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

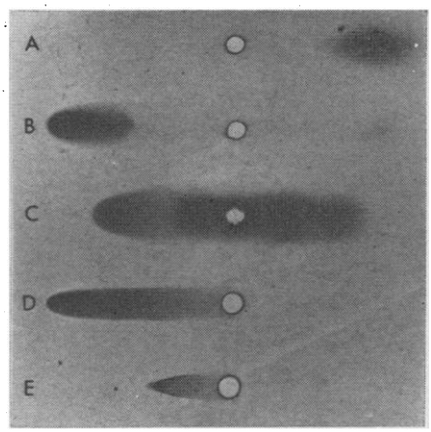

Fig. 1. Agar-gel electrophoresis of urines from a normal and GPC-11 bearing female (BALB/c × NZB) F1 mouse in 1 percent Oxoid Ionagar No. 2, 0.05*M* barbital buffer, *p*H 8.2, 7 volt/cm for 40 minutes. Lysozyme appears as the very dense cathodal (to left) bullet-shaped spot in the tumor urine (B) and is absent from the normal urine (A). Shown for comparison with mouse lysozyme are a concentrated urine from a human monocytic leukemia patient (C) and two concentrations of egg white lysozyme (D, 5 mg/ml; E, 1 mg/ml); these demonstrate the concentration dependence of lysozyme's electrophoretic mobility in agar. The anodally migrating protein in normal and tumor-bearing mouse urine is MUP (mouse urinary protein) (*16*).

(Fig. 1), similar in mobility to lysozyme of egg white and to that from urine of patients with monocytic leukemia (*5*). All three proteins display a characteristic, bullet-shaped spot with trailing toward the origin, and a concentration-dependent mobility. These properties are due to binding of the proteins to the agar gel and are not seen on cellulose acetate.

The lysoplate method of Osserman and Lawlor (*5*) was used to test the GPC-11 protein for lysozyme activity. In this method, sample wells are aspirated in a turbid 1 percent agar gel containing dried *Micrococcus lysodeikticus* (1 mg/ml) in *M*/15 phosphate buffer *p*H 6.3. The sample wells are filled with serums, urines, and a series of standard concentrations of mouse lysozyme. The plate is then incubated for 12 to 24 hours at room temperature. The diameter of the resulting zone of bacterial lysis around each well is proportional to the logarithm of the lysozyme concentration of the sample. Figure 2 shows the elevation of lysozyme in the serums and urines of mice bearing the GPC-11 tumor.

We determined the amounts of lysozzyme in the serums and urines of 20 normal adult (BALB/c × NZB) F1 mice and 14 mice bearing very advanced GPC-11 tumors (Table 1). Concentrations in normal serums were quite low (0.002 mg/ml), and lysozyme was undetectable in normal urines (< 0.0004 mg/ml). The mean concentration of lysozyme in the serums of tumor-bearing mice was 20 times that of normal mice (0.04 mg/ml), but the urines of the tumor-bearing mice contained surprising amounts of lysozyme —as much as 25 mg/ml in the highest instance. Although these extreme levels are reached only in terminal cases, mice with less-advanced tumors still secrete several milligrams of lysozyme per day, so that a gram of lysozyme, adequate material for extensive chemical studies, can be collected over a period of several weeks from a few dozen mice.

Since lysozyme is a normal lysosomal constituent of monocytes, it is likely that the excessive amount of this enzyme in the tumor-bearing animals is the result of its release by tumor monocytes. Extracts of tumor pieces contain larger concentrations of lysozyme than serum does, implicating the tumor mass as a source of the enzyme. Also, a histologic method (*11*), in which dispersed cells are mixed with lysable bacteria and then spread on a slide, demonstrates strong lysozyme activity in individual tumor cells. Bacteria are degraded around the tumor cells and around normal neutrophils and monocytes, while lymphocytes and eosinophils do not show lysozyme action. The production of lysozyme by this tumor raises the possibility that it may also be a rich source of acid phosphatase, glucuronidase, peroxidase, or other components of the azurophilic granule type of lysosome found in young monocytes.

To estimate its size, we compared mouse lysozyme isolated from the urine of mice bearing GPC-11 with bovine serum albumin, human hemoglobin, mouse Bence-Jones protein, and horse myoglobin by thin-layer gel filtration on Sephadex G-75, superfine, in phosphate-buffered saline, *p*H 7.0. Lysozyme moved faster than salts but slower than any of the other proteins including myoglobin, indicating a molecular weight less than 16,900 (myoglobin).

The sedimentation coefficient found

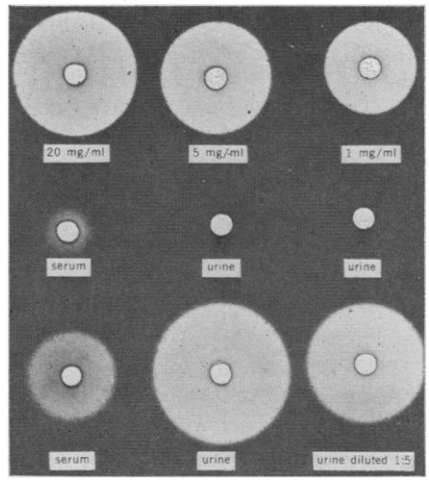

Fig. 2. Lysoplate assay for lysozyme. (Top) Wells contain egg white lysozyme at concentrations of 20, 5, and 1 mg/ml. (Center) One serum and two urines from normal mice; low activity is found in the serum and none is detectable in the urines. (Bottom) One serum and undiluted and fivefold diluted urine from a GPC-11–bearing mouse, demonstrating the greatly increased lytic activity produced by this tumor. The undiluted urine (center) contains only 3 mg per milliliter of mouse lysozyme but gives greater lysis than the solution of egg white lysozyme containing 20 mg per milliliter, making apparent the greater specific activity of the mouse enzyme.

for lysozyme was consistent with the determination of molecular weight by gel filtration. Sedimentation velocity was measured twice in a Spinco Model E analytical ultracentrifuge with sedimentation conditions of 59,780 rev/min and 25°C, at protein concentrations of 1.4 mg/ml in phosphate-buffered saline and of 9.2 mg/ml in 0.9 percent saline. A sedimentation coefficient of 2.0 was obtained in both cases. The $s_{20,w}$ value for human lysozyme is 1.8 to 2.0 (*5*, *12*) and for chicken, quail, and pheasant egg white lysozymes is 1.8 (*13*). The molecular weight of chicken egg white lysozyme is 14,300 (*1*), and human lysozyme from various sources is in the range of 14,000 to 15,000 (*12*). Mouse lysozyme must be approximately this same size.

The lack of antigenic similarity between mouse lysozyme and lysozymes of other species is evident from the fact that a rabbit antiserum raised against an electrophoretically purified fraction of GPC-11 urinary lysozyme gives strong precipitation reactions with mouse lysozyme in the Ouchterlony double diffusion test but does not react with either human or chicken egg

white lysozmes. Rat lysozyme, which might have shown similarity, was not present at high enough concentrations in normal rat serum to allow Ouchterlony analysis.

Testing by the more sensitive method of lysis inhibition confirmed the antigenic disparity of mouse and egg white or human lysozymes, but a similarity between mouse and rat lysozymes was detected. It was necessary to first separate the rabbit antiserum's γ-globulin fraction from its normal serum lysozyme by gel filtration on Sephadex G150. Then the antibody fraction was placed in lysoplate wells adjacent to wells containing purified lysozymes or normal serums from various sources. Inhibition of lysis was seen as a flat indentation in the normally circular lytic zone (*5*). Normal rat and mouse serum lysozymes were strongly inhibited, while the other lysozymes were unaffected.

For chemical studies, mouse lysozyme was readily purified from the urine of mice bearing GPC-11 tumor by ion-exchange chromatography on carboxymethyl cellulose (Whatman CM 32) in 0.05*M* glycine buffer *p*H 9.4. The lysozyme peak was eluted with a NaCl gradient and appeared at approximately 0.2*M* NaCl. The lysozyme preparation was then desalted by passage over Sephadex G-25 in 0.02*M* acetic acid and lyophilized. A solution of commercial chicken egg white lysozyme (Worthington, LYSF) was similarly desalted and lyophilized and used for comparison. The lysozymes were hydrolyzed in 6*N* HCl for 22 hours at 110°C, and the hydrolyzate was then subjected to amino acid analysis (Beckman model 116). Tryptophan was determined by ultraviolet spectrophotometry in 6*M* guanidine hydrochloride as described by Edelhoch (*14*). Values for the composition of human lysozyme were averaged from those reported (*5*, *12*, *15*).

There are at least 36 amino acid differences between mouse and egg white lysozyme and 28 differences between human and egg white lysozymes. The lysozymes of mouse and human are more similar, but have at least 18 amino acid differences. The complement of basic amino acids in all three lysozymes is similar with a total of 18, but acidic residues show wider differences.

While lysozymes from egg white and man have the same total of 26 acidic residues, the mouse protein contains 29. This increase is not, however, reflected in the electrophoretic mobility at neutral *p*H, as mouse lysozyme migrates on cellulose acetate at a rate intermediate to that of the other two, with egg white lysozyme moving to the cathode most rapidly.

Table 1. Lysozyme in normal mice and mice bearing GPC-11 tumors. (BALB/c × NZB) F1 mice were used. Lysozyme concentrations were determined by lysoplate assay (*5*); mouse lysozyme was used as a standard.

Body fluid	Lysozyme (mg/ml)	
	Range	Mean ± S. D.
	Normal	
Serum	0.0018–0.0029	0.0022 ± 0.0004
Urine	< 0.0004	
	GPC-11	
Serum	0.009 –0.081	0.039 ± 0.021
Urine	2.1–25	8.5 ± 9.0

To determine the extinction coefficients of mouse and egg white lysozymes, the desalted lysozymes were dissolved in distilled water, portions were diluted in 0.2*M* phosphate buffer, *p*H 6.5, and their absorbancies were measured at 280 nm, while measured samples were dried to constant weight in a vacuum over P_2O_5 at room temperature. This procedure gave absorbances, $E^{1\%}_{280\ nm}$, for egg white lysozyme of 25.0 and 25.4. These values are approximately 5 percent below that obtained by Canfield by drying in a vacuum over P_2O_5 at 100°C (*1*).

Values for two preparations of mouse lysozyme were 21.6 and 21.9. The lower absorbancy of the mouse lysozyme molecule is explained by its content of amino acid chromophores. At neutral *p*H, tryptophan is the major absorber of ultraviolet light, and mouse lysozyme has only four residues of tryptophan per molecule, compared to six in egg white lysozyme. While mouse lysozyme has seven tyrosines to egg white's three, the absorbancy of this residue below *p*H 10 is only one-fourth that of tryptophan.

The enzymatic specific activities on *M. lysodeikticus* of mouse and human lysozymes are similar to and greater than that of egg white lysozyme. Human lysozyme has a specific activity approximately tenfold higher than that of egg white lysozyme, as measured in the lysoplate assay in phosphate buffer; but it is only two- to threefold higher when measured in a liquid-phase assay with 0.1 percent NaCl added to the buffer (*5*). Mouse lysozyme shows similar behavior, the specific activity being 50-fold higher than that of egg white lysozyme in a standard lysoplate. However, when 0.2*M* NaCl is added to the lysoplate buffer, the activity of egg white lysozyme is increased and that of mouse lysozyme is decreased, so that the mouse activity is only twofold higher.

While compositional and enzymatic analyses show that the lysozymes of mouse and man are more closely related to each other than to chicken egg white lysozyme, these same analyses, in agreement with the antigenic disparity of the enzymes, indicate considerable evolutionary divergence among these proteins.

Lysozyme production by reticulum cell sarcomas in mice has not been previously reported; but it may be a regular property of this class of tumor as it is in human monocytic leukemia. Lysozyme production has also been noted in a transplantable myelomonocytic leukemia induced in BALB/c mice with intraperitoneally administered mineral oil (*7*).

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