Frog Lysozyme

I. ITS IDENTIFICATION, OCCURRENCE AS ISOZYMES, AND QUANTITATIVE DISTRIBUTION IN TISSUES OF THE LEOPARD FROG, *RANA PIPIENS* [1,2,3]

DAVID S. OSTROVSKY,[4,5] JOHN A. SNYDER,[4,6] TAKUZO IWATA,[7] KEN-ICHI IZAKA,[8] DONNA S. MAGLOTT [9] AND GEORGE W. NACE
Division of Biological Sciences and Center for Human Growth and Development, The University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT In the course of examining the etiology of the Lucké renal adenocarcinoma of the frog, *Rana pipiens*, it was found that organs of the normal adult contain bacteriolytic enzymes. These enzymes all satisfied the six criteria for the identification of lysozymes and at least eight forms were separable by polyacrylamide gel electrophoresis. Their qualitative and quantitative distribution was organ-specific. All eight isozymes were found in normal kidney, while liver and spleen contained seven forms; skin, six; ovarian egg, five; and serum, two.

In quantitative assays using a radial diffusion test, spleen had the greatest lysozyme concentration, followed in descending order by kidney, liver, skin, and ovary. Serum contained very low amounts. In terms of enzyme activity per animal, ovary was the highest ranking organ. As such a large number of lysozyme isozymes has not been reported in any other organism, their origins and functions are considered in the context of their presence in an ectotherm.

The discovery of an antigen with a differential distribution in *Rana pipiens* kidney and the Lucké renal adenocarcinoma, and the determination that this was a highly basic protein which possessed bacteriolytic action first suggested the presence of a lysozyme-like enzyme in frogs. It was hypothesized that this enzyme may be related to the etiology of the Lucké tumor (Nace, '61, '62; Nace and Suyama, '65; Nace et al., '65). This hypothesis was based on: (1) the presence of lytic enzyme in normal kidney and its apparent absence from the tumor (Nace et al., '65); (2) the presumed viral etiology of the Lucké tumor (Rafferty, '64; Mizell, '69; McKinnell, '73); (3) the antiviral activity of lysozyme in general (Ferrari et al., '59; Arimura, '73); (4) the antiviral activity of *certain forms* of *R. pipiens* lytic enzyme in particular (Rubin et al., '66); and (5) seasonal and local variations in lytic enzyme level which were related to the life cycle of the tumor and its virus (Nace and Suyama, '65).

Investigation of this hypothesis was complicated by evidence for multiple forms of lytic enzyme (Nace and Suyama, '65; Amano, Iwata, Izaka, unpublished), but gained significance in view of the discovery of differences in the antiviral activity of lytic enzyme preparations from various species of frogs, and from different organs of *R. pipiens* (Rubin et al., '66).

These observations prompted a more detailed study of the specific identification of the enzyme and the distribution of the enzyme among organs of the leopard frog.

Since a variety of enzymes are capable

---

[1] Contribution No. 64 from the Amphibian Facility.

[2] The animals used in this study were maintained in facilities that are fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

[3] Address for reprints: Dr. George W. Nace, Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109.

[4] Adapted, in part, from dissertations submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The University of Michigan.

[5] Present address: Department of Biology, Millersville State College, Millersville, Pennsylvania 17551.

[6] Present address: Biology Department, Furman University, Greenville, South Carolina 29613.

[7] Present address: 1-28 Yamate Dori showa-Ku, Nagoya 466, Japan.

[8] Present address: The Green Cross Corporation, 1/3 Gamau-cho, Joto-ku, Osaka, Japan.

[9] Present address: 1014 Baltimore Road, Rockville, Maryland 20851.

of hydrolyzing bacterial cell walls (Strominger and Ghuysen, '67) the lysis of suspensions of *Micrococcus lysodeikticus (M. luteus)* by frog material is, by itself, insufficient evidence for its classification as lysozyme. To be classified as lysozyme, a protein should satisfy certain criteria: (1) be a basic protein; (2) possess a "low" molecular weight; (3) be stable at acid pH even at high temperatures; (4) be unstable at alkaline pH especially at high temperatures; (5) be active against suspensions of *M. lysodeikticus;* and (6) be capable of releasing specific amino sugars from appropriate substrates (Jollès, '67). The frog lytic enzyme was tested against these criteria and, as described here, was shown to be lysozyme.

The available techniques for the qualitative and quantitative assay of lysozyme were unsatisfactory for frog material. This necessitated the development of new procedures suited to both the characteristics of the enzyme and to the limited quantities available. Electrophoretic resolution on polyacrylamide gels with detection using appropriate exposure to the substrate permitted qualitative assay. Radial diffusion in agar-substrate base permitted quantitative assay (Nace et al., '65). The advantages of this quantitative method over turbidimetric procedures were that it (1) required a smaller volume of sample, (2) could be used with samples which contained colored materials, (3) allowed simultaneous assessment of a large number of samples, and (4) was more sensitive to frog lysozymes.

Applying these procedures, frog lysozyme proved to be present in multiple forms which were found to have organ specific properties in terms of the variety and concentration of isozymes present. Among normal organs, the eye, skin, tongue, skeletal muscle, heart, spleen, serum, gastrointestinal tract, lung, liver, kidney, testis, and gravid ovaries were examined. However, of these organs, only skin, spleen, liver, kidney, gravid ovaries, and serum were found to contain sufficient amounts of lysozyme for qualitative analysis.

The present communication establishes the identity of the frog lytic material as lysozyme, demonstrates its presence in eight isozyme forms, and describes the distribution of these isozymes among normal adult organs. Changes among these lysozyme isozymes during development are described in Ostrovsky ('70) and Ostrovsky and Nace ('76).

MATERIALS AND METHODS

Animals and tissues

The organs analyzed were from wild-caught *R. pipiens* supplied in the fall by C. H. Mumley, Alburg, Vermont. These adult animals were used immediately or were maintained in the University of Michigan Amphibian Facility until the time of use (Nace, '68; Nace et al., '74). The organs were excised from freshly killed animals, washed in saline, and stored at 20°C. Blood was collected and serum prepared in the manner described by Hejmadi ('70).

Extraction of lytic material

Lysozymes are stable at low pH (Jollès, '63). On this basis, a partial purification was achieved by acidifying tissue homogenates. For each acid extraction 5 gm of organ were homogenized in a chilled, power-driven, glass-teflon homogenizer. A small amount of deionized water was added, when necessary, to facilitate the process. Skin was frozen in dry ice and triturated in a Vollrath Tissue Pulverizer. These homogenates were brought to pH 1.0 with 1.0 N HCl and separated into residue (R 1.0) and supernatant (S 1.0) fractions by cold centrifugation at 30,000 *g* for 40 minutes. Three times, the R 1.0 residue was resuspended in HCl, centrifuged and the supernatants added to the S 1.0 fraction. The combined S 1.0 fractions were dialysed against deionized water, effective volume ratio $1:10^4$, using tubing heated at 80°C for 24 hours to decrease its pore size (Callanan et al., '57), brought to pH 6.6 with 0.1 N NaOH and centrifuged to yield precipitate (P 6.6) and supernatant (S 6.6) fractions. The S 6.6 fraction was reacidified and centrifuged, neutralized and centrifuged three additional times to yield the final S 6.6 fraction. The residues from the reacidification and the precipitates from the reneutralization of the S 6.6 fraction were added to the R 1.0 residue and P 6.6 precipitate, respectively. These pools were each dialysed, neutralized and centrifuged to yield final soluble fractions RS 6.6 and PS 6.6, respectively.

The S 6.6, RS 6.6 and PS 6.6 fractions were assayed for lysozyme. The initial analyses were conducted on unconcentrated extracts (Ostrovsky, '70), while later analyses were conducted on extracts which were concentrated either by dialysis against Carbowax 20-M (Union Carbide brand of polyethylene glycol) or by ultrafiltration (Snyder, '71).

Preliminary analyses (Ostrovsky, '70) indicated that the greatest quantity of the enzyme was present in the S 6.6 fraction and that all fractions were qualitatively identical. Thus, the qualitative data reported here were from analysis of the concentrated S 6.6 fraction and the quantitative data from analysis after pooling aliquots of the three fractions.

Qualitative assay

Polyacrylamide gels used for the qualitative assay of lysozyme were made with 30% acrylamide monomer solution, 0.077% TEMED (N, N, N′, N′, tetramethylethylenediamine) catalyst in glycine buffer, 0.64 M, pH 7.3, and 0.14% aqueous ammonium persulfate catalyst. The electrophoresis buffer was a 0.01 M Tris (hydroxymethyl) aminomethane — 0.08 M glycine solution, pH 8.3, selected as optimal for frog lysozyme.

Samples to be analyzed were first dialyzed against a 1:5 dilution of electrophoresis buffer to maximize the "stacking" of proteins prior to their entry into the polyacrylamide gel (Hjerten et al., '65). Horse cytochrome *c* (Sigma brand, Type III), 0.08 mg per 100 μl of sample, served as a marker for the characterization of lysozyme forms. The marker was occasionally omitted to check the possibility of artifact production through an interaction with lysozyme. Sucrose was added in the proportion of 5 mg per 100 μl of sample to increase sample density.

Monomer solution, buffered TEMED catalyst, and ammonium persulfate catalyst were combined in 1:1:1 proportions to yield a 10% gel which was polymerized in 9.5 cm lengths of 4.5 mm ID glass tubing.

Measured amounts of sample, covered with the pH 8.3 buffer, were subjected to electrophoresis at room temperature (22–26°C) for 25 minutes at 350 v and an initial current of 3 mA/gel. Upon completion of each run, the distance from the origin to the middle of the cytochrome *c* band was recorded, and the gels were removed for detection of the lysozyme.

Lysozyme in the gels was detected (Ostrovsky, '70) by allowing the enzyme to diffuse into a surrounding agar-substrate sheath. For this purpose, the polyacrylamide gels were inserted into thin-walled glass tubes containing a molten agar-substrate mixture of the following description. Lyophilized Difco *M. lysodeikticus (M. luteus)* was homogenized in Sorensen's phosphate buffer, 0.067 M, pH 6.6, and added to a 2% Bacto-Difco agar solution, prepared in the same buffer, to yield a final substrate concentration of 1.25 mg/ml and an agar concentration of 1.67%.

The polyacrylamide gels were thus ensheathed in a thin coating of quickly jelling agar substrate mixture. The tubes were then sealed in Saran Wrap and incubated at 37°C. They were examined at 15-minute intervals for lysis bands in the agar (fig. 1).

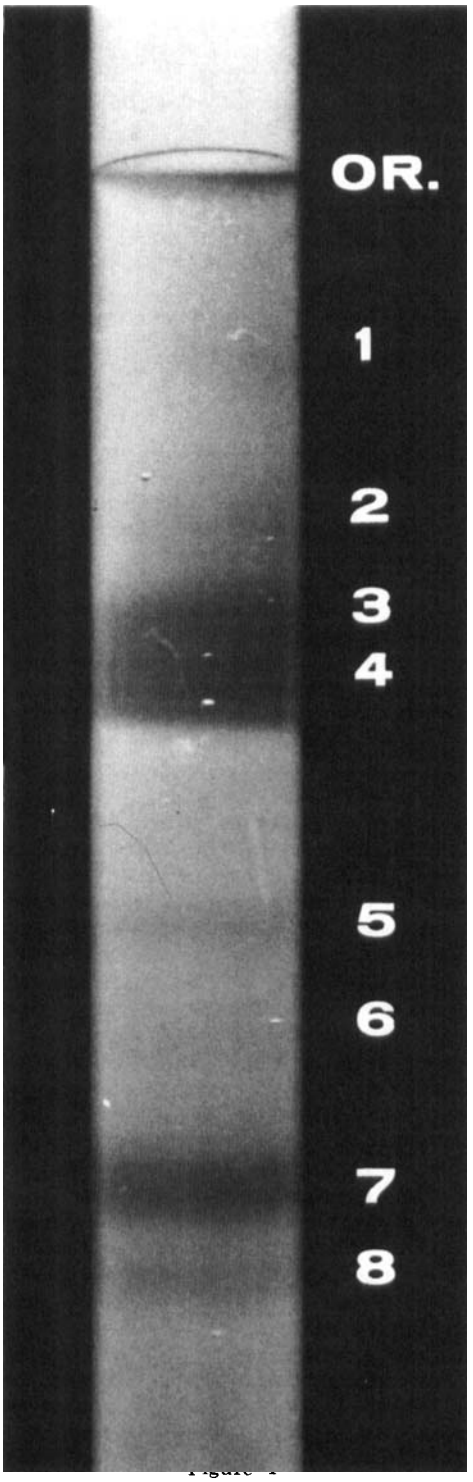
The distance from the origin to the center of each lysis band was used to calculate the mobility of each band relative to cytochrome *c*.

Ten to 17 pooled samples of a given organ, each sample containing tissue from at least two animals, were examined. Each lysozyme sample was tested simultaneously on ten replicate gels. The average relative mobility value for each lysis band from these ten replicates was determined. There was no significant difference at the 95% confidence interval in the relative mobility of a given isozyme among tissue samples.

To determine whether the electrophoresis of frog lysozyme was affected by ammonium persulfate (Brewer, '67; Mitchell, '67), this catalyst was replaced by an equal volume of riboflavin (2.5 mg per 100 ml water) in test gels which were polymerized by 60-minute exposure to light from 15-watt daylight fluorescent bulbs.

Quantitative lysis assay

The intra-agar lysis assay used as a quantitative test for lysozyme was similar in principle to antibiotic assay procedures (Cooper, '63), and was a modification of a lysozyme assay described by Amano ('62) and further modified by Nace et al. ('65). It resembles the assays reported by Osserman and Lawlor ('66) and Bonavida and Sapse ('68).



In this assay, lysozyme, placed in a well, diffused through a plate of agar-substrate mixture. This mixture consisted of lyophilized Difco *M. lysodeikticus (M. luteus)*, homogenized in 2 ml of Sorensen's phosphate buffer, 0.067 M, pH 6.6. This homogenate was combined with 2% Bacto-Difco agar solution, prepared in the same buffer, to yield a final substrate concentration of 1.5 mg/ml and agar concentration of 1.95%. As the lysozyme diffused, it lysed the substrate and produced a clear lysis disc (fig. 14 of Nace et al., '65). Within a given concentration range there was, in accordance with Fick's law of diffusion as applied to radial diffusion through agar (Cooper, '63; Ryan, '67), a linear relationship between the square of the radius (R^2) of these lysis discs and the log concentration of lysozyme in the wells.

In practice, 10 ml aliquots of agar-substrate mixture were pipetted into each leveled 100 × 15 mm Falcon plastic Petri dish. After solidification, a grid of 4.5 mm diameter wells was punched into this 1.5 mm thick plate of agar-substrate mixture. Care was exercised to standardize the orientation and pattern of these wells.

After each sample was pipetted into three different wells dispersed over a plate, the Petri dish was covered, sealed in Saran Wrap, and incubated at 37°C for ten hours. On completion of incubation, each lysis disc was measured to the nearest 0.1 mm on three diameters using a Vernier caliper, and the radius was calculated from the average of these three measurements.

Lysozyme concentration in the test samples was expressed in terms of commercially available (Sigma) chicken egg-white lysozyme (CEWL) units and frog lysozyme units. This dual system was necessitated by an observed difference in the slopes of plots of lysis disc R^2 vs. log lysozyme concentration for lysozyme from the two sources. The CEWL served as a universal standard and frog lysozyme provided a valid comparison among the enzyme concentrations in different organs.

Fig. 1 Lysozyme isozymes of kidney. After electrophoresis, the polyacrylamide gel was incubated with substrate for three hours. The anode was at the top of the tube. The numbers identify isozymes of lysozyme. The marker protein is not seen as only the lysis bands were visualized photographically.

CEWL standards were prepared by serial dilution in deionized water of stock solutions containing 10 mg/ml. A 1 mg/ml concentration was arbitrarily assigned an activity of 1,000 CEWL units/ml. Since the commercial CEWL was not pure, different lots varied in activity per unit weight; thus new lots were calibrated against previous lots and corrections in calculations were made when appropriate.

Frog lysozyme standards were prepared from acid extracted liver, a plentiful tissue rich in lysozyme. Frog standards producing lysis disc R^2 values of 37.5 mm^2 were arbitrarily assigned an activity value of 1,000 frog lysozyme units/ml. This R^2 value was the same as that produced by 1,000 CEWL units/ml. As the concentration of the frog standards was not uniform from preparation to preparation, their concentrations were adjusted to this value. Dilutions of such standards yielded curves against which test extracts were evaluated.

RESULTS AND CONCLUSIONS

Characterization as a basic protein

The original observation suggesting the presence of bacteriolytic substances in frogs was the immunoelectrophoretic detection of basic proteins (Nace et al., '65). The basic protein character of each of the eight forms of lytic material found in the frog was further confirmed by their chromatographic behavior (Nace et al., '65), their cathodal migration during agar or acrylamide gel electrophoresis carried out under conditions for the separation of basic proteins, and by their failure to migrate to the anode under conditions for the separation of acidic proteins.

Molecular weight

Nace et al. ('65) presented some evidence from dialysis that the frog lytic materials were in the low molecular weight range characteristic of lysozyme. This was confirmed by gel filtration (Andrews, '64).

In this case, the gel filtration behavior of horse ferritin, chymotrypsinogen A, chicken egg-white lysozyme, and ribonuclease A used as marker proteins was compared with that of frog lytic materials extracted from liver and ovarian egg. Liver and ovarian egg were the main organs used for the characterization of the frog bacteriolytic enzymes as lysozymes, because they are plentiful and between them they contain all of the electrophoretically separable forms. Sephacex G-75 retards material with a molecular weight below 70,000; thus, ferritin, with a molecular weight higher than this figure, was eluted in the void volume. All of the other proteins were present in the eluent volume. The Kd values ($Kd = (Ve - Vo)/Vi$; Ve the elution volume, Vo the void volume, and Vi the inner volume of the gel) for liver and ovarian egg lysozymes were 0.91 and 0.76 respectively. This indicated a molecular weight of 15,000 for liver lysozyme and 17,500 for ovarian lysozyme. Among the marker proteins, chymotrypsinogen A had a Kd of 0.46; chicken egg-white lysozyme, 0.97; and ribonuclease A, 1.33.

The lytic proteins from both frog organs were of sufficiently low molecular weight to satisfy the lysozyme criterion. In each case, the sample applied to the column contained all of the lytic isozymes normally found in the organ, and, between the two organs, all those found in the frog. Therefore, the values obtained were an average for all of the forms, though liver contained three forms not found in the ovary, and the ovary one form not in the liver.

Heat stability at pH extremes

To confirm earlier observations on the heat stability of frog lysozyme (Nace et al., '65), a sample each of chicken egg-white lysozyme and lytic material from frog kidney was divided into two portions. One portion of each sample was dialyzed against sodium acetate-acetic acid buffer, 0.067 M, pH 4.5; the other portion was dialyzed against boric acid-sodium hydroxide buffer, 0.067 M, pH 9.1. After dialysis for 12 hours

TABLE 1

Heat stabilities of frog (kidney) lytic material and chicken egg-white lysozyme (CEWL) at pH extremes

Preparation	pH	Activity (units/ml) 22°C	Activity (units/ml) 100°C/3 min	% recovery
Kidney lytic material	4.5	1,300	700	54
Kidney lytic material	9.1	1,300	0	0
CEWL	4.5	900	500	55.5
CEWL	9.1	1,050	250	23.8

at an effective volume ratio of $1:10^4$, aliquots of the samples were quantitatively and qualitatively assayed without heating or after heating to 100°C for three minutes.

The quantitative results shown in table 1 indicate that at 100°C frog lytic material and egg-white lysozyme were unstable at pH 9.1 but stable at pH 4.5. Electrophoretic results similarly showed the loss of lytic activity after heating at pH 9.1, and its retention after heating at pH 4.5 for all eight forms of lysozyme in frog extracts.

Lysis of bacteria

The initial evidence that the basic material found in frog preparations might be lysozyme was based on its ability to lyse *M. lysodeikticus* substrate (Nace et al., '65). The efficacy of the intra-agar and electrophoretic techniques confirmed this for each of the eight forms of lytic material found in the frog.

Action on chitobiose

The action of frog lytic materials on chitobiose was examined using a modified Morgan-Elson procedure in which a colored product of free N-acetyl-glucosamine is measured at 585 mμ (Reissig et al., '55; Ghosh and Roseman, '62; Wheat, '66). After 24-hours incubation at 22°C the absorbance of chitobiose solution plus deionized water was 0.675, of chitobiose solution plus chicken egg-white lysozyme was 2.00, and of chitobiose solution plus frog kidney lytic preparations was 0.146.

Comparison with the two controls shows that frog kidney lytic materials produced a decrease instead of the expected increase in free amino sugars. Synthesis may have masked the effect of degradation. Sharon ('67) reported a similar observation when his paper chromatographs of lysozyme digests of an N-acetylhexosamine tetrasaccharide indicated that tri, tetra, and penta oligomers were formed in addition to the expected disaccharide. These observations, together with similar ones by Kravchenko ('67), suggested that lysozyme catalyzed transglycosylation as well as hydrolysis reactions.

Thus, these preliminary tests of the action of frog kidney lytic material on chitobiose, while suggesting synthesis rather than cleavage, were not inconsistent with the identification of this material as lysozyme.

R. pipiens lytic material as lysozyme

All six criteria for lysozyme were met by preparations of frog lytic material, with the reservation that its synthesizing activity under certain conditions appeared to be more rapid than its hydrolytic activity. Thus, there is little room for doubt that the frog lytic material is lysozyme.

As seen below, frog lytic material can be resolved into at least eight forms. That these lytic substances are each lysozymes was indicated by a consideration of the tests for the criteria. All forms showed electrophoretic properties of basic proteins; all lytic activity was in the low molecular weight effluents; all were active against *M. lysodeikticus* in the qualitative assay; all escaped destruction at high temperatures under acid conditions; and none escaped destruction at high temperatures under alkaline conditions. Confirmation of the activity of each form against specific molecular substrates must await their isolation and individual evaluation. Thus, with the exception of the confirmation of individual specific action on molecular substrates, each of the eight forms of lytic material found in *R. pipiens* meets the criteria for lysozyme, and may be considered as a form of lysozyme.

Qualitative assay

A total of eight separate lytic band groups migrating toward the cathode was resolved in frog tissues by polyacrylamide electrophoresis (fig. 2). The mean relative mobilities of these bands ranged from 0.13 to 0.87. Their identity among tissues was confirmed by the electrophoresis of mixtures of extracts of different organs. All eight lytic bands were present in normal kidney; liver and spleen had seven forms; skin, six; ovarian egg, five; and serum, two forms. The time sequence of the appearance of these bands in concentrated samples (fig. 3) demonstrated marked quantitative differences among the various lytic substances in a given tissue. These quantitative differences, as well as the isozyme complement, were characteristic of each organ.

Untreated homogenates of tissues had multiple lysozyme forms which were iden-

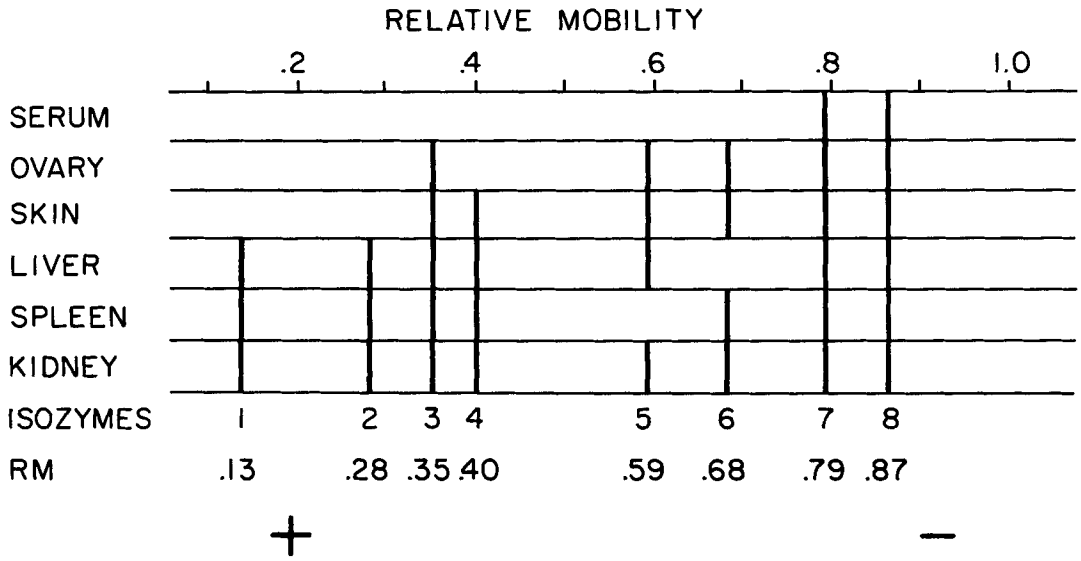


Fig. 2 Summary of the lysozyme isozymes in adult frog tissues. Each isozyme is designated by number, starting at the origin (1–8), and mean relative mobility (RM).

tical in number and relative mobilities to those of tissues which had been acid extracted; however, the bands were wider and not as sharply delineated. Neither elimination of cytochrome *c* nor substitution of riboflavin for ammonium persulfate as gel catalyst changed the number of bands.

The electrophoresis system described above exposed the frog lysozymes to a running pH of 6.6 which is not sufficiently acidic to prevent aggregation of chicken egg-white lysozyme molecules (Sophionopolous and Van Holde, '64; Deonier and Williams, '70). Two tests were applied to determine whether any of the observed bands were attributable to such a phenomenon. First, acid extracted frog lysozyme samples were subjected to the discontinuous electrophoresis system of Reisfeld et al. ('62), at a running pH of 4.3. This pH is below that at which a lysozyme would be expected to polymerize. The frog acid extracts were resolved into the same number of lytic bands under these conditions as was described above; however, they were not as sharply delineated.

The second test for aggregation was based on the principle that polyacrylamide gel pore size is inversely proportional to the gel concentration and that by taking advantage of this property electrophoresis can be used to demonstrate molecular weight differences among a series of polymers (Davis, '64). The higher the molecular weight of a protein, the greater the degree of its retardation as pore size decreases; consequently, the mobilities of proteins of different molecular weights change relative to one another as gel concentration is increased (Raymond and Nakamichi, '64; Zwaan, '67).

Using this procedure, frog skin lysozyme was subjected to electrophoresis in 5%, 7.5%, and 10% polyacrylamide gels. The mobility of each isozyme was calculated relative to the fastest moving band. As anticipated, there was a decrease in the absolute mobility of all bands as the concentration of the polyacrylamide increased. However, no differential change occurred in the relative mobilities of the frog lysozymes. This indicated that these lysozymes were of similar molecular weight and were electrophoretically resolved primarily on the basis of charge differences.

These observations supported the conclusion that the observed multiple forms of frog lysozyme were not artifacts.

From these data we concluded that: (1) *R. pipiens* contains electrophoretically separable lysozymes; (2) these several forms of lysozyme are not artifacts; (3) the constancy of their relative mobilities is suf-

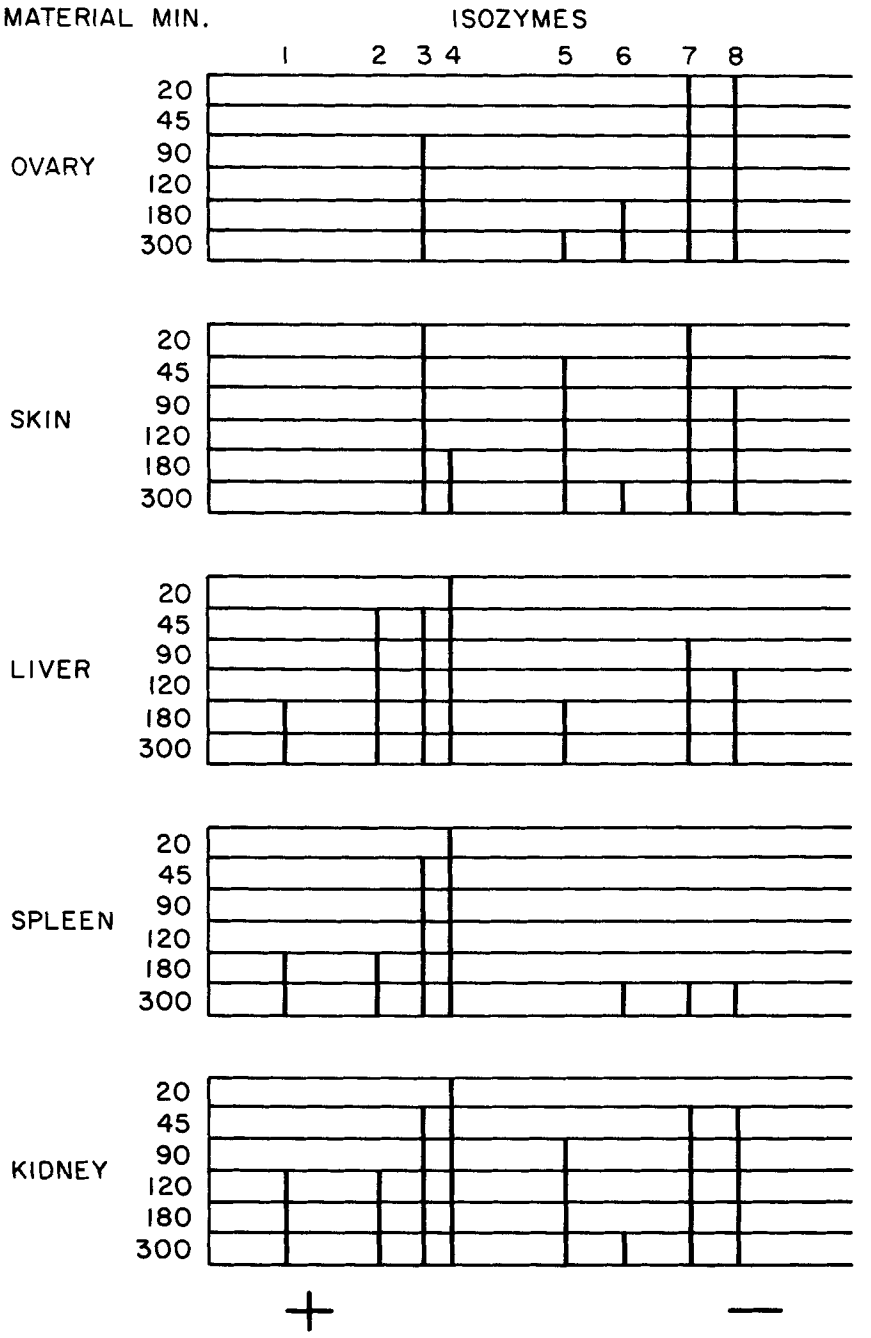


Fig. 3 Electrophoretic patterns of lysozyme isozymes in fractions of adult frog tissues at various times after the beginning of incubation with substrate.

TABLE 2

Lysozyme content [1] *of R. pipiens tissues*

Material	Units per animal	Units per g wet wt	Units per mg dry wt
	(chicken egg-white lysozyme units)		
Ovarian egg	32,900	2,600	5
Skin	13,800	2,500	10
Liver	15,200	13,700	52
Spleen	700	54,900	419
Kidney	8,700	39,000	212
	(frog liver lysozyme units)		
Ovarian egg	7,800	610	1
Skin	3,600	700	3
Liver	6,100	5,500	21
Spleen	190	14,400	100
Kidney	2,800	12,500	68

[1] Both chicken egg-white and frog liver standards were adjusted to yield lysis disc R^2 values of 37.5 mm^2 at arbitrarily assigned activity values of 1,000 units/ml. Differences between tissue values as shown for the two standards arose because the slopes of plots of R^2 vs. log concentration of the standards were different. The reasons for this difference in slopes have not been fully evaluated.

ficient to permit grouping them into a minimum of eight isozymes; and (4) the distribution of these isozymes among the several organs is specific for each organ, both in total number and in relative concentration.

Quantitative lysis assay

Intra-agar lysis assays of tissues pooled from nine mature *R. pipiens* females are summarized in table 2.

The lysozyme content of untreated frog serum was extremely low, and an accurate concentration value could not be determined. However, values which were obtained indicated that serum could account for no more than 0.2% of the total extracted lysozyme in the assayed tissues of an adult animal.

Both liver and kidney contained high concentrations of lysozyme (units/g wet wt and units/mg dry wt). However, spleen was richest. It remains to be determined whether the enzyme is concentrated in erythrocytes or phagocytes, both of which are major components of the spleen. Glynn and Parkman ('64) have demonstrated that rat phagocytes contain large amounts of lysozyme.

Though the greatest quantity of lysozyme per animal was extracted from ovarian eggs, they contained the lowest concentration on the basis of units per mg dry weight. Ovarian eggs were also unique in that the largest proportion of their lysozyme was found in the first residue resulting from acid extraction (fraction RS 6.6) rather than in the supernatant (fraction S 6.6). This may be related to the binding of lysozyme to specific egg proteins.

DISCUSSION

Since its discovery by Fleming ('22) lysozyme has undergone intensive investigation, and enzymes sharing its six properties have been found in a large number of animal and plant species. Thus, its presence in an amphibian is not surprising. Further, it is not unusual to find isozymes of this enzyme. Lysozyme has been shown to exist in two or three forms in rabbit spleen (Jollès, '62), duck egg-white (Jollès et al., '65), snail (Takeda et al., '66), migratory quail egg-white (Baker and Manwell, '67), chicken lung (Jollès, '67) and polymorphonuclear leukocytes (Hindenburg et al., '74), swan egg-white (Arnheim and Steller, '70), and Embden goose bone marrow (Hindenburg et al., '74). Thus we may readily accept evidence that lytic materials found in *R. pipiens* are lysozymes and that they occur in multiple molecular forms. What is unusual is the number of these forms.

The demonstration of eight isozymes in *R. pipiens*, as well as of isozymes of lysozyme in other species, leads to inquiry concerning the genetic control of their synthesis. In the case of duck egg-white, the three forms are distributed among individual eggs in a manner indicating that they are products of alleles at a single locus (Prager and Wilson, '71), and immunological cross reactivity among them indicates structural homology. On the other hand, the two forms seen in swan egg-white, in chicken leukocytes, and in Embden goose bone marrow are immunologically distinct, perhaps indicating that they are products of two non-allelic genes which arose either by gene duplication followed by mutation or by convergent evolution of originally distinct genes (Arnheim and Steller, '70; Hindenburg et al., '74).

Conclusions concerning the genetic control of the eight isozymes of lysozyme in *R. pipiens* must be deferred until the puri-

fied isozymes are examined (Snyder, in preparation) and genetic tests have been conducted (Nace et al., '70). However, the gel filtration studies described in this paper suggest that the predominant isozymes of frog liver and egg differ in size, suggesting in turn, rather large differences in primary structure between at least two of the forms. Such differences would indicate the presence of a set of genes each coding for individual isozymes.

A second question which arises concerning this system of isozymes is that of evolutionary significance and function. If one assumes that their retention in the frog is a result of selective advantage (Asher and Nace, '71) through differences in substrate specificity, catalytic efficiency, or other properties (Arimura, '73), one may expect to find functional variation among the forms. Such variation was suggested by preliminary work (Rubin et al., '66) which indicated that frog kidney lysozyme preparations possessed greater antiviral activity than did skin lysozyme preparations. A selective advantage for such variation was imbedded in the proposal of Nace et al. ('65) that the enzyme may play a role in protecting the frog from the virus implicated in the etiology of the Lucké renal adenocarcinoma.

Other evidence which is relevant to the significance of such large numbers of isozymes of lysozyme in frogs arises from a consideration of the pattern of bacterial colonization in these animals (van der Waaij et al., '74). This pattern of colonization differs from that of mammals (van der Waaig et al., '72) and may be related to differences in defense mechanisms used by endotherms and ectotherms. The variety of isozymes of lysozyme may provide the frog with a wide spectrum of defense against bacteria. Such a defense would compensate for the relatively unreliable antibody defense of these animals and could explain the retention of multiple forms of lysozyme.

The high lysozyme concentration found in frog spleen may be related to the enzyme's antibacterial role since that organ is a site of phagocyte proliferation in the adult *R. pipiens* (Noble, '31; Cowden and Dyer, '71). The skin is an important interface with a bacteria-laden environment; tnus the lysozyme of that tissue may also be primarily associated with antibacterial activity (van der Waaij et al., '74).

The function of lysozyme in several other locations in metazoans remains an enigma. It is found in high concentrations in human earwax, yet many bacteria flourish there (Petrakis et al., '71). It is equally difficult to explain the lysozyme found in specific areas of pre-osseous cartilage on the basis of an antibacterial function (Kuettner et al., '70). Further, it has been reported that human lysozyme has little or no effect upon a number of bacteria normally found in humans (Neu et al., '68) and that no substrate degradable by lysozyme has been identified in animal cells (Jollès, '69). It is possible that frog kidney, liver and ovarian egg are included among those animal tissues which contain lysozyme whose function, though not yet understood, is not exclusively antibacterial. It is noteworthy that at least one mammal has a tissue distribution of lysozyme (Klockars and Osserman, '74) in striking contrast to that reported here for *R. pipiens*. The adult rat has little or no lysozyme in liver, spleen, skin, and ovary; while kidney, small intestines, and lung are areas of high concentration.

The significant amount of lysozyme in mammalian kidney is attributed to filtration from the blood rather than to endogenous production (Perri et al., '64; Cappuccino et al., '64). However, this would not seem to be the case in the frog as its serum contains very little lysozyme and its two isozymes, forms 7 and 8, do not match those of the kidney which contains all eight, with form 4 predominant.

The lysozyme found in ovarian eggs may indeed be maternally contributed rather than being synthesized under the direction of oocyte genes. Hejmadi ('70) and others have demonstrated that maternal serum proteins can be transferred intact to frog oocytes and since the predominant isozymes of serum and egg are identical and egg contains relatively low quantities of lysozyme, transfer of these enzymes seems reasonable. Whatever the source of egg lysozyme, however, its function remains only a topic of speculation aside from noting that these eggs develop outside the protective environment of the female.

ACKNOWLEDGMENTS

These investigations were supported by National Science Foundation grants GB 4677 and GB 8187; National Institutes of Health grants GM 05409 and RR 00572; National Institutes of Health Training Grant 5 TO1 GM0989; National Cancer Institute grant CA 06929; and Damon Runyon Memorial Fund grant DRG 980.

Special thanks are due to Dr. Yasuji Amano of Akita University, Akita, Japan for valuable work conducted during his period as a visitor in this laboratory. Thanks are also due to Dr. David Aminoff, The University of Michigan, for his advice and assistance in the determination of the action of lysozymes upon chitobiose.

LITERATURE CITED

Amano, T. 1962 Rizochimu no meniki kagakuteki kenkyu (Immunochemical study of lysozyme). Tanpakushitsu kakusan koso (Protein, nucleic acid, enzyme), *7:* 697–704 (in Japanese).

Andrews, P. 1964 Estimation of molecular weights of proteins by Sephadex gel-filtration. Biochem. J., *91:* 222–223.

Arimura, H. 1973 Effect of lysozyme from human placenta on ectromelia virus. Acta Virol., *17:* 130–137.

Arnheim, N., and R. Steller 1970 Multiple genes for lysozyme in birds. Arch. Biochem. Biophys., *141:* 656–661.

Asher, Jr., J. H., and G. W. Nace 1971 The genetic structure and evolutionary fate of the parthenogenetic amphibian populations as determined by Markovian analysis. Am. Zool., *11:* 381–398.

Baker, C. M. A., and C. Manwell 1967 Molecular genetics of avian proteins. VIII. Egg white proteins of the migratory quail, *Coturnix coturnix* — new concepts of hybrid vigour. Comp. Biochem. Physiol., *23:* 21–42.

Bonavida, P., and A. T. Sapse 1968 Human tear lysozyme. II. Quantitative determination with standard schirmer strips. Amer. J. Opthalmol., *66:* 70–76.

Brewer, J. M. 1967 Artifact produced in disc electrophoresis by amonium persulfate. Science, *156:* 256–257.

Callanan, M. J., W. R. Carroll and E. R. Mitchell 1957 Physical and chemical properties of protamine from the sperm of salmon (*Onchorhynchus tschawytscha*). J. Biol. Chem., *229:* 279–287.

Cappuccino, J. G., S. Winston and G. C. Perri 1964 Muramidase activity of kidney and spleen in Swiss mice challenged with B.C.G., Zymosan and bacterial endotoxins. Proc. Soc. Exp. Biol. Med., *116:* 869–872.

Cowden, R. R., and R. S. Dyer 1971 Lymphopoietic tissue and plasma cells in amphibians. Amer. Zool., *11:* 183–192.

Cooper, K. E. 1963 The theory of antibiotic inhibition zones. In: Analytical Microbiology. F. Kavanagh, ed. Academic Press, New York, pp. 1–86.

Davis, B. J. 1964 Disc Electrophoresis. 2. Method and application to human serum protein. Ann. N. Y. Acad. Sci., *121:* 404–427.

Deonier, R. C., and J. W. Williams 1970 Self-association of muramidase (lysozyme) in solution at 25°, pH 7.0 and I = 0.20. Biochem., *9:* 4260.

Ferrari, R., C. Callerio and G. Podio 1959 Antiviral activity of lysozyme. Nature, *183:* 548.

Fleming, A. 1922 On a remarkable bacteriolytic element found in tissues and secretions. Proc. Royal Soc. Series B, *93:* 306–317.

Ghosh, S., and S. Roseman 1962 L-glutamine-D-fructose 6-phosphate transamidase from *Escherichia coli.* In: Methods in Enzymology. Vol. 5. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York, pp. 414–417.

Glynn, A. A., and R. Parkman 1964 Studies with an antibody to lysozyme. Immunology, *7:* 724–729.

Hejmadi, P. M. 1970 Transfer of Maternal Serum Protein into the Egg of *Rana pipiens* and Their Role in Development. Ph.D. Thesis. Univ. of Michigan.

Hindenburg, A., J. Spitznagel and N. Arnheim 1974 Isozymes of lysozyme in leukocytes and egg white: Evidence for the species-specific control of egg-white lysozyme synthesis. Proc. Nat. Acad. Sci. (U.S.A.), *71:* 1653–1657.

Hjerten, S., S. Jerstedt and A. Tiselius 1965 Some aspects of the use of "continuous" and "discontinuous" buffer systems in polyacrylamide gel electrophoresis. Anal. Biochem., *11:* 219–223.

Jollès, J., G. Spotorno and P. Jollès 1965 Lysozymes characterized in duck egg-white: Isolation of a histidine-less lysozyme. Nature, *208:* 1204–1205.

Jollès, P. 1962 Lysozymes from rabbit spleen and dog spleen. In: Methods in Enzymology. Vol. 5. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York, pp. 137–140.

——— 1963 Action comparee de trois lysozymes d'origine differente sur des substrats glycopeptidiques isoles de *Micrococcus lysodeikticus.* Biochim. Biophys. Acta, *69:* 505–510.

——— 1967 Relationship between chemical structure and biological activity of hen egg-white lysozyme and lysozymes of different species. Proc. Royal Soc. Series B, *167:* 350–364.

——— 1969 Lysozyme: a chapter in molecular biology. Angew. Chem., *8:* 227–239.

Klockars, M., and E. F. Osserman 1974 Localization of lysozyme in normal rat tissues by immunoperoxidase method. J. Histochem. Cytochem., *22:* 139–146.

Kravchenko, N. A. 1967 Lysozyme as a transferase. Proc. Royal Soc. Series B, *167:* 429–430.

Kuettner, K. C., L. W. Soble, R. D. Ray, R. L. Croxen, M. Passovoy and R. Eisenstein 1970 Lysozyme in epiphyseal cartilage. II. The effect of egg white lysozyme on mouse embryonic femurs in organ cultures. J. Cell. Biol., *44:* 329–339.

McKinnell, R. G. 1973 The Lucké frog kidney

tumor and its herpesvirus. Amer. Zool., *13*: 97–114.

Mizell, M. 1969 State of the art: Lucké renal adenocarcinoma. In: Biology of Amphibian Tumors. M. Mizell, ed. Springer-Verlag, New York, pp. 1–25.

Mitchell, W. 1967 A potential source of electrophoretic artifacts in polyacrylamide gels. Biochem. Biophys. Acta, *147*: 171–174.

Nace, G. W. 1961 Antigenic characterization of frog kidney adenocarcinoma. Transcript Proc. Frog Kidney Adenocarcinoma Conf., Natl. Cancer Inst., Bethesda, Maryland, pp. 91–98.

——— 1962 Discussion. In: Symposium on Specificity of Cell Differentiation and Interaction. J. Cell Comp. Physiol. (Suppl. 1), *60*: 61.

——— 1968 The amphibian facility of the University of Michigan. BioScience, *18*: 767–775.

Nace, G. W., D. D. Culley, M. B. Emmons, E. L. Gibbs, V. H. Hutchison and R. G. McKinnell 1974 Amphibians: Guidelines for the Breeding, Care and Management of Laboratory Animals. Sub-Committee on Amphibian Standards. ILAR (NAS/NRC). Washington, D.C.

Nace, G. W., C. M. Richards and J. H. Asher, Jr. 1970 Parthenogenesis and genetic variability. I. Linkage and inbreeding estimations in the frog, *Rana pipiens*. Genetics, *66*: 349–368.

Nace, G. W., and T. Suyama 1965 The loss of a bacteriolytic enzyme associated with the appearance of a frog kidney tumor. SABCO J., *1*: 1–5 (in Japanese).

Nace, G. W., T. Suyama and T. Iwata 1965 The relationship between a lysozyme-like enzyme and frog adenocarcinoma. Ann. N. Y. Acad. Sci., *126*: 204–221.

Neu, H. C., J. Dreyfus and R. Canfield 1968 Effect of lysozyme on gram-positive and gram-negative bacteria. In: Antimicrobial agents and chemotherapy. G. L. Hobby, ed. Academic Press, New York, pp. 442–444.

Noble, G. K. 1931 The Biology of the Amphibia. McGraw-Hill, Inc., New York.

Osserman, E. F., and D. P. Lawler 1966 Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J. Exptl. Med., *124*: 921–955.

Ostrovsky, D. S. 1970 Lysozyme Ontogeny in *Rana pipiens*. Doctoral Thesis. The University of Michigan.

Ostrovsky, D. S., and G. W. Nace 1976 Frog lysozyme. II. Isozymes of lysozyme during the larval development of the leopard frog, *Rana pipiens*. Devel. Bio., in press.

Perri, G. C., M. Faulk, E. Shapiro and W. L. Money 1964 Role of the kidney in accumulation of egg white lysozyme in experimental animals. Proc. Soc. Exptl. Biol. Med., *115*: 189–192.

Petrakis, N. L., M. Doherty, R. E. Lee, S. C. Smith and N. L. Page 1971 Demonstration and implications of lysozyme and immunoglobulins in human ear wax. Nature, *229*: 119–120.

Prager, E. M., and A. C. Wilson 1971 Multiple lysozymes in duck egg white. J. Biol. Chem., *246*: 523–530.

Rafferty, K. A., Jr. 1964 Kidney tumors of the leopard frog: A review. Cancer Res., *24*: 169–186.

Raymond, S., and M. Nakamichi 1964 Electrophoresis in synthetic gels. 2. Effect of gel concentration. Anal. Biochem., *7*: 22.

Reisfeld, R. A., U. J. Lewis and D. E. Williams 1962 Disc electrophoresis of basic proteins and peptides on polyacrylamide gel. Nature, *195*: 281–283.

Reissig, J. L., J. L. Strominger and L. F. Leloir 1955 A modified colorometric method for the estimation of N-acetylamino sugar. J. Biol. Chem., *217*: 959–966.

Rubin, M. L., D. B. Ar and G. W. Nace 1966 The virus inhibiting action of a lysozyme present in normal frogs but lacking in tumor frogs and eggs. Amer. Zool., *6*: 29.

Ryan, C. A. 1967 Quantitative determination of soluble cellular proteins by radial diffusion in agar gels containing antibodies. Proc. Soc. Exptl. Biol. Med., *19*: 434–440.

Sharon, N. 1967 The chemical structure of lysozyme substrates and their cleavage by the enzyme. Proc. Royal Soc. Series B, *167*: 402–415.

Snyder, J. A. 1971 Characterization of Lysozyme Isozymes in *Rana pipiens*. Doctoral thesis. The University of Michigan.

Sophionopolous, A. J., and K. E. Van Holde 1964 Physical studies of muramidase (lysozyme). II. pH-dependent dimerization. J. Biol. Chem., *239*: 2516–2524.

Strominger, J. L., and J. M. Ghuysen 1967 Mechanisms of enzymatic bacteriolysis. Science, *156*: 213–221.

Takeda, H., G. A. Strasdine, D. R. Whitaker and C. Roy 1966 Lytic enzymes in the digestive juice of *Helix pomatia*. Chitinases and muramidases. Can. J. Biochem., *44*: 509–518.

van der Waaij, D., J. M. Berghuis and J. E. C. Lekkerkirk 1972 Colonization resistance of the digestive tract of mice during systemic antibiotic treatment. J. Hyg., *70*: 605–610.

van der Waaij, D., B. J. Cohen and G. W. Nace 1974 Colonization patterns of aerobic gram-negative bacteria in the cloaca of *Rana pipiens*. Lab. Anim. Sci., *24*: 307–317.

Wheat, R. W. 1966 Analysis of hexosamines in bacterial polysaccharides by chromatographic procedures. In: Methods of Enzymology. Vol. 8. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York, pp. 60–78.

Zwaan, J. 1967 Estimation of molecular weights of proteins by polyacrylamide gel electrophoresis. Anal. Biochem., *21*: 155–168.