

SOME BIOCHEMICAL AND PHYSICAL PROPERTIES OF THE HUMAN PERMEABILITY GLOBULINS

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SERUM kallikrein (Frey, 1925) and PF/dil (Mackay, Miles, Shachter and Wilhelm, 1953) are two activable serum proteins which increase vascular permeability, and have, thus, been termed "permeability globulins". Kallikrein is a protease which, by releasing the polypeptide bradykinin from a plasma α_2 -globulin substrate, causes vasodilation and characteristic changes of inflammation (Werle and Trautschold, 1963; Werle, 1955; Lewis, 1961). Fresh or heated plasma may be used to demonstrate kinin release by kallikrein, *in vitro* (Mason and Miles, 1962). Kallikrein preparations contain esterase activity, hydrolyzing the amino acid ester TAME (Webster and Pierce, 1961). PF/dil, which is also a TAME esterase (Becker, Wilhelm and Miles, 1959) appears to be an activator of a kinin forming system in fresh plasma (Mason and Miles, 1962). It is ineffective in causing kinin release from heated plasma (Mason and Miles, 1962).

Previously we described the separation of two permeability globulins, A and B, from human serum (Kagen, Leddy and Becker, 1963a; 1963b), and suggested, largely on the basis of electrophoretic and chromatographic behaviour, that globulin A was serum kallikrein, and that globulin B was PF/dil. Further to establish this identification, the TAME esterase activity and plasma kinin releasing properties of A and B were studied, and will be reported here. In addition, data on their sedimentation behaviour will be presented.

Individuals with the disease, hereditary angioneurotic oedema, lack a serum permeability globulin inhibitor (Landerman, Webster Becker and Ratcliffe, 1962). Patients with this same disorder recently have been shown to lack C'1 esterase inhibitor (Donaldson and Evans, 1963). It will be shown here that preparations of human C'1 esterase inhibitor inactivate both kallikrein and PF/dil.

MATERIALS AND METHODS

Globulin A was prepared from pooled human serum as previously described (Kagen *et al.*, 1963b). The specific activity of this preparation was 150 times that of starch activated starting material.

Globulin B was prepared from pooled human serum (Kagen *et al.*, 1963b) with 2,000-fold increase in specific activity. For these studies Cohn fraction i + iii (Kagen *et al.*, 1963b) was first prepared, and then chromatographed. The chromatographic and electrophoretic properties of B, thus separated, are identical to those which it has in whole serum. This is in contrast to the earlier finding (Kagen *et al.*, 1963b) that B prepared from the subfraction I + III - 3 or III - I, 2 (Cohn method, Kagen *et al.*, 1963b) had a greater affinity for DEAE-cellulose, and a slightly greater electrophoretic mobility toward the positive pole than it had in whole serum.

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Soy bean trypsin inhibitor (SBTI) 5 times crystallized (Mann Research Laboratories) was used.

p-toluene sulfonyl-L-arginine methyl ester (TAMe) (Mann Research Laboratories) was used as enzyme substrate.

Human C'1 esterase inhibitor (two preparations) with 30 units per ml. and 340–368/units per ml. respectively were kindly supplied by Drs. J. Pinsky and I. H. Lepow.

Di-isopropylfluorophosphate (DFP) (Merck, Sharpe and Dohme Co.) 5 m was used.

Bioassay of vascular permeability was performed in the skin of guinea-pigs, previously given the dye pontamine sky blue intravenously, as described (Kagen *et al.*, 1963*b*; Miles and Wilhelm, 1955). Measurement of the diameter of blue spots arising at the sites of intradermal test injections was used as an assay of permeability increasing activity. The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Ultracentrifugal studies employing the Model E Ultracentrifuge (The Spinco Div., Beckman Instruments Inc.) with the partition cell technique (Yphantis and Waugh, 1956), allowing determination of sedimentation coefficients by direct measurement of permeability activity, were made. Results obtained at 25° were corrected to those expected in water at 20°.

TAMe esterase assay was performed as described by Becker, Fukoto, Boone, Canham and Boger (1963). In this procedure, enzymatic hydrolysis of the substrate liberates carboxyl groups, causing a fall in pH of the reaction mixture. The change in pH over the range measured is linearly related to the enzyme concentration.

Assay of kinin release from plasma was performed with the rat uterus preparation (Elliot, Horton and Lewis, 1960) isolated, and suspended in a 10 ml. oxygenated bath of modified Ringer's solution (Gaddum, Peart and Vogt, 1949) at 29°. The conc. of NaHCO₃ was 0.2 g. per l. The apparatus has been described by Coulson (1953). Aliquots of a pooled sample of outdated, human plasma obtained from two A (+) donors served as substrate, and results were recorded as the size of contraction (magnified 4 times) in mm.

EXPERIMENTAL AND RESULTS

Esterase properties

The enzymatic nature of the permeability globulins was investigated by direct assay of preparations for TAMe esterase, and by noting the effect of esterase inhibitors on their permeability increasing activity.

Assay of esterase activity.—Fig. 1 shows the results of TAMe esterase assay of globulin preparation A. A similar assay of preparation B is shown in Fig. 2. In both cases TAMe esterase activity is demonstrated to be present. Preparation A contained $4.5 \times$ the permeability activity of B. Soy bean trypsin inhibitor (50 $\mu\text{g./ml.}$) was pre-incubated with samples of preparations A and B at each of the three concentrations shown, for 30 min. at 22°, and is seen to have inactivated the TAMe esterase present.

Effect of SBTI.—The inhibitory effect of soy bean trypsin inhibitor on both globulins A and B has been reported (Kagen *et al.*, 1963*a* and *b*). Fig. 3 extends these observations. Solutions of A and B were each adjusted to contain the same amount of permeability activity, and were then incubated with various concentrations of SBTI (30 min., 2°). The incubation mixtures were then bio-assayed, and the ratio of permeability activity remaining in the incubation mixture to that produced by the original solution was subtracted from 100 per cent to determine the per cent inactivation. In this particular assay blue lesions smaller than 30 per cent of the maximum lesion size (11 mm.) could not be differentiated from control values.

Effect of DFP, and substrate protection.—Table I shows the effect of DFP on the permeability activity of both A and B. In both cases, treatment with

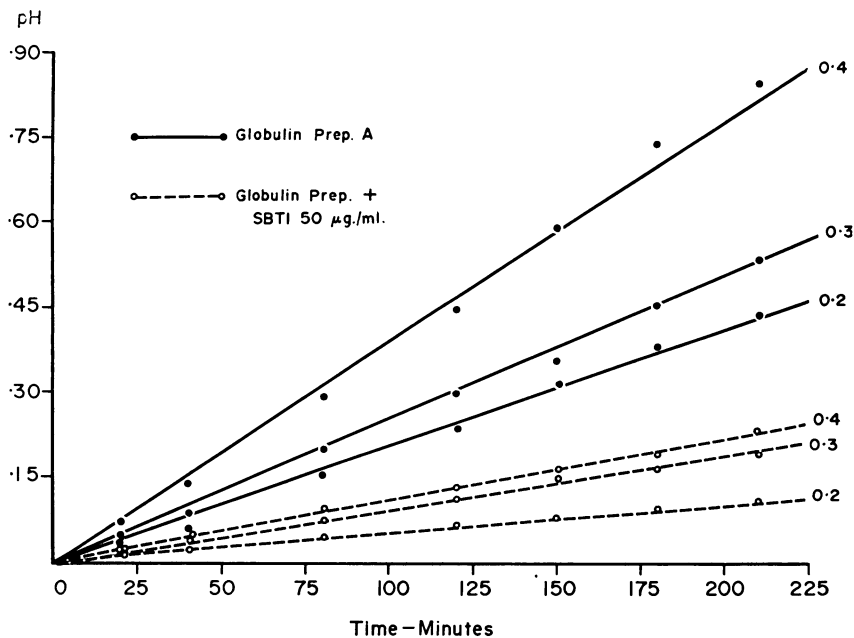


FIG. 1.—The TAME Esterase Activity of Preparation A. Reaction mixture contained 1.0 ml. of 0.04 M-TAME, 0.5 ml. buffer (0.02 M-Tris, 0.04 M-CaCl₂, 0.08 M-NaCl, 0.001 M-Imidazole) and varied amounts of A and water to a final volume of 2.0 ml. A was used in three volumes (0.4, 0.3, 0.2 ml.); temp 22°; SBTI final conc. 50 µg./ml. was preincubated for 30 min. with duplicate samples of A.

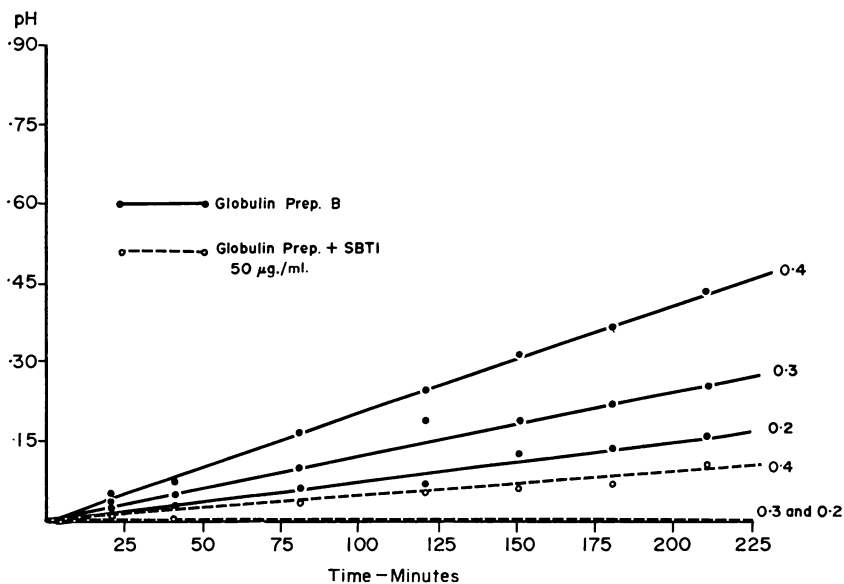


FIG. 2.—TAME Esterase Activity of Preparation B. Reaction mixture contained 1.0 ml. 0.04 M-TAME, 0.5 ml. buffer (0.02 M-Tris, 0.04 M-CaCl₂, 0.08 M-NaCl, 0.001 M-Imidazole) and varied amounts of B and water to a final volume of 2.0 ml. B was used in volumes of 0.4, 0.3, 0.2 ml.; temp 22°; SBTI, final conc. 50 µg./ml., was pre-incubated for 30 min. with duplicate samples of B.

5×10^{-4} M-DFP, inactivates the permeability globulins. It is shown that TAME (0.02 M) when added to such reaction mixtures prevents this DFP inhibition.

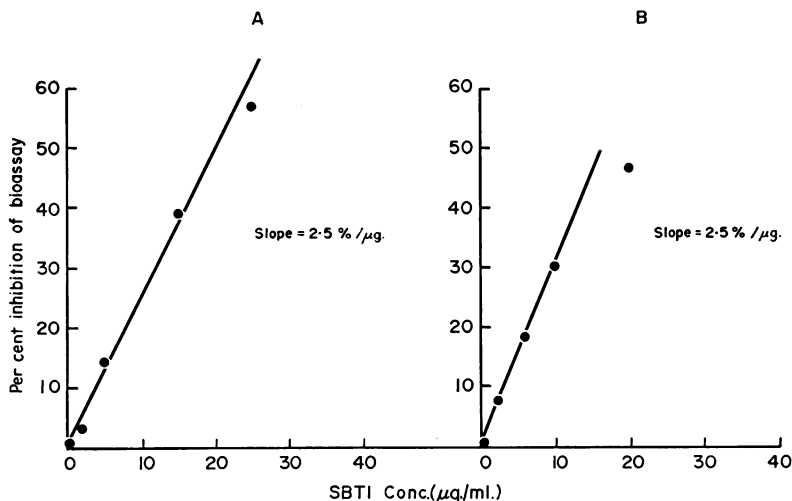


FIG. 3.—Inhibition of a Standard Dose (50 Blueing Units) of Permeability Globulins by Soy Bean Trypsin Inhibitor. Bioassay: average of 12 tests in 6 animals; SBTI incubated with permeability globulins for 30 min. at 2° before bioassay; all solutions were warmed to room temperature (3–4 min.) before bioassay.

TABLE I.—Effect of TAME* on DFP† Inhibition of Permeability Activity

Globulin A		Bioassay‡	Globulin B		Bioassay‡
A alone	.	9.4	B alone	.	8.2
A + DFP	.	1.6	B + DFP	.	3.6
A + DFP + TAME	.	8.6	B + DFP + TAME	.	8.7
A + TAME	.	8.6	B + TAME	.	8.7
TAME alone	.	4.7	TAME alone	.	4.4
Physiological saline	.	1.6	Physiological saline	.	1.5

* Final conc. 0.02 M.

† Final conc. 5×10^{-4} M.

‡ Lesion size in mm.; av. of 6 tests in 3 animals.

Solutions containing both TAME and DFP had TAME added first.

All solutions were incubated for 2 hr. at 4° , then dialyzed for 6 hr. at 4° against physiological saline, three times.

Kinin releasing properties

Kinin releasing activity of both A and B was assayed on the isolated, rat uterus. The results shown in Table II, demonstrate that preparation A on addition to unheated, human plasma causes the immediate release of a kinin. This kininogenase activity is inhibited by pretreatment of the globulin preparation with SBTI (50 $\mu\text{g./ml.}$ for 45 min. at 2°). The addition of A to plasma which has been heated (63° for 1 hr.) results in kinin release after a lag of 50 sec. The total amount of kinin formed, as determined by uterine response, is, however, equivalent to that formed from unheated plasma. Preparation B (Table III) releases kinin from unheated plasma after a 36–50 sec. lag. This effect is inhibited by

TABLE II.—*Kinin Releasing Properties of Globulin Preparation A**

Experiment	Globulin prep (ml.)	Plasma substrate (ml.)		SBTI†	Response (mm.)§
		Heated†	Unheated		
I	—	—	1.0	—	0.5
	0.05	—	1.0	—	25.0
	0.01	—	1.0	—	7.5
	0.01	—	—	—	0.5
II	0.07	—	1.0	+	0.0
	0.07	—	1.0	—	26.0
	0.10	—	1.0	+	6.0
	0.10	—	1.0	—	15.5
	0.10	—	—	—	0.0
III	—	—	1.0	—	4.0
	—	1.0	—	—	8.5
	0.05	—	1.0	—	25.0
	0.05	1.0	—	—	20.0

(lag 50 sec)

* Assayed on rat uterus, 29°.

† 63°, 1 hr.

‡ 50 µg./ml., 45 min., 2°.

§ Magnified 4 times.

TABLE III.—*Kinin Releasing Properties of Globulin Preparation B**

Experiment	Globulin prep. (ml.)	Plasma substrate (ml.)		SBTI†	Response (mm.)§
		Heated†	Unheated		
I	—	—	1.0	—	2.0
	—	1.0	—	—	1.0
	1.0	—	—	—	0.0
	1.0	1.0	—	—	5.0
	1.0	—	1.0	—	15.0
					(lag 40 sec.)
	2.0	—	—	—	0.0
	2.0	1.0	—	—	5.0
	2.0	—	1.0	—	20.0
					(lag 36 sec.)
II	—	—	1.0	—	0.0
	2.0	—	1.0	—	19.0
	2.0	—	1.0	+	0.0

* Assayed on rat uterus, 29°.

† 63°, 1 hr.

‡ 50 µg./ml., 45 min., 2°.

§ Magnified 4 times.

pretreatment of B with SBTI. B is unable to bring about the release of kinin from unheated plasma.

Ultracentrifugal studies

Ultracentrifugal analyses were made of both permeability globulins using the partition cell technique (Yphantis and Waugh, 1956) to characterize these proteins by their permeability activity. Sedimentation coefficients for both A and B were determined by measurement of the ratio of permeability activity remaining in the upper chamber, after a given time of centrifugation, to that of the starting material. The data, obtained from these experiments, appear in

TABLE IV.—*Ultracentrifugal Characterization* of A and B*

Globulin prep.	Time of run (min.)	C_t/C_o †	S_{w20}	Average
A	51	0.425	12.2	11.3S
A	76	0.185	10.1	
A	51	0.410	11.7	
B	51	0.550	6.6	5.7S
B	76	0.580	5.3	
B	51	0.720	5.2	

* performed with the Yphantis-Waugh partition cell at 36,500 rev./min.

† ratio of permeability activity in upper chamber after centrifugation, to that of original sample. average of 12 tests in 6 animals.

Table IV. Globulin A has an S_{w20} of approximately 11S. The S_{w20} of globulin B is approximately 6S. At present neither preparation is pure enough or rich enough in active protein, to allow characterization by optical methods, so that the accuracy of these determinations is chiefly dependent on the accuracy of the bio-assay. It is likely, therefore, that the results represent approximations, and that more exact determination of the S_{w20} for A and B will have to await further purification.

Effect of C'1 esterase inhibitor (Kagen and Becker, 1963)

Partially purified preparations of human C'1 esterase inhibitor (Pensky, Levy and Lepow, 1961) were found to inhibit the permeability increasing activity of both A and B as shown in Fig. 4. In this experiment, globulin A was 70

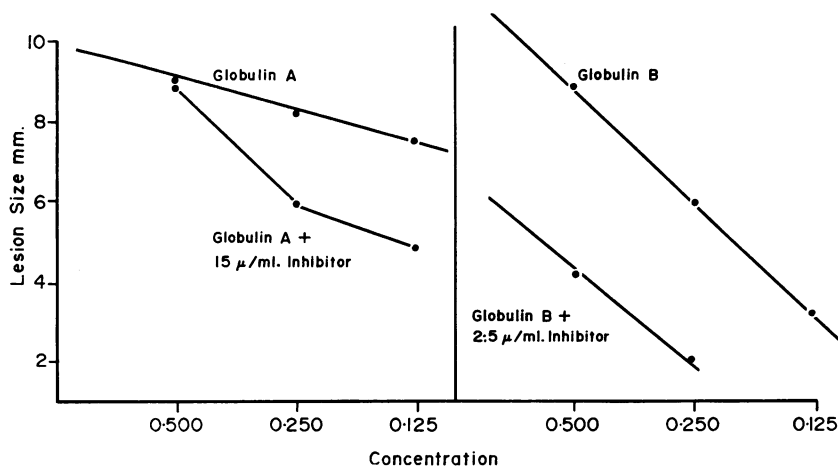


FIG. 4.—Effect of C'1 Esterase Inhibitor. Bioassay: average of 6 tests in 3 animals; inhibitor incubated with permeability globulins for 10 min. at 24° before bioassay.

per cent inactivated by 15 units/ml. of the inhibitor, while an equivalent concentration of B was inactivated to the same extent by only 2.5 units/ml. Each unit of the inhibitor inactivated 6.7 binding units of B, or 1.2 units of A. Similar results were obtained with another inhibitor preparation of greater purity.

DISCUSSION

The evidence strongly suggests that both A and B are esterases. Both are inactivated by esterase inhibitors (SBTI, DFP and C'1 esterase inhibitor); both contain SBTI inhibitable TAME esterase activity, and DFP inhibition of permeability activity is prevented by the amino acid ester TAME. DFP is effective by combining with the enzyme at its active site, so that protection by TAME can be considered as competition by substrate for the active site of the enzyme. Correlation of a protein property with its enzyme function has been made in this fashion for both PF/dil (Becker, 1959) and kallikrein (Webster and Pierce, 1961).

The finding of SBTI inhibitable kininogenase activity of globulin preparation A makes this factor's properties correlate well with those described for serum kallikrein. In addition, the permeability properties, esterase properties, and chromatographic behaviour correspond to those of serum kallikrein (Kagen *et al.*, 1963*b*; Schachter, 1960; Webster and Pierce, 1960, 1961, 1963).

Globulin B seems to be the same as PF/dil. Not only does it possess permeability increasing potency, and esterase activity, but it is a β -globulin which cannot release kinin from heated plasma, all of which corresponds to observations made of PF/dil (Kagen *et al.*, 1963*b*; Miles, 1961; Becker *et al.*, 1959; Mason and Miles, 1962).

Serum kallikrein (globulin A) and PF/dil (globulin B) are different globulins distinguishable by electrophoretic mobility, chromatographic properties, ultracentrifugal sedimentation behaviour, and kininogenase effects.

The inactivation of both permeability globulins by C'1 esterase inhibitor, derived from human serum, is of interest. Landerman and co-workers (1962) concluded that individuals with hereditary angioneurotic oedema lacked an inhibitor to a permeability globulin system. More recently, patients with this same disorder have been found to lack the serum inhibitor of C'1 esterase (Donaldson and Evans, 1963). The findings, presented here, that C'1 esterase inhibitor can function as a permeability globulin inhibitor suggest that the two clinical observations deal with two properties of the same serum component, and that the biochemical lesion of this genetic disease is the lack of a single serum protein. Whether the effects of this disease arise out of unbalanced activation of the permeability globulin system, or the complement system, or both is not known.

SUMMARY

The permeability activity of globulin A is inhibited by C'1 esterase inhibitor, soy bean trypsin inhibitor, and di-isopropylfluorophosphate. TAME protects against this DFP inhibition, and preparations of A contain SBTI inhibitable TAME esterase. Preparations of A are also SBTI inhibitable kininogenases which liberate a smooth muscle contracting factor from both heated (63°, 1 hr.) and unheated plasma. A is estimated to have a sedimentation coefficient of approximately 11 S. It is concluded that globulin A is serum kallikrein.

The permeability activity of globulin B is inhibited by C'1 esterase inhibitor, SBTI and DFP. TAME protects against this DFP inhibition, and preparations of B contain SBTI inhibitable TAME esterase. Preparations of globulin B are ineffective as kinin releasers when heated plasma (63°, 1 hr.) is used as the sub-

strate. B is estimated to have a sedimentation coefficient of approximately 6S. It is concluded that B is PF/dil.

The inhibition of both permeability globulins by C'1 esterase inhibitor indicates that this inhibitor may be the permeability globulin inhibitor lacking in the disease, hereditary angioneurotic oedema.

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