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Valery Ivanovich Petukhov
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INTRODUCTION

The term ‘homeostasis’ (the Greek for ‘staying the same’) in relation to biological systems of self-regulation means a state of unstable (dynamic) equilibrium where the main semantic component is the variability (mobility) of a process exposed to regulatory (homeostatic) factors. The main task of such factors is to keep this inconstancy to a minimum. Therefore, variability (dynamism) is the most essential feature of any homeostasis.

The reliability of self-regulation can be judged by deviations of system parameters from an average value: their minimum size indicates the maximum degree of homeostatic control.

There are two approaches for assessing metal-ligand homeostasis (MLH) in individual and population studies that are far from unambiguous in their practical value and theoretical justification.

One of them, which can be called dynamic, is based on the idea of homeostasis as a constantly changing (dynamic) process. This idea underpins the modern method of assessing the homeostasis of electrogenic metals (EM) whose clinical use is fully justified (although the accuracy of results is far from impeccable). The essence of the method is to determine concentrations of Na^+, K^+ and Ca^{2+} in blood plasma, which for each of these ions should not go beyond a narrow range of normal values. For sodium ions, it is 135-145 mmol/l, for potassium - from 3.6 mmol/l to 6.3 mmol/l and calcium - from 2.23 mmol/l to 2.57 mmol/l.

It should be noted that making a reliable judgment on homeostasis does not necessarily require knowing the total content of metals in the body. It would suffice to have information on the dynamics of the concentration values of EMs in the extracellular fluid. This can be explained by the participation of Na^+, K^+ and Ca^{2+} ions in the work of organ systems consisting of electrically excitable tissues. Such participation implies reliable homeostatic control of the plasma level of EM during the joint work of many organs (cerebral cortex, hypothalamus, pituitary gland, adrenal cortex, kidneys, lungs, stomach, intestines), hormonal systems and working hormones: aldosterone, renin, ACTH, ADH, etc.

In recent years, it has become popular (along with the described dynamic approach) to quantify MLH by the content of metals in hair. Moreover, such

an assessment receives the greatest confidence from those researchers who for the most part are not clinicians.

The non-invasive nature, the possibility of long-term storage of samples, as well as the use of modern high-tech methods of spectrometric analysis: atomic absorption spectrometry (AAS), plasma atomic emission spectrometry (ICP-AES), plasma mass spectrometry (ICP-MS), etc., provided by the approach is undeniably attractive.

It is no coincidence that spectrometric analysis of hair for the assessment of the elemental status of the whole body (not only electrogenic but also other metals and non-metals) was willingly adopted by commercial medicine in many countries. In Moscow, for example, more than a dozen of medical centres emerged where the elemental status of the whole body is judged by the results of hair spectrometry.

The second (quantitative) approach is based on the belief of its proponents that the content of metals (and/or other chemical elements) in a biosubstrate such as hair can be used to judge the homeostasis of these minerals in the whole organism.

It is not excluded that the emergence of such a belief was unwittingly promoted by modern spectrometric analysis technologies themselves, the availability and accuracy of which had a "hypnotic" effect on researchers. Otherwise, it is difficult to explain the *a priori* existence (without preliminary evidence) of the above-mentioned belief among the apologists of the quantitative approach that MLH in the epidermis (hair) is identical to MLH in the whole organism.

It is, of course, legitimate to use the level of chemical elements in any particular biosubstrate to assess the elemental status of the whole organism but only on the condition that you know the answer to the key question: Is it possible to extrapolate the results of the analysis to the whole organism or one should limit the assessment of bioelemental homeostasis to the given substrate alone? There is still no definitive answer to this question in relation to the epidermis and its derivative (hair). But there are serious objections to such extrapolation that require discussion.

Firstly, according to the data of atomic emission spectrometry, concentration values of chemical elements (including but not limited to metals), which are contained in hair, show expressed individual variation: Coefficient of Variation (CV) averages 125.5±17.5% [24]. This very fact suggests that the observed shifts may be caused not so much by ‘hypo- or hyperelementosis’ as by *redistribution* of chemical elements mediated by intra- and extracellular regulators of transmembrane mineral traffic which has practically no effect on the total body elemental composition.

Secondly, evaluation of the elemental status of the cutaneous appendages (as part of the excretory system) must be very accurate when diagnosing impairments in MLH at the level of the body as a whole. Such evaluation is required in cases when the object of study is an excretory product (e.g., urine). Ambiguity in interpretation of results of elemental analyses of biotic substrates of the excretory system (sweat, urine, expired air, epidermis and its appendages) may appear in case of failures in the systems responsible for the 'containment' of essential metals in the body (e.g., heme Fe). As is known, a small (in size) molecule of free (unbound) hemoglobin could easily pass the renal filter and show up in urine (hemoglobinuria). However, it does not happen normally due to the fact that hemoglobin is bound by a molecule of transfer protein, i.e. haptoglobin. The newly formed complex of 'hemoglobin-haptoglobin', because of its sizes, is no longer capable of penetrating through the basal membrane pores, thereby retaining a heme iron pool for resynthesis of hemecontaining proteins.

Under chronic (and often latent) hemolysis, hemoglobin iron may escape from the body with urine in the form of hemosiderin (lead intoxication, Marchiafava-Micheli disease). But can it be an evidence of the increased Fe level in the body? Certainly not! One can rather speak about an inevitable reduction in the total ferrum pool (due to continuous hemosiderinuria). Similar examples may be given with regard to other metals as well.

There are lots of factors to be considered as the most probable causes of quantitative shifts in MLH. Their distinguishing feature is the capacity of activation or deactivation (up to a total block) of ionic channels – hydrous pores of transmembrane proteins, which are in charge of metals transfer. Depending on the way of activation, the ion channels are classified into stretch-activated, voltage-activated and ligand-activated. Activation of ligand-activated channels may take place due to redox-modification of thiol groups of cysteine in the molecule of proteins-transporters. Among the latter ones is the P-type ATPases superfamily, which ensures the transportation of not just electrogenic (Ca, Na, K) but also heavy metals (Cd, Zn, Pb, Cu, Co, Ag) [2-4].

To be objective, it is not the matter of 'trust' to the biosubstrate but the invalidity of extrapolations of data of trace element analysis of hair to the whole body. In other words, the problem is not with the substrate but with interpretation of MLH variations in epidermal cells registered by spectrometry.

The number of publications on this topic that have appeared in recent years (monographs, journal articles, scientific reports) is so large that it is

problematic to cite them in this review. Let us only refer to those that deserve attention [1, 14, 23, 8, 13, 18].

All these works are united by the total assurance of their authors on the adequacy of their chosen criterion (the content of one or another mineral in the hair) for the integral assessment of elemental homeostasis in the whole organism. But what is the basis for such reliance if no necessary evidence is provided to justify this extrapolation?

The inconsistency in quantifying MLH is most easily shown by the example of EM homeostasis, which is, in fact, the objective of this book, although these arguments may also be true with respect for other metals.

At the same time, the monograph presents evidence of homeostatic control of metal traffic at the epidermis level. The explanation of the mechanisms of such control (at least hypothetical) turned out to be possible within the framework of the main provisions of the theory of self-organised criticality (SC), which, according to the authors, allows us to attribute MLH in the epidermis to SC-phenomena.

The authors are grateful to the staff of the Center for Biotic Medicine (Moscow), and first of all to A.R. Grabeklis, for kindly providing a NexION 300D hair mass spectrometry database (Perkin Elmer Inc., Shelton, CT, USA) for Na, K, Ca and other metals.

CHAPTER 1

IS THE LEVEL OF METALS IN THE EPIDERMIS A CRITERION FOR MLH?

1.1. Homeostasis of electrogenic metals (Na, K, Ca) in the human body

It is known that homeostasis of electrogenic metals (EMs) *in toto* occurs in the aquatic environment. Therefore, it is appropriate to give some details of water metabolism in the body.

In humans, the volume of water ingested with food (~2.2 l) and formed as a result of metabolism (~0.3 l) totals ~2.5 l. This basically corresponds to the level of excretion of H_2O (with sweat ~0.6 l, exhaled air ~0.4 l and urine ~1.5 l). These indicators are significantly influenced by the ambient temperature, a rise in which results in an increased water loss from the body and higher water intake (due to thirst). Therefore, the indicated volume of water supplied with food (~2.2 l) should be referred to t°=18°-20°C.

In total, the body of a middle-aged individual, whose body weight is ~70 kg, contains ~42 litres of water (64% inside the cells and 36% in the extracellular space) [21].

The extracellular fluid itself (~15 l) is: a) blood plasma consisting mainly of water (~90%) with a small admixture of chemical ingredients (proteins, ions of electrogenic and other metals, Cl^-, HCO_3^-, etc.); b) interstitium fluid (~12 l or ~17% of body weight), which is similar in chemical composition to plasma but contains less protein; c) transcellular fluid (inside the eye, in the articular and serous cavities, in the gastrointestinal tract, kidneys), which is ~2.5% of the total water volume or ~1.5% of body weight; d) water of crystallization in bone and cartilage tissues (~1.5% of the total amount of H_2O).

The intracellular fluid (~27 l), containing low concentrations of Na^+, Ca^{2+}, HCO_3^-, Cl^- ions (and high concentrations of K^+, protein, organic phosphates) is directly involved in all processes of cellular metabolism, many of which are not completely investigated yet.

EMs, along with other electrolytes, determine the osmotic concentration (osmolality) of the aqueous medium. And the cerebral cortex and, in particular, the hypothalamus (having special osmoreceptors) are very sensitive to changes in osmolality by the feedback mechanism. Therefore, EM homeostasis and water balance, which is more correctly called water-electrolyte, have common mechanisms of homeostatic regulation, including, as already mentioned, many organs and hormonal systems.

The sequence of events occurring in this case is well known: the excess of water losses over its intake leads to an increase in plasma osmolality and activity of the hypothalamic centres responsible for thirst and production of antidiuretic hormone (ADH) by the pituitary gland. This stimulates intake and ADH-dependent fluid retention (due to increased tubular reabsorption of Na^+). The involvement of EMs in this process deserves a more detailed discussion.

The human body (regardless of gender) contains ~3000 mmol of sodium: 70% is the exchange pool, and 30% is in the bone tissue [21]. The extracellular fluid, as already mentioned, normally contains 135-145 mmol/l of sodium, while it is only 4-10 mmol/l in the cells. Na^+ ions are directly involved in the regulation of water and electrolyte balance, being the "main osmotic ions" of the extracellular space, where they make up 90% of all other ions. This is facilitated by the high sensitivity of the hypothalamic osmoreceptors which can be judged by a narrow range of fluctuations in the normal level of Na in plasma and by a change in the volume of extracellular fluid in response to sodium excretion within only 1% (!). It is known that 85% of sodium is excreted in the urine (with a balanced Na diet), and only 15% is excreted through the skin (sweat glands).

The content of K^+ (the main intracellular cation) in the body of an adult weighing 70 kg is ~3500 mmol. And although 90% of potassium is in free form and only 10% in bound form (erythrocytes, brain, bone tissue), the actual size of the exchange pool, due to the predominantly intracellular localization of K^+, is rather modest. In the extracellular space is only 2% of the total amount of potassium (50-60 mmol). The concentration of K^+ in the cytosol is ~110 mmol/l, in the extracellular fluid ~4 mmol/l. However, it should be borne in mind that the total volume of water in the extracellular space is almost half that in the cytosol [21].

Also significant is that even a slight disturbance of the normal fluctuations in the level of K in plasma (for example, a decrease below 3.0 mmol/l) can cause serious disturbances in cardiac activity.

The polar difference in the predominant localization of the Na^+ and K^+ cations relative to the cell membrane is combined with the opposite direction of their electrochemical gradients: for Na^+, it is inside the cell, and

for K^+, it is outward. The separation of cations relative to the cell membrane and the preservation of this “inequality” becomes possible due to the constant work of the ATP-dependent membrane pump - Na^+/K^+-ATPase, which pumps K^+ ions into the cell and removes Na^+ ions (in a ratio of 2:3 resp.) The resulting potential difference on opposite surfaces of the membrane (membrane potential) makes it possible to consider Na^+/K^+-ATPase as electrogenic (along with EMs).

The content of calcium in the human body is noticeably higher than that of other metals (25000 mmol or 1000 g per 70 kg of body weight). 99% of Ca is in the bones of the skeleton and only 22.5 mmol is in the extracellular fluid, including ~9 mmol in blood plasma. The exchange between cellular (bone) and extracellular calcium (tissue fluid, plasma) is very intensive (~500 mmol/day). Daily losses of Ca through the kidneys (~2.5 to 7.5 mmol), the intestine and skin with its appendages, along with the intake of ~12.5 mmol Ca per day (the necessary minimum, which increases during growth period, pregnancy, lactation), account for the fact that the extracellular pool of Ca is renewed about 33 times a day(!). It is clear that such an intensive "circuit" complicates the estimation of the true content of this metal in the body, especially when there is only one indicator, such as the level of calcium in plasma, which can grow with massive osteolysis (plasmocytoma, multiple bone metastases, etc.).

There is very little of this metal inside the cell - only 50 nmol/l - several orders of magnitude less than in plasma or interstitial fluid, where $[Ca^{2+}]$ ~2.5 mmol/l. The impressive “difference” of the electrochemical gradient that exists in Ca^{2+}, which is necessary for the generation of an action potential, is mainly provided by the actively working membrane Ca^{2+}-ATPase due to the energy of ATP molecules.

Thus, EM homeostasis, closely related to the processes in the nervous (excitation-inhibition) and muscle (contraction-relaxation) tissues, as well as the regulation of water and electrolyte balance, already has its own evaluation criterion. This is the concentration of metals in the extracellular fluid (plasma), which has proven to be quite appropriate for clinical use. True, if you rely only on the results of this test, you can make an incorrect conclusion about the homeostasis of EMs. Therefore, additional laboratory studies should be carried out in parallel (plasma osmolality and osmolarity, the level of the main plasma anions - chlorine and bicarbonate, the volume of extracellular fluid, the level of protein, glucose, urea, etc.). Of particular value are clinical data (including case history), as well as the experience and erudition of the doctor. All this allows minimizing the number of diagnostic errors but does not reduce the significance of the analysis of the concentration of EMs in plasma as a criterion for the homeostasis of these

metals *in toto*. To support this point, let us remind you of a very modest spread in each of the EMs of the normal values of their plasma concentration. Let us now address the results of hair spectrometry in healthy individuals taking the scatter of normal values as a criterion for the stability or balance of EM homeostasis.

1.2. Quantitative analysis of electrogenic metals in epidermal cells (hair)

At the Centre for Biotic Medicine (Moscow), measurements of the level of sodium (Na), potassium (K) and calcium (Ca) in hair were made using inductively coupled plasma mass spectrometry (ICP-MS) on a NexION 300D spectrometer (Perkin Elmer Inc., Shelton, CT, USA).

Under observation were practically healthy residents of Moscow aged 20 to 49 years (n=9991). Among them, 4999 (50.04%) are men and 4992 (49.96%) are women. It should be emphasized that all these measurements were carried out in practically healthy individuals with no pathological symptoms. At the same time, the spread of individual values of metals was impressive: for Na, from 0.645 μg/g to 9240 μg/g; for K, from 0.045 μg/g to 6505.1 μg/g; for Ca, from 15.5 μg/g to 19338.9 μg/g. When detecting possible gender-based distinctions in the mineral composition of hair, we found that the level of calcium (Ca) had depended on the gender. Therefore Ca concentration values were registered for males and females separately. Ca content in hair differed significantly between the genders: 15.5 μg/g to 4633.7 μg/g in males, and 107.1 μg/g to 19338.9 μg/g in females [25].

We performed verification of the normal distribution hypothesis by the Jarque-Bera Test [6] and the Kolmogorov-Smirnov Test [19]. As a result of such verification, we managed to disprove, with a higher probability, the hypothesis of normal distribution of EMs (Na, K, Ca).

At a significance level of α=0.05 (confidence level 0.95), the following estimates were obtained in Mathcad software using the X^2 criterion.

Sodium (Na)
a) sum of X-distributions: **8.003**;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Potassium (K)
a) sum of X-distributions: **404.544**;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Calcium (Ca)
a) sum of X-distributions: **8.022**;
b) quantiles of distributions: **7.108** и **7.585**.

Conclusion: the hypothesis of normal distribution cannot be accepted.

Likewise, impressive was the dispersion of individual spectrometry data values that we found in EM and other chemical elements, whose coefficient of variation (CV) ranged from 34.1% to 226.5% (on average, 125.5±17.5%). Moreover, when we were verifying the hypothesis of the spectrometry data distribution normality, we found that the spectrometry data of 25 chemical elements including EMs, had not been distributed in accordance with the normality law. Later on, we did manage to show that the distribution of concentration values was of fractal nature. Therefore, we used alternative approaches (*bootstrap methods*), not requiring normal distribution of *a priori* assembly [9].

Here explanations are required for the spectrometric data, expressed in μg of metal per 1 g of hair. What is behind these numbers? Taking into account the universality of cellular organization, it can be assumed that:

1) for sodium, this is the amount of metal that is located mainly outside the epidermal cell (interstitial fluid), where $[Na^+]$ is ~10 times higher than $[Na^+]$ in the cytosol;
2) for potassium, this is mainly an intracellular pool, because $[K^+]$ in the cell ~30 times higher than extracellular $[K^+]$;
3) $[Ca^{2+}]$ in the cell is so small (50 nmol/l) that the level of calcium in the hair, which the spectrometer shows, actually reflects the size of the extracellular pool of this metal.

How can we explain such “tolerance” (insensitivity) of epidermal cells to pronounced (several orders of magnitude!) quantitative shifts of Na, K, and Ca, which predominantly occur (with the exception of potassium) in the interstitial space of the epidermis? Recall that much more modest fluctuations in the plasma level of the same metals are fraught with serious (fatal) “failures” in the operation of the most important functional systems.

While answering this question, we cannot ignore the fact that epidermal cells, unlike neurons and myocytes, do not have electrical excitability. And the physiological processes in the nervous and muscular tissues with the participation of EM are far from equivalent to those in the epidermis, which, apparently, lacks special “alarm receptors” (similar to the hypothalamic

osmoreceptors), which are highly sensitive to disturbances in EM homeostasis. It cannot be ruled out that throughout the entire period of evolution, tissues that do not have electrical excitability simply did not need this kind of signal structures.

The final conclusion from these considerations can be as follows:
The observed changes in the EM content in the hair, which are not accompanied by any pathological symptoms and do not affect the health of the individual, cannot be used as a criterion for assessing EM homeostasis at the level of the whole organism and should be considered as probable signs of the homeostasis at the local level.

It seems appropriate to cite here the results of testing the hypothesis of normal distribution of other metals (Al, Cd, Fe, Cr, Cu, Li, Pb, V, Zn). The hypothesis of normal distribution could not be confirmed for any of these metals.

At the significance level α=0.05 (confidence level 0.95), the following estimates were obtained in the Mathcad programme using the X^2 criterion.

Aluminium (Al)
a) sum of X-distributions: **8.671**;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Cadmium (Cd)
a) sum of X-distributions: ∞;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Iron (Fe)
a) sum of X-distributions: **64.649**;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Chromium (Cr)
a) sum of X-distributions: **130.836**;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.
Copper (Cu)
a) sum of X-distributions: ∞;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Lithium (Li)
a) sum of X-distributions: ∞;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Lead (Pb)
a) sum of X-distributions: **1.234x10^{5}**;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Vanadium (V)
a) sum of X-distributions: **4.403x10^{8}**;
b) quantiles of distributions: **7.108** и **7.585**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

Zinc (Zn)
a) sum of X-distributions: **7.96**;
b) quantiles of distributions: **6.648** and **8.087**.
Conclusion: the hypothesis of normal distribution cannot be accepted.

1.3 Na/K-homeostasis in the epidermis: relationship with cell bioenergetics

The living cell, as an open dynamic system, depends on energy from outside. However, without preliminary conversion of this energy into reversible forms (ATP, Na^+, H^+) that easily cope with entropy, the cell would not be able to find a rational use for it. In this connection, regulation of the intracellular energy balance (energy expenditure in accordance with adequate energy recovery) is undoubtedly relevant.

EM homeostasis in regenerating tissue cells (epidermis) has a direct connection with cellular energy. At the same time, the key homeostatic mechanisms controlling the level of metals, most likely, should be sought in the peculiarities of intracellular energy transfer, since the existence of common regulatory mechanisms cannot be excluded here.

A possible approach to help analyze successful energy regulation (in micro- as well as in macrosystems), which can help analyze successful energy regulation (in micro- as well as in macrosystems), is provided by the theory of '*self-organized criticality*' (SC) [5]. The theory may be used to explain the statistics of earthquakes (the Gutenberg-Richter earthquake law), numerical patterns of urban growth and urban population, word usage frequency distribution in speech and literary texts (the Zipf's law), fractal

geometry, functioning of neural networks of the brain and other micro- and macrocosmic events, which are referred to as SC-phenomena.

The most visual model of SC, which has become some kind of a "brand" of the theory, is a pile of sand formed by constant addition of minimal quantities of sand. The dynamic relationship between the grains of sand in the pile, resulting in "avalanches" of various calibers is characterized by the power-law of correlation between the size and number of avalanches within a given interval of measurements. Detection of power-law dependence, irrespective of the sizes of objects under study (fractality criterion), helps identify SC-phenomena in a variety of dynamic systems.

Therefore, it is of interest to detect the power-law connection between the EM content in epidermis (spectrometry) and the number of individuals belonging to a given range of quantitative estimates. It is clear that such studies should be carried out on a sufficiently extensive statistical material.

Using mathematical statistics (for more details on the mathematical methods used, see Chapter 5), we have analyzed the results of atomic emission spectrometry of hair samples for Na, K, and Ca content, which were obtained at the Center for Biotic Medicine (Moscow) from 10297 healthy subjects – Moscow and Riga residents (5160 males and 5137 females) aged 2 to 85.

The mathematical substantiation of numerical operations with spectrometric analysis data is given below.

Suppose we have a set of samples $x_1,...,x_n$ of the random variable $x \sim F_x(t)$ and the series of order statistics $x_{(1)} \le x_{(2)} \le \ldots \le x_{(n)}$ is constructed on the basis of this set. The point estimate of the median m of the distribution ($F_x(m) = 0.5$) is a sample median as follows:

$$\tilde{m} = \begin{cases} x_{(k)}, & n = 2k-1; \\ \dfrac{(x_{(k)} + x_{(k+1)})}{2}, & n = 2k. \end{cases}$$

This assessment provides a numerical estimate of the median but gives no indication as regards its accuracy and reliability. The sample median is determined by finite sampling of the population, and it is essentially a random variable. Thus, it may strongly differ from the true median value even with the arbitrarily large *n*. Interval estimations allow us to specify the limits, within which the required parameter lies with a certain probability $p = 1-\alpha$. The search for the interval estimate of median $P(m_{lo} < m < m_{up}) \ge 1-\alpha$ implies the estimation of the sample median distribution. If $n > 50$, one can assume that $m_{lo} = x_{(\lambda)}$ and $m_{up} = x_{(n-\lambda+1)}$,

where $\lambda = 0.5\left(n - \tau_{1-\alpha/2}\sqrt{n} - 1\right)$, $\tau_{1-\alpha/2}$, is a quantile of the standard normal distribution $N(0,1)$.

An alternative approach to building confidence intervals of the sample median is based on the method of repeated samples. The point estimate is just a single realization of the random variable. To get an idea of its distribution, one must have an ensemble of realizations. For this purpose, various artificial methods are used. In this study, we used a technique known as "bootstrapping". It is based on the basic conception of mathematical statistics - the sampling distribution. The method can be formulated as follows.

Assuming the available sample of length n constitutes the general population with the discrete distribution $P(\xi = x_i) = 1/n$, k random samples with replacement of length n are taken from the general population. As a result, we get samples consisting of elements of the original sample, and, since it is the sampling with replacement, there may be repetitive elements. Since the selection is done randomly and the population does not change, any repeated bootstrap samplings can be considered as independent.

The standard approach, which is used for the construction of confidence intervals for the estimated parameter for the specified α, is formulated as follows:

1. Construct an ensemble $\{\tilde{m}_{boot}(i)\}_{i=1}^{k}$ of estimates of the parameter m using repeated bootstrap samples.
2. Construct a series of order statistics $\tilde{m}_{(1)} \le \tilde{m}_{(2)} \le \ldots \le \tilde{m}_{(k)}$.
3. Construct an empirical function of percentiles. Each $\tilde{m}_{(i)}$ is interpreted as a percentile $\frac{i-1/2}{k}\cdot 100$. For the remaining points, the linear interpolation is applied.

Thus, the lower and upper confidence intervals are defined by the following relationships respectively:

$$m_{lo} = \tilde{m}_{(i)} + (\tilde{m}_{(i+1)} - \tilde{m}_{(i)})(n\alpha/2 - i + 1/2),$$
$$m_{up} = \tilde{m}_{(j)} + (\tilde{m}_{(j+1)} - \tilde{m}_{(j)})(n(1-\alpha/2) - j + 1/2).$$

If $\frac{\alpha}{2} < \frac{1-1/2}{n}$, then $m_{lo} = \tilde{m}_{(1)}$ and $m_{ip} = \tilde{m}_{(k)}$.

The energetical capacity of human mitochondria, the main producers of ATP, is not the same throughout the life of an individual. Reduced ATP production in early childhood, when mitochondrial network formation is still in progress, is followed by increased energy production at puberty age achieving its maximum during the active working age (18 to 45 years). As the body ages, there comes a progressive decrease in the number of mitochondria associated with the destructive action of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which ultimately leads to a decrease in the intensity of ATP synthesis [7].

Assuming that K^+ and Na^+ participate in cellular energy exchange, we may be interested not only in the possible age-related changes in the content of the said metals in epidermis, but also in comparing the metals' spectrometry data with mitochondrial activity during different age periods. Therefore, using the hair spectrometry data, we found the median levels of Na and K in different age groups. The results are shown in Table 1 and Figures 1-2.

Table 1. Age-related dynamics in K and Na levels in epidermis

Age	Median K (μg/g)	CI boot low	CI boot up
60-85 years	121.4	87.97	161.5
50-59 years	104.3	95.37	117.56
30-49 years	56.06	53.4	58.46
20-29 years	38.18	35.7	41.04
10-19 years	54.4	41.2	84.8
2-9 years	376.88	231.9	97226

Note: CI – confidence intervals; boot – bootstrap method.

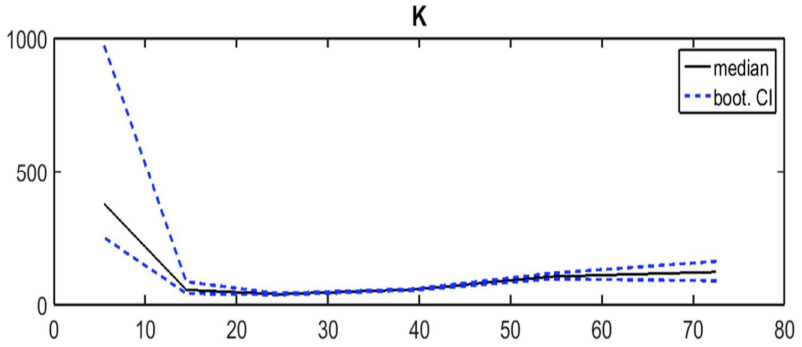

Fig. 1. Age-related potassium levels in epidermal cells of health subjects (n=10297). The X-axis gives age (years); the Y-axis shows the K levels (µg/g). The dotted lines represent confidence intervals (the bootstrap-method).

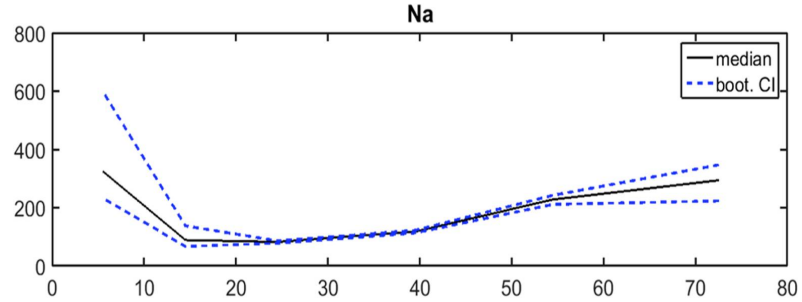

Fig. 2. Age-related sodium levels in epidermal of healthy subjects (n=10297). The X-axis gives age (years), the Y-axis – the Na levels (µg/g). The dotted lines represent confidence intervals (the bootstrap-method).

The age-related dynamics of Na and K content in epidermis shows two periods of significant increase in the level of metals: the first – at the age of 2 to 9 years, the second - after 50 years (Fig.1-2). How can we explain such changes in the EM content?

It seems reasonable to attribute the said changes to the abovementioned insufficient ATP production in childhood and old age. Sodium potential, as a convertible energy "currency", seems to be able of compensating the lack of ATP in the aforesaid age periods. Such increased production of ROS/RNS in elderly and senile persons should lead to more intense activity of membrane pumps (primarily, Na^+/K^+-ATPases) due to oxidative modification and/or S-nitrosylation of these proteins. In this case ROS/RNS

play an important role of signaling molecules that help maintain the required level of power cell and (consequently) an increase in life expectancy of an individual.

The decisive role in prolonging the life of an individual cell and the whole organism is played not only (or rather, not so much) by the presence or absence of a pro-oxidant shift in the redox status, but also by the preservation (as the main condition) of the energy resource at the cellular or organismal level. And if this goal is served by the sodium potential, which is capable of interconversion into other "energy currencies" (ATP and H^+) and in the realization of which ROS/RNS and Na^+/K^+-ATPase activity are involved, then it is reasonable to expect a pro-oxidant shift and an increase in the sodium level in ageing cells of the organism.

This explains the known ability of ROS/RNS to accumulate in old age and calls into question the universality of the free-radical theory of aging, in which free radicals are considered only as the main factors of aging [11] and approaching death, and not as possible prolongers of life.

It is not quite clear what stimulates Na^+/K^+-ATPase activity in children, although there are reports about pro-oxidant shifts in the redox status of children 7-11 years old, which are explained (according to the authors of the reports) by the deficiency of natural antioxidants and adaptation shifts [12].

1.4 Linear relationship between [Na] and [K] in hair

The conclusion about the possibility of synchronous (critical) functioning of Na^+/K^+-ATPases was made by us after we had established a reliable and stable linear relationship (Pearson) between the concentration values of these metals (according to the spectrometry data) [24]. However, the coefficient $r_{K\text{-}Na}$, which was on average 0.6, looked too "modest" for synchronous (critical) operation of membrane pumps. Therefore, it was interesting to find out what leads (directly or indirectly) to a possible "lowering" of the $r_{K\text{-}Na}$ level

It is known that an important parameter of sodium and potassium homeostasis in the human body is the ratio of $[Na^+]$ and $[K^+]$ concentrations inside the cell and in the extracellular medium. Normally, when the volume of water inside the cells is 28 liters (and in the whole organism is 42 liters), the intracellular content of potassium is 110 mmol/l, whereas outside the cell it is only 4 mmol/l. The distribution of sodium (under the same conditions) is the opposite: 135 mmol/l outside the cell, and only 10 mmol/l Na inside the cell [21]. Based on these figures, the total (intra- and extracellular) ratio of $[Na^+]$ / $[K^+]$ in the whole body is ~1.3.

It is significant that the ratio of the average values of [Na] and [K] (bootstrap-method), according to our data, in such a substrate as hair in 947 healthy individuals was almost identical – 1.5 [12].

Determination of sodium (Na) and potassium (K) in hair was done in a laboratory of the Center for Biotic Medicine (Moscow) using mass spectrometry with inductively coupled plasma (ICP-MS) on a NexION 300D spectrometer (Perkin Elmer Inc., Shelton, CT, USA). Practically healthy Moscow residents aged 20 to 49 were under observation (n = 9991), of which 4999 (50.04%) were males, and 4992 (49.96%) – females. Hair samples for the spectrometry study were taken from the subjects following a mandatory informed consent procedure. In the occipital region, a tuft of hair 2 cm long and 0.5 cm thick was cut off close to the scalp. To minimize the possibility of environmental contamination, hair samples were washed with acetone and then rinsed thrice with deionized water with subsequent air drying at 60ºC. Further treatment of the samples was performed using microwave degradation. Specifically, 50 mg hair samples were introduced into a Teflon container and added to 5 ml of concentrated analytical grade HNO_3 (Sigma-Aldrich Co, St. Louis, MO, USA). Decomposition was performed in a *Berghof speedwave four* system (Berghof Products & Instruments, Germany) for 20 min at 170 -180ºC. After decomposition, deionized water was added to get a final volume of 15 ml.

A correlation analysis (Pearson) of the obtained data was carried out with determination of the correlation coefficient $r_{K\text{-}Na}$ (pairwise correlations between the concentration values of K and Na in the substrate). We tested the normal distribution hypothesis using the Jarque-Bera test [6] and the Kolmogorov-Smirnov test [19]. Because of this test, it was possible with high probability to refute the hypothesis of normal distribution of chemical elements. Therefore, an alternative approach (the bootstrap method) was used, which does not require a normal distribution of the priori ensemble [9]. The Matlab software tool was used for statistical data processing.

The distribution of individuals depending on the [Na]/[K] ratio was as follows: [Na]/[K] <1 was detected in 1834 subjects (18.4%); [Na]/[K] from 1 to 5 – in 6884 subjects (68.9%); [Na]/[K] from 5 to 10 – in 893 subjects (8.9%); [Na]/[K]> 10 – in 380 subjects (3.8%). The correlation coefficient ***r*** between [Na] and [K] was found in each of these groups. The results are presented in Table 2.

As shown in Table 2, the values of coefficient ***r*** were higher in all the four subgroups (regardless of the [Na]/[K] ratio value) as compared to the total sample.

It was interesting to find out which one of the presented subgroups contributed the most to the 'understatement' of ***r*** in the general sample as

compared to all the other subgroups. In that respect, the most 'suspicious' was subgroup 1 with the [Na]/[K] ratio<1, which fundamentally distinguished it from all the others.

Therefore, we decided to find out in what way the presence of subgroup 1 in the total sample affected the ***r*** coefficient value alone or in conjunction with each of the subgroups or any combinations thereof (see Table 3).

It is significant that the addition of subgroup 1 (where Na/K <1) to each of the three other subgroups (separately and in various combinations, see Table 3) resulted in a noticeable (almost twofold) decrease in $r_{K\text{-}Na}$. That does not seem accidental. It seems highly likely that it was the presence of individuals from subgroup 1 (Na/K <1) in the total sample that accounted for such an extraordinary 'humble' (0.61) value $r_{K\text{-}Na}$ for general sampling.

The synchronous (critical) mode of operation of Na^+/K^+ -ATPases, as we noted earlier, is combined with a high level of $r_{K\text{-}Na}$. Interestingly, the value of this parameter in subgroup 1 itself ($r_{K\text{-}Na}$ = 0.86; p <0,05) is indicative of synchronous operation of the Na^+/K^+ pump in individuals with an 'inverted' Na/K coefficient (< 1). This fact has little relation to the inhibitory influence of subgroup 1 on the $r_{K\text{-}Na}$, which requires further investigation of the possible causes of such an effect. In this regard, the level of Na and K in the biosubstrate (hair) depending on the value of the Na/K ratio was of special interest.

Using the bootstrap-method [9], which does not require a normal distribution of a priori ensemble, the mean and interval values of these metals were found pursuant to the hair spectrometry data. The results are presented in Table 4.

The results (See Table 4) indicate a marked variability in the Na/K ratio (subgroup average) from 0.65 to 22.6. The mean values of [Na] and [K] differed significantly among the subgroups. The minimum average level of sodium (223.4 μg/g) was in subgroup 1, the maximum (539.4 μg/g) in subgroup 4; whereas for potassium, the average minimum (32.7 μg/g) was in subgroup 4, while the maximum (384.8 μg/g) was in subgroup 1.

When analyzing the presented data, a natural question arises: why does the correlation coefficient $r_{K\text{-}Na}$, while being extremely high (~0.9) in each of the subgroups, become noticeably lower (0.61) when subgroup 1 is added to the total sample?

As already mentioned, the most significant difference of subgroup 1 from the rest is the 'inverted' Na/K ratio. The connection between this 'inversion' and the decrease of $r_{K\text{-}Na}$ may be regarded as one of the possible reasons for the decrease. Let us explain this in detail.

A close relationship between [Na] and [K] in the substrate ($r_{K\text{-}Na}$ ~0.9), indicating the synchronous (critical) nature of the membrane Na^+/K^+-

ATPases, was found in all (without exception) the subgroups studied (including subgroup 1, where Na/K<1). It was combined with significantly ($p<0.05$) different content of sodium and potassium in the biosubstrate for each of these subgroups (Table 4). This allows us to assume the existence of some kind of an external synchronizer for the membrane Na^+/K^+-ATPases, which should be heterogeneous in its frequency characteristics (and/or consisting of several oscillatory systems). In the role of such a synchronizer, one can imagine, at least hypothetically, the brain electrical activity (BEA) with a known set of different-frequency rhythms detected by the electroencephalography (EEG) rhythms.

The physiological frequency ranges of EEG rhythms are known - δ (delta): 0.5–4.0 Hz; θ (theta): 4.0 – 8.0 Hz; α (alpha): 8.0 – 13.0 Hz; β1 (beta 1): 13.0 – 20.0 Hz; β_2 (beta 2): 20.0 – 30.0 Hz. These rhythms differ not only in frequency, but also in other parameters that have an important diagnostic value (amplitude, power, topography, etc.). The main is the α-rhythm, most pronounced in the caudal (occipital and parietal) areas of the cerebral cortex.

It cannot be ruled out that exactly α-rhythm may turn out to be the most demanded synchronization factor Na^+/K^+-ATPases (or *order parameter*) in individuals of subgroup 2 (68.9%) with the average values of basic indicators found in them (according to our data): Na/K = 2.3; [Na] = 260.6 μg/g and [K] = 127.5 μg/g.

An indirect confirmation of the possible effect of BEA on the level of potassium and sodium in the epidermis can be found in our work [25], where we studied the dynamics of the level of Na and K (hair spectrometry) in 10297 healthy individuals (5160 men and 5137 women) of different age groups (2 to 85 years). The results are presented in Table 5.

Table 2. The correlation coefficient $r_{K\text{-}Na}$ for different values of Na/K

Parameters	Subgroups with different Na/K ratios				Total sample
	1	2	3	4	
	Na/K < 1	Na/K (1-5)	Na/K (5-10)	Na/K > 10	
n (%)	1834 (18.4%)	6884 (68.9%)	893 (8.9%)	380 (3.8%)	9991 (100%)
$r_{K\text{-}Na}$	0.86	0.87	0.98	0.90	0.61

Table 3. Correlation coefficient $r_{K\text{-}Na}$ in combined groups

Parameters	Combinations of subgroups with different Na/K ratios									
	1+2	1+3	1+2+3	1+2+4	1+3+4	1+4	2+3+4	2+3	2+4	3+4
n (%)	8718 (87.3)	2727 (27.3)	9611 (96.2)	9098 (91.1)	3107 (31.1)	2214 (22.2)	8157 (81.6)	7777 (77.8)	7264 (72.7)	1273 (12.7)
$r_{K\text{-}Na}$ ($p < 0.05$)	0.69	0.52	0.66	0.64	0.40	0.47	0.79	0.84	0.81	0.98

Table 4. Interval estimate of the average content of Na and K in epidermis

Parameters	Interval estimation of the average in different for Na/K subgroups (μg/g)			
	1	**2**	**3**	**4**
	Na/K < 1	**Na/K (1-5)**	**Na/K (5-10)**	**Na/K > 10**
n	1834	6884	893	380
[Na]	208.9<**223.4**<238.2	249.4<**260.6**<272.4	307.4<**347**<390.5	454.5<**539.4**<635.4
[K]	359.5**<384.8<**410.4	122<**127.5**<133.5	46.5<**52.4**<58.9	27.2<**32.7**<39.3
Na/K	0.64<**0.65**<0.66	2.29<**2.32**<2.35	6.7<**6.8**<6.9	20<**22.6**<25,8

Note: The significance of inter group differences between the mean values of [Na] and [K] ($p<0.05$)

Table 5. Age-related dynamics in K and Na levels in the epidermis [25]

Age (years)	**Median K** (μg/g)	**CI boot** low	**CI boot** up	**Median Na** (μg/g)	**CI boot** low	**CI boot** up
60-85	121.4	87.97	161.5	293.9	222.9	346.7
50-59	104.3	95.37	117.56	228.1	211.8	241.6
30-49	56.06	53.4	58.46	117.71	113.4	122.6
20-29	38.18	35.7	41.04	82.2	78.1	86.2
10-19	54.4	41.2	84.8	88.9	67.1	137.6
2-9	376.88	231.9	972.26	324.9	233	580.7

Note: CI boot – confidence interval (the bootstrap method)

The level of Na and K in the epidermis was found as a median with the boundaries of the confidence interval (the bootstrap method). The ratio of medians (*Me* [Na]/*Me* [K]) in each age group was as follows:

Table 6. The ratio of medians (*Me* [Na] / *Me* [K]) depending on age [25]

Age (years)	***Me*[Na]/*Me*[K]**
60-85	2.4
50-59	2.2
30-49	2.1
20-29	2.1
10-19	1.6
2-9	0.86

In the absolute majority of healthy subjects (Table 6), which we investigated in the cited paper [25], the ratio [Na]/[K] (median) ranged from 2.1 to 2.4 (starting from the age of 20). It is should be noted that it is quite close (2.3) to the same parameter ([Na]/[K]) obtained in the present work for most individuals (68.9%) but using the mean values of [Na] and [K] (Table 4). After replacing the average with the median, the [Na]/[K] ratio was almost unchanged (2.1 *vs* 2.3).

The [Na]/[K] ratio in the younger age group (2 to 9 years) turned out to be less than 1 (0.86), i.e. the same "inverted" ratio as in subgroup 1 (Table 4). In Table 4, this ratio (0.65) is calculated from the mean values of [Na] and [K]. The replacement of the average by the median had practically no effect on the value of this parameter (0.68 *vs* 0.65).

Why is the fact of 'inversion' of [Na]/[K] ratio in children from 2 to 9 years old so important? The answer is that at this age (up to 13 years old) the dominant EEG rhythm is the θ rhythm [10], whose participation in the appearance of the "inverted" [Na]/[K] coefficient in 18.4% of people of mature age (20-49 years) in our observations seems likely. This requires verification and refinement in combined (EEG + spectrometry) studies, but at the same time allows for the possibility of the BEA influence (as an order parameter) on the operation of membrane ATPases. Moreover, this assumption (in addition to the possible "synchronizing" action of BEA on the operation of membrane pumps) will include, as shown by our data, the

probability of a significant effect of BEA on the level and the ratio of [Na] and [K] in the substrate.

It is not very clear why the presence of subgroup 1 ([Na]/[K] <1) in the total sample causes a noticeable decrease in the tightness of the relationship between [Na] and [K], whereas in subgroup 1 itself the $r_{K\text{-}Na}$ turned out to be high (0.86; see Table 2). One possible explanation for such "inconsistency" can be as follows.

As already mentioned, the [Na] / [K] ratio for the whole body is ~1.3 with the predominant localization of sodium in the extracellular space, and potassium - inside the cell. The main membrane pump, Na^+/K^+-ATPase, which works against the electrochemical gradient of these metals, under whose influence Na^+ ions tend to get into the cell and K^+ ions tend to leave it, provides this distribution. An important feature of the Na^+/K^+-ATPase operation is its ability (per unit time) to remove more Na^+ ions from the cell than K^+ ions, which this pump has time to 'pump' into the cell. By the way, this explains the existence and relative constancy of the membrane potential.

The ratio of [Na] and [K] we found earlier (by average values) in such biosubstrate as hair amounted to 1.5, i.e. almost did not differ from that for the whole body (1.3). Therefore, it seemed that distribution of these metals in the epidermis (with a predominance of Na) is normative and may be found in all healthy individuals without exception. However, that is not confirmed by the data we obtained.

In 18.4% of healthy individuals (subgroup 1, Table 2) [Na]/[K] was <1 (average 0.65). In addition, the presence of this subgroup in the general population explains the noticeable decrease in $r_{K\text{-}Na}$ (down to 0.61), compared with the same parameter in each of the fractions and its average value ($r_{K\text{-}Na}$=0.9).

Therefore, we are ready to assume that a decrease in the Na content in a substrate with predominance of K may indicate (at least in some individuals with the lowest [Na] value and the highest [K] significant changes in the distribution of these metals inside and outside the cell. This, in our opinion, may be due to a change in the direction of the electrochemical gradient of Na^+ and K^+ ions or, in other words, the reversal of the pump function of Na^+/K^+-ATPase ("pumping" K^+ ions from the cell and "pumping" Na^+ ions into the cell). In this case, the known proportion of ion exchange for a given pump (3 Na^+ ions *vs* 2 K^+ ions) can be maintained.

It is known that such a reversion (due to changes in the membrane potential and the content of sodium and calcium in the cell) occurs with the sodium-calcium exchanger (NCX) [22].