Intracellular Zinc Movement and Its Effect on the Carbohydrate Metabolism of Isolated Rat Hepatocytes

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The effect of zinc ions on carbohydrate metabolism and intracellular Zn$^{2+}$ was studied in hepatocytes from fed rats. The addition of ZnCl$_2$ to the medium led to an almost 3-fold increase in lactate production and an increase in net glucose production of about 50%. Half-maximal rates occurred at about 40 $\mu$M ZnCl$_2$. These effects were not seen with Mn$^{2+}$, Co$^{2+}$, or Ni$^{2+}$ up to 80 $\mu$M, whereas Cu$^{2+}$ at 80 $\mu$M and Cd$^{2+}$ or Pb$^{2+}$ at 8 $\mu$M exhibited similar effects as 80 $\mu$M ZnCl$_2$. Changes in intracellular Zn$^{2+}$ were followed by single cell epifluorescence using zinquin as a specific probe. Intracellular free Zn$^{2+}$ in isolated hepatocytes was 1.26 ± 0.27 $\mu$M, and the addition of ZnCl$_2$ led to a concentration-dependent increase in epifluorescence. CdCl$_2$ or PbCl$_2$ at 8 $\mu$M was as potent as ZnCl$_2$ at 20–80 $\mu$M, whereas NiCl$_2$ at 80 $\mu$M was without effect. ZnCl$_2$ completely abolished the inhibition of glycolysis by glucagon (cAMP). Glucagon led to a pronounced drop in cytosolic Zn$^{2+}$. Both glucagon and zinc stimulated glycoanalyisis by increasing the phosphorylation of glycogen phosphorylase but acted oppositely on glycolysis. Zinc overcame the inactivation of pyruvate kinase by glucagon without changing the hormone-induced protein phosphorylation. The antagonistic action of zinc and cAMP on glycolysis together with the rapid and marked decrease in free zinc concentration induced by glucagon (cAMP) may indicate an as yet unknown role of zinc as an important mediator of regulation of carbohydrate metabolism.

Glycolysis in the liver is generally assumed to be regulated at the steps catalyzed by hexokinase/glucokinase, phosphofructokinase-1, and pyruvate kinase. We have shown that in vitro a 11.5-kDa Zn$^{2+}$-binding protein (ZnBP), which is identical with parathymosin, interacts in a zinc-specific manner with key enzymes of carbohydrate metabolism, including the liver enzymes aldolase, phosphofructokinase-1, hexokinase, glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-1,6-bisphosphatase (1). ZnBP is present in the liver at high concentrations of about 20 $\mu$M (2), yet its physiological function is unknown, though ZnBP was discovered by its property to inactivate phosphofructokinase-1 in a reversible, zinc-dependent manner (3).

So far only few data are available on the effects of zinc on carbohydrate metabolism. With cytosol from muscle, zinc at half-maximal concentration of about 0.1 $\mu$M stimulated lactate production from glucose-6-phosphate (4). Rognstad (5) reported that zinc markedly inhibited glycogen synthesis in hepatocytes from starved rats; moreover, in hepatocytes from fed rats no effect could be observed on the glycoanalisis. In rat adipocytes zinc stimulated glucose transport (6, 7), incorporation of glucose into lipids (8), and glucose oxidation by both pathways, glycolysis and hexose monophosphate shunt (9).

Zinc is known to play a pivotal role in the regulation of DNA binding and activation of transcription factors or in the regulation of apoptosis. As for Ca$^{2+}$, intracellular zinc levels have been suggested to be under homeostatic control. The latter may be essential to maintain the diversity of biochemical functions in the intermediary metabolism, which are dependent on zinc proteins and enzymes (10). However, little is known about the level of Zn$^{2+}$ in intact cells and to what extent changes in intracellular distribution may influence cellular events. However, the rather high affinity constants of metalloenzymes for zinc have inferred the free zinc concentration in cells to be in the order of $10^{-12}$ M or even lower (11). Recently, Zalewski et al. (12) have introduced the fluorescent indicator zinquin (ethyl-2-methyl-8-p-toluenesulfonamido-6-quinolylxoy) as an intracellular probe for Zn$^{2+}$. Using Jurkat lymphoid cells pretreated with pyrithione and zinc, they estimated the free or “labile” intracellular zinc as detected by zinquin to be in the order of 2 nM/mg (12). Therefore, zinc have inferred the free zinc concentration in cells to be in the order of $10^{-12}$ M or even lower (11). Recently, Zalewski et al. (12) have introduced the fluorescent indicator zinquin (ethyl-2-methyl-8-p-toluenesulfonamido-6-quinolylxoy) as an intracellular probe for Zn$^{2+}$.

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Here we report that incubation of hepatocytes from fed rats with ZnCl₂ leads to a 2–3-fold increase in the rate of lactate production and to an enhanced glucose production, due to an enhanced glycolgenolysis and an increased flux from glucose-6-phosphate through the glycolytic pathway. We show further that zinc counteracts the inhibition of glycolysis by glucagon or cAMP.

In parallel, we measured zinc-dependent epifluorescence of single hepatocytes. The effect of various metal ions and glucagon or cAMP on the rapid exchangeable cytosolic zinc concentration was investigated. The similarity of increase in glycolysis seen with ZnCl₂ as compared with CdCl₂ or PbCl₂ can be explained by their ability to mobilize intracellular zinc. Moreover, by single cell measurement we can show for the first time that glucagon (or cAMP) rapidly decreases the level of intracellular Zn²⁺ to less than 20% of that of control.

**EXPERIMENTAL PROCEDURES**

**Methods**

Preparation and Incubation of Hepatocytes—Livers from male or female Wistar rats (170–220 g) fed on Altromin-R stock diet were perfused and used for isolation of hepatocytes by the two-step procedure as described by Berry et al. (35). For incubation, freshly prepared hepatocytes (about 4 g of wet weight) were washed with 250 ml of an albumin- and P-free incubation medium (4°C, gassed with oxygen) containing 68.44 mM NaCl, 60 mM Hepes, 30 mM Mops, 5.4 mM KCl, 0.70 mM Na₂SO₄, 1.22 mM CaCl₂, and 1.0 mM MgCl₂ adjusted to pH 7.40 with 1 mM NaOH at 20°C (HMS medium). After the last washing step, the hepatocytes were diluted to a final concentration of 100 ± 10 mg of wet weight/ml, and aliquots of 2.5–3.5 ml were transferred to 25 ml conical 25-ml plastic beakers with a diameter of 3.5 cm at the bottom. The vessels contained maltol to give a final concentration of 0.2 mM in the suspension, and, for zinc incubations, the appropriate amounts of ZnCl₂. Hormones or dibutyryl-cAMP (Bl-CAMP), when used, were added at the beginning or at specific incubation times. Incubation time was 10 min. The incubations were performed in a waterbath at 37°C with a shaking frequency of 100 cycles/min under oxygenation. After this the cells were spun down (100 × gfor 1.5 min), the supernatant was discarded, and the pellet was resuspended in the same volume of HMS medium plus 14.5 mM glucose and kept on ice until use. The cells were incubated at 30°C for up to 30 min under conditions as specified in the text. For fluorescence measurements hepatocytes (1–2 mg of wet weight) were withdrawn at time intervals as indicated, suspended in 2 ml of oxygenated HMS medium in a Petri dish (Falcon 3001, with a centered 10-mm glass window), and immediately placed in the light path of the microscope. The fluorescence of single hepatocytes at 290–380 nm excitation was monitored at 480–540 nm (see below). Zn²⁺ was determined from the increase in fluorescence. Usually the epifluorescence of 20–25 single hepatocytes under each condition was averaged. To correct for autofluorescence, control incubations using hepatocytes "preloaded" in the presence of solvent but otherwise treated identically were always run in parallel. The average in epifluorescence of a set of hepatocytes was calculated from the fluorescing values measured in the presence of the dye. The concentration of the solvent(s) in the incubation never exceeded 1% (v/v). The same amount of solvent was added to control incubations.

Fluorescence measurements were performed using a Fura-2 data acquisition system (Luigs & Neumann GmbH, Ratingen, FRG) mounted to an inverted microscope (Zeiss IM 35). The sampling rate was 2 measurements/s. For a more detailed description and evaluation of the equipment see Neher (16).

The concentration of Zn²⁺ was calculated according to Grzymkiewicz et al. (17). Maximal fluorescence was determined in the presence of 40 μM Zn²⁺ plus maltol (0.1 mM), and minimal fluorescence was determined with N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (50 μM) plus maltol (0.1 mM). The stability constant of the Zn²⁺-zincquin complex was determined according to Hagemüller (18) using a medium resembling the intracellular environment (130 mM KCl, 15 mM NaCl, 0.5 mM MgCl₂, 50 mM Mops pH 7.05, and 0.01% bovine serum albumin). The dissociation constant for the 1:1 complex present under our condition was 1.14 ± 0.16 × 10⁻⁹ mol/liter. This value compares favorably with a value of 0.37 × 10⁻⁹ mol/liter given by Zalewski et al. (32).

If not otherwise stated, the values given are the means ± S.E. of 20–25 single cells/dish under each condition. The experiments were repeated at least three times with independent cell preparations. Total zinc and calcium were measured by atomic absorption after acid extraction and appropriate dilution of the sample in 0.1M HCl with 0.3 g of LaCl₃/100 ml.

Determination of Metabolites—Aliquots (0.5 ml) of the suspension were removed and mixed with 0.25 ml of 10.5% HClO₄. After centrifugation, 0.5 ml of the supernatants were neutralized with 0.9 ± KOH/50 mM Trís, kept on ice for 30 min, and then centrifuged. The clear supernatants were used for the determination of lactate and glucose according to Bergmayer (19).

For cross-over studies, at least 6 ml of suspension were used from each incubation. Protein was precipitated by the addition of 0.1 vol of 3M HClO₄ and removed by centrifugation. An amount of again 20–25 single hepatocytes was added to 1.5 ml of a perfusion solution containing 45 mM Hepes, 50 mM NaF, 20 mM K₂HPO₄, 1.8 mM NaN₃, 5.6 mM glucose, and kept on ice until use. Hormones or dibutyryl-cAMP (Bl-CAMP), when used, were added at the beginning or at specific incubation times. Incubation time was 10 min. The incubations were performed in a waterbath (100 cycles/min) under oxygenation. After this the cells were washed once (50 × g for 1.5 min), resuspended in 1 ml of an extraction/stopping solution containing 45 mM Hepes, 50 mM NaF, 20 mM K₂HPO₄, 1.8 mM NaN₃, 1.62 mM CaCl₂, 10.6 mM MgCl₂, and 0.16 mM N,N'-tetrakis (2-pyridylmethyl) ethylenediamine, pH 7.0, and then centrifuged. The supernatant was neutralized with 5 M KOH and centrifuged. The pellet was washed twice with 0.1M KCl. The combined supernatants were treated with Florisil until they became colorless and were lyophilized. The samples were dissolved in 1–1.5 ml of H₂O. Determinations of metabolites and adenine nucleotides were performed essentially as described by Bergmayer (19), but in addition, all assays contained 1 mM diethyl-enetriaminepentaacetic acid.

Determination of Fructose-2,6-bisphosphatase—Fru-2,6-P₂ was determined in hepatocytes as described by Van Schaftingen et al. (20) using activation of pyrophosphate-phosphofructokinase from potato tubers by fru-2,6-P₂. This enzyme was purified according to Ref. 20.

**Materials**

Collagenase "Worthington" (type CLS II) was purchased from Sera-rom Corp. Zinquin acid and zinquin ester were purchased from Luminis.
RESULTS

Set-up of Incubation Conditions—Because hepatocytes had to be incubated in the presence of zinc, anions (bicarbonate, phosphate) maintaining low solubility products with zinc and albumin had to be omitted from the incubation medium. A strongly buffered medium comparable with that reported by Seglen (22) was used but with the omission of phosphate. Under these conditions, hepatocytes from fed rats produced lactate and glucose from endogenous glycogen at a constant rate over the time of incubation. Metabolic rates were constant for at least 30 min under all conditions used (minus or plus ZnCl₂, glucagon, or Bt₂-cAMP). The time was sufficient to reach a constant zinc level in the medium. With hepatocytes from starved rats, the time course of zinc uptake was comparable with that seen with isolated perfused livers, indicating that zinc offered to the cell in the liganded form is better available for biological effect than the unliganded form. Although with hepatocytes a better uptake of zinc was not observed in the presence of liganded zinc to hemoglobin (25). Although with hepatocytes a better uptake of zinc was not observed in the presence of maltol, the effects of zinc were more reproducible, indicating that zinc offered to the cell in the liganded form is better available for biological effect than the unliganded form.

Effect of Zinc on Lactate and Glucose Formation of Hepatocytes from Fed Rats—Hepatocytes from fed rats were incubated without exogenous substrate in the absence or the presence of different ZnCl₂ concentrations up to 0.1 mM. All incubations contained 0.2 mM of the membrane permeable zinc-chelator maltol. Lactate and glucose concentrations were determined, and the rates were calculated as μmol/g of wet weight/h. The results are shown in Fig. 2. The addition of more than 0.05 mM ZnCl₂ to the medium resulted in a doubling of the rate of lactate formation and an increase in the rates of glucose production by 50%. Although the relative increase in each experiment depended on the control rates, distinct effects of zinc were seen in the mean values as given in Fig. 2. In general, incubations with 0.1 mM ZnCl₂ resulted in rates of 30–40 μmol of lactate/g/h. Maltol (0.2 mM) itself had no effect on the metabolic rates in the absence of exogenous zinc. Maltol has been used to facilitate uptake of zinc into erythrocytes, releasing liganded zinc to hemoglobin (25). Although with hepatocytes a better uptake of zinc was not observed in the presence of maltol, the effects of zinc were more reproducible, indicating that zinc offered to the cell in the liganded form is better available for biological effect than the unliganded form.

Under all conditions, the addition of the membrane-impermeable chelating agent diethylenetriaminepentaaetic acid to the medium at concentrations equimolar to those of ZnCl₂ abolished the effects of zinc (data not shown, but see Fig. 3). Moreover, no difference in the oxygen consumption of hepatocytes could be detected in the presence of maltol or maltol plus
Intracellular Zn\(^{2+}\) and Carbohydrate Metabolism

Table I

| Lactate | Glucose |
|---------|---------|
| µmol/g wet wt/h | µmol/g wet wt/h |
| A. Control | 16 ± 4 | 83 ± 4 |
| Glucagon (10\(^{-7}\) M) | 3 ± 3 | 122 ± 4\(^{a}\) |
| ZnCl\(_2\) (0.1 mM) | 32 ± 4 | 127 ± 5\(^{a}\) |
| Glucagon + ZnCl\(_2\) | 28 ± 4 | 125 ± 6\(^{a}\) |
| B. Control | 16 ± 3 | 74 ± 4 |
| Vasopressin (5 × 10\(^{-8}\) M) | 18 ± 3 | 106 ± 9 |
| ZnCl\(_2\) (0.1 mM) | 27 ± 2\(^{b}\) | 104 ± 7\(^{b}\) |
| Vasopressin + ZnCl\(_2\) | 27 ± 3\(^{b}\) | 114 ± 9\(^{b}\) |
| C. Control | 12 ± 3 | 66 ± 3 |
| ZnCl\(_2\) (0.1 mM) | 34 ± 3 | 96 ± 5 |
| Bt\(_2\)-cAMP (0.025 mM) | 4 ± 3 | 100 ± 11\(^{c}\) |
| Bt\(_2\)-cAMP (0.05 mM) | 1 ± 3 | 132 ± 6\(^{c}\) |
| Bt\(_2\)-cAMP (0.1 mM) | 10 ± 4 \(^{d}\) | 174 ± 11\(^{d}\) |
| Bt\(_2\)-cAMP (0.025 mM) + ZnCl\(_2\) | 25 ± 3 \(^{c}\) | 101 ± 7 |
| Bt\(_2\)-cAMP (0.05 mM) + ZnCl\(_2\) | 17 ± 3 \(^{c}\) | 122 ± 8 |
| Bt\(_2\)-cAMP (0.1 mM) + ZnCl\(_2\) | 4 ± 7 \(^{d}\) | 162 ± 10 |

\(^{a}\) p < 0.001.  
\(^{b}\) p < 0.005.  
\(^{c}\) p < 0.01.  
\(^{d}\) Significant difference to the corresponding incubation with Bt\(_2\)-cAMP alone with P < 0.025.

ZnCl\(_2\) (Table I). With glucagon (10\(^{-7}\) M) alone, lactate formation was nearly abolished or was even converted into lactate consumption. However, the stimulatory effect of zinc on lactate production was not overcome by glucagon (Table I).

Glucose formation was increased by glucagon due to enhanced glycolysis. ZnCl\(_2\) had no additional effect at maximally effective concentrations of glucagon, whereas at submaximal rates of glucose formation, it had an additive effect (not shown).

Whereas stimulation of glycolysis by glucagon is caused by increased levels of cAMP, vasopressin acts by increasing the cytosolic calcium concentration via inositol 1,4,5-trisphosphate. Vasopressin (0.5–5 × 10\(^{-6}\) M) itself had no effect on the net lactate production, although the cells were hormone-sensitive as indicated by the enhanced glucose formation (143%). The addition of ZnCl\(_2\) increased the rate of lactate formation in the presence of vasopressin (see Table I) to the rate found with ZnCl\(_2\) alone. It is therefore unlikely that the effect of zinc on glycolysis is due to a mobilization of calcium. This conclusion is strengthened by our observation that in the presence of ZnCl\(_2\) the O\(_2\) consumption of hepatocytes was not different from control incubations (results not shown).

In order to exclude the possibility of an interaction of zinc with the glucagon receptor, experiments were performed with Bt\(_2\)-cAMP. As shown in Table I (part C), 25, 50, and 100 µM Bt\(_2\)-cAMP strongly inhibited lactate formation. The addition of 0.1 mM ZnCl\(_2\) to the medium overcame this inhibition and led to an enhanced glycolysis. With increasing concentration of Bt\(_2\)-cAMP, the zinc-induced stimulation of glycolysis was reduced. Glucose output stimulated by Bt\(_2\)-cAMP was not altered by the addition of ZnCl\(_2\).

Effect of Zinc on Gluconeogenesis—The effect of zinc on gluconeogenesis was examined on hepatocytes from rats starved overnight. Regardless of the gluconeogenic precursor (lactate plus pyruvate or dihydroxyacetone) and the rate of glucone-

Fig. 3. Mobilization of Zn\(^{2+}\) by various cations. Hepatocytes were preloaded with zincin as given under “Experimental Procedures.” Cells (25–30 mg of wet weight/ml) were thermoequilibrated in HAMS medium in the presence of 15 mM glucose at 37 °C under oxygenation in a shaking water bath. After 5 min, chloride salts of the various cations were added at the concentrations indicated, and the incubation was continued for 30 min. An aliquot of 50 µl was withdrawn and mixed with 2 ml of HAMS buffer in a Falcon Petri dish, and epifluorescence of single hepatocytes was determined as given under “Experimental Procedures.” A reference incubation of cells preloaded with solvent instead of zinquin but otherwise treated identically was analyzed in parallel (autofluorescence, open bars). The average (mean ± S.E.) in epifluorescence at 390 nm excitation of 20–30 cells/dish is plotted. DPTA, diethylenetriaminpentacetic acid.

0.1 mM ZnCl\(_2\) as compared with controls (results not shown).

Because we had to use a medium without phosphate or bicarbonate, the zinc effect was compared in parallel incubations using HAMS medium and KRB buffer with 1.2 mM CaCl\(_2\). Because zinc is completely insoluble in KRB buffer alone, maltozol had to be added at much higher concentrations (0.42 mM) to maintain a Zn\(^{2+}\) concentration of 0.1 mM. This titration was verified by atomic absorption spectroscopy. Under these conditions we could show that the zinc effects in both media were comparable. Glycolysis was doubled in both media, the glyco-

Effect of Other Metal Ions on Lactate and Glucose Formation—To evaluate the specificity of zinc, the effect of iron, nickel, cobalt, manganese, copper, cadmium, and lead ions was examined. Calcium and magnesium were always present in the incubation medium at 1.2 and 1.0 mM, respectively. The incubation conditions were as given in the methods section, except that ZnCl\(_2\) was replaced by the other metal ions. Up to a concentration of 80 µM, no effect was observed with Fe\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\). With MnCl\(_2\), a small but inhibitory effect (−20%) on the rates of lactate production was detectable. With CuCl\(_2\), no effect on the lactate formation occurred up to a concentration of 40 µM, but at 80 µM lactate formation increased to values that were comparable with those found in the presence of 80 µM ZnCl\(_2\).

CdCl\(_2\) and PbCl\(_2\) were cell toxic at concentrations above 40 µM. At 8 µM, however, both cations were as effective stimulating the rates of lactate and glucose production as ZnCl\(_2\) at 80 µM.

Hormone Effects in the Presence of Zinc—The effects of glu-

cagon and vasopressin on lactate and glucose formation from glycogen were examined in the presence and the absence of the rates of lactate production was detectable. With CuCl\(_2\), no concentration of 80 µM lactate formation increased to values that were comparable with those found in the presence of 80 µM ZnCl\(_2\).

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Hormone Effects in the Presence of Zinc—The effects of glu-

cagon and vasopressin on lactate and glucose formation from glycogen were examined in the presence and the absence of
production was increased and the glycolytic flux was doubled, with 8-Br-cAMP remaining more stable (Fig. 4). Absence of glucose and in the presence of zinc, the glucose concentration remained at 0.08–0.1 mM ZnCl₂ to the medium resulted in a small (16–18%) but significant inhibition of the rate of glucose formation (Table II). With dihydroxyacetone as the gluconeogenic precursor, glucose production was inhibited by the addition of zinc, but the rate of lactate formation was increased about 100-fold (from 0.18 to 18.9 μmol/g of wet weight/h), demonstrating directly that the flux of triosesphosphates had changed in part from the gluconeogenic to the glycolytic direction.

Measurement of Free Zinc in Hepatocytes—The free zinc concentration in eight independent preparations of rat hepatocytes was 1.26 ± 0.27 μM (range 0.61–2.7 μM). This value increased during prolonged incubation in the absence of zinc, especially in cells showing blebbing and signs of beginning cell death.

Effect of Various Divalent Cations on Zinquin Fluorescence—The addition of ZnCl₂ led to a concentration-dependent increase in fluorescence, which was abolished by the presence of diethylaminoethyl in a manner similar to that of Cd²⁺ or PbCl₂ at 8 μM were as potent as ZnCl₂ at 20 μM in raising Zn-dependent epifluorescence. NiCl₂ at 80 μM was without effect (Fig. 3). This rank of cations resembles their “zinc-like” effects on carbohydrate metabolism and resembles their ability to mobilize zinc from Zn-MT (26). Apart from Zn²⁺, only Cd²⁺ shows a weak reaction with zinquin (12). The quantum yield in emitted light would be less than 30% of that observed in the presence of equimolar Zn²⁺. The observed effect clearly exceeds this proportion.

Short-Term Effects of Hormones or cAMP on Cytosolic Zn²⁺—Glucagon (10⁻⁷ M) induced a rapid decrease in free zinc concentration (Fig. 4A) that could be mimicked by cAMP (0.1 mM) (Fig. 4B). The effect was distinct within 10 min and clearly present at glucagon concentrations as low as 10⁻⁹ M (Fig. 4A, inset) but was partially restored at 20 min. In accordance, Zn²⁺ levels measured in the presence of the slowly degradable analogue 8-Br-cAMP remained more stable (Fig. 4B).

To assess whether the rapid decrease in cytosolic Zn²⁺ was due to an increased binding of the cation by MT, the potential of Cd²⁺ (8 μM) to discharge zinc from MT was investigated. The ability of Cd²⁺ to raise cellular Zn²⁺ was similar in the absence or the presence of cAMP, indicating that a portion of zinc not associated with MT is reduced (not shown).

Cross-over Studies—In order to locate the points of control at which zinc acts on carbohydrate metabolism, intracellular metabolite concentrations were determined in hepatocytes incubated with and without 0.1 mM ZnCl₂ in the presence or the absence of glucagon. The data are given in Table III. In the absence of glucose and in the presence of zinc, the glucose production was increased and the glycolytic flux was doubled, indicating enhanced glycogenolysis. This is supported by the almost 10-fold increase in concentration of hexose-6-phosphates (glucose-6-phosphate plus fructose-6-phosphate) in the presence of ZnCl₂. Under this condition the level of fructose-2,6-bisphosphate was almost 2-fold increased in concentration of hexose-6-phosphates (glucose-6-phosphate plus fructose-6-phosphate) in the presence of ZnCl₂. Under this condition the level of Fru-2,6-P₂ was increased almost 2-fold.

A distinct cross-over point at pyruvate kinase indicates that the conversion of phosphoenolpyruvate to pyruvate was increased in the presence of zinc. Furthermore, the data show that changes in levels of metabolites caused by glucagon are counteracted by the addition of ZnCl₂.

Effect of Zinc on the Concentration of Fructose-2,6-bisphosphate—Fructokinase-1, a positive effector for phosphofructokinase-1 and a negative effector for fructose-1,6-bisphosphatase plays a major role in the control of glycolysis and gluconeogenesis in rat liver (see Ref. 27 for a review). In two experiments with hepatocytes from fed rats, we measured a concentration of
Effect of ZnCl₂ on metabolite concentration of hepatocytes in the presence and the absence of glucagon

Hepatocytes from fed rats were incubated as described under "Experimental Procedures" without and with 0.1 mM ZnCl₂ in the presence or the absence of 10⁻⁷ M glucagon. After 20 min, the incubations were stopped by the addition of 0.1 volume of 35% HClO₄. The values are the means ± S.E. of three independent cell preparations incubated under all four conditions. Further details are given under "Experimental Procedures." The abbreviations are: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenol pyruvate.

| Metabolite concentration | Controls | ZnCl₂ | Glucagon | Glucagon + ZnCl₂ |
|--------------------------|----------|-------|----------|-----------------|
| nmoles/g wet weight      |          |       |          |                 |
| G6P + F6P                | 103 ± 11 | 194 ± 16 | 111 ± 10 | 192 ± 11 |
| FBP                      | 16 ± 2   | 23 ± 4 | 13 ± 3   | 21 ± 4 |
| DHAP + GAP               | 96 ± 18  | 138 ± 28 | 73 ± 10 | 120 ± 21 |
| 3PG + 2PG + PEP          | 616 ± 47 | 537 ± 67 | 661 ± 53 | 586 ± 67 |
| Pyruvate                 | 502 ± 66 | 674 ± 121 | 253 ± 62 | 611 ± 9 |
| Lactate                  | 7,590 ± 1,131 | 15,410 ± 1,253 | 3,468 ± 1,226 | 10,269 ± 1,555 |

Proportion of glycogen phosphorylase a of total activity in hepatocytes incubated with ZnCl₂ or dibutyryl-cAMP

Hepatocytes from fed rats were incubated in the presence or the absence of O.1 mM ZnCl₂ with Bt₂-cAMP. After 15 min of incubation, aliquots (1 ml) of the cell suspension were removed and immediately frozen in liquid nitrogen. Phosphorylase was extracted and determined as given under "Experimental Procedures." The values are the means ± S.D. of four independent cell preparations.

Table IV

| Glycogen phosphorylase activity | a | Total | a of total |
|--------------------------------|---|-------|------------|
| units/g wet weight             |   |       | %          |
| Incubation                     |   |       |            |
| Control                        | 1.6 ± 3.4 | 16.2 ± 1.6 | 34.6       |
| + 0.1 mM ZnCl₂                 | 1.5 ± 0.3 | 16.5 ± 1.5 | 58.8       |
| + 0.1 mM Bt₂-cAMP              | 1.0 ± 0.4 | 16.9 ± 1.5 | 61.5       |

**FIG. 5.** Phosphorylation state of pyruvate kinase, glycogen phosphorylase, and fructose-1,6-bisphosphatase extracted from hepatocytes incubated with and without ZnCl₂ with Bt₂-cAMP and with Bt₂-cAMP plus ZnCl₂. Hepatocytes from fed rats were preincubated with [³²P]Pi (1 mCi/g of wet weight) for 30 min. The medium was changed, and the cells were thereafter incubated without (lanes 1) and with 0.1 mM ZnCl₂ (lanes 2), with 0.1 mM Bt₂-cAMP (lanes 3), and with Bt₂-cAMP plus 0.1 mM ZnCl₂ (lanes 4), as given under "Experimental Procedures." After 20 min of incubation, cells were sedimented and extracted with 0.2% Triton X-100. From aliquots (0.1 ml) of the supernatants pyruvate kinase, glycogen phosphorylase, and fructose-1,6-bisphosphatase were immunoprecipitated, separated by SDS-polyacrylamide gel electrophoresis, and autoradiographed as described under "Experimental Procedures." GP, fructose-1,6-bisphosphatase; PK, pyruvate kinase.
DISCUSSION

We have shown here that incubation of hepatocytes from fed rats with zinc in the medium strongly enhances the formation of lactate and glycogenolysis. The effect depends on the concentration of ZnCl₂ added to the medium and is significant at concentrations of 33 μM and higher. A comparable effect of zinc was also seen with KRB buffer, provided it had been supplemented with enough maltol. The failure of Rognstad (5) to observe this effect was most probably due to the insolubility of zinc in a medium containing phosphate and bicarbonate. Using his medium we were unable to detect zinc in the solution by atomic absorption spectroscopy, even with concentrations as high as 0.5 mM ZnCl₂. Therefore, genuine zinc effects cannot be expected under these conditions without an appropriate membrane-permeable chelator (i.e. maltol).

The change in intracellular free zinc is much lower than the changes of total zinc found in the medium after the equilibration period (Fig. 1). In liver cytosol various proteins (e.g. MT) and low molecular weight compounds will act as buffers by binding large amounts of the added zinc, depending on their stability constants, which in general are higher than that of the maltol-Zn complex, which is 5.62 (26).

We have good evidence that the effects of zinc on glycogenolysis and glycolysis are specific for zinc ions. Ca²⁺, Mg²⁺, Mn²⁺, Fe²⁺, Ni²⁺, and Co²⁺ were ineffective. CuCl₂, however, at comparable concentration and CdCl₂ or PbCl₂ at 10-fold lower concentration showed the same effects as ZnCl₂. Those cations showing the zinc-like effect on metabolism did indeed raise intracellular free zinc (Fig. 3). It seems feasible to conclude that these metal ions mobilize Zn²⁺ from Zn-MTs. This conclusion is supported by in vitro displacement studies (26) that show that Cd²⁺ and Ni²⁺ could not displace zinc from Zn-thionein, whereas Cd²⁺, Pb²⁺, and Cu²⁺ could. The rank of affinities in this study was: Cd > Pb > Cu > Zn > Ni > Co. The metabolic effects observed here as well as the data on intracellular Zn²⁺ mobilization fit exactly into this order of affinities. Furthermore, using the isolated perfused liver, Kingsley and Frazier (28) have shown that exposure to cadmium increases both zinc secretion into the perfusate and biliary excretion of zinc.

Here we have determined the concentration of free Zn²⁺ by monitoring single cell epifluorescence using zinquin as an intracellular probe. The advantage of this technique as compared with bulk measurements of zinquin fluorescence in cell suspension (12, 13) is that specific hepatocytes of uniform size and round shape and lacking signs of cell damage can be examined for zinquin-dependent fluorescence. Recently, Zalewski et al. (12) reported that apoptotic HL-60 cells revealed bright zinquin fluorescence, indicating an increased zinc concentration, membrane blebbing, and often a decrease in cellular volume. Conversely, zinquin seems to escape from necrotic hepatocytes, these cells showing therefore no or a markedly reduced fluorescence. Coyle et al. (13), using zinquin to follow changes in labile zinc in hepatocytes in primary culture or in liver slices treated with dexamethasone and interleukine-6 to induce metallothionein synthesis, have advised not to compare samples with different concentrations of MT. All effects reported here are short term effects (~30 min), where only negligible changes, if any, in MT levels should occur. In freshly isolated rat hepatocytes, induction of MT by Zn²⁺ was detectable after 2 h. Glucagon was less effective increasing MT synthesis only by 28—35% after 5 h (23).

The concentration of free Zn²⁺ reported here (about 10⁻⁸ M) is considerably higher than would be predicted from measurements with enzymes, such as alkaline phosphatase and alcohol dehydrogenase, both being metalloproteins with extremely high affinities for Zn²⁺ (11). Although we cannot exclude that some of the zinc we are detecting could have been derived from Zn-MT, the observed changes in Zn²⁺-dependent fluorescence mediated by glucagon or Cd²⁺ or Pb²⁺ do not support a major interference.

Both zinc and glucagon stimulated glycogenolysis due to an enhanced phosphorylation of glycogen phosphorylase. The mechanism by which intracellular Zn²⁺ activates phosphorylase kinase has to be further investigated.

With respect to the fate of glucose-6-phosphate, zinc and glucagon (or cAMP) acted in completely different ways. Glucagon shifted glucose-6-phosphate mainly in the direction of gluco-ase kinase and inhibited the glycolytic pathway, whereas zinc, in addition to the enhancement of glycogenolysis, also markedly stimulated glycolysis. In combination, Zn²⁺ acted antagonistically to the hormone and was able to overcome the inhibition of glycolysis by glucagon or by Bt₂-cAMP.

The rapid decrease in intracellular Zn²⁺ concentration in the presence of glucagon (Fig. 4) is a new and unexpected observation and offers an explanation for the antagonistic effects of glucagon and zinc on glycolysis. The rapid decrease in intracellular free zinc was also seen with 8-bromo-cAMP (Fig. 4), indicating that the action of glucagon on intracellular Zn²⁺ is mediated via cAMP and most probably catalyzed by protein kinase A. This could lead to a sequestration of the cation. In consequence, in order to locate the points at which zinc acts on carbohydrate metabolism, we focused on points where phosphorylation of enzymes is known to be of regulatory importance. We have analyzed the activity and grade of phosphorylase of glycogen phosphorylase and pyruvate kinase and determined the concentration of fru-2,6-P₂, an indicator of the activity of the bifunctional enzyme fructose-2,6-bisphosphate-phosphofructokinase-2. The proportion of phosphorylase a increased in the presence of Zn²⁺ as well as in the presence of glucagon from 35% to about 60% of total phosphorylase activity (Table IV). This could be verified in intact hepatocytes by [³²P]J incorporation into phosphorylase (Fig. 5). Moreover, the effect of zinc and Bt₂-cAMP on the phosphorylation of glycogen phosphorylase was not additive, suggesting that zinc acts rather via an increased activity of phosphorylase kinase and not by an inhibition of the protein phosphatases 1 or 2a. That the effects of zinc and of Bt₂-cAMP on phosphorylase activation are comparable and not additive can also be calculated from the metabolic rates given in Table I by converting the rates of glucose and lactate production into glycosyl units (not shown). However, the mechanism by which the increase in cytosolic Zn²⁺ activates the phosphorylase kinase remains to be elucidated. A binding of Zn²⁺ by calmodulin has been reported (29), but the measured affinity (Kₜ, about 100 μM) seems to be too low to be of physiological importance.

The mechanism by which zinc activates glycolysis remains obscure. Based on the metabolite measurements reported here, zinc exerts its effect most likely at the steps catalyzed by pyruvate kinase and by phosphofructokinase-1. The substrate concentration (fructose-6-phosphate) of the rate-limiting enzyme phosphofructokinase-1 was doubled, and at the step of pyruvate kinase the inverse changes in substrate and product concentration (cross-over) indicate another control point at this step.

The classical explanation for metabolic regulation by allosteric activators does hold for the antagonistic effect of zinc on the inhibition of glycolysis by glucagon. The most potent activator of phosphofructokinase-1, namely fru-2,6-P₂, was reduced by glucagon from 12 to about 6 mmol/kg of wet weight, whereas in the presence of glucagon plus zinc the concentration of this effector was restored to 9 mmol/kg of wet weight. This
would lead to a higher flux through phosphofructokinase-1 in the presence of glucagon plus zinc as compared with glucagon alone. The effect of zinc on the fru-2,6-P_2 level in the presence of Bt_2-cAMP and the failure to get an effect in the absence of Bt_2-cAMP may well be explained by a counteraction of Zn^{2+} exclusively on the phosphorylated and therefore less active form of phosphofructokinase-2. We have shown that under this condition protein kinase A is as active as in the absence of ZnCl_2 (see phosphorylation of pyruvate kinase, Fig. 5), and phosphofructokinase-2 should be phosphorylated as well, unless there exists an as yet unknown specific and Zn^{2+}-dependent protein phosphatase.

It is also possible that the higher concentration of fructose-6-phosphate, which is almost doubled in the presence of ZnCl_2, has more effect on the phosphorylated than on the unphosphorylated form of phosphofructokinase-2. The mechanism of Zn^{2+} action on this enzyme seems to be different from that of vanadate, which also raises glycolytic flux in hepatocytes (30) but exerts also glycolgenolytic effects by activating glycogen phosphorylase and inactivating glycogen synthase (31). The "insulin-like" effect of vanadate on glycolysis has been related to the increase in fru-2,6-P_2. Rider et al. (32) have found vanadate to be an inhibitor of chicken liver fructose-2,6-bisphosphatase, which offers a likely explanation for the increased flux through phosphofructokinase-1 in the presence of vanadate. However, this explanation is not valid for the pronounced glycolytic effect of zinc on its own, because under this condition the concentration of fru-2-6-P_2 did not change.

The inhibition of glycolysis by glucagon at the step of pyruvate kinase has been explained by the increased protein phosphorylation. The concentration of zinc, however, which overcame the glucagon-induced inhibition of glycolysis, did not affect the glucagon-induced phosphorylation of the enzyme. This makes it also unlikely that a protein phosphatase is involved in the antagonistic effect of zinc. In the presence of zinc alone, pyruvate kinase remains dephosphorylated (Fig. 5) and should be in its active form. Therefore, the elevated fru-1,6-P_2 concentration necessary for half-maximal stimulation is reported to be much lower. K_{D50} values of 0.06 μM and 0.13 μM fru-1,6-P_2 are given for the unphosphorylated and the phosphorylated form of the purified rat liver pyruvate kinase, respectively (33). Even the highest K_{D50} values we could find in the literature, resulting from a study using crude extracts from hepatocytes (4.2 and 9.8 μM fru-1,6-P_2 for control and for hepatocytes incubated with glucagon, respectively (34)), would not readily explain the activation of glycolysis in the presence of zinc alone.

The stimulation of lactate formation in hepatocytes by zinc could also result from a strong inhibition of fructose-1,6-bisphosphatase, a regulatory enzyme in gluconeogenesis. This enzyme is strongly inhibited by zinc in vitro at concentrations below 1 μM (35), but it has also been reported that zinc at 0.5–2 μM counteracts the inhibition of fructose-1,6-bisphosphatase by fru-2,6-P_2, shifting the K_i from 3 μM to values higher than 50 μM (36). In our experiments an inhibition of glucose formation from pyruvate, alanine, or dihydroxyacetone in hepatocytes from starved rats was indeed observed but only at concentrations of 0.1 mM ZnCl_2 or higher, and this inhibition never exceeded 15–20% (see Table I). At lower concentrations of zinc, an inhibition of gluconeogenesis was not observed, although at these concentrations a significant stimulation of glycolysis occurred with hepatocytes from fed rats. In hepatocytes from fed rats, the fructose-1,6-bisphosphatase was already in the phosphorylated state. Although this enzyme is a substrate for protein kinase A in vitro, the incubation with Bt_2-cAMP did not lead to a further incorporation of phosphate into the enzyme (Fig. 5). This observation resembles results from our earlier studies analyzing the phosphorylation of phosphofructokinase-1 in hepatocytes from fed and starved rats. In those experiments the degree of phosphorylation of protein kinase A was clearly determined by the metabolic state rather than by the activation of the protein kinase A (37).

In summary we show here for the first time that effects on carbohydrate metabolism induced by Zn^{2+}, Cd^{2+}, or Pb^{2+} in the medium are accompanied by an intracellular increase in free zinc and that glucagon or cAMP drastically reduce the free zinc concentration. Our observation that zinc counteracts the effect of cAMP on gluconeogenesis as catalyzed by protein kinase A is in line with the concept that Zn^{2+} plays a regulatory role in signal transduction. For instance, zinc has been shown to induce the translocation of protein kinase C to membranes that will convert the enzyme into a more active form due to binding of zinc to the cytosine-rich domains required for the formation of the phorbol ester binding site (38, 39). In Chinese hamster ovary cells overexpressing protein kinase C, phorbol ester activation led to an increased phosphorylation of the insulin receptor. This serinethreonine phosphorylation by excessive protein kinase C seems to inhibit insulin-stimulated responses (40).

We have shown earlier that the 11.5-kDa ZnBP binds in vitro Zn^{2+}-dependently to several enzymes of the carbohydrate metabolism, including phosphofructokinase-1 and fructose-1,6-bisphosphatase. In liver these two regulatory enzymes are both covalently modified by phosphorylation but without measurable effects on the kinetic of these enzymes. It is tempting to speculate that zinc ions could transduce certain effects of glucagon and/or insulin, permitting a crosstalk between these, in many respects, antagonistic hormones. However, there is evidence that insulin-like effects of Zn^{2+} on adipocytes occur by a mechanism unrelated to insulin (9). But in contrast to adipocytes, hepatocytes have the capacity for both glycolysis and gluconeogenesis and have a high zinc content and specific types of phosphorylatable enzymes, so that the regulation mechanisms by insulin in hepatocytes may differ from those in adipocytes.

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