DEVELOPMENT OF AN IMPROVED MEDIUM FOR THE ISOLATION OF LIVER MITOCHONDRIA*

By M. S. C. BIRBECK and E. REID‡, Ph.D.

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

Plates 156 to 159

(Received for publication, June 4, 1956)

For the isolation and biochemical study of mitochondria, the medium in current use is that developed by Hogeboom, Schneider, and Palade (1), *viz.* an isotonic (0.25 M) or hypertonic (0.88 M) solution of sucrose. The advantages of this medium over electrolyte media, as previously developed in the laboratories of Bensley and of Claude, have been discussed elsewhere (2). Hypertonic sucrose, in comparison with isotonic sucrose, furnishes mitochondria of almost natural shape and size as judged by light microscopy, but is now regarded as less convenient for general use (2).

With the more recent application of electron microscopy to the study of isolated mitochondria, it is now generally recognized that the isolation of mitochondria in either hypertonic or isotonic sucrose medium entails considerable damage, with swelling (at least if isotonic sucrose is used) and with loss of structural and other material from the mitochondria. The present paper describes attempts to obviate this damage, by the development of a modified medium as briefly reported elsewhere (3).

A recent report (4) from Dounce's laboratory has described the use of an improved medium, *viz.* 0.44 M sucrose containing a trace of citric acid (pH 6.2). This medium, which was used in conjunction with an improved instrument for homogenizing the liver tissue, can furnish liver mitochondria which have partly, but not completely, retained the internal structure characteristic of mitochondria examined in tissue sections under the electron microscope. Novikoff (5, 6) has recently described a modified sucrose medium which can give liver mitochondria of good appearance. This medium, however, contains polyvinylpyrrolidone which must, of course, be removed before determinations of tissue nitrogen can be performed.

* The investigation was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) 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, United States Public Health Service.

‡ British Empire Cancer Campaign Research Fellow.

The medium which has now been developed is virtually free of nitrogen, and furnishes liver mitochondria the bulk of which are apparently undamaged, and which are relatively free of microsomal material.

Materials and Methods

Chemicals.—The materials purchased from commercial sources included raffinose hydrate (Hopkins and Williams, Ltd., or British Drug Houses, Ltd.), heparin (white, 106 I.U./mg.; Evans Medical Supplies, Ltd.), ATP[1] (disodium salt, "95 per cent pure"; Nutritional Biochemicals Corporation, Inc.), and AMP[1] (Nutritional Biochemicals Corporation, Inc.).

The following materials were donations for which grateful acknowledgement is made. Levan, obtained from grass and having a mean molecular weight of about 8,000 (7), was kindly provided by Dr. D. J. Bell and Dr. D. J. Manners (University of Edinburgh). "Dialyzable" dextran was a sample (number DB. 13) of molecular weight about 3,000, provided by Mr. A. R. Lockwood and Mr. A. E. James (Glaxo Laboratories, Ltd.), to whom thanks are expressed; it was dialyzed and recovered from the dialysate by freeze-drying, a procedure which freed the sample of a small amount of non-dialyzable dextran. Dextrans No. 1 and No. 2 were samples (numbers 318971 and 349779 respectively) of average molecular weight 11,800 and 35,000 respectively, kindly provided by Dr. H. E. Stavely (Commercial Solvents Corporation, Inc.) by courtesy of Dr. Margaret H. Sloan of the National Research Council, Washington, D. C. "Intradex" was a 6 per cent solution, free of salt, of dextran of molecular weight 145,000 (weight average); for this material thanks are expressed to Mr. J. T. Marsh, M. P. S. (Glaxo Laboratories, Ltd.).[2]

Media were prepared just before use, with water purified by distillation and ion exchange treatment; dextran-containing media were, if necessary, centrifuged to remove any insoluble material. Versene (ethylenediamine tetraacetic acid) was kept as a stock solution (0.1 M) neutralized to pH 7.4 with KOH, and was added to media as required. AMP was likewise dissolved at a neutral pH. In general no attempt was made to standardize the pH of the media. This pH was usually between 5 and 6, but was invariably about 7.4 after introduction of the tissue and homogenization.

Enzyme Assays.—The enzyme assays were performed in test tubes which were incubated, with shaking, for 15 minutes at 30° C. or (in acid phosphatase assays) for 20 minutes at 37° C. ATPase (Mg^{++}-activated) was estimated in the presence of ATP (0.003 M, pH 7.4) and $MgCl_2$ (0.002 M) with no added buffer (8). For the determination of "free" activity, the medium contained 0.25 M sucrose. Prior to the determination of "total" activity, the tissue sample was diluted with water and was frozen and thawed once, so as to liberate the "latent" activity (*cf.* reference 4). Glucose-6-phosphatase activity was determined in cacodylate buffer (0.05 M, pH 6.5) in the presence of versene (0.001 M) and glucose-6-phosphate (dibarium salt; 0.0042 M) (9). Acid phosphatase activity was determined at pH 5.0 using β-glycerophosphate (10). In the above assays, the reaction was terminated by addition of trichloracetic acid solution, and a portion of the centrifugate was analyzed for inorganic phosphate.

Other Determinations.—Nitrogen was determined by the micro-Kjeldahl procedure. Ribo-

[1] ATP and AMP denote adenosinetriphosphate and adenosinemonophosphate (adenosine-5′-phosphate) respectively; ATP-ase activity refers to the liberation of inorganic phosphate on incubation of the tissue with ATP in the presence of Mg^{++}.

[2] *Note Added in Proof:* Since this work was commenced, the manufactuers of intradex have introduced glucose (5 per cent) as a routine additive. No adverse effects have been found from this addition, but for many biochemical purposes, it would obviously be desirable to remove this added glucose by dialysis, or to obtain dextran free from glucose.

nucleic acid (RNA) was determined by measuring the extinction, at 260 mμ, of acidified alkaline digests of defatted tissue fractions (11).

Preparation of Mitochondria.—Young male rats, usually fasted overnight, were killed with nembutal and partially exsanguinated. A weighed portion of liver was transferred to the appropriate medium (3 ml./gm. of liver), and was briefly homogenized at high speed (2,000 R.P.M.), in a chilled glass homogenizer, with a teflon pestle, as supplied by Arthur Thomas and Co., Philadelphia. The homogenate was centrifuged at 1,000 *g* for 10 minutes, and the debris was rehomogenized with a further portion of medium (3 ml./gm. liver) and recentrifuged. These and subsequent centrifugations were performed at 0° C. in an angle head, with an angle 13 centrifuge as supplied by Measuring and Scientific Equipment, Ltd., London; the *g* values refer to the highest value attained in the homogenate. The extreme radii, measured horizontally, were 9.6 cm. and (at surface of liquid) approximately 6.4 cm.

After each centrifugation the supernatant fluid was removed by pipette from the underlying nuclear material. The combined supernatant fluids ("cytoplasmic fraction") were further centrifuged, for 15 minutes at 10,000 *g*, to sediment the mitochondrial fraction, which was washed once without removal of the "fluffy layer." Finally (usually 2 hours after sacrifice) the washed fraction was suspended in the appropriate medium (2 ml./gm. of original liver) and samples taken for fixation and for enzymic assays.

Fixation and Examination in the Electron Microscope.—To 0.1 ml. of the mitochondrial suspension 2 ml. of chilled fixative was added. It was found that the standard Palade (12) fixative consisting of 1 per cent OsO_4 buffered to pH 7.4 with veronal acetate buffer was the most satisfactory fixative. No improvement could be detected by the addition of dextran to the fixative or by the use of the fixative described by Novikoff (5). The suspension was centrifuged to produce a pellet, which was left with the supernatant fixative usually for 2 hours. This was considered to be the optimum time and no advantage could be detected by fixing for a longer time (*e.g.* 24 hours). The fixative having been decanted, this pellet was washed with Ringer solution, and then dehydrated with aqueous ethanol, the concentration of ethanol being progressively increased, without dislodging the pellet. While in 100 per cent ethanol the pellet was removed as a solid lump and cut into smaller pieces, each one of which was treated as a lump of tissue for sectioning and examination in the electron microscope. The mitochondria were embedded in a 4:1 mixture of butyl methacrylate and methyl methacrylate which was polymerized at 50°C. after addition of benzoyl peroxide (to 2 per cent).

The block of tissue was sectioned on a Cambridge rocker microtome; the thickness of sections used varied from 200 A to 500 A. It was found necessary to orientate the block so that a section was cut normal to the surface of the pellet. In this way all the strata of the pellet could be observed simultaneously in the section, and therefore the relative distribution of mitochondria and other particulate matter in the suspension might be determined by observing the distribution of particles in the layers from the top to the bottom of the pellet.

The sections were observed and micrographs taken in a Philips or a Siemens (Elmiskop) electron microscope.

EXPERIMENTAL

Morphological Studies

As a guide to the interpretation of electron micrographs of isolated mitochondria, the appearance of mitochondria in thin sections of fixed liver tissue is shown in Fig. 1.

Trial of Sucrose in Different Concentrations.—The medium used for reference purposes was the conventional 0.25 M sucrose solution, usually containing

versene (0.001 M). On biochemical grounds (13, 14) it seemed likely that versene would help maintain the integrity of mitochondria. No such stabilizing effect was evident on examination of mitochondrial fractions by electron microscopy. Nevertheless, versene was added to all media unless otherwise stated.

Examination of mitochondrial fractions isolated in 0.25 M sucrose medium showed that almost all the mitochondria were swollen and damaged, with loss of internal structure (Fig. 2), as previously observed by Witter *et al.* (4). As also found by these authors, mitochondria isolated in 0.88 M sucrose solution in the present study were shrunk and extensively damaged. With 0.40 M sucrose solution, there was again some loss of mitochondrial integrity, some of the mitochondria being apparently shrunk in size and others somewhat swollen.

Trial of Raffinose (in Place of Sucrose) and of Heparin.—When sucrose was replaced by the trisaccharide raffinose, the hydrate of which is sufficiently soluble to give a 0.23 M solution (13.6 per cent) even at 0°C., there was some improvement in the appearance of the isolated mitochondria (Fig. 3).

When 0.25 M raffinose was used in conjunction with heparin (0.1 per cent), most of the mitochondria were found to have retained much of their internal structure, but (as with raffinose alone) some appeared to have undergone loss of contents as indicated by their pale appearance and by breakages in their membranes (Fig. 4).

According to a previous report (15), heparin is effective in preventing gelation of lymph node homogenates prepared in phosphate buffer. With liver homogenates this observation could not be confirmed in Albert's laboratory.[3] In the present study, however, it was found that the clumping of mitochondria which usually occurs in electrolyte-containing media (2) could be obviated if heparin (0.1 per cent) were present. As shown in Fig. 5, mitochondria can be isolated without clumping, and with little loss of morphological integrity, in a heparin-containing electrolyte medium (citric acid, 0.0113 M; KOH, 0.01 M; phosphoric acid, to give pH 7.4; MgO, to saturation), preferably supplemented with levan (10 per cent; see below). However, this phosphate-containing medium is disadvantageous for some enzymic studies, and its use has not been further investigated.

In the further testing of carbohydrate-containing media, heparin was usually present, but in later experiments the concentration of heparin was reduced to 0.035 per cent for reasons which are discussed below. Raffinose was usually employed to provide the requisite tonicity; even with the improved media described below, the electron micrographs of isolated mitochondria suggested that it was advantageous to use raffinose in place of sucrose.

Trial of Supplementation with Polysaccharides.—A study was made of the possibility that improvement in the quality of preservation of the mitochondria

[3] S. Albert, personal communication (*cf.* 16).

might be achieved by the use of a suitable polymer in the medium, preferably a polymer of sufficiently low molecular weight to be finally removed by dialysis if desired. Two types of polysaccharide preparations, both with the requisite good solubility in water, were investigated.

With the preparation of grass levan (8) investigated in the first instance, present at a concentration of 10 per cent in raffinose-containing media, there was definite improvement in the appearance of most of the mitochondria, but there was still a rather high proportion of "empty" mitochondria.

Four dextran preparations differing in molecular weight were then examined, usually at a concentration of 6 per cent. With increasing molecular weight there was improvement in the integrity of the isolated mitochondria. This correlation is illustrated in Figs. 6 and 7. With the dextran preparation of molecular weight 35,000, and particularly with that of molecular weight 145,000 (Fig. 7), the proportion of damaged mitochondria was considerably lower than that with levan or with dialyzable dextran. With a dextran concentration of 2 per cent in place of 6 per cent, greater damage was found. With a concentration of 12 per cent the appearance of the mitochondria was not improved; moreover, in the case of the dextran of molecular weight 145,000, the medium containing 12 per cent dextran was rather viscous.

The mitochondria illustrated in Fig. 7 were obtained with a medium differing from that used for the mitochondria of Fig. 6 not only in the nature of the dextran, but also in having a lower concentration of heparin and in having AMP present. These additional modifications, which were found in other experiments to have no perceptible effect on the integrity of the mitochondria, require some comment.

When liver tissue was homogenized in dextran media containing 0.1 per cent heparin, the homogenate often became remarkably viscous. It was eventually found that this seldom occurred if the heparin concentration was reduced (to 0.035 per cent); this lower concentration being still adequate to prevent agglutination of mitochondria. An interaction of heparin with nuclear material is suspected to be the cause of the rise in viscosity. Preliminary experiments suggest that agglutination is partially attributable to constituents (salts?) of the dextran preparation which can be removed by dialysis prior to use.

The adoption of AMP as a constituent of the medium was based on published observations concerning its effect on certain properties of mitochondria isolated in sucrose media. At a concentration such as has now been used (*ca.* 1 mM), this being of the same order as the AMP concentration in liver tissue (17), AMP preserves the latency of ATPase (18) and prevents mitochondrial swelling, the intramitochondrial concentration of sodium and potassium ions being maintained (14, 19).

Optimal Medium.—The medium adopted in the light of the above observations is a solution of raffinose hydrate (0.23 M; 13.6 per cent), in 6 per cent

dextran solution (intradex) as supplied by the manufacturers, with the following additions: versene (0.001 M), heparin (0.035 per cent), and AMP (0.035 per cent; 1 mM). The versene and the AMP are added as concentrated aqueous solutions at pH approximately 7.4, but no attempt is made to adjust the pH of the medium to 7.4, since a pH close to this value is immediately attained when liver tissue is homogenized in the medium. The versene and AMP are not regarded as absolutely essential. Moreover, with dextran preparations which do not agglutinate mitochondria, the heparin could be omitted without marked loss of mitochondrial integrity. The medium should be freshly prepared, since the effectiveness of heparin in preventing agglutination may be lost if the heparin-containing medium is stored, even for 2 days at 2°C.

It will be noted that mitochondria isolated in this medium, and examined in different regions of the pellet (Figs. 7 and 8), seem not only to have substantially retained their contents but are smaller than those isolated in 0.25 M sucrose solution (Fig. 2), presumably because of swelling in the latter medium. Accordingly, the dextran medium does not, in spite of its relatively high density (*ca.* 1.08 gm./ml. at 20°C. as compared with 1.03 for sucrose) and high viscosity, require more drastic centrifugation conditions than are required to completely sediment mitochondria in 0.25 M sucrose medium.

It will be noted that all the electron micrographs, and particularly Fig. 10, show a diverse population of particles some of which bear little resemblance to mitochondria. The nature of these particles is considered in the Discussion.

Biochemical Studies

In conjunction with the morphological studies described above, certain biochemical aspects were investigated. In particular, determinations were made of ATPase and glucose-6-phosphatase activity, with several considerations in mind. A high level of free ATPase, relative to total ATPase, is commonly regarded as a reflection of damage to the mitochondria; it was thus of interest to ascertain if there was in fact, with different media, a relationship between morphological integrity and free ATPase. Another possibility which has been investigated is that the ATPase which is present, in appreciable amount (20, 21), in the supernatant fluid after sedimentation of mitochondria in sucrose, may be reduced in amount by the use of media which preserve mitochondrial structure. The distribution of glucose-6-phosphatase was likewise examined, since its level in mitochondrial fractions (from which, in the present experiments, the fluffy layer was not separated) may be a measure of contamination with microsomal material which, with improved media, might sediment less readily. Consideration has also been given to the possibility that inactivation or inhibition of certain enzymes might result from the use of modified media.

In assessing the data given in Table I for ATPase and glucose-6-phosphatase, it should be borne in mind that dextran was particularly effective in giving

TABLE I

Comparison of Different Media with Respect to the Distribution of ATP-ase and Glucose-6-Phosphatase Activity in Liver Fractions

All media contained versene. Concentrations of constituents of the media are stated only in instances in which this information is not clear from the text; in the case of dextran preparations, the concentration was 6 per cent unless otherwise stated. The supernatant fraction, being the material unsedimented in the mitochondrial centrifugation, includes microsomal material. Enzyme determinations were made as described in the text, Mg^{++} being present in all ATPase assays.

Activities are expressed as micrograms of P (as phosphate) liberated per minute per 100 mg. original liver tissue. *The values obtained with control liver fractions, prepared in sucrose medium, are given parenthetically* ( ).

Medium	Experiment No.	Cytoplasmic ATPase activity			Cytoplasmic glucose-6-phosphatase activity	
		Mitochondrial fraction		Supernatant fraction (total activity)	Mitochondrial fraction	Supernatant fraction
		Free activity	Total activity			
Electrolytes (see text, p. 00) + levan + heparin (0.1 per cent)	7.7	5 (3)	7 (7)	9 (9)	<0.3 (1)	13 (11)
Raffinose + polyvinylpyrrolidone (6 per cent) + heparin (0.1 per cent)	7.7	2 (3)	13 (7)	5 (9)	0.3 (1)	3 (11)
Raffinose + levan + heparin (0.1 per cent)	7.7 7.10	3 (3) 2 (1.5)	11 (7) 16 (10)	9 (9)	0.3 (1)	11 (11)
Raffinose + dextran (dialysable) + heparin (0.1 per cent)	13.12	5 (3)	14 (17)	7 (14)		
Raffinose + dextran (No. 1) + heparin (0.1 per cent)	7.7 12.7 7.10	1 (3) 3 (4) 1.5 (1.5)	7 (7) 7 (10) 8 (10)	6 (9) 8 (15) 5 (3)	1 (1)	11 (11)
Raffinose + dextran (No. 2) + heparin (0.1 per cent)	7.10	1.5 (1.5)	17 (10)	6 (3)		
Raffinose + dextran (No. 2) (*no* heparin)	7.10	2 (1.5)	17 (10)	6 (3)		
Raffinose + dextran (No. 2) + heparin (0.035 per cent) + AMP	3.1 11.1	2.5 (4) 1 (3)	11 (12) 13 (24)	15 (3)	1 (3)	9 (7)
Raffinose + dextran (intradex) + heparin (0.035 per cent) + AMP (optimal medium)	3.1 11.1 17.1 24.1	3 (4) 3 (3)	11 (12) 21 (24)	2 (11) 14 (3)	1 (3) 3 (6) 7 (3)	11 (7) 6 (6) 11 (10)
Raffinose + dextran (intradex, 2 per cent only) + heparin (0.035 per cent) + AMP	13.12	4 (3)	27 (18)	10 (14)		
Raffinose + dextran (intradex, 12 per cent) + heparin (0.035 per cent) + AMP	3.1 11.1	3 (4) 1 (3)	12 (12) 13 (24)	5 (11) 15 (3)	1 (3)	9 (7)

mitochondria of satisfactory appearance, the effectiveness of the dextran preparations being in the order listed, which corresponds to increasing molecular weight. In view of possible day-to-day variations in technique, values for enzymic activity with experimental media should be compared with the values (tabulated parenthetically) for sucrose fractions prepared and studied simultaneously, rather than with one another. Data obtained in early experiments, with an electrolyte medium (page 612) or with a raffinose medium containing polyvinylpyrrolidone (*cf.* reference 6) or levan, are included in Table I, but need not be considered in detail in view of the later adoption of a raffinose medium containing dextran.

Possible Adverse Effect of Dextran Media on Enzymic Activity.—As is evident from the ATPase values (*total* activity) of Table I, cytoplasmic ATPase activity is usually no lower with dextran media for the isolation than with a sucrose medium. If, however, sucrose fractions are assayed in dextran media rather than in water, ATPase activity is reduced by about 40 per cent. This inhibitory effect, which is presumably not attributable to AMP (18), might account for any difference in free activity between dextran media and the sucrose medium (see below) since in the determination of free activity a substantial amount of the tissue suspension was added to the assay system (0.1 ml. in 0.65 ml., the remaining 0.55 ml. containing 0.25 M sucrose).

The glucose-6-phosphatase activity of cytoplasm is apparently unimpaired by the use, for isolation, of dextran media in place of sucrose medium (Table I), provided that the assays are performed with fresh rather than frozen tissue preparations. Sucrose fractions assayed in dextran media instead of in water show no reduction in activity.

Fractions prepared in dextran media have also been compared with sucrose fractions with respect to succinic dehydrogenase and acid ribonuclease (by Miss B. M. Stevens), to xanthine oxidase (by Dr. I. Lewin) and to oxidative phosphorylation (by Dr. W. W. Aldridge). Even with acid (pH 5) ribonuclease, which may be inhibited by heparin under certain conditions (22), no adverse effect of heparin-containing dextran media has been encountered.

Free ATPase Activity.—With the dextran preparations, other than that of lowest molecular weight, there is evidently a tendency for free ATPase to be lower than that for mitochondrial fractions isolated in sucrose (Table I). This tendency might, however, be attributable merely to an inhibitory effect of the dextran medium, as is discussed above. Moreover, free ATPase values with the dextran preparation No.1 were little different from those with the dextran preparations of higher molecular weight, which were more effective in preserving the morphological integrity of the mitochondria. Neither heparin nor AMP had any marked effect on the level of free ATPase (Table I).

Distribution of ATPase and Glucose-6-Phosphatase Activities.—With the centrifugation conditions employed in this study, the proportion of the cyto-

plasmic ATPase activity found in the mitochondrial fraction was rather variable. There was no consistent difference in this proportion between dextran media and the conventional sucrose medium (Table I). However, the proportion of the cytoplasmic glucose-6-phosphatase activity found in the mitochondrial fraction was usually lower with dextran media than with sucrose medium. It was therefore of interest to extend these distribution studies to include other enzymes, and to modify the centrifugation conditions with the aim of obtaining sharper enzymic distributions.

Further Study of Enzymic Distribution.—In the first instance, enzymic distribution was further studied without modifying the centrifugation conditions for mitochondria, but with a further centrifugation to give microsomal and supernatant fractions (Table II, 1st experiment). In comparison with the sucrose medium (values for which are shown parenthetically), the "optimal" dextran medium gave a similar ATPase distribution, but an altered glucose-6-phosphatase distribution in accordance with the above conclusion that this activity is less readily sedimented in dextran media. The same conclusion evidently holds for acid phosphatase and deoxyribonuclease (Table II), these enzymes being probably located in particles ("lysosomes"; *cf.* reference 23) distinct from mitochondria or microsomal elements.

A further experiment was performed, with modified centrifugation conditions, in an endeavour to obtain three particulate fractions rich in mitochondria, lysosomes, and microsomal elements respectively. As in experiments performed by de Duve and collaborators (23) with sucrose medium, heavy and light mitochondrial fractions were isolated as well as microsomal and supernatant fractions, and were assayed for the mitochondrial enzyme succinic dehydrogenase (succinic-cytochrome *c* reductase), as well as for acid phosphatase and glucose-6-phosphatase (Table II, 2nd experiment). Assays for ATPase were also performed, but the data were of little value in the present connection because of the usual spread of activity among the fractions.

It will be noted that succinic-dehydrogenase activity was quite sharply localized in the heavy mitochondrial fraction, both with dextran medium and with sucrose medium, but that with the former medium this fraction was relatively free of acid phosphatase and glucose-6-phosphatase. Moreover, the light mitochondrial fraction obtained in the dextran medium was relatively free of glucose-6-phosphatase activity, the microsomal fraction being relatively rich in this activity.

Data for nitrogen distribution, also shown in Table II, suggested a shift from the light mitochondrial fraction to the ultracentrifugal supernatant fraction with dextran medium in place of sucrose medium, in accordance with the conclusion that microsomal material sediments less readily. From these data, the enzyme data were recalculated as relative concentrations in terms of nitrogen (Table II). On this basis, the dextran medium was again superior to

sucrose medium with respect to the purity of the particulate fractions, the data for ATPase being disregarded.

TABLE II

Distribution of Enzyme Activities, Nitrogen, and RNA among Cytoplasmic Fractions

The values in parentheses () refer to control fractions prepared in isotonic sucrose medium and centrifuged simultaneously with those prepared in the optimal dextran medium.

The absolute activities or concentrations are not shown; in the case of ATPase and glucose-6-phosphatase these were of the order indicated in Table I. The other absolute values were of the following order (for the whole cytoplasm; per 100 mg. original liver):—N, 1.7 mg.; RNA, 0.45 mg.; succinic dehydrogenase, 0.1 mg. succinate decomposed per minute; acid phosphatase, 0.02 mg. P liberated per minute; deoxyribonuclease, 0.02 mg. mononucleotide liberated per minute.

The centrifugation conditions were as follows, the *g* values being maximum values (see text):—*1st experiment*, 15 minutes at 12,000 *g* (mitochondrial fraction; washed once without removal of the fluffy layer) and 90 minutes at 20,000 *g* (microsomal fraction); *2nd experiment*, 10 minutes at 8,000 *g* (heavy mitochondrial fraction; washed once), 30 minutes at 20,000 *g* (light mitochondrial fraction; washed once), and 60 minutes at 145,000 *g* (microsomal fraction; sedimented in Spinco preparative ultracentrifuge).

	Per cent distribution among fractions (whole cytoplasm = 100 per cent)			Relative concentration, in terms of N (whole cytoplasm = 1.00)		
	Mitochondrial	Microsomal	Supernatant	Mitochondrial	Microsomal	Supernatant
1st Experiment						
ATPase (Mg^{++})	76 (63)	13 (30)	11 (7)			
Acid phosphatase	29 (68)	40 (22)	31 (10)			
Deoxyribonuclease	45 (62)	55 (22)	<1 (16)			
Glucose-6-phosphatase	22 (40)	26 (51)	52 (9)			
2nd Experiment						
	Heavy / Light			Heavy / Light		
N	15 (15) / 8 (15)	18 (21)	59 (49)			
RNA	8 (14) / 9 (39)	36 (25)	47 (22)	0.56 (0.94) / 1.06 (2.7)	2.0 (1.2)	0.78 (0.44)
Succinic dehydrogenase	81 (83) / 14 (11)	7 (5)	<1 (<1)	5.3 (6.0) / 1.6 (0.8)	0.39 (0.31)	(Negligible)
ATPase (Mg^{++})	36 (44) / 22 (29)	38 (26)	4 (1)	2.4 (3.0) / 2.7 (1.9)	2.1 (1.2)	0.07 (0.03)
Acid phosphatase	13 (45) / 29 (26)	37 (9)	21 (20)	0.88 (1.25) / 3.4 (1.7)	2.1 (0.45)	0.36 (0.41)
Glucose-6-phosphatase	9 (21) / 17 (38)	65 (38)	10 (3)	0.56 (1.4) / 2.1 (2.6)	3.6 (1.8)	0.16 (0.07)

Ribonucleic Acid (RNA) in Mitochondrial Fractions.—Novikoff (24) has stated that it is possible to obtain mitochondrial fractions which are virtually free of RNA. Washed mitochondrial fractions prepared in the optimal dextran medium, with low-speed centrifugation so as to minimize contamination with the RNA-rich microsomal elements, usually contain RNA equivalent to at least 2.5 per cent of the amount of protein present. The amount of RNA is,

however, less than with sucrose medium, as is evident from Table II (2nd experiment), whereas the amount in the microsomal fraction and especially in the supernatant fraction is relatively high.

DISCUSSION

Any attempt to isolate mitochondria in good yield and purity entails somewhat rigorous treatment. In particular, the tissue should be homogenized thoroughly so that few cells remain unbroken. With incomplete homogenization, or with a relatively low centrifugal force to sediment the mitochondria, the isolated mitochondria, while only little damaged and little contaminated with other cytoplasmic elements, still might be quite unrepresentative of the mitochondria in the tissue as a whole. With the conditions here employed, the mitochondria were obtained in high yield. Morphological examination of whole homogenates did suggest that the mitochondria suffered some damage during their isolation (the time interval between excision of the liver and fixation of the mitochondria being at least 2 hours), but that this damage was less with dextran-containing media than with the usual sucrose medium. Care was taken in the morphological studies to examine sections obtained at various levels in the homogenate pellet.

In considering the electron micrographs of the isolated fractions, reference must be made to the morphology of the intact rat liver, (25, *cf.* references 26, 27) as revealed in electron micrographs and previously described by Fawcett. His results have been confirmed in this laboratory (Fig. 1). He describes mitochondria seen in thin sections as plump rods or short filaments, each enclosed by a double membrane the inner component of which is folded into ridges or the so called cristae. There is also a considerably greater density of matter inside the mitochondria than in the surrounding cytoplasm.

This internal structure of the mitochondria is poorly preserved with the conventional sucrose medium (0.25 M), which causes the mitochondria to swell and lose their internal structure and contents (Fig. 2). With the dextran-containing medium finally adopted, the mitochondria are in general not swollen, although they are no longer rods or filaments.[4] The internal structure is retained (Fig. 9) but often appears distorted, possibly due to the rounding up of the mitochondria. In general the mitochondria appear dense as though there is no loss of their contents, but there are some "empty" sacs in the mitochondrial fractions (Figs. 7 and 8) which presumably represent damaged mitochondria. Such sacs are distinguishable, both by having double membranes and by the

[4] Similar results have apparently been obtained in unpublished experiments performed in Hogeboom's laboratory (32), with isotonic sucrose containing dextran but apparently no heparin. As mentioned in the Experimental section, agglutination of the mitochondria usually occurred in the absence of heparin with the dextran preparations now examined; but with a suitable dextran preparation the heparin might well be omitted, with only slight adverse effect on mitochondrial morphology (compare Figs. 3 and 4).

absence of peripheral particles, from other sacs which, as discussed below, evidently represent reticular material.

The endoplasmic reticulum in rat liver, as described by Palade and Porter (26) and by Fawcett (25) (*cf.* Fig. 1), consists of flattened sacs which when sectioned may appear as parallel membranes. There are, however, also small vesicles of the same nature which are spherical. All these membranes have associated with them small dense particles which are assumed to consist of ribonucleoprotein. These structures make up the bulk of the microsomal fraction in a cell homogenate (5, 11, 27–29), but they also appear in quantity at the top of the pellet in the mitochondrial fraction (Fig. 10) and to a lesser extent throughout the block (Figs. 7 and 8). It has been suggested (27) that the larger sacs of reticulum are broken during homogenization into smaller units. In 0.25 M sucrose medium these appear as spherical vesicles, but in dextran-containing media some of these are flattened as in sections of the intact cells.

Mitochondrial fractions also contain, in relatively small number, small particles (Fig. 10) of a type recently described by other authors (5, 24, 28). These particles, which may be tentatively identified as lysosomes (*cf.* reference 23), are of about 0.2 μ diameter, and have an external membrane (probably single) and a dense granular interior.

The free ATPase activity of mitochondrial fractions isolated in dextran-containing media is little different from that of sucrose fractions, in accordance with the conclusion of Witter *et al.* (4) that this activity is poorly correlated with mitochondrial integrity. Assays for free succinic dehydrogenase, kindly performed in this laboratory by Miss B. M. Stevens, have likewise shown little difference between sucrose mitochondrial fractions and fractions isolated in a dextran-containing medium.

The "mitochondrial supernatant" fraction, although free of mitochondria, contains appreciable ATPase activity even if the dextran medium is used. It is possible that liver cytoplasm contains two distinct Mg^{++}-activated ATPases, only one of which is located in the mitochondria (30, 31; *cf.* references 20, 21, 33).

Mitochondrial fractions isolated in the dextran medium have a relatively small fluffy layer in comparison with that of sucrose fractions centrifuged at the same speed, and are relatively free of reticular (microsomal) material as judged both by the electron micrographs and by the assays for the microsomal enzyme glucose-6-phosphatase. The RNA content of the fractions is, as would be expected, relatively low; but it is uncertain whether the RNA that remains is situated in contaminating reticular material or in the mitochondria themselves. The mitochondrial fractions are also relatively free of lysosomes, *viz.* the particles which contain hydrolytic enzymes such as acid phosphatase and deoxyribonuclease (23). By centrifugation at a suitable speed (*cf.* Table II), it is possible to obtain a light mitochondrial fraction which is relatively rich

in these particles, and with a considerably higher speed it is possible to sediment microsomal material, the pellet thus obtained being less pink in color than that obtained from sucrose homogenates.

In common with lysosomes and microsomal elements, nuclei may sediment somewhat less readily in the dextran-containing medium than in isotonic sucrose medium, and it is recommended that their centrifugation should be carried out for 10 minutes at 1,000 *g*, rather than at 600 *g* as in experiments with isotonic sucrose medium. The unique behavior of mitochondria in being as readily sedimented in the dextran-containing medium as in sucrose medium, despite the higher density and viscosity of the former medium, may be related to the apparent absence of mitochondrial swelling in the former medium.

It is evident that the new medium now described may find application in in attempts to obtain sharper separations of cytoplasmic particles for biochemical studies, as well as in studies of mitochondrial morphology as related to function.

SUMMARY

In view of the unsatisfactory appearance, under the electron microscope, of liver mitochondria isolated in isotonic sucrose medium, alternative media have been examined. It was found to be advantageous to replace sucrose by raffinose, and to add levan or, preferably, dextran, together with heparin in suitable concentration. With the optimal medium, the constituents of which are raffinose, versene (optional), dextran of high molecular weight, heparin, and AMP (optional), most of the mitochondria in the osmium-fixed pellet are apparently intact, and show the membranes characteristic of mitochondria as seen in cell sections.

The optimal medium has no adverse effect on the activity of the several tissue enzymes which have been studied, except that Mg^{++}-activated ATPase is partially inhibited if the medium is present in high concentration in the assay system. Mitochondrial fractions isolated in the new medium have, in common with sucrose fractions, appreciable "free" ATPase activity, this activity being evidently a poor criterion of mitochondrial integrity. Use of the new medium does not decrease the proportion of cytoplasmic ATPase which fails to sediment with the mitochondria, but does give a mitochondrial fraction low in RNA and in acid phosphatase activity and little contaminated with microsomal material. Particles tentatively identified as "lysosomes" have been seen in certain sections.

BIBLIOGRAPHY

1. Hogeboom, G. W., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, **172,** 619.
2. Schneider, W. C., *Cancer Research*, 1951, **11,** 1.
3. Birbeck, M. S. C., and Reid, E., *Biochim. et Biophysic. Acta*, 1956, **20,** 419.

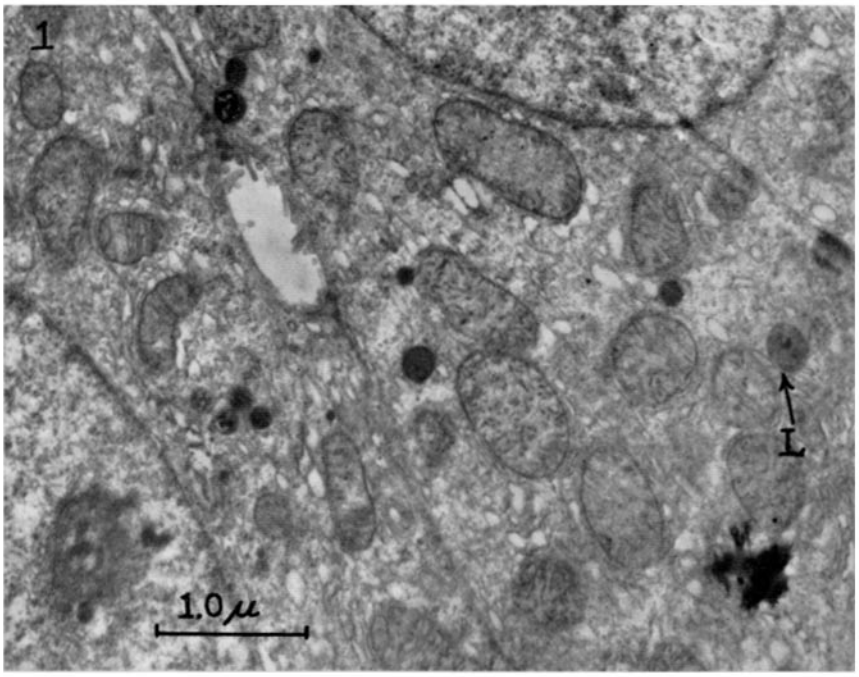
4. Witter, R. F., Watson, M. L., and Cottone, M. A., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 127.
5. Novikoff, A. B., *J. Biophysic., and Biochem. Cytol.*, 1956, **2,** No. 4, suppl., 65.
6. Novikoff, A. B., *Proc. 3rd Internat. Cong. Biochem.*, Brussels, 1955, (C. Liébecq, editor), New York, Academic Press Inc., 1956, 315.
7. Bell, D. J., and Palmer, A., *J. Chem. Soc.*, 1952, 3763.
8. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, 1953, **201,** 357.
9. Duve, C. de, Berthet, J., Hers, H. G., and Dupret, L., *Bull. soc. chim. biol.*, 1949, **31,** 1242.
10. Berthet, J., and Duve, C. de, *Biochem. J.*, 1951, **50,** 174.
11. Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C., *J. Biol. Chem.*, 1955, **217,** 111.
12. Palade, G. E., *J. Exp. Med.*, 1952, **95,** 285.
13. Slater, E. C., and Cleland, K. W., *Nature*, 1952, **170,** 118.
14. Fonnesu, A., and Davies, R. E., *Biochem. J.*, 1955, **61,** p. vi.
15. Hoster, M. S., McBee, B. J., Rolnick, H. A., Winkle, Q. Van, and Hoster, H. A., *Cancer Research*, 1950, **10,** 530.
16. Albert, S., and Johnson, R. M., *Cancer Research*, 1954, **14,** 271.
17. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, 1954, **209,** 23.
18. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, 1951, **191,** 485.
19. Macfarlane, M. G., and Spencer, A. G., *Biochem. J.*, 1953, **54,** 569.
20. Novikoff, A. B., Podber, E., Ryan, J. and Noe, E., *J. Histochem. and Cytochem.*, 1953, **1,** 27.
21. Schneider, W. C., Hogeboom, G. H., and Ross, H. E., *J. Nat. Cancer Inst.* 1950, **10,** 977.
22. Lamirande, G. de, Allard, C., Costa, H. C. da, and Cantero, A., *Science*, 1954, **119,** 351.
23. Duve, C. de, Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, 1955, **60,** 604.
24. Novikoff, A. B., *J. Exp. Biol., Suppl.*, 1956, in press.
25. Fawcett, D. W., *J. Nat. Cancer Inst.*, 1955, **15,** 1475.
26. Palade, G. E., and Porter, K. R., *J. Exp. Med.*, 1954, **100,** 641.
27. Palade, G. E., and Siekevitz, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 171.
28. Kuff, E. L., Hogeboom, G. H., and Dalton, A. J., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 23.
29. Chaveau, J., Gautier, A., Moulé, Y., and Rouiller, C., *Compt. rend. Acad. Sc.*, 1955, **241,** 337.
30. Demers, M., D'Iorio, A., and Robillard, E., *Rev. Canad. Biol.*, 1955, **14,** 89.
31. Thomson, J. F., and Mikuta, E. T., *Arch. Biochem. Biophysic.*, 1954, **51,** 487.
32. Hogeboom, G. H., Kuff, E. L., and Striebich, M. J.: quoted by Hogeboom, G. H., *in* Fine Structure of Cells (Int. Union of Biol. Sciences, Series B, No. 21), Groningen, P. Noordhoff, 1955, 3.
33. Reid, E., O'Neal, M. A., and Lewin, I., *Biochem. J.* 1956, **64,** 730.

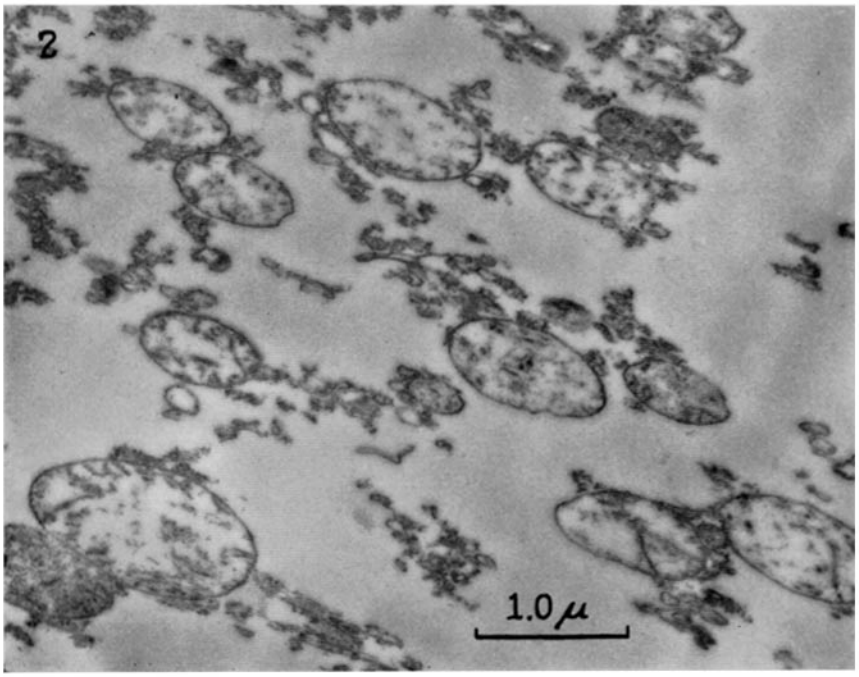
EXPLANATION OF PLATES

PLATE 156

FIG. 1. Electron micrograph of rat liver showing portion of two liver cells with a small bile duct between them. The nuclear membranes of the two nuclei are seen at bottom left and top right. The cytoplasm is filled with mitochondria with their characteristic internal structures. Between the mitochondria are small vesicles of reticulum with their attached RNA-rich particles. There are also several dense bodies smaller than the mitochondria, some of which are assumed to be of a lipide nature, but others such as the one marked (*L*) are similar to the dense particles found at the top of the mitochondrial pellet (Fig. 10) which are probably lysosomes. × 20,000.

FIG. 2. Electron micrograph of a section of a mitochondrial pellet isolated in 0.25 M sucrose solution. Most of the mitochondria are damaged, and much reticular material is present. × 20,000.





(Birbeck and Reid: An improved medium for the isolation of liver mitochondria)

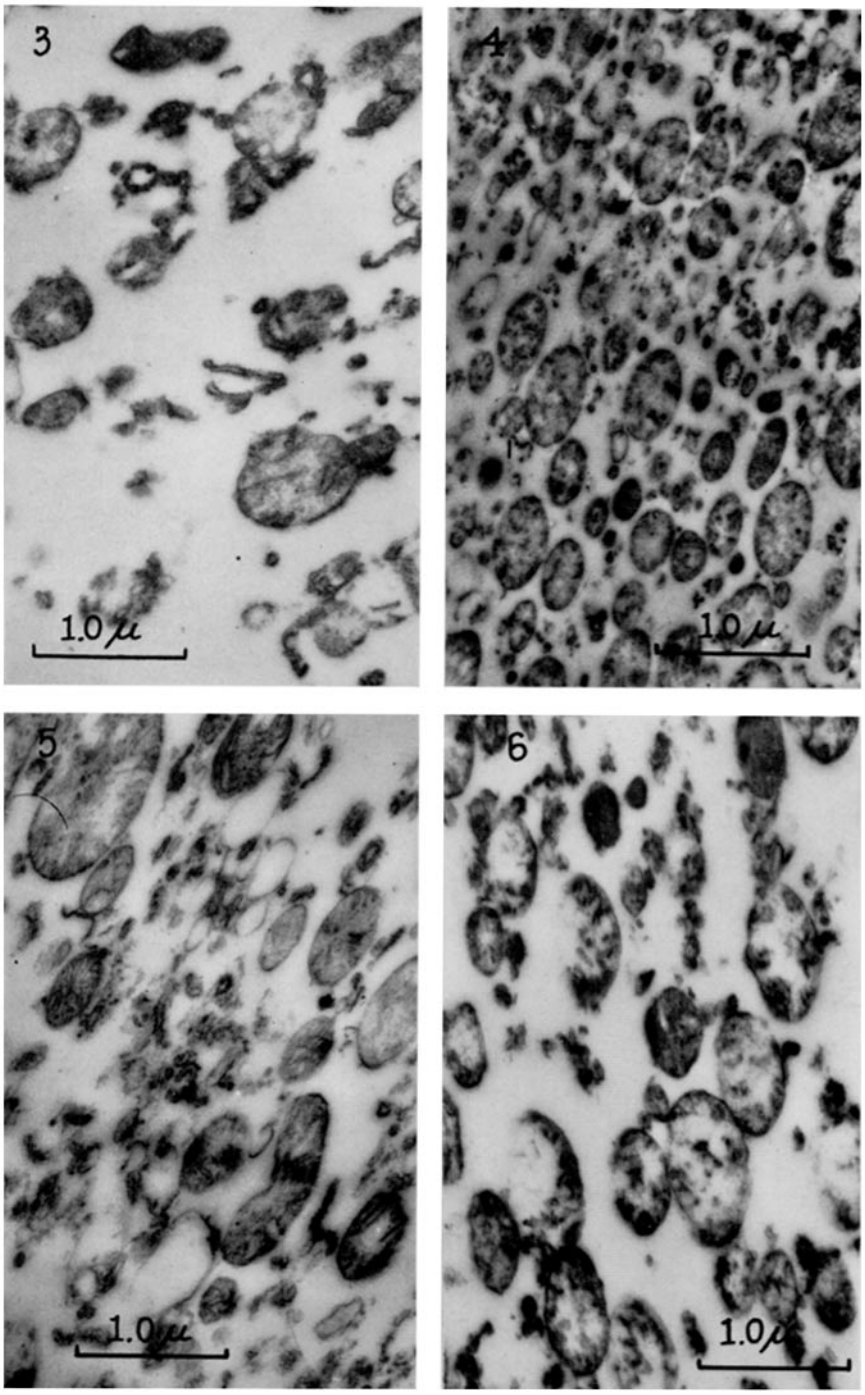
PLATE 157

FIG. 3. Electron micrograph of a section of a mitochondrial pellet isolated in 0.23 M raffinose solution (containing 0.001 M versene). The mitochondria are somewhat less damaged than those shown in Fig. 2. × 20,000.

FIG. 4. Electron micrograph of a section of a mitochondrial pellet isolated in 0.23 M raffinose solution containing (in addition to versene) 0.1 per cent heparin. The mitochondria seem less extensively damaged than those shown in Fig. 3. × 20,000.

FIG. 5. Electron micrograph of a section of a mitochondrial pellet isolated in an electrolyte medium (see text, p. 612) containing heparin (0.1 per cent) and levan (10 per cent). The mitochondria seem little damaged, but there is considerable reticular contamination. × 20,000.

FIG. 6. Electron micrograph of a section of a mitochondrial pellet isolated in a raffinose solution as for Fig. 4, but with the addition of dextran of low molecular weight (6 per cent; dialysable dextran). The mitochondria are very little improved in appearance by the dextran supplement (cf. Fig. 4). × 20,000.

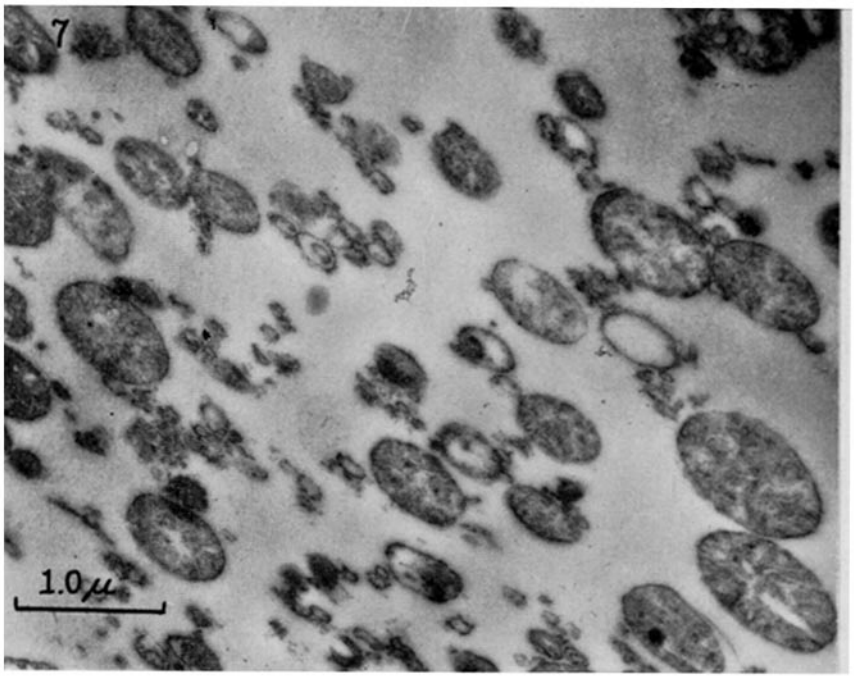


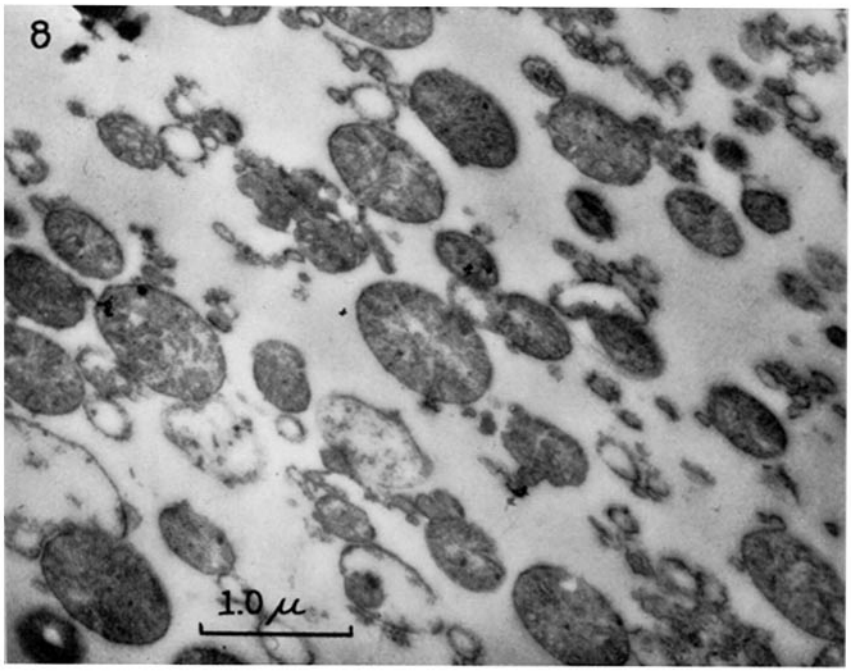
(Birbeck and Reid: An improved medium for the isolation of liver mitochondria)

PLATE 158

FIG. 7. Electron micrograph of a section of a mitochondrial pellet isolated in the optimal dextran medium (see text, p. 8), essentially as for Fig. 6 but with dextran of high molecular weight (intradex). For this figure, as for Figs. 2 to 6, the micrograph represents the appearance half-way down the pellet. The mitochondria are markedly superior to those obtained with dextran of low molecular weight (Fig. 6), and there is less reticular material than with sucrose solution (Fig. 2). × 20,000.

FIG. 8. As for Fig. 7, except that the section was photographed near the bottom of the pellet showing that the appearance of the mitochondria is similar throughout the pellet, but there is less reticular contamination. × 20,000.





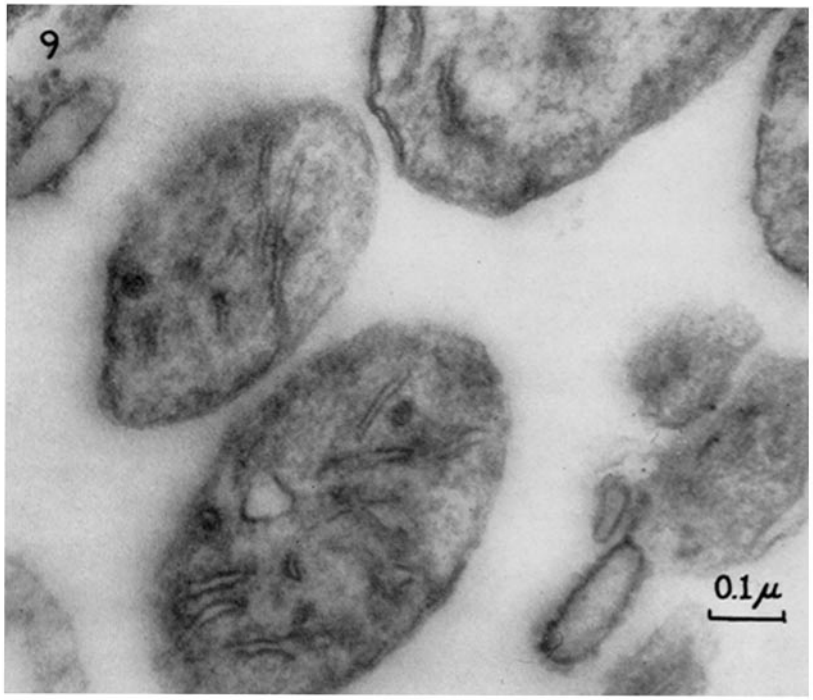
(Birbeck and Reid: An improved medium for the isolation of liver mitochondria)

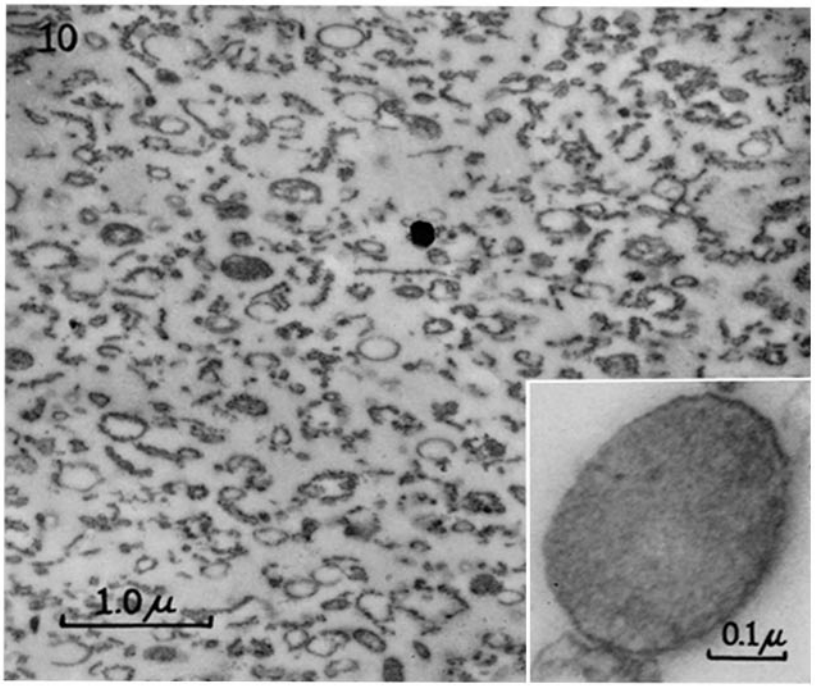
PLATE 159

FIG. 9. A high power view of a few mitochondria (apparently small, because not cut in a central plane) showing the internal arrangement of membranes in those isolated in the optimal dextran medium. There are also a few contaminating reticular elements with their attached RNA-rich particles. × 100,000.

FIG. 10. A micrograph of the extreme top of a mitochondrial pellet isolated in the optimal dextran medium. This micrograph contains no obvious mitochondria but consists almost entirely of reticular elements in this region. There are a few small dense particles (lysosomes?). × 20,000.

Inset: a micrograph of one of the dense particles (lysosomes?). They appear to have an external membrane and a rather granular interior. × 100,000.





(Birbeck and Reid: An improved medium for the isolation of liver mitochondria)