# *N*-ACETYLGLUCOSAMINE AND THE SUBSTRATE-SITE HYPOTHESIS FOR THE CONTROL OF INSULIN BIOSYNTHESIS AND SECRETION

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## 1. Introduction

The means whereby the beta-cell of the islets of Langerhans recognises signals for insulin biosynthesis and secretion and subsequently transduces such signals to activate the biosynthetic and secretory mechanisms are unknown. Two models have been proposed [1] to describe the process of signal recognition. One, the receptor-site model, envisages that the interaction of the signal with a membrane receptor, results in a change in the beta-cell initiating insulin release. The second model, the substrate-site model, proposes that the signal enters the beta-cell where it is metabolised and it is the formation of some metabolite or cofactor which causes initiation of the secretory response. These models have been extended [2] with the development of a dual-site model to encompass the phenomenon of potentiation. There is now an increasing body of evidence to support the substrate-site model. Such evidence includes close correlations between the rates of islet glucose metabolism and the ability of glucose to cause insulin release [2,3], the effects of mannoheptulose to inhibit both glucose phosphorylation and glucose-stimulated insulin release but not glyceraldehyde metabolism or glyceraldehyde-stimulated insulin release [2] and the observation that the anomeric specificity for glucose metabolism by the islet is the same as that for insulin release [4].

The present study is concerned with the metabolism and insulin-releasing activity of *N*-acetylglucosamine in isolated rat islets. The ability of *N*-acetylglucosamine to potentiate insulin release both in isolated rat and mouse islets [2,3] and in the rat in vivo [5] is well documented together with its ability to stimulate insulin biosynthesis [6]. We now find that correlations between the metabolism of *N*-acetylglucosamine and its ability to modulate insulin release and biosynthesis include the dependence on *N*-acetylglucosamine concentration and the effects of caffeine and phloretin. The data support the substrate-site hypothesis as the means whereby *N*-acetylglucosamine is recognised as a signal by the beta-cell.

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## 2. Materials and methods

### 2.1. *Reagents*

Bovine albumin (fraction V), dibutyrylcyclic-3′5′-AMP and collagenase (Type 1) were obtained from Sigma Chemical Co. [$^{125}$I]Insulin and *N*-acetyl-D-[1-$^{14}$C]glucosamine were from the Radiochemical Centre, Amersham. Guinea pig anti-porcine insulin serum was obtained from Wellcome Reagents Ltd, phloretin was from K&K Labs. All other chemicals were from BDH and were of the highest purity available.

### 2.2. *Preparation of islets*

Islets were prepared using collagenase digestion [7] from pancreases of 200–300 g male albino Wistar rats fed ad libitum on a standard laboratory diet.

### 2.3. *Output of $^{14}CO_2$*

The rate of formation of $^{14}CO_2$ from *N*-acetyl-D-[1-$^{14}$C]glucosamine by islets was measured as in [8].

2.4. *Insulin release*

Batches of islets were incubated in vitro for 2 h and insulin release measured as in [2,6]. Radioimmunoassay of insulin was performed as in [5], with the exception that Wellcome guinea pig anti-insulin serum was used as antibody.

2.5. *(Pro)insulin biosynthesis*

Rates of incorporation of L-[4,5-$^3$H]leucine into (pro)insulin and total islet protein were measured as in [6]. The rates of (pro)insulin biosynthesis are given as the insulin index: this parameter is the rate of (pro)insulin to total islet protein synthesis expressed as a fraction of the value of this ratio in the presence of 20 mM glucose in the same experiment. The term '(pro)insulin' is used to denote both insulin and proinsulin since the insulin-binding columns used have affinity for proinsulin as well as insulin [6].

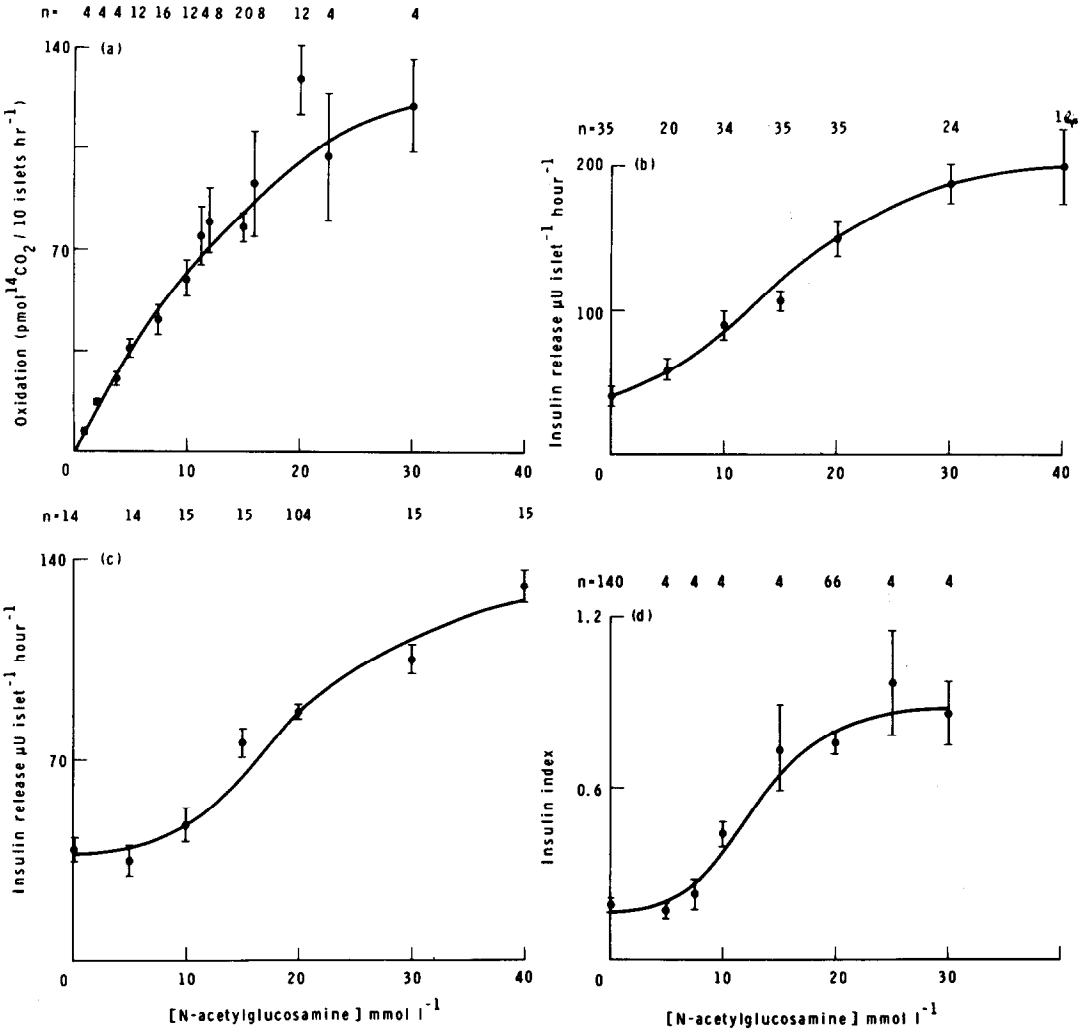


Fig.1. Dose-response curves for (a) *N*-acetylglucosamine oxidation, (b) *N*-acetylglucosamine-stimulated insulin release, (c) *N*-acetylglucosamine-potentiated insulin release and (d) *N*-acetylglucosamine-stimulated insulin biosynthesis. Oxidation, insulin release and insulin biosynthesis were measured as described in the text. Each point is the mean ± SEM of the number of observations (*n*) shown above the point in the diagram. Abscissa: [*N*-acetylglucosamine] (mM): 0; 10; 20; 30; 40. Ordinate: (a) Oxidation (pmol $^{14}CO_2$/10 islets/h): 0; 70; 140. (b) Insulin release (µU/islet/h): 0; 100; 200. (c) Insulin release (µU/islet/h): 0; 70; 140. (d) Insulin index: 0; 0.6; 1.2.

## 3. Results

### 3.1. N-*Acetylglucosamine concentration and rates of oxidation, insulin release and biosynthesis*

The oxidation of *N*-acetyl-D-[1-$^{14}$C]glucosamine to $^{14}CO_2$ was measured over a range of concentrations (fig.1a). A double reciprocal plot of velocity against *N*-acetylglucosamine concentration was linear over the range of concentrations tested. From the intercepts of this plot, a $K_m$ value for *N*-acetylglucosamine of 25.9 mM and a $V_{max}$ value of 214 pmol/h/10 islets were determined.

Two dose–response curves for the effects of *N*-acetylglucosamine on insulin release were measured:

1. A curve for *N*-acetyl-glucosamine-potentiated release obtained by varying the concentration of *N*-acetylglucosamine in the presence of a sub-stimulatory (3.3 mM) concentration of glucose (fig.1b).

2. A curve for *N*-acetyl-glucosamine-stimulated insulin release obtained in the absence of glucose (fig.1c).

The curves were sigmoidal and half-maximal rates of insulin release were observed at concentrations of *N*-acetylglucosamine similar to the $K_m$ for *N*-acetyl-glucosamine oxidation.

The concentration dependence of *N*-acetylglucosamine-stimulated (pro)insulin biosynthesis is shown in fig.1d. The curve was similar to that found for insulin release. Thus the $K_m$ for *N*-acetylglucosamine oxidation, -potentiated insulin release, -stimulated insulin release and -stimulated (pro)insulin biosynthesis are similar. When each of these parameters was expressed as a percentage of its value at 20 mM *N*-acetylglucosamine and plotted against *N*-acetyl-glucosamine concentration, a striking similarity between the concentration dependence of the four processes was observed (fig.2).

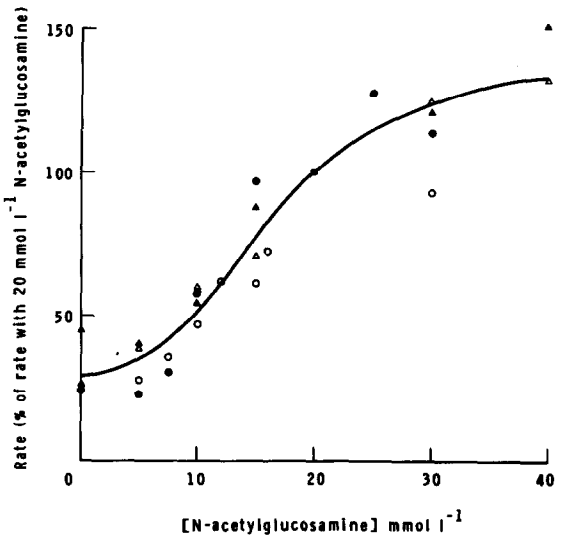


Fig.2. Normalised dose-response curves for *N*-acetylglucosamine oxidation (○), *N*-acetylglucosamine-stimulated insulin release (▲), *N*-acetylglucosamine-potentiated insulin release (△) and *N*-acetylglucosamine-stimulated insulin biosynthesis (●). Parameters were measured as described in legend to fig.1. Points are mean of number of observations (*n*) shown in the corresponding fig.1. Rates of the various processes are normalised by expressing them as a percentage of rate with 20 mM *N*-acetylglucosamine (100%). Abscissa: [*N*-acetylglucosamine] (mM): 0; 10; 20; 30; 40. Ordinate: Rate (% rate with 20 mM *N*-acetylglucosamine).

### 3.2. *Effects of caffeine, 3-isobutylmethylxanthine and dibutyrylcyclic-3′5′-AMP on* N-*acetylglucosamine oxidation and* N-*acetylglucosamine-stimulated insulin release*

The results are shown in tables 1 and 2. Caffeine (5 mM) inhibited *N*-acetylglucosamine-stimulated insulin release and *N*-acetylglucosamine oxidation by approx. 50%. Dibutyrylcyclic-3′5′-AMP (1 mM) did not affect *N*-acetylglucosamine oxidation but increased the rate of *N*-acetylglucosamine-stimulated insulin release. 3-Isobutylmethylxanthine (0.5 mM) inhibited the oxidation of *N*-acetylglucosamine to a similar extent to that found with caffeine.

### 3.3. *Effects of phloretin on islet responses to* N-*acetylglucosamine*

A concentration of phloretin without detectable effect on islet responses to glucose [9] produced marked inhibition of *N*-acetyl-[1-$^{14}$C]glucosamine and *N*-acetylglucosamine-stimulated insulin release (tables 3,4). Similar concentrations of phloretin specifically reduced (pro)insulin biosynthesis: insulin index 0.751 ± 0.044 ($n$ = 12) with 20 mM *N*-acetyl-glucosamine, 0.238 ± 0.051 ($n$ = 6) with 2 μg/ml phloretin plus 20 mM *N*-acetylglucosamine.

Table 1
Effects of caffeine and dibutyrylcyclic-3′5′-AMP on *N*-acetylglucosamine-stimulated insulin release

| Line | Additions to the medium | Insulin release μU/h/islet (mean ± SEM) | No. obs. | Significance |
|---|---|---|---|---|
| 1. | None | 21.4 ± 2.1 | 45 | – |
| 2. | Caffeine 5 mM | 30.5 ± 7.3 | 15 | NS vs 1 |
| 3. | Glucose 10 mM | 163.7 ± 13.2 | 15 | $P \leq 0.001$ vs 1 |
| 4. | Glucose 10 mM + caffeine 5 mM | 301.1 ± 17.7 | 15 | $P \leq 0.001$ vs 3 |
| 5. | *N*-Acetylglucosamine 20 mM | 87.4 ± 3.4 | 45 | $P \leq 0.001$ vs 1 |
| 6. | *N*-Acetylglucosamine 20 mM + caffeine 5 mM | 44.2 ± 3.5 | 45 | $P \leq 0.001$ vs 5<br>NS vs 2 |
| 7. | *N*-Acetylglucosamine 20 mM + dibutyrylcyclic-3′5′-AMP 1 mM | 165.2 ± 6.6 | 10 | $P \leq 0.001$ vs 5 |

Batches of 5 islets were incubated for 2 h at 37°C in Krebs-Henseleit bicarbonate medium containing 2 mg/ml albumin plus the additions shown in the table. Insulin released into the medium was determined by radioimmunoassay. The statistical significance of differences was assessed using Student's *t*-test

Table 2
Effects of caffeine, 3-isobutylmethylxanthine and dibutyryl-cAMP on *N*-[$^{14}$C]acetylglucosamine oxidation

| Line | Additions to the medium | Oxidation pmol $CO_2$/10 islets/h (mean ± SEM) | No. obs. | Significance |
|---|---|---|---|---|
| 1. | *N*-Acetylglucosamine 10 mM | 74.3 ± 4.9 | 24 | – |
| 2. | *N*-Acetylglucosamine 10 mM + Caffeine 5 mM | 38.3 ± 3.4 | 16 | $P \leq 0.001$ vs 1 |
| 3. | *N*-Acetylglucosamine 20 mM | 210.8 ± 26.5 | 8 | – |
| 4. | *N*-Acetylglucosamine 20 mM + caffeine 5 mM | 88.2 ± 6.0 | 8 | $P \leq 0.001$ vs 3 |
| 5. | *N*-Acetylglucosamine 10 mM + isobutylmethylxanthine 0.5 mM | 34.1 ± 1.6 | 8 | $P \leq 0.001$ vs 1 |
| 6. | *N*-Acetylglucosamine 10 mM + dibutyryl-cAMP 1 mM | 66.8 ± 7.4 | 8 | NS vs 1 |

Batches of 10 islets were incubated for 2 h with *N*-acetyl-D-[1-$^{14}$C]glucosamine (1 mCi/mmol). Metabolism was arrested by the addition of 0.2 N HCl and $^{14}CO_2$ absorbed by hyamine and counted using liquid scintillation spectrometry. The statistical significance of differences was assessed using Student's *t*-test

Table 3
Effect of phloretin on the oxidation of *N*-[$^{14}$C]acetylglucosamine

| Line | *N*-Acetylglucosamine (mM) | Phloretin (μg/ml) | Oxidation pmol $CO_2$/10 islets/h (mean ± SEM) | No. obs. | Significance |
|---|---|---|---|---|---|
| 1. | 10 | – | 85.1 ± 9.2 | 8 | – |
| 2. | 10 | 1.6 | 47.0 ± 9.8 | 8 | $P \leq 0.05$ vs 1 |
| 3. | 10 | 2.4 | 30.1 ± 4.8 | 8 | $P \leq 0.001$ vs 1 |
| 4. | 10 | 4.0 | 24.4 ± 3.4 | 8 | $P \leq 0.001$ vs 1 |

The experimental and statistical methods were as described in table 2. All of the above incubations contained 0.017% ethanol

Table 4
Effects of phloretin on *N*-acetylglucosamine-stimulated insulin release

| Line | Additions to the medium | | Insulin release μU/islet/h (mean ± SEM) | No. obs. | Significance |
|---|---|---|---|---|---|
| | Stimulant | Phloretin (μg/ml) | | | |
| 1. | – | – | 29.3 ± 7.2 | 15 | – |
| 2. | 20 mM glucose | – | 237.7 ± 12.0 | 10 | $P \leq 0.001$ vs 1 |
| 3. | 20 mM glucose | 5 | 316.4 ± 17.0 | 10 | – |
| 4. | 20 mM *N*-acetylglucosamine | – | 63.8 ± 5.3 | 15 | $P \leq 0.001$ vs 1 |
| 5. | 20 mM *N*-acetylglucosamine | 5 | 24.5 ± 3.0 | 15 | $P \leq 0.001$ vs 4 |

The experimental and statistical methods were as described in Table 1. All of the above media contained 0.017% ethanol

## 4. Discussion

It has been shown that *N*-acetylglucosamine is oxidised by pancreatic islets [2] which possess a specific *N*-acetylglucosamine kinase [10], and is able to stimulate insulin release [2,5,6] and biosynthesis [6]. The present study has examined in more detail the correlation between the metabolism of *N*-acetylglucosamine and its effects on islet function.

The maximum rate of oxidation of *N*-acetylglucosamine was 214 pmol/h/10 islets. This is considerably less than the total amount of *N*-acetylglucosamine kinase in rat islets (2 nmol/h/10 islets); moreover the $K_m$ for *N*-acetylglucosamine of *N*-acetylglucosamine kinase is 30 μM [10] which is very much lower than the $K_m$ for *N*-acetylglucosamine oxidation. These data suggest that, in contrast to glucose, the rate-limiting step of *N*-acetylglucosamine utilization may be membrane transport. The great sensitivity of *N*-acetylglucosamine oxidation to phloretin, which contrasts with glucose oxidation, supports this view.

The concentration of *N*-acetylglucosamine that gave half-maximum rates of oxidation was similar to that at which half-maximum rates of insulin release (in the presence or absence of 3.3 mM glucose) and biosynthesis occurred. These data are consistent with the hypothesis that *N*-acetylglucosamine metabolism may underlie its recognition by the beta-cell as a signal for insulin release and biosynthesis.

This conclusion is strengthened by the results obtained with caffeine and phloretin. Phloretin has been shown to inhibit the transport of glucose into isolated islets and to inhibit glucose-stimulated insulin release [9,11]. Glucose utilization by the islet is relatively insensitive to phloretin, however, as the transport capacity is greatly in excess of the phosphorylating activity [9]. The converse appears to be true for *N*-acetylglucosamine metabolism. Concentrations of phloretin without detectable effect on islet responses to glucose markedly inhibit *N*-acetylglucosamine oxidation and, in parallel, the biosynthetic and secretory responses to *N*-acetylglucosamine are also inhibited.

Earlier studies [2,6,10] had indicated that *N*-acetylglucosamine was not a stimulator of insulin release unless a low concentration of glucose was also present although insulin biosynthesis was stimulated by *N*-acetylglucosamine in the absence of glucose. However in the earlier studies, 5 mM caffeine was included in the incubation media with the aim of maximizing the sensitivity of insulin release to various sugars tested. The finding of the present study that *N*-acetylglucosamine alone is able to stimulate insulin release (in the absence of caffeine) removes this apparent dichotomy between the biosynthetic and release responses and further supports the view that the two processes are stimulated in the same way. A low glucose concentration is required however for effects of fructose on release and biosynthesis of insulin (I.H.W. unpublished observations). Our studies on the interaction of caffeine with *N*-acetylglucosamine show that caffeine inhibits *N*-acetylglucosamine-stimulated insulin release: since insulin release rates in control incubations without added sugar were marginally higher in the presence of caffeine, the net effect is that in the presence of caffeine *N*-acetylglucosamine elicits no significant stimulation of insulin release. Although surprising in view of its marked potentiatory action on stimulation of insulin release by other sugars, this inhibitory effect of

caffeine permits further strengthening of the substrate-site model for *N*-acetylglucosamine-stimulated insulin release; we find that caffeine inhibits *N*-acetylglucosamine oxidation by islets and suggest that this inhibition of metabolism underlies its impairment of *N*-acetylglucosamine-stimulated insulin release.

The way in which caffeine (or 3-isobutylmethylxanthine) inhibits *N*-acetylglucosamine oxidation and *N*-acetylglucosamine-stimulated insulin release is not completely known. The possibility that the effect is mediated by cyclic-3′5′-AMP is unlikely since the effects of caffeine were not reproduced by dibutyryl cyclic-3′5′-AMP. A direct effect of caffeine on *N*-acetylglucosamine kinase also seems unlikely since, as we have argued above, the kinase is not rate-limiting for *N*-acetylglucosamine utilization, and, in preliminary studies on purified rat liver *N*-acetylglucosamine kinase, we find no effect of caffeine. Thus the only potential sites of action of caffeine are either at the level of *N*-acetylglucosamine transport into the cell or at some point in the metabolism of *N*-acetylglucosamine after the formation of *N*-acetylglucosamine-6-phosphate and before the entry into glycolysis. Further studies on the pathway by which *N*-acetylglucosamine is metabolised by islets are required to resolve this point.

We conclude from these studies that there exists a close correlation between the metabolism of *N*-acetylglucosamine and its ability to stimulate insulin release and biosynthesis; and suggest that the substrate-site hypothesis may apply to this sugar.

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