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A novel, simple and rapid method for the isolation of mitochondria which exhibit respiratory control, from rat small intestinal mucosa

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A method is described for the isolation of functional mitochondria from rat intestinal mucosa. Its novel feature is the removal of mucus from the initial homogenate by treatment with DEAE-cellulose. The preparations exhibited acceptable ADP:O ratios, high State-3 respiration rates, and respiratory control ratios in excess of 3 when succinate, β-hydroxybutyrate, glutamate/malate and glutamine were test substrates.

Introduction

Some two decades ago investigators first achieved success in the development of techniques for the sub-cellular fractionation of intestinal epithelial cells of different species, e.g., rabbit [1] and guinea pig [2]. In more recent years, other workers have attempted to isolate functional, coupled intestinal mucosal mitochondria. Progress in this direction was made with guinea pig small intestine [3], hamster small intestine [4] and rat colon [5]. However, all methodologies were, and are as yet, complicated by the presence of mucus in the mucosal homogenates, particularly when applied to the small intestine of the rat, which contains a large number of Goblet cells [3]. The release of Goblet cell mucus results in delays in preparation, substantial cross contamination of subcellular fractions and the isolation of uncoupled mitochondria with a poor yield [3]. In view of the paucity of knowledge of the functional characteristics of small intestinal mucosal mitochondria and the controversy regarding the substrate requirements of the respiring intestinal mucosa [6,7], we have attempted to develop a rapid and simple method for the isolation of coupled mitochondria relatively free from contamination with mucus and other subcellular components.

In this study, we briefly reevaluated previously published methods of isolation of mitochondria from mucosa of the small intestine before investigating, in rats, a new method involving mucus removal during mucosal homogenization by adsorption to an anion-exchange cellulose, DEAE cellulose. After removal of anion-exchange cellulose with adherent mucus, together with cell debris and nuclear material, by low speed centrifugation, coupled mitochondria were isolated from the supernatant fraction by conventional procedures. Use of this technique may assist workers to resolve some of the problems associated with the transepithelial movement of substrates that are both transported and metabolized.

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Abbreviations: EGTA, 1,2-di(2-aminoethoxy)ethane-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; Hepes, 4-2(hydroxyethyl)-1-piperazineethanesulphonic acid.

Materials and Methods

Animals

Male rats of the Rowett Hooded Lister strain, about 300 g in weight, were used for experimenation. These animals were fed ad libitum (Oxoid pasteurised rat breeding diet, Oxoid, Basingstoke, Hampshire, U.K.) and allowed free access to drinking water. Samples of intestine were also obtained from 9–10 month old steers maintained on a semi-synthetic skimmed milk/oat husk based diet.

Chemicals

Reagents and biochemicals were of analytical grade quality and purchased from either British Drug House (BDH Ltd., Poole, Dorset, U.K.) or Sigma (Poole, Dorset, U.K.) with the exception of bovine serum albumin, fatty acid free, fraction V, which was obtained from Miles Biochemicals (Stoke Poges, Slough, U.K.). Whatman DE32 (diethylaminoethylcellulose) microgranular anion-exchange cellulose was purchased from Chromatography Services Ltd. (Hoylake, U.K.) and heparin from Paines and Byrne (Greenford, Middlesex, U.K.). Kits for acid and alkaline phosphatase assay were supplied by Sigma, and N140 (previously 9N) nylon cloth, for the filtration of mucosal samples, from Henry Simon Ltd. (Stockport, U.K.).

Media for mitochondrial isolation

Subsequent to our initial unsuccessful attempts to isolate viable intestinal mucosal mitochondria, we eventually modified the composition of the isolation solutions to those shown in Table I. As these solutions resulted in the isolation of mitochondria which exhibited satisfactory respiratory function, their composition was left unchanged.

DEAE-cellulose preparation

The DEAE-cellulose, in its original dry form and unwashed, was suspended on the morning of use at a concentration of 10 g anion-exchange cellulose in 100 ml isolation medium A for rat mucosal treatment. Half this concentration was used for steer mucosa (5 g/100 ml). Heparin was added at 7500 units/100 ml, so was dithiothreitol, to give a final concentration of 1 mM. Results of experiments in which anion-exchange cellulose was precycled in 0.5 M acid and 0.5 M alkali and adjusted to pH 7.4 prior to use, as recommended by the manufacturers for normal chromatographic use, resulted in no improvement in the respiratory properties of the preparations and if anything they were marginally inferior.

Isolation of the intestine and separation of the mucosa in the rat

Each rat preparation required the whole in-

TABLE I

COMPOSITION OF THE MEDIA USED FOR FLUSHING THE INTESTINAL MUCOSA IN SITU ('GUT WASH'), ISOLATING MITOCHONDRIA AND RESPIRATION STUDIES

All solutions adjusted to pH 7.4. Isolation media kept at 4°C. Respiration medium used at 30°C. 'Gut wash' oxygenated with 100% O_2 for at least 30 min prior to use.

Compound	Gut wash	Isolation medium A (IMA)	Isolation medium B (IMB)	Respiration medium
Sucrose (mM)	160	70	70	70
Mannitol (mM)	110	220	220	220
Hepes (mM)	2	2	2	2
Tris (mM)	11	–	–	–
EDTA (mM)	–	–	–	0.75
EGTA (mM)	0.25	0.5	–	0.50
PMSF (mM)	0.1	0.1	–	–
$MgCl_2$ (mM)	–	–	–	2.5
KH_2PO_4 (mM)	–	–	–	2.5
Bovine serum albumin (g/100 ml)	–	0.37	1.2	0.13

testine. Rats were rendered unconscious by ether anaestheisa and the abdominal cavity opened. The small intestine was flushed with ice cold 'gut wash' (see Table I) in situ, the ileum cut through near the caecum and the entire small intestine removed and placed in a beaker containing the same medium. The intestine was everted over a metre length stainless steel rod (2 mm diameter) and cut into approximately equal lengths. Each length was subsequently dried by blotting between layers of Whatman No. 50 filter paper and the mucosa separated from the musculature by scraping with a microscope slide. During this procedure the intestine was laid out on a cooled glass plate. The mucosal scrapings from the intestine were combined at this stage and for 'contamination' studies a small sample taken (less than 0.2 g) for homogenisation in 'resin free' medium for enzyme and protein determinations (see Analytical methods).

Anion-exchange cellulose treatment of the mucosa (and liver) and isolation of mitochondria in the rat

Normally a total of about 3 g mucosa was obtained and this was stirred to an almost homogenous state with 30 ml of the anion-exchange cellulose suspension. After standing for approx. 2 min a further 40 ml isolation medium A was added to the suspension, with stirring, and the mixture homogenised using a Potter-Elvehjem homogeniser. Six passes of the pestle were used. The homogenate was diluted further by the addition of a further 50 ml isolation medium A and rehomogenised. This final homogenate was distributed among 4×40 ml tubes and centrifuged at $750 \times g$ for 10 min at 4°C in an MSE 18 refrigerated centrifuge (MSE Ltd., Crawley, U.K.). The resulting supernatant was filtered through a single layer of nylon cloth and recentrifuged at $10\,000 \times g$ for 7 min. The crude mitochondrial pellet obtained still typically contained a layer of mucus above the mitochondrial-rich fraction. This was carefully 'scraped off' with a spatula before resuspension, using a 'cold finger', in a final 35 ml of isolation medium A. Following centrifugation of this suspension at $14\,000 \times g$ for 7 min the resulting pellet was resuspended in isolation medium B prior to a final $14\,000 \times g$ centrifugation. The pellet was then resuspended in 0.8 ml to 1.0 ml isolation medium B. In order to test whether the 'anion-exchange cellulose' resulted in gross mitochondrial damage, liver homogenates were prepared and either treated with or without anion-exchange cellulose. They were subsequently carried through the same procedure as used for isolation of mucosal mitochondria.

Preparation of steer duodenal mitochondria

Steer duodenum was sampled, as soon as possible, from animals slaughtered by captive bolt pistol. Samples of 15 cm were taken approx. 15 cm distal to the pylorus. They were immediately transferred to 'gut wash' solution kept on ice. On arrival in the laboratory (less than 5 min after slaughter) they were slit longitudinally, washed with isolation medium A, blotted and the mucosa removed from the musculature by scraping with a microscope slide. The amount of intestine sampled was more than adequate for provision of 10 g mucosa used for isolation of mitochondria. The 10 g mucosa was stirred with 80 ml of a suspension of 5 g anion-exchange cellulose in 100 ml isolation medium A and after standing 2 min, homogenised. Thereafter the procedure followed was exactly as that for rat mucosal homogenate.

Analytical methods

The final mitochondrial preparation contained a high concentration of bovine serum albumin in its 'suspension medium'. In order, therefore, to estimate mitochondrial protein, a 100 μl aliquot was diluted to 2 ml with mannitol buffer (220 mM) (pH 7.4) and centrifuged for 8 min in a Beckman microfuge B (Beckman Instruments Ltd., Glenrothes, U.K.) at $8000 \times g$. The pellet was washed in the same medium and dissolved in 100 μl 0.25% SDS plus a further 200 μl 0.2 M NaOH before the protein content was estimated using the Bio-Rad dye binding procedure (Bio-Rad GmbH, Munich, West Germany).

Contamination of the mitochondrial fraction by brush borders was assessed by assay for the presence of alkaline phosphatase (EC 3.1.3.1; Sigma kit), microsomal contamination by assay of rotenone insensitive NADPH cytochrome-*c* reductase (EC 1.6.2.4, N.T. Davies, unpublished results) and lysosomal contamination by assay of acid phosphatase (EC 3.1.3.2; Sigma kit). Activity of cytochrome-*c*-oxidase (EC 1.9.3.1) of mitochondria swollen in hypotonic buffer (0.05 M

KH_2PO_4, pH 7.4) was assayed by the procedure previously reported for tissue homogenates (Mills & Dalgarno, 1969 [8]). The enzyme studies described above were also carried out on the mucosal (anion-exchange cellulose-free) homogenate. The nuclear marker DNA, estimated as by Burton (1958) [9], was also determined on both mitochondrial and homogenate fractions.

Respiration studies

Oxygen uptake by isolated mitochondria in the presence of the substrates succinate, β-hydroxy butyrate, glutamate/malate and, with the rat, glutamine, was measured at 30°C in the cell of a Gilson Oxygraph (Anachem, Luton, U.K.) fitted with a Clark electrode. Rates of oxygen consumption, calculated in a similar manner to that described in Ref. 10 were also monitored with ADP added (State 3) and after depletion of the exogenous ADP (State 4 respiration). The respiratory control ratios (State 3/State 4 respiration), an indicator of mitochondrial functional integrity, were calculated, as were the ADP/O ratios.

Results

In preliminary experiments we reevaluated or attempted to modify previously documented procedures as well as investigating techniques successfully applied to other tissues. These included: (1) initial isolation of enterocytes by incubation of everted rat intestine in Krebs–Ringer solution containing hyaluronidase [11], before homogenisation of the cell pellet and differential centrifugation [3]; (2) prior incubation of mucosa, prepared by sampling everted intestines, with hyaluronidase to disperse mucus before differential centrifugation; (3) removal of mucus by treatment of mucosal homogenates with $BaSO_4$ [12]. In addition, attempts were made to isolate mitochondria from mucosa homogenised in different media including sucrose/mannitol (non-ionic) or KCl (ionic). However, none of these approaches nor further variations in methodology as applied to other tissues [13] such as employing different media concentrations of bovine serum albumin, dithiothreitol, heparin, or EDTA and/or EGTA, resulted in isolation of mitochondria exhibiting acceptable respiratory function (respiratory control ratios are greater than 2.0).

Rat intestinal mucosal mitochondria, isolated by the 'anion-exchange cellulose treatment' procedure which we finally adopted could be prepared with reproducible yields and showed consistent respiratory properties. Initial respiration rates in the absence of added substrate (State 1) or after addition of substrate were low, but oxygen consumption increased markedly when ADP was added (State-3 respiration). After the depletion of exogenous ADP (State-4 respiration) the rate of oxygen consumption decreased abruptly.

The results of a series experiments in which a number of preparations were tested with different substrates are shown in Table II, together with result of similar studies on rat liver mitochondria prepared either with or without an 'anion-ex-

TABLE II

OXIDATIVE PHOSPHORYLATION BY RAT INTESTINAL MUCOSAL MITOCHONDRIA, AND RAT LIVER MITOCHONDRIA PREPARED EITHER IN THE PRESENCE OR ABSENCE OF DEAE-CELLULOSE

Mitochondria were prepared as described in the text. RCR, respiratory control ratio. Results: Means ± S.E., $n = 6–12$ preparations (mucosal preparations) or mean of 2 (liver preparations); ND, not determined.

Substrate	Rat intestinal mucosa		Rat liver			
	RCR	ADP:O	without anion-exchange cellulose		with anion-exchange cellulose	
			RCR	ADP:O	RCR	ADP:O
Succinate	3.62 ± 0.16	1.62 ± 0.04	4.10	1.57	3.42	1.52
β-Hydroxybutyrate	3.44 ± 0.19	2.16 ± 0.07	4.11	2.20	3.29	2.22
Glutamate/malate	3.26 ± 0.16	2.33 ± 0.06	4.19	2.26	4.04	1.82
Glutamine	3.27 ± 0.30	2.06 ± 0.07	ND	ND	ND	ND

TABLE III

OXIDATIVE PHOSPHORYLATION BY STEER MUCOSAL MITOCHONDRIA

Mitochondria were prepared as described in Materials and Methods. Results: means ± S.E., $n = 3$. NM, not measurable due to poor coupled response.

Substrate	Respiratory control ratio	ADP:O
Succinate	4.80 ± 0.41	1.60 ± 0.05
β-hydroxybutyrate	NM	NM
Glutamate/malate	3.92 ± 0.18	2.18 ± 0.08

change cellulose treatment' of the initial homogenate. All preparations exhibited acceptable respiratory control and ADP : O ratios. State-3 respiration rates of the mucosal mitochondria were high compared with those of liver (Results with succinate as substrate, ngatoms O/min per mg protein, mucosa 374.3 ± 10.2; liver 108.3 ± 2.9). However, the high metabolic activity of the small intestine has long been recognized [14]. The mucosal mitochondria had similar respiratory control ratios and ADP : O values to those of 'anion-exchange cellulose-treated' liver preparations. Furthermore, a comparison of the respiratory properties of the two liver mitochondrial preparations suggest that the anion-exchange cellulose treatment had either no or only slight, deleterious effects on mitochondrial respiratory function.

Further evidence that intestinal mitochondrial integrity was not adversely affected by anion-exchange cellulose treatment can be inferred from our finding that permeability to exogenous NADH was minimal as assessed from its failure to stimulate oxygen consumption of mitochondria respiring in State I and caused a barely detectable increase upon the subsequent addition of ADP (results not shown).

The results of a limited study on steer mucosal mitochondria are shown in Table III. When succinate or glutamate/malate were substrates respiratory control and ADP : O ratios either were the same as those of rat, or tended to be slightly higher. However, steer mitochondria exhibited extremely low rates of respiration and poor tightness of coupling when β-hydroxybutyrate was tested as substrate.

The results of studies on contamination of the rat mucosal mitochondrial fraction by other subcellular components are shown in Table IV. No evidence was obtained of significant contamination. Thus the recoveries in the mitochondrial fraction, relative to those in an initial (anion-exchange cellulose-free) homogenate, of the activities of alkaline phosphatase, acid phosphatase, NADPH-cytochrome *c* reductase and concentration of DNA, markers, respectively, for brush borders, lysosomes, microsome and nuclei, were less than 5%. In addition, the marked increase in activity of cytochrome oxidase in the mitochondria (results, mean, $n = 3$) 5.06 μmol cytochrome *c* oxidized/min per mg protein compared with the initial homogenate, 0.126, similarly indicates a high degree of purity of these mitochondrial preparations.

Previous attempts to isolate functional mitochondria from rat small intestinal mucosa have

TABLE IV

ASSESSMENT OF THE CONTAMINATION OF THE MITOCHONDRIAL FRACTION OF RAT INTESTINAL MUCOSA BY OTHER SUBCELLULAR COMPONENTS AND THE INCREASE IN ACTIVITY OF CYTOCHROME OXIDASE RELATIVE TO THAT IN THE INITIAL MUCOSAL HOMOGENATE

Alkaline phosphatase, acid phosphatase, NADPH-cytochrome-*c*-reductase (rotenone insensitive) DNA and cytochrome-*c*-oxidase assayed as described in the text and results expressed as activities (or amount) recovered in the mitochondrial fraction as a percent of that present in the initial homogenate. Results are mean values of three determinations.

Subcellular 'marker'	Subcellular fraction	% recovery
Alkaline phosphatase	brush borders	< 0.2
Acid phosphatase	lysosomal	< 0.2
NADPH-cytochrome-*c*-reductase	microsomal	< 5
DNA	nuclear	trace
Cytochrome *c* oxidase	mitochondrial	> 35

been largely unsuccessful [3]. The resultant mitochondrial preparations, although coupled, exhibited poor respiratory control (respiratory control ratios are less than 2.0) and substantial contamination with other subcellular components.

Discussion

During the preparation of this paper a report was published [15] on the isolation of rat mucosal mitochondria relatively free from contamination with other subcellular fractions, which were coupled and showed respiratory control ratio values comparable to those we are reporting. However, since both of these previously published methods require a preliminary separation of isolated epithelial cells either by mechanical means [3] or enzyme treatment [15] of the intestine before homogenisation and differential centrifugation, it is likely that their procedures are not as rapid as the 'anion-exchange cellulose' method reported here.

In conclusion, we describe a method for the isolation of functional mitochondria from rat intestinal mucosa, the principle of which depends upon the removal of mucus from the mucosal homogenate by DEAE cellulose. The procedure is both simple and rapid; requiring neither more expertise nor more preparation time than for the isolation of mitochondria from liver homogenates. That the technique may be of general applicability to mucosa from other species which have fewer Goblet cells and hence less adherent mucus is suggested by its successful application to the preparation of steer mucosal mitochondria.

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