Effect of Endotoxemia on Liver Cell Mitochondria in Man

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IN recent studies [11] on the cellular effects of endotoxin shock, we observed that liver homogenates from Sprague-Dawley rats in which an LD_{50} dose of *Escherichia coli* was injected 15 hours prior to liver extirpation consumed 40% to 50% more oxygen than liver homogenates from noninjected rats. This finding suggested the possibility that endotoxin uncouples phosphate esterification from mitochondrial respiration. To investigate this possibility we added exogenous substrate to the mitochondria and compared oxygen uptake in both the presence and absence of endotoxin. Our primary emphasis was on the toxins of *E. coli* (although others were studied) since this organism is the most common cause of septicemia in man. We observed that lipopolysaccharide (LPS) extracted from *E. coli* had an adverse effect on mitochondria isolated from healthy tissues, that is, both the respiratory rate and oxidative phosphorylation were depressed. We decided, therefore, that it might be of value to study mitochondria isolated from human liver and also the mitochondria of rats that had been rendered endotoxemic by the administration of *E. coli* LPS intraperitoneally.

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Method

Preparation of Mitochondrial Pellet. In Part I of the experiment, liver biopsy specimens weighing 3 to 5 Gm. were obtained from patients undergoing operative procedures for gallbladder disease or duodenal ulcer. These specimens were taken from the liver edge to the right of the gallbladder and were immediately placed in iced 0.25 M sucrose.

In Part II of the study, 1 ml. of saline solution containing approximately 3 mg. of LPS (*E. coli* 026:B6, extracted by the Boivin method [Difco Laboratories]) was injected intraperitoneally into adult male Sprague-Dawley rats weighing 300 Gm. A group of control rats received only saline solution. Eighteen hours after injection of the LPS, the rats were killed by cervical fracture and portions of the liver were excised and chilled in nine volumes of isolation medium.

Homogenization and centrifugation to obtain a mitochondrial fraction were performed in both studies by a modification of the method of Chappell and Perry.[3] The medium used throughout the isolation and final suspension of washed mitochondria was a solution of 0.18 M KCl, 1 mM ethyl-

enediamine tetraacetate, and 10 mM tris buffer (pH 7.4). The entire isolation procedure was conducted at a temperature of 2° to 4° C.

One-half of the human mitochondrial pellet in the assay medium was challenged with LPS and the other half served as the control.

Manometric Assays. A manometric assay on the human mitochondria was carried out at a temperature of 36° C. and a pH of 7.4 by the direct method of Warburg.[12] Each flask contained a total volume of 3 ml. consisting of sucrose medium, phosphate (40 mM), 5 to 10 mg. protein, substrate, and endotoxin. The contents were brought together in the main compartment of the flask after a 10-minute equilibration at a shake rate of 120 cycles per minute. Pressure changes were recorded at 10-minute intervals. The controls (without added substrate) showed negligible endogenous respiration. The pH of the flask contents was routinely determined at the completion of each test to assure that no changes in this variable would influence the experimental outcome. Protein was determined by Miller's[10] method.

Preparation of LPS. The various LPS preparations were obtained commercially (Difco Laboratories, Detroit, Michigan) and used without further purification. The lyophilized powder was taken up in sucrose medium (2 mg. dry weight per ml.) and aliquots of the suspension were added to experimental flasks. The LPS, in the absence of substrate, did not stimulate mitochondrial respiration.

Polarographic Studies. Mitochondrial oxygen consumption was measured polarographically with a Clark platinum electrode (Yellow Springs Instrument Company) at a temperature of 22.5° C. The assay medium contained 2.4 ml. of a solution of 0.25 M sucrose, 10 mM tris (pH 7.4), 8.5 mM potassium acid phosphate and 4 mM tris-alpha ketoglutarate, and 0.6 ml. of mitochondrial suspension containing 1.2 to 1.8 mg. of protein. The adenosine diphosphate-to-oxygen ratio (ADP/O) and respiratory control index (RCI) were calculated according to the method of Chance and Wil-

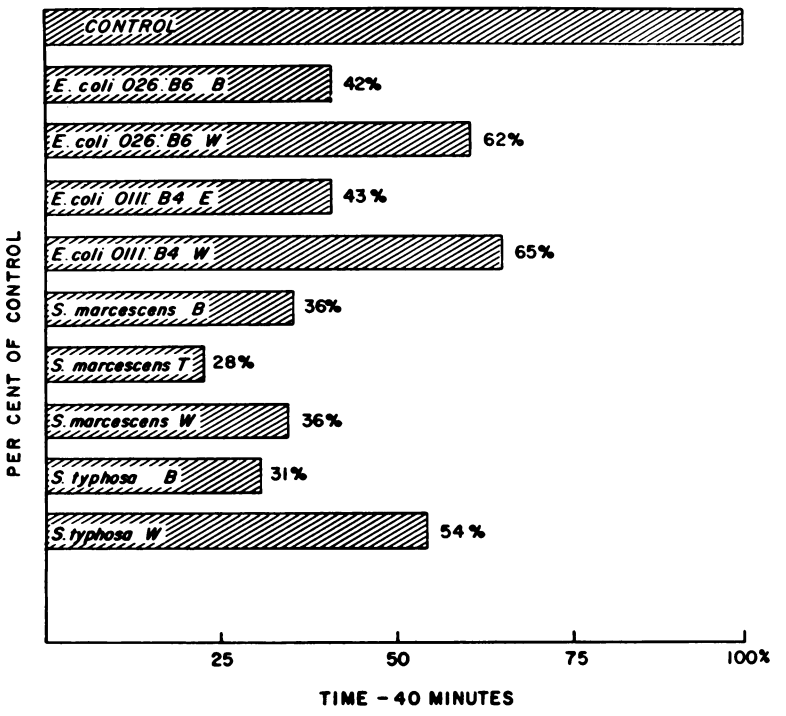

FIG. 1. Warburg studies on isolated human liver mitochondria. There is a depression of mitochondria respiration secondary to incubation with various bacterial lipopolysaccharides.

TABLE 1. *Comparison of Respiration Rates, Respiratory Control Indices, and ADP/O Ratios for Succinate Oxidation by Liver Mitochondria of Control and Endotoxemic Humans and Rats*

	Human Assay—*In Vitro* Challenge				Endotoxemic Rat Assay—*In Vivo* Challenge			
	Respiration Rate				Respiration Rate			
	Without ADP	With ADP	RCI	ADP/O	Without ADP	With ADP	RCI	ADP/O
				Control				
Mean	16.3	45.64	2.8	2.03	17.3	81.31	4.7	4.06
				Endotoxemia				
Mean	15.3	24.86*	1.6*	1.13†	11.1	19.98*	1.8*	2.90†

* Significantly different from control group as determined by the Student *t*-test (unpaired data, degrees of freedom = 12) with a probability $p < 0.01$.
† $p > 0.01 \leq 0.05$.

liams.[2] The RCI is equal to the rate of oxygen uptake stimulated by ADP, divided by the rate prior to addition of ADP. In the present study, both ratios were computed on the basis of results obtained from the second addition of ADP (300 millimicromoles) to the reaction vessel.

Electron Microscopic Studies. In Part I of the study, portions of the human mitochondrial pellet were processed by immersion fixation in cacodylate-buffered glutaraldehyde, then in 2% osmium tetroxide, also cacodylate-buffered, and subsequently were embedded in Epon 812. In Part II of the experiment, a portion of the rat livers was processed and embedded in the same manner.

Results

Figure 1 shows in graphic form the effect of LPS of different bacterial origins and methods of extraction upon succinate-stimulated respiration. Inhibition was most pronounced with the LPS of *E. coli* 026:B6 and 0111:B4 extracted by the Boivin method.[1] Westphal-extracted[14] LPS from the same strains was less inhibitory. LPS extracted by the Boivin method from *Salmonella typhosa* inhibited respiration more than the Westphal-extracted LPS. Endotoxin prepared from *Serratia marcescens* was markedly inhibitory as trypsin-extracted and Westphal-extracted material.

Table 1 shows the comparison of respiration rates, respiratory indices and ADP/O ratios for succinate oxidation in human liver mitochondria challenged *in vitro* and rat liver mitochondria challenged *in vivo* with *E. coli* LPS. In the presence of ADP, the rate of oxygen uptake of LPS-challenged mitochondria isolated from human liver or of endotoxemic rats was significantly lower than in the control group. However, in the absence of ADP there was no significant difference in respiration rates between the two groups. Both the RCI and ADP/O ratio were significantly higher for the control preparations.

Electron microscopic photographs revealed alterations in the mitochondrial pellets isolated from the human liver, that is, swelling and dissolution of the internal architecture. The mitochondrial pellets from the control human mitochondria occasionally showed the same type of swelling and structural alteration, but not to the degree nor with the frequency observed in the pellets obtained from the endotoxin-challenged mitochondria (Fig. 2a & 2b).

Electron microscopic photographs of the control rat liver preparations (Fig. 3a) revealed normal-sized mitochondria distributed throughout the cell, whereas in the LPS-treated rats, the mitochondria were swollen and enlarged (Fig. 3b).

From these observations, there appears

to be a correlation between the polarographic data and the morphologic changes found in the electron microscopic photographs of the mitochondria.

Discussion

Attention has been directed recently to changes in the enzymatic processes of subcellular organelles following endotoxemia. It has been shown that the lysosome is biochemically altered following endointoxication with LPS from gram-negative enteric bacilli.[8, 13] Harris and Green[6] reported an inhibiting effect of *Bordetella bronchiseptica* on rat liver mitochondrial respiration. These authors were, however, unable to show the same effect on mitochondrial respiration from *E. coli* endotoxin. Our studies with different forms of LPS indicate that there is an inhibition of mitochondrial respiration secondary to the *in vitro* challenge of various endotoxins on human mitochondria. They also indicate a differential inhibition, dependent on the strain and type of extraction of LPS. The basis for the differential inhibition probably lies in physical and chemical differences in the toxins attributable to extraction technics. Previous workers[4] have shown that the chemical composition of LPS extracted from *S. marcescens* was markedly influenced by the strain of bacteria, conditions of culture growth, and method of extraction.

The polarographic studies of the isolated human mitochondria also show that the *E. coli* 026:B6 LPS has an uncoupling effect. This is evidenced by the slower succinate respiration rate after the addition of adenosine diphosphate, in contrast to the control human liver mitochondria; that is, a

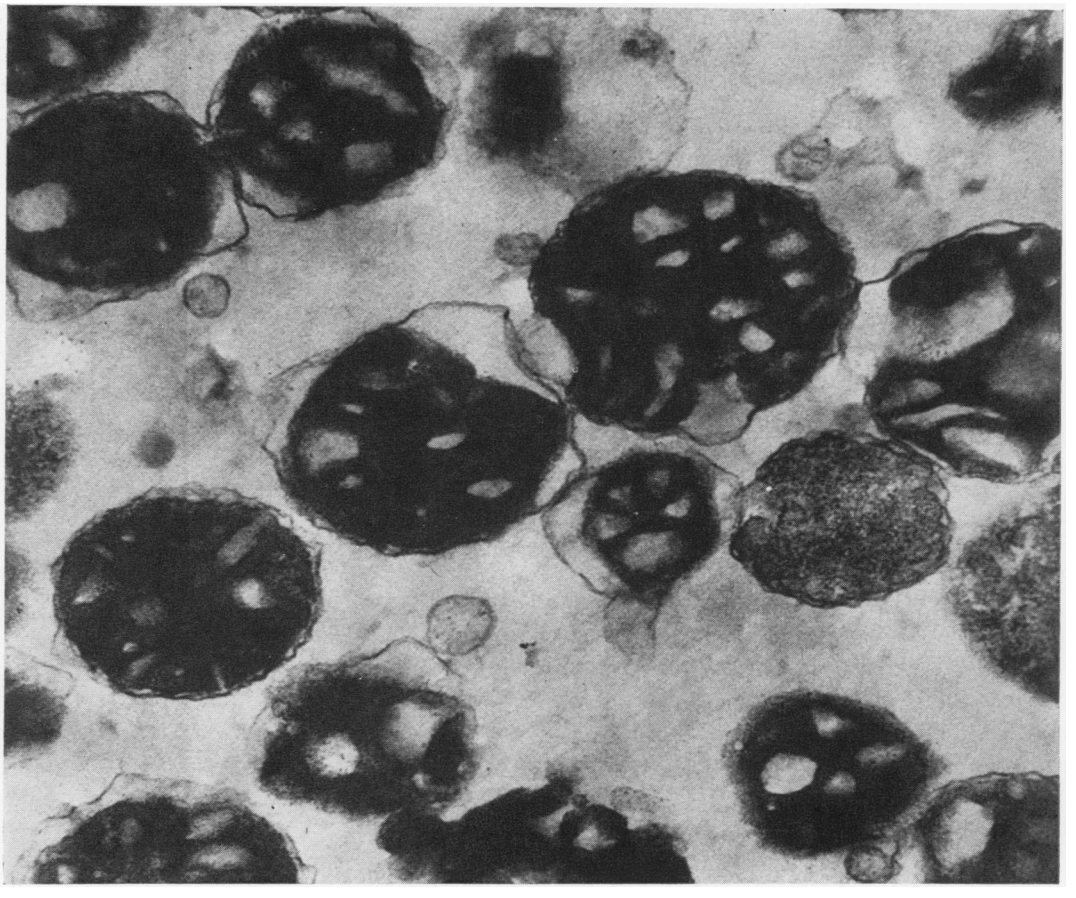

FIG. 2a. Isolated human liver mitochondrial pellet in 0.25 M sucrose showing condensed conformation (×33,000).

lower RCI and lower ADP/O ratio (Table 1). There is a risk in extrapolating this effect clinically; theoretically this effect would translate in an increase in oxygen utilization with less production of energy phosphates (adenosine triphosphate). This picture is similar to the uncoupling effect of thyroxin in thyroid storm and may offer an explanation for the increased febricity and energy deficit seen in humans with endotoxemia.

If LPS has an effect on mitochondrial respiration in an *in vitro* challenge, it should have the same effect in an *in vivo* challenge, to extrapolate these findings to clinical endotoxemia or shock. The second part of the study was designed to determine whether the effect was the same in either type of challenge. It was found that rat liver mitochondria were affected in a manner similar to isolated mitochondria challenged *in vitro*. The ADP/O ratio and RCI were decreased when compared to the controls.

Correlative electron microscopic studies were performed in both parts of the study. The isolated control human mitochondria revealed a typical "condensed" conformation when these mitochondria were incubated in 0.25 M sucrose, described in 1966 by Hackenbrock[5] as decrease in matrical volume, increase in matrical density, irregular pattern of inner membrane folding, and increase in volume in the outer compartment and intracristal space. These mitochondria are said to be in State I, that is, in an environment that has low substrate, low ADP, oxygen present, and a slow respiration rate. These mitochondria with adequate substrate and ADP are capable of oxidative phosphorylation. When the iso-

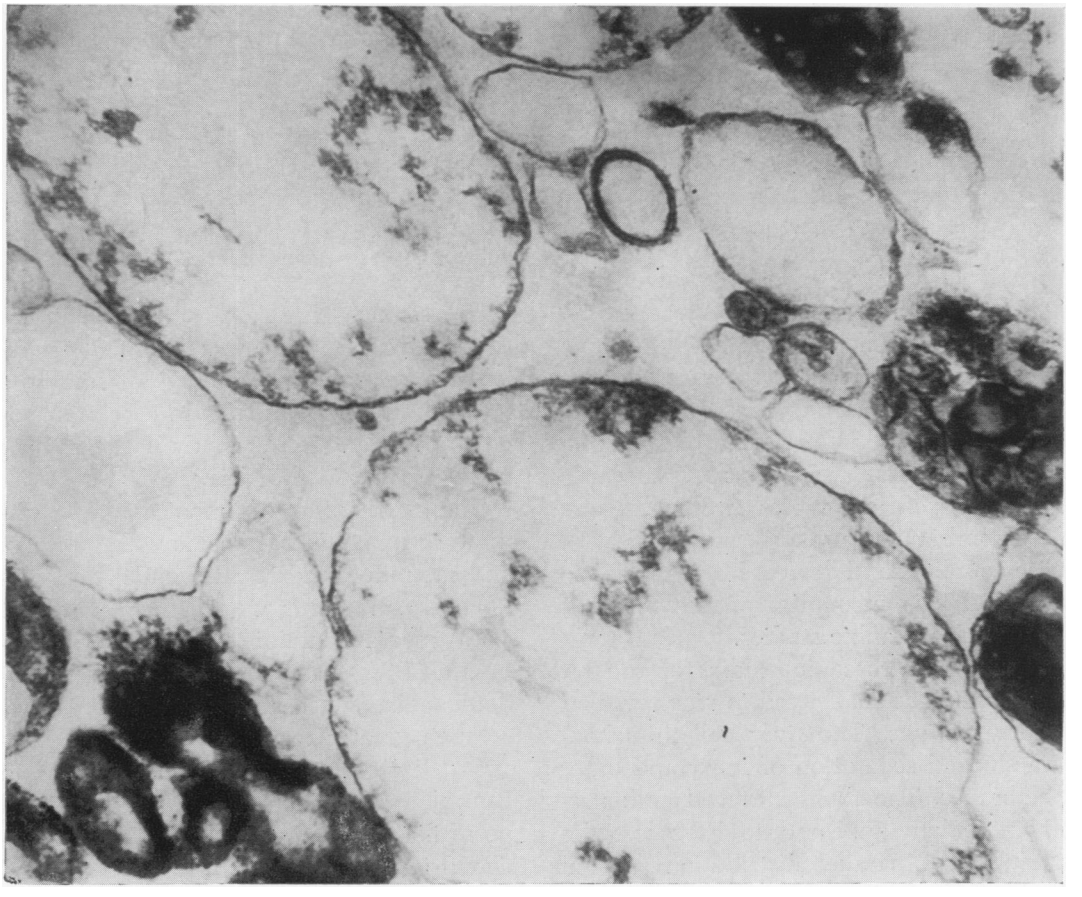

FIG. 2b. *E. coli* LPS 026:B6 effect on isolated human liver mitochondria. There is marked mitochondrial swelling (×33,000).

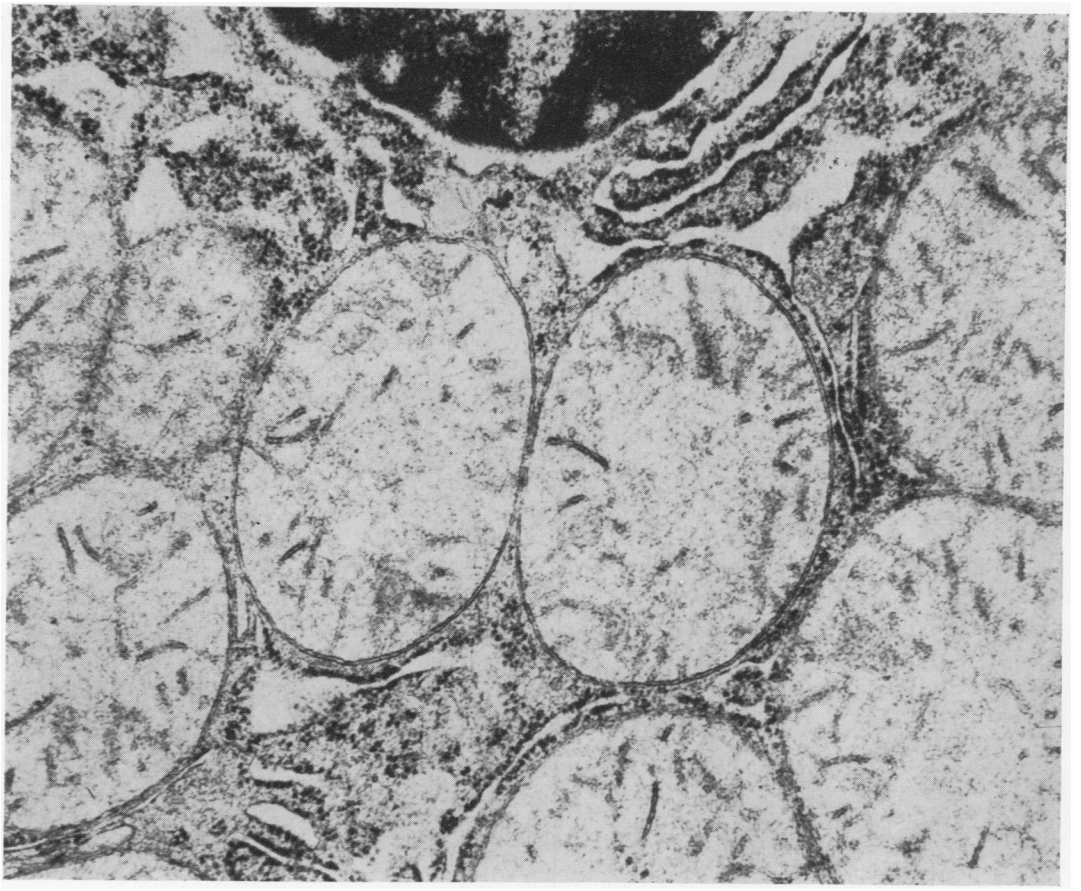

FIG. 3a. Rat liver biopsy specimen of normal liver showing normal mitochondria and endoplasmic reticulum (×24,000).

lated mitochondria are exposed to the *E. coli* LPS, mitochondrial swelling occurs. The inner or limiting membrane is discontinuous. The small matrical granules are widely dispersed. Cristae may either be absent or, if present, may not stain because of the marked amount of swelling. These mitochondria are usually associated with loose coupling, and decreased ADP/O ratio and RCI. This uncoupling effect was noted in our polarographic studies.

Electron microscopic studies of the rat liver specimens challenged *in vivo* revealed similar findings. The liver cell mitochondria showed separation of inner and outer membranes. The rough-surfaced endoplasmic reticulum appeared to have lost the silhouette of parallel distribution seen in the control rat. The frequency of vesicular arrangement of the endoplasmic reticulum was a striking feature. From these studies it appeared that there was a correlation between the polarographic data and the morphologic changes found in electron microscopic photographs of the isolated liver or liver cell mitochondria.

The mechanism of impairment in respiratory function that occurs within human and rat mitochondria after endotoxemia is unknown. However, we recently found that assay media containing bovine serum albumin (BSA) have a salutary effect on endotoxin-challenged mitochondria. BSA has a much greater restorative effect on mitochondria from endotoxemic rats than from control animals and this suggests several possibilities. Restoration of altered mitochondrial function by BSA, as evidenced by an increased efficiency of oxidative phosphorylation, has been reported by Helinski

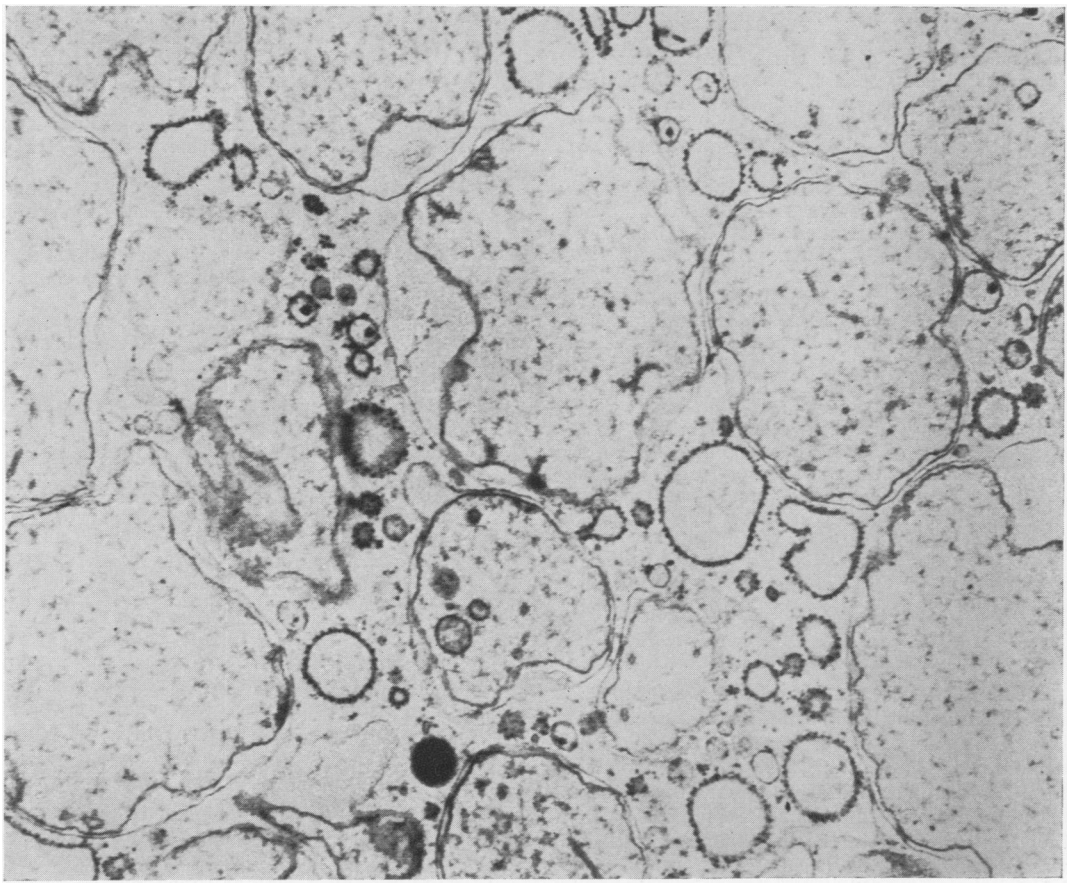

FIG. 3b. Rat liver mitochondria from rats which were rendered endotoxemic after intraperitoneal injection of *E. coli* LPS 026:B6 (×22,000).

and Cooper,[7] who suggested that BSA binds long-chain free fatty acids, thereby removing these inhibitory and uncoupling agents. Mellors and co-workers[9] found evidence that BSA relieves the uncoupling effect of lysosomal membrane fragments. Possibly, free fatty acids or lysosomal membranes, or both, are responsible for altered mitochondrial function and morphology in endotoxemia, thus providing a promising lead for further investigation of the mechanism of the endotoxic effect on mitochondrial respiration.

Summary

1. *E. coli* endotoxin 026:B6 inhibits succinate-stimulated respiration in human and rat liver mitochondria.

2. An *E. coli* endotoxin challenge *in vitro* produces an uncoupling effect on human mitochondria, as shown by polarographic studies. This uncoupling effect is also found in the mitochondria of rats challenged *in vivo*.

3. There is a correlation between polarographic data and the morphological changes found in electron microscopic photographs of the mitochondria.

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Discussion

Dr. George H. A. Clowes, Jr. (Boston): *I* am not sure the term *uncoupling* is correctly used here. It is an inhibition, certainly, that has been demonstrated to us from the production of ATP from ADP and it is caused by endotoxin, apparently, both in the mitochondrial preparation from the liver cells and in the live animal in which it was injected into the peritoneum.

One may say: What difference does this make to a surgeon? If this is true, it gives an explanation we have sought for a long time. The very high cardiac output that is required for survival of patients with serious sepsis has been shown, most of whom had coliform types of infections. As Thal, Siegel, Border, as well as ourselves have shown, there is an inefficient use of the circulation. Not as much oxygen is extracted from the blood that passes through the capillaries as normally would occur, which means that there are functional shunts working in the peripheral circulation. What has been shown this afternoon provides one of several explanations for this process. It means that the tissues, for lack of oxygen or ability to carry out oxidative phosphorylation, are probably producing metabolites causing an opening of vessels in the periphery. This is a wasteful use of the circulation.

This is the first time I have actually seen such a demonstration. The results of this brief examination of the electron photomicrograph would suggest that the lipopolysaccharide (endotoxin) had injured the mitochondria and produced swelling. Failure of energy conversion to produce ATP is exactly what happens when swelling of mitochondria occurs for other reasons. In other words, there has been an interference with the membrane in which enzyme and other biochemical activity of mitochondria is located. If it is true that this interference occurred after putting the material into the peritoneum, then we have the experimental counterpart for patients with severe peritonitis.

We must learn how to block this action and we are nowhere near that knowledge. At present we can only eliminate the source of the endotoxin by treating the infection, and possibly use steroids to restore normal membrane function.

Dr. William Schumer (Closing): The question of uncoupling is one of semantics, of definition. We define *uncoupling* as decreased ADP/C ratios—the ratio of decreased production of energy phosphates to oxygen consumed—and a decrease in the respiratory control index, which is the consumed oxygen with ADP over the consumed oxygen without ADP.

The classic uncoupling that occurs with poisoning is not found here. Rather, this is what we call "non-classic" uncoupling, although it is, in itself, an uncoupling.

I really can not add much to what Dr. Clowes has said—this is a study which probably asks more questions than it answers. We are in the process now of determining the type of effect produced by lipopolysaccharide, that is, whether this is primary or secondary. There are a number of factors that cause uncoupling; for example, increased fatty acids in the system, or lysosomal membranes, which supports Janoff's theory that there is an increase in lysosomal enzymes in endotoxemia.

The fact that uncoupling does not occur until 18 hours after injection of the lipopolysaccharide indicates that this is probably a late manifestation and may be one of the irreversibility factors in endotoxic shock.