

Temperature Dependence of Protein Transport across Lymphatic Endothelium In Vitro

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ABSTRACT The purpose of the work was to develop an in vitro model for the study of lymphatic endothelium and to determine, using this model, whether or not a cytoplasmic process may be involved in transendothelial transport. Segments of canine renal hilar lymphatics were dissected clean, cannulated at both ends, and transferred to a perfusion chamber for measurement of transendothelial protein transport and for ultrastructural tracer studies. The segments were subsequently processed for light and electron microscopy. By both structural and functional criteria the lymphatics were judged to have retained their integrity. At 37°C, 36 lymphatics showed a mean rate of protein transport of 3.51 ± 0.45 (SEM) $\mu\text{g}/\text{min}$ per cm^2 of lymphatic endothelium. The rate was influenced by the temperature of the system, being significantly reduced by $49\% \pm 4.8$, $31\% \pm 5.3$, and $29\% \pm 3.9$ when the temperature was lowered to 4°, 24°, and 30°C, respectively. When the temperature was raised to 40°C, the rate was significantly increased by $48\% \pm 12.2$. The vesicular system and the intercellular regions in vessels with increased or reduced rates of transport were analyzed quantitatively to ascertain whether the rate changes could be correlated with ultrastructurally demonstrable changes in either of these postulated pathways. No significant changes in junctional or vesicular parameters were found between the control lymphatics and those perfused at 24°, 30°, and 40°C. At 4°C, the temperature at which the rate of protein transport was maximally reduced, vesicular size decreased, and the number of free cytoplasmic vesicles increased, whereas the number associated with the abluminal and luminal surfaces decreased. We concluded that isolated perfused lymphatic segments transport protein at a relatively constant rate under control conditions, and that this transendothelial transport comprises both temperature-dependent and temperature-independent mechanisms. The findings were considered in terms of the different theories of lymph formation and were interpreted as providing support for the vesicular theory.

Lymph, by definition, is formed from that component of the interstitial fluid that moves across the endothelium of lymphatic vessels. However, the pathways taken and the forces that control this fluid movement are poorly understood and therefore have become the subjects of considerable controversy. According to one theory the major pathway lies between adjacent endothelial cells (11, 20, 21), and hydrostatic (16) or osmotic pressure (11, 12) provides the necessary force for fluid transport. Integral to this theory are the cyclic changes that occur because lymphatic vessels are alternately compressed and then relaxed within the tissues. For instance, if hydrostatic pressure is the major force, intraluminal pressure

must be greater than atmospheric at times, thus causing lymph to flow downstream, and less than atmospheric at other times, permitting fluid entry from the tissue spaces to the lumen. Comparable fluctuating differences in protein concentration, between capillary lymph and tissue fluid, have been proposed to satisfy the oncotic pressure theory (11, 12).

A second theory holds that cytoplasmic vesicles provide the major route for protein to traverse the endothelium (1, 15, 18, 29, 30). The oncotic gradient created by this protein transport is held responsible for further fluid flow between adjacent cells or through transendothelial channels. This theory does not invoke a cycling mechanism since vesicular

movement is continuous within the endothelium.

Evidence for either of these theories is unfortunately sparse because experiments pertaining to *in vivo* lymph formation are technically difficult to control. Direct evidence of a subatmospheric pressure in initial lymph capillaries is lacking; indeed, measurements in early mesenteric vessels reveal a value that is slightly positive (41). Similarly, unequivocal support for fluctuating protein concentrations in newly formed lymph does not exist; the evidence to date is controversial (12, 25, 39). The vesicular hypothesis receives support from structural experiments with tracers. Horseradish peroxidase (HRP),¹ for instance, when introduced into the general circulation, appears rapidly in cytoplasmic vesicles of lymphatic endothelium (45). Parallel evidence, both structural (7, 27) and functional (28), supports the view that vesicles subserve a transport function across blood vascular endothelium.

The major purpose of the present work lay in the preparation of a model in which lymphatic endothelial transport could be examined under controlled conditions. This model was then applied to study protein transport to analyze the influence of temperature. The reasoning was that if vesicular transport does occur, the rate of protein movement should be temperature dependent in keeping with the known effect of temperature on vesicles (3). Conversely, if protein moves by a noncytoplasmic process, such as diffusion between adjacent cells, then temperature should have comparatively little influence upon the rate. Briefly, these studies showed (a) that isolated, perfused lymphatic segments transport protein at a relatively constant rate under control conditions, and (b) that this transendothelial transport comprises both temperature-dependent and temperature-independent mechanisms.

Simultaneously, we assessed the structural integrity of these lymphatic segments and compared them with lymphatics that had been excised and fixed without perfusion. We also analyzed their interendothelial contacts and their vesicular system morphometrically. The purpose of these procedures was to learn whether this *in vitro* method results in damage to the endothelium and whether changes in the rate of protein transport are accompanied by ultrastructural changes in potential transport pathways.

MATERIALS AND METHODS

Segments of canine renal lymphatic vessels were cannulated at both ends and immediately transferred to a perfusion chamber. The rate of protein transport from the bathing fluid to the lumen was then assessed at different temperatures. The segments were subsequently prepared for ultrastructural analysis.

Preparation of Lymphatic Segments

19 mongrel dogs of either sex were anesthetized with sodium pentobarbital. The renal pedicle was exposed through a left loin incision, and segments of renal hilar lymphatics were dissected cleanly and cannulated at both ends with polyethylene tubing (PE10). The inflow cannulae were about 1 cm long and the outflow cannulae measured between 1.5 and 2.0 cm. Up to five segments, each ~5 mm long and free of tributaries, were prepared in sequence from each animal. The contralateral renal pedicle was then exposed, and right lymphatic segments were prepared in the same manner. On both sides, only one segment at a time was dissected so that the remaining lymphatics, to be used for later cannulations, were left as undisturbed as possible. After cannulation, each segment was transferred to a temperature-controlled perfusion chamber, which contained Dulbecco's modified Eagle's medium and which was mounted on a microscope base between two micromanipulators. The inflow cannula was coupled to a syringe pump (Sage Instruments Div., Cambridge, MA), and perfusion with Dulbecco's medium started at 2 μ l/min. The perfusate was collected into 5- μ l pipettes by capillary action from the end of the outflow

cannula. Whenever a perfusion difficulty became apparent, the lymphatic segment was discarded. The average time between the start of the dissection of a vessel and the onset of its perfusion in the chamber was ~30 min.

Lymphatic Perfusion In Vitro

In all the lymphatics sampled in this study, the rate of protein transport across the vessel wall was measured in a stop-flow mode in order to exclude the influence of hydrostatic pressure. The protein concentration (BSA) of the bathing fluid was set at 30 mg/ml since this approximates the concentration in tissue fluid, whereas the perfusion fluid was devoid of protein. At the beginning of perfusion the first 10- μ l sample was discarded because it contained some lymph and, therefore, protein that had remained within the vessel during cannulation. Thereafter, five or six 10- μ l samples, each consisting of two 5- μ l aliquots, were collected from the outflow cannula into a capillary pipette. In each experiment the first two or three samples were obtained at 37°C and were recorded as controls. The subsequent three samples were collected when the temperature of the chamber had been altered. Temperature control was achieved by thermostatic regulation of a heating element built into the base of the perfusion chamber. The bathing fluid was stirred and oxygenated by gentle bubbling with 95% O₂, and the pH of the medium was monitored.

Each sample was collected in the following manner. Once the perfusion pattern showed the preparation to be satisfactory, and the first 10- μ l sample was discarded, perfusion was discontinued for 5 min. Perfusion was then restarted until exactly 5 μ l had been collected from the outflow cannula. Perfusion was once again shut off for 5 min before the second 5- μ l aliquot was collected. Thus, each aliquot comprised a fluid sample that had occupied the outflow cannula, the lymphatic, and a variable portion of the inflow cannula throughout the stop-flow period. In all, 36 lymphatic segments were investigated. Of these, five were studied at 4°C, six at 24°C, nine at 30°C, six at 37°C (i.e., at 37°C for both the control and experimental samples), and 10 at 40°C.

The duration of each experiment from the beginning of perfusion to subsequent fixation was approximately 90 min.

Collection and Analysis of Data

Protein concentrations were measured by the Bio-Rad colorimetric assay. This test is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs (Bio-Rad Laboratories, Richmond, CA). The protein content of each collection sample was determined and represented the amount of protein that had crossed the lymphatic wall from bath to lumen during the two 5-min stop-flow periods. The surface area of the lymphatic segment was calculated from its length and its circumference. The length was measured prior to fixation, and the circumference was determined from 1- μ m thick sections using a Zeiss Videoplan image analyzer. Thus the rate of protein transport across the endothelial wall under control and experimental conditions was calculated in μ g/min per cm² of endothelium. Statistical analysis was carried out by the paired Student's *t* test for each group of vessels.

Tissue Processing for Electron Microscopy

Immediately after each perfusion was complete, the bathing fluid was quickly changed to cold (4°C) Karnovsky's fixative (19). The vessels were then removed and further fixed for 1–2 h. Thereafter they were postfixed for 30 min at room temperature in 1% OsO₄ in 0.15 M HCl-Na cacodylate buffer, pH 7.4, and dehydrated in graded acetone. All vessels were transversely oriented and embedded in Epon 812. Thin (60 nm) sections were cut with a diamond knife and collected on single-hole grids. In this way the complete perimeter of the lymphatic could be studied, and, since all vessels were cut at right angles to their long axis, comparison among lymphatics could be made. Sections were stained with uranyl acetate and lead citrate and examined with an Hitachi 600 transmission electron microscope operated at 75 kV (Hitachi America, Ltd., NY).

In Situ Controls

To determine whether perfusion altered the structure of the vessels, we excised four additional lymphatics from three dogs without ligation or perfusion and immediately processed them, as described above: these segments were designated as *in situ* controls.

HRP Experiments

HRP was used as a structural tracer in four additional lymphatics, on the assumption that this marker might reveal the pathways taken by transported

¹ Abbreviations used in this paper: HRP, horseradish peroxidase.

protein. These four lymphatic segments were cannulated and perfused in vitro in a routine manner. The bathing fluid was then changed such that it contained HRP (30 mg/ml) without any change in the total fluid protein concentration. After 10 min the HRP-containing fluid was abruptly changed for Karnovsky's fixative (4°C) and the lymphatic was further fixed for 12–15 h. The HRP reaction was achieved with diaminobenzidine and the tissue was then processed for electron microscopy as described above: sections were stained with bismuth. Control reactions were carried out on comparable lymphatic segments perfused in the absence of HRP.

Morphometry

ENDOCYTOTIC VESICULAR SYSTEM: 10 electron micrographs of randomly selected areas from each lymphatic segment were printed at a standard magnification. From these micrographs the diameter (D) and the numerical (N_v) and volume (V_v) densities of the endothelial vesicles were determined with an image analyzer (Zeiss Videoplan). The position of the vesicles, as they appeared in the electron micrographs, was tabulated. Thus, luminal or abluminal vesicles were so designated when they were seen to touch or open on the surface. The rest were classified as cytoplasmic although it was evident that many, or even all, could have connected to the surface through channels absent from the plane of section.

INTERENDOTHELIAL CONTACTS: The frequency of the three forms of intercellular contacts—end-to-end, overlapping, and interdigitating—as well as the incidence of so-called “open” (>30 nm wide) junctions (11, 20, 21) was manually recorded as the perimeter of each vessel was scanned with the electron microscope. Each contact was further characterized by whether it revealed a specialized form of junction. In most instances the particular type of junction was not determined because it was not obvious at the level of magnification used to scan the vessel. Where the presence or absence of a junction was not evident, because of the plane of section, the junctional status was recorded as “unknown.” The size of the intercellular space in 25 randomly selected contacts from each lymphatic was quantified in two ways: (1) the width of the intercellular space at three nonjunctional points was measured using the Zeiss Videoplan and (2) the presence of any dilatations within the contact region was recorded. Statistical analysis of these data included both an F -test of the equality of variance and a Student's t test.

RESULTS

Integrity of Perfused Lymphatics

The initial question to be answered was whether the lymphatic endothelium retained its structural and functional integrity for the extent of each experiment, which was approximately 2 h from the start of the dissection to the end of the perfusion. Support for its sustained integrity was drawn from the comparison between perfused lymphatics and those control (in situ) lymphatics that had been fixed immediately upon removal from the animal. No qualitative differences were observed between these two. Some quantitative differences, however, were present in the data that pertained to vesicles and intercellular regions, though none were found to be significant. Such differences were not considered to be surprising since cells may be expected to respond in some way to the changes that exist between an in vivo and an in vitro environment. Because the procedural circumstances were identical for all perfused lymphatics, those lymphatics perfused at different temperatures were compared statistically to the perfusion controls rather than to the in situ ones. Examples of in situ and perfusion controls as well as vessels perfused at 4° and 40°C are illustrated in Figs. 1, 2, 3, and 4, respectively. In all instances the intima consisted of an unbroken endothelium displaying ultrastructural features now recognized as characteristic of lymphatic endothelial cells (1, 2, 20, 37, 39), including an attenuated basal lamina.

A second important source of evidence for endothelial integrity came from the HRP experiments. This protein, similar in molecular size and weight to albumin, when added to the bathing fluid, entered the endothelial vesicular system (Fig. 5). In these perfused lymphatics the tracer was confined

to the vesicular system and to the intercellular channels. It occupied the luminal as well as cytoplasmic and abluminal vesicles and clothed the luminal membrane (Fig. 5) thereby suggesting that it crossed the endothelial lining of perfused lymphatics in the vesicular system. This appearance is in direct contrast to that seen in endothelial cells that have lost their viability, in which the HRP pervades the cytoplasm in a broad and indiscriminate way.

The third line of evidence was more functional in nature. It stemmed from the control experiments where all the samples were obtained at 37°C. The rate of protein transport was consistent for ~90 min of perfusion in each of these lymphatics (Fig. 6). No change in transport rate occurred with time, as might have been expected if the lymphatic segments had degenerated during the experiments.

Effect of Temperature on Protein Transport

Details pertaining to the perfused lymphatic segments are shown in Table I. On average, these segments measured ~7 mm long and 0.33 mm diam. The mean time taken for each 5- μ l sample to be collected was ~2.3 min. Control values for the rate of protein transport at 37°C were generally consistent within individual experiments as well as among experiments. The mean and control for all the 36 experiments was 3.51 ± 0.45 (SEM) μ g/min per cm^2 of lymphatic endothelium.

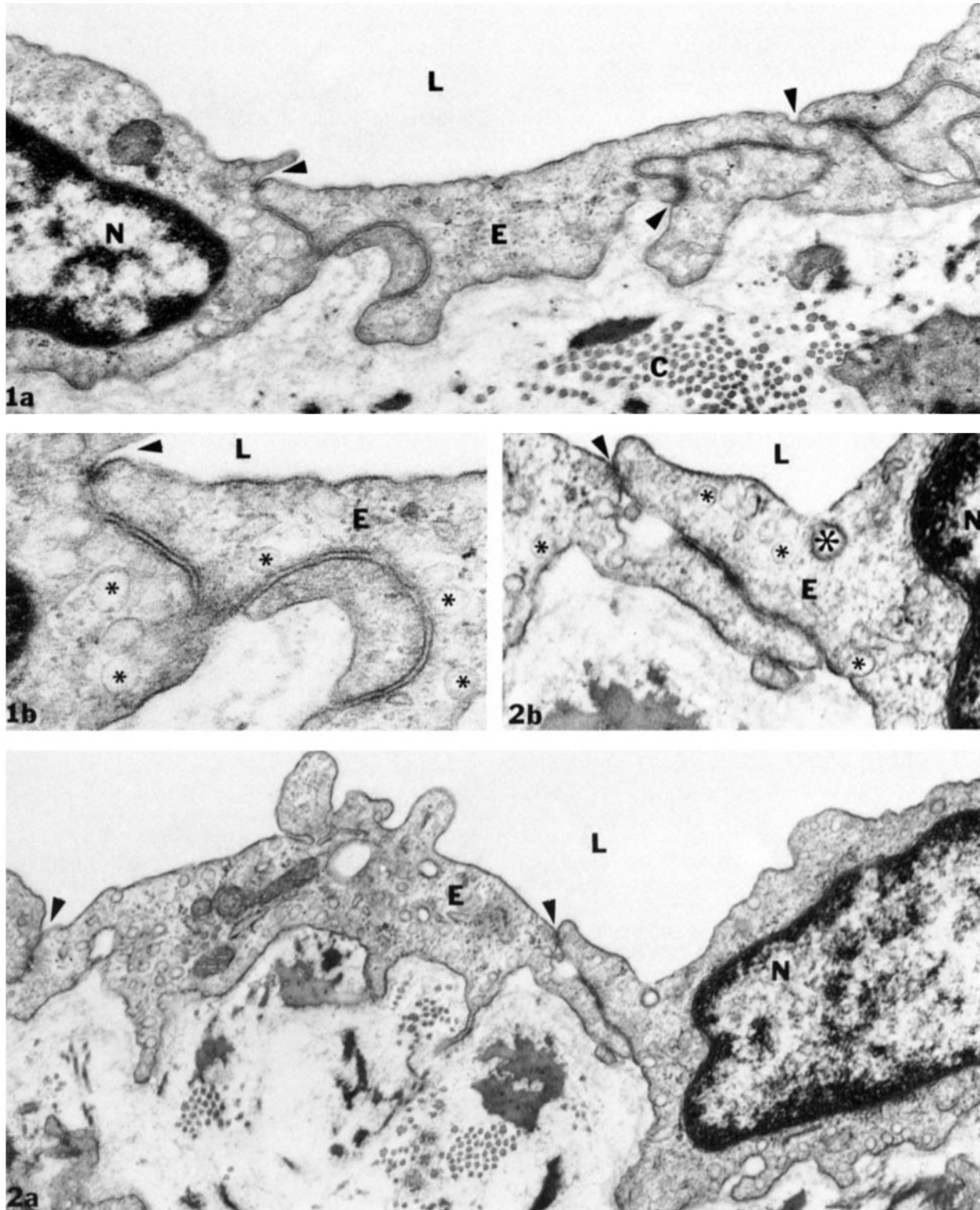
Figs. 7 and 8 and Table II illustrate the effect of temperature upon the rate of protein movement into the lymphatic lumen. Fig. 7 reveals the rate reduction in a representative experiment when the temperature was cooled to 4°C. By contrast, Fig. 8 shows an increase in the rate when the temperature was raised to 40°C in a comparable experiment.

Table II summarizes the effects of temperature in all the groups. Comparison of the means before and after change in temperature does not reflect the real effect on rate because of variation in base values among experiments. However, the percentage change in each experiment reveals a striking temperature effect. The rate of protein entry was decreased by $49\% \pm 4.8$, $31\% \pm 5.3$, and $29\% \pm 3.9$ at 4°, 24°, and 30°C, respectively. No change was evident at 37°C ($1.7\% \pm 10.4$). A rise in temperature to 40°C was accompanied by a $48\% \pm 12.2$ increase in the rate of protein entry. When evaluated by the paired Student t test, all changes were significant (Table II).

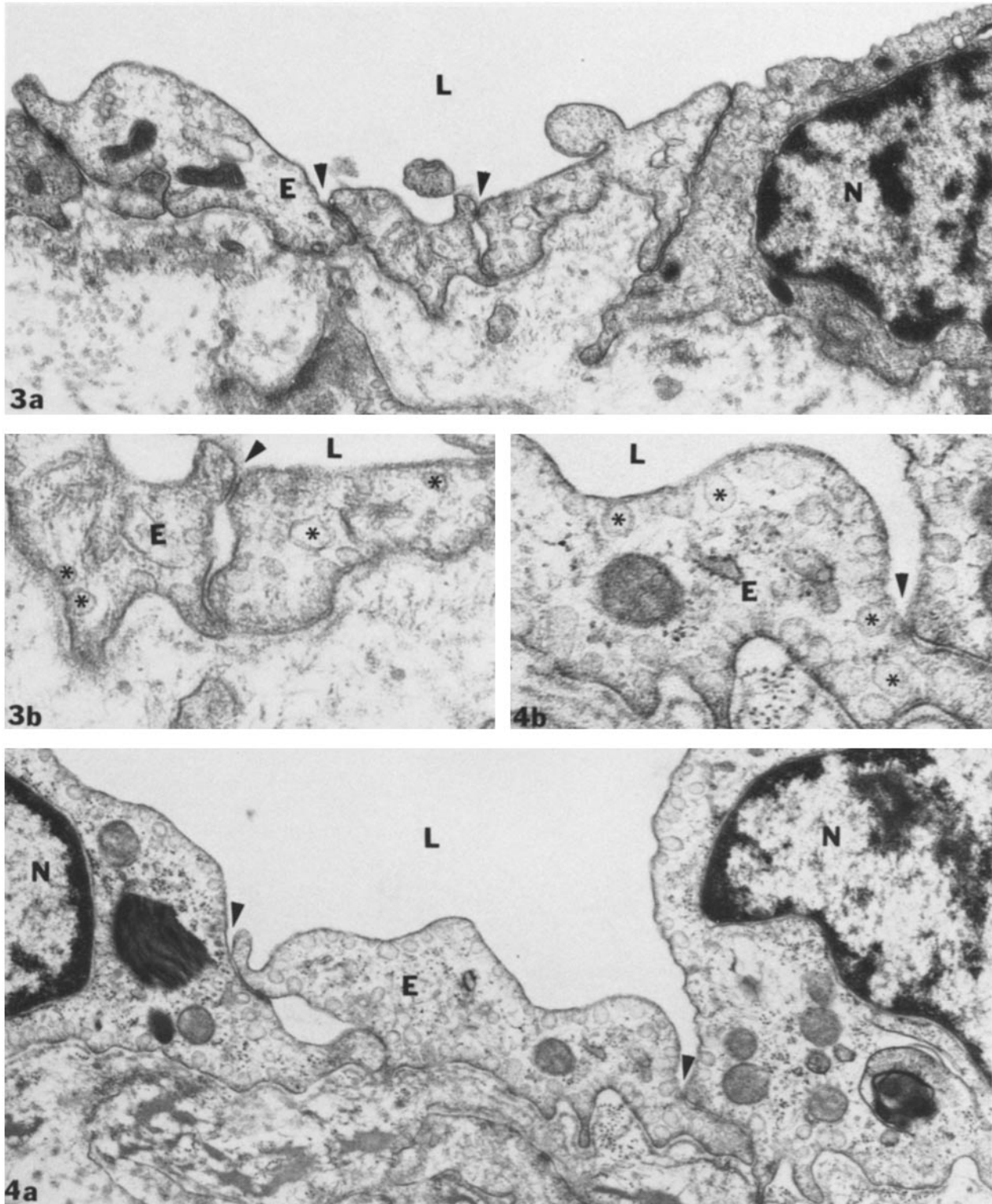
Endocytotic Vesicular System

The lymphatic endothelium contained endocytotic vesicles of both the coated and uncoated types. The latter were small and numerous and bounded by a simple unit membrane (b in Figs. 1–4, small asterisks); the former were larger, bore a fuzzy coating on their cytoplasmic aspect, and were quite rare (Fig. 2*b*; large asterisk). The coated vesicles were not studied further.

DISTRIBUTION: Data on the apparent distribution of the endocytotic vesicles are shown in Table III. In both control lymphatic groups, about a quarter of the vesicles appeared to be free within the endothelial cytoplasm. The rest were equally distributed between the luminal and abluminal surfaces. Lymphatics warmed to 40°C exhibited a similar disposition, as did those cooled to either 30° or 24°C. In those cooled to 4°C, however, the distribution of vesicles revealed a rearrangement. Those designated as abluminal and luminal decreased, from 35 to 27% ($p < 0.05$) and from 33 to 27% ($p < 0.10$),



FIGURES 1 and 2 1a: Portion of the endothelium of an in situ control lymphatic. Parts of several endothelial cells joined by overlapping and interdigitating junctions (arrowheads) are shown. *L*, lymphatic lumen; *E*, endothelium; *N*, endothelial cell nucleus. (Abbreviations are the same in all figures.) $\times 30,000$. 1b: Higher magnification of the same lymphatic at the juncture of two cells (arrowhead). Uncoated (small asterisks) vesicles are present. $\times 60,000$. 2a: Portion of perfusion control lymphatic, demonstrating the structural integrity of the endothelium. Several cells, joined by overlapping junctions (arrowheads), are shown. $\times 30,000$. 2b: Higher magnification of the same lymphatic exhibiting numerous uncoated vesicles (small asterisks) and a coated vesicle (large asterisk) and a portion of an intercellular junction (arrowhead). $\times 60,000$.



FIGURES 3 and 4 3a: Portions of the endothelium from a lymphatic perfused at 4°C and demonstrating the structural integrity of the endothelium. Arrowheads indicate intercellular junctions. $\times 30,000$. 3b: Higher magnification of the same lymphatic at the junction of two cells (arrowhead). Several uncoated vesicles are present (asterisks). $\times 60,000$. 4a: Portions of the endothelium from a lymphatic perfused at 40°C and demonstrating the structural integrity of the endothelium. Arrowheads indicate intercellular junctions. $\times 30,000$. 4b: Higher magnification of the same lymphatic at the junction of two cells (arrowhead). Several uncoated vesicles are present (asterisks). $\times 60,000$.

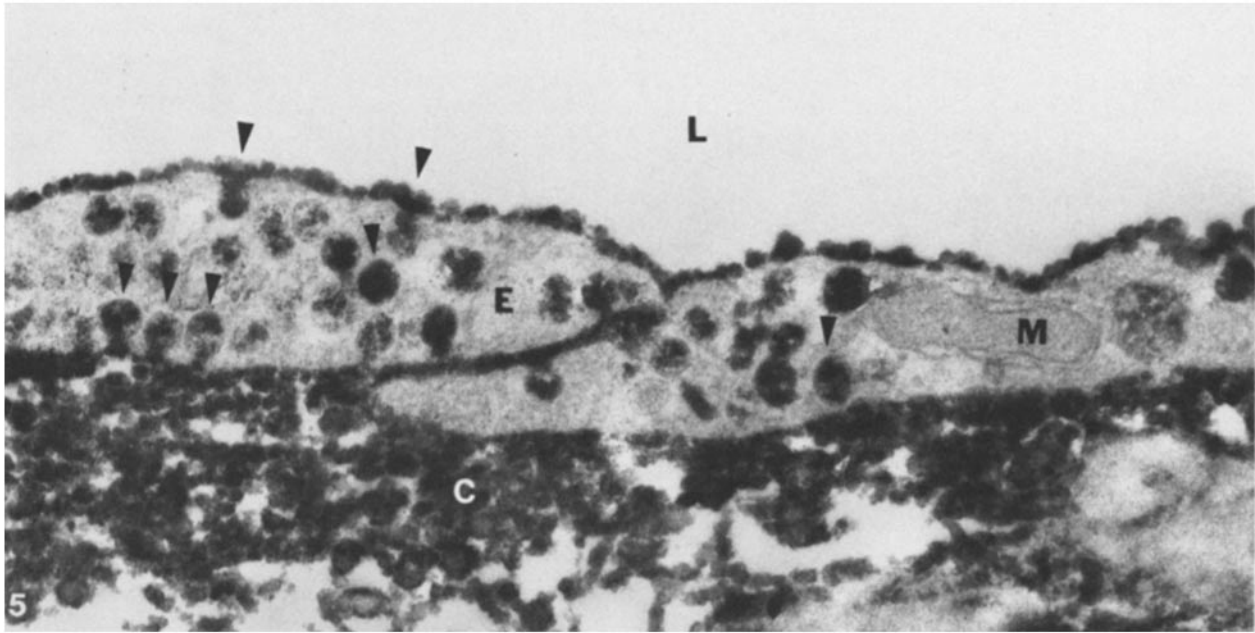


FIGURE 5 Electron micrograph of part of the endothelium of a lymphatic segment perfused in vitro. HRP was added to the bathing fluid for 10 min before fixation. The HRP can be seen in the subendothelial connective tissue, abluminal, cytoplasmic, and luminal vesicles (small arrowheads) and decorating the luminal membrane. *M*, mitochondrion; *C*, collagen. $\times 72,000$.

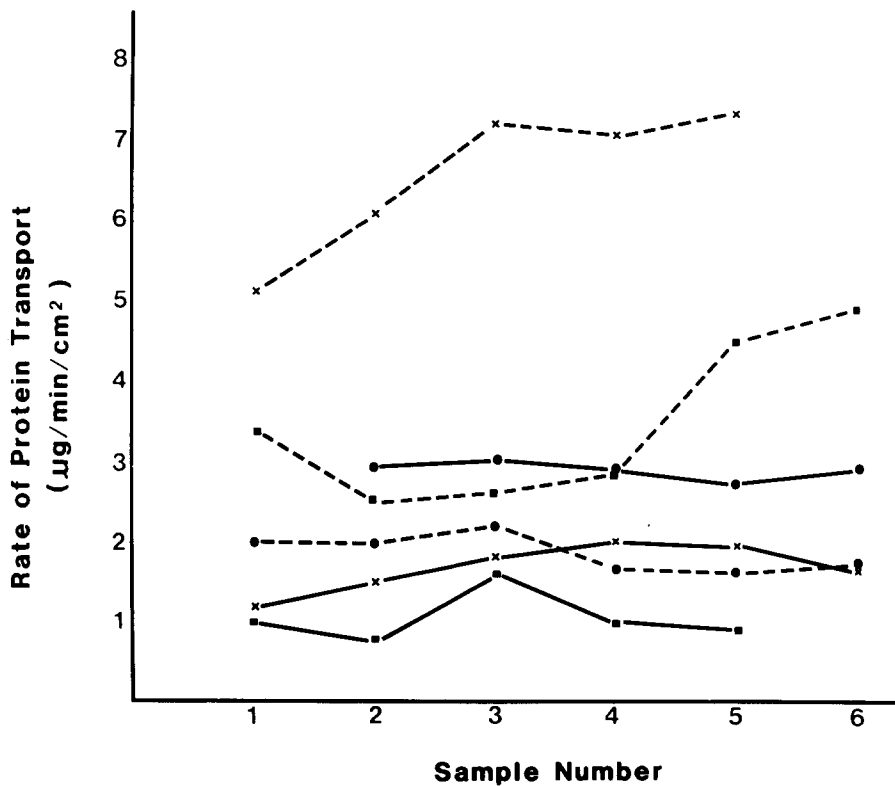


FIGURE 6 Individual results of six control experiments in which the temperature was maintained at 37°C throughout. No decrease in transport rate occurred with time, in contrast to the experiments in which the lymphatic segments were cooled.

respectively, whereas the cytoplasmic vesicles almost doubled, from 24 to 43% ($p < 0.05$).

STEREOLOGY: The vesicles in perfusion control lymphatics had an average diameter of 74.04 ± 2.68 nm, occupied ~5% of the cytoplasm ($V_v = 0.0506 \pm 0.0091$), and numbered about 23 ($N_v = 22.69 \pm 2.61$) per cubic micrometer of cytoplasm. As shown in Table IV, the data for lymphatics from the 24° , 30° , and 40°C groups were not significantly

different. In vessels perfused at 4°C , however, the vesicle diameter was significantly decreased to 64.06 ± 2.32 nm ($p < 0.05$). Although the V_v was somewhat decreased and the N_v increased, these differences were not significant.

More detailed data, related to the cellular disposition of the vesicles, are shown in Table V. Again, values significantly different from the perfusion controls appeared only in lymphatics cooled to 4°C . In these the average vesicular size,

TABLE I
Lymphatic Segments

Group	Length	Diameter	Samples	5- μ l collection time	5-min protein transport
$^{\circ}\text{C}$	<i>mm</i>			<i>min</i>	μg
4	7.7 \pm 1.2	0.54 \pm 0.05	Cont.	2.5 \pm 0.18	2.83 \pm 0.67
			Exper.	2.9 \pm 0.6	1.57 \pm 0.47
24	6.5 \pm 0.43	0.33 \pm 0.02	Cont.	2.2 \pm 0.14	1.16 \pm 0.18
			Exper.	2.1 \pm 0.15	0.76 \pm 0.07
30	7.8 \pm 0.80	0.31 \pm 0.06	Cont.	2.2 \pm 0.26	0.86 \pm 0.15
			Exper.	2.2 \pm 0.28	0.64 \pm 0.13
37	6.2 \pm 1.11	0.38 \pm 0.06	Cont.	2.7 \pm 0.25	1.76 \pm 0.46
			Exper.	2.5 \pm 0.25	1.90 \pm 0.05
40	6.7 \pm 0.80	0.30 \pm 0.04	Cont.	2.32 \pm 0.16	0.87 \pm 0.18
			Exper.	2.41 \pm 0.26	1.40 \pm 0.38

Data are given as mean \pm SEM. The fifth column gives the average time taken to collect 5- μ l aliquots after the 5-min stop-flow periods. The last column gives the average amount of protein transported across the lymphatic segment during the 5-min stop-flow periods. *Cont.*, control; *Exper.* experimental sample.

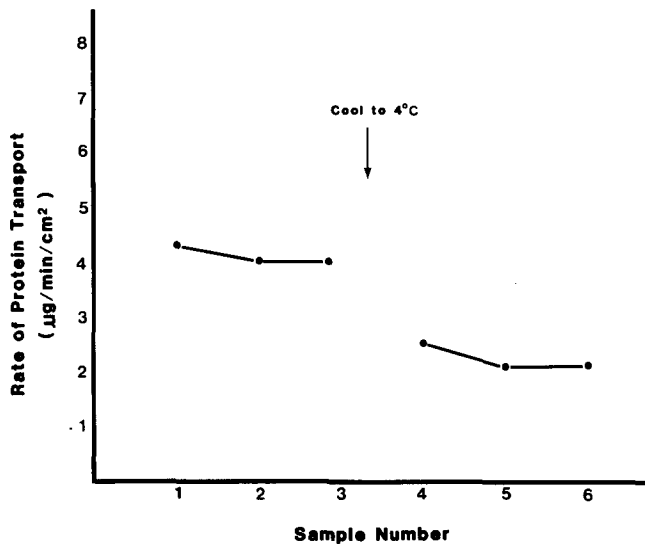


FIGURE 7 Representative experiment demonstrates the effect of cooling a lymphatic segment from 37 $^{\circ}\text{C}$ (first three samples) to 4 $^{\circ}\text{C}$ (last three samples) on the rate of protein movement from bathing to luminal fluid.

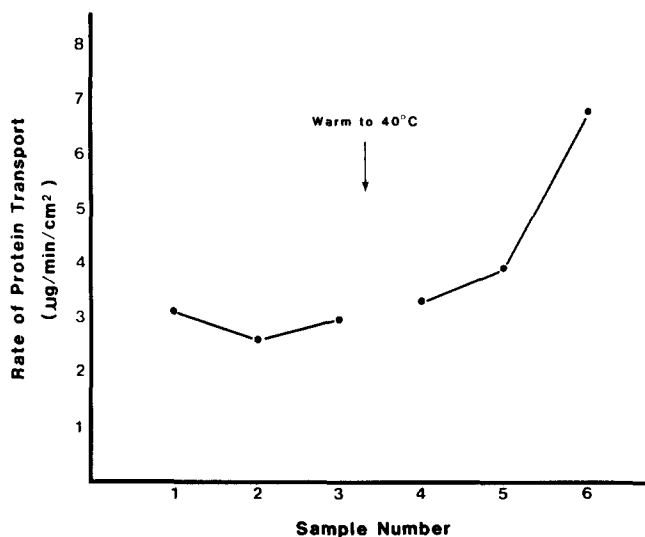


FIGURE 8 Representative experiment demonstrates the effect of warming a lymphatic segment from 37 $^{\circ}\text{C}$ (first three samples) to 40 $^{\circ}\text{C}$ (last three samples) on the rate of protein movement from bathing to luminal fluid.

TABLE II
Rate of Protein Movement into Lymphatic Lumen

Temperature	Rate	% Change	p Value
$^{\circ}\text{C}$	$\mu\text{g}/\text{min per cm}^2$		
37	4.61 \pm 0.42		<0.001
4	2.43 \pm 0.37*	-48.6 \pm 4.8	
37	3.57 \pm 0.50		<0.02
24	2.38 \pm 0.23*	-31.1 \pm 5.3	
37	3.39 \pm 1.24		<0.05
30	2.42 \pm 0.90*	-28.8 \pm 3.9	
37	2.78 \pm 0.73		NS
37	3.09 \pm 0.92	+1.66 \pm 10.43	
37	3.40 \pm 0.85		
40	5.54 \pm 1.69*	+48.4 \pm 12.2	<0.05

Data are given as mean \pm SEM.
* Significantly different.

regardless of position, was decreased as was the volume density of both abluminal and luminal vesicles. However, a more marked change occurred in the numerical density of cytoplasmic vesicles, which doubled from 5.69 \pm 1.32 to 11.81 \pm 1.94 vesicles/ μm^3 of cytoplasm ($p < 0.05$).

Interendothelial Contacts

The types of contact seen between adjacent cells conformed to the well recognized arrangement in endothelium—end-to-end, overlapping, and interdigitating (Fig. 9, *a-c*). The morphometric data are shown in Tables VI, VII, VIII, and IX. The overlapping type was the most common, followed by interdigitating and end-to-end configurations. So called 'open' regions, in which adjacent cells do not come closer to each other than 30 nm, were rarely seen. The numbers of these different types of contact were similar in lymphatics from all the groups except the one perfused at 4 $^{\circ}\text{C}$. In it the numbers of end-to-end junctions were significantly reduced (Table VI).

Two-thirds of the intercellular contacts possessed some form of specialized junction, <10% did not, and in the remaining 20% it was not possible to tell (Table VII). Although examples of both fasciae occludentes and fasciae adherentes were seen, these individual junctions were not quantified for reasons already given. The average width of the intercellular channels throughout most of their extent was 20 nm (Table VIII) although some circumscribed dilatations of

TABLE III
Distribution of Endocytotic Vesicles

Regimen	Number of vessels	Number of vesicles	Abluminal*	Cytoplasmic % ± SEM	Luminal
In situ control	4	1,246	30.2 ± 3.7	37.4 ± 7.9	27.5 ± 2.7
37°C perfusion control	6	1,816	35.1 ± 2.2	23.6 ± 3.3	33.0 ± 2.5
37° → 4°C	5	1,824	26.8 [‡] ± 2.3	42.8 [‡] ± 3.2	27.3 ± 0.6
37° → 24°C	6	2,051	29.8 ± 1.8	31.3 ± 3.7	33.6 ± 2.6
37° → 30°C	9	2,482	33.9 ± 2.3	32.5 ± 2.7	29.8 ± 1.6
37° → 40°C	10	2,741	35.2 ± 2.2	25.6 ± 1.7	31.7 ± 2.4

* Abluminal and luminal vesicles touched or opened onto the abluminal or luminal surface, respectively. Cytoplasmic vesicles had no obvious connection to either surface.

[‡] Values differ significantly from perfusion controls ($p < 0.05$).

TABLE IV
Morphometry of Endocytotic Vesicles

Regimen	Number of vessels	Number of vesicles	D nm ± SEM	V_v $\mu\text{m}^3/\mu\text{m}^3 \pm \text{SEM}$	N_v No./ $\mu\text{m}^3 \pm \text{SEM}$
In situ control	4	1,246	69.4 ± 5.73	0.0608 ± 0.0064	31.74 ± 7.50
37°C perfusion control	6	1,816	74.04 ± 2.68	0.0506 ± 0.0091	22.69 ± 2.61
37° → 4°C	5	1,824	64.06* ± 2.32	0.0436 ± 0.0048	27.62 ± 3.76
37° → 24°C	6	2,051	80.38 ± 1.66	0.0719 ± 0.0069	22.58 ± 2.59
37° → 30°C	9	2,482	81.12 ± 2.38	0.0602 ± 0.0112	17.67 ± 2.26
37° → 40°C	10	2,741	82.58 ± 1.75	0.0727 ± 0.0077	21.35 ± 2.91

D , diameter; V_v , volume density; N_v , numerical density.

* Value differs significantly from perfusion control ($p < 0.05$).

TABLE V
Morphometry of Endocytotic Vesicles by Position

Regimen	Number of Vessels	Parameter*	Abluminal [‡]	Cytoplasmic	Luminal
In situ control	4	D	68.18 ± 5.49	69.12 ± 5.62	71.40 ± 7.02
		V_v	0.018 ± 0.005	0.024 ± 0.006	0.017 ± 0.002
		N_v	9.14 ± 1.35	12.72 ± 4.60	8.68 ± 2.54
37° perfusion control	6	D	76.51 ± 2.43	74.85 ± 3.15	76.51 ± 2.50
		V_v	0.021 ± 0.001	0.014 ± 0.002	0.019 ± 0.002
		N_v	7.73 ± 0.65	5.69 ± 1.32	5.48 ± 1.24
37° → 4°C	5	D	62.47 ± 2.05 [‡]	64.82 ± 2.69 [‡]	64.38 ± 2.39 [‡]
		V_v	0.011 ± 0.001 [‡]	0.019 ± 0.002	0.012 ± 0.002 [‡]
		N_v	7.52 ± 1.18	11.81 ± 1.94 [‡]	7.45 ± 1.01
37° → 24°C	6	D	81.09 ± 2.45	80.01 ± 2.15	80.05 ± 1.21
		V_v	0.022 ± 0.002	0.027 ± 0.004	0.024 ± 0.003
		N_v	6.54 ± 0.56	8.35 ± 1.15	7.61 ± 1.09
37° → 30°C	9	D	79.06 ± 2.80	82.47 ± 2.22	82.19 ± 2.37
		V_v	0.019 ± 0.003	0.021 ± 0.005	0.018 ± 0.003
		N_v	6.03 ± 0.78	5.89 ± 1.18	5.08 ± 0.57
37° → 40°C	10	D	82.93 ± 2.32	80.44 ± 1.12	83.49 ± 2.32
		V_v	0.026 ± 0.003	0.017 ± 0.002	0.024 ± 0.003
		N_v	7.49 ± 0.97	5.48 ± 0.71	6.72 ± 1.04

* D , diameter in nm ± SEM; V_v , volume density in $\mu\text{m}^3/\mu\text{m}^3 \pm \text{SEM}$; N_v , numerical density in No./ $\mu\text{m}^3 \pm \text{SEM}$.

[‡] Abluminal and luminal vesicles opened onto or touched the abluminal or luminal surface, respectively. Cytoplasmic vesicles had no obvious connection to either surface.

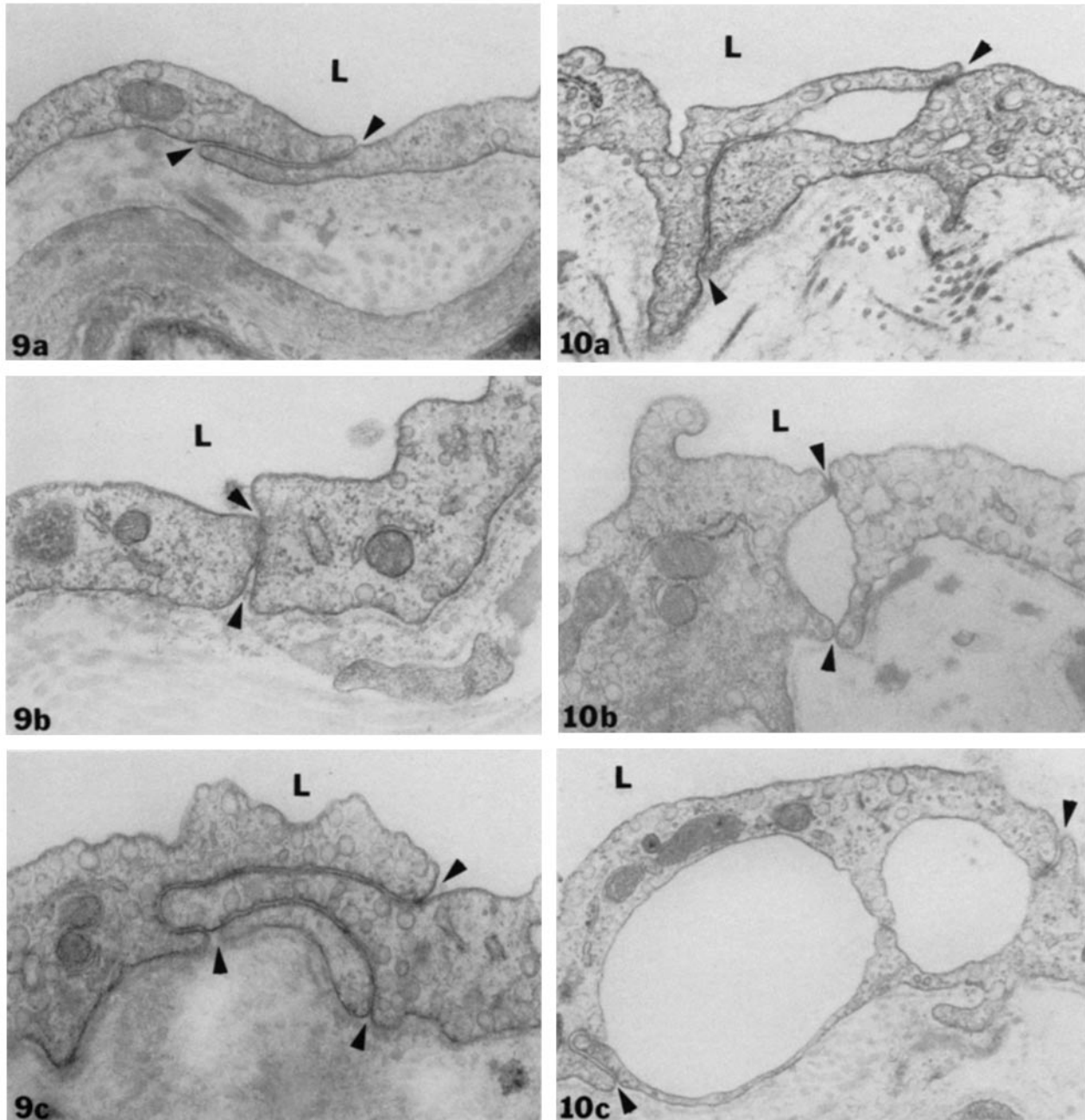
[‡] Values differ significantly from perfusion controls ($p < 0.05$).

these pathways were present in approximately half the contacts (Fig. 10, *a-c*; Table IX).

DISCUSSION

The findings of this study show that perfused lymphatic vessels can provide a useful model for analysis of transport across lymphatic endothelium. Judged by accepted morphological

criteria, these vessels retain their structural integrity while being perfused in vitro (Fig. 2). In addition, the restriction of horseradish peroxidase to the endothelial vesicles and the intercellular channels (Fig. 5) and the influence of temperature upon the rate of protein movement suggest that the lymphatics maintain their functional viability for at least two hours. This model, therefore, provides exciting possibilities for future analysis of lymph formation through the manipu-



FIGURES 9 and 10 Fig. 9: The three classes of intercellular junctions (arrowheads): (a) overlapping; (b) end-to-end; and (c) interdigitating. $\times 32,000$. Fig. 10: Dilations are exhibited in each of the three classes of intercellular junctions (arrowheads): (a) overlapping; (b) end-to-end; and (c) interdigitating. $\times 32,000$.

lation of factors such as hydrostatic and osmotic forces. Isolated microvessels from the blood vascular system have been used in vesicular transport studies (41) and pharmacologic experiments (6, 24) by various authors. However, in those preparations the tissue is subject to potentially damaging isolation procedures before the vessels are obtained for study. In contrast, the technique used in the present work avoids such procedures since the vessels are transferred from the dog to the chamber directly.

In this work, canine renal lymphatics were used because dogs possess exceptionally thin-walled vessels and because sufficient lengths of renal vessels that are free of tributaries can be obtained. Also, detailed analysis of the endothelium of canine hilar lymphatics shows it to be structurally compa-

rable to that of intrarenal lymph capillaries (2). In the experiments included in this study, all samples were monitored in the stop-flow mode. The purpose was to exclude from this particular analysis any effect attributable to hydrostatic pressure across the endothelial wall during the period when protein transport was being measured. To aid in this, the outflow cannula was kept short (~ 1.5 cm) and was positioned horizontally at approximately the same level as the lymphatic segment: thus hydrostatic forces could not account for protein transport from bath to luminal fluid. Similarly, neither cyclical nor continuous osmotic forces could be implicated, since any osmotic gradient would have been in the reverse direction because of the differential protein concentration. Two possibilities to explain the protein transport remain to be consid-

TABLE VI
Distribution of Intercellular Contacts

Regimen	Number of ves- sels	Number of con- tacts	E-E*	OL	ID	Open	% ± SEM	
In situ control	4	368	2.34 ± 0.50	67.52 ± 12.17	28.58 ± 13.47	1.30 ± 0.80		
37° perfusion control	6	804	16.46 ± 5.20	48.81 ± 12.96	28.19 ± 12.35	2.05 ± 1.99		
37° → 4°C	5	959	2.40* ± 0.73	43.88 ± 4.09	53.73 ± 4.19	0.14 ± 0.14		
37° → 24°C	6	375	8.91 ± 2.74	66.49 ± 6.07	23.96 ± 5.45	0.64 ± 0.64		
37° → 30°C	9	405	7.22 ± 2.58	63.22 ± 6.33	29.56 ± 5.19	0		
37° → 40°C	10	707	11.94 ± 2.97	59.89 ± 3.70	27.01 ± 4.20	1.14 ± 1.14		

* E-E, end-to-end; OL, overlapping; ID, interdigitating; open, cells separated by more than 30 nm.

* Value differs significantly from perfusion controls ($p < 0.05$).

TABLE VII
Specialized Junctional Complexes Associated with Interendothelial Contacts*

Regimen	Number of vessels	Number of contacts	Complex present	Complex absent	Unknown	% ± SEM	
In situ control	4	368	74.50 ± 9.71	1.96 ± 1.13	23.54 ± 9.57		
37°C perfusion control	6	804	73.18 ± 3.79	6.67 ± 1.69	20.15 ± 4.64		
37° → 4°C	5	959	61.93 ± 4.84	2.57 ± 1.26	35.51 ± 4.79		
37° → 24°C	6	375	67.11 ± 2.40	7.47 ± 3.28	25.92 ± 3.87		
37° → 30°C	9	405	75.43 ± 4.49	2.86 ± 1.14	21.71 ± 4.35		
37° → 40°C	10	707	71.34 ± 4.17	6.27 ± 2.10	21.24 ± 4.45		

* No values significantly different from the perfusion controls were detectable.

TABLE VIII
Width of Non-junctional Intercellular Channels*

Regimen	Num- ber of vessels	Number of con- tacts	Channel width nm ± SEM
In situ control	4	100	20.43 ± 0.57
37°C perfusion control	6	150	19.46 ± 0.44
37° → 4°C	5	125	19.57 ± 0.44
37° → 24°C	6	150	20.09 ± 0.58
37° → 30°C	9	225	18.92 ± 0.82
37° → 40°C	10	250	19.43 ± 0.65

* No values significantly different from the perfusion controls were detectable.

ered, simple diffusion and a cytoplasmic process. Of these, the former is the simplest and could readily account for the results but only when the control values are considered by themselves.

The evidence against simple diffusion as the complete explanation for the measured protein transport is twofold. The first is the appearance of externally applied HRP peroxidase in luminal endothelial vesicles, thereby suggesting that at least some protein travels by this route (Fig. 5). These tracer findings, however, are not conclusive. They merely show that HRP is present in the vesicles but do not indicate how it gets there, whether by diffusion along interconnecting channels or by endocytotic movement. A more detailed study is now in progress to help solve this problem. The second line of evidence is more direct and is the observed effect of temperature. This major influence of temperature on protein transport (Table II) is not indicative of a diffusion explanation, especially when intercellular pathways are found to be unchanged. Although diffusion itself is temperature dependent, the major

TABLE IX
Distribution of Intrajunctional Dilatations*

Regimen	Num- ber of vessels	Num- ber of con- tacts	Dilatation present	Dilatation absent	% ± SEM	
In situ control	4	100	32.45 ± 16.72	67.56 ± 16.72		
37°C perfu- sion con- trol	6	150	34.13 ± 8.06	66.01 ± 8.01		
37° → 4°C	5	125	54.47 ± 9.75	46.53 ± 9.75		
37° → 24°C	6	150	49.41 ± 7.41	50.59 ± 7.41		
37° → 30°C	9	225	46.25 ± 10.78	53.76 ± 10.79		
37° → 40°C	10	250	27.91 ± 5.94	72.09 ± 5.95		

* No values significantly different from the perfusion control were detectable.

effects apparent in this study are more suggestive of a cytoplasmic process. Pinocytosis in fibroblasts, for instance, is known to be reduced by cooling and almost totally inhibited at 2°C (3, 38). Thus protein transport, if it were to occur by way of vesicles, would be expected to change with temperature—decreased by cold, increased by heat. Indeed, by further analogy with pinocytosis, it may be assumed that vesicular movement is more or less abolished at 4°C. If this is so, then the ~50% reduction in protein transport that occurred at 4°C might be attributable to the vesicular pathway, and the remaining 50%, uninfluenced by temperature, to diffusion between cells or through intracellular channels. If this inference is correct, then nearly half the protein entering the lumen at 37°C was carried in the cytoplasmic system, whereas the remaining half or more was governed by diffusion.

The concept of macromolecular transport by the vesicular

system, first proposed for blood endothelium by Palade (26) and later confirmed by time-dependent tracer studies (13, 33), was originally conceived as a simple shuttling of free vesicles between endothelial surfaces, "like diffusion in quanta" (26). Numerous subsequent studies permit some generalizations concerning the nature of such transport. First, endocytotic vesicles appear to be relatively constant in population (42). Disagreement exists, however, on whether this is due to equality in the rate of de novo vesicle formation through a membrane-consuming process at both abluminal and luminal fronts, or whether vesicles are stable, long-lasting structures whose membrane is highly conserved (32, 42). Second, the process is a passive, dissipative one, apparently requiring no metabolically derived energy (44). Transcytoplasmic movement can be accounted for by Brownian motion (9, 10) and, as might be expected, it is strongly influenced by temperature. Added to the controversy on vesicular dynamics is the conflicting evidence on transport of macromolecules by vesicles of blood or lymphatic endothelium. On the one hand lies evidence that endocytotic vesicles incorporate substances at one surface of the cell and shuttle across to release them at the other (6, 27, 43). For some of the substances at least the uptake appears to be selective, involving a property of the cell membrane as well as the particular substance (43). On the other hand are the reports that question the very existence of discrete vesicles (8, 18). Both reconstruction of vesicular profiles from thin sections (8) and selective staining (18) of surface-exposed membranes suggest that vesicles, which appear to be free in the cytoplasm, in fact communicate with the surface. Thus Bundgaard et al. (8) prefer the term "endothelial plasmalemmal invaginations". At this stage it remains to be seen whether invaginations from opposite surfaces join to form transendothelial channels, as first described by Simionescu et al. (34), or whether they pinch off from one surface before opening to the other as suggested by the membrane staining technique (18). If transendothelial channels exist, transport by diffusion is likely. If invaginations occur and are constantly changing, then cytoplasmic involvement is clear. In either case HRP would enter the system, but the temperature effects seen in the present results would be consistent with a cytoplasmic process. Because of this unanswered structural question, the term 'vesicular system' is used here to denote either alternative.

An obvious question that arises from this work is whether the vesicular system, if it transports protein, can do so in one direction only (i.e., into the lumen) even when protein concentrations are similar on either endothelial surface. Such an arrangement is roughly true in vivo. Although this question was not a topic of the present study it nevertheless is basic to the vesicular theory of lymph formation. Preliminary in vitro experiments with smaller transendothelial protein gradients than the one used in the present study suggest that the rate of protein transport may be increased rather than decreased when the gradient is reduced but not abolished. This surprising finding is difficult to understand. It may be related to bulk flow, because the higher the protein gradient, from high outside to low inside the lumen, the greater the outward flow of fluid by osmotic forces. Such outward fluid movement between the cells or through transendothelial channels would restrict the inward diffusion of protein taking the same pathway. Whether this explanation is correct or not, the effect of membrane charge on transcellular protein movement must also be considered. Much evidence is now accumulating that

the luminal and abluminal membranes have different patterns of charge not only in blood capillary endothelium (35–37) but also in lymphatic endothelium (17). Such differential charge could favor adluminal transport by endocytotic vesicles even when concentration gradients are lacking.

Despite the demonstrated increase in the rate of protein transport with rising temperature, no significant changes in vesicle size, distribution, V_v , or N_v could be detected between 24° and 40°C. These data underline the constancy of the vesicular population. More important, they indicate that if the temperature dependent process reflects vesicular transport, then the alterations in the rate of transport must be attributed to increases in either the rate of vesicular trafficking or transport efficiency rather than in their numbers.

The stereological data obtained in vessels cooled to 4°C are harder to interpret. The change in vesicular distribution, (Table V) without significant change in total V_v or N_v (Table IV) suggests that at this low temperature, accumulation of vesicles within the cytoplasm can occur. Perhaps this is brought about by a reduced ability of vesicles to fuse with the plasma membrane. Indeed, Rubin (31) considers the temperature dependence of vesicular attachment and detachment to the plasma membrane rather than transcytoplasmic vesicle diffusion to be the rate limiting factor in vesicular mediated transport. Citing ultrastructural evidence, Arminski et al. (4) suggest that vesicular attachment and detachment is accompanied by viscoelastic membrane flow, a process which would be radically reduced at 4°C (14). In support of this, studies by Mahoney et al. (23) demonstrate that reducing membrane fluidity significantly reduces endocytosis by macrophages.

A somewhat unexpected finding in the present study was the presence of dilatations (Fig. 10, *a-c*) in many of the intercellular regions. Since the frequency of these structures did not differ between in situ or perfusion controls and any experimental group, it seems unlikely that they were artifacts of the perfusion process. Structures of similar appearance have been described in lymphatics of intestinal villi where they appear to be involved in the transendothelial transport of lipid droplets (5). Whether the dilatations present in renal hilar lymphatics subservise a similar function in protein transport is uncertain, although unpublished in vitro tracer studies in our laboratory suggest that they do not.

Regardless of its relative importance in lymph formation, a question subject to much debate, intercellular transport in vivo is heavily dependent upon adluminally directed convective forces (11, 20, 21). The ultrastructural analyses of the present study showed no essential differences in any intercellular contact-related variable among the groups. Thus it was evident that the changes in protein transport were not the result of changes in the size or pattern of intercellular associations. Similarly, the data show that endothelial discontinuities between adjacent cells (the so-called 'open' junctions; 1, 2, 11, 45) did not provide a route of any consequence for protein transport.

How far the findings of this study can be extrapolated to the in vivo situation is not clear. They do, however, argue strongly for a cytoplasmic mechanism in protein transport, which, when combined with in vivo studies (45), lends credence to the view that vesicles may be a potent force in lymph formation. Although the theories of lymph formation—hydrostatic, osmotic, and vesicular—appear to be widely different, they need not be self-exclusive in the sense that any or all could function simultaneously. Indeed it is probable that

the importance of each varies in different regions of the body and under different physiological conditions. For example, the formation of lymph from peritoneal fluid by the subdiaphragmatic lymphatics is clearly dependent on cyclical pressure changes as well as on the presence of large stomata in the endothelial wall (22, 40). Renal lymph, on the other hand, is formed through an intact endothelial wall that lacks "open" intercellular channels (2). Hence, it would seem that the more complete the endothelial lining is, the more important the role of vesicles in lymph formation may be.

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