Regulation of glycogen synthesis and glycolysis by insulin, pH and cell volume

Interactions between swelling and alkalization in mediating the effects of insulin

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The effects of changes in cell volume and pH on glycogen synthesis and glycolysis and their control by insulin were investigated in hepatocyte cultures. 1. Cell acidification, by increasing $[\text{CO}_2]$ from 2.5 % to 5 %, inhibited glycolysis and stimulated glycogen synthesis. The inhibition of glycolysis was also observed in Na^+ -free media and when K^+ uptake was inhibited, but the stimulation of glycogen synthesis was abolished under these conditions, suggesting that it is secondary to ionic or volume changes. Alkalization had converse effects on glycolysis and glycogen synthesis. 2. In HCO_3^- -containing media, replacement of NaCl with sodium acetate or potassium acetate, like acidification with CO_2 , inhibited glycolysis and stimulated glycogen synthesis. The latter correlated with an increase in cation content. Amiloride, an inhibitor of Na^+/H^+ exchange, inhibited both the increase in cation content and the stimulation of glycogen synthesis, suggesting that the latter is secondary to cell swelling. 3. Hypo-osmotic swelling increased glycogen synthesis in HCO_3^- -containing media, in both the absence and the presence of Na^+ and at both 2.5 % and 5 % CO_2 , but it increased glycolysis in the presence of Na^+ and at 2.5 %, but not at 5 %, CO_2 . In HCO_3^- -free media, during acidification and swelling, glycogen synthesis correlated with pH and not with cell volume, indicating that inhibition by acidification over-rides stimulation by swelling. 4. Stimulation of glycolysis by insulin was not additive with stimulation by alkalization. The stimulation of glycogen synthesis by insulin was partially suppressed under alkaline conditions; it was markedly suppressed in iso-osmolar Na^+ -free media and restored by hypo-osmotic swelling. In hypo-osmolar Na^+ -free media insulin prevented the decrease in glycogen synthesis with decreasing $[\text{HCO}_3^-]$, suggesting that it counteracts inhibition by acidification. 5. It is concluded that glycogen synthesis and glycolysis are both stimulated by cell swelling and inhibited by acidification, under certain conditions, but glycolysis is more sensitive to inhibition by acidification and glycogen synthesis to stimulation by swelling. Consequently, simultaneous swelling and acidification is associated with inhibition of glycolysis and stimulation of glycogen synthesis. Stimuli that cause swelling and alkalization activate both glycogen synthesis and glycolysis, alkalization being more important in control of glycolysis and swelling in control of glycogen synthesis. Both cell swelling and alkalization are components of the mechanism by which insulin controls glycogen synthesis and glycolysis.

INTRODUCTION

The Na^+/H^+ antiporter exchanges extracellular Na^+ for intracellular protons and is important in the regulation of cell pH and cell volume [1,2]. The antiporter is quiescent above a certain cell pH and is activated by acidification of the cytosol, which is consistent with its postulated role in the control of pH homeostasis [1,2]. It is also activated by growth factors, hormones and hyperosmotic shrinking [1–3]. Activation by these stimuli is mediated not by prior acidification of the cytosol, but by an alkaline shift of the pH-dependence of the antiporter, and leads to alkalization of the cytosol above the normal set point [1,2]. The cytosolic alkalization that results from activation of Na^+/H^+ exchange by growth factors is considered to have either an essential or a permissive role in the induction of cell proliferation [2,3]. Insulin activates Na^+/H^+ exchange in muscle [4], and the resultant alkalization is involved in the stimulation of glycolysis [4,5].

The evidence that hypo-osmotic swelling stimulates glycogen synthesis [6] raised the question whether activation of Na^+/H^+ exchange by insulin may be involved in the control of glycogen synthesis through cell swelling [7]. We showed in the preceding paper that insulin increases the hepatocyte cation content (K^+ and Na^+) and thereby cell volume, through a Na^+ -dependent

mechanism, and that a Na^+ -free medium also blocks the stimulation of glycogen synthesis by insulin [8]. Furthermore, hypo-osmotic swelling restored the stimulation of glycogen synthesis by insulin in a Na^+ -free medium, suggesting a role for cell swelling in the stimulation of glycogen synthesis by insulin [8]. This, together with the evidence that alkalization is involved in the control of glycolysis by insulin [4], raises the question of the relative roles of swelling and alkalization in the control of carbohydrate metabolism by insulin.

Changes in cell volume and cell pH are intimately coupled, through Na^+/H^+ exchange, which regulates both cell volume and cell pH [1,2]. In hepatocytes, hypo-osmotic swelling and hyperosmotic shrinking are associated with acidification and alkalization respectively [9]. To what extent these pH changes [9] contribute to the changes in glycogen metabolism [6] or glycolysis [10] that are associated with aniso-osmotic volume changes has not been established. In this study we investigated the effects of changes in pH and cell volume on glycolysis and glycogen synthesis. We determined the effects of changes in cell pH by altering the concentration of CO_2 at constant $[\text{HCO}_3^-]$ and of HCO_3^- at constant $[\text{CO}_2]$, by replacement of Cl^- with acetate, which causes acidification and swelling [11], and by varying extracellular pH in a HCO_3^- -free system. The effects of hypo-osmolarity were investigated over a range of pH to establish

Abbreviation used: MEM, Minimum Essential Medium.

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the interactions between volume and pH changes; and the effects of pH were examined in the presence or absence of extracellular Na^+ , to establish whether the metabolic changes are direct effects of pH or secondary to changes in Na^+ entry. We show that both glycogen synthesis and glycolysis are stimulated by alkalization and swelling, but glycogen synthesis is more sensitive to volume changes and glycolysis to pH changes. Consequently, concurrent swelling and acidification simultaneously stimulate glycogen synthesis and inhibit glycolysis. Differences between the metabolic effects of insulin and of hypo-osmotic swelling can be explained by the inverse pH changes caused by insulin and hypo-osmotic swelling. These findings support the hypotheses that both alkalization [5] and swelling [7,8] are components of the insulin-transduction mechanism, but their relative roles differ for glycolysis and glycogen synthesis.

MATERIALS AND METHODS

Materials

[U- ^{14}C]glucose, UDP-[^3H]glucose and 3-*O*-methyl-D-[^{14}C]glucose were from Amersham International (Amersham, Bucks., U.K.); [3- ^{14}C]pyruvate was from New England Nuclear (Boston, MA, U.S.A.). Insulin (pig), ouabain and amiloride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sources of other reagents were as reported previously [12].

Hepatocyte isolation and culture

Hepatocytes were isolated by collagenase perfusion of whole liver of male Wistar rats (270–340 g body wt.) fed on standard rat chow *ad libitum* [12]. They were cultured in MEM supplemented with 5% (v/v) neonatal-calf serum in 24-well or 12-well plates at a density of 6.4×10^4 cells/cm ^{2} [12]. After cell attachment (4–6 h), the medium was changed to serum-free MEM containing 10 nM-dexamethasone without insulin, and cells were cultured for 16–20 h. Experiments were performed after the 16–20 h pre-culture in fresh medium without dexamethasone [12].

Incubation media

In experiments where we examined the effects of varying $[\text{CO}_2]$ and $[\text{HCO}_3^-]$ and of NH_4Cl and ouabain (Tables 2, 3 and 6), incubations were in MEM (which contains amino acids [13]). In experiments where we investigated the effects of Na^+ -free media, hypo-osmolarity, and replacement of Cl^- with isethionate or acetate, we used balanced salts or modified salts media (without any amino acids) with the composition listed in Table 1 or as described in the text. In experiments where we compared the effects of 5% and 2.5% CO_2 in balanced salts media (Tables 4 and 5), we used 20 mM-Hepes (Na-Hepes in the control medium and K-Hepes in the Na^+ -free medium). In other experiments with Na^+ -free media (Figs. 1 and 2), Hepes was omitted to minimize

[K^+]. In experiments with HCO_3^- -free media, HCO_3^- was replaced by Hepes in experiments at pH 7.4 or by Tris/Mes in experiments involving a range of extracellular pH (6.6–7.4). The latter media were prepared as described previously [11].

Substrates for glycogen synthesis and glycolysis

In experiments where glycolysis, glycogen synthesis and deposition were determined simultaneously, the medium contained 15 mM-[U- ^{14}C]glucose (1.5 $\mu\text{Ci/ml}$). In experiments where only glycogen synthesis and deposition were determined, the medium contained 5 mM-pyruvate in addition to 15 mM-[U- ^{14}C]glucose. Pyruvate was used because it causes a synergistic stimulation of glycogen synthesis relative to glucose alone [12]. In experiments in HCO_3^- -free media rates of glycogen deposition were lower than in the HCO_3^- -containing media, and in some of these experiments (as indicated) the [glucose] was increased to 25 mM.

Glycolysis and glycogen synthesis

These were determined in hepatocyte cultures during a 2 h or 3 h incubation in the media indicated, and incubations were terminated and extracted as described previously [12]. Time-course experiments (1–4 h) of glycogen deposition in balanced salts medium, Na^+ -free and hypo-osmolar media showed similar relative differences between these media over these time points, and rates were linear for about 3 h. Glycogen synthesis was determined from the incorporation of [^{14}C]glucose into glycogen, isolated by ethanol precipitation [12], and glycogen deposition from the incremental increase in cell glycogen, measured enzymically [12]. Glycolysis was determined from the accumulation of lactate [12] in the medium. In the presence of 5 mM-glucose, accumulation of lactate was negligible, suggesting that lactate accumulation during incubation with 15 mM-glucose is largely derived via glycolysis. Glycogen deposition and synthesis are expressed as nmol of glucose units formed or incorporated/time interval per mg of protein, and glycolysis is expressed as nmol of lactate formed/3 h per mg of cell protein.

Glycogen synthase activity

This was determined in hepatocyte extracts with either 0.1 mM- or 4 mM-glucose 6-phosphate (synthase *a* and total activity respectively) as described previously [14], except that Na_2SO_4 was omitted. The activity is expressed as m-units/mg of total cell protein, where 1 m-unit is the amount of enzyme converting 1 nmol of UDP-[^3H]glucose into glycogen/min at 30 °C.

Cell K^+ and Na^+ contents

These were determined after a 60 min incubation in the media indicated, and the water content was determined from the accumulation of 3-*O*-methyl-D-[^{14}C]glucose in glucose-free medium [15], as described in [8].

Cell protein was determined as in [16]. Lactate dehydrogenase

Table 1. Composition of solutions used (mM)

Solution	NaCl	Sucrose	KCl	CaCl_2	MgCl_2	NaH_2PO_4	KH_2PO_4	Na_2SO_4	MgSO_4	NaHCO_3	KHCO_3	Na-Hepes	K-Hepes
A. Balanced salts	116	–	5.1	1.23	1.18	1.23	–	0.80	–	12.5	–	20	–
B. Hypo-osmolar (–80 mosm)	76	–	5.1	1.23	1.18	1.23	–	0.80	–	12.5	–	20	–
C. Na^+ -free	–	232	5.1	1.23	1.18	–	1.23	–	0.80	–	12.5	–	20
D. Na^+ -free hypo- osmolar (–80 mosm)	–	152	5.1	1.23	1.18	–	1.23	–	0.80	–	12.5	–	20
E. Balanced salts	104	–	5.1	1.23	1.18	1.23	–	0.80	–	25.0	–	20	–
F. Na^+ -free	–	257	–	1.23	1.18	–	1.23	–	0.80	–	25.0	–	–
G. Low- Na^+	–	257	–	1.23	1.18	–	1.23	–	0.80	25.0	–	–	–

Table 2. Effects of $[\text{CO}_2]$, $[\text{HCO}_3^-]$ and ouabain on glycogen synthesis and glycogen deposition

Hepatocyte cultures were incubated for 3 h in MEM containing 15 mM-[U- ^{14}C]glucose and 5 mM-pyruvate and the concentrations of NaHCO_3 indicated, without or with ouabain (1 mM) or NH_4Cl (0.5 mM) as indicated. They were equilibrated either with 2.5% CO_2 or with 5% CO_2 . Glycogen synthesis is expressed as nmol of [^{14}C]glucose incorporated into glycogen/3 h per mg of cell protein, and glycogen deposition as nmol of glucosyl units deposited/3 h per mg of cell protein. Values are means $\pm$ S.E.M. for 4 cultures. Statistics (paired *t* test): ^a*P* < 0.05, ^{aa}*P* < 0.005, 5% versus 2.5% CO_2 ; ^b*P* < 0.05, ^{bb}*P* < 0.005, 12.5 mM- HCO_3^- versus 18 mM- or 25 mM- HCO_3^- ; ^c*P* < 0.05, ^{cc}*P* < 0.005, ouabain versus respective control.

$[\text{CO}_2]$ (%)...	Glycogen deposition		Glycogen synthesis	
	2.5	5	2.5	5
12.5 mM- HCO_3^-	105 $\pm$ 8	200 $\pm$ 11 ^{aa}	43 $\pm$ 5	73 $\pm$ 3 ^{aa}
18 mM- HCO_3^-	83 $\pm$ 4 ^b	175 $\pm$ 16 ^{aa,b}	34 $\pm$ 3 ^b	65 $\pm$ 6 ^{aa,b}
25 mM- HCO_3^-	68 $\pm$ 7 ^{bb}	149 $\pm$ 11 ^{aa,bb}	26 $\pm$ 4 ^{bb}	59 $\pm$ 8 ^{a,b}
12.5 mM- HCO_3^- + ouabain	114 $\pm$ 4	109 $\pm$ 5 ^{cc}	43 $\pm$ 5	32 $\pm$ 6 ^{a,cc}
25 mM- HCO_3^- + ouabain	95 $\pm$ 5 ^{cc}	117 $\pm$ 11 ^c	33 $\pm$ 3	32 $\pm$ 4 ^{cc}
12.5 mM- HCO_3^- + NH_4Cl	75 $\pm$ 12	139 $\pm$ 11 ^{aa}	31 $\pm$ 1	64 $\pm$ 2 ^{aa}

activity [17] was determined in the medium on termination of the incubations. None of the experimental conditions reported herein caused increased leakage of lactate dehydrogenase compared with control values. Incubations for metabolic studies were performed in duplicate wells and those for cation or water content in triplicate wells for each condition. Results are expressed as means $\pm$ S.E.M. for the numbers of cell-culture preparations (experiments) indicated. Statistical analysis was by Student's paired *t* test.

RESULTS

Effects of $[\text{CO}_2]$, $[\text{HCO}_3^-]$ and ouabain on glycogen synthesis and glycolysis (Tables 2 and 3)

Cytosolic alkalization by decreasing the $[\text{CO}_2]$ from 5% to 2.7% at constant $[\text{HCO}_3^-]$ stimulates glycolysis in muscle [4]. We determined the effects of 5% and 2.5% CO_2 at different concentrations of HCO_3^- (12.5–25 mM) in the absence and presence of ouabain, an inhibitor of Na^+/K^+ -ATPase [5], on glycogen synthesis (Table 2) and glycolysis (Table 3) in hepatocytes incubated in MEM (containing amino acids).

In the absence of ouabain, glycogen synthesis and deposition were higher at 5% CO_2 than at 2.5% CO_2 at all concentrations of HCO_3^- (Table 2). At each $[\text{CO}_2]$, glycogen synthesis and deposition decreased with increasing $[\text{HCO}_3^-]$, suggesting stimulation by acidification and inhibition by alkalization. Cells are

more permeable to CO_2 than to HCO_3^- ; consequently, for a given $\text{HCO}_3^-/\text{CO}_2$ ratio, cell pH would be more acidic at the higher CO_2 (and HCO_3^-) concentration [18]. At a $\text{HCO}_3^-/\text{CO}_2$ ratio of 5, glycogen synthesis and deposition were higher (*P* < 0.001) at 5% CO_2 (and 25 mM- HCO_3^-) than at 2.5% CO_2 (and 12.5 mM- HCO_3^- , Table 2), indicating that cellular acidification increases glycogen synthesis. Glycolysis, unlike glycogen synthesis, was lower at 5% CO_2 than at 2.5% CO_2 (Table 3), suggesting inhibition by acidification, consistent with the findings in muscle [4].

Ouabain (1 mM) decreased the cell K^+/Na^+ ratio from 9.78 ± 1.32 to 1.75 ± 0.06 (means $\pm$ S.E.M., *n* = 4), consistent with inhibition of Na^+/K^+ -ATPase [5]. Ouabain inhibited glycogen synthesis and deposition at 5% CO_2 , at all HCO_3^- concentrations (45–57% and 22–46% inhibition, at 12.5 mM- and 25 mM- HCO_3^- respectively), but it did not affect glycogen synthesis at 2.5% CO_2 and 12.5 mM- HCO_3^- and it stimulated glycogen synthesis at 2.5% CO_2 and 25 mM- HCO_3^- (Table 2). Because ouabain inhibited the high rates of glycogen synthesis (5% CO_2) and stimulated the lowest rates (2.5% CO_2 and 25 mM- HCO_3^-), it abolished the stimulation of glycogen synthesis by 5% CO_2 as compared with 2.5% CO_2 (Table 2), suggesting that the effect of CO_2 is dependent either on Na^+/K^+ exchange or on the transcellular Na^+ and K^+ gradients.

Ouabain inhibited glycolysis at both 5% and 2.5% CO_2 (Table 3), but did not abolish the inhibition by 5% CO_2 compared with 2.5% CO_2 (20–25% versus 11–17% inhibition in the presence versus the absence of ouabain), suggesting that, unlike stimulation of glycogen synthesis, it is at least in part independent of Na^+/K^+ exchange or the transcellular Na^+ and K^+ gradients.

Effects of Na^+ -free media and hypo-osmolarity on glycogen synthesis and glycolysis at 5% and 2.5% CO_2 (Tables 4 and 5)

To examine whether the stimulation of glycogen synthesis during acidification with CO_2 (5% versus 2.5%) is dependent on Na^+ -entry mechanisms, we determined the effects of 5% CO_2 versus 2.5% CO_2 in a balanced salts medium (without amino acids) and in a Na^+ -free medium, in which NaCl was replaced by sucrose. Additional incubations were performed in hypo-osmolar media (containing 76 mM- instead of 116 mM- NaCl and 152 mM- instead of 232 mM-sucrose) to compare the effects of CO_2 with hypo-osmotic swelling (Tables 4 and 5). In initial experiments we used choline chloride as a replacement for NaCl . However, there was a significant decrease (*P* < 0.01) in cell K^+ content after 60 min incubation in medium containing choline chloride (114 ± 20 nmol of K^+/mg of cell protein; means $\pm$ S.E.M.,

Table 3. Effects of $[\text{CO}_2]$, $[\text{HCO}_3^-]$ and ouabain on glycolysis

Hepatocyte cultures were incubated for 3 h in MEM containing 15 mM-glucose and the concentrations of NaHCO_3 indicated, without or with ouabain (1 mM) as indicated. They were equilibrated either with 2.5% CO_2 or with 5% CO_2 . Glycolysis is expressed as nmol of lactate formed/3 h per mg of cell protein. Values are means $\pm$ S.E.M. for 4 cultures. Statistics (paired *t* test): ^a*P* < 0.05, 5% versus 2.5% CO_2 ; ^b*P* < 0.05, 25 mM- versus 12.5 mM- HCO_3^- ; ^c*P* < 0.05, ^{cc}*P* < 0.005, ouabain versus respective control.

$[\text{CO}_2]$ (%)...	Glycolysis	
	2.5	5
12.5 mM- HCO_3^-	411 $\pm$ 42	344 $\pm$ 31 ^a
25 mM- HCO_3^-	436 $\pm$ 19 ^b	388 $\pm$ 43 ^{a,b}
12.5 mM- HCO_3^- + ouabain	264 $\pm$ 46 ^{cc}	199 $\pm$ 31 ^{a,c}
25 mM- HCO_3^- + ouabain	263 $\pm$ 24 ^{cc}	211 $\pm$ 28 ^c

Table 4. Effects of [CO₂] and hypo-osmolarity on glycogen synthesis and deposition in the absence and presence of extracellular Na⁺

Hepatocyte cultures were incubated for 3 h in Na⁺-containing media without amino acids (Table 1, media A and B) or in Na⁺-free media (Table 1, media C and D). Media B and D were hypo-osmolar (−80 mosm) relative to media A and C. They were equilibrated either with 2.5 % CO₂ or with 5 % CO₂. Media were supplemented with 15 mM-[U-¹⁴C]glucose and 5 mM-pyruvate. Glycogen synthesis is expressed as nmol of [¹⁴C]glucose incorporated into glycogen/3 h per mg of cell protein, and glycogen deposition as nmol of glucosyl units deposited/3 h per mg of cell protein. Values are means ± S.E.M. for 4 cultures. Statistics (paired *t* test): ^a*P* < 0.05, ^{aa}*P* < 0.005, 5 % versus 2.5 % CO₂; ^b*P* < 0.05, ^{bb}*P* < 0.005, hypo-osmolar versus respective control.

[CO ₂] (%) ...	Glycogen deposition		Glycogen synthesis	
	2.5	5	2.5	5
Control	31 ± 3	39 ± 3 ^a	19 ± 3	27 ± 3 ^a
Hypo-osmolar	36 ± 4	48 ± 3 ^{aa,b}	33 ± 3 ^b	41 ± 4 ^b
Na ⁺ -free	25 ± 1	24 ± 2	21 ± 4	17 ± 1
Na ⁺ -free, hypo-osmolar	61 ± 6 ^{bb}	67 ± 3 ^{bb}	60 ± 5 ^b	57 ± 6 ^{bb}

Table 5. Effects of [CO₂] and hypo-osmolarity on glycolysis and gluconeogenesis in the absence or presence of extracellular Na⁺

Hepatocytes were incubated for 3 h in iso-osmolar (316 mosm) or hypo-osmolar (236 mosm) Na⁺-containing media (Table 1, media A and B) or Na⁺-free media (Table 1, media C and D). They were equilibrated with either 2.5 % CO₂ or 5 % CO₂ as indicated. Incubations for determination of glycolysis contained 15 mM-glucose, and those for determination of gluconeogenesis contained 5 mM-glucose and 5 mM-[3-¹⁴C]pyruvate. Glycolysis is expressed as nmol of lactate formed/3 h per mg of protein, and gluconeogenesis as nmol of [¹⁴C]pyruvate incorporated into glucose/3 h per mg of protein. Values are means ± S.E.M. for 4 cultures. ^a*P* < 0.05, ^{aa}*P* < 0.005, 5 % CO₂ versus 2.5 % CO₂; ^b*P* < 0.05, hypo-osmolar versus respective control.

[CO ₂] (%) ...	Glycolysis		Gluconeogenesis	
	2.5	5	2.5	5
Control	357 ± 32	275 ± 32 ^a	366 ± 98	231 ± 43 ^a
Hypo-osmolar	406 ± 30 ^b	285 ± 33 ^{aa}	317 ± 60	272 ± 53
Na ⁺ -free	432 ± 31	294 ± 20 ^a	165 ± 41	117 ± 7
Na ⁺ -free, hypo-osmolar	429 ± 34	312 ± 25 ^a	130 ± 31 ^b	148 ± 13

n = 3) compared with both the control medium containing NaCl (202 ± 10) and the sucrose-containing Na⁺-free medium (192 ± 13). We subsequently used sucrose as a replacement for NaCl.

Acidification (5 % versus 2.5 % CO₂) increased glycogen synthesis and deposition in the control medium and in the hypo-osmolar Na⁺-containing medium, but not in the Na⁺-free media (Table 4), suggesting that the stimulation is dependent on Na⁺-entry mechanisms. Hypo-osmolarity increased glycogen synthesis at both 5 % and 2.5 % CO₂ and also in Na⁺-free media, indicating that the stimulation by hypo-osmolarity is independent of extracellular Na⁺. Furthermore, the stimulation of glycogen synthesis by hypo-osmolarity in the Na⁺-free medium suggests that the failure of 5 % CO₂ to stimulate glycogen synthesis in the Na⁺-free medium is due not to a direct requirement for Na⁺ for glycogen synthesis, but to an indirect mechanism.

Glycolysis (Table 5) was inhibited by 5 % CO₂ both in the presence of Na⁺ (by 23 %) and in its absence (by 32 %), indicating that the inhibition was not Na⁺-dependent. Gluconeogenesis was inhibited by 5 % CO₂ compared with 2.5 % CO₂ (by 37 %). Hypo-osmolarity increased glycolysis at 2.5 % CO₂ (by 14 %), but not at 5 % CO₂, in the Na⁺-containing medium (Table 5), suggesting that stimulation of glycolysis by hypo-osmolarity is favoured by relatively alkaline conditions.

Cell volume, determined after 60 min, was increased by 15 % in the hypo-osmolar Na⁺-containing medium (iso-osmolar 2.19 ± 0.05, hypo-osmolar 2.52 ± 0.09 μl/mg of protein; means ± S.E.M., *n* = 4) and by 10 % in the hypo-osmolar Na-free medium (iso-osmolar 1.97 ± 0.05, hypo-osmolar 2.17 ± 0.08).

Table 6. Effects of [HCO₃[−]] and NH₄Cl on the stimulation of glycolysis and glycogen synthesis by insulin

Hepatocytes were incubated for 3 h in MEM containing 15 mM-[U-¹⁴C]glucose and the concentrations of NaHCO₃ indicated in the absence or presence of 1 nM-insulin and/or 0.5 mM-NH₄Cl and equilibrated with 2.5 % CO₂. Glycolysis is expressed as nmol of lactate formed/3 h per mg of protein, glycogen synthesis as nmol of [¹⁴C]glucose incorporated/3 h per mg of protein, and glycogen deposition as nmol of glucosyl units deposited/3 h per mg of protein. The insulin effect is expressed as a percentage of the respective controls. Values are means ± S.E.M. for 6 cultures. ^a*P* < 0.05, ^{aa}*P* < 0.005, with respect to 12.5 mM-HCO₃[−]; ^b*P* < 0.05 with respect to 12.5 mM-HCO₃[−] in the presence of insulin.

Medium	Glycolysis	Glycogen synthesis	Glycogen deposition
12.5 mM-HCO ₃ [−]	268 ± 64	8.0 ± 1.2	18 ± 3
+ insulin	332 ± 70	11.6 ± 2.1	31 ± 5
Insulin effect (%)	24	45	72
18.0 mM-HCO ₃ [−]	276 ± 67	6.6 ± 1.0 ^a	14 ± 1 ^a
+ insulin	328 ± 66	9.7 ± 1.8 ^b	22 ± 4
Insulin effect (%)	19	47	57
25.0 mM-HCO ₃ [−]	302 ± 67 ^{aa}	5.6 ± 1.1 ^{aa}	10 ± 1 ^a
+ insulin	334 ± 63	8.2 ± 1.3 ^b	17 ± 4 ^b
Insulin effect (%)	11	46	70
12.5 mM-HCO ₃ [−] /NH ₄ Cl	318 ± 58 ^{aa}	5.3 ± 0.6 ^a	10 ± 2
+ insulin	365 ± 52	8.5 ± 1.5 ^b	19 ± 4 ^b
Insulin effect (%)	15	60	90

Effects of alkalization with NH_4Cl and $[\text{HCO}_3^-]$ on the stimulation of glycolysis and glycogen synthesis by insulin (Table 6)

If the effects of increasing $[\text{HCO}_3^-]$ on glycogen synthesis and glycolysis are the result of cell alkalization, then other mechanisms that cause alkalization would be expected to mimic the effects of HCO_3^- . Cells are more permeable to NH_3 than to NH_4^+ ; consequently incubation with NH_4Cl causes cell alkalization because of uptake of NH_3 and intracellular conversion into NH_4^+ . We therefore compared the effects of NH_4Cl and increasing $[\text{HCO}_3^-]$ on glycolysis and glycogen synthesis, and their control by insulin. NH_4Cl stimulated glycolysis by 19% in the absence of insulin and by 10% in its presence. The stimulation of glycolysis by insulin was lower in the presence of NH_4Cl , indicating that the stimulatory effects of NH_4Cl and insulin are not additive. Similarly, the stimulations of glycolysis by HCO_3^- and insulin were also not additive. Increasing $[\text{HCO}_3^-]$ stimulated glycolysis in the absence of insulin, but not in its presence, and the stimulation by insulin diminished with increasing $[\text{HCO}_3^-]$. Glycogen synthesis and deposition decreased with increasing $[\text{HCO}_3^-]$ in both the absence and the presence of insulin (Table 6).

The cell water content was decreased by 7% with NH_4Cl and by 6% with 25 mM relative to 12.5 mM- NaHCO_3 (cell volume relative to control: NH_4Cl , $93 \pm 1\%$; 25 mM- HCO_3^- , $94 \pm 3\%$; means $\pm$ S.E.M., $n = 3$). The decrease in cell volume under alkaline conditions suggests that the effects of NH_4Cl or HCO_3^- could be mediated through changes in cell volume.

Effects of insulin and of hypo-osmotic swelling on glycogen synthesis at different concentrations of HCO_3^- in the absence or presence of Na^+ (Fig. 1)

Insulin causes swelling [8] and alkalization [4], whereas hypo-osmolarity causes swelling [11,19] and acidification [9]. Insulin-induced swelling and hypo-osmotic swelling might be expected to have different effects on metabolic pathways that are affected by swelling if the inverse pH changes (alkalinization and acidification, respectively) amplify and oppose the effects of swelling. We compared the effects of insulin and of hypo-osmolarity on glycogen synthesis at different concentrations of HCO_3^- in three media: a balanced salts medium containing NaCl (Fig. 1a); a Na^+ -free medium in which NaCl was replaced by sucrose (Fig. 1b); and a Cl^- -free medium in which NaCl was

replaced by sodium isethionate (Fig. 1c). The purpose of the latter medium was to establish whether differences between the balanced medium and the sucrose-containing Na^+ -free medium are due to lack of Na^+ or to lack of Cl^- .

At 25 mM- HCO_3^- in the absence of insulin, rates of glycogen synthesis were similar in the three iso-osmolar media (control 12.9 ± 2.3 , Na^+ -free 10.7 ± 2.4 , sodium isethionate, 12.1 ± 4.0 nmol/3 h per mg of protein). However, rates of glycogen synthesis in the Na^+ -containing media (Figs. 1a and 1c) and Na^+ -free media (Fig. 1b) diverged with decreasing $[\text{HCO}_3^-]$ (25 mM to 6 mM), because rates increased with decreasing $[\text{HCO}_3^-]$ ($P < 0.05$, 6 mM versus 25 mM) in the Na^+ -containing media (Figs. 1a and 1c), but decreased ($P < 0.05$, 6 mM versus 25 mM) in the Na^+ -free medium (Fig. 1b). There was a difference ($P < 0.05$) between the control and the sodium isethionate medium at 6 mM- HCO_3^- , because of a greater increase in glycogen synthesis with decreasing $[\text{HCO}_3^-]$ in the isethionate-containing medium (control 18.4 ± 3.1 , sodium isethionate, 22.1 ± 3.8 nmol/3 h per mg protein; Fig. 1).

In the control medium (Fig. 1a), the stimulation of glycogen synthesis by insulin decreased with increasing $[\text{HCO}_3^-]$ (114%, 96%, and 65% at 6, 12 and 25 mM- HCO_3^- respectively), whereas stimulation by hypo-osmolarity increased (26%, 45% and 50% respectively). Consequently insulin caused a greater stimulation than did hypo-osmolarity at 6 mM- HCO_3^- (insulin 116%; hypo-osmolarity 26%), but a similar stimulation at 25 mM- HCO_3^- (65% versus 50%). At all HCO_3^- concentrations insulin caused a similar stimulation in hypo-osmolar media (44–103%) to that in iso-osmolar media (65–114%), indicating additive stimulation by insulin and hypo-osmolarity.

In the Na^+ -free medium (Fig. 1b), there was negligible stimulation by insulin in iso-osmolar conditions, irrespective of the $[\text{HCO}_3^-]$, consistent with a role for Na^+ -entry mechanisms in the control of glycogen synthesis by insulin [8]. Hypo-osmolarity, in contrast with insulin, stimulated glycogen synthesis at all HCO_3^- concentrations (164%, 144% and 67% at 6, 12 and 25 mM- HCO_3^- ; $P < 0.05$). Insulin did stimulate in hypo-osmolar conditions, at all HCO_3^- concentrations, and to a greater extent at the lowest $[\text{HCO}_3^-]$ (66% at 6 mM and 32% at 25 mM; Fig. 1b). This stimulation by insulin was smaller than in hypo-osmolar Na^+ -containing media (103% and 44% respectively; Fig. 1a versus 1b).

In the medium containing sodium isethionate (Fig. 1c), results were similar to those in the control medium, insofar as stimulation

Fig. 1. Effects of insulin and hypo-osmolarity on glycogen synthesis at different concentrations of HCO_3^- in the absence or presence of extracellular Na^+

Hepatocyte cultures were incubated either in media of 317 mosm ($\circ$, $\bullet$, continuous lines) or in hypo-osmolar media ($\triangle$, $\blacktriangle$, broken lines) either without ($\circ$, $\triangle$) or with 10 nM-insulin ($\bullet$, $\blacktriangle$) and with the concentrations of HCO_3^- indicated. The compositions of the iso-osmolar Na^+ -containing (a) and Na^+ -free media (b) containing 25 mM- HCO_3^- were as indicated in Table 1 (media E and F respectively). The composition of the sodium isethionate-containing medium (c) was the same as medium E in Table 1, except that NaCl was replaced by sodium isethionate. The hypo-osmolar media contained 64 mM- instead of 104 mM-NaCl (a) or sodium isethionate (c) and 177 mM- instead of 257 mM-sucrose (b). In the media with the lower HCO_3^- concentrations (6 and 12 mM), NaHCO_3 was replaced by NaCl or sodium isethionate (a and c) and KHCO_3 was replaced by sucrose (b). All media contained 15 mM-[U- ^{14}C]glucose, and glycogen synthesis is expressed as nmol of [^{14}C]glucose incorporated/3 h per mg of cell protein. Values are means $\pm$ S.E.M. for 4 cultures.

by insulin declined (78 %, 61 % and 46 % at 6, 12 and 25 mM) and that by hypo-osmolarity increased (0 %, 29 % and 63 % respectively) with increasing $[\text{HCO}_3^-]$, but they differed from the control medium in that there was no stimulation by hypo-osmolarity at 6 mM- HCO_3^- (Fig. 1c).

Effects of Na^+ -free media containing KCl or potassium isethionate (Fig. 2)

In the above experiments (Fig. 1), constant osmolarity with decreasing $[\text{HCO}_3^-]$ was maintained by replacement of NaHCO_3 with either NaCl (Fig. 1a) or sodium isethionate (Fig. 1c) and of KHCO_3 with sucrose (Fig. 1b). In the experiments in Fig. 2, we compared Na^+ -free media with constant $[\text{K}^+]$ by replacement of KHCO_3 with either KCl (Fig. 2a) or potassium isethionate (Fig. 2b), and in a further medium KHCO_3/KCl was replaced with $\text{NaHCO}_3/\text{NaCl}$ (low- Na^+ medium, Fig. 2c). These experiments show that, as in the preceding experiments in a Na^+ -free medium (Fig. 1b), glycogen synthesis decreased with decreasing $[\text{HCO}_3^-]$, indicating that the change in glycogen synthesis is due to $[\text{HCO}_3^-]$

and not to $[\text{K}^+]$. There was negligible stimulation by insulin in the iso-osmolar Na^+ -free media (Figs. 2a and 2b), but in the low- Na^+ medium containing 25 mM- NaHCO_3 (Fig. 2c) there was 17 % stimulation by insulin. In the hypo-osmolar Na^+ -free media (Fig. 2a and 2b) the stimulation by insulin was greater at low $[\text{HCO}_3^-]$ (82–102 % at 6 mM versus 26–34 % at 25 mM), indicating that stimulation by HCO_3^- and by insulin was not additive. In the hypo-osmolar low- Na^+ medium (Fig. 2c) stimulation by increasing HCO_3^- and insulin was also not additive. These results suggest that in hypo-osmolar Na^+ -free or low- Na^+ media insulin counteracts the decrease in glycogen synthesis that occurs with decreasing $[\text{HCO}_3^-]$, or conversely that insulin mimics the effects of increasing $[\text{HCO}_3^-]$.

Effects of sodium acetate and potassium acetate on glycolysis and glycogen synthesis in HCO_3^- -containing media (Table 7, Fig. 3)

In hepatocyte suspensions, replacement of NaCl with sodium acetate causes cell acidification and swelling [11], because of

Fig. 2. Effects of insulin and hypo-osmolarity on glycogen synthesis at different concentrations of HCO_3^- in the absence or presence of low concentrations of Na^+

Hepatocyte cultures were incubated in media of 317 mosM (○, ●, continuous lines) or in hypo-osmolar media (–80 mosM; △, ▲, broken lines) and without (○, △) or with 10 nM-insulin (●, ▲) with the concentrations of HCO_3^- indicated. The composition of Na^+ -free media containing 25 mM- HCO_3^- (a and b) was as in Table 1 (medium F), and the composition of the low- Na^+ medium (c) containing 25 mM- NaHCO_3 was as in Table 1 (medium G). The hypo-osmolar media contained 177 mM- instead of 257 mM-sucrose. Lower concentrations of HCO_3^- were achieved by replacement of KHCO_3 by KCl (a); by potassium isethionate in (b); and by replacement of NaHCO_3 with NaCl (c). The media contained 15 mM- $[\text{U-}^{14}\text{C}]$ glucose and glycogen synthesis is expressed as nmol of ^{14}C -glucose incorporated/3 h per mg of cell protein. Values are means $\pm$ S.E.M. for duplicate wells from two cultures.

Table 7. Effects of replacement of Cl^- by acetate on glycolysis and glycogen synthesis in HCO_3^- -containing media

Hepatocytes were incubated for 3 h either in a balanced salts medium (Table 1, medium A) or in media in which NaCl was replaced by either sodium acetate or potassium acetate. In the latter case NaHCO_3 was replaced by KHCO_3 . Where indicated, 1 nM-insulin, 0.5 mM- NH_4Cl and 1 mM-amiloride were included. They were equilibrated with 2.5 % CO_2 . Media were supplemented with either 15 mM- $[\text{U-}^{14}\text{C}]$ glucose or 15 mM- $[\text{U-}^{14}\text{C}]$ glucose + 5 mM-pyruvate. Glycolysis is expressed as nmol of lactate formed/3 h per mg of protein, glycogen synthesis as nmol of ^{14}C -glucose incorporated into glycogen/3 h per mg of protein, and cation content as nmol/mg of protein. Values are means $\pm$ S.E.M. for 5–6 hepatocyte cultures. $^aP < 0.05$, $^{aa}P < 0.005$, with respect to control; $^bP < 0.05$, $^{bb}P < 0.005$, with respect to insulin.

Substrates	15 mM-glucose		15 mM-glucose + 5 mM-pyruvate Glycogen synthesis	10 mM-glucose	
	Glycolysis	Glycogen synthesis		$\text{Na}^+ + \text{K}^+$	K^+/Na^+
Control	224 $\pm$ 33	3.9 $\pm$ 0.5	30 $\pm$ 2	213 $\pm$ 11	16.6 $\pm$ 1.1
+ insulin	288 $\pm$ 38	6.0 $\pm$ 0.7	51 $\pm$ 5	—	—
Sodium acetate	118 $\pm$ 15 ^{aa}	18.2 $\pm$ 4.3 ^a	52 $\pm$ 2 ^{aa}	245 $\pm$ 9 ^a	12.0 $\pm$ 0.8 ^a
+ insulin	144 $\pm$ 16 ^b	32.9 $\pm$ 6.2 ^b	81 $\pm$ 1	—	—
Sodium acetate + amiloride	—	10.8 $\pm$ 3.2	40 $\pm$ 5	228 $\pm$ 12	8.2 $\pm$ 0.8 ^{aa}
+ insulin	—	16.4 $\pm$ 4.6	52 $\pm$ 6	—	—
Potassium acetate	—	—	83 $\pm$ 7 ^{aa}	308 $\pm$ 8 ^{aa}	60.3 $\pm$ 6.5 ^{aa}
+ insulin	—	—	103 $\pm$ 9	—	—
NH_4Cl	257 $\pm$ 37 ^{aa}	2.5 $\pm$ 0.4 ^a	—	193 $\pm$ 13 ^a	16.1 $\pm$ 0.5
+ insulin	306 $\pm$ 36	3.3 $\pm$ 0.5 ^{bb}	—	—	—

Fig. 3. Relation between glycogen synthesis and cation ($K^+ + Na^+$) content in HCO_3^- -containing media

For experimental details see Table 7. The cell cation content is expressed as nmol of $K^+ + Na^+$ per mg of protein, and glycogen synthesis as nmol of [^{14}C]glucose incorporated in 3 h per mg of cell protein. Values are means for 5–6 cultures. Abbreviation: Ac, acetate.

entry of undissociated acetic acid, acidification and activation of Na^+/H^+ exchange [11]. We determined the effects of replacement of NaCl with either sodium acetate or potassium acetate in the presence of a CO_2/HCO_3^- buffer (Table 7) to establish whether acidification and swelling by acetate mimics the effects of CO_2 -induced acidification (Tables 2–4).

Replacement of NaCl with sodium acetate increased the cell $K^+ + Na^+$ content by 15%, with a decrease in the K^+/Na^+ ratio, and inhibited glycolysis and stimulated glycogen synthesis (Table 7). The percentage stimulation of glycogen synthesis was greater when this was determined with glucose alone as substrate than with glucose and pyruvate. Amiloride, an inhibitor of Na^+/H^+ exchange, suppressed both the increase in cation content caused by sodium acetate and the stimulation of glycogen synthesis ($P < 0.05$), suggesting that the latter may be secondary to the increase in cation content, and thereby cell swelling. Amiloride caused a further decrease in the K^+/Na^+ ratio ($P < 0.005$) in the presence of sodium acetate (Table 7), suggesting that it may also inhibit Na^+/K^+ exchange, either directly or as a result of cytosolic acidification. In the control medium containing NaCl, amiloride did not inhibit glycogen synthesis (control 3.9 ± 0.5 , amiloride 5.9 ± 1.4 nmol/3 h per mg; means $\pm$ S.E.M., $n = 6$) or lower the

cation content (results not shown), suggesting that inhibition of glycogen synthesis by amiloride in the sodium acetate-containing medium was not due to a non-specific effect. Replacement of NaCl with potassium acetate caused a greater increase in cation content (45%) and in glycogen synthesis than replacement with sodium acetate. NH_4Cl had converse effects to acetate: it increased glycolysis and inhibited glycogen synthesis, and lowered the cell $K^+ + Na^+$ content (Table 7). In these experiments glycogen synthesis correlated positively with the cell cation content (Fig. 3). The cell water content showed similar percentage increases with sodium acetate and potassium acetate (15% and 45%) as the cation content, suggesting that the latter reflects changes in cell volume.

Effects of extracellular pH on glycogen synthesis in HCO_3^- -free media (Fig. 4)

In the absence of a CO_2/HCO_3^- buffer, replacement of extracellular Cl^- with acetate leads to equilibration of intracellular with extracellular pH [11]. We determined the effects of extracellular pH on glycogen synthesis in HCO_3^- -free media containing NaCl, sodium acetate or potassium acetate (Fig. 4). The cell cation content was 44–50% higher ($P < 0.005$) when NaCl was replaced by potassium acetate and 11–16% higher ($P < 0.01$) when replaced by sodium acetate (Fig. 4a). There was a small decline in cation content at pH 6.6 relative to 7.4 (between 3% and 6%) in the three media (Fig. 4a). The cell K^+/Na^+ ratio was higher ($P < 0.01$) in the potassium acetate media (21.6 ± 4.2) and lower ($P < 0.05$) in the sodium acetate media (8.6 ± 0.9) than in the NaCl-containing media (11.3 ± 0.8). At an extracellular pH of 7.4 there was a correlation between glycogen synthesis and cell cation content (Fig. 4b), but rates of glycogen synthesis were lower at pH 7.0 and pH 6.6, and the difference was greater that could be accounted for from the small decrease (3–6%) in cation content, suggesting a direct inhibitory effect of the decrease in pH independent of cell volume. Fig. 4(c) shows the decline in glycogen synthesis with decreasing extracellular pH. At pH 6.6, glycogen synthesis was lower ($P < 0.01$) in the acetate-containing media than in the NaCl-containing medium. This could be explained by equilibration of intracellular and extracellular pH in the presence of acetate [11].

Effects of insulin and hypo-osmolarity in HCO_3^- -free media (Table 8)

When hepatocytes were incubated in a HCO_3^- -free medium containing Hepes, glycogen synthesis (determined during incubation with 25 mM-glucose and 5 mM-pyruvate) was increased

Fig. 4. Effects of extracellular pH on glycogen synthesis in HCO_3^- -free media containing either Cl^- or acetate

Hepatocyte cultures were incubated in HCO_3^- -free media containing (mM): KCl (5.1), $CaCl_2$ (1.2), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), Na_2SO_4 (0.8), Tris/Mes (15) and: (○) NaCl (128), (△) sodium acetate (128) or (□) potassium acetate (128). For determination of glycogen synthesis, media were supplemented with 15 mM-[$U-^{14}C$]glucose and 5 mM-pyruvate and incubations were for 2 h, and for determination of the cation content media contained 10 mM-glucose and incubations were for 1 h. (a) Cell cation content versus pH; (b) glycogen synthesis versus cation content; (c) glycogen synthesis versus pH. Glycogen synthesis is expressed as nmol of [^{14}C]glucose incorporated into glycogen/2 h per mg of protein and the cation content as nmol of $K^+ + Na^+$ per mg of protein. Values are means $\pm$ S.E.M. for 4 cultures. Abbreviation: Ac, acetate.

Table 8. Effects of hypo-osmolarity and extracellular pH on glycogen synthesis in HCO_3^- -free media containing either NaCl or sodium acetate

The compositions of the iso-osmolar media were as described in Fig. 4. The hypo-osmolar media contained 88 mM- instead of 128 mM-NaCl or -sodium acetate. Media were supplemented with 15 mM-[U- ^{14}C]glucose and 5 mM-pyruvate, and incubations were for 2 h. Glycogen synthesis is expressed as nmol of [^{14}C]glucose incorporated into glycogen/2 h per mg of protein. Values are means $\pm$ S.E.M. for 4 cultures. * $P < 0.05$, hypo-osmolar relative to corresponding iso-osmolar.

	Glycogen synthesis (nmol/2 h per mg)		
	pH 7.4	pH 7.0	pH 6.6
NaCl, iso-osmolar	19 $\pm$ 1	18 $\pm$ 1	12 $\pm$ 1
NaCl, hypo-osmolar	22 $\pm$ 1 ^a	14 $\pm$ 1 ^a	5 $\pm$ 1 ^a
Sodium acetate, iso-osmolar	28 $\pm$ 2	15 $\pm$ 1	6 $\pm$ 1
Sodium acetate, hypo-osmolar	19 $\pm$ 1 ^a	7 $\pm$ 2 ^a	2 $\pm$ 1

by insulin ($P < 0.05$), but not by hypo-osmolarity (-80 mosM) (control 64 ± 6 , insulin, 105 ± 24 , hypo-osmolar medium 71 ± 11 nmol of glucose/2 h per mg of protein; means $\pm$ S.E.M., $n = 3$). In a HCO_3^- -containing medium with the same substrates, glycogen synthesis was higher than in the HCO_3^- -free medium (control 77 ± 7 , insulin 138 ± 19 , hypo-osmolar medium 123 ± 21 nmol/2 h per mg; means $\pm$ S.E.M., $n = 3$). Hypo-osmolarity increased ($P < 0.01$) cell volume (determined after 60 min) in both the HCO_3^- -free medium ($112 \pm 4\%$ of control iso-osmolar medium; means $\pm$ S.E.M., $n = 3$) and in the HCO_3^- -containing medium ($112 \pm 4\%$ of control), indicating that the failure of hypo-osmolarity to increase glycogen synthesis in the HCO_3^- -free medium was not due to impaired cell swelling.

Table 8 shows the effects of hypo-osmolarity on glycogen synthesis in HCO_3^- -free media buffered with Tris/Mes. At pH 7.4, hypo-osmolarity inhibited glycogen synthesis in the sodium acetate-containing medium, but not in the NaCl-containing medium. At lower pH, hypo-osmolarity inhibited in both media (Table 8). In two experiments on sodium acetate-containing HCO_3^- -free media, insulin increased glycogen synthesis by about 2-fold at pH 7.4 and pH 7.0 (pH 7.4, sodium acetate 26.9, +insulin 64.3; pH 7.0, sodium acetate 15.1; +insulin 30.8; pH 6.6, sodium acetate 6.5, +insulin 9.8; nmol of glucose/2 h per mg of protein; means of 2 experiments), indicating that insulin stimulates glycogen synthesis in media where hypo-osmolarity inhibits.

Effects of pH on glycogen synthase activity

The effects of pH (7.8–6.8) on the activity of glycogen synthase were determined in hepatocyte extracts. Synthase a and total activity (munits/mg of cell protein; means $\pm$ S.E.M., $n = 3$) decreased with decreasing pH. Synthase a : pH 7.8, 0.48 ± 0.04 ; pH 7.3, 0.40 ± 0.03 ; pH 6.8, 0.32 ± 0.02 . Total activity: pH 7.8, 1.41 ± 0.04 ; pH 7.3, 0.99 ± 0.02 ; pH 6.8, 0.73 ± 0.04 .

DISCUSSION

Effects of cell acidification and alkalization on glycogen synthesis and glycolysis

In skeletal muscle, cell alkalization by decreasing the $[\text{CO}_2]$ from 5% to 2.7% stimulates glycolysis [4]. We show in the present study that in hepatocytes cell acidification by increasing the $[\text{CO}_2]$ from 2.5% to 5% or by replacement of Cl^- by acetate

[11] inhibits glycolysis but stimulates glycogen synthesis. The latter, unlike the inhibition of glycolysis, is abolished by ouabain and in Na^+ -free media, suggesting that it is dependent on ionic changes or transcellular ionic gradients. The inhibition by amiloride of both the increase in cation content caused by sodium acetate and the stimulation of glycogen synthesis (Table 7), and the correlation between glycogen synthesis and cell cation content (Fig. 3), suggests that the stimulation of glycogen synthesis by cell acidification is probably secondary to cell swelling. Further evidence that stimulation of glycogen synthesis by acetate or CO_2 acidification is not a direct effect of pH is the direct (rather than inverse) correlation between glycogen synthesis and pH in HCO_3^- -free media (Fig. 4c). These results together suggest that cell acidification directly inhibits glycolysis, but indirectly stimulates glycogen synthesis via cell swelling.

Effect of hypo-osmotic swelling on glycogen synthesis and glycolysis

Hypo-osmotic swelling stimulates glycogen synthesis in hepatocyte suspensions [6]. We have characterized further the effects of hypo-osmotic swelling on glycogen synthesis and compared them with the effects of insulin. Hypo-osmotic swelling stimulated glycogen synthesis in the presence or absence of Na^+ in HCO_3^- -containing media, but it inhibited glycogen synthesis in HCO_3^- -free media when extracellular pH was lowered or when Cl^- was replaced by acetate (Table 8), which also causes acidification [11]. These findings can be explained by the opposing effects of swelling (stimulation) and acidification (inhibition) on glycogen synthesis. Cell pH is regulated by Na^+/H^+ exchange and by HCO_3^- -exchange mechanisms [18]. Hypo-osmotic swelling is associated with cellular acidification because of inhibition of Na^+/H^+ exchange [9]. The findings that glycogen synthesis correlated with pH in HCO_3^- -free media (Fig. 4c) and that the stimulation of glycogen synthesis by hypo-osmotic swelling was abolished in HCO_3^- -free media under acidic conditions (Table 8) suggest that swelling stimulates glycogen synthesis when it counteracts the inhibitory effects of acidification, and that HCO_3^- -exchange mechanisms are essential for compensating the pH changes. Thus in a HCO_3^- -free system control of cell pH is confined to Na^+/H^+ exchange [18], which is inhibited by swelling [9], and the consequent acidification may have an over-riding inhibitory effect on glycogen synthesis. In HCO_3^- -containing media, regulation of cell pH by HCO_3^- -exchange mechanisms [18] may compensate for the impaired Na^+/H^+ exchange [9]; thus the stimulation of glycogen synthesis by hypo-osmotic swelling is enhanced by increasing the $[\text{HCO}_3^-]$ (Fig. 1a).

Hypo-osmotic swelling increased glycolysis in the presence of Na^+ , but not in its absence, and at 2.5% but not at 5% CO_2 . These effects can also be explained by the opposing effects of swelling (stimulation) and acidification (inhibition) on glycolysis, but they differ from the stimulation of glycogen synthesis, which was observed irrespective of the $[\text{CO}_2]$ and in both the presence and the absence of Na^+ . The differences between glycolysis and glycogen synthesis in their stimulation by swelling can be explained by a greater sensitivity of glycolysis to inhibition by acidification, which counteracts the effect of swelling, as suggested by the inhibition of glycolysis with 5% as compared with 2.5% CO_2 .

Cell swelling and alkalization in the control of glycogen synthesis and glycolysis by insulin

Insulin causes cell swelling [8] and alkalization [4,5]. Glycogen synthesis correlates positively with cell volume in HCO_3^- -containing media ([6] and Fig. 3), but with pH (Fig. 4c) rather than with cell volume (Fig. 4b) in HCO_3^- -free media (Fig. 4c).

Glycolysis is stimulated by alkalization [4] and Table 6) and by swelling under relative alkaline conditions. Since glycolysis and glycogen synthesis are both stimulated by alkalization and by swelling under alkaline conditions, the question arises as to the importance of cell swelling [8] and alkalization [4] in the control of glycogen synthesis and glycolysis by insulin. The present study shows: first, that stimulation of glycolysis by insulin is not additive with the stimulation of alkalization (Table 6), suggesting a role for alkalization in the stimulation of glycolysis; secondly, that stimulation of glycogen synthesis by insulin, unlike that by hypo-osmotic swelling, increases with diminishing $[\text{HCO}_3^-]$ and is not abolished in HCO_3^- -free media. Insulin activates Na^+/H^+ exchange by an alkaline shift of the set point of the Na^+/H^+ exchanger [5]. The diminishing stimulation of glycogen synthesis by insulin under relative alkaline conditions (increasing $[\text{HCO}_3^-]$, Fig. 1a) would be consistent with decreased activation of Na^+/H^+ exchange as cell pH approaches the insulin-activated pH set point of the Na^+/H^+ exchanger. Furthermore, the opposite effects of decreasing $[\text{HCO}_3^-]$ on the stimulation of glycogen synthesis by insulin and hypo-osmolar swelling (Fig. 1a) are consistent with the opposite pH changes caused by hypo-osmotic swelling and insulin (acidification [9] and alkalization [4] respectively), which would be expected to counteract and enhance, respectively, the stimulation by swelling (Fig. 1a).

The effects of insulin in the hypo-osmolar Na^+ -free media as a function of $[\text{HCO}_3^-]$ (Figs. 1b and 2) are of particular interest, because they suggest that insulin opposes the effects of acidification. Since efflux of solute during hypo-osmotic swelling probably involves loss of anions in addition to Cl^- , and possibly HCO_3^- [8], one may speculate that insulin may oppose the effects of low $[\text{HCO}_3^-]$ in hypo-osmolar Na^+ -free media by favouring retention of HCO_3^- in relation to Cl^- during solute efflux.

Conclusions

We conclude the following from these studies (1) Cell acidification in HCO_3^- -containing media by replacing Cl^- by acetate or by increasing the $[\text{CO}_2]$ has converse effects on glycolysis and glycogen synthesis (inhibition and stimulation respectively). The latter is due to secondary ionic changes involving both Na^+ entry and HCO_3^- -exchange mechanisms, leading to cell swelling. The direct effect of acidification on glycogen synthesis is inhibition, as indicated by the correlation of glycogen synthesis with pH in HCO_3^- -free media and the activity of glycogen synthase with varying pH. (2) Hypo-osmotic swelling stimulates glycolysis in HCO_3^- -containing media only under alkaline conditions, but it stimulates glycogen synthesis under a wider range of conditions, though not in HCO_3^- -free media under acidic conditions. Failure of hypo-osmotic swelling to

stimulate either glycolysis or glycogen synthesis is due to the opposing effect of the acidification that accompanies hypo-osmotic swelling [9]. (3) Differences between insulin-induced swelling and hypo-osmotic swelling on glycogen metabolism and glycolysis can be explained by the inverse pH changes caused by insulin and hypo-osmotic swelling (alkalinization and acidification respectively), since alkalization potentiates, whereas the acidification opposes, the stimulatory effect of swelling on glycolysis and glycogen synthesis. (4) The effects of insulin on carbohydrate metabolism are mediated by changes in both cell volume and cell pH, through Na^+ -entry and HCO_3^- -exchange mechanisms. The impaired action of insulin in a Na^+ free medium is due to the lack of swelling and alkalization. The restoration of stimulation by insulin in a hypo-osmolar Na^+ -free medium is probably due to an alkalization mechanism.

We thank the Juvenile Diabetes Foundation (International), Nordisk UK and the M.R.C. for financial support.

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Received 2 September 1991/8 October 1991; accepted 24 October 1991