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Effect of glucagon antibodies on plasma glucose, insulin and somatostatin in the fasting and fed rat

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Summary. A potent high-titre glucagon antibody pool was used to induce a state of acute glucagon deficiency in order to investigate the importance of glucagon in maintaining euglycaemia in the fed and fasted anaesthetised rat. Binding characteristics of the antiserum and evidence of its neutralisation of the biological effects of exogenous glucagon are described. The amount of antibody administered was capable of neutralising up to 12 times the total content of glucagon (approximately 1nmol) in the rat pancreas. The hyperglycaemic response to 1.43 nmol exogenous glucagon was significantly inhibited in the rat by glucagon antibodies given intravenously or intraperitoneally ($p < 0.001$). However, no changes in plasma glucose occurred in rats fasted 16 h (4.35 ± 0.1 mmol/l or 24 h (4.0 ± 0.05 mmol/l) after antibody administration. The same dose of glucagon antibodies produced no change in plasma glucose (6.1 ± 0.2 mmol/l), immunoreactive insulin (1.85 ± 0.05 μg/l) or immunoreactive somatostatin (110 ± 30 ng/l) in rats after antibody administration. Antibody excess, equivalent to a binding capacity for glucagon of 40 nmol/l in the plasma of recipient animals, was demonstrable at all times after passive immunisation. The absence of any affect on glucose concentrations following immunoneutralisation of glucagon suggests that glucagon secretion may not be a major factor in the maintenance of euglycaemia in the rat.

Key words: Glucagon antibodies, euglycaemia, glucose, insulin, somatostatin, rat.

The use of insulin antiserum to induce acute insulin deficiency [1, 2] was one of the first examples of the exploitation of the specificity of the antibody-antigen reaction as a means of neutralising circulating hormones in vivo. Subsequently, antisera to other hormones, including anti-secretin [3], anti-gastric inhibitory polypeptide (GIP) [4], anti-gastrin [5] and anti-somatostatin [6], have been used to produce acute hormone deficiencies specifically, non-surgically and non-pharmacologically in order to study their effects.

Several attempts have been made to produce acute glucagon deficiency using glucagon antibodies [7-10]. The results have been equivocal. In one published account a high titre antiserum had no effect on glucose or insulin concentrations when given to either fed or fasted normal rats [10]. An acute blood glucose-lowering effect of glucagon antiserum was, however, observed in 48-h fasted adrenalectomised rats by Grey et al. [7] though not confirmed by Holst et al. [10]. The importance of glucagon for the maintenance of blood glucose levels in the fasting state remains therefore to be established.

In the present study, an attempt was made to induce acute glucagon deficiency by neutralising endogenous glucagon with an antiserum of higher avidity and potency than those used previously and its effects on basal glucose, insulin, glucagon and somatostatin concentrations were monitored.

Materials and methods

Experimental animals

Male wistar rats (150-200 q) were used throughout the study. All experiments were performed on animals anaesthetised with sodium pentobarbitone (Sagatal, May & Baker, Dagenham, UK; 6 mg/100 g body weight). Each group consisted of six rats, and blood samples were obtained by tail vein bleeding.

Glucagon antiserum

A C-terminal reactive, rabbit antiserum to porcine glucagon (Guildhay Antisera, Guildford, UK) was used throughout the study. An antiserum pool was prepared from several bleeds from the same animal and analysed for specificity and titre. It did not cross-react with insulin, gastrin, secretin, GIP or vasoactive intestinal polypeptide (VIP) and had minimal cross-reactivity (6%) with gut glucagon-like immunoreactants (gut-GLI, 10,000 μg/l). The absence of any insulin

binding capacity in the glucagon antibodies was shown not only by the lack of crossreactivity with unlabelled insulin, but also by the absence of any binding with ^{125}I-insulin. This was considered to be important because the presence of insulin antibodies in the glucagon antiserum might influence pancreatic function directly and would undoubtedly interfere with plasma insulin measurements by radioimmunoassay. The recovery of exogenous insulin added to plasma containing anti-glucagon immunoglobulin was also demonstrated. An immunoglobulin fraction of the antiserum was prepared by ammonium sulphate precipitation and dialysed against saline (0.154 m) over 3 days at 4 °C. The avidity and binding capacity of the resulting antiglucagon immunoglobulin pool were assessed by Scatchard analysis.

Total antibody-bound glucagon originating in the immunised rabbits

In an earlier study, Holst et al. [10] had demonstrated the presence of high concentrations of bound glucagon in their antiserum pool. We therefore assessed, using an acid-ethanol extraction procedure [10], the concentration of antibody-bound glucagon in the immunoglobulin fraction of the undiluted antiserum pool as well as in the immunoglobulin fraction obtained after 3 days dialysis and used in subsequent immunoneutralisation experiments.

Time course binding study (in vitro)

To determine whether the amount of anti-glucagon immunoglobulins infused into rats was sufficient to bind endogenous or exogenous plasma glucagon, a kinetic study on the rate of binding of glucagon to anti-glucagon immunoglobulins was performed.

Equal volumes of glucagon antibodies - at three different dilutions (1:1000, 1:5000, 1:7500) - rat hormone-free plasma, and porcine glucagon dissolved in rat hormone-free plasma (714pmol/l) were incubated at 37 °C. The reaction was stopped by the addition of ice-cold ethanol to a final concentration of 80% (vol/vol) at different time intervals. After 30-min centrifugation (1000 *g*), the supernatent was decanted, lyophilised and the samples reconstituted in 0.04 M phosphate buffer, pH 7.4, and assayed for "free-glucagon". With 80% ethanol, more than 70% of the "free glucagon" was recovered, whilst antibody-bound glucagon was effectively precipitated and did not leak glucagon [11]. Glucagon bound by antibodies was determined by substracting the amount of "free-glucagon" recovered from the total amount of glucagon added. The results are expressed as "percentage bound" glucagon.

Neutralisation of exogenous glucagon by glucagon antibodies

Three groups of fed rats were treated as follows: group A ($n=6$) received 0.5 ml normal rabbit immunoglobulins intravenously and 10 min later were given 1.43 nmol porcine glucagon by the same route (Eli Lilly, Indianapolis, Indiana, USA).

Rats in group B ($n=6$) were given 0.5 ml antiglucagon immunoglobulins intravenously and 10 min later received glucagon (1.43 nmol) by the same route. Group C rats ($n=6$) were injected with 0.5ml saline intravenously, followed 10 min later by anti-glucagon immunoglobulins (0.5 ml) pre-incubated with glucagon (1.43 nmol) for 15 min at 37 °C in a water bath. Blood samples, collected at different time intervals after the injection, were assayed for glucose.

Neutralisation of endogenous glucagon in fasted rats

Two groups of rats were fasted - one for 16 h ($n=6$) and another for 24 h ($n=6$). Each group received 0.5 ml of anti-glucagon immunoglobulins and the animals were bled at different time intervals. Samples were analysed for plasma glucose and insulin concentrations and for anti-glucagon antibody titre.

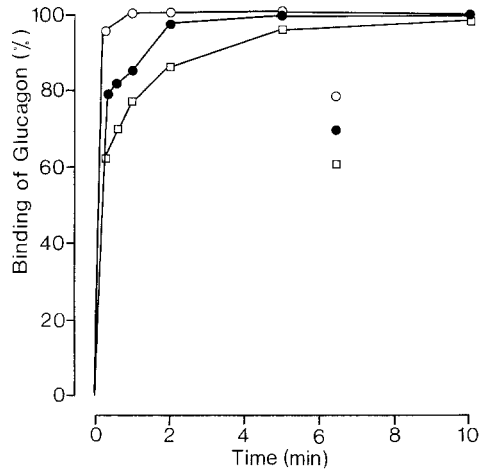

Fig. 1. Time course of binding of glucagon antibodies to glucagon at 37 °C. Antibody dilutions of 1:1000 (○), 1:5000 (●) and 1:7500 (□) were incubated with 714 pmol/l glucagon in rat hormone-free plasma at 37 °C. The reaction was stopped with ethanol and free glucagon assayed by radioimmunoassay. Results are expressed as percentage binding of total glucagon added

Plasma anti-glucagon concentrations following intravenous and intraperitoneal administration of antibodies

Plasma samples obtained at different time intervals after intravenous and intraperitoneal administration of anti-glucagon immunoglobulins (0.5 ml/200 g rat) were serially diluted, incubated with freshly prepared ^{125}I-glucagon and assessed for titre and percentage binding of unbound antibodies. The determination of antibody binding of glucagon by undiluted rat plasma was made by extrapolating the percentage binding of the antibody to zero dilution [4]. From this, the excess glucagon binding capacity in plasma at each time point was determined according to Scatchard analysis of the data.

Plasma glucose, insulin, somatostatin and glucagon following intravenous and intraperitoneal administration of antibodies

Plasma samples, obtained at different time intervals after intravenous and intraperitoneal antibody administration to fed rats, were extracted by the addition of ice-cold ethanol (80%, vol/vol). After centrifugation (1200 *g*) aliquots of the supernatent were lyophilised, reconstituted in 0.04 M phosphate buffer, pH 7.4 and measurement of free glucagon and somatostatin performed by radioimmunoassay. Plasma glucose and insulin measurements were also performed.

Chemical analyses

Plasma glucose was measured using a glucose oxidase method (Beckman glucose analyser). Immunoreactive insulin (IRI) was determined by radioimmunoassay using a dextran-charcoal separation with an antiserum raised against bovine insulin (Guildhay Antisera, Guildford, UK), iodinated bovine insulin (Amersham International, Amersham, Bucks, UK) and a rat insulin standard (Novo, Copenhagen, Denmark). Sensitivity of the assay was 0.3 μg/l and the intra and interassay coefficients of variation were 5.0% ($n=14$), and 6.6% ($n=14$) respectively. Pancreatic glucagon was measured by radioimmunoassay using a C-terminal reactive antiserum. This was specific for glucagon with a 1% cross-reactivity with gut GLI. Porcine glucagon (Novo) was iodinated according to the method of Jorgensen and Larsen [13]. The standard used was the WHO first International Standard for Glucagon, (69/194, Medical Research Council National Institute for

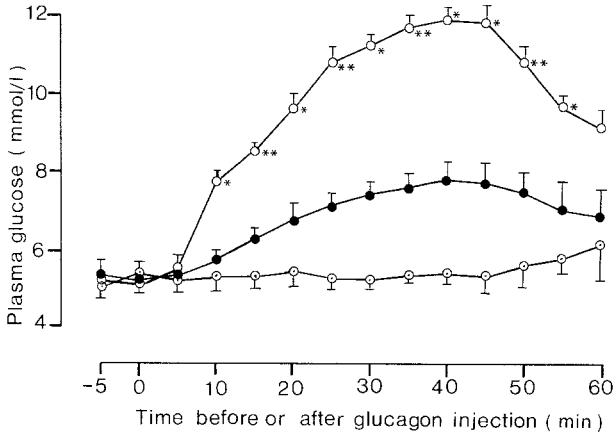

Fig. 2. Effect of immunoneutralisation of exogenous glucagon on plasma glucose levels in fed rats. Group A ($n=6$, ○) received normal rabbit immunoglobulins and 10 min later 1.43 nmol glucagon. Group B ($n=6$ ●) received glucagon antibodies and 10 min later 1.43 nmol glucagon. Group C ($n=6$, ⊙) were injected with saline and 10 min later with the same amount of glucagon antibodies pre-incubated with 1.43 nmol of glucagon. Results are mean ± SEM ($n=6$). Statistical analysis were performed comparing groups A and B (*$=p<$ 0.01; **$=p<$ 0.001)

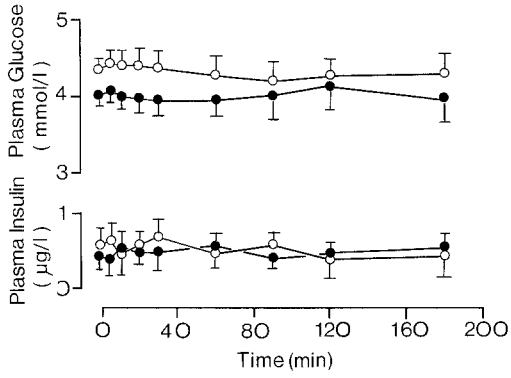

Fig. 3. Effect of glucagon antibodies on plasma glucose and plasma insulin levels in rats fasted for 16 h ($n=6$, ○) or 24 h ($n=6$, ●) prior to injection. Results are mean ± SEM ($n=6$)

Biological Standards and Controls, London, UK). A sequential assay system was employed and plasma samples were extracted with ethanol before assay [11]. Sensitivity of the assay was 60 ng/l and the intra and interassay coefficients of variation were 7.3% ($n=12$) and 15.2% ($n=10$), respectively.

Immunoreactive somatostatin was measured using an antiserum raised against synthetic cyclic somatostatin-14 (Guildhay Antisera, Guildford, UK). The antiserum was specific for somatostatin-14 with a 10% cross-reactivity for somatostatin-28. Synthetic cyclic-somatostatin-14 (Bachem, Marina Del Rey, California, USA) was used as standard and, for the preparation of the tracer, ^{11}Tyr-somatostatin (Bachem) was used. The tracer was purified on octadecylsilyl silica before use. Separation was achieved using dextran-charcoal. Assay sensitivity was 40ng/l with intra and interassay coefficients of variations of 7% ($n=9$) and 15% ($n=10$), respectively.

Statistical analyses

A two-tailed Student's t-test for unpaired data were used. Results are expressed as mean ± SEM ($n=6$).

Results

Anti-glucagon immunoglobulins

The titre of the pool of antiserum was determined to be 1:180,000. Increasing amounts of unlabelled glucagon and 18 pg ^{125}I-glucagon were incubated with antibodies in a volume of 0.45 ml and free and bound hormone separated by second antibody precipitation. The addition of 20 pM glucagon significantly lowered the binding of the tracer ($p<0.001$). From the binding data, Scatchard analysis was performed. The non-linear shape of the Scatchard plot indicated the presence of two classes of glucagon binding sites. The higher affinity class of antibodies was calculated to have an equilibrium constant of 2.9×10^{10} l/mol and a binding capacity of 6030 nmol/l. The lower affinity class of antibodies had an equilibrium constant of 3.7×10^{9} l/mol and a binding capacity of 20.16 µmol/l. The total amount of glucagon present in the antiserum before and after 3 days of dialysis against saline as determined by acid ethanol extraction was 73 nmol/l and 79 nmol/l, respectively. These values are lower than those reported by other workers (i.e. 460 nmol/l [10]) and, should dissociation of glucagon from its antibody have occurred in vivo after administration, would have led to a twenty-threefold increase in plasma glucagon concentration in the recipient animals. It is, however, extremely unlikely that significant dissociation would have occurred, given our findings that the amount of antibody-bound glucagon present in the antiserum after 3 days dialysis was similar to that present before dialysis. When the antibodies are coupled to a solid support, bound glucagon can only be eluted from the antibodies using a glycine-HCl buffer at pH 2.8 and not with a phosphate-saline buffer, pH 7.4 at temperatures up to 50 °C. Furthermore, after administration of the antibodies in vivo and extraction of the free glucagon from the plasma, no significant rise in free circulating glucagon is observed. These observations suggest that the glucagon was very tightly bound to the antibody and effectively neutralised.

Time course binding of glucagon antibodies

At 37 °C, anti-glucagon immunoglobulins, at dilutions of 1:1000, 1:5000 and 1:7500, bound 95%, 80% and 62% of the total glucagon added within 20 s of mixing (Fig. 1). By 2 min, antiserum diluted 1:5000 and 1:7000 had bound 98% and 87% of the glucagon present. Complete binding was achieved within 1 min by antiserum diluted 1:1000.

Neutralisation of exogenous glucagon by glucagon antibodies

Glucagon administration to fed rats given normal rabbit immunoglobulins (group A) produced a marked rise in

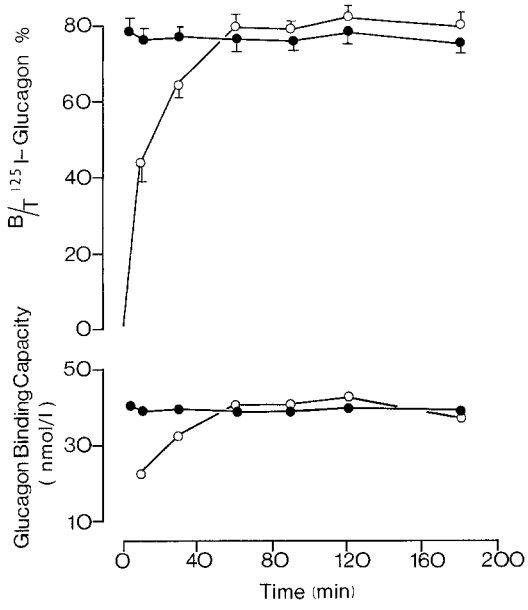

Fig. 4. Plasma antiglucagon levels and binding capacities in fed rats following antibody adminstration via intravenous (●) and intraperitoneal (○) routes. Samples collected at each time point were serially diluted and incubated with 18 pg ^{125}I-glucagon at 4 °C and the percentage binding of neat plasma obtained by extrapolating to zero dilution. From these data, the excess binding capacities at each time point were determined. Results are expressed as mean ± SEM ($n=6$)

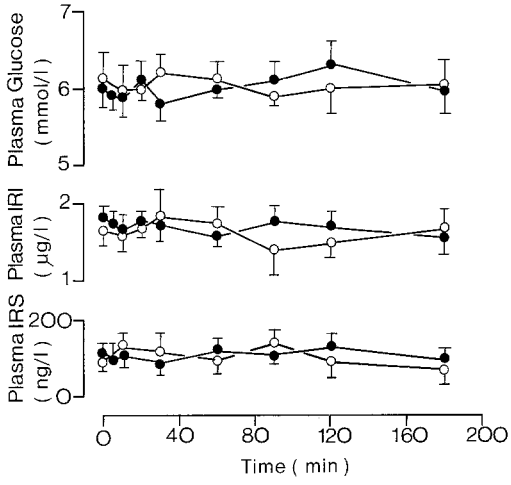

Fig. 5. Effect of glucagon antibodies on plasma glucose, immunoreactive insulin and immunoreactive somatostatin (IRS) concentrations in fed rats following intravenous (●) and intraperitoneal (○) administration of antibodies. Results are mean ± SEM ($n=6$)

blood glucose concentration that persisted for 60 min (Fig. 2). Animals that had received glucagon antibodies (group B) 10 min before the glucagon injection had significantly less hyperglycaemia ($p<0.001$ at 35 min), whilst still showing a significant rise in blood glucose concentration over basal levels. Injection of glucagon antibodies pre-incubated with glucagon for 15 min at 37 °C to animals in group C did not produce any significant change in plasma glucose values.

Neutralisation of endogenous glucagon in fasted rats

Plasma glucose and plasma IRI were measured in two groups of 16 and 24 h-fasted rats (Fig. 3). There was no significant change in plasma glucose concentration during this period in either group (plasma glucose: 4.35 ± 0.1 and 4.0 ± 0.05 mmol/l respectively). Plasma IRI levels did not change significantly over this period (basal plasma IRI: 0.55 ± 0.1 and 0.45 ± 0.1 μg/l respectively). Plasma glucagon concentrations after fasting and before administration of antibodies were 111 ± 26 ng/l for the 16-h fasted animals and 126 ± 23 ng/l for the 24-h fasted rats. Antibody was present in excess at all time points for the next 180 min as determined by the percentage binding to ^{125}I-glucagon (range 75–80% Bound/Total; equivalent to an available binding capacity for glucagon of approximately 40 nmol/l).

Plasma anti-glucagon concentration following administration of glucagon antibodies to fed rats

Intravenous administration of antibodies resulted within 5 min in high antibody binding to ^{125}I-glucagon (Fig. 4). Antibody was present in excess at all time points and at 180 min the percentage binding of ^{125}I-glucagon was 75% of total counts. This was equivalent to a binding capacity for glucagon of approximately 38nmol. The titre of anti-glucagon in the plasma of recipient animals was around 1:1000 throughout the time of study.

The rate of absorption into the circulation of antibodies administered intraperitoneally is also shown in Figure 5. Maximum binding to ^{125}I-glucagon was observed between 45–60 min, by which time percentage binding to ^{125}I-glucagon was 80%; - equivalent to a binding capacity for glucagon of approximately 40 nmol/l. Antibody titre from 45 min onwards approached that achieved after intravenous administration, i.e. 1:1000 and was maintained throughout the period of study.

Plasma glucose and pancreatic hormone concentrations following antibody administration

Plasma glucose, IRI and immunoreactive somatostatin profiles after intravenous and intraperitoneal injection of anti-glucagon immunoglobulins are shown in Figure 5. There were no significant changes in plasma glucose (basal 6.1 ± 0.3 mmol/l), plasma IRI (basal 1.85 ± 0.05 μg/l) or plasma immunoreactive somatostatin (basal 110 ± 30 ng/l) concentrations after intravenous administration of glucagon antibodies nor did intraperitoneal injection of glucagon antibodies produce any change in plasma glucose (6.0 ± 0.2 mmol/l), plasma IRI (1.65 ± 0.15 μg/l) or plasma immunoreactive somatostatin (95 ± 15 ng/l). Basal free glucagon concentrations were undetectable after either intravenous or intraperitoneal antibody administration (detection limit = 60 ng/l).

Discussion

It has long been supposed, mainly on the basis of its known physiological effects, that glucagon is concerned with the maintenance of normoglycaemia in the post absorptive state. The evidence, however, is not entirely conclusive, and indirect. Studies in which glucagon deficiency was induced by infusion of pharmacological amounts of somatostatin have been taken to suggest that glucagon secretion is important in maintenace of euglycaemia because the fall in blood glucose concentration observed under these conditions can be reversed by exogenous glucagon [14–16]. This approach has its problems, however, since somatostatin affects a variety of tissues apart from the endocrine pancreas - including the pituitary, gut and nervous tissues - and is known also to alter portal blood flow and the hepatic response to glucagon and adrenaline in vivo [17]. Moreover, a fall in blood glucose concentration is a far from invariable consequence of somatostatin infusion and rises in blood glucose concentration also occur [18].

Another approach to the problem of producing and observing the effects of acute glucagon deficiency is to produce immunoneutralisation of endogenous glucagon by passive immunisation with glucagon antibodies. Despite its apparent simplicity previous attempts to use this technique have yielded conflicting results possibly due to incomplete neutralisation of circulating glucagon [10]. We decided, therefore, to demonstrate the potency both in vitro and in vivo of the antiglucagon immunoglobulins used to induce a state of glucagon deficiency in recipient animals prior to undertaking a study of the biological effects of acute glucagon deficiency produced by using them.

The quality and quantity of antibodies used in the present study were more appropriate than those used in most previous studies with a titre 4.5 greater than that employed by Holst et al. [10]. The binding capacity and avidity as determined by Scatchard analysis of the antibodies used in our experiments appear to be adequate for the purpose for which they were intended, namely production of complete or virtually complete immunodeficiency. Total binding capacity of the antibodies administered (i.e. 0.5 ml/rat), calculated as the sum of both binding sites was about 13 nmol - in other words up to 12 times the total content of glucagon in the rat pancreas variously estimated by Barling et al. [8] as 1.0 nmol and by ourselves as 1.20 nmol. Even if only the low capacity, high-affinity class of antibodies are taken into account their binding capacity of 3 nmol is still three times larger than the total content of the rat pancreas and very much larger (almost 90 times) than the total amount of glucagon secreted during 60 min by the moderately stimulated rat pancreas which was reported by Luyckx [19] to be 0.032–0.052 nmol/h.

The binding affinity for glucagon of the most avid class of antibodies present in the antiserum used, and which was calculated to be 2.9×10^{10} l/mol, is about 100 times greater than that of the hepatic glucagon receptor of the rat which has been reported by Rodbell et al. [20] to be 2.5×10^{8} l/mol. This would favour binding of circulating glucagon to antibody rather than to target organ receptors. The ability of the antibodies used to neutralise exogenous glucagon is shown by the results of the in vitro binding studies which revealed that they were capable, in the amount and at the concentrations used, of binding approximately 40 pmol glucagon/s. If the glucagon concentration in the portal vein is assumed to be three times that found in the periphery it would take approximately 1 s for the glucagon antibodies injected to combined with half the glucagon present. This time interval is of the same order of magnitude as the time taken for blood to reach the liver from the pancreas through the portal circulation of the rat [8].

Exogenous glucagon administered at a dose of 1.43 nmol (4.7 nmol/kg body weight) produced significant hyperglycaemia, reaching a peak blood glucose concentration of 12 mmol/l in control rats. This effect was markedly inhibited by prior administration of glucagon antibodies. This observation is consistent with data reported by Frohman et al. [21] and by Holst et al. [10] who used much smaller doses of glucagon (28.5 pmol/kg and 30 pmol/kg, respectively) than in the present study. Pre-incubation of 1.43 nmol of glucagon for 15 min at 37 °C with the same amount of glucagon antibodies as were used in the in vivo experiments completely abolished its hyperglycaemic effect suggesting that at least in vitro they were capable of neutralising the total content of glucagon in the rat pancreas.

Despite extensive, if not complete, neutralisation of endogenous glucagon by glucagon antibodies throughout the whole period of observation no appreciable fall in blood glucose concentration occurred in either the 16-or 24-h fasted rats. The lack of effect of glucagon antibodies on blood glucose could mean either (a) that removal of the glycogenolytic and gluconeogenic effects of glucagon was compensated for by other mechanisms, such as diminished insulin secretion or increased adrenomedullary activity or (b) that glucagon is not important for the maintenance of euglycaemia. In the present study, however, no change in plasma insulin or somatostatin concentrations were observed as a result of glucagon antibody-treatment and in a previous study by Holst et al. [10] there was no evidence of a compensatory role for increased adrenomedullary activity. It seems likely, therefore, that glucagon is not as important for the maintenance of normoglycaemia during fasting as has been supposed.

These conclusions are in agreement with those of Holst et al. [10] Barling and Beloff-Chain [8] and Nishino et al. [24] but at variance with those drawn from experiments using somatostatin [14, 16, 22, 25]. The reasons for this discrepancy is unclear. In the elegant experiments of Cherrington et al. [16, 25] demonstrating decreased glucose production rates during glucagon suppression in dogs, glucose production was restored

when glucagon was replaced. However, studies using glucagon antibodies have failed to demonstrate the occurrence of hypoglycaemia. This failure to observe marked and sustained hypoglycaemia could be due to counter-regulatory changes in insulin and/or somatostatin masking the consequences of glucagon deficiency [25]. However, no changes in either plasma insulin or somatostatin were observed in the present study. The role of basal glucagon in the maintenance of plasma glucose remains unresolved. Perhaps the different results are related to species differences (rats versus dogs) and that in the rat at least, glucagon is not that important in the maintenance of normoglycaemia.

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