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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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

Proteolysis by macrophages, normal and accelerated by coumarin; amount of protein fragments (expressed as m*M* glycine)

Time (h)	0	24	Difference (24 h – combined mean 0 h)	*F*-test	*t*-test	Remarks
Without coumarin	1.13 (0.0307) [14]	1.43 (0.0421) [26]	0.26 (0.0507)	+	+++	Normal proteolysis
With coumarin	1.22 (0.0446) [12]	1.74 (0.0522) [24]	0.57 (0.0594)	++	+++	Normal and coumarin stimulated proteolysis
Combined mean at 0 h	1.17 (0.0283) [26]					
Difference between with and without coumarin	0.09 (0.0553)	0.31 (0.0680)	0.31 (0.0862)			Coumarin stimulated proteolysis
F-test	NS	NS	NS			
t-test	NS	+++	+++			

Normal proteolysis by 1 macrophage $\sim 3.7 \times 10^{-10}$ mmoles/24 h; coumarin increases this to $\sim 8.1 \times 10^{-10}$ mmoles/24 h. Results are given as: Mean (S.E.) Number of replicates in brackets. NS, $p > 0.05$; +, $0.05 > p > 0.01$; ++, $0.01 > p > 0.01$; +++, $0.001 > p$.

polyvinyl-pyrolidone molecules of similar diffusion coefficients, under normal conditions, and in burns and lymphoedema[10]. The smaller protein fragments would readily be able to pass into the blood vessels via their intercellular junctions, whereas the larger protein molecules could not, due both to their size and the direction of their concentration gradient. The concentration gradient of the protein fragments would be directed towards the blood vessels, and the fragments would also have quite large diffusion coefficients. In support of this suggested mode of action of coumarin and related compounds, it has been shown that the trichloracetic acid (TCA)-soluble protein fragments in both normal and burned tissue are increased following coumarin treatment[7]. Macrophages are cells specialized for the digestion of proteins[12] and are present in both burned and lymphoedematous tissues. It was therefore decided to investigate the effect of coumarin on proteolysis by macrophages in vitro.

Peritoneal macrophages from male and female mice weighing 20 g were used in this study. The animals were killed by breaking the neck and the abdominal skin was reflected. 2 ml of Hanks medium containing 5% foetal calf serum (Commonwealth Serum Laboratories, Melbourne), 100 units/ml of both streptomycin and penicillin and 5 units/ml of heparin was injected i.p. The abdomen was gently massaged and the fluid was withdrawn and dispensed to a Falcon T-flask containing 2.5 ml of unheparinized medium which had been gassed with 5% CO_2 in air. The flasks were incubated at 37 °C for 1 h to allow the macrophages to adhere to the surface. The fluid was then removed and replaced with 2.5 ml of unheparinized medium, and the flasks were again gassed. After 24 h incubation at 37 °C the flasks were examined and the cells counted. Flasks without sufficient numbers of extended macrophages were discarded. The mean number of macrophages/flask was 1.8×10^6/2.5 ml. The medium was then removed and the flasks washed with 5 ml of Dulbecco phosphate buffer (Commonwealth Serum Laboratories). This was removed and 2.5 ml of Hanks solution containing 10 mg albumin/ml (Bovine Serum Albumin Fraction V) and streptomycin and penicillin was added to each flask. In some flasks the medium also contained 25 µg coumarin/ml. After 24 h incubation at 37 °C the medium was removed and frozen. The TCA-soluble protein fragments in the medium, both before and after incubation with the cells, was determined by the ninhydrin method.

The results are presented in the Table. Proteolysis by stimulated macrophages in vitro is estimated at the equivalent of 3.7×10^{-10} mmoles of glycine per single macrophage in 24 h. The addition of coumarin to the medium increases this to 8.1×10^{-10} mmoles of glycine in 24 h.

These results indicate that it is possible that coumarin acts in oedema by increasing the normal rate of proteolysis by stimulated macrophages of proteins in the oedema fluid. This interpretation is further supported by the observation that in animals injected with silica, which inactivates macrophages, coumarin is ineffective in relieving oedema[13]. COHN et al.[12] have investigated the mechanism of digestion of proteins by macrophages in vitro, and have shown that pinosomes, containing ingested proteins, fuse with Golgi vesicles containing acid hydrolases to form secondary lysosomes[14]. Digesiont of proteins to, or close to, the level of amino acids is probably completed within the lysosomes[15–17]. The lysosomes do not directly regurgitate their contents into the medium[18], so the amino acids must either diffuse

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or be transported through the cytoplasm to the medium, perhaps via vesicles. There is a close relationship between pinocytosis, the formation of lysosomes and the accumulation of acid hydrolases, in that an increase in pinocytosis is followed by an increase in the other two functions[19,20].

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Zusammenfassung. Es wird gezeigt, dass die normale proteolytische Tätigkeit von stimulierten peritonealen Makrophagen der Maus durch das Benzo-pyron-Cumarin in vitro beträchtlich erhöht wird.

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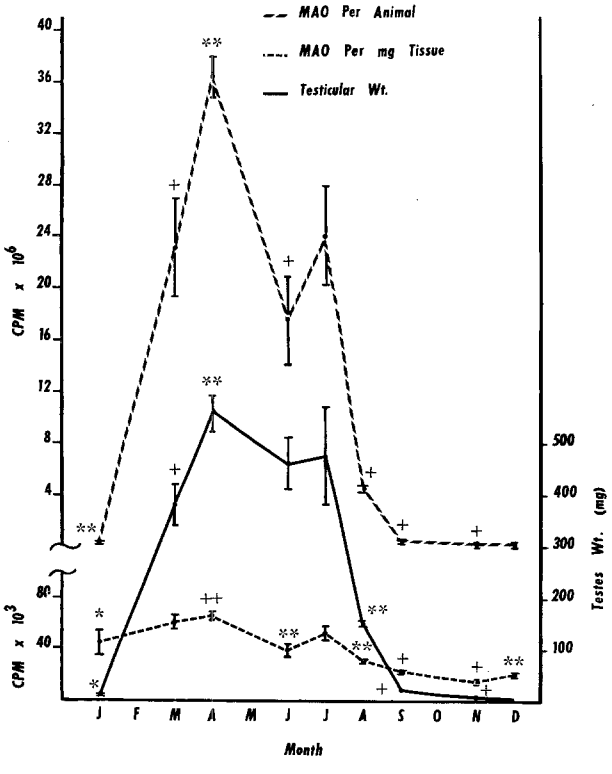
Seasonal Variations in Testicular Monoamine Oxidase in the House Sparrow (*Passer domesticus*) and Uinta Ground Squirrels (*Spermophilus armatus*)[1]

Monoamine oxidase, a deaminating enzyme (MAO, Monoamine: O_2 oxidoreductase (deaminating) EC.1.4.3.4.), and endogenous 5-hydroxy-tryptamine (serotonin, 5-HT), a substrate of testicular MAO, are both normally present in rat testes[2-5]. Testicular MAO activity is low in young rats, increased during sexual development and maturity, and is low again with advanced age[5]. Changes in testicular MAO activity, with respect to aging, were correlated with changes in androgen synthesis and testicular development[5]. No attempt has been made to follow testicular MAO activity in animals that are seasonal breeders with large variations in testicular weight. The purpose of these investigations was to follow testicular MAO activity in house sparrows and ground squirrels to determine the relationship that MAO activity has with testicular development.



Testicular MAO activity and testicular weights of house sparrows trapped in northern Utah from January 1971 through December 1972. Each point represents the mean while the vertical lines are the standard error of mean values (* = $p < 0.05$, ** = $p < 0.01$, + = $p < 0.001$ when compared with the previous value while ++ = $p < 0.05$ when compared with January).

Materials and methods. Male house sparrows (*Passer domesticus*) were collected with a mist net in Cache Valley near Utah State University. 7 sparrows were collected in January, 6 in March, 6 in April, 6 in June, 6 in July, 5 in August, 7 in September, 5 in November, and 6 in December. All sparrows were collected the same day for a given group between 13.00 and 16.30 h. The birds were kept overnight in a wire cage with feed (mixed grains) and water given ad libitum.

Six adult male Uinta ground squirrels (*Spermophilus armatus*) were trapped in early April, soon after emergence (April 19, 1972), at a location in the Logan Canyon area approximately 35 km northeast of Logan, Utah. 5 other squirrels were trapped on May, 25 1972. All animals were weighed immediately after capture and sacrificed by decapitation. Immediately after sacrifice the testes were immediately removed from all animals, weighed, decapsulated and assayed for MAO activity as previously described[3]. A *t*-test was used to test for significance between sample means.

Results. Testicular weights of the house sparrows (Figure) increased from December to April and decreased from July to November. Testicular MAO activity (Figure), calculated on a per animal basis, increased from December to April, but generally decreased from April to December. On a per mg of tissue basis, testicular MAO activity increased from November to April ($p << 0.001$), but decreased from April to November with the exception of July. Increases in MAO activity from November to January preceeded increases in testicular weight.

Ground squirrels captured in May (Table), had significantly higher body weights, but lower testicular weights

[1] We thank the Atomic Energy Commission Grant No. At(11-1)-1602, Utah State University research project No. U-300 and Ecol. Grant No. 19 for their support of this research. R. L. URRY, present address: Division of Urology, University of Rochester, School of Medicine and Dentistry, Rochester, New York, 14642. J. L. FREHN was on sabbatical leave from his present position in the Department of Biology, Illinois State University, Normal, Illinois, 61761. We thank PHYLLIS TAYLER for her technical help in this investigation.

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