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Studies on Glycogen Metabolism in the Human Platelet. I. Characteristics and Regulation of the Glycogen Synthetase *

J. Aguilar and M. Rosell-Pérez

Departamento de Bioquímica
Facultad de Farmacia
Barcelona - 14 (España)

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Human platelets were obtained from plasmapheresis residues. The vells were found to be intact and suitable for metabolic studies. Glycogen content was 0.8±0.3 mg per 10^{10} platelets, phosphorylase activity was 3.0±0.2 μmoles/hour/mg protein when measured in presence of 2.0 mM AMP and glycogen synthetase activity was 5.9±1.4 nmoles/min/mg protein when measured in presence of 10 mM glucose-6-P. Glycogen synthetase showed the two well known forms: I (independent of glucose-6-P) and D (dependent of glucose-6-P).

The energy of activation of human platelet glycogen synthetase was found to be around 11 Kcal for both the «crude extract» and the particulate fraction preparations. K_m and V_{max} for UDP-glucose of both I and D forms of glycogen synthetase were also determined in presence and absence of Mg^{++} as well as the K_a for glucose-6-P of the D form enzyme.

The magnesium divalent cation was found to increase the V_{max} of the enzyme, around 11 Kcal for both the «crude extract» and the particulate fraction preparations. urea-treated enzyme preparation. Phosphate made the I form of the enzyme sensitive to glucose-6-P and sulphate stimulated the D form to maximum at 1 mM concentration, this stimulation being enhanced by the presence of glucose-6-P.

The primary regulatory enzymes of the glycogen synthetase system, the phosphatase and kinase, were detected in our preparations. The phosphatase was estimulated by 50 mM mercaptoethanol and the kinase was normally active when ATP and Mg^{++} were added to the enzyme preparation. The resulting conversion was proportional to the added concentrations of ATP-Mg^{++}. The kinase activity was stimulated by 3',5'-AMP at concentrations of 1 to 100 μM.

The glycogen content of platelets is very similar to that of muscle since each tissue shows a level which varies about 1 % of its wet weight (22, 34). This glycogen, acting as a source of energy, supports many functions in these cells, especially in conditions of the so called «viscous metamorphosis» for which the blood glucose is not available. For this reason much interest has been devoted to the study of the degradation of glycogen in

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platelets in relation to conditions in which energy is rapidly consumed in such processes as aggregation, clot retraction and others (15, 34, 35, 46).

Though the synthesis of glycogen in platelets has been much less studied than glycogenolysis, it is now known that it is produced by UDP-glucose (38) with the aid of glycogen synthetase (16, 43, 44).

This discovery rules out the hypothesis previously held that the synthesis of glycogen and of protein as well was assumed to happen in the megacaryocyte but not in the platelet itself. Some investigators have now demonstrated the synthesis of certain proteins in platelets (3, 4).

The enzyme glycogen synthetase catalyzes the transfer of the glucosyl residue from UDP-glucose to glycogen and is activated by glucose-6-P (17, 18). ROSELL-PÉREZ and LARNER (24, 26) demonstrated that from rat skeletal muscle two forms of this enzyme could be prepared and kinetically differentiated. One is absolutely dependent on glucose-6-P as a cofactor for its activity (D form), the other has some activity even without this cofactor (I form). These two types of enzyme activity have been shown to be interconvertible through a process of phosphorylation and dephosphorylation that is enzymatically catalyzed (7).

Human platelets (30, 44) and lymphocytes (10) possess both forms of glycogen synthetase as well as the interconversion mechanisms, in contrast to polymorphonuclear leukocytes, which until recently were believed to have the D form of the enzyme only (21, 29).

We present in this paper a further characterization of the glycogen synthetase from blood platelets as well as a study of the interconversion mechanisms operating between the two forms.

Materials and Methods

For the preparations of enzymes from platelets the intermediate fraction between the plasma and the red cells obtained in plasmapheresis process was used as source of platelets. This fraction, rich in leukocytes and platelets, was made up to 1/5 of its original volume with saline containing 0.001 M EDTA and centrifuged at $670 \times g$ for 10 minutes. The supernatant constituted the «platelet-rich plasma» which averaged 0.8 to 1.0×10^6 platelets/mm^3 counted in an haemocytometer and had a contamination of less than 4 red cells and 1 leukocyte per 10^4 platelets.

The platelet-rich plasma was centrifuged at $3{,}000 \times g$ to sediment the cells and the sediment was resuspended in 0.05 M Tris - 0.001 M EDTA fuffer (pH 7.8). The cell suspension was sonicated at 4° C for two successive periods of 30 s. Finally the cell debris was removed at $10{,}000 \times g$ for 20 minutes and the supernatant («crude extract») used as an enzyme source.

For some of the experiments described the enzyme of the particulate fraction obtained by centrifugation of the «crude extract» at $100{,}000 \times g$ for one hour was also used. This particulate fraction was purified 50 folds by sucrose density gradient centrifugation in accordance with the method of SCOTT and STILL (36). All procedures were carried out at 4° C.

UDP-glucose: α-1,4-glucan α-4-glucosyl transferase (glycogen synthetase, E.C. 2.4.1.11) activity was measured as radioactivity incorporated into glycogen from (^{14}C) glucose labeled UDP-glucose as described by THOMAS *et al.* (41). The standard assay mixture contained 0.006 M UDP-glucose (specific activity 20,000 counts per minute per μM), 1 % glycogen, 0.05 M Tris - 0.005 M EDTA buffer (pH 7.8) and 0.01 M glucose-6-P when added. For the assay of the enzyme 30 μl of the enzyme source was added to 60 μl of the assay mixture and incubated at 30° C for 15 minutes. The incubation was ended by pipeting 75 μl of the reaction mixture onto a square of filter paper Whatman 31 ET and immediately im-

mersed in 66 % ethanol. The papers containing the radioactive sample were thoroughly rinsed and then counted as described (41).

Phosphorylase activity was measured by the formation of Pi from glucose-1-P in the presence of the enzyme source. The standard assay mixture contained 0.032 M glucose-1-P, 0.05 M FNa, 0.01 M mercaptoethanol, 0.05 M Tris-0.005 M EDTA buffer (pH 6.5) and 0.002 M AMP when added. For the assay 50 μl of enzyme source was added to 50 μl of the assay mixture and incubated at 30° C. The incubation ended with the addition of 0.1 ml of 5 % trichloroacetic acid and Pi was determined by the method of FISKE and SUBBAROW (6).

Glycogen determinations were made by the anthrone method (37). 5×10^9 cells were digested in 2 ml of 30 % KOH at 100° C for 4 hours. Glycogen was precipitated three times in 66 % ethanol containing 1.5 % saturated Na_2SO_4 solution (9), and then measured.

Magnesium determinations were made by atomic absorption spectrophotometry. The assay was performed according to the procedures described by «Evans Electroselenium Ltd.».

The samples were prepared in 0.75 % EDTA and 1/20 (v/v) of 5 % deoxicolate in 17.5 % KOH as a protein solubilizer.

The UDP-glucose, 3',5'-AMP, Tris and glycogen from rabbit liver were purchased from Sigma Chemical Co. (St. Louis). ^{14}C-glucose labeled UDP-glucose was supplied by New England Nuclear Co., or by Radiochemical Center (Amersham). Glucose-6-P was purchased from Boheringer Manheim, mercaptoethanol from Eastman Kodak Co., ATP from Pabst Laboratories and anthrone and urea were obtained from Merck.

Results

Cell preparations. The platelets obtained by plasmapheresis as described in this paper showed a glycogen content of 0.8 ± 0.3 (mean $\pm$ S.E.) mg per 10^{10} platelets and a phosphorylase activity in the presence of 0.002 M AMP of 3 μmoles per hour per mg protein. Both values were in the same range as those described by other investigators who obtained the cells by direct venipuncture which have consequently not suffered the manipulations of plasmapheresis (13, 34, 47).

Glycogen synthetase activity. Within the first twenty minutes of incubation at 30° C, with an enzyme concentration of 1.75 mg protein/ml and under our experimental conditions the activity was linear with the time of incubation. Maximal activity was attained at pH 8.0 for the D form of the enzyme (measured in presence of 0.01 M glucose-6-P) and at pH 7.5 for the I form (measured without the activator) when either Tris-ClH or Tris-maleate was used as buffer (2, 42).

The synthetase activity of our preparations measured in the presence of 0.01 M glucose-6-P, averaged 5.9 ± 1.0 (mean $\pm$ S.E.) nmoles per minute per mg protein for the crude extract and 11.2 ± 1.0 (mean $\pm$ S.E.) nmoles per minute per mg protein for the enzyme in the particulate fraction obtained by centrifugation at $100{,}000 \times g$. The values are also close to those described in the literature for platelets obtained by more direct methods (16, 38, 44).

Effect of temperature. The thermal sensitivity of platelet glycogen synthetase is demonstrated by Arrhenius plots over the range from 15 to 40° C (fig. 1). With the crude extract as well as with the enzyme of the particulate fraction of $100{,}000 \times g$ a linear relationship between log. V and the reciprocal of the temperature was obtained in the presence of 0.01 M glucose-6-P. When the enzyme was preincubated for 60 minutes the plot deflected downward as the temperature rose above 37° C. The energy of activation calculated from these graphs for both enzymes sources is about 11 Kcal per mole.

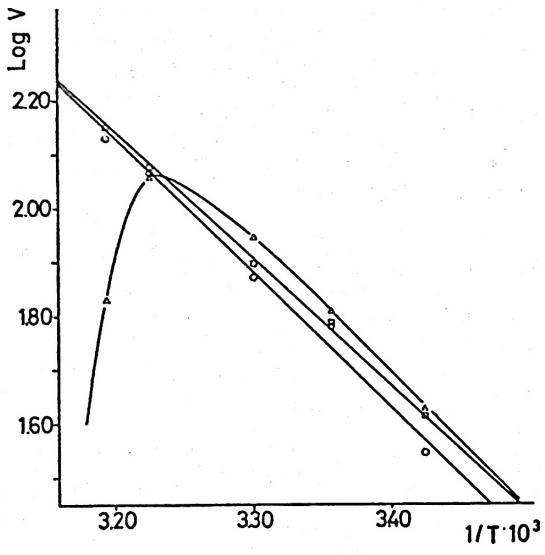

Fig. 1. *Arrhenius plot of human platelets glycogen synthetase.*
The logarithm of velocity has been plotted against the inverse of the absolute temperature in which the enzyme reaction took place. These conditions have been represented: «crude extract» (○), enzyme from the particulate fraction of 100,000 × *g* (□), and «crude extract» after 60 minutes of preincubation at each temperature plotted (△).

The glycogen synthetase from platelets was stable over 30 days when the sample was frozen at —20° C without mercaptoethanol. The presence of this compound being the cause of enzyme inactivation during the freezing (11). The ratio of the enzyme activity —glucose-6-P/enzyme activity +glucose-6-P was unaltered by the frozen state and drastically increased after 5 days when the crude extract was stored at 4° C in presence of 0.05 M mercaptoethanol.

Kinetic characterization of glycogen synthetase. Studies of the UDP-glucose concentration dependence with and without glucose-6-P and magnesium were done with the enzyme of the particulate fraction obtained at 100,000 × *g*.

The enzyme source used had a controlled percent of I or D form with no more than a 10 % contamination of one form with the other. For the preparation of the I form the crude extract was preincubated at 4° C for 2 days in the presence of 0.05 M mercaptoethanol. The resulting preparation containing a high level of the I form was then centrifuged at 100,000 × *g* and the glycogen pellet stored at —20° C. For preparation of the D form, the crude extract was immediatelly centrifuged at 100,000 × *g* in the presence of 0.05 M

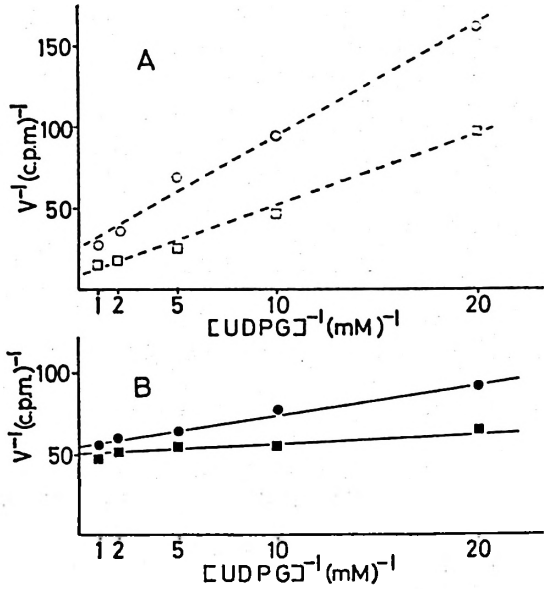

Fig. 2. *Lineweaver-Burk plots of human platelets glycogen synthetase.*
(A) Activity in the absence of glucose-6-P. The inverse values of velocity were plotted against the inverse substrate concentration (UDPG) from an enzyme preparation with high activity independent of glucose-6-P. The I form was then centrifuged at 100,000 × *g* prepared as described in the text. Experiments were done in the absence (○) and presence (□) of 5 mM Mg^{++}. Michaelis constants were calculated from the initial velocities determined as described in the text.
(B) Activity in the presence of glucose-6-P. Plots are the same as in (A) but the enzyme source was a preparation practically without activity in the absence of glucose-6-P obtained as described in the text. Assay without Mg^{++} (●); assay with M^{++} (■).

Table I. *Kinetic data of platelet glycogen synthetase.*

Activity	Mg^{++} 5×10^{-4} M	K_m UDPG M	V_{max} c.p.m.	K_a G-6-P M	V_{max} c.p.m.
In the absence of glucose-6-P	—	2.8×10^{-4}	40	—	—
	+	5.4×10^{-4}	129	—	—
In the presence of glucose-6-P	—	3.6×10^{-5}	18	1.5×10^{-4}	62
	+	1.6×10^{-5}	20	6.0×10^{-5}	89

NaF which completely inhibits the conversion from the D to the I form. The pellet also was stored at —20° C. In every experiment the relative proportions of both forms were checked after the resuspension of the pellets in 0.05 M Tris - 0.005 M EDTA buffer (pH 7.8).

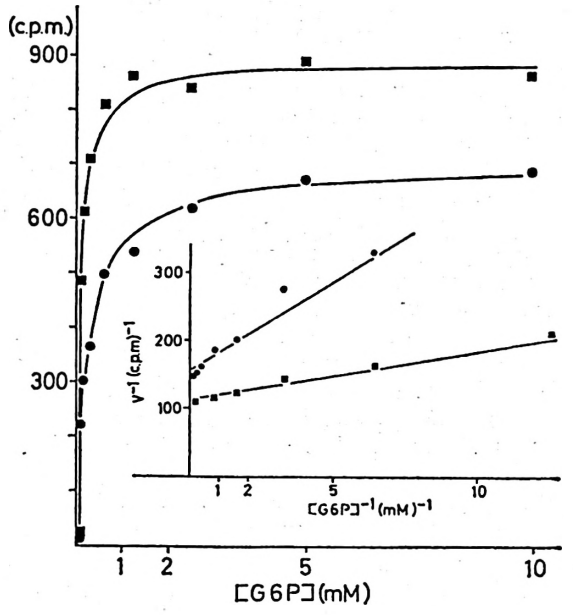

Fig. 3. *The direct and double reciprocal plots for the effect of glucose-6-P on the D form of human platelets glycogen synthetase.* The direct and reciprocal plots of velocity versus glucose-6-P concentration have been represented for an enzyme of the particulate fraction of 100,000 × *g* that was virtually inactive in absence of glucose-6-P. The sample was prepared as described for those samples used in UDPG kinetics. Experiments were done using 5 mM UDPG with specific radioactivity of 300,000 c.p.m./μmole. Assay with 5 mM Mg^{++} (■); assay without Mg^{++} (●).

For every concentration tested the initial velocity was determined and plotted against its concentrations in a double reciprocal plot (Lineweaver burk) (fig. 2).

Table I contains the kinetic data obtained and shows that in the absence of magnesium the D form has a K_m one order of magnitude lower than the I form and that this cation increased about 3 fold the V_{max} of the I form while the V_{max} of the D form remained unchanged.

With the same preparation of the D form used for the kinetics of the UDP-glucose a study of the dependence on the concentration of glucose-6-P was carried out. The direct and the double reciprocal plots obtained (fig. 3) show a K_a of 1.5×10^{-4} M without magnesium and 6×10^{-5} M with 0.005 M magnesium, the V_{max} being increased by 30 % by the same concentration of the divalent cation (table I).

Effect of magnesium, calcium and manganese. As shown in the kinetic studies of UDP-glucose and glucose-6-P, the magnesium increased the V_{max}, specially for the I form of the enzyme. The question arose as to whether other divalent cations could affect the enzyme activity. Attempts to show such an activation with calcium or manganese were unsuccessful and compared to that obtained with magnesium only a slight activation was observed in the experiments performed. The plot of the percent of activation against magnesium concentration (fig. 4) showed a saturation kinetic curve. In this study the concentration of endogeneous magnesium in the sample was measured and taken

into account. The concentration of magnesium in the supernatant which is used as an enzyme source in such experiments was calculated to be 0.001 M when the extract was prepared at a concentration of 1/5 (w/v).

The possible involvement of magnesium in maintaining the enzyme structure was studied in a series of experiments done with a 50 folds purification enzyme source (see methods). To such enzyme preparation, urea was added up to 6 M concentration (pH 7.5). After 4 minutes at 4° C, one aliquot of the enzyme was filtered through a gel of Sephadex G-25 and to another aliquot, magnesium was added up to 0.015 M concentration and after thorough mixing, allowed to stand at 4° C for 30 minutes. Samples were taken at every step and both activities of the synthetase were analyzed.

Figure 5 shows the complete inactivation of both forms of the enzyme after urea treatment and a strong reactivation

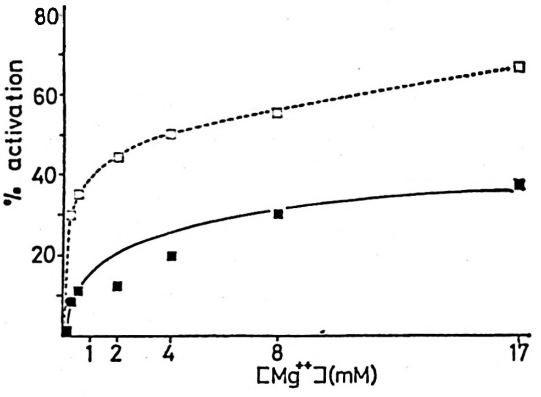

Fig. 4. *Magnesium activation of human platelets glycogen synthetase.*
The percent of increase in activity over the value without magnesium is plotted versus the magnesium concentration. Samples were from a particulate fraction of 100,000 × *g* and were prepared as described for UDPG kinetic experiments. Broken line between open symbols represent the activity independent of glucose-6-P. The solid line between the black symbols depicts the glucose-6-P dependent activity.

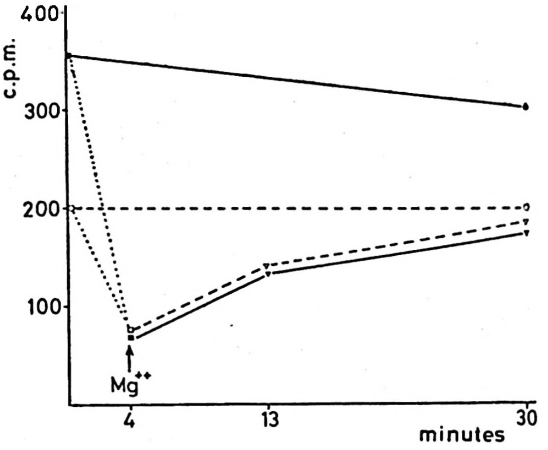

Fig. 5. *Reactivation by Mg^{++} of the urea inactivated enzyme.*
Both activities of glycogen synthetase from human platelets are shown during the experiment. The enzyme source utilized was a preparation purified about 50-fold by differential centrifugation and thereafter sucrose gradient centrifugation. The activities were measured by incubation of aliquot parts of the enzyme during 15 minutes with the assay mixture; the remaining enzyme being kept at 4° C during the experiment. Solid lines between black symbols represent the activity in the presence of 10 mM glucose-6-P, and the broken line between open symbols, the activity in the absence of the hexose-phosphate. The circles (○—●) show the control values, the squares (□—■) the values obtained after the addition of 6 M urea and the triangles (△—▲) those values obtained after the addition of 20 mM Mg^{++} to the urea treated enzyme af the point and times indicated in the graph.

of the I form upon the addition of magnesium even without removal of the urea previously added. In contrast, gel filtration of the inactive enzyme did not promote any reactivation thus demonstrating the role of magnesium in the reactivation.

Effect of inorganic phosphate and sulphate. The implication of phosphate and sulphate in the regulation of glycogen synthetase has been studied in several systems (19, 27, 28), and since their concentration in platelets (14) seem to be of

the same order as in other tissues, we have been interested in the possible modification of the glycogen synthetase by these anions.

An enzyme source like that used for the kinetic studies was used to study the effect of sulphate and phosphate concentration and the same precautions were taken in handling it. The results show that the activity of the I form in these preparations was sensitized to glucose-6-P by the presence of phosphate at concentrations of 0.005 M; the stimulation of the activity of the I form by this anion being enhanced by increasing concentrations of glucose-6-P. Sulphate at concentrations up to 0.005 M had no effect on the activity of the I form in our preparations (fig. 6).

The activity of the D form was stimulated by sulphate, a maximum stimulation being attained at 0.001 M concentration. This stimulation is also enhanced by increasing concentrations of glucose-6-P (fig. 7).

In contrast phosphate, did not stimulate the activity of the D form but decreased it in non saturating concentrations of glucose-6-P. It does seem as if phosphate

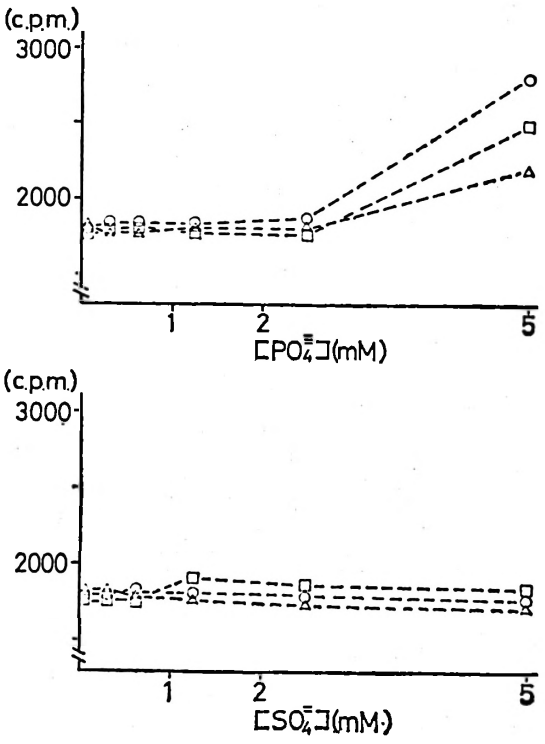

Fig. 6. *Effect of sulphate and phosphate ions on the I form of human platelets glycogen synthetase.*
The c.p.m. incorporated during 5 minutes of incubation were plotted versus phosphate or sulphate concentrations as indicated. The enzyme preparation was a particulate fraction of 100,000 × *g* and was completely in the I form. Experiments were done using 5 mM UDPG with a specific radioactivity of 100,000 c.p.m./μmole. (△) No glucose-6-P added, (□) 0.5 mM glucose-6-P and (○) 10 mM glucose-6-P.

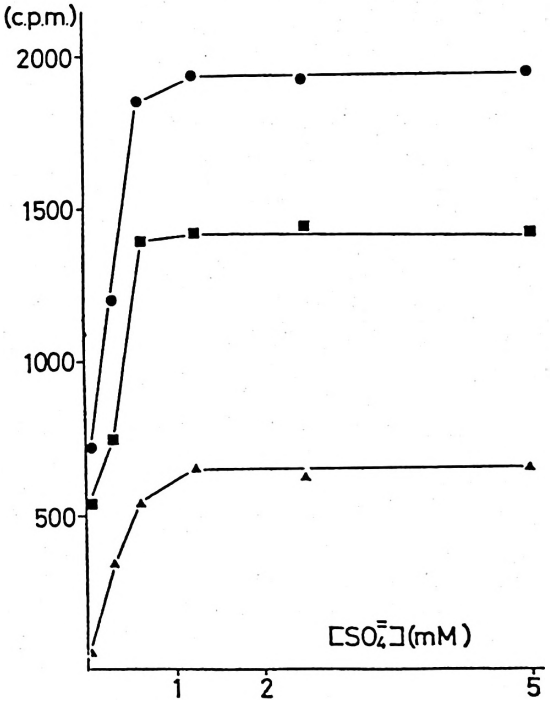

Fig. 7. *Effect of sulphate ion on the D form of human platelets glycogen synthetase.*
Plots were done as in figure 6 but the enzyme preparation was in the D form, prepared as described for the samples used in the UDPG kinetic experiments. (▲) No glucose-6-P added, (■) 0.5 mM glucose-6-P and (●) 10 mM glucose-6-P.

and glucose-6-P competed for the same site in the enzyme molecule.

Primary regulatory enzymes. As we have indicated before platelets exhibit both forms of synthetase activity and the interconversion between them seems to be carried out through a mechanism of phosphorylation and dephosphorylation driven by a system of primary regulatory enzymes as described by ROSELL-PÉREZ and LARNER (25, 31).

Phosphatase. In our crude extracts obtained as described previously, the activity of the I form averaged about 5 % of that of D form. The incubation of this enzyme preparation at 30° C before assay of the enzyme activity revealed a time dependent increase in the percent of I form activity; consequence of the conversion of the D form into I form of the synthetase through a dephosphorylation catalized by the phosphatase of the synthetase D.

We found the phosphatase reaction to be stimulated by 0.05 M mercaptoethanol and a total conversion within 60 minutes could be observed under these conditions (fig. 8). We also found an almost complete inhibition of the conversion by 0.05 M NaF (not shown).

The particulate fraction obtained at 100,000 × *g* from a crude extract of platelets showed a significant rate of conversion provided that Mg^{++} was added to the particulate fraction resuspended in 0.05 M Tris - 0.001 M EDTA buffer. This result indicated that the phosphatase precipitated with the synthetase. To test for phosphatase activity in the supernatant of the 100,000 × *g* particulate fraction, the rate of conversion obtained when the particulate fraction was resuspended in its own supernatant was compared to the rate obtained when this supernatant was maintained at 100° C for 5 minutes, in order to thermically inactivate any enzyme, before being used as resuspension medium. In both cases the same conversion rate was observed indicating that no phosphatase activity remained in the supernatant after centrifugation at 100,000 × *g* and only some thermostable activating factor, such as magnesium, was present in the supernatant.

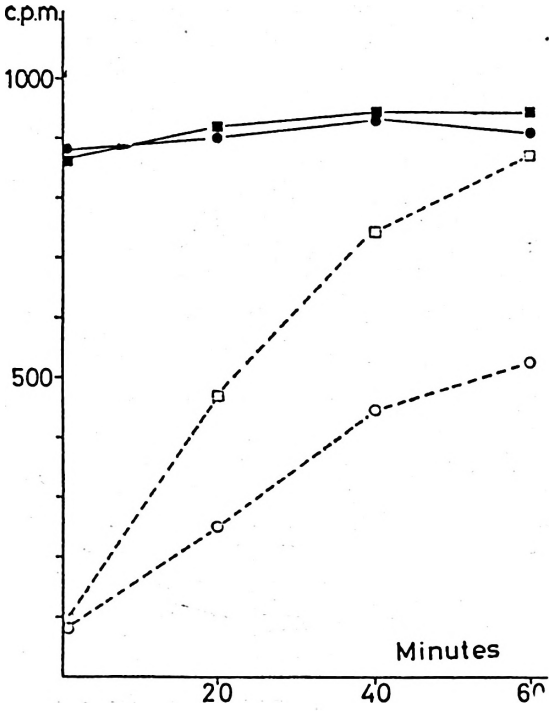

Fig. 8. *Stimulation of human platelets glycogen synthetase phosphatase.*
Both glycogen synthetase activities are plotted versus time of preincubation for the following conditions: (○—●) no additions; (□—■) in presence of 50 mM mercaptoethanol. Open symbols and broken lines represent the activity measured in absence of glucose-6-P and black symbols and solid lines represent the activity measured in presence of glucose-6-P.

The phosphatase seemed to be inactivated when the platelet suspension was homogenized by freezing and thawing as the rate of conversion was much lower in such preparation. This low phosphatase activity was also observed when the preparation was subjected to sonication before the freezing. This result confirms that the low rate of conversion was due to some process inactivating the phospha-

tase activity such as that recently reported for polimorphonuclear leukocytes (21)

Kinase. The glycogen synthetase kinase which catalyzes the phosphorylation of the I form to the D form (8) is also present in platelet extracts and easily tested in the presence of ATP and Mg^{++}.

The addition of 0.001 M ATP - 0.008 M Mg^{++} to a crude extract, previously preincubated at 30° C in the presence of 0.05 M mercaptoethanol, to ensure a high percentage of the I form, promoted a rapid decrease in the activity of the I form of the synthetase without affecting the level of the D form. The degree of the phosphorylation shows a strict dependence on the amount of ATP added to the assay, the maximum effect being obtained at a concentration of 0.005 M (fig. 9). It is important to note that when these high

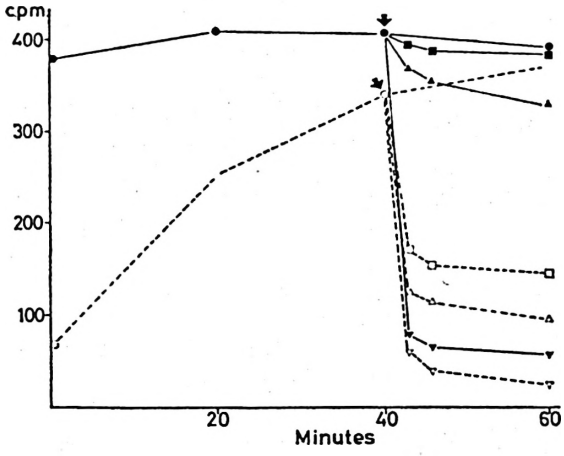

Fig. 9. *Effect of ATP-Mg^{++} on both forms of human platelets glycogen synthetase.*
Both activities of glycogen synthetase are recorded for an enzyme preincubated in presence of 50 mM mercaptoethanol and 8 mM Mg^{++} (○—●). At the time indicated by the arrows, ATP was added to aliquots of the preincubated preparation up to the following concentrations: (□—■) 1 mM ATP, (△—▲) 2.5 mM ATP, (▲—△) 5 mM ATP. Open symbols and broken lines represent the activity measured in absence of glucose-6-P and black symbols and solid lines the activity measured in presence of glucose-6-P.

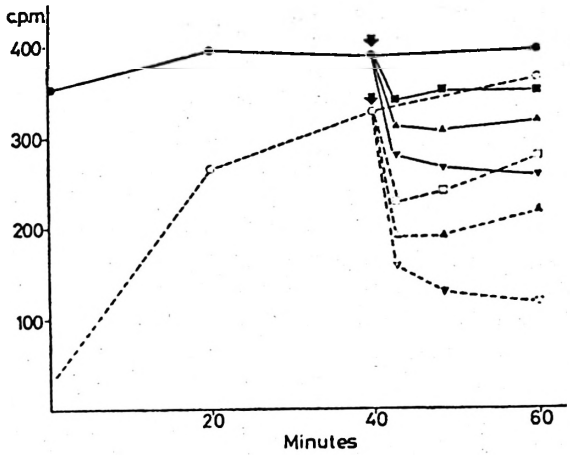

Fig. 10. *Effect of 3′,5′-AMP on human platelets glycogen synthetase kinase.*
Both activities of glycogen synthetase are recorded for an enzyme preincubated in presence of 50 mM mercaptoethanol and 8 mM Mg^{++} (○—●). At the time indicated by the arrows, ATP up to 0.5 mM and cyclic AMP up to 1 μM (□—■), 10 μM (△—▲) and 100 μM (▲—△) concentrations were added to aliquots of the preincubated preparation. Open symbols and broken lines represent the activity measured in the absence of glucose-6-P and black symbols and solid lines, the activitay measured in the presence of glucose-6-P.

concentrations were used, not only the I form disappeared from the assay but the activity of the D form also decreased, eventually becoming inactive (fig. 9). Such inactivation seems to represent an effect of the kinase which besides phosphorylating the D form, is also converting it to another molecular species of the synthetase totally inactive even in presence of glucose-6-P as described by ROSELL-PÉREZ (23).

The synthetase kinase reaction in platelet extracts is sensitive to the addition of 3′,5′-AMP at concentrations ranging from 1 to 100×10^{-6} M, especially in conditions in which the low ATP concentration (i.e. 5×10^{-4} M), does not permit a noticeable degree of phosphorylation. In such conditions the effect of

the cyclic nucleotide is to stimulate and it shows a concentration dependence indicating the participation of 3',5'-AMP in the phosphorylation process (fig. 10).

The kinase of the platelet extract sediments at 100,000 × *g* since the I to D form conversion is shown in the particulate fraction resuspended in 0.05 M Tris - 0.001 M EDTA buffer (pH 7.8). The enzyme seems to be unaffected by freezing and thawing or the sonication either of the cells or the particulate fraction of 100,000 × *g*.

Discussion

The characteristics of the glycogen synthetase system from the blood platelets of human donors have been studied thoroughly using the residue from plasmapheresis obtained from a blood bank. These residues were an excellent supply of cells suitable as sources of enzyme. This is shown by their normal content of glycogen and the levels of activity of the enzymes phosphorylase and glycogen synthetase which were close to the values determined by others (13, 16, 34, 38, 44, 47).

The thermal inactivation values obtained from the Arrhenius plots are practically the same as those obtained for the glycogen synthetase in human polymorphonuclear leukocytes (33), althoung they differ from those reported by STEINER for the enzyme of the liver (39) or muscle (40) in rabbits. If these data suggest a possible difference in the protein structure of the enzymes from liver and muscle, our results would indicate that the enzyme protein structure from the platelets and leukocytes from humans must be similar and would differ from those of liver and muscle from the rabbit.

Although the activity of the glycogen synthetase extracted from the platelets is mainly dependent on glucose-6-P for activity, it has proved possible *in vitro* to transform this D activity into the independent form (I activity) which operates without the sugar phosphate. This transformation of the D to I form was obtained by preincubating the crude extracts or the corresponding particulate glycogen fractions at 30° C in the presence of mercaptoethanol and in the latter preparation was dependent on the presence of Mg^{++} or Ca^{++} ions. In both cases the transformation was totally inhibited by the presence of NaF.

As preparations of both forms of the enzyme *in vitro* were possible, the kinetics of both forms were studied in some detail. The I form showed K_m in the range of $2\text{-}5 \times 10^{-4}$ M, one order of magnitude higher than the D forms ($1.5\text{-}3.5 \times 10^{-5}$ M) either in the absence or in the presence of Mg^{++}. Even taking into account the phosphate concentration of the cells and observing our results *in vitro* about the action of the sulphate and phosphate on both forms, it seems probable that the D form might be active in these cells.

The action of Mg^{++} ions on the enzyme kinetics is very characteristic and defines both forms clearly. The effect on the I form is both a decrease in the K_m and an increase in the V_{max}. On the D form the effect of Mg^{++} is a reduction in the K_m only. The K_a for glucose-6-P also is lowered by an order of magnitude hence making the endocellular concentrations of glucose-6-P more effective as activators.

If one takes into account the effect of Mg^{++} on the aggregation of subunits and stability suggested in the experiments with urea, and also the dependence of the phosphatase on the presence of the cation (which calls for further discussion) the three levels of action for the Mg^{++} ions described previously by ALBERT and ROSELL-PÉREZ (1) have again been shown here.

The observed effects *in vitro* of the phosphate and sulphate anions merit some further comment. While sulphate does not influence the I form whether or not glucose-6-P is present, the phosphate ion

does activate by itself and also sensitizes this I form to a further activation by glucose-6-P. The main difference from the liver systems (45) appears in the action of sulphate on the D form. As shown clearly in figure 7 sulphate stimulates by itself this D form and also, at concentrations as low as 1 mM, promotes a synergistic stimulation by glucose-6-P, the natural activator, even at low concentration of the latter. Although sulphate ions are present in the blood serum, their metabolic significance as regulators of the D activity inside the platelets remains unknown. Their effects, however, showed a clear difference from those on the inactive *b* form of the liver systems (45).

The primary regulatory enzymes have not hitherto been studied in these blood cells although preliminary data on the presence of the two activities have been reported (44).

Under the conditions we have used, the glycogen synthetase D phosphatase sediments in the glycogen particulate fraction of 100,000 × *g*, the supernatant being free of this enzyme system. Its lability to freezing and thawing has already been reported (30) and apparently has also been observed in the phosphatase systems of other blood cells (21). The effect of cations or SH-protecting agents has already been commented.

The kinase that inactivates the glycogen synthetase I is also present in these cells and is quite stable to freezing and thawing. It responds by an increased effect to the increasing concentration of ATP and is sensitive to cyclic AMP. By manipulating the ATP concentration, the transformation of the I to D form can be easily observed, also the partial inactivation of the D form and even the total inactivation of both forms (figs. 9 and 10). This change again exemplifies what appears to be a conversion of the active I or D form of glycogen synthetase into a completely inactive molecular species by extraphosphorylation. This kind of transformation, observed in other enzyme sources such as muscle (20), heart (32), or kidney (12) in rats, and also in human lymphocytes (10) and polymorphonuclear leukocytes (5) are not produced by ATP alone; nor can it be explained by inhibition or the activity by the ATP, since in the case of the D form, its activity has been measured in the presence of sufficiently high concentrations of glucose-6-P to counteract any inhibition by ATP alone.

Resumen

Se obtuvieron plaquetas humanas intactas a partir de residuos de plasmaferesis; su contenido de glucógeno fue de 0,8±0,3 mg por 10^{10} plaquetas, su actividad fosforilasa fue de 3,0±0,2 μmoles/hora/mg de proteína cuando se midió en presencia de 2,0 mM AMP y su actividad glucógeno sintetasa de 5,9±1,4 nmoles/minuto/mg de proteína cuando se midió en presencia de 10 mM glucosa-6-P. El enzima glucógeno sintetasa presentaba ambas formas de actividad: la I (independiente de glucosa-6-P) y la D (dependiente de glucosa-6-P).

Se calculó una energía de activación para el glucógeno sintetasa de 11 Kcal aproximadamente, tanto para el extracto crudo como para el enzima de la fracción particulada. También se determinaron la K_m y V_{max} para el UDP-glucosa de ambas formas, en presencia y ausencia de Mg^{++}, así como la K_a para la glucosa-6-P de la forma D del enzima.

El Mg^{++} incrementa la V_{max} del enzima, especialmente para la forma I, y tiene un efecto positivo sobre la renaturalización de una preparación enzimática tratada con urea.

El fosfato sensibiliza la forma I del enzima a la glucosa-6-P y el sulfato estimula la forma D, alcanzándose un máximo a una concentración de 1,0 mM, y siendo este estímulo mayor en presencia de glucosa-6-P.

Los enzimas reguladores primarios de la glucógeno sintetasa, fosfatasa y kinasa, se hallaban presentes en nuestras preparacoines. La fosfatasa se estimulaba por mercaptoetanol 50 mM y la kinasa se ponía de manifiesto en presencia de ATP-Mg^{++}, siendo la conversión proporcional a la concentración de los mismos. La actividad kinasa se estimulaba además por la presencia de 3',5'-AMP a concentraciones entre 1,0 y 100,0 μM.

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