A METHOD FOR ISOLATING INTACT MITOCHONDRIA AND NUCLEI FROM THE SAME HOMOGENATE, AND THE INFLUENCE OF MITOCHONDRIAL DESTRUCTION ON THE PROPERTIES OF CELL NUCLEI*

By ALEXANDER L. DOUNCE, Ph.D., ROBERT F. WITTER, Ph.D., KENNETH J. MONTY, SIDNEY PATE, and MARY A. COTTONE

(From the Department of Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

(Received for publication, November 10, 1954)

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

In this paper we wish to describe what we consider to be improved techniques that permit the isolation of mitochondria and cell nuclei from the same liver homogenate. In the past it has been necessary to use different types of homogenates in isolating mitochondria and nuclei of acceptable quality. We also wish to demonstrate the importance of maintaining mitochondrial integrity during the isolation of nuclei (1, 2) and we offer evidence indicating that if the mitochondria are disrupted, an enzyme contained within them escapes and enters the nuclei, altering certain of their properties.

The mitochondria produced by the new technique not only are well preserved from the standpoint of morphology, but also fulfill two criteria that have been established for biochemically intact mitochondria (3), namely, that they have latent ATP-ase activity and that they require the presence of a phosphate acceptor for optimal oxidase[1] activity in the presence of those substrates, whose oxidation is coupled to phosphorylation. Previously, mitochondria which satisfied these two criteria have been altered morphologically (4, 5). In this paper a detailed comparison is made of oxidase activities of mitochondria isolated by the new procedure with the corresponding activities of those isolated in 0.25 M sucrose which are biochemically intact but morphologically altered. The ATP-ase activity of mitochondria isolated by the new procedure is described in another publication (6).

The new method has been applied mainly to rat liver, but it would probably work with other soft tissues, although in certain cases special steps to remove fiber would be necessary. The method fails when applied to Walker carcinoma

* We gratefully acknowledge the support of the National Cancer Institute (Grant C-994) and the National Heart Institute (Grant H-1616) of the National Institutes of Health, United States Public Health Service.

[1] By the term oxidase we are referring to succinoxidase, α-ketoglutarate, malate, and hexanoate oxidase.

256, as have other methods involving the use of aqueous solvent, but we have developed an alternative procedure which can be applied both to liver and to the above-mentioned tumor. This latter method does not however provide unaltered mitochondria.

The mitochondrial enzyme referred to above, which affects certain properties of the nuclei, appears to detach desoxyribonucleic acid (DNA) from some protein of the nucleus to which, we maintain, it is otherwise firmly attached. Nuclei that have not been subjected to the action of this enzyme are capable of forming gels in dilute alkali or strong sodium chloride (2, 7, 8), whereas after action of this mitochondrial enzyme, no such gels can be formed. Gel formation is considered to be an indication that the DNA is still firmly bound to nuclear protein, evidently by bonds other than those which are electrostatic in nature. This general concept has been advanced by us previously (8), but apparently has not been widely accepted. We believe that our new work with the action of the mitochondrial enzyme constitutes further evidence in its favor. In an accompanying paper (27) we have summarized the various means which can be utilized to prevent action of the mitochondrial enzyme on the nuclei.

The work described in this paper is to a considerable extent based upon the use of a new type of glass homogenizer (1, 2) which in our opinion offers certain definite advantages over homogenizers previously available for work with soft tissues. The details of the construction and use of this new homogenizer are presented in the section of this paper on experimental procedures. Also the ATP-ase activities[2] of mitochondria prepared with the new homogenizer are compared with those of mitochondria isolated with the aid of an old style homogenizer.

Methods

Description and Construction of the New Homogenizer.—Fig. 1 shows a photograph of the new homogenizers. A description can be found in Vol. 2 of The Nucleic Acids (2). The clearance of the "loosely fitting plunger" is about 0.001 to 0.0015 inch, at the circumference of the ball, and the clearance of the "tightly fitting plunger" is in the neighborhood of 0.0005 inch. The more exactly cylindrical is the barrel, the better the homogenizer will work, at least to the point at which the precision approaches 0.0001 inch, but fair results can be obtained with less precision in the grinding of the cylinder. The ball should *not* have a cylindrical area of appreciable width where it makes contact with the cylinder wall or it will be impossible to move it with a reasonable amount of force. In other words, the inside of the cylinder should be tangent to the ball, and the area of contact should be kept as small as possible. The cross-section of the ball perpendicular to the stem of the plunger at the point of tangency to the cylinder should be circular, but cross-sections of the ball in directions parallel to the stem of the plunger need not be exactly circular.

It would be desirable to construct a lever to operate the homogenizer, but if sizes not greater

[2] In these experiments the term ATP-ase is used to refer to that enzyme or enzyme system responsible for the release of ortho-phosphate from ATP.

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

than the larger of the two shown in Fig. 1 are employed, this is not important. Smaller sizes will work easily by hand, but larger sizes would definitely require mechanical advantage for operation, and the design of the ball would need modification. We have used very heavy glass tubing for constructing the cylinders, but stainless steel might also be suitable. The homogenizer is always surrounded by ice and water while being used.[3]

Isolation of Mitochondria and Nuclei from Rat Liver in 0.44 M Sucrose Adjusted to pH 6.2 with Citric Acid.—In the following procedure great care must be taken to keep the preparation as cold as possible without freezing, especially if mitochondria are to be isolated. All equipment such as mortars, pestles, homogenizers, homogenizer pestles, and centrifuge cups must be thoroughly chilled at 0 to 2° before use. It should be noted that the tissue or any surface upon which the tissue is placed should not be wet if mitochondria are to be isolated, although in the isolation of nuclei only, the addition of a few small pieces of cracked ice during the filtrations through cheese-cloth does no harm. All solutions should be kept at 0 to 2°. This may be most conveniently achieved by packing the reagent bottles in ice in Dewar flasks. For isolation of mitochondria, centrifugation is carried out in the 50 ml. cups of the International centrifuge and in the 25 ml. cups of the high speed head attachment. For simultaneous preparation of nuclei and mitochondria it is necessary to have available two refrigerated centrifuges.

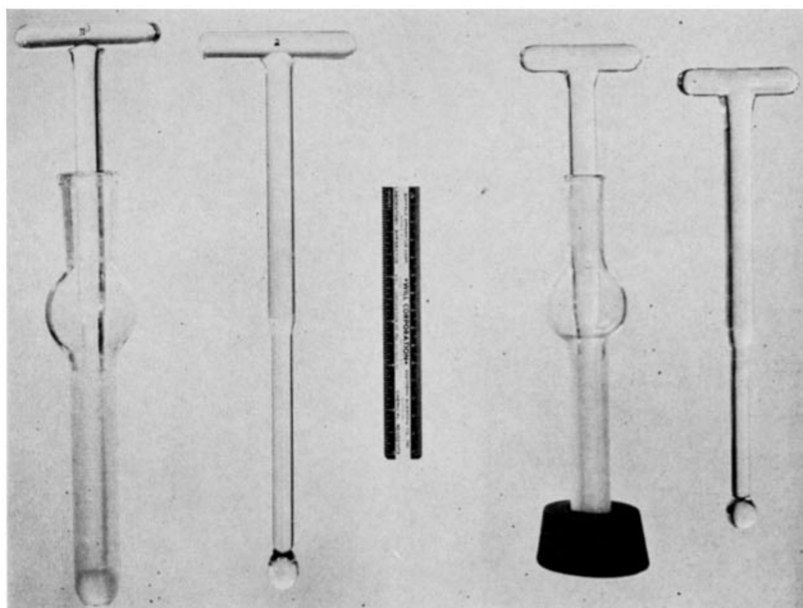

FIG. 1. New ball type ground glass homogenizers with pestles. Two sizes shown with 6 inch ruler for comparison.

A. Preparation of Homogenate.—25 gm. of liver are quickly excised from decapitated rats. This tissue is immediately minced in a chilled Petri dish which is kept in an ice bath. After

[3] Homogenizers of acceptable quality although not of high precision, similar to the large size shown in Fig. 1, are available from Blaessig Glass Specialties, Rochester, New York.

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

the weighed liver has been gently ground up with a pestle in a chilled mortar which is kept in an ice bath, 7 gm. portions of the tissue pulp are transferred to a chilled homogenizer tube immersed in an ice bath. 28 ml. of 0.44 M (15 per cent) sucrose solution containing 0.63 ml. of 0.1 M citric acid is added. The liver is quickly homogenized, with 8 passes of the chilled "loosely fitting" plunger. The pH of an aliquot (0.5 to 1.0 ml.) which has been allowed to warm up to room temperature is measured with the Beckman pH meter, and this small aliquot is then discarded. The pH should not be lower than 6.0 or higher than 6.2. In working with nuclei, pH values close to 6.0 are the safest. If the pH is too low, the homogenate must be discarded. On the other hand, if the pH is too high, a few drops of 0.01 M citric acid in 0.44 M sucrose are added until the proper value is attained. Then the homogenate is transferred to a chilled graduate which is kept in an ice bath. The homogenization and adjustment of pH are repeated with the second and third batches of liver.

B. Isolation of the Mitochondrial Fraction.—For the isolation of mitochondria, one-fifth (25 ml.) of the volume of the combined and thoroughly mixed homogenate is diluted with enough chilled 0.44 M sucrose to bring the final volume to 50 ml. After mixing, this diluted homogenate is centrifuged at 675 *g* for 20 minutes. The supernatant is decanted into chilled graduates immersed in an ice bath. The precipitate is dispersed in 30 ml. of 0.44 M sucrose by 3 or 4 passes of the chilled "loosely fitting" plunger. The suspension is again centrifuged at 675 *g* for 20 minutes. The supernatant is decanted, and nuclear precipitate is discarded. The combined supernatants from the low speed centrifugations are centrifuged at 13,000 *g* for 10 minutes. The Servall centrifuge run in a cold room, or the high speed attachment of a refrigerated International centrifuge is used for this purpose. The supernatants are decanted and can be used for the preparation of the "microsome" and "soluble" phases if so desired. The pellets are resuspended with the aid of a stirring rod in a total of 40 ml. of 0.44 M sucrose. The suspension is centrifuged at 13,000 *g* for 7 minutes. The pellets are again washed with a total of 40 ml. of 0.44 M sucrose. The resulting pellets are resuspended in a total volume of 10 ml. of 0.44 M sucrose with the aid of the new homogenizer. This suspension is equivalent to a 50 per cent suspension of fresh liver. The procedure can be repeated if desired with the other portions of the original homogenate.

C. Isolation of Nuclei.—For the isolation of nuclei the remaining four-fifths (100 ml.) of the homogenate described above under *A* is filtered successively through four layers of Nos. 60, 90, and 120 cheese-cloth (Curity, Kendall Mills Inc., Walpole, Massachusetts). It is not a good plan to squeeze out the cheese-cloth filters, and if the filtration time is to be kept short, it may be necessary to accept a considerable loss of homogenate. For best results, the filtration should be carried out in a cold room, but, in lieu of this, the temperature can be kept down by adding a small amount of cracked ice to the material before filtration *provided the mitochondria are not being isolated* from the supernatant fluid after the first centrifugation of the nuclei.

The next step is to effect a more complete homogenization than is necessary if only mitochondria are to be isolated. This is done by subjecting the combined filtered homogenate to homogenization in the ground glass homogenizer with the tightly fitting pestle. From 12 to 24 passes of the pestle are required, depending upon the behavior of the particular homogenizer being used. When the pestle is being pulled up from the bottom of the cylinder, sufficient time should be allowed for the liquid to run past the ball into the bottom portion of the cylinder. In other words, the ball should not be pulled up into the enlarged portion of the homogenizer until all of the homogenate is below the ball.

The procedure for the isolation of the nuclei from the filtered homogenate is as follows: The filtered homogenate is diluted by adding an equal volume of 0.44 M sucrose (plain, not containing citric acid) and both are thoroughly mixed. The diluted homogenate is then centrifuged in 50 ml. centrifuge bottles (not tapered) at 380 *g* (1700 R.P.M.) in a refrigerated International centrifuge No. 2 for 10 minutes. The supernatant fluid is decanted, and, after

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

microscopic examination to be certain that only a low concentration of nuclei is present, is discarded. It is best to discard also most of the loosely packed sediment at this point, even though appreciable quantities of nuclei may thus be lost.

The sediment of crude nuclei is next suspended in 50 ml. of 0.44 M sucrose by homogenization with 6 passes of the loosely fitting pestle. The pH is adjusted to 6.0–6.1 by adding from 0.1 to 0.5 ml. of 0.01 M citric acid as needed. Only very small portions of the homogenate should be used for testing the pH, and these should subsequently be discarded. The suspension is then centrifuged at 280 g (1520 R.P.M.) for 10 minutes. The supernatant fluid is discarded, and the nuclei are resuspended in 50 ml. of 0.44 M sucrose and homogenized as in the preceding step, and the pH is then adjusted to 6.0–6.1 as before. The suspension is next centrifuged at 195 g (1250 R.P.M.) for 10 minutes. The supernatant fluid is discarded. The nuclei now are largely free from mitochondria but still contain erythrocytes. If the mitochondria must be completely removed without being ruptured, as is necessary in some types of work, a third washing is necessary, using 50 ml. of 0.44 M sucrose, and adjusting the pH if necessary as described previously. In this washing the speed of centrifugation is reduced to 100 g (850 R.P.M.).

All the above steps can be carried out fairly successfully in one 250 ml. centrifuge tube, using approximately the same centrifugation speeds as above. In any case the sediments and supernatant fluids should always be checked until the best centrifugation schedule for the centrifuge at hand is finally determined.

The nuclei obtained by the procedures thus far described will be heavily contaminated with erythrocytes unless the livers have been perfused with 0.9 per cent sodium chloride and sucrose as described by Hogeboom and Schneider (9). The erythrocytes can be rather easily removed however by washing the nuclei twice in a 15 ml. conical centrifuge tube in 1 per cent gum arabic solution previously adjusted to pH 6.0 with NaOH, the tube being nearly filled with gum solution each time. The centrifugations are at 125 g (1000 R.P.M.) and 90 g (800 R.P.M.) respectively. The erythrocytes are laked by this procedure and the stroma remains in the supernatant fluid.

If dry weights of the nuclei are to be determined, the nuclei must then be washed two or three times in water to remove the gum, but this process is not time-consuming, since 5 minutes per spin suffices to bring down all the nuclei if a speed corresponding to 280 g to 513 g (1500 to 2000 R.P.M.) is used.

Additional Notes on Isolation of Nuclei and Mitochondria.—In the procedures just described, the mitochondria and nuclei are isolated from a separate aliquot of the same homogenate. If only mitochondria are to be prepared, 5 to 7 gm. of liver are treated as described in *A* and *B*. This amount usually provides enough mitochondria for one experiment, and a larger amount of tissue merely increases the time and labor involved in the preparation. If only nuclei are to be prepared, a homogenate can be made by adding 20 gm. of the pulped liver to 80 ml. of 0.44 M sucrose containing 1.8 ml. of 0.1 M citric acid, homogenizing this in three portions as described in *A*, and then treating as described in *C*.

Instead of using separate aliquots of liver, mitochondria may be isolated from the first supernatant of the nuclear precipitate mentioned in *C* by the procedure described in *B*. This yields a large quantity of mitochondria, which, however, may not be as suitable for studies of oxidative phosphorylation as those obtained from separate aliquots of homogenates, since the extra handling required for the isolation of nuclei may cause some loss in activity of the enzymes for oxidative phosphorylation. However if more stable mitochondrial enzymes are to be studied, the supernatant from the first nuclear sediment can be used. The mitochondria studied in this paper were prepared from separate aliquots of liver.

It should be stated that the Waring blendor or colloid mill cannot be used for homogenization using 0.44 M sucrose adjusted to pH 6.0 with citric acid, if nuclei are to be isolated. The

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

nuclei will generally be broken, apparently because of the high viscosity of the sucrose. In spite of this, the mitochondria are normal in appearance and probably have been only slightly damaged, so that here we have a case in which the nuclei are less stable to mechanical stress than are mitochondria. It is possible to isolate nuclei at pH 6.0 in 0.25 M sucrose if the Waring blendor or colloid mill is used for homogenization, but in this case the mitochondria are as a rule destroyed. It has been found that if the new homogenizer is used, 0.44 M sucrose is the lowest concentration which can be employed if mitochondrial morphology is to be preserved. It is possible to use higher concentrations of sucrose, but the method does not then work as easily, the yields tend to be less, and the mitochondria tend to contain active ATP-ase (6).

It should be noted that a buffer cannot be used to adjust the pH in the above method, since buffer anions cause the cells to resist breakage at pH 6, and also cause agglutination of the homogenate. At higher pH values they cause more protein to be extracted from the nuclei than if no buffer is used.

Method for the Isolation of Nuclei in Sucrose-Calcium Chloride Solution.—The method just described for isolating mitochondria and nuclei from the same homogenate is excellent for liver, and judging from preliminary experiments will be applicable to certain other tissues. However it fails with Walker carcinoma 256.

We have found that nuclei of fair quality can be isolated from the latter tumor by a procedure in which calcium chloride (2, 10, 11) is used in the first homogenization without pH adjustment. Since the Walker tumor contains much fiber, it is necessary to use a tissue press such as the one designed by Dounce as cited by Witter *et al.* (12) to remove the major part of this fiber before homogenization is possible.

To 20 gm. of the pulped tissue from the tissue press is added 80 ml. of 0.44 M sucrose containing calcium chloride in 0.005 M concentration. The suspension is homogenized and filtered through cheese-cloth in three batches as previously described, except that it may be necessary to use as many as three dozen passes of the tightly fitting plunger of the homogenizer in order to get satisfactory disruption of cells.

The homogenate is next diluted with an equal volume of 0.44 M sucrose containing 0.005 M $CaCl_2$ and the nuclei are centrifuged down as in the method previously described. They are then resuspended in about 100 ml. of 0.44 M sucrose which does not contain any $CaCl_2$. The pH is adjusted to 6.0–6.1 with dilute citric acid, and the isolation thenceforth is carried out in the same manner as described above for liver cell nuclei, except that so few erythrocytes are present that the washings in gum arabic are unnecessary. The nuclei are free from mitochondria, but there is always an easily detectable number of whole cells present. We estimate however by microscopic approximation that this does not represent a contamination of more than 5 to 15 per cent.

A detailed method for isolating cell nuclei from soft tissues with the aid of the new ball type homogenizer, using 0.25 M sucrose containing calcium chloride in 0.005 M concentration, will appear elsewhere (2). The latter method permits the isolation from rat liver of nuclei that form gels, providing the new homogenizer is used. The mitochondria, however, are altered in some aspects by this procedure. We have subsequently found that the use of 0.44 M sucrose is preferable to the use of 0.25 M if the ball type homogenizer is to be used, since the mitochondria in the former case do not tend to swell and since the cells are more easily broken. We have also found that 0.44 M sucrose is very definitely close to the optimal concentration for achieving maximal cell breakdown in the case of the Walker carcinoma. In this work we have tried concentrations of sucrose up to 0.88 M. The specified concentration of $CaCl_2$ also must be strictly adhered to.

Isolation of Mitochondria in Sucrose-Calcium Chloride Solution.—It is possible to isolate mitochondria from the first supernatant fluid remaining after centrifugation of the nuclei in the method involving the use of sucrose–calcium chloride solution for homogenization, by

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

centrifuging as described in the previous method. However, after the first centrifugation, the mitochondria should be resuspended in plain 0.44 M sucrose not containing calcium chloride. The pH is adjusted to 6.0–6.2 and the isolation is completed as described in the previous method. We have not yet had the opportunity to investigate in detail the properties of these mitochondria, but microscopically their appearance is not so good as that of those isolated from liver by the method first described. Mitochondria isolated from liver by the procedure involving sucrose-calcium chloride solutions have high ATP-ase activity (6) and low fatty acid oxidase activities (Table II).

Other Methods.—Mitochondria were isolated from 0.25 M sucrose as previously described (12) and from 0.44 M sucrose at pH 6.2 as outlined previously. Except when noted the new homogenizer was used. The old motor-driven homogenizer was the type described by Dounce and Beyer (13) which gives results comparable to those obtained with the Hagen, Potter, Elvehjem homogenizers. ATP-ase was measured as described by Witter, Watson, and Cottone (6). Centrifugal forces are calculated relative to the center of the tube.

RESULTS

Production of Nuclear Gels.—The nuclear gels referred to in this paper were produced by suspending samples of the appropriate type of nuclei in water and adjusting the pH to a value of 9 to 10 with NH_4OH or NaOH. The gels thus produced are nearly transparent; but opalescent or opaque partial gels can be obtained at pH values ranging from 7 to 8. The presence of salt in concentrations in the neighborhood of 0.9 per cent interferes with gel formation, but transparent or only very slightly opaque strong gels can be obtained at pH values of 7 to 8 by suspending the nuclei in solutions approximately molar in respect to NaCl or even stronger.

Demonstration of an Enzyme in Mitochondria Which Destroys the Gel-Forming Capacity of Nuclei.—Two types of nuclei were used in these experiments, namely those isolated at pH 6.0 in 0.44 M sucrose solution, using the new ball type homogenizer, as already described; and also those at pH 4.0 in distilled water using the Waring blendor for homogenization. Both types of nuclei form strong gels in the presence of dilute alkali. Both types of nuclei gave the same results in this experiment. An extract of mitochondria capable of acting upon nuclei so as to prevent gel formation was prepared in the following manner:—

Mitochondria which had been isolated from normal rat liver in 0.44 M sucrose without pH adjustment, using the ball type homogenizer, were taken up in distilled water at pH 6.5–7.0 and at room temperature. The suspension contained the total quantity of isolated mitochondria corresponding to 0.5 gm. of the liver (wet weight) in a volume of 1 ml. This lysed suspension was subjected to high speed centrifugation and the decanted clear supernatant fluid was used as the source of the "degelling" enzyme. A portion of this fluid was treated for 10 minutes at 100° C. in order to obtain a control solution containing heat-denatured enzyme.

To 0.2 ml. samples of suspensions of "gellable" nuclei previously adjusted to pH 6.5–7.0 (20 to 30 mg./ml. dry weight) were added 0.2 aliquots of the mitochondrial extract and 0.2 ml. of distilled water. The controls contained 0.2 ml. samples of nuclei together with 0.2 ml. aliquots of the heat-treated enzyme solution. Total volumes were again adjusted to 0.6 ml. by

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

the addition of 0.2 ml. portions of distilled water. The test and the control mixtures were then incubated at room temperature (22°C.) for time intervals varying from 15 minutes to 1.5 hours, and then a drop of dilute NH_4OH (concentrated NH_4OH diluted one to five) was added to each tube to induce gel formation. In all cases the nuclei treated with the unheated mitochondrial extract had completely lost their ability to form gels within the maximum incubation time, and in fact 30 minutes sufficed for complete destruction of the gel-forming capacity in most cases. The controls in every case retained their ability to form gels.

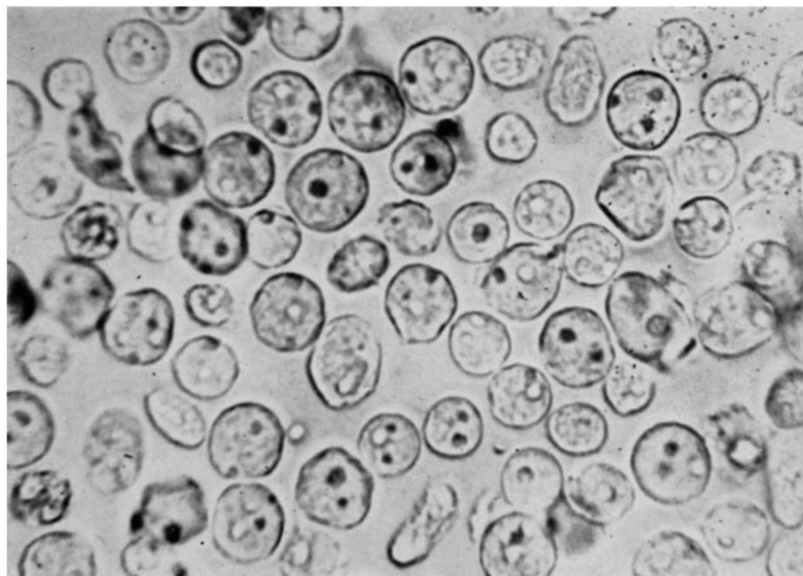

FIG. 2. Liver cell nuclei isolated in 0.44 M sucrose with lowering of the pH to 6.0 by means of dilute citric acid. (First method given in section on Methods.) × 2000. Fresh preparation, unstained, light microscope, 4 mm. objective. (Preparation made by Dr. Arthur J. Emery, Jr.)

The intramitochondrial factor responsible for the destruction of the nuclear gel substance displays a pH optimum in the range of pH 6.0–7.0. Reversible inhibition of the factor occurs at pH 4.0. Inhibition also occurs at elevated pH values, but it is not yet clear whether this inhibition is reversible. Inasmuch as the intramitochondrial factor is heat-labile and displays a pH optimum for its action on the nuclear gel substance, it seems reasonable to assume that the factor is an enzyme.

Morphology of Isolated Nuclei and Mitochondria.—Fig. 2 shows photomicrographs of nuclei isolated from normal rat liver by the first method given in this paper. Nuclei isolated by the procedure involving the use of sucrose–calcium chloride solution are similar in appearance. The slight reticulation is caused by the gum arabic solution used to eliminate erythrocytes. As long

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

as the nuclei remain in the sucrose solution, they are nearly spherical and optically empty, except for the prominent nucleoli. It would be possible to maintain this appearance if it were not necessary to use the gum arabic solutions, and use of the latter could be avoided by perfusing the livers according to the technique of Hogeboom and Schneider (9) to remove erythrocytes.

Fig. 3 shows photomicrographs of nuclei isolated from Walker carcinoma 256. These nuclei do not have a spherical and optically empty appearance under any conditions, and are therefore decidedly different in morphology

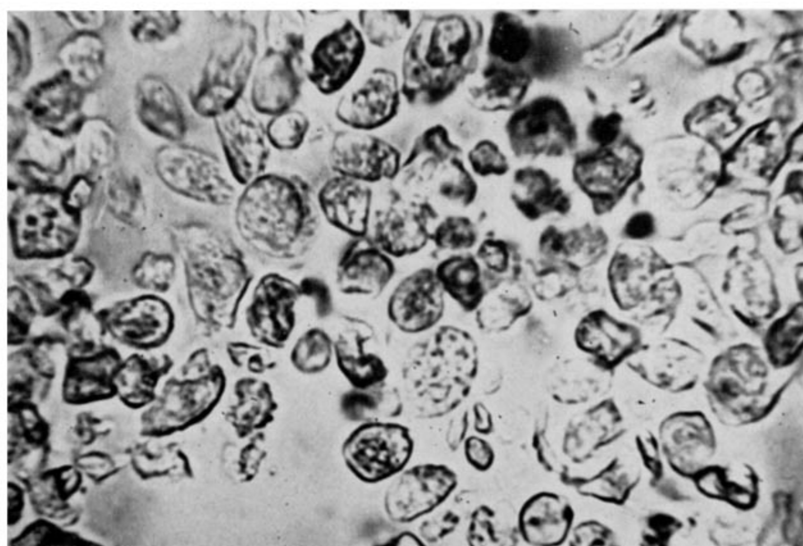

FIG. 3. Nuclei of Walker carcinoma 256 isolated as described in alternate procedure in Methods section, with homogenization in 0.44 M sucrose containing 0.005 M $CaCl_2$. $\times$ 1800. Fresh preparation, unstained, light microscope, 4 mm. objective.

from the liver nuclei. It is well to keep in mind that the spherical "optically empty" nuclei are not characteristic of all cell types. White cell nuclei, for instance, seem to constitute another exception.

Fig. 2 shows that the liver nuclei are relatively free from whole cells, mitochondria, and other types of contamination such as cell membranes and fiber. The tumor nuclei (Fig. 3) do contain some whole cells but far fewer than occur after any other previous mild method for isolating nuclei in aqueous medium. In fact all previous preparations of nuclei from Walker carcinoma 256 isolated in aqueous media have been almost worthless in our experience except when relatively strong citric acid has been used in making the homogenates.

Liver nuclei isolated with the aid of previously described homogenizers have contained far more whole cells in our experience than when isolated by

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

the aid of the new homogenizer. In our opinion, therefore, the new homogenizer is superior to previously existing types, for the isolation of cell nuclei in aqueous media.

In Fig. 2 of a previous publication (6) is shown an electron micrograph of mitochondria isolated at pH 6 in 0.44 M sucrose solution. These mitochondria compare favorably with those fixed *in situ*, and appear to be much better preserved than are mitochondria isolated by other procedures.

TABLE I

ATP-ase Activity of Mitochondria Prepared in Isotonic Sucrose

The conditions of the ATP-ase assay are given by Witter, Watson, and Cottone (6). $MgCl_2$ was added except when 1×10^{-4} M DNP was present. The aged mitochondria were heated at 37° for 20 minutes before use.

Experiment No.	Homogenizer	Condition of mitochondria	PO_4 liberated
			µM/mg. dry weight
1	New	Fresh	0.1
		Fresh plus DNP	1.6
		Aged	0.7
2	New	Fresh	0.05
		Fresh plus DNP	2.0
		Aged	0.9
3	Old	Fresh	0.15
		Fresh plus DNP	1.9
		Aged	1.0
4	Old	Fresh	0.1
		Fresh plus DNP	2.1
		Aged	0.8

DNA Content of Nuclei.—The DNA content of nuclei isolated from liver by the first method described is about 11 per cent as determined by the Schneider technique (14). The DNA content of nuclei isolated from liver by the alternate method involving sucrose-calcium chloride solution in the homogenization is about 14 per cent. The latter nuclei show higher percentages of DNA, owing to a greater loss of protein in the original homogenate, which is at a higher pH than that of the homogenate of the first procedure given in this paper. It has been found that when the pH of the homogenate is increased from 6.0 to 7.0, increasing amounts of protein are lost from the nuclei. This phenomenon has been discussed previously (8, 15). Nuclei of Walker carcinoma 256 isolated by the alternate method using sucrose–calcium chloride for the homogenization contain about 14 per cent DNA.

Comparison of Mitochondria from 0.25 M Sucrose Prepared by New Homo-

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

genizer with Those Prepared by the Old Homogenizer.—In order to determine the effectiveness and gentleness of the new homogenizer in regard to isolation of mitochondria, comparisons have been made of mitochondria isolated with the aid of the new and an older type of homogenizer (13) in 0.25 M sucrose without pH adjustment. These mitochondria are more sensitive to damage than are mitochondria isolated by the new method described in this paper and thus better reflect the amount of damage caused by the homogenization.

TABLE II

Oxidase Activity of Rat Liver Mitochondria

The phosphate acceptor system was the phosphocreatine system described elsewhere (12). The rates are based on first 20 minutes. The reaction conditions have also been given previously (12).

Isolation method	Substrate	μM oxygen per mg. dry weight/10 min. at 30°	
		No phosphate acceptor	Phosphate acceptor
Old homogenizer 0.25 M sucrose	Malate	0.05	0.17
	α-Ketoglutarate	0.07	0.25
	Succinate	0.20	0.64
	Hexanoate	0.15	0.24
New homogenizer 0.25 M sucrose	Malate	0.06	0.15
	α-Ketoglutarate	0.10	0.27
	Succinate	0.25	0.64
	Hexanoate	0.17	0.26
New homogenizer 0.44 M sucrose + Citrate at pH 6.2	Malate	0.06	0.15
	α-Ketoglutarate	0.12	0.30
	Succinate	0.28	0.64
	Hexanoate	0.15	0.27
New homogenizer 0.44 M sucrose + 0.005 M $CaCl_2$	Succinate	0.58	—
	Hexanoate	0.01	—

In Tables I and II are given the ATP-ase and oxidase activities respectively of mitochondria prepared in 0.25 M sucrose by means of the new or the old homogenizer. It can be seen that the ATP-ase of preparations made by either homogenizer is very low but is increased by aging for 20 minutes at 37° or the presence of 1×10^{-4} M DNP (dinitrophenol). Occasionally suspensions of mitochondria prepared with the new homogenizer require more drastic aging to develop the ATP-ase. Also the hexanoate, malate, and α-ketoglutarate oxidases of preparations made by the new homogenizer are similar to those made by the old homogenizer. Both types of mitochondrial suspension re-

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

quired the addition of a phosphate acceptor for maximum rate of oxidase activity. A muscle fraction (16) containing the phosphocreatine transphosphorylase and creatine was added in the experiments given in Table II. The results given in Tables I and II show that the ATP-ase and oxidative enzymes of the mitochondria made with the new homogenizer are as good as those of the organelles made with the old homogenizer and compare favorably with those given in the literature (5, 17–19). As far as the enzymatic activity of the mitochondria is concerned, the new homogenizer has no advantage over the old one. The former is, however, much more convenient to use and results in a more complete homogenization.

Enzymatic Activity of Mitochondria Prepared at pH 6.2.—In a previous paper (6) it is shown that the ATP-ase of these preparations is latent. Also, as is illustrated in Table II, the oxidase[1] activity compares favorably to that of mitochondria isolated in 0.25 M sucrose which are morphologically altered but which have excellent oxidative activity. In addition the mitochondria isolated in 0.44 M sucrose at pH 6.2, like those from 0.25 M sucrose, require the addition of a phosphate acceptor for maximum oxidase activity.

DISCUSSION

This new method for the preparation of nuclei and mitochondria at pH 6.2 is, as far as the authors are aware, the first procedure that can be applied for the isolation of clean suspensions of nuclei and mitochondria from the same homogenate. It is of particular significance that the mitochondria thus isolated not only appear to be superior in morphology to mitochondria obtainable by any of the previous methods (4–7, 18, 20) but also compare favorably in ATP-ase and oxidase activity to previous preparations of mitochondria which were excellent from the standpoint of enzymology but poor from the standpoint of morphology (4–6, 17, 18). Thus the new method should prove to be of value in cytochemical research.

One of the most important features of the new method of isolation is the maintenance of the correct pH during the homogenization by the addition of the exact amount of citric acid prior to homogenization. At pH values from 5.7–4.0 agglutination occurs while at pH values higher than 6.2 some nuclei are destroyed. It is obvious that the ratio of the amount of sucrose and citric acid to the amount of tissue must be determined by preliminary experiments and that this ratio must be kept constant in subsequent preparations. The authors have been able to obtain very consistent results by the procedure described, but it will be necessary for the individual investigator to determine the exact ratio to use under the conditions of his experiments and with the tissue that he wishes to study.

For the Walker carcinoma 256 the method involving sucrose–calcium chloride solution in the initial homogenization can be used for the isolation of nuclei of fair quality, but the mitochondria so produced do not appear to

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

be normal in appearance under the microscope. When this method is applied to liver, the nuclei are of acceptable quality. However, the mitochondria thus produced have a high initial ATP-ase (6) and virtually no fatty acid oxidase (Table II).

A precaution which must always be observed with the citrate method is to discard the small portions of homogenates used to measure the pH, since for accurate results these samples of homogenates must be allowed to warm up to room temperature before being placed in the Beckman pH meter. If this precaution is not observed, the nuclei may agglutinate and the ATP-ase activity of the mitochondrial fraction will be high.

It has been found that no improvement in the enzymatic activity or morphology of the mitochondria was obtained if the pH was kept at 6.0–6.2 through all stages of the preparation. For mitochondria, adjustment of the pH with citric acid appears to be necessary only at the time of homogenization. This may be a reflection of the fact that the pH of suspensions of mitochondria prepared by a variety of methods begins to fall within the range 6.2–6.6 as the mitochondria are washed free of other components of the cell.

The exact mechanism of the protective action of citrate is unknown. Citrate ion has been used in the isolation of nuclei but has not heretofore been employed for mitochondrial preparations. If versene is used at pH 6.2, the mitochondria are abnormal in appearance. Unpublished work of one of us (Witter) shows that citrate may protect mitochondria from swelling in hypotonic solution in much the same manner as versene or ATP (21). It remains to be proven whether this effect of citrate is related to the superior preservation of morphological structure. That this cannot indeed be the whole story is shown by the fact that mitochondria prepared in 0.25 M sucrose adjusted to pH 6.2 with citrate are partially swollen.

The nature of the mitochondrial enzyme which acts upon cell nuclei in such a manner as to prevent subsequent gel formation in dilute alkali is not yet clear. Any enzyme that would degrade either nucleic acid or protein might be the one in question, but it is also possible that a more specific enzyme is involved which attacks whatever linkages bind DNA to protein of the nucleus. Although mitochondria are known to contain DNA-ase (22–24) it does not seem very likely that the mitochondrial enzyme is in fact this enzyme, since neither known form of DNA-ase has a pH optimum close to 6.0, and since the DNA which can be extracted from "non-gellable nuclei" shows by its fibrous nature and stream birefringence (25) that it still possesses a high molecular weight. The DNA-ase of high pH optimum, moreover, tends to be inactivated by citrate, if we may use pancreatic DNA-ase as an example; but liver nuclei isolated in very dilute citric acid at pH 6.0 suffer from the effect of the mitochrondrial enzyme.

The mitochondrial enzyme might however be a protease, since proteases are known to occur in mitochondria (26). Indeed, DNA can be liberated from

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

"gellable" cell nuclei through the action of trypsin, a known protease. More work obviously will be required to determine the nature and action of the enzyme.

In the experiments with lysed mitochondria which demonstrate the presence of the "degelling" enzyme, we have not used unreasonable amounts or concentrations of the mitochondrial extract. The ratio of mitochondria from which the lysed extract was obtained to nuclei was about one to one, on a dry weight basis, whereas in the homogenate the corresponding ratio can be calculated as about 2.5 to 1 (assuming 25 per cent mitochondria and 10 per cent nuclei in the cells). It is true that the concentrations of both the soluble mitochondrial material and of the nuclei were 2 to 2.5 times greater in the incubation mixture than in the usual homogenate, but this concentration difference does not seem to be of sufficient magnitude to cast doubt upon the action of the mitochondrial enzyme on nuclei during isolation procedures in which mitochondria are disrupted.

When nuclei are isolated at pH 6.0 in very dilute citric acid, practically all the mitochondria originally present in the homogenate are disrupted, so that a very large proportion of the mitochondrial enzyme must gain access to the nuclei. These nuclei show no gel formation whatsoever. When nuclei are isolated by methods involving less drastic homogenization, it is sometimes possible to obtain a product that shows some tendency to form gels but that does not show strong gel formation. See footnote in Table I of the accompanying article (27).

SUMMARY

1. An improved type of ground glass homogenizer for soft tissues has been described which brings about a high degree of cell disruption and liberation of nuclei without causing appreciable damage to mitochondria. The gentleness and effectiveness of the new homogenizer in respect to isolation of mitochondria have been ascertained by comparing the ATP-ase activities of mitochondria isolated in 0.25 M sucrose solution without pH adjustment using a previous type of homogenizer with those of mitochondria isolated under the same conditions with the aid of the new homogenizer. In these experiments sucrose of 0.25 molarity without pH adjustment has been used in order to maintain the mitochondria in a rather sensitive state so as to make slightly deleterious effects of homogenization readily apparent.

2. A new method is described for the isolation of morphologically intact mitochondria and cell nuclei from the same homogenate. In this procedure the pH of the homogenate in 0.44 M sucrose is maintained at 6.0–6.2 with citric acid during the homogenization. An alternative method employing 0.44 M sucrose plus 0.005 M $CaCl_2$ is given for the isolation of nuclei from tumor cells. However, the latter method does not produce unaltered mitochondria.

3. The α-ketoglutarate, malate, succinate, and hexanoate oxidases of the

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024

"intact" mitochondria isolated in 0.44 M sucrose adjusted to pH 6.0–6.2 with very dilute citric acid as described in this paper have been investigated, and it has been shown that the mitochondria compare favorably to those isolated in 0.25 M sucrose by a previously described method.

4. Mitochondria have been found to contain an enzyme which causes nuclei to lose their ability to form gels in dilute alkali. This enzyme is released from the mitochondria when the latter are disrupted.

5. Some properties of nuclei isolated by the new method have been brieflv discussed.

BIBLIOGRAPHY

1. Dounce, A. L., Monty, K. J., and Pate, S., *Fed. Proc.*, 1954, **13,** 201.
2. Dounce, A. L., *in* The Nucleic Acids, (E. Chargaff and J. N. Davidson, editors), New York, Academic Press, Inc., 1955.
3. Schneider, W. C., *J. Histochem. and Cytochem.*, 1953, **1,** 212.
4. Schneider, W. C., *J. Biol. Chem.*, 1948, **176,** 259.
5. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, 1951, **191,** 485.
6. Witter, R. F., Watson, M., and Cottone, M., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 127.
7. Dounce, A. L., Science, 1949, **110,** 442.
8. Dounce, A. L., *in* The Enzymes, (J. B. Sumner and K. Myrbäck, editors), New York, Academic Press, Inc., 1950, **1,**—, 222.
9. Hogeboom, G. W., and Schneider, W. C., *J. Biol. Chem.*, 1952, **197,** 611.
10. Schneider, R. M., and Peterman, M. L., *Cancer Research*, 1950, **10,** 751.
11. Hogeboom, G. W., Schneider, W. C., and Striebich, M. J., *J. Biol. Chem.*, 1952, **196,** 111.
12. Witter, R. F., Newcomb, E. H., and Stotz, E., *J. Biol. Chem.*, 1953, **202,** 291.
13. Dounce, A. L., and Beyer, G. T., *J. Biol. Chem.*, 1948, **174,** 859.
14. Schneider, W. C., *J. Biol. Chem.*, 1945, **161,** 293.
15. Dounce, A. L., *Internat. Rev. Cytol.*, 1954, **3,** 199.
16. Racker, E., *J. Biol. Chem.*, 1947, **167,** 843.
17. Potter, V. R., and Recknagel, R. O., *in* Phosphorus Metabolism, (W. O. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 1951, **1,** 297.
18. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, 1953, **201,** 1.
19. Copenhaver, J. H., and Lardy, H. A., *J. Biol. Chem.*, 1952, **195,** 225.
20. Hogeboom, G. W., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, **172,** 619.
21. Raaflaub, J., *Proc. 2nd Internat. Cong. Biochem.*, Paris, July, 1952, 41.
22. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, 1952, **198,** 155.
23. Allfrey, V., and Mirsky, A. E., *J. Gen. Physiol.*, 1952, **36,** 227.
24. Webb, M., *Exp. Cell Research*, 1953, **5,** 16.
25. Dounce, A. L., *J. Biol. Chem.*, 1943, **147,** 685.
26. Maver, M. E., and Greco, A. E., *J. Nat. Cancer Inst.*, 1951, **12,** 37.
27. Dounce, A. L., and Monty, K. J., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 155.

Downloaded from http://rupress.org/jcb/article-pdf/1/2/139/1456217/139.pdf by guest on 25 April 2024