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Adenosine Triphosphate in the *in vitro* Hemolytic Tests for Paroxysmal Nocturnal Hemoglobinuria.* (32013)

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During the performance of the autohemolysis test on the blood of a patient with paroxysmal nocturnal hemoglobinuria (PNH), it was observed that adenosine triphosphate (ATP) was effective in inhibiting the hemolysis. When this finding was extended to the acid-thrombin test of PNH red blood cells, a similar inhibition of hemolysis was observed. Further studies which form the basis of this report, suggest that this inhibition is caused by the effect of ATP as a chelating agent of magnesium (a necessary component of the PNH *in vitro* hemolytic system) rather than by being a source of intracellular energy or by virtue of its effect on the pH of the system.

Methods. The in vitro *hemolytic system.* The *in vitro* system(1) utilized for the acid test in these studies consisted of 0.5 ml normal serum, 0.05 ml 0.2 N HCl, additives in a volume of 0.05 ml and 0.05 ml of PNH (or normal) red blood cells suspended in saline with a hematocrit of approximately 50%. The pH was determined before addition of the red blood cells. Incubation was carried out in a water bath for 15 minutes and the blood specimens were centrifuged for 2 minutes at 1000 rpm. Hemoglobin content was determined in the supernatant by the benzidine or cyanmethemoglobin method (1). In the acid-thrombin test, thrombin 0.05 ml (approximately 50 NIH units) was added before the red blood cells.

Preparation of Mg ATP. Mg ATP was prepared from barium ATP using excess of magnesium sulfate(2). The Mg, adenine, free phosphorus and total phosphorus were determined. The molarity of the Mg ATP solution prepared on 2 separate occasions was approximately 0.20 M/l, as determined by the adenine(3) and total phosphorus content(4). The molarity of Mg in the Mg ATP solution was 0.36 M/l and 0.38 M/l on 2 separate occasions (Mg was determined by A. L. Chaney Chemical Laboratory, Glendale, Calif.). Since the expected molar binding of ATP to Mg is in the ratio of 1:1(5), there was 0.16 and 0.18 M/l of excess magnesium. The free phosphorus(4) of the Mg ATP was less than 1%.

Phosphorolysis of ATP. Although it was not likely that phosphorolysis of ATP by serum phosphatase with release of energy and ADP occurred within the short time

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of incubation (15 min), the possibility was examined in two ways: a) Measurement of "free" phosphorus(4) of acidified serum (no red blood cells) and ATP before and after incubation. In these experiments the amounts of serum (0.5 ml) and ATP (50 µM) incubated would have increased the original serum level of phosphorus at least by 3-fold if 1% phosphorolysis of ATP took place. The sensitivity of the method for phosphorus determination is in the range of 4-40 µg with a precision of ± 0.2 µg(4). b) Chromatography of disodium ATP after incubation with acidified serum for 15 minutes and dilution with saline. In this method an aliquot of 10-15 µM of ATP was subjected to chromatography, using DEAE-Sephadex at room temperature(3). The flow rate of the effluent was approximately 20 ml per hour and the eluate was collected in 6 ml portions. The absorbance of the effluent was determined at a wave-length of 253.7 mµ using a Beckman spectrophotometer model DU-2, and the fractions were pooled in accordance with the observed peaks. The molar adenine phosphorus ratio of each peak was determined.

Results. Effect of disodium ATP on in vitro *hemolysis of PNH cells.* In six experiments using blood of 4 different patients with PNH, disodium ATP was effective in inhibiting the hemolysis of PNH red blood cells in both the acid test and in the acid-thrombin test. Various concentrations of disodium ATP were used, ranging from 2 µM to 60 µM. Concentrations of 5 µM or more inhibited hemolysis completely, concentrations between 2 and 4 µM were occasionally inhibitory; however, concentrations of 40 µM or more depressed the pH below 6.0, a level below the critical pH range for the acid test (6.0-7.2, optimal 6.3 to 6.6)(6).

Effect of ATP related compounds on the in vitro *hemolysis of PNH cells.* Other agents related to ATP, such as adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADE), and inorganic phosphate were tested in the same system. Fig. 1 (upper) shows that adenosine was not inhibitory, whereas ADP and AMP caused inhibition but at higher concentrations than those of ATP. Inorganic phosphate (K_2HPO_4) did not diminish hemolysis of acid-thrombin test significantly unless concentrations of 25 or 50 µM were used, and even then it was not complete (a decrease from 700 mg% hemoglobin to 200 mg%).

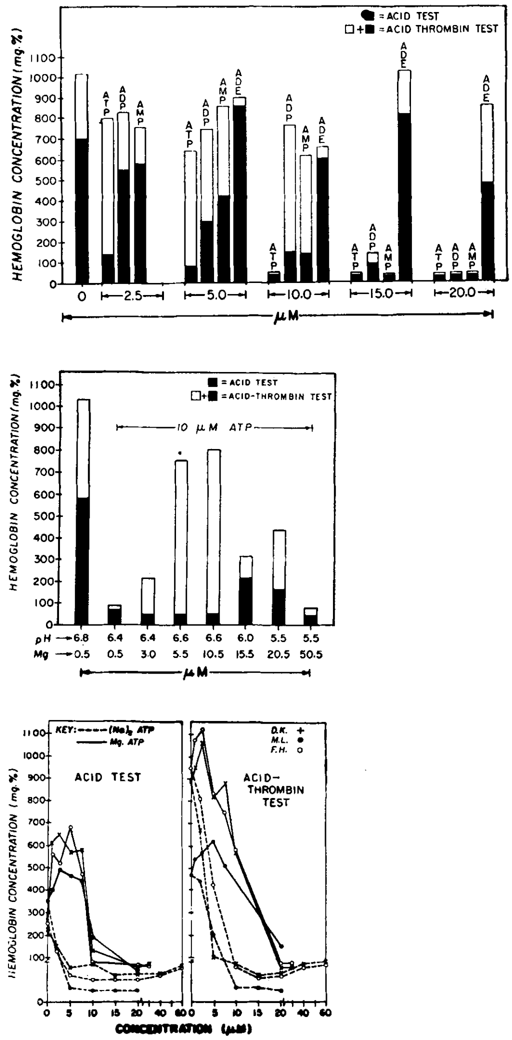

FIG. 1. (Upper). Effect of various concentrations (µM) of ATP, ADP, AMP and adenosine on the acid and the acid-thrombin tests. (Middle): Various concentrations of magnesium (µM) in presence of constant concentration of ATP (10 µM) in the same hemolytic test system. (Lower): Comparison of effects of various concentrations of the sodium and magnesium salts of ATP on the acid and the acid-thrombin tests. Degree of hemolysis is reflected by amounts of serum hemoglobin concentration (mg %) depicted on the ordinate.

Effect of a constant concentration of ATP *in the presence of ranging concentrations of Mg on the PNH hemolytic tests.* Since a critical concentration of magnesium is required for the acid hemolysis test(6), various concentrations of Mg (sulfate or chloride) ranging from 0.5 to 50 μM were tested. Addition of 3 to 10 μM enhanced hemolysis, but 50 μM inhibited it. When various concentrations of Mg were added to the acidified serum and then 10 μM of disodium ATP was added, hemolysis was inhibited by ATP up to a concentration of 10.5 μM of Mg in the acid test and up to a concentration of 0.5 to 3.0 μM in the acid-thrombin test (Fig. 1, middle). Therefore, in the acid test a ratio of ATP to Mg of about 1 (equimolar) seems to inhibit the hemolysis.

Comparison of disodium ATP with Mg ATP in the PNH hemolytic tests. Fig. 1 (lower) shows that disodium ATP is more effective than Mg ATP in inhibiting the hemolysis. A concentration of more than 10 μM of Mg ATP is required to inhibit completely the hemolysis in the acid test and a concentration of more than 20 μM in the acid-thrombin test.

Phosphorolysis of ATP by acidified serum. In 3 experiments where disodium ATP was incubated with acidified serum for 15 minutes the phosphorolysis of ATP was 0.3, 0.4 and 0.8%. In one experiment with Mg ATP, the acidified serum phosphorolyzed 0.7% of the Mg ATP.

Chromatography of disodium ATP. In 4 experiments, the yield of disodium ATP was 78 to 86%. There were 2 main peaks of adenine activity in all experiments except one. The early peak had no phosphorus and represented material from serum alone, and the late peak had adenine phosphorus in a molar ratio of 1:3. In one experiment there were 3 peaks of ATP as indicated by adenine to phosphorus ratio of 1:3. ADP appeared in a concentration of less than 10% in 2 experiments and also in an experiment where ATP was delivered to the column without prior incubation with serum.

Discussion. The acid-thrombin *in vitro* hemolytic test is widely used as a diagnostic test for PNH(1). It is appreciated that a critical pH value and a critical concentration of magnesium(6) and thrombin(1) are necessary for optimal results. The results of the studies have shown that disodium ATP in certain concentrations can inhibit hemolysis without significantly changing the pH. The inhibition of hemolysis could be ascribed to one of two mechanisms. Either the ATP undergoes phosphorolysis in the acidified serum thus providing high energy to protect the red blood cells from hemolysis or the ATP can bind the heavy metal Mg which is an essential component of this hemolytic system. Evidence here is presented to support the latter alternative.

Measurements of free phosphorus before and after incubation of ATP to acidified serum provide no evidence for phosphorolysis. Similarly, chromatography of ATP added to acidified serum indicates no phosphorolysis of tht nucleotide beyond that which occurs as the result of the technique itself. Inorganic phosphate and adenosine, products of ATP phosphorolysis cannot be held responsible for the inhibitory effect of ATP since by themselves they are ineffective in the acid test. On the other hand, ATP and AMP which could be products of ATP hydrolysis inhibit the hemolytic test, but at higher concentration than ATP. AMP has no high energy bond; however, it can bind magnesium in an equimolar ratio similar to the ratio of ATP or ADP binding to the heavy metal(5).

The free energy of magnesium ATP is as much if not more than (Na_2) ATP but within corresponding concentrations was not as effective as the sodium salt. The reason for the inhibition of hemolysis by high concentration of Mg ATP is not clear unless Mg ATP and Mg unbound (total approximity 16 μM or more in the acid test, and 36 μM or more in the acid-thrombin test) behave like the inhibiting high concentration of Mg alone(6).

Summary. Adenosine triphosphate and related compounds inhibit hemolysis in the acid and acid-thrombin tests of paroxysmal nocturnal hemoglobinuria. The mechanism of action is related to the capability of such agents to chelate a heavy metal (Mg) which is essential for these *in vitro* hemolytic tests.

The chelating property of these nucleotides rather than their high free energy, as usually presumed, may also explain their inhibitory action in other *in vitro* hemolytic systems.

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Dactinomycin: Relative Resistance of Green Monkey Kidney Cell Cultures to its Action.* (32014)

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Evidence has accumulated that suppression of DNA dependent RNA synthesis by dactinomycin (U.S. Pharmacopeia official name and also referred to as actinomycin D, actinomycin C_1 and actinomycin IV) begins at about 0.01 μg/ml and maximum inhibition is reached at about 1-5 μg/ml(1-5). In the present study it was unexpectedly observed that while near maximum inhibition of yield of vaccinia virus occurred in HeLa and mouse embryo cells (ME) beginning at 2 μg dactinomycin/ml, in primary green monkey kidney cells (GMK) it was necessary to use a concentration of 200 μg/ml to achieve similar inhibition. The experiments reported here were performed to analyze this phenomenon. Results demonstrated the requirement for unusually high doses of dactinomycin for inhibition of RNA synthesis in GMK cells. A probable explanation for the findings is that GMK cells take up much less dactinomycin than do HeLa cells.

Materials and methods. Cell cultures. HeLa cells, ME, and GMK cell cultures were obtained from Microbiological Associates, Inc., and maintained on Eagle's minimum essential medium in Earle's balanced salt solution (EMEM) containing 2% heated (56°C for 30 minutes) Agamma calf serum (Hyland), 4 mM of glutamine, 100 units of penicillin, 100 μg of streptomycin, 10 μg of kanamycin and 20 μg of polymyxin B per milliliter.

Virus. Vaccinia virus obtained through the courtesy of Dr. Klaus Schell, Microbiological Associates, Inc., Bethesda, Md., was propagated in monolayers of GMK cells and titers of 10^7 plaque forming units (PFU) per ml were regularly obtained. A liquid overlay vaccinia plaque system(6) was used to determine titers. Monolayer cultures of 4×10^5 cells in roller tubes were infected at a multiplicity of 5-10 pfu/cell in medium with or without dactinomycin. After one hour adsorption at 37°C, the medium was removed, the cells were washed 5 times with Earle's balanced salt solution (EBSS), and 1 ml of fresh maintenance medium was added. After 24 hours of incubation at 37°C, the cell-associated virus from each treated group was harvested and pooled after 3 cycles of freezing and thawing.

RNA synthesis. The effect of dactinomycin on uptake of ^{3}H-uridine into nucleic acids of HeLa and GMK cultures was determined.

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