content of the spleen may also play some part; we found that the spleen volume decreased during leptazol induced seizures, but not during electroshock (unpublished) and this may contribute to the increased magnesium concentration in the serum in some cases. Another investigator showed that electrical stimulation of skeletal muscle causes a marked elevation of serum magnesium[11] although no change in the concentration of this ion was found in the skeletal muscle during the present study.

TABLE 3. THE DEGREE OF HAEMOCONCENTRATION DURING EXPERIMENTALLY INDUCED SEIZURES

| | Leptazol | High intensity sound | Electroshock |
|---|---|---|---|
| Control group | 42·0 ± 0·8 | 42·0 ± 0·4 | 41·9 ± 1·2 |
| Experimental group | 45·0 ± 2·2 | 46·8 ± 1·1 | 46·1 ± 2·0 |

Results expressed as mean percentage red blood cells ± S.E.M. Details otherwise as shown in Table 1.

It can be concluded that the raised serum magnesium concentrations are a consequence of peripheral changes which occur irrespective of how the seizure is initiated.

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## Increased vascular permeability and activation of plasminogen by the trypsin-like esterases from the mouse submandibular gland

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THE SUBMANDIBULAR gland of mouse contains a variety of enzymatically and biologically active substances, which include esteroproteinolytic enzymes,[1–9] kallikrein[12,13] and a nerve growth promoting protein (NGF).[14,15] All of these are also capable of splitting *N*-benzoyl-arginine ethyl ester (BAEE) which is a widely used synthetic substrate for the determination of trypsin-like enzymatic activity. The relationships between these substances are not, however, clear-cut and well-established.

Abbreviations used: AMCA = *p*-Aminomethyl-cyclohexane carboxylic acid.

We have recently purified and enzymatically characterized six esterases from the mouse submandibular gland.[10,11] The enzymes were found to resemble kallikreins but their possible identity with salivary kallikrein remained an open question. The present communication deals with the effects of the purified mouse submandibular esterases on vascular permeability and on bovine plasminogen. These effects were studied since the characteristics of the enzymes in kallikrein-kinin and fibrinolytic systems are very similar and the enzymes of both of these systems also participate in similar processes in tissues.[16,17] It is known that, e.g. purified plasma kallikrein which liberates vasoactive bradykinin from kininogens also converts human plasminogen to plasmin.[18]

*Materials and methods*

The effects of vascular permeability were studied as described by Bhoola *et al.*[19] A rabbit back was epilated with an animal clipper between hips and shoulders. The rabbit was injected intravenously with filtered 0·5 per cent Evan's blue (Evan's blue vital T 1824, Gurr Ltd.) in saline. Immediately thereafter the test solutions were injected intradermally in 0·1 ml volumes. The enzyme preparations used were those described by Ekfors *et al.*[10] Histamine (Histamin hydrochloride, Fluka AG) and bradykinin (Bradykinin triacetate, Sigma Chem. Comp.) were used as standards. Saline was used as a control of traumatic blueing due to mechanical injury caused by the injection. The lesion indicated by the blue colour extravasated around the site of injection was recorded 30 min later. The diamater of the blued area is known to correlate to the logarithm of kallikrein concentration.[19]

The fibrinolytic activity was measured using the fibrin plate method of Astrup and Müllertz[20] as presented by Brakman.[21] Seven ml of 2 g/l plasminogen-rich fibrinogen (Grade B1, Kabi, Sweden) solution was clotted with 2 NIH units of Thrombin (Topostasine®, Hoffman la Roche, Switzerland) on Petri dishes (inner diameter 9·0 cm). The proteinase activity was measured by using plate assay after destruction of the plasminogen by heat (1 hr, +75°). The completeness of plasminogen destruction was tested with 2 Ploug units of urokinase (Urokinase reference standard, Leo Pharmaceutical Products, Denmark). Thirty $\mu$l of the enzyme solution was applied on a fibrin plate and incubation was carried out at +37° for 18 hr. The inhibition of plasminogen activation was tested using 1 mM AMCA (Kabi, Sweden) both in fibrin plate and enzyme solutions.

*Results*

Figure 1 shows the effect of the purified esterases on the vascular permeability of the rabbit skin vessels. The components C, D, E (6 $\mu$g) and F (4 $\mu$g) caused approximately the same effect as 10 $\mu$g bradykinin. The diameter of the blue area was 10–12 mm. Histamine (10 $\mu$g) was a little more effective, the diamater being 14–15 mm. The same dose of the components A and B caused the same reaction as saline, i.e. a blue area of 2–3 mm in diameter, but when the dose was increased 4-fold the diameter was significantly greater, 4–5 mm. The vascular permeability increasing activity of all the components was destroyed in 30 min at 100°.

In assays of the plasminogen activation by the purified undiluted enzyme preparations the following lysis areas were obtained (in mm$^2$): A: 205, B: 314, C: 428, D: 82, E: 3 and F: 124. Because the enzyme preparations had very unequal activities against synthetic substrates (BAPA, BAEE), they were diluted equally in regard to their esterase activity (BAEE as substrate about 17 $\mu$moles/ml min). The dilutions required in the different enzyme preparations were: A:B:C:D:E:F = 1:1:13:23:7·5:2·5. The protein concentrations of the enzyme preparations obtained are given in Table 1. The plase minogen activator effect of the diluted enzyme preparations is presented in Fig. 2 and in Table 1. Th-percentages of these enzyme activities from the activity of urokinase and their inhibition by AMCA are also given in Table 1. When plasminogen-free fibrin plates were used no proteinolysis occurred. This confirms our earlier observations about the weak proteinase activity of these purified salivary gland enzymes. AMCA inhibited totally the activator effect of enzyme F, but its inhibitory effect on the activities of the components A, B and C was surprisingly low.

*Discussion*

The results indicate that the esterases from the mouse submandibular gland have activity on vascular permeability. Those components which most actively hydrolysed BAEE were also the most effective in increasing vascular permeability: the components A and B which hydrolyzed BAEE only slowly[11] were almost without vascular effects. It is to be noted that even with doses equal in esterase activity their activity on vascular permeability was significantly smaller.

The vascular effects of the mouse submandibular gland esterases resemble those of the rat submandibular enzymes.[22] The increase in vascular permeability can be assumed to be mediated by plasma kinins liberated by the esterases.[19] If the assumption is correct, it would suggest that the esterases are kallikreins (EC 3.4.4.21). There are, however, a few contradictory findings: (1) The submandibular kallikrein has not been reported to hydrolyse amide substrates, e.g. *N*-benzoyl-arginine-*p*-nitro-anilide (BAPA). The esterases tested in this study readily split BAPA.[11] (2) Vogel[23]

TABLE 1. THE SAME FIBRINOLYTIC ACTIVITIES OF THE ENZYMES A–F AS IN FIG. 2

| Enzyme | Protein content (μg/ml) | Lysis area ($mm^2$) | Per cent of specific activity of urokinase | Inhibition by AMCA (%) |
|---|---|---|---|---|
| A | 60 | 200 | 11 | 55 |
| B | 65 | 270 | 19 | 15 |
| C | 17 | 192 | 38 | 48 |
| D | 6·4 | 0 | — | — |
| E | 8·4 | 0 | — | — |
| F | 15 | 67 | 11 | 100 |

The relative fibrinolytic activity compared with that of urokinase (specific activity), the protein content and the inhibition by AMCA (1 m-mole/l.) are also given.

reported that the bovine basic trypsin-kallikrein inhibitor (Trasylol®) was so species-specific that mouse kallikrein was not inhibited when tested with dog blood pressure as an indicator. We have found that the enzymatic activity of the mouse salivary gland esterases now studied were effectively inhibited by Trasylol® with the exception of the component A. (3) The submandibular kallikrein has been suggested to be located in acini rather than in tubuli.[24–26] The present esterases were all localized immunochemically in the granular ducts of the mouse submandibular gland.[27] The comparison is, however, difficult, since the mouse submandibular kallikrein has not been purified. Studies on the kinin liberating activity of the present mouse esterases are in progress using purified kininogens.

Three of the six mouse salivary gland enzymes seem to be potent plasminogen activators. The specific activity of the enzyme C is over one-third of that of the urokinase preparation tested and it is of the same order as found with cytokinase purified by Ali and Evans,[28] viz. 395 Ploug units/mg protein. It is generally assumed, that the plasminogen activators hydrolyse only synthetic ester substrates.[29–33] Therefore, it is noteworthy that these enzymes have a considerable peptidase activity towards synthetic peptide substrates in addition to their esterase activity.

The enzymes A and B can be considered as "specific" plasminogen activators, because they have only low vascular permeability increasing effect. On the contrary, the component C has, in addition to its high plasminogen activating effect, the ability to increase vascular permeability. The component D, E and F, on the other hand, have a strong permeability increasing effect but only a weak or no plasminogen activating effect. The latter three enzymes also differ from component C with regard to their characteristics towards various chemical modifiers.[11]

It thus appears that one of the mouse salivary gland esterases (component C) affects both plasminogen activation and vascular permeability while two of the enzymes (components A and B) produce only plasminogen activation and three of the enzymes (components D, E and F) affect vascular permeability but not plasminogen activation. Plasminogen activation and vascular permeability effects may thus be characteristics of one and the same kallikrein-like enzyme or both of these effects may be produced separately by different enzyme species.

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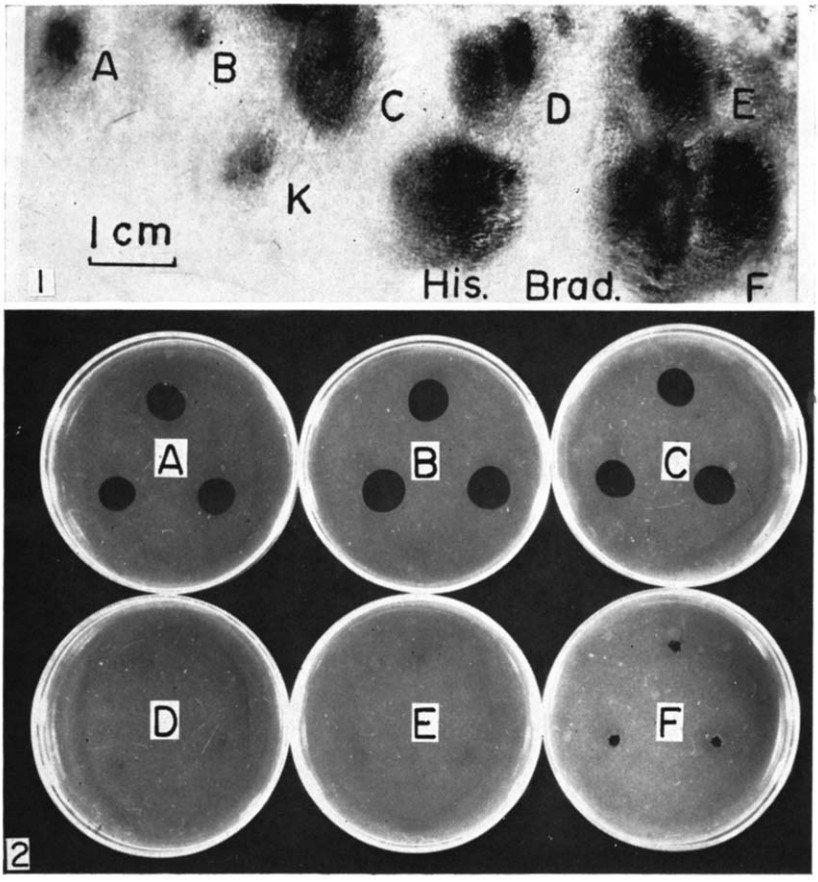


FIG. 1. The vascular permeability increasing effect of the mouse submandibular esterases A, B, C, D and E (6 $\mu$g) and F (4 $\mu$g) compared with the effect of histamine and bradykinin (10 $\mu$g).

FIG. 2. The fibrinolytic activities of the mouse submandibular esterases A–F.

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## Observations on the metabolism of cyclophosphamide

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CYCLOPHOSPHAMIDE (Cytoxan, Endoxan, *N*, *N*-di-2-chloroethyl-*N'O*-trimethylenephosphordiamide[1]), is one of the safest and most widely used agents in the treatment of many cancers. Although originally designed to be activated by tumour cells possessing high levels of a phosphoramidase enzyme[2] it is now well established that cyclophosphamide is activated almost exclusively by the liver. Since this fact was recognised, widespread efforts have been made to isolate and identify the active metabolite. Although metabolites have been isolated from serum[3] and urine[4] and following treatment with liver preparations[5] which activate cyclophosphamide *in vitro*, there is no unequivocal evidence that any one of these compounds is the active cytotoxic metabolite.

We have developed a bioassay method whereby the formation of cytotoxic derivatives of cyclophosphamide in the presence of liver microsomal preparations and an NADPH-generating system can be assayed by incubation with tumour cells.[6] Thereafter aliquots of the incubates, containing known amounts of tumour cells, are injected intraperitoneally into recipient rats. Comparison of the survival times of these animals with those of control animals, receiving an inoculum of tumour cells incubated with cyclophosphamide in the absence of a microsomal activation system, gives a reasonably precise measure of the cytotoxicity of the metabolite formed.

When radioactively labelled [$^{32}$P]cyclophosphamide was used and the incubation mixture extracted with chloroform (efficiency 80 per cent) the radioactive compounds present in the extract were detected