A new medium for the isolation of liver mitochondria

The medium most widely used for the isolation of mitochondria is 0.25 *M* sucrose solution[1], sometimes with the addition of "Versene" (ethylenediaminetetraacetic acid). Mitochondria isolated in sucrose solution have recently been examined under the electron microscope in this laboratory and elsewhere (*e.g.*[2,3]). Such mitochondria show degenerative changes as indicated by swelling, loss of osmium-staining contents, and damage to the membranes (Fig. 1).

These changes have been partially obviated by the use of 0.44 *M* sucrose solution containing citric acid[2], or, more successfully, by the use of 0.25 *M* sucrose solution containing polyvinylpyrollidone[3,4]. Fractions isolated in the latter medium cannot, of course, be analysed for tissue nitrogen unless the polyvinylpyrollidone is removed.

An alternative medium, virtually free of nitrogen, has now been found to give mitochondria little different from those seen in sections of rat liver. The medium is composed of raffinose hydrate, 0.23 *M* (13.6%); dextran, 6% (*vide infra*); Versene, 0.001 *M* (neutralized to pH 7.4 approx.); and heparin, 0.035% ("white", 106 units/mg). Adenosine-5'-phosphate is usually added (0.001 *M*; 0.035%), but its inclusion is not regarded as essential, nor is it considered necessary to adjust the pH of the medium to neutrality (*cf.*[3]).

The aim was to isolate mitochondria in quantitative yield, rather than to isolate a pure fraction of mitochondria as rapidly as possible. Accordingly, the tissue was thoroughly homogenized in the medium, by a Potter-Elvehjem homogenizer with a "Teflon" pestle rotating at 2,000 r.p.m. Nuclei and debris were removed by centrifugation for 10 min at 1,000 *g*, in an angle head at 2°. The cytoplasmic material was centrifuged for 15 min at 10,000 *g* to achieve complete sedimentation of the mitochondria, which were washed once without removal of the "fluffy layer", this being small with the medium now described. The mitochondrial pellet was then re-suspended in the medium.

The mitochondria were fixed, usually 2 hours after excision of the liver, by taking 0.1 ml of the suspension (this amount corresponding to 50 mg of original liver tissue) and adding it to 2 ml of fixative at 0° C. This suspension was then respun to a pellet and allowed to fix for 2 hours. The fixation was performed with the standard PALADE[5] fixative, *viz.* 1% osmium tetroxide buffered to pH 7.4. The addition of dextran or other components did not improve the fixation. The pellet was then washed, dehydrated and embedded in methacrylate using the standard technique for the examination of tissue sections under the electron microscope. Because the pellet showed stratification of components, it was found essential to cut the pellet in such a direction that the layers could be observed, so that a true picture could be offered of the whole mitochondrial fraction. The sections were examined with a Siemens electron microscope (Elmiskop 1).

Fig. 2 shows a representative section of the mitochondrial pellet. With a few exceptions, the mitochondria are unswollen, and double membranes can be discerned both peripherally and internally (see Fig. 2, inset) as in the mitochondria seen in cell sections. As would be expected,

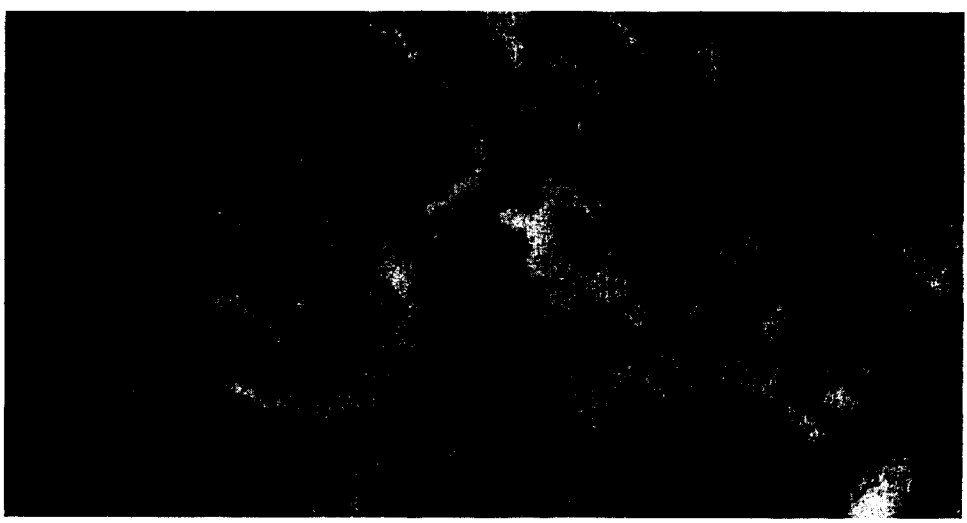

Fig. 1. Mitochondria isolated in 0.25 *M* sucrose medium.

Fig. 2. Mitochondria isolated in new medium (*cf.* Table I).

there appears to be some contamination with "reticular" (microsomal) elements, but this contamination is usually less than with isotonic sucrose medium.

In certain fractions which were rich in acid phosphatase, dense particles (of diameter approx. 0.2 μ) have been seen which resemble those recently observed by NOVIKOFF[3].

The considerations and experiments on which the choice of constituents is based will be fully described elsewhere[6]. Of the several dextrans examined, the most satisfactory has been a product ("Intradex", a sample of which was kindly provided by Mr. J. T. MARSH) of average molecular weight 145,000, supplied by Glaxo Ltd. as a 6% "salt-free" solution. The concentration of heparin is critical; in its presence the mitochondria do not readily agglutinate, even in electrolyte media (*cf.*[7]), but with a concentration of 0.1% or greater the homogenate may become extremely viscous.

Enzymic assays on mitochondrial fractions isolated in this medium have shown that the proportion of magnesium-activated ATP-ase which is "latent" is little different from that of mitochondrial fractions isolated in sucrose media. This finding supports the conclusion of WITTER *et al.*[2] that the latency of ATP-ase is a poor guide to the morphological integrity of mitochondria.

The medium, if present in high concentration in the assay system, has some inhibitory effect on magnesium-activated ATP-ase but not on glucose-6-phosphatase. Fractions prepared in this medium have been assayed not only for ATP-ase and glucose-6-phosphatase, but also (by Miss B. M. STEVENS) for succinic dehydrogenase and ribonuclease (pH 5), and (by Dr. I. LEWIN) for xanthine oxidase. In these assays, in which the diluent was water rather than the medium, there was no evidence for any inactivation or inhibition of the enzymes studied.

NOVIKOFF[8] has stated that it is possible to obtain preparations of mitochondria which are

virtually free of ribonucleic acid (RNA). With the medium now described, washed mitochondrial fractions obtained by low-speed centrifugation* (so as to minimize contamination with microsomal material) do contain RNA, in an amount corresponding to at least 3% of the protein present.

The experiments were performed during the tenure of a British Empire Cancer Campaign Fellowship (E.R.), and were supported by grants to this Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

M. S. C. BIRBECK
E. REID

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London (England)

1 C. H. HOGEBOOM, W. C. SCHNEIDER AND G. E. PALADE, *J. Biol. Chem.*, 172 (1948) 619.
2 R. F. WITTER, M. L. WATSON AND MARY A. COTTONE, *J. Biophys. Biochem. Cytol.*, 1 (1955) 127.
3 A. B. NOVIKOFF, *J. Biophys. Biochem. Cytol.*, 2 (1956) (in press).
4 A. B. NOVIKOFF, *3ème Congr. intern. Biochim., Brussels, 1955, Rapports*, p. 315.
5 G. E. PALADE, *J. Exptl. Med.*, 95 (1952) 285.
6 M. S. C. BIRBECK AND E. REID, to be published.
7 M. S. HOSTER, B. J. MCBEE, H. A. ROLNICK, Q. VAN WINKLE AND H. A. HOSTER, *Cancer Research*, 10 (1950) 530.
8 A. B. NOVIKOFF, *Symposia Soc. Exptl. Biol.*, 10 (1956) (in press).

Received February 21st, 1956

Purification and characterization of the lipemia-clearing factor of postheparin plasma

HAHN[1] first observed that intravenous administration of heparin cleared alimentary lipemia. The active principle of postheparin plasma which possessed clearing ability was named "lipemia-clearing factor" by ANFINSEN and co-workers[2]. Although many of the properties of the clearing factor have been elucidated[2,3,4,5,6,7,8] only very limited purification of this postulated enzyme has been reported. BROWN, BOYLE AND ANFINSEN[9] have achieved a 5-fold purification using ethanol fractionation. NIKKILÄ[10] has reported a 25- to 30-fold purification by repeated precipitation at low ionic strength, pH 5.4 to 5.8. Recently, we have developed a method of purification which yielded a 1480-fold increase in specific activity in the most active fraction, which contained 16% of the original clearing activity.

Method of purification: Postheparin plasma was obtained from dogs which had received 1 mg of sodium heparin* per kg of body weight. The first step in our procedure was similar to that of NIKKILÄ[10]—80 ml of postheparin plasma was diluted 1:15 with cold distilled water; the pH was adjusted to 5.7 by the addition of 0.1 *N* HCl. This mixture was allowed to stand at 4° C for 2 hours and then centrifuged. The precipitate (Fraction I) was made up in 20 ml of *M*/15 phosphate buffer, pH 7.8, and immediately frozen. This fraction is stable and can be kept in the frozen state for several weeks without any loss in clearing activity.

Our next and succeeding steps were carried out using ammonium sulphate saturated at 0° C and pH 5.70. The entire procedure was performed at 0° C. The fractionation was as follows: Fraction I was brought to 25% saturation with ammonium sulphate added dropwise. The mixture was centrifuged and the precipitate (Fraction II) was stored in the deepfreeze at —20° C. Fractionation was continued by bringing the supernatants to 33%, 40%, 50% and 64% saturation with ammonium sulphate. The precipitates at each step were stored as above. The final supernatant was dialyzed in the cold against *M*/15 phosphate buffer for 5 hours. It was then assayed for protein content and clearing ability. The various precipitates were thawed and dissolved in 2.0 ml of *M*/15 phosphate buffer except Fraction II, which required 10 ml of buffer. These were dialyzed as above. Each fraction was assayed for protein content and clearing activity.

It may be pointed out that reprecipitation of Fractions II to V with the corresponding concentrations of ammonium sulphate as described above resulted in a quantitative recovery of their clearing activity in the precipitates obtained. This suggested to us that the clearing factor may be firmly bound to the protein of each particular fraction.

Clearing activity was determined by the method described in an earlier publication[4]. The

* Heparin sodium, 10 mg/ml and 100 units/mg, was furnished through the courtesy of Dr. L. L. COLEMAN, The Upjohn Company, Kalamazoo, Michigan.