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Chorioallantoic membrane assays have been based on diffusion control—Problems arising with a diversity of mass transfers in egg white

Chiu-Lan Hsieh[a,1], Chiung-Chi Peng[b,1], Li-Yun Lin[a,1], Yu-Ting Cheng[c], Kuan-Chou Chen[d,*], Robert Y. Peng[c,e,**]

[a] Department of Food and Nutrition, Hungkuang University, 34, Chung-Chi Rd., Shalu County, Taichung Hsien 43302, Taiwan
[b] Graduate Institute of Rehabilitation Science, College of Health Care, China Medical University, 91 Hsueh-Shih Rd., Taichung 40202, Taiwan
[c] Research Institute of Biotechnology, Hungkuang University, 34, Chung-Chi Rd., Shalu County, Taichung Hsien 43302, Taiwan
[d] Department of Urology, Taipei Medical University Shuang Ho Hospital, Taipei Medical University, 250, Wu-Xin St., Xin-Yi District, Taipei 110, Taiwan
[e] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Wu-Xin St., Xin-Yi District, Taipei 110, Taiwan

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ABSTRACT

The chorioallantoic membrane (CAM) assays have been intensively used to determine angiogenesis and anti-angiogenesis of medicines. In view of bioactivity, this technique should be performed with kinetic control regime in chicken embryos. Whether the dosages ever used had satisfied this requirement, we explored by mathematical analysis. A diffusion-in-egg model was established to describe several medicinal diffusions in egg white that involved the instantaneous transient kinetic behavior, the diffusion of medicines in capping volume (the volume from the air sac to the interface of egg yolk). By reviewing the diffusion of various compounds including the cited and the experimentals in this work, we conclude that all the CAM assays ever cited were performed under diffusion control regime rather than kinetic control, which may bring forth deviations caused by a diversity of constitutes in egg white through various medicine–protein interactions.

1. Introduction

Deficiencies in oxygenation are widespread in solid tumors. The transcription factor hypoxia-inducible factor (HIF)-1α is an important mediator of the hypoxic response of tumor cells and controls the up-regulation of a number of factors important for solid tumor expansion, including the angiogenic factor, vascular endothelial growth factor (VEGF) [1].

Over the last 15 years, considerable progress has been made in the development of therapies based on targeting tumor angiogenesis [2,3]. However, although the induction of the hypoxia inducible factor 1α (HIF-1α) had been confirmed to be a positive factor for solid tumorigenesis, evidences indicate that it is not absolutely related to its regulation of VEGF expression [1]. In a study of two cell lines nullizygous for HIF-1α, one from embryos genetically null for HIF-1α, and the other from embryos carrying loxP-flanked alleles of the gene, which allows for pre-mediated excision, Ryan et al. [1] showed that the loss of HIF-1α negatively affects tumor growth in these two sets of H-*ras*-transformed cell lines, and this negative effect is not due to deficient vascularization. Despite differences in VEGF expression, vascular density is similar in wild-type and HIF-1α-null tumors.

Up to present, a huge number of documents had performed anti-angiogenic test with chorioallantoic membrane assay (CAM) [4–12]. Because of the presence of great variation in composition in egg white [13–15] and the diversity of chemical structures and polarity of the medicines tested [16], the question arises with "Can CAM accurately reflect the inherent response of a biological system to therapeutics with respect to angiogenic status?"

Egg composition varies with genetic selection and feedstocks. Egg Haugh unit (HU) had been altered as a result of genetic selection or by feeding with vanadium (V) to hens. In both altered HU conditions, eggs with low HU values yielded significantly less water-insoluble ovomucin from the thick albumen than eggs with high HU values, whereas the yield of ovomucin from thin albumen

* Corresponding author. Tel.: +886 9 66 739935; fax: +886 2 27294931.
** Corresponding author at: Research Institute of Biotechnology, Hungkuang University, 34, Chung-Chi Rd., Shalu County, Taichung Hsien, Taiwan. Tel.: +886 2 27585767; fax: +886 2 27585767.
E-mail addresses: kc.chen416@msa.hinet.net (K.-C. Chen), ypeng@seed.net.tw (R.Y. Peng).
[1] The three authors have contributed equally.

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did not differ. The amount of ovomucin differed between eggs with high or low HU values as a result of feeding V, but the composition of ovomucin differed in thick albumen was not affected [13]. In comparison, egg white from high HU-line had lower contents of total carbohydrate, sialic acids, hexosamines, and hexoses than genetic lines with low HU. Conversely, thick albumen, whole albumen, and ratio of thick to thin were significantly higher in high HU than low HU line [13]. Purified ovomucin was isolated as an insoluble glycoprotein complex from thick egg white [14]. A homogeneous glycoprotein found in chicken eggs, designated α-ovomucin (molecular weight 210 kD) contains much lower contents of *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, *N*-acetylneuraminic acid and sulfate than β-ovomucin, except mannose [14]. In addition, species-specific compositional variation also exists [15]. Moreover, interaction or the chemicals tested may trigger some signal related mechanism when interacts with glycoproteins in chicken egg-envelope [17]. We suspected that the effective dosage and responsive time in all CAM could be deviated by such many factors, i.e. "Which is actually the true rate-limiting step in a CAM assay?" In this present study, we established a mathematical model and simultaneously performed diffusion studies in egg white using some known authentic coloring matters and herbal extract.

2. Materials and methods

2.1. Diffusion-in-egg-model

2.1.1. Egg characteristics

Fresh chicken eggs and day-3 fertilized chicken embryos were purchased from the local egg wholesale company. The average interior dimension of chicken eggs is shown in Fig. 1.

By referring to Figs. 1 and 2 and assuming that the distance of C_o to C_i is a membrane mimic, the diffusion-in-egg-model can be established according to Fick's First Diffusion Law as

$$\frac{dn}{dt} = \frac{DA(C_o - C_i)}{\ell} \quad (1)$$

where dn/dt is the rate of flow through a plan with cross sectional area of A perpendicular to the abscissa (x-direction) along the longitudinal axis of egg. D is diffusion coefficient, the term $C_o - C_i$ is the concentration gradient across the gap (membrane mimic), ℓ is

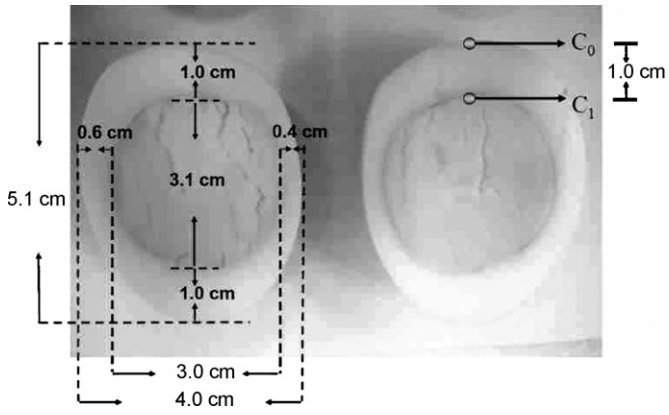

Fig. 1. General interior dimensions of chicken eggs. (Left) Chicken eggs appear as an oval shape, having at average a length of 5.1 cm in the longitudinal direction, 4.0 cm in radial direction. The egg yolk roughly has a diameter of 3.1 cm. (Right) the distance from the air sac to the interface of egg yolk is about 1 cm long. While this cross section part occupies a volume of 5 mL, about 10% of the volume of the egg with a total of 50 mL. The concentration of applied medicine at the initial position was designated as C_o, while that at the interface was C_i.

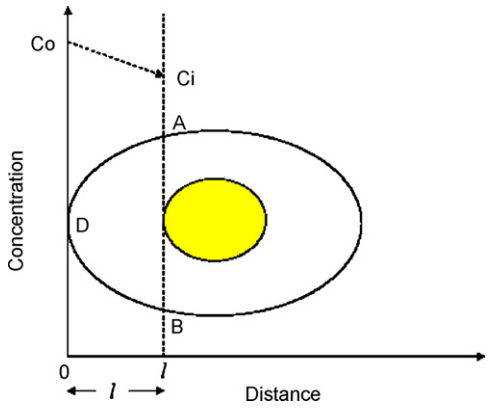

Fig. 2. Diffusion-in-egg model.

the thickness from the point D on the air sac to junction or interface of egg white (EW) and egg yolk (EY). The diffusion coefficient D is therefore calculable from the parameters including dn/dt, A, ℓ, and the concentration gradient $C_o - C_i$ (Eq. (1)). However the egg is oval in shape, the area A varies depending upon the thickness ℓ (Fig. 2); hence Eq. (1) is inapplicable at this moment. Recall that

$$A = r^2\pi \quad (2)$$

On differentiation of Eq. (2) we have

$$dA = 2\pi r\, dr \quad (3)$$

r is the radius at any point from C_o to C_i within the thickness ℓ. Alternatively, values of r are changing with thickness ℓ, i.e. the corresponding volume at certain distance of ℓ is

$$dV_{ABD} = dA\, d\ell \quad (4)$$

Substitution of Eq. (3) into Eq. (4) leads to

$$dV_{ABD} = 2\pi r\, dr\, d\ell \quad (5)$$

Integration of Eq. (5) yields

$$\int_0^v dV_{ABD} = 2\pi \int_0^r \int_0^\ell r\, dr\, d\ell \quad (6)$$

As indicated in Figs. 1 and 2, the total length of ℓ is 1 cm, thus the integral $\int_0^\ell d\ell = 1$ and Eq. (6) reduces to

$$\int_0^v dV_{ABD} = 2\pi \int_0^r r\, dr \quad (7)$$

Or

$$V_{ABD} = 2\pi \int_0^r r\, dr \quad (8)$$

where V_{ABD} is named hereafter as "the capping volume". In addition, we designate an additional terminology C_{inst}, which means the instantaneous concentration achievable in the capping volume V_{ABD}. C_{inst} can be attained provided the volume is very tiny, the egg white is very thin enough and homogeneously isotropic, more importantly the diffusion time should be very short. By definition

$$C_{inst} = \frac{Q_o}{V_{ABD}} \quad (9)$$

Or

$$C_{inst} = \frac{C_o V_a}{V_{ABD}} \quad (10)$$

Here Q_o is the amount of medicine originally applied at the CAM; C_o and V_a are respectively the original concentration and volume of applied test medicine solution.

Further substitution of Eq. (8) into Eq. (10) yields

$$C_{inst} = \frac{C_o V_a}{2\pi} \int_0^r r\,dr \tag{11}$$

Synonymously, C_{inst} also means the theoretical maximum mean concentration of medicine achievable in the capping volume V_{ABD} under condition as above mentioned. Practically, the capping volume occupies 5 mL in eggs having average total volume of 50 mL, which happened to be the actual size used for this experiment. Thus the concentration C_{inst} can be approximately measurable from Eq. (12).

$$C_{inst} = \frac{C_o V_a}{5} \tag{12}$$

Alternatively, assuming that C_i, the concentration of medicine at the EW–EY interface, is responsible for effective therapeutic bioactivity and the reaction is first-order kinetics, the therapeutic rate kinetics, R, is

$$R = kC_i \tag{13}$$

where k is the reaction rate coefficient in EY. By further appropriate simplifying assumption is that the rate of transport of the reacting component onto the EW–EY interface is given by

$$R = (D/\ell)(C_o - C_i), \tag{14}$$

where D is the relevant diffusion coefficient, C_o is the initial concentration of medicine applied at point D on chorioallantoic membrane, and ℓ is the distance between point D and EY surface (Fig. 2). This expression involves various approximations—the neglect of bulk flow for example—but is useful enough for the present purpose.

At steady state, these two rates must rapidly become equal—i.e. as much the chemical diffuses up to the surface as reacts. Hence solving for the concentration C_i, at the interface in terms of the known concentration C_o gives

$$C_i = \frac{[(D/\ell)C_o]}{[k + (D/\ell)]} \tag{15}$$

Substituting Eq. (15) into Eqs. (13) or (14) gives

$$R = \frac{k[(D/\ell)C_o]}{[k + (D/\ell)]} \tag{16}$$

Eq. (16) is applicable to any tiny volume of reacting space or a single particle, provided it is small enough for the concentration C_o to remain reasonably constant.

At low enough temperatures (such as ambient temperature), k tends to be much smaller than the term D/ℓ, and especially when ℓ is small. Under these conditions Eq. (16) reduces to

$$R = kC_o \quad (k \ll D/\ell), \tag{17}$$

which is a Rate-Limiting Process.

Conversely when the increase in k has greatly outgrown the increase in D/ℓ, we have

$$R = (D/\ell)C_o \qquad (k \gg D/\ell), \tag{18}$$

Eq. (18) is named Diffusion- or Mass Transfer-Limiting Equation.

As well known, to evaluate the in vivo kinetic coefficient in egg embryo could be achieved by special techniques including fluorescence or isotope labeling methodology. However the instantaneous kinetic coefficient k_{inst} is calculable from our model. Imagine that the instantaneous kinetics actually measures the true inherent reaction status at the very initial transient stage without being affected by any mass action law and the product interference. By definition, Eq. (13) is modified to give

$$\frac{C_{inst}}{dt} = k_{inst} C_{inst} \tag{19}$$

On rearrangement to yield

$$k_{inst} = \frac{[C_{inst}/dt]}{C_{inst}} \tag{20}$$

Obviously all values of k in Eqs. (13)–(18) practically would be much smaller than values of k_{inst}.

2.1.2. Chemicals

Blue Food Coloring, Brilliant Blue FCF or FD&C Blue No. 1: (disodium bis[4-(*N*-ethyl-*N*-3-sulfonatophenylmethyl)aminophenyl]-2-sulfonatophenylmethylium); Evans Blue: (6,6′-(3,3′-dimethyl (1,1-biphenyl)-4,4′-diyl)-bis(azo)-bis-(4-amino-5-hydroxy)-1,3-naphthalene disulfonic acid, tetrasodium salt; and Red Food Coloring Allura Red AC or FD&C Red No. 40: (disodium 4-hydroxy-3-(2,4-dimethyl-5-sulfonatophenylazo)-1-naphthalenesulfonate) were products of Kiriya Chemical Co. (Tokyo, Japan). Aqua extract of budding leaves of *Psidium guajava* L. (PE) was prepared according the method previously reported [18].

2.2. Experiment

2.2.1. Diffusion medium

Fifty fresh chicken eggs were gently broken to obtain egg white by decantation after removing yolks and suspension cords. The egg white was gently mixed to homogeneous state without disturbing the egg white to entrap air bubbles. The egg white homogenate was subject to aspiration at reduced pressure to drive off all residual tiny gas bubbles. An aliquot of 5 mL egg white was measured and diluted with PBS to a 100-fold diluted egg white solution (solution A), which was kept for establishment of the calibration curves. The remaining undiluted combined egg white was used as the experimental diffusion medium (DM).

2.2.2. Calibration curve

Authentic coloring matters, Color Blue No. 1, Evans Blue and Color Red No. 40, and PE were diluted with egg white solution (1×100 dilution, or solution A) to the desired concentration as indicated. The wavelengths to measure the respective absorbance using a spectrophotometer (Hitachi U-2001 Spectrophotometer, Japan) were 625 nm for both Color Blue No. 1 and Evans Blue, 525 nm for Color Red No. 40, and 450 nm for PE. Egg white solution (1×100 dilution) was used as the blank.

2.2.3. Diffusion experiment

Authentic coloring matters or PE were dissolved to the desired concentrations as indicated with solution A. To 12 cylinders (10 mL), DM was added to the full scale, three cylinders for each test solution. Aliquots of the test solution were measured with a microsyringe and topped on at the middle of DM in each respective cylinder. The entire course of diffusion was performed at $25 \pm 1\,^{\circ}C$ under a relative humidity of 80% in the dark. The diffusion of the target substance was followed by taking the photos automatically using a digital camera (EXZ75, Casio, Japan). The samples were obtained by sucking with a microsyringe at different diffusion distances and corresponding diffusion times as indicated without disturbing the existing diffusion layers. The samples obtained were diluted with phosphate buffer (PBS, pH 7.2) to 10 mL and the absorbance was measured using a spectrophotometer (Hitachi U-2001 Spectrophotometer, Japan) respectively at assigned wavelength. The

Table 1
C_o and C_i values of different chemicals obtained in diffusion experiments with egg white.

Diffusion time (h)	Distance diffused (cm)	Blue No. 1 (mg/mL)	Evans Blue (mg/mL)	Red No. 40 (mg/mL)	PE (mg/mL)[a]
0	1.0 = C_o	1.0 = C_o	1.0 = C_o	1.0 = C_o	10.0 = C_o
7	1	–	–	–	0.2240 = C_i
9	1	0.0460 = C_i	–	–	–
11.5	1	–	0.0300 = C_i	–	–
13.5	1	–	–	0.3620 = C_i	–
24	2.1	0.0073	–	–	–
24	2.3	–	0.0130	–	–
24	1.5	–	–	0.0221	–
24	2.0	–	–	–	0.1087
48	3.1	0.0016	–	–	–
48	4.0	–	0.0030	–	–
48	2.0	–	–	0.0200	–
48	2.7	–	–	–	0.0729

[a] PE: the aqueous extract of *Psidium guajava* L. budding leaves.

Table 2
Parameters collected/or calculated from cited CAM data.

Medicine/C_o	V_o	Q_o	C_t[a]	Reaction time (Δt)	k_{ins}[b] (h^{-1})	Reference
Quercetin/5–10 nmol/1 μL	10 μL/egg	50–100 nmol/egg	10.0–20.0 nmol/mL	9 days	$(4.63–9.26) \times 10^{-3}$	[12]
Safrole oxide/0.11 μmol/μL	100 μL/egg	11 μmol/egg	2.2 μmol/mL	(2 days)	2.08×10^{-2}	[4]
Herbal extract[c]/0.03–1 μg/5 μL	5 μL/egg	0.03–1 μg/egg	0.006–0.2 μg/mL	2 days	2.08×10^{-2}	[5]
Herbal extract[d]/10 μg/μL	5 μL/egg	50 μg/egg	10.0 μg/mL	2 days	2.08×10^{-2}	[6]
Pseudolaric acid B/5 nmol/μL	10 μL/egg	50 nmol/egg	10.0 nmol/mL	2 days	2.08×10^{-2}	[7]
NRG-1/100 ng		100 ng/egg	20.0 ng	3–4 days	$(1.04–1.39) \times 10^{-2}$	[8]
Schiraiachrome A/5–10 nmol/egg		5–10 nmol/egg	1.0–2.0 nmol/mL	2 days	2.08×10^{-2}	[9]
Nano carbon (graphite, fullerene)/100 μg/egg		100 μg/egg	20.0 μg/mL	3 days	1.39×10^{-2}	[11]
Artesunate[e]/0.4–0.8 nmol/μL	100 μL	40–80 nmol/egg	8.0–16.0 nmol/mL.	3 days	1.39×10^{-2}	[12]
Resistin/10–100 ng/mL	1 mL	10–100 ng/egg	2.0–20.0 ng/mL	16–18 h	$(5.56–6.25) \times 10^{-2}$	[19]
RWPCs[f]		5–25 μg/egg	1.0–5.0 μg/egg	48 h	2.08×10^{-2}	[20]

[a] Calculated from Eq. (12).
[b] Calculated from Eq. (20).
[c] Methanol extract of *Ulmus davidiana* Planchon var. Nakai (Ulmaceae) stems and barks.
[d] Aqueous extract of *Cnidium officinale* Makino and *Tabanus bovinus* stems and barks.
[e] Extracted from herb *Artemisia annua*.
[f] RWPCs: Red wine polyphenolic compounds.

concentration for each chemical was calculated from the calibration curves and dilution folds.

3. Results and discussion

As indicated in Table 1, the concentration of the chemicals tested at the interface of egg white and egg yolk only reached 2.24–4.60% of the original applied concentration C_o, implicating diffusion characteristics of chemicals in egg white can be very slow and insignificant in nature. After 24 h-diffusion, fraction of Blue No. 1, Evans blue, Red No. 40 and the PE reached 0.73, 1.30, 2.21, and 1.09%, while at 48 h, the percent of the chemicals under testing was seen to be only 0.16, 0.30, 2.00, and 0.73%, respectively. Overall, the slowest diffusion rate was found for Red No.40 (Table 1).

Whatever medicines applied, the initial concentration applied to CAM was seen to range from 0.03 to 100 μg/egg (or 5–100 nmol/egg) [5,12], which correspondingly yielded instantaneous concentration of 0.006–20 μg/mL (or 1.0–20 nmol/mL) in the "capping volume V_{ABD}" (Table 2). Further estimation indicated

Table 3
Estimated reaction control regime.

Medicine/C_o	C_t[a]	D[b] ($m^2\ s^{-1}$)	D/ℓ[c] (ms^{-1})	k_{inst}[d] (h^{-1})	Control status
Quercetin/5–10 nmol/1 μL	10.0–20.0 nmol/mL	1.5×10^{-8}	1.5×10^{-6}	$(4.63–9.26) \times 10^{-3}$	($k_{inst} \geqslant D/\ell$)
Safrole oxide/0.11 μmol/μL	2.2 μmol/mL	1.5×10^{-8}	1.5×10^{-6}	2.08×10^{-2}	($k_{inst} \geqslant D/\ell$)
Herbal extract[a]/0.03–1 μg/egg	0.06–0.2 μg/mL	1.5×10^{-8}	1.5×10^{-6}	2.08×10^{-2}	($k_{inst} \geqslant D/\ell$)
Herbal extract[b]/10 μg/μL	10.0 μg/mL	1.5×10^{-8}	1.5×10^{-6}	2.08×10^{-2}	($k_{inst} \geqslant D/\ell$)
Pseudolaric acid B/5 nmol/μL	10.0 nmol/mL	1.5×10^{-8}	1.5×10^{-6}	2.08×10^{-2}	($k_{ins\ t} \geqslant D/\ell$)
NRG-1/100 ng	20.0 ng	1.5×10^{-8}	1.5×10^{-6}	$(1.04–1.39) \times 10^{-2}$	($k_{inst} \geqslant D/\ell$)
Schiraiachrome A/5–10 nmol/egg	1.0–2.0 nmol/mL	1.5×10^{-8}	1.5×10^{-6}	2.08×10^{-2}	($k_{inst} \geqslant D/\ell$)
Nano carbon (graphite, fullerene)/100 μg/egg	20.0 μg/mL	1.5×10^{-8}	1.5×10^{-6}	1.39×10^{-2}	($k_{inst} \geqslant D/\ell$)
Artesunate[d]/0.4–0.8 nmol/μL	8.0–16.0 nmol/mL.	1.5×10^{-8}	1.5×10^{-6}	1.39×10^{-2}	($k_{inst} \geqslant D/\ell$)
Resistin/10–100 ng/mL	2.0–20.0 ng/mL	1.5×10^{-8}	1.5×10^{-6}	$(5.56–6.25) \times 10^{-2}$	($k_{inst} \geqslant D/\ell$)
RWPCs[e]	1.0–5.0 μg/egg	1.5×10^{-8}	1.5×10^{-6}	2.08×10^{-2}	($k_{inst} \geqslant D/\ell$)

[a] Calculated from Eq. (12).
[b] Diffusion coefficients of chemicals in egg white varies in range of 2.8×10^{-10} to $3.1 \times 10^{-8}\ m^2\ s^{-1}$), the average value $1.5 \times 10^{-6}\ m^2\ s^{-1}$ was used for calculation.
[c] ℓ = 1 cm = 0.01 m.
[d] Calculated from Eq. (20).

that the instantaneous first-order kinetic coefficients were commonly retained at $2.08 \times 10^{-2}\ h^{-1}$ (Table 2). Longer incubation time might be required for relatively nonpolar polyphenolics such as quercetin that spent 9 days to achieve merely an instantaneous kinetic coefficient of $(4.63–9.26) \times 10^{-3}\ h^{-1}$ (Table 2).

Previously, the diffusion coefficient data had been studied by Peng et al., which exhibited a rather common range of 2.8×10^{-10} to $3.1 \times 10^{-8}\ m^2\ s^{-1}$ (data unpublished). Taking the average value of $1.5 \times 10^{-8}\ m^2\ s^{-1}$ to estimate the reaction control regime (Eqs. (17) and (18)), we arrived at the results shown in Table 3. As can be seen in Table 3, no matter which diffusion coefficient (i.e. from 2.8×10^{-10} to $3.1 \times 10^{-8}\ m^2\ s^{-1}$), values of k_{inst} are consistently exceeding those of D/ℓ, i.e. the relationship $k_{inst} \geqslant D/\ell$ always exists (Table 3), which apparently means a diffusion control regime.

Conclusively, all CAM assays ever cited had been performed under condition of "Diffusion Control" regime. No matter what medicines are used, the instantaneous kinetic rate always excels in magnitude the diffusion term D/ℓ. Thus the problems may arise:

1. In order to access the effective bioactivity, the medicines tested must be used in large excess than normally required for clinical therapeutic uses.
2. The diverse constituents present in egg white could interfere with the diffusion rate of medicine in egg white.
3. Different genetic selection of eggs (or embryos) would deviate the outcome of angiogenesis with CAM assays in this regard.
4. Thus we suggest that in performing the CAM assay, the standardization of egg species would be the most important prerequisite. While the effective concentration at the interface of egg white and embryo that actually affects the onset of anti-angiogenesis has to be determined simultaneously.

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