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An Automated Analysis System of *Limulus* Amebocyte Lysate (LAL)–Endotoxin Reaction Kinetics Using Turbidimetric Kinetic Assay

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An automated analysis system for evaluating reaction kinetics between *Limulus* amebocyte lysate (LAL) and endotoxin has been developed by using a data acquisition program for the Toxinometer. This program collects the time-course data of turbidity change in gel-clotting in the LAL–endotoxin reaction, determines the gelation time, and calculates endotoxin concentration of up to 64 samples simultaneously and independently. Whole time-course data obtained at 12 s increments are displayed on a monitor and reported on a printer both graphically and numerically. LAL–endotoxin reaction kinetics depending upon different LAL preparations or inhibitory or enhancing effects of chemicals, were analyzed with this system by observing the reaction time-course data. By selecting variable threshold values for gelation detection of this program, well correlated standard curves of endotoxin concentration were obtained. Furthermore, by introducing parameters related to the reaction kinetics, gel formation induced by β-1,3-glucan could be distinguished from that by endotoxin, which was impossible with the conventional gel-clot method.

Keywords——endotoxin; *Limulus* amebocyte lysate; turbidimetric kinetic assay; gelation time; automated analysis system; data acquisition program; personal computer; reaction kinetics; Toxinometer

Introduction

Since the discovery that endotoxin induces gel formation in *Limulus* amebocyte lysate (LAL),[1] the gel-clot method for endotoxin analysis has been steadily replacing the rabbit pyrogen test. In the United States, the gel clot method is adopted in USP-NF XXI as [85] Bacterial Endotoxin Test (2).[2] The conventional gel-clot test based on the manual inversion method is semiquantitative and often influenced by environmental conditions and the skill of the operator. Accordingly, some automated methods using turbidimetry have been reported.[3–5] These methods, however, are not widely accepted for routine analysis from the viewpoints of precision and operational efficiency. On the other hand, some chromogenic methods are also currently available.[6,7] But these methods require complicated operations and the linear calibration relationship is obtained within a narrow range of only 1 or 2 orders of magnitude of endotoxin concentration.

We have developed an instrument (Toxinometer ET-201) for automated measurement of endotoxin with excellent precision and efficiency and obtained an extremely wide range of the calibration curve, such as 4 or 5 orders of magnitude of endotoxin concentration, with simple and easy operation.[8] This instrument measures the turbidity change in gel formation of the LAL–endotoxin reaction system and automatically scores the reaction time required to obtain a certain turbidity change as the gelation time. For further detailed analysis of LAL–endotoxin reaction kinetics using this instrument, we have developed a data acquisition program (LABOSOFT-ET), which runs on some personal computers, such as the NEC PC-

9800 or the IBM PC-AT. This program can process on-line or off-line the time-course data transmitted from the Toxinometer, determine the gelation time, and calculate endotoxin concentration, automatically.[9]

In this paper, we present evidence for the basic efficacy of this program in the quantitative determination of endotoxin, and demonstrate its usefulness in monitoring LAL–endotoxin reaction kinetics for analysis of endotoxin.

Materials and Methods

Principle of the Assay——The present system measures the turbidity change at 660 nm in the gelation reaction of LAL–endotoxin solution and automatically scores the gelation time of the sample. Figure 1 shows the definition of the gelation time. The ordinate $R(t)$ is the ratio of the transmittance at time t to the initial transmittance. This transmittance ratio $R(t)$ decreases owing to the turbidity change accompanying gel formation. The gelation time T_g is defined as the reaction time required to obtain a certain turbidity change R_{th}. The present system monitors this ratio $R(t)$ of up to 64 samples simultaneously and independently at 12 s intervals.

Hardware——Figure 2 shows a schematic diagram of this system. A Toxinometer ET-201 is composed of a control module which controls the whole electronic system and an analysis module which monitors the ratio $R(t)$s of

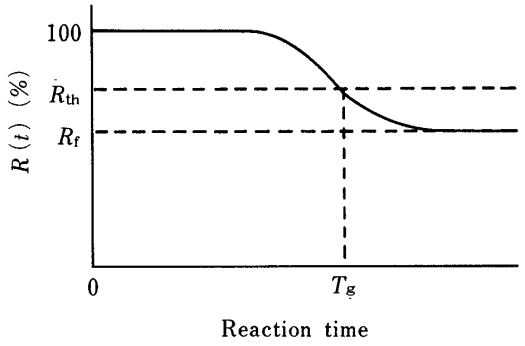

Fig. 1. Definition of the Gelation Time

$R(t)$, Transmittance ratio; R_{th}, threshold value of $R(t)$; T_g, gelation time; R_f, final level of $R(t)$. R_{th} is variable in the present program.

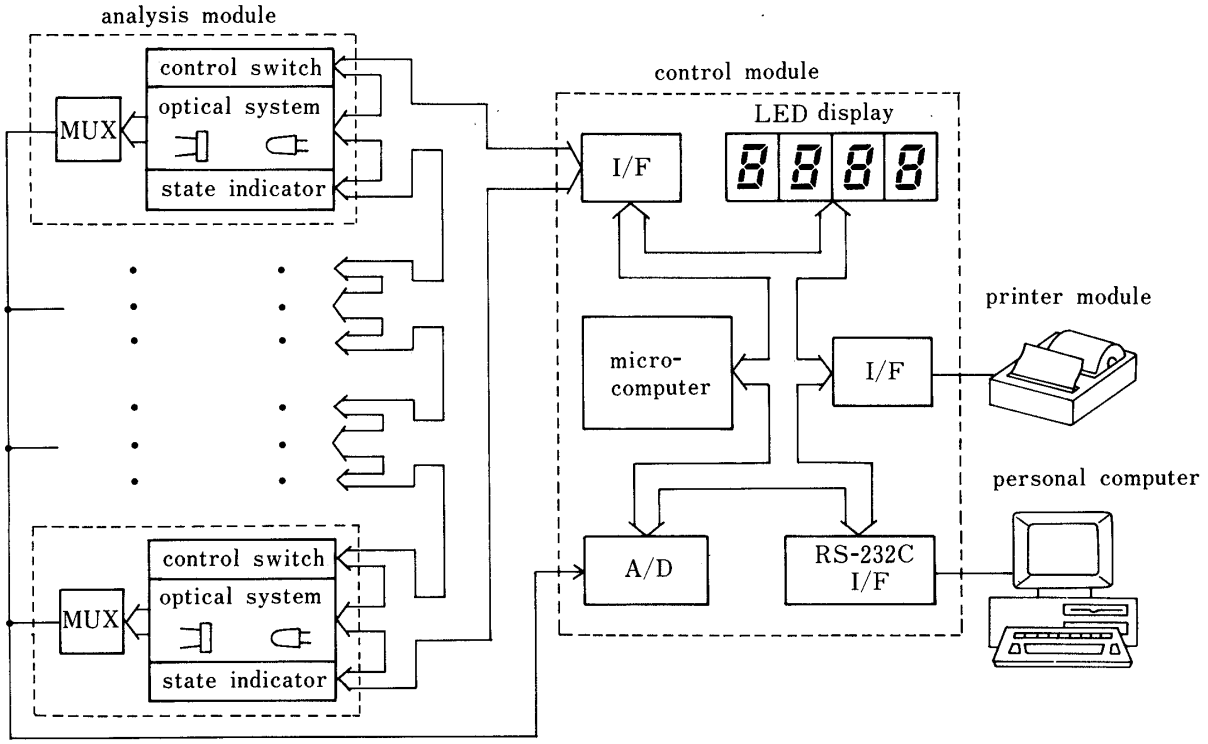

Fig. 2. Schematic Diagram of the Present System

MUX, multiplexer; A/D, A/D converter; I/F, interface circuit.

TABLE I. Hardware for Operation

IBM PC-AT version
1) IBM PC-AT with 640KB RAM
2) Enhanced graphic adapter (256KB on-board memory)
3) Enhanced color display
4) EPSON printer FX-85

NEC PC-9800 version
1) NEC PC-9801 E, F, VX, VM, and U with 640KB RAM
2) High-resolution color display
3) Printer with Chinese character ROM

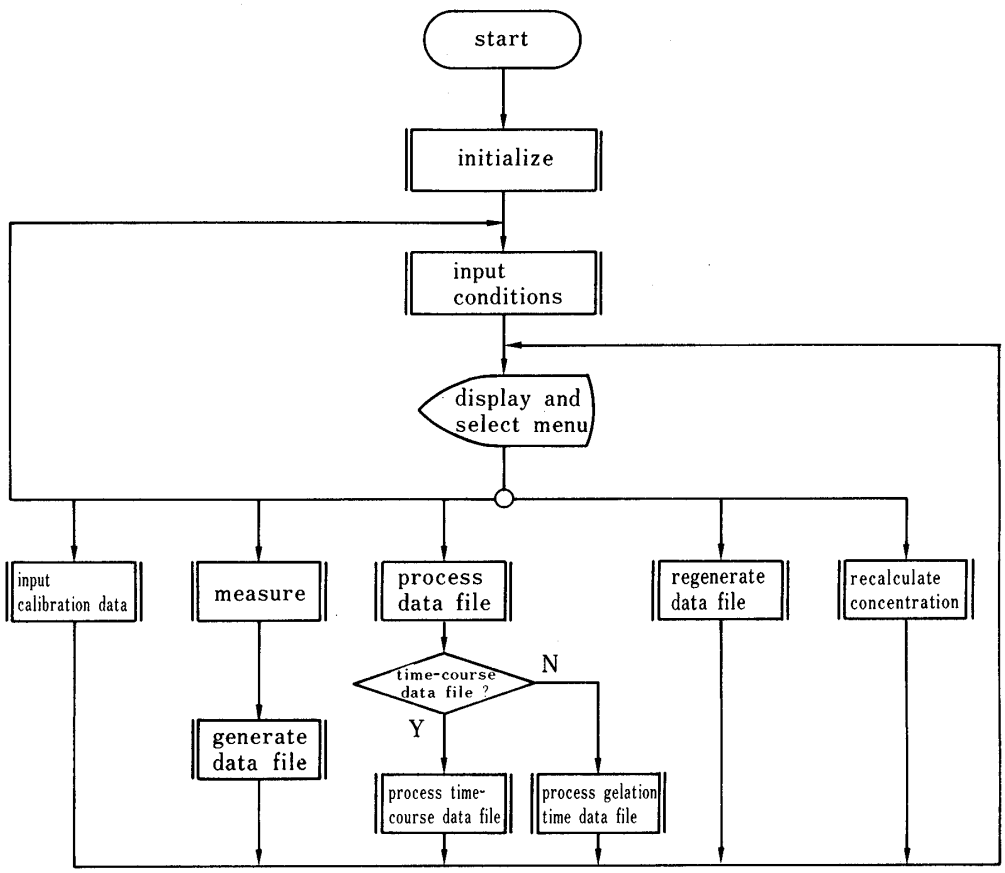

Fig. 3. General Flow Chart of the Program

multiple samples. The same test tubes and same LAL preparations as used in the conventional gel-clot method are utilized in this instrument. As the samples are stationarily incubated at a controlled temperature of 37 ± 0.5 °C, the detection of gelation is not subject to disturbance from sample vibration. The time-course data of turbidity change measured with this instrument are transmitted to an external computer through the RS-232C interface circuit. A personal computer operated with LABOSOFT-ET collects and processes these time-course data. For operation of this program, the equipment listed in Table I is required.

Software——The functions of the present program may be summarized as follows.
1) Collection of the reaction time-course data.
2) Determination of the gelation time.
3) Calculation of endotoxin concentration.
4) Display on a monitor and report on a printer (graphs or numerical tables of the reaction time-course data).
5) Storing the reaction time-course data in a disk.
6) Storing the processed data in a disk.
7) Reevaluation of the stored data.

8) Printing out the full report.
9) Input or modification of the calibration data.

Figure 3 shows the general flow chart of the program. This program is composed of six main functional routines, namely, input calibration data, measure, process data file, regenerate data file, recalculate concentration, and input conditions. Each routine can be selected on the screen menu. At real-time processing for actual measurement, the measure routine is used, and at off-line processing for reevaluation of the stored data in a disk, the process data file routine is used. Data processings of both routines are equivalent except for the difference of data sources.

Reagents——As the LAL preparation, LIMULUS TEST WAKO (lot LAH9610 and CDQ9254, manufactured by Associates of Cape Cod, Inc.) was employed. Other commercially available LALs were obtained from Associates of Cape Cod, Inc. (lot 99-40-383GT), Mallinckrodt, Inc. (lot 4BU), Haemachem Inc. (lot 019GN-1), and Teikoku Hormone Mfg. Co., Ltd. (lot S010) to compare the reaction kinetics and the standard curves for endotoxin concentration. As the control standard endotoxin (CSE), Wako reference endotoxin lot STG9418 and NO9354 (prepared from *E. coli* UKT-B strain) were used. Potency of the CSE was confirmed with US reference standard endotoxin EC-5 using the present instrument. Distilled water for injection (WFI, manufactured by Otsuka Pharmaceutical Corp.) was used in all cases of reconstitution of LAL, dissolution and dilution of samples. Curdlan and DL-sodium lactate were obtained from Wako Pure Chemical Industries, Ltd. Curdlan (5 mg) was dissolved in 5 ml of WFI containing 0.1 N NaOH and diluted with WFI. LAL used in the experiment on curdlan was reconstituted in 0.1 M Tris–HCl buffer solution (pH 7.3).

Methods——Assays were performed by adding 100 μl of test fluid to 100 μl of LAL solution in a 10×75 mm glass tube. After mixing of the reaction mixture for a few seconds, the test tube was inserted into the optical unit of an analysis module and the measurement of turbidity was initiated. The reaction time of each sample was independently counted until the gelation was confirmed.

Results and Discussion

Using the present program, the time-course data of turbidity change of 16 positions was observed simultaneously on a screen with 8 colors. A hard copy of this display was also obtained off-line using a specific printer. Figure 4 shows typical reaction time-course data in which transmittance ratios $R(t)$s of 6 positions were displayed. The gelation time of each position was also displayed on a screen. During monitoring of the reaction kinetics on the screen, endotoxin concentrations of specimens were calculated and reported on a printer. Not only the transmittance ratio but also all raw data measured with the instrument could be called up graphically. Furthermore, numerical tables of all raw data were also available both on the screen and on the printer.

Figure 4 shows the reaction time courses obtained with three different commercially available LALs. The ordinate shows the transmittance ratio $R(t)$, and the abscissa shows the reaction time. The dashed level line shows the threshold value R_{th} for determining the gelation time T_g. Two different concentrations of endotoxin, 10 and 0.05 EU/ml, were prepared for each LAL. These reaction time courses showed different degrees of turbidity change and the

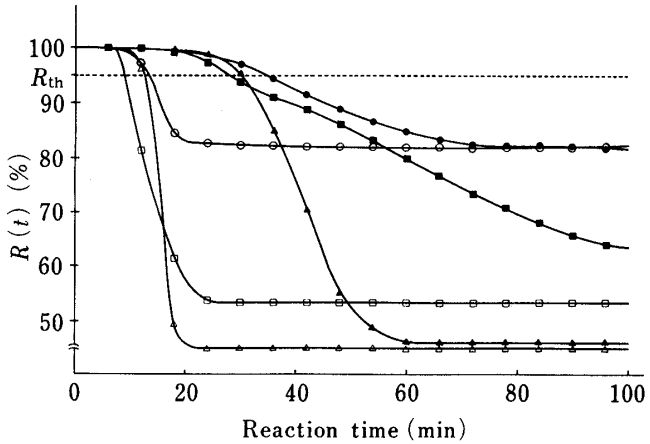

Fig. 4. Typical Time-Course Data of Turbidity Changes

For LAL-A, concentrations of endotoxin are as follows: ○, 10 EU/ml; ●, 0.05 EU/ml. For LAL-B: □, 10 EU/ml; ■, 0.05 EU/ml. For LAL-C: △, 10 EU/ml; ▲, 0.05 EU/ml.

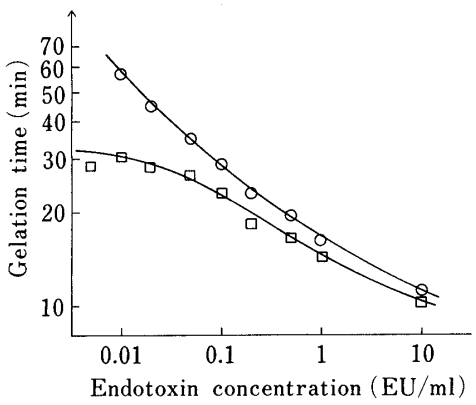

Fig. 5. Effect of Changing the Threshold Value R_{th} with LAL-B

○, R_{th}=90%; □, R_{th}=95%.

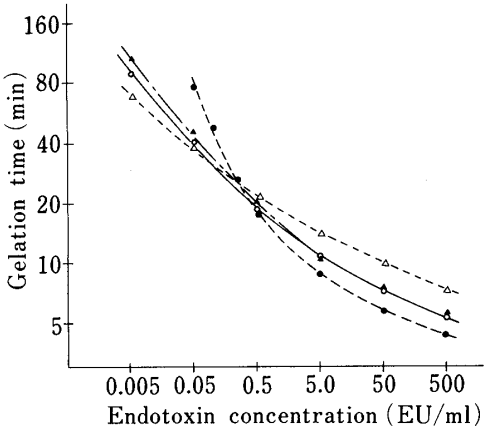

Fig. 6. Comparison of Standard Curves Obtained with Four Different Commercial LALs

○, LAL-A; △, LAL-C; ●, LAL-D; ▲, LAL-E.

TABLE II. Comparison of R_{th}, Calibration Range and Reproducibility Obtained with Five Different Commercial LALs

	R_{th} (%)	Calibration range (EU/ml)	Reproducibility of T_g for a sample Mean T_g (min)	S.D. (min)	C.V. (%)
LAL-A	95	0.005—500	19.3	0.255	1.32
LAL-B	90	0.01 —500	22.0	0.387	1.76
LAL-C	92	0.005—500	21.1	0.473	2.24
LAL-D	95	0.05 —500	17.9	0.342	1.91
LAL-E	95	0.005—500	20.1	0.704	3.50

reaction rates depending upon different preparations of LAL. Namely, LAL-B showed a larger degree of turbidity change than LAL-A for the same endotoxin concentration. LAL-C showed both a larger degree of turbidity change and a higher reaction rate.

Figure 5 shows the comparison of two standard curves which were obtained with LAL-B by changing the threshold value R_{th} for determining the gelation time T_g. At R_{th} of 95%, the lower limit of the calibration was about 0.1 EU/ml due to the flattened standard curve. At R_{th} of 90%, the lower limit of the calibration was extended to 0.01 EU/ml. As LAL-B has a large degree of turbidity change, lowering the R_{th} made the gelation detection more reliable at low concentrations of endotoxin where gel formation is not quite uniform. Consequently, the sensitivity of the measurement with this LAL was improved by employing R_{th} of 90%.

As the R_{th} is variable and the gelation time can be redetermined for the stored data by using a new R_{th}, it is possible to optimize the R_{th} with this program according to characteristics of the reaction kinetics. It should be noted that a higher R_{th} value made it difficult to distinguish optical noise from gelation signal and that a lower R_{th} value reduced the sensitivity of the detection.

In Fig. 6, the standard curves obtained with four different commercial LALs by selecting appropriate R_{th} for each LAL are compared. Although these standard curves had different slopes resulting from different preparations of LAL, each standard curve could be applied to quantitative determination of endotoxin. Selected R_{th} for each LAL, calibration range and reproducibility obtained with these LALs are summarized in Table II.

It is well known that many drugs and chemicals enhance or inhibit the LAL–endotoxin reaction.[10,11] Using the present instrument, enhancement by a drug was expressed quanti-

tatively as shortening of the gelation time T_g, and inhibition as prolongation of T_g.[12]

Figure 7 shows typical enhanced and inhibited reaction time-courses at 0.5 EU/ml of endotoxin. At the concentrations of 1% and 5% sodium lactate, the LAL–endotoxin reaction was inhibited and the gelation times were prolonged compared with the gelation time of the sample without sodium lactate. On the other hand, at the concentration of 10% sodium lactate, the reaction was enhanced and T_g was shortened. It was observed that, while the reaction rate decreased in the inhibited state, both reaction rate and degree of turbidity change increased in the enhanced state. As this turbidity change in the enhanced state was not accompanied with gel formation, the conventional gel-clot method was not applicable to 10% sodium lactate. However, by monitoring the reaction time courses of samples spiked with endotoxin, it was observed that the turbidity change caused in the sample correlated to endotoxin concentration alone. Using this program, it was found that our method was applicable to 10% sodium lactate and a good calibration relationship was obtained without dilution of the sample.

As the reaction kinetics with inhibition or enhancement were observed easily using this program, these effects of various drugs could be quantitatively determined, which is difficult with the conventional gel-clot method. This program makes it possible to evaluate suitable analytical conditions for the affected LAL–endotoxin reaction.

It is known that β-1,3-glucan also induces the gelation of LAL.[13,14] Obayashi *et al.* quantitatively distinguished β-1,3-glucan from endotoxin by using recombined LAL reagent with the chromogenic method.[15] However, gel formation induced by endotoxin and that by β-1,3-glucan could not be distinguished from each other by the conventional gel-clot method. Figure 8 shows examples of the reaction time-courses of endotoxin and curdlan. Though the

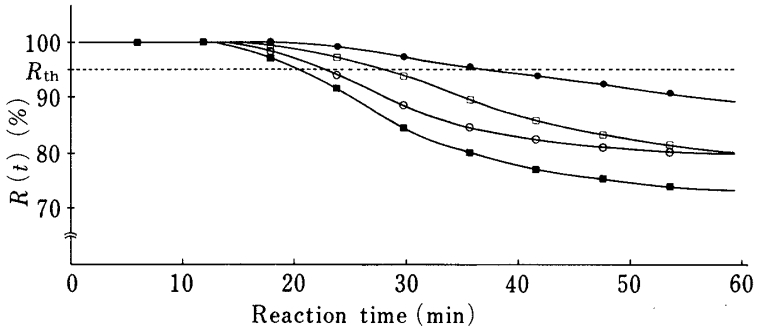

Fig. 7. Reaction Kinetics with or without Inhibition and Enhancement

Concentrations of sodium lactate are as follows: ○, 0%; ●, 1%; □, 5%; ■, 10%. Each sample contains 0.5 EU/ml endotoxin.

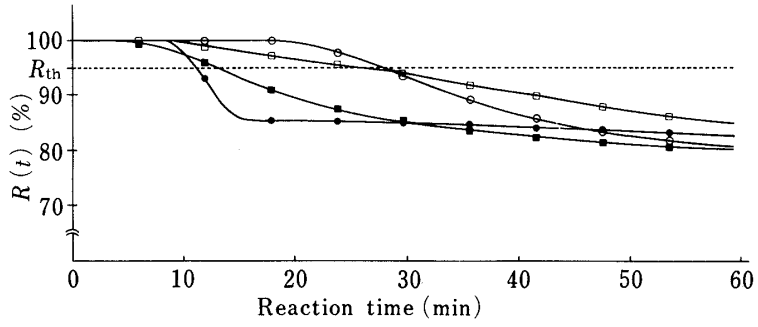

Fig. 8. Comparison of the Reaction Time-Courses between Endotoxin and Curdlan

○, 0.5 EU/ml endotoxin; ●, 50 EU/ml endotoxin; □, 10 ng/ml curdlan; ■, 100 ng/ml curdlan.

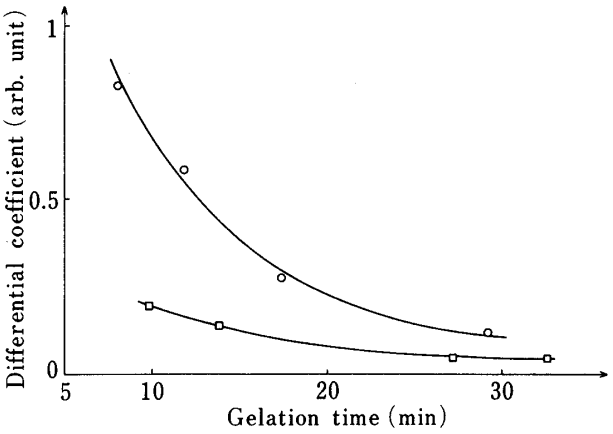

Fig. 9. Plot of the Differential Coefficient against the Gelation Time Obtained with Endotoxin and Curdlan

○, Endotoxin; □, curdlan.

gelation times were similar (obtained T_gs are 11.8, 28.8 min for endotoxin and 13.8, 27.2 min for curdlan), the reaction time-courses of the two substances were quite different. Curdlan showed both an earlier descent of the transmittance ratio and a slower rate of turbidity change than endotoxin. We introduced reaction rate, namely, the differential coefficient of the reaction time-course, as a parameter related to the reaction kinetics. In Fig. 9, differential coefficients of the reaction time-courses of endotoxin and curdlan at gelation time T_g are plotted against the gelation times. Each substance was plotted on a different curve. This shows that endotoxin and β-1,3-glucan, which could not be distinguished from each other by detection of the gelation time alone, can be distinguished by introducing a new parameter such as reaction rate.

Using the present system, LAL–endotoxin reaction kinetics depending upon different LAL preparations, or the inhibitory or enhancing effects of chemicals, were analyzed by observing the time-course data of turbidity change. As all raw data measured with this system were stored in a floppy disk, later reevaluation was easy and versatile. For example, by selecting variable threshold values for gelation detection, well correlated standard curves were obtained with some LAL preparations. Furthermore, new parameters related to the reaction kinetics such as reaction rate or degree of turbidity change could also be introduced to obtain new analytical information on the LAL–endotoxin reaction.

This newly developed analysis system of endotoxin should be very versatile for research purposes and also to improve the methodology of endotoxin detection using the turbidimetric kinetic assay. We are now developing applications of this system to estimate the concentrations of endotoxin and β-1,3-glucan.

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