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# The effects of diosmin (a benzo-pyrone) upon some high-protein oedemas: lung contusion, and burn and lymphoedema of rat legs

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*Abstract*

**Oral diosmin (a benzo-pyrone) was used to treat rats with contused lungs, in doses of 50 or 200 mg/kg/day. The contusion was produced by direct trauma. The lungs were examined with the electron microscope, both qualitatively and quantitatively, at 1, 2 and 4 days. It was found that diosmin considerably reduced interstitial oedema and tissue disorganization. The concentration of protein, both in the interstitial tissue and in the air spaces, was also much reduced. There was a greater effect at the higher dosage than at the lower one. In two other experiments, this drug was used in the usual models of burn oedema of the rat foot and acute lymphoedema of the rat leg – estimating the amount of oedema by weighing the parts. A low dose (50 mg/kg) reduced the burn oedema; a high dose (200 mg/kg) did not. Both doses reduced the acute lymphoedema of the whole leg, or thigh. The high dose did not do this in the foot, but the low one did. At high doses, diosmin has the usual benzo-pyrone property of releasing mediators in the rat-foot. In other tissues (and, no doubt, species) this drug reduces many forms of high-protein oedema, like the other benzo-pyrones.**

*Introduction*

There are many experimental results which show that the benzo-pyrones reduce high-protein oedemas [3, 5, 6]. This group of drugs, which contains many pharmacologically active substances, also have a variety of other actions in the body [3, 5, 6, 13, 15]. They tend to prevent injury to blood vascular endothelium, prevent the desquamation of endothelium (endothelaemia) and act as vitamin P substances in reducing capillary fragility; they also stabilize cellular and lysosomal membranes, increase haemostasis (probably by protecting adrenalin), open arterio-venous anastomoses and relax other smooth muscle (in arteries and veins, the ureter and many glandular ducts); they increase the filling of, and the pumping by, the collecting lymphatics; they tend to prevent the aggregation of erythrocytes and platelets; they stabilize the interstitial tissue. While high doses of many benzo-pyrones actually release histamine and 5-HT in the feet of dextran-reactor rats [17, 18], they usually reduce the effects of all mediators of inflammation – both on the fine structure of the blood capillary and on the resulting high-protein oedema.

Their actions to reduce high-protein oedemas are largely related to their increasing the normal proteolysis by macrophages, and increasing the number of these cells at the site of the oedema [3, 5, 6]. While their other actions also often assist in the lessening of high-protein oedema, these others do not occur with all the benzo-pyrones; indeed some of them may be reversed.

One group of high-protein oedemas which has so far received relatively little attention is contusion following trauma. A mixture of coumarin and troxerutin (both benzo-pyrones) has been shown to considerably reduce the amount of injury and high-protein oedema in contused lung [9]; the specific macrophage poison, silica, abolished this [10]. These benzo-pyrones were also effective in reducing the oedema associated with osteotomy and osteosynthesis [16]. Crushing injury of the rat leg was also made considerably less oedematous by this mixture [24, 25], or by coumarin alone [19]. A few human trials have shown considerable lessening in oedema following trauma, both accidental and surgical [5, 6].

Synthetic diosmin (Hommel) has been shown to have many of the usual properties of the benzo-pyrones [2, 11]. In view of the relative paucity of studies on the effects of the benzo-

pyrones on contusions, and of the few of these drugs tested on the lung, we decided to examine its effects on contused rat lungs. In addition, since diosmin has not been tested in two models which have proved very valuable in the investigation of other high-protein oedemas, we decided to test diosmin in burn and acute lymphoedema of rats' legs.

This last model is particularly important, since the benzo-pyrones are the only medical form of treatment of this disease, from which 100,000,000 people suffer [27]. The benzo-pyrones actually remove the excess fibrosis in this condition, although this takes months [5, 6, 22]. Besides lymphoedema, about one third of a Western, industrialized population each year receive medical treatment for high-protein oedemas, which are far more common than is usually realized [6]. In addition to the pain, swelling and poor oxygenation of the tissues [6], if such oedemas last for two months in rats they cause chronic inflammation [8], just as was hypothesized by WILLOUGHBY and DI ROSA [28]. The alterations in the tissues seen in chronic lymphoedema and elephantiasis are almost entirely caused by this simple accumulation of excess protein [6, 8, 14].

*Materials and methods*

**Lung contusions**

Thirty-six male Wistar hooded rats (250 g ± 25 g) were divided into three equal groups, fed *ad libitum* on rat nuts. Diosmin (Hommel AG, CH-8134 Adliswil, Switzerland) was pulverized in a mortar, suspended in 0.8% Tylose (methyl cellulose) in water, and administrated via a stomach-tube. One ml of the suspension, per rat, was given just after the injury and this was repeated 12 hours later, and each day, until death. One group received 200 mg/kg, another 50 mg/kg, the last just the Tylose. The contusion was made following the method of ECKERT et al. [12], under pentobarbital anaesthesia. Briefly, an incision was made 1–2 cm from the mid-line, two ribs were gently separated, and a portion of the ventero-lateral aspect of the right lungs was gently squeezed with forceps for 10 seconds. The wound was dusted with penicillin and streptomycin powder, and rapidly closed while the lungs were inflated with positive pressure.

One, two and four days later, four rats from each group were anaesthetized with pentobarbital. Fixative was injected via the trachea, which was then ligated to prevent collapse of the lungs. The chest was then opened, and the injured portion of the lung isolated with ligatures, excised and placed in more fixative, for 3 hours, at 4°C. The fixative was 4% glutaraldehyde in MILLONIG's [20] buffer, with 8% glucose and 8g% dextran (mol. wt. 70,000). (These last two were added to prevent hydration changes during fixation – 1.) The tissue was then cut into small pieces. Post-fixation, in 2% osmium tetroxide in the buffer, was performed for 1 hour. The tissue was then stained with uranyl acetate (0.5%, for 1 hour in 70% alcohol), dehydrated in acetone, and embedded in araldite. The contralateral, uninjured, lung was treated similarly in all cases.

Thin sections for electron microscopy, were stained with lead citrate (pH 11) and examined in a Siemens Elmiskop I, equipped with a Faraday cage. The latter was used to estimate protein concentrations [4] in the air spaces and interstitial tissue [17], using that of the plasma in the blood capillaries as a standard, with five estimations of randomly selected regions per site, per section. Because of the three different exposure times, there were 4 rats per subgroup; four blocks were obtained from each; one section was examined per block. The amount of oedema was estimated, on a scale of 0–4, without knowing from which animal the section orginated. The statistical significances of differences were estimated using *t*-tests, since in every case the values were approximately normally distributed.

**Burn oedema**

An inbred strain of hooded Wistar rats were used. While the environmental temperature was maintained constant at 20°C ± 0.5, the amounts of odema in the burnt legs were individually corrected for any variations caused by alterations in this or in the animal's activity, by using the unburnt leg as a control. Diosmin was administered as in the previous experiment. One ml of the suspension, per rat, was given just after the injury and this was repeated 12 hours later. One group received 200 mg/kg, another 50 mg/kg, the last just the Tylose.

Female hooded rats (200 g ± 25 g) were divided into three groups of 15. Under pentobarbital anaesthesia, one foot was mildly burnt, to the ankle, by immersing it in water at 54°C for 60 seconds. The volumes of the burnt and the control feet, up to the ankles, were estimated by cutting off the paws and weighing, after 24 hours. Significances of differences were estimated by *t*-tests, using the ratios of the weights of the {(lymphoedematous site – normal)/(normal)} as independent observations. it was established, graphically, that these were approximately normally distributed.

**Acute lymphoedema**

Three groups of 15 male hooded rats (200 g ± 25 g) were anaesthized with pentobarbital and given lymphostasis in one leg – with the other serving as a control. 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 they were free of the 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, but every other vessel was.

Diosmin was administered as in the previous experiments. One group received 200 mg/kg, another 25 mg/kg, the third just the Tylose. These doses were repeated each day, until death.

The animals were killed at 96 hours after operation, their legs were cut off at the ligatures (and at the equivalent position on the control side) and weighed. The tibio-calcaneal joint was disarticulated and the foot was also

weighed. (This is necessary because sometimes the benzopyrones release so much histamine into the foot that it increases in size, while still reducing the oedema of the thigh.) Significances of differences were estimated as in the previous experiment.

*Results*

**Lung contusion**

The normal, contralateral, lungs corresponded to earlier descriptions [23, 26]. One day after the injury considerable concentrations of protein, many erythrocytes and some phagocytes (mainly neutrophils), together with cell debris, fibrin and granules from Type II alveolar cells, were present in the air spaces (Figs 1 and 2). Many alveolar cells were damaged. The interstitial tissue contained high concentrations of protein, many erythrocytes, and was very oedematous. There were occasional tears in the blood capillary walls. One day later, the picture was much the same, except that the tissue seemed rather more disorganized and some lysis of the erythrocytes had commenced (Figs 3 and 4). The air spaces were often completely occluded by debris. Macrophages were frequently present After four days (Figs 5 and 6), the concentration of protein in both the air spaces and the interstitial tissue was reduced. The oedema was also less. The debris in the air spaces was reduced. The macrophages were very numerous.

Treatment with diosmin (Figs 2, 4 and 6) obviously reduced the amount of oedema and the concentrations of protein in both the air spaces and the interstitial tissue, paticularly on the second and fourth days. While this could be seen qualitatively, it was most evident from the quantitative measurements (Table 1). In the untreated animals all these parameters decreased with time. While on the first day they were mildly reduced by treatment, the effects of diosmin became more and more marked as time progressed – with the higher dose being more effective. The amounts of debris were difficult to estimate quantitatively, but it seemed that the treated animals had less debris and less fibrin in their air spaces, than did the untreated ones – particularly by the fourth day.

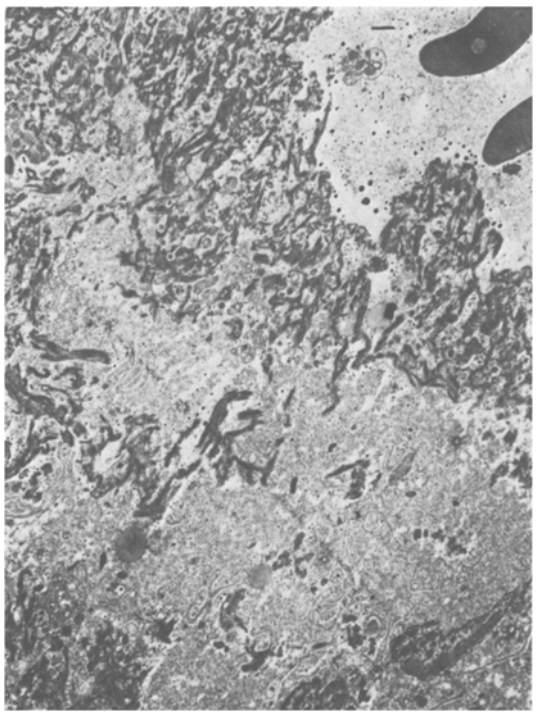

*Figure 1*
A contused lung, after one day; not treated with diosmin. There is much protein in the air space, which includes much fibrin. × 2500. (Bar is 1 $\mu$m.)

*Figure 2*
As for Fig. 1, but treated with diosmin (200 mg/kg). Considerable oedema is present in the interstitial tissue, and the air space contains a high concentration of protein. although rather less than in Fig. 1. × 2500.

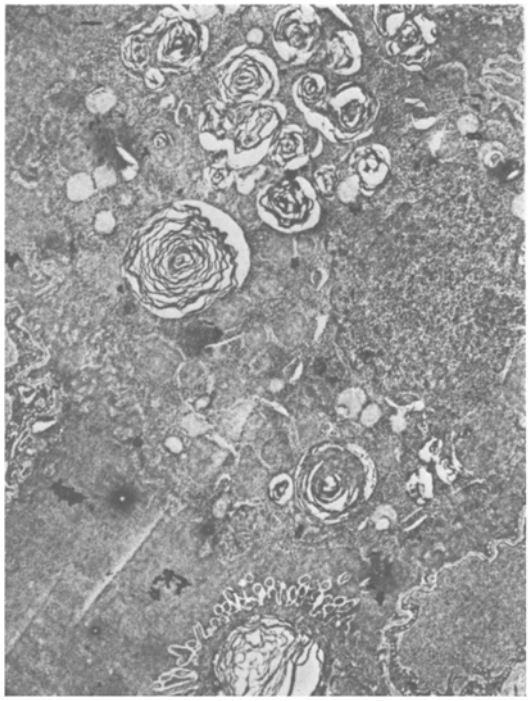

*Figure 3*
A contused lung, after two days; not treated with diosmin. A very high concentration of protein is present in the air space, plus much cellular debris. The disorganization of the tissue is so great that it is difficult to decide what is air space and what is alveolar wall. × 4000.

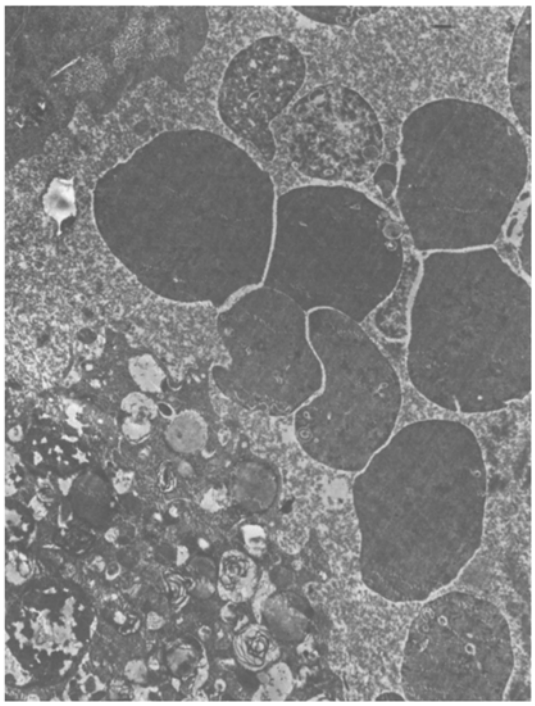

*Figure 4*
As for Fig. 3, but treated with diosmin (200 mg/kg). The concentration of protein in the air space is rather less, as is the amount of tissue disorganization, although it is still considerable. There are many erythrocytes in the air space, as were often found in both treated and untreated animals. They are starting to be lysed. × 4000.

**Burn oedema**

The results for the burn studies are shown in Table 2. There was no alteration in the amount of oedema at the high dose of the drug. Visually, also, the feet looked identical. At the lower dose there was a decrease in the amount of oedema to 75% of the control. This difference, and that between the two doses was significant at the 5% level.

**Acute lymphoedema**

In the experiment using acute lymphoedema it was obvious, visually, that the animals treated with diosmin had less oedema. From Table 3 it can be seen that there were no significant differences between the two doses for the whole leg or thigh, but there was for the foot (significant at the 1% level). The drug reduced the oedema of the whole leg to about 50% of the control value; the oedema of the thigh was reduced to about 35% of the control. Both these reductions were significant at the 1% level. By contrast, at the high dose level of the drug the oedema of the foot was slightly increased, although not significantly so; it was significantly (5% level) reduced by the low dose.

*Discussion*

It is evident that diosmin considerably reduces high-protein oedema, similarly to the other benzo-pyrones. This general effect was demonstrated in these experiments using three different models. In spite of all the actions of this group of drugs on blood vessels and lymphatics, it can be shown that this effect still occurs in a high-protein oedema when the blood vessels are normal and the lymphatics are occluded; it is prevented by selectively poisoning the macrophages [3, 5, 6, 10]. It appears very likely that diosmin may have a similar mode of action in high-protein oedema.

A further similarity, between the actions of the benzo-pyrones in general and those of

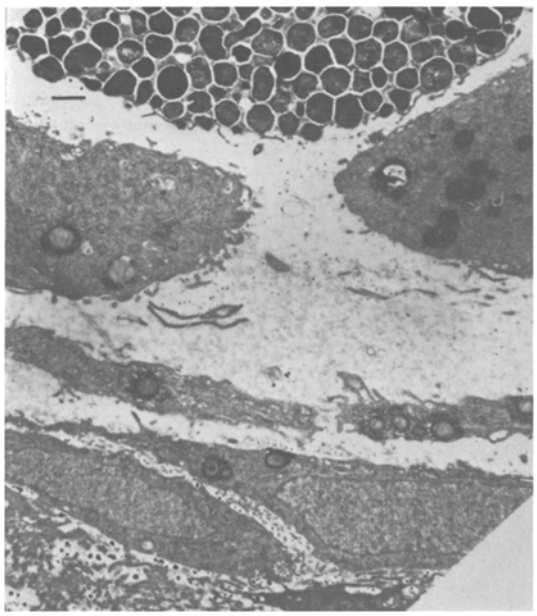

*Figure 5*
A contused lung, after four days; not treated with diosmin. There is still considerable oedema in the interstitial tissue, and much protein in the air space. × 4000.

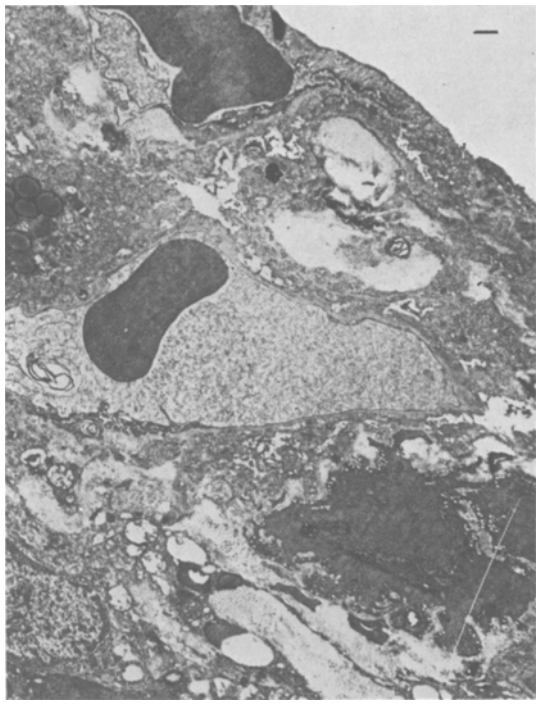

*Figure 6*
As for Fig. 5, but treated with diosmin (200 mg/kg). There is little protein in the air space, and the interstitial tissue has much less oedema. While there are still signs of injury in the cells, tissue organization is much improved. × 2500.

*Table 1*
Quantitative parameters of lung contusion, treated with diosmin.

| Normal, contralateral | | | Contusion | | |
|---|---|---|---|---|---|
| | | | 1 day | 2 days | 4 days |
| Therapy | | Protein concentration (relative to plasma in blood capillaries) | | | |
| In air spaces | None | 0.015 [0.00040] *240* | 1.63 [0.022] *80* | 1.37 [0.035) *80* | 1.08 [0.013] *80* |
| | 50 mg/kg | 0.015 [0.00040] *240* | 1.44 [0.020] *80**** | 1.13 [0.027] *80**** | 0.79 [0.0098] *80**** |
| | 200 mg/kg | 0.015 [0.00040] *240* | 1.35 [0.014] *80**** | 1.07 [0.018] *80** | 0.73 [0.010] *80**** |
| In interstitial tissue | None | 0.25 [0.0020] *240* | 1.24 [0.019] *80* | 0.95 [0.013] *80* | 0.81 [0.014] *80* |
| | 50 mg/kg | 0.25 [0.0020] *240* | 0.84 [0.014] *80**** | 0.75 [0.018] *80**** | 0.44 [0.0099] *80**** |
| | 200 mg/kg | 0.25 [0.0020] *240* | 0.78 [0.018] *80*** | 0.68 [0.012] *80**** | 0.31 [0.012] *80**** |
| | | Amount of interstitial oedema (estimated on scale of 0–4) | | | |
| | None | 0.01 [0.005] *48* | 3.66 [0.12] *16* | 3.20 [0.16] *16* | 2.21 [0.15] *16* |
| | 50 mg/kg | 0.01 [0.005] *48* | 3.31 [0.14] *16** | 2.78 [0.14] *16** | 1.75 [0.11] *16** |
| | 200 mg/kg | 0.01 [0.005] *48* | 3.17 [0.17] *16*$^{NS}$ | 2.50 [0.16] *16*$^{NS}$ | 1.28 [0.10] *16**** |

The means are given, followed by their standard errors ([. . .]), and the numbers of observations (*in italics*). The significance of the differences between a row and the one directly above it are indicated.
NS indicates a significance of less than 5%; * between 1 and 5%; ** between 0.1 and 1%; *** less than 0.1%. The significance of the differences between the columns are not shown, but were usually less than 0.1%, except for oedema, where the means for the various days were significant at the 5% level.

*Table 2*
Effects of diosmin on mild burn oedema of the rat foot, at 24 hours. (Expressed as: (burnt leg – normal)/normal; in g/g.)

| Dose | Saline | Diosmin |
|---|---|---|
| 50 mg/kg | 0.79 [0.051] | 0.58 [0.089]* |
| 200 mg/kg | 0.84 [0.057] | 0.81 [0.062]$^{NS}$ |

There were 15 animals per group; the means are shown, followed by their standard errors [in brackets].
NS implies that the result was not significant at the 5% level; * that it was significant at the 5% level.
There was a significant difference (at the 5% level) between the two dose levels of Diosmin.

*Table 3*
Effects of diosmin on acute lymphoedema at 4 days. (Expressed as: (lymphoedematous site – normal)/normal; in g/g.)

| Site | Saline | Diosmin | |
|---|---|---|---|
| | | 50 mg/kg | 200 mg/kg |
| Whole leg | 0.30 [0.044] | 0.18 [0.019]** | 0.15 [0.020]** |
| Thigh | 0.35 [0.075] | 0.13 [0.021]** | 0.12 [0.023]** |
| Foot | 0.16 [0.021] | 0.10 [0.023]* | 0.19 [0.029]$^{NS}$ |

There were 15 animals per group; the means are shown, followed by their standard errors [in brackets].
NS implies that the difference between the drug and the control was not significant at the 5% level; * that it was significant at the 5% level; ** that it was significant at the 1% level.
The differences between the two dose levels for the whole leg or the thigh were not significant, but that for the foot was significant at the 1% level.

diosmin, is the anomalous effect they produce on the rat's foot (in those strains which are dextran-reactors – [17]). They often reduce oedemas of the foot when given in low doses, but increase them when given in high doses – because high dose levels of benzo-pyrones liberate histamine and 5H-T. This effect was first noted with *O*-(β-hydroxyethyl)-rutosides [18], but occurs also with coumarin [6,21], which is benzo-alpha-pyrone, rather than a benzo-gamma-pyrone. From the contrasting results on acute lymphoedema of the thigh and foot, at different doses, and from the contrasting effects of low and high doses on burn oedema, it can be seen that diosmin also has similar anomalous actions on this unique tissue.

The actions of diosmin on the contused lung show that it has the usual effects of benzo-pyrones on a form of injury quite different to lymphoedea and burn where this similarity has been demonstrated earlier. This action on many forms of high-protein oedema seems to be common to all the benzo-pyrones (see introduction), although varying in degree from one to another. This general action no doubt depends to some extent on the many effects these drugs have on different parts of the microcirculation.

Some of the benzo-pyrones [6] appear to reduce the mediated and direct injuries to blood vessels. Diosmin also does this, probably greatly aided by its rhamnosido-glucose side-chain [2, 11]. However this effect is often difficult to establish, because their other actions may confuse the observations; certainly some benzo-pyrones actually increase vascular injuries (above). However, all that have been tested, act as vitamin P's – reducing the excess capillary fragility caused by a P-deficiency.

Many benzo-pyrones increase the pumping of lymph in the collecting lymphatics; however, they still reduce high-protein oedemas if the lymphatics are completely occluded [3, 6]. It has in fact been shown, in a number of different ways, that the main reason for the reduction of high-protein oedemas by benzo-pyrones is via their enhancement of the normal proteolysis by macrophages, and perhaps other cells [3, 6, 14]. This is partly by their causing increased numbers of macrophages to be present at the sites of high-protein oedema, and partly by their increasing the amount of proteolysis by each cell.

Apart from their other indications, the benzo-pyrones are starting to be used in lymphoedema [3, 5, 6, 14, 22]. What is not often realized is that they are effective in all forms of high-protein oedema – because they hasten the removal of the protein and thus the oedema. If such an oedema lasts for some weeks, it causes chronic inflammation [8], as postulated by WILLOUGHBY and DIROSA [28]. It is now becoming obvious that high-protein oedemas are much more common than has often been thought [6]. Hence the benzo-pyrones probably have many more indications than have been previously realized.

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