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Some derivatives of polyvinylpyridine 1-oxides and their effect on the cytotoxicity of quartz in macrophage cultures

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Summary

1. Poly(2-vinylpyridine 1-oxide) counteracts the pathogenic effects normally produced when quartz is injected into or inhaled by animals and the cytotoxic effects when quartz is added to macrophage cultures. The protective action of this polymer has been attributed variously to the formation of an adsorbed layer on the quartz particles, complex formation with monosilicic acid produced by the dissolution of quartz, and strengthening of the membranes or microstructures of the cells.

2. Stereoregular forms of poly(2-vinylpyridine 1-oxide), some alkyl derivatives of poly(2-vinylpyridine 1-oxide), poly(3-vinylpyridine 1-oxide) and poly(4-vinylpyridine 1-oxide), a copolymer of 2-vinylpyridine 1-oxide and 2-*n*-propenylpyridine 1-oxide, some poly(1-methyl-2-vinylpyridinium) quaternary salts, and poly(1-methoxy-2-vinylpyridinium iodide), which had previously been synthesized and studied with respect to their viscosities and interaction with silicic acid, were tested for their ability to counteract the cytotoxic effects of quartz in macrophage cultures. The tests were effected both by pretreating the quartz with polymers, and by pretreating the cells.

3. Every polymer proved active in one or other of these conditions, but several were active in one test but inactive in the other. Some polymer quaternary salts, which do not contain the N-oxide group, were found to be active. A remarkable difference in activity was found between the two stereoregular forms of poly(2-vinylpyridine 1-oxide). Pretreatment of the quartz with some of the polymers increased its cytotoxicity significantly.

4. Most of the results could be interpreted on the hypothesis that the polymers form an adsorbed layer on the quartz surface, but it is difficult to apply this explanation to two polymers which are inactive when used to pretreat the macrophages but are active when adsorbed on quartz.

Introduction

Schlipköter & Brockhaus (1961) found that intraperitoneal injections of poly(2-vinylpyridine 1-oxide), (I), could almost completely inhibit the fibrogenesis which normally follows the introduction of silica dust into the lung or peritoneal cavity. This polymer is very soluble in water. Poly(2-vinylpyridine), which has a very low solubility, was found to be ineffective (Schlipköter & Brockhaus, 1960); if injected

as the soluble hydrochloride, it was converted into the free base in the body. Other polymers containing a tertiary amine oxide group outside the aromatic ring have been found comparable in activity with polymer (I), and a polymer with the $N(CH_2CH_2)$ group of the morpholine structure is active (Ferruti & Marchisio, 1966 ; Marchisio, Pernis, Vigliani & Ferruti, 1965).

The activity of polymers against the pathogenic action of quartz can also be demonstrated in cultures of macrophages. When incubated with quartz particles, alveolar and peritoneal macrophages are killed, but if polymer (I) is added to the culture, toxic effects are absent or reduced. The protective effects of various substances on macrophages can be measured by several methods (Beck, 1969). The number of living cells in a culture can be determined at intervals and a comparison made between cultures containing quartz alone and similar cultures containing a test substance in addition (Hanks & Wallace, 1958). Two other convenient indicators have been used (Horn & Bruns, 1956): lactic acid production and lactic dehydrogenase. In the absence of toxic substances there is a steady increase in lactic acid in the culture which can be measured enzymatically, but in the presence of quartz the rate of lactic acid production is diminished. The lactic dehydrogenase activity in the supernatant liquid, being a measure of enzyme released from damaged cells, is also used as an indicator. In this series of experiments, the viability test has given consistent and reproducible results. The other two tests confirmed these results. Schlipköter & Beck (1965) have shown that substances which have been proved inactive against silica in cell cultures are inactive in the whole animal but that some substances which show activity in cell cultures may be inactive against silica in the intact animal.

Tests with macrophage cultures can be made in two ways. Polymer may be added first to the culture, the cells washed after an interval and the quartz then added ; or the polymer may be added first to the quartz and the quartz washed after an interval before being added to the macrophage culture. Pretreatment of the quartz allows the polymer to coat the surface before the silica can affect the macrophages ; pretreatment of the macrophages gives an opportunity for the polymer to enter the cytoplasm before the cell can be affected by silica. These two techniques sometimes give different results. This paper describes the activity of a number of alkyl-substituted polyvinylpyridine 1-oxides and quaternary salts of poly(2-vinylpyridine).

Holt & Lindsay (1969a) showed by viscosity studies that all these polymers interact with monosilicic acid. Nasrallah (1968) studied the adsorption of poly(2-vinylpyridine 1-oxide) on quartz ; these other polymers interact with silicic acid, so it is probable that they also will adsorb onto a quartz surface.

Methods

Polymers

The atactic polymers were prepared by polymerizing vinylpyridine monomers derived from alkyl-substituted 2- (or 4-) methylpyridines (Holt & Lindsay, 1969a). Stereoregular poly(2-vinylpyridines) were prepared by the anionic polymerization method of Natta, Mazzanti, Longi, Dall'Asta & Bernandini (1961) for the isotactic, and that of Geuskens, Lubikulu & David (1966) for the syndiotactic polymer. A co-polymer of 2-vinylpyridine and 2-*n*-propenylpyridine was prepared by a method described by Natta, Longi & Nordio (1965). The polymers were oxidized by a

method described by Nasrallah (1968). Details of the synthetic methods used for the polymer oxides and studies of their physical and chemical properties have been described (Holt & Lindsay, 1969a, b) and those used for the quaternary salts will be published. Viscosity determinations were carried out on aqueous solutions at 25° C.

Quartz

The quartz used in the cell tests was a finely ground powder (Dörentruper crystal quartz meal no. 12), particle size less then 3 μ, specific surface 5·7 m^2/g. It was used as a suspension (1 mg/ml.) in culture medium.

Cell tests

Cells pretreated with polymer. Guinea-pigs (300–400 g) were injected intraperitoneally with 20 ml. of physiological saline. After 48 hr the animals were killed, the peritoneum opened, and the exudate washed out. The macrophages were counted and the suspension was so distributed between Leighton tubes that each tube contained $4–5 \times 10^6$ cells. The polymer was added to give a concentration of 0·4 mg/ml. The tubes were incubated at 37° C. After 30 min the cells became attached to the bottom of the tubes; they were then washed by decantation with saline three times and culture medium was added. Details of the method and composition of the culture medium have been published (Beck, Sack & Bruch, 1967). Quartz dust (300 $\mu g/10^6$ cells) was added and the tubes were incubated for a further 3, 6 or 20 hr.

Quartz pretreated with polymer. The procedure was identical with that described above except that no polymer was added directly to the cell suspension and the quartz was incubated for 1 hr with polymer (0·4 mg/ml. saline), then washed with culture medium three times, the quartz being separated each time by centrifugation. The cell viability was determined by the method of Hanks & Wallace (1958) and Boehringer Biochemica Test Kit was used to determine the lactic hydrogenase activity.

Cultures were grown on the medium NCTC 109 (Disco Laboratories, Detroit, Michigan).

TABLE 1

Polymer		$[\eta]$*
Poly(2-vinylpyridine 1-oxide), anionic sample	IA	0·17
free radical sample	IB	0·12
Poly(2-methyl-6-vinylpyridine 1-oxide)	II	0·13
Poly(3-ethyl-6-vinylpyridine 1-oxide), anionic sample	IIIA	0·05
free radical sample	IIIB	0·07
Poly(3-methyl-2-vinylpyridine 1-oxide)	IV	0·19
Poly(2-methyl-5-vinylpyridine 1-oxide)	V	0·51
Poly(3-methyl-4-vinylpyridine 1-oxide)	VII	0·30
Isotactic poly(2-vinylpyridine 1-oxide)	XV	0·13
Syndiotactic poly(2-vinylpyridine 1-oxide)	XVI	0·17
Copolymer (2-vinylpyridine 1-oxide/2-n-propenylpyridine 1-oxide)	XVII	0·08
Poly(1-methyl-2-vinylpyridinium iodide), free radical polymer	XVIII	†
Poly(1-methyl-2-vinylpyridinium iodide), anionic polymer	XIX	†
Poly(1-methyl-2-vinylpyridinium chloride), free radical polymer	XX	†
Poly(1-methoxy-2-vinylpyridinium iodide), free radical polymer	XXI	0·08

* Intrinsic viscosity in water at 25° C. † Polyelectrolyte.
A denotes anionic catalysis; B denotes free radical initiation.

Results

The compounds which were tested are listed in Table 1 and their structures are shown in Fig. 1. Tables 2 and 3 give the viability and lactic dehydrogenase values.

Poly(2-vinylpyridine 1-oxide) (I)

A polymer of high molecular weight, P204 U33, synthesized by Dr. A. Brockhaus of the Med. Institut für Lufthygiene und Silikoseforschung, University of Düsseldorf, was used as a control. Two other samples of poly(2-vinylpyridine 1-oxide) with viscosities considerably lower than that of U33 were tested. One, (IA), $[\eta]=0{\cdot}17$, was prepared by anionic polymerization and would be linear; the other (IB), $[\eta]=0{\cdot}12$, prepared by free radical polymerization, would contain some branching in the chains. Both samples were highly active against silica as judged by viability assessment; when the tests were conducted by pretreating the quartz, (IB) was more active than (IA), but, by pretreating the cells, (IA) appeared to be the more active.

Poly(2-methyl-6-vinylpyridine 1-oxide) (II)

This polymer, $[\eta]=0{\cdot}13$, was prepared by free radical polymerization which would give some branched chains. The polymer had no effect on the toxicity of the quartz when cells were pretreated, but quartz which was pretreated had a diminished cytotoxic activity.

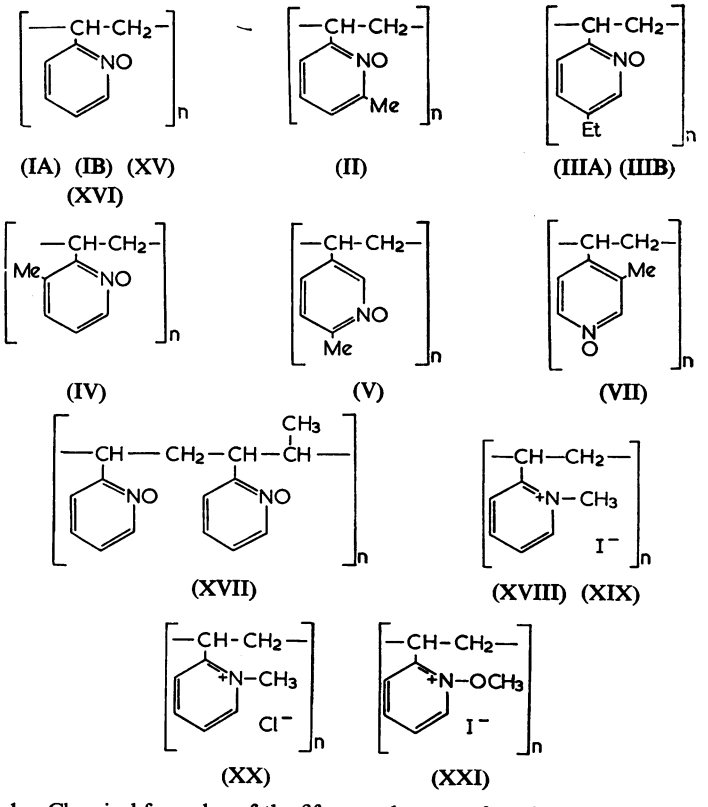

FIG. 1. Chemical formulae of the fifteen polymers referred to in text and tables.

Poly(3-ethyl-6-vinylpyridine 1-oxide) (III)

Two samples were tested, one (IIIA) was prepared by anionic catalysis and the other (IIIB) by free radical initiation. The quartz had little toxic effects on the cells when it was pretreated with either polymer, but the polymer was ineffective when added first to the culture. Both these polymers had low intrinsic viscosities. This polymer has an inverse solubility coefficient and it is precipitated from solution at about 33° C.

Poly(3-methyl-2-vinylpyridine 1-oxide) (IV)

The polymer shows an activity against quartz which is comparable with that of our samples of polymer (I); similar results are obtained whether the quartz or the culture is pretreated.

Poly(2-methyl-5-vinylpyridine 1-oxide) (V)

When the quartz is pretreated, this polymer, $[\eta]=0{\cdot}52$, proved almost as effective against the cytotoxic action of quartz as was polymer (I). When the polymer was added first to the culture, it showed no activity against quartz in either test; indeed, the polymer itself appeared to have a cytotoxic effect since the values were below those for quartz alone. Marchisio *et al.* (1965) found that the parent poly(3-vinylpyridine 1-oxide), $[\eta]^{30°}_{EtOH}=0{\cdot}09$, was likewise inactive when added first to the culture but active if it was used to pretreat the silica. They assumed that the inactivity was due to the low molecular weight. Our sample of polymer (V), however, had a much higher molecular weight than our samples of polymer (I), which were active.

Poly(3-methyl-4-vinylpyridine 1-oxide) (VII)

This polymer, $[\eta]=0{\cdot}30$, was prepared by anionic polymerization which would give unbranched chains. When the culture was pretreated, the polymer was almost

TABLE 2. *Protective action of some polyvinylpyridine derivatives against quartz cytotoxicity*

Substance added	Cells preincubated Time (hr)			Quartz preincubated Time (hr)		
	3	6	20	3	6	20
P204/U33	100	99	96	100	99	95
IA	95	95	88	99	93	67
IB	88	83	69	95	95	92
II	43	27·5	12	88	81	57·5
IIIA	51	50	63	92	93	86
IIIB	68	51	50	93	96	85
IV	97	92	92	90	89·5	65
V	34	12	13	99	97	82
VII	87	86	80·5	45	12·5	0
XV	97·5	92·5	92	88	22·5	1
XVI	82	63·5	61·5	93·5	93	86·5
XVII	95	94	92·5	68	26	4
XVIII	58	54	25	98	98	91
XIX	25	10	6	99	98	88
XX	47	15	10	97	99	93
XXI	98	97	94	97	96	95
Controls: no dust				100	100	98
alumina				98	97	93
quartz				65	35	13

Figures show the number of live cells in a total of 100 cells after the culture had been incubated for the indicated time. Each value is an average from three experiments.

as active against quartz as polymer (I). When the quartz was pretreated with the polymer, however, the toxicity to cells was greater than that of untreated quartz.

Isotactic poly(2-vinylpyridine 1-oxide) (XV)

The isotactic polymer has probably a helical conformation. When the cell culture is pretreated it is as active against the pathogenic effects of quartz as the atactic polymer (I), but when the quartz is pretreated it gives little protection.

Syndiotactic poly(2-vinylpyridine 1-oxide) (XVI)

This polymer has a planar zig-zag conformation. Its behaviour was the reverse of that of the isotactic polymer. When the cell culture was pretreated, no protection was given to the cells against silica: when the quartz was pretreated, the protection was comparable with that given by the atactic polymer previously used.

Copolymer(2-vinylpyridine 1-oxide/2-n-propenylpyridine 1-oxide) (XVII)

In spite of its low viscosity, $[\eta]=0{\cdot}08$, this polymer was highly active against quartz when the cells were pretreated. When the quartz was pretreated its toxicity was increased, as happened in the case of the isotactic poly(2-vinylpyridine 1-oxide).

TABLE 3. *Protective action of some polyvinylpyridine derivatives against the cytotoxic effects of quartz in macrophage cultures: lactate dehydrogenase activity**

	Controls: no polymer		
Dust	3 hr	6 hr	20 hr
None	38	48	210
	22	35	245
	57	86	210
	57	67	217
Alumina	38	57	191
	35	35	165
	29	38	124
	38	48	182
Quartz	124	258	535
	236	263	407
	373	240	516
	193	411	611

	Test: culture+quartz					
	Cells preincubated			Quartz preincubated		
Polymer	3 hr	6 hr	20 hr	3 hr	6 hr	20 hr
P204/U33	57	105	263	29	38	201
IA	29	73	312	29	60	306
IB	16	64	207	19	60	201
II	55	57	96	67	230	363
IIIA	229	322	388	20	70	344
IV	86	86	267	74	87	239
VII	96	124	306	230	449	239
XV	86	58	117	86	134	315
XVI	51	101	250	76	110	259
XVII	67	38	124	76	248	382

*Figures show lactic dehydrogenase (m-u./ml. NCTC 109) after the culture had been incubated for the indicated time. Each value is an average result from three experiments. Alumina is regarded as an inert dust.

Poly(1-methoxy-2-vinylpyridinium iodide) (XXI)

This polymer quaternary halide was highly active in cell tests both when used to pretreat the cells and to pretreat the quartz.

Poly(1-methyl-2-vinylpyridinium halides) (XVIII), (XIX) and (XX)

When the quartz was pretreated with any of these polymers it showed no signs of toxicity. None of them was effective against quartz when the cells were pretreated ; indeed two of the polymers appeared to enhance the toxicity of the quartz, perhaps because they themselves were cytotoxic.

Discussion

Viability tests : cells pretreated

All except polymers (II) and (V) were active against quartz cytotoxicity. Earlier work has shown that, when the cells are pretreated with poly(2-vinylpyridine 1-oxide) (Beck, Bruch & Brockhaus, 1963) or when this polymer is used to counteract the effects of silica in the whole animal (Schlipköter & Brockhaus, 1961) the polymer is ineffective if the molecular weight is low. A molecular weight above about 30,000 seems to be required for maximum activity. Presumably, the macrophages will pinocytose the larger particles and so concentrate the polymer in the cytoplasm, but smaller particles only enter and leave the cell by diffusion and will not be concentrated. A polymer which can potentially affect the cytotoxicity of silica is, then, likely to be inactive if the molecular weight is below a critical value. It must be remembered, however, that the viscosity relates to the average molecular weight and that each polymer has a range of polymer sizes.

The polymers (II) and (V) had high viscosities and their inactivity cannot be due to low molecular weight. Of the active polymers, poly(3-ethyl-6-vinylpyridine oxide) and syndiotactic poly(2-vinylpyridine oxide) were least effective. The former, whether prepared by anionic or free radical polymerization, could be prepared only with a comparatively low molecular weight ; it is reasonable to suppose that, if the molecular weight was as high as that of the other polymers, the activity would be comparable.

The contrast between the relatively low activity of the syndiotactic poly(2-vinylpyridine 1-oxide) and its atactic forms (anionic or free radical polymerization) or the isotactic form needs comment. The syndiotactic had the same viscosity as the anionic polymerized polymer and a higher viscosity than the other two. The syndiotactic form differs in that it has a planar zig-zag structure while the isotactic form is probably helical (Holt & Lindsay, 1969b). The atactic polymer has a chain containing random lengths, some of which have an isotactic and others a syndiotactic conformation.

The two inactive polymers are the only ones in this series in which an alkyl group is adjacent to the N-oxide group. In polymer (II), both positions adjacent to the N-oxide are occupied, so that the considerable steric hindrance may or may not be the explanation of the inactivity. It is unfortunate that polymer (V) is the only example in which the N-oxide has the polymer chain attached to the 3-position, so that the inactivity might be correlated with either the adjacent alkyl group or with the position of the N-oxide relative to the chain.

Quartz pretreated

When the quartz is pretreated, it is in contact with the polymer solution for 1 hr and then it is washed before being added to the cell culture. Its cytotoxicity was considerably diminished by every polymer tested except polymers (VII), (XV) and (XVII); these three polymers appear to make the quartz more toxic to the cells.

It must be supposed that the polymers which affect the cytotoxicity of the quartz become adsorbed to the quartz surface and are carried into the macrophage with the quartz. The degree of adsorption is largely independent of the molecular weight of the polymer, so it is not surprising that the polymers (IIIa) and (IIIb), which have the lowest viscosities and were least active when tested by cell pretreatment, were highly active in these tests. The least active of the active polymers was polymer (II), which has substituent groups either side of the N-oxide and which was inactive when the cells were pretreated.

Quaternary salts

Ferruti & Marchisio (1966), after studying the effects of several polymers on the cytotoxicity of silica, deduced that the N-oxide is the functional group responsible for the protective action, but the fact that all the quaternary salts were about as active as poly(2-vinylpyridine 1-oxide) itself when the quartz was pretreated, indicates that the N-oxide group is not a prerequisite for activity. Presumably all these polymers become adsorbed to the quartz surface and are carried into the cells. When the quaternary polymers were preincubated with the cells, however, only the N-methoxy iodide (XXI) proved active. These N-methyl polymers were made from a polyvinylpyridine with a high average molecular weight ($\overline{M}_w = 1{\cdot}7 \times 10^5$), but it is possible that a polyelectrolyte might not be phagocytosed because of the charge on a cell membrane; this needs to be investigated. In two cases the cell counts were lower than with untreated quartz, suggesting that the quaternary compounds themselves were toxic.

The fact that every polymer oxide and quaternary polymer proved active when tested by one or other of the methods used, suggested that all polymer N-oxides and quaternary salts of high molecular weight are potentially active, but that an alkyl group adjacent to the N-oxide may sometimes cause inactivation. These results can be explained on the hypothesis that the active polymers coat the quartz particles; the surface would then be inaccessible and the rate of dissolution would be reduced. Another suggestion by Marchisio & Comolli (1964) is that the polymer may shield ultrastructural constituents of the cells. Beck & Antweiler (1963) and Schlipköter (1964) suggested that polymers may influence enzyme reactions in the cell or produce a non-specific increased resistance of the cell which stabilizes the cell membrane. Allison, Harington & Birbeck (1966) suggested that, while silicic acid from the quartz normally combines with the lipid protein of the cell membrane, it is prevented from so doing by combining with poly(2-vinylpyridine 1-oxide). These explanations would imply that, when the quartz is pretreated, the polymer is carried into the cell and is then desorbed in the cytoplasm. There is no evidence that this occurs. There are no isotherms available relevant to the low pH which might be found in phagosomes, but Holt & Nasrallah (1968) have shown that poly(2-vinylpyridine 1-oxide) interacts with the hydroxyl groups of monosilicic acid even at pH 1–2 and it is unlikely that the bonds between polymer and the hydroxyl groups of a quartz surface will be broken even at low pH.

There is other evidence against desorption. Kühn & Jung (1957) demonstrated that simple quaternary pyridinum iodides are toxic to cells and our cell tests showed that the poly(vinyl-N-methylpyridinium iodide) salts were also toxic when cells were pretreated. The polymer was active when the quartz was pretreated, but it was not toxic. Presumably, if the polymer were desorbed from the quartz in the cell it would be toxic.

On the other hand, the properties of polymers (II) and (V), which are adsorbed on quartz, as is shown by the tests in which the quartz is pretreated, but are inactive when the cells are pretreated, do not support the hypothesis that the protective action follows the sequence, (*a*) polymer ingested by the macrophage, (*b*) quartz ingested, and (*c*) quartz coated by the polymer in the cell, unless it is assumed that these two polymers are not taken up by macrophages or that they do not adsorb on a quartz surface under the conditions in the cell.

The increased toxicity of the quartz which had been pretreated with polymers (VII), (XV) and (XVII) needs explanation. These polymers are not themselves toxic, as is indicated by the tests in which the cells were pretreated.

The toxicity of quartz to cells has not been satisfactorily explained, but there is increasing evidence that it is correlated directly to the quartz surface. By following the equilibrium of quartz powder with ^{31}Si-labelled silicic acid, Holt & King (1955) showed that a quartz surface is normally coated with an adsorbed layer of silicic acid, and Baumann (1965) studied the rate of dissolution and deposition of this silicic acid on a silica surface. Several workers have shown that treatment of silica with reagents that would remove this layer increases the toxicity. Englebrecht, Yoganathan, King & Nagelschmidt (1958) found that the cytotoxicity of quartz was increased after the outer layer had been leached away with either hydrofluoric acid or alkali. Baumann, Klosterkötter & Robock (1967) found that the cytotoxicity of a leached quartz was reduced when it was treated with monosilicic acid in solution.

If the theory of the involvement of the quartz surface itself in the killing of macrophages is accepted, it seems probable that the increased toxicity of quartz after treatment with polymers (VII), (XV) and (XVII) is due to the removal of the adsorbed silicic acid layer from the quartz by these polymers. The arrangement of the pyridine rings and the position of the 1-oxide groups in the polymer 1-oxide molecules would account for what seems to be poor adsorption on the quartz surface. This would be a reasonable deduction because rings are random about the chain in polymer (VII) and 1-oxide groups are at the extreme edges of the molecule ; the structure of polymer (XV) is probably helical, so that only one-third of the oxide groups could make contact with the surface, and in copolymer (XVII) there is an irregular arrangement of the pyridine rings. The results suggest that hydrated quartz surface adsorbs these polymers, but that they are removed together with the surface layer of silicic acid when the quartz is washed before it is added to the cell culture.

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