EFFECT OF THE SALIVARY GLAND SECRETION OF *Hirudo medicinalis* ON THE EXTRINSIC AND INTRINSIC MECHANISMS OF BLOOD CLOTTING

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The secretion of the salivary glands of the leech *Hirudo medicinalis* can carry out a number of biochemical reactions which prevent the blood from coagulating when sucked out by the leech. One of the most important components preventing blood from clotting is hirudin, which is found in the secretion of the salivary glands of *Hirudo medicinalis* [4]. Hirudin is a highly specific inhibitor of the enzyme thrombin and, consequently, the secretion of leeches can block blood clotting at the stage of conversion of fibrinogen into fibrin.

The aim of this investigation was to study the effect of the secretion of the salivary glands of leeches on activation of the extrinsic mechanism of blood clotting, stimulated by tissue thromboplastin, and on the contact stage, initiated by the intrinsic blood clotting mechanism.

EXPERIMENTAL METHOD

Secretion of the salivary glands of *Hirudo medicinalis* was obtained from mature healthy leeches grown at the Moscow GAPU Biologicals Factory, Ministry of Health of the RSFSR. Antithrombin activity (ATA) of the secretion was determined in a system consisting of 0.2 ml of 0.3% fibrinogen solution, 0.1 ml secretion, and 0.2 ml of thrombin solution. Fibrinogen and thrombin were produced by the Kaunas Bacteriological Preparations Factory. Depending on the physiological state of the leeches, ATA of their salivary gland secretion may vary, as described in detail previously [1]. Preparations of secretion in whose presence fibrinogen did not form a clot with thrombin, and also preparations virtually without ATA, were used. Tissue thromboplastin was obtained from fresh rat brain by the method in [5]. Thromboplastin activity, determined by the method in [6], was 15-18 sec. The plasma recalcification time was determined by the usual method [3]. The contact stage of plasma clotting was activated by glass powder (in the ratio of 300 mg glass to 1 ml plasma;, and also with dextran sulfate from "Fluka" by the method in [7]. Amylolytic activity of the activated plasma was determined by the method in [9] on chromatogenic substrate S-2302, generously supplied by the firm "Kabi Diagnostica."

EXPERIMENTAL RESULTS

The extrinsic mechanism of blood clotting is stimulated by tissue factor, and for that reason tissue thromboplastin is usually used in models of the extrinsic mechanism of blood clotting [2].

TABLE 1. Plasma Recalcification Time (in sec) during Incubation with Leech Salivary Gland Secretion and with Physiological Saline (control)

Volume, ml	Secretion				Physiological saline			
	1 min	5 min	12 min	15 min	1 min	5 min	12 min	15 min
0,05	125	240	230	190	87	85	90	95
0,10	315	270	306	225	60	85	77	80
0,20	660	440	480	300	33	32	43	55

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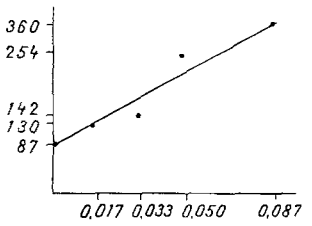

Fig. 1

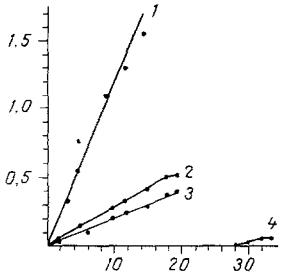

Fig. 2

Fig. 1. Lengthening of recalcification time of plasma activated by dextran sulfate, in presence of increasing quantities of leech salivary gland secretion without ATA. Abscissa, volume of secretion (in ml); ordinate, clotting time (in sec).

Fig. 2. Inhibitory effect of leech salivary gland secretion of amylolytic activity of blood plasma (relative to chromogenic substrate S2302), stimulated by dextran sulfate. 1) Physiological saline, 2, 3, 4) 0.05, 0.1, and 0.2 ml of secretion, respectively. Abscissa, incubation time (in min); ordinate, optical density at 405 nm.

The prothrombin time of rat plasma was determined in a system consisting of 0.2 ml plasma, 0.1 ml secretion (or physiological saline), 0.1 ml brain thromboplastin, and 0.1 ml of 0.025 M $CaCl_2$. Both leech salivary gland with high ATA and secretion without ATA were used. Active secretion lengthened the prothrombin time of plasma from 19-20 sec in the control to 40-48 sec. Secretion with no ATA did not affect the prothrombin time of plasma. Preincubation of inactive secretion with plasma at 37°C for 2-26 min did not change the prothrombin time of plasma, which remained at the control level. These results suggested that secretion of the leech salivary glands does not affect activation of the extrinsic clotting machanism. Lengthening of the prothrombin time of plasma by active secretion was due to the hirudin contained in the secretion.

Freshly frozen bovine plasma in a volume of 1 ml was incubated at 37°C with different volumes of inactive leech secretion and the recalcification time of the plasma was determined. The results of one experiment are given in Table 1. With an increase in the volume of secretion the recalcification time was lengthened. This effect lasted 15 min. Lengthening of the plasma recalcification time in the presence of secretion unable to inhibit the clotting of fibrinogen by thrombin may perhaps take place through blocking of the contact stage of blood clotting. To analyze this phenomenon a series of experiments was undertaken to study the effect of the secretion of leeches on the contact stage of blood clotting.

In the experiments of series I fresh rat plasma was treated with glass powder and incubated for 8 min. Under these circumstances the recalcification time of the plasma was reduced approximately by half. The plasma was separated from the glass and its recalcification time determined in the presence of inactive leech secretion. The system chosen for ananlysis consisted of 0.1 ml of glass-activated plasma, 0.1 ml of leech secretion, and 0.1 ml of 0.025 M $CaCl_2$. Physiological saline was used as the control. In the seven experiments, inhibition of the recalcification time of plasma activated by glass beforehand, by the secretion, was observed. On average the degree of inhibition was 248% ($n = 7$, $P < 0.01$).

In the experiments of series II we evaluated the immediate impact of intermediate leech secretion on the plasma contact activation process. We used dextran sulfate as an activating agent. We determined the specificity of the chromogenic substrate for kallikrein, S-2302, following judgement of the degree of activation of the contact stage and the impact which inactive secretion made on this process during the time of plasma coagulation in the presence of $CaCl_2$ and according to the kallikrein activation of the plasma. We added to 0.2 ml freshly frozen beef plasma secretion deprived of ATA (or physiological solution) and 0.1 ml of dextran sulfate (30 µl in 1 ml water) and incubated the mixture at 37°C for 3 min. We determined the time of coagulation after introduction of 0.1 ml 25 mM $CaCl_2$. It was established that the non-ATA secretion lengthened the coagulation time of the plasma activated

by dextran sulfate, and the effect increased upon increase of the quantity of additional secretion (Fig. 1). We added more secretion to 0.2 ml of freshly frozen beef plasma, this secretion not containing ATA (or 0.1 ml physiological solution), and 0.2 ml of dextran sulfate (30 μg in 1 ml water), and we incubated it for 3 min at 37°C. We brought the volume up to 1.6 ml with 0.05 M Tris buffer, pH 7.9, and added 0.2 ml S-2302 (1 mg per 1 ml water). We observed absorption at 405 nm at 37°C. The secretion sharply stopped generation of kallikrein upon contact with activated plasma in the presence of dextran sulfate (Fig. 2). The inhibiting effect was proportional to the quantity of additional secretion. Upon adding 0.2 ml of secretion the amidolytic activity of kallikrein exhibited development in very weak degrees even after prolonged incubation of the system analyzed in the presence of the substrate.

The results are evidence that inactive salivary gland secretion from *Hirudo medicinalis*, not possessing ATA, did not change the clotting time of blood plasma by brain thromboplastin in the presence of Ca^{++}, i.e., it did not affect activation of the extrinsic mechanism of blood clotting. Meanwhile, the contact stage of blood clotting was blocked by inactive secretion of the leech salivary glands. The inhibitory action of leech secretion on the intrinsic mechanism of blood clotting also is confirmed by experiments to study lengthening of the plasma recalcification time in the presence of secretion. One possibility is that the secretion may contain one or more inhibitors whose action is aimed directly or indirectly at the kallikrein of blood plasma, which is known to play an important role in activation of the contact stage of the intrinsic mechanism of blood clotting [8].

The results showed that the physiological action of the secretion of the salivary glands of the leech *Hirudo medicinalis* is due not only to hirudin, which inhibits the clotting of fibrinogen by thrombin, but also by components which block the contact stage of blood clotting.

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