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# STUDIES ON THE ENZYMATIC SYNTHESIS OF ENTEROCHELIN IN *ESCHERICHIA COLI* K-12, *SALMONELLA TYPHIMURIUM* AND *KLEBSIELLA PNEUMONIAE*

## PHYSICAL ASSOCIATION OF ENTEROCHELIN SYNTHETASE COMPONENTS IN VITRO

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## Summary

Further evidence is presented in support of the proposal made previously (Greenwood, K.T. and Luke, R.K.J. (1976) Biochim. Biophys. Acta 454, 285—297) that components of the *Escherichia coli* enterochelin synthetase system physically associate to form enzyme complexes. Evidence for the existence of three enzyme complexes, designated in order of increasing stability G-D < F-D < F-D-G, has been obtained following gel filtration and chromatography on DEAE-Sephadex. Persistence of the F-D and G-D complexes during chromatography appears to depend on the flow rate of the column.

On the basis of complementation with appropriate *ent* mutants of *E. coli*, activities corresponding to those of the D, E, F and G components of enterochelin synthetase in *E. coli* have been detected in cell-extracts of both *Salmonella typhimurium* and *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) strains. These are designated D′, E′, F′ and G′ activities. Components E′ and G′ are eluted from Sephadex G-100 in similar fashion to their *E. coli* counterparts. Peaks of F′ and D′ activities however, are eluted together at a position corresponding to that of the *E. coli* F component. We suggest that in *S. typhimurium* and *K. pneumoniae*, either a single polypeptide combines the functions of the *E. coli* F and D components, or that separate F′ and D′ components form a stable complex and that activity of uncomplexed D′ component was not detected under the conditions used during chromatography and assay.

## Introduction

Enterochelin, a cyclic trimer of 2,3-dihydroxybenzoylserine, is an essential component of a high-affinity system for transporting iron in *Escherichia coli* [1]. It is also synthesized by *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) [2] and by *Salmonella typhimurium* [3] and has also been called enterobactin [3]. Under conditions of iron deficiency, cells synthesize and excrete increased quantities of enterochelin into the growth medium. The molecule strongly binds iron, and the ferric-enterochelin complex is transported into the cytoplasm where the ester bonds of the ligand are enzymatically hydrolysed to release the mineral nutrient for cellular metabolism [4].

In *E. coli*, the synthesis of enterochelin from 2,3-dihydroxybenzoic acid and L-serine is catalysed by enterochelin synthetase which consists of at least four components, designated D, E, F and G [5]. All four components are necessary for the synthesis of enterochelin [6] and intermediates obtained from partial reactions appear to be protein bound [7]. When a cell-extract containing active forms of all four components is fractionated by means of either gel filtration or ion-exchange chromatography, the D component is eluted as a series of peaks rather than as a single, discrete peak [5]. While one peak of D activity is normally separated from peaks of F and G activities, other peaks of D activity occur coincident with peaks of F or G activity, or in the region of their overlap. It has been proposed that this unusual chromatographic behaviour results from physical association of the D component with the F and/or G components during chromatography [5].

In the present paper, further evidence is presented in support of this proposition. We also compare properties of the enterochelin synthetases in *Salmonella typhimurium* and *Klebsiella pneumoniae* with that of *Escherichia coli* K-12.

## Materials and Methods

*Chemicals.* Whenever possible, chemicals of analytical reagent quality were used. Ethyl acetate was redistilled before use. 2,3-Dihydroxybenzoic acid was obtained from the Aldrich Chemical Company, and L-[U-$^{14}$C]serine from the Radiochemical Centre, Amersham. Iron (III)-enterochelin was prepared as described previously [8] and stored at −10 °C.

*Bacterial strains.* The (Ent$^+$) *E. coli* K-12 strain AN194 [9] is able to convert 2,3-dihydroxybenzoic acid to enterochelin normally and was used as a source of cell-extract containing the complete complement of proteins necessary for synthesis of enterochelin (Ent$^+$ cell-extract). The (Ent$^-$) mutant *E. coli* K-12 strains AN42 (*entD*), AN90 (*entD*), AN41 (*entE*), AN49 (*entF*) and AN462 (*entG*) are not able to convert 2,3-dihydroxybenzoic acid to enterochelin [10,11], and were used as sources of cell-extract lacking particular enterochelin synthetase components (EntD$^-$, EntE$^-$, EntF$^-$ and EntG$^-$ cell-extracts). The *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) strain 62-1 has been described previously [12]. The *S. typhimurium* LT2 strain DG926 (*gal*-1102, *met*E47) was obtained from D.G. MacPhee. All strains were maintained on nutrient agar slopes containing 30 mM glucose; slopes used for storing Ent$^-$

strains also contained 10 mM sodium citrate. All strains were stored at 4 °C.

*Culture media, growth of cells and preparation of purified cell-extract.* The mineral salts mixture used in all media was the medium 56 described by Monod et al. [13], except that no ferrous sulphate was added. 30 mM glucose was used as carbon source. 1 μM thiamine-HCl was included in the minimal medium for the *E. coli* strains; additional supplements were as described previously [5]. For growth of *S. typhimurium* strain DG926, 0.2 mM methionine and 0.1 mM 2,2′-dipyridyl were included, while for *K. pneumoniae* strain 62-1, 0.2 mM L-tryptophan, 0.2 mM L-tyrosine, 0.2 mM L-phenylalanine and 0.1 mM 2,2′-dipyridyl were included in the medium. Under these conditions, synthesis of proteins concerned with the conversion of 2,3-dihydroxybenzoic acid to enterochelin was derepressed. Synthesis of these enzymes was repressed in strain AN194 when 2,2′-dipyridyl was replaced by 20 mM sodium citrate and 50 μM ferrous sulphate. Growth conditions and the procedure for preparing purified cell-extracts were as described previously [5], except that solutions of protamine sulphate used during preliminary purification of cell-extracts derived from *K. pneumoniae* and *S. typhimurium* cells were 0.5 and 0.2%, respectively.

*Gel chromatography.* Samples were applied to Pharmacia columns (2 $cm^2$ × 90 cm or 20 $cm^2$ × 80 cm) packed with either Sephadex G-75 or Sephadex G-100, each of which, unless otherwise specified, was equilibrated with 50 mM Tris-HCl (pH 8.0), containing 5 mM dithiothreitol. Samples were eluted at 4 °C with the same buffer.

*DEAE-Sephadex chromatography.* Samples were dialysed against the starting buffer, 10 mM Tris-HCl (pH 8.0), containing 5 mM dithiothreitol and 0.25 M NaCl, and applied to a 4 $cm^2$ × 30 cm column packed with DEAE-Sephadex A-50 equilibrated with the same buffer. A linear gradient of 0.25—0.5 M NaCl in 1 l 10 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol was applied without preliminary washing with initial buffer. 10 ml fractions; flow rate, 20 ml/h.

*Assay for enterochelin synthetase and its component activities.* Assay conditions for enterochelin synthetase and its component activities have been described previously [5].

*Estimation of protein.* Protein was estimated by the method of Lowry et al. [14] with bovine serum albumin, fraction V, as standard.

*Concentration and storage of enzyme fractions.* When necessary, solutions of protein were concentrated in Diaflo ultrafiltration cells at 4 °C using PM10 membranes. Concentrated enzyme fractions were stored at −10 °C.

## Results

### *Effect of flow rate on the chromatography of D component*

When purified cell-extracts prepared from $Ent^+$ *E. coli* strains were fractionated by gel filtration, one to four regions of D activity could be discerned; the number of regions was found to depend on the rate at which sample moved through the column (flux). The results of slow passage (flux = 0.5 cm/h) through a column of Sephadex G-100 are shown in Fig. 1a where four areas of D activity can be distinguished. The largest (latest eluting) peak is separated from the other three. We propose that this separated peak of D activity reflects the presence of D component which has not been carried forward as a

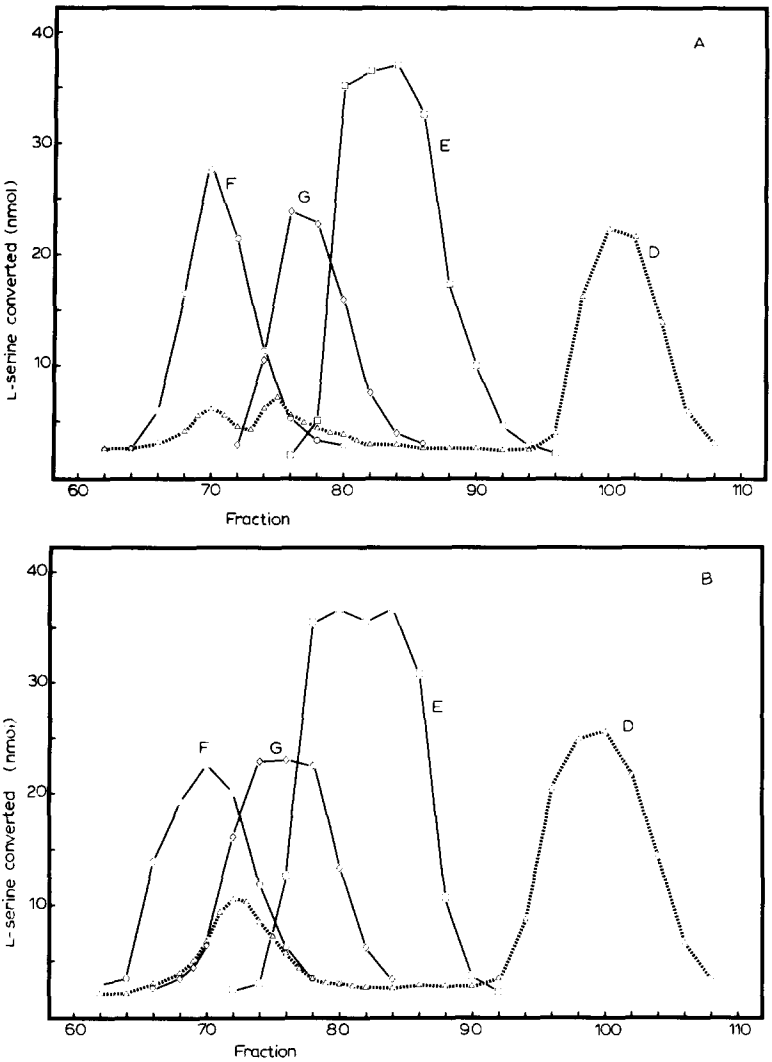


Fig. 1. Chromatography on Sephadex G-100 of two identical samples of purified $Ent^+$ cell-extract prepared from strain AN194. Samples (25 ml) containing 15 mg protein/ml were applied to a 20 $cm^2$ × 80 cm column of Sephadex G-100 equilibrated with 20 mM Tris-HCl (pH 8.0), containing 5 mM dithiothreitol. Fractions (10 ml) were collected at a flow rate of either 10 ml/h (A) or 40 ml/h (B). Samples (100 μl) of fractions were assayed for enterochelin synthetase activity in the presence of 30 μl of cell-extract prepared from an $EntD^-$ (△· · · · ·△) $EntE^-$ (□————□), $EntF^-$ (○————○), or $EntG^-$ (◇————◇) strain.

constituent of a higher molecular weight complex; henceforth such material will be referred to as uncomplexed D component. Two other regions of D activity may be seen associated with peaks of either F or G activity, while the fourth is located in the region of overlap between these two activities. In an earlier report [5], similar results were interpreted as being an indication of physical association between the D component and the F and G components to form higher molecular weight F-D, G-D and F-D-G enzyme complexes.

When an identical sample was chromatographed more rapidly (flux = 2.0 cm/h) on the same column, only two regions of D activity were observed (Fig. 1b). One region (presumed to reflect the presence of uncomplexed D component) was separated from areas of E, F or G activity; the second, thought to reflect the presence of F-D-G complex, was found in the region of overlap between peaks of F and G activities. No D activity was found associated with individual peaks of either F or G activity. This result is consistent with the proposal that the F-D-G complex is more stable than either the F-D or the G-D complex.

*Chromatography of D component in the presence or absence of both the F and G components*

Partially purified D component was obtained from a purified Ent$^+$ cell-extract following rapid elution (flux = 5 cm/h) from DEAE-Sephadex (Fig. 2). A single peak of D activity was eluted at a position corresponding to 0.27 M NaCl on the salt gradient. The D component was separated from the F and G components but not from E component. Fractions containing D activity (20—27) were pooled and concentrated.

Samples of such partially purified D component were mixed separately with two different cell-extracts lacking active D component; the resulting mixtures were then passed rapidly through a column of Sephadex G-75 (flux = 5 cm/h). One sample contained a mixture of partially purified D component and purified cell-extract prepared from cells of strain AN194 grown in medium containing citrate and iron. The synthesis of enzymes concerned with enterochelin biosynthesis is repressed under such conditions, and when the extract alone was fractionated on a column of Sephadex G-75, negligible levels of D, E,

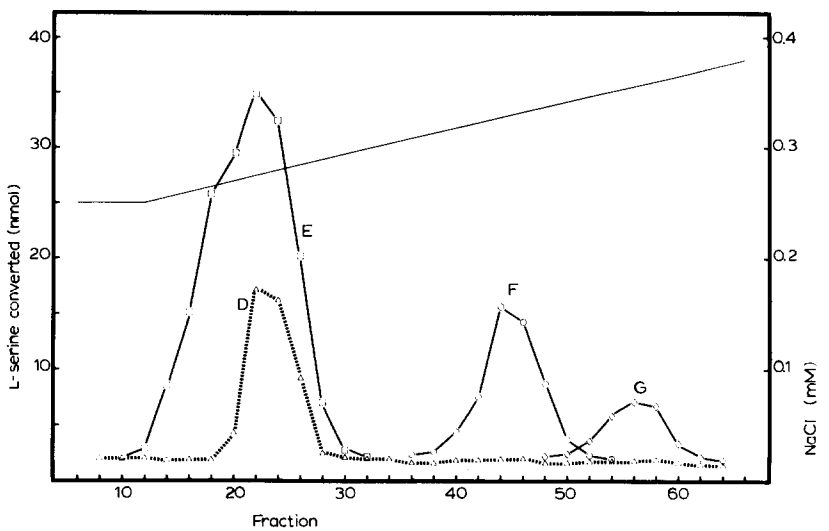


**Fig. 2. Chromatography on DEAE-Sephadex of purified Ent$^+$ cell-extract prepared from stain AN194. The sample (10 ml) contained 20 mg protein/ml. Samples (100 µl) of fractions were assayed for enterochelin synthetase activity in the presence of 30 µl of cell-extract prepared from an EntD$^-$ (△· · · · · △), EntE$^-$ (□———□), EntF$^-$ (○———○), or EntG$^-$ (◇———◇) strain.**

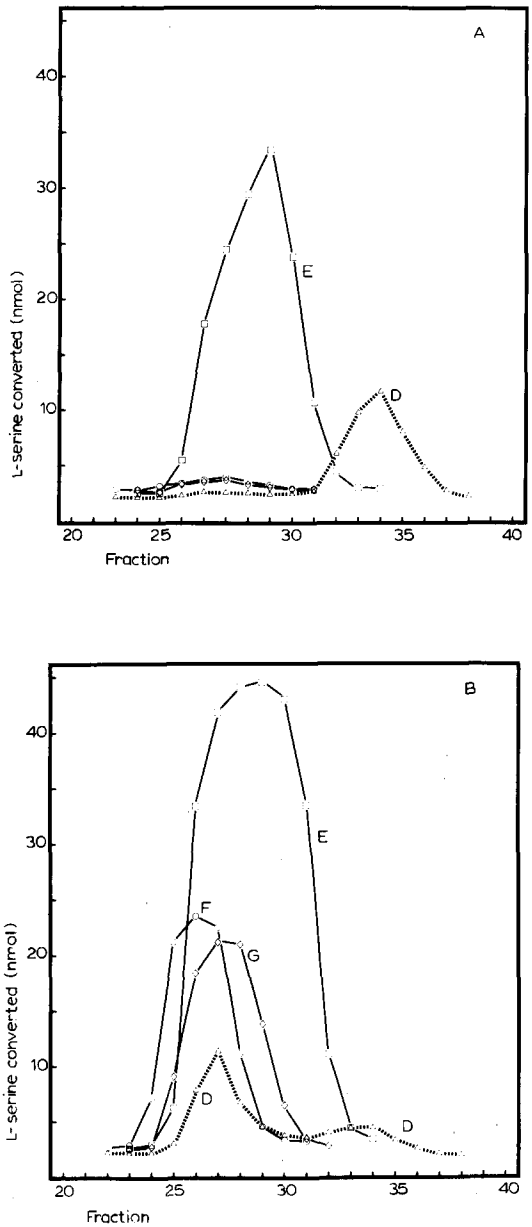


Fig. 3. Chromatography on Sephadex G-75 of partially purified D component, mixed with purified $Ent^+$ cell-extract prepared from cells of strain AN194 grown under repressing conditions (A), or with purified $EntD^-$ cell-extract prepared from strain AN90 (B). Samples (3 ml), containing a mixture of partially purified D component (2 mg protein) and cell-extract (30 mg protein), were applied to a 2 $cm^2$ × 90 cm column of Sephadex G-75 equilibrated with 50 mM Tris-HCl (pH 8.0), containing 5 mM dithiothreitol. Fractions (3 ml) were collected at a flow rate of 10 ml/h. Samples (100 µl) of fractions were assayed for enterochelin synthetase activity in the presence of 30 µl of cell-extracted prepared from an $EntD^-$ (△· · · · · △), $EntE^-$ (□———□), $EntF^-$ (○———○), or $EntG^-$ (◇———◇) strain.

F and G activities were detected (results not shown). Results obtained following chromatography of the mixture with D component are shown in Fig. 3a. Single peaks of E and D activities, but only negligible levels of F and G activities, were detected. We propose that this single peak of D activity represents D component, chromatography of which has not been affected by either the F or the G component. The elution volume of such uncomplexed D component presumably reflects the actual molecular weight of D component.

The second sample contained partially purified D component mixed with purified cell-extract prepared from the EntD$^-$ strain AN90. When the EntD$^-$ cell-extract was passed separately through a column of Sephadex G-75, peaks of F, G and E activities were observed, but negligible D activity was detected (results not shown). The results obtained when the mixture with D component was chromatographed are shown in Fig. 3b. Two peaks of D activity were detected. One peak was eluted at a position corresponding to that of the uncomplexed D component, whilst the other was eluted earlier (reflecting a higher molecular weight), in the area of overlap between the F and G activities. These results are consistent with the view that D component is incorporated into a relatively stable (F-D-G) enzyme complex only when both F and G components are present.

*Chromatography of D component in the absence of either F or G component*

When an Ent$^+$ cell-extract was passed rapidly (flux = 5 cm/h) through a column of Sephadex G-75, two distinct peaks of D activity were obtained (results not shown). One peak was presumed to reflect the presence of uncomplexed D component, and the other to reflect F-D-G complex. When either EntF$^-$ and EntG$^-$ cell-extract was fractionated similarly, only a single peak of D activity was observed; this was located at the position characteristic of the putative uncomplexed D component (Fig. 4a,b). This result is consistent with the proposal advanced above, that both F and G components are necessary for the formation of a stable (F-D-G) complex.

*Chromatography of Ent$^+$ cell-extracts prepared from K. pneumoniae and S. typhimurium*

Results obtained when purified cell-extracts prepared from *S. typhimurium* strain DG926 and *K. pneumoniae* strain 62-1 were passed rapidly (flux = 2.2 cm/h) through a column of Sephadex G-100 are shown in Fig. 5a and Fig. 5b, respectively. In each case, four peaks of activity were observed when fractions were tested to determine whether they were able to complement cell-extracts prepared from Ent$^-$ strains of *E. coli*. Each peak of activity presumably reflected the presence of an active *S. typhimurium* or *K. pneumoniae* polypeptide which was able to substitute for a component of enterochelin synthetase which was either defective in, or missing from, an *E. coli* cell-free extract. These results indicate that enterochelin synthetase components derived from *S. typhimurium* and *K. pneumoniae* are biochemically compatible with components of *E. coli* enterochelin synthetase.

When column fractions were tested for ability to complement cell-extract prepared from the *E. coli* EntE$^-$ and EntG$^-$ strains, peaks of activity (E′ and G′ activities respectively) occurred at positions similar to those at which the E and

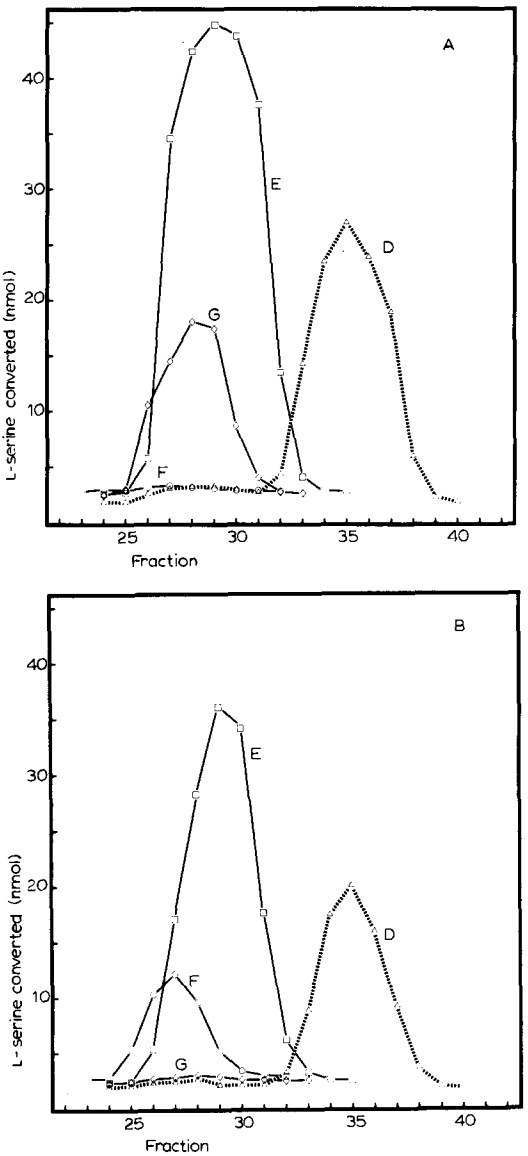


Fig. 4. Chromatography on Sephadex G-75 of purified EntF$^-$ cell-extract prepared from strain AN49 (A) and of EntG$^-$ cell-extract prepared from strain AN462 (B). Samples (3 ml) containing 10 mg protein/ml were applied to a 2 cm$^2$ X 90 cm column of Sephadex G-75 equilibrated with 50 mM Tris-HCl (pH 8.0), containing 5 mM dithiothreitol. Fractions (3 ml) were collected at a flow rate of 10 ml/h. Samples (100 µl) of fractions were assayed for enterochelin synthetase activity in the presence of 30 µl of cell-extract prepared from an EntD$^-$ (△ · · · · · △), EntE$^-$ (□———□), EntF$^-$ (○———○), or EntG$^-$ (◇———◇) strain.

G activities of *E. coli* are normally eluted. These results suggest that the E′ and G′ components of enterochelin synthetase components in *S. typhimurium* and *K. pneumoniae* have similar molecular weights to their *E. coli* counterparts.

When column fractions were tested for ability to complement cell-extract prepared from the *E. coli* EntD$^-$ and EntF$^-$ strains, peaks of activity (D′ and F′

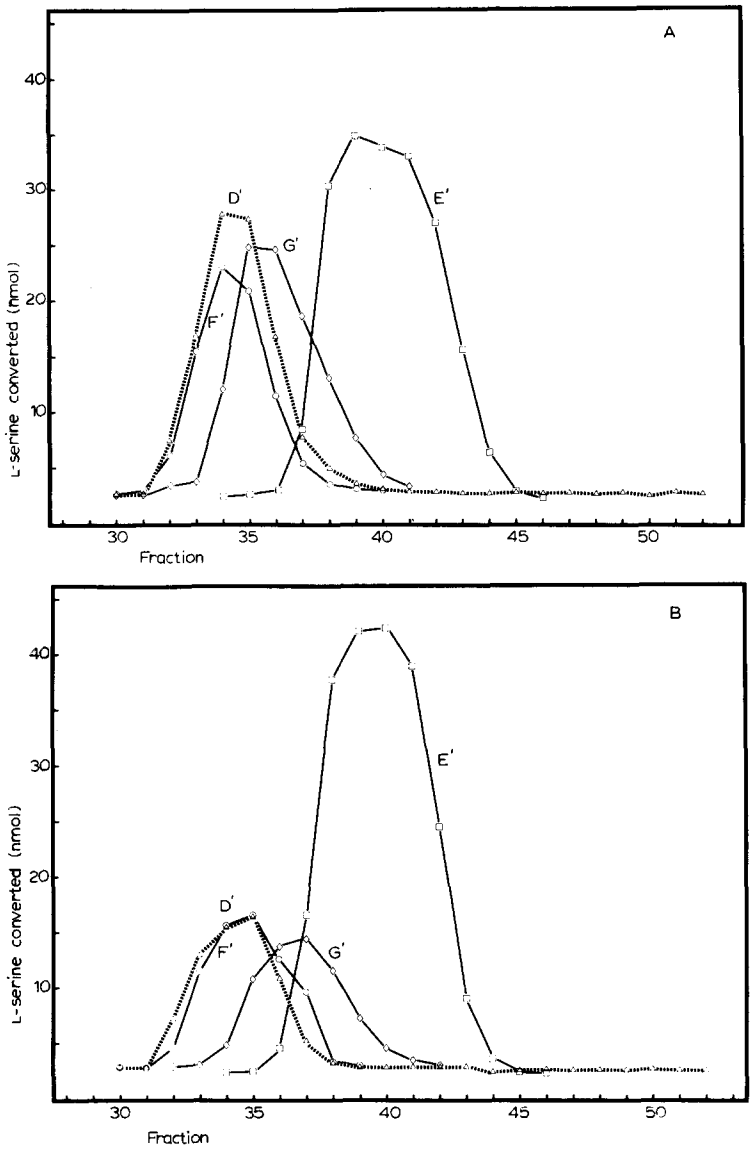


**Fig. 5. Chromatography on Sephadex G-100 of purified Ent$^+$ cell-extract prepared from *Salmonella typhimurium* strain DG926 (A) and purified Ent$^+$ cell-extract prepared from *Klebsiella pneumoniae* strain 62-1 (B). Samples (3 ml) containing 10 mg protein/ml were applied to a 2 cm$^2$ × 90 cm column of Sephadex G-100 equilibrated with 50 mM Tris-HCl (pH 8.0) containing 5 mM dithiothreitol. Fractions (2.5 ml) were collected at a flow rate of 4.4 ml/h. Samples (100 µl) of fractions were assayed for enterochelin synthetase activity in the presence of 30 µl of cell-extract prepared from an EntD$^-$ (△·····△), EntE$^-$ (□———□), EntF$^-$ (○———○), or EntG$^-$ (◇———◇) strain.**

activities respectively) were superimposed on each other (Fig. 5a and 5b). The superimposed peaks of D′ and F′ activities occurred at a position similar to that at which the F component of *E. coli* is normally eluted. These results obtained with *S. typhimurium* and *K. pneumoniae* extracts thus differ from those observed when cell-extracts prepared from *E. coli* are fractionated (see Fig. 1b).

In the latter case, two peaks of D activity are obtained; one of these is separated from the other three components while the other coincides with the region of overlap of the F and G components. One explanation of these results is that in both *S. typhimurium* and *K. pneumoniae*, a single polypeptide is present which combines the functions of the separate D and F components of *E. coli*. Alternatively, there may exist separate D′ and F′ components which are equivalent to their corresponding *E. coli* components in terms of molecular weights and biochemical activities, but which differ in the ways they interact with other components of enterochelin synthetase.

## Discussion

Certain aspects of the biosynthesis of enterochelin from 2,3-dihydroxybenzoic acid and L-serine by *E. coli* cells remain unclear. Neither the molecular nature of the enterochelin synthetase nor the reaction mechanism is completely understood. In a previous report [5], we showed that enterochelin synthetase consisted of at least four components (designated D, E, F and G). We also proposed, on the basis of unusual behaviour of the D component during gel filtration and chromatography on DEAE-Sephadex, that the synthesis of enterochelin is catalyzed by a multienzyme complex. The existence of three enzyme complexes, designated F-D, G-D and F-D-G, was proposed.

We have now shown that the unusual chromatographic behaviour of the D component occurs only when F and/or G components are present and that persistence of complexes during chromatography is influenced by flow rate. The F-D and G-D complexes are able to remain intact during slow chromatography (flux $< 0.5$ cm/h) but are broken up by faster flow rates (flux $> 2$ cm/h). We suggest that the F-D-G complex is more stable than either the F-D or the G-D complex since it remains intact during chromatography at the higher flow rates. This more stable complex is not formed if either the F or G component (or both) is absent.

Enterochelin synthetases of *S. typhimurium* and *K. pneumoniae* differ from that in *E. coli*. The E′ and G′ components of the former organisms appear to be functionally compatible with their *E. coli* counterparts; apparent molecular weights are also similar. However, the single peak of D′ activity observed following gel filtration of cell-extract derived from either *S. typhimurium* or *K. pneumoniae* has been found to coincide with the peak of F′ activity. These results may reflect either the presence of a single polypeptide which combines the functions of the separate F and D components present in *E. coli*; alternatively two separate components (D′ and F′) may form a stable complex. One difficulty with the second explanation concerns the conspicuous absence of D′ activity eluting late from the Sephadex column (corresponding to uncomplexed D component). It is possible that activity associated with late-eluting D′ component may not have been detected under the conditions used for chromatography and assay. In this regard it is of interest that preliminary experiments in our laboratory have shown that the labile D component of *E. coli* is stabilized by the presence of F component. It is therefore conceivable that the D′ components of *S. typhimurium* and *K. pneumoniae* are extremely labile except when complexed with their respective F′ components. Alternatively the D′

component may only be compatible with G and E components in the presence of F′ component.

It seems likely that the in vitro physical association of the F and G components of *E. coli* enterochelin synthetase with the D component may reflect a natural tendency for these components to associate in vivo. Biosynthesis of enterochelin may well be mediated by such an enzyme complex. As has been suggested previously [5], such organization may constitute part of an arrangement whereby biosynthesis and enzymic degradation of enterochelin are kept separate. Much remains to be clarified regarding the properties of such complexes. Biochemical characterization of mutants of *S. typhimurium* or *K. pneumoniae* may help in the gaining of such information, and may also lead to a better understanding of the evolutionary relationships between enteric organisms.

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