Some Effects of Artificial Media upon Glycolysis in Rat Platelets

By ENNIO C. ROssi

With the Technical Assistance of Edith Volk

PLATELETS STORED in artificial media have a shortened Cr${}^{51}$ survival time, decreased adenosine triphosphate concentration (ATP) and decreased activity of glycolytic enzymes. These changes are not observed when platelets are stored in plasma for a comparable period of time. Since storage of platelets in artificial media is associated with decreased activity of some platelet enzymes, incubation of platelets in various media might produce differences in their metabolic activity. This prompted a study of the effects in vitro of artificial media upon platelet glycolysis.

METHODS

All glassware used in this study was sterilized and siliconized. One per cent disodium ethylenediaminetetraacetate (EDTA) in a ratio of 1 part to 4 parts blood was used as anticoagulant. All centrifugation was done at 4°C.

Sprague-Dawley rats were bled by intra-cardiac puncture and the blood was centrifuged for 30 minutes at 150 g. Supernatant platelet rich plasma was removed and recentrifuged for 20 minutes at the same speed. Platelets were separated from plasma by centrifugation for 10 minutes at 7000 g. The platelet buttons were drained free of plasma but not washed in order to avoid elution of platelet enzymes and phosphonucleotides. The platelets were then resuspended in their original plasma or a buffered balanced salt solution (BSS). The BSS contained 3.3 mM NaHCO$_3$, 2.5 mM KCl, 0.4 mM Na$_2$HPO$_4$, 5.0 mM glucose, and 6.7 mM disodium EDTA. A variety of buffers were used including 75 mM imidazole-HCl, 25 mM phosphate, 75 mM phosphate, or 37.5 mM Tris-HCl. Sodium chloride was added in quantities sufficient to achieve an osmolality of 290-310 m Osm. The pH of artificial media were adjusted to 7.4 at room temperature and osmolalities were checked by determination of freezing point depression. The final platelet suspensions contained approximately 0.6-0.7 X 10$^8$ platelets per cubic milliliter with not more than 10 red blood cells and 20 white cells per cubic milliliter.

The platelets suspension were transferred to screw top erlenmeyer flasks and 1000 units penicillin and 1.0 mg. streptomycin were added to each 10 milliliter portion of platelet suspension. The platelet suspensions were then rotated gently at 60 rotations per minute in a water bath at 37°C.

At various intervals over the next few hours, samples of platelet suspension were removed from the flasks. Culture on blood agar, cosin methylene blue, and thioglycollate broth confirmed the absence of bacterial growth. Glucose consumption, lactate production, and acid soluble phosphorus were determined. Platelet counts were performed by the method of Brecher and Cronkite. Glucose was measured by the glucose oxidase method (Glucostat) and lactic acid

From the Department of Medicine, Marquette University School of Medicine, Milwaukee County General Hospital, Milwaukee, Wisconsin.

Research supported by USPHS Grant HE-08792.

First submitted Feb. 15, 1967; accepted for publication June 2, 1967.

ENNIO C. ROSSI, M.D.: Associate Professor of Medicine, Northwestern University School of Medicine, Chicago, Illinois.

* Worthington Biochemical Corp., Freehold, New Jersey.

758
EFFECTS OF ARTIFICIAL MEDIA ON GLYCOLYSIS IN PLATELETS

was determined by the method of Barker and Summerson. Glucose consumption and lactic acid production by platelets were calculated by using a method described previously.

*Acid Soluble Phosphorus*

Two milliliter specimens of platelet rich plasma or platelet suspension were transferred to plastic tubes and centrifuged at 7000 g. for ten minutes. The supernatant plasma or solutions were saved for other studies. The platelets were washed twice in cold isotonic saline in order to remove adherent phosphate derived from the media. Although some phosphate was undoubtedly washed out of the platelets by the saline, platelet samples were treated similarly so that the results were comparable. The platelets were extracted twice with 3.0 ml. 0.3 M perchloric acid. The extracts were pooled and then neutralized with 1.5 ml. 10 per cent KOH. The potassium perchlorate precipitate was removed by centrifugation. A measured aliquot of the neutralized extract was assayed for acid-soluble phosphorus which contains inorganic phosphate, phosphonucleotides, and phosphate esters. The extract was first dried in a vacuum desiccator and then digested in 0.5 ml. 10N H₂SO₄ until white fumes appeared. Repeated digestion after addition of 30 per cent H₂O₂ was necessary to remove the brown color which usually appeared. The phosphorus content was determined by the method of Martin and Doty.

*Adenosine Triphosphate (ATP) and Adenosine Diphosphate (ADP) Concentrations*

In another set of experiments, platelets were extracted with perchloric acid without prior washing. After neutralization with KOH the extracts were subjected to thin layer chromatography and analyzed for ATP and ADP. Measured amounts of unknown and ATP and ADP standards were spotted on glass plates coated with cellulose powder MN 300 (Macherey, Nagel & Co., available from Brinkman) to a thickness of 0.5 mm. The separation was accomplished in glass chambers containing a developing solution composed of 3.5 parts N-butanol, 2.5 parts acetone, 1.5 parts glacial acetic acid, 1.5 parts 5% ammonium hydroxide, and 1 part water. After the plates were dried, standards and unknowns were identified in ultraviolet light and removed by scraping. ATP and ADP were eluted from the cellulose with M/15 phosphate buffer. After centrifugation the eluates were read against blanks in a Beckman DU spectrophotometer at 260 μm (u.v.).

RESULTS

Platelets incubated in isotonic saline did not consume glucose or produce lactic acid effectively (Figure 1). However, when the pH of isotonic saline (approximately 6.0) was adjusted to 7.4 glycolysis proceeded normally for about two hours. Thereafter glycolysis ceased as lactic acid was released and decreased the pH to below 6.0. These preliminary studies demonstrated the importance of adequate buffering systems.

The buffers used in this study maintained the pH above 7.0 throughout a four-hour period of observation. The rate of glucose consumption was similar among platelets incubated in Tris BSS, 25 mM phosphate buffer BSS, and plasma, but was greater in platelets incubated in 75 mM phosphate buffer BSS and 75 mM imidazole BSS (Figure 2). The rates of lactic acid production were consistent with the glucose consumption studies (Figure 3). Platelets incubated in 75 mM imidazole BSS and 75 mM phosphate buffer BSS produced more lactic acid than did the platelets incubated in Tris BSS, 25 mM phosphate buffer BSS, or plasma. Platelets incubated in 37.5 mM and 19 mM imidazole BSS also demonstrated increased glucose consumption and lactic acid production (two experiments).
Fig. 1.—Glucose consumption and lactic acid production by platelets incubated in unbuffered isotonic saline-5.0 mM glucose and in isotonic saline-5.0 mM glucose in which the pH was adjusted to 7.4. Note in particular the acid pH of isotonic saline and the decrease in pH of adjusted saline from 7.4 to 5.5.

Previous studies have shown that anticoagulants can affect platelet glycolysis. In one experiment 16 mM sodium citrate + 9.5 mM citric acid were added to the BSS rather than EDTA. Although citrate appeared to decrease platelet glycolysis slightly, the previously established differences were maintained. When citrate was used platelets incubated in imidazole BSS, Tris BSS, and 25 mM phosphate buffer BSS consumed 0.81, 0.62, and 0.61 μmoles glucose per 10⁹ platelets per hour and produced 1.40, 0.92, and 0.88 μmoles lactic acid per 10⁹ platelets per hour respectively.

The increase in glucose consumption and lactic acid production demonstrated by platelets exposed to imidazole led us to investigate whether imidazole also affected phosphorus metabolism. The acid soluble phosphorus of platelets incubated in plasma and 75 mM imidazole BSS was compared (Figure 4). Platelets in plasma lost phosphorus in linear fashion and contained 60 percent of the original amount after incubation for six hours. However, platelets incubated in imidazole BSS lost phosphorus exponentially and retained only 20 percent of the original amount after six hours.

It seemed possible that the effects of imidazole upon phosphorus metabolism
Fig. 2.—Glucose consumption by platelets incubated in various media. The glucose concentration in the platelet media was measured at hourly intervals for four hours and the rate of glucose consumption per hour (b) was calculated. The results represent the mean glucose consumption rate per hour (b) ± one standard deviation and are based upon the following numbers of determinations of “b”—imidazole (11), .075 M phosphate (4), plasma (6), .025 M phosphate (7), and Tris (10).

Fig. 3.—Lactic acid production by platelets incubated in various media. The methods and numbers of determinations of “b” for lactic acid production are the same as described for glucose consumption (Figure 2).
reflected changes in platelet ATP. Accordingly we investigated the effects of Tris, 75 mM phosphate, and imidazole buffers upon the concentrations of ATP and ADP in platelets (Figure 5). The ATP content of platelets incubated in plasma decreased to 74 percent of the initial concentration in six hours (Figure 5A). Similarly, platelets incubated in Tris BSS and 75 mM phosphate buffer BSS contained 84 percent and 70 percent of the initial concentration of ATP after six hours. However, platelets incubated in imidazole BSS lost ATP rapidly and contained only 38 percent of their initial concentration after six hours. The amount of ADP in platelets incubated in plasma, Tris BSS, and imidazole BSS, remained relatively constant (Figure 5B). However, platelets incubated in 75 mM phosphate buffer BSS showed a definite decrease in ADP.

In one experiment we examined the effects of imidazole upon human platelets. Human platelets incubated in plasma consumed 0.36 μmoles glucose and produced 0.77 μmoles lactic acid per 10⁶ platelets per hour. When incubated in imidazole BSS human platelets consumed 1.16 μmoles glucose and produced 1.55 μmoles lactic acid per 10⁶ platelets per hour. The ATP concentration of human platelets was also affected by imidazole. After incubation for six hours, platelets in plasma contained 67% of their initial ATP concentration while platelets in imidazole BSS retained only 41% of the initial amount.
EFFECTS OF ARTIFICIAL MEDIA ON GLYCOLYSIS IN PLATEETS

Fig. 5.—Concentrations of (a) ATP and (b) ADP in platelets incubated in various media. Each point represents the mean of 6–8 determinations. The vertical lines represent one standard deviation at six hours and the "t" tests refer to the differences at six hours. The concentration of ADP in platelets at 6 hours in 75 mM phosphate is significantly different from the concentration observed with all the other media (p<.01).

DISCUSSION

When platelets were incubated at 37°C in artificial media in which the pH was controlled, glucose consumption and lactic acid production proceeded linearly for up to four hours. However, some buffers used to control pH affected the rate of platelet glycolysis. Twenty-five mM phosphate buffer did not alter glycolytic activity but increasing the phosphate concentration to 75 mM resulted in an increase in both glucose consumption and lactate production by platelets.

Sterk and Zakaria 11 noted that phosphate increased the consumption of glucose by red cells. Recent studies 12-14 have suggested a mechanism for this effect. Hexokinase, which appears to determine the rate of glucose utilization by red cells, is strongly inhibited by glucose-6-phosphate. Rose, Warms, and O'Connell showed that inorganic phosphate increases glucose consumption in red cells by decreasing the inhibitory effect of glucose-6-phosphate upon hexokinase. 13 Since the hexokinase reaction limits glycolysis in platelets also, 15 the stimulation of glucose consumption in platelets by phosphate may be mediated through a mechanism similar to that observed in red cells. The concentration of ATP in both red cells 13 and platelets is unaffected by the increased glucose
consumption observed with large concentrations of phosphate. However, in our studies platelet ADP was distinctly decreased. The reason for the decrease in platelet ADP is not clear.

The effect of imidazole on platelet glycolysis was marked. Glucose consumption and lactate production were greatly increased while total acid soluble phosphorus and ATP were rapidly depleted. Sallis and DeLuca\textsuperscript{16} working with subcellular systems found that imidazole stimulated ATPase. Since the surface membranes of platelets have considerable ATPase activity,\textsuperscript{17} our findings are consistent with the hypothesis that imidazole depletes platelet ATP by stimulating platelet ATPase activity. ADP and Pi have been proposed as regulators of glycolysis in the red cell.\textsuperscript{13,18} If this is true for platelets also, increased generation of ADP and Pi resulting from increased ATPase activity might explain the increase in glucose consumption and lactate production observed in platelets exposed to imidazole. However, this must remain speculative since in our studies the decrease in ATP was not associated with a demonstrable increase of either ADP or Pi in the platelets.

Chernyak\textsuperscript{19} has emphasized that ATP is not vigorously resynthesized in the platelet. Our studies are in accord with this conclusion. Platelets incubated in plasma or artificial media showed a slow decline in ATP content. Stimulation of glycolysis with large concentrations of phosphate did not perceptibly modify the rate of decline in ATP, and a marked increase in glycolytic activity was unable to compensate for the more rapid loss of ATP that occurred with
imidazole. Our data also confirm the suspicion of Sharp\textsuperscript{29} when he questioned whether platelet behavior in different media can be compared. Platelet media may affect platelet metabolism. The effects of imidazole upon platelet ATP are particularly pronounced and must be borne in mind whenever imidazole is used to buffer platelet media.

**Summary**

The effect of artificial media upon glycolysis of rat platelets was studied. Isotonic saline did not support glycolytic activity in platelets because of its acid pH. Although buffering the media to pH 7.4 established platelet glycolysis, the choice of buffer affected the results. Tris buffer or 25 mM phosphate buffer did not alter glucose consumption, lactate production, or adenosine triphosphate (ATP) concentration in platelets from that observed when platelets were incubated in plasma. However, use of 75 mM phosphate buffer produced significant increases in both glucose consumption and lactate production by platelets. A more marked effect was associated with imidazole which not only increased glucose consumption and lactate production but also accelerated the loss of platelet ATP.

**SUMMARIO IN INTERLINGUA**

Esseva studiate le effecto de medios artificial super le glycolyse de plachettos ab rattos. Isotonic solution salin non supportava le activitate glycolytic in le plachettas a causa de su acide pH. Per tamponar le medios de maniera a obtenir un pH de 7,4 resultava in un glycolyse plachettal, sed le typo de tampon afficeva le resultato. Tampon tris o un tampon de 25 mM phosphato non alterava le consumo de glucosa, le production de lactato, o le concentration de triphosphato de adenosina in le plachettas in comparation con lo que esseva observate quando le placettas esseva incubate in plasma. Tamen, le uso de 75 mM phosphato como tampon produceva augmentos significative tanto in le consumo de de glucosa como etiam in le production de lactato per le placettas. Un effecto ancora plus marcate eseva associate con le uso de imidazol le qual non solo augmentava le consumo de glucosa e le production de lactato sed tiam le rapiditate del perdita de triphosphato de adenosina per le placettas.

**REFERENCES**

8. Rossi, E. C.: Effects of ethylenediaminetetraacetate (EDTA) and citrate upon platelet glycolysis. J. Lab. and


15. Waller, H. D., Lohr, G. W., Grignani, F., and Gross, R.: Über den Energie-


Some Effects of Artificial Media upon Glycolysis in Rat Platelets

ENNIO C. ROSSI and Edith Volk