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We analyzed the applicability of high-resolution ^{2}H-HMR spectroscopy for the analysis of microbe metabolism in samples of mitochondrion isolated from rat liver and from aqueous extracts of homogenates of rat liver and other organs and tissues in the presence of high D_2O contents. Such analysis is possible due to the fast microbe adaptation to life in the heavy water. It is also shown that some enzymatic processes typical for the intact cells are preserved in the homogenized tissue preparations. The microbial and cellular metabolic processes can be differentiated via the strategic use of cell poisons and antibiotics.

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Abstract

We analyzed the applicability of high-resolution ^{2}H-HMR spectroscopy for the analysis of microbe metabolism in samples of mitochondrion isolated from rat liver and from aqueous extracts of homogenates of rat liver and other organs and tissues in the presence of high D_2O contents. Such analysis is possible due to the fast microbe adaptation to life in the heavy water. It is also shown that some enzymatic processes typical for the intact cells are preserved in the homogenized tissue preparations. The microbial and cellular metabolic processes can be differentiated via the strategic use of cell poisons and antibiotics.

Key words: Microbial metabolism; High resolution NMR; ^{2}H-HMR spectroscopy; Heavy water; Microbe adaptation;

Introduction

Recent years witnessed an increased interest of researchers in the analysis of various biological fluids. This research is taken now as a fundamental basis of the metabolomics, which studies the metabolic profiles of animals and humans during their normal activity and at various pathological conditions, as well as looks at the effects of various drugs and other substances on specific organ/tissue, the whole organisms, and even on the entire ecosystem (Holmes, Wilson & Nicholson, 2008; Maher *et al*., 2008; Nicholson & Lindon, 2008). Typically, term 'biological fluids' is taken as a synonym to 'body fluids' or 'biofluids' that correspond to liquids originating from inside the bodies of living people, such as urine, blood, saliva, sweat, cerebrospinal fluid, mucus, etc. However, this concept can be extended to include water washouts and aqueous extracts of the homogenates of various organs and tissues of animals (Kutyshenko *et al*., 2007; Kutyshenko *et al*., 2008a; Kutyshenko *et al*., 2008b) and plants (Molchanov *et al*., 2012). Addition of these somewhat artificial biological fluids leads to the noticeable increase in the variability of experimental material suitable for comprehensive analysis and produces substantial information related not only to the organs under study, but also to the interactions of these organs with the remaining organism and with specific microorganisms.

The close connection between plants and animals with specific microorganisms constituting microbiomes or microbiotas is a well-established fact. In fact, animals, including humans, constantly coexist with microorganisms, being involved in numerous symbiotic interactions with various bacteria and yeast that densely populate intestines, skin, and tunica mucosa of airways, pharynx and urinary tract. Furthermore, come microorganisms can get access to various organs through bloodstream or other biofluids leading to the development of various pathologies. The current list of human symbiotic microorganisms includes ~5,000 species that are uniquely distributed between 15 and 18 sites of the permanent habitat in

54 males and females, respectively (Human *et al*., 2012a; Human *et al*., 2012b). Since different
55 organs are biochemically different, sets of symbiotic microorganisms populating them can
56 vary between different organs within the same organism. Many members of the human
57 microbiome are conditionally pathogenic microorganisms that can provoke development of
58 various maladies if appropriate conditions are given (Tancrede, 1992; Riabichenko &
59 Bondarenko, 2007; Y*u et al*., 2012). Under these circumstances, originally harmless and even
60 beneficiary symbiotic microorganisms can go bad and start negatively affect the normal
61 cellular and organ functions of the host organism, secreting specific toxins and ferments and
62 eventually leading to the metabolism distortion and cell death. Furthermore, by destroying the
63 host cells, microorganisms promote the release of the cell content into the extracellular
64 environment, thereby further exacerbating the course of a disease and negatively affecting the
65 overall condition of the host organism. In fact, sometimes, massive cell death can be a
66 self-propagating process, where proteins released from the dying cells affect neighboring
67 cells leading to their death and consequently generating favorable conditions for the
68 propagation of both the “own” symbiotic microorganisms of the microbiome and the
69 microorganisms introduced from the outside. Therefore, under such circumstances, therapy
70 should include both antibacterial and healing strategies.

71 In this work, the mitochondria suspension and the aqueous extracts from the homogenates
72 of several tissues are used to model cell death and organ damage (necrosis) resulting from
73 injuries and pathologies and to experimentally characterize the related processes. We propose
74 here an instrumental approach that can be used to detect and control both microbial and host
75 enzymatic processes taking place within the sites of disease origin. This approach is based on
76 the detection of the deuterium incorporation to the specific metabolism products. Here,
77 deuterium (in a form of heavy water) is added directly to the medium where the ferment
78 action and/or microorganism vital activity takes place. Our earlier analysis revealed that

many microorganisms can easily adapt to the conditions of high heavy water contents, and presence of almost 100% heavy water does not significantly affect normal functioning of certain microorganisms (Kushner, Baker & Dunstall, 1999; Molchanov *et al*., 2012). Under these conditions, deuterium can be incorporated to the substrates due to the existence of efficient exchange between the protons of organic moieties of substrates and deuterium present in media. Next, these deuterated substrates can be used in biochemical reactions leading to the enzymatic incorporation of deuterium to the corresponding metabolism products (Ewy, Ackerman & Balaban, 1988; Kushner, Baker & Dunstall, 1999; Budantsev, Uversky & Kutyshenko, 2010; Molchanov *et al*., 2012). One of the most informative techniques to follow the mentioned processes in biological fluids is the high-resolution NMR at the deuterium nuclei, 2H-NMR (Budantsev, Uversky & Kutyshenko, 2010). In comparison with proton spectra, 2H-NMR spectra are characterized by lower resolution and lower sensitivity. Furthermore, deuterium-deuterium couplings are about 40 times smaller than proton-proton couplings and are therefore not observed. However, the overall shapes of ^{1}H-NMR and ^{2}H-NMR spectra of organic components of are rather similar, except to the fact that the multiplets seeing in ^{2}H-NMR spectra are presented by broader singlets due to the low spin-spin interaction constants and quadrupole broadening (Emsley, Feeney & Sutcliffe, 1966).

In this work, we show the applicability of the high-resolution ^{2}H-NMR spectroscopy for the quantitative analysis of biological fluids using preparations of mitochondria suspension and aqueous extracts from rat liver homogenates as illustrative example. It is important to emphasize here that the proposed approach for studying microbial and host enzymatic activities based on the analysis of deuterium incorporation to the metabolic products can be of wide practical use in many other cases, when high D_2O concentrations do not perturb the physiological processes of the studied (Budantsev, Uversky & Kutyshenko, 2010).

104

105 ## Materials and Methods

106 Mitochondria were isolated from the livers of Wistar rats using the standard protocols
107 (Belosludtse*v et al*., 2009). Mitochondria samples used in our study were a generous gift of
108 Prof. Mironova G.D. The only modification of the isolation protocol in some preparations
109 was substitution of light water by heavy water (OOO Astrochim, Russia, 99.8%) done at our
110 request. The standard functional analysis revealed that the mitochondrion isolated using such
111 modified heavy water-based protocol were active and preserved their activity for several
112 hours after isolation. Part of mitochondrion isolated by a standard, light water-based approach
113 was subsequently treated with heavy water. The concentrations of heavy water in samples
114 were controlled using characteristic features of 1H-NMR spectra.

115 Livers of the Wistar rats were a kind gift of Prof. Kichigina V.F. These animals were
116 sacrificed for the purpose of unrelated experiments (Popova, Sinelnikova & Kitchigina,
117 2008). Aqueous extracts of the rat liver homogenates were prepares using 0.40±0.03 g
118 samples which were first carefully homogenized in the eppendorfs using a special sterile
119 glass spatula and then diluted with 0.75 ml heavy water (CIL, USA, 99.9%). Samples were
120 centrifuged using the microcentrifuge CM-50 (ELMI, USA) prior the NMR measurements.

121 Antibiotics gentomicin (Asparin, Germany) and amphotolecin B (Sigma) were dissolved
122 in 2 ml of D_2O to ensure final dilution of 1:200 (Solovie*va et al*., 2008).

123 NMR spectrometer AVANCE 600 (BRUKER) with the operating frequency 600.13 MHz
124 was used in the experiments. ^{1}H-NMR spectra were measured using the spectral width of
125 8000 Hz, 90^{o} impulse of 11 microseconds, and temperature of 298 K. As a rule, 128
126 accumulations were sufficient to obtain good signal to noise ratio. ^{2}H-NMR spectra were

measured using the 20W field stabilizer at the frequency of 92.12 MHz, 90°-impulse length of 150 microseconds, a spectrum width of 8000 Hz and 500-1000 accumulations. All the measurements were made at 298 K inside the sensor.

Results and discussion

Mitochondria from rat liver

It is believed that the isolated from the rat liver mitochondria preserve their functional activity *in vitro* for 1-3 hrs after isolation (Belosludtse*v et al*., 2009). The proton NMR spectrum of the suspension of mitochondria isolated using the heavy water-based protocol that was collected during this initial time of the sustained mitochondrial activity is shown in Figure 1A. This spectrum is dominated by the rather broad signals typical of the intracellular organic molecules. Note that narrow and very intensive signals correspond in a region from 4.7 to 3.5 ppm to sucrose, which is present in the extracellular medium due to the peculiarities of the isolation protocols (Figure 1A) (Belosludtse*v et al*., 2009). In the absorption region of the aliphatic protons (from 3.0 to 0.5 ppm), the major components are broad signals corresponding to the mitochondrial membranes. After 10-12 hrs of incubation, some sharp signals start to appear (see Figure 1B). These signals correspond to the organic molecules extruded from the mitochondria to medium. With time, the amplitudes and number of these sharp signals increase, whereas the amplitudes of broad signals proportionally decrease. At this moment, spectrum contains signals of free amino acids and other organic components, which are commonly detected in other biological fluids and aqueous extracts from various plant and animal tissues. Figure 1C shows typical 1H-NMR spectrum of the aqueous extracts of the rat liver homogenate. Spectrum contains sharp signals of free amino acids that coincide with signals detected in all major biological fluids. In fact, ^{1}H-NMR spectra of the biological fluids studied so far are quantitatively similar possessing some

fluid/condition-specific qualitative differences. Comparison of Figures 1C and 1B revealed that the majority of sharp signals detected in the ^{1}H-NMR spectrum of the aqueous extract of the rat liver homogenate coincide with those in the ^{1}H-NMR spectrum of the mitochondria. In the ^{1}H-NMR spectrum of the aqueous extract of the rat liver homogenate, the most characteristic signals with highest intensities correspond to glucose. Proton spectra of the aqueous extracts did not change neither qualitatively nor quantitatively during the observation for 3-5 days.

Interestingly, signals in the 2H-NMR spectrum start to appear only after incubation for about 24 hrs. After another day of incubation, the ^{2}H-NMR spectrum is completely formed, and subsequent incubation results in the increase of amplitudes of already existing signals. Figure 2 represents this process by showing normalized integral intensities measured in the range of 3.6-0.0 ppm of proton spectra (black circles) or in the range of 4.2-0.0 ppm of ^{2}H-NMR spectra. The increase in the amplitudes of sharp signals in the proton spectra is related to the gradual release of the intramitochondrial organic compounds resulted from the destruction of mitochondrial membranes. The sharp increase in the amount of these compounds is associated with the massive membrane decomposition. This process starts on the second day after the mitochondrion isolation and continues for another 50 hrs, during which time it slows exponentially. The increase of signals in the ^{2}H-NMR spectra is more gradual. It is related to the activity of the mitochondrial enzymes and to the microbial metabolism. On average, the integral intensities of the ^{2}H-NMR spectra are about 1.3-times lower than amplitudes of peaks in the proton spectra.

During the first 27 hrs after isolation of mitochondrion, the kinetics of the formation of proton- and deuterium-containing metabolites are similar due to the insignificant amounts of the low molecular mass (LMM) compounds released from the destroyed mitochondria. These

LMM compounds serve as substrates for the metabolism of the contaminating microorganisms and for the residual enzymatic activity of the mitochondrial proteins either released to the medium from the destroyed mitochondria or still located inside the damaged mitochondria. At longer incubation times, kinetic parameters of the observed processes become more and more different. This reflects the existence of an active metabolic conversion of the released substrates by microorganisms and by the residual enzymatic activity of mitochondrion. Importantly, the proton spectra of mitochondrion do not qualitatively change with time; i.e., no new signals appear and no old signals completely disappear.

Figures 3A and 3B represent a pair of typical ^{2}H-NMR spectra measured for two mitochondrial isolates randomly selected from a dozen of independent isolation performed during a year using different isolation protocols (sucrose-based and mannitol-sucrose-based), on the basis of D_2O and H_2O, respectively. All the recorded spectra possess close similarity to each other, being mostly different in relative intensities of several peaks. Figure 3 represents signal assignments based on the comparison of chemical shifts with proton spectra of known metabolites from various biological fluids. These assignments took into account the presence of the isotope shift and were performed using a large set of 2H-NMR spectra of samples prepared from various plant and animal sources. The major difference between spectra shown in Figures 3A and 3B is in lesser amounts of ethanol and acetate in mitochondrial preparations utilizing heavy water. Furthermore, in all the cases of heavy water-based isolations, the rightmost signal corresponding to isotopic variant of acetate ($-CD_3$) was always higher than the middle signal corresponding to $-CHD_2$, since the heavy water content in these samples was ~85%, whereas in light water-based isolations with concomitant addition of D_2O, the heavy water content was at the level of 35-40%. The presence of signals

corresponding to ethanol, acetate and formate at 8.43 ppm (not shown) is the reflection of the microbial contamination of the isolated mitochondrion.

Figure 3C represents a typical ^{2}H-spectrum of the aqueous extract of liver homogenate. This spectrum, being corrected for the differences in intensity of some signals, resembles spectrum of the mitochondria isolates. However, since this spectrum possesses signals corresponding to ethanol, formate, and acetate, one can suggest that these samples were contaminated by microorgansims. To identify signals corresponding to the products of the microbial metabolism, some broad-spectrum antibiotics or sodium azide were added during the sample preparation. Similar to antibiotics, sodium azide (low concentration of which are used as preservative in the food industry) possess antimicrobial activities. Sodium azide predominantly affects Gram-negative bacteria, suppressing their growth and development. The application of both bactericides had similar outputs, and the resulting ^{2}H-NMR spectra of the aqueous extract of liver homogenates treated with antibiotics and sodium azide were identical. Figure 3D represents one of the spectra for bactericide-treated sample and shows the lack of signals corresponding to ethanol, formate, and acetate, supporting their bacterial origin. Therefore, resulting spectra contain only signals corresponding to the compounds produced by mitochondrial enzymes under the proton-deuterium exchange conditions. The liver extracts contain both substrates and ferments that participate in the enzymatic reactions uncontrolled by the decomposed cells. The corresponding 2H-NMR spectra contain alanine, glycine, and lactate (Figure 3D), with alanine being the dominating component. It is known that alanine accounts for ~30% of all amino acids delivered to the liver. This explains relatively high concentrations of alanine in the liver preparations (see Figure 2C). In the liver, alanine is converted to pyruvate, which is subsequently used for the glucose synthesis (Malaiss*e et al*., 1996; Burell*e et al*., 2000).

In our experiments, the samples were prepared by the mechanical homogenization of rat livers. Therefore, the resulting homogenate contains some surviving cells that remain functional and continue function more-or-less normally, at least for some time. Therefore, these preparations can be considered as a model of severe tissue damage. Survived cells continue to express proteins and possess metabolic processes supporting cell life activity. Under the oxygen deficiency conditions of our experiments, the only available pathway for energy generation in a cell is anaerobic glycolysis. However, the last stage of this pathway is likely to be failed as evidenced by the lack of the increase in the lactate signal in the corresponding 1H-NMR spectra (see Figure 1C).

Pyruvate produced during glycolysis is converted to the alanine via the transamination reaction. This reaction together with the reversed transformation of alanine to pyruvate is catalyzed by the alanine transaminase also known as alanine aminotransferase (Dolle, 2000; Yan*g et al*., 2009). The activity of this enzyme combined with the protein degradation and membrane decomposition, together with the presence of some free alanine inside the cells give likely explanation for the moderate increase in the alanine signal in the spectra of rat liver homogenates during their long-term observation. The presence of deuterium in the Cα position and in the methyl groups of alanine supports the enzymatic origin of alanine's hydrocarbon skeleton (see Figure 4).

Figure 5 represents the ^{2}H-NMR spectra of mitochondria in samples containing antibiotics. Comparison of spectra measured at different time points after the sample preparation indicates the presence of some kinetic processes. Figure 5C shows signals accumulated during the first 8 hrs of sample incubation. The most intensive signal here is a signal from the glycine deuterons followed by a less intensive signal of deuterated alanine. Furthermore, spectrum contains signals corresponding to the proton-deuterium exchange at

nitrogens of urea (5.7 ppm), glutamine (~7.6 ppm) and asparagine (~6.9 ppm). These signals significantly increase after a one day incubation (see Figure 5C) but did not change much during the more prolonged incubation. However, to the sixth day, spectrum undergoes further changes, and signals of lactate and formic acid appear, whereas signals corresponding to the nitrogen disappear. These changes reflect starting bacterial activity leading to the nitrogen utilization and appearance of own metabolites. Concentrations and ratios of antibiotics were carefully selected to suppress the bacterial activity and not to produce additional damage of the liver cells. In these settings, the bacterial activity was sufficiently suppressed, since in the absence of antibiotics, signals corresponding to lactate and ethanol were easily detectable already after 2-3 days (see Figure 3).

The major glycine biosynthetic pathway in a cell is the one catalyzed by the serine hydroxymethyltransferase, an enzyme that plays an important role in cellular one-carbon pathways by catalyzing the reversible, simultaneous conversions of L-serine to glycine (retro-aldol cleavage) and tetrahydrofolate to 5,10-methylenetetrahydrofolate (hydrolysis) (Appaji Rao *et al*., 2003; Scheer, Mackey & Gregory, 2005; Berdyshev *et al*., 2011). Figure 4 shows that serine is synthesized in a cell from the 3-phosphoglycerate, which is one of the intermediates of the glycolysis, and glutamine, which serves as the source of amine. Serine is subsequently used for the protein biosynthesis and for the synthesis of phosphatidylserine that constitutes typically ~15% of all membrane phospholipids. The transfer of the serine methyl group to tetrahydrofolate in the presence of heavy water can be accompanied by the deuteration of the CH_2-group of the newly synthesized glycine.

Our study revealed that high-resolution ^{2}H-NMR spectroscopy can be successfully used in metabolomics studies. Furthermore, the strategic use of antibiotics helps discriminating microbial activity from enzymatic cellular processes. The major products of microbial

activity are organic acids, such as formate, acetate, lactate, propionate (seeing in spectra of homogenates of heart muscle) and ethanol. It is important to note here that our data suggest that ethanol can originate not only from the classical alcoholic fermentation but can be generated via some other processes. This conclusion is based on the uneven intensities of $–CD_2–$ and $–CD_3$ deuterons reproducible detected in our experiments, whereas these signals would have comparable intensities if ethanol would be exclusively generated via the alcoholic fermentation pathway due to the more efficient deuteration of methylene group (Kutyshenko & Iurkevich, 2000).

The major substrates for the ethanol formation are pyruvic acid and acetaldehyde. There are several biosynthetic pathways for the production of these compounds in the organism, and pyruvate and acetaldehyde can be generated from glucose (as a result of glycolysis), pentoses (via pentose phosphate pathway) or from some amino acids (e.g., due to the catabolism of alanine and threonione) (see Figure 4). Therefore, the ethanol formation is likely a reflection of the successful development of the contaminating bacterial and fungal microbiomes. Based on the characteristic patterns of the hydrogen substitution by deuterium we hypothesize that the significant part of the endogenous ethanol in our settings is synthesized from the deaminated amino acids (see Figure 4). For example, during the processes of alanine transamidation and threonine degradation, the resulting terminal CH_3-groups of pyruvate and acetaldehyde are efficiently deuterated. The subsequent fermentation of pyruvate to ethanol in the presence of heavy water may be accompanied by the deuteration of ethanol's $–CH_2$-group. Resulting ^{2}H-NMR spectra of ethanol derived from these intermediates suggest almost proportional saturation of CH_3- and $–CH_2$-groups, in sharp contrast to the disproportional saturation of these groups in ethanol molecules produced via the glucose fermentation.

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Figure legends

Figure 1. ^{1}H-NMR spectra of biological fluids. **A**. mitochondrion isolated from rat liver. Measurements were done immediately after mitochondrion isolation. **B**. mitochondrion isolated from rat liver. Measurements were done one day after isolation. **C**. Aqueous extract of the rat liver homogenate.

Figure 2. Time courses of changes in the integral intensities of the aliphatic part of ^{1}H-NMR (black circles) and ^{2}H-NMR spectra (open circles).

Figure 3. ^{2}H-NMR spectra of biological fluids. **A**. mitochondrion isolated using D_2O-based protocol. **B**. mitochondrion isolated using D_2O-based protocol. **C**. Aqueous extract of the rat liver homogenate. **D**. Aqueous extract of the rat liver homogenate with sodium azide added.

Figure 4. Various pathways of the metabolite conversion in cytosol and mitochondrion of rat liver at which hydrocarbon skeleton of resulting compounds can be deuterated.

Figure 5. Time course of changes in the ^{2}H-NMR spectra of mitochondrion samples with added antibiotics. A. Spectrum is taken on sixth day after the sample preparation. B. Spectrum is taken on second day after the sample preparation. C. Spectrum is taken 8 hours after the sample preparation.

Figure 1

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^{1} H-NMR spectra of biological fluids. **A**. mitochondrion isolated from rat liver. Measurements were done immediately after mitochondrion isolation. **B**. mitochondrion isolated from rat liver. Measurements were done one day after isolation. **C**. Aqueous extract of the rat liver homogenate.

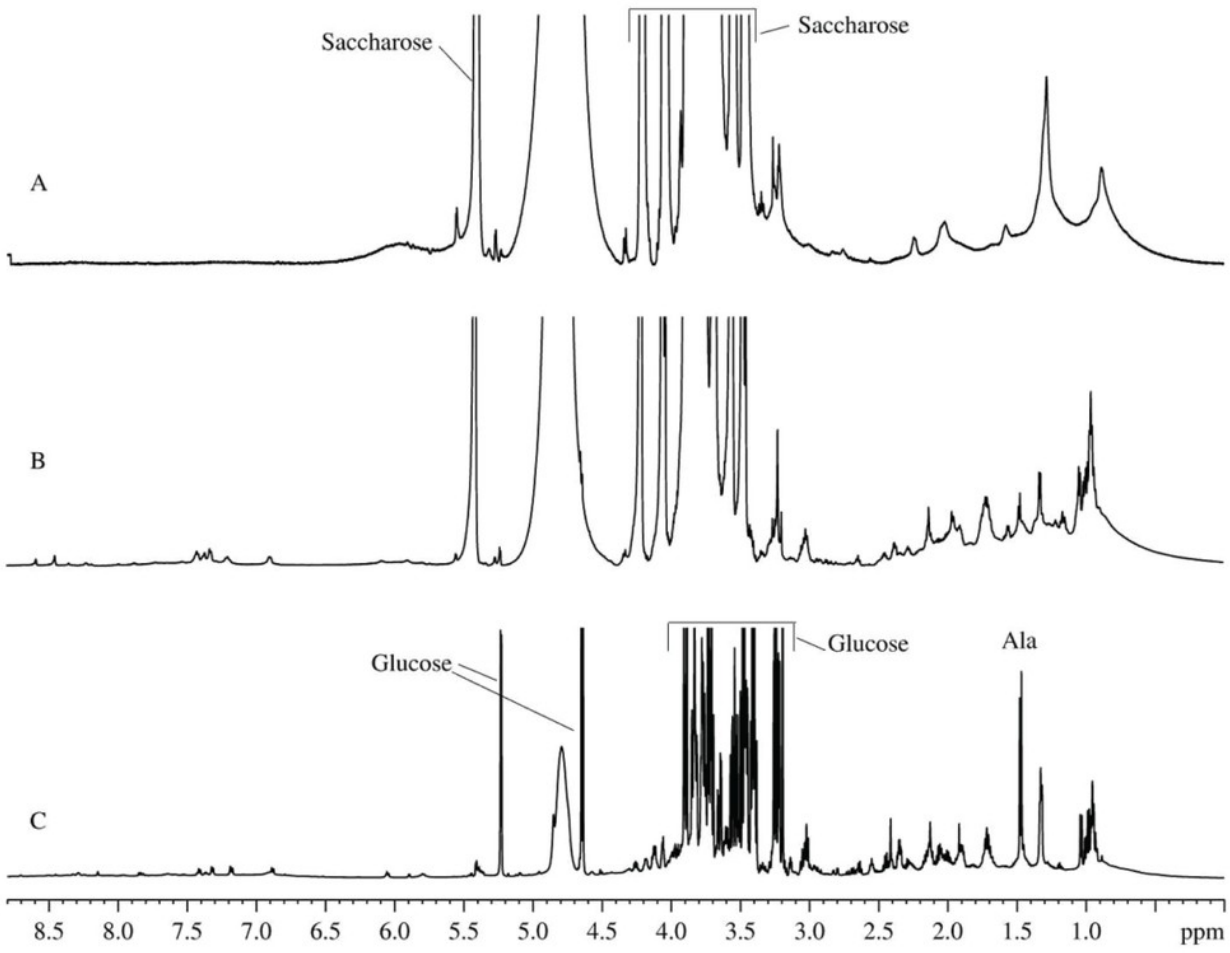

Figure 2

Figure 2

Time courses of changes in the integral intensities of the aliphatic part of ^{1}H-NMR (black circles) and ^{2}H-NMR spectra (open circles).

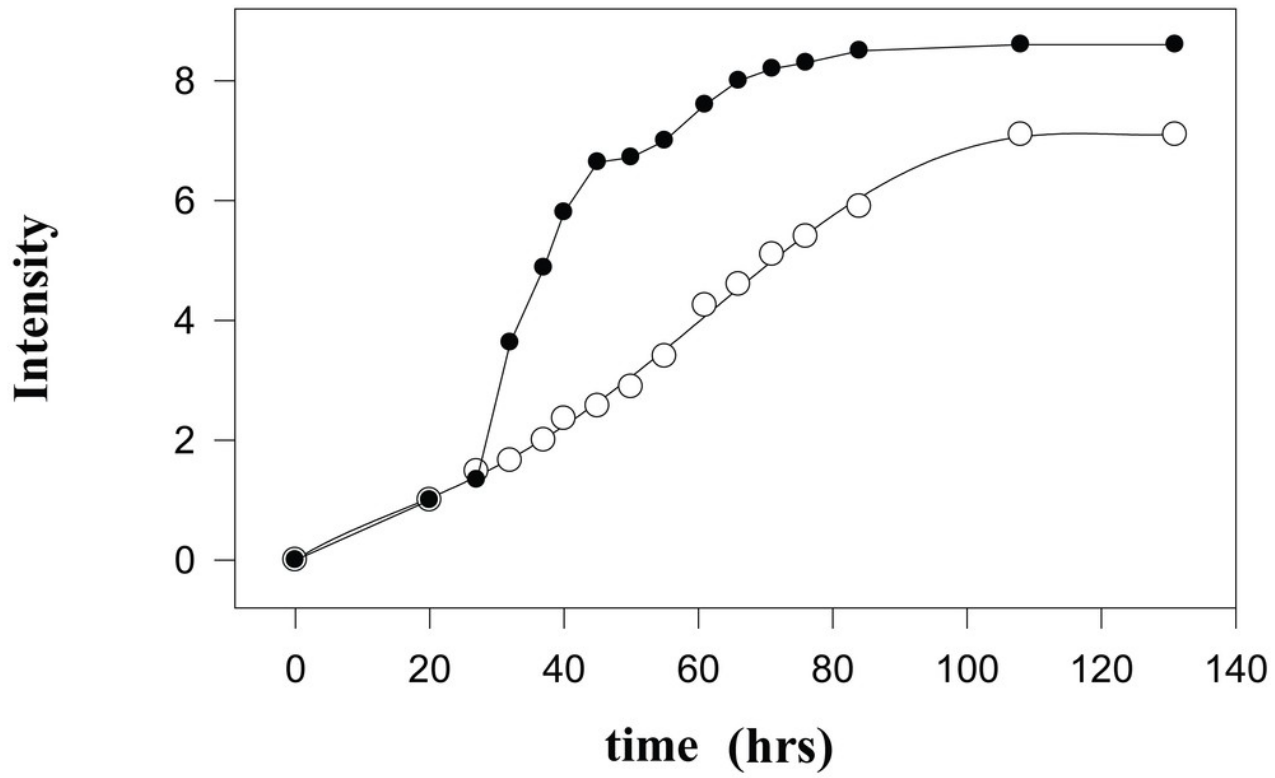

Figure 3

Figure 3

^{2} H-NMR spectra of biological fluids. **A**. mitochondrion isolated using D_2O-based protocol. **B**. mitochondrion isolated using D_2O-based protocol. **C**. Aqueous extract of the rat liver homogenate. **D**. Aqueous extract of the rat liver homogenate with sodium azide added.

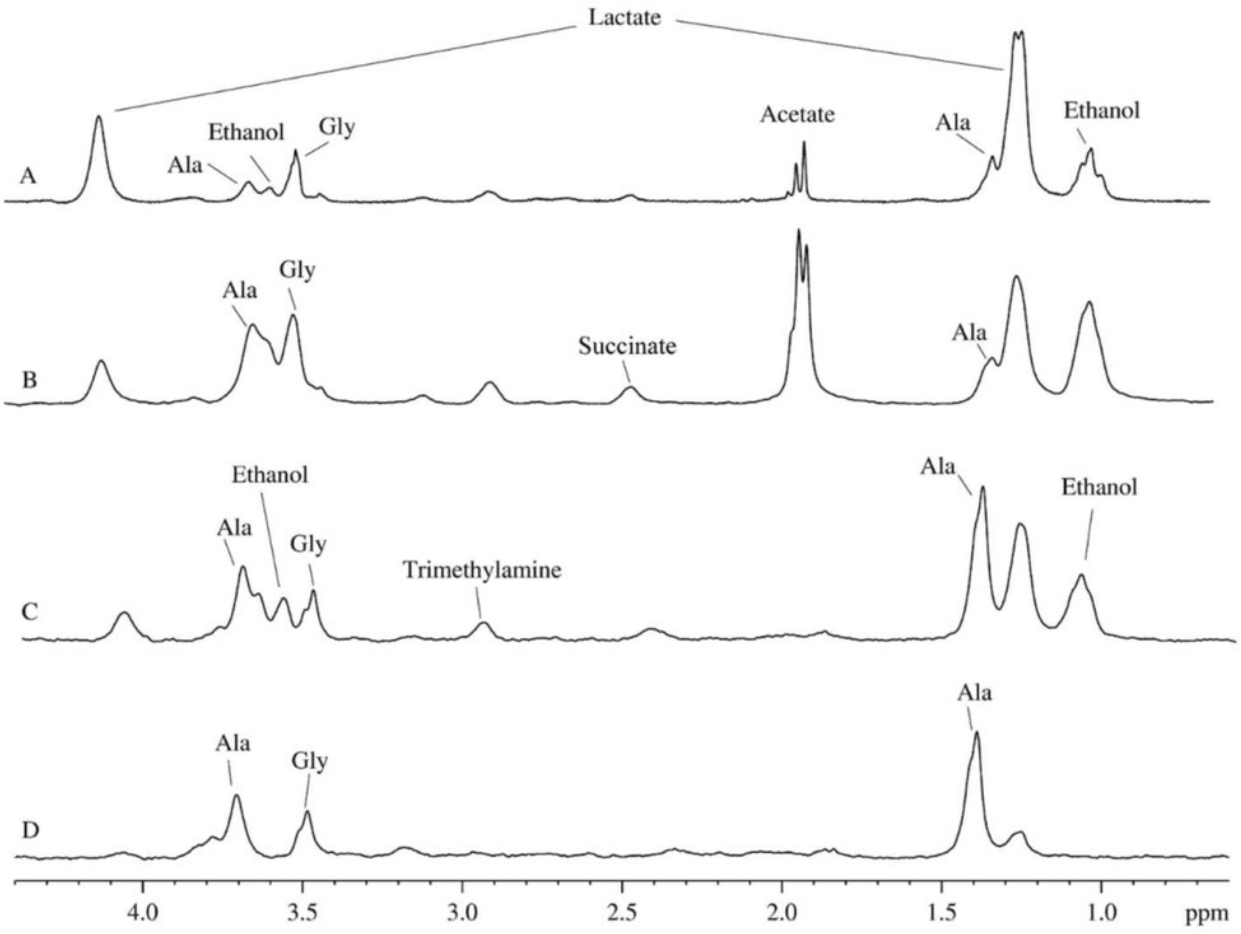

Figure 4

Figure 4

Various pathways of the metabolite conversion in cytosol and mitochondrion of rat liver at which hydrocarbon skeleton of resulting compounds can be deuterated.

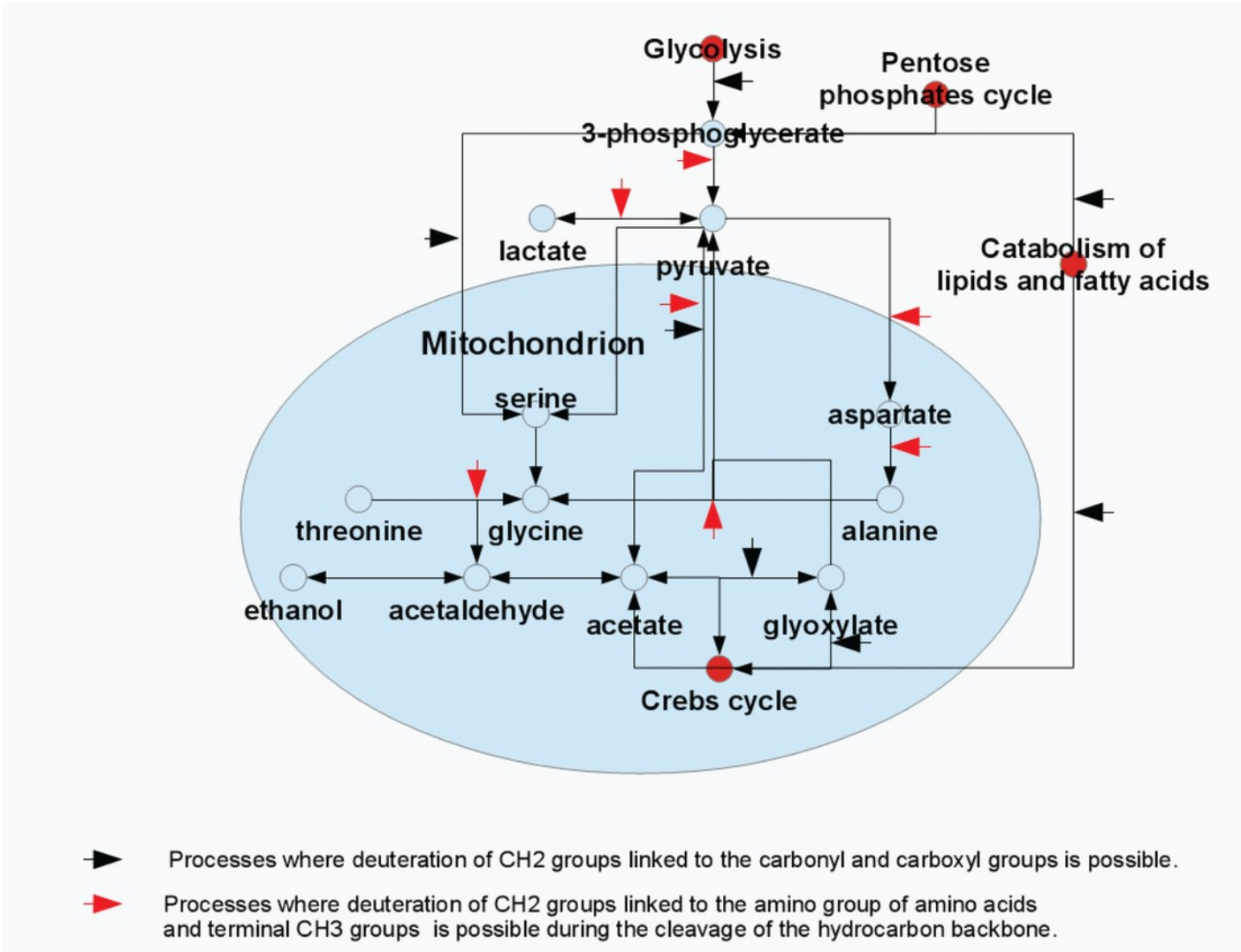

Figure 5

Figure 5

Time course of changes in the ^{2}H-NMR spectra of mitochondrion samples with added antibiotics. A. Spectrum is taken on sixth day after the sample preparation. B. Spectrum is taken on second day after the sample preparation. C. Spectrum is taken 8 hours after the sample preparation.