phopyridine nucleotides have been studied in rat liver after fasting and during oxidation of ethanol. The changes in diphosphopyridine nucleotides have also been studied in baker's yeast during ethanol oxidation. By fasting alone a slight decrease of DPN/DPNH ratio mainly due to a fall in the concentration of DPN is noted. Total DPN + DPNH is also decreased slightly. When the animals are metabolizing ethanol the DPN/DPNH ratio decreases further, due to an increase of DPNH, and especially in the fasted animals a fairly marked increase of the total DPN + DPNH can be noted. Similar changes are observed in baker's yeast during ethanol oxidation. Triphosphopyridine nucleotide is mainly present in the liver in its reduced form, and the TPN/TPNH does not change significantly during ethanol oxidation.

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Inhibition of Reticulocyte Lysis by Alkyl Polyamines. (27375)

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The participation of the alkyl polyamines in many diverse biological and biochemical phenomena has made this class of organic compounds a source of increasing interest. Spermine, spermidine and related substances are widely distributed in animal tissues and microorganisms; yet specific information about their function(s) is lacking. A variety of biological effects has been demonstrated in selected systems(1) including: 1) promotion of growth of microorganisms, 2) stabilization of bacteria, bacterial protoplasts, mitochondria, ribosomes, bacteriophages, and transforming principle, 3) binding to nucleic acids, heparin, and certain lipids, and 4) stabilization of enzyme activity.

This report deals with the stabilizing effect of spermine and related polyamines on the reticulocyte undergoing spontaneous lysis.

Methods. Blood samples, anticoagulated with dry dipotassium versenate, were obtained from human subjects and Sprague-Dawley rats. Reticulocyte-rich blood was obtained from female Sprague-Dawley rats weighing 150-200 g, previously given subcutaneous injections of phenylhydrazine hydrochloride(2). Reticulocyte counts of up to 80% were obtained. Reticulocyte counts were made on all blood samples using the methods of Brecher(3,4).

Dilute suspensions of red blood cells and reticulocytes were incubated in phosphate buffered saline following the osmotic fragility method of Dacie(5). For each blood sample tested, a duplicate series of tubes was used containing 2 ml aliquots of serial dilutions of 0.150 M NaCl in 0.01 M sodium phosphate buffer at pH 7.40. To one series of tubes 0.01 ml of an alkyl amine solution was added so that the final concentration of amine was 0.5 mM. A second series of tubes without alkyl amine served as a control. Whole blood (0.02 ml) was then delivered into each tube and mixed immediately by gentle inversion. Tubes were incubated at room temperature (23°-25°C) for 30 minutes and then centrifuged at 2000 rpm for 5 minutes. The amount of hemolysis (referred to as immediate hemolysis) was determined by reading the optical density of the supernatant solution at 540 mμ in a Coleman Junior Spectrophotometer. The tubes were then reinverted for mixing, covered to prevent evaporation, and incubated at specific temperatures

for varying periods. A second optical density at 540 mμ was then measured on each tube after centrifugation to determine the amount of time-and-temperature dependent lysis (referred to as delayed hemolysis). Paired series of tubes were incubated for varying times at different temperatures with several different alkyl amines.* Corrections for the contribution of the alkyl amine to the osmotic effect of the phosphate buffered saline were calculated on the assumption that complete dissociation of all HCl residues occurred. These corrections were very small, as can be seen from the close proximity of the x-coordinates of the corresponding spermine and control points in Fig. 1. No pH corrections were required because the aqueous solutions of the alkyl amines were neutral.

Quantitative estimation of total endogenous alkyl polyamines was performed on several samples of human and rat blood with high and low reticulocyte counts using the method of Rosenthal and Tabor(6).

Several experiments were performed to determine whether hemoglobin or acid soluble purine and pyrimidine compounds were released selectively from hemolyzing red cells. After the initial release of hemoglobin was measured, the supernatant solutions were precipitated with an equal volume of 10% perchloric acid and centrifuged. The resulting supernatant optical densities were read at 260 mμ in a Beckman DU Spectrophotometer.

Results. The occurrence of delayed hemolysis was found to depend upon the reticulocyte count. Only reticulocyte-rich blood underwent delayed hemolysis; osmotic fragility curves of blood samples containing less than 10% reticulocytes were not shifted measurably following incubation for 24 hours at 37°C, agreeing with the observations of Jaffe *et al.*(7). Delayed hemolysis of reticulocyte-rich blood is illustrated by the dotted line in Fig. 1B as compared with the immediate hemolysis shown by the dotted line in Fig. 1A. Spermine inhibited delayed hemolysis of reticulocyte-rich blood (Fig. 1B) but had no effect upon immediate hemolysis (Fig. 1A). Samples incubated with spermine for 7 hours at 37°C demonstrated markedly less hemolysis than controls; this inhibition occurred only in tubes near the physiologic range of ionic strength. Spermidine and putrescine inhibited delayed hemolysis of reticulocyte-rich blood, but their protective effect was less than that observed with spermine. Incubation at 0°C also completely prevented the hemolysis of reticulocyte-rich blood. Addition of glucose to the incubation mixture in a final concentration of 1 mg/ml altered neither the magnitude of the delayed hemolysis nor the protective action of spermine.

It was found that the optical densities at 260 mμ of supernatant solutions which were precipitated with perchloric acid increased with increasing delayed hemolysis both in tubes that contained spermine and in controls, indicating simultaneous release of hemoglobin and purine-pyrimidine compounds. It appears, therefore, that delayed hemolysis represents true lysis rather than a selective leakage of hemoglobin from red cells and that spermine protects the red blood cell against lysis.

The endogenous polyamine content of whole rat blood corrected for packed cell volume was not significantly different for samples ranging from 1% to 90% reticulocytes.

A crude reticulocyte nuclease was prepared from washed rat reticulocytes and the activity was estimated using the limit polynucleotide substrate of Hilmoe and Heppel(8). Spermine did not inhibit the nuclease activity in this system. Therefore, these data fail to indicate any similarity between the mechanism of the protective effect of spermine on reticulocyte lysis and the osmotic lysis of *Hemophilus parainfluenzae* studied by Herbst and Doctor(9). Rather, the results presented are consistent with the hypothesis of lipid membrane stabilization as the general mechanism of protection by alkyl polyamines against osmotic lysis(10).

Summary. Spermine, putrescine, and spermidine protected rat and human reticulocytes against delayed hemolysis which occurs during incubation in phosphate buffered saline at isotonic and near isotonic ionic strengths. The phenomenon of delayed lysis occurs in

* Spermine, spermidine and putrescine were obtained from California Corporation for Biochemical Research, Los Angeles.

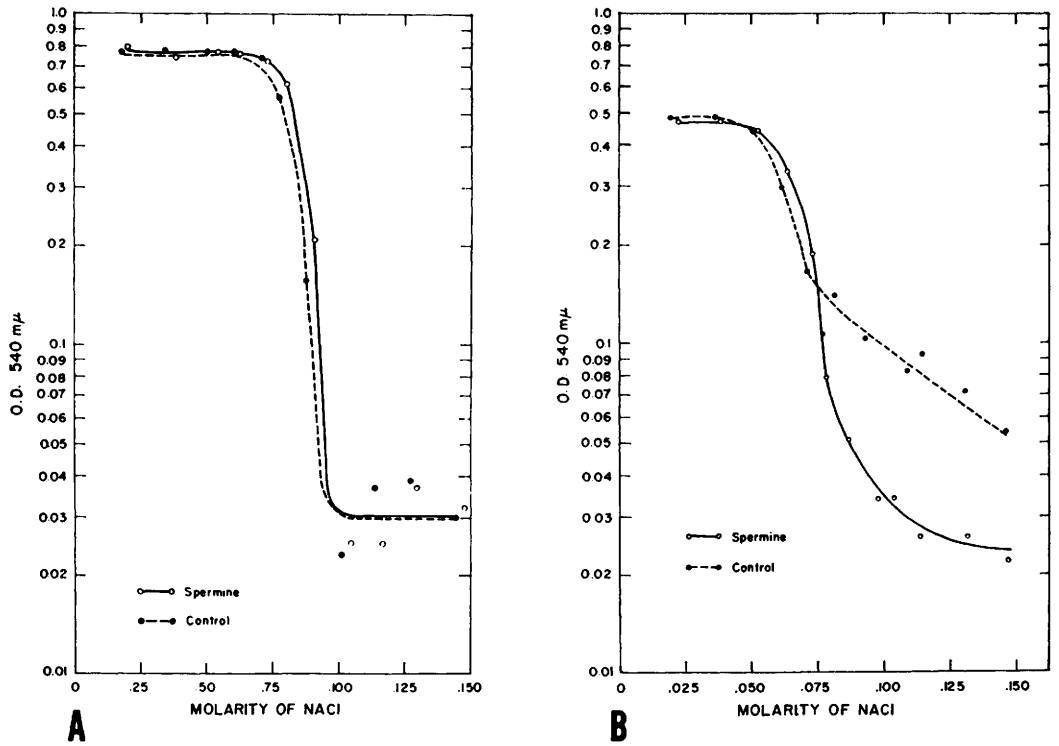

FIG. 1. A. Osmotic fragility curves of rat blood containing 85% reticulocytes with and without 0.5 mM spermine. Optical density 540 mμ was measured 30 min. after mixing (immediate hemolysis). B. Osmotic fragility curves determined on the same samples of reticulocyte-rich rat blood as shown in Fig. 1A after 7 hr incubation at 37°C (delayed hemolysis).

reticulocytes but not in mature red cells. It has a temperature optimum at 37°C and is completely inhibited at 0°C. These data are further evidence of the membrane stabilizing effects of the alkyl polyamines.

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