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The Content of Glycogenolytic Factor in some Insulin Samples

BY GENEVIEVE AUDY AND MARGARET KERLY

Department of Biochemistry, University College, London

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Sutherland & Cori (1948) have reported that a sample of insulin manufactured by the Eli Lilly Research Laboratories contained a glycogenolytic factor, whereas a sample prepared by the Novo Terapeutisk Laboratorium A/S contained none. Later work (Sutherland & Duve, 1948) has shown that the factor is secreted by the pancreas, probably by the α cells, and Sutherland & Cori have suggested that the factor is responsible for the initial hyperglycaemia which may follow an intravenous injection of insulin. The variations in this initial hyperglycaemia reported by different authors, e.g. Burger & Kramer (1930), suggest that the amount of factor associated with different insulin samples may vary. An assay of glycogenolytic-factor content has been carried out on some insulin samples available in this laboratory and has shown that the amount does vary considerably, both in samples prepared by different manufacturers and in samples from the same firm.

The test system was based on that described by Sutherland & Cori (1948) and Sutherland & Duve (1948), where activity was estimated from the increased rate of production of glucose by liver slices, incubated in buffered saline, caused by the addition of the factor. The experimental procedure is de-

scribed somewhat fully, as satisfactory results were only obtained when it was followed in detail. Two methods of assay were used. The first, or single-dose, method gives an approximate estimate of activity after testing at one concentration; the second, or multiple-dose, method requires testing of samples at several concentrations and gives a more accurate assessment of relative potency.

EXPERIMENTAL

The test system

Reference standard. A sample of insulin containing the factor (Eli Lilly, no. 987267) was used as the reference standard for comparison of glycogenolytic-factor content.

Insulin samples. Samples, prepared by a number of manufacturers from the pancreas of ox, calf, sheep and pig, were given to us for assay by Prof. F. G. Young and Dr A. C. Chibnall.

Choice of animal. In preliminary experiments, liver slices from several animals were tested with the reference standard used at a concentration of 53 $\mu\text{g./ml.}$, which was sufficient to give a maximum response with rabbit and rat liver (Table 1). While it would be an advantage to select an animal such as the cat, whose liver gives both a large basic output of glucose and a large response to the factor (especially when testing at concentrations of factor giving a sub-

maximal response), for routine testing a more readily available animal must be chosen and rabbits were used for all the assays reported here. The best results were obtained with liver from young (2-3 months) rabbits fed carrots, in addition to the stock pellet diet, for several days prior to use. Rabbits infected with coccidiosis were generally unsatisfactory and were not used except in very mild cases.

Table 1. *Species variation in response of liver slices to glycogenolytic factor*

(Control liver slices incubated in saline, test slices in saline containing the reference standard (53 $\mu\text{g./ml.}$). Incubation at 37° for 30 min.)

Animal	No. of livers tested	Mean glucose output (mg./g. liver)	
		Control slices	Test slices
Guinea pig	3	5.6	6.6
Rat (hooded)	5	6.3	9.0
Rat (Wistar)	2	11.6	15.8
Rabbit	110	7.0	12.0
Ferret	2	15.1	20.6
Cat (adult)	1	23.1	31.1
Kitten	3	15.9	24.9

After the bulk of the assays had been completed a few experiments were carried out using Wistar rats, the results of which are included in Table 1. Liver slices from this strain produced more glucose than those from the hooded rats used in the preliminary experiments and it is possible that liver from these animals might provide a more consistent test system than rabbit liver, as more uniform animals would be available.

Incubation medium. Phosphate-saline, as recommended by Sutherland & Cori (1948), was used for incubation of the slices; it was prepared by diluting 1 vol. 0.1M-sodium phosphate buffer (pH 7.4) with 4 vol. 0.9% (w/v) NaCl. Media containing the reference standard or the insulin samples to be tested were prepared immediately before use. The insulin was dissolved in 0.0033N-HCl (0.5 ml./mg. insulin) and the solution was diluted with phosphate-saline to give the required concentration.

Preparation of liver slices. The rabbits were anaesthetized by injection of Nembutal and bled from the aorta. The liver

was removed and chilled immediately by placing for 3-5 min. in ice-cold phosphate-saline. A small block of liver was cut into slices, 0.3-0.6 mm. thick and weighing about 200 mg., by means of a tissue slicer (Stadie & Riggs, 1944). The slices were placed on a chilled Petri dish and covered with a moist filter paper. Each slice was cut into several portions, not varying in weight by more than a few mg.; the portions were weighed on a torsion balance and dropped into 3 ml. chilled phosphate-saline, with or without insulin (see below), in 25 ml. conical flasks. (In early experiments test tubes were used, but the slices did not shake adequately in the medium.)

Incubation. The flasks were shaken at 110 oscillations/min. in a water bath at 37°. For the single-dose assays and most of the multiple-dose assays a 30 min. period was used, based on Sutherland & Cori's (1948) results for rat liver. Later it was found that in our conditions 60 min. incubation gave better results both for rabbit and rat (Wistar) liver and this period was used for one of the assays carried out by the multiple-dose method.

Glucose estimation. At the end of the incubation period the flasks were removed from the bath and cooled in ice. Portions of medium (2 ml.) were removed, deproteinized by addition of 1 ml. each of 0.3N-Ba(OH)₂ and 5% (w/v) ZnSO₄, and glucose estimated by Somogyi's (1945) modification of the method of Nelson (1944).

Single-dose assay method

The concentration of reference standard, 53 $\mu\text{g./ml.}$, was chosen to give a maximum response in the test system. Insulin samples were first assayed at the same concentration and those samples which showed little or no activity were retested at higher concentrations. No samples tested showed a higher activity than the reference insulin. In early experiments, liver slices were cut in two portions, one being used to determine the basic rate of glucose output (*G*), the other for the rate of glucose output in the presence of the standard (*S*) or of the sample (*T*). The percentage increase in glucose output was calculated for each slice,

$$100(S \text{ (or } T) - G)/G,$$

and the activity of the sample was expressed as 100 times the ratio of the mean percentage increase for the sample to that for the standard. In later experiments, liver slices were cut into three or four portions and the rates of glucose output

Table 2. *Glycogenolytic-factor content of insulin sample B1 assayed by the single-dose method on several rabbits*

(For experimental details, see 'single-dose assay method'.)

Rabbit no.	Increase in glucose output over control (%)				Activity of sample (Reference standard = 100)
	Individual slices		Mean for each rabbit		
	Standard	Sample	Standard	Sample	
15	95, 83, 64, 64	42, 40, 27, 21	77	33	43
17	63, 73, 77, 84	12, 30, 38, 19	74	25	34
20	138, 151, 152, 217, 282	30, 39, 71	188	47	25
21	72, 75, 83, 85	56, 26, 22, 10, 24	79	28	35
22	94, 107, 130	65, 25, 32, 25	110	37	34
24	47, 89, 94, 71	6, 4, 29, 10	75	12	16
26	59, 45, 33, 31, 48	18, 27, 15, 35	43	24	56
27	70, 30, 75, 72, 40	12, 33, 19, 37	57	25	44
				Mean	36

both under basal conditions and in presence of the standard and sample (or samples) determined on portions of the same slice.

In our experience variation between rabbits is such that several animals should be used to obtain a reasonable assessment of activity. Possibly if an adequate number of rabbits from the same strain had been available, variation would have been less. The activity reported is the mean for all the rabbits used. Results on one sample (Table 2) illustrate the range of variation for slices both from the same and different animals.

Table 3. *Glycogenolytic-factor content of insulin samples*

(Abbreviations used: *B*, Boots Pure Drug Co. Ltd.; *BW*, Burroughs Wellcome Ltd.; *K*, Kry; *N*, Novo Teraeutisk Laboratorium A/S, Denmark; *AH*, Allen and Hanburys Ltd.; *BDH*, British Drug Houses Ltd.; *BP*, Blatherwick Process; *X*, manufacturer unknown. Manufacturers identification number, when known, shown in parenthesis.)

Sample no.	Species	Single-dose method	
		No. of livers used for assay	Activity (Reference standard = 100)
<i>B</i> 1	Ox	8	36
<i>B</i> 2		6	8
<i>B</i> 3 (4840)		3	52
<i>B</i> 4 (4840)		5	74
<i>B</i> 5	Calf	2	29
<i>B</i> 6	Sheep	1	26
<i>B</i> 7 (3091)	Sheep	1	45
<i>B</i> 8	Pig	2	15
<i>B</i> 9	Pig	2	18
<i>B</i> 10	Unknown	4	0
<i>X</i>	Pig	2	94
<i>BP</i>	Unknown	9	55
<i>BW</i> 1 (9726)		2	5
<i>BW</i> 2 (9615)		4	55
<i>AH</i> (2039)	Sheep	2	21
<i>BDH</i> (2065)	Pig	4	19
<i>K</i> 1	Ox	10	64
<i>K</i> 2	Pig	10	12
<i>N</i> 1	Unknown	5	25
<i>N</i> 2	Ox	2	5
<i>N</i> 3	Calf	1	9
<i>N</i> 4	Sheep	1	2
<i>N</i> 5	Pig	2	1

Multiple-dose assay method

A number of samples and the standard were tested at four concentrations which were in geometrical progression. Pilot experiments were carried out to find the approximate activity of each sample, and concentration levels were chosen so that the responses of the standard and test samples were approximately the same at each of the four levels. In each experiment eight liver slices from the same rabbit were cut into three portions, a control portion being incubated in phosphate-saline without addition, while the other two were incubated in phosphate-saline containing either the standard or one of the samples under test at two concentrations. The design as a whole was a randomized block type, each slice being used for testing one sample at

two concentrations. The logarithms of the relative potencies of the samples were calculated from the regressions of the responses on the logarithms of the concentrations.

RESULTS

Twenty-three samples of insulin were tested by the single-dose method (Table 3) at a concentration of 53 $\mu\text{g./ml.}$, the same as that used for the standard. Eight which gave low values were retested at higher concentrations (Table 4); of these four proved to contain no glycogenolytic factor.

Table 4. *Glycogenolytic-factor content of insulin samples tested at high concentrations*

(Single-dose assay method. 30 min. incubation at 37°. Number of observations shown in parenthesis.)

Sample no.	Increase of glucose output over control (mg. glucose/g. liver)	
	Standard (53 $\mu\text{g./ml.}$)	Sample (200 $\mu\text{g./ml.}$)
<i>N</i> 2	4.08 (2)	0 (2)
<i>N</i> 3		0 (2)
<i>N</i> 4		1.36 (1)
<i>B</i> 2		0.06 (1)
<i>N</i> 5	4.48 (3)	0 (2)
<i>BW</i> 1	4.50 (3)	1.74 (1)
<i>B</i> 10	3.55 (10)	0.43 (6)
<i>K</i> 2	3.56 (4)	0.40* (4)

* Sample *K*2 tested at 105 $\mu\text{g./ml.}$

Eleven of the samples were also assayed by the multiple-dose method, three simultaneously with the standard, in assay *A*, the others in a series of separate assays (*C*) against the standard (Table 5).

In assay *A*, the standard was assayed at 2, 4, 8 and 16 $\mu\text{g./ml.}$ samples *B*5 and *N*1 at 20, 40, 80 and 160 $\mu\text{g./ml.}$, and sample *X* at 3, 6, 12 and 24 $\mu\text{g./ml.}$ Four experiments were carried out; in any one experiment each sample and the standard were tested at the lowest and highest concentrations on portions of one liver slice and at the two intermediate concentrations on portions of another slice. In this assay the slopes of the regression lines for all the samples and for the standard were not significantly different so that it is permissible to use the overall slope for calculation of relative potency.

The remaining samples (series *C*) were assayed during the development of this method. Statistical analysis of the results showed that the experiments only permitted the assay of each sample against the standard. However, the experiments treated in this way were well balanced, as each sample was tested simultaneously with the reference standard, at several concentrations, on liver slices from the same rabbit, and the results (except for one sample where the slope of the regression line was significantly

Table 5. *Relative glycogenolytic-factor potencies of insulin samples assayed by the multiple-dose method*

(For explanation of sample numbers see Table 3.)

Sample	Assay A			Assay C		
	Log ₁₀ relative potency (<i>M</i>)	Standard error of <i>M</i> (σ_M)	Relative potency (standard = 1) (10^M)	Log ₁₀ relative potency (<i>M</i>)	Standard error of <i>M</i> (σ_M)	Relative potency (standard = 1) (10^M)
B1	—	—	—	-1.0509	0.1723	0.09
B2	—	—	—	-4.4946	3.6592	0.003
B3	—	—	—	-0.6777	0.1141	0.21
B4	—	—	—	0.0139	0.0976	1.03
B4	—	—	—	-0.0293	0.1641	0.94
B5	-1.2005	0.0632	0.06	—	—	—
B7	—	—	—	-1.0091	0.5921	0.10
B9	—	—	—	-1.9392	0.8844	0.01
X	-0.3944	0.0632	0.40	—	—	—
BP	—	—	—	-0.7331	0.1943	0.18
K1	—	—	—	-0.6825	0.1721	0.21
N1	-1.3630	0.0632	0.04	—	—	—

different from that of the standard) are included in Table 5. One sample, B4, was assayed twice in this series of experiments and also in assay B (see below).

The multiple-dose assay method was also used (assay B) for testing insulin sample B4 and some fractions prepared from it by Dr G. R. Tristram. As the results give a further example of the use of the method they are reported in Table 6.

Table 6. *Assay of insulin sample B4, and of six fractions prepared from it, for glycogenolytic factor by the multiple-dose method*

Fraction no.	Log ₁₀ relative potency (<i>M</i>)	Standard error of <i>M</i> (σ_M)	Relative potency (10^M)
1	-0.6800	0.1234	0.21
2	-0.6344	0.1164	0.23
3	-2.2611	0.1415	0.005
4	-0.4265	0.1156	0.37
5	-1.5103	0.1135	0.03
6	-0.1992	0.1013	0.63
B4	-0.1262	0.1007	0.75

The statistical treatment of these assays is further considered by Mr N. W. Pleas in an Appendix to this paper. In assay C, another three fractions prepared by Dr G. R. Tristram (not shown in Tables 5 or 6) were tested, making up the total of thirteen test samples mentioned in the Addendum.

DISCUSSION

The single-dose method of assay can be carried out rapidly and gives a fair approximation of relative activity except for samples of very low or high glycogenolytic-factor content. In our experience the method suffers from two difficulties. Not only does the increase in glucose output due to the addition of either standard or test sample vary with

individual liver slices from the same rabbit, but the apparent activity of the test substance varies from rabbit to rabbit. Variation in glucose output from parts of a single slice was less than between different slices so that the technique would be improved by always assaying the standard and test sample on portions of the same slice.

Fig. 1. Glycogenolytic activity of insulin samples. Comparison of activity (single-dose method, standard = 100) and relative potency (multiple-dose method, standard = 1.0).

A more accurate assay of relative potency is obtained if samples and standard are tested at several concentrations, arranged so that the responses to samples and standard are of the same order, and the results examined statistically.

After most of our assays had been carried out, Sutherland, Cori, Haynes & Olsen (1949) described a modification of their original method in which

samples were tested at three or more concentrations, usually on the liver from only one rabbit. As we have pointed out, in our experience it is better to use liver from more than one rabbit for accurate results and we think statistical analysis is advisable as an indication of the degree of accuracy.

The numerical values for the activity of samples assayed by the two methods would not be expected to be the same, since in the single-dose method activities are compared at the same concentration of sample, but not of glycogenolytic factor, whereas in the multiple-dose method relative potency is assessed by comparison of concentrations giving similar responses. However, for samples tested by both methods, except for two with high activity, X and B4, there is a relation between the activity assayed by the single-dose method and the relative potency assessed by the multiple-dose method (Fig. 1).

Considerable variation was found in the amount of factor associated with the insulin samples tested. Samples prepared by one firm may contain different amounts of factor, e.g. of the ten samples from Boots Pure Drug Co. Ltd., two contained little or no factor, while others contained an appreciable amount. Two samples from this firm bore the same batch number (4840); of these, B3 (amorphous), received in 1948, contained only a small amount of factor whereas B4 (crystalline), received in 1949, contained about as much factor as the standard sample prepared by Eli Lilly. The reference standard (received in 1947) was the only sample prepared by Eli Lilly which we tested, but Sutherland *et al.* (1949) have since tested Lilly samples which were free from factor. One of the samples (N1) prepared by Novo Terapeutisk Laboratorium contained an appreciable amount of factor, al-

though the other four Novo samples tested were inactive. Two other samples (K2 and BW1), besides two of the Boots samples, were also free from factor.

This variability of samples prepared by the same firm from pancreas from the same species makes difficult the comparison of samples from the pancreas of different animals, but the results suggest there is not much difference in the factor content of pancreas of ox, sheep and pig. We had an opportunity of testing some insulin-inactive fractions, discarded during the manufacture of insulin by Boots, but none of them showed a higher factor content than the standard insulin.

SUMMARY

1. Two methods have been used for the assay of glycogenolytic factor in insulin samples. The first, or single-dose method, allows rapid testing of samples and gives an approximate estimate of activity; the second, or multiple-dose method, requires testing at several concentrations and statistical treatment of results.

2. Twenty-three commercial insulin samples have been assayed. The glycogenolytic-factor content was found to vary considerably; no samples were found to have a higher activity than the sample used as a standard (prepared by Eli Lilly and sent to us in 1947). Several were free from glycogenolytic factor.

We should like to express our thanks to Prof. F. G. Young, F.R.S., for his interest in this work and to him and to Dr A. C. Chibnall, F.R.S., for sending us samples of insulin for testing, as well as to the firms who supplied the samples. One of us (G.A.) is indebted to the Diabetic Association for the award of a Lund Fellowship.

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