*PURIFICATION OF AN ESTROPHILIC PROTEIN FROM CALF UTERUS**

By E. R. De Sombre, G. A. Puca,† and E. V. Jensen

THE BEN MAY LABORATORY FOR CANCER RESEARCH, UNIVERSITY OF CHICAGO

Communicated by Charles Huggins, June 24, 1969

Abstract.—A method has been devised for purifying the estrogen-binding protein ("uptake receptor") of uterine cytosol which circumvents the marked tendency of this receptor to aggregate during attempted isolation. Ammonium sulfate precipitation of the estradiol-receptor complex of calf uterine cytosol in the presence of calcium ions yields both an 8*S* complex and its 4*S* subunit which are stable during further purification by gel filtration and ion exchange chromatography. The major product is the 4*S* complex which does not revert to 8*S* in the absence of salt and which has been purified about 2500-fold. The approximate isoelectric points of these partially purified 8*S* and 4*S* complexes are 5.8 and 6.4, respectively.

Following the demonstration that estrogen-dependent tissues contain characteristic hormone-binding components called "estrogen receptors" or "estrophiles," extensive investigations in many laboratories[1] have contributed to our knowledge about the interaction of estradiol with target tissues, especially the rodent uterus. In this tissue, estradiol first associates with an extranuclear "uptake receptor" (a protein sedimenting at about 8*S* and reversibly dissociated by 0.3 *M* KCl into 4*S* subunits) which then undergoes a temperature-dependent interaction with the nucleus to produce a new entity, extractable by 0.3 *M* KCl as a 5*S* estradiol-protein complex. It appears that the function of the estrogen is to activate a specific uterine protein to enter the nucleus and to do something there; when the exact nature of this intranuclear reaction of the 8*S* protein is elucidated, the mechanism of estrogen action can be established on a firm biochemical basis.

To help delineate the biochemical role and interrelation of the receptor substances of cytosol and the nucleus, we have undertaken the isolation of tangible amounts of these proteins to compare their composition and structure. Most favorable for isolation is the 8*S* uptake receptor of the cytosol, inasmuch as radioactive estradiol, necessary to detect and follow the protein during purification, binds directly to the receptor when hormone is added to the supernatant fraction of a uterine homogenate. Attempts to purify this protein have been hampered by the small amounts present in tissues[4] and its instability in the supernatant fraction where it tends both to decompose and to form large aggregates during storage, salt precipitation, or gel filtration. The partially purified binding protein recently obtained[5] from rat uterine cytosol would appear to consist entirely of aggregate, inasmuch as it was not included by Sephadex G-200.

This paper describes a simple procedure for converting the uptake receptor of calf uterus to a 4*S* subunit which does not revert to 8*S* protein in the absence of salt and which, in association with estradiol, has remained stable through >2000-

fold purification by salt precipitation, gel filtration, and ion exchange chromatography. This phenomenon provides a feasible approach to the isolation of pure receptor substance by available techniques for protein purification.

Materials and Methods.—These investigations used estradiol-6, 7-H^3 (spec. act. 57.4 Ci/mmole), ammonium sulfate (Mann enzyme grade) saturated in water at 2°, pH 7.2, and the following buffers adjusted to pH 7.4 with hydrochloric acid and containing 1 mM NaN_3: Tris-EDTA (10 mM 2-amino-2-hydroxymethyl-1, 3-propanediol with 1.5 mM ethylenediaminetetraacetic acid, disodium salt), TKC (0.1 *M* Tris, 0.4 *M* KCl, 1 mM $CaCl_2$), TKE (10 mM Tris, 0.01, 0.05, or 0.4 *M* KCl, 1 mM EDTA) and TC (0.1 *M* Tris, 1 mM $CaCl_2$).

Fresh calf-uterine horns were stripped of connective tissue and stored at 0° during a 2- to 4-hr collection period at the slaughter house.‡ All purification steps were carried out at 0–2°. Uterine tissue (in two 120-gm batches) was minced in a meat grinder and homogenized in 4 vol Tris-EDTA with a Polytron PT 35 (Brinkmann Instruments) at setting 6, using six 10-sec homogenizations each followed by a 50-sec cooling period. The homogenate was centrifuged for 3 hr at 78,500 × *g* or 2.7 hr at 115,000 × *g* to yield the supernatant fraction, 670 ml of which was treated with 0.67 ml 10^{-5} *M* estradiol-6,7-H^3 and stirred continuously during subsequent additions and incubations. After 1 hr, 13.68 ml 0.2 *M* $CaCl_2$ was added; and 30 min later 170 ml saturated ammonium sulfate was added gradually during a 30-min period. After an additional 30 min, the mixture was centrifuged 15 min at 9,500 × *g*. The precipitate was resuspended by gentle rehomogenization in 67 ml TKC buffer; after stirring for 30 min, undissolved precipitate was removed by centrifugation for 40 min at 105,000 × *g*.

For gel filtration, Sephadex G-200 was swollen in TKE (0.05 *M* KCl) at room temperature and fines removed by aspiration. DEAE-cellulose (Whatman DE 52, preswollen microgranular) was used without precycling after suspension in TKE (0.05 *M* KCl) and removal of fines. Electrofocusing experiments were carried out at 2° in a 110 ml ampholine column (LKB) using 1% ampholytes in 0 to 50% sucrose gradients; protein samples studied were first passed through Sephadex G-25 equilibrated with 10 mM KCl. The 4*S* protein was focused for 24 hr at 500–800 v in a pH gradient of 5 to 8 and the 8*S* protein for 14 hr at 250–400 v in a pH gradient of 3 to 10, after which successive 2 ml fractions were collected and the pH determined at 2°. The tritium content of aliquot portions of fractions from Sephadex, DEAE-cellulose, and ampholine columns was determined with 24% efficiency in 10 ml Triton X-100 Fluor (4 gm PPO, 50 mg POPOP, 250 ml Triton X-100, and 750 ml toluene). Protein concentrations were based on optical density at 280 and 260 mμ.[6] For sedimentation analysis, 0.2 ml samples were layered on 3.6 or 3.8 ml cold preformed 5–20 or 10–30% sucrose gradients in TKE (10 mM KCl); after centrifugation at 2°, successive 100 μl fractions were displaced (by paraffin oil) through a bottom puncture into counting vials and tritium determined as described above.

The sample of uncomplexed 4*S* protein was obtained by precipitation with ammonium sulfate-Ca^{++} in the absence of estradiol, redissolving in TC and passing the product directly through DEAE-cellulose prepared in TKE (10 mM KCl). Under these conditions, the column adsorbs 8*S* protein and aggregate but does not retain the 4*S* material.

Results.—Precipitation with ammonium sulfate: The first step in the isolation of a receptor protein, present in small amount, should effect concentration as well as purification. The precipitation of the receptor at rather low ammonium sulfate concentrations would fulfill this requirement except that this treatment causes the receptor to aggregate (Fig. 1*A*). This aggregation, also reported by Erdos,[7] probably involves interaction with other proteins of the cytosol, inasmuch as 8*S* complex, partially purified by preparative sucrose-density-gradient centrifugation,[8] shows little aggregation with ammonium sulfate (Fig. 1*B*). If

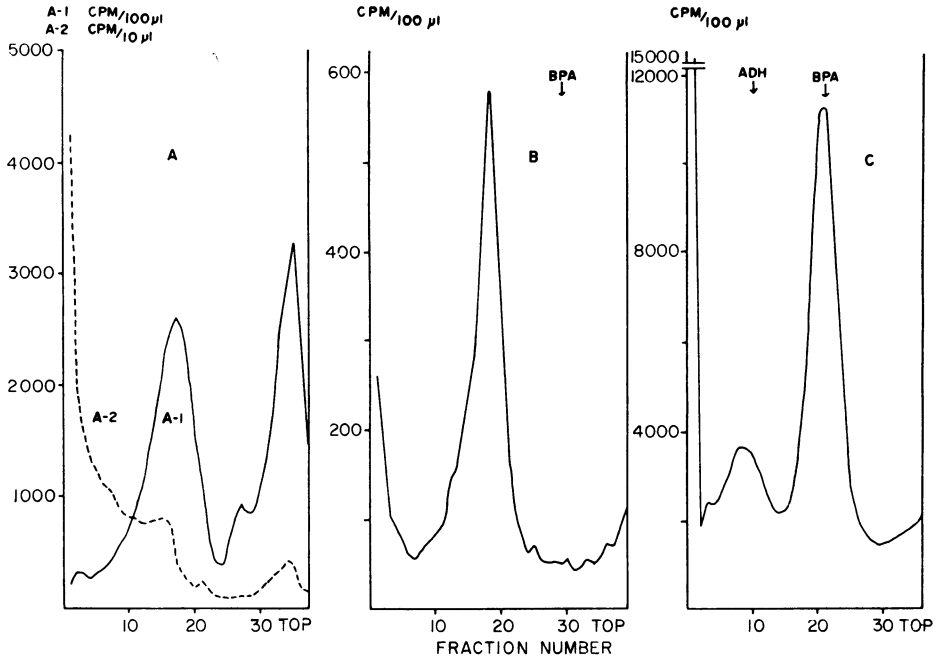

FIG. 1.—Sedimentation patterns of radioactivity from: *A*-1, calf uterine supernatant containing 10 nm estradiol-H^3; *A*-2, Tris-EDTA solution of ammonium sulfate (13% w/v) precipitate of same supernatant; *B*, Tris-EDTA solution of ammonium sulfate (20% w/v) precipitate of 8*S* region of preparative sucrose gradient (48 hr at 75,500 × *g*) of calf uterine supernatant containing 4.6 nm estradiol-H^3; *C*, TKC solution of ammonium sulfate (20% saturation) precipitate of calf-uterine supernatant containing 10 nm estradiol-H^3 and 4 mM $CaCl_2$. Gradients of 5–20% sucrose containing TKE (10 mM KCl) were centrifuged: *A*, 8 hr, 300,000 × *g*; *B*, 7.5 hr, 308,000 × *g*; *C*, 10 hr, 300,000 × *g*. Ammonium sulfate precipitates of *A* and *C* were redissolved in one tenth the original supernatant volume.

the precipitation is carried out with supernatant fraction containing calcium ions (1 mM in excess over that bound by EDTA), most of the precipitated receptor protein which redissolves in TKC sediments at about the rate of bovine plasma albumin even in a salt-free sucrose gradient; some 8*S* protein and aggregate are also present (Fig. 1*C*).

Sephadex G-200 filtration: When uterine supernatant either with or without estradiol is passed through Sephadex G-200, the estradiol-binding component is eluted in or very near the exclusion volume[5, 7]; density gradient analysis of such eluates shows the receptor to be highly aggregated. When the redissolved Ca^{++}-ammonium sulfate precipitate is subjected to such gel filtration, the radioactivity elution pattern (Fig. 2*A*) is remarkably similar to the sedimentation pattern before filtration (Fig. 1*C*). Subsequent density gradient analysis demonstrates that the three radioactivity peaks from G-200 correspond, respectively, to aggregate, 8*S*, and 4*S* complex (Fig. 2*B*). Thus, both the 8*S* and 4*S* complexes obtained after ammonium sulfate-Ca^{++} precipitation can be purified further by gel filtration. Comparison of the elution volumes of the 4*S* and 8*S* peaks with those of protein standards on G-200 indicates that the molecular

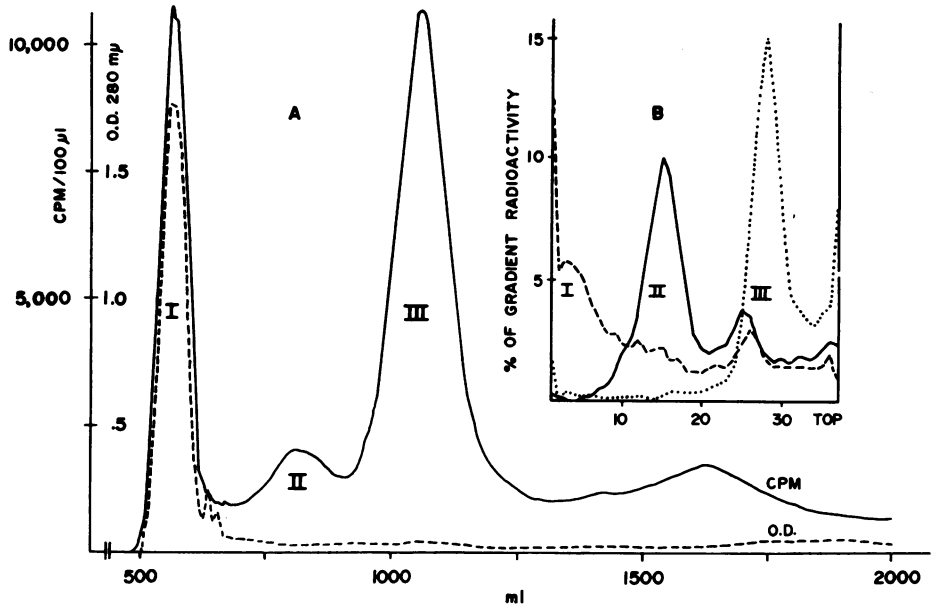

FIG. 2.—*A*, Elution pattern from Sephadex G-200 filtration of TKC solution of ammonium sulfate precipitate of calf uterine supernatant containing 10 nM estradiol-H^3 and 4 mM $CaCl_2$; 61 ml of dissolved precipitate passed through 1570-ml column (flow rate 30 ml/hr). *B*, sedimentation patterns of three radioactive fractions from G-200 column in 10–30% sucrose gradients centrifuged 12 hr at 308,000 × *g*.

weights of the two complexes are about 75,000 and 200,000, respectively (Fig. 3). Although the initial redissolved precipitate slowly forms aggregate on storage, the 4*S* complex purified by G-200 filtration remains stable in prolonged storage at 2°.

DEAE cellulose chromatography: When 4*S* complex (obtained from G-200) is chromatographed on DEAE-cellulose at pH 7.4, radioactivity is eluted with 0.1 *M* KCl slightly ahead of the first peak of contaminating protein (Fig. 4*A*). This radioactivity sediments in a sucrose gradient as a single 4*S* peak. The 8*S* complex, as well as aggregated material, is eluted at higher salt concentration (Fig. 4*B*).

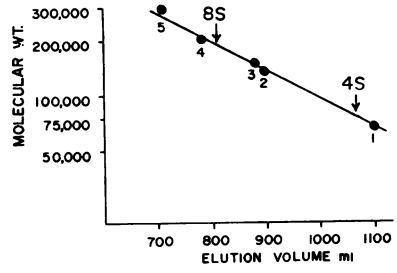

FIG. 3.—Molecular weights of 8*S* and 4*S* estradiol-receptor based on elution volume from Sephadex G-200 (1570-ml column). Protein standards are: 1, 2, and 4, bovine plasma albumin monomer, dimer, and trimer, respectively; 3 and 5, γ-globulin monomer, and dimer. No correction was made for sample volumes inasmuch as nontruncated peaks were obtained in every instance.

Degree of purification: The purification achieved by the various steps of the foregoing procedure is summarized in Table 1. Assuming that one estradiol molecule is bound per 4*S* receptor unit, pure 4*S* complex would be expected to contain

1.7 × 10^9 DPM/mg. After the purification sequence described, 8 per cent of the original receptor is recovered as 4*S* subunit enriched to about 2500 times the level in the original uterine solid and representing 4*S* estradiol-protein complex of about 2 per cent purity.

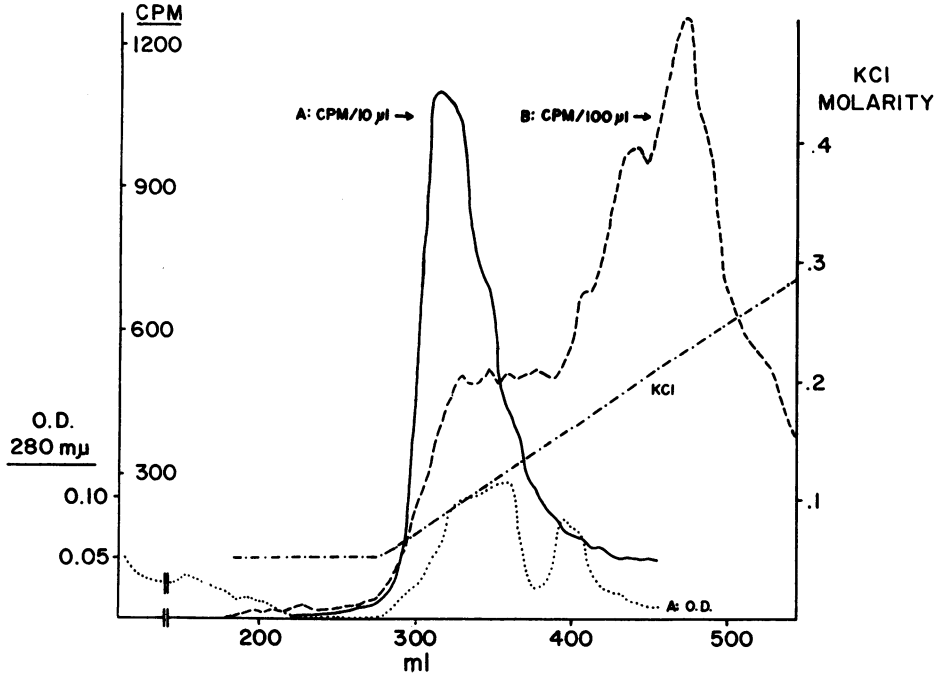

FIG. 4.—DEAE-cellulose chromatography of 4*S* (*A*) and 8*S* (*B*) estradiol-receptor complexes obtained from Sephadex G-200 column. The first 200 ml of eluate consists of the sample applied (185 ml (*A*), 75 ml (*B*)) on a 75-ml column packed in TKE (50 mM KCl) plus wash TKE, after which a linear 50 to 400 mM KCl (in 10 mM Tris-1 mM EDTA) gradient was applied to the top of the column. The KCl concentration of the effluent was determined conductimetrically.

TABLE 1. *Purification and recovery of receptor proteins.*

	Receptor form	DPM/ml (× 10^5)	Protein (µg/ml)	DPM/mg (× 10^4)	Percentage recovered*	Purification†
Homogenate			40,000‡			
Supernatant	8*S*	6.44§	9420	6.8	100	5
Ammonium sulfate ppt.¶	8*S*	7.91§	2570	31	12.3	23
	4*S*	15.92§		62	24.7	45
G-200 eluate	8*S*	0.64	27	238	3.8	174
	4*S*	2.21	26	854	17.5	623
DEAE eluate	4*S*	4.11	12	3400	8.4	2480

* Based on 8*S* plus small amount of aggregate in supernatant fraction as determined by sedimentation analysis. This calculation neglects indeterminable loss in receptor capacity taking place during preparation of the supernatant fraction.

† Based on total uterine solid.

‡ Total uterine solid.

§ Determined by sedimentation analysis.

¶ Redissolved in 0.1 the original supernatant volume.

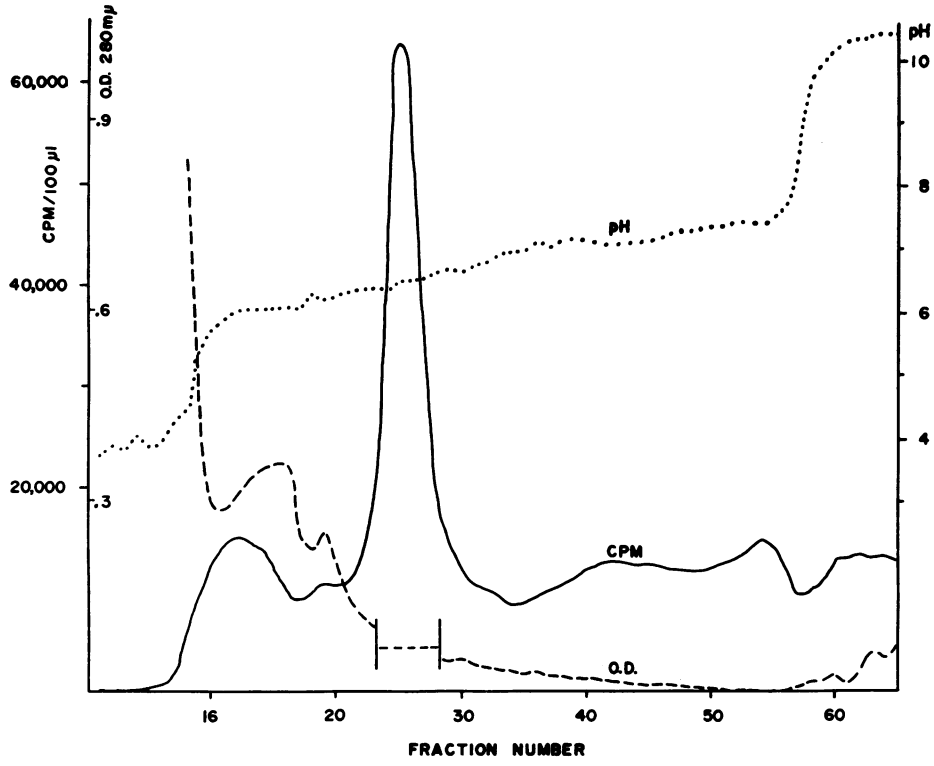

FIG. 5.—Isoelectric focusing of 4S estradiol-receptor complex obtained from a different pool of calf uteri but purified in a fashion similar to that described in Figs. 2 and 4. The run was carried out for 24 hr reaching a final field of 800 v and 0.73 ma. Fractions comprising the peak of radioactivity were combined before optical density was determined.

Isoelectric focusing: The isoelectric point of purified 4S estradiol-receptor complex was determined by isoelectric focusing (Fig. 5). Although a significant background of radioactivity is seen throughout the gradient, a single major peak of radioactivity appeared at about pH 6.4; this was shown to be 4S complex by density gradient centrifugation. Isoelectric focusing of purified 8S complex showed bound estradiol localized at about pH 5.8, confirming the previous indication from DEAE-cellulose chromatography that the 8S protein is more acidic than the 4S unit. Because most of the contaminating protein is localized in the lower pH region, preparative isoelectric focusing offers promise for the further purification of both receptor complexes.

Specificity of binding: The foregoing purification was carried through with tritiated estradiol bound to the receptor, since this complex is considerably more stable than the receptor protein alone. To provide uncomplexed 4S subunit for evaluation of its ability to associate with estradiol, a rapid purification was effected in the absence of estradiol (see *Materials and Methods*). Using the Sephadex G-25 technique of Puca and Bresciani,[9] this preparation was found to show strong, saturable estradiol binding, similar in all respects to that of the uterine supernatant fraction and inhibited by an excess of either hexestrol or nafoxidine

(Upjohn 11,100). The complex formed with estradiol shows a single 4*S* sedimentation peak in sucrose gradients. Thus, this stable 4*S* unit appears to be closely related to the estrophilic portion of the 8*S* protein in the original supernatant; chemical and immunochemical studies of the 4*S* protein, after its further purification, should provide information relevant to the nature and physiological function of the estrogen receptor of uterine cytosol.

Note added in proof: If the uterine supernatant fraction containing estradiol-H^3 is made 1 *M* in KCl (to dissociate the 8*S* complex to 4*S* sub-units) before treatment with ammonium sulfate, the redissolved precipitate gives a higher yield of 4*S* product with practically no aggregate or 8*S* complex. With this modification, 5000-fold purification of the 4*S* complex can be achieved by the 3-step procedure.

* Presented June 29, 1969, at the 51st meeting of The Endocrine Society, New York, New York. These investigations were supported by research grants from the National Cancer Institute (CA-02897), the American Cancer Society (P-422), and the Ford Foundation (690-0109).

† NATO postdoctoral fellow on leave from the Institute of General Pathology, University of Messina.

‡ We are grateful to the Nagle Packing Co., Lansing, Illinois, for their generous cooperation in supplying large quantities of calf uteri.

[1] More detailed description of the estrogen-receptor interaction, with specific citation of the work of Baulieu, Brecher, Bresciani, Eisenfeld, Erdos, Glascock, Gorski, Greenblatt, King, Korenman, Maurer, Stone, Stumpf, Talwar, Terenius, and their associates, as well as our own previous papers, is given in references 2 and 3.

[2] Jensen, E. V., E. R. DeSombre, D. J. Hurst, T. Kawashima, and P. W. Jungblut, in *Colloque International sur la Physiologie de la Reproduction chez les Mammifères, Paris 1966*, ed. A. Jost, *Arch. d'Anat. Microscop.* (Suppl.), **56**, 547 (1967).

[3] Jensen, E. V., T. Suzuki, M. Numata, S. Smith, and E. R. DeSombre, *Steroids*, **13**, 417 (1969).

[4] Assuming one estradiol molecule bound per 4*S* subunit, immature rat uterus contains about 8 ng of uptake receptor per milligram fresh tissue, calf uterus somewhat less.

[5] Soloff, M. S., and C. M. Szego, *Biochem. Biophys. Res. Commun.*, **34**, 141 (1969).

[6] Kalckar, H. M., *J. Biol. Chem.*, **167**, 461 (1947).

[7] Erdos, T., *Biochem. Biophys. Res. Commun.*, **32**, 338 (1968).

[8] The supernatant fraction layered on the preparative sucrose gradient had been lyophilized and redissolved; when precipitated by ammonium sulfate before density gradient purification, this reconstituted supernatant formed an aggregate similar to that illustrated for fresh supernatant (Fig. 1, *A*-2).

[9] Puca, G. A., and F. Bresciani, *Nature*, **223**, 745 (1969).