Tissue Fractionation Studies

10. INFLUENCE OF ISCHAEMIA ON THE STATE OF SOME BOUND ENZYMES IN RAT LIVER

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Cathepsin and other intracellular acid hydrolases have long been suspected of playing an important role in the aseptic autolysis and necrosis of tissues, but no satisfactory explanation has yet been given of their relative lack of activity in intact cells, nor of the processes responsible for their activation in dying or dead cells (see, for instance, the review by Bradley, 1938). As pointed out before (de Duve & Berthet, 1954), the finding that these enzymes are segregated within particles which are impermeable to their respective substrates (lysosomes; see de Duve, Pressman, Gianetto, Wattiaux & Appelmans 1955) provides an obvious answer to the first question. This, however, implies as a necessary corollary and answer to the second question that the rupture of the particles *in situ* and consequent release of their enzymic content must be an early step in the chain of processes leading to the autolytic breakdown of tissue constituents.

The experiments described in this paper were designed to test this hypothesis on a simple and reproducible material, namely on liver tissue entirely deprived of blood supply. Rat liver proved particularly suitable for this kind of study, since the hilum of one lobe can easily be ligated without injuring the remainder of the tissue, which may thus serve as an internal control. The results obtained indicate that lysosomes do indeed suffer a rapid disintegration or increase in fragility in necrotizing liver. The results have been previously reported briefly (Beaufay & de Duve, 1957).

METHODS

Adult rats of either sex were laparotomized under Nembutal anaesthesia. The vascular pedicle of the left liver lobe was ligated firmly with fine thread, care being taken not to injure the adjacent part of the liver, and the abdominal cavity was closed. This operation was well tolerated by the animals, which started moving and feeding normally as soon as they had recovered from the anaesthetic. They were kept in a warm enclosure and killed at times between 0·5 and 48 hr. after the ligature had been placed. The ligated lobe was removed and the remainder of the liver was trimmed of all parts which appeared to have suffered from the operation. This included the left middle lobe, since a collateral vessel irrigating this lobe was frequently enclosed in the ligature.

The two liver fragments were cooled and weighed in ice-cold 0·25 M-sucrose, and then homogenized in the same medium by means of a smooth-glass tube fitted with a polytetrafluoroethylene (Teflon) pestle (manufactured by A. H. Thomas Co., Philadelphia, Pa., U.S.A.). This was done according to a rigorously standardized grinding technique consisting of three complete up-and-down runs of the pestle rotating at about 1000 rev./min. The tubes were kept in a beaker of crushed ice during the whole operation and contained the liver samples in 15 ml. of 0·25 M-sucrose. The homogenized suspensions were subsequently brought to a volume (ml.) corresponding to 10 times the weight (g.) of tissue processed. Part of the homogenates was kept for analysis and the remainder spun for 30 min. at 40 000 rev./min. (3 000 000 g min. in the middle of the fluid) in the no. 40 rotor ($r_{max.}$ = 8·1 cm., $r_{min.}$ = 4·8 cm.) of the Spinco model L preparative ultracentrifuge.

The homogenates were analysed for nitrogen by a micro-Kjeldahl method, for cytochrome oxidase and glucose 6-phosphatase according to de Duve *et al.* (1955), and for total acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease in the presence of 0·1% of Triton X-100 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), as described by Wattiaux & de Duve (1956). Free activities of lysosomal hydrolases were also measured in some cases according to de Duve *et al.* (1955), but these determinations were complicated by high and rapidly changing blanks. For this reason, the degree of binding of the lysosomal hydrolases was assessed by measuring their total activity in the high-speed supernatants of the homogenates.

In the experiments on liver slices, the slices were prepared as described by Vuylsteke & de Duve (1957), placed in stoppered flasks containing the medium and the chosen gas mixture and shaken at 37° in a Warburg bath. After incubation, they were transferred into a homogenizer and treated as described above for the liver samples. The substrate mixtures used to measure the enzymic activities contained all the components of the incubation medium, to prevent an osmotic disruption of the particles in the free activity assays. The experiments on isolated particles were conducted in a similar manner on mitochondrial fractions prepared according to Appelmans & de Duve (1955).

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RESULTS

General findings

Altogether thirty-three animals were operated on and killed, in groups of three, 0·5, 1, 2, 3, 4, 6, 9, 12, 18, 24 and 48 hr. after operation. The ischaemic lobe was seen to darken rapidly after ligation and later became spotted on its surface with haemorrhagic flecks. After a few hours discoloured areas began to appear and to spread out, becoming confluent at the end of 12 hr. At this time the lobe had acquired a uniform greyish colour and a gummy consistency, which persisted without appreciable further change for the next 36 hr. Immediate swelling occurred only if the ligature had not been tight enough, in which case the animal was discarded. A slow progressive swelling developed during the first 8–12 hr. after ligation, presumably owing to imbibition of water from the peritoneal cavity. This was exemplified by a greater resilience of the ligated lobe, a significant increase in the ratio of the total liver weight (ligated + unligated) to the weight of the animal (Table 1) and a lowering of the nitrogen content per unit weight of the ischaemic part with respect to that of the remainder of the liver (Fig. 1). The last phenomenon could be due also to losses of nitrogenous material by diffusion, but, in view of the other evidence, was attributed mainly to gain of water. It was accordingly decided to relate the results of the enzymic determinations to the nitrogen content rather than to the weight of tissue.

Table 1. *Influence of ischaemia on total weight of liver*

Results were obtained 6 hr. or more after ligation. For operated animals, weight = sum of weights of ligated and unligated fragments. Unoperated animals are control group of another experiment performed at the same time.

Group	No. of animals	Mean relative wt. of liver ± S.E.M. (g./100 g. animal)
Unoperated	19	4·23 ± 0·11
Operated	16	4·92 ± 0·16
Difference	—	0·69 ± 0·19
		$P = 0·001$

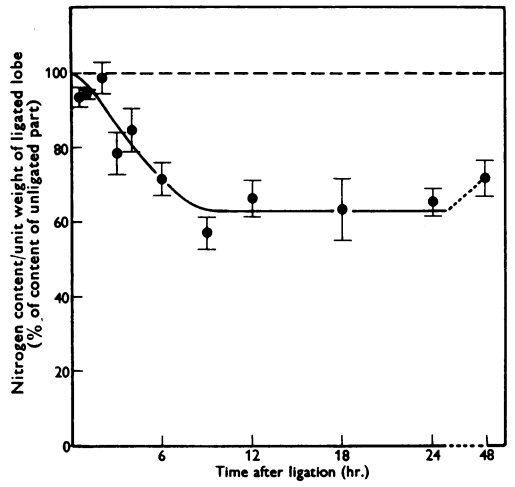

Fig. 1. Influence of ischaemia on nitrogen content of liver tissue. Values, per unit wet weight, for the ligated lobe are given as percentages of corresponding values for the unligated part. Means of three animals, with S.E.M., are given.

Enzymic determinations

In a number of control experiments, it was found that the enzymic activities measured were all identical in the two parts of the liver and that the unligated portion therefore provided a valid internal control for the ligated lobe. In addition, no significant changes were observed in the total activities of the unligated part as a result of the operation. For these reasons, the values (in units/mg. of nitrogen) found for a ligated lobe have simply been expressed as percentages of the corresponding values for the unligated part of the same liver.

In Figs. 2 and 3 are shown the results obtained for cytochrome oxidase and glucose 6-phosphatase. After ligation, both enzymes suffer a progressive inactivation to about one-tenth of their activity in the control fragment. This value is reached in about 6–8 hr. for cytochrome oxidase and 10–12 hr. for glucose 6-phosphatase, and is then maintained almost unchanged, even after 48 hr.

The data gathered on the five lysosomal enzymes are represented graphically in Figs. 4–8. For the total activities, three types of behaviour are seen. Acid phosphatase shows no change for the first 24 hr. and then decreases to half its normal value during the second day after ligature. Cathepsin and the two acid nucleases show a fairly rapid initial decrease, reaching 50–60 % of the control values after about 8 hr. (somewhat earlier with ribonuclease) and followed by an almost imperceptible secondary fall. Finally, β-glucuronidase remains unchanged for the first 6 hr., then increases by about 40 % during the next 12 hr. and remains elevated up to 48 hr. after ligation.

The unsedimentable activities of the lysosomal hydrolases show early changes which are the same for all five enzymes. In the unligated control fragment, they exhibit a slight rise during the first hours after operation and subsequently fall back to their normal values. A much more pronounced increase of these activities occurs in the ligated lobe, reaching a plateau in about 3–4 hr. With

β-glucuronidase, this increase is followed after 6 hr. by a large secondary rise coinciding with the enhancement of the total activity.

In the figures just described, the unsedimentable activities are expressed as percentages of the total activities of the unligated part. Since these do not change as a result of the operation, the curves drawn show the changes suffered by the absolute values of the unsedimentable activities. The actual state of the lysosomes is probably more correctly appreciated by relating the unsedimentable activities to the corresponding total activities. This has been done in Fig. 9, in which the relative values for acid phosphatase, cathepsin and the two nucleases have been further averaged, to provide a condensed

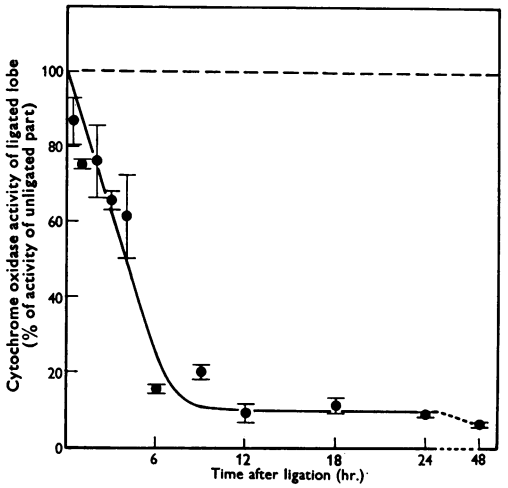

Fig. 2. Influence of ischaemia on cytochrome oxidase activity of liver tissue. Values, in units/mg. of N, for the ligated lobe are given as percentages of corresponding values for the unligated part. Means of three animals, with S.E.M., are given.

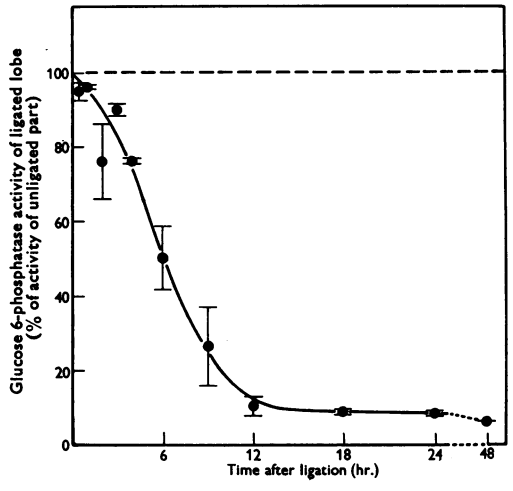

Fig. 3. Influence of ischaemia on glucose 6-phosphatase activity of liver tissue. Values, in units/mg. of N, for the ligated lobe are given as percentages of corresponding values for the unligated part. Means of three animals, with S.E.M., are given.

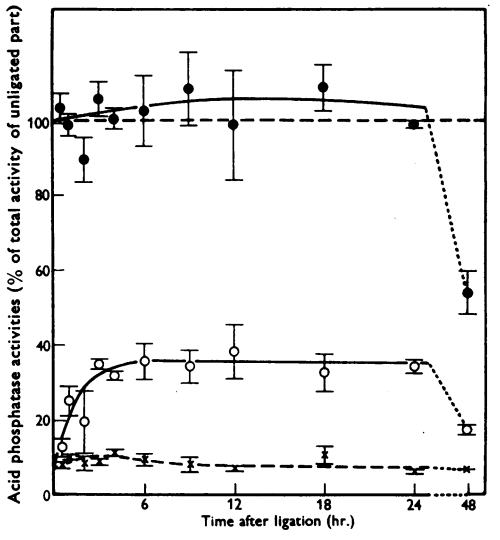

Fig. 4. Influence of ischaemia on acid phosphatase activity of liver tissue. Ligated lobe: ●, total activity; ○, unsedimentable activity. Unligated part: ×, unsedimentable activity. All values, in units/mg. of N, are given as percentages of total activity of the unligated part. Means of three animals, with S.E.M., are given.

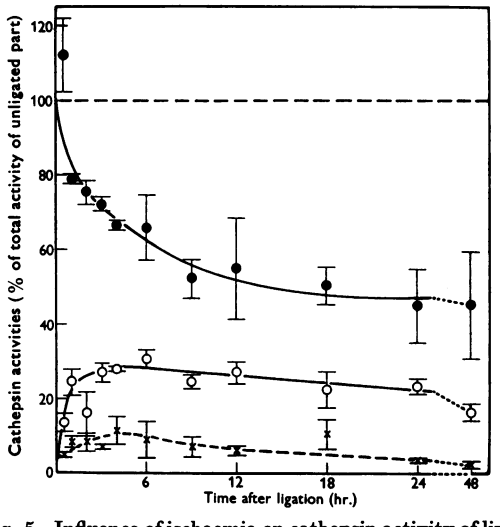

Fig. 5. Influence of ischaemia on cathepsin activity of liver tissue. For explanation of symbols see Fig. 4.

view of the results obtained. The values for β-glucuronidase, which behaves atypically after 6 hr., have been left out of this composite picture.

To clarify the significance of the plateau shown by the upper curve in Fig. 9, free acid phosphatase was measured in addition to the total and unsedimentable activities of this enzyme, on normal livers and on ligated and unligated lobes extirpated 2 and 16 hr. after ligation. The results of these assays are given in Table 2. They show that the release of acid phosphatase, though very extensive, is probably not complete even long after the phenomenon has clearly come to an end. Apparently, some 20 % of the particles containing this enzyme escape destruction under the conditions of our experiments. It will also be noted that the ratio of the free to the unsedimentable activity decreases as more enzyme is released. This is understandable since there will be fewer intact granules capable of contributing to the free activity, and the proportion of adsorbed enzyme will tend to decrease.

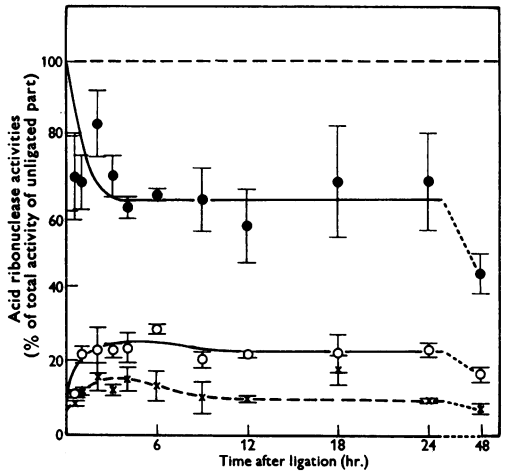

Fig. 6. Influence of ischaemia on acid ribonuclease activity of liver tissue. For explanation of symbols see Fig. 4.

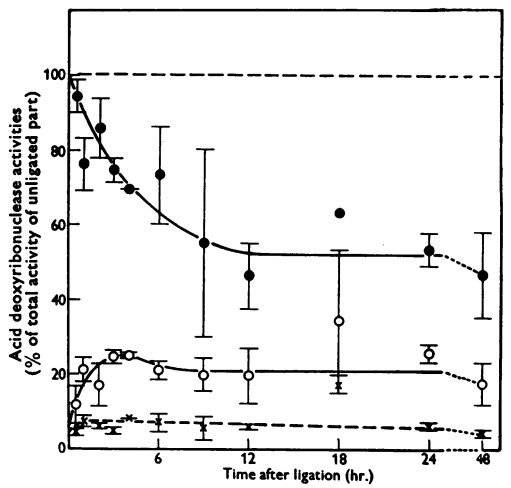

Fig. 7. Influence of ischaemia on acid deoxyribonuclease activity of liver tissue. For explanation of symbols see Fig. 4.

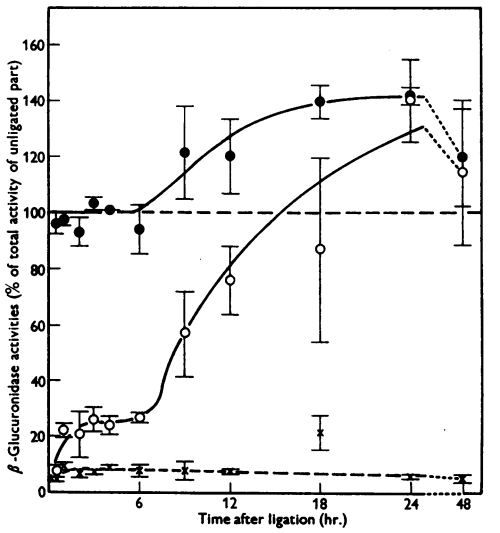

Fig. 8. Influence of ischaemia on β-glucuronidase activity of liver tissue. For explanation of symbols see Fig. 4.

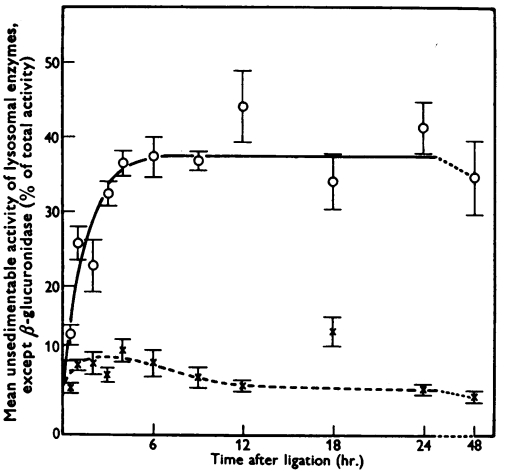

Fig. 9. Influence of ischaemia on partition of lysosomal hydrolases between particulate and soluble phases. Mean unsedimentable activities, with S.E.M., are given for acid phosphatase, cathepsin, acid ribonuclease and acid deoxyribonuclease, expressed as percentages of corresponding total activities: ○, ligated lobe; ×, unligated part.

Table 2. *Comparison of free and unsedimentable acid phosphatase activities in ligated and unligated tissue*

Tissue	No. of expts.	Percentage of total acid phosphatase (mean ± S.E.M.) Free activity	Unsedimentable activity	Ratio (F/U)
Normal	4	20·9 ± 1·0	7·4 ± 0·8	2·82
Unligated lobe from operated animals	8	20·1 ± 0·8	8·4 ± 0·2	2·39
Ligated lobe (2 hr.)	3	47·4 ± 1·7	25·0 ± 0·6	1·90
Ligated lobe (16 hr.)	8	78·7 ± 2·3	42·6 ± 1·2	1·85

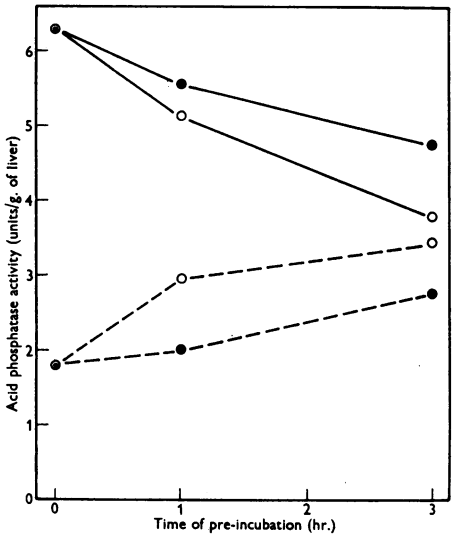

Fig. 10. Influence of anoxia on acid phosphatase in liver slices. Rat-liver slices were incubated at 37° under constant shaking in a medium of pH 7·6, containing 0·11 M-KCl and 0·04 M-$KHCO_3$, equilibrated with 5% of CO_2 in O_2 (●) or in N_2 (○). Continuous lines show total activity, and broken lines free activity, of enzyme in tissue homogenized at times indicated in the abscissa.

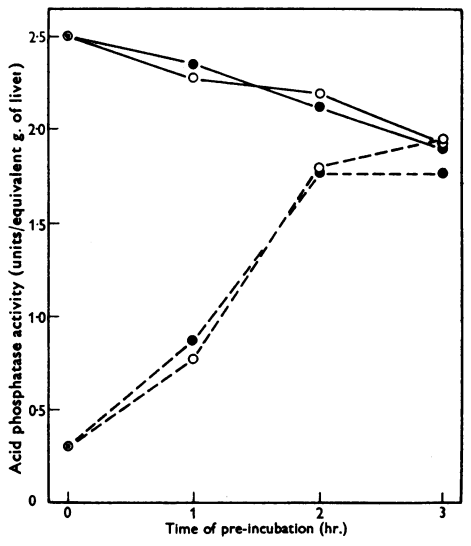

Fig. 11. Influence of anoxia on acid phosphatase in mitochondrial fraction. Particles were incubated at 37° under constant shaking in a medium of pH 7·1, containing 0·25 M-sucrose, 0·175 M-KCl and 0·0125 M-$KHCO_3$, equilibrated with 5% of CO_2 in O_2 (●) or in N_2 (○). Continuous lines show total activity, and broken lines free activity, of enzyme measured at times indicated in the abscissa.

Influence of anoxia

Anoxia represents one of the main consequences of ischaemia and it seemed of interest to find out whether oxygen deprivation alone could cause the release of the lysosomal enzymes. To test this possibility, a number of experiments were performed on rat-liver slices and on mitochondrial fractions, incubated at 37° under constant shaking in a bicarbonate medium, equilibrated with a gas phase containing 5% of CO_2 in either O_2 or N_2. The state of the lysosomes was followed at different intervals by measuring the free and total acid phosphatase activities on homogenates prepared from the slices or on the isolated particles. These experiments were complicated by losses of acid phosphatase, which, when in soluble form, was found to be unstable at 37° and at pH values higher than 6·0. It was also observed that even well-oxygenated slices incubated under conditions which have been found suitable for the study of several metabolic processes show a rapid deterioration of their lysosomes, exemplified by the appearance of acid phosphatase in the incubation medium and by an increase in the free activity of this enzyme in the homogenized tissue. However, as shown in Fig. 10, the release and subsequent loss of acid phosphatase is significantly greater when the slices have been incubated anaerobically. Isolated particles, on the other hand, are not influenced by the availability of oxygen (Fig. 11).

DISCUSSION

The results reported in this paper show that complete ischaemia of the liver modifies the partition of lysosomal hydrolases between the particulate and

soluble phases of the homogenized tissue. As shown most clearly by Fig. 9, the average proportion of activity found in the high-speed supernatants increases from an initial value of less than 10% to a plateau value of about 40%. The proportion of injured lysosomes corresponding to these figures is undoubtedly much greater, since the released enzymes are partly carried down with the sediment owing to adsorption and other trapping phenomena. From the acid phosphatase values given in Table 2 it may be estimated that about 20% of the lysosomal enzymes occur in free form in homogenates of normal tissue and that this proportion reaches 80% in homogenates of tissue maintained ischaemic for 4 hr. or more. Apparently, a small fraction of the lysosomes escape injury, even after prolonged ischaemia. Since about one-tenth of cytochrome oxidase and glucose 6-phosphatase is also spared, it is possible that some of the liver cells survive under the conditions of these experiments.

As compared with other biochemical changes observed in this and other similar investigations, the phenomenon involved in the release of the lysosomal hydrolases appears to be a fairly rapid one, since it comes to an end at a time when the cells still retain more than 60% of their cytochrome oxidase and 75% of their glucose 6-phosphatase activities, and when other morphological and chemical signs of autolysis are still very discreet (Berenbom, Chang, Betz & Stowell, 1955).

The nature of the intracellular change responsible for the lysosomal alterations observed on the homogenates is, of course, not known. It could be a true rupture of the lysosomes, with consequent release of their enzymes within the cells, or an increase in fragility of the particles, causing them to break up more easily upon homogenization. It could even have nothing to do with the particles themselves and consist, for instance, of the formation in other parts of the cells of a lytic substance which causes the rupture of the particles when brought together with them by the homogenization procedure.

With regard to these various hypotheses, it should be pointed out that, with the exception of β-glucuronidase, which will be discussed below, the lysosomal enzymes undergo no further change in partition between 4 and 48 hr. of observation. It seems improbable that the lysosomes are still intact after 2 days of ischaemia, when the tissue is in an advanced state of autolysis, since this would imply not only that the acid hydrolases have actually nothing to do with the autolytic process, but even that the particles in which they are contained are relatively refractory to this process. If therefore we take the homogenate picture after 48 hr. as a fairly faithful representation of the intracellular state, the point at issue becomes whether the intracellular lysosomal rupture does in fact follow the time course indicated by the results on homogenates or whether it is slower. In other words, how near spontaneous rupture must the average lysosome be to break up during homogenization? Whichever the answer to this question, it is clear that the process which brings the particles to the critical stage of fragility is both rapid and early, and it seems therefore reasonable to assume that the intracellular rupture is not too far removed from the point of breakage *in vitro*.

Thus as much by their nature as by their time course and magnitude, the observed lysosomal alterations bring direct support to the hypothesis, outlined in the introduction, that the release of lysosomal enzymes occurs as an early step in autolysis.

Our results also shed some light on the possible mechanism whereby the lysosomes become ruptured. We find that anoxia alone may precipitate the phenomenon in slices, but not in isolated particles. The latter detail is of importance since it suggests that the lysosomes are sensitive, not to the oxygen partial pressure itself, but to some environmental change taking place in anoxic cells. As a possible link in the chain of events one could think of the lowering of the pH, which is known to accelerate considerably the breakdown of lysosomes *in vitro*, by a mechanism which, as shown in the preceding paper (Beaufay & de Duve, 1959), may involve the particles' own cathepsin acting on the membrane from the inside. The properties of this enzyme are compatible with such a role, since it is known to be practically inactive above pH 6·0 and to increase rapidly in activity as the pH falls below this limit.

Whichever the mechanism involved, the data obtained suggest that the autolysis of anoxic cells may start at the site of the lysosomes, possibly with a primary attack of the lysosomal membrane, and spreads out from these centres as the released hydrolases diffuse outwards. Such a process could conceivably play a causal role in cell death, but it could also occur as a consequence of this phenomenon which may itself be due to more rapid alterations, unrelated to the invasion by lysosomal enzymes. Further investigations on the reversibility of the observed changes may possibly help to answer this question. Of interest in this respect is the slight increase in the unsedimentable fraction of lysosomal enzymes which takes place in the control tissue during the first hours after operation, possibly as a consequence of the operative shock. Notable also is the rapid deterioration of lysosomes in isolated liver slices, even under aerobic conditions. The inner layers of cells in such slices are known to be largely deprived of oxygen and the phenomenon is therefore not surprising, but it may

help to explain some of the failures to demonstrate hormonal actions on such systems, in particular with insulin.

In addition to the changes already discussed, our experiments have revealed various alterations in the activity of the enzymes themselves. Most clear-cut is the inactivation of the mitochondrial cytochrome oxidase and of the microsomal glucose 6-phosphatase, which presumably reflects a pathologically accelerated degradation, since a very high turnover would have to be assumed for these enzymes, were their decay simply due to a cessation of synthesis. The partial inactivation suffered by cathepsin and by the nucleases is more puzzling, since it starts fairly rapidly but slows down considerably at a later stage. Finally, the behaviour of β-glucuronidase is particularly striking, since this enzyme undergoes an extensive secondary solubilization, accompanied by a 40 % increase in total activity and starting rather suddenly about 6 hr. after ligation. It may be recalled that, unlike the other acid hydrolases of the group, β-glucuronidase is not exclusively located in the lysosomes but also occurs to a significant extent in the microsomes, where it shows a different pH–activity curve (de Duve *et al.* 1955). It is tempting to relate the secondary rise in total and soluble activity of this enzyme to an autolytic release of the microsomal component. It may be finally pointed out that all five lysosomal hydrolases are still present at half or more of their initial concentration 48 hr. after the blood supply has been cut off. As such, they show a length of survival compatible with their assumed role in the process of autolysis.

SUMMARY

1. The activity and state of a number of bound enzymes have been followed in a rat-liver lobe rendered completely ischaemic by ligation of the vascular pedicle. The unligated part of the same liver was used as an internal control. The enzymic determinations were performed on the homogenized tissue samples and on high-speed supernatants from these homogenates.

2. The earliest change observed after complete ischaemia was a progressive release of the lysosomal hydrolases, acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease. This release reached a plateau, corresponding to the rupture of about 80 % of the lysosomes, in the space of 3–4 hr. Whether the phenomenon actually occurred within the cells or only upon homogenization is not known, but the former interpretation is favoured as providing a satisfactory explanation of how autolytic processes are initiated in necrotizing cells.

3. Changes in total enzymic activities were as follows: cytochrome oxidase lost about nine-tenths of its activity in 6–8 hr. and then remained constant; glucose 6-phosphatase did the same in 10–12 hr.; acid phosphatase remained unchanged during the first day and then slowly decreased to about half its value during the second day; cathepsin and the two acid nucleases suffered a fairly rapid partial inactivation, reaching 50–60 % of their activity after about 8 hr., and then decreased very slowly; β-glucuronidase was unaffected for 6 hr. and then increased by about 40 % during the next 12 hr. and remained elevated. All five lysosomal hydrolases were still present at half or more their initial concentration 48 hr. after the blood supply had been cut off, thus showing a survival time consistent with their assumed role in autolysis.

4. From studies on isolated liver slices and on particles it appears that the ischaemic release of lysosomal hydrolases may be due to an anoxic change in the intracellular environment of the particles, possibly a lowering of the pH.

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