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# THE NATURE OF THE CHANGES IN LIVER MITOCHONDRIAL FUNCTION INDUCED BY GLUCAGON TREATMENT OF RATS

## THE EFFECTS OF INTRAMITOCHONDRIAL VOLUME, AGING AND BENZYL ALCOHOL

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**(1) The effects of changes in the intramitochondrial volume, benzyl alcohol treatment and calcium-induced mitochondrial aging on the behaviour of liver mitochondria from control and glucagon-treated rats are reported. (2) The stimulatory effects of glucagon on mitochondrial respiration, pyruvate metabolism and citrulline synthesis could be mimicked by hypo-osmotic treatment of control mitochondria and reversed by calcium-induced aging of mitochondria or by treatment with 20 mM benzyl alcohol. Hypo-osmotic treatment increased the matrix volume whilst aging but not benzyl alcohol decreased this parameter. (3) Liver mitochondria from glucagon and adrenaline-treated rats were shown to be less susceptible to damage by exposure to calcium than control mitochondria and frequently showed slightly (15%) elevated intramitochondrial volumes. (4) Aging, benzyl alcohol and hypo-osmotic media increased the susceptibility of mitochondria to damage caused by exposure to calcium. (5) Glucagon-treated mitochondria were less leaky to adenine nucleotides than control mitochondria. (6) These results suggest that glucagon may exert its action on a wide variety of mitochondrial parameters through a change in the disposition of the inner mitochondrial membrane, possibly by stabilisation against endogenous phospholipase $A_2$ activity. This effect may be mimicked by an increase in the matrix volume or reversed by calcium-dependent mitochondrial aging.**

## Introduction

Adam and Haynes [1] first demonstrated that liver mitochondria prepared from rats treated with glucagon metabolized pyruvate faster than those from control animals. This effect appears to be mediated by cyclic AMP [2] althought a similar stimulation is induced in a cyclic AMP-independent manner by adrenaline [3]. Since these initial observations it has become apparent that numerous aspects of mitochondrial metabolism are affected by glucagon [4,5]. These include activation of the respiratory chain [6–11], pyruvate transport [7–9], activation of the oligomycin-sensitive ATPase and adenine nucleotide transport [12,13], enhanced citrulline synthesis [14,15], elevated membrane potential, pH gradient, ATP/ADP ratio and total content of adenine nucleotides, phosphate, magnesium and potassium [9,11,15–20], increased ability to retain accumulated $Ca^{2+}$ [18,19], stimulation of glutaminase activity [21], decreased succinyl-CoA concentration and elevated succinate dehydrogenase activity [22–25], and increased rates of respiratory chain and ATP-driven cation accumulation [6,26].

Such a catalogue of effects suggests that some

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Mops, 4-morphilinepropanesulphonic acid.

fundamental change in mitochondrial function is induced by glucagon, and begs the question as to the nature of the primary event in the action of the hormone on the mitochondria. We have suggested that many, if not all the effects, might be explicable in terms of a glucagon-induced structural change in the mitochondrial membrane [5]. This suggestion could allow a single cyclic AMP-mediated effect on the inner mitochondrial membrane to produce all the known effects of glucagon on the mitochondria as primary and secondary consequences of a change in membrane disposition. Previous work from this laboratory has demonstrated that perturbing the mitchondrial membrane by aging the mitochondria, exposing them to benzyl alcohol or decreasing the matrix volume causes changes in the mitochondrial cytochrome spectra exactly the opposite of those induced by glucagon treatment [27]. In this paper, we extend these studies by examining the effects of the same three in vitro treatments of mitochondria on a number of other parameters known to be influenced by glucagon treatment. It is demonstrated that all three treatments also oppose the action of glucagon on mitochondrial pyruvate metabolism, citrulline biosynthesis, ATP concentrations, uncoupled and ADP-stimulated respiration supported by substrates entering the respiratory chain before cytochrome *c* and calcium-stimulated mitochondrial swelling. These observations are consistent with a model for glucagon action which proposes a change in the disposition of the mitochondrial inner membrane as the primary event mediating the numerous other effects of the hormone on the mitochondria. The data are also consistent with the recent suggestion that glucagon-treated mitochondria may be more resistant to endogenous phospholipase $A_2$ [5,72], which could provide a mechanism for the apparent change in membrane disposition.

## Experimental Section

### *Materials*

Rats (female Wistar rats, 200–250 g) were allowed free access to food and water before anaesthetising and intraperitoneal injection of glucagon (0.4 mg/kg body weight), adrenaline (1.2 mg/kg body weight) or saline as described previously [1,9].

Mitochondria were prepared as described previously [9] in sucrose (300 mM), EGTA (2 mM) and Tris-HCl (10 mM), pH 7.6, at 4°C. Unless otherwise stated mitchondria were only washed once before use.

The sources of all chemicals, biochemicals and radiochemicals were the same as those given in Ref.9.

### *Methods*

Measurement of intramitochondrial volumes, respiratory chain activity, pyruvate metabolism and ATP content of mitochondria were performed as described previously [9], but with the modifications described below. In many cases, rates of respiration are expressed as a percentage of the rate of uncoupled oxidation of ascorbate + TMPD, whose rate of oxidation ($242 \pm 7.4$ ngatom/min per mg protein ($n = 11$ at 30°C) is insensitive to glucagon [4–10]. In the measurement of mitochondrial volumes certain refinements were made to previous techniques [9,28]. The protein sedimented by centrifugation of mitochondria was measured by a modified biuret technique [29] in a parallel experiment to the measurement of $^3H_2O$ and [$^{14}$C]sucrose present in the pellet. Great care was taken to avoid $^3H_2O$ loss from the pellet by evaporation. Quench corrections were made for both $^{14}$C overspill into the $^3$H channel and $^3$H overspill into the $^{14}$C channel of the scintillation counter. The same volume of mitochondrial pellet extract and supernatant was counted, the two samples always following one another in sequence in the counter to minimize the effects of variable efficiency. All samples were recycled to give at least three estimates of the intramitochondrial volume for each sample which were then averaged. Four incubations were carried out for each condition. With these precautions the reliability of measuring small intramitochondrial volumes was enhanced greatly.

Citrulline synthesis was studied by following the incorporation of $H^{14}CO_3^-$ into acid-stable material as decribed in Ref. 30. The buffer contained 125 mM KCl, 0.5 mM K-EGTA, 2.5 mM potassium phosphate, 25 mM $KH^{14}CO_3$ (250 dpm/nmol), 2.5 mM $MgCl_2$, 20 mM ornithine, 5 mM $NH_4Cl$, 10 mM Mops, 7 mM Tris base and 0.1 μg/ml rotenone at pH 7.4. Rates were cor-

rected for ornithine-independent incorporation of $^{14}C$ and the values obtained were checked by chemical assay of citrulline [31].

Calcium-dependent swelling of mitochondria was carried out in a Pye Unicam SP 8100 split-beam spectrophotometer. To 5 ml of buffer containing 125 mM KCl, 2.5 mM potassium phosphate, 2.5 mM potassium succinate, 10 mM Mops and 7 mM Tris base, pH 7.4, were added mitochondria (approx. 10 mg protein). After vortex mixing the mitochondrial suspension was divided between two cuvettes in the spectrophotometer and the absorbance at 535 nm was recorded. After obtaining a baseline, $CaCl_2$ (10 nmol $Ca^{2+}$/mg mitochondrial protein) was added to one of the cuvettes and the change in absorbance continuously recorded.

Mitochondrial aging with $Ca^{2+}$ was performed at 37°C for 5 min in sucrose medium ( 300 mM sucrose, 10 mM, Tris-HCl, 2 mM EGTA, 6 mM Ca-EGTA, pH 7.4) at a protein concentration of approx, 75 mg/ml. The free [$Ca^{2+}$] under these conditions was 0.1 $\mu$M. Aging was terminated by addition of a large excess of ice-cold isolation medium and the mitochondria re-sedimented by centrifugation. Control mitochondria were subject to the same treatment in the absence of Ca-EGTA. All manipulations of the osmotic strength of media involved changes in KCl or sucrose concentrations only.

Fluorescence depolarization has been used extensively to study the fluidity of membranes, including mitochondria [32.33], and the most usual probe used is 1,6-diphenyl-1,3,5-hexatriene. Mitochondria were freeze-thawed twice before incubating at a protein concentration of 2 mg/ml for 1 h with 2 $\mu$M 1,6-diphenyl-1,3,5-hexatriene (added as a 2 mM solution in tetrahydrofuran) in 125 mM KCl, 20 mM Tris-HCl and 2 mM K-EGTA at pH 7.4 and 25°C. At this time, fluorescence depolarization measurements were performed with excitation at 342–358 nm and emission at 442–458 nm. A series 8000 spectrofluorometer from SLM Instruments Inc. Urbana, IL, U.S.A., was used which integrated both vertically and horizontally polarized signals for 10 s and calculated the ratio of the vertical to horizontal signal ($R$). The ratio of $R$ obtained with vertically polarized incident light to that obtained with horizontally polarized light was taken as the normalized polarization measurement.

Estimation of the leakage of adenine nucleotides out of the mitochondria was made by observing the extent of extramitochondrial protein phosphorylation that occurred when mitochondria were exposed to [$^{32}$P]phosphate [34]. Under these conditions, extramitochondrial proteins are only phosphorylated when [$\gamma$-$^{32}$P]ATP leaks out of the mitochondria to become available to extramitochondrial protein kinases. Incubations of mitochondria with [$^{32}$P]phosphate were performed as described in Ref. 34. In some incubations, ADP (0.1 mM) was added to allow the synthesis of sufficient extramitochondrial [$\gamma$-$^{32}$P]ATP to give maximal extramitochondrial protein phosphorylation.

## Results

*The effects of various in vitro treatments on respiratory chain activity.*

Inhibition of the rate of oxidation of glutamate + malate and acyl carnitine under conditions of uncoupler-induced matrix condensation has been reported by others [35,36]. In Fig. 1, data are presented demonstrating that the rate of uncoupled oxidation of succinate + rotenone (Fig. 1a) or glutamate + malate (Fig. 1b) is extremely sensitive to changes in the osmolarity of the incubation medium. Oxidation of ascorbate + TMPD was far less sensitive to such changes in osmolarity (Fig. 1b) especially at osmolarities below 300 mosmol (isoosmotic) where changes in the matrix volume were largest (Fig. 1a). Indeed, between 200 and 300 mosmol an increase in the matrix volume of about 15% caused stimulation of the rate of oxidation of succinate or glutamate + malate by about 30% without an appreciable change in the rate of ascorbate + TMPD oxidation. At very low osmolarity the rate of oxidation of both succinate and glutamate + malate declined from its maximal value. At these osmolarities the effect of glucagon on succinate oxidation was reduced from 55 ± 10.5 to 9 ± 4.1% and on glutamate + malate oxidation from 38 ± 18.5 to 8 ± 5.7% (values given as means ± S.E. for eight separate experiments), indicating in both cases that respiration was no longer limited by that process stimulated by glucagon.

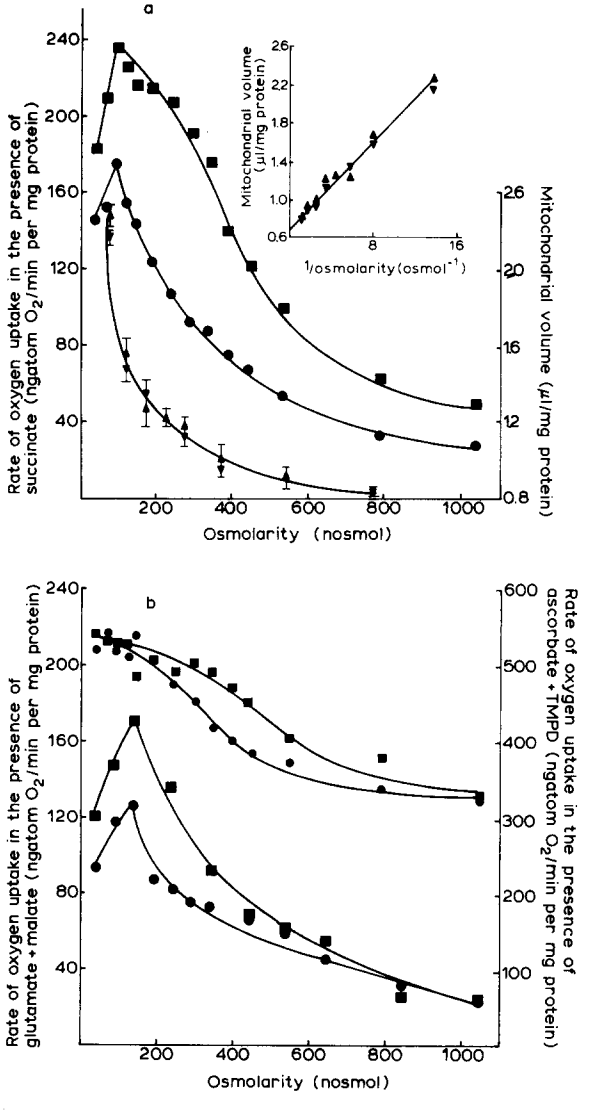


Fig. 1. The effects of osmolarity on intramitochondrial volume and respiratory chain activity. Mitochondria (approx. 1 mg protein) were added directly to 1 ml of medium containing ADP, uncoupler and substrate and rates of oxygen uptake (●, ■) measured as described in the Experimental Section when a constant rate was achieved. The temperature was 30°C. (a) Intramitochodrial volume (▲, ▼) was determined under identical conditions, as described in the Experimental Section. Error bars represent the S.E. for four different incubations on the same batch of mitochondria. The inset shows the same data plotted against the reciprocal of osmolarity. All osmolarities were varied by changing KCl concentrations only. (a) The respiratory substrate was 5 mM succinate; (b) either 5 mM glutamate + 2 mM malate (lower traces) or 10 mM ascorbate + 0.3 mM TMPD (higher traces) were present. Mitochondria from both control (●, ▼) and glucagon-treated animals (■, ▲) were used throughout.

We have also studied the effects of altering the intramitochondrial volume without changing the osmolarity of the incubation medium. In media containing low $K^+$ concentrations such as those based on sucrose or choline chloride the matrix volume is considerably reduced as shown in Table I and Ref. 9. This reflects the role of $K^+$ in the regulation of the intramitochondrial volume [38–40]. In these media the volume may be further reduced by the addition of adenine nucleotides in the presence of oligomycin [41]; such treatment caused a decrease in volume of 25 ± 5% (mean ± S.E. of three separate observations on different batches of mitochondria). Shrinkage of mitochondria by any of the above treatments inhibited respiration in exactly the same way as did changing the osmotic strength of the medium (data not shown). Conversely, the matrix volume could be increased in KCl medium by incubation for 5 min with $10^{-9}$ M valinomycin or 1 mM ADP in the presence of oligomycin (10 μg/ml) before measuring the rate of uncoupled respiration. The respective increases in volume were 15.4 ± 4.5 and 19.5 ± 2.0% (values given as the means ± S.E. of four separate observations) and again the rate of uncoupled respiration increased by the amount expected from experiments where the volume was increased by the use of hypotonic media. To establish that neither the effects of changing the matrix volume nor the effects of glucagon treatment were due to changes in the internal $[Mg^{2+}]$ or $[Ca^{2+}]$, incubations were performed in the presence of the divalent metal ion ionophore A23187 at various concentrations of $Mg^{2+}$ and $Ca^{2+}$. Under no conditions did these additions abolish the effects of volume changes or glucagon treatment on the rate of uncoupled respiration.

Rapid aging of mitochondria may be achieved by incubating them at 37°C for 5 min in isolation buffer (containing 2 mM EGTA) with 6 mM Ca-EGTA (see Experimental Section and Ref. 27). In Table I it is shown that such limited exposure to low concentrations of $Ca^{2+}$ inhibited the oxidation of succinate and glutamate + malate but not ascorbate + TMPD. After more prolonged exposure the rate of ascorbate + TMPD oxidation was also impaired, probably as a result of calcium-dependent swelling and subsequent loss of cytochrome *c* [27,42]. The conditions described for

TABLE I

THE EFFECTS OF GLUCAGON TREATMENT AND AGING ON MITOCHONDRIAL VOLUME, PYRUVATE METABOLISM AND ON CALCIUM-DEPENDENT SWELLING

Aging of mitochondria and measurement of mitochondrial volumes, oxygen uptake, pyruvate metabolism and calcium-induced swelling were performed as described in the Experimental Section. Measurement of mitochondrial volume under State 4 conditions was made in the presence of 5 mM succinate in the oxygen electrode medium described in the Experimental Section. No absolute values are given for swelling experiments but only the ratio of the rate of swelling of glucagon mitochondria to those of controls. Absolute values varied considerably from day to day depending on endogenous calcium levels. Statistical significance of experimental results when compared to the relevant controls was calculated by paired Student's *t*-test.

| Parameter | Nature of medium or respiratory substrate | Control | Glucagon | Glucagon as % control | Number of experiments | Aged glucagon | Aged glucagon as % of glucagon | Number of experiments |
|---|---|---|---|---|---|---|---|---|
| 1. Volumes (μl/mg protein) during: | | | | | | | | |
| (a) State 4 | KCl | 1.08 ±0.04 | 1.25 ±0.05 | 116.7± 4.2[b] | 13 | – | – | – |
| (b) Pyruvate metabolism | KCl | 1.18 ±0.05 | 1.32 ±0.05 | 112.9± 2.3[c] | 28 | 1.04±0.10 | 80 ± 5.6[a] | 5 |
| (c) Pyruvate metabolism | Sucrose | 0.577±0.026 | 0.698±0.040 | 121.4± 5.5[c] | 23 | – | – | – |
| 2. Pyruvate metabolism (nmol pyruvate/ min per mg protein) | KCl | 35.5 ±2.4 | 52.8 ±2.7 | 155 ± 6.3[c] | 28 | 20.2 ±5.2 | 43.5±10.2 | 5 |
| | Sucrose | 12.4 ±1.4 | 35.0 ±3.3 | 320 ±25[c] | 23 | – | – | – |
| 3. Rate of calcium-induced swelling | KCl | – | – | 33.7± 3.5[c] | 27 | – | 262 ±37[b] | 10 |
| 4. Rate of uncoupled oxygen uptake as a percentage of the rate of ascorbate +TMPD oxidation | Succinate | 48.4 ±2.7 | 66.8 ±3.0 | 143 ± 4.4[c] | 36 | 49.5 ±4.4 | 71.9± 2.6[c] | 36 |
| | Glutamate +malate | 47.6 ±1.7 | 59.4 ±2.1 | 125 ± 3.5[c] | 16 | 30.6 ±2.5 | 52.2± 4.5[c] | 16 |

[a] $P < 0.02$.
[b] $P < 0.01$.
[c] $P < 0.001$.

calcium-induced aging in the Experimental Section routinely produced maximal effects on succinate oxidation without inhibition of ascorbate + TMPD oxidation. Data are only shown in Table I for aged mitochondria from glucagon-treated rats, since aging of control mitochondria caused similar changes and was not routinely performed.

In Fig. 2, the effect of increasing concentrations of benzyl alcohol on uncoupled succinate oxidation and membrane fluidity measured by the fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene is shown. Benzyl alcohol is known to perturb membrane structure and to increase membrane fluidity [43]. It was found that coincident with changes in fluidity measured by fluorescence depolarization, benzyl alcohol caused substantial inhibition of mitochondrial succinate oxidation without affecting the oxidation of ascorbate + TMPD. Similar inhibition of the oxidation of glutamate + malate was also observed (data not shown). At 20 mM benzyl alcohol inhibition of uncoupled succinate oxidation was 31 ± 2.5% (mean ± S.E. of four observations on different mitochondrial preparations).

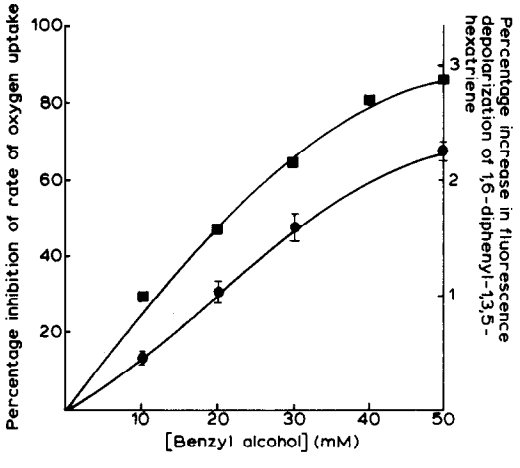


Fig. 2. The effects of benzyl alcohol on succinate oxidation by rat liver mitochondria and the degree of fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene. Rates of succinate oxidation (●) were studied polarographically under uncoupled conditions as described in the Experimental Section. Values given represent the mean of four separate experiments with the S.E. indicated by an error bar. Fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (■) was measured as described in the Experimental Section, the value shown being the mean of two separate experiments agreeing within 10%. No inhibition of ascorbate + TMPD oxidation was observed at any of the benzyl alcohol concentrations used.

*The effects of intramitochondrial volume, aging and benzyl alcohol on mitochondrial metabolism*

Data are shown in Figs. 3 and 4 on the effects of aging, intramitochondrial volume and benzyl alcohol (20 mM) on mitochondrial pyruvate metabolism and citrulline biosynthesis. In the Dixon plots of the inhibition of pyruvate metabolism by α-cyano-4-hydroxycinnamate, it is apparent that hypo-osmotic media caused control mitochondria to behave like glucagon-treated mitochondria (Fig. 4a) whereas aging or 20 mM benzyl alcohol caused glucagon-treated mitochondria to behave more like control mitochondria (Fig. 3b). The same pattern was observed in the time course of citrulline synthesis by mitochondria and associated mitochondrial ATP content (Fig. 4a, b). These effects of intramitochondrial volume on the metabolism of pyruvate by mitochondria may partially explain the slower rates of metabolism seen in sucrose medium than in KCl medium, since the intramitochindrial volume is considerably greater in KCl medium than in sucrose medium (Table I). Data are only given for the effects of aging and benzyl alcohol on mitochondria from glucagon-treated animals, since control mitochondria behaved similarly. Excessive aging or benzyl alcohol treatment inhibited rates of citrulline synthesis and pyruvate metabolism to levels too low to measure with accuracy. However, even at these greatly inhibited rates a glucagon effect was still apparent.

*Effects of glucagon and various in vitro treatments on the intramitochondrial volume of isolated liver mitochondria*

In Table I, data are presented to show that mitochondria from glucagon-treated rats exhibit matrix volumes 10–20% greater than those from control rats under a variety of incubation conditions with either KCl or sucrose as osmotic support. Although this increase in matrix volume may appear small, it must be realised that there is an osmotically inactive component of the matrix volume of 0.6–0.8 μl/mg mitochondrial protein as indicated in the inset to Fig. 1a and reported elsewhere [44,45]. Thus, the changes in osmotically

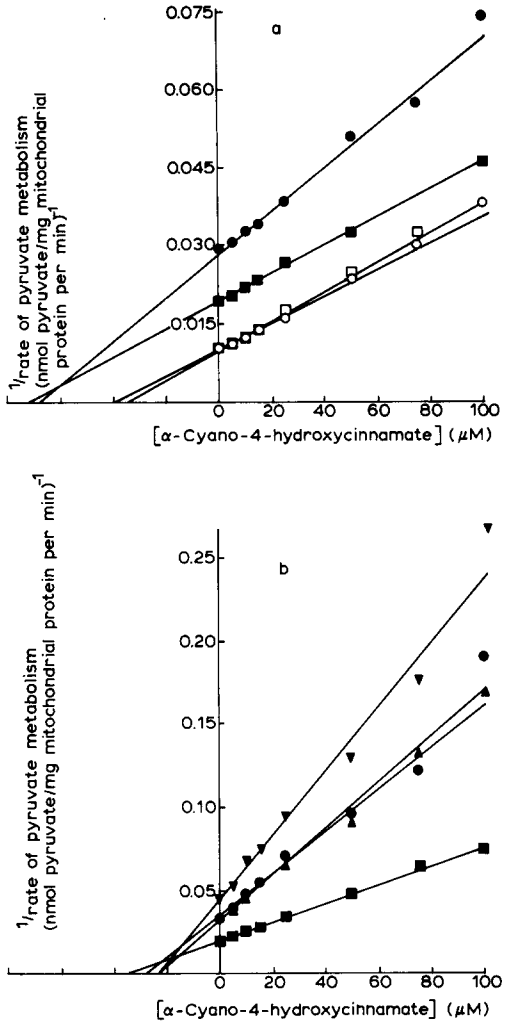


Fig. 3 The effects of glucagon treatment, aging, hypo-osmotic media and benzyl alcohol on mitochondrial pyruvate metabolism. The rate of pyruvate metabolism by mitochondria was measured by the disappearance of pyruvate from the medium as described in the Experimental Section. The data were fitted to the equation for non-competitive inhibition using non-linear least-squares regression as described previously [9]. Rates of pyruvate uptake were corrected for carrier-independent pyruvate transport (normally less than 10% of the carrier-mediated rate) as described by Thomas and Halestrap [76]. (a) Data are shown for control mitochondria (●, ○) and glucagon-treated mitochondria (■, □) in buffer of normal (●, ■) and half-normal (○, □) osmolarity. (b) Data are given for control mitochondria (●), glucagon-treated mitochondria (■), aged glucagon-treated mitochondria (▲) and glucagon-treated mitochondria in the presence of 20 mM benzyl alcohol (▼). The difference in $K_i$ values between experiments a and b is within the range found previously [9] and probably reflects minor pH perturbations due to loss of $CO_2$ from the medium.

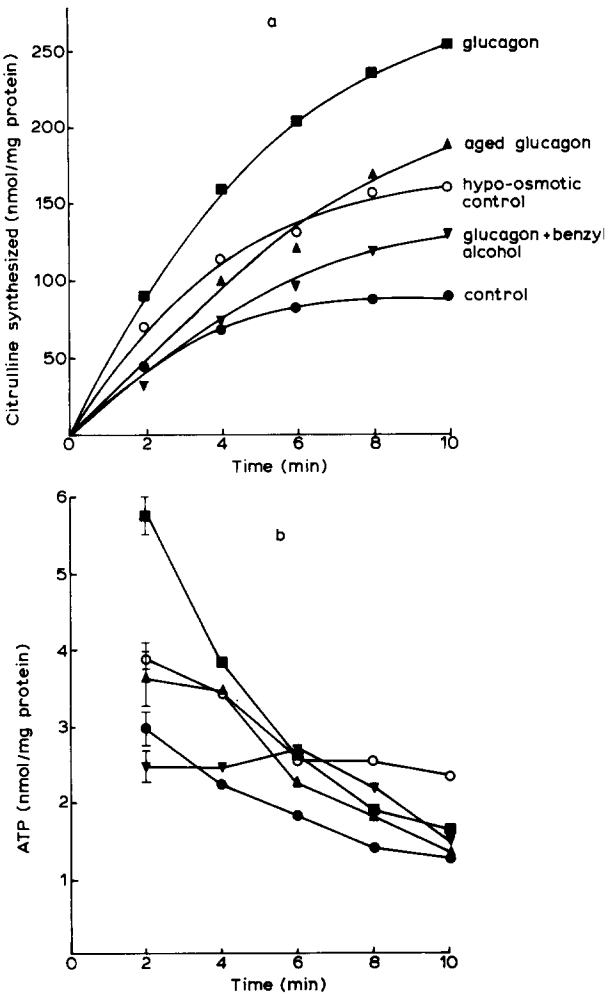


Fig. 4 The effects of glucagon treatment, hypo-osmotic media and benzyl alcohol on citrulline synthesis by liver mitochondria. Citrulline synthesis was measured at 37°C by the incorporation of $H^{14}CO_3^-$ into acid-stable material as described in the Experimental Section. ATP was measured in the $HClO_4$ extract using luciferase. All measurements are the means of two observations on the same batch of mitochondria except where error bars are given. These represent the S.E. of four observations. The symbols used are the same as given in the legend to Fig. 3.

active volume may be more significant than the changes in total volume suggest. Previous data from this laboratory included the measurement of intramitochondrial volumes [9] and following glucagon treatment small increases in the matrix volume were observed provided measurements were made in the absence of valinomycin. How-

ever, the changes were not measured with sufficient accuracy to be statistically significant. In the present experiments, the accuracy of the measurements was considerably enhanced by using the modifications outlined in the Experimental Section, and even with these precautions an effect of glucagon on intramitochondrial volume was not detected in every experiment. Liver mitochondria from rats treated with adrenaline also showed an increase in matrix volume ($14.7 \pm 4.3\%$, $n = 6$) only on those days that glucagon treatment also caused an increase in matrix volume ($9.7 \pm 4.8\%$). It should be stressed, however, that there were days when neither hormone appeared to affect the matrix volume even though other of the hormones were apparent. This may explain the inability of Titheradge and Coore [8] to observe intramitochondrial volume changes after glucagon treatment although we cannot account for the decrease in matrix volume that these workers observed after adrenaline treatment, nor for the very low values (about 0.4 $\mu$l/mg protein) that they measured.

Table I also provides data to demonstrate that mitochondrial aging decreases the matrix volumes of mitochondria from glucagon-treated rats to values similar to those from control animals. However, we have been unable to show any effect of 20 mM benzyl alcohol on this parameter.

*The effects of glucagon and various in vitro treatments on calcium-dependent swelling and adenine nucleotide leakage*

Exposure of liver mitochondria to low concentrations of calcium in the presence of phosphate can cause massive, deleterious swelling of the mitochondria in the absence of adenine nucleotides [5,27,42,46–49]. Additional data presented in Table I demonstrate that glucagon treatment inhibits this swelling process whilst aging enhances it. We have also demonstrated that hypo-osmotic conditions and 20 mM benzyl alcohol enhance calcium-dependent swelling whilst hyper-osmotic conditions or adrenaline treatment of rats inhibits this phenomenon (data not shown).

Calcium-dependent swelling is accompanied by a general increase in mitochondrial permeability and loss of adenine nucleotides and magnesium [46–53]. Liver mitochondria from glucagon-treated rats exhibit a higher endogenous content of adenine nucleotides, phosphate, magnesium and potassium than control mitochondrial [9,16,18–20]. It was therefore of interest to see whether mitochondria from glucagon-treated mitochondria are less leaky to adenine nucleotides than control mitochondria. The data presented in Fig. 5 suggest that this is the case. In the presence of [$^{32}$P]phosphate mitochondria generate intramitochondrial [$\gamma$-$^{32}$P]ATP which can only phosphorylate extramitochondrial proteins if it leaks out of the mitochondria. The radioautograms shown in Fig. 5 demonstrate that considerably greater extramitochondrial protein phosphorylation occurred when control mitochondria were used (tracks 1,3,5) than when glucagon-treated mitochondria were used (tracks 2,4,6). When excess extramitochondrial [$\gamma$-$^{32}$P]ATP was provided the extent of protein phosphorylation was the same in both cases (tracks 7,8).

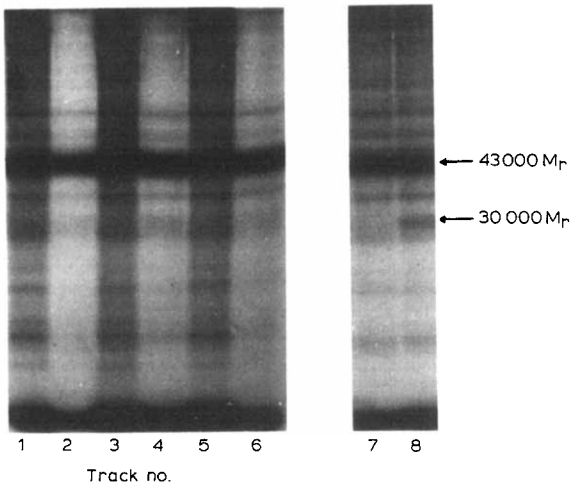


Fig. 5 Radioautograph of mitochondrial proteins phosphorylated in the absence of added ADP to demonstrated ATP leakage from mitochondria. Mitochondria were incubated with [$^{32}$P] phosphate as described in the Experimental Section in the absence of added ADP or EGTA. Tracks 1–6 represent separate batches of mitochondria from three control and three glucagon-treated animals in the order control 1, glucagon 1, control 2, etc. Tracks 7 and 8 represent experiments in the presence of ADP for both control (7) and glucagon-treated (8) mitochondria.

## Discussion

The results presented in this paper show that both changing the intramitochondrial volume and perturbing the mitochondrial membrane by aging or addition of benzyl alcohol influence many of the parameters affected by glucagon treatment. This supports our previous suggestion [5,27] that the numerous effects of glucagon on mitochondrial function could be explained by a subtle change in the disposition of the inner mitochondrial membrane which may be mimicked or reversed by these in vitro treatments of mitochondria. Other workers have reported stimulatory effects of intramitochondrial volume on mitochondrial glutaminase activity and uncoupler-stimulated ATPase [36,54]. However, although a small increase in intramitochondrial volume is apparent in mitochondria from glucagon-treated animals (Table I), it would not appear to be sufficiently large or consistent to account for all the effects of glucagon treatment on the function of isolated mitochondria. Furthermore, effects of glucagon on respiratory chain function are apparent in disrupted mitochondria and submitochondrial particles [11,27]. It is well established that the amounts of phosphate, $Mg^{2+}$, $K^+$ and adenine nucleotides in mitochondria from glucagon-treated rats are greater than in those from control animals [9,16,18–20], and the increased matrix volume may simply be and osmotic consequence of this. This in turn may be the result of glucagon-treated mitochondria being less leaky than control mitochondria as indicated by their enhanced ability to retain adenine nucleotides (Fig. 5). In this context, it is of interest to note that maturation of neonatal mitochondria involves a change from very leaky, inefficient, adenine nucleotide-deficient organelles to adult, efficient, sealed mitochondria through a process totally dependent on glucagon [55,56].

Leakage of adenine nucleotides and $Mg^{2+}$ from mitochondria is known to be a consequence of mitochondrial aging, a process thought to involve phospholipase $A_2$ and $Ca^{2+}$ [57–64]. Calcium-dependent swelling also appears to involve mitochondrial phospholipase $A_2$ and to cause accumulation of minute amounts of lysophospholipids and unsaturated fatty acids which perturb the membrane structure sufficiently to produce increased permeability to small ions [59,60,65–68]. Since both adenine nucleotide leakage and calcium-dependent swelling are inhibited by glucagon treatment (Fig. 5 and Table I), it seems possible (as we have speculated previously [5]) that mitochondria from glucagon-treated animals are less susceptible to the activity of endogenous phospholipase $A_2$ present in the inner mitochondrial membrane. A similar conclusion has recently been reached by others [69] although direct assay of this enzyme activity has not been achieved by us or others [66,69].

This hypothesis is support by data which demonstrate that aging of mitochondria caused glucagon-treated mitochondria to revert to those resembling control mitochondria with respect to their pyruvate metabolism (Fig. 3), citrulline synthesis (Fig. 4), respiratory chain activity (Table I), calcium-dependent swelling (Table I) and volumes (Table I). In addition to this recent work, this laboratory has demonstrated that the effects of glucagon and aging of mitochondria on the respiratory chain and its inhibition by colletotrichin persist in disrupted mitochondria (where changes in matrix volume are not appropriate). Under these conditions the effect of glucagon can be reversed by exposure of the disrupted mitochondria to added snake venom phospholipase $A_2$ [27]. Furthermore, it has been established that the lysophospholipids and fatty acids produced by phospholipase $A_2$ action on mitochondria can inhibit the respiratory chain, adenine nucleotide translocation and oligomycin-sensitive ATPase activity [70–75]. As outlined in the Introduction these are all processes which are activated by glucagon.

In the light of the evidence we have presented it seems possible that the primary locus of action of glucagon on the mitochondria may be to perturb the mitochondrial inner membrane, perhaps by inhibiting an endogenous phospholipase $A_2$ known to be associated with the inner mitochondrial membrane [55,69,70]. It is thus pertinent to ask whether the majority of the effects of glucagon treatment seen in isolated mitochondria are merely a result of their stabilization during preparation. This has been suggested recently by Siess et al. [69] and might be taken to indicate that such effects of

glucagon are not apparent in vivo. However, we have demonstrated that mitochondrial pyruvate transport is activated by glucagon in the intact hepatocyte [76]. Furthermore, it is known that glutaminase activity [77], urea synthesis [15], mitochondrial ATP/ADP ratios [12,15,17], oxygen consumption [78,79] and mitochondrial $NADH/NAD^+$ ratios [80] are increased by glucagon in the intact hepatocyte. It is therefore quite clear that something is happening to the mitochondria in situ which resembles the changes seen in isolated mitochondria. Whether changes is phospholipase $A_2$ activity are responsible for these in vivo changes in mitochondrial function, and how such regulation may be achieved remain to be established.

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