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PATHOLOGICAL PROGNOSTIC FACTORS IN PROSTATE CANCER

Ronald Joseph Cohen

A thesis submitted to the Faculty of Medicine, University of the Witwatersrand, for the degree of doctor of Philosophy in Medicine.

Johannesburg 1991

ABSTRACT

Adenocarcinoma of the prostate is the commonest cancer of the western male. Each year more than 120 000 new cases are reported in the United States alone, together with more than 28 000 deaths. Autopsy studies suggest that the majority of prostatic cancer remains clinically undetected, therefore propagating the concept of latent versus aggressive disease. More recent studies have challenged this concept and related tumour aggression to tumour size and grade. These studies however concede that even the most anaplastic large cancers began as a small probably well differentiated tumour. Why some small cancers progress and others do not , has not been established. Long term prognosis and response to therapy can also not be predicted by the histology of the tumour.

The purpose of this thesis is to explore new parameters which may provide more reliable data with regard to prognosis, and explain why some tumours progress while others do not. The thesis will also attempt

to explain why some tumours respond well to therapy and others not.

Nucleolar Organizer Regions (NORs) which represent specific nucleic acid regions will be assessed with respect to benign and malignant prostatic epithelium. NORs will also be investigated as a prognostic indicator.

Neuro-endocrine cells in prostatic cancer, a poorly recognized entity will be studied. These cells will be assessed as a prognostic factor in prostatic cancers. Small tumours will be compared to large tumours with respect to their neuro-endocrine status. Finally the biochemistry and distribution of the neuro-endocrine cell in normal prostates will be examined.

It is proposed that the prostatic neuro-endocrine cell plays a major role in the development and progression of aggressive prostate cancer, and that this cell be addressed in future studies in this field.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor in Philosophy in Medicine to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to any other University.

Ronald Joseph Cohen

19th day of August, 1991.

DEDICATION

TO MY FATHER DAVID COHEN

PREFACE

The following publications have formed part of this thesis:

Cohen R.J., Glezerson G., Zenobia Haffejee, Afrika D.,Prostatic Carcinoma: Histological and Immunohistochemical Factors Affecting Prognosis. Brit. J. Urol., 66: 405-410, 1990.

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Cohen R.J., Glezerson G., Zenobia Haffejee, Neuro-Endocrine Cells a New Prognostic Parameter in Prostate Cancer. Brit. J. Urol., IN PRESS (Accepted for Publication 21/11/90).

Cohen R.J., Glezerson G., Zenobia Haffegee, Prostate Specific Antigen And Prostate Specific Acid Phosphatase in Neuro-Endocrine Cells of Prostate Cancer. Arch. Path. Lab. Med., IN PRESS (Accepted for Publication 6/6/91)

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CHAPTER I

INTRODUCTION

The incidence of prostatic carcinoma in most western societies is second only to that of lung cancer, yet cancer of the prostate ranks only third as a cause of cancer deaths (1). Recent literature suggests that in the United States of America and Western Europe prostate cancer has now become the commonest malignant tumour and the second most common cause of cancer deaths in males. In 1987 among malignant diseases affecting American males 20% were due to cancer of the prostate. Carcinoma of the prostate, however, accounts for only 10% of cancer deaths in American males (2). This disparity between the incidence and mortality is an indication of this tumour's wide range of biological activity. Some patients with prostatic cancer progress rapidly and die within several months while others have a much slower progression and ultimately die of unrelated causes. Autopsy studies suggest that the majority of prostate cancers remain clinically undetected (3). These findings have led to the concept of latent cancer versus aggressive cancer. A corollary of this theory is that the latent and aggressive forms of the disease are histologically indistinguishable

(4). Many studies have attempted to identify histological features which would indicate a poor prognosis, so that the patient may be treated accordingly. Over the past 2 decades the progression of tumour, related to tumour volume has been meticulously studied by McNeal et al (5). While most researchers accept that as tumour volume increases, the likelihood of clinically manifest cancer increases, the rate of tumour growth , varies considerably from one tumour to another. In addition once a tumour reaches proportions enabling it to produce clinical symptoms the overall prognosis will once again vary from one patient to another. In a large study by Gleason et al (6), it was convincingly demonstrated that those tumours which were poorly differentiated and had an anaplastic growth pattern had a significantly worse prognosis than tumours which were well differentiated and closely resembled normal prostatic acinar structures. The vast majority of tumours in that study (2 585 of 2 911) were graded major pattern 2, 3 and 4, i.e., intermediate grade with an intermediate or unpredictable outcome. Therefore, those patients who fell into group 1 (histologically well differentiated carcinoma) and group 5 (anaplastic carcinoma) in which the prognosis could be reliably predicted, represented only a small minority.

CRITIQUE OF THE GLEASON SCORE

In 1977 a study (7) at The Armed Forces Institute of Pathology examined 584 cases of prostatic carcinoma. These cases were graded according to the Gleason system, and observations including those by Gleason himself were compared.There was an overall concordance of only 38%. A workshop in 1979 (8) further addressed the issue of grading. The conclusion of this workshop was to adopt the Gleason score for grading of all prostate cancer. The workshop however recommended some modifications to the Gleason scoring system.

Other grading systems that have been utilized include Mostofi (9), the Gaeta Grading System (10,11), The M D Anderson (12), The Mayo Clinic Grading System (13) and the University of Freiburg Grading System (14).

LIMITS OF GRADING SYSTEMS

Prostatic adenocarcinoma is usually graded from tissue removed at the time of transurethral resection or by transrectal or transperineal needle biopsy. Several studies have shown a poor concordance between tumour grade on biopsy

specimens compared to radical prostatectomy (15, 16).

As trans-urethral biopsies (TURP) sample the transition zone and central portion of the gland, this may reflect poorly on the peripheral subcapsular zone where most investigators agree the tumour arises. A spuriously low grade of tumour may therefore be given.

Prostate cancer has been shown to dedifferentiate with time. With dedifferentiation the Gleason score increases (17). The rate of dedifferentiation is unpredictable and is an unknown factor in tumour grading.

In view of the problems in grading systems newer techniques have been utilized to predict the prognosis in prostate cancer. One of these , flow cytometry (18), which with the determination of ploidy may prove to be of value in the determination of prognosis in prostate cancer. Ploidy refers to the amount of chromatin material present in cell nuclei. The term aneuploid refers to a chromatin content that is not a multiple of the normal haploid (23) or diploid (46) chromosome number. The data available is however, not concurrent. Correlation between ploidy and capsular

penetration has been shown (19). Several studies have shown a correlation between ploidy and grading (20,21). A further study (22) examined ploidy of fresh tissue from 34 radical prostatectomies and found all organ confined tumour to be diploid. However, of 25 diploid cancers 8 had spread outside the gland and 6 had nodal metastasis. Stevenson et al (23) showed that ploidy correlated statistically with survival in stage D1 cancers. The authors however, were reluctant to recommend adjuvant therapy for aneuploid tumours. A large number of related studies (24,25,26) confirm a relationship between prognosis and ploidy although in all studies a significant number of diploid tumours had a poor prognosis. These studies also indicate a good correlation between survival in localized disease treated by radical surgery and ploidy. The correlation with survival in more advanced disease treated with anti-androgens was poor.

Other recently developed histological techniques used to predict the outcome in prostate cancer include nucleolar surface area (27) and nuclear roundness factor (28,29,30,31). These parameters, particularly the latter, require complex image analysis equipment and is at present only being performed by one group of investigators at Johns Hopkins University. As in the case of ploidy these

grading systems appear to be of most benefit in predicting prognosis in early localized disease, rather than in advanced cancer. Response to hormone manipulation in advanced cancer remains unpredictable.

In spite of the variety of grading systems and newer techniques in predicting the outcome of prostate cancer it is the opinion of Catalona et al. (15) that grading of prostate cancer including newer methods have serious deficiencies. These new techniques appear to be no better than good histological grading. What they achieve is effective removal of observer subjectivity. These systems are however, at present beyond the financial reach of most institutions. In addition they do not provide new data on prostate cancer biology, nor do they supply any new approach to the treatment of the disease.

Probably, the most important prognostic parameter in prostate cancer is tumour stage. The TMN system (16) was previously utilized and this has been superseded by the Whitmore\Jewett staging system (33,34,35). Tumour stage in prostate cancer is certainly of major prognostic value and determines what treatment is administered. However, within each tumour stage, prediction of long term survival

in the individual case as well as response to hormone manipulation is unreliable.

In view of the unsatisfactory results of histological prognostication in prostate cancer a variety of biochemical parameters have been attempted. These include serum testosterone levels and serum alkaline phosphatase (36). In early prostate cancer (stage A/B) tumour grading is thought to be most useful, while in more advanced stages C and D serum Prostate Specific Antigen ("PSA"), an antigen specific to prostate epithelium is thought to be more important (37,38,39). Serum PSA determination following therapy enables fairly accurate prediction of tumour recurrence. The height of the serum PSA prior to therapy does not, however, reliably predict prognosis and is only weakly associated with tumour stage. Other parameters including tumour androgen receptor assay (40,41), 5-alpha-reductase determination (42), and a variety of growth factors (43,44) have proved to be disappointing as predictive prognostic indices. Clinical non-specific parameters, including haemaglobin determination, patients performance status, bone pain and the extent of bone metastases, have proved to be of prognostic value in advanced cancer after initial hormonal manipulation (45,46,47).

In 1943 Huggins et.al. (48) confirmed the inhibitory effects of orchidectomy and exogenous oestrogen on the growth of prostate cancer. Despite the initially encouraging results of luteinizing hormone-releasing hormone ("LHRH") agonists (49) very recent and yet unpublished studies have shown this form of therapy to be little better then orchidectomy.

What is required in prostate cancer today is an accurate,reliable and readily reproducible method of prognostication. This method must not only determine prognosis in the individual patient, but must also indicate a possible new approach to the treatment of this disease, which has not altered significantly in over 50 years. Ideally this parameter should be within the financial reach of most prostate cancer patients.

CHAPTER II

PROPOSED NEW PROGNOSTIC PARAMETERS.

NUCLEOLAR ORGANIZER REGIONS

Nucleolar organizer regions ("NOR") are loops of ribosomal RNA occurring in the nucleoli of cells which ultimately process RNA genes (50). NORs have been demonstrated by silver staining techniques ("AgNOR") and have been studied in numerous malignant tumours, namely malignant lymphoma (51), cutaneous melanocytic lesions (52), and a small series of prostatic tumours (53).The numbers and/or size of NOR's may reflect the synthetic activity of cells, or even their malignant potential (54). In a pilot study AgNOR counts in malignant and benign prostatic tissue will be compared. Should this study reveal a significant difference between the two groups, this parameter may be of value in predicting long term survival in this disease.

NEURO-ENDOCRINE CELLS

Neuro-endocrine or paracrine cells of the human

prostate have been identified by various methods,predominantly silver stains and immunocytochemistry (55,56,57,58,59). It is believed that the prostatic Amine Producing Uptake and Decarboxylase ("APUD") cells like those of the gastro-intestinal tract arise by divergent differentiation, rather than from a neural crest primordial cell (60,61). Although these cells have been identified in benign prostatic tissue and in prostate cancer the precise localization of the neuro-endocrine cell in the normal prostate at the various stages of prostatic growth, development and hyperplasia has not knowledge been studied. The function of these cells remains unknown, although they have been shown to contain copious amounts of serotonin and a somatostatin-like peptide (57).

A single report by DiSant'Agnese et al. (57) has indicated that the neuro-endocrine cell is scattered randomly throughout the entire prostate, although present in somewhat greater numbers in the main prostatic ducts. No neuro-endocrine cells have been found in the seminal vesicle or ejaculatory duct. This report indicated that the number of neuro-endocrine cells appear to vary from one individual to another. No explanation was given for this variation in the cell population, but one explanation may be the duration of fixation.

Although, as mentioned in this report, some of the material examined was well fixed, there was no mention as to the duration of fixation. It is well established that proteins stored in formalin fixative progressively lose their antigenicity (62). The antigenic loss may begin within 24 hours of fixation, and many markers of neuro-endocrine cells are lost within 72 hours of formalin fixation.

The studies by DiSant'Agnese et al as indicated were performed on prostate glands obtained at radical cystectomy. The method of section of these glands was not described, and there was no mention as to the patients' age. It is presumed that these cases represented an elderly population with a significant incidence of prostatic hyperplasia and hypertropy, and it is questioned how many of the prostates examined were in fact "normal". The number of neuro-endocrine cells in the hyperplastic tissue was also not commented upon. The authors did not refer to the distribution of neuro-endocrine cells in the prostatic peri-urethral gland zone,or central zone. In addition a group of younger patients was selected from an autopsy series. The tissues, unlike the previous cases, were so autolysed that immunoperoxidase stains could not to be performed, the authors relying on relatively

insensitive silver stains. The immunoperoxidase stains selected were based on polyclonal antibodies which may cause significant background staining, particularly on autopsy material. Background staining and non-specific precipitation can result in misinterpretation of these stains. The author did not characterize these cells with known biological markers of prostate epithelium including PSA and Prostatic Acid Phosphatase ("PAP").

ANATOMY OF THE PROSTATE

The prostate gland consists of three major glandular regions, a peripheral zone, a central zone and a transition zone (Fig. 1). The central zone is related to the ejaculatory duct and is resistant to the development of prostate cancer (63,64). The peripheral zone comprises approximately 70% of the prostate mass. It consists predominantly of acinar structures which drain by a series of ducts to the prostatic urethra. These ducts penetrate the postero-lateral recesses of the urethral wall as well as the urethra from the base of the verumontanum to the prostatic apex (63). The peripheral zone is most susceptible to inflammation (65) and is the site of origin of most carcinomas (66). The periurethral zone comprises less than 5%

of the normal prostate. It consists of tiny ducts and acinar structures scattered along the length of the proximal urethra. This zone appears to end abruptly at the verumontanum. The transition zone consists of two lobes immediately adjacent to the verumontanum. The periurethral gland zone forms a portion of the transition zone. The transition zone and periurethral regions are exclusive sites of benign nodular hyperplasia ("BPH") (63). The aim of this study is to accurately localize, quantitate and document the distribution of neuro-endocrine cells in the normal prostate. In addition prostatic tissue from patients of different ages will be compared. The effects of puberty and ageing on this sub-population of cells can then be documented.

NEURO-ENDOCRINE CELLS IN PROSTATE CANCER

With the utilization of modern immunohistochemical techniques neuro-endocrine cells have been identified in approximate 50% of prostate cancers (59). This study will assess the observation and attempt to establish whether or not the neuro-endocrine cell is of any prognostic value as has been suggested by a single report (67). Neuro-endocrine or paracrine cells produce a variety of peptides (68,69) some of which may stimulate the growth of cells. Small cell carcinoma

of the prostate which has been described in isolated case reports (70,71) appears to be in part a dedifferentiated neuro-endocrine tumour. These tumours have a uniformly poor prognosis and frequently do not elaborate significant amounts of PSA and PAP the usual markers of prostate cancer. The tumours which will be studied are not small cell ancers but usual prostatic adenocarcinomas. In addition the neuro-endocrine status of the tumours will be compared to currently utilized prognostic parameters including clinical stage and Gleason score. The neuro-endocrine cell population of clinically manifest as well as occult cancers will be studied. The incidence of occult cancer is approximately 50% in males aged 50 years (72). As the vast majority of these tumours will remain occult and will not become clinically manifest it will be of value to compare these cases to those with manifest carcinoma.

PSA first isolated by Wang et al (73) is a glycoprotein with a molecular weight of 34,000 daltons and is specific to prostate tissue (74). PSA has proven to be a reliable marker of prostate cancer and may be an indicator of effective treatment (75). The effect of hormone therapy appears to drastically decrease the production of PSA and it seems that PSA may be androgen dependent

(75,76). The production of PSA and PAP in neuro-endocrine cells in otherwise unrmarkable adenocarcinoma has not been determined.One report has documented PAP and PSA in a single prostatic carcinoid tumour (60). In this report comparative immunohistochemical staining was not undertaken and it is questionable whether every cell present was neuro-endocrine.

Virtually all prostate cancers will demonstrate PSA (69,77,78) albeit focally, on direct immunoperoxidase staining. Serum PSA levels appear to increase with advancing clinical stage (78). Considerable overlap, however, exists within clinical stages, and in the individual case, PSA levels are of questionable accuracy in predicting stage of disease. It is well described that patients with advanced cancers may have only minimally raised PSA values (76,79), the explanation for which is poorly addressed in the literature.

The aim of this portion of the study is to determine some of the biochemical characteristics of the neuro-endocrine cell and to determine if this cell produces PSA and PAP.

17-B-OESTRADIOL

17-B-OESTRADIOL is an oestrogen produced from circulating testosterone by peripheral tissues including prostatic epithelium.The function of this oestrogen molecule in androgen sensitive tissues is not known. In several studies (80,81,82) it was shown that 17-B-OESTRADIOL increased the number of testosterone receptors in dog prostatic tissue and was necessary for normal prostatic growth. The measurement of 17-B-OESTRADIOL in the cell cytoplasm and nucleus may indicate indirectly those prostatic cells which are binding testosterone.This may indicate those cells requiring testosterone for cell metabolism. With the use of a new anti-17-B-OESTRADIOL polyclonal antibody developed for use in breast tumours (83), 17-B-OESTRADIOL can be assessed in prostatic cells. Positivity in prostate cancer may thus indicate tumour dependence on testosterone and predict response to hormone therapy.

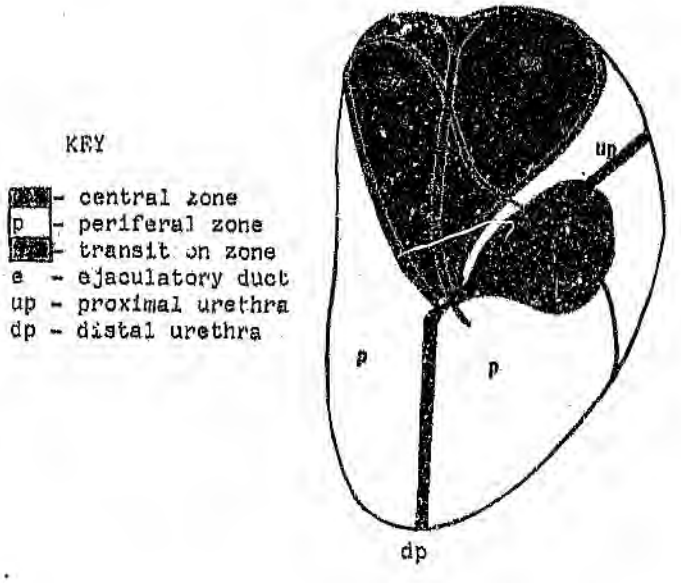


Figure 1. Sagittal section of the prostate (adapted from McNeal (63))

CHAPTER III

STUDY I : NUCLEOLAR ORGANIZER REGIONS IN BENIGN AND MALIGNANT PROSTATE TISSUE

MATERIALS AND METHODS

Eleven cases of benign prostatic enlargement and 11 cases of prostatic carcinoma were randomly selected from the Johannesburg Hospital records from January 1989 through to April 1989. Two 3 micron sections were cut and were taken to aqueous solution via xyline and graded alcohols.

All sections were submitted to the AgNOR procedure at room temperature for 50 minutes (53). The reaction mixture comprised a 1% formic-acid solution in 2% gelatin. This was combined in a proportion of 1-2 volumes with a 50% silver nitrate solution under darkroom conditions to prevent

precipitation. Counter staining was not performed, and the sections were dehydrated and mounted in a synthetic medium.

The specimens were stained under oil immersion lens at X1000 magnification. One hundred nuclei were examined and a simple graticule used to prevent recounting. The number of AgNORs present in lining epithelial cells was documented. AgNOR precipitation in stromal, endothelial,and inflammatory cells was disregarded.

At the same time AgNOR precipitation granules were assessed, sections from the malignant prostates stained with haematoxylin and eosin ("H&E") were examined independently by two pathologists. The tumours were graded using the histological score proposed by Gleason (6), and according to the major and minor patterns,a total score (Gleason Score) out of 10 was allocated to each tumour (Table 1). As indicated by Gleason, the best differentiated tumours with a tubular growth pattern were graded 1. The anaplastic tumours and those with single cell infiltration were graded 5. The predominant or major pattern was then added to the lesser or minor pattern and a total value out of ten was obtained. The lower the score the better differentiated the tumour and according to Gleason the better the

ultimate prognosis.

RESULTS

In all specimens the AgNOR staining was clearly visible as black nuclear dots of varying size. In general they were present throughout the nucleus often obliterating the nucleolus. The largest number of NORs were seen in the highest grade of prostatic malignancy (Fig. 2) where as the fewest NORs were seen in benign prostatic tissue (Fig. 3).

The greatest number of AgNORs seen in benign prostatic tissue was 373 per 100 nuclei (3.73 per cell) with a mean AgNOR count of 3,06 per cell (Table 1).

The greatest number of AgNORs seen in prostatic cancer was 600 per 100 nuclei (6 per cell) with a mean AgNOR count of 4,63 per cell (Table 2).

Only 2 of the malignant tumours had AgNOR counts below 400 per 100 cells. These subjects (cases 516 & 780) had AgNOR counts of 230 and 278 respectively, but the AgNORs were unduly large - 2 or 3 times the size of NORs present in the other cases of adenocarcinoma (Fig. 4).

The 2 series examined, showed statistically significant differences in AgNOR staining. When applying the "t" test via the Fischer Behrns formula a value of 3,95 was obtained ($P < 0,01$). There is thus a significant difference in the staining patterns between the 2 series.

DISCUSSION

Two factors emerge from this pilot study. The first is that there is a significant difference between the AgNOR staining of benign as compared to malignant prostatic epithelial cells. The second is that this may prove to be a useful aid in distinguishing well differentiated carcinoma from atypical hyperplasia. The AgNOR count may also prove to be a useful aid to the grading of cancer and may have prognostic importance.

The 2 cases in the malignant series demonstrating low AgNOR counts, with unusually large granules probably represent fused AgNor granules. The overall count is therefore probably far higher than estimated by light microscopic methods.

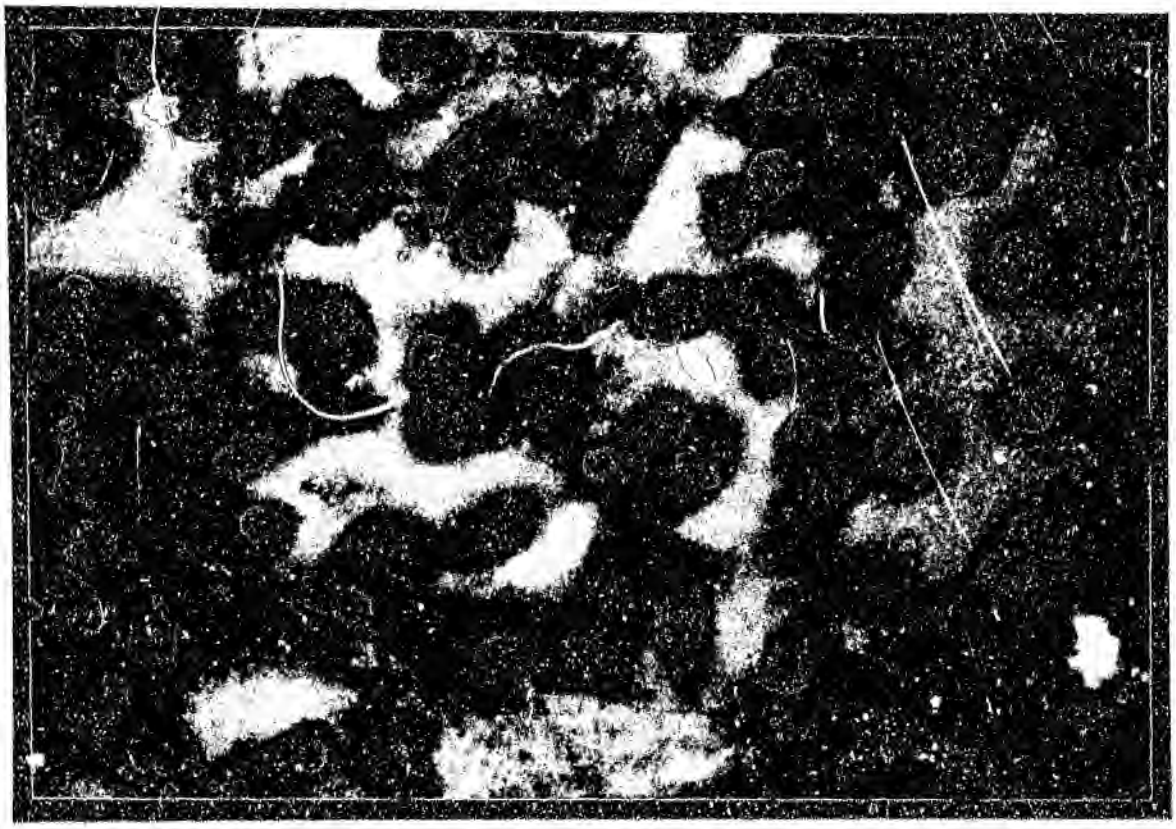


Figure 2. Black silver precipitation granules or AgNOR noted in a prostatic adenocarcinoma (X 1650).



Figure 3. AgNOR precipitation granules in benign prostatic epithelium (X 1650)

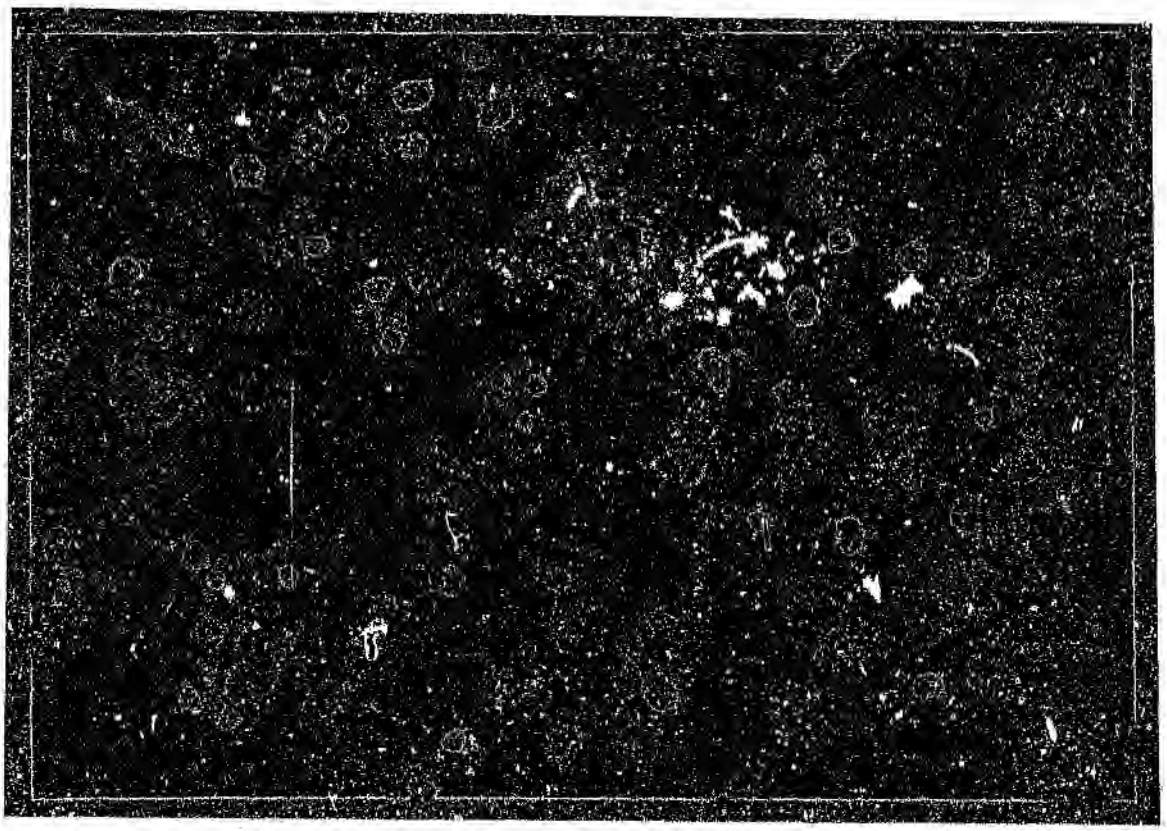


Figure 4. Large AgNOR precipitation granules noted in 2 cases (X 1650).

BENIGN PROSTATIC ENLARGEMENT
AGNOR SIZES

	CASE NO.	DIAGNOSIS	AGNOR NO.
1	2963	BENIGN	267
2	2910	" "	350
3	2939	" "	341
4	137	" "	316
5	2912	" "	220
6	584	" "	278
7	637	" "	372
8	646	" "	297
9	451	" "	373
10	527	" "	255
11	459	" "	302

Mean = 306.45
Standard Deviation = 49.5

Table 1. AgNOR counts in benign prostatic epithelium

ADENOCARCINOMA OF THE PROSTATE
AGNOR SIZES

	CASE NO.	DIAGNOSIS	TOTAL NO.
1	2910	Carcinoma	410
2	3568	" "	481
3	3435	" "	541
4	3215	" "	589
5	249	" "	514
6	322	" "	403
7	15	" "	600
8	585	" "	465
9	645	" "	536
10	516	" "	278
11	780	" "	230

Mean = 463.8
Standard Deviation = 118.95

Table 2. AgNOR counts in prostatic carcinoma.

CHAPTER IV

STUDY 2 : NUCLEOLAR ORGANIZER REGIONS AND NEURO-ENDOCRINE CELLS IN PROSTATE CANCER

METHODS AND MATERIALS

Fifty-two Patients were selected sequentially from the Johannesburg Hospital records from October 1981 to June 1983. The records indicated that all of these patients were subjected to prostatic biopsy because of symptoms related to the local complications of the tumour. In all cases the pre-operative diagnosis was carcinoma stage B, C or D. Twenty-one patients were immediately excluded from the trial for the following reasons:

(i) Eight had died within 1 month of surgery. These deaths were attributed to medical or surgical complications unrelated to the malignant disease. A significant number had died of cardio-vascular complications.

(ii) Five were excluded because no follow-up history was available.

(iii) In 5 cases no tissue was available for examination.

(iv) In 3 cases the original diagnosis was incorrect (transitional cell carcinoma)

The remaining 31 patients were followed-up to April 1990 or to date of death. In each case the cause of death had been documented and, where available, post mortem tissu ...equent surgical specimens were examined. ... had died 4, 6 and 6.5 years aft... diagnosis. Post mortem material showed ... had died of unrelated causes, and there ... evidence of residual prostatic carcinoma. These post mortem examinations were routine and only 1 tissue block of each prostate was submitted for histological examination. These 3 patients were thus included in the surviving group.

The specimens had been fixed in 10% phosphate buffered formalin and processed to wax paraffin. Four 2 micron sections were cut and taken to aqueous solutions through graded alcohols. One section was submitted to the AgNOR staining procedure at room temperature for 50 minutes (53). Two sections were submitted to immunoperoxidase staining, namely polyclona..., Neuron Specific

Enolase ("NSE") (DAKO) and monoclonal Chromogranin A ("ChA") (ENZO). Both of these peroxidase stains represent markers of neuro-endocrine cells (84,85). NSE represents a highly sensitive marker of neuro-endocrine cells, while ChA, although less sensitive, appears more specific (86).

AgNOR Technique

The AgNOR precipitation granules were assessed as in Study 1. This assessment was performed without prior knowledge of the patients history. An AgNOR count for each case was noted.

Immunoperoxidase Technique

Sections stained for ChA and NSE were examined by an independent observer with no knowledge of the clinical history, and the staining was recorded as negative, (no neuro-endocrine cells seen) or positive (individual cells or groups of cells showing positive staining) (Fig. 5). In 3 cases non-specific staining was noted; this was assessed by a second independent pathologist and recorded as negative.

RESULTS

At the time of follow up (1989), 11 patients (36%) were alive and well with no clinical evidence of disease progression: all were shown to be negative for NSE and ChA. The three patients who died of unrelated disease were also negative for NSE and ChA immunoperoxidase staining. Of the 17 patients who died of prostate cancer, 15 were positive for NSE and 14 for ChA; 2 of the 17 were negative for both peroxidase stains.

Survival graphs were plotted, comparing patients with positive staining tumours to those with negative results. The total population survival over 7 years (Fig. 6) was documented. The survival of patients whose tumours were positive for NSE were compared with those negative for the stain (Fig. 7). Similarly survival of patients with tumours positive for ChA was compared with those negative for ChA staining (Fig. 8). It should be noted that the very significant but less striking survival curve noted for ChA may be related to its lower sensitivity compared with NSE. The striking association between survival and tumours negative for neuro-endocrine markers ($P<0,001$) was noted (Fig. 9). The intensity of the staining for NSE and ChA did not appear to be related to outcome.

AgNOR counts performed had a mean count of 579.5\100 nuclei in the patients dying of prostate cancer (SD 157.5). The mean AgNOR count in the surviving patients was 487.8 (SD 116.7). Using the pooled data formulae ("t" test), a t value of 1,69 was obtained, indicating no significant difference at P = 0,05. This indicated that although AgNOR may be useful in distinguishing prostatic carcinoma from benign prostatic epithelium (study 1), it appears to have no value as a prognostic indicator.

DISCUSSION

The occurrence of neuro-endocrine cells in prostatic carcinoma is variable, depending on the method used to demonstrate these cells. In the present study, approximately 60% of the tumours showed neuro-endocrine differentiation; none histologically resembled a carcinoid tumour or small cell carcinoma. The aggressive behavior of these tumours is noted from the above statistics and is, therefore, of therapeutic and diagnostic importance. It has been suggested that chemotherapy similar to that used for small cell carcinoma of the lung (85) be used for small cell carcinoma of the prostate. The influence of this new prognostic

marker on the interpretation of the results of treatment and the selection of treatment modalities needs to be investigated.

Finally, we propose that although the presence of neuro-endocrine cells in other tumours (87,88) may be of no prognostic importance, it is of prognostic significance in prostatic carcinoma. An additional factor to emerge from the study was the finding that AgNOR staining appear to have no prognostic significance in prostatic carcinoma.

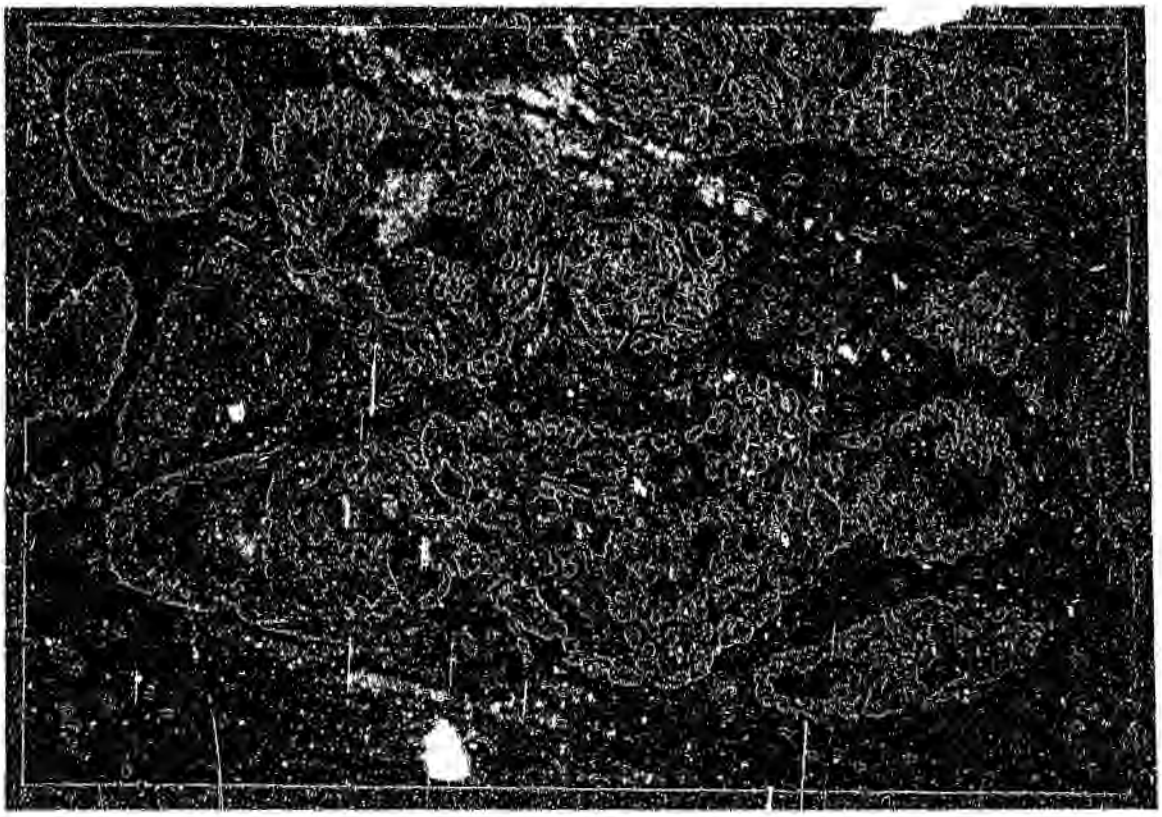


Figure 5. Immunoperoxidase staining for Chromogranin A (ENZO) shows the presence of neuro-endocrine cells scattered in an otherwise well to moderately-well differentiated carcinoma (X 600).

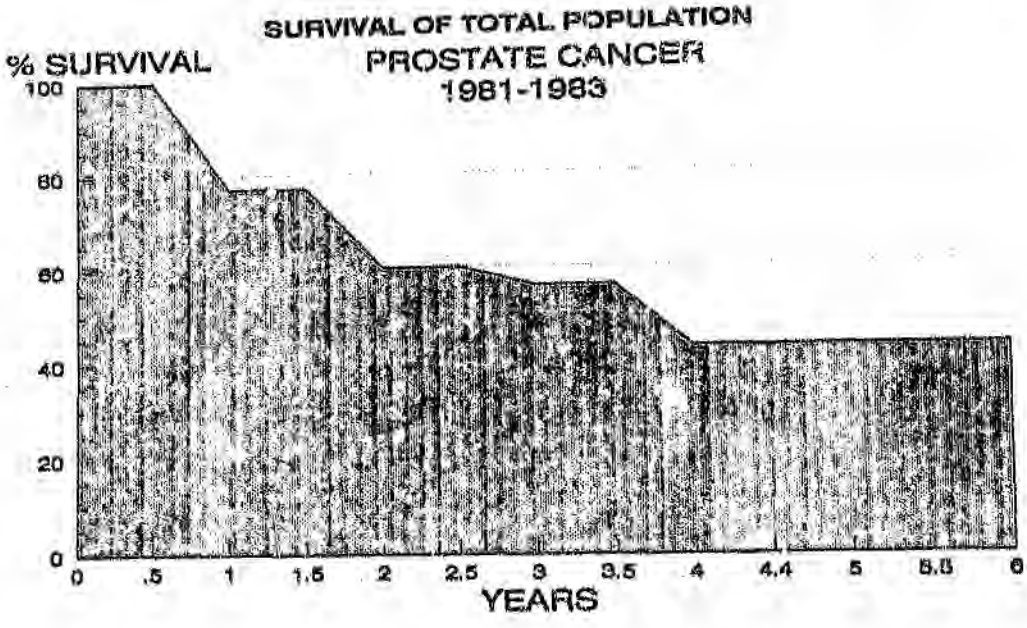


Figure 6. Survival of total population

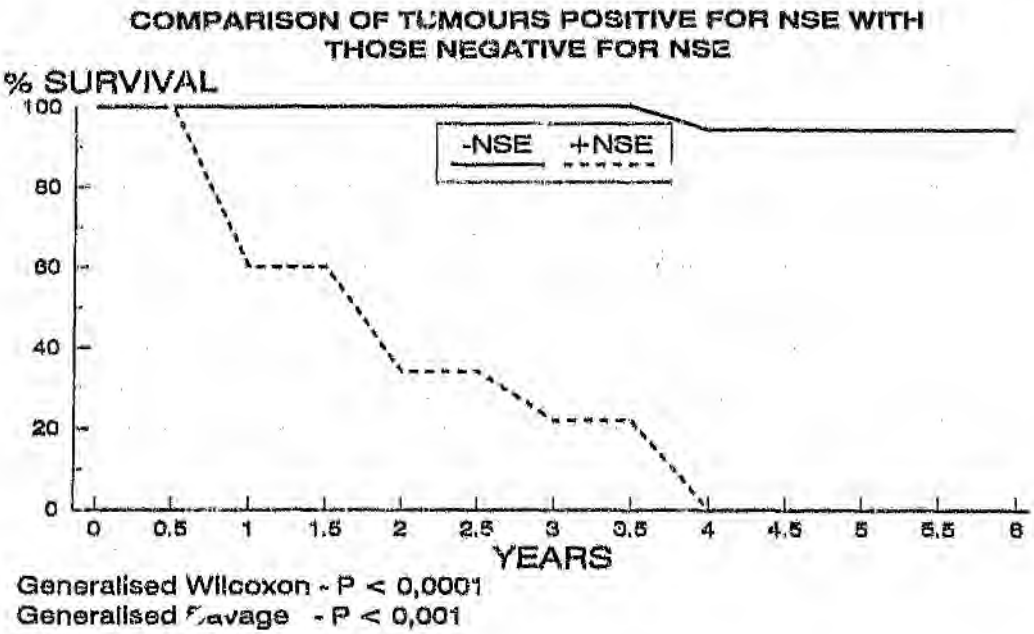


Figure 7. Comparison of tumours positive for NSE with those negative for NSE.

Generalized Wilcoxon - P<0,0001

Generalized Savage - P<0,001

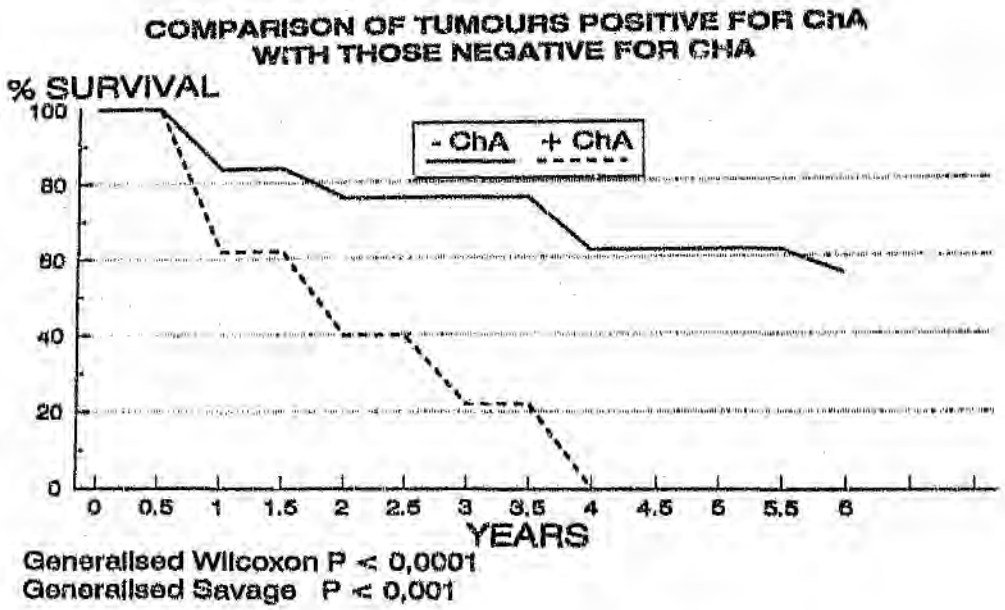


Figure 8. Survival of patients positive for Chromogranin A with those who were negative for Chromagranin A.

Generalized Wilcoxon - P<0,0001

Generalized Savage - P<0,001

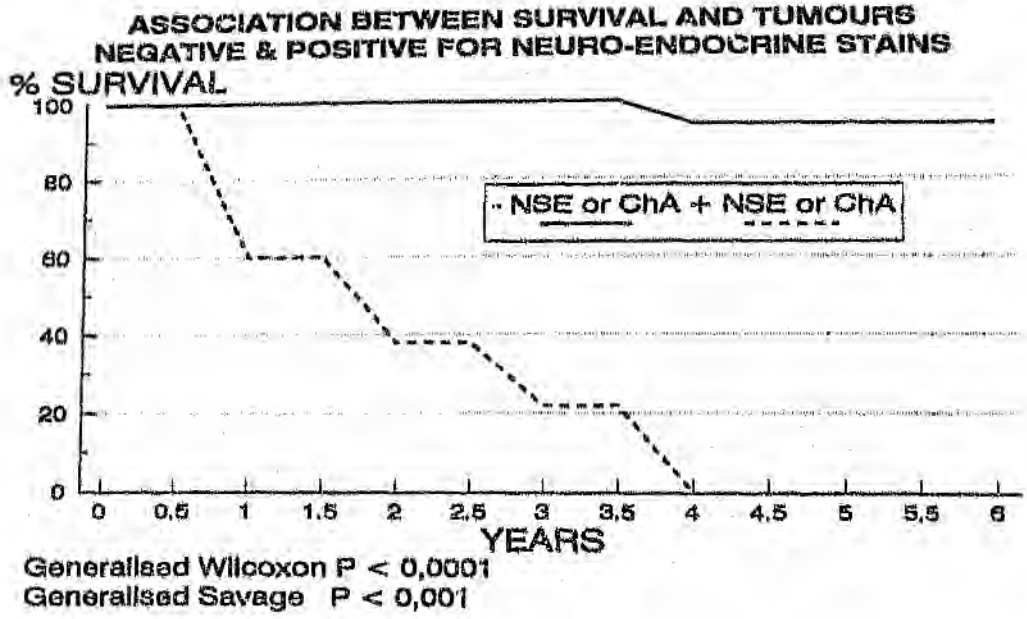


Figure 9. Association between survival and tumours negative for neuro-endocrine markers,

Generalized Wilcoxon - P<0,0001

Generalized Savage - P<0,001

CHAPTER V

STUDY 3 : NEURO-ENDOCRINE CELLS ; A NEW PROGNOSTIC PARAMETER IN PROSTATE CANCER

INTRODUCTION

Prognostic indicators in current use in prostate cancer include tumour stage and tumour grade (35,6). Tumour staging appears to be readily reproducible and correlates well with patient survival. By contrast, grading systems are prone to individual subjectivity (7,8,9) but are most accurate at predicting prognosis at the extremes of grade (well differentiated and poorly differentiated tumours). The vast majority of tumours fall into the intermediate grades with an unpredictable prognosis.

The aim of this study is to correlate the prognosis of prostatic carcinoma with the presence of neuro-endocrine cells and to compare this variable with presently utilized prognostic indices, namely,

stage and histological grade. Neuro-endocrine cells are indistinguishable on routine light microscopy from non neuro- endocrine cells and can be detected only with silver stains or preferably immunoperoxidase.

METHODS AND MATERIALS

One hundred and ten patients were selected sequentially from hospital records at this institution, from October 1981 through June 1986. The records indicated that all patients were subjected to prostatic biopsy, either by trans-urethral resection or by needle biopsy (transrectal). In all cases the diagnosis was that of carcinoma stage B, C or D. Thirty-one cases from Study II were included. No single patient had received any form of therapy prior to biopsy.

Twenty cases were excluded from this study for the following reasons:

1. Five patients had died within one month of surgery. These deaths were attributed to pulmonary thrombo-embolic disease or to medical complication unrelated to the prostatic cancer.

2. Six patients were excluded due to lack of

follow-up history.

3. In 4 cases there was insufficient tissue-for-histological examination.

4. In 5 cases the original diagnosis was incorrect (transitional cell carcinoma [4], metastatic colo-rectal carcinoma [1]).

The remaining 90 patients were followed-up to July 1990 or date of death. In each case the cause of death had been documented. Six patients (including the 3 from Study II) died more than 4 year after the original diagnosis. In all 6 patients, postmortem examination confirmed that they had died of causes unrelated to their prostate cancers. These 6 cases were included in the surviving group.

Seventy-seven of the 90 cases studied were subjected to trans- urethral resection and at least 2 representative blocks were selected for examination. In 9 cases needle biopsy material only was available for assessment. A further 4 cases underwent initial needle biopsy and due to obstructive symptoms, this was followed by trans-urethral resection. In these 4 cases both needle biopsies and trans-urethral resection

specimens were examined.

Thirty-one further cases of (A1) occult adenocarcinoma were selected sequentially from the hospital records, from December 1988 through June 1990. All cases were found incidentally at trans-urethral resection performed for benign prostatic enlargement. In all cases tumour volume was less than 4% of the total tissue and was graded "Gleason" score 7 or less. Ten of these 30 cases were excluded as further sections of the tissue block failed to demonstrate any malignant cells.

All patients with manifest local and regional disease (B and C) were initially treated by radio-therapy. All cases with disseminated disease (D2) were subjected to an endocrine manipulation (orchidectomy, "LH-RH" agonists, or stilbesterol). No patient in the series died without some form of endocrine manipulation.

All specimens were fixed in 10% phosphate buffered formalin and processed to wax paraffin. Three 2 micron sections were cut and taken to aqueous solutions through graded alcohols. One section was stained with routine haematoxylin and eosin for diagnostic purposes and histological grading. Two

sections were submitted to immunoperoxidase staining, namely, polyclonal Neuron-Specific-Enolase ("NSE": DAKO) and monoclonal Chromogranin A ("ChA": ENZO), both markers of neuro-endocrine cells.

Without knowledge of clinical history or the patient's records the following information was recorded:

1. Haematoxylin and eosin stained sections, were graded according to "Gleason" as major grade and minor grade, and a Gleason score was allocated to each case.

2. Sections stained for NSE and ChA were regarded as negative (no neuro-endocrine cells present) or positive (individual cells or groups of cells showing positive staining) (Fig. 10, 11 & 12).

Two of the 4 needle biopsies in which further material was available for examination showed no evidence of neuro-endocrine differentiation. Neuro-endocrine cells, however, were demonstrated on evaluation of the trans-urethral biopsy material. These 2 case therefore, were considered positive for neuro-endocrine cells. This observation confirmed the focal distribution of

neuro-endocrine cells in prostate cancer. A single needle biopsy may therefore be inadequate in assessing neuro-endocrine differentiation in a particular tumour. Two other important observations were noted, namely, the presence of neuro-endocrine cells in benign prostatic glands (Fig. 13) as well as positive staining of nerve twigs by NSE (Fig. 14 & 15). These positive areas were noted but disregarded in tumour assessment.

RESULTS

At the time of follow-up (July 1990), 38 patients from the clinically manifest group were alive at least 4 years after the original diagnosis. Another 6 patients had died of unrelated causes and were included in the total surviving population of 44. Forty-six patients had died of their prostate cancer. Of the 44 survivors, 5 patients demonstrated neuro-endocrine cells in their tumours. Of the 46 that had died as a result of their tumours, 42 demonstrated neuro-endocrine cell differentiation (Fig. 16). Of the 4 negative cases, two cases were represented by a single needle biopsy. The patients were classified according to stage (Fig. 17), Gleason major grade (Fig. 18), Gleason minor grade (Fig. 19) and Gleason score (Fig. 20). Clinical stage and the presence of

neuro- endocrine cells were the most significant prognostic indicators (P< 0,0001) while the histological grading (Gleason) was of less significance (P= 0,1241). Neuro-endocrine cells were assessed for each stage and proved to be a reliable prognostic indicator in all stages (Fig. 21).

Neuro-endocrine differentiation was demonstrated in 2 cases (10%) of the 20 occult (A1) carcinomas examined. Follow-up of these cases is, however, limited to a maximum of 2 years with no tumour progression in any single case.

DISCUSSION

This study confirms the findings of Study II as well as ther authors (58,59,67) that approximately 50% of clinically manifest prostate cancer show neuro-endocrine differentiation. No case resembled a carcinoid tumour or small cell (oat cell) carcinoma on histological examination. In early prostate cancer, (pre-clinical A1 tumours) the incidence of neuro-endocrine cell differentiation is approximately 10%. This suggests that the neuro-endocrine cells may be important in tumour progression. In addition, the prognostic importance

of neuro-endocrine cells in stage-matched disease is demonstrated. This prognostic factor was significantly superior to Gleason's grading system.

In view of these findings it is suggested that the presence of neuro-endocrine cells be considered as an independent prognostic variable. The precise role of these particular tumour cells is unknown, but it is speculated that they like neuro-endocrine cells in other tissues, secrete peptide hormones which then influence the growth of tumour cells and allow these to escape androgen blockade. Alternatively neuro-endocrine cells may simply indicate the presence of androgen resistant cell clones. As neuro-endocrine cells are influenced by other peptide hormones including somatostatin (89) this should be considered in future therapeutic trials.

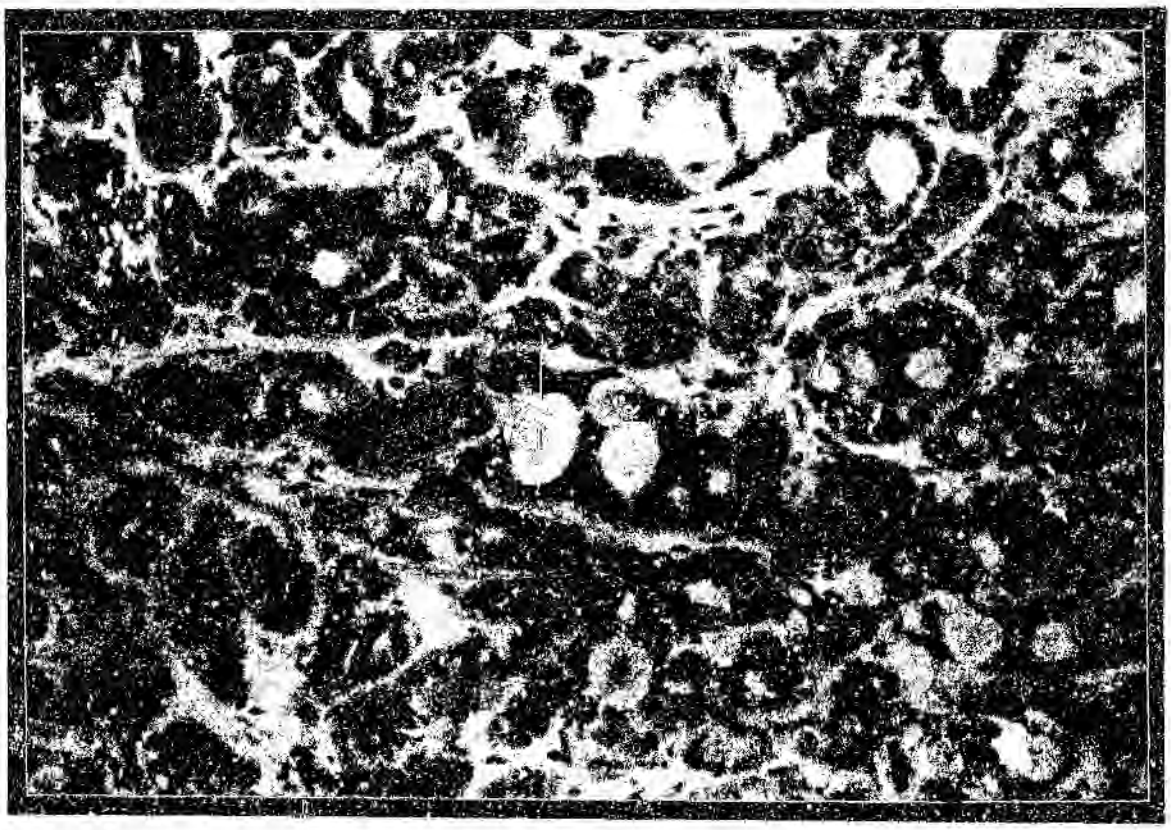


Figure 10. Immunoperoxidase staining for NSE (DAKO) showing positive staining cells in an otherwise unremarkable carcinoma (X 820).



Figure 11. Immunoperoxidase staining for ChA (ENZO). positive staining cells have identical cell morphology as compared to unstained cells. No small cell or oat cell morphology is noted (X 1650).



Figu' 12. Electron Micrograph demonstrating dense cor: granules in the cytoplasm of prostatic neuro-endocrine cells (X 6820).

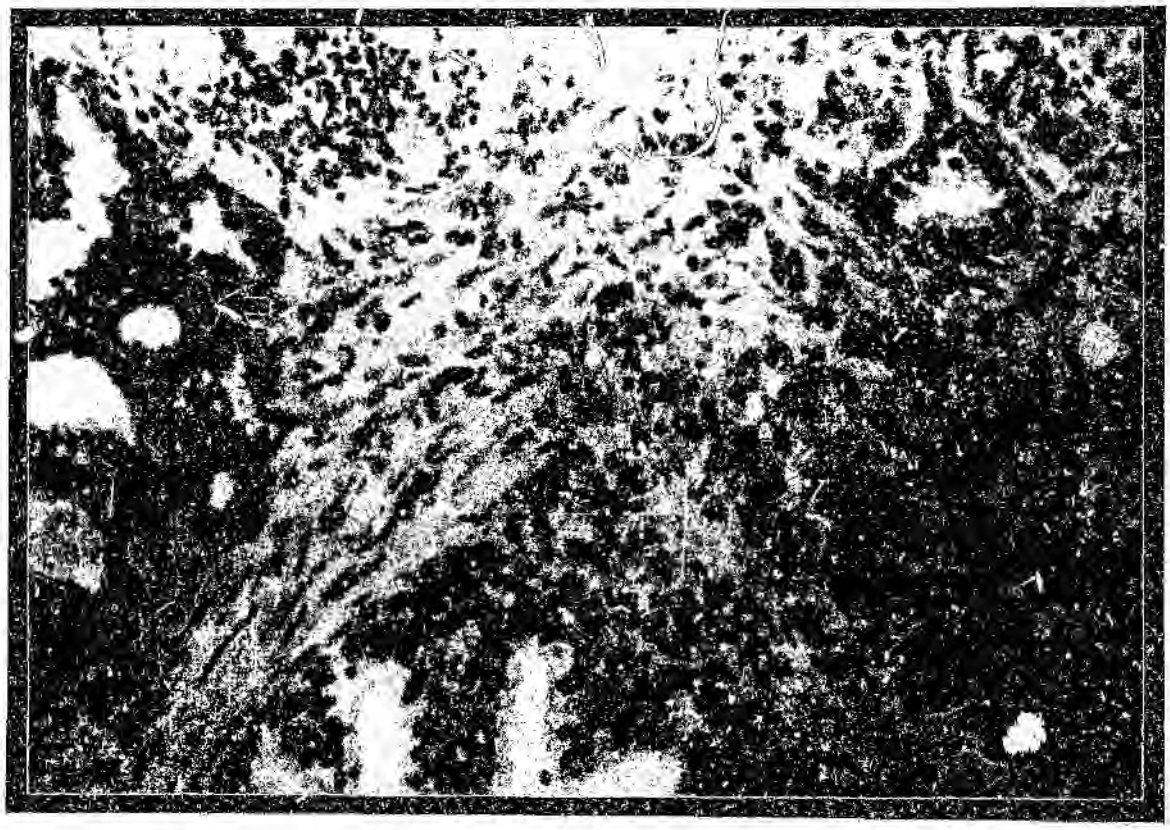


Figure 13. Benign neuro-endocrine cells stained with Chromogranin A noted in entrapped glands (arrow). The Tumour (right) shows no staining with Chromogranin A (X600).

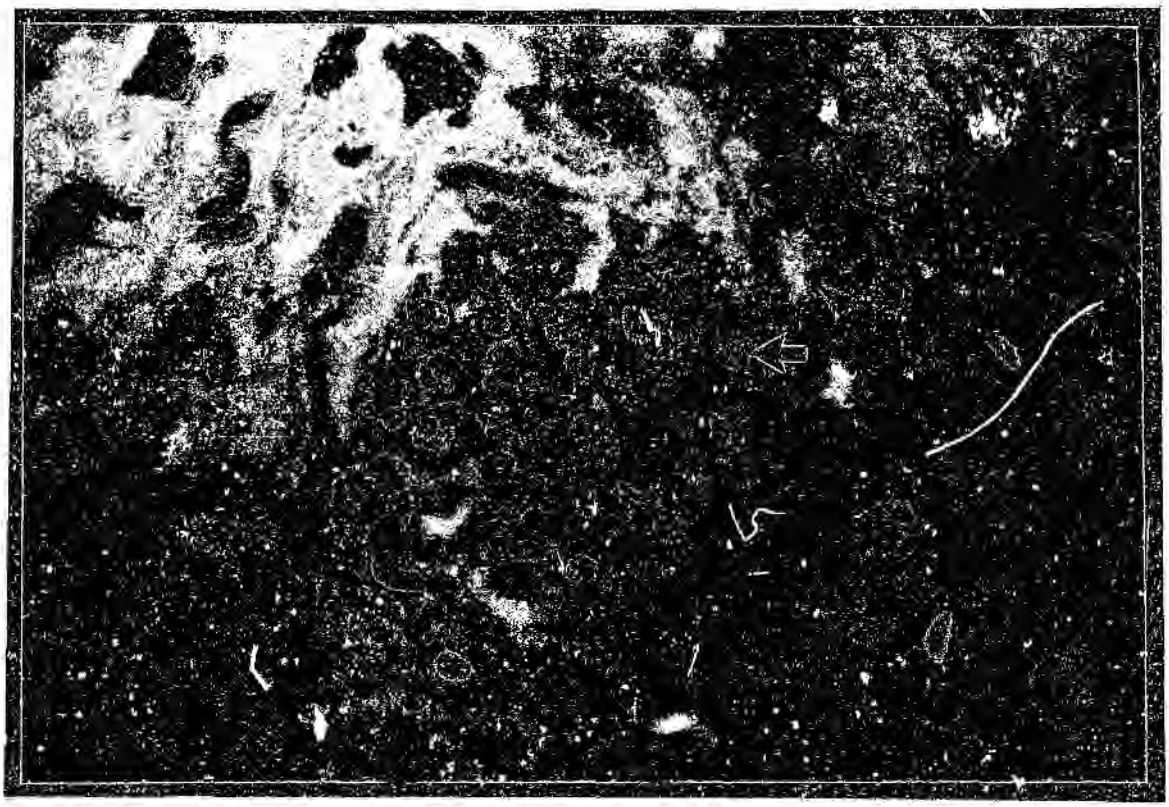


Figure 14. Nerve twigs staining with NSE, surrounded by unstained tumour cells (X 1650).



Figure 15. Neuro-endocrine negative cells infiltrating positive staining nerves (NSE; X 165).

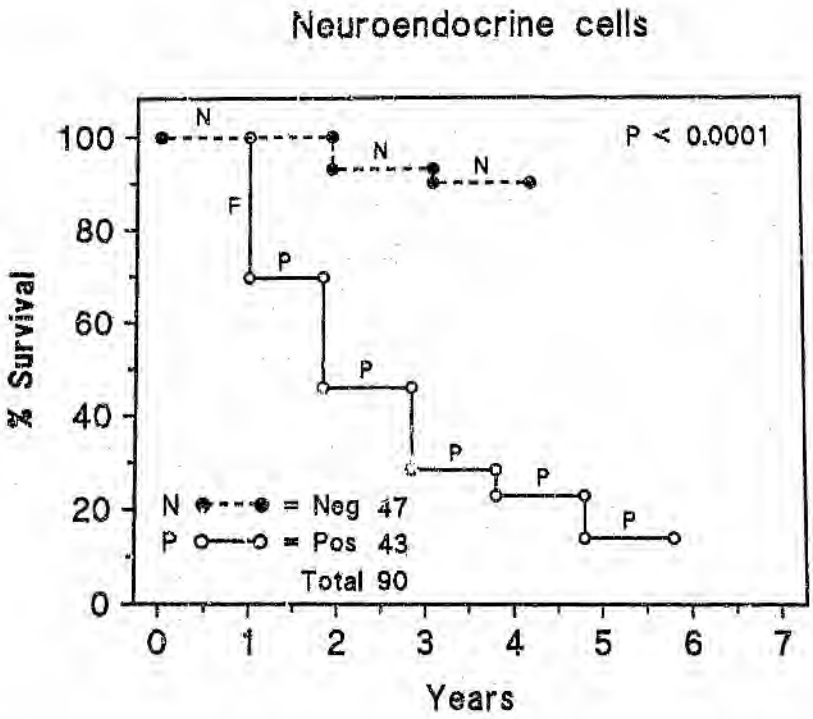


Figure 16. Survival of Patients with neuro-endocrine negative tumours as compared to patients with positive tumours.

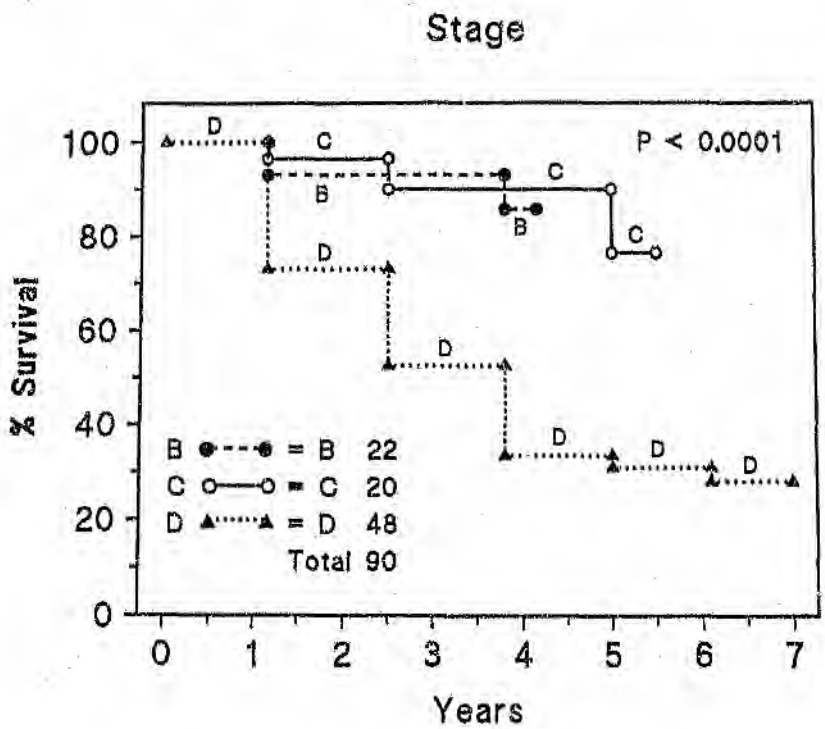


Figure 17. Patient survival according to clinical stage.

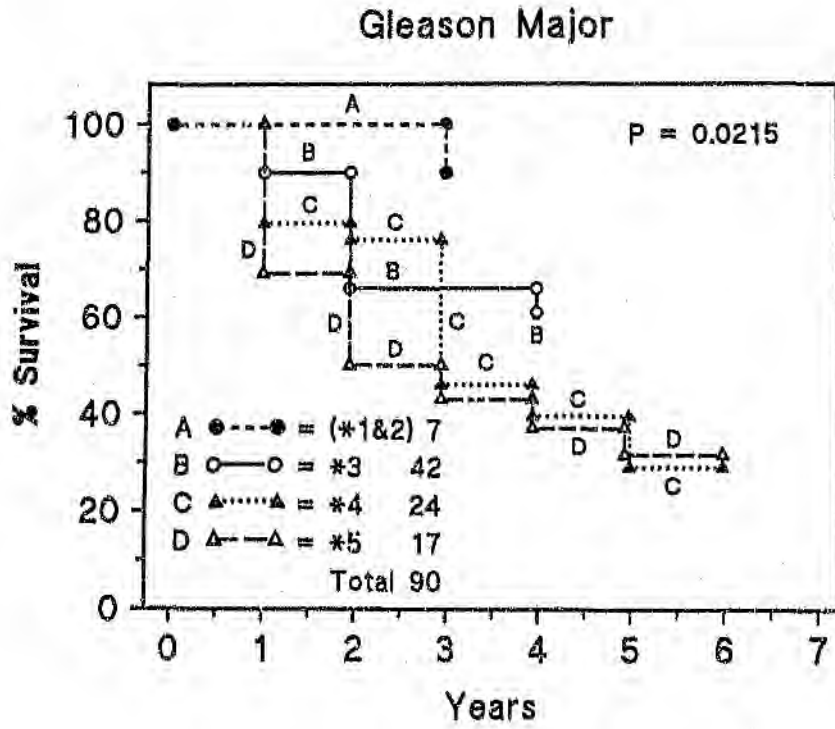


Figure 18. Patient survival according to Gleason major grade.

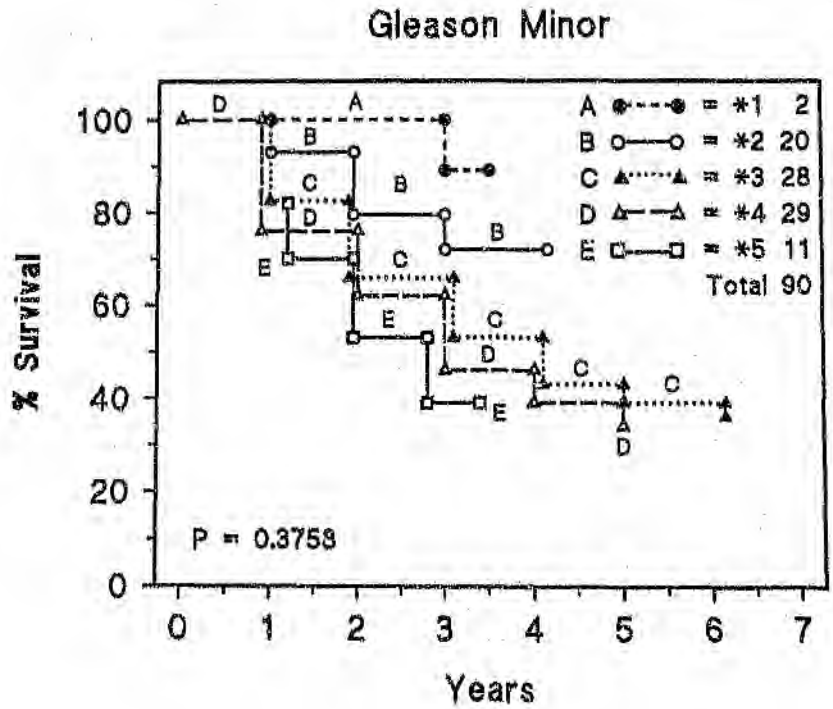


Figure 19. Patient survival according to Gleason minor grade.

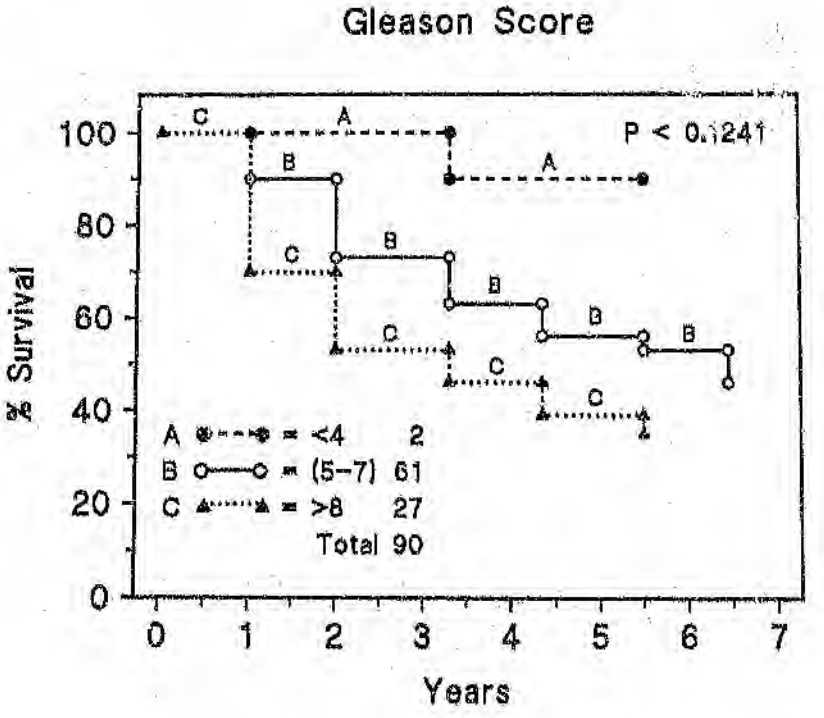


Figure 20. Patient survival according to Gleason score.

NEURO-ENDOCRINE CELLS IN STAGED MATCHED CANCER

90 CASES OF PROSTATE CANCER WITH 5 Yr FOLLOW UP

No.	STAGE	CANCER DEATHS	ALIVE/NON CANCER DEATHS
22	B	4 (3)*	18 (3)*
20	C	5 (5)*	15(2)*
48	D	37 (34)*	11 (0)*

()* Positive for Neuro-Endocrine Cells

Figure 21. Survival of patients with prostate cancer according to stage and neuro-endocrine status of tumours.

ADDENDUM

Abrahamsson et al (Abrahamsson PA, Wadstrom LB, Alumets J, Falkmer S, and Grimelius L, Peptide-hormone-and-serotonin-immunoreactive tumour cells in carcinoma of the prostate, Pathology Research and Practice 182: 298-307, 1987) have suggested that if the tissues are preserved in Bouin's fixative, neuroendocrine differentiation is universal in prostate cancer. This study utilises silver stains, which it claims to be positive in all cases studied. These cases, in addition, where stained with a battery of polyclonal antisera and confirmed positive immuno-precipitation in 0-90% of tumours. Several antibodies used, are not specific to neuroendocrine cells ie. HCG found in placental tissue and some germ cell tumours and leu-enkephalin and beta- endorphin found in peripheral nerve tissues. Silver techniques used in this study are not truly comparable to immunohistochemical techniques, and silver salts stain a variety of materials including reticulin fibres, nerves, and inflammatory cells. Other investigators (67), staining in excess of 300 cases of prostate cancer, documented neuroendocrine cells in only 10% of tumours, using the Grimelius stain on tissues fixed in formalin or Bouin's solution.

Polyclonal neuron specific enolase is a sensitive stain although not always highly specific. In this thesis we utilise both monoclonal and polyclonal sera, and analyze these separately. In addition, no mention is made in the article by Abrahamsson of a major pitfall of interpretation, documented in

this thesis, ie. benign neuroendocrine cells and small nerve twigs entrapped between non-neuroendocrine carcinoma cells, simulating true neuroendocrine differentiation of the tumour. This is a frequently observed occurrence and requires careful histological assessment in distinguishing benign glands from carcinoma cells. This would be almost impossible to achieve using fluorescent techniques utilised in Abrahamsson's study. Peroxidase methods used in this thesis enable a far better assessment of cytological and architectural detail, necessary to distinguish benign from malignant glands.

Bouin's fixative containing picric acid (a), is thought by some authors to preserve dense core granules,(important in the Grimelius stain) and their peptide hormones better than 10% buffered formalin (b). Neuron specific enolase used as a neuroendoorine marker in this thesis represents a beta-enolase isomer specific to nerve tissues and neuroendocrine cells, but unrelated to the dense core granule. Neuron specific enolase is a highly sensitive marker of neuroendocrine cells present in the majority of neuroendocrine tumours (b). Beta-enolase appears to be denatured by 10% buffered formalin only after 4 days (62) of fixation. A recent article (c) reviewed 20 cases of breast carcinoma containing cells showing silver precipitation using the Grimelius technique. Bouin's fixative or formalin was utilised. All 20 cases were positive for neuron specific enolase and in more than half the cases a greater percentage of cells stained positively for neuron specific enolase than with the Grimelius technique. This applied to both formalin fixed tissues and

Bouin's fixation. In this thesis only 50% of cases studied were positive for neuron specific enolase. In radical prostatectomy specimens, neuro-endocrine stains are performed on frozen sections, and neuroendocrine features are observed in only 30%-50% of cases (unpublished data).

If the article by Abrahamsson is accurate and all prostate cancers show neuroendocrine differentiation with silver staining, this may indicate a critical degree of neuro-endocrine differentiation that is not detected in routinely fixed material. Tumours negative in this study may have shown very focal positivity if processed according to the article by Abrahamsson. This may represent a degree of neuroendocrine differentiation critical in prognostic evaluation. Other studies utilising methods of fixation similar to that used in this thesis have all yielded an incidence of neuroendocrine differentiation of only 40-50% (57,58,59,60,67,68).

The article by Abrahamsson may certainly add a new dimension to what is currently known about neuroendocrine cells in prostate cancer. His techniques differ significantly to those used in this thesis, and the incidence of neuroendocrine detection is in contrast to that of other investigators. This article requires further evaluation with parallel immunoperoxidase studies.

References

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1st ed. Edinburgh-London-New York, Churchill Livingstone, 1977, 16-28.

(b) Rosai J, Ackerman's Surgical Pathology, 7th ed. St Louis-Toronto-Washington-Washington D.C., C.V. Mosby Company, 1989, Ch.3, 31-51.

(c) Bussolati G, Papotti M, Sapino A, Gugliotta P, Ghiringhello B, Azzopardi J.G., Endocrine markers in agyrophilic carcinomas of the breast. Am.J.Surg.Pathol., 11: (4), 248-256,1987.

CHAPTER VI

STUDY IV: THE BIOCHEMICAL ASSESSMENT OF THE PROSTATIC NEURO-ENDOCRINE CELL

INTRODUCTION

Prostate specific antigen (PSA), a serine protease, is a highly specific marker of benign and malignant prostatic epithelium. Virtually all prostate cancers, (69,77,78) will demonstrate this antigen, albeit focally, on direct immunoperoxidase staining. Serum levels of PSA are to an extent proportional to advancing clinical stage (74). Considerable overlap, however, exists between stages, and in the individual case PSA levels are of questionable accuracy in predicting stage of disease. It is well described that patients with advanced cancers may only have minimally raised PSA values (74,79). The explanation of this phenomenon is poorly addressed in the literature.

Neuro-endocrine cells are a well recognized component of prostatic ducts and acini (79), and

approximately 50% of prostate cancers will contain neuro-endocrine cells. Neuro-endocrine cells in prostate cancer have been shown to be of independent prognostic importance (90,91). The aim of this study is to correlate on direct immunohistochemical staining, the PSA content of prostatic carcinoma cells with neuro-endocrine differentiation. Prostate specific acid phosphatase (PSAP) is also to be assessed in these neuro-endocrine cells. To the best of our knowledge the only previous study that addressed this issue was a single case report (60) that described PSAP and PSA in a single prostatic carcinoid tumour. Careful review of this article, however, confirmed that only acid phosphatase had been conclusively demonstrated in the neuro-endocrine cells. PSA was demonstrated in the tumour cells but no attempt had been made to prove these very same tumour cells to be of neuro-endocrine type. The report simply indicated that PSA containing cells were morphologically identical on light microscopy to the neuro-endocrine cell. In our experience (Study 2 and 3) the neuro-endocrine cell in prostatic adenocarcinoma is morphologically identical to the non neuro-endocrine tumour cell. We therefore question the observation that neuro-endocrine in this case report produced PSA.

17-B-Oestradiol is an oestrogen molecule produced from circulating testosterone by prostatic epithelial cells. As previously mentioned (CHAPTER I) this molecule is necessary for the normal development of the prostate and may reflect androgen receptor status of prostate cancer cells. Androgen receptors are extremely labile structures and studies (40,41) have shown these to be of little prognostic importance in prostate cancer. In a study related to the biochemical nature of the neuro-endocrine cell in prostate cancer we wish to assess the cell content of this molecule. This study is facilitated by the development of a new anti-17-B- Oestradiol polyclonal antibody (83).

METHODS AND MATERIALS

Ten cases of neuro-endocrine positive prostate cancer were selected randomly from the hospital records at this Institution. In all 10 cases neuro-endocrine cells represented between 10% and 30% of the total tumour cell population. Four serial three micron sections were prepared from each case. The first section was stained with haematoxylin and eosin to confirm the diagnosis of prostatic adenocarcinoma; the second section was stained with a monoclonal anti-PSA antibody (AMERSHAM) and developed with Diaminobenzadine

("DAB") substrate; the third section was stained with monoclonal anti-PSAP (AMERSHAM) and likewise was developed with the DAB substrate. The fourth section was stained with anti-17-B-Oestradiol. This too was developed with DAB and counterstained with haematoxylin.

Positive areas were compared in slides 2 and 3 and photographed. Section 3 (anti-PSAP) was resubmitted to immunoperoxidase staining with a specific marker of neuro-endocrine cells, monoclonal anti-Chromagranin A (ENZO). The stain, however, was modified by the addition of cobalt chloride to the DAB solution (92). This technique confers a blue-black colour to the DAB precipitate. This colour is readily discernible from the golden-brown colour of unaltered DAB.

RESULTS

In all 10 cases of prostate carcinoma, neuro-endocrine cells positive for Chromogranin A failed to stain with PSA immunoperoxidase (Fig. 22 & 23). Immunoperoxidase stains for PSAP confirmed the presence of this antigen within the neuro-endocrine cell population (Fig. 24 & 25) in all cases. A significant number of cells negative for neuro-endocrine marker Chromogranin A, stained

positively for PSA and PSAP (Fig. 26, 27 & 28).

Stains for 17-B-Oestradiol could not be assessed due to excessive background precipitation. These stains were repeated several times utilizing a variety of blocking agents, varying antibody titres, and enzyme digestive techniques. Despite these manipulations these stains could not be interpreted.

DISCUSSION

This study has demonstrated that in 10 cases of prostate cancer, neuro-endocrine cells in all cases failed to secrete PSA in levels detectable on tissue immunoperoxidase staining. These cells, however, produced detectable quantities of PSAP.

This finding may explain, in part, the variation of serum PSA in all stages of prostate cancer. Neuro-endocrine cells require further evaluation in prostate cancer subjected to hormonal manipulation. In this setting PSA levels decline significantly. This finding, however, may not indicate changes in the neuro-endocrine cell population. Immunohistochemical studies of tumours altered by hormone therapy are needed to fully evaluate the effects of this form of therapy on the

neuro-endocrine cell population in prostate cancer.

It is of some interest that PSAP has been detected in non-prostatic neuro-endocrine tumours, particularly of the gastro-intestinal tract (93). As the neuro-endocrine cell of the prostate is thought to be of endodermal origin there may be a closer link than was previously realized between the prostatic neuro-endocrine cell and the gastro-intestinal APUD cell. As the function of neither cell is in fact known, this biochemical link may be the first step in establishing the function of these cells.

17-B-Oestradiol staining was unfortunately not successful. This was primarily due to non-specific staining by the polyclonal anti-body. We hope to overcome this problem in future studies with the use of a recently developed probe to 5-alpha-reductase, another indirect method of assessing the androgen receptor.



Figure 22. A neuro-endocrine positive tumour stained with monoclonal prostate specific antigen (X 660).



Figure 23. A serial section of Figure 22 stained with monoclonal Chromogranin A. The cells positive for this marker fail to stain with prostate specific antigen (X 660).

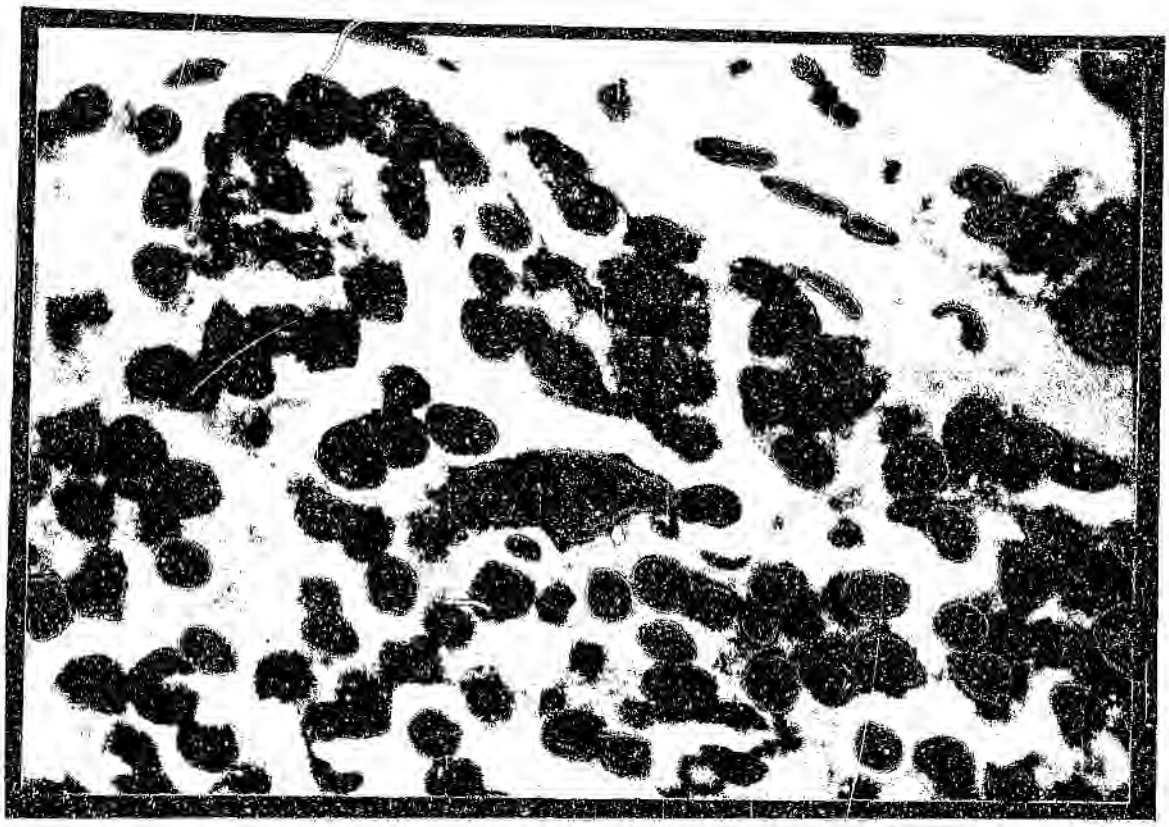


Figure 24. A neuro-endocrine positive cancer stained with monoclonal prostatic acid phosphatase. DAB substrate was utilized (X 1650).

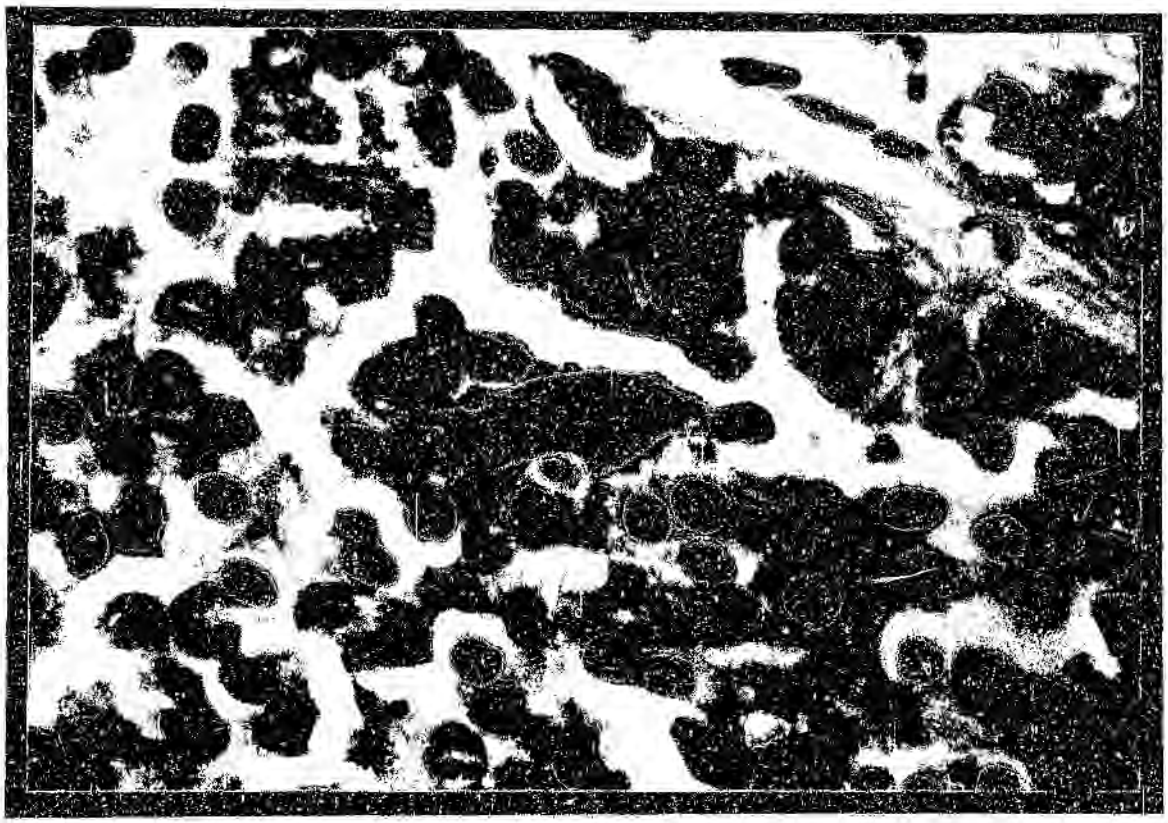


Figure 25. Section (Figure 24) restained with monoclonal Chromogranin A. DAB / cobalt chloride substrate was utilized. The neuro-endocrine cell is identified by blue-black staining. (arrow) (X 1650).

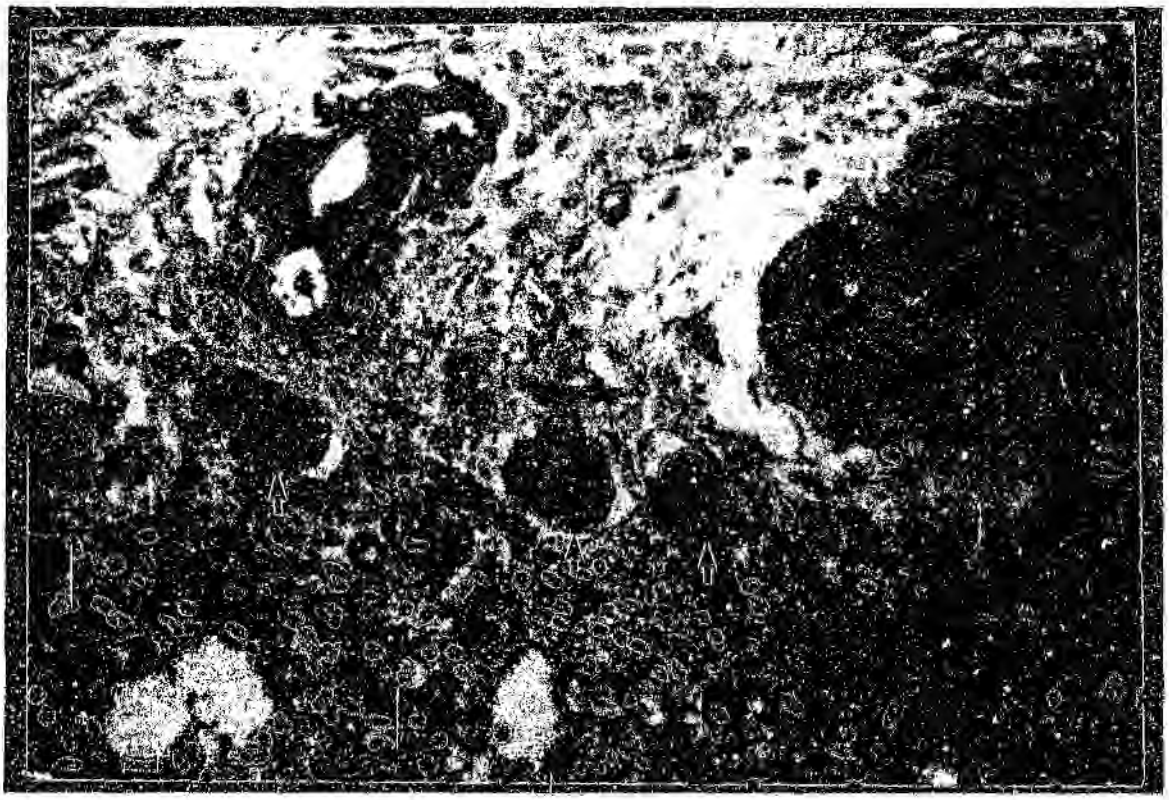


Figure 26. Prostatic adenocarcinoma with neuro-endocrine cells stained with monoclonal prostate specific antigen. Groups (arrows) of tumour cells fail to stain with this marker (X 660).

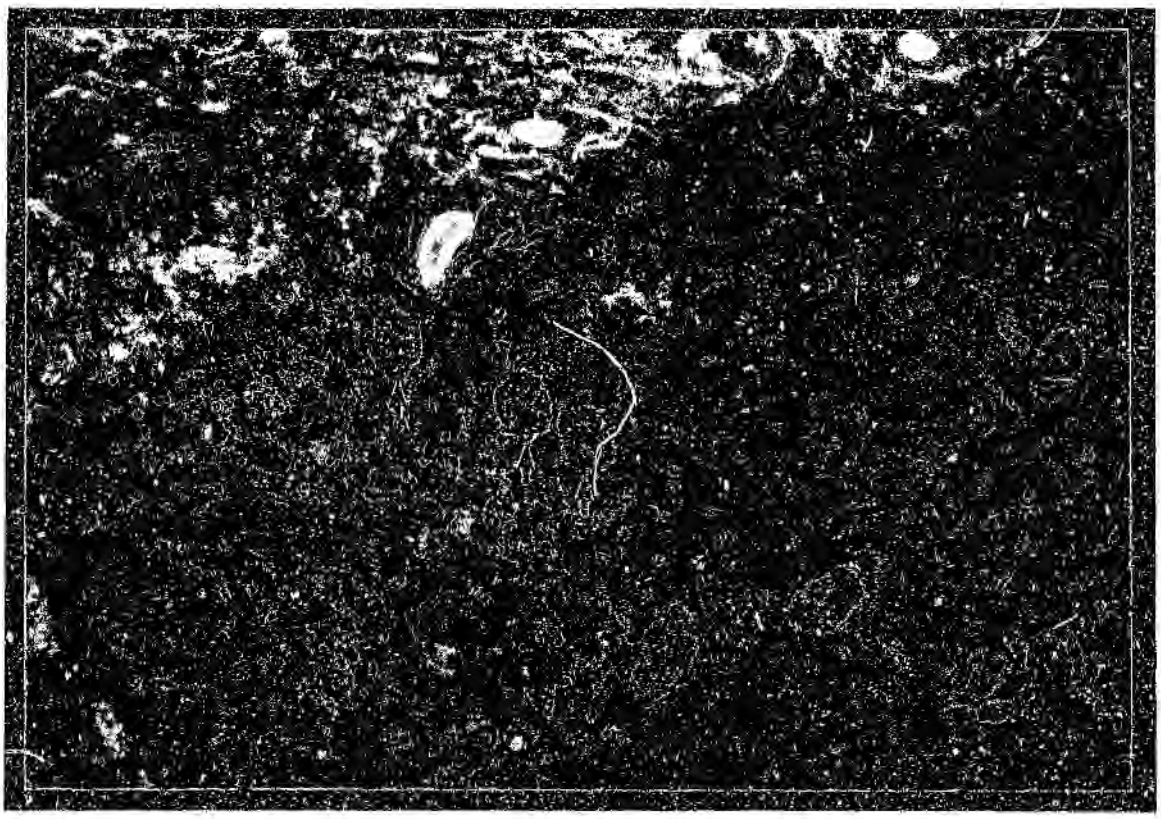


Figure 27. Serial section of tumour shown in Figure 26. Section was stained with monoclonal Chromogranin A. The groups of tumour cells noted in Figure 26 stain with this marker (X 380).



Figure 28. Serial section of tumour shown in Figure 26. Section was stained with monoclonal prostatic acid phosphatase. The groups of tumour cells noted in Figure 27 stain with this marker (X 660).

CHAPTER VII

STUDY 5 : THE NORMAL NEURO-ENDOCRINE CELL POPULATION

INTRODUCTION

As discussed in CHAPTER 1, there has been no adequate study addressing the subject of the normal occurrence and distribution of the neuro-endocrine cell through all stages of prostate development. Such a study requires the assessment of well preserved prostatic tissue, fixed in 10% formalin for a maximum of 24 hours to prevent excessive cross linkage and denaturing of antigenic sites. In addition, the material must be of such a nature that all zones of the gland are available for histological and immuno-histological assessment and comparison. The antibody, unlike those used in previous studies (57), should be monoclonal, in order to avoid background precipitation. Large numbers of prostate glands need to be examined, to allow comparison of the of neuro-endocrine cells in different glands at different stages of development. Finally, an accurate and objective counting method is necessary to compare the numbers

of neuro-endocrine cells in different zones and in different prostate glands.

A variety of peptides, including calcitonin (68) and somatostatin (94), have been demonstrated in the neuro-endocrine cells of the prostate gland. These studies do not document the distribution of calcitonin, or somatostatin producing cells. The present study assesses the normal occurrence and distribution of the neuro-endocrine cell through all stages of prostate development, and attempts to overcome the above problems.

MATERIALS AND METHODS

Sixty-three intact prostate glands were removed at autopsy from patients who died of unnatural deaths, unrelated to any prostatic disease. These patients ranged in age from 1 month to 70 years. The autopsies were performed within 12 hours of death and the prostate glands were fixed in 10% buffered formalin for 18 to 24 hours. The glands were sectioned sagittally (Fig. 29) to allow all zones to be examined simultaneously (fig 30). This sagittal section was cut in such a manner that the urethra and ejaculatory duct were present in a single histological preparation.

These large tissue sections (approx. 4.5 x 5.5 cm) were processed through to wax paraffin over a 24 hour period, and then sectioned on a rotary microtome at 3 microns. To facilitate this procedure, it was necessary to design a special tissue block (registered patent number 913715) which allowed tissue sections of this magnitude to be prepared (Fig. 31, 32, 33, & Annex 1). Two serial sections were cut, one stained with haematoxylin and eosin, the other with monoclonal Chromogranin A (ENZO) (Fig. 34).

The tissue sections were then examined under a dissecting microscope and the peripheral zone, central zone, and the peri-urethral glands were delineated.

The 57 cases were divided into the following groups: [*() = number of cases examined]

i) Infants less than 3 months of age *(2)

ii) Pre-pubertal males (3 months to 13 years) *(5)

iii) Pubertal males (14 to 18 years) *(5)

iv) Young adults (18 to 35 years) *(27)

v) Middle-aged males (36 to 50 years) *(10)

vi) Elderly males (50 to 80 years) *(9)

Five cases were excluded because of :

a) Acute Suppurative Prostatitis - 3 cases (In which most of the gland had been destroyed by an acute inflammatory process)

b) Poor Tissue Preparation - 3 cases

Ten high power (H/P) fields were then randomly selected in each zone and the number of neuro-endocrine cells per ten H/P fields was recorded. The radius of each field measured 0,25 mm; the surface area 0,196 mm2‡ ; and a total of ten fields were assessed (1,96 mm2‡). If no neuro-endocrine cells were noted in 10 H/P fields the entire zone in that tissue section was examined under high power magnification and the number of positively staining cells recorded. The number of high power fields examined per zone in each section ranged from 36 to 145, with a mean of 58.

Thirteen cases from the series of 58 prostate glands were selected according to their age distribution, as indicated below.

[*() = number of cases]

i) Infants less than 3 months of age *(1)

ii) Pre-pubertal males (3 months to 13 years) *(3)

iii) Pubertal males (13 years to 18 years) *(1)

iv) Young adults (18 years to 34 years) *(4)

v) Middle-aged males (35 years to 50 years) *(3)

vi) Elderly males (50 years to 80 years) *(1)

Two further serial sections were stained with polyclonal anti-calcitonin (DAKO) and polyclonal anti-somatostatin (DAKO) antibody. These sections were examined in the same manner as those stained for Chromogranin A. Where no staining was noted in 10 fields, the entire zone was examined, following the same protocol as outlined for Chromogranin A staining.

With respect to staining for Chromogranin A, the

peri-urethral glands, including the transition zone, were, for the purposes of this study, termed "NE1"; the ductal structures linking the peripheral acini to the verumontanum were termed "NE2"; and the acini in the peripheral zone were termed "NE3". With regard to calcitonin staining the same zones were termed "C1", "C2" and "C3" respectively.

RESULTS

The number of positively staining cells is represented in Table 3. Values in NE1 and NE2 are similar and do not significantly differ ($p = 0,76$). The values in NE3 are significantly lower than those in either NE1 or NE2 ($p = 0,01$ / $p = 0,04$ respectively). As indicated in the table, where no cells were observed in ten fields the entire zone in the section was examined and the number of cells in the total zone was noted. Where no positively stained cells were observed in an entire zone this, too, was noted.

Neuro-endocrine Cells

Neuro-endocrine cells were seen in the peripheral zones (NE3) in pre-pubertal males only during the first few months of life. Between 4 and 13 years no neuro-endocrine cells were seen in this peripheral

zone (NE3). These cells, however, re-appeared at approximately 14 years of age. Following puberty, every case demonstrated a number of neuro-endocrine cells in the peripheral zone. Conversely, neuro-endocrine cells were seen in relatively constant numbers in the peri-urethral glands (NE1) and in the prostatic ducts (NE2) in all age groups, including pre-pubertal males. It should also be noted in this group of pre-pubertal males, that although neuro-endocrine cells were not observed in the peripheral zones of the prostate gland, large numbers of neuro-endocrine cells were recorded in NE1 and NE2, verifying the adequacy of the stain.

Neuro-endocrine cells in zones NE1 and NE2 as well as the prostatic urethra (not assessed for this study) were shown to exhibit four morphological types. One cell type appeared as a basally orientated, elongated cell with dendritic processes (Fig. 35). The second cell type was superficial, larger and rounded without visible processes (Fig. 36). The third type of cell, a smaller cuboidal cell, was closely applied to the basal lamina (Fig. 37). All the neuro-endocrine cells in the peripheral zone (NE3) resembled the third basal type of NE1 and NE2 (Fig. 38 & 39). A fourth cell type was identified in only 2 cases. This cell appeared as an elongated spindle shaped stromal

cell, with slender cytoplasmic processes (Fig. 40).

Calcitonin

Stains for calcitonin demonstrated larger numbers of cells in areas C1 and C2 as compared to area C3. When comparing C1 with C2, by means of the Wilcoxon test, the "p" value of 0,17 was not significant. When comparing C1 and C2 with C3 respectively, significant "p" values were obtained (0,001 & 0,002).

Somatostatin

Stains for somatostatin revealed no staining in any zone of the prostate glands.

Incidental Schistosomiasis was documented in 15,5% of cases (Fig. 41).

DISCUSSION

Although DiSant'Agnese (57) noted large numbers of neuro-endocrine cells in the peri-urethral area of prostate glands removed at radical cystectomy, this is the first study to demonstrate the zonal

distribution of the neuro-endocrine cell in the normal prostate gland. In addition, prostate glands from pre-pubertal and post-pubertal males have not been compared in other studies.

Neuro-endocrine cells in the peri-urethral glands (NE1), and prostatic ducts (NE2) remain constant throughout life. The fluctuating levels of androgens at birth, puberty and old age do not appear to influence the the number of cells in these areas of the gland. Neuro-endocrine cells in the peripheral zone, however, are postulated to be present only when androgen levels are raised, namely, the neonatal period under the influence of maternal progesterone, and post-pubertal period under the influence of testicular androgens. The intervening years of childhood are characterized by low circulating androgen levels. The presence of an apparent androgen dependent and an androgen independent neuro-endocrine cell raises the possibility, of their being two types of neuro-endocrine cells in the different zones of the prostate gland.

Four, previously undescribed, morphological variants of the neuro-endocrine cell in the areas designated NE1 and NE2 were recognized. The presence of stromal neuro-endocrine cells has no'

been previously described in prostatic tissues, although it has been noted in the mucosa of the gastro-intestinal tract (95). As these stromal neuro-endocrine cells were so infrequently encountered,it is possible that they may represent a pathological process, such as a stromal metaplastic response to an as yet unidentified stimulus.

Calcitonin staining confirms the findings of DiSant'Agnese (68), but again shows a zonal distribution with little staining in the peripheral zone. DiSant'Agnese demonstrated positive somatostatin staining in less than 50% of his cases (94). This study has failed to confirm this finding.

Other studies have postulated the presence of more than one type of neuro-endocrine cell in the gastro-intestinal tract (95), this raising the possibility of 2 cell types of different embryonic origin; the one as indicated by Pearse et al (96) to be of endodermal origin, the other of possible neural crest derivation. The present observation in prostate glands favors the theory of divergent differentiation of an epithelial stem cell to form the neuro-endocrine cell in the peripheral zone. The neuro-endocrine cells of the other 2 regions

may well represent a mixed population, part of which may be of neural crest origin.

The function of the neuro-endocrine cell remains to be determined. The presence of neuro-endocrine cells with dendritic processes may indicate intimate association with nerve tissues which could conceivably provide the stimulus for degranulation of these cells. A large number of growth factors are secreted from nerve tissue (97) and these may be related not only to the function of the normal prostate gland but also to the tumour modulating function of the prostatic neuro-endocrine cell (91).

Calcitonin receptors in Leydig cells of the testis have been documented (98), and may be related in part, to the secretion of this hormone by the prostate gland.

In conclusion it is of importance that prostatic neuro-endocrine cells and their products be further studied as they may provide the solution to the problem of hormone relapse prostate carcinoma.

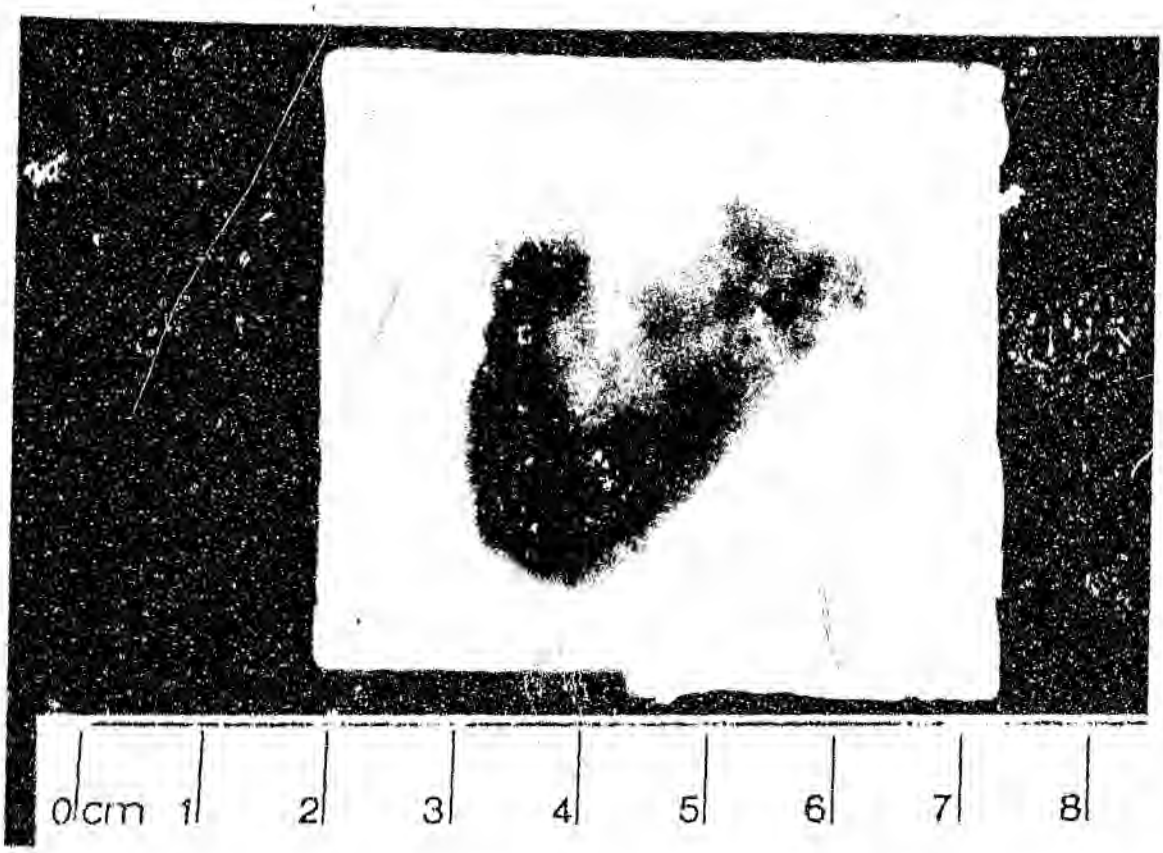


Figure 29. Sagittal section of an entire prostate gland embedded in paraffin wax.

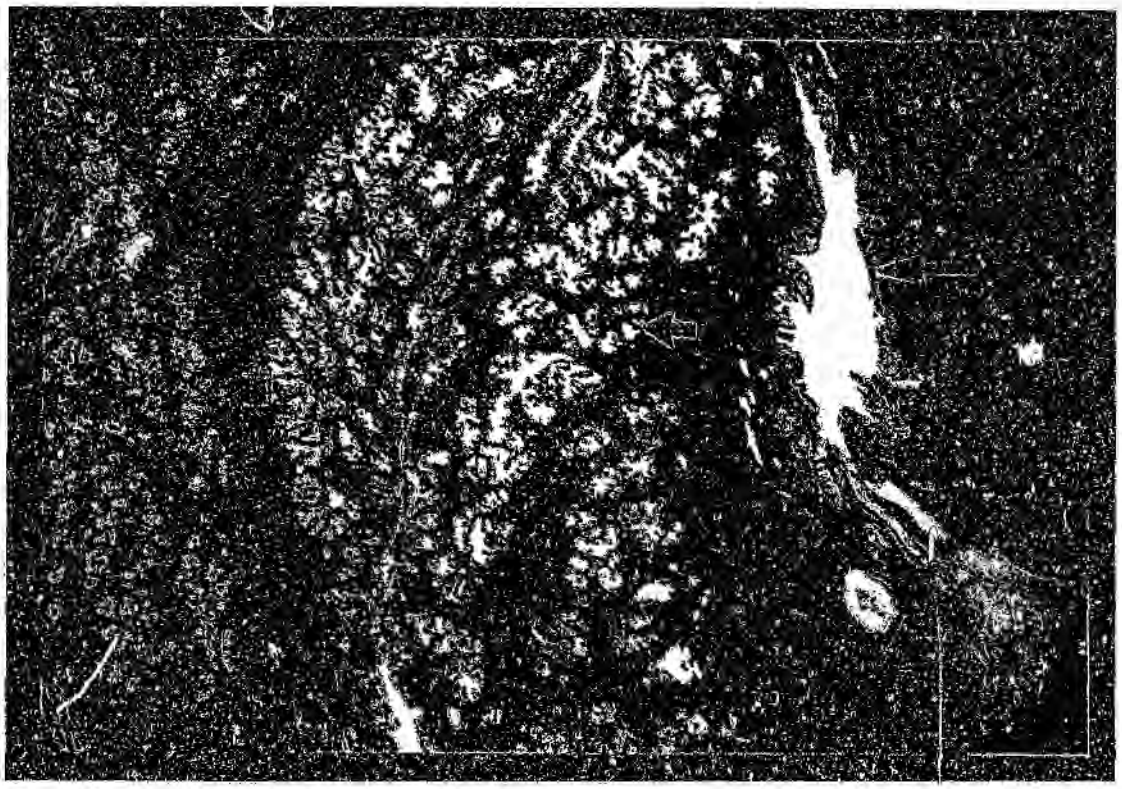


Figure 30. A low power field (X 16,5) of a prostate gland from a child aged 3 months. The prostatic urethra (long arrow); prostatic duct system (short arrow); and peripheral acini (open arrow) can be identified in this single section (Haematoxylin & eosin).

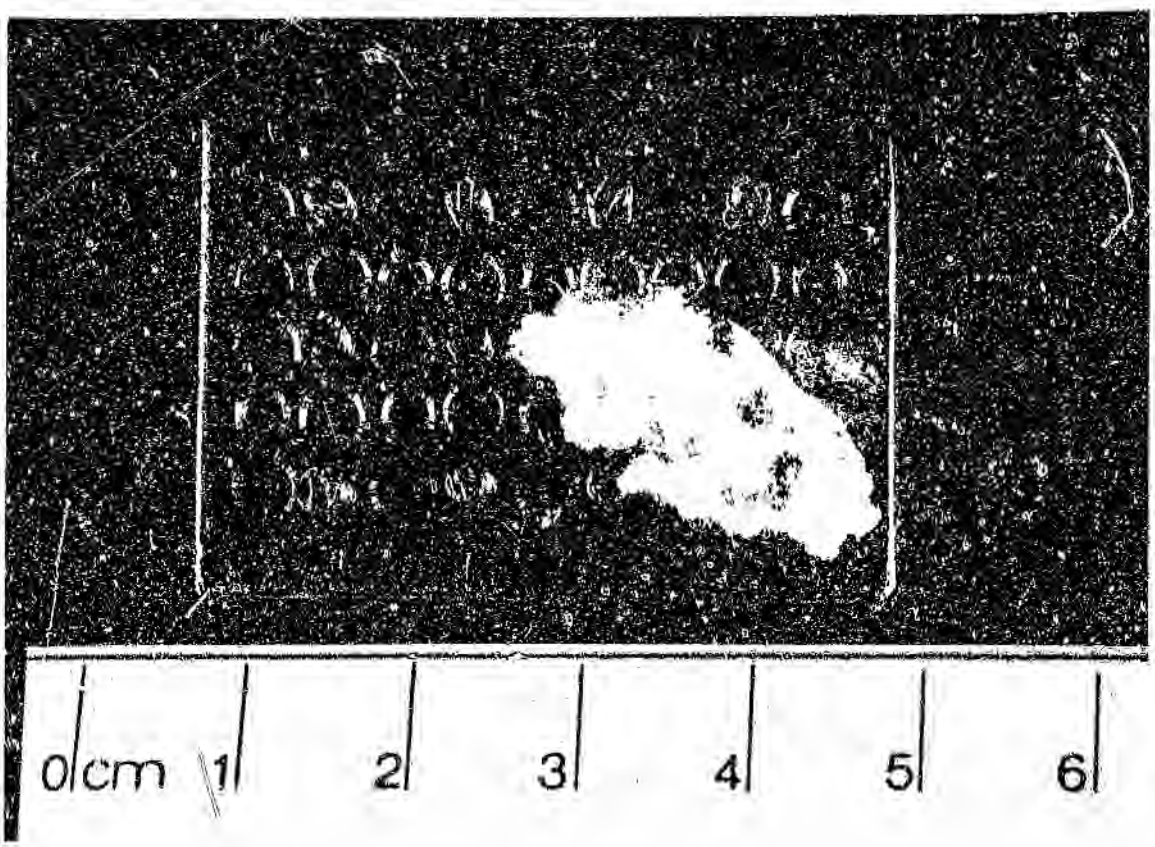


Figure 31. Prototype block designed to cut large prostate sections

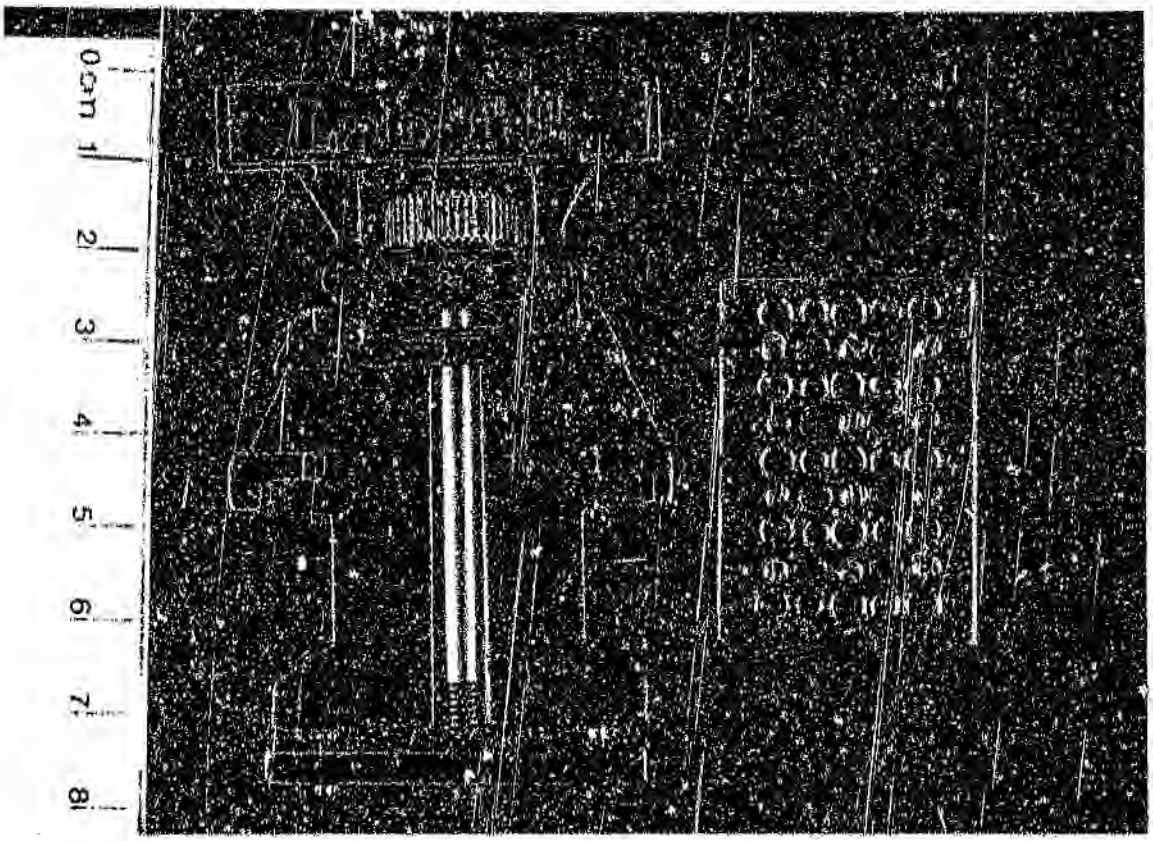


Figure 32. Prototype block together with the standard microtome head.

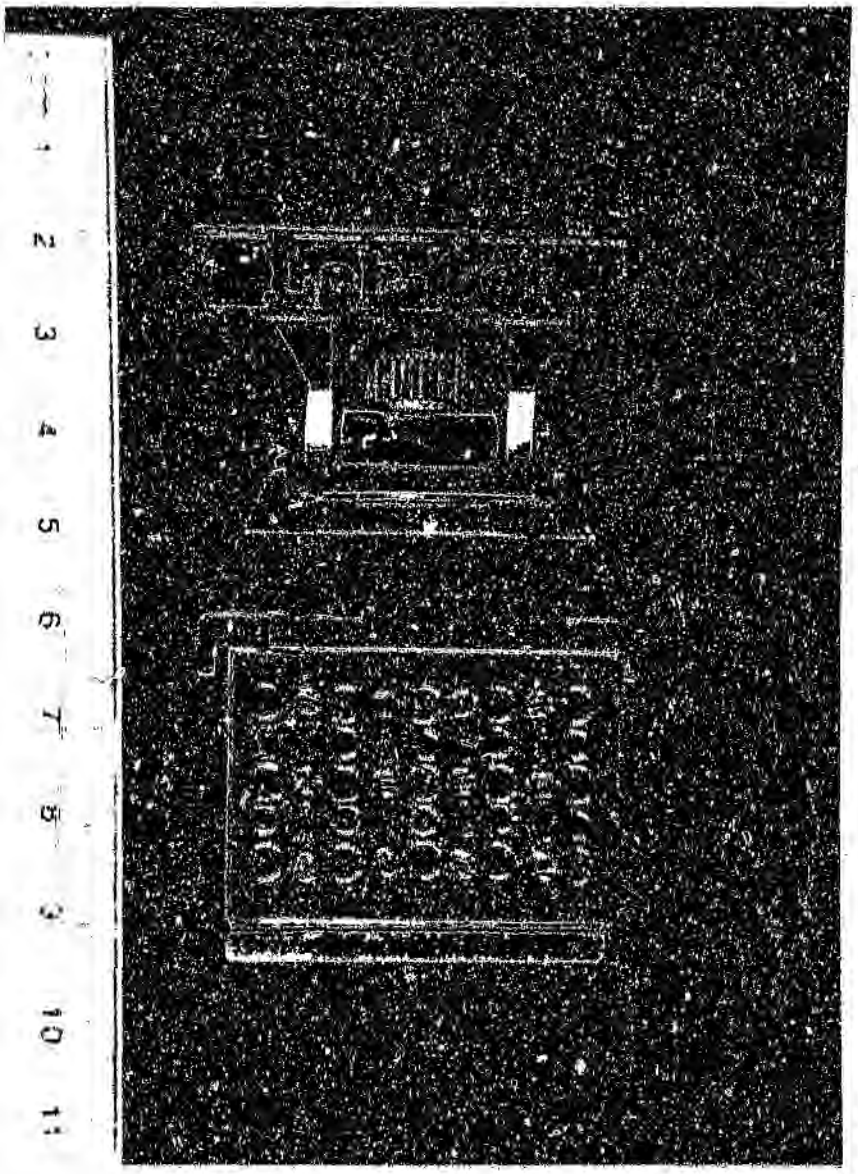


Figure 33. Prototype block clamped firmly into the standard microtome head.

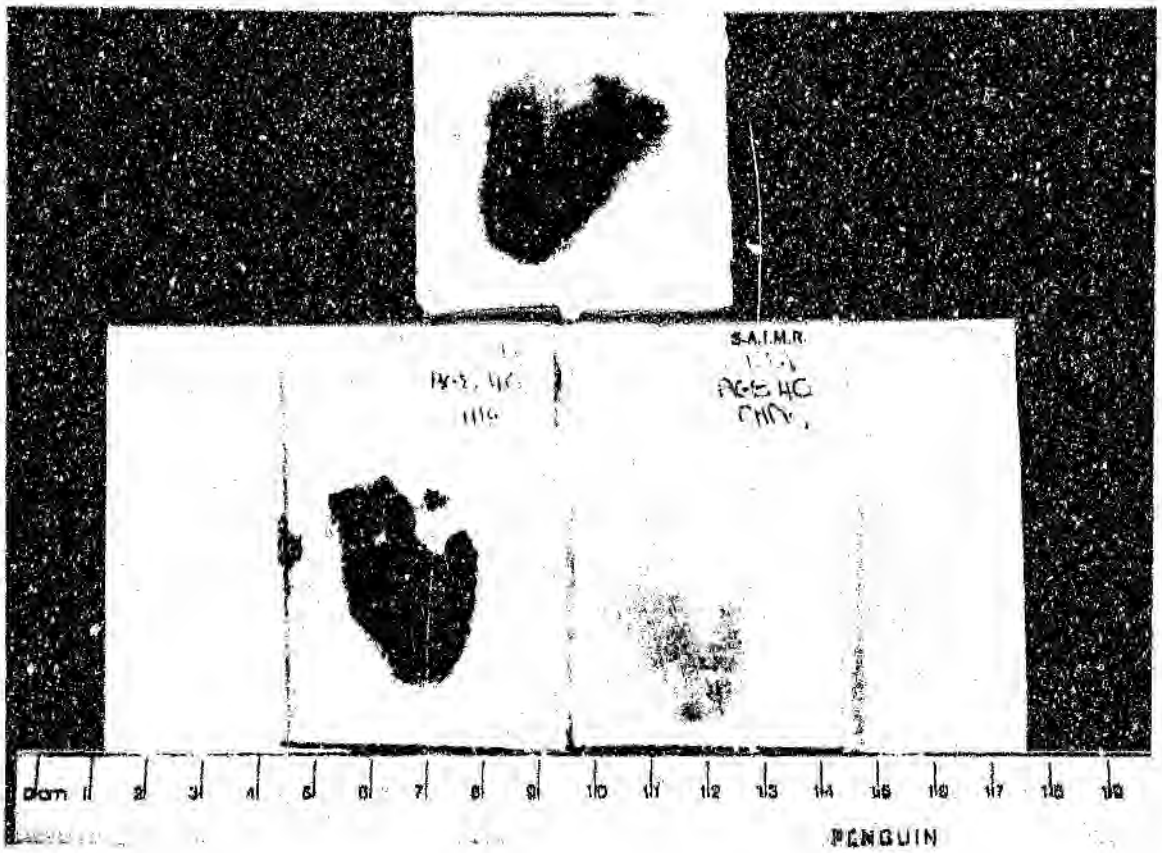


Figure 34. Paraffin block together with 2 large slides one stained with Haematoxylin and eosin, the other stained with Chromogranin A.

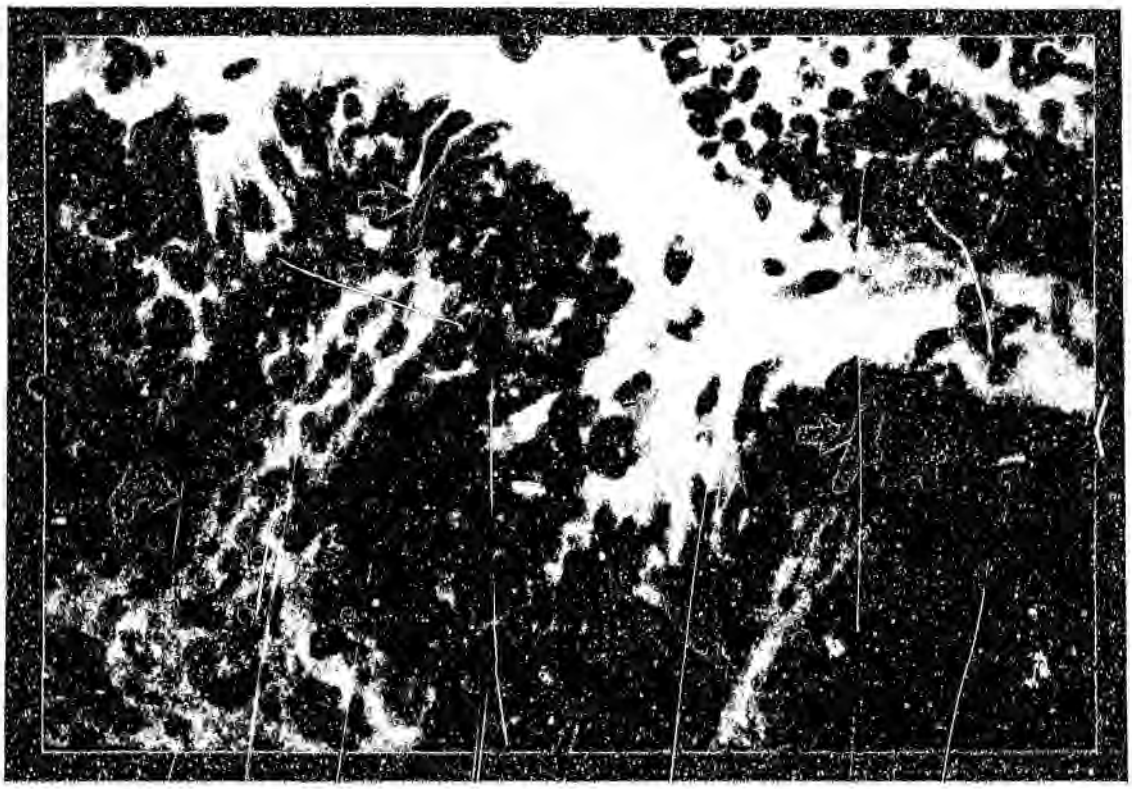


Figure 35. Prostatic epithelium stained with Chromogranin A which demonstrates basally orientated, elongated cells with dendritic processes (arrows) (X 660).

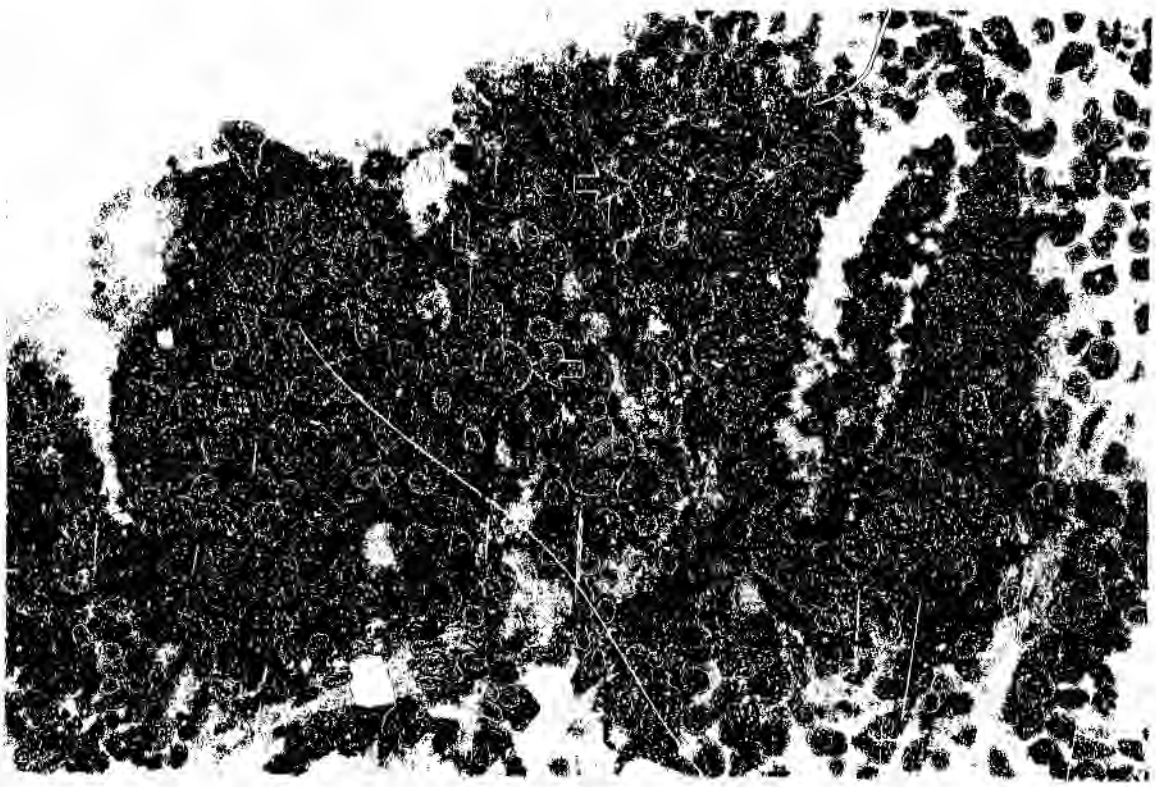


Figure 36. Prostatic epithelium stained with Chromogranin A which demonstrates a rounded cell without cytoplasmic processes (arrow) (X 660).

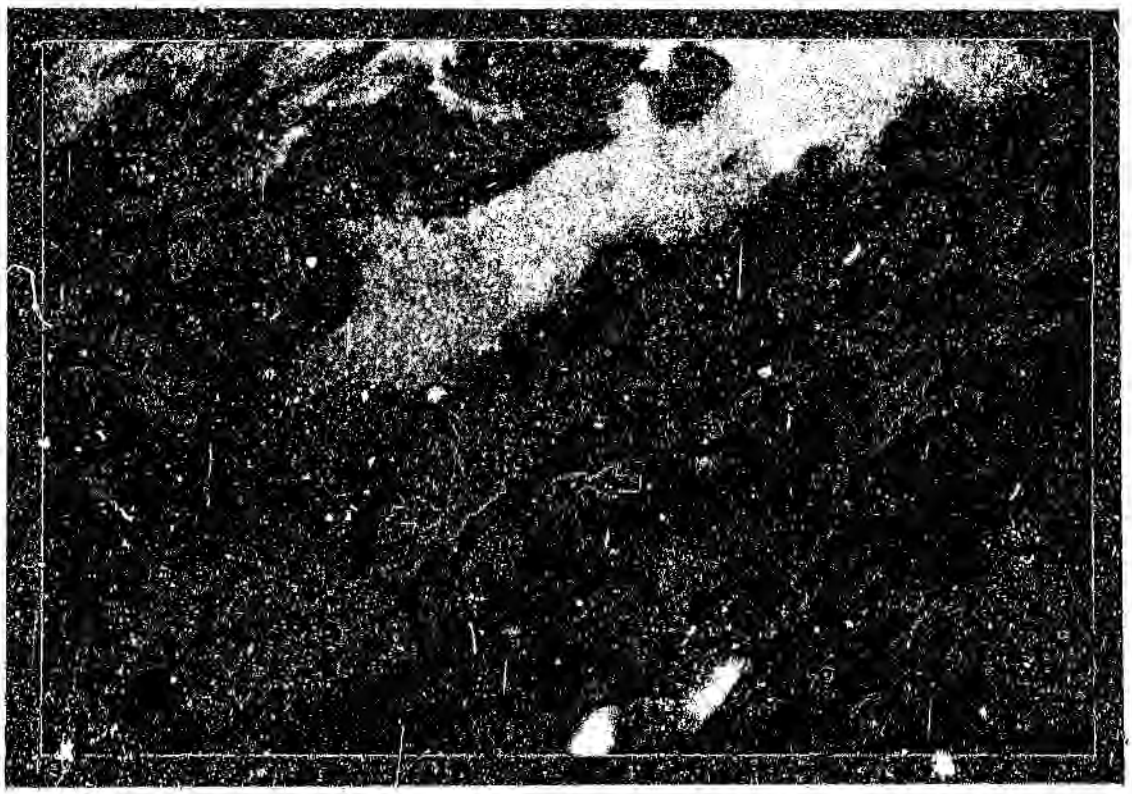


Figure 37. Prostatic epithelium stained with Chromogranin A demonstrating small basal neuro-endocrine cells (arrow) (X 660).

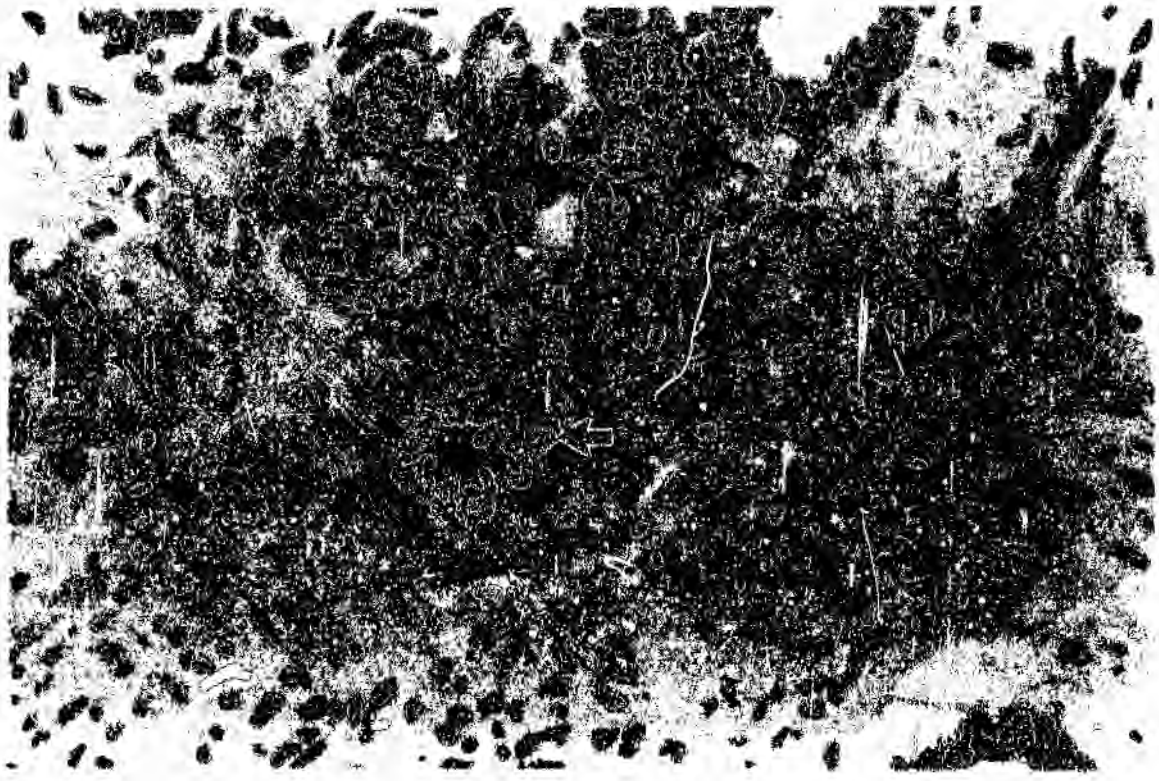


Figure 38. Peripheral zone of prostate stained wi. ı monoclonal Chromogranin A. Small basal cells are identified (arrow) (X 660).

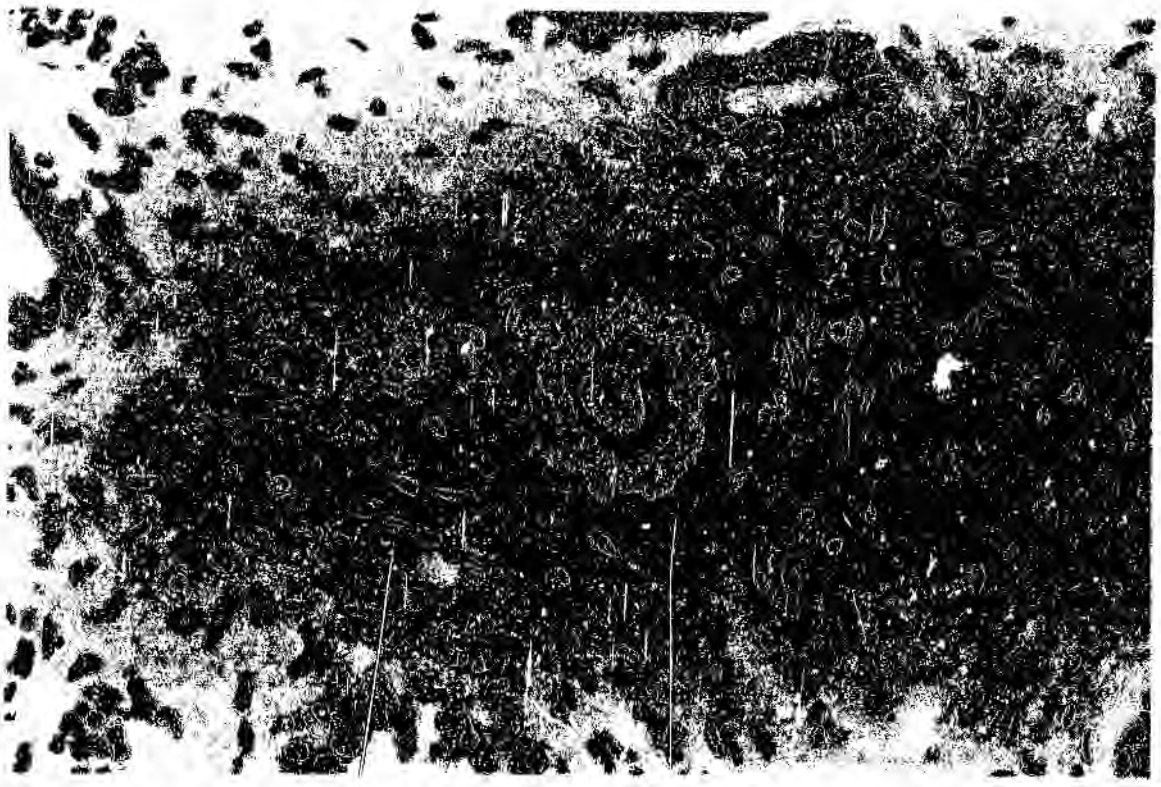


Figure 39. Prost :e from infant aged one month demonstrating well-formed prostatic concretions (X 660).

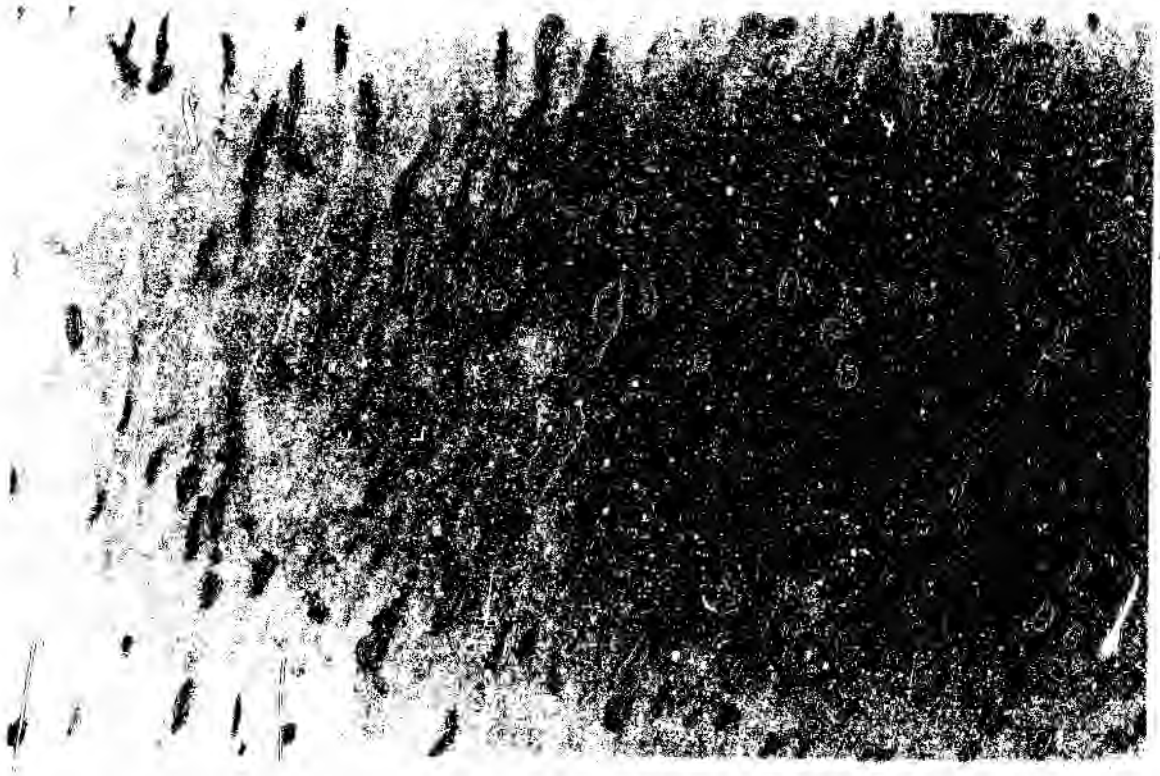


Figure 40. Stromal neuro-endocrine cells stained with monoclonal Chromogranin A (X 660).

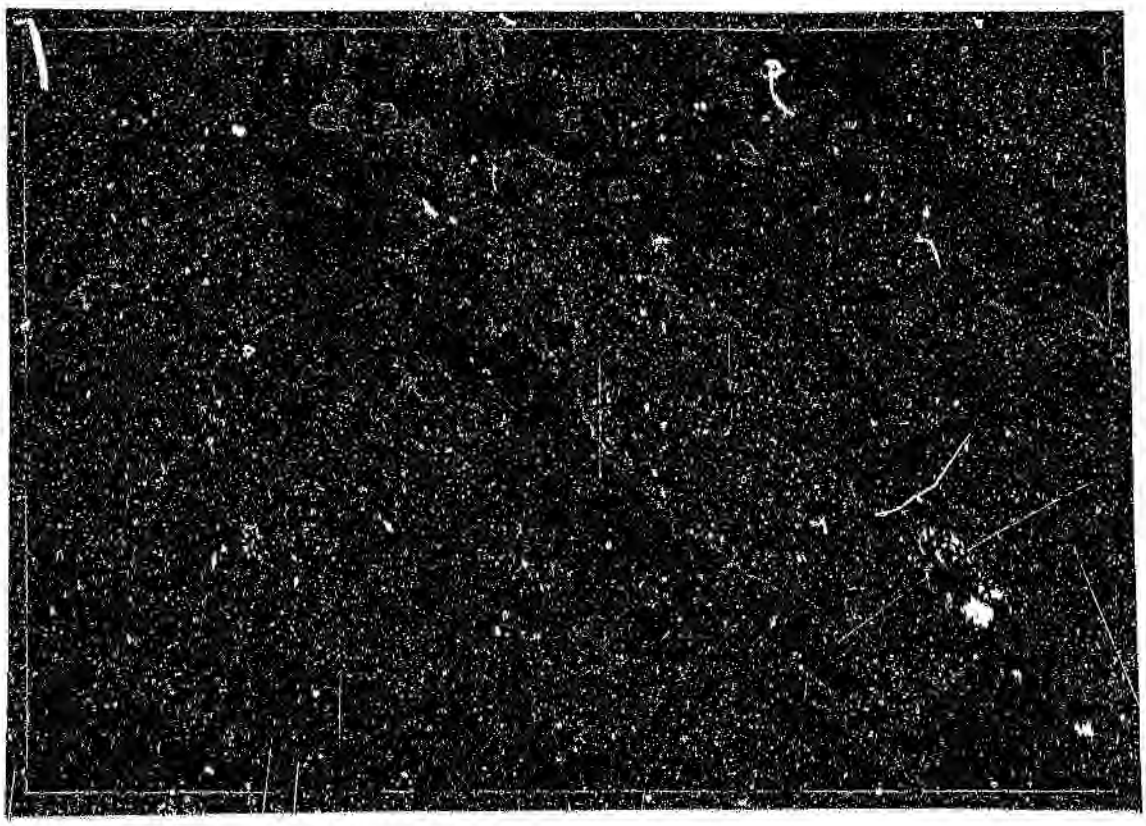


Figure 41. Bilharzia ova identified in 15,5% of cases (X 1650, Haematoxylin and eosin).

TABLE 3

TABLE 3

No.	CASE No.	AGE	NE-1	NE-2	NE-3	C-1	C-2	C-3	BILHARZIA
1	3	1m	49	37	17				-
2	21	3m	34	40	3	16	17	4	-
3	79	4yr	57	63	0*	23	19	0*	-
4	10	5yr	96	82	0*	35	23	0*	-
5	73	5yr	75	42	0*				-
6	84	9yr	19	14	0*				-
7	71	13yr	83	36	0*				-
8	40	16yr	66	60	6	70	49	4	-
9	81	16yr	113	53	10				-
10	72	17yr	209	103	15				-
11	70	18yr	189	142	3				-
12	75	18yr	100	29	5				-
13	77	19yr	212	303	17				-
14	98	20yr	33	24	2				-
15	60	21yr	NA	48	3				+
16	59	21yr	186	110	3				-
17	61	21yr	259	172	2				-
18	25	22yr	180	153	5				+
19	36	22yr	189	150	6	0*	0*	0*	-
20	65	22yr	NA	36	1				+
21	31	22yr	NA	62	6				+
22	20	24yr	13	22	6	0*	0*	0*	-
23	4	25yr	236	129	11				-
24	5	25yr	106	69	29	35	23	0*	-
25	48	25yr	59	40	1				-
26	68	26yr	NA	25	1				+
27	28	26yr	57	31	2				-
28	34	27yr	34	35	5				-
29	52	28yr	65	48	6				-
30	54	30yr	190	214	*				+
31	63	30yr	NA	38	7				+
32	41	30yr	71	122	6				-
33	8	30yr	103	74	6				-
34	14	31yr	79	38	5	13	4	0*	-
35	42	32yr	39	70	5				-
36	6	33yr	116	79	10				-
37	1	35yr	60	27	0(14,	8	0(2)	0*	-
38	49	35yr	199	104	*				-
39	39	35yr	75	147	2				-
40	29	38yr	239	220	37				-
41	55	38yr	178	42	9				-
42	53	39yr	NA	119	2				-
43	44	39yr	55	27	5				-
44	13	40yr	41	7	0(14)	1	2	0*	-
45	66	30	12	3					-
46	15	41yr	171	94	31				+
47	9	48yr	100	109	2	86	118	0*	+
48	38	49yr	101	30	4				-
49	46	50yr	124	87	11				-
50	33	51yr	93	72	14				-
51	17	56yr	84	106	7				-
52	2	60yr	77	48	20	8	0(15)	0*	-
53	26	61yr	33	16	2				-
54	7	61yr	196	NA	NA				-
55	22	67yr	108	124	1				-
56	51	68yr	52	24	3				-
57	69	70yr	12	8	3				-

NOTE

\+ ..... Indicates A Positive Value
\- ..... Indicates A Negative Value
NA ..... Indicates Tissue That Could Not Be Assessed (Inflamed or ulcerated)
() ..... The Number Of Cells In The Entl a Zone (1 Tissue Section)
* No Cells Noted In The Entire Zone (1 Tissue Section)

TABLE 3 CONTINUED

NEURO-ENDOCRINE CELL AND CALCITONIN
DISTRIBUTION IN THE NORMAL PROSTATE GLAND

	NE 1	NE 2	NE 3	C 1	C 2	C 3
Mean	105.0	77.0	7.4	22.0	20.0	0.6
Std.Deviation	69.8	55.9	8.0	26.5	32.6	1.5
Maximum	289.0	220.0	37.0	93.0	118.0	4.0
Minimum	12.0	7.0	0.0	0.0	0.0	0.0

ANNEXURE A

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT

AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) - Regulation 22)

The granting of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate

OFFICIAL APPLICATION No.			S & F REFERENCE
21	01	913715	JP/S 936

FULL NAME(S) OF APPLICANT(S)

71	RONALD JOSEPH COHEN

ADDRESS(ES) OF APPLICANTS(S)

	11 NIELSON DRIVE, BLAIRGOWRIE, RANDBURG, TRANSVAAL

TITLE OF INVENTION

54	"APPARATUS FOR PREPARING MICROTOME SPECIMENS"

~~THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. THE EARLIEST PRIORITY CLAIMED IS~~

COUNTRY: NONE	NUMBER: NONE	DATE: NONE

~~THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.~~

21	01	

~~THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND IS BASED ON APPLICATION NO.~~

P1	01	

THIS APPLICATION IS ACCOMPANIED BY:

- [X] 1. A single copy of a provisional ~~or two copies of a complete~~ specification of 5 pages.
- [X] 2. Drawings of 1 sheets.
- [ ] 3. Publication particulars and abstract (Form P.6 in duplicate).
- [ ] 4. A copy of Figure ..... of the drawings (if any) for the abstract.
- [ ] 5. Assignment of invention.
- [ ] 6. Certified priority document(s) (State number)
- [ ] 7. Translation of the priority document(s).
- [ ] 8. An assignment of priority rights.
- [ ] 9. A copy of the Form P.2. and the specification of S.A. Patent Application No.
- [X] 10. A declaration and power of attorney on Form P.3.
- [ ] 11. Request for ante-dating on Form P.4.
- [ ] 12. Request for classification on Form P.9.
- [X] 13. P2 in duplicate

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Dated this 16 Day of MAY 1991

SPOOR AND FISHER
APPLICANTS PATENT ATTORNEYS

RECEIVED
OFFICIAL DATE STAMP

REGISTRAR OF PATENTS

FIG. 1

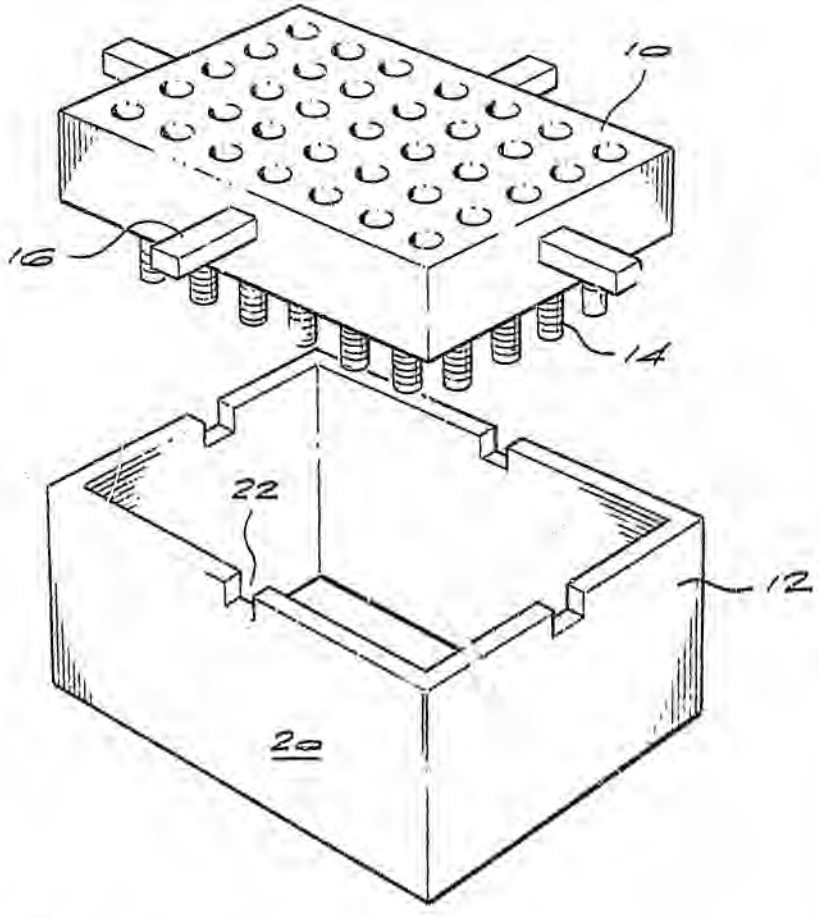
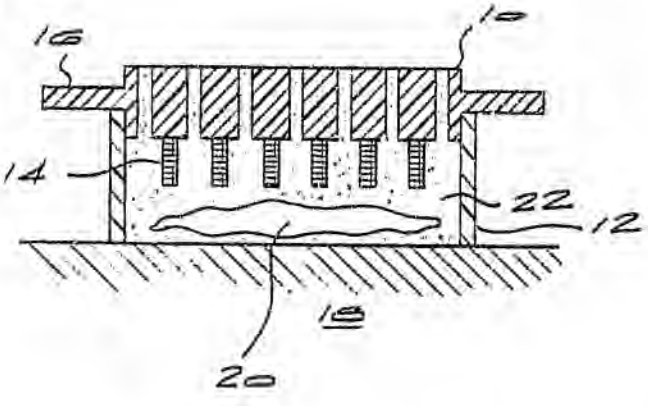


FIG 2



CHAPTER VIII

CONCLUSION

Prostatic adenocarcinoma is one of the major causes of death by malignant disease and will remain so well into the next century. Fifty years ago hormonal manipulation was found to alter the clinical course of the disease, and while some early investigators believed in hormonal cure, later studies confirmed tumour relapse in almost all cases. It also became apparent that some patients relapsed within months while others had a disease-free period lasting many years. Prognostic parameters including stage, histological grade, and a variety of new indices have failed to explain why patients with disease of similar stage and grade have such different responses to therapy and ultimately a different long-term prognosis. This study has attempted to explore two parameters not previously investigated, namely nucleolar organizer regions and the prostatic neuro-endocrine cell.

Nucleolar organizer regions (NOR) are indicators of chromatin content of the cell and have been used in the prognostic assessment of many tumours. This study confirmed a correlation between high value

NOR counts and malignant prostatic epithelium. NOR counts may therefore be of use in the histological assessment of atypical prostatic epithelium and the distinction between carcinoma and regenerative atypia. NOR counts, did not, however, prove to be a reliable prognostic index.

Although the prostatic neuro-endocrine cell has been recognized for almost a quarter of a century, very little is known about this cell population. This project has attempted to define this population, in tumours as well as in the normal prostate gland. This cell has been shown to provide accurate prognostic information on patients with clinically manifest carcinoma. The study has also confirmed that occult carcinomas contain fewer neuro-endocrine cells than do more advanced tumours. The reason for this is unknown, but implies an involvement of the neuro-endocrine cell in tumour progression.

The biochemical nature of the neuro-endocrine cell was investigated and it was demonstrated that this population unlike most other prostatic tumour populations (prior to hormone therapy) fails to produce prostate specific antigen (PSA) but secretes prostatic acid phosphatase (PSAP). It is of interest that PSA is less frequently detected in

tumour cells following anti-androgen therapy. The presence of neuro-endocrine cells in such recurrent tumours has not been addressed, primarily due to lack of appropriate tissues. It is suggested, however, that through future multi-centre collaborative studies this factor be further investigated.

The final chapter of this thesis suggests the possibility that there may be more than one type of neuro-endocrine cell and furthermore that these cells may be of different embryonic origins. It is clear that the treatment of advanced prostatic carcinoma, which has over the past 50 years remained unaltered, may not significantly improve, until the functions of the various neuro-endocrine cells are better understood.

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