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Calcium Uptake and Release by Rat Liver Mitochondria in the Presence of Rat Liver Cytosol or the Components of Cytosol

Juzu Hayati Arshad and Eric S. Holdsworth*
Biochemistry Department, University of Tasmania, Hobart, Tasmania 7000, Australia

Summary. A study has been made of factors present in rat liver cytosol that might regulate the calcium content of mitochondria. A cytosol preparation containing all the components of molecular weight greater than 10,000 prevented uptake and caused early release of accumulated calcium. These effects were due to free long-chain fatty acids and their coenzyme A derivatives present in the cytosol, and these inhibitory effects were controlled by inclusion of Mg^{2+}, carnitine, and adenosine triphosphate at physiological levels in the incubation medium. Palmitoyl carnitine was a good substrate for calcium uptake and did not cause release of calcium from mitochondria. A specific fatty acid-binding protein was found in cytosol which may be the intracellular transport protein for fatty acids.

It is widely believed that the concentration of Ca in cell cytosol is maintained at a level of 1 μM or less by the activity of mitochondria (reviewed by [7]). This belief is based largely on observations on the uptake of Ca^{2+} by isolated mitochondria suspended in isotonic sucrose or KCl solutions [23, 24, 31] and the observations that a number of cellular reactions are sensitive to Ca^{2+} in the μM range. No reliable methods have been found that can measure cytosolic Ca^{2+} concentration. If the redistribution of Ca that takes place during homogenation is prevented by carrying out the homogenation at 28° in 0.25 M sucrose or at 2° with sucrose in the presence of 10 mM ruthenium red, it was found that intestinal mucosal cells had an extramitochondrial Ca between 20–30% of the total cell Ca [16]. Similar amounts of extramitochondrial Ca were found in rat hepatocytes [13]. Cytosol prepared from intestinal mucosal cells caused a rapid release of Ca from intestinal mitochondria [16]. In heart mitochondria it was shown that Na^+ caused the efflux of Ca [10]. Since no similar effects have been found for liver, the work reported here was an attempt to study whether there are substances in rat liver cytosol that control Ca movements.

* To whom reprint requests should be made.

Materials and Methods

Preparation of Mitochondria

Male hooded Wistar rats of approx 180–200 g weight were decapitated and livers removed into ice-cold medium containing 0.25 M sucrose, 2.5 mM HEPES, 1 mM $MgCl_2$ adjusted to pH 7.4 with Tris base. The livers were minced and homogenized at a ratio of 1 g liver to 7 ml of this medium with the addition of 1 mM EGTA and 0.1% wt/vol bovine serum albumin (BSA). The homogenate was layered over 0.5 M sucrose and centrifuged at $1,500 \times g$ for 10 min. The layer above 0.5 M sucrose was removed and layered over 0.34 M sucrose and centrifuged at $1,500 \times g$ for 10 min. The layer above 0.34 M sucrose was removed and centrifuged at $15,000 \times g$ for 10 min. The sedimented mitochondria were suspended in the above sucrose medium (without EGTA or BSA) and centrifuged at $12,000 \times g$ for 10 min, and the washing was repeated. The washed mitochondria were suspended in 0.25 M sucrose, protein estimated, then BSA added to 0.01% wt/vol. The mitochondrial suspension was stored as a stock suspension at 0°. Before each experiment the ratio of phosphate esterified to O_2 consumption was measured as described by Chance and Williams [8] using succinate as substrate. The P/O ratios obtained were in the range 1.6–1.8.

Preparation of Rat Liver Cytosol

Rat livers were perfused *in vivo* with 25–30 ml oxygenated 0.25 M sucrose at 35° then washed and cooled in ice-cold 0.25 M sucrose. The livers were crushed by passing through a Harvard Tissue Press (Harvard Apparatus Co., Inc., Millis, Mass.) then homogenized at ratio 1 g to 7 ml 0.25 M sucrose. The homogenate was centrifuged first at $40,000 \times g$ for 20 min, then $250,000 \times g$ for 60 min. The supernatant was concentrated over a PM10 DIAFLO ultrafilter (Amincon. Corp., Lexington, Mass.) to a weight corresponding to the original weight of liver. It was stored at −15 °C. Analyses were made of the supernatant before and after being concentrated.

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Chromatography of Rat Liver Cytosol

Cytosol was labeled with either ^3H (9:10^3H) palmitic acid 10 μCi, 30 μmol per g cytosol alone, or ^3H palmitic acid plus ^{45}Ca, 2 μCi, 0.4 μmol per g. The labeled cytosol was fractionated on columns 2 × 50 cm of one of the following molecular sieve gels: AcA44 (LKB Produkter, A.B. Stockholm), Biogel P30 (BIORAD Laboratories, Richmond, Calif.) or Sephacryl 300 superfine (Pharmacia, North Ryde, N.S.W., Australia). Columns were eluted with 50 mM Tris-HCl at pH 7.6 and the absorption of the eluate scanned at 280 nm for protein. Fractions (~10 ml) were collected for measurement of radioactivity. Fractions containing radioactivity were concentrated to small volumes by ultrafiltration (PM10 DIAFLO membrane). The different columns were calibrated with proteins of known molecular weight.

Calcium Uptake and Release by Mitochondria Studied by Means of a Ca-Electrode

The Radiometer calcium-sensitive electrode type F2112 Ca was used with a "non-flow" reference electrode (Ionode, type RNFM, Selby Scientific Ltd., Hobart, Tasmania). A special high performance amplifier was built around a LH0022 National semiconductor (National Semiconductor Corp., Santa Clara, Calif.). The characteristics of this amplifier were: thermal drift less than 5 μV/°C, impedance 10^{12} Ω, and capacitance 4 pF. These characteristics are required to ensure stability and sensitivity in the μM range of Ca^{2+} concentration [33]. The Ca-electrode was specific for ionic Ca and gave no response to any of the constituents in the incubation medium or to the substrates used. The amplifier was connected to a 10 mV recorder. The Ca-electrode, its reference electrode, and an O_2-electrode (type 506 Titron Pty. Ltd., Braeside, Victoria, Australia) were placed into a 6-ml vessel which was maintained at 25°. Water-saturated O_2 at 25° was directed across the surface of the fluid in the 6-ml vessel. The O_2 supply was adjusted to maintain O_2 saturation of the fluid as monitored by the O_2-electrode. The log concentration range was 0.5×10^{-6} M to 10^{-4} M Ca^{2+}. The electrodes were standardized with Ca buffers [27]. To a final volume of 5 ml of 0.25 M sucrose, 2.5 mM HEPES, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 72 mM KCl, pH 7.4, were added the various substrates and the reaction started by the addition of mitochondria (9–30 mg mitochondrial protein).

The water and sucrose solutions for this work were freed from contaminating Ca by filtration through columns of Chelex-100, 100–200 mesh (BIO-RAD, Richmond, Calif.). This method allows continuous monitoring of ionic Ca in the medium and makes it possible to measure rapid changes in Ca concentration. The disadvantage is that only one test situation can be investigated at any one time.

Calcium Uptake and Release by Mitochondria Using the Radioisotope Technique

^{45}Ca was used to monitor the movement of Ca^{2+} into and out of mitochondria. The incubation medium was that described above. Substrates were added to a final volume of 5 ml. Known amounts of mitochondria were added and preincubated 1 min before the addition of ^{45}Ca and in different experiments amounts of 100 to 1,000 nmol $CaCl_2$ (0.1 μCi) were used. A flow of O_2 was maintained over the surface of the fluid. Incubation was at 25° and 0.5 ml samples were removed at various time intervals into icecold quench medium containing 1 mM EGTA and 2 μM ruthenium red [27]. The mitochondria were separated by centrifugation, and ^{45}Ca in the supernatant was measured by liquid scintillation counting. This quench technique allows measurement of Ca^{2+} taken into the mitochondrial matrix as distinct from that bound to the outer membrane [27] and has the advantage that many tests can be carried out simultaneously.

Estimation of Adenosine 5′-Nucleotides

Samples of 1 ml incubation mixture, containing both medium and mitochondria were acidified with 0.2 ml 5 N perchloric acid, centrifuged and neutralized with KOH. For estimation of nucleotides in mitochondria, the 1-ml sample was layered over 0.4 ml Silicone oil (Silicone Fluid MS 704, Midland Silicone Ltd., Barry, Glamorgan) and centrifuged at 12,000 × *g* through the oil into a lower layer of 0.25 ml 1 N perchloric acid in 12.5% wt/vol sucrose. This lower layer was removed and neutralized with KOH. ATP was determined enzymatically [21], as was AMP and ADP [19].

Removal of Free Fatty Acid from Protein

Free fatty acids were removed from rat albumin using activated charcoal [9]. A column 0.4 × 5 cm containing 600 mg Florisil (100–200 mesh) was prepared and 6 ml rat liver cytosol filtered through the column. One passage served to remove 50% of the free fatty acids.

Analytical Methods

Protein was determined by the Biuret method [14], inorganic phosphate by the method of Taussky and Shorr [32], and potassium was estimated by means of the Beckman Model 105 flame photometer. For the determination of Mg and Ca, the tissues were digested with 1 ml conc. HNO_3 and 0.5 ml 60% wt/vol $HClO_4$, taken to dryness and dissolved in distilled water. A Varian Techtron Model 1000 atomic absorption spectrophotometer was used to determine Mg at 254.2 nm and Ca at 422.7 nm in these solutions. Calcium was also determined by the reaction with Arsenoazo III [15] using the purified dye [20]. Free fatty acids were estimated by a colorimetric method [30].

The enzymes, substrates, and bovine albumin (electrophoretically pure and free from fatty acids) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Rat serum albumin was Pentax fraction V (Miles Laboratories, Inc., Ekhart, Indiana). Goat Antiserum to rat albumin was from U.S. Biochemical Corp. (Cleveland, Ohio) and the donkey antiserum to goat antibody was from Wellcome Reagents, Ltd. (Beckenahm, England).

Immunoelectrophoresis was performed in model 2117 Multiphor apparatus in accordance with the LKB handbook (LKB Produkter, Bromme, Sweden) and using agarose electrophoresis film (type 470–10–000 Corning Universal, Palo Alto, Calif.).

Results

Properties of Rat Liver Cytosol

The cytosol preparation before concentration had the composition shown in Table 1. The values for protein, Mg^{2+}, P_i are approx half those obtained by organic solvent separation of mitochondria and cytosol [31] and suggest that only 50% of the cells were broken by the method of preparation.

Uptake of Ca^{2+} by Isolated Rat Liver Mitochondria

Figure 1 shows that in 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i, 1 mM $MgCl_2$, 72 mM KCl, and 2 mM Na succinate, mitochondria rapidly reduced the concentration of Ca^{2+} to a value corresponding to approx 0.5 μM (calibrated with Ca-nitrilotriacetic acid buffers [28]. When 2 ml ultrafiltrate of rat liver cytosol (equivalent to 0.3 g wet liver) was included in the medium, a similar recording was observed (not shown). When concentrated rat cytosol, 1.5 ml (equivalent to 1.5 g wet liver), was included in the medium, approximately 66% of the added Ca^{2+} was taken up rapidly by mitochondria; however, this was almost immediately released, as shown in Fig. 2. The inhibitory effect of cytosol on Ca^{2+} could also be shown when studying the uptake of ^{45}Ca by the radiometric method (results not shown). The rat cytosol did not contribute significantly to the total Ca^{2+} concentration. If 25 mM D-L-carnitine hydrochloride was added to the medium containing concentrated rat liver cytosol, then Ca^{2+} was taken up rapidly by mitochondria, but the mitochondria began to release this Ca^{2+} gradually after 4 min as shown in Fig. 3. Further additions of carnitine did not cause reuptake of Ca^{2+}, but addition of 0.2 μmol BSA did cause reuptake (Fig. 3). In other experiments defatted rat albumin aided reten-

Table 1. The concentration of some components of the 250,000 × *g* rat liver supernatant which may influence mitochondrial calcium transport

Cytosolic components	Per kg wet wt liver
Protein	
Cytosolic protein	40 g
Cations	
Ca^{2+}	150 μg atoms
Mg^{2+}	440 μg atoms
K^+	75×10^3 μg atoms
Anions	
Inorganic phosphate	2.5 mmol
Adenine nucleotides	
ATP	0.108 mmol
ADP	0.156 mmol
AMP	0.108 mmol
Fatty acids	0.4 mmol

Rat livers were perfused with 0.25 M sucrose and sytosol cytosol prepared and analyzed as described in the Methods section

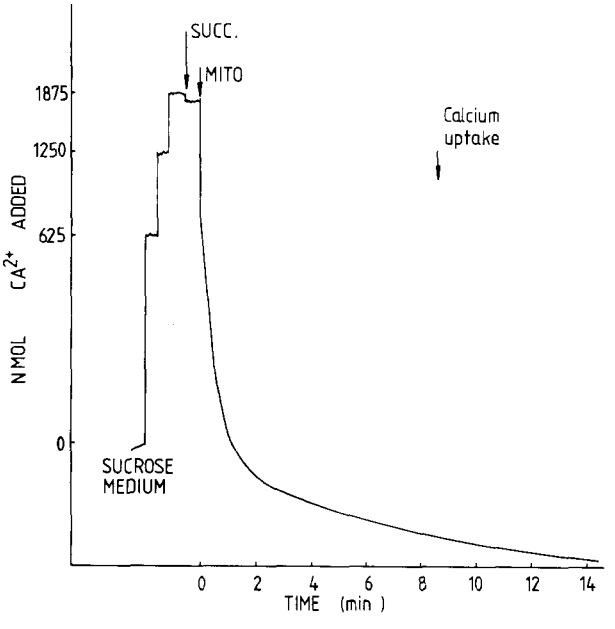

Fig. 1. Uptake of Ca^{2+} by rat liver mitochondria in the presence of sucrose medium, using a Ca^{2+}-sensitive electrode. Rat liver mitochondria, 20 mg, were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES Tris at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl and 2 mM Na succinate at 25 °C. The Ca^{2+} added is shown on a log scale. The electrode was also calibrated with Ca-NTA buffers

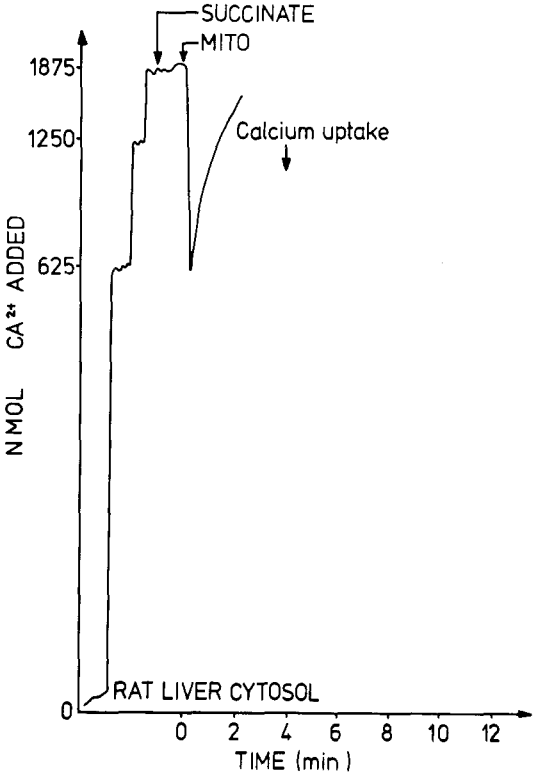

Fig. 2. Uptake of Ca^{2+} by rat liver mitochondria in the presence of concentrated rat liver cytosol using a Ca^{2+} sensitive electrode. Rat liver mitochondria, 20 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES Tris at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 1.5 ml concentrated rat cytosol (equivalent to 1.5 g wet wt liver) and 2 mM Na succinate of 25 °C. The Ca^{2+} added is shown on a log scale. The electrode was also calibrated with Ca-NTA buffers

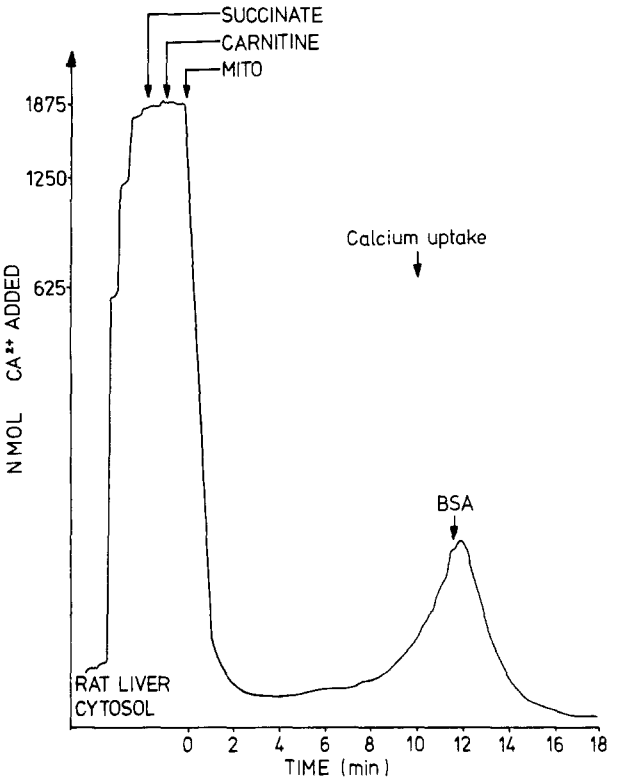

Fig. 3. Uptake of Ca^{2+} by rat liver mitochondria in the presence of concentrated rat liver cytosol plus 25 mM carnitine HCl using a Ca^{2+}-sensitive electrode. Rat liver mitochondria, 20 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES Tris at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 1.5 ml concentrated rat cytosol (equivalent to 1.5 g wet wt liver), 25 mM carnitine HCl and 2 mM Na succinate at 25 °C. The Ca^{2+} added is shown on a log scale. The electrode was also calibrated with Ca-NTA buffers. 0.2 μmol BSA was added after Ca^{2+} release

tion of Ca^{2+} by mitochondria. Carnitine alone at a conc. 25 mM had no effect on uptake or release of Ca^{2+} in the medium used in Fig. 1, i.e., in the absence of cytosol.

When 50 mM carnitine, 1 mM ATP, and 2 mM succinate were present together with rat liver cytosol, ^{45}Ca was taken up, and this ^{45}Ca was retained for 35 min which is longer than the control experiment (Fig. 4). If the fatty acid content of the concentrated cytosol was reduced from 200 to 100 μm by filtration through florisil, then ^{45}Ca was retained for up to 55 min in the presence of succinate and ATP. In these experiments larger than physiological amounts of carnitine were used, but this concentration itself had no effect on Ca^{2+} uptake or release. Later experiments used carnitine at 1 or 2 mM.

The Effect of Palmitic Acid, Palmitoyl CoA, Palmitoyl Carnitine on Ca^{2+} Uptake or Release

For these experiments the uptake of ^{45}Ca by the radiometric method was used so that several substances could be tested at the same time. Figure 5 shows that with 2 mM β hydroxybutyrate and 1 mM ATP present as energy sources, 70 nmol Ca^{2+} were taken up per mg mitochondrial protein. The presence of 250 μM palmitate inhibited this uptake. This amount of palmitate is comparable to the estimated free fatty acid content of rat liver cytosol.

Although 10 μM palmitate did not inhibit ^{45}Ca uptake, it did cause release of ^{45}Ca before the control

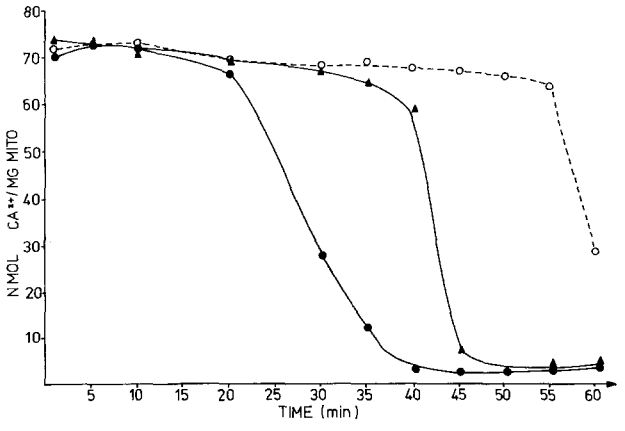

Fig. 4. Mitochondrial calcium uptake in the presence of rat liver cytosol or florisil-treated rat liver cytosol by the radioassay technique. Rat liver mitochondria, 29 mg, were added to 1.5 ml rat liver cytosol in a total of 5 ml medium; 1.6 mM ATP, 1.6 mM Na succinate, 50 mM carnitine HCl and 2500 nmol Ca^{2+}. Control (●); 1.5 ml concentrated rat liver cytosol (▲); 1.5 ml florisil-treated concentrated rat liver cytosol (○)

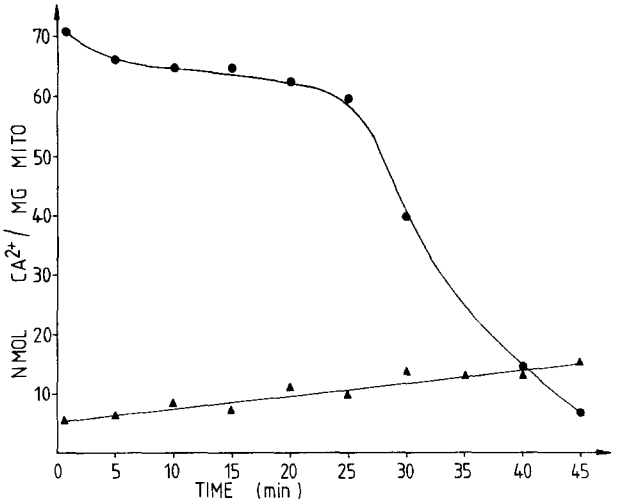

Fig. 5. Mitochondrial calcium uptake in the presence of potassium palmitate by the radioassay technique. Rat liver mitochondria, 4.6 mg, were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 1 mM ATP, 2 mM β-hydroxybutyrate, and 400 nmol Ca^{2+}. Control (●); +250 μm potassium palmitate (▲)

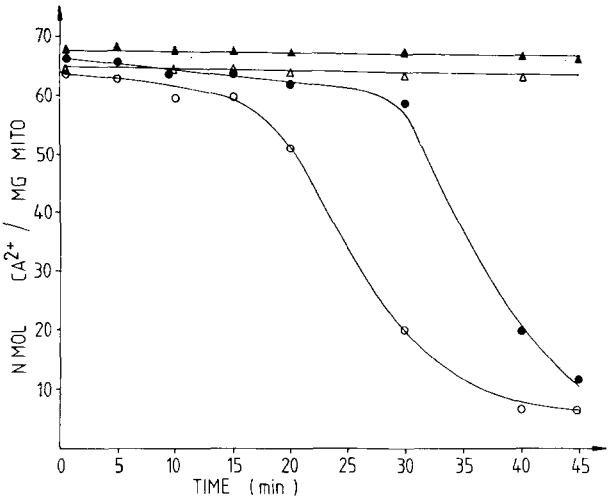

Fig. 6. The effects of bovine serum albumin or carnitine HCl on calcium efflux from mitochondria induced by potassium palmitate by the radioassay technique. Rat liver mitochondria, 4.8 mg, were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i, at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 1 mM ATP, 2 mM β-hydroxybutyrate and 400 nmol Ca^{2+}. Control (●); +10 μm potassium palmitate (○); +10 μM potassium palmitate +1 mM carnitine HCl (△); 10 μM potassium palmitate +10 mg BSA (▲)

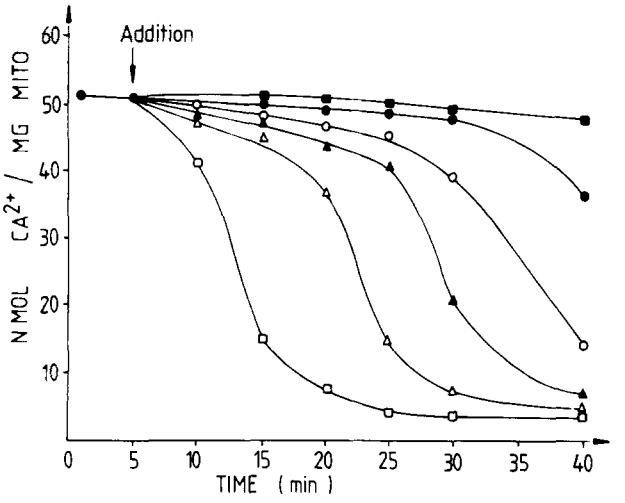

Fig. 7. The effects of potassium palmitate, palmitoylcarnitine and varying concentrations of palmitoyl CoA on calcium transport in rat liver mitochondria by the radioassay technique. Rat liver mitochondria, 6 mg, were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 1 mM ATP, 2 mM β-hydroxybutyrate and 400 nmol Ca^{2+}. The following additions were made at 5 min: control (●), +20 μM potassium palmitate (○), +5 μM palmitoyl CoA (▲), +10 μM palmitoyl CoA (△), +20 μM palmitoyl CoA (□), +10 μM palmitoyl-carnitine (■)

Table 2. Change in total adenosine 5′-nucleotide concentrations during incubation with different substrates

Substrate	Incubation time 10 min				Incubation time 30 min			
	Ca^{2+} in mitochondria (nmol/mg)	Total adenine nucleotide[a] (μmole)			Ca^{2+} in mitochondria (nmol/mg)	Total adenine nucleotide[a] (μmole)		
		ATP	AMP	ADP		ATP	AMP	ADP
Control	57	0.66	0.05	0.47	38	0.44	0.19	0.58
10 μM palmitoyl CoA	22	0.58	0.16	0.47	17	0.22	0.39	0.58
10 μM palmitoyl carnitine	59	0.69	0.05	0.47	51	0.50	0.14	0.58
10 μM palmitate plus 1 mM carnitine	59	0.69	0.05	0.47	51	0.50	0.14	0.58
10 μM palmitate plus 10 mg BSA	59	0.69	0.05	0.47	51	0.50	0.14	0.58

Calcium uptake by rat liver mitochondria by the radioassay technique. Rat liver mitochondria, 4.8 mg, were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 2 mM β-hydroxybutyrate, 1 mM ATP and 400 nmol $CaCl_2$. Substrates shown in the table were added to 5 min after Ca^{2+} uptake. Samples of the incubation mixtures were taken at 10 and 30 min for the estimation of total adenine nucleotides.

[a] Total adenine nucleotides (μmol) in the incubation mixtures containing 1 mg mitochondrial protein

mitochondria (Fig. 6). The presence of either 1 mM D-L-carnitine hydrochloride or 30 μM BSA prevented early release of ^{45}Ca, and ^{45}Ca was retained up to 60 min at which time the experiment was terminated (Fig. 6).

As little as 5 μM palmitoyl CoA caused ^{45}Ca release earlier than the control, and this effect was greater than 20 μM palmitate (Fig. 7). Palmitoyl carnitine, 10 μM, had no releasing effect and, on the contrary, helped the retention of ^{45}Ca (Fig. 7).

Adenine Nucleotide Concentrations during Ca Release

Samples of the incubation medium from the experiment shown in Fig. 7 were examined for adenine nucleotides. Table 2 shows that at 30 min when Ca^{2+}

Table 3. The effect of alteration in adenosine 5′-nucleotide concentrations on Ca release

Substrates added	Ca^{2+} in mitochondria (nmol/mg)	Total adenine nucleotide[a] (μmol)			Ca^{2+} in mitochondria (nmol/mg)	Total adenine nucleotide[a] (μmol)		
		ATP	AMP	ADP		ATP	AMP	ADP
		Sampling time = 15 min				Sampling time = 45 min		
Control	47	0.37	0.06	0.22	10	0.09	0.19	0.22
Expt. A								
+ PEP + pyruvate kinase	47	0.64	0.02	0.09	36	0.53	0.01	0.13
		Sampling time = 17 min				Sampling time = 32 min		
Control	60	0.51	0.17	0.33	22	0.29	0.30	0.33
Expt. B								
+ PEP	57	0.41	0.24	0.30	12	0.13	0.47	0.30
		Sampling time = 15 min				Sampling time = 30 min		
Control	59	0.49	0.13	0.30	55	0.40	0.21	0.33
Expt. C								
+ glucose + hexokinase	51	0.35	0.15	0.47	8	0.12	0.49	0.37

Ca^{2+} uptake by rat liver mitochondria by the radioassay technique. Rat liver mitochondria, 5.8, 5.0, and 5.4 mg (for expts. A, B, and C, respectively) were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 2 mM β OH butyrate, 1 mM ATP and 400 nmol $CaCl_2$. 1 ml of incubation mixtures was taken at various time intervals for the estimation of total adenine nucleotides and for the determination of Ca^{2+}.
Experiment A – 4 mM PEP and 15 U pyruvate kinase were added; Experiment B – 1 mM PEP was added; Experiment C – 5 mM glucose and 15 U hexokinase were added.

[a] Total adenine nucleotides (μmol) in the incubation mixtures containing 1 mg mitochondrial protein

Table 4. Mitochondrial adenosine 5′-nucleotide concentrations during Ca^{2+} uptake and release

Substrates added	Ca^{2+} in mitochondria (nmol/mg)	Sampling time = 17 min Total adenine nucleotides per mg mitochondrial protein			Ca^{2+} in mitochondria (nmol/mg)	Sampling time = 32 min Total adenine nucleotides per mg mitochondrial protein		
		ATP	AMP	ADP		ATP	AMP	ADP
Control	60	0.012	0.012	0.03	22	0.018	0.015	0.042
Expt. B								
+ 1 mM PEP	57	0.012	0.018	0.03	12	0.009	0.021	0.036

Ca^{2+} uptake rat liver mitochondria by the radioassay technique. Rat liver mitochondria 5 mg were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 2 mM β OH butyrate, 1 mM ATP and 400 nmol $^{45}CaCl_2$. 1 ml of incubation medium was taken at 17 and 32 min for the estimation of mitochondrial adenosine 5′ nucleotides and for determination of Ca^{2+}.

was released by palmitoyl CoA the ATP concentration had decreased and AMP increased. Palmitoyl carnitine and BSA both helped Ca^{2+} retention and maintained the ATP concentration. If ATP concentrations were decreased by the inclusion of glucose plus hexokinase in the incubation mixture, then an early release of Ca^{2+} occurred but uptake was not affected since there was 1 mM ATP present in the early stages of the experiment (Table 3). Regeneration of ATP during the incubation helped retention of Ca^{2+} for longer than the control experiment (Table 3). The results in Table 3 were total adenine nucleotides in medium plus mitochondria. The mitochondria contained only 5–10% of these nucleotides and changed in the same direction as the medium when Ca^{2+} was released (Table 4).

ATP Requirement for Ca^{2+} Uptake

The Ca-ion electrode was used to study the influence of adenine nucleotides on the initial uptake of Ca^{2+} by mitochondria. Incubation of the mitochondria with Ca^{2+} in the absence of substrates led to only

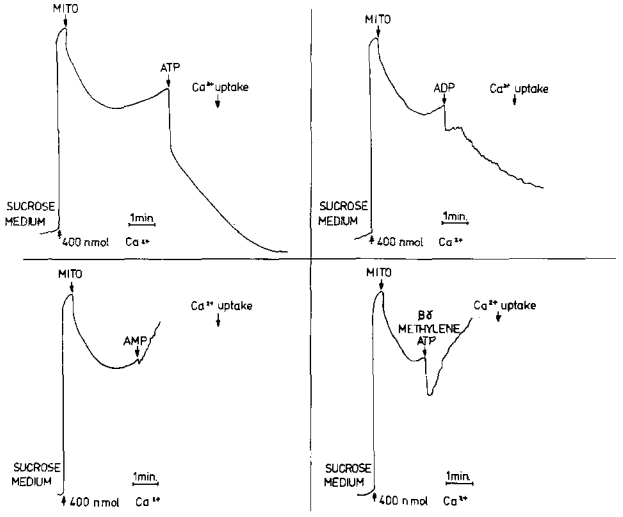

Fig. 8. ATP requirement for Ca^{2+} uptake studied by means of the Ca^{2+}-sensitive electrode. Rat liver mitochondria, 4 mg, were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES Tris at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl and 400 nmol Ca^{2+}. The Ca^{2+} added is shown on a log scale. 1 mM final concentration of ATP, $\beta\gamma$ methylene ATP, ADP and AMP was added to the respective experiments at the point of Ca^{2+} release

a small accumulation of Ca^{2+}, and when the endogenous substrates were exhausted Ca^{2+} release commenced. At this point adenine nucleotides were added. Figure 8 shows that addition of ATP caused an immediate decrease in ionic Ca due to combination with ATP and this was followed by a decrease due to uptake by the mitochondria. The $\beta\gamma$ methylene analogue of ATP showed the fall in ionic Ca due to combination with the analogue, but Ca^{2+} uptake into mitochondria did not take place. Similar results were obtained with the $\alpha\beta$ methylene analogue. Mitochondria accumulated Ca^{2+} when ADP was the substrate but the rate of uptake was slow compared to that with ATP. The ability of ADP to chelate Ca^{2+}, 150 nmol, was less than that of ATP which chelated 275 nmol of the total of 400 nmol Ca^{2+} added. The chelating ability of AMP for Ca^{2+} was small and AMP did not support Ca^{2+} uptake.

The Presence of a Fatty-Acid Binding Protein in Rat Liver Cytosol

Rat liver cytosol was prepared from livers perfused with sucrose to remove blood. The cytosol had been concentrated by membrane filtration and contained molecules greater than 10,000. ^{45}Ca and ^{3}H palmitate were added and the labeled cytosol was fractionated on an AcA44 column. A broad peak of protein was eluted between 30 to 120 ml (Fig. 9) and at 140 ml a 280 nm peak of adenine nucleotides was eluted. A small peak of ^{45}Ca-binding protein of mol wt approximately 400,000 eluted at 40 ml and free ^{45}Ca eluted from 150 ml onwards with a peak at 240 ml. Two peaks of ^{3}H palmitate occurred, one at 75 ml with a mol wt between 35,000 to 60,000. Most palmitate eluted at 130 ml, which corresponded to a mol wt less than 13,000 (cytochrome C reference) and was probably free palmitate. A column of Biogel P30 established that the protein-bound palmitate eluted at the front while the free palmitate eluted later. The protein-bound palmitate fraction was rechromatographed on Sephacryl 300 and was eluted at a position giving a calculated mol wt between 30,000–50,000. This fraction was concentrated to 2 ml and 0.1 ml goat antiserum to rat serum albumin was added and

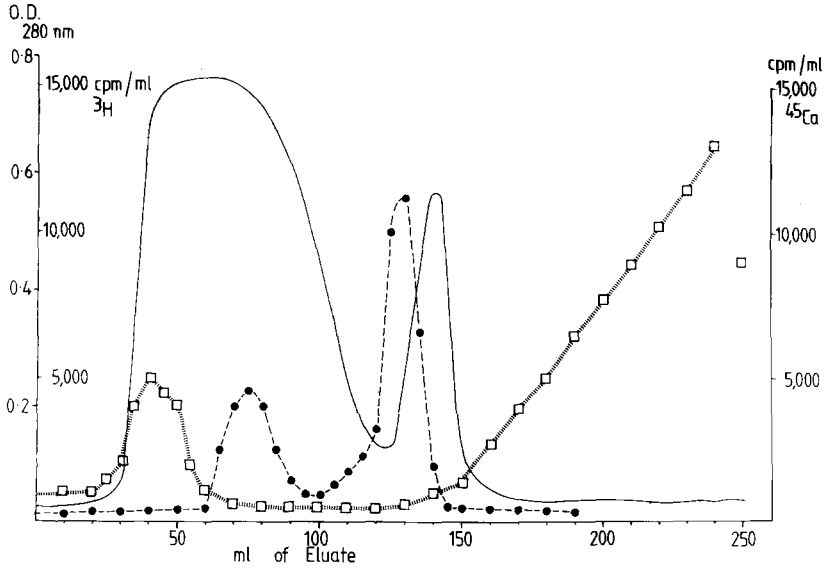

Fig. 9. Gel-chromatography of rat liver cytosol. A column of AcA44 2 × 50 cm was used and eluted with 50 mM Tris-HCl at pH 7.4. 5 g rat liver cytosol was labeled with 50 μCi (150 μmol) ^{3}H palmitic acid plus 10 μCi (2 μmol) $^{45}CaCl_2$ and passed through the column. ^{45}Ca (□) and ^{3}H (●) were counted by a triple-channel scintillation counter. The solid line was a continuous monitoring of absorption at 280 nm

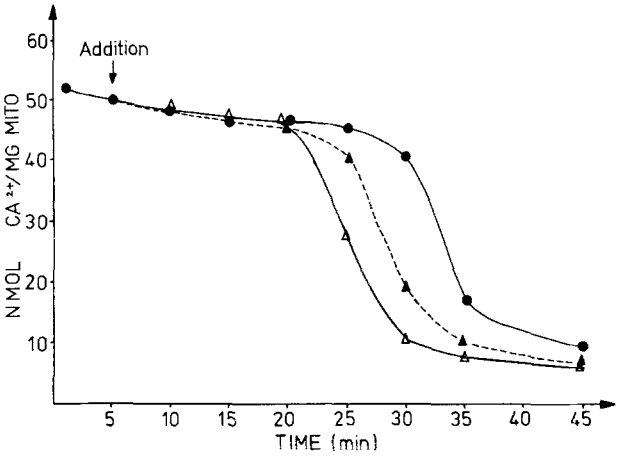

Fig. 10. Mitochondrial Ca^{2+} movement in the presence of ^3H-palmitate binding protein isolated from rat liver cytosol studied by means of the radioassay technique. Rat liver mitochondria, 6.4 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES at Tris pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 2 mM hydroxybutyrate, 1 mM ATP and 400 nmol Ca^{2+}. Additions of 50 µl (equivalent to 0.25 g wet wt liver) ^3H-palmitate binding protein fractions were made at 5 min. (*Note:* the ^3H-palmitate binding protein fractions were obtained from fractionation of the conc. rat cytosol on Sephacryl column.) Control (●); +50 µl ^3H-palmitate binding protein (△); +50 µl florisil-treated ^3H-palmitate binding protein (▲)

Table 5. The influence of Mg^{2+} on the uptake and release of Ca^{2+} by rat liver mitochondria

	natoms of Ca^{2+} or Mg^{2+} per mg mitochondria protein							
	5 min		15 min		25 min		40 min	
	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
No Mg^{2+}	51	31	49	30	16	18	1	15
1 mM Mg^{2+}	51	53	50	51	38	48	7	32
3 mM Mg^{2+}	51	63	56	63	45	62	26	54

Ca^{2+} uptake by rat liver mitochondria was by the radioassay technique. Rat liver mitochondria, 16 mg, was added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 72 mM KCl, 1 mM ATP, 2 mM β-hydroxybutyrate and 1000 nmol $CaCl_2$. Either no Mg^{2+}, 1 mM or 3 mM $MgCl_2$ was added to the incubation medium. Mitochondria pellets for Mg^{2+} estimation were obtained by centrifugation of 0.5-ml samples obtained at various time intervals through a layer of silicone oil. The silicone and supernatant layers were removed and the side of the centrifugation tube cleaned with cotton wool to remove any Mg^{2+} contamination. Mg^{2+} was estimated in the ashed pellets by atomic absorption spectrophotometry.

left at 2° for 16 hr. Donkey antiserum to goat serum, 0.1 ml in 1 ml 0.15-M NaCl solution was added, left for 12 hr at 2°, then centrifuged at 12,000 × *g* for 5 min. This double antibody precipitation would remove any rat albumin from the solution, but it did not precipitate protein-bound ^3H palmitate. The bound palmitate was also examined by immuno-electrophoresis, and although there were several protein bands in the fraction from the Sephacryl 300 column none was rat albumin and the ^3H in the fraction was at a position different from rat albumin as shown by counting 2-mm portions of the gel. The effect of the fatty acid-binding protein on mitochondrial Ca transport was examined by means of the radiometric method. As shown in Fig. 10, the binding protein (saturated with ^3H palmitate) added after Ca uptake caused release of Ca earlier than the control. Partial removal of palmitate by florisil treatment allowed Ca to be retained for a further 5 min.

The Influence of Mg^{2+} on the Uptake and Release of Ca^{2+} by Rat Liver Mitochondria Studied by the Radiometric Assay

As can be seen from Table 5, rat liver mitochondria contained 31 natoms Mg^{2+} per mg mitochondrial protein and were able to accumulate extra Mg^{2+} when incubated in the presence of ATP and β hydroxybutyrate as energy sources. Although extra Mg^{2+} did not increase or inhibit Ca^{2+} uptake, the presence of Mg^{2+} did help the retention of Ca^{2+} by mitochondria.

Discussion

The object of the investigation was to study factors that might be present in rat liver cytosol and which could regulate the uptake or release of Ca^{2+} by mitochondria. For this reason the media we used contained 1 mM Mg^{2+}, 2 mM P_i and 72 mM K^+, amounts similar to those expected in cytosol. The media were stirred and oxygenated with a stream of O_2 over the surface to ensure that the system did not become anaerobic, which can cause Ca^{2+} release from mitochondria [2]. Although Mg^{2+} was not needed in the medium to obtain maximum Ca^{2+} uptake, the presence of Mg^{2+} caused the retention of Ca^{2+} for a longer period. The isolated mitochondria contained Mg^{2+} and were able to accumulate further Mg^{2+} (Table 5). The Mg^{2+} content of mitochondria agrees with that previously reported [5]. Ca^{2+} was stated to cause a rapid efflux of Mg^{2+} from rat liver mitochondria, but in those experiments the medium did not contain either P_i or a source of energy [4] and may not reflect the physiological situation. Similarly in a test system containing succinate plus rotenone

but not ATP or P_i it was reported that Mg^{2+} decreased the uptake of Ca^{2+} by rat liver mitochondria [1]. The cytosol contained 150 μg atoms of Ca per kg wet liver. This is in agreement with an extramitochondrial Ca component forming 20–30% of the total Ca [13, 16]. The concentration of ionic Ca^{2+} in cells is believed to be in the μM range [7]. If the total soluble Ca in cells was 0.15–0.4 mM, based on the above analyis, then in the presence of 1–2 mM Mg^{2+}, the ionic Ca^{2+} concentration would be reduced to the μM range due to the presence of 6 mM ATP present in cytosol [29]. The stability constants for the Mg^{2+} and Ca^{2+} complexes with ATP vary considerably in different media and are profoundly affected by the presence of Na^+ and K^+ ions [25]. The extrapolated values of the constants under physiological conditions were 15×10^3 M^{-1} for $MgATP^{2-}$ and 6.6×10^3M^{-1} for $CaATP^{2-}$ [25]. Therefore, at physiological concentrations of Mg^{2+} and ATP the Ca^{2+} would largely be complexed to ATP and thus the ionic Ca^{2+} would be in the μM range. It has previously been suggested that ATP is an excellent Ca-buffer in the μM range [27]. In the medium used in our experiments, with 1 mM Mg^{2+} and 1 mM ATP, 275 ng atoms of Ca^{2+} of the 400 ng atoms Ca^{2+} added, were immediately complexed by ATP (Fig. 8) and the mitochondria then accumulated Ca^{2+} until the concentration reached the 0.5–1.0 μM range.

As produced by us, the cytosol preparation contained approximately half the concentration of K^+, Mg^{2+}, P_i and protein in cytosol prepared by methods designed to find the concentration of metabolites in cytosol [30]. The concentrated cytosol preparation contained all the materials above 10,000 mol wt plus 0.25 M sucrose. This cytosol preparation prevented the uptake of Ca^2 by mitochondria. Substances of less than 10,000 mol wt would be left in the concentrate at only $^1/_7$ of the concentration present in the original homogenate. One such substance would be carnitine present in amounts of 2 mM in liver [6]. Additions of excess carnitine to the concentrated cytosol preparation enabled mitochondria to accumulate Ca^{2+} (Fig. 3). The preliminary experiments with rat cytosol used unphysiological levels of carnitine (25 mM), but the experiments on the effect of fatty acids and their derivatives were done at 1–2 mM. That a long chain of fatty acid could cause inhibition of Ca^{2+} uptake or early release was shown in Figs. 5 and 6. Palmitoyl CoA was more potent than the free fatty acid in causing release of Ca^{2+}. These results obtained with liver mitochondria resemble those obtained with rat heart mitochondria where palmitoyl CoA slowed the rate of uptake of Ca^{2+} and caused its release [2]. With heart mitochondria, carnitine was able to prevent the effects of palmitoyl CoA, and it was suggested that this was due to the formation of palmitoyl carnitine, which rapidly entered the mitochondria [2]. This suggestion is supported by the results shown in Fig. 7 where palmitoyl carnitine enable mitochondria to retain Ca^{2+} longer than the control mitochondria. This would explain why the presence of ATP and carnitine enable mitochondria to retain their Ca^{2+} for 35 min in the presence of rat cytosol (Fig. 4) and when the fatty acid content of the cytosol was decreased retention was extended to 55 min. The effect of palmitoyl CoA on heart mitochondria has previously been shown not to be uncoupling of mitochondria respiration but was consistent with the idea that palmitoyl CoA was a potent inhibitor of adenine nucleotide translocase [2]. Substances present in cytosol in addition to carnitine would be the adenine nucleotides. The presence of ATP has been shown [11] to enable mitochondria to retain Ca^{2+}. When Ca^{2+} was being released rapidly from rat liver mitochondria, then it was observed that the concentration ATP decreased and that of AMP increased. This was shown in Table 2, where palmitoyl CoA caused a marked rise in AMP and a fall in both ATP and ADP concentrations. During the large changes in nucleotide concentration in the medium, the intramitochondrial concentrations of the adenine nucleotides altered only slightly (Table 4). Palmitoyl carnitine caused only a slight increase in AMP and the ATP concentration was greater than in the control mitochondria. The presence of ATP in the extramitochondrial compartment seems necessary to obtain long retention times for Ca^{2+} [11]. If ATP was removed by glucose plus hexokinase, Ca^{2+} was released. When ATP was regenerated by means of phosphoenolpyruvate plus pyruvate kinase then Ca^{2+} was retained (Table 3). The very short retention times and the effect of ageing of mitochondria reported by some authors may be due to lack of ATP or ADP in the external medium [18]. Many of the results in this paper agree with the theory [23] that whenever the [ATP]/[ADP][P_i] declines in the cytosol then there is a shift to a more oxidized state of NAD in mitochondria, which leads to Ca^{2+} release. It has already been shown the ATP is required for Ca^{2+} uptake in the absence of respiratory substrates [3]. However, this work was done in the presence of inhibitors of the respiratory chain, with or without oligomycin. By means of the Ca electrode it was possible to directly monitor Ca^{2+} uptake without using inhibitors to show that when endogenous substrates were depleted, ATP supported uptake. Analogues of ATP that could not be hydrolyzed [12] were unable to support uptake.

Work concerned with the effect of Ca^{2+} on fatty acid oxidation by mitochondria has shown that Ca stimulates the metabolism of palmitate complexed to

albumin to form ketone bodies and the concentration of Ca^{2+} required for half-maximum stimulation of ketone body production was 82 nmol per mg mitochondrial protein [26]. These workers found that carnitine, ATP, CoA and Mg were required for these effects. The secondary effect of this increased fatty acid oxidation was an increase in the NADH/NAD ratio, and this would result in a longer retention of the accumulated Ca^{2+} [23] which agress with the findings reported in this paper.

Another substance that protected liver mitochondria from the effects of palmitoyl CoA or free fatty acids was bovine serum albumin, and this effect was distinct from the effect of carnitine (Fig. 3). Albumin was able to reverse the release of Ca^{2+}, causing reuptake. Similar results were obtained with heart mitochondria, and the effect was ascribed to binding of fatty acids and lyso-compounds produced by the phospholipases of mitochondria [17]. Since bovine albumin was hardly a physiological substrate for rat liver mitochondria, a search was made of a similar protein in liver. Rat serum albumin, which is synthesized in liver, binds fatty acids and its behavior was similar to bovine serum albumin. When rat livers were thoroughly perfused with 0.25 M sucrose to flush out blood plasma, the cytosol prepared from these livers contained only traces of rat albumin. However, a different protein of mol wt 30,000–50,000 was isolated which combines with free fatty acids. A recent report also found a specific fatty acid binding protein in rat cytosol [28]. Unfortunately, that report concerned a "cytosol" prepared from rat livers containing unknown contamination by blood plasma, and therefore three peaks of fatty acid were found during chromatography of cytosol labeled with radioactive fatty acid, one of which was rat plasma albumin [28]. The fatty-acid binding protein in liver cytosol may be the physiological transport protein for fatty acids in the liver cell. Fatty acids bound to this protein caused early release of Ca^{2+} from mitochondria (Fig. 10), but in the presence of carnitine and ATP, Ca^{2+} would be retained (Figs. 4 and 6).

This study has shown that cytosol from rat liver contains many factors that influence Ca^{2+} uptake and release by mitochondria. The free fatty acid in cytosol prevented Ca^{2+} uptake and caused early release. Mitochondria were protected from the effect of fatty acids by the presence of carnitine and ATP in cytosol. A specific fatty acid binding protein may be the protein that transports fatty acids within the cell. Physiological levels of Mg^{2+} in the presence of ATP and P_i enabled mitochondria to retain Ca^{2+} for longer periods.

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