References and Notes

1. J. W. Everett, in *Sex and Internal Secretion*, W. C. Young, Ed. (Williams & Wilkins, Baltimore, 1961), p. 497.
2. E. R. Smith, R. F. Weick, J. M. Davidson, *Endocrinology* **85**, 1129 (1969); A. P. Labhsetwar and J. G. Bainbridge, *J. Reprod. Fertil.* **27**, 445 (1971); F. Döcke, G. Dörner, K. H. Voight, *J. Endocrinol.* **41**, 353 (1968); J. L. Pasteels and F. Ectors, in *Basic Action of Sex Steroids on Target Organs*, P. O. Hubinont, F. Leroy, P. Galand, Eds. (Karger, Basel, 1971), p. 201.
3. H. G. Spies, K. R. Stevens, J. Hillard, C. H. Sawyer, *Endocrinology* **84**, 277 (1969).
4. P. V. Malven and R. Ruiz-Diaz, *J. Anim. Sci.* **32**, 919 (1971).
5. G. Dörner and F. Döcke, *Endocrinol. Exp.* **2**, 65 (1967).
6. S. Kanematsu and C. H. Sawyer, *Endocrinology* **76**, 691 (1965).
7. J. B. Powers, *Brain Res.* **48**, 311 (1972).
8. K. Seiki, M. Miyamoto, A. Yamashita, M. Kotami, *J. Endocrinol.* **43**, 129 (1969); K. Seiki and M. Hattori, *ibid.* **51**, 793 (1971); R. E. Whalen and W. G. Luttge, *Brain Res.* **33**, 147 (1971); G. N. Wade and H. H. Feder, *ibid.* **45**, 525 (1972); C. A. Iramain and C. A. Strott, in *Program* (55th annual meeting of the Endocrine Society, Chicago, 1973), abstr., p. 117.
9. E. Milgrom, M. Atger, E. E. Baulieu, *Steroids* **16**, 741 (1970).
10. E. Milgrom, M. Perrot, M. Atger, E. Baulieu, *Endocrinology* **90**, 1064 (1972).
11. P. D. Feil, S. R. Glasser, D. O. Toft, B. W. O'Malley, *ibid.* **91**, 738 (1972).
12. M. Sar and W. E. Stumpf, in *Program* (6th annual meeting of the Society for Study of Reproduction, Athens, Ga., 1973), abstr., p. 32; W. E. Stumpf and M. Sar, *J. Steroid Biochem.* **4**, 125 (1973).
13. W. E. Stumpf, *Endocrinology* **85**, 31 (1969); *Science* **162**, 1001 (1968); M. Sar and W. E. Stumpf, *Experientia* **28**, 1364 (1972); *Endocrinology* **92**, 251 (1973); W. E. Stumpf, *Z. Mikrosk. Anat. Zellforsch. (Leipz.)* **92**, 23 (1968); M. Sar and W. E. Stumpf, *Science* **179**, 389 (1973).
14. W. E. Stumpf, *Acta Endocrinal. Suppl.* **153**, 205 (1971).
15. In the quantitative evaluation of autoradiograms, the average number (mean ± standard error) of silver grains in nuclei and cytoplasm of neurons of the arcuate nucleus in estrogen-primed guinea pig is 35.60 ± 2.40 and 6.1 ± 0.4; in nonprimed guinea pig, 11.30 ± 0.99 and 3.4 ± 0.5; and in guinea pig previously treated with cortisol, 33.70 ± 3.20 and 5.6 ± 0.5, respectively. When the distribution of silver grains is calculated per unit (square micrometer) of the nuclear and cytoplasmic area of the cell with labeled nuclei, and assuming the cells are round, the following counts were obtained for estrogen-primed animals: nuclei, 1.26, cytoplasm, 0.21; for nonprimed animals: nuclei, 0.40, cytoplasm, 0.12; and for animals pretreated with cortisol: nuclei, 1.20, cytoplasm, 0.19. The estimation of mean value in the nuclear and cytoplasmic concentration of silver grains is based on the counting of silver grains in 50 randomly selected labeled neurons after correcting for background.
16. B. R. Rao, G. W. Wiest, W. M. Allen, *Endocrinology* **92**, 1229 (1973).
17. E. Terasawsa and C. H. Sawyer, *Exp. Neurol.* **27**, 359 (1970).

18. Supported by PHS grants NS 09914 and HD 05700, and by a grant from the Rockefeller Foundation to the Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill. We thank Ms. Anu Turnbull for technical assistance.

20 June 1973; revised 10 September 1973

Phosphate-Induced Protein Chromatography

Abstract. *High phosphate concentration is shown to cause a large proportion of the proteins of a cell-free extract of* Escherichia coli *to bind to agarose columns to which* L*-valine is attached. With a decreasing concentration gradient of potassium phosphate, the proteins elute in relation to their solubility in concentrated ammonium sulfate. This column technique appears to provide a general tool for the purification of proteins.*

To date, only a relatively few properties of proteins have been used, advantageously, for the purification of this class of macromolecules in their native state. These techniques are largely dependent on charge, size, shape, solubility, and physical and chemical adsorption properties of these macromolecules (*1–3*). Techniques designed to exploit the hydrophobic properties of macromolecules for their purification have not been highly developed. This report describes a new technique that appears to provide a general method for the purification of proteins, which may operate primarily on the basis of the hydrophobic characteristics of proteins.

It is shown in this report that in the presence of high potassium phosphate concentrations (1.0*M*) over half of the proteins in a cell-free extract of *Escherichia coli* are retarded on agarose columns to which aliphatic substituents have been attached. It is further demonstrated that it is possible to elute and chromatograph these proteins by utilizing a decreasing potassium phosphate concentration gradient.

The soluble proteins from a dialyzed cell-free extract of *E. coli* strain K12 were placed on a column of L-valine substituted agarose in the presence of 1.0*M* potassium phosphate, *p*H 7.5. The elution profile of these proteins observed during the development of a 1.0 to 0.1*M* potassium phosphate concentration gradient is shown in Fig. 1a. About half of the proteins applied to this column chromatographed throughout the concentration gradient. In addition, a large amount of material that absorbs strongly at 260 nm (presumably polynucleotides) also eluted throughout the course of the concentration gradient (Fig. 1a). In order to determine whether this 260-nm absorbing material affected the chromatographic behavior of these proteins, a dialyzed cell-free extract of *E. coli* K12 was treated with 2 percent streptomycin sulfate to remove the nucleic acids. This dialyzed extract was chromatographed as before. This procedure removed over 90 percent of the 260-nm absorbing material from the extract, and the chromatography of the proteins was not significantly altered (Fig. 1b). The nature and chromatographic behavior of this 260-nm absorbing material requires investigation.

To determine whether a significant amount of protein remained on the column after completion of the decreasing gradient, the column was washed with several column volumes of a high-ionic-strength solution of a mild chaotropic agent (1.0*M* potassium bromide in 0.05*M* potassium phosphate, *p*H 7.5). That this treatment did not further remove any protein from the column suggests that retention of the proteins on the column due to ion exchange at the low potassium phosphate concentrations is unimportant. However, a measurable amount of denatured protein was removed from the column by 0.1*N* sodium hydroxide (Fig. 1b).

Figure 1c shows that the proteins from these extracts elute within the void volume of an unsubstituted agarose column developed under the same decreasing potassium phosphate concentration gradient conditions.

Since phosphate and sulfate ions are structure-forming ions, which are known to decrease the solubility of proteins and to stabilize hydrophobic bonds between nonpolar molecules (*4*), we attempted to establish a relationship between the solubility of the proteins of an *E. coli* cell-free extract in concentrated ammonium sulfate and the position at which these proteins appear in a decreasing potassium phosphate concentration gradient. Accordingly, three nucleic acid–free ammonium sulfate fractions (0 to 35 percent, 35 to 50 percent, 50 to 65 percent of saturation) of a cell-free extract of *E. coli* K12 were dialyzed free of ammonium sulfate and placed on separate columns. The 0 to 35 percent fraction, which contained the proteins that were least soluble and, therefore, presumably the most hydrophobic (*4*), were chromatographed across the entire decreasing concentration gradient (Fig. 1d). A greater proportion of these proteins was eluted at the lower potassium phosphate concentrations than was observed for the 35 to 50 percent fraction (Fig. 1e). The most soluble, least hydrophobic, proteins, which are in the

50 to 65 percent fraction, were only slightly retarded on the column at the higher salt concentrations (Fig. 1f). Therefore, the more soluble the protein is in ammonium sulfate, the earlier it elutes from the L-valine agarose column during the development of the decreasing potassium phosphate concentration gradient.

In order to demonstrate the utility of this chromatographic method for the purification of specific proteins, an *E. coli* strain 2000 cell-free extract containing β-galactosidase activity was applied to an L-valine agarose column. Approximately 3 to 4 percent of the protein of this extract was β-galactosidase. The purification of this enzyme is shown in Fig. 1g. The β-galactosidase activity applied was quantitatively recovered from the column. Column fractions containing 71 percent of the total activity applied to the column were combined. The purity of the β-galactosidase in these fractions was approximately 77 percent (*5*). This represents one of the easiest and most efficient purification methods known for this enzyme (*5–7*). When β-galactosidase was applied to an L-valine agarose column in the presence of 0.05*M* potassium phosphate, the enzyme activity was eluted within the void volume of the column. Also, the enzyme was not retarded on unsubstituted agarose in the presence of 1.0*M* potassium phosphate. Since it has been shown here that β-galactosidase has a much higher affinity for L-valine agarose than for unsubstituted agarose in the presence of high potassium phosphate concentrations, it may be that it is the long hydrocarbon chain, in addition to the attached galactoside, that causes β-galactosidase to bind to the affinity column developed for this enzyme by Steers *et al.* (*6*). This situation may occur in other cases of affinity chromatography.

The biosynthetic L-threonine deaminases from cell-free extracts of *Bacillus subtilis* and *E. coli* (*8*) have also been partially purified by this method. The purification obtained with the *B. subtilis* enzyme is shown in Fig. 2. More than 95 percent of the enzyme activity applied was recovered. Over 90 percent of the enzyme activity was recovered in the fractions pooled from the column and the purification was 12-fold. It should be noted that the *B. subtilis* enzyme elutes very early in the gradient (0.88*M* potassium phosphate), whereas the *E. coli* enzyme elutes near the end of the gradient (0.2*M*). This difference is consistent with known structural differences between these two L-threonine deaminases (*9*).

The phosphate-induced binding of proteins to L-valine agarose reported here may be due primarily to hydrophobic interactions between the proteins and the aliphatic moiety of L-valine, although there is insufficient evidence at the present time to establish that this is the case. Such phosphate-induced hydrophobic binding could be caused by a specific salt effect whereby phosphate ions stabilize hydrophobic bonds (*4*). Preliminary evidence of others (*10*) suggests that the binding of alkaline phosphatase isozymes to L-leucine or L-phenylalanine agarose does depend on the type of salt in the buffer and does involve hydrophobic sites on the enzyme. That high potassium phosphate concentrations are needed to cause the enzyme α-isopropylmalate synthase to bind to aliphatic amino acid agarose derivatives has been interpreted by Doellgast and Kohlaw (*11*) as being due to a salt-induced conformational change in the protein. On the other hand, binding

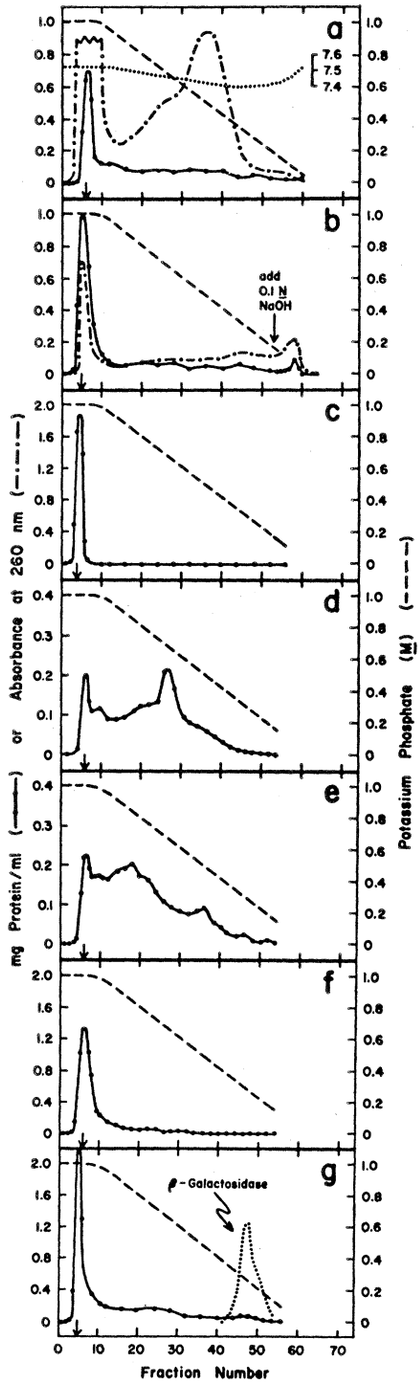

Fig. 1. Chromatography of cell-free extracts of *E. coli* on L-valine agarose. Columns were prepared with 20 ml of settled gel (1.5 by 11.4 cm) at 2°C. Each column was washed with 1*M* standard buffer (potassium phosphate, *p*H 7.5, and 4×10^{-4}*M* 2-mercaptoethanol). After the addition of 4 to 6 ml of cell-free extract in 1*M* standard buffer to the column, a decreasing gradient of potassium phosphate stabilizing buffer was started. For the gradient, 150 ml of 1*M* standard buffer was placed in the mixing chamber and 150 ml of 0.05*M* standard buffer (from dilution of 1*M* potassium phosphate, *p*H 7.5, and addition of 2-mercaptoethanol) was placed in the reservoir. The concentration of salt in the fractions collected was determined from conductivity measurements (dashed line). The dotted line in (a) represents the *p*H of the buffer, which varies slightly with temperature and with ionic strength for a fixed ratio of added buffer salts (*15*). The protein content of the fractions (solid line) was monitored by the method of Lowry *et al.* (*16*). Material absorbing at 260 nm is denoted by a dot-dashed line. The arrow at the base of each panel indicates the void volume. The crude extract used for the chromatography depicted in (a) to (f) was obtained from *E. coli* strain K12 that was grown on Kornberg-Littauer medium (*17*); that used for the chromatogram shown in (g) was obtained from *E. coli* strain 2000 that was grown on double-strength Davis minimal salts (*18*) (without citrate and glucose) containing 0.4 percent lactose and 0.08 g of thiamine per liter. Thirty milligrams of protein was placed on columns (a) and (b) and 40 mg on the rest. All protein solutions except that used on column (g) were dialyzed free of small contaminating molecules before application to the columns. (a) Cell-free extracts; (b) treated with 2 percent streptomycin sulfate in order to precipitate nucleic acids; (c) control with streptomycin-treated extract on unsubstituted Sepharose 4B; (d) to (f) 0 to 35, 35 to 50, and 50 to 65 percent, respectively, of saturation ammonium sulfate fractions (*p*H 7.5) of dialyzed streptomycin-treated extract (containing 19, 22, and 45 percent, respectively, of the protein); (g) β-galactosidase (dotted line) assayed by use of *o*-nitrophenyl-β-D-galactopyranoside reagent (*6*).

of proteins may be brought about by a nonspecific salt effect whereby high potassium phosphate concentration shields repulsive interactions between charges on the proteins and the gel and, thereby, allows hydrophobic interactions to occur. Also, since it is known that at moderate concentrations phosphate ions bind to proteins and substantially lower their isoelectric points (*12*), it is possible that this binding influences chromatographic behavior and that as the ionic strength of the buffer entering the column decreases, ionic interactions between the proteins and the positively charged gel linkage (*2, 13*) may become significant. In fact, recent investigations of protein binding to alkylamino agarose and aminoalkylamino agarose derivatives at low ionic strength indicate that, while hydrophobic interactions play a major role, ionic interactions are an important factor (*14*). However, the carboxylate group of the L-valine used here should tend to neutralize the positive charge of the gel linkage and thereby diminish the importance of these electrostatic effects. Experiments utilizing different buffer salts, column temperatures, and agarose substituents may help elucidate the mechanism by which this new form of chromatography operates.

In summary, it has been shown that high potassium phosphate concentrations cause a large proportion of the proteins in bacterial cell-free extracts to bind to L-valine substituted agarose. When columns of this material are developed under conditions of a decreasing potassium phosphate concentration gradient, these proteins elute roughly in the order of their decreasing solubilities. Enzyme activity has been quantitatively recovered from columns. The high capacity of these columns for protein is illustrated by the fact that as much as 40 mg of protein per milliliter of L-valine agarose can be loaded on a column.

It has recently been observed in several laboratories that the purification of certain specific enzymes by affinity chromatography can be enhanced by increasing either the phosphate or sulfate salt concentrations in the elution buffers (*8, 10, 11*). However, these reports have been concerned with isolated observations and have not considered the general applicability of this technique. This report presents in a more formal fashion the general utility of chromatography of proteins on agarose substituted with an aliphatic ligand in high concentrations of structure-forming salts such as potassium phosphate or ammonium sulfate and demonstrates its usefulness as a new protein chromatography method.

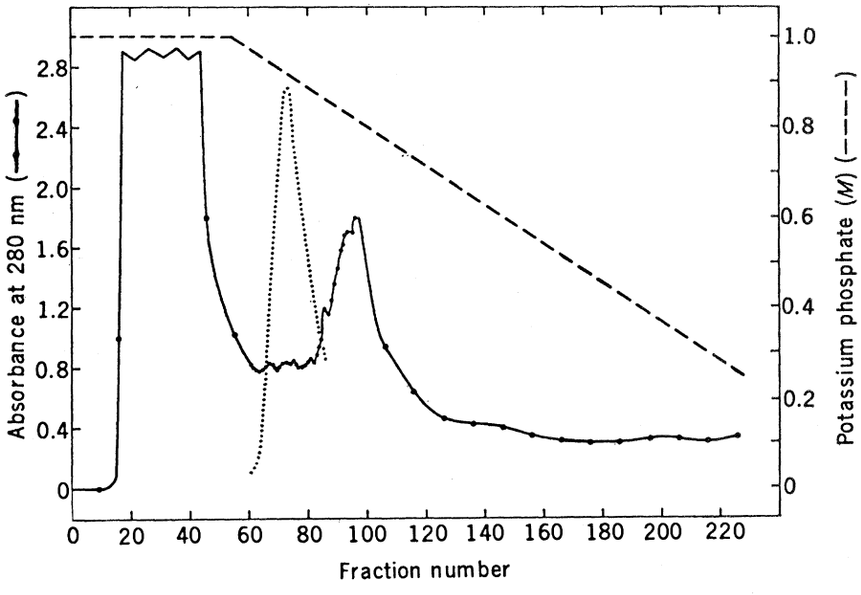

Fig. 2. Chromatography of L-threonine deaminase on L-valine agarose. The substituted Sepharose 4B was prepared by the method of Cuatrecasas (*19*), except that the cyanogen bromide was initially dissolved in a small volume of dioxane in order to increase the rate of the activation reaction. For the reaction 0.2 g of cyanogen bromide and 2 mmole of L-valine were used per milliliter of Sepharose 4B. A 5 by 19 cm column of the gel at 5°C was washed with 1*M* stabilizing buffer (*p*H 7.5, $10^{-3}M$ 2-mercaptoethanol, $10^{-4}M$ pyridoxal 6-phosphate). The column flow rate was 250 ml/hour, and 21-ml fractions were collected. An ultracentrifuged *B. subtilis* cell-free extract (240 ml) [Hatfield and Umbarger (*9*)] containing 1.93 g of protein (*16*) in 1*M* stabilizing buffer was applied. After 800 ml of the 1*M* buffer was added, a decreasing potassium phosphate gradient was started. For the gradient 2 liters of 1*M* stabilizing buffer was placed in the mixing chamber and 2 liters of 0.1*M* stabilizing buffer was added to the reservoir. When equal volumes of a high and a low phosphate buffer are used, the container of low phosphate buffer must be slightly higher than the chamber with the more dense buffer so that no net flow occurs initially. It is probably simpler to use equal weights of the two buffers, in which case the bases of the two containers are maintained at equal heights. L-Threonine deaminase activity (dotted line) was detected by use of the dinitrophenylhydrazine colorimetric assay (*20*).

RONALD A. RIMERMAN
G. WESLEY HATFIELD
Department of Medical Microbiology, California College of Medicine, University of California, Irvine 92664

References and Notes

1. C. H. W. Hirs, *Methods Enzymol.* **1**, 113 (1955); S. P. Colowick, *ibid.*, p. 90.
2. J. Porath, *Nature (Lond.)* **218**, 834 (1968).
3. A. A. Green and W. L. Hughes, *Methods Enzymol.* **1**, 67 (1955); P. Cuatrecasas and C. B. Anfinsen, *Annu. Rev. Biochem.* **40**, 259 (1971).
4. P. H. von Hippel and T. Schleich, in *Structure and Stability of Biological Macromolecules*, S. N. Timasheff and G. D. Fasman, Eds. (Dekker, New York, 1969), p. 417.
5. G. R. Craven, E. Steers, Jr., C. B. Anfinsen, *J. Biol. Chem.* **240**, 2468 (1965).
6. E. Steers, Jr., P. Cuatrecasas, H. B. Pollard, *ibid.* **246**, 196 (1971).
7. A. V. Fowler, *J. Bacteriol.* **112**, 856 (1972); M. R. Villarejo and I. Zabin, *Nat. New Biol.* **242**, 50 (1973).
8. I. Rahimi-Laridjani, H. Grimminger, F. Lingens, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **30**, 185 (1973).
9. G. W. Hatfield and H. E. Umbarger, *J. Biol. Chem.* **245**, 1736 (1970); D. H. Calhoun, R. A. Rimerman, G. W. Hatfield, *ibid.* **248**, 3511 (1973).
10. G. J. Doellgast and W. H. Fishman, *Fed. Proc.* **32** (Abstr.), 582 (1973).
11. G. Doellgast and G. Kohlaw, *ibid.* **31** (Abstr.), 424 (1972).
12. I. Tinoco, Jr., *Arch. Biochem. Biophys.* **68**, 367 (1957).
13. P. Cuatrecasas and C. B. Anfinsen, *Methods Enzymol.* **22**, 351 (1971).
14. Z. Er-el, Y. Zaidenzaig, S. Shaltiel, *Biochem. Biophys. Res. Commun.* **49**, 383 (1972); S. Shaltiel and Z. Er-el, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 778 (1973); B. H. J. Hofstee, *Biochem. Biophys. Res. Commun.* **50**, 751 (1973).
15. R. G. Bates, *Determination of pH* (Wiley, New York, 1964), pp. 78, 101, and 112; G. Mattock and D. M. Band, in *Glass Electrodes for Hydrogen and Other Cations*, G. Eisenman, Ed. (Dekker, New York, 1967), p. 29.
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
17. V. Z. Littauer and A. Kornberg, *ibid.* **266**, 1077 (1957).
18. B. D. Davis and S. Mingioli, *J. Bacteriol.* **60**, 17 (1950).
19. P. Cuatrecasas, *J. Biol. Chem.* **245**, 3059 (1970).
20. G. W. Hatfield, *Biochem. Biophys. Res. Commun.* **44**, 464 (1971).

21. This work was supported by a research grant from the National Science Foundation (NSF-GB 36587). R.A.R. is a U.S. Public Health Service postdoctoral trainee (AI-00444-01). G.W.H. is the recipient of a National Institutes of Health career development award (GM 70530).

30 July 1973