Submitochondrial Location of Ruthenium Red-Sensitive Calcium-Ion Transport and Evidence for its Enrichment in a Specific Population of Rat Liver Mitochondria

By FYFE L. BYGRAVE, THOMAS P. HEANEY and CHIDAMBARAM RAMACHANDRAN
*Department of Biochemistry, Faculty of Science, Australian National University,
Canberra, A.C.T. 2600, Australia*

(Received 6 March 1978)

1. Seven fractions sedimenting at between 3000 and 120000g-min were prepared from a rat liver homogenate by differential centrifugation in buffered iso-osmotic sucrose. The following measurements were carried out on each of these fractions: Ruthenium Red-sensitive Ca^{2+} transport in the absence and in the presence of P_i as well as in the presence of *N*-ethylmaleimide to prevent P_i cycling, succinate-supported respiration in the absence and in the presence of ADP, the ΔE and $-59 \Delta\text{pH}$ components of the protonmotive force, cytochrome oxidase, uncoupler-stimulated adenosine triphosphatase, α -glycerophosphate dehydrogenase, P_i content and the effect on the 'resting' rate of respiration of repeated additions of a fixed Ca^{2+} concentration. 2. Ca^{2+} transport either in the presence or in the absence of added P_i and in the presence of *N*-ethylmaleimide exhibits significantly higher rates in the fraction sedimenting at 8000g-min. By contrast, respiration in the presence or in the absence of added ADP and the values for ΔE and $-59 \Delta\text{pH}$ were similar in those fractions sedimenting between 4000 and 20000g-min, indicating that the driving force for Ca^{2+} transport was similar in each of these fractions. 3. Experiments designed to determine the capacity of the individual fractions for Ca^{2+} , as measured by the effect of repeated additions of Ca^{2+} on the resting rate of respiration, showed that fraction 2, i.e. that sedimenting at 8000g-min, also exhibited the greatest tolerance towards the uncoupling action of the ion. 4. Of the three enzyme activity profiles, only that of α -glycerophosphate dehydrogenase was similar to that of Ca^{2+} transport. Because previous workers have assigned this enzyme to loci in the inner peripheral membrane [Werner & Neupert (1972) *Eur. J. Biochem.* **25**, 379–396], it is concluded that the Ruthenium Red-sensitive Ca^{2+} -transport system also is located in this domain of the inner membrane. The relation of these findings to the mechanisms of mitochondrial Ca^{2+} transport and the biogenesis of mitochondria is discussed.

Considerable evidence shows that mitochondria of rat liver are heterogeneous both in terms of their anatomical distribution in the intact liver tissue (Loud, 1968; Novikoff & Holtzman, 1970; Van Berkel & Kruijt, 1977) and in terms of the structure, function and biosynthesis of the inner mitochondrial membrane (Gear, 1965; Lusena & Depocas, 1966; Pette, 1966; Pollak & Munn, 1970; Werner & Neupert, 1972; Weiss *et al.*, 1974; Swick *et al.*, 1975; Satav *et al.*, 1976). In the course of studying the biogenesis of mitochondrial Ca^{2+} transport activity in rat liver (Bygrave & Ash, 1977) we observed (G. R. Ash & F. L. Bygrave, unpublished work) that

'heavy' mitochondria, i.e. those isolated at relatively low centrifugal forces in buffered iso-osmotic sucrose, accumulated Ca^{2+} much more rapidly than 'light' mitochondria, an observation we felt warranted further investigation.

We describe in the present paper a procedure with which we prepared, by differential centrifugation, a number of mitochondrial fractions from rat liver. We assessed the ability of each fraction to carry out a range of energy-linked reactions, including Ruthenium Red-sensitive Ca^{2+} transport. The data show that although most of the fractions possess similar values for the ΔE and $-59 \Delta\text{pH}$ components of the protonmotive force and exhibit similar abilities to carry out respiration in the presence and absence of ADP, they differ markedly both in their ability to transport Ca^{2+} as determined by initial-rate measurements and in their susceptibility to the uncoupling action of Ca^{2+} . Other experiments provide evidence that the

Abbreviations used: ATPase, adenosine triphosphatase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; butyl-PBD, 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole; Δp , protonmotive force; ΔE , membrane potential; $-59 \Delta\text{pH}$, transmembrane pH gradient.

distribution of Ca^{2+} -transport activity follows closely that of α -glycerophosphate dehydrogenase activity, but not that of cytochrome oxidase nor uncoupler-stimulated ATPase activity.

These findings raise the possibility that Ruthenium Red-sensitive Ca^{2+} transport may be enriched in specific populations of mitochondria from rat liver and that in these populations the Ca^{2+} -transport system is concentrated in that region of the inner membrane that is in close proximity to the outer membrane, i.e. the inner peripheral membrane (Werner & Neupert, 1972).

Experimental

Female Wistar rats, aged 8–12 weeks and fed *ad libitum* were used in all experiments. They were not starved overnight before killing. Rats were first stunned, and then killed by cervical dislocation.

Fractionation of mitochondria

Livers from two rats were used in all experiments to provide sufficient mitochondria. They were rapidly excised and pooled in ice-cold isolation medium (250 mM - sucrose / 5 mM - Hepes / 0.5 mM-EGTA, adjusted to pH 7.4 with KOH). The combined livers were minced with scissors and washed several times to remove excess blood and fat. All subsequent operations were carried out in the cold-room (4°C). The livers were homogenized in the isolation medium (20%, w/v) with a Thomas C homogenizer, with two up-and-down passages of a motor-driver pestle. The homogenate was then made up to 10% (w/v) and filtered through a double layer of cheesecloth to remove unbroken cells. The volume of the homogenate was then noted, and a measured sample kept for assays. Differential centrifugation was carried out in a Sorvall RC 2-B centrifuge with an SS34 angle rotor. Each centrifugation step was of 10 min duration. The remaining homogenate was centrifuged at 400g. The supernatant from this step was poured into two centrifuge tubes and the pellet was resuspended in isolation medium and re-centrifuged at 300g. The combined supernatants were then centrifuged at 400g. While this step was in progress, the first pellet (designated 'nuclear') was resuspended in about 35 ml of isolation medium and was then kept on ice in the cold-room. The pellet from the 400g step was resuspended in a total of 35 ml of isolation medium, and was then kept on ice in the cold-room. This was designated fraction 1. Similarly, the pellets from the 800, 1200, 2000, 3000 and 12000g centrifugation steps were obtained and designated fractions 2–6 respectively. All fractions were then centrifuged at 14500g and the supernatants discarded. Each fraction was then resuspended in wash medium (250 mM-sucrose/5 mM-Hepes, pH 7.4), and gently

homogenized with one up-and-down stroke of a Thomas C homogenizer. The fractions were centrifuged at 14500g and the pellets resuspended in the minimum possible volume, with three up-and-down strokes of a Thomas AA homogenizer, and then transferred to an Eppendorf tube. The final volume of each fraction was then determined.

Protein estimation

Protein was determined by the biuret method (Szarkowska & Klingenberg, 1963), with bovine serum albumin as standard.

Calcium transport

Ca^{2+} transport was measured at 4°C in a KCl medium by the EGTA–Ruthenium Red quench technique (Reed & Bygrave, 1975a,b). All incubations were performed in a temperature-controlled, water-jacketed incubation vessel, with continuous stirring. The basic medium contained 150 mM-KCl, 5 mM-sodium succinate, 20 mM-Hepes (adjusted to pH 7.4 with KOH). The final volume was 2.0 ml. The effect of phosphate was studied by (a) adding phosphate to a final concentration of 2 mM, and (b) having no added phosphate present. Ca^{2+} transport in the presence of the phosphate-transport inhibitor *N*-ethylmaleimide was also studied (see below). The quench medium contained 0.5 mM-EGTA, 2 μ M-Ruthenium Red and 150 mM-KCl.

Mitochondria (1 mg of protein/ml) were pre-incubated in the reaction medium for 1 min before $^{45}\text{Ca}^{2+}$ (100 nmol of Ca/mg of protein) was added. Samples (100 μ l) were taken from the incubation at intervals of 10 s, and were immediately mixed with 100 μ l of cold (4°C) quench medium contained in Eppendorf tubes. These tubes were then centrifuged for 2 min in an Eppendorf Microfuge (12000g). A 100 μ l sample of the supernatant was added to 10 ml of methylCellosolve scintillant (6 gm of butyl-PBD in 600 ml of toluene and 400 ml of 2-methoxyethanol). The vials were counted for radioactivity on the $^3\text{H}+^{14}\text{C}$ channel of a Packard refrigerated scintillation counter. The total amount of radioactivity added to the incubation was determined by mixing a 100 μ l sample of the incubation medium with 100 μ l of quench medium, and then adding 100 μ l of the uncentrifuged mixture to a vial containing scintillant.

Initial rates of Ca^{2+} transport, expressed as nmol of Ca^{2+} /min per mg of mitochondrial protein, were calculated by extrapolating to 60 s the rate measured during the initial 10 s.

Ca^{2+} transport in the presence of *N*-ethylmaleimide

In these experiments Ca^{2+} concentration in the medium was continuously measured using an ion-sensitive calcium electrode (Radiometer, Copenhagen, Denmark) with a combination pH electrode as

reference (see Bygrave *et al.*, 1977). The incubation medium (4ml, pH 7.4, 25°C) contained 150mM-KCl, 3mM-Hepes, 1 μ M-rotenone, and 200nmol of *N*-ethylmaleimide/mg of protein. Mitochondria (4mg) were added at least 1 min before 20nmol of calcium/mg of protein was added. The reaction was started by the addition of 5mM-sodium succinate.

Respiration

Respiration in the absence and in the presence of 200 μ M-ADP was measured polarographically using a Clark-type oxygen electrode in a medium containing 50mM-KCl, 100mM-sucrose, 15mM-Tris/HCl, 10mM- KH_2PO_4 , 2mM- MgSO_4 , 1mM-EDTA and 10mM-succinate. Mitochondria were added at 1mg/ml. The total volume was 2.4ml and the temperature was 25°C.

Ca^{2+} -stimulated respiration

The influence of repeated additions of Ca^{2+} on the resting rate of respiration was determined by adding 40nmol amounts of Ca^{2+} to the vessel soon after the rate of respiration had returned to the resting rate after a 'calcium jump' (see Rossi & Lehninger, 1964). The basic incubation contained 200mM-sucrose, 25mM-KCl, 10mM-Hepes/KOH, pH 7.4, 1mM-phosphate, 1 μ M-rotenone and 5mM-sodium succinate. Mitochondria were added at 1mg/ml. The temperature was 25°C and the total volume 2.0ml.

Determination of the components of the proton electrochemical gradient

The ΔE and $-59\Delta\text{pH}$ components of the proton-motive force, were determined by the method of Nicholls (1974). The basic incubation medium contained 150mM-LiCl, 3mM-Hepes, 0.5mM-KCl, 1 μ M-rotenone, 0.5 μ M-valinomycin and 5mM-succinate. The indicators [^3H]acetate, [^{14}C]methylamine and $^{86}\text{Rb}^+$ were added to a final concentration of 10 μ M. Then 2min after addition of mitochondria (1mg of protein/ml), 400 μ l samples were taken and filtered. Standards (15 μ l) were added directly to a vial containing 10ml of butyl-PBD/10% Bio-solv. Vials were counted for radioactivity for 10min in a Packard Tri-Carb liquid-scintillation spectrometer, and the results calculated by computer program on a Univac 1100 computer, which included corrections for cross-over and background. A limiting matrix volume of 0.4 μ l/mg of protein was assumed for calculation of the protonmotive force. The components of the protonmotive force are not very sensitive to changes in mitochondrial volume.

Cytochrome *c* oxidase assay

The method used was a modification of that of Tolbert (1974); 75 μ l of 2% Triton X-100 and 1.5ml of 200mM- KH_2PO_4 , pH 7.4, were added to a cuvette.

The volume was made up to 3.0ml with water. Cytochrome *c* (100 μ l) (39mg/ml) that had been reduced with KBH_4 was added. Once the absorbance had stabilized, 100–900 μ g of protein was added and the decrease in A_{550} was followed on a Varian Techtron.

Uncoupler-stimulated ATPase activity

ATPase activity was measured by a modification of the method of Veldsema-Currie & Slater (1968). Measurements were made in a reaction mixture (1.5ml) containing 75mM-KCl, 50mM-sucrose, 0.5mM-EDTA, 50mM-Tris/HCl buffer, 1 μ M-carbonyl cyanide *m*-chlorophenylhydrazine and 6mM-ATP. The final pH was 7.4 and the temperature 25°C. The mitochondria (1mg of protein/ml) were added 1 min before the reaction was started by the addition of ATP. Exactly 2min after the addition of ATP the reaction was stopped by the addition of 1.5ml of 10% (w/v) trichloroacetic acid. The protein was separated by centrifugation and the P_i in the supernatant determined by the detergent modification (Dulley, 1975) of the method of Baginski *et al.* (1967).

α -Glycerophosphate dehydrogenase activity

α -Glycerophosphate dehydrogenase activity was measured exactly by the method of Lee & Lardy (1969).

Materials

$^{45}\text{Ca}^{2+}$, $^{86}\text{Rb}^+$, [^3H]acetate and [^{14}C]methylamine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Nucleotides were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, West Germany. Ruthenium Red was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and was recrystallized before use. All other chemicals used were of analytical grade.

Results

Ca^{2+} transport in mitochondrial fractions isolated from rat liver

Data in Fig. 1 show results obtained from seven individual experiments in which the initial rate of Ca^{2+} transport by the rat liver fractions was measured in the absence and in the presence of 2mM- P_i . The rate of transport clearly is significantly different in each fraction, being greatest in fraction 2 with or without P_i ; all other fractions exhibited considerably lower rates of transport. The second point to note is that the degree of stimulation by P_i of Ca^{2+} transport was maximal also in fractions 2 and 3.

Recent studies have shown that Ca^{2+} transport by mitochondria is diminished when P_i cycling is prevented (Harris & Zaba, 1977; Bygrave *et al.*, 1977), and that this leads subsequently to a spontaneous

Fig. 1. Initial rates of Ca^{2+} transport by rat liver fractions. Reaction media in a final volume of 2.0 ml included: 150 mM-KCl, 5 mM-sodium succinate, 20 mM-Hepes (adjusted to pH 7.4 with KOH), 2 mg of liver fraction protein and 200 nmol of Ca^{2+} (containing $0.3 \mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$). P_i was added as indicated to a final concentration of 2 mM. The temperature was 4°C . For further details see the Experimental section. N refers to nuclear fraction. ○, No added P_i ; ●, 2 mM- P_i . Data are presented as the means $\pm$ S.E.M. for seven independent experiments.

efflux of Ca^{2+} from the mitochondria (Bygrave *et al.*, 1977; Ramachandran & Bygrave, 1978). Having found that Ca^{2+} transport is maximal in fraction 2, both in the absence and in the presence of P_i , it was decided to measure Ca^{2+} transport in the various mitochondrial fractions when P_i cycling was prevented by preincubation of mitochondria with *N*-ethylmaleimide.

Data in Fig. 2, obtained from following the movements of Ca^{2+} by using the Ca^{2+} -electrode technique (see the Experimental section), were from one of two experiments with independent mitochondrial preparations, each giving identical results; several important features are seen. First, the initial rate of transport is again greatest in fraction 2 (Fig. 2b). Secondly, this fraction also accumulates Ca^{2+} to the greatest extent and thirdly, the rate of Ca^{2+} efflux that occurs spontaneously after approx. 1 min of incuba-

tion with Ca^{2+} (Bygrave *et al.*, 1977) also is greatest in this fraction (Fig. 2b).

The data of Figs. 1 and 2 are important too in that they establish that the lower temperature at which Ca^{2+} transport is measured in the quench technique (see Fig. 1) is not responsible for the observed differences in the initial rates of Ca^{2+} transport in the different mitochondrial fractions. It seems significant that the rates of influx and efflux are greatest also in identical mitochondrial fractions.

It is important to mention at this point that the contribution of, for example, contaminating vesicles from the endoplasmic reticulum to Ruthenium Red-sensitive Ca^{2+} transport as measured in the present work, is minimal. Ca^{2+} transport by these vesicles is Ruthenium Red-insensitive, has an absolute requirement for ATP (Bygrave, 1978b) and exhibits relatively low initial transport rates by comparison with mitochondria (Bygrave & Tranter, 1978). Finally, observations by electron microscopy of the individual fractions revealed little contamination by vesicles of non-mitochondrial origin (results not shown).

Functional integrity of mitochondria in fractions isolated from rat liver

It could be argued that the relative ability of the mitochondrial fractions to transport Ca^{2+} as seen above, simply reflect the extent to which the mitochondria in these fractions have been damaged in the course of their isolation. The experiments that follow therefore were designed to assess the integrity of the mitochondria in the isolated fractions.

Data in Fig. 3(a), obtained from three individual experiments, show the rates of respiration in the different mitochondrial fractions in the absence (State 4) and presence (State 3) of ADP. Little difference in the rate of State-4 respiration is evident among fractions 1–5, but that in fraction 6 is significantly less. Moreover, State-3 respiration is similar in fractions 1–4; that in fraction 5 is slightly less and in fraction 6 about 30% of the maximum rate. The acceptor control ratio, i.e. the rate of respiration in State 3 divided by that in State 4 (Fig. 3b), is similar for fractions 1–5 and less in fraction 6.

Thus the similarity in rates of respiration between fractions 1–4 in particular, and especially the complete absence in all fractions of high rates of State-4 respiration, provide strong evidence that the mitochondrial populations in fractions 1–5 do not differ significantly in terms of organelle integrity.

Further direct evidence for this conclusion is seen in the experiments in Fig. 4, in which the two components of the protonmotive force were measured in the different fractions. Clearly both the ΔE and $-59 \Delta\text{pH}$ are virtually identical in fractions 1–4 and only slightly less in fraction 5. In accordance with the data of Fig. 4, values for these components in fraction 6 are lower. It should be noted also that the values for

Fig. 2. Initial rates of Ca^{2+} influx and efflux by mitochondrial fractions in the absence of P_i cycling

The reaction mixture contained in a final volume of 4.0 ml: 150 mM-KCl, 3 mM-Hepes/KOH, pH 7.4, 1 μM -rotenone and 800 nmol of *N*-ethylmaleimide. Mitochondria (4 mg of protein) were added before the addition of 80 nmol of Ca^{2+} and 5 mM-sodium succinate. Ca^{2+} movements were followed by using the Ca^{2+} -sensitive electrode at 25°C (see the Experimental section). Data are replotted from one of two experiments that gave identical results. The mitochondrial fractions studied were: (a) 1; (b) 2; (c) 3; (d) 4; (e) 5; (f) 6.

Fig. 3. Rates of respiration and acceptor control ratios in the mitochondrial fractions

Respiration in the absence (●) or in the presence (○) of 200 μM -ADP was measured as described in the Experimental section. The acceptor control ratios (3b) were calculated from these data, which are given as the means $\pm$ S.E.M. of three independent experiments.

the Δp obtained in this study are in quantitative agreement with those obtained by Mitchell & Moyle (1969) and by Nicholls (1974).

P_i content in mitochondrial fractions isolated from rat liver

In light of the influence that P_i has on mitochondrial Ca²⁺ transport (see, e.g., Lehninger, 1974; Bygrave, 1977) it was important to measure the content of P_i in each of the mitochondrial fractions. Data in Table 1 show that fractions 1–6 contained similar

amounts of P_i; the values obtained agree well with those reported by Rossi & Lehninger (1964) and Coty & Pedersen (1974), who obtained values of 12 and 17 nmol/mg of protein respectively.

Correlation of Ca²⁺-transport activity in mitochondrial fractions with some enzymic components of the energy-transducing assembly of rat liver mitochondria

These experiments were undertaken to assess whether the profile of Ca²⁺-transport activity seen in Fig. 1 could be correlated with the activity of enzymes known to be integral components of the energy-transducing assembly of rat liver mitochondria. The enzyme activities chosen were cytochrome oxidase, reflecting a typical component of the electron-transport chain, uncoupler-stimulated ATPase, reflecting the ATP synthetase component of the inner-membrane complex; both components presumably abundant in all regions of the inner membrane where respiration-supported ATP synthesis is carried out. The third enzyme assayed was α -glycerophosphate dehydrogenase, an enzyme considered to be enriched

Fig. 4. Components of the protonmotive force in mitochondrial fractions

The ΔE (●) and $-59 \Delta pH$ (▲) components of the Δp (○) were measured as described in the Experimental section. Data given are the means $\pm$ S.E.M. of three independent experiments, two of which were carried out in triplicate and one in duplicate giving a total of eight separate values for each point.

Table 1. P_i content of mitochondrial fractions
The P_i content in each mitochondrial fraction was determined as described in the Experimental section. Data are the means $\pm$ S.E.M. of four independent experiments each carried out in duplicate.

Fraction no.	P _i content (nmol of P _i /mg of protein)
1	16.19 $\pm$ 0.95
2	14.76 $\pm$ 0.48
3	15.24 $\pm$ 1.19
4	13.57 $\pm$ 1.43
5	12.62 $\pm$ 0.95
6	10.95 $\pm$ 1.67

Fig. 5. Cytochrome oxidase, uncoupler-stimulated ATPase and α -glycerophosphate dehydrogenase activity in mitochondrial fractions

Each enzyme assay was carried out on the individual mitochondrial fractions as described in the Experimental section. Data presented are the means $\pm$ S.E.M. of three independent experiments. (a) Cytochrome oxidase; (b) ATPase in the presence of 1 μ M-carbonyl cyanide phenylhydrazine; (c) α -glycerophosphate dehydrogenase.

Fig. 6. Influence of repeated additions of Ca^{2+} on the resting rate of respiration by the mitochondrial fractions

The resting rate of respiration was determined as described in the Experimental section after the addition of pulses of 40 nmol of Ca^{2+} /mg of protein. The fractions were 1 (●), 2 (○), 3 (▲), 4 (△), 5 (■) and 6 (□). The data are from one experiment, which is representative of four independent experiments.

respiration of repeated additions of a fixed amount of Ca^{2+} (see Rossi & Lehninger, 1964; Thorne & Bygrave, 1974).

Fig. 6 shows that after the addition of 80 nmol of Ca^{2+} /mg of protein, the rate of resting respiration in all fractions increased by approx 50%. After the addition of a further 80 nmol of Ca^{2+} /mg of protein, the rate of resting respiration in fractions 3 and 4 increased by about 4-fold to the maximum rate. That in fractions 1 and 2 remained low until about 200–240 nmol of Ca^{2+} /mg of protein had been added, reflecting a greater resistance of these mitochondrial populations to the deleterious (uncoupling) effects of the ion. Although fraction 5 appeared to require a higher Ca^{2+} concentration to induce an increase in the resting rate of respiration, the degree of stimulation was only intermediate between that of fractions 4 and 6.

These data thus show that the mitochondrial fractions differ not only in terms of the initial rate of Ca^{2+} transport, but also in terms of their resistance to the uncoupling action of the ion.

Discussion

The experiments presented in this report extend knowledge about several aspects of Ca^{2+} transport by mitochondria isolated from liver; these include its submitochondrial location as well as certain features of its mechanism, biogenesis and physiological role.

Submitochondrial location of the carrier system

Evidence that the Ca^{2+} -transport system resides in the inner mitochondrial membrane has been reviewed (Bygrave, 1977). Also in that review it was suggested that the transport system might logically be located in that part of the inner membrane in closest proximity to the outer membrane, i.e. in the inner peripheral membrane, a view expressed by Brdiczka *et al.* (1974).

The data in the present paper clearly show that the distribution of Ca^{2+} -transport activity in rat liver mitochondria correlates well with that of α -glycerophosphate dehydrogenase, but not with that of cytochrome oxidase or uncoupler-stimulated ATPase. The importance of this finding lies with the report of Werner & Neupert (1972) who concluded from their detailed study of the distribution of enzymes in the inner membrane that α -glycerophosphate dehydrogenase activity is enriched in the inner peripheral membrane.

Striking evidence supporting the view (Carafoli & Sottacasa, 1974) that the mitochondrial Ca^{2+} carrier might be a glycoprotein was obtained by Panfili *et al.* (1976), who found that an antibody to the purified glycoprotein inhibited mitochondrial Ca^{2+} transport. The assumed glycoprotein nature of the carrier is important in the present context because Brdiczka *et al.* (1974) observed that the inner membrane is

in the inner peripheral membrane (Werner & Neupert, 1972; Brdiczka *et al.*, 1974).

Fig. 5 shows results from several individual experiments in which the above three enzymes were assayed in the mitochondrial fractions. Fig. 5(a) shows that the distribution profile of cytochrome oxidase activity closely resembles that of the Δp ; fractions 1–4 exhibit maximal and similar specific activities. Data in Fig. 5(b) show that the distribution of uncoupler-stimulated ATPase, like that of cytochrome oxidase activity, also does not correlate with Ca^{2+} -transport activity; the highest activity is located in fraction 1. However, as can be seen in Fig. 5(c), there is a significant similarity between the distribution profile of Ca^{2+} transport activity and that of α -glycerophosphate dehydrogenase. Maximum activity is seen in fraction 2 with lower activities in both the 'heavier' and 'lighter' fractions; once again fraction 6 exhibits much lower activities than the other fractions.

Relative capacities of mitochondrial fractions for Ca^{2+}

Having established that the initial rate of Ca^{2+} transport differed in each of the mitochondrial fractions, it was relevant to examine whether the fractions differed also in their capacity to retain Ca^{2+} . This was assessed by measuring the effect on resting

heterogeneous with respect to the glycoprotein composition; the inner peripheral membrane is especially rich in several species of glycoprotein, one of which these workers suggested may possibly be the Ca^{2+} carrier.

The collective weight of the above data provides strong evidence that the system in rat liver mitochondria responsible for transporting Ca^{2+} into the organelle is located in the inner peripheral membrane. Evidence has been presented that the atractyloside-sensitive adenine nucleotide translocase also is located in the inner peripheral membrane (Winkler, 1969).

Mechanism of mitochondrial Ca^{2+} transport

A report from this laboratory (Reed & Bygrave, 1975a) provided evidence that the initial rate of Ca^{2+} transport is not limited by the 'driving force' for the process, i.e. the membrane potential. By contrast with this conclusion, Heaton & Nicholls (1976), Hutson *et al.* (1976) and Hutson (1977) concluded from studies of uncoupler A23187-induced Ca^{2+} cycling, that the rate of respiration is the rate-limiting step in the mechanism of mitochondrial Ca^{2+} transport. However, one problem associated with the use of the ionophore in studies of this nature is that it can induce an efflux of Mg^{2+} from mitochondria (Binet & Volfin, 1975) and this ion is known to inhibit mitochondrial Ca^{2+} transport (Åkerman *et al.*, 1977; Hutson *et al.*, 1976; Hutson, 1977; Crompton *et al.*, 1976).

The data reported in the present paper are in full agreement with our earlier proposal (Reed & Bygrave, 1975a). Thus a comparison of Figs. 1 and 2 with Figs. 3 and 4 clearly shows that Ca^{2+} transport is significantly greater in fraction 2 by comparison with fractions 1, 3 and 4, yet all fractions have practically identical rates of State-3 and -4 respiration and values for the Δp . Indeed several other observations from this laboratory provide further substantial evidence that the driving force cannot be the rate-limiting step. First, additions of P_i , which do not lead to an increase in Δp , but rather to a decrease (Nicholls, 1974), lead to an increase in the rate of Ca^{2+} transport (Fig. 1). Secondly, Ca^{2+} transport in developing flight-muscle of *Lucilia* changes dramatically with little or no change in ability to generate a Δp as judged from measurements of respiration and the P/O ratio (Bygrave *et al.*, 1975; Smith & Bygrave, 1978). Thirdly, Ca^{2+} transport in mitochondria isolated from hormone-challenged liver is altered under conditions where the rates of State-3 and -4 respiration are unchanged (Dorman *et al.*, 1975; V. Prpic, T. L. Spencer & F. L. Bygrave, unpublished work). Fourthly, concentrations of tributyltin that inhibit State-3 and -4 respiration in *Lucilia* flight muscle have no effect on Ca^{2+} transport (Bygrave & Smith, 1978). Fifthly, dietary-induced modification of mitochondrial Ca^{2+} transport can occur

with no change in ability of the mitochondria to respire (Bygrave & Smith, 1977). Sixthly, the initial rates of Ca^{2+} transport by rat liver mitochondria are not significantly greater than those from heart (see, e.g., Bygrave *et al.*, 1977), yet it is well known that the rate of respiration in heart mitochondria is much greater than that in liver mitochondria.

The finding that the P_i content was similar in all fractions rules out the possibility that this compound alone is associated with the rate-limiting step (cf. Reed & Bygrave, 1975a). Further evidence for this is seen in the observation that fraction 2 exhibited maximum rates both when P_i cycling was prevented (Fig. 2) or when it was allowed to proceed (Fig. 1).

The findings do raise the possibility that the different rates of Ca^{2+} transport is attributable to a difference in the number of Ca^{2+} carrier molecules in the inner peripheral membrane in each of the fractions.

Biogenesis of liver mitochondria

The data in the present paper consolidate evidence already in abundance, that the mitochondrial population in rat liver is heterogeneous (see references in the introduction). In particular considerable evidence exists that adult rat liver contain both fully-functional (mature) and non-functional (immature) mitochondria (see Gear, 1965; Pollak & Munn, 1970; Pollak, 1975; Satav *et al.*, 1976).

We suggest that the mitochondria obtained in fraction 6 and perhaps also in fraction 5 [i.e., those exhibiting a low acceptor control of respiration and cytochrome oxidase activity (see Pollak, 1975; Katyare *et al.*, 1970)] represent 'light', immature mitochondria. That these mitochondria are 'leaky' to H^+ (Pollak, 1975) is reflected also in their low value for the Δp . These mitochondria also exhibit low rates of Ca^{2+} transport and an inability to retain significant amounts of Ca^{2+} (Figs. 1 and 6). Significantly, foetal liver contains a higher proportion of 'light' mitochondria than does adult liver (Pollak, 1975), and these mitochondria exhibit poor acceptor control ratios (Pollak, 1975) and low rates of Ca^{2+} transport (Bygrave & Ash, 1977).

Thus it would appear that mitochondrial Ca^{2+} transport is fully functional in only those mitochondria that have reached maturity. This study then has raised the possibility of examining the biogenesis of Ca^{2+} transport in mitochondria from knowledge of the precursor-product relationship of 'light' and 'heavy' mitochondria (Gear, 1965; Pollak & Munn, 1970; Pollak, 1975, 1977; Katyare *et al.*, 1970; Satav *et al.*, 1976).

Physiological role of mitochondrial Ca^{2+} transport

Evidence that mitochondria in a range of tissues and species play a major task in controlling cell Ca^{2+} has

been reviewed (Bygrave, 1977, 1978a). Because of the possibility that certain hormones exert a fundamental controlling influence over cell Ca^{2+} (Bygrave, 1978a,c) and because many of these hormones probably enter the liver via the hepatic artery, it might be anticipated that the 'heavy' mitochondria, enriched in ability to transport Ca^{2+} are located predominantly in the peripheral lobular cells. In fact, electron micrographs of Loud (1968) show that the appearance of the mitochondria found in this region of the liver is different from those located in centrilobular cells adjacent to the central vein.

The logical extension of this argument is that those mitochondria most susceptible to the actions of hormones *in vivo* (cf. Dorman *et al.*, 1975) would be those isolated in the 'heavy' fraction described in the present paper.

This work was supported by a grant to F. L. B. from the National Health and Medical Research Council of Australia.

References

- Åkerman, K. E. O., Wikström, M. K. F. & Saris, N.-E. (1977) *Biochim. Biophys. Acta* **464**, 287–294
- Baginski, E. S., Foa, P. P. & Zak, B. (1967) *Clin. Chim. Acta* **15**, 155–158
- Binet, A. & Volfin, P. (1975) *Arch. Biochem. Biophys.* **170**, 576–586
- Brdiczka, D., Dolken, G., Krebs, W. & Hofmann, D. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 731–743
- Bygrave, F. L. (1977) *Curr. Top. Bioenerg.* **6**, 259–318
- Bygrave, F. L. (1978a) *Biol. Rev.* **53**, 43–79
- Bygrave, F. L. (1978b) *Biochem. J.* **170**, 87–91
- Bygrave, F. L. (1978c) *Trends Biochem. Sci.* In the press
- Bygrave, F. L. & Ash, G. R. (1977) *FEBS Lett.* **80**, 271–274
- Bygrave, F. L. & Smith, R. L. (1977) *Biochem. Biophys. Res. Commun.* **79**, 154–158
- Bygrave, F. L. & Smith, R. L. (1978) *Biochem. J.* **174**, 1075–1077
- Bygrave, F. L. & Tranter, C. J. (1978) *Biochem. J.* **174**, 1021–1030
- Bygrave, F. L., Daday, A. A. & Doy, F. A. (1975) *Biochem. J.* **146**, 601–608
- Bygrave, F. L., Ramachandran, C. & Smith, R. L. (1977) *FEBS Lett.* **83**, 155–158
- Carafoli, E. & Sottocasa, G. L. (1974) in *Dynamics of Energy-Transducing Membranes* (Ernster, L., Estabrook, R. W. & Slater, E. C. eds.), pp. 455–469, Elsevier, Amsterdam
- Coty, W. A. & Pedersen, P. L. (1974) *J. Biol. Chem.* **249**, 2593–2598
- Crompton, M., Sigel, E., Salzmann, M. & Carafoli, E. (1976) *Eur. J. Biochem.* **69**, 429–434
- Dorman, D. M., Barritt, G. J. & Bygrave, F. L. (1975) *Biochem. J.* **150**, 389–395
- Dulley, J. R. (1975) *Anal. Biochem.* **67**, 91–96
- Gear, A. R. L. (1965) *Biochem. J.* **97**, 532–539
- Harris, E. J. & Zaba, B. (1977) *FEBS Lett.* **79**, 284–290
- Heaton, G. M. & Nicholls, D. G. (1976) *Biochem. J.* **156**, 635–646
- Hutson, S. M. (1977) *J. Biol. Chem.* **252**, 4539–4545
- Hutson, S. M., Pfeiffer, D. R. & Lardy, H. A. (1976) *J. Biol. Chem.* **251**, 5251–5258
- Katyare, S. S., Fatterpaker, P. & Sreenivasan, A. (1970) *Biochem. J.* **118**, 111–121
- Lee, Y. P. & Lardy, H. A. (1965) *J. Biol. Chem.* **240**, 1427–1436
- Lehninger, A. L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1520–1524
- Loud, A. V. (1968) *J. Cell Biol.* **37**, 27–45
- Lusena, C. V. & Depocas, F. (1966) *Can. J. Biochem.* **44**, 497–506
- Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471–484
- Nicholls, D. G. (1974) *Eur. J. Biochem.* **50**, 305–315
- Novikoff, A. B. & Holtzman, E. (1970) *Cells and Organelles*, Holt, Reinehart and Winston, London
- Panfili, E., Sandri, G., Sottocasa, G. L., Lunazzi, G., Liut, G. & Graziosi, G. (1976) *Nature (London)* **264**, 185–186
- Pette, D. (1966) in *Regulation of Metabolic Processes in Mitochondria* (Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C., eds.), pp. 28–50, Elsevier, Amsterdam
- Pollak, J. K. (1975) *Biochem. J.* **150**, 477–488
- Pollak, J. K. (1977) *Biochem. Soc. Trans.* **5**, 341–348
- Pollak, J. K. & Munn, E. A. (1970) *Biochem. J.* **117**, 913–919
- Ramachandran, C. & Bygrave, F. L. (1978) *Biochem. J.* **174**, 613–620
- Reed, K. C. & Bygrave, F. L. (1975a) *Eur. J. Biochem.* **55**, 497–504
- Reed, K. C. & Bygrave, F. L. (1975b) *Anal. Biochem.* **67**, 44–54
- Rossi, C. S. & Lehninger, A. L. (1964) *J. Biol. Chem.* **239**, 3971–3980
- Satav, J. G., Katyare, S. S., Fatterpaker, P. & Sreenivasan, A. (1976) *Biochem. J.* **156**, 215–225
- Smith, R. L. & Bygrave, F. L. (1978) *Biochem. J.* **170**, 81–85
- Swick, R. W., Tollaksen, S. L., Nance, S. L. & Thomson, J. F. (1975) *Arch. Biochem. Biophys.* **168**, 281–288
- Szarkowska, I. L. & Klingenberg, M. (1963) *Biochem. Z.* **338**, 674–697
- Thorne, R. F. W. & Bygrave, F. L. (1974) *Biochem. J.* **144**, 551–558
- Tolbert, N. E. (1974) *Methods Enzymol.* **31**, 734–746
- Van Berkel, T. J. C. & Kruijt, J. K. (1977) *Eur. J. Biochem.* **73**, 223–229
- Veldsema-Currie, R. D. & Slater, E. C. (1968) *Biochim. Biophys. Acta* **162**, 310–319
- Weiss, G., Lamartiniere, C. A., Muller-Ohly, B. & Seubert, W. (1974) *Eur. J. Biochem.* **43**, 391–403
- Werner, S. & Neupert, W. (1972) *Eur. J. Biochem.* **25**, 379–396
- Winkler, H. H. (1969) *Biochim. Biophys. Acta* **189**, 152–161