CoA synthetase is an enzyme specific for the double bonds in $\Delta^{9,12}$ position, and the $\Delta^{9}$ double bond is a main determinant of the specificity. Because we did not investigate the optimum cofactor requirement of long-chain acyl-CoA synthetase for each isomer of linoleic acid, the possible existence of different enzymes for each substrate was not excluded. However this possibility seems remote with fatty acids which do not occur, or occur only as minor constituents in rat liver.

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We are greatly indebted to Dr. Ralph T. Holman, in whose laboratory at the Hormel Institute were synthesized the radioactive-labeled octadecadienoic acids, and for his continued interest in this work. The technical assistance of Mr. Richard Meadows is gratefully acknowledged.

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# Studies on the Enzymatic Synthesis of the Cyclic Trimer of 2,3-Dihydroxy-*N*-benzoyl-L-serine in *Escherichia coli*†

Graeme F. Bryce* and Nathan Brot

ABSTRACT: The enzyme system in *Escherichia coli* $K_{12}$ responsible for the synthesis of the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine from L-serine, ATP, and 2,3-dihydroxybenzoic acid has been resolved into three separate protein components, each essentially free of the other activities. Two covalently bound enzyme intermediates have been isolated and characterized as thio ester linked L-serine and 2,3-dihydroxy-*N*-benzoyl-L-serine, representing the first two stages of the overall synthesis. In addition another enzyme has been resolved which hydrolyzes this trimer, through intermediates, to 2,3-dihydroxy-*N*-benzoyl-L-serine.

The cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine (DBS)[1] and its breakdown products (Figure 1) are found in the cell-free growth medium of iron-deficient cultures of *Escherichia coli* (Brot *et al.*, 1966; Brot and Goodwin, 1968; O'Brien and Gibson, 1970a), *Salmonella typhimurium* (Pollack and Neilands, 1970) and *Aerobacter aerogenes* (O'Brien and Gibson, 1970b). That this compound may be implicated in iron transport is suggested by the observation that the enzymes responsible for the synthesis of the cyclic trimer were repressed when the organism was grown in an iron-sufficient medium (Brot and Goodwin, 1968; Bryce and Brot, 1971a). Additional evidence is provided by the isolation of iron-transport mutants which are defective in the enzymatic synthesis of *cyclo*-$(DBS)_3$ in *S. typhimurium* (Pollack *et al.*, 1970) and *Escherichia coli* (Cox *et al.*, 1970).

A previous report from this laboratory (Bryce *et al.*, 1971) showed that at least two enzymes were required for the synthesis of *cyclo*-$(DBS)_3$ from 2,3-dihydroxybenzoic acid,

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[1] Abbreviations used are: DBS, 2,3-dihydroxy-*N*-benzoyl-L-serine; *cyclo*-$(DBS)_3$, *cyclo*-tris(2,3-dihydroxy-*N*-benzoyl-L-seryl); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DON, 6-diazo-5-oxonorleucine.

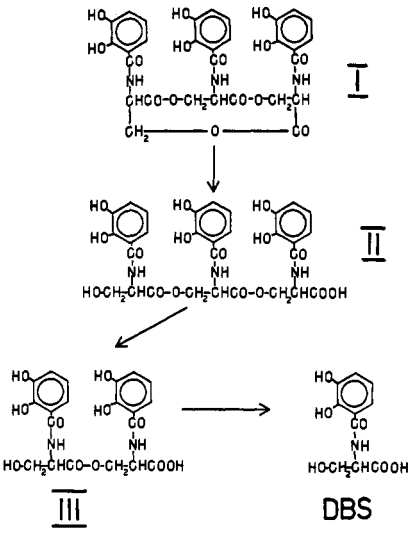



FIGURE 1: DBS-containing compounds formed in cell-free supernatants of iron-deficient cultures of *E. coli*. (I) *cyclo*-Tris(2,3-dihydroxy-*N*-benzoyl-L-seryl) or *cyclo*-$(DBS)_3$, (II) *N,N',N''*-tris(2,3,-dihydroxy-*N*-benzoyl)-*O*-seryl-*O*-serylserine, (III) *N,N'*-bis(2,3-dihydroxy-*N*-benzoyl)-*O*-serylserine, and DBS, 2,3-dihydroxy-*N*-benzoyl-L-serine. The arrows indicate the sequence of reactions catalyzed by a hydrolase found in *E. coli* when grown in iron-free medium.

L-serine, and ATP and that DBS was not a free intermediate in this synthesis. In addition, it was found that crude cell-free extracts were capable of converting the cyclic trimer, through intermediates, to DBS and that this hydrolytic activity was repressed if the cells were grown in the presence of iron. This unique feature of being repressed by iron suggested that this enzyme was specifically involved in the metabolism of *cyclo*-$(DBS)_3$.

The present paper describes the further resolution of the crude system into four distinct fractions, three of which are required for *cyclo*-$(DBS)_3$ synthesis, and the fourth for its hydrolysis, eventually to DBS. We also report the isolation of two covalently bound enzyme–substrate complexes, one containing L-serine and the other containing DBS, both being bound to the enzyme through thio ester linkage.

A preliminary account of some of this work has previously been reported (Bryce and Brot, 1971b).

## Materials and Methods

*Organism. E. coli* $K_{12}$, strain 2276, a methionine cyano $B_{12}$ auxotroph, was used as a source of the enzymes. Cultures were grown with forced aeration in 5-gal. carboys in the medium described previously (Brot *et al.*, 1966).

*Materials.* Uniformly labeled L-[$^{14}$C]serine and generally labeled L-[$^{3}$H]serine were purchased from New England Nuclear Corp. Dihydroxybenzoic acid was purchased from K & K Laboratories. DBS was synthesized by Dr. H. Gurien of the Chemical Research Department of Hoffmann-La Roche using a procedure utilizing dicyclohexylcarbodiimide. It was crystallized from benzene and had a melting point of 135–137°. Tritium was incorporated into both dihydroxybenzoic acid and DBS, by catalytic exchange, by the New England Nuclear Corp. They were purified prior to use by thin-layer chromatography on MN300 cellulose and developed with 5% ammonium formate–0.5% formic acid.

*cyclo*-$(DBS)_3$ was isolated from the cell-free growth medium of an iron-deficient culture of *E. coli* and purified by

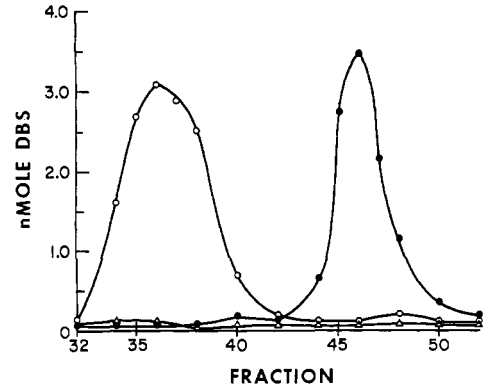



FIGURE 2: Sephadex G-100 chromatography of a 0–50% ammonium sulfate fraction. 23 ml at 65.5 mg of protein/ml was applied to a 85 × 5 cm column equilibrated with 0.01 M Tris-chloride (pH 8.0) containing 5 mM dithiothreitol. Fractions (20 ml) were collected at a flow rate of 10 ml/hr. (△) Activities of fractions assayed alone; (○) activities of 0.05-ml aliquots assayed with 0.05 ml of fraction 46; (●) activities of 0.05-ml aliquots assayed with 0.05 ml of fraction 36. Conditions of the assay are described in the text.

the method described by Pollack and Neilands (1970). Elemental analysis and melting point determination were in excellent agreement with the published data. A 0.05 M stock solution of the trimer was prepared in dimethyl sulfoxide and the concentration was checked by dilution in ethyl acetate and measurement of absorbance at 316 nm using an extinction coefficient of 9390 $M^{-1}$ $cm^{-1}$ (O'Brien and Gibson, 1970b).

The ferric complex of the trimer was prepared by mixing equal volumes of 0.05 M *cyclo*-$(DBS)_3$ in dimethyl sulfoxide and 0.05 M $FeCl_3$ in dimethyl sulfoxide. [$^{14}$C]-*cyclo*-$(DBS)_3$ was prepared using purified preparations of the enzymes involved in its synthesis. The incubations were carried out as previously described and after extracting the cyclic trimer into ethyl acetate it was further purified by thin-layer chromatography on MN300 cellulose developed with 5% ammonium formate–0.5% formic acid. The purified compound was eluted from the plate with 15% acetic acid and then extracted into ethyl acetate.

Protein was determined by the method of Lowry *et al.* (1951).

*Enzyme Purification.* Cells (60 g) were washed twice with 0.01 M Tris-chloride (pH 8.0) and then suspended in three volumes of 0.1 M Tris-chloride (pH 8.0) containing 5 mM dithiothreitol. The cells were then disrupted by sonication and centrifuged at 18,000*g* for 10 min. The supernatant was then further centrifuged at 40,000 rpm in a 60 Ti Spinco rotor for 1 hr to yield a clear supernatant fraction. Ammonium sulfate was added to 50% saturation and the precipitate was dissolved in 0.01 M Tris-chloride (pH 8.0) containing 5 mM dithiothreitol and dialyzed overnight against the same buffer.

*Sephadex G-100 Chromatography.* The above solution (23 ml; 65.5 mg of protein/ml) was then applied to an 85 × 5 cm column of Sephadex G-100 equilibrated with the above buffer. Fractions (20 ml) were collected at a flow rate of 10 ml/hr and assayed for their ability to synthesize *cyclo*-$(DBS)_3$. Two complementary peaks of activity were found and designated $E_1$ and $E_2$ (see legend to Figure 2). They were then pooled and concentrated by ultrafiltration (PM-10 membranes, Diaflo, Amicon Corp.).

*DEAE-Sephadex Chromatography.* Each of the above pooled fractions was dialyzed against 0.01 M Tris-chloride (pH 8.0) containing 5 mM dithiothreitol and 0.2 M NaCl and

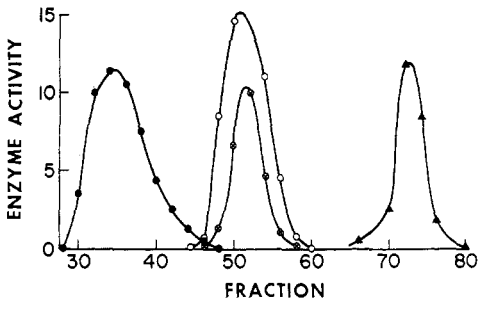



FIGURE 3: DEAE-Sephadex chromatography of the $E_2$ fraction. 8.7 ml at 34 mg of protein/ml of the pooled fractions from the Sephadex G-100 columns were applied to a 40 × 2.5 cm column equilibrated with 0.01 M Tris-chloride (pH 8.0) containing 5 mM dithiothreitol and 0.2 M NaCl. Elution was carried out with a linear salt gradient (see Methods section); 10-ml fractions were collected at a flow rate of about 20 ml/hr. (○) Activities of fractions assayed with 0.05 ml of fraction 72 and 20 μg of $E_1$; (▲) activities of fractions assayed with 0.05 ml of fraction 52 and 20 μg of $E_1$; (⊗) ATP–pyrophosphate-exchange activity dependent upon dihydroxybenzoic acid; (●)—*cyclo*-$(DBS)_3$ hydrolase activity. For assay conditions, see Methods section.

applied to columns of DEAE-Sephadex (40 × 2.5 cm) equilibrated with the same buffer. The columns were washed with this buffer until no more uv-absorbing material was eluted and then a linear gradient was applied consisting of 500 ml of 0.01 M Tris-chloride (pH 8.0), 5 mM dithiothreitol, and 0.2 M NaCl in the mixing chamber and 500 ml of 0.01 M Tris-chloride (pH 7.0), 5 mM dithiothreitol, and 0.6 M NaCl in the reservoir. Fractions (10 ml) were collected at a flow rate of about 20 ml/hr. Active fractions (see legend to Figure 3) were pooled and concentrated as described above.

*Enzyme Assays—Synthesis of DBS-Containing Compounds.* The incubation mixture employed to measure the synthesis of DBS-containing compounds (referred to as the overall reaction) contained, in a total volume of 0.2 ml, enzyme, 10 μmoles of Tris-chloride (pH 7.4), 100 nmoles of dihydroxybenzoic acid, 100 nmoles of L-[$^{14}$C]serine (about 2000 cpm/nmole), 1 μmole of ATP, 2 μmoles of $MgCl_2$, and 2 μmoles of dithiothreitol. The reaction mixture was incubated for 15 min at 37° and the reaction was stopped by the addition of 0.8 ml of 0.05 M HCl. DBS-containing compounds were extracted with 3 ml of ethyl acetate and 2 ml was removed and assayed for radioactivity in a Beckman LS-100 scintillation counter in a scintillation fluid described by Bray (1960).

*ATP–[$^{32}$P]$P_i$ Exchange.* ATP–pyrophosphate exchange was assayed using the following incubation mixture in a final volume of 0.1 ml: enzyme, 0.2 μmole of ATP, 1 μmole of $MgCl_2$, 10 μmoles of Tris-chloride (pH 8.0), 0.5 μmole of dithiothreitol, 0.2 μmole of sodium [$^{32}$P]pyrophosphate (about 300 cpm/nmole), and 0.1 μmole of either dihydroxybenzoic acid or L-serine, depending upon which reaction was being studied. The reactions were incubated at 37° for 15 min and terminated by the addition of 0.4 ml of 0.5 M perchloric acid. Sodium pyrophosphate (0.4 ml of 0.1 M) was added and [$^{32}$P]ATP was adsorbed onto charcoal by adding 0.2 ml of 4% Norit. The samples were mixed thoroughly, centrifuged, and the Norit precipitate was washed four times with 4 ml of water and finally suspended in 1 ml of water and added to 10 ml of Bray's solution.

The purified enzyme preparations were tested for hydrolysis of pyrophosphate and ATP and both were found to be free of these activities, thus eliminating the possibility of an ATP–orthophosphate exchange.

*Hydrolysis of cyclo-(DBS)$_3$.* The enzymatic breakdown of *cyclo*-$(DBS)_3$ was assayed by two methods. For the assay of column fractions, each incubation contained, in a volume of 0.2 ml: enzyme, 20 nmoles of [$^{14}$C]-*cyclo*-$(DBS)_3$ (specific activity 500 cpm/nmole), and 10 μmoles of Tris-chloride (pH 8.0). After 15 min at 37°, 0.8 ml of 0.01 M Tris-chloride (pH 7.4) was added, the tubes were shaken with 3 ml of ethyl acetate, and 2 ml of the organic layer was counted in 10 ml of Bray's solution. At this pH the cyclic trimer is neutral and can be extracted into ethyl acetate, while the negatively charged products will remain in the aqueous phase. Thus the difference in the amount of radioactivity found at zero time and at the end of the incubation reflects the amount of the trimer which has been hydrolyzed. Under these conditions nonenzymatic hydrolysis was negligible.

Qualitative detection of *cyclo*-$(DBS)_3$ hydrolysis was carried out by extracting the acidified reaction mixtures into ethyl acetate and applying this extract to MN300 cellulose thin-layer plates developed with 5% ammonium formate–0.5% formic acid. The various DBS-containing compounds were located by uv fluorescence and compared to known standards.

*Formation of Trichloroacetic Acid Insoluble Enzyme-Bound Intermediates.* Each 0.05-ml incubation contained: enzyme ($E_1$), 2.5 μmoles of triethanolamine buffer (pH 7.5), 0.25 μmole of dithiothreitol, 0.25 μmole of KCl, 0.5 μmole of $MgCl_2$, 0.5 μmole of ATP, and 0.5 nmole of L-[$^3$H]serine (specific activity 1440 cpm/pmole). After incubation at 37° the reactions were stopped by the addition of 4 ml of cold 10% trichloroacetic acid. After 20 min at 0°, the solutions were filtered through nitrocellulose filters (HAWP, 0.45 μ, Millipore Corp.) and washed with four 2-ml portions of cold 5% trichloroacetic acid. The bound L-[$^3$H]serine was assayed by counting the filters in 10 ml of Bray's solution.

To form dihydroxybenzoic acid containing complexes, the above incubation mixture was used with the addition of $E_2$ and 1.25 nmoles of dihydroxybenzoic acid. In some experiments (see text) incubations contained 0.5 nmole of unlabeled L-serine and 1.25 nmoles of [$^3$H]dihydroxybenzoic acid (specific activity 5760 cpm/pmole). In addition, in this type of experiment, 2.5 μmoles of unlabeled DBS and *cyclo*-$(DBS)_3$ was added to the reaction mixture in order to dilute out any radioactive products (these were present as a result of a low level of catalytic turnover) which might bind nonspecifically to trichloroacetic acid insoluble protein. Separate experiments indicated that, at the level of turnover observed, the unlabeled products represented a 2000-fold excess.

In both sets of experiments, nonspecific binding to protein and filters by L-[$^3$H]serine and [$^3$H]dihydroxybenzoic acid was corrected for by inclusion of controls to which no ATP was added.

When the L-[$^3$H]serine binding assay was used to determine specific activities of $E_1$ in crude extracts, the enzyme solution was first incubated with 200 μg/ml of pancreatic RNase (Worthington) for 15 min at 37° to digest endogenous tRNA.

*Characterization of Enzyme-Bound Species.* $E_1$·SERINE. This complex was prepared, as described above, by trichloroacetic acid precipitation in a conical centrifuge tube and washed with 4-ml volumes of 5% trichloroacetic acid until negligible radioactivity was detected in the supernatant. The precipitate was dissolved in 0.2 ml of 0.2 M bicarbonate buffer (pH 10) and the mixture was placed into boiling water for about 30 min. After cooling and acidification, 5 μl of 0.05 M unlabeled L-serine was added and the solution was passed through a 4 × 0.5 cm column of Dowex 50 equilibrated with 0.05 M HCl. The

column was washed with 5 ml of water and the serine was eluted with 3 ml of 3 M $NH_4OH$. This solution was then evaporated to dryness and chromatographed on a MN300 cellulose thin-layer plate developed with pyridine–methanol–water (70:15:15, v/v). Radioactivity was located by counting directly portions of the plate in 10 ml of Bray's solution.

E·DBS. To isolate enzyme-bound DBS the procedure was modified slightly. The trichloroacetic acid insoluble precipitate, after washing, was dissolved in 0.2 ml of 0.2 M bicarbonate buffer (pH 10) containing 5 mM borax. The latter was necessary to protect the compound from oxidation by formation of a complex with the 1,2-diol function. Two microliters of 0.05 M unlabeled DBS was added and after incubation at 60° for 30 min the solution was acidified and extracted into ethyl acetate. Chromatography of the concentrated ethyl acetate extract was performed on MN300 cellulose thin-layer plates developed with 5% ammonium formate–0.5% formic acid.

## Results

*Fractionation of the Enzymes.* Figure 2 illustrates the separation, by Sephadex G-100 chromatography, of a 0–50% ammonium sulfate fraction into two complementary fractions, each inactive alone, which are required for the synthesis of DBS-containing compounds from L-serine, dihydroxybenzoic acid, and ATP. These peaks of activity are labeled $E_1$ and $E_2$ corresponding to their order of emergence from the column. Both fractions catalyzed ATP–pyrophosphate-exchange reactions dependent upon L-serine in the case of $E_1$ and dependent upon dihydroxybenzoic acid in the case of $E_2$; however, it is difficult to quantitate how much of this L-serine-dependent exchange was due to L-serine tRNA synthetase. In addition, $E_1$ was assayed by the formation of a RNase-resistant trichloroacetic acid insoluble complex when incubated with L-[$^3$H]-serine as outlined in the Methods section. When this procedure was carried out the enzyme was pretreated with pancreatic RNase, to eliminate any insoluble radioactivity which could be present as a result of contaminating L-serine tRNA synthetase. When the $E_1$ fraction from the Sephadex G-100 column was further purified by chromatography on DEAE-Sephadex as described in the Methods section, no further resolution was noted and the activity emerged as a single peak at a position corresponding to 0.34 M NaCl on the salt gradient. This step increased the specific activity of $E_1$ by about sevenfold.

In contrast to $E_1$ when $E_2$ was chromatographed on DEAE-Sephadex it was found that no single fraction was active by itself in complementing $E_1$ in the synthesis of DBS-containing compounds. This is shown in Figure 3. However, upon combining various fractions, two peaks of activity were found which were required for the synthesis of *cyclo*-$(DBS)_3$ in addition to $E_1$. One of these peaks was identified as $E_2$ by virtue of its coincidence with an ATP–pyrophosphate-exchange activity dependent upon dihydroxybenzoic acid. The other peak is designated $E_3$ and was assayed by its complementation of $E_1$ and $E_2$ in the overall synthesis of *cyclo*-$(DBS)_3$ from L-serine, dihydroxybenzoic acid, and ATP.

The positions of $E_2$ and $E_3$ relative to the salt gradient were 0.34 and 0.42 M NaCl, respectively.

Also shown in Figure 3 is the peak of activity responsible for the hydrolysis of *cyclo*-$(DBS)_3$. This fraction is designated $E_4$.

A summary of the purification data is presented in Table I. Two points should be emphasized here: (a) when the overall reaction is used to measure specific activities, the values obtained are subject to some uncertainty since a multienzyme system is involved. In Table I the values quoted are, therefore, those values which represent the maximum specific activities obtained over a wide range of enzyme concentrations. After resolution on Sephadex G-100, $E_1$ and $E_2$ were assayed against a saturating level of the other enzyme fraction. Thus it is difficult to compare specific activities between two preparations, one in which the enzymes are separate and the other in which they are mixed in a constant proportion. (b) A possible solution to this problem would be to assay the individual enzymes using an assay for the partial reactions catalyzed by these enzymes. Serine covalently bound to $E_1$ is difficult to measure in crude systems due to possible tRNA contamination remaining even after RNase treatment; moreover, at the levels of protein required, the solutions are difficult to filter and wash adequately. The $E_2$-catalyzed dihydroxybenzoic acid dependent ATP–pyrophosphate-exchange activity in crude systems is not very much different from the nondihydroxybenzoic acid dependent control reaction and hence subject to uncertainty.

Thus the values obtained for the purification of $E_1$ and $E_2$ shown in Table I should be regarded with caution, since the

TABLE I: Purification of Two of the Enzymes Responsible for *cyclo*-$(DBS)_3$ Synthesis.

| Step | Specific Activity: Overall Reaction[a] | Specific Activity: Individual Reaction[b] | Units[c] | Purification: Overall Reaction | Purification: Individual Reaction |
|---|---|---|---|---|---|
| | | | $E_1$ | | |
| Sonicate | 14 | 0.01 | 50,500 | 1 | 1 |
| 0–50% Ammonium sulfate | 56 | 0.06 | 84,500 | 4 | 6 |
| Sephadex G-100 | 91 | | 39,800 | 6.5 | |
| DEAE-Sephadex | 668 | 0.84 | 22,500 | 48 | 84 |
| | | | $E_2$ | | |
| Sonicate | 14 | 0.06 | 50,500 | 1 | 1 |
| 0–50% Ammonium sulfate | 56 | 0.30 | 84,500 | 4 | 5 |
| Sephadex G-100 | 76 | 1.67 | 22,500 | 5.4 | 28 |
| DEAE-Sephadex | 338 | 3.42 | 21,000 | 24 | 57 |

[a] Synthesis of DBS-containing compounds from dihydroxybenzoic acid, L-serine, and ATP. Specific activity is defined as nmoles of DBS-containing compounds synthesized per mg of protein in 15 min at 37°. [b] $E_1$ was assayed by L-[$^3$H]-serine binding as described in the Methods section. Specific activity is defined as pmoles of L-[$^3$H]serine bound per μg of protein in 15 min at 37°. $E_2$ was assayed by the dihydroxybenzoic acid dependent ATP–[$^{32}$P]$P_i$-exchange assay as described in the Methods section. Specific activity is defined as nmoles of [$^{32}$P]ATP synthesized per μg of protein in 15 min at 37°. [c] Units are defined as specific activity × total mg of protein. Specific activities are those determined from the assay of the overall reaction. After the Sephadex G-100 step, overall synthesis was measured as a function of enzyme concentration in the presence of an excess of the other enzyme or enzymes involved.

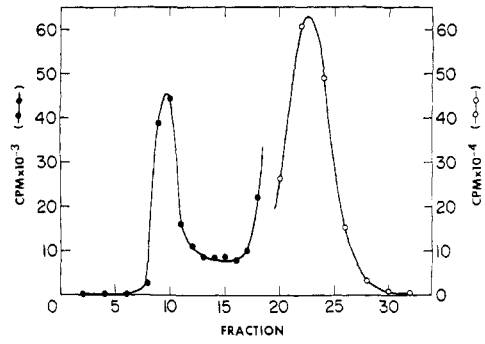



FIGURE 4: Chromatographic isolation of $E_1$·serine complex. A 30 × 0.3 cm Sephadex G-50 column was equilibrated with 0.02 M triethanolamine buffer (pH 7.5) containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2 mM EDTA. $E_1$ (0.1 mg) and L-[$^3$H]serine (2 nmoles; 1440 cpm/pmole) were incubated in a final volume of 0.1 ml with the other reaction mixture components as described in the Methods section. After 15 min at 37°, the sample was applied to the column in the cold and 0.12-ml fractions collected. Aliquots of each fraction were withdrawn and counted in 10 ml of Bray's solution.

data depend entirely upon the results determined for the crude extract.

It can be seen in Table II that in the complete system no cyclic trimer is detected at the end of the incubation, although DBS-containing compounds are synthesized. These compounds have been characterized as being mostly monomeric DBS and small amounts of the dimer and trimer of DBS. On the other hand when $E_4$ is omitted from the incubation it is only the cyclic trimer which accumulates in the reaction mixture. This and other evidence suggest that $E_1$, $E_2$, and $E_3$ are responsible for the synthesis of the cyclic trimer while $E_4$ catalyzes its hydrolysis to DBS *via* various intermediates. Table II also shows that there is virtually an absolute requirement for ATP, dihydroxybenzoic acid, and $Mg^{2+}$ and that the reaction is stimulated about sixfold by the presence of 5 mM dithiothreitol.

*Properties of $E_1$.* When $E_1$ was incubated with ATP, $MgCl_2$, and radioactive L-serine, a complex was formed which could be precipitated from 10% trichloroacetic acid. The time course of complex formation showed a rapid reaction which was essentially complete after 10 min. The enzyme was saturated at a L-[$^3$H]serine concentration of 25 μM and the $K_m$ for the binding was approximately 1 μM. Magnesium ion was required and maximum complex formation occurred at 10 mM $Mg^{2+}$. In addition, it was found that the requirement for ATP was absolute and that the amount of L-[$^3$H]serine bound was proportional to the amount of $E_1$ present in the incubation.

Complex formation was unaffected by pretreatment of the enzyme with pancreatic ribonuclease so that the trichloroacetic acid insoluble radioactivity was not due to a contamination with tRNA and small amounts of L-serine tRNA synthetase.

The complex could be isolated by gel filtration on Sephadex G-50. Figure 4 shows the elution profile. When the peak fractions were pooled the total radioactivity emerging with the protein was found to be about 85% of the total trichloroacetic acid insoluble radioactivity contained in the sample before application to the column. However, only 25% of the radioactivity in the eluate was trichloroacetic acid insoluble and over an interval of several hours at 0° this value decreased to about 10% indicating a slow breakdown of the complex.

Under the conditions described in the text the complex forms relatively rapidly and is stable for at least 1 hr at 37°.

TABLE II: Dependencies for the Enzymatic Synthesis of *cyclo*-(DBS)$_3$ and DBS-Containing Compounds.[a]

| Omissions | nmoles of DBS-Containing Compounds | nmoles of *cyclo*-(DBS)$_3$ |
|---|---|---|
| None | 7.13 | <0.1 |
| DBA | 0.00 | <0.1 |
| ATP | 0.00 | <0.1 |
| $Mg^{2+}$ | 0.41 | <0.1 |
| Dithiothreitol | 1.18 | <0.1 |
| $E_1$ | 0.00 | <0.1 |
| $E_2$ | 0.04 | <0.1 |
| $E_3$ | 0.07 | <0.1 |
| $E_4$ | 7.13 | 6.77 |

[a] Each incubation contained, where indicated, 17.5 μg of $E_1$, 92.5 μg of $E_2$, 30.6 μg of $E_3$, and 63.0 μg of $E_4$, and the other components of the incubation mixture as described in the text. To obtain total DBS-containing compounds the reaction mixtures were acidified prior to extraction with 3 ml of ethyl acetate. Aliquots (2 ml) of the organic layer were counted in 10 ml of Bray's solution. Chromatographic analysis of the products by the procedure outlined in the Methods section indicated that, when $E_4$ was omitted, greater than 95% of the radioactivity was associated with *cyclo*-(DBS)$_3$.

However, when a 2000-fold excess of unlabeled serine was added to such a reaction mixture, trichloroacetic acid insoluble radioactivity decreased rapidly to about zero with a half-time of about 1.5 min. This behavior is indicative of a rapid exchange of free and bound serine by spontaneous breakdown and subsequent resynthesis of the complex and provides an explanation of the instability of the complex to gel filtration in which removal of reactants (ATP and L-[$^3$H]serine) leads to dissociation of the complex.

*Nature of the Bond between $E_1$ and L-Serine.* The finding that serine bound to protein could be precipitated by trichloroacetic acid suggested that a covalent bond had been formed between $E_1$ and serine. Thus it was of importance to ascertain the nature of this bond.

The complex was prepared as described in the text, precipitated with 10% trichloroacetic acid and washed several times with cold 5% trichloroacetic acid. The precipitate was redissolved in 0.1 M Bistris buffer (pH 6.5) and aliquots were withdrawn for incubation, under a variety of conditions, at 37°. It was found that the complex was stable for at least 20 min at neutral pH but that 80% of the trichloroacetic acid insoluble radioactivity was lost when incubated at pH 10. A similar instability of the complex was observed when incubated in a neutral solution of 1% mercuric acetate. Under the same conditions, 3 M hydroxylamine at pH 6.5 completely destroyed the complex.

These data are consistent with a thio ester mode of linkage between $E_1$ and L-serine. As further confirmation of this it was observed that preincubation of the enzyme with *N*-ethylmaleimide completely abolished complex formation. Moreover, treatment of the $E_1$·serine complex, even in the presence of all the reactants, with *N*-ethylmaleimide led to a rapid loss of trichloroacetic acid insoluble radioactivity indicating that the complex was constantly being destroyed and resynthe-

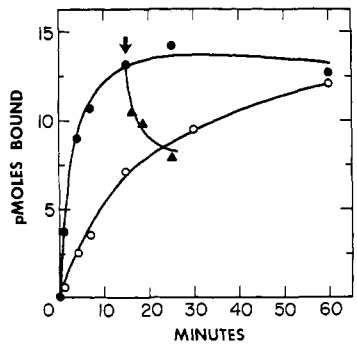



FIGURE 5: Kinetics of formation of a trichloroacetic acid insoluble complex upon incubating $E_1$, $E_2$, L-serine, and dihydroxybenzoic acid. Conditions are described in the Methods section. $E_1$ (10.5 $\mu$g) and $E_2$ (92 $\mu$g) were used. (●) L-[$^3$H]Serine and unlabeled dihydroxybenzoic acid as substrates; (○) [$^3$H]dihydroxybenzoic acid and unlabeled L-serine as substrates. At $t$ = 15 min, indicated by the arrow, a 500-fold excess of unlabeled L-serine was added and residual trichloroacetic acid insoluble radioactivity (▲) was determined.

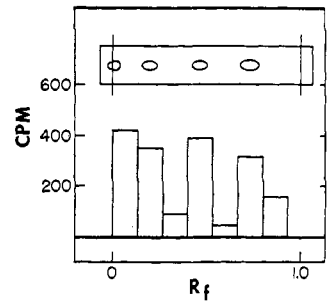



FIGURE 6: Thin-layer chromatography of protein-bound radioactivity liberated by alkaline treatment. Details of the incubation are described in the text. The upper portion shows the uv fluorescence of the markers which were obtained by partial hydrolysis of *cyclo*-(DBS)$_3$ by $E_4$. In order of increasing $R_F$, they are *cyclo*-(DBS)$_3$, linear trimer of DBS, linear dimer of DBS and DBS. The lower portion of the figure indicates the radioactivity which was scraped off the various areas of the plate and counted directly in 10 ml of Bray's solution after correction for background.

sized. This is further evidence of the labile nature of the bond in the complex. Other experiments have shown that when the radioactivity was released from the complex by alkali treatment and chromatographed only free serine was found.

*Properties of $E_2$.* As observed previously, $E_2$ catalyzed an ATP–pyrophosphate exchange dependent upon dihydroxybenzoic acid. The $K_m$ value was calculated to be 16 $\mu$M, while the 2,4 and 2,5 isomers of dihydroxybenzoic acid had $K_m$ values of 250 and 480 $\mu$M, respectively. Relative $V_{max}$ values were 100, 17.5, and 8 for the 2,3, 2,4, and 2,5 isomers, respectively. Other derivatives tested were 2,6-, 3,4-, and 3,5-dihydroxybenzoic acid and 2,3,4-trihydroxybenzoic acid; none were substrates in the ATP–pyrophosphate-exchange assay and only 2,3-dihydroxybenzoic acid was a substrate in the overall synthesis of *cyclo*-(DBS)$_3$. It was found that *N*-ethylmaleimide completely inhibited the dihydroxybenzoic acid dependent ATP–pyrophosphate exchange indicating that a thiol group was involved in this reaction; however, no evidence of a trichloroacetic acid insoluble complex analogous to $E_1$·serine was observed. Thus these data indicate that the sole function of $E_2$ may be to form a dihydroxybenzoic acid–adenylate complex.

*Formation of an E·DBS Complex.* When $E_1$ and $E_2$ were incubated together with unlabeled serine and [$^3$H]dihydroxybenzoic acid (in addition to ATP, $Mg^{2+}$, and dithiothreitol) it was found that a radioactive trichloroacetic acid insoluble material was formed, which was dependent upon the presence of all of the above components. However, the kinetics of this reaction were different depending upon whether the radioactive label was in the L-serine or the dihydroxybenzoic acid (Figure 5). Although the rates were faster with labeled L-serine, the total amount of complex formed at the end of one hour was the same as when labeled dihydroxybenzoic acid was used. This suggested the formation of a complex containing 1:1 stoichiometric amounts of L-serine and dihydroxybenzoic acid.

That the difference in the rates of radioactive complex formation was due to the rapid synthesis of an $E_1$·serine complex, to which was added an activated dihydroxybenzoic acid moiety, is illustrated in Figure 5. When a 500-fold excess of unlabeled L-serine was added after 15 minutes to an incubation a rapid loss of a portion of the trichloroacetic acid insoluble radioactivity was observed. The residual radioactive L-serine bound after the dilution was complete was approximately equal to the amount of [$^3$H]dihydroxybenzoic acid bound at the corresponding time in the parallel experiment.

*Nature of the E·DBS Bond.* The complex was prepared using [$^3$H]dihydroxybenzoic acid as labeled substrate and subjected to the same treatments as described previously for the $E_1$·serine complex. Very similar results were obtained and the data are consistent with a thio ester mode of linkage between enzyme and DBS.

When the radioactivity, liberated by alkaline treatment, was chromatographed according to the procedures described in the text, a pattern of radioactivity was obtained as shown in Figure 6. Areas of the plate to be assayed for radioactivity were chosen so as to coincide with markers corresponding to DBS, the dimer of DBS, the linear trimer of DBS and *cyclo*-(DBS)$_3$ (see Figure 1). The figure shows that there is radioactivity associated with DBS, however, there was also appreciable radioactivity found in areas corresponding to the dimer and linear trimer of DBS as well as substantial amounts of radioactivity remaining at the origin. When control experiments were run in which [$^3$H]DBS was subjected to the same alkaline treatments as used to hydrolyze the complex, the chromatogram contained only one fluorescent spot identified as DBS but 10% of the radioactivity remained at the origin. It appears that the treatment used in these experiments caused the conversion of DBS to material which remains at the origin.

The question now arises as to the identity of the enzyme in the DBS-containing complex. On the basis of the above data, it has been assumed that the dihydroxybenzoic acid moiety, previously activated by the formation of an $E_2$·dihydroxybenzoic acid·adenylate intermediate, is transferred to the $\alpha$-amino group of the L-serine residue bound to $E_1$ through thio ester linkage; this linkage is presumed to remain intact and the site is now occupied by a DBS moiety.

To verify this presumption, the following experiments were performed; the rate and extent of [$^3$H]dihydroxybenzoic acid binding, in the presence of L-serine, $E_1$ and $E_2$, was measured at two different levels of $E_2$ in conditions analogous to those employed in Figure 5. These data are shown in Figure 7 in which it is seen that only the rate, and not the extent, of enzyme-bound DBS was influenced by $E_2$. When a threefold increase in $E_2$ concentration was used, the increase in rate was only about 80% which indicated that the system was near

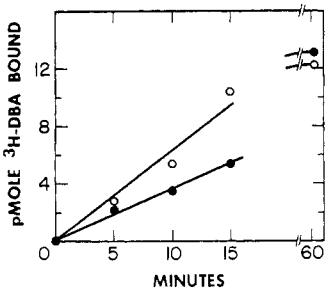



FIGURE 7: The kinetics of formation of an $E_1$·DBS complex at two different concentrations of $E_2$·[$^3$H]dihydroxybenzoic acid was the labeled substrate and 10.5 $\mu$g of $E_1$ was used in each assay. (●) 92 $\mu$g of $E_2$ and (○) 276 $\mu$g of $E_2$. Other conditions are described in the text.

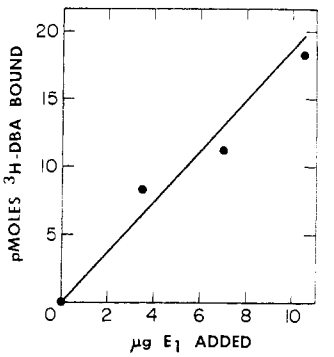



FIGURE 8: The effect of $E_1$ concentration on the extent of formation of an $E_1$·DBS complex. Each incubation contained 92 $\mu$g of $E_2$ and was carried out for 60 min using [$^3$H]dihydroxybenzoic acid as the labeled substrate. Other conditions are described in the text.

saturation with respect to the latter enzyme. Thus, $E_2$ appears to be acting catalytically in the system.

Figure 8 shows that $E_1$ is involved in the stoichiometric binding of DBS. Here the extent of complex formation was proportional to the amount of $E_1$ present, which was carried out in the presence of an excess of $E_2$ to ensure that the slower reaction catalyzed by $E_2$ was not rate limiting.

## Discussion

Previous studies (Brot *et al.*, 1966; Brot and Goodwin, 1968; Bryce and Brot, 1971a) have shown that both the synthesis of DBS-containing compounds and the enzymes required for their synthesis are repressed when *E. coli* is grown in medium containing 5 $\mu$M iron. It has been proposed that the biological function of *cyclo*-(DBS)$_3$ is to chelate extracellular iron and transport it into the cell under special conditions (Pollack *et al.*, 1970; Cox *et al.*, 1970; Bryce *et al.*, 1971; Young *et al.*, 1971; Wang and Newton, 1971; O'Brien *et al.*, 1971). This suggestion correlates well with the fact that this compound only appears under conditions of iron depletion and when other means of transporting iron into the cell are required. The biosynthesis of the cyclic trimer represents a system in which an enzyme-bound amide bond is formed (between L-serine and dihydroxybenzoic acid) by two ATP-dependent reactions. This monomer of the final product is finally cyclized to a trimer by ester formation between the carboxyl group of one serine and the hydroxyl group of a second serine residue. Thus, it appears to have the components of a polymer synthesis system, namely, initiation, elongation, and termination (cyclization).

This system of peptide-bond formation, as well as other characteristics, resembles very closely the peptide antibiotic biosynthesis typified by gramicidin S (Gevers *et al.*, 1968, 1969) and tyrocidin (Roskoski *et al.*, 1970a,b). Additional similarities to the latter two systems can be extended by the observed involvement of thio ester bound intermediates, two of which were identified as $E_1$·serine and $E_1$·DBS. Thus, the first can be considered the initiation complex, and the latter, as well $E_1$·(DBS)$_{2-3}$, is visualized as the consequence of an elongation-type process, since it appears that the serine residue remains bonded to $E_1$ during this process. Because of the lability of the complexes it has not been possible to isolate either $E_1$·serine or $E_1$·DBS in sufficient quantities and free from reactants to utilize them as substrates for the subsequent reactions. This is seen in the spontaneous breakdown of the $E_1$·serine complex during Sephadex G-50 chromatography described in Figure 4 and almost identical behavior was found for the $E_1$·DBS complex. Despite this lack of stability, the evidence in favor of a thio ester bond is strongly suggested by the nature of the conditions which lead to the hydrolysis of the complex. Thus, it appears that these thio ester complexes are less stable than those in gramicidin S biosynthesis (Gevers *et al.*, 1968) and tyrocidin biosynthesis (Gevers *et al.*, 1969). However, it is to be noted that the proposed glutamyl thio ester bound intermediate of CTP synthetase (Levitski and Koshland, 1971) was also labile to gel filtration, and the thiol group responsible could be reacted with DTNB and the affinity label DON even in the presence of excess glutamine.

The above experiments demonstrate the roles of both proteins, $E_1$ and $E_2$, in the synthesis of *cyclo*-(DBS)$_3$. However, the function of $E_3$ is more enigmatic.

By analogy, with the gramicidin S and tyrocidin systems, it is envisioned that $E_3$ catalyzes the condensation of $E_1$-bound DBS residues by a series of transesterifications which leads to the formation of a linear dimer and linear trimer of DBS. The final condensation would effect a cyclization with the consequent liberation of the product, *cyclo*-(DBS)$_3$. The chromatogram in Figure 6 showed evidence for these compounds in addition to DBS. The system does have a small contamination of $E_3$ (see below) and it is possible that these compounds were derived from thio ester bound enzyme intermediates, analogous to DBS, by the process outlined above. It is not known whether this elongation process occurs on $E_1$ or whether transthiolations occur mediated by a pantetheine thiol group located on $E_3$, the analog of which has been elegantly demonstrated by Kleinkauf *et al.* (1971) in the case of gramicidin S and tyrocidin biosynthesis. It has been observed that $E_3$ is inhibited by *N*-ethylmaleimide suggesting the involvement of an essential thiol group. Investigations of the final steps in *cyclo*-(DBS)$_3$ biosynthesis are in progress.

The resolution of the enzyme system for *cyclo*-(DBS)$_3$ synthesis into three components has already been reported from this laboratory (Bryce and Brot, 1971b) and indirect evidence has recently been obtained by Luke and Gibson (1971) for three gene products which convert dihydroxybenzoic acid to *cyclo*-(DBS)$_3$.

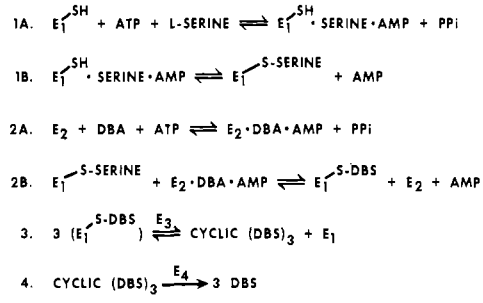
Table II demonstrates that these enzymes have been obtained essentially free of each other. However, in systems dealing with the stoichiometric binding of substrates the possibility of low levels of catalytic turnover must be carefully evaluated. In particular, it must be noted that one of the enzyme-bound intermediates proposed is a monomer of the final product. Therefore, identification of this complex by measure-

ments of the ratio of serine to dihydroxybenzoic acid depends crucially upon elimination of traces of product which may bind nonspecifically to protein in the preparation and which may not be removed by washing with trichloroacetic acid.

Under experimental conditions employed in these studies we have found low levels of catalytic turnover due to traces of $E_3$ in either $E_1$ or $E_2$. This has been quantitated, and we have eliminated any contribution of product by the following criteria. (a) We have measured the binding of [$^3$H]DBS in the presence of the reaction components to the relevant amounts of $E_1$ and $E_2$ employed in the experiments described and found it to be much less than the amount of trichloroacetic acid insoluble complex generated. (b) The latter nonspecific binding was further reduced by addition of a 2000-fold excess of unlabeled DBS and *cyclo*-$(DBS)_3$ over the amount calculated to be produced by catalytic turnover. Thus, the amount of nonspecific binding was reduced to less than 0.06 pmole of DBS.

We propose the following sequence of reactions leading to *cyclo*-$(DBS)_3$ from dihydroxybenzoic acid, L-serine, and ATP (Scheme I).

SCHEME I: Proposed Scheme for the Synthesis and Breakdown of *cyclo*-$(DBS)_3$.




In a previous communication (Bryce *et al.*, 1971) it was suggested that the role of $E_4$ might be hydrolyze the ferric *cyclo*-$(DBS)_3$ complex in order to release the iron and make it available to the cell.

However, we have been unable to detect any hydrolysis by $E_4$ of the ferric complex. When substrate was added as the ferric complex no hydrolysis was observed. Moreover, when a slight excess of ferric ion was added to a hydrolytic reaction in progress the hydrolysis promptly ceased. Finally, when excess free *cyclo*-$(DBS)_3$ was added to an incubation containing the ferric complex hydrolytic products were observed in amounts approximately equal to the ferric complex initially present. Dimethyl sulfoxide (2.5%; originating from the substrate solutions) was present in all the incubations described above so that the failure to observe hydrolysis of the ferric complex was probably not due to inhibition of the enzyme by the solvent.

These results are in marked contrast to the studies of O'Brien *et al.* (1971) in which the ferric complex was used as substrate. The reasons for this discrepancy are not clear at the moment but slight differences in the methods of preparation of the substrate may provide an explanation.

Acknowledgments

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