Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

Carlo Gregolin, Liliana Manzi, Renzo Deana, Marina Valente

Respiratory activities of isolated rat liver cells

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **54** (1973), n.4, p. 637–647.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1973_8_54_4_637_0>

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Accademia Nazionale dei Lincei, 1973.

Chimica biologica. — *Respiratory activities of isolated rat liver cells* (*). Nota di CARLO GREGOLIN, LILIANA MANZI, RENZO DEANA e MARINA VALENTE, presentata (**) dal Corrisp. N. SILIPRANDI.

RIASSUNTO. — È stata studiata la respirazione delle cellule isolate di fegato di ratto in presenza di substrati e cofattori delle ossidazioni mitocondriali, allo scopo di accertare la permeabilità di questi attraverso la membrana plasmatica.

Le cellule sono state isolate mediante perfusione del fegato di ratto con ialuronidasi, collagenasi ed EDTA e dispersione dell'organo in soluzione isotonica. La respirazione è stata misurata polarograficamente con elettrodo di Clark su cellule sospese in messo isotonico contenente fosfato, a 22° C.

La velocità di respirazione endogena corrispondeva a 4,3 *n* atomi/min/mg di proteina. L'aggiunta di substrati ossidabili attraverso il ciclo degli acidi tricarbossilici, alla concentrazione di 10 mM, causava in tutti i casi una piccola stimolazione della respirazione. La respirazione tuttavia raggiungeva quote molto elevate a seguito dell'ulteriore addizione di disaccoppianti. I substrati ossidati più velocemente erano succinato (50 *n* atomi/min/mg di proteina), glutammato (29,8 *n* atomi), α–chetoglutarato (20,2 *n* atomi), piruvato in presenza di malato (19,2 *n* atomi), citrato (18,2 *n* atomi).

La respirazione in presenza di substrati mitocondriali non era stimolata dall'aggiunta di ADP, fino alla concentrazione 1 mM. Era stimolata invece dall'aggiunta di Ca^{2+}. Con l'aggiunta di quantità limitate di Ca^{2+} in presenza di mezzo isotonico in cui il fosfato era sostituito da Tris–HCl, era possibile ottenere una stimolazione transitoria, seguita da ritorno della respirazione ad uno stato controllato. Ciò suggerisce che la stimolazione della respirazione indotta dal Ca^{2+} nelle cellule isolate sia essenzialmente un fenomeno mitocondriale e che il trasporto del Ca^{2+} attraverso la membrana plasmatica avvenga, sotto le condizioni impiegate, per un processo di diffusione, non controllato da meccanismi di trasporto attivo.

INTRODUCTION

Isolated rat hepatocytes have been used in several laboratories to study morphological and metabolic problems in an integrated manner. Earlier methods of isolation of the cells, such as dissociation by the use of calcium binding agents and mechanical disruption of the liver [1–10] have been superseded by enzymatic procedures, in which liver disgregation is achieved essentially through perfusion with collagenase, hyaluronidase or lysozyme, occasionally combined with EDTA [11–18]. The use of enzymes and chelating agent in the disintegration of the liver appears superior to other procedures with respect to reproducibility, yield, cell viability and metabolic activities in the presence of various substrates.

On the other hand, little is known about the mechanisms controlling the access of substrates to the site where they are metabolized in the rat liver cell.

(*) Lavoro eseguito nell'Istituto di Chimica Biologica dell'Università di Padova con contributo del Centro per lo Studio della Fisiologia dei Mitocondri del CNR, Padova.

(**) Nella seduta del 14 aprile 1973.

Ross *et al.* [19] and Hems *et al.* [20] reported that glutamate, aspartate and intermediates of the tricarboxylic acid cycle, if added to the perfused rat liver or to liver slices, are removed at a rate slighter than that of many other metabolites. Thus a major permeability barrier would restrict the entry of these substances into the liver cell.

With the purpose of shedding some light on the question of the conditions which regulate the flow of metabolites through the liver cell membrane, the present paper reports the results of an investigation on the properties of the oxidation of mitochondrial substrates by isolated rat liver cells.

Methods and materials

Male rats of the Wistar strain, weighing 180 to 220 g, fed *ad libitum* on ordinary laboratory chow, were used for all except the gluconeogenesis experiments, for which the rats were fasted for 24 hr. Liver cells were isolated by using essentially the procedure of Berry and Friend [14]. Departures from the original method were as follows. Livers were washed with 10 ml of perfusion medium, removed from the rat, and placed on a proper support in the thermostated cabinet (37° C) of a Passoni perfusion apparatus, type EC3–A. The perfusion medium was comprised of calcium-free Hanks' solution [21] containing 1 % bovine serum albumin. 0.06 % collagenase and 0.12 % hyaluronidase. The enzyme medium (60 ml) was recycled through the liver for 15 min before starting the perfusion with the EDTA–containing medium described by Berry and Friend [14]. Manipulation of the liver at the end of the perfusion and of the liver fragments at the filtration stage was avoided in order to prevent cell damage. Cells were washed three times with Hanks' solution (without calcium, bicarbonate or glucose) containing 10 mM sodium phosphate, pH 7.4, and then resuspended in the same medium. The final suspension was routinely diluted to a concentration corresponding to 60 to 80 mg of cell protein per ml. Protein concentration was determined according to Lowry *et al.* [22] using bovine serum albumin as the standard. Respiration rates were measured at 22° in a 3 ml stirred cell using a Clark oxygen electrode. Unless otherwise indicated, the incubation mixture (2 ml total volume) included 1.73 ml of the medium used to wash and resuspend the cells, 0.25 ml of cell suspension and 0.2 ml of substrate solution. Unless stated otherwise, substrates were used at 10 mM final concentration, as their sodium salts; ADP was added routinely at 0.25 mM final concentration. ADP, ATP (sodium salts), DNP (1), FCCP and inhibitors were used in concentrated solutions, and their volumes (at most 10 µl) were neglected in the calculation of the results. Antimycin A, rotenone, oligomycin and ouabain were dissolved in ethanol. Inhibitors were used in all the experiments

(1) Abbreviations: DNP, 2,4–dinitrophenol; FCCP, carbonyl cyanide *p*–trifluoromethoxyphenylhydrazone; RLC, rat liver cells; BSA, bovine serum albumin.

at the following final concentrations: DNP, 5×10^{-5} M, FCCP, 1×10^{-6} M, antimycin A, 5 μg/ml, rotenone 0.5 μg/ml, oligomycin, 5 μg/ml, atractyloside 0.2 mM, ouabain, 10 μg/ml. The ratio between intracellular and extracellular 260–*nm* absorbing material, extracted with cold perchloric acid, was measured as described by Hommes *et al.* [15]. Gluconeogenesis from lactate was measured according to Berry and Kun [23], and leakage of lactate dehydrogenase from the cells according to Ontko [18]. ^{14}C–leucine incorporation into trichloroacetic acid insoluble proteins of the cells was determined as described by Mookerjea and Marai [24] for incorporation into microsomal proteins of liver slices, using about 10 mg of cell protein and 0.5 mCi L–U–^{14}C–leucine (344 mCi/mM) per flask; incubation were continued for one hr. Collagenase, hyaluronidase, bovine serum albumin (fraction V), oligomycin, antimycin A and rotenone were obtained from Sigma Chemical Co., nucleotides from Boehringer Mannheim Gmbh, ouabain from Merck A.G., radiactive leucine from the Radiochemical Centre. Atractyloside was a kind gift of Dr. A. Bruni.

RESULTS

Integrity of the isolated rat liver cells.

The preparation described above yielded routinely about 5 ml of packed cells (7 to 8×10^7 cells/ml), or 700 to 800 mg of cell protein from a liver of 7 to 8 g (net weight). In the various preparations, at least 85 % of the cells excluded trypan blue. Light microscope examination showed that the cell population consisted of about 90 % free parenchymal cells with apparently uninterrupted plasma membranes. The remaining cells had broken membranes. Determination of 260–*nm* absorbing material in the cell suspension and in the medium as a measure for leakage of nucleotides and breakage of cells gave a ratio of 11.9, in close agreement with that reported by Hommes *et al.* [15]. Gluconeogenesis proceeded linearly for at least one hour (longer times not tested) at a rate of 0.8 n moles of glucose sinthesized/min/mg protein. This value is of the order of rate reported by Berry and Kun [23]. The leakage of lactate dehydrogenase from the isolated liver cells corresponded to 18 % of the total activity. Leucine incorporation was linear for one hour (longer time not measured).

Respiratory activities of the isolated cells.

Endogenous respiration rate, as tested polarographically in the normal incubation medium, was 4.3 *n* atoms of oxygen consumed/min/mg protein (fig. 1). Respiration was not affected by the addition of ADP, but was stimulated markedly by DNP. In agreement with the results of Howard and Pesch [12], the basal rate was higher when 1.25 mM Ca^{2+} or 10 mg/ml bovine serum albumin was added to the normal medium (fig. 1).

When oxidizable substrates were added to the incubation misture, oxygen uptake was stimulated at various but in general modest extents above the basal rate, the highest stimulation having been produced by succinate and pyruvate *plus* malate (Table I). ADP brought about sporadically a further stimulation, that was, in any case, just noticeable. This poor response remained unaltered when ADP concentration was raised to 1 mM. It was however completely abolished by atractyloside (results not shown), like in isolated mitochondria [25, 26].

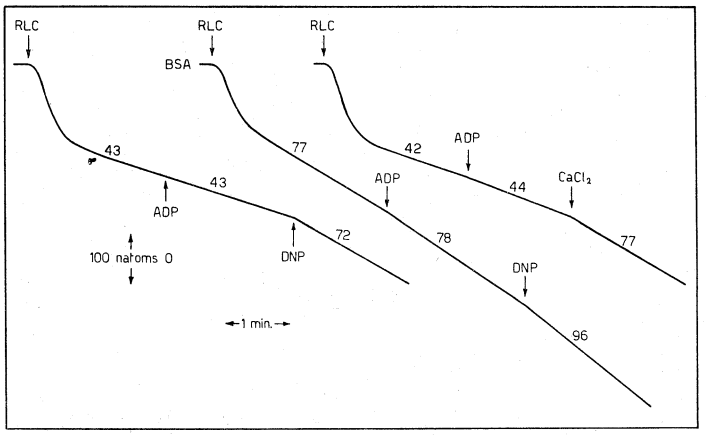

Fig. 1. – Oxygen electrode traces of the endogenous respiration of isolated rat liver cells. Cells corresponded to 10 mg of protein. Other conditions as indicated under "Methods and Materials". The number on the traces refer to *n* atoms of oxygen consumed/min. BSA (10 mg/ml) and $CaCl_2$ (1.25 μmoles/ml) were added where indicated.

A marked stimulation of the oxidation was constantly caused by the addition of DNP or FCCP. Thus in the presence of an uncoupler it was possible to measure accurately the relative order of oxidizability or the specific substrates by the liver cells. Under this condition, isolated cells very actively oxidized succinate, glutamate, α–oxoglutarate, pyruvate plus malate, citrate and β–hydroxybutyrate. Oxygen uptake above the basal rate was very modest or insignificant in the presence of malate, lactate, α–glycerophosphate, ethanol and NADH. Octanoate and oleate slightly stimulated oxygen uptake in the presence of malate.

The respiration of liver cells in the presence of different substrates was stimulated by arsenate. For experiments with arsenate the standard reaction mixture was replaced by a mixture containing 15 mM Tris–HCl and 20 mM arsenate in place of phosphate. Cells were added to the reaction mixture from a suspension containing either phosphate or Tris–HCl (so that the final concentration of phosphate in the assays was 1 nM or nil). In no case was 20 mM arsenate sufficient to release maximal respiration, regardless of the presence of ADP. Maximal respiration was achieved only with the addition of DNP or FCCP.

TABLE I

Oxidative activities of isolated rat liver cells.

SUBSTRATE	No addition	Plus 0.25 mM ADP	Plus 5×10^{-5} M DNP
None	4.3	4.3	7.2
Succinate	10.1	11.6	40.4
Glutamate	6.2	6.7	29.8
α–oxoglutarate	6.7	7.2	20.2
Malate	8.2	9.1	10.6
Pyruvate with malate	10.1	13.0	19.2
Citrate	5.8	6.2	18.3
β–hydroxybutyrate	7.2	7.7	15.4
DL–lactate	6.2	8.2	13.0
α–glycerophosphate	8.6	8.6	12.0
Ethanol	6.8	7.2	11.0
NADH	7.7	7.7	9.6
None, BSA	7.7	7.8	9.6
Malate, BSA	7.2	8.2	9.6
Malate, octanoate, BSA	10.1	10.1	14.4
Malate, oleate, BSA	9.1	9.1	11.6

Substrates were 10 mM, except NADH (0.5 mM), octanoate and oleate (1 mM). Results are expressed in *n* atoms of oxygen consumed/min/mg of cell protein, at 22° C.

The characteristics of cell respiration remained essentially unaltered when cells were washed, resuspended and tested in media differing in various respects from the standard solution. Media tested include: *all-sodium medium*, in which potassium chloride of the standard solution was replaced by an equivalent molarity of sodium chloride; *all-potassium* medium, in which sodium chloride was replaced by an equivalent molarity of potassium chloride; *no-phosphate medium*, in which phosphate buffer was replaced by 15 mM Tris–HCl, pH 7.4; *no-mineral medium*, in which 15 mM Tris–HCl, pH 7.4, was the buffering agent and osmolality was assured by 0.25 M mannitol. When all-sodium or all-potassium media were employed, substrates were neutralized with the corresponding cations.

Properties of succinate and glutamate oxidation.

Succinate and glutamate, which are most actively oxidized by the liver cells, were used to investigate in greater detail the characteristics of the respiration depending on FAD and NAD–containing dehydrogenases. The results obtained were in agreement with the properties of the oxidation of the substrates by mitochondria. Oligomycin slowly inhibited oxygen uptake in the presence of succinate, to levels lower than the basal rate observed in the absence of exogenous substrates; the inhibition was relieved by DNP or FCCP (fig. 2, trace A). Succinate oxidation was sensitive to antimycin A

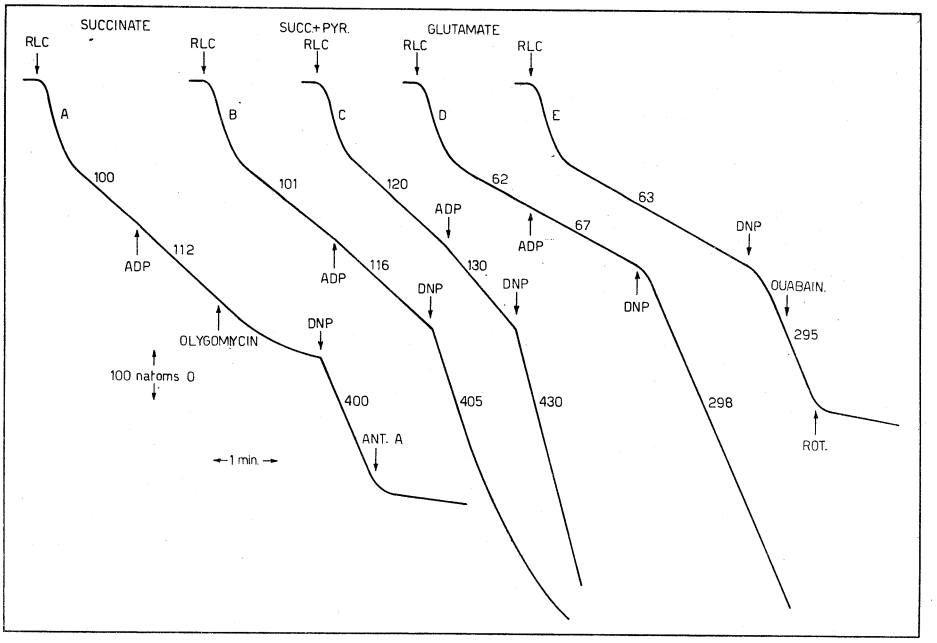

Fig. 2. – Oxidation of succinate and glutamate by rat liver cells. Conditions as indicated under fig. 1.

inhibition, which was immediate at concentrations of inhibitor comparable to those effective on isolated mitochondria. Similar properties were exhibited by the respiratory activity in the presence of glutamate (fig. 2, trace D). Rotenone, ineffective on succinate oxidation, was strongly inhibitory on glutamate oxidation (fig. 2, trace E).

When cells were respiring succinate, addition of DNP caused an abrupt of oxygen uptake, which proceeded linearly for a few seconds. Then the oxidation rate decreased steadily in a curvilinear manner (fig. 2, trace B). The same progression of events, although less pronounced, was caused by FCCP. The addition of 1 mM ATP together with oligomycin was without effect. On the contrary, oxidation of succinate proceeded linearly till oxygen exhaustion if pyruvate was simultaneously present in the reaction mixture

(fig. 2, trace C). Since it has been reported [27] that uncouplers compete with succinate for entry into the mitochondria, it has been tested whether the oxidation of succinate by isolated cells present different kinetics in the absence and in the presence of FCCP. Rates of oxidation were determined in the presence of different concentrations of succinate before and after addition of FCCP (fig. 3). The point of curve B, fig. 3, represent the linear initial

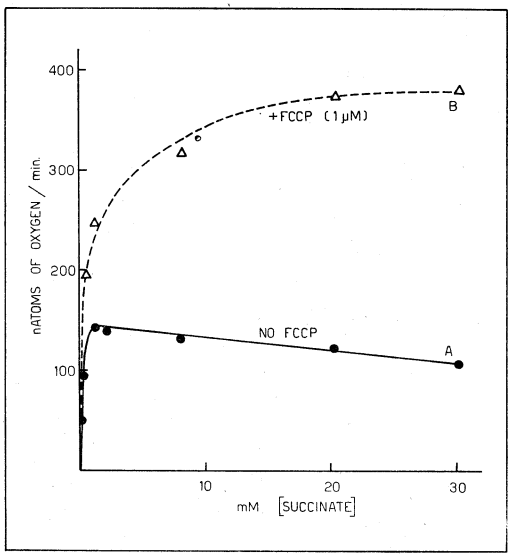

Fig. 3. – Saturation curves of succinate oxidation in the absence and presence of uncoupler. Rates of oxygen uptake were corrected for endogenous respiration. ADP (0.25 mM) was present in curve A experiments. Cell protein corresponded to 9.2 mg.

velocities following FCCP addition. It appears that, in the absence of uncoupler, succinate oxidation is fully saturated at 2 mM succinate, while, in the presence of FCCP, saturation was reached only at 30 mM succinate.

It has been also explored whether liver cell respiration was influenced by ouabain, a known inhibitor of transport of Na^+ and K^+ [28] and of amino acids and sugars [29, 30] in many types of cells. No effect has been observed with glutamate (fig. 2, trace E) or with any other of the substrates listed in Table I, used at substrate concentrations ranging from 0.5 to 10 mM.

Effect of calcium ions on cell respiration.

It has been reported above that 1.25 mM Ca^{2+} added to the incubation mixture stimulates the basal rate of respiration of the liver cells. An analogous stimulation has been obtained on the oxidation of exogenous substrates. However, maximal stimulation could be obtained with a concentration of Ca^{2+} as low as 0.15 mM added during the course of the oxidation. With low concentrations of Ca^{2+} the stimulation was transitory and the extra-amount of oxygen consumed was proportional to the amount of Ca^{2+} added. This phenomenon is reminiscent of Ca^{2+} stimulated oxygen uptake in mitochondria, linked to active Ca^{2+} accumulation [31]. The effect of Ca^{2+} on cells respiring

succinate or glutamate, was best observed in an incubation mixture in which 15 mM Tris–HCl was substituted for phosphate as the buffering component (fig. 4). A Ca^{2+}/O ratio equal to 4.1 was obtained with succinate and to 5.25 with glutamate. These ratios gives a Ca^{2+}/O ratio of (4.1/2) 2.05 per phosphorylation site with succinate and (5.25/3) 1.75 with glutamate, with an average

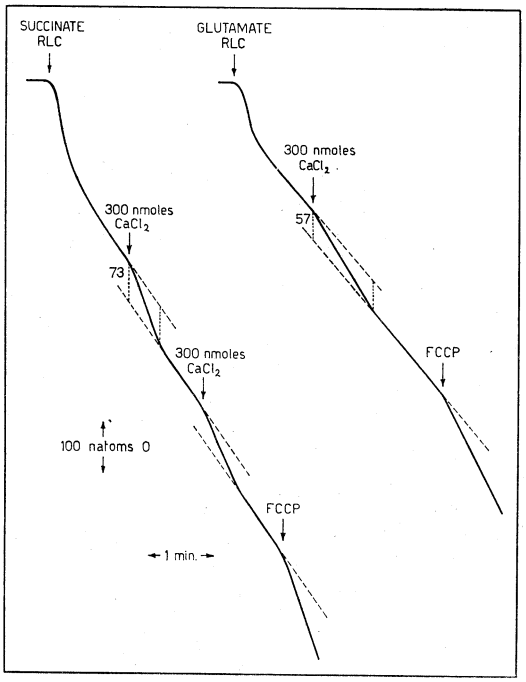

Fig. 4. – Effect of Ca^{2+} addition on the oxidation of succinate and glutamate by rat liver cells. The incubation mixture contained 15 mM Tris–HCl, pH 7.4, in place of phosphate. Cells corresponded to 12.6 mg of protein. The numbers close to the traces refer to the extra-amount (natoms) of oxygen consumed following Ca^{2+} addition. Other conditions as indicated under "Methods and Materials".

of (2.05+1.75)/2 1.90. This figure is in good agreement with the value of 1.79 observed with liver mitochondria [32]. The resting respiration following the burst of oxygen uptake induced by Ca^{2+} could be again stimulated by the addition of uncoupler.

Discussion

The properties described above indicate that the preparation employed in the present work satisfies the commonly accepted parameters of integrity of isolated liver cells. The reported rate of endogenous respiration compares quite favourably with the Q_{O_2} of 8.4 to 12.1 obtained by Berry and Friend with a manometric technique at 37°C [14].

The rate of respiration observed in the presence of exogenous metabolites does not reflect the true capacity of the cells to oxidize the individual substrates unless an uncoupler is added. Indeed intact cells appear to respire under tightly controlled conditions, but, at variance with the behaviour of tightly coupled mitochondria, are unreactive to ADP addition, and their respiration can be fully released, under the present experimental conditions,

only by the addition of an uncoupler. Very probably, in the majority of the cells the exchange between extracellular ADP and intracellular ATP is hindered by the same accessibility barrier which prevents loss of 260–*nm* absorbing material by the intact plasma membrane. Thus exogenous ADP does not gain access to the mitochondrial phosphorylation system. It appears that the lack of response to ADP can be one of the most stringent tests of integrity of the isolated cell plasma membranes. The reported occasional and small response to ADP, which has the characteristics of a stimulation of the mitochondrial respiration, is attributable to the small percentage of cells with damaged plasma membranes.

The action of oligomycin, antimycin A and rotenone on cell oxidations resembles rather closely the effect of these inhibitors on mitochondrial oxidation. This can be understood on the basis of the prevailing hydrophobic properties of these substances, which make their transport through cell membrane easy.

The interpretation of the effect of the uncouplers can be readily found in a release of the control of the mitochondrial respiratory chain. But it is possible that this mode of action is accompanied also by a modification of the permeability of the plasma membrane *versus* various metabolites, caused by the lipophylic nature of the uncoupler. The rate of oxidation of some externally added substrates, e.g. succinate and glutamate, as measured in the presence of an uncoupler, is of the order of magnitude which can be theoretically calculated assuming that mitochondrial protein represents 20 per cent of the cell protein. It is clear that, although rat liver seems to possess a permeability for succinate and glutamate [20], in isolated cells such a barrier does not prevent succinate and glutamate from very quickly reaching the active sites of the corresponding dehydrogenases at the concentrations used in this work and in the presence of uncoupler. The same is essentially true for the other substrates actively oxidized by the cells.

The effect of the uncoupler on the kinetics of succinate oxidation by the cells is very probably complex. The steady decrease following the high initial rate of oxidation is observed also on isolated mitochondria, and can be interpreted as inhibition of succinate dehydrogenase by accumulation of oxaloacetate [32] or alteration of the kinetic parameters of succinate dehydrogenase in the uncoupled state of the mitochondria [33]. The fact that pyruvate addition is sufficient to preserve a linear rate of succinate respiration in the presence of uncoupler suggests that the major component of the observed phenomenon is the accumulation of products of succinate oxidation. However the apparent decrease in substrate affinity caused by uncouplers on the systems oxidizing succinate in the cell can be in part explained on the basis of the competition between succinate and uncoupler for entry into the mitochondria [27].

None of the reactions studied here was inhibited by ouabain. This should apparently exclude participation of a Mg^{2+}, Na^{+}, K^{+} dependent ATPase in the transport of the metabolites employed. However, two alternative possi-

bilities seem more plausible. Firstly, it is possible that the conditions used to measure oxidation activities are not suitable to evidentiate dependence of entry of the substrates into the cells on active transport. Secondly, the action of enzymes employed to disintegrate the liver could modify in some way the plasma membranes so that a tight control of ion and metabolite transport is at least in part altered. This is in agreement with the report of Berry and Friend [14] that cells prepared by their method progressively lose potassium ions.

The stimulation of respiration by 1.26 mM Ca^{2+} has been explained by Howard and Pesch [12] by assuming that Ca^{2+} stabilize the plasma membranes of the cells or antagonize a metabolic inhibitor. In the present work, the burst of oxygen uptake following addition of low concentrations of Ca^{2+} to respiring cells appears to be due to release of the respiratory control accompanying Ca^{2+} accumulation in mitochondria. It is probable that this explanation holds also for the stimulatory effect of comparatively high concentrations of Ca^{2+} on cell respiration, although Ca^{2+} may have important effects on other aspects of cell metabolism. The fact that with the addition of small amounts of Ca^{2+} it was obtained with the cells a Ca^{2+}/O ratio of the order of that exhibited by isolated mitochondria suggests that under the present conditions Ca^{2+} is transferred through the plasma membrane by simple diffusion, independently from mechanisms of active control of the cytoplasmic Ca^{2+} concentration.

References

[1] Anderson W. G., « Science », *117*, 627–628 (1953).
[2] Kaltenbach J. P., « Exp. Cell Res. », *7*, 568–571 (1954).
[3] Longmuir I. S. and Rees W. A., « Nature », *177*, 997 (1956).
[4] Laws J. O. and Stickland L. H., « Nature », *178*, 309–310 (1956).
[5] Branster M. V. and Morton R. K., « Nature », *180*, 1283–1284 (1957).
[6] Jacob S. T. and Bhargava P. M., « Exp. Cell Res. », *27*, 453–467 (1962).
[7] Berry M. N., « J. Cell Biol. », *15*, 1–8 (1962).
[8] Berry M. N. and Simpson F. O., « J. Cell Biol. », *15*, 9–17 (1962).
[9] Takeda Y., Ichimara A., Tanioka H. and Inove H., « J. Biol. Chem. », *239*, 3590–3596 (1964).
[10] Ontko J. A., « Biochim. Biophys. Acta », *137*, 13–22 (1967).
[11] Howard R. B., Christensen A. K., Gibbs F. A. and Pesch L. A., « J. Cell Biol. », *35*, 675–684 (1967).
[12] Howard R. B. and Pesch L. A., « J. Biol. Chem. », *243*, 3105–3109 (1968).
[13] Jezick P. F. and Liberti J. P., « Arch. Biochem. Biophys. », *134*, 442–449 (1969).
[14] Berry M. N. and Friend D. S., « J. Cell Biol. », *43*, 506–520 (1969).
[15] Hommes F. A., Draisma N. I. and Molenaar I., « Biochim. Biophys. Acta », *222*, 361–371 (1970).
[16] Capuzzi D. M., Rothman V. and Margolis S., « Biochem. Biophys. Res. Comm. », *45*, 421–429 (1971).
[17] Ingebretsen W. R. Jr. and Wagle S. R., « Biochem. Biophys. Res. Comm. », *47*, 403–410 (1972).
[18] Ontko J. A., « J. Biol. Chem. », *247*, 1788–1800 (1972).

[19] ROSS B. D., HEMS R. and KREBS H. A., « Biochem. J. », *102*, 942–951 (1967).
[20] HAMS R., STUBBS M. and KREBS H. A., « Biochem. J. », *107*, 807–815 (1968).
[21] HANKS J. H. and WALLACE R. E., « Proc. Soc. Exp. Biol. and Med. », *71*, 196–200 (1949).
[22] LOWRY O. H., ROSEBROUGH N. J., FARR A. L. and RANDALL R. J., « J. Biol. Chem. », *193*, 265–275.
[23] BERRY M. N. and KUN E., « Eur. J. Biochem. », *27*, 395–400 (1972).
[24] MOOKEREJA S. and MARAI S., « J. Biol. Chem. », *246*, 3008–3017 (1971).
[25] KLINGENBERG M. and PFAFF E. in *Regulation of Metabolic Processes in Mitochondria* (Tager J. M., Papa S., Quagliarello E. and Slater E. C., eds.), pp. 180–201, Elsevier, Amsterdam 1966.
[26] BRUNI A. in *Regulation of Metabolic Processes in Mitochondria* (Tager J. M., Papa S., Quagliarello E. and Slater E. C., eds.), pp. 275–292, Elsevier, Amsterdam 1966.
[27] QUAGLIARELLO E. and PALMIERI F. in *Energy Transduction in Respiration and Photosynthesis* (Quagliarello E., Papa S., and Rossi C. S., eds.), pp. 205–235, Adriatica Editrice, Bari 1971.
[28] GLYNN I. M., « Pharmacol. Rev. », *16*, 381–407 (1964).
[29] CSAKY T. Z., « Am. J. Physiol. », *201*, 999–1001 (1961).
[30] SCHULTZ S. G., FUISZ R. E. and CURRAN P. F., « J. Gen. Physiol. », *49*, 849–866 (1966).
[31] LEHNINGER A. L., ROSSI C. S. and GREENAWALT J. W., « Biochem. Biophys. Res. Comm. », *10*, 444–448 (1963).
[32] ROSSI C. S. and LEHNINGER A. L., « Biochem. Biophys. Res. Comm. », *11*, 441–446 (1963).
[33] GREVILLE G. D. in *Regulation of Metabolic Processes in Mitochondria* (Tager J. M., Papa S., Quagliarello E. and Slater E. C., eds.), pp. 86–107, Elsevier, Amsterdam 1966.
[34] SINGER T. P., GUTMAN M. and KEARNEY E. B. in *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone G. F., Carafoli E., Lehninger A. L., and Siliprandi N., eds.), pp. 41–65, Academic Press, New York 1972.