$See \ discussions, stats, and author \ profiles \ for \ this \ publication \ at: \ https://www.researchgate.net/publication/19756835$

Development of an in Vitro Technique for Measuring Elastic Properties of Vocal Fold Tissue

Article in Journal of Speech and Hearing Research \cdot July 1988

DOI: 10.1044/jshr.3102.288 · Source: PubMed

Project

CITATIONS 30	5	reads 33	
2 autho	rs:		
0	Adrienne L Perlman University of Illinois, Urbana-Champaign 63 PUBLICATIONS 2,099 CITATIONS SEE PROFILE		Ingo R Titze University of Utah 424 PUBLICATIONS 21,266 CITATIONS SEE PROFILE
Some of	f the authors of this publication are also working on these related projects:		
Some of	and authors of this publication are also working on these related projects.		

Project An Optimizer-Simulator for Phonosurgery View project

Voice disorders and assessment View project

Research Note

DEVELOPMENT OF AN IN VITRO TECHNIQUE FOR MEASURING ELASTIC PROPERTIES OF VOCAL FOLD TISSUE

ADRIENNE L. PERLMAN

Veterans Administration Medical Center, Iowa City

INGO R. TITZE The University of Iowa, Iowa City

The purpose of this investigation was to develop an *in vitro* technique for measurement of elastic properties of isolated vocal fold tissue. Larynges were excised from anesthetized dogs, immediately submerged in a Krebs-Ringer solution, and aerated with 95% oxygen and 5% carbon dioxide. The tissue was maintained in the aerated electrolyte solution throughout the experiment. The vocalis muscle was carefully dissected, with attachments to the arytenoid and thyroid cartilages maintained. The preparation was then subjected to isometric (constant length) force-elongation measures, which were converted to stress-strain values. Stage I of the investigation identified the dog model with the least intrastudy variability relative to the age, sex, and breed of the research animal, as well as to the effects of curare. Stage II investigated the effects of different instrumentation, definitions of reference length, methods of elongation, and effects of electrical stimulation. Once the procedure had been refined, the effects of age and sex were retested. There was a significant interaction between sex and strain and between age and strain. The least variability was obtained with curarized tissue from one sex of young, mixed-breed dogs, where an arbitrary 1 gram of initial force was the criterion for establishing "zero" strain. Problems associated with determination of reference length and various approaches to this problem are discussed.

This paper deals with development of a methodology for measurement of some physical properties underlying the control of fundamental frequency in phonation. In particular, the longitudinal elastic properties of vocal fold tissue are measured. To conceptualize the relationship between length, tension, mass and fundamental frequency of vocal fold vibration, consider the simple string model. The fundamental frequency is known to be

$$f = (T/M)^{1/2}/(2L)$$
(1)

where T is the tension of the vocal folds, M is the mass per unit length, and L is the length of the folds.

Assuming that this simple string model, or any of a number of more complex biomechanical models (e.g., Ishizaka & Flanagan, 1972; Titze & Talkin, 1979) can predict the fundamental frequency of the vocal folds, it is imperative to quantify the effective tension to mass ratio for various phonatory conditions. Previous investigations on the elasticity of vocal fold tissue have shown considerable interstudy variability with respect to the type of tissue preparation, condition of the tissue, method of measurement, and time post mortem for cadaver preparations (Bauer & Konig, 1967; Damste & Wienke, 1973; Hast, 1966; Kakita, Hirano, & Ohmaru, 1981; Lecluse, 1977; Perlman, Titze, & Cooper, 1984; van den Berg & Tan, 1959).

Until the study by Perlman et al. (1984), all studies except that by Hast (1966) used post mortem specimens for the investigation of vocal fold elasticity. Hast reported on the *in situ* active contractile properties of the canine vocal fold; his report included data on the passive lengthtension curve for the vocalis muscle from one dog. Perlman et al. compared the passive elastic properties of viable and dead vocal fold tissue in vitro. They found differences in the length-tension curves as a function of viability and time post mortem. Additionally, they reported a dependence of the tension on elapsed time immediately after step-elongation (commonly called stress relaxation), which was then quantified mathematically by Alipour-Haghighi and Titze (1985) with a socalled reduced relaxation function. Perlman et al. also pointed out a possible ambiguity with regard to the choice of a reference length if the normalized elongation (strain) was to be computed. Concern was expressed about the great variability in stress values that occurred across samples at each level of strain. This concern was also apparent in the previous work by Kakita et al. (1981).

The predictive power of a fundamental frequency model is no better than the accuracy of the physical properties from which it is constructed. Recognizing that variability is always present in biological tissue, we nevertheless believed that some of the causes of variability in length-tension could be identified and possibly removed (or at least controlled) with more refined experimental procedures.

The purpose of this research was to identify factors that contribute to large interstudy variability, and to subsequently develop a technique for acquisition of data on the elastic properties of isolated vocal fold tissue. Because it was not practical to attempt to develop a protocol using viable human larynges without vocal fold pathology, it

© 1988, American Speech-Language-Hearing Association

288

was necessary to use an animal model. Use of the appropriate animal model depends on anatomical and physiological similarity between the human and the animal, and the availability of the research animal. Although studies on the anatomy, morphology, and histochemistry of the vocal folds of various species have shown certain differences between the species (Abo El-ene, 1975; Hall-Craggs, 1968; Hast, 1969; Hirose, Tatsujiro, Kobayashi, & Sawashima, 1969; Kurita, Nagata, & Hirano, 1983; Negus, 1929, 1949; Rossi & Cortesina, 1965; Zealear, 1983), the similarity in size and gross morphology of the canine larynx suggested it to be an acceptable model for studying the elastic properties of the vocalis muscle.

This investigation compared the differences in tissue stiffness as a function of age, sex, and breed of the research animal, as well as differences in the biomechanical properties of those samples with and without curare in the protocol. These variables were studied using conventional methods for measuring tissue elasticity. The protocol was then refined using more modern, electronically controlled equipment.

Rationale for the In Vitro Technique

Lawton (1957) stated: "The primary purpose of isolating a biological system for study is the control of the many variables which alter its behavior *in situ*" (p. 6). A basic assumption in this approach, however, is that isolated preparations *in vitro* demonstrate the *in vivo* performance of that substructure; and, as the isolated substructures are gradually combined, understanding of the total system will be more complete.

Mechanically, the canine vocal fold can be viewed as a two-layer system containing a body (the vocalis muscle) and a cover (the epithelium and the lamina propria) (Hirano, 1977). The body has both active contractile and passive elastic properties, whereas the cover has only passive elastic properties. Length and tension of the cover are determined by contractions of the cricothyroid and vocalis muscles which act as an agonist-antagonist pair. Thus, control of fundamental frequency is achieved by adjusting the length (and thereby the tension) of the vocal folds through various combinations of cricothyroid and vocalis muscle contraction. Except for the studies by Kakita et al. (1981), and Perlman et al. (1984), other attempts to study the elastic properties of the vocal folds have treated the body and cover as a single unit.

The technical aspects of measuring active and passive properties of isolated *in vitro* muscle tissue are discussed in the pharmacological literature (Greenspan & Edmands, 1971; Levy, 1971; Stephens, 1975), and the validity of using *in vitro* procedures to study the response characteristics of skeletal muscles under various pharmacological conditions has been demonstrated by Moulds and Denborough (1974). In addition, the appropriateness of comparing active muscle function of an *in vitro* skeletal muscle sample with *in vivo* muscle function was studied by Moulds, Young, Jones, and Edwards (1977). These authors reported that biomechanical and histochemical analyses showed the properties of the isolated preparations to be compatible with those from the *in vivo* study by Edwards, Young, Hosking, and Jones (1977). Moulds et al. concluded that for many purposes, isolated preparations may be regarded as normal muscle and that observations *in vitro* may be considered relevant to *in vivo* function. Thus, it appeared to us that the traditional use of the *in vitro* model for pharmacological and physiological research, along with the evidence of the validity reported by Moulds et al., supported the approach taken here.

The use of curare in *in vitro* muscle studies is universal. Curare is routinely used to inhibit the post-synaptic motor endplate receptors from reacting to acetylcholine (ACh). Without curare, electrically stimulated contractions of *in vitro* muscle could induce action potentials in small motor nerve terminals; this would result in the release of ACh at the motor endplate, thus enhancing the contraction (McIntyre, 1947). The use of curare with non-stimulated muscle insures that any slow leakage of ACh at the motor endplate does not excite the muscle.

This investigation was conducted in two stages. As previously indicated, Stage I compared the differences in tissue stiffness as a function of age, sex, and breed of the research animal, using conventional instrumentation for physiological research. Differences in the stiffness characteristics of those samples with and without curare were also evaluated. Using more modern instrumentation, Stage II attempted to refine the procedure with special attention to the method of elongation and effects of electrical stimulation on tissue stiffness. Once the final protocol was determined in Stage II, comparison by sex and age of the research animal was repeated.

STAGE I METHODS

Animal Selection

The data for Stage I were gathered on 24 tissue samples from 13 dogs; two samples were damaged during the preparatory stages and consequently were not included in the study. This stage used three groups of animals. Seven samples were from young beagles, eight samples were from young mixed-breeds, and nine samples were from old mixed-breeds. Attempts were made to match the groups according to sex; however, the match could not be made in any precise manner because of the limitations in resources. Consequently, nine samples were from females and 15 samples were from males. Additionally, the inaccessibility of old beagles precluded their use in this investigation. Animals with respiratory infections were not suitable for this investigation. All animals weighed no less than 10 kilograms.

The young beagles were all 10 months of age and were to be sacrificed as the control group in a cleft palate dental research study. The completion of the dental research project coincided with this investigation; consequently, they were easily incorporated into our research. They were healthy animals who had experienced no treatment. The mixed-breeds were animals acquired from the University of Iowa Animal Care Unit. Age of these animals was estimated by a University Veterinarian on the basis of dental and sexual development. Young mixed-breeds were estimated to be no older than one and one half years, and old mixed-breeds no younger than ten years of age.

Tissue Preparation

The animals were anesthetized with 30 mg/kg of sodium pentobarbital, following which the larynx was excised and immediately submerged in an aerated Krebs-Ringer solution consisting of 115.50 mM NaCl, 4.64 mM KCl, 2.47 mM CaCl₂, 21.19 mM NaHCO₃, 1.16 mM MgCl₂· $6H_{2}0$, 1.17 mM NaH₂PO₄, and 13.26 mM glucose. This solution was continuously aerated with a mixture of 95% oxygen and 5% carbon dioxide. The pH of the solution was 7.4, and the temperature was maintained at $37^{\circ} \pm 1^{\circ}$ C with an immersion circulator (Fisher Circulation, Model 73) (Levy, 1971; Perlman, 1985; Stephens, 1975). The animals were immediately sacrificed with an intramuscular injection of euthanasia drug.

All dissection was performed in a heated bath (Tekbath) with the larynx submerged in the aerated Krebs-Ringer solution. Extrinsic muscles were removed from the thyroid cartilage and all structures above the level of the ventricles of Morgagni were cut away, thus exposing the true vocal folds.

The thyroid cartilage was hemisected at the anterior notch and a wedge-shaped portion of the thyroid cartilage remained attached to the vocal fold. A similar attachment was retained to the arytenoid posteriorly, thus isolating the vocal folds and allowing for natural anterior and posterior boundary conditions. Using a dissecting microscope, the epithelium and lamina propria were carefully dissected from the underlying vocalis muscle. A 3–0 teflon impregnated, polyester suture (Deknatel, Tevdek II, 7–913) was then inserted through each cartilage wedge and tied. As reported earlier (Perlman et al., 1984), the standard braided silk suture has dynamically changing elastic properties that can affect the validity of the measurement, whereas polyester suture maintains more constant and predictable properties.

Instrumentation

Each tissue preparation was mounted in a water-jacketed organ bath chamber containing Krebs-Ringer solution and continuously aerated with oxygen and carbon dioxide. The gas mixture was filtered through a sintered glass disc built into the base of the chamber (Figure 1); 1cc/50ml of curare was added to the physiological salt solution of one half of the tissue samples. Approximately one half of the right and left samples received curare and one half of the right and left samples did not. The suture from one cartilage was fixed at one end of the chamber, and the suture from the other cartilage was fixed to a Grass FT.03 force-displacement transducer held by a rack and pinion with a micrometer screw for manual length adjustment. The transducer signal was then amplified via a custom fabricated preamplifier, and amplified and displayed on a Gould 260 multichannel analog recorder. In order to provide field stimulation to the muscle preparations, a Grass S48 stimulator was connected to platinum ring electrodes placed in the chambers. Contractions resulting from stimulation were used to assess tissue viability, and in the determination of reference length.

Determination of Reference Lengths

Fung (1967) presented a detailed discussion of the problems in selecting the appropriate means for determining resting length. Because resting length appeared to us to be a physiological state that might best be defined in relation to sarcomere length, the term "physiological length" was employed in this study to refer to the zero strain state. This does not necessarily correspond to zero passive stress, but rather to maximum active contractile stress when the muscle is electrically stimulated. The procedure for establishing this physiological length will be discussed below.

Another reference length was of importance. Because it had been determined that the *in situ* length was less subject to measurement uncertainty than either the dissected length (Perlman et al., 1984) or the physiological length (Perlman, 1985), the *in situ* reference length was used to normalize the elongations for strain calculations. The *in situ* length was defined as the distance from that point where the vocal fold attached to the thyroid cartilage to the angle formed by the corniculate cartilage. This measurement could be made with the greatest reliability of any vocal fold length measurements with the use of calipers.

The physiological length was not measured in an absolute sense, but set according to the following criterion. A baseline tension of 1 gram of force was first established. Beginning at 80 Hz, the stimulus frequency was increased until a maximum contraction was obtained. The mode frequency of maximum contraction was approximately 110 Hz. Using that frequency and a current that provided supramaximal stimulation (to all fibers), the tissue was elongated in 1 mm increments until a maximum force was obtained. This then established the physiological zero strain condition. Because the force maximum was rather broad, its location was quite error prone ($\pm 1-2$ mm uncertainty). Typically, this zero strain condition corresponded to a passive tissue force of 1.4 to 10.5 grams.

Length changes were made by manually adjusting the micrometer in 1 mm increments above the physiologic length. To verify the viability of the preparations, electrical stimulation was applied shortly after the preparation had been secured in the organ bath chamber and at the end of the experiment. If there was no response to the stimulation, the tissue was presumed dead and not included in the study. The tissue samples were allowed to



FIGURE 1. Schematic of the water-jacketed organ bath chamber.

equilibrate for one half hour following attachment to the transducer, and for one half hour following adjustment of physiological length. One half of the preparations were treated with d-tubocurarine (curare) preceding the second equilibration and following the measurement procedure.

Data Analysis

Raw data were collected on the recorder in the form of isometric (constant length) force as a continuous function of time; following each stepwise elongation, the data were then interpreted as force per elongation. An example of the raw data is shown in Figure 2.

For purposes of this discussion, "short-term" changes in the force are on the order of milliseconds, "mediumterm" changes are on the order of one to two seconds, and "long-term" changes are on the order of several minutes. Based on these somewhat arbitrary definitions, two discrete time samples were then taken to evaluate this force function in Stage I. The first sample was taken at the end of the one to two second time interval required for manual elongation. This was essentially the peak value in Figure 2, termed MTR (medium-term relaxation), because some relaxation takes place during the act of manual elongation. The second force sample was taken after five minutes of stress relaxation. This was termed LTR (long-term relaxation). As seen in Figure 2, it essentially represented the asymptotic force value before another step elongation occurred. A short-term relaxation (STR) value, which we would define as occurring in the order of milliseconds after a very sudden step elongation, could not be evaluated with this manual technique, but will be discussed in Stage II.

In order to normalize for the differences in mass m and cross-sectional area A of the preparations, the two samples of the force F (at MTR and LTR) were converted to stress measures at every elongation, where stress σ is defined as

$$\sigma = F/A \tag{2}$$
$$= F_{\rm D}L_o(1+\varepsilon)/m$$

The second part of the equation follows directly from a consideration of the total mass of an assumed cylindrical tissue sample

$$m = {}_{\rho}AL \qquad (3)$$
$$= {}_{\rho}AL_o(1+\epsilon),$$

where ρ is the tissue density $(1.1g/\text{cm}^3)$ (Greenspan & Edmands, 1971), *L* is any specified tissue length, L_o is the *in situ* reference length, and υ is the tissue strain defined as $\Delta L/L_o$.

Mean stress-strain curves were plotted for MTR and LTR time samples over a number of tissue samples. Comparisons were made between the variability at strain



FIGURE 2. An example of the change in force resulting from a step elongation followed by stress relaxation.

levels from 10% to 50%. These comparisons were made using the coefficient of variation $(S\bar{x}, \text{ where } S \text{ is the standard deviation and } \bar{x} \text{ is the mean}).$

Analysis of the separate effects of age, breed, sex, and curare on stress was conducted using a multiway repeated measures analysis of variance (Winer, 1971) where the repeated factors ("within subjects") were stress and curare, and the "between subjects" factors were age, breed, and sex. This analysis was performed with Statistical Analysis System (SAS) PROC GLM and by calculating the appropriate error terms as described by Winer. A log transformation of stress values was used to equalize variances across strain levels. Because there were no old beagles, the factors of age and breed were combined as "age/breed" and differences between the means were compared with Tukey's studentized range test. The presence of interactions between the various factors was also checked.

STAGE I RESULTS

Composite Stress-Strain Curves

Figure 3a represents the averaged stress-strain curves for the 24 tissue samples at two time samples (MTR and LTR). The differences in the curves at various levels of strain represent the peak-to-asymptote differences in Figure 2.

The values in Figure 3a are higher than those values reported earlier by Perlman et al. (1984). In the earlier study, an initial tension of 0.5 gram was used to define zero strain. We will return to such a definition (for comparative purposes) in Stage II. Differences in definition of zero strain can account, at least in part, for major differences in the stress-strain curves reported in the literature. More will be said about that later.

Figure 3b shows the results obtained from calculating the coefficient of variation at each level of strain. These plots indicate that stress values were approximately 10%



less variable after MTR than after LTR. It was decided that for future data analysis, we would use the MTR stress values.

Use of Curare

Comparison of the stress-strain curves for the 11 tissue samples that had been treated with curare and the 13 tissues that received no treatment, showed almost identical curves in both shape and magnitude. Since the curves represent samples equally distributed by sex, and with a difference of only one sample according to age group and breed, the similarity between the drug-treated and non-treated samples suggested that the drug had no effect. These findings were in agreement with those of Moulds et al. (1977). However, when the coefficient of variation was calculated (Figure 4b), the groups behaved differently. Variability seemed to decrease as a function of strain with curare and increase without curare. One possible explanation for this difference is that the muscle samples did contain varying amounts of terminal nerve fibers; and, with the inclusion of curare in the bath, once the preparations were elongated, the released neuromuscular transmitter was not effective.

Variability with Regard to Breed and Age

Figure 5a represents the MTR stress-strain curves of vocalis muscle tissue from young beagles (YB), young mixed-breeds (YM) and old mixed-breeds (OM). Tissue from the old mixed-breeds was observed to be the least stiff, and that obtained from the young beagles was most stiff. When the groups were compared relative to the variability at each level of strain (Figure 5b), the samples from the old dogs showed the greatest variability, the young mixed-breeds showed the least variability, and samples from the young beagles were intermediate, but most consistent across strain levels.



FIGURE 3. (a) Mean MTR and LTR stress-strain curves. (b)The coefficient of variation. No control over sex, breed, age, or curare. Zero strain was at physiological length.



FIGURE 4. (a) Mean MTR stress-strain curves and (b) the coefficients of variation, comparing the effect of curare. No control over sex, breed, or age. Zero strain was at physiological length.



FIGURE 5. (a) Mean MTR stress-strain curves and (b) the coefficients of variation, comparing age and breed. No control for sex. Zero strain was at physiological length. YB = young beagles, YM = young mixed-breeds, OM = old mixed breed.

Differences between the young mixed-breeds and young beagles are somewhat confusing. Since the beagles were purebred animals born within days of one another, often from the same litter, and raised under the same laboratory conditions, it was expected that the beagles would show less variability than the mixed-breeds. One explanation might be that the young beagles were generally the smallest of the animals used. They ranged from 11 to 14 kilograms. The length of the vocal fold *in situ* was often close to 1.0 cm; consequently, a .5 mm error in measurement would make a greater error than that occurring with the larger mixed-breeds with vocal folds ranging from 1.2 to 1.7 cm.

Informal laboratory observation revealed age-related differences in the morphology of the larynges. Of specialinterest to this study was the underdeveloped vocal process of the young animals. This would result in a different orientation of the medial thyroarytenoid muscle fibers. Consequently it was not unexpected that the tissue samples from the old dogs would exhibit different mechanical properties from the tissue samples from the young dogs. Other changes occur in muscle as a function of exercise, and nutritional status (Chesky, 1978; Mc-Carter, 1978) but these were not controlled.

Variability with Regard to Sex

Comparison of all samples by sex (Figure 6a) showed male dogs to have stiffer vocal folds than females, but the coefficient of variation was also higher for the males than for the females (Figure 6b). However, these results were confounded by the effects of unequal age distribution and breed. At the completion of Stage II a study will be described that was designed to resolve this issue.

Analysis of Variance

The repeated measures analysis of variance showed no effect of curare, a marginal effect of sex, and significant



FIGURE 6. (a) Mean MTR stress-strain curves and (b) the coefficients of variation, comparing males and females. No control for age and breed. Zero strain was at physiological length.

effects of age/breed and strain on the log stress response (Table 1).

The interaction term between age/breed and strain approached significance (p = 0.08). However, since the age/breed variable was a composite of two factors, the interaction was uninterpretable, and required further investigation. On the other hand, the Tukey multiple comparison test between the means for age/breed determined each mean to be significantly different from the other two (p < .05).

STAGE II METHODS

The results of Stage I demonstrated that the subgroup showing the least intrastudy variability consisted of curarized vocalis muscle tissue from young, mixed breeds of the same sex. It was the intent of this portion of the investigation (Stage II), to address the issue of variability resulting from two definitions of zero strain, method of

 TABLE 1. Repeated measures analysis of variance for joint effects of age, breed, sex, and curare.

Variable	Degrees of freedom	Sum of squares	F	p
Age/Breed	2	8.34	5.36	0.0400
Sex	1	3.13	4.02	0.0800
Age/Breed*Sex	2	0.69	0.44	0.6600
Error (between subjects)	7	5.44		
Curare	1	0.00	90.00	0.9600
Error (within subjects-curare)	10	3.38		
Strain	4	33.89	366.39	0.0001
Age/Breed*Strain	8	0.37	2.01	0.0800
Sex*Strain	4	0.07	0.81	0.5300
Error (within subjects-strain	28	0.65		

elongation, presence or absence of electrical stimulation, and selection of instrumentation.

In an ideal experimental design, the same tissue samples would have been tested for all conditions. However, the deterioration of the tissue over time, and the effects of conditioning and plasticity, precluded such a design. Therefore, results from this investigation will be compared with those from Stage I. Because the issue of sex and age differences was not satisfactorily resolved in Stage I, stress-strain measures were again made with young males and females and with old and young males.

Animal Selection

This portion of the investigation used primarily young, mixed-breed, females. In those portions of the investigation where males were used, the change in subject selection is explained. Age was determined in the manner similar to Stage I. From our past experience with the young beagles (Stage I), it was found that animals weighing less than 12 kg generally had very small larynges and therefore vocal folds that were too short. Consequently, in this portion of the investigation (Stage II) only animals weighing a minimum of 15 kg were acceptable.

Tissue Preparation

In general, the method for tissue preparation in Stage II was identical to that of Stage I. In this study, curare (1cc/50ml) was used with all preparations. Because only one force-displacement transducer was available (as opposed to two in Stage I), both vocal folds could not be studied simultaneously. Hence, the right vocal fold was always studied before the left. Data were first gathered on 18 tissue samples from nine young, mixed-breed female dogs. Four preparations from the left vocal fold were not included because they showed poor response characteristics to electrical stimulation.

In the initial phases of Stage II (as in Stage I), both folds were dissected before beginning the measurement process. That method was later changed because it became apparent that the active contractile forces of the second preparations were generally much weaker than those of the first vocal fold preparations. When dissection of the second vocal fold was postponed until completion of data collection on the first fold, the active contractile forces of the second preparations were then compatible with those of the first preparations.

Instrumentation

The tissue preparations were mounted in the waterjacketed organ bath chamber (Figure 1). Whereas ring electrodes were used in Stage I, it was determined in this investigation that plate electrodes, 10 mm wide x 25 mm long, closely approximating (but not touching) the muscle, gave a better response to stimulation. Silver electrodes were first used, but because of a buildup of negative ions on the silver electrodes, we have since returned to the use of platinum. The plate configuration has been retained ever since.

In Stage II the upper suture was fixed to the arm of a dual mode ergometer (Cambridge Technology, Model 305). The ergometer system is electronically controlled via a precision servomechanism and was thought to be ideal for future studies where arbitrary wave shapes might be used as force or displacement inputs. Whereas tissue displacement in Stage I was performed by manually turning a micrometer screw in 1 mm increments, elongations in Stage II were initially performed by a step function generator. Later in Stage II, there was a return to manual elongation for reasons to be explained. In Stage I, the force values were plotted onto an analog recorder. In Stage II, the signals were fed into a PDP 11/44 computer for analog to digital conversion. The analog signal was sampled at a rate of one thousand samples per second for five seconds. Output from the ergometer also went into a data logger (Fluke, 2240C) for A/D conversion at a sampling rate of one sample per second. Force values at discrete times following long term relaxation could be obtained via the data logger. However, the sampling rate was too slow for accurate short-term or medium-term stress measurements; hence the additional recording by computer.

Whereas in the first study density was assumed to be 1.1g/cm³, in this investigation, the density of each preparation was determined experimentally (Mettler balance). Mean tissue density was 1.043g/cm³ with a standard deviation of .005g/cm³. This measurement was taken at the completion of the experiment, after the cartilage had been carefully dissected from the muscle sample and the mass had been determined.

It took from one to two seconds for completion of a manual elongation in Stage I. In Stage II, stress values were sampled at three different times: (a) within 10 ms post elongation (subsequently called short-term relaxation, or STR), (b) two seconds post elongation (mediumterm relaxation, or MTR), and (c) five minutes post elongation (long-term relaxation, or LTR).

In Stage I, there were five tissue samples that came from young females. Three of these samples were from beagles and two from mixed-breeds. Although the mean stress values of the female beagles were somewhat greater than those of the female mixed-breeds (which, when combined, would affect the overall variability), it was nevertheless decided to combine the values in order to have a sizeable comparison with the samples in Stage II.

In order to verify the results of Stage I, two separate analyses were undertaken. These experiments were done on 10 male and 11 female samples from young, mixbreeds and on six old and 10 young samples from mixedbreed males. Reexamination of the effects of age and of sex were conducted using the procedures that developed from the experiments in Stages I and II.

STAGE II RESULTS

Method of Elongation

Stress-strain curves were obtained less than 10 ms following the step elongation (STR), as well as two seconds post elongation (MTR) and five minutes post elongation (LTR); these curves are shown in Figure 7. Note that the difference between MTR and LTR is on the order of 15–25% for most levels of strain, as in Figure 3. Recall that this difference represents the peak-to-asymptote difference in Figure 2. A greater difference is noted



FIGURE 7. Mean stress-strain curves for young female mixedbreeds. STR = < 10 ms post elongation, MTR = 2 s post elongation, LTR = 5 minutes post elongation. Zero strain was established at physiological length.

between the STR curve and either of the others, suggesting that large stresses are developed when the tissue is elongated very rapidly. These stresses are to a large part velocity-dependent (viscous) stresses that relax very rapidly when the steady length has been achieved (Alipour-Haghighi & Titze, 1985).

According to Martensson and Skoglund (1964), the dog thyroarytenoid has a contraction time of approximately 14 ms and the cricothyroid has a contraction time of approximately 35 ms. It was hypothesized that the 1 mm length changes imposed by our ergometer system in a rise time of 7 ms were perhaps too rapid for normal dynamic behavior of the vocalis muscle. The unnatural "shocking" of the muscle may disturb the fibers and cause more variability in the length-tension characteristics. Elongations were subsequently performed manually via the ergometer's servo driver (by adjusting a 10-turn potentiometer). Absolute values of displacement were then determined with the data logger, and the error in length adjustment was determined to be about $\pm .01$ mm.

In order to assess the time for each manual elongation in Stage II, a random group of force-displacement curves was analyzed. Time for this displacement was between .8 sec and 1.5 s with a mean of 1.0 s. This is comparable to the MTR criterion set in Stage I.

Effects of Electrical Stimulation

The purpose of this study was to test the effect of periodic stimulation on tissue elasticity. We felt this was important for future studies when active and passive muscle properties would be compared. Eight tissue samples were divided into two groups. One group received stimulation in the manner compatible with all previous experiments in this research, and one group had passive elongation only. Results indicated no major differences in the stress-strain curves, although the group that received no stimulation had slightly higher stresses (about 20%) at strains above 40%. Below 40%, there were no significant differences. Variability was also comparable.

Retest of Sex and Age Differences and Revised Zero-Strain Definition

Once the method of elongation, the use of electrical stimulation, and the choice of instrumentation had been determined, the differences in the stress-strain curves as a function of sex and age were retested. Also, to test a more reliable zero-strain condition, a one gram force was used here to establish zero strain.

All samples were curarized and treated with the same protocol. Results are shown in Figure 8. Although on the average there were no major sex differences in the stress magnitudes across all strains, the young female tissue samples showed more linearity than the young male samples. For low strains, female tissue was stiffer. Whereas for high-strains, male tissue was stiffer. In Stage I, the male tissue showed an overall greater stiffness (Figure 6a). As previously noted, however, there was no control for age. The young-to-old sample ratio was 1.0 for females and 2.5 for males. Thus, a disproportionate amount of younger males was used, and since younger tissue has been shown to be stiffer (Figure 5), there is likely to have been a bias.

Comparison of Figures 8 and 6b further indicate that the variability of both sexes was less in Stage II than in Stage I. The coefficient of variation was about 0.2 instead of 0.4. Also, the males showed slightly greater variability than the females in Stage I. This was not the case in Stage II. These differences are likely to be attributable to better controls in the experiment.

As in Stage I (Figure 5a), the old tissue samples were consistently less stiff than those samples from young dogs (Figure 8a). The change in the slope of the curve at 40% suggests the tissue may have reached the point of plasticity. The variability of the old tissue samples (Figures 5b and 8b) was generally much greater than that of the young samples.

Statistical analysis of the stress-strain relationships for the male and female vocalis muscle tissue samples (Table 2) found a significant interaction between sex and strain (p <.001) indicating that the stress-strain relationship was different between males and females. The greater nonlinearity for the males in Figure 8a is therefore an important observation. With regard to age, statistical



FIGURE 8. (a) Mean MTR stress-strain curves and (b) the coefficients of variation, comparing curarized tissue by sex (young mixed breeds) and age (mixed-breed males). Zero strain was established at F = 1g. The tissue had been conditioned with electrical stimulation.

31 288–298 June 1988

TABLE 2. Repeated measures analysis of variance for separate effects of age and sex.

Variable	Degrees of freedom	Sum of squares	F	p
Age	1	2.27	21.1	0.0040
Error (Age)	6	0.64		
Strain	4	61.00	219.5	0.0001
Age*Strain	4	0.61	2.2	0.0800
Error (Strain)	63	4.38		
Sex	1	0.88	4.9	0.0500
Error (Sex)	10	1.79		
Strain	4	64.09	439.1	0.0001
Sex*Strain	4	2.74	18.8	0.0001
Error (Strain)	85	3.10		

analysis of the stress-strain relationships for tissues from old and young male vocalis muscle confirmed the observed difference in Figures 5a and 8a, the main effect of age being significant at the .004 level.

As a result of the study by Perlman et al. (1984), the interpretation of the data from Stage I, and the generations of studies within Stage II, it was concluded that the most consistent measurements are obtained with curarized tissue from young, mixed-breed dogs of the same sex, where the 1 gram initial force was the criterion for establishing zero strain. It is important to remember, however, that biological tissue is not consistent within or across specimens and that some variability will always exist.

DISCUSSION

"A common problem in all scientific endeavor is the making of measurements without the act of measurement influencing the measurement itself" (Lawton, 1957, p. 1). The problems associated with accurate determination of sample geometry still present a major challenge. First, there is a nonuniformity in the cross-sectional area resulting from the manner in which the vocalis muscle inserts onto the arytenoid cartilage. This would argue for the use of thin samples, that is few fibers in parallel. Using the relationship between length, density, and mass to determine the cross sectional area, the average area appeared to be approximately 8 mm² for the tissue samples in Stage II; however, due to some remaining nonuniformity, error is certain to exist in this effective cross-sectional area. Secondly, since reference length is a factor in the calculation of stress, error is likely to be introduced in normalizing the elongations. In the study by Perlman et al. (1984), measurement of the *in situ* length was found to show less error than measurement of the length after dissection. Additionally, the in situ length has been found to show somewhat less variability than the *in vitro* measurement made in the organ bath chamber (Perlman, 1985). Although determination of in-chamber length is the more appropriate, unless more sophisticated methods of measurement (such as microscopic techniques) are incorporated into the methodology, it appears advisable to continue to use the *in situ* length.

The decision to use a minimum threshold force for definition of zero strain has certain benefits. By using a common force and a nearly common cross-sectional area, it is felt that a more meaningful comparison can be made across samples. Although maximum active force may be indicative of a physiological reference state, it may not be indicative of the force exerted by the vocalis during phonation. This remains to be seen. Therefore, the physiological reference length or a minimum force of some predetermined amount are both in some sense arbitrary. Since the minimum force shows the lesser variability, it may prove more useful.

With regard to instrumentation, it was advantageous to be able to use an electronically-controlled servo system connected to a computer for more rapid data collection and analysis. Because of the sophisticated nature of this servo system, calibration was performed by the manufacturer, who assured us that the equipment met the specifications (0.1% linearity of force output signal, 1 V/mm \pm 2% position signal output). However, when we attempted to test the accuracy of the force measurement, we suspected greater error at the minimum and maximum deflections than in the midposition. Although such variability would not be as significant at high levels of tissue force, at the negative displacement this error could have been important. It was therefore inadvisable to use the extreme negative position. We acknowledged that this was not an ideal solution, but it was the best we could do. It does help explain, however, the increased variability in force at low levels of strain. Although the ergometer error would also contribute to variability at higher levels of strain, tissue damage must be considered as a major contributor to variability at that level.

Still, it is likely that the ergometer was more precise than the method we employed with the mechanical force-displacement transducer used in Stage I. With the mechanical transducer, displacement was measured by a micrometer that marked 1 mm increments. Thus, error in displacement could be occurring. With the ergometer, displacement was measured as a voltage and the tradeoff appeared to be in favor of the electronic transducer.

The findings relative to differences in the longitudinal stiffness of the vocalis muscle as a function of sex and age have important implications to the development of mathematical models of phonation and to theories of motor control. If this is also true in the human larynx, the decreased tension in the aged vocalis may be providing less support for the vocal fold cover. This could result in changes in the glottal configuration and pattern of oscillation. Such changes could describe, at least in part, some of the changes in the acoustic signal that occur with aging. In future research with animal models, one needs to consider the appropriateness of homogeneous grouping by age and/or sex.

The act of stretching a piece of vocal fold muscle and recording the tension of that tissue is not an end unto itself. Observing the behavior of vocal fold tissue *in vitro* does allow, however, for more control of the environment and specific conditions of that tissue. In future studies, questions about the effects of medications, radiation treatment, and certain surgical procedures can be addressed. However, additional information on the physiological properties of healthy vocal folds is still needed, particularly with regard to tissue viscosity.

SUMMARY

The larynx was excised from anesthetized dogs and immediately submerged in an aerated Krebs-Ringer solution. The preparation was maintained in solution throughout the experiment. The vocalis muscle was carefully dissected with attachment to the arytenoid and thyroid cartilages remaining. The preparations were subjected to isometric force-elongation measures and were later converted to stress-strain values.

Analysis of the data in Stage I, which employed conventional manual (micrometer) methods of tissue elongation, suggested the presence of significant differences related to the age of the laboratory animal. Old tissue was less stiff than young tissue. The tests of significance performed at the conclusion of Study II confirmed the age differences and in addition revealed a sex difference in tissue linearity. Stress-strain curves of females were more linear than those of males. The inclusion of curare in the organ bath chamber was found to have no effect on the shape or magnitude of the stress-strain curves. However, the differences in variability between those samples with and without any drug treatment, suggested the advisability of continuing with the curare. The presence of curare insured no effect from release of ACh. Additionally, the data suggested that pure breeds (e.g., beagles) and mixed breeds produced different results. The availability and lower cost of mixed breeds, however, made them the more desirable choice, unless specific breed information is needed. Lastly, at this time, the use of in situ reference length and a predetermined force, such as 1 gram, as the definition for zero strain are recommended for data comparison.

ACKNOWLEDGMENTS

We wish to thank Dr. David G. Reynolds, Director of Surgery Research at the University of Iowa, for use of his facilities and his numerous suggestions. We also thank Dr. Fari Alipour-Haghighi for writing the computer programs, Brenda Booth for her assistance with the statistical design (Iowa Health Services Research and Development Field Program, Iowa City VA Medical Center) and Paul Durham, laboratory research assistant.

This research was supported in part by Veterans Administration Medical Research Funds and in part by the National Institutes of Health, NINCDS-Grant #NS 16320-03.

REFERENCES

- ABO EL-ENE, M. A. (1975). Significance of the pattern of motor innervation of the intrinsic laryngeal muscles of cat. Acta Anatomica, 93, 543-555.
- ALIPOUR-HACHICHI, F., & TITZE, I. R. (1985). Viscoelastic

modeling of canine vocalis muscle in relaxation. Journal of the Acoustical Society of America, 78, 1939–1943.

- BAUER, H., & KONIG, W. (1967). Physikalische und histologische Untersuchungen an Normalen and einem weiblichen virilisierten Kehlkopf (Physiological and histological research on normal and a female virilized larynx.). Archiv fur klinische und experimentelle Ohren- Nasen- und Kehlkopfheilkunde, 188, 358–363.
- CHESKY, J. A. (1978). Comparative aspects of declining muscle function. In G. Kaldor & W. J. DiBattista (Eds.), Aging (Vol. 6, pp. 179–210). New York: Raven Press.
- DAMSTE, P. H., & WIENEKE, G. H. (1973). Experiments on the elasticity of the vocal folds. *Journal of the South African* Speech and Hearing Association, 20, 14–21.
- EDWARDS, R. H. T., YOUNG, A., HOSKING, G. P., & JONES, D. A. (1977). Human skeletal muscle functions: Description of tests and normal values. *Clinical Science and Molecular Medicine*, 52, 283–290.
- FUNG, Y. C. (1967). Elasticity of soft tissue in simple elongation. American Journal of Physiology, 213(6), 1532–1544.
- GREENSPAN, K., & EDMANDS, R. É. (1971). The use of microelectrode techniques in muscle. In A. Schwartz (Ed.), *Methods in Pharmacology* (Vol. 1, pp. 553–581). New York: Appleton-Century-Crofts.
- HALL-CRAGGS, E. C. B. (1968). The contraction time and enzyme activity of two rabbit laryngeal muscles. Journal of Anatomy, 102, 241-255.
- HAST, M. H. (1966). Physiological mechanisms of phonation: Tension of the vocal fold muscle. Acta oto-laryngologica (Stockholm), 62(30), 309-313.
- HAST, M. H. (1969). The primate larynx. Acta oto-laryngologica (Stockholm), 67, 84–92.
- HIRANO, M. (1977). Structure and vibratory behavior of the vocal folds. In M. Sawashima & F. Cooper (Eds.), *Dynamic aspects* of speech production (pp 13–30). University of Tokyo Press.
- HIROSE, H., TATSUJIRO, U., KOBAYASHI, T., & SAWASHIMA, M. (1969). An experimental study of the contraction properties of the laryngeal muscles in the cat. Annals of Otology, 78, 297–307.
- ISHIZAKA, K. & FLANAGAN, N. G., (1972). Acoustic properties of a two-mass model of the vocal folds. *Journal of the Acoustical Society of America*, 51, 91.
- KAKITA, Y., HIRANO, M. & OHMARU, K. (1981). Physical properties of the vocal fold tissue: Measurements on excised larynges. In K. Stevens & M. Hirano (Eds.), Vocal fold physiology (pp. 377–397). University of Tokyo Press.
- KURITA, S., NAGATA, K., & HIRANO, M. (1983). A comparative study of the layer structure of the vocal fold. In D. M. Bless & J. H. Abbs (Eds.), Vocal fold physiology (pp. 3–21). San Diego: College-Hill Press.
- LAWTON, R. W. (1957). Some aspects of research in biological elasticity. In J.W. Remington (Ed.), *Tissue elasticity* (pp. 1–11). Washington, DC: American Physiological Society.
- LECLUSE, F. L. E. (1977). *Elektroglottografie*. Utrecht, Netherlands: Drukkerijelinkwijk B.V.
- LEVY, J. V. (1971). Isolated atrial preparations. In A. Schwartz

(Ed.), *Methods in pharmacology* (Vol. 1, pp. 77-104). New York: Appleton-Century-Crofts.

- MARTENSSON, A., & SKOGLUND, C. R. (1964). Contraction properties of intrinsic laryngeal muscles. Acta Physiologica Scandanavica, 60, 318-336.
- MCCARTER, R. (1978). Effects of age on contraction of mammalian skeletal muscle. In G. Kaldor & W. J. DiBattista (Eds.), Aging (Vol. 6, pp. 1–21). New York: Raven Press.
- MCINTYRE, A. R. (1947). Curare: Its history, nature, and clinical use. Chicago: University of Chicago Press.
- MOULDS, R. F. W., & DENBOROUGH, M. A. (1974). The study of the action of caffeine, halothane, potassium chloride, and procaine on normal human skeletal muscle. *Clinical and Experimental Pharmacology and Physiology*, 1, 197–209.
- MOULDS, R. F. W., YOUNG, A., JONES, D. A., & EDWARDS, R. H. T. (1977). A study of the contractility, biochemistry, and morphology of an isolated preparation of human skeletal muscle. Clinical Science and Molecular Medicine, 52, 291–297.
- NEGUS, V. E. (1929). The mechanism of the larynx. St. Louis: C.V. Mosby Co.
- NEGUS, V. E. (1949). The comparative anatomy and physiology of the larynx. New York: Hafner.
- PERLMAN, A. L. (1985). A technique for measuring the elastic properties of vocal fold tissue. Unpublished doctoral dissertation. University of Iowa, Iowa City, IA.
- PERLMAN, A. L., TITZE, I. R. & COOPER, D. S. (1984). Elasticity of canine vocal fold tissue. *Journal of Speech and Hearing Research*, 27, 212–219.
- ROSSI, G., & CORTESINA, G. (1965). Morphological study of the laryngeal muscles in man. Insertions and course of the muscle fibers, motor-end-plates, and proprioceptors. Acta otolaryngologica (Stockholm), 59, 575–592.
- STEPHENS, N. L. (1975). Physical properties of contractile systems. In E. E. Daniel & D. M. Paton (Eds.), *Methods in pharmacology* (Vol. 3, pp. 265–296). New York: Plenum Press.
- TITZE, I. R., & TALKIN, D. T. (1979). A theoretical study of the effects of various laryngeal configurations on the acoustics of phonation. Journal of the Acoustical Society of America, 66, 60-74.
- VAN DEN BERG, J. & TAN, T. S. (1959). Results of experiments with human larynges. *Practica oto-rhino-laryngologica*, 21, 425–450.
- WINER, B. J. (1971). Statistical principles in experimental design (2nd ed.). New York: McGraw Hill.
- ZEALEAR, D. L. (1983). The contractile properties and functions of single motor units of the thyroarytenoid muscle in the cat. In D. M. Bless & J. H. Abbs (Eds.), Vocal fold physiology (pp. 96–106). San Diego: College Hill Press.

Received June 2, 1986 Accepted August 28, 1987

Requests for reprints should be sent to Adrienne L. Perlman, Audiology Speech Pathology Service, VA Medical Center, Iowa City, IA 52240.