saccharides are observed in reactions to injury, and neutral mucopolysaccharides predominate in stabilized situations as observed in the repaired state.

Viewed in this way, the HCP treatment of cholesterol-fed rabbits protected them from aortic intimal and medial injury and its consequences to a significant degree, although under the experimental conditions of massive and prolonged cholesterol feeding it did not completely prevent cholesterol damage.

Although the biological mechanisms of this protective effect evidently involved are not yet clear, our studies, when considered with results from biochemical and toxicological studies (*6*, *7*), indicate the participation of a molecular subunit of hepatocatalase in the homeostatic control of lipid matabolism and suggest the potential pharmacological value of the isolated peroxidase subunit in the control of atherosclerosis.

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## Prevention of Protein Denaturation during Exposure to Sterilization Temperatures

Abstract. *Firefly luciferase exposed to a temperature of 135°C for 36 hours retained up to 40 percent of its original activity. Prerequisites for heat stability were the use of a molecular sieve (Sephadex G-25 or Biogel P-300) and a high vacuum ($5 \times 10^{-4}$ mm-Hg). These studies present a possible solution to the problem of sterilization for exobiological experiments.*

The necessity of sterilizing components of planetary experiments by dry heat (135°C) presents a problem of some magnitude to experimenters concerned with systems, in which enzymes are involved, for the detection of life. The problem derives from the phenomenon of protein denaturation at elevated temperatures. Preliminary experiments which indicate a possible solution to this problem are described here.

Although denaturation of protein is still a phenomenon that has not yet been rigorously elucidated, there has emerged over the years a general concept. This denaturation involves changes in the secondary and tertiary configuration which are manifested by changes in solubility, chemical accessibility of certain functional groups, and in activity, if the protein is an enzyme. There are indications that denaturation proceeds in two stages, the first being an unfolding or swelling of peptide chains and the second consisting of a rupture of intramolecular bonds, that is, hydrogen and sulfur bonds, followed by a random re-formation of bonds both intra- and intermolecularly (*1*). This concept has led us to the hypothesis that the presence of a physical barrier between individual protein molecules may prevent random re-formation of secondary and tertiary bonding; thus, an isolated protein molecule, when exposed to stress that does not rupture primary structure (peptide linkage), would return to its original state after removal of stress. The probability of a return to the original molecular configuration is supported by the observation of Epstein *et al.* (*2*) that the most stable energy state of a protein is that corresponding to the original configuration. The validity of our hypothesis is possibly indicated by the experiments described below.

The model enzyme selected for these studies was firefly luciferase, which has a molecular weight of 100,000. Activity of this enzyme may be measured by the intensity of light emitted as the result of the addition of luciferin, adenosine triphosphate (ATP), and $MgSO_4$ (*3*).

The materials selected for their ability to provide intermolecular barriers were Sephadex G-25 and Biogel P-300. These compounds are molecular sieves that have the property of segregating individual molecules in their matrix structure. This matrix contains pores of controlled dimensions. Sephadex G-25 (Pharmacia Fine Chemicals, Inc.) is a cross-linked dextran gel with a pore size that corresponds to a protein with a molecular weight of approximately 5000; Biogel P-300 (Bio-Rad Laboratories), a polyacrylamide gel with pore size corresponding to a protein with a molecular weight of approximately 400,000. Both of these gels are chemically inert.

The experimental procedure was as follows: 2 ml of luciferase solution [containing partially purified luciferase, 1 mg of protein per milliliter; luciferin, 0.5 mg/ml; $MgSO_4$, 0.01*M*; and tris buffer (*p*H 7.4), 0.05*M*] was added to separate tubes of Sephadex G-25, Biogel P-300, dextran, bovine serum albumin, and diethylaminoethyl (DEAE) cellulose. These five mixtures were quick-frozen in liquid nitrogen and lyophilized for 24 hours. Then, in replicates of three, the following treatments were imposed: (i) storage at −80°C for 36 hours, (ii) exposure in air at 135°C for 36 hours, and (iii) exposure to 135°C for 36 hours with an initial chamber pressure of $5 \times 10^{-4}$ mm-Hg which rose to $4 \times 10^{-3}$ mm-Hg during heating. After these treatments samples were prepared for assay by the addition of 5 ml of distilled water. Activi-

Table 1. Retention of activity by firefly luciferase after heating.

| Compound added | Retention of activity (%) | | |
|---|---|---|---|
| | Unheated | Heat plus air | Heat with vacuum |
| Sephadex G-25 | 100 | 0 | 10 |
| Biogel P-300 | 100 | 0 | 40 |
| Dextran | 100 | 0 | 0 |
| Serum albumin | 100 | 0 | 0 |
| DEAE | 100 | 0 | 0 |

ties of the samples were then compared on the basis of the light emitted when 10 $\mu$g of ATP in solution (0.1 ml) were added to 0.3 ml of the sample suspension. Results are given in Table 1.

While the preliminary nature of these observations should be stressed, the protective action of the molecular sieve material, in conjunction with a high vacuum, is readily apparent. It is assumed that the necessity for a high vacuum indicates a requirement for the complete removal of oxygen and water. The higher recovery of activity from the Biogel P-300 relative to Sephadex G-25 can be attributed to the ability of the luciferase molecules with a molecular weight of 100,000 to enter more readily into its larger pores. Total loss of activity after the addition of dextran, serum albumin, and DEAE indicates that the protective effect of the molecular sieve is not due merely to a macromolecule or an electrostatic binding effect. A complete validation of our hypothesis must await further definitive studies, which are now in progress.

An important ramification of these studies is their application to the particular requirements for sterilization of components for planetary experiments (*4*). These preliminary observations may represent a significant step toward sterilization for a biological experiment to be conducted on the surface of Mars.

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## Oxidative Phosphorylation in Experimental Bilirubin Encephalopathy

Abstract. *Mitochondria from the whole brain or cerebellum of newborn guinea pigs with experimental bilirubin encephalopathy failed to exhibit uncoupling of oxidative phosphorylation. The pigment concentrations required to initiate uncoupling in vitro are much higher than those found in the brain of neurotoxic animals.*

Experimental work has shown that unconjugated bilirubin interferes with vital cellular functions (*1*), and this toxicity probably is responsible for the encephalopathy encountered in severe unconjugated hyperbilirubinemia of the newborn. Studies of normal liver and brain mitochrondria to which bilirubin was added in vitro suggested that the toxicity of the pigment may result from uncoupling of oxidative phosphorylation (*2*). However, the pigment concentrations required to elicit this phenomenon in vitro are much higher than those found in the central nervous system of animals with experimental bilirubin encephalopathy (*3*). This observation suggests that in the intact brain the neurotoxic effects of the pigment may not be related to the mitochrondrial damage observed under conditions in vitro. To examine this possibility we studied brain mitochondria from newborn guinea pigs after infusion of unbound bilirubin at an hourly rate of 150 $\mu$g per gram of body weight for 60 minutes. Under these conditions the animals develop manifestations of bilirubin encephalopathy similar to those in infants with severe hyperbilirubinemia, and the extent of the neurologic injury is related directly to the concentration of pigment in the brain (*3*). The animals were killed 30 minutes or 5 to 16 hours after completion of the infusions, and mitochondria were prepared from the whole brain or from the separated cerebellum (*4*). Untreated animals of the same age served as controls. To the mitochrondrial pellet was added 0.5 ml of suspending solution per gram of brain. The functional stability of the suspensions during and beyond the time of subsequent incubation was ascertained by the absence of mitochondrial swelling in aliquots added to 0.25*M* sucrose in 0.02*M* tris-HCl, *p*H 7.4 (*4*).

Aliquots of the mitochondrial suspensions (0.1 ml) containing approximately 0.4 mg of protein (*5*) were incubated at 32°C in 1 ml of a solution, *p*H 7.4, containing 10 m*M* substrate (either succinate, glutamate, glutamate and 0.1 m*M* 2,4-dinitrophenol, or pyruvate and 1 m*M* L-malate) and 25 m*M* glucose, 25 m*M* sucrose, 0.025 mg of yeast hexokinase (Sigma Type III, practical grade), 5 m*M* adenosine diphosphate, 5 m*M* of $MgCl_2$, 1.25m*M* phosphate, and 20 m*M* tris-HCl. In all experiments oxygen consumption was followed polarographically (*6*) and utilization of inorganic phosphate was determined spectrophotometrically (*7*). Measurements usually were made for 3 to 5 consecutive minutes or until at least 30 percent of the oxygen was utilized (*8*).

The cerebellum is one of the areas of the central nervous system most vulnerable to bilirubin (*9*) and in experimental bilirubin encephalopathy may contain the highest concentration of pigment (*3*). Therefore, mitochondria obtained from the cerebellum would be most likely to exhibit pigment toxicity. We found, however, that on incubation with glutamate, cerebellar mitochondria from neurotoxic animals showed a P/O ratio similar to that of preparations from control animals (Fig. 1). Similar P/O ratios were obtained whether the mitochondria were prepared 30 minutes after completion of the bilirubin infusion or 5 to 16 hours later, at a time when the guinea pigs showed advanced neurotoxicity or were moribund (Fig. 1). Furthermore, mitochondria prepared from the whole brain of neurotoxic animals also showed normal P/O ratios with various substrates as shown by the representative results in Table 1. By electron microscopy (*10*) no structural differences could be detected between brain mitochondia from treated

Table 1. Oxidative phosphorylation in mitochondria from the whole brain of a newborn guinea pig 30 minutes after completion of bilirubin infusion. Control: untreated animal.

| Substrate | $\Delta$ $\mu$mole $P_i$ liter$^{-1}$ sec$^{-1}$ | | $\Delta$ $\mu$atom $O_2$ liter$^{-1}$ sec$^{-1}$ | | P/O ratio | |
|---|---|---|---|---|---|---|
| | Bilirubin | Control | Bilirubin | Control | Bilirubin | Control |
| Succinate | 2.19 | 1.89 | 0.87 | 0.75 | 2.52 | 2.52 |
| Glutamate | 1.52 | 1.66 | .75 | .69 | 2.11 | 2.41 |
| Glutamate and 2,4-dinitrophenol | 0 | 0 | .92 | .75 | 0 | 0 |
| Pyruvate and malate | 2.11 | 1.42 | .79 | .67 | 2.67 | 2.12 |