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PERFORMANCE REPORT

Research efforts during the past four months of this project have been concentrated in two areas: 1) testing of tripolyphosphate as a possible agent useful in optimizing the oxygen binding affinities of the hemoglobin contained in liposome-encapsulated hemoglobin (LEH) and 2) the synthesis of LR16.

As described in previous reports, the LR16 agent that we initially proposed to use to optimize the oxygen binding properties of LEH is capable of diffusing out of liposomes. The end result of this diffusion process is a less than optimal P_{50} value and physiological perfomance for the preparation. Through discussions with Dr. Enrico Bucci, Department of Biological Chemistry, University of Maryland School of Medicine, a well recognized expert in the area of hemoglobin structure and function, the notion emerged that tripolyphosphate may be a useful hemoglobin effector molecule to employ in our studies. Like LR16, tripolyphosphate can bind hemoglobin and shift the oxygen dissociation curve to the right; however, tripolyphosphphosphate is a polyanion which is membrane-impermeable. Thus we became interested in the potential of tripolyphosphate anion in optimizing the oxygen affinity of LEH.

Our initial experiment was to compare the abilities of LR16 and tripolyphosphate to modulate the P_{50} value of hemoglobin in solution free of membrane. Figure 1, found on page 3, compares the oxygen affinities of hemolysate solution with: a sample containing no effector (control sample); a sample with 0.5 mM LR16 present; and a sample with 10 mM tripolyphosphate present. The P_{50} values (mm Hg) of these solutions were 14.5, 21.5, and 22.5 mm, respectively. It thus appears that much higher concentrations (some 20-fold higher) of tripolyphosphate are required to achieve the same effect as LR16.

Then we tried to make LEH with tripolyphosphate present at the 10 mM level using the lipid mixture that we have employed previously [dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC), cholesterol and dimyristoyl phosphatidylglycerol (DMPG). The ratio of lipids were 1:1:1:2.7:0.3 for DMPC:DPPC:DSPC:cholesterol:DMPG, respectively. Our initial attempts to make LEH in the presence of such a high concentration of tripolyphosphate have been unsuccessful. We are uncertain of the factors responsible for this finding; at present we are varying the lipid composition in order to possibly achieve membrane encapsulation of the material.

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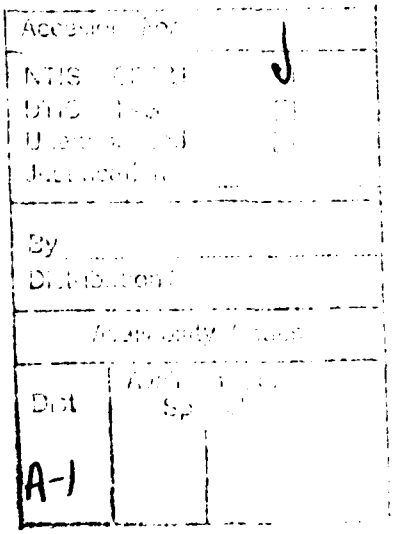
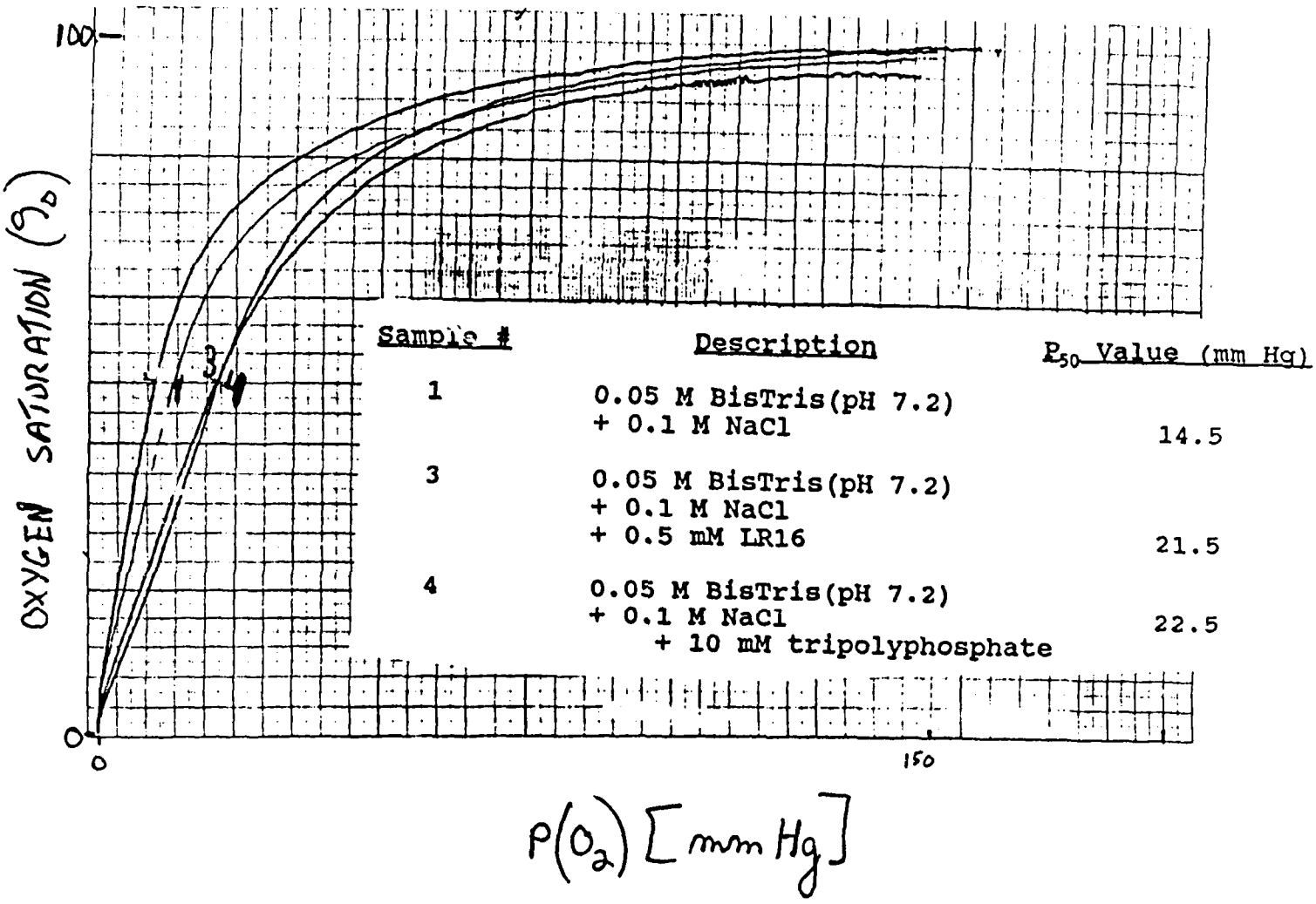
Thus it still appears that our best approach to optimizing the oxygen affinity of LEH remains the development of an active but membrane-impermeable LR16 analogue. To this end, we have been involved actively in the synthesis of LR16. Our overall strategy is to synthesize LR16, and then go on and pursue the development of less membrane-permeable analogues of LR16 that also exhibit biological activity. The synthetic work is being conducted at the University of Texas M.D. Anderson Medical Center, in the laboratory of Dr. Waldemar Priebe. Pages 4 and 5 of this report summarize information concerning the methodologies that have been recently employed in that laboratory to synthesize LR16.





Sample #	Description	P_{50} Value (mm Hg)
1	0.05 M BisTris(pH 7.2) + 0.1 M NaCl	14.5
3	0.05 M BisTris(pH 7.2) + 0.1 M NaCl + 0.5 mM LR16	21.5
4	0.05 M BisTris(pH 7.2) + 0.1 M NaCl + 10 mM tripolyphosphate	22.5

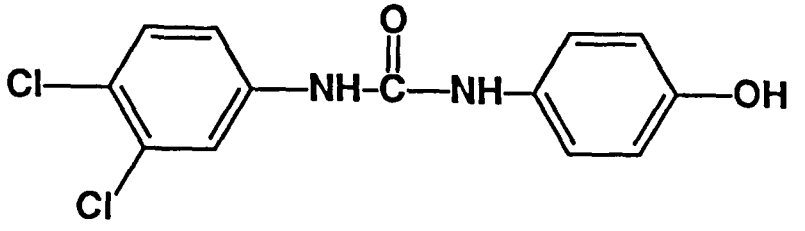
Figure 1. Oxygen dissociation curves comparing the abilities of LR16 and tripolyphosphate to modulate the oxygen affinity of hemoglobin at 20 °C.

Preparation of LR16

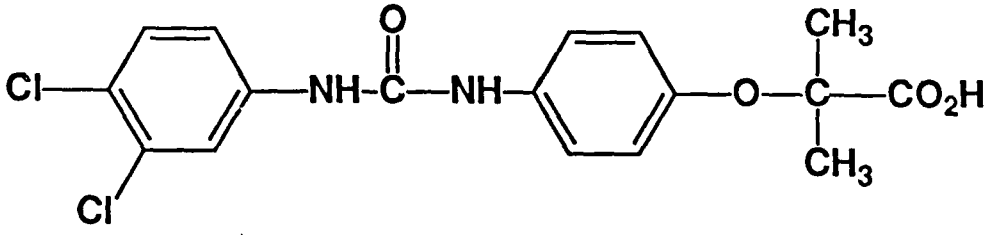
1-(3,4-Dichlorophenyl)-3-(4-hydroxyphenyl)urea. 3,4-Dichlorophenyl isocyanate (1.9 g, 10 mmol) and 4-aminophenol (1.1 g, 10 mmol) were stirred intensively in pyridine (10 mL) at room temperature during 0.5 h. Then cold water (80 mL) was added. The pyridine was neutralized with a small excess of 10% HCl. The precipitate was separated by filtration, dissolved in methanol (20 mL) and the insoluble white solid - 1-(3,4-dichlorophenyl)-3-{4-[[(3,4-dichlorophenyl)amino]carbonyl]oxo-phenyl}urea - was filtered off. The filtrate was concentrated and dried on oil pump to give 2.8 g (94 %) of the urea derivative: m.p. 206-208°C; ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6 - 10:1) 8.18 (s, 1 H, NH), 7.72 (s, 1 H, NH), 7.52 (d, J = 2.4 Hz, 1 H), 7.02 (d, J = 8.7 Hz, 1 H), 6.98-6.82 (m, 3 H), 6.50 (d, J = 8.7 Hz, 2 H).

2-[4-[[(3,4-Dichlorophenyl)amino]carbonyl]amino]phenoxy-2-methyl propionic acid, LR16. To a refluxing mixture of 1-(3,4-dichlorophenyl)-3-(4-hydroxyphenyl)urea (1.5 g, 5 mmol) and NaOH (1.0 g, 26 mmol) in acetone (12 mL) chloroform (2 mL, 25 mmol) was added dropwise and the reaction was continued for additional 4 h in reflux. Then, the solvents were evaporated and the residue was dissolved in water (30 mL), warmed with charcoal, filtered through celite and washed with water (30 mL). The filtrate was shaken with ethyl acetate (50 mL) and then acidified to pH ~2. The precipitate was isolated by filtration to give after drying 1.18 g (62%) of the product: m.p. 175-177°C; ^{1}H NMR (300 MHz, DMSO-d_6) 8.96 (s, 1 H, NH), 8.68 (s, 1 H, NH), 7.87 (d, J = 2.4 Hz, 1 H), 7.51 (d, J = 8.8 Hz, 1 H), 7.33 (d, J = 8.9 Hz, 2 H), 7.31 (dd, J = 8.8, 2.4 Hz, 1 H), 6.81 (d, J = 8.9 Hz, 2 H), 1.47 (s, 6 H, 2xCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) 175.1 (CO_2H) , 152.4 (CO), 150.4, 140.1, 133.6, 131.0, 130.5, 123.0, 119.9 (2xC), 119.8 (2xC), 119.2, 118.2 (**C**-Ar), 78.7 (**C**CO_2H), 25.1 (2xCH_3).

Attempts to recrystallized the LR16 from aqueous acetone and ethanol failed. Purity of this product will be compared with sample obtained from Dr. Burke. Product will be further purified, if required.



1-(3,4-Dichlorophenyl)-3-(4-hydroxyphenyl)urea



2-[4-[[(3,4-Dichlorophenyl)amino]carbonyl]amino]phenoxy-
2-methyl propionic acid, LR16