Comparative Studies of the Fibrinolytic System of Sera of Various Vertebrates*)

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Introduction

Comparative studies on the coagulation and fibrinolytic components of various animal sera may be of practical and theoretical importance. An exact knowledge of the fibrinolytic system is needed for choice of the experimental animal, when the activation of fibrinolysis is to be studied under physiological and pathological conditions and after injection of fibrinolytic enzymes. Also a comparison of various fibrinolytic systems might reveal a new scheme of activation as it already has been the case (Müllertz and Lassen [24], Müllertz [21—23], Sherry [26]).

The purpose of this work was to examine the fibrinolytic system and its components in a number of species of vertebrates. It was hoped that convenient models could be found for the studies on the activation of fibrinolysis and that more information concerning the fibrinolytic system in general might be obtained.

Material and methods

The *sera* not plasmas of the following 18 species have been examined: man, monkey, dog, cat, ox, horse, ram, pig, rabbit, mouse, rat, hamster, guinea-pig, hen, duck, turkey, frog, fish.

The preparations used in this study were as follows:

1. Streptokinase (SK) Mfd by WWSS, Warsaw.
2. Human plasminogen (HP) prepared by modif. Kline's method (13).
4. The mixture of Streptokinase and human plasminogen (SKHP) was used as a source of activator.
5. Bovine thrombin prepared by modif. Lewis and Ferguson method (18).

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6. Casein — Commercial. Purified
7. Euglobulins ppt. from diluted serum (pH 5.3; temp. + 4° C; 24 hour).
8. Bovine fibrinogen prepared by modif. K e k w i c k et al. method (12).

It should by noted that the preparations of fibrinogen and thrombin used in this study did not contain any appreciable amounts of plasminogen and proactivator. The clot formed from fibrinogen after addition of SK did not dissolve in 2 days.

Fibrinolysis and *proteolysis* have been examined in the following systems:

Determinations of fibrinolysis:
0.2 ml euglobulin or serum (various dilutions)
0.2 ml SK (400 u/ml) or SKHP or 0.9% NaCl
incubation 3 min. at 37° C
0.1 ml thrombin (5 u/ml)
0.4 ml 0.2% fibrinogen:
lysis time measured in 8 mm test tubes at 37° C
Determination of proteolysis:
0.5 ml euglobulin
0.5 ml SK 1000 u/ml or SKHP or 0.9% NaCl
1.0 ml 4% casein.

After various times of incubation 3 ml of 10% TCA was added and "tyrosine" was determined using Folin-Ciocalteu reagent. Fibrinolysis activity was expressed as fibrinolytic index $F = \frac{1000}{t}$ (t = time of fibrinolysis in seconds).

Total protein in serum and euglobulin was determined by the W e i c h s e l b a u m method using the biuret reagent (11). The averaged values of protein in euglobulin of all species were 0.7—1.1%; of fish, frog and rabbit — 0.3—0.4%, of turkey — 1.8%.

Results

1. *Spontaneous fibrinolytic activity in euglobulins of various species.* It is well known that by isoelectric precipitation the fibrinolytic precursors can be separated from most of the inhibitors. The fibrinolytic activity of the euglobulin precipitate may be an indicator of fibrinolytic potential of serum.

T a b . 1 : Spontaneous activity of euglobulin precipitated from serum of various animals

Species	Fibrinolysis time	Fibrinolytic index
Guinea-pig	1—4 min.	4.17—16.66
Dog, Hamster	1—5 hours	0.05—0.27
Monkey, Cat, Mouse, Fish	5—24 hours	0.01—0.05
Human, Rabbit, Bovine, Horse, Pig, Ram, Duck, Hen, Rat, Turkey, Frog	above 24 hours	< 0.01

It can be seen from table 1 that in the conditions of this experiment only guinea-pig euglobulin shows any appreciable spontaneous fibrinolytic activity. Attention should be drawn to the fact that all experiments have been performed using serum euglobulin. If plasma euglobulin is used instead of serum euglobulin the fibrinolysis time is considerably shorter. This is in agreement with the results obtained by K o w a r z y k (15) and C o p l e y et al. (6). However serum and plasma euglobulin of guinea-pig reached almost the same activity.

We conclude from this experiment that only guinea-pig euglobulin contains active plasmin in any considerably amount.

2. *Fibrinolytic activity of euglobulin induced by SK or SKHP mixture.* This experiment was performed adding SK or SKHP to the euglobulin solution. The fibrinolytic activity is recorded on figure 1. It can be seen that SK activates euglobulins from 4 species, namely man, monkey, cat and dog. Euglobulins from further 8 species can be activated by SKHP (hamster, rat, mouse, rabbit, bovine, horse, pig, ram). The euglobulins of remaining 5 species (hen, duck, turkey, frog, fish) can be activated neither by SK nor by SKHP to any considerable value.

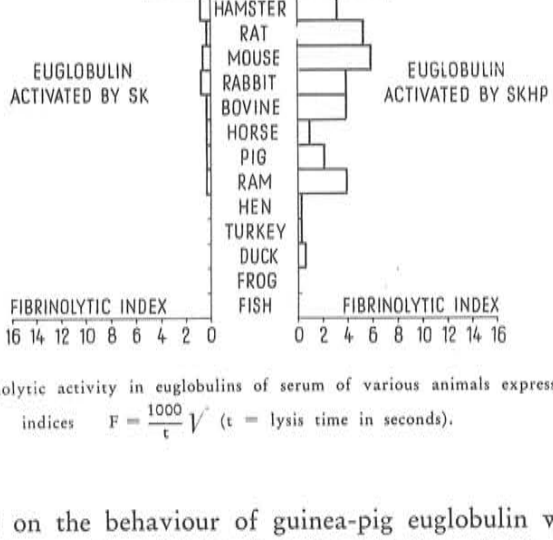

Fig. 1: Fibrinolytic activity in euglobulins of serum of various animals expressed in fibrinolytic indices $F = \frac{1000}{t}\sqrt{}$ (t = lysis time in seconds).

Some details on the behaviour of guinea-pig euglobulin will be presented later. It should be mentioned here that the addition of SK did not increase the fibrinolytic activity of the euglobulin. SKPH increased this activity only if guinea-pig euglobulins were used in higher dilutions.

3. *Proteolytic activity of bovine, guinea-pig and human euglobulin.* Some data obtained on the basis of the experiments in which the fibrinolytic method was employed, were also confirmed by studying the proteolytic activity of euglobulin against casein.

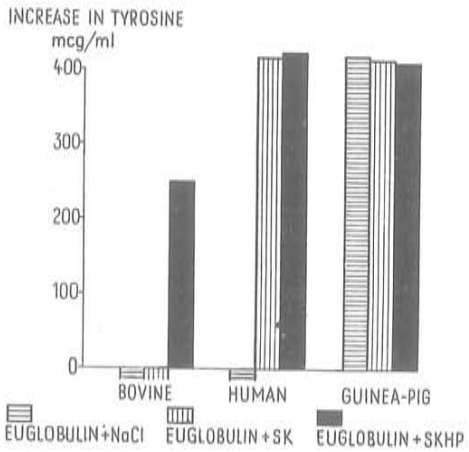

Fig. 2: Comparison of proteolytic activity of euglobulin from bovine, human and guinea-pig serum. (values expressed as increase in trichloroacetic acid soluble tyrosine after 60 minutes incubation of euglobulin with casein).

It can be seen from figure 2 that the proteolytic activity of guinea-pig euglobulin, activated by SK or SKHP did not exceed the activity of euglobulin without activator. In human euglobulin activation by SK and by SKHP produced activities of the same order, but no spontaneous activity was found. In bovine euglobulin marked proteolytic activity could be produced only after addition of SKHP.

4. "*Proactivator activity*" of various animal sera. In order to explain some differences between the examined animal sera, a following experiment was performed (see table 2). Serum was diluted 1 : 20, mixed with an equal part of SK and the ability of this mixture to activate bovine plasminogen was examined. It can be seen that the same four species, which are directly activated by SK, show fibrinolytic activity. This experiment confirms the existence of proactivator in the species of human, monkey, dog and cat, if proactivator is defined as a substance which reacts with SK to activate bovine plasminogen.

5. *Inhibition of SKHP mixture by animal sera.* In this experiment (table 3) a mixture of SK and HP with relatively high activity has been incubated with serum in various dilutions and the fibrinolytic activity of this mixture has been

tested against purified fibrinogen. It can be seen that most of the examined animals except the first four species showed inhibitory activity.

Tab. 2: Proactivator activity of the serum of various animals

Serum diluted	Fibrinolytic index
Human	5.45
Monkey	4.30
Cat	4.86
Dog	2.03
Guinea-pig, Horse, Pig, Ram, Hamster, Mouse, Rabbit, Rat, Hen, Duck, Turkey, Frog, Fish	0.1

Tab. 3: Inhibition of human SK-activated plasmin by serum of various animals

Species	Lysis time (minutes)		
	100 per cent serum	25 per cent serum	12.5 per cent serum
Human	3.5	3	2.5
Monkey	3	2	1.5
Dog	3	3	3
Cat	3	1.8	2.0
Guinea-pig	3	3.8	2.7
Hamster	> 600	> 600	6.5
Rat	> 600	7.3	8.5
Mouse	6.5	4.0	3.5
Rabbit	> 600	9.0	4.7
Bovine	> 600	9.0	4.9
Horse	> 600	> 600	8.2
Pig	> 600	4.2	—
Ram	> 600	8.7	4.0
Hen	15	6.0	4.0
Turkey	> 600	> 600	4.3
Duck	> 600	> 600	4.5
Frog	24	6.5	5.5
Fish	8	4.3	3.5
SKHP (control)		3.5 — 4.3	

6. *Activation of different bovine plasminogen (BP) preparations by SK and SKHP mixture.* At this point of our experiments we were not able to decide whether the low activity of various species is caused by lack of proactivator of plasminogen or by the presence of inhibitor or by both.

Another approach to this problem consisted in purifying bovine plasminogen using the method of K l i n e (13). In the course of purification BP acquires the ability to be activated by SK. This is shown on figure 3 and table 4 where the fibrinolytic and proteolytic activities of bovine serum, euglobulin and plasminogen activated by SK or SKHP were compared. Without activators no fibrinolytic activity was detected. These experiments could be explained assuming that the inhibitor is lost during purification of bovine plasminogen

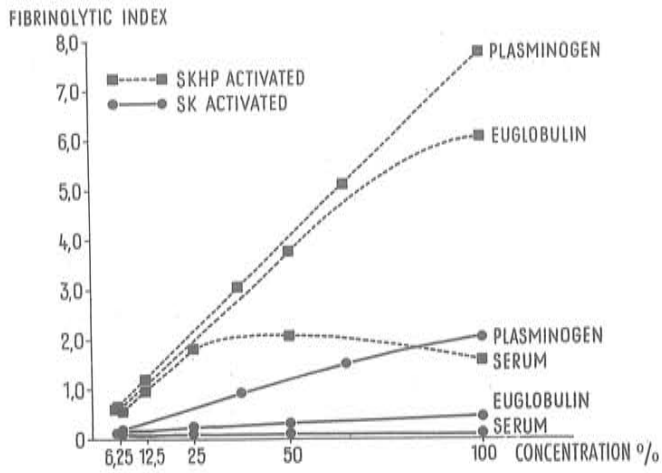

Fig. 3: Activation of bovine serum, euglobulin and purified plasminogen by SK and SKHP.

7. *Activation of plasmin in guinea-pig euglobulin.* Because of the unique position of the guinea-pig among all tested animals it was desirable to obtain more data about the fibrinolytic system of this animal. The guinea-pig serum was stored after dilution at pH 5.3 (t + 4° C), and at various time intervals up to 24 hours, fibrinolytic activity was determined in the dissolved euglobulin precipitate. The results of this experiment are shown on figure 4. It can be seen that spontaneous fibrinolytic activity generates slowly in guinea-pig euglobulin precipitate and reaches a maximum after 24 hours. No more activity appeared on longer storage. It is possible to obtain, at any time within 24 hours, almost the same maximal activity with SKHP. SK alone gives only a trace of

activation. Other animals did not show, under the conditions of this experiment, such a phenomenon.

Tab. 4: Proteolytic activity of bovine serum, bovine euglobulin and purified bovine plasminogen (BP) (Kline method) after addition of 0.9% NaCl, SK and SKHP mixture*)

System	Increase in TCA soluble tyrosine, mcg/ml		
	Incubation time		
	0 min.	30 min.	60 min.
serum + NaCl	0	0	0
serum + SK	0	0	0
serum + SKHP*)	0	90	110
euglobulin + NaCl	0	0	0
euglobulin + SK	0	30	30
euglobulin + SKHP*)	0	300	480
BP + NaCl	0	40	140
BP + SK	0	200	340
BP + SKHP*)	0	520	680

*) Values after substraction of blank for SKHP

It seems that the most adequate explanation of our findings is that guinea-pig serum contains plasmin bound with antiplasmin in a complex which dissociates at pH 5.3.

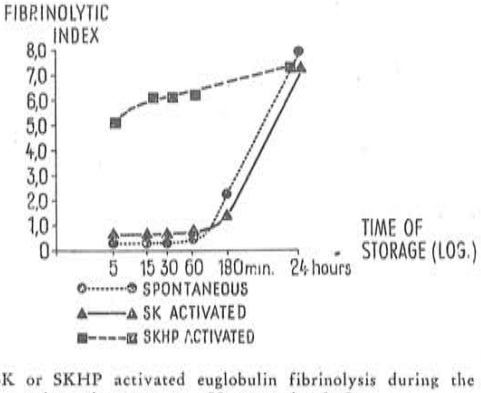

Fig. 4: Spontaneous and SK or SKHP activated euglobulin fibrinolysis during the storage of diluted guinea-pig serum at pH 5.3 and 4° C.

Discussion

A number of investigators studied the fibrinolytic system and its activation in various animals.

Guest et al. (9) found that the sequence of decreasing plasma antiplasmin activities by species is: alligator, guinea-pig, ox, rat, rabbit, frog, human, cat, chicken, opossum, pigeon and dog. Gerheim and Ferguson (8) found that SK activates only human plasminogen while the staphylokinase is capable of activating various kinds of plasminogen prepared not only from human but also from other animal plasma (dog, rabbit, guinea-pig). Using the proteolytic and fibrinolytic methods, Clifton and Canamella (5) found that SK activates euglobulin of human, dog and guinea-pig, while euglobulin of pig, rabbit, monkey, rat, chicken and cow were refractory. A slight spontaneous activity in euglobulin of dog and guinea-pig has been found.

Hayashi and Maekawa (10) found that SK prepared by them activates only human plasminogen. Commercial SK (Lederle and Co., Wellcome and Borrough) and staphylokinase were active also against dog, sheep, rabbit and horse plasminogen. Bovine plasminogen was activated by SK only in the presence of human serum.

Geiger (7) using a mixture of streptokinase and human euglobulin heated to 50° C was able to induce a proteolytic activity in the euglobulin of rabbit, guinea-pig, duck and chicken.

These facts may be explained by the findings of Müllertz (21—23) and Müllertz and Lassen (24) who postulated that SK does not activate plasminogen directly. They accepted the existence of proactivator in human serum which reacts with SK to form an activator which converts plasminogen to plasmin.

The fact that bovine plasminogen may be activated by SKHP mixture was confirmed also by other authors (14, 16, 26, 27). Subsequently Meyer and Burdon (19) found that SKHP mixtures activate plasminogen of mice, rat, rabbit, dog, sheep and monkey. The kinetics of activation of guinea-pig euglobulin by SKHP was studied by Norman (25).

According to Mohler et al. (20) streptokinase activates only plasminogen of human, dog, cat and rabbits.

It is difficult to see from the above results whether any regular pattern of the activation of fibrinolytic system exists among various animals. On the basis of our results, all animals investigated may be divided into four groups shown on table 5.

To group 1 belongs only guinea-pig serum. Its unique fibrinolytic system shows spontaneous activity after storage of euglobulins at its isoelectric point.

Tab. 5: Activation of fibrinolytic system in various vertebrates species

Spontaneously activated	SK and SKHP activated	SK resistant SKHP activated	SK and SKHP resistant
Guinea-pig	Man Monkey Cat Dog	Ox Ram Pig Horse Rabbit Hamster Mouse Rat Guinea-pig	Hen Duck Turkey Frog Fish

The euglobulin of the animals belonging to group 2 can be activated by SK alone. They do not show any considerable spontaneous activity.

The euglobulins precipitated from serum of animals belonging to group 3 are SK resistent but can be activated by SKHP.

It should be noted that guinea-pig is also included to group 3 because serum of this animal could be activated by SKHP but not by SK.

It differs, however, from other animals in this group by the spontaneous activity of its euglobulin fraction. The euglobulin of lower vertebrates sera (group 4) are relatively SK and SKHP resistant.

The differences between the fibrinolytic systems of various animals, presented in this paper, can be explained in three possible ways. They may be due to 1. the existence of species specificity of different fibrinolytic precursors; 2. The existence of inhibitors of the activating agents; 3. The lack of one or more of the components of fibrinolytic systems in the serum of a particular group of animals.

Ad 1. It is not possible at the present state of our experimental evidence to decide whether species specificity plays any role in the observed differences. It might be that small differences exist in the structure of the proteins of the fibrinolytic system among various groups of animals. Knowledge of the exact chemical structure and of amino-acid sequences in the studied proteins is needed to elucidate this question.

Ad 2. It was possible to show in the above experiments that the role of inhibitors is probably more important than it has been postulated previously. Purification of bovine plasminogen renders it far more sensitive to SK activation. In this case the existence of an inhibitor of SK activation may be postulated; this inhibitor of activation present in a third group of animals and

lacking in a second group, may be destroyed by SKHP or removed by purification.

In guinea-pig serum the inhibitor may be of antiplasmin nature. We have suggested previously (Latallo et al. [17]) that in guinea-pig serum plasmin is bound to an antiplasmin in a complex which dissociates slowly at pH 5.3 and at low ionic strenght. This would not exclude the possibility that SKHP destroys or neutralizes this inhibitor.

It is possible that the action of peptone and of heparin (28) on guinea-pig serum, may be of the same nature (Astrup [2]). Heparin is known to inactivate antiplasmin (4).

Ad 3. Another possible explanation of our findings may be the acceptance of a multiplicity of the components of the fibrinolytic system. According to the hypothesis of Müllertz (23) and Astrup (1, 2) human plasminogen preparations contain a proactivator necessary for conversion of plasminogen into plasmin. A second group of animals would contain both proactivator and plasminogen, the third group of animals would posses plasminogen but no proactivator; in the fourth group both factors would be absent.

We were able to show the so called "proactivator activity" only in the euglobulin of the second group of animals. However it is not known whether proactivator and plasminogen are two separate substances (Astrup [1, 2], Müllertz [23]) or activator is a plasminogen SK complex (Sherry [26, 27], Kline and Fishman [14]).

At the present state of knowledge it is not possible to decide which explanation is true. Further work is needed to resolve this question.

Acknowledgement

We are indebted to Mr. Zenon Wegrzynowicz for his valuable technical assistance.

Summary

1. Comparative studies have been performed on the fibrinolytic system of sera of 18 vertebrates: man, monkey, dog, cat, ox, horse, ram, pig, rabbit, mouse, rat, hamster, guinea-pig, hen, duck, turkey, frog, fish.

2. Spontaneously active plasmin in a considerable amount has been found only in guinea-pig euglobulin.

3. The euglobulins from human, monkey, cat and dog sera can be activated by SK and by SKHP mixture.

4. The euglobulin from hamster, rat, mouse, rabbit, bovine, horse, pig and ram sera can be activated by SKHP but not by SK.

5. The euglobulins of lower vertebrates species cannot be activated by neither SK nor SKHP to any considerable value.

6. The activation of fibrinolytic system runs parallel with the activation of proteolysis in human, bovine and guinea-pig euglobulin.

7. The proactivator activity has been found in the species of human, monkey, dog and cat.

8. Streptokinase activated human plasmin (SKHP) has been inhibited by most of the examined animals sera except of the human, monkey, dog and cat sera.

9. It has been found that in the course of purification bovine plasminogen acquires the ability to be activated by SK. An inhibitor of activation occuring in bovine serum and euglobulin is probably removed by purification of plasminogen.

10. The activation of plasmin in guinea-pig serum has been studied, it was found that spontaneous fibrinolytic activity generates slowly in guinea-pig euglobulin and reaches a maximum after 24 hours. Guinea-pig serum and shortly precipitated euglobulin could be activated by SKHP mixture only.

11. Several possible explanations of the different behaviour of fibrinolytic systems in various animals have been discussed.

Résumé

1. Nous avons conduit des études comparatives sur le systéme fibrinolytique des sérums de 18 vertebrés: homme, singe, chien, chat, boeuf, cheval, mouton, porc, lapin, souris, rat, hamster, cobaye, poule, canard, dindon, grenouille, poisson.

2. Les euglobulines isolées à partir du serum animal ne contiennent que des quantités négligables de plasmine. Nous avons demontré une activité fibrinolytique en quantité considérable dans les euglobulines du cobaye.

3. Les euglobulines de l'homme, du singe, du chat, et du chien sont activées par la streptokinase (SK) et par le mélange de streptokinase et plasminogène humain (SKHP).

4. SKHP produit l'activité fibrinolytique dans les euglobulines des animaux suivants: boeuf, cheval, mouton, porc, lapin, hamster, rat, souris. Les euglobulines de ces animaux resistent á la streptokinase.

5. Les euglobulines des oiseaux, des grenouilles et des poissons résistent à la streptokinase et à SKHP.

6. L'activation de la fibrinolyse dans les euglobulines de l'homme, du boeuf et du cobaye est liée à l'activation paralléle de la protéolyse.

7. Nous avons trouvé "l'activité de proactivateur" dans le sérum de l'homme, du singe, du chien et du chat.

8. Nous avons constaté l'inhibition de SKHP par les sérums des animaux examinés sauf ceux de l'homme, du singe, du chien et du chat.

9. Nous avons trouvé que le plasminogéne du boeuf, purifié par la méthode de K l i n e , est devenu sensible à SK. L'inhibiteur de l'activation présent dans le sérum et dans les euglobulines est détruit ou, eliminé au cours de l'isolement du plasminogéne de boeuf.

10. Nous avons examiné l'activation de la plasmine chez le cobaye. La formation spontanée de la plasmine a lieu dans le sérum dilué du cobaye, précipité à pH 5.3 et à 4° C pendant 24 heures.

11. Nous avons discuté les hypothéses avancées pour expliquer l'existence de différents types de l'activation de la fibrinolyse chez les animaux examinés.

Zusammenfassung

1. Es wurden vergleichende Untersuchungen über das fibrinolytische System in den Seren von folgenden 18 Vertebraten ausgeführt: Mensch, Affe, Hund, Katze, Rind, Pferd, Schaf, Schwein, Kaninchen, Maus, Ratte, Hamster, Meerschweinchen, Huhn, Ente, Truthahn, Frosch, Fisch.

2. Spontan aktives Plasmin in größeren Mengen konnte nur in Meerschweinchen-Euglobulinen nachgewiesen werden.

3. Serum-Euglobuline von Mensch, Affe, Katze und Hund können durch SK oder von einer SKHP-Mischung aktiviert werden.

4. Euglobuline von Hamster, Ratte, Maus, Kaninchen, Rind, Pferd, Schwein und Schaf können durch SKHP, aber nicht durch SK aktiviert werden.

5. Euglobuline von niederen Vertebraten können weder durch SK noch durch SKHP aktiviert werden.

6. Die Ergebnisse, die bei Mensch, Rind und Meerschweinchen mit der fibrinolytischen Methode erzielt wurden, konnten auch mit proteolytischen Methoden bestätigt werden.

7. Ein Proaktivator konnte bei Mensch, Affe, Hund und Katze nachgewiesen werden.

8. SK-aktiviertes menschliches Plasmin (SKHP) konnte von der Mehrzahl der tierischen Seren gehemmt werden, mit Ausnahme der Seren von Menschen, Affen, Hunden und Katzen.

9. Es konnte nachgewiesen werden, daß Rinderplasminogen im Verlauf seiner Reinigung die Fähigkeit gewinnt, durch SK aktiviert zu werden. Es ist möglich, daß im Verlauf der Reinigung von Rinderplasminogen ein Inhibitor der SK-Aktivierung, der in Rinderserum und Euglobulin vorkommt, entfernt wird.

10. Die Aktivierung von Plasmin in Meerschweinchenserum wurde untersucht. Es wurde nachgewiesen, daß in Meerschweinchen-Euglobulinen sich spontan fibrinolytische Aktivität entwickelt, die ein Maximum in 24 Stunden erreicht. Meerschweinchenserum und Euglobuline können kurze Zeit nach ihrer Präzipitation nur durch eine SKHP-Mischung aktiviert werden.

11. Es werden Möglichkeiten einer Erklärung des unterschiedlichen Verhaltens des fibrinolytischen Systems bei den verschiedenen Tiergattungen diskutiert.

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