together they may represent over 90 % of the products. There is present about 5 % of the epoxy acid, but little or no monoacyl esters and little or no coloured, resinous oils and the acids forming the scission products also appear to be absent or nearly so.

5. *At all the above temperatures* the products give evidence for the presence of β -ketonic acids and considerable volumes of carbon dioxide and water vapour are evolved. This apparent chain oxidation and degradation is regarded as being brought about

by peroxides present in small amounts and formed from the unsaturated keto acids which are the initial products whose formation is probably catalysed by certain metals.

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Studies on the more Soluble Complexes of Soil Organic Matter

2. THE COMPOSITION OF THE SOLUBLE POLYSACCHARIDE FRACTION

By W. G. C. FORSYTH,* *The Macaulay Institute for Soil Research, Aberdeen*

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In the first paper of this series (Forsyth, 1947a) a method of fractionation of the soluble complexes of soil organic matter was presented and the observation made that polysaccharide fractions containing uronic acid groups could be readily isolated in reasonable yield from diverse types of soil. Because of the important effect that such polysaccharides might well have on the physical properties (Martin, 1946) and the biochemical properties (Forsyth, 1948a) of the soil, their composition has been studied and to some extent elucidated.

EXPERIMENTAL

Purification of the polysaccharide complex

The polysaccharide fraction was isolated by the selective adsorption procedure previously described (Forsyth, 1947a).

* Now at the Colonial Microbiological Research Institute, Trinidad, British West Indies.

It was first purified by precipitation into 4 vol. of ethanol containing 2 % (v/v) acetic acid followed by further purification by the addition of excess CuSO_4 to an alkaline solution of the gum. The precipitated copper complex after washing with water was decomposed with ethanol containing HCl and washed free from Cu^{++} and Cl^- with ethanol. The polysaccharide remained as a white powder which, after a further ethanol precipitation, could be dried by washing with ethanol followed by ether to give an ash-free powder readily soluble in water. Polysaccharide complexes were prepared in this manner from the heath, pine-forest, and agricultural soils previously described (Forsyth, 1947b), and also from deciduous woodland and permanent pasture soils, all from north-east Scotland. A similar fraction was prepared from a garden soil in Trinidad, British West Indies. All the preparations from the diverse sources had a slight negative rotation $[\alpha]_D^{20} - 2^\circ$ to -10° in water (c, 1.0, as Na salt) and a nitrogen content of 0.1-0.5%. However, on titration with 0.02N-NaOH equivalents were obtained ranging from 1000 to 1300.

*Qualitative identification of the constituent
sugars*

The polysaccharides from all sources gave the same sugars on hydrolysis. The sugars were identified by paper chromatography (Partridge, 1946, 1948), confirmation being obtained by spraying papers with phenolic reagents (Forsyth, 1948*b*; Partridge, 1948). It was found advisable because of the complexity of the hydrolysate to chromatograph with four different solvents on one-dimensional chromatograms. Two-dimensional chromatograms were not very helpful with the sugar mixture present. The solvents used were water-saturated *n*-butanol, phenol, *s*-collidine, and amyl alcohol. Although the relative movements of the sugars with respect to the solvent boundary (R_F value) varied to some extent, it was found that their relative movement with respect to one another was remarkably constant in spite of variations in apparatus, temperature, etc. Xylose is the most readily identified of the sugars present in the polysaccharides. The rate of movement of the sugars is therefore expressed with reference to the rate of movement of xylose on the same paper. The factor used (R_X) is the ratio of the distance moved by a sugar to the

distance moved by xylose. R_X is much more readily determined than R_F when the spots are very slow moving, as in butanol or amyl alcohol, and may require up to 1 week for full development, by which time the solvent boundary has moved off the paper.

The polysaccharides (5–10 mg.) in $N-H_2SO_4$ (0.5 ml.) were heated in a sealed capillary tube in a boiling water bath for 5 hr. The seal was then broken, the hydrolysate carefully neutralized with $BaCO_3$ to congo red paper and the precipitate centrifuged down while still in the capillary. The neutral supernatant liquor was then spotted on many chromatogram strips for development with different solvents and spraying with various reagents after development. The polysaccharides from the different soils gave qualitatively identical chromatograms. The sugars identified are shown in Table 1. Spraying with resorcinol, etc. (Forsyth, 1948*b*), showed that ketoses were absent, and spraying with acetylacetone and *p*-dimethylaminobenzaldehyde (Partridge, 1948), demonstrated that no hexosamines were present. Fucose, lyxose, rhamnose, and deoxyribose were chromatographed alongside the hydrolysates and shown to be absent. Galactose, glucose, mannose, arabinose, xylose, and ribose spots were identified. Spots correspond-

Table 1. *One-dimensional chromatograms of polysaccharide hydrolysate
and reference sugars*

Spot no.	R_X of spot	Reference sugars	R_X of reference sugars	Phenolic spray
<i>n</i> -Butanol				
1	0.50	Galactose	0.50	Hexose
2	0.58	Glucose	0.58	Hexose
3	0.83	Mannose	0.80	Pentose
		Arabinose	0.85	Pentose
4	1.00	Xylose	1.00	Pentose
5	1.50	Ribose	1.50	Pentose
Amyl alcohol				
1	0.31	Galactose	0.29	Hexose
2	0.38	Glucose	0.38	Hexose
3	0.66	Mannose	0.65	Hexose
4	0.81	Arabinose	0.81	Pentose
5	1.00	Xylose	1.00	Pentose
6	1.30	Ribose	1.31	Pentose
<i>s</i> -Collidine				
1	0.68	Galactose	0.68	—
2	0.78	Glucose	0.78	—
3	0.88	Mannose	0.92	—
		Arabinose	0.86	—
4	1.00	Xylose	1.00	—
5	1.20	Ribose	1.12	—
Phenol				
1	0.87	Glucose	0.89	—
2	1.00	Galactose	1.00	—
		Mannose	1.02	—
		Xylose	1.00	—
3	1.20	Arabinose	1.23	—
4	1.36	Ribose	1.34	—

ing to uronic residues were invariably found close to the starting line. Moreover, faint traces of glucurone were identified (R_x in collidine, 1.44; in amyl alcohol, 1.90).

Quantitative estimation of the constituent sugars

For the methods used see Flood, Hirst & Jones (1947, 1949). For comparison of the polysaccharides from different sources an internal sugar standard was not used but only the ratios of the sugars present determined. About 10 mg. of each polysaccharide was hydrolysed and neutralized as in the qualitative examination. The sugar solution was spotted across a 5 in. chromatogram strip and, after developing, the individual sugars were extracted and estimated by Somogyi's micro sugar reagent (Somogyi, 1945) as described by Flood *et al.* (1947). Unfortunately, it was not realized when the estimations were made that both mannose and arabinose were present and butanol was used as the solvent. The mannose and arabinose were therefore estimated together and the molar ratios for this spot are calculated in Table 2 by division by 165, the mean of a hexose and pentose molecule. It is probable that the mannose is generally present in greater amount than arabinose.

Hydrolysis with *N*-sulphuric acid

The polysaccharide complex (2 g.) was heated with $N-H_2SO_4$ (100 ml.) at 90–95°, the reaction being followed by polarimetric observations. $[\alpha]_D^{20}$ (c, 2, in $N-H_2SO_4$) –5° (initial value); +21° (1 hr.); +43° (2 hr.); +45° (3 hr.); +45° (5 hr.). Iodometric titration of samples after 5 hr. gave a value for reducing sugars corresponding to about 80% of the original polysaccharide. The cooled hydrolysate was neutralized with $BaCO_3$ and filtered. The neutral solution was concentrated under reduced pressure to a small volume and poured into methanol. The precipitated Ba salts were collected (0.8 g.), washed with methanol and dried. Concentration of the methanol filtrate gave a sugar syrup (1.3 g.).

(a) *The sugars.* The syrup was dissolved in water (25 ml.) and volumes of the order of 2–3 μ l. spotted on to paper strips along with reference sugars as before. Galactose, glucose, mannose, arabinose, xylose and ribose were again detected. Neither ketoses, hexosamines, rhamnose, fucose, lyxose nor deoxyribose were present. To 10 ml. of the sugar solution the phenylhydrazine reagent of Hirst, Jones & Woods (1947) was added and the mannose estimated as phenyl-

Table 2. *Sugar ratios in the polysaccharide complexes from diverse soils*

(A, Amount of sugar estimated (μ g.); B, molar ratio.)

Sugars	Heath soil		Pine forest soil		Deciduous woodland soil		Permanent pasture soil		Trinidad soil	
	A	B	A	B	A	B	A	B	A	B
Galactose	121	1.8	370	1.9	256	1.8	314	1.8	291	1.9
Glucose	139	2	385	2	281	2	360	2	314	2
Mannose, arabinose	225	3.5	474	2.6	462	3.6	457	2.8	381	2.7
Xylose	197	3.4	619	3.9	434	3.1	421	2.8	392	3.0
Ribose	25	—	24	—	76	—	49	—	15	—

Larger scale investigation

The work described below was carried out entirely on the polysaccharide complex prepared in the usual manner from a large sample of a humus-rich agricultural soil. About 10 g. of the purified soil polysaccharide was prepared from 10 kg. of air-dried soil containing 8.5% organic matter. The air-dried polysaccharide contained 10–20% water. It did not reduce Fehling solution and had a slight negative rotation $[\alpha]_D^{20}$ –5° in water (c, 1.0, as Na salt). After dialysis for 1 week against distilled water the polysaccharide had a titration equivalent of 1185 (0.02 *N*-NaOH). (Found: OMe, nil; N, 0.34; furfural, 18.1, estimated as phloroglucide after treatment of the polysaccharide with boiling 12% (w/v) HCl in the usual way; methylfurfural, nil; hexosamine, nil; uronic anhydride, 15.8%, calc. from the CO_2 liberated on boiling with 12% (w/v) HCl.) A substance containing 15.8% uronic anhydride and no other acidic residues should have an equiv. wt. of 1114 (found by titration, 1185). This proportion of uronic anhydride accounts for 3.5% furfural, leaving 14.6% furfural contributed by the pentosans. Therefore the calculated pentosan—assuming xylose and arabinose to be present in the ratio 3:1 (*vide infra*)—is 23.8% (found 24.7%, by chromatographic estimation).

hydrazone (found, 15.2% mannose). The remainder of the sugar solution was used for the estimation of xylose by the method of Breddy & Jones (1945) as modified by Wise & Ratliff (1947) (found, 16.5% xylose).

(b) *Chromatography on a cellulose column.* Using the technique introduced by Hough, Jones & Wadman (1948), a column of Whatman no. 1 cellulose, 20 $\times$ 2 cm., was prepared by breaking up the filter paper in water-saturated collidine in a Waring blender and transferring to the column with gentle suction. The sugar syrup from a hydrolysate of 1 g. of polysaccharide, prepared as before, was added to the top of the column which was then eluted with water-saturated collidine under slight pressure at the rate of 10 ml./hr. Each 10 ml. portion was collected separately, concentrated to about 1 ml. and spotted on to paper-strip chromatograms using the four solvents and also phenolic sprays. Because of the partial resolution of the complex mixture the identification of the constituent sugars was more precise and the absence of other sugars definitely confirmed. The volumes of the fractions and the sugars identified in them were as follows: first 200 ml., nil; 60 ml., ribose; 70 ml., ribose + xylose; 20 ml., xylose; 150 ml., xylose + mannose + arabinose; 20 ml., mannose + arabinose + glucose; 60 ml., mannose + arabinose + glucose + galactose; 170 ml., glucose + galactose; 30 ml., galactose; 20 ml., nil. In the last two sugar fractions glucose could be identified

as the osazone and galactose as the phenylmethylhydrazone, since no interfering sugars such as mannose or arabinose were present. All the fractions containing arabinose (less than half the sugars) were combined and evaporated to a syrup which was taken up in 2 ml. of water, and the arabinose was estimated as the benzoylhydrazone (Hirst *et al.* 1947). No interfering sugars were present in these fractions (found 5.9% arabinose).

(c) *Quantitative paper chromatography.* Polysaccharide (10.205 mg., in 0.5 ml. of $N-H_2SO_4$) was heated in a sealed capillary in a boiling water bath for 5 hr. The seal was broken and rhamnose (3.120 mg.) weighed in as internal standard. The hydrolysate was then carefully neutralized with $BaCO_3$ and centrifuged while still in the capillary. The supernatant liquor was spotted on 'quantitative' strips and developed with amyl alcohol for 1 week. The results are shown in Table 3.

Table 3. *Quantitative analysis of hydrolysate of soil polysaccharide complex*

	Sugar estimated from paper ($\mu g.$)	Sugar present in hydrolysate (mg.)	Hexosan or pentosan (%)	Approx. molar ratio
Rhamnose*	280	3.120	—	—
Polysaccharide	—	10.205	100	—
Galactose	136	1.51	13.3	2
Glucose	140	1.56	13.8	2
Mannose	147	1.64	14.5	2
Arabinose	82	0.91	7.8	1
Xylose	163	1.82	15.7	3
Ribose	12	0.14	1.2	—

* International standard.

(d) *Barium salts.* The precipitated Ba salts (0.8 g.) from the hydrolysis with $N-H_2SO_4$ had $[\alpha]_D^{20} + 57^\circ$ in water (c, 0.3, as Ba salt). (Found: Ba, 17.1 (estimated as $BaSO_4$); furfural, 8.9%. The Ba salt of a hexose aldobionic acid requires furfural, 10.0; Ba, 16.2%.) The Ba salt (10 mg.) was hydrolysed in a sealed tube in a water bath with $2N-H_2SO_4$ (0.5 ml.) for 7 hr., giving glucose and glucuronic acid (or glucurone, depending on the solvent) as shown by chromatography. Quantitative chromatography confirmed that the acid and the sugar were present in equimolar quantities.

Table 4. *Calculated and observed values for soil polysaccharide*

(Calculated values are based on the molecular proportions of sugars given below.)

	Calculated (%)	Observed (%)
Galactan	14.8	13.3*
Glucosan	14.8	13.8*
Mannan	14.8	{ 14.5*
		{ 15.2†
Araban	6.1	{ 7.8*
		{ 5.9†
Xylan	18.2	{ 15.7*
		{ 16.5‡
Uronic anhydride	16.2	15.8
Furfural	18.5	18.1
Equivalent	1088	1185

* By paper chromatography. † As phenylhydrazone.

‡ As benzoylhydrazone.

§ As dibenzylidene dimethylacetal.

(e) *Composition of soil polysaccharide.* The results indicate (Tables 3 and 4) that the soil polysaccharide complex contains the following sugars in the proportions indicated: D-galactose (2 mol.); D-glucose (2 mol.); D-mannose (2 mol.); L-arabinose (1 mol.); D-xylose (3 mol.) and 2 mol. of an aldobionic acid containing D-glucose (1 mol.) and glucuronic acid (1 mol.).

Oxidation of the polysaccharide with potassium metaperiodate

(a) Soil gum (39.346 mg.) was weighed into a dry steamed 100 ml. stoppered bottle, dissolved in 10 ml. of distilled water and neutralized. 0.3 M-sodium metaperiodate (5 ml.) was then added, followed by KCl (2.5 g.) and water to 40 ml. The bottle was then shaken for 9 days in diffuse light, 5 ml. samples being withdrawn on the seventh, eighth and

ninth days. Excess ethylene glycol was added to destroy the residual periodate and the formic acid produced was titrated with 0.01 N-NaOH using methyl red as indicator. The reaction was found to be complete by the seventh day showing that uronic acid was not present as an end group (Brown, Dunstan, Halsall, Hirst & Jones, 1945):

39.346 mg. polysaccharide $\equiv$ 7.45 ml. 0.01 N-NaOH,
hence 522 g. polysaccharide $\equiv$ 1 mol. formic acid.

(b) In another set of identical bottles the whole 40 ml. was titrated after the eighth day for excess periodate using standard sodium arsenite and I_2 . From the results it was calculated that the 522 g. of polysaccharide which liberated 1 mol. of formic acid consumed 3.58 mol. of periodate.

(c) About 50 mg. of the polysaccharide were oxidized as in (a). After the excess periodate had been removed with ethylene glycol the solution was dialysed till free from salts. The polymeric aldehyde was then precipitated with ethanol, filtered, washed with ethanol and then ether and dried. About 20 mg. were hydrolysed in a sealed capillary tube on a boiling water bath with 0.5 ml. $2N-H_2SO_4$ for 5 hr. The neutralized solution was chromatographed and only glucose and glucuronic acid could be detected.

Autohydrolysis of soil polysaccharide

Aqueous solutions of the polysaccharide are sufficiently acid to bring about a partial hydrolysis of the polysaccharide when heated in a boiling water bath for 24 hr. Soil polysaccharide (2 g.) was heated with water (30 ml.) on a boiling water bath for 24 hr., by which time the rotation had become constant ($[\alpha]_D^{20} + 24^\circ$) and the iodometric titration of samples gave a value for reducing sugars corre-

sponding to about 15% of the original gum. The cooled solution when poured into ethanol (100 ml.) gave an alcohol-insoluble polysaccharide *B* (1.7 g.), which was washed with ethanol and then ether and dried. The filtrate from *B* was concentrated under reduced pressure to a syrup. Paper chromatography showed that arabinose was present in quantity. Considerable amounts of ribose had also been produced by hydrolysis and traces of galactose and xylose were detected. No glucose, mannose or uronic acid had been liberated. The syrup was made up to 2 ml. with water, and arabinose was estimated as the benzoyl-hydrazine by the method of Hirst *et al.* (1947). (Found, 6.7; calc., 6.1%, if all the arabinose were liberated.)

Polysaccharide B

Polysaccharide *B* was a white powder easily soluble in water; $[\alpha]_D^{20} + 20^\circ$ in water (*c*, 1). The equiv. wt. (by titration with 0.02N-alkali) was 974. Furfural, 16.2% (estimated as phloroglucide after boiling with 12% (w/v) HCl under the standard conditions). Uronic anhydride, 17.9% (calc. from the CO₂ liberated on boiling with 12% (w/v) HCl). (A substance containing 17.9% uronic anhydride with no other acidic residue should have an equivalent weight of 983; found by titration, 974. This proportion of uronic acid accounts for about 4% of the furfural, leaving 12.2% furfural contributed by the pentosan.) Polysaccharide *B* (12.394 mg. in 0.5 ml. of N-H₂SO₄) was heated in a sealed capillary in a boiling water bath for 5 hr. The seal was broken and rhamnose (4.316 mg.) weighed in as reference sugar. The hydrolysate was neutralized and chromatographed and the sugars estimated as before. No arabinose or ribose were detected. The aldobionic acid fraction was present as before. For analysis, see Table 5.

Table 5. *Calculated and observed values for polysaccharide B*

(Calculated values are for a polysaccharide containing galactose (2 mol.); glucose (2 mol.); mannose (2 mol.); xylose (3 mol.) and aldobionic acid (2 mol.; each consisting of glucose and glucuronic acid).)

	Calculated (%)	Observed (%)
Galactan	15.8	13.7
Glucosan	15.8	16.0
Mannan	15.8	15.4
Xylan	19.4	18.3
Uronic anhydride	17.2	18.9*
Furfural	16.3	17.9
Equivalent	983	16.2
		974

* From furfural estimation.

Polysaccharide *B* was also oxidized with potassium metaperiodate, and the formic acid produced and the periodate consumed estimated as before. 430 g. of *B* liberated 1 mol. of formic acid and consumed 2.84 mol. of periodate. The polymeric aldehyde produced again gave only glucose and glucuronic acid on hydrolysis. Once again the uronic acids were not end groups.

DISCUSSION

A striking feature of the results is that all the polysaccharide preparations, from several diverse types of soils, have very similar properties. Although differences in optical rotations and titration equivalents were noted, all the polysaccharides gave the same sugars on hydrolysis. The sugars identified were galactose, glucose, mannose, arabinose, xylose, and a uronic acid fraction containing glucuronic acid. Ribose was also invariably present in small amount. Estimation of the sugars present in the polysaccharides showed that the sugars, especially the pentoses, varied to some extent in the different samples, but it is considered that the unexpected similarity is much more striking than the minor discrepancies noticed and is a remarkable reflexion of the uniformity in total carbohydrate metabolism which must exist in widely different soil types (Forsyth, 1948a).

The degree of homogeneity of the polysaccharide complex from any one soil is not known. Although all attempts to separate significantly different fractions by partial ethanol and acetone precipitation were unsuccessful, this is no proof of homogeneity, since the complex polysaccharides are particularly difficult to separate. The presence of traces of ribose and nitrogen in the gums suggests that they have not been completely freed from ribonucleic acid. This is frequently the case with polysaccharides synthesized by soil bacteria (Forsyth & Webley, 1949) and strengthens the hypothesis that the polysaccharide complex is a synthetic microbial product. Polysaccharide mucilages approaching the degree of complexity of the soil polysaccharide complexes have been isolated from soil bacteria growing in artificial media (Forsyth & Webley, 1949). The highly selective method of isolation (adsorption at pH 3 and elution at pH 6-7 on charcoal) might be thought to lead to a fairly homogeneous product. Neutral polysaccharides such as bacterial levans and dextrans, when added to the soil extract and treated under the isolation conditions used, are not adsorbed by the charcoal and therefore do not contaminate the soil polysaccharide complex.

Even although the degree of homogeneity of the polysaccharide fraction is not proved, and absolute homogeneity may be considered unlikely, the same complex can be isolated from a soil repeatedly, and similar complexes from a very wide variety of soils. The complex is therefore a characteristic fraction of soil organic matter, and as such was isolated in a relatively large amount from a rich agricultural soil and further investigated. It was hydrolysed and the constituent sugars identified and estimated both by chromatographic analysis and with specific precipitants. The results indicate that this soil polysaccharide complex contains the following sugars in

the proportions indicated: D-galactose (2 mol.); D-glucose (2 mol.); D-mannose (2 mol.); L-arabinose (1 mol.); D-xylose (3 mol.); and an aldobionic acid containing D-glucose (1 mol.) and glucuronic acid (1 mol.). Considering the equivalent weight and other quantities determined, the polysaccharide has therefore a repeating unit which may be represented by the following symbols:

where Ga = D-galactose, A = L-arabinose, G = D-glucose, X = D-xylose, M = D-mannose and U = D-glucuronic acid. By estimating the formic acid produced and the periodate consumed on oxidation of the polysaccharide with potassium metaperiodate (Brown *et al.*, 1945; Halsall, Hirst & Jones, 1947), it was found that the above repeating unit liberates 4 mol. of formic acid with the consumption of 14–15 mol. of periodate. Since, under the mild conditions of oxidation used, there is no continual rise in formic acid titre followed by liberation of iodine, the glucuronic acid residues are not end groups. Examination of the polymeric aldehyde produced by periodate oxidation showed that glucose and glucuronic acid were the only sugars not attacked and therefore are the only sugar residues not containing two adjacent hydroxyl groups.

The slightly acidic gum undergoes autohydrolysis when its aqueous solution is heated, the products being arabinose, ribose, traces of galactose and xylose, and a polysaccharide *B* composed of galactose, glucose, mannose, xylose and an aldobionic acid in the approximate molar ratios 2:2:2:3:2. All the arabinose residues are removed under conditions which indicate that they are attached to the polysaccharide by furanoside links. The traces of galactose and xylose found are probably derived from 'over-hydrolysis' of polysaccharide *B*. By estimating the formic acid produced and periodate consumed during metaperiodate oxidation of *B* it appears that

the repeating unit of *B* required 13–14 mol. of periodate and produced 4.7 mol. of formic acid. Glucose and glucuronic acid were again the only sugars not oxidized.

SUMMARY

1. Ash-free polysaccharide complexes have been obtained from widely different pedological, vegetational and climatic types of soil.

2. All the polysaccharide preparations have very similar properties. They all contain the same sugars, galactose, glucose, mannose, arabinose, xylose, and glucuronic acid, although the proportions, especially of the pentoses, vary to some extent in the different samples.

3. They all contain small amounts of nitrogen and ribose, presumably as ribonucleic acid, suggestive of a microbial origin.

4. From a humus-rich agricultural soil a polysaccharide complex has been isolated in relatively large quantity and the constituent sugars determined both by paper chromatography and by specific precipitants. The results agree well with a repeating unit containing galactose (2 mol.); glucose (2 mol.); mannose (2 mol.); arabinose (1 mol.); xylose (3 mol.); 2 mol. of an aldobionic acid consisting of glucose and glucuronic acid.

5. Mild hydrolysis of the polysaccharide gives an arabinose-free polysaccharide containing galactose (2 mol.); glucose (2 mol.); mannose (2 mol.); xylose (3 mol.); the aldobionic fraction as before.

6. The oxidation of both the original and the degraded polysaccharides with potassium metaperiodate has been studied.

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