REFERENCES

[1] A. T. WILSON, *Nature*, 182 (1958) 524.
[2] R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 28 (1944) 224.
[3] A. A. BENSEON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS AND W. STEPKA, *J. Am. Chem. Soc.*, 72 (1950) 1710.
[4] K. E. WILZBACH, *J. Am. Chem. Soc.*, 79 (1957) 1013.
[5] I. P. CRAWFORD, *Stanford University*, private communication.

Biochim. Biophys. Acta, 40 (1960) 522–526

VITAMIN D AND THE STRUCTURE OF KIDNEY MITOCHONDRIA*

HECTOR F. DELUCA, SHELDON REISER, HARRY STEENBOCK AND PAUL KAESBERG

University of Wisconsin, Department of Biochemistry, Madison, Wisc. (U.S.A.)

(Received October 23rd, 1959)

SUMMARY

An electron microscopic examination has been made of thin sections of kidney mitochondria isolated in 0.44 *M* sucrose from vitamin D deficient rats and from vitamin D treated rats. Vitamin D deficiency resulted in swollen and morphologically damaged mitochondria characterized by large intercristal spaces and disrupted cristal systems. These changes were prevented by vitamin D.

INTRODUCTION

The well-established accumulation of citrate in tissues following vitamin D administration[1] prompted our investigation of Krebs cycle oxidations in vitamin D treated and deficient rats[2]. It was found that vitamin D specifically reduced the oxidation of citrate and isocitrate by kidney homogenates and mitochondria[2,3]. This effect was also produced by the addition of vitamin D *in vitro*[4]. Further studies[5] to be published in detail later, suggested that the reduction in oxidation was not necessarily due to an enzymic inhibition, but was possibly due to physical inhibition of citrate penetration of the mitochondria. In support of this, it has now been found that the morphology of mitochondria isolated from vitamin D deficient rats differs markedly from that of vitamin D supplemented rats.

METHODS

Young, male Holtzman rats weighing 70–80 g were fed *ad libitum* an adequate Ca and P semisynthetic diet with and without vitamin D for 21–28 days as described earlier[1,2]. They were killed by a sharp blow on the head followed by decapitation. The kidneys

* Published with the approval of the director of the Wisconsin Agricultural Experiment Station. We are indebted to the Wisconsin Alumni Research Foundation for financial support.

were quickly immersed in ice-cold 0.44 *M* sucrose solution. A 10 % homogenate of the chilled tissues in 0.44 *M* sucrose was prepared with a Potter-Elvehjem homogenizer fitted with a Teflon pestle (A. H. Thomas Co., U.S.A.) and 10 ml of the homogenate was layered over 10 ml of 0.52 *M* sucrose in a lusteroid tube at 0°. The layered preparation was centrifuged in an International PR-2 refrigerated centrifuge equipped with a high speed rotor No. 296 at 800 × *g* for 12 min to remove nuclei, cellular debris, unbroken cells, etc. The upper layer was centrifuged at 3500 × *g* for 12 min to sediment the mitochondria. The resulting pellet, which was washed once with 0.44 *M* sucrose was a relatively pure mitochondrial preparation, as was revealed by both electron and phase-contrast microscopy. This method was a modification of the method used for the preparation of liver mitochondria in 0.44 *M* sucrose[6], since with kidney tissue that technique gave poor yields and impure preparations.

The mitochondria were fixed for 2 h in a buffered, 2 % osmium tetroxide-hypertonic sucrose solution (0.16 *M* veronal–acetate buffer, pH 7.2, 0.60 *M* sucrose, 0.08 *M* osmium tetroxide), essentially according to the method of PALADE[7]. The fixed pellet was broken up and washed 3 times with 0.32 *M* veronal–acetate buffer, pH 7.2. It was dehydrated by washing consecutively with ethyl alcohol of gradually increasing concentrations and finally with butyl methacrylate. Samples of the pellet were embedded at 49° for 36–48 h in a mixture of butyl methacrylate–methyl methacrylate (4:1) with 0.8 % benzoyl peroxide as the catalyst. Sections were cut with a Servall Porter-Blum microtome, and examined with an RCA EMU-2 or a Siemens Elmiscop I electron microscope.

RESULTS AND DISCUSSION

Preliminary observations with a phase-contrast microscope revealed that kidney mitochondria from vitamin D deficient rats were larger than those isolated from corresponding rats which had received vitamin D. Furthermore they indicated differences in structure. These indications have been confirmed and extended by observations with the electron microscopes on preparations made in parallel from vitamin D treated and vitamin D deficient rats.

In the vitamin D deficient rats there was a consistent increase and a greater variation in the size of mitochondria. Table I presents data obtained by measuring all the mitochondria in the central area of comparable electron micrographs. At least 25 mitochondria were measured for each experiment without selective elimination. Figs. 1 and 2 also show the difference in size and in addition a difference in structure. The preparations from the deficient rats are characterized by large intercristal spaces or vacuoles (C). In many cases the cristae are withdrawn from the outer mitochondrial membrane (E). Fragmentation or disruption of the cristal organization occurs with some degree of frequency (D). In most cases, however, the outer double membranes and cristae are still present.

By way of contrast, the mitochondria from the vitamin D treated rats are relatively intact structures (Fig. 2). The outer double membrane closely envelops a tightly and uniformly arranged cristal system in which few intercristal vacuoles appear. Disrupted cristal systems or cristae withdrawn from the outer membrane are rarely encountered.

To our knowledge, electron micrographs of isolated kidney mitochondria have

TABLE I

THE EFFECT OF VITAMIN D ON THE DIMENSIONS OF ISOLATED KIDNEY MITOCHONDRIA

The rats were fed an adequate Ca and P containing diet for 21–28 days. Where indicated the rats received 75 I.U. of vitamin D every 3 days. Each figure represents an average measurement of 25 mitochondria from a given experiment.

Group	*Expt. No.*	*Greatest diameter* μ	*Smallest diameter* μ
No vitamin D	1	0.91 ± 0.25*	0.47 ± 0.12*
	2	0.98 ± 0.25	0.58 ± 0.23
	3	1.00 ± 0.31	0.61 ± 0.15
	4	1.05 ± 0.36	0.51 ± 0.12
	5	0.95 ± 0.36	0.52 ± 0.18
Average		0.98 ± 0.31	0.54 ± 0.16
Plus vitamin D	1	0.62 ± 0.18	0.39 ± 0.09
	2	0.85 ± 0.19	0.61 ± 0.12
	3	0.77 ± 0.18	0.43 ± 0.10
	4	0.84 ± 0.22	0.51 ± 0.12
	5	0.87 ± 0.18	0.51 ± 0.09
Average		0.79 ± 0.19	0.49 ± 0.10

* Standard deviation = $\sqrt{\Sigma d^2/n}$.

not yet appeared in the literature, although SJÖSTRAN AND RHODIN[8] and PALADE[9] have published micrographs of kidney mitochondria *in situ*. On the other hand, numerous micrographs of liver and heart mitochondria, both isolated and *in situ*, have been published[6,8–10]. WITTER *et al.*[6], found that liver mitochondria isolated in 0.44 *M* sucrose solutions closely approached the morphology of mitochondria in intact tissue. ZIEGLER *et al.*[10], also showed that the use of hypertonic sucrose solutions resulted in the isolation of nearly intact structures. Similarly, the isolation of kidney mitochondria (Fig. 2) from 0.44 *M* sucrose solutions yields relatively intact mitochondria as judged by their similarity to *in situ* micrographs[8].

The now abundant morphological studies on isolated mitochondria opens the opportunity for a correlation of structure with the ability to oxidize externally added citrate. It has been known[11] for some time that heart mitochondria cannot oxidize added citrate, but can oxidize internally generated citrate. This finding has been reaffirmed[12] recently and correlated with mitochondrial structure. It is also well established[9] that heart mitochondria contain a large number of cristae per unit of mitochondrial volume arranged in a tightly-knit branching system. In contrast to the heart particles, liver mitochondria readily oxidize external citrate and have comparatively few cristae which are widely separated from each other[9]. Furthermore, kidney mitochondria from normal rats (Fig. 2), which oxidize added citrate very slowly[5], approximate heart particles in their compact and dense cristal arrangement while those from vitamin D deficient rats, which oxidize citrate more rapidly, have more widely separated cristae (Fig. 1). From these spatial relations it might be permissible to assume that the citrate molecules are oxidized slowly because they have more difficulty in gaining access to the oxidative sites in the more densely packed particles. This is supported by the observation of ATP inhibition of citrate oxidation[5,11,13], since ATP is known to reverse or prevent mitochondrial swelling[14,17].

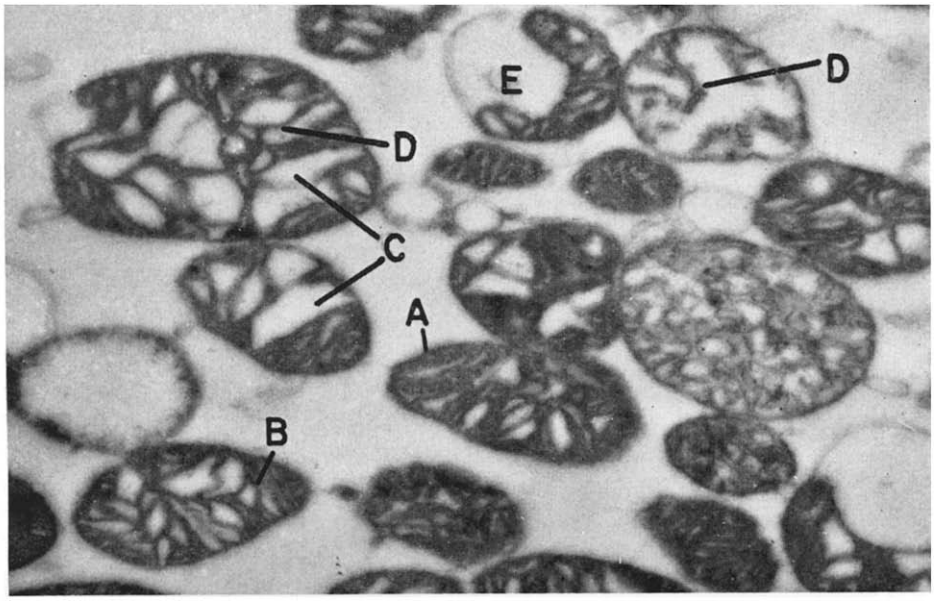

Fig. 1. Electron micrograph of kidney mitochondria isolated in 0.44 *M* sucrose from a vitamin D deficient rat (adequate Ca and P diet). The outer double membranes are clearly discernible (A), as are the cristae (B). Note: the large intercristal spaces (C); the swollen appearance of most mitochondria; the disrupted cristal arrangements (D), and the separation of the cristae from the outer membrane (E). Magnification: 34,000 ×.

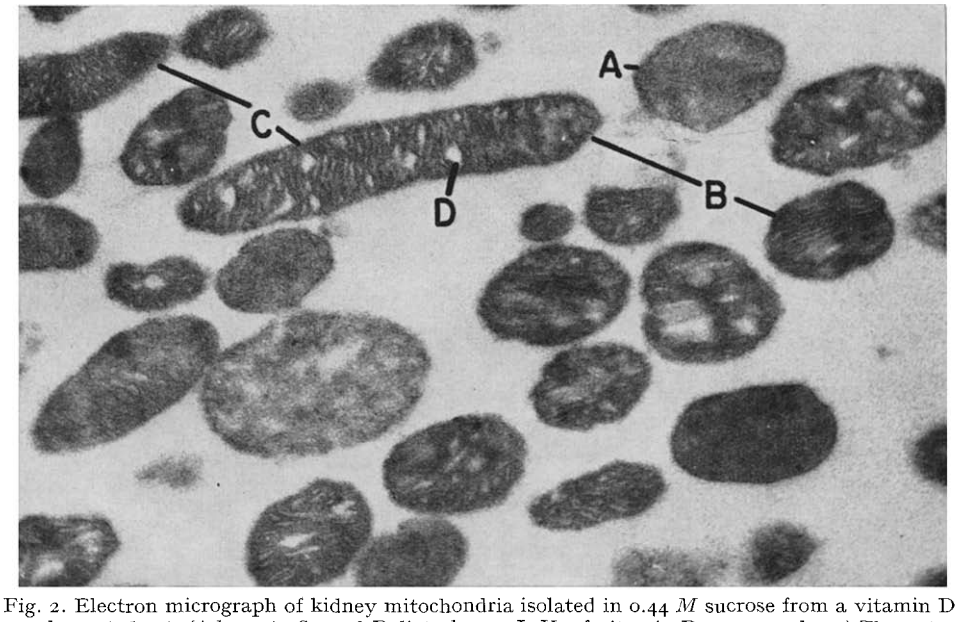

Fig. 2. Electron micrograph of kidney mitochondria isolated in 0.44 *M* sucrose from a vitamin D supplemented rat. (Adequate Ca and P diet plus 75 I. U. of vitamin D_2 every 3 days.) The outer double membrane (A) envelops a tightly packed and highly organized cristal system (B). Note the rods (C) and the small intercristal spaces (D) which are poorly visible in most cases. Magnification: 34,000 ×.

It is not certain that the morphological differences observed here occur in intact tissue as it is entirely possible that the deficiency in vitamin D so changes the mitochondria that they become susceptible to injury by the technique used for their isolation. This nevertheless means that vitamin D had effected some change in mitochondrial structure. There is a possibility that a difference in homogenate composition may also account for these changes, since numerous agents which promote or prevent mitochondrial swelling are now known[16,18]. This now appears unlikely, since suspending the mitochondria from vitamin D treated rats in the supernatant fraction from vitamin D deficient homogenates and vice versa resulted in no change in citrate oxidation.

Because of the intimate relation between vitamin D and Ca metabolism, the role of Ca and other cations should be considered with regard to mitochondrial damage. On the basis of our present knowledge it does not appear that these changes could have been effected through Ca, since vitamin D would be expected to elevate kidney Ca^2 and Ca is a known swelling agent for isolated mitochondria[16]. These studies on mitochondrial structure are being extended to give a more thorough understanding of factors affecting morphology.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Dr. H. HIFT for her generous help in the phase-contrast microscope work and to S. SACHS for her invaluable sectioning work.

REFERENCES

1 H. STEENBOCK AND S. A. BELLIN, *J. Biol. Chem.*, 176 (1948) 1075.
2 H. F. DELUCA, F. C. GRAN AND H. STEENBOCK, *J. Biol. Chem.*, 224 (1957) 201.
3 H. F. DELUCA, F. C. GRAN, H. STEENBOCK AND S. REISER, *J. Biol. Chem.*, 228 (1957) 469.
4 H. F. DELUCA AND H. STEENBOCK, *Science*, 126 (1957) 258.
5 H. F. DELUCA, S. REISER AND H. STEENBOCK, *Federation Proc.*, 18 (1959) 212.
6 R. F. WITTER, M. L. WATSON AND M. A. COTTONE, *J. Biophys. Biochem. Cytol.*, 1 (1955) 127.
7 G. E. PALADE, *J. Exptl. Med.*, 95 (1952) 285.
8 F. S. SJÖSTRAND AND J. RHODIN, *Exptl. Cell Research*, 4 (1953) 426.
9 G. E. PALADE, in O. H. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, Inc., New York, 1956, p. 185.
10 D. M. ZIEGLER, A. W. LINNANE, D. E. GREENE, C. M. S. DASS AND H. RIS, *Biochim. Biophys. Acta*, 28 (1958) 524.
11 G. W. E. PLAUT AND K. A. PLAUT, *J. Biol. Chem.*, 199 (1952) 141.
12 D. M. ZIEGLER AND A. W. LINNANE, *Biochim. Biophys. Acta*, 30 (1958) 53.
13 H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 190 (1951) 215.
14 J. RAAFLANB, *Helv. Physiol. et Pharmacol. Acta*, 11 (1953) 142.
15 F. E. HUNTER, JR. AND F. FORD, *J. Biol. Chem.*, 216 (1955) 357.
16 D. F. TAPLEY, *J. Biol. Chem.*, 222 (1956) 325.
17 K. W. CLELAND, *Nature*, 170 (1952) 407.
18 F. E. HUNTER, JR., J. F. LEVY, J. FINK, B. SCHUTZ, F. ONERRA AND A. HURWITZ, *J. Biol. Chem.*, 234 (1959) 2176.