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THE FOURTH INTERNATIONAL STANDARD FOR INSULIN

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SYNOPSIS

The composition, analysis and distribution of crystalline insulin for the Fourth International Standard for Insulin is described. In an international collaborative assay 20 laboratories compared this Standard with the Third International Standard, and the statistical analysis of the results is reported.

On the basis of the results the Fourth International Standard for Insulin is established with a potency of 24.0 IU/mg. The International Unit is thus defined as the activity contained in 0.04167 mg of the Standard preparation.

The Third International Standard for Insulin consisted of one batch of 25 g of soluble insulin prepared from bovine pancreas and was established in 1952 with a potency of 24.5 IU/mg. It soon became apparent that, owing to the advent of the several new delay media being used for insulin for clinical purposes, the consumption of the International Standard would increase considerably, since it was desirable for the purposes of the assay that the Standard should in some instances be made up in the delay media.

In view of these two factors and of the relatively small amount of the Third International Standard available, the Expert Committee on Biological Standardization of the World Health Organization in September 1952 authorized the Department of Biological Standards of the National Institute for Medical Research, London, in consultation with the Insulin Committee of the University of Toronto, Canada, and the United States Pharmacopeia Revision Committee, to obtain a quantity of insulin suitable for establishment as the Fourth International Standard, and to proceed with arrangements for its assay in terms of the existing Standard (World Health Organization, 1953). This quantity of insulin was to be large enough to act as the International Standard and also as laboratory working standards. Since for this purpose a quantity of 1 kg of crystalline insulin, with a potency of approximately 24 International Units per mg was necessary, it was agreed that the standard should be prepared from a pool of contributions from a number of laboratories.

The Fourth International Standard

By September 1954, a total of 1460 g of crystalline insulin had been donated by 13 laboratories in seven countries. This starting material contained 52% bovine and 48% porcine insulin and was sent for further processing to Eli Lilly & Company, Indianapolis, Ind., USA. The material was pooled and recrystallized twice from ammonium acetate buffer and the zinc insulin crystals dried to a moisture content of 5.65%. Although the biological potency was satisfactory, analysis showed the presence of protamine-splitting enzyme, and it was decided to purify the material still further. The crystals were subjected to the process developed by A. M. Fisher (Toronto) for the elimination of the contaminating enzyme, except that recrystallization was from ammonium acetate buffer. The crystals were finally dried in a humidity-controlled atmosphere, and analysis gave their composition as follows:

Water content	4.79% ± 0.58% (weight loss in oven at 105°-110°C for 16 hours, average of 16 samples)
Nitrogen	16.4%, calculated on moisture-free basis
Ash	0.533%, calculated on moisture-free basis
Zinc	0.382%, calculated on moisture-free basis
Protamine-splitting enzyme	insignificant concentration
Glucagon [1] (hyperglycaemic-glycogenolytic factor or enzyme)	0.069% ± 0.02% in the filled Standard.

The final yield, of 1060 g of crystals, was distributed into ampoules in a humidity-controlled room. The distribution took two days to complete; 1424 ampoules were filled on the first day and 5870 on the second. Each ampoule contains 110-125 mg of crystals. The ampoules used are of commercial neutral glass; they are not of the thick-walled hard-glass type usually used for International Standards and care must be taken when opening them that no small glass splinters fall into the crystals. Since the ampoules were sealed in air (unlike the majority of International Standards which are normally sealed in nitrogen), it is particularly advisable to store them at or below 0°C to avoid any possible loss of potency. It should be stressed that the crystals have not been extensively dried over P_2O_5, contain 5.65% moisture, and are nevertheless hygroscopic.

The Collaborative Assay

Twenty-two laboratories in nine countries were invited to take part in the collaborative assay of the Proposed Fourth International Standard in terms of the current (Third) International Standard, and of those invited, twenty laboratories in eight countries agreed to co-operate. Throughout

[1] Tested by the method of Staub & Behrens (1954).

the text of this report, laboratories are referred to by numbers which do not necessarily correspond to the order in which laboratories are listed in the Annex. A brief description of the material and suggestions on the assay were sent, with two ampoules each of the Third and Proposed Fourth International Standard, to each participant. It was suggested that at least one of the conventional methods which had been almost exclusively used in the assay of the Third International Standard should be used. The methods, full details of which are laid out in Annex 1 of the memorandum on the Third International Standard for Insulin (Miles et al., 1952), may be summarized as follows.

(a) The mouse-convulsion test: A (2 + 2) assay, using four groups of 24 mice, the whole assay being repeated at least five times. The response to each treatment is expressed in terms of the proportion of mice convulsing within 1½ hours of injection.

(b) The rabbit blood-sugar twin cross-over test: A (2 + 2) assay, using four groups of 6 rabbits. Every group receives each of the four doses at intervals of a week. The (time × rabbit-group × dose) injection programme is arranged as a 4 × 4 Latin square. Blood sugars are determined before injection (in duplicate) and at 1½, 3 and 5 hours after injection.

(c) The rabbit blood-sugar triplet cross-over test: A (3 + 3) assay, using six groups of at least 4 rabbits, which receive each of the six doses according to an injection programme arranged as a 6 × 6 Latin square. Blood-sugars are recorded at 1½ hours after injection.

In addition, participants were invited to use any other method of which they had had experience and in which they had confidence.

Results

Different assay methods

The number and type of assays carried out by each laboratory are recorded in Table 1, which shows that the 20 participating laboratories carried out a total of 292 assays. Apart from one set of 31 mouse cross-over tests from Laboratory 7, in which two dose levels of the Proposed Standard were compared with a single dose of the Third International Standard, all assays were based on the suggested methods. There were, however, variations in the numbers of animals used and one laboratory extended the mouse-convulsion test to include an extra dose of each preparation.

In many of the rabbit assays, blood-sugars were estimated at times other than those suggested in the memorandum and several laboratories dispensed with the Latin square arrangement and arranged their assays as simple cross-over tests. For this reason the number of rabbit assays, listed in Table 1, represents the number of simple cross-over tests which were done;

TABLE 1. NUMBER OF ASSAYS RECEIVED FROM PARTICIPATING LABORATORIES

Laboratory No.	Mouse-convulsion	Rabbit twin cross-over	Rabbit triplet cross-over	Other methods
1	—	18	—	—
2	—	2	—	—
3	12	—	—	—
4	—	2	—	—
5	—	2	—	—
6	—	9	—	—
7	10	—	—	31
8	9	—	—	—
9	—	4	3	—
10	—	22	—	—
11	8	—	—	—
12	—	4	—	—
13	30	—	—	—
14	47	—	12	—
15	5	2	—	—
16	—	18	—	—
17	18	—	—	—
18	7	—	—	—
19	11	—	—	—
20	6	—	—	—
Total	163	83	15	31

a complete comparison as described under (*b*) above counted as two assays, while the design (*c*) above counted as three.

Results of assays by the mouse method

The results of 163 assays were received from 11 laboratories. Laboratory 13 used three doses of each preparation in each assay and the other laboratories used a (2 + 2) design.

Each assay was analysed by the standard method (Gaddum, 1933) after transforming the responses to normal equivalent deviations, and sixteen assays were rejected as invalid (Table 2). In two assays from Laboratory 7 and five from Laboratory 13, the regression was not significant; four from Laboratory 13 showed significant departures from linearity, and five from Laboratory 14 significant departures from parallelism.

TABLE 2. RESULTS OF ASSAYS BY MOUSE-CONVULSION METHOD

Laboratory No.	Number of valid assays	Potency (IU/mg)	Weight	Limits of error (P = 0.95)	
				IU/mg	percentage of potency
3	12	26.0	2 213	23.6-28.6	90.8-110.0
7	8	21.9	2 029	19.8-24.2	90.5-110.3
8	9	23.7	3 895	22.0-25.4	93.1-107.4
11	8	23.3	4 277	21.8-25.0	93.6-107.3
13	21	25.3	8 477	24.1-26.6	95.4-105.1
14	42	23.5	18 318	22.8-24.3	96.8-103.3
15	5	25.8	3 999	24.0-27.7	93.0-107.4
17	18	24.7	4 525	23.1-26.4	93.6-106.9
18	7	23.4	2 873	21.5-25.5	91.9-109.0
19	11	24.1	1 854	21.7-26.8	90.0-111.2
20	6	25.9	1 383	22.9-29.2	88.4-112.7
Total	147		53 843		
Weighted mean		24.1		23.7-24.6	98.1-102.0

A weight equal to the reciprocal of the variance of the log-potency was assigned to each estimate of log-potency. Since there was no sign of heterogeneity within any laboratory, the separate estimates were combined to give the values in Table 2. The limits of error have been calculated as:

$$\text{antilog } [\overline{M} \pm t s_{\overline{M}}],$$

where $s_{\overline{M}}$ is estimated from the total weight for the laboratory in question.

The potencies for the Proposed Standard, obtained by different laboratories, are fairly consistent ($\chi^2 = 18.56$, $P \simeq 0.05$) and combine to a weighted mean potency of 24.14 IU/mg, with confidence limits (P = 0.95) of 23.68-24.62 IU/mg.

The unweighted mean potency, calculated directly from the distribution of 147 log potencies, is 24.29 IU/mg, with limits of 23.77-24.82 IU/mg, i.e., 97.9%-102.2% of the estimated potency. Since these limits are only 0.4% wider than those estimated from the internal evidence of the assays, the slight heterogeneity between potencies estimated by different laboratories may be ignored.

Results of assays by the rabbit twin cross-over method

Ten laboratories contributed to the total of 83 assays done by the suggested twin cross-over method of assay in the rabbit. Laboratories 6, 10 and 16 recorded blood-sugar levels at 1 and 2½ hours in a series of separate cross-over tests, while Laboratory 1 recorded the sum of the responses at 1 and 2½ hours. The other laboratories used the Latin square design, and recorded initial blood levels and responses at 1½, 3 and 5 hours. In an attempt to standardize the analysis of the results, the Latin squares have been ignored and each cross-over treated as a single assay. The sum of the blood-sugars at 1 and 2½ hours or 1½ and 3 hours has been used as response and analysed by the method of Smith, Marks, Fieller & Broom (1944).

One assay from each of Laboratories 15 and 16 was rejected as the slopes of the log-dose response lines for the test and standard preparations were significantly different. The remaining 81 log-potencies were weighted and combined by the same method as for the mouse assays.

There was no heterogeneity between estimates of potency obtained by the same laboratory, but the mean potencies for different laboratories (Table 3) vary significantly ($\chi^2 = 29.0$, $P < 0.001$). This variation is due to the relatively high potencies obtained by Laboratories 5 and 15. Their exclusion reduces χ^2 to 13.4 but makes little difference to the weighted

TABLE 3. RESULTS OF ASSAYS BY RABBIT TWIN CROSS-OVER METHOD

Laboratory No.	Number of valid assays	Potency (IU/mg)	Weight	Limits of error (P = 0.95)	
				IU/mg	percentage of potency
1	18	23.4	17 585	22.6-24.2	96.6-103.5
2	2	25.0	1 721	22.3-28.0	89.4-111.9
4	2	26.6	1 647	23.7-29.8	89.2-112.2
5	2	29.0	1 521	25.8-32.7	88.7-112.7
6	9	23.8	9 254	22.7-24.9	95.4-104.8
9	4	23.5	6 114	22.1-24.9	94.3-106.1
10	22	23.4	26 553	22.7-24.0	97.0-102.6
12	4	21.7	2 102	19.6-23.9	90.5-110.5
15	1	29.5	444	23.5-37.1	79.6-125.6
16	17	24.8	9 722	23.7-25.9	95.5-104.7
Total	81		76 663		
Weighted mean		23.8		23.4-24.2	98.4-101.6

mean potency which becomes 23.7 IU/mg, with confidence limits (P = 0.95) of 23.3-24.1 IU/mg, as compared with the weighted mean potency of 23.8 IU/mg and limits of 23.4-24.2 IU/mg obtained when all valid assays by this method are included.

Results of assays by the rabbit triplet cross-over method

All the assays from Laboratory 9, and three of those from Laboratory 14, were invalid. In each case there were two assays with significant departures from linearity of the log-dose response lines and one with a significant departure from parallelism.

The remaining nine assays formed a homogeneous set with a weighted mean potency of 23.4 IU/mg and confidence limits (P=0.95) of 21.0-26.1 IU/mg, i.e., 89.6%-111.6% of the estimated potency.

Other methods

Since only a single dose level of the current (Third) International Standard was used in the 31 mouse assays from Laboratory 7, tests of validity were not possible. The mean potency (and its limits) were estimated directly from the distribution of individual log-potencies and came to 22.8 IU/mg, with confidence limits (P=0.95) of 20.9-24.9 IU/mg, or 91.5%-109.3% of the estimated potency.

Combined results of assays by all methods

The final results obtained by each of the four methods employed are summarized in Table 4. They are quite consistent (χ^2=2.58, P=0.3-0.5) and combine to an over-all weighted mean potency of 23.912 IU/mg with limits of error, based on the total weight, of ± 1.2%.

TABLE 4. COMBINED RESULTS OF ASSAYS BY ALL METHODS

Method	Number of assays	Potency (IU/mg)	Limits of error (P = 0.95)	Log potency	Total weight
Mouse-convulsion	147	24.1	23.7-24.6	1.38280	53 843
Rabbit twin cross-over	81	23.8	23.4-24.2	1.37664	76 663
Rabbit triplet cross-over	9	23.4	21.0-26.1	1.36920	1 688
Others	31	22.8	20.9-24.9	1.35800	2 784
Total	268				134 978
Weighted mean		23.9	23.6-24.2	1.37862	

Comparison of assay methods

The relative precision of the different assay methods has been assessed from consideration of the average weights contributed by each animal (Table 5). In the cross-over assays the same rabbits are used on two or more occasions, and the design of the assays is such that this is recorded.

TABLE 5. RELATIVE WEIGHTS CONTRIBUTED BY EACH ANIMAL IN DIFFERENT TYPES OF ASSAY

Type of assay	Number of animal appearances	Total weight	Average weight per animal appearance
Mouse-convulsion	20 956	53 843	2.6
Rabbit twin cross-over	4 158	76 663	18.4
Rabbit triplet cross-over	432	1 688	3.9

In the quantal response assays the same mice may be used in several assays but cannot be identified. For this reason the average values in Table 5 are based on animal appearances rather than on actual numbers of animals; e.g., a simple twin cross-over test, where four groups of 6 rabbits each receive two doses of insulin, would contribute 48 animal appearances.

The average weight of 2.6 contributed by a single mouse should be fairly reliable as it is based on a very large number of assays. It is also quite close to the value of 3.2 which was obtained from the collaborative assay of the Third International Standard for Insulin (Miles et al., 1952). There is undoubtedly no real difference between these two estimates, for the weight per mouse obtained by different laboratories in the present study ranges from 1.1 to 8.3, the higher values coming mostly from insulin manufacturers who use the mouse test as a routine measure.

The average weight per rabbit appearance obtained by laboratories using the twin cross-over test ranges from 9.3 to 31.8, but it seems likely that the over-all estimate of 18.4 per rabbit appearance is significantly greater than the weight of 19 per rabbit estimated in the earlier collaborative assay. On that occasion, the average weight was estimated by counting each rabbit as one animal (regardless of the number of times it was used in an assay), and since a rabbit must have appeared at least twice in an assay a fair comparison with the present estimate of 18.4 would be with some value less than one-half of 19.

Little importance can be attached to the weight of 3.9 for the triplet cross-over test, as this was based on only nine assays from a single laboratory.

Comparison of the methods of assay thus shows that a twin cross-over test on 24 rabbits each used twice (with an average weight per appearance

of 18.4, total weight 886) gave about three times the precision yielded by the convulsion test on 96 mice (average weight 2.6, total weight 250). The simplicity of the latter method, however, is such that it may be more convenient, practically and economically, for a laboratory to perform three mouse quantal assays than to estimate blood-sugar levels on 24 rabbits maintained during the course of a twin cross-over test.

Redefinition of the Unit for the Proposed Fourth International Standard

A draft report, describing the combined results of the collaborative assay, was circulated among those who had taken part in the assay. Their attention was drawn to the fact that the over-all weighted mean potency of 23.9 IU/mg (with confidence limits of 23.6-24.2 IU/mg) was not significantly different from 24.0 IU/mg, and they were asked to express a preference for one of these values to define the unitage of the Insulin Standard.

Of the twenty participants, the majority were of the opinion that a potency of 24.0 IU/mg should be adopted. The Expert Committee on Biological Standardization of the World Health Organization accepted the majority view and in September 1958 the Fourth International Standard for Insulin was established with a potency of 24.0 IU/mg (World Health Organization, 1959).

The International Unit for Insulin is therefore defined as the activity contained in 0.04167 mg of the Fourth International Standard preparation.

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The following manufacturers of insulin generously provided material for the Fourth International Standard :

Australia :

Commonwealth Serum Laboratories

Canada :

University of Toronto

Denmark :

Nordisk Insulinlaboratorium
Novo Terapeutisk Laboratorium

Germany :

Farbwerke Hoechst A. G.

Netherlands :

N. V. Organon

United Kingdom of Great Britain and Northern Ireland :

Allen & Hanburys
Boots Pure Drug Co. Ltd.
British Drug Houses Ltd.
Burroughs Wellcome & Co.

United States of America :

Eli Lilly & Co.
Merck, Sharpe & Dohme Inc.
E. R. Squibb & Sons

The pooling, recrystallization and distribution of the material were performed under the direction of Dr G. B. Walden of Eli Lilly & Co., Indianapolis, Ind., USA.

Annex

PARTICIPANTS IN THE INTERNATIONAL COLLABORATIVE ASSAY OF THE PROPOSED FOURTH INTERNATIONAL STANDARD FOR INSULIN

AUSTRALIA	Dr K. C. Porter, Mr A. H. Mengoni and Miss E. M. Wadeson Commonwealth Serum Laboratories Parkville, Victoria
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	Dr W. E. Gaunt E. R. Squibb & Sons New Brunswick, N.J.

RÉSUMÉ

Le Quatrième Etalon international d'Insuline destiné à remplacer le troisième, en voie d'épuisement, est maintenant établi. Pour satisfaire aux demandes, il a été prévu de préparer une quantité de l'ordre de 1 kg d'insuline cristallisée, ce qui a nécessité la réunion de plusieurs lots de diverses provenances. Treize laboratoires de sept pays y ont participé. Le matériel initial contenait 52% d'insuline bovine et 48% d'insuline porcine. Après un premier traitement de purification, on s'aperçut de la présence d'un enzyme décomposant la protamine, qui fut ensuite éliminée au cours d'une seconde purification.

Le produit cristallisé a été réparti en ampoules de 0,110-0,125 g chacune, scellées à l'air. Il est recommandé de les conserver à une température de 0° C ou inférieure.

L'activité a été calculée d'après les résultats d'essais comparatifs effectués dans 20 laboratoires de 8 pays. Un mg de la substance contient 24,0 Unités internationales. L'Unité internationale d'Insuline correspond donc à 0,04167 mg du Quatrième Etalon international d'Insuline.

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