4. The monoethenoid fatty acids are calculated by difference.

5. Replicate analyses of two samples of pooled human sera indicate a good reproducibility. Values for normal human blood serum are given.

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The Effect of Soluble Silica on the Metabolism of Rat-Tissue Preparations

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The free silicas (amorphous, quartz, cristobalite and tridymite), in a finely divided form, are strongly fibrogenic in the tissues of man and experimental animals (see, for example, Gardner, 1938; King, Mohanty, Harrison & Nagelschmidt, 1953). Solubilities of about 14 mg. of silica/100 ml. in Ringer solution and blood plasma have been observed with the free silicas. Silicates (e.g. feldspar, mica, sericite) at the same particle size are weakly fibrogenic, if at all, and their silica solubilities are comparatively low, 1 mg. of silica/100 ml. or less. This correlation has been taken as evidence that soluble silica is the toxic agent responsible for the pathogenicity of silica dusts (King, 1950). Other evidence in support of the solubility theory of silicosis is assembled and critically discussed by King (1947, 1950).

The present study concerns the effect of soluble silica on the metabolism of rat-tissue preparations. Many possibilities must be considered for the mode of action of a tissue poison. For example, it might inhibit the activity of one or more enzymes (King, Schmidt, Roman & Kind, 1956, have observed considerable inhibitions of acetylcholine esterase, succinic dehydrogenase, liver esterase and hyaluronidase with 0.025 M-silicic acid), in which case it

would need first to penetrate to the site of activity, or it might change the permeability of cell membranes or protoplasm towards certain substrates. With such considerations in mind the effect of soluble silica on aspects of metabolism of rat-tissue slices has been investigated. The effect on the respiration of tissue homogenates is also described.

Preliminary reports of this work have been published (Rowsell & Leonard, 1957*a, b*).

EXPERIMENTAL

Soluble-silica preparations. Finely ground Madagascar quartz, 99.8% SiO₂, was supplied by the Safety in Mines Research Establishment, Sheffield; 0.75 g. was intimately mixed with 4.5 g. of anhydrous Na₂CO₃ in a platinum crucible. The mixture was heated until a clear melt was obtained, and extracted with water, with heat to facilitate solution, and made up to 100 ml.

A column (45 cm. long, 2.5 cm. in diameter) of the cation-exchange resin Zeo-Karb 225 (The Permutit Co. Ltd., London) was prepared in the H⁺ ion form. After standing with 2N-HCl for 30 min. it was washed with water until neutralization of 100 ml. of effluent required less than 1 ml. of 0.02N-NaOH, with methyl orange as indicator. The sodium silicate solution was poured on the column and silicic acid sols were obtained when the effluent was in the range pH 3.3-3.7. The sols were gassed with N₂ or O₂ to

remove dissolved CO_2 and stored in polythene containers. Visual flame comparisons showed that the concentration of sodium in the sols was less than 0.004%. Total silica was estimated in each sol gravimetrically; usually 6.0–6.5 mg. of SiO_2 /ml. was found.

Silica sols contain mono- and di-silicic acids which react rapidly with molybdic acid, higher polymers of silicic acid which react very slowly and colloidal silica which does not react. It is therefore possible to measure the concentration of mono- and di-silicic acids in the presence of higher polymers (Alexander, 1953, 1954).

Some sols were analysed for total silica by the colorimetric method of Case (1944), as modified by Paterson & Wheatley (1955), and for molybdate-reactive silica (King, Pash & Weedon, 1954). Measured portions of these sols were neutralized (bromothymol blue as indicator) with a few drops of 0.1N-NaOH, diluted with homogenate medium or tissue-slice saline, and incubated at 37° in polythene vessels (see Fig. 1). Samples were removed at different times and the concentration of molybdate-reactive silica was estimated by the method of King *et al.* (1954) so that the phosphate present would make no contribution to the development of blue colour. Optical densities of the solutions were measured at 800 m μ . Silica standards were prepared from sodium silicofluoride according to King (1939). A stable level of about 200 μg . of molybdate-reactive silica/ml. was always attained. This was independent of the original level of molybdate-reactive and polymerized silica in the undiluted sol, where the total silica exceeded 200 μg /ml. (Fig. 1).

The concentration of molybdate-reactive silica in freshly prepared undiluted sols was in the range 1000–1500 μg /ml. This diminished over a period of days to the stable level of 200 μg /ml.

Pre-incubations with soluble silica in metabolic experiments. In all the metabolic studies soluble silica was pre-incubated with saline and substrates for 100 min. at 37°, to ensure that this stable level of molybdate-reactive silica had been reached, before homogenate or slices were added. Similar pre-incubations were included in control experiments, with water replacing silica sol. Sols (6 mg. of SiO_2 /ml.) were diluted 1:5 for the metabolic experiments to give 1.2 mg. of SiO_2 /ml. (0.02M-total SiO_2). From the results presented in Fig. 1 it is evident that the 0.02M-total SiO_2 included

Fig. 1. Effect of dilution of silica sols with phosphate-saline, and incubation at 37°, on the molybdate-reactive silica. The undiluted sols had a total SiO_2 concentration of 7 mg./ml.; 1 vol. of sol was mixed with saline: ●, 19 vol.; ■, 4 vol.; ○, 1.5 vol.

200 μg /ml. or 0.003M-molybdate-reactive silica, and 0.017M-polymerized silica.

Siliceous dusts. Finely divided tridymite, quartz, mica and felspar, graded by differential centrifuging to include only particles in the range 0.5–2.0 μ , were prepared at the Safety in Mines Research Establishment, Sheffield.

Tissue preparations and incubation conditions

Laboratory-stock albino rats have been used throughout. After stunning they were killed by severing the blood vessels of the neck.

Homogenates. Livers were removed immediately into ice-cold medium (13 vol. of 1.15% KCl, 2 vol. of 0.1M-sodium phosphate buffer, pH 7.3, 0.5 vol. of 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). Liver samples (2 g.) were homogenized with 8.5 ml. of medium in a cold steel Potter-Elvehjem (1936) homogenizer and strained through gauze to remove fibrous material.

Warburg vessels contained 0.5 ml. of homogenate, 0.2 ml. of 0.2M-substrate, 0.2 ml. of diphosphopyridine nucleotide (DPN) (10 mg./ml. when added), 0.8 ml. of SiO_2 (6 mg./ml.) or of water, and medium to a total of 4 ml.; NaOH (0.2 ml. of 10N-solution) and filter paper were added to the centre wells. The gas phase was O_2 .

In some experiments substrates were added to the side bulbs and mixed with the main vessel contents after equilibration in the water bath. However, incubation of the tissue preparation at 37° for 8 min. in the absence of substrate gave considerable diminution in the respiration rates, observed after mixing, with or without silica. In most experiments therefore the cold homogenate was added to the medium already containing substrate, and the first manometric readings were noted when the vessels had been in the bath 7–8 min.

Slices. For tissue-slice experiments the phosphate saline normally used (a modification of medium III, Krebs, 1950) was made up as follows:

0.9% NaCl, 103 vol.	} Solution A.
1.15% KCl, 4 vol.	
2.11% KH_2PO_4 , 1 vol.	
3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 vol.	
1.3% NaHCO_3 , 3 vol.	

2.8 vol. of sodium phosphate, 0.1M, pH 7.3, and 2.6 vol. of 0.11M- CaCl_2 , were added to 100 vol. of solution A whilst gassing with O_2 .

Organs were removed into ice-cold saline. Slices were cut with a Stadie-Riggs (1944) slicer and collected in saline. They were weighed on a torsion balance after adherent saline had been drained off by touching the slices against a clean Petri dish.

Respiration studies. About 100 mg. of liver or spleen, and 50 mg. of kidney or lung, were taken for individual incubations at 37°. Warburg vessels contained 0.2 ml. of 0.2M-substrate when added, 0.8 ml. of SiO_2 (6 mg./ml.) or of water, and phosphate saline to 4 ml. The gas phase was O_2 .

Acetoacetate synthesis. The same conditions applied in studying the effect of soluble silica on the synthesis of acetoacetate in liver slices (about 130 mg.) from 0.02M-acetate. Rats starved for 17 hr. were employed in these experiments. After the incubations (120 min.) slices were removed and acetoacetate in the medium was estimated manometrically, with aniline citrate to catalyse decarboxylation (Edson, 1935).

Glycolysis. Slices of liver (about 100 mg.), spleen (50 mg.) and lung (80 mg.) suspended in 4 ml. of phosphate-saline (with or without silica) containing 0.01M-glucose were incubated in 25 ml. conical flasks accommodated in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill., U.S.A.) at 37°, with air as the gas phase. Lactic acid accumulating in the medium was estimated by the method of Barker & Summerson (1941), as modified by LePage (1949). Irregular values with standards (18 μ g. of lactic acid as zinc lactate) were first obtained. Consistent results followed when the amounts of reagents added were increased to 0.1 ml. of 4% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.15 ml. of 1.5% *p*-hydroxydiphenyl in 0.5% NaOH.

Urea synthesis. For studies on urea synthesis the Warburg vessels contained 3 ml. of bicarbonate medium (Krebs & Henseleit, 1932) with or without silica, 0.01M- NH_4Cl and liver slices (about 200 mg.). In some experiments DL-lactate was also present, 0.007M with each stereoisomer. Incubations were for 90 min. at 37° with $\text{O}_2 + \text{CO}_2$ (95:5) as gas phase. Urea accumulating in the medium was estimated manometrically with jack-bean-urease (Krebs, 1942).

Silica at the concentration used in the slice experiments had no effect on the estimation of acetoacetate [prepared from ethyl acetoacetate (Ceresole, 1982)], lactic acid or urea.

Substrates. DPN (95% pure) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other substrates were of analytical grade. Organic acids and NH_4Cl were neutralized to pH 7.3 with NaOH before use.

RESULTS

Effect of silica on the respiration of rat-liver homogenates

Aged silica sols (more than 7 days after preparation) invariably inhibited O_2 consumption (30–70% measured on the first 15 min. of observations) in rat-liver homogenates with succinate, fumarate, L-malate, β -hydroxybutyrate or DL-lactate as substrate. DPN was added with all substrates except succinate.

Some fresh sols gave strikingly different results. With succinate, β -hydroxybutyrate or DL-lactate as substrate the rates of O_2 uptake were not

significantly affected. With fumarate and L-malate there was little difference in the initial respiration rate, but whereas this diminished in the controls the rate with silica was maintained and the total O_2 consumption was thereby much increased (more than twofold in some experiments) (Fig. 2). This effect was observed only with fumarate and L-malate as substrates and was given with six out of nine fresh sols examined (Fig. 2). The other fresh sols all gave 30–50% inhibition with all substrates tested.

When initial tests with a fresh silica sol failed to show this maintenance of respiration with fumarate and L-malate, then repeated experiments with the same sol and other rat-liver preparations were also negative in this respect. Apparently the inconsistency was to be attributed to variation in the nature of the silica sols, perhaps in the molecular size of the silicic acid polymers.

In all experiments the tissue homogenate was precipitated within 2–3 min. of its addition to medium containing 0.02M-silica. No effect was observed on respiration with succinate, fumarate or β -hydroxybutyrate when the silica concentration was 0.003M, 180 μ g./ml. (i.e. with only molybdate-reactive silica).

The effect of 25 mg. of tridymite or quartz dust has been examined on the respiration of rat-liver homogenates with fumarate or L-malate as substrate under the same conditions. The maintenance of respiration obtained with fresh silica sols was never observed. In some experiments tridymite and quartz gave 20% inhibition of respiration, but in parallel incubations greater inhibition (40–60%) was observed with 25 mg. of mica or feldspar (non-pathogenic dusts). It was therefore concluded that the inhibitions observed with tridymite and quartz had no direct bearing on the problem of silicosis.

Effect of silica on the metabolism of rat-liver slices. Soluble silica (0.02M) was without effect on the respiration of liver-, spleen-, kidney- or lung-tissue slices. For all tissues investigated, observations on O_2 consumption have been continued for periods up to 3 hr. Respiration rates diminished, but not more so in the presence of silica than in the controls. Where the addition of a suitable substrate produced an increased respiration (e.g. L-glutamate with kidney and succinate with spleen and lung slices), the same increase was observed where silica was present. This is taken to signify that the silica did not prevent the penetration into the tissue cells of the substrates concerned.

Soluble silica had no effect on the formation of urea from ammonia and CO_2 , or on the formation of acetoacetate from acetate by rat-liver slices.

There was always a slight increase in the aerobic rate of lactate production from glucose in the presence of soluble silica, with slices of liver, spleen

Fig. 2. Effect of soluble silica on the respiration of rat-liver homogenates with 0.01M-fumarate or L-malate. DPN was present in each incubation. ○, Controls; ●, with 0.02M- SiO_2 .

Table 1. *Effect of soluble silica on aerobic glycolysis in rat-tissue slices*

Glucose (0.01 M) was present in each incubation. In each experiment individual slices were cut into two approximately equal pieces. One half was used as a control, the other with SiO_2 medium.

		Lactic acid formation ($\mu\text{mole/g. of wet slice/hr.}$)	
		Control	With 0.02 M- SiO_2
(i)	Liver	(a) 7.0	8.5
		(b) 8.3	9.3
		(c) 6.0	7.5
	Spleen	(d) 12.6	14.4
		(e) 13.5	14.4
		(f) 16.2	17.4
(ii)	Liver	(g) 5.7	6.3
		(h) 5.5	6.5
	Lung	(i) 8.8	9.6
		(j) 10.4	12.4

and lung (Table 1). The same results were obtained in the various tissue-slice investigations with fresh and aged sols. Sols have been used from 1 to 11 days after preparation.

DISCUSSION

In the present investigation a far higher concentration of soluble silica has been employed than is ever likely to occur *in vivo*. Even under the most favourable conditions, i.e. maintained dispersion of fine dust with continuous shaking, the rate at which silica dissolves is very slow, e.g. 20 $\mu\text{g.}$ of molybdate-reactive silica/ml./hr. from 6.2 g. of quartz or tridymite in 100 ml. of Ringer solution (Paterson & Wheatley, 1955), and that dissolving from silica dust introduced into the tissues (e.g. lungs) of experimental animals is quickly excreted in the urine (King, 1950). Until experimental evidence is obtained indicating localized accumulations *in vivo* it must be assumed that these do not occur.

Furthermore, there is no evidence that silicic acid coming into solution from solid particles of silica is every highly polymerized. Paterson & Wheatley (1955) found that tridymite, quartz, cristobalite and fused silica did not give any colloidal polymerized silica on treatment with Ringer solution. They attribute positive findings in this respect (Clelland, Cummings & Ritchie, 1952) to incomplete sedimentation of very fine particles of crystalline solids, and to the employment of analytical techniques which would not differentiate between finely divided solid silica in suspension and silica in true colloidal solution. There is need to extend this work, with discriminating techniques to investigate the amount and form of silica dissolving from dusts in media more closely approximating in composition to those found *in vivo*.

Provisionally it must be assumed that only low polymers of silica (molybdate-reactive) dissolve from silica dusts, and that these do not accumulate *in vivo* in sufficient concentration to condense to form highly polymerized silicic acid. Effects obtained with highly polymerized silicic acid must be regarded as having doubtful relevance to the aetiology of silicosis.

Nevertheless, it was decided to employ as high a concentration of soluble silica as could be conveniently introduced, in the metabolic investigations, so that any effect silica might have would be more readily observed. The maximum attainable level of molybdate-reactive silica (about 200 $\mu\text{g./ml.}$) has been employed, together with highly polymerized silica, to make a total of 1200 $\mu\text{g.}$ of silica/ml. (0.02 M). These two forms of silica have been simultaneously tested.

Fumarate, L-malate, β -hydroxybutyrate and DL-lactate all require DPN for their oxidation in animal-tissue preparations. The observation that colloidal silica maintains respiration only with fumarate and L-malate (Fig. 2) makes it unlikely that the silica could have produced this effect by maintaining the DPN level, e.g. by inhibiting the liver enzyme catalysing the hydrolytic breakdown of DPN (Handler & Klein, 1942).

When fumarate and L-malate are oxidized enzymically oxaloacetate accumulates and inhibits the reaction. The possibility that silica in some way facilitates the removal of oxaloacetate may be considered. A demonstration of a specific interaction between soluble silica and oxaloacetate (a key metabolite) would be of great interest.

Inconsistency in the results obtained has hindered the development of this line of work. It seems that there is some critical unstable state in which fresh silica sols behave differently from aged sols. Moreover, not all fresh sols prolonged respiration with fumarate and L-malate. A standardized procedure has been followed in the preparation of silica sols (see Experimental section), but there are variables (e.g. the room temperature at which sols were prepared and kept), possibly of importance, which have not been taken into account. Before this investigation can be profitably pursued, it will be necessary to find conditions for preparation of silica sol where this biochemical effect can be consistently observed.

Soluble silica had no effect on the respiration of slices of rat tissues. Liver, spleen and lung were included in this study since extensive fibrosis in response to silica dusts has been observed in these organs in many investigations, in experimental animals or in human post-mortem material (e.g. in rat lung, King *et al.* 1953; in rabbit liver, Gardner, 1938; in human spleen, Belt, 1939).

Aspects of metabolism have been studied which involve the integrated action of many individual

enzymes: glucolysis, urea synthesis (see, for example, Ratner, 1951) and acetoacetate synthesis (e.g. Kennedy & Lehninger, 1952). Thus in each experiment the effect of silica on many metabolic processes, some of vital importance to the cell, was being observed. Silica had no effect on urea or acetoacetate formation in rat-liver slices, and slightly increased the aerobic glycolysis rate in liver, spleen and lung slices.

These results indicate that soluble silica did not diminish the permeability of the cells to a variety of substances (e.g. oxygen, carbon dioxide, ammonia, lactate, glucose, urea, acetate, acetoacetate) and that a considerable number of enzymes catalysing a great diversity of reactions (including oxidative phosphorylation) are immune to the influence of the silica penetrating the cells.

An investigation is being made on the extent of penetration of soluble silica into tissue slices. Preliminary results indicate that there is no barrier to the diffusion of molybdate-reactive silica into the cells of rat-liver and -kidney slices. The concentration attained in the total tissue water is that of the surrounding medium (Rowell & Leonard, 1957b).

Marks, Mason & Nagelschmidt (1956) have observed a toxic effect of silica dusts on leucocytes *in vitro*, and they suggest that injury to the phagocytic cells by dust is the stimulus for formation of fibrous tissue. Leucocytes ingest silica dusts in the animal body, and this is the only situation *in vivo* where silica dusts make direct contact with cell cytoplasm. It is conceivable that polymerized silica comes into solution in this particular environment but evidence is lacking. James & Marks (1956) found that soluble silica (0.005M, a lower concentration than has been employed in the present work) severely depressed the respiration of guinea-pig leucocytes. (This total silica concentration presumably included 0.003M-molybdate-reactive silica and 0.002M-polymerized silica; see Experimental section.) They found no inhibition of leucocyte respiration when only molybdate-reactive silica was present.

The results of the present study suggest that soluble silica is not a toxic substance affecting the tissues generally. However, it may be that more complex metabolic activities, e.g. synthesis of protein or nucleic acid, are inhibited by soluble silica or it may be that it can affect specifically only one cell type, the leucocyte.

SUMMARY

1. Soluble silica (0.02M), which includes 0.003M-mono- and -di-silicic acids, and higher polymers (0.017M), prolonged the respiration of rat-liver homogenates with fumarate and L-malate. Highly

polymerized silicic acid was responsible for this effect.

2. Soluble silica had no effect on the respiration of slices of rat liver, kidney, spleen or lung, nor on urea or acetoacetate formation in liver slices.

3. A slight increase in aerobic glycolysis was observed with slices of liver, spleen and lung in the presence of soluble silica.

4. Implications of these negative findings with tissue slices for the solubility theory of silicosis are discussed.

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