A CRITICAL EVALUATION OF THE CONTAMINATION, BY LYSOSOMES, OF PREPARATIONS OF OUTER MEMBRANE OF MITOCHONDRIA.

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Several methods have been recently set up (cf. Quagliariello et al., 1967) to separate inner and outer membranes of mitochondria by density gradient centrifugation. Although these methods are powerful tools for the study of mitochondrial compartmentation, their validity must still be critically examined on morphological and enzymatic criteria. In this line of idea, Parsons et al. (1967) have evaluated the degree of microsomal contamination of mitochondrial membrane fractions. Other membranes such as lysosomal membranes may also be contaminant. In the course of a study undertaken in this laboratory to specify the localization and to understand the role of phospholipase A in mitochondrial membranes it became necessary to assess the purity of mitochondrial membrane preparations and especially to avoid as much as possible a contamination by lysosomes.

We thought it useful to present here some observations we made when using enzymic markers to evaluate the degree of contamination of membrane preparations which would appear homogeneous under the electron microscope. The results presented in this paper show that on discontinuous sucrose gradient lysosome membranes gather at the same place as outer mitochondrial membranes. Based on a previous work by Wattiaux, Wibo and Baudhuin (1963), a procedure is described which leads to a preparation of outer membranes of mitochondria practically devoid of lysosomes.

Materials and Methods

Isolation of membranes : Four days prior to sacrifice, rats were injected

intraperitoneously with 170 mg of Triton WR-1339 (Rohm and Haas, Philadelphia, Pa., U.S.A.) (Wattiaux, Wibo and Baudhuin, 1963). Rat liver mitochondria were then isolated by the classical differential centrifugation method from 10% liver homogenate in 0.27 M sucrose buffered with 2 mM Tris, pH 7.3. The nuclear fraction was sedimented at 10,000 g.min., mitochondria were sedimented from the supernatant by centrifugation at 53,000 g.min. in SS-1 servall centrifuge and washed twice with half the initial volume of 0.27 M sucrose. Five ml of the mitochondrial suspension (at circa 50 mg/ml) was placed on top of a 2-layer gradient formed by successively layering 22 ml of 41.4% (w/v) sucrose and 22 ml of 39.3% (w/v) sucrose and then centrifuging in a Spinco SW 25.2 bucket rotor at 23,500 rev./min. (55,000g) for 2 1/2 hrs. Up to this point all sucrose solutions were prepared with 2 mM Tris buffer, pH 7.3. Two fractions were obtained : the H-fraction floating on top of the gradient and mainly composed of lysosomes and the pellet (Γ' composed of mitochondria. The small amount of material which gathered at the interface was discarded. The mitochondrial pellet E was then treated as described by Parsons et al. (1967) for the isolation of inner and outer membranes : after swelling in 20 mM phosphate for 20 min. at 0°, the membranes were gathered by centrifugation at 7.10^5 g.min. then separated by differential centrifugation (Spinco Rotor 30). The inner membranes were spun first at low speed (45,000 g.min.) and outer membranes at high speed (7.10^5 g.min.). The inner membrane fraction was resuspended in 20 mM phosphate, pH 7.3, to be centrifuged a second time in a sucrose gradient in a SW 25.2 Spinco rotor. The re-run gradient consisted of 20 ml of 51.3% (w/v) sucrose, 12 ml of 37.7% (w/v) sucrose and 12 ml of 23.2% (w/v) sucrose ; the input consisted of 5 ml of inner membrane suspension (30 to 50 mg prot./ml) and was spun at 23,500 rev./min. (55,000g) for 90 min.. The outer membrane fraction was usually re-run in a SW-39 Spinco rotor. The re-run gradient consisted of 1.2 ml of 51.3% (w/v) sucrose, 1.2 ml of 37.7% (w/v) sucrose, 2.0 ml of 23.2% (w/v) sucrose and 1 ml of the input, it was spun at 38,000 rev./min. for 40 min..All these sucrose solutions were made in 20 mM phosphate (Parsons et al., 1967). Rat-liver microsomes were obtained by differential centrifugation in 0.27 M sucrose made in 2 mM Tris, pH 7.3, nuclei being eliminated at 6,000 g.min., mito-

chondria at 50,000 g.min. and lysosomes at 220,000 g.min.. The lysosomes used in the experiment described in Fig. 1 were isolated first by differential centrifugation between 53,000 g.min. and 220,000 g.min. in 0.27 M sucrose from rats which had received an injection of Triton WR-1339. They were purified on the two-layer sucrose density gradient described above to separate lysosomes from mitochondria, the lysosomes being obtained in the H-fraction. The protein content of the different fractions was estimated according to Lowry et al. (1951).

Enzymic determinations : Acid phosphatase (Ac.Ptase) was estimated with p-nitrophenylphosphate as substrate (Linhardt and Walter, 1965) and activity measured at 37° after unmasking by Triton X-100. Arylsulfatase was estimated with nitrocatechol sulfate as substrate at pH 5.2 and at 37°. (Dodgson, Spencer and Thomas, 1955). Glucose 6-phosphatase (G 6-Ptase) was determined by the amount of inorganic phosphate formed at 37° and at pH 6.5 according to de Duve et al. (1955). Monoamine oxidase (MAO) was measured at pH 7.5 and at 25° by following either the accumulation of benzaldehyde (Tabor et al. 1965) or the disappearance of kynuramine (Weisbach et al 1960). Cytochrome oxidase was assayed spectrophotometrically as described by Appelmans et al. (1955) ; since the oxidation of reduced cytochrome c is practically of first order with respect to its concentration, the rate-constant k was deduced from a logarithmic plot of the kinetics of the reaction. The rate of oxidation of reduced cytochrome c was calculated from the rate constant.

Monoamine oxidase was used as marker for outer mitochondrial membrane (Schnaitman et al., 1967), cytochrome oxidase for inner mitochondrial membrane, acid phosphatase for lysosomes and glucose 6-phosphatase for microsomes (de Duve et al., 1955). It is worth noting that a similar distribution of MAO activity in subcellular particles or membranes is found when using either the benzaldehyde or the kynuramine test. However since the latter is less sensitive, only MAO activity determined by the benzaldehyde test will be recorded.

Results

In preliminary experiments a relatively high acid phosphatase activity was found to be associated with outer membranes of mitochondria prepared

according to Parsons et al. (1967). Since acid phosphatase activity is located in lysosomes (de Duve, 1955), its presence in outer mitochondrial membrane preparations was taken as a proof of lysosomal contamination. Experiments to be described now show that actually lysosomal membranes obtained from purified lysosomes sediment exactly at the same place as outer membranes of mitochondria. A fraction enriched in Triton-loaded lysosomes (H-fraction) was prepared from rat liver as described in Materials and Methods. The H-fraction was then treated exactly as were mitochondria for a preparation of inner and outer membranes, according to Parsons et al. (1967) ; it was homogenized in 20 mM phosphate, pH 7.3, left for 20 min. at 0°, then placed on a three-layer sucrose gradient similar to the one used for the purification of outer mitochondrial membranes and spun at 38,000 rev./ min. for 40 min. (SW-39 swinging bucket). Most of the resulting material (O-fraction) was found at the first interface of the gradient, i.e. between the 25.2% and the 37.7% sucrose layers where outer mitochondrial membranes gather. As shown in Fig.1, the O-fraction is characterized by a very high acid phosphatase activity when tested either with p-nitrophenylphosphate or β-glycerophosphate as substrate.

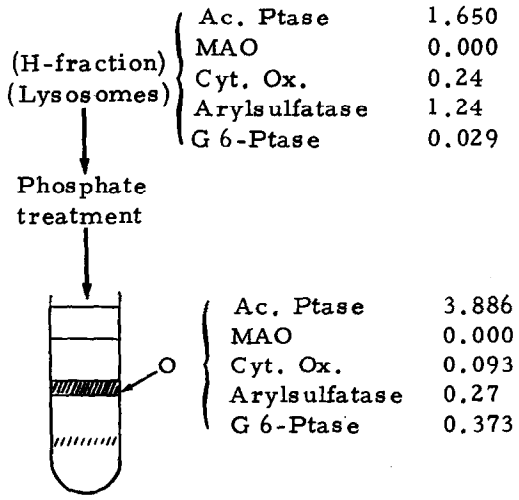

Fig.1 Enzymic activities of a lysosome fraction purified on a three-layer sucrose gradient (cf. Materials and Methods).
Activities are expressed as μmoles of substrate decomposed/min./mg of protein except for arylsulfatase (μmole/hr/mg of protein).

Because of presence of phosphate in the gradient and of necessary corrections when using β-glycerophosphate as substrate, the acid phosphatase activity was routinely assayed with p-nitrophenylphosphate. It is noteworthy that whereas the O-fraction is richer than the H-fraction in acid phosphatase, its arylsulfatase activity is lower. This is in agreement with Sawant et al. (1964) who found that arylsulfatase is a 100% soluble lysosomal enzyme while acid phosphatase is 44% particulate. In keeping with this finding, it is inferred that lysosomes are easily broken down by treatment with 20 mM phosphate and that the resulting lysosomal membranes have a density similar to that of the outer mitochondrial membranes. An important conclusion is that outer membranes obtained from mitochondria prepared by the classical differential centrifugation (which are therefore highly contaminated by lysosomes) are themselves contaminated by lysosomal membranes. It must be added that outer mitochondrial membranes can hardly be discriminated from lysosomal membranes on a morphological basis by electron microscopy (André J., personal communication).

The glucose 6-phosphatase activity which is found in the O-fraction (Fig.1) likely belongs to microsomes present as contaminants in this fraction. Actually, it was shown that when microsomes prepared by the classical differential centrifugation in 0.25 M sucrose are homogenized in a 20 mM phosphate medium, pH 7.3, in the same manner as mitochondria or lysosomes (cf. above) and are afterwards centrifuged through the three-layer sucrose gradient already described, the specific activity of glucose 6-phosphatase of the material which gathered at the first interface of the gradient is much higher (0.70 µmole P_i/min./mg prot.) than the one of the starting microsomes (0.24 µmole P_i/min./mg prot.). As shown in thin-section micrographs and in agreement with Parsons et al. (1967), this fraction mainly consisted of smooth vesicles of cytoplasmic reticulum (Vignais et al., 1968).

Preparations of mitochondria freed of lysosomes may be obtained from liver of rats which have been previously injected with Triton WR-1339 (Wattiaux, Wibo and Baudhuin, 1963) as described in Materials and Methods. As shown in Fig.2, the lysosomes present in the mitochondrial preparation are eliminated (H-fraction) by centrifugation on a discontinuous gradient. The E-fraction, characterized by a low

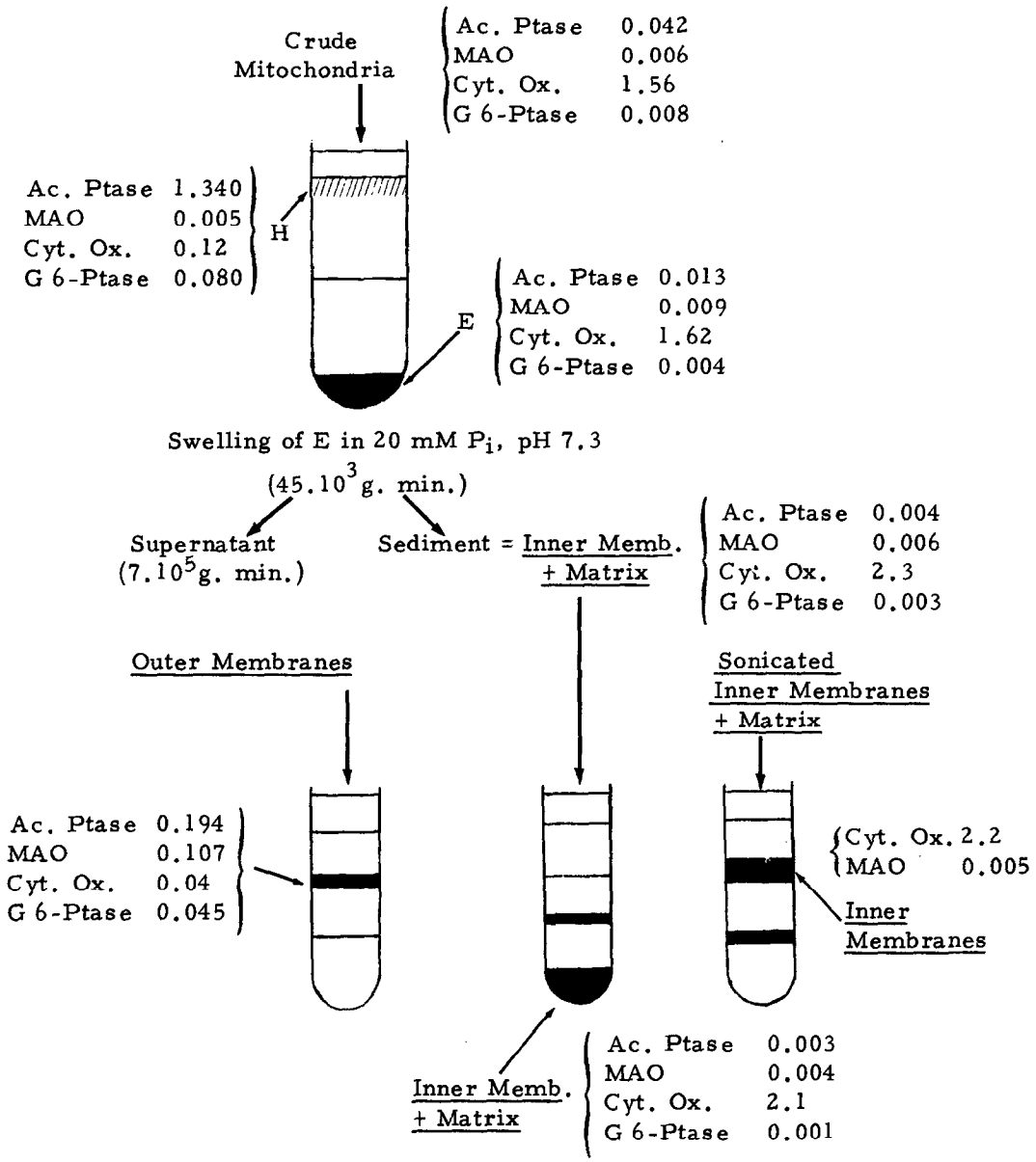

Fig.2 Purification of inner and outer mitochondrial membranes. The first gradient allows a purification of mitochondria from lysosomes (H-fraction). The outer membrane fraction and the "inner membranes + matrix" fraction were prepared according to Parsons et al. (1967). The inner membranes + matrix were resuspended in 0.25 M sucrose (35 mg protein/ml), one ml was placed directly on the gradient, another was first sonicated in a Branson sonifier at 10 Amp. for 2 min. prior to centrifugation.

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acid phosphatase activity, consists of mitochondria practically devoid of lysosomes (Vignais et al., 1968). Although being markedly purified

from lysosomal contaminants, these mitochondria have nevertheless lost their respiratory control. Mitochondria of the E-fraction treated by 20 mM phosphate buffer, pH 7.3, and centrifuged through the three-layer sucrose gradient, yield a fraction of outer membranes which gathers at the first interface of the gradient. Since the above fraction is considerably enriched in monoamine oxidase activity and practically devoid of acid phosphatase activity, it may be considered as a satisfactory preparation of outer mitochondrial membranes. The particles which sediment at the bottom of the tube and which consist mainly of inner membranes and matrix keep most of their cytochrome oxidase activity. It may be seen (Fig.2) that disruption of these particles by sonication results in a particulate material which, after centrifugation in a three-layer sucrose gradient at 39,000 rev./min. for 45 min., gathers at the first interface of this gradient. According to its cytochrome oxidase activity and its origin, the material is identified as the inner membranes of mitochondria.

In summary, it can be concluded that membranes obtained from various subcellular particles sediment at the same level in the three-layer sucrose gradient described by Parsons et al., (1967). This observation calls attention to the necessity of extensive purification of mitochondria before separation of their membranes. As shown in the accompanying paper, (Nachbaur, J. and Vignais, P.M., 1968) the ability to separate effectively mitochondria from lysosomes has allowed to demonstrate unambiguously that mitochondria contain a Phospholipase A_2 localized in the outer membrane.

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Références

Appelmans, F., Wattiaux, R. and de Duve, C., Biochem. J., (1955) 59, 438.

de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F., Biochem. J., (1955) 60, 604.

Dodgson, K.S., Spencer, B. and Thomas, J., Biochem. J., (1955) 59, 29.

Linhardt, K. and Walter, K., in "Methods of Enzymatic Analysis", (Bergmeyer, H.U., Ed.) Verlag Chemie, Weinheim, (1965) 2nd Edn, p. 783.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. J. Biol. Chem., (1951), 193, 265.

Nachbaur, J. and Vignais, P.M., Biochem. Biophys. Res. Comm., (1968), this volume.

Parsons, D.F., Williams, G.R., Thompson, W. Wilson, D. and Chance, B., in "Mitochondrial Structure and Compartmentation" (Quagliariello, E., Papa, S., Slater, E.C. and Tager, J.M., Eds) Adriatica Editrice, Bari, (1967), p.29.

Quagliariello, E., Papa, S., Slater, E.C. and Tager, J.M. (Eds) "Mitochondrial Structure and Compartmentation", Adriatica Editrice, Bari, (1967).

Sawant, P.L., Shibko, S., Kumta, U.S. and Tappel, A.L., Biochim. Biophys. Acta, (1964), 85, 82.

Schnaitman, C., Erwin, V.G. and Greenawalt, J.W., J. Cell. Biol., (1967), 32, 719.

Tabor, C.W., Tabor, H., Rosenthal, S.M., in "Methods in Enzymology" (Colowick, S.P. and Kaplan, N.O., Eds), Academic Press, Vol.II, (1955), p.390.

Vignais, P.M., Nachbaur, J., André, J. and Vignais, P.V., Symposium on Mitochondria-Structure and Function, Vth F.E.B.S. Meeting, Prague, (1968).

Wattiaux, R., Wibo, M. and Baudhuin, P., in "Lysosomes" (de Reuck, A.V.S. and Cameron, M.P., Eds), Ciba Foundation Symposium, J. and A. Churchill, Ltd, London, (1963), p.176.

Weisbach, H., Smith, T.E., Daly, J.W., Witkop, B. and Udenfriend, S., J. Biol. Chem., (1960), 235, 1160.