A Study of Stabilization of Gluconeogenic Activity in Rat Liver Slices by Calcium and Manganese Ions

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1. The effect of some bivalent cations on gluconeogenesis by the rat liver-slice preparation has been investigated. 2. Ca^{2+} and Mn^{2+} stimulated glucose production from a range of substrates but not from glycerol. Mg^{2+} had no effect on the rate of glucose production. 3. Ca^{2+} were required to maintain phosphoenolpyruvate carboxylase activity in the slice preparation. 4. Ca^{2+} and Mn^{2+} , but not Mg^{2+} , retarded the release of lysosomal enzymes from the slice into the incubation medium. 5. It is proposed that Ca^{2+} and Mn^{2+} stimulate glucose production by stabilizing the lysosome system in the liver-slice preparation. 6. The value of the liver-slice preparation as a means of measuring hepatic gluconeogenesis is discussed.

Hepatic and renal gluconeogenesis may have different modes of control and respond in different ways to various dietary and hormonal states. Before carrying out experiments in which hepatic and renal gluconeogenesis were measured simultaneously it was thought that the value of the liver-slice preparation as a means of measuring hepatic glucose production should be assessed. The slice preparation provides a convenient method for estimating the liver's capacity for gluconeogenesis, but the rates of glucose production are often lower than those obtained with the perfused organ (Krebs *et al.*, 1966; Wagle *et al.*, 1966), although Schimmel & Knobil (1969) have reported that their slice preparation yields rates comparable with perfused liver.

It is also known that variation in the ionic composition of the saline medium used for perfusion or slice incubation can have a marked effect on the gluconeogenesis rate. In particular, the presence of Ca^{2+} or Mn^{2+} in the medium enhances both hepatic and renal glucose production (Hems *et al.*, 1966; Krebs *et al.*, 1963; Friedmann & Rasmussen, 1970; Rutman *et al.*, 1965; Papavasiliou *et al.*, 1968), but their mode of action is as yet unclear.

The studies described here examine the effects of these and other ions on gluconeogenesis, relevant enzyme activities and other aspects of liver-slice metabolism. A mechanism is postulated through which hepatic gluconeogenesis is altered *in vitro*.

Experimental

Methods

Animals and tissue handling. Male adult Sprague-Dawley rats of a locally bred strain were used. Unless

otherwise stated, the animals were starved for 18–22 h before the experiment and then killed by cervical fracture. The liver was rapidly removed and chilled on an ice-cold Petri dish. Slices were cut freehand with a Stadie–Riggs microtome blade and dropped into ice-cold 0.9% NaCl. After rinsing in a further portion of ice-cold saline, four random slices were taken for initial glycogen estimation by KOH digestion, ethanol precipitation and acid hydrolysis (Johnson & Fusaro, 1966). Each of the remaining slices (30–60 mg wet wt.) was placed in a 50 ml Erlenmeyer flask containing 5 ml of incubation medium, which was then flushed with $\text{O}_2 + \text{CO}_2$ (95:5), stoppered and incubated for 20–120 min in a shaking water bath (120 cycles/min) at 37–38°C.

Composition of the incubation medium. For experiments in which various Ca^{2+} concentrations were used, the Krebs bicarbonate buffer contained 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM- MgSO_4 , 1.2 mM- KH_2PO_4 and 25 mM- NaHCO_3 , and CaCl_2 was added to give a final concentration in the range 0–5 mM. To avoid precipitation when Mn^{2+} was included in the medium, a 'basic saline' mixture containing 120 mM-NaCl, 4.8 mM-KCl and 25 mM- NaHCO_3 was used, to which MnCl_2 or CaCl_2 was added to give a final concentration of 2.5 mM. The rates of glucose formation from lactate or pyruvate observed for Krebs bicarbonate buffer with 2.5 mM- CaCl_2 and for the 'basic saline' mixture plus 2.5 mM- CaCl_2 were not significantly different, i.e. the omission of MgSO_4 and KH_2PO_4 had little effect on the rates of glucose production. After gassing with $\text{O}_2 + \text{CO}_2$ (95:5) the pH of these media was 7.35–7.40 at 37°C. To produce media of pH 7.05–7.10, the bicarbonate concentration was lowered to 11.8 mM.

Substrates, when present, were at a final concentration of 10mM with the exception of DL-lactate, which was at 20mM.

Gluconeogenesis measurements. At the end of the incubation period, protein was precipitated with perchloric acid and the glucose content of the medium assayed by the glucose oxidase method (Huggett & Nixon, 1957). When Mn^{2+} was present in the medium, an appropriate concentration of $MnCl_2$ was added to the glucose standards, since Mn^{2+} itself enhances the colour of the glucose oxidase reagent (Rutman *et al.*, 1965). The slices were rinsed, blotted, wet-weighted and their glycogen content was determined. Net glucose produced was calculated as the change in medium glucose minus the change in slice glycogen content, and the glucose formed from added substrate as the difference in net glucose production with and without substrate.

Schimmel & Knobil (1969) and Kamm & Cahill (1969) included a high glucose concentration (80–180mg/100ml) in the medium to minimize the effects of variable glycogenolysis on medium glucose concentration. Table 1 shows the effect of varying initial medium glucose content between 0 and 100mg/100ml on gluconeogenesis from alanine and on associated changes in the glycogen content of slices from the liver of a fed rat. Glycogenolysis was significantly decreased by a medium containing glucose at a concentration >50mg/100ml, with no effect on the gluconeogenesis rate. After the rats had been starved for 18h, liver glycogen concentration fell to 2–10mg of glucose equivalents/g wet wt. so that the contribution of glycogenolysis to the glucose in the medium was small compared with that of gluconeogenesis. Further, in the experiments reported here, the concentration of glucose in the medium never attained values sufficient to inhibit glycogenolysis significantly. Glucose was therefore always omitted from the initial incubation medium.

Enzyme activities in slice and medium. When enzyme

activities in a slice were to be measured, the slice, after incubation, rinsing and weighing, was homogenized in 4vol. of ice-cold extraction buffer, 0.02M-tris-HCl, pH7.5, containing 0.25M-sucrose. A portion of the homogenate was retained for glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase; EC 3.1.3.9) assay, and the remainder was centrifuged at 100000g for 1h in an MSE SS65 refrigerated ultracentrifuge. The post-microsomal supernatant was retained for phosphoenolpyruvate carboxylase [GTP-oxaloacetate carboxy-lyase (transphosphorylating); EC 4.1.1.32] and fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase; EC 3.1.3.11) assays.

Before assaying the medium for phosphoenolpyruvate carboxylase activity, the sample was dialysed for 4h against 2×50 vol. of extraction buffer at 4°C. When the medium content of lysosomal marker enzymes was to be determined, it was dialysed against 2×50 vol. of 50mM-sodium acetate buffer, pH5.0, for 4h at 4°C. After both dialysis procedures the samples were briefly centrifuged to remove large debris and precipitated protein.

Phosphoenolpyruvate carboxylase was assayed by the method of Flores & Alleyne (1971) and activity expressed as μ mol of phosphoenolpyruvate formed/min per g wet wt. or as nmol of phosphoenolpyruvate formed/min per mg of protein. Fructose 1,6-diphosphatase assays were done by the method of Pontremoli (1966) at pH7.5 in the presence of 1mM-MgCl₂. A Unicam SP. 8000 recording spectrophotometer was used for both these assays. The glucose 6-phosphatase assay was as described by Nordlie & Arion (1966).

Acid phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.2) was measured in the presence and absence of 10mM-L-(+)-tartrate (Wootton 1964). The lysosomal activity was taken as the tartrate-inhibited portion of total acid phosphatase activity, the remainder being attributed to the erythrocyte enzyme (Sawant *et al.*, 1964). The unit of activity

Table 1. *Effect of medium glucose concentration on slice glycogen content and gluconeogenesis from alanine by liver slices*

Liver slices from well-fed rats were incubated in Krebs bicarbonate buffer, pH7.4, containing 0–100mg of glucose/100ml for 90min at 37°C. Changes in glycogen content of the slices and glucose formed from added substrate (alanine 10mM) were estimated as described in the text. Results are means $\pm$ S.E.M. of five observations.

Concn. of glucose (mg/100ml of incubation medium) ...	0	25	50	100
Change in glycogen content (mg of glucose equivalents/g wet wt.)				
No substrate	31.0 $\pm$ 2.6	32.5 $\pm$ 3.0	30.1 $\pm$ 2.1	27.5 $\pm$ 0.4
Alanine (10mM)	29.5 $\pm$ 1.7	29.2 $\pm$ 1.6	28.5 $\pm$ 0.7	24.4 $\pm$ 0.2
Glucose formed from alanine (μ mol/h per g wet wt.)	18.0 $\pm$ 3.2	15.2 $\pm$ 3.5	21.0 $\pm$ 4.0	20.6 $\pm$ 1.2

was μg of phenol liberated/h. The β -glucuronidase (β -D-glucuronide glucuronohydrolase; EC 3.2.1.31) was assayed by the method of Musa *et al.* (1965) and activity was expressed as μg of phenolphthalein released/h per mg of protein.

All enzymes were assayed at 37°C.
Other determinations. Protein was determined by the method of Lowry *et al.* (1951).

Materials

All chemicals were AnalaR grade. Biochemicals and enzymes were purchased from the Sigma Chemical Co. with the following exceptions: malate dehydrogenase, Koch-Light Laboratories, Colnbrook, Bucks., U.K.; disodium phenyl phosphate, BDH Chemicals Ltd., Poole, Dorset, U.K.; sodium pyruvate, C. F. Boehringer, London, U.K.; bovine serum albumin, fraction V from bovine plasma, Armour Pharmaceutical Co., Chicago, Ill., U.S.A. Dialysis tubing was purchased from Arthur H. Thomas Co., Philadelphia, Pa., U.S.A. and boiled in two changes of 1 mM-EDTA before use.

Results

Concentration of Ca^{2+} in the medium

The effect of various concentrations of Ca^{2+} in the medium on glucose production from alanine (10mM)

Fig. 1. Effect of Ca^{2+} on gluconeogenesis from alanine by liver slices

Liver slices from well-fed rats (●) and rats that had been starved for 18–22h (■) were incubated in Krebs bicarbonate buffer, pH7.40, containing 0.5mM- CaCl_2 for 90min at 37°C. Glucose formed from alanine (10mM) was determined as described in the text. Each point is the mean $\pm$ s.e.m. of four observations.

Table 2. Effect of Ca^{2+} and pH on gluconeogenesis by liver slices from a range of substrates

Liver slices from rats that had been starved for 18–22h were incubated in Krebs bicarbonate buffer, pH7.40 or 7.10, with or without 2.5mM- CaCl_2 , for 90min at 37°C. Substrates, when present, were at 10mM with the exception of DL-lactate, which was at 20mM. Glucose formed from added substrate was determined as described in the text. Results are means $\pm$ s.e.m. of five observations. The data for perfused liver are means $\pm$ s.e.m. with the no. of observations in parentheses. The perfusion medium included Krebs bicarbonate buffer.

	Incubation buffer (Krebs bicarbonate)		Net glucose formed from added substrate ($\mu\text{mol/h per g wet wt.}$)							
	Concn. of Ca^{2+} (mM)	pH	Substrate	...	Alanine	Pyruvate	Lactate	Serine	Glutamine	Glycerol
Slice	2.5	7.40			29.8 $\pm$ 3.2	48.0 $\pm$ 8.2	52.0 $\pm$ 5.0	21.0 $\pm$ 4.6	8.0 $\pm$ 1.5	44.0 $\pm$ 2.0
	0	7.40			4.7 $\pm$ 5.7	7.0 $\pm$ 5.0	8.0 $\pm$ 6.7	0.2 $\pm$ 4.3	1.9 $\pm$ 1.7	36.0 $\pm$ 2.6
	2.5	7.10			21.5 $\pm$ 2.9	38.0 $\pm$ 4.8	39.0 $\pm$ 5.1	13.7 $\pm$ 2.7	0.95 $\pm$ 1.5	28.9 $\pm$ 6.3
	0	7.10			4.0 $\pm$ 1.5	8.5 $\pm$ 4.8	10.5 $\pm$ 3.0	0.5 $\pm$ 3.6	1.06 $\pm$ 1.1	29.0 $\pm$ 3.1
Perfused liver	2.5	7.40			40* $\pm$ 7(6)	62† $\pm$ 6(5)	64* $\pm$ 5(12)	59* $\pm$ 2(3)	18.6* $\pm$ 0.5(4)	43† $\pm$ 8(4)

* Ross *et al.* (1967).
† Exton & Park (1967).

Fig. 2. Time-courses of glucose production by liver slices with and without Ca^{2+}

Liver slices from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.40, with and without 2.5 mM- CaCl_2 for the indicated times at 37°C , with 10 mM substrates: (a) alanine; (b) pyruvate; (c) glycerol; (d) endogenous. The glucose formed was calculated from the change in medium glucose content minus the change in slice glycogen content, which were determined as described in the text. Each point is the mean $\pm$ S.E.M. of four observations. Glucose formed: $\bullet$ 2.5 mM- CaCl_2 present; $\blacktriangle$, Ca^{2+} omitted. Slice glycogen content: $\blacktriangledown$, 2.5 mM- CaCl_2 present; $\blacksquare$, Ca^{2+} omitted.

over a 90 min incubation period was first investigated: the relationship obtained is shown in Fig. 1. This marked dependence on the external Ca^{2+} concentration held for a range of substrates, with the exception of glycerol (Table 2). The rates of gluconeogenesis from all substrates tested, other than glycerol, were lower than those reported for the perfused liver.

The effect of medium Ca^{2+} concentration on gluconeogenesis from glycerol, alanine and pyruvate was studied in more detail. When Ca^{2+} was present in the medium, a fairly linear rate of glucose production from all three substrates was maintained over a 2 h period (Fig. 2). In the absence of Ca^{2+} , linearity still held for glycerol, but glucose production from alanine or pyruvate virtually ceased after 1 h. The glycogen content of the slices always decreased during incubation, in contrast with the observation of Schimmel & Knobil (1969) that there was no change in glycogen content over a 2 h period in liver slices from rats that had been starved for 24 h.

The effect of Ca^{2+} on gluconeogenesis, and in particular on the time-courses of glucose production, suggested that Ca^{2+} might be required to maintain some enzyme activity (or activities) in the gluconeogenic sequence between oxaloacetate and the triose phosphate level. The activities of three rate-limiting enzymes involved in gluconeogenesis were assayed in slices that had been incubated for 2 h at 37°C with 0 or 2.5 mM- CaCl_2 in the medium. The activity of phosphoenolpyruvate carboxylase in slices incubated without Ca^{2+} was significantly lower (65%) than in those where Ca^{2+} had been present. There were no significant changes in fructose 1,6-diphosphatase and glucose 6-phosphatase activities.

The possibility that Ca^{2+} decreased the loss of cytosol protein from the slice was investigated by assaying the phosphoenolpyruvate carboxylase activity in the incubation medium. There were no consistent findings (Table 3). However, the total activity (medium plus slice) in the presence of Ca^{2+} was always greater

Fig. 3. Time-course of the effect of Ca^{2+} on phosphoenolpyruvate carboxylase activity in liver slices

Liver slices from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.40, with and without 2.5 mM- CaCl_2 at 37°C for the indicated times. Phosphoenolpyruvate carboxylase activity in the slices was determined as described in the protocol of Table 3. Each point is the mean of two observations. Slice phosphoenolpyruvate carboxylase activity: ●, 2.5 mM- CaCl_2 present; ▲, Ca^{2+} omitted. Slice protein content: ▼, 2.5 mM- CaCl_2 present; ■, Ca^{2+} omitted.

than without Ca^{2+} , though less than in a fresh tissue extract of the same liver, suggesting that the metal ion was protecting against some inactivation process. This conclusion was supported by an experiment in which the activity of phosphoenolpyruvate carboxylase in slices was monitored over a 2 h period (Fig. 3). In the absence of Ca^{2+} there was a marked decrease in the enzyme activity after 1 h, correlating with the decline in glucose production rates from alanine and pyruvate.

The simplest system in which this protection by Ca^{2+} could be demonstrated was the slice preparation. When the liver was homogenized in suitable media with and without 2.5 mM- CaCl_2 , and incubated in the manner described by Gevers & Krebs (1966), in both cases phosphoenolpyruvate carboxylase activity decreased to 40 % of the initial value over a 2 h period. A process showing similar structural requirements is that of intracellular protein degradation (Schimke *et al.*, 1965). It seemed possible that the protection afforded by Ca^{2+} was associated in some way with the activity of this degradation system and perhaps mediated by changes in lysosomal stability.

The integrity of the lysosomes was assessed by monitoring the appearance of marker enzyme activities (acid phosphatase and β -glucuronidase; Gianetto & de Duve, 1955) in the slice incubation medium. The results of such an experiment are shown in Fig. 4: the activities are expressed as units/mg of protein to nullify any effect Ca^{2+} may have had on the general leakage of proteins from the slice.

Table 3. Effect of Ca^{2+} and pH on phosphoenolpyruvate carboxylase activity in slice and incubation medium

Liver slices from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.40 or 7.10, with or without 2.5 mM- CaCl_2 , for 120 min at 37°C. After incubation the slices were homogenized in 4 vol. of ice-cold 0.02 M-tris-HCl, pH 7.5, containing 0.25 M-sucrose. Samples of the incubation medium were dialysed against the tris-sucrose buffer for 4 h at 4°C. Phosphoenolpyruvate carboxylase in the cytosol fraction of the homogenates and in the dialysed medium was assayed as described in the text. Results are means $\pm$ S.E.M. of four observations. Phosphoenolpyruvate carboxylase activity in the cytosol fraction of fresh tissue homogenates was 7–9 units/g wet wt. n.s., Not significant.

	Incubation buffer (Krebs bicarbonate)		Phosphoenolpyruvate carboxylase activity (units/g wet wt.)		
	Concn. of Ca^{2+} (mM)	pH	Slice	Medium	Total
Expt. 1	2.5	7.40	6.34 $\pm$ 0.41 *	1.69 $\pm$ 0.06	8.03
	0	7.40	4.06 $\pm$ 0.18	2.17 $\pm$ 0.12	6.23
			$P < 0.005$	$P < 0.02$	
Expt. 2	2.5	7.40	6.72 $\pm$ 0.05	1.30 $\pm$ 0.07	8.02
	0	7.40	4.52 $\pm$ 0.02	1.39 $\pm$ 0.09	5.91
			$P < 0.005$	n.s.	
Expt. 3	2.5	7.10	5.64 $\pm$ 0.45 *	1.35 $\pm$ 0.07	6.99
	0	7.10	4.10 $\pm$ 0.31	1.41 $\pm$ 0.06	5.51
			$P < 0.05$	n.s.	

* Not significant.

Fig. 4. Effect of Ca^{2+} on the release of lysosomal enzymes from liver slices into the incubation medium

Liver slices from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.40, with and without $2.5 \text{ mM } \text{CaCl}_2$, at 37°C for the times indicated. At the end of the incubation the medium content of lysosomal enzymes was determined as described in the text: (a) acid phosphatase; (b) β -glucuronidase. Each point is the mean of two observations. ▲, $2.5 \text{ mM } \text{CaCl}_2$ present; ●, Ca^{2+} omitted.

Concentration of Mn^{2+} in the medium

Mn^{2+} , like Ca^{2+} , markedly stimulated gluconeogenesis from pyruvate and lactate: 39.8 ± 3.3 and 41.5 ± 7.0 respectively, compared with a value of $9.0 \pm 3.4 \mu\text{mol/h}$ per g wet wt. in the absence of bivalent cation. A linear glucose production was maintained over a 2 h period in the presence of $2.5 \text{ mM } \text{MnCl}_2$. The data in Table 4 show that Mn^{2+} , like Ca^{2+} , retarded the appearance of lysosomal enzymes in the incubation medium. This obtains in the presence or the absence of substrate.

Changes in other components of the medium

Omission of Mg^{2+} from the incubation medium had no significant effect on the amount of glucose formed from pyruvate over 90 min and the rates of release of lysosomal marker activities in the absence of Mg^{2+} were the same as observed in the presence of standard Krebs bicarbonate buffer ($1.2 \text{ mM } \text{MgSO}_4$) (Table 5).

Lowering the bicarbonate concentration, and consequently pH, of the medium caused some decrease in glucose production from most substrates (Table 2), but phosphoenolpyruvate carboxylase activity in the slice was unchanged (Table 3). The effect of Ca^{2+} on gluconeogenesis and on slice phosphoenolpyruvate carboxylase activity at pH 7.10 was similar to that observed at pH 7.40.

Discussion

The liver-slice preparation described here gives rates of glucose formation from most substrates that are lower than those obtained with the perfused liver, but are rather higher than reported for other slice preparations (Krebs *et al.*, 1966; Wagle *et al.*, 1966). This may be due to the use of small amounts of tissue—about 30–60 mg wet wt./flask. The reason for the unusually high rates of gluconeogenesis in the slice preparation of Schimmel & Knobil (1969), is not clear. They used Diazyme (Johnson & Fusaro, 1966) for glycogen estimation, which gives higher values than the ethanol precipitation–acid hydrolysis method, and this is particularly so for the final glycogen concentration in incubated slices (A. Roobol, unpublished work), presumably because the Diazyme procedure detects oligosaccharides that are not precipitated by ethanol. This higher value for final glycogen content would decrease the estimate of the change in glycogen during incubation and consequently raise the value for net glucose production. However, when glycogen concentrations are determined by the enzymic procedure, the values for net glucose production from alanine are only 20–25% higher than when glycogen is determined after ethanol precipitation, which is insufficient to account for the rates given by Schimmel & Knobil (1969).

A qualitative similarity to the perfused organ is, however, maintained in the liver slice in that the

Table 4. *Effect of Ca^{2+} , Mn^{2+} and lactate on release of lysosomal enzymes from liver slices into the incubation medium*

Liver slices from rats that had been starved for 18–22h were incubated in ‘basic saline’ (210mm-NaCl, 4.8mm-KCl and 25mm- $NaHCO_3$), pH7.40, with or without 2.5mm- $CaCl_2$ or 2.5mm- $MnCl_2$, for 30 and 60 min at 37°C. Samples of the incubation medium were dialysed against 50mm-sodium acetate buffer, pH5.0, at 4°C for 4h and then were assayed for lysosomal enzyme activities as described in the text. Results are means of two observations.

	Incubation buffer	Lysosomal enzyme activity in the medium (units/mg of protein)			
		Incubation time (min)			
		...	...	30	60
(1) Acid phosphatase	‘Basic saline’ ‘Basic saline’ + 2.5mm- $CaCl_2$ ‘Basic saline’ + 2.5mm- $MnCl_2$	Substrate	...	None	20mm-DL-Lactate
				17.5	15.7
				9.5	7.7
				2.8	5.7
(2) β -Glucuronidase	‘Basic saline’ ‘Basic saline’ + 2.5mm- $CaCl_2$ ‘Basic saline’ + 2.5mm- $MnCl_2$	Substrate	...	131	149
				71	68
				48	60
					173
					109
					111

relative rates of gluconeogenesis from a range of substrates vary in a comparable way (Table 2) and the response to starvation is similar. Further, glucose production from glutamine by the slice responds to a decrease in medium pH in much the same way as the perfused liver (Lueck & Miller, 1970). The small decrease in rates of glucose formation from most substrates incurred by decreasing the pH of the medium to 7.05–7.10 is consistent with the decrease in hepatic gluconeogenesis in slices from rats with induced metabolic acidosis reported by Kamm & Cahill (1969).

Glycogenolysis frequently complicates measurements of gluconeogenesis in the perfused liver or the liver slice (Newsholme & Gevers, 1967). As shown in Table 1, a high concentration of glucose in the medium is required to decrease the extent of glycogenolysis, so high in fact that accurate determination of changes in glucose concentration due to gluconeogenesis becomes difficult. Since a glucose concentration of approx. 100mg/100ml is required to inhibit glycogenolysis significantly, it is unlikely that the amount of glucose accumulating in the medium during an incubation would be sufficient to alter the rate of glycogenolysis. It therefore seems to no advantage to have a high glucose concentration in the medium for slice incubations. The contribution by gluconeogenesis to total medium glucose is sufficiently high even in slices from fed rats (27% with lactate as substrate) to permit accurate determination. Thus glycogenolysis does not severely hinder measurement of hepatic gluconeogenesis in slice preparations.

There are several factors that may contribute to the relative impairment of gluconeogenesis in liver slices. The availability of substrate could be one factor, since in the slice this depends on simple diffusion whereas in the perfused organ substrate is presented under pressure via the capillary system. It has been suggested that energy resources are limiting for gluconeogenesis in the slice preparation, since slicing itself decreases the ATP concentration by about 60%, in comparison with freeze-clamping of liver (Krebs, 1970). However, it seems unlikely that the stimulation of gluconeogenesis by bivalent cations is due to their ability to maintain the ATP concentration, since a linear glucose production is observed over a 2h incubation period with Ca^{2+} or Mn^{2+} present, whereas there is no significant difference in the ATP concentrations in slices incubated for 90min with and without these metal ions added to the medium (A. Roobol, unpublished work). The slice can support satisfactory urea production (Ross *et al.*, 1967), which also argues against energy production being a limiting factor.

The proposition remains that certain key enzyme activities are impaired in the slice. The present data show this is true for phosphoenolpyruvate carboxylase, which is always less active in slices than in a

Table 5. *Effect of Mg^{2+} on gluconeogenesis from pyruvate and on release of lysosomal enzymes from liver slices into the incubation medium*

Liver slices from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.40, or with $MgSO_4$ or $CaCl_2$ omitted from the buffer. Glucose formed from pyruvate over 90 min at 37°C was determined as described in the text. The results are means $\pm$ S.E.M. of five observations. The content of lysosomal enzymes in the medium was determined after 30 and 60 min of incubation at 37°C as described in the protocol of Table 4.

Incubation buffer	Glucose formed from pyruvate (μ mol/h per g wet wt.)	Incubation time (min) ...	Lysosomal enzyme activity in the medium (units/mg of protein)			
			Acid phosphatase		β -Glucuronidase	
			30	60	30	60
Krebs bicarbonate	54.5 $\pm$ 5.2		5.8	18.8	38.9	59.2
Krebs bicarbonate without $MgSO_4$	51.7 $\pm$ 4.8		6.9	19.2	40.0	58.1
Krebs bicarbonate without $CaCl_2$	7.0 $\pm$ 5.0		16.3	29.4	69.3	85.6

fresh tissue extract. The activities of glucose 6-phosphatase and fructose 1,6-diphosphatase are unimpaired, which is consistent with the observation that glucose formation from glycerol is only moderately affected. The relative lability of phosphoenolpyruvate carboxylase is compatible with its short half-life (I. D. Longshaw, G. A. O. Alleyne & C. I. Pogson, unpublished work) and probably results from some feature of the tertiary structure of this protein, which renders it rather susceptible to the intracellular protein-degradation system (Schimke, 1970).

The stabilization of slice phosphoenolpyruvate carboxylase by Ca^{2+} and Mn^{2+} is consistent with the effects these ions have on gluconeogenesis. There might appear to be a discrepancy in the magnitude of these two effects: only about 40% of the enzyme activity is lost whereas gluconeogenesis from most substrates shows an almost complete dependence on the presence of the metal ion. This is probably due to the fact that no substrate was added to the medium when slice phosphoenolpyruvate carboxylase activities were measured. Intracellular protein degradation is an energy-dependent process (Simpson, 1953; Steinberg & Vaughan, 1956) and so it is reasonable to suppose that the loss of enzyme activity would be greater if the experiment were repeated in the presence of substrate. Also, the impairment of other enzyme activities is not excluded, and it is possible that these metal ions are affecting glucose production in more direct ways as cofactors or inhibitors of regulatory enzymes in the gluconeogenic and glycolytic sequences (Wimhurst & Manchester, 1970; Kim-mich & Rasmussen, 1969; Bygrave, 1966), or by modifying substrate permeability.

There are at least three ways in which the metal ions could protect phosphoenolpyruvate carboxylase against intracellular degradation: by stabilizing the lysosome, by stabilizing the enzyme against proteolysis or by inhibiting catheptic attack. The two last-named seem unlikely since activity is lost in the crude homogenate regardless of the presence of Ca^{2+} . The first possibility is supported by the rates of appearance of lysosomal marker enzymes from the slice during incubation. The mechanism by which the metal ions stabilize the lysosome is not understood, but it can be said that the process requires intact tissue and is not maintained at the homogenate level.

If, as is suggested by the present data, the impaired gluconeogenic capacity of hepatic tissue after slicing is due to increased lability of the lysosome system, then there must be some doubt about the usefulness of the liver slice for studying hepatic gluconeogenesis and its control. It is true that the slice responds in a manner qualitatively resembling the perfused liver, but any factor altering lysosomal stability in the slice will most likely affect the rate of glucose production without necessarily having any relevance to regulation *in vivo*. This may be the basis for the stimulation *in vitro* of gluconeogenesis by glucocorticoids (Haynes, 1962; Ashmore *et al.*, 1964), which are known to stabilize lysosomes (Weissmann & Thomas, 1964; Weissmann, 1964).

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