The Uptake of Silicic Acid by Rat Liver Mitochondria

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1. To gain insight into a putative role for mitochondria in silicon metabolism, mitochondrial uptake (by which it is meant the removal from the medium) of silicic acid $[\text{Si}(\text{OH})_4]$ was studied under conditions minimizing $\text{Si}(\text{OH})_4$ polymerization. 2. Measurements of mitochondrial respiration and swelling indicated indirectly a significant uptake of $\text{Si}(\text{OH})_4$ as a weak acid, but this was not confirmed when $^{31}\text{Si}(\text{OH})_4$ was used as a tracer. $^{31}\text{Si}(\text{OH})_4$ occupied a mitochondrial volume similar to that of $^3\text{H}_2\text{O}$ and was relatively unaffected by mitochondrial energy status and by the pH gradient across the mitochondrial inner membrane. 3. Uptake was directly proportional to $\text{Si}(\text{OH})_4$ concentration in the range 0–3 mM. 4. The uptake consisted of two components: under all conditions examined, the greater quantity, amounting to 1–2 nmol of $\text{Si}(\text{OH})_4$ /mg of mitochondrial protein, was bound, a major portion of it external to the inner membrane, with the lesser quantity free within the matrix space. 5. Equilibration of $^{31}\text{Si}(\text{OH})_4$ between medium and matrix was a slow process, having a half-time of approx. 10 min at 22°C. 6. Mersalyl and *N*-ethylmaleimide inhibited the uptake by preferentially lowering the amount of $\text{Si}(\text{OH})_4$ bound. Their action was somewhat variable, depending on the precise nature of the suspending medium, and suggesting that the bound material may represent polymerized forms of $\text{Si}(\text{OH})_4$. 7. It is concluded that $\text{Si}(\text{OH})_4$ may penetrate the mitochondrial inner membrane by a simple diffusion mechanism.

Although the siliceous nature of the cell wall of diatoms has been recognized for decades, that silicon is required nutritionally by organisms widely distributed throughout both biological kingdoms, and especially by higher animals, has been established only recently [see Carlisle (1974) and Schwarz (1974) for reviews]. In particular, in rats and chicks, silicon has been shown to be necessary for normal bone formation, and the lesion induced by silicon deprivation has been traced to an insufficient synthesis of mucopolysaccharides needed for connective-tissue assembly before calcification (Carlisle, 1974). Further, a role for mitochondria in silicon metabolism has been suggested by the finding of a relative abundance of silicon in mitochondria from diatoms (Mehard *et al.*, 1974), osteoblasts (Carlisle, 1976) and rat liver, kidney and spleen, silicon-containing granules being observed in the matrices of isolated mitochondria from the last group (Mehard & Volcani, 1976).

The present study seeks to elucidate the mechanism by which silicon in water-soluble form as orthosilicic

acid $[\text{Si}(\text{OH})_4]$ enters the mitochondrial matrix, and in this way attempts to extend previous work on the transport of $\text{Si}(\text{OH})_4$ into intact diatom cells (Azam *et al.*, 1974). For this purpose, $\text{Si}(\text{OH})_4$ has been used under conditions minimizing its polymerization (see Iler, 1955), since silicon is found in body fluids at concentrations not exceeding 5 p.p.m. (Carlisle, 1974), i.e. equivalent to approx. 0.2 mM- $\text{Si}(\text{OH})_4$, which at physiological pH probably exists as the monomeric species entirely. In addition, since it is difficult to isolate intact mitochondria in quantity from cells (e.g. diatoms) and tissues associated with mineralization, and since a physiological role for liver mitochondria is suggested by the finding of silicon-containing granules within their matrix space (Mehard & Volcani, 1976), the use of isolated rat liver mitochondria was considered appropriate for the present study.

The results suggest that $\text{Si}(\text{OH})_4$ diffuses slowly into the mitochondrial matrix compartment, possibly as the undissociated acid. However, the interpretation of the data is made less certain by the limited nature of the overall uptake (i.e. removal from the medium by whatever mechanism), by the finding that the major component of the overall uptake is bound to sites of low affinity, and by the variable effects of certain inhibitors, implying uptake of more than one species of $\text{Si}(\text{OH})_4$.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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Experimental

Methods

Preparation of mitochondria. Rat liver mitochondria were prepared by the method of Chappell & Hansford (1972) in a medium comprising 0.25M-sucrose / 10mM-Tris / Hepes / 1mM-EGTA, pH 7.4. Mitochondrial protein was measured by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Incubation of mitochondria. Two types of incubation medium were used. In experiments where $\text{Si}(\text{OH})_4$ was unlabelled, the medium contained 0.25M-sucrose/20mM-Tris/Hepes plus either 3mM- Na_2SiO_3 or 6mequiv. of Na^+ /litre as Cl^- or SO_4^{2-} depending on whether the $\text{Si}(\text{OH})_4$ -containing medium was adjusted to pH 7.2 with HCl or H_2SO_4 . When $^{31}\text{Si}(\text{OH})_4$ was used, initially a medium containing 0.129M-LiCl / 25.8mM-Tris / Hepes / 12.9mM-sucrose of pH 7.1 (approx.) was prepared, to which was added sufficient $^{31}\text{Si}(\text{OH})_4$ solution (see below) to give finally 0.1M-LiCl/20mM-Tris/Hepes / 10mM-sucrose/3mM- $\text{Si}(\text{OH})_4$ plus 17mequiv. of Na^+ /litre. After adjustment of the pH to 7.2 with HCl, [^{14}C]-sucrose and $^3\text{H}_2\text{O}$ were added to give approx. 0.1 $\mu\text{Ci}/\text{ml}$ and 0.5 $\mu\text{Ci}/\text{ml}$ respectively. The latter is referred to in the text as basal medium. It was varied in only one instance (see Fig. 3), when it was mixed with a corresponding solution lacking $\text{Si}(\text{OH})_4$ but containing 17mM-NaCl to produce concentrations of $\text{Si}(\text{OH})_4$ lower than 3mM. Unless stated otherwise, the temperature of incubation was 25°C.

Measurement of O_2 partial pressure. A Clark-type oxygen electrode monitored the O_2 partial pressure in a thermostatically controlled closed cell for quantitative purposes, and in an open cell where it was used only in a qualitative manner.

Measurement of mitochondrial swelling. The time-course of the absolute change in mitochondrial volume is reflected in the change in absorbance of the mitochondrial suspension (Tedeschi & Harris, 1955). By using a Beckman-Gilford recording spectrophotometer fitted with a thermostatically controlled cell housing and a wavelength of 650 nm, changes in mitochondrial volume were monitored continuously.

Separation of mitochondria from the suspending medium and measurement of associated radioactivity. Mitochondria were separated from the suspending medium by one of two methods. When it was desired to preserve a particular energetic state, the silicone-oil-centrifugation procedure of LaNoue *et al.* (1973) was used as described previously (Johnson & Hansford, 1977). In other experiments where rotenone-poisoned mitochondria were used, centrifugation of 300 μl samples was carried out for 1 min in a Beckman microcentrifuge (model 152) or with 1 ml samples in an Eppendorf microcentrifuge (model

3200) for 1 min. Larger samples (2 ml or greater) were separated rapidly in a Sorvall centrifuge (model RC2-B) by accelerating maximally to 19000g and immediately braking maximally. After removal of the supernatant fraction as completely as possible, all pellets were resuspended in 2.5 ml of water in vials, 8 ml of Aquasol was added and the resulting gels were analysed for ^{31}Si , ^{14}C and ^3H in a Beckman scintillation counter (model LS-230). Since ^{31}Si has a short half-life (156 min) and a β -particle emission of high energy, its radioactivity was counted first, and a discriminator was used that excluded ^{14}C and ^3H completely. After 18 h had elapsed (by which time the ^{31}Si contribution was negligible), the samples were analysed for ^{14}C and ^3H . All counts were corrected for background and quenching; in addition the ^{31}Si counts were corrected for decay and the ^3H counts for cross-contamination from ^{14}C .

Measurement of mitochondrial spaces and assessment of $^{31}\text{Si}(\text{OH})_4$ uptake. By including standard samples containing a known volume of medium (usually 25 μl), experimental radioactivities (c.p.m.) were readily expressed as an equivalent volume of medium or space. Since [^{14}C]sucrose is excluded by the mitochondrial inner membrane and $^3\text{H}_2\text{O}$ distributes rapidly throughout the entire mitochondrial water (see Chappell, 1968), the volume of mitochondrial matrix water was obtained by difference. Similarly the $^{31}\text{Si}(\text{OH})_4$ space was corrected for the [^{14}C]sucrose contribution to give the extent of $^{31}\text{Si}(\text{OH})_4$ uptake.

Measurement of the pH gradient across the mitochondrial inner membrane. The ion-distribution method of Nicholls (1974) was used with the single modification that the mitochondria were separated by centrifugation through silicone oil as described above. Duplicate incubations were performed for each condition: one contained [^{14}C]sucrose and $^3\text{H}_2\text{O}$ so that the matrix volume could be derived (see above), and the other contained 25 μM -[^{14}C]methylamine (approx. 0.1 $\mu\text{Ci}/\text{ml}$) and 10 μM -[^3H]acetate (approx. 0.3 $\mu\text{Ci}/\text{ml}$). Spaces were calculated for each radioactive isotope (see above), giving the values c for methylamine, h for acetate and m for the matrix spaces respectively. By analogy with a previously described approach (Johnson & Hansford, 1977), an absolute value (N) was calculated for $(h-c)/m$. The magnitude of ΔpH was then given exactly by $\log[(N+\sqrt{N^2+4})/2]$, and the sign of ΔpH depended on the sign of $(h-c)/m$: defining ΔpH as $\text{pH}_{\text{medium}} - \text{pH}_{\text{matrix}}$, for $(h-c)/m > 0$, $\Delta\text{pH} < 0$, and vice versa.

Preparation and characterization of submitochondrial fractions. The mitochondrial inner membrane was removed preferentially by treatment with digitonin essentially as described by Schnaitman *et al.* (1967) and by Hoppel & Cooper (1968). Thus mitochondria (90 mg of protein) were suspended in 1 ml of 0.25M-sucrose to which was added 100 μl of a

digitonin solution [10% (w/v) in 0.25M-sucrose]. The material was stirred for 20 min at 0°C, then diluted to 5 ml with ice-cold 0.25M-sucrose and centrifuged at 12000g for 10 min. The supernatant fraction was carefully removed for analysis and is referred to as fraction I. The pellet was resuspended in 3 ml of 50 mM-potassium phosphate, pH 7.6, and sonicated by using a Branson sonifier operated at maximum power output on setting 2: three 15 s bursts of sonication were used, 1 min elapsing between each burst, and an ice/water mixture provided cooling throughout. The sonicated material was centrifuged at 100000g for 40 min, and the supernatant fraction (fraction III) and pellet (fraction II) were separated. All fractions were assayed for malate dehydrogenase (representative of the mitochondrial matrix), cytochrome *c* oxidase (representative of the mitochondrial inner membrane) and monoamine oxidase (representative of the outer membrane) essentially as described by Schnaitman *et al.* (1967). Recoveries of enzyme markers were close to 100%, except for the cytochrome *c* oxidase activity, which showed some loss (20%) after sonication. This may have been due to partial inversion of the particles causing the oxidase to become latent. In any event little cytochrome *c* oxidase was recovered in fractions other than fraction II, so that any error of this sort was insignificant. Since it was desired to measure $^{31}\text{Si}(\text{OH})_4$ binding in this type of experiment and considerable dilution of material was involved in the fractionation procedure, samples were run in parallel to correct for any simple reversibility of binding that otherwise would contribute to fraction I. Thus samples lacking digitonin were treated in a similar fashion and analysed for ^{31}Si and ^{14}C so as to correct the data for fraction I. Other fractions were not corrected in this manner, since most of the $^{31}\text{Si}(\text{OH})_4$ was recovered in fraction I, and error elsewhere would not have affected the conclusions.

Preparation of $\text{Si}(\text{OH})_4$ solutions. Unlabelled $\text{Si}(\text{OH})_4$ was prepared by dissolving $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ in the incubation medium to give a final concentration of 3 mM. The medium was then adjusted to pH 7.2 by dropwise addition of 1 M-HCl with constant stirring. $^{31}\text{Si}(\text{OH})_4$ was prepared as previously described (Azam *et al.*, 1974), except that the stock solution was made up to 100 ml and contained 13.3 mM- $\text{Si}(\text{OH})_4$. Neutralization of the concentrated stock solution gave rise to polymerization of $\text{Si}(\text{OH})_4$, which was apparent for several hours after dilution to 3 mM. In general therefore the stock solution was kept at alkaline pH until diluted to 3 mM in the incubation medium, when it was adjusted to pH 7.2. The medium prepared in this way contains 17 mequiv. of Na^+ /litre arising from the stock $\text{Si}(\text{OH})_4$. The activity of the 3 mM- $^{31}\text{Si}(\text{OH})_4$ solution immediately after preparation was approx. 0.2 $\mu\text{Ci}/\text{ml}$ or 70 mCi/mol. Samples were analysed within 5 h of the

solution's preparation, i.e. within two half-lives of ^{31}Si .

Assay of $\text{Si}(\text{OH})_4$. $\text{Si}(\text{OH})_4$ was assayed by the method of Tüma (1962). Although the method relies on a reaction with molybdate at low pH, Alexander (1953) has pointed out that this is only relatively specific for monomeric $\text{Si}(\text{OH})_4$ and does not exclude the possibility of reaction with the dimeric species. Species of higher degrees of polymerization were assayed by the same method (Tüma, 1962) after treatment with 2 M-NaOH and heating at 100°C for 15 min to effect depolymerization.

Materials

Carbonyl cyanide *m*-chlorophenylhydrazone was obtained from Calbiochem, Los Angeles, CA, U.S.A., and rotenone and valinomycin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; these compounds were added as ethanolic solutions. Digitonin was from K & K Laboratories, Irvine, CA, U.S.A. Mersalyl [*o*-(3-hydroxymercuri-2-methoxypropyl)-carbamoylphenoxycetic acid] and *N*-ethylmaleimide were from Sigma Chemical Co. Radioactive chemicals were from ICN, Irvine, CA, U.S.A., except for $^{31}\text{SiO}_2$, which was obtained by subjecting a sample of Specpure-grade SiO_2 from Johnson Matthey Chemicals, London E.C.1, U.K., to neutron bombardment carried out by Gulf Energy and Environmental Systems, San Diego, CA, U.S.A. Also ^{68}Ge and Aquasol were obtained from New England Nuclear, Boston, MA, U.S.A. Dextran T-40 and Ficoll were from Pharmacia, Uppsala, Sweden. Crystalline bovine serum albumin was obtained from Sigma Chemical Co.

Except as noted above, all reagents were dissolved in double-glass-distilled water, and were of the highest grade available. Wherever possible plastic ware was used for handling and storing solutions, to minimize the contribution of stray $\text{Si}(\text{OH})_4$.

Results

Effects of $\text{Si}(\text{OH})_4$ on mitochondrial respiration and swelling

Since the effects of $\text{Si}(\text{OH})_4$ on mitochondrial metabolism have not been studied extensively (but see Kersten *et al.*, 1958, for some early observations), initial experiments were directed towards establishing whether $\text{Si}(\text{OH})_4$ could interact in any way with the enzymes involved in oxidative phosphorylation, as suggested for the bacterium *Proteus mirabilis* (Heinen, 1967). It should be emphasized that in the present work $\text{Si}(\text{OH})_4$ was used at a concentration not exceeding 3 mM to minimize its polymerization (see Rowsell & Leonard, 1958; confirmed in this laboratory) and participation of multiple species of $\text{Si}(\text{OH})_4$. Also, it is worth noting that although

Fig. 1. Measurements of rates of mitochondrial respiration and swelling

For the measurement of respiration rate (a) approx. 7 mg of mitochondrial protein (M) was added to 1.9 ml of a sucrose medium (see the Experimental section) saturated with air at 25°C and containing 5 mM-sodium succinate, 4 µg of rotenone and 0.5 µM-valinomycin. After 1.2 min, K⁺ ions (as Cl⁻ or SO₄²⁻, as appropriate) were added to a final concentration of 5 mequiv./litre. The final steady-state rate of respiration was measured as indicated by the broken line. For the measurement of swelling (b) the conditions were as for (a) except that approx. 2 mg of mitochondrial protein were suspended in a final volume of 2.5 ml. After a steady absorbance reading was achieved, K⁺ ions were added to a final concentration of 5 mequiv./litre at arrow 1 and rapid swelling ensued. The initial rate of swelling was measured directly by eye (----) and by calculating the absorbance change in the first 20 s, i.e. between arrows 1 and 2.

solid Na₂SiO₃ was the form of silicate added to the incubation media in these experiments, the meta-silicate ion SiO₃²⁻ does not exist in solution, the simplest silicate ion in an aqueous environment being the ortho form, SiO₄⁴⁻ (Carman, 1940).

With glutamate plus malate, β-hydroxybutyrate or succinate as respiratory substrate, no effect of Si(OH)₄ was discerned on the phosphorylation of added ADP or on the uncoupled oxidation of these substrates. However, in experiments designed to test whether Si(OH)₄ might be translocated into the mitochondrial matrix as a weak acid species like acetate or P_i (Chappell & Crofts, 1966), some small differences caused by the presence of Si(OH)₄ were observed. The experimental system made use of the fact that when added to respiring mitochondria K⁺ ions, in the presence of valinomycin, plus a penetrant weak acid species cause rapid mitochondrial respiration and swelling owing to salt accumulation (Chappell & Crofts, 1966). Sucrose was used as the bulk osmotic support, since in ionic media more rapid rates of respiration were observed, possibly masking any increment caused by Si(OH)₄. In addition, the control media contained an equivalent concentration

Table 1. Effect of Si(OH)₄ on mitochondrial respiration and swelling

Rates of respiration and swelling were measured as described in Fig. 1 in media containing 3 mM-Na₂SiO₃ neutralized with either HCl or H₂SO₄; the corresponding control media contained either 6 mM-NaCl or 3 mM-Na₂SO₄ as appropriate. The values presented are means ± S.E.M. for the increment in rate caused by Si(OH)₄ expressed as a percentage of the control; they are derived from means of duplicate determinations on each of five mitochondrial preparations. *P* values were determined by Student's *t* test.

Measurement	Increment by Si(OH) ₄ (%)	<i>P</i>
Respiration	12.4 ± 0.7	<0.001
Swelling	30.6 ± 5.5	<0.01

of NaCl or Na₂SO₄, depending on whether the Si(OH)₄-containing medium was neutralized with HCl or H₂SO₄; correspondingly K⁺ ions were added as Cl⁻ or SO₄²⁻ to maintain anionic homogeneity. The methods of measurement are illustrated in Fig. 1 to clarify their nature and time relations: when respiration was monitored (Fig. 1a), a slowly

increasing rate was observed, reaching a steady state, which was readily measured, approx. 2 min after the addition of K^+ ; the most reproducible characteristic of the swelling experiments was the initial rate, which was measured by two methods (Fig. 1b), which gave very similar results. Since there was no appreciable difference between experiments conducted in the presence of Cl^- and those with SO_4^{2-} , consistent with a specific effect of $Si(OH)_4$, the data were pooled and are presented as such in Table 1. Both types of measurement gave a significant increment, due to the presence of $Si(OH)_4$ (Table 1), suggesting that active accumulation of $Si(OH)_4$ was responsible; although respiration was affected to a lesser extent, it was the more accurate of the two types of measurement and the more reproducible.

Direct measurement of $Si(OH)_4$ uptake

The experiments described above indicated indirectly that $Si(OH)_4$ was transported to a limited extent and in a manner dependent on mitochondrial energy status. This was investigated directly by using a radioactive tracer of $Si(OH)_4$. Initially $^{68}Ge(OH)_4$ was used, since this radioactive isotope is available commercially, and in diatoms has been found to mimic $Si(OH)_4$ (Azam, 1974). However, in the mitochondrial system it was unsuitable for the following reasons: (i) $^{68}Ge(OH)_4$ uptake was independent of $Si(OH)_4$ concentration in the range 0–3 mM; (ii) $^{68}Ge(OH)_4$ uptake showed little dependence on the degree of structural integrity of the mitochondria or on the amount of mitochondrial protein present in the incubation medium. For these reasons, it became necessary to use $^{31}Si(OH)_4$, a radioactive isotope of short half-life (156 min) and obtainable only at a low specific radioactivity.

Care was required in the handling of the stock $^{31}Si(OH)_4$ solution to avoid polymerization of the $Si(OH)_4$. The effect of polymerization was most dramatically illustrated when frozen-thawed mitochondria were incubated with $^{31}Si(OH)_4$ (Fig. 2). When the $^{31}Si(OH)_4$ -containing medium was prepared in such a manner (see the Experimental section) that polymerization was readily detected (at least 20% of total silicon as a polymer) the amount of $^{31}Si(OH)_4$ taken up was large and relatively independent of protein concentration (Fig. 2). However, when the $^{31}Si(OH)_4$ -containing medium had an undetectable (less than 5%) content of polymerized species, the amount taken up was much less and was now directly proportional to protein concentration (Fig. 2). In both cases, the $[^{14}C]$ sucrose and 3H_2O spaces were identical and gave values very similar to those of unpolymerized $^{31}Si(OH)_4$ when expressed in terms of occupied volume (Fig. 2), indicating the lack of a permeability barrier for sucrose and the

Fig. 2. Effect of $Si(OH)_4$ polymerization on $^{31}Si(OH)_4$ associated with frozen-thawed mitochondria

A mitochondrial suspension containing approx. 65 mg of protein/ml was frozen and thawed slowly three times. Appropriate volumes of this suspension were added to 2 ml of basal medium (see the Experimental section) containing 3 mM $^{31}Si(OH)_4$ prepared under conditions giving appreciable (■) or undetectable (●) polymerization as judged by molybdate reactivity (see the Experimental section). The medium contained $[^{14}C]$ sucrose (Δ) and 3H_2O ($\square$) as markers of excluded and total volumes respectively. Samples (300 μ l) were withdrawn, and centrifuged in a Beckman microcentrifuge after 1, 5 and 10 min. The pellets were analysed for ^{31}Si , ^{14}C and 3H as described in the Experimental section, and the displayed values are means of the pooled determinations. The lines through the points were drawn by eye. Note the discontinuity in the ordinate.

presumption of extensive binding of the polymerized species. Thus in all succeeding experiments, the stock $^{31}Si(OH)_4$ solution was handled in such a way that polymerization was minimized (see the Experimental section for further details).

Effect on $Si(OH)_4$ uptake of mitochondrial energy status and pH gradient across the mitochondrial inner membrane

The effect of mitochondrial energy status on $Si(OH)_4$ uptake was investigated by using a silicone-

oil-centrifugation technique, since such a method has been shown to preserve mitochondrial energy states until complete separation of organelles from the suspending medium has been accomplished (Davis & Lumeng, 1975; Johnson & Hansford, 1977). Control experiments with rotenone-treated mitochondria showed that neither the silicone oil nor the dextran added to the medium (LaNoue *et al.*, 1973) affected the results appreciably. However, for ease of separation of the mitochondria it was not possible to use the sucrose-based medium of the respiration experiments described above. Accordingly LiCl was substituted for sucrose, since it was desired to manipulate the pH gradient across the mitochondrial inner membrane (ΔpH), which may be done successfully in a medium almost free of K^+ ions. The use of NaCl as a bulk osmotic support was avoided, since Na^+/H^+ -exchange activity in liver mitochondria is marked (Mitchell & Moyle, 1969) and might have compromised attempts to alter ΔpH (cf. Johnson & Hansford, 1977). By comparison, Li^+/H^+ exchange activity, as judged by mitochondrial swelling in iso-

osmolar lithium acetate, is very much slower (R. N. Johnson, unpublished work).

Two conditions were used extensively to assess the effect of energy status and ΔpH : the 'high-energy' state comprised a medium containing a low concentration of K^+ and valinomycin, designed to cause internal alkalinization (Nicholls, 1974), whereas in the 'low-energy' state, valinomycin and uncoupling agent were added to give internal acidification (Nicholls, 1974). The effect of these manoeuvres on $^{31}\text{Si}(\text{OH})_4$ uptake and mitochondrial matrix volume is shown in Table 2. In the energized condition ($\Delta\text{pH} = -0.91 \pm 0.04$ unit; mean $\pm$ S.E.M.; $n = 4$), the uptake of $\text{Si}(\text{OH})_4$ increased with time in a bi-phasic manner and was approximately twice that found in the de-energized condition after 10 min of incubation (Table 2). The de-energized condition ($\Delta\text{pH} = 0.60 \pm 0.01$ unit; mean $\pm$ S.E.M.; $n = 4$) showed a similar rapid uptake phase in the first 1 min, but apparently reached equilibrium soon afterwards (Table 2). Evidence adduced below is consistent with the notion that at least the initial rapid phase of uptake (in the first 1 min) was due to binding of $\text{Si}(\text{OH})_4$.

However, the effect of energy status on mitochondrial matrix volume was more dramatic, with matrix expansion in the energized and matrix shrinkage in the de-energized condition (Table 2). Thus the effect of energy on $\text{Si}(\text{OH})_4$ uptake may have been a consequence of more general salt accumulation and increased intramitochondrial volume rather than a specific effect on $\text{Si}(\text{OH})_4$ itself. In any event, the effect of energization and of the sign of ΔpH was not large and, in both conditions examined, the $^{31}\text{Si}(\text{OH})_4$ volume approximated to the $^3\text{H}_2\text{O}$ volume, emphasizing the limited extent of the observed uptake.

As a consequence of these findings, all subsequent experiments were performed with rotenone-poisoned mitochondria, since they provide a more uniform system on a day-to-day basis and are more easily amenable to experimental analysis. Table 2 shows that in the presence of rotenone alone the values tended to be similar to those in the de-energized condition, although ΔpH increased from -0.46 at 1 min to 0.05 unit at 10 min, in contrast with the other conditions, where it was stable throughout. In addition, a direct centrifugation procedure (i.e. without silicone oil) was adopted, since technically this is easier to carry out.

Although the effect of varying ΔpH was investigated as described above, a systematic study of the effect of varying the pH of the suspending medium was not done, as interpretation of such an experiment is not straightforward: inevitably, ionization of both $\text{Si}(\text{OH})_4$ and mitochondrial components will be changed and differences in uptake cannot be attributed to one rather than the other. Thus an external pH of 7.20–7.25 was used throughout as

Table 2. Effect of mitochondrial energy status on $^{31}\text{Si}(\text{OH})_4$ uptake

Mitochondria (9 mg of protein) were added to 2 ml of basal medium (see the Experimental section) containing 60 mg of Dextran T-40/ml. Samples were obtained at the stated times by centrifuging the mitochondria from 300 μl of incubation fluid through a layer of silicone oil (see the Experimental section); the times indicate when centrifugation was begun. In the 'energized' condition, the medium was saturated with O_2 and contained 5 mM-succinate, 4 μg of rotenone, 1 mM-ATP, 0.5 μM -valinomycin and 0.5 mM-KCl. Aerobiosis was ensured by blowing O_2 at the surface of the incubation medium and by monitoring the O_2 content with an oxygen electrode. In the 'de-energized' condition, the medium contained 4 μg of rotenone, 0.5 μM -valinomycin and 1 μM -carbonyl cyanide *m*-chlorophenylhydrazone. When rotenone alone was used, the medium contained 4 μg of rotenone and no other addition. The values are means $\pm$ S.E.M. for $^{31}\text{Si}(\text{OH})_4$ and $^3\text{H}_2\text{O}$ spaces corrected for [^{14}C]sucrose contribution from four mitochondrial preparations, except for the condition with rotenone alone, where mean values from two mitochondrial preparations are shown.

Time (min)	Marker	Space (μl)		
		Energized	De-energized	Rotenone
1	$^{31}\text{Si}(\text{OH})_4$	0.50 ± 0.08	0.46 ± 0.23	0.73
	$^3\text{H}_2\text{O}$	0.88 ± 0.09	0.46 ± 0.04	0.57
5	$^{31}\text{Si}(\text{OH})_4$	0.90 ± 0.15	0.73 ± 0.08	1.34
	$^3\text{H}_2\text{O}$	1.06 ± 0.06	0.42 ± 0.02	0.48
10	$^{31}\text{Si}(\text{OH})_4$	1.21 ± 0.09	0.63 ± 0.10	0.86
	$^3\text{H}_2\text{O}$	1.07 ± 0.05	0.38 ± 0.04	0.37

Fig. 3. Uptake of $^{31}\text{Si}(\text{OH})_4$ as a function of $^{31}\text{Si}(\text{OH})_4$ concentration

Mitochondria (20mg of protein) were added to 4ml of incubation fluid comprising appropriate volumes of stock basal media (see the Experimental section) with or without 3mM- $\text{Si}(\text{OH})_4$ plus 4 μg of rotenone. Samples (1ml, in triplicate) were centrifuged in an Eppendorf microcentrifuge after 10min of incubation. The pellets were analysed for ^{31}Si and ^{14}C (added as sucrose) as described in the Experimental section. The values shown are means of the triplicate determinations corrected for $[^{14}\text{C}]\text{sucrose}$ contribution (see the Experimental section) and are derived from two mitochondrial preparations. The line was constructed by using linear regression analysis ($r = 0.911$).

representative of that found for liver cytosol (Iles *et al.*, 1977).

Uptake of $^{31}\text{Si}(\text{OH})_4$ as a function of $\text{Si}(\text{OH})_4$ concentration

The overall uptake of $^{31}\text{Si}(\text{OH})_4$ measured after 10min of incubation was proportional to the concentration of $\text{Si}(\text{OH})_4$ in the suspending medium (Fig. 3). The line through the points was drawn by using linear regression analysis and is displaced from the origin chiefly because of a single determination with 1mM- $\text{Si}(\text{OH})_4$. It should be borne in mind that each of the displayed values was derived after subtraction of the $[^{14}\text{C}]\text{sucrose}$ volume and is subject to error on this account. The tentative conclusion was that the uptake appeared to be of low affinity, and clearly was most accurately measured at the highest concentration of $\text{Si}(\text{OH})_4$ compatible with lack of polymerization, namely 3mM. This concentration of $\text{Si}(\text{OH})_4$ was used in all succeeding experiments.

Fig. 4. Dependence of $^{31}\text{Si}(\text{OH})_4$ uptake on mitochondrial volume

Mitochondria (8.6mg of protein) were suspended in each of three tubes containing 2ml of medium of a particular osmolarity plus 4 μg of rotenone. The osmolarity of the suspending medium was varied in a systematic manner by mixing appropriate volumes of 0.12M-LiCl/20mM-Tris/Hepes/10mM- $[^{14}\text{C}]\text{sucrose}$ /17mM-NaCl/3mM- $^{31}\text{Si}(\text{OH})_4$, pH7.2, and a similar solution lacking LiCl. After 10min of incubation at room temperature (22°C), the mitochondria were separated by using a Sorvall centrifuge as described in the Experimental section. The pellets were analysed for ^{31}Si , ^{14}C and ^3H (as $^3\text{H}_2\text{O}$), and after calculation of the contribution of each radioactive isotope in the pellet as a space (see the Experimental section), the $[^{14}\text{C}]\text{sucrose}$ space was subtracted from both the total $^3\text{H}_2\text{O}$ and the total $^{31}\text{Si}(\text{OH})_4$ spaces. The resulting values are expressed as means $\pm$ S.E.M. for the triplicate determinations of $^3\text{H}_2\text{O}$ space ($\square$) and $^{31}\text{Si}(\text{OH})_4$ space ($\bullet$), and the lines through the points were drawn by linear regression analysis. (See Table 3 for further details.)

Assessment of free and bound components of $\text{Si}(\text{OH})_4$ uptake

Isolated mitochondria behave as osmometers (Chappell & Crofts, 1966), and use of this property was made in assessing the extent of binding of $\text{Si}(\text{OH})_4$ to mitochondria as well as in providing an estimate of the $\text{Si}(\text{OH})_4$ concentration free within the matrix compartment. Fig. 4 illustrates an experiment in which the osmolarity of the sus-

Table 3. Separation of $^{31}\text{Si}(\text{OH})_4$ uptake into free and bound components

Linear regression analysis was performed on the data in Fig. 4 and on data from a duplicate experiment with a different mitochondrial preparation. The intercepts on the ordinate (corrected for protein concentration), the gradients and the regression coefficients of the lines for $^{31}\text{Si}(\text{OH})_4$ and $^3\text{H}_2\text{O}$ are presented. Bound $\text{Si}(\text{OH})_4$ is considered to be given directly by the intercept of the line for $\text{Si}(\text{OH})_4$ on the ordinate when expressed as an amount, whereas the concentration of $\text{Si}(\text{OH})_4$ free within the matrix space is calculated as the ratio of the gradients of the lines, $\text{Si}(\text{OH})_4/\text{H}_2\text{O}$, multiplied by the external concentration of $\text{Si}(\text{OH})_4$, namely 3 mM.

Expt. no.	Intercept ($\mu\text{l}/\text{mg}$ of protein)		Gradient (μosmol)		Regression coefficient		$\text{Si}(\text{OH})_4$ uptake	
	$^{31}\text{Si}(\text{OH})_4$	$^3\text{H}_2\text{O}$	$^{31}\text{Si}(\text{OH})_4$	$^3\text{H}_2\text{O}$	$^{31}\text{Si}(\text{OH})_4$	$^3\text{H}_2\text{O}$	Bound (nmol/mg of protein)	Free in matrix (mM)
1	0.39	0.61	0.33	0.66	0.95	0.99	1.2	1.5
2	0.28	0.52	0.25	0.41	0.90	0.95	0.8	1.8

pending medium was varied systematically, causing a proportional change in matrix volume shown by the line for $^3\text{H}_2\text{O}$. The line does not pass through the origin, the intercept on the ordinate giving the osmotic dead-space, i.e. non-solvent water probably accounted for by the hydration sphere of macromolecules (Bentzel & Solomon, 1967). Determination of $^{31}\text{Si}(\text{OH})_4$ in the same samples produced a less-well-defined line (Fig. 4) caused by the inability to measure with sufficient accuracy the $^{31}\text{Si}(\text{OH})_4$ space. The line for $^{31}\text{Si}(\text{OH})_4$ does not pass through the origin either, in this case presumably because of $\text{Si}(\text{OH})_4$ binding. In addition, a comparison of the gradients of the two lines, i.e. a comparison of the degree to which the respective spaces are affected by osmolarity, gives an estimate of the degree of equilibrium of $\text{Si}(\text{OH})_4$ between the matrix space and the suspending medium. These results are presented in Table 3 together with data from a duplicate experiment performed at another time. Thus it was found (Table 3) that after 10 min of incubation at 22°C , approx. 1 nmol of $\text{Si}(\text{OH})_4$ was bound per mg of protein and that $\text{Si}(\text{OH})_4$ was equilibrated to the extent of approx. 55% in the matrix compartment relative to the bulk phase.

Two questions arose directly from this type of experiment: the location of the sites of $\text{Si}(\text{OH})_4$ binding and the reason for the lack of equilibration of $\text{Si}(\text{OH})_4$ between the medium and the matrix space. An attempt to localize the sites of binding was made first, as the bound $\text{Si}(\text{OH})_4$ represented the majority of the total uptake at the customary osmolarity used in other experiments (approx. 0.27 osM).

Localization of $^{31}\text{Si}(\text{OH})_4$ within submitochondrial fractions

The procedure adopted was simply to fractionate mitochondria previously exposed to 3 mM- $^{31}\text{Si}(\text{OH})_4$ for 10 min into outer membrane, inner membrane and matrix, and to examine each fraction for

$^{31}\text{Si}(\text{OH})_4$ content, measured relative to [^{14}C]sucrose as before. [The alternative approach of incubating $^{31}\text{Si}(\text{OH})_4$ with relatively pure samples of outer membrane, inner membrane and matrix was not attempted, since in the membrane fractions the problem of distinguishing free and bound material remains, and there is the more general concern of how the properties of the purified material relate to those of the isolated organelle. Admittedly, the procedure used here does not provide unambiguous information on inner-membrane and matrix fractions, owing to the likelihood of including unbound $\text{Si}(\text{OH})_4$, so that care was needed in interpreting the results.] The purity of each of the fractions was judged on the basis of marker enzymes (see the Experimental section) so that each fraction could be assigned a definite proportion of each of the three loci. These data from two independent experiments are shown in Table 4(a). One further correction was applied to the $^{31}\text{Si}(\text{OH})_4$ appearing in fraction I, since simple dilution of the starting material resulted in a loss of $^{31}\text{Si}(\text{OH})_4$, causing $^{31}\text{Si}(\text{OH})_4$ in fraction I to be overestimated. This correction was large (38% in Expt. 1 and 61% in Expt. 2) and is recorded (in Table 4b) as the proportion of the initial $^{31}\text{Si}(\text{OH})_4$ represented by the final values. Also shown in Table 4(b) are the results of solving the three simultaneous equations involving values from fractions I–III in Table 4(a) for the amount of $^{31}\text{Si}(\text{OH})_4$ associated with each locus. Although in one instance this procedure resulted in a negative quantity, possibly because $^{31}\text{Si}(\text{OH})_4$ did not follow precisely the position of the enzyme markers, the conclusion was that most of the bound material was in the outer-membrane fraction.

Effect of prolonged exposure to $^{31}\text{Si}(\text{OH})_4$

A second feature of the osmometer experiments detailed in Table 3 was the lack of equilibration of $^{31}\text{Si}(\text{OH})_4$ between the medium and the mitochondrial matrix. Conceivably this was a kinetic

Table 4. *Localization of $^{31}\text{Si}(\text{OH})_4$ within the mitochondrion*

Mitochondria were incubated in basal medium containing $0.6\text{ }\mu\text{g}$ of rotenone/ml at a protein concentration of 5 mg/ml . After 10 min of incubation at room temperature (22°C) the mitochondria were sedimented by centrifugation at $6000g$ for 10 min. The pellet was fractionated and the fractions were analysed for outer membrane, inner membrane and matrix in terms of marker-enzyme distribution as explained in the Experimental section. ^{31}Si and ^{14}C (as sucrose) were measured in each fraction, and the ^{31}Si -space was corrected for the ^{14}C space contributing to it. In one instance this procedure resulted in a negative corrected space for ^{31}Si , which is recorded as not detectable (n.d.). The data from two such experiments using different mitochondrial preparations are shown in part (a). In part (b) are recorded the results of solving the three simultaneous equations involving values from fractions I, II and III in part (a) for the ^{31}Si associated with each site. Note that simple dilution of the pellet material resulted in an appreciable loss of ^{31}Si from the pellet, which would increase the amount of ^{31}Si associated with fraction I. The values for each fraction I have been corrected for this non-specific removal of ^{31}Si (see the Experimental section) and the extent of the correction is indicated by the proportion of the corrected ^{31}Si represented by the sum of the three fractions relative to that in the initial pellet. Abbreviation: n.d., not determined.

Expt. no.	Fraction	Proportion of			$^{31}\text{Si}(\text{OH})_4$ (nmol/mg of mitochondrial protein)
		Outer membrane	Inner membrane	Matrix	
1	I	0.85	0.03	0.15	0.7
	II	0.15	0.97	0.30	0.2
	III	0	0	0.55	0.2
2	I	0.79	0.16	0.41	0.7
	II	0.21	0.84	0.18	0.1
	III	0	0	0.41	n.d.

Expt. no.	$^{31}\text{Si}(\text{OH})_4$ (nmol/mg of mitochondrial protein) associated with			Proportion represented (%)
	Outer membrane	Inner membrane	Matrix	
1	0.8	0.0	0.4	62
2	0.9	-0.1	n.d.	39

Table 5. *Effect of prolonged incubation on free and bound components of $^{31}\text{Si}(\text{OH})_4$ uptake*

Duplicate experiments using different mitochondrial preparations were performed exactly as described in Fig. 4, except that centrifugation was begun after 30 min of incubation rather than at 10 min. Linear regression analysis was carried out on the experimental values and the resulting values are presented as in Table 3.

Expt. no.	Intercept ($\mu\text{l/mg}$ of protein)		Gradient (μosmol)		Regression coefficient		Si(OH) $_4$ uptake	
	$^{31}\text{Si}(\text{OH})_4$	$^3\text{H}_2\text{O}$	$^{31}\text{Si}(\text{OH})_4$	$^3\text{H}_2\text{O}$	$^{31}\text{Si}(\text{OH})_4$	$^3\text{H}_2\text{O}$	Bound (nmol/mg of protein)	Free in matrix (mM)
1	0.48	0.62	0.44	0.42	0.91	0.98	1.4	3.1
2	0.58	0.66	0.36	0.44	0.93	0.98	1.9	2.5

limitation, and this proposal was tested by repeating the experiment described in Fig. 4 with an incubation time of 30 min rather than 10 min. The results of two such experiments are shown in Table 5. By using the assumptions described above, the analysis shows that the bound component was increased relative to that calculated at 10 min, but more noteworthy is the finding that $^{31}\text{Si}(\text{OH})_4$ was more nearly equilibrated (approx. 95%) after the prolonged incubation. Thus it appeared that $\text{Si}(\text{OH})_4$ diffusion into the matrix space was a slow

process and that 10 min of incubation was insufficient time to allow equilibration.

Inhibition of $\text{Si}(\text{OH})_4$ uptake

Initially as an attempt to examine whether the mitochondrial phosphate carrier was involved in $\text{Si}(\text{OH})_4$ permeation, the thiol-blocking reagents mersalyl and *N*-ethylmaleimide were tested as inhibitors of $\text{Si}(\text{OH})_4$ uptake, since these compounds are known to inactivate the phosphate carrier (Tyler,

Fig. 5. Inhibition of $^{31}\text{Si}(\text{OH})_4$ uptake by thiol-blocking reagents

Mitochondria (20 mg of protein) were preincubated at room temperature (22°C) with 250 μl of 0.12 M-LiCl/20 mM-Hepes, pH 7.2, containing various amounts of mersalyl (a) or *N*-ethylmaleimide (b) for 2 min. The total preincubation mixture was then added to 4 ml of basal medium containing 8 μg of rotenone, 1 ml samples were removed in triplicate and centrifuged 2 min later. The $^{31}\text{Si}(\text{OH})_4$ uptake (●) was corrected for [^{14}C]sucrose space, and the space inaccessible to $^{31}\text{Si}(\text{OH})_4$ (○) was obtained by subtracting the $^{31}\text{Si}(\text{OH})_4$ space from the $^3\text{H}_2\text{O}$ space. The data are mean values derived from the triplicate samples and were obtained with different mitochondrial preparations at different times.

1969; Johnson & Chappell, 1973). Incubations carried out for 10 min in the presence of mersalyl indicated substantial (approx. 50%) inhibition of $\text{Si}(\text{OH})_4$ uptake, with no greater effect when 100 nmol of mersalyl/mg of protein was used rather than 15 nmol/mg of protein. A more careful titration carried out at low concentrations of mersalyl for a shorter time (2 min) revealed the pattern of inhibition illustrated in Fig. 5(a), and a similar experiment with *N*-ethylmaleimide gave the results shown in Fig. 5(b). In both instances $^3\text{H}_2\text{O}$ was present in the incubation medium, so that inhibition could be judged by two criteria, namely the amount of $^{31}\text{Si}(\text{OH})_4$ taken up and the volume of the pellet water accessible to $^{31}\text{Si}(\text{OH})_4$. The opposite behaviour of these two indices gave a very clear indication of inhibition of $\text{Si}(\text{OH})_4$ uptake, maximal at a concentration of approx. 5 nmol of mersalyl/mg of protein (Fig. 5a), and at approx. 12 nmol of *N*-ethylmaleimide/mg of protein (Fig. 5b). Indeed, the inhibitory characteristics of the two compounds were strikingly similar to those found for the inhibition of the phosphate carrier (by mersalyl: Tyler, 1969; Meijer *et al.*, 1970; by *N*-ethylmaleimide: Johnson & Chappell, 1973; Coty & Pedersen, 1974). However, in the titration with *N*-ethylmaleimide (Fig. 5b) a systematic error in the determination of ^{31}Si may have been responsible for the overall displacements of the $^{31}\text{Si}(\text{OH})_4$ uptake to lower values and of the space inaccessible to $^{31}\text{Si}(\text{OH})_4$ to higher values (cf. Fig. 5a). Such an error may result from the three radioactive isotopes being assayed at different times (see the Experimental section).

Further experiments showed that by incubating mitochondria for 8 min with $^{31}\text{Si}(\text{OH})_4$ followed by 2 min exposure to mersalyl, a degree of inhibition (approx. 50%) was observed similar to that seen when mitochondria were preincubated with mersalyl and then exposed to $^{31}\text{Si}(\text{OH})_4$ (as in Fig. 5a). The interpretation was that mersalyl was capable of displacing $^{31}\text{Si}(\text{OH})_3$ previously taken up, and presumably from an external location, since $^{31}\text{Si}(\text{OH})_4$ was released into the external phase. In addition, it was demonstrated in an experiment of the type shown in Fig. 4 that mersalyl exerted a greater effect at high osmolarity (69% inhibition) than at low osmolarity (35% inhibition), the bound component being lowered to 0.3 nmol of $\text{Si}(\text{OH})_4$ /mg of protein with a calculated 1.1 mM- $\text{Si}(\text{OH})_4$ free within the matrix compartment (cf. Table 3). Thus mersalyl appeared to affect the bound component to an extent greater than that found for the component identified with the matrix space.

In all of the experiments with inhibitors reported above, there was a degree of variability that could not be controlled satisfactorily. Experiments with mersalyl were the most reproducible and account for most of the observations described; by contrast, inhibition by *N*-ethylmaleimide was found in far fewer instances, so that, for example, with a given preparation of mitochondria it was possible to demonstrate inhibition of $^{31}\text{Si}(\text{OH})_4$ uptake by mersalyl, but not by *N*-ethylmaleimide. Further, it was demonstrated that the variability was due to the particular $^{31}\text{Si}(\text{OH})_4$ -containing medium used for a given experiment: incubations done with the

same mitochondrial preparation in two identical media prepared independently from the same stock $^{31}\text{Si}(\text{OH})_4$ solution resulted in inhibition of $^{31}\text{Si}(\text{OH})_4$ uptake by mersalyl in one case and not in the other. The possible implications of these findings are explored in the Discussion section.

Discussion

The purpose of the present work was to determine the mode of penetration of $\text{Si}(\text{OH})_4$ into mitochondria to assess better the role of mitochondria in silicon metabolism. Although it is recognized that the behaviour towards silicon of mitochondria from soft tissues and from mineralizing cells may not be the same, if liver mitochondria are considered as generally representative, the results suggest that $\text{Si}(\text{OH})_4$ diffuses slowly into the matrix space in a manner relatively independent of mitochondrial energy status (Table 2) and of the pH gradient across the mitochondrial inner membrane, ΔpH . Considering that $\text{p}K_1$ of $\text{Si}(\text{OH})_4$ is 9.8 at 25°C (Greenberg & Price, 1957), the penetrating species is probably undissociated, and the lack of dependence of the uptake on ΔpH (with an external pH of 7.2 and an internal pH estimated to be not more than 8.2) is easily comprehended: in both phases, the undissociated acid will be the dominant form and, in mechanisms of accumulation driven by ΔpH , the concentration of an uncharged weak acid at equilibrium is the same on both sides of the inner membrane (Chappell & Crofts, 1966; Mitchell & Moyle, 1969). By contrast, the experiments in which unlabelled $\text{Si}(\text{OH})_4$ was used (Table 1) suggested that $\text{Si}(\text{OH})_4$ was responsible for an increased rate of mitochondrial swelling after salt uptake, and for an increased rate of respiration, probably as an indirect effect of salt accumulation, since this observation was made at a time when swelling was complete (Fig. 1). In view of the extremely limited uptake of $^{31}\text{Si}(\text{OH})_4$, it seems probable that these findings derive from a $\text{Si}(\text{OH})_4$ -induced change in the anion permeability of the mitochondrial inner membrane (perhaps increasing Cl^- and SO_4^{2-} permeability), possibly analogous to the deleterious effects of silica on erythrocytes (see Allison, 1968). However, proton permeability did not appear to be affected, as judged by the retention of coupling and respiratory control.

The rate of permeation of $\text{Si}(\text{OH})_4$ was judged to be slow (showing a half-time for equilibration of approx. 10 min at 22°C) in experiments where free and bound components could be determined (see Tables 3 and 5). In these experiments binding represented the greater portion of the uptake, and evidence from two different sources indicated that most of the binding was external to the inner membrane: (i) direct

fractionation of the mitochondria showed that the majority of $\text{Si}(\text{OH})_4$ recovered was in the outer-membrane fraction (Table 4); (ii) when mersalyl was added to mitochondria previously incubated with $\text{Si}(\text{OH})_4$, the displacement of $\text{Si}(\text{OH})_4$ from the pellet into the external phase indicated competition between $\text{Si}(\text{OH})_4$ and mersalyl for an external site. In addition, the limited association of $\text{Si}(\text{OH})_4$ with components within the inner membrane is consistent with a slow rate of $\text{Si}(\text{OH})_4$ penetration into the matrix compartment.

The experiments in which the mitochondria were fractionated to determine bound $\text{Si}(\text{OH})_4$ were limited in their resolution, owing to the removal of significant amounts of $^{31}\text{Si}(\text{OH})_4$ on dilution of the mitochondria (Table 4). This behaviour is compatible with the finding of a proportionality between overall uptake and external concentration (Fig. 3), consistent with binding of low affinity on the one hand and of simple diffusion into the matrix on the other.

Although mersalyl and *N*-ethylmaleimide, the inhibitors of $\text{Si}(\text{OH})_4$ uptake, showed characteristics (Fig. 5) similar to those observed for inhibition of the phosphate carrier, the variation found within a single experiment (i.e. inhibition by mersalyl but not by *N*-ethylmaleimide) argues against a role for the transporter. The variability noted both within a series and between experiments carried out at different times can best be explained on the basis of different polymeric forms of $\text{Si}(\text{OH})_4$ being present in differing proportions from incubation medium to another. Such polymers may result from the neutralization of the media owing to local acidity as HCl is added (see the Experimental section). Considering that the total $\text{Si}(\text{OH})_4$ uptake represents approx. 0.1% of the $\text{Si}(\text{OH})_4$ available, microscopic variations of this sort are quite conceivable. However, if this explanation is correct, doubts are raised about the nature of the $\text{Si}(\text{OH})_4$ being studied. The weight of evidence suggests that the inhibitors affect the bound component rather than the diffusible component, since (i) they are as effective at 2 min as at later times, (ii) mersalyl was able to displace $\text{Si}(\text{OH})_4$ previously taken up (discussed above), and (iii) mersalyl caused a greater degree of inhibition at high osmolarity than at low osmolarity, causing the bound component to be decreased to an extent greater than determined for the diffusible component. Thus the proposal of microscopic variability may be relevant only to the bound component; the same, presumably monomeric, species may be responsible for permeation in all experiments.

To summarize, the uptake of $\text{Si}(\text{OH})_4$ into mitochondria appears to proceed by two mechanisms: the permeation of $\text{Si}(\text{OH})_4$ into the matrix appears to be due to a slow, simple diffusion, being independent of energy provision and probably lacking a specific

carrier in the mitochondrial inner membrane; however, under all conditions examined, the greater portion of the total uptake is not free in solution, but is bound, probably to sites external to the inner membrane. This behaviour is in marked contrast with that seen in intact diatoms, which show an energy-dependent, high-affinity transport of Si(OH)_4 , with the ability to maintain a concentration gradient 30-fold between the cytosol and the suspending medium (Azam *et al.*, 1974). Such a profound difference may indicate their respective requirements *in vivo*: whereas diatoms depend absolutely on silicon, the role of mitochondria in silicon metabolism is suggested only by circumstantial evidence and may be relatively minor.

The relationship between these findings and the uptake of Si(OH)_4 by mitochondria observed experimentally in whole cells *in vitro* and *in vivo* is now assessed. The simple detection of silicon in mitochondrial fractions of whole cells does not necessarily imply permeation of silicon into the matrix space even when steps are taken to correct for surface adsorption (cf. Mehard *et al.*, 1974). However, in other instances (Mehard & Volcani, 1976; Carlisle, 1976), silicon has been detected in the matrix compartment, and in association with granular material (Mehard & Volcani, 1976). The observation of silicon-containing granules is particularly intriguing, since it implies an asymmetric distribution of silicon between the matrix and the cytosol, a feature notably lacking in the present study. Even recognizing the ability of diatoms to concentrate Si(OH)_4 in the cytosol (Azam *et al.*, 1974) does not explain granule formation within the mitochondrion, unless sites within the matrix act as centres for nucleation. It is known that mitochondrial silicon-containing granules can be produced by the injection of silica gel into rats (Policard *et al.*, 1961), possibly a result of polymerized Si(OH)_4 , and in the present study it was shown that polymerized Si(OH)_4 was avidly bound by disrupted mitochondria (Fig. 2). However, it is doubtful whether high concentrations of Si(OH)_4 are normally encountered *in vivo*, particularly in higher animals, where the concentration of silicon in the blood does not exceed the equivalent of 0.2 mM- Si(OH)_4 (see the introduction). Clearly, the present work does not offer an explanation of how intramitochondrial silicon granules might be formed, and the nature and physiological relevance of the mitochondrial silicon stores must await further investigation.

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