Isolation and Characterization of Pigments from Protein–Carbonyl Browning Systems. Models for Two Insulin–Glucose Pigments

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Limit-peptide pigments of approximately 7800 and 1500 molecular weight were isolated from a humidified insulin-glucose-U-^{14}C reaction system. Gel chromatographic analysis of the intact model system and analyses of the amino acid and carbohydrate compositions of the limit-peptide pigments indicated cross-linking between peptide chains. This cross-linking was associated with extensive condensation of sugar residues, some of which have already reacted with amine groups on the protein, into aggregates of up to 31 residues. These results give credence to the hypothesis that extensive carbohydrate condensation is required for the formation of brown chromophores [Hurd, C. D., Buess, C. M., *J. Amer. Chem. Soc.* **78,** 5667 (1956); Liggett, R. W., Deitz, V. R., *Advan. Carbohyd. Chem.* **9,** 247 (1954)].

We have previously reported (Clark and Tannenbaum, 1970, 1973) a general procedure for the isolation and purification of brown limit-peptide pigments (LPP's) from protein–carbonyl browning systems. A difficulty of studying the LPP's isolated from casein-glucose and milk systems is that the isolated pigment is heterogeneous. This heterogeneity arises from the fact that the protein molecules have several reactive amino acid residues, each with a different, unknown amino acid sequence adjacent to it. Studies of structure are complicated by the facts that it is difficult to determine the number of peptide chains in a pigment (Bradbury, 1958a,b) and that the reacted amino acids are not usually 100% regenerable by acid hydrolysis.

Insulin, however, is a protein whose amino acid sequence is known (*cf.* Klostermeyer and Humbel, 1966). It contains only one lysine and two N-terminal amino acids per mole, and has been previously investigated in the type of model system used in these studies (Schwartz and Lea, 1952). Use of radioactive tracers to measure glucose incorporation and amino acid analyses give information on the stoichiometry of the sugar and amino interactions after extensive browning. This provides a basis for understanding the structure of brown pigments.

VISIBLE ABSORPTION SPECTRA OF BROWN PIGMENTS

Illustrated in Figure 1 is the typical shape of the absorption curve of a brown pigment. As indicated in the figure, the total absorption could be the sum of the absorption of several discrete chromophores. In this model there is lesser absorption by chromophores which absorb light at the higher wavelengths. Calculation of the components which could make up this absorption spectrum would require a more complex computer program than that of Deisseroth and Dounce (1967) to generate the number of contributing absorption curves and their absorption maxima.

Several authors have discussed the brown pigment absorption curve in terms of the chemical mechanisms which would be required to fit a model similar to that just discussed. Liggett and Deitz (1954) indicated that the formation of the pigment is a polymerization process. The generation of chromophores with progressively higher wavelengths of maximum absorption could also be a consequence of polymerization, *i.e.*, polymerization in such a way that chromophore conjugation is extended. Molecules with the highest degree of conjugation would be produced with the least frequency.

Hurd and Buess (1956) suggested that one of the major pathways of color formation is repeated aldol condensation, which would give conjugated carbonyls and other conjugated systems which they feel are responsible for color. Many conjugated unsaturated chromophores are yellow (Hodge, 1967); brown is considered to be a red-yellow of low lightness (Munsel value; MacKinney and Little, 1962). The observed increase in color intensity with increasing pH (Liggett and Deitz, 1954) suggests the presence of carbonyl groups (which have been demonstrated by Burton *et al.*, 1962) and other negative auxochromes as part of the chromophores of interest.

It would be reasonable to expect variations in the slope of the log transform of the absorption spectrum (Clark and Tannenbaum, 1970; Liggett and Deitz, 1954) for different browning systems, depending upon the available reactive compounds and catalyst. The linear relationship of log λ *vs.* log A would not be predicted *a priori* from the chemical model. It would, in fact, have been difficult to predict a continuum rather than a curve with small but distinct absorption maxima (shoulders).

The fact that Hannan and Lea (1952) were able to separate materials from the early stages of browning of an N-acetylysine–glucose system which had different ultraviolet absorption maxima suggests that the simultaneous formation of many chromophores is possible. However, no one has reported the separation of brown pigments into the red, green, blue, etc. colored pigments suggested in Figure 1. Thus, if the mixed chromophore hypothesis is true, then each polymeric unit may have all of the groups required for the brown color bound into one molecule. It is the purpose of this paper to demonstrate that the extensive cross-linking which is required for these models can occur in protein–carbonyl browning reaction systems.

EXPERIMENTAL SECTION

Materials. Insulin, bovine pancreas, recrystallized (25.1 units/mg), was obtained from Mann Research Laboratories, New York, N. Y. It appeared homogeneous by gel filtration (12,000 mol wt dimer at pH 7.0 on Bio-Gel P-10). Gel filtration after partial cleavage with dithiothreitol (Cleland, 1964) showed fragments of 6000 mol wt (monomer) and 3000 mol wt (A and B chains). The amino acid analysis agreed with the expected composition. Sources of other chemicals and radiochemicals are described in earlier publications (Clark and Tannenbaum, 1970).

Sample Preparation. One gram of insulin and 0.410 g of glucose-U-^{14}C (specific activity, 11 μCi/mmol) were dissolved in 75 ml of water at pH 7.0. The sample was freeze-dried, equilibrated in an evacuated chamber at 75%

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Table I. Amino Acid Composition of High Molecular Weight Insulin LPP

Amino acid	Molar[a] ratio	Molar ratio[a] (calcd from model, Fig 5)	Comments
Asp	1.62	1.8	Probably enzymatic cleavage losses of A-21 would improve agreement still further
Thr	1.03	1.0	"Internal standard" (B-27), suggests lysine = 1.0
Ser	1.91	2.0	A-9 present, B-9 and A-12 both probably contribute to second mole
Glu	3.76	3.8	Suggests 0.8 arginine stoichiometry
Pro	1.00	1.0	"Internal standard," suggests lysine = 1.0
Gly	3.45	3.6	B-8, A-1, 2 × 0.8 in arginine peptide (B-20 and -23)
Ala	1.62	2.0	B-30 enzymatic cleavage is highly probable and would improve agreement
Val	2.96[b]	3.0	Good agreement
Cys	4.5[c]	5.6	Discrepancy probably due to cysteine destruction during storage
Ile	0.91[d]	1.0	Good agreement, slight error could be in variability of A-2,3 acid cleavage
Leu	0.93	1.0	Must be B-6
Tyr	0.06	0	Indicates loss of central regions of A and B chains
Phe	0.50	1	Reasonable, depends on B-1 regeneration (B-24 may be present)
Lys	0.36	1	Reasonable, depends on lysine regeneration from acid hydrolysis
His	0.69	1	Reasonable, depends on B-5 reactivity and regeneration, possibly some B-10
Arg	0.19	0.8	Reasonable, depends on regeneration from acid hydrolysis
NH_3	3.11	3.0	Reasonable (corrected for A-21 as aspartic acid)

[a] Proline = 1.0. [b] Corrected for expected 50% recovery in A-2,3 dipeptide. [c] Corrected for losses to cysteic acid and for incomplete recovery from insulin. [d] Corrected for 50% recovery from insulin. A and B refer to the respective insulin chains.

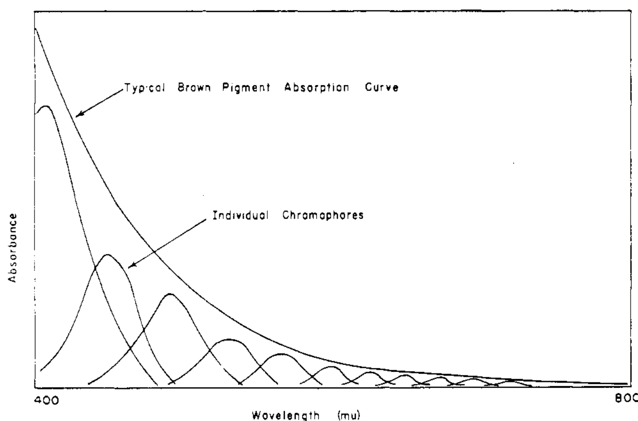

Figure 1. Absorption curve for a brown pigment.

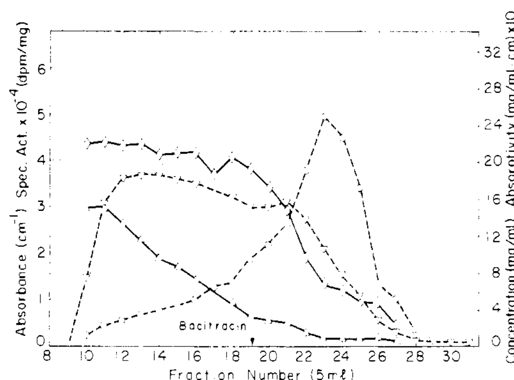

Figure 3. Characteristics of insulin-glucose LPP's eluted from Bio-Gel P-4: (○) concentration; (△) absorbancy (a_{420}); (▽) A_{420}; (◇) ^{14}C specific activity.

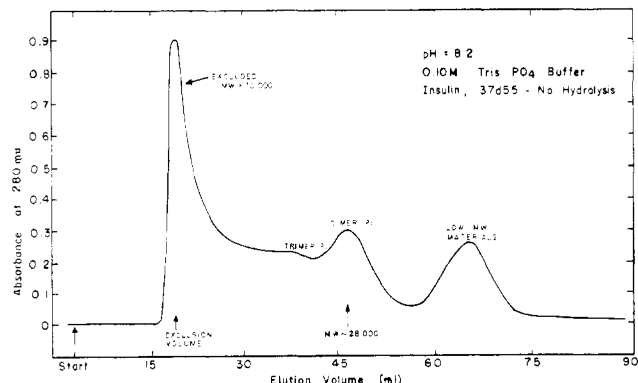

Figure 2. Elution profile of insulin-glucose reaction products on Sephadex G-75.

relative humidity, and 55° for 4 hr, stoppered, and stored for 37 days at 55°.

Method of Procedure. Limit-peptide pigments were prepared by exhaustive treatment with proteolytic enzymes and isolated as previously reported (Clark and Tannenbaum, 1970). Purification was on a 1.5 × 92 cm column of Bio-Gel P-4. The material eluted at the exclusion volume of the column (about 25 mg with a_{420} = 1.4) was used without further purification. The mean molecular weight was approximately 7800 by gel filtration on Bio-Gel P-10, fractions 11 and 14 from Figure 3. Material of approximately 1500 mol wt was further purified on a 1.5 × 92 cm column of carboxymethylcellulose (Clark and Tannenbaum, 1970).

Determination of glucose incorporation into LPP's was by scintillation counting (Packard Instruments, Co., La-Grange, Ill.) using toluene-^{14}C as an internal standard.

Samples for amino acid analysis were prepared by hydrolysis in 6 N HCl for 20 hr at 110°. Analyses were performed on the Technicon Amino Acid Analyzer (Technicon Corp., Ardsley, N. Y.). Suitable correction factors were applied to serine and threonine for losses during hydrolysis. No peaks were observed on the amino acid chromatogram which could be related to the pigment or its degradation products.

RESULTS AND DISCUSSION

Chromatography of a portion of the browned insulin-glucose system on Sephadex G-75 showed that polymers of

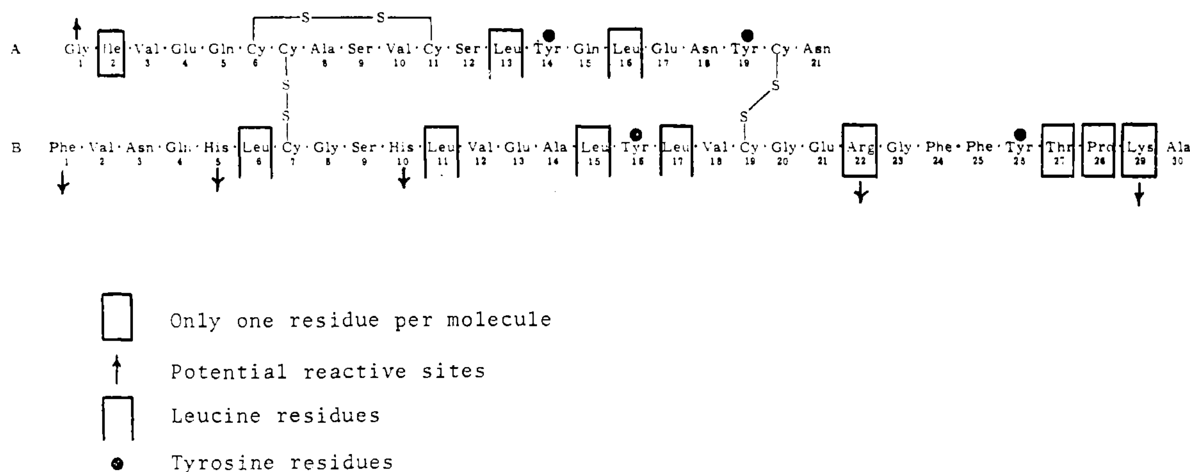

Figure 4. The secondary structure of insulin with potential reactive sites.

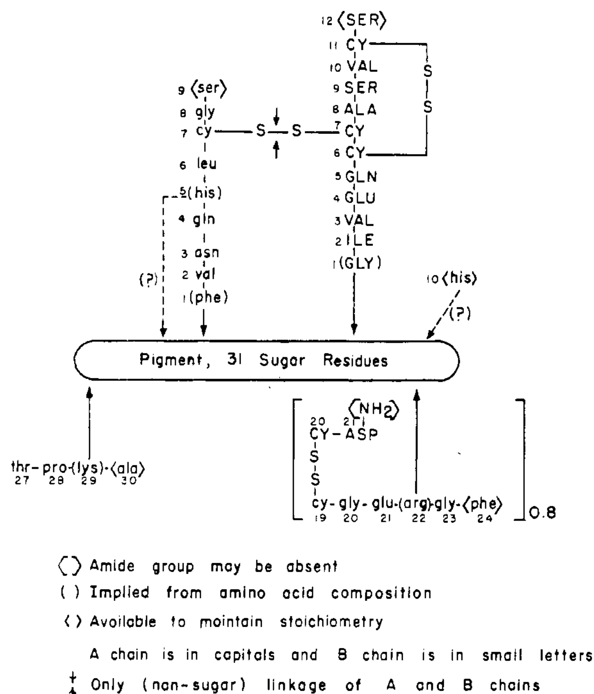

Figure 5. Structural features of a 7800 mol wt LPP.

the dimeric insulin molecule had been formed on browning (Figure 2). This observation confirms the occurrence of interchain cross-linking as discussed by Hannan and Lea (1952) and Mohammed *et al.* (1949a,b).

The elution data for the insulin–glucose LPP's from the Bio-Gel P-4 column are shown in Figure 3. As with previous experiments (Clark and Tannenbaum, 1970, 1973) the darkest materials are those with the highest molecular weights.

High Molecular Weight Pigment from Insulin. The amino acid composition of the high molecular weight pigment derived from insulin is shown in Table I. The amino acid composition, molecular weight, and sugar residue content of the pigment have been used to assist in deducing the pigment structure. The amino acid sequence of insulin given in Figure 4 is coded to emphasize several pertinent features, particularly the importance of lysine, arginine, histidine, and N-terminal amino acids as loci for attachment of pigment to peptide.

Isoleucine, proline, and threonine are present in equimolar amounts (A chain) suggesting that the lysine and N-terminal glycine are also equal. Tyrosine and leucine values are extremely low compared to the starting material, indicating that enzymatic digestion has resulted in cleavage of the middle of the A and B chains in such a way that the N and C terminal ends are no longer associated with each other through the peptide chain. Comparing the results of the amino acid analysis of the high molecular weight pigment and the known sequences of the insulin chains, it is possible to determine which pieces of the protein chain are intact, and to estimate their ratios. Carbon-14 data on the sample indicate that there are approximately 31 sugar residues present for each mole of isoleucine, proline, or threonine.

These considerations suggest the limit-peptide pigment structure shown in Figure 5. The observed molecular weight (~7800) and amino acid composition of the pigment indicate that the N-terminal portions of the A and B chains are still cross-linked by cystine. There is also cross-linking through sugar residues, since the lysine and arginine peptides must be cross-linked in this manner. For instance, if the Thr-Pro-Lys-Ala peptide was not cross-linked to the other peptides present, a model giving a mol wt of 7800 would require the binding of 40–50 sugar residues to the ϵ-amino group of the lysine, a less likely possibility.

The stoichiometry indicates that the N-terminal glycine, N-terminal phenylalanine, and lysine residues are equally involved in pigment structure. The arginine appears to be less completely reacted (80% of lysine) which is reasonable considering that it is lost more slowly from browning reaction systems (Lea and Hannan, 1950) and is thought to be involved almost entirely through reaction with carbonyls released from lysine (Mohammed *et al.*, 1949a). It is not possible to say to what extent the histidine has reacted.

The particular configuration of any one LPP depends on the number of sugar residues bound together, the number and type of peptide chains bound to one group of sugar residues, and the possible binding of the two N-terminal amino acids to separate groups of sugar residues. The structure shown in Figure 5 is thus only an average. To further support this "average" structure, the observed and predicted amino acid molar ratios of the model are given (based on proline = 1.0) in Table I. Comments on the agreement between the observed and predicted values are also given.

The large number of sugar residues associated with the limit peptide indicates that sugar residues have self-condensed as well as combined with the amino groups of the protein. A ratio of about 8 sugar residues per amine group is observed in this highly purified pigment.

The model system used here contained a glucose to amine ratio of 4.4:1. Lea and Hannan (1949) found that

Table II. Amino Acid Composition of Low Molecular Weight Insulin LPP

Amino acid	Wt %[a]	Molar[b] ratio	Molar ratio[b] (calcd from model, Fig 6)	Comments
Asp	10.60	1.26	1.1	B-3; A-21
Thr	1.50	0.20	0.2	B-27 (therefore lysine is probably 0.2)
Ser	5.32	0.82	0.9	A-9, slight cleavage of A-12 or B-9
Glu	26.3	2.79	2.8	A-4, A-5, B-4, and B-21
Pro	1.50	0.21	0.2	Therefore lysine is probably 0.2
Gly	9.41	2.25	2.3	A-1 plus some B-20, B-23, and B-8
Ala	2.26	0.44	0.5	A-8 and B-30
Val	13.2	1.83[c]	1.9	B-2, A-3, and A-10
Cys	9.69	1.3	2.2	Discrepancy probably due to cysteine destruction during storage
Ile	8.27	1.00[c]	1.0	Internal standard
Leu	2.52	0.30	0.3	
Tyr	0.83	0.07	0	
Phe	3.29	0.31	0.6	B-1 agrees with 50% regeneration of high molecular weight pigment
Lys	0.81	0.08	0.2	Reasonable, depends on regeneration during acid hydrolysis
His	2.53	0.25	0.3	Probably little reacted
Arg	1.16	0.10	0.5	Reasonable, depends on regeneration during acid hydrolysis
NH_{39} % recovered as amino acid	0.77	1.19	1.9	

[a] Per cent of total amino acids recovered. [b] Isoleucine = 1.0. [c] Corrected for recovery losses in A-2,3 dipeptide.

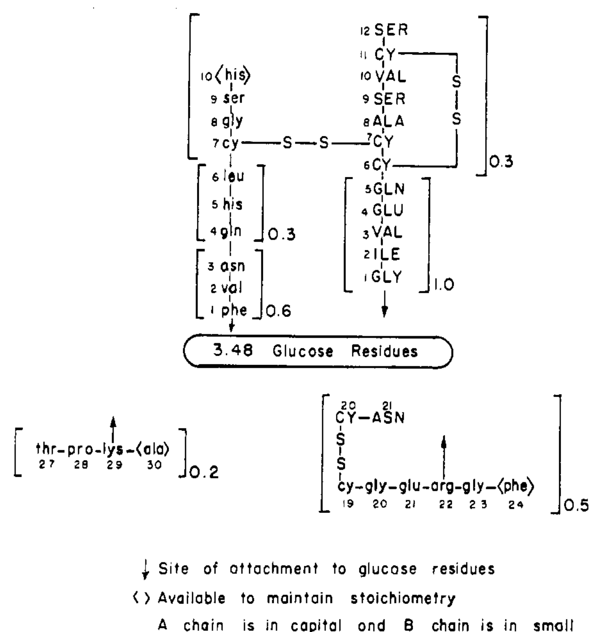

Figure 6. Structural features of a 1500 mol wt LPP.

increasing the glucose-amino ratio in the casein-glucose system to 8:1 resulted in an increase in the amount of bound sugar (up to 2.2 mol/reacted amine after 30 days at 37°). Our results confirm their findings that sugar is a limiting substrate. Mohammed *et al.* (1949a,b) noted that in acetaldehyde-protein browning, acetaldehyde combines at 3-4 mol/mol of amine (in contrast to their observations of glucose combining at about 1:1 with amine groups). Carson and Olcott (1954), using model systems composed of acetaldehyde and various aliphatic amines (pH 6-7, 3-25°), formed water-soluble brown pigments which had a 4:1 (molar, aldehyde to amine) condensation ratio, with loss of 2 mol of water. Hurd and Buess (1956) reported that acetol-phenylalanine (2.5:1) gave a pigment with a 4:1 molar ratio of acetol to phenylalanine residues. The data of Stinson and Willits (1965) indicate a 6:1, sugar residue-amino acid ratio for an approximate 10,000 mol wt polymer isolated from maple syrup.

Table III. Comparison of Sugar Content and Absorptivity of 1500 and 7800 Mol Wt LPP's from Insulin-Glucose

Mol wt	Sugar content $\times 10^3$, mmol/mg	a_{420}	(a_{420}/sugar content) $\times 10^{-3}$
7800	1.71	1.4	0.82
1500	1.48	0.29	0.20

Our data cannot indicate to what extent the interaction of the sugar residues is essential for color formation or cross-linking. The high sugar-amine ratio supports a theory (Hurd and Buess, 1956) and model (Liggett and Deitz, 1954) that pigment color is a consequence of a mixture of chromophores produced by aldol condensation or similar reactions of the unsaturated molecules which develop in the browning system.

1500 Mol Wt Pigment from Insulin. It is possible to partially construct a model or skeleton of this LPP from the amino acid composition of the 1500 mol wt fraction (Table II). The amino acid ratios which fit the model (Figure 6) and the agreement to the observed values are presented in Table II. Carbon-14 incorporation indicates the presence of about 3.5 residues of glucose per isoleucine residue or about 1.5 mol of glucose for each mole of reacted amine.

It is possible to put the components together in several ways to give molecular weights in the range of 1500. The much lower absorptivity for a given sugar content in the 1500 than in the 7800 mol wt insulin LPP (see Table III) and the lower degree of sugar condensation in the lower

molecular weight peptides (contrast Figures 5 and 6) suggest that condensation of sugar residues is a requisite for color formation. This conclusion is also strongly supported by our earlier observations (Clark and Tannenbaum, 1973) that dehydration of glucose at the hydroxymethyl carbon is strongly correlated with color formation.

Nutritional Significance. While the enzymes and model systems used are not those encountered *in vivo*, some comments can be made on the expected reduction in the nutritive value of foods through losses of amino acids other than lysine, arginine, and histidine. The models of the insulin pigments (Figures 5 and 6) demonstrate the incompleteness of the *in vitro* enzymatic digestion of the peptide near the bound carbohydrate. The low molecular weight pigment (Figure 6) apparently has undergone extensive cleavage of bonds B-6 to -7, B-3 to -4, and A-5 to -6. These bonds appear to be intact in the higher molecular weight pigment which contains a greater number of nearby sugar residues that can sterically interfere with hydrolysis.

Boctor and Harper (1968) demonstrated that the nutritional value of heat treated egg-albumin-glucose was lower than that which could be explained on the basis of the loss of fluorodinitrobenzene (FDNB) reactive lysine, and also that some FDNB-reactive lysine was excreted. Similarly, Dvorak (1968) also found that there could be FDNB-reactive lysine that was not nutritionally available. These observations agree with the results of Ford and Salter (1966) who found that the availability of several amino acids in heated cod fillets was lower than could be accounted for by direct losses. Thus, the observed difference in the susceptibility of the insulin pigments to enzymatic hydrolysis is a direct example of a nonspecific loss of amino acids (to undigested peptides) that contributes to deterioration of the protein-nutritive value of foods.

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Metabolism of Limonoids. Isolation and Characterization of Deoxylimonin Hydrolase from *Pseudomonas*

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A new limonoid-metabolizing enzyme, deoxylimonin hydrolase, was isolated from cell-free extracts of *Pseudomonas* 321-18 by $(NH_4)_2SO_4$ precipitation followed by three applications of DEAE-cellulose column chromatography. This enzyme catalyzes the hydrolysis of deoxylimonin to form deoxylimonic acid and apparently attacks only the closed D ring of deoxylimonin. The enzyme requires no cofactor and its activity is optimal at pH 8.0–8.5. The enzyme possesses sulfhydryl groups, which are involved in its catalytic action.

Limonin (I) is the intensely bitter triterpenoid dilactone present in citrus seeds (Arigoni *et al.*, 1960; Barton *et al.*, 1961) or formed during the delayed bittering of citrus juices (Maier and Beverly, 1968).

Because of a limonin bitterness problem in certain citrus juices and other processed products, the metabolic pathways of limonoids in plants and microorganisms have been intensively investigated (Nomura, 1966; Flavian and Levi, 1970; Chandler, 1971; Maier *et al.*, 1971; Hasegawa *et al.*, 1972a,b, 1974a,b). Limonoate A-ring lactone (II), the major limonoid in citrus fruit tissues, decreases in concentration with advancing maturity of the fruits (Higby, 1938; Scott, 1970) or during post-harvest treatments of navel oranges with ethylene or its analogs (Maier *et al.*, 1971), indicating that the fruit tissues possess limonoid-degrading systems. Recently, we have isolated the metabolite 17-dehydrolimonoate A-ring lactone (III) from various parts of citrus (Hsu *et al.*, 1973) and have also detected limonoate dehydrogenase activity in albedo tissues of navel oranges (Hasegawa *et al.*, 1974a).

In bacteria, limonoate and limonoate A-ring lactone have been shown to be metabolized through two pathways: one through 17-dehydrolimonoate or 17-dehydrolimonoate A-ring lactone, and the other through deoxylimonin (Hasegawa *et al.*, 1972a,b; 1974a,b). During the course of our investigation on bacterial limonoid metabolism, we have found and isolated a new enzyme, deoxyli-

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