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The Effect of Ischaemia on Acid Phosphatase, ß-Glucuronidase and Lactic Acid Dehydrogenase in Lymph from Hind Paw of the Rabbit

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Summary

The effect of ischaemia of the hind paw of the rabbit on the release of the lysosomal enzymes, acid-phosphatase and β-glucuronidase, and of lactic acid dehydrogenase (LDH) was assessed by measuring the activities of these enzymes in the lymph from the paw before and at varying intervals after the release of a tourniquet applied for 4 hr just above the ankle.

Histologically, this degree of ischaemia produced considerable necrosis of the plantar muscles with ultimate regeneration within 4 weeks.

The levels of activity of the lysosomal enzymes in lymph from the normal paw were less than those in plasma. The mean lymph:plasma ratios were 0.38 for total protein, 0.28 for acid-phosphatase and 0.08 for β-glucuronidase, suggesting that these enzymes in the lymph were derived mainly from the plasma with a degree of molecular sieving at the blood capillary wall. Ischaemia increased the permeability of the capillaries to all these macromolecules, but there was no evidence of any appreciable release of lysosomal enzymes from the injured cells or from the invading phagocytic cells.

During the period of muscle degeneration from 1 to 7 days after release of the tourniquet, the level of activity of β-glucuronidase was significantly less in lymph from the affected paw than in lymph from the normal paw. It seems likely that in the areas of muscle necrosis the enzyme in the tissue fluid originating from the blood plasma was involved in the lytic processes of the damaged muscle cells.

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In contrast to the changes in levels of activity of the lysosomal enzymes, the activity of LDH in the lymph increased to a level many times greater than in the plasma. The levels of activity of LDH were the same in the venous blood coming from the paw as in arterial blood, indicating that when an enzyme is released into the tissue fluid from the injured cells it is returned to the blood stream by way of the lymphatic vessels.

In hypovolaemic shock following tissue injury or haemorrhage, the levels of activity of lysosomal enzymes in circulating plasma increase (3, 6, 28, 29, 35). Evidence suggests that the most likely sources of the increased enzyme activity in the plasma are the cells of the liver and intestine. The circulatory changes in shock give rise to a condition of severe hypoxia of the liver and gut which leads to disintegration of the lysosomal membrane with dispersal of the enzymes in the cells and ultimately in the tissue fluid (4, 28, 33, 40). The resulting increased levels in the tissue fluid are first reflected in the levels in the thoracic duct lymph (21, 40), although *Barankay* et al. (1a) have not been able to confirm this. It has been postulated that the lysosomal enzymes in the extracellular fluid might lead to changes in the microcirculation which adversely affect the course of the clinical shock state (27).

Janoff et al. (28) have also reported that ischaemia of the intestine leads to an increase in lysosomal enzymes in the circulating plasma. In other conditions, for example the surgical procedures involved in transplantation, a period of severe hypoxia of the tissue concerned is unavoidable. It was decided, therefore, to study further the levels of activity of lysosomal and other enzymes in the extracellular fluid following a period of complete ischaemia of various tissues.

The present experiments concern the changes in the levels of activity of the lysosomal enzymes, acid phosphatase and β-glucuronidase, and of lactic acid dehydrogenase in blood plasma and in lymph from the hind paw of the rabbit at intervals after a period of ischaemia. It was thought that any changes in the rate of release of enzymes from the cells of the ischaemic tissues into the tissue fluid would first be reflected in the levels of activity in the lymph draining the affected region. The tissues concerned here are mainly skin and muscle which are not as in rich lysosomes as the intestinal mucosa (19, 34, 38).

Methods

Rabbits of either sex and of 2.5 to 4 kg body weight were used. Ischaemia of one hind paw was produced by a tourniquet applied just above the ankle with the animal under nembutal anaesthesia. In preliminary experiments the tourniquet was released after ½, 1, 2, 4 or 6 hr but in the experiments to be reported the period of ischaemia was maintained for 4 hr.

In one group of animals, blood and lymph samples were collected under nembutal anaesthesia over a period of 2 hr immediately after release of the tourniquet. In other groups the rabbits were allowed to recover after release of the tourniquet and blood and lymph samples were then collected 1, 3, 7 or 28 days later. For this purpose the animals were again anaesthetized with nembutal intravenously. Blood samples were obtained from a cannula in the carotid artery and in some experiments blood samples were also taken from the vein draining the hind paw. Lymph was collected over a period of 2 hr

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from the afferent vessels to the popliteal node about midway between the ankle and knee joints and lymph flow was attained by passive movement of the legs. Clotting was prevented in both blood and lymph samples by powdered heparin and all samples were centrifuged to remove the cells. They were then kept on ice until analysed on the same day.

At the end of each experiment, the popliteal node together with samples of skin from the dorsum of the paw and of the plantar muscle were removed from both the normal and affected sides and fixed in formol saline. Paraffin sections of these tissues were stained with haematoxylin and eosin.

The total nitrogen concentrations in the lymph and plasma samples were determined by the microkjeldahl method. After allowing for non-protein nitrogen, protein concentration was calculated assuming a mean nitrogen content of protein of 16 per cent.

The levels of activity of lactic acid dehydrogenase and of the lysosomal enzymes, acid-phosphatase and β-glucuronidase, were measured in blood plasma and in lymph by the following methods:

Lactic acid dehydrogenase (LDH) (L-lactate: NAD-oxidoreductase EC 1.1.1. 27.) was determined by the method of *Wrôblewski* and *La Due* (44). The assay was carried out in 0.05 M phosphate buffer, pH 7.5, containing 3×10^{-4} M sodium pyruvate and a final concentration of 1.3×10^{-4} M β-dihydronicotinamide adenine dinucleotide (NADH). The decrease in optical density was read at 340 nm at minute intervals extending over a total period of five minutes.

A unit of LDH activity was expressed as the amount of LDH which changes the optical density of NADH at 340 nm by 0.001 in one minute in a 3 ml assay mixture at 24–27 °.

Acid phosphatase (orthophosphoric acid monoester phosphohydrolase, EC 3. 1. 3. 2.) was determined by using the method of *Bessey* et al. (5). Activity was assessed by the amount of p-nitrophenol released from the disodium salt of p-nitrophenol phosphate at pH 5.0 in 0.05 M citrate buffer. Samples, sample blanks and reagent blanks were incubated for 30 minutes at 37°, the hydrolysis was stopped and the chromagen was developed on the addition of alkali. The amount of p-nitrophenol released was determined from a standard curve.

Activity was expressed as the amount of p-nitrophenol (μg) released per hr, per ml of plasma or lymph.

β-*D glucuronidase* (β-D-glucuronide glucuronohydrolase EC 3. 2. 1. 3. 1.) was determined by a modification of the method of *Talalay* et al. (4), using phenolphthalein glucuronide as substrate at a final concentration of 2×10^{-4} M. Blood plasma and lymph plasma samples were diluted with 0.1 M acetate buffer, pH 4.6. Samples and blanks were incubated for 16 hr at 37 °. Phenolphthalein released by the enzyme was developed by the addition of alkaline-glycine buffer pH 11.2, the removal of proteins by TCA was found not to be necessary. The amount released was determined from a standard curve of phenolphthalein.

Activity was expressed as the amount of phenolphthalein (μg) released per hr of incubation, per ml of sample.

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Results

Histological Changes

The preliminary experiments showed that the histological changes in muscle and skin were in general related to the time that the paw was made ischaemic. In the experiments reported here, however, a period of 4 hr was used because this degree of ischaemia resulted in considerable degeneration of the plantar muscles followed ultimately by almost complete regeneration.

Immediately after release of the tourniquet applied for this time the paw became somewhat oedematous and the flow of lymph from the affected paw was two or three times greater than that from the normal paw. The red blood cell content of the lymph also increased to reach values of 5,000 to 100,000 per mm^3. The histological changes in muscle and skin at the end of lymph collection, 2 hr after release of the tourniquet, are shown in Figs. 1a and 1b. Red blood cells were evident in the tissue fluid of both muscle and skin, but at this stage polymorphonuclear neutrophils were not present.

One day after removal of the tourniquet red blood cells were still present in the tissue fluid in both muscle and skin and in the lymph at about the same levels as observed in the first 2 hr. The popliteal lymph node of the injured leg also contained numerous red cells, some free in the lymph sinuses and some in macrophages; these red cells had been transported by the lymphatic vessels from the paw. The muscle showed evidence of degeneration and often numerous polymorphonuclear leucocytes were seen (Fig. 1c), although in the skin this infiltration with polymorphs was not observed (Fig. 1d). Three days after removal of the tourniquet, there was no longer evidence of increased polymorphonuclear leucocytes or red blood cells in the muscle; red cells were also no longer present in the tissue fluid of the skin or in the popliteal lymph nodes. Muscle degeneration, however, and the beginning of regeneration were observed. These changes were strikingly seen 1 week after removal of the tourniquet. Figs. 2a, b, and c show areas of extensive degeneration with evidence of regeneration. By four weeks after release of the tourniquet, most of the muscles had regenerated although in some areas the muscle was not completely restored to normal, Fig. 2d.

Concentrations of Total Protein and Levels of Activity of Lysosomal Enzymes in Plasma and in Lymph from the Normal Paw

The mean levels of protein and of activity of the lysosomal enzymes, acid-phosphatase and β-glucuronidase in blood plasma and in lymph from the normal paw in a group of

Fig. 1a Section of plantar muscles, 2 hr after release of tourniquet, showing the extravasation of red blood cells into the tissue fluid. Haematoxylin and eosin, 350×.

Fig. 1b Section of skin, 2 hr after release of tourniquet, showing extravasation of red blood cells into the tissue fluid and dilated lymphatic vessels. Haematoxylin and eosin, 350×.

Fig. 1c Section of plantar muscles, 1 day after release of tourniquet, showing infiltration with polymorphonuclear neutrophils. Red blood cells are still present in the tissue fluid. Haematoxylin and eosin, 350×.

Fig. 1d Section of skin, 1 day after release of tourniquet, showing presence of red blood cells in the tissue fluid, but there are no appreciable numbers of neutrophils. Haematoxylin and eosin, 350×.

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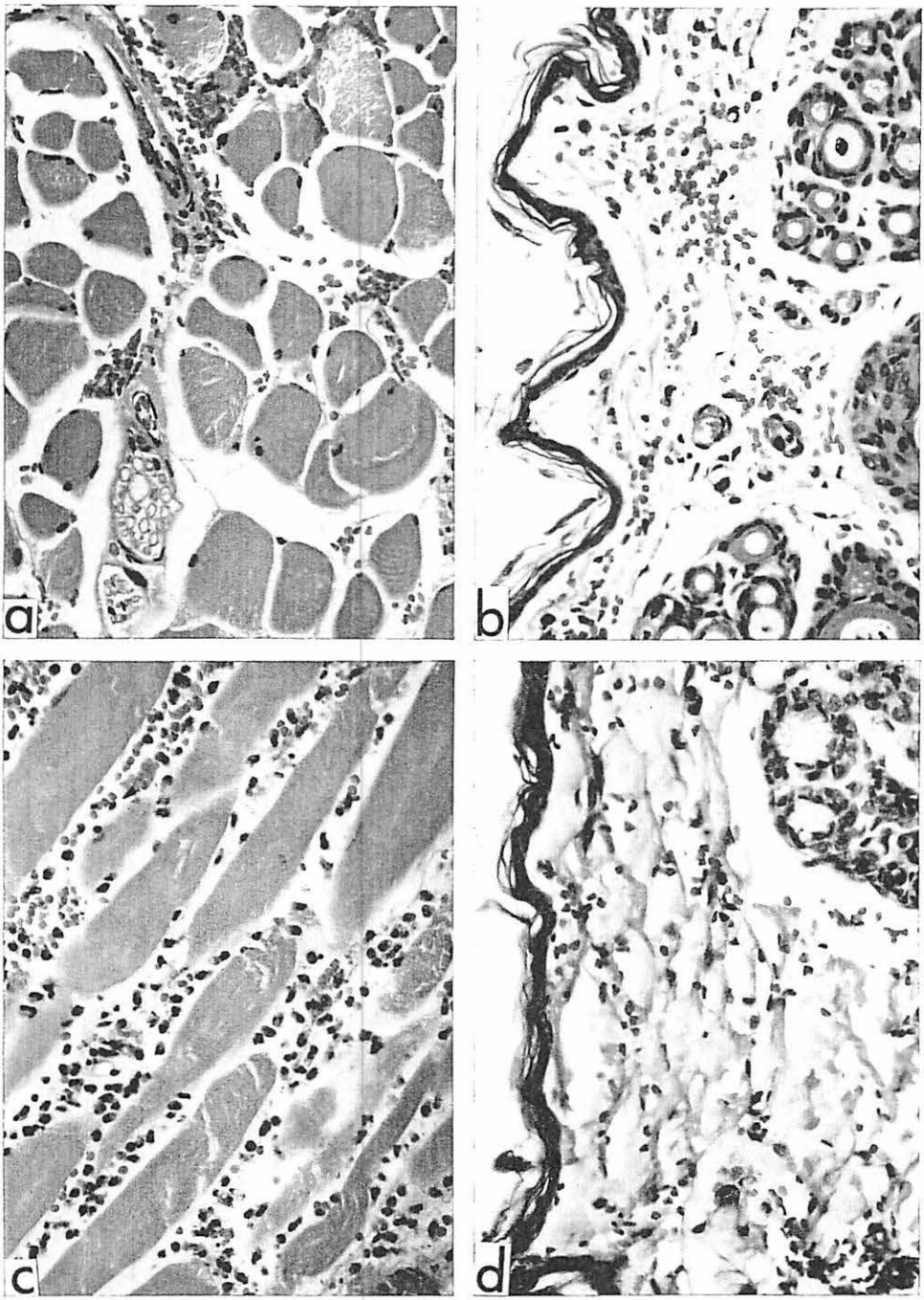

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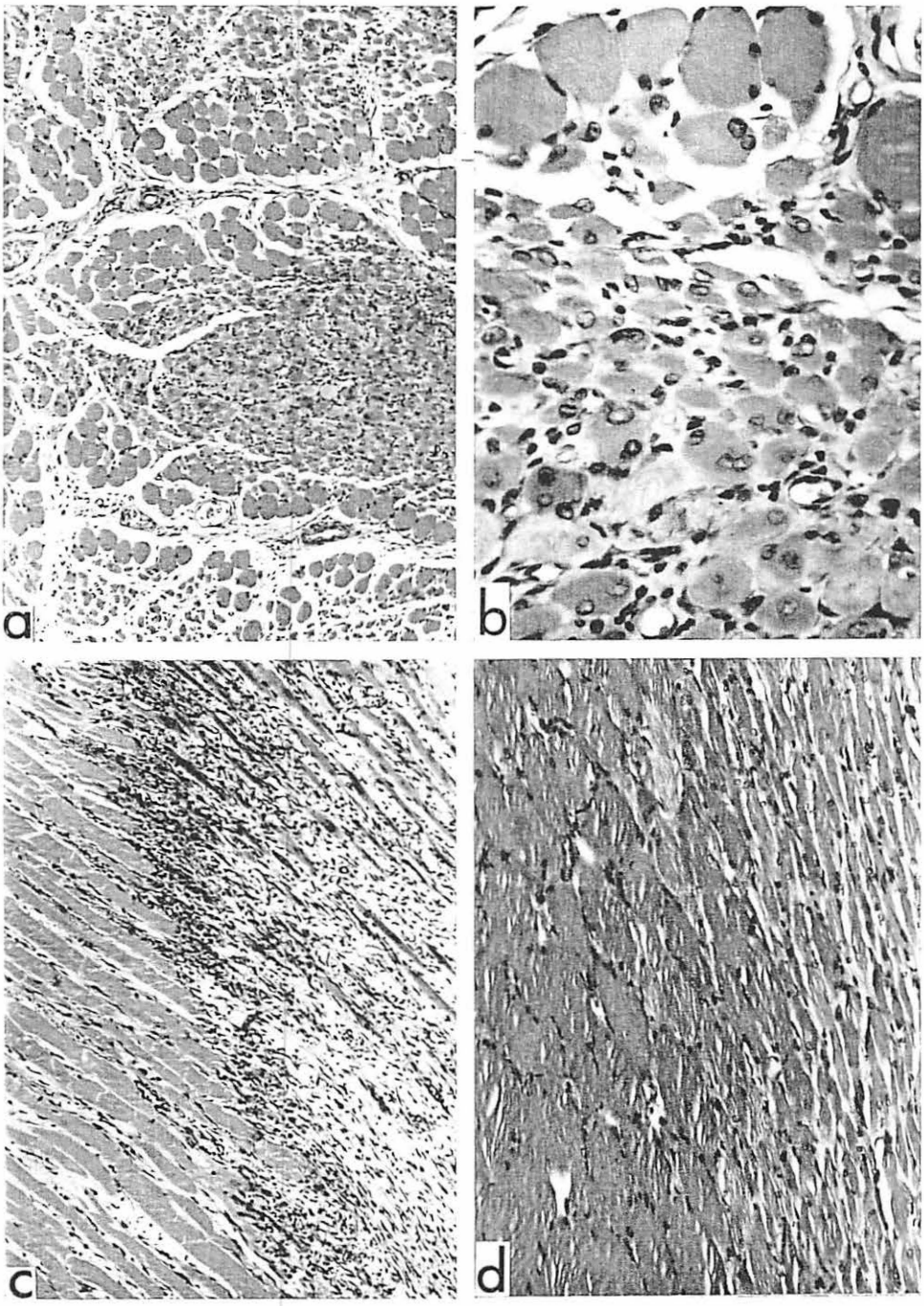

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Table 1 Concentrations of total protein and levels of activities of acid phosphatase and β-glucuronidase in arterial blood plasma and in lymph from the normal hind paw in a group of 35 rabbits. Values are the means and standard error of the means.

	Total protein g/100	Acid phosphatase Units	β-glucuronidase Units
Plasma	5.73 ± 0.12	78.3 ± 6.4	18.2 ± 1.7
Lymph	2.18 ± 0.09	20.8 ± 1.5	1.5 ± 0.2

35 animals are given in Table 1. In all cases the values are much lower for lymph than for plasma. There is, however, a relationship between the levels in lymph and plasma as shown in Fig. 3. This relationship is very clearly depicted when the individual lymph : plasma ($C_L : C_P$) ratios are plotted as in Fig. 4. The means of these ratios were 0.38 for total protein, 0.28 for acid-phosphatase and 0.08 for β-glucuronidase. These results are consistent with the view that the enzymes in the lymph arise mainly from the plasma and undergo a process of molecular sieving at the blood capillary wall.

The Effect of Ischaemia of the Paw for 4 hr on the Levels of Total Protein and of Activity of the Lysosomal Enzymes in Lymph and Plasma

The mean values in plasma and in lymph from the normal and affected hind paws at different times after removal of the tourniquet are given in Table 2. In those experiments in which lymph was collected for 2 hr immediately after release of the tourniquet the concentration of total protein and the levels of activity of acid phosphatase and β-glucuronidase in the lymph increased rapidly to reach a plateau within the first ½ hr; in no case did the levels reach those in the blood plasma. If the increase in the concentration of total protein in the lymph immediately after release of the tourniquet is regarded as an effect of increased permeability of the small blood vessels to macromolecules, the increases in the levels of activity of the lysosomal enzymes could be explained in the same way. The results show that for total protein and acid phosphatase the altered permeability was restored to normal in 3 to 7 days. This is clearly shown in Fig. 5 where the lymph : plasma ratios are depicted.

The changes in the levels of activity of β-glucuronidase in lymph during the recovery phase were somewhat different from those of total protein and acid phosphatase. In all 12 experiments in which lymph was collected 1, 3 and 7 days after release of the tourniquet the level of activity in lymph from the affected paw was considerably less than that in lymph from the normal paw. A paired "t" test shows that this difference is signi-

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Fig. 2a Section of plantar muscles, 1 week after release of tourniquet, showing areas of degeneration. Haematoxylin and eosin, 88×.

Fig. 2b same as (a), 350×.

Fig. 2c Section of plantar muscles, 1 week after release of tourniquet, showing an extensive area of degeneration with active regeneration. Haematoxylin and eosin, 88×.

Fig. 2d Section of plantar muscles, 4 weeks after release of tourniquet, showing almost complete regeneration. Haematoxylin and eosin, 140×.

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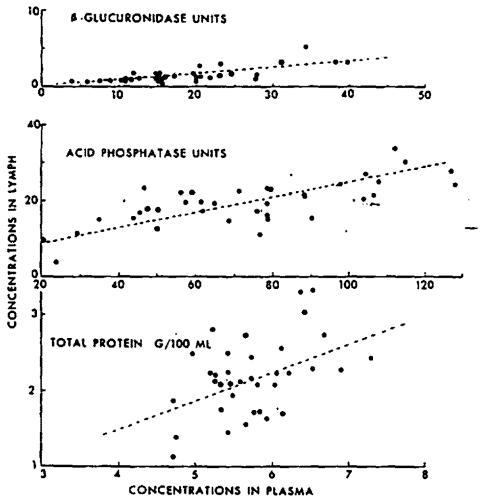

Fig. 3 The relationship between the concentration of total protein and the levels of activity of acid phosphatase and β-glucuronidase in plasma and in lymph from the normal paw. The broken lines represent the regression lines.

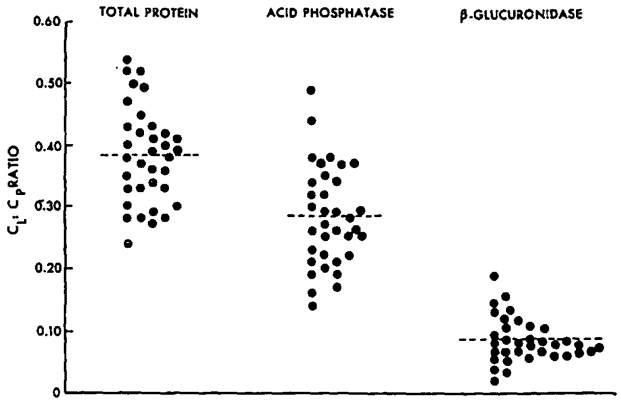

Fig. 4 The normal lymph: plasma ratios, C_L:C_P, of the concentration of total protein and of the levels of activity of the lysosomal enzymes, acid phosphatase and β-glucuronidase in 35 rabbits.

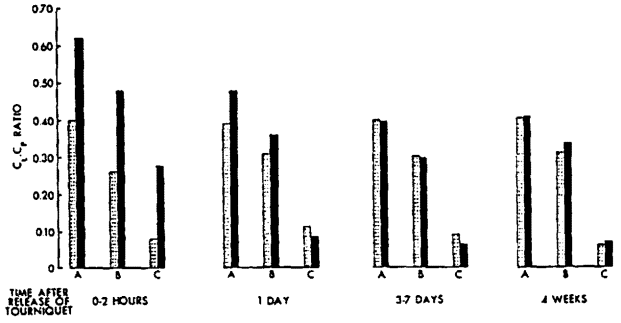

Fig. 5 The mean C_L:C_P ratios for total protein (A), acid phosphatase (B) and β-glucuronidase (C) at different intervals after release of the tourniquet calculated from data in Table 2.
normal paw
affected paw

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Table 2 Concentrations of total protein and levels of activities of acid phosphatase and β-glucuronidase in plasma and in lymph from the normal hind paw and from the affected hind paw at different times after release of tourniquet applied for 4 hr. The figures in brackets are the number of animals in each group. Values are the means and standard errors of the means.

Time after release of the tourniquet		Protein g/100 ml	Acid phosphatase Units	β-glucuronidase Units
	Plasma	5.61 ± 0.18	83.2 ± 8.5	19.2 ± 1.7
0–2 hr (8)	Lymph from normal paw	2.26 ±-0.20	20.6 ± 2.2	1.5 ± 0.3
	Lymph from affected paw	3.46 ± 0.24	38.8 ± 7.1	7.8 ± 1.7
	Plasma	6.08 ± 0.36	80.6 ± 25.8	22.3 ± 6.2
1 day (6)	Lymph from normal paw	2.42 ± 0.29	23.3 ± 6.4	2.2 ± 0.7
	Lymph from affected paw	2.92 ± 0.30	27.1 ± 6.5	1.5 ± 0.4
	Plasma	5.51 ± 0.17	64.7 ± 2.2	17.9 ± 1.3
3–7 days (6)	Lymph from normal paw	2.21 ± 0.12	19.2 ± 1.0	1.6 ± 0.3
	Lymph from affected paw	2.16 ± 0.14	18.7 ± 1.2	1.1 ± 0.1
	Plasma	5.78 ± 0.19	51.0 ± 3.8	14.5 ± 2.6
4 weeks (5)	Lymph from normal paw	2.32 ± 0.13	16.0 ± 1.5	0.8 ± 0.13
	Lymph from affected paw	2.34 ± 0.06	17.4 ± 1.9	0.9 ± 0.15

ficant ($0.01>P>0.001$). During this time there was considerable degeneration of the plantar muscles. By 28 days the levels of activity of β-glucuronidase in lymph from the affected paw was not significantly different from that in lymph from the normal paw.

The Effect of Ischaemia on Levels of Lactic Acid Dehydrogenase in Lymph and Blood Plasma

The effect of ischaemia on the levels of activity of LDH in the lymph was different from that on the levels of activity of the lysosomal enzymes. In normal lymph the level of activity of LDH was variable, being sometimes less and sometimes greater than the level in the plasma. The mean level in 35 animals was 336 ± 26 units compared with 206 ± 17 in the plasma. After release of the tourniquet, however, the level of activity of LDH in the lymph increased considerably and this activity increased further during

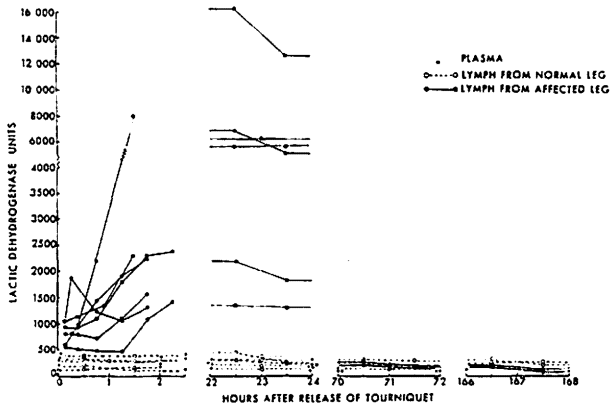

Fig. 6 The levels of activity of lactic dehydrogenase in plasma, lymph from the normal paw and lymph from the affected paw. Samples were collected over a period of 2 hr immediately after, 1 day, 3 days and 7 days after release of the tourniquet.

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the first day. These levels were always many times greater than the values in the plasma. By the third day, however, the levels in the lymph had returned to normal. These changes are depicted in Fig. 6.

The Concentration of Protein and Levels of Activities of the Enzymes in Leg Vein Blood

In all the experiments described so far the blood samples were obtained from the carotid artery. In ten experiments, blood samples were also taken from the vein draining the paw immediately after the sample was obtained from the carotid artery at the end of the period of lymph collection. Table 3 shows that the levels of activity of lactic dehydrogenase in leg vein blood plasma were not significantly different from those in arterial blood plasma. In the leg lymph, however, the levels were usually much greater depending on the time after release of the tourniquet.

Table 3 The levels of activity of lactic acid dehydrogenase in arterial blood plasma, in leg vein blood plasma and in leg lymph in ten animals. The lymph was collected for 2 hr and the blood samples were taken almost simultaneously at the end of lymph collection.

	Arterial plasma Units	Leg vein plasma Units	Leg lymph Units	Time after release of tourniquet
	326	324	400	Normal
	138	146	288	Normal
	296	218	830	0–2 hr
	248	270	1144	0–2 hr
	146	166	1320	1 day
	450	444	6100	1 day
	326	316	5680	1 day
	180	130	200	3 days
	138	144	440	3 days
	234	254	296	4 weeks
Mean and S.E. of mean	248 ± 32	241 ± 32	1670 ± 714	

Table 4 Mean levels (with SE of mean) of total protein and activities of acid phosphatase and β-glucuronidase in arterial blood plasma, leg vein blood plasma and in leg lymph. The samples were from the same experiments as in Table 3.

	Total protein g/100 ml	Acid phosphatase Units	β-glucuronidase Units
Carotid Artery	5.53 ± 0.18	48.6 ± 1.9	25.0 ± 4.0
Leg Vein	5.39 ± 0.17	52.3 ± 2.4	22.7 ± 4.2
Leg Lymph	2.58 ± 0.17	17.6 ± 1.4	1.6 ± 0.4

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The mean concentration of total protein and of the levels of activity of acid phosphatase and of β-glucuronidase were also not significantly different in arterial and leg vein blood plasma, Table 4. In the leg lymph the corresponding values were much lower as already described.

Discussion

In the present series of experiments the degree of ischaemia used affected the small blood vessels in both skin and muscle. In both these tissues, red cells escaped from the blood stream into the tissue fluid and were ultimately removed by the lymphatic vessels, passing through the popliteal node on their way to the blood stream. The permeability of these vessels to proteins was also increased as shown by local oedema and an increase in protein concentration in the lymph. Skin has a rich lymphatic network whereas in muscle the lymphatic vessels are not so prominent and are confined to the perimysium and epimysium. What proportions of the lymph collected in the present experiments were derived from the skin and the muscle of the paw could not be assessed. There is some evidence to suggest, however, that normally the concentration of protein in lymph from the muscle is similar to that in lymph from the skin (26). The most severe damage to cells, assessed histologically, occurred in the muscle. The degenerative changes followed by regeneration as seen by light microscopy were similar to those previously described for various types of muscle injury (8, 9, 22, 24, 25). Studies by electron microscopy which further support these findings have recently been reviewed by *Pellegrino* and *Franzini-Armstrong* (34).

Many enzymes with properties similar to those within cells are normally present in the extracellular fluid. The level of activity of any particular enzyme in the blood plasma remains fairly constant, suggesting that the entry from cells is counterbalanced by removal from the extracellular fluid (23, 39). In the lymph the level of activity of an individual enzyme is usually less than that in the plasma. Together with other proteins, enzymes continually move throughout the extracellular fluid from plasma to tissue fluid to lymph (c. f. 1, 11, 12, 45). In general, therefore, the level of activity of an enzyme in the lymph from any region reflects that in the plasma, but is also dependent upon capillary pressure, the fine structure of the blood capillary barrier and the size of the molecule concerned.

The results of the present series of experiments are consistent with this concept of the movement of macromolecules throughout the extracellular fluid. In lymph from the normal leg, the lymph : plasma ratios of protein and of the lysosomal enzymes vary inversely with molecular size. Although in these experiments the concentration of albumin has not been specifically determined, mean results for similar experiments in the rabbit have previously shown that if the mean lymph : plasma ratio for total protein is 0.38 that for albumin would be approximately 0.42 (10, 14, 36). Therefore, ratios of 0.28 for acid phosphatase and 0.08 for β-glucuronidase should be compared to 0.42 for albumin. The molecular weights of these three proteins are approximately 69,000 for albumin, 100,000 for acid phosphatase and 200,000 for β-glucuronidase (2).

The increase in the lymph : plasma ratios of the activities of the lysosomal enzymes immediately after the release of the tourniquet could be explained by the increased

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permeability of the capillary wall to these macromolecules. The relatively greater increase in this ratio for the larger molecules in lymph after injury is consistent with the findings for other macromolecules of different size (13, 14). The results suggest that the cells of the tissues concerned or the invading phagocytes are not the origin of any appreciable amount of lysosomal enzyme activity in the lymph. It would seem, therefore, that unlike the gut and liver where the cells are rich in lysosomes, ischaemia of the paw does not lead to the release into the tissue fluid of significant amounts of lysosomal enzymes, even though there is considerable damage to the muscle.

An interesting finding in these experiments is the lower level of activity of β-glucuronidase in lymph from the affected paw 1, 3 and 7 days after release of the tourniquet during the process of muscle degeneration. It seems possible that in these areas the β-glucuronidase that filters from the blood plasma is involved in the process of necrosis of the damaged muscle cells, so that less will enter the lymph.

The possible mode of action of the hydrolytic enzymes within the lysosomes has been extensively discussed in recent years (c. f. 15, 16, 17, 18, 20, 42, 43). These enzymes are optimally active in the acid pH range and their substrates are most, if not all, of the major constituents of the cells and connective tissue. When a tissue suffers injury, damaged cells may undergo lytic processes by the lysosomal enzymes that they contain themselves or by the enzymes of the phagocytic cells which migrate into the injured tissue. It would seem that in the present experiments considerable ischaemic degeneration and subsequent regeneration of muscle can occur without any significant release into the tissue fluid of lysosomal enzymes from the damaged cells or from the invading phagocytes. The evidence suggests, however, that the β-glucuronidase that filters from the blood plasma may be specifically involved in the lytic processes of the damaged cells. Other hydrolytic enzymes of the lysosomal system may be similarly involved.

Although the intensity of the injury in these experiments was severe, the extent was confined to a small region of the body so that a condition of hypovolaemic shock did not result. The levels of activity of the lysosomal enzymes in the blood plasma, therefore, did not significantly alter during the course of the experiments. The results would suggest, however, that if the extent of the injured area was greatly increased to produce hypovolaemic shock, any increase in the levels of activity of lysosomal enzymes in the plasma could not be explained by release from the cells of the damaged skin and muscle.

When there is an appreciable release of enzymes from cells into the tissue fluid, much higher levels are observed in the lymph from the region concerned (7, 30, 31, 32, 37). This has been shown to be true in the present experiments for lactic acid dehydrogenase, where levels of activity in the lymph were very much higher than in the plasma for 24 hours or more after release of the tourniquet. The results also clearly showed that the level of this enzyme in the venous blood plasma leaving the paw was the same as that in the arterial plasma. This confirms the view that, in general, when an enzyme is released from cells into the tissue fluid, it is returned to the blood stream indirectly by way of the lymphatic vessels.

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The Cultured Lymphocyte in Clinical and Experimental Medicine*

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Our understanding of the nature of the lymphocyte and its role in health and disease has undergone dramatic changes in the last decade. Known for almost one hundred years, peripheral lymphocytes had traditionally been considered short-lived with limited biologic activity and of little significance to the economy of the host. Recent studies, however, have clearly demonstrated that the circulating lymphocyte pool is composed of a spectrum of cells of varying origins, lifespans, fine structural features and capacities to mediate immunologic responses (1). A fortuitous observation by *Nowell* in 1960 (2) was a major impetus for this continuing series of investigations in lymphocyte biology. He noted that phytohemagglutinin (PHA), a crude extract of the common red kidney bean, had the remarkable ability to cause normal small lymphocytes from peripheral blood to undergo a series of morphologic changes to "blast like" cells in tissue culture. A variety of immunologic and non-immunologic stimuli were subsequently shown to initiate similar morphologic alterations in small lymphocytes associated with new RNA, protein, and DNA synthesis, followed by mitosis and cell division (3). This process, termed lymphocyte transformation, confirmed that the lymphocyte is a resting cell capable of further differentiation and proliferation and provided an exceedingly ver-

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