xylyl-3-methyl-5-pyrazolone melting at 164° together with a small amount[4] of a compound, more soluble in alcohol, melting at 150–151°, which showed the same percentage composition but which did not give the pyrazole blue test. It is suggested that this low melting derivative may be the imine[5] form of pyrazolone formed by a reaction of the hydrazine with the enol form of acetoacetic ester.

Compound	Crystalline structure	M. p., °C.	Nitrogen, % Calcd.	Found	
1-*p*-Xylyl-3-methyl-5-pyrazolone[6]	Needles from alcohol or high test gasoline	164	13.86	13.92	14.05
1-*p*-Xylyl-2,3-dimethyl-5-pyrazolone[7] (*p*-xylylantipyrine)	Lustrous plates from high test gasoline	97.5	12.96	13.02	13.22
1-*p*-Xylyl-3-methyl-5-benzoyl-5-pyrazolone[8]	Colorless needles from alcohol or pet. ether	119	9.15	9.25	9.23
N-Acetyl-N(2,5-dimethylphenyl)-hydrazine	Leaflets from water	104–106	15.72	15.55	
1-*p*-Xylyl-5-methyl-3-pyrazolone[9]	Cream-colored plates from alcohol or ligroin	180–181	13.86	13.77	13.61
1-*p*-Xylyl-3-benzoyl-5-methyl-3-pyrazolone[8]	Cream-colored rhomboids from ligroin	74	9.15	9.17	9.22

(4) In some earlier runs this compound was the only product isolated.

(5) Knorr, *Ber.*, **28,** 706 (1895).

(6) Prepared by method of Knorr, *Ber.*, **16,** 2597 (1883). Heated for eight hours at 150–160°. A concentrated alcoholic solution of the crude product was boiled with an excess of high test gasoline until solution was complete and then cooled.

(7) Prepared by method of Knorr, *Ber.*, **17,** 550, 2037 (1884). Heated for eight hours at 130°.

(8) From pyrazolone, benzoyl chloride and pyridine.

(9) Prepared by the method of Michaelis, *Ann.*, **238,** 310 (1887).

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Decomposition of Polyuronides by Fungi and Bacteria. I. Decomposition of Pectin and Pectic Acid by Fungi and Formation of Pectolytic Enzymes[1]

BY SELMAN A. WAKSMAN AND MELVIN C. ALLEN

Polyuronides or uronic acid containing complexes are synthesized extensively by higher and lower plants. When the residues of these plants undergo decomposition in the soil, in composts or in the sea, the various carbohydrates, including the polyuronides, are readily decomposed by numerous fungi and bacteria; the rate and nature of the decomposition is not always the same, however. Some of these compounds are utilized by a great variety of microörganisms, while others are highly specific in nature and can be acted upon only by certain limited groups of fungi or bacteria.

Among the various polyuronides so far described, including the polymers of a single uronic acid and combinations of uronic acids with hexose and

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

pentose sugars, as well as with other compounds, pectins and their derivatives received most consideration. Pectic acid, the major constituent of the pectin molecule, is considered by Ehrlich[2] as a triacetylarabino-galacto-dimethoxy-tetragalacturonic acid; the central nucleus of the pectic acid molecule is polygalacturonic acid, a polymer of *d*-galacturonic acid. On mild alkali hydrolysis of the pectin, a tetragalacturonic acid, methyl alcohol, acetic acid, a molecule of galactose and one of arabinose are produced. The tetragalacturonic acid complex makes up 68% of the total pectin molecule and 94% of the pectic acid. Ehrlich[3] at first distinguished three forms of the tetragalacturonic acid, which he designated as *a*, *b* and *c*; later, however, only two forms were recognized as natural isomers, the *c* and *b* forms. He proposed to designate the first as *pectolic acid;* it forms the nucleus of the pectin molecule and contains the galacturonic acids in a ring-like arrangement. The *b* form was called *pecto-lactonic acid*, and was looked upon as a decomposition product of pectolic acid; it was believed to represent an open chain of four galacturonic acid molecules, having, however, only three free titratable carboxyl groups and one saponifiable lactone group. Pectolic acid was thus represented as $C_{20}H_{30}O_{17}(COOH)_4$ and pectolactonic acid as $C_{20}H_{29}O_{15}\begin{matrix}CO\\ \diagup\\ \diagdown\\ O\end{matrix}(COOH)_3$.

On hydrolysis of pectolic acid with dilute sulfuric acid, monomolecular *d*-galacturonic acid is always formed, with pectolactonic acid as the first decomposition product

$$C_{24}H_{34}O_{25} = C_{24}H_{32}O_{24} + H_2O \qquad C_{24}H_{32}O_{24} + 4H_2O = 4C_6H_{10}O_7$$

On acid hydrolysis, about 35–40% of the theoretical amount of *d*-galacturonic acid was obtained.

The enzymes acting upon pectins have been commonly classified into three groups: *protopectinase* (*pectosinase*), *pectinase* and *pectase*. The first is said to bring about the dissolution of the middle lamella in plant tissues and to change the insoluble pectin into a soluble form; the second hydrolyzes the soluble pectin to simpler compounds, including reducing substances; the third changes the soluble pectins into insoluble compounds which come out as gelatinous precipitates, due to the splitting off of a molecule of methyl alcohol from the pectin molecule and the precipitation of the pectic acid (or the tetragalacturonic acid) as the calcium salt. Ehrlich[4] recognized three new enzymes, which he designated as *propectinase*, *arabanase* and *pectolase*. The first is similar in its action to that of hot water and hydrolyzes the cold water insoluble pectin to hydrate pectin, namely, a water-soluble mixture of araban and of the Ca–Mg salt of pectic acid; the second splits the araban from pectin and changes it to *l*-arabinose; the pectolase hydrolyzes the most important constituent of the pectin

(2) Ehrlich and Schubert, *Ber.*, **62**, 1974 (1929); Ehrlich, *Cellulosechemie*, **11**, 140, 161 (1930).
(3) Ehrlich, *Z. angew. Chem.*, **40**, 1305 (1927); *Schles. Gesell. Vaterl. Cultur.*, **103**, 57 (1930).
(4) Ehrlich, *Biochem. Z.*, **250**, 525 (1932); **251**, 204 (1932).

molecule, namely, the tetragalacturonic acid, to monomolecular *d*-galacturonic acid.

The enzyme pectolase seems to be similar in its action to pectinase. This enzyme was not found in yeasts but could easily be demonstrated in taka-diastase and especially in a species of *Penicillium*, which has been isolated from macerated sugar beets and described as *P. ehrlichii*. At 30° and at a P_H of 4.5–6.3, without buffer but only in a solution of the acid neutralized with $N/10$ sodium hydroxide, the pectolic acid was changed to pectolactonic acid in a day; the enzyme then proceeded during the next few days to hydrolyze this complex to monogalacturonic acids. An enzyme preparation was obtained by precipitation of the extract of autolyzed mycelium with alcohol. This preparation hydrolyzed, at 30° and in one hour, twenty times its weight of pectolic acid to pectolactonic acid; the latter was changed, within three to four days, almost completely to *d*-galacturonic acid (70–80% of theoretical).

Experimental

For the following investigations, pectin and certain of its derivatives, namely, pectic acid and polygalacturonic acid, obtained from citrus fruits, were supplied by the California Citrus Growers Exchange. The polygalacturonic acid is obtained from lemon pulp by hydrolysis with dilute alkali solution, precipitating as the calcium salt and washing with a dilute acid. The particular preparation was found to analyze, on a dry basis, as follows:

Ash, %	1.02
Pentose, calcd. from the furfuraldehyde yield, %	41.5
Reducing sugar, as glucose, obtained on hydrolysis for five hours with 2% HCl in flowing steam, %	30.4
Uronic acid anhydride, %	89.3

A study has first been made of the decomposition of polygalacturonic acid in soil, which has been adjusted to a moisture content favorable for aerobic decomposition, as well as in sand and solution media which have been inoculated with a soil suspension; all the cultures were incubated at 28°. A liquid medium containing 20 g. of the polygalacturonic acid per liter and various nutrient salts, with sodium nitrate as a source of nitrogen, was prepared; the reaction was adjusted to neutrality by dissolving the polyuronide in dilute sodium hydroxide solution. The nutrient solution was distributed, in 100-cc. portions, in Erlenmeyer flasks and sterilized for fifteen minutes at 15 lb. pressure. This medium was used for the enrichment cultures and later for the growth of the pure cultures of the organisms. The sand flasks also received the nutrient salts, in addition to the polyuronide.

The soil cultures, as well as the sand and liquid media inoculated with soil, gave an abundant growth of fungi and bacteria. This was accompanied by extensive decomposition of the polygalacturonic acid, as measured by the carbon dioxide evolution. After twenty-four days of incubation, the controls and the inoculated cultures were analyzed for uronic acid, using the method of Dickson, Otterson and Link,[5] and for pentose by the furfuraldehyde method. The polygalacturonic acid preparation gave only about half as much pentose as uronic acid anhydride. The results of the decomposition (Table I)

(5) Dickson, Otterson and Link, THIS JOURNAL, **52**, 775 (1930).

show that the polygalacturonic acid is rapidly attacked by microörganisms, both in liquid and in solid media. In the liquid medium more than half of the uronic acid has disappeared. In the sand medium, practically all the polygalacturonic acid was decomposed, measured both as uronic acid and as pentose. These results lead one to conclude that the soil harbors organisms capable of active decomposition of polygalacturonic acid.

TABLE I

DECOMPOSITION OF POLYGALACTURONIC ACID BY CRUDE CULTURES OF SOIL MICROORGANISMS, IN LIQUID AND IN SAND MEDIA[a]

Nature of medium		Liquid	Liquid	Sand	Sand
Treatment of culture		Control	Soil inoculum	Control	Soil inoculum
Uronic acid anhydride	left, mg.	1378.0	665.4	1389.6	96.8
	dec., mg.	....	712.6	....	1292.8
Pentose	left, mg.	730.4	181.2	712.8	0
	dec., mg.	....	549.2	....	712.8

[a] Two grams of polygalacturonic acid, containing 12.1% moisture, added to each culture.

A number of fungi and bacteria were isolated from the sand cultures. Three fungi, namely, *Asp. niger*, *Penicillium* sp.[6] and *Fusarium* sp. proved to be very active in decomposing the polyuronide and were selected for further study. These organisms were inoculated into sterile media and the carbon dioxide liberated during growth absorbed in standard barium hydroxide solution. At the end of the incubation period, some of the cultures were removed and immediately filtered through paper. The residue left on the paper consisted partly of the mycelium and spores of the fungus and partly of a certain sediment which was always found to be produced in the decomposition of the polygalacturonic acid; the nature of this sediment will be discussed later. The amount of undecomposed polygalacturonic acid left in the cultures was determined by precipitating an aliquot portion with calcium chloride solution; the precipitated calcium polygalacturonate was filtered, washed with water, dried to constant weight, then ignited and ash determined. In some cases the uronic acid in the total culture and in the filtrate from the polygalacturonate, as well as the total reducing substance, were also determined.

The results brought out in Table II show clearly that different microorganisms do not attack the polygalacturonic acid in the same manner. The culture of *Penicillium* was found to hydrolyze the polyuronide very rapidly to simpler compounds which are no longer precipitated as calcium salts. The rapidity of this hydrolysis is also brought out by the increase in the amount of reducing substances formed in the cultures. During the first seven days of decomposition, most active hydrolysis of the polyuronide took place; this was accompanied, however, only by limited decomposition and energy utilization, as shown by the comparatively small amount of carbon dioxide produced and of microbial cell substance synthesized. The reducing substances produced in the culture and calculated as glucose were just about a half of the uronic acid content of the culture which was not precipitated by calcium. This points to the fact that, during the first few days of the growth of the organism, the tetragalacturonic acid is

(6) This is a typical soil Penicillium belonging, according to Dr. Thom, to the *P. janthinellum* group.

TABLE II

DECOMPOSITION OF POLYGALACTURONIC ACID BY FUNGI

2-g. portions of air-dry material used in 100 cc. of culture; theoretical amount of dry material 1,758 mg., containing 1,549 mg. of uronic acid anhydride.

Period of incubation, days	Residue after filtr. of culture, mg.	CO_2 liberated, mg. of C	Ppt. produced with $CaCl_2$ ash-free, mg.	Uronic acid anhydride in total culture, mg.	Uronic acid anhydride in filtr. from $CaCl_2$ ppt., mg.	Red. sugar as glucose, in total culture, mg.	Red. sugar, as glucose, in filtr. from $CaCl_2$ ppt., mg.
Control	29	0	1,612	1,540	88	20	9
			Penicillium sp.				
3	50	11.0[b]	36	1,451	1,080	692	530
7	188	49.3	1	1,209	972	656	581
15	247	201.8	263	556	557	152	121
21	271[a]	252.4	213	655	...	50	...
			Aspergillus niger				
3	66	33.4	1,136	1,425	20	206	0
7	217	83.2	528	791	317	279	261
15	356	216.3	409	237	108	44	0
21	352[c]	247.8	187	185	...	29	...
			Fusarium sp.				
7	52	18.9	214	1,522	915	572	503
15	313	157.0	73	1,319	885	272	186
21	433[d]	235.0	101	1,267	...	18	...

[a] Nitrogen content of residue, 12.0 mg.; nitrate left in culture, 15.8 mg. N; nitrate consumed by organism or reduced, 19.2 mg. [b] Four days incubation. [c] Nitrogen content of residue, 15.7 mg.; nitrate left in culture, 14.0 mg. N; nitrate consumed by organism or reduced, 21.0 mg. [d] Nitrogen content of residue, 22.3 mg.; nitrate left in culture, 3.4 mg. N; nitrate consumed by organism or reduced, 31.6 mg.

hydrolyzed to simple galacturonic acids, containing the free reducing group. With the further development of the organism, the simple uronic acids are rapidly decomposed, as shown by the total reduction both in the uronic acid content and in the reducing groups; the energy liberated during this decomposition is used by the organism for its metabolic needs, as shown by the rapid increase in the amount of carbon liberated as carbon dioxide and in the synthesis of microbial cell substance. It is important to note here that, although the calcium precipitated complex has completely disappeared within seven days' decomposition, it reappeared again after that period; this is probably due to the synthesis by the fungus or by its enzymes of new complexes which are also precipitated by the calcium salt.

The second fungus, *Aspergillus niger*, decomposed the polygalacturonic acid in a totally different manner, as shown by the difference in the mechanism of the transformation of the substrate and its decomposition products. This organism did not allow the accumulation of hydrolytic products, namely, simple uronic acids, but rapidly decomposed these further to carbon dioxide. This resulted in liberation of considerable energy which

was utilized for the synthesis of somewhat larger amounts of fungus cell substance than in the case of the *Penicillium*. The rate of hydrolysis of the polyuronide by *A. niger* was much slower than in the case of the *Penicillium:* the latter organism destroyed practically all the calcium precipitable polyuronide within three days, while the *Aspergillus* attacked less of the substrate and allowed much less reducing substances to accumulate in the culture. This tends to emphasize the fact that the *Aspergillus* hydrolyzed the polygalacturonic acid more slowly, while the products of hydrolysis were oxidized more rapidly to carbon dioxide than in the case of the *Penicillium*.

The third organism, namely, the *Fusarium* sp., acted upon the polygalacturonic acid in a manner similar to that of the *Penicillium*. It also hydrolyzed the polyuronide rapidly and allowed at first the accumulation of a considerable amount of simpler uronides, as shown by the increase of the uronic acid in the filtrate from the calcium precipitate; this was accompanied also by an increase in the amount of reducing substances in the culture. However, the *Fusarium* seemed to be slower in bringing about the complete hydrolysis of the polyuronide than the *Penicillium*.

In order to study the influence of the reaction of the medium upon the decomposition of the polygalacturonic acid by fungi, four quantities of the above culture solution were prepared and adjusted to P_H 3.0, 4.0, 5.5 and 7.0, using sodium hydroxide solution. Each flask contained in 100 cc. of solution 2 g. of the air-dry polygalacturonic acid, equivalent to 1,758 mg. of the ash-free dry material and to 1,549 mg. of uronic acid anhydride, with sodium nitrate as a source of nitrogen. The reaction of the medium was found (Table III) to have a very decided effect upon the growth and enzyme production of the fungus *Penicillium*. At the most acid reaction, namely, at P_H 3.0, a considerable amount of the polygalacturonic acid in the medium was hydrolyzed during the process of sterilization, as shown by the increase in the uronic acid and sugar content in the filtrate from the calcium chloride precipitate and in the content of reducing substances. The organism brought about very rapid hydrolysis of the complex polyuronide into simple uronic acids, as shown by the complete disappearance within three days of the polyuronide which could be precipitated as the calcium salt, and by the rapid accumulation of reducing substances. During the first two days of decomposition, the rate of hydrolysis of the polyuronide was much greater at the higher acidity. The amount of reducing substances left after twelve days of decomposition was greater with a decrease in acidity. It is of special interest to note that at P_H 3.0 and 4.0 the amount of reducing substances measured as glucose was less, with one exception, than the uronic acid content; however, at neutrality the amount of the reducing substances was, at the end of the decomposition period, greater than that of the uronic acid anhydride. This can only be due to the decarboxylation of the uronic acid under those conditions. The optimum reaction for the growth of *Penicillium* was P_H 4.0 to 5.5. This experiment was repeated using the *Fusarium* culture. This organism again proved to be weaker in its action upon the polyuronide and had its optimum at P_H 5.5.

Finally a comparative study was made of the ability of different fungi to decompose both pectin and polygalacturonic acid. A series of flasks containing the synthetic culture solution as well as 2% of the air-dry pectin or 2% of the polygalacturonic acid were inoculated with three different fungi and incubated for varying periods of time (Table IV). The decomposition of the pectin, as measured by the reduction of the total

TABLE III

INFLUENCE OF REACTION UPON THE DECOMPOSITION OF POLYGALACTURONIC ACID BY *Penicillium* SP.

Reaction P_H	Period of incubation, days,	Residue after filtration, mg.	Ppt. produced with $CaCl_2$, ash-free, mg.	Uronic anhydride in filt. from $CaCl_2$ ppt., mg.	Red. sugar, as glucose in total culture, mg.
3.0	Check	61	1,266	299	133
3.0	1	45	1,256	381	231
3.0	2	33	122	1,346	1,134
3.0	3	68	14	1,346	938
3.0	12	331	112	106	179
4.0	Check	27	1,428	198	68
4.0	1	29	1,276	422	300
4.0	2	43	174	1,399	803
4.0	3	138	4	1,310	919
4.0	6	293	116	1,126	481
4.0	12	320	188	440	138
4.0	15	292	224	255	131
5.5	Check	50	1,684	142	74
5.5	1	24	1,462	242	286
5.5	2	31	375	965	710
5.5	3	91	0	1,055	963
5.5	6	183	32	1,442	842
5.5	12	296	254	287	269
7.0	Check	66	1,622	101	49
7.0	1	31	1,462	194	205
7.0	2	43	1,226	598	491
7.0	3	96	24	844	666
7.0	12	179	122	135	654

uronic acid, was also accompanied by the accumulation of hydrolytic products, as shown by the increase of the reducing sugar. The fungus *Trichoderma* proved to be more active in decomposing the polyuronide than the Penicillium. The different fungi attacked the pectin and its derivatives in different manners, as brought out by the fact

TABLE IV

COMPARATIVE DECOMPOSITION OF PECTIN AND POLYGALACTURONIC ACID BY FUNGI

		Pectin		Polygalacturonic acid		
Nature of organism	Period of incubation, days	Total uronic acid anhydride, mg.	Red. sugar, as glucose, mg.	Ppt. with $CaCl_2$, ash-free, mg.	Total uronic acid anhydride, mg.	Red. sugar, as glucose, mg.
Check	.	1,471	133	1,716	1,660	57
Aspergillus niger	4	...	...	27	1,120	778
Aspergillus niger	8	...	...	294	457	327
Penicillium sp.	3	1,003	305	...	...	...
Penicillium sp.	5	900	409	...	...	...
Penicillium sp.	9	723	390	...	...	...
Trichoderma sp.	3	976	120	...	...	...
Trichoderma sp.	5	765	240	...	...	...
Trichoderma sp.	9	430	308	...	...	...
Trichoderma sp.	4	...	...	31	1,290	619
Trichoderma sp.	8	...	...	14	528	289

that in the case of the polygalacturonic acid the action of *Trichoderma* resulted in the formation of products of hydrolysis which had a ratio of reducing sugar to uronic acid of about 1:2, while the growth of *A. niger* resulted in hydrolytic products in which the above ratio was only 2:3 or even less.

In order to study these relationships under more controlled conditions, it was deemed essential to obtain enzymes from these fungi and determine their action upon the polyuronides. The interfering action of the metabolism of the organism could thus be eliminated. The organisms were at first grown in liquid media, containing polygalacturonic acid and nutrient salts; after three to seven days of incubation, the fungus mycelium was removed by filtration, and both mycelium and filtrate tested for the enzyme. Later the fungi were grown on a solid medium consisting of a mixture of rice hulls and grape-pomace; this was moistened with the above culture solution. At the end of the incubation period, the mass of substrate and fungus mycelium was extracted with several volumes of water and the enzyme precipitated from the extract with 2 volumes of 95% alcohol; in the case of the liquid cultures, only the filtrate was used. The precipitated enzyme was washed with acetone and dried *in vacuo* over sulfuric acid. The liquid cultures gave the most active enzyme after three days of incubation; on further development, the enzyme content gradually diminished (Table V). The enzyme produced by the organisms grown upon the solid medium was much more active than when grown upon the liquid medium; here, as well, the highest activity of the enzyme was obtained after three days of incubation of the culture and diminished on further growth of the organism.

TABLE V

INFLUENCE OF AGE OF PENICILLIUM CULTURE UPON THE ACTIVITY OF THE ENZYME PRESENT IN THE LIQUID MEDIUM

Age of culture,[a] days		Boiled control	2	4	7	9	12	16
Time of enzyme action (30°), hours		24	16	24	24	24	24	24
Polygalacturonic acid	residual, mg.	938	625	343	537	588	657	755
	hydrolyzed, mg.	0	313	595	401	350	281	183

[a] Ten cc. of liquid culture, except in two day old, when 20 cc. was used, added to 50 cc. of a 2% polygalacturonic acid solution containing the nutrient salts.

When the liquid culture was used as a source of enzyme, it was necessary to introduce the nutrient salts in order to produce the maximum action of the enzyme upon the substrate. However, in the case of the powdered preparation obtained from the culture grown on the solid medium, the presence of the salts was not essential. The action of the enzyme in liquid culture was measured as follows: 10-cc. portions of the culture and some toluene were added to 50 cc. of a 2% polygalacturonic acid solution, and the flasks placed in a thermostat, at 30°, for twenty-four hours. At the end of that period, a solution of calcium chloride was added, the precipitate was filtered off, washed, dried, weighed, ignited and weighed again; the loss in weight is taken as the residual unhydrolyzed polygalacturonic acid.

The influence of enzyme concentration, time of action and reaction of substrate, using dry enzyme preparations, is brought out in Tables VI and VII. An increase in enzyme concentration brings about a proportional increase in the amount of hydrolysis of the polygalacturonic acid. The optimum reaction for the action of the pectolytic enzyme is found to be at P_H 4.0 to 6.0. With an increase in alkalinity, namely, at P_H 7.0 or above, the action of the enzyme rapidly diminishes.

TABLE VI

INFLUENCE OF CONCENTRATION OF PECTOLYTIC ENZYME AND LENGTH OF TIME UPON ITS ACTION

Penicillium enzyme used (100 mg.), 1 g. polygalacturonic acid, temp. 40°

Enzyme, mg.	Enzyme added[a]	Time of action, hours	Polygalacturonic acid Residual, mg.	Hydrolyzed, mg.
25	−	4	877	0
25	+	4	730	147
50	−	8	843	0
50	+	4	508	335
50	+	8	287	556
100	−	8	838	0
100	+	4	220	618
100	+	8	39	799

[a] − indicates boiled control.

TABLE VII

INFLUENCE OF REACTION UPON THE ACTION OF THE PECTOLYTIC ENZYME

100 mg. of dry enzyme added to 1 g. of polygalacturonic acid, 40°, eight hours

Reaction, P_H	Enzyme added	Polygalacturonic acid Residual, mg.	Hydrolyzed, mg.
4.0	−	828	0
4.0	+	35	793
5.0	−	822	0
5.0	+	27	795
6.0	−	811	0
6.0	+	51	760
7.0	−	791	0
7.0	+	714	77
8.0	−	795	0
8.0	+	768	27

The action of the *Penicillium* enzyme upon pectin is similar to its action upon polygalacturonic acid, although the rate is somewhat slower, as shown in Table VIII.

TABLE VIII

ACTION OF PECTOLASE UPON PECTIN AND POLYGALACTURONIC ACID

100 mg. of enzyme, 1 g. of substrate, seven hours at 40°

Substrate	Enzyme	Unhydrolyzed residue, mg.	Polygalacturonic acid pptd. by $CaCl_2$, mg.	Uronic acid anhydride in soln., mg.	Red. sugar as galactose, mg.
Pectin	Boiled control	45	562	176	171
Pectin	Enzyme	104	231	396	325
Polygalacturonic acid	Boiled control	...	874	106	62
Polygalacturonic acid	Enzyme	...	23	865	448

The use of the polygalacturonic acid for standardizing the activity of pectolytic enzymes suggested itself. For this purpose different amounts of the precipitated enzyme were allowed to act, for varying periods of time at constant temperature, upon 1-g. portions of the substrate, and the residual unhydrolyzed polygalacturonic acid determined by precipitation with calcium chloride. The results obtained (Table IX) show considerable variation because of the insufficient number of duplicates; they permit, however, of drawing a certain definite conclusion, namely, that on the average one milligram of the enzyme preparation brought about, at 40° and in one hour, the hydrolysis of 4 milligrams of the substrate. One may be justified, therefore, in recommending the following unit system for standardizing pectolytic enzymes: *one pectolytic unit is that amount of enzyme which hydrolyzes 1 milligram of polygalacturonic acid in one hour at 40°*. One gram of the enzyme preparation which has been used in this experiment contains, therefore, 4000 pectolytic units.

TABLE IX

THE INFLUENCE OF ENZYME CONCENTRATION AND TIME OF ACTION UPON THE HYDROLYSIS OF POLYGALACTURONIC ACID AT 40°

Enzyme, mg.	Time of action, hours	Resid. polygalacturonic acid, mg.	Substrate hydrolyzed, mg.	Enzyme, mg.	Time of action, hours	Resid. polygalacturonic acid, mg.	Substrate hydrolyzed, mg.
Boiled control	.	754	0	25	2	586	168
10	1	708	46	25	5	338	416
10	2	659	95	50	1	609	145
10	5	577	177	50	2	411	343
25	1	622	132	50	5	69	685

Previous experiments brought out the fact that both during the growth of the fungus in a liquid culture containing pectin or polygalacturonic acid and as a result of the action of the enzyme upon these substrates, a sediment invariably is formed. In order to determine the nature of this sediment, 20 g. of the polygalacturonic acid was hydrolyzed by sufficient enzyme obtained from the *Penicillium* culture, and the sediment produced as a result of hydrolysis collected by filtration through paper. The yield of the dry sediment was 1.527 g. or 7.63% of the total original material. This particular sediment probably contained some unhydrolyzed polygalacturonic acid, since the usual yield of precipitate was 3.5 to 5% of the substrate. A comparative chemical analysis of this sediment with that of the original substrate is given in Table X. The results show that the sediment is much lower in uronic acid and has a higher methoxyl content. Whether this sediment is due to the presence of a lignin-like substance in the polygalacturonic acid complex or to a certain amount of polyuronide not acted upon by cold 80% sulfuric acid and by hot 5% solution of sulfuric acid, remains to be determined. The important point to be emphasized is that such a group exists in the original pectin preparation; it seems to be liberated only as a result of the action of the pectolytic enzyme and is then precipitated.

TABLE X

COMPARATIVE CHEMICAL COMPOSITION (IN PER CENT. OF DRY MATERIAL) OF THE POLYGALACTURONIC ACID AND OF THE SEDIMENT FORMED AS A RESULT OF ITS HYDROLYSIS BY A PECTOLYTIC ENZYME

Constituent complex	Polygalacturonic acid	Sediment
Total uronic acid anhydride	89.0	41.5
Residue left on treatment with 80% sulfuric acid in cold (lignin?)	0	12.6
Reducing sugar, as glucose, on hydrolysis with 80% sulfuric acid	65.3	30.0
Methoxyl	3.26	3.97

Summary and Conclusions

Several fungi were isolated from the soil and were found capable of decomposing pectin and polygalacturonic acid.

In the decomposition of the polyuronides, the simple uronic acids are first produced; these are then decomposed further by the organism.

Different fungi vary in the rate and nature of decomposition of the uronic acid complexes.

These fungi produce pectolytic enzymes which hydrolyze pectin and polygalacturonic acid.

It is proposed to designate as a unit of pectolytic enzyme that amount of enzyme which will hydrolyze 1 milligram of polygalacturonic acid in one hour at 40° and at P_H 4.0 to 6.0. Using this unit of measurement, 1 g. of a dry enzyme preparation of certain fungi contained about 4000 pectolytic units.

As a result of the action of the enzyme upon pectin and polygalacturonic acid, a small amount of sediment is formed which seems to contain either a lignin-like complex or a higher polyuronide not hydrolyzed by cold 80% sulfuric acid or by hot 5% sulfuric acid.

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Anisohydroxamyl Chloride

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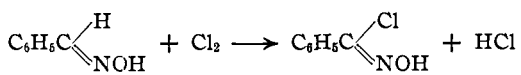
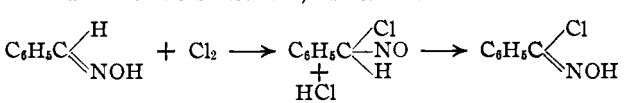
Werner and Buss[1] prepared the first aromatic hydroxamyl chloride by chlorinating benzaldoxime in chloroform solution.



Contrary to their expectations, based on the Hantzsch–Werner theory, the two isomeric benzaldoximes did not yield two isomeric hydroxamyl chlorides.

Later, Piloty and Steinbock[2] discovered that the benzaldoximes could be chlorinated in a fairly concentrated hydrochloric acid solution. A blue intermediate was observed which was unstable and which quickly changed into the benzohydroxamyl chloride isolated by Werner and Buss. Since the intermediate compound had a blue color and since the chlorination mixture, following Werner and Buss's procedure, took on a blue-green color Piloty and Steinbock assumed that the intermediate in the chlorination reaction had a nitroso structure, as follows



(1) Werner and Buss, *Ber.*, **27**, 2193 (1894).
(2) Piloty and Steinbock, *ibid.*, **35**, 3112 (1902).