Action of Pectic Enzymes on Oligogalacturonic Acids and Some of Their Derivatives

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Introduction

Enzymes which attack pectic substances have received considerable attention in the past few years. In general, these enzymes may be divided into two broad classes—those possessing esterase activity and those attacking glycosidic linkages.

There is general agreement that the esterase activity can be accounted for by a single enzyme, pectinesterase (PE), whose action on pectin results in the production of pectic acid and methyl alcohol. Reid (1) reported that a fungal PE preparation was resolved into two active preparations, and suggested that PE's from different fungi differ in the total amount of de-esterification of pectin which they can accomplish and in their reaction velocities. Lineweaver and Jansen (2) have suggested that an ester bond between two adjacent esterified residues may not be attacked by PE, or may be attacked at a reduced rate compared to an ester bond adjacent to free carboxyl groups. A portion of this paper is concerned with the preparation of the diester of methyldigalacturonic acid, the diester of digalacturonic acid, the half esters of digalacturonic acid, and the triester of methyltrigalacturonic acid, and with the action of citrus PE on them. Citrus PE failed to de-esterify any of these compounds.

Polygalacturonase (PG), the best known enzyme of the group which attack the glycosidic linkages of polygalacturonic acid, ultimately yields the monomer (3). Ayres *et al.* (4) report that the degradation of pectic acid to galacturonic acid involves at least two enzymes. Dingle *et al.* (5) claim that the PG of Kertesz is a complex of at least three enzymes.

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Schubert (6) reports at least four different polygalacturonases in a culture extract of *Aspergillus niger*. The availability of crystalline model oligogalacturonic acids such as digalacturonic and trigalacturonic acids (7), and some simple derivatives therefrom, has suggested an investigation of whether more than one polygalacturonase is needed to effect the complete conversion of polygalacturonic acid to the monomer. Using these substrates, further information has been obtained on the structural requirements of substrates attacked by PG. Purified fungal PG attacked all substrates which possessed a glycosidic galacturonide bond between two adjacent free carboxyl groups, but failed to hydrolyze a glycosidic galacturonide linkage where one or both adjacent carboxyl groups were esterified. It behaved as if it were a single enzyme.

Materials and Methods

Paper Chromatography

Paper chromatograms were carried out using ascending irrigations for 16–20 hr. on Whatman No. 1 filter papers[2] (not equilibrated with solvent vapors) with a miscible solvent of 5 vol. ethyl acetate, 3 vol. water, and 2.5 vol. acetic acid. The dried, acetic acid-free paper sheets were sprayed with 0.04% bromcresol green in 95% ethanol adjusted to pH 6 with sodium hydroxide to reveal the positions of the acidic substances. The hydroxylamine–ferric chloride reagents were used as sprays to reveal the position occupied by the carboxylic methyl esters (8). Dry chromatograms were dipped in a solution of 2% aniline and 2% trichloroacetic acid in ethyl acetate, dried and heated at 100°C. for 5 min. to reveal the reducing substances. The ratio of the distances of migration on paper chromatograms of a particular substance compared with galacturonic acid is the R_{GA} value.

Polygalacturonic acid was made by heating 100 g. of purified citrus pectin in 2 l. of N sulfuric acid for 1 hr. at 100°C. The suspension was cooled and the acid-insoluble pectic acid removed by filtration and discarded. To the clear solution was added 2 vol. of 95% ethanol, and the precipitated polygalacturonic acid was separated by filtration. The wet cake of polygalacturonic acid was suspended in 70% ethanol and washed free from sulfuric acid in three treatments. It was further dehydrated with 95% ethanol and dried in air. A yield of 18 g. of dry material was obtained. After humidification and drying, its anhydrouronic acid content determined by a carbazole method (10) was 98%, and its average degree of polymerization (determined by a hypoiodite method (9) for reducing groups) was 10.

Digalacturonic and trigalacturonic acids produced by partial enzymic hydrolysis of polygalacturonic acid with PG were isolated by chromatography on thick filter paper and crystallized as their brucine salts (7). Brucine was removed from 2% solutions of these oligogalacturonides with cation-exchange resin Dowex 50, and

[2] Mention of manufacturers or of trade names of products or equipment does not imply that they are recommended by the Department of Agriculture over others of a similar nature not mentioned.

the solutions of the free acids were evaporated at 25°C. to dryness. The digalacturonic acid had a molar ratio of —COOH/—CHO of 2.02. Its equilibrium specific rotation was +153° (C = 2) and it gave an orange precipitate with Ehrlich's lead acetate test (11). The R_{GA} was 0.50 and it reacted both as a reducing substance and also as an acid.

Trigalacturonic acid had a molar ratio of —COOH/—CHO of 3.01. Its specific rotation was +186° (C = 2) and it gave a light-orange precipitate in the lead acetate test. The R_{GA} was 0.25 and it reacted both as an acid and as a reducing substance.

Polymethyl ester of polygalacturonic acid was made from the polygalacturonic acid (D.P. of 10) previously described by refluxing 3.0 g. of the material in 0.1 N dry hydrochloric acid in methanol for 5 hr. The insoluble polymethyl ester was removed by filtration, washed free of hydrochloric acid with methanol, and dried in air. A yield of 2.0 g. of material was obtained. Analyses showed 81% of the carboxyl groups esterified with methanol. No movement of the material on paper chromatograms was observed after 20 hr. irrigation.

Dimethyl ester of digalacturonic acid was made by the method of Jansen and Jang (12). Digalacturonic acid (0.8 g.) was dissolved in 50 ml. of 0.02 N dry hydrochloric acid–methanol solution and permitted to react for 66 hr. at 5°C. At the end of this period 50 ml. of water was added and the hydrochloric acid and the unreacted digalacturonic acid were removed with anion-exchange resin Permutit A. The neutral solution was evaporated to a sirup and then to dryness, but attempts to crystallize the ester were unsuccessful. Qualitative paper chromatograms revealed that most of the substance believed to be the dimethyl ester of digalacturonic acid was a neutral reducing ester (R_{GA} of 1.33) which gave a positive lead acetate test. There was a trace of neutral, nonreducing ester in the mixture with an R_{GA} of 1.83; this was believed to be the dimethyl ester of methyldigalacturonic acid. The dimethyl ester of digalacturonic acid was purified further by chromatography on heavy filter paper, eluted, freed from salts with Dowex 50 and Permutit A, and evaporated to dryness at 25°C. A yield of 0.65 g., or 74% of theory, was obtained. The molar ratio of —CH_3O/—CHO was 2.08. Its equilibrium specific rotation was +145° (C = 1) and it gave an orange precipitate in the lead acetate test. Its R_{GA} was 1.33 and it reacted as a neutral reducing ester.

Dimethyl ester of methyldigalacturonic acid was prepared by refluxing 0.79 g. of digalacturonic acid with 75 ml. of dry 0.1 N hydrochloric acid–methanol solution for 3 hr. The methanol solution was cooled, diluted with an equal volume of water, and then treated with Permutit A. A qualitative paper chromatogram showed only the presence of a neutral, nonreducing ester, believed to be the dimethyl ester of methyldigalacturonic acid. The solution was evaporated to a sirup and then to dryness, but attempts to crystallize either the α- or β-forms were unrewarding. A yield of 0.76 g., or 87% of theory, of glassy dimethyl ester of methyldigalacturonic acid was obtained. As the preparation contains both the α- and β-forms of the compound, no measurements of rotations were made. The molar ratio of ester —CH_3O/anhydrouronic acid was 1.01. It was a neutral, nonreducing ester having an R_{GA} of 1.83 and giving a negative lead acetate test.

Monomethyl esters of digalacturonic acid were prepared by partial alkaline deesterification of 0.10 g. of the dimethyl ester of digalacturonic acid in 10 ml. of

solution at pH 9.7–10.3 for 15 min. at 25°C., followed by addition of 1 g. of Dowex 50 to exchange sodium for hydrogen ions. Qualitative paper chromatograms revealed the presence of unreacted dimethyl ester of digalacturonic acid (R_{GA} = 1.33), a reducing ester (R_{GA} = 0.90) and digalacturonic acid (R_{GA} = 0.50) in the reaction mixture. The reducing ester (R_{GA} = 0.90) was also an acid, and was assumed to be a mixture of the two possible monomethyl esters of digalacturonic acid. The reaction mixture was concentrated to 1 ml. and placed on a 45 × 55 cm. sheet of Whatman No. 3MM filter paper and dried. After irrigation, the paper sheet was again dried and the band occupied by the monoesters (revealed by test strips) was excised and eluted with 10 ml. of water by capillary flow. The solution was treated with Dowex 50 and evaporated to a sirup. The half esters failed to crystallize. The amorphous glass weighed 0.063 g. and reacted as a reducing ester with acidic properties. It gave a positive lead acetate test and its R_{GA} was 0.90.

Methyl-α-D-digalacturonic acid was made from the amorphous mixed dimethyl esters of α- and β-methyldigalacturonic acids. To 0.55 g. of this material dissolved in 18 ml. of solution was added 5 ml. of 0.5 *N* barium hydroxide. Saponification of the ester groups was complete in 15 min. and 2 vol. of 95% ethanol was added and the precipitate of methyldigalacturonic acid barium salts removed by centrifugation. The precipitate was washed twice with 66% ethanol and then dissolved in 20 ml. of water. The barium ions were removed with Dowex 50, and the acid solution was permitted to evaporate slowly to a sirup. Crystals of methyl-α-D-digalacturonic acid (see *Results* section for proof of configuration) were separated from the sirup, washed once with water, and air-dried. The crystalline acid weighed 0.16 g., or 31% of theory. Its specific rotation was +205° (*C* = 1) for the anhydrous acid. By chromatography it was a nonreducing acid with an R_{GA} of 0.83 and its lead acetate test was negative.

α-D-Galacturonosyl-L-galactonic acid was made by reducing 0.28 g. of digalacturonic acid in 25 ml. of solution at pH 7.5–9.5 with 0.25 g. of sodium borohydride at 25°C. for 15 min. Lead acetate tests and tests for reducing sugars were negative. Excess sodium borohydride was destroyed by adding acetic acid to pH 4.0. Barium acetate was added, followed by 2 vol. of 95% ethanol, and the barium salts of the reduced uronic acid precipitated from solution. The precipitate was collected by centrifugation and washed twice with 60% ethanol, and the barium ions were removed by Dowex 50. Upon evaporation, the free acid, 4-(α-D-galacturonopyranosyl)-L-galactonic acid crystallized from solution. The air-dried crystals weighed 0.27 g. They gave a negative lead acetate test for free uronic acids. The R_{GA} was 0.81, and the crystals reacted as a nonreducing acid.

Trimethyl ester of methyltrigalacturonic acid was made by refluxing 0.8 g. of amorphous methyltrigalacturonic acid (prepared under the same reaction conditions described for the methyldigalacturonic acid) with 50 ml. of 0.1 *N* dry hydrochloric acid in methanol for 3 hr. It was cooled, diluted with 50 ml. of water, and the acids were removed with Permutit A. The neutral solution was evaporated at 25°C. to dryness. The amorphous, dried material weighed 0.60 g. It gave a negative lead acetate test for free uronic acids. Its R_{GA} was 1.70 on paper chromatograms, and it reacted as a nonreducing, neutral ester.

Fungal polygalacturonase enzymes used in this study were Pectinol 100 D, and purified PG made therefrom by Miss Rosie Jang of this laboratory by the method

of Jansen and MacDonnell (9). The reaction course of the glycoside-splitting enzymes on various substrates at pH 5.0 and 25°C. was followed quantitatively by a hypoiodite method (9) for determining the increase in reducing groups. (A trace of the preservative phenyl mercuric nitrate was added when experiments ran overnight or longer.) Activities have been expressed as millimoles of bonds split per gram of enzyme per minute $(PG_u)_g$. The activity of the purified PG was $(PG_u)_g = 20$, and that for Pectinol 100 D was $(PG_u)_g = 0.55$.

Purified orange pectinesterase (PE) was made from the flavedo of Valencia oranges by L. R. MacDonnell of this laboratory, by the method of MacDonnell *et al.* (13). The activity of the PE expressed as millimoles of ester groups hydrolyzed per gram of enzyme per minute was $(PE_u)_g = 2.3$. The conditions of assay, 20 ml. of 0.5% substrate, pH 7.5, 0.15 *M* sodium chloride, and 30°C., as recommended by MacDonnell *et al.* (14), were used.

RESULTS

A. Pectinesterase

The *dimethyl ester of digalacturonic acid* failed to react when it was substituted for pectin in the conventional pH titration method of analysis for PE (14); no detectable increase in free carboxyl groups was observed when 0.002 g. (0.0046 PE_u) of PE was used. No digalacturonic acid could be detected on paper chromatograms after permitting 0.01 g. (0.023 PE_u) of PE to act on 1 % solution of the dimethyl ester of digalacturonic acid for 3 hr.

Similarly, the *trimethyl ester of methyltrigalacturonic acid* failed to show a detectable increase in free carboxyl groups with 0.002 g. of PE.

The *dimethyl ester of methyldigalacturonic acid* also failed to produce free carboxyl groups using 0.002 g., and later 0.01 g. of PE. Furthermore, qualitative paper chromatography revealed only the presence of the original, neutral, nonreducing material moving at $R_{GA} = 1.83$.

To carry out a preliminary test on the reactivity of the *half esters of methyldigalacturonic acid*, 20 ml. of a 0.5 % solution of the dimethyl ester of methyldigalacturonic acid was made alkaline at pH 10 for 10 min. to partially hydrolyze off the ester groups. When the resultant mixture (later analysis by complete alkali saponification revealed that 31 % of the ester groups had been removed by the first alkaline hydrolysis) was partially neutralized to pH 7.5, and was treated with 0.002 g. (0.0046 PE_u) of PE, no increase in free carboxyl groups could be detected in 1 hr. This result was confirmed by treating a sample of the monomethyl esters of digalacturonic acid which had been isolated by paper chromatography, with 1 mg. PE/ml. of 1 % solution at pH 7. Hydrolysis of the methyl ester group of either of the two possible monoesters would be expected

to yield digalacturonic acid, but no digalacturonic acid could be detected on a chromatogram of the reaction mixture after 24 hr. or more exposure to PE.

The *polymethyl ester of polygalacturonic acid* (average D.P. of 10) reacted with PE, but the rate of production of carboxyl groups fell off with time, dropping to roughly 50% of the original value in about 15 min. when the substrate was treated with 0.002 g. of PE. Under these conditions, the rate of hydrolysis of pectin remains essentially constant for at least 15 min.

B. Polygalacturonase

Polygalacturonic acid (average D.P. of 10) under the standard PG assay conditions, was attacked at the same rate as was ordinary citrus pectic acid; $(PG_u)_g = 20$ for this substrate.

Di- and Trigalacturonic Acids. The rate of attack of PG on these two substrates was very slow under standard assay conditions. To make the number of substrate bonds available for enzyme attack more nearly comparable to that found in pectic acid, substrate concentrations were increased to 1%. But the rate was still so slow that it was necessary to use 30 times as much PG as was used for polygalacturonic acid. Under these conditions, $(PG_u)_g$ is equal to 1.0 for a digalacturonic acid substrate, and $(PG_u)_g$ is equal to 0.82 for a trigalacturonic acid substrate, indicating that these two substrates are attacked at a rate roughly 5% as great as that for polygalacturonic acid.

Methyl-α-D-digalacturonic Acid. When tested as for digalacturonic acid, this substrate gave $(PG_u)_g = 1.4$. Qualitative paper chromatography of the reaction mixture showed the presence of galacturonic acid and a nonreducing acid moving at $R_{GA} = 1.45$, identical with an authentic sample of methyl-α-D-galacturonic acid. It has already been established that methyl-α-D-galacturonic acid is not hydrolyzed by PG (15). The configuration of the methylgalacturonic acid moiety in methyldigalacturonic acid was demonstrated by allowing the PG hydrolysis to proceed to completion, adding HCl to make the solution 1 N with respect to this acid, and determining the specific rotation, which was $+94°$ ($C = 0.9$). Allowing for the slight dilution with the mineral acid, the rotation would have been $+95°$ ($C = 0.9$) for a mixture containing 1 mole each of galacturonic acid and of methyl-α-D-galacturonic acid. Upon heating this acidic solution at 100°C., the rotation dropped in less than 1 hr. to $+74°$. Quantitative acid hydrolysis of the methyl glycoside

had not occurred, but the downward mutarotation during acid hydrolysis demonstrates the α-configuration of the methylgalacturonic acid hydrolysis product of PG action. The α-configuration can therefore be tentatively assigned to the methyldigalacturonic acid.

α-D-Galacturonopyranosyl-L-galactonic acid in 1 % solution at pH 5 was slowly hydrolyzed by PG to give products tentatively identified on paper chromatograms as galacturonic acid and L-galactonic acid.

The *monomethyl esters of digalacturonic acid* were not hydrolyzed under the conditions of reaction previously described even when massive amounts (1 mg. of PG/ml. of substrate solution) of PG were used. No trace of the methyl ester of galacturonic acid (R_{GA} = 1.60) or galacturonic acid, the expected products of hydrolysis, could be observed by chromatography after 24 hr. or more exposure to PG. Both monoesters would yield identical hydrolysis products.

As expected (9), the *dimethyl ester of methyldigalacturonic acid*, the *dimethyl ester of digalacturonic acid*, and the *trimethyl ester of methyltrigalacturonic acid* were not hydrolyzed under the reaction conditions previously described, even when massive amounts (1 mg. of PG/ml. of substrate solution) of PG were used. No trace of the expected products of hydrolysis could be observed by chromatography after 24 hr. or more exposure to PG.

TABLE I

Susceptibilities Of Substrates To Attack By Pectic Enzymes

Substrate	Enzyme PG	PE
Methyl ester of galacturonic acid	—	No (17–19, 13)
Methyl galacturonide	No (15)	—
Methyl ester of methyl galacturonide	No (15)	No (17–19, 13)
Digalacturonic acid	Yes	—
Methyl-α-D-digalacturonic acid	Yes	—
Dimethyl ester of digalacturonic acid	No	No
Monomethyl esters of digalacturonic acid	No	No
Dimethyl ester of methyldigalacturonic acid	No	No
α-D-Galacturonosyl-L-galactonic acid	Yes	—
Trigalacturonic acid	Yes	—
Trimethyl ester of trigalacturonic acid	No	No
Polygalacturonic acid (average D.P. of 10)	Yes	—
Polymethyl ester of polygalacturonic acid (average D.P. of 10)	—	Yes

Pectinol 100 D activity was determined on polygalacturonic acid (D.P. of 10) and on digalacturonic acid under the same conditions as previously used for the purified fungal PG, except that 35 times as much by weight of the Pectinol 100 D was required. Under these conditions $(PG_u)_g$ is equal to 0.55 for the polygalacturonic acid substrate, and $(PG_u)_g$ is equal to 0.03 for digalacturonic acid, so that the ratio of the rates of attack of these two substrates by Pectinol 100 D is about 18, or essentially the same as the ratio of the rates of attack of these two substrates by purified PG.

These results are summarized in Table I.

Discussion

A. Mode of Pectinesterase Action

PE fails to de-esterify three completely esterified oligogalacturonic acids, the *diester of digalacturonic acid*, the *diester of methyldigalacturonic acid*, and the *triester of methyltrigalacturonic acid*. These results support the Lineweaver-Jansen hypothesis (2) that an ester bond between two adjacent esterified residues may not be attacked by PE, or may be attacked at a reduced rate compared to an ester bond adjacent to free carboxyl groups. However, the failure of the half esters of digalacturonic acid to react suggests that molecular size of the substrate may also be a factor. Accordingly, one might further speculate that at least below a certain limiting chain length, the rate of attack of PE decreases with decreasing molecular weight. An esterified polygalacturonic acid with an average D.P. of 10 was attacked by PE, but the rate of ester hydrolysis decreased rapidly with time, in marked contrast with pectin substrates, whose rates of hydrolysis remain essentially constant over comparable periods of time. As the esterified polygalacturonic acid probably contains a mixture of molecules of varying chain lengths, it is possible that PE more rapidly de-esterifies the larger molecules (D.P. above 10) than the smaller molecules (D.P. below 10). With continued action of the enzyme, the effective average D.P. of the remaining esterified molecules of the mixture might be lowered to the point where the rate of attack of PE would be appreciably slower.

To sum up, it would appear that PE preferentially attacks substrates which possess ester groups adjacent to free carboxyl groups, but that below a certain limiting molecular weight, somewhere in the neighborhood of a D.P. of 10, the rate of hydrolysis is also dependent upon the chain length of the molecule. For completely esterified substrates, the rate drops to zero for a D.P. of 3 or perhaps somewhat above; for a

partially esterified substrate, the rate drops to zero for a D.P. of 2 or perhaps somewhat above.

On the basis of the modified hypothesis, the hydrolysis of the methyl ester groups of pectin by PE in a mixture of fungal enzymes could be quite complicated. Polymethylgalacturonase (PMG) is able to hydrolyze the glycosidic galacturonide linkages of highly esterified substrates (16). If PMG were present, it could rapidly reduce pectin to molecules of such short chain length that, while they still possessed ester groups, they would be only slowly attacked by PE, or perhaps not at all. On the other hand, if the PE source contained only PG, or if it contained no enzyme capable of splitting galacturonide linkages, the de-esterification could be rapid and practically complete. Thus different PE preparations could show differing rates and extents of de-esterification, depending on the amounts of PMG and PG that they contained.

B. Mode of Polygalacturonase Action

In view of the failure of PE to attack the methyl esters of digalacturonide and trigalacturonide, and the apparent falling off of the rate for those lower oligouronide esters which are attacked, it is interesting to note a somewhat similar phenomenon for polygalacturonase. Although the uronide bonds of digalacturonic acid, methyl-α-D-digalacturonic acid and trigalacturonic acid are completely hydrolyzed by PG, the rate is only about 5% of that for pectic acid.

The specificity apparently is not influenced by the reducing end of the molecule, as reduction of the aldehyde group to an alcohol with sodium borohydride did not destroy the lability of the glycosidic linkage of the digalacturonic acid. The fact that the monomethyl esters of digalacturonide are not attacked by PG shows that both carboxyl groups adjacent to a glycosidic 1,4-linkage must be free for the linkage to be labile to PG.

It is possible that a typical fungal preparation of pectic enzymes might contain two different polygalacturonases, one of which attacked polygalacturonic acid and the larger molecular weight hydrolysis products therefrom, but which failed to attack lower oligogalacturonic acids such as digalacturonic acid; and a second enzyme which attacked only the lower oligogalacturonic acids (4, 5). It is not likely that a mixture of two distinct polygalacturonases could undergo the drastic purification procedures employed to prepare purified PG from Pectinol 100 D without an appreciable change in the ratio of their activities. Hence it appears

that a single enzyme, purified fungal PG, is able to effect the complete transformation of polygalacturonic to galacturonic acid.

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Summary

Purified citrus pectinesterase (PE) does not hydrolyze the ester groups of (*a*) the diester of digalacturonic acid, (*b*) the diester of methyldigalacturonic acid, (*c*) the triester of methyltrigalacturonic acid, or (*d*) the half esters of digalacturonic acid. It readily attacks polygalacturonic acid polymethyl esters with D.P.'s of 10 and above.

Purified fungal polygalacturonase (PG) does not hydrolyze the glycosidic galacturonide bonds of the three completely esterified oligogalacturonic acids, dimethyl ester of methyldigalacturonic acid, dimethyl ester of digalacturonic acid, or trimethyl ester of methyltrigalacturonic acid. It does not attack the glycosidic linkage of the half esters of digalacturonic acid. It hydrolyzes digalacturonic acid, methyl-α-D-digalacturonic acid, and trigalacturonic acid, but only at about 5% of the rate at which it attacks polygalacturonic acid. It attacks α-D-galacturonopyranosyl-L-galactonic acid. Thus PG attacks all polygalacturonide substrates which possess a glycosidic uronide bond between two adjacent free carboxyl groups, but fails to attack those where one or both of the adjacent carboxyl groups is esterified.

References

1. Reid, W. W., *Nature* **166,** 569 (1950).
2. Lineweaver, H., and Jansen, E. F., *Advances in Enzymol.* **11,** 267 (1951).
3. Kertesz, Z. I., "The Pectic Substances," p. 337. Interscience Publishers, Inc., New York and London, 1951.
4. Ayres, A., Dingle, J., Phipps, A., Reid, W. W., and Solomons, G. L., *Nature* **170,** 834 (1952).
5. Dingle, J., Reid, W. W., and Solomons, G. L., *J. Sci. Food Agr.* **4,** 149 (1953).
6. Schubert, E., *Nature* **169,** 931 (1952).
7. McCready, R. M., and McComb, E. A., Abstracts of 123rd Meeting Am. Chem. Soc., p. 2*D*. Los Angeles, 1953.
8. Whittaker, V. P., and Wijesundera, S., *Biochem. Soc. Proc.*, p.xlv (bound with *Biochem. J.*, Vol. 49) (1951).
9. Jansen, E. F., and MacDonnell, L. R., *Arch. Biochem.* **8,** 97 (1945).
10. McComb, E. A., and McCready, R. M., *Anal. Chem.* **24,** 1630 (1952).
11. Ehrlich, F., *Ber.* **65B,** 352 (1932).

12. JANSEN, E. F., AND JANG, R., *J. Am. Chem. Soc.* **68,** 1475 (1946).
13. MACDONNELL, L. R., JANG, R., JANSEN, E. F., AND LINEWEAVER, H., *Arch. Biochem.* **28,** 260 (1950).
14. MACDONNELL, L. R., JANSEN, E. F., AND LINEWEAVER, H., *Arch. Biochem.* **6,** 389 (1945).
15. LINEWEAVER, H., JANG, R., AND JANSEN, E. F., *Arch. Biochem.* **20,** 137 (1949).
16. SEEGMILLER, C. G., AND JANSEN, E. F., *J. Biol. Chem.* **195,** 327 (1952).
17. KERTESZ, Z. I., *J. Biol. Chem.* **121,** 589 (1937).
18. LINEWEAVER, H., AND BALLOU, G. A., *Federation Proc.* **2,** 66 (1943).
19. LINEWEAVER, H., JANSEN, E. F., MACDONNELL, L. R., AND JANG, R., *Federation Proc.* **5,** 145 (1946).