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Effects of Glucose on the Cytosolic Ratio of Reduced/Oxidized Nicotinamide–Adenine Dinucleotide Phosphate in Rat Islets of Langerhans

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The maximal extractable activity of 'malic' enzyme (EC 1.1.1.40) in rat islets of Langerhans was similar to that reported for liver. Thus 'malic' enzyme may catalyse a near-equilibrium reaction in the cytosol of islets of Langerhans. Measurements of islet content of malate and pyruvate, the metabolite substrate and product of 'malic' enzyme, were therefore used to calculate the cytosolic ratio of [NADPH]/[NADP$^+$]. This ratio was higher in islets incubated with 20mM-glucose than in islets incubated with 2mM-glucose.

Several lines of evidence have suggested that the extent of reduction of nicotinamide nucleotides in the pancreatic β-cell may be an important controlling factor in stimulus–secretion coupling. Effects on insulin release of agents known to modify the extent of reduction of nicotinamide nucleotides were noted by Ammon & Steinke (1972) and by Deery & Taylor (1973). An increase in the extent of reduction of nicotinamide nucleotides in intact perifused islets of Langerhans was observed when the concentration of glucose in the perifusion medium was increased from 3 to 20mM (Panten *et al.*, 1973). Such changes have been correlated with rates of insulin release under various conditions (Panten *et al.*, 1973, 1974). However, with the technique used, it was not possible ascertain the cellular location and identity of the reduced nicotinamide nucleotides. Malaisse and co-workers have demonstrated changes in whole-tissue concentrations of reduced nicotinamide nucleotides in islets exposed to stimulatory concentrations of glucose (Malaisse *et al.*, 1979*a*,*b*) or pyruvate (Sener *et al.*, 1978*b*). A limitation of these studies is that measurement of whole-tissue concentrations of nicotinamide nucleotides cannot give information on the changes in any particular cellular compartment, and ratios of [NAD(P)H]/[NAD(P)$^+$] are known to differ by several orders of magnitude between cytosol and mitochondria (Veech *et al.*, 1969). A possible role for the availability of reduced nicotinamide nucleotides in stimulus–secretion coupling was further supported by the use of agents such as menadione and NH_4^+, which lowered islet content of reduced nicotinamide nucleotides and inhibited insulin release (Malaisse *et al.*, 1978*a*,*b*; Sener *et al.*, 1978*a*). Again, however, these studies could not give unequivocal information on the identity and cellular location of the redox couple(s) affected. In the present study we have attempted to provide such information.

The principle and limitations of the approach have been discussed in detail by Williamson *et al.* (1967) and by Veech *et al.* (1969). In brief, measurement of whole-tissue concentrations of substrate and product of a nicotinamide nucleotide-linked dehydrogenase can be used to compute the ratio [NAD(P)H]/[NAD(P)$^+$] in a particular cellular compartment provided that certain conditions are fulfilled, namely: (1) the dehydrogenase should exist solely in one cellular compartment; (2) the dehydrogenase should be present at high activity such that the reaction it catalyses is close to equilibrium; (3) measurement of whole-tissue concentrations of the metabolite substrate and product of the reaction should be a reasonable estimate of their concentrations in the compartment of interest. In this study we have used measurements of substrate and product concentrations of 'malic' enzyme (EC 1.1.1.40) to compute the ratio [NADPH]/[NADP$^+$] in the cytosol of islets of Langerhans incubated at non-stimulatory and stimulatory concentrations of glucose.

It was first necessary to investigate the cellular location of 'malic' enzyme in pancreatic islets, and to verify its presence at such an activity that it can be assumed to catalyse a near-equilibrium reaction. The islets were prepared by collagenase digestion (Coll-Garcia & Gill, 1969) from the pancreases of adult male Wistar rats fed *ad libitum* on standard laboratory diet. Islets (250) were collected in 200μl of Tris buffer (5mM, pH 7.4) containing sucrose (250mM), and homogenized in a hand-held homogenizer with a Teflon pestle. Half the homogenate was retained for assay of 'malic' enzyme and citrate synthase (a mitochondrial marker) as described below. The remaining homogenate was centrifuged for 1 min in an Eppendorf 3200 centrifuge to obtain a mitochondrial fraction. The supernatant was aspirated and the pellet taken up in 100μl of

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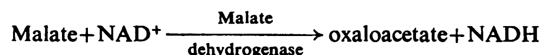
sucrose/Tris medium. The homogenate and the mitochondrial fraction were frozen and thawed three times to disrupt the mitochondria. Citrate synthase activity was assayed spectrophotometrically at 30°C on 10 μl portions of whole homogenate and fractions by a modification of the method of Srere *et al.* (1963) as described by Coore *et al.* (1971). 'Malic' enzyme was assayed spectrophotometrically at 30°C on 30 μl portions of homogenate or fractions. The reaction medium (0.5 ml) contained triethanolamine hydrochloride buffer (50 mM, pH 7.4), L-malate (1 mM), $NADP^+$ (0.1 mM), $MnCl_2$ (3 mM) and albumin (0.1 mg/ml). The activities of 'malic' enzyme and citrate synthase in the homogenate were 11.0 and 67.2 pmol/min per islet respectively. 'Malic' enzyme was confined almost entirely to the supernatant fraction, less than 10% of the total appearing in the mitochondrial pellet. The latter, however, contained 85% of the total citrate synthase activity. These data show that in islets, as in liver (Rutter & Lardy, 1958) but unlike in heart (Frenkel, 1971), 'malic' enzyme is predominantly localized in the extramitochondrial compartment. It was further shown that neither the homogenate nor the supernatant fraction catalysed reduction of NAD^+ under the conditions used for assay of 'malic' enzyme, excluding the possibility that the activity observed was residual activity of malate dehydrogenase with $NADP^+$ (Ashcroft & Randle, 1970).

The islets used in the present studies have a mean net weight of 7 μg (I. H. Williams, 1978). The activity of 'malic' enzyme is therefore 1.6 mol/min per g at 30°C. The activity of 'malic' enzyme in liver is 1.27 μmol/min per g at 25°C (Veech *et al.*, 1969), and this activity has been shown to be sufficient to catalyse a near-equilibrium reaction. It was concluded therefore that in islets of Langerhans, 'malic' enzyme is suitable for determination of the cytosolic [NADPH]/[$NADP^+$] ratio. This was accomplished as follows. Batches of 30–40 islets were incubated in Krebs–Henseleit bicarbonate medium (Krebs & Henseleit, 1932) containing albumin (2 mg/ml) and glucose (2 or 20 mM). The incubation vessels were Beckman Microfuge tubes containing 20 ml of 5% (w/v) $HClO_4$ with 50 μl of silicone oil (specific gravity 1.02) layered on top; 100 μl of incubation medium was layered on top of the silicone oil. The tubes were incubated for 30 min, in a 37°C water bath situated in a metabolic incubator containing an atmosphere of air/CO_2 (19:1). After incubation, the tubes were spun on a Beckman Microfuge for 10 s. The islets were sedimented through the layer of silicone oil into the quenching solution of $HClO_4$. The incubation

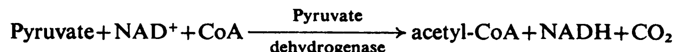
Table 1. *Cytosolic ratios of [NADPH]/[$NADP^+$] in rat islets of Langerhans*

Five separate preparations of islets were used. For each preparation batches of 30–40 islets were incubated in 100 μl of bicarbonate medium containing albumin (2 mg/ml); for half the batches the incubation medium contained 2 mM-glucose and for the other half 20 mM-glucose. After 30 min incubation at 37°C, the islets were rapidly separated from incubation medium, and metabolism was arrested by centrifugation through silicone oil into 5% (w/v) $HClO_4$. Islets were extracted, and endogenous NADPH and NADH destroyed by heating for 30 min at 60°C. After neutralization, extracts were assayed for malate and pyruvate. Malate was assayed as NADH formed in the reaction:



$$\text{Malate} + \text{NAD}^+ \xrightarrow[\text{dehydrogenase}]{\text{Malate}} \text{oxaloacetate} + \text{NADH}$$

The reaction was pulled over to the right by using a following enzyme system of aspartate aminotransferase plus glutamate. Pyruvate was assayed as NADH formed in the reaction:



$$\text{Pyruvate} + \text{NAD}^+ + \text{CoA} \xrightarrow[\text{dehydrogenase}]{\text{Pyruvate}} \text{acetyl-CoA} + \text{NADH} + \text{CO}_2$$

NADH was measured by a luciferase assay. The assays were quantified by standard curves for malate and pyruvate taken through the same procedure. Islet malate and pyruvate contents were converted from pmol/islet to μM by assuming a mean intracellular space of 3 nl/islet (Sener & Malaisse, 1978). Cytosolic [NADPH]/[$NADP^+$] ratios were calculated from the equation:

$$\frac{[\text{NADPH}]}{[\text{NADP}^+]} = \frac{[\text{malate}]}{[\text{pyruvate}]} \frac{K'}{[\text{CO}_2]}$$

where K' is the apparent equilibrium content for 'malic' enzyme at pH 7. A value of 3.44×10^{-2} M was taken for K' and [CO_2] was assumed to be 1.16 mM (Veech *et al.*, 1969). Results are means ± S.E.M. for the number of determinations shown. The significance of differences of mean values at 20 mM-glucose versus those at 2 mM-glucose was assessed by Student's *t* test: *$P \leq 0.05$; **$P \leq 0.001$.

Glucose concn. (mM)	*n*	Malate concn. (pmol/islet)	Malate concn. (μM)	Pyruvate concn. (pmol/islet)	Pyruvate concn. (μM)	[Malate]/[pyruvate]	[NADPH]/[$NADP^+$]
2	20	0.66 ± 0.09	220	1.34 ± 0.19	447	0.53 ± 0.10	15.7 ± 3.0
20	27	1.31 ± 0.17**	437	1.69 ± 0.15	563	0.91 ± 0.12*	26.9 ± 3.6*

medium was removed and the tubes were stored frozen before assay. The tubes were then heated at 60°C for 30min to extract the islet contents and to destroy endogenous reduced nicotinamide nucleotides. The $HClO_4$ extract was neutralized with 5μl of 4M-KOH in 0.5M-triethanolamine, and the tubes were spun on a Beckman Microfuge for 20s to sediment the precipitate of $KClO_4$. The neutralized extract was now less dense than the silicone oil and became the upper phase.

The malate and pyruvate in the neutralized extract were assayed as follows. Malate was assayed by adding 10μl of extract to 40μl of 2-amino-2-methylpropan-1-ol buffer (0.1M, pH9.9) containing NAD^+ (1mM), glutamate (6.7mM), malate dehydrogenase (Boehringer; 1.6 units) and aspartate aminotransferase (Boehringer; 0.11 unit). Pyruvate was assayed by adding 10μl of extract to 40μl of Tris/HCl buffer (0.1M, pH7.8) containing $MgSO_4$ (1mM), EDTA (0.5mM), mercaptoethanol (5mM), NAD^+ (0.45mM), CoA (0.13mM) and thiamin pyrophosphate (1mM). The malate and pyruvate assays went to completion within 60min at 22°C. Then 40μl of each reaction medium was assayed for NADH by the luciferase method (Ashcroft *et al.*, 1973). Pyruvate and malate standards (10–100pmol in 5% $HClO_4$) were taken through the procedure from the neutralization step onwards. Incubation media containing no islets were also taken through the entire procedure to serve as blanks. Results are given in Table 1. On increasing the glucose concentration from 2 to 20mM, the mean pyruvate content of islets was unchanged, but the malate content was approximately doubled. Thus between 2mM- and 20mM-glucose there was an increase in the concentration ratio of malate/pyruvate. From the equilibrium constant of 'malic' enzyme, the cytosolic ratio of [NADPH]/[$NADP^+$] was calculated to be 15.7 ± 3.0 at 2mM-glucose and 26.9 ± 3.6 at 20mM-glucose. In the absence of glucose the islet content of pyruvate and malate was not significantly different from that at 2mM-glucose.

It has been conclusively shown for liver that the redox couple [NADPH]/[$NADP^+$] is considerably more reduced in the cytosol than in the mitochondria (Veech *et al.*, 1969; Siess *et al.*, 1976; Tischler *et al.*, 1977). Our values for rat islet cytosolic [NADPH]/[$NADP^+$] are of the same order as the reported values for liver and are clearly markedly different from the whole-islet ratios of [NADPH]/[$NADP^+$] (1.3–2.7) measured by Malaisse *et al.* (1978*b*). This underlines the compartmentation of these cofactors and the need for the type of approach used in the present study to assess the possible role of the redox state of nicotinamide nucleotides in stimulus–secretion coupling. The data demonstrate that the cytosolic [NADPH]/[$NADP^+$] in islets is increased on raising the extracellular glucose concentration from a non-stimulatory to a stimulatory value. Assuming that the data are representative of the β-cells, which constitute approx. 70% of rat islets of Langerhans (Carpenter, 1966), the results provide some support for the view that an increase in the cytosolic [NADPH]/[$NADP^+$] ratio may be involved in coupling β-cell glucose metabolism to insulin release and/or biosynthesis. The change observed, however, is relatively modest; it is necessary to consider whether other redox couples may exhibit more dramatic changes. The present method should permit investigation of this problem: thus, for example, measurement of islet lactate and pyruvate contents can be used to calculate cytosolic [NADH]/[NAD^+] ratios, since lactate dehydrogenase is likely to catalyse a near-equilibrium reaction in islet cytosol.

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