ALTERATION OF SUBCELLULAR ORGANELLES INDUCED BY COMPRESSION

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1. Introduction

Rat liver mitochondria may suffer extensive biochemical and morphological alterations during high-speed centrifugation in a sucrose gradient [1]. The particular conditions under which these alterations occur point towards the hydrostatic pressure generated by the centrifugal force as the main causal factor [2]. Lysosomes and peroxisomes appear also sensitive to high-speed centrifugation [3]. These observations have led us to investigate directly the effects of pressure on subcellular organelles by hydraulically compressing rat-liver granules under various conditions. Hydraulic compression was preferred to centrifugation because, in the latter case, the pressure as well as other factors that may act upon the stability of organelles (centrifugal force, concentration of sucrose) vary throughout the liquid column. After compression of crude subcellular preparations, the structural integrity of various organelles was monitored separately by measurement of the structure-linked latency of marker enzymes included within: the mitochohdrial matrix (malate dehydrogenase [4]); the intermembrane space of mitochondria (sulfate cytochrome *c* reductase [5]); peroxisomes (catalase [6]); lysosomes (acid phosphatase [7]); and *N*-acetyl-β-glucosaminidase [8]) and microsomal vesicles derived from endoplasmic reticulum (nucleoside diphosphatase [9, 10]). All these enzymes are largely latent in intact granules, either because they are inaccessible to external substrates owing to the impermeability of the limiting membrane, or because the rate of the reaction is limited by the rate of diffusion of the substrate molecules [11]. We have observed that they become unmasked by compression at 500–1000 $kg \cdot cm^{-2}$. Suppression of the structure-linked latency results from the release of the enzymes in the surrounding medium and reflects alterations of the membrane limiting the corresponding compartment. The sensitivity of organelles to compression increases with decerasing temperature from 27 to 3°C.

2. Experimental

2.1. *Tissue fractionation*

Rat livers were homogenized in 0.25 M ice-cold sucrose (4 ml/g tissue) buffered at pH 7.4 with 3 mM imidazole–HCl. Nuclei and tissue debris were sedimented by 10 min centrifugation at 1700 rpm (rotor no. 252, International centrifuge, model PR-2, International Equipment Company, Boston, Mass.,), rehomogenized in 3 ml of 0.25 M buffered sucrose per g tissue and pelleted by 10 min centrifugation at 1400 rpm (fraction N).

Fifty ml samples of the combined supernatants (cytoplasmic extract = fraction E) were then quickly freed of soluble proteins and low molecular weight constituents by gel chromatography on Sepharose 2B at 4°C, on a 8 × 5 cm bed (total volume = 250 ml) elution being performed with buffered 0.25 M sucrose at 120 ml/hr flow rate. The elution curves of protein and various enzymes are shown in fig. 1. The first peak of protein corresponds to subcellular organelles excluded from the gel. Microsomes were included within this material, as shown by the elution curve of glucose 6-phosphatase [12]. In agreement with results of tissue fractionation, catalase [13] and malate dehydrogenase [4] separated into soluble and particle-bound enzymes. In practice, the 50 ml eluted following the void volume were combined into a cytoplasmic granules (G) fraction which contained about 2/3 of the granules peak and was cleared of soluble constituents. The material eluted afterwards was recovered

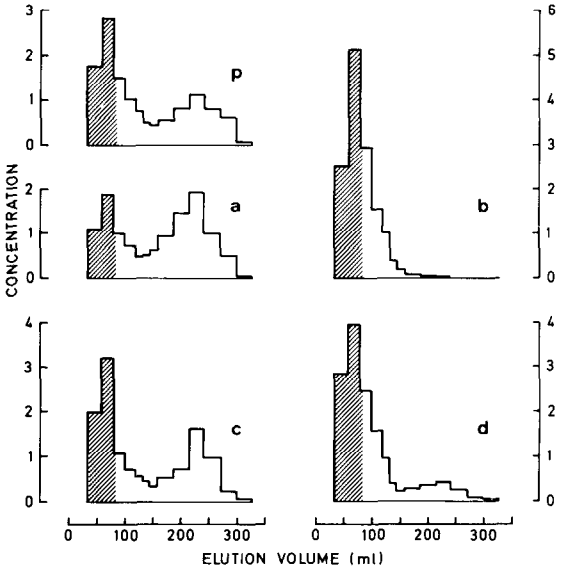

Fig. 1. Gel chromatography of a cytoplasmic extract on Sepharose 2B. Concentrations are expressed in a standardized form as % of recovered constituent/% of recovered volume. Elution curves of: (a) total malate dehydrogenase; (b) glucose 6-phosphatase assayed according to [15]; (c) total catalase; (d) acid phosphatase and (p) protein. Cross-hatching indicates the material selected in the G fraction. Experimental procedure is given in the text.

(R fraction) to establish the balance-sheet of constituents. The distributions of several constituents are reported in table 1.

2.2. *Compression procedure*

For compression, the samples, in stoppered flexible polyallomer tubes, were introduced into a thick-wall chamber machined from hardened steel, filled with water and connected to a hand-operated hydraulic pump (Enerpac P-228, Applied Power Industries Inc., Butler, Wisconsin). The compression unit was immersed in a water-bath at constant temperature. Temperature within the high pressure chamber was monitored throughout the experiment by a pressure-resistant thermocouple (Thermocoax TCA, Sodern SA, France) feeding into a digital millivoltmeter (Keithley model 163, Keithley Instruments Inc., Cleveland, Ohio) connected with a voltage recorder (Varian model A-25, Varian Associates, Palo Alto, California). Due to the partially adiabatic character of the system a temperature rise of the order of 1–3°C occurred within the chamber when the pressure was established. In order to compensate for this increase, the system was precooled to a temperature Δt°C below that set for the experiment, Δt° being read from an empirical calibration curve relating it to pressure. Pressure was then established progressively, at a rate of 800 kg·cm^{-2} min^{-1}. In this way temperature remained constant

Table 1
Distribution of constituents after fractionation of liver.

Constituent	No. of expts.	Absolute values (E + N)	Percentage values			
			N	G	R	Recovery
Protein	19	224 ± 16	20.8 ± 3.0	25.5 ± 3.6	53.7 ± 4.5	100.0 ± 8.5
Malate dehydrogenase	15	548 ± 87	17.0 ± 3.9	25.9 ± 4.7	51.0 ± 5.5	92.9 ± 9.0
Sulfite cytochrome *c* reductase	9	11.6 ± 2.1	20.4 ± 5.3	40.4 ± 6.7	34.0 ± 4.8	94.8 ± 10.1
N-acetyl-β-glucosaminidase	13	6.1 ± 0.7	14.8 ± 3.0	47.2 ± 10.2	33.6 ± 10.9	95.6 ± 9.8
Catalase	13	53.0 ± 8.7	11.3 ± 4.7	30.1 ± 10.5	43.6 ± 11.2	85.0 ± 8.8
Nucleoside diphosphatase	10	88.7 ± 20.3	17.2 ± 4.9	41.0 ± 9.7	32.7 ± 11.6	90.9 ± 14.2

Results are given as means ± S.D. Absolute values refer to one g fresh weight of liver; they are expressed in mg for protein and in units for total activities of enzymes. One unit of activity is defined as the amount of enzyme causing, in 1 min and under the assay conditions, the destruction of 90% of the H_2O_2 (catalase) or the formation of 1 μmole of reaction product (other enzymes). Percentage values refer to the sum of absolute values in fractions E and N.

Table 2
Free activity of enzymes in fractions E and G.

Enzyme	Fraction E	Fraction G
Malate dehydrogenase	40.3 ± 7.9	5.9 ± 2.1
Sulfite cytochrome *c* reductase	21.1 ± 6.0	3.5 ± 1.0
N-acetyl-β-glucosaminidase	22.9 ± 9.6	19.3 ± 4.3
Catalase	40.1 ± 8.3	8.7 ± 2.1
Nucleoside diphosphatase	10.5 ± 3.2	9.5 ± 4.1

Free activities are given as means ± S.D. in percent of total activity. The number of experiments is that reported in table 1.

within ± 0.3°C as long as the maximum pressure was maintained.

2.3. *Enzyme assays*

Free enzymic activities were determined under conditions designed to preserve the structural integrity of subcellular organelles; in particular osmotic protection was achieved by making the incubation medium 0.25 M in sucrose. Total enzymic activities were determined under identical conditions, except that the membrane barriers were suppressed by addition of Triton X-100 or sodium deoxycholate to the incubation medium. The structural latency of an enzyme is taken to be the difference: total minus free activity.

Malate dehydrogenase was assayed by the rate of decrease over 5–10 min of absorbance at 340 nm, in a mixture containing: the enzyme; 0.2 mM NADH; 0.25 mM oxaloacetate; 20 mM Tris–HCl buffer, pH 8.5, and 0.1% Triton X-100 for determination of total activity. Methods described elsewhere were followed for determination of sulfite cytochrome *c* reductase [5], catalase [13], acid phosphatase [14], *N*-acetyl-β-glucosaminidase [15] and nucleoside diphosphatase [15]. In some experiments the reaction of *N*-acetyl-β-glucosaminidase was stopped by addition of 5 vol of 0.5 M $NaHCO_3$ buffer at pH 10.7 and 2 vol of chloroform:amyl alcohol (5:1); after centrifugation *p*-nitrophenol was measured at 400 nm in the clear supernatant.

3. Results and discussion

The percentages of free activity reported in table 2 show that the markar enzymes studied are largely

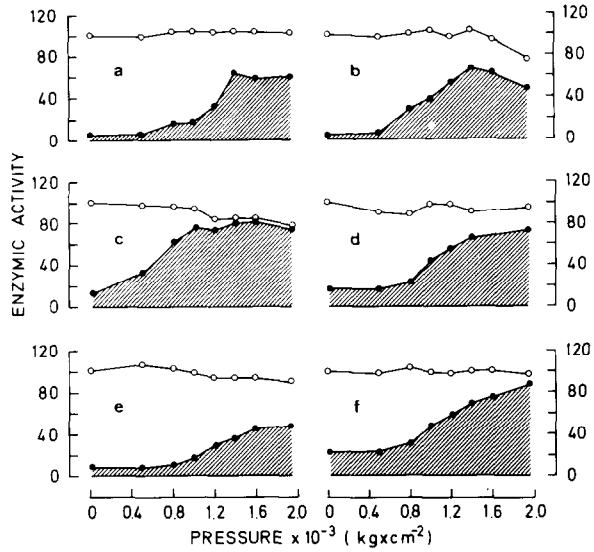

Fig. 2. Effect of compression on free and total activity of enzymes. G fractions were compressed at various pressures (abscissa) during 30 min at 3.5°C. Free (●—●—● cross-hatching) and total (○—○—○) activities are given in percent of the total activity in the untreated sample: (a) malate dehydrogenase; (b) sulfite cytochrome *c* reductase; (d) *N*-acetyl-β-glucosaminidase; (e) nucleoside diphosphatase; (f) acid phosphatase.

latent in untreated G fractions. The higher degree of latency in that fraction with respect to cytoplasmic extract results from the removal of soluble enzymes by gel chromatography.

Compression of G fractions at 3.5°C had little influence on the total activities, except for some loss of catalase and sulfite cytochrome *c* reductase at the higher pressures (fig. 2). In contrast, free activities were greatly enhanced after compression. However, suppression of the structure-linked latency followed a distinct pattern in most cases. Peroxisomes showed the greatest sensibility to hydrostatic pressure, as indicated by the ready release of catalase, whereas the highest resistance to compression was exhibited by microsomes, judging from the latency of nucleoside diphosphatase; 40% of this activity remained latent after compression at 2000 kg·cm^{-2}. An intermediate behavior was observed for other marker enzymes. Latency of sulfite cytochrome *c* reductase was somewhat more easily suppressed than that of malate dehydrogenase, in

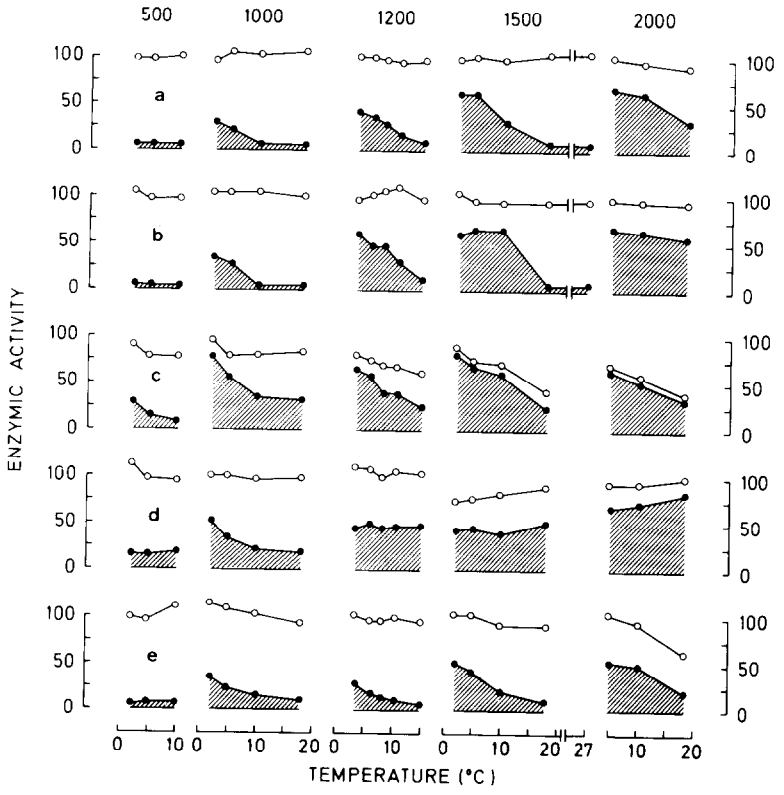

Fig. 3. Temperature-dependence of the compression effects on free and total activity of enzymes. G fractions were compressed at various temperatures (abscissa) and pressures (given in $kg \cdot cm^{-2}$ above the corresponding column). Free (●——●——● and cross-hatching) and total (○——○——○) activities are given in percent of the total activity in the untreated sample: (a) malate dehydrogenase; (b) sulfite cytochrome *c* reductase; (c) catalase; (d) *N*-acetyl-β-glucosaminidase; (e) nucleoside diphosphatase.

agreement with the distinct submitochondrial location of these enzymes. The two lysosomal acid hydrolases behaved similarly.

Temperature during compression is a critical factor in the suppression of structure-linked latency of enzymes (fig. 3). At a given pressure, the percentage of latent activity after compression was consistently greater at high than at low temperature. This relationship appears most clearly at 1500 $kg \cdot cm^{-2}$ for the mitochondrial and microsomal enzymes and at 1000 $kg \cdot cm^{-2}$ for catalase (peroxisomes) and *N*-acetyl-β-glucosaminidase (lysosomes). The opposite effect was observed for the loss of total catalase activity, which was more pronounced at high temperature.

Electron microscope examinations have shown that structural alterations of mitochondria (swelling, loss or aggregation of matrix material) were induced by compression. Thet paralleled closely the unmasking of mitochondrial enzymes and exhibited the same dependence upon temperature.

The suppression of structure-linked latency may result either from the release of the enzyme in the surrounding medium, or from the permeabilization of membranes to the substrates of the enzymic reaction. To settle this point, G fractions were submitted to gel chromatography on Sepharose 2B after compression (fig. 4). An important part of the enzymic activities was then retarded and separated from the particles excluded from the gel. Furthermore the enzymic activities associated with the particles were largely latent, whereas those eluted with the retarded material were completely unmasked. These observations demonstrate that the suppression of structural latency is caused by the release of enzyme into the surrounding medium.

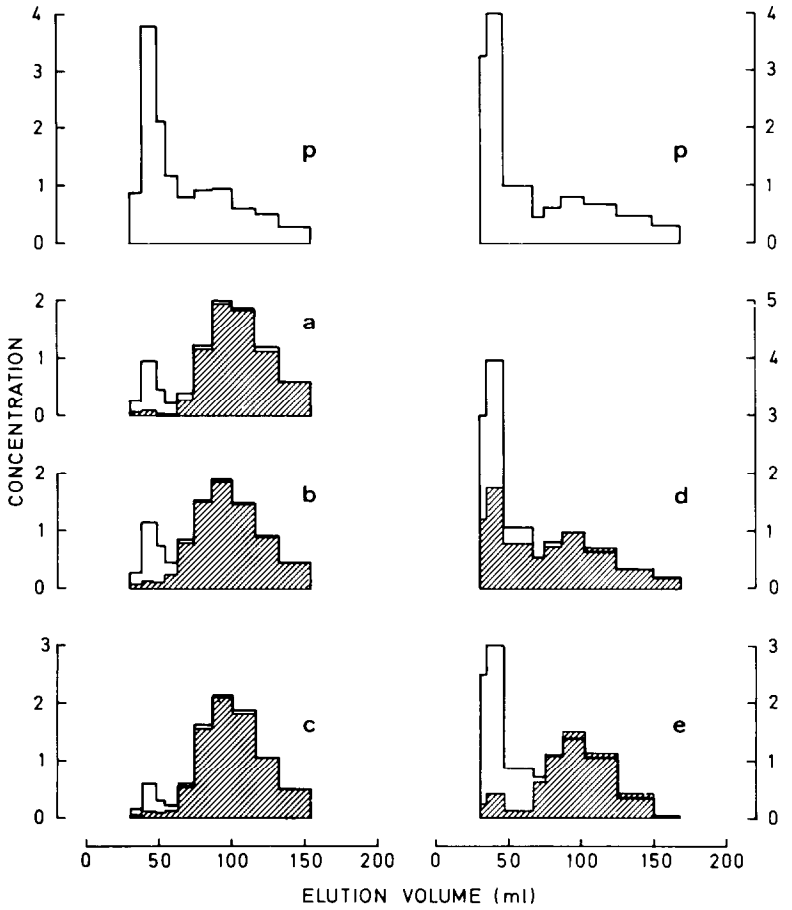

Fig. 4. Effect of compression on the linkage of enzymes to subcellular organelles. G fractions were compressed at 1600 kg·cm^{-2}, during 30 min at 3.5°C. After decompression KCl was added at 50 mM final concentration to lower adsorption of soluble proteins and the precipitation was chromatographed on Sepharose 2B (6 × 5 cm bed) at 4°C, with 0.25 M sucrose, 50 mM KCl as eluent. Results of a distinct experiment are reported in each column. The thick lines give total activities expressed as in fig. 1; the cross-hatched areas give free activities referred to the total activities recovered. Recoveries range from 78–93%. Elution curves of (a) malate dehydrogenase; (b) sulfite cytochrome *c* reductase; (c) catalase; (d) *N*-acetyl-β-glucosaminidase; (e) nucleoside diphosphatase and (p) protein.

The release of enzymes from subcellular organelles is largely attributable to the pressure itself and not to decompression. It increases with the duration of compression, but is unaffected by the rate of decompression.

The mechanism whereby pressure damages cytomembranes is now being investigated. Preliminary data indicate that the resistance of organelles is increased by substituting D_2O for H_2O as solvent. The different sensitivity of enzymes associated with distinct subcellular entities is probably related to the particular composition and structure of their limiting membrane. Our results strengthen previous conclusions [2] calling attention to the danger of excessive hydrostatic pressure in centrifugation of subcellular granules. The hydrostatic pressure generated at the periphery of rotors in preparative ultracentrifuges fall well within the range of values capable of disrupting subcellular entities [16]. Complex enzymic systems integrated into cellular membranes may also be sensitive to pressure. In this respect, it may be advisable to isolate microsomes from post-mitochondrial supernatants by gel chromatography rather than by high-speed centrifugation.

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