MINI-REVIEWS

STRATEGIES OF SUBCELLULAR FRACTIONATION
SUITABLE FOR ANALYSIS OF PEROXISOMES AND
MICROPEROXISOMES OF ANIMAL TISSUES

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Abstract—Strategies of subcellular fractionation are reviewed from the perspective of their utility in the analysis of peroxisomes. The considerable potential inherent in the method of rate dependent banding in zonal rotors is emphasized. The use of various density gradient solutes is considered.

INTRODUCTION

De Duve (1969a,b) has defined peroxisomes as organelles containing catalase together with one or more H2O2 producing oxidases. The peroxisomes of liver have been much studied by biochemical, histochemical and whole organ spectrophotometric methods. Relatively little attention has been devoted to other tissues. However, Novikoff & Novikoff (1972) introduced the term “microperoxisome” to describe “elongate or roughly spherical cytoplasmic organelles, ranging in size from approx. 0.1 μ-1.0 μ, containing a moderately electron-opaque matrix, lacking a nucleoid and delimited by a tripartite membrane which is continuous via numerous slender connections...with the tripartite smooth ER (endoplasmic reticulum) membrane...and generally giving a positive “histochemical” reaction for catalase” (Novikoff et al., 1973). Histochemistry demonstrating catalase at the electron microscope level revealed that particles fitting this definition are ubiquitous in mammalian cells (Hruban et al., 1972; Novikoff et al., 1973)*. This data built upon the earlier observations of Hruban & Rechcigl (1969).

In recent years there has been a quickening of interest in animal peroxisomes because concrete evidence has been supplied of their involvement in lipid metabolism. Thus liver peroxisomes have been shown to oxidise fatty acid (Lazarow & De Duve, 1976) and to be the site of dihydroxyacetone phosphate acyltransferase activity (Hajra et al., 1979), and toad bladder microperoxisomes were shown to contain the glyoxylate cycle enzyme malate synthase (Goodman et al., 1980). In view of these recent findings two questions arise: do tissues other than liver have lipid metabolising enzymes in their catalase particles (or microperoxisomes) and can these particles universally be described as peroxisomes in stricto sensu? The answer to these questions depends largely on suitable subcellular fractionation studies being performed.

Several reviews have appeared pertaining to various aspects of the biology of peroxisomes (Baudhuin, 1969; De Duve & Baudhuin, 1966; Tolbert & Essner, 1981; Tolbert, 1981; Masters & Holmes, 1977; Hogg, 1969; Richardson, 1974; De Duve, 1969a; Vigil, 1973; Seis, 1974; Muller, 1975; Reide et al., 1980) and some (e.g. Bock et al., 1980; Baudhuin, 1974) have devoted attention to the methods available for subcellular fractionation and subsequent analysis of these organelles. However, most of these latter articles have been almost exclusively concerned either with plant or with mammalian hepatic and renal peroxisomes. The purpose of the present brief review is to highlight fractionation strategies suitable for the analysis of peroxisomes and microperoxisomes of various animal tissues. Only centrifugation methods will be considered and the terms defined by Reid & Williamson (1974) will be used. We will not reiterate the general principles of analytical subcellular fractionation detailed by De Duve, Beaufay & co-workers (e.g. De Duve, 1971; De Duve & Beaufay, 1981; Beaufay & Amar-Costese, 1976) other than to point out here the importance of calculating balance sheets and of bearing in mind the assumptions embodied in the postulates of “unique localisation” and of “biochemical homogeneity” when using markers for subcellular organelles.

DESI RABLE PROPERTIES OF FRACTIONATION

STRATEGIES

The study of liver peroxisomes has emphasised that numerous peroxisomal enzymes are not exclusive to these organelles but may be shared with mitochondria, microsomes or cytosol. For apparently cytosolic activities it is generally uncertain whether the activity has been released from damaged organelles or...
whether it is a *bona fide* representative of the cell sap. De Duve (1967) has emphasised that because both peroxisomes and mitochondria are respiratory (O₂ consuming) organelles it must be expected (and in fact has been demonstrated) that they will share several enzyme activities. In view of these considerations it is clear that the primary requisite of a fractionation strategy designed to allow the analysis of peroxisomes is that these organelles be well separated especially from mitochondria but also from microsomes and cytosol (the cytosol, of course, presents few problems). Further desirable properties are minimal damage to organelles so as to prevent enzyme leakage, yield of organelles sufficient for biochemical study, cheapness of gradient materials and ease and speed of operation.

A. Sucrose Media

1. Differential pelleting

Of available techniques the now classical differential pelleting scheme (De Duve et al., 1955) which separates homogenate into 5 subcellular fractions (N, M, L, P and S) represents the simplest in terms of equipment required. It is capable of large yields. Its major disadvantages are the need for repeated pelleting and resuspension of organelles which is likely to be injurious and to cause aggregation, that it is relatively time consuming, and that when unsupplemented by subsequent procedures gives a generally poorer resolution than other methods. The scheme was first developed for rat liver and De Duve has cautioned against indiscriminate adoption for other tissues. Most mammalian tissues contain microperoxisomes of considerably smaller size than the peroxisomes of liver or of rat renal tubules and consequently in differential pelleting these require greater integrals of time and centrifugal force to sediment them. Consequently, to separate microperoxisomes from microsomes by this method resuspension and recentrifugation would need to be repeated several times. Resuspension of such hard pelleted microperoxisomes is likely to be traumatic and is best avoided. Tolbert (1974) has even advocated the avoidance of pelleting in analysis of liver peroxisomes.

2. Rate dependent banding

(Reid & Williamson, 1974). This is an alternative procedure which, like differential pelleting, exploits the differences in sedimentation rate between peroxisomes on the one hand and mitochondria and microsomes on the other. It can be done with tubes or with zonal rotors but the latter are far superior and more convenient. Preferably a long path length zonal rotor (e.g. B-XIV) is used. It has been found using this procedure that intestinal microperoxisomes of various species including guinea-pig (Connock et al., 1974) mouse, (Small et al., 1981) goldfish (Connock, 1978...
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and rat (Peters & Shio, 1976) (Fig. 1) can be reasonably well separated from microsomes (which sediment more slowly) and especially well separated from mitochondria (which sediment more quickly). Similar separations can be achieved for lung (Goldenberg et al., 1978) and for brown adipose tissue (BAT) microperoxisomes (Fig. 2).

Most mammalian tissues contain microperoxisomes of similar size to those of lung, BAT and intestine and these, in theory, should be nicely separated by this method. Fibroblasts also contain microperoxisomes which have a slower sedimentation rate than mitochondria (Tulkens et al., 1974) and this has been exploited in an alternative procedure using the Beaufay (short path length) zonal rotor (Sun & Poole, 1975). Baggioili et al. (1978) have designed an adapter for the B-XIV zonal rotor that halves its capacity while retaining full path length characteristics. This adaptation makes the rotor more suitable for rate dependent banding with preparations capable of only small yield of starting homogenate (e.g. cell culture samples).

Rate dependent banding in a zonal rotor also gives a good separation of peroxisomes from mitochondrial markers when mouse liver or kidney are analysed (Figs 3 and 4). Because of the larger size of these peroxisomes smaller integrals of centrifugal force and time are required compared with tissues containing smaller microperoxisomes. In practice the best rate dependent banding separations are achieved if the sample layer is introduced slowly by perfusion pump at no more than 5 ml/min so as to create a discrete sample band (Cline, 1971). Also a long shallow gradient should be used (e.g. 15 to 30% w/v sucrose) so that particles are not slowed by approaching their equilibrium density; particles will only be arrested if they have traversed the whole gradient and reached the dense cushion. The advantages of rate dependent
Density dependent banding of peroxisome-enriched fractions from a rate dependent banding experiment like that shown in Fig. 3 except that pyrophosphate was used (2 ml of 100 mM pyrophosphate, pH 8.2) was mixed with 28 ml of post-nuclear supernatant just before loading the zonal rotor for the rate separation. A small volume (40 ml) sucrose gradient (30 to 52% w/w) was used with a 50% w/w sucrose cushion. Centrifugation was at 35,000 rev/min for 4.5 hr with a 200 ml 15% w/w sucrose overlay and a 150 ml sample band. Details as in Fig. 3 except that acid phosphatase has been used as a marker for lysosomes.

Fig. 5. Mouse kidney. Density dependent banding of peroxisome-enriched fractions from a rate dependent banding experiment like that shown in Fig. 3 except that pyrophosphate was used (2 ml of 100 mM pyrophosphate, pH 8.2) was mixed with 28 ml of post-nuclear supernatant just before loading the zonal rotor for the rate separation. A small volume (40 ml) sucrose gradient (30 to 52% w/w) was used with a 50% w/w sucrose cushion. Centrifugation was at 35,000 rev/min for 4.5 hr with a 200 ml 15% w/w sucrose overlay and a 150 ml sample band. Details as in Fig. 3 except that pyrophosphate was used as a marker for lysosomes.

Densit y dependent banding

Density dependent banding in sucrose gradients has been the principal procedure for obtaining useful peroxisome-enriched fractions. The now classical method reported by Leighton et al. (1968) yielded peroxisomes estimated to be 95% pure from the livers of rats treated with Triton WR-1339 so as to allow separation from lysosomes. The density banding step was preceded by differential pelleting to produce the "L" fraction greatly depleted in microsomes and enriched ~2 times for peroxisomes with respect to mitochondria. If a tube held gradient (instead of one held in a zonal rotor) is used for the final step then purity is reduced [purification factor for catalase of 27 compared with 36 (Baudhuin, 1974)]. A similar procedure, but without Triton WR-1339 treatment, has been used by Goldenberg et al. (1978) to purify fish (carp) liver peroxisomes ~40 times. Triton WR-1339 should be used only with the realisation that the levels of peroxisomal enzyme activities are likely to be decreased (Ishi et al., 1979).

Tolbert (1974) has advocated the use of a single density dependent banding of homogenate or post-nuclear supernatant in a zonal rotor for the analysis of hepatic and renal peroxisomes. Obviously, the purity of peroxisome fractions so obtained will be less than that if the density step is preceded either by differential pelleting (Leighton et al., 1968) or by rate dependent banding in a zonal rotor. Because of the generally appreciable difference in equilibrium density between peroxisomes and mitochondria from liver and kidney this single banding procedure is reasonably efficient with these tissues and allows the detection of peroxisomal enzyme locations if the proportion of activity in the peroxisomes is of the order of 15-20% or greater. Lower proportions in peroxisomes would probably require greater purification of peroxisomal fractions for unequivocal demonstration of location. On the other hand microperoxisomes of tissues analysed so far (e.g. Peters & Shio, 1976; Connock et al., 1974) do not differ so much from mitochondria in their equilibrium density, therefore, 8
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A single density-dependent banding step is less satisfactory for analysis of microperoxisomes and is best coupled with a preliminary rate-dependent banding or differential pelleting step. The relative merits of these two preliminary procedures, in our opinion, weigh in favor of rate-dependent banding in a zonal rotor except where very high yields are required. The major advantages being lack of damage (especially in the case of microperoxisomes) and superior separation from mitochondria. However, contamination of peroxisomes with microsomes may be somewhat greater than when differential pelleting is used. It is, therefore, advantageous in the subsequent density-dependent step to reduce the "time x centrifugal force" integral as much as possible so that the slower sedimenting microsomes do not reach equilibrium and thereby cause less contamination of peroxisomal fractions. An alternative and/or additional strategy is to pretreat the payload (or homogenate) with EDTA or pyrophosphate (see Fig. 5) which Amar-Costesec et al. (1974) have shown to reduce the equilibrium density of endoplasmic reticulum elements by stripping them of ribosomes and protein. This procedure was exploited by Dobrota & Hinton (1980) to aid the large-scale purification of hepatic lysosomes from untreated rats (peroxisomes were removed by appropriate rate-dependent procedures prior to the final density-dependent banding).

B. OTHER MEDIA

Two other media have been introduced recently which allow separation of peroxisomes of reasonable purity. Wattiaux et al. (1978) found that a linear metrizamide density gradient gave as good a separation of peroxisomes from other liver organelles as did the sucrose gradient method of Leighton et al. (1968). An advantage of this procedure is that it obviates the use of Triton WR-1339 to ensure a good separation from lysosomes. Metrizamide gradients have been used successfully in the investigation of human liver peroxisomes (Bronfman et al., 1979). We observed reasonable separation of mouse renal peroxisomes using density-dependent banding on a metrizamide gradient (Small et al., 1984). Does metrizamide hold the same promise from microperoxisomes as it does for renal and hepatic peroxisomes? Figure 6 and 7 show that this is the case for microperoxisomes of hamster BAT and goldfish intestine, and we have observed similar results with insect fat body (Connock et al., 1982) and guinea pig small intestinal microperoxisomes (Small et al., 1980). In all instances the use of a metrizamide gradient results in a good separation of peroxisomes from lysosomes thus eliminating the use of Triton WR-1339 or other lysosome perturbing agents. So far metrizamide gradients have only been used with post-nuclear supernatant or crude homogenate or in combination with preliminary differential pelleting. In theory we would expect the best purifications to be achieved by combining pyrophosphate treatment and rate-dependent banding through a sucrose gradient in a zonal rotor with subsequent den-
sity dependent banding in a metrizamide gradient. The latter step, because of the large volume of the sample to be loaded, would also be best performed in a zonal rotor. However, the relative expense of metrizamide might rule out this strategy. Information so far available indicates that for peroxisomes and microperoxisomes metrizamide gradients are equal or superior to those of sucrose with density dependent banding procedures. Unfortunately, the expense of metrizamide means that its resolving power might not be fully exploited by using zonal rotors.

Neat et al. (1981) and Appelkvist et al. (1981) recently reported procedures for isolating purified rat liver peroxisomes in self generated Percoll gradients held (in the former method) in a vertical tube rotor. The advantages of the method are its speed, the fact that Percoll gradients can be made isosmotic, and the facility of analyzing several samples simultaneously in the multi-place rotor. The main disadvantages appear to be the moderate purification achieved and the fact that percol must be removed prior to some biochemical analyses being performed. Percoll gradients as yet seem not to have been used for isolating microperoxisomes.

CONCLUSION

In this brief review we have aimed to highlight fractionation strategies likely to be useful for analysis of peroxisomes and microperoxisomes from a variety of animal tissues. The considerable resolution between mitochondria and peroxisomes that is achieved by rate dependent banding in a zonal rotor appears to us to hold considerable promise and to have been under-exploited. Hinton & Dobrota (1980) have similarly advocated a procedure they call the "s-p" approach for analysis of the heterogeneity of organelles. It would appear to us that by coupling available fractionation strategies with oxidative assays that exploit sensitive methods for detection of H$_2$O$_2$ (e.g. Guilbault et al., 1968; Kochli & Von Wartburg, 1978) and with histochemistry using diaminobenzidine (Hand, 1979) and cerous ion methods (Briggs et al., 1979) it should be possible to answer some of the outstanding questions concerning the nature of the microperoxisomes that are ubiquitous in mammalian cells.

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REFERENCES


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