285, and 275 (shoulder) nm in ethanol] diminished, suggesting that they may be precursors of trisporic acids. In addition, a new component with an $R_F$ of 0.66 appeared in the neutral fraction with a principal maximum at 234 nm and a second maximum at about 295 nm. Separate experiments revealed that the isolated acid fraction from the culture medium of the (+) strain did not stimulate the formation of trisporic acid by cultures of the (−) strain, whereas the isolated neutral fraction was over 90 percent as effective as the total fraction.

To determine whether the neutral fraction from culture media of the (+) strain contained precursors or an inducer, or both, cultures of the (−) strain with neutral fraction from cultures of the (+) strain added were incubated with and without actidione (58 μg/ml). Trisporic acids were formed equally well in the presence and absence of actidione (Fig. 2). Control experiments in which cultures of the (−) strain were incubated with 2.5 μc of [$^{14}$C]leucine for 1 hour with and without actidione revealed that incorporation of leucine was inhibited over 97 percent by actidione. A similar experiment with actidione and [$^{14}$C]leucine has been reported (*4*). These observations suggest that (i) the culture medium of the (+) strain contains a precursor, or precursors, which cultures of the (−) strain convert to trisporic acid; and (ii) the enzymes necessary for formation of trisporic acid in cultures of the (−) strain are constitutive.

Because cultures of the (−) strain of *B. trispora* synthesize trisporic acid C from precursors made by cultures of the (+) strain, the observation (*3*) that trisporic acid synthesis was proportional to the amount of inoculum from the (+) strain used in combined cultures of the (+) and (−) strains must now be interpreted that precursor formation by cultures of the (+) strain is the rate-limiting step in trisporic acid synthesis. In a similar fashion, van den Ende's (*5*) observation that cultures of the (+) strain but not of the (−) strain of *B. trispora* produce trisporic acids when incubated with *Z. moelleri* can be interpreted that *Z. moelleri*, while being able to convert precursor to trisporic acids, is either unable to make precursor or (and more likely) synthesizes such small quantities of precursor that trisporic acid synthesis was not detected under the conditions employed. Consistent with these views is the observation (*6*) that cultures of the (−) strain of *B. trispora* but not those of the (+) strain synthesized trisporic acids or trisporone, or both, when incubated in filter-sterilized culture medium from combined cultures of (+) and (−) strains of *B. trispora.*

Dutch investigators (*5, 6*) have reported that a single trisporic acid, isolated from combined cultures of (+) and (−) strains of *B. trispora,* induced the formation of zygophores in cultures of both the (+) and (−) strains of *M. mucedo,* implying that a single mating-type produces a hormone that induces the formation of zygophores for both mating-types. In contrast, German investigators (*7, 11*) postulated that (i) separate sex hormones induce zygophore formation in cultures of (+) and (−) strains of *M. mucedo*; (ii) cultures of the (+) strain produce the hormone which induces the formation of zygophores in cultures of the (−) strain; and (iii) cultures of the (−) strain produce the hormone which induces the formation of zygophores in cultures of the (+) strain. It should be possible to determine which of the above two hypotheses is correct—that is, determine whether trisporic acids made by a single mating-type induce zygophores in both mating-types or in only the opposite mating-type—by utilizing the extract of trisporic acids isolated from cultures of the (−) strain.

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## Bulk Isolation in Nonaqueous Media of Nuclei from Lyophilized Cells

Abstract. *Intact lyophilized nuclei are obtainable from a variety of tissues, either in situ or in culture, by freezing at −156°C, drying at −25°C, and mechanical disassociation in glycerol at 2°C. Centrifugal separation of nuclei is accomplished in an 85 : 15 by volume mixture of glycerol and 3-chloro-1,2 propanediol at 2°C. The method gives homogeneous nuclear preparations in high yield with preservation of labile and water-soluble constituents.*

Cell fractionation for nuclear isolation is most conveniently processed in aqueous media (*1*). Conventional homogenization of isolated tissue in aqueous media, however, introduces unavoidable analytical artifacts rendering assays for many important biological materials invalid. Catabolic events associated with anoxia, extraction of water-soluble nuclear constituents, and cross-contamination between nuclei and cytoplasm compromise aqueous nuclear preparative methods (*2*). Despite these deficiencies, nuclear isolation in water remains a standard procedure, since alternative nonaqueous preparative methods have been cumbersome and destructive of both nuclear morphology and enzymatic activity (*3*). Microdissection of nuclei from individual lyophilized cells circumvents these difficulties, but is tedious, applicable only to large nuclei, and necessarily of low yield. This report describes a bulk method, with preliminary steps based on the system of Lowry (*4*) for lyophilization of tissue sections, but extended and adapted to permit efficient isolation of remarkably clean, lyophilized nuclei in high yield.

Though technical maneuvers involved in nonaqueous nuclear isolation methods are similar, certain modifications are critical for reproducible results. Tissue or cells must be frozen rapidly, not only to minimize ice artifacts, but to limit redistribution of diffusible intracellular components and degradation of

labile compounds as well. Immediately excised tissue, or tissue culture material, is immersed without delay in Freon-12 ($CCl_2F_2$) chilled to its freezing point (−156°C) by liquid nitrogen. Specimens with a volume of approximately 1 $cm^3$ or dimensions of an adult mouse liver can be satisfactorily frozen by this method. Cultured cells adhering to the floor of small plastic bottles are frozen immediately after removal of incubation media and the bottle roof. Other freezing techniques including liquid nitrogen alone, Dry Ice, and slow freezing at −80°C result in serious ice artifacts. These large specimens of frozen tissue (mouse liver) are coarsely powdered in a mortar chilled with liquid nitrogen prior to drying. Frozen cultured cells are scraped from the plastic bottle floor with a spatula in a cryostat at −30°C. Tissue once frozen is stored under vacuum at −80°C or dried at −25°C (5 μm-Hg vacuum for 48 hours) with a $P_2O_5$ trap. Lyophilization at these conditions progresses rapidly over the first 24 hours, but removal of an additional 2 to 3 percent of tissue water over an ensuing 24 hours is essential for optimal cell comminution. Since tissue dried in this manner is exceptionally hygroscopic, exposure to room air should be limited. We have noted during microdissection that tissue brittleness predisposes to intracellular fracture about the nuclear-cytoplasmic junction.

Cell disruption of dried avian erythrocytes, murine liver and hepatoma, and murine glioblastoma in tissue culture has been accomplished by homogenizing samples suspended in chilled glycerol (2°C, analytical grade; 1 to 2 g of tissue to 30 ml of glycerol). Two commercially available milling devices are satisfactory. The Polytron (Kinematica GMBH, Lucerne, Switzerland, model PT 10) effectively disintegrates lyophilized cells by combining sonication and shearing in viscous glycerol. A pulse-frequency of approximately 2500 cycle/sec for 45 seconds liberates free nuclei from about half the suspended cells, but two additional pulse periods of 30 to 45 seconds each are usually necessary to assure fields containing 90 to 95 percent free nuclei devoid of cytoplasmic tabs. The Sorvall Omni-Mixer (setting 5.5, approximately 20,000 rev/min for about 4½ minutes) will also effectively homogenize lyophilized tissue in chilled glycerol. The temperature of the suspension should be maintained at 2°C during homogenization. Diffusion and solubility of a variety of compounds having small molecular weights (adenosine triphosphate, lactate, glucose, inorganic phosphate, glucose-6-phosphate) have been found negligible in glycerol at 2°C. Cytological monitoring of preparations, either by phase or bright-field microscopy, shows the extent of nuclear liberation. It is noteworthy that lyophilized nuclei are remarkably resistant to prolonged sonic and shearing forces in chilled glycerol. The homogenized suspension is maintained at 2°C, filtered through glass wool to remove fibrous material, and a 4-ml sample is layered over 1.0 ml of an 85 percent glycerol, 15 percent 3-chloro-1,2-propanediol (α-chlorohydrin) mixture chilled to 2°C in a cellulose nitrate tube. The density of this mixture is 1.312 at 2°C and 1.324 at 4°C. Nuclear sedimentation with flotation of other cytoplasmic components occurs with a single centrifugation. Tubes are centrifuged at 30,000 rev/min in a Spinco 39 SWL swinging bucket rotor for 30 minutes at 4°C (120,000*g*). Densities of lyophilized nuclei from the specimens used in these experiments exceed 1.33 (*5*).

Typical nuclear preparations are gelatinous pellets. This consistency is in-

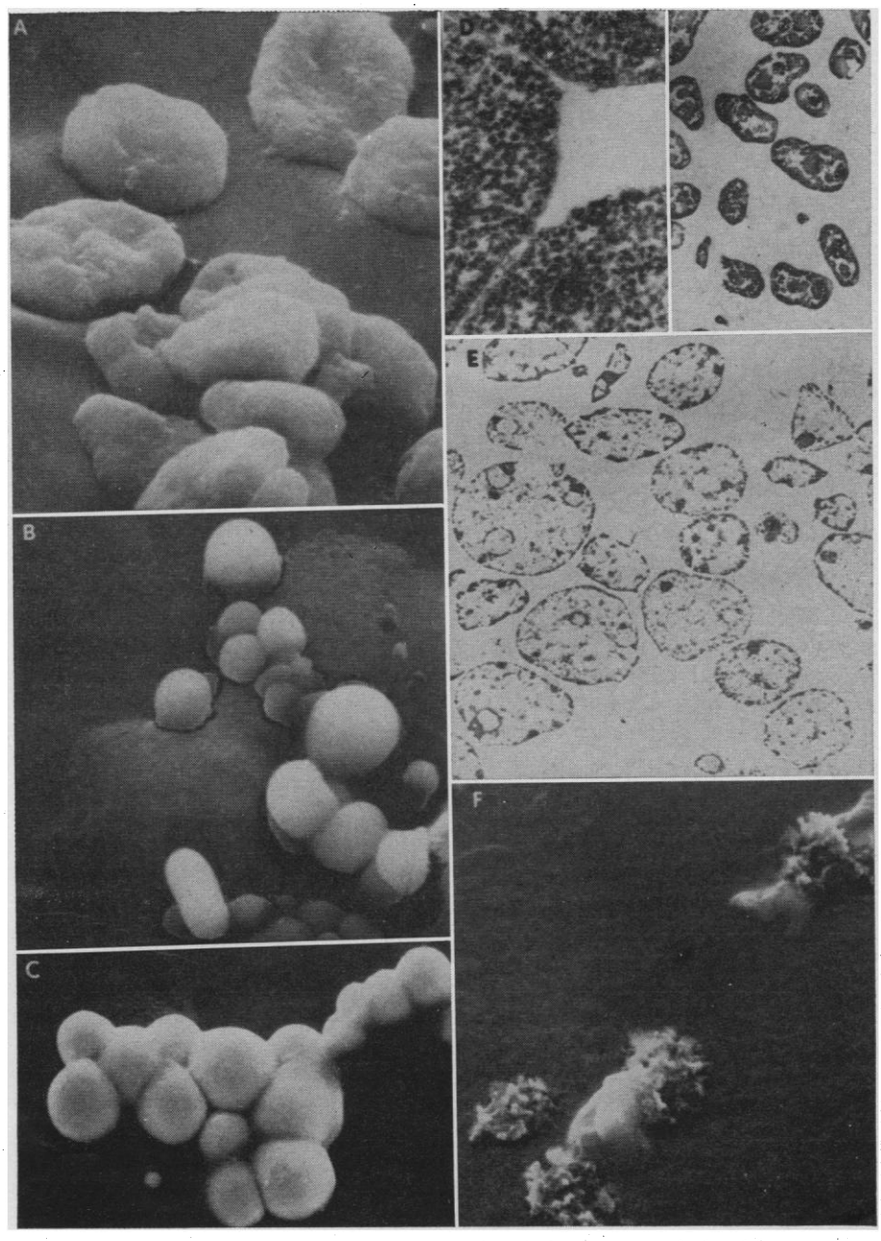


Fig. 1. Representative fields of lyophilized nuclei and chromosomes isolated by our nonaqueous method. A, B, C, and F are scanning electron micrographs; D and E transmission electron micrographs. A and D are hen erythrocyte nuclei: A, ×5000; D, ×40,000 (left), ×2000 (right). B and E are murine liver nuclei: B, ×2500; E, ×3000. C is murine hepatoma nuclei, ×2000. F is chromosomes from thymidine "blocked" glioblastoma cells in culture, lower left anaphase, upper right metaphase, ×6000.

Table 1. Levels of RNA, DNA, and certain intermediates of glycolysis in glycerol homogenates and isolated nuclear preparations from three different cell types.

| Tissue* | | Nucleic acids† | | | Intermediates of glycolysis‡ | | | |
|---|---|---|---|---|---|---|---|---|
| | | RNA | DNA | RNA/DNA | ATP | Glucose | Glucose-6-phosphate | Lactate |
| Chicken erythrocytes | H | 6.8 ± 0.4 | 71.3 ± 7.6 | 0.09 | 3.8 ± 0.04 | 6.3 ± 0.07 | 0.15 ± 0.02 | 10.8 ± 0.7 |
| | N | 37.0 ± 3.0 | 416.1 ± 20.0 | 0.09 | 0.1 ± 0.01 | 0.33 ± < 0.01 | 0.06 ± < 0.01 | 7.2 ± 0.4 |
| Mouse§ liver | H | 46.3 ± 1.0 | 21.1 ± 0.9 | 2.20 | 6.1 ± 0.9 | 92.4 ± 6.0 | 4.8 ± 0.5 | 29.8 ± 0.7 |
| | N | 64.7 ± 2.6 | 452.2 ± 28 | 0.14 | 2.8 ± 0.4 | 24.9 ± 3.6 | 0.92 ± 0.1 | 39.9 ± 2.4 |
| Mouse¶ hepatoma | H | 56.8 ± 1.1 | 42.2 ± 3.7 | 1.35 | 3.7 ± 0.5 | 16.3 ± 1.4 | 1.6 ± 0.05 | 147 ± 8.7 |
| | N | 74.8 ± 4.8 | 488.5 ± 31 | 0.15 | 1.4 ± 0.4 | 3.7 ± 0.6 | 0.3 ± 0.07 | 43.8 ± 5.0 |

*Mouse liver and hepatoma frozen immediately after excision, chicken erythrocytes prepared from defibrinated blood, washed with isotonic saline, and frozen hours after removal. H refers to glycerol homogenate (broken cell suspension); N, isolated nuclear pellet. Animals fed freely (ad libitum) prior to excision of tissue. †Nucleic acids assayed as in text and expressed as micrograms per milligram of homogenate or nuclear protein ± S.E.M. Each value is an average of at least five separate samples, and in some cases as many as eight. ‡Concentration of substrates expressed as millimicromoles per milligram of homogenate or nuclear protein ± S.E.M. Substrates measured in the acid-soluble pool by fluorimetric methods of Lowry *et al.* (*18*). Each value is the average of at least five determinations, and in many cases more, of separate samples. §Female C-57 mice, 6 weeks old. ¶Mouse hepatoma BW-7756, Jackson Laboratories, Bar Harbor, Maine in C-57 female mice, 6 weeks old.

dicative of minimal autolysis (*6*). Practically all of the DNA in homogenized chicken erythrocytes sediments with the nuclear pellet. Recoveries of DNA from mouse liver and hepatoma homogenates average 90 and 85 percent respectively. Microscopic examination of the supernatant shows infrequent contamination with free nuclei, though occasional intact cells are present. In tissue culture material, 100 mg of lyophilized glioblastoma cells (approximately $5 \times 10^6$ cells) yields a 10-mg nuclear pellet. High yields are attributable to flotation of light constituents in chilled glycerol and negligible trapping of nuclei at the glycerol : glycerol, $\alpha$-chlorohydrin interface. After a single centrifugation, the supernatant is discarded and the interior of the tube above the visible nuclear precipitate is wiped clean. The pellet is resuspended in 0.2 ml of glycerol at 2°C for further study. Quantitative volumetric transfers of chilled glycerol are accomplished with a No. 13 needle and tuberculin syringe.

Biochemical and morphological studies of lyophilized nuclei isolated by our system have confirmed the remarkable purity and residual biological activity of the preparations. Conventional electron micrography and scanning electron microscopy demonstrate negligible contamination with endoplasmic reticulum or broken nuclei. For electron microscopy, nuclear pellets in glycerol are impregnated with hydroxyethyl methacrylate, and subsequently with Epon 812, and polymerized at 60°C for 48 hours. Ultrathin sections are stained with aqueous uranyl acetate and lead citrate and viewed with a Philips electron microscope (EM-200).

Material for scanning electron microscopy is prepared by drying nuclear suspensions in glycerol on membrane-coated grids (60°C, 5 $\mu$m-Hg vacuum for 48 hours), followed by a uranium vapor coating. Samples are viewed with the Jeolco scanning electron microscope (*7*). Outer nuclear membranes and contiguous ribosomes have been completely stripped from virtually all nuclei (Fig. 1). Nuclear suspensions in glycerol are amenable to assays for both acid-extractable and acid-precipitable constituents. An aliquot of the dispersed nuclear suspension is taken for protein measurement by the method of Lowry *et al.* (*8*). Acid-soluble substances are extracted by pooling successive extractions with 0.6 and 0.3*M* perchloric acid chilled to 0°C. Alkaline digestion of the acid-precipitable material in 0.1 ml of 0.3*M* KOH at 37°C for 18 hours solubilizes nucleic acids. Deoxyribonucleic acid is precipitated at 0°C upon acidification with 0.1 ml of 0.6*M* perchloric acid, and measured by the method of Ceriotti (*9*) with calf thymus DNA as a standard. Supernatant RNA is measured by the orcinol method (*10*) with yeast RNA as a standard. No cross-contamination of RNA and DNA fractions is detectable.

Comparing RNA : DNA ratios of glycerol homogenates from whole tissue to isolated nuclear preparations gives an index of nuclear purification (*11*) (Table 1). In the hen erythrocyte, a highly differentiated cell, virtually all nucleic acids are localized in the nucleus. The observed RNA : DNA ratio of 0.09 for chicken erythrocyte nuclei closely agrees with literature values for aqueous nuclear preparations (*12*). Lyophilized mouse liver nuclear pellets isolated in glycerol contain approximately 6 percent of the total tissue RNA, corresponding to reported values of 4 to 6 percent for pure aqueous rat liver nuclear preparations (*13*). Higher percentages of total tissue RNA in nuclear preparations suggest contamination with outer nuclear membranes and contiguous ribosomes. Aqueous nuclear preparations are customarily treated with detergents in order to separate outer nuclear membranes, thus introducing additional morphological and solubilization artifacts (*13*). Controlled disintegration of lyophilized tissue in chilled glycerol has the advantage of quantitatively removing outer nuclear membranes without resorting to special treatment. Isolation of clean nuclei from neoplastic tissue or tissue culture material is considered a difficult analytical procedure (*14*). The RNA : DNA ratio of 0.15 in hepatoma nuclei isolated by our method is several fold less than previously reported values of 0.63 and 0.35 attained by aqueous methods (*15*).

Reported assays of glycolytic intermediates in nonaqueous rat liver nuclear preparations (isolated in carbon tetrachloride and cyclohexane) have shown no significant differences in nuclear-cytoplasmic distribution of these compounds (*16*). In contrast, our data for glycerol-isolated nuclei demonstrate that adenosine triphosphate, glucose, and glucose-6-phosphate are present in significantly lower concentrations in the nuclear component. The concentration of lactate in normal liver nuclei is higher than in cytoplasm, but equivalent to that found in hepatoma nuclei. Lactate levels in hepatoma cytoplasm greatly exceed those found in nuclei, thus indicating an extranuclear site for the enhanced glycolysis characteristic of this tissue.

Incubation of glycerol-isolated lyophilized liver, hepatoma, and glioblastoma nuclei in 0.1*M* tris buffer, *p*H 7.6, restores lactate production and glucose degradation. Thus nuclear enzymes and cofactors of glycolysis have withstood freeze-drying, exposure to glycerol, and homogenization. The RNA polymerase

activity of rehydrated lyophilized nuclei has been examined by the method of Pogo (*17*) and found to be present. In the case of lyophilized glioblastoma nuclei, RNA polymerase activity is, in fact, several fold greater than in control aqueous sucrose preparations.

This method of supercooling, prolonged lyophilization, and cell disintegration in neutral nonaqueous media of high density provides unique opportunities for quantitative cytochemical studies at the subcellular level. Nuclear subfractionation, for example, has been attained by comminution of thymidine "blocked" glioblastoma cell cultures, giving quantitative yields of intact chromosomes in glycerol (Fig. 1). Minor modifications of the centrifugation procedure and gradient media may permit the preparative isolation of other lyophilized subcellular organelles.

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## Mouse Lysozyme Production by a Monocytoma: Isolation and Comparison with Other Lysozymes

Abstract. *A transplantable mouse tumor, GPC-11, produces large amounts of lysozyme. The tumor is a reticulum cell sarcoma, type A, and is a neoplasm of monocytes. The lysozyme was purified from mouse urine in quantities sufficient for structural analysis. Comparison of mouse lysozyme with lysozymes from chicken egg white and patients with monocytic leukemia reveals similarities in size and electrophoretic mobility and, with human lysozyme, in functional properties; but considerable differences are found in antigenic characteristics and amino acid composition.*

Chicken egg white lysozyme (muramidase), a hydrolytic enzyme which degrades certain bacterial cell walls, is a well-studied, small protein whose complete primary, secondary (*1*, *2*), and tertiary structures (*3*) have been elucidated. Several mammalian lysozymes have been analyzed for amino acid composition (*4*), but sufficient quantities for sequence determination were not readily available until Osserman and Lawlor (*5*) discovered that patients with monocytic leukemia excrete up to several grams of lysozyme daily in their urine. Purification from leukemic urine is extremely easy, making human lysozyme an excellent subject for structural studies (*6*). We report here an analogous phenomenon in mice, that is, a transplantable monocytic tumor which secretes large quantities of lysozyme via the urine. Transplantation of this tumor into modest numbers of mice furnishes gram amounts of mouse lysozyme, so that details of structure of the two mammalian enzymes may be compared. We now report the characterization of the mouse monocytic tumor and its lysozyme product. A probably related type of mouse tumor, myelomonocytic leukemia, has been reported to produce lysozyme (*7*).

The GPC-11 is one of a series of tumors induced by Goldstein *et al.* (*8*) in (NZB × BALB/c) F1 hybrid mice. Mice were inoculated intraperitoneally with 0.4 ml of medicinal paraffin at 6 weeks of age and again at 10 and 14 weeks of age. When they were swollen with ascites, the mice were killed, and their tumors were transplanted. This treatment in BALB/c mice induces mainly immunoglobulin-producing plasma cell tumors; in DBA/2 mice it induces reticulum cell sarcomas (*9*), which are malignancies of the tissue phagocytes. Dunn classified these tumors (*10*) and defined the reticulum cell sarcoma type A as a pure line of phagocytic cells related to histiocytes and monocytes. This tumor type has also been described as a monocytoma. In the NZB × BALB/c hybrid mice, tumors begin to arise in the ninth month of age, and the first ten tumors were plasmacytomas. The eleventh, GPC-11, arose sometime after the mice were 14 months of age and was quite unlike the previous tumors.

The GPC-11 tumor grows rather slowly—14 months in the initial passage, 6 to 11 months for the second, and 2 to 5 months for all ensuing generations. When passaged intraperitoneally, the tumor grows as white nodules in the mesentery, as deposits on the liver, and most strikingly, in females, as massive spongy invasions of the ovaries. Subcutaneous transfer results in massive local growth, regular spread to the liver, and occasional obvious metastases to the spleen, kidney, ovary, or thymus. A few presumptive tumor cells can be found in blood smears, but the differential white count is within normal limits.

Histological examination of GPC-11 sections reveals a cell population heterogeneous in size and morphology. The tumor includes large and small round mononuclear cells, multinucleated giant cells, and strands and whorls of spindle cells. Nuclei are strongly basophilic and vary from round through kidney and U shapes to rings. Cells with two or more nuclei are common. The cytoplasm is lightly staining, often vacuolated, and sometimes shows engulfed red cells. In Giesma-stained ascites smears, the principal cell is a distinctive monocyte showing pseudopods, extensive vacuolation, an eccentric round or kidney-shaped nucleus, and strongly basophilic cytoplasm. The phagocytic aspect of this monocytoma, its varying cellular morphology and ascitic monocytes, and its slow growth and predilection for liver metastases clearly distinguish it as a reticulum cell sarcoma, type A (*10*).

Agar electrophoresis of the serum or urine from a mouse bearing a GPC-11 tumor discloses an unusual basic protein migrating toward the cathode