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THE EFFECTS OF CALCIUM DOBESILATE ON ACUTE LYMPHEDEMA (WITH AND WITHOUT MACROPHAGES), AND ON BURN EDEMA

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ABSTRACT

Calcium dobesilate ("Doxium", O.M. Laboratories, Geneva) was tested in two high-protein edemas. While at a high dose level it had no effect on burn edema of the ratfoot, it did at a low dose. It greatly reduced (to 26%) acute lymphedema in the rat thigh (although not in the foot). Electron microscopy confirmed these findings, and showed that the drug increased the number of macrophages in the tissues and reduced the protein concentrations. If, however, the macrophages were destroyed (by silica) this effect on acute lymphedema was lost (as with benzo-pyrones).

Calcium dobesilate also reduces high-protein edemas in other models, and in many ways its actions resemble those of benzo-pyrones. Hence it is suggested that this substituted benzene ring may be the basic structure responsible for this action in high-protein edema, and that the pyrone ring (and any side-chains) in the benzo-pyrones are not essential to their enhancement of proteolytic activity by macrophages.

Globally, some 300,000,000 people are likely to suffer from lymphedema during their lifetime (34). Besides lymphedema, it has been estimated that about one-third of a Western, industralized population each year receive medical treatment for high-protein edemas, which are far more common than is usually realized (7). In addition to the pain, swelling and poor oxygenation of the tissues (7), if such edemas last for two months in rats they cause chronic inflammation (9), just as was hypothesized by Willoughby and Di Rosa (35). Indeed, it appears that the alterations in the tissues seen in chronic lymphedema and elephantiasis are almost entirely initiated by this simple accumulation of excess protein (7, 9, 11, 14).

Because benzo-pyrones reduce high protein edemas, these agents should play a central role in their treatment. To date, these drugs have been successfully used in a wide range of disorders (reviewed: 6, 7): lymphedema; trauma, accidental and surgical; radiation reactions; varicose veins, hemorrhoids and chronic venous insufficiency; diabetic retinopathy and hyphema; hepatitis and cirrhosis; pancreatitis; asthma and allergies; capillary fragility; peripheral arterial disease; migraine; to increase lymph flow in the collecting lymphatics during lymphangiography; venous and arterial thrombosis, embolism; smooth muscle spasm in a number of regions.

Calcium dobesilate (marketed as "Doxium", Laboratories O.M., Geneva), is calcium dihydroxy-2,5 benzenesulphonate, i.e. it is a benzene ring, with OH groups at the 2nd and 5th positions and a SO_3 group at position 1, through which two of these molecules are linked, via calcium. Thus it resembles a benzo-pyrone, except that the pyrone ring is missing. When one examines its biological properties, there are many

remarkable similarities with those of the benzo-pyrones. It reduces high protein edemas caused by many mediators, inhibiting both fine structural damage and high-protein edema (12, 21, 24, 31, 33); it prevents the desquamation of endothelium (12); it reduces capillary fragility and permeability in scurvy (which is often linked with a vitamin P deficiency) and in other conditions which cause this (12); it stabilizes the interstitial tissue (12); it increases the filling of collecting lymphatics in their pumping (1); it promotes hemostasis (probably by favoring platelet-vessel wall interaction — 12); it lowers increased tendencies for platelet and erythrocyte aggregation (17-19, 31). In human studies it has been used successfully in: diabetic retinopathy (2, 3, 15, 20, 29, 30); increased blood capillary permeability in inflammation (33); varicose veins and chronic venous insufficiency, including flaccid paraplegic peripheral vascular decompensation (17, 18, 21); tendencies towards platelet aggregation (19) and blood hyperviscosity (2, 20).

Thus, where examined, calcium dobesilate's actions and those of the benzo-pyrones seem similar. (One must of course remember that the actions of the benzo-pyrones are far from identical between different members of this vast group, so this is only a general impression). However, many actions of the benzo-pyrones have not been tested for calcium dobesilate. Specifically, while its actions have been tested in a variety of high-protein edemas caused by injury to blood vessels, it has not been tested in lymphedema. The latter situation is a particularly valuable test, because it allows isolation of the proteolysis-increasing capacity of the benzo-pyrones because the blood capillaries are normal and the lymphatic system is blocked. Accordingly, we tested calcium dobesilate in a lymphedema model and when macrophages were rendered nonfunctional by silica, — a phenomenon that blocks the action of benzo-pyrones (7, 10). Mild burn edema was also examined because this abnormal state is a highly reproducible form of high-protein edema.

MATERIALS AND METHODS

An inbred strain of hooded Wistar rats were used. In all the experiments tests of significance were by t-tests, or using the Normal Distribution — when the degrees of freedom warranted it. While the environmental temperature was maintained constant at 20° C±0.5, the amounts of edema in the injured legs were individually corrected for any variations caused by slight alteration in temperature or in rat activity, by using the uninjured leg as a control.

Acute lymphedema

For the acute lymphedema experiments, three groups of 16 male hooded rats (200g±25) were anesthesized with Sagital. Their left legs were given lymphstasis by a modification of a previous technique (27). The skin was transversely incised on the medial aspect of the thigh, 1 cm distal to the inguinal ligament. The fascia overlying the femoral vessels was removed and the vessels undercut with a scalpel until freed of muscle for a distance of 0.5 cm. Care was taken to dissect along, but to avoid cutting, any major tributaries. All collateral lymphatics were obstructed by a pair of ligatures; these were passed, beneath the femoral vessels, through the musculature of the thigh, and around the skin in both medial and lateral directions. Thus the femoral vessels were not occluded when the ligatures were tightened, but every other vessel was.

This form of acute lymphedema is maximal at 90-100 hours. Hence the rats were killed at 96 hours after operation. Then their legs were cut off at the ligatures (and at the equivalent position on the control side) and weighed. A second weighing was also performed: the tibio-calcaneal joint was disarticulated and the foot was weighed. (This was necessary because sometimes the benzo-pyrones release so much histamine in the foot that it actually increased in size, while that of the thigh was still reduced — see above). The drugs were injected at the time of operation, and daily thereafter. One group was given just saline; another re-

ceived 50 mg/kg/day of calcium dobesilate — intraperitoneally, in physiological saline (1ml/200g body weight); the third group received 200 mg/kg/day of the drug.

"Poisoning" Macrophages

Four groups of 10 male hooded rats (400g±25) were given acute lymphedema in one leg, as in the previous experiment. Two of the groups were daily injected with tridymite silica for 8 days before and after operation. Silica was intraperitoneally injected, each day (100mg/kg in a 10g/dl solution in physiological saline — 10). One of the silica groups and one of the non-silica groups received calcium dobesilate (200 mg/kg/day) from the time of lymphstasis. The legs were then treated as before.

Thermal Injury

For the burns, female hooded rats (200±25g) were divided into groups of 16. Each animal was anesthesized with Sagital. (It is of interest that those treated with calcium dobesilate recovered from anesthesia in about half the time taken by the saline-injected group). One foot was mildly burned to the ankle, by immersing it in water (continually changed) at 54° C for 60 seconds. At this time, each rat was injected intraperitoneally with 1ml/200 g body weight of physiological saline. The control group received only saline; the others received calcium dobesilate in doses of 50, or 200 mg/kg body weight, in this injectant. The volumes of the burned and the control feet, up to the ankles, were estimated by weighing, 24 hours after injury.

Electron Microscopy

The subcutaneous tissue, of the leg and foot, of the rats with acute lymphedema for 4 days, was prepared for electron microscopy by the usual 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 16 rats in each group, both qualitatively and by stereology, using the techniques set out in Casley-Smith and Gaffney (9). 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 — 9). The incidence of the points on the various tissue features were used for estimating V_V , and the numbers of macrophages per standard field (120 um) for their N_V(9). 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 term represents the tissue which is not occupied by vessels, cells or collagen fibers. It is thus predominantly ground substance in normal tissues (although it also includes tissue channels, with some plasma protein); in edema the "empty" tissue is largely fluid with protein. Hence increases in this "space" is a measure of the increase of tissue fluid. Because so much is ground substance in normal tissue, comparisons between normal and edematous tissues are difficult. Nonetheless, it is a good measure of edema, both with treatments and under various conditions. Moreover, 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 sections, from each tissue, of the 16 rats in each group. This was done using a Faraday cage attached to the electron microscope (8). Internal standards were provided by the plasma in the blood vessels, external ones were polystyrene

spheres. While it is relatively easy to measure just the protein in the initial lymphatics and in edematous interstitial tissues, it is more difficult to do so in normal interstitial tissue, where tissue channels are small and infrequent. 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. The former value is far more meaningful (9) than the latter, which is what is commonly used. The standard errors of these means were obtained from those of the parameters and of the volumes of the tissue, using large number theory (9).

RESULTS

In the experiment using acute lymphedema it was grossly obvious that rats treated with calcium dobesilate had less edema. In fact, an extra 15 rats were used in the saline-group, but they chewed

Table 1
Effects of Calcium Dobesilate on Acute Lymphedema at 4 Days Expressed as (Lymphedematous site - Normal)/Normal; in g/g (Mean ± SE)+

Site(n)	Saline	Calcium dobesiltate	
		50 mg/kg	200 mg/kg
Whole leg (16)	0.28[0.045]	0.14[0.023]**	0.12[0.019]**
Thigh (16)	0.36[0.071	0.13[0.026]**	0.095[0.015]***
Foot (16)	0.14[0.018)	0.18[0.032)NS	0.20[0.027]NS

+Tests of significance were between each treated group and that treated with saline; there were no significant differences between the two treated groups. In this, and the following Tables, NS stands for no significant difference; * for significance at the 5% level; ** at the 1% level; and *** at the 0.1% level.

Table 2
Effects of Calcium Dobesilate on Acute Lymphoedema - without Macrophages
Expressed as: (Lymphoedematous site - Normal)/Normal; in g/g

	(Saline - Normal)	(Drug - Normal)	Sig of
	Normal	Normal	Difference
LEGS + THIGHS			
No Silica	0.31[0.043]	0.11[0.020]	***
Silica	0.45[0.032]	0.35[0.036]	*
Sig. of Difference	**	***	
FEET			
No Silica	0.19[0.061]	0.27[0.044]	NS
Silica	0.39[0.075]	0.45[0.074]	NS
Sig. of Difference	*	**	

There were 10 animals per group; the Means are shown, followed by their Standards Errors [in brackets].

through ligatures around their legs and had to be discarded from the final results, a phenomenon not seen in the calcium dobesilate group. This difference was significant at the 0.1% level, and implies that the latter group was much more comfortable.

Table 1 shows that at the higher dose, calcium dobesilate reduced edema of the whole leg to 43% of the control value; that of the thigh was reduced to 26% of the control. By contrast, edema of the foot was unaffected.

Table 2 shows the results of "poisoning" the macrophages with silica. Again, the lymphedematous legs treated with calcium dobesilate were considerably reduced, but this effect is largely blocked with injury to the macrophages. Indeed, macrophage blockage increased the amount of lymphedema in rats receiving saline. An increase also occurred in the feet, but even with intact macrophages (i.e. no silica) calcium dobesilate did not reduce edema.

The results for the burn studies are shown in Table 3. There was no alteration in the amount of edema at high dosage of the drug. Visually, as well, the feet looked similar. At the lower dose, edema was decreased to 75% of the saline-control.

Electron microscopy of acutely lymphedematous rats confirmed the reductions in the amount of edema. The normal tissue, including that from rats treated with calcium dobesilate but not from the uninjured contralateral limb, did not differ from that found in other studies (5, 7). The lymphedematous, but untreated, tissues (Fig. 1) were similar to that usually found in acute lymphedema (5, 7). The initial lymphatics (Fig. 1) were widely dilated, contained much protein, and had many open junctions. There was much edema, with a high protein content, and numerous macrophages in the interstitium (Fig. 1). While no major differences were seen between tissues treated with either dose level of calcium dobesilate, this drug considerably improved the side-effects of lymphostasis (Figs. 2-4). Thus, calcium dobesilate reduced the protein concentrations in both the initial lymphatics (Figs. 2, 3) and the tissues, reduced tissue edema and further increased the already raised number of macrophages (Fig. 4).

This qualitative data was echoed in the quantitative observations (Table 4). Protein concentrations were reduced (in both the interstitum and initial lymphatics); macrophages, which were increased in numbers (as is usual in lymphedema and other high-protein edemas — 7, 9, 11), were further increased by calcium dobesilate. While the higher dose tended to accentuate these differences, no statistical differences were detected between the two dose levels.

DISCUSSION

Calcium dobesilate considerably reduces high-protein edemas and in this regard acts similar to benzo-pyrones. These latter agents decrease edema by increasing normal proteolysis by macrophages and possibly by other cells (reviewed: 5, 7, 14, 27). In spite of the action of benzo-pyrones on blood

Table 3

Effects of Calcium Dobesilate on Mild Burn Oedema at 24 Hours Expressed as: (Burned leg - Normal)/Normal; in g/g

Saline	Calcium dobesiltate	
	50 mg/kg	200 mg/kg
0.81[0.055]	0.57[0.090]*	0.79[0.060]NS

There were 16 animals per group; the Means are shown, followed by their Standard Errors [in brackets]
The tests of significance were between each treated group and that treated with saline; there was a significant difference (at the 5% level) between the two dose levels of Calcium dobesilate.

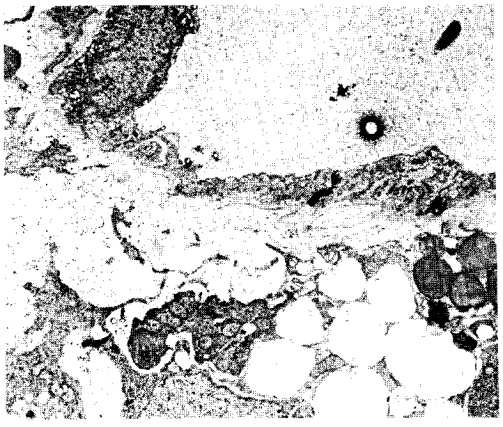

Fig. 1: A dilated initial lymphatic in an untreated rat with lymphstasis. The tissue is edematous and contains much protein and a number of macrophages, some of which contain much lipid. 5000X

Fig. 2: A small collecting lymphatic in a rat treated with 200 mg/kg/day of calcium dobesilate. While the vessel is dilated (as expected with lymphstasis), there is less protein in both lymph and in the interstitium — which appears better organized. 5000X (compare with Fig. 1, 3).

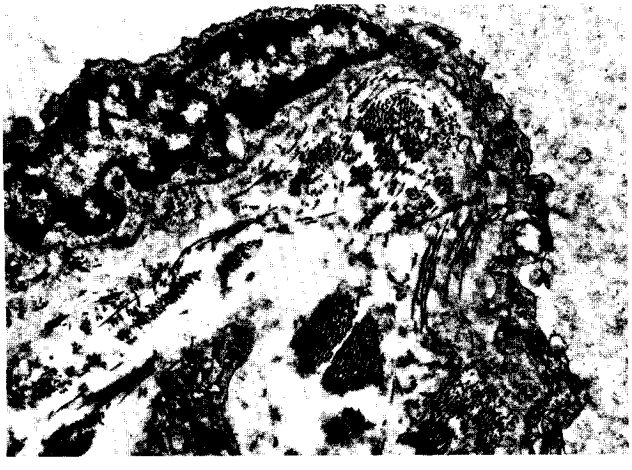

Fig. 3: An initial lymphatic in a rat treated with 50/mg/kg/day of calcium dobesilate. The protein content of both lymph and interstitial tissue is reduced, while the latter is less edematous. 30,000X (compare with Fig. 1, 2).

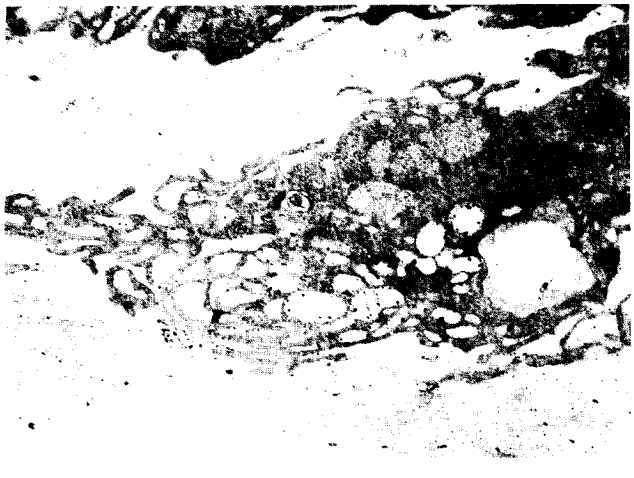

Fig. 4: A portion of a macrophage in the interstitial tissue of a rat with lymphedema treated with 200 mg/kg/day of calcium dobesilate. There are many pseudopodia and vacuoles. The protein in the interstitial tissue is reduced. 35,000X (compare with Fig. 1-3).

Table 4
Fine Structural Tissue & Lymphatic Parameters, in Acute Lymphoedema, with Calcium Dobesilate

	LEGS					FEET				
	Normal	Lymphoedema				Normal	Lymphoedema			
		Saline	Calcium dobesilate 50 mg/kg		Calcium dobesilate 200 mg/kg		Saline	Calcium dobesilate 50 mg/kg		Calcium dobesilate 200 mg/kg
INTERSTITIAL TISSUE										
"Empty Space"[b] in ml/ml; S.E.	.112 .0102	***	.440 .0147	.229 .0365	***	.198 .0289	.151 .0188	***	.345 .0501	NS .411 .0402	NS	.438 .0513
Total " in region in ml[c]; S.E.	.648 .104	***	3.71 .412	*** 1.60 .257	***	1.38 .176	.271 .0321	***	.819 .0411	NS 1.06 .142	*	1.39 .178
Protein Concn., in g/dl; S.E.	.682 .119	***	4.89 .702	** 2.45 .379	***	2.11 .332	.712 .114	***	4.77 .713	** 2.43 .232	**	2.33 .317
Total in region in g[d]; S.E.			.201 .0422	*** .0423 .00991	***	.0395 .00875			.0424 .00653	*** .0168 .00238	***	.0152 .00327
Macrophage Nos. $\times 10^{-6}$/ml; S.E.	5.77 1.49	**	123 42.1	* 267 57.8	*	284 63.2	7.12 2.31	***	89.3 23.2	* 207 53.9	*	289 74.2
Total in region $\times 10^{-6c}$; S.E.	46.1 8.45	**	862 271	* 1780 376	*	1960 391	8.88 3.46	***	211 58.9	* 521 128	*	609 158
INITIAL LYMPHATICS												
Dilation (0-5 scale); S.E.	.58 .21	***	4.5 .66	NS 4.3 .54	NS	4.4 .72	.65 .19	***	4.1 .79	NS 3.7 .88	NS	4.0 .83
Protein Concn. in g/dl; S.E.	2.08 .319	***	5.01 .781	* 3.15 .412	**	2.71 .431	2.05 .289	***	4.61 .674	* 2.93 .306	**	2.74 .281

a. The Standard Errors of the Means (S.E.) are shown below them. The degrees of freedom for each mean are 799, except for the normal values which are combined — since there were no statistically significant variations between the untreated animals and those treated at either dose level — which have 2,397 degrees of freedom. The significance of the difference between the untreated mean and the normal are shown between them; the results of comparing the untreated mean with the mean for each dose level is shown to its left.
b. In normal tissue this is largely ground substance, invisible in the electron microscope, plus protein in tissue channels; in oedematous tissue it is largely oedema fluid, which also contains an amorphous mass of precipitated protein.
c. Obtained by multiplying the previous one by the volume of the tissue (see text).
d. Obtained by multiplying the concentrations by the second row (see text). This overestimates the amount of protein in non-oedematous regions. Hence it can be used to compare the effects of treatment on these, but they should not be compared with normal tissue — which are omitted.

vessels and lymphatics, this ėdema sparing effect still occurs in high-protein edema when blood vessels are normal and lymphatics are occluded (e.g. in acute lymphedema): it is prevented by selectively "poisoning" the macrophages (7, 10). From the observed increase in the numbers of macrophages and the reductions in protein concentrations observed here, it appeared likely that calcium dobesilate has a similar action. This probable action on the macrophages was confirmed by failure of calcium dobesilate to decrease edema when macrophages were selectively "poisoned" by silica.

A further similarity between the actions of the benzo-pyrones and those of calcium dobesilate is the anomalous effect on the rat's foot (in those strains which are dextran-reactors — 22). As mentioned

earlier, while low doses of benzo-pyrones reduce high-protein edema in this tissue, high doses release histamine and 5 hydroxy-triptamine even in normal feet. Thus, in injured feet high doses tend to increase paradoxically the edema. This effect first noted with O-(β-Hydroxyethyl)-rutosides (23), also occurs with coumarin (26), which is a benzo-*alpha*-pyrone, rather than a benzo-*gamma*-pyrone. From the reported actions of calcium dobesilate in reducing high-protein edema (mentioned earlier, and the results on acute lymphedema — reported here) and from the contrasting effects of low and high doses on burn edema (reported here), similar anomalous actions like benzo-pyrones are seen. Thus, low dose reduces and high dose aggravates high-protein edema in the rat foot; the latter phenomenon perhaps is related to release of histamine. Elsewhere in the rat both doses reduce high-protein edema with the higher dose more effective.

It is also of interest that rats injected with calcium dobesilate recovered from the anesthetic more quickly than controls, a phenomenon noted using benzo-pyrones which act on the liver to antagonize barbiturates (7, 13).

Although various benzo-pyrones differ in their actions, both quantitatively and, at times, qualitatively (5, 7, 13, 16); overall their activity in improving high-protein edema, is similar. It is noteworthy, therefore, that calcium dobesilate functions like benzopyrones but without a pyrone ring. Whereas, calcium ions may assist in these actions (31), a similar molecule in which this ion is replaced by diethylammonium ("Etamsylate") acts similarly (30). Perhaps, it is the benzene-ring, appropriately modified by substitution, which is essential to both these groups.

Modifying the side-chains of benzo-pyrones alters certain of its biological properties (4, 7). A drug which treats high-protein edema without undesired side effects and without losing potency is ideal. Perhaps calcium dobesilate fits the bill.

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