Thermochimica Acta, 163 (1990) 71–80
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

APPLICATION OF CALORIMETRIC METHODS TO CELLULAR PROCESSES: WITH SPECIAL REFERENCES TO QUANTITATIVE EVALUATION OF DRUG ACTION ON LIVING CELLS

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SUMMARY

This paper is a review article of the research works conducted in the author's group and deals with a method of calorimetric characterization of the biological effects of drugs and other chemicals on metabolic activities of living cells. The effects of various drugs and chemicals on different microbial systems were quantitatively studied in a calorimeter and by using the results drug potency curves were successfully drawn for each drugs. The method was also applied to the study of microbial activity in soil and it was shown that the method can be a powerful technique for the quantitative characterization of pollutants in ecological systems.

INTRODUCTION

Much work has been made by various investigators to study quantitatively the biological activities of living cells and tissues by observing their metabolic heats(refs. 1-3). The method is mainly based on the fact that the heat effect is strictly proportional to the metabolic activity and in most cases a magnitude of calorimetric signal which is given by power unit (μwatt) is simply employed as an index to express the biological activities.

However, it seems that less attention has been paid to the time course of the calometric signals (the growth thermogram*1), although it reflects most directly the growth behavior of cells in culture vessel.

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*1 For this term, the notation "power-time-curve" is commonly used, in accordance with the recommendation by Interunion Commission on Biothermodynamics(*Pure & Applied Chemistry*, 54, 671-679(1982)). It is true that if one observes a heat evolution process in a calorimeter whose time constant is very short, the calorimeter output signal would be very close to the thermogenesis that is expressed in the unit of power(=watt). However, it should be pointed out that what one actually observes with any type of calorimeter is the temperature difference between the calorimetric vessel and its surroundings (or the reference vessel), and is never the "power dq/dt". The signal is proportional to the power only in particular case where the instrumental time constant is practically zero (the heat leakage modulus is infinite). This relation will be clear when one considers about the use of an isoperibol (Dewar vessel) calorimeter or an adiabatic calorimeter. Since with the adiabatic calorimeter the heat leakage modulus is practically zero, the heat evolution in the calorimetric vessel results in the

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It has been long my view that the biocalorimetry as an analytical tool is most effectively applied to the kinetic analysis of microbial cultures. Recently we have developed a high sensitive multiplex batch-calorimeter which is applicable to the detection of small heat effects due to cellular metabolism and applied to the analysis of growth behavior of microbial cells. Heat evolution processes were observed with microbial cultures grown on different culture media containing various antimicrobial drugs and the growth behaviors were analyzed in terms of growth kinetics to obtain the viable activity of the microbes and the effect of the drugs on them. This enabled one to determine parameters which characterize the drug action and to draw calorimetrically the drug potency curve.

The same principle was also adapted to a quantitative estimation of food putrefaction and of environmental pollution studies. In the former, an attempt has been made to determine quantitatively the action of food preservatives on actual foodstuffs and in the latter study a method to evaluate the effect of pollutants on microbial activity in soil has been proposed.

EXPERIMENTAL

Calorimeter

A multiplex batch calorimeter involving the conduction principle was constructed in this laboratory. The apparatus has 25 calorimetric units which are arranged in an aluminum heatsink with a dimension of 350 x 700 x 120 mm. The basic structure and its operation are essentially the same as those of our previous design with six-membered calorimetric units(ref. 4).

Method of Analysis

The heat evolution process $f(t)$ during incubation of microbial cells was obtained from the apparent calorimetric output signal $g(t)$ at an incubation time of t by using the relation(refs. 5,6):

$$f(t) = g(t) + K\int g(t)dt \qquad (1)$$

where K is the heat leakage modulus (the heat conduction constant) of the

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temperature rise whose extent is directly proportional to the amount of heat evolved. Thus the calorimetric signal (deg or K) gives the amount of heat evolved(J) when it is multiplied by the heat capacity(J/deg or J/K) of the system.

Strictly speaking, with calorimeters of existing design (except that working ideally in adiabatic principle) the signal (K) is converted to the power unit by the following procedure; the signal (K) is converted to "Joule unit(J)" after correcting for the heat leakage and being multiplied by the heat capacity and then differentiated with respect to time(s) to give the "power(J/s=watt)". In another word, a simple graphical integration of the calorimeter recording does never give the true thermokinetical curve and we believe that the use of the term "power-time-curve" may lead to erroneous conclusions. For this reason we disagree with the use of this term, but rather use "growth thermogram" or simply "calorimetric recording".

calorimetric unit which is determined on the basis of Newton's heat conduction law(refs.5,7).

$f(t)$ is a quantity which one would observe when the calorimetric unit were placed in a hypothetical adiabatic condition and corresponds to the true thermal process occurring in the calorimetric vessel.

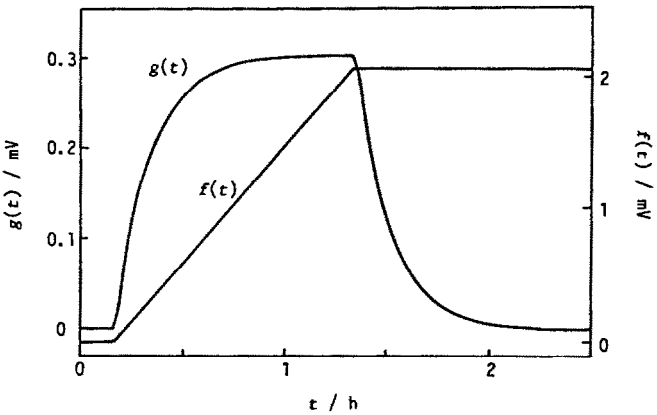

Fig. 1 Electrical calibration mark $g(t)$ and its conversion to $f(t)$ curve

The relationships between these two quantities $g(t)$ and $f(t)$ are shown in Fig. 1 for an electrical calibration experiment conducted on one of the calorimetric units and in Fig. 2 for a practical measurement on a fungi culture.

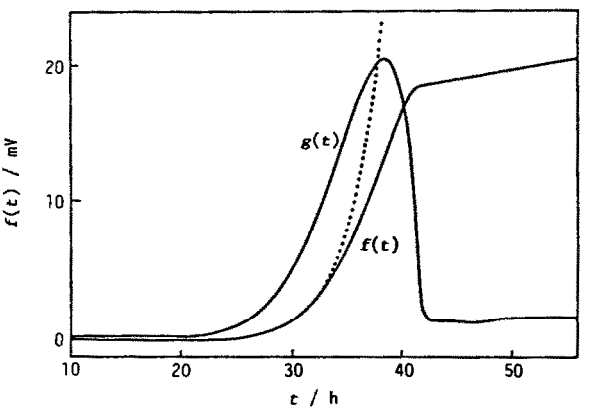

Fig. 2 Growth thermogram $g(t)$ observed for the growth of *Asp. oryzae* grown at 30°C on bran and its conversion to $f(t)$ curve. The dotted curve is the one fitted on eq. (2).

In the electrical calibration experiment, a constant electric power of 330.8 μW was sent on a heater mounted on the calorimetric unit. The calorimeter record $g(t)$ gave a typical calibration mark and the corresponding $f(t)$ curve was obtained by computation using eq. (1) as shown in Fig. 1. It can be seen that the $f(t)$ curve gives a straight line with a definite slope until the heat power is off. Thus the $f(t)$ curve determined from the calorimeter recording after correcting for the heat leakage well reflects the temperature change of a calorimetric unit which one would observe when it were placed in a hypothetical adiabatic condition. This fact obviously indicates that the computational procedure employed here on the basis of eq. (1) is a reasonable one and applicable to practical measurements.

In Fig. 2 a typical calorimetric recording $g(t)$ (the growth thermogram) observed for the growth of a fungi, *Asp. oryzae* grown on solid medium bran at 30 °C. In this measurement, the culture vessel was tightly sealed with a gastight cap and when all the oxygen present in the vessel was consumed, the signal rapidly returned to the baseline. The corresponding $f(t)$ curve which was ob-

tained by computation using eq. (1) is also included in the figure. The curve thus obtained gives a typical growth curve of microbes.

It is very interesting to see that even after the oxygen is consumed (near at 40 h incubation), $f(t)$ curve gradually goes up, indicating the involvement of a minute heat evolution without the oxygen consumption.

Determination of growth rate constant

We have previously reported that the heat evolution process during the exponential growth phase of microbes is given by a simple exponential function;

$$f(t) = AN_0\exp(\mu t) + BN_0 \qquad (2)$$

where μ is the growth rate constant, N_0 is the total number of viable cells at the start of incubation (the inoculum size) and A and B are the constants(refs. 5,6).

Using this relation a regression analysis was made on the $f(t)$ curve and the growth rate constant, μ, was determined as a best-fit value. The dotted line in Fig. 2 is the curve fitted by the regression analysis and in this particular case the growth rate constant was obtained to be

$$\mu = 5.32 \times 10^{-3}\ \mathrm{min}^{-1} \qquad (3)$$

Here, it should be noted that the curve fitting was made just for a limited incubation period, since the growth deviates from the exponential phase as the oxygen concentration becomes low.

EXPERIMENTAL RESULTS AND DRUG POTENCY CURVES DRAWN

Fig. 3(a) shows growth thermograms observed for the growth of *Escherichia coli* grown at 30 ℃ on bouillon medium containing various amounts of antibiotics novobiocin (ref.8). The result indicates that with increasing the concentration of novobiocin in the medium , the growth thermogram shifts toward a longer incubation period with a slight decrease in the steepness of their initial slopes. Such the effect of the drug becomes more clear when the observed growth thermogramsare converted to $f(t)$ curves.

The corresponding $f(t)$ curves obtained by computation using eq. (1) are shown in Fig.3(b). Obviously there is a good correlation between the change in growth curves and the novobiocin concentrations.

A similar result was also obtained with the growing culture of *Asp. oryzae* grown on bran containing different amounts of *p*-hydroxybenzoic acid (PHBA) and the results are shown in Figs. 4(a) and 4(b) (ref. 9).

In order to characterize this feature induced by the drug action more quanti-

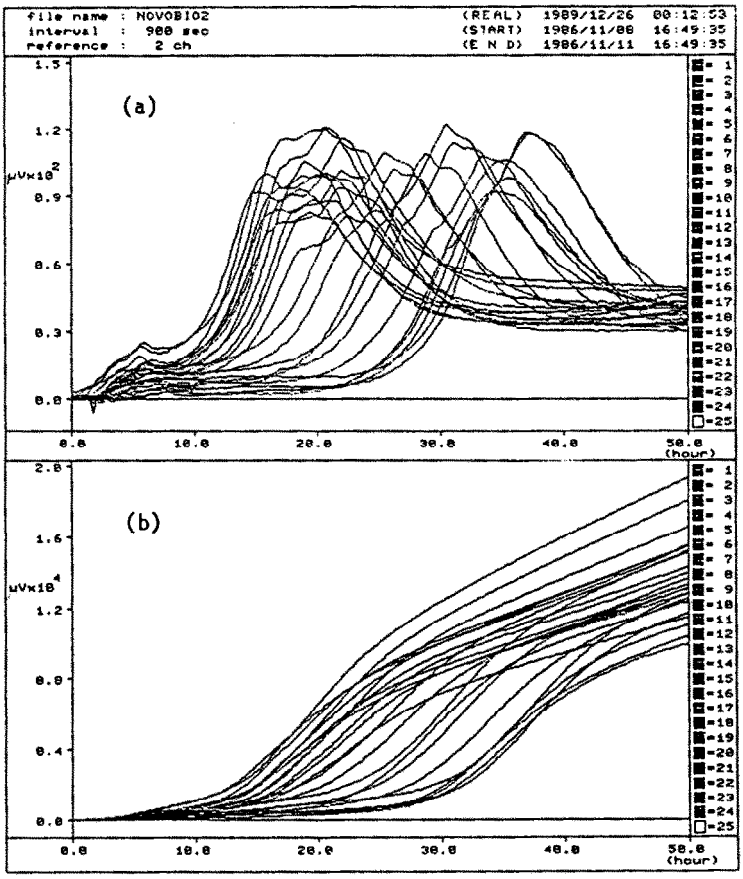

Fig. 3 (a) Growth thermograms of *E. coli* grown at 30°C on bouillon medium containing novobiocin at various concentrations and (b) the corresponding $f(t)$ curves computed according to the method described in the text.

tatively, we have proposed a method of analysis based on the following scheme (ref.10);

$$\begin{aligned} V + nS &\rightleftharpoons VS_n \rightharpoonup 2V + P \\ V + mI &\rightleftharpoons VI_m \\ VS_n + mI &\rightleftharpoons VS_nI_m \end{aligned} \qquad (4)$$

where a viable microbial cell V takes up n moles of nutrient (substrate) S to form an intermediate VS_n which produces a new viable cell together with the possible formation of metabolic by-product P. The drug I inhibits the viable activity of the cells by forming nonviable states VI_m and VS_nI_m where m is the mean number of molecules of the drug needed to inhibit the reproduction of new cells.

If we denote the substrate constant and the dissociation constant of the drug as $K_S=[V][S]^n/[VS_n]$ and $K_d=[V][I]^m/[VI_m]=[VS_n][I]^m/[VS_nI_m]$, respectively, then we have the growth rate equation;

$$\mu_i = \mu_m/(1+K_S/s^n)(1+i^m/K_d) \qquad (5)$$

where μ_i is the growth rate constant found in the presence of a drug at concentration $i(=[I])$ and μ_m is the maximum growth rate constant in the absence of the drug and in the presence of excess nutrient.

Since in actual measuremnts, the following relationship holds, to a good approximation,

$$K_S \ll s^n \qquad (6)$$

eq.(5) is reduced to eq.(7);

$$\mu_i = \mu_m/(1+i^m/K_d) \qquad (7)$$

The values of K_d and m are determinable by a regression analysis on the basis of eq.(7) and moreover a drug potency curve will obtained by plotting the specific growth activity μ_i/μ_m against the drug concentration i.

The drug potency curve obtained by the above procedure for the action of PHBA on the growth of *Asp. oryzae* grown on bran at 30 ℃ is shown in Fig.5.

Measurements were also made on the drug actions on the growth of *Asp. oryzae* grown at 30 ℃ on bran containing various food preservatives as test drugs and the drug

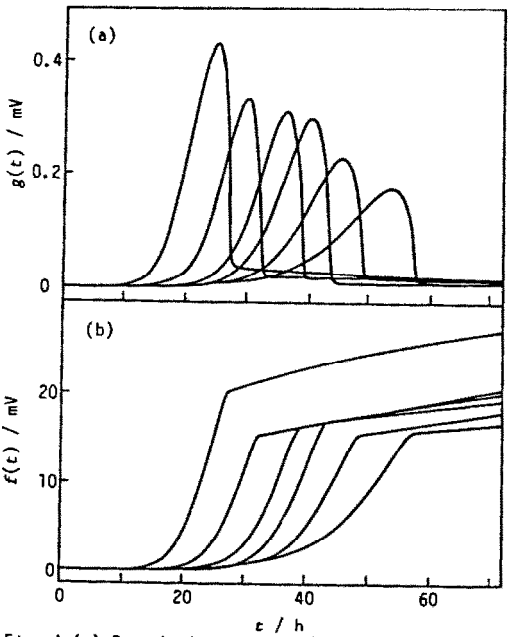

Fig. 4 (a) Growth thermograms of *Asp. oryzae* grown at 30°C on bran containing *p*-hydroxy benzoic acid at various concentrations and (b) the corresponding $f(t)$ curves.

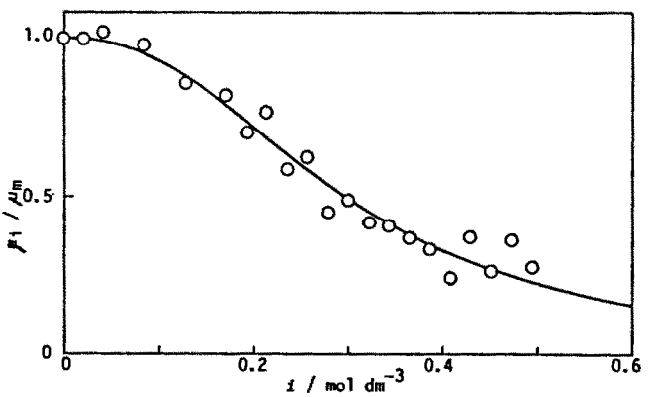

Fig. 5 Drug potency curve drawn for the actions of *p*-hydroxy benzoic acid on *Asp. oryzae* growth. The open circles denote the experimental data. The curve was drawn by regression analysis on the basis of eq.(7).

potency curves were calorimetrically drawn and are shown in Fig. 6. In the plots, the drug concentrations are given in the logarithmic unit so that they cover a wide concentration range.

As understood from eq.(7), the values of $K_i = \sqrt[m]{K_d}$ corresponds to the drug concentration at which the growth activity is repressed by 50 %, while the value of m reflects the cooperative nature of the drug actions. Thus the two parameters can be an index of the effective and dynamic concentration range in the drug action. These parameters determined by the present calorimetric method are summarized in Table I (ref.9).

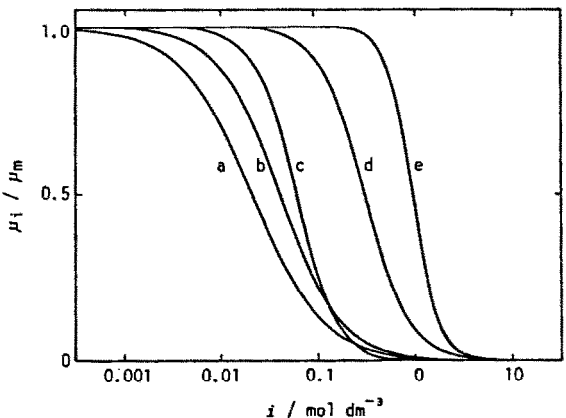

Fig. 6 Drug potency curves for the action of various food preservatives on *Asp. oryzae* drawn calorimetrically. Drugs are; (a)potassium sorbate, (b)calcium propionate, (c)sodium benzoate, (d)p-hydroxy benzoic acid and (e)ethyl alcohol.

In Fig. 7, the drug potency curves obtained calorimetrically for the action of three antibiotics, streptomycin, tetracycline and chloramphenicol, on *E. coli* growth are shown. The corresponding parameters m, K_d and K_i are also summarized in Table I (ref.10).

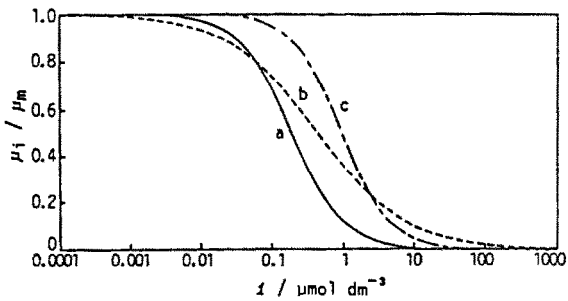

Fig. 7 Drug potency curves for the action of (a)streptomycine, (b)tetracycline and (c)chloramphenicol on *E. coli* growth drawn calorimetrically.

It turned out that the change of growth thermograms in the presence of certain drugs can not be explained quantitatively by the noncompetitive inhibition model which is defined by scheme (4). These drugs are penicillin G, amipicillin and polymyxin B (ref.11) which are known to inhibit the biosynthesis of bacterial cell walls. With these three antibiotics it was found that the growth thermograms are affected in such a manner that they shift toward a longer incubation period, while the growth rate constant remains unchanged, even when the drugs are present in the incubation medium.

This characteristic feature may be explained by the drug action that the effective inoculum size is lowered due to their specific binding to the cell to inhibit the cell division. On the basis of a scheme that the decrease in the

effective inoculum size is proportional to the m_N-th power of drug concentration, the observed changes in growth thermograms were analyzed to obtain the drug potency curve. The results are shown in Fig. 8 (ref.11).

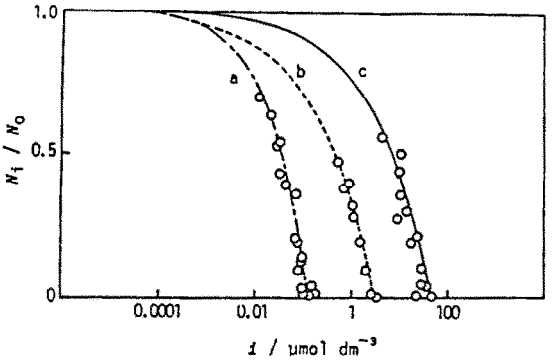

Fig. 8 Drug potency curves for the action of (a)penicillin G, (b)ampicillin and (c)polymyxin B on *E. coli* growth drawn calorimetrically.

TABLE I Inhibitory parameters of some drugs on the growth activity of microbes as measured by the calorimetric method

Drugs	Microbes	m	K_d	K_i
		molecule cell^{-1}	(mol dm^{-3})^m	mol dm^{-3}
ethyl alcohol	*Asp. oryzae*	3.3	0.815	0.94
p-hydroxybenzoate	*Asp. oryzae*	1.9	0.102	0.300
sodium benzoate	*Asp. oryzae*	2.1	0.00278	0.0607
calcium propionate	*Asp. oryzae*	1.4	0.0113	0.0408
potassium sorbate	*Asp. oryzae*	1.2	0.00964	0.0209
chloramphenicol	*E. coli*	1.3	1.46×10^{-8}	$0.94 \times 10^{6-}$
tetracycline	*E. coli*	0.7	3.49×10^{-5}	0.43×10^{-6}
streptomycin	*E. coli*	1.2	8.69×10^{-9}	0.19×10^{-6}

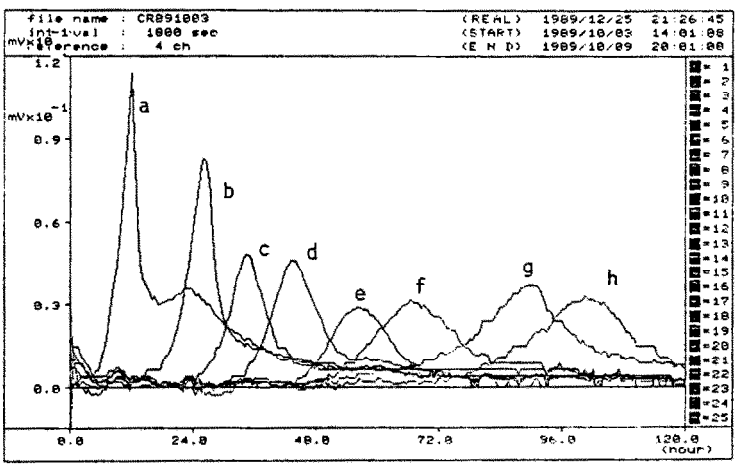

Fig. 9 Microbial degradation thermograms of glucose in soil containing various amounts of chromium at 30°C. Chromium concentrations / ppm are; (a) 0, (b) 25, (c) 50, (d) 75, (e) 100, (f) 125, (g) 150 and (h) 200.

Fig. 9 shows the results of experiments conducted for microbial degradation of glucose in soil containing various amounts of chromium as a model compound of pollutant (ref.12).

The specific degradation rate of glucose was determined in the same order of the above procedure and plotted against the chromium concentration with the results shown in Fig. 10.

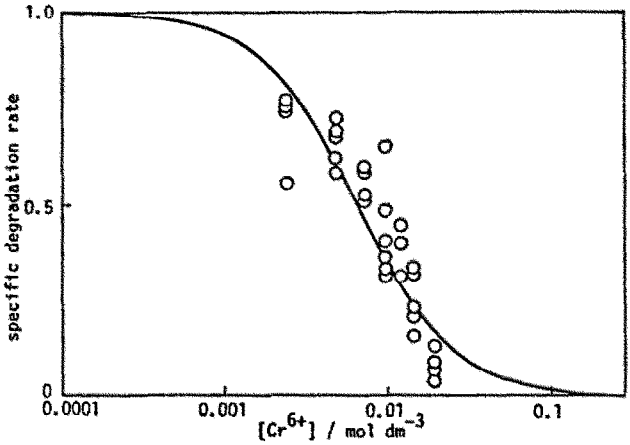

Fig. 10 The effect of chromium concentration on microbial degradation of glucose in soil at 30°C.

It will be clear that the present calorimetric method is useful for the quantitative characterization of the pollution study on ecological systems.

Although the actual biochemical processes in living cells are more complicated than what we assumed here and the parameters defined above have limited biological significance in themselves, the drug potency curves drawn are the ones which are obtained by fitting the experimental data and therefore, we believe, that they are fully reliable and that the method given here will be a powerful technique to characterize quantitatively the biological effects of various drugs and foreign substances on living cells.

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