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# UPTAKE AND PHOSPHORYLATION OF D-GLUCOSAMINE-1-$^{14}$C IN THE HUMAN PLATELET

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**Abstract**—Human platelets accumulate and phosphorylate D-glucosamine-1-$^{14}$C. The accumulation process is saturable, suppressed by iodoacetic acid and other hexoses, and apparently not mediated by phosphorylation. Neither uptake nor phosphorylation is influenced by L-glucose. Platelet homogenate, supplemented with ATP, phosphorylated the compound with an apparent $K_m$ of $3{\cdot}3 \times 10^{-4}$ M. Such phosphorylation is inhibited by other hexoses but not by L-glucose. D-glucosamine kinase in the platelet appears specific since the apparent $K_i$ values for other sugars, including D-glucose, were substantially higher than the apparent $K_m$ of D-glucosamine. No D-glucosamine-6-phosphatase was found in the homogenate.

IN RAT liver glucosamine ($G_m$) is metabolized to glucosamine-6-phosphate, *N*-acetylglucosamine, *N*-acetylglucosamine-6-phosphate, *N*-acetylglucosamine-1-phosphate, UDP-*N*-acetylglucosamine, and thence to glycoprotein.[1,2] Glucosamine is also accumulated against a concentration gradient and metabolized to glucosamine-6-phosphate in yeast[3] and similarly, phosphorylated in ascites tumor cells.[4–6] Although a mucopolysaccharide, containing glucosamine, has been isolated from the blood platelet, relatively little is known concerning the mode whereby this sugar enters the cell.[7] The present study characterizes the accumulation and phosphorylation of $G_m$ by the human platelet.

## EXPERIMENTAL SECTION

Platelet-rich plasma was obtained from healthy donors as previously described.[8] Approximately 35 mg wet weight of cells were sedimented, washed twice with tris-buffered saline (pH 7·5) containing 1·3 mM EDTA, and resuspended in 0·9 ml $Ca^{2+}$ and $Mg^{2+}$ free Krebs–Ringer bicarbonate buffer (pH 7·4). Samples were incubated for varying periods of time at 37° in a Dubnoff metabolic shaker with 0·1 ml of D-glucosamine-1-$^{14}$C hydrochloride ($G_m$) solutions. After incubation, the platelets were sedimented, supernatants decanted and the tubes swabbed with a cotton-tipped applicator. The samples were weighed and lysed in 1 ml of distilled water. $G_m$, D-glucosamine-6-phosphate ($G_m$-6-P), UDP-*N*-acetylglucosamine (UDPAGA) and *N*-acetylglucosamine-6-phosphate ($AG_m$-6-P) in the supernatants and lysates were separated on thin layers of cellulose (TLC) utilizing solvent systems I, II and III (Table 1). "Carrier" $G_m$, $G_m$-6-P, UDPAGA and $AG_m$-6-P were added to the platelet lysate and medium prior to chromatographic separation. $G_m$ and $G_m$-6-P were visualized with ninhydrin, UDPAGA with u.v. light and $AG_m$-6-P with ammonium molybdate and stannous chloride.[9] Thereafter the cellulose was scraped from the plates, suspended in gelled scintillation medium and the radioactivity determined.[10]

TABLE 1. CHROMATOGRAPHIC SEPARATION OF GLUCOSAMINE AND METABOLITES ON THIN LAYERS OF CELLULOSE

| Solvent system | $R_f$ values of standard compounds | | | |
|---|---|---|---|---|
| | UDPAGA | $G_m$ | $G_m$-6-P | $AG_m$-6-P |
| I. Ethanol–water–$NH_4OH$ (65:35:5) | 0·83 | 0·72 | 0·35 | 0·25 |
| II. Ethanol–water–acetic acid (65:35:5) | 0·68 | 0·68 | 0·31 | 0·18 |
| III. *N*-propanol–water–acetic acid (70:30:5) | 0·45 | 0·64 | 0·34 | 0·34 |

The distribution ratio ([I]/[0]) of $G_m$ was expressed as the ratio of disintegrations per minute per millilitre of platelet water to disintegrations per minute per millilitre of incubation medium. Total platelet water was 76 per cent and extracellular water 27 per cent of the wet weight of the platelet pellet.[11] The concentration of $G_m$ in the intraplatelet water was corrected for that trapped within the extracellular space of the platelet pellet according to the formulation of Helmreich and Kipnis.[12] Radioactivity of all samples exceeded 10 times background. Counting efficiency determined by the channels ratio method was 72–76 per cent. D-glucosamine-1-$^{14}$C hydrochloride (sp. act. 52 mc/m-mole), obtained from New England Nuclear Corp., was found to be greater than 96 per cent pure by thin-layer chromatography.

In some experiments the influence of various sugars on the uptake of $G_m$ by the platelet was studied; similarly, the influence of iodoacetic acid on the uptake of $G_m$ was evaluated.

Further experiments evaluated the enzyme, $G_m$ kinase, in platelet lysate. Approximately 100 mg (wet weight) of human platelets were lysed in 1·5 ml of distilled water

TABLE 2. APPARENT INHIBITORY CONSTANTS ($K_i$) FOR VARIOUS COMPOUNDS WHICH INHIBIT THE CONVERSION OF GLUCOSAMINE TO GLUCOSAMINE-6-PHOSPHATE BY HUMAN PLATELET LYSATE*

| Inhibitor | Concentration of inhibitor (M) | $K_i$ (M) |
|---|---|---|
| *N*-Acetyl-D-glucosamine | $1 \times 10^{-3}$ | $5{\cdot}0 \times 10^{-4}$ |
| D-Glucose | $2 \times 10^{-4}$ | $1{\cdot}0 \times 10^{-3}$ |
| D-Mannose | $2{\cdot}5 \times 10^{-4}$ | $1{\cdot}2 \times 10^{-3}$ |
| D-Mannosamine | $5 \times 10^{-4}$ | $2{\cdot}4 \times 10^{-3}$ |
| 2-Deoxy-D-glucose | $1 \times 10^{-3}$ | $4{\cdot}2 \times 10^{-3}$ |
| D-Fructose | $1 \times 10^{-3}$ | $4{\cdot}8 \times 10^{-3}$ |
| D-Mannoheptulose | $1 \times 10^{-3}$ | $4{\cdot}8 \times 10^{-3}$ |

* Results of representative experiments are shown. Parallel controls accompanied each experiment.

and 0·3-ml aliquots incubated in $Ca^{2+}$-free, Krebs–Ringer bicarbonate buffer modified to contain $Mg^{2+}$, 3·0 mM; NaF, 1·0 mM; ATP, 1·0 mM; and various concentrations of $G_m$-1-$^{14}$C. Such incubations were carried out in total volumes of 0·7 ml in a metabolic shaker under 95% $O_2$–5% $CO_2$ for 30 min at 37°. Other sugars (Table 2) were added to some incubates to obtain their apparent inhibitory constants for $G_m$ kinase; parallel controls accompanied each of these determinations. The intercepts, for calculation of the apparent $K_i$ values, were obtained by the method of least squares. The reaction was stopped by immersing the tubes in solid $CO_2$–acetone mixture. Subsequently "carrier" $G_m$, $G_m$-6-P, UDPAGA and $AG_m$-6-P were added to the lysate and the compounds separated on thin layers of cellulose as described above.

The lysate was also incubated with D-glucosamine-1-$^{14}$C-6-phosphate, disodium salt (sp. act. 40 mc/m-mole) at a concentration of $3{\cdot}4 \times 10^{-7}$ M for 1 hr and subsequently analyzed for $G_m$-1-$^{14}$C.

## RESULTS

*Uptake of $G_m$ by the intact platelet.* Human platelets, incubated with $^{14}$C-$G_m$ ($2 \times 10^{-6}$ M) for 15 min, accumulated free $G_m$ in the intraplatelet water with a distribution ratio ([I]/[O]) of 3·0 (Table 1). Accumulation decreased progressively with increasing concentration of $G_m$ in the medium such that the distribution ratio declined to 1·0 at $1 \times 10^{-4}$ M.

Since the total uptake of $^{14}$C-$G_m$ was in excess of that attributable to unchanged $G_m$, phosphorylation was considered and confirmed by TLC. The rates of penetration and phosphorylation of $G_m$ in the platelet were virtually linear during the initial 15 min of incubation (Fig. 1). During this phase the rate of entry approximated 0·91

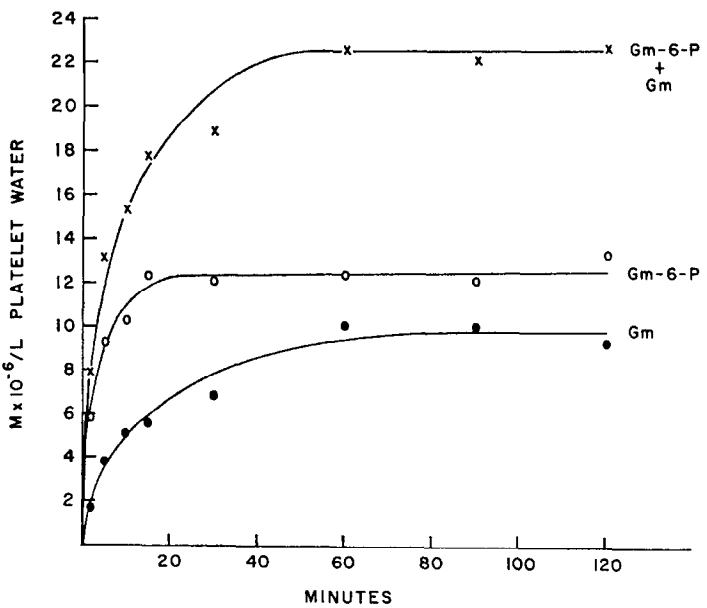


FIG. 1. Total rate of penetration of glucosamine ($G_m$) and rates of accumulation of free $G_m$ and glucosamine-6-phosphate ($G_m$-6-P) by the human platelet. Platelets were incubated at 37° with $G_m$ at an initial concentration of $2 \times 10^{-6}$ M. Results of a representative experiment are shown.

$\pm 0{\cdot}19 \times 10^{-12}$ moles $G_m$ per mg platelet per min (mean $\pm$ S.E. of five experiments) and phosphorylation, $0{\cdot}63 \pm 0{\cdot}15 \times 10^{-12}$ moles $G_m$/mg platelet per min. Between the 30th and 60th min of incubation further entry and phosphorylation of $G_m$ ceased (Fig. 1). At all times more than 90 per cent of the intraplatelet radioactivity was accounted for as $G_m$ and $G_m$-6-P; less than 5 per cent appeared, respectively, as UDPAGA and $AG_m$-6-P.

The influence of iodoacetic acid ($10^{-3}$ M) on the intracellular concentrations of $G_m$ and $G_m$-6-P is presented in Table 3. Iodoacetic acid diminished the intracellular

TABLE 3. INFLUENCE OF CHANGES IN CONCENTRATION OF GLUCOSAMINE ($G_m$) ON ITS DISTRIBUTION RATIO IN THE HUMAN PLATELET AFTER 15 min OF INCUBATION*

| Initial $G_m$ concn in medium (M) | Free $G_m$ [I]/[O] |
|---|---|
| $2 \times 10^{-6}$ | $3{\cdot}0 \pm 0{\cdot}6$ |
| $1 \times 10^{-5}$ | $2{\cdot}8 \pm 0{\cdot}4$ |
| $5 \times 10^{-5}$ | $1{\cdot}6 \pm 0{\cdot}3$ |
| $1 \times 10^{-4}$ | $1{\cdot}0 \pm 0{\cdot}1$ |

* Results expressed as mean $\pm$ S.E. of four experiments.

concentration of $G_m$ when the initial concentration of the amino sugar was $2 \times 10^{-6}$ M and $1 \times 10^{-5}$ M but not $1 \times 10^{-4}$ M. This inhibitor also diminished the intracellular concentration of $G_m$-6-P at initial $G_m$ concentrations of $1 \times 10^{-4}$ M and $1 \times 10^{-5}$ M but not at $2 \times 10^{-6}$ M.

Accumulation and phosphorylation of $G_m$ by the intact platelet were also influenced by other hexoses (Table 4). The intracellular concentrations of $G_m$ and $G_m$-6-P were

TABLE 4. INFLUENCE OF IODOACETIC ACID ($10^{-3}$ M) ON THE INTRAPLATELET CONCENTRATIONS OF GLUCOSAMINE ($G_m$) AND GLUCOSAMINE-6-PHOSPHATE ($G_m$-6-P) AFTER 15 min OF INCUBATION*

| Initial $G_m$ concn in medium | Treatment | Intraplatelet conc. moles/l. platelet $H_2O$ | | % Decrease from control | |
|---|---|---|---|---|---|
| (M) | | $G_m$ | $G_m$-6-P | $G_m$ | $G_m$-6-P |
| $2 \times 10^{-6}$ | Iodoacetate | $2{\cdot}4 \times 10^{-6}$ | $10{\cdot}3 \times 10^{-6}$ | 54·0 | 0·0 |
| | Control | $5{\cdot}2 \times 10^{-6}$ | $10{\cdot}3 \times 10^{-6}$ | | |
| $1 \times 10^{-5}$ | Iodoacetate | $0{\cdot}8 \times 10^{-5}$ | $2{\cdot}5 \times 10^{-5}$ | 70·1 | 73·0 |
| | Control | $2{\cdot}7 \times 10^{-5}$ | $9{\cdot}3 \times 10^{-5}$ | | |
| $1 \times 10^{-4}$ | Iodoacetate | $1{\cdot}0 \times 10^{-4}$ | $2{\cdot}9 \times 10^{-4}$ | 0·0 | 64·0 |
| | Control | $1{\cdot}0 \times 10^{-4}$ | $6{\cdot}3 \times 10^{-4}$ | | |

* Results of representative experiments and parallel controls are shown.

diminished by D-glucose, D-mannose, and 2-deoxy-D-glucose. L-glucose, *N*-acetyl-D-glucosamine, D-fructose, 6-deoxy-D-glucose, and D-mannoheptulose at $1 \times 10^{-3}$ M did not inhibit accumulation or phosphorylation of $G_m$ by the platelet.

*Phosphorylation of $G_m$ by platelet homogenates.* When incubated with $^{14}$C-$G_m$ and ATP, human platelet lysate incorporated radioactivity into $G_m$-6-P. The rate of phosphorylation of $G_m$ was dependent upon its concentration and linear during the first 30 min of incubation. Figure 2 reveals the apparent $K_m$ for $G_m$ to be $3{\cdot}3 \times 10^{-3}$ M

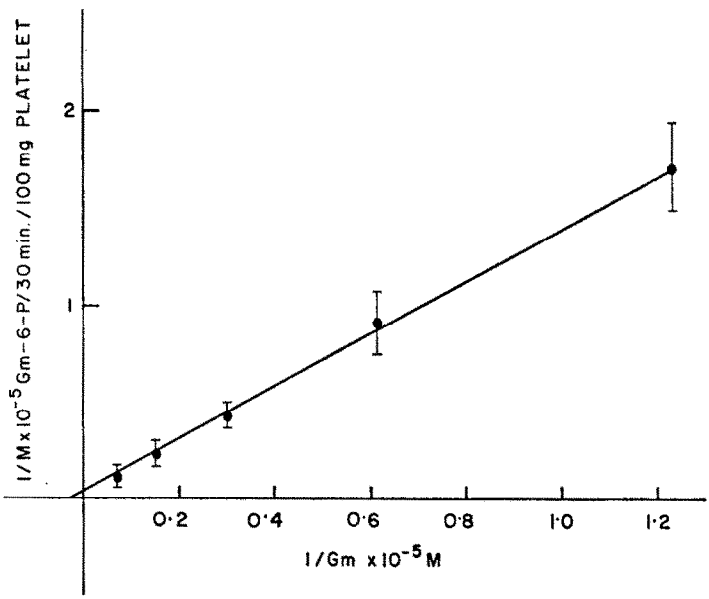


FIG. 2. Lineweaver–Burk plot of concentration of glucosamine ($G_m$) versus rate of formation of glucosamine-6-phosphate ($G_m$-6-P) by human platelet lysate. See text for composition of incubation medium. Results expressed as mean ± S.E. of four experiments.

and $V_{max}$, $2{\cdot}0 \times 10^{-4}$ moles $G_m$-6-P/30 min/100 mg platelets. A variety of sugars inhibited the phosphorylation of $G_m$ and apparent inhibitory constants for these compounds are presented in Table 5. Of the compounds studied *N*-acetyl-D-glucosamine was the most potent inhibitor with an apparent $K_i$ of $5{\cdot}0 \times 10^{-4}$ M; the value for D-glucose was $1{\cdot}0 \times 10^{-3}$ M. Phosphorylation of $G_m$ by platelet lysate was not inhibited by the following compounds at a concentration of $1 \times 10^{-3}$ M: D-glucosamine-6-phosphate, 6-deoxy-D-glucose, L-glucose, D-fructose-6-phosphate, and D-fructose-1, 6-diphosphate.

Platelet lysate was also incubated with D-glucosamine-1-$^{14}$C-6-phosphate at a concentration of $3{\cdot}4 \times 10^{-7}$ M for 1 hr and then analyzed for $G_m$-1-$^{14}$C by TLC. In excess of 90 per cent of the radioactivity was recovered as intact compound and none as $G_m$-1-$^{14}$C; hence no $G_m$-6-phosphatase was present.

## DISCUSSION

Human platelets incubated with $G_m$ at a concentration of $2 \times 10^{-6}$ M accumulate this compound against a gradient by a process which is restrained by iodoacetic acid

TABLE 5. INFLUENCE OF VARIOUS HEXOSES ($10^{-3}$ M) ON THE INTRAPLATELET CONCENTRATIONS OF GLUCOSAMINE ($G_m$) AND GLUCOSAMINE-6-PHOSPHATE ($G_m$-6-P) AFTER 15 min OF INCUBATION*

| Inhibitor | Intraplatelet concn moles/l. platelet $H_2O$ | | % Decrease from control | |
|---|---|---|---|---|
| | $G_m$ | $G_m$-6-P | $G_m$ | $G_m$-6-P |
| D-Glucose | $2{\cdot}1 \times 10^{-6}$ | $2{\cdot}7 \times 10^{-6}$ | 62·0 | 77·4 |
| Control | $5{\cdot}5 \times 10^{-6}$ | $12{\cdot}0 \times 10^{-6}$ | | |
| D-Mannose | $1{\cdot}8 \times 10^{-6}$ | $5{\cdot}7 \times 10^{-6}$ | 65·0 | 59·0 |
| Control | $5{\cdot}2 \times 10^{-6}$ | $13{\cdot}8 \times 10^{-6}$ | | |
| 2 Deoxy-D-glucose | $2{\cdot}2 \times 10^{-6}$ | $5{\cdot}9 \times 10^{-6}$ | 56·0 | 52·0 |
| Control | $5{\cdot}0 \times 10^{-6}$ | $12{\cdot}3 \times 10^{-6}$ | | |
| L-Glucose | $5{\cdot}9 \times 10^{-6}$ | $11{\cdot}2 \times 10^{-6}$ | 3·3 | 0·0 |
| Control | $6{\cdot}1 \times 10^{-6}$ | $11{\cdot}2 \times 10^{-6}$ | | |

* In all studies the initial concentration of $G_m$ was $2 \times 10^{-6}$ M. Results of representative experiments and parallel controls are shown.

and various hexoses including D-glucose, D-mannose, and 2-deoxy-D-glucose. Such accumulation exhibits specificity since L-glucose ($1 \times 10^{-3}$ M) had no effect. The decrease in the distribution ratio from 3·0 to 1·0 with increasing concentrations of $G_m$ (Table 1) suggests that the uptake process is saturable. $G_m$, at the highest concentration ($1 \times 10^{-4}$ M), apparently enters the platelet by passive diffusion since its rate of penetration is not diminished by iodoacetic acid.

Although a gradient for free $G_m$ was established at an extracellular concentration of $2 \times 10^{-6}$ M, approximately 52 per cent of the total sugar within the platelet was present as $G_m$-6-P (Fig. 1). The absence of $G_m$-6-phosphatase suggests that $G_m$ transport is not mediated by phosphorylation. The capacity for uptake exceeds that of phosphorylation since free $G_m$ is present in the platelet. When the intracellular concentration of $G_m$-6-P approached $1{\cdot}2 \times 10^{-5}$ M the rates of $G_m$ accumulation and phosphorylation markedly decreased. Such decreases in rates are apparently not due to inhibition of $G_m$ kinase by $G_m$-6-P since a much higher concentration of this compound ($1 \times 10^{-3}$ M) did not inhibit the enzyme in platelet homogenate. The data rather suggest feedback inhibition of $G_m$ transport at the membrane by $G_m$-6-P. A similar hypothesis holds relative to uptake of 2-deoxy-D-glucose by the platelet.[13]

Iodoacetic acid ($10^{-3}$ M) inhibits the phosphorylation of $G_m$ at initial concentrations of $10^{-5}$ M and $10^{-4}$ M but not at $2 \times 10^{-6}$ M (Table 3). Presumably, at the lowest concentration of $G_m$, sufficient ATP remains available within the platelet for phosphorylation despite the presence of iodoacetic acid.

Studies with homogenates of other cells are consistent with nonspecific hexokinases which, at the expense of ATP, phosphorylate a variety of sugars including $G_m$. For

example, in beef brain, the $K_m$ for D-glucose is 0·1 mM and for $G_m$, 0·6 mM[14]. In rat adipose tissue, the values are 0·03 mM and 0·5 respectively.[15] Though these hexokinases lack specificity, they prefer D-glucose over $G_m$. In contrast, the enzyme responsible for phosphorylation of $G_m$ in human platelet lysate seems to prefer $G_m$ since the apparent $K_i$ values for other sugars including D-glucose (Table 5) are much larger than the apparent $K_m$ of $G_m$. Failure of L-glucose to inhibit the phosphorylation of $G_m$ by platelet lysate further documents the enzyme's stereospecificity.

Hindrance of $G_m$ phosphorylation by D-glucose is probably not due to competition for ATP since inhibition occurred in the presence of $2 \times 10^{-4}$ M glucose and $10^{-3}$ M ATP. Furthermore the velocity of $G_m$ phosphorylation was not diminished in the absence of glucose when ATP was reduced to $8 \times 10^{-4}$ M. Such a concentration of ATP would have resulted if all the glucose at $2 \times 10^{-4}$ M had been phosphorylated. ATPase activity in the lysate was precluded by the addition of NaF ($10^{-3}$ M).

The penetration and phosphorylation of $G_m$ by the intact platelet is not influenced by *N*-acetyl-D-glucosamine ($1 \times 10^{-3}$ M). In contrast this compound, at the same concentration, is a competitive inhibitor of the phosphorylation of $G_m$ by platelet lysate. These results suggest that the membrane of the intact platelet is impermeable to *N*-acetyl-D-glucosamine. Similarly rat adipocytes, Ehrlich ascites tumor cells and sarcoma 180 ascites tumor cells do not permit entry of this compound.[4,16]

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