ATP-ase as an *in vitro* Inhibitor of Surface Factor in the Blood-Clotting Mechanism.* (29928)

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Having recently(1) presented evidence that a key trigger mechanism (surface factor (SF) or activation product) for blood clotting is present in the plasmatic atmosphere of blood platelets and presumably formed from plasma factors XII and XI during platelet alterations, it was logical to explore possible relations between SF activity and certain metabolic processes involving ATP-ase and organic phosphates, since these are known to occur in altering platelets. A number of workers (cited in Roskam's(2) review) have studied these processes in relation to platelet changes. The following have been salient contributions. Gaarder *et al*(3) identified ADP as the factor R which Hellem(4) had described as a substance for inducing *in vitro* platelet aggregation. Others confirmed this(5,6,7). One of the chief biochemical alterations during viscous metamorphosis (2) is a sudden acceleration of platelet glycolysis associated with the synthesis of ATP, which is then split to ADP and mineral phosphate by ATP-ase ('thrombosthenine'(8)). Clearly there is a ripe new field fully to explore all possible connections between platelet metabolic functions, their alterations, and the clotting mechanisms. The present report is a preliminary approach to a small part of this broad topic. It is concerned with the unexpected discovery of an inhibitory effect of ATP-ase on *in vitro* assays of surface factor activity(9,10). Brief mention of the organic phosphates (ATP, ADP, AMP) and lack of detectable ATP in the SF preparation are relevant in excluding possible roles of these phosphates in the inhibitions demonstrated.

Materials and methods. 1 mg ATP-ase (Apyrase form, Sigma†) hydrolyzes 1.34 mM ATP per minute. Dilutions were made from a stock solution of 1 mg/ml in imidazole-buffered NaCl (b. sal.), pH 7.3. Ten mM stock solutions, in b. sal., were freshly prepared from Sigma ATP, ADP, and AMP. The luciferase bioassay for ATP(11) was performed on the following SF preparations. Surface factor (SF, or activation product (12)) was prepared by the technique previously described(9) and was free from other known clotting factors. The test mixtures contained 30,000-60,000 units per ml. Iatridis and Ferguson's(10) two stage assay for SF is based on the Hicks-Pitney (1957) "thromboplastin screening test," modified by use of a Hageman (XII-) plasma as the activator-detecting substrate. Results (see Tables) are expressed as percentage (%) of the original SF unitage added. Inhibitions (in parentheses) are given as difference, *i.e.*, 100—residual %. Ferguson's(13) two-stage enzymic thrombin generation tests employed Milstone's thrombokinase and Burroughs Wellcome Stypven. Partial thromboplastin time (PTT) tests, after Langdell *et al* (14), used human brain cephalin and specific factor-deficient plasma substrates.

Results. Table I shows results of testing ATP-ase in the 2-stage assay for surface factor activity.

Section A shows mean values and standard deviations of the inhibitory effect of (50 μg/ml) ATP-ase on 12 different SF preparations. The p values (a) with respect to the control and (b) of the difference obtained at the zero and 10 minutes of incubation are very significant.

Section B shows the progressive inhibitory effect of (50 μg/ml) ATP-ase on SF in relation to time of incubation.

Section C shows the immediate and progressive effects of different concentrations of ATP-ase. This experiment is significant in showing that even 5 μg/ml of ATP-ase causes 83% inhibition of SF after 10 minutes of incubation.

Table II shows the results of testing ATP-ase in 2-stage thrombin-generation systems (13) employing the enzymes thrombokinase

* Aided by N.I.H. Research Grant HE-01510-12.
† Sigma Chemical Co.

TABLE I. A. *In vitro* Inhibition of SF by 50 μg/ml ATP-ase, B. Progressive Inhibition of SF by 50 μg/ml ATP-ase in Relation to Time of Incubation, C. Varying ATP-ase Concentrations tested at 0 and 10 min.

A. *In vitro* Inhibitory Effect of ATP-ase on SF (12 Tests).

Incubation time	0′	10′
Inhibition (%)	61 ± 24.22	86 ± 8.7
p value with respect to control	<.001	<.001
p value of difference obtained at 0′ and 10′ of incubation	<.001	

B. SF Activity (%)

Incubation time	0′	1′	2′	3′	4′	5′	10′
Activity (%)	40	32	30	18	14	12	11
Inhibition (%)	(60)	(68)	(70)	(82)	(86)	(88)	(89)

C. SF Activity (%).

ATP-ase (μg/ml)	pH	Incubation time 0′		10′	
5	7.00	92	(8)	27	(83)
50	7.00	42	(58)	7	(93)
250	7.00	26	(74)	6	(94)
500	6.98	19	(81)	4	(96)

Numbers in parentheses are inhibition per cent.

and stypven (RVV), with control of cofactors, Ca^{++}, prothromboplastic phospholipid (cephalin) and AcG (factor V). These enzymes bypass any need for surface factor and, therefore, afford a means of testing for possible effects of ATP-ase on other components of the prothrombin-activation mechanisms. Tests were carried out after 1 minute and optimal (endpoint) incubation. The complete lack of any ATP-ase effect is clearly indicated. It may be concluded that ATP-ase has no significant rate or yield effects on (a) thrombin ("antithrombic"), (b) calcium, (c) prothromboplastic phosphatide ("antithromboplastic"), (d) the procoagulant (proteolytic) enzymes ("anti-enzyme"), or (e) factor V. The last is particularly demonstrated by the tests (Nos. 3 and 4) without added AcG, where the activations are incomplete and rely upon the minute traces of factor V contaminating the eluate prothrombin and/or the thrombokinase preparation(13). There is good reason to believe that thrombokinase is a natural component in normal blood clotting, which needs only the cofactors mentioned. Stypven is an artificial "thromboplastic enzyme" requiring an additional cofactor, namely, factor X, which is provided in the eluate prothrombin. It may be deduced further (f) that ATP-ase has no "anti-factor X" activity. In addition, ATP-ase was demonstrated to lack any inhibitory effect on factors V, VIII, IX, and X in specific PTT-type(14) clotting test systems.

Table III shows results of testing heated and unheated ATP-ase in the two-stage assay for surface factor activity. This experiment is significant since the enzyme heated for 10 minutes at 90°C shows no inhibitory effect at all and it may therefore be assumed that

TABLE III. Effects of 50 μg/ml ATP-ase, Heated and Unheated, in 2-Stage Assay for SF Activity (see text).

Inc time (min)	SF-activity (%) Saline pH 7.3	ATP-ase pH 7.0	ATP-ase (heated) pH 7.1
0	100	56	100
	(0)	(44)	(0)
10	100	20	(93)
	(0)	(80)	(7)

Numbers in parentheses are inhibition per cent.

TABLE II. Lack of ATP-ase Effects in Two-Stage Thrombin-Generation Test Systems, Employing (a) Thrombokinase or (b) Stypven.

Clotting-times (seconds) at stated incubation periods (minutes), for 0.2 ml fibrinogen + 0.2 ml incubate, 21°C.

Test	ATP-ase	AcG	T. kinase	Stypven	1 min	Endpoint (min)
1	—	+	2 μg/ml	—	20.6″	14.0″ (4 m)
2	20 μg	+	″	—	22.5″	14.4″ (4 m)
3	—	—	″	—	65.0″	25.0″ (7 m)
4	20 μg	—	″	—	62.8″	24.3″ (9 m)
5	—	+	—	4 μg/ml	23.5″	13.9″ (3 m)
6	20 μg	+	—	″	26.3″	13.9″ (3 m)
7	100 μg	+	—	″	25.5″	14.4″ (5 m)

its effect is not due to any heat-stable impurity.

The luciferase bioassay(11), which is sensitive to less than 1 μg (0.002 mM) ATP, was completely negative with our surface factor preparations. Effects of ATP, ADP, AMP, and H_3PO_4 were tested on the two-stage SF assay over a range of 0.5 to 5 mM, with accompanying pH shifts from 6.42 to 3.45. There were no significant inhibitory or adjuvant effects of 0.5 mM ATP or ADP. Inhibitions at higher concentrations and those with AMP or H_3PO_4 could be dismissed as pH effects (acidic) technically invalidating the SF bioassay (cf. 10).

Discussion. It is clear from the data presented that ATP-ase significantly inhibits SF activity, even in concentrations of a few μg/ml. It does this without changing the pH. Since platelets are a source of ATP-ase(8) this might be of physiological interest. In previous reports(1) we showed that Hageman factor is activated on the altered surface of platelets during the preparations of platelet suspension, and we postulated that it is the interrelationship of SF precursors and platelet surfaces which provides the trigger for a hypercoagulable state. Since ATP-ase inhibits active SF, we may speculate that after factors XII and XI have been activated on the surface of platelets, the SF is then subject to inhibition by the thrombostenine (ATP-ase) of platelets. This could probably be an important protective mechanism *in vivo*. The role of SF has been emphasized by ourselves and by others (cited(15)) as the key factor for the *in vivo* triggering of the clotting mechanism, thus predisposing to vascular thrombosis. We(15) have also described a powerful plasmatic anti-SF mechanism, but its nature has not been elucidated. A number of problems remain to be solved before any complete hypothesis can be set up concerning the endogenous inhibition of SF. It is too early to speculate whether any major role can be assigned in the body to the ATP-ase type of inhibitor demonstrated by the *in vitro* experiments we have described. The negative results of luciferase bioassays for ATP in the surface factor preparations, and the nonsignificant effects (apart from pH) of small quantities of ATP or ADP, indicate that (a) SF activity cannot be related to ATP, and (b) the inhibitory action of ATP-ase is unrelated to these organic phosphates, but must be a special phenomenon in its own right.

Summary. As little as 5 μg/ml ATP-ase, at pH 7.0, shows a significant inhibitory effect on surface factor (SF or activation product) *in vitro*. ATP-ase does not inhibit thrombin, tissue thromboplastin, thrombokinase, trypsin, stypven, factors IX, VIII, X or V. The specific anti-SF effect of ATP-ase cannot be related to ATP or ADP. It may be significant *in vivo*, since platelets are a source of ATP-ase (thrombosthenine), and active SF is present in the platelet plasmatic atmosphere.

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Received October 1, 1964. P.S.E.B.M., 1965, v118.