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A Mechanical Cell Disintegrator and its Application for the Preparation of Mitochondria

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A mechanical cell disintegrator is described, using Ballotini glass beads. The capsule (34 ml) is surrounded by an ice cap, which keeps the preparation at 1–1.5°C during the procedure. The performance is 4200 cycles/min with a stroke of 14 mm.

Ehrlich ascites tumour cells were totally disintegrated in 6 sec. The mitochondria isolated from this preparation satisfied biochemical criteria of being intact.

The isolation of mitochondria from cells requires a mild disintegration method. The homogenization with a Potter-Elvehjem homogenizer is effective for disrupting a number of animal cells under conditions which do not destroy the mitochondria. Other cell types, however, have very resistant cell walls, which require more effective methods. Nossal[1] has described a mechanical shaker which disintegrates 2 g wet weight yeast cells in 4.5 min, using Ballotini glass beads as the abrasive. This method was further developed by Merkenschlager *et al.*,[2] and the samples were kept at a temperature below 5°C by cooling with liquid carbon dioxide; however, the cooling device is difficult to control. For this reason an effective mechanical shaker where the preparation is kept at 1–1.5°C during the procedure was developed in our laboratory. The cooling is achieved by an ice cap surrounding the stainless steel capsule which contains the preparation.

This work describes the construction of the cell disintegrator, as well as the properties of mictochondria isolated from Ehrlich ascites tumour cells.

EXPERIMENTAL

Materials. *N*,*N*′,*N*′,*N*-Tetramethyl-*p*-phenylenediamine (TMPD) was obtained from Fluka AG. Hexokinase (type III from yeast), ATP, cytochrome *c* (type III from horse heart), rotenone, antimycin A, and bovine serum albumin fraction V were products of the Sigma corporation. The other reagents were of highest purity, analytical grade.

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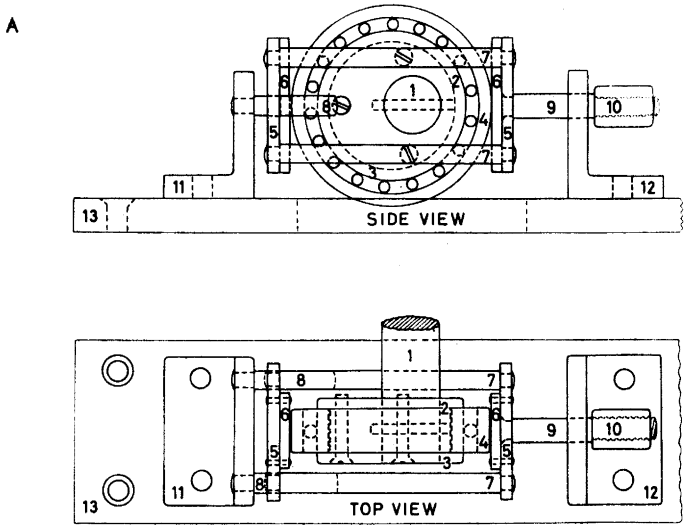
Ballotini glass beads (The English Glass Co., Leicester, England), No. 12 (0.17—0.18 mm diam.) were used.

The Ehrlich ascites tumour cells were obtained from Dr. L. Révész, Karolinska Institutet, Stockholm, and maintained by intraperitoneal inoculations into hybrid Swiss mice of both sexes, according to the technique of Klein *et al.*[3] The cells were washed twice in saline before use.

Fig. 1. The machine consists of three main parts which are mounted on a steel base (Fig. 1 A): the driving unit, the eccentric bolt and the cylindrical capsule with aluminum frame (Fig. 1 B).

A 1 hp electric motor 220 V, 1450 rpm is powering the drive shaft by a V-belt. The drive shaft can attain a speed of 4200 rpm, and is mounted in two ball bearings (SKF—S.—1505). A balance weight is mounted on the drive-shaft. The eccentric bolt, with 7 mm between the center of the drive shaft and the eccentric bolt, is mounted on the drive-shaft. The eccentric bolt causes the steel frame to oscillate with a stroke of 14 mm. The steel frame in turn moves the aluminum frame to which the capsule is mounted.



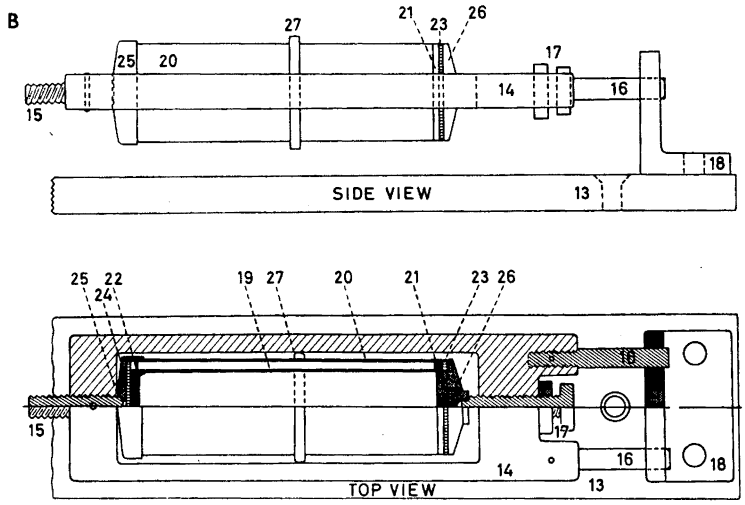


Fig. 2, A and B. All dimensions in mm. 1. Drive-shaft: steel. 20 diam × 44. 2. Eccentric bolt: 50 diam × 19, turned down to 40 diam × 15. Mounting hole for drive-shaft 20 mm, 7 mm out of center. 3. Retaining ring: brass, 50 diam × 4, screwed down to eccentric bolt. 4. Single row ball bearing: SKF series 60, No. 6008. 40 – 68 – 15 mm. 5. 2 flanges: 4 × 45 × 45, steel. 6. 2 flanges: 3 × 25 × 25, steel, hardened. 7. 3 spacers: steel, 7 diam × 84, for connecting flanges No. 5 together. 8. 2 guide pins: steel, 8 diam × 36 (mounted to No. 11). 9. Guide and connecting rod: steel, 9 diam × 50, mounted to No. 5 in one end, the other end has an M8 thread. 10. Sleeve: steel, 16 diam × 20, M8 internal thread. 11. Bracket: brass angel profile, 40 × 30 × 6.5 × 50. Provided with mounting holes for guide pins No. 8. 12. Bracket: As No. 11. Provided with guide hole for No. 9 (guide and connecting rod). 13. Mounting plate: steel, 10 × 60 × 425. 14. Aluminum frame: 12 × 48 × 168. Cut out 38 × 120. 15. Screw: M8 × 30, turned down to 5 diam × 2 at tip. The screw is fixed so that 13 mm with M8 thread protrudes outside the frame, for mounting to No. 9 with sleeve No. 10. 16. 2 guide pins: steel, 7 diam × 45, mounted to the aluminum frame. 17. Screw: M8 thread, with a locking nut. 18. Bracket: As No. 11, provided with guide holes for guide pins No. 16. 19. Capsule: Stainless steel, external 22 diam × 105, internal 21 diam × 100. Polished interior. 20. Tubing: Stainless steel. Wall thickness 0.5. External 32 diam × 105. Welded to flench at on end (No. 21), and welded to spacers (three) at the other end (No. 22). 21. Flench for capsule, stainless steel. 22. 3 radial spacers. 23. Rubber gasket: 0.5 mm thick. For No. 26. 24. Leather gasket: for No. 25, 1 mm thick. 25. Cover: Brass, for tubing No. 20, provided with diam 5 hole for matching with screw No. 15. 26. Cover: Stainless steel, turned down to fit inside of capsule No. 19, and polished. Provided with 5 diam hole for matching with screw No. 17. 27. Stabilizing ring: Stainless steel, 37 diam × 3. Welded to tubing No. 20.

The mechanical cell disintegrator. Fig. 1 shows a photograph of the apparatus, and Fig. 2, A and B a brief description of the parts. 4200 cycles/min are obtained by a 1 hp electrical motor. The cylindrical stainless steel capsule (Figs. 1 B and 2 B) has an inner volume of 34 ml and a space between the double walls of 35 ml. Before use, the space between the double walls is filled with water to about 5 mm below the rim and frozen at −20°C. It is convenient always to keep 2 or 3 capsules in the freezer ready for use. 1/3 of the inner volume is filled with glass beads and 1/3 with cell suspension, which are both precooled in the icebath. The cell suspension contains about 5 g wet weight of cells. The capsule is sealed with caps at the bottom and the top, and mounted on an aluminium frame, which again is mounted on the slide, as shown in Figs. 1 A and 2 B.

The machine is usually run for 4—10 sec. Even after 60 sec the temperature does not exceed 1—1.5°C inside the capsule. It is important to lubricate all sliding and moving parts with molybdenum oil.

Preparation of mitochondria. The ascites tumour cells were suspended in a medium containing mannitol 0.25 M and EDTA 0.1 mg/ml.[4] The final concentration was 40 % (v/v). 11 ml of cell suspension and the same volume of glass beads were placed in the cylindrical capsule according to the above description and shaken for 6 sec. The cells were almost completely disrupted by this procedure (0.3—0.5 % cells remaining). The homogenate was decanted and centrifuged for 5 min at 450 *g*, and the supernatant centrifuged for 10 min at 20 000 *g* in a Sorwall centrifuge RC2-B, rotor No. SS34. The mitochondrial preparation was washed twice.

Oxygen uptake. The oxygen uptake was measured at 30°C by the standard Warburg technique, using 15 ml flasks and 0.2 ml 10 % KOH on a roll of filter paper in the center well. The total volume of the incubation was 2 ml. The reaction was stopped by addition of perchloric acid (0.3 M final concentration), and the perchloric acid precipitated with KOH.

Respiratory control (R.C.) was measured in this system as the ratio of oxygen uptake in the presence of hexokinase, glucose, and ATP, to the uptake without these compounds.

Oxidative phosphorylation. Oxidative phosphorylation was determined at 30°C by measuring oxygen uptake and ATP formation in a medium of the following composition: hexokinase, 0.2 mg/ml; glucose, 0.05 M; ATP, 0.8 mM; (^{32}P), 15 μC/ml; bovine serum albumine, 0.2 %; cytochrome *c*, 2.5×10^{-3} mM; mannitol, 100 mM; EDTA, 0.14 mM; $MgCl_2$, 6 mM; KCl, 40 mM; potassium phosphate pH 7.4, 40 mM; Tris-HCl, 20 mM. To this was added the mitochondrial suspension with 2—8 mg protein in a total volume of 2 ml.

The oxidative phosphorylation of site II, and site I+II including substrate phosphorylation was assayed by measuring the reduction of 2.5 mM ferricyanide in the presence of 1 mM KCN at 25°C, as well as the phosphorylation coupled to this reduction.[5] The reduction of ferricyanide was determined spectrophotometrically at 420 mμ and corrected for that value in the presence of antimycin A (1 μg/mg protein). The coupled phosphorylation was corrected for any phosphate uptake in the absence of ferricyanide.[6] Thus, only the antimycin-sensitive ferricyanide reduction was used.

The concentrations of the substrates and inhibitors are described in the legends to the tables.

Phosphate uptake was determined according to Conover *et al.*[7] Their method is a slight modification of that of Lindberg *et al.*[8] Inorganic phosphate was measured after extraction by isobutanol/benzene,[9] and a Packard liquid scintillation counter was used for the counting of radioactive phosphate in Bray's solution.[10]

Protein was determined using the biuret method.[11]

RESULTS AND DISCUSSION

Optimal conditions for cell disintegration were obtained using 1:1:1 proportion of cell suspension – glass beads – air. Different sizes of glass beads (Ballotini 8—10 and 12) were tested, of which No. 12 was shown to give the best results in our system. A stroke of 14 mm was found to be the most effective. Using a shaking time of 6 sec, less than 1 % whole cells was left in the preparation.

Table 1 shows that oxidative phosphorylation at the three sites of the respiratory chain is intact. Thus, satisfactory values for oxygen uptake, phosphate uptake, and P/O ratio are obtained in the presence of pyruvate + malate (site I + II + III), in the presence of succinate + rotenone (site II + III), and in the presence of ascorbate + TMPD (site III). Moreover, respiratory control (R.C.) is a sensitive indicator of the intactness of the mitochondrial membrane. The high respiratory control (R.C.) with pyruvate + malate as

Table 1. Specific oxidation rates, oxidative phosphorylation and respiratory control of ascites cell mitochondria. The specific oxidation rates are given as natoms oxygen/min/mg mitochondrial protein. R.C.: The respiratory control.

Substrate	Specific oxidation rate	P/O	R.C.
6 mM pyruvate +12 mM malate	48.7	2.4	40
12.5 mM succinate [a]	75.6	1.4	2.2
16 mM ascorbate [b] +0.4 mM TMPD	69.2	0.6	1.3

[a] in the presence of 0.3 nM rotenone.
[b] in the presence of 0.3 nM rotenone and 1 ng antimycin per mg protein.

substrates indicates that phosphorylation is tightly coupled to oxidation in this preparation and that the mitochondrial membranes are intact. The values for this parameter are comparable to those of Wu *et al.*[12]

When ferricyanide is used as electron acceptor and site III is blocked by KCN, satisfactory respiratory control is also obtained for sites I+II with puryvate+malate as substrates and for site II with succinate as substrate (Table 2).

The antimycin inhibition of ferricyanide reduction is an additional indicator for intact mitochondrial membranes. Damage to the membrane leads to rerouting of the initial dehydrogenases (pyruvate or succinate) *via* ferricyanide, by passing the antimycin-sensitive site in the complete system.[5]

The fact that the mitochondria do not need externally added NAD for oxidation of pyruvate+malate also supports this view, because damaged mitochondrial membranes would result in a leakage of NAD, which would have to be supplemented in the incubation mixture in that case. Table 2 shows

Table 2. Reduction of ferricyanide by KCN-inhibited ascites cell mitochondria. The specific oxidation rates are given as equivalents of nmoles ferricyanide reduced/min/mg mitochondrial protein. The measurements were made in the presence of 1 mM KCN for the inhibition of site III. R.C.: The respiratory control.

Substrate	Specific oxidation rate	antimycin added [a]	R.C.
6 mM pyruvate +12 mM malate	50.1	4.5	6.4
6 mM pyruvate +12 mM malate +50 mM NAD	36.9		8.9
12.5 mM succinate [b]	55.3	5.2	5.3

[a] 1 ng/mg mitochondrial protein.
[b] in the presence of 0.3 nM rotenone.

that NAD inhibits the oxidation slightly. Moreover, oxygen uptake is independent of addition of cytochrome c, which would leak through mitochondrial membranes damaged by mild heat treatment.[13] These experiments show that after determination of a shaking time sufficient to break the particular cell species, mitochondria are obtained which satisfy biochemical criteria of intact membranes. The advantages of the machine are short breaking times and control of the cooling during shaking.

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