two small droplets of equal size (5 to 25 μl), one consisting of Bchl protein (8 to 10 g/liter), 1*M* NaCl, and 0.01*M* phosphate buffer (*p*H 7.8) and the other of 13 percent $(NH_4)_2SO_4$ solution weight per volume (*10*). The droplets of each pair, juxtaposed in acrylic depression slides, were united by a long, narrow liquid bridge drawn from the droplet containing the Bchl protein. The preparations, when completed, were covered, sealed, and stored in the refrigerator (~5°C). Spherulites which appeared after a period of several hours to several days apparently formed at sites favorable to their nucleation in the numerous gradients occurring during and after droplet union.

Typical Bchl protein spherulites in various stages of growth are shown in Fig. 1. In the early stage (Fig. 1, a and b) dissimilarity in the length of the extending microtubules gives a fuzzy appearance to the outer edge of conical lobes formed by their growth; the sheaflike structure from which they originate appears to be twinned or symmetrically expanded in two directions. However, bilobate, twinned spherulites do not always occur; the single-lobed, conical form (half structure) is also present. The next stage in the growth of the Bchl protein spherulites involves the rounding up and enlargement of one of the conical lobes. During this process, bundles of microtubules and the originating sheaflike structure gradually disappear until only one lobe is left (Fig. 1c). The edges of these larger lobes are less fuzzy and indicate a more uniform length of the radiating microtubules. The final stage in the development of Bchl protein spherulites is reached when sharp resolution of the outer surface of the lobe (Fig. 1f) indicates that the shorter microtubules have grown in length to fill in the spaces between the longer ones. The microtubular texture can be nearly resolved by absorption and birefringence microscopy (Fig. 1, f and g). The weak birefringence and low density of tubule packing in Bchl protein spherulites are indicated by the polarization color, which remains blue (first order) even in the larger, mature forms.

The final stages of spherulite development in Bchl protein differ from that in most other materials. In most spherulites complete rounding involves the expansion of both fibrillar lobes until they meet and fill a spherical space. This often leaves two fibril radiation centers which, when the originating sheaf structure is incorporated, become cavities (*3*, *5*). The predominance, in bilobate spherulites, of one lobe developing at the expense of the other, as well as the competence to originate single-lobed structures, appears to be unique to proteins. These characteristics of Bchl protein may be linked with the different long-range order effects involved in an assemblage composed of large single molecules as opposed to those involved with atoms or small molecules. The occurrence of microfibrillar spherulites of fraction 1 protein in the chloroplast stroma of *Avena* and *Phaseolus* (*11*) indicates that under certain intracellular conditions this crystal habit can assemble even in the presence of other macromolecules.

Although fully developed protein spherulites are rarely seen in vivo, the considerable order in the arrangement of the quaternary structured elements of proteins which comprise the paracrystalline microtubules, fibrils, lamellae, and so forth, of many organelles suggests that a spherulitic ordering may exist in the living cell. Many of these structures have been separated, disassembled, and then reconstituted in vitro; a few have been reassembled in crystal habits different from the originals, including one which resembles spherulitic crystals (*12*). In view of my observations, Bchl protein appears well qualified as a material for the study of the molecular forces involved in the assemblage of macromolecules to form functional organelles.

RODNEY A. OLSON
Laboratory of Physical Biology, NIAMD, National Institutes of Health, Bethesda, Maryland 20014

References and Notes

1. R. A. Olson, W. H. Jennings, C. H. Hanna, *Arch. Biochem. Biophys.* **130**, 140 (1969).
2. J. E. Coleman, B. J. Allan, B. L. Vallee, *Science* **131**, 350 (1960).
3. A. Keller and J. R. S. Waring, *J. Polymer Sci.* **28**, 447 (1955).
4. B. Popoff, *Latv. Farm. Zurn.* **1934**, 1 (1934).
5. H. W. Morse and J. D. H. Donnay, *Amer. Mineral.* **21**, 391 (1936).
6. J. P. Thornber and J. M. Olson, *Biochemistry* **7**, 2242 (1968).
7. J. M. Olson, D. F. Koenig, M. C. Ledbetter, *Arch. Biochem. Biophys.* **129**, 42 (1969).
8. L. W. Labaw and R. A. Olson, *J. Ultrastruct. Res.*, in press.
9. R. A. Olson, W. H. Jennings, J. M. Olson, *Arch. Biochem. Biophys.* **129**, 30 (1969).
10. Bchl protein was kindly provided by J. M. Olson, Brookhaven National Laboratory, Long Island, New York.
11. B. E. S. Gunning, M. W. Steer, M. P. Cochrane, *J. Cell Sci.* **3**, 445 (1968).
12. B. Poglazof, *Structure and Functions of Contractile Proteins* (Academic Press, New York, 1966), pp. 164–230; D. Abram and H. Koffler, *J. Mol. Biol.* **9**, 168 (1964); R. E. Stephens, *Quart. Rev. Biophys.* **1**, 377 (1969).

26 February 1970

Bronchograms and Tracheograms of Seals under Pressure

Abstract. *Radiograms of the upper portion of the respiratory system were obtained at pressures up to 31.6 atmospheres absolute in the Weddell seal,* Leptonychotes weddelli, *and the northern elephant seal,* Mirounga angustirostris. *The trachea was considerably compressed but not fully collapsed at the highest pressures. No measurable change in the size of the bronchioles and smaller bronchi was observed. Measurements of total lung volume obtained simultaneously showed that the seals consistently dived with a small volume.*

All explanations of the effects of pressure on marine mammals have been hypothetical and based on anatomical characteristics and inference. The subject has been reviewed recently (*1*); with the exception of heart rate (*2*) no physiological parameters have been directly measured, although Ridgway *et al.* (*3*) have measured expiratory gas composition, after diving, in a porpoise trained to dive to 300 m. We now describe some observations of the effects of pressure on the upper respiratory passageways of two species of seal. The Weddell seal, *Leptonychotes weddelli,* and the northern elephant seal, *Mirounga angustirostris,* were selected for this study for two reasons: (i) *Leptonychotes* is a deep diver (*4*), and general information that we have gathered on *Mirounga* indicates that it can endure great pressures as well; (ii) tracheal cartilage rings in *Leptonychotes* are highly modified which suggests that they are an adaptation to pressure (*1*, *5*), whereas the trachea of *Mirounga* is not so unusual. Consequently, we had subjects that could withstand high pressures, which in turn would enhance our chances of observing any gross changes in the bronchi and trachea. Comparison of changes in these two species might aid us in interpretation of the significance of certain anatomical configurations of the trachea and bronchi.

We used a 7-month-old female Weddell seal (109 kg) collected in the Antarctic in December 1968 and a male elephant seal (78 kg) estimated to be about 15 months old. The animals were anesthetized with halothane, and

then powdered tantalum was insufflated onto walls of the trachea, bronchi, and bronchioles by means of a tracheal tube and small catheter. The radiopaque tantalum provides better contrast on the x-rays and is reported not to irritate mucous membranes nor to be taken up by the cells (*6*). Placement of the catheter and the degree of coating were determined by fluoroscopy. After applying the tantalum we placed the seals on a steel restraining board and put them into a specially built "wet" pressure chamber (61 cm inside diameter). Scout films were exposed to determine the lung field being x-rayed through aluminum ports (0.64 cm thick, 9.53 cm in diameter). The x-ray unit was a Philips 1000-ma tube equipped with exposure-timing accessory. Two plastic film holders (20 by 25 cm) with medium-speed intensifying screens and medium-speed film were sealed in thin plastic and placed firmly against the animal before each dive. The portion of the trachea examined was 5 to 8 cm posterior to the glottis; that of the bronchi was at about the midsection of the lung. At least 3 hours after the cessation of anesthesia, when the animals were fully awake, we simulated dives by filling the chamber completely with fresh water. For each seal diving was simulated at least three times at ambient pressure of 1 atmosphere absolute (ATA) before simulated deep dives were carried out. Deep dives were accomplished by rapidly filling the chamber with water and then hydraulically increasing pressure the desired amount.

The volume of water required to achieve a particular pressure was determined. The difference between this volume and values for the chamber alone, which represent chamber expansion gave us the net compression volume of the seal. Using the expression $V_1 = P_2V_2/(P_2 - P_1)$, where V_1 equals the initial gas volume of the seal, V_2 equals the compression volume minus the chamber expansion factor, and P_1 and P_2 are the initial and final pressures, respectively, we calculated the gas volume of the seal before the pressurization. If we assume that there is no significant resistance to compression by the thorax and that gas absorption from lungs by the blood is negligible during pressurization, then V_1 equals the gas volume of the respiratory system and gastrointestinal tract before pressure is applied. The size of the lumen of the intestinal tract of these species indicates that the amount of gas here would not be large; consequently, the values for gas volume in Table 1 represent the volume of the respiratory system primarily. If the gastrointestinal volume were large, then the actual lung volume during the dive would be even more remarkable because it would be the difference between total and gastrointestinal gas volumes. To make sure that the volumes measured represented gas volumes in the seal and not expelled air after submergence and pressurization we always bled a small amount of water from a valve located in the uppermost portion of the chamber just before depressurizing. In no case did we note gas escaping into the reservoir mounted over this bleed-off valve; thus the seal did not exhale once pressurization began.

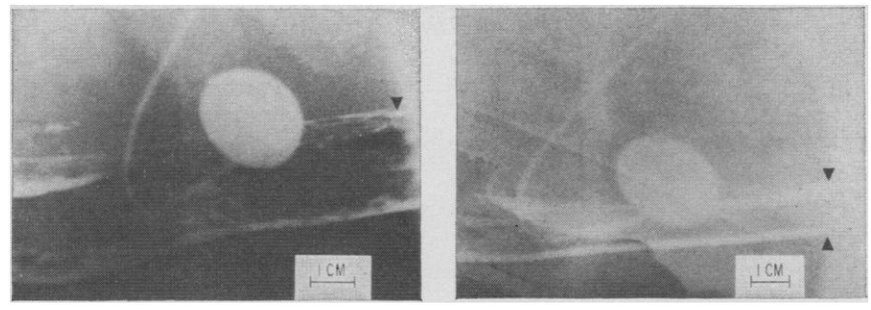

Fig. 1. Radiograms of the lateral portion of the trachea just posterior to the glottis in the Weddell seal, *Leptonychotes weddelli*. (Left) The photo was taken when the seal was submerged at 1 ATA; (right) this picture was taken when the seal was submerged at 31.6 ATA. Anterior is to the left; arrowheads delineate the boundaries of the trachea; the circular object is an electrode for monitoring heart rate.

It has been reported that seals exhale before diving (*7*), and that exhalation is so extensive that they often dive with little more than residual lung volume. Based on reports of the relationship of lung volume to body weight in seals (*8*), the total lung volume of our animals is probably about 8 to 10 liters. Our measurements of the total lung volume (total gas volume) (Table 1) during the dives, in agreement with earlier work, show that these animals exhale before diving and dive with a reduced volume of air in the lungs, in some instances probably only slightly more than residual. In the case of both seals the volume varied after the first pressure dive. The lung volume of *L. weddelli* increased slightly whereas that of *M. angustirostris* decreased. This may mean that there are volumetric variations depending on whether a deep or a shallow dive is anticipated.

Table 1. Estimations of total gas volume of the seals at the beginning of the dive and gas volume at maximum pressure, as calculated from the final compression volume. Pressure is expressed in atmospheres absolute (ATA). For every 10 m of seawater depth the pressure increases approximately 1 atmosphere; therefore, 31.6 ATA equals a depth of 306 m.

Pressure (ATA)	Gas volume (liter)		
	Compression	Total	At pressure
	Leptonychotes weddelli		
6.4	0.87	1.03	0.16
11.2	1.75	1.92	0.17
31.6	2.02	2.08	0.06
	Mirounga angustirostris		
6.4	2.27	2.69	0.42
11.8	2.07	2.26	0.19
31.6	1.47	1.52	0.05

Measurements from the x-ray plates (Fig. 1) showed that the trachea is definitely compressed in both animals (Table 2). Lateral pictures were taken because we assumed that the major portion of tracheal compression would occur in this plane since the dorsal aspect of the trachea is without cartilaginous support. Indeed, the tracheal cartilages of Weddell seals are flattened bows that permit the tracheal lumen to close in the relaxed state. However, we noted no exceptional difference between animals. The diameter of the trachea from the lateral view in both seals is reduced to less than half its original dimension at 31.6 ATA, which is equal to the pressure in seawater at 306 m. The bronchi from the tertiary branching to the bronchioles (5th branching) show no or little change in size at pressures up to 31.6 ATA. We also observed that when pressurizing, the major portion of the volume of water required was pumped into the chamber between 0 and 3 ATA gauge pressure. Since this is a measure of change in total body volume, which in turn is a function of the compressible portions of the body, it represents a decrease primarily in total lung volume. Alveolar volume is the major portion of this value; thus, the absorptive area of the lung must be considerably reduced at depths of less than 30 m. If we assume that there has been no

Table 2. Sizes of trachea and bronchi as determined from x-ray plates obtained at different pressures and taken at a constant position. ATA, atmospheres absolute.

Pressure (ATA)	Trachea lateral diameter (mm)	Bronchi-bronchioles branching level (mm)		
		3	4	5
	Leptonychotes weddelli			
1	17.3–22.0			1.4
6.4	15.6		1.9	1.3
11.2	11.6		1.8	1.2
31.6	9.0		1.8	1.1
	Mirounga angustirostris			
1	13.9	3.4–4.9	1.6	
6.4	10.4	3.8	1.7	1.0
11.8		3.2	2.3	1.7
31.6	6.3	4.0	1.9	0.9

resistance to compression at pressures as great as 31 ATA, then there is less than 100 cm^3 of gas distributed throughout the respiratory tree. Once such high pressures are achieved, continued increases in pressure require only slight decreases in total gas volume for equilibration. Consequently, only small changes in configuration of the tracheal and bronchial lumens are necessary if the seal dives even deeper.

Based on our results the sequence of events within the respiratory system during deep diving in these two species of seals is: (i) considerable decrease in size of the alveoli at pressures less than 4 ATA, (ii) beginning compression of the trachea at less than 6 ATA pressure, but not fully collapsed at 31 ATA, (iii) no measurable change in size of the bronchi and bronchioles at 31 ATA.

An anatomical comparison of these various structures provides us with a clue to the reasons for this sequence of compression. Unlike those of terrestrial mammals, the structurally weak alveoli of seals are enclosed in a much more flexible rib cage. Also, the trachea in these two species, particularly *Leptonychotes*, is remarkably compressible. In contrast, the bronchial tree of marine mammals is more rigid than that of land mammals as a result of a more extensive distribution of cartilage (*9*). Apparently, the end result is a bronchial tree less compressible than either the alveoli or trachea, which consequently retains its size and is the recipient of any gases that may be squeezed out of the other structures as they become smaller.

In view of the pressures involved, it seems important that most of the gases of the respiratory system are contained in slow or nonabsorptive areas, otherwise they would be quickly taken up by the blood and be a liability to the seal upon rapid ascent. Also, if the gases were absorbed their value as a space filler for pressure compensation would be lost. Furthermore, as long as the trachea remains open it is able to function in sound production, a feature that may be of some value at great depths. Especially pertinent with regard to this latter point is the work of Piérard (*10*) whose anatomical evidence for Weddell seals indicates that sound production is a result of air moving between the trachea and larynx, and vibratory movements of the rostral portion of a gas-filled trachea. Another experiment where helium was substituted for nitrogen resulted in a frequency shift of the major harmonics of the underwater vocalizations of a California sea lion, *Zalophus californianus*, which demonstrates that a gas-filled portion of the respiratory system is also necessary for sound production in this species (*11*). Piérard (*10*) further observed that the structures important for closure of the glottis are remarkably well developed. If the larynx and upper portion of the trachea do function as a device for underwater sound production then we believe that tight closure of the glottis would be important in preventing air from escaping into the buccal cavity and possibly being lost completely.

It is also noteworthy that these two species of seal do not have cranial air sinuses. This must be a considerable asset, since in humans these cavities are easily occluded and impose depth limitations. Finally, the apparent ability of the middle ear of seals to be reduced in air volume by the expansion of venous sinuses lining it (*12*) suggests that there need be no communication between the middle ear and the respiratory system for pressure equilibration. This would further simplify volumetric compensation of gases during deep dives because congested communication passages, such as the eustachian tube, would be of no consequence.

G. L. KOOYMAN, D. D. HAMMOND
J. P. SCHROEDER
Physiological Research Laboratory, Scripps Institution of Oceanography, University of California, San Diego, La Jolla 92037

References and Notes

1. P. F. Scholander, in *Handbook of Physiology*, D. B. Dill, E. F. Adolph, C. G. Wilber, Eds. (American Physiological Society, Washington, D.C., 1964), p. 729; G. L. Kooyman and H. T. Andersen, in *The Biology of Marine Mammals*, H. T. Andersen, Ed. (Academic Press, New York, 1969), p. 65.
2. R. J. Harrison and J. D. W. Tomlinson, *Mammalia* **24**, 386 (1960).
3. S. H. Ridgway, B. L. Scronce, J. Kanwisher, *Science* **166**, 1651 (1969).
4. G. L. Kooyman, *ibid.* **151**, 1553 (1966).
5. ———, *Sci. Amer.* **221**, No. 2, 100 (1969).
6. S. A. Nadel, W. G. Wolfe, P. D. Graf, *Invest. Radiol.* **3**, 229 (1968).
7. P. F. Scholander, *Hvålradets Skrifter, Norske Videnskaps Akad., Oslo* **22** (1940); G. L. Kooyman, *Antarctic Res. Ser.* **11**, 227 (1968).
8. L. Irving, in *Handbook of Physiology*, W. O. Fenn and H. Rahn, Eds. (American Physiological Society, Washington, D.C., 1964), vol. 1, p. 177; H. T. Andersen, *Physiol. Rev.* **46**, 212 (1966).
9. L. F. Belanger, *Amer. J. Anat.* **67**, 437 (1940).
10. J. Piérard, *Can. J. Zool.* **47**, 77 (1969).
11. R. W. Brauer, R. A. Jennings, T. C. Poulter, *Proc. 3rd Annual Conf. on Biol. Sonar and Diving Mammals* (Stanford Research Institute, Menlo Park, 1966), p. 68.
12. A. Denker, *Vergleichend-Anatomische Untersuchungen über das Gehörorgan der Säugethiere* (Verlag Von Veit, Leipzig, 1899), p. 1.
13. Supported by NSF grant GA 4038 to G. L. Kooyman, NIH grant 10521 to P. F. Scholander, and NIH grant HE 08323 to R. Elsner. We thank P. F. Scholander and W. M. Hanafee for support and advice and for reviewing the manuscript; J. H. Grollman and P. Campbell for taking and interpreting the radiograms; H. Hill for assistance in design and construction of the pressure chamber; and B. E. Baron, D. J. Foster, D. H. Kerem, and J. J. Wright for assistance during the experiments; and the California Department of Fish and Game for permission to use the elephant seal in this experiment.

1 April 1970

Vacuolar Perfusion Technique for Nitella Internodal Cells

Abstract. *A method has been developed for continuous control of the vacuolar composition of the giant internodal cells of the alga* Nitella. *During perfusion of the vacuole, cyclosis and spontaneous action potentials were evident, while the membrane potential and resistance were 86 and 58 percent of normal, respectively. The membrane system remained intact and functional during perfusion.*

Our present understanding of the function of natural membranes owes much to the large, convenient internodal cells of the freshwater Characeae, particularly *Nitella* (*1*). Recent investigations into metabolically coupled transport, electroosmosis, and current-voltage relationships have produced a large amount of data concerning membrane transport and behavior in these algal cells. The new data have raised new questions which, together with some older unresolved problems, would seem to assure the future use of *Nitella* in membrane research. In view of this situation it would be desirable to be