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.

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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.

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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

able to control vacuolar composition, and an intracellular perfusion technique for these cells which did not destroy normal membrane function would be of value.

The success of Blinks (*2*) with continuous vacuolar perfusion of *Valonia* and *Halicystis* was never repeated with the more delicate *Nitella* cells. Tazawa (*3*) reported exchanging the vacuolar contents of *Nitella* by tying off a segment of a cell under perfusion and allowing the segment to recover, but this procedure permits only a single change of composition per cell. Alternatively, continuous perfusion of the vacuole of *Nitella* with glass cannulae, while it allows several changes of composition, also presents some unique difficulties. These arise, in spite of a tough cell wall, because slight mechanical deformations readily induce collapse and permanent loss of turgor. I now describe a successful method and report electrical parameters and activity of *Nitella* under continuous-flow perfusion.

The technique consisted of removing the ends of a cell of *Nitella clavata* and inserting glass perfusion pipettes (150 μm, tip diameter) into either end. Two major problems belie the apparent simplicity of this approach: (i) how to make the cell wall–pipette junction leak-proof under pressure; and (ii) how to prevent collapse of the cell when severing the ends.

Many attempts to prevent leakage around the cell-pipette junction (for example, by ligature) were unsatisfactory, but I discovered a simple solution. After insertion of the pipette the cell wall would adhere naturally to the glass shoulder if allowed to dry. Careful blotting of the junction with fine pieces of filter paper ensured a rapid, firm adhesion to the glass. This natural seal was water tight and so tenacious that the cell wall had to be scraped from the glass at the end of an experiment if the pipette were to be used again. It withstood pressure to the extent that it occasionally forced leaks at weaker physical junctions of the gravity-fed perfusion system.

The loss of turgor was avoided with the following procedures. Cells 2 to 3.5 cm long were mounted in a Lucite chamber consisting of three pools with a Vaseline-filled groove in the partitions and a Lucite cover which was sealed over the grooves (Fig. 1). Once the cell was in the grooves and the cover in place, a microelectrode was inserted well into the vacuole for electrical measurements, and the cytoplasm was

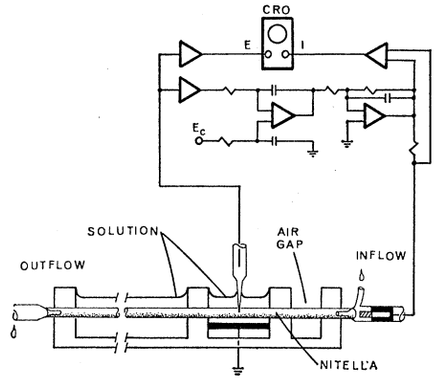

Fig. 1. Cross section of the experimental chamber with perfusion pipettes in place. The central pool was grounded and both inflow and outflow systems had regulating valves. The left-hand pool was large to accommodate the major portion of the cell. The voltage-clamp circuit employed electrometers and operational amplifiers, and the dual beam oscilloscope allowed display of both membrane current and potential. E_c is the command potential.

allowed to seal around the insertion. If a cell were immersed in solution when the ends were severed, the cell collapsed with an outrush of contents. However, if the cell were "dry mounted" (that is, with the pools empty) the ends of the cell could be removed without collapse and loss of contents. This is in accord with the observations of Tazawa (*3*). Thus, with the cell secure in the chamber, all of the pools were rapidly drained and one of the protruding ends was removed with iridectomy shears. The inflow pipette was quickly introduced into the open end with a micromanipulator until the cell fitted tightly around the glass shoulder. The cell-pipette junction was blotted and rapidly dried. The far end of the cell was then cut off, the outflow pipette was inserted, and the junction was dried. Flow was begun as gently as possible by carefully applying a slight pressure through the perfusion system, and only then were the central and left-hand pools of the chamber flooded. The right-hand pool was left empty as a high-resistance air gap for voltage clamping. Initial flow rates of 50 to 100 μm/sec (linear velocity down the vacuole) were gradually increased to 100 to 200 μm/sec. A 3.5-cm cell would thus experience one complete exchange of solution about every 3 to 6 minutes.

The object of the first series of experiments was simply to determine whether the preparation would function and, accordingly, conditions were chosen to be as nearly normal as possible. The perfusion solution was made to duplicate the natural vacuolar composition of *Nitella clavata* (*4*). This artificial vacuolar solution (AVS) contained 31.0 m*M* NaCl + 77.0 m*M* KCl, and the bathing solution, an artificial pond water (APW), contained 1.0 m*M* NaCl + 0.1 m*M* KCl + 0.1 m*M* $CaCl_2$. When experience with the technique

Table 1. Properties of perfused *Nitella* cells. The membrane potential, E_r, and the membrane resistance, R_m, are given along with the time during which E_r remained relatively constant and/or cyclosis was evident. The composition of APW and AVS is given in the text. Both the Na_2SO_4 and K_2SO_4 solutions were 0.05*M*. The presence of spontaneous action potentials (*) and cyclosis (+) are also noted.

Cell	Time (min)	R_m (kohm cm²)	E_r (mv)	Solution composition		Activity	
				Bath	Vacuole		
			Continuous flow				
1	90			APW	AVS	+	
2	45		− 80	APW	AVS	+	*
3	15		−130	APW	AVS	+	
4	30		−110	APW	AVS	+	
5	20		−105	APW	AVS		*
6	60	15	−105	APW	AVS	+	*
7	60	2	− 75	APW	AVS	+	*
8	70	26	−124	APW	AVS	+	*
			Flow stopped				
9	15		−120	APW	AVS	+	
10	30		−115	APW	AVS	+	*
11	30	6.3	− 60	APW	AVS	+	
			Solutions changed				
6	25		− 18	Na_2SO_4	Na_2SO_4		*
7	30		− 50	Na_2SO_4	Na_2SO_4	+	*
11	15		− 19	Na_2SO_4	Na_2SO_4	+	
12	15		− 70	APW	K_2SO_4		
12	15		− 20	K_2SO_4	K_2SO_4		
13	20		− 60	APW	Na_2SO_4	+	
13	15		− 30	Na_2SO_4	Na_2SO_4	+	
14	45		− 75	Na_2SO_4	Na_2SO_4		
15	25		0	APW	APW		

permitted a higher frequency of functioning cannulations, the composition of the perfusion solution was changed in several trials.

The success of the technique depended upon the retention of normal membrane function. With regard to electrical properties, this was achieved. The rate of cyclosis was measured in each cell before perfusion and only cells with rates in excess of 50 μm/sec were used. If the cell began to show physiological deterioration during perfusion, either through irreversible depolarization or loss of chloroplasts, it was discarded. Of the 15 cells upon which measurements were made, 11 exhibited cyclosis during perfusion, eight displayed spontaneous action potentials, and in six cells both were observed (Table 1). The presence of cyclosis would argue for an intact tonoplast, and since the origin of the action potential in *Nitella* has been established as the plasmalemma, the six cells exhibiting both activities would represent preparations with both membranes intact. Furthermore, cyclosis is immediately arrested in normal cells when an action potential occurs and, if this is associated with the increased ionic permeability of the plasmalemma during activity, cyclosis may indicate that the normal permeability of the plasmalemma was not drastically altered by perfusion.

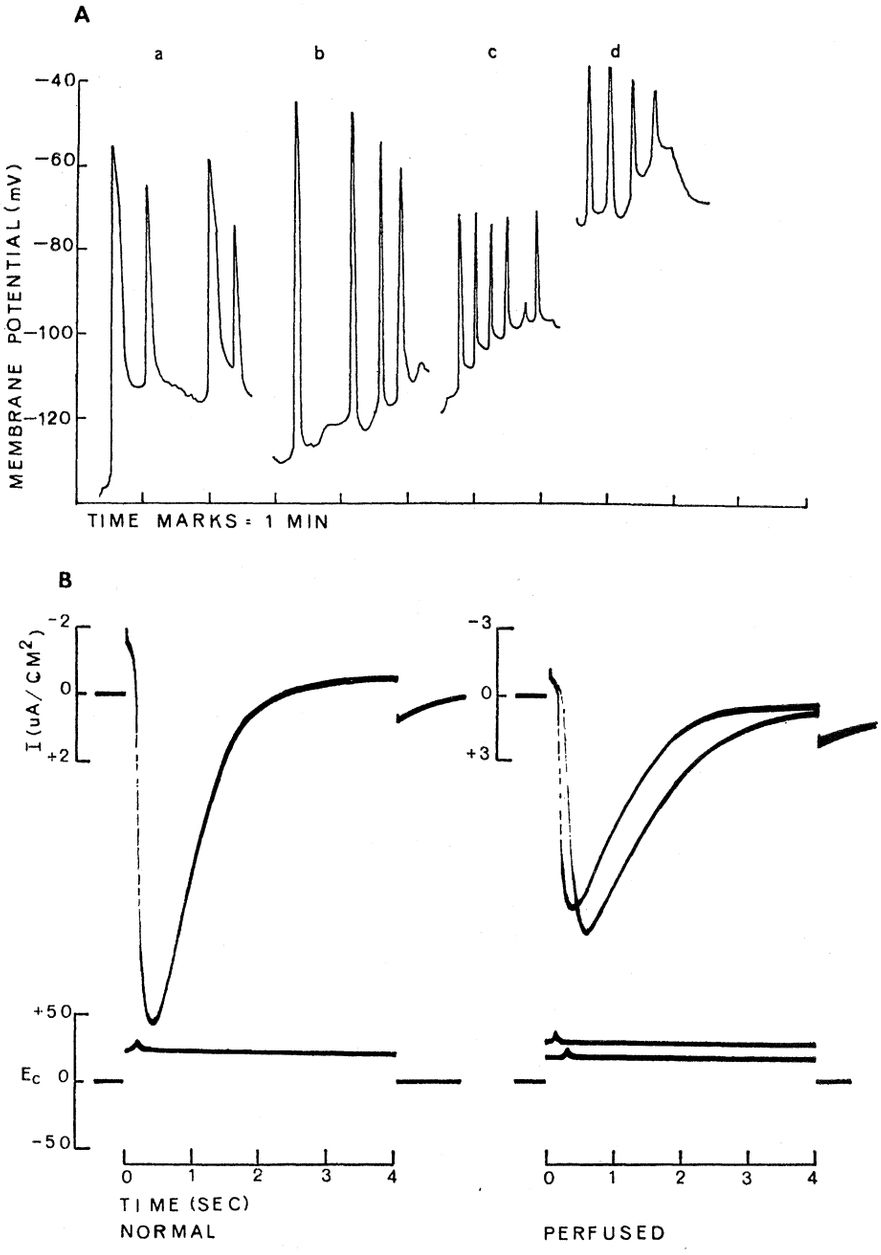

Fig. 2. (A) Examples of spontaneous electrical activity from four different *Nitella* cells. Cells a and c are normal, nonperfused preparations, cell b was perfused with AVS, and cell d was perfused with 0.05*M* Na_2SO_4. All four cells were bathed in APW. (B) Upper trace: membrane current during voltage clamp of induced action potentials in normal and perfused cells (note dissimilar current scales). Lower trace: membrane potential during step depolarizations. The two recordings are from two different cells.

The average resting potential (± standard error of the mean) of seven cells under continuous perfusion with AVS was −104 ± 7 mv, whereas the value from six normal cells was −121 ± 6 mv. The membrane resistance of three cells measured under continuous perfusion was 14.3 ± 5 kohm cm^2 and compares with 24.8 ± 0.9 kohm cm^2 averaged from 20 cells. This last value includes 14 cells of the same clone but from a subculture of slightly younger cells. The time during which both the membrane potential remained relatively constant and cyclosis was present averaged 37 minutes, lasting only 15 minutes in two cells and lasting 1 hour or more in four others (Table 1). In three cells flow was stopped after some 15 minutes of perfusion by shutting off the outflow. Under a hydrostatic head of 50 cm of water, the resting potential in two continued for 30 minutes more with no deterioration, while one cell slowly depolarized.

In three cells, initially perfused with AVS and bathed in APW, both solutions were changed to 0.05*M* Na_2SO_4 to observe the effects of an absence of Cl^- upon electrical activity and also the effects of identical solutions in the vacuole and bath upon the resting potential. The membrane potential decreased by an average of 61 percent, but two cells continued to show action potentials under these conditions. However, with the same 0.05*M* K_2SO_4 solutions or with APW solutions on both sides of the membrane system, depolarization was almost complete, and both cyclosis and firing were arrested. The mean resting potential, with identical solutions in the vacuole and bath, was −30 ± 8.8 mv.

No consistent differences in duration, amplitude, or frequency of spikes could be established between preparations of perfused and normal cells (Fig. 2A). For example, given a normal cell a and a perfused cell b with spikes of comparable amplitude, it would appear that the duration of spikes is longer and the frequency lower

in the normal preparation. However, an examination of cell c (normal) and d (perfused) would indicate that the reverse was true.

The membrane potential of perfused and normal cells was held constant during excitation while the membrane current was measured with the circuit shown in Fig. 1. In perfused cells, the inflow pipette contained a platinized silver chloride electrode which was used to pass current down the vacuole. Initially under these conditions, I was unable to confirm any marked difference in the time course of the membrane current between the two. There does appear to be a delay in the outward current from perfused cells, but the data are not sufficient to warrant firm conclusions (Fig. 2B). The vacuolar composition of *Nitella* can be continuously controlled without detriment to either the tonoplast or the plasmalemma.

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References and Notes

1. K. S. Cole, *Membranes, Ions, and Impulses* (Univ. of California Press, Berkeley, 1968), pp. 51–158.
2. L. R. Blinks, *J. Gen. Physiol.* **18**, 409 (1935).
3. M. Tazawa, *Plant Cell Physiol.* **5**, 33 (1964).
4. C. E. Barr and T. C. Broyer, *Plant Physiol.* **39**, 48 (1964); C. E. Barr, *J. Gen. Physiol.* **49**, 181 (1965).

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DNA Polymerase Activities Associated with Smooth Membranes and Ribosomes from Rat Liver and Hepatoma Cytoplasm

Abstract. *Deoxyribonucleic acid polymerase activity that prefers denatured DNA primer concentrates with the smooth membranes during sucrose gradient centrifugation of rat liver and hepatoma cytoplasmic extracts. The activity in this fraction is eightfold higher in hepatoma tissue. Deoxyribonucleic acid polymerase activity that prefers native DNA primer concentrates with the free ribosome fraction from both tissues.*

Most investigations of DNA polymerase in normal rat liver and hepatomas, with the exception of a few specific studies of nuclear (*1*) or mitochondrial (*2*) DNA polymerase, have utilized the postmicrosomal supernatant solution as the starting material for enzyme isolation. In general, these studies have shown that DNA polymerase activity present in the postmicrosomal supernatant solution of normal adult and regenerating rat liver has a preference for native DNA as primer and is of relatively low molecular weight (*3*). It has recently been reported that the postmicrosomal supernatant solutions from fetal rat liver and hepatomas also contain a high-molecular-weight DNA polymerase with a preference for denatured DNA primer (*4*). The results presented here show that the latter DNA polymerase activity can be isolated from the postmicrosomal supernatant solution of normal adult rat liver, as well as hepatoma tissue. This activity is associated with the smooth membrane fraction in both tissues, but the activity is higher in hepatoma. By contrast, the DNA polymerase activity that prefers native DNA primer is concentrated in the free ribosome fraction obtained from either liver or hepatoma.

Morris hepatoma 7777 (generation No. 55) was transplanted intramuscularly in male, Buffalo strain rats at Howard University, Washington, D.C., and the animals were shipped by air express. Normal (Holtzman) and tumor-bearing rats were permitted to feed ad libitum, but were fasted overnight before sacrifice. A 20 percent homogenate of the perfused liver or hepatoma was prepared in 0.25*M* sucrose-TKMM (0.05*M* tris-HCl, *p*H 7.5 at 20°C, 0.025*M* KCl, 0.005*M* $MgCl_2$, 0.001*M* 2-mercaptoethanol) as described by Blobel and Potter (*5*). Nuclei (P-1) and mitochondria (P-2) were removed by successive centrifugation at 600*g* for 10 minutes and 20,000*g* for 15 minutes. The postmitochondrial supernatant solution (S-2) was centrifuged at 105,000*g* for 1 hour to remove microsomes (P-3). The postmicrosomal supernatant solution (S-3) was centrifuged at 105,000*g* for 15 hours to assure complete sedimentation of the free ribosomes and smooth membranes and thereby to separate these particulates as completely as possible from soluble materials. The resultant solution (S-4) was removed by aspiration, and the pellet (P-4) was resuspended in TKMM buffer by ten strokes with a loose-fitting Dounce homogenizer. The free ribosome and smooth membrane fractions were separated in a discontinuous sucrose gradient (0.8 to 2.0*M* sucrose in TKMM buffer) prepared according to Murray *et al.* (*6*). Centrifugation was performed in the No. 30 rotor at 30,000 rev/min for 24 hours. The sucrose layers were carefully removed with a pipette and diluted with three volumes of TKMM buffer. The pellet (free ribosomes) was resuspended in TKMM buffer by ten strokes with a loose-fitting Dounce homogenizer. The resuspended pellet and the diluted sucrose layers were recentrifuged at 105,000*g* for 15 hours. The supernatant solutions (soluble material) were decanted and the free ribosome and membrane pellets were drained and resuspended in TKMM buffer. All procedures were carried out at 0° to 4°C.

Deoxyribonucleic acid polymerase activity in the postmicrosomal supernatant solution (S-3) of normal rat liver is low and has no preference for native or heat-denatured DNA as primer (Table 1). In contrast, S-3 from hepatoma tissue has a higher polymerase activity, and heat-denatured DNA is preferred as primer, as previously reported (*4*).

Centrifugation of the S-3 from either normal liver or hepatoma at 105,000*g* for 15 hours concentrates the denatured primer–preferring activity in the pellet (P-4). The specific activity with heat-denatured DNA primer remained considerably higher in the hepatoma preparation, whereas the specific activity with native DNA primer was equal in P-4 fractions isolated from liver and hepatoma tissue. The supernatant solution (S-4) from hepatoma exhibited higher polymerase activity with native DNA primer than did S-4 from liver.

Sucrose gradient centrifugation of resuspended P-4 from liver or hepatoma separated the DNA polymerase activity into three fractions: that associated with the free ribosomes which sedimented through the 2.0*M* sucrose layer, that with the membrane fraction that banded at the interface of the 0.8 to 1.3*M* sucrose layers and subsequently pelleted after dilution and recentrifugation for 15 hours, and that in the soluble fraction from the gradient that did not pellet after dilution and recentrifugation for 15 hours. Nearly all of the denatured primer–preferring polymerase activity in the P-4 fractions from liver or hepatoma tissue was concentrated with the membrane fraction after sucrose gradient centrifugation. However, the