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DISCLAIMER

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SUMMARY

L-Isoleucyl t-RNA synthetase was purified to 97% purity in a single step by affinity chromatography on a column carrying L-isoleucinyl 5'-adenylate as the insolubilized ligand.

INTRODUCTION

The purification of aminoacyl t-RNA synthetases by conventional methods is often long and tedious and results in low yields of the purified enzyme. The great specificity these enzymes show for their respective substrates made them seem ideal candidates for purification by the technique of affinity chromatography.

Initially we chose to investigate the purification of L-isoleucyl t-RNA synthetase from E. coli B, a well characterized enzyme first purified to homogeneity by Baldwin and Berg.[1] As the insolubilized ligand, we chose to employ analogs of L-isoleucine and L-isoleucyl adenylate rather than $tRNA^{ile}$, since the latter is difficult to obtain in homogeneous form and is subject to degradation during use.[2]

Affinity columns bearing L-isoleucine esters or amides afforded little or no purification. Apparently, these ligands bound the enzyme too weakly.[3] We therefore turned to the powerful inhibitor, L-isoleucinyl 5'-adenylate ($K_i = 7 \times 10^{-9}$ M).[4] Inhibition studies with model compounds indicated that of the possible methods of attachment to agarose, linkage via a spacer on the 6-amino group of the adenine would least impair the compound's ability to bind the synthetase. Using columns with this inhibitor so attached, we have obtained 97% pure L-isoleucyl t-RNA synthetase without any additional procedures for protein fractionation.

MATERIALS AND METHODS

Preparation of the Adsorbent. Succinyl diaminodipropylamino agarose was prepared by activating Biogel A-15, 100-200 mesh (BioRad Laboratories) with cyanogen bromide, coupling to 3,3'-iminobispropylamine, and succinylating the resulting material with succinic anhydride, following the procedures of Cuatrecasas and Parikh.[5] The resulting beads carried about 12 μmoles of side chain per ml of gel, as determined by ninhydrin assay of hydrolysates.[6] Labeled N-t-BOC-L-isoleucinyl N^6-(2-aminoethyl)-5'-adenylate was prepared using N-t-BOC-[(U)-^{14}C]-L-isoleucinol following the procedure outlined in Figure 1. This was coupled to the succinyldiaminodipropyl agarose using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in 3:1 dioxane-water. Radioassay of the beads suspended in scintillation fluid indicated about 4 μmoles of inhibitor bound per ml of gel. The t-BOC group was removed by shaking with 1 N HCl in 1:1 ethylene glycol-water. After thorough washing, the gel retained the inhibitor at a level of 1 μmole per ml. Details of the procedure will be published elsewhere.

Crude E. coli B extract was prepared by blending E. coli B cell paste (Miles Laboratories) with glass beads, centrifuging, and autolysing according to Baldwin and Berg.[1] The autolysate was then dialyzed against several changes of 0.02 M potassium phosphate (pH 7.5) containing 0.02 M 2-mercaptoethanol.

Protein was determined by the method of Lowry et al.[6] Enzyme activity was measured by the ATP-[^{32}P]PPi exchange assay of Norris and Berg.[7] One unit of activity is defined as the formation of 1 μmole of [^{32}P]ATP from [^{32}P]PPi in 15 min at 37° under the given conditions.

Ultrafiltration was carried out in an ultrafiltration cell with Diaflo PM-10 membranes (Amicon Corp.). Operating pressure was 15-20 psi. Buffer exchange was carried out under the same conditions, displacing the old buffer through the membrane by continuous introduction into the cell of the new buffer.

SDS-acrylamide gel electrophoresis was carried out on 10% gels following the method of Laemmli[8] with minor modifications. Gels were fixed and stained with 0.2% Coomassie Brilliant Blue R-250 in 20% acetic acid and diffusion destained in 20% acetic acid.

RESULTS AND DISCUSSION

When crude E. coli extract was passed through a column of the adsorbent, only 10-25% of the [^{32}P]-pyrophosphate exchange activity was recovered in the effluent, while more than 95% of the total protein passed through unretarded. The percent of activity in the effluent was roughly constant for a given batch of extract and was independent of the ratio of the amount of extract to the amount of adsorbent. Presumably, this activity represents modified or partially denatured synthetase which does not bind strongly to the ligand or it represents some other enzyme capable of catalyzing the exchange. It does not

appear to be a saturation effect.

The retained activity could be recovered in varying yield by the usual techniques for desorption--pH shift, increased ionic strength, or elution with a soluble competitive ligand. However, the enzyme recovered by simple application of any of these techniques was invariably contaminated by several other proteins.

The following procedure was developed to circumvent this difficulty: Two columns were prepared. The affinity column contained the adsorbent diluted with two or more parts of unmodified agarose beads. Sufficient affinity adsorbent was used to provide at least fifty binding sites per molecule of enzyme to be applied. A precolumn contained a volume of succinyldiaminodipropylamino agarose equal to two-thirds the volume of the affinity column. These columns were linked in tandem with the non-specific one first. The crude E. coli extract was passed onto the columns at such a rate as to give 30 minutes of contact time with the affinity column. The columns were then washed with 0.02 M potassium phosphate containing 0.02 M 2-mercaptoethanol until the OD_{280} of the effluent ceased to decrease. The precolumn was then detached and washing of the affinity column only was continued. The pH of the irrigant buffer was raised to 9.2 via a gradient over 3-4 column volumes. Washing then proceeded at pH 9.2 until the OD_{280} of the effluent again ceased to decrease. Incomplete washing at this stage will result in less pure preparations. The pH of the irrigant buffer was returned to 7.5 and elution proceeded until the effluent returned to pH 7.5. The irrigant was then replaced by one which contained as well 0.02 M L-isoleucine. The flow rate was reduced to give a contact time of 1 hour or more. Twenty or more column volumes of effluent were collected.

A typical elution pattern is shown in Figure 2. In this instance, 22% of the applied synthetase activity passed through the column unretarded and 57% of the activity was recovered in purified form. The normal recovery of purified enzyme is 50-60%.

The purified enzyme is obtained in rather dilute form. This is the result of the low partition ratio of the synthetase between the L-isoleucine in solution and the more powerful insolubilized ligand. Reduction of the effective concentration of the gel-bound inhibitor by dilution of the adsorbent with unmodified agarose ameliorates this situation somewhat and has the added advantages of reducing non-specific adsorption of protein and improving flow properties. The use of more potent soluble ligands, such as free L-isoleucinyl 5'-adenylate, or L-isoleucyl adenylate generated in situ from L-isoleucine and ATP, is precluded, because their use results in elution of undesired (apparently AMP-specific) proteins from the column.

The fractions containing the purified synthetase have been pooled and concentrated by ultrafiltration with little or no loss of activity. After concentration, the elution buffer may be replaced by diafiltration with 20 mM potassium phosphate (pH 7.5) containing 2 mM dithiothreitol if this is desired.

The concentrated L-isoleucyl t-RNA synthetase preparations have had specific activities of 650-800 units per milligram of protein. The specific activity reported for enzyme purified by classical techniques is 650 units/mg protein.[1,9] However, SDS-acrylamide gel electrophoresis indicated the presence of another protein in our preparations (see Fig. 3). Quantitative densitometry revealed the latter to have an integrated absorbance of ca. 3% of that of the L-isoleucyl t-RNA synthetase. This ratio remained essentially constant when individual fractions were compared.

We have reused the adsorbent many times over a period of six months without any apparent loss in ability to retain the synthetase. During this period there was a very slow loss of insolubilized ligand which could be minimized by cold (3°C) storage of the gel at pH 6. Before reuse, both the precolumn and the affinity column were thoroughly washed with at least ten volumes each of 0.2 M potassium phosphate (pH 9.5), 4 M urea, and 0.2 M potassium dihydrogen

phosphate to remove any proteins which remained bound to the gels.

Others have reported affinity purifications of certain aminoacyl tRNA synthetases using either the cognate tRNA[2,10,11] or the amino acid amide as the insolubilized ligand.[12] Our procedure has the advantages that it does not require a partially purified enzyme preparation as the starting material, that a small amount of adsorbent can process a large volume of crude extract, and that the adsorbent can be reused many times.

In principle, the L-isoleucinyl 5'-adenylate agarose should be capable of purifying L-isoleucyl t-RNA synthetase from any source. Purification of other aminoacyl tRNA synthetases should be possible using appropriate analogous adsorbents. We are currently investigating these possibilities.

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Fig. 1. Synthesis of N-t-BOC-L-isoleucinol N^6-(2-aminoethyl)-5'-adenylate.

Fig. 2. Affinity chromatography of L-isoleucyl t-RNA synthetase. The chromatography was carried out on columns of succinyldiaminodipropylamine (1.27 cm x 7 cm) and of one part L-isoleucinol 5'-adenylyl agarose mixed with two parts of unmodified agarose (1.27 cm x 9 cm) following the procedure outlined in the text: A. Crude E. coli extract (12.3 units/ml of synthetase; 6.62 mg/ml of protein; 360 ml total volume) was passed through the tandem columns. B. The columns were washed with 0.02 M potassium phosphate (pH 7.5) containing 0.02 M 2-mercaptoethanol. C. The affinity column only was washed with a pH gradient from 7.5 to 9.2. D. Washing was continued at pH 9.2. E. The pH of the irrigant was returned to 7.5. F. Irrigant was switched to 0.02 M potassium phosphate (pH 7.5) containing 0.02 M L-isoleucine and 0.02 M 2-mercaptoethanol. Flow rate was 23.2 ml/hr for fractions 1-37 and 10.7 ml/hr for fractions 38-50. Fraction size was 23.2 ml for 1-37 and 21.4 ml for 38-50. Protein (•) and enzyme activity (o) were measured as described under Methods.

Fig. 3. SDS-acrylamide electrophoresis of purified L-isoleucyl tRNA synthetase. The main band has an apparent molecular weight of 106,000. The reported weight of the synthetase is 112,000.[1] The arrow indicates the location of a faint second band.

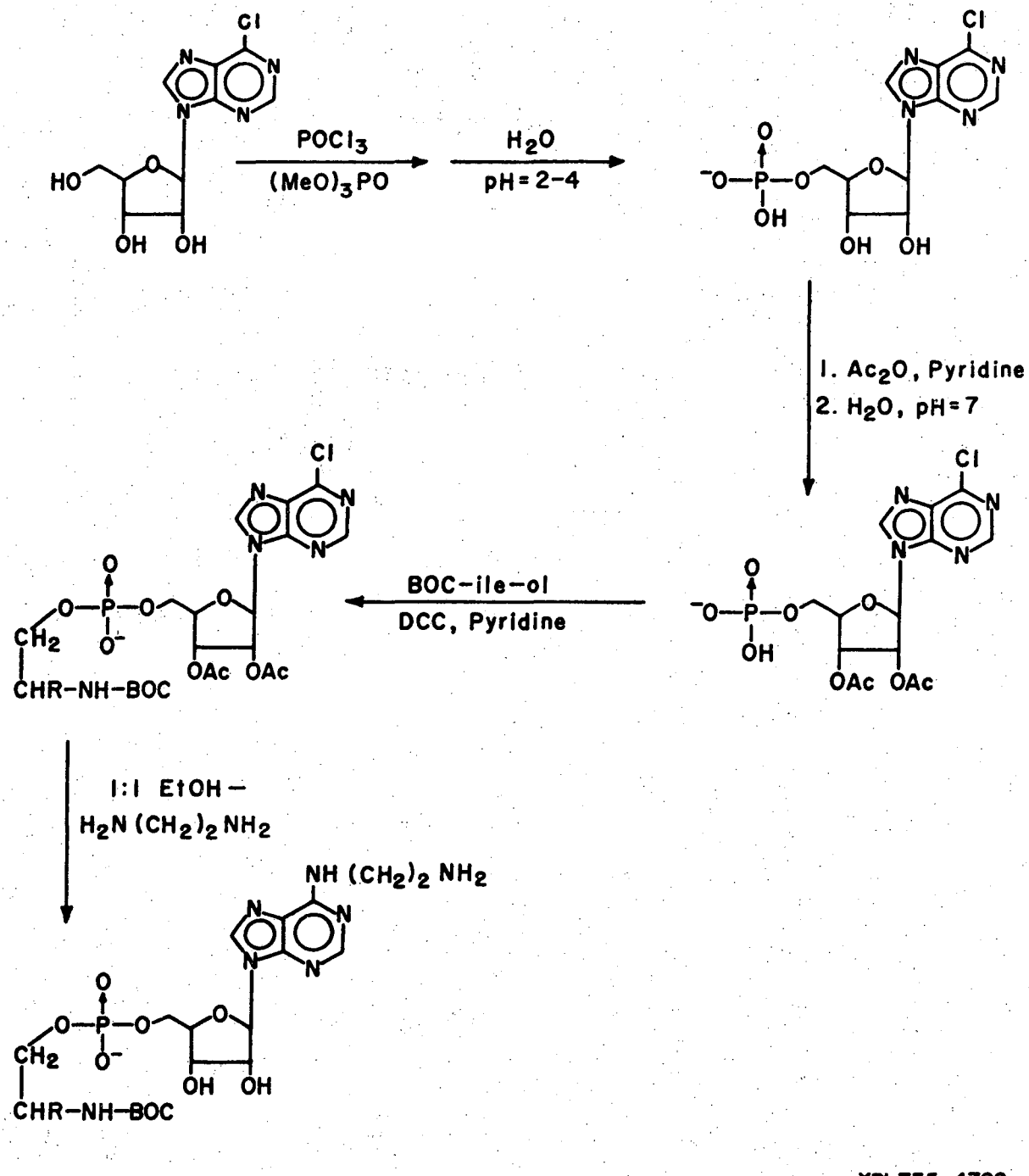
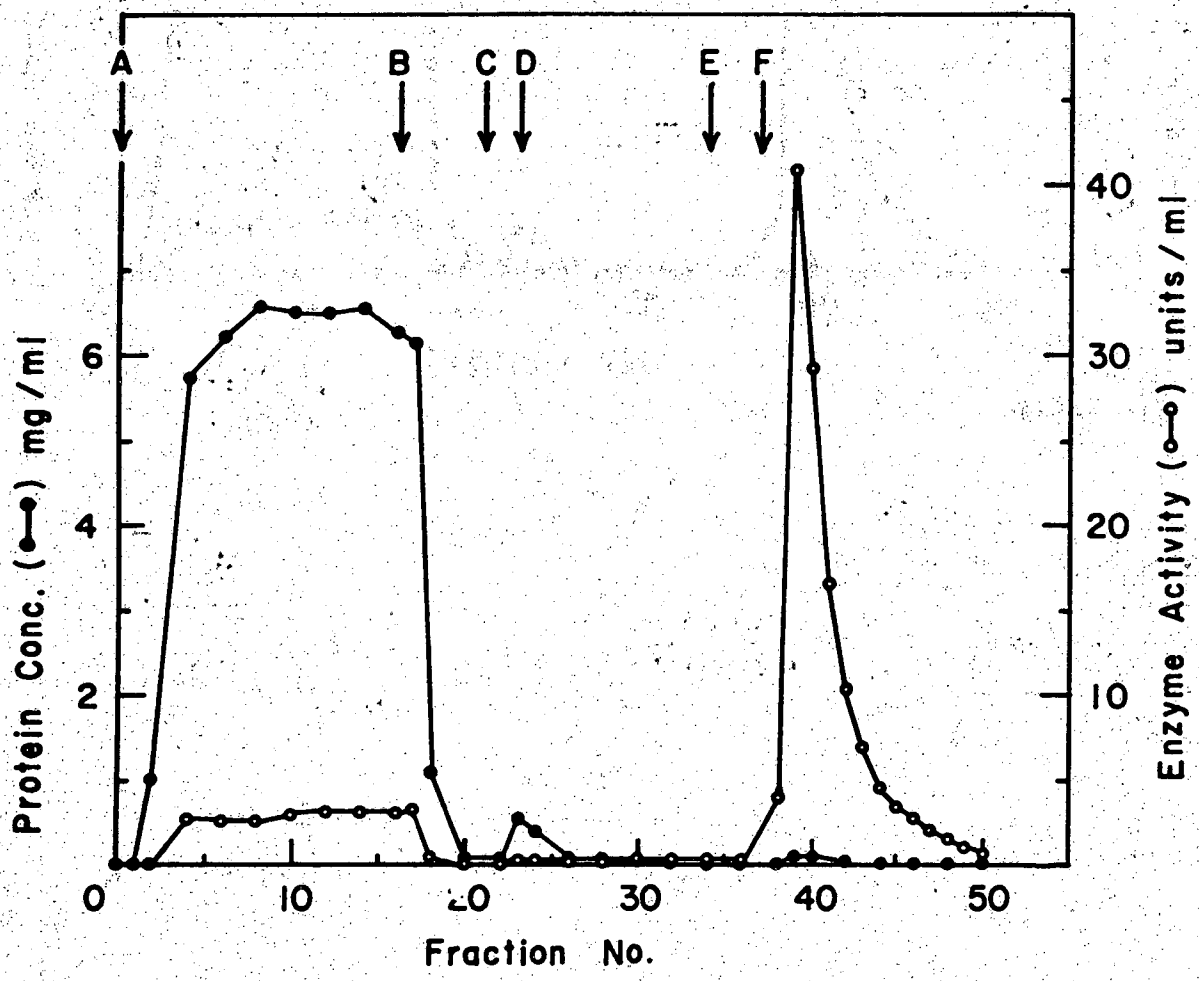

Cl
HO
OH OH
POCl3
(MeO)3PO
H2O
pH=2-4
O
-O—P—O
OH
1. Ac2O, Pyridine
2. H2O, pH=7
OAc OAc
BOC-ile-ol
DCC, Pyridine
CH2
CHR-NH-BOC
1:1 EtOH—
H2N(CH2)2NH2
NH(CH2)2NH2
XBL735-4792

Fig. 1.


A
B
C
D
E
F
Protein Conc. (●—●) mg/ml
Enzyme Activity (o—o) units/ml
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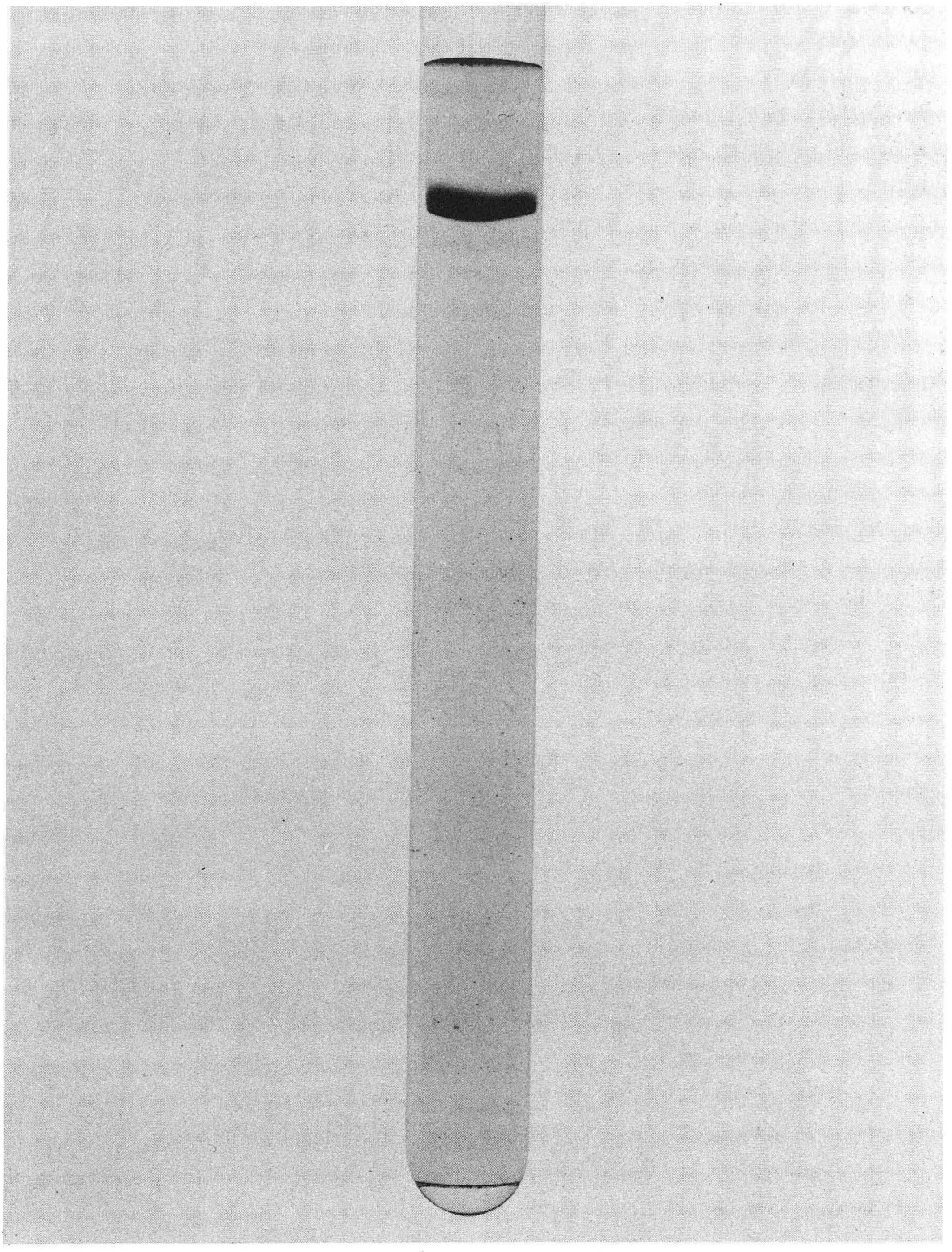
10
20
30
40
50
2
4
6
10
20
30
40
XBL743-5082

Fig. 2.



XBB 743-1732

Fig. 3.

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