ACCUMULATION OF 5-FORMYLPYRROLE-2-CARBOXYLIC ACID IN CULTURES OF *ERWINIA AROIDEAE* GROWN IN THE PRESENCE OF PECTIC ACID AND AMMONIUM SALTS*

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When *Erwinia aroideae* was grown with pectic acid and ammonium salts as substrates, the culture fluid turned reddish brown after a few days of incubation. At the same time, an unknown pyrrole showing an absorption peak at 300 mμ was found in the culture fluid. This pyrrole was also formed when pectic acid and ammonium salts were incubated with the extract of acetone-dried cells of this organism. By using this extract, conditions for the formation of this pyrrole were investigated.

The formation of the pyrrole in the incubation mixture was always accompanied by the appearance of the reddish brown color. The coloring matter might be the same as that found in vegetables infected with some species of *Erwinia* or in fermented plant food products. However, no detailed study was made here as to this coloring matter.

The chemical structure of the pyrrole was investigated too. It was found to be 5-formylpyrrole-2-carboxylic acid.

MATERIALS AND METHODS

Pectic acid, 4, 5-unsaturated digalacturonic acid and 4-deoxy-5-ketofructuronic acid were prepared as described in previous papers (1~3).

Washed cells and enzyme solution. A strain of *Erwinia aroideae* isolated from rotting roots of Japanese radish (4) was maintained on slopes containing 0.5% of peptone, 0.1% of KH_2PO_4, 0.5% of $Na_2HPO_4 \cdot 12H_2O$ and 2% of agar agar in potato extracts. The medium used for growth consisted of pectic acid, 1%, $Na_2HPO_4 \cdot 12H_2O$, 0.5%, K_2HPO_4, 0.1%, $(NH_4)_2HPO_4$, 0.4% and $MgSO_4 \cdot 7H_2O$, 0.05%, the pH being 7.0. A loopful of the organisms from the freshly grown slope was suspended in 100 ml of the medium in a 500 ml flask and cultured with shaking at 27°C. After 20 hours' growth, the cells were harvested by centrifugation, and part of them rinsed twice in distilled water and once in 0.02 M phosphate buffer, pH 7.0, to remove medium constituents. They were then suspended in the same buffer (washed cells). The rest of the cells were washed twice with water and dehydrated with acetone in the usual manner. The acetone-dried cells so obtained were suspended in 100 parts of water and allowed to stand at 4°C for 20 hours with occasional stirring. The suspension was centrifuged to

remove the cells and the supernatant was used as the enzyme solution without purification (cell-free extract).

Paper chromotography. Samples (0.1 ml) were placed on Toyo Roshi No. 51 filter paper (2 × 40 cm) and run as ascending chromatograms at room temperature. The solvent system used was *n*-butanol·acetic acid·water (4 : 1 : 2). The paper was sprayed with aniline·hydrochloric acid or 0.1% alcoholic solution of bromophenol blue. Rf values for 4, 5-unsaturated digalacturonic acid, 4-deoxy-5-ketofructuronic acid and the unknown pyrrole were 0.25, 0.32 and 0.85, respectively.

Optical densities were measured on a Hitachi Model 139 Spectrophotometer, employing cuvettes with 1 cm light path. Samples (reaction mixtures) were diluted 1 : 100, before optical density readings were taken.

EXPERIMENTAL AND RESULTS

1.   *Formation of the Unknown Pyrrole from Pectic Acid and its Degradation Products, and Ammonium salts*

By the chromatography of the culture fluid of *Erwinia aroideae* and the subsequent examination of the extract from the section of paper corresponding to the spot, the unknown substance was demonstrated to be a derivative of pyrrole (5~8). With the washed cells and the cell-free extract as well as with the growing cells, this unknown pyrrole was formed from pectic acid and ammonium salts. By using the cell-free extract and washed cells, conditions for the formation of the pyrrole and other subjects were investigated.

Effect of pH on the Formation of the Pyrrole.

Fig. 1 shows the effect of pH on the formation of the pyrrole by the cell-free extract. Toluene was added to the incubation mixtures. After incubation the rate of formation of the pyrrole was measured by ultraviolet absorption (300 mμ). As shown in Fig. 1, optimum pH for the formation of the pyrrole was found to be 7.0 to 7.3.
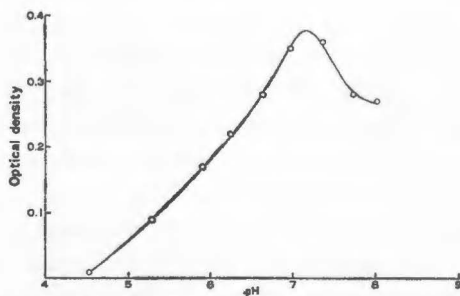

Fig. 1.   Effect of pH on the formation of the unknown pyrrole.   Incubation mixture: pectic acid, 0.5%; (NH₄)₂HPO₄, 0.4%; phosphate buffer, 0.01 M; cell-free extract, 2.0 ml; total volume, 10 ml; toluene, 0.1 ml. Temp., 27°C. Time, 24 hr.

Effect of Shaking and Addition of Toluene on the Formation of the Pyrrole.

The effect of shaking and addition of toluene on the formation of the pyrrole and coloring matter (measured at 300 and 420 mμ respectively) by the cell-free extract is shown in Table 1. When incubation was made with reciprocal shaking, the amounts of the pyrrole and coloring matter formed decreased sharply. In contrast, they were increased remarkably by the addition of toluene. For these findings an explanation will be offered later.

TABLE 1

Effect of shaking and addition of toluene on the formation
of the unknown pyrrole

Method of incubation*	Toluene**	Optical density		Paper chromatogram		
		300 mμ	420 mμ	Unknown pyrrole	4, 5-Unsaturated digalacturonic acid	4-Deoxy-5-keto-fructuronic acid
Stationary	0	0.13	0.27	+	+ +	+ +
	+	0.36	0.71	+ +	+ +	+
Shaking	0	0	0.05	−	±	−
	+	0.10	0.20	+	±	+

Incubation mixture: pectic acid, 0.5%; (NH$_4$)$_2$HPO$_4$, 0.4%; phosphate buffer, 0.01M; cell-free extract, 2 ml; total volume, 10.0 ml; pH, 7.0. Temp., 27°C; time, 24 hr.
* Rubber-stoppered flasks (200 ml) containing the incubation mixture were placed on a shaker of 7 cm amplitude and 130 rpm.
** Volume of toluene added, 0.1 ml.

The products present in large amounts are indicated by + +, those present in moderate amounts by +, those present in traces by ± and compounds not detected by −. This grading is based mainly on the appearance of spots on paper chromatograms and not on quantitative determinations. The same signs were used for indicating the amounts of the products in Tables 2—4.

Relationship between Sort of Carbohydrate and Formation of the Pyrrole.

The carbohydrates tested were pectic acid, 4, 5-unsaturated digalacturonic acid, D-galacturonic acid and D-glucose. The pyrrole and coloring matter were formed from the first two, but not from the other two (Table 2). Such was also the case with 4-deoxy-5-ketofructuronic acid. When ammonium phosphate (secondary) was omitted in the incubation mixture, both the pyrrole and the coloring matter did not arise even from pectic acid and 4, 5-unsaturated digalacturonic acid. In contrast, 4-deoxy-5-ketofructuronic acid was formed from these two carbohydrates without ammonium phosphate.

Relationship between Sort of Nitrogenous Substance and Formation of the Pyrrole.

Five sorts of nitrogenous substances were tested, including L-glutamic acid and glycine. The pyrrole and coloring matter were rapidly formed from pectic

TABLE 2

Relationship between sort of carbohydrate and formation
of the unknown pyrrole

Carbohydrate	(NH₄)₂HPO₄*	Optical density		Paper chromatogram		
		300 mμ	420 mμ	Unknown pyrrole	4, 5-Unsaturated digalacturonic acid	4-Deoxy-5-keto-fructuronic acid
Pectic acid	0	0.02	0.05	±	+ +	+ +
	+	0.42	0.82	+ +	+ +	+
4, 5-Unsaturated digalacturonic acid	0	0.04	0.05	±	+	+ +
	+	0.41	0.80	+ +	+	+
Galacturonic acid	+	0.01	0.04	−	−	−
Glucose	+	0.01	0.04	−	−	−

Incubation mixture: carbohydrate, 0.5%; phosphate buffer, 0.01M; cell-free extract, 2.0 ml;
total volume, 10.0 ml; pH, 7.0; toluene, 0.1 ml. Temp., 27°C; time, 24 hr.
* Concentration of (NH₄)₂HPO₄, 0.4%.

TABLE 3

Relationship between sort of nitrogenous substance
and formation of the unknown pyrrole

Nitrogenous substance	Optical density		Paper chromatogram		
	300 mμ	420 mμ	Unknown pyrrole	4, 5-Unsaturated digalacturonic acid	4-Deoxy-5-keto-fructuronic acid
(NH₄)₂HPO₄	0.34	0.63	+ +	+ +	+
(NH₄)₂SO₄	0.24	0.45	+ +	+ +	+ +
NH₄NO₃	0.14	0.21	+	+ +	+ +
L-Glutamic acid	0(0.16)	0(0.23)	−(+)	+ +(+ +)	+ +(+ +)
Glycine	0(0.14)	0(0.25)	−(+)	+ +(+ +)	+ +(+ +)

Incubation mixture; pectic acid, 0.5%; nitrogenous substance, 0.085%* (expressed as con-
centration of nitrogen); phosphate buffer, 0.01M; cell-free extract, 2 ml; total volume, 10.0
ml; pH, 7.0; toluene, 0.1 ml. Temp., 27°C; time, 24 hr. The values in parentheses were
obtained after 10 days' incubation,
* Nitrogen concentration of 0.4% (NH₄)₂HPO₄.

acid and the ammonium salts tested. With the amino acids, however, formation
of both substances were very slow: they were detected 10 days after the start
of incubation, but not after 20 hours (Table 3). The formation of 4-deoxy-5-
ketofructuronic acid was rapid not only with the ammonium salts but also with the
amino acids.

*Non enzymatic Formation of the Pyrrole from 4-Deoxy-5-ketofructuron-
ic Acid.*

4-Deoxy-5-ketofructuronic acid and ammonium phosphate were incubated
with or without the cell-free extract. As shown in Table 4, the pyrrole and
coloring matter were produced whether the extract was added to the incubation

TABLE 4

Formation of the unkown pyrrole from 4-deoxy-5-ketofructuronic acid

Addition	Optical density	Paper chromatogram	
	300 mμ	Unknown pyrrole	4-Deoxy-5-ketofructuronic acid
None	0.15	+	++
Cell-free extract	0.20	++	++
Cell-free extract heated for 5 min. at 80°C	0.15	+	++

Incubation mixture: 4-deoxy-5-ketofructuronic acid, 0.5%; (NH₄)₂HPO₄, 0.4%; phosphate buffer, 0.01M; cell-free extract or heated cell-free extract, 2 ml; total volume, 10.0 ml; pH, 7.0; toluene 0.1 ml. Temp., 27°C; time, 24 hr.

mixture or not. The results indicate that the formation of the pyrrole and coloring matter from 4-deoxy-5-ketofructuronic acid and ammonium salts is brought about nonenzymatically.

Effect of Ammonium Phosphate on the Oxidation of Pectic Acid by the Washed Cells.

The effect of ammonium phosphate on the rate of oxidation of pectic acid by the washed cells was studied by the Warburg manometric method (Fig. 2). The
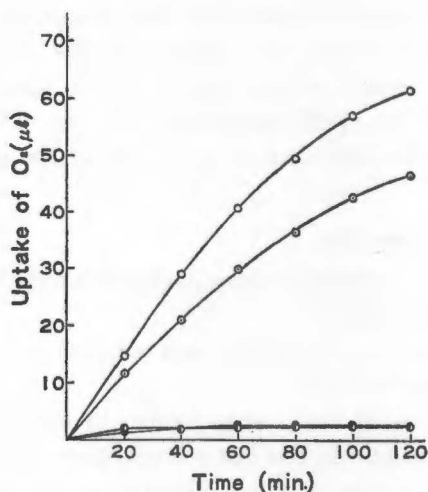

Fig. 2. Oxidation of pectic acid and the unknown pyrrole by washed cell suspension. Manometer flasks contained 0.5 ml of washed cells (0.88 mg dry wt.) in 0.1M phosphate buffer (pH 7.0), 0.5 ml of 0.01 M (NH₄)₂HPO₄ or water and 0.5 ml of water in the main compartments, 1 ml of 0.1% pectic acid or 0.005 M solution of the unknown pyrrole (mol. wt., 139.1) in the side bulbs, and strips of Toyo No. 2 filter paper and 0.2 ml of 20% KOH in the center wells. Incubation in air at 30°C. Substrates were added at zero time.

○ Pectic acid,   ⊙ pectic acid + (NH₄)₂HPO₄,   ◖ the unknown pyrrole
● endogenous respiration.

oxygen consumption by the cells was more rapid in the absence of $(NH_4)_2HPO_4$ than in its presence. An experiment was also made to determine whether the pyrrole is further metabolized by the cell suspension. As shown in Fig. 2, no consumption was observed : further metabolism of the pyrrole did not occurred. This was confirmed by the paper chromatogram of the incubated medium. The decrease in the rate of respiration caused by the presence of $(NH_4)_2HPO_4$ may be attributed to the partial conversion of pectic acid to this unutilizable pyrrole.

2.   *The Chemical Structure of the Unknown Pyrrole*

Isolation of the Pyrrole.

A medium containing about 3 g of washed cell paste of *Erwinia aroideae*, 7.5 g of pectic acid, 3 g of ammonium phosphate and 7.5 mmoles of phosphate buffer in total volume of 750 ml, pH 7.0, was incubated at 30°C under a layer of toluene (1 ml). After 48 to 72 hours of incubation, the medium was filtered through a layer of diatomaceous earth on a Büchner funnel to remove the cells. The clear filtrate was made slightly acid by adding a few drops of 1 N H_2SO_4 and concentrated to about 100 ml under reduced pressure. After being brought to pH 1.8 with 1 N H_2SO_4, the concentrate was extracted with ether for about 20 hours. When the ether extract was evaporated, grayish brown crystals appeared. The crystals were collected by filtration and dissolved in hot 50 % ethanol. The solution was treated with activated charcoal and filtered through a funnel with a fritted disk. When the clear filtrate was kept overnight in the ice chest, yellow crystals separated. On several recrystallizations from warm 50 % ethanol, they formed colorless needles, the yield being about 200 mg. Chromatographic behavior of the colorless needles was identical with that of the pyrrole in the original incubated medium.

Properties of the Pyrrole.

Melting point— The colorless needles darkened slowly from 180°C and melted at 207°C with decomposition.

Water of crystallization— The water was not lost on heating at 20 mm. Hg over phosphoric anhydride at 80°C.

Solubility— The crystals were very soluble in ethanol, methanol, acetone and 5 % sodium bicarbonate. In the last solvent, they dissolved forming bubles. They were considerably soluble in ether, chloroform and water. The pH of 0.1 % solution in 50% ethanol was 3.0.

Elementary analysis— This gave C 52.33 %, H 3.74 %, N 10.20 % (nitrogen was determined by the Kjeldahl method). Theory demands for $C_6H_5NO_3$, C 51.80%, H 3.62%, N 10.07%.

Neutralization equivalent— The pyrrole was dissolved in 50 % ethanol and titrated with phenolphthalein as indicator. One gram of the pyrrole required 724.63 ml of 0.01N NaOH. For $C_6H_5NO_3$, theory requires 718.83 ml.

Ultraviolet absorption spectrum. As shown in Fig. 3, there is an absorp-

tion peak at 300—302 mμ.

Infrared spectrum of the pyrrole.   The infrared spectrum of the pyrrole in KBr disk is given in Fig. 4.   The absorption bands at 1690 and 1658 cm⁻¹ are attributable to carboxyl and formyl groups, respectively.   The bands at 1550 and 1503 cm⁻¹ suggest the presence of double bonds.
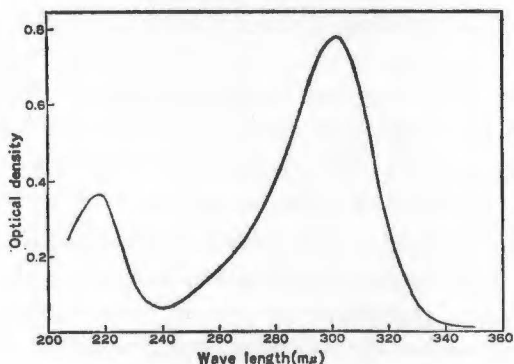

Fig. 3.   Ultraviolet absorption spectrum of the unknown pyrrole.   The optical density was measured at a concentration of 4 μg of the pyrrole/ml of 5 % ethanol.
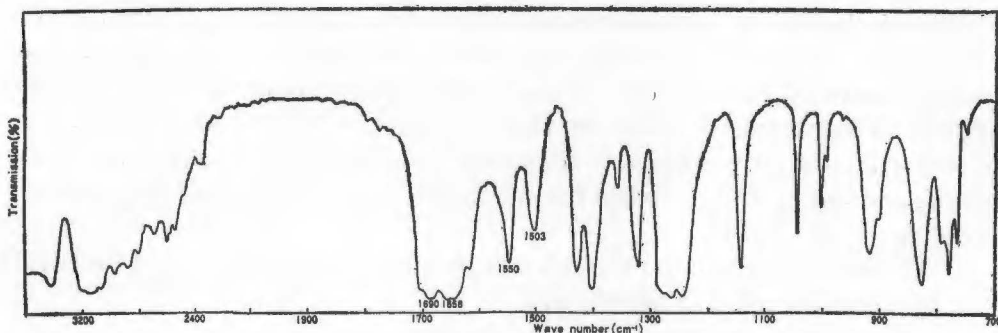

Fig. 4.   Infrared spectrum of the unknown pyrrole (KBr disk).

Reactions.   The unknown substance reacted as a derivative of pyrrole : the pine splinter (5), Ehrlich's (6), Liebermann-Burchard's (7) and the ninhydrin (8) tests were all positive.   It was strongly reducing toward Tollens's and Bertrand's reagents, and gave a positive *o*-dianisidine test (9).   When treated with dinitro-phenylhydrazine, it gave the hydrazone.   It was oxidized readily by potassium permanganate in the presence of sulfuric acid and by bromine water.   One gram of the pyrrole consumed 6.68 mmoles of bromine : on the basis of a molecular weight of 139.1, the amount of bromine consumed per 1 mole of the pyrrole was calculated to be 0.93 mole.

Decarboxylation of the pyrrole to α-pyrrole-aldehyde.   When a powder-ed mixture of 1 part of the pyrrole and 10 parts of sodium carbonate, anhydrous,

was distilled at 22°C under reduced pressure, colorless needles were deposited on the inner wall of the receiving flask. The crystals were identified as α-pyrrole-aldehyde from the following results: They had m. p. 42—43°C and there was no depression of the mixed m. p. with a sample of α-pyrrole-aldehyde prepared from pyrrole by the dimethyl formamide method (10). When 20 mg of the crystals were mixed with 1.5 ml of a phenylhydrazine solution (this was prepared by dissolving 2 ml of freshly distilled phenylhydrazine in enough water to make 100 ml of solution), the crystals of the phenylhydrazone formed immediately. They were recrystallized from ligroin as colorless needles. Their m. p. 139.0—139.5°C agrees with that of phenylhydrazone of α-pyrrole-aldehyde recorded by Bamberger and Djierdjian (11). The above results indicate that the formyl group of the unknown pyrrole is present in the α-position of the pyrrole nucleus.

Oxidation of the pyrrole to pyrrole-2, 5-dicarboxylic acid. The ease with which the pyrrole undergoes decarboxylation suggests the presence of carboxyl group in the α or α'-position of the pyrrole nucleus (see above). This was confirmed by the results from the following experiment (11). By adding 10 ml of water and about 2 ml of 1N NaOH, 250 mg of the pyrrole was brought into solution. To the solution was added the powder of potassium permanganate in small portions for oxdizing the pyrrole. After being kept at 40°C for about 1 hour, the solution was mixed with ethanol to reduce the remaining potassium permanganate and the manganese dioxide formed was filtered away. The filtrate was cooled, acidified and extracted with ether. Evaporation of the ether layer obtained gave colorless crystals. After several recrystallization, the m. p. was 260°C. This agrees with that reported by Ciamician (12, 13) for pyrrole-2, 5-dicarboxylic acid. It was titrated with sodium hydroxide and phenolphthalein as a dibasic acid, the equivalent weight found being 80, which is about half the required molecular weight 151.1.

All these results lead to the conclusion that the chemical structure of the pyrrole is 5-formylpyrrole-2-carboxylic acid.

DISCUSSION

From the results of the present study, the unknown pyrrole which was found in the cultures of *Erwinia aroideae* grown with pectic acid and ammonium salts as substrates was concluded to be 5-formylpyrrole-2-carboxylic acid. This pyrrole was formed nonenzymatically when the mixture containing 4-deoxy-5-ketofructuronic acid and ammonium salts were allowed to stand at room temperature for 24 hours. By the action of oligogalacturonide transeliminase (1, 14~16) or a certain type of exopolygalacturonase (17~19), 4-deoxy-5-ketoglucuronic acid is produced from pectic acid and its partial degradation products. This is converted into 4-deoxy-5-ketofructuronic acid by an isomerase (20). 5-Formylpyrrole-2-carboxylic acid found in the culture fluid of this organism must have been derived from the nonenzymatic reaction of 4-deoxy-5-ketofructuronic acid with ammonium ions.

The postulated scheme of the route by which 5-formylpyrrole-5-carboxylic acid is formed from pectic acid is as follows:

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Pectic acid

↓

4-Deoxy-5-ketoglucuronic acid

↓

4-Deoxy-5-ketofructuronic acid

↓

5-Formylpyrrole-2-carboxylic acid

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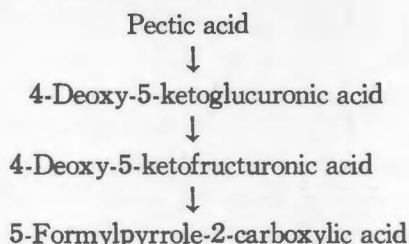
It has been known that 2, 5-dimethylpyrrole is produced when acetonyl acetone is heated with ammonium carbonate at 100°C (21). The nonenzymatic formation of 5-formylpyrrole-2-carboxylic acid is probably similar in mechanism to this reaction.

From pectic acid, 5-formylpyrrole-2-carboxylic acid is formed by the extract of the acetone-dried cells as well as by the growing cells. Conditions for the production of the pyrrole were investigated by the use of this extract. The shaking and the omission of toluene from the incubation mixture retarded the production of the pyrrole. Preiss and Ashwell (20) have reported that oxidation of 4-deoxy-5-ketofructuronic acid by bacteria proceeds through 2-keto-3-deoxygluconic acid. The above finding may be interpreted as indicating that the reaction of 4-deoxy-5- ketofructuronic acid with ammonium ions is prevented by the rapid oxidation which results from the shaking or omission of toluene from the incubation mixture.

When amino acids were used in place of ammonium salts, the pyrrole was also found in the reaction mixtures, though the time required was much more prolonged. It is reasonable to assume that the liberation of ammonium ions from the amino acids leads to the formation of the pyrrole.

5-Formylpyrrole-2-carboxylic acid was not metabolized by the washed cells of *Erwinia aroideae.* The rate of oxidation of pectic acid by the cells was lower in the presence of ammonium phosphate than in its absence. This is probably due to the fact that pectic acid is converted in part into this unutilizable pyrrole. Previous work from our laboratory (22)* showed that, when this organism was grown on pectic acid, glutamic and aspartic acids were better sources of nitrogen than was ammonium phosphate. As 5-formylpyrrole-2-carboxylic acid is far more rapidly formed from pectic acid and ammonium salts than from pectic acid and glutamic acid or glycine (Table 3), the above finding is also explained in terms of the waste of pectic acid, that is, its conversion into the pyrrole. In conclusion, we may safely say from the foregoing results that, in some cases, ammonium salts

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* Stationary culture was carried out at 27°C for 70 hours in 10 ml of the medium in a 50 ml conical flask, the medium containing pectic acid, 1%, glutamic acid, aspartic acid or $(NH_4)_2$ HPO_4, 0.087% (nitrogen content), $MgSO_4 \cdot 7H_2O$, 0.05%, KH_2PO_4, 0.2%, Na_2HPO_4 and NaH_2PO_4 (the last two were added to the incubation mixture so that the final concentration of phosphoric acid was 0.0573 M and the pH was 7.0).

act as inhibitors in pectic acid metabolism by *Erwinia aroideae*.

The formation of 5-formylpyrrole-2-carboxylic acid was always accompanied by the appearance of the reddish brown color. When an aqueous solution of 5-formylpyrrole-2-carboxylic acid was set aside at room temperature, a coloring matter was produced which gave a similar spectrum to that of the reddish brown coloring matter described. However, whether the coloring matter found in the culture fluid or incubation mixture is produced by way of 5-formylpyrrole-2-carboxylic acid can not be decided from the present results. In our laboratories, further work is being designed to determine the properties of this coloring matter and the mechanism of its formation.

SUMMARY

1) An unknown pyrrole was found in cultures of *Erwinia aroideae* grown with pectic acid and ammonium salts as substrates. It was also formed when pectic acid and ammonium salts were incubated with the extracts of acetone-dried cells of this organism.

2) With the extracts of the acetone-dried cells, the optimum for the production of this pyrrole was found to be at pH 7.0—7.3. The addition of toluene enhanced the production of the pyrrole and the shaking of the incubation mixture retarded it. The pyrrole was formed from pectic acid and 4, 5-unsaturated digalacturonic acid, but not from galacturonic acid and glucose. When amino acids were used in stead of ammonium salts, the production of the pyrrole was much delayed.

3) The pyrrole was produced nonenzymatically from 4-deoxy-5-ketofructuronic acid, an intermediate in the metabolism of pectic acid by *Erwinia aroideae*, and ammonium salts.

4) Further metabolism of this pyrrole was not observed with the washed cells. It is probable that, in the metabolism of pectic acid by *Erwinia aroideae*, ammonium salts acts as inhibitors in some cases.

5) The chemical structure of the pyrrole was investigated. It was found to be 5-formylpyrrole-2-carboxylic acid.

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