BBA 45760

CLOT RETRACTION AND ENERGY METABOLISM OF PLATELETS. EFFECT AND MECHANISM OF INHIBITORS*

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(Received October 21st, 1968)

SUMMARY

1. Clot retraction in a system containing buffered saline, fibrinogen, washed blood platelets and thrombin was inhibited by addition of 6–20 ng antimycin or oligomycin per mg platelet protein. The inhibition was counteracted by glucose.

2. Platelet respiration was inhibited 80–90 % by addition of 6–20 ng antimycin or oligomycin per mg platelet protein.

3. Monoiodoacetic acid inhibited retraction almost completely, while 2-deoxy-D-glucose and NaF inhibited it partially. Glycolytic inhibitors and antimycin added in combination completely blocked retraction.

4. Washed platelets labeled with radioactive phosphorus and incubated under different conditions were studied. Addition of thrombin caused a sharp drop in radioactivity in ATP and a minor drop in ADP.

5. Incubation with deoxyglucose caused an accumulation of radioactivity in deoxyglucose 6-phosphate; with monoiodoacetic acid, accumulation in fructose 1,6-diphosphate and secondarily in fructose 6-phosphate; with fluoride, accumulation in 3-phosphoglyceric acid and secondarily in fructose 1,6-diphosphate.

6. Incubation with antimycin in the presence or absence of glycolytic inhibitors caused a shift of radioactivity to inorganic phosphate.

7. Incubation with inhibitors in all cases reduced the radioactivity of ATP and ADP to trace amounts.

8. It was concluded that the effect of inhibitors of respiration and glycolysis on clot retraction was caused by the reduction in ATP production in the presence of these agents.

INTRODUCTION

An energy requirement has been demonstrated for several of the blood platelet's functions[1–9]. GRETTE[10], however, claimed that the functions studied by him were not energy-requiring, but at the same time assigned a definite role to ATPases. The role

Abbreviations: Fru-1,6-P_2, fructose 1,6-diphosphate; Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate.

* Results in Table II were presented to the Colloquium on Biochemistry of Blood Platelets, 3rd Federation European Biochem. Soc. Meeting, Warsaw, 7th April, 1966. Part of this work was also presented to the 4th Federation European Biochem. Soc. Meeting, Oslo, 1967, Abstr. 298, Universitetsforlaget, Oslo, Norway, p. 75.

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of glucose as substrate for clot retraction was already established in 1956 by LÜSCHER[5,6]. LÖHR, WALLER AND GROSS[7] correlated the level of glycolytic enzymes with the efficiency of platelets to undergo "viscous metamorphosis".

Inhibitors of energy production were valuable tools in several studies[1,2,4]. WEBER AND UNGER[11] have demonstrated a relationship between the depletion of platelet glycogen and clot retraction. Both were inhibited by monoiodoacetate and by EDTA. A similar relationship has been demonstrated between platelet functions and the production of lactic acid[1,3,6,12].

A decrease in the platelets' ATP content caused by exposure to thrombin or collagen also suggests that energy is required[13–15], but should be corrected for the release of metabolically inert adenine nucleotides, which accounts for the major part of the total decrease[16–18]. Later studies have correlated the uptake of oxygen with platelet functions[12,19–21].

CHERNYAK[22] isolated mitochondrial structures from platelets, but failed to obtain evidence of oxidative phosphorylation. She therefore concluded that this plays no important role in platelets. Likewise inhibitors of oxidative metabolism failed to influence platelet functions, while glycolytic inhibitors always seemed to have some effect[1,7,23,24]. The theory was therefore put forward that the energy for the functions studied was almost entirely dependent upon the glycolytic steps of glucose breakdown. HARRISON, EMMONS AND MITCHELL[4] found that neither oxidative (KCN) nor glycolytic inhibitors (monoiodoacetic acid) influenced platelet aggregation. HEMPLING[25], however, has demonstrated an oligomycin- and antimycin-induced inhibition of K^+ and Na^+ transport in tumor cells, which was overcome by glucose, thus showing that conclusions based on inhibition of only one pathway for energy production might be erroneous. Likewise HARARY AND SLATER[26] have shown that a combination of iodoacetate and oligomycin is necessary to inhibit beating in cultured heart cells. CARROLL AND GORSTEIN[27] and GORSTEIN, CARROLL AND PUSZKIN[28] showed that a combined inhibition of glycolysis and oxidative metabolism is necessary to destroy the K^+ gradient in human platelets. The same necessity for a combination of inhibitors has been demonstrated for platelet aggregation and adhesion to glass[29] and for adhesion, aggregation and clot retraction in platelet-rich plasma[30,31].

In the present study the roles of the separate pathways of energy production have been evaluated by comparing the effect of inhibitors on energy metabolism and on clot retraction. Since the effect of anaerobic glycolysis and oxidation coupled to phosphorylation have been studied separately, the choice of inhibitors has been restricted to specific blockers of the metabolic system: 2-deoxy-D-glucose[32], fluoride and monoiodoacetic acid for glycolysis, and antimycin[33] and oligomycin[34] for the oxidative mitochondria-linked pathway.

Inhibition of respiration was measured directly as the reduction in oxygen uptake, while the specific inhibition of glycolysis was measured chromatographically by the accumulation of intermediates of glycolysis in the acid-soluble fraction of platelets labeled with ^{32}P.

The experiments to determine the inhibitory effect on glycolysis were all done with platelets which were exposed 5 min to thrombin under conditions where aggregation would normally take place. This was the amount of time the suspension of platelets and fibrinogen was exposed to thrombin before the clot was released from the vessel wall and allowed to retract.

MATERIALS AND METHODS

Materials

Oligomycin (15 % A and 85 % B), antimycin A, type 3, hexokinase, type C, glucose-6-phosphate dehydrogenase, type V and 2-deoxy-D-glucose were from Sigma Chemical Co., St. Louis, Mo., U.S.A., and monoiodoacetic acid from the British Drug-houses, Poole, Dorset, Great Britain.

As chromatographic markers: Fructose 6-phosphate, NADP, glucose 1-phosphate, glucose 6-phosphate, fructose 1,6-diphosphate, 2,3-diphosphoglyceric acid, 3-phosphoglyceric acid, AMP, ADP, ATP, adenosine tetraphosphate, ribosyl 1-phosphate, ribosyl 5-phosphate, IMP, CDP, CTP, phosphoethanolamine, phosphoserine and phosphocholine were from Sigma.

Carrier-free $^{32}P_i$ (type FO) was supplied by Institutt for Atomenergi, Kjeller, Norway.

Fibrinogen was prepared from human platelet-poor plasma according to the procedure of BLOMBÄCK AND BLOMBÄCK[35] (fraction I-O). The concentration was determined by the biuret method[36].

Thrombin was a bovine Hoffmann–LaRoche (Basle, Switzerland) preparation (Topostasin), 50 NIH units per ml saline with 25 mM Tris buffered to pH 7.4 with HCl, and stored at −15°.

Platelet suspension. 40-ml samples of blood drawn from healthy donors into 0.07 vol. of 0.077 M EDTA (pH 7.4), were centrifuged at $G_{max} = 300 \times g$ for 15 min at 4–10°. The platelets were isolated by centrifugation at $G_{max} = 900 \times g$ for 20 min at 4–10°, pooled, washed twice with 12.5 ml of 0.154 M NaCl per sample and finally suspended in 0.154 M NaCl to a concentration of 3–5 mg protein per ml. (Only occasionally did the mixing of platelets from different donors result in aggregates which did not resuspend readily in saline. Such platelet preparations were not used.)

Methods

Paper chromatography (descending) was performed with Whatman No. 1 paper and in: (1) isobutyric acid–1 M NH_3–0.1 M EDTA (250:150:4, by vol.)[37] and (2) *n*-butanol–acetone–acetic acid–conc. NH_3–water (45:15:10:2:28, by vol.)[38]. The chromatograms were developed for 22 h two dimensionally with System 1 as the first direction, and radioautograms (on Ilford Red Seal photographic film) were made of the dried chromatograms. The radioactive spots were cut out and counted in a Beckman Lowbeta II counter.

Determinations of ^{32}P-labeled compounds in trichloroacetic acid extracts from platelets. Labeling of platelets with ^{32}P was achieved by incubation of platelet-rich plasma from one 40-ml blood sample with 10–100 μC $^{32}P_i$ for about 2 h before separation and washing of platelets. Labeled platelets were washed as described for non-labeled platelets and incubated as described in Table V. The incubated platelets were extracted for 15 min at room temperature with 1 ml 10 % trichloroacetic acid, centrifuged 15 min at $G_{max} = 1200 \times g$ and re-extracted with 0.5 ml trichloroacetic acid. All centrifugation took place at 4–10°. The combined extract was shaken with ether and the resulting trichloroacetic acid-free extract used for further examination.

The distribution of radioactivity among organophosphates in the trichloroacetic acid-soluble fraction was determined by chromatography. The chromatographic spots were identified by co-chromatography with internal standards.

TABLE I

CLOT RETRACTION

Platelets (2–3 mg protein) and 2 mg fibrinogen in 4.45 ml 0.154 M NaCl were added to tubes (1.5 cm internal diameter) graduated in 0.1 ml to 10 ml. To this was added 1.5 ml 0.1 M Tris–HCl (pH 7.4) glycolytic inhibitors and substrate with a concomitant reduction in the NaCl volume (30 μmoles 2-deoxy-D-glucose in 0.154 M NaCl, 6 μmoles monoiodoacetic acid in 60 μl water, 20 μmoles sodium pyruvate in 0.2 ml 0.154 M NaCl (pH 7.0) and 10 μmoles sodium phosphate as a 0.2 M solution (pH 7.4 when diluted 20 ×), and antimycin and oligomycin in 5 to 10 μl 95 % ethanol. (Separate experiments showed that concentrations of ethanol below 0.5 % did not interfere with clot retraction.) 50 μl of the thrombin solution were then added, the tubes were immediately inverted twice and placed in an air incubator at 37°. After a 5-min incubation (necessary to produce a solid clot), the clot was released from the tube wall with a thin glass rod or by gentle shaking, and allowed to retract. Later a 37° water bath was used instead of the air incubator. After the end of the retraction period (20–40 min) the clot was removed from the medium with a glass hook, and the volume of remaining liquid was determined in the graduated glass. The decrease in liquid volume was taken as clot volume. Preincubation was performed by shaking in 25-ml erlenmeyer flasks in a Dubnoff metabolic incubator. In Expts. I, II, and 3, III the incubation medium was made isotonic by the addition of NaCl. 6.7 mM glucose was added as a 0.4 M aqueous solution. Glc stands for glucose, deGlc for 2-deoxy-D-glucose.

Addition	*Clot size (ml)*			*Addition*	*Clot size (ml)*		
	I	*II*	*III*		*I*	*II*	*III*
No preincubation				*Preincubated 30 min*			
				Added before preincubation			
1. No	0.1	0.2		3. deGlc	0.6	0.4	
0.8 mM NaF	0.1	0.4		Monoiodoacetic acid	3.8	4.1	
2.5 mM NaF	0.15	0.8		deGlc (antimycin after)	3.5		3.8
10 mM NaF	1.5	2.2		Monoiodoacetic acid (antimycin after)	3.9		4.2
Antimycin	1.1	1.8		EDTA	4.0		3.6
Antimycin + 0.8 mM NaF	1.4	1.7					
Antimycin + 2.5 mM NaF	3.1	3.6		*Added after preincubation*			
Antimycin + 10 mM NaF	2.8	4.5		3. No addition	0.05		0.6
Antimycin + Glc		0.3		deGlc	0.7	0.4	
Antimycin + 0.8 mM NaF + Glc		0.3		Monoiodoacetic acid	1.9	1.8	2.7
Antimycin + 2.5 mM NaF + Glc		3.0		Antimycin	2.5		3.4
Antimycin + 10 mM NaF + Glc		4.0		Antimycin + deGlc	3.7		4.0
2. No	0.2			Antimycin + monoiodoacetic acid	4.3		4.6
Antimycin	1.2						
Oligomycin	0.8			4. No addition	0.9	0.5	
Antimycin + oligomycin + glucose	0.1			P_i	0.3	0.2	
				Pyruvate	0.5	0.4	
3. No	0.1	0.05		Pyruvate + P_i	0.2	0.1	
deGlc	0.3	0.2		deGlc	1.6	0.9	
Monoiodoacetic acid	3.1	3.0	4.2	deGlc + P_i	1.3	0.4	
Antimycin	1.0		1.6	Pyruvate + deGlc	0.9	0.6	
Antimycin + deGlc	2.0		2.2	Pyruvate + deGlc + P_i	0.6	0.1	
Antimycin + monoiodoacetic acid	4.3		4.6				

When the spots contained a considerable part of the available radioactivity, care was taken to establish unequivocally the identity of the spot.

Special identifications. Fru-1,6-P_2 was only partially broken down by boiling for 1 h with 10% trichloroacetic acid, and the radioactive fraction corresponding to Fru-1,6-P_2 on the chromatograms was broken down in the same manner.

2-Deoxy-D-glucose 6-phosphate was identified as follows: ATP, commercial 2-deoxy-D-glucose and hexokinase were incubated together in a buffered aqueous solution (pH 7), and the product applied on a chromatogram together with the trichloroacetic acid extract of platelets treated with 2-deoxy-D-glucose. The two spots assumed to be 2-deoxy-D-glucose 6-phosphate overlapped when developed two dimensionally.

RESULTS

Antimycin and oligomycin caused a partial inhibition of clot retraction with washed platelets suspended in buffered saline (Table I). The inhibition was completely reversed by the addition of 6.7 mM glucose.

A combination of mitochondrial and glycolytic inhibitors was necessary to produce complete inhibition of clot retraction. Preincubation of the washed platelets was necessary for maximal inhibition with 2-deoxy-D-glucose (routinely a 30-min preincubation was employed). The preincubation was effective even when 2-deoxy-D-glucose was added afterwards. Preincubation in the absence of inhibitor reduced the inhibitory effect of monoiodoacetic acid, even though the effect of monoiodoacetic acid *plus* antimycin was still maximal, as was the effect of monoiodoacetic acid, when the inhibitor was present during preincubation (Table I).

TABLE II

TITRE OF INHIBITION OF OXYGEN UPTAKE AND CLOT RETRACTION

Clot retraction was measured as described in Table I in a medium of 4.5 ml 0.154 M NaCl and 1.5 ml 0.1 M Tris–HCl (pH 7.4), containing 15 μmoles potassium phosphate (pH 7.4 when diluted to concentration in medium), 2 mg fibrinogen, unwashed platelets (5.5 mg protein, corresponding to about 3 mg protein in washed platelets), 12 μmoles $CaCl_2$ and 2.5 NIH units thrombin. Oxygen consumption was measured by the conventional Warburg manometric technique in a medium of 1.2 ml 0.154 M NaCl and 0.5 ml 0.1 M Tris–HCl (pH 7.4), containing 15 μmoles Tris–succinate and unwashed platelets (5.5 mg protein); the center well contained 0.2 ml 20% KOH. A 5-min preincubation was allowed before the system was closed and the reading started. Consumption was determined after 175 min in the oligomycin experiment, after 165 min in the antimycin experiment. Antimycin and oligomycin were added in 95% ethanol.

Expt. No.	*Inhibitor added (ng/mg protein)*	*Oxygen consumed (μl/mg protein)*	*Clot retraction (ml clot volume)*
1	—	12.1	0.1
	Oligomycin (1)	11.2	0.2
	Oligomycin (3)	3.8	0.2
	Oligomycin (10)	2.3	0.8
2	—	11.9	0.1
	Antimycin (1)	10.9	0.1
	Antimycin (3)	2.2	0.5
	Antimycin (10)	1.4	0.6

10 mM NaF gave a significant inhibition of clot retraction in the absence of antimycin, while 2.5 mM NaF gave a similar inhibition when the respiratory inhibitor was added simultaneously (Table I).

Clot retraction was somewhat activated by the addition of phosphate and pyruvate in the absence of inhibitor or when 2-deoxy-D-glucose was used as inhibitor (Table I).

When respiration was inhibited with antimycin and time of retraction kept constant, the degree of retraction depended directly on the time of addition of glucose (Table III).

As little as 6 to 20 ng antimycin or oligomycin per mg platelet protein resulted in maximal partial inhibition of clot retraction (Table II). Increasing the oligomycin concentration 10 times did not significantly increase the inhibitory effect. Parallel experiments showed that the uptake of oxygen was maximally inhibited at levels similar to those which inhibited clot retraction (Table II). Several of the components of the incubation media used in the experiments described in Table II were not necessary for the process and were therefore omitted in the later studies.

TABLE III

SIZE OF CLOT AS FUNCTION OF TIME EXPOSED TO SUBSTRATE

All retractions took place in the presence of 50 ng antimycin. Incubation medium and performance as described in Table I, in the presence of 40 μmoles glucose. Glucose was added at different times during retraction. Results in ml clot size after 20-min clot retraction.

Exposure to substrate (min)	*Clot size (ml)*	
	I	*II*
0	2.4	2.8
5	1.5	2.2
10	1.1	1.5
15	0.9	0.9
20	0.7	0.5

In the trichloroacetic acid extract of ^{32}P-labeled platelets incubated for 35 min at 37°, the distribution of radioactivity was about 20% for ATP, 7% for ADP and 50% for P_i. When thrombin was added 5 min before the end of incubation, a sharp drop in [^{32}P]ATP and a smaller, but consistent drop in [^{32}P]ADP could be observed, the bulk of radioactivity being recovered as $^{32}P_i$ (Table IV).

When monoiodoacetic acid was added during incubation the bulk of radioactivity accumulated in Fru-1,6-P_2 and Fru-6-P, while the radioactivity of ADP and ATP was reduced to less than 2%. Thrombin addition did not alter the pattern (Table IV).

Incubation with 2-deoxy-D-glucose resulted in an accumulation of radioactivity in 2-deoxy-D-glucose 6-phosphate, while the label was significantly reduced in ADP and ATP (Table IV).

An unidentified spot appeared in the presence of monoiodoacetic acid, which moved slowly in Solvent 1 and rapidly in Solvent 2 (Fig. 1, Table IV). Incubation especially with 2-deoxy-D-glucose frequently resulted in an unidentified spot (containing up to 10% of total radioactivity) which did not move in either system (Table IV).

Incubation with NaF resulted in an accumulation of radioactivity in 3-phosphoglyceric acid in 10 mM NaF, while reduction of the concentration of inhibitor reduced the degree of accumulation drastically. In 10 mM NaF the concentration of labeled ATP and ADP was reduced to 3 % or less (Table IV).

Incubation with antimycin resulted in a shift of label to P_i. This shift was also evident in the presence of glycolytic inhibitors (Table IV). An increase in NaF-induced accumulation into [^{32}P]Fru-1,6-P_2 could also be noted. The addition of antimycin alone decreased the amount of [^{32}P]ATP by 30–60 % (Table IV).

The addition of thrombin increased the inhibitory effect of 2-deoxy-D-glucose and of 2.5 mM NaF (Table IV).

Several tentatively identified chromatographic spots, as well as two spots identified as phosphocholine and phosphoethanolamine, did not undergo significant alterations by the inclusion of inhibitors or thrombin during incubation.

Addition of unlabeled P_i during incubation did not significantly alter the distribution of radioactivity.

TABLE IV

DISTRIBUTION OF ^{32}P AMONG METABOLITES SEPARATED BY TWO-DIMENSIONAL CHROMATOGRAPHY

Platelets labeled with ^{32}P as described under MATERIALS AND METHODS were used. The platelets (6–10 mg protein) were incubated in the same medium as for clot retraction (Table I) (no fibrinogen added) with or without additions for 35 min at 37°. Thrombin was added 5 min before the end of incubation, which was stopped by cooling in icewater, and the platelets were isolated by centrifugation at $G_{max} = 900 \times g$ for 15 min; % distribution of radioactivity in the metabolites was determined as described under MATERIALS AND METHODS. 0.16 mM $CaCl_2$ was routinely added during incubation, since the platelets from preincubated, EDTA-containing platelet-rich plasma would not aggregate by addition of thrombin in the absence of added $CaCl_2$ (possibly because of influx of EDTA during incubation). In Expts. 2 and 3 the incubation mixture was made isotonic with NaCl. Results in % of total counts. Roman numbers refer to the numbering used in Fig. 1.

Expt. No.	*Conditions*	*Application point*	*Fru-1, 6-P_2*	*Glc-6-P*	*3-Phosphoglyceric acid*
1	No addition	0.2	7.8	5.1	1.2
	Thrombin	0	7.6	1.8	0.6
	Thrombin + monoiodoacetic acid	1.2	49.0	1.6	10.2
	Thrombin + deGlc	2.7	0.0	2.9	1.4
2	Monoiodoacetic acid	0.1	52.4	2.0	3.0
	Thrombin + monoiodoacetic acid	0	54.0	1.6	2.5
	deGlc	10.3		3.4	3.2
	Thrombin + deGlc	6.7		3.2	2.3
3	No addition		8.4	3.9	1.3
	Thrombin		6.2	3.9	1.3
	Thrombin + 10 mM NaF		8.4	1.8	62.5
	Thrombin + antimycin + 10 mM NaF		18.3	1.4	37.9
	Thrombin + antimycin		3.8	2.7	0.8
	Thrombin + antimycin + 2.5 mM NaF		4.9	1.3	18.1
	Thrombin + 2.5 mM NaF		8.1	2.0	33.1
	2.5 mM NaF		9.5	2.2	14.4
	10 mM NaF		8.8		58.7

Abbreviations: deGlc, 2-deoxy-D-glucose; deGlc-6-*P*, 2-deoxy-D-glucose 6-phosphate.

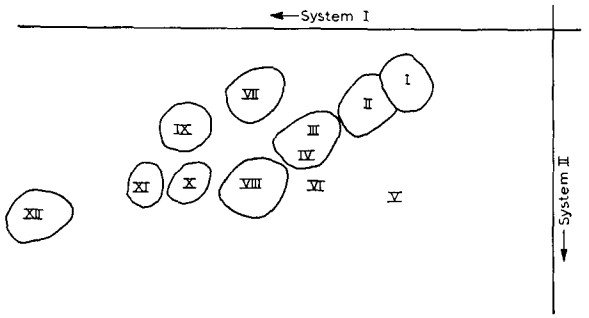

Fig. 1. Radioautographic distribution of radioactivity on a two dimensionally developed chromatogram. The chromatogram was developed in System 1 as the first direction. The figure represents the experiment presented in Table VI, 5, platelets incubated in the absence of thrombin and inhibitors. The spots were identified as follows: I, Fru-1,6-P_2; II, Glu-6-*P*; III, 3-phosphoglyceric acid; IV, Fru-6-*P*; V, unidentified spot which appeared on incubation with monoiodoacetic acid; VI, 2-deoxy-D-glucose 6-phosphate which appeared on incubation with 2-deoxy-D-glucose; VII, ATP; VIII, P_i; IX, ADP; X, unidentified spot (possibly a breakdown product of phosphatidyl inositol); XI, phosphoethanolamine; XII, phosphocholine. Phosphoserine was found between III and IV and may constitute part of the counts in these spots. 2,3-diphosphoglyceric acid was found at spot I. Ribose 1-phosphate, CTP, CDP, IMP, Glc-1-*P*, NADP, adenosine tetraphosphate, and AMP did not correspond to any of the spots in the chromatogram. Solvent systems 1 and 2 are as described under MATERIALS AND METHODS.

Fru-6-P	*deGlc-6-P*	*V*	*ATP*	P_i	*ADP*	*Phospho-ethanolamine Phospho-choline*	*X*
1.5			16.1	54.7	6.3	4.7	2.4
1.9			7.0	67.0	5.9	5.1	2.9
0.0		2.5	1.2	26.6	1.6	4.2	1.8
3.5	39.4		1.7	34.6	2.4	3.5	3.2
11.6		2.0	1.4	18.5	1.7	4.6	2.3
11.1		2.4	1.7	18.0	1.8	4.4	2.2
2.8	36.1		3.8	27.2	3.3	5.2	4.8
2.6	48.3		2.1	23.4	3.1	5.2	3.1
1.2			17.8	50.5	7.7	5.2	4.0
1.7			6.6	65.0	5.6	6.0	3.4
2.0			2.0	13.8	2.8	4.0	2.8
4.6			3.4	29.4	2.4	3.9	2.6
1.3			4.2	75.8	4.0	4.1	3.2
1.5			5.2	57.2	4.2	4.6	3.0
2.0			3.5	37.2	4.1	5.8	4.1
1.6			14.6	40.7	7.0	5.6	4.1
2.8			2.8	16.2	3.1	4.2	3.2

DISCUSSION

There is a simple relationship between the effect of metabolic inhibitors on clot retraction and their effect on platelet energy metabolism.

Antimycin and oligomycin clearly inhibit clot retraction by blocking the synthesis of ATP from ADP linked to uptake of oxygen, as can be seen from the concomitant inhibition of oxygen uptake and of clot retraction in a glucose-deficient medium or when glycolysis is inhibited (Tables I and II), and also from the shift in ^{32}P from ATP to P_i when labeled platelets are incubated with antimycin (Table IV).

The effect of antimycin and oligomycin on clot retraction gives strong evidence that the mitochondria are the main sites of oxygen-consuming energy production in platelets. Oligomycin causes an 80% reduction in oxygen uptake by platelets at concentrations where retraction was inhibited when glycolysis was suppressed. This indicates that at least 80% of the oxygen uptake by platelets is linked to oxidative phosphorylation, and that this is the part of platelet respiration which is linked to production of energy for clot retraction, thus contradicting the conclusion put forward by CHERNYAK[22] that mitochondrial activities play no role in platelet functions.

Deoxyglucose, monoiodoacetic acid and fluoride inhibit retraction by blocking specific steps in the production of ATP by the anaerobic glycolytic pathway. This inhibition takes place simultaneously with an accumulation of phosphorus in a form which is inactivated by the blocking of the metabolic process, and with a dramatic reduction in metabolically active adenine nucleotides (Table IV).

The combined results indicate that glycolysis and mitochondrial oxidative phosphorylation are the only pathways which supply energy for clot retraction. When one pathway is inhibited, the remaining one produces sufficient energy to support retraction, but with both pathways inhibited, clot retraction is stopped altogether. This leads to the conclusion that ATP is the direct source of energy for clot retraction and that continuous ATP production is necessary. This is further supported by the finding that the continuous presence of glucose is necessary during retraction in an antimycin-inhibited system.

It seems that the only natural substrates for clot retraction are glucose[6] and glycogen[11,39]. One can postulate that the breakdown of glycogen in platelets is much slower than the utilization of native glucose, and that therefore most of the glycogen is metabolized by the mitochondria-linked oxidative pathway. However, as pointed out by SCOTT[39], several factors influence the rate of glycogenolysis in platelets (*e.g.*, manipulation of platelets, treatment with thrombin) and thus the speed by which glycogen is made available as a source of energy. This may be the reason for the variation in the effect on platelet functions of the inhibition of one of the pathways for energy production.

Pyruvate (unlike succinate, E. H. MÜRER, unpublished results) has a small activating effect on clot retraction; this supports the findings of WARSHAW, LASTER AND SHULMAN[20] that this substrate can participate in platelet metabolism. The additional activation by P_i gives further support to the evidence that part of the inhibitory effect of monoiodoacetic acid and 2-deoxy-D-glucose was the removal of intracellular P_i from active metabolism.

The effect of monoiodoacetic acid on clot retraction is much greater than would

be expected if it affected only glycolysis (and also differs from the effect of 2-deoxy-D-glucose by its response to preincubation), but SH-reagents have previously been shown to have a general effect on platelet functions[40], so that apart from the inhibition of triose-phosphate dehydrogenase (*i.e.*, of ATP-production by one pathway) monoiodoacetic acid exerts an effect on the processes energized by ATP. In this way it may look as if monoiodoacetic acid inhibits both pathways of energy production, or as if only one pathway is used.

For this reason the use of monoiodoacetic acid for the evaluation of the role of glycolysis in energy production should be avoided.

The decrease in [^{32}P]ATP caused by the addition of thrombin is significant and further supports the view that platelet functions induced by thrombin are supported by energy released from ATP breakdown. Only the high-energy phosphate groups are labeled during the 2-h incubation with $^{32}P_i$, and only the ATP label is significantly reduced by the addition of thrombin. This supports HOLMSEN's findings[17] on the influence of collagen on the breakdown of ^{32}P-labeled ATP in platelets in platelet-rich plasma. However, the effect is much greater in washed platelets where the contents of labeled ATP are reduced by up to 60%, as IRELAND[18] has also found using [^{14}C]adenosine as isotopic precursor (*vs.* the 28% breakdown demonstrated by HOLMSEN). This ATP shows a labeling pattern different from the bulk of metabolically active ATP. HOLMSEN[17] has therefore suggested a third pool which exclusively provides energy for release processes. This third pool is labeled slower than the pool for general metabolism, so that only preparations which have been incubated for the same time with $^{32}P_i$ are comparable. It will be of great interest to determine which pool provides energy for clot retraction.

Further studies by IRELAND[18] and by the author (unpublished results) have revealed that the main drop in [^{32}P]ATP takes place within the first minute after addition of thrombin, again stressing that the energy for continuous retraction of the clot must be provided by a continuous synthesis of ATP. As HOLMSEN[16] has shown, the unlabeled ATP is not available for energy-requiring processes.

One point which should be stressed is the possibility of distinguishing between the glycolytic and mitochondrial way of energy production with the aid of antimycin and glucose addition. With these tools possible errors in the platelet enzyme system of either glycolysis or oxidative phosphorylation should be detectable. This might have practical applications in clinical research.

ACKNOWLEDGEMENTS

These studies were supported by grants from C. H. HOMANS legat and Legatet til Henrik Homans Minde, Det Videnskapelige Forskningsfond av 1919, and Anders Jahres Fond til Vitenskapens Fremme.

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