Cytochemical Fractionation of the Lettré-Ehrlich Ascites Tumor*

LEONARD A. SAUER, ARLENE P. MARTIN, AND ELMER STOTZ

(Deportment of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.)

SUMMARY

A method for cytochemical fractionation of the Lettré-Ehrlich ascites tumor has been presented. The cells were exposed to dilute Ringer solution which increased the cell size and rigidity and allowed them to be homogenized in 0.44 M sucrose with the use of the Dounce all-glass “ball and cylinder” homogenizer. Nuclear, mitochondrial, and microsome + “soluble” fractions were obtained and assayed for cytochrome oxidase and peroxidase activities. A good nuclear fraction was obtained from this tumor as shown by microscopic examination and as indicated by the low cytochrome oxidase content. The mitochondrial fraction was evaluated only by enzymatic methods. As would be expected, both cytochrome oxidase and peroxidase occurred predominantly in the mitochondrial fraction.

Spectral data indicated that the predominant pigment of the mitochondria was cytochrome c.

The technic of tissue homogenization and differential centrifugation is now widely used. Most animal tissues yield to this technic with ease, and large amounts of cellular particulates can be quickly obtained. Ascites tumor cells, however, have tended to resist successful homogenization by the methods in common use, since the membrane and matrix of these cells are especially strong and cannot be ruptured by procedures designed for liver cells (9). This has resulted in methods with media of high viscosity (1, 15), cellular disruption by shaking with quartz sand (9), lysis with digitonin (8), and a mechanical device (8). The mechanical methods require equipment that is elaborate and costly for small preparations. The technic of shaking with quartz sand has, in this laboratory, given low yields of homogenate and requires subsequent separation of the sand. The method with high viscosity media yields only a well defined nuclear fraction.

The studies to be presented in this paper describe a method for homogenization of the Lettré-Ehrlich ascites tumor cells that yields from the same homogenate nuclear and mitochondrial fractions as well as a supernatant fraction designated as the microsomal + “soluble” fraction. The ascites cells were suspended in quarter strength ascites Ringer solution in preparation for homogenization. The homogenizer used was the all-glass “ball and cylinder” type described by Dounce (4), which can be operated manually. The distribution of cytochrome oxidase and peroxidase in the cell particulates is also presented.

MATERIALS AND METHODS

Tumor transplantation and harvest.—Male Swiss Ha/ICR strain mice, between the ages of 8 weeks and 6 months, were given implants intraperitoneally, by syringe, of 0.1 ml. of undiluted ascites fluid under semi-sterile conditions. There were no restrictions on the food and water given. Ten to 15 days after the implantation, when the mice were distended, two animals were sacrificed by cervical fracture. Twenty to 40 ml. of pooled ascites fluid were collected, with a syringe, and were transferred immediately to a round-bottomed 50-ml centrifuge tube in an ice bath.

Enzyme assays.—Cytochrome oxidase was determined as described by Neufeld et al. (12), by a modification of the method of Smith and Stotz (14). A unit of enzyme activity is expressed as that amount of enzyme which causes a Δ log I per minute of 1.0 under the specified conditions of the test. Peroxidase was determined by measuring the rate of oxidation of leuco 2,6-dichlorobenzenoindole-3'-chlorophenol, with the Lumetron colorim...
eter, as described by Lucas and co-workers (10).
2,4-Dichlorophenol was included in the assay mixture to inhibit catalase as described by Neufeld et al. (12). A unit of peroxidase activity is defined as that amount of enzyme which causes a $\Delta \log I$ per minute of 1.0 under the specified conditions of the test.

The enzyme assays were carried out within 1–2 hours after preparation of the cellular fractions. Protein concentration was determined by measuring the dialyzed dry weight of duplicate 1.0-ml. aliquots of each fraction, except in the more dilute fractions, such as the waste and microsomal †+ soluble,” when 2- or 3-ml. aliquots were dialyzed.

Reagents.—2,4-Dichlorophenol and sodium 2,6-dichlorobenzenenoindo-3′-chlorophenol were purchased from the Eastman Kodak Co. Cytochrome c was purchased from Sigma Chemical Co. Crystalline beef liver catalase was prepared according to the method of Dounce (3). Ascites Ringer solution, Solution A (6), was diluted, 1 volume plus 3 volumes of distilled water, and is hereafter referred to as dilute Ringer solution.

Cytochemical fractionation.—A modification of the method of Dounce et al. (4), using 0.44 M sucrose solution, pH 6.0, for the separation of a mitochondrial and nuclear fraction from the same homogenate, was employed. The initial homogenate was made in 0.44 M sucrose with enough 0.1 M citric acid added to adjust the pH of the homogenate to 6.0–6.2. The amount of citric acid required depended upon the number of cells obtained from the two mice. The procedure was therefore modified to overcome these difficulties, and the following describes in detail the methods used to prepare the fractions. Centrifugal forces were calculated relative to the center of the tube.

Homogenate.—Ascites fluid obtained from two mice was centrifuged in an International Refrigerated Centrifuge Model PR-2 (temperature setting at $-2.5^\circ$ C.) with the low-speed head No. 253 at 2200 r.p.m. (960 × $g$) for 5 minutes to separate the cells from the ascites fluid. The ascites fluid was discarded. To prepare the cells for homogenization they were suspended in about 40 ml. of cold dilute Ringer solution with a rubber policeman. This suspension was then allowed to stand, with occasional stirring, in an ice bath for 30 minutes. The suspension was then centrifuged at 2200 r.p.m. (960 × $g$) for 5 minutes. This soaking process markedly increases the volume of the packed cells and lyses any erythrocytes present. The swollen cells were then mixed with a rubber policeman to break up any clumps and resuspended in enough cold dilute Ringer solution to give a final volume of 40 ml. It is important to have complete dispersion of the cells with no visible clumps. The suspension was returned to the ice bath.

By taking a 1.0-ml. aliquot of this suspension it was found that a correlation existed between the turbidity of the suspension and the amount of citric acid needed to bring the pH of the homogenate to approximately 6.0. A 1.0-ml. aliquot of the 40-ml. suspension was removed and placed in a small flask with 19 ml. of dilute Ringer solution and mixed. The optical density of this dilute suspension was measured at 520 m$\mu$ with a Bausch and Lomb colorimeter against a blank of dilute Ringer solution. The correct amount of citric acid was then added to 20 ml. of cold 0.44 M sucrose to be used subsequently (see Chart 1).

The remaining 39 ml. of suspension were allowed to remain in the ice bath for 15–30 minutes for maximum swelling. When a drop of this suspension is examined under the microscope (high dry objective) and the cells roll across the field, optimal swelling has been reached. The suspension was then centrifuged at 2200 r.p.m. (960 × $g$) for 5 minutes and the supernatant fluid discarded. The swollen cells were resuspended in the 20 ml. of cold sucrose containing the citric acid and immediately

1 Another criterion for recognition of optimal swelling is the microscopic appearance (high dry objective), in the majority of the cells, of apparent Brownian motion of the small cellular particles.
transferred to a pre-cooled homogenizer. Cells that adhered to the side of the tube were washed off with 25 ml. of cold sucrose solution and added to the homogenizer. The cells were homogenized immediately with 20–25 passes of the tight-fitting rod (clearance, 0.0005 inches). A drop of this suspension was examined under the microscope for the presence of unbroken cells and more passes made if necessary. The pH of the suspension was measured while cold with the Beckman pH meter and adjusted to 6.0–6.2 with 0.01 M citric acid if necessary. This suspension is called the homogenate.

**Nuclear fraction.**—The homogenate was decanted into 50-ml. tubes and was centrifuged at 2200 r.p.m. (960 X g) for 20 minutes. The precipitate from this centrifugation is the crude nuclear fraction, and the supernatant fluid contains the mitochondria and microsomes. This supernatant fluid was stored in the ice bath and the crude nuclear fraction was resuspended in 30 ml. of cold sucrose solution containing 0.05 ml. of 0.1 M citric acid. This suspension was homogenized with 5–8 passes of the tight-fitting rod. The pH was measured and adjusted to 6.0–6.2 with 0.01 M citric acid if necessary. This suspension was centrifuged at 2200 r.p.m. (960 X g) for 20 minutes and the supernatant fluid combined with that obtained earlier.

The precipitate of nuclei was washed extensively with cold sucrose solution in the following manner: (a) resuspension to 40 ml. with 1–2 passes of the tight-fitting rod and the pH measured and adjusted with 0.01 M citric acid if necessary, followed by centrifugation at 1700 r.p.m. (580 X g) for 15 minutes and the supernatant fluid discarded; (b) resuspension without homogenization to 40 ml. and centrifugation at 1500 r.p.m. (450 X g) for 15 minutes and the supernatant fluid discarded; (c) resuspension without homogenization to 40 ml. and centrifugation at 1200 r.p.m. (290 X g) for 15 minutes and the supernatant fluid discarded. This precipitate of washed nuclei was resuspended in 10 ml. of cold sucrose solution, and the pH measured and adjusted if necessary. This represents the nuclear fraction.

**Mitochondrial fraction.**—The centrifuge temperature setting was adjusted to −7.5°C. and the high-speed head No. 296 installed. The combined supernatant fluids from the preparation of nuclei were centrifuged at 11,500 r.p.m. (7,800 X g) for 10 minutes. The precipitate is the crude mitochondrial fraction, and the supernatant fluid containing microsomes and “soluble” fraction was saved. The precipitate was suspended in cold sucrose solution with a rubber policeman and adjusted to a final volume of 40 ml. and centrifuged at 10,500 r.p.m. (6,400 X g) for 10 minutes. This washing and centrifugation procedure was repeated once more. The final precipitate was resuspended in 10 ml. of cold sucrose solution. This represents the mitochondrial fraction.

**Microsome + “soluble” fraction.**—The supernatant fluid saved from the first centrifugation of the mitochondria was not subjected to further centrifugation and is therefore called the microsome + “soluble” fraction.

**Waste fraction.**—The supernatant discards from the nuclear and mitochondrial washes were pooled and saved for enzymatic analysis and were designated the waste fraction.

**RESULTS**

The swelling of the ascites cells that occurs in dilute Ringer solution is readily seen by contrasting Figures 1 and 2. The cells in Figure 2 are, however, slightly flattened by the coverslip. Figure 3 shows the nuclear fraction isolated as described in the text. A cytoplasmic tag often adheres to the nucleus which resembles the cytoplasmic clump seen in the swollen cell.

The DNA content of the isolated nuclear fraction was determined by the Schneider technic (13). The DNA analysis represents a triplicate determination on five nuclear fractions and was found to average 6.5 per cent, with a range of values from 5.5 to 8.2 per cent. These low values may perhaps be due to the presence of the attached cytoplasmic tag.

Since cytochrome oxidase is of mitochondrial origin, the distribution of this enzyme was investigated to serve as an index of the cytochemical fractionation. All attempts were made to obtain fractions of the highest purity. A waste fraction was saved only for assay purposes. As shown in Table 1, cytochrome oxidase was found in its highest concentration in the mitochondrial fraction, representing a five-fold average increase in purity for seven experiments. A small and nearly constant amount of activity was found in the nuclear fraction, which may be contained in the cytoplasmic tag. The microsomal + “soluble” fraction was at all times free of cytochrome oxidase activity. The wash fraction contained a variable amount of activity generally directly proportional to the total activity of the preparation. Table 1 also shows the recovery of cytochrome oxidase activity in each fraction, with the homogenate set at 100. In the seven experiments there was an average total recovery of 114 per cent, with 55 per cent recovered in the mitochondrial fractions. The waste fractions contained an average value of 42 per cent.
The peroxidase activity of this tumor was investigated because of the interest aroused by the presence of this enzyme in proliferating tissues (11). The data shown in Table 2 indicate that this tumor does contain a peroxidase localized in the mitochondria. The distribution of this enzyme followed that of the cytochrome oxidase, with the exception that in two experiments some peroxidase activity was found in the microsomal + "soluble" fractions, evidently owing to some mitochondrial spillage. The experiments are listed in the order in which they were performed. It is seen that the purity of the homogenate for peroxidase increased while the cytochrome oxidase purity remained nearly constant. This increase in the peroxidase purity may indicate a change in this tumor during the 2-year period it has been maintained in this laboratory.

Shown in Chart 2 is the difference spectrum of the mitochondrial fraction. The predominant pigment was found to be cytochrome c, as seen by the upward deflection at 550 and 525 m\(\mu\). There was also an increase in absorbancy in the region of 555–570 m\(\mu\), which may be owing to cytochrome b, c\(_1\), or both, and in the region of 590–605 m\(\mu\), owing to cytochrome oxidase.

In other types of ascites tumor cells, Chance and Castor (2) have demonstrated the predominance of cytochrome c, and Hess (7) has given difference spectra of whole cells and mitochondria which show the presence of cytochromes a\(_3\), b, and c.

**DISCUSSION**

The ascites fluid when collected was frequently visibly bloody. Soaking the cells in quarter-strength ascites Ringer solution lysed the erythrocytes, and the discarded supernatant fluid contained the hemoglobin. The homogenate, therefore, contained little residual hemoglobin and was white in appearance.

**TABLE 1**

<table>
<thead>
<tr>
<th>EXP. NO.</th>
<th>HOMOGENATE</th>
<th>NUCLEI</th>
<th>MITOCHONDRIA</th>
<th>MICROSOME + &quot;SOLUBLE&quot;</th>
<th>WASTE</th>
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<td></td>
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<td>(%)†</td>
<td>(units/ mg)*</td>
<td>(%)†</td>
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<tr>
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* Purity in units/mg dry weight. Units as described in the text.
† Per cent recovery with the homogenate set at 100.

**TABLE 2**

<table>
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<tr>
<th>EXP. NO.</th>
<th>HOMOGENATE</th>
<th>NUCLEI</th>
<th>MITOCHONDRIA</th>
<th>MICROSOME + &quot;SOLUBLE&quot;</th>
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<td>2.4</td>
<td>1.50</td>
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* Purity in units/mg dry weight. Units as described in the text.
† Per cent recovery with the homogenate set at 100.
The soaking of the tumor cells increased the turgor of the cells and the rigidity of the cell membrane so that rupture occurred during homogenization. It was found that the hypotonic solution made by a 1:4 dilution of the ascites Ringer solution would cause swelling without lysis for periods up to 1-1/2 hours at 0-5° C. When distilled water was used, lysis of the cells as well as of the cellular particles occurred after only 5-15 minutes at 0-5° C. Since the lysis was so rapid, there was insufficient time for centrifuging the cells and for resuspending them in the sucrose solution. After lysis, the integrity of the cellular particles is destroyed, and fractionation is impossible.

Neufeld et al. (11) have shown that the ratio of peroxidase to cytochrome oxidase exceeds 5.0 for tissues which exhibit cell renewal. In this ascites tumor we have found this ratio to be variable; the first three experiments gave a ratio less than unity, and the last four experiments gave a ratio greater than unity, but never higher than 2.8. The cytochrome oxidase purity has remained nearly constant. The shift in the ratio is associated with the increase in peroxidase purity that this tumor has experienced since its maintenance began in this laboratory. The reason for this change is unknown. All other factors involved in the care of the animals and transplantation of the tumor have remained constant.

Preliminary results with isolated mitochondrial fractions indicate that oxidative phosphorylation does occur with succinate as a substrate. Further investigations are being carried out.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. Theodore S. Haushka, Roswell Park Memorial Institute, Buffalo 3, New York, for supplying the Lettré-Ehrlich ascites tumor for transplantation, and to Dr. Alexander L. Dounce of the Department of Biochemistry for his guidance in the cytochemical fractionation process.

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Fig. 1. — Lettré-Ehrlich ascites tumor cells suspended in normal ascites fluid. X790.
Fig. 2. — Lettré-Ehrlich ascites tumor cells after being sus-
pended for 1 hour in dilute Ringer solution. X790.
Fig. 3. — Isolated nuclei suspended in 0.44 M sucrose. X790.
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