Similar qualitative results were obtained when nitrobenzene, nitrofluorene, and *p*-nitrocresol (or their nitroso and hydroxylamino derivatives) were carried through analogous investigations.

Zusammenfassung. Eine elektrochemische Sonde wurde entwickelt, um Arylhydroxylamin direkt in mikrosomalen Suspensionen festzustellen. Dieser Sensor ermittelte die Bildung von Arylhydroxylamin während der enzymatischen Reduktion von aromatischen Nitroverbindungen durch Mikrosomen der Kaninchenleber. Die Reduktion der Nitrogruppe wurde durch beide Enzyme, Cytochrom P-450-abhängige und P-450-unabhängige, katalysiert.

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Release of Hydroxyproline from Rat Hearts Perfused with Collagenase

Various enzymes, i.e. collagenase, trypsin and hyaluronidase, have been used extensively for dissociating tissues. During preliminary experiments in this laboratory we found that perfusion of rat hearts with some preparations of hyaluronidase resulted in partial inactivation of 5'-nucleotidase; for example, 20 min of perfusion with Sigma Type I hyaluronidase caused a greater than $^1/_3$ loss of enzyme activity[1]. There have been a number of reports of other functional losses due to similar treatments of various tissues with dispersing enzymes[2–4]. We were therefore interested in identifying methods which effect dissociation of hearts while keeping functional losses to a minimum. As tissue dissociation was not readily quantitated by direct observation, a more objective result of enzyme activity was monitored. We measured the release of hydroxyproline, an amino acid which is contained almost exclusively in collagen and elastin. In this communication we report the rate of release of hydroxyproline by rat hearts exposed to collagenase alone or in combination with hyaluronidase and trypsin.

Materials and methods. Sources of enzymes: Collagenase Type III, hyaluronidase, Worthington; trypsin 1:250, Difco. Chemicals were reagent grade from various sources; C^{14}-hydroxyproline was purchased from Amersham.

Using the Langendorff technique, rat hearts were perfused for 5 min with complete Hank's solution to wash out blood and then perfused for 5 min with Hank's solution from which Ca^{++} was omitted. Recirculating perfusion with Ca^{++}-free Hank's solution containing enzymes was then started (30 °C) and 1 ml samples of this perfusate were taken for hydroxyproline assays as required. The hydroxyproline content of the samples was determined by the method of PROCKOP and UDENFRIEND[5]

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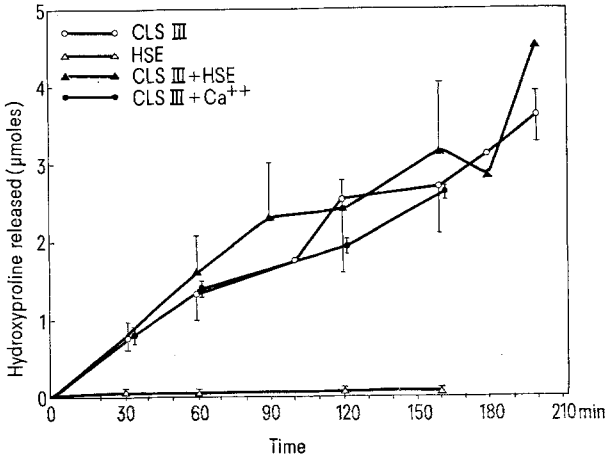

Fig. 1. Enzymatic release of hydroxyproline from perfused rat hearts. Rat hearts were perfused with collagenase (1 mg/ml CLS Worthington, Type III) and/or hyaluronidase (2 mg/ml HSE, Worthington) in Hank's solution from which Ca^{++} was omitted. The effect of 1.25 m*M* Ca^{++} is shown by the closed circles. A recirculating perfusion system was used and aliquots of the perfusate were taken for hydroxyproline determination at the times indicated. The data are expressed as μmoles of hydroxyproline released per heart at the perfusion times indicated and each point with a vertical bar represents the mean of 2 to 6 hearts ± S.D.

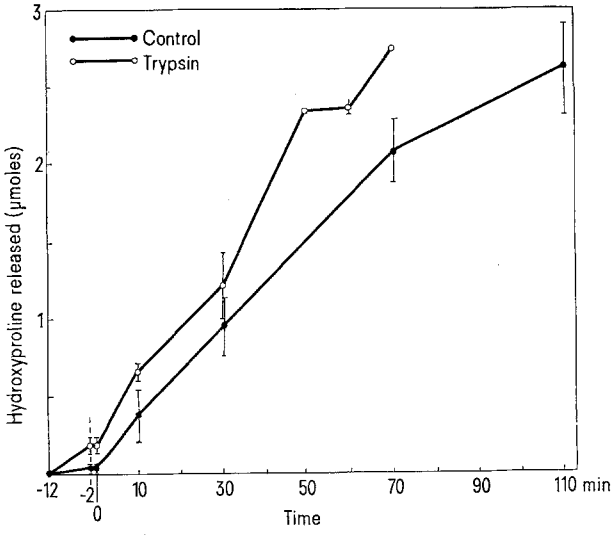

Fig. 2. Hydroxyproline relaese from perfused rat hearts after preperfusion with trypsin. Hearts were preperfused with trypsin (1 mg/ml) in Ca^{++}-free Hank's solution for 10 min and then the trypsin was washed out prior to perfusion with collagenase. Control hearts were preperfused with Ca^{++}-free Hank's solution. Other conditions same as for Figure 1.

with the following modification. The borate buffer of KIVIRIKKO, LAITINEN and PROCKOP[6] was used instead of pyrophosphate and C^{14}-hydroxyproline was added at the start of the assay to allow calculation of yield. The latter was necessary because our yield (usually 80%) was lower than previously reported (about 90%)[6].

Results and discussion. When hearts of 200–250 g rats were perfused with collagenase alone, 1 mg/ml, the hydrolysis of collagen was constant for the first 120 min. During this time the rate of appearance of hydroxyproline in the perfusate was about 1.34 µmoles/h and after 120 min this rate decreased by about 30% (Figure 1). After establishing the rate of collagen hydrolysis by collagenase alone, we studied the effects of treating hearts with hyaluronidase, Ca^{++} and trypsin in conjunction with collagenase.

Several years ago MATEYKO and KOPAC[7] reported that hyaluronidase increased the effectiveness of trypsin in dissociating fibrous ovarian tumors. They attributed this to one enzyme aiding the permeation of the other by unmasking reactive groups. By analogy a similar relationship would explain the basis for the widely accepted use of collagenase in combination with hyaluronidase. Collagen is considered to be in intimate contact with acidic mucopolysaccharides which are substrates for hyaluronidase[8]. It seems reasonable then that removal of acidic mucopolysaccharide by this enzyme should increase the effectiveness of collagenase. Our data however do not support such a prediction. In our experiments there was little or no effect on the rate of hydroxyproline release from rat hearts by collagenase when hyaluronidase (2 mg/ml) was added to the perfusing solution (Figure 1). After 60 min of perfusion 1.34 $\pm$ 0.32 µmoles of hydroxyproline were released by collagenase alone compared to 1.58 $\pm$ 0.50 µmoles when the perfusion solution contained both collagenase (1 mg/ml) and hyaluronidase (2 mg/ml). From Figure 1 it is also evident that hyaluronidase alone did not cause any release of hydroxyproline. Our observation that hyaluronidase did not alter the rate of release of hydroxyproline was consistent over a range of animal sizes. The amount of hydroxyproline released in 60 min by hearts of 135–175 g rats was 0.86 $\pm$ 0.25 µmoles with collagenase alone and 1.04 $\pm$ 0.13 µmoles with collagenase plus hyaluronidase. When 340–510 g rats were used the values were 1.34 $\pm$ 0.82 and 1.92 $\pm$ 0.42 respectively. Thus, hearts from large rats release hydroxyproline faster than those of small rats but the effect of hyaluronidase was not statistically significant.

Ca^{++} is an activator of collagenase[9], therefore we compared the rate of hydroxyproline release from hearts perfused with Hank's solution which contained collagenase and 1.26 m*M* Ca^{++} with that from hearts perfused with Hank's solution which contained collagenase but no added Ca^{++}. This cation had essentially no effect on collagen hydrolysis during the first 60 min of perfusion; the rate of hydroxyproline release in its presence was not different from that in Ca^{++}-free Hank's solution (Figure 1).

Experiments with trypsin could not be reasonably conducted in the same manner as those with hyaluronidase because prolonged exposure to trypsin causes considerable functional damage[2]. Therefore we perfused hearts with trypsin (1 mg/ml) for 10 min, washed out the trypsin and then started treatment with collagenase, instead of exposing them to both enzymes simultaneously. In Figure 2 it can be seen that trypsin pretreatment results in a small increase in hydroxyproline release; this difference was not statistically significant for the 30 min samples.

In summary, the hydrolysis of rat heart collagen by perfusion with collagenase was enhanced to a small degree by pretreatment with trypsin but was not significantly enhanced by hyaluronidase or Ca^{++}. This may indicate that in cases in which hyaluronidase has been shown to enhance tissue dissociation by collagenase, the mechanisms of enhancement may not involve greater accessibility of substrate to collagenase.

Résumé. La libération de l'hydroxyproline dans le cœur du rat a été déterminée en perfusant le cœur avec la solution de Hank's contenant de la collagénase. Le taux de libération par la collagénase n'a pas été augmenté significativement quand la hyaluronidase ou le Ca^{++} ont été ajoutés à la solution utilisée pour la perfusion. Une préperfusion avec de la trypsine a augmenté légèrement le taux de libération de l'hydroxyproline.

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23 September 1974.

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An in vitro Demonstration of Proteolysis by Macrophages and its Increase with Coumarin[1]

Coumarin and benzo-pyrones in general have been shown to be very effective in reducing high protein oedemas, and especially lymphoedema[2–7]. Fine structural studies of lymphoedematous tissues have shown that coumarin[8], and the related compound troxerutin[9], reduce the amount of protein in both the connective tissues and the lymphatics[6]. Due to the reduction in the protein's osmotic effect, the fluid and hence the oedema are also reduced.

That the drugs do not reduce the protein by increasing lymph flow is shown both by the fact that they are effective in experimental lymphoedema, when the lymphatics have been ligated[7,10], and by the fact that coumarin does not increase either lymph flow or its removal of protein in the thoracic duct of rats with a burnt leg[11]. It is also unlikely that the drugs work by decreasing the protein outflow from the blood vessels, since it has been shown that a number of them slightly injure the blood vascular intercellular junctions in normal conditions, lymphoedema, and thermal injury[2,7,10].

That the removal of the protein is probably brought about by its increased catabolism in the tissues is suggested by the fact that coumarin greatly increases the removal of proteins, but not that of non-metabolizable