Spleen Acid Phosphomonoesterase (1)

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A very highly purified acid phosphomonoesterase has been prepared from hog spleen by Chersi, Bernardi, and Bernardi (1). The enzyme is obtained as a by-product during the preparation of acid deoxyribonuclease.

Spleen acid phosphomonoesterase is very similar in both its physical and enzymological properties to acid prostatic phosphomonoesterase (2).

ASSAY

Reaction

p-Nitrophenyl phosphate ⟶ *p*-Nitrophenol + Phosphate

The phosphomonoesterase activity is assayed by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate.

Reagents

1. The reaction mixture (total volume 1.1 ml) contains
   a. 1 μmole of *p*-nitrophenyl phosphate, disodium salt
      150 μmoles of acetate buffer, pH 5.0
      10 μmoles of EDTA
   b. Enzyme—this is diluted, if necessary, with 0.15 M acetate buffer + 0.01 M EDTA, pH 5.0, containing 0.05% Armour bovine serum albumin.
2. 2 N $(NH_4)OH$

Procedure

After 10 min of incubation at 37 C, the reaction is stopped by adding 0.2 ml of 2 N $(NH_4)OH$. The absorption at 400 mμ is measured and a suitable blank is subtracted from the reading. The concentration of liberated *p*-nitrophenol is calculated from the corrected reading taking an $E_{400} = 12{,}000$ for *p*-nitrophenol (3). One activity unit is defined as the amount of enzyme that catalyzes the liberation of 1 μmole of *p*-nitrophenol per minute under the above conditions. Assays are performed using enzyme concentrations to obtain OD_{400} readings not higher than 5.0. Under these conditions a linear relationship is obtained between enzyme concentration and *p*-nitrophenol liberation. The specific activity is calculated by dividing the activity by the OD_{280} of the enzyme solution.

CHROMATOGRAPHIC PURIFICATION

The starting material is crude spleen nuclease II (p. 105). If acidification to pH 2.5 is omitted, and the fraction precipitated between 0.3 and 0.6 saturation (20 C) of $(NH_4)_2SO_4$ is collected, a much higher acid phosphomonoesterase activity is found (Table 2, p. 147). Most of this activity belongs, however, to a different acid phosphomonoesterase, which is not retained by hydroxyapatite equilibrated with 0.05 M phosphate buffer pH 6.8. The chromatographic purification is patterned on Procedure C described for spleen acid deoxyribonuclease (p. 113) and summarized in Table 1.

STEP 1. DEAE-SEPHADEX A-50 (FIG. 1). This step has been described (p. 113). Acid phosphomonoesterase activity is not retained by the column equilibrated with 0.05 M phosphate buffer, pH 6.8.

A second minor activity peak, belonging to a different acid phosphomonoesterase is eluted by 0.5 M phosphate buffer, pH 6.8.

STEP 2. HYDROXYAPATITE (FIG. 2). This step has been described (p. 113). Acid phosphomonoesterase is eluted by a 0.05–0.5 M linear gradient of phosphate buffer, pH 6.8, at a molarity of about 0.25.

The second peak of acid phosphomonoesterase activity was not investigated further (see, however, step 2 in the purification of acid ribonuclease, p. 40).

STEP 3. SEPHADEX G-100 (FIG. 3). The acid phosphomonoesterase activity from the previous step is loaded on a Sephadex G-100 column equilibrated with 0.1 M acetate buffer, pH 5.6. All the activity is eluted before the main protein peak.

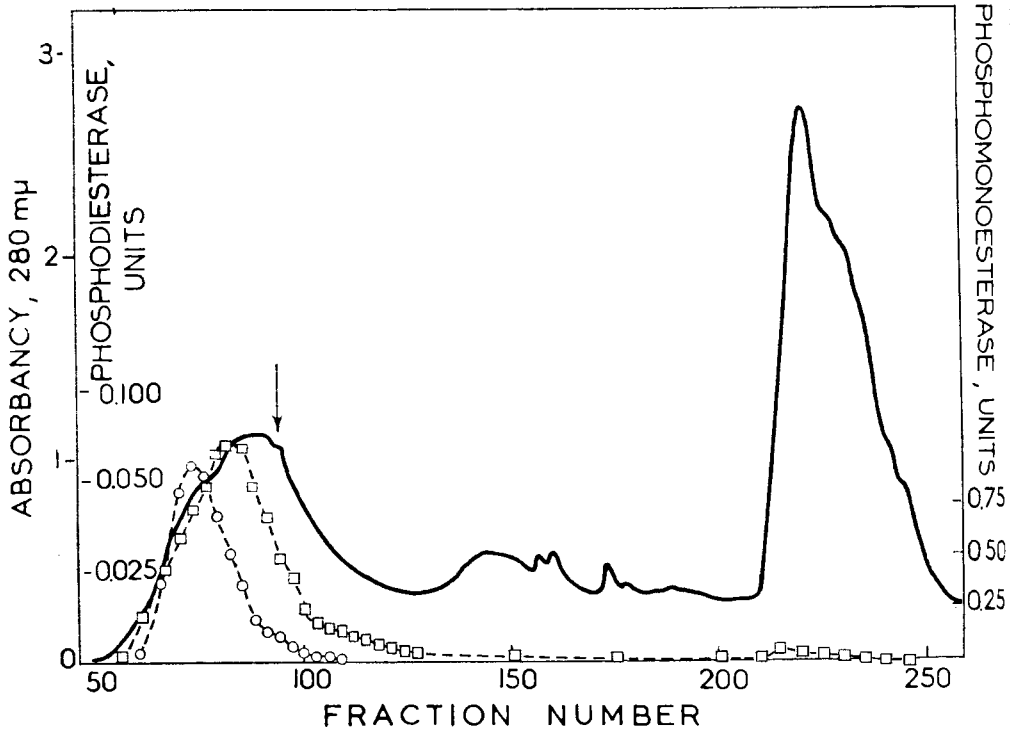

Fig. 1. Chromatography on DEAE-Sephadex A-50 of crude spleen nuclease II (see also p. 112) (Step 1). 330 ml of preparation HS 9 ($OD_{260} = 10.3$; $OD_{280} = 6.9$) were loaded on a 8- × 80-cm column of DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer, pH 6.8. This buffer was also used to elute the first protein peak. 0.5 M phosphate buffer, pH 6.8, was loaded at the fraction indicated by the arrow. 24-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Squares indicate the acid phosphomonoesterase activity (right-hand scale); circles, the phosphodiesterase activity, which is due to nucleoside polyphosphatase (left-hand inner scale). Acid deoxyribonuclease, cytochrome c, acid and basic ribonuclease were also assayed; the results are shown on pp. 39 and 112.

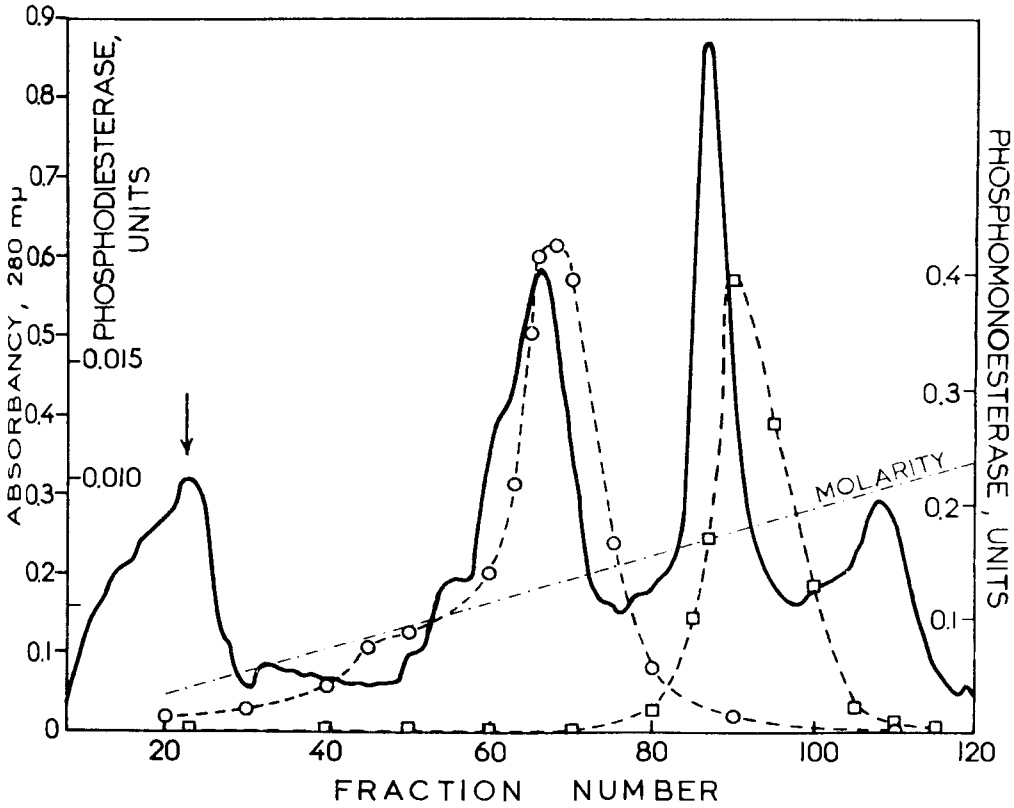

Fig. 2. Chromatography on hydroxyapatite of fractions 50–65 from Step 1. 370 ml ($OD_{280} = 1.18$) were loaded on a 2- × 40-cm column of hydroxyapatite equilibrated with 0.05 M phosphate buffer, pH 6.8. A molarity gradient (0.05–0.5) was started at the fraction indicated by the arrow (at fraction 120 M = 0.35). 24-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Squares indicate the acid phosphomonoesterase activity (right-hand outer scale); circles, the phosphodiesterase activity, which is due to nucleoside polyphosphatase (left-hand inner scale). Acid deoxyribonuclease, acid and basic ribonuclease, and cytochrome c were also assayed (see Fig. 4, p. 114).

TABLE 1. *Chromatographic Purification of Spleen Acid Phosphomonoesterase*[a]

	Volume (ml)	Total OD_{280}	Total Units	Specific Activity
Crude spleen nuclease II				0.048[b]
Step 1. DEAE-Sephadex[c]	160	950	1,090	1.15
Step 2. Hydroxyapatite	120	170	834	4.86
Step 3. Sephadex G-100	130	10	544	54.4
Step 4. CM-Sephadex	65	1	256	256
CM-Sephadex	12	0.45	128	280

[a] All values reported refer to the fractions that were processed further, or to the final product. The side fractions of the activity peaks were processed separately.

[b] This value was found for preparation HS 11.

[c] This product was formed by the phosphomonoesterase-rich fractions obtained from the first peak of the DEAE-Sephadex and from the subsequent hydroxyapatite chromatography (Steps 1 and 2 of Procedure C in the acid deoxyribonuclease purification).

STEP 4. CM-SEPHADEX C-50 (FIG. 4). The activity is loaded on a CM-Sephadex C-50 column equilibrated with 0.1 M acetate buffer, pH 5.6. The activity is eluted by a molarity gradient, 0.1–0.3 M of acetate buffer at a molarity of about 0.26. The active fractions are diluted with one volume of water and rechromatographed on CM-Sephadex C-50 as above (Fig. 5). Phosphomonoesterase is again eluted at about 0.26 M acetate; the specific activity is constant through the peak. The active fractions are pooled, frozen, and stored at −60 C. This product is referred to as the final product in Table 1.

Properties of the Enzyme

PHYSICAL PROPERTIES. A sedimentation constant equal to 5.6 was determined when the acid phosphomonoesterase activity was centrifuged in a sucrose gradient as described by Bernardi and Griffé (4), using cytochrome c or acid deoxyribonuclease as reference proteins.

PURITY. The following possible contaminating activities were assayed in the final product: acid deoxyribonuclease, acid ribonuclease, exonuclease, and phosphodiesterase; this latter activity was determined using Ca [bis(*p*-nitrophenyl) phosphate]$_2$ in 0.15 M acetate buffer + 0.01 M EDTA, pH 5.0, and in 0.25 M succinate buffer, pH 6.5. In every case 0.1 ml of the final solution was used and digestions were carried out for

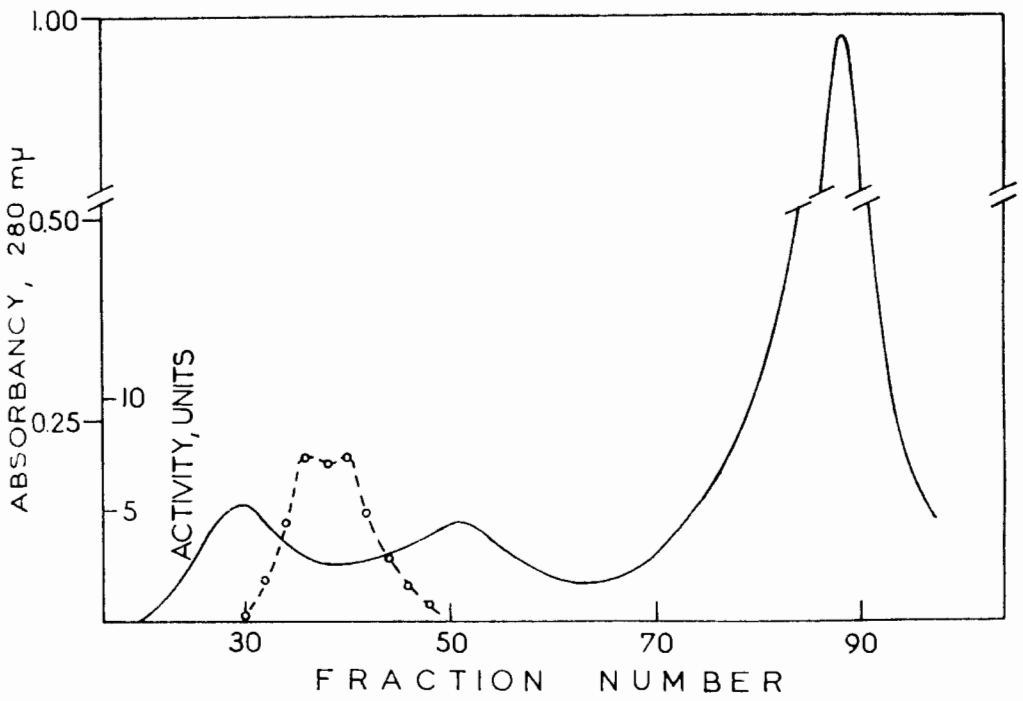

Fig. 3. Gel filtration on Sephadex G-100 of acid phosphomonoesterase–rich fractions from a hydroxyapatite chromatography. 120 ml (OD_{280} = 1.40) were loaded on a 4- × 95-cm column of Sephadex G-100 equilibrated with 0.1 M acetate buffer, pH 5.6; the same solvent was used to elute the protein from the column. 13-ml fractions were collected. The continuous line indicates the absorption at 2.80 mμ. Circles indicate the acid phosphomonoesterase activity (left-hand inner scale). Fractions 34–42 were processed further.

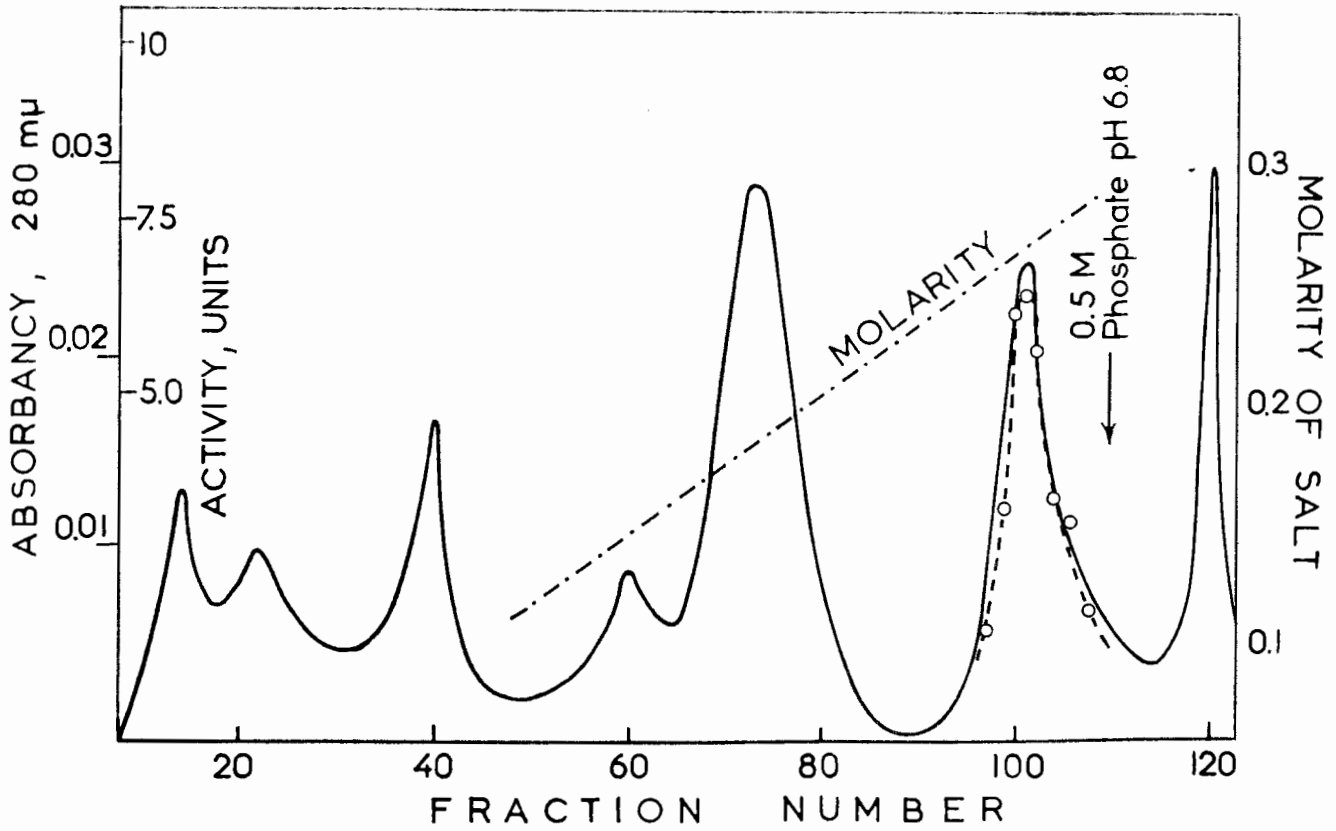

Fig. 4. Chromatography on CM-Sephadex C-50 of the acid phosphomonoesterase fraction from Sephadex G-100. 130 ml ($OD_{280} = 0.080$) were loaded on a 2- × 38-cm CM-Sephadex C-50 column equilibrated with a 0.1 M acetate buffer, pH 5.6. A molarity gradient (0.1–0.3) of acetate buffer was started at the fraction indicated with an arrow (right-hand ordinate). 0.5 M phosphate buffer, pH 6.8, was loaded at the fraction indicated by the arrow. 5-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid phosphomonoesterase activity (left-hand inner scale). Fractions 97–109 were diluted with 1 volume of water and processed further.

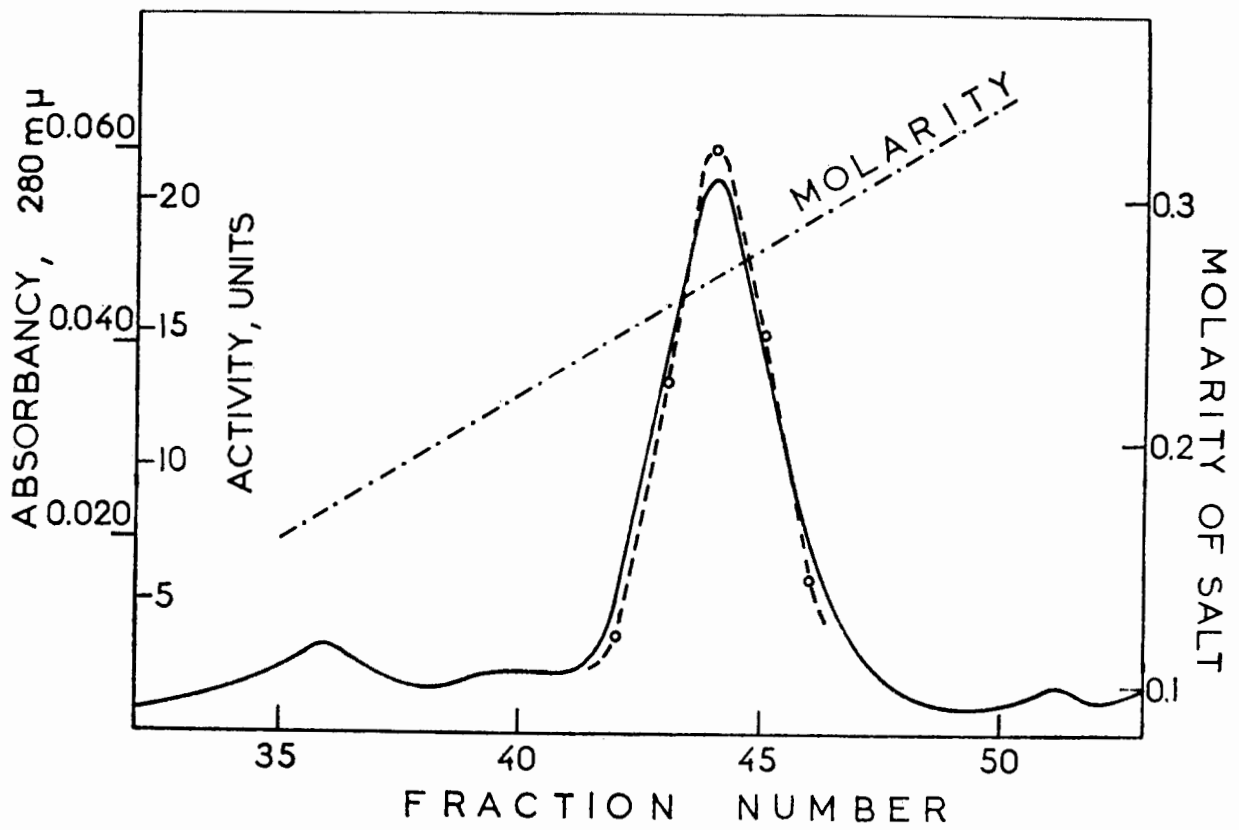

Fig. 5. Rechromatography on CM-Sephadex C-50 of the acid phosphomonoesterase fraction. 130 ml were loaded on a 0.9- × 16-cm column of CM-Sephadex C-50 equilibrated with 0.125 M acetate buffer, pH 5.6. A molarity gradient (0.125–0.5) of acetate buffer was used to elute the enzyme (right-hand scale). 3.2-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid phosphomonoesterase activity (left-hand inner scale).

2 hr at 37 C. No contaminating activities were detected. When using a 1:100 dilution of the same enzyme solution, the liberation of 0.058 μmoles of *p*-nitrophenol from *p*-nitrophenyl phosphate was obtained in 10 min.

ENZYMOLOGICAL PROPERTIES. With *p*-nitrophenyl phosphate as the substrate, at $\mu = 0.1$ between pH 2.0 and 6.0 the pH-activity curve shows a very broad optimum between pH 3.0 and 5.0. The activity falls much more rapidly on the acid than on the basic side. Cysteine and versene do not exert any relevant effect on the enzyme at a 0.01 M level. Magnesium is very slightly inhibitory. L(+) tartaric acid, but not D(−) tartaric acid, F^-, Cu^{2+}, and Mo^{6+} are powerful inhibitors at a 0.001 M level. The relative rates of hydrolysis of several substrates are given in Table 2.

TABLE 2. *Relative Rate of Hydrolysis of Several Substrates by Spleen Acid Phosphomonoesterase*

Substrates[a]	Relative Rate of Hydrolysis (%)
p-Nitrophenyl phosphate	100
2′-3′-AMP	96
5′-AMP	63
Riboflavinphosphate	29
Thiaminphosphate	21
Phosphothreonine	6
Phosphoserine	4
ATP	0
Bis(*p*-nitrophenyl) phosphate	0

[a] The substrate concentration was 1.5 mM. The liberation of inorganic phosphate at 37 C was determined at different incubation times, and the initial rates were used.

The enzyme is competitively inhibited by inorganic phosphate.
The enzyme is very easily adsorbed by glass surfaces.

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