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**Research Article**

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# The Stimulus-Secretion Coupling of Glucose-Induced Insulin Release

## METABOLIC AND FUNCTIONAL EFFECTS OF $NH_4^+$ IN RAT ISLETS

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ABSTRACT $NH_4^+$ caused a dose-related, rapid, and reversible inhibition of glucose-stimulated insulin release by isolated rat islets. It also inhibited glyceraldehyde-, $Ba^{2+}$-, and sulfonylurea-stimulated insulin secretion. $NH_4^+$ failed to affect glucose utilization and oxidation, glucose-stimulated proinsulin biosynthesis, the concentration of ATP, ADP, and AMP, and the intracellular pH. $NH_4^+$ also failed to affect the ability of theophylline and cytochalasin B to augment glucose-induced insulin release. However, in the presence and absence of glucose, accumulation of $NH_4^+$ in islet cells was associated with a fall in the concentration of NADH and NADPH and a concomitant alteration of $^{86}Rb^+$ and $^{45}Ca^{2+}$ (or $^{133}Ba^{2+}$) handling. These findings suggest that reduced pyridine nucleotides, generated by the metabolism of endogenous or exogenous nutrients, may modulate ionophoretic processes in the islet cells and by doing so, affect the net uptake of $Ca^{2+}$ and subsequent release of insulin.

## INTRODUCTION

In the preceding reports in this series (1, 2), we have used menadione to lower the concentration of reduced pyridine nucleotides (NADH and NADPH) in isolated pancreatic islets, and obtained data to suggest that the generation of such nucleotides plays a key role in the process of glucose-induced insulin release, by coupling metabolic events (e.g., glycolysis, mitochondrial oxidation) to subsequent cationic events (e.g., $K^+$ and $Ca^{2+}$ accumulation in islet cells). The latter concept was already proposed as a working hypothesis by several investigators (3–6).

The present study, reported in abstract form elsewhere (7), aims at further exploring the validity of such a concept. For this purpose, we have used $NH_4^+$, which is known to lower the concentration of reduced pyridine nucleotides in liver cells, by regulating the activity of glutamate dehydrogenase (8–10). Previous reports have already indicated that $NH_4^+$ is an inhibitor of insulin release (11–13). However, to our knowledge, the mode of action of $NH_4^+$ upon islet function had not as yet been explored in any great detail.

## METHODS

*Insulin release by isolated islets.* Pancreatic islets were isolated from the pancreas of fed female albino rats (150–200 g, body wt) by the collagenase technique. They were incubated in groups of eight islets each in a 1.0-ml bicarbonate-buffered medium containing 5 mg/ml albumin; the release of insulin was measured as described elsewhere (14). The chloride salt of $NH_4^+$ was invariably used in the present study.

*Insulin release by the perfused pancreas.* Pancreases removed from fed rats were placed in an open circuit extracorporeal perfusion unit, as described elsewhere (15). The perfusate contained 5 mg/ml albumin and 40 mg/ml dextran; as previously outlined (16). The $Ca^{2+}$ concentration of the perfusate (2 mM) was twice that of the medium used for incubation of islets.

*Proinsulin biosynthesis.* The method used to assess the biosynthetic activity of the islets was previously described (17). Briefly, groups of 100 islets each were incubated for 90 min in the presence of [$^3$H]leucine. After repeated washes, the islets were homogenized in subgroups of eight islets each and examined for their content in both TCA-precipitable and insulin-like, immunoreactive tritiated peptides.

*Uptake of $NH_4^+$ by the islets.* Groups of 10 islets each were incubated for 20 min in 60 $\mu$l of incubation medium placed in small polythene tubes (Beckman microfuge tubes, Beckman Instruments, Inc., Palo Alto, Calif.). The islets were then separated from the incubation medium by passing the

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This paper is the 32nd in a series.

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latter through a layer of silicon oil (0.2 ml; Versilube F50; General Electric Co., Bergen-op-Zoom, Netherlands). The islets were immediately mixed with 1.0 μl of HCl (50 mM) in a 20% (wt/vol) sucrose solution, which was introduced into the tube through the oil layer using a microsyringe. The tubes were centrifuged and heated at 80°C for 5 min. The upper phase (i.e. the incubation medium and part of the oil layer) was aspirated. For the assay of $NH_4^+$, a 5-μl reaction mixture, which consisted of a 50 mM tris buffer (pH 8.3) containing sucrose (20%, wt/vol); 250 μM NADH, 10 mM α-ketoglutarate, and 18 U/ml beef liver glutamate dehydrogenase (glycerol suspension), was introduced into the tube using a microsyringe and mixed with the solution in the tip of the tube (18). The tubes were then incubated for 60 min at 37°C, and the reaction stopped by addition of HCl (0.1 M; 75 μl). After a 10-min incubation at 25°C to ensure destruction of NADH, a 60-μl sample of the aqueous phase was mixed with 1 ml NaOH (6 N) in a fluorimeter tube, and heated at 60°C for 10 min to develop the NAD fluorescence, which was determined using a fluorometer (Aminco Chew-Glow Photofluorometer; American Instrument Co., Travenol Laboratories Inc., Silver Spring, Md.). Standard solutions of $NH_4Cl$ in 50 mM HCl-20% (wt/vol) sucrose were treated in the same way.

*Glucose metabolism in the islets.* The methods used for the measurement of glucose oxidation (19) and glucose utilization (i.e. the conversion of [5-$^3$H]glucose to $^3H_2O$; see reference 20) are described elsewhere.

*Assay of adenine and pyridine nucleotides.* The procedures used for the assay of adenine and pyridine nucleotides in the islets were previously described (2).

*Measurement of intracellular pH.* Groups of 10 islets each derived from the same batch of islets were incubated for 30 min in 0.1 ml of medium containing [6,6'(n)-$^3$H]sucrose (1.0 mM; 20 μCi/ml) and either (5,5-dimethyl[2-$^{14}$C]-oxazolidine-2,4-dione ([$^{14}$C]DMO);[1] 1.17 mM; 10 μCi/ml) or [$^{14}$C]urea (10 mM; 10 μCi/ml), so that the apparent intracellular space of distribution of [$^{14}$C]DMO or [$^{14}$C]urea could be measured in the same experiment(s). From these two series of values, the intracellular pH was calculated (21, 22), the relative error (SEM/mean) on each series of measurement being summed together. The islets were separated from the incubation medium as described elsewhere (23). The validity of the experimental design was assessed by measuring the intracellular pH of islets exposed to an acidic medium (pH 6.25–6.28), which was prepared by reducing the $NaHCO_3$ concentration from 24.0 to 2.4 mM, replacing the missing $NaHCO_3$ by an equimolar amount of NaCl, and equilibrating against the usual mixture of $O_2$ (95%) and $CO_2$ (5%).

*Cationic fluxes.* For measurement of $^{86}Rb^+$ efflux, groups of 100 islets each were preincubated for 60 min in 0.6 ml of a medium containing 2.8 mM glucose and $^{86}Rb^+$ (0.1–0.2 mM; 80 μCi/ml). The islets were then washed twice with an Rb-free medium and placed in a perifusion chamber, the effluent radioactivity being continuously monitored (24).

The net uptake of $^{86}Rb^+$ was measured in groups of 10 islets each, as described elsewhere (23, 24). The same technique was used for measuring the net uptake of $^{133}Ba^{2+}$ or $^{45}Ca^{2+}$. In each case, the islets were incubated in the presence of radioactive cation as well as [$^3$H]sucrose and, thereafter, separated from the incubation medium, by passing the latter through a layer of di-*n*-butyl phthalate. The efflux of $^{45}Ca^{2+}$ was measured by a method previously described (25).

*Presentation of results.* All results are expressed as the mean (±SEM) together with the number of individual observations (*n*). Comparison of experimental and control data is restricted to measurements collected within the same experiment(s), all islets in each experiment being derived from the same initial batch.

[1] *Abbreviation used in this paper*: DMO, 5,5-dimethyl oxazolidine-2,4-dione.

## RESULTS

*(1) Effect of $NH_4^+$ upon glucose-induced insulin release.* Ammonium caused a dose-related inhibition of insulin release evoked by 16.7 mM glucose in isolated pancreatic islets (Fig. 1A). A 50% inhibition was observed at a $NH_4^+$ concentration close to 1.0 mM. At $NH_4^+$ 5.0 mM, the rate of insulin release was close to its basal value. The inhibitory effect of 5.0 mM $NH_4^+$ upon glucose-stimulated insulin release was unaffected by raising the sugar concentration up to 27.8 mM (Fig. 1B). In the isolated perfused rat pancreas, the effect of $NH_4^+$ was rapid and rapidly reversible, a biphasic secretory pattern being noticed upon removal of $NH_4^+$ (Fig. 2). The effect of $NH_4^+$ upon insulin release was also reversible in the isolated islets. During a first 60-min incubation, the secretion of insulin evoked by 16.7 mM glucose was reduced by 5.0 mM $NH_4^+$ from a control value of 177.4±14.2 to 6.4±10.4 μU/islet per 60 min, the latter value being comparable to that found in islets incubated in the absence of

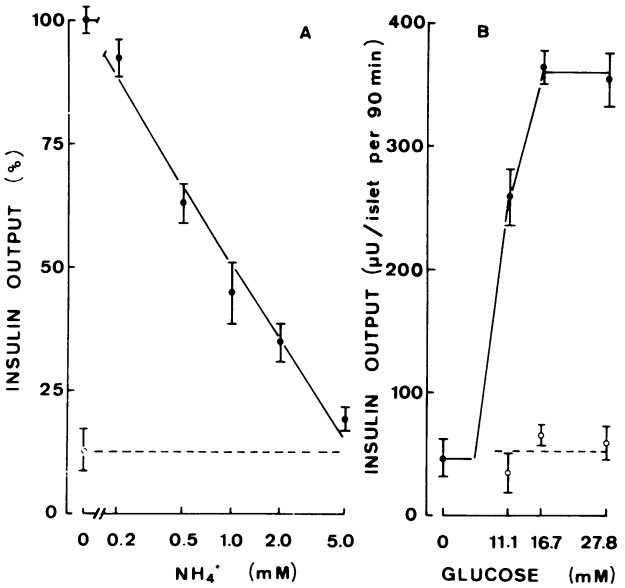


FIGURE 1 (A) Dose-related effect of $NH_4^+$ (logarithmic scale) upon insulin release evoked by 16.7 mM glucose in isolated islets. All data, including basal insulin output (○), are expressed in percent of the control value found within the same experiment in the presence of glucose. Such a control value averaged 364±13 μU/islet per 90 min. Mean values (±SEM) refer to 10–53 individual observations. (B) Effect of 5.0 mM $NH_4^+$ (○) upon insulin release at various glucose levels; (●), control data (no $NH_4^+$). Mean values (±SEM) refer to 8–10 individual observations.

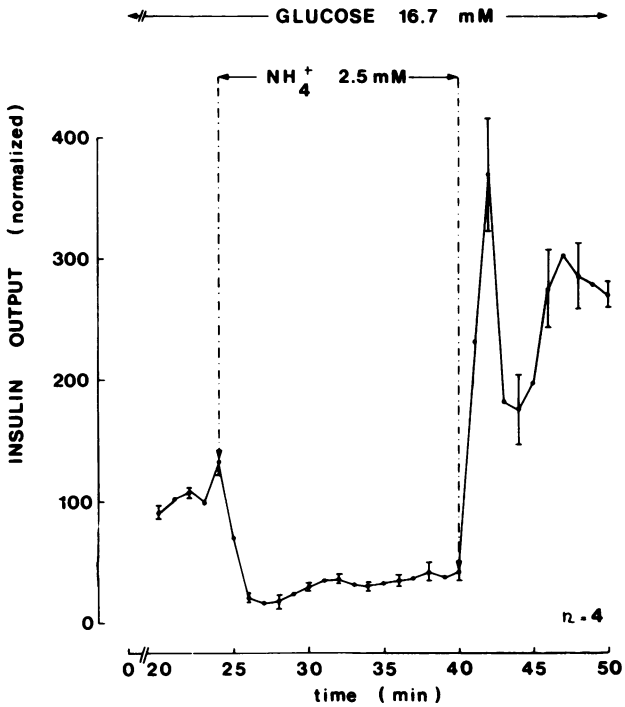


FIGURE 2 Effect of $NH_4^+$ upon insulin release evoked by glucose in the isolated perfused rat pancreas. All data are expressed as percent of the mean control value found between the 20th and 23rd min of perfusion within the same experiment. Such a control value averaged 440±76 μU/pancreas per min ($n$ = 4).

glucose. During a second 60-min incubation in the sole presence of 16.7 mM glucose, the islets which had been first exposed to $NH_4^+$ secreted insulin at a rate (171.0±22.9 μU/islet per 60 min) comparable to that found in the islets first incubated in the absence of both glucose and $NH_4^+$ (181.8±15.2 μU/islet per 60 min; $n$ = 12 in all cases). In perifused islets, $NH_4^+$ inhibited both the early and late phases of glucose-induced insulin release (Fig. 5).

*(2) Effect of $NH_4^+$ upon insulin release evoked by other secretagogues.* Ammonium inhibited in a dose-related fashion the release of insulin evoked by other nutrients than glucose, such as glyceraldehyde (Table I), leucine, and α-ketoisocaproate (data not shown).

The release of insulin provoked, in the isolated perfused pancreas, by gliclazide at a low glucose concentration (2.8 mM) was also inhibited by $NH_4^+$. In the absence of $NH_4^+$ and in response to administration of gliclazide (0.05 mM), the rate of insulin release increased from a basal value of 2±1 μU/min per pancreas to a peak value of 143±19 μU/min and, within 4 min, reached a plateau averaging 15±5 μU/min ($n$ = 6). In the presence of 5.0 mM $NH_4^+$, the gliclazide-induced peak value did not exceed 27±6 μU/min per pancreas and the later steady-state insulin output amounted to

TABLE I
*Effect of $NH_4^+$ upon Glyceraldehyde-Induced Insulin Release by Isolated Islets*

| Glyceraldehyde | $NH_4^+$ | Insulin output* |
|---|---|---|
| *mM* | *mM* | *μU/islet/60 min* |
| — | — | 21.0±4.5 (51) |
| — | 10.0 | 10.0±8.9 (10) |
| 10.0 | — | 99.5±6.3 (35) |
| 10.0 | 0.5 | 70.1±6.2 (36) |
| 10.0 | 5.0 | 30.6±4.6 (20) |

* Mean values (±SEM) are shown with the number of individual determinations (in parentheses).

only 3±2 μU/min ($n$ = 6). Over a 15-min exposure to gliclazide, the integrated output of insulin averaged 514±87 and 96±24 μU/pancreas in the absence and presence of $NH_4^+$, respectively ($P < 0.001$).

In the absence of both glucose and $Ca^{2+}$, the secretory response to the combination of 2.0 mM $Ba^{2+}$ and 1.4 mM theophylline was reduced ($P < 0.001$) from a control value of 175±14 μU/islet per 90 min ($n$ = 9) to 89±11 and 54±14 μU/islet per 90 min ($n$ = 12 in both cases), respectively in the presence of 0.5 and 5.0 mM $NH_4^+$.

In contrast with these findings, 1.0–2.0 mM $NH_4^+$ apparently failed to affect the capacity of theophylline or cytochalasin B to enhance glucose-induced insulin release (Table II). Thus, relative to the appropriate control values, the fractional increment in insulin output attributable to either theophylline or cytochalasin B was not reduced by $NH_4^+$. Even when glucose-induced insulin release was reduced by $NH_4^+$ to a value close to basal, the administration of theophylline still provoked a progressive and rapidly reversible escape from the inhibitory action of $NH_4^+$ (Fig. 3).

*(3) Effect of $NH_4^+$ upon glucose-stimulated proinsulin biosynthesis.* In the absence of $NH_4^+$ but presence of 16.7 mM glucose, the incorporation of radioactive leucine in TCA-precipitable material averaged 154±12 fmol/islet over a 90-min incubation in the presence of 0.84 μM [$^3$H]leucine. About 26.5±1.1% of these labeled peptides represented immunoreactive material (Table III). As judged by the latter ratio, which is representative of the preferential stimulant action of glucose upon the synthesis of proinsulin as distinct from other islet peptides (26), 0.5 and 5.0 mM $NH_4^+$ failed to affect glucose-stimulated proinsulin biosynthesis. The mean absolute amount of [$^3$H]leucine incorporated in both TCA-precipitable and immunoreactive material was slightly increased in the presence of 0.5 mM $NH_4^+$.

The failure of $NH_4^+$ to affect the biosynthetic activity of the islets was confirmed by use of a lower glucose concentration (8.3 mM), in which case the ratio of im-

TABLE II

*Effect of Glucose, Theophylline, Cytochalasin B, and $NH_4^+$ upon Insulin Release by Isolated Islets*

| Glucose | Theophylline | Cytochalasin B | $NH_4^+$ | Insulin output* | Theophylline- or cytochalasin-induced increment | |
|---|---|---|---|---|---|---|
| | *mM* | | | *μU/islet/90 min* | | *% of control* |
| 16.7 | — | — | — | 454±34 (10) | +326±36 | +71.8±7.9 |
| 16.7 | 1.4 | — | — | 780±11 (10) | | |
| 16.7 | — | — | 1.0 | 204±29 (10) | +390±42 | +191.2±20.4 |
| 16.7 | 1.4 | — | 1.0 | 594±30 (10) | | |
| 16.7 | — | — | — | 403±27 (10) | +212±43 | +52.6±10.6 |
| 16.7 | — | 0.02 | — | 615±33 (10) | | |
| 16.7 | — | — | 2.0 | 151±16 (10) | +143±39 | +94.7±25.9 |
| 16.7 | — | 0.02 | 2.0 | 294±36 (10) | | |

* Mean values (±SEM) are shown with the number of individual determinations (in parentheses).

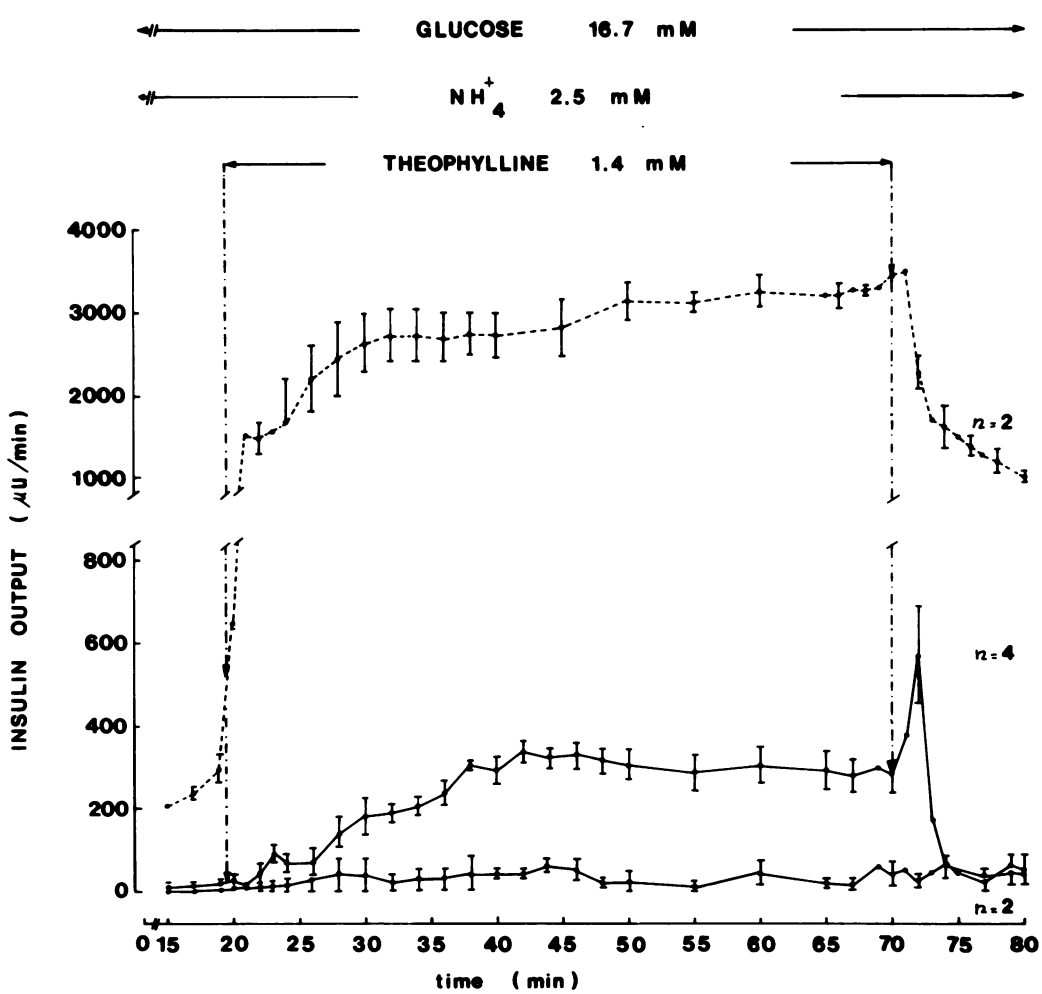


FIGURE 3 Effect of 1.4 mM theophylline upon insulin output by isolated perfused pancreases exposed to 16.7 mM glucose (upper curve) or to the combination of 16.7 mM glucose and 2.5 mM $NH_4^+$ (middle curve). In the lower curve, the pancreases were also exposed to both glucose and $NH_4^+$, but no theophylline was administered. In the present system, theophylline fails to affect insulin release in the absence of glucose (35).

TABLE III

*Effect of $NH_4^+$ upon the Incorporation of [$^3H$]Leucine in TCA-Precipitable Islet Peptides ([$^3H$]TCA) and the Ratio of Immunoreactive ([$^3H$]IRI) to TCA-Precipitable Peptides*

| Glucose | $NH_4^+$ | [$^3H$]TCA | [$^3H$]IRI/[$^3H$]TCA |
|---|---|---|---|
| *mM* | | *cpm/islet** | *%* |
| 8.3 | — | 5,844±469 (10) | 26.6±1.7 (10) |
| 8.3 | 0.5 | 5,469±587 (10) | 26.3±2.2 (10) |
| 8.3 | 5.0 | 5,571±204 (10) | 24.7±1.4 (10) |
| 16.7 | — | 7,325±581 (14) | 26.6±1.1 (14) |
| 16.7 | 0.5 | 8,993±740 (14) | 28.9±1.0 (14) |
| 16.7 | 5.0 | 8,188±437 (14) | 27.9±0.9 (14) |

* Mean values (±SEM) are shown with the number of individual determinations (in parentheses).

munoreactive to TCA-precipitable tritiated material, although not different from that seen at 16.7 mM glucose, would inevitably fall if the stimulant action of glucose upon proinsulin biosynthesis were to be impaired by $NH_4^+$ (26).

*(4) Uptake of $NH_4^+$ by isolated islets.* The amount of $NH_4^+$ present in the islets after a 20-min incubation in the presence of 16.7 mM glucose increased from a negligible value (0.3±6.9 pmol/islet) in islets not exposed to $NH_4^+$ to 20.8±8.5, 58.3±7.4, and 115.8±7.9 pmol/islet ($n$ = 8 in each case) as the concentration of $NH_4^+$ in the incubation medium was raised to 0.1, 0.5, and 2.5 mM, respectively. These values are much higher than those expected from the simple diffusion of $NH_4^+$ in the intracellular water space, and also much higher than those attributable to extracellular contamination. Indeed, the intracellular $H_2O$ content of the islets averages 2–3 nl/islet (Table VI) and, under the present experimental conditions, the contaminating extracellular space also represents 2–3 nl/islet (24). It would thus appear that the islets are able to concentrate $NH_4^+$ intracellularly, with an apparent $K_m$ of 0.7–0.8 mM and a maximal value of 150–160 pmol/islet for the net uptake of $NH_4^+$ as a function of its extracellular concentration.

*(5) Effect of $NH_4^+$ upon glucose metabolism.* At a 16.7-mM glucose concentration, $NH_4^+$ failed to affect significantly the utilization and oxidation of the sugar by the islets. The conversion of [5-$^3$H]glucose to $^3H_2O$ averaged 200±31 ($n$ = 25), 195±40 ($n$ = 25), and 239 ±47 ($n$ = 15) pmol/islet per 120 min in the absence of $NH_4^+$ and in the presence of 0.5 and 5.0 mM $NH_4^+$, respectively. The oxidation of [U-$^{14}$C]glucose averaged 42.6±3.6 ($n$ = 30), 56.5±8.2 ($n$ = 23), 48.0±3.4 ($n$ = 30), and 45.1±2.4 ($n$ = 7) pmol/islet per 120 min in the absence of $NH_4^+$ and in the presence of 0.5, 2.0, and 5.0 mM $NH_4^+$, respectively.

*(6) Effect of $NH_4^+$ upon the concentration of adenine nucleotides.* The concentration of ATP, ADP, and AMP was measured in groups of eight islets each after a 30-min incubation in the presence or absence of 16.7 mM glucose and 5.0 mM $NH_4^+$. In the absence of $NH_4^+$, the total concentration of adenine nucleotides was not significantly affected by glucose (0–16.7 mM), and averaged 13.1±0.6 pmol/islet ($n$ = 16). Whether in the absence or presence of glucose, $NH_4^+$ failed to affect the total concentration of adenine nucleotides, the mean value recorded in islets exposed to $NH_4^+$ amounting to 12.6±0.6 pmol/islet ($n$ = 16).

Relative to the total amount of adenine nucleotides, 16.7 mM glucose increased the concentration of ATP from 51.2±3.1 to 73.1±4.1%, whereas reducing the concentration of ADP from 27.1±2.3 to 14.9±2.5% and that of AMP from 21.4±1.3 to 12.0±1.8% ($n$ = 8 in each case). In the absence of $NH_4^+$ the ATP/ADP ratio and the adenylate charge (i.e. [ATP + 1/2 ADP]/[ATP + ADP + AMP] ratio) were both increased by glucose from a basal value of 2.07±0.36 to 6.18±1.22 (ATP/ADP) and 0.649±0.021 to 0.805±0.028 (adenylate charge). Ammonium failed to exert any significant effect upon the relative concentration of each adenine nucleotide (data not shown). In the islets exposed to 5.0 mM $NH_4^+$, the ATP/ADP ratio averaged 2.05±0.31 and 6.53±0.85 and the adenylate charge 0.635±0.027 and 0.806±0.018 in the absence and presence of glucose, respectively ($n$ = 8 in each case).

*(7) Effect of $NH_4^+$ upon the concentration of reduced pyridine nucleotides.* The total concentration of reduced pyridine nucleotides (NADH + NADPH) was measured by the luciferase method in groups of eight islets incubated for 30 min in the presence or absence of 16.7 mM glucose and 0.5 and 5.0 mM $NH_4^+$. Glucose significantly increased ($P < 0.001$) the concentration of reduced pyridine nucleotides from a basal value of 112±12 to 179±12 fmol/islet ($n$ = 20 in each case). Ammonium invariably lowered the concentration of reduced pyridine nucleotides, whether in the absence

TABLE IV

*Effect of Glucose and $NH_4^+$ upon the Total Concentration of Reduced Pyridine Nucleotides in Isolated Islets*

| Glucose | $NH_4^+$ | NADH + NADPH |
|---|---|---|
| *mM* | *mM* | *fmol/islet** |
| — | — | 112.4±11.5 (20) |
| — | 0.5 | 79.2±8.0 (10) |
| — | 5.0 | 73.6±9.2 (10) |
| 16.7 | — | 179.3±12.0 (20) |
| 16.7 | 0.5 | 140.6±17.6 (10) |
| 16.7 | 5.0 | 111.9±10.6 (10) |

* Mean values (±SEM) are shown with the number of individual determinations (in parentheses).

TABLE V

*Effect of Glucose and $NH_4^+$ upon the Concentration of Pyridine Nucleotides in Isolated Islets Preincubated for 30 min in a Glucose-Free Medium and, thereafter, Either Maintained in such a Medium (First column) or Exposed for 3 min to Glucose in the Absence (Second column) or Presence (Third column) of $NH_4^+$*

| Glucose, *mM* | — | 16.7 | 16.7 |
|---|---|---|---|
| $NH_4^+$, *mM* | — | — | 5.0 |
| NADH, *fmol/islet** | 66±12 | 116±18 | 75±6 |
| $NAD^+$, *fmol/islet* | 590±17 | 766±61 | 605±62 |
| NADH + $NAD^+$, *fmol/islet* | 656±29 | 882±79 | 680±68 |
| NADH/$NAD^+$, *ratio* | 0.111±0.019 | 0.152±0.021 | 0.126±0.008 |
| NADPH, *fmol/islet* | 72±11 | 101±10 | 90±11 |
| $NADP^+$, *fmol/islet* | 53±2 | 50±3 | 52±6 |
| NADPH + $NADP^+$, *fmol/islet* | 125±11 | 151±13 | 142±17 |
| NADPH/$NADP^+$, *ratio* | 1.357±0.217 | 2.004±0.147 | 1.807±0.217 |
| NADH + NADPH, *fmol/islet* | 138±23 | 218±22 | 166±14 |

* Mean values (±SEM) invariably refer to six individual determinations.

or presence of glucose (Table IV). As little as 0.5 mM $NH_4^+$ was sufficient to reduce the level of (NADH + NADPH) to 74.4±6.0% of its appropriate mean control value.

We have explored whether the $NH_4^+$-induced decrease in the concentration of reduced pyridine nucleotides occurred promptly enough to account for a rapid inhibition of glucose-induced insulin release. For this purpose, groups of 12 islets each were preincubated for 30 min in the absence of glucose and, thereafter, exposed for 3 min to either glucose alone (16.7 mM) or the combination of glucose and $NH_4^+$ (5.0 mM). The concentration of pyridine nucleotides was measured by the enzymatic cycling technique (2). As indicated in Table V, within 3 min of incubation, glucose significantly increased the concentration of NADH ($P < 0.05$) and $NAD^+$ ($P < 0.02$), the NADPH/$NADP^+$ ratio ($P < 0.05$), and the total amount of both diphosphopyridine nucleotides ($P < 0.025$) and reduced pyridine nucleotides ($P < 0.05$). In the presence of $NH_4^+$, glucose failed to exert such effects, the results obtained in the concomitant presence of glucose and $NH_4^+$ being not significantly different from the basal values found in the islets maintained in a glucose-free medium.

In the absence of glucose, and after a 3-min exposure to 5.0 mM $NH_4^+$, there was not yet any major change in the concentration of pyridine nucleotides (data not shown). A trend toward a more oxidized state was nevertheless noticed, the NADH/$NAD^+$ and NADPH/$NADP^+$ ratios averaging, in the presence of $NH_4^+$, respectively 88.7±10.2 and 89.1±10.5% ($n = 12$ in each case) of their appropriate mean control values, i.e., 0.102±0.026 for NADH/$NAD^+$ and 1.337±0.110 for NADPH/$NADP^+$ ($n = 12$).

*(8) Effect of $NH_4^+$ upon the intracellular $H_2O$ space and pH.* In the presence of 5.0 mM $NH_4^+$ or at low extracellular pH (6.3), the intracellular $H_2O$ space (as judged from the difference between the [$^{14}$C]urea and [$^3$H]sucrose spaces of distribution) was not significantly different from the control value found at a normal pH in the absence of $NH_4^+$. Pooling all measurements, the intracellular $H_2O$ space averaged 2.56±0.13 nl/islet ($n = 61$). The presence of $NH_4^+$ also failed to affect the difference between the [$^{14}$C]DMO and [$^3$H]sucrose spaces of distribution, and the calculated intracellular pH (Table VI). In contrast, exposure of the islets to an acidic extracellular medium resulted in a significant fall ($P < 0.05$) of the intracellular pH.

TABLE VI

*Effect of $NH_4^+$ and Extracellular Acidosis upon the Intracellular pH of Isolated Islets Exposed to 16.7 mM Glucose*

| $NH_4^+$ | Extracellular pH | DMO space | Urea space | Intracellular pH |
|---|---|---|---|---|
| *mM* | | *nl/islet** | *nl/islet** | |
| — | 7.35–7.40 | 1.88±0.20 (16) | 2.33±0.24 (23) | 7.18±0.09 (16) |
| 5.0 | 7.35–7.40 | 2.04±0.33 (15) | 2.95±0.20 (23) | 7.20±0.11 (15) |
| — | 6.25–6.28 | 6.17±0.94 (7) | 2.31±0.17 (15) | 6.81±0.14 (7) |

* The DMO and urea spaces are corrected for extracellular contamination. Mean values (±SEM) are shown with the number of individual determinations (in parentheses).

*(9) Effect of $NH_4^+$ upon $^{86}Rb^+$ handling.* In the absence of glucose, 2.0 mM $NH_4^+$ increased ($P < 0.005$) the fractional outflow rate of $^{86}Rb^+$ from a control value of 5.40±0.17 ($n$ = 28) to 7.48±0.61%/min ($n$ = 2). The ability of glucose to provoke a rapid and reversible fall in $^{86}Rb^+$ efflux was still detectable in the presence of 2.0 mM $NH_4^+$ (Fig. 4). At a higher concentration of $NH_4^+$ (5.0 mM), the basal value for $^{86}Rb^+$ fractional outflow was again increased (7.29±0.45%/min; $n$ = 2), and the effect of glucose upon $^{86}Rb^+$ efflux was abolished. Consistent with these findings, $NH_4^+$ caused a dose-related decrease in the glucose-stimulated net uptake of $^{86}Rb^+$. Thus when expressed as $K^+$ with the same specific activity ($^{86}Rb^+/^{39}K^+$) as that of the incubation medium (24), the net uptake of $^{86}Rb^+$ averaged 359±34, 309±49, 297±45, and 226±38 pmol/islet ($n$ = 16 in each case) in the sole presence of 16.7 mM glucose and in the concomitant presence of glucose and $NH_4^+$, 0.5, 2.0, and 5.0 mM, respectively. The value found at the highest concentration of $NH_4^+$ represented 63.0±10.6% of the mean control value and was close to that normally seen in the absence of glucose. Indeed, in the present series of experiments, the basal net uptake of $^{86}Rb^+$ averaged 65.8±3.9% ($n$ = 34) of the glucose-stimulated reference value. In the presence of 16.7 mM glucose, the $NH_4^+$-induced decrease in the $K^+$ pool was approximately matched by the amount of $NH_4^+$ accumulated in the islet cells.

*(10) Effect of $NH_4^+$ upon $^{133}Ba^{2+}$ net uptake.* In the presence of millimolar concentration of $Ba^{2+}$ and in the absence of $Ca^{2+}$, glucose is known to stimulate $^{133}Ba^{2+}$ net uptake which, however, is unaffected by theophylline (27). In media deprived of both $Ca^{2+}$ and

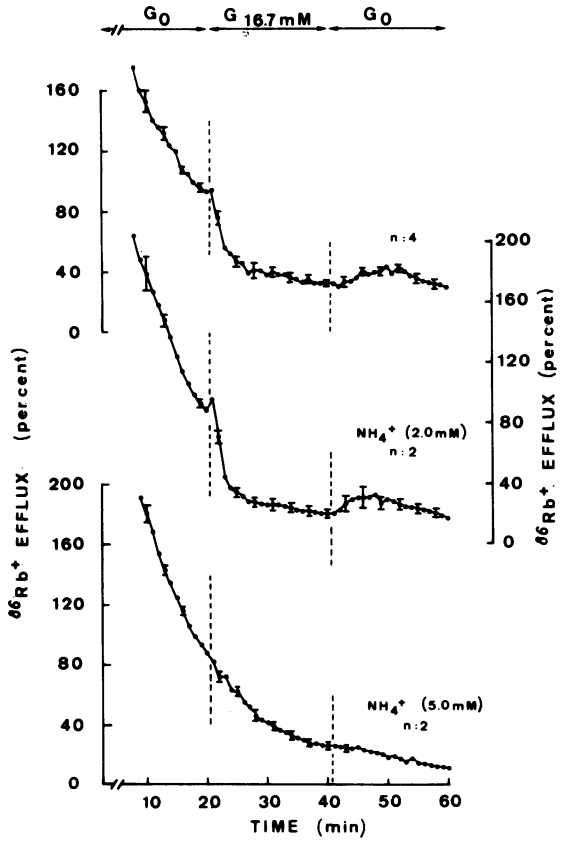


FIGURE 4 Effect of glucose upon $^{86}Rb^+$ efflux in the absence (upper curve) or presence of $NH_4^+$ (2.0 mM: middle curve; 5.0 mM: lower curve). The efflux of $^{86}Rb^+$ is expressed in percent of the mean control value found, within the same experiment, between the 16th and 20th min of perifusion. Glucose was added ($G_{16.7\ mM}$) or removed ($G_0$) at the time shown by the vertical dotted lines. Mean values (±SEM) refer to two to four individual experiments.

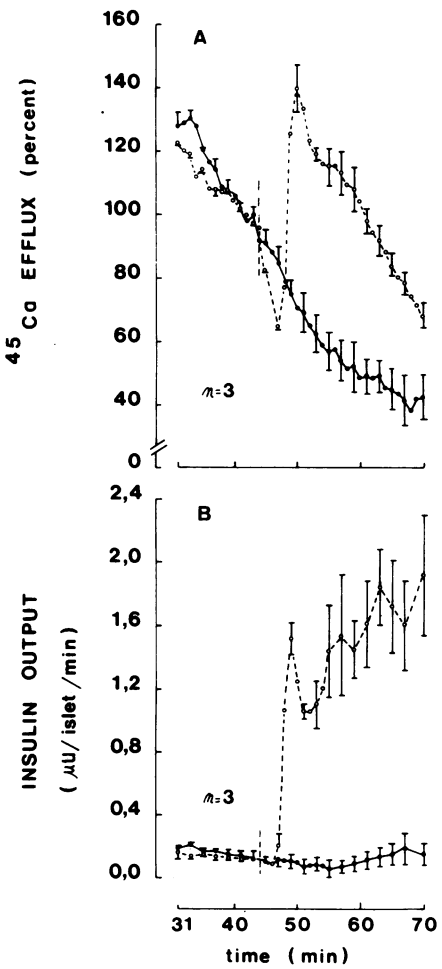


FIGURE 5 Effect of 16.7 mM glucose (introduced at the time shown by the vertical dotted line) upon $^{45}Ca$ efflux (A) and insulin output (B) from isolated islets perifused in the absence (dotted line) or presence (solid line) of 5.0 mM $NH_4^+$. The efflux of $^{45}Ca$ is expressed in percent of the mean control value found, within the same experiment, between the 40th and 44th min of perifusion. The experiments were performed at a normal calcium concentration (1.0 mM). Mean values (±SEM) refer to three to four individual experiments.

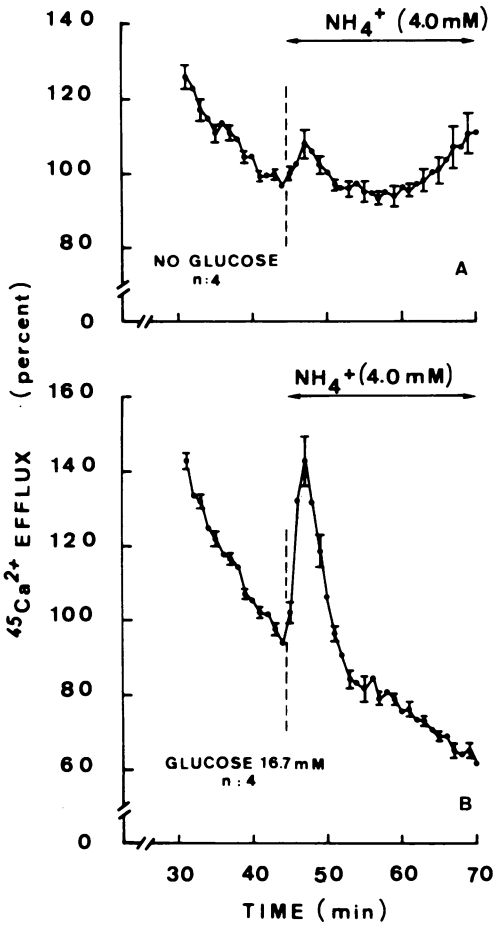


FIGURE 6 Effect of $NH_4^+$ (introduced at the time shown by the vertical dotted line) upon the efflux of $^{45}Ca$ from islets perifused in the absence (A) or presenee of 16.7 mM glucose (B). Same presentation as in Fig. 5 (A). The experiments were performed in the absence of extracellular calcium.

glucose but containing 1.4 mM theophylline, 5.0 mM $NH_4^+$ decreased the net uptake of radioactive 2.0 mM $Ba^{2+}$ from a control value of 43.1±3.6 to 28.5±1.9 peq/islet per 90 min ($P < 0.005$; $n = 10$ in both cases).

*(11) Effect of $NH_4^+$ upon $^{45}Ca^{2+}$ handling.* 5.0 mM $NH_4^+$ suppressed the ability of glucose to provoke both an initial fall and a secondary rise in the efflux of $^{45}Ca$ from islets perifused at a normal $Ca^{2+}$ concentration (Fig. 5). At a lower concentration of $NH_4^+$ (1.0 mM), the early inhibitory effect of glucose upon $^{45}Ca$ efflux was still discernible, but the secondary rise appeared both blunted and retarded, reaching a peak value of 116.0±6.9% at the 57th min (see Fig. 5 for comparison with control data). When the perifusate contained no $Ca^{2+}$, to prevent the release of $^{45}Ca^{2+}$ usually associated with the exocytosis of secretory granules (25), the addition of $NH_4^+$ provoked an immediate increase in $^{45}Ca^{2+}$ efflux, suggesting a facilitation of $^{45}Ca^{2+}$ outflow from the islet cells (Fig. 6). The latter phenomenon was seen both in the absence and presence of glucose. In the latter case, the increase in $^{45}Ca^{2+}$ efflux occurred in a much more brisk manner as that seen when glucose itself is removed from the perifusate (Fig. 6, right panel in reference 25).

In the 0.5–5.0-mM range, $NH_4^+$ caused a dose-related inhibition ($P < 0.01$ or less) of glucose-stimulated $^{45}Ca^{2+}$ net uptake (Fig. 7A). In the presence of $NH_4^+$, the relationship between insulin release and $^{45}Ca^{2+}$ net uptake displayed a pattern (Fig. 7B) comparable to that usually seen in the present system (1, 28–30). Such a relationship is characterized by the fact that insulin release increases above its basal value whenever the net uptake of $^{45}Ca^{2+}$ exceeds a critical threshold level representing approximately half of the reference value normally found at high glucose concentration (16.7 mM).

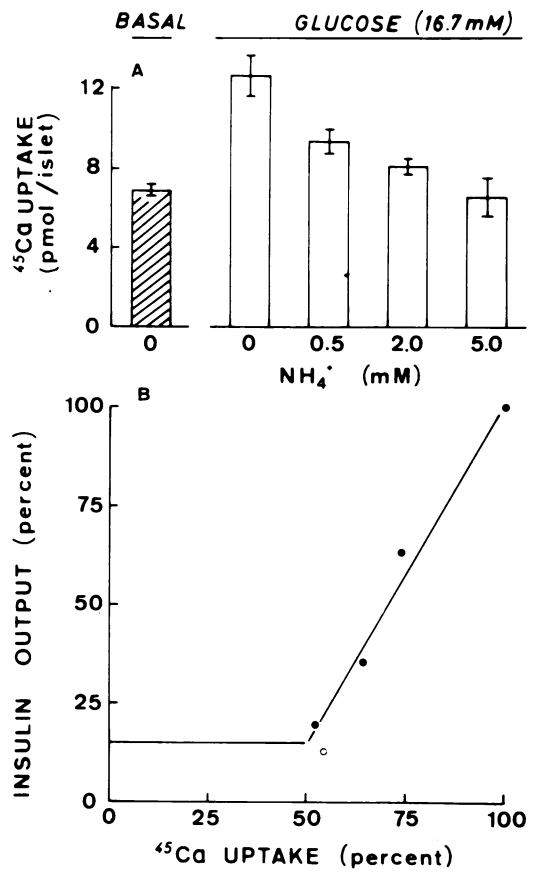


FIGURE 7 (A) Effect of $NH_4^+$ upon glucose-stimulated (open columns) calcium net uptake after a 90-min incubation. The basal uptake (no glucose present) is shown in the shaded column. Mean values (±SEM) refer to 12±2 individual observations. (B) Relationship between $^{45}Ca$ net uptake and insulin output in isolated islets incubated in the absence of glucose (○) or presence of 16.7 mM glucose and increasing concentrations of $NH_4^+$ (●). Mean values (Fig. 1A and Fig. 7A) are expressed in percent of the reference value found in the presence of glucose but absence of $NH_4^+$.

## DISCUSSION

The present findings confirm that $NH_4^+$ is a potent inhibitor of insulin release. Previous reports have indicated that 0.1–1.0 mM $NH_4^+$ inhibits glucose-stimulated insulin release both in vitro using pieces of pancreas removed from the golden hamster (11) and in vivo in rats (13) and men (12) infused with $NH_4^+$. A role for $NH_4^+$ in the abnormality of islet function seen in cirrhotic patients (12, 31) was also documented.

The aim of the present study was to elucidate the mode of action of $NH_4^+$ upon insulin release, with emphasis on the possible role of NAD(P)H in such a process.

The inhibitory effect of $NH_4^+$ upon glucose-induced insulin release cannot be ascribed to any major anomaly in the rate of glycolysis or glucose oxidation in the islets (Results, section 5). The concentration of adenine nucleotides was unaffected by $NH_4^+$ (Results, section 6). There was no obvious alteration of the intracellular pH in the islets exposed to $NH_4^+$ (Table VI). The stimulant action of glucose upon proinsulin biosynthesis was also unaffected by $NH_4^+$ (Table III). The $NH_4^+$ ion did not suppress the capacity of theophylline to augment glucose-induced insulin release (Table II, Fig. 3), an effect currently attributed to an intracellular translocation of $Ca^{2+}$ from the vacuolar pool into the cytosolic compartment (30). Last, our data suggest that $NH_4^+$ fails to alter the responsiveness of the effector system which controls the translocation and extrusion of secretory granules, and which is thought to be activated by intracellularly accumulated $Ca^{2+}$. Indeed, on one hand, $NH_4^+$ failed to affect the ability of cytochalasin B to augment glucose-stimulated insulin release (Table II). The latter effect if currently ascribed to the interference of cytochalasin B with the microfilamentous cell web known to control the final access of secretory granules to the plasma membrane (15). On the other hand, and in contrast to what is observed with poisons of the pancreatic $\beta$-cell microtubular-microfilamentous system, $NH_4^+$ did not modify the normal relationship between $^{45}Ca^{2+}$ net uptake and insulin release (Fig. 7B).

The sole abnormalities encountered in the islets exposed to $NH_4^+$ were (*a*) a dose-related fall in the concentration of reduced pyridine nucleotides (Table IV), (*b*) a dose-related alteration of $^{86}Rb^+$ (Fig. 4) and $^{45}Ca^{2+}$ (Fig. 7) handling, and (*c*) the already mentioned dose-related inhibition of insulin release (Fig. 1, Table I). We propose that these three abnormalities are sequentially linked with one another.

A primary effect of $NH_4^+$ upon the concentration of reduced pyridine nucleotides is consistent with the effect of $NH_4^+$ in other tissues. For instance, in liver cells, $NH_4^+$ lowers the concentration of both NADH and NADPH, which act as reducing equivalents in the reductive amination of $\alpha$-ketoglutarate (8–10). A similar effect of $NH_4^+$ in islet cells is compatible with the finding that the dose-related effect of $NH_4^+$ upon the concentration of reduced pyridine nucleotides (Table IV) coincided with a dose-related increase in the net uptake of $NH_4^+$ by the islets (Results, section 4). The effect of glucose to increase the concentration of reduced pyridine nucleotides is sufficiently rapid to account for the early phase of glucose-induced insulin release (32). $NH_4^+$ prevents the effect of glucose to rapidly increase the concentration of NADH and NADPH (Table V), and suppresses the early secretory response to this sugar (Fig. 5). Our data, however, do not permit us to decide which parameter of the pyridine nucleotides profile is most relevant to the secretory function of the islets.

The altered handling of both $K^+$ (as judged from the $^{86}Rb^+$ data; [24]) and $Ca^{2+}$ (Figs. 5–7) in the islets exposed to $NH_4^+$ could well be the consequence of the $NH_4^+$-induced change in the concentration of reduced pyridine nucleotides. As far as $K^+$ is concerned, the $NH_4^+$-induced decrease in $^{86}Rb^+$ net uptake could be due not solely to the observed increase in $^{86}Rb^+$ fractional outflow rate and to the dose-related inhibition of the glucose effect upon $^{86}Rb^+$ outflow, but possibly also to a reduced rate of $^{86}Rb^+$ (and $^{39}K^+$) inflow, since $NH_4^+$, $Rb^+$, and $K^+$ may compete for the $(Na^+ + K^+)$-activated ATPase (33). In the case of $Ca^{2+}$, the existence of a tight link between the altered handling of $^{45}Ca^{2+}$ and the change in NAD(P)H concentration is supported by the highly significant correlation ($r = 0.9989$, $n = 4$, $P < 0.002$) observed between the mean concentration of reduced pyridine nucleotides (Table IV) and the mean corresponding value for $^{45}Ca^{2+}$ net uptake (Fig. 7A). The 34.5% decrease in the concentration of NAD(P)H, as evoked by 5.0 mM $NH_4^+$ in the absence of glucose (Table IV), also coincided with a 33.8% reduction in the net uptake of $^{133}Ba^{2+}$ by the islets exposed to $NH_4^+$ in a glucose-free medium (Results, section 10). As outlined in greater detail elsewhere (34), the concentration of NAD(P)H could affect the affinity for cations of native ionophoretic systems located in B-cell membranes. Incidentally, $NH_4^+$ itself fails to directly affect A23817-mediated $Ca^{2+}$ ionophoresis in an artificial system.[2]

In view of the key role ascribed to divalent cations in the process of insulin secretion (see reference 34 for review), it is reasonable to assume that, in the presence of $NH_4^+$, the inhibition of insulin release is mainly due to the altered cationic response of the islets to different secretagogues.

In conclusion, the present data are compatible with the view that the $NH_4^+$-induced change in the concen-

[2] Unpublished observation.

tration of reduced pyridine nucleotides is mainly responsible for the concomitant abnormality in both cationic and secretory events. Along this line of thought, it is remarkable that, even under conditions in which the islet cells derived most or all of their energy from endogenous nutrients, $NH_4^+$ was still able to markedly lower the concentration of NAD(P)H (Table IV), to alter the handling of $^{86}Rb^+$ (Fig. 4), $^{45}Ca^{2+}$ (Fig. 6A) and $^{133}Ba^{2+}$ (Results, section 10), and to impair the secretory response to secretagogues such as sulfonylurea and $Ba^{2+}$ (Results, section 2). Thus, the postulated influence of reduced pyridine nucleotides upon the handling of cations by native ionophoretic systems may be operative over a wide range of NAD(P)H concentrations.

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