INHIBITION OF THE ANAEROBIC GLYCOLYSIS IN PIGEON HEMOLYSATES BY MULTIVALENT ANIONS

by

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It has been reported in previous papers[1,2] that multivalent intracellular ions (Ca^{++}, Mg^{++}, HPO_4^{--}, Ribonucleate, mono- and diphosphoglycerate) strongly inhibit the aerobic glycolysis in hemolysates of nucleated red cells. It was found later that the same ions also inhibit the O_2 uptake of the hemolysate to which neither oxidation substrate nor glucose had been added. It seemed reasonable to assume that the obigatorily aerobic glycolysis in the hemolysate was due to a synthesis of ATP (or another active P carrier) which was coupled with the oxidative processes and was followed by a transfer of P to glucose. In order to find the mechanism of this inhibitory effect of ions it seemed first of all necessary to decide whether the inhibition of aerobic glycolysis was due only to the slowing down of the oxidative processes or whether the simultaneous synthesis of ATP and the consecutive P transfer to glucose were directly affected. We have studied, therefore, in a series of experiments the effect of multivalent ions on anaerobic glycolysis and phosphorylation of glucose by ATP in hemolysates of pigeon red cells. The present report deals with the results of these experiments.

EXPERIMENTAL

General experimental procedure

Suspensions of red cells of pigeon free of any significant amount of glucose were prepared as described in a previous paper[2]. The cells were hemolyzed by adding 1–$1\frac{1}{2}$ parts of $N/100$ HCl. The pH of the hemolysate was found to lie between 6.8 and 6.9. One series of experiments was carried out with this hemolysate containing all the ghosts, which will be referred to as full hemolysate. For other experiments hemolysates were used from which all ghosts were removed by centrifugation in a high speed centrifuge for 45′ at 0°. This completely transparent hemolysate will be referred to as cytoplasmic hemolysate. In all experiments 2 ml of the hemolysate in a 15 ml centrifuge tube was treated with 0.4 ml of solutions of various substances. With every sample which contained a substrate for glycolysis (glucose, hexosephosphate) a control sample prepared in an identical way but without substrate was run simultaneously. At the end of the experiment all samples were deproteinized by adding 10 ml of 7% TCA, and after centrifugation the respective reaction products were estimated.

Analytical procedures

Lactic acid was determined in TCA filtrates by the BARKER-SUMMERSON procedure[3]. Hexosemono- and diphosphate were determined as fructose; triosephosphate was estimated as dihydroxyacetone by the carbazole cysteine reaction[4] and the results checked as a rule by a second method.

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This consisted in treating the TCA filtrate with 1 N NaOH for 10 minutes, neutralizing with 6 N H_2SO_4, and determining the lactic acid produced. This method could be used only on samples in which all enzymic production of lactic acid was suppressed. Glucose and HARDEN-YOUNG ester when treated with 1 N NaOH produced in the BARKER-SUMMERSON procedure some color the intensity of which was considerably less than 10% of that produced by an equivalent amount of triosephosphate. Controls containing approximately as much glucose or HARDEN-YOUNG ester as the hemolysate filtrates were run in every determination of triosephosphate and the values for the latter corrected accordingly.

RESULTS

Inhibition of the anaerobic glycolysis of glucose in hemolysates

a. *Anaerobic glycolysis in the full and cytoplasmic hemolysates*

When all the oxygen uptake in the full hemolysate is eliminated by $M/500$ NaCN a certain formation of lactic acid from added glucose can be observed. The rate of glycolysis decreases rapidly with the dilution of the hemolysate. If the latter is only 25% greater than that prevailing in our experiments, the anaerobic glycolysis drops almost to zero. In the cytoplasmic hemolysate 10 to 30 γ/ml of lactic acid is produced in 3 hours without the addition of any substrate. No glycolysis from glucose can be observed, except when $M/2000$ to $M/500$ ATP is added. Whereas the full hemolysate shows an intense ATPase activity, the activity of this enzyme in the cytoplasmic hemolysate is so low that after 3 hours at room temperature no more than 30% of the added ATP is destroyed. As glycolysis of glucose increased less than the ATP concentration in the range used, the drop in glycolysis during the experimental period did not exceed 10%.

As the effect of ions on respiration had to be studied on the full hemolysate, it was necessary first of all to study the effect of ions on the anaerobic glycolysis in it. To locate, on the other hand, the point of attack of the ionic inhibitors on the anaerobic glycolysis, it was necessary to use the cytoplasmic hemolysate as a medium, because only in this preparation were the conditions, such as the level of ATP and the accessibility of enzymes, well defined.

b. *Effect of ions on the anaerobic glycolysis in the full hemolysate*

The effects of a series of multivalent anions and cations which were found to inhibit the respiration of the full hemolysate are listed in Table I. As can be seen, multivalent anions inhibit the lactic acid production from glucose at about the same concentration as those at which they were shown to produce a definite inhibition of the O_2 uptake and aerobic glycolysis[2]. Anions which are always present in free or combined form in the cell have an inhibitory effect in concentrations which can be regarded as physiological.

Among cations two different groups can be distinguished. Ions of Ca, Ba and La not only do not inhibit but, in general, increase the rate of glycolysis (Table I). Mn^{++} and Zn^{++}, on the other hand, inhibit considerably at $M/1000$, probably by virtue of their general denaturing action on proteins. Fe^{++}, which is not a general denaturant, does not affect the oxidative metabolism of red cells at $M/1000$ but inhibits the anaerobic glycolysis by about 50% at this concentration.

c. *Inhibition of glycolysis of glucose by multivalent anions*

Lactic acid production from glucose in cytoplasmic hemolysates was measured

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TABLE I

INFLUENCE OF POLYVALENT IONS ON ANAEROBIC LACTIC ACID (L.A.) PRODUCTION FROM ENDOGENOUS SUBSTRATE AND FROM ADDED GLUCOSE IN THE FULL HEMOLYSATE

2 ml of hemolysate containing $M/500$ NaCN plus 0.2 ml H_2O or 0.6% glucose plus 0.2 ml H_2O or salt solution. Incubation 3 h at R.T.

Exp. No.	*Salt added*	*L.A. formed from glucose γ/ml*	*Inhibition %*
I	0	41.0	
	Na_2SO_4 $M/25$	6.5	84
	Na oxalate $M/240$	0	100
II	0	15.0	
	$CaCl_2$ $M/2000$	14.3	5
	$BaCl_2$	25.0	−60
	$MnCl_2$ $M/1000$	1.2	92
	$ZnSO_4$ $M/1000$	10.1	33
	$FeSO_4$ $M/1000$	6.9	54
III	0	16.0	
	$BaCl_2$ $M/250$	23.8	−48
	$La_2(SO_4)_3$ $M/1000$	28.0	−75
	$CaCl_2$ $M/2000$	19.3	−21
IV	0	14.0	
	$CaCl_2$ $M/1000$	19.0	−36
	Guanidine HCl $M/1000$	17.0	−26
V	0	31.0	
	Na Phosphate $M/50$	11.2	64
	Ribonucleate 0.1%	15.8	48

in presence of $M/2000$ to $M/500$ ATP. With $M/500$ ATP, 17–47 γ/ml of lactic acid were formed from glucose in 3 hours at room temperature. In these determinations, the lactic acid produced by triose phosphate in alkaline hydrolysis during the precipitation with copper lime, was neglected as it was too small to be determined accurately. In the $M/2000$–$M/500$ range of ATP concentration, glycolysis did not increase proportionally to the ATP concentration. Doubling of the amount of ATP increased glycolysis by about 50%. Glycolysis from glucose proceeds continuously during the period of 3 hours, though with decreasing speed.

Most of the multivalent anions showed an inhibitory effect on the original lactic acid formation in absence of glucose as well as that from added glucose. The effect varies considerably with the nature of the ion. Ribonucleate and phosphate show a considerable inhibition in concentrations which can be regarded as physiological. NaCl shows no inhibition when $M/12$ is added. Cl^- ions, therefore, do not inhibit at all in physiological concentrations. The very strong inhibitory effect by oxalate cannot be due to its known effect on enolase as in presence of excessive ATP and pyruvate the activity of this enzyme does not control the rate of glycolysis. The specificity of the inhibitory effect of multivalent anions is illustrated by the fact that malonate 1/100 does not show any inhibitory effect.

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TABLE II

INFLUENCE OF POLYVALENT IONS ON LACTIC ACID (L.A.) FORMATION FROM PREFORMED SUBSTRATE AND GLUCOSE IN PRESENCE AND ABSENCE OF PYRUVATE IN A CELL-FREE HEMOLYSATE TO WHICH ATP WAS ADDED TO A FINAL CONCENTRATION OF $M/500$

Incubation 3 h at R.T.

Exp. No.	*Concentration of pyruvate*	*Salt added*	*L.A. found without glucose γ/ml*	*Inhibition by ions in %*	*L.A. formed from glucose γ/ml*	*Inhibition %*
I	0	0	30.7		39	
	0	Na_2SO_4 $M/25$	6.0	81	20.1	48
II	$M/1200$	0	26.0		28	
	$M/1200$	Na_2SO_4 $M/25$	15.5	36	11.6	59
III	$M/1200$	0	40.5		33.9	
	$M/1200$	Na_2SO_4 $M/50$	22.5	45	29.1	14
	$M/1200$	Na_2SO_4 $M/25$	19.5	51	18.7	45
	$M/1200$	$CaCl_2$ $M/1000$	40.1	1	30.2	11
	$M/1200$	$CaCl_2$ $M/1000$/ Na_2SO_4 $M/50$	24.5	55	23.5	30
IV	$M/800$	0			20.5	
	$M/800$	Ribonucleate 0.3 %			14.2	30
	$M/800$	Phosphate $M/50$			7.4	64
	$M/800$	$K_4Fe(CN)f$ $M/250$			9.0	56
	$M/800$	Malonate $M/100$			20.6	0
	$M/800$	Inosital hexa-phosphate $M/1000$			18.5	10
V	$M/800$	0			27.5	
	$M/800$	Na Phosphate $M/50$			20.0	127.5
	Pyruvate $M/800$	Ribonucleate 0.3 %			18.5	32.5
	Pyruvate $M/800$	Inosital hexa-phosphate $M/250$			18.4	32.5
	Pyruvate $M/800$	NaCl $M/12$			27.5	0
VI	Pyruvate $M/800$	0			20.5	
	Pyruvate $M/800$	Oxalate $M/240$			3.1	85
	Pyruvate $M/800$	Oxalate $M/20$			1.8	91

d. *Absence of inhibition by cations*

Ca^{++}, on the other hand, which at $M/1000$ inhibits respiration of the full hemolysate by 50–70 % and aerobic glycolysis by 90–100 %, has no significant effect on the glycolysis of the cell free hemolysate. In the presence of Na_2SO_4 $M/50$–$M/25$, $M/1000$ Ca did not show any significant inhibitory effect; this is in contrast to the marked synergistic effect of these two ions on the aerobic glycolysis of the full hemolysate.

Localization of the inhibitory effect of anions

In any attempt to establish which constituents of the glycolytic system are inhibited by the anions, it seemed reasonable to assume that the mechanism of the glycolysis in the cytoplasmic hemolysate is identical with that known to occur in most of the other tissues. It was, therefore, assumed that glucose is first phosphorylated by ATP to hexose-6-phosphate and the latter in turn to the HARDEN-YOUNG ester which is split by an aldolase and the glyceraldehyde-3-phosphate thereby formed is oxidized to

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diphosphoglycerate. If this assumption is correct, then after inhibition of this dehydrogenase by bromoacetate, an accumulation of HARDEN YOUNG ester and of triose phosphate should follow. It was found indeed that after addition of $M/4000$ sodium bromoacetate, a mixture of two sugars accumulated which in the cysteine carbazole reaction[5] reacted like fructose and triose. On hydrolysis with N NaOH at room temperature, the latter substance disappeared and lactic acid was produced. A substance which reacted like fructose was precipitated as Ba salt by ethanol. The total amounts, however, of fructose and triose accumulated in 3 hours at room temperature, was considerably lower than the total amount of lactic acid produced without bromoacetate in the same period. This unexpected result is apparently due to an inhibitory effect of bromoacetate on the phosphorylation of glucose, as increase in the concentration of bromoacetate reduced still more the amounts of triose and fructose formed.

a. *Effect of multivalent anions on lactic acid formation from Harden Young esters*

To localize the point of attack of the anions inhibiting the glycolysis of glucose, it was first necessary to decide whether the phosphorylation of glucose to hexose diphosphate or the breakdown of the latter is inhibited. The cytoplasmic hemolysate was found to glycolyze HARDEN YOUNG ester in presence of pyruvate much more rapidly than glucose itself. At a concentration of $M/1500$, the transformation of the ester to lactic acid was complete after about 30 minutes at 25°. After 10 minutes about 40% of the ester was transformed into lactic acid. Na_2SO_4 at $M/25$, Na oxalate at $M/250$ and phosphate at $M/50$ all inhibited the lactic acid production in the first 10 minutes, the inhibition varying between 40 and 50%. When the inhibition, however, was investigated during the longer time interval, it always appeared smaller than during the first 10 minutes. This may be due, at least partly, to the fact that the glycolysis of HARDEN YOUNG ester at concentrations below $M/1500$ increases with the concentration. In the inhibited sample, therefore, the concentrations of the HARDEN YOUNG ester after the first 10 minutes must be higher than in the sample without the inhibitor and the inhibition therefore appears lower in the following time intervals (Table III).

b. *Effect of anions on the formation of triose phosphate from glucose*

The fact that the anions inhibited the glycolysis of the HARDEN YOUNG ester to a lesser degree than that of glucose suggested that an enzymic process preceding the stage of triose phosphate in the anaerobic glycolysis was also inhibited. To test this conclusion the effect of several anions on the formation of triose phosphate from glucose in presence of ATP was determined. The oxido-reductive processes in these experiments were eliminated by $M/4000$ bromoacetate The sample was incubated for 3 hours at room temperature, under which conditions as was mentioned above, triose phosphate and fructose-diphosphate accumulate. As can be seen from Table IV, the amount of triose phosphate was strongly depressed by $M/50$ sodium sulfate as well as by 1% Na ribonucleinate. That this inhibition was not due to a higher dephosphorylation of hexose-diphosphate in presence of sodium sulfate, was shown in control experiments in which hexose-diphosphate was incubated for three hours with the hemolysate in presence of $M/4000$ sodium bromacetate with and without Na_2SO_4.

c. *Effect of anions on the aldolase*

The inhibition of the formation of triose phosphate by anions could be due to their

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TABLE III

INFLUENCE OF $CaCl_2$, Na_2SO_4, AND $Na_2(COO_2)$ ON LACTIC ACID (L.A.) FORMATION FROM FRUCTOSE-1,6-DIPHOSPHATE (H.Y. ESTER) IN PRESENCE OF $M/660$ PYRUVATE

Exp. No.	*Substrate and additions*	*Inhibitor*	*Time of incubation in minutes*	*L.A. formed from H.Y. ester in μM/ml*	*Inhibition in %*
I	H.Y. ester 1 μM/ml + pyruvate 2 μM/ml	0	7.5	0.61	0
	H.Y. ester 1 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	7.5	0.42	31
	H.Y. ester 1 μM/ml + pyruvate 2 μM/ml	0	15	0.90	0
	H.Y. ester 1 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	15	0.88	2
II	H.Y. ester 1 μM/ml	0	15	0.54	0
	H.Y. ester 1 μM/ml	Na_2SO_4 $M/25$	15	0.29	46
	H.Y. ester 1 μM/ml	$CaCl_2$ $M/1000$	15	0.55	0
III	H.Y. ester 1 μM/ml	0	15	0.69	0
	H.Y. ester 1 μM/ml	Na_2SO_4 $M/25$	15	0.42	40
	H.Y. ester 1 μM/ml	Na_3AsO_4 $M/600$	15	0.81	0
	H.Y. ester 1 μM/ml	Na_3AsO_4 + Na_2SO_4 $M/25$	15	0.48	41
IV	H.Y. ester 0.667 μM/ml	0	15	0.48	0
	H.Y. ester 0.667 μM/ml	Na_2SO_4 $M/25$	15	0.24	50
	H.Y. ester 0.667 μM/ml	0	30	0.70	0
	H.Y. ester 0.667 μM/ml	Na_2SO_4 $M/25$	30	0.63	10
V	H.Y. ester 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	0	10	0.39	0
	H.Y. ester 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	10	0.25	36.0
	H.Y. ester 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	Na oxalate $M/240$	10	0.28	28.0

TABLE IV

INHIBITION OF TRIOSEPHOSPHATE FORMATION FROM GLUCOSE IN THE CYTOPLASMIC HEMOLYSATE IN PRESENCE OF ATP AND $M/4000$ MONOBROMOACETATE

Incubation 3 h at R.T.

Exp. No.	*Concentration of ATP*	*Inhibitor*	*Triosephosphate formed as P in γ ml of hemolysate*	*Inhibition %*
I	$M/500$	0	20.0	
	$M/500$	Na_2SO_4 $M/25$	0.06	97
II	$M/1000$	0	8.5	
	$M/1000$	Na_2SO_4 $M/25$	2.5	70
III	$M/1000$	0	11.2	
	$M/1000$	Na_2SO_4 $M/25$	2.5	78
	$M/1000$	Ribonucleate 0.3 %	8.0	30

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effect on the breakdown of ATP, on the phosphorylation of glucose to hexose-diphosphate, or on the activity of the aldolase. The first possibility was eliminated by determining the liberation of inorganic phosphate in presence and absence of $M/25$ sodium sulfate from ATP added to the hemolysate. The effect on the aldolase was determined with $M/25$ sodium sulfate and $M/250$ sodium oxalate. The HARDEN YOUNG ester was added to the hemolysate to a final concentration of $M/1500$, and ATP to a final concentration of $M/1000$, and the amount of the fructose which disappeared and triose which was formed, were measured after 5 and again after 20 minutes when the equilibrium was reached. Sodium sulfate decreased the extent of the breakdown of fructose phosphate in 5 minutes by about 20%, but the final equilibrium was also shifted, the concentration of the HARDEN YOUNG ester being about 20% higher in the equilibrium mixture than in the control without sodium sulfate. This shift in the equilibrium is not surprising, as MEYERHOF[6] had shown for the aldolase of the non-nucleated red cells that hemoglobin shifts this equilibrium, and this property of hemoglobin might be easily affected by sodium sulfate Sodium oxalate did not affect significantly either the equilibrium or the speed of the formation of triose phosphate.

d. *The effect of multivalent anions on the glycolysis of hexose-6-phosphate*

As the inhibition of the formation of triose phosphate from glucose is due neither to an increase of ATPase nor to inhibition of aldolase, either the phosphorylation of glucose to hexose-6-phosphate or the phosphorylation of the latter to fructose-di-phosphate must be affected. To decide this question, the effect of multivalent anions on the glycolysis of hexose-6-phosphate was studied in presence of ATP and pyruvate. As the speed of the phosphorylation of hexose-6-phosphate could be dependent on the activity of hexosephosphomutase, exploratory experiments were carried out in which the influence of multivalent anions on this second enzyme was tested. These indicated that the equilibrium between glucose-6-phosphate and fructose-6-phosphate was reached at room temperature after about 3 minutes and the anions had no effect on this enzyme. Under the condition adopted, the glycolysis is as high as in controls containing HARDEN YOUNG ester. The rate of glycolysis is proportional to the concentration of glucose-6-phosphate in the range of concentrations prevailing in the experiments. These two facts show that the phosphorylation of glucose-6-phosphate to fructose-di-phosphate is a much faster process than the glycolysis of the diphosphate. Sulfate at $M/25$ and oxalate at $M/240$ were both found to inhibit the glycolysis of glucose-6-phosphate, like that of the HARDEN YOUNG ester, by 40 to 50%. Inhibition of the phosphorylation of glucose-6-phosphate in these experiments could not have affected the overall rate of the glycolysis.

e. *Effect o anions on the glycolysis of glucose-6-phosphate in absence of ATP.*

Our experiments so far demonstrate that the inhibitory effect of multivalent anions is directed not only against the enzymes which phosphorylate glucose, but also against the enzymic system involved in the formation of lactic acid from triose phosphate. It therefore seemed interesting to investigate the effect of anions on a third group of enzymes involved in glycolysis, namely those which catalyze the phosphate transfer from 1,3-diphosphoglyceric acid and phosphopyruvic acid to ADP and the conversion of monophosphoglyceric acid to pyruvate. This question could easily be decided because hexose-6-phosphate was found to be glycolyzed by the cytoplasmic hemolysate even without addition of ATP and pyruvate, although the rate in this case is lower than in

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presence of ATP (Table V). The inhibition by $M/25$ sulfate as well as $M/240$ oxalate is much higher when ATP is absent while the presence of pyruvate does not significantly affect the degree of inhibition. This indicates that the transformation of monophospho-

TABLE V

INFLUENCE OF Na_2SO_4 AND $Na_2(COO)_2$ ON LACTIC ACID (L.A.) FORMATION FROM HEXOSE-6-PHOSPHATE IN THE CELL-FREE HEMOLYSATE IN PRESENCE AND ABSENCE OF PYRUVATE AND ATP

Exp. No.	*Substrate and additions*	*Inhibitor*	*Time of incubation in minutes*	*L.A. formed from hexose-6-phosphate in M/ml*	*Inhibition in %*
Ia.	Glucose-6-phosphate 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	0	10	0.38	0
	Glucose-6-phosphate 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	10	0.23	40.0
	Glucose-6-phosphate 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	Na oxalate $M/240$	10	0.23	37.5
	Glucose-6-phosphate 0.333 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	0	10	0.20	0
	Glucose-6-phosphate 0.333 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	10	0.13	36.0
	HARDEN YOUNG ester 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	0	10	0.39	0
	HARDEN YOUNG ester 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	10	0.25	36.0
	HARDEN YOUNG ester 0.667 μM/ml + ATP 1 μM/ml + pyruvate 2 μM/ml	Na oxalate $M/240$	10	0.28	28.0
b.	Glucose-6-phosphate 0.667 μM/ml + pyruvate 2 μM/ml	0	15	0.61	0
	Glucose-6-phosphate 0.667 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	15	0.14	77.0
	Glucose-6-phosphate 0.667 μM/ml + pyruvate 2 μM/ml	Na oxalate $M/240$	15	0.08	87.0
c.	Glucose-6-phosphate 0.667 μM/ml	0	15	0.63	0
	Glucose-6-phosphate 0.667 μM/ml	Na_2SO_4 $M/25$	15	0.08	87.0
	Glucose-6-phosphate 0.667 μM/ml	Na oxalate $M/240$	15	0.07	91.0
II	Fructose-6-phosphate	0	15	0.85	0
	Fructose-6-phosphate	Na_2SO_4 $M/25$	15	0.11	87.0
III	Fructose-6-phosphate 0.667 μM/ml + pyruvate 2 μM/ml	0	7.5	0.32	0
	Fructose-6-phosphate 0.667 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	7.5	0.10	69.0
	Fructose-6-phosphate 0.667 μM/ml + pyruvate 2 μM/ml	0	15	0.90	0
	Fructose-6-phosphate 0.667 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	15	0.30	67.0
IV	Fructose-6-phosphate + ATP 1 μM/ml + pyruvate 2 μM/ml	0	15	0.47	0
	Fructose-6-phosphate + ATP 1 μM/ml + pyruvate 2 μM/ml	Na_2SO_4 $M/25$	15	0.35	25.0
	Fructose-6-phosphate + ATP 1 μM/ml + pyruvate 2 μM/ml	NaCl $M/12$	15	0.48	0
	Fructose-6-phosphate + ATP 1 μM/ml + pyruvate 2 μM/ml	0	30	0.96	0

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glycerate to pyruvate, if it is affected at all, does not control the rate of glycolysis of glucose-6-phosphate. As on the other hand this glycolysis can be assumed to proceed only when glucose-6-phosphate is phosphorylated by ATP, and as the latter is practically absent in the cytoplasmic hemolysate, we have to assume that the rate of formation of ATP during glycolysis controls the overall speed of the process. The higher inhibition, therefore, in absence of ATP can be explained by inhibition of the synthesis of ATP due to phosphate transfer from 1,3-diphosphoglyceric acid. The inhibition of this transphosphorylation would depress the level of ATP with a corresponding decrease in the speed of phosphorylation of glucose-6-phosphate.

DISCUSSION

Our results indicate that at least 3 different constituents of the glycolytic system are affected by the anions. The strongest effect apparently involves the enzyme which catalyzes the phosphorylation of glucose and therefore corresponds to the hexokinase in other living cells. This can be deduced from the fact that the inhibition of the lactic acid production from glucose in presence of an excess of ATP and pyruvate is stronger than the inhibition of the lactic acid production from hexosemonophosphate under the same conditions, and also from the fact that the glycolysis of hexose-6-phosphate proceeds at a concentration of the substrate of $M/1500$ about 15 times more rapidly than the glycolysis of glucose and at a rate proportional to the concentration of the ester. As after 30 minutes at room temperature all of the hexose-6-phosphate has been converted to lactic acid, it is clear that inhibition of the phosphorylation of hexose-6-phosphate by the anions, with no corresponding inhibitory effect on the hexokinase, would only result in an increase of the concentration of the hexose-6-phosphate produced from glucose to such a level that the inhibition by the anions would be completely compensated in a few minutes.

A second point of attack by the anions is the system which catalyzes the oxidation of triosephosphate. As addition of $M/600$ of sodium arsenate has no effect on the inhibitory action of the anion and the activity of the aldolase does not decrease in presence of anions, either the triosephosphate dehydrogenase or the lactic acid dehydrogenase, or both, are apparently affected. Apart from these two directly established effects, we obtained indirect evidence that at least one more transphosphorylating enzyme is inhibited by the anions. This inference results from the observation that in absence of ATP the degree of inhibition of glycolysis of hexose-6-phosphate is about twice as high as in presence of ATP. As the presence of pyruvate is without influence, we must conclude that this difference does not depend on any effect on the transphosphorylation between phosphopyruvate and ADP.

The third constituent of the glycolytic system which is inhibited by enzyme, therefore, can be either Bucher's enzyme or phosphohexokinase; the latter conclusion, however, would be valid only if we assume that inhibition appears only at low concentrations of ATP or ADP and is eliminated by an excess of these nucleotides.

From the biological point of view, the inhibition of hexokinase appears most significant as this enzyme may be assumed to control the overall rate of the metabolism of glucose in tissues which do not utilize glycogen. The multiplicity of points of attack of the anions in their inhibitory effect on the anaerobic glycolysis suggests that this effect may be related to factors which the inhibited enzymes of the glycolytic system have in

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common. This seems to be, in our case, the participation of mono- and dinucleotides in the enzymic reaction. In the transphosphorylating enzymes, ATP functions as donor, or ADP as acceptor of phosphoric acid, and in the enzymes involved in the oxidation of triose phosphate, DPN is an essential constituent. As these nucleotides are in a sense multivalent anions, the inhibitory effect of the anions on the activity of these enzymes may be due to an interference with the fixation of the nucleotides on the enzyme surface.

It was shown in 1941[7] that the phosphorylation of glucose and hexose-6-phosphate, as well as the phosphate transfer from the monophosphoglycerate and phosphopyruvate to ADP in human red cells are strongly inhibited by 3-phospho- and 2,3-diphosphoglycerate at $M/300$ and by phosphopyruvate at $M/1000$ while -glycero-phosphate, even in considerably higher concentrations, had no such effect. As the phosphoglycerates and phosphopyruvate possess a higher valency than glycero-phosphate, it may well be that we are dealing here with the same phenomenon as in the case of inhibition of the anaerobic glycolysis in hemolysates of nucleated red cells.

The fact that other multivalent anions which in nucleated red cells inhibit glycolysis either have not this effect at all in nonnucleated red cells, like phosphates or inhibit glycolysis in significantly higher concentrations like oxalate, does not contradict this explanation of the inhibitory effect of phosphoglycerates and phosphopyruvate. These differences may be due to varying specificities of these inhibitory effects which were also found in nucleated cells as demonstrated by the lacking inhibition by malonate and low inhibition by imositolhexaphosphate.

The observations here reported raise two questions, namely, how far these inhibitory effects will be observed on enzymes of the glycolytic system of nucleated red cells after purification. Experiments on purified enzymes from other sources which can be easily obtained would not necessarily decide this question because of the possible tissue specificity of these effects. The red cells in general are supposed to be involved in the regulation of the ionic environment of the body and it may be that inhibitory effects of ions on the metabolism are characteristic for the red cell and perhaps the other tissues of similar function or activities. The possibility, however, cannot be discarded that we are here confronted with a more general phenomenon and that these ionic effects are part of a system related to regulation of the metabolic rates in various functional states of living cells.

SUMMARY

1. Multivalent anions inhibit acid formation from glucose in the complete and cytoplasmic hemolysate of pigeon red cells.

2. Ca, Ba and La either do not inhibit or even accelerate the glycolysis of glucose.

3. The inhibition of anions is due to the inhibition of at least three different constituents of the glycolytic system, of which the most pronounced is the inhibition of hexokinase.

4. The possible mechanism of this inhibition is discussed.

RÉSUMÉ

1. Les anions polyvalents inhibent la formation d'acide lactique à partir de glucose dans des hémolysats complets et cytoplasmiques d'érythrocytes de pigeon.

2. Le Ca, le Ba et le La n'inhibent pas ou même accélèrent la glycolyse du glucose.

3. L'inhibition par les anions est due à l'inhibition d'au moins trois constituants différents du système glycolytique, et plus particulièrement à l'inhibition de l'hexokinase.

4. Le mécanisme possible de cette inhibition est discuté.

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ZUSAMMENFASSUNG

1. Durch mehrwertige Anionen wird die durch hämolysierte rote Blutkörperchen der Taube, sowie durch das Zytoplasma von deren Hämolysaten bewirkte Milchsäurebildung aus Glukose gehemmt.

2. Ca, Ba und La ihrerseits hemmen diese Glykolyse der Glukose nicht und beschleunigen sie in manchen Fällen sogar.

3. Die durch Anionen bedingte Hemmung setzt mindestens an drei Stellen des glykolytischen Systems ein. Die ausgeprägteste Hemmung ist die des Hexokinase-Systems.

4. Der wahrscheinliche Mechanismus dieser Hemmung wird erörtert.

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