ESTRAMUSTINE BINDING IN RAT, BABOON AND HUMAN PROSTATE MEASURED BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

R.Y. Kirdani, J.J. Corrales, P.A. Høisaeter, J.P. Karr, G.P. Murphy and A.A. Sandberg

Roswell Park Memorial Institute, Buffalo, N.Y. 14263

Received 6-6-80

ABSTRACT

High pressure liquid chromatography (HPLC) was used to determine ^{3}H-estramustine (estradiol-17β3N-bis-[2-chlorethyl] carbamate), ^{3}H-17β-hydroxy-5α-androstan-3-one (^{3}H-dihydrotestosterone or ^{3}H-DHT), ^{3}H-estradiol-17β (^{3}H-E_2) and ^{3}H-3β-hydroxy-5-pregnen-20-one (^{3}H-pregnenolone) binding in 50µl of cytosol utilizing a column which separates proteins in the molecular weight range of 2,000 to 70,000 daltons. The rat prostate contains a protein in considerable concentration and with the highest affinity for estramustine (375,000dpm ^{3}H-estramustine per mg. cytosol protein) among the substances tested. Operationally, we have named this protein "estramustine binding protein" (EBP), though it is very likely similar to other previously described prostatic proteins (e.g., α-protein, prostatein, prostatic binding protein). The sensitivity of the HPLC method disclosed EBP-like proteins, but in much lesser concentrations, in some of the other tissues tested. The concentration of these proteins in the human and baboon prostates was much lower (average for the baboon cranial lobe 4800dpm/mg cytosol protein, with a somewhat higher value for the caudal lobe) than that in the rat gland. The amount of the EBP-like protein was higher in prostatic cancer than in that of benign prostatic hypertrophy (BPH) (range 9350 - 25,900 vs. 2200 - 18,900 dpm/mg cytosol protein). In the human, the highest value was found in one normal prostate tested (106,000dpm/mg cytosol protein).

INTRODUCTION

The terms "α-protein" (1), "prostatic binding protein" (2) and "prostatein" (3) appear to designate the same steroid binding protein found in high concentration in rat ventral prostate (20-40% of the cytosol protein) and in the prostatic fluid of that gland. The synthesis of this protein is under hormonal control; dihydrotestosterone (DHT) is bound to this protein with a lower affinity (10^{-6}-10^{-7}) than that steroid to its receptor (1-3).

The above three groups of workers (1-3) have characterized this binder in its affinity for steroids. Independently, however, a fourth

group of investigators (4) found that the synthetic compound, estramustine, localized in the rat ventral prostate and subsequently were able to isolate and characterize this "estramustine binding protein" showing that its amino acid composition, among other properties, was very similar to that of "prostatic binding protein" of Heyns and De Moor (2). As compared to steroid binding however, the binding of estramustine to this protein was of a higher magnitude ($K = 1.7 \times 10^{-8}$). Forsgren et al. (5) were also able to show that antibodies to rat estramustine binding protein cross-reacted with human prostatic cytosol, indicating that the human gland may contain a similar protein.

If EBP and the protein found in the human and baboon glands are similar, then it would seem that measurement of a protein having the aforementioned properties, viz., high concentration, hormonal dependancy, as well as a moderately high affinity for binding estramustine could be of practical significance as a marker in conditions of the prostate. In this paper, we present data on the binding of estramustine to cytosol from prostates of the rat (ventral prostate), baboon and human. For the determination of the estramustine binding protein (EBP) and the EBP-like protein, we have used recently available high pressure liquid chromatography (HPLC) protein analysis columns. Advantages of this technique include good resolution, small sample size and rapidity of measurement, as compared to sucrose density gradient centrifugation (SDGC).

MATERIALS AND METHODS

Chemicals and Radioactive Compounds: Tris-(hydroxymethyl)-aminomethane and the disodium salt of EDTA were purchased from Eastman Kodak Co. (Rochester, N.Y.), sucrose from Schwarz/Mann Co. (Orangeburg, N.Y.), D-dithiothreitol and protease from Sigma Chemical Co. (St. Louis, Mo), sodium sulfate (analytical reagent) from Mallinckrodt Inc., (Paris, Kentucky), scintillation liquid (Complete Counting

Cocktail 3a70) from Research Products International Co. (Elk Grove Village, Ill.), and blue dextran 2000 from Pharmacia Fine Chemicals Inc. (Piscataway, N.J.).

Tritiated estramustine labeled at positions 6 and 7 of the steroid moiety with a specific activity of 59Ci/mmole was a gift from the Hoffman LaRoche Co. (Nutley, N.J.). Tritiated dihydrotestosterone (Sp.Ac. 50.6Ci/mmole), tritiated estradiol-17β (E_2) (Sp. Ac. 94Ci/mmole) and tritiated pregnenolone (Sp.Ac. 24.2Ci/mmole) were purchased from New England Nuclear Co. (Boston, Mass.). Unlabeled E_2 and estrone (E_1) were obtained from Sigma Co. (St. Louis, Mo.). Unlabeled estramustine and estromustine (estrone 3N-bis[2-chloroethyl] carbamate) were gifts from AB Leo (Helsingborg, Sweden).

High Pressure Liquid Chromatography (HPLC): Fifty microliters of the cytosol incubation mixture (see below) was injected into the port of the liquid chromatograph (Waters Associates, Milford, Mass., model ALC/GPC-244). Two protein analysis columns (Waters Associates, model I-125) were used in series. The elution was accomplished at 1ml per minute using the same buffer as that employed for the cytosol preparations. Fractions of 1 ml were collected in scintillation vials for determination of radioactivity. The protein profile of the cytosol was analyzed by recording the absorbance at 280nm.

The columns were washed extensively between each analysis to free them of any contaminating radioactive ligand. The columns were first washed with water for 15 minutes at 3ml/min., followed by 50% methanol in water for 15 min. at the same rate, and finally with methanol at 4ml/min. for 15 min. To prepare the columns for the following analysis, 50% methanol in water was passed through the column for 10 min. at 3ml/min., followed by water and then buffer for 10 min., each at 3ml/min. After reducing the flow rate to 1ml/min., the instrument was ready for the next injection.

To establish the time relationship between the O.D. recording of the eluate and its final collection, a solution of blue dextran was injected. A 3-min. lag-time between optical density read-out of the sample and its collection into the scintillation vials was observed. In all the curves presented here, corrections have been made for this time difference between protein profile and radioactivity measurements.

Human Samples, Animals and Cytosol Preparation: Prostatic samples were obtained from 17 patients: 1 normal (autopsy immediately following death), 10 with BPH (2 autopsy, 8 surgical) and 6 with cancers of the prostate (6 surgical). In addition, studies were performed on three human abdominal wall muscle samples and one serum.

Five-month-old Wistar-Furth rats were supplied by Microbiological Associates, Inc. (Bethesda, Md.). The rats were maintained under standard conditions, fed a standard diet (Teklad animal pellets, Windfield, Iowa) and given water ad libitum. The animals were killed under ether anesthesia by cervical dislocation and the ventral prostates dissected out and weighed.

Adult male baboons (Papio cyanocephalus) weighing 18-25kg were caged individually and, along with a daily supplement of fresh fruits and vegetables, fed Teklad primate diet ad libitum. Cranial and caudal prostatic lobes from these baboons were stored at -70°C before homogenization.

For HPLC, prostate, muscle and liver tissue samples (280-490mg)

were minced with scissors in 24 volumes of a buffer containing 0.1M Na_2SO_4 and 0.1M Tris-acetate(pH 7.0). The homogenization was carried out in Kontes glass Dual-21 homogenizers with 10 strokes. All tissue handling procedures were performed in a cold room with the samples on ice. The homogenates were ultracentrifuged at an average of 105,000×g for 60 min. in a Beckman L2-65B ultracentrifuge and the supernatants carefully removed. The cytosol samples were stored at -20°C before use.

For sucrose density gradient centrifugation (SDGC), the rat ventral prostates were minced in 6 volumes of TED buffer (0.05M Tris, 0.0015M EDTA and 0.00025M D-dithiothreitol, pH 7.4) and the procedures used were the same as those described above.

EBP Measured by SDGC: 0.5ml aliquots of cytosol from the rat ventral prostate were incubated with 40nM ^{3}H-estramustine for 1 hr. at 0°C and 0.4ml then layered on the top of 5-15% sucrose gradients (Density Gradient Former, Beckman Instruments, Palo Alto, Ca.) prepared in 0.05M Tris containing 0.00025M D-dithiothreitol and 10% glycerol. The gradients were ultracentrifuged at an average of 180,000×g for 20 hours at 1°C using an SW-41 rotor in a Beckman L2-65B ultracentrifuge. Fractions were collected from the bottom of the tubes in a Universal Fraction recovery system (Beckman Instruments, Palo Alto, Ca.). Bovine serum albumin (4.6S) was used as a reference for S-value estimations. After the addition of 10ml of scintillation liquid to each vial, the amount of radioactivity was determined in Packard Liquid Scintillation Spectrometers, models 3375 or 2450, at an efficiency of approximately 40%.

EBP Measured by HPLC: For tissue specificity studies, ^{3}H-estramustine (40nM) was added to 0.5ml aliquots of serum and cytosol from ventral prostate, muscle and liver and incubated for 1 hr. at 22°C before analysis by HPLC (as described above). For steroid specificity studies, 0.5ml of cytosol from ventral prostate was incubated for 1 hr. at 22°C with H-estramustine (40nM), ^{3}H-E_2 (40nM), ^{3}H-DHT (43nM) and ^{3}H-pregnenolone (40nM) before being injected into the HPLC column.

Saturation analyses were carried out using 0.5ml of cytosol incubated for 1 hr. at 22°C with concentrations of ^{3}H-estramustine ranging between 1 and 160nM, and a recycling experiment was performed as described in the "Results" section.

Protein Determination: The protein concentrations of the various samples were determined according to the method of Lowry et al. (6). Measurements were performed on the whole cytosol.

Thin Layer Chromatography (TLC): This was performed using polysilic acid gel impregnated glass fiber sheets (Gelman Instruments Co., Ann Arbor, Mich.) developed in chloroform:methanol (98:2).

RESULTS

Identification of EBP: In Fig. 1 are shown the results from SDGC experiments performed on rat ventral prostate cytosol incubated with

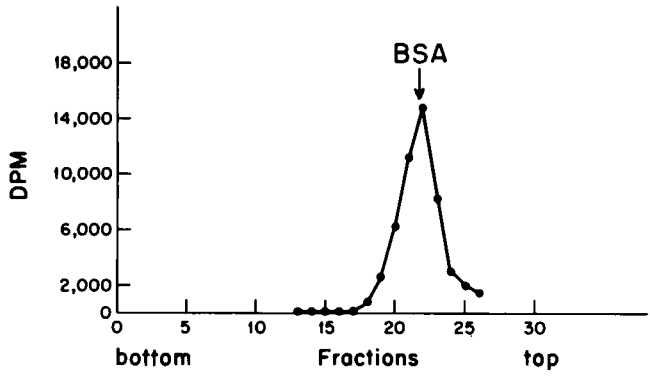

Fig. 1. Sucrose density gradient analysis of ventral prostate cytosol incubated one hour with 1nM ^{3}H-estramustine.

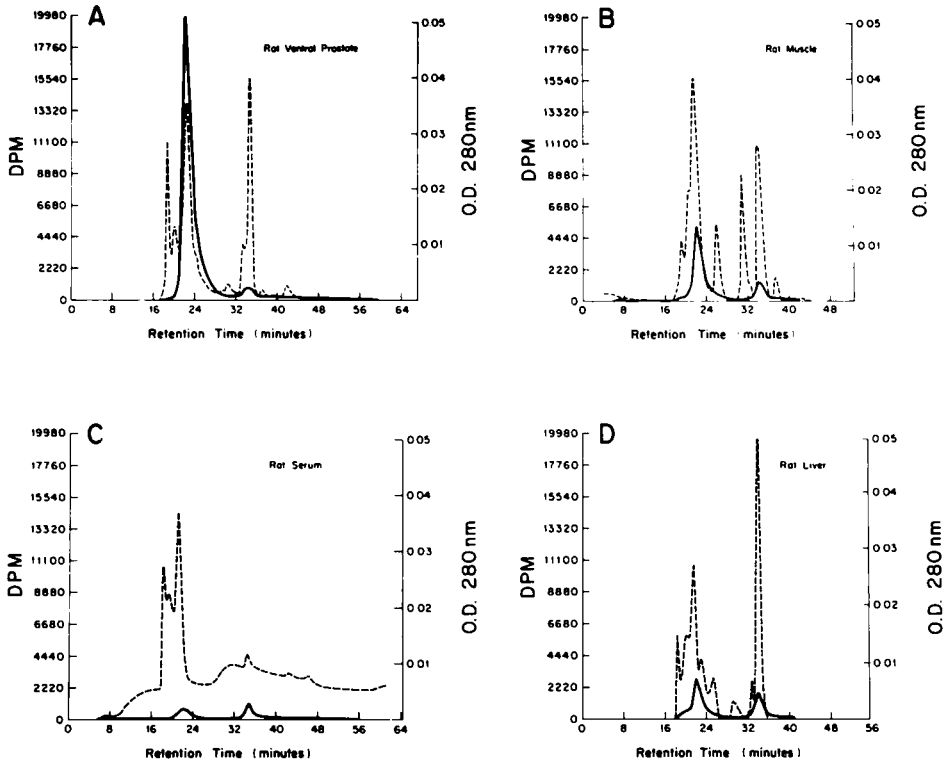

Fig. 2. Tissue specificity studies of the estramustine binding protein. Diluted serum and aliquots of cytosol from rat ventral prostate, liver and muscle were all incubated with ^{3}H-estramustine (40nM) and analyzed by HPLC. The protein profile (dashed line) and radioactivity pattern (continuous line) are represented.

1nM ^{3}H-estramustine. A peak was observed in the 3-4S region. No free radioactivity was shown on the top of the gradient. This is probably due to the adherence of this ligand to the walls of the incubation and centrifugation tubes (4).

In Fig. 2A is shown the protein profile and distribution of radioactivity of ventral prostate cytosol after HPLC. Several protein peaks appeared. The two most prominent peaks occurred with retention times (R_t) of 21-23 min. and 33-35 min.

Only two radioactive peaks were seen (Fig. 2A) which corresponded with the two prominent protein peaks with molecular weights of 60,000 (peak 1) and 32,000 daltons (peak 2), respectively; albumin and cytochrome C were the reference standards.

When 1.25mg of protease was added to 0.5ml of the incubation mixture and the results compared to those of the control specimen, a decrease in the amount of radioactivity associated with both peaks was observed, i.e., peak 1 contained only 52% of the radioactivity when compared to the control and in peak 2 no radioactivity was observed. Thus, this proteolytic enzyme totally abolished the binding of peak 2, but only partially that of peak 1.

TLC analysis of radioactive metabolites in peaks 1 and 2 was performed on the sample of cytosol from the normal human prostate fractionated by HPLC. The radioactivity in both peaks (cytosol incubated with 133nM of ^{3}H-estramustine) was extracted separately and analyzed by TLC. Peak 1 consisted of 70% estramustine, 2.7% E_2, 2.9% E_1 and 2.2% estromustine, with the remaining part being polar metabolites. The second peak consisted of 9.5% estramustine, 6.7% E_2, 6.8% E_1, 6.7% estromustine and the rest polar metabolites. Thus, the

main component of peak 1 was estramustine, whereas in peak 2 there was no preponderance of any particular compound.

Tissue Specificity Studies: The results of HPLC analysis of cytosols from rat muscle and liver and of serum incubated with 40nM ^{3}H-estramustine are shown in Figs. 2B, 2C and 2D. The protein profile is different among these tissues. However, in all cases the two radioactive peaks coincided with the protein peaks with an R_t of 21-23 and 33-35 min., respectively.

When the radioactivity in peak 1 in various tissues was expressed in terms of the protein concentration in the sample and calculated as a percentage of that found in the ventral prostate, the results were: muscle, 22.3%; liver, 17.5% and serum, 6.2%. Similar calculations performed on peak 2 showed the following results relative to ventral prostate: muscle, 105%; liver, 198%; serum 127%. Thus peak 1 exhibited a high degree of tissue specificity for estramustine, whereas no such specificity could be recognized in peak 2. From here on, we confined our investigation to peak 1.

Ligand Specificity Studies: Aliquots from a cytosol prepared from rat ventral prostate were incubated with H-estramustine (40nM), ^{3}H-17β-E_2 (40nM), ^{3}H-DHT (43 nM) and ^{3}H-pregnenolone (40nM). The results are shown in Fig. 3. Assigning the radioactivity/mg protein in peak 1 from the incubation with ^{3}H-estramustine as a control (100%), the values for the other steroids were: E_2, 2.3%; DHT, 3.5%; and pregnenolone 1.5%. Thus, peak 1 showed a high specificity for estramustine binding.

Recycling of the EBP: Cytosol from rat ventral prostate (0.5ml) was incubated with ^{3}H-estramustine (80nM) and fractionated by HPLC. The

radioactivity in aliquots from fractions in the region of peak 1 was

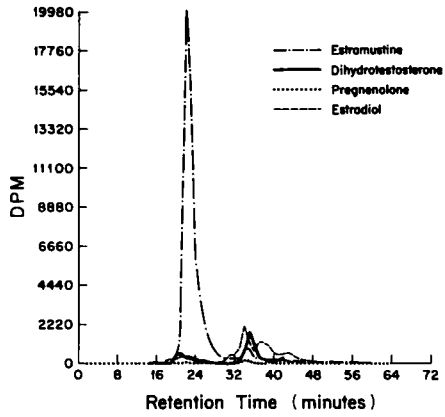

Fig. 3. Steroid specificity studies of the estramustine binding protein. Aliquots of cytosol from rat ventral prostate were incubated with ^{3}H-estramustine (40nM), ^{3}H-pregnenolone (40nM), ^{3}H-E_2 (40nM) and ^{3}H-DHT (43nM) and analyzed by HPLC. The values were corrected for the specific activity of each steroid.

determined and the sample containing the highest amount rechromatographed. HPLC analysis of the reinjected sample showed only one peak of radioactivity which coincided with the protein peak having an R_t of 21-23 min. From these experiments, it can be deduced that EBP is stable with respect to retention time during HPLC.

Studies in the Baboon: Cytosols from two cranial and one caudal

Table I

HPLC ANALYSIS OF THE ESTRAMUSTINE BINDING PROTEIN IN THE CRANIAL AND CAUDAL PROSTATIC LOBES FROM TWO BABOONS. VALUES REPRESENT TOTAL AMOUNT OF BINDING WITHIN EACH PEAK EXPRESSED PER MG OF PROTEIN.

Prostatic Lobe	dpm/mg. Protein in Peak I
Cranial	4080
	5530
Caudal	5240

prostatic lobes were prepared as described above (Methods) and incubat-

ed with 40nM of ^{3}H-estramustine. The results are presented in Table I. The amounts of EBP-like protein in the cranial and caudal prostates of the baboon were considerably lower than the values for the ventral prostate of the rat, whereas the values of the second peak were similar to those of the rat ventral prostate, muscle, liver and serum.

Studies in the Human: The HPLC pattern of ^{3}H-estramustine labeled cytosol from the normal human prostate is given in Fig. 4. The protein profile was different from that of the rat and baboon, but two prominent peaks with R_t of 21-23 min. and R_t of 33-35 min. were

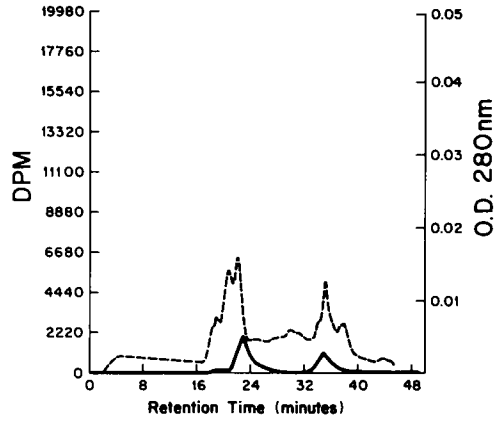

Fig. 4. Cytosol from normal human prostate incubated with 40nM of ^{3}H-estramustine for one hour (room temperature) was subjected to HPLC analysis. The protein profile (dashed line) and the radioactivity pattern (continuous line) are presented.

identified and two peaks of radioactivity were found which coincided with the above mentioned protein peaks. Thus, HPLC analysis of prostates from several species showed a remarkable qualitative similarity with respect to protein bound radioactivity (peaks 1 and 2) of labeled estramustine.

Results of studies on estramustine binding specificity in human tissues are illustrated in Fig. 5. The amounts of estramustine bound

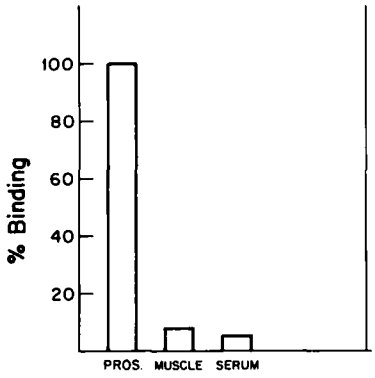

Fig. 5. Binding of estramustine in cytosol from various human tissues. 40nM of ^{3}H-estramustine was incubated for one hour at room temperature. 50µl of the incubation mixture were subjected to HPLC analysis. Total amount of radioactivity in each peak is expressed per mg. of cytosol protein. The amount of estramustine bound in prostate cytosol represents 100%.

in peak 1 of human serum and the male muscle sample with the highest binding value of the samples tested were compared to the respective binding peaks in the normal prostate. Assigning 100% binding to the prostate, we found that in peak 1 the muscle exhibited 7.7% and serum 5.5%.

To determine steroid specificity, cytosol samples from the normal human prostate were incubated with ^{3}H-estramustine (40nM), ^{3}H-E_2 (40nM) and ^{3}H-DHT (43nM). The calculations were performed as previously described and the results are shown in Fig. 6. It is evident that the affinity of peak 1 for estramustine was much higher than for E_2 and DHT.

The results of studies conducted on samples from one normal prostate, ten BPH samples and six prostatic carcinomas are represented in Fig. 7. The protein profiles of the BPH samples were different

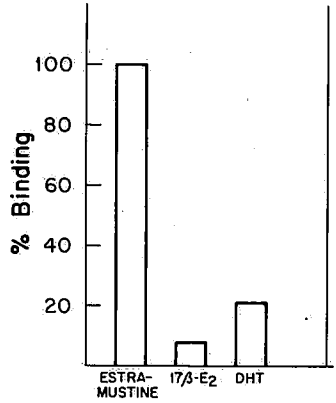

Fig. 6. Binding of various steroids in cytosol from normal human prostate. 40nM of ^{3}H-estramustine, 40nM of ^{3}H-E_2 and 43nM of ^{3}H-DHT were incubated for one hour at room temperature. 50µl of the incubation mixture was subjected to HPLC analysis. Total amount of radioactivity in each peak is expressed per mg. of cytosol protein. The binding of estramustine was taken as 100%

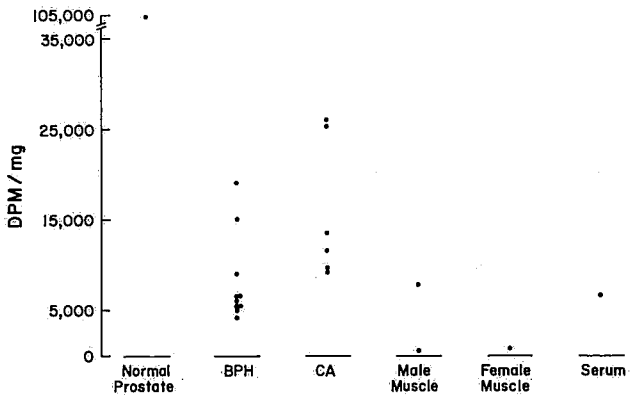

Fig. 7. Estramustine binding protein (total dpm off peak 1 per mg. of cytosol protein) in normal, BPH and cancerous prostate were incubated with 40nM of ^{3}H-estramustine for one hour at room temperature.

from those of the carcinomatous tissues and both were different from that of the normal prostate. Even within each series there were variations in the protein profile. Still, we were always able to recognize two prominent protein peaks which coincided with the two

radioactive peaks (peaks 1 and 2). In fact, we observed in all human samples studied the same two radioactive peaks as had previously been recognized in the rat and baboon.

Fig. 7 illustrates that the value of the EBP-like protein in the normal prostate was 106,100 dpm/mg protein. In the BPH samples the range was between 2200 and 18,900 dpm/mg protein and in the cancerous tissues between 9350 and 25,900 dpm/mg protein. When the results of estramustine binding (Fig. 7) in BPH and cancers of the prostate are compared, there appears to be a trend suggesting that the concentration of EBP-like protein is higher in cancer than in BPH. The normal prostate showed much higher values than either the cancers or BPH.

DISCUSSION

The current thinking is that α-protein (1), prostatic binding protein (2) rostatein (3) and EBP are the same protein; whether the spermine-binding protein of the rat ventral prostate is to be included in this group has not been settled rigorously (7). Thus, Chen et al. (8) have stated that their α-protein appears to be identical to prostatein or prostatic binding protein, and that it may be involved in the recycling and/or regulation of the DHT-receptor complex in prostatic cells. α-Protein is found in prostatic fluid and, therefore, is a secretory product of the epithelial cells. Forsgren et al. (5) isolated EBP from rat ventral prostate and showed that its amino acid composition is identical to that of prostatic binding protein of Heyns and De Moor (2). The high affinity with which this protein binds estramustine, a synthetic drug consisting of a conjugate of E_2 and an alkylating agent (9), though fortuitous, may have important thera-

peutic implications in prostatic cancer. In fact, EBP binds this drug with a higher association constant than that of the naturally occurring steroids (2,3,10,11) and may explain, in part, the mechanism by which estramustine is localized in the prostate and produces its effects on cancer of that organ. Operationally, we have named this protein, estramustine binding protein (EBP).

EBP has previously been measured by SDGC (12), gel filtration and dextran-coated charcoal (DCC) procedures. For its purification, Forsgren et al. (4) used extensive chromatography and polyacrylamide electrophoresis. The HPLC method, as applied in our studies, is particularly useful since small volumes of cytosol are used (50µl) and, thus, reliable data can be obtained on small prostatic samples (e.g., needle biopsies).

In the rat, EBP is found almost exclusively in the prostate (4), where its concentration is highest in the ventral lobe, constituting a remarkable portion of the total cytosol proteins (as much as 50%)(13). The presence of EBP in prostatic secretion is undoubtedly associated with a physiologic role for this protein; the exact nature of this role is still unkown. In the human and baboon prostate EBP-like protein is present in lower concentration than EBP in the rat gland; some of the characteristics of the proteins appear to be the same. The need is to collect data on the EBP-like protein on many more samples from normal and abnormal human prostates and to correlate the findings with the biochemical (e.g. steroid receptors), pathological, clinical and therapeutic aspects of the cases. Thus, measurement of the EBP-like protein may offer another important marker in prostatic disease.

ACKNOWLEDGEMENTS

This study has been supported in part by a grant to J.J.C. from the U.S.-Spanish Joint Committee for Scientific and Technological Corporation, a Fogarty International Fellowship (Award # TW027801) to P.A.H., and grants CA20459 and CA15346 from the National Cancer Institute through the National Prostatic Cancer Project.

REFERENCES

1. Liao, S. and Fang, S. IN Some Aspects of Aetiology and Biochemistry of Prostatic Cancer. Griffiths, K., Pierrepoint, C.G. (Eds.). Alpha Omega Alpha Publishing, Cardiff, U.K. 1970. p. 105-108.
2. Heyns, W. and De Moor, P. Eur. J. Biochem. 78:221-230 (1977).
3. Lea, O.A., Petrusz, P. and French, F.S. Fed. Proc. 36:780 (1977).
4. Forsgren, B., Bjørk, P., Carsltröm, K., Gustafsson, J.A., Pousette, A. and Högberg, B. Proc. Natl. Acad. Sci. USA 76:3149-3153 (1979).
5. Forsgren, B., Bjørk, P., Carlström, K., Gustafsson, J.A., Högberg, B. and Pousette, A. Cancer Treatment Reports 63:1186 (1979).
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. J. Biol. Chem. 193:265-275 (1951).
7. Mezzetti, G., Loor, R. and Liao, S. Biochem. J. 184:431-440 (1979).
8. Chen, C., Hiipakka, R.A. and Liao, S. J. Steroid Biochem. 5:401-405 (1979).
9. Sandberg, A.A. The Fate and Biochemical Effects of Estracyt in the Human and Baboon. IN: "Cytotoxic Estrogens in Hormone Receptive Tumors". Eds. Raus, J., Martens, H., and Leclercq, G. Academic Press, London, 1980. pp. 219-242.
10. Heyns, W., Peeters, B., Mous, J., Rombauts, W. and De Moor, P. Eur. J. Biochem. 89:181-186 (1978).
11. Lea, O.A., Petrusz, P. and French, F.S. J. Biol. Chem. 254: 6196-6202 (1979).
12. Høisaeter, P.A. Scand. J. Urol. Nephrol. 11:135-141 (1977).
13. Peeters, B.L., Mous, J.M., Rombauts, W.A. and Heyns, W.J. J. Biol. Chem. 255:7017-7023 (1980).