The Modernization of the Autopsy: Application of Ultrastructural and Biochemical Methods to Human Disease* **

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The information gained through the autopsy has played an important role in the evolution of knowledge of human disease. At the time of Semmelweiss, bedside symptoms and signs plus postmortem examinations were the only investigative tools available to study and understand the causes and progression of disease, to assess the efficacy of therapy and to monitor the quality of medical care. Feedback provided by postmortem examination was highly instrumental in the development of physical diagnosis. The autopsy was virtually the sole means of classifying disease. In the process, the autopsy spawned and furthered the development of almost every contemporary technique for the diagnosis of disease of which radiology and electrocardiography are notable examples. Once developed and refined, such clinical sciences have assumed a direction and ideology aimed at the living patient and paradoxically, the contribution of the autopsy to patient care has steadily diminished. Advances in the clinical sciences have depended more on achievements made in biologic science and less on necropsy in large part because modern biological techniques have not been employed in postmortem examinations. As a result, the clinical sciences have been cut loose from the conceptual base previously provided by information obtained at autopsy.

The autopsy has provided, and still provides, the stimulus for many attempts to reproduce disease in experimental animal models. This approach has become increasingly difficult, however, in the case of human disease, principally shock. The study of some pathological states in animal models requires testing in several species and final confirmation in man before this knowledge can be applied to living patients. In our studies the application of cell biology techniques at autopsy has permitted the generation of new hypotheses which are more amenable to further exploration in experimental models and can be more precisely related to human disease.

The chief limitation for the interpretation of observations made in the routine autopsy is caused by the delay in obtaining tissue following somatic death. The resultant autolytic changes invalidate most ultrastructural, biochemical and functional studies. Investigations utilizing contemporary refined techniques from the experimental laboratories of university pathology departments are, however, the very ones needed to restore the postmortem examination to a primary role in patient care and in quality con-

* Presented by Dr. Trump at the 44th Annual McGuire Lecture Series, March 23, 1973, at the Medical College of Virginia, Richmond.

** Supported by the following grants from the National Institutes of Health: GM-00431-12, AM-15440-02 and GM-15700.

trol of health care. The use of biopsy obviates time delay. While much valuable information can be and is obtained from biopsy, there are distinct limitations: a) The small size of biopsies restricts the number of analytical techniques that can be applied; b) Often, medical indications for biopsy do not outweigh the risks involved for seriously ill patients; c) Finally, the fact that complete organ sampling cannot be performed make this contribution of limited value in the study of systemic disease.

A potential limitation in applying an immediate autopsy technique is the selection of patients for any contemplated multidisciplinary approach. The voluminous data generated by intensive care units have made valuable contributions to medical knowledge and patient care, yet little of these data has been related to phenomena at the tissue and cellular level. It seems logical then to direct an intensive approach to this population of patients. Especially needed, we feel, are morphologic, biochemical and functional observations on tissues removed immediately after death in order to correlate them with clinical data.

The initial objective of the immediate autopsy is directed to the evaluation of cellular and subcellular changes in major organ systems in cases of shock. Shock frequently accompanies trauma and constitutes a significant determinant of the posttraumatic morbidity and mortality, a major cause of death. Antecedent diseases resulting in shock are legion and shock is a component of the terminal phase of numerous illnesses of many causes. Morphologic techniques used in the routine autopsy do not permit separation of the cellular effects of shock from the effects of certain underlying diseases which cause shock. For example, in acute myocardial infarction with cardiogenic shock it is often impossible to distinguish the extent of primary myocardial injury from the damage secondary to shock. Although we know that there are common pathways of cellular reaction to a diversity of injurious agents, variations exist. Our studies have demonstrated that a distinction can be obtained between the effects of antecedent disease and the effects of shock when precise clinical data are correlated with ultrastructural, biochemical and functional assays at immediate autopsy. This permits better understanding of the cellular shock, which is of importance in the utilization of autopsy technique to the study of other diseases. Without knowledge of the changes resulting from shock, adequate evaluation of autopsy data in other diseases may be difficult or impossible.

The Immediate Autopsy Program at the University of Maryland is conducted by the Pathology Department in conjunction with the Maryland Institute for Emergency Medicine. The primary purpose of the program is to document and clarify the pathological changes leading to organ failure in cases of shock and trauma. This paper demonstrates that tissues obtained at immediate autopsy yield valid observations on the pathogenesis of cellular injury in shock. Methods have been discussed in detail elsewhere (5); the autopsy procedure, however, will be briefly outlined below.

Immediate autopsy is made possible by two legal instruments existing in the State of Maryland: a) the Medical Examiners' Law which charges the Medical Examiner with the responsibility of determining the cause of death and investigating medicolegal and biological factors in deaths due to violence, traffic accidents and unexplained natural death; b) the Anatomical Gift Act, similar to laws enacted in many states and aimed at obtaining organs for transplantation, whereby the patient or his next of kin can give permission for autopsy prior to death.

All the individuals studied include cases of "brain death" as determined by the Harvard criteria (1). Once somatic death is determined by the absence of heart beat, pulse and respiration, as well as an isoelectric EKG, the rapid sampling phase of the immediate autopsy is begun. The prosector, together with the ten-member team, does a rapid sampling of several organs for study by light and electron microscopy, immunofluorescence, enzyme histochemistry and studies of organelles after homogenization and differential centrifugation. Following the rapid sample phase, a routine autopsy dissection is performed.

Simultaneously, the intracranial contents are perfused *in situ* via polyethylene tubing inserted through the right common carotid and after ligation of the three other major cervicocranial arteries. The fixative is composed primarily of aldehydes.

Concepts of Cell Injury. Since Rudolph Virchow's famous papers of about one hundred years ago, we think of disease in terms of cellular changes, and define disease as the summation of the effects of injury as well as the responses of cells to injury. Injury is defined as an event that alters cellular homeostasis. If the injury is lethal, such as complete ischemia or anoxia, there will be a reversible phase prior to the time of cell death. If the blood supply or oxygen are restored during this reversible stage the cell can recover, return to the normal state of homeostasis and

continue functioning. At some point, however, damage becomes irreversible and the cell is said to die; even if the blood supply is restored recovery does not occur. Instead, the cell undergoes necrosis and gradually approaches equilibrium with the environment. Thus there is a reversible and an irreversible phase following a lethal injury. Shown in figure 1 are some of the key organelle changes which characterize these

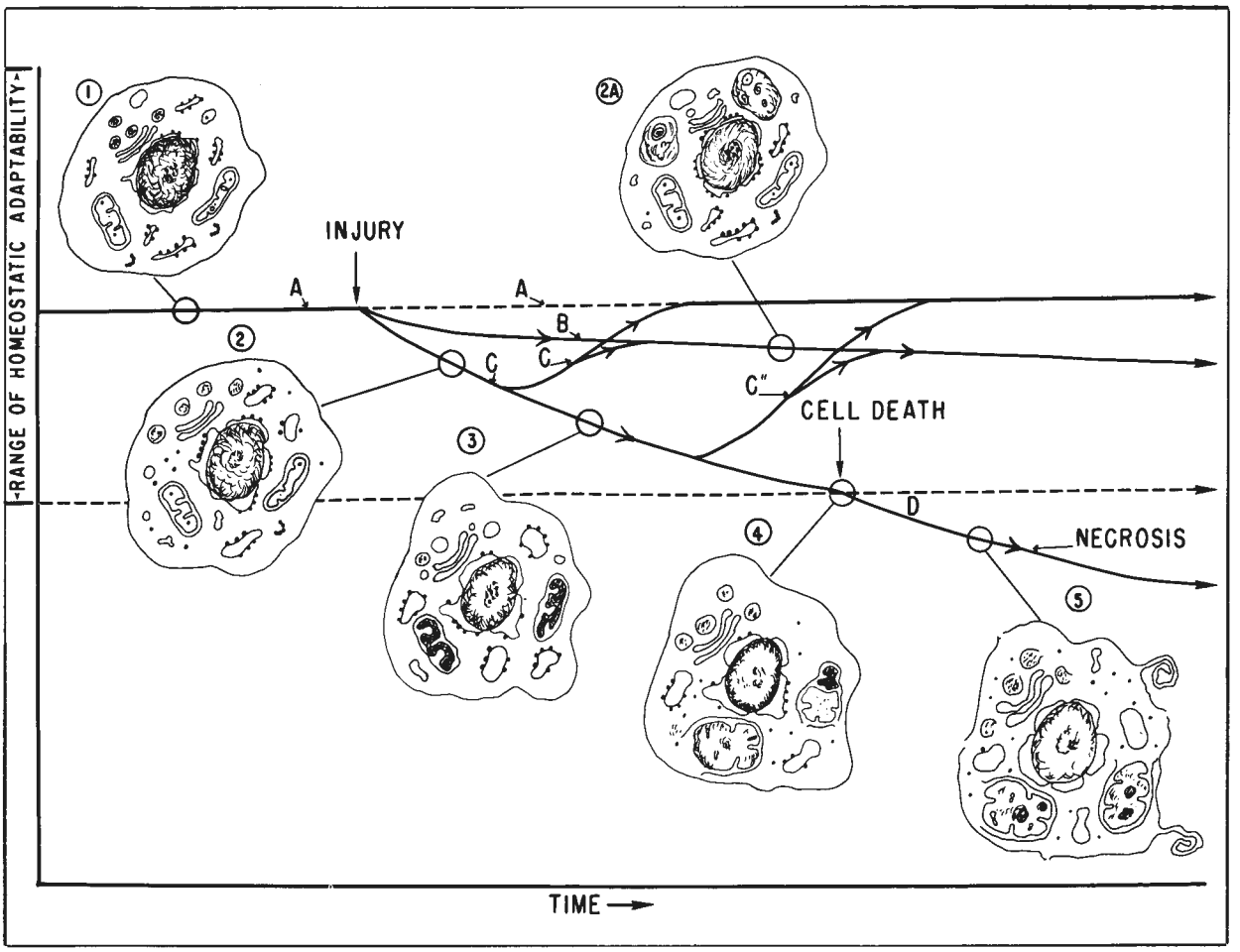

Fig. 1—Stages of cell injury. This diagram represents a conceptualization of the results of cell injury. Time is plotted along the abscissa and the range of homeostatic ability along the ordinate. An injury is applied at the arrow. This injury may be sublethal or acutely lethal. Lethal injury will be considered first. Curve C represents a homeostatic ability. Recovery can occur, however, if the injurious stimulus is removed prior to the point of cell death. Such recovery may proceed along the curve C′ or C″. Stages 2 and 3 represent progressive changes during this period. Stage 2 is characterized by dilatation of the endoplasmic reticulum and slight clumping of the nuclear chromatin. Some ribosomes may also be detached from the endoplasmic reticulum and the entire cell may be slightly swollen. Condensing of the mitochondria, additional swelling of the cell and the appearance of blebs along the cell membrane are characteristic of stage 3. After the point of cell death, recovery can not occur even if the injurious stimulus is removed and the cell is said to enter the phase of necrosis. Stages similar to those in stage 3 occur in stage 4. In stage 4, however, some mitochondria are markedly swollen, others have portions that are condensed and other portions that are swollen. Stage 5 depicts a typical morphologic picture of cell necrosis during which the cell undergoes degenerate degradation by autolysis and denaturation. In this and later stages, myelin figures appear along the cell membrane, intracellular membrane systems are fragmented, interruptions occur in continuity of the plasma membrane and the mitochondria show high amplitude swelling of the inner compartment with prominent flocculent densities. Lysosomes are probably beginning to leak at this point although they may still appear intact. In some injuries, the cell is able to adapt even to the presence of continued injury by attaining some altered steady state. This is depicted as curve B. A common sublethal adaptation in which numerous secondary lysosomes are filled with digestive debris is represented in stage 2A. Note that incomplete recovery during the reversible phases after lethal injury might also result in a new steady state depicted by the right-hand limb of curves C′ and C″. From Trump, B. F., *et al.* (6). Used by permission.

phases as observed in studies of experimental animals. For example, one of the earliest changes that occurs following injury is dilatation of the endoplasmic reticulum (ER), which is associated with early ion and water shifts within the cell. These constitute a manifestation of reversible injury. Thereafter, the inner compartment of the mitochondria undergoes condensation probably due to potassium loss from that compartment; this, too, is reversible and if blood supply is restored, the cell can survive. Cells that are past the "point of no return" or have entered the phase of necrosis typically show massive mitochondrial swelling with formation of dense matrical inclusions in the inner compartment, presumably due to denaturation of matrix protein. The permeability of the cellular plasma membrane leads to increased levels of intracellular enzymes in the plasma. These enzymes include glutamic oxalacetic transaminase and lactate dehydrogenase. Nuclei swell and undergo karyolysis. In late phases of necrosis lysosomal enzymes such as acid phosphatase can be demonstrated in the serum.

Immediate autopsy vs. routine autopsy. The state of preservation of tissue, in this case liver, removed at immediate autopsy within minutes of death (fig. 2), is contrasted with a sample of liver removed from the same patient after the customary somatic death-fixation interval (12 hours) had elapsed (fig. 3). A "differential necrosis" of centrilobular zones is noted in the later specimen which is not present in the immediate autopsy sample. Given only the routine sample, this could lead to the erroneous conclusion that a terminal episode of shock in this patient together with two earlier episodes had resulted in extensive central and midzonal hepatic necrosis.

Instead, chronic injury over a one-month period had resulted in a differential accumulation of lysosomes in centrilobular hepatocytes. Lysosomes were precisely localized with an enzyme marker, acid phosphatase, in the immediate specimen, but diffuse reaction characterized the routine sample. Study of the immediate sample by electron microscopy revealed enlarged lysosomes in this human liver (fig. 4). The remarkable degree of good ultrastructural preservation obtained is evident and demonstrates the feasibility of applying this technique to the study of human disease. In a series of such patients sustaining repeated episodes of shock, rather striking increases in the amount of chemically assayed acid phosphatase have been found in liver homogenates as compared with patients not sustaining or in cases with a single acute injury or shock (7). The numerous large lysosomes in liver cells contain various types of debris much of which presumably resulted from what is called autophagocytosis, a situation whereby normal organelles, such as mitochondria, are segregated within the lysosomal system and are digested. This phenomena of autophagocytosis occurs through budding of portions of cytoplasm into

Fig. 2—(left) Light micrograph of centrilobular cell of liver from a 48-year-old man who had suffered several episodes of shock over a 32-day period. Note the excellent state of preservation of hepatic parenchymal cells. Note, however, that many of these cells contain easily visible, eosinophilic cytoplasmic inclusions. Paraffin embedding, H & E, × 345. Fig. 3—(right) Portion of liver from the same case from the routine autopsy taken 12 hours later. Note that in this picture typical coagulation necrosis of some of the centrilobular hepatic cells can be seen. Paraffin embedding, H & E, × 345.

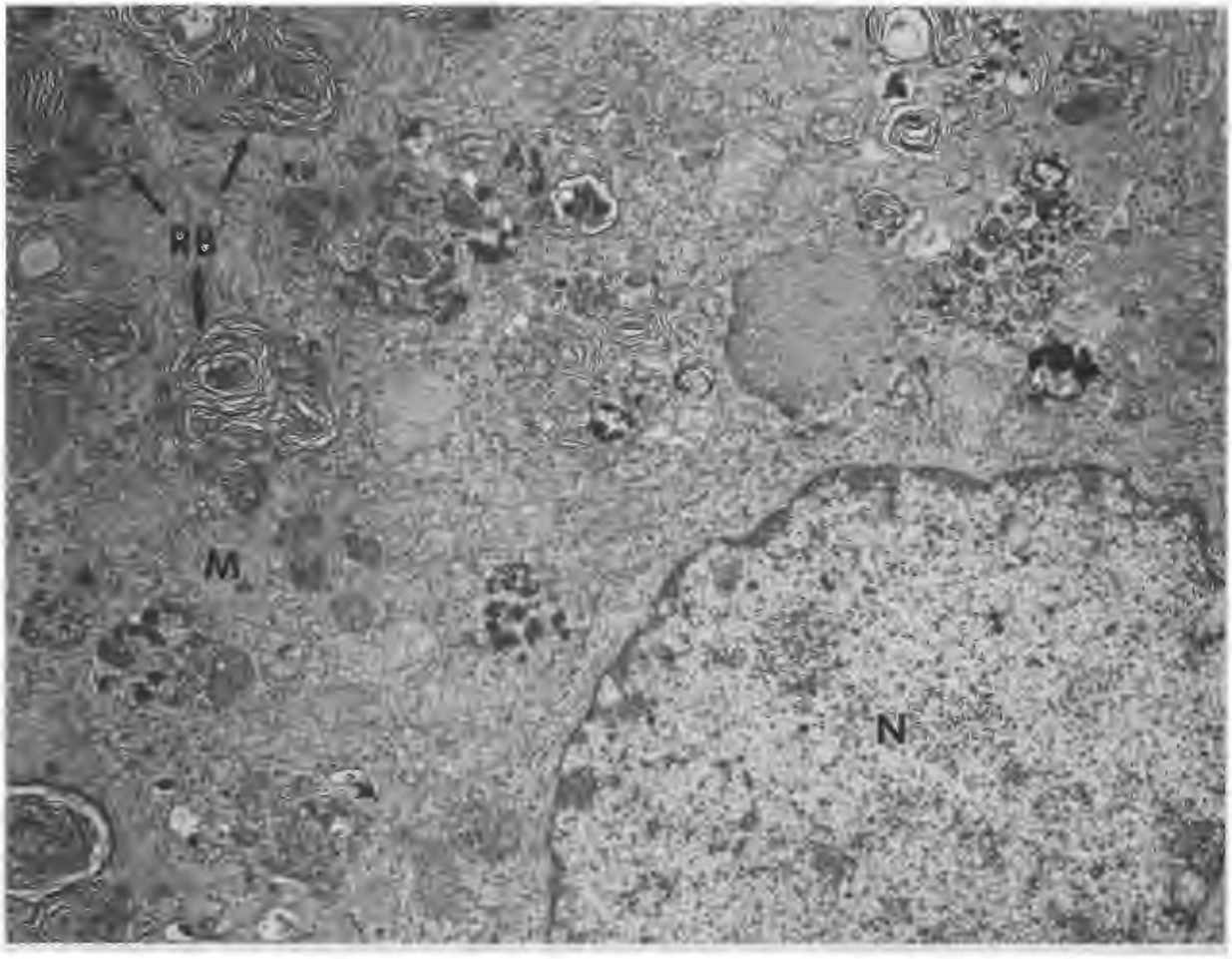

Fig. 4—Electron micrograph taken from a specimen fixed at immediate autopsy from the same case shown in figure 2 illustrating numerous large single membrane-bound inclusions in the hepatic parenchymal cells. These inclusions contain a variety of debris principally lamellar and are interpreted as residual bodies (RB) resulting from incomplete digestion of material entering the lysosomal system by autophagy and/or heterophagy. Nucleus (N), mitochondria (M), × 12,000.

intracellular cavities such as the endoplasmic reticulum followed by pinching off of the bud releasing the segment of cytoplasm containing sequestered organelles and fusion with primary and/or secondary lysosomes. This rather complex process involving cell membrane movements is ATP-dependent and can be reproduced experimentally by administering glucagon.

In the case of the rat model it appears that the glucagon-induced autophagy is mediated through cyclic AMP. It is entirely conceivable, therefore, that in this particular patient, as well as in others with shock, the increased autophagy is the result of changes in hormonal levels; for example, increased levels of glucagon in the plasma. With digestion, lipids are released giving rise to phospholipid bilayers sometimes called "myelin figures." The lysosomes in this patient superficially resemble those seen in many of the "lipid storage" diseases, such as the gangliosidoses in which various types of cellular debris accumulate on the basis of a lysosomal enzymatic defect. In this case presumably there is no enzymatic defect, but rather an overloading of the system due to induced autophagocytosis. This results in cells which are not killed but are perturbed by the initial injury. Autophagocytosis can be defined as a manifestation of sublethal injury. Only an occasional hepatocyte was "necrotic" in the immediate specimen, a fact which cannot be validly assessed from the routine sample. The observation that necrosis was more advanced in the routine (delayed) sample is interpreted as the effect of increased digestion by lysosomal enzymes following postmortem ischemic necrosis. In a sense, therefore, these

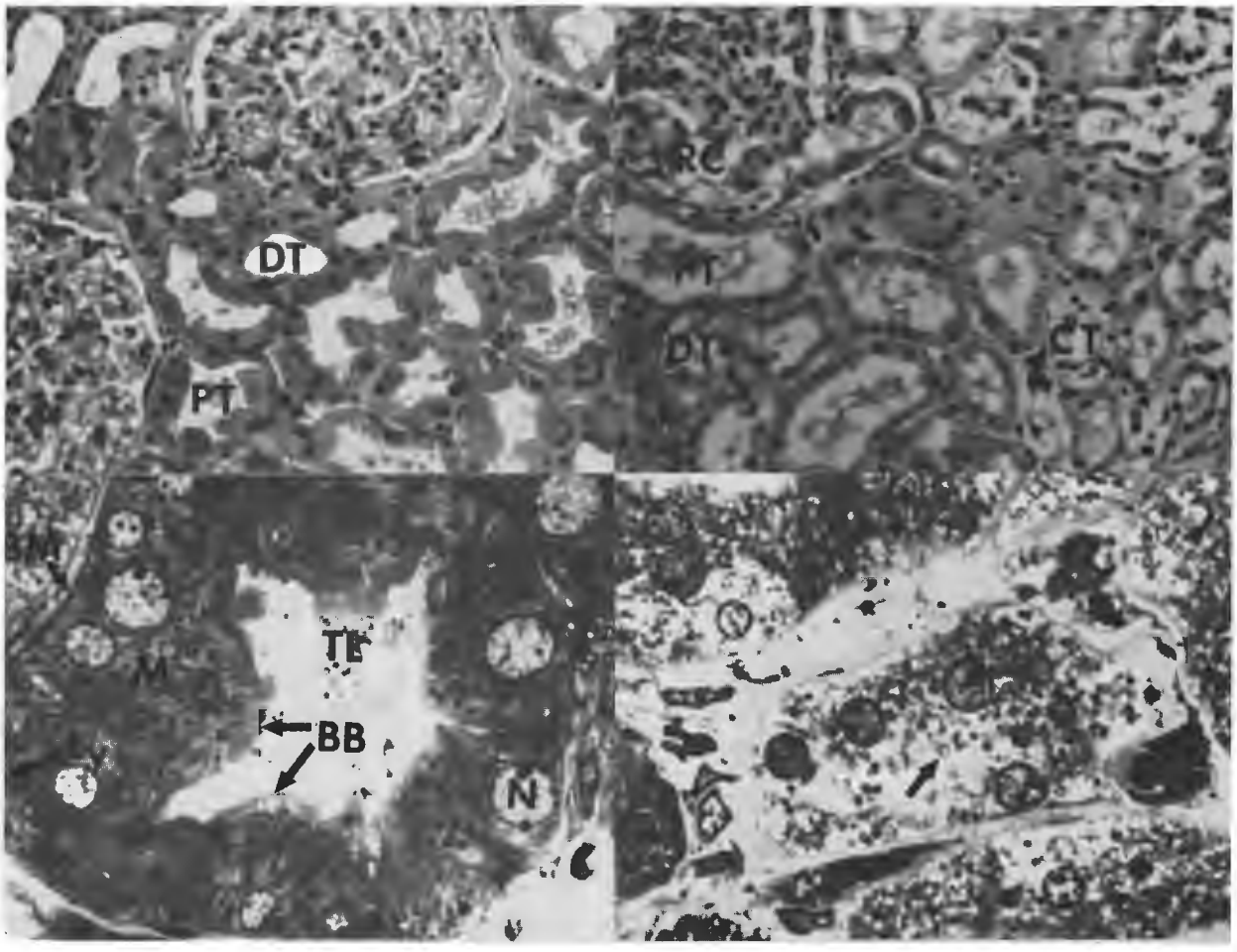

Fig. 5—(left, top) Light micrograph of renal cortex from a 28-year-old man who did not have shock, but died following severe head injuries. Note good preservation of renal corpuscle from the proximal tubules (PT) and distal tubules (DT). Paraffin embedding, H & E, × 345. Fig. 6—(left, bottom) Semithin section from the same case shown in figure 5 illustrating the increased cytologic detail visible with this technique. Note that now the brush border (BB) can be fairly delineated and numerous granules, most of which are mitochondria (M), can be seen in the cytoplasm. Note also the lysosomes (Ly). The basement membrane (BM) can be clearly seen along the outside of the tubule. Tubular lumen (TL), nucleus (N). Epon embedding, toluidine blue, × 865. Fig. 7—(right, top) Light micrograph of the renal cortex from a 16-year-old girl who died six hours after an anoxic episode. Note the normal appearance of the renal corpuscle (RC), proximal tubules (PT) and distal tubules (DT). One collecting tubule (CT) can be seen. With this technique the tubules appear within normal limits. Paraffin embedding, H & E, × 345. Fig. 8—(right, bottom) Semithin section from the same case. Note again the increased detail. Now, however, it is evident that the tubule is not within normal limits but that the mitochondria are swollen and have small extrinsic densities (free arrow) apparently equivalent to the flocculent densities seen in electron micrograph. Epon embedding, toluidine blue, × 865.

liver cells with more lysosomes become somewhat like the normal pancreas which is known to undergo much more rapid autolysis following death.

High Resolution Light Microscopy. Much additional information can be obtained when tissue for light microscopy is embedded in epoxy resins as a supplement to routine paraffin embedding. The structure seen in paraffin, as opposed to plastic embedded immediate samples of kidney from a patient who did not die in shock (figs. 5, 6), are contrasted with a similar sample from a patient having a single acute terminal shock episode (figs. 7, 8). Generalized cell swelling (both cytoplasmic and nuclear) can be noted in either paraffin or plastic embedded kidney from the shock patient but can be more precisely assessed in the plastic embedded specimen. Paraffin embedding results in comparatively poor preservation of cellular structures. Numerous rounded bodies are

noted within the cytoplasm of the plastic embedded proximal tubule cells in both specimens. These are mitochondria but are not readily visualized in the paraffin sections. Furthermore, the mitochondria in the shock kidney cells (fig. 8) are notably larger and even exhibited punctate densities in epoxy semithin sections. Using electron microscopy (fig. 9), mitochondria are markedly swollen and exhibit flocculent matrix densities; there are features characterizing acute lethal cell injuries as derived from varied model experiments *in vivo* or *in vitro* (5). The close correlation between light and electron microscopy is then obvious and the light microscope can thus be employed to evaluate tissue samples larger than those that can be meaningfully studied by electron microscopy.

Correlation of Organelle Structure and Function. The immediate autopsy approach further permits a valid assessment of mitochondrial function which is of profound importance in the pathogenesis of cell injury in shock because mitochondria often seem to be the deciding factor for cell survival. Whether or not the mitochondria can recover and make ATP following treatment of the cause of injury seems to be the crucial question.

Mitochondria can readily be prepared from the immediate autopsy specimens. Tissue is removed and shortly thereafter homogenized and centrifuged to obtain a mitochondrial suspension. Mitochondrial function is then assayed using an oxygen electrode. The chamber is designed so that substrates and ADP can be added to the mitochondria suspension

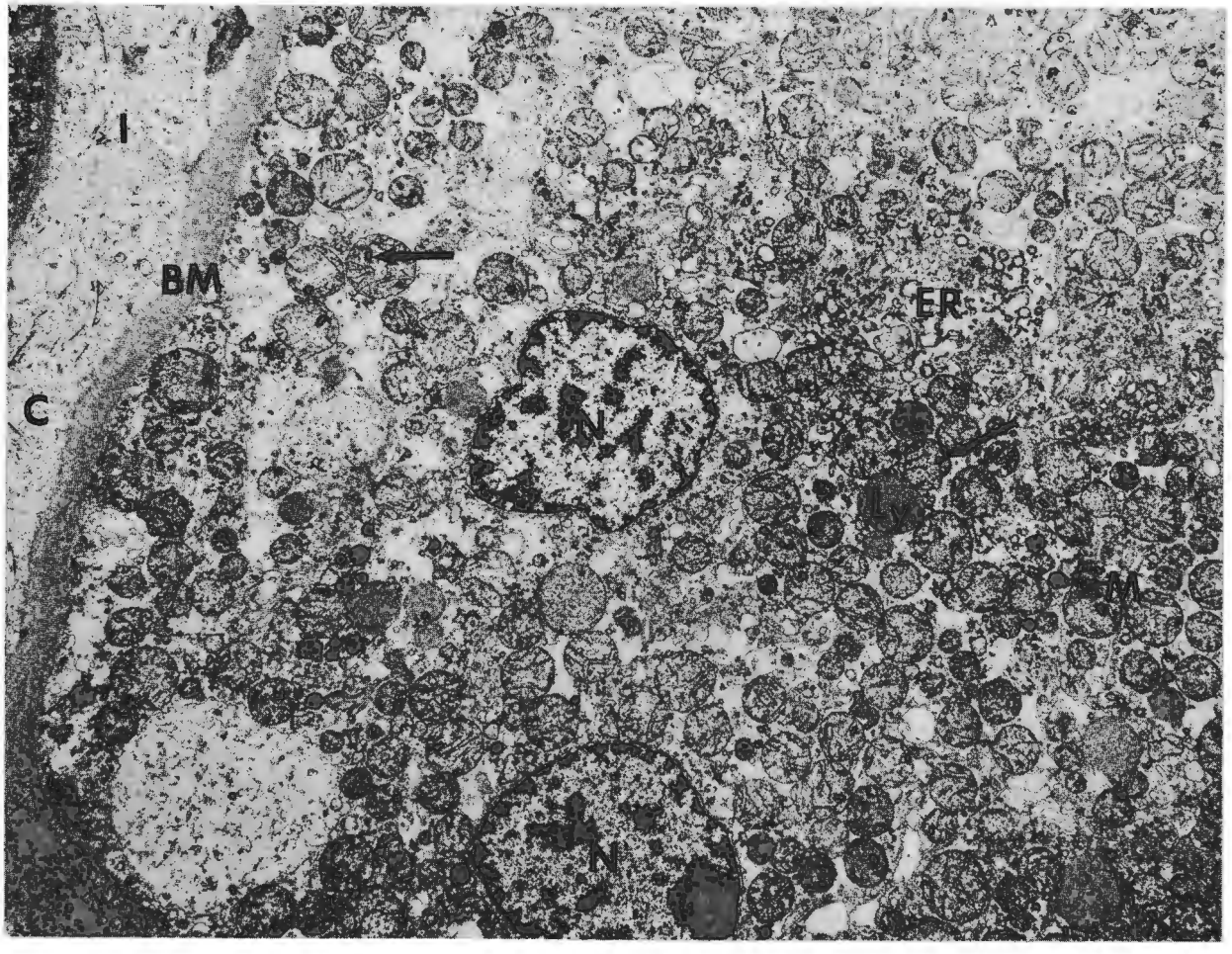

Fig. 9—Electron micrograph of proximal tubules from the same case showing stage 5 changes as depicted in diagramatic form in figure 1. Note the markedly swollen mitochondria (M) with flocculent densities (free arrow). Fragmentation of cisternae of endoplasmic reticulum (ER); nuclei (N) which show marked clumping of chromatin and several apparently intact lysosomes (Ly). Basement membrane (BM), interstitium (I), collagen fibers (C), × 10,000.

to stimulate respiration. With mitochondrial respiration, a downward slope as a function of time depicts the oxygen consumed. Given the amount of mitochondrial protein added, oxygen consumption per milligram protein can be computed. Normal mitochondria exhibit tightly coupled respiration, meaning that they do not respire in the presence of a substrate like succinate unless ADP is present. In other words, the rate of respiration is limited either by the amount of substrates or ADP which is going to be phosphorylated; addition of mitochondria to the chamber results in a gradual slope due to endogenous substrates. When a substrate, for example, succinate, is added there is only slight respiration, due to the absence of ADP which is phosphorylated. With addition of ADP, there is a great increase in the rate of respiration which continues until completion of the phosphorylation of the ADP at which point the slope of oxygen consumption levels off again. By comparing the phosphorylation slope with the resting slope, a ratio is obtained which is a measure of the efficiency of the mitochondria. If the mitochondria are completely damaged, there will be no change in rate on adding the ADP. If they are normal mitochondria or normally functioning, the rate will increase three- to ten-fold. Plotting phosphorylation rate over resting rate is termed the respiratory control index which is utilized as a measure of mitochondrial functional integrity. A P/O ratio or an ADP/O ratio is computed from the amount of ADP added, divided by the atoms of oxygen consumed. When the amount of ATP that formed in the oxygen electrode per milligram protein in both the liver and kidney from a series of patients is plotted on a two dimensional scale, one can discriminate two groups of patients, one group with head injury which did not have shock and one group of patients sustaining shock (fig. 10). In shock, apparently there is a marked reduction in the ability of mitochondria to make the ATP; this fact correlates with the morphology of mitochondria studied in plastic embedded preparations and by light and electron microscopy (figs. 6, 7). P/O ratios which should be around 2.5-3 for glutamate and about 1.5-2 for succinate, are significantly reduced in shock (4).

Studies of the Central Nervous System. The final two cases are examples of the improved results obtained in the interpretation of microscopic abnormalities of the brain whenever intravascular perfusion fixation and electron microscopic methods are used according to the systems mentioned above. The first patient, neurologically normal for 72 years, died after a prolonged period of marked hypotension with a ruptured abdominal aneurysm. The second patient, who had a lengthy history of inappropriate belligerent behavior, was stabbed and developed suppurative peritonitis and pulmonary edema. In both instances perfusion fixation of the brain was started about 25 minutes after somatic death.

Fixation of the central nervous system which allows adequate cytological evaluation is difficult to accomplish in part due to the relative inaccessibility of the tissue. In other animals, it has been shown that handling of the normal brain frequently results in abnormalities referred to as "dark" neurons (2). Thus, avoidance of tissue handling before fixation has distinct advantages in the fixation of neuronal and glial elements in the human brain. As for the effects of prolonged death-fixation interval, we have studied the ultrastructural effects of total ischemia in feline cerebral cortex and have determined that pro-

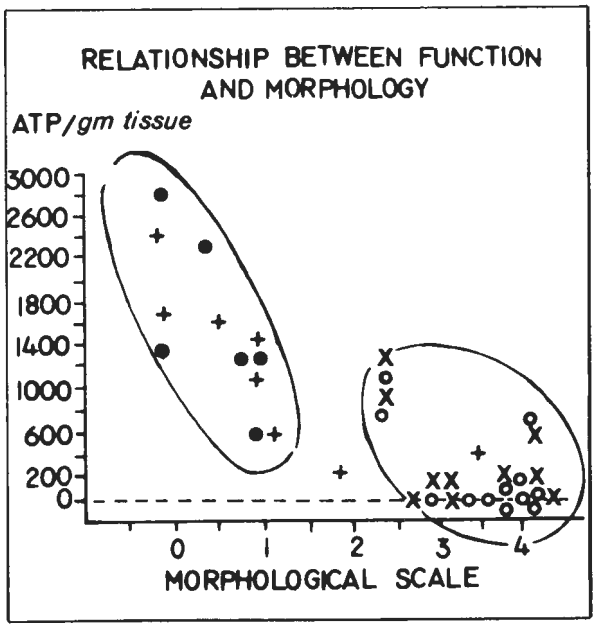

Fig. 10—Two dimensional scatter plot of morphological changes in mitochondria compared with ability of the mitochondria to synthesize ATP *in vivo*. On the morphological scale, 0 represents mitochondria showing normal morphology and 4, mitochondria with the most advanced changes. One, 2 and 3 are intermediate. Note that two clusters are formed; the patients dying without shock have better morphology and higher rates of ATP production in contrast to mitochondria from individuals with shock. + = succinate with pure head injuries; ● = glutamate with patients with head injuries without shock; X = succinate with shock and ○ = glutamate with shock.

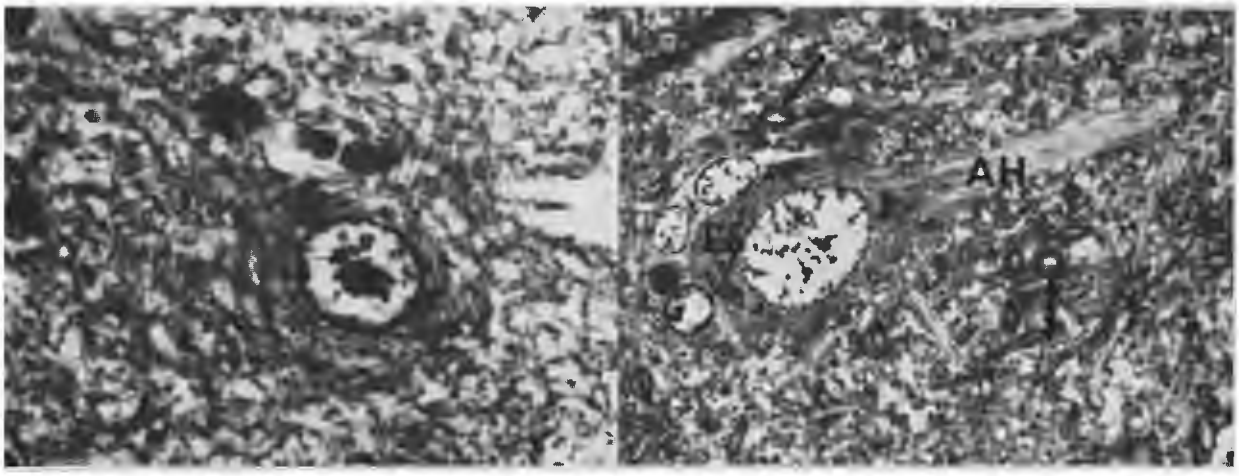

Fig. 11—(left) Light micrograph of tissue from the cerebral cortex of a 72-year-old man who died with irreversible brain damage following an 18-hour ischemic episode subsequent to a ruptured abdominal aneurysm. Note that in this pyramidal cell we can appreciate the nucleus and cytoplasm. Only rather indistinctly can portions of the chromidial substance be seen. Paraffin embedding, H & E, × 865. Fig. 12—(right) Semithin section of cerebral cortex from the same case as in figure 11 showing greatly improved cytologic detail. Now we can recognize an axon hillock (AH), cytoplasmic lysosomes (Ly), and in the adjacent neuropil, myelinated nerve fibers (free arrows). Epon embedding, toluidine blue, × 865.

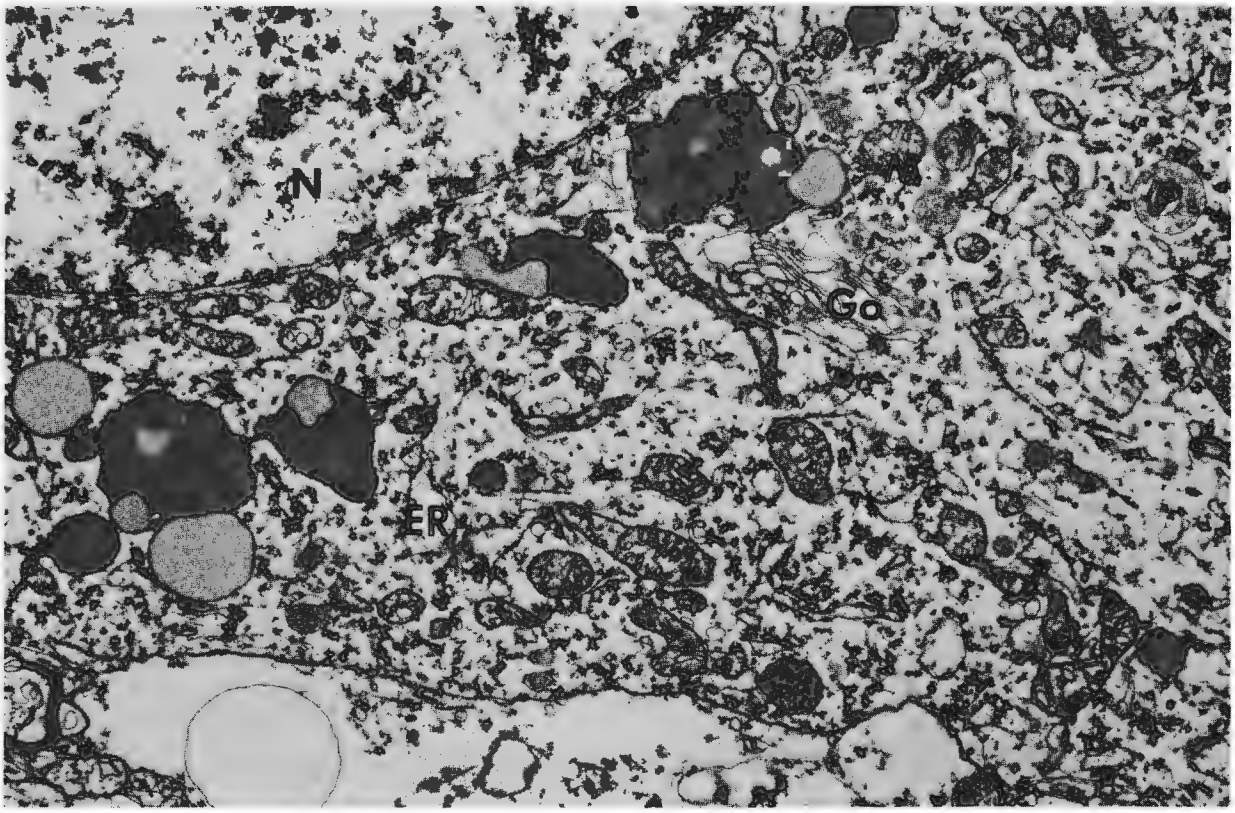

Fig. 13—Electron micrograph from the same case as in figures 11 and 12 showing a portion of a cortical neuron which exhibits stage 5 changes. The mitochondria (M) show flocculent densities, the endoplasmic reticulum (ER) is dilated as are the saccules of the Golgi apparatus (Go). Several residual bodies (RB) which are presumably related to the patient's age are also seen; nucleus (N), × 15,000.

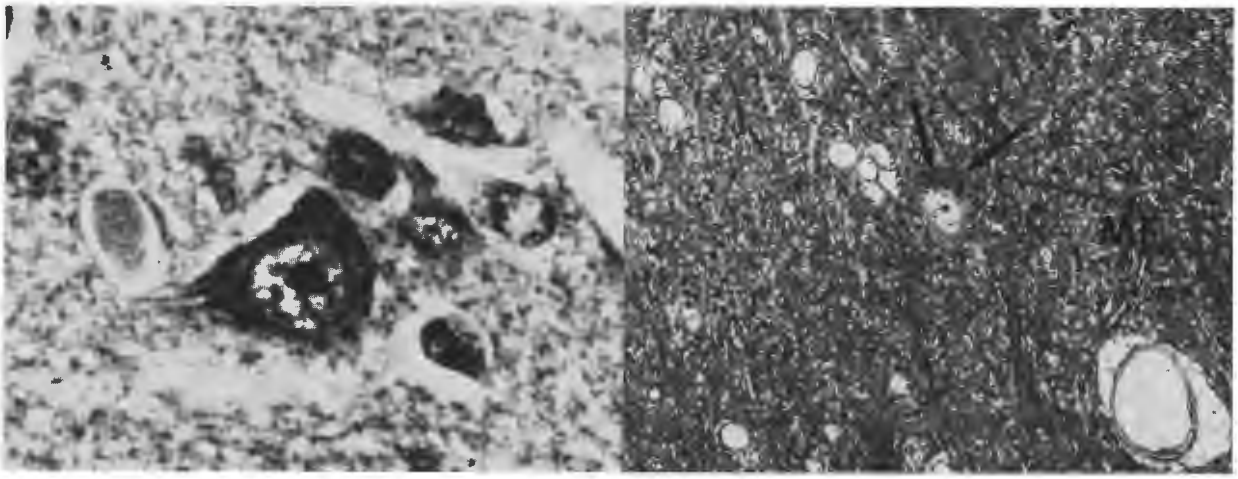

Fig. 14—(left) Light micrograph of pyramidal cell from the cerebral cortex of a 50-year-old man who died with peritonitis and pulmonary failure. At the time of autopsy, severe atrophy of the brain was grossly apparent. In contrast to the patient shown in figures 11–13, this patient had only a brief terminal episode of hypotension. In this preparation, the neuron has an appearance which is within normal limits. Paraffin embedding, H & E, × 865. **Fig. 15**—(right) Light micrograph of a semithin section from the same patient. Note the large amount of lysosmal granules (free arrow) in the neuron, which correspond to brownish pigment seen in hematoxylin-eosin preparations. Note also numerous myelinated fibers (MF). Epon embedding, toluidine blue, × 345.

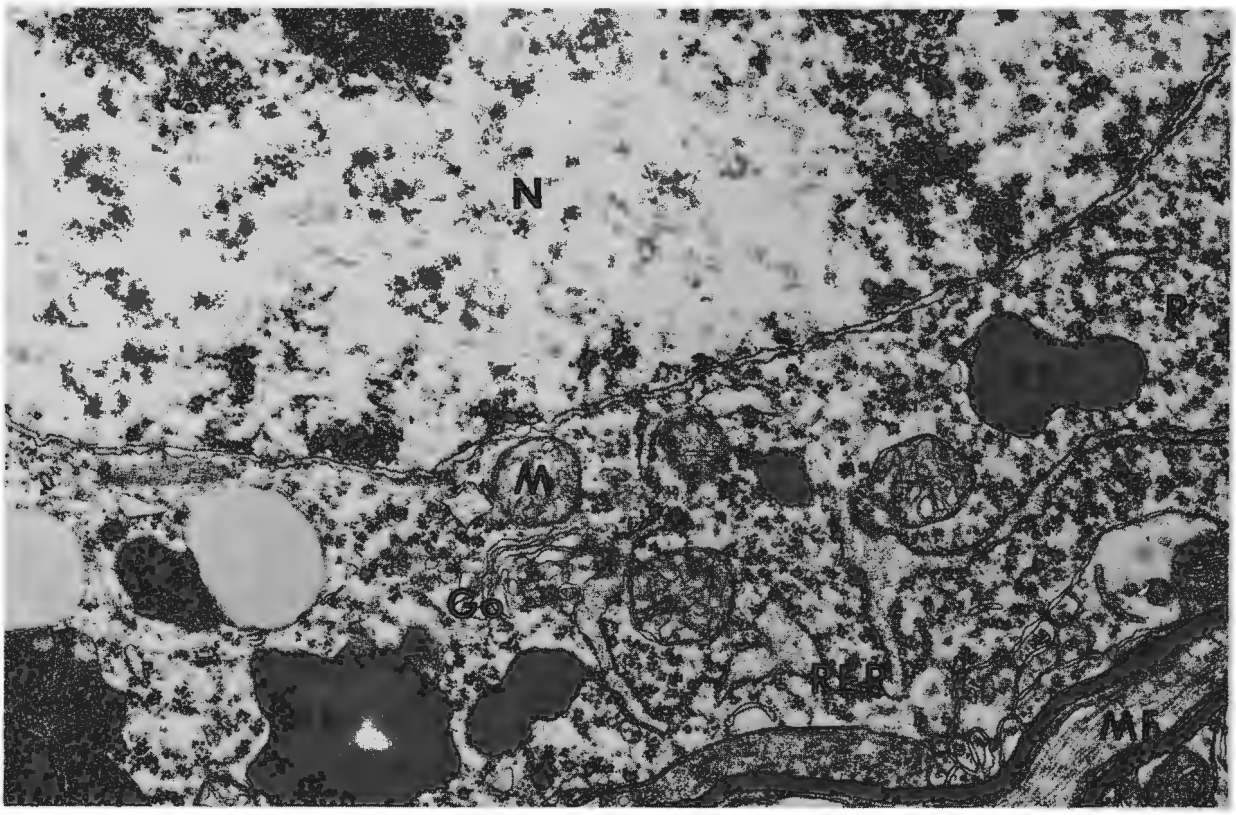

Fig. 16—Electron micrograph of same case as in figures 14 and 15 showing numerous residual bodies (RB), profiles of rough endoplasmic reticulum (RER), Golgi apparatus (Go), mitochondria (M) and numerous free ribosomes (R). All of these appear within normal limits. The numerous residual bodies which are much too frequent for this patient's age presumably reflect a diffuse metabolic disorder of nerve cells. At the bottom of the picture a normal-appearing myelinated fiber (MF) can be seen; nucleus (N), × 20,000.

found structural changes occur after 60 minutes, but that these may be minimal before 30 minutes (3).

Finally, the improved resolution and, therefore, information gained through electron microscopy can also be appreciated by comparing the images obtained after paraffin embedding and processing, Epon embedding and ultrastructural evaluation. Note that in this instance electron microscopy not only reveals alterations invisible in the paraffin-embedded material, but also permits separation between two different degrees of circulatory injuries to the brain—one lasting up to 18 hours before death (figs. 11, 12, 13) and one probably lasting less than one hour before death (figs. 14, 15, 16).

The application, therefore, of concepts and investigative techniques generated in research laboratories for studying cellular response to injury, provide powerful methods which will markedly improve our understanding of human disease. It is possible to conclude, on the basis of our studies to date, that human shock has important and often disasterous effects on cell function level. The cellular pathology of shock has been a mystery for a long time. The principle reason for the continuing mystery surrounding the pathology of shock is that the changes appear to be mainly at the organelle level. In the future we may think of shock more as a mitochondrial, lysosomal or cell membrane disease than as a disease with primary organ targets.

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