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In 1975 one of us (M.S.S.) [5] described a method of determining the activity of total kallikrein in blood plasma.

The object of the present investigation was to determine the precise mechanism of the reaction on which the method is based and also to improve and modify some of its stages and to assess its suitability for clinical diagnostic purposes.

EXPERIMENTAL METHOD

The method is based on the property of plasma kallikrein to form kinins, i.e., the kininogenase action of the enzyme. The conditions leading to inactivation of kininases and kallikrein inhibitors, and measures promoting the transformation of plasma prekallikrein into kallikrein were elaborated in experiments *in vitro*. Experiments were carried out with blood plasma from 68 clinically normal subjects (children and adults) and 231 patients (children and adults) with various diseases. In the course of the work a preparation of high-molecular-weight kininogen (HMWK) was obtained from human blood serum [8]. The specific activity of the preparations was equivalent to 2.5-4.0 μ g bradykinin. The method in [6] was used for assay of α_1 -antitrypsin, and the method described previously in [4] was used to determine α_2 -macroglobulin.

Of the reagents used in the work bradykinin triacetate was obtained from Reanal (Hungary); unithiol (5% solution in ampuls), kaolin, and Tris were of Soviet origin.

EXPERIMENTAL RESULTS

A series of experiments *in vitro* showed that heating blood plasma in acid medium (pH 3.0) at 61°C for 15-30 min leads to activation of α_1 -antitrypsin and α_2 -macroglobulin, and also of kininases I and II and plasmin. However, prekallikrein, kallikrein, low-molecular-weight kininogen, and HMWK still remained intact. Hence, plasma deprived of two components of the kinin system (kallikrein inhibitors and kininases) thus still preserve the functionally united protein complex essential for realization of the kininogenase reaction. Kaolin, added to plasma after its neutralization, performs the role of a specific prekallikrein activator. The activating dose of kaolin under these circumstances is 0.5 mg/ml of mixture (Table 1), i.e., twenty times smaller than that used to determine kallikrein in native plasma [1, 2, 7, 9-11].

It will be clear from Table 1 that if kallikrein comes into contact with high doses of kaolin (5-20 mg/ml) its kininogenase activity is reduced. This was explained by adsorption of kallikrein on the kaolin: The enzyme was eluted from kaolin and kinins formed after incubation with HMWK preparations. Kallikrein from patients was adsorbed on kaolin to a much greater degree than that from clinically healthy subjects (Table 2). This property of kallikrein can be used as an additional indicator of the qualitative state of its molecule.

The results suggest the following modification of the kininogenase method of assay of blood plasma kallikrein.

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TABLE 1. Kininogenase Activity of Kallikrein in Native Plasma from Human Blood Donors on Contact with Different Doses of Kaolin (plasma from 6 donors mixed in equal parts was used in the experiments)

Parameter	Samples of plasma								
	without kaolin	with kaolin, mg/ml							
		0,125	0,25	0,5	1,0	2,0	5,0	10,0	20,0
Kallikrein, $\mu\text{g}/\text{bradykinin}/\text{ml}/\text{h}$	0,93	0,94	0,91	1,57	1,57	1,42	0,94	0,10	0
Activation (+) or adsorption (-) of kallikrein by kaolin, %	—	0	0	+70	+70	-10	-40	-90	-100

To 0.6 ml plasma 0.6 ml of physiological saline and 0.15 ml of 1 N HCl (pH 3.0) are added, the mixture is heated for 17-18 min at 61°C, cooled, neutralized with 0.5 N NaOH (0.27 ml), and treated with 1.7 ml of Tris-HCl (pH 7.6-7.8). Equal volumes of the mixture (1.1 ml) are poured into three test tubes, to two of which kaolin (in 0.1 ml Tris-HCl buffer) is added at the rate of 0.5 and 5.0 mg/ml mixture. The third test tube is left without kaolin. The samples are kept for 10 min at room temperature and then incubated for 1 h at 37°C. The reaction is stopped by the addition of 0.4 ml of 10% TCA, proteins are separated by centrifugation, and the quantity of kinins formed is determined in supernatants of the neutralized TCA-filtrates on the rat uterine cornu or a strip of cat jejunum. Synthetic bradykinin is used as the standard. Kallikrein activity is expressed in μg bradykinin/ml/h. By determining the kininogenase activity of samples without kaolin and with different concentrations of kaolin it is possible to obtain the characteristics of the following forms of kallikrein: 1) kallikrein in complex with inhibitors (sample without kaolin); 2) total kallikrein (sample with 0.5 mg kaolin/ml); 3) prekallikrein (difference between activity of samples with kaolin in a dose of 0.5 mg/ml and without it); 4) index of adsorption of kallikrein on kaolin, in percent (difference between activity of samples with kaolin in doses of 0.5 and 5.0 mg/kg),

The results of experiments with addition of purified HMWK preparations (200 $\mu\text{g}/\text{ml}$ mixture) to plasma from healthy subjects (24 persons) and patients with inflammatory-allergic diseases of the heart, respiratory organs, and hepatobiliary system (altogether 55 patients), neutralized after heating in an acid medium, showed that the intensity of kinin production in the blood can be judged from the level of kallikrein activity. On addition of HMWK preparations to plasma from blood donors or clinically healthy subjects whose kallikrein activity was 1.57-1.59 $\mu\text{g}/\text{ml}/\text{h}$, the increase in the content of kinins in the TCA-filtrates was very small, and averaged 16% in samples without kaolin and 8% in samples with an activating dose of kaolin (0.5 mg/ml) (Fig. 1). This indicates that the plasma samples contained HMWK necessary for the kininogenase reaction and for the formation of considerable quantities of kinins by kallikrein, and its preservation during treatment of the plasma under these experimental conditions. The smaller increase in the kinin content in samples of plasma with kaolin, it was found, was due to competition between HMWK and kaolin for prekallikrein. Addition of HMWK preparations to plasma from patients with total kallikrein levels reduced by 10, 25, 44, and 66% in samples without kaolin, revealed an increase in kinin content by 70, 110, 135, and 260%, respectively (Fig. 1). These findings indicate a sharp decline in the HMWK reserves in the test plasma despite preservation of the kininogenase activity of kallikrein. Addition of HMWK preparations to plasma samples in which kallikrein activity was reduced by more than 70% to 0.4 $\mu\text{g}/\text{ml}/\text{h}$ (Fig. 1, origin of broken lines), led to dissimilar results: In 50% of cases there was no rise of the kinin level, in the other 50% kininogenase activity of kallikrein was increased (to 850%). The increase in kininogenase activity of kallikrein in samples with kaolin depended on the prekallikrein content. The relationship was direct: the lower the prekallikrein content, the smaller the rise in kinin content in the TCA filtrates after addition of HMWK preparations to the plasma (Fig. 1).

TABLE 2. Components of Blood Kinin System in Children and Adults under Normal and Pathological Conditions Showing Increased (1) and Reduced (2) Kinin Production ($M \pm m$)

Parameters	Children under three years old								Children aged 7-12 years								Adults							
	healthy (n = 38)				sick				healthy (n = 10)				sick				healthy (n = 8)				sick			
	with empyema (n = 35)		with acute pneumonia (n = 42)		with asthmatic bronchitis (n = 26)								with chronic cholecystitis and cholangitis (n = 13)		with chronic hepatitis (n = 26)						with chronic bronchitis due to dust (n = 74)		with diabetes mellitus (n = 15)	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Frequency of changes in kinin production, %	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Free blood kinins, ng/ml	2,37±0,30	10,8±1,04	37±0,15	5,24±0,81	55±1,53	45±0,14	52±8,55	48±1,70	—	—	—	—	40±4,7±0,38	60±1,95±0,21	30±4,8±0,42	70±1,09±0,11	—	—	—	—	40±3,55±0,33	60±0,40±0,03	70±4,58±0,03	30±1,4
Kallikrein, µg/ml/h	1,58±0,08	4,16±0,73	1,08±0,12	2,90±0,22	2,90±0,15	0,97±0,62	2,90±0,18	0,94±0,10	1,60±0,10	1,60±0,10	1,60±0,10	1,60±0,10	2,15±0,19	0,78±0,11	2,17±0,20	0,85±0,12	1,59±0,16	2,51±0,22	0,81±0,10	1,84±0,10	1,84±0,10	0,81±0,10	1,84±0,10	0,99±0,09
bound with inhibitors	1,05±0,16	3,26±0,40	0,80±0,10	1,70±0,13	1,70±0,11	0,62±0,35	1,70±0,16	0,52±0,09	1,0±0,09	1,0±0,09	1,0±0,09	1,0±0,09	1,59±0,13	0,34±0,04	1,35±0,14	0,64±0,08	0,95±0,12	1,40±0,11	0,42±0,05	1,66±0,05	1,66±0,05	1,40±0,05	1,66±0,05	0,78±0,21
prekallikrein	0,52±0,08	0,90±0,12	0,28±0,09	0,90±0,12	0,35±0,04	0,35±0,04	1,20±0,11	0,34±0,05	0,60±0,08	0,60±0,08	0,60±0,08	0,60±0,08	0,56±0,07	0,44±0,05	0,82±0,09	0,21±0,04	0,64±0,10	1,11±0,10	0,41±0,05	0,18±0,05	0,18±0,05	0,41±0,05	0,18±0,05	0,21±0,05
Adsorption of kallikrein on kaolin, %	38	—	—	85±7	80±8	94±9	83±8	94±9	38±3	38±3	38±3	38±3	66±6	74±7	71±7	85±8	40±4	60±5	53±5	65±6	53±5	65±6	53±5	59±5
Kininases, µg/ml/min	0,82±0,06	0,53±0,04	0,81±0,08	1,50±0,11	1,53±0,14	3,69±0,42	3,50±0,31	3,69±0,42	0,93±0,10	0,93±0,10	0,93±0,10	0,93±0,10	0,48±0,04	0,48±0,05	0,79±0,06	0,93±0,10	0,88±0,09	1,69±0,20	2,15±0,21	0,56±0,07	2,15±0,21	0,56±0,07	2,15±0,21	0,70±0,10

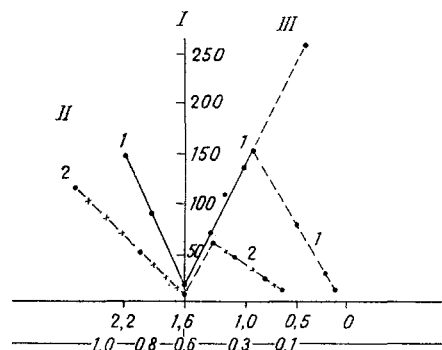


Fig. 1. Changes in kininogenase activity of kallikrein (1) and prekallikrein (2) of blood plasma (in $\mu\text{g/ml/h}$) on addition of HMWK preparations to plasma from healthy subjects (I) and patients with increased (II) and depressed (III) kinin formation. Abscissa: top line — kallikrein activity, bottom line — prekallikrein activity; ordinate, increase in kininogenase activity (in percent).

If kallikrein activity was increased by 20-40% the addition of HMWK to samples without kaolin led to an ever greater increase in the kinin content — by 90 and 150%, respectively. Changes of the same character in kinin production were found in samples with kaolin. It can be concluded from these results that with an increase in the total kallikrein content true activation of kinin production takes place as a result of stimulation of kallikrein and prekallikrein activity if the blood HMWK level is sufficiently high. With a fall in total kallikrein and prekallikrein levels by 50 and 45%, respectively, kinin production is depressed because of a decrease in the content of HMWK and prekallikrein in the blood. A decrease in the total kallikrein and prekallikrein content by 70% or more is reflected in depression of kinin production as a result of reduced activity of kallikrein and prekallikrein and a sharp decline in the HMWK reserves.

Since no purified preparations of HMWK are available for the investigator and since chromogenic substrates are expensive, and the method of determination of kallikrein which we suggest is based on preservation of the whole complex of proteins necessary for the kininogenase reaction, in our opinion the kininogenase method of kallikrein determination which we have developed and modified can be used to estimate the intensity of kinin production at every stage of the course of a disease in the most objective manner.

The intensity of kallikrein production is known to be one of the principal factors determining the concentration of free kinins in the blood and responsible for the physiological or pathogenetic influence of the kinin system. The result of an investigation of 68 healthy subjects and 231 patients with acute and chronic inflammatory, inflammatory-allergic, and allergic diseases of the lungs, pleura, bronchi, liver, and biliary tract, and also with endocrine diseases (diabetes mellitus), are given in Table 2. They show that the blood kinin system and kinin production undergo dissimilar changes in different stages of the course of the pathological process. In the acute phases of the disease activation of kinin production predominates (in more than 60% of cases) and is accompanied by hyperkininemia; in the chronic stage a state of depressed kinin production predominates (60-70% of cases) and is accompanied by hypokinemia.

The method we suggest for determining three forms of kallikrein and interpretation of the intensity of kinin formation give a deep insight into the pathogenesis of diseases; for example, low values of prekallikrein activity in patients with diabetes are evidence of depression of the prekallikrein synthesizing function of the liver. Our method permits the choice of therapeutic tactics for the prescription of remedies correcting pathologically changed kinin production to be established on a sound basis in each individual case. Parameters of the qualitative state of the kallikrein molecule, which constitute a delicate and sensitive test for the presence of a pathological process, enlarge an arsenal of diagnostic tests.

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USE OF SCATTERING OF LIGHT TO RECORD ERYTHROCYTE DESTRUCTION BY HEAT

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The study of resistance of erythrocytes to harmful factors is widely used in biological and medical research because it provides important information on the physicochemical properties of erythrocytes. The best known methods of determining resistance are osmotic, acid, mechanical, and certain others [1, 2-4]. Each of these methods provides different information and each has its advantages and disadvantages. For example, in osmotic hemolysis the erythrocyte ghosts remain in suspension, indicating incomplete destruction of the cells. From our point of view, the soundest method of studying erythrocyte resistance is that involving the use of heat, which also causes hemolysis and much more complete destruction of the cell membranes [2, 3, 5].

In the investigation described below, to determine the resistance of erythrocytes they were destroyed by heat and a method based on scattering of light was used to measure the course of the process.

EXPERIMENTAL METHOD

The method is essentially as follows. A suspension of erythrocytes in a solution of NaCl (or any other substance) is exposed to a constant high temperature (58-60°C) and changes in scattering of light by the suspension are recorded over a period of time. In this way the resistance of erythrocytes can be studied in different salt concentrations (i.e., at different osmotic pressures), including in isotonic solutions, in the presence of any other substances, over a wide range of pH values, and so on. Erythrocyte destruction during osmotic and acid hemolysis can also be recorded automatically by this method.

In Fig. 1 a diagram of the apparatus for recording erythrocyte destruction by the scattering of light method is shown. The tube containing the specimen, 0.5 ml in volume, is placed in a constant temperature cuvette 1. The cuvette is made of duralumin and blackened electrolytically. Temperature is kept constant by the U-3 ultrathermostat. The suspension is stirred by the mixer 7. A beam from the source of light (OI-19 illuminator) passes through the condenser lens 2, red filter 3, and light guide 4 and falls on the test object. Scattering of light is recorded at an angle of 20° to the incident beam by a cadmium sulfide (type SF-3-1) photoresistor 6 through the light guide 5, protecting the photoresistor against heat. The light guides are made from transparent plastic, covered with black nitro-enamel; the ends of the light guides are polished with GOI paste.

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