A Filtration Method for the Separation of Cell Sap from the Large-Particle Fraction of Rat Liver Suspensions, Enabling the Rapid Measurement of Nucleotide Distributions

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(Received 6 June 1969)

A simple method is described that allows a rapid separation of a cell-sap fraction from the large-particle fraction of rat liver suspensions. The method is based on the filtration under suction of liver suspensions through Millipore filters that retain nuclei, mitochondria and some of the endoplasmic-reticulum fraction, but allow quantitative passage of cell sap into a collecting tube. The cell sap may be separated in this manner within 2 min of the death of the rat. The method was applied to study the intracellular distribution of ATP and of the nicotinamide-adenine dinucleotides and the results obtained were compared with those obtained after separating the cell sap by a rapid centrifuging procedure. The percentage of total liver ATP in the cell sap was found to be 46% by the filtration method and more than 70% by the centrifuging procedure. Corresponding figures found for the distribution of $\text{NADP}^+ + \text{NADPH}$ were 40 and 49% respectively.

It is known from studies with isolated mitochondrial fractions that the mitochondrial membrane is permeable to some extent to exogenous ATP; exchange between the suspending medium and the mitochondrial particles may be essentially complete in a few minutes (Amoore, 1958; Brierley & Green, 1965; O'Brien & Brierley, 1965). It may therefore be expected that when a tissue is homogenized in a suspending medium like 0.25 M-sucrose the distribution of ATP between the various intracellular fractions undergoes a rapid change resulting from the dilution of the soluble phase in which the mitochondria are suspended. As a consequence, the distribution pattern obtained some 10–20 min after preparation of the homogenate and separation of the various fractions by centrifuging may be considerably different from the distribution of ATP among these fractions in the intact cell. In order to see if any correlation exists between the distribution of ATP in liver fractions and bile flow rate (Slater & Delaney, 1970) it was necessary to develop a method for the separation of intracellular fractions of liver by a procedure considerably more rapid than that involving centrifuging; a suitable method is described below. Brief details have been reported previously (Delaney & Slater, 1967).

METHODS AND RESULTS

Female albino rats (body wt. approx. 150 g) were used and were fed on standard diet (modified M.E.C. diet 41B; Oxo Ltd., London S.E.1, U.K.) and water *ad libitum*.

Rats were obtained from two different sources: (A) Twyford Laboratories, Park Royal, London N.W.10, U.K., and (B) Carworth (Europe), Alconbury, Hunts., U.K.

Nicotinamide-adenine dinucleotides were measured by the method of Slater, Sawyer & Straüli (1964).

ATP was measured by a modification of the spectrophotometric method of Lamprecht & Trautschold (1964). When centrifuging was used as the means of intracellular fractionation the composition of the reaction mixture in the spectrophotometer cuvettes was as follows: 0.05 M-triethanolamine-HCl buffer, pH 7.6, 2.5 ml; 0.1 M-magnesium chloride, 0.15 ml; 6.5 mM-NADP⁺, 0.06 ml; glucose 6-phosphate dehydrogenase, 0.04 ml containing 0.56 unit; 0.5 M-glucose, 0.1 ml; acid extract of homogenate or supernatant, 0.2 ml; hexokinase, 0.1 ml containing 1.4 units; total volume in cuvette, 3.45 ml. The enzymes (hexokinase and glucose 6-phosphate dehydrogenase) used in the ATP assay and ATP were from Boehringer Corp. (London) Ltd., London W.5. When the micro-filtration method was used to separate the supernatant fraction, the volume of buffer used was decreased to 2.5 ml (125 μmol) and the volume of acid extract used was increased to 0.5 ml. Readings were made at 340 nm with 1 cm silica cells and an SP.800 recording spectrophotometer. The results obtained by the spectrophotometric method were compared directly with the values obtained with the luciferin-luciferase method of Strehler (1964). The luciferin assays were kindly performed by Professor D. Wilkie, University College London. In five experiments the luciferin-luciferase method gave a mean value of $112 \pm 4\%$ compared with the hexokinase procedure.

Of several suspending media tested, the most satisfactory ('standard medium') in terms of inhibition of ATP breakdown during a 15-min period at 0°C was 0.25 M-sucrose–0.1 M-sodium fluoride–0.1 mM-EDTA that had

been pre-gassed with nitrogen for 15 min (see Frunder, Blume, Thielmann & Börnig, 1961). ATP determinations were made at various times after preparation of liver suspensions in this medium and were compared with the ATP values in intact liver obtained from the same rats. The mean ATP values in the homogenate ($\pm$ S.E.M.) expressed as percentages of the corresponding ATP values for intact liver were: time 2 min, $98 \pm 3\%$ (six rats); time 10–20 min, $90 \pm 4\%$ (nine rats).

Homogenate fractionation

Centrifuging procedure. Rats were killed by cervical dislocation. Pieces of liver (1–2 g) were quickly removed, weighed and homogenized (1:10, w/v) in the ice-cold standard medium. The homogenate (10 ml) was centrifuged at 10000g for 8 min at 5°C (M.S.E. 13 centrifuge with angle head). The supernatant ('cell sap' for the purpose of comparison in Table 2) was sucked off with a pipette with a bent tip and made up to 10 ml with ice-cold standard medium. The mitochondrial pellet was re-suspended in the standard medium, rehomogenized and made up to a final volume of 10 ml to produce the mitochondrial suspension. Samples (2 ml) of homogenate, mitochondrial suspension and 'cell sap' were taken and each was added to 2 ml of perchloric acid at the same time. The entire procedure from the death of the animals to the final mixing with perchloric acid took 15–20 min. Control experiments showed that the sedimentation behaviour of total protein, cytochrome oxidase and succinate-neotetrazolium reductase (EC 1.3.99.1) was similar in the standard (fluoride-containing) medium and in 0.25 M-sucrose.

ATP analyses were carried out on the homogenate, mitochondrial pellet and supernatant fractions obtained by the centrifuging scheme described above. The percentage of ATP found in the cell sap compared with the value for the whole homogenate ranged from 64 to 89% (mean $\pm$ S.E.M. $72 \pm 3\%$, from eight experiments). It was believed that this figure was artificially high owing to

diffusion of ATP from the mitochondrial fraction into the cell sap during centrifuging (see the Discussion section) and an attempt was made to minimize such diffusion by the addition to the standard medium of various drugs known to stabilize biomembranes. Agents tried were promethazine (see Judah, 1960; McLean, 1960) bovine plasma albumin (see Lehninger, 1962; Weinbach & Garbus, 1966), cortisone (see Weissmann & Dingle, 1961), *p*-fluoro- γ -[4-hydroxy-4-($\alpha\alpha\alpha$ -trifluoro-*m*-tolyl)piperidino]-butyrophenone (Triperidol, see Janssen, 1966) and potassium atractylate (Brierley & O'Brien, 1965). Table 1 shows the results obtained. From this, it is apparent that 1% bovine plasma albumin in 0.44 M-sucrose, and 1.5 mM-promethazine in the standard medium, were both partially effective in decreasing the amount of ATP in the liver cell-sap fraction, whereas promethazine treatment *in vivo*, or cortisone, Triperidol or potassium atractylate (40 mg/100 ml of standard medium) *in vitro* were either less effective or without effect.

Filtration procedure. Immediately after the rat had been killed by cervical dislocation a piece of liver (approx. 0.8 g, but not weighed) was homogenized in 25 ml of standard medium. The homogenate (2 ml) was pipetted into a metal filter holder (Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.) containing a porous metal disc upon which two Millipore filters of pore sizes 0.2 μ m and 5.0 μ m were superimposed. The filter holder drained into a test tube containing 1 ml of M-perchloric acid and standing in ice. Suction from a water pump was applied via a side-arm connection. The filtrate was washed through into the perchloric acid with 2 ml of medium. All filtrates were made up to a final volume of 5 ml in volumetric flasks. As soon as the filtrate passed through the filters into the perchloric acid, 2 ml of homogenate was added directly to 1 ml of M-perchloric acid and made up to a final vol. of 5 ml to measure the total-homogenate ATP value. ATP analyses were carried out on the homogenate and the filtrate. The entire procedure from the death of the animal to mixing with perchloric acid took approx. 2 min. The concentration of the homogenate in g wet wt. of

Table 1. *Effect of various additives to the standard medium on the distribution of ATP in liver cell fractions separated by a centrifuging procedure*

The centrifuging scheme used for the separation and preparation of the mitochondrial suspension (M) and the cell-sap fraction (S) is described in the text. ATP was determined by the hexokinase procedure. ATP in fraction S is expressed as a percentage of that in fractions M+S combined. In Expts. 3(a) and 3(b) the two rats were each pre-treated 30 min before death with promethazine (2.5 mg/100 g body wt. by intraperitoneal injection). The rats were obtained from source A.

Experiment	Medium	ATP distribution (S/M+S) $\times$ 100%
1(a)	0.44 M-Sucrose + 1% (w/v) bovine serum albumin	55
1(b)		51
2(a)	Standard medium + 1.57 mM-promethazine	62
2(b)		60
3(a)	Standard medium + promethazine <i>in vivo</i>	67
3(b)		73
4(a)	Standard medium + 0.51 mM-cortisone	79
4(b)		78
5(a)	Standard medium + 1.25 mM-Triperidol	65
6(a)-(b)	Standard medium	72 $\pm$ 3

liver/ml of suspension was found by measuring the DNA content of the homogenate and comparing it with the DNA content of a homogenate of known concentration prepared from the same liver. The DNA content of liver samples was measured by the method of Greenbaum & Slater (1957) with a short boiling time to decrease interference by other carbohydrate-containing materials (Slater, 1961).

Control experiments showed that the filter assembly retained all of the DNA and cytochrome oxidase activity of the liver homogenate. Glucose 6-phosphate dehydrogenase activity was not retained by the filters except for a very small amount due probably to the presence of unbroken cells in the tissue suspension. Glucose 6-phosphatase activity was partially retained by the filters, indicating that large fragments of endoplasmic reticulum were trapped on the filter membranes whereas smaller fragments passed through. Methods used for these analyses were: for glucose 6-phosphatase, that of Slater & Sawyer (1969), with PP_i replaced by sodium glucose 6-phosphate (0.013M); for glucose 6-phosphate dehydrogenase, that of McLean (1958); for cytochrome oxidase, that of Slater & Planterose (1960); for DNA, that of Slater (1961).

Known amounts of ATP added to liver suspensions

Table 2. *Comparison of the filtration and centrifuging procedures for separating liver cell-sap fraction in terms of the distributions of NADP and ATP*

For experimental details see the Methods and Results section. The numbers of animals used are given in parentheses. Mean values are given $\pm$ S.E.M.

Separation procedure	Component in cell-sap fraction (%)		
	NADP+ + NADPH	ATP	
Source of rats ...	(A)	(A)	(B)
Filtration	40 $\pm$ 2 (6)	49 $\pm$ 3 (7)	40 $\pm$ 3 (11)
Centrifuging	49 $\pm$ 5 (6)*	72 $\pm$ 3 (8)	—

*Results from Slater (1967).

Table 3. *Effects of aging liver suspensions at 0°C on the percentage of ATP found in the cell-sap fraction obtained by the filtration procedure*

For experimental details see the text. The rats were obtained from source B.

Experiment	Duration of aging (min)	Percentage of ATP in filtrate
a	3	36
	17	45
b	2	22
	20	52
c	2	43
	20	72
d	0.8	34
	15	75

were quantitatively recovered in the membrane filtrate; in two experiments the percentage recoveries were 99 and 109%.

The intracellular distributions of the nicotinamide-adenine dinucleotides and ATP obtained by each of the two methods of cell fractionation were compared, and the results obtained are given in Table 2, which shows that the percentage of ATP present in the cell-sap fraction was 72% when centrifugation was used, whereas a value of 46% was obtained when filtration was employed.

When homogenates were 'aged' by standing in ice for periods up to 15 min, it was found that the amount of ATP present in the cell-sap fraction increased compared with the values obtained by the filtration procedure carried out immediately after homogenization. Table 3 gives the results obtained from four experiments. These results suggest that the ATP in the mitochondrial fraction undergoes rapid equilibration with the cell sap.

DISCUSSION

It is known that isolated mitochondria are readily permeable to small molecules at 0°C (for references see Brierley & Green, 1965). Amoore (1958) has shown that exogenous ATP penetrates the mitochondrial membrane although about four times less readily than does sucrose. About 30–50% of the total mitochondrial water space is apparently freely accessible to exogenous ATP and the nucleotide in this compartment is readily removed by washing the mitochondrial fraction (O'Brien & Brierley, 1965). Some mitochondrial ATP is resistant to removal by washing procedures and part of this 'bound ATP' is exchangeable with exogenous ATP (Pressman, 1958). Brierley & O'Brien (1965) found that the exchange between the bound ATP fraction and external labelled ATP was very rapid and was essentially complete in 1 min at 2°C. It is obvious from the results in Table 2 that net transfer of ATP from the mitochondrial fraction into the cell sap is a slower process than exchange. It can be concluded that the filtration procedure gives a distribution pattern of ATP that is closer to the situation *in vivo* than do procedures based on centrifuging. The results in Table 3 on aging of tissue suspensions and those of Slater & Delaney (1970) for the effect of sodium malonate administration show that the ATP distribution can be varied in either direction by the appropriate experimental procedures.

The work described above was done with a commercially available filter unit (Fisons) constructed of aluminium and with a phosphor-bronze filter-support grid. A similar assembly was constructed in our laboratories from Perspex and with a porous glass support. This assembly gave similar results in terms of ATP distribution when tried with liver suspensions. The Perspex-glass assembly may, however, be preferable if measurements on the filtrate are affected by trace amounts of metal ions.

It is evident that the filter procedure gives a

reliable and rapid method for separating cell sap from a large-particle fraction. The procedure could be used for the determination of the intracellular distribution patterns of other key intermediates such as citrate, fructose 6-phosphate, phosphoglycerate etc. Further, by varying the pore size of the filters used in the assembly it should be possible to remove whole cells and nuclei preferentially from smaller particles (e.g. mitochondria). Combinations of appropriate filters and analysis of the different filtrates so produced enables the distribution of small and rapidly diffusible molecules among the various intracellular fractions to be obtained by difference.

We are grateful to the Medical Research Council for a grant that has made this work possible. Gifts of atracylate by Dr G. Acocello, Professor R. Santi and Professor T. Ajello, and of Triperidol by Dr N. W. Shephard of Janssen Pharmaceuticals, Saunderton, High Wycombe, Bucks., U.K. are gratefully acknowledged. We thank Professor D. Wilkie of University College London for facilitating the luciferin assays of ATP.

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