PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 174, 350–355 (1983)

Rat Liver Nuclei Contain Receptors for a Folate Binding Protein (41747)

MARIA DA COSTA, SHELDON P. ROTHENBERG, AND STEPHEN J. BECKMAN

Department of Medicine, SUNY-Downstate Medical Center and Brooklyn Veterans Administration Medical Center, 800 Poly Place, Brooklyn, New York 11209

Abstract. A folate binding protein purified from the cytoplasm of human chronic myelogenous leukemia cells and saturated with [^{3}H]pteroylglutamic acid, and the same protein labeled with ^{125}I and saturated with pteroylglutamic acid, binds to the nuclear fraction of rat liver. EDTA inhibits this binding and this inhibition is reversed by Ca^{2+} but not by Mg^{2+}. The nuclear fraction binds very little free [^{3}H]pteroylglutamic acid, and the cytoplasm from which the nuclei have been removed does not bind the protein–folate complex. A K_d of 0.7 nM and a value of 1000 unsaturated binding sites per nucleus were obtained by Scatchard analysis. The translocation of folate to the nuclear membrane or nucleus by this soluble cytoplasmic folate binder may be the mechanism for the induction of enzyme(s) required for the metabolism of the folate ligand attached to the protein.

Folate binding proteins (FBP) have been identified in milk (1), plasma (2), leukemia cells (3), intestinal brush borders (4), liver (5, 6), kidney (7, 8), choroid plexus (9), and malignant tumors (10). The functions of cellular forms of FBP(s) have not been defined except for one purified from rat liver which appears to have enzymatic activity (11, 12). Membrane-bound FBP may have some role in folate transport (13): those binding proteins located in the cytoplasm may serve to retain folate in the cell (14), or modulate folate biosynthetic pathways by sequestering the folate cofactor as a nonfunctional folate–protein complex (15); and the FBP in milk has been shown to enhance the uptake of folate by isolated mucosal cells from rat small intestine (16).

Two observations in our laboratory led us to test the hypothesis that the FBP may function to translocate folate cofactors to the nucleus of the cell. First, we observed that the concentration of the FBP in the nuclear fraction of chronic myelogenous leukemia cells was considerably higher when measured using a radioimmunoassay (which assays both apo and holo binder) (17), than when measured by the binding of [^{3}H]folic acid (pteroylglutamic acid, PteGlu), which measures only apo binder (unpublished observations). Second, we observed by immunofluorescent cytology using a monospecific antiserum against the FBP purified from chronic myelogenous leukemic (CML) cells (18), that fluorescence could be seen around the nucleus of these cells, in addition to cytoplasmic fluorescence (Fig. 1).

We could not extend these studies using CML cells to determine the direct binding of ligand saturated FBP to isolated nuclei because it is extremely difficult to prepare the nuclei of these cells free of major contamination by cytoplasmic organelles. We, therefore, used rat liver nuclei, which are more readily separated from cytoplasmic components, to examine our hypothesis.

Materials and Methods. Sprague–Dawley rats, food-fasted for 14–72 hr, were decapitated and exsanguinated. The liver was immediately excised, placed in cold 0.15 M sodium chloride, minced into small pieces, rinsed free of blood, and then suspended in approximately 5 vol of 0.02 M Tris buffer, pH 7.4, containing 0.32 M sucrose, 0.02 M KCl, 0.003 M $MgCl_2$, phenylmethylsulfonyl fluoride (PMSF) (3.5 mg/liter), and Trasylol (10,000 KIU/liter). All these and subsequent steps were carried out at 4°C. The liver was homogenized with four to five up-and-down strokes of a motor-driven pestle turning at 1000 rpm. The nuclei and intact cells were pelleted by centrifugation at 100g for 5 min and then resuspended in 50 ml of buffer. The supernate from this step, containing the cytoplasmic organelles, were used as the "granule" fraction in experiments described below. The whole cells mixed with the nuclear suspension were separated by gravity sedimentation for 60 min. This step was repeated twice and the nuclei, which re-

0037-9727/83 $1.50

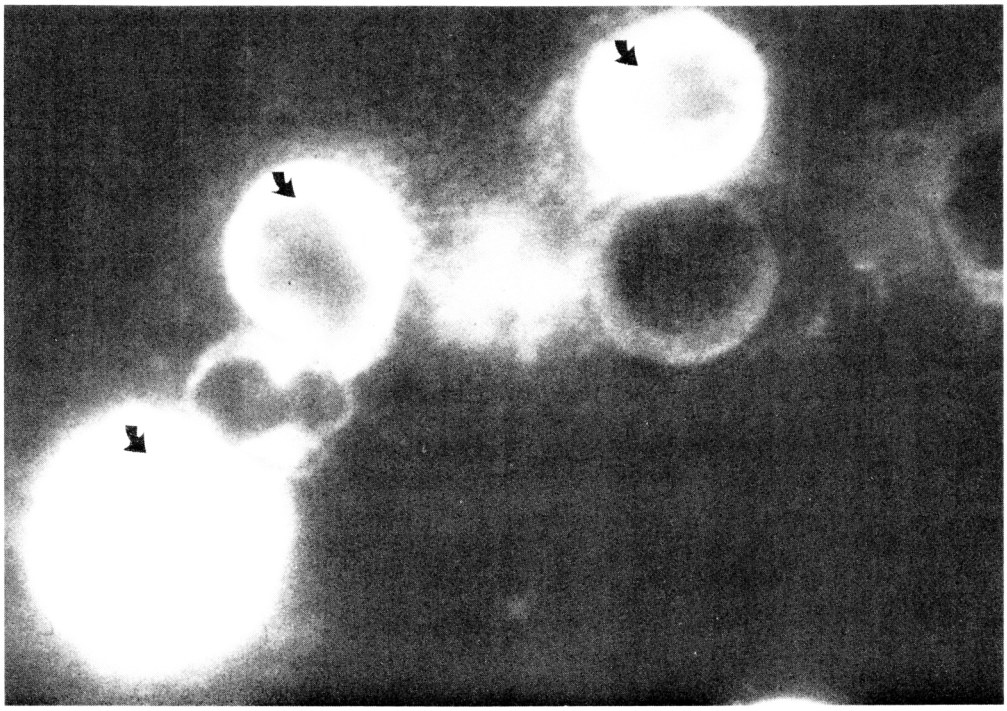

FIG. 1. Immunofluorescent cytology for the FBP in human chronic myelogenous (CML) leukemia cells. Cytocentrifuge slides were prepared with washed CML cells from heparinized blood and fixed in absolute methanol for 5 min. The area of cells was overlayed with 10 μl of the rabbit antiserum to the FBP for 30 min, washed thoroughly, and then overlayed with isothiocyanate-labeled goat antiserum to rabbit γ globulin. After 30 min incubation, the slides were washed, mounted in 90% glycerol under a coverslip, and examined for fluorescence using the Leitz Ortholux II fluorescent microscope. Photographs were made with 100X oil immersion objective. The fluorescence observed with the normal rabbit serum and antiserum absorbed with purified FBP was too low to photograph.

mained in the supernate, were then pelleted by centrifugation at 100*g*, washed twice, and suspended to a final concentration of 400 $\times$ 10^6 nuclei/ml in Hank's balanced salt solution (HBSS) containing the PMSF and Trasylol. The final suspension of nuclei usually contained approximately 5% intact cells. There were also contaminating granules which were quantified by acid phosphatase activity (*vide infra*).

One hundred microliters of this nuclear-rich fraction containing 40 $\times$ 10^6 nuclei, were incubated separately with 200 pg of free [^{3}H]PteGlu (Amersham) and with 200 pg of [^{3}H]PteGlu of which 80% was bound to the purified FBP from chronic myelogenous leukemia cells (18), in a total volume of 250 μl of HBSS at 4°C. To determine the binding of these ligands to the non-nuclear components, an aliquot of the granule fraction, adjusted to contain the same acid phosphatase activity as the nuclear fraction, was similarly incubated with the [^{3}H]PteGlu and the FBP–[^{3}H]PteGlu

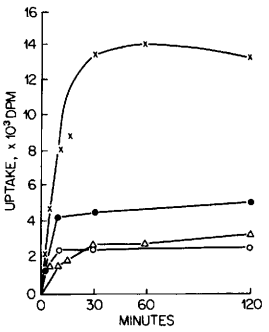

FIG. 2. Uptake of the FBP–[^{3}H]PteGlu complex by the nuclear fraction of rat liver. $\times$, nuclear rich fraction + FBP–[^{3}H]PteGlu; $\triangle$, nuclear-rich fraction + [^{3}H]PteGlu; $\bullet$, granule fraction + FBP–[^{3}H]PteGlu; $\circ$, granule fraction + [^{3}H]PteGlu.

TABLE I. UPTAKE OF FBP-[^{3}H]PteGlu AND FREE [^{3}H]PteGlu BY SUBCELLULAR FRACTIONS OF RAT LIVER

Subcellular fraction (g)[a]	Number of nuclei (per reaction)	DNA[b] (OD)	Acid phosphatase[b] (OD)	Alkaline phosphatase[b] (OD)	Binding of [^{3}H]PteGlu (dpm)[c]	Binding of FBP-[^{3}H]PteGlu (dpm)[c]
100	12×10^6	0.31	2.7	0.12	2046	3391
3,000	7.5×10^5	0.05	2.4	0.15	2338	1763
12,000	0	0.027	2.9	0.29	3212	310
27,000	0	0.001	3.1	0.30	4842	0

[a] The pellet was obtained by centrifugation at the indicated g force for 5 min and then suspended in 1–2 ml of HBBS and analyzed for each of the listed parameters.

[b] These optical density (OD) values represent the absorbancy of the respective assay reactions for DNA, acid phosphatase, and alkaline phosphatase.

[c] These parameters were determined by incubating 200 μl of each suspension with free [^{3}H]PteGlu or FBP-[^{3}H]PteGlu, in a total volume of 250 μl. After 120 min of incubation at 4°C, the suspension was centrifuged at the fractionation speeds, the pellets were washed twice, dissolved in NCS solubilizer, and the radioactivity determined. The results are the mean of triplicate reactions.

complex. After incubation, the nuclei and granule pellets were washed and then solubilized in 1 ml of NCS solubilizer (Amersham) and mixed with the scintillation fluor containing 7 g PPO and 150 mg POPOP/liter of toluene. The radioactivity was determined using a liquid scintillation counter (Packard Tricarb) and sufficient counts were accumulated for an error of ±3%.

DNA was measured as described by Burton (19). Acid and alkaline phosphatase were measured as described by Michell and coworkers (20). The FBP saturated with PteGlu was labeled as previously described with ^{125}iodine by the chloramine T method (17).

Results. The uptake of the FBP-[^{3}H]PteGlu complex at 4°C by the nuclear fraction reached a maximum at 30 min and remained constant for the 120 min of observation (Fig. 2). The uptake of free [^{3}H]PteGlu by both the nuclear and granule fractions, and the uptake of the FBP-[^{3}H]PteGlu complex by the granule fraction was substantially lower.

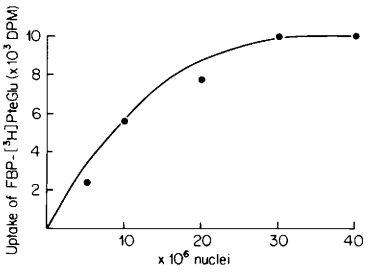

FIG. 3. Uptake of the FBP-[^{3}H]PteGlu complex as a function of the number of nuclei in the incubation mixture.

In another experiment, subcellular fractions of liver homogenate were prepared by differential centrifugation and the uptake of FBP-[^{3}H]PteGlu by each fraction was correlated with the number of nuclei, the DNA concentration, and the activity of acid phosphatase and alkaline phosphatase. We selected acid phosphatase as a marker enzyme because it has been found by a number of investigators (21, 22) to be associated with heavy and light mitochondrial and microsomal fractions (73–84%) and less with nuclei (3–9%) following differential centrifugation and would thus serve as a control for the binding of the FBP by contaminating organelles. We selected alkaline phosphatase as a marker because it is associated with heavy membrane components (23) that could contaminate the nuclear preparation. The highest uptake of the FBP-[^{3}H]PteGlu complex occurred in the fraction containing the greatest number of nuclei and the highest concentration of DNA (Table I). There was no correlation of acid phosphatase activity with the uptake of FBP-[^{3}H]PteGlu. The uptake of free [^{3}H]PteGlu did correlate directly with the activity of alkaline phosphatase in each fraction indicating that there is an insoluble unsaturated FBP associated with heavy and light membrane fragments sedimenting at each g force used for this experiment.

The uptake of the FBP-[^{3}H]PteGlu complex as a function of the number of nuclei is shown in Fig. 3. The uptake of the complex was clearly proportional to the number of nuclei in the incubation mixture. EDTA inhib-

TABLE II. EFFECT OF EDTA ON THE UPTAKE OF FBP-[^{3}H]PteGlu BY THE NUCLEAR-RICH FRACTION OF RAT LIVER

Experiment[a]	Radioactivity bound to nuclear pellet (dpm)[b]
1. FBP-[^{3}H]PteGlu, alone	5550 (5162–5843)
2. [^{3}H]PteGlu, alone	1845 (1704–2091)
3. FBP-[^{3}H]PteGlu + EDTA (4 mM)	737 (669–805)
4. FBP-[^{3}H]PteGlu + EDTA (4 mM)+ $CaCl_2$ (10 mM)	6107 (5642–6444)
5. FBP-[^{3}H]PteGlu + EDTA 4 (mM) + $MgSO_4$ 10 (mM)	730 (605–824)

[a] There were 20 × 10^6 nuclei incubated in each experimental reaction as described under Materials and Methods.
[b] The values are the means of triplicate reactions; the ranges are in parentheses.

ited the uptake of binding of the FBP-[^{3}H]PteGlu complex (Table II) and this inhibition was reversed by Ca^{2+} but not Mg^{2+}. Thus, this system, like many receptor–protein interactions (24) requires calcium.

The results of saturation kinetic studies are shown in Fig. 4. For these experiments, nonspecific binding was determined by measuring the uptake of FBP-[^{3}H]PteGlu in the presence of an excess of FBP saturated with unlabeled PteGlu and this value was subtracted from the total uptake of radioactivity to determine the specific uptake. Scatchard plot (25) analysis of these data (inset of Fig. 4) shows an apparently linear plot indicating that the FBP-[^{3}H]PteGlu is reacting with an homogenous class of binding sites, computed to number 1000 sites per nucleus and having a K_d of 0.7 nM.

Direct evidence that the nuclei were binding (or taking up) the FBP moiety of the complex was obtained by incubating the nuclear rich (and control granule fraction) with the purified FBP which was labeled with ^{125}iodine. Ninety percent of the radioactivity was immunoreactive with monospecific rabbit antiserum to the FBP (17). Like the FBP-[^{3}H]PteGlu complex, the ^{125}I-labeled FBP-PteGlu also binds to the nuclear-rich and not to the granule fraction, and EDTA and an excess of FBP (apo and holo) inhibited this binding (Table III).

Figure 5 shows the dose–response curves for the inhibition of the nuclear binding of ^{125}I-labeled FBP-PteGlu by unsaturated (apo) and PteGlu saturated (holo) FBP. There appears to be no difference in the affinity of the apo and holo forms of the FBP for the nuclear binding site.

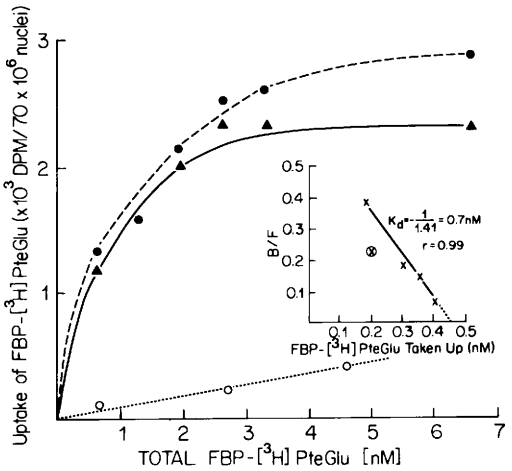

FIG. 4. Saturation kinetics for the binding of the FBP-[^{3}H]PteGlu complex by the nuclear-rich fraction of rat liver. Aliquots of the nuclear-rich fraction containing 70 × 10^6 nuclei were incubated in triplicate with 0.55–6.62 nM FBP saturated with [^{3}H]PteGlu in a total volume of 250 μl for 2 hr at 4°C. Nonspecific binding was determined by incubating FBP-[^{3}H]PteGlu and nuclear suspension in the presence of a 15-fold excess of the FBP saturated with unlabeled PteGlu. The specific uptake (▲) was determined by subtracting the nonspecific uptake (○) from the total uptake determined in the absence of the excess FBP (●). For the Scatchard analysis, shown in the inset, the total amount of the FBP-[^{3}H]PteGlu taken up by the nuclei was computed as the product of the percentage uptake of FBP-[^{3}H]PteGlu times the total concentration of FBP-[^{3}H]PteGlu in the incubation mixture. This was expressed as nanomole bound per liter of reaction mixture (i.e., nM) in order to facilitate the direct derivation of the K_d from the reciprocal of the slope of the line (1.41 liter/mole), which is the association constant. The number of binding sites per nucleus was obtained from the number of molecules of FBP-[^{3}H]PteGlu bound to the nuclei (Avogadro's number × moles bound to the nuclei) divided by the number of nuclei in the reaction mixture. The point ⊗ was considered an experimental error.

TABLE III. UPTAKE OF ^{125}I-LABELED FBP BY THE NUCLEAR-RICH AND GRANULE FRACTION OF RAT LIVER

Fraction	Counts per minute bound to pellet[a]				
	^{125}I-FBP	^{125}I-FBP + EDTA	^{125}I-FBP + unsaturated FBP (640 ng)	^{125}I-FBP + FBP (640 ng) saturated with PteGlu	^{125}I-FBP + free PteGlu (6.4 ng)
Nuclei[b]	2042	433	394	328	1971
Granule	199	131	—	—	—

[a] The values are the mean of triplicate experiments. Each reaction mixture contained 10,000 cpm of radioactivity.
[b] The number of nuclei per incubation was 40×10^6.

This binding of FBP–[^{3}H]PteGlu by the nuclear rich fraction was not observed if the nuclei were prepared using the detergent Triton X-100 (26) or citric acid (27) to separate the contaminating granules. Since our control experiments using the nuclear free cytoplasmic fractions containing organelles and membrane fragments sedimenting up to 27,000*g* did not bind the FBP–[^{3}H]PteGlu complex, it is likely that the nuclear site is damaged by the citric acid and by the Triton X-100. Coetzee and co-workers have shown that this detergent does alter the phospholipid and protein components of hepatic nuclear membranes (28).

Discussion. This report describes for the first time a phenomenon which may be important in folate metabolism. The binding of this cytoplasmic FBP to the nucleus of the cell satisfies the criteria of a specific protein–receptor interaction; i.e., the binding follows saturation kinetics with a very low K_d of 0.7 n*M;* the binding sites appear to be of a single order of activity; and the binding is calcium dependent. The binding of the human FBP by rat liver nuclei is similar to other types of protein–receptor interactions which are not species specific (29).

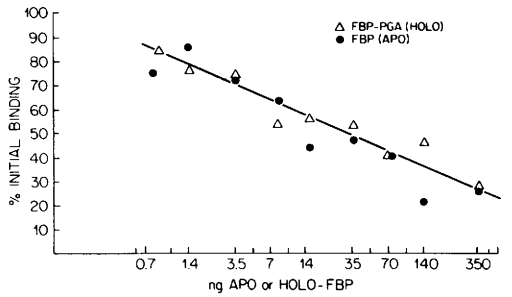

FIG. 5. The inhibition of binding of ^{125}I-labeled FBP by saturated FBP (holo, △) and by unsaturated FBP (apo, ○).

The mechanism by which gene expression is initiated in eukaryotic cells is poorly understood but it is axiomatic that some "message" must reach the nucleus from the cytoplasm if there is to be the induction of protein synthesis which is required for cellular metabolism. A FBP, by transporting a specific folate cofactor to the cell nucleus, could activate the gene for the synthesis of the specific enzyme(s) required for the metabolism of that cofactor. It is known that derepression of a structural gene by a nonhistone acidic protein in the nucleus (30) may be one mechanism for gene activation and since the FBP is an acid glycoprotein (18) it may serve this function.

The role of the FBP as a carrier of the folate cofactor to the nucleus would be analogous to the cytoplasmic steroid receptor proteins which bind and translocate steroid hormones to the nucleus to initiate protein synthesis (31). For example, by this hypothesis, H_2PteGlu, which accumulates in the cell following the inhibition of dihydrofolate reductase (DHFR) by antifolates, would be bound and translocated to the nucleus by the FBP and initiate the induction of DHFR synthesis (32).

The similar affinity of the apo and holo forms of the FBP for nuclear binding sites does not mitigate this hypothesis because this protein, though found in virtually all tissues, is generally not present free in the cell cytoplasm in the unsaturated apo state. Thus, apo-FBP would not be a competitive inhibitor of the binding of the holo-FBP to the cell nucleus.

1. Ghitis J. The folate binding in milk. Amer J Clin Nutr **20**:1–4, 1967.
2. Metz J, Zalusky R, Herbert V. Folic acid binding by serum and milk. Amer J Clin Nutr **21**:289–297, 1968.

3. Rothenberg SP. A macromolecular factor in some leukemic cells which binds folic acid. Proc Soc Exp Biol Med **133**:428–432, 1972.
4. Leslie GI, Rowe PB. Folate binding by the brush border membrane proteins of small intestinal epithelial cells. Biochemistry **11**:1696–1703, 1972.
5. Correcher R, de Sandre G, Pacor ML, Hoffbrand AV. Hepatic protein binding of folate. Clin Sci Mol Med **46**:551–554, 1974.
6. Zamierowski M, Wagner C. High molecular weight complexes of folic acid in mammalian tissues. Biochem Biophys Res Commun **60**:81–87, 1974.
7. Kamen BA, Casten JD. Identification of a folate binder in hog kidney. J Biol Chem **250**:2303–2305, 1975.
8. Selhub J, Rosenberg IH. Demonstration of high-affinity folate binding activity associated with the brush border membranes of rat kidney. Proc Natl Acad Soc USA **75**:3090–3093, 1978.
9. Spector R. Identification of folate binding macromolecule in rabbit choroid plexus. J Biol Chem **252**:3364–3370, 1977.
10. Rothenberg SP, da Costa M, Fischer C, Cohen J. Immunoreactive heterogeneity of folate binding proteins from human tissues. In: Kisliuk RL, Brown GM, eds. Chemistry and Biology of Pteridines; Developments in Biochemistry. Vol 4:pp581–586. Amsterdam, Elsevier, 1979.
11. Wittwer AJ, Wagner C. Identification of folate binding protein of mitochondria as dimethylglycine dehydrogenase. Proc Natl Acad Sci USA **77**:4484–4488, 1980.
12. Wittwer AJ, Wagner C. Identification of folate binding protein of rat liver mitochondria as dimethylglycine dehydrogenase-sarcosine dehydrogenase. Flavoprotein nature and enzymatic properties of purified protein. J Biol Chem **256**:4109–4115, 1981.
13. Huennekens FM, Vitols KS, Henderson GP. Transport of folate compounds in bacterial and mammalian cells. Advan Enzymol **47**:313–346, 1978.
14. Waxman S. Folate binding proteins. Brit J Haematol **29**:23–29, 1975.
15. Rothenberg SP, Fischer C, da Costa M. Binding of N^5N^{10}-methylene tetrahydrofolate and inhibition of thymidylate synthesis by a folate binding protein. Biochim Biophys Acta **543**:340–348, 1978.
16. Colman N. Hettiararchy N, Herbert V. Detection of a milk factor that facilitates folate uptake by intestinal cells. Science **211**:1427–1429, 1982.
17. da Costa M, Rothenberg SP, Fischer C, Rosenberg Z. The identification and measurement of a folate binding protein in human serum by radioimmunoassay. J Lab Clin Med **91**:901–910, 1978.
18. Fischer C, da Costa M, Rothenberg SP. Properties of purified folate-binding proteins from chronic myelogenous leukemia cells. Biochim Biophys Acta **533**:328–339, 1978.
19. Burton K. Study of conditions and mechanism of diphenylamine reaction for colorimetric estimation of deoxyribonucleic acid. Biochem J **62**:315–323, 1956.
20. Michell RH, Karnofsky MF, Karnofsky ML. The distribution of some granulocyte-associated enzymes in guinea pig polymorphonuclear leucocytes. Biochem J **116**:207–216, 1970.
21. de Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. Biochem J **60**:604–617, 1955.
22. Cornbleet PJ, Vorbeck ML, Lucas FV, Esterly JA, Morris HP, Martin AP. Differences in distribution pattern of marker enzymes among subcellular fractions from Morris hepatoma 16. Cancer Res **34**:439–446, 1974.
23. Hubscher G, West GR, Brindley DN. Studies on the fractionation of mucosal homogenates from the small intestine. Biochem J **97**:629–642, 1965.
24. Razin S. Reconstruction of biological membranes. Biochim Biophys Acta **265**:241–296, 1972.
25. Scatchard G. The attractions of proteins for small molecules and ions. Ann NY Acad Sci **51**:660–672, 1949.
26. Berkowitz DM, Kadefuda T, Spron MB. A simple and rapid method for the isolation of enzymatically active HeLa cell nuclei. J Cell Biol **42**:851–854, 1969.
27. Lawson GM, Tsai MJ, Tsai SY, Minghetti PP, McClure ME, O'Malley BW. Nuclei and chromatin: Isolation, characterization and structure. In: O'Malley BE, Schrader WT, eds. Laboratory Methods Manual for Hormone Action and Molecular Endocrinology. Houston, Texas, Baylor College of Medicine pp7–7 to 7–8, 1981.
28. Coetzee ML, Spangler M, Morris HP, Ove P. DNA synthesis in membrane-denuded nuclear fraction from host liver and Morris hepatomas. Cancer Res **35**:2752–2761, 1975.
29. Feldman D, Do A, Burshell A, Stathis P, Loose DS. An estrogen-binding protein and endogenous ligand in *Saccharomyces cerevisae:* Possible hormone receptor system. Science **218**:297–298, 1982.
30. Stein GS, Spelsberg TC, Kleinsmith LF. Nonhistone chromosomal proteins and gene regulation. Science **183**:817–824, 1974.
31. O'Malley BW, Means A. Female steroid hormones and target cell nuclei. Science **183**:610–620, 1974.
32. Jackson RC, Harrap KR. Studies with a mathematical model of folate metabolism. Arch Biochem Biophys **153**:827–841, 1973.

Received May 2, 1983. P.S.E.B.M. 1983, Vol. 174.