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Kinetic Study on the Effects of Acidic Polysaccharides on the Interaction of Fibrinogen and Thrombin

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Summary

Retardation effect of acidic polysaccharides on the interactions of fibrinogen with thrombin was kinetically investigated by measuring the turbidity as a measure of the concentration of fibrin polymer formed in the system. To this aim, the fibrinogen-fibrin conversion curve was analyzed kinetically based on the elementary reactions, and the slope and the induction period of the conversion curve were expressed as functions of the concentrations of fibrinogen, thrombin, and polysaccharide. The strong retardation effect of dextran sulfate and heparin was quantitatively interpreted. Seven kinds of polysaccharides were examined.

Introduction

According to the accumulated information (WAUGH and PATCH 1953, SCHERAGA and LASKOWSKI 1957, DOOLITTLE 1973, CONIO *et al.* 1976, PALMER and FRITZ 1979) on the fibrinogen-fibrin conversion, fibrin formation is considered to proceed *via* three stages. The first is the initiation stage during which time (the induction period) fibrinogen (designated as F) is activated by thrombin(T), and splitted into fibrin monomer (f) and fibrinopeptide (P). The second stage is the propagation phase in which the fibrin monomers polymerize and rapidly grow in size. During this time, particle dissymmetry increases rapidly. Finally, in the third stage, gelation takes place. These reactions may be written as

$$\mathrm{F} + \mathrm{T} \rightleftarrows \mathrm{FT}^* \longrightarrow \mathrm{f} + \mathrm{P} + \mathrm{T}$$

$$n\ \mathrm{f} \rightleftarrows \mathrm{f}_n$$

$$m\ \mathrm{f}_n \rightleftarrows (\mathrm{f}_n)_m$$

where FT^*, f_n, and $(\mathrm{f}_n)_m$ denote the activated complex, the fibrin n-mer, and the structural network, respectively.

Heparin (POOLE 1959), dextran sulfate (WALTON 1952), and chondroitin sulfate (THOMAS *et al.* 1977) are known as the antithrombogenic agents for preventing the blood clotting. However, quantitative investigation on the inhibitory activity of heparin and others seems by no mean to be sufficient, even *in vitro*.

Among few papers hitherto published, noticeably, SHEPPARD and WRIGHT (1954), and SHEPPARD *et al.* (1956) explained the inhibitory activity of heparin on the basis of an electrostatic interaction mechanism from zeta potential and light scattering measurements, and pointed out that heparin blocks the activation and

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early propagation stages of fibrin polymerization at lower ionic strength level as 0.05, and at pH 7.4. Besides, the effects of hydroxyl compounds on the conversion of fibrinogen to fibrin was discussed by FERRY and SHULMAN (1949), SHULMAN (1953), and SHULMAN *et al.* (1953) based on the molecular structure of the added components.

The objective of this study is to elucidate kinetically the effects of active polysaccharides, such as heparin (Hep), chondroitin sulfate (CHS), hyaluronic acid (HA), dextran sulfate A (DSA), dextran sulfate C (DSC), sulfated methyl cellulose (SMC), and carboxy methyl cellulose (CMC), on the fibrinogen-fibrin conversion in the presence of thrombin in low ionic strength phosphate buffer at pH 7.0, by measuring the turbidity of the mixed system. Since SASAKI *et al.* (1964) have pointed out that formation of solid complex between fibrinogen and polysaccharide is responsible for the injurious effects of heparinoid, high molecular weight dextran sulfate (SASAKI and NOGUCHI 1959) and cellulose sulfate (NAKANISHI *et al.* 1980) were not used here.

Materials and Methods

Bovin fibrinogen (Lot. 21, Code 82-022-4) was purchased from Miles Laboratories Inc. Its protein content as determined by the nitrogen analysis was 80%. The clottability as determined by Laki's method (1951) was 90%. The weight average molecular weight and the isoelectric point of fibrinogen are 340,000 and 5.5, respectively. Fibrinogen is an amphoteric polyelectrolyte including 124.7 mole cationic groups and 119.0 mole anionic groups per 10^5 g (MIHALYI 1970). Thrombin (No. T-6132) was purchased from Sigma Chemical Co., and has the activity of 300 units per *g*. The weight average molecular weight is 30,000 (ESMON *et al.* 1974).

Heparin-Na (Lot. M6G5365), chondroitin sulfate C -Na and hyaluronic acid-Na were purchased from Nakarai Chemical Co. Dextran sulfate A -Na (Lot. LK-1004, M= 1,100) was purchased from Meito Sangyo Co.; dextran sulfate C -Na (Lot. 48C-0241, M=40,000) from Sigma Chemical Co. Carboxy methyl cellulose having the degree of substitution of 0.91 was supplied by Daiichi Kogyo Seiyaku Co. Sulfated methyl cellulose was prepared by us (NAKAJIMA and SATO 1974) from methyl cellulose supplied by Matsumoto Yushi Co. Lauryl benzene sulfonate (M=348) (LBS) purchased from Nakarai Chemical Co. was used as a low molecular weight reference.

Fibrinogen solutions were prepared by dissolving the stock powder in 0.05 M phosphate buffer, containing 4.4775 g Na_2HPO_4

TABLE I Polysaccharides Used

Polymer	Nominal Mol.Wt.	No.of Ionizable group/Pyranose	Ionic Groups
Hep	9,000	1.5	$-COO^-$, $-OSO_3^-$, $-NHSO_3^-$
DSA	1,100	2.13	$-OSO_3^-$
DSC	40,000	1.59	$-OSO_3^-$
CHS	45,000	1.0	$-COO^-$, $-OSO_3^-$
SMC	50,000	0.855	$-OSO_3^-$
CMC	50,000	0.91	$-COO^-$
HA	850,000	1.0	$-COO^-$

and 1.3601 g KH_2PO_4 per 450 ml aqueous solution, at a concentration of about 0.06 g/dl for 2 hours, and clarifying by filtration through filter pads. The concentrations of the fibrinogen solutions were determined by the optical density measurements at 280 nm with a Hitachi Spectrophotometer Model EPS-3T. The numerical value of the specific extinction of 0.1% fibrinogen used was E_{cm} =1.506 (MIHALYI 1968). The pH and the ionic strength of the fibrinogen solutions were 7.0 and 0.106, respectively. Thrombin solution was prepared by dissolving the stock powder in the 0.05 M phosphate buffer at pH 7.0, at a concentration of 40 units/ml.

Acidic polysaccharide solutions were prepared by dissolving the materials in the 0.05 M phosphate buffer at pH 7.0, at various concentrations. The numbers of ionizable groups in these polysaccharides were determined from conductometric titration by using a Yanagimoto Conductivity Outfit Model MY-8.

Polysaccharide solution was mixed with fibrinogen solution. After the reaction reached the equilibrium, 3 ml of mixture was put into a quartz cell for the UV measurement. Now 0.025 ml of 1 unit thrombin solution was added to the mixture with a microsyringe, and at the same time, the turbidity of the system was measured at time intervals. The time at which thrombin was added was taken as zero time. Throughout the entire measurement, the initial concentrations of fibrinogen and of thrombin were constant. The turbidity measurement was carried out at room temperature.

Reaction Kinetics

To begin with, we consider elementary reactions as shown below in the earlier stages of fibrinogen-fibrin conversion in the absence of polysaccharide component.

$$F + T \underset{k_1}{\overset{k_2}{\rightleftharpoons}} FT^* \xrightarrow{k_3} f + P + T \qquad (1)$$

$$f + f \xrightarrow{k_p} f_n \qquad (2)$$

where, k_1, k_2, k_3, and k_p denote the respective rate constants. The fibrin monomers f formed in stage (1) immediately polymerize into fibrin polymer f_n. In stage (2), the reverse reaction was not taken into account because of its minor contribution.

Now we assume that the formation of active complex FT* obeys the second-order reaction and the complex produces f by the first-order reaction. The latter would be the rate determining step. We denote the concentrations and the initial concentrations of F and T by [F] and [T], and $[F_o]$ and $[T_o]$, respectively. Experimentally, $[F_o] \gg [T_o]$. In the stage (1), when the stationary state holds, the concentration [FT*] of FT* becomes independent of time, and thus we have

$$\frac{d[FT^*]}{dt} = k_2[F_o][T_o] - k_1[FT^*_e] - k_3[FT^*_e] = 0 \qquad (3)$$

because $[F_o] \gg [FT^*_e]$, and [T] is allways approximated by $[T_o]$. Accordingly, the concentration of FT^*_e in the stationary state, $[FT^*_e]$, is given by eq (4).

$$[FT_e^*] = \frac{k_2[F_o][T_o]}{k_1 + k_3} \tag{4}$$

Further we assume that the polymerization reaction of f proceeds by the second-order reaction. Fibrin monomers are formed in stage (1) and consumed in stage (2). Therefore,

$$\frac{d[f]}{dt} = k_3[FT_e^*] - k_p[f]^2 \tag{5}$$

From eq (4) and $k_2k_3/(k_1 + k_3) \equiv k_i$, we have

$$\frac{d[f]}{dt} = k_i[F_o][T_o] - k_p[f]^2 \tag{6}$$

Integration of eq (6) by the use of the initial condition, [f]=0 at t=0, yields

$$\text{arc tanh}\,([f]/\lambda) = k_p\lambda t \tag{7}$$

$$\lambda = \left\{(k_i/k_p)[F_o][T_o]\right\}^{1/2} \tag{8}$$

By transforming the inverse function arc tanh to tanh, we obtain

$$\tanh(k_p\lambda t) = [f]/\lambda \tag{9}$$

On the other hand, the accumulated concentration of fibrin polymer, $\Delta[f_n]$, is given by

$$\Delta[f_n] = \int_o^t k_p[f]^2 dt$$

$$= k_p\lambda^2\left\{k_p\lambda t - \tanh(k_p\lambda t)\right\} \tag{10}$$

For t except for very small t region, $\exp(2k_p\lambda t) \gg 1$, and hence $\tanh(k_p\lambda t)$ is approximated by unity. Accordingly,

$$\Delta[f_n] = k_p^2\lambda^3 t - k_p\lambda^2 \tag{11}$$

In Figure 1, $\Delta[f_n]$ is shown schematically as a function of time by using eqs (10) and (11). From the slope S and the intercept I_p on the abscissa (designated as the induction period), we obtain

$$S = k_p^2\lambda^3 \tag{12}$$

$$I_p = (k_p\lambda)^{-1} \tag{13}$$

Now we consider the effect of acidic polysaccharides on the fibrinogen-fibrin conversion. As mentioned earlier, polysaccharide

was mixed with fibrinogen prior to the addition of thrombin. So, we first refer to the interaction of polysaccharides with fibrinogen. SHEPPARD (1956) pointed out the formation of links similar to these involved in the antigen-antibody reaction for the reaction of fibrinogen with heparin at pH 7.4. Here we assume such an interaction as indicated by the Freundlich adsorption isotherm for the reaction between fibrinogen and polysaccharides, designated as X

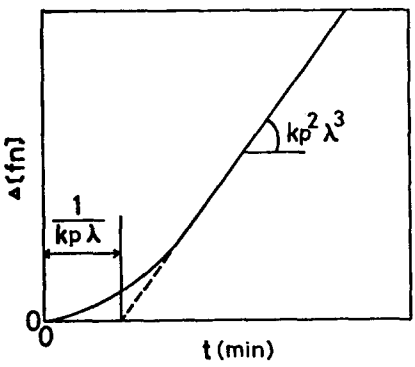


Fig. 1

$$\frac{[X_o] - [X_e]}{[F_{oo}]} = k[X_o]^{\nu} \tag{14}$$

where $[F_{oo}]$ is the initial concentration of fibrinogen, $[X_e]$ is the concentration of X at adsorption equilibrium, and k and ν are the constants. Eq (14) is rewritten as

$$[F_{oo}] - [F_e] = \text{const}\cdot k[F_{oo}][X_o]^{\nu} \tag{15}$$

or

$$[F_e] = [F_{oo}](1 - K[X_o]^{\nu}) = [F_{oo}] \exp(-K[X_o]^{\nu}) \tag{16}$$

where K =const$\cdot k$. Experimentally, thrombin was added after the adsorption equilibrium attained, and hence $[F_e]$ in eq (16) is equal to $[F_o]$ in eq (3). According to Li *et al.* (1974, 1976), and FEINMAN and LI (1977), heparin can bind with thrombin but can not suppress the thrombin activity. So, here we assume that acidic polysaccharides affect only on fibrinogen.

Such assumption results in eqs (17) and (18), by combining simply eqs (12) and (13) with eq (16), for the system of fibrinogen, polysaccharide, and thrombin.

$$\log S = \log S_o - A[X_o]^{\nu} \tag{17}$$

$$\log I_p = \log I_{po} + B[X_o]^{\nu} \tag{18}$$

where, S_o and I_{po} are the S and I_p values for the system including no thrombin, and A and B are the constants. Thus the logarithms of the slope and induction period for the system containing polysaccharide are proportional to the νth power of the initial concentration of the polysaccharide.

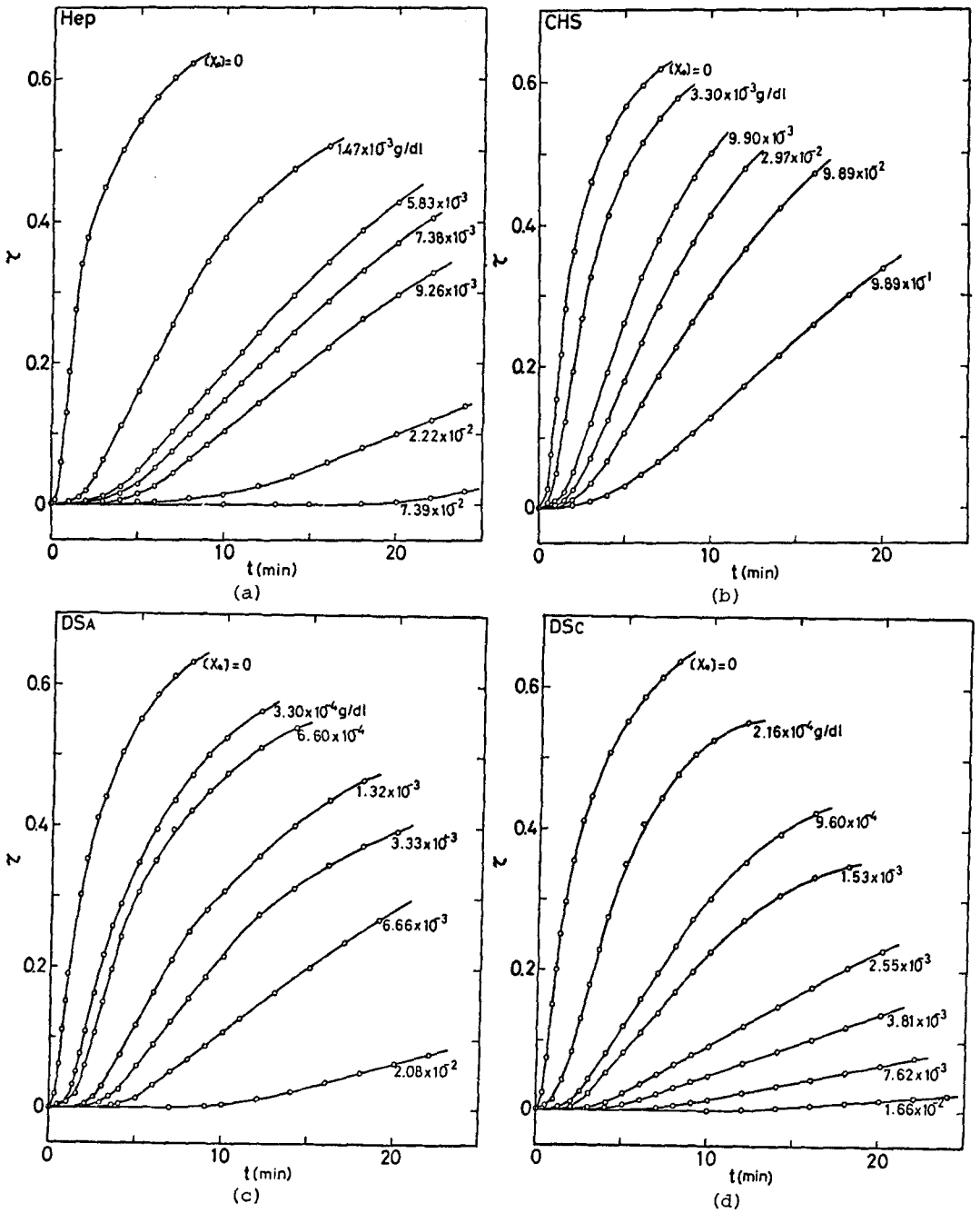
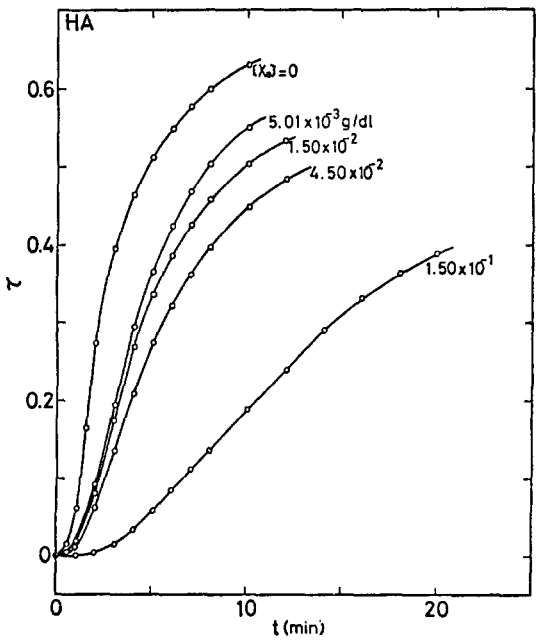
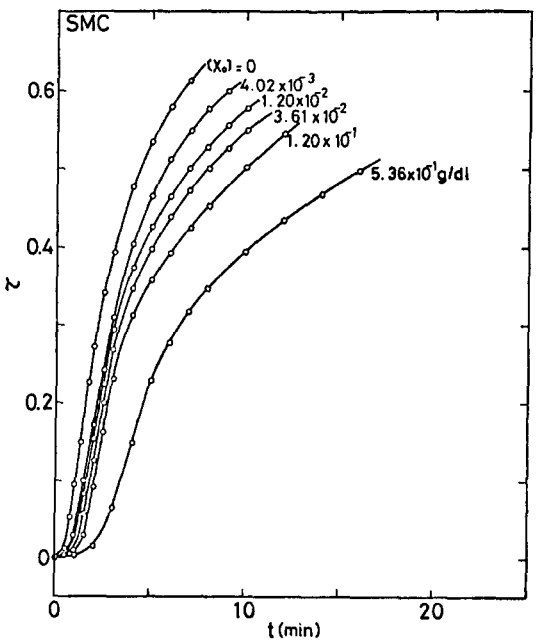


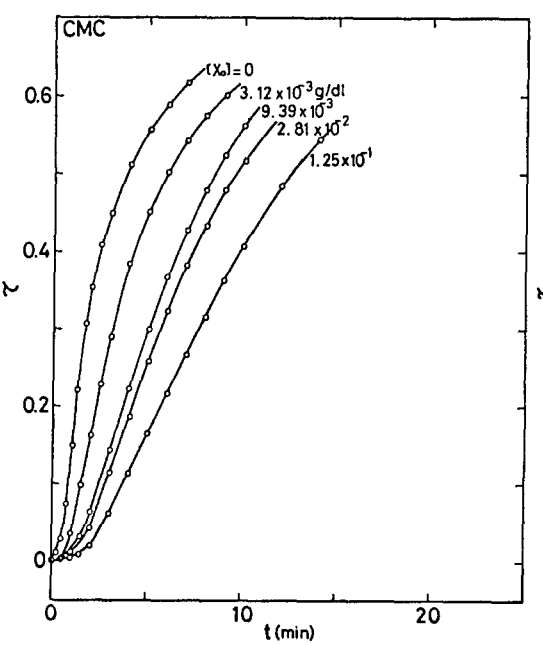
Fig. 2 Turbidity τ plotted against to time t at pH 7.0.
$[F_{oo}]$=0.03 g/dl; $[T_o]$=1.0 unit/3ml; ionic strength=0.106



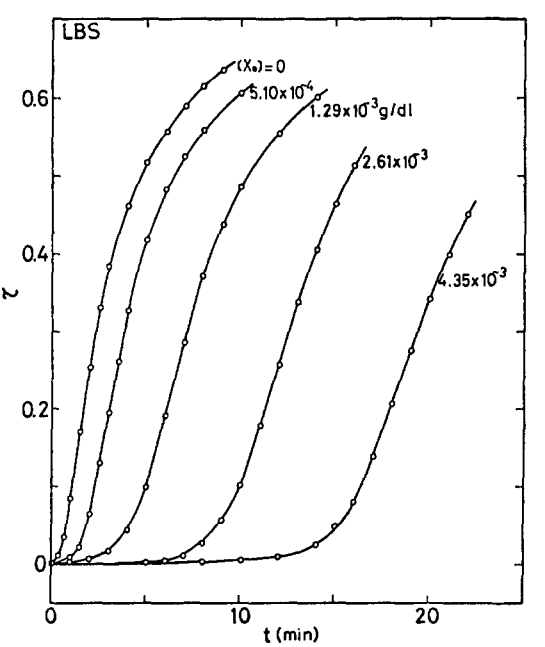
(e)



(f)



(g)



(h)

Fig. 2 Continued.

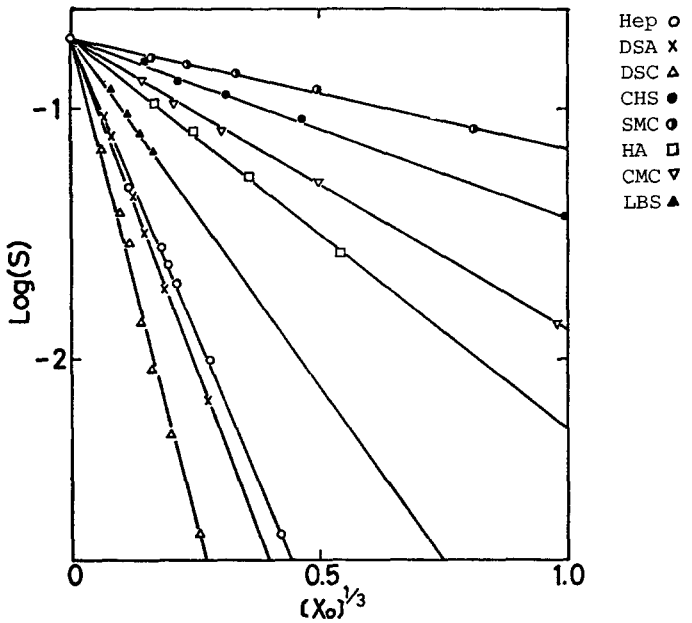


Fig. 3 Logarithm of slope plotted against $[X_o]^{1/3}$.

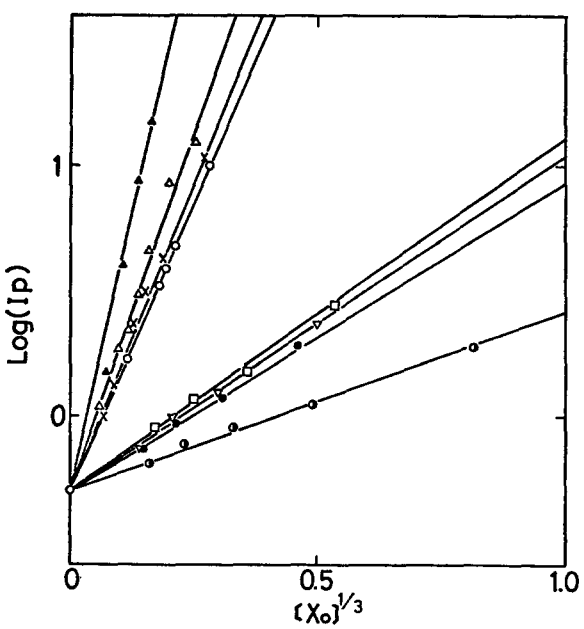


Fig. 4 Logarithm of induction period plotted against $[X_o]^{1/3}$.

Results and Discussion

Fibrin polymer formation was measured by turbidity after FERRY and MORRISON (1947), FERRY and SHULMAN (1949) and SHULMAN (1953). In Figure 2, the turbidity τ of the mixture is plotted against time t for Hep, DSA, DSC, CHS, SMC, CMC, HA, and LBS systems. As obvious from the figure, the τ-t curves are well represented by Figure 1. Now the slope S and the induction period I_p were determined from the curves. In Figures 3 and 4, log S and log I_p are plotted against the 1/3 power of the initial concentration $[X_o]$ of polysaccharides. Obviously, linear relationships are obtained for both log S vs. $[X_o]^{1/3}$ and log I_p vs. $[X_o]^{1/3}$. In other words, eqs (17) and (18) became valid by assigning 1/3 to ν. The value of ν may slightly deviate from 1/3, depending on the kind of polysaccharides. In conclusion, we point out that acidic polysaccharides may react with fibrinogen in obedience with a scheme similar to the Freundlich-type adsorption isotherm, and the fibrinogen-fibrin conversion may proceed by the mechanism kinetically proposed in this study. Thus, retardation effect of polysaccharides is indicated by the values of A and B in eqs (17) and (18). The retardation effect of polysaccharides reflected in A is in the following order.

$$\text{DSC} > \text{DSA} > \text{Hep} > \text{LBS} > \text{HA} > \text{CMC} > \text{CHS} > \text{SMC}$$

According to Figures 3 and 4, the retardation effect is classified to two groups; the stronger one and weaker one. It is noteworthy that DSC, DSA, and Hep which belong to the former group have α-1,4 linkage; contrary, the latter group has β-1,4 linkages. The stronger retardation effect of α-1,4 chains may be due to their more flexible nature of the backbone chain than β-1,4 chains.

Kinetic study will be continued in more detail for the system of thrombin, fibrinogen, and retardants.

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