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Isolation of Lysosomes from Brain Tissue

A SEPARATION METHOD BY MEANS OF ALTERATION OF MITOCHONDRIAL AND SYNAPTOSOMAL SEDIMENTATION PROPERTIES

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1. A crude mitochondrial–lysosomal preparation from brain tissue was layered on a sucrose gradient containing 20mM-succinate, 10mM-Tris and 1mM-disodium EDTA at pH 7.4. The lysosomes were separated from the mitochondria and synaptosomes by means of a two-steps centrifugation procedure. In a first low-speed step (40min at 5300*g* at 15°C) the sedimentation rate of mitochondria and mitochondria-containing synaptosomes was enlarged due to passage of these subcellular structures through the sucrose gradient with the above-mentioned chemicals (called 'chemical field'). That part of the gradient which contained the mitochondria and synaptosomes was removed and substituted by a gradient suitable for isopycnic isolation of lysosomes in a second centrifugation step. The achieved purification for bovine brain lysosomes was 5–8-fold, for rat brain lysosomes 7–10-fold, over the homogenate. 2. The enlargement of the sedimentation rate of mitochondria and synaptosomes was brought about by the presence of succinate, but also by one of the following salts in the chemical field: malonate, fumarate, pyruvate, phosphate and chloride. 3. Comparison of the chemical-field method with other methods for the isolation of lysosomes showed that (*a*) with the chemical-field method a 2–3-times higher purification of the rat and bovine brain lysosomal fraction can be achieved than with the procedure described by Koenig, Gaines, McDonald, Gray & Scott [(1964) *J. Neurochem.* **11**, 729–743], and that (*b*) similar purification results for rat liver lysosomes were obtained when the chemical-field method and the procedure described by van Dijk, Roholl, Reijngoud & Tager [(1976) *FEBS Lett.* **62**, 177–181] were compared.

The usual methods for purification of brain lysosomes include a combination of differential and isopycnic centrifugation. Koenig *et al.* (1964) worked out these procedures for rat brain tissue. These authors found mitochondrial contamination of the lysosomal fraction which was low because of the different buoyant densities of both subcellular structures. The purification, based on the relative specific activity of the lysosomal marker enzyme acid phosphatase, was 4 times with respect to the homogenate. That procedure appeared to be unsuitable for the isolation of bovine brain lysosomes, since lysosomes and mitochondria of bovine brain possess equal density (Overdijk *et al.*, 1978).

Several attempts have been undertaken to improve the purification results for lysosomes by changing the density of subcellular particles. In some isolation procedures the buoyant density of lysosomes is changed by filling them with Triton WR 1339 (Wattiaux *et al.*, 1963; Trouet, 1964), Dextran 500 (Thinès-Sempoux *et al.*, 1966), or colloidal iron (Arborgh *et al.*, 1973, 1974*a*,*b*). These methods are not applicable to bovine brain tissue because the necessary injections with the undigestible materials are impossible in practice. Another approach is raising the density of mitochondria by deposition of insoluble dense formazan inside the mitochondria (Davis & Bloom, 1973; van Dijk *et al.*, 1976). However, bovine brain lysosomal enzymes were solubilized when the procedure described by van Dijk *et al.* (1976) was followed.

Therefore a new technique has been introduced, the preliminary results of which were published recently (Overdijk *et al.*, 1978). In this procedure the sedimentation rate rather than the density of mitochondria and mitochondria-containing synaptosomes is increased. The increased sedimentation rate was obtained during low-speed centrifugation of a crude mitochondrial–lysosomal suspension which had been layered on a discontinuous sucrose gradient in which buffered succinate was present. This gradient was called a 'chemical field'. Mitochondria and mitochondria-containing synaptosomes were separated from lysosomes, and ended up in the dense part of the sucrose gradient. After removal of this part and sub-

stitution of it by a sucrose gradient of higher density without succinate, the lysosomes were partly separated from other subcellular particles in an isopycnic run. The purification factors of four lysosomal marker enzymes were about 2–3 with respect to the original homogenate.

In the present report some modifications of our technique are described, which result in an improvement of the purification of the lysosomal fraction. In a second section the mechanism of the chemical field is studied. The last part describes experiments related to the applicability of the chemical-field method to tissues other than brain.

Experimental

Chemicals

The following materials were purchased from the suppliers indicated: 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside, 4-methylumbelliferyl β-D-glucopyranoside, 4-methylumbelliferyl β-D-galactopyranoside, 5′-AMP (disodium salt), NADH (disodium salt) and 4-hydroxyquinoline (Koch–Light Laboratories, Colnbrook, Bucks., U.K.); 2-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (J. T. Baker Chemicals B.V., Deventer, The Netherlands); kynuramine dihydrobromide (Sigma Chemical Co., St. Louis, MO, U.S.A.); human serum albumin, fraction V (Calbiochem, San Diego, CA, U.S.A.); Folin–Ciocalteu phenol reagent (Merck, Darmstadt, Germany); Triton X-100 (Rohm and Haas Co., Philadelphia, U.S.A.).

Other reagents were of analytical grade. All solutions were prepared with glass-distilled water.

Methods

Preparation of primary fractions. Adult bovine brain tissue was obtained from the local slaughterhouse. Time between killing the animal and storage of the brain tissue at 4°C never exceeded 2h. All further processing (dissection, homogenization, centrifugation) was carried out at 4°C. Grey matter was dissected from the white matter. Liver and brain tissue from male Wistar rats were immediately stored at 4°C. Homogenates (10%, w/v) in 0.32M-sucrose were prepared in a Potter–Elvehjem homogenizer (three strokes up and down) at 1400rev./min of the Teflon pestle; the clearance was 0.17mm. Centrifugations were carried out in a Sorvall RC-5 centrifuge with a SS-34 rotor. Depending on the kind of tissue, one of the following centrifugation schemes was followed.

Brain tissue. Nuclei and debris were removed by centrifuging the homogenate (H) for 10min at 500*g*. The pellet (P_1) was discarded and the supernatant (S_1) was centrifuged for 20min at 10000*g*. The resulting pellet was resuspended in 0.32M-sucrose, and recentrifuged for 20min at 10000*g*. The combined supernatants of these centrifugations (S_2) were discarded. The pellet P_2 (the crude mitochondrial–lysosomal fraction) was suspended in a volume (ml) of 0.32M-sucrose equal to the weight (g) of the fresh tissue.

Liver tissue. After removal of nuclei and debris (10min at 500*g*), and of the heavy mitochondria in a second centrifugation step (10min at 3000*g*), the crude lysosomal pellet (P_3) was obtained by centrifuging the supernatant from the foregoing step for 20min at 10000*g*. The resulting pellet was suspended in a volume (ml) of 0.32M-sucrose equal to the weight (in g) of the fresh tissue.

Isopycnic centrifugation in a discontinuous sucrose gradient. The P_2 fractions of brain tissue were subfractionated on a discontinuous sucrose gradient prepared as described by Koenig *et al.* (1964). In polycarbonate tubes the sucrose gradients were prepared at 4°C by successive layering of 3.5ml of each of the following solutions: 1.4M-, 1.2M-, 1.0M- and 0.8M-sucrose. On top of each gradient 3.5ml of the P_2 suspension was layered. Centrifugation was carried out for 2h at 46000*g* with a 3 × 25ml swing-out rotor in a MSE-65 centrifuge. The following fractions were collected: a clear upper layer (fraction 1); four distinct white–yellow bands at the interfaces of the different sucrose layers [fractions 2–5, corresponding to the fractions A–D described by Koenig *et al.* (1964)]; a clear layer (fraction 6); and a pellet (fraction 7, comparable with fraction E described by Koenig *et al.*, 1964). The pellet was suspended to a volume of 10ml in water.

Chemical-field centrifugation of the brain P_2 suspension. In polycarbonate tubes the following sucrose gradient was prepared: 2ml of 2M-sucrose as a cushion; 38ml of 0.8M- and 10ml of 0.5M-sucrose, which solutions comprised the chemical field. This field consisted of 20mM-sodium succinate, 10mM-Tris and 1mM-disodium EDTA, and was adjusted to pH7.4 with concentrated HCl. Then 2ml of the P_2 suspension was layered on top of the gradient. In experiments concerning the study of the mechanism of the chemical field, the sodium succinate was substituted by Na_2HPO_4, NaCl, and the sodium salts of malonic acid, fumaric acid and pyruvic acid respectively. The concentration for each salt was 20mM.

After a low-speed run (40min at 5300*g*) at 15°C with a 3 × 70ml swing-out rotor in a MSE-65 centrifuge, the tubes were partly unloaded from the dense side of the gradient; 26ml was collected as one fraction (fraction I). The tubes were reloaded with a discontinuous sucrose gradient starting with the less dense part of it. The gradient consisted of 3ml each of the following sucrose solutions: 0.85, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 2.0M. After centrifugation for 2h at 64000*g* and 4°C 22 fractions of 2.4ml were collected. The fractions 1–5, 6–7 and the fraction I were used for electron-microscopic examination.

Chemical-field centrifugation of the liver P_3 suspension. In polycarbonate tubes the following gradient was prepared: 2ml of 2M-sucrose as a cushion; 13ml of 1.4M-, 25ml of 1.0M- and 10ml of 0.6M-sucrose. These three layers contained the chemical field consisting of 20mM-sodium succinate, 10mM-Tris and 1mM-disodium EDTA, and were adjusted to pH7.4 with concentrated HCl. On top of the gradient 2ml of the P_3 suspension was layered.

After centrifuging for 25min at 5000*g* and 15°C with a 3 × 70ml swing-out rotor in a MSE-65 centrifuge the tubes were partly unloaded from the dense side of the gradient; 32ml was collected in 17 fractions. The tubes were reloaded with a discontinuous sucrose gradient consisting of 3ml each of the following sucrose solutions: 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8 and 2.0M. After centrifugation for 90min at 64000*g* at 4°C 28 fractions of 2ml were collected from each tube.

Electron microscopy. Fraction I, resulting from the chemical-field centrifugation, and the fractions 1–5 and 6–7, resulting from the isopycnic centrifugation, from bovine brain were diluted with an equal volume of 1.1M-sucrose, and centrifuged for 1h at 37000*g* and 4°C in a Sorvall RC-5 centrifuge with a SS-34 rotor. The resulting pellets of the different fractions were covered overnight with freshly prepared 2% glutaraldehyde in 1.1M-sucrose. The glutaraldehyde was decanted, and the pellets were rinsed with 1.1M-sucrose. The pellets were post-fixed for 2h with a 2% osmium tetroxide solution in Millonig (1962) buffer; this buffer was composed as follows: 0.012M-NaH_2PO_4, 0.01M-NaOH and 0.003M-glucose. After post-fixation the pellets were dehydrated through a series of graded ethanol concentrations ranging from 50 to 100%. The dehydrated pellets were then treated two times for 15min with a 1:1 mixture of 100% ethanol and propylene oxide, and two times for 30min with propylene oxide, followed by an overnight immersion in a mixture of equal parts of propylene oxide and Araldite. After this the pellets were immersed in Araldite for 2h. Finally they were embedded in Araldite and polymerized for 48h at 60°C. Thin sections were cut with a LKB Ultratome III to a thickness of 35nm (350Å). The sections were mounted on 200 or 300 mesh naked copper grids, and then stained with uranyl acetate and lead citrate by the method of Reynolds (1963). Observations of the specimens were carried out with a Philips model EM 301 electron microscope.

Enzyme assays. (1) Lactate dehydrogenase (EC 1.1.1.27). This was measured with the aid of the Vitatron automatic kinetic enzyme system in a Vitatron MPS photofluorimeter (McQueen & King, 1975). Samples of the fractions were preincubated 30min at 0°C in the presence of 0.5% (v/v) Triton X-100 in order to liberate membrane- or organelle-enclosed lactate dehydrogenase. The enzyme sample, usually 100µl, was then added to 2ml of a buffer/substrate mixture, pH7.5, containing the following components: 3.38mM-Na_2HPO_4; 1.07mM-KH_2PO_4; 0.2mM-sodium pyruvate; 0.2mM-disodium NADH. The decrease of the absorbance at 340nm, due to the oxidation of NADH, was measured at 25°C.

(2) Succinate dehydrogenase (EC 1.3.99.1). This was measured by the method described by Pennington (1961).

(3) Monoamine oxidase (EC 1.4.3.4). The incubation mixture contained: 0.25ml of 0.1M-borax/3mM-disodium EDTA, adjusted to pH8.2 with concentrated HCl; 0.2ml of 0.384mM-kynuramine dihydrobromide; 0.25ml of water; 0.05ml of enzyme sample. After 30min at 37°C the reaction was stopped with 1.5ml of 1.5M-NaOH. After 30min at 20°C the fluorescence of the formed 4-hydroxyquinoline was measured, with a primary filter of 313nm and a secondary filter U5 (maximum transmission at 365nm).

(4) Acid phosphatase (EC 3.1.3.2). The incubation mixture contained: 0.4ml of 0.2M-sodium acetate buffer, pH5.0, containing 0.25% (v/v) Triton X-100; 0.1ml of 1.0M-sodium β-glycerophosphate, adjusted to pH5.0 with concentrated acetic acid; 0.5ml of enzyme sample. After 30min at 37°C the reaction was stopped with 1.0ml of 10% (w/v) trichloroacetic acid, after which the mixture was cooled in ice, diluted to 2.5ml with 5% (w/v) trichloroacetic acid, and centrifuged 5min at 3000rev./min in a Sorvall GLC-2 centrifuge. In the clear supernatant the liberated P_i was determined by the method described by Chen *et al.* (1956).

(5) 5′-Nucleotidase (EC 3.1.3.5). The incubation mixture contained: 0.3ml of 0.2M-Tris/12.5mM-$MgCl_2$, adjusted to pH7.7 with concentrated HCl; 0.1ml of 0.05M-5′-AMP adjusted to pH8.0 with concentrated HCl; 0.1ml of 0.1M-sodium L(+)-tartrate to inhibit acid phosphatase activity (Goodlad & Mills, 1957; Neil & Horner, 1964); 0.5ml of enzyme sample. After incubation for 30min at 37°C the reaction was stopped as described for acid phosphatase, and P_i was determined as described by Chen *et al.* (1956).

(6) Glucose 6-phosphatase (EC 3.1.3.9). The incubation mixture contained: 0.4ml of 0.014M-histidine/2mM-disodium EDTA, adjusted to pH6.5 with concentrated HCl; 0.1ml of 0.1M-sodium glucose 6-phosphate; 0.5ml of enzyme sample. After 30min at 37°C the reaction was stopped as described for acid phosphatase and P_i was determined as described by Chen *et al.* (1956).

(7) β-Glucosidase (EC 3.2.1.21). The buffer/substrate solution was prepared as follows: 4-methylumbelliferyl β-D-glucopyranoside and Triton X-100 were dissolved in 0.143M-sodium acetate buffer, pH4.7, in a final concentration of 1.43mM and 2.86% (v/v) respectively. The incubation mixture contained: 0.7ml of buffer/substrate solution; 0.3ml of enzyme

sample. After 30 min at 37°C the reaction was stopped, and the liberated 4-methylumbelliferone was measured as described by Overdijk *et al.* (1975).

(8) β-Galactosidase (EC 3.2.1.23). The buffer/substrate solution was prepared as follows: 4-methylumbelliferyl β-D-galactopyranoside and Triton X-100 were dissolved in 0.143 M-sodium acetate buffer, pH 3.8, in a final concentration of 1.43 mM and 1.43% (v/v) respectively. The incubation mixture contained: 0.7 ml of buffer/substrate solution; 0.3 ml of enzyme sample. After 30 min at 37°C the reaction was stopped, and the liberated 4-methylumbelliferone was measured as described by Overdijk *et al.* (1975).

(9) β-Glucosaminidase (β-*N*-acetyl-D-glucosaminidase, EC 3.2.1.30). This was assayed as described by Overdijk *et al.* (1975). All absorbance and fluorescence values were measured with a Vitatron MPS photofluorimeter.

Other assays

(1) Protein was determined by the method of Lowry *et al.* (1951). Crystalline human serum albumin was used as a standard.

(2) The concentration of the sucrose solutions was determined by measuring the refractive indices at 20°C with an Abbe refractometer.

(3) Since sucrose interferes in the assay of enzymes and protein (Hinton *et al.*, 1969) corrections had to be made for the different assay values. For the enzyme assays of acid phosphatase and glucose 6-phosphatase the correction factors given by Hinton *et al.* (1969) were used. Experiments analogous to those of Hinton *et al.* (1969) were done to obtain these factors for the other assays. In Table 1 these data are summarized.

The data described in the Results section are the mean values for four experiments in which each assay has been performed in duplicate.

Table 1. *Correction factors for the assays due to sucrose interference*

The correction factors for the assay of lactate dehydrogenase and monoamine oxidase were not determined. These factors can be neglected because the sucrose concentrations which can maximally be reached under the assay conditions are 0.05 and 0.13 M respectively. '$[Sucrose]_{max.}$' is the maximum sucrose concentration that can be reached in the assay mixture. The correction factors are those by which the absorbance and fluorescence values, measured in the presence of maximum sucrose concentration, have to be multiplied in order to correct the sucrose interference. The corresponding factors for lower sucrose concentrations can be deduced from the linear relationship between the sucrose interference and the concentration of sucrose in the assay mixture.

Assay	$[Sucrose]_{max.}$ (M)	Correction factor
Succinate dehydrogenase	1.00	2.27
Acid phosphatase	1.00	1.18
5'-Nucleotidase	1.00	1.34
Glucose 6-phosphatase	1.00	2.00
β-Glucosidase	0.60	1.19
β-Galactosidase	0.60	0.79
β-Glucosaminidase	0.33	1.60
Protein	0.33	2.50

Results

Chemical-field procedure for purification of brain lysosomes

The principle of the chemical field method for the isolation of bovine brain lysosomes has been described in preliminary form (Overdijk *et al.*, 1978). This principle was not changed, but some modifications with regard to the homogenization and centrifugation circumstances were introduced in order to optimize the purification conditions. By using a 10% instead of a 25% (w/v) homogenate a higher yield of lysosomal marker enzyme activities was obtained in the P_2 fraction of the primary fractionation (Table 2). For rat brain these results were similar to those given by Koenig *et al.* (1964). The small differences for the distribution percentages of fractions P_1 and P_2 are probably caused by the fact that we did not wash fraction P_1, whereas Koenig *et al.* (1964) did so twice. The substitute sucrose gradient was discontinuous instead of continuous, and the time needed for isopycnic centrifugation with the Sorvall SZ-14 rotor (17 h) was reduced to 2 h.

The results of the chemical-field and isopycnic centrifugations are given in Fig. 1 and Table 3. The fractions 1–5 are designated as the lysosomal fraction. The top of the lysosomal marker enzyme activity is clearly separated from the peaks of lactate dehydrogenase activity (synaptosomal marker enzyme) and of 5'-nucleotidase activity (plasma-membrane marker enzyme). Hardly any mitochondrial marker enzyme activity could be detected in these fractions. The percentages of nearly all non-lysosomal marker enzyme activities (with the exception of 5'-nucleotidase) in the lysosomal fraction were reduced when compared with our previous results.

Fraction I contained the following amounts of enzyme activity expressed as percentages of the total activity present in the P_2 suspension applied to the gradient: 98% succinate dehydrogenase, 92% monoamine oxidase, 90% lactate dehydrogenase and 70–85% of the acid hydrolase activities. In control experiments without chemical field none of these enzymic activities were present in a comparable part of the gradient. The explanation for these results is that, due to passage through the chemical field, mitochondria and synaptosomes reach a higher sedimentation rate. Some synaptosomes enclose, besides mitochondria, also lysosomes (Gordon *et al.*, 1968). Such

Table 2. *Distribution of marker enzyme activity and of protein in primary fractions of brain homogenates*
Experimental details are given in the Experimental section under 'Preparation of primary fractions'. Values are means ±S.E.M. for four experiments in which the enzyme activities are determined in duplicate. Activity in the homogenate was taken as 100%. Relative specific activity is the percentage of total enzyme activity in a fraction recovered from the homogenate/percentage of total protein in that fraction recovered from the homogenate.

	Recovery (%)	Percentage distribution			Relative specific activity		
		P_1	P_2	S_2	P_1	P_2	S_2
(*a*) Bovine brain							
β-Glucosaminidase	90 ± 5	36 ± 4	42 ± 1	22 ± 2	1.39 ± 0.01	1.00 ± 0.02	0.76 ± 0.06
β-Glucosidase	110 ± 9	28 ± 4	60 ± 3	12 ± 2	1.35 ± 0.13	1.73 ± 0.03	0.51 ± 0.09
β-Galactosidase	99 ± 10	37 ± 6	50 ± 5	13 ± 2	1.59 ± 0.08	1.28 ± 0.08	0.51 ± 0.01
Acid phosphatase	102 ± 6	28 ± 1	49 ± 7	23 ± 5	1.26 ± 0.09	1.31 ± 0.14	0.81 ± 0.35
Monoamine oxidase	100 ± 11	26 ± 2	68 ± 12	6 ± 1	1.15 ± 0.20	1.77 ± 0.24	0.24 ± 0.05
Succinate dehydrogenase	94 ± 6	20 ± 3	76 ± 7	4 ± 1	0.85 ± 0.22	1.84 ± 0.16	0.24 ± 0.07
Lactate dehydrogenase	62 ± 8	10 ± 1	42 ± 4	48 ± 7	0.27 ± 0.07	0.69 ± 0.13	1.46 ± 0.39
5′-Nucleotidase	105 ± 4	26 ± 7	31 ± 6	43 ± 8	1.14 ± 0.23	0.86 ± 0.19	1.48 ± 0.15
Glucose 6-phosphatase	91 ± 7	32 ± 9	37 ± 1	31 ± 1	1.46 ± 0.20	0.99 ± 0.08	1.12 ± 0.29
Protein	87 ± 6	26 ± 3	44 ± 2	30 ± 4	—	—	—
(*b*) Rat brain							
β-Glucosaminidase	84 ± 1	21 ± 3	55 ± 3	24 ± 2	0.96 ± 0.19	1.13 ± 0.06	0.63 ± 0.11
β-Glucosidase	96 ± 2	13 ± 2	65 ± 1	22 ± 1	0.49 ± 0.17	1.56 ± 0.05	0.69 ± 0.11
β-Galactosidase	86 ± 2	19 ± 3	65 ± 5	16 ± 1	0.85 ± 0.13	1.38 ± 0.07	0.44 ± 0.07
Acid phosphatase	96 ± 4	27 ± 8	45 ± 6	28 ± 6	1.04 ± 0.38	1.14 ± 0.09	0.92 ± 0.19
Monoamine oxidase	82 ± 8	22 ± 2	68 ± 7	10 ± 2	0.98 ± 0.21	1.38 ± 0.09	0.27 ± 0.07
Succinate dehydrogenase	81 ± 6	8 ± 5	87 ± 11	5 ± 2	0.37 ± 0.22	1.80 ± 0.17	0.18 ± 0.01
Lactate dehydrogenase	80 ± 4	10 ± 3	53 ± 3	37 ± 4	0.37 ± 0.07	1.10 ± 0.01	0.94 ± 0.15
5′-Nucleotidase	99 ± 10	25 ± 7	39 ± 2	36 ± 2	1.29 ± 0.29	0.95 ± 0.09	1.13 ± 0.06
Glucose 6-phosphatase	92 ± 1	19 ± 3	39 ± 1	42 ± 5	0.98 ± 0.29	0.69 ± 0.16	0.79 ± 0.24
Protein	91 ± 5	31 ± 9	38 ± 4	31 ± 5	—	—	—

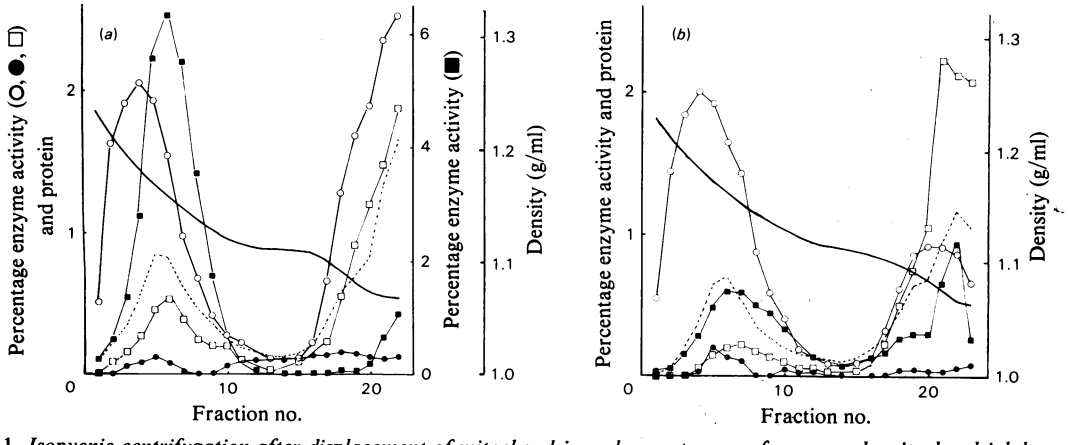


Fig. 1. *Isopycnic centrifugation after displacement of mitochondria and synaptosomes from a crude mitochondrial–lysosomal preparation of bovine (a) and rat (b) brain*
The details of the low-speed centrifugations whereby the sedimentation rate of mitochondria and synaptosomes is enlarged (chemical-field centrifugation) and of the isopycnic centrifugations are given in the Experimental section. The enzyme activities β-glucosaminidase (○), succinate dehydrogenase (●), lactate dehydrogenase (□) and 5′-nucleotidase (■), and protein (----) are expressed as percentages of the total activity present in the crude mitochondrial–lysosomal preparation applied to the sucrose gradient. ——, Density (g/ml).

Table 3. *Isolation of bovine and rat brain lysosomes: recoveries of marker enzyme activity and of protein after chemical-field centrifugation of the mitochondrial–lysosomal fraction P_2*

The experimental details of the chemical-field centrifugation are given in the Experimental section. The data given under 'Recovery' are the percentages of the marker enzyme activity or of protein recovered from the applied sample (100%) on the gradient. The 'Recovered amount in the lysosomal fraction' is the percentage of the total enzyme activity or of protein recovered in the lysosomal fraction (fraction 1–5, Fig. 1). Values are the means ±S.E.M. for four experiments in which the enzyme activities are determined in duplicate.

	Recovery (%)		Recovered amount in lysosomal fraction (%)	
	Bovine	Rat	Bovine	Rat
β-Glucosaminidase	93 ± 6	101 ± 2	8.4 ± 0.6	9.4 ± 1.5
β-Glucosidase	112 ± 12	—*	7.8 ± 1.5	—*
β-Galactosidase	108 ± 7	108 ± 5	6.7 ± 0.5	6.3 ± 0.4
Acid phosphatase	105 ± 4	121 ± 11	5.5 ± 0.6	6.2 ± 0.6
Monoamine oxidase	114 ± 10	96 ± 1	1.9 ± 0.3	2.2 ± 0.1
Succinate dehydrogenase	58 ± 5	152 ± 18	0.2 ± 0.1	0.1 ± 0.05
Lactate dehydrogenase	231 ± 69	243 ± 47	0.4 ± 0.2	0.2 ± 0.1
5′-Nucleotidase	19 ± 7	29 ± 10	10.7 ± 1.1	1.0 ± 0.2
Glucose 6-phosphatase	178 ± 44	90 ± 39	2.0 ± 0.7	3.6 ± 1.3
Protein	113 ± 15	112 ± 6	1.0 ± 0.3	0.9 ± 0.1

* Enzyme activities were too low to permit calculations.

synaptosomes are responsible for the 70–85% lysosomal marker enzyme activities in fraction I of the chemical-field centrifugation. A similar percentage of hydrolase activity was present in the synaptosomal fractions obtained by discontinuous sucrose gradient centrifugation by the method of Koenig *et al.* (1964). About 10% of the lysosomal enzyme activities was present in the fraction of the free lysosomes in both methods.

This new procedure has led to a 5–8-fold purification of the lysosomal fraction, being an improvement with a factor 2–4 in comparison with the previously published results (Overdijk *et al.*, 1978).

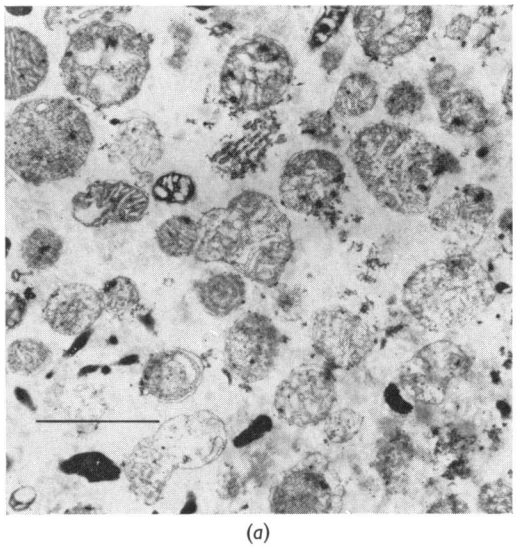
Electron microscopy

In Plate 1 the results of a typical experiment with bovine brain are given. A crude mitochondrial–lysosomal preparation was subjected to chemical-field centrifugation and isopycnic centrifugation successively. Plate 1(*a*) is an electron micrograph of fraction I. In this fraction numerous mitochondria and synaptosomes are present, and also some lipofuscine granules are visible. Plate 1(*b*) is a representative picture of the fraction designated the lysosomal fraction. Besides free lysosomes this fraction also contains the dumb-bell-shaped structures of the postsynaptic membranes (Cohen & McGovern, 1973; Garey *et al.*, 1972). No mitochondria could be detected in this fraction. Only a few mitochondria-like structures are visible, but their diameter (0.02 μm) appears to be too small for mitochondria. Plate 1(*c*) is an electron micrograph of the fraction with highest synaptosomal marker enzyme activity (fraction 6–7). Besides free lysosomes numerous postsynaptic densities, some synaptosomes and lipofuscine granules are present.

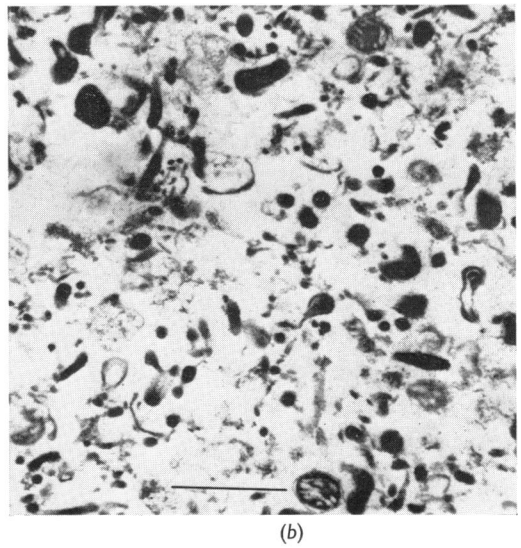
Mechanism of the chemical field

In the isolation procedures for rat brain synaptosomes (Davis & Bloom, 1973) and rat liver lysosomes (van Dijk *et al.*, 1976) a crude mitochondrial–lysosomal preparation was treated with succinate/2-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride to enlarge the density of mitochondria. This resulted in a separation of these particles from other subcellular organelles. In our previous report (Overdijk *et al.*, 1978) it was shown that the presence of 2-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride in the chemical field was not a compelling condition for the change in sedimentation properties of mitochondria. The mere presence of succinate was sufficient to accomplish this effect. The proposed mechanism was that succinate permeates the mitochondrial membrane, and that consequently the mitochondria will swell. This swelling should cause the acceleration of the sedimentation rate. If this explanation were to be true, other salts which permeate the mitochondrial membrane should show the same effect as succinate. On the other hand, if it was a specific effect of succinate and if succinate dehydrogenase activity were involved, then those salts which prevent succinate dehydrogenase activity or which are not at all involved in mitochondrial enzyme activities should not influence the sedimentation rate of mitochondria.

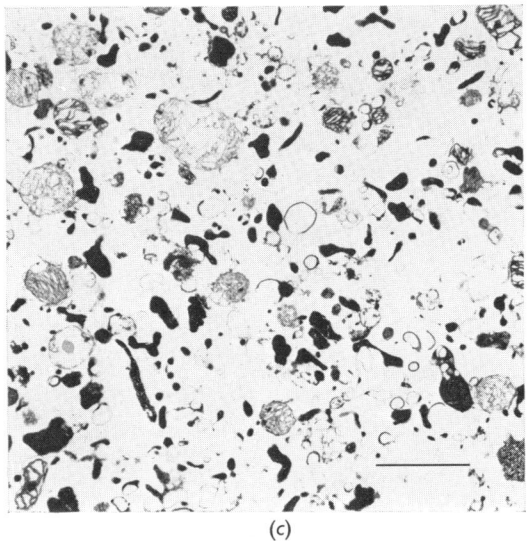
To check these possibilities the following salts were tested for their effectiveness in the chemical field: malonate (competitive inhibitor of succinate dehydro-



(*a*)



(*b*)



(*c*)

EXPLANATION OF PLATE 1

Electron micrographs of subcellular fractions from bovine brain isolated by chemical-field and isopycnic centrifugation The experimental details for the preparation of fraction I, 1–5 and 6–7 are described in the Experimental section. (*a*) Fraction I, showing mitochondria and synaptosomes; (*b*) fraction 1–5, containing free lysosomes and some postsynaptic membranes; (*c*) fraction 6–7, with postsynaptic membranes and some free lysosomes. The bars in (*a*) and (*c*) represent 1 μm; the bar in (*b*) represents 0.5 μm.

Table 4. *Isolation of bovine and rat brain lysosomes: comparison of the chemical-field centrifugation and the discontinuous sucrose gradient centrifugation*

The experimental details of both centrifugation methods are given in the Experimental section. The fractions which were compared are fractions 1–5 of the chemical-field centrifugation and fraction 7 of the discontinuous sucrose gradient centrifugation. Values are means ± S.E.M. for four experiments in which the enzyme activities are determined in duplicate. 'Recovery' is the percentage of the activity recovered from the applied sample on the gradient. 'Relative specific activity' is the percentage of total enzyme activity recovered in a fraction/percentage of total protein recovered in that fraction.

	Chemical-field centrifugation		Discontinuous sucrose gradient centrifugation	
	Recovery	Relative specific activity	Recovery	Relative specific activity
(*a*) Bovine brain				
β-Glucosaminidase	93 ± 6	7.06 ± 0.64	87 ± 4	2.69 ± 0.65
β-Glucosidase	112 ± 12	7.99 ± 1.79	77 ± 4	2.42 ± 0.50
β-Galactosidase	108 ± 7	6.10 ± 1.40	87 ± 5	2.45 ± 0.05
Acid phosphatase	105 ± 4	5.23 ± 1.08	82 ± 10	2.42 ± 0.12
Monoamine oxidase	114 ± 10	1.85 ± 0.45	79 ± 9	5.17 ± 0.74
Succinate dehydrogenase	58 ± 5	0.05 ± 0.01	93 ± 3	7.21 ± 1.18
Lactate dehydrogenase	231 ± 69	0.49 ± 0.09	102 ± 17	0.24 ± 0.07
5′-Nucleotidase	19 ± 7	0.22 ± 0.12	143 ± 69	0.21 ± 0.10
Glucose 6-phosphatase	178 ± 44	3.15 ± 0.56	79 ± 7	1.13 ± 0.09
Protein	113 ± 15	—	93 ± 8	—
(*b*) Rat brain				
β-Glucosaminidase	101 ± 2	9.46 ± 1.50	99 ± 5	5.43 ± 1.25
β-Glucosidase	—*	—*	75 ± 9	2.95 ± 0.61
β-Galactosidase	108 ± 5	6.76 ± 0.30	97 ± 4	7.14 ± 1.41
Acid phosphatase	121 ± 11	7.56 ± 1.30	93 ± 20	3.21 ± 0.62
Monoamine oxidase	96 ± 1	2.12 ± 1.14	104 ± 4	4.81 ± 0.50
Succinate dehydrogenase	152 ± 18	0.05 ± 0.01	47 ± 17	0.14 ± 0.03
Lactate dehydrogenase	243 ± 47	0.37 ± 0.32	114 ± 30	0.31 ± 0.03
5′-Nucleotidase	29 ± 10	0.31 ± 0.16	114 ± 2	0.23 ± 0.04
Glucose 6-phosphatase	90 ± 39	3.22 ± 1.58	136 ± 14	2.68 ± 0.35
Protein	112 ± 6	—	96 ± 1	—

* Enzyme activities were too low to permit calculations.

genase); fumarate (product of succinate dehydrogenase reaction); pyruvate; phosphate; chloride. All these salts brought about the same effect as succinate. These results suggest that not enzymic reactions, but rather physicochemical processes, are the cause of the acceleration of the sedimentation rate.

General applicability of the chemical-field method

Brain tissue. By isopycnic centrifugation with a discontinuous sucrose gradient Koenig *et al.* (1964) were able to purify rat brain lysosomes (fraction E) 4-fold over the homogenate. This value was based on the relative specific activity of acid phosphatase.

With the following approach the value of the chemical-field method was compared with that of the method described by Koenig *et al.* (1964). Crude mitochondrial–lysosomal suspensions of rat and of bovine brain were purified with both methods. The lysosomal fractions to be compared are the fractions 1–5 of the chemical-field centrifugation and fraction 7 of the discontinuous sucrose gradient centrifugation.

The results of these experiments are shown in Table 4. With the chemical-field method the purification factors (relative specific activity values) of the lysosomal marker enzymes of both tissues were 2–3 times higher, with the exception of rat brain β-galactosidase. The recovery of the lysosomal marker enzymes with respect to the applied activities on the gradients was always about 100%. The relative specific activities of the mitochondrial marker enzymes in the lysosomal fraction were strongly decreased by using the chemical-field method instead of the method of Koenig *et al.* (1964). For bovine brain the relative specific activity of succinate dehydrogenase was even decreased to less than 1% of the value obtained with the method of Koenig *et al.* (1964): 0.05 and 7.21 respectively. The relative specific activities of the other non-lysosomal marker enzymes in the lysosomal fraction were nearly equal for both methods.

Liver tissue. A crude rat liver lysosomal preparation was purified with the chemical-field method in order to compare the results obtained by this method

with those obtained by the procedure of van Dijk *et al.* (1976). The *g*-min value and the sucrose gradient for the low-speed run were coarsely adapted to the sedimentation characteristics of rat liver subcellular particles. Under these suboptimal conditions we found a relative specific activity for β-glucosaminidase by the chemical-field method of 6.0, whereas van Dijk *et al.* (1976) achieved a factor of 8.5.

Discussion

Purification results

The effectiveness of the chemical field for displacing mitochondria out of a crude mitochondrial–lysosomal preparation has already been proven (Overdijk *et al.*, 1978). The conclusions based on the biochemical data of marker-enzyme activities, especially with respect to hardly detectable mitochondrial enzyme activities, were sustained by the electron-microscopic observations. Fraction I consisted of mitochondria and synaptosomes, fractions 1–5 predominantly contained free lysosomes, but also synaptic structures, whereas in the fractions 6–7 numerous synaptic densities besides some free lysosomes were visible.

The cause of the higher purification of lysosomes achieved with the present procedure can be attributed only partly to the modified centrifugation circumstances: a discontinuous substitute gradient and shorter centrifugation time for isopycnic centrifugation. However, the lower percentage of mitochondrial enzyme activity in the lysosomal fraction cannot be explained in this way, since the centrifugation conditions of the low-speed run with the chemical field were not changed. The radial distances in the Sorvall SZ-14 zonal rotor and in the MSE 3 × 70ml rotor tubes are almost identical, and so are the centrifugal force profiles. The composition of the chemical field was also identical.

An explanation for the present improvement could be a temperature effect during the low-speed run in the ultracentrifuge. Only a 1°C increase of the temperature results in a 3.3% lowering in viscosity of the sucrose solutions. Consequently the sedimentation rate of subcellular particles becomes higher. In this respect it should be considered that maintaining the temperature of rotor and rotor chamber in the case of an ultracentrifuge is less effective than with a high-speed centrifuge without vacuum, because (1) the heat content of the SZ-14 rotor is higher than that of the MSE 3 × 70ml swing-out rotor, and (2) the cooling in a vacuum chamber is less effective.

In a previous report (Overdijk *et al.*, 1978) it was argued that mitochondrial swelling was probably the basis for the acceleration of the sedimentation rate. The uptake of succinate and of the other salts is due to the passive temperature-dependent process of diffusion. At higher temperature the diffusion increases so that the mitochondrial swelling also will increase. Although this effect may be relatively small, the ultimate effect on the sedimentation rate cannot be neglected since the sedimentation rate of a particle is directly proportional to the square of its radius.

In bovine brain the ratio of the relative specific activities for the two mitochondrial marker enzymes (succinate dehydrogenase and monoamine oxidase) shows disparity when the results of both centrifugation methods are compared (Table 4). The relative specific activities for both enzymes are also different in the chemical-field centrifugation, but nearly equal in the discontinuous sucrose gradient centrifugation. These results are difficult to explain. There might be mitochondrial heterogeneity with different enzyme content, but equal density for the various populations. These different kinds of mitochondria would not accelerate to the same extent in the chemical-field centrifugation. Another explanation might be that a difference in latency becomes manifest for the respective mitochondrial marker enzymes due to the conditions used in the chemical field, giving rise to the mentioned discrepancy.

Mechanism of the chemical field

The mechanism of the chemical field was already discussed in our preliminary report (Overdijk *et al.*, 1978). The mitochondrial membrane is permeable for succinate, and in the presence of succinate significant mitochondrial swelling occurs (Rendon & Packer, 1976). It was argued that the swelling was probably the basis for the acceleration of the sedimentation rate. This explanation for the mechanism of the chemical field was supported by the results of the experiments in which the chemical field contained salts other than succinate. The mitochondrial membrane is permeable for all these salts. By the choice of the salts an enzymic involvement in the chemical-field mechanism could be ruled out.

The lysosomal membrane is impermeable for the salts in the chemical field (Reijngoud & Tager, 1977). The sedimentation rate of these particles when passing through the chemical field therefore does not change.

General applicability

The following conclusions can be drawn with respect to the general applicability of the chemical-field method for the isolation of lysosomes: (1) the method results in a 2–3-fold higher purification for brain lysosomes than can be achieved with the known methods (Koenig *et al.*, 1964); (2) the method is not restricted to application to brain tissue, but can be used as a general one for the isolation of lysosomes, although the *g*-min values have to be adapted to the actual kind of tissue, especially for the low-speed run with the chemical field.

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