

Electron Microscopy of the Effects of Unguentum Lymphaticum on Acute Experimental Lymphedema and Various High-protein Edemas

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Summary

Rats' legs and feet were studied by qualitative and quantitative electron microscopy, including mass-densitometry of protein in the tissues and in the initial lymphatics. The tissues were either normal, or had been made edematous by lymphostasis, moderate burns, or dextran. It was found that *Unguentum lymphaticum* very greatly reduced the amount of edema in the legs with lymphostasis. Since the concentrations of plasma protein in the tissues and initial lymphatics, and its total amounts in the tissues were all greatly reduced, it appears that the cream's anti-lymphedematous activity is via a removal of the excess protein. Since the macrophages were greatly increased in number by the cream (and previous work shows that selectively poisoning these cells prevents much of the cream's effectiveness in lymphedema), very probably it is this increase in their numbers (and possibly their individual proteolytic activities) which is how the cream causes the removal of the excess protein — via an increased proteolysis. The cream also causes an increase in the amount of edema in the rat-foot (after all the injuries, including lymphedema); however the concentration and amount of protein in the tissues is reduced. This is consistent with an inflow of low protein fluid, caused by a vaso-dilatation of the blood microcirculation of this rather specialized tissue. All of these characteristics are very similar to those of many of the benzo-pyrone group of drugs.

Introduction

High-protein edemas in general, and lymphedema in particular, are far more common than is often realised. In the previous paper (Casley-Smith and Casley-Smith 1983) we give some statistics about their frequency. If a high-protein edema persists for more than

two months it will cause chronic inflammation (Casley-Smith and Gaffney 1981); chronic lymphedema is almost certainly caused in this way (Casley-Smith et al. 1980; Földi and Casley-Smith 1978). Hence it is of considerable importance that high-protein edemas be treated both early and adequately. Since the treatment of these conditions, especially of lymphedema, is notoriously difficult — although it is also often needlessly neglected (Casley-Smith and Casley-Smith 1983; Clodius and Gibson 1982; Földi 1982), it is evident that any therapy which offers improvements in this deserves careful and energetic investigation.

One such therapy is the cream: "*Unguentum lymphaticum*" (Pharmazeutische Gesellschaft mbH & Co., München). Its constituents, and a brief review of animal experiments and clinical findings, are given in the previous paper. While both kinds of studies are very favorable for this mixture of drugs, regrettably they are all too few. It was in the hope of finding out more about what this cream does, and how it does it, that the present work was undertaken.

In the previous paper it was shown that the cream produced almost complete protection against the lymphedema caused by acute lymphostasis in the legs of rats. Curiously, it increased the edema produced by dextrans or moderate burns. It appears that the cream has two actions (at least): it reduces lymphedema in the leg, but increases it in the foot (possibly by causing vaso-dilatation in the rather unusual dermal blood vessels of the rat-foot).

It was of considerable interest that its protective effect against the leg lymphedema was lost if the macrophages were selectively destroyed by injecting silica for a week before the lymphostasis was produced. In this, it greatly resembled the benzo-pyrone group of drugs which also reduce lymphedema, almost certainly by increasing the numbers of macrophages in lymphedema and other high-protein edemas, and by increasing their normal proteolytic activity (reviewed: *Casley-Smith* 1976, 1983a, b).

In order to learn more of the actions of this cream, specimens were prepared for electron microscopy from many of the animals used in the previous paper. The subcutaneous tissue of the legs and feet, in lymphedema and in dextran and burn edemas, were examined qualitatively and quantitatively, paying particular attention to the interstitial tissue and the initial lymphatics. In addition, a group of normal animals were studied. Each tissue had been treated either with *Unguentum lymphaticum* or with its drug-free base.

Material and Methods

Treatment with Unguentum lymphaticum

After each injury the animals were immediately treated with *Unguentum lymphaticum* on one foot, using about 0.75 g gently spread over the foot and massaged in, again very gently. The other foot was treated identically with the base of the cream, which contained no drugs. This treatment was repeated twice every day for the duration of the experiments. Because of the adherence of saw-dust to the creams, the rats were placed in cages with plain plastic floors (without saw-dust); these were changed at each treatment. The animals were kept in air-conditioned surroundings ($20 \pm 0.5^\circ\text{C}$) and fed on standard rat-nuts.

Normal Rats

Ten hooded S.P.E. rats (200 ± 20 g) were treated with the creams for four days. They were then killed (with chloroform) and their tissues were processed for electron microscopy.

Dextran Edema

Ten hooded rats (S.P.F.: 500 ± 25 g) were given an intraperitoneal injection of 12 g/dl dextran (m.w. 70,000) in physiological saline — 1 ml/kg. Fifteen hours later they were killed and their tissues were processed for electron microscopy.

Thermal Edema

Ten hooded rats (S.P.F., 350 ± 25 g) were anesthetized with Sagital, and each hind foot was burnt, up to the Achilles tendon, by immersing it in water (continually changed) at 57°C for 60 seconds. While many of the feet treated with the drug-free base of the cream were gangrenous only those without obvious gangrene were included in the present study. After three days they were killed and their tissues were processed for electron microscopy.

Acute Lymphedema

Ten hooded rats (S.P.F.; $450 \text{ g} \pm 25$) were anesthetized with Sagital. Both legs were given lymphostasis by a modification of the method of *Piller and Casley-Smith* (1975) — see *Casley-Smith and Casley-Smith* (1983). The animals were killed, with chloroform, at four days after the operation, and their tissues were processed for electron microscopy.

Electron microscopy

The subcutaneous tissue, of the leg and foot, was prepared for electron microscopy by the normal techniques of glutaraldehyde fixation, (4 % in Millonig's buffer for four hours). Osmium post-fixation, embedding in araldite, and staining with Lead citrate. Two random sections were studied, from each tissue, of the ten animals in each group, both qualitatively and by stereology, using the techniques set out in *Casley-Smith and Gaffney* (1981). For the stereological studies, 25 random fields were observed, per section — using a 7-point grid inscribed on the fluorescent screen of the electron microscope. The magnification was 7300x (checked with a grating replica of 2160 lines per mm, and found to be reproducible to within 0.5 %). Sections were 50–60 nm thick (checked individually with a Faraday-cage — *Casley-Smith and Crocker* 1975). The incidences of the points on the various tissue features were used

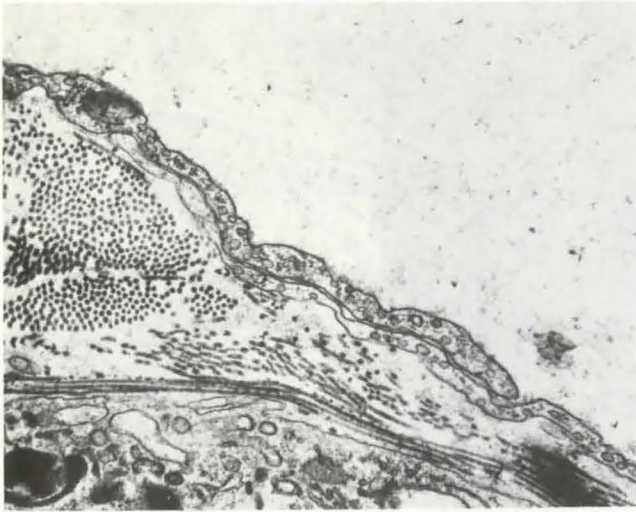


Fig. 1 Normal leg subcutaneous tissue, treated with *Unguentum lymphaticum*, showing an initial lymphatic with a closed endothelial intercellular junction. 20,000 x

for estimating V_V , and the numbers of macrophages per standard field ($120 \mu\text{m}^2$) for their N_V (Weibel 1969). The Standard Errors of the Means were estimated from the Binomial Distribution.

The main features studied in this way were the numbers of macrophages in the tissues, and the amount of "empty" tissue. This latter is the tissue which is not occupied by vessels, cells or collagen fibres. It is thus largely ground substance in normal tissues (although it also includes the tissue channels, with some plasma protein in them); in edematous tissues it is largely edema fluid and the protein it contains. Hence increases in it are a measure of the increase of fluid in a tissue, although it is not an accurate one, since so much is ground substance in normal tissue. However, it is a good measure of variations in edema, both with treatments and under various conditions. Similarly, one can measure its protein concentration and thus compare total protein contents of one edematous region with another, or one form of treatment with another, but one cannot compare an edematous tissue with a normal one, using this parameter.

Mass-densitometric estimations of protein concentration in the tissues and initial lymphatics was performed, again using 25 random measurements (per parameter), on two random sec-

tions, from each tissue, of the ten animals in each group. This was done using the technique set out in Casley-Smith and Crocker (1975), using a Faraday-cage attached to the electron microscope (a Siemens Elmiskop I). Internal standards were provided by the plasma in the blood vessels, external ones were polystyrene spheres. While it is easy to measure just the protein in the initial lymphatics and in edematous interstitial tissues, it is much more difficult to do so in normal interstitial tissue. This is because one has to find the relatively small and infrequent tissue channels (Casley-Smith 1982a). Hence the figures for normal tissue are not as reliable as those for edematous ones.

Whenever possible the stereological and mass-densitometric parameters were related to the total volumes of the tissues, rather than just to their unit volumes. This is a far more meaningful value than the latter, which is what is usually used (Casley-Smith and Gaffney 1981). The Standard Errors of these Means were obtained from those of the parameters and of the volumes of the tissue (Casley-Smith and Casley-Smith 1983), using large number theory (Kendall and Stuart 1966, p. 231).

Results

Qualitative

The normal tissue did not differ from that

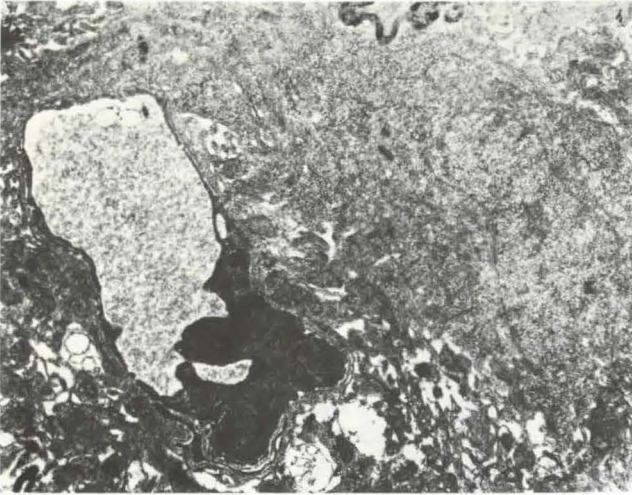


Fig. 2 Leg subcutaneous tissue with lymphedema, treated with the drug-free base. There is much edema, with a high concentration of protein in it (but this can only be accurately quantified by mass-densitometry in the electron microscope). The initial lymphatic, which is quite dilated, has much protein in the lymph. Some open junctions are just visible in the endothelium at this magnification. 4,000 x



Fig. 3 As in Fig. 2. An open junction is visible between two endothelial cells (top and bottom), leading from the tissue (at right) to the lymphatic lumen (left). It contains much protein. 100,000 x

found in other studies (Fig. 1 - reviewed: Casley-Smith 1982a). Those subjected to lymphedema and treated with the placebo (the drug-free base of *Unguentum lymphaticum*), had similar alterations to those found with other lymphedemas in subcutaneous tissue (Figs. 2, 3, 6 - Casley-Smith et al. 1974; reviewed: Casley-Smith 1982b, 1983a). The essential changes were also studied quantitatively (see below). In brief, the acute lymphedema caused the initial lymphatics to be widely dilated, with many open endothelial intercellular junctions and a high concentra-

tion of protein in the lymph; the interstitial tissues were very edematous, with a high concentration of protein, and contained many macrophages.

Dextran and burn edema were also similar to previous reports (Casley-Smith et al. 1973; Hammersen 1972; reviewed: Casley-Smith 1982b, 1983a). Again the tissues were very edematous (especially with the burns) and the initial lymphatics were very dilated, with many open junctions, but the protein concentrations, although greater than normal, were less than those seen in lymphedema. In burns,

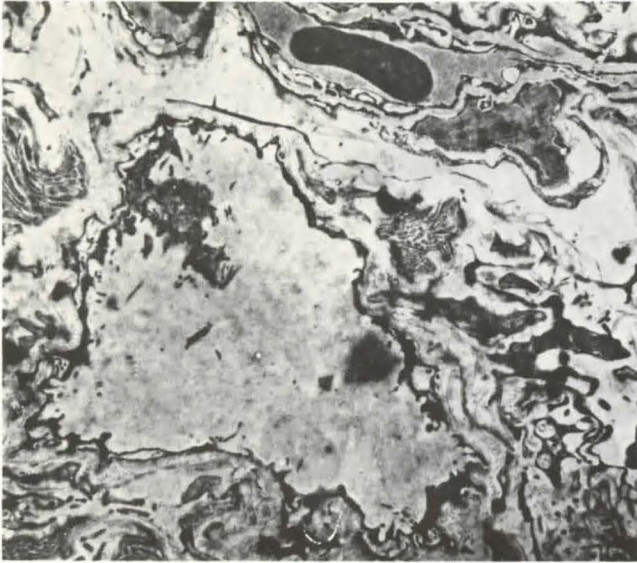


Fig. 4 Leg subcutaneous tissue with lymphostasis, but treated with *Unguentum lymphaticum*. The initial lymphatic is still quite dilated, but it contains a much lower concentration of protein, as does the interstitial tissue. 4,000 x

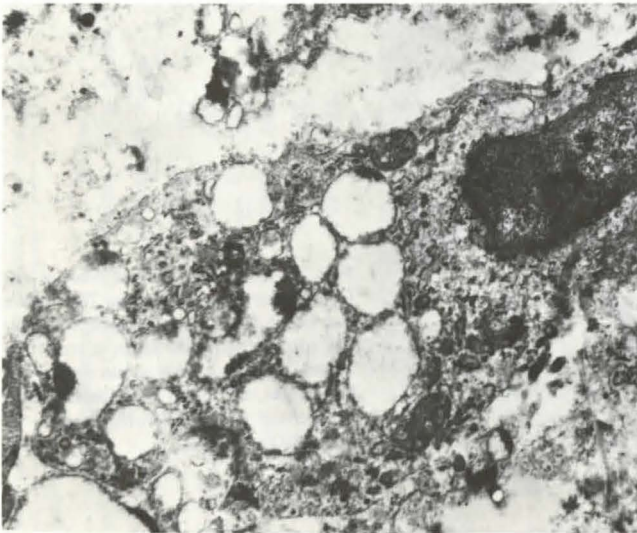


Fig. 5 As for Fig. 4, showing part of a macrophage, with many vacuoles. 20,000 x

many of the small blood vessels (capillaries and post-capillary venules) contained thrombi — especially in those feet treated with the placebo rather than with the drugs. The vessels in these feet also were much more frequently damaged, with dark, broken endothelium, and open junctions.

The main effect noticed with *Unguentum lymphaticum* was to reduce the amounts of protein seen in the edematous tissues (both legs and feet), no matter what caused the edema (Figs. 4, 5, 7). In the lymphedematous legs, it was remarkable how little edema there was (Figs. 4, 5): in the feet, no matter what

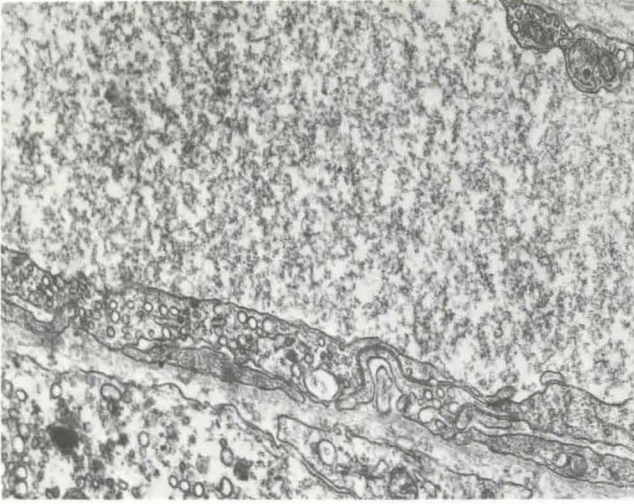


Fig. 6 Subcutaneous tissue in the foot, with lymphedema, and treated with the drug-free base. A dilated lymphatic (with more pericytes than are usual in an initial lymphatic, implying it is tending towards being a collecting lymphatic) contains much protein. 8,000 x

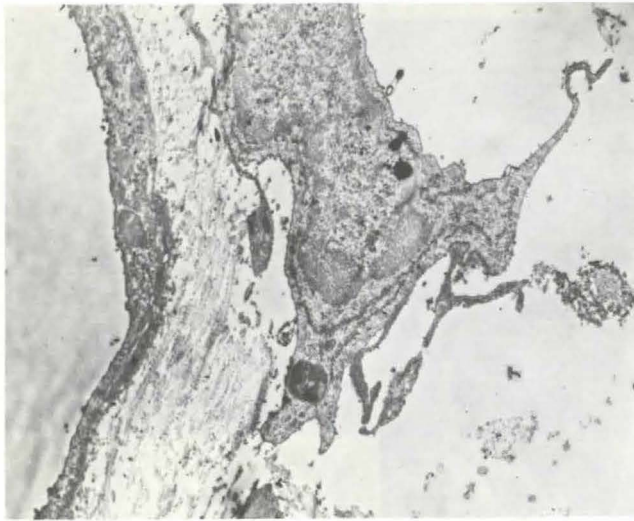


Fig. 7 As for Fig. 6, but treated with *Unguentum lymphaticum*. The interstitial tissue is still very edematous, but the protein concentration is much reduced — both in the tissue and the lymphatic, although mass-densitometry is essential to establish this quantitatively. 6,000 x

the cause of the edema, the cream somewhat increased the amount of edema (Fig. 7). (This latter may have even included the normal feet, as was suggested — but not shown significantly — by quantitation.) The cream also increased the numbers of macrophages, in both legs and feet, under all the conditions, and these cells gave the visual impression of being more active (more pseudopodia, large vacuoles, etc. — Fig. 5). The dilatation of the initial lymphatics did not seem to be greatly

altered by the cream. The numbers of open junctions were also largely unaffected, except in the lymphedematous legs, where they were fewer.

Quantitative

These results are summarized in the Table, where results of t-tests, between the drug- and placebo-treated tissues are shown for each parameter. It can be seen that the cream greatly reduced the amount of edema in the

Table 1 Several tissue and lymphatic parameters, under various conditions, treated with *Unguentum lymphaticum*^{a)}

Treatment	Legs				Feet							
	Normal		Lymphedema (96 hrs)		Normal		Lymphedema (96 hrs)		Dextran (15 hrs)		Burn (72 hrs)	
	Placebo	Ung. lymph.	Placebo	Ung. lymph.	Placebo	Ung. lymph.	Placebo	Ung. lymph.	Placebo	Ung. lymph.	Placebo	Ung. lymph.
"Empty space" ^{b)} in ml/ml; S.E.	.104 NS	.124	.435***	.128	.149NS	.187	.326 NS	.402	.204 **	.298	.513 NS	.637
Total in region in ml(c); S.E.	.623 NS	.772	3.53 ***	.760	.282 *	.387	.841 *	1.11	.422 **	.629	1.48 *	2.16
Protein concn., in g/dl; S.E.	.721 NS	.610	5.21 ***	2.14	.780 NS	.620	4.98 ***	1.54	1.69 **	.871	2.26 **	.764
Total in region in gdl; S.E.	?	?	.184***	.0163	?	?	.0419***	.0171	.227	.139	.483	.190
Macrophage Nos.x10 ⁻⁶ /ml. S.E.	6.12 *	20.3	115 **	.344	4.95 **	18.3	89.3 *	.242	3.27 **	13.7	34.2 *	87.4
Total in region x10 ⁻⁶ c); S.E.	36.7 *	126	934	*2043	1.78	4.65	23.2	56.7	1.03	3.77	8.01	20.5
Dilatation (0-5 scale) S.E.	.53 NS	.91	4.9 NS	4.8	.79 NS	.99	4.3 NS	4.7	1.7 NS	2.5	3.9 NS	4.6
Protein concn. in g/dl; S.E.	.198 NS	1.53	5.16 **	2.76	2.37 NS	1.99	4.53 **	1.74	2.21 NS	1.63	2.97 **	1.56
Open junctions (0-5 scale); S.E.	.723 NS	1.12	4.31 *	2.53	.923NS	1.3	4.57 NS	4.89	2.35 NS	3.29	3.12 NS	3.87
	.130	.232	.786	.455	.321	.378	.864	.966	.560	.782	.659	.688

a) The degrees of freedom for each mean are 499, since they are the results of 50 fields observed in 10 animals. The results of a t-test of the significance of the difference between each pair of means (treated with placebo or *Unguentum lymphaticum*) is shown between them. "NS" stands for no significant difference, "*" for significance at the 5% level, "**" at the 1% level, and "***" at the 0.1% level. The Standard Errors of the Means (S.E.) are shown below them.

b) In normal tissue this is largely ground substance, invisible in the electron microscope, plus protein in tissue channels; in edematous tissue it is largely edema fluid, which also contains an amorphous mass of precipitated protein.

c) This row was obtained by multiplying the previous one by the volume of the tissue (see text).

d) This row was obtained by multiplying the concentrations by the total volumes of "empty space" in the rows above. This greatly overestimates the amount of protein in non-edematous tissue (because of the volume occupied by the ground substance), but is probably approximately correct in very edematous regions. Hence it can be used to compare the effects of treatment on these, but they should not be compared with normal tissue.

legs with lymphostasis (both per ml of tissue and, more importantly, for the whole region); the reverse of this occurred in the feet, under all conditions. (While this was often not significant per ml, it was when the total amount was considered.)

Again, the drugs very greatly reduced the amount of protein in both the legs and feet in lymphedema (both per ml and totally). While the concentrations were significantly reduced in the feet with dextran and burn edemas, the reductions were not significant when the total protein in the region was considered; this decreased concentration could occur if the edema was increased by low-protein fluid leaving the blood vessels, e.g. by the cream causing their vaso-dilatation. In the initial lymphatics, the lymph was normally considerably more concentrated than the tissue fluid (significant at the 0.1% level), and the protein concentration changes approximately paralleled those in the tissues. However, the two fluids were in approximate equilibrium in lymphedema; the concentrations in both of them were about equally reduced by the drugs, as might be expected in tissue with lymphostasis. In burn and dextran edema, the lymph was only a little more concentrated than the tissue fluid.

Macrophage numbers (both per ml of tissue and total) were considerably increased above normal by lymphedema and by burning (but not by dextran – possibly because this injury only lasted 15 hours). This corresponds to findings by others (reviewed: *Casley-Smith* 1982b, 1983a). It was of considerable interest to observe that in the normal tissues, and in all the edemas, *Unguentum lymphaticum* considerably increased the numbers of these cells (again, both relatively and absolutely). Thus the effect of their increased numbers, even at this early stage of lymphedema, was augmented still more.

The dilatation of the initial lymphatics was very significantly increased (0.1% level) in lymphedema and burn, and to a lesser extent by dextran. These amounts were not significantly altered by the drugs – nor would one expect this, even if the edema was much re-

duced in lymphedematous legs (because lymphostasis was still present), or if the edema was increased in the feet (for the same reason, or because dilatation was considerable already).

Similarly, the numbers of open junctions in the initial lymphatics were much increased (significant at the 0.1% level) in lymphedema, or burn or dextran edema, as compared with normal. This was significantly reduced by the reduction of edema in the lymphedematous legs by the drugs, but was not significantly altered in the other conditions.

Discussion

As mentioned earlier, the qualitative and quantitative findings confirm previous observations about the alterations produced in the interstitial tissues and initial lymphatics by lymphedema, moderate burns and dextran. They also confirm that in normal tissue the concentration of protein in the lymph is greater than that in the tissues (*Casley-Smith* 1982a, c). This difference was less in the tissues made edematous by burns or dextran, probably because here the initial lymphatics acted more as conduits, rather than force-pumps (*Casley-Smith* 1982c). The concentration was also approximately equal in the lymphedematous tissues, because of the equilibration of the tissue fluid with the motionless lymph.

The most important result to emerge from the present study was the increased numbers of macrophages (both per ml and total) produced in both sites, under all conditions, by *Unguentum lymphaticum*. This correlates well with the finding that selectively poisoning these cells with silica largely prevents this cream having its normal anti-lymphedematous effect (*Casley-Smith* and *Casley-Smith* 1983). It also provides a good explanation for this effect.

It would appear that one or more of the components of the cream act similarly to the benzo-pyrone group of drugs. It has been shown that the benzo-pyrones, while they have many actions in the body, almost certainly possess the ability to reduce lymphedema because they increase the numbers and proteolytic activity of the macrophages (reviewed: *Casley-Smith* 1976, 1983a, b; *Földi* and *Casley-*

Smith 1978). It was thus of considerable interest that *Unguentum lymphaticum* also increases their numbers, has a greatly reduced action if they are destroyed, and – qualitatively at least – give the appearance of stimulating them. When these data are linked with the present observations that the cream causes a considerable reduction in both the concentration of protein in the interstitial tissue and initial lymphatics, and in the total amount in the tissues, it is difficult to avoid the conclusion that one or more of the cream's components greatly increases normal proteolysis in the tissues, and hence is responsible for most of its anti-lymphedematous activity.

The present results also confirm the previous findings that *Unguentum lymphaticum* increases the edema of rat-feet, whether this is caused by lymphedema, dextran, or moderate burning. In this characteristic, also, they are very similar to some of the benzo-pyrones. It has been shown that 0-(β -Hydroxyethyl)-rutosides, while they protect rats' feet against various high-protein edemas – when given in small doses, increase their edemas by releasing histamine and 5-HT – when given in higher doses (Lecomte 1971; Lecomte and van Cauwenberge 1972, 1974; Lecomte et al. 1971). This action is blocked by anti-histamines, and does not occur in non-reactor rats (Lazar et al. 1977). Probably coumarin and rutin (also benzo-pyrones) have this property in rabbit skin (Piller 1976).

In the previous paper some evidence was presented which suggested that the cream might have a vaso-dilatory action upon the specialized blood vessels of the rat foot (Casley-Smith and Casley-Smith 1983). Thus it was shown that, while many of the burnt feet which were treated with the drug-free base of the cream became gangrenous, none of the feet treated with the cream itself did this. In addition, the feet of lymphedematous animals treated with the complete cream were much redder than those treated only with its base. Finally, other, animal and clinical studies suggested that the cream has a vaso-dilatory action. The present findings confirm this. The alterations in the protein concentration and total pro-

teins in the rat feet are what would be expected if a vaso-dilatation of the blood microcirculation permitted the tissues to be flooded with excess fluid with a low protein concentration. Unfortunately, the electron microscope, alone, is not a suitable instrument for studying vaso-dilatation. This is because it shows *all* the blood vessels, not just those in which blood is actually flowing at any given time; this can be overcome by using tracers, but it has not yet been done for this cream. Hence there was no point in doing quantitative studies on the blood vessels in the present experiments.

It is of interest to note that the benzo-pyrones also often have an activity on parts of the blood microcirculation, although this is not constant throughout the group (Casley-Smith 1983a). The usual effect of the benzo-pyrones appears to be to dilate the arterio-venous anastomoses and constrict the precapillary sphincters. This explains why coumarin and Sodium rutin sulphate greatly reduce the amounts of gangrene (Casley-Smith et al. 1977; Clodius and Földi 1976) – because oxygen is supplied largely from the arteriolar part of the microcirculation (reviewed: Casley-Smith 1983a); similar actions by the cream would explain its action in preventing gangrene. However, it would not explain the increase in edema it causes in rat feet (but not in rat legs). A generalized vaso-dilatation would explain this, but it would tend to reduce the supply of oxygen to the tissues (because of the increase in edema) – thus worsening any gangrene. Perhaps both actions occur. This would not be surprising since the cream contains a mixture of many drugs, and even single, purified, benzo-pyrones have many actions in the body (Casley-Smith 1976, 1983a).

Thus it is evident, from the present experiments, that *Unguentum lymphaticum* has an excellent effect on acute lymphedema of the rat's leg. This correlates well with other experimental and clinical data (Casley-Smith and Casley-Smith 1983). It appears highly probable that this is produced through increased proteolysis by macrophages. It also appears that the cream has another, anti-gangrenous, activity which may occur via vaso-dilatation.

There is some evidence that this activity occurs in tissues other than the rather abnormal ones of the rat's foot. If so, it resembles the benzo-pyrones in this characteristic also, and this property may well be of use therapeutically.

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