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Complete Loss of Heparin-Releasable Triacylglycerol Lipase Activity after Collagenase Treatment of the Rat Liver

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Lipolytic activity at pH 8.5–9.5 was lowered by approx. 80% in homogenates from livers perfused with collagenase or heparin. No heparin-releasable lipase activity was detected in hepatocytes isolated by collagenase treatment. It is concluded that crude collagenase completely inactivates the plasma-membrane-bound heparin-releasable lipase.

Intravenous injection of heparin in the intact rat or perfusion of the isolated rat liver with heparin results in the release of a triacylglycerol lipase (EC 3.1.1.3) from the liver (La Rosa *et al.*, 1972; Krauss *et al.*, 1973; Assmann *et al.*, 1973). Tissue-fractionation studies have shown that the enzyme, characterized by a pH optimum of 9.5, is primarily associated with the plasma-membrane fraction (Assmann *et al.*, 1973). The function of the lipase is still unknown. As the liver is a major site of lipoprotein catabolism (for references see Dreven *et al.*, 1977), it is conceivable that the enzyme might facilitate hepatic uptake and degradation of lipoproteins or their remnants by promoting their delipidation before entry into the liver cells.

In the course of our experiments on hepatic lipolysis we observed that perfusion of the liver with crude collagenase (EC 3.4.24.3) for the isolation of hepatocytes caused a complete loss of heparin-releasable lipase activity. The fact that suspensions of freshly isolated hepatocytes are increasingly used in experiments on hepatic lipoprotein uptake (Nilsson & Åkesson, 1975; Nakai *et al.*, 1976; Nilsson, 1977; Dreven *et al.*, 1977) prompted us to report these findings.

Materials and Methods

Materials

Collagenase (type I from *Clostridium histolyticum*), soya-bean trypsin inhibitor and bacitracin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; heparin (Liquémine) was from Hoffmann-La Roche, Basle, Switzerland.

Animals

Male Wistar rats weighing 150–200 g were used. They were fed on a standard laboratory diet.

Liver perfusion and isolation of hepatocytes

The operative technique of Hems *et al.* (1966) was used, except that the animals were not treated with heparin. Instead, after insertion of the portal cannula, the inferior caval vein was cut at a point below the kidney and the liver was immediately flushed with 20 ml of Krebs–Henseleit bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932). The perfusion apparatus has been described in detail previously (Mannaerts *et al.*, 1974).

For the isolation of hepatocytes livers were perfused with Ca^{2+} -free Krebs–Henseleit bicarbonate buffer for 15 min at 37°C. Collagenase was then added to a final concentration of 0.25 mg/ml of perfusion medium and perfusion continued for another 20 min. The liver was gently minced, the cells were suspended in 1.5% (w/v) gelatin in Ca^{2+} -free Krebs–Henseleit bicarbonate buffer, filtered through nylon mesh and washed by centrifugation at 75g for 1 min. Cell viability was more than 90%, as estimated by the Trypan Blue-exclusion test.

In preliminary experiments lipase activity was measured in homogenates prepared from the hepatocyte pellets. In later experiments the suspending and washing procedures were omitted and lipase activity was measured in homogenates prepared directly from the whole collagenase-treated liver and compared with lipase activity associated with a liver lobe taken from the same liver during the pre-perfusion period without collagenase. In experiments where the effects of heparin were studied, livers were perfused with Krebs–Henseleit bicarbonate buffer supplemented with 2.6 g of bovine serum albumin per 100 ml to provide maximal protection for the lipase released into the perfusion medium. Heparin was added to a concentration of 50 units U.S.P./ml of perfusion medium after 5 min, and perfusion was continued for another 10 min. Under these conditions essentially

all the heparin-releasable lipase activity appeared in the perfusion medium within 5 min after heparin addition. At the end of the experiments livers were homogenized and lipase activity was measured in the homogenates and perfusates. As in the collagenase experiments, lipase activity was also measured in homogenates from liver lobes taken before heparin addition. Collagenase- or heparin-treated livers were always flushed with 20 ml of Krebs–Henseleit bicarbonate buffer to remove excess collagenase or heparin before homogenization.

Preparation of homogenates and measurement of triacylglycerol lipase activity

Homogenates (5%, w/v) in Krebs–Henseleit bicarbonate buffer were prepared from liver lobes taken before addition of collagenase or heparin, and 10% (w/v) homogenates were prepared from collagenase- or heparin-treated livers. The tissue was homogenized in a Dounce homogenizer with six strokes each of a loose- and a tight-fitting plunger. Homogenates from collagenase-treated livers were always examined under the microscope for cell destruction. Triacylglycerol lipase activity over the pH range 4–9.5 was measured as described previously (Debeer *et al.*, 1977). Direct addition of appropriate amounts of collagenase or heparin to the lipase assay mixtures did not affect the measurements. Results are expressed as μmol of non-esterified fatty acid released/h per mg of liver protein. Lipase activity recovered in the perfusates was converted into the

same units. Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and Discussion

Preliminary experiments had shown that homogenates from isolated hepatocytes displayed very little triacylglycerol lipase activity in the alkaline pH range. Since the 75g cell pellets used for the preparation of these homogenates contained less than 10% non-parenchymal cells, the possibility was envisaged that the alkaline lipase activity might have been associated with these cells. However, centrifugation of the whole-cell preparation at 2000g for 5 min, during which all the cells sedimented, did not increase the specific activity of the lipase. In another experiment the cells of the 2000g pellet were resuspended in Krebs–Henseleit bicarbonate buffer and incubated at 37°C for 10 min in the presence of 5–50 units of heparin/ml. No heparin-releasable triacylglycerol lipase activity could be detected. Thus these experiments suggested that the heparin-releasable lipase was either released or inactivated during collagenase treatment.

Fig. 1(a) shows the pH profile of lipolytic activity in homogenates prepared from a liver lobe taken before addition of collagenase and from the liver after collagenase treatment. Whereas after perfusion of the liver with collagenase acid lipase activity, which is associated with the lysosomes (Mahadevan & Tappel, 1968; Assmann *et al.*, 1973), was not affected to any

Fig. 1. pH profile of lipolytic activity in homogenates and perfusates of livers perfused with collagenase or heparin (a) Livers perfused with 0.25 mg of collagenase/ml. ○, Liver lobe before collagenase addition; ●, liver 20 min after collagenase addition; ▲, perfusate 20 min after collagenase addition. Results are means for five experiments. (b) Livers perfused with 50 units of heparin/ml. ○, Liver lobe before heparin addition; ●, liver 10 min after heparin addition; ▲, perfusate 10 min after heparin addition. Results are means for three experiments.

major extent, there was a dramatic decrease in lipase activity in the alkaline pH range. No lipase activity was recovered in the perfusates. Control perfusions carried out under identical conditions, but without addition of collagenase or with addition of collagenase that had been inactivated at 100°C for 2 min, caused a loss of hepatic lipase activity of only 6.6 and 5.6% at pH 8.5 and 9.5 respectively (mean for three experiments).

Fig. 1(b) illustrates the release of hepatic lipase activity by heparin. Comparison of Figs. 1(a) and 1(b) demonstrates that collagenase treatment induced an apparently complete loss of the heparin-releasable fraction of the hepatic alkaline lipase activity, a finding consistent with the observation that no heparin-releasable lipase activity could be detected on incubation of isolated hepatocytes with heparin (see above). Since in contrast with the heparin experiments no lipase activity was recovered in the perfusates of collagenase-treated livers, the enzyme was apparently inactivated during collagenase treatment. This was further substantiated by the fact that lipase activity completely disappeared from 'post-heparin' perfusates incubated with 0.25 mg of collagenase/ml for 20 min at 37°C. Although our experiments do not rule out the presence of a heat-labile and slowly dissociating (since no alkaline lipase activity was found in washed hepatocytes) inhibitor in the crude collagenase preparation, it is more likely that the lipase was destroyed by contaminating proteinases or perhaps glycosidases. Addition to the perfusion medium, however, of albumin (2.6 g/100 ml) or albumin plus classical proteinase or peptidase inhibitors, such as soya-bean anti-trypsin (5 or 50 mg/100 ml) and bacitracin (100 mg/100 ml), did not increase lipase recoveries either in the post-collagenase homogenates or in the corresponding perfusates.

The loss of the heparin-releasable lipase was apparently not accompanied by any extensive damage to the plasma membranes of the cells. Indeed, as repeatedly shown by others, the isolated hepatocytes responded to glucagon and adrenaline (results not shown), indicating that the membrane receptors for these hormones were largely intact. Thus it seems that the heparin-releasable lipase is particularly sensitive to the damaging effects of crude collagenase.

The fact that the pH profiles obtained with untreated liver and 'post-heparin' perfusate consistently displayed a maximum at pH 8.5 suggested that more than one lipase might have been released by heparin (and inactivated by collagenase). In view of the recent identification of an adipose-tissue-like lipoprotein lipase in perfusates of chicken liver (Bensadoun & Koh, 1977), post-heparin perfusates were also assayed in the presence of 1 M-NaCl, 10% (v/v) starved-rat serum or a combination of both. Absolute lipase

activities, as well as the shape of the pH curves, did not change, suggesting that the maximum at pH 8.5 was not due to a lipoprotein lipase.

In recent years isolated hepatocytes have been increasingly used in studies on hepatic lipoprotein catabolism (Nilsson & Åkesson, 1975; Nakai *et al.*, 1976; Nilsson, 1977; Drevon *et al.*, 1977). Since the heparin-releasable triacylglycerol lipase might be involved in this process, our results suggest that isolated hepatocytes might not be the best-suited preparation for experiments on hepatic lipoprotein uptake and degradation. As long as the exact role of the heparin-releasable lipase remains unknown, the perfused liver, although more cumbersome, should be preferred. Since the possibility exists that the heparin-releasable lipase would reappear in cultured hepatocytes, the use of hepatocytes brought into maintenance culture could be an elegant alternative.

Finally, our results demonstrate the complete disappearance of a plasma-membrane-associated enzyme activity after collagenase treatment. The question then arises whether there are other ecto-enzymes or membrane proteins whose function is lost or profoundly altered during the isolation of cells from liver or other tissues by the collagenase technique.

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References

- Assmann, G., Krauss, R. M., Frederickson, D. S. & Levy, R. I. (1973) *J. Biol. Chem.* **248**, 1992–1999
- Bensadoun, A. & Koh, T. L. (1977) *J. Lipid Res.* **18**, 768–773
- Debeer, L. J., Thomas, J., Mannaerts, G. & De Schepper, P. J. (1977) *J. Clin. Invest.* **59**, 185–192
- Drevon, C. A., Berg, T. & Norum, K. R. (1977) *Biochim. Biophys. Acta* **487**, 122–136
- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
- Krauss, R. M., Windmueller, H. G., Levy, R. I. & Frederickson, D. S. (1973) *J. Lipid Res.* **14**, 286–295
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- La Rosa, J. C., Levy, R. I., Windmueller, H. G. & Frederickson, D. S. (1972) *J. Lipid Res.* **13**, 356–363
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mahadevan, S. & Tappel, A. L. (1968) *J. Biol. Chem.* **243**, 2849–2854
- Mannaerts, G., Debeer, L. & De Schepper, P. J. (1974) *Biochem. Pharmacol.* **23**, 75–87
- Nakai, T., Otto, P. S., Kennedy, L. & Whayne, T. F. (1976) *J. Biol. Chem.* **251**, 4914–4921
- Nilsson, Å. (1977) *Biochem. J.* **162**, 367–377
- Nilsson, Å. & Åkesson, B. (1975) *FEBS Lett.* **51**, 219–224