CHANGES IN ACTIVITIES OF RESPIRATORY ENZYMES IN LUNGS OF GUINEA-PIGS EXPOSED TO SILICA DUST*

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Changes in enzyme activity due to *in vivo* dusting with quartz were investigated in guinea-pig lungs before the formation of collagen. Of the three enzymes investigated, succindehydrogenase and succinoxidase are increased, and cytochrome c oxidase is not affected by inhalation of quartz dust. The increases tend to reach a maximum which is more rapidly approached by succindehydrogenase than by succinoxidase. The results are discussed in relation to results obtained by other workers on the *in vitro* effects of silicon compounds. The lung weight increased markedly with increased period of inhalation of dust. This increase is discussed in relation to fat and ash content.

This investigation forms part of a plan to study the changes that occur in the pneumoconioses in the amounts and activities of respiratory enzymes in the lungs.

The testing of the *in vivo* effect of quartz dust on enzymes in lungs by using homogenates of lungs from animals exposed to quartz dust does not necessarily give the same results as are obtained with *in vitro* tests on homogenates or isolated enzymes to which silica or silicic acid has been added. The *in vivo* reaction includes the whole organism and is affected by factors such as the inflammatory state engendered by foreign bodies as well as possible general systemic effects on the organism in regard to detoxifying action on the silica.

Since most authors have ignored the early stages of silicosis, we have studied the effect of silica inhalation on respiratory enzymes in the lung at different stages, before the formation of collagen. Whole lungs were assayed in preference to dissected silicotic nodules. The number of animals used for each period of dusting was sufficient to make results statistically significant.

Experimental

Methods of Dusting Guinea-pigs.—The animals were exposed to quartz dust, and were kept in the same room, size 10 × 10 × 10 ft., throughout the period of dusting,

The quartz dust was blown into the room during six or seven hours for about five days a week at a concentration of 23,000 to 25,000 particles/ml. for all groups except for those 26 and 63 "days dusted" (see Tables 1 and 2), which were dusted at a concentration of about 30,000 particles/ml. Most of the air-borne dust was in the range of 0.1 to 1.0 μ in diameter, according to thermal precipitation counts. The quartz used was acid-washed and no more than 0.1 mg. of iron per gramme of washed quartz dissolved on warming for 15 minutes with 1N HCl. Controls of the same age were kept in another room out of contact with quartz dust.

Lung Material.—A 20% homogenate (fresh weight basis) was prepared from guinea-pig lungs as previously described (Kilroe-Smith and Breyer, 1960) except that for later homogenizations a Duall tissue homogenizer† was used and the homogenization time reduced from five to two minutes. A 20% homogenate when kept on ice retains full enzyme activity for the three enzymes tested for at least five hours.

Enzyme Assays.—These were carried out as follows.

Succindehydrogenase.—This was assayed spectrophotometrically with neotetrazolium, as previously described (Kilroe-Smith and Breyer, 1960), except that a zero-time blank was substituted for the no-substrate blank. A no-substrate blank is unnecessary under the conditions described, since it does not give a meaningful result (Slater and Planterose, 1960; Reiner, 1959). However, the zero-time blank is necessary if the lung is not well

*A preliminary report has been made covering some of the material presented in this paper (Kilroe-Smith and Breyer, 1960).

†Kontes Glass Co., Vineland, New Jersey, U.S.A.

perfused since an appreciable amount of extraneous colour is extracted when blood is present. The homogenate was used undiluted.

Cytochrome c Oxidase.—In the modified method of Neufeld, Levay, Lucas, Martin, and Stotz (1958), the reagents were added to the cuvette in the following order:

1. 2.0 ml. phosphate buffer 0.1M, pH 7.4
2. 0.3 ml. cytochrome c (Nutritional Biochemicals Corporation) solution 0.32%
3. 0.1 ml. catalase (Sigma lyophilized) solution 5 mg. per 100 ml. water
4. 0.5 ml. leucodye, prepared as follows: 25 ml. 0.001M 2,6-dichlorobenzenone-endo-3'-chlorophenol (Eastman) standardized against ascorbic acid was reduced with 15 mg. 5% palladized asbestos (Fisher). The latter was used unwashed since washing removed too much palladium. After adding 4 drops of 0.2M McIlvaine buffer, pH 6.0, hydrogen was bubbled through until the blue indophenol was completely reduced, which requires about 15 minutes. After rapid filtration through No. 5 Whatman filter paper, the reduced dye was put into a burette fitted with an alkaline pyrogallol guard tube. The air in the apparatus was subsequently replaced by hydrogen. These precautions are necessary because the rate of oxidation of leucodye increases with increased amount of oxidized dye (Smith and Stotz, 1949).

The pink colour referred to by Smith and Stotz (1949) does not appear to be due to over-reduction but rather to decomposition products formed in the solid dye before reduction. We found that no pink colour develops with either an excess of catalyst or an excess of hydrogen. Pink colour does appear when the solid dye is old stock and has been irreversibly decomposed, but does not appear when undecomposed dye is used for the preparation of reduced dye solution.

5. 0.1 ml. 10% homogenate.

The final volume of the reaction mixture was 3 ml. Readings of extinction at 645 m μ were done for 1 minute at 20-second intervals, starting 105 seconds after addition of the homogenate. This was necessary because the reaction rate did not become linear immediately after addition of the homogenate. The autoxidation was determined separately, replacing homogenate with 0.25M sucrose and subtracting this figure from the assay. All reagents were kept in a 30°C. bath and the temperature in the spectrophotometer chamber kept as near to this as possible.

The enzyme activity is expressed as extinction change per 100 mg. ash-free dry tissue per minute.

If desired, it is possible to do three assays simultaneously by suitable staggering.

Using this method for guinea-pig lungs, maximum activity was obtained between pH 7.0 and 7.5 and a final phosphate buffer concentration in the reaction mixture of 0.060 to 0.073M.

Succinoxidase.—This was assayed manometrically as previously described (Kilroe-Smith and Breyer, 1960; Slater, 1949). The homogenate was used undiluted.

Fat Estimation.—The fat content was determined by

extracting oven-dried powdered lung tissue with 2:1 chloroform-methanol mixture in a micro continuous extraction apparatus for 4 hours and determining the loss of weight.

Results and Discussion

The results are given in Tables 1 and 2 and Figs. 1 and 2. The figures recorded in Table 2 refer to the same lungs as those used in Table 1.

The dusted and control animals in each group were all 6 months old at the start of dusting except the groups 26 and 63 "days dusted" (see Tables 1 and 2), which were 3 months old when dusting was commenced. All assays were done in pairs, one dusted and one control on any particular day, to eliminate possible variation due to methodology. When the assays were done in this way a high statistical correlation was obtained between pairs whereas there was often quite a large variation between results obtained on different days.

The results did not appear to be dependent on the sex of the animal, but for all assays the same sex was used for pairs of controls and dusted animals.

The results were obtained from animals whose lungs did not yet show advanced collagenization because at the maximum period of exposure to dust the tissue had not yet advanced beyond the formation of reticulin, as based on histological staining. Histological examination revealed no abnormality in the lungs apart from the effects due to dusting with quartz.

It is possible to express enzyme activities in three ways based on (a) total activity per lung, (b) ash-free dry lung tissue, and (c) wet lung tissue. Method (a) is misleading because it gives a composite figure in which increases due to increase in lung size are combined with increases in specific activity. Method (b) as used separates these two factors. Method (c) gives similar results to method (b) but is less reliable because of the introduction of another variable, namely the moisture content of the wet tissue.

The mean values of the differences between animals exposed to quartz dust and control animals (D-C in Tables 1 and 2) were statistically analysed and the variances calculated. These variances were used to test whether the values of D-C are significantly different from zero.

The trends are very similar whether "days dusted" or "days in dust room" is used as the independent variable. The results are plotted against "days dusted" as the independent variable. Smooth curves were fitted to the experimental data. The curve fitted to the succindehydrogenase data (Fig. 1) is exponential. No curve fits the succinoxidase

TABLE 1
EFFECT OF EXPOSURE TO INHALATION OF QUARTZ DUST ON THE ACTIVITY OF
ENZYME SYSTEMS IN LUNGS OF GUINEA-PIGS

Days Dusted	Days in Dust Room	Ash-free Dry Weight of Lung (g.)			Succindehydrogenase (μ g. diformazan/mg. A.F.D.T./15 min.)			Cytochrome c Oxidase (u.) $\dagger$			Succinoxidase (Qo ₂)		
		D	C	D-C	D	C	D-C	D	C	D-C	D	C	D-C
6	14	0.55	0.52	0.03	3.62 (5)*	4.49 (5)	-0.87†	1.3	2.4	-1.1†	16.0	16.4	-0.4†
13	19	0.75	0.67	0.08	4.22 (5)	3.00 (2)	1.22	—	—	—	9.9	10.1	-0.2†
25	44	0.80	0.61	0.19	5.45 (7)	4.66 (7)	0.79†	5.4	5.0	0.4†	12.1	10.4	1.7
26	49	0.77	0.61	0.16	3.98 (5)	3.17 (5)	0.81	—	—	—	8.6	8.2	0.4†
63	133	0.97	0.63	0.34	7.17 (7)	5.03 (7)	2.09	—	—	—	9.3	7.9	1.4
88	166	1.09	0.73	0.36	6.53 (5)	5.66 (5)	0.87	5.8	6.3	-0.5†	17.4	15.5	1.9
97	189	1.27	0.68	0.59	4.85 (5)	3.27 (5)	1.58	—	—	—	11.1	9.5	1.6
132	242	1.27	0.54	0.73	6.83 (5)	4.22 (2)	2.61	—	—	—	13.4	11.3	2.1
142	255	1.40	0.57	0.83	7.24 (7)	5.75 (7)	1.49	5.2	5.5	-0.3†	15.8	13.5	2.3
180	318	1.79	0.63	1.16	6.36 (7)	4.99 (7)	1.37	7.1	6.3	0.8†	15.8	13.2	2.6

D = animals exposed to quartz dust

C = control animals

A.F.D.T. = ash-free dry tissue

*Figures in brackets refer to the number of animals on which averages are based and apply to all the enzyme assays and ash estimations done on dusted and control animals respectively; duplicate assays and estimations were done on each animal. For methods used see text.

†Differences statistically not significant at 95% level

‡Modified Neufeld *et al.* method: extinction change per 100 mg. A.F.D.T./min.

TABLE 2
EFFECT OF EXPOSURE TO INHALATION OF QUARTZ DUST ON WEIGHT, MOISTURE, FAT,
AND ASH CONTENT OF GUINEA-PIG LUNGS

Days Dusted	Weight of Animals when Killed (g.)		Wet Weight of Lungs (g.)		Moisture Content (%)		Fat Calculated on Dry Tissue (%)		Fat Total Weight in Lungs (g.)			Ash Calculated on Dry Tissue (%)		Ash Total Weight in Lung (g.)		
	D	C	D	C	D	C	D	C	D	C	D-C	D	C	D	C	D-C
6	517 (5)*	518 (5)	4.07	3.99	85.5	86.0	20.1	20.6	0.12	0.12	0	7.45	7.07	0.044	0.040	0.004
13	717 (5)	718 (2)	4.46	4.27	82.1	83.4	21.6	—	0.17	—	—	6.93	5.78	0.055	0.041	0.014
25	810 (7)	821 (7)	5.34	4.18	83.8	84.4	24.3	22.4	0.21	0.15	0.06	8.02	5.96	0.069	0.039	0.030
26	546 (5)	520 (5)	4.61	3.62	82.1	82.1	27.0	26.7	0.22	0.17	0.05	7.37	6.26	0.061	0.042	0.020
63	570 (7)	732 (7)	5.60	3.73	81.3	81.7	21.9	19.5	0.23	0.13	0.10	7.65	6.78	0.080	0.046	0.034
88	766 (5)	776 (5)	7.21	4.61	83.6	83.1	22.9	21.2	0.27	0.17	0.10	7.56	6.39	0.089	0.050	0.039
97	733 (5)	879 (5)	7.10	4.10	80.5	82.2	21.9	17.4	0.30	0.13	0.17	7.63	6.63	0.105	0.048	0.057
132	799 (5)	653 (2)	7.30	3.26	81.0	82.1	16.2	17.3	0.23	0.10	0.13	8.53	6.93	0.118	0.040	0.078
142	863 (7)	668 (7)	7.64	3.45	80.1	82.4	19.9	22.8	0.30	0.14	0.16	7.54	6.89	0.115	0.043	0.072
180	920 (7)	757 (7)	8.92	4.23	78.4	83.9	22.1	19.5	0.43	0.13	0.30	7.13	7.02	0.137	0.048	0.089

D = animals exposed to quartz dust

C = control animals

*Figures in brackets refer to the number of animals on which averages are based and apply to all estimations done on dusted and control animals respectively; duplicate estimations were done on each animal. For methods used see text.

uniquely. The curve chosen was an exponential curve and the 95% confidence intervals are shown (Fig. 2). This gives some idea of the accuracy of conclusions drawn from the figures. No curve was

fitted to cytochrome c oxidase. Statistically significant changes in specific enzyme activities occur very soon after guinea-pigs have begun to inhale quartz dust.

FIG. 1.—Effect of different periods of exposure to inhalation of quartz dust on the activity of succinic dehydrogenase in guinea-pig lung. For method of assay see text.
 Δ = activity of dusted lungs minus activity of control lungs.
 Units used — μ g. diformazan per mg. ash-free dry tissue per 15 min. Other symbols as in Fig. 2

Succindehydrogenase.—After 13 days' dusting succindehydrogenase shows a significant increase in enzyme activity (Fig. 1). The value of D-C levels off after 30 days' dusting.

It has been shown (Marasas and Harington, 1960) that chemically quartz can bring about a number of oxidative and hydroxylative conversions, many of which have been previously demonstrated in biological systems. This is not the cause of higher succindehydrogenase values for dusted animals as compared to control animals because the presence of silica in the lung has no activating effect on succindehydrogenase in the lung (see Table 3). The addition of boiled dusted lung homogenate to control lung homogenate does not increase the activity beyond that of the figures in column 2 plus column 4.

Cytochrome c Oxidase.—There is no significant change at all.

FIG. 2.—Effect of different periods of exposure to inhalation of quartz dust on the activity of succinoxidase in guinea-pig lungs. For method of assay see text.
 Δ = activity of dusted lungs minus activity of control lungs.
 Units used — Q_{O_2} based on ash-free dry tissue.
 ● = significantly different from zero (95% limit)
 × = not significantly different from zero
 — = "fitted" exponential curve
 --- = 95% confidence intervals

Succinoxidase.—After 25 days' dusting succinoxidase shows a significant increase in activity which is slower than that of succindehydrogenase but has not levelled off after 180 days' dusting (Fig. 2).

The figures for percentage fat (Table 2) in lungs are so erratic that it is impossible to draw any conclusions about possible small increases due to dusting. The figures for total fat in lungs increase with duration of dusting, but, as pointed out above for enzyme activities, this method of expressing the results gives a false impression because the increase in total fat may be merely parallel to the increase in lung weight (see Table 2). The increase in total fat accounts for only about 23% of the total increase in dry weight of the lung. This is in general agreement with the findings of Marks and Marasas (1960), whose figures indicate a value of about 22%.

Although there is no definite increase in weight of the control lungs, the weight of dusted lungs in-

TABLE 3
 EFFECT OF SiO_2 IN DUSTED LUNG ON REDUCTION OF NEOTETRAZOLIUM CHLORIDE
 BY SUCCINDEHYDROGENASE
 (Optical Density at 510 $m\mu$)

0.5 ml. Dusted + 0.5 ml. Water	0.5 ml. Control + 0.5 ml. Water	0.5 ml. Boiled Dusted + 0.5 ml. Control	0.5 ml. Boiled Dusted + 0.5 ml. Water
1.359	1.000	1.040	0.061

The figures represent an average of two estimations by the succindehydrogenase method referred to in the text, except that the final volume of the reaction mixture was 3.5 ml.

"Dusted" refers to an homogenate of lung obtained from a guinea-pig dusted for 121 days.

creases with the increase in number of days dusted (see Tables 1 and 2). The percentage ash is always higher in the dusted lungs than in the controls, but this difference exhibits no definite trend. The total weight of ash per lung increases with duration of dusting (see Table 2). As for fat, this increase is parallel to the increase in lung weight but represents only about 10% of the weight increase of the dry lung.

The investigations of other workers, which show an *in vitro* inhibition of succindehydrogenase (James and Marks, 1956; King, Schmidt, Roman, and Kind, 1956), of succinoxidase (James and Marks, 1956; Rowsell and Leonard, 1957; 1958), and of cytochrome c oxidase (James and Marks, 1956) after addition of silicic acid or silica-containing materials to homogenates or normal tissue, do not necessarily contradict our results. They measured the immediate effect of these materials on the enzymes, whereas we measured a composite long-term effect of continuous contact *in vivo* of lung tissue with silica dust.

Engelbrecht and Burger (1961), using polymerized silicic acid, found different reactions, depending on whether they used Keilin and Hartree preparations or liver homogenate. Cytochrome c oxidase was inhibited in both; succindehydrogenase was unaffected in the enzyme preparation whereas in liver homogenate slight inhibition occurred, but succinoxidase, although it showed inhibition in the enzyme preparation, showed an increase in activity in the liver homogenate.

A result analogous to ours has been obtained with esterase. Engelbrecht and Paul (1959) demonstrated histochemically that there was an increase of esterase activity at the sites of the very early reactions to

injected tridymite. *In vitro* studies (King *et al.*, 1956) of the effect of colloidal silicic acid on liver homogenates showed an inhibition of esterase activity. Silica particles do not affect esterase activity *in vitro*; thus it appears that this kind of *in vivo* effect, which is in the opposite direction to the *in vitro* effect, is a compensatory reaction of the organism to the silica stimulus.

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