REFERENCES

[1] J. J. WOLKEN, *Ann. N.Y. Acad. Sci.*, 56 (1953) 873.
[2] J. J. WOLKEN AND F. A. SCHWERTZ, *J. Gen. Physiol.*, 37 (1953) 111.
[3] J. J. WOLKEN, A. D. MELLON AND C. L. GREENBLATT, *J. Protozool.*, 2 (1955) 89.
[4] J. J. WOLKEN AND A. D. MELLON, *J. Gen. Physiol.*, 39 (1956) 675.
[5] S. H. HUTNER AND L. PROVASOLI, *The Biochemistry and Physiology of Protozoa*, Academic Press, Inc., New York, 1951, p. 27.
[6] E. I. RABINOWITCH, *Photosynthesis and Related Processes*, I, Interscience Publishers, Inc., New York, 1945, p. 538.
[7] E. L. SMITH, *J. Gen. Physiol.*, 24 (1941) 565.
[8] E. L. SMITH AND E. G. PICKELS, *J. Gen. Physiol.*, 24 (1941) 753.
[9] J. J. WOLKEN AND F. A. SCHWERTZ, *Nature*, 177 (1956) 136.
[10] J. J. WOLKEN, *J. Cellular Comp. Physiol.*, 48 (1956).
[11] J. H. C. SMITH AND V. M. K. YOUNG, *Radiation Biol.*, 3 (1956) 393.
[12] L. N. M. DUYSENS, *Ann. Rev. Plant Biochem.*, 7 (1956) 25.
[13] J. J. WOLKEN, *Abstr. Papers, 130th Natl. Meeting Am. Chem. Soc.*, Atlantic City, New Jersey, September 16–21, 1956, p. 48C; and J. J. WOLKEN AND R. A. EVERSOLE (unpublished data).
[14] F. H. JOHNSON, H. EYRING AND M. S. POLISSAR, *The Kinetic Basis of Molecular Biology*, John Wiley & Sons, Inc., New York, 1954, p. 272.
[15] C. N. HINSHELWOOD, *The Chemical Kinetics of the Bacterial Cell*, Oxford University Press, 1946, p. 257.
[16] R. C. ST. GEORGE, *J. Gen. Physiol.*, 35 (1952) 495.
[17] F. S. SJÖSTRAND, *J. Cellular Comp. Physiol.*, 42 (1953) 15.
[18] K. MÜHLETHALER, *Intern. Rev. Cytol.*, 4 (1955) 197.
[19] R. HUBBARD, *J. Gen. Physiol.*, 37 (1953–1954) 381.
[20] R. S. LYTHGOE AND J. P. QUILLIAM, *J. Physiol.*, 93 (1938) 24.
[21] S. HECHT, *J. Gen. Physiol.*, 3 (1920–1921) 285.
[22] H. S. DUTTON, W. M. MANNING AND B. M. DUGGAR, *J. Phys. Chem.*, 47 (1943) 308.

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SOME CYTOCHEMICAL CHARACTERISTICS OF A PHOSPHORYLATING DIGITONIN PREPARATION OF MITOCHONDRIA

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The mechanism and even the constituents of the oxidative phosphorylating system of mitochondria are largely unknown. Earlier work[1] has given indications that mitochondrially-bound nucleotides, mostly of the adenine type, are involved in this process, since when mitochondria are treated so that they lose their phosphorylating ability, they also lose their bound nucleotides. It has recently been shown[2,3,4] that extracts of mammalian mitochondria could be prepared which contained the enzymes necessary for oxidative phosphorylation, but there was no indication of the presence or absence of possible co-factors such as the intra-mitochondrial nucleotides. This paper describes the properties of a digitonin preparation of broken mitochondria, and in brief it shows that this preparation contains no whole mitochondria, that it has oxidative phosphorylation ability, and that it contains the same nucleotides, and in the same proportions to each other, as are found in whole mitochondria.

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METHODS

Mitochondria from the livers of fed, 150–200 g Sprague-Dawley rats were obtained from 0.44 *M* sucrose homogenates as previously described[5]. Digitonin extracts of these mitochondria were made by a modification of the method of LEHNINGER and co-workers[2]. The mitochondria from approximately 60 g wet weight rat liver were homogenized in 60 ml of a 1% water solution of digitonin (Merck and Co. water-soluble product). The somewhat cleared suspension was stored at 0° C for 30 min and was then centrifuged at 13,000 × *g* for 10 min. The supernatant from this was then centrifuged at 25,000 × *g* for 20 min and a final centrifugation of this supernatant was made at 105,000 × *g* for 60 min. The reddish-brown, translucent pellets formed at 25,000 × *g* and at 105,000 × *g* were washed with several changes of water and finally resuspended in small volumes of cold water. Aliquots were removed from each suspension for phosphorylation studies and the remainder was used for the extraction and the ion-exchange chromatography of the acid-soluble nucleotides as previously described[1]. The tentative identification of the nucleotides contained in the extract was made as described earlier[1]. Oxygen consumption was determined manometrically, while phosphate uptake was determined by the disappearance of inorganic phosphate as measured by the Fiske-Subbarow method. The digitonin preparation from the mitochondria of approximately 3 g wet weight rat liver was enough to give an oxygen consumption (with succinate as substrate) of 120–140 μl O_2 in 30 min, with a phosphate uptake of from 8 to 10 μmoles, both at 30° C. The reaction mixture contained 0.02 *M* succinate, 0.01 *M* phosphate buffer, pH 7.0[2], 0.002 *M* adenosine diphosphate (ADP), 0.02 *M* glucose, 0.005 *M* $MgCl_2$, $1 \cdot 10^{-5}$ *M* cytochrome *c*, and 0.1 ml hexokinase (Sigma Co, St. Louis).

RESULTS

When isolated mitochondria are suspended in 1% digitonin, they lose 60–80% of their ability to oxidize succinate and apparently all of their phosphorylative ability. After an initial centrifugation to remove whole mitochondria, the supernatant is centrifuged at 25,000 × *g* and the supernatant from this at 105,000 × *g* to give two pellets. Both these pellets contain succinoxidase activity (*cf.*[6]), but only the 25,000 × *g* pellet has any phosphorylative ability. The P/O ratio of this pellet is decreased by 20–50% if Mg^{++} is omitted from the test medium. The 25,000 × *g* pellet could be re-suspended in water and re-centrifuged at the same speed to give a washed pellet with unimpaired phosphorylative ability and with the same partial requirement for added Mg^{++}.

These observations are in disagreement in two points with those reported by LEHNINGER and co-workers[2] who applied the digitonin in a slightly different manner. In their work phosphorylation activity was found in a fraction sedimented at 105,000 × *g* and the P/O ratio of this pellet was maximal when Mg^{++} was omitted from the test medium. In our preparation, no phosphorylation occurred in the 105,000 × *g* fraction and that which was recovered in the 25,000 × *g* fraction was enhanced by the addition of Mg^{++} (*cf.*[1]). The reasons for these differences are not understood, but may lie in differences in the particular digitonin preparation used and in the way in which it was applied.

The 25,000 × *g* pellet obtained from digitonin-treated mitochondria was fixed, embedded, sectioned, and examined in the electron microscope following procedures described earlier[5]. The contents of the pellet (Fig. 1) proved to be in the form of aggregates of small (approx. 500 A) elements. Between these aggregates are threads (about 100 A in diam.) of material which is less dense than the material comprising the aggregates. The aggregates appear to be composed mainly of vesicles 200–500 A in diameter, together with masses of ill-defined material. No intact mitochondria were observed in the sections of the pellet.

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In an earlier study[5] of mitochondria disrupted with deoxycholate, it was possible to identify the resulting material as being membranous and as being derived from the membranes of the mitochondria. This identification rested mainly on experiments in which pellets of isolated, intact mitochondria were overlaid with deoxycholate. In sections cut through these pellets the gradual disruption of the mitochondria could be traced. Intact mitochondria, unexposed to the reagent, were found at the bottom of the pellet, while elements on the surface, which were fully exposed, resembled the membrane pellet obtained by differential centrifugation of a suspension of mitochondria treated with 0.3% deoxycholate. Between these two extremes was a wide region of intermediate steps. Similar procedures were tried in the present work, but no such gradual disruption was observed. Instead, the process appeared to take place with little or no intermediate zone between intact mitochondria and the material seen in Fig. 1. It was therefore impossible to establish on morphological grounds the source within the mitochondria of the elements observed after treatment with digitonin. However, the fact that this material has succinoxidase activity, and that in the previous studies[6] this enzyme complex was found to reside in the mitochondrial membranes, suggests that some of the material in the 25,000 × *g* pellet after digitonin treatment consists of membranes. It is therefore presumed that the vesicular elements seen in Fig. 1 are derived from mitochondrial membranes.

The digitonin pellet also contains phosphorylative ability, but whether this enzyme system is derived from or is a part of the mitochondrial membrane, or is attached to the membrane by an action of digitonin, cannot be decided at present (*cf.*[6]). The deoxycholate preparation was made up of mostly large vesicular structures with no dense material inside the vesicles[5], while the digitonin preparation (Fig. 1) contains many small vesicles with much dense material inside. Whether this dense material is digitonin which, according to LEHNINGER and co-workers[2], comes down with the phosphorylating pellet and cannot be washed out, or whether it is mitochondrial material, is not known.

The extraction procedure was not always successful in giving good phosphorylating preparations (*cf.*[2]). Unlike the earlier digitonin preparation[2], the present preparation does not show an increased specific phosphorylating activity over that of whole mitochondria. This preparation contains approximately 10% of the protein-N, 10% of the phosphorylative ability, and 10% of the bound nucleotides of the whole mitochondria. Thus it would appear that upon disintegrating the mitochondria digitonin is, at least by the present method, not a selective agent, as deoxycholate is with respect to membranes[5,6]. It is probable that the present preparation contains parts of the mitochondrial membranes together with parts of the other constituents in the matrix of the mitochondria, and thus digitonin appears to act in our hands by disrupting the mitochondria without separating one constituent from another.

The striking finding with these preparations of disintegrated mitochondria is that they still contain acid-soluble nucleotides. This fact supports an earlier belief[1] that these nucleotides are bound to some of the mitochondrial proteins and probably to the mitochondrial structure in such a way that they resist solubilization by digitonin*. That the presence of these nucleotides can be correlated with oxidative phosphorylation is indicated in the data in Table I. This table gives a comparison of the proper-

* After treatment with digitonin, approximately 50% of the mitochondrial proteins are solubilized, that is, cannot be sedimented at 105,000 × *g* for 60 min.

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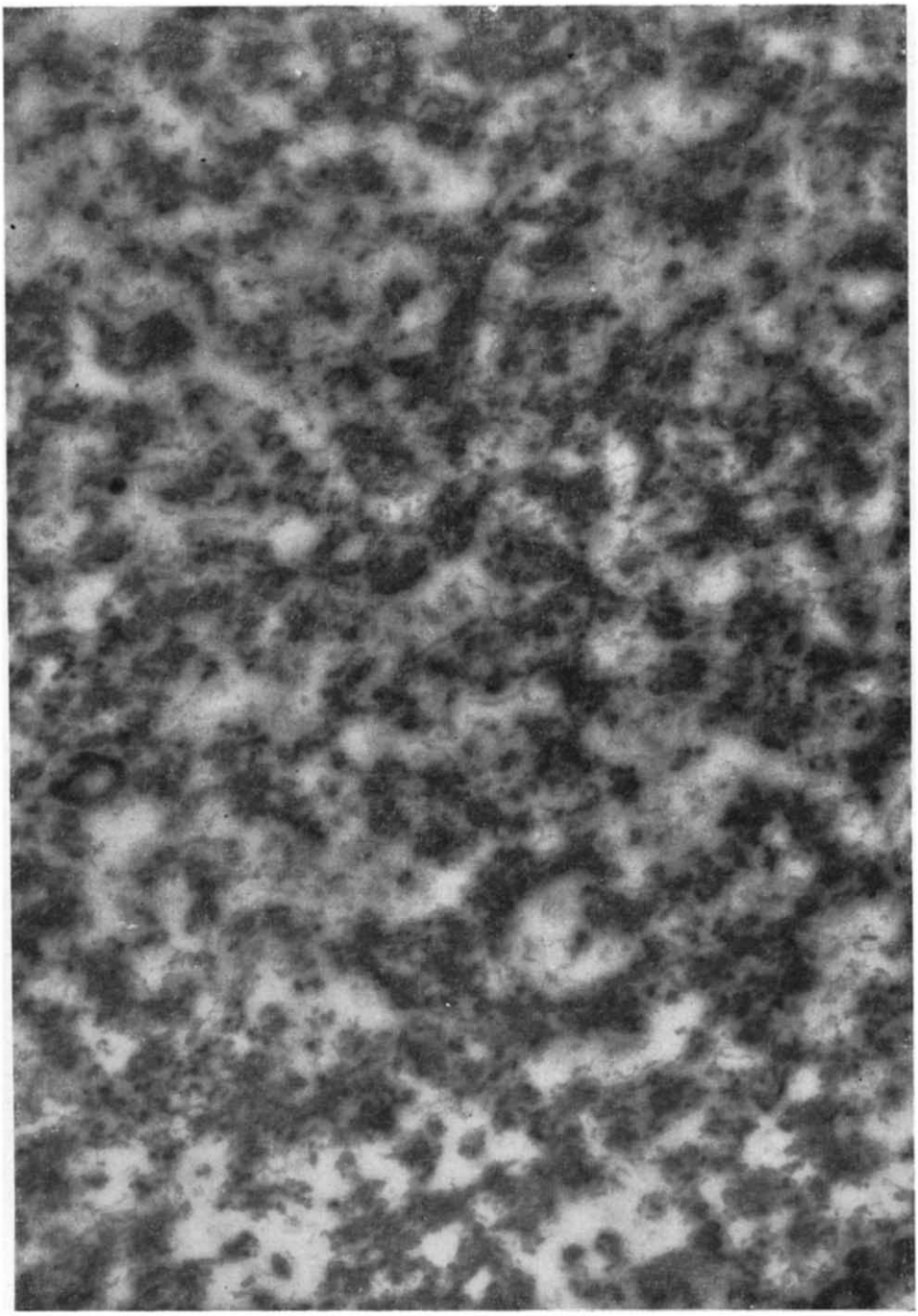

Fig. 1. Electron micrograph of a section through 25,000 × *g* pellet from a suspension of isolated mitochondria treated with 1 % digitonin. Nothing resembling intact mitochondria could be found in this pellet. The material present is mostly in the form of aggregates containing many vesicular elements 200–500 A in diameter. Also present is amorphous or unresolved material. Between the aggregates are pale, thread-like elements 100–200 A in width. × 31,500.

TABLE I

COMPARISON OF P/O RATIOS AND NUCLEOTIDE CONTENTS OF MITOCHONDRIA AND OF DIGITONIN PREPARATIONS OF MITOCHONDRIA

Nucleotides (Peak No.)	*Expt. 1*	*Expt. 2*	
	Digitonin ppn.	*Digitonin ppn.*	*Mitochondria**
	—μatoms O_2/60 min/mg prot.-N		
	30.7	23.6	30.9
	—μmoles IP/60 min/mg prot.-N		
	9.3	17.4	36.4
	P/O		
	0.30	0.74	1.18
	μmoles nucleotide/mg prot.-N/10^{-3}		
1	2.0	15.4	7.7
2	< 0.5	8.0	8.5
3 (DPN)	< 0.5	6.3	2.6
4 (AMP)	5.8	35.4	48.3
5	< 0.5	2.9	3.3
6	< 0.5	5.9	16.1
7 (ADP)	7.6	32.0	41.4
8	1.5	10.9	14.1
9	1.5	9.1	4.0
10	< 0.5	23.4	14.3
11 (ADX)	4.1	18.9	37.4
12 (ATP)	4.7	13.1	23.8
13	< 0.5	2.3	3.8

* The mitochondria used in this study were left in distilled water at 0° C for the length of time (usually 75 min) that it took to prepare the digitonin preparation from the same batch of mitochondria. The digitonin preparation and the mitochondria were then tested for oxidative phosphorylation at the same time. Suspending the mitochondria in water for this length of time could account for their lowered P/O ratio.

ties of different digitonin preparations, with different P/O ratios, and in a qualitative positive correlation, with different concentrations of all of these nucleotides. The digitonin preparation with a higher P/O ratio has the same nucleotides, and in approximately the same proportions to each other, as do the mitochondria from which this preparation was derived. The digitonin extract with a lower P/O ratio has much less of these nucleotides, with only adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), and ADX (*cf.*[1]) being predominant. Also, in Expt. 2 it can be seen that with a 40% loss in the P/O ratio in the digitonin preparation as against the whole mitochondria, there is a similar loss this time in only a few nucleotides; namely cpd. 6, ATP, and ADX*. These last two compounds have previously been found[1] to be reduced in concentration in whole mitochondria when these mitochondria have been damaged by pre-incubation with fluoride so as to lower their phosphorylating efficiency.

It appears, circumstantially at least, that the presence of these intramitochondrial nucleotides in some bound form, particularly ATP and ADX, is an essential requisite for coupled phosphorylation. Mitochondria, or digitonin preparations, with

* Compound ADX is thought to be a derivative of triphosphopyridine-nucleotide (personal communication from VAN R. POTTER).

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a relatively high P/O ratio contain these nucleotides; some digitonin extracts having a low P/O ratio contain less of these compounds; and it has been found that the deoxycholate extracts of mitochondria, which have no phosphorylating ability, contain very little or none of these compounds. A comparison (Table I) of the properties of the digitonin preparations indicates that the presence of not all of these nucleotides is necessary for oxidative phosphorylation to occur, and of those which are necessary, only a small proportion of the amount contained in whole mitochondria are of vital importance for coupled phosphorylation. The evidence so far[1] (*cf.* above) indicates that only 10–20% of the bound nucleotides of whole mitochondria are concerned in coupled phosphorylation, and of all these compounds, perhaps only 10–20% of the ATP and of the ADX are of prime importance.

The difference between the phosphorylating digitonin preparations and the non-phosphorylating deoxycholate preparations[6] could be that the former contains while the latter lacks the phosphorylating enzymes. However, it is possible that both preparations contain the necessary enzymes, and the real difference between them lies in the fact that deoxycholate extracts the bound nucleotides from the mitochondria, while digitonin does not.

SUMMARY

1. Rat-liver mitochondria were disintegrated with 1% digitonin to produce a cleared suspension from which an enzymically active preparation could be isolated by differential centrifugation.

2. This preparation was examined with the electron microscope and was found to consist of aggregates of small vesicles and other material, with no intact mitochondria. The vesicles are thought to be derived from the membranes of the mitochondria.

3. This preparation contained oxidative phosphorylation ability and all of the acid-soluble bound nucleotides which are found in intact mitochondria, these nucleotides being in approximately the same proportions to each other as they are in the mitochondria. Circumstantial evidence was presented that some of these compounds are necessary for phosphorylation to occur.

REFERENCES

[1] P. SIEKEVITZ AND V. R. POTTER, *J. Biol. Chem.*, 215 (1955) 221; *ibid.*, 215 (1955) 237.
[2] C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 489;
T. M. DEVLIN AND A. L. LEHNINGER, *ibid.*, 219 (1956) 507;
C. COOPER AND A. L. LEHNINGER, *ibid.*, 219 (1956) 519.
[3] D. ZIEGLER, R. LESTER AND D. E. GREEN, *Biochim. Biophys. Acta*, 21 (1956) 80.
[4] W. W. KIELLEY AND J. R. BRONK, *Biochim. Biophys. Acta*, 23 (1957) 448.
[5] M. L. WATSON AND P. SIEKEVITZ, *J. Biophys. Biochem. Cytol.*, 2 (1956) 639.
[6] P. SIEKEVITZ AND M. L. WATSON, *J. Biophys. Biochem. Cytol.*, 2 (1956) 653.

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