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Activation and Inhibition of Succinate-Dependent Ca^{2+} Transport in Liver Mitochondria during Adaptation

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Abstract—Succinate-dependent Ca^{2+} accumulation was investigated in rat liver mitochondria and in operation biopsies of patients under states either with prevalence of adrenergic influences (administration of activating doses of adrenaline, acute phase of immobilization stress, initial period after allogenic transplantation, and acute phase of myocardial infarction) or with prevalence of the reciprocal mediator acetylcholine (late period after transplantation, chronic ulcer disease of stomach and duodenum). Adrenaline prevalence leads to increase of succinate-dependent ATP synthesis and Ca^{2+} accumulation, which is due to known activation of succinate oxidation. However, together with activation, inhibition of these processes was revealed. The inhibition phase prevails under chronic pathology. Therefore, reciprocal regulation of succinate oxidation and succinate-dependent Ca^{2+} transport in mitochondria occurs in the body in the course of adaptation. The reciprocal regulation of mitochondrial processes is considered as a mechanism of reciprocal regulation of physiological functions.

Key words: mitochondria, adrenaline, acetylcholine, stress, antigenic stress, Ca^{2+} transport, succinate oxidation, adaptation

Regulation of physiological functions of the body and their adaptation to current conditions occurs with the participation of two reciprocal parts of the vegetative nervous system, sympathetic and parasympathetic. Their mediators, adrenaline and acetylcholine, regulate a number of metabolic processes. The level of these hormones in blood and tissues changes reciprocally. Under excitation, the level of catecholamines is raised while acetylcholine decreases. In contrast, in quiescent state acetylcholine level is elevated while adrenaline decreases [1, 2]. Catecholamines as well as α-agonists and glucagon specifically increase phosphorylating oxidation of succinate [3-8], succinate dehydrogenase activity [9-11], transport of added Ca^{2+} [6, 7, 12], and NAD reduction [13].

Regulation of phosphorylating succinate oxidation under immobilization stress involves the reciprocal interaction of adrenaline activation and inhibition due to the increase of cholinergic influences and a synergistic effect of serotonin [3]. Activation of succinate oxidation by adrenaline is coupled with inhibition of α-ketoglutarate oxidation. Acetylcholine reciprocally activates oxidation of α-ketoglutarate and inhibits oxidation of succinate [14-16].

In this study, investigation of reciprocal regulation of coupled succinate oxidation is continued using a more sensitive test, succinate-dependent Ca^{2+} transport and accumulation in mitochondria of animals and patients.

More intensive short-term processes were investigated which are mainly influenced by adrenaline, whereas as more prolonged processes are related to increased cholinergic effects that compensate hyperactivity. Hyperactive states, e.g., arterial hypertension, are also compensated by adrenoblockers. However, it is known that increased cholinergic regulation can in turn lead to hypertrophy of the acetylcholine effect with its transition to a pathogenic state. In such states, particularly with duodenal ulcer, cholinoreceptor blockers are used and even section of the vagus nerve.

It remains unclear what happens in mitochondria in such states.

The presented investigation shows that a similar response is developed under some experimental influences and in human pathologies, i.e., progressive activation of succinate-dependent energy supply followed by its progressive inhibition.

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MATERIALS AND METHODS

States with prevalence of the influence of adrenaline (injection of stimulating doses of adrenaline, acute phase of immobilization stress, initial stages after transplantation of allogenic tissues, and acute phase of myocardial infarction) and with prevalence of the influence of acetylcholine (late terms after tissue transplantation, chronic duodenal ulcer) were studied.

Male Wistar rats weighting 200-220 g were used in the experiments. Adrenaline was injected intraperitoneally at 25 or 35 μg per 100 g body weight 30 min before decapitation. Immobilization was carried out on a special support with fixation of the head for 1 h. Myocardial infarction was modeled by occlusion of the left coronary artery for 2 days. Antigenic stress was modeled in two ways: 1) transplantation to Wistar rat of heart taken from August rat [17]; 2) transplantation to August rat lymphocytes ($2 \cdot 10^7$ cells in 1 ml of saline) taken from Wistar rat after the allogenic transplantation of the heart. Rats after allotransplantation of heart or lymphocytes were decapitated after 2 and 7 days. In addition, operational liver biopsies of patients with chronic duodenal ulcer disease were investigated.

In cases with adrenaline administration, investigations were performed using liver homogenates. In the other cases, mitochondria were prepared from tissue homogenates by a method increasing preservation of the native state of the organelles [3]. In each experiment a pair of animals was used, control and experimental, with a 1-h interval between the first (decapitation at 10 a.m.) and the second.

Visual examination of the thymus and stomach mucous was used as a test of the state of animals. In control animals in normal physiological state the thymus is a well-formed gland of elastic opalescent tissue weighing 540 ± 20 mg. Under stress a dotted hemorrhage appeared on the thymus, the glandular tissue looks flabby and of cyanotic color, and the mass was decreased to 400 ± 40 mg.

The stomach mucous in control animals was of pink color and had soft folds. Under the influence of adrenaline, the mucous color turned to cyanotic and the folds become rough.

Preparation, storage, and sampling of homogenate. After decapitation of an animal, the liver was quickly excised and put in ice-cold homogenization medium containing 120 mM KCl, 10 mM HEPES, pH 7.5, 1 mM EGTA. The liver was weighed and washed from of blood with ice-cold homogenization medium. Then the tissue was crushed through an iron-steel press with 1 mm holes. The minced tissue was homogenized after addition of 1 ml of medium per 1 g of tissue in a loosely fitting Potter-Elvehjem homogenizer with about 0.2 mm radial clearance, rotating at 200 rpm, making 3-4 up and down strokes. Then the homogenate was filtered through a double layer of kapron. The preparation was ready for measurements within 10 min after decapitation and kept stable responses of phosphorylating oxidation of substrates for several hours of storage on ice. Samples were taken with an ice-cooled pipette with wide obliquely cut tip. For H^+/Ca^{2+}-exchange measurements, 200 μl of homogenate containing 60-70 mg protein/ml was used.

Accumulation and release of Ca^{2+} by mitochondria. These parameters were registered either with a hydrogen electrode (by the opposite change of H^+ in the incubation medium) or with Ca^{2+}-selective electrode. $CaCl_2$ was added in 200-nmol portions [18-21]. The total amount of cation accumulated is a measure of the Ca^{2+} capacity. Spontaneous efflux of accumulated Ca^{2+} occurs when a certain limit is reached.

Calcium capacity was measured during oxidation of different added substrates, e.g., succinate, succinate with glutamate, succinate with ADP, and succinate with glutamate and ADP, as well as during oxidation of only endogenous substrates [20, 21]. The curves of accumulation and release of Ca^{2+} were compared. ADP addition increases Ca^{2+} capacity due to prevention of Ca^{2+} release (pore opening) [22, 23]. Increase of the level of intramitochondrial ATP is probably also important. Use of glutamate, which reveals oxaloacetate inhibition of succinate oxidation, increases considerably the sensitivity of detection of the state of mitochondria. We found that succinate-dependent Ca^{2+}-accumulation in homogenate is completely prevented with inhibitors of mitochondrial processes such as ruthenium red and malonate.

Measurement of the rate of ATP synthesis. The rate of ATP synthesis was measured by H^+ utilization after ADP addition.

Measurement of endogenous succinate content. This was measured as the fraction of Ca^{2+} capacity sensitive to malonate under oxidation of endogenous substrates.

The incubation medium for isolated the mitochondria contained 100 mM sucrose, 60 mM KCl, 1.5 mM Tris-buffer, 1.5 mM KH_2PO_4 (pH 7.4), 8 mM succinate, 1 mM glutamate, 3 mM malonate, and 5 mg protein per 2 ml medium. The incubation medium for homogenates contained 120 mM KCl, 1 mM KH_2PO_4, 1 mM HEPES (pH 7.35), 2.5 mM succinate, 0.6 mM glutamate, and 8-10 mg protein per 1.6 ml medium. In all cases, 200 μM ADP and 200 μM $CaCl_2$ were added; the temperature was 26°C. Measurements in mitochondria and homogenates were carried out during 30-40 min after their preparation. The Ca^{2+} capacity and the rate of ATP synthesis under succinate oxidation in control animals were taken as the 100% values.

Protein content was measured using the method of Lowry [24].

Statistical treatment. Results were treated using the *t*-criterion of Student and the pair comparison U-test of Wilcoxon [25].

RESULTS

Acute stress—prevalence of adrenergic influences. In Fig. 1, curves of Ca^{2+} accumulation and release are compared in control and adrenaline-activated animals as measured by H^+ registration. In control animals, Ca^{2+} accumulation with only succinate is minimal, and after the third addition all Ca^{2+} was released. ATP synthesis increases the level of Ca^{2+} accumulation with respective to its level with succinate and diminishes its subsequent release. Additional supply with glutamate does not increase Ca^{2+} capacity, which indicates the absence of oxaloacetate inhibition.

In adrenaline-activated animals, Ca^{2+} accumulation with succinate is 2-fold higher than control animals. Its level is higher than in the presence of ATP in control animals. In contrast to control animals, ADP addition does not increase Ca^{2+} accumulation.

The loss of the stimulating effect of ADP might indicate the appearance of hidden oxaloacetate inhibition of succinate oxidation together with its activation. ADP increases this [26].

Glutamate addition indeed leads to a pronounced rise of Ca^{2+} capacity, which is more than 2-fold higher than the control. This strong activation by glutamate reveals the great value of oxidation inhibition by oxaloacetate. This shows the appearance of oxaloacetate inhibition under adrenaline activation. However, an activating effect is also obvious. This is also observed with only succinate. In addition, the considerably greater Ca^{2+} capacity with glutamate also suggests that in hidden form stimulation of a succinate-dependent process is present in mitochondria of the adrenaline-activated animals. Therefore, the data presented show simultaneous development of activation and inhibition of succinate-dependent processes under the influence of adrenaline.

The data given in Fig. 2 for mitochondria of adrenaline activated animal are similar to that presented in Fig. 1 but registered with Ca^{2+} electrode. As in Fig. 1 ADP addition does not increase Ca^{2+} capacity. Glutamate addition as well as in the presence of succinate and particularly in the presence of succinate with ADP leads to a considerable (up to 3-fold) rise of Ca^{2+} accumulation which corresponds to data in Fig. 1. These results confirm that increase of Ca^{2+} capacity by glutamate evidences appearance not only inhibition but also activation of succinate oxidation.

In Table 1, mean data are given for ADP phosphorylation rates and Ca^{2+} capacity in liver homogenates under the influence of two adrenaline doses (25 and 35 μg per 100 g body weight) as well as under immobilization stress in rats. All these influences accelerate ATP synthesis and increase Ca^{2+} capacity. In all variants, increase of Ca^{2+} capacity is greater for the lower dose of adrenaline. Increase in dose leads to 2-fold decrease of stimulation. This probably indicates the appearance of an inhibitory

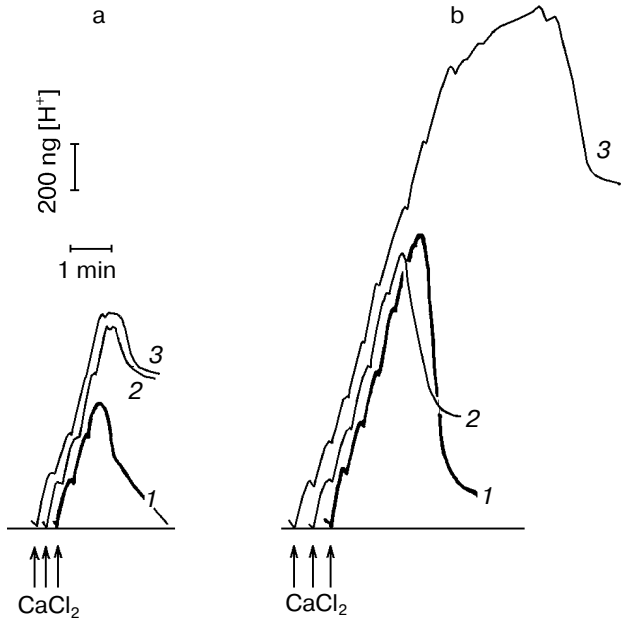

Fig. 1. Activation and inhibition of Ca^{2+} transport in mitochondria of rat liver homogenate under the influence of adrenaline (registration with pH electrode): a) control rat; b) adrenaline administration, 25 μg per 100 g body weight. Ca^{2+} capacity on addition of succinate (*1*), succinate and ADP (*2*), succinate, glutamate, and ADP (*3*). Conditions of incubation are given in "Materials and Methods".

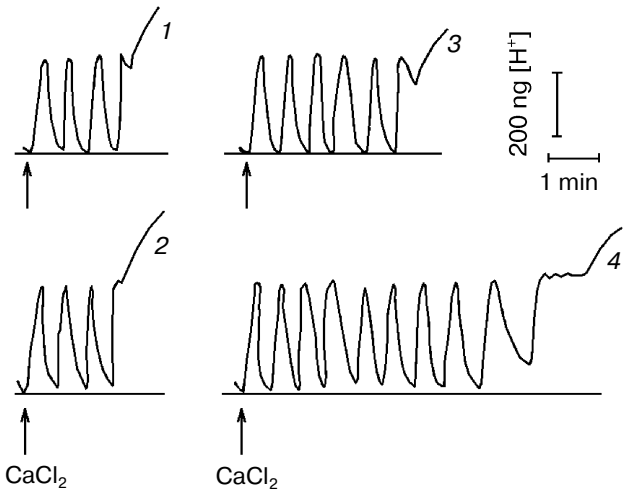

Fig. 2. Activation and inhibition of Ca^{2+} transport in mitochondria of rat liver homogenate under the influence of adrenaline (registration with Ca^{2+}-sensitive electrode). Adrenaline administration, 25 μg per 100 g body weight. Calcium capacity on addition of succinate (*1*), succinate and glutamate (*2*), succinate and ADP (*3*), succinate, glutamate, and ADP (*4*).

Table 1. Rate of ADP phosphorylation and Ca^{2+} capacity in liver homogenates under the influence of different doses of adrenaline or immobilization stress in rats

Experiment	ATP synthesis, ng H^+/min per mg protein		Ca^{2+} capacity, nmol H^+/mg protein			Increase in capacity, %	
	S + ADP	S + GL + ADP	S	S + ADP	S + GL + ADP	S + ADP/S	S + GL + ADP/S + ADP
Control (n = 10)	28 ± 1.2 100%	32 ± 1.7 113	93 ± 4 100%	138 ± 9* 148	150 ± 8* 162	148	109
Adrenaline: 25 μg per 100 g body weight (n = 12)	40 ± 2* 144	45 ± 2.8* 161	165 ± 11 180	163 ± 14 177	289 ± 10* 315	99	177
35 μg per 100 g body weight (n = 6)	34 ± 0.5 110	35 ± 0.4 113	124 ± 20 132	133 ± 11 142	185 ± 10* 201	108	142
Immobilization stress (1 h, n = 5)	32 ± 2 118	37 ± 4* 128	144 ± 11 157	144 ± 14 157	234 ± 17* 254	100	163

Note: Effects are presented in percent of ATP synthesis or Ca^{2+} capacity with succinate only taken as % for the respective group. S, succinate; GL, glutamate.
* p = 0.01.

effect of higher adrenaline dose as shown before [7]. The respective parameters under 1 h immobilization stress are intermediate between the effects of the two tested doses of adrenaline. The fact that adrenaline stimulation is abolished by ADP and the strong stimulating effect of glutamate suggest that oxaloacetate inhibition causes the decrease in the stimulating effect of adrenaline.

Increase in Ca^{2+} capacity was observed in early periods of pathogenic influences presented in the next paragraph. It will be shown that activation is followed by inhibition at later periods.

Prolonged pathological states—prevalence of cholinergic influences. We have observed the above-described simultaneous development of activation and inhibition of succinate-dependent Ca^{2+} accumulation under the development of all investigated pathologies. In initial steps activation prevailed over inhibition, and later *vice versa*.

The changes in Ca^{2+} capacity under allogenic transplantation of heart are presented in Fig. 3. As shown, these data for control animals are completely consistent with those for the control animal given in Fig. 1. Calcium capacity is minimal with only succinate; this is increased of addition of ADP and is not activated by glutamate. On the second day after transplantation, responses of mitochondria are close to those under adrenaline administration: Ca^{2+} accumulation under only succinate oxidation considerably rises, ADP does not stimulate this, and some glutamate stimulation appears. This indicates that together with activation of succinate oxidation, its inhibition appears. On the 7th day after transplantation, prevalence of inhibition over activation is obvious: Ca^{2+} capacity under succinate activation returns to a level close to the initial level, while glutamate activation is increased. It is worth of mentioning that the activating effect of ADP is restored, this possibly indicating a protective role of inhibition.

Calcium capacities after transplantation of allogenic lymphocytes are given in Table 2. Strong activation of

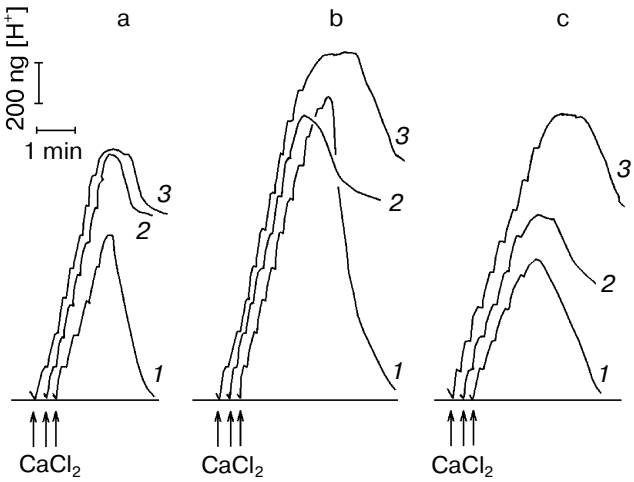

Fig. 3. Activation and inhibition of Ca^{2+} transport in rat liver mitochondria under transplantation of allogenic heart (registration with pH electrode): a) control rat; b, c) 2nd and 7th day after transplantation, respectively. Ca^{2+} capacity on addition of succinate (*1*), succinate and ADP (*2*), succinate, glutamate, and ADP (*3*).

Table 2. Calcium capacity in liver mitochondria after transplantation of allogenic activated lymphocytes in rat

Experiment	Ca^{2+} capacity, nmol H^+/mg protein				Increase in capacity, %	
	endogenous substrate	S	S + GL	S + ADP	S + GL/S	S + ADP/S
Control (n = 10)	80 ± 8 100%	213 ± 29 100%	240 ± 11 113	399 ± 33* 187	113	187
Lymphocyte transplantation:						
2nd day (n = 7)	273 ± 35 441	497 ± 30 233	564 ± 46 265	498 ± 32 234	113	101
7th day (n = 8)	112 ± 11 140	250 ± 24 117	397 ± 35* 186	291 ± 18 137	159	116

Note: Calcium capacities with either endogenous substrates or succinate in control animals are taken as 100% for the respective groups. Effect in % of the control is given on the second lines.

* p = 0.001.

Ca^{2+} accumulation is observed on the second day of the pathogenic influence. This is pronounced with added succinate, more than 2-fold, and even greater with endogenous substrates, more than 3-fold (Table 3). At the stage of activation, ADP stimulation disappeared as well as under adrenaline administration. Simultaneously with the described activation, Ca^{2+} capacity in the presence of glutamate is increased, which also suggests the development of inhibition of succinate oxidation. On the 7th day activation is considerably decreased, the value of Ca^{2+} capacity approaching the initial value. This is a consequence of increase of inhibition because the stimulating effect of glutamate rises.

The most pronounced activation of Ca^{2+} accumulation on the 2nd day after transplantation of allogenic tissues (Table 3) suggests a rise of endogenous succinate, which serves as the main substrate supporting Ca^{2+} transport [19]. Indeed the 3.5-4.5-fold increase of endogenous Ca^{2+} with succinate was found on the 2nd day after transplantation of both tissues, followed by its decrease almost to the initial level on the 7th day. Activation of succinate-dependent Ca^{2+} transport under short-term pathological states corresponds to changes under adrenaline administration, which suggests the prevalence of adrenergic influences in this period. Increase of endogenous succinate formation by adrenaline is carried out through activation of transamination [8]. The effect of adrenaline includes activation of succinate dehydrogenase [9-11], which increases the utilization of endogenous succinate.

On the 2nd day of experimental myocardial infarction, some increase of Ca^{2+} accumulation under only succinate oxidation is observed (Table 4). Glutamate produces 2-fold increase of this accumulation without ADP

Table 3. Pool of endogenous succinate and the rate of ATP synthesis in liver mitochondria under antigenic stress in rats

Experiment	Succinate pool		ATP synthesis	
	ng H^+/mg protein	%	ng H^+/min per mg protein	%
Control (n = 15)	28 ± 4	100	107.6 ± 8	100
Heart transplantation:				
2nd day (n = 16)	126 ± 9.5*	450	165 ± 19*	153
7th day (n = 19)	35 ± 7	125	109 ± 7	102
Control (n = 8)	36 ± 5	100	107 ± 7	100
Lymphocyte transplantation:				
2nd day (n = 7)	137 ± 10*	350	174 ± 21*	163
7th day (n = 7)	45 ± 7	125	125 ± 15*	117

* p = 0.01.

Table 4. Calcium capacity in liver mitochondria under myocardial infarction in rat

Experiment	Ca^{2+} capacity, nmol H^+/mg protein				Increase in capacity, %	
	S	S + GL	S + ADP	S + GL + ADP	S + GL/S	S + GL + ADP/S
Control (n = 2)	212	220	348	357	104	168
Myocardial infarction, 2nd day (n = 2)	296	632	430	875	214	296

and 3-fold with ADP. Therefore, in this case inhibition is developed earlier than under the transplantations.

The deepest inhibition of succinate-dependent Ca^{2+} accumulation was observed in mitochondria of liver biopsies in patients with chronic duodenal ulcer. A typical example for this pathology is given in Fig. 4. In this case, even the rate of accumulation of Ca^{2+} portions is lowered. Glutamate gives 3-fold activation of the process in the sample with succinate and 5-fold in the presence of ADP. Such magnitude of glutamate activation and consequently the depth of oxaloacetate inhibition were not observed in the states described above. Ulcer disease is a classic example of hypertrophy of cholinergic influences. The most pronounced oxaloacetate inhibition in this state is the additional indication of its relevance to the action of acetylcholine.

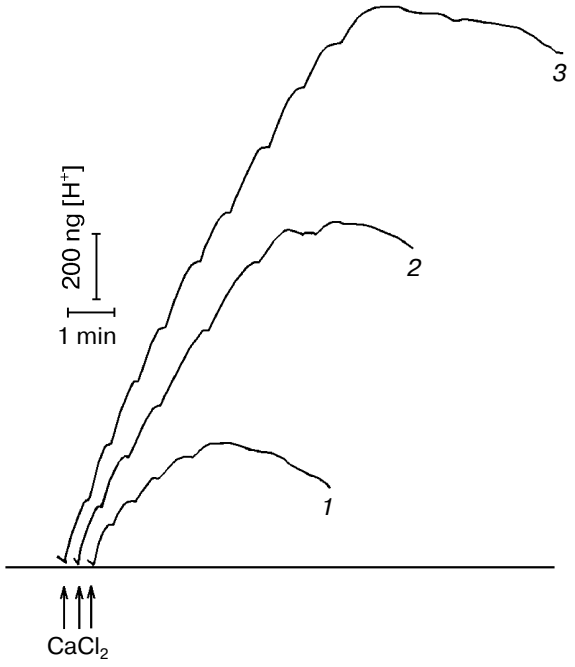

Fig. 4. Activation and inhibition of Ca^{2+} transport in human liver mitochondria in chronic duodenal ulcer. Ca^{2+} capacity on addition of succinate (*1*), succinate and glutamate (*2*), succinate, glutamate, and ADP (*3*).

DISCUSSION

Our investigations confirmed simultaneous development of activation and inhibition of succinate oxidation under states with prevalence of either adrenergic or cholinergic influences reported earlier for 1-day stress [3]. It is important to note that the magnitude of changes in Ca^{2+} capacity under similar states is considerably greater than in phosphorylating oxidation [20, 21, 27]. Results reported here also indicate greater functional sensitivity compared to phosphorylation (Tables 1-3). The increase in the rate of ADP phosphorylation under the influence of adrenaline is 44 and 61%, while that in Ca^{2+} capacity is 77 and 215%, respectively. The increase in ADP phosphorylation under transplantation of allogenic heart and lymphocytes is 53 and 63%, while that in Ca^{2+} capacity is 97 and 133%, and even 205 and 241% with endogenous substrates.

The greater sensitivity of Ca^{2+} capacity compared to ADP phosphorylation is probably due to two causes. Even a single (but even more several) addition(s) of Ca^{2+} serves as a considerably stronger load on the respiratory chain than ADP. The stronger load reveals more sensitively the working abilities of the tested object. It is necessary also to consider that Ca^{2+} mediates the effect of adrenaline on mitochondria [28-31]. Under conditions for measurement of phosphorylation, Ca^{2+} is absent. Therefore, the effect of the hormone on mitochondria is not manifested completely. The greater sensitivity of the Ca^{2+} test should be considered in investigations of physiological and pathological states in the body, which are usually formed by hormone regulation. This test is very convenient as an informative diagnostic criterion for clinical study of tissue biopsies [21, 32].

A new phenomenon—simultaneous development of activation and inhibition of succinate oxidation—is reported here. How can the simultaneous appearance of these two alternative, reciprocal processes in mitochondria be explained? The conclusion that this phenomenon exists is based on glutamate activation of succinate oxidation. Glutamate activation of respiration by elimination of oxaloacetate inhibition is well known. However, earlier this phenomenon was investigated in mitochondria of control animals during ageing of the preparation, addi-

tion of uncoupler, or other deenergizing influences *in vitro*. In such cases glutamate increased the rate of respiration only to the initial level in freshly prepared control mitochondria. In the present paper and in the other reporting the same effect [3] different animals were compared—control and under different influences. Such comparison revealed that in excited animals succinate oxidation or succinate-dependent Ca^{2+} transport in the presence of glutamate is considerably higher than in control. Similarly, Ca^{2+} accumulation with succinate was increased, but not decreased. Thus, changes detected indicate development of both activation and inhibition of succinate oxidation. The mechanism of such a double regulation can be considered as follows. Adrenaline activates succinate oxidation. Due to this, oxaloacetate formation is increased. Oxaloacetate inhibition limits activation. The state of succinate dehydrogenase in excited animal is different compared with control: enzyme is activated, and this activation is partially restricted and the enzyme is in "stress" conformational state.

Under prolonged influence of different factors *in vivo*, products of biogenic amine oxidation can participate in the inhibition of succinate dehydrogenase.

In the total population of mitochondria, organelles appear with the higher and lower activity than normal. This corresponds to the cytochemical measurements of succinate dehydrogenase activity in blood lymphocytes during pathologies [33]. Development of inhibition as a result of more strong activation (hyperactivation) [3] corresponds to the principle of *optimum* and *pessimum* of regulation stated by Vvedensky: stimulation increase leads to inhibition instead of activation of functions. This principle is effectively used in selection of mild physiological doses of adrenaline for the treatment of different diseases [34]. The dose of adrenaline should be diminished, not increased, for treatment of more severe pathological states. We suggest that the transition to succinate oxidation inhibition is not only impairment of mitochondria but can also provide restorative processes by switching over to α-ketoglutarate oxidation, GTP formation, and activation of GTP-dependent biosynthetic processes [14-16, 35]. Corrective (homeostasis) effect of α-ketoglutarate on succinate oxidation is indeed coupled with restoration of physiological functions and increase survival of animals under pathology [21]. Alternation of activity and rest of functional subunits in tissue is a general principle [2] that provides stability of organ function. Alternative efflux of catecholamines and acetylcholine is an initiating signal in this regulation.

The reciprocal regulation of mitochondrial processes reported here corresponds to the principle of reciprocal regulation of physiological functions discovered by Sherrington in the case of coordinated activity of muscle antagonists. Teppermen and Teppermen extended this principle on the activity of biochemical systems, revealing the existence of metabolic pathway antagonists, for example, gluconeogenesis and lipogenesis [36].

We investigated the action of hormones, adrenaline and acetylcholine, which can be considered as antagonists. Their reciprocal effect on succinate-dependent Ca^{2+} capacity was revealed. This phenomenon in essence corresponds to the principle of reciprocal regulation of metabolism. Moreover, this is not the only example of such regulation, but the mechanism of the Sherrington principle at the level of mitochondria because reciprocal activity of muscle antagonists is regulated by the interaction of adrenaline and acetylcholine. As this interaction is a general mechanism of formation of states in the organism, the phenomena reported can be considered as mechanisms of general adaptation reactions at the level of mitochondria.

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