Trans **β-(p-Bromobenzoyl)-dibromoacrylic Methyl Ester, XIX.**—Dibromofumaric monomethyl ester monochloride (4 g.) was added slowly with mechanical stirring to a mixture of carbon disulfide, 10 cc. of bromobenzene, and 5 g. of aluminum chloride. The mixture was stirred for one hour and heated for fifteen minutes, and then decomposed in ice and hydrochloric acid. The carbon disulfide solution was evaporated and the residue crystallized from ligroin; m. p. 102°; yield 66%.

Anal. Calcd. for $C_{11}H_7O_3Br_3$: C, 30.9; H, 1.68. Found: C, 30.9; H, 1.54.

The above preparation goes equally well using the crude acid chloride–phosphorus oxychloride mixture.

Hydrolysis of the ester using 80% ethanolic sodium hydroxide (standing at room temperature for two days) gave a nearly theoretical yield of *p*-bromobenzoic acid.

Summary

The nitric–glacial acetic acid oxidation of the di-(*p*-bromophenyl)-dihalogenofurans gives *cis* unsaturated 1,4-diketones, which may be rearranged into the stable *trans* isomers. The oxidation of di-(*p*-bromophenyl)-furan is described.

The di-(*p*-bromophenyl)-dihalogenofurans are formed by the action of phosphorus pentahalides on saturated and unsaturated 1,4-diketones and on the di-(*p*-bromophenyl)-furans.

Dibromofumaric monomethyl acid ester and its acid chloride are described.

UNIVERSITY, VIRGINIA RECEIVED SEPTEMBER 10, 1934

[CONTRIBUTION FROM THE NEW JERSEY AGRICULTURAL EXPERIMENT STATION]

Decomposition of Polyuronides by Fungi and Bacteria. II. Decomposition of Alginic Acid by Bacteria and Formation of the Enzyme Alginase[1]

BY SELMAN A. WAKSMAN AND MELVIN C. ALLEN

Alginic acid or algin, one of the carbohydrate constituents of marine algae, has attracted for many years the attention of chemists because of its abundance in nature and its specific chemical structure.[2] Kylin,[3] distinguished between algin and fucin, the first being soluble in hot water and the second only in dilute sodium carbonate solution; algin was believed to be the calcium salt of alginic acid and its solubility in hot water was explained by the substitution of an alkali base for the calcium. On hydrolysis with mineral acids, alginic acid was found to give reducing substances, which were at first thought to be pentoses; however, it was shown later that the furfural is produced not from a pentose group, but from an acidic nucleus, since, on boiling with hydrochloric acid, the algin or alginic acid preparations lost 20% of their weight as carbon dioxide. The constituent uronic acid was believed[4,5] to be glucuronic, but, more recently, it was found[6,7] to be *d*-mannuronic acid. According to Schmidt,[5] the polyuronic acid of *Fucus serratus* consists of two forms, *a*, highly resistant to chemical reagents, and *b*, readily hydrolyzed by mineral acids.

Very little is known concerning the fate of this polyuronide in the process of decomposition of marine algae in the sea and in other natural substrates; the fact that it makes up nearly 20 to 30% of the total organic matter of many of the algae would make a study of its decomposition under natural conditions of considerable interest. It has been definitely established[8,9] that the polymer of galacturonic acid, namely, pectic acid of fruits and vegetables, is readily decomposed by a great variety of microörganisms, especially fungi; these produce an enzyme which hydrolyzes the polyuronide into simpler units; the latter are readily oxidized further by the organisms in the process of their metabolism. It remained to be determined whether the process of decomposition of the polymer of mannuronic acid is similar in nature.

Experimental

As a source of alginic acid, freshly collected *Fucus vesiculosus* was used. The material was treated in the cold with 0.5% hydrochloric acid, to remove the bases, washed with water and extracted with approximately 0.5% solution of ammonia; the extract was precipitated with hydrochloric acid, giving a crude algin precipitate, which was washed with water, alcohol and ether, and dried. By the use of the chlorine dioxide reagent of Schmidt,[5] a fairly pure alginic acid was obtained, as shown by the presence of only a small amount of ash and nitrogen;

(1) Journal Series paper of the New Jersey Agricultural Experiment Station, Department of Soil Microbiology.

(2) E. C. C. Stanford, *Chem. News*, **47**, 254, 267 (1883).

(3) H. Kylin, *Z. physiol. Chem.*, **83**, 171 (1913); **94**, 337 (1915).

(4) Atsuki and Tomada, *J. Soc. Chem. Ind. Japan*, **29**, 509 (1926).

(5) E. Schmidt and F. Vocke, *Ber.*, **59**, 1585 (1926).

(6) W. L. Nelson and L. H. Cretcher, THIS JOURNAL, **51**, 1914 (1929); **52**, 2130 (1930).

(7) J. M. Bird and P. Haas, *Biochem. J.*, **25**, 403 (1931).

(8) F. Ehrlich, *Biochem. Z.*, **250**, 525 (1932); **251**, 204 (1932).

(9) S. A. Waksman and M. C. Allen, THIS JOURNAL, **55**, 3408 (1933).

the purified preparation contained 88.5 to 90.7% uronic acid anhydride and had a base-combining power of 51.6 cc. of $N/10$ sodium hydroxide per 1 g. of dry material. When a neutralized alkali solution of the alginic acid was treated with calcium chloride solution, the acid was precipitated to the extent of 96%. If, in the presence of preparations of the alginic acid, hot water was used for washing the material, especially after the preliminary treatment of the algal material with the mineral acid, the purified alginic acid could not be precipitated quantitatively as the calcium salt, but only to the extent of 58.4%, due to its partial hydrolysis by the residual acid. The "pentosan" content of the alginic acid preparations varied from 27.9 to 29.4%; however, on hydrolysis with hot 2% hydrochloric acid, only 15.4 to 26.1% reducing sugar was obtained. About 17 to 20% of the alginic acid preparation was in the *b* form, which was left unhydrolyzed after treatment with 80% sulfuric acid for two hours in the cold, then diluting with 15 volumes of water and heating at 100° for five hours.

The first experiment deals with the decomposition of alginic acid by a series of fungi, previously found[9] capable of decomposing pectic acid actively, also by a suspension of fresh soil and by a pure culture of a bacterium isolated from the soil. The alginic acid was dissolved in a slight excess of sodium hydroxide solution and the reaction adjusted to *p*H 7.0. Sodium nitrate was used as a source of nitrogen and mineral nutrients in concentrations equal to that of Czapek's solution added; one hundred cc. portions of the medium were placed in long-necked flasks, sterilized, inoculated and incubated for twenty-four days. The carbon dioxide given off during the growth of the organisms was absorbed in standard barium hydroxide solution and measured (Table I). The fungi and the pure culture of the bacterium acted upon the alginic acid only to a very limited extent. The soil infusion, however, brought about active decomposition of the complex, as shown by the evolution of carbon dioxide and by the amount of uronic acid left in the culture. The fact that the residual total uronic acid anhydride was greater than the calcium-precipitated alginic acid points to the production by the organisms of lower uronides, which are no longer precipitated by calcium. Only a limited amount of reducing substance was found in the culture.

TABLE I

DECOMPOSITION OF ALGINIC ACID BY VARIOUS MICRO-ORGANISMS

Nature of organism	CO_2 given off, mg. C	Uronic acid anhydride Left, mg.	Uronic acid anhydride Decomposed, mg.	Ca-precipitated alginic acid Left, mg.	Ca-precipitated alginic acid Decomposed, mg.
Check	4.1	879.6	...	717.0	...
Penicillium	32.4	822.3	57.3	549.0	168.0
Aspergillus	18.6	886.2	0	681.0	36.0
Trichoderma	20.4	867.6	12.0	721.5	0
Fusarium	27.4	889.4	0	711.0	6.0
Soil infusion	144.2	626.6	253.0	127.5	589.5
Bacterium A	16.4	872.3	7.3	673.5	43.5

These results prove that (1) certain fungi actively decomposing polygalacturonic acid cannot attack polymannuronic acid at all or do so only to a very limited extent; (2) the soil contains organisms which can attack alginic acid actively; (3) the ability of the soil organisms to decompose this substance is probably limited to specific bacteria; (4) in the decomposition of alginic acid by bacteria, the polymer is first hydrolyzed to simpler groups of uronic acids which are no longer precipitated by calcium, and the latter are then decomposed further or oxidized to carbon dioxide; (5) simple uronic acids, as shown by the formation of reducing substances, are either not formed at all in the decomposition of alginic acid or only to a very limited extent.

Further studies resulted in the isolation from the soil of an active alginic acid decomposing bacterium, designated as *B. terrestralginicum*.[10] This organism was found capable of attacking alginic acid not only in the purified form but also in the crude algin preparations and in the untreated *Fucus* material. Six-gram portions of *Fucus* were placed in 250-cc. flasks and 100 cc. of the mineral solution containing 50 mg. of nitrogen as sodium nitrate added (Table II). The water-insoluble portion of the *Fucus* was either not attacked at all or only to a limited extent; the increase in this portion is due to the synthesis of bacterial cell substance, as shown by the increase in total nitrogen. The water-soluble part of the *Fucus*, especially the alginic acid complex, has undergone extensive decomposition both by the mixed and pure culture of bacteria. The combined uronic acid content of the soluble and in-

TABLE II

DECOMPOSITION OF FUCUS VESICULOSUS BY A SOIL INFUSION AND BY A PURE CULTURE OF A SOIL BACTERIUM

Nature of organism	Period of incubation days	Water-soluble portion: Total organic matter Left, mg.	Total organic matter Dec., mg.	Uronic acid anhydride Left, mg.	Uronic acid anhydride Dec., mg.	Ca-ppt. alginic acid Left, mg.	Ca-ppt. alginic acid Dec., mg.	Water-insoluble portion: Total organic matter Left, mg.	Total organic matter Dec. (−) or accumulated (+) mg.	Uronic acid anhydride Left, mg.	Total nitrogen mg.
Control	..	1880	...	841	...	609	...	2340	...	582	27.7
Soil infusion	9	1500	380	680	161	...	...	2216	−124	605	..
	18	1308	558	603	246	366	243	2342	+ 2	576	42.2
Bact. terrestralginicum	9	1059	821	543	298	...	...	2423	+ 83	614	..
	18	912	954	467	382	254	355	2400	+ 60	586	40.3

(10) S. A. Waksman, C. L. Carey and M. C. Allen, *J. Bact.*, **28**, 213 (1934).

soluble portions of the *Fucus* amounted to 1423 mg., or 25.5% of the dry material; this accounted for all the original uronic acid, which was 24.7%. Only the polyuronide of the soluble portion was decomposed by the bacteria, but not that of the insoluble portion, thus pointing to a chemical difference between the two forms of the complex in the *Fucus*.

Crude algin cannot be looked upon as merely the calcium salt of alginic acid but can easily be shown to consist of two complexes: (1) alginic acid, and (2) a dark-colored, readily oxidizable substance, which has the properties of lignin, except for a very low methoxyl content. The second algin constituent can be considered as a type of "primitive lignin," which is similar to lignin in higher plants in its physical and chemical behavior, in its combination with proteins, and its resistance to bacterial decomposition. When algin was allowed to undergo bacterial decomposition (Table III), only the alginic acid constituent was attacked, while the accompanying "lignin" complex was precipitated out and allowed to accumulate, in the form of dark-colored, humus-like material. Thus humus was found to be considerably richer in carbon and in nitrogen than the original algin. The higher carbon content of the residue is due to the increase in the "lignin fraction" which is resistant to bacterial decomposition; the higher nitrogen content is due to the increase in protein content of the residue, largely as a result of bacterial synthesis. The original *Fucus* contained 24.7% "lignin," which had a uronic acid anhydride content of 12.5%; the algin contained 38.8% "lignin" with 32.1% uronic acid; the humus formed in the decomposition of algin by the bacterium contained 68.4% of ash-free residue, of which 55.1% was lignin and 13.3% of protein. The decomposition of algin is thus shown to lead to the formation of a dark colored complex which had all the properties of humus, commonly found in soils and in the sea bottom.

TABLE III

DECOMPOSITION OF CRUDE ALGIN[a] BY AN ALGINIC ACID DECOMPOSING BACTERIUM

10 g. of air-dry algin in 500 cc. of culture solution containing mineral nutrients.[b] Results on per cent. of dry material.

Nature of constituent	Original algin	Precipitate formed in the decomposition of algin by bacteria
Uronic acid anhydride	51.8	13.2
Ash	2.1	17.3
Total nitrogen	1.66	3.7
Total carbon	48.63	62.2
Treatment with 80% H_2SO_4		
Sugar produced	22.6	Trace
Residue unacted upon	46.1	71.0
Ash in residue	1.5	2.6
Nitrogen in residue	2.03	3.1
Uronic acid anhydride in residue	32.1	..

[a] Crude algin = acid precipitate of ammoniacal extract of *Fucus* previously washed with dilute acid and water.

[b] Total dry weight of algin in original culture, 9.1 g.; total dry precipitate in decomposed culture, 2.58 g.

For the purpose of comparing the action of the alginic acid decomposing bacterium upon the two fractions of alginic acid, namely *a* and *b*, referred to above, 10 g. of the purified acid was added to a liquid medium and allowed to decompose by the bacterium for eighteen days. The culture was then filtered through paper and the filtrate acidified with hydrochloric acid; the precipitate was washed and dried. Both the original alginic acid and the precipitate obtained after the growth of the bacterium were treated with cold 80% sulfuric acid for three hours, then diluted with 15 volumes of water and steamed for five hours. The residue was filtered off, washed, dried, weighed, ignited and weighed. The non-hydrolyzable alginic acid fraction was found to be somewhat more resistant to decomposition by the bacterium than the hydrolyzable fraction; it was not, however, absolutely resistant, since it has also undergone extensive decomposition.

No enzyme hydrolyzing alginic acid has ever been demonstrated to be formed by microorganisms. Oshima[11] found an enzyme of this type in the aqueous extracts of the "liver" of lower marine animals; he designated this enzyme as *alginase*. However, various fungi, the extracts of pig's pancreas and the livers of crabs and other animals did not contain this enzyme. The action of the enzyme was measured by a change in viscosity of the substrate and by an increase in reduction with Fehling's solution.

The alginic acid decomposing bacterium isolated from the soil was grown in the alginic acid medium, and different preparations obtained from the culture; these were tested for the presence of the specific enzyme. For this purpose, 500-mg. portions of alginic acid were dissolved as the sodium salt, and the reaction adjusted to *p*H 7.0. Toluene was used to ensure sterility and the enzyme preparations allowed to act for twenty hours at 40°. Calcium salt was used to precipitate the residual unhydrolyzed alginic acid. The results (Table IV) show that the bacterium produced an active alginase. However, the enzyme hydrolyzed the alginic acid only to smaller groups of mannuronic acid which were no longer precipitated as the calcium salt but not to simple mannuronic acid units, as shown by the lack of formation of a reducing substance.

The composition of the medium was found to have an effect upon the production of alginase by bacteria. For this experiment, a marine organism[10] was utilized. It

(11) H. Oshima, *Bull. Agr. Chem. Soc. Japan*, **7**, 332–340 (1931).

TABLE IV

THE ACTION OF ALGINASE PRODUCED BY A SOIL BACTERIUM

Nature of enzyme preparation	Amount of enzyme used	Residual alginic acid[a]	Alginic acid hydrolyzed Mg.	Alginic acid hydrolyzed % of total	Reducing substance
Control	0	318	0	0	0
Liquid culture	10 cc.	214	104	32.7	0
Control	0	502	0	0	0
Alcohol pptd. enzyme	200 mg.	272	230	45.8	0
Control	0	380	0	0	0
Bacterial cells, acetone treated	200 mg.	188	192	50.5	0

[a] Precipitated by calcium chloride and calculated on ash free basis.

was grown upon a sea water medium containing the necessary minerals and different sources of energy. In the case of the starch containing medium, both sea water and salt water (3.5% sodium chloride) were used. The organism was grown in 500-cc. portions of the different media for five days; the cultures were then treated with toluene and allowed to autolyze for several hours. Enzyme preparations were obtained by precipitating the autolyzed cultures with alcohol, washing with alcohol and with ether, and drying *in vacuo* (Table V). The results show that the enzyme alginase was produced in all media containing the different carbohydrates, but least of all in the alginic acid medium. With starch as a source of energy, the sea water medium gave nearly four and one-half times as much yield of enzyme, since the preparation obtained from the salt water medium had only a third of the enzyme activity.

TABLE V

INFLUENCE OF CARBOHYDRATE IN MEDIUM UPON THE PRODUCTION OF ALGINASE BY A MARINE BACTERIUM

Carbohydrate used	Yield of enzyme preparation, mg.	Activity of alginase[a]	Reducing sugar as glucose in alginic acid hydrolyzate, mg.
Starch in sea water medium	1800	84	None
Starch in salt water (NaCl) medium	400	238	64
Glucose	1200	232	None
Alginic acid	900	14	None

[a] Alginic acid hydrolyzed, in milligrams, by 50 mg. of enzyme preparation added to 50 cc. of 1% alginic acid solution containing 3.5% sodium chloride and incubated for sixteen hours at 40°.

A quantity of the enzyme was prepared by growing the organism on a starch–salt water medium. The effects of salt concentration, of reaction and temperature upon the action of the alginase are given in Tables VI, VII and VIII. The results show that an increasing concentration of salt, up to 2.0% in case of sodium chloride and to 1% in case of potassium chloride, is highly favorable to the specific action of the enzyme obtained from the marine bacterium. A *p*H of 7.0 and a temperature of 40° are optimum for the action of this enzyme. At *p*H 8.5 the enzyme is only slightly injured; on the acid side, however, its action diminishes very rapidly.

TABLE VI

EFFECT OF SALT CONCENTRATION ON THE ACTIVITY OF ALGINASE

Nature of salt	Concn., %	Alginic acid hydrolyzed,[b] mg.	Reducing sugar, as glucose, produced, mg.
None	0[a]	None	None
None	0	93	None
NaCl	0.5	189	Trace
NaCl	1.0	354	82
NaCl	2.0	396	107
NaCl	4.0	218	89
NaCl	6.0	56	None
KCl	0.5	164	None
KCl	1.0	240	Trace
KCl	2.0	218	None
KCl	4.0	36	None
KCl	6.0	None	None

[a] Boiled control. [b] 50 mg. of the alginase preparation was added to 50 cc. of 1% alginic acid containing various concentrations of salt. The *p*H was adjusted to 7.0 and the flasks were incubated at 40° for eighteen hours. Toluene was used as a preservative.

TABLE VII

INFLUENCE OF REACTION ON THE ACTIVITY OF ALGINASE[a]

*p*H value	Boiled control	4.0	5.5	7.0	8.5
Residual alginic acid, $CaCl_2$ precipitate, ash-free, mg.	447	369	302	40	69
Alginic acid hydrolyzed, mg.	None	78	145	407	378

[a] Fifty mg. of the alginase preparation was added to 50 cc. of 1% alginic acid, containing 2% sodium chloride and adjusted to various *p*H values, and incubated eighteen hours at 40°. Toluene was used as a preservative.

TABLE VIII

INFLUENCE OF TEMPERATURE ON THE ACTIVITY OF ALGINASE[a]

Temp., °C.	Boiled control	3	20	28	40	55	70
Residual alginic acid, $CaCl_2$ precipitate, ash free, mg.	454	454	400	176	25	336	390
Alginic acid hydrolyzed, mg.	None	None	54	278	429	118	64

[a] Fifty mg. of alginase was added to 50 cc. of 1% alginic acid, containing 2% sodium chloride and adjusted to *p*H 7.5, and incubated for eighteen hours at various temperatures. Toluene was used as a preservative.

Summary and Conclusions

The decomposition of alginic acid, or the polymer of mannuronic acid which occurs abundantly in marine algae, is carried out in nature through the action of specific bacteria.

These bacteria, before decomposing the polyuronide, hydrolyze it first into smaller groups of mannuronic acid, but not into the simple units.

Crude algin contains, in addition to alginic acid, a lignin-like complex. When the alginic acid

in the algin preparation is decomposed by the bacteria, the lignin-like material is precipitated in the culture in a form similar to natural humus, commonly found in soil and in the sea bottom.

Alginic acid decomposing bacteria attack readily both the acid hydrolyzable and the nonhydrolyzable fractions of the polymannuronic acid, the former to a somewhat greater extent, however.

Alginic acid decomposing bacteria isolated from soil and from sea water produce active enzymes which hydrolyze alginic acid, starch and various other polysaccharides.

The enzyme alginase hydrolyzes the alginic acid largely to simpler groups of mannuronic acid but not to simple units.

The optimum reaction for the action of alginase produced by a marine bacterium was *p*H 7.0, the optimum temperature 40° and the optimum salt concentration 2% sodium chloride.

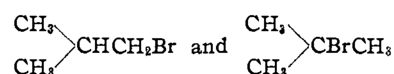
NEW BRUNSWICK, N. J. RECEIVED SEPTEMBER 12, 1934

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY]

Catalytic Dehydration of Butyl Alcohols

BY V. KOMAREWSKY, W. JOHNSTONE AND P. YODER

The catalytic dehydration of alcohols first studied by Ipatieff[1] is a simple reaction of the first two representatives of this class, ethyl and propyl alcohols. However, in the case of the butyl alcohols, the production of different isomeric butenes has been reported by different investigators.[2] These differences were caused partly by the different catalysts used, but mainly, as shown in the present work, by inaccurate methods of identification of the butenes produced. Usually the butenes obtained were identified by converting them into the corresponding dibromides, but this reaction is not suitable for the identification of the isomeric butenes. When butenes are passed into cold bromine there is some formation of tribromides[3] as well as dibromides, along with simultaneous evolution of hydrogen bromide, which then reacts with butenes to form monobromides. The final product therefore is a mixture of mono-, di- and tribromides. In the distillation of this product there is always a certain amount of hydrogen bromide evolved and the formation of higher boiling residues (tribromides). For this reason it was decided to base the determination and identification of the butenes upon their physical properties. Due to the development of distillation technique in recent years, it was possible to identify the butenes by distilling them in the low temperature apparatus of Podbielniak.[4] The identification of isobutene, whose boiling point (−6.3° at 760 mm.) is very close to that of 1-butene (−5° at 760 mm.), was checked by the formation of the two isomeric monobromides



by the action of hydrogen bromide in glacial acetic acid, according to the method of Ipatieff and Ogonowsky,[5] and subsequent hydrolysis of the tertiary monobromide to the corresponding tertiary butyl alcohol.

Using the above methods of determination, the catalytic action of phosphoric acid on the dehydration of normal, secondary and isobutyl alcohols was studied. In order to follow the usual procedure of passing the alcohol vapors at constant speed through a heated tube filled with catalyst, a solid catalyst was prepared by mixing phosphoric acid with pure dried alumina. The experiments of Ipatieff[6] and Pines[7] have proved that pure alumina has no isomerizing action on butenes. The dehydration of butyl alcohols over such a phosphoric acid–alumina catalyst has shown that phosphoric acid has an isomerizing effect on 1-butene, but the yields of 1- and 2-butenes reported by other investigators[8] are not exact. The dehydration of isobutyl alcohol resulted in the formation of pure isobutene and not a mixture of the three isomers as found by Mitchell.[9]

(1) Ipatieff, *Ber.*, **34**, 596, 3579 (1901).
(2) Le Bel and Greene, *Am. Chem. J.*, **2**, 23 (1880); Nef. *Ann.*, **318**, 211 (1901); Harries, *ibid.*, **383**, 181 (1911); Senderens, *Bull. soc. chim.*, [4] **1**, 693 (1907); Senderens, *Ann. chim. phys.*, [8] **25**, 497 (1912).
(3) Faworsky, Dissertation, 1890.
(4) Podbielniak, *Oil and Gas Jour.*, October, 1929.
(5) Ipatieff and Ogonowsky, *Ber.*, **36**, 1988 (1903).
(6) Ipatieff, *ibid.*, **36**, 2011 (1903).
(7) Pines, THIS JOURNAL, **55**, 3892 (1933).
(8) See Reference 10.
(9) Whitmore, THIS JOURNAL, **54**, 3278, Footnote 14 (1932).