THE PREPARATION AND TESTING OF THE PROVISIONAL BRITISH STANDARD FOR GLOBIN ZINC INSULIN

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INTRODUCTION

From the time of the introduction of globin zinc insulin in 1939, and up to the date of issue of the Provisional British Standard for Globin Zinc Insulin on January 1st, 1949, the potency of batches of globin zinc insulin was measured in terms of either soluble insulin or of protamine zinc insulin by their action on the blood sugar level in experimental animals. The measurement of potency of one kind of preparation of a biologically active substance in terms of another kind of preparation of the same substance, is in general undesirable in the light of the requirements for valid biological assay. It cannot be assumed that insulin bound in a complex with globin for the express purpose of altering its rate of action, will always have the same effect relative to other preparations of insulin in the test animal.

For this reason, the British Insulin Manufacturers' Biological Sub-Committee, in collaboration with the Department of Biological Standards of the National Institute for Medical Research, have established a standard preparation of globin zinc insulin for use in the assay of globin zinc insulin. This has been prepared by the British Insulin Manufacturers, distributed in ampoules in the freeze-dried state and presented to the Medical Research Council for custody in the Department of Biological Standards. The preparation has been designated The Provisional British Standard for Globin Zinc Insulin. In this connection it must be emphasised that the qualification "Provisional" does not imply any doubt about the suitability of the preparation as such. The standard was established provisionally solely to avoid unduly prejudicing any action that may in the future be taken to establish an International Standard for the substance.

Before a freeze-dried globin zinc insulin preparation could be adopted as a standard it was important to show that the freeze-drying and subsequent reconstitution had produced no changes in its properties.

It was felt that this point could be established by showing:—

1. That the potency was unchanged by freeze-drying and reconstitution and did not vary from ampoule to ampoule.
2. That the ratio of potencies as determined respectively by the rabbit and mouse assays was unchanged by these processes.
3. That the shape of the rabbit blood sugar curve before and after freeze-drying and reconstitution was unchanged.

Before the freeze-dried globin zinc insulin preparation could be used as a standard it was essential to show also that it was sterile and was sufficiently stable when stored under reasonable conditions.

I. Preparation and Properties of the Standard

Method of Preparation

The British standard for globin zinc insulin was prepared by freeze-drying a solution of globin zinc insulin made from a concentrated solution of crystalline insulin from ox pancreas. Globin and zinc chloride were added to bring the concentration of globin to 3·8 mg. and of zinc to 0·3 mg. for each 100 units of insulin. To this solution *o*-cresol was added to give a final concentration of 0·2 per cent. The *p*H of this solution before freeze-drying was 3·1.

The potency of the concentrated insulin solution used in the preparation of the original globin zinc insulin solution was not determined directly. The rest of this same concentrated solution was, however, by simple dilution converted into 9 commercial batches of insulin which were separately assayed by the mouse convulsion method. The results obtained in these assays are given in Table I. From them it can be calculated that the amount of soluble insulin converted to globin zinc insulin and present in each ml. of the original globin zinc insulin solution was 39·73 I.U. with limits of error (P = 0·95) from 38·45 to 41·05 I.U. This result did not differ significantly from the intended value of 40 I.U./ml. which was therefore accepted as the concentration of soluble insulin present and converted to globin zinc insulin.

The globin zinc insulin solution prepared as above was freeze-dried

TABLE I

POTENCY ASSAYS BY THE MOUSE CONVULSION METHOD OF COMMERCIAL BATCHES PREPARED FROM THE SAME CONCENTRATED SOLUTION AS THE GLOBIN ZINC INSULIN STANDARD

Dilution No.	Labelled Potency I.U./ml.	Assayed Potency I.U./ml.	Log Potency	Weight = Reciprocal of Variance
1	40	41·1	1·6135	4075
2	40	38·7	1·5877	2469
3	40	41·6	1·6194	1802
4	20	20·9	1·3211	1239
5	80	78·8	1·8964	1295
6	20	19·0	1·2785	1419
7	20	19·7	1·2943	1570
8	20	19·5	1·2904	1827
9	20	19·2	1·2839	3257

and the solid material distributed in ampoules each containing approximately 40 mg.

A quantity of the globin zinc insulin solution before freeze-drying, termed hereafter the "original liquid," was set aside in order that the freeze-dried material, when reconstituted, could be assayed in terms of the former to ascertain whether a loss in potency had occurred during the freeze-drying process.

Calculated Potency from Weight Yield

Assuming no loss of potency on freeze-drying, the potency of the freeze-dried material could be calculated from the weight of freeze-dried globin zinc insulin obtained from a given volume of the original globin zinc insulin solution. This was determined in the following manner.

10 weighing bottles were packed with strips of absorbent paper and dried *in vacuo* over silica gel to constant weight. 8 of the bottles were then taken and 10 ml. of the original liquid was delivered into each. The 10 bottles were then redried *in vacuo* at 20° C. until the 8 bottles had attained constant weight and the 2 remaining bottles were the same weight as at the commencement of the experiment. This latter precaution was taken to ensure that a similar state of dryness was obtained at the end as at the beginning of the experiment.

The weight obtained from 10 ml. (400 units) of liquid globin zinc insulin was 44·02 mg. Therefore, the potency of the anhydrous material, assuming no potency loss was $400{\cdot}0/44{\cdot}02 = 9{\cdot}09$ U./mg.

To obtain the potency of the standard from this figure it was necessary to know its moisture content. This was determined by drying the contents of 4 ampoules of the standard preparation to constant weight in the desiccator used in the above experiment. Before drying the weight was 167·1 mg. and the loss in weight due to moisture 7·4 mg. The British standard for globin zinc insulin therefore has a moisture content of 4·43 per cent. Its potency calculated from that of the anhydrous material by correcting for the moisture content of the former was therefore

$$\frac{9{\cdot}09\ (100{\cdot}0 - 4{\cdot}43)}{100} = 8{\cdot}69 \text{ U./mg.}$$

II. Proof of the Identity of the Original Liquid and the Reconstituted Freeze-dried Solid

As explained in the introduction a highly accurate determination of the identity of the original liquid and the reconstituted freeze-dried solid was absolutely necessary to the successful assignment of a potency to the British standard for globin zinc insulin. The present section describes the tests undertaken to prove this. The assays were carried out on both rabbits and mice.

Rabbit Assays

The rabbit assays were planned to a design which consisted of three triplet cross-over tests embodied in a 6×6 Latin Square. Similar designs have been discussed by Emmens[2] and Smith[3]. An example of one such square is shown in Table II.

TABLE II

6 × 6 LATIN SQUARE DESIGN CONSISTING OF 3 TRIPLET CROSS-OVER TESTS

	Day					
Group	1	2	3	4	5	6
1	T_3	S_1	S_2	T_2	T_1	S_3
2	S_1	T_2	T_3	S_3	S_2	T_1
3	T_1	S_3	S_1	T_3	S_3	T_2
4	S_3	T_1	T_2	S_1	T_3	S_2
5	T_2	S_2	T_1	S_2	T_2	S_1
6	S_2	T_3	S_3	T_1	S_1	T_3

S_1 = low dose—original liquid
S_2 = medium dose—original liquid
S_3 = high dose—original liquid
T_1 = low dose—freeze-dried reconstituted material
T_2 = medium dose—freeze-dried reconstituted material
T_3 = high dose—freeze-dried reconstituted material

The rows represent groups of rabbits, 3 or 4 animals being used per group, and the columns represent the different days of the test. The 6 treatments are provided by the 3 doses of original liquid and 3 doses of reconstituted material. In addition to the Latin Square, however, each pair of 2 days provides a complete triplet cross-over test in its own right. This design makes for flexibility in practice, since if anything occurs to invalidate the results on one particular day, the whole of the experiment is not rendered useless, as 2 complete cross-over tests can still be analysed; in addition, the analysis of the results can be carried out in both ways and the method having the smaller error used for estimating the potency. 6 such squares were worked through in 3 separate laboratories, providing 18 cross-over tests in all. Each square was randomised independently in each laboratory and the example given is one of those actually used. In addition all rabbits were allocated in the first place to the different groups at random, making use of a table of random numbers.

Methods. The methods used in the different laboratories were based on those in use for the routine assay of insulin on rabbits (Smith[3]). Initial blood sugar levels of fasting rabbits, determined from the mean of two separate bleedings, were recorded on each day of the test. The animals were then injected with the appropriate solution for each group and further bleedings were carried out at hourly intervals for 6 hours, single blood sugar determinations being carried out independently on each blood sample. It had been suggested that duplicate estimations of blood sugar should be done for each sample but Smith has shown (*loc. cit.*) that 12 rabbits with one determination of blood sugar at each time are equivalent to 10 rabbits with duplicate blood sugar determinations; it was therefore decided to carry out only single estimations. An interval of one week was allowed between each day of the experiment.

Results. As it would be impracticable to reproduce tables giving the detailed data for all the experiments in this collaborative assay, the data have been collected and bound, and are available for inspection in the library of the National Institute for Medical Research, Mill Hill, London, N.W.7, U.K.

Analysis. The functions of the data used for the estimation of the potency of the freeze-dried preparation in terms of its own original liquid were chosen so as to minimize the fiducial limits of error of that estimate. It became evident at an early stage of the analysis that nothing was to be gained by analysing the results of a complete Latin Square instead of by treating the individual cross-over tests separately. In fact, the fiducial limits of error obtained by analysing the whole square were wider than those obtained by analysing the cross-over tests separately. This is attributable to the fact that during a total period of 6 weeks the variation within animals is so large that the additional advantage gained by the use of a Latin Square design is largely offset. The analysis was, therefore, based solely on the individual cross-over tests. The various functions of the data used in the analysis were:

1. The mean percentage reduction of blood sugar over the hours 1 to 6 inclusive.
2. The mean percentage reduction of blood sugar over the hours 1 to 6 inclusive adjusted by covariance for the initial blood sugar.
3. The mean blood sugar level over the hours 1 to 6 inclusive.
4. The mean blood sugar level at selected hours only. The hours selected varied in different laboratories.

As explained earlier it was also necessary to ensure that the shape of the blood sugar curve was unaltered by the processes of freeze-drying and reconstitution. For this purpose graphs were drawn of all the individual curves. It was apparent that the biggest discrepancies occurred at different times in the different laboratories. The analysis mentioned in 4 above was, therefore, carried out at those hours at which the discrepancies were greatest. Such analysis would bring out any significant difference in the shape of the curves. It was apparent from the analyses that the best estimate of potency, that is the one giving the narrowest fiducial limits of error, was obtained from the analysis using as the variate the mean percentage reduction of blood sugar over the hours 1 to 6 inclusive, adjusted by covariance for the initial blood sugar. Table III shows a summary of the results, using this variate for analysis, for each laboratory separately; each estimate was weighted by the reciprocal of its own variance and all values for log. potency ratio (M) have been adjusted to refer to an assumed potency of 10 U./mg.

There was no heterogeneity of the estimates of potency calculated from the different cross-over tests either within any one laboratory or

TABLE III

ESTIMATE OF POTENCY BY THE RABBIT ASSAY

Laboratory	M	Weight	Potency U./mg.	Limits of Error P = 0·95
A	−0·07096	2191	8·49	7·71—9·36
B	−0·04600	2195	9·00	8·15—9·92
C	−0·08623	2206	8·22	7·44—9·04
Mean	−0·06776		8·56	8·09—9·04

between the different laboratories. The results of the tests which illustrate this are given in Table V.

Mouse Assays

Method. The mouse assays were carried out by the usual mouse-convulsion method and the data subjected to probit analysis. A (2 + 2) assay design was used throughout.

Results. The results of the individual mouse assays are not reproduced here, but may be inspected at the National Institute for Medical Research.

Analysis. Each assay was analysed by the method described by Bliss[4]; the combination of the results was carried out by the simplest method; namely, by obtaining the weighted mean potency, each estimate being weighted by the reciprocal of its own variance. This method tends to underestimate the limits of error (Perry[5]). Table IV shows the summarised results; all values for log. potency ratio (M) have been adjusted to refer to an assumed potency of 10 U./mg.

TABLE IV

ESTIMATE OF POTENCY BY THE MOUSE ASSAY

Laboratory	M	Weight	Potency U./mg.	Limits of Error P = 0·95
A	− 0·05651	2553	8·78	8·03—9·60
C	− 0·08323	2265	8·26	7·51—9·08
D	− 0·06208	3634	8·67	8·04—9·34
Mean	− 0·06607		8·59	8·18—9·02

There was no heterogeneity of the estimates of potency between the different individual assays within the laboratories nor between the different laboratories; the results of the χ^2 tests showing this are given in Table V.

TABLE V

CONSISTENCY OF ASSAY RESULTS

Test	Laboratory	χ^2	Degrees of freedom	P
Consistency with laboratories				
(*a*) Rabbit assays	A	2·7	5	0·7—0·8
	B	1·9	5	0·8—0·9
	C	1·7	5	0·8—0·9
(*b*) Mouse assays	A	12·09	12	0·3—0·5
	C	9·52	8	0·3—0·5
	D	21·85	16	0·1—0·2
Consistency between laboratories				
(*a*) Rabbit assays		1·81	2	0·3—0·5
(*b*) Mouse assays		0·96	2	0·5—0·7
Consistency between mouse and rabbit assays		2·78	1	0·05–0·1
Total ..		55·31	56	0·49

Assignment of Potency to the Standard.

From the 6 values for M and W (Tables III and IV) an overall estimate potency of the freeze-dried, reconstituted globin zinc insulin in terms of the original liquid was obtained by using the equation $\overline{M} = \frac{SWM}{SW}$;

this gave a value of 8·57 U./mg. with limits of error (P = 9·95) of 8·26 and 8·90 U./mg.

The consistency of the results was tested by making use of the approximate relationship $\chi^2 = SW (M - \bar{M})^2$ and Table V shows the results of all such comparisons. Since there is thus no evidence of heterogeneity of the estimates it is assumed that the combined potency of 8·57 U./mg. is the best available estimate of the true potency of the British standard for globin zinc insulin.

The criteria set out in the introduction for proof of the identity of the freeze-dried, reconstituted material with its original liquid have thus been satisfied. It was shown in Section I that, on the basis of the weight yield, the potency of the freeze-dried material should be 8·69 U./mg. assuming no loss on freeze-drying. The assay results are in full accord with this expectation, indicating that there is no significant loss of potency. The shapes of the blood sugar curves were compared in two ways; namely by graphical representation, which showed a close similarity in general appearance, and by analysis of the selected hours at which the most noticeable divergence in the shapes of the curves existed; the analysis showed no significant change in the potency ratio at these selected hours. Finally, the homogeneity of the potency ratios in different species indicated in the χ^2 tests, lends strong support to the conclusion that the original liquid and the freeze-dried, reconstituted preparation are identical in properties.

It was agreed, therefore, with the concurrence of the Medical Research Council, to establish this freeze-dried globin zinc insulin as the Provisional British Standard for Globin Zinc Insulin, and to define it as having a potency of 8·6 British units of globin zinc insulin per mg., one British unit of globin zinc insulin being the activity of one International unit of soluble insulin after conversion to the Provisional British Standard for Globin Zinc Insulin.

III. Stability and Bacteriological Tests

Stability

Samples of the standard were stored for 6 months both at room temperature and at 50° C. and thereafter assayed on mice against the same material stored in the cold. Assays were carried out by three laboratories. The results were as follows.

(*a*) *Storage at room temperature for 6 months*

Table VI gives the results. The combined weighted mean potency is 8·38 U./mg. with P = 0·95 limits of error 7·99 and 8·76 U./mg. A χ^2 test indicates that there is no heterogeneity between these three estimates. $\chi^2 = 1{\cdot}6261$ on 2 degrees of freedom. Assuming 8·57 U./mg. to be the potency at the beginning of the experiment, there has been a net loss of potency of 2·30 per cent. during storage at room temperature for a period of 6 months. This potency loss, however, is not significant: t = 0·772 P = 0·4 — 0·5.

TABLE VI

STORAGE AT ROOM TEMPERATURE FOR 6 MONTHS

Laboratory	Potency U/mg.	Log. potency	Weight
A	8·60	0·93450	3715
D	8·08	0·90741	3919
C	8·57	0·93278	1906
Mean	8·38	0·92303	

(*b*) *Storage at 50° C. for 6 months*

Table VII shows the results. The combined weighted mean potency is 6·74 U./mg. with P = 0·95 limits of error 6·47 — 7·02 U./mg. A χ^2 test indicates that there is no significant heterogeneity between the estimates at the P = 0·99 level, although there is significant heterogeneity at the P = 0·95 level. χ^2 = 7·9545 on 2 degrees of freedom. Assuming homogeneity there has been a net loss of potency of 21·41 per cent. during storage at 50° C. for 6 months.

TABLE VII

STORAGE AT 50° C. FOR SIX MONTHS

Laboratory	Potency U./mg.	Log. potency	Weight
A	7·09	0·85065	6822
D	6·36	0·80346	4403
C	6·07	0·78297	891
Mean	6·74	0·82852	

(*c*) *Estimated Stability of the Standard at — 10° C.*

The probable rate of deterioration of the standard at — 10° C. has been calculated from the above results. It has been assumed that "room temperature" may be taken as 18° C., and that the most probable values for the rates of deterioration at 50° C. and 18° C. may reasonably be derived from the estimated potencies after storage at these temperatures. Moreover, the comparisons were made against material stored at 0° C., and no allowance has been made for any deterioration at this temperature which would tend to lesson the apparent decay. The calculation of the probable loss of potency at — 10° C. is based upon the theoretical linear relationship between the reciprocal of the absolute temperature and the velocity of the reaction. On this basis at — 10° C. the standard would be expected to lose potency at the approximate rate of 0·5 per cent. per annum. The stock of standard held will not, at present rates of usage, last for more than 10 years; and in this period the total loss of potency should not exceed 5 per cent. Although the loss of potency has not been considered sufficiently serious to invalidate the use of the material as a standard, it is large enough to merit further investigation.

Bacteriological Tests

(*a*) *Sterility.* 6 ampoules of the standard preparation, reconstituted with distilled water, passed the sterility tests described in the British Pharmacopoeia 1948.

(*b*) *Bacteriostatic powers.* The contents of 5 ampoules of the standard preparation were mixed, reconstituted and distributed into tubes in 1·0 ml. lots. These were seeded with serial 10-fold dilutions in 0·85 per cent. saline solution of washed cultures of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, and of *Bacillus subtilis* spores. The liquid, the *p*H of which was below 3·0, was bactericidal within 24 hours at 20° C. and 37° C. to the 3 vegetative bacteria added to make a final concentration of 10^4 living cells per millilitre. A small proportion of the *Bacillus subtilis* spores was killed, and the remainder failed to germinate in the globin zinc insulin solution even after 1 week at 37° C. The reconstituted material thus has bactericidal and bacteriostatic properties and it is unnecessary to add any antimicrobial substance to it.

IV. Conclusions and Recommendations for the use of the Standard

Suitability of the Freeze-dried Preparations for use as a Standard

From the tests already described it may be concluded that there was no evidence of a loss of potency during freeze-drying of the preparation; its potency did not appear to vary from ampoule to ampoule; and it was found to be bacteriologically sterile and sufficiently stable. That is, it satisfied the requirements for a stable biological standard.

Potency of the Standard

International unit potency for soluble insulin, which is the activity of 0·0455 mg. of the 2nd International Standard for Insulin, cannot be used directly to describe the activity of globin zinc insulin. Moreover, the potency of a preparation cannot be described in terms of the units of soluble insulin used in its manufacture without making unwarrantable assumptions about the molecular state of globin zinc insulin. An independent unit of globin zinc insulin potency is therefore required. However, globin zinc insulin defined in terms of the International unit of soluble insulin has already been in extensive clinical use, so that it was highly desirable to ensure that the units of soluble and of globin zinc insulin, although formally distinct, were related in such a way that the association between the two, already established clinically, was maintained. Consequently, unit potency of globin zinc insulin is defined as the activity of a weight of the Provisional British Standard for Globin Zinc Insulin and not in terms of the International Standard for Soluble Insulin. The unit of activity is the activity of 0·1163 mg. of the Provisional British Standard for Globin Zinc Insulin (i.e. the standard contains 8·6 British Units per milligram.); but this unit is related to the unit of soluble insulin in that one British Unit has the activity of one International Unit of soluble insulin after conversion to the British standard for globin zinc insulin.

Use of the Standard

The sealed standard preparation should be kept in the dark at temperatures of − 5·0° C. or below. Each ampoule of the preparation contains approximately 40 mg. of dried globin zinc insulin, and should be reconstituted for use by adding distilled water.

(*a*) *Potency Estimation.* The mouse convulsion or the rabbit cross-over method is recommended for the estimation of the potency of globin zinc insulin preparations in terms of the standard. The assay should be designed so that the fiducial limits of error of the estimated potency can be calculated from the data.

(*b*) *Estimates of Delayed Action.* No routine method of estimating the delayed action of globin zinc insulin can be recommended since a detailed investigation revealed that the rate of recovery of rabbit blood sugar following the decrease induced by globin zinc insulin is biometrically indistinguishable from that induced by soluble insulin unless an impracticably large number of animals is used in a cross-over test.

SUMMARY

1. In the light of the requirements for valid biological assay it seemed desirable to establish a standard globin zinc insulin preparation. A suitable solution prepared by the British Insulin Manufacturers has been freeze-dried and tested in collaboration with the National Institute for Medical Research. The solid has been designated the Provisional British Standard for Globin Zinc Insulin.

2. Various tests, both chemical and biological, were carried out on the soluble insulin solution from which the globin zinc insulin solution was made and on the globin zinc insulin preparation before and after freeze-drying, in order to ensure the suitability of the freeze-dried material as a standard preparation and to determine its potency.

3. There was no evidence of a loss of potency during the freeze-drying of the standard; its potency did not appear to vary from ampoule to ampoule; and it was found to be bacteriologically sterile and sufficiently stable.

4. The unit potency of globin zinc insulin has been defined as the activity of 0·1163 mg. of the Provisional British Standard for Globin Zinc Insulin. That is, the solid contains 8·6 British Units per mg. This unit is related to the unit of soluble insulin in that one British Unit of activity is the activity of one International Unit of soluble insulin after conversion to the British standard for globin zinc insulin.

5. The use of the Provisional British Standard for Globin Zinc Insulin in assays of globin zinc insulin intended for therapeutic use has been discussed. Potency may be estimated by the mouse convulsion or the rabbit cross-over method. The assay should be designed so that the fiducial limits of error of the estimated potency can be calculated from the data. No method of estimating the delayed action of globin zinc insulin has been recommended owing to the fact that the rabbit blood sugar curve induced by globin zinc insulin is biometrically indistinguishable from that induced by soluble insulin unless an impracticably large number of animals is used.

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