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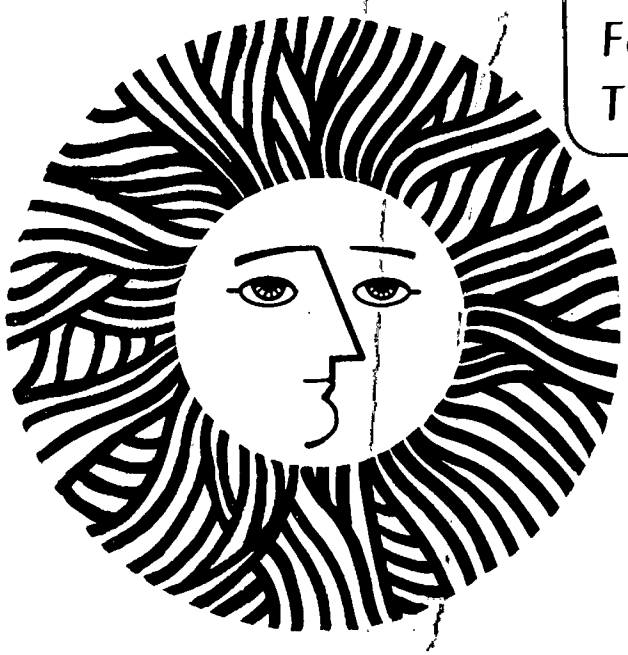

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DISCLAIMER

DEVELOPMENT OF A METHODOLOGY TO ASSESS ORGANOMETALLIC EFFECTS ON BIOENERGETIC SYSTEMS *

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We are developing a methodology for assessing the impact of subacute concentrations of organometallic agents on bioenergetic and oxidative damage processes in animals, cells and energy transducing subcellular organelles. Several of the assays are non-invasive and thus lend themselves to human tests.

At the whole-animal level we utilize a treadmill chamber where physiological parameters of exercising animals are monitored. These include parameters of whole animals' work performance such as oxygen consumption, carbon dioxide evolution and endurance. Oxidative damage can be monitored in experiments by analyzing expired air of the animals for ethane and n-pentane. These alkanes correlate with lipid peroxidation in vivo.

At the cellular and subcellular levels, respiratory activity, lipid peroxidation and free radical species are assayed. Respiratory activity is measured in muscle homogenates and isolated mitochondria using substrates which feed into different segments of the electron transport chain.

To demonstrate how these assay procedures correlate, iron deficiency anemia in rats was analyzed. Physiologically, iron deficiency caused a 90% decrease in endurance which correlated with an 80% decrease in pyruvate-malate oxidation rates in muscle homogenates. Significant but smaller effects were seen in hemoglobin/hematocrit levels (50% decrease) and in maximal oxygen consumption (50% decrease). Tissue free-radical signals observed by ESR at room temperature increased with exercise.

There is increasing evidence that some organometallics exert their deleterious effects by interfering with mitochondrial respiration and by promoting oxidative reactions. Since the respiratory organelles have been shown to be a major source of endogenous free-radicals which initiate tissue oxidation it is plausible that our exercise system will aggravate the effects of organometallics which act as pro-oxidants. Thus, the proposed animal exercise system may be particularly appropriate for evaluating the potential impact of these substances on humans.

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1. BACKGROUND

Much is known about acute toxicity mechanisms of hazardous substances in the environment. Our knowledge of the biological effects of chronic exposure is relatively sparse, yet, as our understanding of human aging, carcinogenesis and other disease processes evolves we are becoming increasingly aware that some hazardous substances exert deleterious effects that may not express themselves for years or even decades. Hence we are discovering a need for short term biological assays that will warn us of such latent harmful agents in the environment. Microbial assays, particularly the Ames test, continue to serve a useful function in screening potential carcinogens but it is now clear that a significant number of potent carcinogens elude detection with the Ames system. We are presently developing an assay system which will evaluate substances which may not exert their effect directly on DNA, but, rather, may react with other biological target molecules, possibly potentiating carcinogenic agents. A particularly interesting group of substances in this category is chemicals which initiate or promote oxidative free-radical reactions.

There is a strong correlation between oxygen tension and the lifespan potential of human cells in culture [1]. In mammals a linear correlation between inverse metabolic activity and lifespan has been demonstrated provided that tissue levels of the antioxigenic enzyme superoxide dismutase are taken into account [2]. These and other data illustrate the hazard posed to aerobic life by oxygen and respiratory activity. A model of oxidative damage has been evolved. This model focuses upon free radicals as well as the excited singlet species as being responsible for the destructive effects of oxygen. Endogenous free radicals, arising during normal respiratory activity are viewed as the initiators of free-radical chain reactions in which a variety of biomolecules are oxidized. Antioxidants like vitamin E protect against such damage by terminating free-radical chain reactions. Other protective mechanisms in cells include three enzymes: catalase, which decomposes hydrogen peroxide and thus eliminates a potential source of hydroxyl radicals, superoxide dismutase, which decomposes superoxide radicals, and glutathione peroxidase which decomposes both organic hydroperoxides and hydrogen peroxide. Within the framework of this model of oxidative damage, chronic effects of hazardous substances would be manifested in terms of interfering with cellular protective machinery or by acting as initiators of oxidative reactions.

1.1 MECHANISMS OF METAL TOXICITY

A brief survey of toxicity mechanisms of metals and metalloids reveals a prevalence of mitochondrial and oxidative reactions. For example, mercury is a potent sulfhydryl reagent. Methyl mercury has been shown to inhibit coupled mitochondrial respiration (3) and to cause the appearance of characteristic porphyrin precursors in the urine of treated animals (4). Selenium also reacts with sulfhydryl groups, possibly acting as a crosslinking reagent in the formation of selenotrisulfides (5). Selenium also has the potential to substitute for sulfur within essential amino acids (6), thus causing possible errors in protein structure. Arsenicals exert destructive effects in at least two ways: inorganic pentavalent arsenates compete with phosphate in a number of biosynthetic reactions (7) including oxidative phosphorylation. Trivalent arsenic is a sulfhydryl reagent, having been shown to combine particularly avidly with lipoic acid (8) of the pyruvate dehydrogenase enzyme complex. Thus, arsenicals interfere with respiratory energy coupling mechanisms. Trialkyl-tin compounds act as lipid-soluble ion-pair formers with anions (9,10) and thus exert their toxic action in cells by exchanging chloride ions for hydroxyl ions. In mitochondria this causes pH gradients to collapse and thus interferes with energy coupling mechanisms. These examples of metal toxicity illustrate the prevalence of reactions that interfere with mitochondrial metabolism and thus strengthens our assertion that aerobic metabolic functions are an excellent assay system for potential destructive effects of hazardous metals and metalloids in the environment.

An example of an environmental agent of concern, particularly with the probable forthcoming exploitation of oil-shale resources, is arsenic. Epidemiological studies strongly implicate arsenic as a carcinogenic agent yet no definitive studies have demonstrated that arsenic compounds can cause cancer in laboratory animals, despite extensive experimentation (11). Thus it may well be that arsenic acts as an agent that potentiates the action of other carcinogens, perhaps by promoting oxidative reactions.

The assay system we are developing will examine the antioxigenic potential of a biological system. This antioxigenic potential will be stressed by means of the relatively harmless protocol of forced exercise. In this preliminary report we describe one example of how our assay system is used - that of iron deficiency anemia.

2. PROCEDURES, RESULTS AND DISCUSSION

2.1 WHOLE ANIMAL STUDIES

Physiological parameters are measured in environmentally controlled exercise chambers for rodents. The animals are forced by means of electric prods at the rear of the chamber to run on a treadmill which moves at a predetermined rate. The chamber currently in use, (access generously provided by Professor Brooks of the Exercise Physiology Laboratory with whom we are collaborating) is equipped for measuring oxygen consumption, carbon dioxide evolution and electrocardiograms. It is planned to construct modified chambers which will provide opportunities for collecting substance in the air expired by the animals using a cold-finger trap. Of particular interest are pentane, which is a breakdown product of peroxidized polyunsaturated lipids and volatile metabolites of metalloid substances ingested by the animals. Pentane evolution from mammalian lungs (but not necessarily originating in the lung) has been shown to be correlated with the vitamin E status of animals and to increase with exercise. A physiological parameter which has proven useful as an indicator of whole-animal integrity is maximum endurance, i.e. the time required for an animal to become totally exhausted as a result of running on a treadmill at submaximum speed. Another useful parameter is response to exercise training. As shown in the table, endurance can be increased about five-fold by training in unstressed rats.

Table: Effect of Endurance Training

Group	Mitochondrial Content of Muscle mg/g	Muscle Cytochrome oxidase moles/min/g	VO_2 max mg $kg^{-1} \cdot min^{-1}$	Maximal Endurance min.
Control	18.2 ± 0.7	37.4 ± 3.1	76.6 ± 1.2	36.3 ± 2.2
Endurance Trained	36.2 ± 1.6*	75.9 ± 5.4*	87.7 ± 2.0*	182.6 ± 10.4*

*$P<0.01$ controls vs. endurance trained (t-test), n = 10.

Summary -- 100% increase in mitochondrial content of muscle, 100% increase in muscle oxidative capacity, 14% increase in VO_2 max, 403% increase in endurance. Thus endurance is highly correlated with mitochondrial content of muscle, and muscle oxidative capacity, but only moderately correlated with VO_2 max. Endurance training provides a useful and physiologic tool for the study of muscle energetics and whole-animal respiration.

2.2 TISSUE, CELLULAR AND SUBCELLULAR STUDIES

Respiratory activity of tissue, particularly muscle homogenates, is performed in a Clark-type electrode using succinate and pyruvate-malate as substrates. A full battery of bioenergetic parameters, including respiratory control and VO_2 max, is measured. Similar assays are conducted with mitochondria isolated from these tissues. Mitochondrial yields are related to muscle weight. Lysosomal membrane integrity is evaluated with latency assays of intralysosomal enzymes. Lipid peroxidation is measured by means of the thiobarbituric acid procedure (12). Endogenous free-radicals are monitored both in tissue and homogenates with room temperature EPR techniques. The status of protective enzymes and antioxidants in isolated mitochondria is evaluated by detecting free radicals with nitroxide and nitrone spin traps under conditions where free-radical reactions are induced with exogenous initiators.

2.3 EFFECT OF IRON DEFICIENCY ON ENDURANCE CAPACITY AND RESPIRATORY ACTIVITY

Iron deficiency anemia in rats was used as a model system to study the correlation between physiological dysfunction and subcellular bioenergetic parameters (13). It was of interest to determine whether gross, noninvasive exercise parameters might yield evidence of tissue damage of comparable sensitivity to measurements of respiratory enzyme activities.

Rats were made anemic by means of dietary iron deficiency over a period of three weeks. The blood hematocrits of the deficient rats were about 20% compared to those of normal rats with 40% hematocrits. The most striking effect of iron depletion was a 90% reduction in the endurance capacity, i.e. the time period that endurance trained rats ran until total exhaustion. A comparable but less dramatic decline was seen in the pyruvate-malate oxidation rates, where an 80% reduction was seen for muscle homogenates and a 70% decline was noted for isolated muscle mitochondria. Whole animal maximal oxygen consumption, VO_2 max, diminished by 50%. When hematocrits of the deficient rats were increased by means of blood transfusion, VO_2 max values reached nearly normal levels whereas no significant changes occurred in endurance capacities. The latter result demonstrated that oxygen utilization at the level of the respiratory chain rather than oxygen delivery via hemoglobin was limiting in endurance capacity.

Effects of exercise and iron deficiency are summarized in the figure.

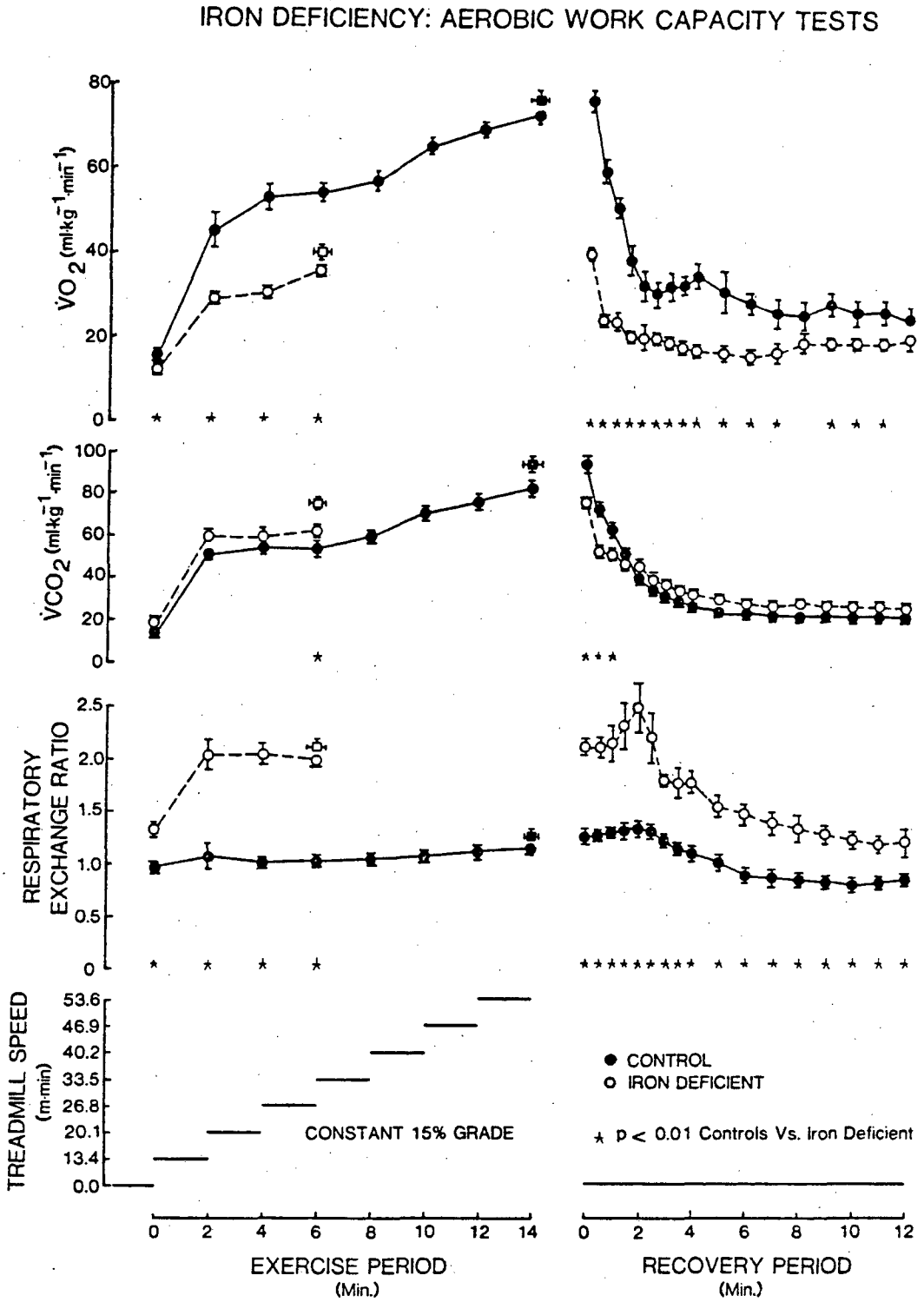

FIGURE. Demonstration that exercise enhances the effects of iron depletion upon VO_2 and the respiratory exchange ratio. A substantial reversal of these enhancements occurs within a few minutes of subsequent rest (]4).

2.4 RELATIONSHIP BETWEEN EXERCISE, VITAMIN E STATUS, FREE-RADICAL SIGNALS AND DAMAGE

Vitamin E is a lipid soluble antioxidant which is found in biological membranes. Dietary vitamin E manipulations have provided an important tool for demonstrating suspected free-radical reactions involving membranes. Usually the most effective procedure for testing in-vivo free-radical involvement is to compare vitamin E-deficient animals with supplemented ones, since normal diets contain adequate concentrations of this vitamin. In the study described below, such animals were used to examine the correlation between vitamin E status, exercise and damaging free radical-reactions.

Two groups of rats, a control group and another group which had been fed a vitamin E-deficient diet for six months were forced to run in the exercise chambers to exhaustion (15). The control group had about twice the endurance of the deficient rats. Both liver and muscle homogenates exhibited larger free-radical ESR signals in the exercised rats than in non-exercised animals, regardless of vitamin E status. Results in non-homogenized tissues were similar and free-radical signals varied in intensity over a range of about three among the different preparations. Lipid peroxidation, respiratory activity and lysosomal latency all revealed a correlation between increased free-radical signals and tissue damage. Concerning the effect of vitamin E deficiency, the most marked effects were increased lipid peroxidation and a greater fragility of lysosomal membranes as reflected in decreased latency of alkaline phosphatase activity in the deficient rats.

These results show that the stress of forced exercise exacerbates free-radical mediated tissue oxidative processes and lends credence to our proposal that this regimen will enhance the biological effects of organometallics.

2.5 SUSCEPTIBILITY OF TISSUE TO FREE RADICAL-ATTACK

Our recent ESR data suggests that increased metabolic activity during exercise gives rise to increased generation of endogenous free-radicals. Thus a number of oxidative reactions are likely to be promoted during exercise, as has been shown for evolution of pentane during lipid peroxidation in the lung. Therefore pentane production is expected to increase as a function of exercise duration. During the

course of these oxidative reactions the antioxidant defenses of tissues will be progressively depleted and hence their susceptibility to further oxidative ractions will be increased. In view of these considerations cellular studies of free-radical propagation may also provide sensitive assays of organometallic damage.

To study tissue susceptibility to free-radical attack isolated mitochondrial preparations will be treated with free-radical initiators. Subsequent propagation reactions will be assayed with nitrone and nitroxide free radical traps (16) and by means of the TBA assay for lipid peroxidation. The extent of free-radical propagation caused by these initiators will provide a measure of the antioxidant status of the tissue.

2.6 RELATIONSHIP OF IRON DEFICIENCY STUDY TO ORGANOMETALLIC TOXICITY ASSAYS

Organoarsenicals and perhaps other organometallics react chemically with sulfhydryl compounds in the mitochondrial enzyme pyruvate dehydrogenase. The iron deficiency study revealed a good correlation between loss of pyruvate-malate oxidation rates and maximum endurance, suggesting that endurance capacity might be similarly impaired when animals are exposed to certain organometallics. This suggestion is being tested in the exercise system. The coupling of exercise to the measurement of alkanes and organometalloids in breath expired by animals and humans offers a new biological assay system which may provide sensitive information about biological damage occurring during exposure to subacute doses of hazardous environmental agents. Further detailed information which is required for an understandin of the biological transformations of metalloids and hence their toxicity mechanism is the chemical speciation of these expired substances by means of state-of-the-art chromatography with element-specific detectors.

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