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ATPase Na,K-dependent activity and water–matrix interactions in cerebella of aged mice

M.L. Antonelli[a,*], R. Curini[a], S. de Angelis Curtis[a], G. Palladini[b]

[a]*Department of Chemistry, University "La Sapienza", Box 34, Roma 62, P. le A. Moro 5, 00185 Rome, Italy*
[b]*Department of Neurological Sciences, University "La Sapienza", Rome, Italy*

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Abstract

A new analytical approach to the chemical behaviour of mouse cerebella during ageing is described. The microcalorimetric determination of Na^+/K^+-ATPase activity and the thermogravimetric investigation of the water–matrix interactions were performed on cerebella of mice of 2, 10 and 22 months (young, adult and aged mice). The results are compared with a preceding work regarding the same determinations performed on cerebella of ouabain-treated mice (spongy-state induced in cerebella of mice as those affected by the Jacob–Creutzfeld disease) and also with similar studies on ageing of brain in mice reported in the literature. The results here obtained on cerebella of aged mice show a substantial difference with respect to those reported for analogous samples of mice affected by a spongy-state.

Keywords: Na^+/K^+-ATPase; Thermoanalytical techniques; Mice

1. Introduction

After studying cerebellum tissues of ouabain-treated mice [1] in order to correlate the spongy-state of brain with the water content and the Na^+/K^+-ATPase activity in the Jacob–Creutzfeld disease, we have investigated the same kind of tissues in naturally aged mice, in order to study the cerebral behaviour during ageing. Some studies are reported [2–4] regarding the Na^+/K^+-ATPase levels in ageing rats, related to the RNA messengers. As they say, in normal ageing of brain there is not only a neuronal degeneration but also an alteration in the glial activity that could be accompanied by some variations in the Na^+/K^+-ATPase expression. In fact we know that the cation transport across the cellular membranes depends upon the Na^+/K^+-ATPase and Ca^{2+}-ATPase activities, influencing also membrane potentials, neurotransmitter re-uptake and similar phenomena [5]. Also during Alzheimer's disease in human brain some evidences have been reported [6] correlated with the Na^+/K^+-ATPase activity variations.

During cellular ageing, variations occur in the normal state of membrane potential [7,8]. Such situations causes some decrease of the calcium flux in the induced depolarisation and a reduced release of acetylcholine. Altered levels of membrane potentials could be associated with variations of neurone functions due to the ageing [9]. As the ionic equilibrium of the intra- and extra-cellular spaces is governed by the ATPase activity, the changed equilibrium in the membrane potentials (pointed out by in vivo experiments) could be due to variations in the catalytic activity of this specific enzyme. The Na^+ and K^+ transport across

* Corresponding author. Tel.: +39-06-49913618; fax: +39-06-490631.
E-mail address: marta.antonelli@unirama1.it (M.L. Antonelli).

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cellular membranes involves many water molecules, in order to maintain the osmotic equilibrium. Because of the variations in the relative ionic concentrations between inside and outside of cells, a continuous change in the water arrangement comes out due to variations in the ionic solvation spheres and in the dimensions of ionic channels. Therefore, it is clear that the Na^+/K^+ pump plays an important role in the regulation of the intra-cellular water content, especially in the nervous cells.

The present study has been addressed to research experimental evidences, from a chemical point of view, which could suggest an ATPase activity variation during ageing of the cerebral tissues. We tried to find out an experimental proof that the possible causes of found symptoms could really be related in some way to the ATPase activity.

Some authors put in evidence that Na^+, K^+-ATPase activity decreases during the synaptic ageing [10,11], but they also say that this decrease is probably due to: "...the age-related alterations in the microenvironment constructed by phospholipids...".

As already done we would like to verify, by means of a very sensible, direct and accurate methodology (enzymatic microcalorimetry) [12], if in the brain aged tissues there is a real decrease of the Na^+, K^+-ATPase activity; otherwise if it could be only an apparent malfunction of the enzyme itself, due to some altered surrounding conditions, which should be necessary to the protein for working properly.

To this end the already used experimental conditions [1], regarding the study of a possible model of the Jakob–Creutzfeldt disease in mice, have been reproduced. In particular some modifications of ATPase activity and of water–matrix interactions have been studied in mice, in which that pathology was simulated by means of ouabain treatment [13]. The results of the preceding study confirmed that selective inhibition of the Na^+/K^+-ATPase, caused by ouabain injection, determines many metabolic effects which go on with time. In fact, the variations of enzymatic activity and water arrangement in cerebral tissues goes on increasing the period of time from the ouabain injection to the mouse death. Therefore the same studies regarding the Na^+/K^+-ATPase activity and water arrangement in cerebral tissues of mice have been carried out in order to verify if analogous evidences could be found also in human aged subjects [14].

Many experiments have been performed in order to determine directly the Na^+/K^+-ATPase activity and in parallel the water arrangement in cerebral tissue samples of mice.

Many experimental steps have been carried out as follows:

- improvement of a microcalorimetric method of high sensitivity useful for the analytical study of enzymatic activity [15,16];
- microcalorimetric investigation of cerebellum tissues of different aged mice 2, 10 and 22 months old mice have been chosen because they are so considered young, adult and aged subjects, respectively;
- use of the thermoanalytical techniques (thermogravimetric analysis (TG), derivative one DTG also coupled with Fourier transformed IR spectroscopy) [17] in order to go into details about the water–matrix interactions occurring in the same samples just analysed for the enzymatic activity by microcalorimetry.

2. Experimental

2.1. Instrumentation

A microcalorimeter LKB Model 2107, isothermal batch instrument of heat conduction type [18,19], equipped with two gold vessels of about 7 cm^3 total volume, a multi-temperature cooling circulator (LKB Model 2209), a control unit (LKB Model 2210), was used. All measurements were performed at 25 ± 0.001°C and all instrumentation was housed in a thermostated room at 25 ± 0.5°C. The calorimetric apparatus was calibrated by measuring the heat of a known reaction: the sucrose dilution, as in the literature [19]. A Model TGA-7 Thermogravimetric Analyzer (Perkin Elmer) coupled to an FTIR Spectrometer 1760 X (Perkin Elmer) and an UNIX 433 DX LP computer was used.

2.2. Auxiliary instrumentation

A Polytron homogeniser PCU Power with a control unit Kinematica, Kriens, Luzern, Switzerland and an ultrasonic bath AGE Electronica, Italy were

employed. A Metrhom 605 pH-meter equipped with a calomel reference electrode and a glass measuring electrode was used. A 4226 Nuova Criotecnica Amcota centrifuge and a B6 Mettler analytical assay balance were used.

2.3. Materials

All solutions were prepared by using deionized water, distilled twice on potassium permanganate. All chemicals were of analytical grade (from Merck). ATPase enzyme Na^+, K^+ activated was a lyophilised powder (EC 3613) (Sigma from porcine cerebral cortex). ATP disodium salt was obtained from Sigma; Tris (tris(hydroxymethyl) aminomethane) for analysis and HCl (concentrated ampoules) from Merck.

The lyophilised 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. The buffer used was always Tris–HCl 0.05 M at pH 7.4 for the ATPase determination.

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.

2.4. Sample treatments

In view of the need to use reproducible samples, to avoid corrupting of the data as a result of non-controllable anatomical differences, and bearing in mind the size characteristics of thermoanalytical instrument (in particular the maximum size of sample for the sample holder of the thermogravimetry), it was deemed advisable to use mice cerebellum, since the brain hemisphere was too large to fit into the holder. The cerebella analysed were of mice of three different ages 2, 10 and 22 months.

Mice were sacrificed, after treatment with ether as anaesthetic, by decapitation by a guillotine device, the two lobes of 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 change in the water–matrix interactions, six animals were sacrificed with guillotine device without anaesthesia and six others after anaesthesia by ether. Fresh cerebella hemispheres were first analysed, while the controlaterals were placed in liquid nitrogen. The consistency of the analytical results obtained using fresh and refrigerated samples of anaesthetised and non-anaesthetised mice allowed this treatment to be validated and showed, at the same time, that the use of ether as anaesthetic was satisfactory.

For the TG experiments the mice cerebella hemispheres were used untouched without any treatment. For the calorimetric measurements each hemisphere (after weighing) was treated with 5 cm^3 of Tris–HCl buffer and then homogenised and then placed for 5 min in the ultrasonic bath; 2 cm^3 were used for the calorimetric analysis. For each age range, six animals have been tested in double (i.e. one analysis for each cerebellum hemisphere).

2.5. Methods

2.5.1. Microcalorimetry

The microcalorimetric method, which has been successfully applied to other enzymatic systems [20,21], employs the enzymatic hydrolysis of the 5′-ATP in the presence of the sodium, potassium-5′-adenosinetriphosphatase (Na^+/K^+-ATPase or simply ATPase) by the reaction below:

$$5'\text{-ATP} + H_2O \overset{Na^+,K^+,Mg^{2+}}{\underset{5'\text{-ATPase}}{\rightarrow}} 5'\text{-ADP} + P_i + \Delta H_R$$

where P_i is the phosphate residue and ΔH_R the enthalpy variation associated with the enzymatic reaction.

This enzyme is Na^+ and K^+-activated and also Mg^{2+}-dependent; moreover, the $[Na^+]/[K^+]$ and the $[Mg^{2+}]/[ATP]$ ratios are very critical for the enzymatic activity.

The results reported here have been obtained as described elsewhere [1,16]; in any case the measurements have been performed in pseudo-zero order kinetics with respect to the substrate (ATP), i.e. in excess of [ATP] (at least 10 times the value of the Michaelis–Menten constant of the ATPase enzyme investigated, K_m). Bearing in mind that $V = K\Delta(q/t)$ (where V is the thermopile voltage of the calorimeter = output of the instrument, K an instrumental calibration constant and q the exothermic heat quantity evolved during the reaction in a period of

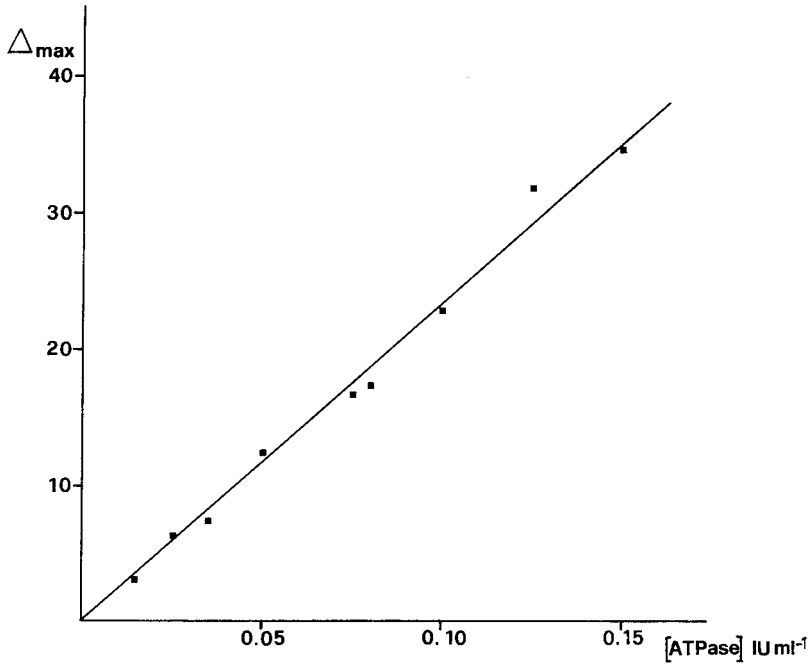


Fig. 1. Calibration curve for the Na^+/K^+-ATPase activity determination by microcalorimetry.

time t), the following equation was considered: $V_i = K'[E_i]\,\Delta H_R$. In this case, K' is constant depending upon the enzymatic reaction and $[E_i]$ represent the different enzymatic activities. Under these conditions (pseudo-zero order kinetics with respect to the substrate), the recorded thermograms are curves (V in function of time) which reach a steady state at different values of V_i depending on different $[E_i]$. In particular, those V_i correspond to the maximum deviation of the thermopile voltage = maximum height of the calorimetric curves (Δ_{max}). This is the experimental datum reported in the calibration graph (Fig. 1), because it is linearly related with the ATPase activity. All the experiments were run three times and for each $[E_i]$ value (expressed in terms of IU per millilitre, international units of enzyme). The reported values in the Fig. 1 (Δ_{max}) represent the average values of the maximum deviation of the calorimetric curves.

2.6. Experimental conditions

2.6.1. Microcalorimetry

The following experimental conditions were used: $T = 25.00 \pm 0.01$°C; $[ATP] = 5$ mM; $[Na^+] = 80$ mM and $[K^+] = 10$ mM; $[Mg^{2+}] = 5$ mM; the recorder full scale was 100 mV and the calorimeter amplification was 10 times.

In each calorimetric run 2 cm^3 of the sample homogenised in buffer and sonicated were put in one part of the measuring vessel and 2 cm^3 of the substrate solution (containing all the reagents indicated before prepared in Tris–HCl buffer) in the other part of the vessel, the reference vessel was filled in one part with 2 cm^3 of buffer solution and in the other part with 2 cm^3 of the same substrate solution as before.

The ATPase activity values relative to the samples analysed were obtained by means of a calibration curve (Fig. 1), plotted by using enzyme standard solutions as already described [16]. The period of time necessary for equilibrating the temperature inside the calorimetric unit after filling was about half an hour, meanwhile the calorimetric run took about 5 min time.

2.6.2. Thermogravimetry

Each mouse cerebellum hemisphere was placed in the thermobalance sample pan and heated over the temperature range 25–800°C, at a scanning rate of 10°C min^{-1}, in a flow of air of 50–100 ml min^{-1}.

To verify the water loss in the tissue studied, the TG results were accompanied by IR spectra, useful to identify the gases evolved during the thermogravimetric analysis. The TG-FTIR analyses were performed in a thermobalance coupled by transfer line to an IR spectrophotometer operating in Fourier transform mode. The IR spectra were performed over the temperature range 25–220°C in an air flow.

3. Results and discussion

Thermoanalytical techniques provide detailed information on the cooperative activity of all the various water–matrix interactions and they allow all the different types of water present in a system to be accounted for. Different types of water include those bound to the matrix with different energies. Thermogravimetric measures can be used to make a detailed analysis of the water contained in the matrix, making both qualitative and quantitative distinctions among the different types of water.

It has been demonstrated that water can be bound to the matrix with different energies and so when the matrix is heated, the water is lost in successive stages, depending on the amount of activation energy required to break the bonds (hydrogen bonds, Van der Waals forces, London forces, etc.) formed between water and matrix. The temperatures at which the various activation energies are reached can even be much higher than the boiling point of water.

Examination of the thermogravimetric curves and their first derivatives shows that water loss always occurs through two main, partially overlapping processes, corresponding to interactions between water and different components of the matrix over the temperature range 25–220°C. Two different types of water can thus be identified: free water and bound water.

Free water, that is water bound with less energy to the matrix, is released over the temperature range between 25 and 90–110°C, corresponding to first slight thermogravimetric step.

Bound water, that is water more strongly linked to the matrix, is lost through a more extensive process over the temperature range 110–220°C.

In Fig. 2 (a and b), as an example, the TG and DTG curves of mouse cerebellum relative to 2 and 22 months subjects are reported, respectively.

The observed trend of the weight loss is sufficiently different during the age increase of the examined subjects. In particular it can be observed that the total loss of the water molecules (free and bound) occurs between 25 and 220°C, which corresponds to 83.5% for the younger and about 79% for the aged subject.

In Fig. 3 the TGIR spectra relative to the gases evolved during the thermogravimetric analyses (temperature between 25 and 220°C) are reported. The figure shows the loss of water and the beginning of loss relative to some other substances, which cannot be quantified.

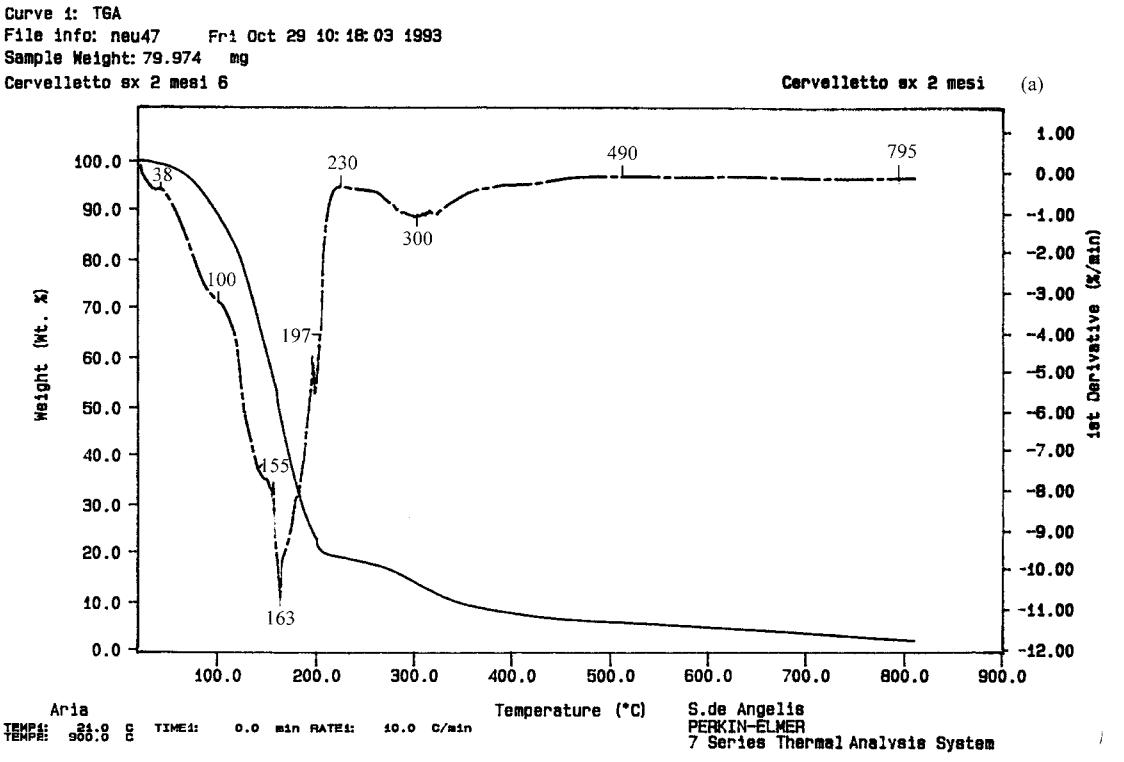
The obtained results are listed in Table 1, as it can be seen that the percentage of total water released during the temperature rise slightly decreases by increasing the sample age. The percentage of the free water released decreases with ageing, while the percentage of bound water slightly increases. This fact is probably due to the transformation of water from free to bound with the age of subjects. Moreover, the discrepancy of data is higher in the results relative to the young subjects with respect to the aged mice. This result may be ascribed to the biological variability, which mainly affects the younger subjects.

In any case, the total water loss in the cerebral tissues of mice resulted higher in aged subjects than in the younger ones, and this fact could be related with the senescence of cerebella of aged mice together with the ATPase activity in the same tissues.

Fig. 4 shows, as an example, the calorimetric response relative to the ATP/ATPase reaction in a 10 months subject. In Table 2 the calorimetric results relative to all the subjects analysed are listed. In fact it must be noticed that there is no real difference in the ATPase activities of subjects of different ages.

As already reported [10,11] some studies stress a decrease in the ATPase activity during ageing, but they all are based on the spectrophotometric determination of the P_i released from ATP and therefore they are less accurate and less precise with respect to the calorimetric method proposed here, especially because they use an indirect assay.

It is our opinion that the optical analysis is in effect less reliable and moreover we notice that the common methods of analysis [22–24] feel the effect of the varied surrounding environment of reaction. Those methods in fact do not use a unique specific reaction as in the calorimetric one. So that the decrease in the



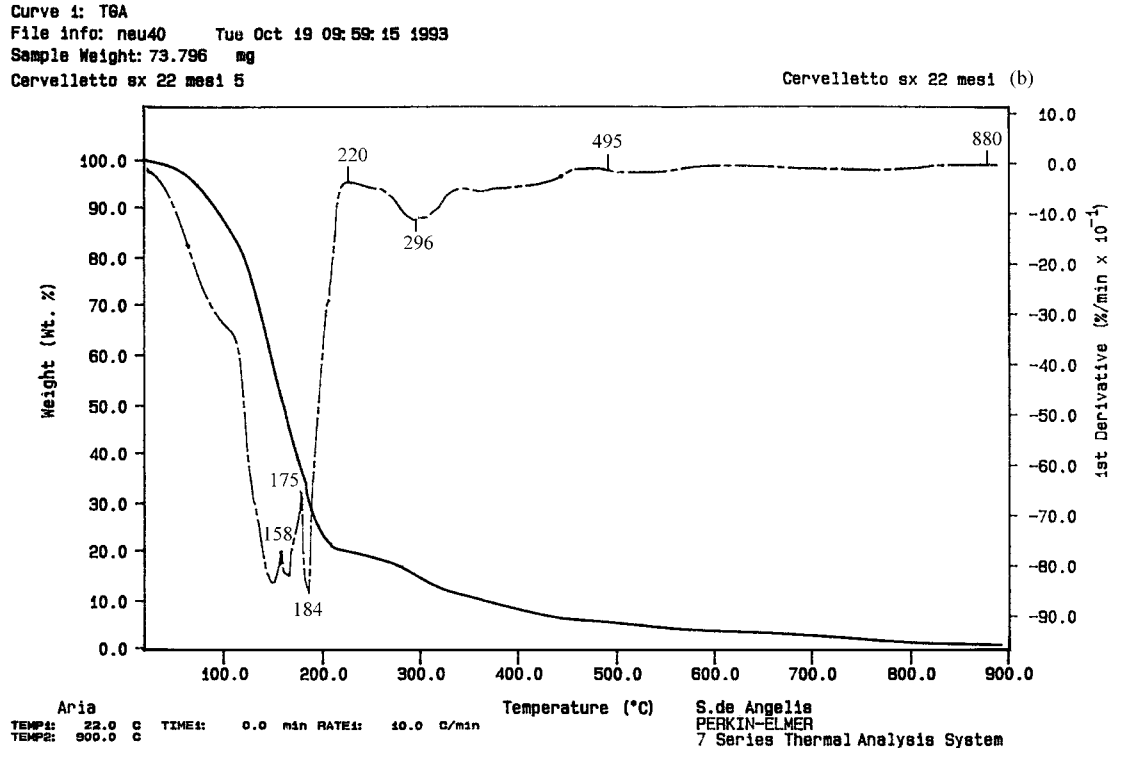


Fig. 2. TG and DTG curves of cerebellum mouse at different ages: (a) 2 months; (b) 22 months. Atmosphere: air, heating rate: 10°C min^{-1}.

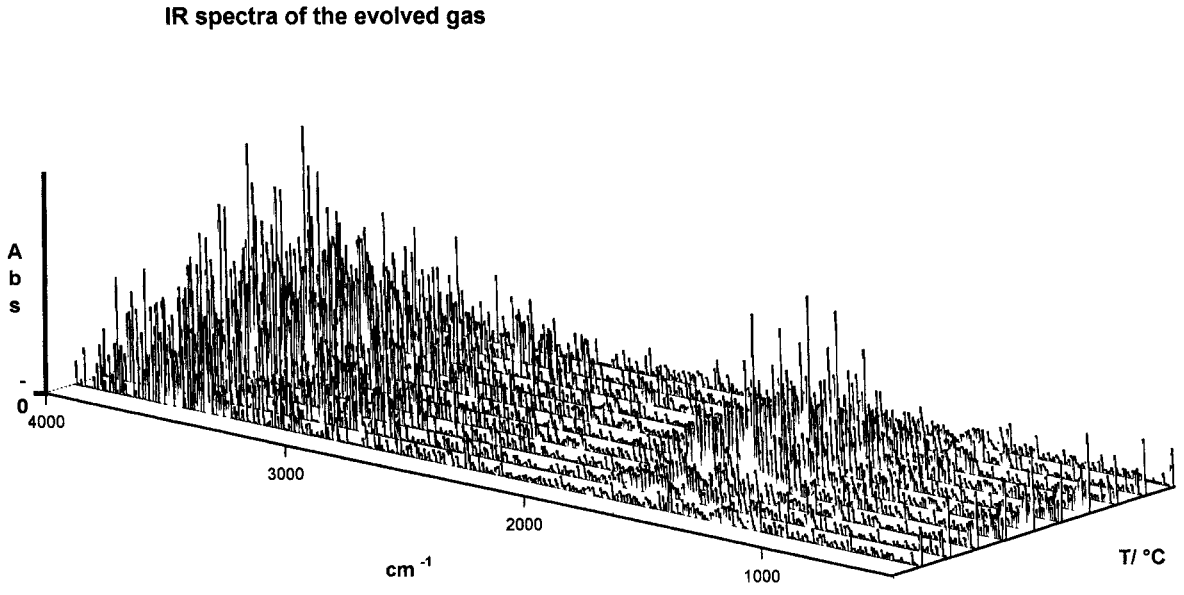


Fig. 3. Example of an IR spectra relative to the water loss in the temperature range 25–220°C.

ATPase activities, observed by spectrophotometry, could be due to some interference by the ADP accumulation in the reaction batch and/or to the influence of other substances, which concentrations could vary with age and then affect the activities investigated. In other words, the chemical environment in the cerebral tissues could affect the enzymatic activity investigated.

Table 1
Percentage of water loss (*free, bound* and total) in cerebral tissues of mice at different ages, obtained by thermogravimetric technique (TG and DTG)

Mice	Free water (%)	Mean ± S.D.	Bound water (%)	Mean ± S.D.	Total water (%)	Mean ± S.D.
2 months	18.1	16.5 ± 0.8	65.4	65.5 ± 0.8	83.5	82.0 ± 0.4
	18.2		64.3		82.5	
	14.7		67.0		81.7	
	18.1		62.8		80.9	
	17.6		64.0		81.6	
	14.6		67.0		81.6	
	–		–		–	
10 months	11.0	10.6 ± 0.4	69.8	69.9 ± 0.3	80.8	80.5 ± 0.4
	10.0		69.2		79.2	
	12.1		69.3		81.4	
	10.2		70.3		80.5	
	10.3		70.2		80.5	
22 months	8.9	8.3 ± 0.2	71.0	71.6 ± 0.2	79.9	80.0 ± 0.1
	9.0		70.8		79.8	
	8.4		72.1		80.5	
	9.1		71.0		80.1	
	8.8		71.1		79.9	
	8.4		71.2		79.6	

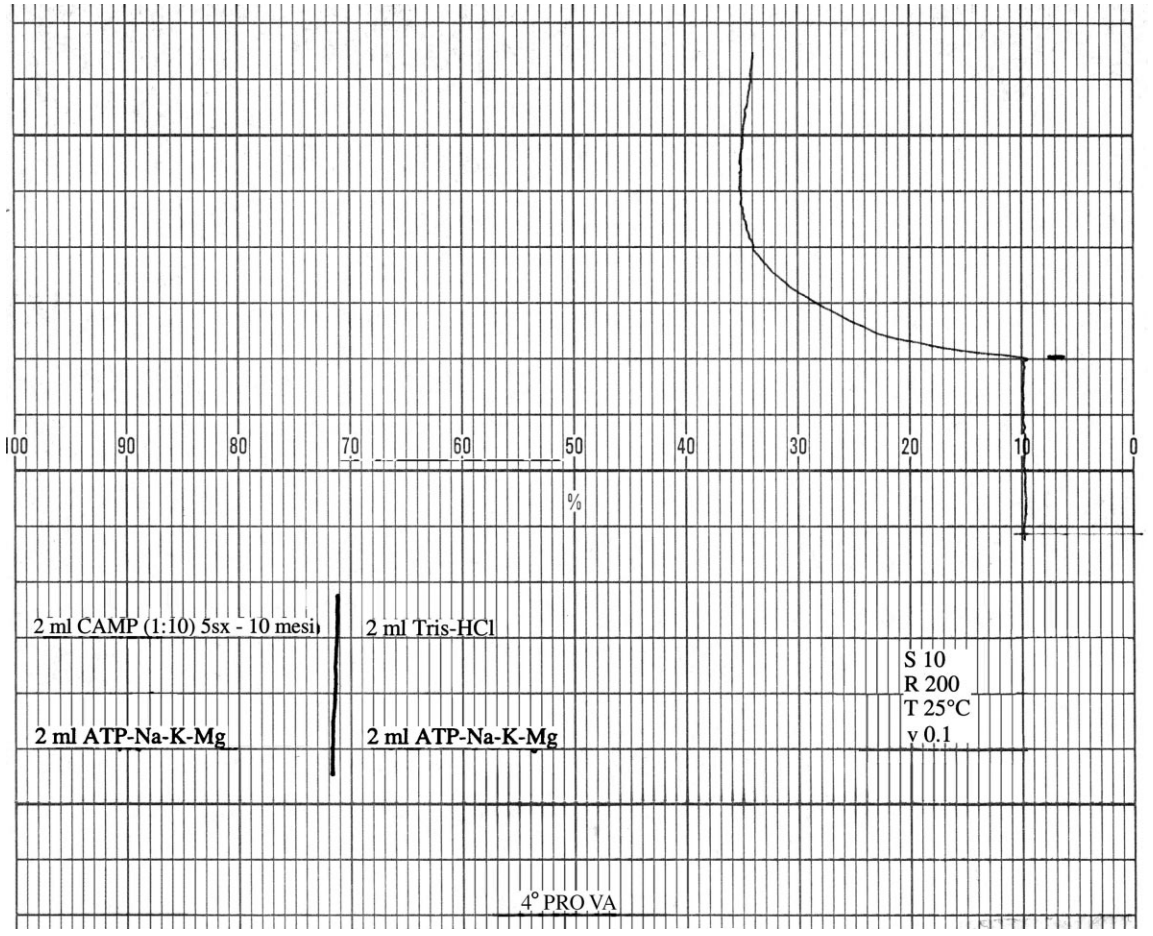


Fig. 4. Microcalorimetric output relative to the analysis of the Na^+/K^+-ATPase activity in a cerebellum tissue of a 10 months old mouse.

The calorimetric results indicate that the ATPase activity does not change during the ageing of the cerebral tissues in mice, this experimental evidence seems to be confirmed also by the investigation on the water arrangement of the same samples. During ageing tissues become wrinkled and as a matter of fact their total water content slightly decreases; on the contrary the cells would swell up if the ATPase enzyme reduces its catalytic action upon the sodium/potassium pump at the membrane level.

4. Conclusions

From the experimental evidences described herein, we can suggest that the behaviour of cerebral tissues in naturally aged mice is not the same as that in cerebella of ouabain-treated mice, before studied [1,13]. In fact from our results it seems that the Na^+, K^+-ATPase activity does not decrease in the former case. In fact also the cited papers regarding the ATPase levels in RNA messengers [2,3] show that the ATPase activity decreases in the α_3-isoform but increases in the α_1-isoform. Therefore it would be likely that the total ATPase catalysis in cerebellum tissues would remain almost constant. Other authors [10,11] who found, on the contrary, a substantial decrease in ATPase activity also in brain of naturally aged mice speculate about a varied microenvironment of lipids in the cellular membrane which could be responsible of the fact.

The method used in the reported studies is indirect and lacking in accuracy [22] because it is based on the

Table 2
ATPase activity (expressed in International Units per millilitre per milligram) in cerebral tissues of mice at different ages, measured by microcalorimetry

Mice	[ATPase] IU ml^{-1} mg^{-1}	Mean ± S.D.
2 months	0.040	0.045 ± 0.0034
	0.035	
	0.055	
	0.050	
	0.047	
	0.045	
10 months	0.040	0.045 ± 0.0024
	0.045	
	0.050	
	0.042	
	0.040	
	0.053	
22 months	0.043	0.043 ± 0.0021
	0.040	
	0.047	
	0.040	
	0.050	
	0.037	

measurement of the total inorganic phosphate in synaptic material after administration in vivo of ATP, as substrate, and of ouabain as enzyme inhibitor after a period of time.

Otherwise it must be noticed that the analytical method employed here to determine the Na^+, K^+-ATPase activity is direct and really specific: it is based on the simple and high sensible measure of the enthalpy involved in the enzymatic hydrolysis of ATP. We hypothesise a fundamental difference in the behaviour of ATPase action in naturally aged mice with respect to that observed in mice showing a spongy-state.

In particular we suppose that the natural ageing of mouse cerebella also accompanied by different arrangement of water in tissues (compare also the different content of water observed, Table 1) could be really responsible of an ''apparent'' decrease of the Na^+, K^+-ATPase activity. In this case the enzyme could really work properly, but the substrate and its environment are varied, therefore they are less available for the enzymatic catalysis.

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