Figure 2 Hypothetical scheme of the mechanism of action of 6-phosphogluconate dehydrogenase

Abbreviation: Ru5P, ribulose 5-phosphate.

It has now been found that 6PG or d6PG also activate the enzymic decarboxylation of the keto intermediate (Figure 1); 50% of the highest rate of decarboxylation is obtained when 6PG or d6PG are present at concentrations almost equal to their K_m for the enzyme (Table 1). At concentrations higher than 0.07 mM, 6PG produces a lower activating effect (Figure 1a), due to competition with 3kd6PG for the same protein site [5]. 6-Sulphogluconate, 6-phosphogalactonate, sorbitol 6-phosphate, ribulose 5-phosphate (even at a 50 mM final concentration) and, of course, 3kd6PG do not activate the decarboxylation reaction, indicating that an activator must contain six carbon atoms, a carboxyl at C-1, a phosphate group at C-6, and hydroxyls at C-3 and C-4 (with the same configuration as in 6PG), while the presence of hydroxyl at C-2 is not essential.

Since each subunit has only one substrate binding site, these results indicate that the binding of 6PG (or d6PG) to one site, through a conformational change involving both subunits, activates the decarboxylation of the 3kd6PG bound to the other site.

If decarboxylation, the rate-limiting step of oxidative decarboxylation [13], is triggered by 6PG, then, while one subunit is involved in decarboxylation, the other subunit can catalyse the oxidation of 6PG by NADP^+ ; thus both subunits are catalytically active at the same time, one in the redox and the other in the decarboxylation reaction. The 3kd6PG, formed from 6PG in the subunit which catalysed the redox reaction, must now be decarboxylated. The more simple (and likely) hypothesis is that decarboxylation is catalysed by the same subunit, while 6PG is bound and oxidized by the other subunit. According to this hypothesis, the two equal subunits reverse their role during each turnover of oxidative decarboxylation: each subunit alternately catalyses the redox and decarboxylation reactions (as illustrated in Figure 2), and during catalysis there is a fluid functional asymmetry triggered by intersubunit communications and positive co-operativity [6,14]. The validity of this hypothesis could be tested by determining whether the release of NADPH precedes or follows the decarboxylation. An alternative, less likely, hypothesis foresees the dimer with one permanently catalytic subunit and the other with only a regulatory role; in this case the turnover number would be lower.

NADPH also activates (Table 1) the decarboxylation of 3kd6PG, probably bound to the other subunit [5], but at a 5-fold lower rate; the two activating effects (by 6PG and NADPH) are not additive. NADPH and 6PG, even if they induce the conformational changes required for decarboxylation, have different effects on the enzyme; indeed, 6PG (tested at concentrations ranging from 0.004 to 4 mM) does not activate the ^3H exchange reaction between the medium and ribulose 5-phosphate, a

reaction activated instead by NADPH. The mechanism of NADPH-induced decarboxylation is also different: if NADPH activates decarboxylation, this does not allow the simultaneous binding of 6PG and NADP^+ [6] (and thus the redox reaction) in the other subunit. In this case, there is not the alternating site co-operativity, and only one of the two subunits is catalytically active.

The finding that activation of decarboxylation by NADPH is lower than that by 6PG raises the question whether the level of cellular NADPH could determine the reaction mechanism and thus the turnover, regulating the activity of the enzyme.

It would be interesting to check if this hypothesis of alternating sites is also valid for other homodimeric and homopolymeric enzymes.

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- 1 Miller, S. M., Massey, V., Williams, C. H., Ballou, D. P. and Walsh, C. D. (1991) *Biochemistry* **30**, 2600–2612
- 2 Silverberg, M. and Dalziel, K. (1973) *Eur. J. Biochem.* **38**, 229–238
- 3 Silverberg, M. and Dalziel, K. (1975) *Arch. Biochem. Biophys.* **168**, 646–651
- 4 Adams, M. J., Gover, S., Leabach, R., Phillips, C. and Somers, D. O'N. (1991) *Acta Crystallogr. Ser. B* **47**, 817–820
- 5 Hanau, S., Dallochio, F. and Ripa, M. (1992) *Biochim. Biophys. Acta* **1122**, 273–277
- 6 Hanau, S., Dallochio, F. and Ripa, M. (1992) *Biochim. Biophys. Acta* **1159**, 262–266
- 7 Topham, C. M., Matthews, B. and Dalziel, K. (1986) *Eur. J. Biochem.* **156**, 555–567
- 8 Lienhard, G. E. and Rose, I. A. (1966) *Biochemistry* **3**, 190–195
- 9 Ripa, M., Signorini, M. and Dallochio, F. (1972) *Biochem. Biophys. Res. Commun.* **48**, 764–768
- 10 Kalnitsky, G. and Tapley, D. F. (1958) *Biochem. J.* **70**, 28–34
- 11 Hanau, S., Dallochio, F. and Ripa, M. (1991) *Biochem. Int.* **25**, 613–620
- 12 Villet, R. H. and Dalziel, K. (1972) *Eur. J. Biochem.* **27**, 244–250
- 13 Rendina, A. R., Hermes, J. D. and Cleland, W. W. (1984) *Biochemistry* **23**, 6257–6267
- 14 Bild, G. S., Janson, C. A. and Boyer, P. D. (1980) *J. Biol. Chem.* **255**, 8109–8115

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An Avrami analysis of the effects of serum and human albumin on calcium hydroxyapatite crystal growth

The nucleation and growth of hydroxyapatite (HAP) from low supersaturated aqueous solutions is of considerable interest to the biomedical field. Both the calcification of tissue and that of implants are serious problems that are poorly understood. The areas of biocompatibility and biomimetic processing are also keenly interested in the nucleation and growth kinetics, and the effect of proteins in moderating the precipitation reaction.

The recent paper by Garnett and Dieppe [1] presented results showing that albumin and other serum proteins significantly slowed the growth of HAP from low supersaturations. The results are explained in terms of the specific adsorption of the proteins, and the subsequent lowering of the surface growth rate. The data presented, though, can be analysed using fairly simple models to suggest that other mechanisms may be possible.

The modelling of the nucleation, growth and agglomeration of particles from a solution has been described for a number of various conditions in some detail [2–6]. All are based on the population distribution function and include various approximations for creation and annihilation of particles and agglomerates. Most of these later models are so complicated and include

Figure 1 Transformation versus time for constant nucleation rate and constant growth rate

The points are data taken from reference [9] and the solid curve is the fitted Avrami curve, $n = 4$ and $k = 3.5 \times 10^{-14}$.

so many terms as to be intractable for many experiments [2]. They are however useful for a quantitative description of the process, and can provide insight into the precipitation reaction.

A much simplified version of these analyses is the Avrami equation [7], or to some the Johnson-Mehl equation [8]. This equation is derived using the general geometry of the growing precipitate and includes, to leading order, the effect of depletion of the parent phase during the reaction. As such, it provides a quantitative description of the kinetics of a transformation in a medium where the total amount of reactants is restricted, but is simple enough to be tractable for the analysis of most experimental data. The equation follows the general form:

$$W = 1 - e^{-f(t)} \quad (1)$$

where W is the fraction transformed and the function $f(t)$ is dependent on the system. For the ideal nucleation and growth of spherical particles at constant nucleation rate, N , and constant surface growth rate, v , the equation is:

$$W = 1 - e^{-\frac{4}{3}\pi N v^3 t^4} \quad (2)$$

The resulting curve is sigmoidal in shape, showing very little growth for early times. If the shape of the precipitates is non-spherical, the v^3 and t^4 terms are modified to reflect the proper Hausdorf (or fractal) dimension of the particle.

An example of these is shown in Figure 1, fitted to the homogeneous nucleation and growth data for HAP originally published by Boskey and Posner [9] (including an approximate 24 h so-called 'incubation time' [9] or induction time for nucleation). As can be seen in the Figure, the sigmoidal shape of the curve reproduced the apparent induction time phenomenon fairly well. The data of Boskey and Posner [9] are consistent with constant nucleation and growth rates in a limited system, and no induction time for nucleation is necessarily observed. For more complicated geometries and heterogeneous nucleation problems the appropriate Avrami equation can be derived to describe the system (see for example [10]). The structure and composition of the nuclei is unimportant to the analysis, and is generally expected to be significantly different from that of the growing phase [11]. Agglomeration and/or ripening of the growing phase are obviously ignored in the simple cases but could be included for analysis.

Later authors [12,13] have used the induction time conclusion of Boskey and Posner [9] to analyse their data by presentation of induction times calculated graphically from the inflection points of the fraction transformed (titrant added) versus time plots.

Assuming $f(t)$ in eqn. (1) has the simplified form kt^n , the inflection point in the plot will occur at time:

$$t_1 = \left(\frac{n-1}{nk} \right)^{\frac{1}{n}} \quad (3)$$

or more conveniently:

$$kt_1^n = \frac{n-1}{n} \quad (4)$$

The induction time is related to the inflection point by the relation:

$$I_t = t_1 \left[\frac{n - e^{\frac{n-1}{n}}}{(n-1)} \right] \quad (5)$$

The term n is only dependent of the geometry of the system and is unaffected by changes in the nucleation and growth rates. Unless the geometry of the transforming system changes then this should be invariant and can be used to calculate n . For turbidity experiments, an induction time is expected because the particles of very small size do not interact strongly with light of comparable wavelength.

For the system described by Boskey and Posner [9] this reduces to:

$$I_t = 0.6277 t_1 \quad (6)$$

The exact inflection point is generally difficult to determine on an experimental curve, and almost impossible on a published plot, which makes this a difficult method of determining the n and k values for a system. In essence, though, for a series of carefully controlled experiments, where constant n can be fairly safely assumed, I_t , the so-called induction time, is related to the nucleation and growth rates of the transformation. If the system has complicating geometry, or if the geometry is changing in the reaction, the induction times can change dramatically with no real change in the nucleation or growth rates at the surface. The reporting of an induction time, then, is insufficient to adequately describe the evolution of the system during precipitation.

For the analysis of seeded growth, the size of the seed must be included as an initial condition, but also the geometry of the seed is very important. The seed particles used in [1] were sized at $\sim 500 \mu\text{m}$ using sieves. BET analysis showed the specific surface areas to be $\sim 30.8 \text{ m}^2/\text{g}$, giving an average particle size of $\sim 70 \text{ nm}$. The growth problem in this system becomes the infiltration and occluded growth of particles in an agglomerate. Assuming spherical seed particles, the $f(t)$ for this condition can be shown to be

$$f(t) = V_0 + 4\pi M r_0^2 v t + (4-m)\pi M r_0 v^2 t^2 + (4-m)\frac{\pi}{3} M v^3 t^3 \quad (7)$$

where V_0 is the initial volume of the seeds, r_0 is the initial particle size in the agglomerates, v is the growth rate, M is the total number of particles in the agglomerate and m is the average number of nearest neighbours for a particle in the agglomerate.

Converting all units to μmol of KOH (the units used in [1]) and normalizing to the total amount of the HAP product gives the fraction transformed during the reaction. For the experimental data, the $f(t)$ can be calculated as $-\ln(1-W)$. Figure 2 shows the $f(t)$ calculated for the growth of HAP in serum, dialysed serum and a control taken from Figure 2 in [1]. Figure 3 shows similar results for albumin and heat-denatured albumin, the data of Figure 4 in [1]. The solid lines show the result of the model for early growth with the parameters in Table 1.

As can be seen in the Figures, the calculated values for the

Figure 2 Avrami function for seeded growth of hydroxyapatite for data taken from reference [1], Figure 2

The points mark the experimental data for: control ($\square$), serum ($\triangle$) and dialysed serum ($\blacksquare$). The solid lines are the fitted results from Table 1.

Figure 3 Avrami function for seeded growth of hydroxyapatite for data taken from reference [1], Figure 4

The points mark the experimental data for: control ($\square$), albumin ($\triangle$) and heat-denatured albumin ($\blacksquare$). The solid lines are the fitted results from Table 1; the line without points represents the fit suggested by equation [8].

parameters fit the experimental data reasonably well (considering the data was read directly from the Figures in the journal) for all but one case. This strongly suggests that the infiltration model correctly describes the growth phenomenon of the experiment. The growth rates given in Table 1 follow the same trends that the authors of [1] point out in their own conclusions, that the growth rate calculated for the system decreases with the addition of serum proteins.

The one exception is the case of heat-denatured albumin, which exhibits a greater inhibitory effect than this geometric model can predict. By assuming a growth surface area for the particles in the form of a fractal:

$$S_{\text{growth}} = k(4\pi r^2)^n \quad (8)$$

the occluded growth model above can be modified to include the

Table 1 Seeded growth data from Avrami analysis

Values were assumed for ζ ($0.340 \mu\text{mol KOH}$), $M^{1/3}r_0$ [$0.434 (\mu\text{mol KOH})^{1/3}$] and m (6.5).

Solution	$M^{1/3}v$ [($\mu\text{mol KOH}$) $^{1/3}$ /min]
Control (Fig. 2)	0.00314
Dialysed serum	0.00201
Serum	0.00168
Control (Fig. 4)	0.00241
Albumin	0.00192
Heat-denatured albumin	0.0094

growth process dimensionality. An estimate of the resulting model is:

$$f(t) = V_0 + k(4\pi Mr_0^2)^n vt + (4-m)k^*(\pi M^{2/3}r_0 vt)^n M^{1/3}vt \\ + (4-m)k^*\left(\frac{\pi}{3}M^{2/3}v^2 t^2\right)^n M^{1/3}vt \quad (9)$$

which is identical to the earlier model for $k = n = 1$. Assuming $n = 0.5$ and $k = 2\sqrt{\pi}$ for this model, the equivalent of plate-like growth, a value of $M^{1/3}v = 0.00045 (\mu\text{mol KOH})^{1/3}/\text{min}$ for the apparent growth rate gives the line without points on Figure 3 for the heat-denatured albumin sample. With this many parameters, the fit should be very good. This does suggest, though, that the growth mechanism for the heat-denatured albumin sample is distinct from the other samples and warrants further detailed study. Using the less quantitative analysis of the original authors, this change in mechanism was overlooked.

The nucleation and growth of HAP in solution is a very important topic. Many excellent experiments have been performed to determine the effects of supersaturation, temperature and proteins on the process in order to better understand the mechanisms and potentially control the process. The analysis of the data, though, is as important as careful experiment design. Thoughtful analysis can allow for better comparison of results and more insight into the mechanisms of the transformation.

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- Garnett, J. and Dieppe, P. (1990) *Biochem. J.* **266**, 863–868
- Santacesaria, E., Tonello, M., Storti, G., Pace, R. C. and Carra, S. (1986) *J. Colloid Interface Sci.* **111**, 44–53
- Alam, M. K. and Flagan, R. C. (1984) *J. Colloid Interface Sci.* **97**, 232–246
- Rusanov, A. I. and Kuni, F. M. (1984) *J. Colloid Interface Sci.* **100**, 264–277
- Chiang, P., Donohue, M. D. and Katz, J. L. (1988) *J. Colloid Interface Sci.* **122**, 251–265
- LaMer, V. K. and Dinegar, R. H. (1950) *J. Am. Chem. Soc.* **72**, 4847–4854
- Avrami, M. (1939) *J. Chem. Phys.* **7**, 1103–1112; **8** (1940) 212; **9** (1941) 177–184
- Johnson, W. A. and Mehl, R. F. (1939) *Trans. AIME* **135**, 416–458
- Boskey, A. L. and Posner, A. S. (1976) *J. Phys. Chem.* **80**, 40–45
- Cahn, J. W. (1956) *Acta Met.* **4**, 449–459
- Reiss, H. (1950) *J. Chem. Phys.* **18**, 840–848
- Williams, G. and Salis, S. D. (1979) *Biochem. J.* **184**, 181–184
- Williams, G. and Salis, S. D. (1982) *Calcif. Tissue Int.* **34**, 169–177

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