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The Effect of Calcium Ions on the Glycolytic Activity of Ehrlich Ascites-Tumour Cells

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1. Added Ca^{2+} inhibited lactate formation from sugar phosphates by intact Ehrlich ascites-tumour cells. Lactate formation from glucose by these cells was unaffected by added Ca^{2+}. 2. The Ca^{2+} inhibition of lactate formation by intact cells occurred in the extracellular medium. 3. Intact ascites-tumour cells did not take up Ca^{2+} *in vitro*. 4. Glycolysis of sugar phosphates by cell extracts as well as pyruvate formation from 3-phosphoglycerate and phosphoenolpyruvate was inhibited by Ca^{2+}. 5. It was concluded that Ca^{2+} inhibited the pyruvate-kinase (EC 2.7.1.40) reaction. Further, Ca^{2+} inhibition of pyruvate kinase could be correlated with the overall inhibition of glycolysis. 6. Concentrations of Ca^{2+} usually present in Krebs–Ringer buffers, inhibited glycolysis and pyruvate-kinase activity by approx. 50%. 7. The inhibition of glycolysis by added Ca^{2+} could be partially reversed by K^{+} and completely reversed by Mg^{2+} or by stoicheiometric amounts of EDTA. 8. The hypothesis is advanced that the inability of tumour cells to take up Ca^{2+} is a factor contributing towards their high rate of glycolysis.

It has been observed that Ca^{2+} inhibits the glycolytic activity of tissue slices (Dickens & Greville, 1935; McIlwain, 1952) and of cell extracts (Geiger, 1940). However, little is known about the mechanism of this cationic inhibition. The present paper reports a detailed investigation into the mechanism of this inhibition by using a glycolytic system prepared from Ehrlich ascites-tumour cells. It is concluded that added Ca^{2+} inhibits glycolysis in extracts of Ehrlich ascites-tumour cells by inhibiting the pyruvate-kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) reaction.

A preliminary report of part of this work has appeared (Bygrave & Macdonald, 1963).

MATERIALS AND METHODS

Ascites-tumour cells. The Ehrlich ascites tumour used in the current studies was a hyperdiploid-strain ascites carcinoma supplied by Dr E. L. French, Animal Health Research Laboratories, Parkville, Victoria, Australia, and obtained originally from Dr T. S. Hauschka, Buffalo, N.Y., U.S.A. The ascites tumour was propagated by weekly intraperitoneal inoculations of 0·2ml. of ascitic fluid into adult mice of a mixed laboratory strain. The tumours were harvested about 10 days after inoculation, by which time significant growth had occurred.

The ascites-tumour cells were harvested and prepared at room temperature. The animals were killed by cervical fracture and the ascitic fluid was allowed to drain from an abdominal incision into a centrifuge tube containing about 0·3ml. of heparin to minimize coagulation. This procedure was carried out as rapidly as possible. The mouse ascitic fluid usually contained relatively few blood cells and most of these remained suspended after centrifuging at 800*g* for 1min. The supernatant was discarded. The unwashed cells were suspended in about 5vol. of 0·9% NaCl and then centrifuged briefly (800*g* for 15sec.) to remove any solid material that may have been present. The cell suspension was then centrifuged at 1700*g* for 3min. and the packed cells were washed twice more with 0·9% NaCl to remove ascitic fluid. After a final washing with Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, the volume of the packed cells was noted and a 20% (w/v) suspension made in the bicarbonate buffer. At this stage the tumour cells were chilled in an ice bath. The cell suspensions had an average dry weight of 30mg./ml. when corrected for salt content.

An MSE ultrasonic cell disintegrator (60w, frequency 20kcyc./sec., 1·5A, with a titanium stub) was used to disrupt the ascites cells. Good results were obtained when 10–12ml. of the chilled cell suspension was exposed for 15sec. in a conical centrifuge tube surrounded by ice. Examination of the resulting dispersion (referred to below as the 'homogenate') with a phase-contrast microscope showed that over 90% of the cells were disrupted.

The soluble supernatant fraction of the cell was prepared from the homogenate by centrifuging at 97000*g* for 50min. in a Spinco model L refrigerated centrifuge.

Chemicals. The following (as their sodium or potassium salts unless otherwise noted) were products of the Sigma Chemical Co., St Louis, Mo., U.S.A.: NADH, glucose

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6-phosphate, glucose 1-phosphate, fructose 1,6-diphosphate, ribose 5-phosphate, 3-phosphoglyceric acid and phosphoenolpyruvate. Fructose 6-phosphate (barium salt), a product of Schwartz Laboratories, New York, N.Y., U.S.A., was converted into the sodium salt by treatment with Na_2SO_4. D-Glucose was obtained from the British Drug Houses Ltd., Poole, Dorset. Metal salts and EDTA were commercial products (A.R. grade) obtained from the British Drug Houses Ltd. Carrier-free $^{45}CaCl_2$ of specific activity 12mc/ml. was a product of Oak Ridge National Laboratories, Oak Ridge, Tenn., U.S.A.

Oxidation of NADH. This was followed by measuring the change in extinction at 340mμ in a Unicam SP. 500 spectrophotometer. Reaction mixtures were contained in silica cuvettes having a 1cm. light-path. The temperature was 25°.

Incubation medium and measurement of glycolytic activity. Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4 (NaCl, 118mM; KCl, 4·7mM; KH_2PO_4, 1·2mM; $MgSO_4$, 1·2mM; $NaHCO_3$, 25mM; Umbreit, Burris & Stauffer, 1964), comprised the basic ionic medium. The reaction medium contained 2·0ml. of Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 1·0ml. of enzyme suspension and $CaCl_2$ (2·8mM) (where indicated). These were all added to the main compartment of conventional Warburg flasks. The substrate (5·6mM) was added to the side arm. The total volume was 3·5ml. Before incubation at 37° flasks were gassed with N_2+CO_2 (95:5) for 7min. The incubation was terminated by transferring the incubation mixture to an equal volume of 10% (w/v) trichloroacetic acid contained in a graduated centrifuge tube. The mixture was centrifuged at 1700***g*** for 3min. and portions of the water-clear supernatant were assayed for products.

Quantitative determinations of lactate (Barker & Summerson, 1941), pyruvate (Friedemann & Haugen, 1943), aldopentose (Mejbaum, 1939), ketopentose (Dickens & Williamson, 1956) and ketoheptose (Dische, 1953) were carried out as described in the references cited. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of ^{45}Ca. Portions (1·0ml.) of the tumour-cell suspension were added to a 10ml. conical flask containing Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, $CaCl_2$ (labelled with $^{45}Ca^{2+}$) and other components in a final volume of 3·5ml. The flask was stoppered and shaken at 37° for the times shown. After incubation, the assay solutions were transferred to 10ml. glass centrifuge tubes and centrifuged (at room temperature) at 1700***g*** for 5min. to pack the cells. A 0·5ml. portion of the supernatant liquid was transferred into an aluminium planchet (2·0cm. diam.), 10 drops of an asbestos suspension were added to assist spreading and the sample was then evaporated to dryness under an infra-red lamp. No correction for self-absorption was made owing to the infinitely thin film that was formed. The radioactivity was measured with an end-window Geiger–Müller tube. The amount of Ca^{2+} taken up by the cells was determined by measuring the loss of radioactivity from the supernatant liquid.

RESULTS

The effect of Ca^{2+} on lactate production from various sugar phosphates and from glucose by ascites-tumour cells is shown in Table 1. Significant amounts of lactate were formed when glucose 6-phosphate, fructose 1,6-diphosphate and ribose 5-phosphate were incubated with ascites cells. In each case added Ca^{2+} (final concn. 2·8mM) markedly decreased the production of lactate. However, added Ca^{2+} in the concentration range 1·4–8·4mM did not inhibit lactate production when glucose was incubated with ascites cells.

Table 1. *Effect of* Ca^{2+} *on lactate production by intact ascites-tumour cells incubated with various sugar phosphates and with glucose*

Each flask contained: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0ml.; sugar phosphate, 10μmoles, or glucose, 5μmoles; Ca^{2+}, where indicated, 10μmoles; ascites-cell suspension, 1·0ml. The final volume was 3·5ml. Incubation was for 120min. (for sugar phosphates) or for 25min. (for glucose) at 37°.

Expt.	Substrate	Added Ca^{2+}	Total lactate formed (μmoles)	Lactate formed from substrate (μmoles)
1	Glucose	–	6·8	5·5
	6-phosphate	+	4·9	3·6
	Ribose	–	4·5	3·2
	5-phosphate	+	2·3	1·0
	None	–	1·3	—
2	Glucose	–	6·7	5·2
	6-phosphate	+	3·1	1·6
	Fructose	–	3·7	2·2
	1,6-diphosphate	+	1·7	0·2
	None	–	1·5	—
3	Glucose	–	10·0	8·8
		+	10·1	8·9
	None	–	1·2	—

The following observations are relevant in this context: (*a*) glucose is both rapidly taken up and rapidly metabolized by Ehrlich ascites-tumour cells (Crane, Field & Cori, 1957); (*b*) mammalian cells are generally considered to be impermeable to phosphorylated sugars (Wu, 1959); (*c*) Ehrlich ascites-tumour cells can release enzymes of glycolysis and of the pentose phosphate cycle to metabolize ribose 5-phosphate extracellularly (Wu, 1959). In view of these observations it was considered that Ca^{2+} prevented the formation of lactate from sugar phosphates by inhibiting one or more of the released enzymes concerned with their conversion into lactate. It was further considered that Ca^{2+} did not inhibit lactate formation from glucose because this process takes place inside the cell where the Ca^{2+} may be unable to penetrate. Therefore the ability of the ascites cells to take up Ca^{2+} *in vitro* was investigated.

Incubation of $^{45}Ca^{2+}$ *with intact ascites-tumour cells* in vitro.

Table 2 shows the results of experiments in which $^{45}Ca^{2+}$ was incubated with ascites cells in the presence and absence of glucose. No uptake of $^{45}Ca^{2+}$ was observed during the 90min. of incubation with glucose absent or present. Even with higher concentrations of ascites cells in the medium no loss of radioactivity from the supernatant could be demonstrated.

Calcium transport in preparations from the small intestine is, at least in part, an active process that requires energy from cell metabolism and is stimulated by vitamin D_2 and parathyroid hormone (De Luca, Engstrom & Rasmussen, 1962). Further, Thomason & Schofield (1959) have shown that ascites-tumour cells can take up Ca^{2+} *in vivo* in the presence of ascitic fluid. It therefore appeared possible that Ca^{2+} uptake by the ascites cells *in vitro* might be stimulated by the addition of ATP, vitamin D_2, parathyroid hormone or ascitic fluid to the incubation medium and as a result produce an inhibition of glycolysis (glucose to lactate). None of these factors when added to the incubation medium affected glycolysis either in the presence or absence of added Ca^{2+}. Uptake of $^{45}Ca^{2+}$ was similarly unaffected. On the basis of this and of the results of the experiment described in Table 2 it was concluded that no uptake of Ca^{2+} by the ascites-tumour cells was taking place *in vitro*.

Mechanism of the Ca^{2+} *inhibition of glycolysis by extracts of ascites-tumour cells.*

The experiments described so far suggested that the added Ca^{2+} was affecting the conversion of sugar phosphate into lactate by intact ascites-tumour cells by one or both of the following: (*a*) by inhibiting the activity of one or more of the released enzymes concerned with the conversion of sugar phosphates into lactate; (*b*) by influencing the release of glycolytic and of pentose phosphate-cycle enzymes from the cells, thus influencing the extracellular enzymic conversion of sugar phosphates into lactate.

That the Ca^{2+} was in a large part directly inhibiting the activity of one or more of the released enzymes concerned in the breakdown of sugar phosphate to lactate is shown in the following experiment. Ascites cells were preincubated in buffer and subsequently removed by centrifuging. Portions of the resulting supernatant were then incubated with ribose 5-phosphate and the production of lactate was determined. The results of this experiment (Table 3) confirm the observation by Wu (1959) that ascites-tumour cells metabolize ribose 5-phosphate outside the cells, and also show that when Ca^{2+} is present in the incubation medium lactate production is markedly decreased.

In view of these observations, the first of the two possible modes of action of Ca^{2+} mentioned above was investigated. The supernatant fraction prepared from ascites cell-free extracts was used to eliminate any effects that Ca^{2+} might have on cell-membrane permeability.

Effect of Ca^{2+} *on the utilization of glycolytic substrates by extracts of ascites-tumour cells*

The effect of Ca^{2+} on the glycolysis of various sugar phosphates is shown in Table 4. The glycolysis of hexose monophosphates, ribose 5-phosphate and

Table 2. *Incubation of* $^{45}Ca^{2+}$ *with intact ascites-tumour cells* in vitro

Ascites tumour cells (30mg. dry wt.) were incubated with: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0ml.; Ca^{2+} (containing $^{45}Ca^{2+}$), 10μmoles; glucose, where indicated, 20μmoles. The final volume was 3·5ml. Incubation was at 37°. For other details see the Materials and Methods section.

Incubation time (min.)	Radioactivity in supernatant after incubation (counts/min./ml.)	
	Glucose absent	Glucose present
0	1980	1990
15	1914	1923
30	2042	2020
60	2000	2015
90	2084	2000

Table 3. *Effect of* Ca^{2+} *on the extracellular production of lactate by intact ascites-tumour cells incubated with ribose 5-phosphate*

Ascites cells (4g. wet wt.) were preincubated anaerobically with 7ml. of Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, containing NADH (7μmoles) at 37° for 75min. The cells were removed by centrifuging and 1·0ml. portions of the supernatant transferred to Warburg flasks containing: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0ml.; ribose 5-phosphate, 30μmoles; Ca^{2+}, where indicated, 10μmoles; NADH, 1·4μmoles; ATP, 20μmoles. The final volume was 3·5ml. Incubation was for 60min. at 37°. Each value (expressed as μmoles of lactate produced/5mg. of protein/hr.) is the mean obtained from the contents of duplicate flasks.

Substrate	Added Ca^{2+}	Total lactate formed (μmoles)	Lactate formed from ribose 5-phosphate (μmoles)
Ribose	−	6·5	3·2
5-phosphate	+	4·3	1·0
None	−	3·3	—

Table 4. *Effect of* Ca^{2+} *on the glycolysis of various substrates by the supernatant fraction of ascites-tumour cells*

Each flask contained: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0 ml.; Ca^{2+}, where indicated, 10 μmoles; substrate, 20 μmoles; supernatant fraction (8·0 mg. of protein), 1·0 ml. The final volume was 3·5 ml. Incubation was for 80 min. at 37°. Values are expressed in μmoles.

Substrate	Added Ca^{2+}	Lactate formed	Pyruvate formed	Aldopentose formed	Ketopentose formed	Ketoheptose formed
Glucose 6-phosphate	−	11·6	5·0	3·3	1·4	1·6
	+	6·3	1·1	4·4	1·9	2·5
Ribose 5-phosphate	−	8·1	2·7	2·6	0·7	0·9
	+	3·9	0·9	4·6	1·0	1·0
Glucose 1-phosphate	−	11·0	4·4	3·3	1·7	1·6
	+	7·2	1·0	4·2	2·2	1·7
Fructose 6-phosphate	−	4·2	0·8	1·5	0·5	0·1
	+	2·2	0·3	2·0	0·7	0·3
Fructose 1,6-diphosphate	−	5·7	0·8	1·8	0·4	0·2
	+	3·6	0	2·0	0·6	0·2
None	−	1·0	0	1·7	0·5	0·2

Table 5. *Effect of* Ca^{2+} *on pyruvate formation from 3-phosphoglycerate and from phosphoenolpyruvate*

Each flask contained: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0 ml.; 3-phosphoglycerate or phosphoenolpyruvate, 10 μmoles; Ca^{2+}, where indicated, 10 μ moles supernatant fraction, 1·0 ml. The final volume was 3·5 ml. Incubation was for 30 min. at 37°.

Substrate	Added Ca^{2+}	Pyruvate formed (μmoles)
3-Phosphoglycerate	−	6·5
	+	3·9
Phosphoenolpyruvate	−	7·0
	+	4·9
None	−	0·1

Table 6. *Inhibition of pyruvate-kinase activity by added* Ca^{2+}

Each cuvette contained: tris–HCl buffer, pH 7·4, 100 μmoles; 3-phosphoglycerate, 2 μmoles, or phosphoenolpyruvate, 1 μmole (added last); ADP, 0·2 μmole; NADH, 0·125 μmole; Mg^{2+}, 10 μmoles; K^{+}, 75 μmoles; Ca^{2+}, where indicated, 10 μmoles; supernatant fraction, 0·1 ml. The final volume was 2·0 ml. Incubation was at 25°. The blank cuvette contained the same mixture without added substrate.

Substrate	Added Ca^{2+}	Reaction velocity (ΔE_{340}/min.)
3-Phosphoglycerate	−	0·124
	+	0·060
Phosphoenolpyruvate	−	0·080
	+	0·031

fructose 1,6-diphosphate was inhibited by about 50% by added Ca^{2+}. Quantitative determinations on the incubation mixtures after removal of protein showed that decreased concentrations (60–80%) of pyruvate, but increased concentrations (30–45%) of ketopentose, ketoheptose and aldopentose, were present in those flasks that contained added Ca^{2+}.

Since the glycolytic sequence and pentose phosphate cycle possess a common metabolic pathway below and including glyceraldehyde 3-phosphate, these results suggested that Ca^{2+} was inhibiting one or more of the enzymic reactions localized in the glycolytic pathway below glyceraldehyde 3-phosphate.

Inhibition by Ca^{2+} *of pyruvate-kinase activity in ascites-tumour cells*

The effect of Ca^{2+} on the formation of pyruvate from 3-phosphoglycerate and from phosphoenolpyruvate was determined. The presence of Ca^{2+} in the incubation medium inhibited the formation of pyruvate by about 50% from each substrate (Table 5). This was consistent with findings by Boyer, Lardy & Phillips (1943) and Kachmar & Boyer (1953), with the purified mammalian muscle enzyme, that the added Ca^{2+} was inhibiting the activity of pyruvate kinase.

A spectrophotometric assay (Bücher & Pfleiderer, 1955) was used to confirm that the added Ca^{2+} was inhibiting pyruvate kinase in the tumour-cell supernatant. The oxidation of NADH, coupled with the reduction of the resulting pyruvate by lactate dehydrogenase, was followed. As shown in Table 6, with 2 mM-3-phosphoglycerate and with 1 mM-phosphoenolpyruvate, a concentration of 5 mM-Ca^{2+} inhibited the oxidation of NADH in each case by about 60%.

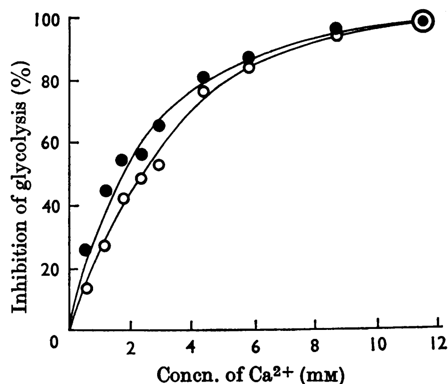

Fig. 1. Effect of Ca^{2+} concentration on glycolysis by the supernatant fraction and by the homogenate prepared from ascites-tumour cells. Each flask contained: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0 ml.; glucose 6-phosphate, 10 μmoles; supernatant fraction or homogenate as indicated, 1·0 ml. (containing 8 and 12 mg. of protein respectively). The Ca^{2+} concentration was varied as indicated. The final volume was 3·5 ml. Incubation was for 30 min. at 37°. Each plot (corrected for endogenous activity) is the mean value obtained from two individual experiments. ○, Supernatant fraction; ●, homogenate.

Kinetic studies on the Ca^{2+} *inhibition of glycolysis*

Effect of Ca^{2+} *concentration on glycolysis of ascites-cell extracts.* The influence of Ca^{2+} concentration on glycolysis (glucose 6-phosphate to lactate) is shown in Fig. 1. The degree of inhibition varies according to the concentration of Ca^{2+} in the medium. Glycolysis is inhibited by about 50% when the concentration of Ca^{2+} in the medium is 2·5 mM.

Effect of Ca^{2+} *concentration on pyruvate-kinase activity.* Table 7 shows the effect of Ca^{2+} concentration on the activity of pyruvate kinase. Increasing the Ca^{2+} concentration decreases the activity of pyruvate kinase. At a Ca^{2+} concentration of 2·5 mM pyruvate-kinase activity is inhibited by about 51%. Almost complete inhibition of enzyme activity is obtained with a concentration of 10 mM-Ca^{2+}. Results from Fig. 1 (showing the effect of Ca^{2+} concentration on glycolysis) are included for comparison. As the Ca^{2+} concentration is increased in both systems the degree of inhibition of glycolysis correlates very closely with the degree of inhibition of pyruvate kinase.

Effect of Ca^{2+} *on the time-course of lactate production.* The rate of glycolysis in the absence and presence of added Ca^{2+} is linear during the initial 15 min. of the reaction but then gradually falls off to a minimal rate (Fig. 2). The presence of Ca^{2+} inhibits the rate of glycolysis by about 70% over the initial linear portion of the curve. After about 35 min. of incubation the degree of inhibition of glycolysis by added Ca^{2+} approaches a constant value of approx. 40%.

Table 7. *Effect of* Ca^{2+} *concentration on the activity of pyruvate kinase from the supernatant fraction of ascites-tumour cells*

Each cuvette contained: tris–HCl buffer, pH 7·4, 100 μmoles; phosphoenolpyruvate (added last), 2·5 μmoles; ADP, 0·2 μmole; NADH, 0·125 μmole; Mg^{2+}, 10 μmoles; K^{+}, 75 μmoles; supernatant fraction, 0·1 ml. The Ca^{2+} concentration was varied as indicated. The final volume was 2·0 ml. Incubation was at 25°.

Concn. of Ca^{2+} (mM)	Reaction velocity (ΔE_{340}/min.)	Inhibition of pyruvate-kinase activity (%)	Inhibition of supernatant glycolysis (%) (data from Fig. 1)
0	0·095	0	0
0·1	0·092	3	4
0·2	0·085	10	8
1·0	0·065	32	27
2·5	0·048	51	53
5·0	0·035	63	63
10·0	0·016	83	97
15·0	0·007	93	100

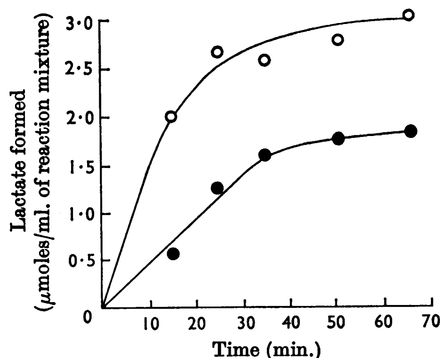

Fig. 2. Effect of Ca^{2+} on the rate of glycolysis by the supernatant fraction of ascites-tumour cells. Each flask contained: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0 ml.; glucose 6-phosphate, 20 μmoles; Ca^{2+}, where indicated, 10 μmoles; supernatant fraction, 1·0 ml. (containing 8 mg. of protein). The final volume was 3·5 ml. Incubation was at 37°. Each plot (corrected for endogenous activity) is the mean value obtained from two individual experiments. ○, Ca^{2+} absent; ●, Ca^{2+} present.

Effect of added Mg^{2+} *and* K^{+} *on the inhibition of glycolysis by* Ca^{2+}. Addition of Mg^{2+} to the incubation medium decreases the inhibitory action of Ca^{2+} on glycolysis (Table 8). The antagonism by Mg^{2+} is maximal in the concentration range

Table 8. *Effect of* Mg^{2+} *and* K^+ *on the inhibition of glycolysis by added* Ca^{2+}

Each flask contained: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0 ml.; glucose 6-phosphate, 10 μmoles; Ca^{2+}, where indicated, 10 μmoles; supernatant fraction, 1·0 ml. (containing 8 mg. of protein); K^+ and Mg^{2+}, as indicated. The final volume was 3·5 ml. Incubation was for 20 min. (Expt. 1) or for 30 min. (Expt. 2) at 37°.

Expt. no.	Added Ca^{2+}	Metal ion added	Final concn. of metal ion (mM)	Lactate formed (μmoles)
1	+	None	—	1·6
	+	Mg^{2+}	2·8	2·9
	+	K^+	2·8	2·1
	−	Mg^{2+}	2·8	4·4
	−	K^+	2·8	4·6
2	+	None	—	3·0
	+	Mg^{2+}	1·4	3·8
	+	Mg^{2+}	2·8	4·5
	+	Mg^{2+}	4·2	4·4
	+	Mg^{2+}	5·6	4·5
	+	Mg^{2+}	8·4	4·0
	−	Mg^{2+}	4·2	4·2

2·8–5·6 mM. Higher concentrations of Mg^{2+} are themselves inhibitory. Added K^+ could partially offset the inhibition of glycolysis by Ca^{2+}.

Reversibility of the Ca^{2+} *inhibition of glycolysis by EDTA.* Preliminary studies showed that the inhibition of glycolysis by added Ca^{2+} is decreased maximally and almost completely when stoicheiometric quantities of EDTA and Ca^{2+} are present together in the incubation medium. In the experiment described in Fig. 3, glycolysis was allowed to proceed for 10 min. before EDTA (10 μmoles) was added to the medium containing added Ca^{2+} (10 μmoles). Addition of EDTA in this manner also reverses the inhibitory effect of Ca^{2+} on glycolysis. Addition of Ca^{2+} (after the elapse of a similar time-interval) to the incubation medium initially free of added Ca^{2+} decreases the rate of lactate formation (Fig. 3).

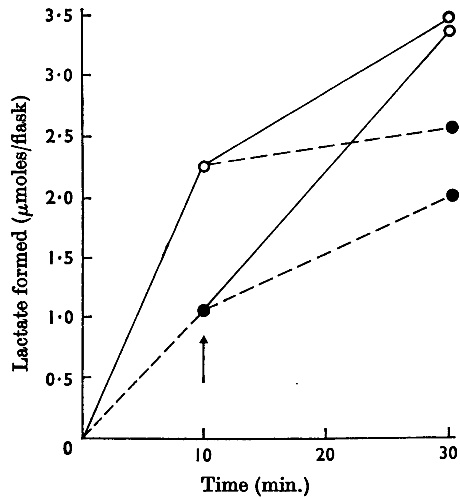

Fig. 3. Demonstration of the reversal of the Ca^{2+} inhibition of glycolysis by addition of EDTA. Each flask contained: Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7·4, 2·0 ml.; glucose 6-phosphate, 10 μmoles; Ca^{2+}, where indicated, 10 μmoles; supernatant fraction, 1·0 ml. (containing 7·5 mg. of protein). EDTA (10 μmoles) or Ca^{2+} (10 μmoles) was added, where indicated, at the arrow. The final volume was 3·5 ml. Incubation was at 37°. To minimize glycolytic activity before incubation, flasks were surrounded by ice during addition of the reaction components and during gassing. ●, Ca^{2+} present; ○, Ca^{2+} absent or EDTA present.

DISCUSSION

It has been reported that Ca^{2+} can inhibit glycolysis in brain slices (Dickens & Greville, 1935; McIlwain, 1952) and can also profoundly influence the pattern of ketogenesis in liver slices (Mellanby & Williamson, 1963). Although it is not known if these reported ionic effects reflect an actual uptake of Ca^{2+} by the cells, Judah & Ahmed (1963) have demonstrated a net uptake of Ca^{2+} when liver slices were incubated in a normal Ringer solution. It is noteworthy that Krebs (1950) observed that Ca^{2+} could influence the respiratory rate of brain-tissue slices more than that of liver slices.

In the present studies, on the other hand, it was found that added Ca^{2+} does not affect glycolysis (glucose to lactate) by suspensions of ascites-tumour cells. Experiments with $^{45}Ca^{2+}$ showed that uptake of Ca^{2+} by ascites-tumour cells does not take place *in vitro*. Nor was it possible, on the assumption that uptake of Ca^{2+} by the whole cells would cause an inhibition of glycolysis, to stimulate Ca^{2+} uptake by factors known to influence transport of Ca^{2+} in other biological systems. Thomason & Schofield (1959), however, have observed Ca^{2+}-exchange by ascites-tumour cells *in vivo*. It therefore seems possible that the apparent differences between uptake of Ca^{2+} *in vivo* and *in vitro* by ascites-tumour cells might involve a factor or factors intrinsically located in the ascitic fluid. Preliminary experiments have indicated that Ca^{2+} uptake by mitochondria prepared from the ascites-tumour cells is significantly less than that taken up by mitochondria from rat kidney. It remains to be established that, for this tumour, the failure of the whole cells to take up Ca^{2+} is a reflection of the failure of the mitochondria to take up Ca^{2+} (see Bartley, Davies & Krebs, 1954). It is of note that cations other than Ca^{2+} are taken up by Ehrlich ascites-tumour cells

in vitro. With experimental conditions similar to those used in the present studies, active transport of Na^+ and K^+ by suspensions of these cells has been demonstrated (Grobecker, Kromphardt, Mariani & Heinz, 1963; Hempling, 1962).

Ca^{2+} has been shown to inhibit the glycolytic activity of brain-cortex slices (Dickens & Greville, 1935; McIlwain, 1952) cell-free extracts of brain (Geiger, 1940) and nucleated red cells (Ashwell & Dische, 1950). In none of these studies, however, was the mechanism of the Ca^{2+} inhibition established.

The present studies show that addition of low concentrations of Ca^{2+} to the incubation medium exert an inhibitory effect on the anaerobic glycolytic activity of extracts prepared from Ehrlich ascites-tumour cells. The concentration of Ca^{2+} required to produce 50% inhibition of glycolysis was about 2·5mM (Fig. 1), about the same concentration as is usually present in Krebs–Ringer bicarbonate and phosphate buffers (Umbreit *et al.* 1964). The reversal of the Ca^{2+} inhibition of glycolysis by EDTA (Fig. 3) suggested that the action of Ca^{2+} did not involve irreversible inactivation of any of the glycolytic enzymes.

Inhibition of the pyruvate-kinase reaction of Ehrlich ascites cells by added Ca^{2+} is consistent with previous findings with purified pyruvate kinase from rat muscle (Boyer *et al.* 1943) and rabbit muscle (Kachmar & Boyer, 1953). Kachmar & Boyer (1953) showed that Ca^{2+} antagonized the stimulatory effect of K^+, an obligatory activator of this enzyme. In view of the well-known antagonism between Ca^{2+} and Mg^{2+} and between Ca^{2+} and K^+ (Heilbrunn, 1947) it was not surprising that added Mg^{2+} or K^+ reversed the inhibition of glycolysis by Ca^{2+} (Table 8). Since glycolytic activity and the activity of pyruvate kinase were inhibited to similar degrees over a wide range of Ca^{2+} concentrations (compare Fig. 1 and Table 7) it is concluded that inhibition of the pyruvate-kinase reaction by Ca^{2+} accounts in large part for the overall inhibition of glycolysis in extracts of Ehrlich ascites-tumour cells.

Other sites of inhibition by Ca^{2+}, however, cannot be overlooked. Other enzymes of the glycolytic sequence, concerned with phosphoryl group transfer, could be inhibited by Ca^{2+}. Further, phosphoenolpyruvate-hydratase (EC 4.2.1.11) activity in lysed erythrocytes (Boszormenyi-Nagy, 1955), yeast (Malmstrom, 1955) and *Escherichia coli* (Utter & Werkman, 1942) is known to be inhibited by Ca^{2+}.

The action of Ca^{2+} on intact cells is probably more complex. Since the normal impermeability of the mammalian cell is believed to depend on the presence of calcium (Shanes, 1958), it is conceivable that Ca^{2+}, by inducing changes in the permeability of the ascites-cell membrane, could inhibit the release of glycolytic and of pentose phosphate-cycle enzymes (Table 3).

Possible significance of the decreased calcium content of tumour cells. Tumour cells in general are considered by a number of workers to have lower calcium concentrations than normal cells (see Cowdry, 1953; Abercrombie & Ambrose, 1962). Further, it has been suggested (Coman, 1953, 1960) that tumour cells have little or no ability to bind calcium and that the invasiveness of cancer cells depends largely on a loss of adhesiveness that is associated with, if not due to, local calcium deficiency (see also Abercrombie & Ambrose, 1962).

In the present proposal, Ca^{2+} is implicated as a controlling factor in glycolysis. It is suggested that the concentration of intracellular Ca^{2+} in the normal cell is sufficiently high to maintain a normal glycolytic rate. However, because of the apparent decreased capacity for Ca^{2+} uptake the concentration of Ca^{2+} in the tumour cell is lower than in the normal cell. This decreased concentration of Ca^{2+} in the tumour cell produces an effect on glycolysis similar to that of releasing an inhibition. As a consequence glycolysis proceeds at a rate faster than that characteristic of an average normal cell.

The following lines of evidence support this hypothesis. Tumour cells in general (*a*) have a decreased calcium content (Cowdry, 1953), (*b*) are unable to take up or bind calcium (Coman, 1953; Thomason & Schofield, 1959; Table 2) and (*c*) possess unusually high rates of both aerobic and anaerobic glycolysis (Warburg, 1956; Kit & Griffin, 1958). Also, as the present work with cell extracts shows, if Ca^{2+} is allowed to come into contact with the enzymes and cofactors of the glycolytic sequence, it does inhibit the rate of glycolysis. That the activity of pyruvate kinase could play a key role in the regulation of gluconeogenesis in rat liver was advanced by Krebs & Eggleston (1965). Finally, in view of the well-known relationship between the Ca^{2+} and inorganic phosphate, it is noteworthy with regard to the present proposal that Wu & Racker (1959) have considered that an altered regulation of inorganic phosphate transport in tumour cells may contribute to their high rate of glycolysis.

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