	11	1	13	0	10	1
68/162	12	2	13	1	10	2
68/163	12	2	13	1	10	1
68/164	12	3	13	2	10	1
68/165	12	3	13	2	10	2
68/166	13	3	14	2	11	1
All sera	113	18	120	14	93	10

[a] At 5% probability level of statistical significance.

TABLE 8

MEAN WEIGHTED [a] RELATIVE POTENCY OF EACH SERUM WITH RESPECT TO THE STANDARD SERUM 67/86 AND THE CORRESPONDING 95 % CONFIDENCE INTERVAL

Serum	Number of potency estimates	Between laboratory x^2 heterogeneity test [b]	Weighted mean relative potency	Total weight	Average weight per potency estimate	95 % confidence interval for the relative potency
			Immunoglobulin G			
67/95	14	51.706 **	1.011	9 936	709.7	0.966–1.058
67/97	14	30.254 **	1.023	49 648	3 546.3	1.003–1.044
68/159	13	223.550 **	0.242	2 127	163.6	0.220–0.267
68/160	11	137.234 **	1.013	22 598	2 054.3	0.983–1.044
68/162	12	119.941 **	0.956	13 284	1 107.0	0.919–0.994
68/163	12	119.167 **	1.234	20 467	1 705.6	1.195–1.273
68/164	12	80.885 **	0.952	11 565	963.8	0.912–0.992
68/165	12	152.647 **	0.847	7 860	655.0	0.805–0.891
68/166	13	33.290 **	1.259	10 834	833.4	1.206–1.315
			Immunoglobulin A			
67/95	14	6.192	1.007	138 883	9 920.2	0.995–1.020
67/97	14	8.638	1.007	122 941	8 781.5	0.994–1.020
68/159	13	>300.000 **	0.239	472	36.3	0.194–0.294
68/160	13	28.040 **	0.746	42 215	3 247.3	0.730–0.763
68/162	13	55.481 **	0.978	4 800	369.2	0.917–1.044
68/163	13	21.315 *	1.384	26 973	2 074.8	1.346–1.422
68/164	13	15.192	0.873	98 068	7 543.7	0.860–0.885
68/165	13	27.966 **	1.511	19 613	1 508.7	1.463–1.560
68/166	14	37.287 **	0.929	99 059	7 075.7	0.916–0.942
			Immunoglobulin M			
67/95	11	6.466	1.018	66 337	6 030.6	1.000–1.036
67/97	11	12.929	1.015	53 590	4 871.9	0.995–1.035
68/159	10	154.172 **	0.275	612	61.2	0.229–0.329
68/160	10	57.606 **	1.170	3 516	351.6	1.084–1.263
68/162	10	113.752 **	0.619	2 671	267.1	0.567–0.675
68/163	10	34.530 **	2.020	18 059	1 805.9	1.954–2.089
68/164	10	26.839 **	1.039	12 088	1 208.8	0.998–1.083
68/165	10	95.665 **	0.829	13 726	1 372.6	0.798–0.862
68/166	11	34.279 **	1.415	63 015	5 728.7	1.390–1.441

[a] With weights adjusted for between-laboratory heterogeneity when required.

[b] * Significant heterogeneity at 5 % probability level. ** Significant heterogeneity at 1 % probability level.

Table 9 there was a frequent tendency for a particular laboratory to provide estimates which were higher or lower than the general mean. This explains the high between-laboratory heterogeneity seen in Table 8.

The heterogeneity of results between laboratories implied that some aspect of the procedure adopted differed between laboratories. One possible reason for this was the use of different antisera. Some evidence was available on this question, since laboratories D, E and K made estimates of relative potencies using 2 different antisera for each immunoglobulin. An additional study was also carried out by laboratory P, which measured the potency of 6 different samples of fresh human sera relative to 67/95, using 2 different antisera to IgG and IgA and 3 different antisera to IgM. Table 10 shows the frequency with which heterogeneity of potencies was observed in the results obtained by the use of different antisera. The antisera used in these studies differed in various ways, including the species of animal immunized, the nature of the immunogen (monoclonal or polyclonal) and the materials used for absorption. The available data did not permit the conclusion that any one of these factors was related to the heterogeneity of potency estimates but it was noteworthy that when 3 antisera to IgM prepared against 3 different M-macroglobulins of Waldenström were used, estimates of the potencies of 5 out of 6 serum samples showed heterogeneity. These results suggest that the use of different antisera may be a factor responsible for between-laboratory heterogeneity. However, since the differences of potency estimates between laboratories were much larger than the differences of potency estimates between antisera in the same laboratory (Table 8), it appeared unlikely that the use of different antisera was a major factor responsible for between-laboratory heterogeneity.

The homogeneity of results for IgA and IgM in 67/95 and 67/97, both of which were derived from the same pool as 67/86, implied, at least in respect of

TABLE 9

NUMBER OF LABORATORY MEAN POTENCY ESTIMATES LOWER (<) AND HIGHER (>) THAN THE OVER-ALL MEAN POTENCY

Laboratory code	Immunoglobulin G		Immunoglobulin A		Immunoglobulin M		All immunoglobulins		Total no. of laboratory means
	<	>	<	>	<	>	<	>	
A	3	6	4	5	8	1	15	12	27
B	2	7	2	7	7	2	11	16	27
C	6	3	4	5	8	1	18	9	27
D (1)	—	—	7	2	—	—	7	2	9
D (2)	—	—	6	2	—	—	6	2	8
D	1	8	—	—	8	1	9	9	18
E (1)	9	—	5	4	—	—	14	4	18
E (2)	7	2	8	1	—	—	15	3	18
F	2	2	3	3	3	2	8	7	15
I	4	5	3	6	5	4	12	15	27
J	7	2	—	8	—	8	7	18	25
K (1)	1	8	3	6	1	8	5	22	27
K (2)	1	8	2	7	3	6	6	21	27
L	3	5	1	7	—	8	4	20	24
N	—	2	—	—	—	—	—	2	2
P	7	2	1	8	5	4	13	14	27
All laboratories	53	60	49	71	48	45	150	176	326

TABLE 10

NUMBER OF POTENCY ESTIMATES FOR IMMUNOGLOBULINS SHOWING HETEROGENEITY [a] WHEN 2 OR MORE ANTISERA WERE USED WITHIN THE SAME LABORATORY

Laboratory code	IgG	IgA	IgM
D	—	0 of 6	—
E	2 of 5	2 of 6	—
K	0 of 6	0 of 6	1 of 6
P	2 of 6	2 of 6	5 of 6 [b]

[a] Potencies were considered heterogeneous if they differed significantly at the 5 % probability level. Results of laboratories D, E and K taken from Table 6.

[b] Heterogeneity between the potencies obtained using 3 different antisera. In all other results the potencies obtained using 2 different antisera were compared.

these proteins, that between-laboratory heterogeneity did not arise from some trivial cause, such as imprecision in reconstituting the dried powder or in the details of statistical handling. This heterogeneity appeared to be related to the differences between the characteristics of immunoglobulins in 67/86 and its related materials, and the characteristics of immunoglobulins in the other serum samples. The results do not permit a further evaluation of the nature of these differences. Immunoglobulins are renowned for their heterogeneity; features which may affect the single-radial-diffusion technique include antigenic variability and variability of degree of polymerization. The heterogeneity of immunoglobulins in serum therefore sets a limit to the accuracy with which measurements can be made. This limitation applies to the proposed standard (67/86) as well as to other serum samples.

The material 67/159 was included in these studies as a preparation of known potency relative to the proposed standard. As noted above 67/159 was prepared as a 1 in 4 dilution of the pool from which 67/86 was also prepared. Allowing for the effect of reconstitution of freeze-dried 67/86 (see above) the relative potency of 67/159 would be $0.25 \times 100/94.5 = 0.265$. The weighted mean relative potency estimates from all laboratories (Table 8) did not significantly differ from this value. However, the weighted mean relative potencies from individual laboratories often departed considerably from the expected value and within-laboratory heterogeneity was frequently observed. These unsatisfactory results may be attributable to the relatively low immunoglobulin content of 67/159; values of ring diameters for this material were at the lower end of the dose–response curve for 67/86, and the precision was therefore less.

Comparisons of materials 67/95 and 67/97, with the proposed standard 67/86

The materials 67/95 and 67/97 were from the same source as 67/86, and were processed in similar ways. They were compared with 67/86 by quantitative immunochemical analysis and by weighing the ampoule contents. The relative mean weighted potencies from all laboratories were close to 1.00 for all immunoglobulins—they differed significantly only for IgG in 67/97 (Table 8). The dry weight of the contents of 30 ampoules of 67/86 was estimated by opening each ampoule in a dry box, weighing in a dry atmosphere, then washing out the contents of the ampoules, drying and re-weighing. The mean weight estimated in the Division of Biological Standards in this way was 81.47 mg and the range was 80.55 mg to 82.35 mg. Estimates of the dry weights of contents were also made by 9 of the laboratories taking part either in the comparative assay reported here or in the collaborative assay to be reported in a later paper. The means and ranges were as follows:

Material	*Weight per ampoule (mg)*	
	Mean	*Range*
67/86 (4 estimates)	81.2	(79.5-84.3)
67/95 (9 estimates)	84.6	(81.5-86.2)
67/97 (13 estimates)	83.7	(77.0-85.7)

Thus comparison both by immunochemical tests and by weighing showed that 67/95 and 67/97 were similar to 67/86 as regards the amount of activity per ampoule and per milligram. Because the immunochemical tests provided more direct estimates of the relative potencies of these preparations and because of the possibility that variation of weight of ampoule contents might be due to different amounts of retained water, the values finally accepted for the relative potencies of 67/95 and 67/97 have been based on the results of the immunochemical analyses alone. The relative potencies are, therefore, those given for the values of weighted mean relative potencies in Table 8.

DISCUSSION

Quantitative immunochemical tests for the measurement of serum immunoglobulins have been widely used for several years. There are, however, many variables in these tests; these may include the

heterogeneity of immunoglobulins, the heterogeneity of antisera and the sensitivity of tests to diffusion constant and to molecular size of antigens. It has become generally recognized that the comparison of estimations of immunoglobulins made in different laboratories is unsatisfactory (Robbins, 1968). Since measurements of immunoglobulin concentrations of serum and other body fluids are important for clinical and research purposes, this attempt has been made to provide a biological standard for human immunoglobulins in the hope of improving the reproducibility of measurements between laboratories.

When 13 laboratories used the material 67/86 as a common standard for the measurement of immunoglobulins in 6 samples of serum a rather narrow range of relative potencies was obtained. Moreover, there was evidence that those potency estimates that differed most from the mean were usually those in which technical aspects were least satisfactory since in general their values carried the least statistical weight (Table 6). This relative uniformity of results obtained in many laboratories by the use of 67/86 is in contrast with the results obtained by present methods of immunoglobulin estimation. Many different standards for immunoglobulins are in current use. These usually consist of human serum samples calibrated for immunoglobulin content by immunochemical comparisons with isolated, purified immunoglobulins at known concentration. It appears that this calibration may introduce important variability since when such a calibration was carried out on 67/86 as a collaborative assay by specialist laboratories, a wide range of values for the weight contents of immunoglobulins was obtained (to be reported in a later paper). The variability of estimates due to calibration is eliminated if all results are expressed relative to a common standard. The results of the collaborative assay by 13 laboratories, as well as the demonstrated stability of immunoglobulins (Table 2) indicate that 67/86 is suitable for use as such a standard. The results also show that the techniques employed, chiefly based on the single-radial-diffusion method, are appropriate for immunoglobulin estimations for use in clinical medicine.

This study also demonstrates a limitation to the reproducibility of immunoglobulin estimations that is probably related to the heterogeneity of immunoglobulins and of anti-immunoglobulin antisera. In spite of the relatively narrow range of relative potency estimates of the 6 serum samples obtained in the different laboratories and the low values calculated for within-laboratory heterogeneity (Table 6) highly significant values of heterogeneity of potency estimates between laboratories were the rule (Table 8). Possible causes for this have already been discussed. Therefore, there is a limit to the precision with which results between laboratories can be compared even when an identical standard is used. Since the techniques employed in this study were sufficiently precise to detect dissimilarities it is unlikely that improvements in the precision of techniques would contribute to improved between-laboratory reproducibility.

Two aspects of immunoglobulin heterogeneity which were not investigated in this work were the effects of differences of molecular size and diffusion constants within individual classes and the effects of differences between normal immunoglobulins and M-components. It is recognized that valid comparisons cannot be made between molecules of different diffusion constants using single-radial-diffusion methods (Mancini, Carbonara & Heremans, 1965). This precludes the use of these techniques employing 67/86 as a standard for measurements of immunoglobulins such as secretory IgA, IgM subunits and the IgG fragments found in urine. It is recognized that M-components differ in their antigenic characteristics from normal immunoglobulins; for example M-components consist of molecules of one subclass only. These molecules may, therefore, be antigenically deficient as compared with proteins of the same class in other serum samples, i.e., fewer antibodies reactive with these molecules may be present in a specific antiserum. Immunochemical comparisons of M-components with 67/86 may, therefore, be invalid.

The degree of homogeneity of estimated dry weights of contents of the ampoules suggests that in using these preparations it is sufficient for most purposes to assume that the weights of contents of all ampoules of any one batch are the same. This makes it unnecessary to estimate the weight of contents of individual ampoules.

The relative potency of a sample assayed against a standard may be expressed as a proportion of the potency of the standard, but it is more convenient to assign arbitrary units to the standard and to express the relative potency of a test material as the number of units per given volume or weight of the test material. The material 67/86 has been freeze-dried under what are considered to be the best conditions and has, therefore, been the basis of the investigations reported here. The material 67/86 has been established in the United Kingdom as the British

research standard for IgG, IgA and IgM. The unit of activity of each immunoglobulin is defined in the United Kingdom as the activity present in 0.8147 mg of the freeze-dried powder. Since the mean weight of contents of 67/86 is 81.47 mg per ampoule, each ampoule of 67/86 contains on average 100 units of activity of each of the 3 immunoglobulins. As calculated from the weighted mean relative potencies (Table 8) each ampoule of 67/95 contains on average 101 units of activity of IgG, 101 units of activity of IgA and 102 units of activity of IgM. Similarly, each ampoule of 67/97 contains 102 units of activity of IgG, 101 units of activity of IgA and 102 units of activity of IgM.

This preparation will be presented to the WHO Expert Committee on Biological Standardization for possible establishment as an international standard and definition of an international unit of activity.

It is recognized that the current practice is to express the concentration of immunoglobulin in body fluids in terms of mg/ml. The estimation of the number of mg of each immunoglobulin in 1 unit of activity is considered in a later paper where it will be shown that this estimate is as yet imprecise. Immunoglobulin concentrations can, therefore, be most precisely expressed as a relative potency in terms of units of the activity in a given volume, in relation to the standard.

ACKNOWLEDGEMENTS

This work was made possible by the ready collaboration of the investigators listed in Annex 2. Plasma was provided by courtesy of the British National Blood Transfusion Service and the supply of the samples was arranged by Dr W. D'A. Maycock, Dr T. E. Cleghorn and Dr W. J. Jenkins. We are indebted to the following for discussions or for technical assistance: Dr J. L. Fahey and Dr W. D. Terry of the National Institutes of Health, Bethesda, USA; Dr D. R. Bangham, Dr J. H. Humphrey, Miss M. V. Mussett and Miss J. Skegg of the National Institute for Medical Research, Mill Hill, London, England; Mr R. Drew and Miss S. Smith of the Department of Experimental Pathology, University of Birmingham, England; and Dr H. D. Welscher, Mrs H. Darling and Mrs I. Mikes of the WHO International Reference Centre for Immunoglobulins, Lausanne, Switzerland. We are especially indebted to Dr I. Addison and Dr P. J. Campbell of the National Institute of Medical Research, Mill Hill, London, England, for the preparation of the reference materials.

RÉSUMÉ

ÉTALON DE RECHERCHE POUR LES IMMUNOGLOBULINES SÉRIQUES HUMAINES IgG, IgA ET IgM

On a organisé une étude collective en vue de définir une préparation étalon pouvant servir à mesurer la teneur en immunoglobulines IgG, IgA et IgM des sérums humains. Le matériel choisi à cet effet, consistant en un mélange de sérums de donneurs sans antécédents de paludisme ou d'hépatite, a été réparti en ampoules, lyophilisé et soumis à un titrage comparatif dans 13 laboratoires de 8 pays.

La plupart des laboratoires ont employé pour leurs analyses la technique de la diffusion radiale en gel de gélose. Les mesures effectuées en prenant comme étalon le matériel à l'examen ont donné des résultats légèrement divergents suivant les laboratoires. Ces discordances sont attribuées à l'hétérogénéité des immunoglobulines et à l'emploi d'antisérums différents. On ne peut donc espérer obtenir par les techniques actuellement en usage une uniformité parfaite des résultats des titrages.

Après analyse statistique des données recueillies lors de l'étude collective, le matériel examiné (préparation 67/86) a été considéré comme pouvant servir de préparation de référence et a été constitué en étalon britannique de recherche pour les immunoglobulines sériques humaines IgG, IgA et IgM. L'unité d'activité a été définie au Royaume-Uni comme l'activité contenue dans 0,8147 mg de poudre sèche. Chaque ampoule de la préparation renferme en moyenne 100 unités de chacune des immunoglobulines IgG, IgA et IgM.

REFERENCES

Bliss, C. I. (1952) *The statistics of bioassay with special reference to vitamins*, New York, Academic Press, pp. 580-582

Bull. Wld Hlth Org., 1964, **30**, 447

Claman, H. N. & Merrill, D. (1964) *J. Lab. clin. Med.*, **64**, 685

Finney, D. J. (1964) *Statistical methods in biological assay*, London, Charles Griffin & Co. Ltd, p. 195

Humphrey, J. H., Mussett, M. V. & Perry, W. L. M. (1953) *Bull. Wld Hlth Org.*, **9**, 15

Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry*, **2**, 235

Robbins, J. B. (1968) *Birth defects, vol. 4, Immunological deficiency states in man*, New York, National Foundation, p. 338

Annex 1

AVAILABILITY OF MATERIAL

Preparations related to the proposed standard, 67/86, are now generally available for investigators wishing to use them for measuring concentrations of IgG, IgA and IgM in their own laboratories.

Workers in the Americas should request batch 67/95, which is available from:

Dr J. L. Fahey
National Cancer Institute
Immunoglobulin Reference Centre
Bethesda
Md. 20014, USA

Workers in the United Kingdom should request batch 67/97 from:

The Director
Division of Biological Standards
National Institute of Medical Research
Mill Hill, London, N.W. 7, England

Workers elsewhere should request batch 67/95 from:

The Director
WHO International Reference Centre for Immunoglobulins
21, rue du Bugnon
1005 Lausanne, Switzerland

Annex 2

PARTICIPANTS IN THE COLLABORATIVE ASSAY

BELGIUM

Dr J. P. Vaerman
University of Louvain
Louvain

CZECHOSLOVAKIA

Dr F. V. Skväril
Research Institute of Immunology
W. Pieck Street 108
Prague 10

FEDERAL REPUBLIC OF GERMANY

Dr G. Schwick
Behringwerke Aktiengesellschaft
355 Marburg-Lahn

FRANCE

Dr P. Burtin
Chef du Laboratoire d'Immunochimie
Institut de Recherches Scientifiques sur le Cancer
Villejuif

NETHERLANDS

Dr R. E. Ballieux
Department of Medicine
University Hospital
Utrecht

Dr W. Hijmans
Research Laboratories
Department of Rheumatology
University Hospital
Leiden

Dr Ph. Rümke
The Netherlands Cancer Institute
Sarphastraat 108
Amsterdam

SWITZERLAND

Dr D. S. Rowe
WHO International Reference Centre for Immunoglobulins
Université de Lausanne
21, rue du Bugnon
1005 Lausanne

UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND

Dr J. Hobbs
Postgraduate Medical School
Ducane Road
London W.12

UNITED STATES OF AMERICA

Dr M. Allansmith
Department of Surgery/Ophthalmology
Stanford Medical Center SO18
Palo Alto, Calif.

Dr C. E. Buckley
Allergy and Clinical Immunology Laboratory
Duke University Medical Center
Durham, N. C. 27706

Dr H. N. Claman
Assistant Professor of Medicine
Head, Division of Allergy and Immunology
University of Colorado Medical Center
5200 East Ninth Avenue
Denver, Colo. 80220

Dr W. F. Hymes
NCI Immunoglobulin Reference Center
6715 Electronic Drive
Springfield, Va. 22150

Annex 3

RECOMMENDATIONS FOR THE USE OF THE REFERENCE PREPARATIONS

(1) On receipt the reference preparation should be stored at −20°C.

(2) One ampoule of the reference preparation should be reconstituted by the addition of 1 ml of distilled water. The powder should dissolve readily on standing for 1 hour at room temperature to give a slightly turbid solution. An appropriate series of dilutions of this solution should be prepared and used on the same day as the material was reconstituted.

(3) The total volume of the solution of this preparation made in this way will exceed 1 ml. It has been calculated from the wet and dry weights of the ampoule contents and from specific gravity measurements that the average volume of this solution will be 1.06 ml; this solution will, therefore, contain the number of units [1] of immunoglobulin in the standard in 1.06 ml; i.e., for 67/86 this would be 100 units in 1.06 ml = 94.4 units per ml. The immunoglobulin contents of other batches may be calculated in the same way, i.e., the IgG content of reconstituted 67/97 would be 102 units in 1.06 ml = 96 units per ml.

(4) The concentration of each immunoglobulin in the solution under test should be compared with that of the standard, by using techniques which have been validated statistically. The potencies of immunoglobulins in the test solutions in relation to the standard should be estimated by a valid statistical analysis of the results of the comparative experiments.

(5) The relative potency should be expressed as units of activity of each immunoglobulin per ml of solution.

[1] The units of activity are defined on p. 549.

Annex 4

PREPARATION OF DILUTION SERIES FOR DOSE-RESPONSE CURVES: THE EFFECTS OF DIFFERENT DILUENTS

To assay immunoglobulins in comparison with the standard it is necessary to compare dose–response curves of a series of dilutions of each material. The nature of the diluent used might affect the results; accordingly a series of different diluents were compared for their effects on the apparent potency of the standard. This work was carried out in laboratory K.

Ampoules of 67/86 were reconstituted with 1 ml of distilled water. A doubling dilution series to 1 in 32 was prepared with each diluent using the contents of 2 ampoules separately. The diluents were as follows: 0.14 M NaCl, 0.14 M NaCl containing 1% bovine serum albumin (BSA), normal rabbit serum and normal sheep serum. Single-radial-diffusion tests were carried out and the results were assessed by parallel-line assay. Apparent potencies of IgG, IgA and IgM were expressed relative to the potency of dilutions prepared with 0.14 M NaCl (Table 11).

The saline and the saline-BSA diluents gave very similar potency values for all 3 immunoglobulins, values differing significantly only for IgG in 1 assay. Assays using rabbit serum diluent gave higher relative potencies for IgG and examination of the data showed that the differences were due to larger values of ring diameters given by the higher dilutions of 67/86. This may have been due to cross-reactivity between rabbit and human IgG in respect of the sheep antiserum used in the tests. When this antiserum was tested for reactivity against rabbit serum by Ouchterlony analysis no precipitin lines were, however, observed. None the less, replacement of the rabbit serum by sheep serum as a diluent gave relative potency values for IgG, which did not differ from those given by the use of a saline diluent.

A similar effect of the rabbit serum as diluent was suggested by the higher apparent potency value for IgA, but the difference was not significant.

These results suggest that either saline or saline-BSA were appropriate diluents for the single-radial-diffusion technique. The use of sera of animal species as a diluent may introduce differences in apparent potency, which are probably due to cross-reactivity. There seems little reason to use animal sera as diluents; however, if such sera are used they should preferably be from the same species as that used to prepare the specific anti-immunoglobulin antiserum.

TABLE 11

APPARENT RELATIVE POTENCIES OF 67/86 USING DIFFERENT DILUENTS

Diluent	Assay no.	Apparent potency relative to 0.14 M NaCl diluent	95% confidence limits
	IgG		
1% bovine serum albumin in 0.14 M NaCl	1	0.95	0.90–0.99
	2	0.95	0.91–1.00
Rabbit serum	1	1.08	1.03–1.13
	2	1.07	1.02–1.13
Sheep serum	1	0.99	0.97–1.01
	2	0.99	0.96–1.01
	IgA		
1% bovine serum albumin in 0.14 M NaCl	1	1.01	0.96–1.06
	2	0.98	0.94–1.02
Rabbit serum	1	1.05	1.00–1.10
	2	1.04	1.00–1.08
	IgM		
1% bovine serum albumin in 0.14 M NaCl	1	1.13	1.00–1.17
	2	0.93	0.90–1.02
Rabbit serum	1	1.08	0.96–1.21
	2	0.96	0.82–1.21

[a] All antisera were prepared in sheep.