SOME OBSERVATIONS ON A REMARKABLE
FIBRINOLYTIC ACTIVITY IN THE
EXTRACT OF NASAL TISSUES
AND THE RELATED TISSUES

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INTRODUCTION

Chemical aspects of lung have been studied minutely by a great number of
workers, very few of whom, nevertheless, have noticed the significant relation
of chemical activity of lung to that of nasal tissues. Jobling & Petersen(1),
Weiss(2), Rocha e Silba(3), Ungar(4), Collumbine & Rydon(5) and others investi-
gated proteolytic activity of lung tissues, relating with the pathogenesis of lung
diseases, and obtained the results that proteolytic activity in lung might play an
important role in the process of inflammation and allergic change of lung.

In nasal tissues, however, any investigations have not been undertaken yet
of the proteolytic system, although nasal tissues might be regarded as a part of
the respiratory system. We are facing problems characteristic inflammations
or allergic disorders of nasal tissues seem to present, and trying to solve them
with favour of advanced study of proteolysis of lung.

MATERIALS AND METHODS

Tissue materials used: Fresh tissues used in the preparation were taken
out, on the occasion of operations, of patients ailing such diseases as sinusitis,
tonsillitis, gastritis, stomach ulcer and stomach cancer.

Mucous membrane of maxillary sinus and of inferior turbinate, faucial tonsil,
pharyngeal tonsil and stomach mucous membrane taken from the patients, were
washed in isotonic saline solution, dried on filter paper, and kept in a deep

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freezer at $-20^\circ$C.

Crude preparation: The tissues were suspended in ten times its wet weight of ice-cold isotonic saline solution and homogenized by glass-homogenizer of a Potter type. After centrifugation of the homogenate, the supernatant fluids obtained were used as crude enzyme solution. These treatments were carried out in a cold room at $0^\circ$C.

Tissue fractions(6): The tissues were suspended in ten times its wet weight of ice-cold 0.25 M sucrose solution, and homogenized. The homogenate was then submitted to differential centrifugation. The homogenate was first centrifuged for 10 minutes at 600 g. The sediment was suspended in 0.25 M sucrose solution and also centrifuged for 10 minutes at 600 g. The supernates summed up were centrifuged for 10 minutes at 8,500 g. That sediment was again suspended in 0.25 M sucrose solution, and then centrifuged for 10 minutes at 8,500 g, which was known as mitochondria. The remained supernate, separated from mitochondria, was centrifuged for one hour at 18,000 g. The sediment was suspended in 0.25 M sucrose and also centrifuged for one hour at 18,000 g, at last microsome obtained.

Fibrinogen: The preparation of Armour Laboratories was dissolved in isotonic saline solution, making 0.2% of solution.

Thrombin: The preparation of Parke Davis & Co. was dissolved in isotonic saline solution, making 10 u/cc of solution.

Plasminogen: As a source of plasminogen, the globulin fraction of human plasma was used. The human plasma was diluted with distilled water, adjusted to pH 5.2 with 0.5 per cent acetic acid to precipitate the euglobulin fraction. After centrifugation, the precipitate was redissolved in saline buffer at pH 7.4 (1/15 M phosphate buffer) to the original volume.

Determination of the fibrinolytic activity: Slightly modified Ungar's method(7) was adopted. Each crude tissue extract was divided into three samples, usually of 1.8 cc each. The samples of crude extracts were mixed with 1.8 cc of 1/15 M phosphate buffer (pH 7.4). Sample A and B were added to 2.0 cc of 0.2 per cent fibrinogen solution. Sample C was added to 2.4 cc of isotonic saline solution. Sample A was mixed with 0.4 cc of 10 u/cc thrombin solution. As a control was used the mixture of 3.6 cc of 1/15 M phosphate buffer, 2.0 cc of 0.2 per cent fibrinogen solution and 0.4 cc of 10 u/cc thrombin solution. After these treatments, Sample A and the control formed fibrin clots. Therefore, these four samples were placed for 30 minutes in a hot water bath at $37^\circ$C. After incubation, 0.4 cc of thrombin solution was added to clot sample B remaining fibrinogen. All these samples were filtered and the filtrate read on the Beckmann
spectrophotometer. The optical density reading of sample A against C, corrected by subtracting the absorption of a fibrinogen-thrombin blank, gave the fibrinolytic activity. The reading of sample B against C, corrected by subtracting the absorption of a fibrinogen thrombin blank, gave the fibrinogenolytic activity.

RESULTS

1. Effect of the crude extract of maxillary mucous membrane on fibrin or fibrinogen.

Twenty-five minutes was dissolution time by the extract in ten times its wet weight of isotonic saline solution on fibrin clot. It took solution of two times its crude original extract of isotonic solution 28 minutes; the solution of four times its crude original extract of isotonic solution 30 minutes (Table 1). Sample B formed fibrin clot after addition of thrombin.

Table 1

<table>
<thead>
<tr>
<th>Action of the Extract from the Mucous Membrane Affected by Maxillary Sinusitis for Fibrin at 37°C</th>
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</thead>
<tbody>
<tr>
<td>Extract of maxillary mucous membrane</td>
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<tr>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Dissolution time of fibrin clot</td>
</tr>
</tbody>
</table>

1/15 M phosphate saline solution (pH 7.4) 1.8 cc + extract 1.8 cc + 2.0 cc of 0.2 % fibrinogen + 0.4 cc of thrombin (10 units per cc).

The optical density reading of fibrin clot lysis was as seen in Figure 1 and Figure 2. The fibrinogenolysis was found not to occur, judging from the optical density readings. The optical density reading of fibrinolysis were either high concentration of extract with large value of absorbance or high concentration of extract with smaller value of absorbance than the low one. It was found that fibrinolysis times did not always keep pace with the absorbances. This relation should be investigated furthermore.

2. Effect of the crude extract of inferior turbinate on fibrin or fibrinogen.

The dissolution time by the extract in ten times its wet weight of isotonic saline solution on fibrin clot was 15 minutes; the solution in two times its crude original extract of isotonic solution 18 minutes, in four times extract 20 minutes, in eight times extract 25 minutes. Sample B formed fibrin clot after addition of thrombin (Table 2).

The optical density reading of fibrinolysis product is presented in Figure 3.
Fig. 1 & 2. Action of the extract from the mucous membrane affected by maxillary sinusitis for fibrin for 30 min at 37°C. Wave length (m\(\mu\))

1/15 M phosphate saline solution (pH 7.4) 1.8 cc + extract (from isotonic saline solution with 10 times weight of the wet original mucous membrane) 1.8 cc + 2.0 cc of 0.2% fibrinogen + 0.4 cc of thrombin (10 units per cc).

(1): Original extract.
(2): Isotonic saline solution with 2 times weight of the original extract.
(3): Isotonic saline solution with 4 times weight.
(4): Isotonic saline solution with 8 times weight.

The fibrinogenolysis did not occur in the optical density readings. The high concentration of the extract showed the short dissolution time of fibrin clot but low value of absorbance. The relation of the optical density readings of the maxillary mucous membrane extract to the inferior turbinate one was as follows Figure 4.

**Table 2**

*Action of the Extract from the Mucous Membrane of Inferior Turbinate for fibrin at 37°C*

<table>
<thead>
<tr>
<th>Extract of inferior turbinate mucous membrane</th>
<th>Extract from isotonic saline solution with 10 times weight of the wet original mucous membrane</th>
<th>Isotonic saline solution with 2 times weight of the original extract</th>
<th>Isotonic saline solution with 4 times weight of the original extract</th>
<th>Isotonic saline solution with 8 times weight of the original extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolution time of fibrin clot</td>
<td>15 min</td>
<td>18 min</td>
<td>20 min</td>
<td>25 min</td>
</tr>
</tbody>
</table>

1/15 M phosphate saline solution (pH 7.4) 1.8 cc + extract 1.8 cc + 2.0 cc of 0.2% fibrinogen + 0.4 cc of thrombin (10 units per cc).
3. Effect of the crude extract of faucial tonsil, pharyngeal tonsil and stomach mucous membrane on fibrin or fibrinogen.

In these experiments fibrinolysis and fibrinogenolysis were not observed after incubation for 30 minutes at 37°C, and the optical density readings were not measured.

4. Effect of addition of cystein to the extract of mucous membrane of maxillary sinus on fibrinolysis.

As such enzyme as cathepsin is activated by cystein which inhibits plasmin it was by addition of cystein that the fibrinolytic activity was analyzed in the isotonic saline extract of the mucous membrane affected by maxillary sinusitis. According to the stated modification of Ungar’s method, the effect of cystein, the final concentration of which was adjusted to 1%, was observed. The dissolution time of fibrin clot by addition of cystein was markedly delayed long and the
optical density reading decreased, that is, the fibrinolytic activity was decreased to a certain extent (Figure 5).

![Graph showing effect of addition of cystein to the extract from maxillary mucous membrane on fibrinolysis.](image)

Fig. 5. Effect of addition of cystein to the extract from maxillary mucous membrane on fibrinolysis. Wave length (mμ)

(1): 1/15 M. phosphate saline solution (pH 7.4) 1.8 cc + extract (from isotonic saline solution with 10 times weight of the wet original mucous membrane).

(2): The solution to which cystein was added to (1) (final concentration 1%).

This fact showed that the fibrinolytic activity in the mucous membrane affected by maxillary sinusitis differed from cathepsin activity.

5. Effect of addition of ε-aminocaproic acid to the extract of mucous membrane affected by maxillary sinusitis.

According to the stated Ungar's method, the effect of ε-aminocaproic acid was observed, the final concentration of which was adjusted to 1%. It has been known that ε-aminocaproic acid is a plasmin inhibitor.

Results obtained indicated that ε-aminocaproic acid inhibited completely the fibrinolytic activity in the extract of mucous membrane affected by maxillary sinusitis.

Comparison of the fibrinolytic activities found in the mucous membrane of maxillary sinus, inferior turbinate and nasal polyp

Preceding experiments showed that the nasal tissues had fibrinolytic activity, each of them a little different activity. We adopted the Astrup's plate\(^{(8)}\) method.
to make distinct the differences of the fibrinolytic activity of them: the mucous membrane affected by maxillary sinusitis, the mucous membrane of inferior turbinate and nasal polyp.

The fibrinogen was contaminated with bovine plasminogen, thereby rendering the fibrin plate (0.1 per cent solution of bovine fibrinogen) susceptible to the action of plasminogen activator. The product of two perpendicular diameters of the lysed zone was used as a measure of proteolytic or activator activity (Figure 6).

![Figure 6. Action of the extract from the mucous membrane of inferior turbinate (1), maxillary sinus (2) and nasal polyp (3) (extract from isotonic saline solution with 10 times weight of the wet original material).](image)

Ordinate: Product (in mm²) of two perpendicular diameters of the lysed zones (average of 3 single determinations).

Abscissa: Reaction time.

The fibrinolytic activity in the mucous membrane of inferior turbinate was more potent than that in the mucous membrane affected by maxillary sinusitis which was more potent than that of nasal polyp which was active.

In the experiment adopting the heated (for 45 minutes at 85°C) fibrin plate method, the lysed zone by the extract in ten times its wet weight of isotonic saline solution was not observed.

But by the extract in three or four times its wet weight of isotonic saline solution, the lysed zone was observed to some degree. It will be made certain by advanced experiments whether this fibrinolytic activity is plasmin proper or plasminogen activator.

*Effect of fractions from maxillary mucous membrane extract on plasminogen*
According to the Tagnon's method(9) a clot was formed, when the fibrinogen solution was mixed with plasminogen solution, tissue fractions (mitochondria, microsome and last supernate), and thrombin added. The time of dissolution of fibrin clot was measured, which told that the activity of the extract was found not in the fraction of microsome, supernate, but in the fraction of mitochondria.

Table 3

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Reagents in CC</th>
<th>Dissolution time of Clot (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total extract</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>1</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

All fraction were suspended in a volume equal to that of the original extract.
Fibrinogen 0.2 cc+globulin solution 0.1 cc+1/15 M phosphate buffer to make a total of 1 cc. Thrombin (on glass rod) added to each tube pH 7.4 temperature 37°C.

But also an experiment, where fibrinogen solution was mixed with mitochondria from extract, thrombin added, but plasminogen was not added, showed fairly fibrinolytic activity (Table 3).

Three similar fractionation experiments of faucial tonsil, were carried out, in all of which it was proved that fibrinolytic activity was absent in two particulated fractions and supernate.

DISCUSSION

It would be thought that there are differences in the physico-chemical process of morphological tissue. In the oto-rhino-laryngological field, there are also the differences in that of mucous membrane of maxillary sinus, faucial tonsil, pharyngeal tonsil (and stomach mucous membrane). When these tissues become morbid in inflammation, the differences are possibly more accelerated. The pathologic-physiological study of the inflammatory tissues would be of considerable help to that of physico-chemical mechanisms of the normal ones.

To investigate the proteolytic activity of each inflammatory tissue, a test was made for substrate of fibrin and fibrinogen. Fibrinolytic activity was observed in isotonic saline extracts of such tissues as mucous membrane of inferior turbinate and of maxillary sinus, but in those of faucial tonsil, pharyngeal tonsil and stomach mucous membrane it was not observed. It was found
that all tissues did not contain fibrinogenolysis.

By the plate method the fibrinolytic activity in nasal tissues was analyzed. Nasal polyp was one of the proper nasal tissues severely destroyed by inflammation or allergy. The mucous membrane affected by maxillary sinusitis had still the morphological structure of a nasal tissue, though it was considerably influenced by inflammation. The mucous membrane of inferior turbinate possessed its normal nasal tissue. It was examined in Astrup's plate method how the morphologically modified tissues changed the proteolytic activity. The result indicated that the mucous membrane of inferior turbinate is more potent than that affected by maxillary sinusitis which is more active than nasal polyp.

As for proteolytic enzymes there are plasmin, trypsin, pepsin, cathepsin and papain, etc., which have each its own varied characters. The peculiarities are as follows. (1) KCN, H₂S and others which activate cathepsin inhibit plasmin activity; (2) such inhibitory substances of cathepsin as H₂O₂ clearly activate plasmin activity. Accordingly plasmin is an enzyme with different nature from those of cathepsin, papain and others. We tested, to make the property clear, how the fibrinolytic activity in nasal tissues was changed by the addition of cysteine, and found the fibrinolytic activity goes down at that time. It was also found that ε-aminocaproic acid inhibits the activity completely.

According to Astrup, Müllerz(8) and Sherry(10), the bovine fibrin used as a substrate in the method contains bovin plasminogen as a contamination, because no procedure is available which may produce bovine fibrinogen free from plasminogen.

Therefore the method of fibrin clot dissolution responds to fibrinolytic enzymes as well as to activators which are able to transform bovine plasminogen into plasmin. But it is possible to destroy the plasminogen by means of a heat denaturation, still with most of the sensitivity of the fibrin towards the proteolytic enzymes and to make it insensitive to fibrinolytic activator. Using the heat fibrin plate method by Astrup et al, we observed whether the tissue extracts dissolved the fibrin or not, and found the extract also dissolved the fibrin to some degree. Characteristics of the fibrinolytic activity are under investigation.

By Tagnon(9), and by Lewis and Ferguson(11) it was confirmed that animal tissues contained a fibrinolytic activator existing particularly in the microsome fraction. But Astrup(12) could not find the fibrinolytic activity in microsome by the method of Tagnon. And using potassium thiocyanate he succeeded in isolation of a soluble fibrinolytic activator from animal tissues. In order to ascertain the view, we designed an experiment of function in tissue fraction. Results obtained showed, contrary to Tagnon's test, that fibrinolytic activity
existed in mitochondria, not in microsome, and moreover mitochondria presented
the fibrinolytic activity without plasminogen. This experimental fact might
signify that the contaminant plasminogen in bovine fibrinogen was activated by
mitochondria or any enzyme in mitochondria which dissolved the fibrin. This
nature is to be researched into. The active agent in tonsil was not detected
in mitochondria, microsome and supernatant respectively.

Spector(13), Duthie and Chain(14) reported that polypeptide by the action of
proteolytic enzyme offered a reasonable mechanism for the locally increased
capillary permeability, and leucocyte infiltration was observed whenever tissue
was injured. Also Collumbine and Rydon(5) stated that a substance or substances,
similar to Menkin's leucotaxine, was found in blister fluids produced thermally
or by the action of the vesicants mustard gas, and in phosgene lung oedema
fluids.

According to Ungar et al(4), the addition of the specific antigen to slices
of lung taken from a sensitized guinea pig, or the addition of anaphylactoid
agents (tween 20, Octadecylamine, morphine, and 48/80) to tissue slices from
normal animals, or the perfusion of lung with these agents, caused protein
breakdown and liberation of histamine and heparin.

Suppression of histamine and heparin released by inhibition of proteolysis
suggested that the latter was the more fundamental reaction.

Buhrmester, Wenner(15), Baxter and Rose(16) fixed quantity of histamin
in nasal tissue, and pointed out that nasal polyp and normal nasal mucous
membrane contain histamine. They also investigated the variation of quantity
of histamine in case of the tissue injured.

Whenever plasmin system in nasal mucous membrane was activated by
antigen-antibody or kinase function derived from bacterial infection, such
product as polypeptide increased in quantity and might accelerate to make locally
capillary permeability, leucocyte infiltration and oedem, and would cause a tissue
disturbance. The inhibition of plasmin activity to a certain low concentration
would obstruct the cleaning function by plasmin activity and bring deposition
of fibrin to tissue, fibrinous hypertrophie.

Astrup(17) stated that the tissue containing fibrinokinase is an organ with
tendency of bleeding. And it is characteristic that nose has frequent opportuni-
ties of bleeding.

Astrup(18) confirmed that there was fibrinokinase in lung of human. It is
of great interest that nose has a plasmin-like substance as in lung, when we
recall that nasal tissue might be regarded as a part of the respiratory system
organ. On the other hand, there is not fibrinolytic activity in stomach mucous
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membrane of the digestive organ.

We hope the facts and the considerations which we detailed would make an useful offer for future investigations.

CONCLUSION

1. Fibrinolytic activity was measured in isotonic saline extract of such fresh tissue as (1) the mucous membrane of inferior turbinate and maxillary sinus (2) faucial tonsil and pharyngeal tonsil, and (3) stomach mucous membrane, which were all taken from patients on the occasion of their operations.

2. Results obtained indicated that: (1) the strongest activity was observed in the mucous membrane of inferior turbinate, (2) stronger activity in the mucous membrane affected by maxillary sinusitis, (3) fibrinolytic activity was weaker in nasal polyp, and (4) fibrinolytic activity was not observed at all in faucial tonsil, pharyngeal tonsil and stomach mucous membrane.

3. Fibrinolytic activity of nasal tissues was decreased to a certain extent by the addition of cystein; and completely inhibited by /-aminocaproic acid, which has been known as a specific inhibitor of plasmin.

4. Fibrinolytic activity was found in the mitochondria fraction obtained from nasal tissues, but not in that from tonsil.

5. The nature of this fibrinolytic activity in detail are under further investigation.

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REFERENCES