INHIBITION OF THE METABOLISM OF NUCLEATED RED CELLS BY INTRACELLUAR IONS AND ITS RELATION TO INTRACELLULAR STRUCTURAL FACTORS

by

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INTRODUCTION

The fundamental investigations of MEYERHOF, EMBDEN, WARBURG, CORI and others on the anaerobic metabolism of the skeletal muscle, yeast and blood cells, and the discovery of the role of dicarboxylic and tricarboxylic acids in the oxidative metabolism of animal cells by SZENT-GYÖRGI AND KREBS and of the mechanism of the hydrogen transfer to oxygen by KEILIN AND WARBURG laid the foundations of our knowledge of the nature of chemical reactions providing the energy for cell activities. MEYERHOF's work elucidated the correlation between certain oxidative and anaerobic enzyme reactions and certain phases of muscle activity. In general, however, our knowledge of the integration of enzyme reactions involved in aerobic metabolism into the organisation of the cell and its mechanism is rather inadequate.

The cell metabolism is not a static phenomenon. Any increase in cell activity following stimulation is accompanied by a very considerable increase of the oxidative cell metabolism. The latter goes on mainly at the expense of glucose taken up from the environment or glycogen present in the cell. There is some evidence scattered in the literature that the mechanism of this part of the oxidative metabolism of sugar, which appears after stimulation may not be completely identical that with of the oxidative metabolism of the resting cell. This evidence was obtained from the study of the metabolism of cells stimulated *in vitro*. In 1936 DEUTSCH AND RAPER[1] made the important observation that slices of glandular tissue (salivary gland, pancreas, liver) increase their O_2 uptake several times, when treated with certain hormones like acetyl choline, adrenaline and secretin, which *in vivo* stimulate the specific activities of those glands. Specific pharmacological stimulants of glands like pilocarpine showed the same effect. The increase is temporary, lasting about 30–60 minutes. It can, however, repeatedly be fully reproduced by a new dose of a stimulant some times after the preceding stimulation. Adrenaline provokes the increase in respiration only with salivary glands which can be physiologically stimulated by the sympathetic and adrenaline.

This fact, the reproducibility of the metabolic response to stimulants after a period of recovery and its temporary character, strongly suggest that this metabolic process

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in vitro is essentially with the metabolic response to stimulation *in vivo*. This is further borne out by observations of BROCK, DRUCKEREY AND HERKEN[2] who confirmed the findings of DEUTSCH AND RAPER. They calculated the metabolic turnover of the whole salivary gland from the values obtained *in vitro* on slices and found after stimulation values which agreed well with values obtained by BARCROFT AND PEPER[3] on the salivary gland stimulated *in vivo* by chorda tympani. They found, furthermore, that the "stimulation metabolism", as they call the metabolic response of tissue slices to stimulants, depends on the ionic equilibrium in the RINGER solution in which the slices are suspended. Complete removal of the Ca from the RINGER suppresses completely the stimulation response, which can be restored by the subsequent addition of Ca. The removal of K ions does not suppress the first response but prevents the recovery. The ionic equilibrium in the medium is essential for the structural integrity of the cell or at least its surface membrane. It is therefore clear that the stimulation response requires the integrity of the cell structure and cannot be a consequence of injury and structural disintegration.

The stimulation metabolism shows two significant features as compared with the basic or rest metabolism: 1. the latter has a *R.Q.* below 1 while the excess respiration after stimulation has a *R.Q.* of 1, indicating a pure carbohydrate metabolism; 2. the increase in O_2 uptake is always paradoxically accompanied by a production of free acids, of which at least half was shown by DEUTSCH AND RAPER to be lactic acid[1]. BROCK, DRUCKEREY, AND HERKEN[4] have shown that this production of acid does not occur when K ions are removed from the surrounding medium, although the increase in respiration appears unchanged in size after the first stimulus.

The characteristic metabolism response to hormonal or pharmacological stimuli is by no means a peculiarity of glandular tissues. The increased respiration of the sea urchin egg after fertilization shows all the characteristic properties of the stimulation metabolism of glands[5]. The production of free acid in this case was found by RUNNSTROM, although the nature of the acid was not definitely established. As in glands there is also a marked difference in the sensitivity towards HCN between the respiration of the unfertilized and that of the fertilized egg. And according to BROCK *et al.* a hormonal extract of the anterior pituitary which influences the division of the egg provokes the same characteristic metabolic response in it as fertilization. This cannot be obtained with extracts which do not influence the cleavage of the egg.

Finally a similar metabolic response was observed in 1937 by GOTTDENKER AND MARCHI[6] on mammalian heart lung preparations. They found that adrenaline, which is a heart stimulant, increased the O_2 uptake of these preparations and at the same time provoked an intensive lactic acid production.

The fact that the increased respiration in stimulated tissue slices goes on at the expense of carbohydrates and is accompanied by formation of lactic acid only under physiological conditions of the medium suggests a certain interpretation of the mechanism of this metabolic phenomenon. The anaerobic glycolysis of the glands is completely suppressed by the basic respiration due to the PASTEUR effect. Any factor leading to a deteriorization of the structural integrity of the cell tends to provoke an aerobic glycolysis. This is the case for instance with liver or brain slices when K is removed from the medium. The aerobic glycolysis accompanying the stimulation response differs in this respect fundamentally in being dependent on the presence of K ions in the medium and is suppressed completely after their elimination. This indicates clearly that aerobic glycolysis of stimulation is not due to structural damage or increase of per-

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meability, but to a specific coupling between the oxidative breakdown of sugar and glycolysis. Now it is reasonable to assume that phosphorylation of glucose to hexosediphosphate constitutes the first steps in glycolysis. Any coupling between glycolysis and respiration therefore will consist primarily in a coupling between certain oxidative processes and phosphorylation of glucose. It is well known that the oxidation of pyruvic acid in the KREBS cycle is coupled with an intensive phosphorylation of glucose and adenylic acid (to ATP). Certain individual enzyme reactions in the KREBS cycle, like oxidation of the succinic and α-ketoglutaric acid, have been shown to be coupled with phosphorylation of glucose and adenylic acid[7]. Quite recently the same was shown for the electron transfer from dihydrocozymase to the cytochrome system[8]. OCHOA[9] has shown for heart muscle extracts that complete oxidation of one molecule of pyruvate can be coupled with the phosphorylation of 9 molecules of glucose to hexosediphosphate. The oxidation of 1 molecule of glucose over the KREBS cycle therefore can phosphorylate 18 molecules of glucose. That this excess phosphorylation does not appear in resting cells must be ascribed to the coupling of the phosphorylation of glucose with oxidative processes in such a way that the speed of these processes does not exceed the maximal speed of oxidation of pyruvate. If the KREBS cycle is operating and these controls are eliminated, aerobic glycolysis or accumulation of hexosephosphate must result. All these considerations suggest that the metabolic response to stimulation in organs may be due to a release or increase of the activity of the tricarboxylic acid system and accompanying phosphorylation. In the metabolism of resting cells this system may play only a minor role or be lacking altogether. This view appears supported by the fact that cells like embryonic and tumor cells, *et al.*, which according to BROCK do not show any stimulation response *in vitro*, show only very weak activity of enzymes belonging to the tricarboxylic acid system.

Turning to the consideration of possible mechanisms involved in the release of the metabolic response to stimulation we must keep in mind that every cell responds to stimulation by the electric current essentially in the same way as to that by nervous impulses or hormonal and pharmacological stimuli. The primary effect of the electric stimulus consists in shifts of intracellular ions. It is generally assumed that such shifts, with consecutive accumulation of certain ions on intracellular membranes, are responsible for the functional response to stimulation. It may reasonably be assumed that such shifts of intracellular ions are also instrumental in provoking the metabolic response. As the latter can be more protracted than the functional response the effects of ionic shifts must be more complex in this case and consist in a chain of reactions released by the primary shift. The ions could exert their influence either directly on enzymes involved in the stimulation metabolism or indirectly by changing the permeability of intracellular membranes and thus facilitating the access of substrates to certain enzymes.

It was observed recently[10] that hemolysates of nucleated red cells of pigeon glycolyse only in presence of oxygen. This aerobic glycolysis disappears in presence of M/500 NaCN. It was further found that all intracellular polyvalent ions like Mg, Ca, orthophosphate, ribonucleate inhibit the aerobic glycolysis in physiological concentration. COLOWICK, KALCKAR AND CORI[11] found in 1941 a similar obligatorily aerobic glycolysis in kidney extracts and showed that it is dependent upon the oxidation of succinic acid. As it was known that nucleated red cells are able to oxidise pyruvic acid to CO_2 and that their respiration is coupled with the synthesis of ATP it seemed reasonable to assume that the aerobic glycolysis in hemolysates of these cells is the result of the coupling

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of phosphorylation of glucose with the oxidative processes of the KREBS cycle. The inhibitory effects of ions on the aerobic glycolysis suggested that we are here in presence of an enzymatic system displaying this sensitivity towards ions which underlies the mechanism of the metabolic response to cell stimulation.

The possible general physiological significance of this phenomenon invites closer investigation of its mechanism. The present report deals with experiments in this direction.

EXPERIMENTAL

A. Preparation of the material

Red blood cells of pigeons were used for the experiments. The animals were kept fasting for at least 12 hours preceding the bleeding, which was carried out by cutting the throat on one side after removal of feathers. The blood was caught in a dish containing 0.3 ml of 3.6% sodium citrate. It was centrifuged and the upper stratum of the sediment, containing the white cells, was removed as far as possible by pipetting. The remaining red cells were first washed twice with a fivefold volume of a mixture of 1 part 3.6% sodium citrate and 9 parts of 0.9% NaCl and then 3 times with the NaCl solution. The washed cells were hemolyzed by adding 1.5 parts of distilled water to 1 part of cells. The p_H of these hemolysates was found to vary between 7.25 and 7.15. As it was intended to investigated the effect of salts on the metabolism of the hemolysate it was not possible to use buffers in our experiments and we had to rely for the stabilization of p_H during the experimental period on the considerable buffering capacity of hemoglobin. Orienting experiments, however, showed that the shift of p_H due to acid formation during 4 hours at 25° did not exceed 0.2. The optimal p_H for the aerobic metabolism was found to be about 6.8. In most of our experiments the p_H at time 0 was therefore that of the original hemolysate or slightly lower, *i.e.*, 6.9–7.0. The latter was obtained by adding an appropriate amount of diluted HCl to the water used for hemolysis.

B. Analytical methods

In a certain number of experiments a complete balance of O_2 uptake, CO_2 production, and glucose consumption was carried out. In these and most of the other experiments the total volume of either water or of respective solutions added to the hemolysate was 0.2 ml per 1 ml of the original hemolysate. The final dilution of the original cell suspension was therefore threefold. All experiments were done at 25° and lasted as a rule four hours. The O_2 uptake was measured on 2 ml of the hemolysate in standard BARCROFT-WARBURG manometers with absorption of CO_2 and NH_3. This shifted the p_H of the hemolysate no more than 0.1 to the alkaline side. CO_2 production was determined by the direct method. To account for the retention of CO_2 by the hemolysate the manometer in which CO_2 was not absorbed contained in a second sidearm 0.4 ml of diluted H_2SO_4. At the end of the experiment the acid was tipped in from the sidearm into the hemolysate. The p_H of the latter was then shifted below 4. The hemolysate became very viscous at this p_H but came into the equilibrium with the gas phase after about 30 minutes. As the hemolysate contained from the beginning a certain amount of bound CO_2 the same procedure was carried out on a sample of the hemolysate at time 0. The difference of the increase in gas volume after addition of acid in the two samples gave the amount of CO_2 produced by oxidation and retained by the hemolysate. At the end of the experiment 1 ml of the hemolysate was pipetted out of the manometer vessels, deproteinezed with 4 ml of 7.5% trichloracetic acid. The centrifugate served for the determination of lactic acid and glucose and phosphate fractions. The lactic acid determination was carried out by the procedure of BARKER AND SUMMERSON[12], glucose by the new spectrophotometric micromethod of DISCHE, SHETTLES AND OSNOS[13] based on a specific reaction of hexoses with cysteine in H_2SO_4. In this reaction fructose gives only 12% more absorption than the equivalent of glucose, so that the phosphorylation of a small amount of the latter to HARDEN-YOUNG ester will not influence significantly the accuracy of the determination. In some experiments we tested for this ester and triosephosphate by a new highly sensitive reaction with carbazole, which allows the determination of fructose and triosephosphate in the same sample. Inorganic and the labile phosphate were determined by the FISKE-SUBBAROW method in the modification of KING, ribose and adenosine-5-phosphate by the orcinol reaction.

C. Results

In a first series of experiments the aerobic metabolism of the hemolysate was examined to obtain information about the nature of enzyme reactions involved in this

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metabolism. In a second series the influence of various cations and anions on those reactions was investigated.

1. *The aerobic metabolism in the hemolysate*

a) *O_2 uptake, lactic acid formation in the hemolysate in absence of glucose.* The hemolysate to which 0.2 ml of liquid per ml was added shows a marked respiration which varied in our experiments between 19 and 92 cmm per 1 ml and 4 hours. The respiration is in general much higher during the first hour and drops afterwards to a lower but constant level. The *R.Q.* varies considerably between 0.82 and 1 (Table I). The erythrocytes contain very little hexoses soluble in trichloracetic acid. Less than 1 γ/ml of hexose (calculated as glucose) was found in the hemolysate. This amount does not change during the 4 hours of the experiment. On the other hand there is a considerable decrease in the amount of adenosine-5-phosphate. In experiment VI (Table I) 84 γ/ml of this compound, corresponding to 35 γ/ml pentose, disappeared in 4 hours. If all of this pentose had been oxidized to CO_2 half of the total O_2 uptake in this experiment would be accounted for. The breakdown of adenosine-5-phosphate can be explained by the fact that it is formed in the hemolysate by the ATPase and dephosphorylated to adenosine which, as was shown for human erythrocytes, can be split, with phosphorylation, to form triosephosphate and hexosediphosphate. One part of the respiration of the hemolysate in absence of glucose must be due to the oxidation of either fat or protein. The hemolysate contains from the beginning very small amounts of lactic acid (about 5 γ/ml). In some cases small amounts of this acid are formed during incubation, but not more than about 5 γ/ml.

b) *The tricarboxylic acid cycle in the hemolysate.* The presence of this enzyme system in the hemolysate can be demonstrated after addition of citrate or one of the dicarboxylic acids metabolised by the system. When M/1200 of succinic, fumaric, malic, oxaloacetic, citric and α-ketoglutaric acid is added the O_2 uptake increases considerably (Table I). In presence of ketoglutaric and citric acid much more than in that of other acids this additional O_2 uptake increases with the concentration of the acid. It is about twice as great in presence of M/600 succinate than of M/1200. At the same time lactic acid is formed in significant amounts. This increases with the concentration of succinate or malate. The amount of lactic acid varies with the nature of the acid in the following sense: malate, fumarate > succinate > α-ketoglutarate > citrate. This can be explained by the assumption that oxaloacetic is formed from malic acid, with reduction of coenzyme I to dihydrocoenzyme I. One part of the oxaloacetic acid is decarboxylated to pyruvate and CO_2. As the cytochrome system is not able to oxidize dihydrocozymase rapidly enough, one part of it reduces pyruvate to lactate. The same sequence of reactions was observed by E. A. EVANS[14] in liver extracts. As the increase in succinate increases the O_2 uptake as well as lactic acid formation the cytochrome system apparently competes with the pyruvate for dihydrocozymase. Thus the fact that lactic is formed from citrate indicates that the whole series of reactions from citrate to oxaloacetates goes on in the hemolysate. Pyruvic acid also increases the respiration and lactic acid formation, though less than any one of the polycarboxylic acids, and the increase is observed only during the last 3 hours of the 4 hour period.

2. *Aerobic metabolism in presence of glucose*

When 50 mg % of glucose is added to the hemolysate it is broken down at a rate

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TABLE I

INFLUENCE OF $MgCl_2$ M/250 ,OF PYRUVIC, CITRIC AND DICARBOXYLIC ACIDS OF THE KREBS CYCLE ON THE O_2 CONSUMPTION OF THE HEMOLYSATE IN PRESENCE AND ABSENCE OF GLUCOSE AND ON AEROBIC GLYCOLYSIS. TIME OF EXP.: 4 h THE BRACKETED VALUES REPRESENT THE O_2 UPTAKE IN THE LAST 3 h

Exp. No.	Substance added	O_2 uptake in 1/ml of hemolysate in μl				Aerobic glycolysis	
		by hemolysate itself	change %	glucose in the hemolysate	change %	γ lactic acid γ/ml of hemolysate	change %
I	o	43.5 (31)		8.2 (3.0)		168	
	a. $MgCl_2$ M/250	54 (32.4)	+ 24 (+ 5)	15 (12.5)	+ 84 (+ 320)	245	+ 46
	b. Na succinate M/1540	52 (35.6)	+ 20 (+ 16)			218	+ 30
	a + b	75 (47.5)	+ 70 (+ 53)			259	+ 54
II	o	34.2 (23.8)		8.3 (2.4)		220	
	$MgCl_2$ M/250	45 (28.5)	+ 31.6 (+ 19.9)	13.9 (11)	+ 67 (+ 360)	265	+ 21
	Na succinate M/770	60.2 (34)	+ 76 (+ 43)				
III	o	29 (18.7)		6.7 (8)		139	
	$MgCl_2$ M/250	34.3 (25.5)	+ 18 (+ 36)	12.6 (10.1)	+ 88 (+ 26)	185	+ 33
	Na Pyruvate M/1200	21.7 (21.7)	— 25 (+ 16)	14 (5)	+ 10.9 (— 37)	156	+ 12
IV	o	44.3 (27)					
	Na succinate M/1200	54 (37)	+ 22 (+ 37)				
	Na citrate Na /1200	60 (41)	+ 35 (+ 51)				
	Na α-keto glutarate M/1 200	67 (42.5)	+ 52 (+ 57)				
V	o	32.4 (21)					
	Na succinate M/1 200	49 (34.3)	+ 51 (63)				
	Na citrate M/1 200	55.4 (41)	+ 71 (+ 95)				
	Na α-keto glutarate M/1 200	64 (43)	+ 100 (+ 102)				
VI	o	45.6 (30.1)		6 (10.5)		262	
	Na pyruvate M/1 200	48 (34.6)	+ 5.3 (+ 15)	o (o)	— 100 (— 100)	258	— 1.5
VII	o	35 (19.1)		17.5 (12.3)		164	
	Na succinate M/1 200	55 (36.1)	+ 57 (+ 89)	13.3 (12.4)	— 24 (+ 1)	218	+ 33
	Na pyruvate M/1 200	46.5 (29.9)	+ 33 (+ 55)	6.7 (6.1)	— 62 (— 50)	169	+ 3
VIII	o	75.6 (38.6)		8.6 (20.7)		276	
	Na pyruvate M/1 200	78.7 (47)	+ 4.1 (+ 22)	14.3 (8.9)	+ 66 (— 58)	246	— 11
IX	o	27.8 (18.7)		13.3 (10.3)		159	
	NaCN M/500	2.5 (2.9)	— 91 (— 87)	3.3 (1.2)	— 75 (— 88)		
	NaCN (M/250	o (o)	— 100 (— 100)	o (o)	— 100 (— 100)	13.1	— 92

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of 75–150 γ/ml per hour. The O_2 consumption increases at the same time considerably by 13–50% in 4 hours. At the same time an intensive aerobic glycolysis and sometimes esterification of inorganic P to difficultly hydrolyzable esters is observed. Up to 260 γ/ml of lactic is produced in 4 hours. The rate of O_2 consumption during the first hour is different from the rate in the following 3 hours during which it remains almost constant. The rate of glycolysis is in general smaller during the first hour than later. If we assume that the additional O_2 consumption in presence of glucose is due to the total oxidation of the latter and calculate the total breakdown of glucose by oxidation and glycolysis the latter turns out to be considerably smaller than the amount of glucose which really disappeared. The *R.Q.* of the additional respiration due to glucose is only about 0.7 (Table VI). The discrepancy between the observed values and those calculated for glucose which disappears indicates that only one part of it is completely oxidized while another part is oxidized either to phosphogluconic or pyruvic acid.

3. *The coupling between aerobic glycolysis and respiration*

The glycolysis of the hemolysate is obligatorily aerobic and disappears almost completely when the oxidation processes in the hemolysate are suppressed either by inhibitors or by elimination of O_2. Thus NaCN at M/250 almost completely suppresses the glycolysis and 90% of the total O_2 consumption. (Table I) Further increase of the concentration does not have any significant effect. The small residual glycolysis amounts to only a few per cent of the total and is probably due to the leucocytes which were not removed. The leucocytes which are siphoned off in the beginning of the blood washing display in fact a powerful anaerobic glycolysis which is partly suppressed in aerobiosis. That the effect of cyanide on glycolysis is due to the blocking of respiration could be shown in experiments in which O_2 was removed from the hemolysate. These were carried out in the following way. 4 ml of the hemolysate + 0.8 ml of 0.3% glucose solution were pipetted into a 500 ml flask which was closed by a ground stopper with stopcock. The flask was weighed and then evacuated first with a water pump. When the foaming of the fluid became too intense the evacuation was interrupted until the foam broke down and the evacuation then resumed until no more gas escaped. The evacuation was continued with the oil pump until a vacuum of about 1 mm Hg was obtained. The flask was then weighed again to determine the loss in water. The hemolysate was kept *in vacuo* for 4 hours at room temperature and then the flask opened, the evaporated water replaced and the hemolysate deproteinized simultaneously with a control, which stayed during the same period in presence of oxygen and one to which NaCN M/500 was added. The determination of lactic acid showed that the glycolysis was suppressed in the sample *in vacuo*, though not quite as far as in the sample with NaCN.

While suppression of the O_2 consumption inhibits the glycolysis in our hemolysate any increase of O_2 consumption after addition of pyruvate, citrate and dicarboxylic acids of the KREBS cycle is accompanied by a strong increase of glycolysis (Table II). If the final dilution of the hemolysate is no more than the threefold of the original volume of the suspension, α-ketoglutarate is most effective, with succinate and fumarate following, and pyruvate the least effective. It was found for the succinate that the promoting effect on glycolysis increases with the concentration, as also does the O_2 consumption.

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TABLE II

INFLUENCE OF PYRUVATE, CITRATE AND DICARBOXYLIC ACIDS OF THE KREBS CYCLE ON AEROBIC GLYCOLYSIS IN THE HEMOLYSATE. TIME 2 HOURS, T 25°

Experiment No.	Substance added	mg lactic acid/ml of hemolysate	Change %	pH
I	o	46		7.0
	M/600 $MgCl_2$	73	+ 58	
	M/1 200 succinate	96	+ 109	
	M/600 $MgCl_2$ + M/1 200 succinate	131	+ 185	
II	o	58		7.0
	M/600 $MgCl_2$	81	+ 40	
	M/1 200 succinate	100	+ 72	
	M/600 + M/1 200 succinate	120	+ 108	
III	o	42		7.0
	M/1 200 succinate	54	+ 29	
	M/300 $MgCl_2$	80	+ 90	
	M/1 200 Na pyruvate	39.5	— 6	
	M/1 200 Na pyruvate + M/1 200 succinate	53	+ 29	
IV	o	103		6.8
	M/1 200 succinate	151	+ 46	
	M/800 succinate	158	+ 53	
V	o	35		7.1
	M/400 succinate	47	+ 34	
	M/400 α-ketoglutarate	44	+ 26	
VI	o	7.5		7.0
	M/400 succinate	29.5	+ 300	
	M/1 000 pyruvate	15	+ 100	
VII	o	48		7.2
	M/1 200 succinate	123	+ 156	
	M/800 succinate	132	+ 175	
VIII	M/800 α-ketoglutarate	168	+ 250	
	M/1 200	100	+ 108	
IX	o	42		7.2
	M/450 succinate	86	+ 105	
	M/450 malate	64	+ 57	
X	o	24		7.2
	M/450 succinate	52	+ 116	
	M/900 succinate	38	+ 58	
	M/450 malate	41	+ 70	
	M/900 malate	28	+ 17	

4. *Influence of ions on the aerobic metabolism in presence and absence of glucose*

Two different effects of ions on the aerobic metabolism in the hemolysate can be observed. The first is specific for magnesium ions and the second is common to all multivalent ions. In this second group, the nature and the charge of the ion is important for the intensity of the effect.

a) *Magnesium.* In concentrations up to M/200 $MgCl_2$ increases the basic O_2 consump-

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tion in the hemolysate as well as the additional uptake in presence of glucose and the dicarboxylic acids. The increase ranges from 18 to 24% for the basic respiration and from 67 to 88% for the additional respiration due to glucose (Table I). At the same time there is an increase of the aerobic-glycolysis amounting to 21–46% of the original value (Table I, Exp. I–III). This effect of Mg reaches its maximum at M/200 to M/150. The additional O_2 uptake as well as the accompanying aerobic glycolysis are inhibited by M/500 NaCN to the same extent as is the case without addition of Mg.

b) Univalent cations. When so much KCl is added to the hemolysate that the concentration of the added salt in the hemolysate becomes 1/11 M and the hemolysate therefore isotonic no inhibition of the basic O_2 uptake with and without glucose can be observed. The aerobic glycolysis is in general somewhat decreased. In some cases, however, a decrease of 60% was observed. NaCl at the same concentration decreases the O_2 uptake moderately and inhibits the aerobic glycolysis 33–50%. It must be noted that this concentration of Na ions cannot be considered any more as physiological. If the concentration of the added NaCl was only M/25 no significant inhibition of the O_2 uptake or aerobic glycolysis could be observed. These observations indicate that Cl ions in physiological concentrations do not have any significant effect on the aerobic metabolism of the hemolysate.

c) Calcium and other multivalent cations. When the concentration of Mg exceeds M/150 the aerobic glycolysis in the hemolysate begins te decline. At M/80 an inhibition of about 15–25% appears. This inhibitory effect is a property of all multivalent cations. (Table IV). Of all the cations investigated Ca shows the strongest inhibitory effect. M/1000–M/1500 shows almost complete inhibition of the aerobic glycolysis. Sr is almost as strong but Ba^{++}, Ce^{+++} and La^{+++} are ten times weaker inhibitors. However, our figures merely correlate the strength of the inhibition with the overall concentration of the salt. The latter is almost identical with the concentration of the bivalent ions for the earth alkalis and rare earth but not for the other metals, the hydroxides of which possess low second dissociation constants. The ion Mn^{++} and Cd^{++} as such are, therefore, probably stronger inhibitors than Ca^{++}. This however does not seem of any physiological significance. The inhibitory effect of Ca on the glycolysis is still perceptible at M/8000. After having ascertained that the inhibitory effect of Mg and Ca on glycolysis is related to their multivalence the effects on the O_2 consumption of those two as representatives of multivalent cations were studied. The basic O_2 consumption was inhibited 28–52% by Ca M/1000. The oxidation due to glucose, however, may completely disappear at this concentration while that of succinate and α-ketoglutarate is reduced to about the same extent as the basic respiration (Table III).

d) Anions. All multivalent anions inhibit strongly the aerobic glycolysis (Table III). The importance of valency is more marked with anions than cations. The bivalent HPO_4^{--} and SO_4^{--} show a significant inhibition only at M/100 and M/50 respectively, while the tetravalent $Fe(CN)^{----}$ at M/250, ribonucleate, diphosphoglycerate and inositolhexaphosphate strongly at M/1500, M/700 and M/1000 respectively. The nature of the ion plays, however, also a considerable role. The bivalent oxalate for example shows at M/1000 a stronger inhibition than malonate at M/200. The physiological polycarboxylic acids like succinate and citrate, which up to M/500 increase the aerobic glycolysis, inhibit at higher concentrations. At M/50 the inhibition is considerable with citrate. That multivalency is only one of the factors promoting the inhibitory effect on the metabolism is shown by the behaviour of the CNS^- ion. While KCl at M/11 and

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TABLE III

EFFECT OF KCl, NaCl, $MgCl_2$ AND OF MULTIVALENT IONS ON THE O_2 CONSUMPTION BY THE HEMOLYSATE ITSELF AND BY GLUCOSE, SUCCINATE, α-KETOGLUTARATE IN THE HEMOLYSATE. TIME 4 HOURS

Experi-No.	Substance added	By hemolysate itself		By glucose		By succinate M/1 200		By α-keto glutarate M/1 200	
		O_2 used	Inhibition %	O_2 used	Inhibition %	O_2 used	Inhibition %	O_2 used	Inhibition %
I	Mg M/250	54		15					
	Mg M/250 + Ca M/2000	48.5	10	8.2	45				
II	Mg M/250	45		13.6					
	Mg M/250 + Ca M/1000	21.5	52	2	93				
III	0	92				21.8		40.2	
	Ca M/1000	50.6	45			14.3	34	25.4	37
IV	0	45.6		5.9					
	Ca M/1000	24.7	46	0.3	95				
	Mg M/250	45.6		21.3					
	Mg M/250 + Ca M/1000	23.6	48	11.5	46				
V	0	35		17.5		20			
	Ca M/1000	18.9	46	4.3	76	5.9	70		
VI	0	75.6		8.7					
	Ca M/1000	34	55	4.1	53				
VII	0	32.1		10.5					
	Ca M/1000	23	28	0.0	100				
VIII	0	27.8		13.4					
	KCl M/12	29.4	—	14	—				
IX	0	48		11		29.4			
	PO_4 M/500	40.3	16	1	90				
	Oxalate M/500	41.6	14	6.2	38	16.3	45		
X	0	44.3				10.1		23.3	
	Oxalate M/500	29.5	34			19	—	32.5	—
XI	0	32.4		10		16.8		31.4	
	Oxalate M/250	17.8	46	0.7	93	11	34	32.6	—
XII	0	60.2		21		19.2		29.5	
	Na_2SO_4 M/24	47.4	21	4.1	80	16	17	26.6	10
XIII	0	56.3							
	NaCl M/12	51.6	8						
	NaCl M/25	68.4							
	KCl M/12	56.3							
XIV	0	56		14.2					
	Ribosenucleic acid M/1500	43.1	23	3	80				

PH 7.2 does not inhibit at all or only little, KCNS at the same concentration completely inhibits glycolysis (Table III).

The O_2 consumption is suppressed by anions to about the same extent as glycolysis.

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TABLE IV

INHIBITION OF AEROBIC GLYCOLYSIS BY VARIOUS CATIONS AND ANIONS. TIME: EXPERIMENT I–X 4 HOURS, EXPERIMENT XI 2 HOURS

Experiment No.	Substance added	γ Lactic acid formed in 1 ml of hemolysate	Inhibition %	pH
I	0	62		7.25
	HCN M/500	11.2	82	
	$CaCl_2$ M/1 000	11	82	
	$BaCl_2$ M/333	20.7	66	
	SO_4 M/333	18.6	70	
	$MnCl_2$ M/333	11	82	
	$CaCl_2$ M/333	11	82	
II	0	71.8		7.1
	$SrCl_2$ M/800	5	93	
	$FeSO_4$ M/333	11.9	83	
	$CdSO_4$ M/333	5.8	92	
	HCN M/500	0.07	99	
		0.64		
III	0			
	2,3 diphosphoglycerate M/500	39	39	7.2
	Inositol hexaphosphate M/700	22.1	65.5	
IV	0	37.2		
	$CaCl_2$ M/4 000	19.4	48	
	Phosphate M/50	28.3	23	
	Na_2SO_4 M/50	18.4	50	
	Na Citrate M/50	5.2	86	
V	0	46.5		
	Ribosenucleic acid M/1 600	37.2	20	7.25
	Yeast adenylic acid M/400	48	—3	
VI	0	164		
	$CaCl_2$M/1 000	30	82	7
	Na Succinate M/1 200	218		
	$CaCl_2$ M100 + Na Succinate M/1 200	27	83	
VII	0	64		7.2
	NaCl 1/11	65		
	KCl 1/9	33	48	
VIII	0	36		
	KCl 1/11	35	3	7.2
	$MgCl_2$ M/250	45		
	$MgCl_2$ M/250 + KCl 1/11	37	17	
IX	0	227		6.9
	NaCl M/11	128	43	
	KCl M/11	85	63	
X	0	290		6.9
	KCl M/11	158	45	
	NaCl M/11	163	43	
	Ribonucleate M/1 540	169	42	
XI	0	103		
	M/300 $MgCl_2$	177		7.0
	M/1 200 Na Succinate	151		
	M/1 000 $CaCl_2$	34	97	
	M/1 000 $CaCl_2$ + $MgCl_2$ M/300	13.6	87	
	M/1 000 $CaCl_2$ + M1 200 Succinate	16	85	

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Different oxidation processes, however, are influenced to a very different degree. The oxidation of glucose suffers much more than the basic oxidation. M/480 sodium oxalate suppresses the additional respiration by glucose 80–100%, the basic only 0–15%. Essentially the same relation is valid for M/25 Na_2SO_4, M/50 Na phosphate and M/1700 Na ribonucleate. The oxidation of succinate is less suppressed than that of glucose but more so than that of α-ketoglutarate and citrate.

e) Synergy between Mg and Ca and anions in their inhibitory effects. Effects of ions on colloidal particles are in general counteracted by ions of opposite charge if the effects are due to neutralization of electric charges It is, therefore, surprising that inhibitory effects of anions on the metabolism of red cells are not eliminated or decreased, but on the contrary strongly enhanced by Mg and Ca (other multivalent cations

TABLE V

SYNERGY BETWEEN Mg^{++} AND Ca^{++} AND MULTIVALENT ANIONS IN THEIR EFFECTS ON THE AEROBIC GLYCOLYSIS OF THE HEMOLYSATE IN PRESENCE OF GLUCOSE. TEMP. 25°. TIME: EXP. I–V 4 HOURS, EXP. II 2 HOURS

Experiment No.	Inhibitor	Lactic acid formed in mg/ml of hemolysate	Inhibition %	PH
I	a. o	36		7.2
	b. Na phosphate M/50	25	30	
	c. $CaCl_2$ M/4000	34	6	
	b. + c.	4.3	88	
	d. $MgSO_4$ M/150	38.5		
	e. KCl M 1/10	35	3	
	d. + e.	33	8	
II	a. o	71		7.15
	b. $CaCl_2$ M/4000	50	30	
	c. Na phosphate M/50	55.4	22	
	b. + c.	28.3	60	
III	a. o	46.5		7.2
	b. $CaCl_2$ M/4000	34	27	
	c. Inositol hexaphosphate M/1000	31	33	
	b. + c.	6.6	86	
	d. Na_2SO_4 M/100	41.4	11	
	b. + d.	9.2	80	
	e. $MgCl_2$ M/250	62		
	e. + d.	38.1	38	
IV	a. o	33		7.2
	b. phosphate M/50	41	— 24	
	c. $MgCl_2$ M/250	66		
	b. + c.	40	40	
V	a. o	202		7.25
	b. $K_4Fe(CN)_6$ M/1000	17.5	13	
	c. KCNS M/90	20.4	— 1	
	d. $MgCl_2$ M/250	56.3		
	b. + d.	33.8	40	
	c. + d.	33.8	40	
VI	a. o	112		6.8
	b. $CaCl_2$ M/4000	99	12	
	c. phosphate M/90	90	20	
	b. + c.	41	63.5	

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were not investigated). M/100 Na_2SO_4 and Na phosphate, M/1000 $K_4Fe(CN)_6$ and M/90 KCNS which by themselves show little or no inhibition of aerobic glycolysis, strongly inhibit in presence of M/250 $MgCl_2$, which by itself increases the glycolysis. The inhibition by M/4000 Ca in presence of M/100 Na_2SO_4 or Na phosphate is much stronger than corresponds to the sum of inhibitions of the two kinds of ions (Table IV). This synergy manifests itself also towards the oxidation of glucose as well as towards the original O_2 consumption of the hemolysate. On the other hand no synergy was found between K and Na_2SO_4 or Na phosphate.

f) Reversibility of the inhibitory effect of Ca against the aerobic glycolysis. That the inhibition of the metabolism in the hemolysate by ions is not due to an irreversible destruction of enzymes is clearly indicated by the fact that the degree of the inhibition does not increase with the time even when the inhibition was not complete. The reversibility of the inhibition was, furthermore, demonstrated directly for Ca in the following way. Two samples of washed red cells were taken. One sample, hemolysate I, was hemolyzed with 1.5 volumes of water containing enough Ca to yield a final concentration of 2 mg % in the hemolysate. The other sample, hemolysate II, was hemolyzed with 1.5 volumes of water. 4 samples of 1 ml each were pipetted from every hemolysate. 0.03 ml of a 2% glucose solution were added to samples of hemolysate I and 1 sample of hemolysate II (glucose samples) while to the remaining five samples 0.03 ml of water was added (water samples). All samples were left for 2 hours at 25° and then the glucose sample and one water sample of II and one of the glucose samples of I and one water sample were deproteinized (2 hours samples). 0.6 ml of water was now added to the glucose and water samples of I and to the one of the water samples of II while the other water sample of II received 0.6 ml of a glucose solution of 0.1%. The Ca concentration in I was thus reduced from 2 to 1.2 mg %. If the inhibition of the aerobic glycolysis by Ca was reversible then the reduction of the Ca concentration in I should result in a decrease of the inhibition in the following 2 hours. This was in fact the case.

It must be noted that the 4 hour glucose sample of I contained more lactic acid in the second 2 hour period than the corresponding sample of II. This tended to make the inhibition by Ca rather stronger than weaker.

TABLE VI

BALANCE OF GLUCOSE CONSUMPTION, O_2 UPTAKE AND LACTIC ACID FORMATION IN THE HEMOLYSATE. 4 HOUR EXPERIMENTS AT 25° pH 7.0

Exp. No.	O_2 uptake in γ/ml of hemolysate			Increase of CO_2 production by glucose	Glucose consumed in γ/ml	Lactic acid formed	C O_2 uptake due to glucose in μmol	D mol glucose oxydized	Ratio $\frac{C}{D}$
	A by hemolysate itself	B hemolysate + glucose	B–A						
33	50	75	25		255	163	0.78	0.51	1.53
35	46	61	15		250	207	0.47	0.24	1.96
40	86	115	29	21.5	525	320	0.9	1.14	0.79

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DISCUSSION

5. *Mechanism of the aerobic glycolysis in the hemolysate*

On the basis of our experiments we can draw the conclusion that the aerobic metabolism in nucleated erythrocytes consists of several distinct enzymatic systems. If no glucose is added to the hemolysate no significant amounts of preformed hexoses are available for oxidation, but adenosine-5-phosphate, derived from ATP, breaks down and its ribose disappears. This process and oxidation of fat and protein should be responsible for the observed respiration of the hemolysate in absence of glucose. The increase after addition of glucose can be traced again to at least two different reactions, namely, complete oxidation to CO_2 and oxidation of glucose to a phosphoric ester, whereby one atom of oxygen combines with one mol of glucose. It is probable that the latter reaction consists in the oxidation of glucose to phosphogluconic acid.

The powerful aerobic glycolysis in the hemolysate in presence of glucose can be due to the fact that the oxidation of one molecule of glucose is coupled with the phosphorylation of many molecules of this sugar and the triosephosphate dehydrogenase is much more efficient in the hemolysate than the system oxidizing pyruvate. The excess of the latter is therefore reduced to lactic acid. As the hemolysate contains the enzyme system of the tricarboxylic acid cycle it is reasonable to assume that the oxidation of glucose to CO_2 goes over this cycle. It is known from experiments on other tissue extracts that the oxidation of 1 mol of glucose in this way can be coupled with the phosphorylation of 18 molecules of glucose to hexose diphosphate. This would explain the fact that the addition of all those acids which increase the turnover of the KREBS cycle, and of Mg which is an activator of the oxidation of pyruvic acid, considerably increases the aerobic glycolysis.

The inability of the hemolysate to glycolyse anaerobically can be explained easily. The hemolysis of nucleated erythrocytes is accompanied by an explosive increase in the activity of ATPase. At room temperature practically all of ATP originally present in the cells is dephosphorylated in a few minutes; the glycolysis of one molecule of glucose can maximally resynthesize 2 molecules of ATP. As long, therefore, as the speed of the simple dephosphorylation of ATP exceeds the speed of transphosphorylation with glucose the latter process must stop in anaerobiosis due to the total disappearance of ATP. The efficiency of the oxidative breakdown of glucose as far as synthesis of ATP is concerned makes it possible to keep up under aerobic conditions a certain minimum concentration of ATP necessary for the phosphorylation of glucose. This amount, however, is very small, even under aerobic conditions, and not detectable by the usual colorimetric procedures of determination.

Point of attack of ions

The realization of this multitude of enzymatic processes involved in the aerobic metabolism is important for the consideration of the possible mechanism of the inhibitory effects of ions on this metabolism. It appears significant that all ions, cations as well as anions, are able to suppress not one but many of the enzyme reactions constituting the oxidative metabolism. On the other hand, the degree of inhibition is different for different enzyme reactions or systems of reactions. The aerobic glycolysis is in general more strongly inhibited than the oxidation of glucose, which in turn suffers more than the O_2 consumption without glucose. The oxidation of succinate and α-ketoglutarate

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are least affected. It is very significant that this sequence in the susceptibility to inhibitory effects is the same for all kinds of ions and the reactions affected are of very different types. The oxidation of glucose, for example, is as was shown due to two completely different reactions. It appears most improbable that so many and so different reactions should be influenced in the same way by all the ions. We have rather to assume that the ions exert their influence on a substrate the activity of which is again correlated is some way with the activities of all the enzymes of the oxidative system. Such a substrate for example is the cytochrome system, which serves as H_2 carrier to the oxidation of the preformed substrates of the hemolysate as well as that of added glucose. It seems impossible, however, to consider the cytochrome system as the point of attack in the ionic inhibition, because of the great differences between various enzymes in their sensitivity towards the ions. M/1000 of Ca almost completely inhibits the oxidation of glucose, but the inhibition of the basic respiration of the hemolysate is not complete even at M/200. M/500 HCN, on the other side, inhibits both to the same extent. All this suggests that the inhibitory action of ions is directed against one single substrate which in changing its physicochemical properties influences in its turn all the enzymes of the oxidative system. The enzymes are in fact not in solution inside the cell, but are attached to insoluble particles, the mitochondria. These contain, apart from proteins, considerable amounts of lipids and ribosenucleic acids. In these subcellular structural and functional units the enzyme proteins are probably attached to a stroma consisting of lipo- and nucleoproteins and may be surrounded by a surface membrane. One way to explain the effects of ions on the oxidative processes would therefore be to assume a decrease in the permeability of such a surface membrane under their influence. The fact that the aerobic glycolysis coupled with the oxidations is quite generally more strongly inhibited than the oxidative processes themselves is in agreement with this concept. This glycolysis depends on the coupled phosphorylation of a phosphate carrier which transfers the phosphate to glucose. Any decrease in the permeability of the surface membrane will decrease the speed of the penetration not only of the substrate but also of the phosphate carrier and the speed with which it leaves the particle after being phosphorylated. The amounts of the phosphate carrier available for the reaction with glucose must decrease to a much higher degree than the corresponding oxidative process. It could also be that the ions change not the permeability but the physical properties of the hypothetical stroma to which the enzyme proteins are attached. Any change in the water binding capacity or shape of the protein molecules of the stroma would have a considerable influence on the shape and arrangement of the respective enzyme proteins and tend to change their activity.

If we assume that in one way or the other the proteins of mitochondria are the point of attack of inhibiting ions the most probable mechanism of this inhibitions appears to be elimination of local electric fields on the surface of this protein, due to the adsorption of the ions. Thus CNS^- which forms stable complexes with proteins, inhibits the aerobic glycolysis at low concentrations, whereas Cl^- is ineffective. This can also explain the characteristic synergy between cations and anions in their effects. Even at the isoelectric point of a protein the charged groups on its surface will exert con-

* More recent experiments on the mechanism of the inhibition of the oxidative enzymes by Ca^{++} suggest, that the specific protein in the mitochondria, affected by ions, influences the energy transfer during the enzymereactions of the KREBS cycle rather, than the enzymes themselves or the access of substrates. The results of these new experiments will be reported in a subsequent paper.

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siderable forces of repulsion on ions of the same charge and thus counteract their adsorption. This repulsion will obviously be decreased by the simultaneous presence in solution of ions of opposite charge of great surface activity. The adsorption of cations will therefore be facilitated by the presence of easily adsorbable polyvalent anions, and vice versa, and thus a higher degree of elimination of polarized groups on the protein surface may be achieved. This again will affect the water binding capacity and the shape of the respective protein molecule.

This view appears supported by the rather striking analogy between the inhibition of the aerobic metabolism by ions and the effect of certain ions on proteins like myosin, actin, actomyosin and the so called structural proteins of kidney and brain investigated by SZENT-GYÖRGYI and his associates[12]. These proteins adsorb physiological cations (Na, K, Ca, Mg) from solutions of physiological concentrations. Ca is more strongly adsorbed than Mg and this again more strongly than the monovalent cations. This adsorption neutralizes charges of polar groups on the protein surface and changes the affinity to water and in the case of actin the ability to polymerize. A striking analogy to the synergy between ions in our case can be seen in the influence of the cations (K, Ca) on the adsorption of the polyvalent ATP ion by myosin. In this case the anion of ATP does not counteract the effect of K on myosin but enhances it.

The affinity of structural proteins to ions depends upon a certain specific state of the protein surface and is easily suppressed by procedures tending to denature the protein. The adsorption of cations by myosin for example decreases strongly during 24 hour storage at 0°[13]. This may be the reason why such general inhibitory effects of ions on oxidative enzymes have not yet been observed in tissue homogenates. In this case the subcellular structural units may suffer considerable injury by the mechanical crushing of the tissue. Hemolysis on the other hand appears as a much milder procedure for getting access to a little altered inner parts of the cell.

SUMMARY

1. The hemolyzed nucleated erythrocytes of the pigeon show considerable O_2 consumption, which is considerably increased by $MgCl_2$ M/250, glucose and constituents of the tricarboxylic acid cycle and completely inhibited by NaCN M/250.

2. This oxidative metabolism is coupled with a strong aerobic glycolysis.

3. All multivalent cations and anions inhibit the O_2 consumption as well as the aerobic glycolysis.

4. $CaCl_2$, orthophosphate and ribonucleate inhibit strongly at physiological concentrations.

5. Different oxidative reactions in the hemolysate are inhibited by ions to a different degree.

6. These inhibitory effects of ions may be due to disturbances of the local electric fields of proteins which are constituents either of membrane or stroma of subcellular structural units which are carriers of enzymes of the oxidative system of the cell.

RÉSUMÉ

1. Les nucléo-érythrocytes hémolysés du pigeon montrent une consommation d'oxygène considérable, qui est encore fortement accrue par $MgCl_2$ M/250, le glucose et les constituants du cycle des acides tricarboxyliques, mais complètement inhibée par NaCN M/250.

2. Ce métabolisme d'oxydation est couplé avec une forte glycolyse aérobique.

3. Tous les cations et anions plurivalents inhibent la consommation d'oxygène aussi bien que la glycolyse aérobique.

4. Le $CaCl_2$, l'ion orthophosphorique et l'ion ribonucléique sont de forts inhibiteurs aux concentrations physiologiques.

5. Différentes réactions d'oxydation, dont l'hémolysat est le siège, sont inhibées par les ions à des degrés différents.

6. Ces effects inhibitoires d'ions sont peut-être dus à des perturbations des champs électriques locaux des protéines qui sont des constituants soit de la membrane, soit du tissus conjonctif d'unités structurales subcellulaires, supports d'enzymes du système d'oxydation de la cellule.

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ZUSAMMENFASSUNG

1. Die hämolysierten, kernhaltigen Erythrocyten der Taube zeigen einen bedeutenden O_2-Verbrauch, welcher durch $MgCl_2$ M/250, Glucose und Bestandteile des Tricarboxylsäure-Zyklus beträchtlich erhöht, durch NaCN M/250 dagegen völlig unterbunden wird.

2. Dieser oxydative Metabolismus ist mit starker aerober Glykolyse gekuppelt.

3. Alle mehrwertigen Kationen und Anionen hemmen den O_2-Verbrauch sowohl als die aerobe Glykolyse.

4. $CaCl_2$, Orthophosphat und Ribonukleinat hemmen bei physiologischer Konzentration stark.

5. Verschiedene oxydative Vorgänge im Hämolysat werden durch Ionen verschieden stark gehemmt.

6. Diese hemmenden Wirkungen der Ionen beruhen vielleicht auf Störungen lokaler elektrischer Felder von Proteinen, welche Bestandteile sind von Membran oder Bindegewebe von subcellularen Struktureinheiten, die Träger von Enzymen des oxydativen Systems in der Zelle sind.

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