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Stimulation of Erythrocyte Glycolysis by Nicotinamide Adenylates. (30657)

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The purine nucleosides, particularly adenosine and inosine, have been repeatedly demonstrated to diminish the progressive fall in the glycolytic activity of human erythrocytes which occurs under the conditions of blood storage. These compounds also possess the capacity to replenish the ATP level and thereby restore glucose utilization of cells that have been metabolically depleted(1). The nucleosides have been shown to be freely accessible to the metabolic machinery of the cell whereas the nucleoside phosphates are not generally considered to be as readily permeable and consequently it is not to be expected that they would influence metabolism significantly(2). However, in the course of an investigation of the metabolism of labelled yeast adenylic acid compounds *in vivo*, it was noted that the red cells of the rat and other animals accumulated the label to an unexpected extent. It was of interest to reassess the ability of the adenine nucleoside phosphates to contribute to the support of the metabolism of red cells. This investigation is concerned with effects of these compounds on the glucose metabolism of human erythrocytes. For this purpose the nicotinamide salts of the adenine nucleotides possess distinct advantages in that they do not contribute excess metallic cation and also because nicotinamide has been shown to restore the synthesis of pyridine nucleotides and particularly the pyridine coenzyme levels of aged cells. In addition the nucleotide-organic base combination possesses a potentially useful buffering capacity that could be of significance in maintaining the hydrogen-ion concentration of metabolizing cell suspensions.

That the glycolytic activity of mature erythrocytes is sensitive to the hydrogen-ion concentration is well substantiated(3). As a result of the progressive decrease of pH from the accumulation of lactic acid, the rate of glucose utilization diminishes rapidly. Bishop (4) has shown that if the hydrogen-ion concentration of a suspension of cells is continuously adjusted, the glycolytic activity could be maintained for several days. Although several enzyme reactions of the glycolytic pathway have been implicated, it appears certain that the acid-sensitive reaction is above the glyceraldehyde-3-phosphate step because the addition of nucleosides stimulates glycolysis with an increase of lactate production de-

rived from the ribose moiety of adenosine.

While the findings of the present report indicate that the nicotinamide salts of the adenylic acids are also effective in maintaining *in vitro* the glycolysis of human erythrocytes and restoring the activity of depleted cells, this effect could not be ascribed solely to a buffering action of the adenylates nor could the effect be accounted for by the nicotinamide moiety since under the conditions of the experiments this substance did not influence the glycolytic activity.

Materials and methods. Nicotinamide 5′ adenylate (N5′A)* and nicotinamide 2′, 3′ adenylate (N2′, 3′A)* have a composition of 2 moles nicotinamide to 1 mole adenylic acid. The composition and purity of the compounds, particularly with respect to presence of adenine and adenosine, were examined by descending paper chromatography with the following solvent systems: a) butanol, acetic acid, cellosolve, water (7:2:7:4, pH 2.8); b) ethyl acetate, pyridine, water (2:2:2, pH 7.0); c) 1.5 M sodium phosphate buffer, pH 7.0; d) saturated ammonium sulfate, sodium acetate, isopropanol (80:18:2); e) t-pentanol, formic acid, water (2:2:1). Systems c, d and e, and also thin layer chromatography (DEAE-cellulose with 0.01 M HCl solvent), adequately separated the adenylic acids. With these systems, the compounds were found to be contaminated by not more than 1% with adenine plus adenosine with any of the systems used. Preparatory to use in the experiments, the adenylates were dissolved in sterile Krebs-Ringer solution (KR)(5) without calcium and the pH adjusted to 7.4 with sodium hydroxide. The solution was filtered (Millipore filter, 0.45 micron) and stored frozen in sterile ampules.

Human red blood cells were used in all experiments. Blood was collected aseptically from normal fasting donors with the aid of heparin, centrifuged immediately at 2000 rpm for 15 minutes at 4°C, and the plasma drawn off together with the white cell layer and the top 5% of the packed red cells. The cells were washed a minimum of three times with Krebs-Ringer solution buffered at pH 7.4 with either phosphate (KRP) or bicarbonate (KRB). They were then resuspended in one of these media containing 100 mg per liter each of potassium penicillin G and streptomycin sulfate.

The glycolytic activity of the erythrocytes was estimated manometrically using a Gilson differential respirometer(6). Unless otherwise noted the washed cells were suspended in the ratio of 1.0 ml packed red cells to 6 ml of KRB containing 250 mg% glucose. A volume of 3.0 ml of cell suspension was pipetted into Warburg flasks. The flasks were equilibrated at 37°C and gassed with a N_2-CO_2 mixture (95%:5%) for 15 minutes before the system was closed. The CO_2 evolution due to lactic acid production was measured over a 90-minute period. Glycolytic activity was expressed as μmoles lactic acid produced per ml packed red blood cells.

The nicotinamide 2′, 3′ adenylate-8-C^{14} used in the experiments was prepared from 2′, 3′ adenylic acid-8-C^{14} (31 μcuries per mmole) obtained from Schwarz BioResearch Inc. Radioactivity was estimated with a Nuclear-Chicago low background beta counting system. Aliquots of the suspending media and of the cell hemolysates were plated with gelatin on copper planchets. The CPM of the samples were corrected for self absorption by the method of Berson and Yalow(7) and for hemoglobin content.

Results. Maintenance of glycolysis of fresh cells. The ability of the adenylates to modify the glycolytic activity of mature erythrocytes was explored by incubating freshly drawn cells in either KRP or KRB mediums for periods up to 20 hours with measurements of the glycolytic activity made at intervals.

The effects of N2′, 3′A and also nicotinamide on the glycolysis of erythrocytes incubated at 37°C are illustrated in Fig. 1 for a representative experiment. The results shown were observed consistently. The procedure for these experiments was as follows. Aliquots of 2.5 ml of a cell suspension with an hematocrit of 20% in KRB medium containing 250 mg% glucose were placed in Warburg flasks. The flasks were arranged in sets of four, the sidearms of which contained 0.5 ml of either nicotinamide, nicotinamide ade-

* Investigational drugs made available by Union Carbide Corp., New York.

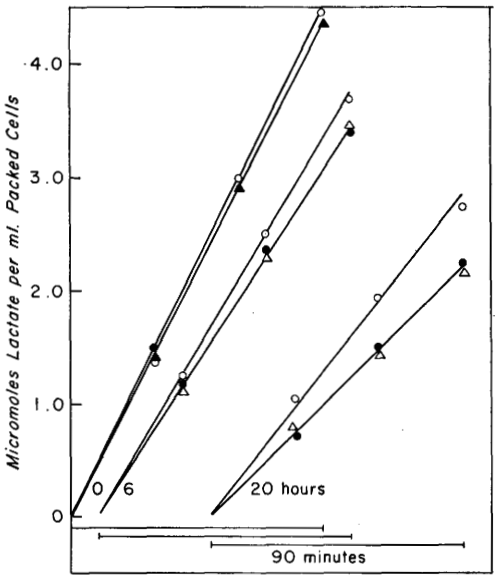

FIG. 1. Rate of glycolysis of erythrocytes (measured over 90-min periods) after 0, 6 and 20 hr incubation in bicarbonate medium in presence of nicotinamide adenylate 2.8 mM, ○—○; nicotinamide 2.8 mM, △—△; control, ●—●. The pH of all flasks was initially 7.4 and terminal pH after 20 hr was nicotinamide adenylate 6.95, nicotinamide 7.09 and control 7.13.

nylate or the medium. After equilibration and gassing with 95% N_2-5% CO_2, the system was closed, the sidearms were dumped and the initial rate of glycolysis measured. The flasks were never opened to the atmosphere thereafter during the total experimental period; but they were maintained in constant equilibrium with a large common reservoir flask containing the gassing mixture except during the measurement periods.

From the Figure it may be seen that the initial rate of glycolysis was 2.90 μmoles lactate per hour and although glucose was not limiting, the rate decreased steadily, as the pH fell, to 1.50 μmoles per hour at 20 hours. The presence of nicotinamide influenced neither the initial nor the final glycolytic rate. In contrast, N2′, 3′A measurably decreased the progressive fall in glycolysis so that the final rate was significantly higher than the untreated cells, (1.93 μmoles lactate per hour). The metabolic support given by the N2′, 3′A did not appear to be due to a buffering effect since the final pH of the suspension was lower than that of the nicotinamide or the control. Essentially no hemolysis occurred in any of the cell suspensions; the initial hematocrits of 16% compared with the final value of 17% indicated slight increase in cell volume.

Experiments carried out in KRP medium containing glucose provided similar results (Fig. 2). For these experiments, cells washed as outlined were suspended to an hematocrit of 15% and incubated in air at 37°C with gentle agitation. To portions of this suspension were added either nicotinamide or N2′, 3′A. At designated intervals, aliquots were removed from the cell suspensions. They were centrifuged, the cells washed once with KR solution, twice with KRB and finally resuspended in this medium with 250 mg% glucose. The ability of the cells to metabolize glucose was measured with the respirometer.

It is apparent from the Figure, which summarizes the results of a representative experiment, that as expected the pH of the cell suspension declined steadily. Concurrently, the rate of glycolysis of the cells decreases even though the glycolytic activity was measured after the cells were restored to the initial pH. It was clear that raising the pH was not sufficient to restore glycolysis to normal. The pH of the N2′, 3′A suspension decreased at a rate greater than either the nicotinamide or

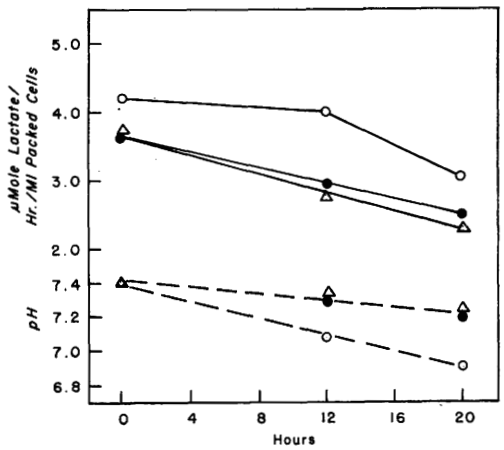

FIG. 2. Rates of glycolysis of erythrocytes incubated in phosphate buffered medium and resulting pH changes. Additions were nicotinamide adenylate 2.8 mM, ○—○; nicotinamide 2.8 mM, △—△; without addition, ●—●.

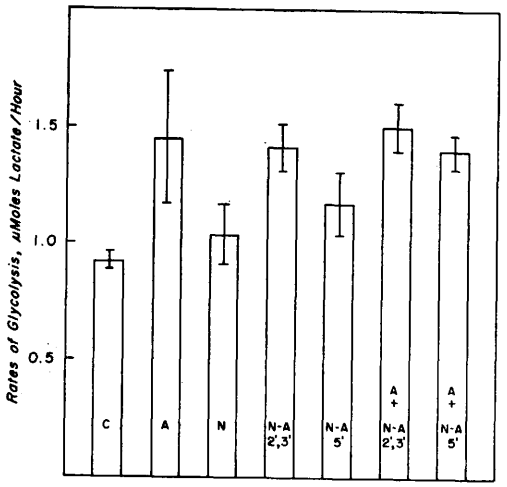

FIG. 3. Glycolytic activity of human erythrocytes after aging in phosphate buffered medium without glucose but with additions of N, nicotinamide 9.0 mM; A, adenosine 5.5 mM; N2′, 3′A, nicotinamide 2′, 3′ adenylate 2.8 mM; N5′A, nicotinamide 5′ adenylate 2.8 mM; and C, no addition. The washed cells were resuspended in bicarbonate medium containing glucose. Rate of glycolysis was determined on 3.0 ml cell suspension, containing 0.25 ml packed cells, placed into Warburg flasks and measuring CO_2 production. Bars represent standard error of mean of 6 experiments.

the control suggesting a greater rate of glycolysis during the incubation and this was confirmed by the greater glycolytic rates found for the N2′, 3′A suspension. The presence of nicotinamide did not maintain the capacity of the cells to utilize glucose over that of the control.

Restoration of glycolysis of aged cells. It was of interest to determine the effect of N2′, 3′A on the glycolysis of erythrocytes which had been metabolically depleted of endogenous substrates. Washed red blood cells were suspended in KRP and incubated without glucose at 37°C for 16-18 hours. The aged cells were then centrifuged, washed twice with KRP, and resuspended in this medium with 250 mg% glucose to an hematocrit of 16%. To portions of this suspension were added either adenosine, N2′, 3′A or combinations of adenosine and these adenylates. The individual suspensions were incubated at 37°C in air for 4 hours. At this point the ability of the suspensions to metabolize glucose was determined. They were centrifuged, washed in KRB containing 250 mg% glucose and finally resuspended to an identical hematocrit in this medium and their glycolytic activity measured with the respirometer.

The results of a series of experiments are summarized in Fig. 3. As has been noted by others also, the untreated control cells demonstrate decreased ability to utilize glucose, while the addition of adenosine was capable of accelerating the glycolytic rate to its maximum (57%). It should be noted that cells treated with N2′, 3′A increased their glycolytic capacity to virtually the same extent (53%) while N5′A stimulated to a significant but lesser degree (27%). The effect of the adenylates was not additive to that of a maximally effective concentration of adenosine. Nicotinamide by itself did not significantly influence the metabolic activity.

Assimilation of nicotinamide adenylate. The results of the preceding experiments suggest that N2′, 3′A and N5′A can be metabolized by human erythrocytes. Experiments were undertaken to determine whether an assimilation of these compounds occurred under our conditions. Washed cells were resuspended to an hematocrit of 18% in KRP containing glucose and this cell suspension divided into equal portions. Each portion was centrifuged, resuspended, and washed twice more with KRP buffered at pH 7.0 and 6.0 and then finally resuspended to the original hematocrit. Nicotinamide 2′,3′ adenylate-8-C^{14} was added to provide a final concentration of 2.6 mM. Immediately prior to incubation at 37°C in air, the pH of the cell suspensions were found to be 7.03 and 6.37. At intervals, duplicate aliquots were withdrawn from each suspension, centrifuged, the cells washed with KRP of the appropriate pH, and the radioactivity determined, as CPM per ml of incubating medium and per ml cells. The results were expressed as the ratio of these values.

The datum of one experiment is summarized in Fig. 4. The cells incubated at pH 7.0 accumulated an appreciable amount of radioactivity from the ring-labelled adenylate in the suspending medium. This occurred in a relatively linear manner over the 4-hour incubation period. The cell to medium ratio did not reach an equilibrium value within the

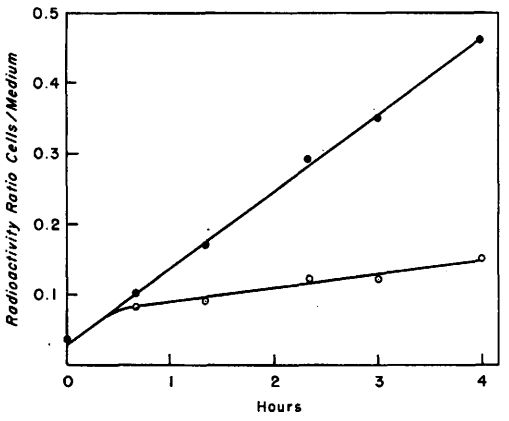

FIG. 4. Distribution of radioactivity of nicotinamide 2′, 3′ adenylate-8-C^{14}, 2.6 mM, between cells and suspending medium in a cell suspension of 18 vol %. Erythrocytes were incubated with glucose at 37°C in KRP medium at pH 7.03, ●—● and 6.37, ⊙—⊙.

experimental period although in other experiments an equilibrium ratio of 1.0 was observed occasionally. A small amount of radioactivity was associated with the cells immediately after the adenylate-C^{14} was added to the suspension. This radioactivity appeared to be the result of an adsorption of the compound onto the membrane. Under the conditions of the experiment, calculation of the apparent rates of transfer indicate that at pH 7.03 and 6.37, radioactivity equivalent to 250 and 42 μmoles of adenylic acid per liter of packed cells per hour respectively was assimilated by the erythrocytes. The transfer activity of erythrocytes from different donors gave values of 235, 250, 445, and 550 μmoles per liter per hour assimilated at neutral pH while at acid pH only minimal assimilation was observed.

Discussion. The ability of the adenylates to decrease the gradual decline of the glycolytic activity of human erythrocytes incubated for extended periods *in vitro* represents a substantial effect on cell metabolism. The activity of fresh untreated cells incubated in KRB, initially 2.90 mmoles lactate per liter cells per hour, diminished to 48.3% of this value while cells in the presence of N2′, 3′A fell to only 65.5% of the initial glycolytic rate after 20 hours. This effect could not be accounted for by either the nicotinamide moiety of the compound or by a buffering property. Similar results were determined from cells incubated in KRP. In addition, these latter experiments showed that it was not possible to fully rejuvenate the cells to their initial glycolytic activity by merely restoring them to their normal pH.

It is well recognized that blood cells stored at pH 7.0 or lower lose their intracellular substrates with a concomitant fall in ATP to very low levels even in the presence of glucose. These changes have been shown to occur for experimental conditions and for periods of incubations similar to ours(1). By the addition of certain purine nucleosides the depleted cells can be reprimed metabolically with sufficient ATP to allow increased rates of glycolysis. These nucleosides provide substrate for the shunt pathway and thereby increase ATP synthesis and concentration(1). In agreement with these observations, cells that were depleted by our procedure demonstrated a marked loss of glycolytic activity but responded to the addition of adenosine. Of particular interest, however, was the finding that the addition of the nicotinamide adenylates also had the effect of stimulating glycolysis. For the 2′,3′ compound, the response was comparable to that of adenosine although the 5′ isomer was less effective. The combination of adenosine together with the adenylate compounds did not produce a response which was additive. Since, in these experiments, the concentration of adenosine was more than twice that necessary to provide a maximal response by this agent, it is possible still that the modes of action of adenosine and the adenylates may be exerted through a similar metabolic action.

Phosphorylated compounds, such as the adenine nucleotides have not been noted to be as readily accessible to the metabolism of the cell as are the ribosides. Under the conditions of the present experiments, it was found that the labelled moiety of the 2′, 3′-nicotinamide adenylate-8-C^{14} was slowly acquired by the erythrocytes at a rate which increased as the pH of the suspending medium was increased to neutrality. The accumulation of the label of the adenylate compound increased linearly over a 4-hour period, a finding which is not consistent with a

binding or an adsorption to the cell membrane because such processes would be expected to occur at considerably more rapid rates. Although it is possible that the nicotinamide adenylate passed the membrane barrier in an unchanged molecular form, the experiments do not provide evidence to this point. On the other hand, the assimilation can be accounted for by the phosphomonoesterase activity associated with this cell. This enzyme has been shown by several investigators to act on yeast adenylic acid to yield adenosine. Tsuboi and Hudson(8) have assayed the esterase activity of hemolysates of human red cells and found a value of 6.5 mmoles phosphorus liberated per hour per liter RBC at 37°C. This value was measured at pH 5.5, the optimum hydrogen-ion concentration reported for this enzyme. The data of these investigators and others(9,10) show that considerable enzyme activity is present at pH 7.0 particularly in the presence of magnesium which markedly increased enzyme activity at neutrality. In the present experiments the esterase activity, as indicated by assimilation of radioactivity, was somewhat lower and of a magnitude which might be expected at neutral pH. Therefore it is possible that by the action of this enzyme amounts of adenosine sufficient to contribute to ATP formation were made available to the cells. But, contrary to what might be expected through the action of this esterase, only minimal assimilation of the labelled compound occurred at the more optimal pH of 6.4. However, at this pH, membrane nucleoside phosphorylase activity is minimal (8), and consequently adenosine may not be able to enter the cell readily. At neutral pH, the activity of nucleoside phosphorylase is maximal and thereby by its action facilitates the entry of adenosine and its incorporation into cell substrates.

Summary. The addition of nicotinamide adenylate compounds was found to maintain, to a significant extent, the glycolytic capacity of fresh human erythrocytes incubated *in vitro* for 20 hours. The compounds appeared to act also to restore the metabolism of aged cells. Although it is not believed that nucleotides readily enter the intracellular compartment, evidence is presented that erythrocytes can slowly assimilate adenine nucleoside phosphates. The possible role of erythrocyte phosphomonoesterase is discussed.

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In vitro Inhibition of Na^+-K^+ and Mg^{2+} ATPases by Mono, Di and Trivalent Cations.* (30658)

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It is now generally accepted that mammalian cells maintain their ionic composition by pumping potassium inward and sodium outward. Evidence that active cation transport is intimately involved with the enzymatic hydrolysis of ATP is available(1). Observations that both the Na^+-K^+ ATPase and active cation transport systems share common characteristics, have led to the hypothesis that

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