In vitro Action of Silicogenic and Non-Silicogenic Dusts on Macrophage Metabolism.* (28344)

ROBERTO COMOLLI AND ANTONIO PERIN (Introduced by Guido Guidotti)
Istituto di Patologia Generale dell'Università, Milano, Italy

Necrosis of silica laden lung macrophages is regarded as the first biological effect of inhaled silica particles in humans. Several *in vitro* investigations have shown that silica particles display a necrotic action on macrophages(1,2,3) and bring about an impairment of their metabolism(4). Furthermore, the action of silica on tissues appears in some way related to the surface structure of particles(5), since pure crystalline forms of silica, like tridymite and cristobalite, are more toxic *in vitro* and fibrogenic *in vivo* than other silica forms like mineral quartz or amorphous (vitreous) silica(1,6,7,8).

The purpose of the present investigation was to gain information on the biochemical basis of silica action on macrophages. The effect of the engulfment of various forms of silica particles differing by surface structure on oxygen uptake, glycolysis and amino acid incorporation into protein has been investigated in macrophages harvested from the peritoneal cavity of the albino rat. Dusts tested were tridymite, tridymite coated with a layer of amorphous silica (silicic acid) and amorphous (vitreous) silica particles. The action of anthracite engulfment on macrophages was also investigated for comparison.

Materials and methods. The procedure for obtaining the peritoneal macrophages has been described(9). Endogenous oxygen uptake and anaerobic glycolysis (in presence of glucose 1.5×10^{-3} M) were measured at 38°C by conventional Warburg manometric techniques, with air as gas phase for aerobic and N_2 95% + CO_2 5% as gas phase for anaerobic conditions. Each flask contained, in a total volume of 3 ml saline solution(9), 2.5×10^7 cells, 2 mg dust (except for the controls) and in the incorporation experiments 1.0 μc (2.67 μmoles) of DL-Leucine-1-C^{14}. Incorporation experiments were run in aerobic as well as in anaerobic conditions. For measurements of radioactivity, cell protein was obtained and purified according to Rabinovitz *et al.*(10) and plated as infinitely thin sample. Radioactivity was measured beneath a mica window G.M. counter. Counting error was less than 3%. All data have been referred to mg nitrogen determined by nesslerization. Viability of cells during the incubation was determined at various times with the nigrosin-staining method(11). Particle engulfment by cells during the incubations was tested by phase-contrast microscopy. Dusts used and their characteristics are reported in Table I. Dusts were prepared by the Laboratorio di Mineralogia della Clinica del Lavoro 'L. Devoto' dell'Università di Milano, Italy.

Results. Mononuclear cells (macrophages) account for 85-90% of the total peritoneal cells, as microscopically checked; other cells are small lymphocytes, eosinophils and mast cells. The percentage of cells found to engulf the particles increases rapidly during the first 45 minutes of incubation and reaches 70% within 2 hours' incubation.

Fig. 1 and 2 show the behavior of macrophage viability of control and engulfing cells in aerobic and anaerobic conditions, as tested with the nigrosin-staining method. The presence of silica dusts causes an impairment of macrophage viability shown by the increase

TABLE I. Characteristics of Dust Particles.

Dusts	Diameter range (μ)	Dvs (μ)*
Tridymite	.5-2.0	.75
Amorphous (vitreous) silica	.5-1.0	.75
Tridymite coated with a layer of amorphous silica (silicic acid)	.5-2.0	.75
Anthracite	.5-3.0	1.25

* Dvs $= \frac{\Sigma N d^3}{\Sigma N d^2}$, where d is diameter of a particle and N is number of particles having that diameter.

* Aided by grant from Haute Autorité de la Communauté Européenne du Charbon et de l'Acier.

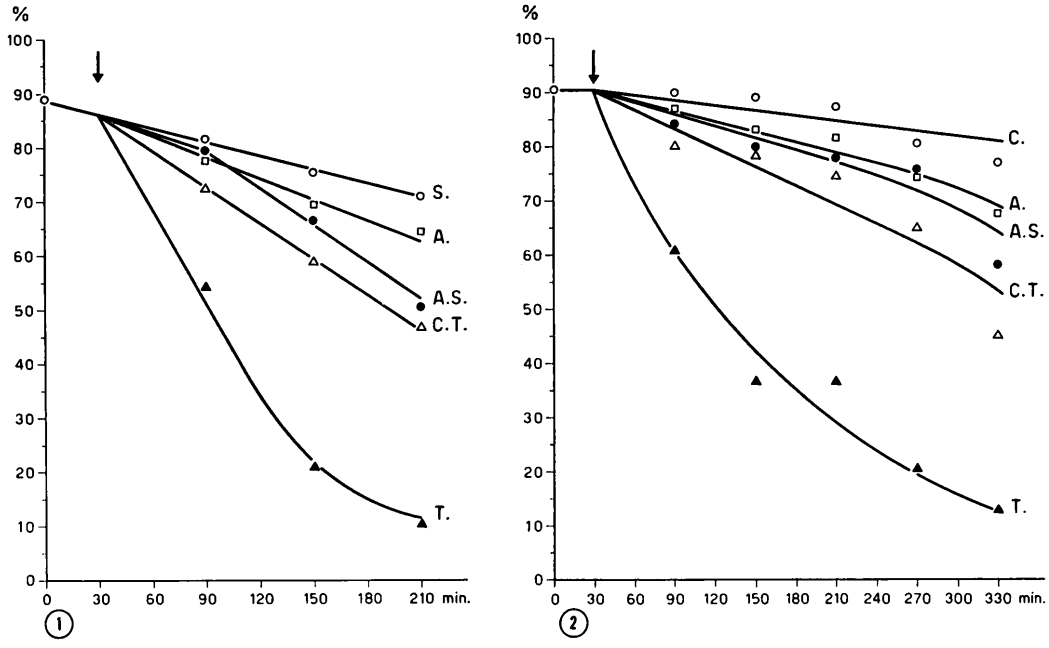

FIG. 1. Nigrosin staining of incubated cells. Aerobiosis. Points represent % of cells found unstained by nigrosin. C. = control; T. = tridymite; A.S. = amorphous (vitreous) silica; C.T. = coated tridymite; A. = anthracite. Additions of dusts and leucine-C^{14} to the system is indicated by arrow.

FIG. 2. Nigrosin staining of incubated cells. Anaerobiosis. Points represent % of cells found unstained by nigrosin. Details as in Fig. 1.

in number of nigrosin-stained cells, tridymite being the most effective. The effect is greater in aerobic than in anaerobic conditions.

The experimental values of oxygen uptake, measured after every 30 minutes incubation, are given in Table II, columns E. These data show that the engulfment of silica dusts induces a progressive lowering of oxygen consumption which stops within 3 hours in the presence of tridymite. However, when values of oxygen uptake are referred to viable cells present at the various periods of incubation (columns C), no definite departure from a linear rate of oxygen consumption is seen and inhibition of macrophage respiration disappears almost completely.

The experimental values of glycolytic CO_2 output, measured after every 30 minutes' in-

TABLE II. Oxygen Uptake. Figures represent μliters of oxygen uptake, after every 30 minutes incubation per mg cell nitrogen. Values of a typical experiment are reported.

	0-30†		1*: 30-60†		1*: 60-90†		2*: 90-120†		2*: 120-150†		3*: 150-180†		3*: 180-210†	
	E‡	C§	E‡	C§	E‡	C§	E‡	C§	E‡	C§	E‡	C§	E‡	C§
Control	42.4	42.8	44.3	46.4	34.8	37.5	38.4	42.6	34.9	40.2	35.2	42.1	29.0	35.6
Tridymite	39.0	39.6	37.8	42.9	23.7	34.0	19.8	38.2	9.2	27.6	5.6	26.5	2.2	29.0
Amorphous silica	40.6	41.2	38.3	40.3	33.9	37.4	32.4	37.9	30.0	38.1	27.1	38.3	18.1	29.2
Coated tridymite	41.5	42.2	38.5	40.9	30.0	35.3	31.8	40.9	27.9	39.8	23.0	36.6	21.4	37.7
Anthracite	42.2	42.8	36.1	38.0	34.3	38.2	33.6	40.5	33.2	40.0	27.9	36.4	26.0	36.0

* Hours after addition of dusts. Dusts added 30 minutes after beginning of readings.
† Minutes after beginning of readings.
‡ E = experimental values.
§ C = values corrected for cell viability.

TABLE III. Anaerobic Glycolysis. Figures represent CO_2 output in μliters after every 30 minutes incubation per mg cell nitrogen. Values of a typical experiment are reported.

	0-30†		1* 30-60†		60-90†		2* 90-120†		120-150†	
	E‡	C§	E‡	C§	E‡	C§	E‡	C§	E‡	C§
Control	114.3	114.3	103.1	103.4	107.7	109.0	99.1	101.2	99.3	102.5
Tridymite	112.5	112.5	96.4	106.1	76.0	99.8	65.4	100.7	53.7	95.4
Amorphous silica	110.8	110.8	106.3	107.4	98.7	101.3	88.4	92.8	78.7	84.4
Coated tridymite	109.9	109.9	109.1	110.1	88.4	93.1	79.6	87.2	69.5	79.5
Anthracite	109.5	109.5	115.7	116.3	109.3	113.2	105.1	111.1	94.3	102.3

	3* 150-180†		180-210†		4* 210-240†		240-270†		5* 270-300†		300-330†	
	E‡	C§	E‡	C§	E‡	C§	E‡	C§	E‡	C§	E‡	C§
Control	92.1	96.1	88.7	93.1	86.5	91.7	83.3	89.3	76.7	83.0	69.9	76.4
Tridymite	47.7	99.2	41.3	98.2	42.1	106.0	29.2	93.7	21.0	92.0	23.7	101.2
Amorphous silica	68.9	75.5	68.3	76.3	63.8	72.9	53.1	72.3	46.2	75.5	50.0	73.3
Coated tridymite	66.7	79.6	63.0	78.1	60.2	78.0	54.7	74.7	43.7	72.4	44.9	78.2
Anthracite	91.3	101.5	93.3	106.3	82.8	96.6	75.0	90.2	74.5	91.8	68.5	87.4

* Hours after addition of dusts. Dusts added 30 min. after beginning of readings.
† Minutes after beginning of readings.
‡ E = experimental values.
§ C = values corrected for cell viability.

cubation, are given in Table III, columns E. Also in this case the engulfment of silica dusts produces a progressive decrease of macrophage glycolysis, the effect being more pronounced in the presence of tridymite. However, as shown for oxygen consumption, the inhibition of macrophage glycolysis disappears almost completely when the experimental values of CO_2 output are referred to cells still viable at the various incubation times.

Tables IV and V show the increments of labelled leucine incorporation into macrophage protein, measured at 60-minute incubation intervals. Experimental (columns E) and calculated (columns C) values corrected for cell viability are reported. In control cells the rate of incorporation of labelled leucine into protein is higher in aerobic than in anaerobic conditions, according to previous findings(9). In aerobic conditions, tridymite strongly decreases the incorporation of leucine into protein. This inhibition occurs chiefly during the first hour of incubation and is not dependent on the decrease in number of viable cells. As compared to tridymite, coated tridymite and amorphous (vitreous) silica inhibit leucine incorporation into protein to a lesser extent. In anaerobic conditions the incorporation of labelled leucine into protein is markedly impaired by tridymite, although less so than in aerobiosis, in a way still not dependent on the decrease of the number of viable cells. The inhibitory action is lower with the other silica dusts.

Anthracite induces a slight inhibitory effect on the macrophage metabolism in aerobic conditions and a moderate stimulatory effect in anaerobiosis.

Discussion. Observations here reported confirm that the engulfment of silica particles by macrophages causes an impairment of their metabolism and viability. Among the silica dusts tested, tridymite, a crystalline purified form of quartz highly fibrogenic *in vivo,* has the most profound effects *in vitro* in our experiments. This agrees well with the results reported by Marks and Nagelschmidt(8). When tridymite is coated with a layer of amorphous silica (silicic acid) the metabolic impairment is much lower and resembles that caused by amorphous (vitreous) silica. This seems to indicate that the surface structure of the silica particle is related to the observed metabolic changes of macrophage metabolism. Silicic acid released by the engulfed particles should not play a role

TABLE IV. Leucine-C^{14} Incorporation into Protein in Aerobic Conditions. Each figure is the mean of 5 experiments ± S.E. (c.p.m./mg nitrogen).

	1*		2*		3*		Total radioactivity	% of control
	E†	C‡	E†	C‡	E†	C‡	E†	
Control	3775 ± 514	4012 ± 546	2875 ± 364	3252 ± 387	1539 ± 307	1861 ± 326	8189	100.0
Tridymite	1923 ± 561	2436 ± 711	0	0	0	0	1923	23.5
Amorphous silica	3155 ± 879	3399 ± 947	2009 ± 342	2444 ± 416	294 ± 52	443 ± 78	5458	66.7
Coated tridymite	2563 ± 365	2873 ± 409	1524 ± 502	2059 ± 678	695 ± 91	1160 ± 152	4782	58.4
Anthracite	3611 ± 735	3904 ± 795	1296 ± 261	1565 ± 315	1299 ± 258	1723 ± 342	6206	75.8

* Hours after addition of dusts and of radioactive leucine. † E = experimental values. ‡ C = values corrected for cell viability.

TABLE V. Leucine-C^{14} Incorporation into Protein in Anaerobic Conditions. Each figure is the mean of 4 experiments ± S.E. (c.p.m./mg nitrogen).

	1*		2*		3*		Total radioactivity	% of control
	E†	C‡	E†	C‡	E†	C‡	E†	
Control	1960 ± 114	1976 ± 115	1269 ± 110	1303 ± 113	1635 ± 284	1709 ± 297	4864	100.0
Tridymite	1549 ± 255	1855 ± 305	145 ± 36	240 ± 60	225 ± 73	500 ± 162	1919	39.5
Amorphous silica	1704 ± 210	1736 ± 214	1397 ± 94	1492 ± 100	779 ± 190	862 ± 211	3880	79.8
Coated tridymite	1318 ± 107	1365 ± 111	1368 ± 34	1532 ± 38	1602 ± 150	1948 ± 182	4288	88.2
Anthracite	2239 ± 741	2292 ± 758	2375 ± 719	2473 ± 771	2121 ± 213	2510 ± 240	6735	138.5

* Hours after addition of dusts and of radioactive leucine. † E = experimental values. ‡ C = values corrected for cell viability.

in determining the metabolic damage since it acts as a cellular poison only in a polymeric form which does not seem to occur in living tissues(12), in spite of recent conflicting results(13).

The progressive inhibition of oxygen consumption and glycolysis found in macrophages after the engulfment of tridymite and other silica dusts seems to be correlated with the decreasing number of viable cells during the incubation. Thus, we believe that these phenomena are the expression of cell death and that the specific locus at which silica exerts its toxic action is not at the level of these metabolic reactions. Indeed, a decrease of the oxygen uptake which clearly correlates with the number of viable cells occurs also in the presence of anthracite, a non-silicogenic dust(14). On the contrary, the inhibition of leucine incorporation into protein by crystalline silica dusts seems to depend neither on the decrease in number of viable cells nor on the impairment of energy-yielding reactions. Indeed, in the presence of tridymite aerobic and anaerobic incorporation of leucine into protein is completely inhibited at the end of the first hour of incubation when oxygen uptake, glycolysis and cell viability are far from completely inhibited.

These results suggest that crystalline silica dusts act primarily on protein synthesis, decreasing their rate. This impairment could bring about a derangement of other metabolic activities of the macrophage which in turn might be followed by cell death. The persistence of oxidative and glycolytic reactions when the incorporation of labelled leucine is inhibited shows that this inhibition cannot be explained on the ground of a decreased energy availability, unless uncoupling of oxidative phosphorylization occurs in aerobic conditions.

Summary. The effect of the engulfment of crystalline and non-crystalline silica dusts and anthracite on oxygen uptake, glycolysis and amino acid incorporation into protein of rat peritoneal macrophages was studied. A marked inhibition of these processes occurs in presence of silica dusts, tridymite being the most effective. The effects on oxygen consumption and glycolysis but not on amino acid incorporation almost disappear when results are correlated to the number of viable cells. Anthracite slightly stimulates macrophage metabolism in anaerobic conditions. It is suggested that protein synthesis is the primary site at which silica particles exert their toxic action.

1. Marks, J., Mason, M. A., Nagelschmidt, G., *Brit. J. Indust. Med.*, 1956, v13, 187.

2. Pernis, B., Vigliani, E. C., Monaco, L., *Med. Lavoro,* 1960, v51, 3.

3. Ebina, T., Takahashi, Y., Hasuike, T., *Am. Rev. Resp. Dis.*, 1960, v82, 516.

4. Marks, J., James, D. M., *J. Path. Bact.*, 1959, v77, 401.

5. King, E. J., Zaidi, S. H., Nagelschmidt, G., *Arch. Indust. Health,* 1956, v13, 133.

6. Zaidi, S. H., King, E. J., Harrison, C. V., Nagelschmidt, G., *ibid.*, 1956, v13, 112.

7. Engelbrecht, F. M., Yoganathan, M., King, E. J., Nagelschmidt, G., *ibid.*, 1958, v17, 287.

8. Marks, J., Nagelschmidt, G., *ibid.*, 1959, v20, 383.

9. Comolli, R., *Nature,* 1962, v195, 178.

10. Rabinovitz, M., Olson, M. E., Greenberg, D. M., *J. Biol. Chem.*, 1954, v210, 837.

11. Kaltenbach, J. P., Kaltenbach, M. H., Lyons, W. B., *Exp. Cell Res.*, 1958, v15, 112.

12. Vigliani, E. C., Pernis, B., *Bull. Hyg.*, 1961, v36, 1.

13. Engelbrecht, F. M., Burger, F. J., *S. Afr. J. Lab. Clin. Med.*, 1961, v7, 16.

14. Attygalle, D., King, E. J., Harrison, C. V., Nagelschmidt, G., *Brit. J. Indust. Med.*, 1956, v13, 41.

Received February 11, 1963. P.S.E.B.M., 1963, v113.