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Modifications induced on water–matrix interactions and on ATPase, Na,K-dependent activity in ouabain-treated cerebella of mice

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Abstract

In a biological system, water can be bound to the matrix with different energies and thus, when the latter is heated, be lost at subsequent stages according to the activation energies required to obtain the release of the different types of water. In this sense, particularly when combined with ancillary systems such as FT-IR spectroscopy, thermoanalytical techniques allow us to distinguish all the different types of water contained in a biological system. Using this approach, the water in the cerebellum of mice treated by subdural inoculation of ouabain (G-strophanthin) was studied.

At the same time, the activity of Na,K-ATPase was followed in treated and untreated mice cerebella by means of calorimetry. The activity decreases as a function of the time elapsing between inoculation and the animal's death. Experimental data are discussed in terms of inhibition of Na,K-ATPase and in terms of the modified water–matrix interaction, consequent to the changed mechanism of the diffusion transport deriving from the gradient in Na-ion concentration between the inside and outside of the cell wall.

Keywords: Na,K-ATPase; Thermoanalytical techniques; Water

1. Introduction

The role of water in biochemical reactions and cellular events has been underestimated for a long time. In biological systems, water is conventionally viewed as an

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unstructured filling in which the biochemical reactions can take place without its being involved, unless hydrolysis reactions are present.

Clegg et al. [1] stressed that this model was constructed using NMR (Nuclear Magnetic Resonance) spectroscopic data and dielectric relaxation techniques, which provide information on the mean rotational motion of individual molecules, but do not reveal collective long-term processes. Watterson [2–4] pointed out that the thermodynamic interpretation of colligative properties displays two fundamental flaws when transported to the molecular scale. One of these consists of the fact that, while classical thermodynamics claims that colligative phenomena are not dependent on solute molecule size, this is contradicted by experimental verification, particularly for large-size solutes; the other flaw consists of the fact that the thermodynamic interpretation rules out intermolecular interactions, while researches in the fields of colloid and interphase physics and in biological chemistry stress the importance of solvent/solute and solvent/solvent interactions.

Watterson has thus proposed an alternative model in which this type of interaction is cooperative. This leads to well-defined kinetics of local structural variations as the vicinal molecules mutually influence each other when they combine with, or separate out of, transient aggregates.

In any case, whatever the model selected, in a biological system water can be bound to the matrix with different energies and thus, when the latter is heated, be lost at subsequent stages according to the activation energy required to obtain the release of the different types of water.

In this sense, particularly when combined with ancillary systems such as FT-IR (Fourier Transform Infrared) spectroscopy, thermoanalytical techniques allow us, as has already been pointed out [5–8], to obtain detailed information concerning the different water–biological-matrix interactions and thus to distinguish all the different types of water contained in a biological system implying that different types of water are bound to the matrix with different energies.

With this in mind, this study has been addressed to water–matrix interactions in the nervous system (cerebella) of mice treated by subdural inoculation of ouabain (G-strophantin). Ouabain is a specific inhibitor of Na,K-ATPase and the cerebellum, as shown by Harik et al. [9], is an elective site of ouabain action. The effects on Na,K-ATPase activity induced by ouabain inhibition could lead to a modification of the water–biological-matrix interaction. To support this hypothesis, a parallel study of water behaviour and of Na,K-ATPase activity in mice cerebella has been carried out.

Water–matrix interactions were studied by thermoanalytical techniques, thermogravimetry (TG) and derivative thermogravimetry (DTG), also coupled with FT-IR spectroscopy, while the ATPase activity was determined by calorimetric techniques.

2. Experimental

2.1. Instrumentation

A model TGA-7 Thermogravimetric Analyzer (Perkin-Elmer) coupled to an FT-IR Spectrometer 1760 X (Perkin-Elmer) and 7700 Computer were used. NMR spectra were measured with an Outsuka 4.7 Tesla Instrument.

A microcalorimeter LKB Model 2107, isothermal batch instrument of heat conduction type, equipped with two gold vessels of about 7 cm^3 total volume, a multitemperature cooling circulator (LKB Model 2209), a control unit (LKB Model 2107-350) and a potentiometric recorder (LKB Model 2210), were used.

A Polytron homogenizer-Kinematica, Krieus-LU Switzerland, and an Ultrasonic bath, Model Uniset (AGE electronica, Italy).

2.2. Materials and methods

In view of the need to use reproducible samples to avoid corrupting the data as a result of non-controllable anatomical differences, and bearing in mind the size characteristics of the instruments (in particular, the maximum usable volume determined by the thermobalance sample holder), it was deemed advisable to use the cerebellum of six-month-old Swiss race mice (Nossan) since the volume of the brain hemisphere was two large to fit into a sample holder and also because cerebellar cells have a high binding capacity for ouabain [9].

After administering ether as an anaesthetic, ouabain was introduced directly into the subdural space using a very short needle, taking care not to damage the brain matter and perforating the occipital squama 3 mm to the right of the median line. The glycoside (octohydrate SIGMA ouabain) dosage used was 0.5 mg/0.25 cm^3 of physiological solution (solution pH 6.8), while controls were injected with 0.25 cm^3 of saline (pH 6.7). The animals were sacrificed by decapitation by a guillotine device at fixed intervals after injection. The two lobes of the cerebellum were removed immediately and frozen in liquid nitrogen, in which they were kept until the time of analysis.

In order to ensure that any treatment was not responsible for any changes in the water–matrix interactions, six animals were sacrificed by the guillotine device without anaesthesia and six after anaesthesia by ether; fresh cerebellar hemispheres were first analysed while the controlaterals were placed in liquid nitrogen. The consistency of the thermoanalytical results obtained using fresh and perfrigerated samples of anaesthetized and non-anaesthetized mice allowed this treatment to be validated and showed, at the same time, that ether used as an anaesthetic does not induce any change in the water–matrix interactions.

All solutions were prepared using deionized water, distilled twice over potassium permanganate.

All chemicals were of analytical-reagent grade (Merck). ATPase enzyme Na,K-activated was lyophilized powder (E.C. 3.6.1.3.) (Sigma from porcine cerebral cortex), ATP disodium salt was obtained from Sigma, and Tris (tris(hydroxymethyl) aminomethane) for analysis and HCl (concentrated ampoules) from Merck. The buffer used was always Tris–0.05 M HCl of pH 7.4. The lyophilized enzyme was stored at −20°C; fresh solutions were prepared daily, stored at 4°C, and just before the experiments were thermostated at 25°C for 10 min.

Stock solutions of $MgSO_4$ (0.1 M), NaCl (0.5 M) and KCl (0.05 M) were prepared by weight; in the same way ATP solutions were prepared daily, taking into account the water content that was measured by thermal analysis.

All concentrations refer to the reactants in the calorimetric vessels before mixing.

3. Results

3.1. Thermal analysis

In the first instance the two cerebellar hemispheres of ouabain-untreated mice were subjected to thermochemical analysis so that the thermogravimetric curves (TG) and derivative thermogravimetric curves (DTG) corresponding to these animals could be used as reference states. In any case, for the ouabain-untreated mice the experimental data, especially the DTG curves, point to two processes, first a smaller one, located between room temperature and 65°C and then a larger one, located between 65 and 185°C. The second peak of the DTG curve often displays a dissymmetry and the onset of an inflection. The two processes were also monitored by means of continuous analysis of the gases released during the processes themselves, using an FT-IR spectrometer coupled to the thermobalance.

These tests showed that the corresponding gases were composed of water vapour containing traces of volatile organic substances that were negligible with respect to the water mass. Subsequently, two other processes appeared on the TG and DTG curves, corresponding to the break down of the organic matrix.

By way of example, the curves referring to the two cerebellar hemispheres of one of the untreated animal are shown in Figs. 1 and 2. The percentages of water released during the first and the second processes and of the total water are listed in Table 1.

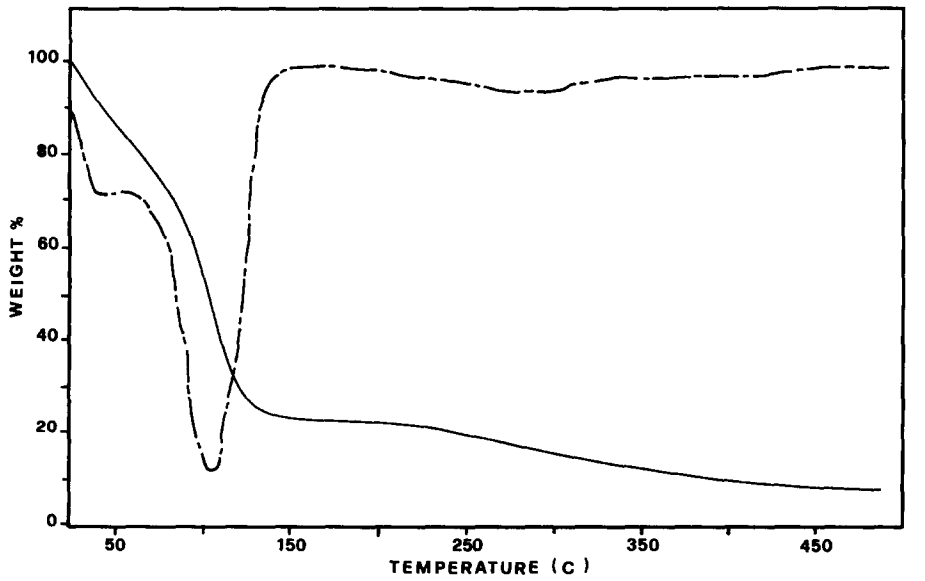

Fig. 1. TG and DTG curves corresponding to the left cerebellar hemisphere of one of the untreated animals: air atmosphere; heating rate 10°C min^{-1}.

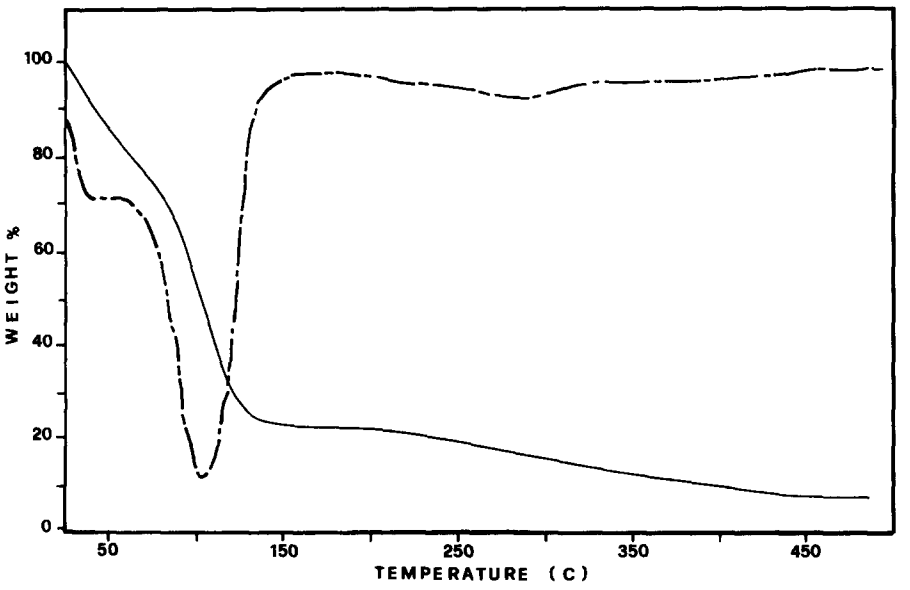

Fig. 2. TG and DTG curves corresponding to the right cerebellar hemisphere of one of the untreated animals (same as Fig. 1): air atmosphere; heating rate 10°C min^{-1}.

Table 1
Effect of ouabain treatment on the water percentage and water peak temperature shift of mice cerebella. Values are mean ± SD of 6 animals

Animals	Process I		Process II		Total water
	% Water	Peak shift	% Water	Peak shift	
Untreated	13.8±0.8		60.1±0.7		73.8±0.8
Ouabain treated and sacrificed at:					
5 min	14.5±0.9	1.5±0.5	61.1±0.8	1.5±0.6	75.8±0.9
30 min	15.1±0.9	5.6±0.7	63.8±1.0	10.5±0.5	78.9±0.9
60 min	17.0±0.7	6.9±0.7	66.2±0.7	15.7±0.8	83.2±0.8

Six saline-treated animals sacrificed at 30 and 60 min, respectively, were then examined. In all cases, both cerebellar hemispheres were analysed. The TG and DTG curves for one specimen sacrificed after 60 min are shown in Figs. 3 and 4. The 30 min curves are superimposable on the 60 min ones and both are comparable with those of the untreated animals. Small deviations can be accounted for in terms of biological variability. In particular, it should be emphasized that modifications in the temperatures of the DTG peaks are negligible. The percentages of water corresponding to the two separate processes and to the total water lie within the range of percentages obtained for untreated samples.

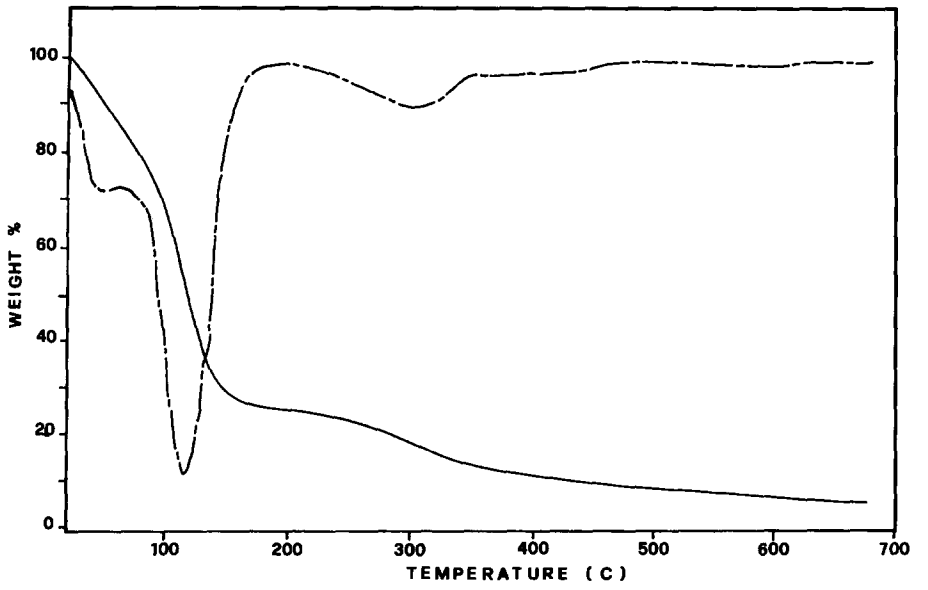

Fig. 3. TG and DTG curves corresponding to the cerebellar hemisphere of one of the saline-treated animals sacrificed at 30 min: air atmosphere; heating rate 10°C min^{-1}.

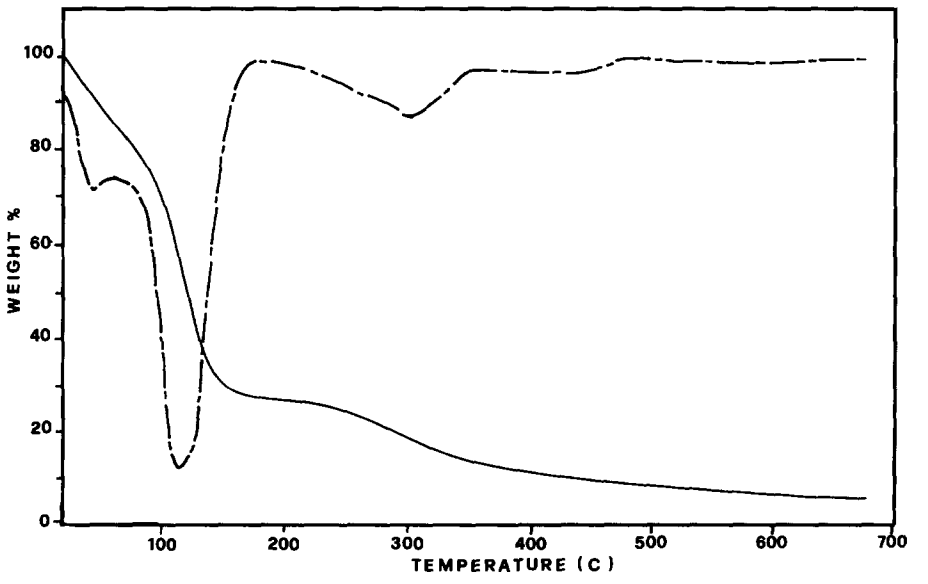

Fig. 4. TG and DTG curves corresponding to the cerebellar hemisphere of one of the saline-treated animals sacrificed at 60 min: air atmosphere; heating rate 10°C min^{-1}.

The cerebellar hemispheres of animals injected with ouabain and sacrificed at fixed intervals after inoculation were then subjected to thermoanalytical analysis. The TG and DTG of the two cerebellar hemispheres of an animal sacrificed 30 min after inoculation, followed by the predicted motor hyperactivity and then convulsions [10], are shown in Fig. 5. The TG and DTG curves of the left hemisphere (Fig. 5a,b) are superimposable on those of the controls. Conversely, the same curves referring to the right hemisphere, i.e. the site of the inoculation (Fig. 5c,d) show that the DTG peak corresponding to the first process of water loss increases slightly, while the peak corresponding to the second process increases perceptibly in area and, at the same time, the minimum shifts towards higher temperatures. Furthermore, the second process displays an inflection on the descending branch of the DTG curve.

Figure 6 shows the TG and DTG curves corresponding to the animals sacrificed at 5, 30 and 60 min, respectively, after the inoculation with ouabain.

The referred modifications of the TG and DTG curve profiles of the cerebellar hemispheres are common to all the treated animals in which the described clinical symptoms occurred, and the extent of the shift is a function of the time elapsing between inoculation and the animal's death.

The TG and DTG curves of an animal that displayed no clinical symptoms are shown in Fig. 7. Curve 7a, referring to the treated hemisphere, displays only a slight increase in the DTG peak concerning the water with a lower bonding energy and a very slight widening of the second DTG peak. Curve 7b, referring to the untreated hemisphere, is fully superimposable on the controls.

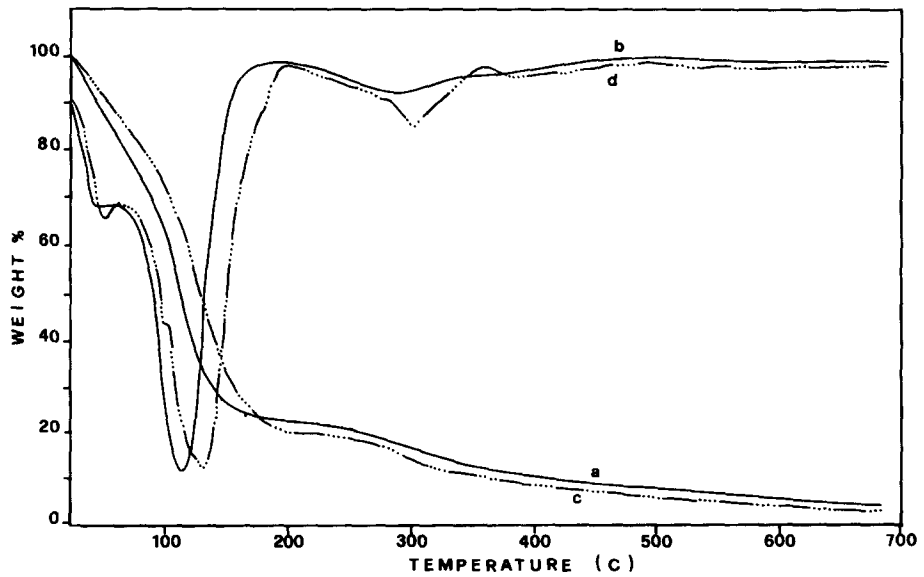

Fig. 5. a,b, TG and DTG curves corresponding to the left hemisphere of one of the animals sacrificed 30 min after inoculation with ouabain; c,d, TG and DTG curves corresponding to the right hemisphere (site of inoculation) of the same animal. Air atmosphere; heating rate 10°C min^{-1}.

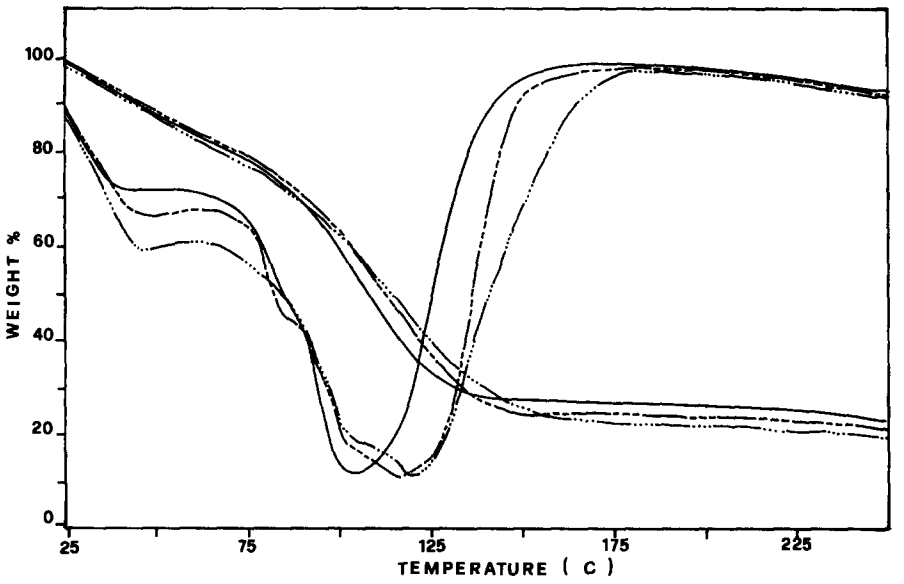

Fig. 6. TG and DTG curves corresponding to the right cerebellar hemisphere of the animals sacrificed at: —, 5 min after inoculation with ouabain; - - - —, 30 min after inoculation with ouabain; ····· —, 60 min after inoculation with ouabain. Air atmosphere; heating rate 10°C min^{-1}.

3.2. Calorimetry

Sodium, potassium-5′-adenosinetriphosphatase (Na^+,K^+-ATPase or simply ATPase) is a membrane enzyme which catalyses 5′-ATP hydrolysis inside living cells in the presence of magnesium ions

$$5'\text{-ATP} + H_2O \xrightarrow[\text{'5'-ATPase'}]{Na^+,K^+,Mg^{2+}} 5'\text{-ADP} + P_i + \Delta H_R$$

P_i represents the phosphate residue and ΔH_R is the enthalpy variation associated with the enzymatic reaction. This enzymatic hydrolysis, and also all other reactions involving ATP, is magnesium dependent and sodium and potassium activated. In particular, the $[Na^+]/[K^+]$ and $[Mg^{2+}]/[ATP]$ ratios are very important with respect to the enzymatic activity.

The microcalorimetric method, which has been successfully applied to other enzymatic assays [11–13], meets the necessary requirements for a reliable method and can also be applied to solutions that are not clear, for example homogenized tissues. In fact, enzymatic calorimetry makes it possible to measure the variation in enthalpy associated with the enzymatic reaction in a direct way and also to relate the instrumental output linearly to the catalytic activity. Calorimetry has already been employed to study some processes involving ATPase, with special regard to the physiological aspects such as cellular metabolism, but not for analytical purposes [14–17].

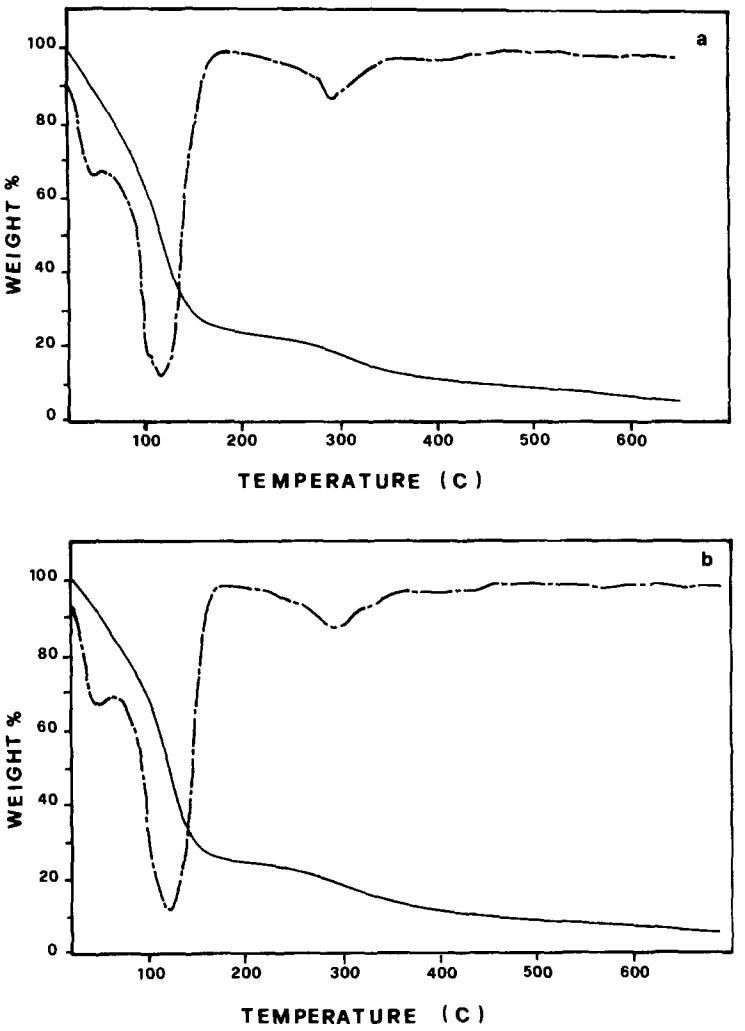

Fig. 7. TG and DTG curves corresponding to the cerebellar hemispheres of an animal that displayed no clinical symptoms, sacrificed after 60 min: a, right treated hemisphere; b, left untreated hemisphere. Air atmosphere; heating rate 10°C min^{-1}.

The results reported here were obtained as described elsewhere [18]. In particular, the heat produced in the main enzymatic hydrolysis of ATP (expressed in terms of thermopile voltage) can be related linearly to the ATPase activity

$$V = K\Delta(q/t)$$

where V is the thermopile voltage, K is the calibration constant of the instrument and q is the quantity of heat evolved during a period of time t.

In order to measure the enzymatic activity, pseudo-zero-order kinetics (with respect to the substrate) may be assumed, and therefore an excess concentration of substrate was used (at least ten times the Michaelis–Menten constant, K_m, of the ATPase enzyme). Under these conditions, the enzymatic activity can be correlated linearly with the instrumental output

$$V = K'(EU)\Delta H_r$$

where K' is a constant depending on the enzymatic reaction, (EU) is the enzyme activity and ΔH_r is the enthalpy change due to the enzymatic reaction. In practice, the recorded thermogram (V vs. t) reaches a steady state and the maximum thermopile voltage (height of the curve = Δ_{max}) is the instrumental parameter which has been considered and correlated with the enzymatic activity to plot the calibration graph for the ATPase activity assay, Δ_{mass} vs. (IU cm^{-3}).

All the experiments were run three times for each IU value of ATPase, and the reported points in the figures (Δ_{max}) are the resulting average maximum values of the calorimetric curve (thermopile voltage V vs. time) (Fig. 8a,b).

For the measurements of the Na,K-ATPase activity in this study, where cerebella samples are used, a calibration curve was obtained using Na,K-ATPase standard enzyme (Sigma, from porcine cerebral cortex). The graph is relative to an activity range of 0.015–0.200 IU cm^{-1} (Fig. 9): the trend is always linear and all samples show an activity value consistent with the calibration plot.

The ATPase assay was then performed on the cerebellar tissues of the mice, untreated or injected with ouabain and sacrificed at fixed times after inoculation. Before the analysis each cerebellar hemisphere (about 10 mg weight) was homogenized by means of a Polytron system in 5 cm^3 of buffer solution and then sonicated for 5 min in the ultrasonic bath.

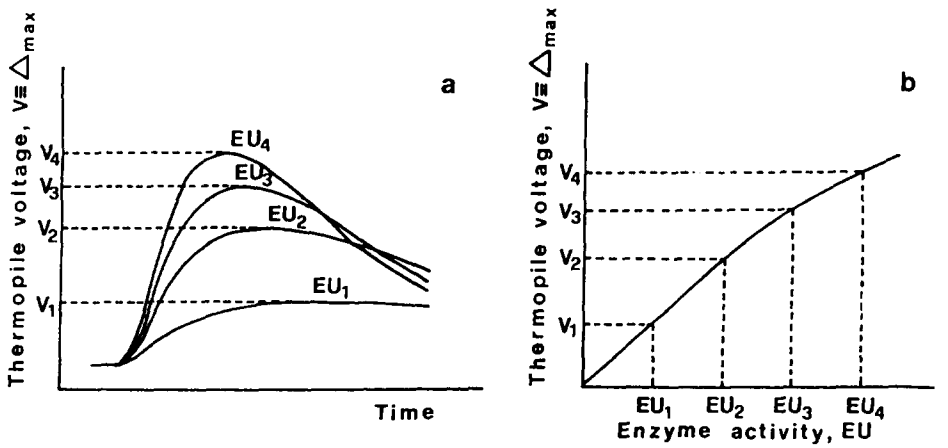

Fig. 8. a. Calorimetric response vs. time at different enzymatic activity (EU_i). b, Calibration curve for enzymatic activity determination.

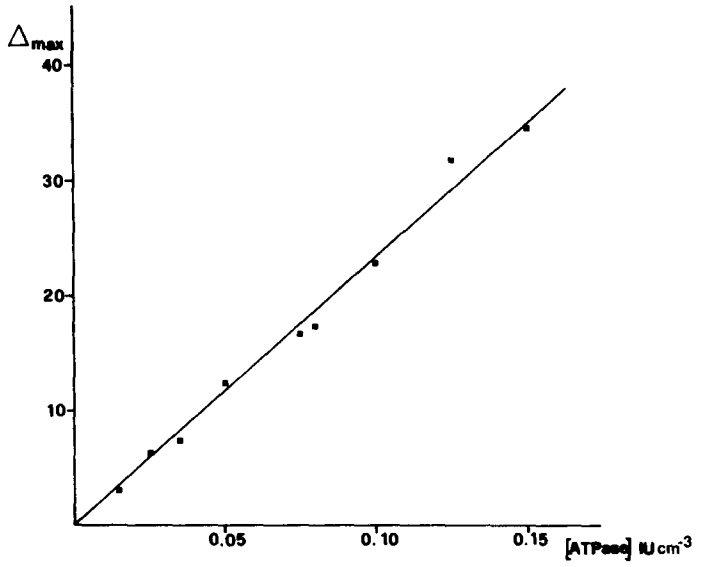

Fig. 9. Calibration curve of the Na,K-ATPase activity in the range 0.015–0.200 IU cm^{-1}.

The samples were divided into two parts and two separate measurements were performed in the microcalorimeter. In each calorimetric run, 2 cm^3 of sample were put in one part of the measuring vessel and 2 cm^3 of the substrate solution ([ATP] 5mM, [Na^+] 80 mM, [K^+] 10 mM, [Mg^{2+}] 5 mM in buffer solution) in the other part; the reference vessel was filled with 2 cm^3 of the buffer solution and 2 cm^3 of the substrate solution in the two compartments respectively.

Each value of the ATPase activity is obtained by plotting the mean value of Δ_{max} (two measurements for each sample) versus the enzyme activity (IU cm^{-3} mg^{-1}) taking into account the weight of each sample and the buffer volumes used.

As shown in the data collected in Table 2, the ATPase activity decreases as a function of the time elapsed between the ouabain inoculation and the death of the animals.

Table 2
Assay of the Na,K-ATPase activity on the cerebellar hemispheres of untreated mice (time 0) or mice injected with ouabain and sacrificed at 5, 30 and 60 min after inoculation. Values are means ± SD of six animals, for the homogenized sample, corresponding to each hemisphere, divided in two parts and two different measurements performed in the calorimeter. The mean value of all the data corresponding to unaffected hemispheres, see right hemispheres at time 0 plus all left hemispheres, is $\bar{\Delta}_{max} = 10.59 \pm 0.77$

Time/min	Δ_{max}		% Change	%Change vs. $\bar{\Delta}_{max}$
	Right hemispheres	Left hemispheres		
0	10.50±0.76	10.71±0.81	1.96	0.85
5	10.05±0.79	10.30±0.98	2.43	5.01
30	8.02±0.65	10.60±0.77	24.33	24.26
60	5.57±0.55	10.85±0.96	48.66	47.40

4. Discussion

Analysis of the TG and DTG curves in untreated animals or animals treated with saline alone shows that two processes involving water loss are always present: a smaller one referring to water bound at lower energy to the organic matrix and a second one, involving a much higher percentage of the total water, referring to water bound to the matrix with greater energy. The dissymmetry and inflection located on the descending branch of the DTG curve indicate that the peak in question represents the convolution of at least two continuously superimposed processes corresponding to the breakdown of bonds at very similar energy levels between water and the various components of the biological matrix; the continuity comes to an end at the point of inflection of the peak.

In the case of the hemisphere corresponding to the inoculation zone, the TG and DTG curves referring to the cerebella of the treated animals show that the processes of water release undergo considerable modifications:

(i) The water bound with weaker energy to the matrix increases, as shown by the TG and DTG curves; the extent of the modifications is dependent on the time elapsing between inoculation and sacrifice;

(ii) The water bound with higher energy to the matrix increases and the extent of the increase is greater than for the first process;

(iii) The corresponding process shifts towards higher temperatures; also here the extent of the modification is a function of the time elapsing between inoculation and sacrifice; the dissymmetry and inflection of the DTG curves are located on the descending branch of the DTG curves.

The irreversible inhibition of the Na,K-ATPase induced by the glycoside [19], and demonstrated by our calorimetric data (Table 2), brings about the blocking of active sodium ion transport towards the outside of the cell wall and its consequent intracellular accumulation as a result of the superimposition of the diffusion transport mechanism deriving from the gradient in Na ion concentration between the inside and outside of the cell wall [19]. The accumulation of sodium ions leads to the formation of aquoions in which water is bound at high energy, especially at the level of the inner sphere of hydration.

When the water bonds to the Na ion, there is a decrease in the water used as "solvent system" and water is thus attracted through the concentration gradient from outside to the inside of the cell. This phenomenological hypothesis is in perfect harmony with the experimental data, which indicate that the process of loss is shifted towards higher temperatures by the water/sodium interaction with a consequent increase in the water interaction energy. As shown by the TG and DTG curves, the total water increases as a result of the attraction due to concentration gradients. The modification of the peaks follows a trend that is a function of time as it is dependent on the ouabain diffusion process. The inflection of the DTG curves is shifted towards the descending part of the curve, i.e. in the continuum of the convolution of the processes corresponding to the different water/matrix interactions represented by the DTG peak, the water bound with a higher energy increases compared with that bound with lower energy.

In the case of the first process, in view of the low temperature at which it is released and in agreement with the NMR signals, the corresponding water may be said to be unbound.

If we examine the formation equilibrium of the type

$$Na^+ + nH_2O \rightarrow Na(N_2O)_n$$

we see that, in order to achieve equilibrium, it is necessary for free water to be attracted from the surrounding systems towards the cells involved. This attraction increases as the number of cells involved in the enzymatic blocking increases, and thus as a function of time.

Atypical situations are rare: one refers to an animal displaying no clinical signs after ouabain inoculation and in which the two hemispheres give practically identical responses superposable on those of the controls; this is ascribable to an injection error (inoculation in the epidural space, in the transverse sinus, etc.) as observed in previous research [10]. The results of the present research are of potential usefulness in the interpretation of pathologies involving neuronal damage consequent to the reduction of Na,K-ATPase activity [20] and shows that nuclear magnetic resonance could be useful tool in the early diagnosis of some pathologies involving changes in the water–biological-matrix interaction, NMR water signals being the basis of the NMR image [21].

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