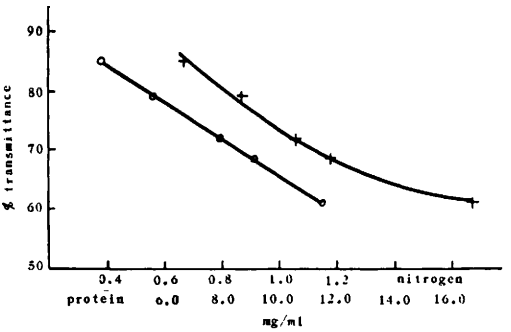
Structure and Function of Mitochondria in Irreversible Hemorrhagic Shock. II.* (24001)

H. HIFT AND J. G. STRAWITZ† (Introduced by P. P. Cohen)
Cardiovascular Research Laboratory and Department of Surgery, University of Wisconsin Medical School, Madison

The first paper of this series cited reasons for our belief that the phenomenon of irreversibility in hemorrhagic shock was associated with defects in energy metabolism. Consequently, it seemed promising to study structural and functional state of mitochondria from dogs subjected to prolonged hypotension. Results reported in earlier papers have justified these assumptions: heart mitochondria from dogs in irreversible hemorrhagic shock yielded low P/O ratios and were altered morphologically(1). Furthermore, they did not undergo swelling and "crescent" formation as readily as normal controls under a variety of *in vitro* conditions(2). These findings suggested the possibility that, in shock, heart mitochondria acquired some unusual component or lost some normal constituent. The problem was approached by obtaining absorption spectra and chemical analyses on various kinds of mitochondrial extracts which differed in protein and coenzyme contents. The data to be reported here indicate that the alterations in composition associated with hemorrhagic shock in dogs are probably of a quantitative rather than a qualitative nature.

Methods. Procedures used for production of irreversible hemorrhagic shock in dogs and for isolation of heart mitochondria from experimental and paired control animals have been described(1). Briefly, dogs were bled and maintained at an arterial pressure of 40 mm Hg for 5 to 6 hours. Mitochondria were isolated by differential centrifugation in 0.38 M sucrose. The final pellets were suspended in 0.5 M sucrose. They were standardized turbidimetrically by diluting aliquots 1:100 in sucrose and reading the % transmittance at 680 mμ against water in a Coleman Universal Spectrophotometer. Each preparation was examined by phase contrast microscopy to ascertain its purity and structural integrity as judged by the absence of myofibrils and swollen mitochondria. *Severity of shock* was appraised routinely on the basis of (1) behaviour of the animal during the hypotensive period, particularly its ability to maintain the established hypotensive level; (2) degree of congestion and hemorrhage into the vital organs observed at necropsy; (3) extent to which mitochondria had undergone the morphological alterations characteristic of shock (1). *"Desoxycholate solutions"* of mitochondria were prepared by exposing the suspensions to 1% Na-desoxycholate. *Water extracts* were obtained by diluting the suspension with 5 or more volumes of distilled water, incubating in a 37°C water bath overnight and collecting the clear supernatants by centrifugation. For *perchloric acid extracts* the particles were exposed to 3 or 6% perchloric acid at 6°C overnight. After neutralization, volumes were adjusted (dil. 1:4 or 1:5) and supernatants collected by centrifugation. *Proteins* were determined by Gornall's method (3) in either desoxycholate solutions or water extracts. *Total nitrogen* was estimated by Johnson's method(4) or by titration after digestion and steam distillation of the ammonia formed into boric acid. *Phosphate* levels in the various supernatant fractions were determined essentially by the Fiske-Subbarow method(5) before and after a 15 minute hydrolysis in 1 N sulfuric acid in boiling water. Total pellet phosphate was estimated in the same way in the original suspensions after 5 hours' digestion with conc. sulfuric acid in the presence of a copper-selenite catalyst. *Absorption spectra* were determined using a Beckman DU Spectrophotometer. The data were *evaluated statistically* by applying the t-test to the differences between the individual pairs comprising each series of experiments.

* Supported by grants from Wis. Heart Assn., Department of Army, and Nat. Heart Inst. U.S.P.H.S.
† Fellow of Am. Heart Assn.



A fresh mitochondrial suspension was diluted with varying amounts of 0.5 M sucrose

FIG. 1. Correlation of turbidity measurements with the protein and nitrogen contents of a suspension of dog heart mitochondria.

Results. Turbidity measurements may be taken as rough estimates of the number of particles unit volume. This assumption is supported by the observation that, within a critical range, successive dilutions of a mitochondrial suspension yield protein and nitrogen values which vary approximately linearly with % transmittance (Fig. 1). In each experiment, therefore, the paired preparations from shocked and control dogs were adjusted to give the same turbidity reading in the general range of 72 to 80%. Their protein and nitrogen contents were usually found to be very similar and apparently unaffected by the severity of shock. In 25 experiments the average transmittance for both the normal and abnormal preparations was 77.5%, the average protein levels were 7.14 and 7.13 mg/ml respectively and the nitrogen contents 0.82 mg/ml for both.‡

This gross similarity of the 2 types of mitochondria was further borne out by the absorption spectra of desoxycholate solutions: one of 7 such pairs is given in Fig. 2a. These preparations were considered to represent more or less the whole mitochondrion in a dispersed, if not dissolved, state. They exhibited large protein peaks between 270 and 280 mμ and distinct Soret bands between 410 and 416 mμ, presumably mostly due to cytochromes. Absorption in the flavin and adenine regions was not pronounced.

In spite of this apparently close resemblance in protein and coenzyme contents, the total phosphate level in mitochondria derived from shocked dogs was abnormally high, especially so in cases of severe shock (Table I).

TABLE I. Total Pellet Phosphate in Heart Mitochondria from Normal and Shocked Dogs.

Shock	μM/mg N		
	Normal	Shocked	% increase
Slight	2.96	2.90	.0
	2.49	2.84	14.0
Moderate	2.16	2.54	17.6
	3.08	3.94	27.9
Severe	3.05	3.42	12.1
	2.64	3.09	17.0
	2.65	3.42	29.1
	2.94	4.24	44.2
	2.00	3.10	55.0
	P <.003		

These differences were found to be highly significant. Unfortunately, since these values were obtained after digestion, they yielded no clue as to the form in which this surplus phosphorus was present in the original particle.

Treatment of mitochondria with water left behind an insoluble residue of lipoidal and proteinatious material. Such extracts were, therefore, less concentrated than the desoxycholate solutions. Table II lists the percentages of the original protein and nitrogen recoverable in the aqueous supernatants. Fig. 2b gives a representative pair of spectra. Preparations derived from shocked dogs showed some tendency to be more water sol-

TABLE II. Percent Protein and Nitrogen Extracted into Water from Suspensions of Heart Mitochondria from Normal and Shocked Dogs.

% protein		% nitrogen	
Normal	Shocked	Normal	Shocked
23.2	59.0	14.8	44.8
14.4	14.4	37.9	27.5
17.3	23.8	29.6	47.0
19.8	29.5	29.1	54.7
30.6	27.1	35.6	50.8
17.7	22.9	20.2	10.8
18.5	29.2	16.5	7.9
22.0	23.5		
13.5	30.4	38.5	48.6
22.0	18.8		
		23.8	55.6
P <.059		P <.084	

‡ For unknown reasons, however, daily variations were quite great, so that any particular transmittance value could not be correlated with any standard protein or nitrogen level.

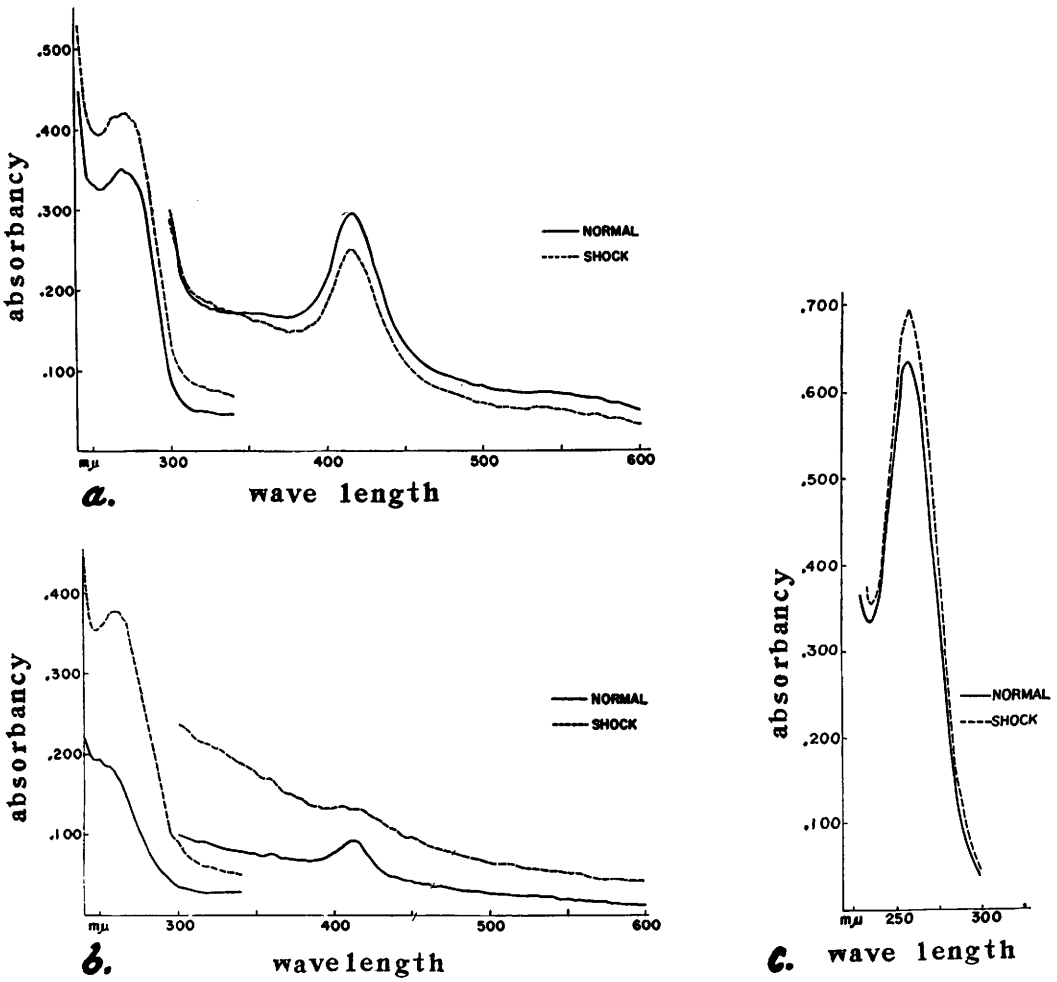


FIG. 2. Pairs of absorption spectra of heart mitochondria from normal and shocked dogs. a. Desoxycholate solutions: mitochondrial suspensions representing 0.94 mg/ml N (normal) and 1.08 mg/ml N (shocked) cleared in 1% Na-desoxycholate and diluted with water 1:5 (visual range) and 1:20 (U.V. range). b. Water extracts: normal = .32 mg/ml protein, .03 mg/ml N; shocked = .66 mg/ml protein, .08 mg/ml N. For U.V. spectrum the preparations were further diluted 1:3. c. Perchloric acid extracts: N-contents = app. 6-7 γ/ml.

uble. However, the extracted material did not appear to be grossly abnormal, for the pairs of absorption spectra became almost superimposable if reduced to the common denominator of 1 mg nitrogen. The peaks at 258 to 260 mμ corresponded to roughly 1 μM adenine/mg nitrogen. General absorption in the flavin region and the Soret band at 410 to 412 mμ also revealed nothing abnormal. The number of experiments was too small, however, to demonstrate any correlation between degree of solubility and severity of shock.

Perchloric acid extracts of mitochondria were free of the bulk of protein- and lipid-bound material and contained the dissociable purine and pyrimidine derivatives. The 2 types of mitochondria appeared very much alike under these conditions, both qualitatively and quantitatively. The extracts contained roughly 4% of the original nitrogen and exhibited clean peaks at 258 mμ (Fig. 2c). The spectra were not altered qualitatively when the mitochondria were preincubated in the presence of either the complete phosphorylating system or of the hexokinase-glucose trap alone.

Phosphate determinations run on these extracts revealed a tendency on the part of the

TABLE III. Phosphate Levels in Perchloric Acid Extracts of Heart Mitochondria from Normal and Shocked Dogs.

	μM/mg N of original suspension			
	Normal		Shocked	
	$P_{inorganic}$	$P_{acid\text{-}labile}$	$P_{inorganic}$	$P_{acid\text{-}labile}$
	.10	.04	.20	.01
	.20	.12	.23	.11
	.20	.18	.33	.18
	.19	.03	.20	.03
	.49	.28	.52	.31
	.17	.04	.30	.05
	.76	.37	1.16	.49
	.74	.11	1.27	.09
Avg	.36	.15	.53	.16
			$P < .042$	

Phosphates determined before ($= P_{inorganic}$) and after 15 min. hydrolysis in 1 N H_2SO_4 at 100°C. Diff. between the 2 determinations $= P_{acid\text{-}labile}$.

abnormal mitochondria to be richer than the normal in "inorganic" but not in acid-labile phosphate (Table III): Some of the surplus pellet phosphate apparently was present in the inorganic or a closely related form.

Discussion. On the whole, and within the limitations of the technic used, the spectral data suggest that heart mitochondria of shocked dogs do not contain any grossly abnormal constituent nor do they seem to lack any normal component. They tend to accumulate abnormal amounts of phosphate, some of it in a form which is extractable with perchloric acid and behaves like inorganic phosphate under Fiske-Subbarow conditions. Simultaneously, their proteins seem to undergo some physico-chemical alterations which result in a greater solubility in water. The protein fraction involved appears to contain bound flavins and possibly other substances absorbing at 260 mμ. This bound material is apparently not dissociable in perchloric acid.

The accumulation of phosphates within the mitochondria of shocked dogs complements our earlier observations on lowered P/O ratios. In view of the fact that some of this phosphate is in a form which reacts as inorganic phosphate under Fiske-Subbarow conditions, it is likely that the particles take up inorganic phosphate from the medium quite normally. The defect, therefore, appears to involve either the synthesis of ATP within the mitochondrion or the delivery of ATP to an external receiver system such as the hexokinase trap in Warburg experiments or the myofibrils in living muscle.

Summary. Heart mitochondria from dogs in irreversible hemorrhagic shock and from paired control animals were analyzed for protein, nitrogen and phosphate and their absorption spectra were compared. Similar analyses were done on water and perchloric acid extracts. The results indicate that, in shock, mitochondria accumulate abnormal amounts of phosphate, some of it in a form which is soluble in perchloric acid and reacts as inorganic phosphate under Fiske-Subbarow conditions. Simultaneously, some of the intramitochondrial proteins become more readily extractable with water.

1. Strawitz, J. G., Hift, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 641.

2. ———, *Surg. Forum,* 1957, v7, 11.

3. Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.,* 1949, v177, 751.

4. Johnson, M. J., in Umbreit, Burris, Stauffer: *Manometric Techniques and Tissue Metabolism,* Burgess Publishing Co., 1951, 191.

5. Fiske, C. H., Subbarow, Y., *J. Biol. Chem.,* 1925, v66, 375; 1929, v81, 629.

Received February 5, 1958. P.S.E.B.M., 1958, v98.