Responsiveness to Glucagon by Isolated Rat Hepatocytes Controlled by the Redox State of the Cytosolic Nicotinamide–Adenine Dinucleotide Couple Acting on Adenosine 3':5'-Cyclic Monophosphate Phosphodiesterase

By MICHAEL G. CLARK* and IVAN G. JARRETT†

*Clinical Biochemistry Unit, Flinders University Medical School, Bedford Park, South Australia 5042, and †CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide, South Australia 5000, Australia

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1. The effects of changes in the cytoplasmic $[NADH]/[NAD^+]$ ratio on the efficacy of glucagon to alter rates of metabolism in isolated rat hepatocytes were examined. 2. Under reduced conditions (with 10 mM-lactate), 10 nM-glucagon stimulated both gluconeogenesis and urea synthesis in isolated hepatocytes from 48 h-starved rats; under oxidized conditions (with 10 mM-pyruvate), 10 nM-glucagon had no effect on either of these rates. 3. The ability of glucagon to alter the concentration of 3':5'-cyclic AMP and the rates of glucose output, glycogen breakdown and glycolysis in cells from fed rats were each affected by a change in the extracellular $[lactate]/[pyruvate]$ ratio; minimal effects of glucagon occurred at low $[lactate]/[pyruvate]$ ratios. 4. Dose-response curves for glucagon-mediated changes in cyclic AMP concentration and glucose output indicated that under oxidized conditions the ability of glucagon to alter each parameter was decreased without affecting the concentration of hormone at which half-maximal effects occurred. 5. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.05 mM) significantly reversed the inhibitory effects of pyruvate on glucagon-stimulated glucose output. 6. For exogenously added cyclic $[^3H]$ AMP (0.1 mM), oxidized conditions decreased the stimulatory effect on glucose output as well as the intracellular concentration of cyclic AMP attained, but did not alter the amount of cyclic $[^3H]$ AMP taken up. 7. The effects of lactate, pyruvate, NAD^+ and $NADH$ on cyclic AMP phosphodiesterase activities of rat hepatocytes were examined. 8. $NADH$ (0.01–1 mM) inhibited the low- K_m enzyme, particularly that which was associated with the plasma membrane. 9. The inhibition of membrane-bound cyclic AMP phosphodiesterase by $NADH$ was specific, reversible and resulted in a decrease in the maximal velocity of the enzyme. 10. It is proposed that regulation of the membrane-bound low- K_m cyclic AMP phosphodiesterase by nicotinamide nucleotides provides the molecular basis for the effect of redox state on the hormonal control of hepatocyte metabolism by glucagon.

It has been previously reported that the ability of glucagon to stimulate glucose release by isolated rat hepatocytes and perfused liver is affected by the redox state of the cytosolic NAD^+ couple (Clark *et al.*, 1977). In hepatocytes from fed rats an increase in pyruvate, NH_4^+ or O_2 concentration or a decrease in the $[lactate]/[pyruvate]$ or $[sorbitol]/[fructose]$ ratios decreased the ability of 1 μ M-glucagon to stimulate glucose release without significantly altering the control rate. Since these changes correlated with a decrease in the measured $[lactate]/[pyruvate]$ ratio of the cell suspension and did not result from diminished concentrations of ATP, it was concluded that the efficacy of glucagon as a stimulant of glucose release depended on the cytoplasmic redox state represented by the cellular $[lactate]/[pyruvate]$ ratio.

In the present work an attempt was made to

elucidate the mechanism at the cellular level by which the efficacy of glucagon is altered by changes in redox state.

Materials and Methods

Materials

D- $[5-^3H]$ Glucose, cyclic $[8-^3H]$ AMP (21 Ci/mmol) and cyclic AMP assay kits were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Glucagon (lot 258-VO16-36) was a generous gift from Dr. W. Bromer, Eli Lilly, Indianapolis, IN, U.S.A., and freshly prepared in 0.9% (w/v) NaCl (0.1 mg/ml). Cyclic AMP, NAD^+ , $NADH$, L(+)-lactic acid, 3-isobutyl-1-methylxanthine, sodium pyruvate and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.,

and were used as freshly prepared aqueous solutions (pH 7.4). Collagenase (type CLS, 190 units/mg) was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Dowex AG1-X8 (200–400 mesh; Cl^- form) resin was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Dow-Corning silicone oil was obtained from Ajax Chemicals, Bankstown, N.S.W., Australia. All other chemicals were of analytical-reagent grade.

Hepatocyte isolation and incubation

Isolated hepatocytes were prepared from fed or 48 h-starved rats (180–220 g) essentially as described by Berry & Friend (1969), with the omission of hyaluronidase. Buffers were equilibrated with O_2/CO_2 (19:1) before use.

Unless indicated otherwise, incubations were conducted at 37°C with shaking (90 oscillations/min) in stoppered vials of 20 ml capacity with substrate (in 0.02 ml of 0.9% NaCl solution) and hormone (in 0.05 ml of 0.9% NaCl solution) in a total volume of 1.2 ml of Krebs–Henseleit buffer, pH 7.4 (Dawson, 1969), containing 1.3 mM- Ca^{2+} . After addition of the cells (approx. 65 mg wet wt.) each vial was gassed with the O_2/CO_2 mixture and stoppered. The reactions were terminated by the addition of 0.5 ml of 6% (w/v) HClO_4 .

The method for the separation of hepatocytes from medium was based on the silicone-oil centrifugation method of La Noue *et al.* (1972). A sample of the cell-suspension incubation medium (0.2 ml) was layered on the surface of 0.5 ml of silicone-oil mixture (7:1, v/v: type 550 fluid + type 200 fluid, viscosity $1.5 \times 10^{-4} \text{ Pa}\cdot\text{s}$) placed above 0.05 ml of 6% (w/v) HClO_4 contained in an Eppendorf Microfuge tube (total capacity 1.5 ml). Each sample was centrifuged for 30 s (Eppendorf Zentrifuge), after which time separation was complete. A sample (0.04 ml) was carefully removed from the bottom HClO_4 layer and neutralized to pH 7.0 with 1 M-KOH.

Analytical determinations

Glucose output (cells from fed rats) and gluconeogenesis (cells from starved rats) were determined by measuring the total glucose content of the cells plus medium. Analyses for glucose were conducted on the acid supernatant fluid by using the glucose oxidase/peroxidase method (Werner *et al.*, 1970). Since the rate of gluconeogenesis from 10 mM-lactate was approx. $0.6 \mu\text{mol}/\text{min}$ per g wet wt. for cells from fed rats (results not shown), the rate of glucose output of 2–2.5 $\mu\text{mol}/\text{min}$ per g wet wt. essentially represented glucose output from substrates other than the added lactate or pyruvate (e.g. glycogen). Urea was determined by the urease method of Fawcett & Scott (1960). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Glycogen breakdown was assessed by measuring the radioactivity retained in glycogen after 10 min incubation of liver cells that contained prelabelled glycogen. Cellular glycogen was prelabelled by incubating the freshly prepared liver cells (2.5 g wet wt.) with constant gassing (O_2/CO_2 mixture) and shaking (90 oscillations/min) at 37°C for 1 h in 33 ml of Krebs–Henseleit buffer containing 1.3 mM- CaCl_2 , 30 mM-glucose and 100 μCi of $[5\text{-}^3\text{H}]\text{glucose}$. Cells were harvested and washed by centrifugation (400 g for 75 s) before use and resuspended in 15–20 ml of Krebs–Henseleit saline. Incubations were as described above. The radioactivity retained by glycogen was determined as follows: 0.8 ml of the HClO_4 extract of cells plus incubation medium was added to 0.1 ml of 0.1% (w/v) glycogen and 4 ml of ethanol. The precipitate was collected by centrifugation (10000 g for 2 min), and washed twice with 65% (w/v) ethanol and then dissolved in 0.2 ml of 2% (w/v) HClO_4 for counting of radioactivity. The extent to which glycogen was metabolized by reactions of glycolysis was assessed by measuring the yield of $^3\text{H}_2\text{O}$ which was isolated from HClO_4 extracts of cells+medium as described by Clark (1978). Cyclic AMP was determined on neutralized HClO_4 extracts of cells+medium or cells alone by the method of Gilman (1970).

Preparation of hepatocyte fractions containing cyclic AMP phosphodiesterase activity

The method used was essentially as described by Loten *et al.* (1978). Briefly, 5 ml of hepatocyte suspension (approx. 400 mg wet wt.) was added to 5 ml of ice-cold buffer (0.25 M-sucrose, 10 mM-Tris/HCl, 2 mM-EDTA, pH 7.5) and centrifuged at 5°C to separate cells (40 g for 75 s). The supernatant fluid was discarded and the cells were resuspended in 5 ml of ice-cold buffer and homogenized in a Dounce homogenizer by using 35 strokes of the A pestle (microscopic examination indicated that more than 90% of the cells were broken under these conditions). The homogenate was centrifuged for 30 min at 20000 rev./min in a JA 21 rotor in a Beckman JA 21B centrifuge (4°C). The supernatant from this centrifugation, which contained the cytosolic high- and low- K_m enzyme activities, was retained. The pellet was resuspended in 7 ml of 10 mM-Tris/HCl/1 mM-EDTA, pH 7.5, and homogenized for 5 s with a Polytron homogenizer. The homogenate was centrifuged for 30 min at 20000 rev./min as before and the supernatant fluid retained (membrane low- K_m enzyme activity).

Enzyme assays

Cyclic AMP phosphodiesterase (EC 3.1.4.17) in cytosolic and membrane fractions was assayed at 30°C by the radiochemical procedure of Arch & Newsholme (1976). The concentrations of cyclic AMP

Fig. 1. Effects of extracellular lactate and pyruvate on glucagon-mediated changes in (a) gluconeogenesis and (b) urea synthesis by isolated hepatocytes

Isolated hepatocytes were prepared from 48h-starved rats as described in the Materials and Methods section. Additions were 0.9% NaCl (●) and 10nM-glucagon (○). Incubations contained 50mg wet wt. of cells/ml and were for 30min. Glutamine (5mM) was included in the incubations for urea synthesis. For gluconeogenesis, mean values from five cell preparations are shown and the bars indicate $\pm$ S.E.M. For urea synthesis mean values from two cell preparations are given. Rates for incubations to which neither lactate nor pyruvate was added are also shown.

used for the high- and low- K_m enzyme activities were 200 and 0.1 μ M respectively. 5'-Nucleotidase and glucose 6-phosphatase were assayed at 30°C as described by Touster *et al.* (1970) and de Duve *et al.* (1955) respectively.

Results

It has previously been reported by three groups (Zahlten *et al.*, 1973; Garrison & Haynes, 1973; Rognstad, 1975) that glucagon and cyclic AMP do not stimulate gluconeogenesis from 10mM-pyruvate in isolated hepatocytes. Such results were unexpected, as in parallel experiments glucagon stimulated gluconeogenesis from 10mM-lactate. The data of Fig. 1(a) confirm those observations and show that for hepatocytes from 48h-starved rats incubated with lactate and pyruvate (combined concentration of 10mM) the maximal rate of gluconeogenesis and the maximal stimulation by glucagon occurred when the initial extracellular concentrations were 8mM-lactate and 2mM-pyruvate. As the proportion of pyruvate increased and exceeded that of lactate, the effect of glucagon changed from stimulatory to slightly inhibitory. Similar effects of lactate and pyruvate combinations on glucagon-mediated ureogenesis from 5mM-glutamine were evident (Fig. 1b). A maximal stimulation of urea synthesis occurred at 8mM-lactate + 2mM-pyruvate, even though the maximal control rate occurred at 10mM-lactate. For both gluconeogenesis and ureogenesis the inhibitory effect produced by glucagon in the presence of 10mM-pyruvate was not statistically significant.

Since both gluconeogenesis and ureogenesis involve reactions in which reducing equivalents are consumed or produced and can thereby be influenced by redox state, attention was focused on glycogenolysis, a reaction sequence that does not appear to involve a flow of reducing equivalents. The data of Fig. 2 extend our previous observations (Clark *et al.*, 1977) and show that glucagon-mediated effects on glucose output (Fig. 2a), glycogenolysis (assessed by loss of radioactivity from prelabelled glycogen; Fig. 2c) and glycolysis (Fig. 2b) were decreased as the extracellular ratio of [pyruvate]/[lactate] increased. The maximal stimulation of glucose output and glycogen breakdown, as well as the maximal inhibition of glycolysis, occurred in the presence of 10mM-lactate.

The next group of experiments (Figs. 3–6) were conducted in an attempt to locate the possible site, between glucagon–receptor interaction and change in glycogenolytic rate, where redox state (in particular pyruvate) exerted its influence. Fig. 3 shows the effects of 10mM-lactate and 10mM-pyruvate on glucagon-mediated changes in cyclic AMP concentration. Glucagon (1nM) increased cyclic AMP approx. 8-fold in 1 min (Fig. 3c). This increase was

Fig. 2. Effect of extracellular lactate and pyruvate on glucagon-mediated changes in (a) glucose output, (b) $^3\text{H}_2\text{O}$ formation and (c) loss of radioactivity from $[^3\text{H}]$ glycogen by isolated hepatocytes

Isolated hepatocytes were prepared from fed rats and intracellular glycogen was labelled with $[5\text{-}^3\text{H}]\text{-glucose}$ as described in the Materials and Methods section. In addition to lactate and pyruvate the cells (approx. 45mg wet wt.) were incubated with 10nM-glucagon (○) or an equal volume of 0.9% saline (●) for 10min in a total volume of 1.5ml of buffer, at which time HClO_4 was added to give a final concentration of 2% (w/v). Glucose output, $^3\text{H}_2\text{O}$ and radioactivity in glycogen were determined as described in the text. Results from a single representative liver cell preparation are given. Pre-incubation radioactivity values are also shown (■) together with values for incubations to which neither lactate nor pyruvate was added.

Fig. 3. Effects of extracellular lactate and pyruvate on glucagon-mediated changes in cyclic AMP

Isolated hepatocytes (approx. 130mg wet wt.) from fed rats in a total volume of 2ml were preincubated for 5min with 10mM-pyruvate (a), 10mM-lactate (b) or 0.9% NaCl solution (c) before the additions of 1nM-glucagon (○) or an equal volume of 0.9% NaCl solution (●). Samples (0.2ml) were removed at the times shown and added to 0.1ml of 6% (w/v) HClO_4 . The vials were re-gassed with the O_2/CO_2 mixture, stoppered and returned to the water bath. Mean values from three cell preparations are shown and the bars indicate $\pm$ S.E.M.

further augmented by the inclusion of 10mM-lactate (Fig. 3b). In the presence of 10mM-pyruvate virtually no glucagon stimulation of cyclic AMP production occurred. Basal cyclic AMP concentrations were not significantly affected by either lactate or pyruvate.

A diminished production of cyclic AMP by glucagon under oxidized conditions suggested one of two possibilities: (a) an effect of redox state on the receptor-adenylate cyclase complex or (b) an effect of redox state on the enzymes concerned with cyclic AMP metabolism. To investigate the first possibility, the dose-response curves for glucagon-mediated changes in cyclic AMP concentration and

Fig. 4. Dose-responses curve for glucagon-mediated changes in (a) cyclic AMP content and (b) glucose output by isolated hepatocytes

Isolated hepatocytes (approx. 65mg wet wt.) were prepared from fed rats and preincubated for 5 min with 0.9% NaCl solution ($\circ$), 10mM-lactate (Δ), 10mM-pyruvate ($\square$) or 10mM-pyruvate+0.05mM-3-isobutyl-1-methylxanthine ($\blacksquare$) before the addition of glucagon. Cyclic AMP was determined in neutralized HClO_4 extracts of cells+medium after incubation with glucagon for 1 min. Glucose output was determined after a total of 25 min. Data for pyruvate+3-isobutyl-1-methylxanthine are from a single experiment. For the rest, mean values from three preparations are shown and the bars indicate $\pm$ S.E.M.

glucose output were examined (Fig. 4). Most significantly the data showed that the concentration of glucagon required to achieve half-maximal production of cyclic AMP or glucose output was unaffected by redox state, even though oxidized conditions (produced by adding 10mM-pyruvate) markedly decreased the overall response to glucagon. In addition, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine when added with pyruvate significantly increased glucagon-stimulated glucose output (Fig. 4b) by decreasing by approximately one order of magnitude the concentration of hormone required to produce half-maximal effects.

An experiment similar to that of Fig. 4(b) was undertaken to determine the effect of extracellular lactate and pyruvate on extracellular cyclic AMP-mediated glucose output (Fig. 5). In the presence of 10mM-pyruvate there was no significant effect of the cyclic nucleotide on the rate of glucose output. On this basis an effect of redox state on a reaction after adenylate cyclase that affected cyclic AMP concentration seemed likely. Fig. 6 shows the effect of extracellular lactate and pyruvate on (a) the intracellular concentration of cyclic AMP, (b) the uptake of radioactivity and (c) the rate of glucose output that occurred after the addition of 0.1mM-cyclic

Fig. 5. Effects of extracellular lactate and pyruvate on cyclic AMP-mediated glucose output by isolated hepatocytes. Isolated hepatocytes from fed rats were incubated for 10min with cyclic AMP at the concentrations shown. Additions were 0.9% NaCl solution ($\circ$), 10mM-lactate (Δ) and 10mM-pyruvate ($\square$). Data from a single representative experiment are shown.

[8-³H]AMP to the cells. As noted above (Fig. 5), cyclic AMP-stimulated glucose release occurred under reduced conditions and essentially no stimulation occurred under oxidized conditions (Fig. 6c). Whereas a change in the extracellular [lactate]/[pyruvate] ratio had no apparent effect on the uptake of cyclic [³H]AMP (Fig. 6b), a pronounced effect on the intracellular cyclic AMP concentration occurred (Fig. 6a). In the presence of 10 mM-pyruvate the intracellular concentration of cyclic AMP arising from extracellular addition of 0.1 M-cyclic AMP was 13% of that produced in the presence of 10 mM-lactate. No attempt was made to identify the radioactive material found inside the cell and which was not cyclic AMP.

Since the apparent uptake of cyclic [³H]AMP was unaffected by the extracellular [lactate]/[pyruvate] ratio, this suggested that cyclic AMP degradation may be the site of action. On this basis it was considered germane to examine the effects of lactate and pyruvate as well as NAD⁺ and NADH (compounds closely related to the [lactate]/[pyruvate] ratio through the equilibrium enzyme lactate dehydrogenase) on cyclic AMP phosphodiesterase activity of liver. As reported by Gumaa *et al.* (1977), three activities were detected in crude preparations from liver: cytosolic low-*K_m*, cytosolic high-*K_m* and

Fig. 6. Effects of extracellular lactate and pyruvate on (a) the intracellular concentration of cyclic AMP and (b) uptake of [³H]cyclic AMP and (c) cyclic AMP-stimulated glucose output by isolated hepatocytes

Isolated hepatocytes were prepared from fed rats and preincubated with lactate and pyruvate at the concentrations shown for 5 min. At this time 0.1 mM-cyclic [8-³H]AMP (specific radioactivity 60000 c.p.m./nmol) or an equal volume of 0.05 ml of 0.9% NaCl solution was added. After an additional 2 min a 0.2 ml sample of the incubation mixture was removed and the cells were separated from the medium by rapid centrifugation through silicone oil into 6% (w/v) HClO₄ (see the Materials and Methods section). Uptake of cyclic AMP was assessed by counting the radioactivity of a portion of the HClO₄ extract. Intracellular cyclic AMP was determined on the neutralized HClO₄ extract. Both values were corrected for the quantity of medium that was centrifuged with the cells through the layer of oil (0.15 μl of medium/mg wet wt. of cells). After a further 8 min the incubations were stopped by the addition of HClO₄ to give a final concentration of 0.2 M; glucose was determined on the HClO₄ extract. Mean results from two liver cell preparations are given for (a) and (c); means ± S.E.M. from three cell preparations are given for (b). Additions were 0.9% NaCl solution (●) and 0.1 mM-cyclic [³H]AMP (○). Values are also shown for incubations to which neither lactate nor pyruvate were added.

Fig. 7. Effects of NADH on hepatocyte membrane low- K_m phosphodiesterase activity: (a) time-response curve; (b) dose-response curve

Hepatocyte membrane fraction containing low- K_m phosphodiesterase was prepared from isolated hepatocytes and assayed as described in the Materials and Methods section. The initial substrate concentration was 0.1 μ M. Other additions for (a) were none (●), 0.01 mM-NADH (Δ) and 1 mM-NADH (○).

Table 1. Effects of nicotinamide nucleotides, lactate and pyruvate on cyclic AMP phosphodiesterase activities of isolated rat hepatocytes

Cytosolic and membrane fractions containing phosphodiesterase activities were prepared from isolated rat hepatocytes as described in the Materials and Methods section. Cyclic AMP phosphodiesterase activity was determined as described in the text. Values given are the means $\pm$ S.E.M. for three preparations.

Additions	Cyclic AMP phosphodiesterase activity (pmol/min per mg of protein)		
	Cytosolic high- K_m enzyme	Cytosolic low- K_m enzyme	Membrane low- K_m enzyme
None	911 $\pm$ 81	25.0 $\pm$ 2.1	34.9 $\pm$ 1.9
1 mM-NADH	1013 $\pm$ 97	18.0 $\pm$ 1.9	13.0 $\pm$ 1.5
1 mM-NAD ⁺	1090 $\pm$ 93	24.2 $\pm$ 1.9	34.2 $\pm$ 1.7
10 mM-Lactate	1207 $\pm$ 120	24.0 $\pm$ 2.0	35.7 $\pm$ 2.0
10 mM-Pyruvate	1034 $\pm$ 87	25.3 $\pm$ 1.8	35.1 $\pm$ 1.7

membrane-bound low- K_m . Table 1 shows that both low- K_m activities were affected by one of the four compounds tested. Most significantly, NADH strongly inhibited the membrane enzyme, with relatively less effect and no effect on the cytosolic low- and high- K_m enzymes respectively. The inhibitory effect was apparent directly after mixing with NADH (Fig. 7a), and concentrations as low as 5 μ M-NADH produced significant inhibition of the membrane low- K_m activity. Half-maximal inhibitory effects by NADH were obtained at approx. 20 μ M-NADH (Fig. 7b). To assess the possibility that the inhibitory effect of NADH was due to contaminants, a small portion (0.1 ml of 10 mM-NADH) was made to react with an equal quantity of sodium pyruvate and 2 units of crystalline lactate dehydrogenase; the small inhibitory effect of this reaction mixture on phosphodiesterase activity corresponded to remaining assayable content of NADH (results not shown). Further, NADH (1 mM) did not inhibit the snake-venom 5'-nucleotidase, a component of the phosphodiesterase assay system (Arch & Newsholme, 1976).

Table 2 shows the effects of NADH and related compounds on the activity of the low- K_m membrane AMP phosphodiesterase. Whereas 1 mM-NADH inhibited the enzyme by 65–70%, the same concentration of NAD⁺, NADP⁺ or lactate had no effect. A 1 mM solution of NADPH, dithiothreitol, reduced glutathione or 2-mercaptoethanol had slight effects (15–24% inhibition). The inclusion of 1.3 μ M-Ca²⁺ produced insignificant change in the control rate or the inhibition produced by NADH. 3-Isobutyl-1-methylxanthine (0.05 mM) inhibited the enzyme by 87%.

To establish whether the inhibitory effect of 1 mM-NADH was reversible for the membrane low- K_m

Table 2. *Effects of NADH, related substances, Ca^{2+} and 3-isobutyl-1-methylxanthine on the activity of membrane low- K_m cyclic AMP phosphodiesterase from isolated rat hepatocytes*

The membrane fraction containing cyclic AMP phosphodiesterase activity was prepared from isolated hepatocytes and assayed as described in the Materials and Methods section. Unless indicated otherwise, Ca^{2+} was not added to the assay. Where appropriate, means $\pm$ S.E.M. have been calculated and the numbers of experiments are given in parentheses.

Additions	Cyclic AMP phosphodiesterase activity (pmol/min per mg of protein)
None	34.8 $\pm$ 1.7 (9)
1 mM-NADH	12.2 $\pm$ 2.3 (9)
1 mM-NADPH	29.6 $\pm$ 1.7 (4)
1 mM-Dithiothreitol	26.1
1 mM-Lactate	33.7 $\pm$ 2.0 (3)
1 mM-Reduced glutathione	26.7
1 mM-2-Mercaptoethanol	28.4
1.3 μM - Ca^{2+} (free)	29.3
1.3 μM - Ca^{2+} (free) + 1 mM-NADH	12.7
0.05 mM-Isobutylmethylxanthine	4.5 $\pm$ 0.4 (8)

enzyme, a mixture of NADH and enzyme in 10 mM-Tris/HCl + 1 mM-EDTA, pH 7.5, was applied to a Sephadex G-25 (superfine, 10–40 μm) column (28 cm $\times$ 1.5 cm) and eluted with the same buffer. Column fractions (0.6 ml) were assayed for enzyme activity, protein and A_{340} . The specific activities of the enzyme were 13.0 (in the presence of 1 mM-NADH) and 39.7 pmol of cyclic AMP hydrolysed/min per g of protein before and after gel filtration respectively. Since the specific activities were 33.0 and 40.5 pmol of cyclic AMP hydrolysed/min per mg of protein (no NADH) before and after passage through a Sephadex G-25 column respectively, the results overall indicated that the inhibitory effect of NADH was reversible.

The data of Fig. 8(a) show the substrate-saturation kinetics for the low- K_m membrane cyclic AMP phosphodiesterase in the presence and absence of the inhibitor (1 mM-NADH). Measurement of enzyme rate for the range 0.025–1 μM -cyclic AMP showed that this enzyme preparation contained mainly low- K_m activity, although some contamination with an activity of high K_m was also noted. Thus the inhibitory effects of NADH were examined mainly in the substrate range 0.025–0.1 μM (Fig. 8b). These results indicated the apparent K_m of the enzyme to be approx. 0.2 μM and that NADH inhibited the enzyme by decreasing the V_{max} .

Subcellular fractionation of the hepatocytes was undertaken to ascertain whether the low- K_m phosphodiesterase activity was associated with the plasma

Fig. 8. *Effect of NADH on the activity of hepatocyte membrane low- K_m phosphodiesterase*

A solubilized hepatocyte membrane fraction containing low- K_m phosphodiesterase was prepared and assayed as described in the Materials and Methods section. The initial velocity was measured over a 5 min period, during which the rate was linear and less than 25% of the substrate was consumed. Additions were none (▲) and 1 mM-NADH (●). The data are from duplicate assays for two liver cell-membrane preparations and are presented in (b) by a Lineweaver-Burk plot, where the lines have been fitted by eye.

membrane and whether NADH inhibition occurred for a more pure preparation of the enzyme. Fractionation of the microsomal pellet by discontinuous-sucrose-density-gradient centrifugation (Table 3) by the method of Touster *et al.* (1970) showed that the low- K_m cyclic AMP phosphodiesterase activity was

Table 3. *Cyclic AMP phosphodiesterase activities in subcellular fractions of isolated hepatocytes*

Isolated hepatocytes (approx. 2 g wet wt.) were homogenized as described by Wisher & Evans (1977) for the isolation of plasma membranes. Fractionation of the homogenate and the microsomal pellet was conducted as described by Touster *et al.* (1970). Methods for the determination of protein, cyclic AMP phosphodiesterase, 5'-nucleotidase and glucose 6-phosphatase were as described in the text. The terms 'homogenate' and 'microsomal (P) fraction' are as used by Touster *et al.* (1970). Values are the means from two hepatocyte preparations.

Fraction	Enzyme activity (nmol/min per mg of protein)			Protein (mg/ml)	Inhibition of cyclic AMP phosphodiesterase with 1 mM-NADH (%)
	Cyclic AMP phosphodiesterase	Glucose 6-phosphatase	5'-Nucleotidase		
Homogenate	0.035	3.1	2.9	18.2	55.7
Microsomal (P) fraction	0.060	5.0	8.3	2.4	48
8% (w/v) sucrose layer	0.222	nil	28.0	0.05	73.7
8/34% (w/v) sucrose interface	0.151	2.2	133.3	0.09	52.6
34/57% (w/v) sucrose interface	0.069	18.5	4.2	1.5	51.9

located in two adjacent fractions: (a) the 8/34% (w/v) sucrose interface, which contained predominantly plasma membranes and was enriched with 5'-nucleotidase; (b) the 8% (w/v) sucrose layer, which was also partially enriched with 5'-nucleotidase. NADH (1 mM) inhibited the cyclic AMP phosphodiesterase activity in each fraction; the highest-specific-activity fraction was inhibited to the greatest extent (Table 3).

Discussion

Redox state and glucagon action

Thus far the involvement of redox state in hormone action has received little attention. Parrilla *et al.* (1975, 1976) have noted that the glucagon-mediated enhancement of gluconeogenesis in isolated perfused livers from starved rats was accompanied by a more reduced cytosolic and mitochondrial redox state of the NAD system. Siess *et al.* (1977) confirmed these observations and also noted that glucagon lowered the cytosolic [NADP⁺]/[NADPH] ratio and the mitochondrial [NAD⁺]/[NADH] ratio. However, the basis by which glucagon alters the redox state is as yet uncertain. Since fatty acid administration to liver results in a more reduced redox state, the proposal by Struck *et al.* (1965) that the stimulation of gluconeogenesis by glucagon is the result of an enhanced lipid mobilization may account for observed changes in redox state. Such a hypothesis has been further developed by Williamson (1969). However, the finding that glycodiazine, an inhibitor of lipolysis (Menahan & Wieland, 1969), does not prevent the glucagon stimulation of gluconeogenesis (Frolich & Wieland, 1971) has cast some doubt on this hypothesis. Thus the mechanism by which the cell becomes more reduced after glucagon administration remains to be established, and as yet there are no data to indicate whether a change in redox state

results from, or leads to, the stimulation of gluconeogenesis by this hormone.

From an entirely different viewpoint Löw, Crane and their co-workers have examined a hormone-regulated redox function in plasma membranes (Löw & Crane, 1976; Crane & Löw, 1976; Löw & Werner, 1976). They have reported that: (i) NADH inhibits adenylate cyclase in fat-cell plasma membranes; (ii) NADH dehydrogenase is present in plasma membranes; (iii) the NADH dehydrogenase activity in the liver cell plasma membrane is activated by glucagon at concentrations of the hormone that stimulate adenylate cyclase activity; (iv) the addition of 3 mM-lactate (preincubated with the cells for 45 min) together with 3-isobutyl-1-methylxanthine to isolated hepatocytes resulted in a decreased production of cyclic AMP by glucagon (Zederman *et al.*, 1977). In the present studies without the addition of a phosphodiesterase inhibitor, or an extensive preincubation period with lactate, no inhibitory effect of lactate on glucagon-mediated cyclic AMP production was noted.

In one other system (Ehrlich ascites-tumour cells) it has been observed that quinacrine, an agent that inhibits plasma-membrane NADH reductase, inhibited methylaminoisobutyrate uptake (Garcia-Sancho *et al.*, 1977), an effect that could not be simulated by agents such as rotenone, cyanide and azide, which each appreciably decreased cellular ATP. These workers proposed that the NADH dehydrogenase present in plasma-membrane preparations (Samudio & Canessa, 1966; Ferber *et al.*, 1972; Löw & Crane, 1976; Crane & Löw, 1976) participates in the energization of both Na⁺-dependent and Na⁺-independent amino acid transport, allowing the utilization of reducing equivalents which can be made available by a shuttle from the mitochondria to the cytoplasm. It remains to be seen whether hormonal effects on amino acid transport are related

to changes in the plasma NADH dehydrogenase activity.

In the present work data are presented to show that glucagon-mediated glycogenolysis in hepatocytes from fed rats is controlled by the redox state and that this modification of glucagon efficacy may be regulated by NADH acting to inhibit the membrane-bound low- K_m cyclic AMP phosphodiesterase. The present observations extend the findings of Zahlten *et al.* (1973) with hepatocytes from starved rats, where glucagon was noted to inhibit gluconeogenesis from pyruvate while stimulating gluconeogenesis from lactate, and earlier ones with the isolated perfused rat liver, where glucagon consistently stimulated gluconeogenesis from lactate to a greater extent than from pyruvate (Garrison & Haynes, 1973; see their Table VI for a summary of various groups' data). Zahlten *et al.* (1973) explained their observations on the basis that, since glucagon inhibited pyruvate dehydrogenase (determined from effects on the decarboxylation of $[1-^{14}\text{C}]$ pyruvate), the production of reducing equivalents for gluconeogenesis became limiting. This explanation appeared likely for gluconeogenesis in cells from starved rats, particularly since it was overcome by the inclusion of ethanol (Garrison & Haynes, 1973; Zahlten *et al.*, 1973), but was considered unsatisfactory for a similar related loss of glucagon efficacy in stimulating glycogenolysis in cells from fed rats (Clark *et al.*, 1977). In that previous work (Clark *et al.*, 1977) we have shown that changes in the hepatocyte cytosolic redox state (indicated by the $[\text{lactate}]/[\text{pyruvate}]$ ratio) affected by a variety of conditions correlated with changes in the efficacy of glucagon to alter the rate of glycogenolysis. In particular, glucagon-mediated glycogenolysis was favoured by a reduced state, indicated by a $[\text{lactate}]/[\text{pyruvate}]$ ratio >10 for the cells+media. Values for this ratio of less than 5 gave rise to a marked loss of glucagon efficacy. The present experiments were conducted in an attempt to determine the site(s) in the cascade mechanism at which redox state affected the ability of glucagon to stimulate glycogenolysis. Dose-response curves indicated that the concentration of glucagon required to produce half-maximal increases in cyclic AMP and glycogenolysis were unaffected by an oxidized redox state (10mM-pyruvate), even though under these conditions the maximal effect was markedly decreased. Similar effects of pyruvate on cyclic AMP-stimulated glycogenolysis were noted and thus suggested that redox state did not affect either receptor binding or adenylate cyclase leading to the formation of cyclic AMP. Since oxidized conditions decreased the intracellular cyclic AMP concentration without affecting uptake of the cyclic nucleotide, attention was directed towards the effects of redox state on cyclic AMP phosphodiesterases.

Redox-state control of the membrane-bound low- K_m cyclic AMP phosphodiesterase

It is generally accepted that there are at least two forms of cyclic AMP phosphodiesterase in rat liver. The two forms differ in a number of aspects, but primarily in their affinity for the substrate: the high- K_m (low-affinity) enzyme is usually soluble and the low- K_m (high-affinity) enzyme is both soluble and particulate. In the present study three cyclic AMP phosphodiesterase activities were detected: a soluble high- K_m enzyme, a soluble low- K_m enzyme and a membrane-bound low- K_m enzyme. Contamination of the membrane fraction with high- K_m activity was assumed to result from incomplete disruption of the cells during isolation, although some high- K_m activity has been reported to be associated with the membrane fraction (Tria *et al.*, 1976; Gumaa *et al.*, 1977). Both the membrane and the soluble low- K_m enzymes were inhibited by 1mM-NADH, although the membrane enzyme was more markedly inhibited. The specificity of the inhibitory effect appeared to be high; only slight inhibitory effects of NADPH, dithiothreitol, lactate, reduced glutathione and 2-mercaptoethanol were noted. In addition, the inhibitory effect by NADH was reversible and kinetic examination indicated that 1mM-NADH decreased the maximal velocity without affecting the K_m . Furthermore the low- K_m NADH-sensitive enzyme appeared to be associated with the plasma-membrane fraction of hepatocytes.

To our knowledge NADH has not been previously reported as an inhibitor of cyclic AMP phosphodiesterase (for example, see the review by Amer & Kreighbaum, 1975). The present data indicate that the concentration at which half-maximal inhibition is attained is approx. 20 μM . The total hepatic concentrations of NADH and NAD^+ (free+bound) are in the ranges 40–500 and 500–800nmol/g per wet wt. respectively (Bücher, 1970). However, since the calculated $[\text{free NAD}^+]/[\text{free NADH}]$ ratio of cytosol for normal liver is approx. 1000 (Krebs & Veech, 1970), the concentration of free NADH is of the order of 1 μM , which is well below the minimum concentration to affect phosphodiesterase. Thus the proposal for a physiologically important regulation of the membrane-bound cyclic AMP phosphodiesterase by NADH is based on the assumption that there is a constant equilibrium between the redox state of free and bound nicotinamide nucleotides. Indeed, several groups have shown that the $[\text{total NAD}^+]/[\text{total NADH}]$ ratio changes in concert with the $[\text{free NAD}^+]/[\text{free NADH}]$ ratio (e.g. see Bücher, 1970).

Regulation by NADH of both adenylate cyclase (Lów & Werner, 1976) and cyclic AMP phosphodiesterase in an inhibitory capacity might appear contradictory. The present data, together with those

of Löw and co-workers, suggest the possibility of a 'redox optimum' as an overriding control of glucagon efficacy, where both highly reduced conditions and highly oxidized conditions decrease the response. Under normal conditions and in the absence of phosphodiesterase inhibitor, the effect of NADH on cyclic AMP phosphodiesterase would appear to be the most significant [i.e. no significant inhibitory effect of 10mM-lactate on glucagon stimulated glycogenolysis was noted previously (Clark *et al.*, 1977) or in the present studies (Figs. 2 and 4)].

Various laboratories have reported on the control of the intracellular concentration of cyclic AMP through hormonal modulation of a specific phosphodiesterase (see Appleman *et al.*, 1973, for review). Insulin has been reported to increase in adipose tissue the activity of a particulate low- K_m cyclic AMP phosphodiesterase (Loten & Sneyd, 1970; Manganiello & Vaughan, 1973; Zinman & Hollenberg, 1974; Sakai *et al.*, 1974; Lovell-Smith *et al.*, 1977) and in some liver tissue a plasma-membrane-bound low- K_m enzyme has been reported to be activated by insulin after treatment *in vivo* (Thompson *et al.*, 1973) or *in vitro* (House *et al.*, 1972). More recently, isolated hepatocytes incubated in the presence of insulin showed increased phosphodiesterase activity in a plasma-membrane-containing fraction (Tria *et al.*, 1976; Loten *et al.*, 1978). Even though insulin also lowers the glucagon-mediated increase in reduction of the redox state (Parrilla *et al.*, 1975, 1976), there is no evidence as yet that this relates to the present observed inhibition of the enzyme by NADH.

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