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Factors Affecting the *in Vitro* Dissolution of Cobalt Oxide

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In a recent interspecies comparison of the lung clearance of cobalt oxide ($^{57}Co_3O_4$), differences of up to 4-fold were found in the translocation rates of ^{57}Co to blood between seven different animal species, including man. This study investigated some factors that could influence the dissolution of this material *in vitro*. The effect of bicarbonate and citrate concentrations (over physiological ranges) and medium pH on *in vitro* dissolution of ^{57}Co from $^{57}Co_3O_4$ particles was measured in a simple noncellular system. pH levels of 4.5, 6.1, and 7.2 were used to correspond to those in the alveolar macrophage lysosome, its cytoplasm, and the extracellular lung fluid. Measurements of the fractional dissolution rate were made weekly for 3 months. pH had the greatest effect on dissolution rates, with particles suspended in the lowest pH medium (4.5) dissolving at a significantly faster rate than at higher pH values. Increasing citrate concentrations resulted in slightly higher dissolution rates, but there was no effect of bicarbonate concentration. There was no evidence of synergism between the factors studied.

Introduction

The purpose of this study was to identify a factor in the dissolving environment that could account for the species differences in translocation rates of ^{57}Co from lungs to blood observed after inhalation of cobalt oxide ($^{57}Co_3O_4$) (*1*). A simple system was used, and no attempt was made to estimate the magnitude of the dissolution rates *in vivo* from those obtained *in vitro*.

Studies of the factors involved in particle dissolution have established the characteristics of the environment that affect particle dissolution. Of these factors the most important are the pH, concentration of complexing agents, such as citrate and bicarbonate, and the extent of hydrolysis, absorption, and precipitation (*2,3*). This study compared the effect of three factors, pH, citrate concentration, and bicarbonate concentration, on the dissolution of ^{57}Co from $^{57}Co_3O_4$. The effect of pH on the dissolution of ^{57}Co from ^{57}Co-labeled fused aluminosilicate (^{57}Co-FAP) was also studied.

After inhalation, particles are deposited on the surfaces of the lung. Alveolar macrophages rapidly ingest particles deposited within the alveolar region of the lung (*4*). After phagocytosis, fusion between the phagocytotic vacuole and the primary lysosomes occurs to form the phagolysosomes. The lysosomes release their digestive enzymes into the vacuole containing the particle (*5*). Hence, after inhalation, particles are subjected to at least three different environments that may affect their solubility: the lysosome, the cytoplasm of the alveolar macrophage, and the extracellular lung fluid. Translocation of material dissolved from the particles occurs by transfer through these environments (Fig. 1). The most probable route, following dissolution in the lysosomes of the alveolar macrophages, is through the cytoplasm of the macrophage and into the surrounding lung fluid. From there transfer to the blood is via the blood–air barrier. Three levels of citrate, bicarbonate, and pH were used in this study to reflect the levels in the extracellular lung fluid, the cytoplasm of the macrophage, and the phagolysosome.

Materials and Methods

The materials used in this study were the 1.7-μm CMD "porous" $^{57}Co_3O_4$ particles, which were used in the interspecies comparison study (*1*), and ^{57}Co-FAP, which was used to study lung clearance kinetics in rats (*6*). Solutions of deionized, distilled water containing citrate at 0, 0.067, 0.13, and 0.2 mM and bicarbonate at 0, 18, 27, and 30 mM were adjusted to pH 4.5, 6.1, and 7.2 using 4M HCl. These solutions were placed in 1-L Pyrex bottles that had been autoclaved before use. The composition of each of the solutions is given in Table 1. Suspensions of the particles (1–2 kBq, 1×10^6 particles) in 2 mL of the appropriate solution were placed in sections of dialysis tubing (Visking tubing, 19 mm diameter, Medicell International Ltd., London, UK). Each section was measured for ^{57}Co activity and placed in the bottle containing the appropriate solution, which was sealed and shaken. A bottle containing a section of tubing with no particles in a solution of citrate (0.2 mM) and bicarbonate (30 mM) was used to provide blank samples.

After 1 week, the tubing containing the particles was removed from the bottles and the ^{57}Co activity measured against a known

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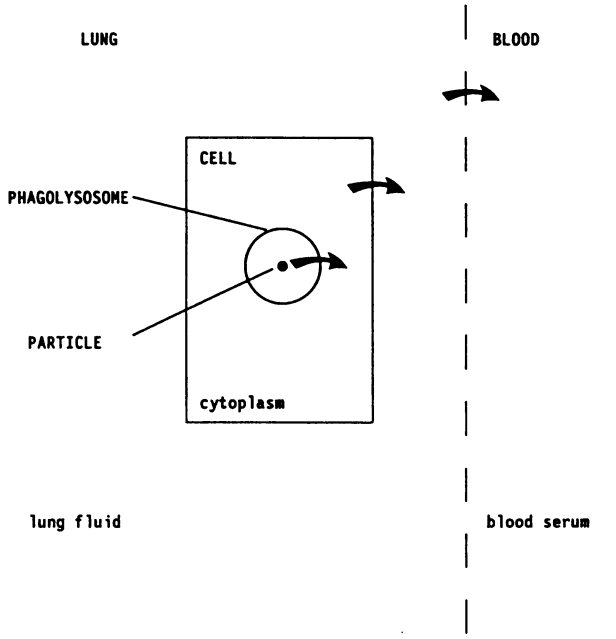

FIGURE 1. Environments encountered by material during its translocation from alveolar macrophage to blood.

standard in a scintillation vial on an automatic γ counter using the sample containing no particles as a background. The solution was removed from each bottle and replaced with fresh solution of the appropriate composition. After counting, the particles were returned to the appropriate bottles. The solution that had been removed was dried down and the residue dissolved in 4 M HNO_3 (20 mL), placed in a scintillation vial, and the ^{57}Co activity measured on an automatic γ counter, as before, using the solution from the particle-free bottle as a background. The weekly changing of solution and measurement of activity as particles dissolved in the solution continued for 3 months.

Results

Less than 0.5% of the $^{57}Co_3O_4$ and less than 1.8% of the ^{57}Co-FAP dissolved over the study period. The activity dissolving and removed to the solution is therefore expressed as a percentage of the initial particle activity in that sample. The fractional dissolution rate per day with time for each sample is given in Tables 1 and 2. In all cases, dissolution in the first week was considerably higher than in following weeks, generally by a factor of 3, but in some cases as much as 20-fold higher. The initial dissolution rate for the ^{57}Co-FAP was about 25 times that of the $^{57}Co_3O_4$, but at later times the rates for the two materials were not dissimilar. For example, over the period of 56–91 days, the mean dissolution rate of $^{57}Co_3O_4$ at all concentrations of citrate and bicarbonate was $1.85 \pm 0.2 \times 10^{-4}$/day (mean $\pm$ SE, n[number of samples] = 60) and at the same pH, was 1.83×10^{-4}/day ($n = 6$) for ^{57}Co-FAP.

The statistical computer package GENSTAT (*7*) was used to fit an exponential curve to the dissolution rate with time for each sample. This procedure assumed a Poisson distribution of the errors. The scaled deviance statistic gave an approximate estimate of the goodness of fit (*7*). This statistic has approximately a chi-square distribution. For the fits to Co_3O_4 dissolution, the scaled deviance was in the range 0.02–0.15. For the ^{57}Co-FAP dissolution, the scaled deviance was in the range 1.2–2.3. Given that the degrees of freedom were small (df = 11), examination of the scaled deviance suggests that the use of a single exponential gave a good fit to the data ($p > 0.99$).

Analysis of variance of the fitted parameters was then possible. Comparing all measurement times, the majority (88%) of samples with the same citrate and bicarbonate concentrations showed the highest dissolution rates of ^{57}Co from $^{57}Co_3O_4$ at the lowest pH and the lowest dissolution rates at the highest pH. These effects were significant ($p < 0.001$). The converse was true for the ^{57}Co-FAP, with the highest dissolution rates occurring at the highest pH. The dissolution rates with time for $^{57}Co_3O_4$ and ^{57}Co-FAP at 30 mM bicarbonate and 0.2mM citrate are plotted in Figure 2.

The effect of citrate concentration was less marked. Higher citrate concentrations resulted in higher dissolution rates, and the effect was significant ($p < 0.02$). Different bicarbonate concentrations had no significant effect on dissolution rates ($p > 0.32$). There was no evidence of synergism between any of the factors ($p > 0.4$).

Discussion and Conclusions

The pH in the lysosome of the rabbit alveolar macrophage is low (4.5–5) (*8*), and although no measurements from other species were available at the time of the study, the levels in rat, dog, and baboon alveolar macrophage lysosomes have since been shown to be similar (*9*). The intracellular (cytoplasmic) pH of lung tissues has been measured by Nicholls and Drew (personal communication) in tissue slices from rat, guinea pig, and rabbit. Values of 6.5, 6.3, and 6.9, respectively, were reported, which were similar to the value of 6.8 reported for extravascular pH of dog lung. The lung fluid outside the cells was considered by Kanapilly (*3*) to have a similar pH to that of plasma (pH 7.4). Hence, the pH levels studied here represented lung fluid, cytoplasmic, and intraphagolysosomal pH.

Citrate has been shown to enhance the *in vitro* dissolution of some particles (*2*). No direct measurements of citrate concentration in the alveolar fluid are available, but the permeability of the blood–air barrier would suggest a similar concentration to that in serum (*2*). Serum citrate levels in different animal species range from 0.1 to 0.159 mM in man and 0.1 to 0.52 mM in guinea pig, with most species falling between 0.1 and 0.3 mM (*10*). Previous studies by Kanapilly and Goh (*2*) have used 0.2 mM citrate, based on the blood serum level listed by Gamble (*11*). Citrate is formed in the mitochondria of all cells as a part of the tricarboxylic acid cycled and transport from the mitochondria to the cytoplasm occurs via the tricarboxylate transport system (*12*). The cytoplasmic concentration of citrate depends upon the energy status of the individual cell. However, for the purposes of this study, it was assumed that the cytoplasmic citrate concentration would not differ greatly from the serum levels. The sodium citrate concentrations used in this study gave a range of concentrations from 0 to 0.2 mM, covering those encountered in the lung fluid, cytoplasm, and phagolysosome.

Bicarbonate is present in the alveolar fluid both as a result of formation from CO_2 in the lungs and from HCO_3^- in the blood (*3*).

Table 1. *In vitro* dissolution rates of ^{57}Co from $^{57}Co_3O_4$ and ^{57}Co-FAP, percent initial activity dissolved/day × 10^3.

pH		4.5	6	7.2	4.5	6	7.2	4.5	6	7.2	4.5	6
Bicarbonate, mM		18	18	18	18	18	18	18	18	18	27	27
Citrate, mM	Day	0.07	0.07	0.07	0.14	0.14	0.14	0.2	0.2	0.2	0.07	0.07
$^{57}Co_3O_4$	7	66.1	57.2	49.1	86.0	53.9	52.4	79.8	82.0	104.8	109.8	73.9
	14	21.9	9.06	24.0	18.8	29.5	37.0	42.0	99.7	17.5	24.0	11.8
	21	33.1	7.56	7.54	11.4	3.86	8.30	23.9	22.7	18.9	40.4	6.05
	28	25.1	7.25	3.61	20.1	7.82	6.98	28.0	17.3	8.46	41.2	12.5
	35	25.1	3.15	2.87	8.39	4.99	3.88	34.6	29.8	5.40	31.0	5.20
	42	16.4	3.31	1.56	20.0	5.75	7.95	39.9	32.3	4.28	21.8	3.50
	49	17.6	2.21	1.56	12.8	ND	12.5	43.4	25.8	4.28	20.5	4.98
	56	8.82	2.60	0.74	13.6	3.96	5.83	23.3	14.1	5.30	16.7	2.55
	63	6.96	ND	ND	9.11	3.20	ND	17.9	14.9	2.45	15.2	2.02
	70	6.96	ND	ND	9.92	3.58	ND	17.9	11.3	1.43	9.94	ND
	77	5.50	1.10	ND	11.72	6.78	0.44	23.8	7.27	2.45	8.07	3.06
	84	7.37	1.89	0.82	10.82	3.58	2.91	28.9	9.72	5.40	9.84	2.53
	91	7.61	2.84	1.72	12.2	ND	3.71	36.4	8.33	8.15	12.3	3.29

pH		7.2	4.5	6	7.2	4.5	6	7.2	4.5	6	7.2	4.5
Bicarbonate, mM		27	27	27	27	27	27	27	30	30	30	30
Citrate, mM	Day	0.07	0.14	0.14	0.14	0.2	0.2	0.2	0.07	0.07	0.07	0.14
	7	97.7	83.5	54.3	65.8	75.0	94.8	59.3	99.1	62.5	62.1	123.1
	14	30.7	32.4	21.3	26.5	36.8	9.5	23.5	1.47	7.0	19.9	13.2
	21	56.3	30.3	3.97	17.9	29.0	27.9	2.60	8.14	10.5	12.1	38.0
	28	21.2	23.2	8.49	10.5	34.2	17.2	25.7	26.6	9.19	7.26	42.2
	35	8.86	35.1	5.22	4.05	43.1	11.7	13.5	30.4	2.95	3.46	25.6
	42	7.49	25.9	2.57	1.98	34.9	12.5	7.42	27.8	3.54	5.25	45.0
	49	15.0	38.3	5.53	7.03	38.4	12.0	14.8	32.0	3.54	3.13	37.2
	56	17.0	37.4	3.66	2.73	36.3	8.11	10.5	25.0	1.60	2.12	30.4
	63	9.07	55.8	1.87	2.40	23.0	2.94	2.22	17.5	1.60	ND	24.5
	70	5.06	61.8	1.87	3.14	20.6	10.8	11.6	14.6	1.18	ND	18.1
	77	3.06	43.4	ND	1.98	13.3	2.94	3.672	12.8	0.84	ND	13.4
	84	2.53	65.0	3.35	2.40	11.5	4.87	6.21	10.8	ND	2.12	18.5
	91	3.27	46.5	3.66	2.56	10.2	4.26	4.82	10.7	3.96	3.46	14.4

pH		6	7.2	4.5	6	7.2	4.5	6	7.2
Bicarbonate, mM		30	30	30	30	30	0	0	0
Citrate, mM	Day	0.14	0.14	0.2	0.2	0.2	0	0	0
	7	126.5	42.9	75.3	70.5	61.7	81.8	93.6	32.2
	14	14.7	17.7	17.7	5.52	31.4	16.3	5.04	4.51
	21	24.1	10.2	10.0	3.55	10.3	26.3	ND	33.3
	28	13.9	4.16	21.1	8.99	8.91	24.2	5.17	3.42
	35	21.1	3.21	12.1	4.57	5.17	58.1	2.39	2.02
	42	10.3	4.45	18.1	5.60	9.71	27.1	ND	1.40
	49	6.79	4.16	17.0	5.20	3.74	37.8	11.3	41.8
	56	4.04	1.75	26.6	6.31	5.08	10.7	2.52	1.40
	63	1.44	1.02	25.3	2.68	3.38	5.20	ND	ND
	70	1.44	1.39	5.20	1.50	2.14	15.6	1.33	3.73
	77	1.44	1.02	9.39	0.39	1.25	4.23	ND	ND
	84	3.47	2.77	26.58	3.78	4.72	6.72	1.86	1.55
	91	5.20	3.07	19.2	5.00	3.74	7.37	3.45	1.55

pH		4.5	6	7.2
Bicarbonate, mM		30	30	30
Citrate, mM	Day	0.2	0.2	0.2
^{57}Co-FAP	7	1546.8	1346.2	1534.9
	14	47.8	87.5	94.6
	21	20.3	50.5	83.6
	28	33.6	36.0	81.8
	35	24.2	24.2	56.3
	42	25.1	24.5	43.9
	49	26.3	19.8	45.4
	56	19.4	22.2	35.6
	63	14.6	10.9	222.6
	70	34.4	14.6	47.4
	77	16.6	14.7	27.0
	84	11.4	9.76	20.5
	91	13.2	16.5	30.8

Abbreviations: FAP, fused aluminosilicate particles; ND, activity in sample below detection limit (0.15 Bq).

Table 2. Summary of dissolution rates *in vitro* and translocation *in vivo* for ^{57}Co from ^{57}Co-FAP and $^{57}Co_3O_4$, fractional rates/day.

Material	Rate	System/reference
^{57}Co-FAP	1.8×10^{-4}	This study
	$8\text{–}12 \times 10^{-4}$	*In vivo* (*6*)
	1×10^{-3}	*In vitro* (*2*)
	$5\text{–}15 \times 10^{-4}$	*In vitro* (*18*)
$^{57}Co_3O_4$	1.8×10^{-4}	This study
	0	Cell culture medium (*9*)
	9×10^{-4}	Cell culture medium (*17*)
	2.1×10^{-3}	Dog alveolar macrophage culture (*9*)
	1.6×10^{-3}	Human macrophage culture (*9*)
	2.4×10^{-3}	Baboon macrophage culture (*17*)

FAP, fused aluminosilicate particles.

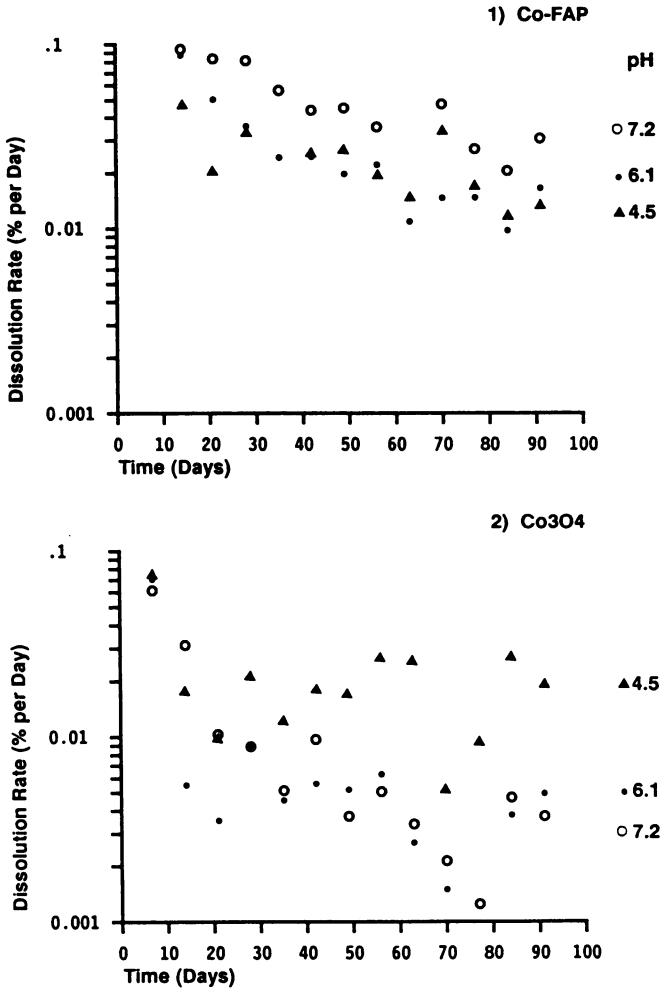

FIGURE 2. Effect of pH on the *in vitro* dissolution of $^{57}Co_3O_4$ and ^{57}Co-FAP (fused aluminosilicate particles) in 30 mM bicarbonate and 0.2 mM citrate.

Both of these sources represent large reservoirs for HCO_3^-, so its concentration is relatively constant and similar to that in the blood. Alveolar macrophage cell membranes have been shown to be permeable to HCO_3^- (*13*), so the assumption that cytoplasmic concentrations are similar to serum levels is not unfounded. Serum levels of HCO_3^- of 30–40 mM (*14*) and of 19–30 mM (*10*) have been measured for different animal species including man, dog, and rat. Simulated serum used for *in vitro* dissolution studies in flow-past systems contain HCO_3^- at 27 mM (*2*). For the purposes of this study, $NaHCO_3$ concentrations of 0, 18, 27, and 30 mM were used, covering the levels observed in the lung fluid, cytoplasm, and phagolysosome.

The factor that had the greatest effect on dissolution rates *in vitro* was pH. Citrate concentration had a small effect, and there was no effect of bicarbonate concentration. There was no evidence of synergism between these three factors. These results are in agreement with those of Kanapilly and Goh (*2*), who found that both pH and complexing agent concentration were factors affecting *in vitro* dissolution rates. The dependence of dissolution on pH was different for the two materials, with $^{57}Co_3O_4$ showing higher dissolution and ^{57}Co-FAP showing lower dissolution at low pHs. The effect of pH on $^{57}Co_3O_4$ dissolution is in agreement with the results of Kreyling and Ferron (*15*). Kanapilly and Goh (*2*) found that dissolution of FAP was higher at low pH. However, at high pHs, the dissolution of aluminosilicates increases (*16*), and so the observed differences between the pH dependency of the two materials are not unexpected.

This study was not designed to model dissolution *in vivo*, but some comparison of the absolute values of dissolution rates obtained *in vitro* with *in vivo* measurements is possible. The measurements of dissolution *in vitro* for ^{57}Co-FAP at pH 4.5 were lower than the measurements of urinary excretion of ^{57}Co seen after inhalation of ^{57}Co-FAP (*6*), which were taken to represent the rate of translocation of ^{57}Co from the lung. They were also lower than the results presented by other authors for *in vitro* dissolution rates.

The *in vitro* dissolution rate of $^{57}Co_3O_4$ at pH 4.5 and all citrate and bicarbonate concentrations was also lower than measured translocation rates of ^{57}Co from this material *in vivo* (*1*). Kreyling et al. (*9*) have found no further dissolution of $^{57}Co_3O_4$ particles beyond 10 hr in RPMI cell culture media. Lisarc et al. (*17*) have observed low dissolution rates of the same particles in cell culture medium. In their study of $^{57}Co_3O_4$ dissolution, Kreyling et al. also measured dissolution of these particles within cultured alveolar macrophages from both dogs and humans (*9*). The dissolution rates measured were in good agreement with the translocation rate of ^{57}Co from this material measured *in vivo* (*1*). Similar results have been reported by Lisarc et al. (*17*) for dissolution of the same particles in baboon macrophages. Thus, indications are that $^{57}Co_3O_4$ is relatively insoluble both in culture medium and the simple solutions studied here. Since considerably higher solubility of the particles is seen in alveolar macrophages in culture or *in vivo*, factors in addition to pH and complexing agents are likely to be involved in particle dissolution within phagocytic cells.

In conclusion, these experiments have shown that pH can significantly affect dissolution rates *in vitro* and may be important in determining translocation rates *in vivo*, although other factors must also be involved.

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