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THE EFFECT OF SODIUM VALPROATE ON THE SUBCELLULAR PARTICLES OF THE RAT LIVER

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Abstract—Sodium valproate, 200 mg/kg, i.p., for three weeks produced an increase in the mean sedimentation coefficient of liver mitochondria, while no changes were observed in lysosomes and peroxisomes. As the mean density of the mitochondria, the lysosomes and the peroxisomes of the control and treated animals were not different, it was concluded that the size of the mitochondria had increased in the liver of the treated rats. The mean sedimentation coefficient of the mitochondria returned to normal after the administration of the drug was interrupted for 8 days, indicating a reversible phenomenon. The mechanical and osmotical fragility of the mitochondria and the peroxisomes were not affected by sodium valproate. The osmotic stability of the lysosomes was increased in the *in vivo* experimental conditions.

Sodium valproate is generally considered as an effective drug against convulsions. Although the basis of its action remains unknown, many authors have related its effect to the GABA system [1].

Adverse effects of valproate during its therapeutic use have been reported and the cases of hepatotoxicity with death have received the utmost attention [2]. Although the mechanism of this toxicity is still elusive, several hypotheses have been put forward, some of them related to mitochondrial metabolism. Valproate has been shown to have an inhibitory effect on fatty acid oxidation [3]. Hyperammonaemia with inhibition of urea production associated with a ornithine carbamyl transferase deficiency has also been reported [4]. Finally, inhibition of pyruvate oxidation and oxidative phosphorylation have been found in isolated mitochondria [5, 6].

Morphological studies of the valproate-induced toxicity have reported a zonal necrosis around the central veins and the presence of megamitochondria with vesiculation in the matrix [7, 8]. An increase in the number and the volume of the peroxisomes with flocculent matrices was also noticed [9]. These observations were thought to be related to the toxic effects of valproate on the liver [9]. Since the morphological reports were of clinical cases of overt toxicity and no quantitative data are available on the subcellular particles, it was deemed of interest to complement these initial observations with biochemical data. The purpose of the present investigation was to obtain information on the relationship between enlarged mitochondria and the toxicity of sodium valproate. The effect of valproate on the physical characteristics of rat liver subcellular particles and in particular mitochondria and peroxisomes was studied. After chronic treatment with the drug in apparently non-toxic clinical conditions, we were indeed able to demonstrate the presence of enlarged mitochondria.

MATERIALS AND METHODS

The experiments were carried out on Sprague–Dawley female rats. The animals received an intraperitoneal injection (i.p.) of 200 mg/kg of sodium valproate (a gift from Labaz-Sanofi, Brussels) once a day. This dosage was adopted because its effects are well-documented and it was previously used as a treatment in experimental epilepsy in the rat [10–12]. The treatment was continued for 3 weeks. Control animals received an identical injection of an isoosmotic sodium chloride solution during a same period of time. After this period, the animals were killed by decapitation 24 hr after the last injection and the liver was excised and homogenized in 0.25 M sucrose with a Potter–Elvejhem homogenizer.

Plasma was collected when the animal was killed for glutamate pyruvate transaminase (GPT) and glutamate oxalacetate transaminase (GOT), assay using a Merckotest kit. No differences were observed between the activities of GPT (controls: 30.17 ± 4.27; treated: 37.33 ± 4.73) and GOT (controls: 135.56 ± 19.38; treated: 174.73 ± 21.24) of treated and control rats. The activities are given as means ± standard error of the mean. It was concluded on the basis of these activities, on light microscopic examinations of rat liver specimens and also on the general clinical condition of the animals, that the dosage used had no apparent toxic effects.

The homogenate, the total extract and the M + L fraction were checked by phase contrast microscopy.

Differential centrifugation experiments. Two types of experiments were carried out. First, a subcellular fractionation was performed in 0.25 M sucrose as previously described by de Duve *et al.* [13]. Four fractions were isolated: a nuclear fraction (N), a mitochondrial fraction (M + L), a microsomal fraction (P) and a supernatant (S). In a second type of experiment, the centrifugation was performed on a sucrose gradient produced from 2 samples of a

postnuclear extract containing 0.25 M and 0.50 M sucrose [14]. The densities at all levels of the gradient were lower than the densities of the subcellular particles and the centrifuging conditions were chosen so that sedimentation was incomplete. The only purpose served by the gradient was to stabilize the preparation [15]. The sample was a postnuclear total extract corresponding to 10 ml (g of liver)$^{-1}$. The preparation was centrifuged at 6000 rpm^{-1} for a short time using a Beckman SW 65 Ti rotor in a Beckman L2-65B centrifuge. The time was adjusted so that the time integral of the square of the velocity ($W = \int_0^t \omega^2 dt$) was 2.6×10^8 rad^{2}sec^{-1}. The 2 samples used to make the gradient, were mixed in equal proportions and centrifuged at $3 \times 10^6 g$/min in order to sediment the subcellular particles (M + L) from the non-sedimentable components of the homogenate (S).

Isopycnic density gradient centrifugation experiments. Glycogen gradients (5–20% w/w) were prepared with 0.25 M sucrose in water as solvent. Isopycnic centrifugation experiments were performed according to Beaufay *et al.* [16] in a Beckman L5-65 centrifuge and a SW 65 Ti rotor. In the experiments, the postnuclear total extract was placed on top of the gradients.

Activation experiments by hypo-osmoticity. In order to check upon the fragility of the subcellular particles in experimental and control animals, a M + L fraction was submitted to hypo-osmotic conditions for 10 min at 0°. Then, a concentrated sucrose solution was added to restore iso-osmoticity [17]. Free activities of the mitochondrial marker enzymes, malate dehydrogenase (matrix) and sulphite cytochrome *c* reductase (intermembranous space) were measured in 0.25 M sucrose and the total activities were assayed in the presence of 1 mg·ml^{-1} Triton X-100. A similar procedure was used to estimate the fragility of the lysosomes using β-galactosidase and acid phosphatase and the peroxisomes using catalase as marker enzymes. In order to compare the results obtained from the experiments on the subcellular particles with a well-documented cellular model [18], the fragility of the erythrocytes was tested and the free and total haemoglobin were measured at 540 mμ under different hypo-osmotic conditions. The same activation experiments were also performed *in vitro* on the subcellular particles and erythrocytes from control animals with different concentrations of sodium valproate added to the incubation medium. Sucrose concentration was decreased *pari passu* with the increase of sodium valproate in order to keep the osmotic pressure constant. The osmotic pressure was checked by a Fiske-Associates osmometer.

Enzyme assays. Marker enzymes, chosen for their specific location in different subcellular particles were assayed according to the following references: in the mitochondria, cytochrome oxidase (inner membrane) [19], sulphite cytochrome *c* reductase (intermembranous space) [20], malate dehydrogenase (matrix) [21]; in the lysosomes, β-galactosidase [22] and acid phosphatase [13]; in the peroxisomes, catalase [23].

Calculation of the mean sedimentation coefficients. The mean sedimentation coefficients in a sucrose medium were calculated according to Deter and de Duve [14]. In brief, the assumption was made that there is a linear relationship between the radial distance and the sedimentation coefficient. A diagram was constructed with the radial distance on the X axis and the relative concentration (C/Ci) on the Y axis. C refers to the observed enzymic activity after the centrifugation; Ci is the activity measured before the centrifugation and thus homogeneously distributed in the gradient. The unsedimentable activities assayed on the high speed supernatant were plotted on the diagram so as to indicate the partition between particle-bound and soluble enzymic activity in each fraction and so give the baseline of the diagram. The sedimentation curves were drawn and extrapolated to the origin. The relative concentration (C/Ci) at the origin on the ordinate is higher than the unsedimentable activity.

The mean sedimentation coefficient was obtained by using the following equation: $\ln X/X_0 = s \int_{t=0}^{t} \omega^2 dt$. X_0 stands for the radial distance to the meniscus (cm), X for the mean radial distance of the analysed fraction (cm), ω for the angular velocity (rad. sec^{-1}), t for the centrifugation time in sec and s for the Svedberg unit (10^{-13} sec). The value of X was read on the abscissa at the point situated at midway between value 1.0 and the baseline of unsedimentable enzymic activity (Fig. 2). The sedimentation profiles determined from the activities of tracer enzymes allowed us to calculate the sedimentation coefficients of the related particles. The values of the sedimentation coefficients were analysed in both, control and treated animals by an analysis of variance. F values have been determined according to Snedecor [24].

RESULTS

Differential centrifugation in 0.25 M sucrose

The enzyme distribution in the subcellular fractionation experiments was essentially identical in the treated and the control animals (Fig. 1). There was no increase in soluble activity for the different enzymes assayed in the valproate-treated rats. Thus, sodium valproate did not affect the representativity of the different subcellular fractions. Moreover, in the centrifugation experiment at $3 \times 10^6 g$ min in which the subcellular particles were separated from the non-sedimentable elements, no differences in soluble enzymic activities were noticed.

Differential centrifugation in a sucrose gradient

Free and total activities in the total postnuclear cytoplasmic extract were assayed first in an iso-osmotic sucrose medium. No differences were observed between treated and control animals for the sulphite cytochrome *c* reductase, β-galactosidase and catalase-free activities.

When stabilized by a sucrose gradient, the differential centrifugation experiments on a total postnuclear cytoplamsic extract showed no difference in the sedimentation profile for β-galactosidase and catalase, respectively tracer enzymes of lysosomes and peroxisomes. Cytochrome oxidase, a tracer enzyme for the mitochondria, exhibited a shift of the sedimentation boundary in the rats chronically treated with sodium valproate. In Table 1, one can

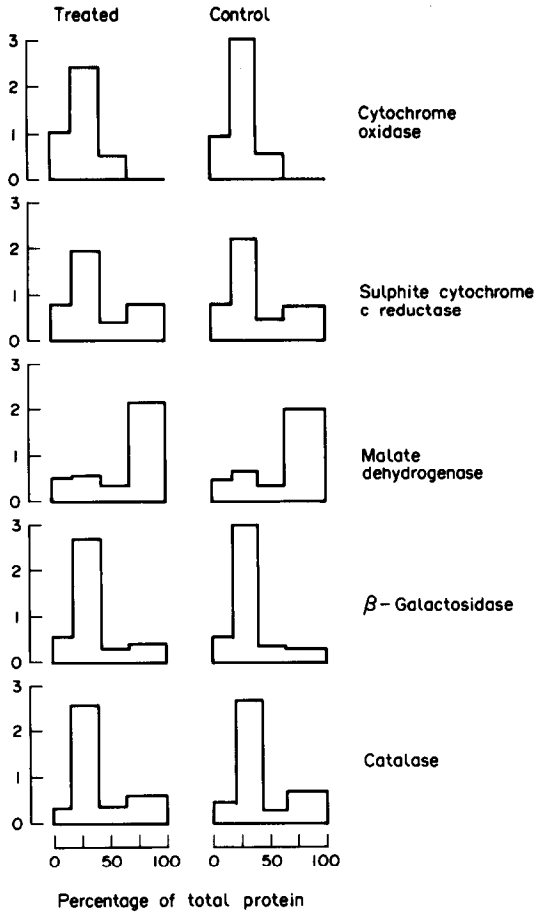

Fig. 1. Distribution pattern after liver fractionation by differential centrifugation of control and valproate-treated rats. From left to right fractions N, M + L, P and S are depicted. Each fraction is presented separately in the ordinate scale by the relative specific activity of the enzyme (percentage of total activity/percentage of total recovered proteins). In the abcissa scale, each fraction is represented cumulatively from left to right by its percentage of proteins. The mean recoveries in percentage for cytochrome oxidase, sulphite cytochrome *c* reductase, malate dehydrogenase, β-galactosidase and catalase were respectively in control and treated animals of 92.66 ± 1.42 and 94.45 ± 1.75, 98.60 ± 1.84 and 98.23 ± 2.15, 95.50 ± 1.79 and 94.20 ± 3.07, 95.24 ± 1.91 and 97.28 ± 2.06, 82.85 ± 0.82 and 89.60 ± 1.71. The mean recoveries in percentage for proteins in control and treated rats were 97.85 ± 1.84 and 96.81 ± 1.71.

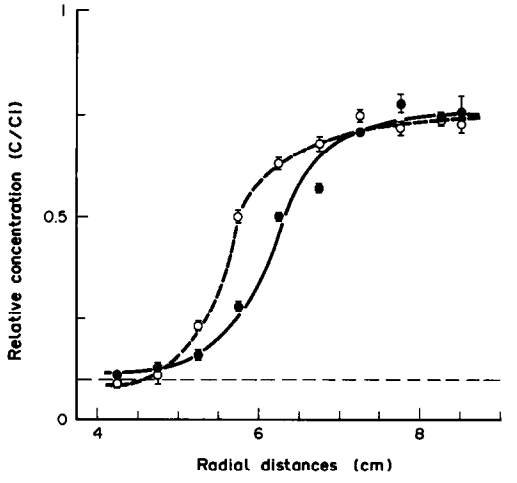

Fig. 2. Mean sedimentation boundary of particle bound cytochrome oxidase in control and treated animals. Mean control (N = 16) and treated (N = 10) curves are depicted respectively by dotted and solid lines (○ control, ● treated). The experiments were carried out on a linear gradient of 0.25–0.5 M sucrose over a radial distance of 3.8–8.9 cm with $W = 2.6 \times 10^8$ rad.^{2} sec^{-1}. The diagram represents the radial distance on the X axis and the relative concentration on the Y axis. C_1 and C refer respectively to enzyme concentrations before and after centrifugation. The vertical bars indicate the SEM.

see the calculated mean sedimentation coefficients of the mitochondria, lysosomes and peroxisomes in treated and control animals. As shown, the sedimentation coefficient of the mitochondria in the experimental rats is significantly increased ($F = 9.25$; $P < 0.01$). There were no significant differences between the mean sedimentation coefficients of the lysosomes ($F = 0.53$) and peroxisomes ($F = 0.05$) in treated and control animals (Table 1).

In order to elucidate the importance of the density factor of the mitochondrial population in the displacement of the boundary curves, isopycnic glycogen gradient experiments were performed with a total postnuclear extract. As shown in Table 2, the mean densities of control and treated mitochondria, lysosomes and peroxisomes were identical.

The changes of the mean sedimentation coefficient observed for the mitochondria disappeared when the sodium valproate treatment was interrupted for 8 days ($s = 12{,}650 \pm 330$).

Hypo-osmotic activation experiments on mitochondria and lysosomes

Sodium valproate did not change the internal and external cohesiveness of the mitochondrial membranes as shown by the free to total activity curves of malate dehydrogenase and sulphite cytochrome *c* reductase (Fig. 3). Negative results were also obtained when the experiments were carried out *in vitro* by adding increasing amounts of sodium valproate in the different sucrose media.

As shown in Fig. 4 the lysosomal osmotic stability was increased in experimental animals. No further increase in lysosomal stability could be obtained by increasing the dosage. *In vitro*, no changes in the osmotic stability of the lysosomes were noticed.

The erythrocytes showed no alteration of their osmotic fragility *in vivo*. *In vitro*, however, the erythocytes submitted to different concentrations of sodium valproate followed a fragility course similar to the ones described previously for local anaesthetics by Seeman [18].

DISCUSSION

As shown in this report, the sedimentation coefficient of liver mitochondria was significantly increased in valproate treated rats. The sedimentation pattern of other subcellular particles was

Table 1. Median sedimentation coefficients (Svedberg)

	Mitochondria Cytochrome oxidase	Lysosomes β-Galactosidase	Peroxysomes Catalase
Control	13,500* ± 440 (16)	9960 ± 680 (15)	5940 ± 450 (10)
Valproate	15,970* ± 760 (10)	10,950 ± 1360 (8)	5720 ± 240 (5)

The mean sedimentation coefficients were calculated according to the following equation: $\ln X/X_0 = s \int_{t=0}^{t} \omega^2 dt$. X_0 = radial distance to the meniscus (cm), X = mean radial distance of the analysed fraction (cm), ω = angular velocity (rad. sec^{-1}), t = centrifugation time in seconds, s = Svedberg unit (10^{-13} sec). The X values were estimated from boundary curves at a relative concentration midway between 1.0 and the baseline of unsedimentable activity. Results are given as means ± standard error of the mean. Number of experiments are placed between parentheses.

* Significantly different ($P < 0.01$).

Table 2. Mean equilibrium density of different tracer enzymes of subcellular particles in control and treated rats

	Controls	Treated
Cytochrome oxidase	1.100 ± 0.003	1.102 ± 0.005
β-Galactosidase	1.102 ± 0.003	1.104 ± 0.005
Catalase	1.074 ± 0.003	1.071 ± 0.008

Results are given as means (g/cm^3) ± SEM. Values are the mean of four experiments. The mean densities of particles bound enzymes were calculated after an isopycnic centrifugation of a rat postnuclear fraction in a glycogen gradient (d = 1.045–1.110 g/cm^3) with 0.25 M sucrose solution as solvent. The centrifugation was performed at 39,000 rpm^{-1} for 2.30 hr in a Beckman SW 65 Ti rotor at 4°. Particles suspended in 0.25 M sucrose were layered on top of the gradient.

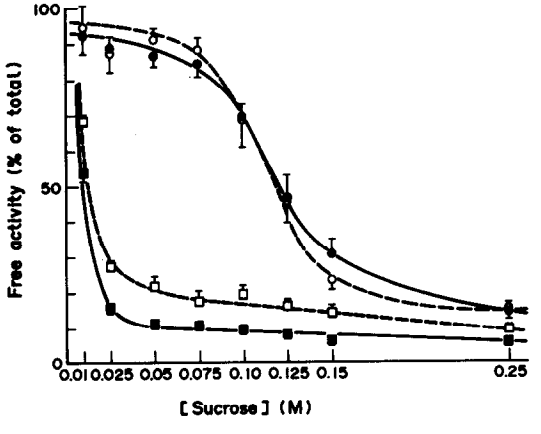

Fig. 3. Malate dehydrogenase in control □ and treated animals ■, sulphite cytochrome *c* reductase in control ○ and experimental animals ●. Samples of a liver mitochondrial fraction prepared in 0.25 M sucrose were diluted with water to give the indicated sucrose concentrations and kept for 10 min at 0°. After that, a concentrated sucrose solution was added to restore iso-osmoticity. Free activity was determined in 0.25 M sucrose, and total activity in 1 mg·ml^{-1} Triton X-100. The results are shown as means ± SEM of eight experiments.

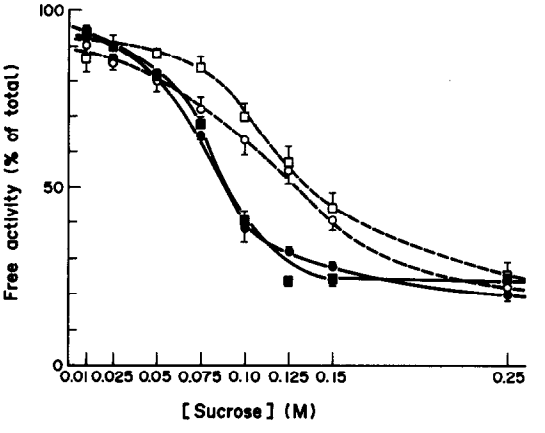

Fig. 4. Acid phosphatase of control □ and valproate treated ■ rats. β-galactosidase of control ○ and treated ● animals. Samples of a mitochondrial fraction prepared in 0.25 M sucrose were diluted with water to give the indicated sucrose concentrations and kept for 10 min at 0°. After that, a concentrated sucrose solution was added to restore isoosmoticity. Free activity was determined by assaying for 10 min in 0.25 M sucrose, and total activity in 1 mg·ml^{-1} Triton X-100. The results are shown as means ± SEM of ten experiments.

not affected. Sodium valproate did not change the density and fragility of the enlarged mitochondria.

Assuming that the subcellular particles are spherical, the increment of the sedimentation rate in the differential gradient centrifugation experiments can be mainly related to an increase in the size or an increase in density of the subcellular components. In the experiments with the isopycnic density gradient centrifugation, the densities of the mitochondria, the lysosomes and the peroxisomes were identical in control and experimental animals. Since, as revealed by the examination by phase contrast microscopy, there was no agglutination of particles in the total postnuclear cytoplasmic extract, it is not likely that this artefact would be responsible for the sedimentation pattern of the mitochondria. Moreover, only the mitochondria sedimentation rate increased in the experimental animals; the other particles, and lysosomes and the peroxisomes of the treated rats showed no change in their sedimentation. As shown,

this phenomenon is reversible when the administration of the drug is interrupted for 8 days.

Our results are in accordance with the clinical morphological findings of megamitochondria of intoxicated human hepatocytes in sodium valproate treatment [7, 9]. Nevertheless, it cannot be excluded that a change in shape of the mitochondria is related to our results.

Since no clinical signs of hepatic intoxication, as determined by the transaminase activities were present in our experimental animals, we would suggest that the enlarged mitochondria are a non-toxic consequence of sodium valproate administration. We did not observe increased size of the peroxisomes as previous reports indicated in liver toxicity [9]. We therefore would propose that the increased-sized peroxisomes would be related to valproate toxicity.

It is well known that larger particles are more susceptible to damage in homogenization procedures. In addition to this mechanical fragility, an osmotic fragility related to an inbalance between inner over outer osmotic pressure causing swollen particles, had to be considered. An increase in lateral tension resulting from the increase in radius could favor membrane rupture under certain experimental conditions. Under our conditions, however, no increase of unsedimentable activity was observed for the tracer enzymes after the homogenization procedure, in the fractionation and in the high speed centrifugation ($3 \times 10^6 g$ min) experiments. Moreover, the osmotic stability of the mitochondria was unchanged *in vivo* and *in vitro* in hypo-osmotic conditions. Suprisingly, the stability of the lysosomes was increased in our *in vivo* experiments. The significance of this phenomenon is still not understood. Sodium valproate did not change the osmotic fragility of the erythrocytes in our *in vivo* experimental conditions.

No detailed metabolic data of sodium valproate are available at the present time in our experiments in order to estimate whether the effect of the drug is directly related to the component or to its metabolites. Since 97% of sodium valproate is eliminated from the rat tissues after 24 hr [25], it is likely that a metabolite or a lasting metabolic effect is responsible for the present observations.

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