# Dephosphorylation of Human Erythrocyte Membranes Induced by Sendai Virus†

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ABSTRACT: Phosphorylated human erythrocyte membranes were prepared by incubation of either intact cells with $^{32}P$-labeled inorganic phosphate or erythrocyte ghosts with [$\gamma$-$^{32}P$]ATP. Forty to seventy percent of total $^{32}P$ counts found in membrane preparations were bound to membrane proteins. Incubation of phosphorylated erythrocyte ghosts with Sendai virus at 37 °C causes rapid membrane dephosphorylation which releases between 20 and 50% of the bound phosphate from the membranes. Under these conditions, Sendai virus induces leakage of the residual hemoglobin left in the erythrocyte ghosts after the hemolysis process. The initial rates of dephosphorylation obey a first-order kinetics and rate constants of $1 \times 10^{-7}$ $s^{-1}$/HAU to $2.5 \times 10^{-7}$ $s^{-1}$/HAU were calculated for the viral process. The membrane's endogenous dephosphorylation rates were found to be about five times slower than virus-induced dephosphorylation. A specific virus–cell interaction is essential for stimulation of membrane dephosphorylation as the virus fails to induce dephosphorylation either of neuraminidase-treated erythrocyte ghosts or in the presence of specific antiviral serum. $Mg^{2+}$ and $Ca^{2+}$ ions are required for induction of membrane dephosphorylation by the virus. In the presence of EDTA both self- and virus-induced dephosphorylation are abolished, while in the presence of EGTA and $Mg^{2+}$ only the virus-induced dephosphorylation is inhibited. It appears that $Ca^{2+}$ affects the maximal rate of dephosphorylation obtained at saturating $Mg^{2+}$ concentration. $Ca^{2+}$ does affect, but to a much lesser extent, the apparent $K_{m(Mg)}$. The ionophore A-21387 + $Ca^{2+}$, as well as various hemolytic reagents such as prymnesin (5000 hemolytic units) and Triton X-100 (0.5%), fail to mimic the virus-specific membrane dephosphorylation.

Interaction between viruses of the paramyxovirus group and eukaryotic cells results in cell agglutination, membrane fusion, and partial lysis (Okada, 1969). During this process, the virus envelope fuses with the host cell membrane, thus permitting the entry of the viral nucleoprotein complex to the cell interior (Klenk, 1974). The connection between fusion and hemolysis has not yet been clarified, although interrelationship between these two processes has been claimed (Yanovsky and Loyter, 1972).

It has been shown that intracellular ATP is required for viral-induced fusion and that interaction of Sendai virus with ATP depleted cells induces complete lysis without fusion (Okada, 1969). Plasma membrane proteins of ATP depleted cells were shown to be dephosphorylated (Gazitt et al., 1975; Hermoni and Milner, 1975), and it is conceivable that this renders them more sensitive to the viral hemolytic activity. Intracellular ATP may, on the other hand, increase membrane integrity by causing phosphorylation of its proteins and in this way reduce the membrane susceptibility to the viral lytic activity (Gazitt et al., 1975; Frisch et al., 1973), thus increasing the fusion potentiality of the cells. That intracellular ATP might control the integrity of cell membranes has also been concluded from experiments showing that ATP depleted cells are more susceptible to various nonviral lytic agents (Frisch et al., 1973).

Recently it has been shown that the interaction of Sendai virus with either human or chicken erythrocytes induces clustering of intramembrane particles as revealed by freeze-etching techniques (Bachi et al., 1973; Volski et al., 1976). Changes in the normal intramembrane particle distribution pattern have also been observed during nonviral membrane fusion (Zakai et al., 1976; Chi et al., 1976). ATP depletion of red blood cells from various sources, as well as of transformed cells, also induces clustering of intramembrane particles (Gazitt et al., 1976; Voldavsky and Sachs, 1976). The above two phenomena can be linked by assuming that interaction of the virus with the cell membrane may induce its dephosphorylation, causing aggregation of intramembrane particles. This process exposes areas of naked phospholipids, at which membrane fusion may be initiated (Gazitt et al., 1975; Zakai et al., 1976).

In the present investigation we have tested the ability of Sendai virus to induce membrane dephosphorylation using $^{32}P$-labeled human erythrocyte ghosts (HEG).[1] Erythrocyte ghosts are a suitable system for this type of study as dephosphorylation processes would not be obscured by rephosphorylation reactions which require intracellular ATP.

In the present work we show that Sendai virus is able to induce rapid dephosphorylation of both open and resealed HEG. The relation between the dephosphorylation process and the virus-induced fusion phenomena is discussed.

## Materials and Methods

*Cells.* Human blood was obtained from volunteers by venipuncture. The cells were washed with solution Na (see Media) as previously described (Peretz et al., 1974) and finally suspended in solution Na to give a suspension of 10–20% (v/v).

*Virus.* Sendai virus was isolated and its hemagglutinin titer was determined as previously described (Peretz et al., 1974).

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; HEG, human erythrocyte ghosts; HAU, hemagglutinating units of Sendai virus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N'*-tetraacetic acid; $P_i$, inorganic phosphate; UV, ultraviolet.

*Media.* The following media were used throughout the present work: Solution Na contained (mM): NaCl, 140; KCl, 5; $MgCl_2$, 0.9; and Tris-HCl (pH 7.4), 10.

Solution K contained (mM): KCl, 140; NaCl, 5; $MgCl_2$, 0.9; Tris-HCl (pH 7.4), 10.

*Antiviral Glycoprotein Antibody.* Viral glycoproteins were obtained after solubilization of the virus in ether and Tween-20 according to Hosaka et al. (1960). The envelope glycoproteins were injected into rabbits and antiserum was collected as described by Seto et al. (1974). The antibody prevented virus-induced agglutination, hemolysis, and fusion of intact red blood cells.

*Preparation of Phosphorylated Intact Human Erythrocyte Cells.* Erythrocytes were first depleted of ATP by incubating 50–100 mL of a 10% (v/v) cell suspension in solution Na containing 15–20 mM NaF for 12 h at 37 °C. Cells were then washed twice with 20 volumes of solution Na and intracellular ATP was restored by incubating a 10% (v/v) suspension of depleted cells in solution Na containing 2 mM adenine, 0.5 mM inosine, 15 mM glucose, 3–5 mM [$^{32}$P]$P_i$ (specific activity 1 mCi/$\mu$mol), 0.05 mM phenylmethanesulfonyl fluoride. After 8 h of incubation at 37 °C, cells were washed with solution Na as described above and used immediately. The phosphorylation procedure results in incorporation of 4–15 nmol of [$^{32}$P]$P_i$ per mg of membrane protein (see below).

*Preparation of Phosphorylated Human Erythrocyte Ghosts (HEG).* The phosphorylated intact red blood cells were hemolyzed by addition of 20 volumes of a cold solution containing 2 mM ATP, 2 mM sodium phosphate (pH 7.4), 0.05 mM phenylmethanesulfonyl fluoride, and 5 mM Tris-HCl (pH 7.4). The hemolyzed cells were centrifuged at 4 °C (10 000*g*, 15 min) and washed once with the above solution. The erythrocyte ghosts obtained were resealed by suspending the pellet in 10 volumes of solution K and incubated for 30 min at 37 °C.

The resealed erythrocyte ghosts were washed once with 20 volumes of solution Na. The final pellet was suspended in a minimal volume of solution Na containing 5 mM $NaN_3$ and was kept at 4 °C. These phosphorylated HEG were used within 4 days. Usually, about 0.4 mL of packed HEG (approximately 6 mg of membranous protein and 0.5–1 mg of hemoglobin) were obtained from each mL of packed cells.

*Preparation of Phosphorylated Leaky (Frozen-Thawed) HEG.* Intact red blood cells were depleted of intracellular ATP as described above. ATP depleted cells were hemolyzed by addition of 20 volumes of cold 10 mM Tris-HCl (pH 7.4) containing 0.05 mM phenylmethanesulfonyl fluoride. After washing with the above buffer, the erythrocyte ghosts were suspended in 1 volume of 10 mM Tris-HCl (pH 7.4), frozen in liquid nitrogen, and immediately thawed at 37 °C. The freezing and thawing procedure was repeated twice. Phosphorylation was performed essentially as described by Guthrow et al. (1972), using 5–10 nmol of [$\gamma$-$^{32}$P]ATP per mg of membranous protein (2–5 × $10^5$ cpm/nmol). The phosphorylation reaction mixture contained, in a final volume of 3 mL, the following (mM): Tris-HCl (pH 7.4), 50; $MgSO_4$, 1; cAMP, 0.02; NaCl, 50. After 15 min at 37 °C the membranes were washed twice with 20 volumes of 10 mM Tris-HCl (pH 7.4) containing 2 mM sodium phosphate and 2 mM NaATP.

Phosphorylated membranes were kept frozen at −20 °C until use. Phosphorylation level ranged between 5 and 8 nmol of bound [$^{32}$P]P/mg of protein.

*Determination of $^{32}$P Bound to Cell Membranes in Inorganic Phosphate and in ATP.* Samples (usually between 10 and 25 $\mu$L) of phosphorylated HEG or intact phosphorylated cells were dissolved by mixing with equal volumes of 5% sodium dodecyl sulfate in 0.05 M acetate (pH 5.0). Mercaptoethanol (2 $\mu$L) was added and then the dissolved membranes were heated for 5–10 min at 60 °C. Aliquots of 10 $\mu$L were applied to DEAE-cellulose paper (DE-81, Whatman) prespotted at the origin with unlabeled carriers ATP and inorganic phosphate (about 1–2 $\mu$L of 0.1 M solutions of each). The papers were subjected to descending chromatography using 0.2 M ammonium formate (pH 5.5) as a solvent. After 3–4 h the chromatography papers were dried with hot air. ATP spots were revealed by UV light and inorganic phosphate by the Hanes–Isherwood spray (Hanes and Isherwood, 1949). The points of sample application, ATP and $P_i$ spots, were cut and counted in toluene–Triton scintillation fluid. By this method, the distribution of $^{32}$P in membrane proteins and phospholipids (origin of the paper), ATP, and inorganic phosphate could be estimated simultaneously. Concentrations of each reactant in the incubation mixture were calculated from the percent distribution of the counts between the three components. The above method was verified by using the phosphomolybdate extraction method (Avron, 1960) for measuring inorganic phosphate and by precipitation of phosphorylated proteins with 10% $Cl_3CCOOH$ for determination of $^{32}$P in the precipitate.

The degree of absolute phosphorylation and dephosphorylation was somewhat variable from experiment to experiment due to difficulties in accurate sampling of membrane preparations. Therefore, all the results in the present work are expressed as percent values and not in terms of concentration.

*Release of Residual Hemoglobin from Resealed Ghosts.* Release of residual hemoglobin was determined by withdrawing 50–100-$\mu$L samples of reaction mixtures to 1.0 mL of solution Na, centrifugation at 9000*g* for 15 min, and checking 0.5–0.7 mL of supernatant in 1 N NaOH for hemoglobin at 405 nm.

Total hemoglobin release was obtained by incubation of resealed HEG with 50 $\mu$L of 28% $NH_4OH$.

*Materials.* [$^{32}$P]$P_i$ carrier free was obtained from KAMAG, Beer-Sheva, Israel.

[$\gamma$-$^{32}$P]ATP was synthesized by modification of the Glynn and Chappell method (1964).

$Ca^{2+}$ ionophore A-23187 was kindly supplied by Eli Lilly & Co.

Prymnesin (25 000 hemolytic units/mL in ethanol) was purchased from MAKOR Chem. Ltd., Israel.

Neuraminidase (*Vibrio cholerae*) was obtained from Beringwerke AG, Berlin.

All chemicals used were of analytical grade.

## Results

*Dephosphorylation of Resealed Human Erythrocyte Ghosts (HEG).* Incubation of Sendai virus with phosphorylated intact human erythrocytes causes dephosphorylation of cell protein (not shown). However, quantitative studies of the dephosphorylation phenomena using phosphorylated intact cells (see Materials and Methods) are difficult because of the high [$^{32}$P]$P_i$ background and because of the presence of labeled ATP which might rephosphorylate cell proteins. Due to its lack of cytoplasm and the ease of performing phosphorylation using [$\gamma$-$^{32}$P]ATP (Guthrow et al., 1972), human erythrocyte ghosts were thought to serve as an ideal system for the study of virus-induced dephosphorylation.

Results of viral dephosphorylation of resealed HEG are presented in Figure 1A. As can be seen, the virus causes about a fourfold increase in the dephosphorylation rate of resealed HEG above the rate of self-dephosphorylation. The above data

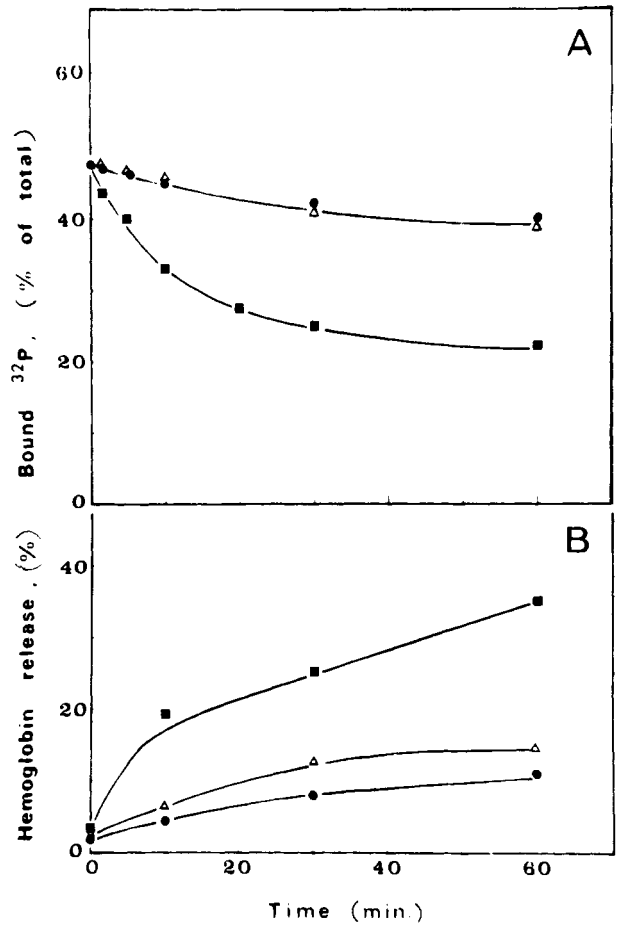


FIGURE 1: Time course of virus-induced dephosphorylation and hemoglobin release in resealed HEG. The reaction mixture contained 4 mg of membrane protein and 4000 HAU of Sendai virus suspended in a final volume of 0.6 mL of a solution containing (mM): NaCl, 140; Tris-HCl (pH 7.4), 25; $MgCl_2$,[2] 8; $CaCl_2$,[2] 3; $NaN_3$, 1. Incubation was performed at 37 °C with gentle shaking and, at the time intervals indicated, samples of 10 μL were withdrawn and mixed immediately with 10 μL of 5% sodium dodecyl sulfate to stop the reaction. Determinations of membrane phosphate and inorganic phosphate are as described in Methods. The membrane contained 5.5 nmol of phosphate/mg of protein which represents $1.1 \times 10^6$ cpm/mg of protein. At zero time, the counts were distributed as follows: 48% membrane bound phosphate, 32% in ATP, and 20% in inorganic phosphate. Membrane sialic acid was hydrolyzed by incubating 4.5 mg of membrane protein with 15 units of soluble neuraminidase (*V. cholerae*) at pH 5.5 (50 mM sodium acetate buffer) during 30 min at 37 °C (Warren, 1959). After two washings with 20 volumes of solution Na, the neuraminidase-treated HEG were suspended in solution Na and then were incubated with Sendai virus as described above. (A) Dephosphorylation kinetics. (B) Release of residual hemoglobin from resealed HEG induced by Sendai virus. Samples of 50 μL were withdrawn from the incubation mixture described above and centrifuged, and hemoglobin was estimated in the supernatant. (● — ●) HEG (control), no virus was added; (△ —△) neuraminidase treated HEG + virus; (■ — ■) HEG + virus.

were plotted in a semilogarithmic fashion. This led to first-order kinetic plots from which a first-order constant of $4 \times 10^{-4}$ $s^{-1}$ for 4000 HAU and of $2 \times 10^{-4}$ $s^{-1}$ for 2000 HAU could be derived (not shown). Titration of virus concentration shows that initial rates of dephosphorylation are linear between

[2] Experiments indicated that the addition of $Mg^{2+}$ and $Ca^{2+}$ significantly stimulated the dephosphorylation reaction (see below). However, their effects were somewhat variable with different concentrations of membrane proteins or with resealed or frozen-thawed (leaky) erythrocyte ghosts. Therefore, slightly different concentrations of $Ca^{2+}$ and $Mg^{2+}$ were used in different experiments. For further details on the effect of bivalent metals, see Metal Requirements in Results.

TABLE I: Inhibition of Virus-Induced Dephosphorylation by Antiviral Serum.[a]

| System | pmol of $^{32}P$ released/min | Net phosphatase units per system |
|---|---|---|
| HEG | 5.50 | |
| HEG + virus | 26.40 | 10800 |
| HEG + virus + antiviral serum | 5.71 | 112 |

[a] Sendai virus (0.2 mL of 40 000 HAU/mL) was incubated for 20 min at 37 °C with 500 μL of antiviral serum. Aliquots of the virus-antivirus complex or of active virus (equivalent to 900 HAU of virus) were incubated with 2.5 mg of protein of phosphorylated membranes (containing 14 nmol of $^{32}P$/mg of protein, $5 \times 10^5$ cpm/nmol in a final volume of 0.2 mL). The composition of the reaction mixture and conditions of incubation are as described in Figure 1. Rates of dephosphorylation were obtained as described in Figure 1A. Release of $^{32}P$ was measured as described in Materials and Methods. Unit of phosphatase is defined as pmol of $^{32}P$ released per 60 min per 1 HAU virus. Endogenous dephosphorylation rate was subtracted from the rate in the viral systems.

100 and 4000 HAU. Under these conditions one can calculate a rate of 0.083 pmol of phosphate hydrolyzed per min per 1 HAU at 37 °C. However, rates of phosphate hydrolysis are not first order over the whole time course of incubation. After 30 min the rate deviates sharply from first- or even second-order kinetics. It should be added that, under these conditions, the virus causes release of residual ghost hemoglobin from HEG (Figure 1B), as previously observed (Lalazar et al., 1977). Leakage of hemoglobin from the ghosts did not, by itself, result in stimulation of the self dephosphorylation (see section Dephosphorylation and Hemolysis).

The virus fails to induce dephosphorylation when incubated with neuraminidase-treated HEG (Figure 1A), demonstrating the necessity for specific virus-cell interaction. Indeed, the neuraminidase-treated HEG underwent neither viral-induced agglutination nor appreciable hemolysis (Figure 1B). A further indication that the dephosphorylation activity is associated with the virus particles or is a virus-induced phenomenon is obtained from experiments using antiviral serum. Addition of antiviral serum completely prevents the viral-induced dephosphorylation of HEG (Table I).

*Dephosphorylation of Leaky Human Erythrocyte Ghosts (HEG).* A slow and seemingly linear dephosphorylation is observed when frozen-thawed HEG are incubated with the virus (Figures 2A and 2B). The relation between bound and free $^{32}P$ is clearly seen from inspection of Figure 2 where the decrease in bound phosphate is accompanied by a release of equal amounts of inorganic phosphate. The distribution of counts in relation to residual, labeled ATP as well as some of the characteristics of virus-induced membrane dephosphorylation are shown in Table II. One can see that nearly all of the counts appearing as $P_i$ originate in the membrane fraction, whereas ATP hydrolysis contributes very little. It appears, also, that the addition of $Ca^{2+}$ to a system containing $Mg^{2+}$ slightly enhances the dephosphorylation. It is evident from the experiments that the presence of membranous sialic acid is a prerequisite for the dephosphorylation in ruptured membranes as well as in resealed HEG (see above). In neuraminidase-treated membranes, viral dephosphorylation is abolished, even in the presence of $Ca^{2+}$ (Table II). The requirement of membrane sialic acid for virus-induced dephosphorylation is also evident from the experiment shown in Figure 3. It can be seen in the figure, which depicts an elution pattern of phosphorylated membrane proteins from Bio-Gel column, that the virus causes dephosphorylation of peptide peaks of apparent mo-

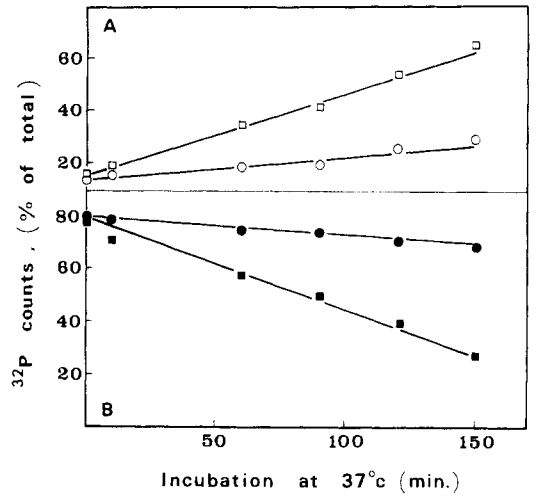


FIGURE 2: Kinetics of virus-induced dephosphorylation and [$^{32}$P]$P_i$ release in frozen-thawed HEG. Incubation was performed at 37 °C with 400 $\mu$g of phosphorylated membrane protein and 1200 HAU of Sendai virus suspended in a final volume of 0.1 mL of a solution containing (mM): Tris-HCl (pH 7.4), 10; $MgCl_2$, 2; $CaCl_2$, 1. At zero time of incubation, the phosphorylated membranes contained 7 nmol of $^{32}$P/mg of protein ($2 \times 10^6$ cpm/mg of protein). Counts were distributed as follows: 80% were membrane bound phosphate, 15% inorganic phosphate, and 5% in ATP. (A) Release of inorganic phosphate vs. time. (○ — ○) HEG (control), no virus was added; (□ — □) HEG + virus. (B) Disappearance of membrane-bound $^{32}$P. (● — ●) HEG without added virus; (■ — ■) HEG + virus.

TABLE II: Dephosphorylation of Frozen-Thawed Human Erythrocyte Ghosts by Sendai Virus.[a]

| Addition | Time (min) | $^{32}$P counts (% of total) Membrane | ATP | $P_i$ |
|---|---|---|---|---|
| 1. None | 0 | 63.0 | 6.2 | 30.8 |
| | 60 | 55.0 | 5.1 | 39.9 |
| 2. Virus | 0 | 62.6 | 8.5 | 29.9 |
| | 60 | 41.7 | 6.7 | 51.6 |
| 3. Virus + $Ca^{2+}$ (0.5 mM) | 0 | 64.3 | 4.5 | 31.2 |
| | 60 | 32.5 | 2.5 | 65.0 |
| 4. $Ca^{2+}$ (0.5 mM) | 0 | 64.0 | 4.5 | 31.5 |
| | 60 | 55.6 | 4.1 | 40.3 |
| 5. Neuraminidase treated membranes + virus | 0 | 43.3 | 16.1 | 40.6 |
| | 60 | 36.5 | 14.3 | 49.2 |
| 6. Neuraminidase treated membranes + virus + $Ca^{2+}$ (0.5 mM) | 0 | 55.4 | 13.8 | 30.8 |
| | 60 | 47.4 | 9.6 | 43.0 |

[a] Preparation of phosphorylated, frozen-thawed HEG and incubation with Sendai virus are as described in Figure 2. All systems contained 2 mM $MgCl_2$. Measurement of $^{32}$P in the various components was conducted by mixing samples of 25 $\mu$L of the reaction mixture with 25 $\mu$L of 5% sodium dodecyl sulfate which were then subjected to paper chromatography (see Materials and Methods). Treatment with neuraminidase as described in Figure 1.

lecular weights of 100 000, 70 000, 35 000, and 15 000. The elution pattern of phosphorylated proteins from neuraminidase-treated membranes incubated with the virus (Figure 3) is identical with the elution pattern of the proteins of control untreated phosphorylated membranes (not shown).

*Metal Requirements.* The Michaelian kinetics of viral dephosphorylation as a function of $Mg^{2+}$ concentration, in the presence and in the absence of 2 mM $Ca^{2+}$, are seen in Figure 4A. The initial velocity of dephosphorylation is enhanced by

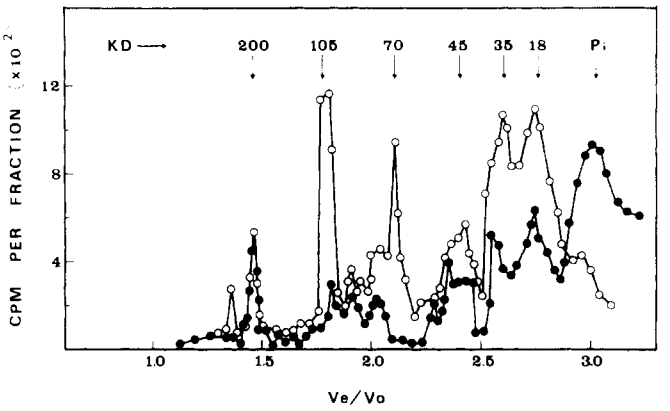


FIGURE 3: Elution of phosphorylated erythrocyte membrane proteins from Bio-Gel column; effect of Sendai virus treatment. Reaction mixtures for virus treatment contained, in 0.6 mL final volume (mM): Tris-HCl (pH 7.4), 50; $MgCl_2$, 8; $CaCl_2$, 3; 4000 HAU of Sendai virus and 8 mg of membrane proteins (ruptured membranes obtained by freezing and thawing) containing 9 nmol of $^{32}$P per mg of protein with a specific activity of $2 \times 10^5$ cpm/nmol. Membranes were treated with neuraminidase as described in Figure 1. After 65 min of incubation with the virus at 37 °C, the entire mixtures were dissolved in 1.0 mL of 8.0 M urea in 0.05 M sodium acetate (pH 5.0) containing 20 mM mercaptoethanol and heated for 15 min at 60 °C. The samples (0.5 mL) of dissolved mixtures were loaded on a 2.5 × 45 cm Bio-Gel A-15m column equilibrated with 7.0 M urea in 0.05 M sodium acetate pH 5.0 and 10 mM mercaptoethanol. The protein peaks were eluted by the above solvent and 1.0-mL fractions were collected and monitored for radioactivity. Molecular weights of the peptides were estimated from a calibration curve constructed from similar column runs with bovine serum albumin (mol wt 68 000), $\beta$-galactosidase (mol wt 130 000), glyceraldehyde-3P dehydrogenase (mol wt 35 000), and chymotrypsinogen (mol wt 23 000) as markers: (● — ●) membranes + virus; (○ — ○) neuraminidase treated membranes + virus; $V_e$ = elution volume; $V_0$ = void volume.

$Ca^{2+}$ at all $Mg^{2+}$ concentrations. It can be estimated that the apparent Michaelis constants for $Mg^{2+}$ and $Mg^{2+}$ in the presence of $Ca^{2+}$ are similar ($K_{mapp(Mg^{2+}+Ca^{2+})}$ = 1 mM) and, therefore, the effect of $Ca^{2+}$ is on the $V_{max}$ of the reaction ($V_{max(Mg^{2+})}$ = 2.15 nmol of $P_i$ per 30 min per 1000 HAU; $V_{max(Mg^{2+}+Ca^{2+})}$ = 3.2 nmol of $P_i$ per 30 min per 1000 HAU).

As membrane-bound $Ca^{2+}$ could affect the viral-induced dephosphorylation, the effects of $Mg^{2+}$ and $Mg^{2+}$ + $Ca^{2+}$ were studied in the presence of EDTA and EGTA. Results of $Mg^{2+}$ and $Mg^{2+}$ + $Ca^{2+}$ back titrations (Figure 4B) show that no significant dephosphorylation could be detected after addition of $Mg^{2+}$ ions alone up to 7 mM. Neither did the addition of $Ca^{2+}$ (free $Ca^{2+}$ concentration 2 mM) in the absence of $Mg^{2+}$ result in any viral dephosphorylation activity. However, when a concentration as low as 1 mM free $Mg^{2+}$ was present, the addition of 3 mM $Ca^{2+}$ induced almost maximal dephosphorylation (Figure 4B). The $Ca^{2+}$ effect is most dramatically expressed in the $V_{max}$ of the dephosphorylation reaction.

Figure 4C shows that addition of EGTA in the presence of excess $Mg^{2+}$ ions abolished the viral activity and only membrane self-dephosphorylation was observed. Addition of excess EDTA abolishes both viral and self-induced dephosphorylation. $Mn^{2+}$ could substitute for $Ca^{2+}$ in stimulating virus-induced dephosphorylation, but was only about 50% active on concentration basis. $Co^{2+}$ and $Tb^{3+}$ were without any effect (not shown).

*The Effect of $Ca^{2+}$ Ionophore.* It has been previously suggested that the virus facilitates $Ca^{2+}$ penetration into the cell (Volski et al., 1976). The high intracellular $Ca^{2+}$ concentration might then induce direct dephosphorylation or indirectly cause rapid turnover of trapped ATP (via the ($Ca^{2+}$, $Mg^{2+}$)AT-

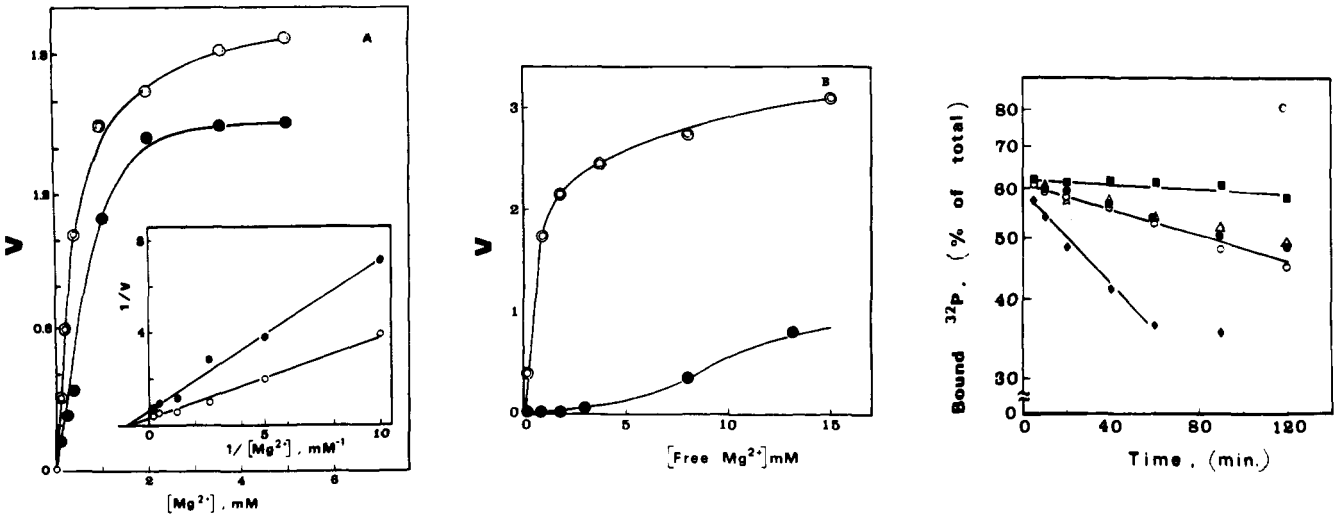


FIGURE 4: The effect of $Ca^{2+}$ and $Mg^{2+}$ on the virus-induced process of dephosphorylation. (A) Michaelis-Menten and Lineweaver-Burk plots of virus-induced membrane dephosphorylation for $Mg^{2+}$ and $Mg^{2+}$ + $Ca^{2+}$. Dephosphorylation of resealed HEG was performed and estimated as described in Figure 1. Membrane protein, 5 mg, and 1000 HAU of Sendai virus were used. (● — ●) $Mg^{2+}$ alone; (○ — ○) $Mg^{2+}$ + $Ca^{2+}$ (2 mM). $v$ and $1/v$ were calculated from initial rates and expressed as nmol of [$^{32}$P]$P_i$ released per 30 min per 1000 HAU. Control values of dephosphorylation were obtained from systems incubated without virus and were subtracted from the rates presented in this figure. The kinetic constants recorded: $V_{max(Mg^{2+})}$ = 2.15 nmol of $P_i$ per 30 min per 1000 HAU; $V_{max(Mg^{2+}+Ca^{2+})}$ = 3.2 nmol of $P_i$ per 30 min per 1000 HAU; $K_{m(Mg^{2+}+Ca^{2+})} = K_{m(Mg^{2+})}$ = 1 mM. (B) Saturation curves of viral dephosphorylation for $Mg^{2+}$ and $Ca^{2+}$ in the presence of EGTA and EDTA. Experimental details are similar to those reported in Figure 1, using resealed HEG (4 mg of protein, 8 nmol of $^{32}$P per mg of protein). Concentrations (mM): EDTA, 1; EGTA, 1; 1200 HAU Sendai virus and $CaCl_2$, 4. $V$ is expressed as in A. The free $Mg^{2+}$ and $Ca^{2+}$ concentrations were calculated according to dissociation constants of $EDTA_{Ca^{2+}} = 1 \times 10^{-11}$; $EDTA_{Mg^{2+}} = 1 \times 10^{-11}$; $EGTA_{Ca^{2+}} = 1 \times 10^{-11}$; $EGTA_{Mg^{2+}} = 3 \times 10^{-5}$. Kinetic constants derived were: $V_{m(Mg^{2+})}$ = 0.55 nmol of $P_i$ per 30 min per 1000 HAU; $V_{m(Mg^{2+}+Ca^{2+})}$ = 3.57 nmol of $P_i$ per 30 min per 1000 HAU; $K_{m(Mg^{2+})}$ = 4 mM; $K_{m(Mg^{2+}+Ca^{2+})}$ = 2 mM. (○ — ○) $Mg^{2+}$ + $Ca^{2+}$; (● — ●) $Mg^{2+}$ alone. (C) Kinetic plot of dephosphorylation reaction in resealed HEG—effect of EDTA and EGTA + $Mg^{2+}$. Experimental details of the dephosphorylation reaction are as described in Figure 1, except that 6 mg of membrane protein (8 nmol of $^{32}$P per mg of protein) and 3000 HAU of Sendai virus were used. (◆ — ◆) HEG + $Ca^{2+}$ + $Mg^{2+}$ + virus; (● — ●) HEG + $Ca^{2+}$ + $Mg^{2+}$ without virus; (○ — ○) HEG + $Mg^{2+}$ + EGTA without virus; (△ — △) HEG + $Mg^{2+}$ + EGTA + virus; (■ — ■) HEG + EDTA + virus. Concentrations (mM): $Ca^{2+}$, 4; $Mg^{2+}$, 8; EGTA, 10; EDTA, 10. Rate constants recorded: $k_{virus(Mg^{2+}+Ca^{2+})} = 1.4 \times 10^{-4}$ s$^{-1}$; $k_{virus+EGTA} = 0.34 \times 10^{-4}$ s$^{-1}$.

Pase), which would lead to rapid turnover of the membranous $^{32}$P. This assumption could be tested directly by incubating resealed HEG with external $Ca^{2+}$ and $Ca^{2+}$ ionophore. It is clear from the data presented in Figure 5A that the addition of $Ca^{2+}$ + ionophore has very little effect on the dephosphorylation rate. In fact, the presence of 40 $\mu$g of $Ca^{2+}$ ionophore together with the virus somewhat decreases the virus dephosphorylating activity (Figure 5A).

*Dephosphorylation and Hemolysis.* The dephosphorylation observed might be due to latent membranous phosphatase which is activated during hemolysis induced by the virus (see Figure 1B). An attempt to mimic virus hemolysis by the action of non-ionic detergents and the hemolytic toxin prymnesin is shown in Figure 5B. Clearly, the treatment of resealed HEG with various hemolytic agents does not induce dephosphorylation, despite extensive membrane rupture. Control experiments, in which HEG were treated by a combination of virus and Triton X-100, showed that virus dephosphorylation was stimulated by the detergent (data to be published). It is noteworthy that, in the experiment shown in Figure 5B, the virus preparation was very active in causing rapid dephosphorylation of resealed HEG. The rate constant measured (for 2200 HAU of virus) was $k_{virus} = 5.5 \times 10^{-4}$ s$^{-1}$, while the endogenous rate constant was $k = 0.5 \times 10^{-4}$ s$^{-1}$.

## Discussion

The data presented clearly show an extensive dephosphorylation of phosphorylated human erythrocyte membrane as a result of interaction with Sendai virus. Accurate quantitative measurements of the dephosphorylation reaction were feasible as a result of the procedure developed in the present work, which involves extensive washing of membrane preparations with nonlabeled ATP and $P_i$. The washings removed labeled ATP which could obscure viral dephosphorylation by rephosphorylation of the membrane proteins, a reaction catalyzed by the endogenous membranous protein kinase (Guthrow et al., 1972; Avruch and Fairbanks, 1974; Rubin and Rosen, 1975).

The HEG preparations used were variable, to some extent, with regard to both phosphorylation levels and the degree of viral dephosphorylation. Phosphorylation levels of membrane preparations ranged from 4 to 15 nmol of bound phosphate per mg of membranous protein. These preparations underwent variable viral dephosphorylation ranging from 20 to 70% of the total bound phosphate. This is probably due to the heterogeneous nature of the ghost population, as well as to variable contamination by cellular or membranous phosphatase adhering to the membranes after washing. The virus preparations used were also heterogeneous in regard to size and membrane integrity (Homma et al., 1976). However, initial rates of dephosphorylation were quite similar, regardless of the extent of dephosphorylation. First-order kinetic constant ranged from $1 \times 10^{-7}$ s$^{-1}$/HAU (Figure 1) to $2.5 \times 10^{-7}$ s$^{-1}$/HAU (Figure 5B). The virus-induced dephosphorylation could be interpreted in terms of a simple, unsaturated enzyme-substrate relationship in a Michaelian system.

About 90% of the bound $^{32}$P in the HEG represents phos-

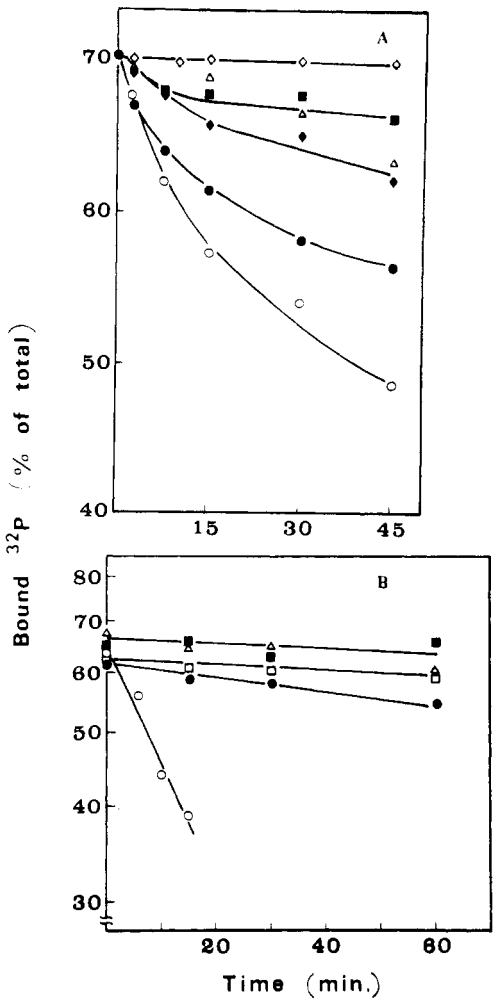


FIGURE 5: (A) The effect of $Ca^{2+}$ and the ionophore A-23187 on self and virus-induced dephosphorylation of resealed HEG. The reaction mixture compositions and determination of dephosphorylation rates were as described in Figure 1. (◇ — ◇) HEG + EDTA + virus (no metals); (■ — ■) HEG + ionophore A-23187 (40 μg); (△ — △) HEG + ionophore A-23187 (20 μg); (◆ — ◆) HEG (control, without added virus); (● — ●) HEG + virus + 40 μg of ionophore A-23187; (○ — ○) HEG + virus. Virus 4000 HAU/system; concentrations (mM): EDTA, 2; $MgCl_2$, 8; $CaCl_2$, 3. (B) The effect of virus and various hemolytic agents on the dephosphorylation reaction in resealed HEG. Phosphorylated resealed HEG (4 mg of protein containing 6 nmol of $^{32}P$/mg) were incubated in a final volume of 0.5 mL in the absence or in the presence of Sendai virus as described in Figure 1. The hemolytic agents were added to HEG suspension before incubation at 37 °C. (● — ●) HEG; (□ — □) HEG + prymnesin (5000 hemolytic units); (△ — △) HEG + Triton X-100 (0.5%); (■ — ■) HEG + Tween 40 (0.5%); (○ — ○) HEG + virus (2200 HAU). In this experiment almost all the residual hemoglobin left in the HEG was released by incubation with the above hemolytic agents, while incubation with the virus caused only 30% leakage of the residual hemoglobin.

phoryl moieties covalently bound to membranous protein, while 10% represents phosphorylated phospholipids (Milner, unpublished results). There was no appreciable dephosphorylation of the phospholipids upon incubation of the HEG with Sendai virus.

In gel electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (data to be published) and on Bio-Gel columns (Figure 3), the bound phosphate migrated with distinct protein bands, indicating the covalent nature of the bound phosphate. The phosphorylated membranes were obtained by either phosphorylation of intact human erythrocytes ($[^{32}P]P_i$ restoration) or internally trapping [$\gamma$-$^{32}P$]ATP in ghosts. It is therefore probable that the phosphoryl moieties are located only on the internal side of the membrane. The ability of the virus to induce hydrolysis of internally located phosphoryl groups can be explained by the following alternatives. The virus preparations are contaminated with soluble phosphatases which penetrate into the ghosts during viral-induced "hemolysis." This alternative seems to be unlikely since virus absorption is necessary for inducing the dephosphorylation, even in leaky, frozen-thawed HEG. Alternatively, the virus might stimulate a membrane phosphatase or contain a phosphatase which interacts with the internal phosphoryl groups concomitantly with or after the viral envelope fuses with the cell membrane.

The presence of a phosphatase activity in erythrocyte preparations has been reported by others (Fairbanks et al., 1976) and can also be inferred from the slow endogenous dephosphorylation observed in the present work. This activity may be stimulated by the structural changes accompanied by the virus-cell interaction. On the other hand, the presence of both phosphatase and ATPase (Silberstein and Aughust, 1973; Kohn, 1970) has been reported in various virus strains. At the present stage of the work, we cannot exclude either of these two possibilities. This is currently being pursued in our laboratory using $^{32}P$-phosphorylated proteins as substrates for the putative enzyme system.

A specific virus-cell interaction is essential for induction of viral dephosphorylation. This can be inferred from the experiments showing that neuraminidase treatment of membranes and viral antiserum completely prevents viral dephosphorylation.

It is known that $Ca^{2+}$ ions are involved in virus-cell interactions, as is reflected by $Ca^{2+}$ stimulation of viral-induced membrane fusion and inhibition of viral-induced hemolysis (Okada, 1969; Yanovsky and Loyter, 1972; Poste and Allison, 1973). The experiments showing that $Ca^{2+}$ significantly stimulates the dephosphorylation process also indicate that virus-cell interactions are essential for the dephosphorylation. The $Mg^{2+}$ probably functions as a cofactor of the enzyme that dephosphorylates membranous proteins as it does for many transphosphorylating enzymes (Morrison and Heyde, 1972).

This is the first time, to our knowledge, that specific permanent dephosphorylation of membranous proteins has been demonstrated by a virus of this group. The dephosphorylation observed could be related to other functions of the viruses, such as infectivity and membrane fusion. The dephosphorylation may be restricted to membrane regions close to virus attachment and induce a change in the membrane ultrastructure, such as migration of intramembrane particles, resulting in the appearance of protein-free phospholipid areas. It is at these areas that fusion of either adjacent membranes or the viral envelope with the cell membrane might occur. It has been suggested by Vos et al. (1976) and demonstrated by us, as well as by others (Zakai et al., 1976; Chi et al., 1976; Gratzl and Dahl, 1976), that fusion occurs in areas devoid of intramembrane particles. In this context, it should be mentioned that ATP depletion from red blood cells was shown to cause clustering of intramembrane particles, while restoration of intracellular ATP induced redistribution of the clustered IMP (Gazitt et al., 1976). Hence, it seems logical that rephosphorylation of dephosphorylated proteins is required to redistribute the intramembrane particles. However, the possibility that ATP depletion leads to other chemical changes in membrane proteins, such as SH group oxidation resulting in intramembrane particles aggregation, cannot be excluded. In addition, it is possible that ATP depletion and aggregation of intramembrane particles are two independent processes.

The data presented are consistent with the hypothesis that

a dephosphorylation–phosphorylation cycle controls virus-induced cell fusion. We would also like to suggest that interaction of Sendai virus with eukaryotic cells other than erythrocytes may cause similar dephosphorylation of membrane proteins. It will be of great interest to find the site of viral action. Experiments to verify this hypothesis are under way in our laboratory.

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