JOURNAL OF PHARMACEUTICAL SCIENCES

FEBRUARY 1978
VOLUME 67 NUMBER 2

RESEARCH ARTICLES

Kinetics of Plasma Coagulation and Lysis I: Basic Kinetic Model for Time Course of Coagulation–Lysis Systems and Its Potential Application to Clinical Studies

EDWARD R. GARRETT *x, **JEAN DAVER** §, **PIERRE DESNOYERS** ‡, **MARTINE KOSSER** ‡, and **MICHEL VINCENT** ‡

Received February 22, 1977, from the *Beehive, College of Pharmacy, University of Florida, Gainesville, FL 32610, the ‡Servier Research Institute, F. 92150 Suresnes, France, and the §Biomedical Laboratory, F. 81100 Castres, France. Accepted for publication April 29, 1977.

Abstract □ The time courses of coagulation and coagulation–lysis were spectrophotometrically monitored after the addition of thrombin or thrombin–streptokinase to plasma, diluted 1:5 with normal saline, obtained from normal and presumably abnormal subjects. The kinetics of clotting, after an initial lag period of 0.5–1.5 min, demonstrated essentially first-order dependence on the amount of fibrinogen available to form the clot, and the asymptotic absorbance was independent of thrombin concentration. The rate of clotting was a function of added thrombin, and the ratios of the rate constants at 2.5 and 1.25 units of thrombin/ml of undiluted plasma were 1.65 ± 0.03 *SEM*. At early times, the coagulation–lysis curve with thrombin–streptokinase could be superimposed on the clotting curve with thrombin alone for a given plasma with minor compensation for variable lag times. Subsequently, the curves diverged; lysis was monitored by the decrease in absorbance of the coagulation–lysis system. The rate of fibrinolysis increased with streptokinase concentration and was a function of the extent of lysis, and it permitted the description of the kinetics of lysis by a pseudoautocatalytic mechanism where the bimolecular rate constant appears proportional to streptokinase concentration. Ranges of clotting and lytic parameters for the plasma of normal subjects are given, and their potential use in diagnosing abnormalities is described.

Keyphrases □ Coagulation and coagulation–lysis—time courses after addition of thrombin or thrombin–streptokinase to plasma, kinetic model developed □ Models, kinetic—coagulation and coagulation–lysis, time courses after addition of thrombin or thrombin–streptokinase to plasma □ Kinetic models—coagulation and coagulation–lysis, time courses after addition of thrombin or thrombin–streptokinase to plasma

The effects of pathological states on the rates of plasma coagulation and lysis are well recognized. Measurements of coagulation parameters such as partial thromboplastin time, prothrombin time, Howell time, and whole blood coagulation time are often employed to adjust dosage regimens of anticoagulant drugs and to diagnose certain liver dysfunctions, circulatory disorders, and other diseases. Nevertheless, quantitative information on the kinetics of blood coagulation and lysis is scarce (1–5). In fact, the parameter normally used to estimate lytic rates is a one-value response, the lysis time, a time taken as the interval between the addition of thrombin and an empirical observation of dissolution of the fibrin network. Prior postulated kinetic models have been based on this one-value response (1–8).

These present investigations are a continuation of prior studies on coagulation–lysis system kinetics, with the ultimate objective of searching for "normocoagulating" drugs (8) that could maintain the equilibrium of such systems. The results of kinetic studies of plasma coagulation and lysis of diluted plasma from various normal subjects are given as a function of various concentrations of thrombin and streptokinase. The continuously spectrophotometrically monitored time courses of formation and lysis of the clot, a method proposed by Bouvier and colleagues (9–11), were used to determine the pertinent kinetic parameters and to establish kinetic models. The potential use of these derived kinetic parameters to diagnose abnormalities is described.

EXPERIMENTAL

Platelet-poor plasmas were obtained from whole citrated blood[1] from presumed normal subjects[1] and subjects with possible thromboembolic diseases[2] by centrifugation at 4° for 10 min at 1500×*g*. They were then frozen at −20° and thawed in a water bath at 37° when used. The monitoring of the time course of clotting and lysis at 350 min was similar to that previously reported (9–11); details of effects of storage time and treatment of blood and plasma were given (9–11).

[1] Nancy Regional Blood Transfusion Center, Nancy, France.
[2] Hotel Dieu Hospital, Paris, France.

Table I—Reproducibility of Parameters of Blood Clotting Model (Eq. 1) at a Fixed Concentration of Plasma (20%) and Varying Concentrations of Thrombin [a]

	Thrombin, Units/ml of Undiluted Plasma						
	1.25			2.5			
Replicate	k_c, min^{-1}	10^2A_∞	t_{lag}, sec	k_c, min^{-1}	10^2A_∞	t_{lag}, sec	$\frac{k_c\ (2.5)}{k_c\ (1.25)}$
1	0.94	53	62	1.42	55	27	1.51
2	1.15	49	55	1.60	51	26	1.39
3	0.97	53	58	1.48	55	27	1.53
4	1.00	53	65	1.51	54	28	1.51
5	0.95	52	76	1.37	55	30	1.44
6	0.93	51	54	1.51	57	29	1.62
7	1.02	54	56	1.56	52	31	1.53
8	1.01	49	67	1.54	52	27	1.52
9	0.99	51	59	1.33	50	32	1.34
10	1.00	48	68	1.40	54	34	1.40
Mean	1.00	53	66	1.5	55	28	1.48
SD	0.084	2	7.8	0.09	2	2	0.08

[a] Data fitted by a Wang digital computer and the Simplex program to $A = A_\infty[1 - e^{-k_c(t-t_{lag})}]$, where A is the absorbance at time t. The plasma was from an individual subject.

A 2-ml aliquot of each diluted plasma, previously diluted 1:5 with normal saline (0.9%), was added to a 1 × 1-cm 25° thermostated quartz cell. The coagulation was initiated by adding 0.1 ml of Michaelis buffer and 0.1 ml of thrombin[3] solution in Michaelis buffer containing 1.25 or 2.5 units of thrombin/ml of undiluted plasma by calibrated syringes and immediately mixing on a vortex mixer.

The Michaelis buffer was prepared from a mixture of 1 volume of a stock buffer solution (I) and 4 volumes of 0.85% NaCl, which was adjusted to pH 7.42. The stock buffer solution (I) at pH 7.42 contained 5 volumes of a solution prepared to contain 9.714 g of sodium acetate trihydrate and 14.714 g of barbital sodium in 500 ml of freshly boiled distilled water, 5 volumes of 0.1 *N* HCl, 2 volumes of 8.5% NaCl, and 15 volumes of distilled water. The increasing absorbance due to coagulation was monitored as a function of time against a similarly treated blank in a continuously recording UV spectrophotometer[4].

The 2.0 ml of diluted plasma was also mixed in the cell with the same amount of thrombin solution (0.1 ml) and with 0.1 ml of a streptokinase[5] solution in isotonic saline containing 25 or 50 Christensen units/ml of undiluted plasma. The initially increasing and subsequent decreasing absorbances due to coagulation and lysis were monitored spectrophotometrically as a function of time. The thrombin and streptokinase solutions were kept refrigerated prior to use.

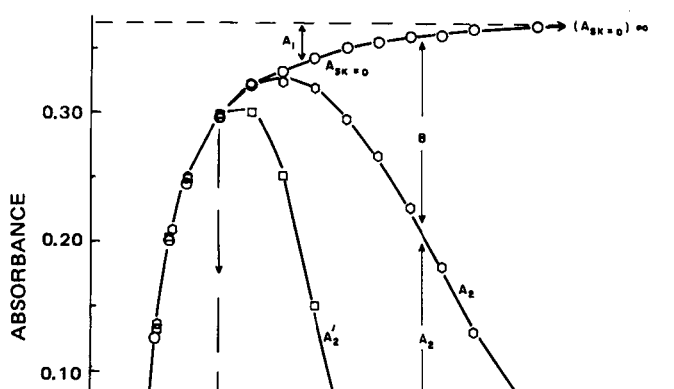


Figure 1—*Typical plots of the observed absorbance of clots from 20% human plasma in isotonic saline with time with 2.5 units of thrombin/ml of undiluted plasma (○) and with 25 (○), and 50 (□) units of streptokinase added per milliliter of undiluted plasma. The initial portions of the curves were superimposed so as to be independent of slightly variable t_{lag} times. The values of* B *for Eq. 5 can be determined from the difference between the values at a specific time of* $A_{SK=0}$ *and* A_2 *(or* A_2'*). The values of* A *can be determined from the sum of* A_2 *(or* A_2'*) and* A_1*, the difference between the asymptotic value of* $(A_{SK=0})_\infty$ *and* $A_{SK=0}$ *at the same time. The arrow at* t_s *represents the limit of coincidence among the various absorbance–time curves.*

RESULTS AND DISCUSSION

Kinetics of Clotting—A typical spectrophotometric absorbance, A, against time plot for the clotting of isotonic saline-diluted plasma at a thrombin concentration of 2.5 units of undiluted plasma/ml is given in Fig. 1. A lag time, t_{lag}, was observed before significant clotting was apparent as monitored by absorbance. The increase in thrombin concentration appeared to lessen this lag time but had no significant effect on the final magnitude of the absorbance. Qualitatively, the curve, on correction for lag time, appeared to be generated by a first-order process, where the rate of absorbance increase appeared to be proportional to the concentration of a precursor, P. Apparently, the amount of this precursor diminished with time and was directly related to the amount of clot yet to be formed, $A_\infty - A$. It follows that:

$$-dA/dt = k_cP = k_c(A_\infty - A) \qquad \text{(Eq. 1)}$$

Thus, for $t > t_{lag}$:

$$\ln (A_\infty - A) = \ln A_\infty - k_c(t - t_{lag}) \qquad \text{(Eq. 2)}$$

Various models for the clotting time curves, A *versus* time, t (Fig. 1), were challenged by fitting them by nonlinear regression with digital computerization to the models $P \rightarrow A$ (with compensation for lag where $A = 0$ at $t = t_{lag}$) and $P' \rightarrow P \rightarrow A$ (where lag time was and was not considered). Appropriate fitting was satisfactorily obtained by the former model in accordance with Eqs. 1 and 2.

As expected, the absorbance data at the very early times (<0.5 min) showed slight deviations from the semilogarithmic plot of the data in accordance with Eq. 2. This result is a consequence of the inability to monitor technologically the absorbance accurately during the initial fast rates of clot formation. The validity of Eq. 2 depends on the accurate determination of the final values, A_∞, of the absorbance on cessation of clotting. The clotting system is a heterogeneous one, and secondary and tertiary cross-linking of fibrin polymer may give a consistently elevating final absorbance for longer periods of time.

Such slight elevations were observed, and the appropriate A_∞ was chosen to give complete linearity of the semilogarithmic plots of $A_\infty - A$ against time. Actually, only minor decreases of 2–5% in the experimentally observed A_∞ values were necessary to obtain complete linearity in such plots. The clotting rate constant, k_c, was obtained from the slope. Although graphical methods are applicable to determine the clotting parameters t_{lag}, A_∞, and k_c from Eq. 2, it was more practical to fit the nonlogarithmic transformation of this equation:

$$A = A_\infty[1 - e^{-k_c(t-t_{lag})}] \qquad \text{(Eq. 3)}$$

by nonlinear regression to the data of Fig. 1 using a digital computer[6] and

[3] Thrombin (Hoffmann-La Roche), vial of 100 mg at 45 NIH units/mg, Batch B 938 K 91 A.

[4] Beckman (Acta III), Fullerton, Calif.

[5] Kabikinase (AB Kabi, Stockholm), vials of 5000 Christensen units.

[6] Wang.

Table II—Plasma (Diluted 1:5 with Isotonic Saline) Clotting Parameters [a] for Clinically Appraised Normals of Set A

Subject	Thrombin, Units/ml of Undiluted Plasma: 1.25 t_{lag}, sec	1.25 $100\,A_\infty$	1.25 k_c, min^{-1}	1.25 $t_{1/2}$, min	2.5 t_{lag}, sec	2.5 $100\,A_\infty$	2.5 k_c, min^{-1}	2.5 $t_{1/2}$, min	$\frac{k_c\ (2.5)}{k_c\ (1.25)}$
1	30	35.7	0.91	0.75	15	32.1	1.51	0.45	1.66
2	40	32.5	0.93	0.74	118	34.3	1.83	0.37	1.97
3	15	33.8	0.79	0.87	40	34.4	1.53	0.45	1.94
4	63	43.6	0.75	0.92	30	45.1	1.26	0.54	1.68
5	83	41.3	0.73	0.94	0	39.9	1.18	0.58	1.62
6	84	32.7	0.77	0.89	55	32.9	1.45	0.47	1.88
7	60	37.7	0.55	1.25	27	37.7	0.84	0.81	1.52
8	80	38.2	0.78	0.87	34	40.8	1.38	0.49	1.77
9	80	38	0.64	1.07	45	39.9	1.00	0.69	1.56
10	93	36.6	0.59	1.16	28	37.3	1.08	0.63	1.83
Average *SD*	62.80 ± 26.35	37.07 ± 3.40	0.74 ± 0.12	0.94 ± 0.16	69.20 ± 31.62	37.44 ± 4.08	1.30 ± 0.29	0.56 ± 0.13	1.74 ± 0.15
Average ± *SD* with 7 and 10 omitted	59.37 ± 27.34	36.97 ± 4.05	0.78 ± 0.094	0.88 ± 0.10	42.12 ± 35.17	34.92 ± 7.57	1.39 ± 0.25	0.50 ± 0.09	1.76 ± 0.15

[a] Obtained by a Wang digital computer and the Simplex program fitting to the equation $A = A_\infty[1 - e^{-k_c(t-t_{lag})}]$, where A is the absorbance at time t.

a Simplex algorithm (12). Some terminal absorbance values were ignored in obtaining the best fit.

Reproducibility of Parameters of Clotting Rates—The reproducibilities of the clotting rate constants, k_c, the lag times, t_{lag}, for the same plasma diluted to 20% by isotonic saline, and the estimated infinite clotting absorbances are demonstrated in Table I for 10 replicate studies of the same plasma from a particular individual for two thrombin concentrations. There was a significant difference between the t_{lag} of the two thrombin concentrations; the lag time at twice the thrombin concentration was practically halved. There was no significant difference in the A_∞ values at the two thrombin concentrations, but the ratio of the clotting rate constants was 1.5 for the higher thrombin concentration to the lower.

Two sets (Tables II and III) of plasma from normal subjects were kinetically evaluated with respect to clotting parameters. The sets differ in that their studies were conducted 6 months apart. Table II presents the data for the plasma of the 10 presumed normal subjects of Set A. Mean k_c rate constants for plasma at 1:5 dilution were 0.74 min^{-1} ± 0.12 *SD* ($t_{1/2}$ 0.94 min) with a range of 0.55–0.93, the average t_{lag} was 63 sec ± 26 *SD*, and the average A_∞ value was 0.371 ± 0.034 *SD* at 1.25 units of thrombin/ml of undiluted plasma. The average k_c was 1.30 min^{-1} ± 0.29 *SD* ($t_{1/2}$ 0.52 min) with a range of 0.84–1.83, the average t_{lag} was 39 sec ± 32 *SD*, and the average A_∞ value was 0.374 ± 0.041 *SD* at 2.5 units of thrombin/ml. The average of the individual ratios of the k_c values at the two thrombin concentrations was 1.74 ± 0.15, whereas the ratio of the average k_c values was 1.76.

Of course, Subjects 7 and 10 may not be representative of the normal population. Subjects classified as normal by the classical tests of coagulation and lysis could be considered abnormal when their blood properties are subjected to careful kinetic analysis (13).

Table III presents the data for the plasma of 20 presumed normal subjects of Set B where the clotting parameters were obtained similarly. The mean k_c rate constant at 1.25 units of thrombin/ml of undiluted plasma was 0.85 min^{-1} ± 0.14 *SD* ($t_{1/2}$ 0.84 min) with a range of 0.54–1.04, the average t_{lag} was 90 sec ± 19 *SD*, and the average A_∞ value was 0.367 ± 0.028 *SD*. The mean k_c rate constant at 2.5 units of thrombin/ml of undiluted plasma was 1.37 min^{-1} ± 0.22 *SD* ($t_{1/2}$ 0.52 min) with a range of 1.02–1.67, the average t_{lag} was 50 sec ± 8 *SD*, and the average A_∞ value was 0.375 ± 0.039 *SD*. The average of the individual ratios of the k_c values at the two thrombin concentrations was 1.62 ± 0.17 *SD*, whereas the ratio of the average k_c values was 1.61.

Kinetics of Fibrinolysis—The absorbance, A, of plasma diluted with isotonic saline and with both thrombin and streptokinase added was monitored with time; typical plots of the data obtained are given in Fig. 1. The possibilities that such coagulation–lysis data could be fit to various kinetic models were challenged graphically and by digital computer estimates. They included:

$$P \xrightarrow{k_1} A \xrightarrow{k_2} B$$

Scheme I

$$P_1 \xrightarrow{k_1} P_2 \xrightarrow{k_2} A \xrightarrow{k_3} B$$

Scheme II

Table III—Plasma (Diluted 1:5 with Isotonic Saline) Clotting Parameters [a] for Clinically Appraised Normals of Set B

Subject	Thrombin, Units/ml of Undiluted Plasma: 1.25 t_{lag}, sec	1.25 $100\,A_\infty$	1.25 k_c, min^{-1}	1.25 $t_{1/2}$, min	2.5 t_{lag}, sec	2.5 $100\,A_\infty$	2.5 k_c, min^{-1}	2.5 $t_{1/2}$, min	$\frac{k_c\ (2.5)}{k\ (1.25)}$
1	52	35.5	0.97	0.71	38	32.2	1.67	0.41	1.72
2	64	32.0	1.04	0.67	37	36.0	1.58	0.44	1.52
3	68	37.2	0.84	0.83	38	33.2	1.51	0.46	1.80
4	61	36.2	0.92	0.75	42	34.6	1.50	0.46	1.63
5	99	40.7	0.99	0.70	52	44.6	1.48	0.47	0.49
6	90	37.4	1.00	0.69	47	38.5	1.44	0.48	1.44
7	78	39.5	0.94	0.74	43	40.3	1.11	0.62	1.18
8	88	32.7	1.00	0.69	48	32.2	1.58	0.44	1.58
9	91	39.1	0.87	0.80	53	37.8	1.57	0.44	1.80
10	94	34.8	0.91	0.76	55	35.3	1.56	0.44	1.71
11	100	38.9	0.73	0.95	57	42.4	1.12	0.62	1.53
12	103	33.3	0.84	0.83	59	34.8	1.50	0.46	1.79
13	119	42.1	0.83	0.83	52	44.7	1.53	0.45	1.84
14	85	38.6	0.92	0.75	52	39.4	1.34	0.52	1.46
15	84	32.1	0.80	0.87	54	33.0	1.37	0.51	1.71
16	89	36.2	0.67	1.03	54	36.6	1.03	0.67	1.54
17	101	37.8	0.82	0.85	52	41.8	1.26	0.55	1.54
18	103	37.2	0.63	1.10	69	39.3	1.02	0.68	1.62
19	124	37.1	0.54	1.28	53	36.8	1.03	0.67	1.91
20	118	35.5	0.68	1.02	49	36.3	1.12	0.62	1.65
Mean ± *SD*	90 ± 19	36.7 ± 2.8	0.85 ± 0.14	0.84 ± 0.16	50 ± 8	37.5 ± 3.9	1.37 ± 0.22	0.52 ± 0.09	1.62 ± 0.17

[a] Based on nonlinear fitting by a Wang digital computer using a Simplex algorithm to the equation $A = A_\infty[1 - e^{-k_c(t-t_{lag})}]$, where A is the absorbance at time t.

Table IV—Reproducibility of Coagulation–Lysis Parameters [a] at a Fixed Plasma Concentration of 20% and a Thrombin Concentration of 2.5 Units/ml of Undiluted Plasma

	Streptokinase, Units/ml of Undiluted Plasma					
	25			50		
	$10^2 k_1$	A_0	$10^2 B_0$	$10^2 k_1$	A_0	$10^2 B_0$
	2.95	0.61	0.12	4.49	0.61	0.086
	3.0	0.56	0.3	4.83	0.56	0.12
	2.1	0.61	0.32	3.76	0.61	0.17
	2.34	0.59	0.34	4.19	0.59	0.19
	1.89	0.6	0.63	3.71	0.6	0.19
	2.27	0.63	0.44	4.12	0.63	0.14
	2.64	0.58	0.33	4.76	0.58	0.12
	2.63	0.6	0.37	4.23	0.6	0.14
	2.21	0.56	0.47	3.73	0.57	0.21
	2.36	0.6	0.38	4.23	0.61	0.13
Mean ± *SD*	2.44 ± 0.36	0.59 ± 0.02	0.37 ± 0.13	4.21 ± 0.4	0.59 ± 0.02	0.15 ± 0.04
Range	1.9–3.0	0.56–0.63	0.12–0.63	3.7–4.8	0.56–0.63	0.09–0.21

[a] Obtained from slope and intercept of appropriate plots in accordance with $\ln A/B = -(A_0 + B_0)k_1 t + \ln A_0/B_0$.

$$P \xrightarrow{k_1} A \cdots\cdots \xrightarrow[\text{lag}]{k_2} C$$

Scheme III

$$P \xrightarrow{k_1} A \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \cdots\cdots \xrightarrow[\text{lag}]{k_3} D$$

Scheme IV

All of these rate constants were considered to be first order, and a delay or lag time was introduced into Schemes III and IV. Scheme IV also considered an equilibrium between the extent of clotting as represented by the absorbance, A, and a nonobservable equilibration with a reversible product, C. None of these kinetic models was properly applicable to fit the data.

When the coagulation–lysis curves with added streptokinase were superimposed on the coagulation curve, where compensation was made for the time of commencement of the coagulation process, *i.e.*, A was plotted against $t - t_{lag}$ for the same plasma (Fig. 1), some striking features were apparent. For most of the streptokinase concentrations, the curves of absorbance were superimposable on the clotting curve for plasma without streptokinase for a considerable length of time. Only subsequent to this point did the absorbance due to the clot decrease. This result implied a delay or lag in the lytic action of streptokinase. Since the rate of lysis initially increased and then decreased, it was hypothesized that the kinetics of lysis simulated an autocatalytic reaction that could be expressed by:

$$A + B \xrightarrow{k_1} C$$

Scheme V

so that:

$$-dA/dt = k_1 AB \qquad \text{(Eq. 4)}$$

and:

$$\ln A/B = -(A_0 + B_0)k_1 t + \ln A_0/B_0 \qquad \text{(Eq. 5)}$$

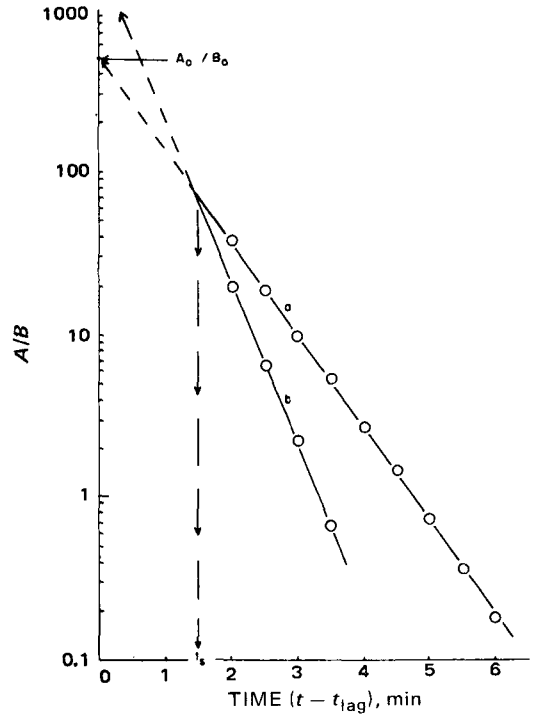


Figure 2—*Example of the linearity of semilogarithmic plots in accordance with Eq. 5, where the slopes are* $-[(A_0 + B_0)k_1]/2.303$ *and the intercept is ln* A_0/B_0. *Time* t_s *is that of coincidence of the plots of absorbance* versus *time for the thrombin plus plasma with and without streptokinase. Curves a and b are for 20% plasma in isotonic saline containing 2.5 units of thrombin/ml of undiluted plasma and 25 and 50 units of streptokinase/ml of undiluted plasma, respectively.*

after some period of time, t_s, when the absorbance, A, deviates from the clotting time curve obtained for $A_{SK=0}$ *versus* time, *i.e.*, the curve without any added streptokinase given by Eqs. 1–3. These same equations are presumed to be valid for any streptokinase concentration up to time t_s.

It was initially presumed that A in Scheme V and Eqs. 4 and 5 represented the observed absorbance, A_2 (or A_2') (Fig. 1), at any streptokinase concentration and was directly proportional to the extent of clotting. If this absorbance with added streptokinase, $A_2 = A_{SK>0}$, were proportional to the amount of clot yet to be lysed at any time, the product B that catalyzes the lysis could be obtained from:

$$B = A_{SK=0} - A_2 \qquad \text{(Eq. 6)}$$

Thus, a plot of ln A/B *versus* time, t, should give a slope of $-(A_0 + B_0)k_1$ and an intercept of ln A_0/B_0 in accordance with Eq. 5. Obviously, specific values of A_0, B_0, and k_1 cannot be obtained by this method. The $A = A_2$ and B values could be obtained from plots similar to those shown in Fig. 1. Unfortunately such plots, although linear for data in the terminal

Table V—Plasma (Diluted 1:5 with Isotonic Saline and with 2.5 Units of Thrombin/ml of Undiluted Plasma) Fibrinolytic Kinetic Parameters [a] for the Clinically Appraised Normals [b] of Set A

	Streptokinase, Units/ml of Undiluted Plasma						
	25			50			
Subject	$10^2 k_1$	$10^2 A_0$	$10^2 B_0$	$10^2 k_1$	$10^2 A_0$	$10^2 B_0$	$\frac{k_{SK50}}{k_{SK25}}$
1	4.07	34.3	0.76	8.36	34.1	0.90	2.05
2	2.52	38.5	0.46	4.84	38.3	0.68	1.92
3	1.51	36.8	1.75	4.03	37.6	0.90	2.67
4	2.35	47.1	0.87	4.46	47.2	0.79	1.90
5	3.35	42.5	0.49	5.88	42.6	0.37	1.76
6	3.40	36.5	0.49	6.52	36.5	0.54	1.92
7	2.50	38.1	1.36	6.58	38.0	1.52	2.63
8	0.76	43.0	2.97	3.25	45.3	0.67	4.28
9	2.70	42.5	0.48	5.80	42.5	0.52	2.15
10	2.62	39.6	0.40	4.77	39.3	0.68	1.82
Average ± *SD*	2.58 ± 0.94	39.9 ± 3.9	1.00 ± 0.82	5.50 ± 1.49	40.1 ± 4.1	0.76 ± 0.32	2.31 ± 0.76[c,d]
Range	1.51–3.96[e]	34–47	0.40–1.75[e]	3.25–7.50[e]	34.–47.	0.37–1.52	1.8–2.7[e]

[a] $\ln A/B = -(A_0 + B_0)k_1 t + \ln A_0/B_0$. [b] Same set as in Table II. [c] 2.09 ± 0.33 when Subject 8 was omitted. [d] $\bar{k}_{SK50}/\bar{k}_{SK25} = 2.11$. [e] Subject 8 was omitted.

Table VI—Plasma (Diluted 1:5 with Isotonic Saline and with 2.5 Units of Thrombin/ml of Undiluted Plasma) Fibrinolytic Kinetic Parameters [a] for the Clinically Appraised Normals [b] of Set B

Subject	Streptokinase, Units/ml of Undiluted Plasma: 25 $10^2 k_1$	25 $10^2 A_0$	25 $10^2 B_0$	50 $10^2 k_1$	50 $10^2 A_0$	50 $10^2 B_0$	$\frac{k_{SK50}}{k_{SK25}}$
1	3.97	33.8	1.25	8.38	34.1	0.88	2.11
2	2.46	38.5	0.54	5.39	38.7	0.34	2.19
3	4.05	34.9	0.08	9.21	35.0	0.03	2.27
4	3.06	35.8	1.16	8.14	36.5	0.49	2.66
5	0.74	46.8	3.23	2.39	48.6	1.43	3.24
6	1.69	43.7	1.32	3.60	44.4	0.62	2.13
7	1.47	46.3	1.68	2.72	46.6	1.37	1.85
8	1.69	35.3	1.68		No available data		
9	2.51	41.9	1.08	5.20	42.3	0.69	2.07
10		No available data		3.47	39.0	0.95	
11	1.81	48.3	1.70	3.57	48.8	1.25	1.97
12	1.40	38.0	2.00	3.46	39.0	0.95	2.47
13	2.19	48.4	1.64	3.96	48.3	1.70	1.81
14	2.83	44.0	1.02	4.94	43.9	1.13	1.75
15	3.16	39.4	0.62	5.63	39.5	0.46	1.78
16	2.29	40.1	1.87	6.20	40.8	1.17	2.71
17	0.63	46.0	4.00	2.63	48.6	1.39	4.20
18	2.71	44.3	0.75	5.41	44.7	0.32	2.00
19	3.10	39.8	1.20	6.15	39.5	1.46	1.98
20	2.84	39.3	0.69	6.56	39.6	0.43	2.31
Average ± *SD*	2.35 ± 0.95	41.3 ± 4.6	1.45 ± 0.92	5.11 ± 2.00	42.0 ± 4.7	0.90 ± 0.48	2.31 ± 0.61[c,d]
Range	1.4–4.0[e]	33.8–48.4	0.08–2.0[e]	2.4–9.2	34.1–48.8	0.032–1.7	1.8–2.7[e]

[a] $\ln A/B = -(A_0 + B_0)k_1 t + \ln A_0/B_0$. [b] Same subjects as in Table III. [c] 2.13 ± 0.30 when Subjects 5 and 17 were omitted. [d] $\bar{k}_{SK50}/\bar{k}_{SK25} = 2.17$. [e] Subjects 5 and 17 were omitted.

phase, were not linear in the phase after time t_s to the time shown by the double-headed vertical arrows.

However, linear semilogarithmic plots against time were obtained for essentially the entire time course after t_s in accordance with Eq. 5 (Fig. 2) when the A value was taken as the amount of total clotting capacity that had not yet been lysed. This value was determined from the difference between the absorbance of the final total clot formed without streptokinase, $(A_{SK=0})_\infty$, and the deficit absorbance that could be assigned to the lytic product, B (Fig. 1), as in:

$$A = (A_{SK=0})_\infty - B = A_1 + A_2 = a(\alpha_1 + \alpha_2) = a\alpha \qquad \text{(Eq. 7)}$$

where α is the total concentration of clotting sites, both yet to be clotted ($\alpha_1 = A_1/a$) and already clotted ($\alpha_2 = A_2/a$), and a is a proportionality constant.

This sum, A, is proportional to an amount of material in the plasma, available both prior to and after clotting, that is consumed during lysis. It would be presumed that the initial amount of this material, A_0, is directly proportional to the plasma content but is diminished by lysis. The intercept of plots (Fig. 2) in accordance with Eq. 5 and the definitions of B and A in Eqs. 6 and 7, respectively, is $\ln A_0/B_0$. The A_0 values were estimated from the asymptotic absorbance in the absence of streptokinase, and the B_0 values were calculated from this value and the intercept. The k_1 values were determined from the quotient of the negative of the slope and $A_0 + B_0$.

Thus, A_0 is the same as A_∞ in Eqs. 1–3 when there is no streptokinase added and:

$$A_0 = a\alpha_0 = (A_{SK=0})_\infty \qquad \text{(Eq. 8)}$$

where α_0 is the total concentration of yet-to-be-clotted clotting sites at time zero and is equivalent to the number of clotted sites at infinite time, A_∞/a, when streptokinase is not added.

The concentration of lytic cofactor β at any time is the sum of an intrinsic concentration of lytic agent β_0, initially in the plasma, and that concentration produced from the apparent concentration of lysed clotting sites:

$$\beta = \beta_0 - (\alpha_0 - \alpha) = \beta_0 + [\alpha_0 - (\alpha_1 + \alpha_2)] \qquad \text{(Eq. 9)}$$

Thus, the actual bimolecular reaction monitored by application of Eq. 5 is between unlysed clotting sites α (whether clotted, α_2, or yet to be clotted, α_1) and a lytic cofactor, β, either inherent in the plasma as β or derived from the lysed clot: $\alpha + \beta \rightarrow \gamma$.

Thus, if $\beta = aB$, *i.e.*, each unit of lysed clotting site α resulted in a unit of cofactor β:

$$-d\alpha/dt = k_1'\alpha\beta = \frac{dA/a}{dt} = k_1'AB/a^2 \qquad \text{(Eq. 10)}$$

so that:

$$-dA/dt = \frac{k_1'}{a}AB \qquad \text{(Eq. 11)}$$

when the observed k_1 of Scheme V and Eqs. 4 and 5 is equivalent to k_1'/a and the observed $B_0 = a\beta_0$ where the proportionality factor a is unobtainable from the given data.

Reproducibility of Parameters of Fibrinolytic Rates—The reproducibilities of the lytic rate constants, k_1, and the A_0 and B_0 pa-

Table VII—Estimated Normal Parameters

Parameter	Average	Normal Range	Presumed Normals Outside Range: Lower	Higher	Range to Include All Presumed Normals[a]
t_{lag}, sec at 1.25 units of thrombin/ml[b]	81	37–110	2/30	3/30	30–125
t_{lag}, sec at 2.50 units of thrombin/ml[b]	57	27–60	2/30	1/30	15–90
k_c, min^{-1} at 1.25	0.80	0.59–1.05	2/30	0/30	0.4–1.20
k_c, min^{-1} at 2.5	1.35	1.0–1.7	1/30	1/30	0.7–2.0
(k_c at 2.5)/(k_c at 1.25)	1.65	1.4–2.0	1/30	0/30	1.1–2.3
$10^2 A_\infty$ and $10^2 A_0$	37	32–45	0/30	0/30	26–52
$10^2 k_1$ (conc.-min)$^{-1}$ at 25 units of streptkinase/ml[b,c]	2.43	1.4–3.5	3/30	3/30	0.6–4.2
$10^2 k_1$ (conc.-min)$^{-1}$ at 50 units of streptokinase/ml[b,c]	5.24	3.0–8.4	3/30	1/30	2.0–12.0
(k_1 at 50)/(k_1 at 25)	2.3	1.7–2.9	0/30	3/30	1.1–4.3
$10^2 B_0$ at 25 units of streptokinase/ml[b,c]	1.3	0.4–3.0	1/30	2/30	0–5.0
$10^2 B_0$ at 50 units of streptokinase/ml[b,c]	0.8	0.3–1.6	1/30	1/30	0–2.4

[a] Estimated to cover more than 95% confidence limits. Except for t_{lag} at both thrombin concentrations where 1/30 was less than the given ranges, none of the presumed normals out of 30 was outside the ranges given for the parameters. [b] Per milliliter of undiluted plasma. [c] At a thrombin concentration of 2.5 units/ml of undiluted plasma.

Table VIII—Plasma (Diluted 1:5 with Isotonic Saline) Clotting Factors [a] for Subjects Treated with Heparin and 2.5 Units of Thrombin/ml of Undiluted Plasma [b]

Set	t_{lag}, sec	$100\,A_\infty$	k_c, min^{-1}	$t_{1/2}$, min
A	240	69.7	0.095	7.22
B	235	62.7	0.038	17.90

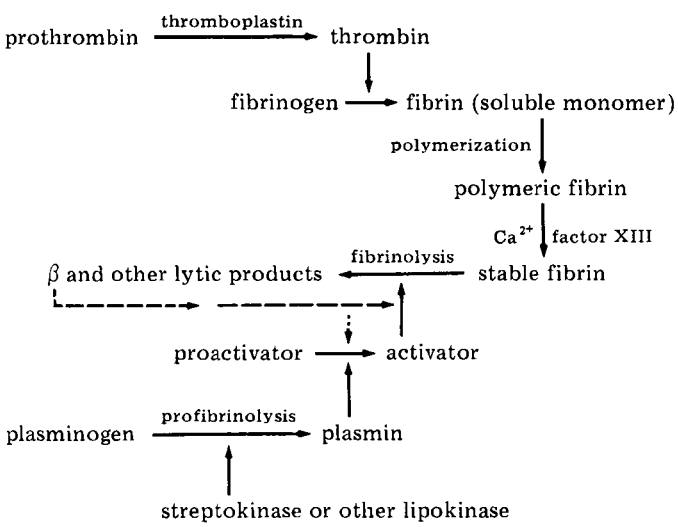
[a] Based on fitting to the equation $A = A_\infty[1 - e^{-k(t-t_{lag})}]$. [b] Clotting rates were too slow at 1.25 units of thrombin/ml of undiluted plasma to be determined satisfactorily.

rameters for the same plasma as considered in Table I are summarized in Table IV. The wide variation in B_0 is anticipated since it is a small number and is derived from extrapolation to obtain the intercept of plots in accordance with Eq. 5. The B values at the initial times are subject to the error obtained when one takes small differences of large numbers (Eq. 6). Nevertheless, the B_0 values for the two concentrations of streptokinase were significantly different and decreased with increasing streptokinase concentration. The lytic rate constant, k_1, increased 1.7 times for an apparent doubling of the streptokinase concentration.

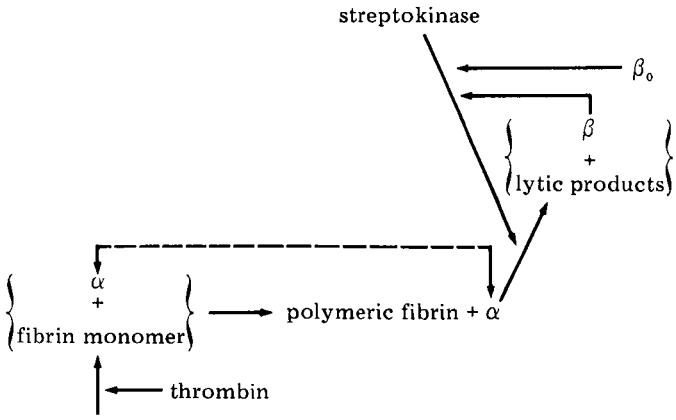
The two sets of plasma from the normal subjects considered with respect to clotting rates in Tables II and III were kinetically evaluated for fibrinolysis in Tables V and VI, respectively. Table V gives the data for the plasma of 10 presumed normal subjects and shows mean lytic constants, k_1, for plasma at 1:5 dilution and 2.5 units of thrombin/ml of undiluted plasma of 2.58 ± 0.94 *SD* and 5.45 ± 1.5 *SD* (ratio 2.11) at the concentrations of streptokinase of 25 and 50 units/ml of undiluted plasma, respectively. There were no significant statistical differences between the means of the A_0 and B_0 parameters, respectively, for the two streptokinase concentrations. The only anomalous ratios of k_{SK50}/k_{SK25} and $B_{0(SK50)}/B_{0(SK25)}$ were in Subject 8. Subject 1 had inconsistently high lytic constants.

Table VI lists the data for the plasma of 20 presumed normal subjects and shows mean lytic constants, k_1, for similarly treated plasmas of 2.35 ± 0.95 *SD* and 5.11 ± 2.0 *SD* (ratio 2.17) at the concentrations of streptokinase of 25 and 50 units/ml of undiluted plasma, respectively. Similar to the results of Table V, there were no statistically significant differences between the means of the A_0 and B_0 parameters for the two streptokinase concentrations. The parameters for this set of normals (Table VI) were not consistently different from those of Table V; Subjects 5 and 17 showed anomalous ratios of k_{SK50}/k_{SK25}. Subjects 1 and 3 had inconsistently high lytic constants, whereas Subjects 5, 7, and 17 had inconsistently low lytic constants.

Characterization of Subjects on Basis of Established Normal Ranges for Clotting and Lytic Parameters—On the basis of the evaluated parameters for presumed normals of Set A (Tables II and V) and Set B (Tables III and VI), Table VII was constructed to give estimated averages, normal ranges, and possible extended ranges for the designated parameters of clotting and lysis under the described conditions. The normal ranges given cover the entire population of 30 presumed normals with exceptions of one to three individuals in several categories. The extended ranges included all, with a minor exception, of the values from the 30 presumed normals.



Scheme VI—Accepted scheme of blood coagulation and lysis is given by solid arrows.



Scheme VII—Simplistic scheme of blood coagulation and lysis that incorporates the observed kinetic dependencies. Arrows perpendicular to the coagulation–lysis process or to a catalytic action represent catalytic or cofactor action and can be mediated by sequential processes. The α produced by thrombin action is considered the same as that consumed in the lytic process. The kinetic information is insufficient to state whether the β formed on lysis or the intrinsic β_0 is a necessary catalyst or cofactor in the ultimate sequence of streptokinase action or is consumed by the lytic process.

The clotting parameters of two cardiovascular risk subjects treated with heparin are given in Table VIII. The lag times and final absorbances on clotting were greatly in excess of normals. The clotting rate constants were extremely low, 1:10 or less that of the normals. Three other subjects, diagnosed as high cardiovascular risks and on moderate heparin therapy, had final absorbances on clotting that were also greatly in excess of normals, *i.e.*, 77, 64, and 170.

Studies are in progress to correlate the results of kinetic appraisals with the careful diagnoses of clinically appraised subjects.

Relation of Established Mechanisms to Observed Kinetics—It is generally recognized that blood clotting and lysis mechanisms are complex and intricate. However, Scheme VI is widely employed to illustrate the probable mechanisms.

The present studies showed that the rate of formation of polymeric or stable fibrin as monitored by the spectrophotometric absorbance of clot formation is generally dependent on the concentration of thrombin.

The rate of fibrinolysis ultimately depends on the concentration of added streptokinase. However, fibrinolysis does not occur immediately after the addition of streptokinase. Thus, streptokinase action on lysis must be mediated by other processes such as the formation of activator. Apparently, a cofactor or catalyst for these processes must be released by the phenomenon of lysis. This pathway is indicated by the dashed arrow in Scheme VI. It may catalyze the activator action on lysis or may be a necessary cofactor for activator production. Either a small concentration of β, β_0, can be present at zero time or a slow rate of spontaneous lysis can occur to promote the accumulation of β that significantly catalyzes lysis at the time, t_s, when the absorbances of the coagulation and lytic curves diverge (Fig. 1).

The kinetics of lysis (Fig. 2 and Eq. 5) depend not only on β but the apparent concentration of some factor, α, that accompanies and is proportional to unlysed fibrin, whether soluble or polymerized. This factor can be rationalized as a concentration of proactivator or derived activator, which is consumed during the lytic process.

A simplification of Scheme VI that incorporates these observations is given in Scheme VII.

The procedures and kinetic analyses described in this paper may be useful in diagnosing abnormalities in clinical subjects and in monitoring the effect of therapy. They may also be found to correlate the time course of drug action with the concomitantly studied pharmacokinetics of administered therapeutic agents in subjects. These *in vitro* studies might serve as screens for possible agents that inhibit coagulation and enhance fibrinolysis, actions that may be favored in proper therapeutic treatment of cardiovascular disease.

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ACKNOWLEDGMENTS

The authors are grateful to Dr. Kwang Eun Kim and Mlle. Francoise Chamboine for assistance in evaluating the data and to Dr. Richard Süverkrup for the programming of the Simplex algorithm on the computer. Appreciation is also expressed to Dr. Jean Claude Arnaud, Director of Research of the Servier Research Laboratories, whose support and encouragement made this collaborative endeavor possible.

Physicochemical Properties of Glycyrrhizic Acid in Aqueous Media II: Effect on Flocculation–Deflocculation Behavior of Suspensions of Sulfathiazole and Graphite

AKINOBU OTSUKA ˣ, YORINOBU YONEZAWA, and YASUHIKO NAKAMURA

Received January 19, 1977, from the *Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya, Japan.* Accepted for publication April 22, 1977.

Abstract □ The flocculation–deflocculation behavior of sulfathiazole and graphite in aqueous solutions of glycyrrhizic acid was studied by measuring the sedimentation volume and turbidity of supernates. The dispersing effect of glycyrrhizic acid on suspensions of sulfathiazole showed a maximum in the pH 3–4 region, the same pH region where the ζ-potential of sulfathiazole particles showed a negative maximum. The results were explained by the variation of degrees of ionization of glycyrrhizic acid and sulfathiazole with pH. With graphite suspensions, the pH region where the dispersing effect of glycyrrhizic acid showed a maximum shifted to a higher pH compared with sulfathiazole. This result can be attributed to the fact that graphite is a nonpolar substance so the surface properties are not affected by a pH change. Hence, the adsorption of glycyrrhizic acid occurs even in a fairly high pH range.

Keyphrases □ Glycyrrhizic acid—effect on flocculation–deflocculation behavior of sulfathiazole and graphite, aqueous solutions, various pH values □ Sulfathiazole—flocculation–deflocculation behavior, effect of glycyrrhizic acid, aqueous solutions, various pH values □ Graphite—flocculation–deflocculation behavior, effect of glycyrrhizic acid, aqueous solutions, various pH values □ Flocculation–deflocculation behavior—sulfathiazole and graphite, effect of glycyrrhizic acid, aqueous solutions, various pH values □ Antibacterials—sulfathiazole, flocculation–deflocculation behavior, effect of glycyrrhizic acid, aqueous solutions, various pH values

A previous report from this laboratory (1) dealt with the surface-active properties and the formation of molecular aggregates of glycyrrhizic acid, a triterpenoid acidic glucoside extracted from *Glycyrrhiza glabra* L. This paper describes the dispersing effect of this substance on aqueous suspensions of sulfathiazole and graphite. While the use of natural products for suspending agents or emulsifying agents has attracted special interest recently, studies of this kind on glycyrrhizic acid have not been published.

EXPERIMENTAL

Materials—Monopotassium glycyrrhizinate[1] was used as received. Graphite[2] and sulfathiazole[3] were obtained as commercial samples. The specific surface area of the graphite determined by the air permeation method[4] was 0.25 m^2/g. The sulfathiazole was finely ground, and the sample with a specific surface area of 0.62 m^2/g was used. All other reagents were analytical grade.

Sedimentation Measurements—To 2.50 g of sulfathiazole or 2.00 g of graphite in a 50-ml graduated test tube was added 50 ml of an aqueous solution of monopotassium glycyrrhizinate whose pH had been adjusted with hydrochloric acid or potassium hydroxide solutions. The mixture was shaken in a constant-temperature bath at 25° for 24 hr and subsequently allowed to stand for 24 hr at the same temperature; the sedimentation volume was then measured.

Turbidity Tests—After the measurements of sedimentation volume, the turbidity of the cloudy liquid phase above the sediment was measured at 500 nm according to:

$$\tau = -\ln T/l \qquad \text{(Eq. 1)}$$

where τ is the turbidity, T is the transmittance, and l is the cell path length.

Adsorption Measurements—After the sedimentation measurements and/or the turbidity tests, the suspensions were centrifuged quickly. The glycyrrhizic acid concentration in the supernate was determined as follows. For the sulfathiazole–glycyrrhizic acid system, 10 ml of supernate was transferred into a test tube, and then 1 ml of concentrated hydrochloric acid and 5 ml of water were added. The mixture was heated to 95° and then allowed to cool. The formed precipitate was dissolved in 5 ml of chloroform, and the absorbance was measured at 258 nm.

For the graphite–glycyrrhizic acid system, the absorbance of the su-

[1] Maruzen Seiyaku Co., Tokyo, Japan.
[2] Yoneyama Chemical Industries, Osaka, Japan.
[3] Sankyo Co., Tokyo, Japan.
[4] Model SS-100, Shimadzu Seisakusho, Kyoto, Japan.