Activation of Glycogen Phosphorylase in Blood Platelets

By Robert B. Scott

With the technical assistance of LaVerne W. Cooper

BLOOD PLATELETS contain relatively large amounts of glycogen, as demonstrated by biochemical analyses and electron microscopy. For example, platelets contain as much glycogen as skeletal muscle on a gram for gram basis. Furthermore, it has been reported that during in vitro clotting of porcine platelets, the glycogen content, as measured by chemical methods, decreased.

In order to initiate glycogenolysis, preexisting glycogen phosphorylase molecules must be utilized since the anucleate platelet no longer possesses the mechanisms for inducing new enzyme synthesis. For this reason the platelet is a relatively simplified system in which to study aspects of the control of glycogen metabolism. Given a complement of enzymes somehow protected from proteolytic degradation, there are a number of possible mechanisms for the maintenance of a glycogen reserve and its degradation for needed energy. The enzyme may be in a totally inactive form waiting to be used. The balance of functioning glycogen synthetase and phosphorylase may maintain a constant glycogen level. Necessary co-factors or substrates may be unavailable to the active enzyme until needed, or some combination of these mechanisms could be utilized.

In muscle, phosphorylase exists in a tetrameric a form which is active without 5'-AMP, and a dimeric b form which requires 5'-AMP. Transformation from the b to the a form is mediated by the enzyme phosphorylase b kinase, and this serves as an activation mechanism during muscular contraction. Still another enzyme, phosphorylase phosphatase, serves to reduce the active a form to the b form. Liver phosphorylase differs in that its molecular weight is more like the a form only, although the majority of its activity is independent of the presence of 5'-AMP and similar enzymes activate and deactivate it. Epinephrine, glucagon, and electrical stimulation of muscle contraction also influence the activity of phosphorylase, either through AMP or by other influences on phosphorylase b kinase.

In the present study the activity of phosphorylase has been measured in human platelets incubated in vitro under conditions favoring glycogenolysis—hence, phosphorylase activation. Additional experiments were performed to determine whether glycogen synthesis continued during active glycogenolysis.
and to determine the relative dependence of coagulation and clot retraction on glycogen stores.

**METHODS**

All glassware was siliconized. Normal donor blood was obtained through plastic tubing into iced centrifuge tubes with one-tenth volume of 2 per cent disodium ethylenediaminetetraacetate (EDTA). All operations prior to incubation were performed at 4°C. Platelet rich plasma (PRP) was removed after centrifuging the blood at 200 g for 20 minutes. A few lymphocytes were usually present in the plasma.

The PRP was mixed well, samples taken for platelet counts, and the remainder then divided into separate aliquots for incubation. Separate aliquots were incubated for each time interval to be measured. A pair of 1 or 2 ml. samples were incubated for each interval glycogen determination and a 10 to 20 ml. aliquot for each enzyme assay. When incubations were to be made in buffered saline or Hanks solution, the aliquots of PRP were centrifuged at 10,000 g for 20 minutes (to insure complete recovery of the platelets) and pellets gently resuspended in the salt solution. Hanks solution included 0.2 ml. EDTA per 10 ml. solution. Samples for glycogen assay were resuspended in 1 or 2 ml. volumes of salt solution and samples for enzyme assay were resuspended in 2 ml. volumes. Incubations were carried out in a 37°C water bath and terminated by icing the samples. Platelets were then centrifuged from suspension at 10,000 g.

Isotonic saline solution for suspension was buffered to pH 7.4 with 0.01 M Tris-HCl and sterilized by membrane filtration. Complete Hanks solution was obtained commercially (Baltimore Biological Laboratory). Glucose-free Hanks solution was prepared in the laboratory and sterilized by membrane filtration.

**Glycogen Determination**

Duplicate samples of platelets which had been centrifuged from suspension were treated with 1 ml. of 30 per cent KOH at 100°C for 20 minutes and the glycogen was precipitated with 1.1 ml. ethanol. The precipitate was centrifuged at 1000 g and resuspended in distilled water. Glycogen was estimated by the method of Seifter, using glucose standards.

**Phosphorylase Assay**

Platelets which had been centrifuged from suspending media were resuspended in 0.4 ml. of a solution containing 0.34 M sucrose, 0.001 M EDTA, 0.1 M NaF, at pH 7.0. Platelets were disrupted by 10 seconds sonication at 3 amp. in a Branson Model S-75 sonifier and were quickly iced. Previous experiments showed that this period of sonication was the minimum sufficient to release 97 per cent of the glycogen into a 1000 g supernate. After disruption, the sonicate was spun at 2000 g for 10 minutes and the supernate assayed for enzyme activity and protein. Except in early experiments, the supernate was treated with acid-washed charcoal to remove 5'-AMP. This was found to reduce the activity without added 5'-AMP by about 15 per cent. Phosphorylase assay was performed in the direction of glycogen synthesis following the method of Steiner. The reaction mixture contained 0.02 M sodium glucose-1-phosphate, 1 per cent glycogen (charcoal-treated) 0.05 M sodium glycerophosphate, pH 6.5 (total volume 0.5 ml.) to which 0.05 ml. of sonicate was added. Each assay was performed both with and without 0.002 M 5'-AMP. Assays were performed immediately after incubation of platelet suspensions since the enzyme loses most of its activity overnight at −15°C. Reaction mixtures were incubated at 37°C for 20 minutes. The inorganic phosphorous liberated from the glucose-1-phosphate was estimated by the Comori method. Enzyme and reagent blanks were run concurrently with each platelet extract and a control run without glycogen in the mixture showed insignificant activity. Enzyme activity was found to be linear throughout the range of protein concentrations used. Results were expressed in micromoles inorganic phosphate (P1) per hour per milligram of protein.

Protein was determined according to Lowry et al. Because of turbidity due to glycogen,
samples of the sonicate were first precipitated with trichloracetic acid (5 per cent final concentration) and redissolved in 0.1 N NaOH.

Topical thrombin, 100 NIH units per ml., was dialyzed against 2 changes of distilled water overnight to remove part of the anthrone-reacting material in it. Dialyzed samples were treated with hot alkali and ethanol precipitation to obtain correction factors for the glycogen-like material it contained.

Glucose-C\(^14\) (uniformly labeled with specific activity 182 mc. per mM) was dissolved in water after removal of alcohol in a vacuum dessicator. The final concentration was 500 \(\mu\)c. per ml. For incubation, platelets from 1 ml. aliquots of plasma were resuspended in 0.5 ml. of Hanks solution made without glucose. After incubation, 1 mg. carrier glycogen was added, and glycogen was isolated by KOH treatment and reprecipitated once from distilled water. Aliquots of the final solution were dried on copper planchets and radioactivity counted in a low background gas flow counter. Appropriate corrections were made for self-absorption. Additional samples were incubated and assayed for glycogen content.

Statistical analyses were performed according to Snedecor.\(^27\)

**RESULTS**

**Effect of Incubation Medium on Phosphorylase Activation and Glycogen Depletion**

**Total Phosphorylase Activity.** Enzyme activity in the presence of 5'-AMP is designated “total” activity since it also includes that activity which would be present without added 5'-AMP. True total activity would be that activity measured if every phosphorylase molecule were maximally activated. Of particular interest in these experiments, however, is the determination of the relative state of activation of phosphorylase prior to glycogenolysis and after active glycogenolysis has begun.

When platelets were isolated, carefully resuspended from platelet-rich plasma, and incubated in salt solutions, the “total” activity increased progressively during 1 hour incubation at 37 C. (Fig. 1). Similar activation occurred whether the platelets were resuspended in Tris-buffered saline (pH 7.4) or in Hanks buffered salt solution. In Figure 1 it is seen that phosphorylase activation was negligible when platelets were incubated in plasma and significant when incubated in buffered saline or Hanks solution. The lowest curve in Figure 1 represents assays made on platelets which were incubated in plasma, centrifuged at the end of incubation, and not washed to remove contaminating plasma protein before enzyme assay. Since the contaminating protein interfered with specific activity calculations, corrections were made from simultaneously washed and unwashed platelet preparations. The curve designated “plasma” (washed platelets) represents assays on platelets incubated in plasma, centrifuged, and washed once in Hanks solution. Although little activation took place during either group of plasma incubation experiments, the zero time activity was greater in the washed platelets. The initial activity in plasma, which probably most closely represents the in vivo value, is 0.944 ± 0.187 (SE) \(\mu\)moles P\(_1\) per hour per mg. protein (range 0.605-1.389).

**Phosphorylase Activity without 5'-AMP.** Figure 2 represents additional data from the same experiments shown in Figure 1. In this case the activity without 5'-AMP (presumed to be the active form of phosphorylase) is recorded as a percentage of the “total” activity at intervals of incubation at 37 C. Only results from charcoal-treated homogenates are shown. In untreated homogenates the
starting values were slightly higher but the curves were qualitatively similar. During the incubation the proportion of activity not requiring AMP increased to a maximum at about 20 minutes. Further incubation resulted in a decrease in this fraction even though the total activity (Fig. 1) continued to increase throughout the 60 minutes, at least in the saline or Hanks incubation media.

In platelets in plasma and unincubated, the enzyme is only about 10 per cent as active without 5'-AMP as when it is present.

Glycogen Degradation. Table 1 represents the glycogen assays of the experiments in Figure 2.

The difference between zero and 60-minute values in each experiment was greater in saline than in plasma, correlating with greater enzyme activation in the salt solution. The t test revealed the differences in plasma to be insignificant, whereas glycogen depletion in saline was statistically significant (p < 0.025).

In the preceding experiments there was no platelet agglutination, fibrin formation, or clot retraction; yet glycogenolysis occurred. Additional experiments (not shown) were performed in which coagulation was initiated by recalcifica-
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Table 1.—Degradation of Platelet Glycogen during Incubation in Various Media

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>No. of Experiments</th>
<th>Zero Time</th>
<th>60 Min.</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4</td>
<td>91.0 ± 16.6 (SE)*</td>
<td>57.9 ± 12.0*</td>
<td>33.0*</td>
</tr>
<tr>
<td>Plasma</td>
<td>5</td>
<td>101.1 ± 11.1</td>
<td>89.2 ± 5.7</td>
<td>11.9</td>
</tr>
</tbody>
</table>

* µg. glycogen/10^9 platelets.

...phosphorylase activation occurred, since the enzyme was already activated in the unclotted suspension under these experimental conditions.

Evidence for Continued Glycogen Synthesis during Glycogenolysis. In addition to glycogenolysis due to the activation of phosphorylase, glycogen depletion would be further favored by cessation of glycogen synthesis. To investigate this possibility, platelets were suspended in glucose-free Hanks solution, 5 µg. per ml. glucose-C\textsuperscript{14} was added, and the mixture was incubated at 37°C. Glycogen concentration was determined on parallel incubations. The amount of radioactivity incorporated into duplicate samples at 10, 30, and 60 minutes was determined after the glycogen had been isolated. Radioactivity was incorporated into platelet glycogen at all intervals and the final specific activity was 4.96 × 10\textsuperscript{5} c.p.m. per mg. glycogen (Table 2). When the same experiment was performed with thrombin (5 NIH units per ml.) added at zero time, the results were qualitatively similar. Thus, there was not a cessation of glycogen synthesis during the period of glycogenolysis.

Coagulation and Retraction Time during Glycogenolysis. A measure of the relative dependence of coagulation and retraction on glycogen stores was made by incubating platelets in glucose-free Hanks solution (to insure maximum glycogenolysis) and recalciifying aliquots at intervals. Glycogen content, coagulation time, and retraction time were measured, and the results shown in Figure 3. It can be seen that during 4 hours of incubation the coagulation time changed from 2.5 to 3.5 minutes, but time taken for complete retraction lengthened from 13 to 25 minutes. This was correlated with a decreasing glycogen content. Thus the coagulation time remained relatively constant although the glycogen concentration was reduced to 15 per cent of normal.

DISCUSSION

In these experiments, it was seen that transfer of platelets to a relatively unphysiologic environment, such as buffered salt solutions, resulted in the activation of platelet phosphorylase. This indicates that even slight manipulation results in detectable increases in phosphorylase total activity. The higher initial activity of the samples suspended in Hanks solution or saline represents activation occurring in platelets during resuspension. Accordingly, the lower curve (Fig. 1) may also represent somewhat greater activity than present in undisturbed platelets in vivo. It may be that platelets are more sensitive to such trauma than some other cells. The platelet is such a highly specialized cell that it, like the mature erythrocyte, no longer has the capacity to alter its metabolism...
Table 2.—Incorporation of Glucose-C\(^{14}\) into Platelet Glycogen during Incubation in Hanks Solution and after Thrombin Addition

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Glucose-Free Hanks Solution Specific Activity</th>
<th>Hanks Solution Plus Thrombin Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min.</td>
<td>2.55 \times 10^6</td>
<td>5.98 \times 10^6</td>
</tr>
<tr>
<td>30 min.</td>
<td>2.91 \times 10^6</td>
<td>7.75 \times 10^6</td>
</tr>
<tr>
<td>60 min.</td>
<td>4.96 \times 10^6</td>
<td>14.35 \times 10^6</td>
</tr>
</tbody>
</table>

* Counts per minute per mg glycogen.

Fig. 3.—Coagulation and clot retraction times correlated with glycogenolysis during incubation of platelets in Hanks solution. Clotting was instituted by recalcification of the EDTA-containing medium. \( \Delta \) clotting time; ■ retraction time; ○ glycogen concentration. Incubation temperature was 37°C.

except in a limited way. Degradation of glycogen to provide a burst of ATP may be one of a limited number of ways the platelet can react to stress.

When measured prior to incubation in plasma, 90 per cent of phosphorylase activity required AMP. In this respect the enzyme resembles the muscle enzyme, where prior to contraction nearly all of the enzyme is in the \( b \) form.\(^5\) Activation of platelet phosphorylase occurred both through an increase in "total" activity and the fraction independent of AMP. If the platelet enzyme is indeed similar to muscle phosphorylase, then decreasing dependence on AMP indicates that a phosphorylase kinase is present and contributing to the activation. During the course of activation in saline, both phosphorylase activities increased initially. Later the AMP-independent fraction began to decrease. Since the AMP-dependent activity continued to increase, more than one activation mechanism must be implicated. Activation mechanisms other than the \( b \) to \( a \) transition are known to exist since in certain mice whose skeletal muscle lacks phosphorylase \( b \) kinase, near-normal glycogenolysis takes place during muscle contraction.\(^1^8\) The final activity (with AMP) at 60 minutes still may not be the maximum activation possible, since this was not followed further. Whether the late decline in AMP-independent activity represents the action of phosphorylase phosphatase or some other mechanism could not be ascertained from the data.

As pointed out by Morgan and Parmeggiani,\(^1^9\) levels of ATP, AMP, glucose-6-phosphate, and inorganic orthophosphate also may play a role in the regulation of the activities of phosphorylase \( b \) and \( a \). Another possible consideration is that platelets contain a mixture of liver and muscle types of phosphorylase. If some liver type of enzyme were present, this might explain the increase in AMP-dependent activity shown in Figure 1. Leukocytes, in which phosphorylase
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resembles the liver type, show increased AMP-dependent activity after incubation with ATP and magnesium ions.20

The experiments reported utilizing the incorporation of C\(^{14}\)-glucose into glycogen were done primarily to learn if platelet glycogen were in a state of constant turnover, as has been reported for liver glycogen.21 Although no quantitative interpretation of the data should be attempted, it is evident that glycogen synthesis is still active both during glycogenolysis in Hanks solution and while coagulation and retraction were taking place. The possibility that some of the glycogen synthesis measured during clotting may be in platelets not fully taking part in the coagulation process has not been ruled out.

Weber and Unger\(^4\) showed (under somewhat different conditions) that retraction of porcine platelets utilized larger amounts of glycogen than did clotting. Similarly, by preincubating platelets until much of their glycogen was depleted, the greater dependence of retraction on adequate glycogen stores was evident. Since glycogen breakdown is probably an attempt to maintain ATP levels, it is not surprising that lessened retraction also is correlated with lowered ATP levels.\(^2\)

Under conditions of high platelet concentration compared with fibrinogen concentration, relatively less additional ATP may be required for retraction to take place. Under in vivo conditions with vastly greater fibrin formation, perhaps a great deal more glycogen would be degraded. Also, the contribution of intracellular platelet fibrinogen\(^23\) and fibrinogen absorbed to the outer platelet surface in these experiments could not be ascertained.

The fact that much glycogen degradation is associated with retraction is consistent with the present concept of a contractile protein in platelets causing the retraction. The contractile protein, thrombosethin, has many of the properties of muscle myosin and both have ATPase activity.\(^24\) Phosphorylase activation and glycogen degradation are concomitants of the action of both these ATP requiring contractile systems. Both platelets and muscle are of mesodermal origin. It is possible that the platelet and muscle enzymes will be shown to be similar; this suggestion is supported by the properties described in these experiments. The muscle enzyme is believed to differ from the liver\(^ 25\) and Hela cell enzyme,\(^26\) both of which are tissues derived from ectodermal structures.

Although clotting and clot retraction may be the most important platelet functions which require glycogenolysis, there may be other circumstances which may result in platelet glycogenolysis. Platelets have been demonstrated to phagocytose particulate matter,\(^27\) and, assuming analogy to leukocyte phagocytosis, this would require energy\(^28\) and may result in glycogen breakdown. Also, since platelets contain lysosomal granules,\(^29\) some of these granules may well contain amylase. If so, then part of the glycogen breakdown occurring during clotting would result from lysosomal rupture and disintegration of platelets in the clot.

These studies have described ways in which a single enzyme behaves during changing glycogen requirements in a specialized cell. A more complete understanding of the mechanisms involved will require data on all of the enzymes which are intimately associated with particulate glycogen in the platelet.
instance, what happens to glycogen synthetase activity during glycogenolysis? Evidence presented suggests that some glycogen synthesis takes place, perhaps even during clotting. Obviously the effective activity of phosphorylase is greater than that of glycogen synthetase under these conditions.

SUMMARY

1. In platelets incubated in vitro, glycogen phosphorylase was activated, and the degree of activation roughly corresponds to the degree of manipulation of the platelets and is greatest in the least physiologic incubation medium.
2. Activation proceeds with an increasing proportion of enzyme which is active in the absence of AMP, suggesting the action of the phosphorylase-activating enzyme, phosphorylase kinase, in platelets.
3. Total enzyme activity (activity with AMP) likewise increases and continues to increase while the AMP-independent fraction begins to decrease again, suggesting more than one activating mechanism.
4. During glycogen breakdown, and during coagulation and clot retraction induced by thrombin, glycogen synthesis was shown to continue in vitro. Clot retraction was found to be more dependent on adequate glycogen stores than was coagulation.

SUMMARIO IN INTERLINGUA

1. Phosphorvlase (IC glycogeno esseva activate in plachettas incubate in vitro. Le grados de activation corresponde grossierrnmente al grados de manipulation del plachettas. Illos es le plus marcate in le medio de incubation le minus physiologic.
2. Le activation procecle con un crescente proportion de enzyme active in le absentia de monophosphato de adenosina, lo que suggestiona que que le enzyme phosphorylaso-activatori, kinase de phosphorylase es active in le plachettas.
3. Le activitate enzymatic total (i.e., activitate con monophosphato de adenosina) accresce similemente. Illo continua accrescer quando le fraction non (iepen(ientc (i.e, monophosphat() (1#{128}'(ellOSlflll recomencia declinar, lo que suggestiona le existentia de plus que un sol mechanismo de activation.
4. Esseva monstrate in vitro que le synthese de glycogeno continua durante le decom- position de glycogeno e durante le processos de coagulation e de retraction del coagulo induce per thrombina. Esseva trovate, plus specificamente, que le retraction del coagulo dependeva plus que le coagulation de un adequate reserva de glycogeno.

ACKNOWLEDGMENTS

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