JMEA 2477

X 8 80

Inactivation by polymers of hemolytic microparticles in vitro

Dr. R.J. Schnitzer and Sachi Senrui
Environmental Sciences Laboratory
Mount Sinai School of Medicine
The City University of New York
New York, N. Y.

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Abstract

Selected polyanions, sodium alginate, carboxymethylcellulose, chondroitin sulfate, pyran-copolymer NSC 46015 and to a lesser degree polystyrene sulfate are adsorbed to asbestos (UICC Standard Reference Samples of chrysotile B and amphibole asbestiform fibers) by exposure of the asbestos dusts (100 mg) to the polymers (1.0 - 50 mg) for 30 minutes at 37°C. Stable mineral - polymer complexes with markedly reduced lytic properties for sheep erythrocytes are formed although the not adsorbed excess of the inactivators is removed by washing and no free polymers or only not measurable traces are available in the hemolytic system. The non-ionic polyvinylpyrrolidone (PVP) does not inactivate asbestos fibers with the exception of anthophyllite. Silica, both fibrous microquartz and microfine precipitated powders are inactivated by PVP also in the range of 1.0 - 50 and 5.0 - 50 mg while polyanions are generally not effective. An exception is chondroitin sulfate which inactivates microquartz but not the microfine dusts.

I. Introduction

Earlier studies on the prevention of hemolysis caused by minerals e.g. silica of various forms or the asbestos variety chrysotile were based on an experimental arrangement in which polymers were present as antagonists in the hemolytic system (Stalder and Stöber, 1965; Nash et al. 1966; Macnab and Harington 1967; Koshi et. al., 1968; Schnitzer and Pundsack, 1970; Schnitzer and Bunescu, 1970; Schnitzer et al. 1971). Schnitzer and Pundsack (1970) have also shown that the anionic polymer carboxymethylcellulose (CMC) forms apparently a stable complex with chrysotile fibers, which lose their hemolytic property after the excess of the polyanion is removed by centrifuging and repeated washing with comparatively large amounts of saline. This technique has also been used by Harington et al. (1971) to show the lack of inhibition of asbestos hemolysis by polyvinylpyridine-N-oxide (PVPNO) and the marked antagonism of adequate concentrations of sialic acid. Schlipköter (1968) has also studied the process of adsorption of PVPNO on asbestos; he mentions that PVPNO is bound to the fibers without, however, eliminating the lytic effect. Similar procedures were employed to demonstrate the fact that the polymer is bound to silica particles but not to red cells./ (Nash et al. 1966) This observation has been confirmed in this laboratory with several chrysotile antagonists of the series of polyanions and the silica antagonist polyvinylpyrrolidone (unpublished).

The generally high specificity of the antihemolytic activity of poly-

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anions for chrysotile and to a certain degree also for amphibole asbestiform fibers (amosite, crocidolite, anthophyllite) and the prevention of silica hemolysis by nonionic polymers prompted systematic quantitative experiments on the binding of polymers to minerals followed by removal of excess of nonadsorbed antagonists. The purpose of these studies was the preparation of inactivated minerals which fail to show hemolytic activity in a system in which no free antagonists or only not measurable traces of them are present. The experiments are described in this report.

II. Materials and Methods

1. Hemolytic agents

a. Asbestos varieties (UICC Standard Reference Samples)

Chrysotile B: hammer-milled; surface area: 4.4 m^2/g.

Amosite: hammer-milled; surface area: 2.5 m^2/g.

Crocidolite: hammer-milled; surface area: 2.4 m^2/g.

Anthophyllite: hammer-milled; surface area: 2.9 m^2/g.

The values for surface area were obtained by the permeability method (Rendall, 1970).

b. Silica specimens

Microquartz (MQ): obtained from Johns-Manville; code 16; mean fiber diameter 0.6 μ.

Microfine precipitated silica (Philadelphia Quartz Company) Quso

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G30 (G30); particle size: 14 mμ; surface area 300 m^2/g; bulk density 10 lb/ft^3.

Microfine precipitated silica (Philadelphia Quartz Company) Quso G32 (G32); particle size 14 mμ; surface area 300 m^2/g; bulk density 4 lb/ft^3.

The physical data on Quso G30 and G32 are taken from the manufacturer's data sheet.

2. Antagonistic polymers

a. Anionic polymers

Sodium alginate (SA): commercial product "Kelkosol". Code number: 20-462.

Carboxymethylcellulose ether sodium salt (CMC); commercial product (Matheson Coleman & Bell).

Chondroitin sulfate (CS) (mucopolysaccharide): commercial product (General Biochemicals).

Pyran-copolymer (NSC 46015): sample obtained from National Cancer Institute. Mol. Weight 475,000. Pyran - 3,4, dicarboxylic anhydride, tetrahydromethyl - 6 (tetrahydro - 2,5 - dioxo - 3 - furyl) polymer. Structural formula: see De Clercq (1970).

Polystyrene sulfate (RS 781): product of the Esso Research Laboratories.

b. Nonionic Polymers

Polyvinylpyrrolidone (PVP) "Plasdone" of GAF Lot No: 20-680-01

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Code: K - 29 - 32.

Methyl cellulose (MC): commercial product (Matheson, Coleman and Bell).

3. Cells

3 times washed red cells from defibrinated sheep blood.

4. Procedures

The results of the experiments to be described here are based on tests with a comparatively large amount of 100 mg of the various materials, but tests with smaller amounts were also carried out. Fibers and particles(G30, G32) were expose in large plastic centrifuge tubesto graded amounts of antagonists of 0.5 to 100 mg in a volume of 20 ml isotonic veronal buffer (pH 7.3 - 7.4) and kept in a shaking waterbath at 37°C for 30 minutes. The excess of antagonist solution was removed by centrifuging and the sediments washed 3 times in 40 ml saline. Between the washings the sediments were resuspended in saline by manual shaking and, if necessary, by breaking up of clumpled sediments by stirring with glass rods. To the drained sediments were added 4 ml buffer solution and 4 ml 4% suspension of 3 times washed sheep blood erythrocytes. The tubes were then shaken for 2 hours at 37°C, centrifuged and the hemoglobin concentration determined colorimetrically. Every test contained 2 positive controls, one of them the untreated lytic agents, the other one containing

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saponin (1.25 mg/ml). The negative controls consisted only of 2% cell suspension in buffer. Degree of hemolysis was determined in a Photovolt Colorimeter #401 and calculated as percentage of the lysis of the untreated materials. Chrysotile B 100 mg always produced 100% lysis; the lytic concentration of 100 mg of the amphiboles varied. Amosite had an average of 50% lysis (min. 19.5%, max. 85%), crocidolite at 100 mg an average of 47.6% lysis (min. 27.5%, max. 75%). The average value of 100 mg anthophyllite was 80% (min. 45%, max. 95%).

In the group of silica specimens microquartz was of moderate hemolytic activity, 100 mg causing an average of 81% lysis (min. 60%, mas. 95%). The microfine silica powders were highly active hemolytic agents: G30 (50% hemolytic concentration [HC_{50}] 0.085 mg/ml) and G32 (HC_{50} 0.036 mg/ml) tested at a 50 mg dose produced 100% hemolysis.

III. Results

1. Inactivation of Asbestos

As examples of the effect of adsorption of polymers on the hemolytic action of asbestos experiments with chrysotile B and anthophyllite are given in Tables 1 and 2. They present the observations of tests carried out with the anionic polymers SA, CMC, CS, NSC 46015 and RS 781 and the nonionic compound PVP. From the inhibition values listed in Tables 1 and 2 the following conclusions can be drawn.

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a. The sheep cell hemolysis of the asbestos varieites tested can be inhibited by the anionic polymers, but there exist quantitative differences. These differences are in the most instances not significant, but it is evident that the polystyrene RS 781 is less effective than the other polyanions. Anthophyllite also appears to be less sensitive to SA and NSC 46015, which are as a rule potent inhibitors.

b. The range of active concentrations of the antagonists is surprisingly wide. It may be seen from the Tables 1 and 2 that in spite of certain more or less unavoidable (different batches of sheep blood) irregularities the mineral fibers were equally well inactivated after exposure to 2.5 - 50 mg of the antagonists. This is shown by the comparatively small standard errors (generally < 10%) of the means, as given in Table 3 in which the inactivation values for the range of active amounts are averaged. Inactive amounts - mostly 1.0 mg - have not been included in these calculations.

c. Chrysotile B fibers are binding sufficient amounts of antagonists to show an inactivation of approximately 80 - 90% particularly after treatment with SA, CMC, NSC 46015 and CS. Anthophyllite responds particularly to CMC, CS and PVP.

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d. The wide range of binding of antagonists by comparatively large amounts of mineral fibers (100 mg) leading to a high degree of inactivation suggests that small amounts of antagonists, approximately 5 - 10% of the weight of the fibers will be adequate for producing inactivated material. As a rule not the entire amount of antagonists is adsorbed by the fibers. In a similar group of experiments the residual concentration of the polymers was determined by measuring the inhibiting titer of supernatants and washwaters in hemolytic tests with chrysotile B or silica G30 or G32 as lytic indicators. All supernatants contained active concentrations of the antagonists, but only small amounts were occasionally found in the first washings. The second or third washwaters did not contain measurable amounts. Tests with amosite and crocidolite showed generally similar conditions as may be seen in Table 3, although the level of inactivation of crocidolite is somewhat lower than that of the other varieties. If one considers the low hemolytic potency of crocidolite and of amosite the results are not unsatisfactory, since in the actual experiments hardly any lysis can be detected after exposure to the polyanions.

Anthophyllite is characterized by a marked response to the nonionic polymer PVP. The general principle of specificity according to which polyanions antagonize and inactivate chrysotiles and other asbestos varieties, whereas the nonionic polymers prevent

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silica hemolysis (Schnitzer et al., 1971) is not valid in all instances. Schlipköter (1968) has mentioned that amphiboles respond to the antagonistic property of PVPNO, but in our tests in which PVP was used this could only be demonstrated with anthophyllite. The recent binding experiments confirm these observations. As shown in Tables 2 and 3 anthophyllite is markedly inactivated following exposure even to 2.5 mg. Also nonionic MC inactivated anthophyllite. In this experiment (not tabulated) a higher dose of the amphiboles was used, 200 mg causing 95 - 100% lysis. The average of inactivation in the range of 2.5 - 50 mg MC was 75% ± 9.8. Otherwise the larger amount of anthophyllite did not change essentially the activity of polyanion antagonists; for instance the percentage of inactivation in the range of 1.0 - 50 mg CMC was 87% ± 7.8, of CS 77% ± 10.8.

The question arose whether differences in the inactivation of asbestos varieties by polymers particularly the general lower level of binding by crocidolite and the marked response of anthophyllite to PVP and MC could be explained by specific adsorptive properties of the fibers.

In earlier experiments it had indeed been observed that such differences exist and that they can be demonstrated by dye stuff adsorption. The principal result of the earlier experiments was

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the finding that chrysotiles show a much higher adsorption rate to trypan blue (a benzidinetetrazo dye with 4 anionic sulfonate groups) than to the nonionic methylene blue. Amphiboles had not been tested at that time.

Comparative tests have been carried out now; adsorption of methylene blue and trypan blue in aqueous solution by chrysotile B, amosite, crocidolite, and anthophyllite (all UICC material) was tested under experimental conditions similar to the lysis-inactivation tests. A constant amount of fibers (100 mg) was exposed to 20 ml dye stuff solution under agitation at 37°C for 30 minutes. After centrifuging the color of the supernatant was noted and its density determined using a filter #580. The color of the sediments was also recorded. The results are shown in Table 4.

The higher adsorption rate of chrysotile for the anionic dye is obvious. It is also evident that anthophyllite adsorbed both the anionic and the nonionic dye, the latter more than 2 to almost 5 times better than the former one.

Amosite and crocidolite show generally low adsorptive capacity of approximately 50% of the control and strictly limited to methylene blue. These finding suggest a parallelism of dye stuff adsorption and inactivation of hemolysis by polymers in case of chrysotile and anthophyllite and offer a basis for interpretation

for the lack of inactivation by PVP in case of chrysotile and the response of anthophyllite to both polyanions and nonionic polymers. The reaction of amosite and crocidolite which is limited to methylene blue is not in agreement with the inactivation of hemolysis produced preferentially by polyanions. This is perhaps an example to show that the inactivation of amphiboles by polyanions is a complex process in which other factors in addition to adsorption are participating.

2. Inactivation of Silica

It is evident from the data in Table 5 that the nonionic polymer PVP is markedly bound to the fibrous as well as to the microfine precipitated silica. After removal of the original polymer solution and thorough washing with saline the hemolytic property of the silica sediments is 80 - 90% reduced after exposure to small amounts of the antagonist, such as 0.25 mg (microquartz), or 5.0 mg (G30, G32). The anionic polymers were ineffective with the exception of CS which exerted an inactivating effect limited to microquartz. As had been found in the corresponding experiments with asbestos varieties there was only an insignificant difference of inactivation in the range of the different active concentrations of the antagonists:

microquartz/PVP:	range 0.25 - 10 mg:	87% ± 1.9	inactivation
microquartz/CS:	" 5.0 - 50 mg:	87% ± 6.8	"
G30/PVP:	" 5.0 - 40 mg:	94% ± 4.5	"
G32/PVP:	" 5.0 - 50 mg:	95% ± 3.0	"

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Another exception from the rule of specificity has been observed: the effect of the anionic CS on microquartz. It has been known that CS acts as an inhibitor of microquartz hemolysis, if present in the lytic system (50% inhibiting concentration 0.35 mg/ml. See Schnitzer et al., 1971); while it failed to antagonize significantly hemolysis of G30 and G32 at a concentration of 10.0-50.0 mg/ml. The surface structure of the fibrous material seems to possess specific sites of adsorption for certain polyanions lacking in the fine powders.

IV. Discussion

The experiments described in the earlier sections of the report show the inactivation of the hemolytic potency of 4 asbestos varieties by a group of anionic and nonionic polymers following the binding of the antagonists to the fibers and the removal of unbound excess by repeated washing. Identical observations were made on the elimination of hemolytic properties of fibrous and microfine silica after exposure to the nonionic PVP and in one instance (microquartz) also to anionic CS. It has been mentioned in Section I that similar methods of binding have been carried out before with silica and PVPNO and with sialic acid and chrysotile (Harington et al., 1971). In other studies inactivation of mineral dusts by serum proteins was achieved by their adsorption to dust particles. Stalder and Stöber (1965) mention that silica suspension was pretreated with serum. Aronova et al. (1968) studied the adsorption of human serum proteins on modified (treatment with silanes)

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quartz surfaces. [illegible] and Sakabe (1972) adsorbed rat serum on asbestos dust and found inactivation of the toxic effect on macrophages after removal of excess serum, thus confirming that inactivation of cytotoxic properties of minerals can be produced by stable antagonist-mineral complexes.

The principal result of the present studies is the fact that approximately 80 - 90% of the lytic potency of the asbestos or silica can be eliminated by some of the polymers. Moreover the active concentrations cover a rather wide range, from 50 to 2.5 to occasionally even 1.0 mg of the effective antagonists. Although the inactivation fails to show essential differences of the various antagonists, a certain degree of specificity exists. The antagonists of the asbestos varieties could perhaps be arranged in decreasing order in the following series:

Chrysotile B: (CS = NSC 46015) > (CMC = SA) > RS 781 > PVP

Amosite: CS > (CMC = SA = NSL 46015) > RS 781 > PVP

Crocidolite: (CS = CMC = SA = NSC 46015) > RS 781 > PVP

Anthophyllite: CMC > (CS = PVP) > SA > NSC 46015 > RS 781

These series showing a kind of order of the inhibition of hemolysis may indicate that the different asbestos varieties possess specific optimal inactivators. For instance, CS was found somewhat more active in chrysotile B, and amosite and CMC in anthophyllite. In no case seem the differences to be sufficiently marked to be considered of practical

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importance. It is likely that CS, CMC, SA and NSC 46015 are all suitable for inactivation of the hemolysis by asbestos.* The only striking deviation from this general aspect is anthophyllite with its marked sensitivity to the nonionic polymers PVP and MC and a suprisingly low sensitivity to the otherwise quite active pyran-copolymer NSC 46015. These observations suggest the existence of physical and/or chemical binding factors or structural surface differences. They cannot yet be explained biologically.

It should be pointed out that inactivation of fiber hemolysis by polymers is a quantitative process not only dependent on the concentration of the inactivator as evaluated in the present experiments, but also on the amount of fibers as shown in the following experiments with chrysotile and SA.

Chrysotile B	100 mg	SA	1.0 mg	:	inactivation	40%
"	40 mg	"	1.0 mg	:	"	91%
"	20 mg	"	1.0 mg	:	"	94%
"	10 mg	"	1.0 mg	:	"	95%
"	20 mg		-	:	"	0
"	10 mg		-	:	"	15%

* Polystyrene sulfate (RS781) was of lower activity. However, marked, though not optimal inactivation was observed in experiments with air-jet-milled chrysotile Jeffrey treated with RS 781 at a ratio of 25% of the weight of the fibers (Dr. J.P. Leineweber).

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An identical experiment was carried out with highly lytic silica G32 and an inactive (see Table 5) amount of PVP 2.5 mg:

G32 mg	PVP mg	% inactivation
50	2.5	0
25	2.5	55.0
10	2.5	95.5
5	2.5	97.3
5	-	0

Summarizing the observations on inactivated hemolytic minerals one may state that both asbestos and silica can be deprived of hemolytic action by comparatively small amount of polymers. The lytic agents retain only fractions of their erythrocytolytic potency even in systems in which if any or only traces of inactivators are present.

The mechanisms of mineral hemolysis and its prevention cannot yet be definitively interpreted, because the lytic process is a very complex phenomenon in which proteins, phospholipids (Stalder and Stöber, 1965; Nash et al., 1966) polysaccharides, mucodisaccharides (sialic acid; Harington et al., 1971) and mucopolysaccharides can be involved. The specificity of antagonists for asbestos and silica respectively could serve as a guide, if not the exceptions of the rule mentioned by Schnitzer et al. (1971) and described in this report would require explanations which could only be hypothetical at the present time.

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The possibility to inactivate the cytotoxic action of fibrous and particulate minerals seems, however, established. The correlation of hemolytic effects with other cytotoxic phenomena e.g. in macrophages (Parazzi et al, 1968; Pernis et al.,1966; Koshi and Sakabe, 1972)has been discussed by Harington et al.(1971) and may contribute to a rational approach to experimental work in vivo and eventually to clinical applications in silicosis and asbestosis.

V. Summary

1. Hemolytic fibers of UICC samples of chrysotile, amosite, crocidolite, and anthophyllite can be inactivated by binding of polyanion - antagonists. After extensive washing are no or not measurable traces of the inhibitors available in the system.

2. Polyanions are preferentially inactivating 80 - 90% of the lytic action of asbestos varieties in a wide range of concentrations covering amounts 2.5 mg - 50 mg in case of sodium alginate, carboxymethylcellulose, chondroitin sulfate and pyran-copolymer NSC 46015. Polystyrene sulfate was as a rule less effective.

3. Anthophyllite was the only variety to show marked inactivation after binding nonionics such as polyvinylpyrrolidone and methylcellulose in addition to most of the anionic polymers.

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4. Silica, both fibrous (microquartz) and microfine dusts, is inactivated by adsorbing polyvinylpyrrolidone resulting in stable mineral-polymer complexes of 80 - 90% reduced lytic activity in a system in which no free antagonist is available.

5. Microquartz, but not the microfine silica dusts, is also inactivated by the anionic chondroitin sulfate.

Acknowledgements

The authors gratefully acknowledge the collaboration of Victor Baden, Ph.D. and Mr. Igor Gonda, Jr. who participated in earlier stages of this work. We also thank Dr. Walter L. Schleyer of Philadelphia Quartz Company for generously supplying us with the silica materials used in these studies. To Dr. Irving J. Selikoff, Professor of Community Medicine, Mount Sinai School of Medicine we are greatly indebted for his encouraging interest in this work and his always helpful advice and guidance.

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Table 1

Inactivation by Polymers of Chrysotile B (UICC)

in Sheep Cell Hemolysis

Constant amount of chrysotile: 100 mg

Antagonist	% Inactivation by mg:					
	50	25	10	5	2.5	1.0
SA[a]	77	85	85	76	78	25
CMC	77	82	85	79	81	15
CS	83	86	86	87	84	15
NSC 46015	84	90	91	80	80	63
RS 781	73	72	68	68	63	-
PVP	< 5[b]					

a: SA = sodium alginate
CMC = carboxymethylcellulose
CS = chondroitin sulfate
NSC 46015 = pyran-copolymer
RS 781 = polystyrene sulfate (high molecular weight)
PVP = polyvinylpyrrolidone

b: 40 mg PVP tested against a small chrysotile quantity of 20 mg

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Table 2

Inactivation by Polymers of Anthophyllite (UICC)

in Sheep Cell Hemolysis

Constant amount of anthophyllite: 100 mg

Antagonist	% Inactivation by mg:						
	100	50	25	10	5	2.5	1.0
SA[a]	-	75	75	51	46	27	-
CMC	-	90	90	91	92	88	92
CS	-	90	92	84	44	19	-
NSC 46015	-	65	45	24	17	13	-
RS 781	-	41	30	26	11	-	-
PVP	88	79	85	78	82	78	-

a: see Table 1

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Table 3

Average of Inactivation by Polymers of Sheep Cell

Hemolysis caused by Asbestos Fibers

Antagonist	range mg	Average % Inactivation of Lysis by Chryso B	Amosite	Crocidolite	Anthophyllite
SA[a]	2.5 - 50	80 ± 4.4	81 ± 5.4[b]	75 ± 5.2	62 ± 15.4
CMC	2.5 - 50	81 ± 3.1	81 ± 3.5	70 ± 9.5	91 ± 1.3[c]
CS	2.5 - 50	85 ± 1.6	90 ± 4.5	72 ± 6.2	82 ± 9.2[d]
NSC 46015	2.5 - 50	85 ± 5.3	79 ± 4.4[c]	72 ± 6.0	65.0[e]
RS 781	2.5 - 50	69 ± 4.2	43 ± 2.4	25 ± 7.0	0
PVP	2.5 50	0	29 ± 3.6	0	82 ± 5.0

a: see Table 1

b: 100 mg SA included

c: 1.0 mg CMC or NSC 46015 included

d: 5.0 mg CS and less omitted: < 50%

e: 2.5 - 25 mg omitted: < 50%

Table 4

Dye Stuff Adsorption by Chrysotile B and Amphibole Asbestiform Fibers

Fiber UICC 100 mg	Percent Adsorption of Methylene Blue 2 mg %[a]	Trypan Blue 2 mg %[a]	Trypan Blue 5 mg %[b]
Chrysotile B	23.0	100.0	100.0
Amosite	52.5	0	0
Crocidolite	48.0	0	0
Anthophyllite	90.8	42.5	18.6
Control	0	0	0

a: total amount: 0.4 mg

b: total amount: 1.0 mg

Table 5

Inactivation by Polymers of Silica in Sheep Cell Hemolysis

Silica	Inactivator	% Inactivation by mg[a]: 50-40	25-20	10	5.0	2.5	1.0	0.5	0.25	0.13	0.06
Micro-Quartz 100 mg[a]	PVP	-	-	89	89	86	89	86	85	64	48
	CS	93	91	84	78	54					
	SA	0	-	-	-	-	-	-	-	-	-
	NSC 46015	-	0	-	-	-	-	-	-	-	-
G30	PVP	95	95	97	87	64	12.5	-	-	-	-
50 mg[a]	CS	0	0	0	-	-	-	-	-	-	-
	SA	-	0	-	-	-	-	-	-	-	-
G32	PVP	96	96	96	90	0	0	0	-	-	-
50 mg[a]	CS	0	0	0	-	-	-	-	-	-	-
	SA	-	0	-	-	-	-	-	-	-	-

a: total amount of silica or inactivator respectively in the exposure (binding) system

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