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A Fine Structural Study of Variations in Protein Concentration in Lacteals during Compression and Relaxation

J.R. Casley-Smith

Electron Microscope Unit, University of Adelaide, South Australia

Summary

The ratios of plasma proteins and ferritin (introduced into the gut) were determined between the jejunal lacteals and the interstitial channels in puppies. This was done by fine structural densitometry and the counting of molecules, respectively. In the normal state this ratio was ~2. In portions of tissue whose smooth muscle had been relaxed by atropine the ratio was ~1; in others, where the muscle had been contracted by Carbacol, it was ~3. These latter correspond approximately to the filling-phase and the emptying-phase of the initial lymphatic cycle, respectively. Thus the evidence was strengthened for an hypothesis, wich holds that the filling is caused by the high effective colloidal osmotic pressure of the concentraded lymph.

Introduction

There is considerable controversy about how material is caused to enter the initial lymphatics (reviews: 5, 6, 8, 11). There is no doubt that in oedema (and in a few regions, e. g. the kidney and the testes), when the tissue hydrostatic pressure is considerably above atmospheric, there is a net hydrostatic pressure gradient from the tissues to the initial lymphatics (5). However, normally this gradient is probably in the reverse direction, because the tissue hydrostatic pressure is considerably less than in the initial lymphatics (5, 6, 11). Other suggested mechanisms are most unlikely (5, 11), so that the only one which now appears still possible is the effective colloidal osmotic pressure of the lymph (2, 5 11).

The operation of this mechanism involves a somewhat complicated cycle which has been described in detail elsewhere (5, 11). The basic concept is that the lymph is concentraded during the emptying-phase by the ultrafiltration of fluid through the close junctions (which are permeable to fluid, but impermeable to proteins). This is because, during compression, the total tissue pressure rises considerably higher than the tissue hydrostatic pressure, and is transmitted nearly in full to the lymph. This causes any open junctions to shut; they act as flap-valves. While some lymph is forced into the collecting vessels, it can be calculated (11) that much larger amounts will be ultrafiltered – because of the relative hydraulic conductivities of the two paths. The total tissue pressure is caused to increase by the contractions of adjacent muscles, etc. When these later relax, this pressure falls, as does the intralymphatic pressure. Material once more enters the vessels via the newly opened endothelial intercellular junctions, with the motive force being the high effective colloidal osmotic pressure of the lymph. This tissue fluid, however, is much less concentrated than the lymph, and dilutes it – thus necessitating its reconcentrations during the next emptying-phase.

The most direct evidence in favour of this hypothesis has come from fine structural evidence that the mean concentration of protein in the lymph is considerably greater than in the tissue channels (6, 8), and that this increases greatly during the emptying-phase and decreases greatly – to become equal to that in the tissues – during the filling-phase (6). This evidence was gained by estimating the concentrations in both the initial lymphatics and the tissue channels by counting ferritin or lipoprotein molecules, by autoradiography of RISA, and by micro-densitometry of protein. The determinations of the mean lymphatic/tissue concentration ratios were performed in the ear, the villi and the diaphragm (6, 8), but the evidence that these varied during the cycle was only obtained in the diaphragm (6). While in the ear and the villi, normal proteins

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(plasma proteins and lipoproteins) could be estimated together with microinjected ferritin, in the diaphragm ferritin was introduced into the peritoneal cavity – which hardly corresponds to the normal tissue channels.
I therefore decided to take advantage ot the well-established fact, that macromolecules readily traverse the intestinal epithelium of neonates via vesicles, to test the hypothesis once more. Ferritin, which was administered to the intestinal lumen, and the normal plasma proteins were both studied. They both pass to the lymphatics via the tissue channels. Using smooth muscle relaxants and contractants, it was possible to rapidly fix the lacteals during relaxation or compression – which would mimic the filling – or the emptying-phases of the initial lymphatic cycle. Puppies were used since their finger-like villi seemed more likely to contract uniformly than the leaf-like ones of rodents (23).

Materials and Methods

Eight, 12-hour old, Gordon Setter/Golden Retriever-cross puppies were anaesthetised with aether, a 2 cm segment of jejunum was isolated between ligatures – being careful to preserve its blood and lymphatic supply – and injected with 0,25 ml of 5 g/100 ml ferritin (Cadmium-free, N.B.C.) in gassed-Kreb's solution. They were allowed to recover for 1 hour, then reanaesthetised and the segment was excised and placed in fresh Kreb's solution where it was cut into three parts. One of these was left; one was placed in fresh medium to which 100 μg/ml of Carbacol (British Drug Houses) had been added to cause contraction of the smooth muscle; one was placed in solution to which 10 μg/ml of atropine had been added to cause relaxation of the smooth muscle. Thirty seconds were allowed to elapse, then all the pieces were placed in fixative. The material used for micro-densitometry of protein was treated similarly, without ferritin.
The fixative was 4% glutaraldehyde in Millonig's buffer (17), and was allowed to act for 1 hour. The pieces were then cut into 1 mm^2 segments, dehydrated in acetone, embedded in araldite, and the sections stained with lead citrate (pH 11). They were examined in a Siemens Elmiskop I fitted with a Faraday-cage which was used to estimate the plasma protein concentrations from their electron-opacities, as described elsewhere (6). (These were taken to be the fine, grey, irregular fibrils in the vessels and tissues). The ferritin concentrations were estimated by counting the molecules per unit area (6). In both of these estimations particular care was taken to avoid areas in the interstitial tissue where there were cells or collagen fibres. For each animal 10 random micrographs were measured (for each kind of estimation) for each of 2 blocks; thus 160 micrographs were studied for each condition. In order to avoid the problem of sections differing in thickness, for each micrograph the ratio of the protein concentration in the lumen was compared with that in the tissue and used as an independant variable. These ratios for each micrograph were estimated as the mean of 10 random measurements of concentrations in the lumen and in the tissue in the case of the densitometry of protein. The ratio of the sums of ten random squares (~0.05 μm^2), for each of the sites, was obtained for the ferritin counting.

Results

The ferritin penetrated readily into the lamina propria of the villi (Figs. 1, 2) and was found in the fenestrae of the blood capillaries and in their lumens. However it was obviously not possible to estimate the relative amounts which were removed by this route as compared with those going via the lymphatics. The lymphatics themselves (Figs. 1–4) appeared very similar to those described in adult rats (1, 19) and mice (8). Ferritin could be seen within the open endothelial intercellular junctions observed in the relaxed and normal tissues (Fig. 1), but not in the closed junctions
In the contracted tissues, no open junctions were seen, and there was little fer-

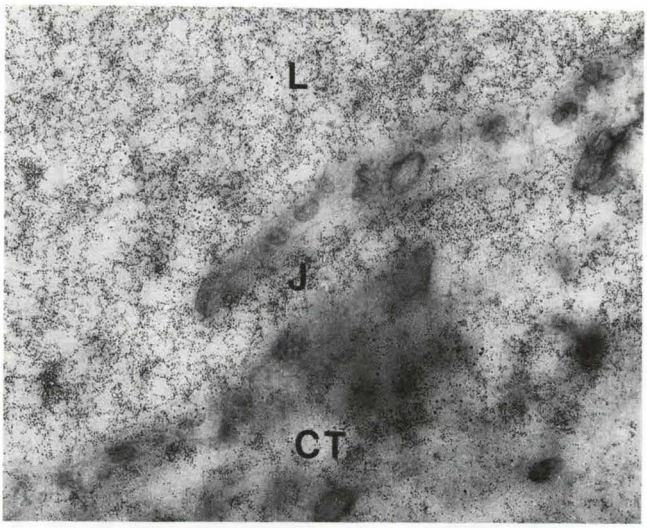

Fig. 1 A lacteal in a relaxed villus. There is an open junction (J). The concentration in this, in the lumen (L), and in the connective tissue (CT) are all approximately equal. 37,000x

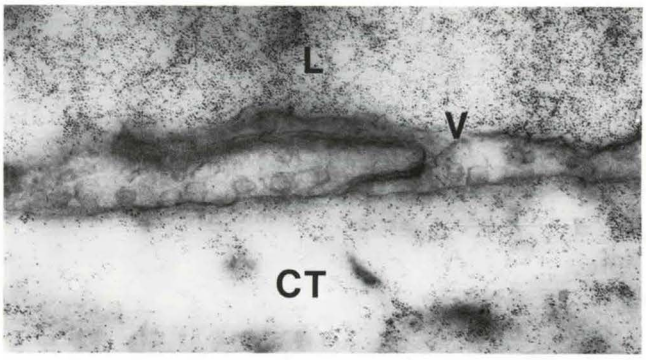

Fig. 2 A lacteal in a contracted villus. The concentration in the lumen (L) is considerably greater than that in the connective tissue (CT). There is a closed junction, with very little ferritin in it. Some is present in small vesicles (V). 30,000x

ritin within their closed junctions (Fig. 2). When the native proteins (not ferritin) were observed, the lumen contained similar concentrations to the tissues in the relaxed villi (Fig. 3), in the contracted ones the lumenal concentration was considerably increased (Fig. 4).

The ratios of the ferritin concentrations in the lumens as compared with those in the interstitial tissue channels are shown in the Table, as are those measured by densitometry of the proteins. It can be seen that these ratios are about 2 in the normal tissue, that these decrease to 1 or slightly less in the filling-phase, and increase to rather more

Table The ratios of Protein Concentration in the lumens compared within the tissues[1]

	Ferritin	Plasma Proteins
Normal[2]	1.87 (0.050)	2.03 (0.026)
"Filling-phase"[3]	0.967 (0.022)	0.912 (0.011)
"Emptying-phase"[4]	3.41 (0.073)	3.18 (0.051)

[1] The Means are given, followed by their Standard Errors (in brackets); the numbers of observations are 160 for each group.
[2] These represent the means for villi, both relaxed and contracted.
[3] These were tissues with the smooth muscle relaxed by atropine.
[4] These were tissues with the smooth muscle contracted by Carbacol.

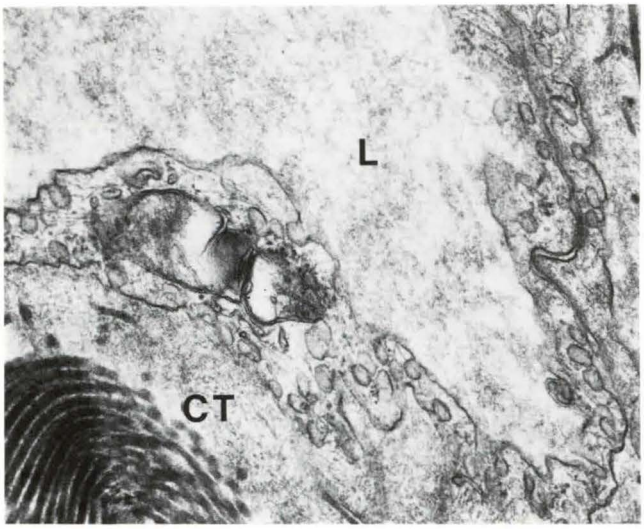

Fig. 3 A lacteal in a relaxed villus, without ferritin. The concentrations of protein in the lumen (L) and in the tissue (CT) are approximately equal. 30,000x

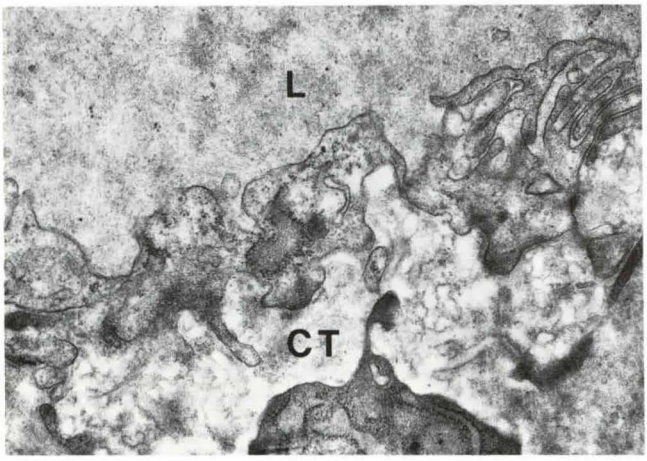

Fig. 4 A lacteal in a contracted villus, without ferritin. The protein concentration in the lumen (L) is considerably greater than in the tissue (CT). 25,000x

than 3 in the emptying-phase. All these differences are highly significant ($p < 0.001$), by t-tests.

Discussion

As was the case with the experiments using the diaphragm (6), the present ones can not completely represent what happens during life, because the fixative takes some 30 seconds to work (6). So only the relatively late stages of the two phases are represented. Also, no doubt many of the lacteals were in their filling-phases and a few, some 20% (23), were in their emptying-phases, when the relaxant or contractant was applied. Hence the values obtained here for the "filling" or "emptying-phases" can not be exactly what occurs in an individual lacteal during its cycle. Also, the fixative may well cause the muscles to alter their tension while they are being fixed, hence while the tissues were relaxed or contracted for some 30–60 seconds, these states probably altered during the final seconds. Nevertheless, it is clear that there are very substan-

tial alterations in the concentrations of proteins in the vessels during the cycle, in the directions predicted by the hypothesis.
Possible errors in the techniques of ferritin-counting and protein-densitometry have been discussed elsewhere (6). It was concluded that these were not likely to be significant compared to such alterations as have been found here. This is especially the case, seeing that the two methods gave such similar results, not only here but in other sites, and to those obtained by other methods (5, 6, 8).

One particular objection to the method has been that the interstitial tissue gel would exclude the larger molecules and that hence a ratio > 1 would be expected. Even if the avoidance of the regions with collagen in them (where most of the gel-phase occurs $\frac{\alpha}{M}$6) did not largely overcome this effect, it has been shown (6,8) that it would only amount to 1.1 for albumin. In addition, a lymph/tissue ratio of 2–3 is found even in oedema (6), where the exclusion effect would be much less. Also, such an effect could not possibly account for the variation found during the cycle. Hence it is evident that the results can not be attributed to this exclusion.

A number of workers have performed experiments where one might have expected them to observe a similarly elevated ratio between the lymph concentrations and those in the tissues, or a difference between the ratios in the initial lymphatics and those in the collectors. (According to the hypothesis the concentrated lymph expelled into these latter would be rediluted). In fact, their results have not been as predicted by the hypothesis. However, it would appear that in each case there is a quite reasonable alternative explanation.

Rutilli and *Arfors* (22) examined initial lymphatic lymph and adjacent tissue fluid and found no significant difference; however the animals had been anaesthetised and immobilised for some 30 minutes. Hence there could not have been the variations in total tissue pressure which are an essential feature of the hypothesis and are essential to normal lymphatic functioning. *Hargens* and *Zweifach* (12) and *Witte* and *Zenzes-Geprägs* (26) found only an increase in concentration as one passed from the initial lymphatics centrally along the collectors, not an initial redilution. However this was in the exteriorised mesentry, where again there were no variations in total tissue pressure. (No doubt such increases in concentration do normally occur along the collecting vessels, in addition to the initial redilution, as the intralymphatic pressures increase). *Nicolaysen* et al. (18) found no dilution between the initial lymphatics and the collecting vessels in the lung. However, as explained in detail elsewhere (6), these were "adjacent collectors" and subjected to very similar total tissue pressures as the initial lymphatics, hence no dilution would be expected until the "remote collectors", outside the organ, were reached. The theoretical objections by *Taylor* et al. (24, 25) have been answered elsewhere (5, 11), using much more detailed calculations. The objections mainly consisted of misconceptions about where the ultrafiltered fluid went, the conditions in the tissue channels, and not appreciating the very large amount of ultrafiltered fluid. Other theoretical objections relating to the effects of such colloidal osmotic pressures across large gaps (16) have also been shown to be incorrect, both theoretically (4, 20) and by experiment (5, 7).
By contrast, results supporting the hypothesis have been presented by a number of workers, in addition to the fine structural estimations of protein concentration in a number of sites and by a number of methods, mentioned earlier (6, 8). *Rusznyak* et al. (21) reported increased concentrations in the initial lymphatics compared with the tissues, as have others (14, 26). *Johnson* and *Richardson* (13) found that this hypothesis was necessary to explain their macrophysiological results. A mathematical model (11) has shown it to be quite physically possible, and in vitro studies (5, 7) show it is likely to work.

These various results, and the ones presented here, do not necessarily mean that the hypothesis is correct, but had they not confirmed its essential preconditions and predictions they would have made it very unlikely. Similarly, the fact that they do confirm these makes it that much more likely. The present results are of considerable importance since the mean ratio of concentrations being greater than 1, and its variation during the cycle in the directions predicted, are essential elements of the hypothesis.

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J.R. Casley-Smith, D.Sc., M.B., Electron Microscope Unit, University of Adelaide, Adelaide, South Australia