Biological Assay of Glucagon in Rabbits

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Summary. Bioassay of glucagon based on the hyperglycaemic activity was investigated in normal rabbits. — It was shown that the index of precision (λ) derived from the dose response curve is about 1.2, which means that several hundred animals are required per assay in order to obtain a reasonably narrow confidence interval. Triple cross-over design improved the index of precision to 0.37, about 50 rabbits then being required to obtain a 95% confidence interval of $\pm 35\%$. The weight and sex of animals, as well as the fasting time and the route of administration of glucagon, were shown not to influence the quality of the assay. — Pretreatment of rabbits with cortisone improves the blood sugar response to glucagon. With a single dose of 25 mg of cortisone per rabbit, a considerable increase in the response to glucagon was observed with only a moderate elevation of the fasting blood sugar level. It was shown that the cortisone potentiation of the glucagon effect is at a maximum around 2–4 days after the cortisone administration, and that a cross-over assay can be performed within this period. — Further, it was shown that the cortisone treatment of the animals can be repeated several times without causing any changes in the glucagon response or in the animals as such. — Variables in an assay, such as sampling time, dose ratio, and response calculation, have been evaluated. — Based on these results, an assay procedure for the determination of the biological potency of glucagon employing cortisone-pretreated rabbits has been designed. — With this procedure, a 95% confidence interval of $\pm 20\%$ is ordinarily obtained in assays employing 32 rabbits.

Dosage biologique du glucagon chez des lapins

Résumé. Les auteurs ont étudié le dosage biologique du glucagon basé sur son activité hyperglycémisante chez des lapins normaux. — Il a été montré que l'index de précision (λ) établi d'après la courbe effet-dose est d'environ 1.2, ce qui signifie qu'il faut plusieurs centaines d'animaux par dosage pour obtenir un intervalle de confiance suffisamment étroit. Un plan expérimental de cross-over triple améliorait l'index de précision jusqu'à 0.37, il fallait alors 50 lapins environ pour obtenir un intervalle de confiance 95% de $\pm 35\%$. Il a été montré que le poids et le sexe des animaux, de même que la durée du jeûne et la voie d'administration du glucagon n'avaient pas d'influence sur la qualité du dosage. — Le traitement préalable des lapins à la cortisone améliore la réponse de la glycémie au glucagon. Avec une seule dose de 25 mg de cortisone par lapin, on observe une augmentation considérable de la réponse au glucagon avec seulement une élévation modérée du taux de la glycémie à jeun. Il a été montré que la potentialisation de l'effet du glucagon par la cortisone est à son maximum 2–4 jours après l'administration de cortisone et qu'un dosage en cross-over peut être

effectué pendant cette période. — En outre, il a été montré que le traitement des animaux par la cortisone peut être répété plusieurs fois sans provoquer de modifications dans la réponse au glucagon ou chez les animaux tels quels. — Les variables du dosage, telles que l'heure de prélèvement, le rapport des doses et le calcul de la réponse ont été évaluées. — Sur la base de ces résultats, on a élaboré un procédé de dosage pour déterminer l'activité biologique du glucagon, en employant des lapins traités préalablement à la cortisone. — Avec ce procédé on obtient généralement un intervalle de confiance 95% de $\pm 20\%$ dans des dosages utilisant 32 lapins.

Ein biologisches Glucagon-Bestimmungsverfahren am Kaninchen

Zusammenfassung. Die Autoren untersuchten den Nachweis für Glucagon auf Grund seiner hyperglykämisierenden Wirkung bei Kaninchen. Es konnte gezeigt werden, daß der Präzisionsindex (λ), wie er sich aus der Dosis-Wirkungskurve ergibt, bei 1.2 liegt, was bedeuten würde, daß mehrere hundert Kaninchen pro Versuch notwendig wären, um einen annehmbaren Vertrauensbereich zu erhalten. Eine Versuchsanordnung mit 3fachem „cross-over“ führte zu einer Verbesserung des Präzisionsindex auf 0.37; dabei waren 50 Tiere erforderlich, um den 95% Vertrauensbereich auf $\pm 35\%$ zu reduzieren. Es ließ sich zeigen, daß Gewicht und Geschlecht der Kaninchen, sowie die Dauer der Fastenperiode und die Applikationsart des Glucagons den Ausfall des Tests nicht beeinflussten. — Eine Vorbehandlung der Versuchstiere mit Cortison verstärkte die Blutzuckerreaktion auf Glucagon. 25 mg Cortison/Kaninchen führten zu einer deutlichen Verbesserung des Glucagon-Effektes auf den Blutzucker bei nur geringfügiger Erhöhung des Blutzucker-Nüchternspiegels. Die Verstärkung der Glucagonwirkung erreicht nach 2–4 Tagen ein Maximum und der cross-over Versuch kann während dieses Zeitraumes durchgeführt werden. Weiter ließ sich nachweisen, daß die Cortisonbehandlung der Tiere wiederholt werden kann, ohne zu Veränderungen des Glucagon-Effektes oder der Tiere selbst zu führen. — Der Einfluß, den geänderte Abnahmezeiten oder Variationen des Dosierungsverhältnisses bzw. die Berechnung des Effektes ausüben, wurde überprüft. — Auf Grund dieser Ergebnisse wurde eine biologische Bestimmungsmethode für Glucagon am mit Cortison vorbehandelten Kaninchen entwickelt. Mit diesem Verfahren läßt sich in der Regel ein 95% Vertrauensbereich von $\pm 20\%$ bei Verwendung von 32 Tieren erzielen.

Key-words: Glucagon, bio-assay, cortisone-treated rabbits, biological standardization, cross-over assay, glucagon patency estimation.

The production and clinical use of pancreatic glucagon have set a requirement for reliable biological methods usable for routine potency estimations of the hormone.

Attempts to elaborate such assay systems have been carried out, prior to the success of STAUB et al. [19, 20] in preparing crystalline glucagon, by SUTHERLAND and CORI [22] and by SUTHERLAND and DE DUVE

[23], who investigated pancreatic extracts in order to determine the hyperglycaemic principle in the gland. They employed a method using the ability of glucagon *in vitro* to stimulate glycogenolysis in rabbit liver slices. Modifications of this method have been elaborated by AUDY and KERLY [1], PLEASE [13], VUYLSTEKE et al. [27], DE DUVE and VUYLSTEKE [5], and TYBERGHEIM and WILLIAMS [24]. The most extensive investigation of this method has been published by VUYLSTEKE and DE DUVE [28]. The liver slice method, however, occasionally yields extreme variability in response, besides calling for a high degree of purity of the test and the standard preparations.

BERTHET et al. [4] used homogenates of dog or cat liver for a glucagon assay, measuring the activation of phosphorylase b. The method is reported to be more sensitive and more accurate than the liver slice method.

FODDEN and READ [7] and Ue et al. [25] have published a combination of the aforementioned techniques, in which glucagon is determined by incubation of liver sections, with subsequent homogenization and determination of phosphorylase activity.

A method employing a pure enzymatic system has been developed by MAKMANN and SUTHERLAND [12], who use the ability of glucagon to activate adenylyl cyclase, and determine the cyclase-activated formation of cyclic 3'5'-AMP from ATP.

ROWLINSON and LESFORD [16] proposed an assay *in vivo* based on the increase in blood sugar level subsequent to administration of glucagon to rabbits. Based on these results, a potency determination of glucagon as described by ROWLINSON and LESFORD will necessitate the use of about 300 rabbits to obtain a confidence interval ($P = 0.95$) of about $\pm 25\%$.

An assay in cats has been published by STAUB and BEHRENS [21] and SCIAN et al. [17]. They use anaesthetized cats, and administer the test substance and the glucagon standard by intravenous injections. The assay is performed according to a cross-over design with 2 h intervals. According to STAUB and BEHRENS [21] a confidence interval ($P = 0.95$) of about $\pm 20\%$ necessitates the use of 16 cats.

The use of chickens [2, 3] and of cortisone-primed adrenalectomized rats [10] in a glucagon assay has also been suggested.

Pretreatment of rabbits with cortisone has been used by HELMER [8] and by LAZARUS et al. [11] in connection with the demonstration of a glucagon content in insulin preparations. VOLK et al. [26] have suggested that the cortisone pretreatment of rabbits could be used in an assay of glucagon.

The purpose of the present study is to investigate and evaluate the possibilities of an assay *in vivo* of glucagon in rabbits based on the hyperglycaemic effect of the hormone. The investigations are focussed on improving the precision of the assay, e.g., by pretreatment of the animals with cortisone, in order to render it useful for a routine control of the biological activity of glucagon produced by pancreatic extraction.

Materials and Methods

Rabbits of the White Danish strain, weighing between 1.3 and 4.2 kg were used. They were fed 90 g of pellets once daily at 11 a.m., and had free access to water at all times. The animals were housed in individual cages in a room maintained at a constant temperature of $20 \pm 1^\circ\text{C}$ and a humidity of approximately 60%. The air was changed seven times per hour. Eighteen hours prior to commencement of experiments, the rabbits were moved to the experimental area with identical conditions. Between experiments, the rabbits were allowed a 15–30 day period of rest. Starvation periods of 5–18 h were employed in the individual experiments.

Blood samples of 0.1 ml were taken from a marginal ear vein, and the glucose content was determined with a Technicon Autoanalyzer, using HOFFMANN's ferricyanide method [9]. In all the experiments use was made of crystalline glucagon (Novo BD 63, 3 times recrystallized), which served as internal glucagon standard¹. Just prior to the commencement of the experiments, the hormone was dissolved in distilled water which had been acidified with hydrochloric acid to pH 3. The same medium was used in the dilution of stock solutions. When administered intravenously, the glucagon solution was injected in a volume of 0.5 ml into an ear vein. Subcutaneous injections were given dorsally in a volume of 1.0 ml. In the experiments where cortisone acetate was used, it was suspended in 0.5% carboxymethyl-cellulose (7 LF, Hercules) and administered intramuscularly.

The hyperglycaemic response was expressed as the differences (ΔBS_t) between the initial fasting blood sugar concentration and the blood sugar level measured at various intervals (t) after the injection of glucagon. Although the response is not a single number but a set of numbers where the ΔBS_t for each of the various intervals should be a parameter valid for use in an assay, it is preferred to calculate a single number that utilizes all the ΔBS_t 's. The sum or the weighted average of the individual ΔBS_t 's has been employed; the latter corresponds to the area between the fasting blood sugar level and the blood sugar versus time graph, where the individual points are connected by straight lines. For example, based on ΔBS at 10, 20, 40 and 80 min after the injection, the above-defined area would be:

$$10 \cdot \Delta BS_{10} + 15 \cdot \Delta BS_{20} + 30 \cdot \Delta BS_{40} + 20 \cdot \Delta BS_{80}$$

measured in blood sugar concentration units times minutes.

The quality of bioassays, where the response can be expressed as a linear function of the log-dose, is

¹ The internal glucagon standard has been assayed against the USP Glucagon Reference Standard by the assay procedure described in this paper. The potency estimation gave 101% with a 95% confidence interval of 93–111%.

usually judged by the standard deviation of the estimated log-potency ratio (m) between the test and standard; if the average responses obtained with the standard and test preparations are $\bar{y}_s$ and $\bar{y}_t$, respectively, and the true slope of the response-log-dose line is β , we have the following formula for m :

$$m = (\bar{y}_t - \bar{y}_s)/\beta.$$

The variance of m is equal to $\left(\frac{1}{N_T} + \frac{1}{N_S}\right) \sigma^2/\beta^2$, where N_T and N_S are the number of responses obtained with the test and standard preparations, respectively, and σ^2 is the variance of the response. From this it follows that with a given number of observations, the assay will be the more accurate (i.e., smaller standard deviation of m) the smaller the quantity

more detailed calculation of an assay using cross-over designs and Fieller's theorem for obtaining confidence limits, reference is made to standard textbooks such as SMITH [18] or FINNEY [6].

Results

1. *Untreated rabbits.* The question primarily investigated was whether controllable factors such as weight and sex of the animals, duration of fasting prior to the injection of glucagon, and the mode of injection could be shown to influence the hyperglycaemic response to glucagon in doses ranging from 5 to 500 μ g per rabbit. In these experiments, blood samples were taken immediately before, and 10, 20, 40, and

Table 1. Hyperglycaemic responses (ABS_t) obtained with different combinations of controllable factors

No. of rabbits	Sex	Average weight, kg	Hours of fasting	Glucagon dose μ g	Mode of injection	Hyperglycaemic response $\pm$ S.E.M. (mg/100 ml)			
						10 min	20 min	40 min	80 min
6	M	2.13	18	50	s.c.	33.5 $\pm$ 2.7	50.5 $\pm$ 5.2	51.7 $\pm$ 9.6	12.7 $\pm$ 11.6
6	M	2.05	18	50	i.v.	58.2 $\pm$ 10.9	77.2 $\pm$ 14.6	44.3 $\pm$ 13.1	3.2 $\pm$ 5.8
6	M	2.19	5	50	i.v.	52.7 $\pm$ 3.2	70.7 $\pm$ 4.2	55.0 $\pm$ 8.1	20.8 $\pm$ 4.8
3	F	2.11	18	50	i.v.	53.0 $\pm$ 4.6	73.3 $\pm$ 4.7	49.0 $\pm$ 2.3	10.3 $\pm$ 8.4
6	mixed	1.58	5	50	s.c.	32.2 $\pm$ 1.8	61.7 $\pm$ 7.5	64.0 $\pm$ 13.2	27.8 $\pm$ 3.8
6	mixed	2.45	5	50	s.c.	42.7 $\pm$ 4.5	62.0 $\pm$ 7.5	63.7 $\pm$ 10.1	23.2 $\pm$ 10.3
6	mixed	1.63	5	50	i.v.	42.5 $\pm$ 3.2	65.2 $\pm$ 6.5	39.8 $\pm$ 10.4	11.3 $\pm$ 8.8
6	mixed	2.71	5	50	i.v.	52.3 $\pm$ 5.9	82.2 $\pm$ 6.9	64.3 $\pm$ 5.7	16.3 $\pm$ 2.3

Fig. 1. Average hyperglycaemic responses $(10 \cdot ABS_{10} + 15 \cdot ABS_{20} + 30 \cdot ABS_{40} + 20 \cdot ABS_{80}) \pm$ S.E.M. to different doses of glucagon. The figures at the points are the number of animals in the corresponding groups

σ^2/β^2 , or σ/β . This means that the quantity $\lambda = s/b$, where s is the estimated standard deviation and b is the estimated slope in a given assay, will be an expression of the quality of the assay. Regarding the

80 min after the glucagon injection. Table 1 shows the average hyperglycaemic responses to 50 μ g of glucagon/rabbit with various combinations of the above-mentioned factors. It is seen that the hyperglycaemic

response after 10 and 20 min was lower following subcutaneous injection compared with intravenous administration, but the variation of the response does not seem to depend on the mode of administration. Neither the starvation periods of 5 to 18 h nor the weight of the animals in the range used had any influence on the response. Similar results were obtained at the dose levels of 5, 15.8, 158, and 500 $\mu\text{g}/\text{rabbit}$.

Fig. 1 shows the hyperglycaemic response to various subcutaneous glucagon doses expressed as the area defined previously. In this experiment, rabbits of both sexes and with a weight range of 1.7–2.6 kg have been used. Fasting period prior to the glucagon administration was 18 h.

The linear part of the fitted curve has a slope of 20.2 $\text{g} \cdot \text{min}/\text{l}$ (area units per $\log_{10}$ -dose) and the standard deviation of the response is 23.4 $\text{g} \cdot \text{min}/\text{l}$. This corresponds to a value for $\lambda = 1.16$, which means that several hundred animals must be employed in an assay in order to obtain reasonably narrow confidence limits on the estimated potency.

Some improvement can be made by utilizing the cross-over principle. In this way, the standard deviation can be appreciably reduced, as it is then measured as intra- rather than inter-animal variation as was the case in the dose-response analysis. Table 2 shows the

Table 2. Average "area" responses ($30 \cdot \Delta BS_{20} + 20 \cdot \Delta BS_{60}$) measured in $\text{g} \cdot \text{min}/\text{l}$ obtained in a triple cross-over assay of glucagon

No. of rabbits	June 13		June 17		June 20	
	Dose μg	Average response	Dose μg	Average response	Dose μg	Average response
12	2	4.3	20	13.6	2	4.1
12	20	18.5	2	3.8	20	20.2
12	2	4.8	20	15.8	2	6.0
12	20	18.1	2	4.2	20	14.6

average hyperglycaemic responses obtained in a triple cross-over assay, where the glucagon standard was assayed against itself. The two dose levels were 2.0 and 20 μg glucagon per rabbit. For practical reasons, only three determinations of the blood sugar concentration were performed: the first one just prior to the subcutaneous injection of glucagon, the second one 20 min after, and the third one 60 minutes after the injection. Other experimental conditions were as described in the preceding dose-response experiment. The response given in the table and used in the calculations is the hyperglycaemic "area" defined as $30 \cdot \Delta BS_{20} + 20 \cdot \Delta BS_{60}$ and expressed in $\text{g} \cdot \text{min}/\text{l}$.

Regarding the detailed calculations of such an assay see FINNEY [6]. The intra-animal standard deviation on a single response was 4.4 ($\text{g} \cdot \text{min}/\text{l}$). The slope was 11.9 ($\text{g} \cdot \text{min}/\text{l}$) per $\log_{10}$ -dose. This gave an improved value of $\lambda = 0.37$. An assay of the above size employing 48 animals in a triple cross-over design will give 95% confidence limits of about $\pm 35\%$.

2. *Cortisone-pretreated rabbits.* As mentioned previously [26], cortisone treatment of rabbits increases their sensitivity to glucagon. The cortisone effect, however, is sustained, meaning that the time that elapses between the cortisone treatment and the glucagon injection, as well as the size of the cortisone dose might be of importance.

Fig. 2. Average blood sugar concentrations $\pm$ S.E.M. after subcutaneous injection of 20 μg of glucagon into a group of 23 untreated rabbits and a group of 10 rabbits treated with 50 mg of cortisone per day for 5 days prior to the glucagon injection

Fig. 3. Average hyperglycaemic responses (ΔBS_{60}) $\pm$ S.E.M. to glucagon in groups of six rabbits at various intervals after a single dose of 25 mg cortisone given on day zero

The hyperglycaemic response to 20 μg of glucagon injected subcutaneously into rabbits pretreated for 5 days with 50 mg of cortisone per day is shown in Fig. 2,

which represents the average of 10 rabbits. For comparison, the hyperglycaemic response to 20 μ g of glucagon injected subcutaneously into untreated rabbits is included in the graph. It is seen that the blood sugar response, as well as the fasting blood sugar concentration, was appreciably increased in the cortisone-treated group. A similar result was obtained on days

the ΔBS_{60} showed a maximum at about 2 to 4 days after the cortisone injection, thereafter it gradually decreased. By comparison with Fig. 2, it can be inferred that the blood sugar response to glucagon is not diminished when the reduced cortisone load is used. The fasting blood sugar, however, was reduced to 140 mg/100 ml compared with 258 mg/100 ml in the

Fig. 4. Effects of four months of treatment with a weekly dose of 25 mg of cortisone. The dashed lines show the averages (6 rabbits) of weight, hyperglycaemic response to 2 μ g of glucagon given 2 days after the cortisone treatment, and fasting blood sugar concentration (blood sugar determinations performed at 0, 20 and 60 min after the glucagon injection). The dotted lines show similar responses in another group of 6 rabbits, instead of glucagon the animals were injected saline. The full lines show the corresponding responses in a control group of 6 rabbits not treated with cortisone, the glucagon responses were tested as in the first group

2 and 4 after termination of the cortisone treatment. A cortisone load of the above-mentioned size, however, is stressing for the animals, and their continuous use for assay might be doubtful. Therefore it was attempted to reduce the cortisone dose.

The glucagon response in relation to time was then investigated in rabbits given a single dose of 25 mg of cortisone. The result is shown in Fig. 3. As it appears,

5-days-cortisone-treated group. The duration of maximal cortisone effect indicated that a cross-over assay can be performed within this period, without a repeated cortisone treatment.

The influence of long-term cortisone treatment on the fasting blood sugar concentration, the glucagon response, and the weight of the rabbits is shown in Fig. 4. Two groups of rabbits received a weekly cor-

tisone injection of 25 mg per animal. In the first group, the hyperglycaemic response to 2 μ g of glucagon was tested two days after the cortisone injection. In the second group, the response to injections of a similar volume of saline was tested. A third group of rabbits, not treated with cortisone, received 2 μ g of glucagon only. The testing was repeated once a week over a period of four months. No reduction in the glucagon response was seen during this period. Both the fasting blood sugar concentration and the weight of the animals remained stable. It was noticed that the fasting blood sugar concentration in the cortisone-treated groups was only slightly elevated in contrast to the elevation seen in the rabbits treated for 5 days with cortisone (Fig. 2). Glucagon administration in a dose of 2 μ g to untreated rabbits, did not give an effect distinguishable from the effect seen after saline in the cortisone-treated animals.

The hyperglycaemic response to glucagon doses of from 2 to 64 μ g per animal was then investigated in rabbits pretreated with 25 mg of cortisone per animal two-and-a-half days before the glucagon administration. Animals of both sexes, weighing 2–2.5 kg, were used. The glucagon was given subcutaneously. The fasting period amounted to 18 h, and the "area" ($30 \cdot \Delta BS_{20} + 20 \cdot \Delta BS_{60}$) was used as a measure for the response. The results are shown in Fig. 5.

The linear part of the fitted curve has a slope of 76.9 g $\cdot$ min/l, which is almost 4 times as steep as that seen in the experiment with untreated animals. The standard deviation is 14.2 g $\cdot$ min/l, corresponding to a λ -value of 0.18. An assay performed according to a cross-over design will probably reduce this standard deviation. As it further appears from Fig. 5, it is possible to use a dose ratio of up to 5.

For the further evaluation of a biological assay of glucagon, the following procedure, based on the above-mentioned results, has been used.

Suitable, healthy rabbits weighing between 2.5 and 3.5 kg were kept in the stable for at least one week before use, and maintained on an adequate uniform diet.

Forty-eight hours before the commencement of the assay, the animals were injected intramuscularly with 1 ml of a suspension of cortisone acetate containing 25 mg/ml.

Food was withdrawn from the rabbits eighteen hours prior to the first test day; they were allowed free access to drinking water. Then, after the last blood sample had been drawn, they were fed again until 18 hours before the second test day.

The assay was performed according to the standard twin cross-over design with two dose levels [18]. The rabbits were divided randomly into four equal groups, and injected subcutaneously with glucagon in doses of, e.g., 6 and 24 μ g of glucagon per animal of the standard and the test. Just before, and at 20 and 60 min after the glucagon injection, blood samples were drawn from a marginal ear vein for blood sugar deter-

mination. The second part of the assay was made on the day after the first injection.

The response was calculated for each rabbit as the "area" ($30 \cdot \Delta BS_{20} + 20 \cdot \Delta BS_{60}$). The potency and confidence interval were determined according to standard methods for twin cross-over assays [18]. The time required between the two parts of a cross-over assay has been examined further. Assays performed with a 4 h interval between the two parts of the test, with no feeding of the animals in between, and assays with a 6 day interval between the two test parts have been compared with assays carried out with the above-mentioned procedure. The results appear in Table 3,

Fig. 5. Average hyperglycaemic responses ($30 \cdot \Delta BS_{20} + 20 \cdot \Delta BS_{60}$) $\pm$ S.E.M. in groups of 12 rabbits to various doses of glucagon. The rabbits were given 25 mg of cortisone 48 h prior to the glucagon injection

from which it is seen that a cross-over assay can be performed with an interval as short as 4 h without any overlapping of the responses or any appreciable loss in precision.

It has been examined whether sampling at 0, 60, and 90 min after the glucagon injection resulted in a higher precision than sampling at 0, 20 and 60 min. Table 4 shows the results of three assays where blood samples were taken at 0, 20, 60 and 90 min after the glucagon injection, and separate calculations have

Even if in view of the quality of the assay the initial sample does not contribute to the precision of the potency estimate, it provides a control of the homogeneity of the animal material used.

For the routine assay of glucagon, it was decided to modify the calculation of the response by omitting

Table 3. Average blood sugar concentrations at 20 and 60 min after glucagon injections in twin cross-over assays. The standard errors include the inter-animal variation, whereas the λ -values are calculated on the basis of the intra-animal variation. With 5 and 24 h intervals, only one cortisone treatment was given 2 days preceding the first glucagon injection. With the one week interval the cortisone treatment was given 2 days before each glucagon injection

		Average blood sugar concn. $\pm$ S.E.M.						λ -value
	Glucagon dose μ g	No. of rabbits	1. injection		2. injection			
			20 min	60 min	20 min	60 min.		
5 h interval	4	16	168 $\pm$ 8	166 $\pm$ 11	142 $\pm$ 4	137 $\pm$ 4		
	20	16	237 $\pm$ 6	289 $\pm$ 11	226 $\pm$ 4	243 $\pm$ 11	0.16	
	6	16	204 $\pm$ 9	212 $\pm$ 11	164 $\pm$ 8	156 $\pm$ 11		
24 h interval	24	16	229 $\pm$ 8	284 $\pm$ 13	222 $\pm$ 5	267 $\pm$ 8	0.14	
	4	16	150 $\pm$ 6	148 $\pm$ 7	168 $\pm$ 8	166 $\pm$ 11		
1 week interval	20	16	212 $\pm$ 5	231 $\pm$ 12	237 $\pm$ 6	289 $\pm$ 11	0.16	

Table 4. Results of three assays of glucagon each utilizing 32 rabbits in a twin cross-over design. Blood sugar concentrations were determined just prior to, and 20, 60, and 90 min after the glucagon injection. For each assay, the calculations of the λ -value and the 95% confidence interval were made on the basis of either the blood sugar values from 0, 20, and 60 min or from 0, 60, and 90 min. The blood sugar values were in both instances combined ("area"-response).

Date of assay	Blood sugar concentrations 0 — 20 — 60 min		Blood sugar concentrations 0 — 60 — 90 min	
	λ -value	confidence interval	λ -value	confidence interval
17.6	0.15	84—119%	0.22	77—130%
25.6	0.15	84—119%	0.22	78—129%
28.6	0.18	81—124%	0.28	72—139%

been performed on the 0, 20 and 60, and on the 0, 60 and 90 min values. It appears that the maximal precision is obtained by sampling up to 60 min after the glucagon injection.

Table 5 shows the results of a series of assays in which the blood sugar concentrations at 20 and 60 min (BS_{20} and BS_{60}) were used as the response, either alone or after subtraction of or division by the fasting blood sugar concentration. The sum of the two blood sugar concentrations and the "area" were also used as responses in a similar way. As can be seen, the highest precision is obtained by using the combined responses. Moreover, it is seen that the 20 min and the 60 min samples give approximately equal contributions to the precision.

The highest precision with combined responses was obtained in all thirteen assays without the correction for the fasting blood sugar concentration. Therefore, the samples at zero time can be omitted with an advantage for the assay.

Table 5. Average λ -values obtained from calculations on 13 twin cross-over assays of glucagon. In each of the 13 assays, the twelve different definitions of the blood sugar response were used to estimate λ

Response	Average λ -value	min λ	max λ
BS_{20}	0.21	0.14	0.42
ΔBS_{20}	0.21	0.15	0.35
BS_{20}/BS_0	0.23	0.16	0.36
BS_{60}	0.17	0.12	0.25
ΔBS_{60}	0.20	0.14	0.29
BS_{60}/BS_0	0.21	0.16	0.28
$BS_{20} + BS_{60}$	0.16	0.11	0.22
$\Delta BS_{20} + \Delta BS_{60}$	0.18	0.12	0.25
$(BS_{20} + BS_{60})/BS_0$	0.19	0.13	0.28
$30 \cdot BS_{20} + 20 \cdot BS_{60}$	0.16	0.11	0.22
$30 \cdot \Delta BS_{20} + 20 \cdot \Delta BS_{60}$	0.18	0.12	0.26
$(30 \cdot BS_{20} + 20 \cdot BS_{60})/BS_0$	0.19	0.13	0.28

the fasting blood sugar value, and using the sum of the blood sugar values at 20 and 60 min after the glucagon injection.

Fifty glucagon assays, with 32 rabbits each, performed according to this procedure during the period 1966—1968, gave an average 95% confidence interval from 83 to 120%. This corresponds to an average λ -value of 0.16.

The influence of insulin contamination of the glucagon to be assayed was tested by assaying a glucagon preparation to which varying amounts of insulin were added. The glucagon standard used had an insulin content of 0.2%. This amount is included in the figures for added insulin in Table 6, from which it appears that an insulin content of up to 10% does not have

any disturbing influence on the potency determination of a glucagon preparation. This is in accordance with previous findings [26], where cortisone-pretreated rabbits have been used for estimation of a glucagon content in commercial insulin preparations.

sufficient for use in a glucagon bioassay. The effect is dependent on the lapse of time after the administration of cortisone (Fig. 3), so that potentiation of glucagon response is at its maximum 2–4 days after intramuscular administration of cortisone. Thus the,

Table 6. Results of a triple cross-over assay with one standard and two tests performed on three consecutive days with rabbits treated with cortisone two days before the first glucagon injection. The two test preparations were the glucagon standard + 2% or 10% insulin. The potency calculations were based on the sum of BS_{20} and BS_{60} without correction for BS_0 . The λ -value of the assay was 0.22

	Average blood sugar concn. $\pm$ S.E.M. in groups of 17 or 18 rabbits			Potency with 95% confidence limits
	0 min	20 min	60 min	
6 μ g glucagon standard	114 $\pm$ 3	168 $\pm$ 6	177 $\pm$ 11	
24 μ g glucagon standard	117 $\pm$ 3	201 $\pm$ 6	254 $\pm$ 10	
6 μ g glucagon + 0.12 μ g insulin	114 $\pm$ 3	167 $\pm$ 4	176 $\pm$ 8	
24 μ g glucagon + 0.48 μ g insulin	116 $\pm$ 4	199 $\pm$ 6	258 $\pm$ 6	99.2% (80.9 – 121.6%)
6 μ g glucagon + 0.6 μ g insulin	116 $\pm$ 4	177 $\pm$ 6	187 $\pm$ 13	
24 μ g glucagon + 2.4 μ g insulin	119 $\pm$ 5	201 $\pm$ 6	268 $\pm$ 10	114.0% (93.1 – 139.9%)

Discussion

The use of untreated rabbits for biological assays of glucagon has proved to be unsuitable in the present experiments because the response was too low and there was too much intra- and inter-animal variation. This is in accordance with previous publications in which normal rabbits have been used for biological determination of glucagon [16, 8, 11, 26].

It is well known that cortisone increases the glycogen content of the liver. On the basis of the assumption that an increased glycogen depot in the liver will improve the glucagon response, the utilization of cortisone-pretreated rabbits in measurements of glucagon has been investigated by HELMER and ROOT [8] and by LAZARUS and VOLK [11, 26].

From the findings of Helmer and Root it is concluded, however, that the response to a certain dose of glucagon is not increased through pretreatment of the rabbits with cortisone (20 mg/animal for 3 days); only an increment in the fasting blood sugar concentration is found. ACTH pretreatment, on the other hand, was found in the studies to potentiate the effect of glucagon.

VOLK et al. found a clear potentiation of the glucagon effect with a cortisone-pretreatment dosage of 50 mg/day for 10 days [11] and, in a later investigation, with 5 mg/kg for 7 days [26]. According to these authors, the discrepancy is due to the dose level of cortisone and the extended period of treatment.

The present investigation shows that the use of a single dose of 25 mg cortisone per rabbit ($\sim$ 10 mg/kg) gives a clear potentiation of the glucagon effect *in vivo*,

duration of cortisone effect is long enough to permit a cross-over assay with an interval of 24 h, starting 2 days after a single cortisone injection.

In our experiments, the increase in blood sugar in cortisone-treated rabbits was at maximum around 60 min after a subcutaneous glucagon administration. The lack of potentiation observed by HELMER and ROOT [8] may be due to the late sampling times (90 and 120 min) that had been used. At these time intervals, hardly any glucagon effect could be registered in the present study.

With the present assay procedure, repeated cortisone treatments over a period of several months seem neither to change the size of the glucagon response in the rabbits nor to influence the animals in a way that would prohibit their continuous use. This is considered important for the routine set-up of a glucagon assay. In our laboratory, the same animals are used at average intervals of 3 weeks for over a year.

The assay procedure described does not take full advantage of the experimental results. A dose ratio of 5, which — as it appears from Fig. 2 — should be usable, is in the assay procedure set to 4. This is due to the fact that, seasonal variations for example, and variations in the rabbit material supplied can give rise to changes in the position of the dose response curve. This will interfere with a potency estimation, especially when the potency of the preparation to be assayed differs more than 10–20% from that of the glucagon standard. This risk is diminished when the dose ratio is decreased.

The minimum effective dose in the present experiment is about 2 μ g of glucagon/rabbit, and the maxi-

mum effective dose is about 50 μ g. Accordingly, the doses usable in an assay will be of the order of 6 and 24 μ g for low and high dose, respectively.

The investigation of the time interval required between the two parts of the test showed that an interval of as little as 4 h is sufficient. Although the cross-over then, can be performed on the same day, it has been decided to use a time lapse of 24 h between the two parts of the test. This is justified by the fact that the intra-animal variation is about the same irrespective of whether the responses are measured on the same day or with an interval of up to 6 days (Table 3), and because, for practical reasons, it is more convenient to perform the two parts of the assay on separate days.

Investigation of the influence of a contamination with insulin of the glucagon preparation to be assayed, showed that a content of at least up to 10% insulin does not interfere with the potency determination. This insulin content is believed to be far greater than the contamination that will exist in samples labelled "glucagon".

The finding corresponds to what is known from the insulin assay, i.e., that glucagon contamination does not invalidate the biological assay of insulin preparations [15].

The assay procedure presented here is primarily designed for the control of the biological potency of glucagon preparations; a reasonable amount of material (~ 1 mg) is always available in this situation. The principle for the assay, however, can possibly be used in an assay of glucagon where mice are the experimental animals. Blood samples from each mouse are easily obtained from the orbital venous plexus [14]. The amount of glucagon needed for a potency estimation will thereby be reduced to 20–40 μ g.

The glucagon assay described in the present paper yields a precision which is comparable with what is ordinarily obtained in biological assays, e.g. of insulin, performed in mice or in rabbits according to the procedures given in official formula indices (e.g. British Pharmacopoeia 63).

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