8 December 1992

Scientific Officer
Patrick Curran, CAPT, MC, USN
Naval Medical R&D Command
Director of Research and Development
Bethesda, MD 20814-50440

Ref: N00014-91-C-0044

Dear Captain Curran:

Enclosed is the Sixth Triannual Report for Contract No.: N00014-91-C-0044, which is entitled "Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite". This Report covers the period from September - November, 1992. If you have any questions about the Report or the research, please contact Kelvin G.M. Brockbank at 404-952-1660.

Sincerely yours,

Kelvin G.M. Brockbank, Ph.D.
Director, Research and Development
for
John F. Carpenter, Ph.D.
Senior Scientist

cc: Mrs. Mellars, DCMDS-GAACA, DCMAO Atlanta
Administrative Contracting Officer (1 Copy)
Director, Naval Research Laboratory (2 Copy)
Defense Technical Information Center (2 Copies)
Metabolic perturbation and enzyme structure-function studies. We have now almost completed our investigation of the structure-function relationships that are thought to be involved with regulating activity of phosphofructokinase (PFK) in vivo. Specifically, we set out to study how alterations in quaternary structure account for enzyme inhibition in red blood cells under cold, acidotic conditions. Prior in vitro studies have shown that phosphofructokinase from erythrocytes (and several other mammalian cells and tissues) dissociates from active tetrameres into inactive dimers and monomers at low Ph. In addition, we have previously demonstrated that this reversible phenomenon accounts for the inhibition of PFK activity in vivo in ischemic myocardium.

However, we have now clearly documented that this is not the case for human red blood cells. We incubated cells under conditions that we have shown previously to inhibit the enzyme's activity greatly in vivo and which, according to the prevailing dogma, should cause the enzyme to dissociate into inactive dimers/monomers. Surprisingly, we found that there is no disassembly of the enzyme, even when the cells are homogenized in Ph 6.5 buffer. With purified PFK, these buffer conditions induce rapid depolymerization.

Our hypothesis was that the presence of the high concentration of hemoglobin in erythrocytes and in the cell homogenates inhibits dissociation of PFK. There is a substantial body of biophysical evidence that large, bulky polymers (hemoglobin in this case) serve to stabilize the assembled forms of proteins because the polymers are preferentially excluded, due to steric hindrance, from the surface of the protein. Preferential exclusion of solute leads to an increase
in the chemical potential of the protein, an effect that is thermodynamically unfavorable. This effect is greater for dissociated proteins because the constituent dimers/monomers have a greater surface area than the fully assembled protein.

To test the role of bulky solutes in maintaining PFK tetramers in red blood cells, we purified PFK from human red cells. The purified enzyme does undergo pH-induced dissociation in vitro, and this process is reversible upon restoration of alkaline pH. However, in the presence of 40% (wt/vol) bovine serum albumin (which approximates the protein content in red blood cells), dissociation is partially inhibited. Thus, the presence of a high concentration of protein alone appears to not account totally for the lack dissociation in vivo and in the cell homogenates. The substrate for the enzyme, fructose-6-phosphate, also can reduce the degree of dissociation noted under acidotic conditions. When PFK activity is inhibited in red blood cells, this metabolite reaches high levels. We have found that 30 uM fructose-6-phosphate alone provides minimal stabilization to purified PFK in vitro at low pH. However, when 40% bovine serum albumin and 1 mM fructose-6-phosphate are used in combination, the purified enzyme does not dissociate, even after 1 hour at pH 6.5 and 0°C. Thus, these results demonstrate that the conditions found in vivo in acidotic red cells are sufficient to prevent dissociation of PFK. We have now located a commercial source of native hemoglobin and will soon repeat the above experiments using hemoglobin as the bulky solute.

That the effect of the proteins ultimately derives from steric hindrance is documented by our results using polyethylene glycols (PEG). 10% (wt/vol) PEG with a molecular weight of 8000 completely inhibits pH-induced dissociation of red cell PFK. In contrast, to achieve similar effects takes 25% (wt/vol) of PEG with a molecular weight of 1000. The larger PEG is a more effective stabilizer because it excluded from the domain of the protein to a greater degree.

Finally, we have tested a related hypothesis that the enzyme can be inhibited by low pH, even when in the fully assembled state. That is, we have found that
the Ph profile for PFK activity in the presence of the 10% PEG-8000 is essentially the same as that seen in the absence of the solute. In the presence of the bulky solute, inhibition is due solely to the acute protonation of key histidyl residues in the stable tetramer.

Role of metabolic perturbation in damage to vascular endothelial cells. We have started to apply the techniques that we have developed with red cells to study this sensitive cell type. In our initial experiments with bovine endothelial cells we have found that their metabolic heat dissipation rate is acutely inhibited at low pH, presumably due to inhibition of phosphofructokinase. Our first detailed experiments will test how this pH-induced metabolic inhibition affects cell survival under normoxic and hypoxic conditions. In the latter case, the sole source of metabolic energy comes from glycolysis. Hence, at low pH the cells should rapidly be depleted of metabolic energy.

In addition to measuring metabolic rate and energy status of the cells, we will determine intracellular calcium levels using fluorescent dyes. These experiments will determine the relative contributions to cell damage of disruption of calcium homeostasis and of perturbation of energy metabolism.

Membrane perturbation studies. The most interesting results in this area of effort have come from our comparison of two human lymphocyte cell lines that have remarkably different sensitivities to hypothermia. Previously we had found that with one line (MOLT-4) 80-90% cells were destroyed within 24 hours at 4°C. In contrast, with the other line (K562) more than 72 hours at 4°C was required to induce similar damage. This observation provided us with an opportunity to test the hypothesis that thermotropic membrane phase transitions are primary lesions during chilling injury to cells. Using Fourier transform infrared (FTIR) microspectroscopy, we found that the average, main transition temperature of the membrane phospholipids from liquid crystalline to gel state was more than 10°C higher for the MOLT-4 than for the K562 cells. Thus, for these two cell lines increased sensitivity to chilling damage correlates with a higher membrane phase transition temperature.
We have now found that the reduction in the number of viable cells in a sample during hypothermia correlates with an increase in the level of intracellular calcium. This could arise from leakage of calcium into the cells due to membrane defects induced by the phase transition. The high calcium levels could activate proteases and lipases, which would lead to cellular destruction. Alternatively, however, the calcium influx could be the effect of cell death, not the cause. To differentiate between these two possibilities, we will carefully compare the time courses of alterations of calcium homeostasis and loss of cell viability.

We also found that if vitamin E, a membrane fluidizer, is added to the culture medium, the resistance of the MOLT-4 cells to hypothermia could be greatly improved. The beneficial effects of vitamin E were not as apparent for the more chilling-resistant cell line, K562. Our hypothesis is that vitamin E increases the membrane fluidity of the MOLT-4 cells, thereby lowering the thermotropic phase transition temperature. We will test this proposal by measuring effect of vitamin E on the phase transition temperature with Fourier transform infrared spectroscopy. We will also test whether other membrane fluidizers increase resistance of MOLT-4 cells to chilling damage.

Effect of acidosis and ionic environment on sarcoplasmic reticulum. Our original experiments on calcium uptake and release were designed to be carried out on SR-enriched membrane vesicles. Calcium uptake must be supported by intravesicular oxalate in order to be large enough to be measured with FURA-2 as the calcium indicator. We have found that oxalate transport into the vesicles is sensitive to Pi, and probably to temperature as well. This dependency has a dramatic effect on calcium transport. Therefore, SR vesicles are not the best preparation in which to study the Pi and temperature dependence of calcium uptake. We have developed a saponin-skinned skeletal muscle preparation in which to carry out these experiments. Saponin skinning permeabilizes the sarcolemma but leaves the SR membrane intact. Calcium uptake and release can be measured from the amount of calcium remaining in the SR (measured by the contractile response to a high concentration of caffeine, which releases all calcium in the SR) without the need for bathing in an oxalate medium. We
will now use this model system to measure the effects of pH, Pi, ADP and temperature on calcium uptake and release from the SR. These experiments will provide information on how the stresses encountered during hypothermia and ischemia impact on the calcium sequestering activity of these organelles.

**Effect of hypothermia and acidosis of smooth muscle survival.** We will investigate vascular smooth muscle survival and function during and after prolonged exposure to cold, anoxia and acidosis using a rabbit saphenous vein model. These studies will assign a causal relationship between loss of smooth muscle contractility and the experimental conditions employed. The objective of these studies is to define the length of exposure (to a given set of conditions) that results in irreversible smooth muscle function and the time course of recovery of veins subjected to shorter periods of insult. These studies will set the stage for future metabolic and ion transport studies in identically treated vein segments with the objective of defining which lesion(s) lead to irreversible damage of smooth muscle and from which lesion(s) smooth muscle cells can recover.