served in animals given high doses of this polymer is due, in part, to the action to these amines, then animals previously depleted of these amines would be less sensitive to the action of a drug which presumptively acted *via* this pathway. Such may be the case with hexadimethrine.

Summary. Animals partially depleted of their histamine and serotonin by pretreatment with 48/80 were rendered less sensitive to the toxic effects of hexadimethrine. Conversely, pretreatment of mice with imidazole increased the toxic effects of a subsequent injection of hexadimethrine. These results, coupled to our previous findings(1,2) suggest that some of the responses to hexadimethrine in certain laboratory animals may be mediated *via* histamine, serotonin and heparin release. It is probable that there are other factors which may be concerned in the over-all toxicologic and pharmacologic picture of this antiheparin agent.

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Diphosphopyridine Nucleotide Binding Effect of Sickle Cell Erythrocytes On *in vitro* Growth of *Hemophilus influenzae.** (26522)

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The recent demonstration that erythrocytes from patients with sickle cell anemia fail adequately to support *in vitro* growth of certain hemophilic bacteria(1) suggests the existence of an unidentified inhibitor in these erythrocytes. The present investigations demonstrate and identify the inhibitory system.

Materials and methods. Blood from 8 patients with sickle cell anemia, 6 patients with sickle cell trait, and 5 normal controls was collected under sterile conditions in standard (A.C.D.) anticoagulant. The blood cells were immediately washed 3 times with sterile 0.85% saline solution. The washed cells were resuspended in physiological saline to a hematocrit value of 40% and incorporated in culture media.

Trypticase soy agar and broth (BBL) was employed for agar plates and broth cultures. Erythrocyte suspensions were added to the cultures in a concentration of 4%.

Lyophilized cultures from the American Type Culture Collection, Washington, D.C., were used: *Hemophilus influenzae* type *A* (9006); *H. influenzae* type *B* (9334); *Diplococcus pneumoniae* type *III* (6303); and *Streptococcus salivarius* (9756). Hospital laboratory cultures of *H. influenzae* type *A; H. influenzae* type *B*; *Salmonella montevideo*; *S. oranienberg*; and *S. bareilly* were also employed.

Inocula were prepared from 24 hour broth cultures. A single 0.04 ml drop of a 1:200 di-

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lution of the broth culture was placed in the center of each Petri plate and was spread with a sterile glass stirring rod. Ten replicate platings were made each time the growth of the organism was studied. Plates were observed and growth recorded at 24 hours. Tube broth cultures containing 10 ml of media were inoculated with 0.04 ml of a 1:200 dilution of a 24 hour broth culture and growth recorded at 24 hours. The size of surface colonies was estimated as + (barely visible) to ++++ (larger than 1 mm in diameter). Since replicate inocula resulted in almost exactly the same number of colonies (100), no report of colony numbers was necessary. Growth of broth cultures was estimated as + (barely visible) to ++++ (heavy turbidity).

Chocolating of erythrocytes was carried on at 80°C. Peptic digestion was done according to the method of Fildes(2). Osmotic fragility and alkali denaturation were determined by the methods of Chernoff(3). Diphosphopyridine nucleotide (Assay 95%) was purchased from Sigma Chemical Co.

Results. Preliminary screening studies were conducted with surface plate cultures containing washed erythrocytes from patients with sickle cell anemia (SS), sickle cell trait (SA) and normal adult blood (AA). Representative organisms of the genus *Hemophilus*, *Diplococcus*, *Streptococcus*, and *Salmonella* were cultured. *H. influenzae* type *A* and *B* organisms grown on media containing SA or AA erythrocytes grew well (+++ or ++++), however, when grown on media with SS erythrocytes, growth was poor (+). No similar differential growth pattern was consistently found with the other microorganisms. The observations with the genus *Hemophilus* were confirmed with blood samples from several different patients and with freshly isolated cultures. Identical results were found when blood samples were defibrinated instead of being treated with anticoagulant. Because of this inhibition of growth on media containing SS erythrocytes, all further investigations were conducted with the *H. influenzae* type *B* organism.

The physical-chemical properties of representative samples of SS, SA and AA erythrocyte suspensions were determined. Resistance to hemolysis in hypotonic salt solutions was greatest for SS erythrocytes and least for AA erythrocytes. Microscopic examination of agar and broth culture media containing washed erythrocytes showed no greater hemolysis for SS than SA and AA erythrocytes. Resistance to alkali denaturation was greatest for SS erythrocytes (5-7%) and least for SA and AA erythrocytes (3.2-3.8%).

The loss of the ability of the SS, SA, and AA erythrocytes to support the growth of *Hemophilus* organisms under various conditions was studied with the surface plate technic. Samples of each type of washed erythrocyte suspension were permitted to stand both at room temperature and at 0°C for variable periods of time before being incorporated into culture medium. It was found that while SS erythrocytes were capable of supporting minimal (+) bacterial growth when immediately incorporated into media, this capacity disappeared completely if the erythrocytes were permitted to stand one hour or more before being added to the media. Similarly, SA and AA erythrocytes completely lost their ability to support growth if permitted to stand 6 hours or more. Hemolysis of any of the 3 types of erythrocytes by hypotonic salt solution, shaking, freezing, Meckle disintegration, or sonic oscillation immediately eliminated the bacterial growth supporting potential.

Methods of protecting and enhancing the ability of the SS, SA, and AA erythrocytes to support growth of *Hemophilus* organisms were demonstrated in several ways by the surface plate technic. Immediate chocolating of the washed erythrocyte suspension resulted in ++++ growth of the microorganisms for SS as well as SA and AA preparations. This was true even when several hours elapsed between the time of chocolating and incorporation of the material in the media. Peptic digestion of SS, SA, or AA erythrocytes as long as 5 days after the original preparation of the suspension resulted in ++++ growth of the microorganisms in each case. Furthermore, peptic digestion completely restored the growth supporting ac-

TABLE I. Stimulation of Growth of *H. influenzae* Type *B* by Treated Suspensions of Erythrocytes.

Conc. of erythrocyte suspension†	Erythrocyte suspensions* Immediate chocolating SS	SA	AA	Peptic digestion SS	SA	AA
1:250	++++	++++	++++	++++	++++	++++
1:500	++++	++++	++++	++++	++++	++++
1:1000	+++	+++	+++	+++	+++	+++
1:2000	++	++	++	++	++	++
1:4000	+	+	+	+	+	+
1:8000	0	0	0	0	0	0
1:16000	0	0	0	0	0	0

* Washed erythrocytes, growth stimulating activity of which had been inactivated by standing 6 hr. Bacterial growth 0 to ++++.

† Concentration in terms of original erythrocyte suspension after addition to 10 ml of broth —equivalent in chocolated erythrocytes or peptic digestate. Hemin (1 mg) added to each culture tube.

tivity of inactive (hemolyzed) SS, SA, or AA erythrocytes so that media prepared with any of these preparations resulted in ++++ bacterial growth. Table I reveals that restoration of activity with peptic digestion was equal to that activity found with immediate chocolating of the erythrocyte suspensions. Erythrocyte stroma prepared from hemolyzed SS, SA, and AA erythrocytes by washing 3 times with saline, filtering, and peptic digestion were similarly capable of supporting ++++ bacterial growth while the supernatant of preparations so treated was inactive. Addition of DPN 1 mg% with or without Hemin 10 mg% to fresh, old or hemolyzed suspensions of SS, SA or AA erythrocytes resulted in ++++ bacterial growth. Table II demonstrates the greater binding effect of SS erythrocytes when compared to SA and AA erythrocytes.

TABLE II. Inhibition of Growth of *H. influenzae* Type *B* through DPN Binding by Erythrocytes.

DPN added (μg)	Erythrocyte suspension* SS	SA	AA
0	0	0	0
10	0	+	+
20	0	++	++
40	0	+++	+++
80	+	++++	++++
100	++	++++	++++
200	+++	++++	++++
400	++++	++++	++++
800	++++	++++	++++

* Washed erythrocytes, growth promoting activity of which had been inactivated by standing 6 hr. Bacterial growth 0 to ++++.

DPN added 2 hr before suspension was introduced into broth.

Discussion. The results of the experiments demonstrate that DPN required for growth of *H. influenzae* type *B* is normally present in SS, SA, and AA erythrocytes in amounts sufficient to support heavy bacterial growth when added to suitable media. However, in the case of SS erythrocytes DPN is almost completely removed shortly after the sample is obtained from the patient and is totally unavailable after standing for one hour. DPN is initially available from SA and AA erythrocytes in quantities capable of stimulating heavy bacterial growth but becomes completely bound in 6 hours. Differnces in availability of DPN would appear to be associated with the greater binding capacity demonstrated to be present in SS erythrocytes.

The site of binding is the stroma of the erythrocyte. Immediate chocolating of any of the fresh preparations alters the erythrocytes so that binding is prevented. The bound DPN can be completely released by peptic digestion of the preparation. Only minimal quantities of bound DPN are released by chocolating and only from the SA and AA erythrocytes. Hemolysis greatly augments binding, apparently by exposing stromal sites of binding. Spontaneous hemolysis however does not appear to be responsible for the increased binding of DPN by SS erythrocytes as these cells are more resistant to hemolysis by hypotonic salt solu-

tions and they show no greater number of hemolyzed erythrocytes when observed directly in the culture medium. Interestingly, incorporation of SA and AA erythrocytes into culture media apparently inhibits binding of DPN sufficiently to permit release for bacterial growth.

Inactivation of DPN (V factor) in blood of animals has been discussed by Lwoff and Lwoff(4). These observations were extended to include several animal species by Krumwiede and Kuttner(5). Further information comparing various species was reported by Waterworth(6). These observations have lead to the hypothesis that DPN is enzymatically destroyed(6) and evidence for a DPN splitting enzyme has been reported by Denstedt(7) and others.

The information reported here concerning the effect of human SS, SA, and AA erythrocytes on growth of *H. influenzae* type *B* does not appear compatible with the hypothesis of a DPN destroying enzyme. Instead, the presence of a system of reversible erythrocyte stromal binding of DPN is supported by the fact that: 1. Hemolysis immediately removes DPN, 2. Chocolating prevents removal of DPN, and 3. Peptic digestion completely releases DPN. The differences between SS erythrocytes and SA or AA erythrocytes are due to the more rapid and extensive binding of DPN which occurs with the SS cells. The actual mechanism of the bond requires further investigation. Preliminary experiments suggest that the binding is not enhanced, retarded or reversed by the presence of human albumin or pH changes between 4.5 and 8.5.

Summary. Experiments are reported which demonstrate that DPN required for growth of *H. influenzae* type *B* is normally present in equal amounts in erythrocytes from patients with sickle cell anemia, sickle cell trait, and normal adults. This material disappears within one hour from sickle cell anemia erythrocyte preparations and in 6 hours from sickle cell trait and normal adult erythrocyte preparations. The difference in rate of disappearance is caused by the greater DPN binding capacity of sickle cell anemia erythrocytes. Hemolysis stimulates binding of DPN; chocolating prevents binding; and peptic digestion frees bound DPN. The site of the bond is the stroma of the erythrocyte.

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