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ESCHERICHIA COLI B N^5-METHYLTETRAHYDROFOLATE–HOMOCYSTEINE COBALAMIN METHYLTRANSFERASE: GEL-FILTRATION BEHAVIOR OF APOENZYME AND HOLOENZYMES

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SUMMARY

1. Sephadex G-200 chromatography revealed a significant difference in the gel-filtration behavior of urea-resolved apomethyltransferase compared to three forms of the enzyme containing bound cobalamin (B_{12}). The initial (unalkylated) B_{12} holoenzyme, a cobalt-propyl-B_{12} enzyme, and reconstituted methyl-B_{12} holoenzyme each eluted as if their molecular weights were 150 000; whereas, urea-resolved apoenzyme appeared to have a molecular weight of 205 000. In addition, the following average Stokes radii were obtained: initial B_{12} holoenzyme, 54.2 Å; urea-resolved apoenzyme, 62.9 Å; reconstituted methyl-B_{12} holoenzyme, 53.6 Å; and propyl-B_{12} enzyme, 55.1 Å. Sephadex G-200 chromatography of apoenzyme in the presence of 6.0 M urea + 10 mM 1,4-dithiothreitol indicated that dissociation into subunits is not essential in order to remove the B_{12} from holoenzyme with urea.

INTRODUCTION

Escherichia coli B grown in the presence of cyano-B_{12} utilizes a cobalamin methyltransferase to synthesize methionine by the reaction: 5-methyltetrahydrofolate + homocysteine → methionine + tetrahydrofolate. Our preparations of the B_{12} methyltransferase (initial B_{12} holoenzyme*) contain firmly bound, but non-methylated cobalamin[2,3]. In the presence of *S*-adenosylmethionine and a reducing system, this bound B_{12} functions cyclically as a methyl-carrier prosthetic group in

Abbreviations: B_{12} is used to denote various cobalamins, *e.g.* cyano-B_{12}, cyanocobalamin; methyl-B_{12}, methylcobalamin; propyl-B_{12}, propylcobalamin. K_2HPO_4 buffer, potassium phosphate buffer, pH 7.4.

* Four forms of the enzyme[1] are defined as follows: Initial B_{12} holoenzyme, a salmon-colored, unalkylated form of holoenzyme purified from cells grown with cyano-B_{12}; apoenzyme, apoprotein-derived by urea + dithiothreitol treatment of the initial B_{12} holoenzyme; reconstituted methyl-B_{12} holoenzyme, holoenzyme formed by the binding of methyl-B_{12} to apoenzyme; propylated B_{12} enzyme, inactive cobalt-propyl-B_{12} enzyme obtained by alkylation with propyl iodide.

the catalysis of the above reaction[4–7]. Recently, it was observed that the bound cobalamin could be removed from the initial B_{12} holoenzyme by selective treatment with urea[1]. Apoenzyme, prepared by urea resolution, recombined spontaneously with methyl-[^{3}H]B_{12} at 37° to form a reconstituted methyl-[^{3}H]B_{12} holoenzyme. In a sucrose gradient, the initial and the reconstituted holoenzymes yielded sedimentation coefficients of 7.0 S; whereas, the apoenzyme was found to have a sedimentation coefficient of 6.2 S (ref. 1). A gel-filtration study was therefore undertaken to estimate the Stokes radius of each form[1] of the enzyme and to ascertain the influence of urea resolution on its relative size.

MATERIALS AND METHODS

Initial B_{12} holoenzyme was isolated[1,2] from extracts of cyano-B_{12} grown *E. coli* B. It contained 1.4 nmoles of bound cobalamin per mg of protein[2,3] and was 85–90% in the form of holoenzyme as opposed to apoenzyme[1]. Assuming only 1 mole of B_{12} per 140 000 g of enzyme[2], its estimated purity was 20%. Unlabeled 5-methyltetrahydrofolate was synthesized[8] and mixed with 5-[^{14}C]methyltetrahydrofolate (79 000 counts/min per nmole), Nuclear-Chicago, to give a specific radioactivity of 2500 counts/min per nmole. Unlabeled methyl-B_{12}, unlabeled propyl-B_{12}, methyl-[^{3}H]B_{12} (generally labeled with ^{3}H except for the cobalt-methyl group; 44 000 counts/min per nmole), urea-resolved apoenzyme (90–95% resolved), and reconstituted methyl-[^{3}H]B_{12} holoenzyme (70–75% reconstituted) were prepared as described earlier[1]. Cobalt-propyl-B_{12} enzyme was formed by treating initial B_{12} holoenzyme with propyl iodide in a $FMNH_2$ + dithiothreitol reducing system[9]. L-Homocysteine thiolactone, dithiothreitol, 2-mercaptoethanol, urea (A-grade), bovine serum albumin (Pentex, A-grade), pig heart lactate dehydrogenase, yeast alcohol dehydrogenase, and beef liver catalase were purchased from Calbiochem. Crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and tritiated water ($1.5 \cdot 10^6$ counts/ml) were kindly provided by Drs. T. E. Smith and J. Koranda, respectively, in this Division. Human apotransferrin was obtained from the Sigma Chemical Co. Horse heart cytochrome *c*, sperm whale myoglobin, beef pancreas chymotrypsinogen A, ovalbumin, human γ-globulins, and horse spleen apoferritin were obtained in a molecular weight marker kit (No. 20900-8109) from the Mann Research Laboratories. Blue Dextran 2000 (mol. wt. $2.0 \cdot 10^6$) and Sephadex G-200 (40–120 μm) were purchased from Pharmacia Fine Chemicals, Inc.

Sephadex G-200 chromatography

Gel filtration was carried out at 4° basically according to the procedure of ANDREWS[10]; although, the buffer was either 0.1 M K_2HPO_4 or 0.1 M K_2HPO_4 + 6.0 M urea + 10 mM dithiothreitol at pH 7.4 (Fig. 6 only). The gel-bed dimensions for both types of columns were 2.5 cm × 90 cm. For each filtration the enzyme, reference protein, Blue Dextran 2000, or ^{3}HOH was incorporated into the column buffer and layered onto the sample applicator (Pharmacia) in a volume of 1.5 ml. A constant downward flow rate of 15 ml/h was maintained for each run. Fractions of 3.9–4.1 ml were collected. Protein markers were detected in the column fractions by the methods used by ANDREWS[10]. The only exception was rabbit muscle glyceraldehyde-3-phosphate dehydrogenase which was assayed as described by VELICK[11].

Molecular weights for dilute solutions of the protein markers were taken from refs. 10 and 12–14.

Folate methyltransferase activity

A radioactive tracer assay was used. Assay mixtures (0.2 ml) contained 60 nmoles 5-[^{14}C]methyltetrahydrofolate (2500 counts/min per nmole); 100 nmoles homocysteine; 10 nmoles *S*-adenosylmethionine; 40 μmoles 2-mercaptoethanol; enzyme; and either 10 nmoles methyl-B_{12}, or 10 nmoles propyl-B_{12}. Enzymatically formed [*Me*-^{14}C]methionine was determined by means of an ion-exchange, column procedure[2,15]. All radioactivity measurements were made in a Packard Tri-Carb Model 3320 liquid scintillation spectrometer with the use of a naphthalene–dioxane counting fluid[16]. A unit of transmethylase activity was defined as that amount of enzyme catalyzing the synthesis of 1 nmole of [*Me*-^{14}C]methionine per 15 min at 37°.

The above assay system contained either methyl-B_{12} or propyl-B_{12} in place of cyano-B_{12}[2] for two reasons. First, from the ratio of the activities given by duplicate samples of enzyme in the presence of these two alkyl-B_{12} compounds, respectively, one obtains the percentage of holoenzyme in a given enzyme preparation[17]. Secondly, in the presence of propyl-B_{12}, one can obtain the elution profile of holoenzyme alone without any interference due to apoenzyme[1]. Both propyl-B_{12} and methyl-B_{12} bind tightly to urea-resolved apoenzyme to form a stable complex[1]. However, in the dark, only the methyl-B_{12} enzyme complex is active in folate transmethylation[1,5,7]. Therefore, folate transmethylase activity in the presence of propyl-B_{12} is due to preexisting holoenzyme; whereas, activity observed in the presence of methyl-B_{12} is due to the total amount of functional (or potentially functional) protein, *i.e.* apoenzyme + holoenzyme.

Enzyme–protein was determined by the method of LOWRY *et al.*[18].

RESULTS

Sephadex G-200 profiles of apoenzyme and holoenzymes relative to bovine serum albumin (monomer)

Fig. 1 shows a typical elution of the initial B_{12} holoenzyme in which the peak concentration of B_{12}–protein was observed at 200 ml. Upon subsequent passage of bovine serum albumin through the column, the dimer and monomer forms were maximal at 200 ml and 256 ml, respectively. Recovery of the propyl-B_{12} assayed (METHODS) activity from the column (Fig. 1) ranged from 84 to 98% (av. = 89%) for six separate runs .When urea-resolved apoenzyme was chromatographed (Fig. 2), the 7% of residual initial B_{12} holoenzyme also eluted with its peak concentration at or very near 200 ml. However, the 93% fraction of apoenzyme (methyl-B_{12} assayed activity) eluted maximally at 183 ml. Bovine serum albumin (monomer) which was chromatographed simultaneously again peaked at an elution volume of 256 ml. For three separate filtrations of urea-resolved apoenzyme the recovery was 74–82% (av. = 78%).

The foregoing recovery of apoenzyme activity was consistent with the results of a 4° stability study (Fig. 3). Storage of a dilute solution of urea-resolved apoenzyme in K_2HPO_4 buffer (pH 7.4) alone resulted in a 20% decrease of the zero time activity over the first 6 h. Thereafter, no further loss occurred up to 23 h. The inset (Fig. 3)

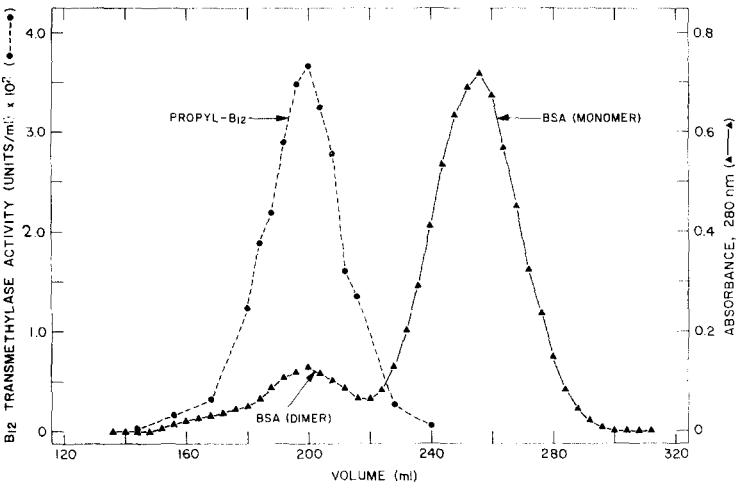


Fig. 1. Sephadex G-200 filtration of the initial B_{12} holoenzyme in relationship to bovine serum albumin (BSA). Initial B_{12} holoenzyme, 5.1 mg, was chromatographed with 0.1 M K_2HPO_4 buffer (pH 7.4) and then fractions were assayed in the presence of propyl-B_{12} (METHODS). Subsequently, 50 mg of BSA were chromatographed (280 nm absorbance).

shows that the 21-h stability of apoenzyme was influenced very little by protein concentrations between 0.1 and 0.6 mg/ml. This range spans the concentrations of apoenzyme that were in the peak column fractions (Fig. 2). In all of the gel filtrations of urea-resolved apoprotein (Fig. 2), the apoenzyme was eluted after 15 h and all assays were completed within 21 h after application of the sample to the column.

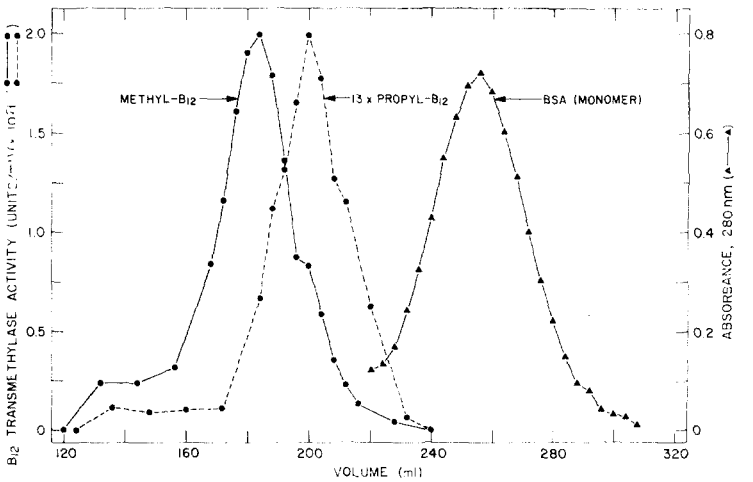


Fig. 2. Sephadex G-200 filtration of urea-resolved apoenzyme in relationship to bovine serum albumin (BSA). Urea-resolved apoenzyme, 8 mg, was mixed with 50 mg of BSA and chromatographed with 0.1 M K_2HPO_4 buffer (pH 7.4). Fractions giving folate transmethylase activity in the presence of methyl-B_{12} (apoenzyme + 7% residual initial B_{12} holoenzyme) and propyl-B_{12} (7% residual initial B_{12} holoenzyme alone), respectively, (METHODS) are indicated. Transmethylase activity observed with propyl-B_{12} was multiplied 13-fold so that the partial separation between the apoenzyme and 7% residual initial B_{12} holoenzyme could be readily seen.

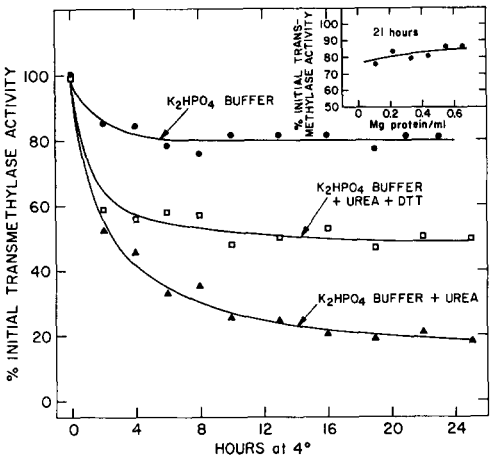


Fig. 3. Stability of urea-resolved apoenzyme at 4°. Samples of apoenzyme (6% residual initial B_{12} holoenzyme) were stored at protein concentrations of 0.3–0.7 mg/ml in the indicated buffer systems. Aliquots from each buffer system were assayed in the presence of methyl-B_{12} (METHODS). Data are plotted as percentages of the zero time activity. Inset: the 21 h recovery of apoenzyme activity in 0.1 M K_2HPO_4 buffer (pH 7.4) *vs.* protein concentration. DTT, dithiothreitol.

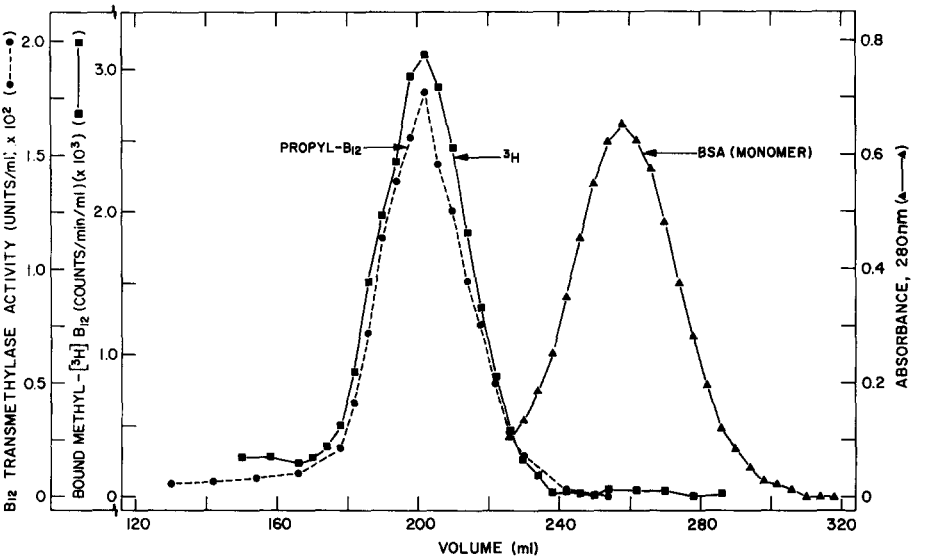


Fig. 4. Sephadex G-200 filtration of reconstituted methyl-[^{3}H]B_{12} holoenzyme in relationship to bovine serum albumin (BSA). 54 mg of urea-resolved apoenzyme (5% residual initial B_{12} holoenzyme, METHODS) were prepared and chromatographed as in Fig. 2. Apoenzyme eluting between 170 and 190 ml was pooled, concentrated, and reconstituted with methyl-[^{3}H]B_{12} (ref. 1). The final product contained 0.67 nmole of bound methyl-[^{3}H]B_{12} per mg of protein and had a transmethylase specific activity of 3400 units/mg in the presence of propyl-B_{12} (METHODS). 4 mg were mixed with 50 mg of BSA and rechromatographed in dim light. Depicted are the elution of the holoenzyme activity (propyl-B_{12} curve, METHODS), the bound methyl-[^{3}H]B_{12}, and BSA monomer.

Interestingly, the apoenzyme was relatively stable in K_2HPO_4 buffer (pH 7.4) containing 6.0 M urea + 10 mM dithiothreitol (Fig. 3). Approximately 50% of the zero-time activity was still present after 10–25 h. This buffered mixture is equivalent to the urea + dithiothreitol resolution system[1]. In the absence of dithiothreitol, 6.0 M urea decreased the apoenzyme activity to 18% of the initial amount at 25 h.

As evidence for B_{12}-mediated reversibility, a reconstituted methyl-[^{3}H]B_{12} holoenzyme was found to chromatograph like the initial B_{12} holoenzyme, with a peak elution at 202 ml (Fig. 4). Fig. 4 also shows a very close correspondence between the elution of bound methyl-[^{3}H]B_{12} and the holoenzyme activity assayed in the presence of propyl-B_{12} (METHODS). For three separate filtrations, the recovery of reconstituted holoenzyme activity was 74–99% (av. = 83%). Recovery of the applied ^{3}H associated with holoenzyme activity ranged from 72 to 94% (av. = 83%).

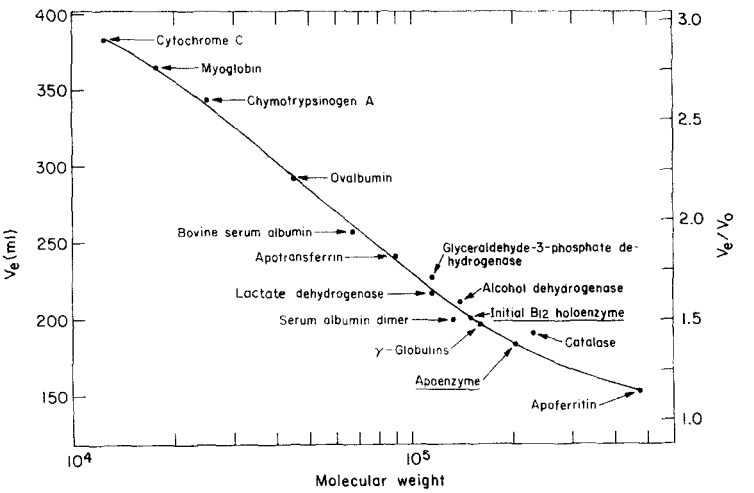


Fig. 5. Sephadex G-200 elution volume against log mol. wt. The data are mean values for 3–6 determinations. Points for the initial B_{12} holoenzyme and apoenzyme were placed on the standard curve only to indicate their elution relative to a wide range of marker proteins.

Estimation of molecular weight

Gel filtration with a Sephadex G-200 column generally is a useful method to estimate the size of proteins over a wide range in molecular weights[10]. Calibration of our Sephadex G-200 column with thirteen protein markers yielded a standard curve (Fig. 5) with the same shape as that published by ANDREWS[10]. Catalase was disregarded in drawing the curve because it behaves quite abnormally on gel filtration[10]. Relative to the protein markers (Fig. 5), the initial B_{12}, methyl-[^{3}H]B_{12}, and propyl-B_{12} forms of the enzyme each chromatographed as a protein with a molecular weight of 150 000. However, the more excluded apoenzyme (Fig. 2) eluted at a volume corresponding to a molecular weight of 205 000.

Determination of the Stokes radii

ACKERS[19] has validated that the distribution coefficient (K_D) of a protein is dependent upon its Stokes radius (a) and the effective pore radius (r) of the molecular sieve in accordance with the equation:

$$K_D = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right]$$

The effective pore radius of the gel (r) was calculated from the preceding equation by using the experimentally observed K_D and the known Stokes radius (a) of 36.1 Å[19–23] for bovine serum albumin. For six determinations, the effective pore radius was 179–186 Å (av. = 184 Å). Using 184 Å for r, the mean Stokes radii in Table I were obtained for the apoenzyme and the three formes of holoenzyme. Only

TABLE I

STOKES RADII OF UREA-RESOLVED APOENZYME AND THREE HOLOENZYMES

Values are the mean ±S.D. K_D is calculated from the equation given by GELOTTE[24] for which the elution maxima of 133 ml (Blue Dextran) and 452 ml (^{3}HOH) were assumed to be the void and liquid volumes, respectively. Stokes radii were calculated by the method of ACKERS[19]. f/f_0 was calculated by use of the equation[20]:

$$f/f_0 = \left(a \Big/ \sqrt[3]{\frac{\bar{V}M}{4\pi N}}\right)^{1/3}.$$

Form of the enzyme	*Number of experiments*	K_D	*Stokes radius (Å)*	f/f_0
Initial B_{12} holoenzyme	6	0.215	54.2±1.1	1.55
Urea-resolved apoenzyme	4	0.156	62.9±0.3	1.79
Reconstituted methyl-[^{3}H]B_{12} holoenzyme	4	0.219	53.6±0.4	1.53
Propyl-B_{12} enzyme*	4	0.209	55.1±0.3	1.57

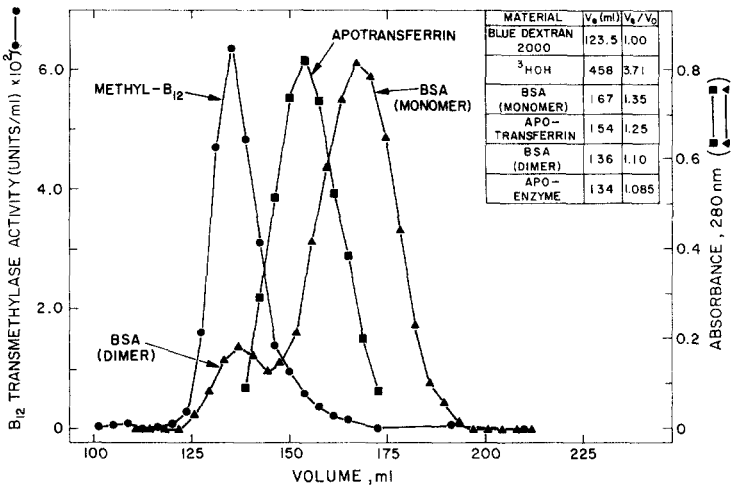
* Prepared by cobalt alkylation with propyl iodide and 90% propylated[9]. It was chromatographed in the dark and then column fractions were assayed after photolysis of the cobalt-propyl bond[9].

apoenzyme had a decidedly different radius. To estimate the frictional coefficients (f/f_0), a molecular weight of 150 000 (Fig. 5 and ref. 2) and a partial specific volume of 0.725 (ref. 25) were used.

Sephadex G-200 chromatography in urea + dithiothreitol

In view of the 4° stability (Fig. 3) of apoenzyme in K_2HPO_4 buffer (pH 7.4) + urea + dithiothreitol, it was of interest to carry out gel filtration in this resolution system. From Fig. 6 it can be seen that apoenzyme still eluted slightly ahead of the bovine serum albumin dimer (mol. wt., 134 000) and considerably earlier than apotransferrin (mol. wt., 90 000) and bovine serum albumin monomer (mol. wt., 67 000). Assays of the percentage holoenzyme (METHODS) in the peak transmethylase fractions showed only 2.5–3.0% of residual, initial B_{12} holoenzyme. Recovery of the applied apoenzyme activity (Fig. 6) was 56–62% (av. = 59%), in agreement with the stability data (Fig. 3).

A series of thiol-reduced polypeptides (*e.g.* bovine serum albumin and transferrin), in denaturing solvents, have been shown to chromatograph predictably with respect to their Stokes radii and their molecular weights[26]. Consequently, one would expect identical apoenzyme subunits with a molecular weight of about 75 000 to elute close to or between bovine serum albumin (monomer) and apotransferrin. On the



MATERIAL	V_e (ml)	V_e/V_0
BLUE DEXTRAN 2000	123.5	1.00
^{3}HOH	458	3.71
BSA (MONOMER)	167	1.35
APO-TRANSFERRIN	154	1.25
BSA (DIMER)	136	1.10
APO-ENZYME	134	1.085

Fig. 6. Sephadex G-200 chromatography in 0.1 M K_2HPO_4 buffer + 6.0 M urea + 10 mM dithiothreitol at pH 7.4. Initial B_{12} holoenzyme, 12 mg, was incubated 15 min at 37° in 1.5 ml of the resolution system[1] and immediately chromatographed. Column fractions were assayed for apoenzyme activity in the presence of methyl-B_{12} (METHODS). Then 50 mg of bovine serum albumin and 30 mg of apotransferrin were processed in the same manner along with Blue Dextran 2000 and ^{3}HOH. Inset table: mean data for three determinations. BSA, bovine serum albumin.

other hand, a dissociation of apoenzyme into very dissimilar subunits (one large and one very small) would have yielded (Fig. 6) a marked loss of transmethylase activity. The loss would have been much greater than the 50% loss observed merely upon storage (Fig. 3) in this buffer system. Clearly, neither of these possible dissociations were observed. Thus, in both the absence (Figs. 1, 2, and 4) and presence (Fig. 6) of urea + dithiothreitol we find no indication that, upon chromatography, *E. coli* B methyltransferase loses an essential 3000 mol. wt. subunit as reported for *E. coli* K-12[27].

DISCUSSION

Although urea-resolved apoenzyme has a smaller sedimentation coefficient than either the initial B_{12} or the reconstituted methyl-[^{3}H]B_{12} holoenzyme[1], its greater exclusion from Sephadex G-200 is indicative of a conformational change rather than a decrease in molecular weight. If the methyltransferase is, in fact, composed of tightly-linked subunits, apparently their reversible dissociation is not essential in order to remove and bind back the B_{12} prosthetic group. A decrease in the Stokes radius of 9.3 Å (Table I) accompanied the binding[1] of a methyl-B_{12} prosthetic group[7]. However, methylcobinamide can not effect the binding transformation of urea-resolved apoenzyme into holoenzyme[1]. These observations indicate that an attachment between the 5,6-dimethylbenzimidazolyl nucleotide and the apoprotein is required in order to return the loosely-folded apoenzyme to a more compact structure.

Similar results have been published for two physiological B_{12}-transport proteins. Free human intrinsic factor (S-form) has a sedimentation coefficient of 3.7 S and a

Stokes radius, by Sephadex G-200 chromatography, of 36.5 Å[28]. Upon binding cyano-B_{12}, the intrinsic factor–cyano-B_{12} complex yielded a sedimentation coefficient of 4.1 S, but a Stokes radius of only 32.5 Å[28]. This 4 Å decrease in its Stokes radius has been independently confirmed[23]. A comparable decrease in the Stokes radius was also observed upon binding aquo-B_{12}, 5'-deoxyadenosyl-B_{12}, methyl-B_{12}, and carboxymethyl-B_{12} to the S-form of human intrinsic factor[23]. Likewise, human (plasma) transcobalamin II underwent a decrease of 1.4 Å (26.7 to 25.3 Å) in its Stokes radius when cyano-B_{12} was bound to it[22,23].

In two recent publications RÜDIGER AND JAENICKE[29,30] reported the purification, directly from *E. coli* B extracts, of a cobalt-methyl-B_{12} holoenzyme. A molecular weight estimate of 255 000 was made from its Sephadex G-200 elution relative to bovine liver catalase[30]. Thus, the size of their methyl-B_{12} enzyme would appear to be much greater than the methyl-B_{12} holoenzyme that this author has studied (see RESULTS and refs. 1 and 4–7). However, they[29,30] have not yet stripped[4] the B_{12} chromophore from their new enzyme in order to identify it definitively as methyl-B_{12}. In addition, ANDREWS[10] pointed out that catalase behaves quite abnormally on Sephadex G-200 filtration. It chromatographs as a globular protein with a molecular weight of 190 000, instead of about 240 000[10].

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REFERENCES

1 R. T. TAYLOR, *Arch. Biochem. Biophys.*, 137 (1970) 529.
2 R. T. TAYLOR AND H. WEISSBACH, *J. Biol. Chem.*, 242 (1967) 1502.
3 R. ERTEL, N. BROT, R. T. TAYLOR AND H. WEISSBACH, *Arch. Biochem. Biophys.*, 126 (1968) 353.
4 R. T. TAYLOR AND H. WEISSBACH, *Arch. Biochem. Biophys.*, 123 (1968) 109.
5 R. T. TAYLOR AND H. WEISSBACH, *Arch. Biochem. Biophys.*, 129 (1969) 728, 745.
6 R. T. TAYLOR AND M. L. HANNA, *Biochem. Biophys. Res. Commun.*, 38 (1970) 758.
7 R. T. TAYLOR AND M. L. HANNA, *Arch. Biochem. Biophys.*, 137 (1970) 453.
8 J. C. KERESZTESY AND K. O. DONALDSON, *Biochem. Biophys. Res. Commun.*, 5 (1961) 286.
9 R. T. TAYLOR AND H. WEISSBACH, *J. Biol. Chem.*, 242 (1967) 1509.
10 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
11 S. F. VELICK, in S. P. COLOWICK AND N. O. KAPLAN (Eds.), *Methods in Enzymology*, Vol. I, Academic Press, New York, 1955, p. 401.
12 R. JAENICKE AND G. PFLEIDERER, *Biochim. Biophys. Acta*, 60 (1962) 615.
13 G. A. JAMIESON, *J. Biol. Chem.*, 240 (1965) 2914.
14 V. D. HOAGLAND, JR. AND D. C. TELLER, *Biochemistry*, 8 (1969) 594.
15 H. WEISSBACH, A. PETERKOFSKY, B. G. REDFIELD AND H. DICKERMAN, *J. Biol. Chem.*, 238 (1963) 3318.
16 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
17 H. WEISSBACH, B. REDFIELD AND H. DICKERMAN, *Biochem. Biophys. Res. Commun.*, 17 (1964) 17.

18 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
19 G. K. ACKERS, *Biochemistry*, 3 (1964) 723.
20 C. TANFORD, in *Physical Chemistry of Macromolecules*, Wiley, New York, 1961, pp. 356–360.
21 K. S. ROGERS, L. HELLERMAN AND T. E. THOMPSON, *J. Biol. Chem.*, 240 (1965) 198.
22 B. L. HOM, *Biochim. Biophys. Acta*, 175 (1969) 20.
23 E. HIPPE, *Biochim. Biophys. Acta*, 208 (1970) 337.
24 B. GELOTTE, *J. Chromatogr.*, 3 (1960) 330.
25 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
26 W. W. FISH, J. A. REYNOLDS AND C. TANFORD, *J. Biol. Chem.*, 245 (1970) 5166.
27 J. GALIVAN AND F. M. HUENNEKENS, *Biochem. Biophys. Res. Commun.*, 38 (1970) 46.
28 R. GRÄSBECK, *Scand. J. Clin. Lab. Invest. Suppl.*, 95 (1967) 7.
29 H. RÜDIGER AND L. JAENICKE, *Eur. J. Biochem.*, 10 (1969) 557.
30 H. RÜDIGER AND L. JAENICKE, *Eur. J. Biochem.*, 16 (1970) 92.