evaluation of post-heparin plasma lipolytic activity in intact animals.

Summary. Following intravenous injection of S^{35}-labelled heparin in doses from 0.1-0.6 mg/kg, in rabbits, the time course of heparin radioactivity and lipolytic activity was measured. After 10 minutes, both S^{35} and lipolytic activity decay curves follow an essentially similar course whether prolonged by larger doses of heparin or a previous 24-hour fast. These data suggest a circulating combination between these two substances. The data are not compatible with previous studies which suggested a much faster rate of turnover for heparin.

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Inhibition of Enzymic (*Thrombokinase*) Activation of Blood Clotting by Organic Phosphates (ATP, ADP, AMP), Involving a Calcium Complex.* (29927)

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Pilgeram and Loegering(1) recently reported inhibition of certain blood-clotting test systems by additions of adenosine triphosphate (ATP) and other organic phosphates. Questions as to how much this might be related to pH, to calcium ions, and to other possible variables, were raised but were not easily answered by these test systems.

In our weakly-buffered 2-stage test system for activating prothrombin to thrombin enzymatically(2), *Sigma's*† ATP, ADP, or AMP, in 1-8 mM stable acidic solutions with plain physiological saline, reduced the pH and proved markedly inhibitory to the enzymic thrombin formation. We have attempted to quantitate this in terms of thrombin yields at controlled concentrations of prothrombin substrate, pH, and calcium ions. Even more interesting than the specific results with these organic phosphates, are the broad generalizations uncovered with respect to the roles of these 3 variables in connection with the enzymatic activation of prothrombin to thrombin.

Materials and methods. ATP, $Na_2 \cdot 3H_2O$ (610), ADP, $Na_{1.5} \cdot 3H_2O$ (524), AMP, $Na \cdot 2H_2O$ (406, apparent molec wt), and ATP-ase, of high quality, were commercially available (Sigma). Reagents and methodology for the 2-stage thrombin-generating system have been described(2). Briefly, (a) 5 ml incubation mixture containing canine eluate (prothrombin, with factor X), factor V (bovine serum AcG), prothromboplastic phospholipid (human brain cephalin), thromboplastic enzyme (2 μg/ml Milstone's(3) thrombokinase), any additive, and finally calcium, in imidazole-buffered 0.85% NaCl (at stated pH), is sampled at minute inter-

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† Sigma Chemical Co., St. Louis, Mo.

vals and tested (b) with an equal (0.2 ml) volume of bovine fibrinogen (Armour's Fraction I, containing citrate), until the endpoint or shortest clotting time reached.

Log-log plots of these endpoints against at least a 10-fold range of prothrombin (eluate) dilutions are rectilinear. Parallel plots with all the cited test mixtures permit precise quantitation of thrombin yield or activity. This is expressed as EPA, or equivalent of prothrombin activated, by reference to the percentage dilutions of the selected standard. Absolute standards refer to incubations containing 15 or 16 mM $CaCl_2$, at pH 7.05. The slight deviation from routine pH of 7.3(2) is dictated by considerations with respect to control of instability of the organic phosphates.

EPA quantitates not only prothrombin, but if this is fixed, also any other factor affecting thrombin yield. Here, we specifically explore controlled variations of prothrombin, pH, and calcium concentration, with and without stated additions of organic phosphates or ATP-ase.

Effects of varying pH. Successive additions of N/10 NaOH shifted pH to 10.8, while acidifications to pH 5 were achieved with (a) N/10 acetic acid, (b) N/10 phosphoric acid, or (c) 1-8 mM acidic disodium ATP. EPA measurements were made at representative intervening pH levels over the cited range, and parallel tests were run at 2 calcium concentrations, (a) 15 mM (optimal) and (b) 1.5 mM (suboptimal). All other components, including initial strength of prothrombin, were constant throughout. The results clearly showed a zone of optimal pH in the approximate range of 6.2-7.3, with a tendency to peak somewhat on the acid side of neutrality. The main results were: (a) significant reduction in thrombin yields (EPA) on either side of the optimum, (b) only minor differences with the various acidifications, and (c) close similarity of the optimum, whether using optimal or suboptimal calcium. As far as pH optimum is concerned, therefore, this seems independent of the Ca^{++} concentration.

Second stage controls. pH measurements on the defibrinated step-*two* mixtures showed shifts of no more than 0.3 pH at the extremes of acidity and alkalinity tested. This is negligible in terms of the known wide pH optimum, 5.5-10, for the thrombin-fibrinogen reaction(4). Thus, it may be accepted that the test systems were satisfactorily buffered in the final clotting test. Conductivity meter readings were also essentially unchanged in the various mixtures. It may fairly be concluded, therefore that the observed pH effects are those exerted upon the enzymic activation of prothrombin to thrombin and indeed serve to establish the approximate pH optimum (hitherto undetermined) for *thrombokinase* enzyme.

Effects of varying Ca^{++} concentration, at constant pH. Calcium was varied (2, 4, 8, 16, 32 mM), at constant pH (7.05), in tests on 4 prothrombin dilutions (potential EPA: 20, 50, 100, 200, respectively), other components being constant. The results clearly showed an optimum for Ca^{++} close to 16 mM, and independent of the substrate concentration. The valid conclusion must be that the calcium ion concentration directly determines the endpoint of the enzymic activation.

Fig. 1 shows the rectilinear log-log plots of the clotting-time endpoints with varying prothrombin dilutions (EPA), at pH 7.05, and varying Ca^{++} (2, 4, 8, 16 mM) in 4 test series. The parallel plots must mean that each suboptimal amount of calcium lessens the thrombin yield by a specific fraction, which is independent of the initial prothrombin concentration. Because all possible factors affecting Ca^{++} activities and hence this specific fraction are not yet determined,

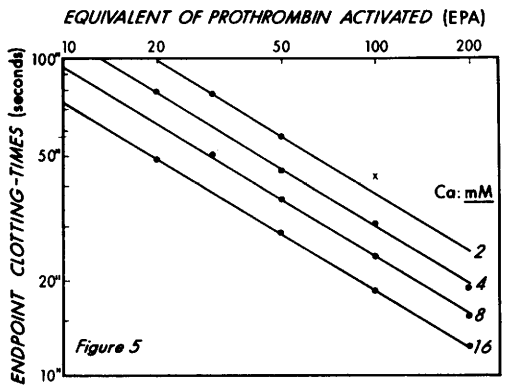

FIG. 1. Thrombin yields, as endpoint clotting times, from prothrombin dilutions at varying concentrations of Ca^{++}.

TABLE I.

(A) Average Thrombin Yields, as Percentage of Potential EPA, Referred to Each Standard at Its Particular pH.

pH	Ca (mM) 16	8	4
7.05	100%	61%	45%
6	100%	83%	52%

(B) Divergence of Results at the Two pH Values, When Both Series of Data Are Referred to the Routine Standard at pH 7.05.

pH	M	N	O	P
(1) 7.05	200	100	50	20
(2) 6	165	76	36	13
(2)/(1) × 100	83	76	72	65

we prefer to set up controls for each day's tests, as seen in experiments to follow.

Combined effects of varying the pH and calcium are illustrated in Table I. Calcium was 16 mM (optimal), 8 or 4 mM (suboptimal). pH was either (a) 7.05 or (b) 6, the latter achieved by adding acetic acid. Test series (M, N, O, P) were performed over the usual 10-fold range of prothrombin concentrations (200, 100, 50, 20). Thrombin yields are recalculated *relative* to the control (100%) using 16 mM Ca, at the particular pH. Table I (A) shows that these percentages were unquestionably better at the lower pH, when using the suboptimal amounts of calcium. This, indeed, confirms the expectation that Ca^{++} ion activities should be improved by lowering the pH. Here this was achieved by acetic acid, which does not combine with calcium ions in dilute solutions.

Table I (B) shows the divergence of results at the two pH values, when *absolute* thrombin yields are obtained by referring all endpoints to the routine standard at pH 7.05 and 16 mM Ca. This clearly indicates that even with optimal, as well as with suboptimal, calcium, the absolute yields are significantly reduced by the acid pH. The percentage ratios in the last line show this point.

Effects of varying ATP on relative and absolute thrombin yields, at varying calcium concentrations, (A) with constant pH (7.05 ± .05) or (B) with varying pH. These results are shown in Table II. ATP was 0.3, 0.6, or 1.2 mM (final), and Ca was 16, 8, or 4. Values (EPA) are given (1) as absolutes, *i.e.*, referred to the standard control at 16 mM Ca and pH 7.05, and (2) as relative, *i.e.*, referred to controls at the stated Ca and pH (in parentheses). The observed reductions in thrombin yields are partly dependent upon concentration of ATP, and partly upon the molarity of calcium. II (B) shows only minor modifications of these inhibitions, for any one molarity of ATP and calcium, when there is an acid shift in pH due to use of unneutralized ATP.

The really significant result, however, is the marked inhibition demonstrable with low molarities of ATP, in the presence of which the stoichiometry must indicate a considerable excess of ionized calcium. Hence we are faced with the situation of an inhibitory mechanism which is undoubtedly calcium dependent, and yet evidently occurs despite persistence of much free calcium. Not only does this indicate a complex mode of action of ATP, but all the data point to a yield-limiting calcium dependency (even without the ATP) in the enzymic conversion of prothrombin to thrombin.

Effects of ADP and AMP, at constant pH. These results need not be cited in detail, since they were qualitatively like the ATP data (Table I), but quantitatively different in requiring more ADP, and even more AMP, for comparable degrees of inhibitory effect.

Effects of ATP-ase. Table III shows effects of 100 μg/ml of the enzyme ATP-ase (apyrase form) on test systems at 8 mM Ca^{++}, with and without 1 mM ATP. Calcium was added after a 3-minute preincubation of all the other ingredients. The effect of ATP-ase

TABLE II. Effects of Varying Calcium and ATP, on Thrombin Yields, at (A) Constant pH, (B) Varying pH. 23°C.

	pH	ATP	mM Ca 16	8	4
(A)	7.05	0	100	80	50
	7.05	0.3 mM	95	69 (*86*)	11 (*22*)
	7.05	0.6 mM	88	50 (*63*)	1 (2)
	7.05	1.2 mM	81	2 ($2^{.5}$)	0
(B)	6.87	0.3 mM	90	70 (*87*)	12 (*24*)
	6.72	0.6 mM	68	46 (*58*)	2 (*4*)
	6.35	1.2 mM	62	5 (*6*)	0

Percentages of the respective controls, without ATP, in parentheses.

TABLE III. *ATP-ase* Reduction of ATP Inhibition of Thrombin Generation, at pH Stated; Calcium : 8 mM. 25°C.

pH	Preine* with	Endpoint C.T. (sec)	Incub time	Relative thrombin
7.01	B. Sal	31.1″	5 min	100
7.06	*ATP-ase*	33.2″	5 min	86
6.48	ATP	84.0″	18 min	19
6.09	ATP + *ATP-ase*	34.0″	6 min	84

* 3 min.

alone is negligible (86 *vs* 100). ATP shows the usual inhibition (19), but after incubation with ATP-ase is ineffective (84 *vs* 86 for ATP-ase alone). This result indicates that ATP is in no way protected in our test system, but suffers its typical degradation by ATP-ase; the resulting ADP, according to the previous series of experiments, being virtually noninhibitory in this concentration range. The only relevance of ATP-ase, therefore, is when its presence reduces the inhibitory effectiveness of ATP.

Discussion. ATP, ADP, and AMP (but not ATP-ase) are inhibitory to the enzymic activation of prothrombin by thrombokinase, (with factor V, prothromboplastic phospholipid, and Ca^{++}). Their relative order of effectiveness might be correlated with known data(5,6) on the same order with respect to combining and chelating with calcium ions. Marked inhibitions at organic phosphate concentrations well below stoichiometric equivalence, however, must mean persistence of free Ca^{++} ions. Hence, the inhibition is only partly dependent on the calcium chelation, and some other factor must be involved.

Varying the Ca^{++} alone (without organic phosphates) markedly affects the thrombin yield, which is surprising for an enzymatic activation. Some preliminary results suggest independence of any excess of phospholipid or enzyme (thrombokinase), but a calcium complex might involve certain clotting factor proteins. Presumably, the inhibitory effects of the studied organic phosphates are related to this unknown calcium complex in the clotting activation. There is no intent to suggest, at present, any physio-pathological role of the organic phosphates. Rather, they are used here as tools for some *in vitro* experiments made possible by an extension of certain new quantitative methodologies in the field of blood clotting. It is in the broader context of certain fundamental calcium-dependent mechanisms uncovered by these studies that the reported results are of more than casual interest.

Summary. Reduction of thrombin yields in an enzymic (thrombokinase)-activated 2-stage clotting system is achieved *in vitro* by mM additions of organic phosphates (ATP, ADP, AMP). This is investigated in relation to pH and Ca^{++} concentration, optima for which are defined in control systems. The evidence indicates that the major inhibition must be related to some unknown calcium complex, which is a yield-determining factor in thrombin generation, even without the organic phosphates. ATP-ase degrades ATP and largely removes its inhibitory action.

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