Some studies were made of the influence of dietary molybdenum on intestinal alkaline phosphatase activity. It was observed that after one week on the experimental regimen 5 rats receiving 1200 p.p.m. molybdenum had significantly less ($P<0.01$) intestinal phosphatase activity than 5 controls. Control and toxic values were $16.58 \pm 1.64$ and $4.46 \pm 0.73$ $\mu$M phosphate released/30'/mg protein respectively. Because of this rapid and pronounced effect, the influence of sodium molybdate on the intestinal phosphatase assay system was tried. Molybdate concentrations up to $10^{-4}$ M had no influence on a semi-purified preparation of intestinal phosphatase. At $10^{-3}$ M molybdate, a 20% inhibition of activity was observed. This is in agreement with experiments on liver preparations(10) and suggests that the decrease in activity observed in toxicity is probably a reflection of altered synthesis of the enzyme rather than a direct influence of the mineral on the enzyme assay system.

*Summary.* 1. High levels of dietary molybdenum did not alter ability of rat to acetylate p-aminobenzoic acid or to conjugate benzoic acid with glycine. 2. A depression of food intake and growth was evident within 24 hours after addition of 0.12% molybdenum to the diet. 3. Liver alkaline phosphatase activity was significantly increased in molybdenum-toxic rats whereas the activities of the kidney and intestinal alkaline phosphatases were significantly reduced.

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## Structure and Function of Mitochondria in Irreversible Hemorrhagic Shock, I.*† (22359)

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Irreversible hemorrhagic shock is seen in humans and experimental animals who have suffered a prolonged period of hemorrhagic hypotension and are refractory to blood volume replacement therapy. Battlefield experience with massive blood transfusions in World War II and the Korean conflict(1) strengthened the belief of some workers that an irreversible intracellular metabolic change was taking place. Several studies involving tissue analyses have appeared during the last decade and have been ably summarized by Engel(2). In general, high levels of glucose lactate, pyruvate, amino acids and inorganic

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phosphate were found in the blood while the tissue stores of glycogen, ATP‡, and coenzymes appeared depleted. Edwards *et al.*(3) recently repeated some of the key measurements in dogs. Early enzymatic studies were conducted by Russell, Long and Wilhelmi(4, 5) Burdette(6) and Hannon and Cook(7) using liver and heart slices of rats subjected to hemorrhagic shock: in some of these experiments lowered oxygen uptakes were observed. Thus, both the analytical and the enzymatic data suggested failure at the mitochondrial level. Harman(8-11) described a series of degenerative changes occurring in mitochondria when isolated by differential centrifugation and exposed to various environmental conditions. These alterations in structure (swelling and "crescent" formation) were accompanied by functional changes *e.g.* low P/O ratios and variations in oxygen uptakes.

The two lines of thought, *i.e.* the evidence suggesting mitochondrial involvement in the phenomenon of irreversibility in clinical hemorrhagic shock and the interesting correlation between mitochondrial structure and function observed in *in vitro* experiments raised the following questions: Are mitochondria morphologically and functionally altered in irreversible hemorrhagic shock and do such *in vivo* changes resemble the *in vitro* changes described?

*Methods. Production of shock.*§ Healthy mongrel dogs maintained on a standard *ad libitum* diet of dried dog biscuits were paired as nearly as possible as to sex, age, breed and weight and anaesthetized with 30 mg/kg pentobarbital sodium. One animal served as a control while the other was subjected to hemorrhagic shock by a modification of the Wigger's technic(12). Blood was withdrawn rapidly so that an arterial pressure of 40 mm Hg was produced in approximately 5 minutes. Subsequently blood was withdrawn and reinfused as necessary to maintain the initial hypotensive level.‖ The animals were sacrificed after 3 to 5 hours by removing the left ventricular musculature surgically. The members of the pair died within 15 minutes of each other. *Formalin-fixed preparations.* 5 g of heart muscle cut into thin slices were transferred to a semi-micro Monel cup containing 1 g sodium acetate in 50 ml of cold 10% formaldehyde and were ground on a Waring blendor base at full speed for 45 seconds. Liver and kidney samples were prepared in the same manner but blendorized at half speed for 15 seconds. The homogenates were strained through cheese cloth and examined under oil immersion in a Spencer phase contrast microscope using Dark-M contrast.

*Isolation of mitochondria.* 15 g heart were minced and ground for 90 seconds in 75 ml semi-frozen 0.38 M sucrose containing 100 $\mu$M $K_2HPO_4$ using a semi-micro Monel cup in a standard Waring blendor base. Another 75 ml of the sucrose solution were stirred in and the suspension strained through cheese cloth. The preparations from the control and the shocked dogs were thus made within 15 minutes of each other and centrifuged simultaneously in a refrigerated international centrifuge. The mitochondria were sedimented between the speeds of 450 and 1300 x g, suspended in 0.5 M sucrose, dispersed by means of a glass homogenizer and the densities of the suspensions adjusted nephelometrically. The total nitrogen concentration was estimated by Johnson's method(13). The mitochondria were thus isolated in hypertonic sucrose because of Harman's findings(10,11) that under such conditions degenerative changes ("crescent" formation) were negligible, an observation which we have been able to confirm. *Enzymatic activities* were estimated by the conventional Warburg method at limiting levels of mitochondria under the conditions outlined in Fig. 4. In order to determine P/O ratios the incubation mixtures were fortified with 50 $\mu$M fructose and 2 mg

‡ The following abbreviations will be used: AMP = adenosine – 5′ – phosphate; ATP = adenosinetriphosphate; P/O ratios = micromoles of inorganic phosphate esterified per microatom of oxygen consumed.

§ The experiments described were carried out between July and Nov. 1955.

‖ Baxter blood bottle with ACD solution 1.38% Na-citrate, 1.58% dextrose and 0.5% citric acid were used to collect the blood.

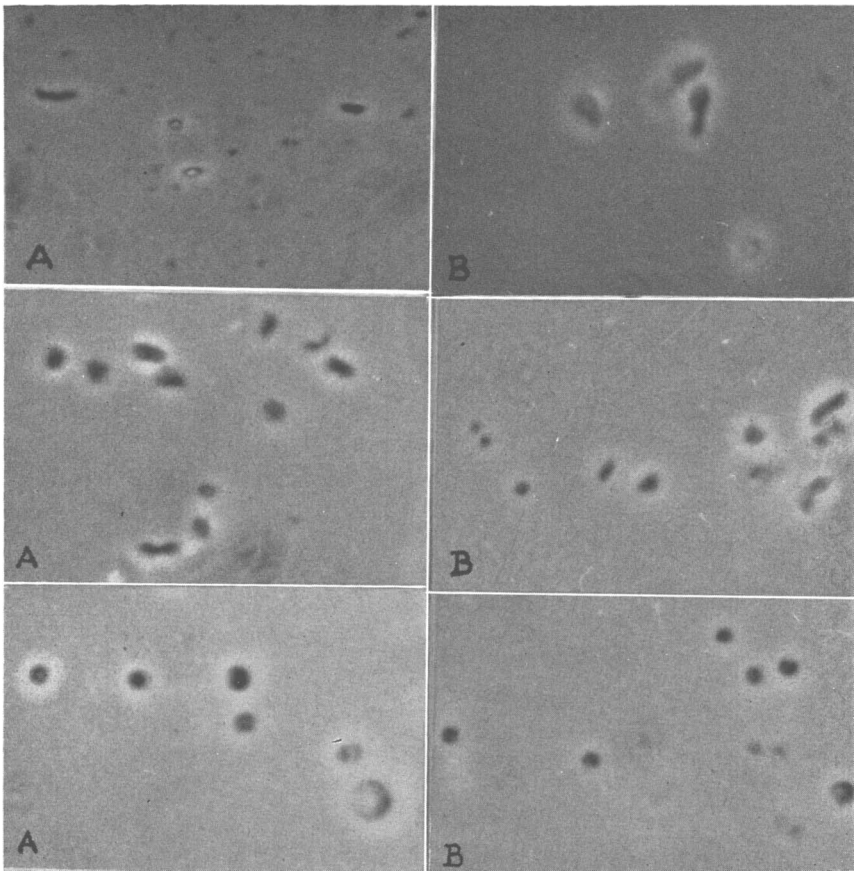


FIG. 1 (top). Heart mitochondria from normal and shocked dogs prepared in formaldehyde and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

FIG. 2 (middle). Heart mitochondria from normal and shocked dogs isolated by differential centrifugation, suspended in 0.5 M sucrose and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

FIG. 3 (bottom). Heart mitochondria from normal and shocked dogs aged in 0.5 M sucrose and examined under phase-contrast. a, normal; b, shock. Magnification app. 2300 ×.

yeast hexokinase (Sigma, type II) which were poured in from the side arm shortly before the taps were closed. Inorganic phosphate levels were determined by a modification of the Fiske-Subbarow method. In a few experiments fluoride was added as a phosphatase inhibitor but the results were not affected materially by its presence. In general the experimental mixtures were kept as simple as possible in an effort to maintain the mitochondria in as nearly their native state as feasible.

*Results. Morphology of formalin-fixed preparations* may be observed in Fig. 1. The grinding breaks up the long chains of mitochondria which accompany the strands of myofibrils in the undisturbed tissue. The free floating normal particles tend to be square to spindle-shaped rods with sharp boundaries and dense to moderately translucent centers. At times they may be slightly knobby. The particles encountered in preparations from shocked dog hearts are swollen and irregular in shape with indistinct boundaries and often have translucent centers. Blendorates from livers and kidneys also showed abnormal mitochondria. The swollen spheres and "crescents" which are associated with degenerative changes in Harman's *in vitro* experiments have not been observed in formalin-fixed preparations.

*Structure of mitochondria isolated in su-*

TABLE I. Oxygen and Phosphate Uptakes of Heart Mitochondria from Normal and Shocked Dogs in the Presence of the Hexokinase System.

| Substrate | Normal | | | Shock | | | Hr of shock |
|---|---|---|---|---|---|---|---|
| | $O_2$ uptake μatoms /mg N* | P uptake μmoles /mg N* | P/O | $O_2$ uptake μatoms /mg N* | P uptake μmoles /mg N* | P/O | |
| α-Ketoglutarate | 12.1 | 19.5 | 1.6 | 10.3 | 11.8 | 1.1 | 2 |
| | 9.9 | 16.9 | 1.7 | 10.4 | 20.7 | 2.0 | 3 |
| | 9.8 | 25.9 | 2.6 | 5.9 | 13.8 | 2.3 | 3½ |
| | 11.4 | 23.4 | 2.1 | 5.1 | 8.3 | 1.6 | 5 |
| | 9.8 | 22.7 | 2.3 | 6.8 | 10.5 | 1.5 | 5 |
| | 8.6 | 14.8 | 1.7 | 4.9 | 3.6 | .7 | 5 |
| | 14.8 | 30.9 | 2.1 | 8.8 | 14.8 | 1.7 | 5½ |
| Succinate | 10.4 | 19.6 | 1.9 | 11.7 | 13.2 | 1.1 | 3 |
| | 14.3 | 31.3 | 2.2 | 13.5 | 20.6 | 1.5 | 3 |
| | 10.8 | 22.5 | 2.1 | 11.0 | 23.0 | 2.1 | 5 |

* Avg of duplicate or triplicate flasks.

Main compartment: 20 μM $MgCl_2$, 3 μM AMP, 50 μM phosphate buffer, pH 7.5, 40 μM substrate, mitochondria (app. 1 mg N) and 0.5 M sucrose to 1.8 ml; sidearm: 50 μM fructose and 2 mg hexokinase in 0.2 ml; center well: 0.2 ml 2 N NaOH; temperature: 30°C; gas phase: oxygen.

*crose suspension* is demonstrated in Fig. 2. Rods persist in varying numbers, often as high as 80%: the remaining mitochondria appear as spheres of varying sizes. "Crescents" are not seen in fresh suspensions. Preparations from shocked dogs generally have a lower rod count and show a preponderance of the small and medium sized spheres while the normal tends to contain large forms. This difference in size is often very marked and is observed also in the incubation mixtures at the end of Warburg experiments. A comparison of Fig. 1 and 2 reveals that the normal mitochondrion tends to be small in formalin but large in sucrose whereas the abnormal behaves in an opposite manner.

On aging at room temperature swollen spheres and "crescents" will be formed in both types of preparations. However, this *in vitro* change tends to occur less readily and less extensively in the particles isolated from shocked dogs (Fig. 3). *Enzymatic data.* Fig. 4 shows that the oxidation of succinate, malate and α-ketoglutarate was essentially unimpaired even after 5 hours of shock; in many experiments the 2 curves were superimposable. Experiments with acetate and butyrate were also negative. No requirements for coenzyme I, guanosine diphosphate¶ or cytochrome c could be demonstrated; in fact, added coenzymes often were found to be inhibitory. On the other hand, Table I indicates that in the presence of the hexokinase system oxygen uptake and phosphate esterification were often decreased: thus in seven experiments with α-ketoglutarate the normal consumed an average of 11 microatoms of oxygen per mg nitrogen and 22 micromoles of phosphate resulting in a P/O of 2.0; this was reduced in shock to 7.5 microatoms oxygen and 12 micromoles phosphate

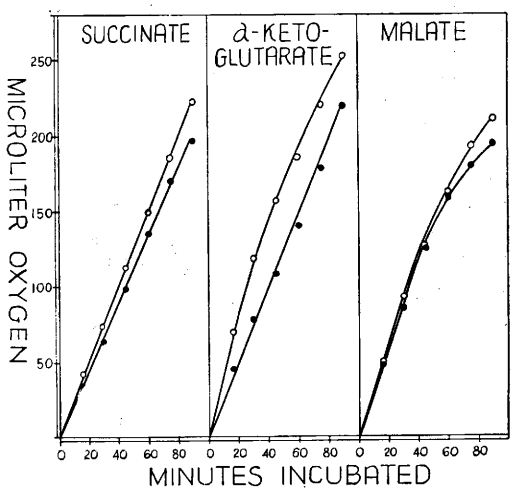


FIG. 4. Oxygen uptakes of heart mitochondria from normal and shocked dogs. Main compartment: 20 μM $MgCl_2$, 3 μM ATP, 50 μM phosphate buffer, pH 7.5, 40 μM substrate, mitochondria (app. 1 mg N) and 0.5 M sucrose to 2.0 ml; center well: 0.2 ml 2 N NaOH; temperature: 30°C; gas phase: oxygen. ○ = normal, ● = 5 hr shock.

¶ Generously presented to us by Dr. D. M. Gibson of the Enzyme Institute.

giving an average P/O of 1.6.

*Discussion.* It may be concluded, therefore, that the mitochondria are structurally altered in hemorrhagic shock and that these *in vivo* changes differ from the *in vitro* degenerations described by Harman, for in the formalin-fixed preparations the basic rod shape is maintained despite the swelling and distortion.

Upon isolation in sucrose solution, however, the serial transformations observed by Harman are found to take place, *i.e.* the rods change into spheres which increase in size and ultimately form "crescents." Under these conditions the spheres derived from the abnormal mitochondria are smaller and exhibit less of a tendency to swell and to turn into "crescents." This phenomenon suggests that shock alters the particles in a way which renders them less responsive to environmental conditions. It will be of interest to study their submicroscopic internal structure.

The observation that the *in vitro* oxidative activities of the abnormal mitochondria are affected by the presence of the hexokinase system has raised an interesting point. It is possible that the classical Warburg methods, which supply an optimal environment as far as cofactors and substrate requirements are concerned, obscure or reverse early enzymatic defects. The negative results of Fig. 4 could thus be explained. In the living organism, on the other hand, the mitochondria are called upon to furnish an undiminishing supply of energy and building materials for use by other cell constituents. It may well be that the hexokinase trap which sets up a constant extramitochondrial demand for ATP mimics to some extent such *in vivo* situations. The results of the enzymatic studies reported here would then indicate that the heart mitochondria of shocked dogs are capable of generating enough energy to maintain themselves in a functional state but begin to fail when exposed to stress conditions which force them to produce ATP at rates beyond those which will suffice for their maintenance. Future work will have to elucidate the correctness of this hypothesis.

*Summary.* Dogs were subjected to irreversible hemorrhagic shock. Their hearts, livers, and kidneys contained mitochondria which differed in size and shape from those of normal dogs. Isolated by differential centrifugation the mitochondria from shocked animals were found to retain the normal oxidative capacities for Krebs cycle substrates but tended to have impaired activities in the presence of the hexokinase system.

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