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Estimation of Postmortem Interval Using Kinetic Analysis of the Third Component of Complement (C3) Cleavage

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ABSTRACT: To estimate postmortem interval (PMI), spontaneous cleavage of the third component of complement (C3) was studied in aged blood and cadaveric blood by crossed immunoelectrophoresis. Using the kinetics of C3 cleavage in vitro described as $dC/dt = -kC$, where C is the concentration of native C3 at time t and k is a first-order rate constant, Arrhenius' equation, and another equation which assumes a linear drop of body temperature after death, the percentages of C3 cleavage were calculated. There was a significant positive correlation between the calculated percentages and the measured percentages of up to 10% in cadaveric blood. We found that the comparison between the calculated percentage of C3 cleavage for each optional postmortem interval and the measured percentage of up to 10% in cadaveric blood leads to the estimation of PMI. This approach is one step towards the development of an accurate method for determining PMI based on C3 cleavage, that is, on a first-order reaction.

KEYWORDS: pathology and biology, postmortem interval, blood, electrophoresis, third component of complement (C3)

To estimate the postmortem interval (PMI), various objective methods such as the analysis of the level of enzyme activities and organic and inorganic constituents of almost all body fluids have been used over the past 50 years [*1-4*]. Of these various objective means, postmortem accumulation of potassium (K^+) in vitreous humor is a widely used gauge for assessing PMI. A number of studies on the relationship between PMI and vitreous humor K^+ established that K^+ accumulates with increasing PMI, but the accuracy is quite variable. Some investigators concluded that the standard error of the estimate of time of death was 4.7 h [*5*], while others reported that there was such individual variation that the confidence limits of this method exceeded ± 10 h [*6*]. One of the problems in using the various objective methods, including that of vitreous humor K^+, is the presence of individual variations in

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antemortem values. Therefore, only substances that are not influenced by individual antemortem values are suitable for the estimation of PMI.

One possible substance that can be used to estimate PMI is the third component of complement (C3). It is known that C3 is spontaneously cleaved into several fragments in nonactivated plasma, which cannot be detected in circulation, thus the ratio of C3-derived fragments among individuals remains constant. C3 is composed of two disulfide-linked polypeptide chains, an alpha chain of Mr 120 000 and a beta chain of Mr 75 000 [7]. Immunoelectrophoretic analysis showed only one precipitin line in fresh serum, named beta 1C (native C3), and two additional major precipitin lines in aged normal serum, named beta 1A and alpha 2D [8]. Recently it was shown that the intramolecular thioester bond of C3 is altered by water hydrolysis, which changes it to C3(H_2O) without hemolytic activity [9]. This is followed by two proteolytic steps: the first cleavage is from C3(H_2O) to iC3(H_2O) with factor I and factor H [10], and the second slow cleavage is from iC3(H_2O) to C3c + C3d,g with other proteases such as plasmin [11,12]. In vivo, the modified C3 is removed from circulation. Therefore, C3-derived fragments cannot be detected in circulation with the exception of patients with complement activation such as acute glomerular nephritis [13], systemic lupus erythematosus, and sepsis.

In a previous study [14], we showed using crossed immunoelectrophoresis (CIE) that in cadaveric blood the percentage of C3 derived fragments in relation to native C3 increased with time. These strongly suggest that the rate of water hydrolysis determines the rate of fragmentation of native C3 to C3-derived fragments, since the rates of proteolytic cleavages are, as a rule, much faster than that of water hydrolysis. Therefore, the kinetics of C3 cleavage may be described as $dC/dt = -kt$, where C is the concentration of native C3 at time t and k is a rate constant. In this paper we show that the percentage of C3 cleavage in cadaveric blood can be calculated by using the above equation, Arrhenius' equation, and another equation, according to the rule of thumb method [15], which assumes that the drop of the body temperature is linear up to the time when the blood samples are taken. We then show that PMI can be estimated by comparing the percentage of C3 cleavage which is measured in cadaveric blood by CIE with that which is calculated using the three equations.

Materials and Methods

Blood

The ratio of C3 cleavage was measured in vitro and in cadaveric blood. Blood samples drawn from 20 healthy people were incubated at 37, 30, 23, and 14°C. Serum from each sample was removed at various times; every 12 h at 37 and 30°C until 72 h, every 24 h at 23°C until five days, and every 48 h at 14°C until ten days. These were immediately stored at −80°C, thawed within two weeks, and analyzed using crossed immunoelectrophoresis (CIE).

Cadaveric blood was obtained from autopsy cases in which the time of death had been determined from eyewitness accounts, including bystanders or attending physicians. In addition, the rectal temperature was simultaneously recorded. Blood samples were drawn from the hearts or the thoracic cavities, stored at 0°C within 3 h, centrifuged at 3000 *g* for 30 min to remove sera, and stored at −80°C. The blood sample storage and the freeze-thaw do not have any significant effect on C3 cleavage.

Antiserum to Beta 1A

Antiserum to beta 1A, which precipitated beta 1C as well as beta 1A, was kindly provided by Professor N. Tamura of the Department of Immunology, Institute of Basic Medical Sciences, University of Tsukuba.

Crossed Immunoelectrophoresis (CIE)

The method described by Weeke [16] was used to examine beta 1A and beta 1C qualitatively and quantitatively and modified by using 50-mm gelbond film for agarose gels (LKB, USA). First electophoresis was carried out from the right (cathode) to the left (anode) at 5 V/cm for 2 h at 15°C and then electrophoresed into antiserum-charged agarose from bottom to top at 2 V/cm for 15 h at 15°C. After electrophoresis, the gel was pressed, dried, and stained in 0.25% Coomassie brilliant blue. For quantitative analysis, tracings of two loops of precipitate were made from twofold enlarged copies of the immunoelectrogram, and then the areas under them were measured by tablet digitizer in an HP 1000 system.

Calculation of Data

Since the water hydrolysis of the intramolecular thioester bond in C3 may be the rate-determining step in the conversion of beta 1C to beta 1A, and furthermore since it may be dependent upon the first-order reaction, the conversion rate of beta 1C to beta 1A is assumed to be proportional to the concentration of beta 1C. Thus, the conversion rate at a given time can be represented by the equation:

$$dC/dt = -kC \quad (1)$$

where C is the concentration of beta 1C at time t and k is the rate constant.

The solution of Eq 1 at the initial condition, $C(t_0) = C_0$, is

$$C = C_0 \times \mathrm{EXP}(-kt) \quad (2)$$

and then the concentration of beta 1A, A, is represented by the equation:

$$A = C_0 - C \quad (3)$$

From Eqs 2 and 3, the following equation is obtained:

$$x = A/(A + C) = 1 - \mathrm{EXP}(-kt) \quad (4)$$

The values of x, the ratio of C3 cleavage, were determined by measuring the areas under beta 1C and beta 1A and by calculation using Eq 4.

Equation 4 can be converted to the following equation:

$$-\log(1 - x) = kt \quad (5)$$

The value of the rate constant (k) was determined by calculating the slope of the regression line in a plot of the negative logarithm of the ratio of beta 1C, $-\log(1 - x)$ versus time t.

The rate constant (k) is dependent on temperature, as represented by the equation (Arrhenius' equation):

$$\log k = -E/RT + a \quad (6)$$

where T is the absolute temperature, E is the activation energy, R is the gas constant, and a is the residual constant. The values of the ratio activation energy: gas constant (E/R) and of the residual constant (a) were determined by calculating the slope and y intersection of the regression line in a plot of the logarithm of the rate constant, log k, versus the inverse temperature.

Results

Spontaneous Cleavage of C3

To determine the kinetics of the cleavage, whole blood was incubated for various lengths of time at 37°C, and the ratios of beta 1C (native C3) and beta 1A (C3-derived fragments) were monitored simultaneously as a function of time by crossed immunoelectrophoresis (CIE) analysis. Slow cleavage of C3 was observed upon incubation at 37°C, as indicated by a decrease of beta 1C and an increase of beta 1A (Fig. 1 top). A semilog plot of the kinetic data from Fig. 1 (top) is shown in Fig. 1 (bottom). The linear relationship between −log (ratio of beta 1C) and the elapsed time at 37°C was consistent with a first-order reaction. This result suggests that the water hydrolysis of the intramolecular thioester bond in C3 determines the rate of C3 fragmentation, since water hydrolysis depends on the first-order reaction, and the rate of water hydrolysis may be slower than those of proteolytic cleavages in C3 fragmentation. A first-order rate constant of 0.515%/hour was calculated for C3 at 37°C.

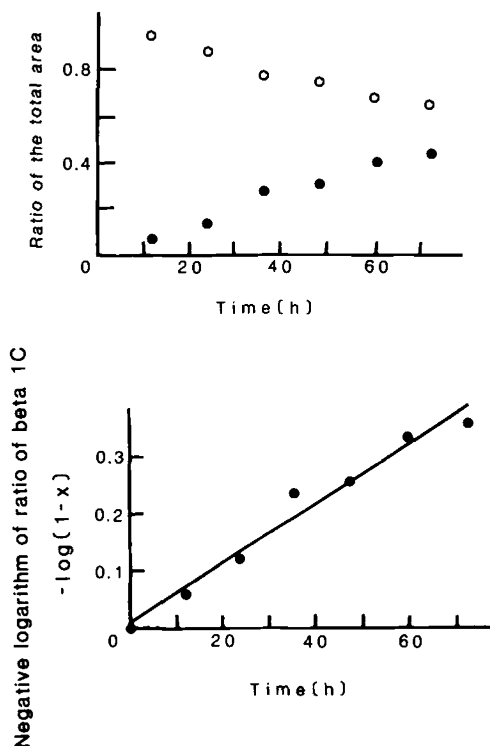

FIG. 1—*Spontaneous cleavage of C3 at 37°C. Whole blood was incubated at 37°C for 0, 12, 24, 36, 48, 60, or 72 h. Sera were taken and stored at −80°C. C3 cleavage was measured as a function of time by CIE analysis. The areas under the loops corresponding to beta 1C* (open circle) *or beta 1A* (closed circle) *in the immunoelectrogram are expressed as a ratio of the total area, shown in the top fig., though the ratio of beta 1A corresponds to the ratio of C3 cleaved* (x), *which is calculated by using the relationship: ratio of C3 cleaved* (x) = *(area under beta 1A)/(area under beta 1A and beta 1C). Therefore, a ratio of beta 1C represents 1* − x. *In the bottom fig. the results shown in the top fig. were used to calculate the negative logarithm of ratio of beta 1C by using the relationship: negative logarithm of ratio of beta 1C* = *−log(1* − x*). In the bottom fig. regression analysis of the points creates a line with the equation:* $-\log(1 - x) = 0.00864 + 0.515 \times t/100$. *The correlation coefficient is 0.988.*

Spontaneous C3 Cleavage: Temperature Dependence

The kinetics of spontaneous cleavage of C3 in whole blood were measured at temperatures of 14, 23, 30, and 37°C (Fig. 2). The spontaneous cleavage of C3 at each temperature follows first-order kinetics. The first-order rate constants calculated at 37, 30, 23, and 14 were 0.515, 0.384, 0.186, and 0.079%/h, respectively. From the rate constants, an Arrhenius plot was constructed (Fig. 3), and a ratio of activation energy: gas constant (E/R) of 7790K and a residual constant of 19.7/h here calculated. Whole blood drawn from another 19 healthy people was analysed as described above. The averages for the first-order rate constant (k), the ratio of activation energy: gas constant (E/R), and the residual constant (a) are given in Table 1. Although there were individual variations among the values, the rate constants (k) at the same temperature were within a relatively narrow range. Comparison among the rate constants (k) at different temperatures shows that incubation at a higher temperature results in a higher rate constant of C3 cleavage. The average rate constant (k) at 37°C was about five times higher than that at 14°C. In the calculation below, the averages of the ratio of activation energy: gas constant (E/R) and the residual constant were used.

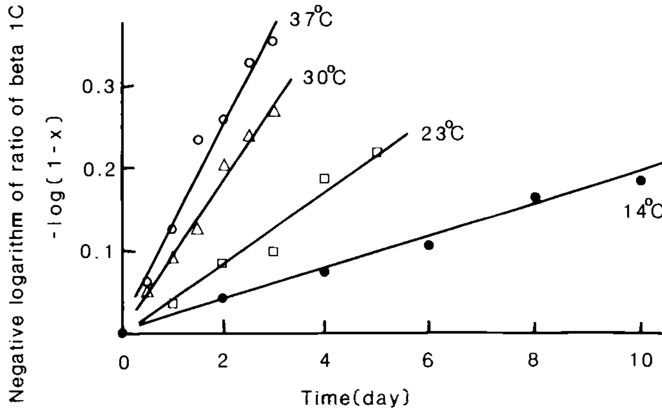

FIG. 2—*Spontaneous C3 cleavage: temperature dependence. The kinetics of spontaneous cleavage of C3 were measured as a function of temperature. Rate constants were calculated for each temperature.*

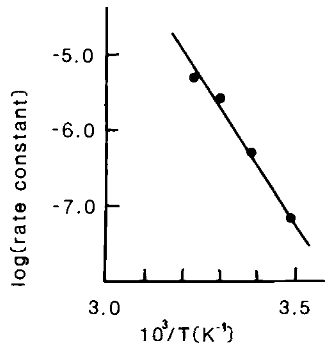

FIG. 3—*Spontaneous C3 cleavage: temperature dependence, An Arrhenius plot is depicted. The ratio of the activation energy: gas constant* (E/R) *and the residual constant* (a) *were calculated from the slope and* y *intersection of this line.*

TABLE 1—*The averages*[a] *of first-order rate constant* (k) *at each temperature, a ratio of an activation energy: gas constant* (E/R), *and a residual constant* (a).

	Rate Constant, %/h					
	37°C	30°C	23°C	14°C	*E/R*, *K*	*a*, /h
Average	0.560	0.334	0.215	0.103	$6.69*10^3$	16.4
Standard deviation	0.171	0.074	0.068	0.026	$1.10*10^3$	3.65

[a]Whole blood drawn from 20 healthy people was analyzed as described in the text.

C3 Cleavage in Cadaverous Blood

The percentages of C3 conversion (x) in cadaveric blood were measured by calculating the proportion of the area under beta 1A to the total area. All data, including the cause of death, rectal temperature [$T(0)$], PMI (time between death and storage of blood sample at 0°C), and the measured percentage of C3 cleavage (x) in cadaveric blood are given in Table 2. Figure 4 shows a positive correlation ($r = 0.724$) between the postmortem intervals (PMIs) and percentages of C3 cleavage. The 95% confidence [*17*] of predicting PMI from an experimentally determined percentage of C3 conversion is ± 9.2 h for all data. The cause of death did not influence the relationship between PMI and the ratios of C3 cleavage.

Calculated Percentage of C3 Cleavage in Cadaverous Blood

The manner in which the body temperature falls after death has an effect on C3 cleavage in cadaverous blood, because the rate constant (k) of C3 cleavage depends on temperature. Although this is unknown in almost all cases, it may be assumed that the drop of the body temperature is linear up to the time when the blood samples are drawn and stored at 0°C. This may lead to the determination of the value of the body temperature at a given time, followed by the determination of the value of the rate constant (k). Then the body temperature (absolute temperature) at a given time can be represented by the equation:

$$T(t) = (T(0) - 37.6)*t/I + 310.6 \qquad (7)$$

where $T(t)$ is the body temperature at time t, $T(0)$ is the body temperature when the cadaverous blood was drawn, and I is the postmortem interval. From Eqs 1 and 6 as described in the Materials and Methods section and Eq 7, the percentages of C3 cleavage were calculated using the ratio of an activation energy: gas constant (E/R) of 6690K and a residual constant of 16.4/h (Table 2). The data in Table 2 were plotted as calculated percentage versus measured percentage in cadaverous blood (Fig. 5). As can be seen in Fig. 5, the calculated percentages increased in proportion with the measured percentages up to a C3 cleavage of 10% and reached a plateau thereafter. Therefore, Eq 1 may be valid within C3 cleavage of 10% in cadaverous blood, and it is possible to calculate postmortem intervals from measured percentages below 10%.

Calculated Postmortem Interval (Calculated PMI)

Postmortem intervals were estimated using rectal temperatures [$T(0)$] and percentages of C3 cleavage below 10% measured in cadaverous blood. From Eqs 1, 6, and 7 the percentage of C3 cleavage that corresponds to each optional value of interval (I) was calculated using a ratio of an activation energy: gas constant of 6690K and a residual constant of 16.4/h. Each

TABLE 2—*Age, cause of death,[a] postmortem interval (PMI), rectal temperature (RT), measured percentage of C3 cleavage, calculated percentage,[b] and calculated PMI.[b]*

No.	Age, years	COD	RT, °C	PMI, h	Percentage of C3 Cleaved	Calculated Percentage	Calculated PMI, h
1	66	OHD	31	4	2.4	1.86	5
2	86	OHD	33	4	5.1	1.99	10
3	73	OHD	23	21	8.2	7.36	23
4	50	DA	33	10	5.9	4.89	12
5	47	asthma	31	14	6.8	6.36	15
6	53	OHD	29	17	9.8	7.20	23
7	79	Br	31	19	9.7	8.53	22
8	42	un	32	12	6.7	5.65	14
9	55	OHD	32	12	8.5	5.65	18
10	14	un	32	4	3.3	1.92	7
11	62	SAH	21	24	9.3	7.90	28
12	83	DA	30	16	7.3	7.00	17
13	42	OHD	32	7	7.5	3.34	16
14	75	SAH	28	21	9.3	8.55	23
15	55	asphyxia	26	15	6.9	5.44	18
16	48	gunshot	28	11	7.4	4.57	18
17	43	OHD	34	12	7.1	6.03	14
18	49	OHD	33	8	6.2	3.93	13
19	44	drowned	28	15	7.1	6.19	17
20	43	un	28	8	6.0	3.35	15
21	1	brain contusion	18	24	5.5	7.26	18
22	65	ICH	29	19	7.5	8.01	18
23	48	SAH	20	14	5.3	4.55	16
24	55	OHD	20	22	7.2	7.06	22
25	77	un	20	18	6.6	5.82	21
26	79	OHD	34	20	10.6	9.85	...
27	54	bleeding	22	22	10.7	7.48	...
28	70	pneumonia	28	23	13.3	9.33	...
29	76	OHD	23	16	10.5	5.66	...
30	50	un	23	21	13.0	7.36	...
31	70	OHD	26	19	10.7	7.31	...
32	62	OHD	26	21	15.6	8.05	...
33	63	drowned	30	15	10.1	6.58	...
34	86	OHD	26	22	16.3	8.41	...
35	65	asthma	24	23	10.8	8.27	...
36	43	bleeding	16	25	12.3	7.15	...
37	39	un	26	27	15.7	10.2	...

[a]Cause of death abbreviations: OHD = organic heart disease, Br = bronchitis, DA = dissecting aneurysm, Tx = transection, SAH = subarachnoideal hemorrhage, and un = unknown cause.

[b]Calculated PMI and calculated percentage were determined using a ratio of activation energy : gas constant *(E/R)* of 6690K and a residual constant of 16.4/h.

measured percentage of C3 cleavage in cadaveric blood was compared to the calculated percentages. From the closest corresponding calculated percentage, the interval I was then determined, which in turn enabled the calculation of PMI (Table 2). The calculated postmortem interval is positively correlated ($r = 0.872$) with the actual postmortem interval (Fig. 6). The 95% confidence of predicting PMI from experimentally calculated PMI was ± 9.1 h.

Discussion

The hypothesis that the cleavage rate of native C3 to its derived fragments is determined by water hydrolysis accords well with the experimental data obtained from the incubation of

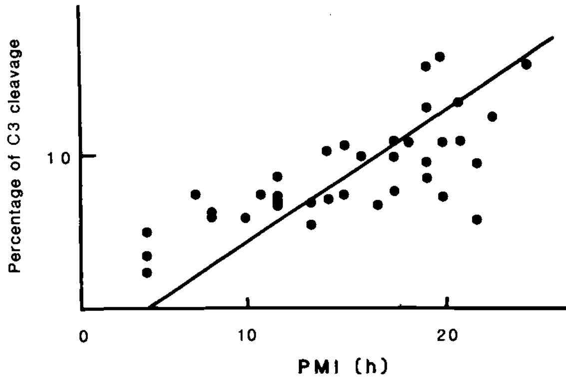

FIG. 4—*Percentage of C3 cleavage* (x) *in cadaveric blood. The measured percentage of C3 cleavage* (x) *in cadaveric blood was plotted against the respective PMI (hours). Regression analysis of the points creates a line with the equation: PMI (hours) = 4.21 + 1.39* x. *The correlation coefficient is* r = *0.721.*

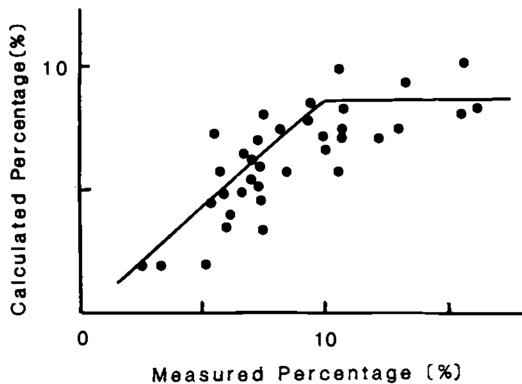

FIG. 5—*The relationship between measured percentages and calculated percentages of C3 cleavage. The data from Table 2 were plotted as calculated percentage versus measured percentage of C3 cleaved in cadaveric blood.*

whole blood. Thus, the kinetics of C3 cleavage may be described as $dC/dt = -kC$, where C is the concentration of native C3 at time t and k is a rate constant. The rate constant (k) is the conversion rate of C3 to its derived fragments which have no hemolytic activity. We show here that the average of the rate constant (k) at 37°C is 0.56%/h, which is in agreement with the 0.5%/h at 37°C determined in an earlier study on a loss of C3 hemolytic activity [*9*].

Several studies have been performed to relate C3 cleavage with earlier immunoelectrophoretic analyses. Beta 1A has been identified with C3c for a long time [*18*]. Nagasawa et al. have shown that C3b, iC3b, and C3c migrate similarly on immunoelectrophoresis [*11*], and Lachman et al. recently reported that the C3 product known as beta 1A is iC3b [*19*]. Considering that the conversion rate of beta 1C to beta 1A is proportional to the concentration of beta 1C, beta 1C should consist of only native C3, and therefore, beta 1A should consist of C3-derived fragments, such as iC3(H_2O) and C3c.

Moreover, it is shown that a comparison between the calculated percentage of C3 cleavage that corresponds to each optional postmortem interval at a recorded rectal temperature

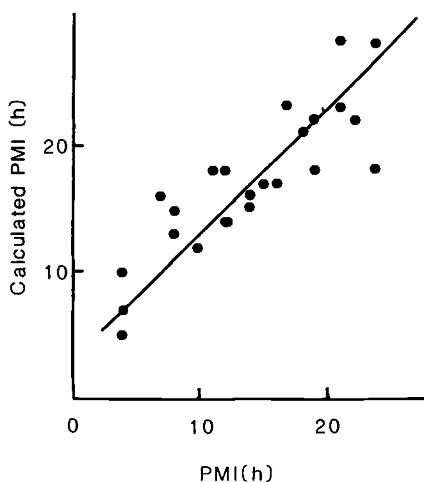

FIG. 6—*The comparison between actual PMI and calculated PMI (cPMI). The calculated PMI (hours) are plotted against their respective actual PMI (hours). Regression analysis of the points creates a line with the equation: PMI (hours) = 1.01*cPMI − 3.07. The correlation coefficient is* r = *0.866.*

and the measured percentage in cadaveric blood leads to the estimation of PMI. A 95% confidence of predicting experimentally calculated PMI based on the ratio of activation energy:gas constant (E/R) and the residual constant determined in vitro was ± 9.1 h.

Because body temperature drops after death, it is expected that the rate of C3 cleavage decreases with increasing PMI, and eventually the ratio of C3 conversion reaches a plateau. However, the ratio of C3 conversion in cadaveric blood rose for a longer time than predicted, and the calculated percentages increased in proportion with the measured percentages up to 10% and then reached a plateau. These findings suggest that in cadaveric blood C3 cleavage is accelerated by additional factors such as proteases, which include trypsin [*20*], plasmin [*21*], or neutrophil elastase [*22*], and the growth of bacteria in the heart. In sudden death, plasminogen activator is released from the vascular wall [*23*], so that C3 cleavage may be accelerated, and the acceleration may depend much more on the lethal process than the cause of death.

In practice, different bodies cool at greatly differing rates; but the general manner of cooling is the same for all bodies [*15*]. There is an initial lag period, then a period in which the drop is fairly linear, and finally a period for which the drop is nearly exponential. The relative length of these different periods varies from case to case, and they are not represented in Eq 7. In addition, this equation is not suitable in cases with antemortem hyperthermia. Therefore, many factors, such as proteases or bacteria in cadaveric blood, and the difference between actual manner of cooling of the body and the manner represented in the equation affect the difference between actual PMI and calculated PMI.

C3 cleavage in nonactivated plasma is affected by proteases or bacteria or both, particularly in the conversion of C3 to C3a and C3b. When such factors are introduced into blood, C3 cleavage may occur in two ways, so that the activation energy which is calculated based on CIE using antiserum to beta 1A appears to be less than the actual value.

From the results in this study, it is probable that the method for estimating PMI based on a first-order reaction can be useful in estimating the time of death. The lower activation energy of a reaction, the less effect the temperature has on the first-order rate constants or on the rate of the reaction. Thus, substances with lower activation energy seem more suitable for the estimation of PMI.

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