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# The influence of the ketogenic diet on the elemental and biochemical compositions of the hippocampal formation

Joanna Chwiej [a,*], Agnieszka Skoczen [a], Katarzyna Matusiak [a], Krzysztof Janeczko [b], Agnieszka Patulska [a], Christophe Sandt [c], Rolf Simon [d], Malgorzata Ciarach [b], Zuzanna Setkowicz [b]

[a] AGH University of Science and Technology, Faculty of Physics and Applied Computer Science, Krakow, Poland
[b] Jagiellonian University, Institute of Zoology, Department of Neuroanatomy, Krakow, Poland
[c] SOLEIL, St Aubin, France
[d] Institut fur Synchrotronstrahlung, Research Centre Karlsruhe, Karlsruhe, Germany

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ABSTRACT

A growing body of evidence demonstrates that dietary therapies, mainly the ketogenic diet, may be highly effective in the reduction of epileptic seizures. All of them share the common characteristic of restricting carbohydrate intake to shift the predominant caloric source of the diet to fat. Catabolism of fats results in the production of ketone bodies which become alternate energy substrates to glucose. Although many mechanisms by which ketone bodies yield its anticonvulsant effect are proposed, the relationships between the brain metabolism of the ketone bodies and their neuroprotective and antiepileptogenic action still remain to be discerned.

In the study, X-ray fluorescence microscopy and FTIR microspectroscopy were used to follow ketogenic diet-induced changes in the elemental and biochemical compositions of rat hippocampal formation tissue. The use of synchrotron sources of X-rays and infrared allowed us to examine changes in the accumulation and distribution of selected elements (P, S, K, Ca, Fe, Cu, Zn, and Se) and biomolecules (proteins, lipids, ketone bodies, etc.) with the micrometer spatial resolution.

The comparison of rats fed with the ketogenic diet and rats fed with the standard laboratory diet showed changes in the hippocampal accumulation of P, K, Ca, and Zn. The relations obtained for Ca (increased level in CA3, DG, and its internal area) and Zn (decreased areal density in CA3 and DG) were analogous to those that we previously observed for rats in the acute phase of pilocarpine-induced seizures.

Biochemical analysis of tissues taken from ketogenic diet-fed rats demonstrated increased intensity of absorption band occurring at 1740 $cm^{-1}$, which was probably the result of elevated accumulation of ketone bodies. Moreover, higher absolute and relative (3012 $cm^{-1}$/2924 $cm^{-1}$, 3012 $cm^{-1}$/lipid massif, and 3012 $cm^{-1}$/amide I) intensity of the 3012-$cm^{-1}$ band resulting from increased unsaturated fatty acids content was found after the treatment with the high-fat diet.

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## 1. Introduction

Epilepsy is one of the most common diseases of the central nervous system (CNS). People at risk of epilepsy can often be identified; however, there is still no prophylactic treatment that would prevent the development of the disease [1,2]. The possible solution could be the ketogenic diet (KD) which is a broadly effective therapy for intractable epilepsy. Fats are the predominant caloric source of the ketogenic diet, while carbohydrate and protein intake is restricted. Catabolism of fats results in the production of ketone bodies, and they become an alternative substrate to glucose for energy utilization [3,4].

Although KD has been used clinically for nearly a century, the mechanisms of its anticonvulsant and neuroprotective action are still not fully understood [5–9]. Elemental and biochemical changes occurring in selected areas of rat brain as a result of the treatment with KD may throw some new light on this problem. Therefore, they were done in this study using highly spatially resolved techniques utilizing synchrotron radiation.

Studies of the molecular structure of nervous tissue using synchrotron radiation are very few (PubMed database search), and those exploring the mechanisms underlying the brain susceptibility to seizures, seizure-induced degenerative changes, or epileptogenesis itself are very occasional. Thus, at this stage of the newly developing research field, the studies aims not only to better knowledge of the mechanisms

* Corresponding author at: AGH University of Science and Technology, Al. Mickiewicza 30, 30-059 Krakow, Poland. Tel.: +48 12 617 41 55; fax: +48 12 634 00 10.
*E-mail address:* joanna.chwiej@fis.agh.edu.pl (J. Chwiej).

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at the molecular level, but also to a constant improvement of this methodological approach.

Synchrotron radiation is produced by charged particles moving at speeds close to those of light when their paths are altered through the magnetic field. Its unique features such as high intensity, collimation, and wide spectral range enable the examination of most subtle biomolecular changes occurring at ranges even less than micrometer [10–12]. Therefore, synchrotron radiation-based techniques play an increasing role in biomedical research [13–21].

In the present paper, two characterization techniques that use synchrotron radiation were utilized. For the analysis of distribution and accumulation of selected elements within the hippocampal formation, X-ray fluorescence microscopy (XRFM) was used, while biochemical evaluation of particular hippocampal areas was done by applying synchrotron Fourier transform infrared microspectroscopy (FTIRM). Both techniques have been successfully applied in our previous studies concerning the mechanisms leading to epilepsy which were carried out mostly on the pilocarpine model of seizures [22–29]. Examining seizure-induced elemental changes of the hippocampal formation, we were able to follow the processes of excitotoxicity and mossy fiber sprouting occurring in this brain area [18–20]. In turn, the use of FTIRM for biochemical analysis allowed us to detect the areas where abnormalities in the accumulation of main biomolecules (proteins, lipids, and compounds containing phosphate group(s)) and their structure (protein conformation and saturation and unsaturation levels of lipids) occurred [21,30].

## 2. Animals

Male Wistar rats came from an animal colony of the Department of Neuroanatomy (Institute of Zoology, Jagiellonian University). All animal-use procedures were carried out there and were approved by the Bioethical Commission of the Jagiellonian University (agreement no. 65/2012) in accordance with international standards.

On the day 30th of postnatal development, the rats were divided into two groups. The first group of animals, through the next 30 days of their lives, was fed with the ssniff® ketogenic diet E15149-30 (K group), while the second one was fed with the standard laboratory diet in the form of Labofeed (N group). The content of main nutrients and fatty acids in both diets was compared in Table 1.

On the 60th day of postnatal life, animals were perfused with physiological saline solution of high analytical quality. The brains were excised, frozen, and cut using a cryomicrotome into 12-μm thick sections. Three neighboring slices of the dorsal part of the hippocampus were mounted on the three different sample carriers. The tissue dedicated to elemental analysis was put on the ultrapure, thin (4 μm) and transparent for X-rays, Ultralene® foil. For biochemical analysis, MirrIR slides were used as sample carriers, while routine histopathological analysis was carried out on standard microscope slides.

## 3. Methods

Two modern techniques using synchrotron radiation were applied in the study. These were X-ray fluorescence microscopy (XRFM) and Fourier transform infrared microspectroscopy (FTIRM). The first method was used for the topographic and quantitative analysis of elements such as the following: P, S, K, Ca, Fe, Cu, Zn, and Se in the hippocampal formation, while the second one was used for the biochemical analysis of its selected areas. The data obtained using synchrotron FTIRM provided information on the accumulation and distribution of main biomolecules (lipids, proteins, and compounds containing phosphate bands) and their structural changes.

**Table 1**
The content of main nutrients (in [%]) and fatty acids (in [g/kg]) in the dry mass of ketogenic and standard diets.

| Nutrient | Ketogenic diet | Standard diet |
|---|---|---|
| Lipids | 79 | 5 |
| Carbohydrates | 1 | 63 |
| Proteins | 8 | 25 |
| Others | 12 | 7 |
| SFAs[a] | 329 | – |
| MUFAs | 330 | – |
| PUFAs | 86 | – |

[a] Saturated, monounsaturated, and polyunsaturated fatty acids.

### 3.1. Elemental analysis

The measurements using XRFM were done at the FLUO beamline of ANKA synchrotron facility. The excitation energy of 17 keV was selected, and the X-ray beam was focused with polycapillary optics. The size of the beam impinging on the sample was 12 μm × 18 μm (vertically × horizontally). Silicon drift detector was used to detect the fluorescence radiation from the sample. The detector was positioned at the angle of 45° in respect to the sample and 90° in respect to the exciting beam. The samples were mapped in two dimensions, and the time of single fluorescence spectrum acquisition was 8 s.

### 3.2. Biochemical analysis

The measurements using FTIRM were done at the SMIS beamline of SOLEIL in transreflection mode using an infrared microscope continuum XL coupled to a FTIR spectrometer Thermo Nicolet 5700. The samples deposited on MirrIR slides were analyzed using the IR beam of 10 μm in diameter. The spectral resolution was set to 6 $cm^{-1}$, and 38 scans were averaged per single sample spectrum. In turn, each background spectrum was the mean value of 128 scans. The data acquisition as well as the spectral analysis were performed with OMNIC software (version 8.0). Similarly, as in elemental analysis, the sample was mapped in two dimensions by changing its position in respect to infrared beam.

## 4. Results

### 4.1. KD-induced elemental changes of hippocampal formations

The elemental analysis of hippocampal formations showed the presence of the elements such as the following: P, S, K, Ca, Fe, Cu, Zn, Se, Br, and Rb, which can be easily seen in Fig. 1 presenting cumulative XRF spectrum typical for this brain area.

The analysis of single X-ray fluorescence spectra as well as the batch processing of large data sets were carried out using PyMCA software freely available for noncommercial use. The obtained net peak areas of K-α lines of the analyzed elements and elemental sensitivities evaluated

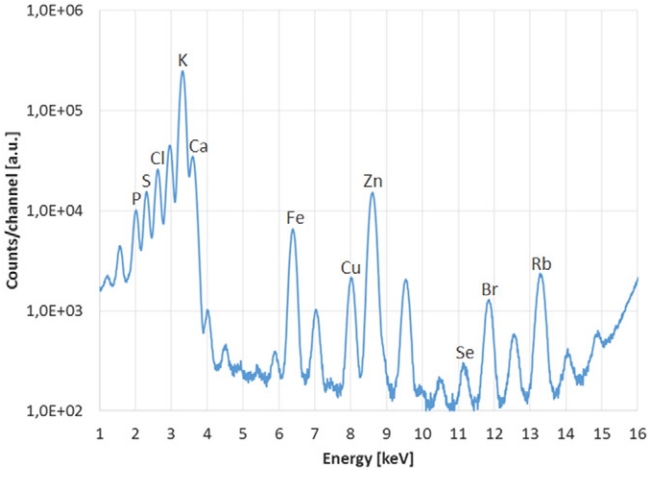


**Fig. 1.** The typical X-ray fluorescence spectrum recorded in hippocampal formations.