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Subcellular Fractionation of Brown Adipose Tissue

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The present study proposes a technique, using Metrizamide, which permits the preparation of brown adipose tissue plasma membranes from the crude mitochondria as well as from the crude microsome fraction. These plasma membranes have high relative specific activities of their marker enzyme, 5′-nucleotidase (15 ± 3 and 14 ± 2 respectively) and, particularly those originating in the crude microsomes, are relatively free of mitochondria contamination. This study also shows the influence of the mode of cell disruption on microsome integrity. When cell disruption was achieved by grinding in liquid nitrogen the purified microsome NADPH cytochrome c reductase specific activity was found to be 3.5 times greater than that of microsomes obtained after homogenization of the tissue.

Key words: subcellular fractionation, brown adipose tissue, plasma membranes, microsomes, Metrizamide

The study of the metabolic events occurring in the plasma membrane of brown adipose tissue, such as metabolite and ion transports, or hormone-receptor interactions, would benefit from a convenient technique for preparing purified plasma membranes from the tissue. One of the main difficulties encountered in separating plasma membranes from brown adipose tissue is that their quantity is so much smaller than that of the mitochondria. This difficulty was overcome in a former study [1] by preparing plasma membranes from only the crude microsome fraction. This technique, however, yielded a relatively low recovery of plasma membranes because those sedimenting with the mitochondria were lost. It was also time consuming because it involved dialysis of the crude microsome fraction against a pH 8.6 solution containing divalent cations. This operation, which had been shown by Kamat and Wallach [2] to increase the differences in volume and aggregation state between plasma membrane and microsome vesicles, is necessary for separation with Ficoll.

Recently, however, a new substance has been proposed for the isopycnic centrifugation of biological particles [3]. This substance, Metrizamide, has the advantage of being chemically inert, non-ionic, and of having relatively low viscosity in aqueous solutions and it seems to permit the separation of particles of relatively similar densities.

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In the present study, the use of Metrizamide made it possible to separate plasma membranes not only from the microsomes but also from the mitochondria, thus increasing the quantity of plasma membranes recovered, and also to eliminate the dialysis step, thus gaining time in the plasma membrane preparation. The present study also attempted to determine conditions of separation which would result in microsome fractions of good quality.

METHODS

Sprague-Dawley male rats, about 2 months old, weighing 200–250 gm and fed Nafag chow (St-Gall, Switzerland) ad libitum, were used. The rats were decapitated and the brown adipose tissue was excised.

In the first set of experiments, the brown adipose tissue was placed at 4°C in a homogenization medium at pH 7.5 containing $CaCl_2$ 0.5 mM, $NaHCO_3$ 1 mM and $MgSO_4$ 0.2 mM. This medium was found to increase the yield and enzymatic activity of the plasma membranes isolated from liver [4]. The tissue was cut with scissors into small pieces of about 1 mm^3 and homogenized in a Potter-Elvejhem homogenizer at 1,800 rev/min (Teflon pestle, 8 up-and-down strokes, clearance 0.3 mm) in 10 volumes of the homogenizing medium described above. The homogenate was centrifuged at 1,300 × g for 10 min and the supernatant was recentrifuged at 35,000 × g for 20 min. The resulting pellet (mitochondria + plasma membranes) was resuspended in 1.7 ml of the homogenization medium (protein concentration, about 5 mg/ml) and layered on 2.5 ml of Metrizamide 18.5% (w/v). The 35,000 × g supernatant was centrifuged at 105,000 × g for 60 min. The resulting pellet (microsomes + plasma membranes) was resuspended in 1.7 ml of the homogenization medium containing bovine serum albumin 4% (w/v) and layered on 2.5 ml of Metrizamide 18.5% (w/v). The addition of bovine serum albumin to the crude microsome resuspension medium seemed to decrease microsome enzyme solubilization. The concentrations of Metrizamide to be used were determined by preliminary experiments with discontinous gradients. Both fraction mixtures (mitochondria + plasma membranes and microsomes + plasma membranes) were then centrifuged at 156,000 × g for 90 min. For the first fraction mixture, the plasma membranes (referred to as plasma membranes $_{(mit)}$) were found in a layer above the Metrizamide and the purified mitochondria formed a pellet. For the second, the plasma membranes (referred to as plasma membranes $_{(mc)}$) were found in a layer above the Metrizamide and the purified microsomes formed a pellet. The membrane layers were collected and centrifuged at 156,000 × g for 30 min. The pellets were resuspended in a solution containing sucrose 0.25 M, Tris-HCl 10 mM and EDTA 1 mM at pH 7.4.

In the second set of experiments, the excised brown adipose tissue was placed in a mortar containing liquid nitrogen and ground with a pestle to obtain a fine powder. This was homogenized in 10 volumes of the homogenization medium at 1,800 rev/min (Teflon pestle, 2 up-and-down strokes, clearance 0.3 mm). The subcellular fractionation was performed as described above except that a Metrizamide 15% (w/v) solution was used for the separation of the plasma membranes $_{(mc)}$ from the purified microsomes.

The 5′-nucleotidase (E.C.:3.1.3.5) activity was measured according to the method of Heppel and Hilmoe [5] and inorganic phosphorus by the method of Taussky and Shorr [6]. NADPH cytochrome c reductase and succinate dehydrogenase (E.C.:1.3.99.1) activities were measured spectrophotometrically according to the methods described by Sottocasa et al [7] and Bachmann et al [8] respectively. Under the conditions of the present study, the enzymatic activities were found to increase linearly with the protein concentration. The proteins were measured according to the method of Lowry et al [9].

RESULTS

The results of the first set of experiments are given in Tables I and II. Table I shows the quantity of proteins in the different subcellular membrane fractions obtained from brown adipose tissue. It can be seen that the mitochondria fraction made up to 80% of the total subcellular membrane proteins.

Table II shows both the specific and total activities of each of three marker enzymes in the homogenate or in the different subcellular membrane fractions obtained from brown adipose tissue after cell disruption by homogenization. The relative specific activity (ie specific activity of the fraction divided by the specific activity of the original homogenate) of the plasma membrane marker enzyme 5′-nucleotidase was found to be 15 ± 3 and 14 ± 2 for the plasma membranes $_{(mit)}$ and for the plasma membranes $_{(mc)}$ respectively. The 5′-nucleotidase specific activity of the plasma membranes$_{(mit)}$ was 14 ± 4 and 33 ± 11 times higher than that of the microsomes and the mitochondria respectively and the 5′-nucleotidase specific activity of the plasma membranes$_{(mc)}$ was 13 ± 4 and 32 ± 10 times higher than that of the microsomes and mitochondria respectively. Table II also shows that 31 ± 10% of the total membrane 5′-nucleotidase activity was found in the purified mitochondria indicating a quantity of plasma membranes remaining in the large mitochondria pellet that was significant. The relative specific activity of the mitochondria marker enzyme succinate dehydrogenase in the mitochondria was 1.7 ± 0.6 and as much as 94 ± 4% of the total membrane succinate dehydrogenase activity was found in the purified mitochondria. However, the succinate dehydrogenase specific activities of the plasma membranes $_{(mit)}$ and of the microsomes were found to be 34 ± 4% and 43 ± 3% respectively of that of the mitochondria indicating that both fractions were still contaminated with mitochondria. Only the plasma membranes$_{(mc)}$ were relatively free of mitochondria contamination as shown by a succinate dehydrogenase specific activity value which was 15 ± 1% of that of the mitochondria. The relative specific activity of the microsome marker enzyme NADPH cytochrome c reductase in the microsomes was only 3.6 ± 0.4. Furthermore, the NADPH cytochrome c reductase specific activity value was not higher for the microsomes than for the two plasma membrane fractions.

The results of the second set of experiments, in which cell disruption was achieved by grinding in liquid nitrogen rather than by homogenization, show a comparable distribution of proteins among the different subcellular membrane fractions. They also show lower 5′-nucleotidase relative specific activity values (6.4 ± 1.4 and 5.4 ± 0.9 for the plasma membranes $_{(mit)}$ and $_{(mc)}$ respectively) than those obtained in the first set of experiments. The purified microsome fraction, on the other hand, was found to have an NADPH cyto-

TABLE I. Quantity of Proteins Found in the Membrane Subcellular Fractions Obtained From the Pooled Brown Adipose Tissues of 10 Rats after Cell Disruption by Homogenization

Plasma membranes $_{(mit)}$	1.04 ± 0.18
Plasma membranes $_{(mc)}$	0.65 ± 0.11
Microsomes	2.61 ± 0.25
Mitochondria	17.2 ± 5.2

The subcellular fractions were prepared according to the first technique described under Methods. The values represent the average of 3 experiments ± S.E.M. They represent the total quantity of proteins (expressed in mg) found in the subcellular fractions obtained from the pooled brown adipose tissues of 10 rats. The wet weight of the pooled brown adipose tissues of 10 rats is 3.7 ± 0.3 gm.

TABLE II. Marker Enzyme Activities of Membrane Subcellular Fractions Obtained From Brown Adipose Tissue After Cell Disruption by Homogenization

	5'-nucleotidase		NADPH cytochrome c reductase		Succinate dehydrogenase	
	Spec.act.	Total	Spec.act.	Total	Spec.act.	Total
Homogenate	19.1± 2.7	1203 ± 274	0.046 ± 0.006	2.89 ± 0.94	47.3 ± 13	2980 ± 882
Plasma membranes (mit)	277 ± 59	238 ± 68	0.240 ± 0.028	0.207 ± 0.050	27.7 ± 4.7	23.8 ± 1.3
Plasma membranes (mc)	273 ± 38	158 ± 32	0.198 ± 0.036	0.114 ± 0.031	12.5 ± 1.8	7.25 ± 2.3
Microsomes	20.5± 3.2	48.5 ± 9.5	0.164 ± 0.014	0.389 ± 0.050	35.0 ± 8.1	83.0 ± 33
Mitochondria	8.50± 4.3	198 ± 130	0.048 ± 0.028	1.12 ± 0.80	82.2 ± 13	1915 ± 265

The subcellular fractions were prepared according to the first technique described under Methods. The values represent the average of 3 experiments ± S.E.M. Specific activities (spec. act.) are expressed as nmoles of reaction products formed/mg proteins per min. The corresponding total activities are expressed as nmoles of reaction products formed in 1 min in the total homogenate or in the total subcellular membrane fractions obtained from the pooled brown adipose tissues of 10 rats.

chrome c reductase specific activity that was 3.5 ($P < 0.02$) times greater than that obtained in the first set of experiments. Contamination of the purified microsomes with plasma membranes and mitochondria was similar to that observed in the first set of experiments.

DISCUSSION

The results of the present study indicate that the technique using Metrizamide for subcellular fractionation of homogenized brown adipose tissue can be recommended for the preparation of purified plasma membranes. It has been shown to yield plasma membranes that have a high 5′-nucleotidase relative specific activity and that are (the plasma membranes originating in the microsomes, in particular) relatively free of mitochondria contamination. This technique seems not suitable for the preparation of microsomes, however, since it resulted in microsomes with an NADPH cytochrome c reductase activity that was even lower than that of the plasma membranes. Similar results have been obtained in white adipose tissue, when subcellular fractionation was performed starting from whole tissue, which showed that the microsome enzymes were solubilized and subsequently adsorbed on other membranes [10]. In the same study, it was found that this solubilization could be prevented by starting from isolated fat cells instead of whole adipose tissue. In the present study with brown adipose tissue, however, the use of isolated fat cells obtained by collagenase digestion instead of whole adipose tissue, did not result in increased NADPH cytochrome c reductase activity of the microsomes and, as also reported for white adipose tissue [10], caused a sharp decrease of plasma membrane marker enzyme activity (results not shown). Although cell disruption in liquid nitrogen is not generally considered a suitable technique for subsequent subcellular fractionation, the present study with brown adipose tissue showed it to be effective in preserving microsome integrity. This observation indicates the importance of choosing the mode of cell disruption as a function of the object of the study, ie, whether the desired purpose is to obtain plasma membranes or microsomes with high marker enzyme specific activity.

It can be concluded that the Metrizamide techniques described in the present study are preferable to the previously described technique for subcellular fractionation of brown adipose tissue [1] because they allow a better recovery of plasma membranes, a shorter preparation time and adaptibility to the experimental needs.

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