Isolation of a Cobalamin Containing 5-Methyltetrahydrofolate–Homocysteine Transmethylase from Mammalian Kidney*

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ABSTRACT: The enzyme (5-methyltetrahydrofolate–homocysteine transmethylase) that catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine to form methionine has been purified approximately 1800-fold from pig kidney. The purified transmethylase was shown spectrally to contain a cobalamin prosthetic group. The cofactor requirements of the purified enzyme were also examined.

The terminal reaction in methionine biosynthesis involves the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine (Sakami and Ukstins, 1961; Larrabee *et al.*, 1963). The cofactors which have been implicated in methionine biosynthesis include $FADH_2$ (Hatch *et al.*, 1959) and *S*-adenosylmethionine (Mangum and Scrimgeour, 1962). Enzyme systems catalyzing this reaction have been partially purified from *Escherichia coli* (Stavrianopoulos and Jaenicke, 1967; Taylor and Weissbach, 1967) and from mammalian (Buchanan *et al.*, 1964) and avian liver (Dickerman *et al.*, 1964). The transmethylase isolated from *Escherichia coli* has been shown both spectrally (Takeyama and Buchanan, 1961; Taylor and Weissbach, 1967; Stavrianopoulos and Jaenicke, 1967) and by the extraction of the prosthetic group (Taylor and Weissbach, 1968) to contain a bound form of vitamin B_{12}. In addition, Taylor (1970) has succeeded in resolving and reconstituting the bacterial enzyme. There is also evidence that methionine biosynthesis in animal tissues requires a vitamin B_{12} prosthetic group. The dietary requirement of rats for methionine can be replaced by homocysteine and vitamin B_{12} (du Vigneaud *et al.*, 1950). A nutritional deficiency of vitamin B_{12} in young chicks resulted in a marked reduction of the transmethylase activity (Dickerman *et al.*, 1964). A partial restoration of the activity occurred when the animals were subsequently provided vitamin B_{12}. Methionine biosynthesis in cultured mammalian cells has also been shown to be dependent on vitamin B_{12} (Mangum *et al.*, 1969). Loughlin *et al.* (1964) demonstrated that there was an excellent correlation between transmethylase activity and vitamin B_{12} content (determined microbiologically) of fractions of their most highly purified preparation of a pig liver enzyme as it eluted from columns of DEAE. A cobalamin derivative has been extracted from a partially purified 5-methyltetrahydrofolate–homocysteine transmethylase obtained from pig kidney (Burke *et al.*, 1970). However, this transmethylase, as well as the other mammalian enzyme preparations have not been purified to the extent that it was possible to demonstrate by spectral observation the existence of a vitamin B_{12} prosthetic group. In this communication, a scheme is described which results in an 1800-fold purification of the transmethylase of pig kidney. This preparation is free of contaminating heme proteins and an examination of the visible region of the absorption spectrum provides additional direct evidence that 5-methyltetrahydrofolate–homocysteine transmethylase contains a bound form of vitamin B_{12}.

5-methyltetrahydrofolate + homocysteine ⟶ methionine + tetrahydrofolate

Materials and Methods

Chemicals were obtained from the following sources: DL-homocysteine and protamine sulfate (Salmine) from Nutritional Biochemical Corp.; [^{14}C]formaldehyde from New England Nuclear Corp.; FAD from Sigma Chemical Co., DEAE-Sephadex and Sephadex G-200 from Pharmacia, Inc.; and *S*-adenosylmethionine, folic acid, and Dowex 1-Cl from California Corp. for Biochemical Research.

Tetrahydrofolate was prepared by the catalytic hydrogenation of folic acid (Hatefi *et al.*, 1960) and [*methyl*-^{14}C]5-methyltetrahydrofolate was chemically synthesized by reducing a mixture of [^{14}C]HCHO and tetrahydrofolate with potassium borohydride (Keresztesy and Donaldson, 1961). The desired product was purified by chromatography on DEAE-cellulose. $FADH_2$ was prepared by the catalytic reduction of FAD (Loughlin *et al.*, 1964). The procedure of Keilin and Hartree (1938) was followed in the preparation of the calcium phosphate gel. The assay system for methionine synthesis consisted of the following components present in a total volume of 1 ml: [*methyl*-^{14}C]5-methyltetrahydrofolate, 0.25 μmole; *S*-adenosylmethionine, 0.10 μmole; homocysteine, 2.5 μmoles; $FADH_2$, 0.08 μmole; potassium phosphate buffer (pH 7.4), 50 μmoles; and the transmethylase. Incubations were carried out in 5-ml wide-mouthed stoppered serum bottles at 37° for 15 min under a hydrogen atmosphere. An 0.2-ml aliquot of the reaction mixture was placed on a small Dowex 1-Cl$^-$ column (6 × 30 mm) which retained the 5-methyltetrahydrofolate but not the methionine (Weissbach *et al.*, 1963). The column was washed with 1.8 ml of water, and after the addition of 10 ml of naphthalene–dioxane scintillation fluid (Bray, 1960), the radioactive solution was counted with a Packard Tri-Carb spectrometer. All values are reported

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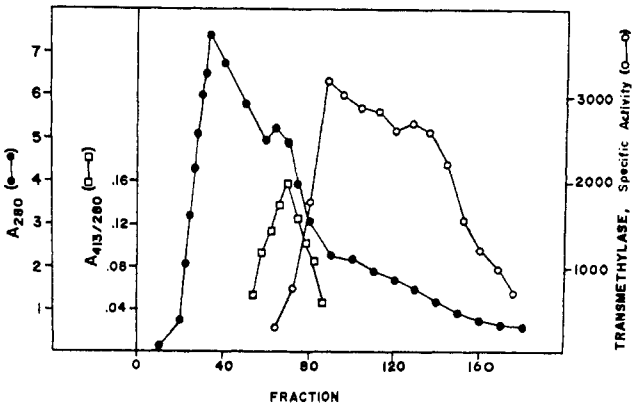

FIGURE 1: Chromatography of the 28–42% ammonium sulfate fraction on DEAE-Sephadex.

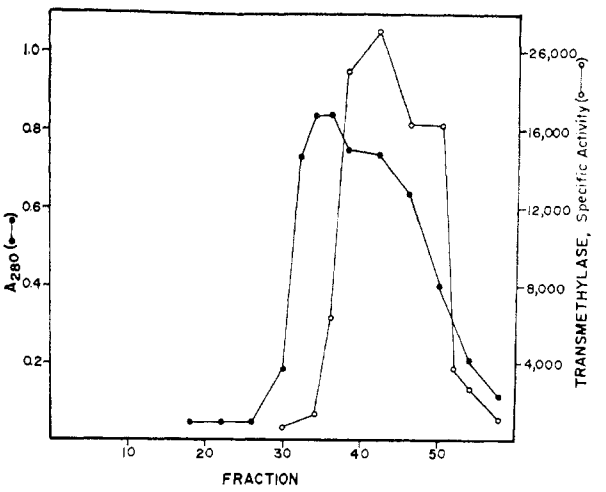

FIGURE 2: Chromatography of the second DEAE-Sephadex fraction on Sephadex G-200.

in terms of specific activity which is defined as the millimicromoles of methionine synthesized per hour per milligram of protein.

Protein concentrations were determined either by the method of Warburg and Christian (1941) or by the biuret procedure described by Gornall *et al.* (1949). Absorption spectra of the different fractions were determined with a Cary Model 15 recording spectrophotometer.

Purification of the Enzyme. Initial Extract. Frozen pig kidneys were chopped into small cubes and placed in a commercial Waring Blendor (1200 g of kidney and 3000 ml of 0.03 M potassium phosphate buffer, pH 6.5) and homogenized for 1 min. The suspension was centrifuged at 48,000*g* for 30 min and the rotor temperature during centrifugation was allowed to raise to 20°. The clear supernatant fraction was carefully removed with a syringe in such a way that both the lipid at the top of the centrifuge tubes and the loosely packed material at the bottom of the tubes were excluded.

Protamine Sulfate Fractionation. This and all subsequent steps were performed at 0–5°. A 1% suspension of protamine sulfate in 0.03 M potassium phosphate buffer, which had been adjusted to pH 6.5, was added dropwise with stirring to the initial extract. The protamine sulfate was added in two increments: first, 4.5 ml; and then 3.5 ml for each 100 ml of the initial extract. After each addition, the suspension was stirred for 15 min and then centrifuged at 40,000*g* for 15 min. The first precipitate was discarded. The second precipitate was resuspended in 0.5 M potassium phosphate buffer (pH 7.4) and in all subsequent steps potassium phosphate buffers (pH 7.4) at the indicated concentrations were used. These buffers contained 10^{-2} M homocysteine in all of the ammonium sulfate fractionations and in all other procedures the homocysteine concentration was maintained at 10^{-3} M.

Ammonium Sulfate Fractionation. Saturated ammonium sulfate (pH 7.4) was added dropwise with stirring to the protamine sulfate fraction until the fraction was 28% saturated. Stirring was continued for an additional 10 min and the precipitated protein was removed by centrifugation at 27,000*g* for 15 min. An additional volume of ammonium sulfate was added to the supernatant fraction until a final concentration of 42% saturation was reached. After stirring for 10 min, the precipitate was collected by centrifugation and the ammonium sulfate paste was frozen and stored at a −20°.

First DEAE-Sephadex Chromatography. When ten of the above ammonium sulfate preparations had been accumulated, they were resuspended in 0.05 M potassium phosphate buffer and dialyzed for 6 hr in 18 l. of the same buffer. The dialyzed material was centrifuged for 10 min at 40,000*g* and then carefully layered on a DEAE-Sephadex A-50 column (3.8 × 21 cm) which had previously been equilibrated with 0.05 M phosphate buffer. After the protein had passed into the Sephadex bed, the column was washed with the 0.05 M phosphate buffer until the protein concentration of the eluent had dropped below 0.15 mg/ml. Absorbed protein was then eluted with a linear gradient of 0.05–0.5 M phosphate buffer. Both the reservoir and the mixing chamber contained 500 ml of buffer. Fractions (10 ml) were collected at a flow rate of 120 ml/hr. A comparison of the elution profiles of protein, a contaminating cytochrome, and transmethylase activity is given in Figure 1. Those tubes which had a specific activity that exceed 2000 (tubes 81–144) were pooled, and an equal volume of saturated ammonium sulfate (pH 7.4) was added and the precipitated protein was centrifuged for 10 min at 40,000*g*. The ammonium sulfate pastes were either stored at −20° or immediately resuspended in 0.05 M phosphate buffer and dialyzed against the same buffer for 6 hr prior to being applied to the second DEAE-Sephadex column.

Second DEAE-Sephadex Chromatography. The dialyzed material obtained from the first DEAE-Sephadex column was centrifuged for 10 min at 40,000*g* and then placed on a column (2.8 × 11 cm) of DEAE-Sephadex A-50 which had been equilibrated with 0.05 M potassium phosphate buffer. The column was washed with 0.1 M phosphate buffer until the protein concentration eluting from the column was less than 0.15 mg/ml. Elution of the remaining protein was effected with a linear gradient which resulted from mixing 250 ml of 0.5 M phosphate buffer with 0.1 M phosphate buffer. Fractions of 12 ml were collected at a flow rate of 90 ml/hr. Fractions with a specific activity which exceeded 5000 were pooled, and the protein was concentrated by adding saturated ammonium sulfate as described in the previous step.

Sephadex G-200 Chromatography. The ammonium sulfate pastes obtained from the second DEAE-Sephadex step were resuspended in 0.05 M potassium phosphate buffer and was dialyzed for 2 hr in 6 l. of this buffer. This same buffer was also used to equilibrate a Sephadex G-200 column (2.5 × 85 cm). The protein was allowed to flow into the bottom of the column and elution was achieved by allowing 0.05 M potassium phosphate buffer to percolate up the column. Fractions of 5

TABLE I: Calcium Phosphate Gel Fractionation.[a]

Fraction	Protein (mg/ml)	Vol (ml)	Sp Act.
Initial supernatant	0.056		2,857
0.005 eluate	0.305	5	4,130
0.01 eluate	0.434	5	6,976
I–0.025 eluate	0.977	5	24,898
II–0.025 eluate	0.398	5	37,000
0.05 eluate	0.560	5	30,179
0.75 eluate	0.430	5	54,419
0.10 eluate	0.326	5	25,510
0.20 eluate	0.254	5	8,627
0.50 eluate	0.084	5	4,524

[a] The activity of the 0–50% $(NH_4)_2SO_4$ fraction of the III–DEAE-Sephadex was 24,286 and the protein concentration was 9.8 mg/ml, and the total volume 2 ml.

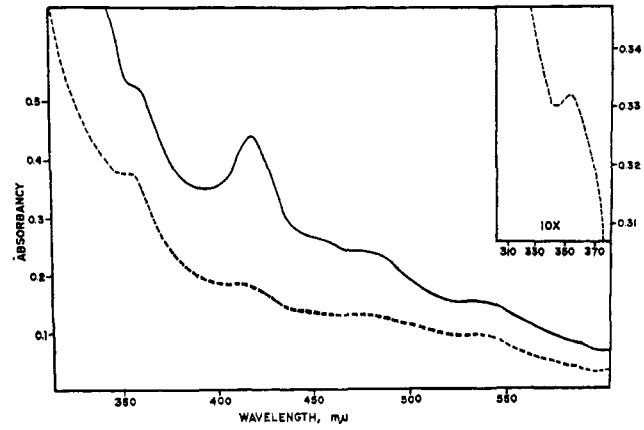

FIGURE 3: Absorption spectrum of purified 5-methyltetrahydrofolate–homocysteine transmethylase: dashed line, 10 mg/ml of the 0.075 $(Ca_3PO_4)_2$ gel eluate, and solid line, 30 mg/ml of the third DEAE-Sephadex fraction.

ml were collected at a flow rate of 20 ml/hr. Those fractions with a specific activity of 10,000 or higher were pooled. The protein and transmethylase elution patterns are shown in Figure 2.

Third DEAE-Sephadex Chromatography. The pooled enzyme from the above step was placed directly on a column (2.2 × 9 cm) of DEAE-Sephadex A-50 and eluted as described in the second DEAE-Sephadex chromatography step with the modification that only 100 ml of 0.05 and 0.5 M potassium phosphate were allowed to mix to form the linear gradient which was used to elute the protein from the column. Tubes containing peak activity were again pooled and the protein was concentrated by an ammonium sulfate precipitation as described previously.

Calcium Phosphate Gel Fractionation. The ammonium sulfate precipitates from the third DEAE-Sephadex fractionation were dialyzed in a 100-fold excess of 0.005 M potassium phosphate buffer. The dialyzed enzyme was centrifuged for 10 min at 40,000*g* and then 5 mg of calcium phosphate gel was added for each mg of protein. The slurry was stirred 20 min and centrifuged at 40,000*g* for 5 min. The precipitated gel was successively resuspended in 5 ml of each of the following potassium phosphate buffers: 0.005, 0.01 M; twice in 0.025, 0.05, 0.075, 0.1, 0.2, and 0.5 M. Resuspension was accomplished using a glass Teflon homogenizer. Following each resuspension, the gel–buffer mixture was centrifuged 5 min at 40,000*g*. Table I contains the results of this fractionation.

Results and Discussion

5-Methyltetrahydrofolate–homocysteine transmethylase has been highly purified from *Escherichia coli* by Stavrianopoulos and Jaenicke (1967) who reported that their preparation was homogeneous. However, only a partial purification (250-fold) of a mammalian transmethylase (Loughlin *et al.*, 1964) has previously been achieved. By using a combination of the procedure described by Loughlin *et al.* (1964) and Taylor and Weissbach (1967) this enzyme has now been purified 1800-fold from pig kidney (Table II). An analysis of this preparation by analytical ultracentrifugation and polyacrylamide gel electrophoresis demonstrated some heterogeneity. A cytochrome which spectrally resembles microsomal cytochrome b_5 (Mangum *et al.*, 1970) has been found to be major contaminant of the transmethylase. The intense absorption peak at 413 mμ has made it difficult to observe the spectrum of the bound vitamin B_{12}. Figure 3 illustrates that even the third DEAE-Sephadex fraction after having been concentrated by ammonium sulfate (specific activity 18,400) still showed a major absorption maximum at 413 mμ. However, by manipulating the calcium phosphate gel fractionation the majority of the cytochrome could be separated from the transmethylase. The cytochrome was selectively removed from the gel by three extractions with 0.04 M potassium phosphate buffer. These fractions were contaminated with transmethylase (specific activity 15,000–22,000). The gel was next treated with 0.08 and 0.12 M potassium phosphate buffer. These two fractions were combined (specific activity 30,000) and concentrated by ultrafiltration until a protein concentration of 10 mg/ml was reached. In order to obtain 10 mg of the nearly cytochrome-free transmethylase it was necessary to process 30 kg of kidney. This was three times the amount of kidney used in the purification scheme outlined in Table III. The purified transmethylase (Figure 3) had an absorption maximum at approximately 250–355 mμ and pleateaus at from 460 to 480 mμ and from 530 to 550 mμ. A trace of residual cytochrome most likely contributed to this absorption in the 400- to 420-mμ region. The spectrum of the mammalian enzyme is very similar to that observed for the *E. coli* transmethylase (Takeyama and Buchanan, 1961; Stavrianopoulos and Jaenicke, 1967; Taylor and Weissbach, 1967). However, with the limited amount of the purified kidney transmethylase it was impossible to establish the nature of the cobalamin prosthetic group.

The pig kidney transmethylase was found to be stabilized by one of its substrates, homocysteine. Therefore, homocysteine (10^{-2} M) was added before each ammonium sulfate fractionation, and to all fractions (10^{-3} M) before they were placed on columns. Without the addition of homocysteine to the protamine sulfate fractions essentially no increase in the specific activity was obtained by fractionation with ammonium sulfate (Table IV). This table also demonstrates that protective effect was specific for homocysteine since cysteine was ineffective. Ammonium sulfate pastes containing homocysteine could be stored and stockpiled at −20° for an indefinite period of time without any loss of activity. The stabil-

TABLE II: Purification Procedure for N^5-Methyltetrahydrofolate–Homocysteine Transmethylase from Pig Kidney.

Fraction	Vol (ml)	Total Act. $\times 10^6$	Protein (mg/ml)	Sp Act.	Yield (%)	Rel Improvement
Initial extract	17,000	15.28	31	29	100	1
Protamine sulfate	764	4.97	30	217	32.5	7.5
Ammonium sulfate 28–42%	210	2.25	23	465	14.4	16
I-DEAE-Sephadex	240	1.25	1.90	2,745	8.2	95
Ammonium sulfate 0–50%	25	1.10	9.80	4,480	7.2	155
II-DEAE-Sephadex	218	0.79	0.606	5,947	5.2	205
Ammonium sulfate 0–50%	3.2	0.70	31	7,036	4.6	243
G-200 Sephadex	76	0.67	0.628	14,012	4.4	483
III-DEAE-Sephadex	87	0.61	0.387	18,166	4.0	626
Ammonium sulfate 0–50%	2	0.48	9.8	24,286	3.1	837
$Ca_3(PO_4)_2$ 0.075 M eluate	5	0.12	0.43	54,419	0.8	1877

TABLE III: Ammonium Sulfate Fractionation of N^5-Methyltetrahydrofolate–Homocysteine Transmethylase Protective Effect of Homocysteine.[a]

Addition	Specific Activity	
	Expt 1	Expt 2
None	234	210
Homocysteine		
1×10^{-3} M	341	
1×10^{-2} M	356	360
Cysteine		
1×10^{-2} M		241

[a] The specific activity of the protamine sulfate fraction was 206 in expt 1 and 275 in expt 2.

TABLE IV: Cofactor Requirements for 5-Methyltetrahydrofolate–Homocysteine Transmethylase.[a]

Omission	Methionine Formed (nmoles)	
	Expt 1	Expt 2
None	55	70
S-Adenosylmethionine	31	54
$FADH_2$	7	7

[a] The Sephadex G-200 fraction and the $Ca_3(PO_4)_2$ gel fraction were used in expt 1 and expt 2, respectively.

ity of these frozen pastes as well as the protective effect of homocysteine in the various fractionation procedures were the most important factors in the successful purification of the pig kidney transmethylase.

Both a Sephadex G-200 fraction (specific activity 14,012) and a calcium phosphate gel fraction (specific activity 45,738) had very similar cofactor requirements. These highly purified transmethylase fractions showed nearly an absolute dependency upon a reducing system, however, they were only partially stimulated by *S*-adenosylmethionine (Table IV). The fact that *S*-adenosylmethionine would replace the requirement for ATP and Mg^{2+} in methionine biosynthesis was first demonstrated with a crude mammalian transmethylase preparation (Mangum and Scrimgeour, 1962). It was somewhat surprising, therefore, to find that the purified kidney tramsmethylase was only partially stimulated by *S*-adenosylmethionine. The explanation that the enzyme as isolated contained bound methylcobalamin would not seem too likely, since, at all times the enzyme fractions contain either 10^{-2} or 10^{-3} M homocysteine. Under these conditions it would be expected that methylcobalamin would transfer its methyl group to homocysteine to form methionine and the demethylated cobalamin as has been shown to occur with the bacterial transmethylase (Jaenicke, 1969). It would appear, therefore, that the kidney transmethylase is capable of catalyzing the transfer of the methyl group from N^5-methyltetrahydrofolate directly to the cobalamin in the absence of *S*-adenosylmethionine. However, this transfer is stimulated by *S*-adenosylmethionine. This represents a fundamental difference in the mechanism of methionine biosynthesis in kidney and in *E. coli* where an absolute dependency on *S*-adenosylmethionine exists (Taylor and Weissbach, 1967; Rosenthal and Buchanan, 1963), and this difference is currently under investigation in this laboratory.

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Estrogen-Binding Proteins of Calf Uterus. Partial Purification and Preliminary Characterization of Two Cytoplasmic Proteins*

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ABSTRACT: The larger (~8 S) estrogen-binding protein (EBP) which is found in the soluble fraction of uterine cytoplasm has been purified about 500-fold from prepuberal calf. The smaller (~4 S) cytoplasmic EBP has also been purified about 1500-fold by a previous method. Using these partially purified preparations, the following molecular characteristics were assessed by sucrose gradient centrifugation combined with "gel filtration" on Sephadex G-200 and by electrofocusing. Larger EBP: $s_{20,w}$ = 8.6; Stokes radius, 67 Å; mol wt 238,000 (for $\bar{v}$ = 0.725); f/f_0 = 1.65; homogeneous at electrofocusing, with isoelectric point at 6.2. Smaller EBP: $s_{20,w}$ = 4.5; Stokes radius, 33 Å; mol wt 61,000 (for $\bar{v}$ = 0.725); f/f_0 = 1.25; heterogeneous at electrofocusing: two major components with isoelectric point at 6.6 and 6.8, respectively. As estimated by a method based on gel filtration, K_{ass} with estradiol of both EBPs is 1.5–3.5 × 10^9 l./mole at +4°. Relative affinity for ligands is as follows: diethylstilbestrol > estradiol > estrone > estriol; in comparison, no significant affinity for progesterone, deoxycorticosterone, testosterone, and cortisol is detected. Estrogen-binding activity of EBPs is irreversibly destroyed by acidic pH, +65° for 5 min at pH 7.5, cold ethanol or ether, 1.5–6 M urea, and 1.5–2.5 M guanidine hydrochloride at pH 7.5. In contrast, aspecific binding of estradiol by plasma albumin increases with increasing pH and temperature. It is suggested that the larger 8.6S EBP is a tetrad consisting of four 4.5S subunits and that the subunits possibly belong to at least two different types. Some data suggest that binding of estradiol is cooperative. The estrogen-binding subunit of cytoplasmic origin and the partially purified EBP which is extracted from the nuclear fraction of calf uterus homogenate were compared to find no significant difference in molecular and other properties as investigated by present methods.

The idea that the action of a hormone must involve specific interaction with a component (receptor) of the target cell is old (Ehrlich, 1902). Only recently, however, the binding of estrogens to target tissues was discovered (Jensen and Jacobson, 1962). It has been shown that the estrogenic molecule is bound by specific macromolecules in the cytoplasm (Talwar *et al.*, 1964; Toft and Gorsky, 1966; Jensen *et al.*, 1967; Erdos, 1968) and that the complex moves to the nucleus (Brecher *et al.*, 1967; Jensen *et al.*, 1968; Gorsky *et al.*, 1968) where an increased RNA synthesis takes place (Hamilton, 1968). These macromolecules are protein in nature (Toft and Gorsky, 1966) and there is ample evidence of their highest affinity for estrogenic ligands (Toft *et al.*, 1967; Puca and Bresciani, 1969a; Best-Belpomme *et al.*, 1970; Korenman, 1970).

Several estrogen-binding proteins (EBPs)[1] have been described in target tissue homogenates. In prepuberal calf uterus, which is a convenient source of these proteins, a larger and a smaller cytoplasmic EBP have been identified. They are also referred to as 8 S and 4 S, respectively, according to their approximate sedimentation coefficients on a sucrose gradient (De Sombre *et al.*, 1969). The larger EBP shows a marked

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[1] Abbreviations used are: EBP, estrogen-binding protein; BPA, bovine plasma albumin; RTF, receptor transforming factor.