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1. Introduction

In the fields of biochemistry and medicine there is growing interest to the crucial role of mitochondria in changing of physiological states and disease development. This interest is fueled by rapid progress in the modern branches of mitochondriology: Mitochondrial Physiology (MiP) and Mitochondrial Medicine [1-8]. Numerous investigations demonstrated mitochondrial dysfunctions in different experimental models of stress or disease. These results were obtained under clearly pronounced functional disorders induced by strong external stimuli or pathogenic mutations. These observations increased interest in the detection of mitochondrial dysfunctions in the organism as early biomarkers of pathogenic processes. The detection of mitochondrial dysfunction is crucial at subclinical stages of disease because it can lead to more effective prevention and earlier intervention. In spite of this benefit to date the available testing methods cannot properly measure the state of mitochondria in the organism in ex vivo observations. Past successes of respiration measurements in isolated mitochondria distracted attention from obvious, methodological shortcoming of the existing method of mitochondria isolation: intentional destroy of their native structure organization into entire network. It is known nowadays that the responses of mitochondria to various physiological challenges are based on the reversible dissociation
of such subtle network structure. In the network state mitochondrial respiration proceeds at a low rate while excitation leads to the dissociation of the network and an increase in respiration. We demonstrated this shortcoming in simultaneous polarographic and microscopic studies of network fragments in KCl rat liver homogenate that contains a higher density of tissue than is usual (1:1 tissue:solution) [9-11].

Since the first step of isolation of mitochondria is network destruction, this method artificially mimics the physiological signal of the activation of respiration. Thus, mitochondria isolated from non-active tissues using the standard methodology do not maintain low levels of respiration typical of the quiescent state and show rapid respiration.

A matter of great concern of biochemists is primarily not to inhibit the enzyme activity; therefore, rapid respiration of isolated mitochondria did not cause apprehension. However, the hyperactivation of functions as an initial step of their alteration preceding inhibition is well known in physiology. That is why artificially accelerated respiration of isolated mitochondria means in essence the LOSS of data for norm in biochemical investigations. The importance of preservation of the native mitochondrial network in ex vivo observations was recently highlighted in a special issue of IJBCB assembled under the editorial guidance of R. Rossignol [12].

The modern investigations clearly demonstrate strong correspondence in functional state of mitochondria and their native organization in the network. This is formulated by the impressive motto: “To be in good shape - to survive” [13]. At the beginning of mitochondria isolation coryphaei of mitochondriology considered possible loss of native properties caused by preparation. H. Krebs attracted the attention to the acceleration of respiration of mitochondria by dilution. He preferred to study mitochondria in homogenate as more native. A. Lehninger mentioned that effects observed in mitochondria in vitro can often reflect their “preparative story” but not native properties. N. Kaplan and collaborators carried out the study just of the SDH activity by the most sensitive function: the reversed electron transfer, which was at that time recently discovered by B. Chance [14]. They explained discrepancies between data of some leading laboratories by the dependence of the effects on the common experimental additions and conditions. Chance participated in discussion of results and all his life developed technics to measure mitochondria inside the organism.

However, the convenient method to study mitochondria ex vivo was not found. Meanwhile the advantages of work with isolated mitochondria by rapid recording technics stimulated biochemists to wide studies and doubts on properties of preparations were neglected.

In order to overcome the crucial methodological shortcoming of traditional procedures, we have created a novel, CytoBioChemical (CBCh) method which preserves mitochondrial network ex vivo using glass-adhered blood lymphocytes. Studies of various physiological and pathological states using the novel CBCh method have shown greater responses of the mitochondria to functional changes in the organism compared with mitochondria isolated in the form of single granules.
An additional, clinical benefit of the CBCh method is the ability to measure mitochondrial function from a drop of blood, substantially reducing the invasiveness of muscle biopsy that is currently required for mitochondrial disease diagnosis. Besides, CBCh method abolishes problem of liquid blood sample changing during work with a group of patients. The state of glass-adhered cells is stable for several hours and after that all samples can be activated simultaneously by insertion into media for measurement of enzyme activity.

In this review we briefly summarize our previous results on elaboration the method, focusing on recent data on its application in physiological and clinical studies. A special attention is paid to interpretation of the data because some unknown phenomena were revealed. Perspectives of further development of our method will be also considered.

2. A brief description of the cytobiochemical method.
Succinate dehydrogenase and α-ketoglutarate dehydrogenase as markers of adrenaline or acetylcholine regulation in the organism

The CBCh method for the study of mitochondrial dehydrogenases was developed to avoid the loss of native mitochondrial network structure in ex vivo experiments. The CBCh method was based on the combination of cytochemical techniques of blood smear fixation on the glass with the use of modern biochemical media for incubation of mitochondria. The composition of the used medium was modified to resemble more closely the intracellular medium to better preserve ex vivo the quiescent state of mitochondria inside lymphocytes. We measure dehydrogenase activity by reduction of nitroblue tetrazolium (NBT) to blue formazan. The image of lymphocytes after succinate (SUC) oxidation is shown in Figure 1. Blue mitochondria are located by periphery. The bulk of the cell is occupied by large nuclei stained with neutral red after SDH measurement for identification of lymphocytes in the smear.

![Figure 1. Images of human glass-adhered lymphocytes after SUC oxidation.](image)

Images were collected using an oil immersion lens (100x/1.25) under a light microscope (Leica, DM 2000) equipped with a camera (Leica, DFC-425) connected to a computer. SDH activity is registered by blue staining with formazan in mitochondria. Red staining is used for nuclei visualization.

In the search for the best conditions for network preservation, we have shown that common substances and conditions obligatory for the investigation of oxidative phosphorylation, such as inorganic phosphate, ADP, Ca\(^{2+}\), pH 7.4, evidently sucrose, as well as dilution of tissue are not only biochemical activators but they also dissipate assemblies of mitochondria.
Because the test of NBT reduction is more sensitive to electrons than oxygen consumption, the CBCh method first allowed us to measure low-rate respiration without artificial high concentrations of stimulators. This characteristic corresponds to a real Quiescent State of mitochondria in the cell. It is of great importance because additions distort the physiological regulation. For instance, ADP results in a shift of the ADP/GDP ratio in mitochondria and inhibits SDH. At the dawn of the polarography era, some leading mitochondriologists believed this extreme stimulation to be artificial, and the measurement of respiration without any external additions to be more physiological and desirable. However, the advantages of rapid-recording polarography were so attractive, compared with the previous monumental and slow manometric epoch that the polarographic method spread quickly over the world. Considering the aforesaid, the possibility to measure the real Quiescent State respiration is one more crucial advantage of the CBCh method. Therefore, besides the preservation of network by fixation, the quiescent state is protected by exclusion of the artificial addition of exogenous activators. Due to the combination of cytological and biochemical advantages, the novel method was called Cytobiochemical.

The measurements of the dehydrogenase (DH) activity are carried out using the cytochemical technique of glass-adhered cells by nitroblue tetrazolium reduction after 1-h incubation at 37 °C. The basal medium contains: 125 mM KCl, 10 mM HEPES, 1.22 mM NBT, pH 7.2 ± 0.05.

As distinct from the cytochemical method according to which only one sample with substrate is usually studied for DH activity measurement, the several selected samples with different additions are investigated according to CBCh procedure. The additions will be mentioned and explained further. These additions allow one to analyze the biochemical mechanisms of DH activity regulation. The regulation of the SDH activity by isocitric acid (ISC) and its combination with KGL activity were found to be most informative. A set of separate samples forms a CBCh pattern of DH activity and serves as a sensitive marker of the state of the DHs in the organism and, therefore, of the state of the organism itself. Examples of patterns and their interpretation will be described further.

The special interest to SDH is due to a great domination of SUC over other substrates in the rate of oxidation, known more than a century, and crown by the brilliant Chance’s discovery of even greater domination in energy-dependent NAD+ reduction [15, 16].

As cited below, our group demonstrated numerous effects of stimulation of mitochondrial and physiological functions by SUC. The crucial role of SDH in tumor resistance and cardioprotection and disease development is recently reported [17-20]. The special properties of KDH are less known and will be considered further.

It was shown in previous studies by our group that the oxidation of SUC and selective activation of SDH are involved in adrenergic (ADR) regulation, while the oxidation of α-ketoglutarate (KGL) and selective activation of KDH is involved in cholinergic (ACH) regulation (see also for more details [10], section 3.2.).
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Figure 2. Substrate - hormonal system. Participation of mitochondria in physiological regulation. Reciprocal adrenergic - cholinergic regulation as a whole with oxidation of succinate - α-ketoglutarate. Link between sympathetic and parasympathetic nervous system and mitochondria, or link between non-neuronal intracellular hormones and substrates.

Therefore, the measurement of the activity of the chosen DHs, SDH and KDH, provides also information on the level of reciprocal ADR/ACH regulation in the organism. Substrates of the two DHs, SUC and KGL, were shown to influence the ADR and ACH level as well as support physiological functions under load and in recovery. It is of importance that the regulatory effects of SUC and KGL in the organism are well pronounced in concentrations far below than necessary for substrate supply. To differentiate these two activities of substrates, the low concentrations manifesting general regulatory action in the organism were called “signal” suggesting that it is realized via specific receptors [21-34]. It was an exciting event for our group when later on such receptors were found indeed, and also for only the two of many mitochondrial substrates. As the authors of the first paper stated, the cause of this selectivity was not clear [35-40]. However, it is well explained from the standpoint that just a pair of substrates is needed as a counterpart of also the paired ADR/ACH regulatory system. The novel mechanism of physiological regulation was named by our group Substrate-Hormonal System. It is presented in Figure 2. It serves as a link between the vegetative nervous system and mitochondria. This system may exist also inside cells with no innervation: in embryos, yeasts, plants, and as was shown recently, in lymphocytes [41-45].

As was mentioned above, the SDH/KDH ratio reflects in mitochondria the balance of ADR and ACH regulation in the organism.

3. Methodological meaning of statistical analysis in modern biomedical investigations

Usual desire of physician to find unique for a certain patient individual diagnosis and treatment become to be an advanced direction of the modern probative medicine. Some statistical algorithms appeared which substantiate individual conclusions made earlier
intuitively. The latter usually expelled these conclusions from the area of science, although this does not correspond to the truth. Misunderstanding between practical physicians and experts in statistics is based on the mistakes in applications of statistical methods in biomedical investigations.

Very interesting and useful monograph by Thomas A. Lang and Mishelle Secic: “How to report Statistics in Medicine. Annotated Guidelines for Authors, Editors, and Reviewers” [46] has the purpose to fill the gap between mathematicians and physicians. The chapter: “The difference between clinical and statistical significance” substantiates the importance of a single observation, for example, life-saving of one person, in spite of its statistical unreliability.

In this respect the statistical validity of CBCh method for individual measurement is of importance and will be briefly considered below. We share also the idea of the authors that statistical analysis in biomedical investigations should be actively used for design of experiment but not only for evaluation of the results. We can illustrate this by our experience.

1. **Statistical treatment**

Besides its crucial advantage in preservation of native structure organization of mitochondria CBCh method possesses one more important property. This is high statistical validity of a single result because it is obtained by computer quantitation of a multitude of microscopic images, which measure enzyme activity. This kind of calculation finds an ever increasing application in biomedical studies. In the case of dehydrogenase activity determination by CBCh method, both in rats and in people, even in a small part of experimental samples with minimal enzyme activity, the number of objects measured was about a hundred. In most cases it was 300 - 500 images, while in a considerable part of samples with maximal activity, the number of images was more than 700. According to the standard determination of statistical significance, it is practically maximal, as judged by measurements even a hundred of images. Therefore all the data obtained in our work by computer quantitation are statistically significant at the level of 0.001 (99.99%).

We did statistical analysis for our data and illustrate this result in Fig. 3 by putting in the bars presenting ± S.D. As seen, S.D. is negligible in contrast to non-computer treatment which deals with a considerably smaller number of measurements. Therefore, we believe it possible do not overload all the figures by designation of minor bars after preliminary explanation in Methods for the presented data sampling. Such description may be convenient for presentation of other data obtained by computer quantitation.

In case of more than single individual measurement, such as data presented in Fig.8, we indicate ± S.D. in diagrams.

2. **Design of experiment for the purpose to diminish “uncontrolled” statistical variations of data.**

The preliminary design directed to diminishing of “uncontrolled” statistical variations of data was considered in the Guidance cited and in the valuable monograph by Glanz [47]. In these handbooks, protocols of comparison of results of the action of different substances are
described, convenient even in examination of people for which the selection of conditions is limited compared with animals. We can add to them examples from our experience. A pair comparison of results in one person in contrast to group comparison belongs to these protocols. This list is continued by: performance of measurements at maximal possible identical time. Comparison with healthy control, taken on the same day and at the same hour, or at least for four seasons separately, for which the difference of reactions is known. Performance of a comparison with controls, taken in the same laboratory simultaneously with examined group instead of using averaged data for norm, which increases variations.

**Figure 3.** Changes in the activity of SDH and KDH and ROS formation in glass-adhered blood lymphocytes in rats during short-term painless psychoemotional stress (PES) in a box. Data are presented as a total area (S) of formazan granules in 30 cells. Owing to a great amount of objects (200-800) the presented values are statistically significant at the level of 0.001 (99.99%).
In connection with data in animal models, described in this paper, we shall consider in more detail the protocol of the selection of really statistical homogenous groups of animals, which was elaborated in our laboratory. The necessity to form statistically homogenous groups of animals for application of statistical analysis for treatment of results is well known, although often it is practically ignored. We found that, in the selection of animals assumed to be similar many factors still exist, that cause uncontrolled variations of the state of animals and preparations obtained. We select animals bred in the vivarium of our Institute within 1-3 days after birth. We do not use for one group animals from only one brood because it is known from physiological data and found by the CBCh method that at least three subgroups can be differentiated in the one brood according to physiological activity [48]. These subgroups correspond to weight distribution. For standard investigations we take animals with greater weight, close to each other on the day of selection - 4 weeks, the time of transfer of young rats to dry feed and taking off from mother milk suckling (100-110 g). It was shown in the special experiments that the prolongation of suckling to 6 weeks considerably improves CBCh data and weight increase as compared with 160-180 g, when suckling was stopped at 4 weeks. The differences are found also in animals at the age of 6 week, before maturation and at the age of 8 week, just after maturation (190-210 g). According to physiological data the differences between these ages are due not only to maturation but also to domination of adrenergic regulation before maturation and cholinergic regulation after maturation. We found manifestation of these physiological phenomena by the ratio of SDH and KDH activities. For standard investigations we have chosen the age of 8 weeks, however other ages can be used but in separate groups. Usually these details are not considered in experiments, which lead to the increase in variations of the results.

Besides careful choice of animals, maintenance of conditions of treatment of isolated tissue preparation is of importance. Usual measurement of data in so called “parallel” samples for analysis taken from liquid preparation: suspension of mitochondria, tissue homogenate or liquid blood is indeed not simultaneous but subsequent in time. It is carried out subsequently, often during prolonged time of storage, while preparation is changed, which gives variations of results. These variations reflect not only changes in the state of the preparation itself but also its response to the pronounced changes in time of cosmophysical environment. This problem is fundamentally investigated in many year studies of S. Shnoll [49]. Many investigators confess that they meet such variations when make repeated measurements. However, observed deviations seem to be obscure and are neglected. Our method of preparation of fixed smears, which are more stable in time, allows one to carry out analysis much more close to simultaneous. Elimination of the considered sources of variations affords the possibility to observe practically completely identical results for different animals in the same physiological state within the selected group of 6-8 species. Such example is given at the upper panel of Figure 3. As shown under 120 min PES CBCh data for two animals are identical. Statistical probability of data for individual organism, considered in paragraph 1, allows one to compare the influence of investigated factors on individual animals in the same group. Comparison the results obtained in one group of
animals (upper panel) completely agree with results in the other group (lower panel), selected in different time. In both cases we observe maximal activation of dehydrogenases in the initial stage of stress - alarm reaction at 30 and 60 min, their further decline approaching the initial level to 120 min, however in the both cases with a small excess, which is considered in the description. Prolongation of stress to 180 min, presented at lower panel, shows increase in inhibition in time. On the reasons considered for upper panel we did not show minor bars in the lower panel as well as in the further similar figures in the text. However the number of images measured and statistical significance are given in legend.

The attention to the creative search of causes of result variations and to the real possibilities of their diminishing will improve efficiency of studies. This affords making smaller number of experiments with more definite results. Such approach presents more deep penetration of statistical analysis into biomedical investigations.

4. Demonstration of high physiological sensitivity of the CBCh method in models of increased adrenergic regulation. SDH/KDH ratio as a distinct borderline between intensive functioning and early dysfunction

Over a long initial period, the biochemical mechanisms of stress were studied under action of strong chemical or physical stressors, which are beyond the range of physiological adaptation. In spite of this, the responses of isolated mitochondria studied under pain immobilization stress for a long time, 24 h, were not pronounced, accounting for no more than 20% inhibition of succinate oxidation measured by the polarographic method [11, 50, 51]. The considerably higher sensitivity of the CBCh assay to changes in the physiological state is evident, as it reveals a great-range difference of SDH activity, 2-3 times and more, under the influence of much milder stressors compatible with life, such as the administration of ADR in submaximal dose or painless short-time restraint of rat in a narrow box for 30-120 min, psychoemotional stress (PES). A comparison of mitochondrial responses observed in isolated mitochondria and in mitochondria inside lymphocytes is convincing, considering that the state of animals after strong or physiological stress differed greatly. After strong stress, animals were exhausted and could not move, while animals after short-term stress looked like intact.

The changes in the SDH and KDH activities after the administration of ADR are presented in the upper panel of Figure 4. As shown, these activities are moderate and nearly equal in the initial quiescent state. Under the action of ADR, the initial acute phase of stress, an alarm reaction arises. It is clearly manifested by a pronounced (more than twofold!) increase in the SDH activity, and by a fall (about twentyfold!) in the KDH activity. Under 24-h restraint in a box, a stress phase of exhaustion develops. It is also clearly pronounced by a decrease in the SDH activity approximately to the initial level. It is very important that this decline does not mean a restoration of the initial state because the KDH activity nearly disappears. Therefore, the dynamics of SDH activity develops according to a bell-shaped curve with a maximum at early stages of stress. This corresponds to the classic stress dynamics followed by physiological data.
As mentioned, the SDH activity serves as a marker of ADR regulation, which supports the external work of tissues, while the KDH activity is a marker of ACH regulation which is responsible for restoration of expenditures for work and underlies the general immune resistance of the organism, including the participation of lymphocytes. Therefore, we consider the critical fall in the KDH activity as a manifestation of stress that exceeds the range of physiological adaptation. It is worth mentioning that the 24- h PES when so dramatic changes in the DH activity were observed by the CBCh method is not as severe as the widely investigated rough form of immobilization when only small changes in respiration were found in isolated mitochondria.

An extremely interesting, quite novel area of real adaptive changes in the DH activity opens during even milder PES of shorter duration: 30, 60, 120 min. As seen in the lower panel of Figure 4, during 30 and 60 min a gradual hyperactivation of the both SDH and KDH activity are observed without the critical fall of the latter. Changes in the KDH activity are only of somewhat less extent than that of SDH. By 120 min hyperactivation of the both DHs is followed by the return to the quiescent state.

The status of DHs at 120-min PES is of crucial importance.

Let us remind that in the initial completely quiescent state, the activity of both DHs is low and nearly equal. This is time to note that the KDH activity is somewhat higher than SDH, which is well reproduced in the independent observations. This small excess of KDH activity over SDH is of great importance because it corresponds to the domination of ACH regulation over ADR regulation in the quiescent state. As shown in the lower panel of Figure 4, by 120 min both DHs return nearly to the initial level. Two wonderful details of this state should be noticed. The first is that a slight prevalence of KDH activity over SDH appears which is typical of the quiescent state. The second is that the level of both is somewhat higher than in the initial state before stress.

These specific features evidence a real return to the well balanced quiescent state in spite of continuation of stress. In essence this decrease in DH activity is a real adaptation to the load during training.

Such phenomenon is well known in physiological observations and only after repeated training.

But it has been never observed in a single short-term experiment, the more so for mitochondrial processes. A search for biochemical markers of such a desirable state was only a dream of physicians and trainers. The CBCh method revealed such changes in a short-term experiment and in a wide range.

A finding these two types of early changes in responses of mitochondria to the pathogenic influence detected for the first time a distinct borderline between stimulating, adaptive action of load and the transition to its adverse action, which increases the risk of pathology.

The reliable detection of this border is particularly important for the prevention of disease or a more effective intervention.
Figure 4. Changes in SDH and KDH activity and their ratio in rats.

Upper panel. Administration of moderately increased dose of adrenaline (50 μg/100 g) for 30 min and long-term (24-hour) psychoemotional stress in a box. SDH activity is expressed as a mean area (S) of formazan granules in 30 cells.

Low panel. Short-term psychoemotional stress in a box. SDH activity is expressed as a total area (S) of formazan granules in 30 cells.

Owing to a great number of objects calculated (200-800) the presented data are statistically significant at the level of 0.001 (99.99%).

As shown in the last example in the lower panel of Figure 4, the prolongation of PES to 180 min exceeds adaptive reserves as it leads to a fall in the activity of both DHs. This inhibition corresponds to the stage of exhaustion of stress. Thus the dynamics of SDH and KDH activity changes presented in both panels of Figure 4 for the first time clearly reveals mitochondrial mechanisms that underlie the physiological development of stress.

In contrast to the results obtained on isolated mitochondria, the CBCh method allows one to observe a broad range of mitochondrial states from deep quiescence up to strong excitation followed by pathological exhaustion in the model of wholly physiological stress. This area of states of mitochondria is not available under the conditions of standard biochemical investigations and is Terra Incognita for mitochondriologists.
Further we shall describe some other unknown phenomena in this unexplored area.

5. Other CBCh characteristics of mitochondria besides SDH and KDH

5.1. Endogenous substrates (ES), SDH true and SDH latent

As mentioned in Methods, for the CBCh characteristic of SDH we used additional samples besides the basal with the addition of millimolar substrate concentrations of SUC. The main of them are shown in Figure 5 and 7, which present more completely the experiments demonstrated in Figure 4. Some additional samples are also given in Figure 6.

The first column from the left shows the reduction of NBT by only ES oxidation without addition of exogenous substrates. This indicator is ignored in most cytochemical investigations. Meanwhile its consideration provides additional valuable information.

The first important characteristic which is determined with the help of ES is a more precise measurement of SDH activity with subtraction of the contribution of substrates other than SUC. As shown in Figure 5, in intact animal the value of reduction in sample SDH is close to that in the sample ES. When subtracting the data for ES from the data for SDH, as it is accustomed in biochemistry, it turned out that the addition of 5 mM SUC absolutely does not increase the reduction in the least. This means that the reduction in the sample with SUC is not caused by SDH activity but is due to other processes. These data show that SDH in physiological quiescence of the organism is not active, keeping a latent state. In contrast, after ADR administration reduction due to ES is lower, the SDH activity is considerably higher, and the difference SDH-ES is great. We call this calculated value true SDH activity (SDHtru) because the subtraction eliminates the contribution of other substrates. To distinguish this corrected value from the common SDH activity measured without subtraction we term the latter the total SDH (SDHtot). The loss of SDH activity in the quiescent state of the organism is well explained from the standpoint of physiology. In the quiescent state, the ADR regulation is switched off reciprocally to ACH regulation. Apparently, SDH as a target of ADR in mitochondria follows hormone signal, switching activity off. As shown in the last example under prolonged -24 h stress corresponding to the exhaustion stage, the activity of both DHs declined.

The state of non-active SDH can also be termed latent or “sleeping” because it does not function in spite of SUC addition. The finding of this state of SDH is one of the most important attainments of the CBCh method. Such a state is not known in biochemical investigations of mitochondria because they lose the quiescent state during preparation and measurements. Biochemists are familiar only with initially activated SDH. The wide range of states from quiescent state to activation is lost for current observation. The loss of the initial basal level of respiration, i.e. the quiescent state, is in essence the absence of data for norm in current biochemical investigations.

We have found that the crucial difference in SDH activity between healthy and sick people or between calm and anxious animals is just the loss of the quiescent state. This means that,
in the state of relative physiological quiescence, sick people has no quiescent state in mitochondria. This will be demonstrated further.

Figure 5. CBCh patterns of mitochondria measured in glass-adhered lymphocytes from intact and stressed rats. Adrenaline administration, 30 min, and PES 24 h. NBT reduction is expressed as a mean area of formazan granules in 30 lymphocytes.

Owing to a great number of objects calculated (200-800) the presented data are statistically significant at the level of 0.001 (99.99%).

5.2. KDH activity measurement. Addition of malonate (MAL)

The pair of columns in Figure 5 next to SDH presents the KDH activity measurements without and with the addition of MAL. These samples contained also NAD$^+$ as a soluble cofactor of KDH. It is worth reminding that MAL should be obligatory added for correct determination of KDH activity. It was well known but undeservedly forgotten in many polarographic measurements that SUC generated in mitochondria during KGL oxidation may amount to a considerable part of respiration in the presence of KGL added. The SUC-dependent portion of respiration should be subtracted from total respiration with KGL. The data presented show that the SUC-dependent portion of KDH activity is absent in the intact state, when SDH is non-active. In contrast, this portion rises in excited animals. It is evident from this example that the own KGL oxidation is negligible under strong excitation. The elimination of the SUC-dependent portion of NBT reduction by MAL allows one to measure KDH activity more precisely. Reciprocal changes in KDH and SDH activities in the presence of MAL become also more evident.

5.3. Signal action of SUC and ADR

The last pairs of columns in Figure 5 presents the data on NBT reduction with the addition of 5 μM, “Signal” concentration of SUC or 5 μM of ADR with substrate concentration of
SUC - 5 mM. The effects of signal concentrations were negligible or absent when measured with subtraction of sample with MAL in the intact animal. However, both effects rise similar to the SDH activity in ADR-activated animal. Under prolonged stress, they both decline also in accordance with SDH activity. These data are the first evidence of a great SDH activity in the presence of low, signal concentration of SUC, far below the substrate ones, under the influence of ADR.

Signal concentrations of ADR act synergetically to SUC. These data serve as a further evidence for synergism between SUC and ADR.

5.4. Addition of MAL: Endogenous SUC (ESUC = ES - MAL), reactive oxygen species (ROS) formation

MAL is commonly used to exclude the contribution to oxidation of SUC, either added or endogenous (ESUC), formed in mitochondria from other substrates, particularly from KGL. Although the amount of ESUC is very low compared with that of added KGL, SUC dominates in competition for the respiratory chain due to the dominating reducing power of SDH. ESUC has additional advantage over added SUC, because it forms inside mitochondria and is better available to SDH [16]. In CBCh measurements, a decrease in formazan formation corresponds to the inhibition of respiration. The difference in reduction between sample ES and (ES+MAL) gives a measure of ESUC.

We observed an increase in ESUC content in animals excited by endogenous ADR (stress) or ADR administration. ESUC also increases in a spontaneously anxious individuals, animals or people. 12 examples of typical patterns of MAL effects on NBT reduction with ES are presented in Figure 6. They were selected from more than 20 volunteers examined. Among them we have found four types of repeating patterns and no more variations. Each type is presented by three examples. The form of pattern is well reproduced for each type. Therefore we believe the pattern to be a regular relationship but not random combination of characteristics. All volunteers were in the relatively calm state. Blood samples were taken in the laboratory between 9-10 a.m. after a very weak breakfast at about 8 at home. All of them were practically healthy, however, the level of health and the state on the day of examination varied. Probably, in accordance with these differences, we found four types of repeating patterns.

Ranging the patterns according to the increase in the SDH activity from low to high, we differentiated the following States: Deep Quiescence, Operative Quiescence, Activity, and Activation with Inhibition.

The decrease in NBT reduction, caused by MAL, is particularly pronounced in the subgroup Activation, in which the SDH activity is the highest. The members of this subgroup have personal reasons for anxiety and activity. The same type of pattern but with a lower amount of ESUC was found in the subgroup named Operative Quiescence, because the SDH activity was considerably lower than in the subgroup Activity. The decrease in NBT reduction caused by MAL evidences the contribution of ESUC to respiration when SDH is active.
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Figure 6. Four (and no more) types of specific CBCh patterns of SDH found in healthy volunteers and presented by three examples. Owing to a great number of objects calculated (200-800) the presented data are statistically significant at the level of 0.001 (99.99%)

A. Deep Quiescence
Quiescent State transition to activity from which is hard.
ES NBT reduction is low, MAL increases it per 10-20%, SDH activity with 5mM SUC is low, considerably lower than ES and ES+MAL, the state of SDH - latent, “sleeping”, switched off.

B. Operative Quiescence
Quiescent State transition to activity from which is easy.
ES reduction is low, MAL decreases it per 20-25 %, SDH activity with 5mM SUC is low. The State of SDH is also latent as in Operative Quiescence, as measured with 5 mM SUC. However, the appearance of ESUC among ES evidences switch on fine activation of SDH and SUC oxidation.

C. Activation
Activated state before work.
ES reduction is higher than in the two previous States, MAL markedly inhibits ES reduction showing great contribution of ESUC in ES, high SDH activity with 5 mM SUC.

D. Activation with inhibition
Stressed, restrained Quiescence which can transform to pathology.
In such a relative, not complete Quiescence excitation is not switched off, but is suppressed by internal mechanisms and in case of their insufficiency by drugs (β - adrenoblocker for example 20).
ES reduction is low for except of example 20 where it is high, probably due to the SDH inhibition by adrenoblocker. MAL increases ES reduction 2 - 4 fold, SDH activity with SUC 5 mM is moderate and high for the example 20.
Together with this traditional effect of MAL, we revealed the opposite action of MAL, namely, an increase in NBT reduction. It was most pronounced in the subgroup Activation with Inhibition, which was distinguished according to a decreased SDH activity relative to the Activation subgroup. One cause of the inhibition (example 20) could be an elder age and initial hypertension demanding the intake small doses of adrenoblockers, which also inhibit SDH. In this case, an increase in NBT reduction by MAL is the highest. This effect is also well pronounced relatively to ES in two other examples in this subgroup. This unknown effect of MAL on NBT reduction is also observed in Deep Quiescence, in which all activities are at the lowest level.

Thus, MAL manifests the ability to increase NBT reduction in case of low SDH activity, in contrast to its traditional inhibition of respiration is great when SDH activity is high.

In our opinion, the increase in NBT reduction by MAL is a marker of ROS formation in mitochondria. As shown in Figure 7, ROS formation sharply rises at the beginning of stress. During stress, the rise of ROS formation declines as evidenced by our measurements with MAL. This agrees with other results [52]. As shown in the last example in Figure 7, ROS rise again by the exhausting stage. The biochemical mechanism of this effect of MAL is in deep inhibition of SDH, which leads to a fall of coenzyme Q reduction from full reduction to free radical form. Free radical form of Q produces superoxide in interaction with surrounding oxygen [53].

5.5. Dual regulatory effect of ISC on SDH activity in the area of physiological regulation. GTP-recoupling but not oxaloacetate (OAA)-inhibition regulates SUC oxidation under physiological conditions

ISC is a natural efficient intramitochondrial antioxidant [54]. Reducing equivalents produced by ISC NAD(P)-dependent dehydrogenases protect the most sensitive enzymes aconitase and KDH from oxidation and increase the stability of mitochondria to oxidative stress, which accompanies any stress. It was shown long ago that ISC abolishes OAA inhibition of SDH in aged mitochondria or under severe stress [50, 55]. This effect is due to OAA elimination by reduction into malate because of increase in the reduction of the respiratory chain. A similar effect is usually attained by the addition of glutamate, which eliminates OAA via transamination. As distinct from glutamate, the application of ISC for the elimination of SDH inhibition was nearly forgotten. This may be due to the fact that its fully active natural isomer is commercially hardly available, while the available chemical racemate is less active. Fortunately, due to the collaboration with microbiologists manufacturing natural ISC, we had an opportunity to investigate this substance [56].

Our investigation of the influence of natural ISC on SDH activity during PES under more physiological conditions of the CBCh method discovered an unknown effect of this substrate. In spite of acceleration of inhibited SUC oxidation, ISC strongly suppresses hyperactivated SDH. This effect is shown in Figure 7. It is clearly seen that ISC addition suppresses greatly SDH activity in the hyperactivated state, 30, 60, 120 min of PES.
However, ISC preserves the ability to stimulate inhibited SDH under 180-min PES. Besides, we have found that ISC does not influence the SDH activity in the Quiescent State. The two opposite effects of ISL: an increase and a decrease in SDH activity are easily explained by the single antioxidant mechanism. The increase in NBT reduction by MAL at the beginning of PES shows the development of oxidative stress. This agrees with other observations. The level of oxidative stress is regulated by well-balanced interactions of several processes occurring in mitochondria. Among them are initial uncoupling and its secondary recoupling by GTP [52].

Recoupling effect of GTP can explain our observation that preserving the KDH, which is a real source of GTP-formed in substrate-level phosphorylation, protects SDH from hyperactivation followed by inhibition.

In this metabolic environment, protection of KDH from oxidation by ISC allows one to support GTP generation and to diminish the uncoupling that was a cause of initial SDH hyperactivation. This explanation agrees well with our repeated observations that the preservation of high KDH activity simultaneously with activation of SDH is typical of activity within physiological adaptation area. The “scissors” between SDH rise and KDH fall are observed in severe stress or pathology. Therefore, three different effects of ISL on SDH activity: suppression of hyperactivation, activation of inhibition, and absence of influence in norm, allow one to differentiate clearly functional states of SDH. The area of

Figure 7. CBCh patterns of mitochondria measured in glass-adhered lymphocytes from intact and stressed rats under mild PES of different duration. NBT reduction is expressed as a total area of formazan granules in 30 lymphocytes. Owing to a great number of objects calculated (200-800) the presented data are statistically significant at the level of 0.001 (99.99%).
Dehydrogenases

SDH regulation involving KDH-dependent GTP – recoupling - is not known in biochemical investigations because SDH is initially hyperactivated under the experimental conditions. The hyperactivation of SDH elevates the risk of inhibition by OAA due to the increase in its formation. Indeed, OAA inhibition is often observed, when SUC oxidation is accelerated, for example, by uncoupling. Therefore, OAA inhibition is considered to be the only mechanism of SDH control. However, this is true for in vitro conditions in biochemical experiments in isolated mitochondria. The preservation of the area more close to physiological conditions in the CBCh method opens Terra Incognita of unknown regulation of the SDH activity by GTP-recoupling instead of usual OAA inhibition.

The last but not least, it is worth mentioning that the mutual reciprocal regulation of SDH and KDH activity is further evidence of ADR and ACH regulation in mitochondria. In essence, it corresponds to the reciprocal influence of ADR and ACH on physiological functions: stimulation, and restriction with restoration, respectively.

Before continuation, let us put in a brief insertion to memorize the better approach of CBCh determination of SDH activity to mitochondrial processes compared to pure enzymatic measurements.

**Interlude:** CBCh activity of SDH is not so much a characteristic of abstract DH, as a measure of the rate of SUC oxidation in mitochondria

What is the interest of physiologists and physicians in measurement of enzyme activities? Evidently they need to learn how the enzymes work in the organism in different states. Many purely enzymatic methods give exact information, although rather abstract in respect of the conditions in vivo. Preservation of the native state and native surrounding of SDH and KDH under CBCh conditions permit measurement of SUC oxidation, that approaches the real rate of respiration in mitochondria in vivo rather than an abstract activity of SDH. This is one more crucial advantage of CBCh measurements of DHs activity.

6. Revealing fine physiological differences in the state of the organism in norm by the activity of dehydrogenases as measured with the CBCh method. Differences in the activity and responses of SDH in rabbits with opposite behaviour

The CBCh method clearly detects some fine physiological differences in the state of the organism that were earlier not observed in mitochondria. Among them are some age-related differences in rats at different stages of ontogenesis, namely, in newborn animals, depending on the time of feeding with mother milk, prior to or immediately after maturation, as well as individual differences of pattern types between healthy humans that are related to the current condition or temperament [48, 57]. Both age-dependent changes and differences of temperaments reflect different ratios of ADR/ACH regulation. In the earlier age, ADR regulation dominates; after maturation, it is more balanced by ACH.
Figure 8. Responses of ES and SDH to activation by administration of adrenaline or succinate measured in lymphocytes of rabbits with different behavior.
A - calm rabbit, B - restless rabbit. SDH activity is expressed as a mean area of formazan granules in 30 cells. The mean of three measurements in different days for each rabbit are presented, M ± SEM. * - p<0.05, ** - p<0.01, comparing to the respective initial state, # - p<0.05, comparing to the calm rabbit.

As one of the models of domination of these regulation types we studied individual differences of CBCh patterns in rabbits with different behaviour.

One rabbit was calm during the experiment; another rabbit escaped and was anxious and restless even when it was not exposed to treatment. They represented characteristic types of a cholinergic and adrenergic, respectively. Figure 8 demonstrates significant differences between the calm and restless rabbits in the level of ES and in the SDH activity.

The reserve of ES in the calm rabbit is much greater than in the restless. After the injection of ADR, the level of ES in the calm rabbit decreases; however, this level is higher than in the restless animal under the influence of ADR. In the intact restless animal, the level of ES is 15 times lower than that of the intact calm rabbit and 5 times lower than in the calm animal after the injection of ADR. This indicates an enhanced expenditure of substrates in the restless animal.

The SDH activity in the calm rabbit is low, and the injections of ADR or SUC strongly stimulates it. The stimulation of SUC is somewhat stronger, which increases the risk of the transition from hyperactivation to inhibition. By contrast to ADR, the injection of SUC also increases the level of ES.
SDH in the intact restless rabbit is hyperactivated nearly up to the level of calm under ADR influence. Thus, as judged by ES level and the SDH activity, the restless rabbit has no quiescence in the quiet state without external excitations. The same state of continuous internal metabolic excitation is inherent in all patients examined in our laboratory in contrast to healthy volunteers. The data will be considered in the next sections.

At the background of the initially high SDH activity in restless rabbit ADR administration leads to only small stimulation, which only somewhat exceeds the level under the influence of ADR in calm rabbit. The influence of SUC, which was somewhat stronger activator when injected to the calm rabbit, leads to realization of risk of transition to inhibition in restless animal.

The difference in ADR and SUC effects on ES can be explained by different mechanisms of their influence on SDH activity. ADR only activates SDH through protein kinase. The influence of SUC is more complicated. SUC is an allosteric activator of SDH [58]. Simultaneously the rise of SUC level increases OAA formation, which inhibits SDH according to the mechanism of the negative feedback.

These effects are better pronounced in a sample without SUC addition, namely ES, because SUC excess partially abolish OAA inhibition. Therefore in sample ES SDH inhibition is better observed than in sample for SDH measurement, containing 5 mM SUC, because it is not masked by the addition of substrate. Probably, the main part of ES under SUC injection is ESUC, which is accumulated due to OAA inhibition. It should be considered that measurements are made for an hour. Insight into dynamics of the processes can be achieved by a comparison of data for calm rabbit, in which oxidation is slower with those for restless one, in which oxidation is accelerated. As shown for calm rabbit, at the beginning rapid oxidation of SUC occurred, resulted in an increase in OAA, which is evidenced by the initial accumulation of ESUC. The next stage is presented by data for restless rabbit in which stronger inhibition of SDH by OAA is already developed, which is not completely abolished by SUC addition and therefore ESUC accumulation is higher than in calm animal.

The data presented show great difference of the two CBCh properties of the state of mitochondria in animals with opposite behaviour, which corresponds in magnitude to the pronounced individual peculiarities in behaviour.

We have also found the pronounced difference in the SDH and KDH activity in rats before maturation, 6 week old with domination of ADR regulation, and just after maturation, 8 week old with increase in ACH regulation. These changes are close to differences between calm and restless rabbits.

It was shown also that complicated menopause in rats and women, which is manifested under the decrease in ADR regulation in the organism, is related to the increase in the SDH activity and can be corrected by metabolic regulators, containing SUC - “Amberen” [59].

The data considered support the view of existence of the link between activity of studied DHs and the level of ADR and ACH regulation.
7. The changes in SDH and KDH activity in patients with metabolic syndrome and arterial hypertension

The stages of the SDH and KDH activity changes found under stress were observed in patients with metabolic syndrome and arterial hypertension.

Disturbance of normal metabolic flexibility between oxidation of glucose and fatty acids is the basis of many diseases. High dietary fat intake and low physical activity provoke this disorder. Complex of accompanied biochemical changes is called metabolic syndrome. Metabolic syndrome underlies various pathological symptoms, such as obesity, insulin resistance, type 2 diabetes, risk of hypertension, heart diseases and others [1, 60-65].

Metabolic syndrome is caused by increased intensity of life accompanied with persistent sympathetic hyperactivation and deficit of physical activity that leads to PES. That is why we have studied the participation of mitochondria in development of PES. The importance of mitochondria in development of metabolic syndrome is caused by their crucial role in intracellular oxidation. Aerobic fibers of skeletal muscle mitochondria make a major contribution to regulation of metabolic syndrome and support metabolic health of the whole organism.

Figure 9. True SDH without and with ISC in not severe patients suffering from food allergy accompanied by arterial hypertension.

Data are expressed in mean area (S) of formazan granules in 30 cells. Owing to a great number of objects calculated (200-800) the presented data are statistically significant at the level of 0.001 (99.99%) 

Inscription above - types of patterns.

SDHtru is calculated as the difference between samples SUC 5 mM - MAL 5 mM.

The changes of SDH and SDHisc activity are presented in per cents respective to conditional norm of patient 1 (100%). The low row for SUC 5mM, the upper row for SUC 5 mM + ISC 5 mM.
The changes in the SDH activity in non-severe, non-hospitalized and workable patients with allergy to some food products are given in Figure 9. In this case for more precise determination of the SDH activity we subtracted ES contribution from the data for the sample with SUC addition. This value was termed as a true SDH (SDHtrue). It was determined under addition of SUC and also SUC+ISL, modulator of the SDH activity. This provides determination of the functional state of SDH as considered in section 5.5. Calculation of SDHtrue help to find clearly pronounced similarity between some patients, which is masked by ES contribution. According to SDHtrue the patients can be joined in subgroups with nearly identical data for members inside one subgroup and clearly different from the other.

The first example from the left is for healthy person. The next one, 1, is taken as reference control to other patients. He used diet containing no allergenic products for more than 7 years and for this period his body weight, AD and other physiological characteristics were greatly normalized. Other patients have some pathological symptoms and used recommended diet for only several months. Among them we have found increase in SDH activity.

For healthy people full absence of SDHtrue - complete quiescence state is observed. Low SDHtrue was determined for patient with normalization of the state. In all others patients different levels of activation or hyperactivation of SDH were found. In the state of hyperactivation without inhibition, ISL addition results in only the tendency to stimulation. Progressive hyperactivation is masked by internal inhibition of OAA. This is revealed by the increase in activity with ISL. For the patients with greater symptoms, presented in the right part of the Figure 9, the increase in the internal inhibition is typical, which is manifested in both, the SDH activity decrease and strong increase in activation by ISL.

Hyperactivation of the SDH activity without inhibition is more typical of patients hospitalized with hypertension. In Figure 10 data are presented on the total SDH activity (SDH tot) without subtraction of ES contribution. It is seen also, as in Figure 9, that for the healthy control very low activity of SDH is typical. The rise in the activity can be considered as hyperactivation without inhibition by both, their greater value and because ISL does not stimulate it but, in contrast, decreases. ISL addition serves as unique test of the functional state of SDH developed by our group. Additionally to the explanations of ISL influence in section 5.5 it should be mentioned that effect of ISL is dose-dependent. It can be seen from the example of patient N2. Addition of 5mM of ISL decreases hyperactivation of SDH per 2/3, while 50μM only per 1/3.

The extent of the deflection of the SDH activity from norm should be quantitatively determined using different concentrations of ISL. In Figure 10 (as in Figures 5 and 7) the KDH activity is also given, as well as the response of mitochondria to the signal concentration of SUC - 5μM. In norm the KDH activity is low and nearly equal to the SDH activity. At hyperactivation of SDH in patients N2 and N3, KDH is also greatly increased, however less than SDH. Such coupled changes in the two DH activities in patients are similar to those, which were found in rats under PES within the range of physiological
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adaptation. However, the changes in more severe patient, N4 are already beyond the physiological range and are analogous to that for damaging stress in Figure 4. It is also seen that the response to signal concentration of SUC corresponds to the SDH activity, measured with substrate concentration of SUC, as shown in Figure 5.

The presented examples show that the loss of full value quiescence, which is manifested in the complete switching SDH off, is a general difference of all examined patients from healthy people. Therefore mitochondria of patients in the relatively quiet state of the organism have no metabolic quiescence.

According to the influence of ISL functional state of SDH in patients with hypertension differs from that of patients with allergy. Hidden inhibition of SDH is typical of allergy, while hyperactivation of SDH is more typical of hypertension. This coincides with more pronounced sympathetic hyperactivation in hypertensive patients, demanding intake of adrenoblockers.

Figure 10. CBCh patterns of patients hospitalized with hypertonic disease. The data are expressed as the total area of formazan granules in 30 lymphocytes. AP, upper line - the data just before blood taking, low line - more stable data from History of the disease. The gender and age are indicated between.

Owing to a great number of objects calculated (200-800) the presented data are statistically significant at the level of 0.001 (99.99%)
8. Conclusion

The experience of the reviewed study evidences reliability to approach ex vivo more closely to the state of investigated tissue in the organism. In order to maintain native state of isolated tissue it is necessary to join the both, care of biochemical processes and physical structure. Only combined biochemical and biophysical approaches will provide attaining the desirable goal to study biochemical mechanisms of physiological processes ex vivo.

This approach opens unknown areas in biochemical regulation in the organism, as shown for instance, by SDH and KDH regulation. One of the mostly known phenomenons, OAA inhibition of SDH, is considered as the main regulator of the SDH activity. However, according to investigations under more physiological conditions, it seems to be in many cases rather artifact of “preparative story” of mitochondria. The wide range of SDH states under the physiological and pathological conditions in the organism, compatible with life, are DH hyperactivation, which is far from the inhibition. This state is probably controlled by GTP, produced due to KDH activity in substrate-level phosphorylation during KGL oxidation. This restriction of SDH by KDH is in essence identical to reciprocal interrelationship between parasympathetic a sympathetic nervous regulation of physiological functions.

A very convincing observation of this substrate-hormonal interaction, as judged by the CBCh method, which cannot be achieved in investigation of isolated mitochondria, can be also mentioned as the crucial result of preservation of the native structure of mitochondria under study.

9. Perspectives of the CBCh method development

The wide application in clinical practice of the proposed very sensitive method to penetrate the state of human mitochondria is the most near, reliable and useful perspective. This penetration is even deeper than in the current biochemical studies. The procedure of the measurement is not the main difficulty on this way. It is within the range of the commonly used clinical tests and may be advanced for the convenience.

In order to the CBCh analysis will bring benefit to patients, physicians should acquire deep knowledge of the investigated biochemical processes. Fundamental data from handbooks are not sufficient for this purpose. It is necessary to learn more special area, which is developed in current studies by the CBCh method. This area is advanced even in modern mitochondriology, because it is created during novel experiments. The authors are preparing the special guidance to facilitate the entrance in the new area. We are also glad to help to overcome individual difficulties in the procedure and analysis of data.

Radical approach to the state in the organism through to the preservation of the native structural state of the tissue, found in the study oh DHs, encourages widening the developed conditions on the great variety of the cyto- and histochemical determinations.
Earlier we have stated that the main advantage of the CBCh method lies in the use of lymphocytes as indicators of the overall metabolic status of the entire organism, which is especially useful as an early diagnostic tool of various mitochondrial dysfunctions. This method can be also used for individual selection of drugs in sample of blood of a certain person.

However, the CBCh method may also be used as a tool for the study of lymphocytes as an independent object of study, which carries important functions in the immune response. The use of the CBCh method for the study of dehydrogenase activity, as well as other indicators, is likely to lead to a better understanding of the dependence of the immune response on mitochondrial functions.

The novel area of CBCh investigations of lymphocytes can be related with their use as cells possessing recently discovered pronounced ACH regulation, both vagus-dependent and, particularly interesting, self-dependent, intracellular, which is based on the internal pool of non-neuronal ACH. The simultaneous study of ACH level and dehydrogenase activity in lymphocytes can provide further penetration into substrate-hormonal regulatory system that was pioneered in our laboratory.

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