specific for type 1 cPDE.

The inhibitor, theophylline (50 mM) was not as potent an innibitor as MIX (100 uM) which was as expected and in accordance with reports in the literature (see Introduction). Of interest, though, theophylline showed differential inhibition of the cPDE activity bands in murine mammary tumour tissue depending on which substrate was used (see Figs. 2:1a and 2:1b). Of the two inhibitors for type I cPDE, trifluoroperazine at a concentration of 1 mM was found to substantially inhibit the soluble cPDE forms in all the crude tissue cytosols suggesting that most of the soluble cPDE activity bands observed were calmodulin-dependent. It is postulated that trifluoroperazine binds reversibly to the active form of calmodulin and thus blocks the activation of the type I cPDE (Wolff and Brostrom 1979). Although calmidazolium is considered to be a much more potent inhibitor of type I cPDE than trifluoroperazine in the order of about 500 fold (Van Belle 1984), there was little inhibition of the cPDE activity in crude cytosols at the concentration tested (5 uM), particularly in the case of cGMP hydrolysis (Tables 2:5, 2:6, 2:7 and 2:8).

Although the majority of the soluble cPDE activity was ascertained to be calmodulin-dependent (the type I cPDE classification) there was no further activation of cPDE activity when calmodulin in the presence of calcium ion was added either to crude cytosols or to the incubation medium, suggesting that the cytosols contained sufficient endogenous

calcium/calmodulin. Furthermore, activated calmodulin remained bound to type I cPDE during both the polyacrylamide electrophoresis stage and incubation step.

Finally, in the overall comparison of the molecular masses of the six cPDE migrating forms there were no enzymes that had molecular masses of less than 169000 daltons (Table 2:11) either calculated from Ferguson plot of polyacrylamide gels or by linear polyacrylamide gradients or by gel filtration. Clearly, the soluble cPDE enzymes in the five murine tissues, when examined simultaneously, not previously done to my knowledge, possess both similarities and differences. It would therefore be of value to discuss the findings of the cPDE activities in the individual tissues and compare my results with findings in the literature. Since cPDE enzymes are both present in small quantities, in addition to being labile, there has been very little published on the enzyme in murine tissues and thus most comparisons will be based on the same tissues but in different mammalian species.

Murine brain:-

The tissue which exhibited the highest specific activity for soluble cPDE (both cAMP and cGMP hydrolysis at concentrations of 1 and 100 uM) was the murine brain. This was also true for the cPDE activity stain observed in the non-denaturing polyacrylamide electrophoresis gels and in the cPDE enzymes eluted from the excised activity band. This observation is consistent with comparative findings in brain tissue of bovines and rats (Strada *et al.* 1984). Indeed, since the soluble cPDE

activity in mammalian brain is so substantially greater, most of the homogenous preparations of cPDE enzymes have been attempted on cerebellum (Morril *et al.* 1979; Sharma *et al.* 1980; Hansen and Beavo 1982; Kincaid and Vaughan 1983). The non-denaturing polyacrylamide gel electrophoresis experiments consistently demonstrated one large, somewhat diffuse band associated with soluble murine brain fraction with an Rf range of 0.29-0.35 which, in fact, represented a range that was common to the other four tissue soluble cPDE forms studied (Table 2:4). This large cPDE activity band was observed over a polyacrylamide concentration range of 5% to 8%. When linear-gradient polyacrylamide gel electrophoresis was performed on the extracts of murine brain, however, three forms of soluble cPDE were clearly visible (Fig. 2:7) and they had a range of molecular masses of 169000 to 209000 daltons (Table 2:11).

Early studies on the soluble cPDE forms in rat cerebellum, using preparative non-denaturing polyacrylamide electrophoresis, demonstrated six peaks of cPDE activity, only two of which were calmodulin-dependent (Uzonov and Weiss 1972; Weiss *et al.* 1974). Unfortunately, ultrasonication was used in the preparation of the tissue by Uzonov and co-workers. A later study by Pledger and associates (Pledger *et al.* 1974), using isoelectricfocusing, demonstrated only three peaks of soluble cPDE activity in non-sonicated rat cerebellum as averse to 6 bands in sonicated tissue. These three other forms that arise as a result of sonication, which is a harsh procedure, might

represent membrane-bound cPDE enzymes and/or proteolytic forms derived from the presence of lysosomal enzymes released from the ruptured membranes of lysosomes. No molecular mass determinations of the cPDE forms were reported (Pledger *et al.* 1974), although the three respective pI values were shown to be 4.8, 6.1 and 8.2.

The properties of the purified soluble type I cPDE forms in bovine brain which represent the majority of the soluble enzyme activity has been extensively covered (see section on Type I cPDE enzymes in the Introduction). The most recent work indicates the presence of at least two subunits of 60000 and 63000 daltons in the native enzyme of the soluble type I cPDE that can exist in three dimer forms, that is, two homodimers or one heterodimer (Sharma and Wang 1985). The theoretical molecular masses, assuming 16000 daltons per molecule of calmodulin and two molecules of calmodulin in the holoenzyme would fall within the range of 152000 to 158000 daltons. It should be noted at this point that since brain tissue is composed of different cell types and exhibits marked differences in cPDE activity in various brain areas (Yang *et al.* 1981) the three *in vitro* isoenzymes might not reflect the *in vivo* situation in the different cell types. Nevertheless, in mouse brain, I have detected at least three soluble forms of cPDE activity by *in vitro* methods and, in addition to being strongly inhibited by trifluoroperazine (1 mM) suggesting that the majority of the cPDE activity to be type I, the molecular mass range was 169000 to 209000 daltons. My findings do not, therefore, differ much

from those reported in bovine and ovine brain.

Murine kidney:-

There has been very little reported in the literature on the soluble cPDE enzymes in kidney tissue. The most notable reports are those of Thompson and associates (Thompson *et al.* 1979; Epstein *et al.* 1982 (b)) which have described the purification of the soluble type IV (cAMP-specific) cPDE which is usually reported to be membrane-bound (see section on type IV cPDE in the Introduction). The type IV enzyme which preferentially hydrolyzes cAMP and has a molecular mass of about 60000 daltons was not detected by the methods used in my studies on murine kidney cytosols. I did, however, detect at least three soluble kidney cPDE forms (Fig. 2:6) on non-denaturing polyacrylamide gel electrophoresis and calculated, by Ferguson plot, the molecular masses to be 245000, 282000 and 385000 respectively (Table 2:12). These masses were somewhat higher than those calculated from non-denaturing polyacrylamide gradient gel electrophoresis (Table 2:11). Later, similar experiments based on human tissue which exhibited similar Rf ranges of cPDE activity to those of the kidney activity bands but, where I used special protein standards (Sigma) for Ferguson plot analysis, showed lower molecular mass values more closely resembling the data derived from the gradient gels (Table 2:11).

It could be argued that band 1 of kidney (Rf range 0.095-0.11) represents an aggregate of band 3, whereas the band 2 kidney form (Rf range 0.24-0.28) was most probably a discrete enzyme

because of its lack of inhibition by EDTA (Fig. 2:6). The crude cytosolic kidney cPDE enzyme activity was strongly inhibited by trifluoroperazine (1 mM) suggesting the majority of the soluble kidney cPDE enzyme activity to be calmodulin-dependent.

Murine Liver:-

I detected at least two forms of cPDE in cytosols of murine liver (Fig. 2:4a). The calculated molecular masses by Ferguson plot were 226000 and 233000 daltons respectively (Table 2:12) although, as I stated previously for the kidney forms, the molecular masses are probably somewhat lower. The cPDE activity of crude liver cytosols was strongly inhibited by trifluoroperazine indicating the majority of the cPDE activity to be type I. Nonetheless, the band 1 form of liver (Rf range 0.24-0.28) was relatively impervious to the presence of EDTA (1 mM), a chelating agent, whereas band 2 was largely inhibited. Much work on liver cPDE activities has been focussed on the particulate forms (Loten 1983; Pyne *et al.* 1987) although there have been some fairly recent publications on the soluble cPDE enzymes of liver which will be summarized as follows.

Four forms of cPDE have been described in either rat hepatoma or normal rat liver (Turnbull *et al.* 1984) although closer inspection of their methodology revealed that Turnbull and associates had subjected the liver homogenates to sonication. Others have reported 2 forms of cPDE in rat liver supernatant, one of them a type I cPDE with a mass of 150000 daltons and the other a calmodulin-independent form with a mass of 325000

daltons (Strewler *et al.* 1983).

One form of liver cPDE has been purified to apparent homogeneity and it resembles the Type II cPDE. The enzyme is cGMP-stimulated, calmodulin-independent with a molecular mass of about 201000 daltons (Yamomoto *et al.* 1983).

I am unable, at present, to comment on the possible presence of a type II cPDE form in one of the two migrating forms of liver cPDE I observed on polyacrylamide gels although it might be the band 1 form (impervious to EDTA). However, the maximum stimulation of type II cPDE occurs at cAMP concentrations of 10 uM (Martins *et al.* 1982) which I found to be too low to give adequate staining of all the cPDE forms using my methodology (see lower right plate Fig. 2:4b) which would preclude this possibility. By loading a higher protein concentration on the gels (5 mg protein/ml of murine heart cytosol instead of 2 mg/ml in 50 ul aliquots), however, I was able to perform experiments at substrate concentrations of 10 uM in heart cytosols (see below).

Murine heart:-

Early studies of the soluble cPDE enzymes in bovine heart demonstrated two forms (Goren and Rosen 1972). A later report by Donnelly and associates (Donnelly *et al.* 1978) concluded that, although three forms of soluble cPDE were eluted from DEAE-cellulose, the one peak represented a mixture of the other two enzymes and was therefore not a discrete enzyme. Since those early publications, individual forms of soluble cPDE enzymes from mammalian heart tissues have been purified to

apparent homogeneity: a soluble cPDE type I enzyme from bovine heart (Ho *et al.* 1977; La Porte *et al.* 1979) with apparent molecular mass range of 114000-150000 daltons; and a type II cPDE enzyme from bovine heart with a molecular mass of 240000 daltons (Martins et al. 1982). In addition to the purification studies, there have been some reports based on isoelectricfocusing of crude rat heart cytosols (Nemoz *et al.* 1981; 1983). The isoelectricfocusing studies, however, yielded somewhat ambiguous findings in that the earlier report demonstrated several forms of soluble bovine heart cPDE enzymes only three of which were shown not to be interconvertible. Whereas the later study (Nemoz *et al.* 1983), which described a second isoelectricfocusing step of the three pooled pI peaks 4.9, 5.45 and 5.6-6.0, showed 4 major cPDE forms with respective pI values of 4.9, 5.75, 5.9 and 6.2. In the latter study (Nemoz *et al.* 1983), it was deduced that the pI=4.9 band represented a type I cPDE and the pI bands 5.9 and 6.2 were found to be the cAMP-specific type IV cPDE enzymes.

In murine heart, I was unable to detect any type IV enzymes (that is, only hydrolyzing cAMP) as has been reported in rat hearts (Moses *et al.* 1987) but at least three distinct forms of soluble cPDE enzymes were demonstrated. The band i heart form (Fig. 2:4a) with Rf range 0.12-0.14 (Table 2:4) was unique to murine heart tissue. The calculated molecular masses from bands i to 3 were 246000, 225000 and 227000 daltons respectively. The latter two enzymes were of similar molecular mass but exhibited different charges and, furthermore, closely resembled the two

cPDE forms observed in the murine liver cytosols with the band 2 form impervious to EDTA (Fig. 2:6). Notable, was the observation that the cPDE activities of the crude cytosols of murine heart were least inhibited in the presence of 1 mM trifluoroperazine (for both cAMP and cGMP) compared to the other murine tissue cytosols (Tables 2:5 and 2:6) suggesting a portion of the cPDE activity to be calcium/calmodulin-independent. Indeed, the heart band 1 form of molecular mass 246000 daltons is similar in mass to the purified type II cPDE of bovine heart tissue calculated to be 240000 daltons by Martins *et al.*(1982) which was shown to be calmodulin-independent. Of interest, I was not only able to detect increased cAMP hydrolysis in the presence of cGMP but, in fact, at 10 uM cAMP + 1 uM cGMP and 10 uM cGMP + 1 uM cAMP there was increased cPDE activity in one and three bands (by larger peaks on densitometry scans) respectively compared to 10 uM cAMP and 10 uM cGMP (Figs 2:5e and 2:5f).

Murine mammary tissue:-

The description of soluble mammary cPDE enzymes in both normal and tumour tissues has been dealt with extensively in the introductory sections of Chapters 2 and 4 of this thesis. Suffice to say, there has been no purification of any of the soluble cPDE enzymes in mammalian tumour mammary tissues and in normal mammary tissue. Only one particulate form has been purified (Aitchison *et al.* 1984) and three soluble forms partially characterized (Mullaney and Clegg 1984). Four forms of soluble cPDE were consistently detected in the murine

mammary tumours using a 5% to 8% polyacrylamide concentration range (Savage *et al.* 1983). The two faster migrating forms (see Fig. 2:4b) with respective Rf ranges of 0.34-0.36 and 0.37-0.41 (Table 2:4) were unique to the murine mammary tumour tissue. Of note, in the human mammary tumour cytosols using the identical techniques, however, I was able to detect at least 6 forms (see Chapter 4).

The apparent molecular masses of the four murine tumour cPDE activities calculated from Ferguson Plot (Table 2:12) (bands from the top of the gel) were respectively 219000, 226000, 234000 and 229000 daltons. The small variation in molecular mass between the four cPDE forms indicated that the observed Rf values between the different enzymes were based on charge rather than molecular mass differences. The activity stain indicated that band 1 of the tumour (see Fig. 2:4b) had a higher staining intensity than the other cPDE forms. When identical protein concentrations were loaded on non-denaturing polyacrylamide gels two faint cPDE activity bands at positions 1 and 2 were noticed for tumour cytosols at 1 uM cAMP concentration (lower right Fig. 2:4b) suggesting that these two enzymes exhibited a higher affinity for cAMP than bands 3 and 4. Most notable in this experiment was the complete lack of staining activity for brain cytosols at 1 uM cAMP compared to the comparatively intense band observed at 100 uM cAMP (top left Fig. 2:4b).

Further evidence that characterized the band 1 form of tumour as being discrete (and incidentally, it shared the same Rf

range 0.24-0.28 with murine kidney, liver and heart) was the lack of inhibition of this form by the chelating agent EDTA (lower right Fig. 2:6). In later experiments, when I worked with human mammary and uterine tissues, I was able to show a cPDE band in these tissues with a similar Rf range of 0.25-0.27 to that of position 3 in murine cytosols (Tables 2:4, 6:1 and 6:2) that was impervious to EGTA (2 mM) indicating a calmodulin-independent cPDE.

My attempts to purify and characterize the soluble cPDE enzymes of mouse mammary tumours were to prove disappointing insofar as large losses of enzyme activity were incurred during the purification stages especially the chromatofocusing step (Table 2:13). Nonetheless, although activities for cPDE at 100 uM cAMP (low affinity) were undetectable, three isoelectric peaks of cPDE activity at 1 uM cAMP were observed with respective pI ranges of > 7.7, 5.2-5.6 and 4.1-4.2 (Fig. 2:9) (Robinson et al. 1983). These pI values are not dissimiliar from those described in the non-sonicated fractions of rat cerebellum which yielded PI values of 8.2, 6.1 and 4.8 on electrofocusing gels coupled to the cPDE activity stain (Pledger *et al.* 1974). Finally, gel filtration of the pooled pI peaks (active for cPDE) indicated the molecular masses to be greater than 200000 daltons since all the activity was eluted in the void volume (Fig. 2:10) which is consistent with my molecular mass determinations from Ferguson plot.

In conclusion, from the polyacrylamide-gel electrophoresis technique, it was evident that there were multiple forms of

soluble cPDE in the different murine tissues studied. Some of the cPDE bands were unique to specific tissues particularly the two faster migrating bands observed in the mammary tumour tissue. These two tumour-specific forms appeared to differ in charge from the other cPDE bands rather than on a mass basis. Also, no type IV cAMP-specific cPDE activities were evident nor were there any low molecular mass cPDE forms detected. The interesting findings derived from this study, especially the tumour-specific cPDE enzymes, was to prompt further investigation of these cPDE forms, both soluble and particulate, in human mammary tumours.

CHAPTER THREE

ULTRACYTOCHEMICAL LOCALIZATION OF cPDE IN HUMAN MAMMARY TISSUES.

Objectives

The aim of this study was to examine cPDE enzymes that are membrane-bound in human mammary tissues. This was achieved by utilizing an enzyme-specific stain for cPDE on whole mammary tissue sections and then studying the stained sections under an electron microscope. The method employed enabled a reasonable sample size to be analyzed and, furthermore, the technique required little tissue which is a necessary prerequisite in studies based on human tissues. However, because of lack of tissue, the research was confined to using cAMP as the substrate and thus the effect of cGMP remained unknown.

Introduction

The control of abnormal multiplication and differentiation of cells in cancer or premalignant states remains to be elucidated at the eukaryote level. The substrate cAMP, which is a potent regulator of cell function, is implicated in cell proliferation (Ryan and Heidrich, 1974). The study, therefore, of the enzyme

forms of cPDE are of interest with respect to their role in the control of cell growth and transformation (see introduction of Chapter 2 for an in-depth review). There exist soluble and particulate forms of cPDE in a variety of tissues (Beavo *et al.* 1982). The membrane-bound forms have been purified to homogeneity in rat liver membranes (Marchmont *et al.* 1981(a); Pyne *et al.* 1986).

To date, three functionally different forms of soluble cPDE have been described in rat mammary tissue (Larner and Rutherford 1982; Mullaney and Clegg 1984). A membrane-bound cAMP-specific cPDE (type IV cAMP PDE) which is hormone-dependent (insulin-stimulated) has been described, although not purified, in isolated acini from mammary tissue of lactating rats (Aitchison *et al.* 1984). In neoplastic mammary tissue, Singer *et al.* (1976) showed that in the human breast tumours, in addition to overall higher levels of cPDE activity (only cAMP was tested) there was present relatively more of a low Km (high affinity form) than the low affinity form. Other workers have also shown that there are higher levels of soluble cPDE in malignant mammary tumours compared to normal tumours provided that results were expressed on a per protein basis as averse to a per DNA basis (Kung *et al.* 1977). Sheth *et al.* (1980), using murine normal and neoplastic mammary gland, reported that the tumour tissue possessed higher activities of a particulate or membrane-associated form of cPDE when compared to the normal tissue.

In an attempt to detect the membrane-bound cPDE enzymes in

normal and neoplastic human mammary tissues, I used an ultracytochemical technique. I present evidence that in neoplastic tissues a specific form of cPDE appears to be localized within the nucleus. This form could not be detected in the normal tissue examined. Associated plasma membrane activity was observed in both normal and neoplastic cells. The malignant murine mammary tissue of the F1 hybrid (described in Chapter 2 of this thesis) was also studied. The effects of two known phosphodiesterase inhibitors, theophylline and papaverine, are also discussed.

Materials and Methods

Tissue collection.

Tissues were obtained from human subjects during routine operative procedures in accordance with the Ethical Procedures stipulated by the University of Witwatersrand Senate Human Subjects Research Committee. A total of seventeen cases were examined comprising five infiltrating duct carcinomas of the breast, eight with fibrocystic disease of the breast and four cases of normal mammary tissues. All the tissues were documented histologically by Dr. Ninin to be either malignant, benign or normal. The murine mammary tissue was processed in the same way as that for human mammary tissues described on the next page.

Cytochemical procedure for localization of cPDE.

The membrane-bound cPDE forms were localized using methods described by Florendo *et al.* (1971) and further modified by Kalderon and Ravenshenas (1974), Farnham (1975) and Duma and Moraczewski (1980). Essentially, the rationale behind the cytochemical procedure is that endogenous membrane-bound cPDE hydrolyzes cAMP to 5'AMP. The 5'AMP is converted to adenosine plus Pi using an excess of exogenous 5'-nucleotidase in the incubation medium. The byproduct, Pi, forms a precipitate present in the incubation medium, thus forming an electron dense product at or near the site of cPDE activity.

Within 3 minutes of biopsy, the specimen was placed in buffer containing 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, and then carefully cut into approximately 1 mm sections with a surgical blade. The sections were stored in this buffer at room temperature for 30 minutes. The buffer solution was then replaced with 0.05 M cacodylate buffer pH 7.4 without glutaraldehyde, gently rinsed twice and the specimens were left in the refrigerator overnight. The following day the tissue was incubated for 30 minutes at 37^{o} C in a solution of 80 mM Tris/maleate buffer, pH 7.4, containing 2 mM $MgCl_2$ and 50 units/ml 5'-nucleotidase (Grade IV - Sigma Chemical Company). Thereafter, the tissue was placed in 80 mM Tris/maleate buffer, pH 7.4, containing 2 mM $MgCl_2$, 1.5 mM lead nitrate, 3mM cAMP and 30 units/ml 5'-nucleotidase for 1 hour at 37^{o} C. This was followed by a 5 minute rinse in 80 mM Tris/maleate, pH 7.4, at

room temperature. The tissue was them fixed for 2 hours at 0^{o} C in 2% OSO_4 cacodylate buffer pH 7.4. Dehydration was achieved by increasing concentrations of ethanol viz 50%, 70%, and 95% for 10 minute periods and finally 100% for two 15 minute periods. The specimens were then placed in Epon overnight. The following day the tissues were set into blocks by placing them in Epon at 60^{o} C for 48 hours. Sections were then cut using a Reichert-Jung Ultracut OMU-4 and viewed unstained in an Electron microscope, Jeol 100s. Control incubations were carried out with solutions containing 1) only lead ions 2) without cAMP 3) without 5'-nucleotidase 4) full incubation medium which contained 10 mM theophylline and 6) full incubation medium with 15 mM papaverine (papaverine hydrochloride). The latter two are known cPDE inhibitors. Thick sections adjacent to that examined ultracytochemically, were also cut, stained with toludine blue and viewed under the light microscope to determine the histology of the tissue.

Results

The results were somewhat marred by the fact that there was incomplete penetration of lead staining (that is cPDE activity) in the 1 mm sections of the various mammary tissues studied. Ideally, much thinner sections of the fresh tissues during the incubation stage for cPDE activity would have circumvented this problem. The equipment for such a step, however, was not available. Nonetheless, assuming incomplete penetration of the incubation products to the central core, there were some very interesting findings observed in the outer perimeter of the tissue sections. Studies, therefore, of all the different mammary tissue sections were confined to that area.

The most significant observation of the study was that in some of the malignant cells of carcinoma of the human breast there was considerable membrane-bound cPDE activity localized in the nuclear membrane, chromatin and nucleoli (only cAMP was tested)(Plates 3:1, 3:2, 3:7). In addition, there was cytoplasmic membrane activity although this staining was not observed in the cells which exhibited nuclear activity. The five cases of infiltrating duct carcinoma of the breast all exhibited identical nuclear activity (Plates 3:1, 3:2). Eight cases of fibrocystic disease, a benign condition, were examined (particularly areas of epithelial proliferation) and these exhibited nuclear reaction product but to a much lesser degree (Plate 3:4). Plasma membrane activity was also observed. Four normal breast tissues reported as being histologically normal

were examined and found to exhibit no nuclear activity (Plate 3:5) but cytoplasmic staining was evident (Plate 3:6). The tumour controls on all the above tissues with no 5'-nucleotidase added showed little or no reactivity stain in nuclear areas of the tissues which exhibited the phenomenon (Plate 3:3). The controls with 10 mM theophylline added did not appear to inhibit the detectable cPDE activity associated with the nuclear or plasma membranes, nucleoli and chromatin (Plates 3:6, 3:8). The controls without cAMP or with 15 mM papaverine showed no evidence of any staining activity (Plates 3:9, 3:10). The identical nuclear activity was also observed in the malignant murine mammary tissue (Plate 3:11).

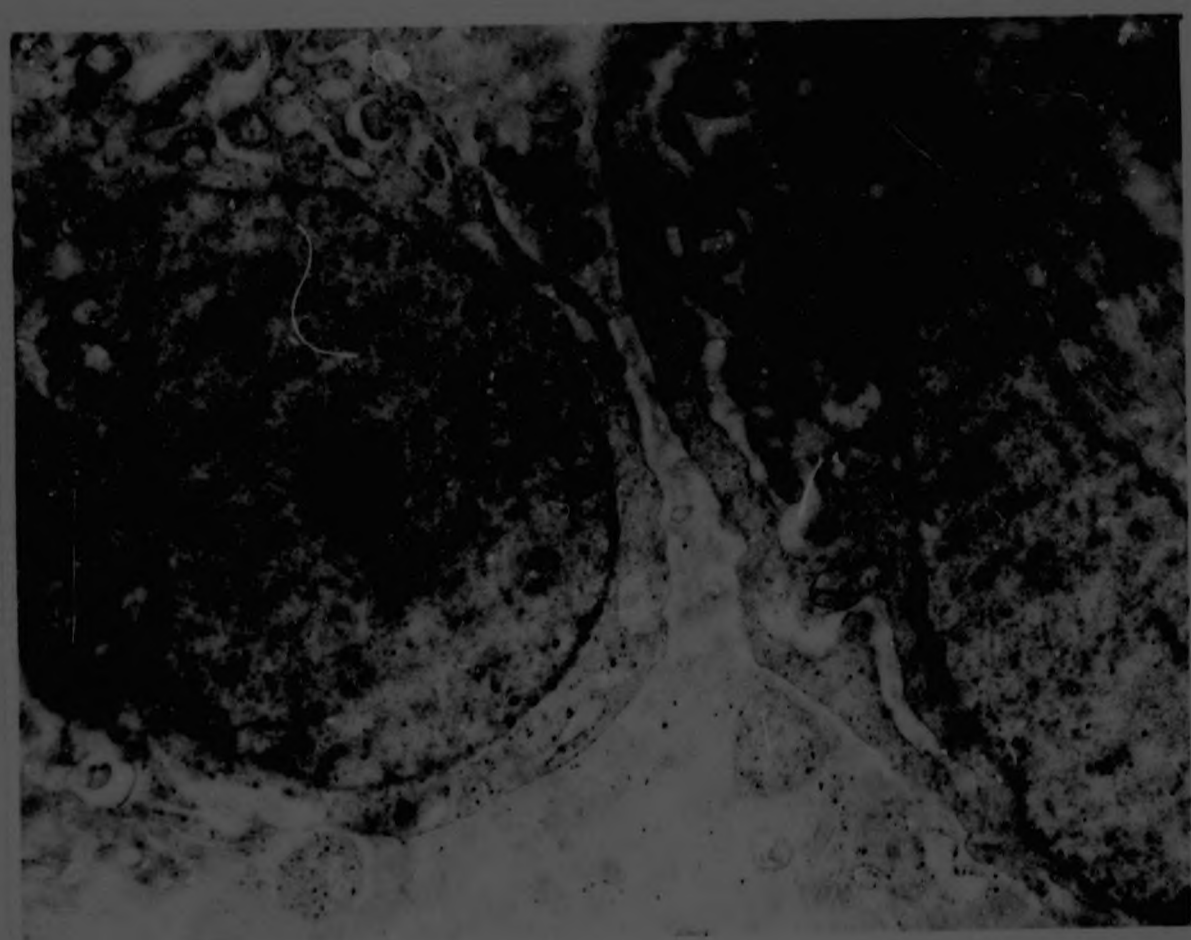


Plate 3:1. Human mammary cancer tissue in complete incubation medium. Cytochemical localization of cPDE activity is particularly concentrated in the nuclei and nucleoli (see arrows). Note slight scatter of lead deposit in surrounding organelles.

E.M. magnification = 5000X

Negative mag. = 2X

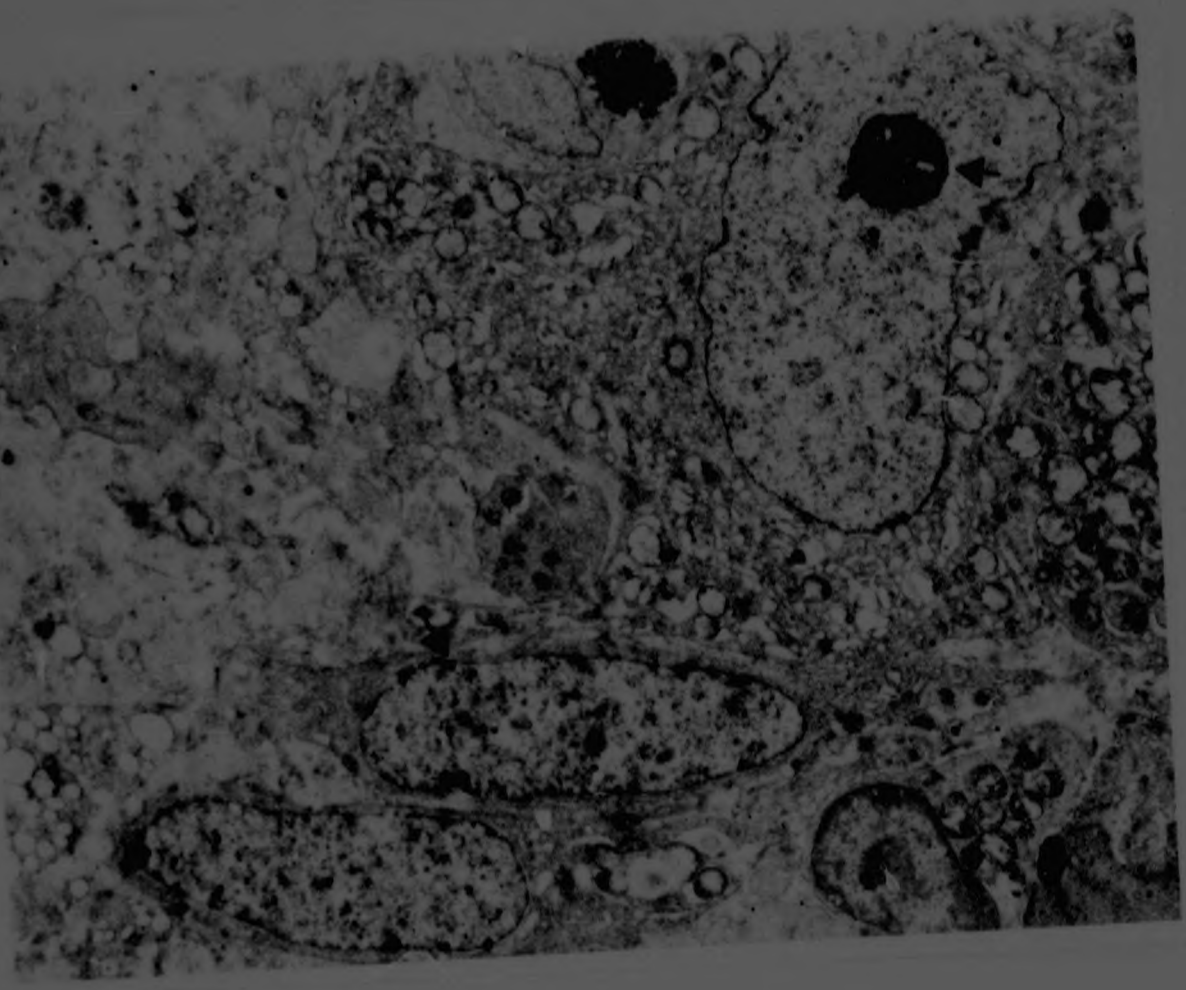


Plate 3:2. Human mammary tissue in complete incubation medium. Lower magnification of cytochemical localization of cPDE activity showing lead deposit concentrated in the nuclei and nucleoli (see arrows).

E.M. mag. = 4000X

Neg. mag. = 2X

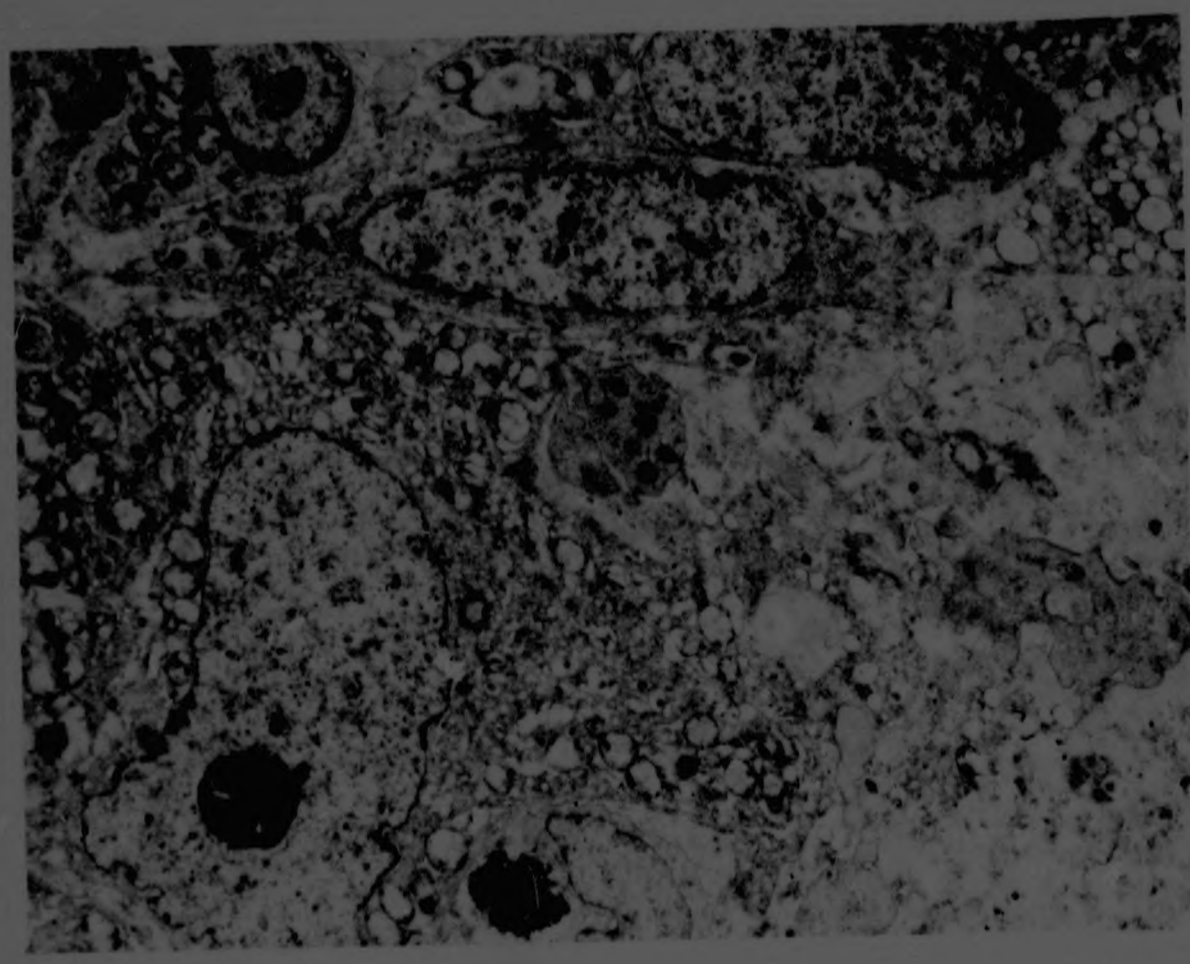


Plate 3:3. Human carcinoma of the breast in control incubation medium without 5'-Nucleotidase. In the five malignant mammary tissues studied there was very little nuclear activity observed in these controls.

E.M. mag. = 2000X

Neg. mag. = 2X

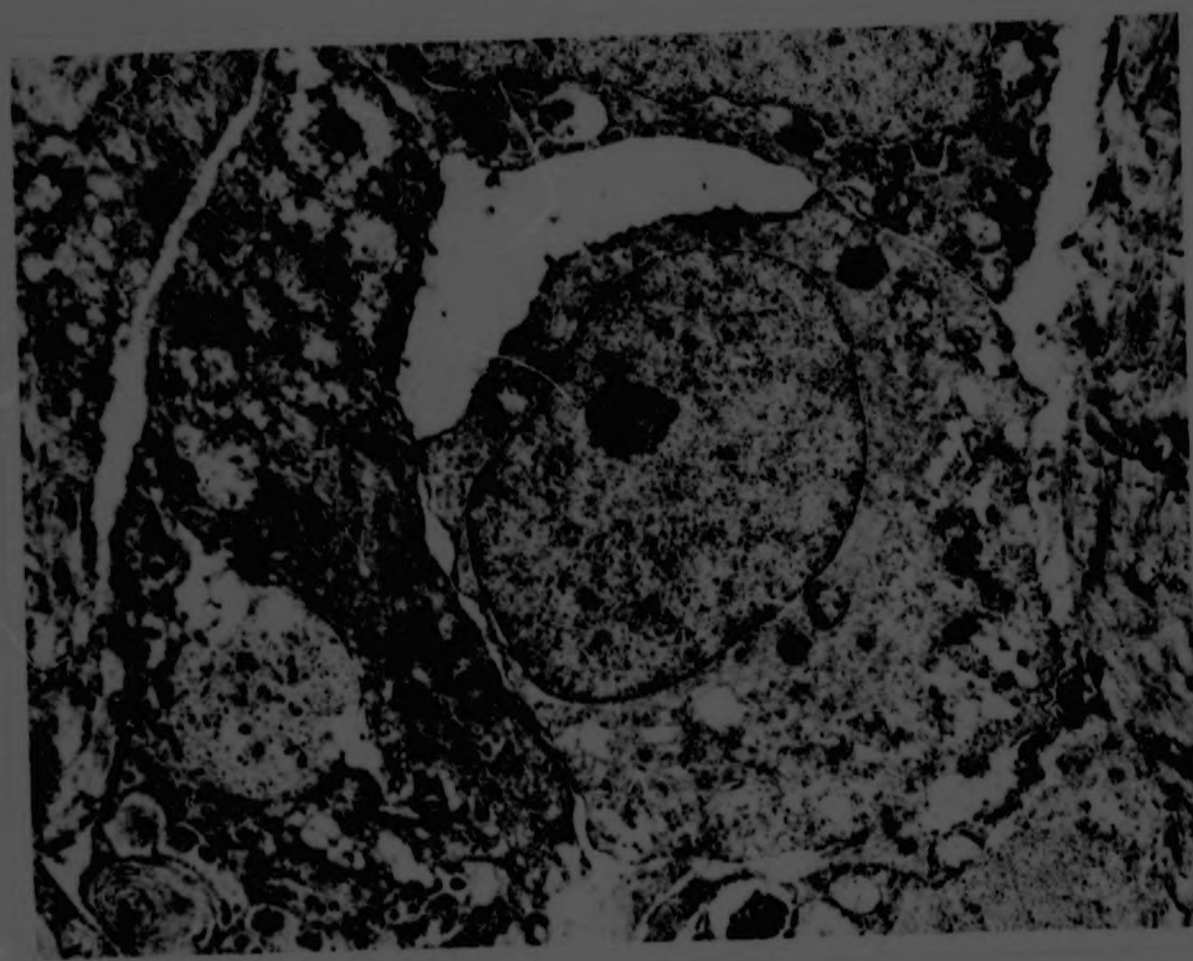


Plate 3:4. Human mammary benign fibroadenoma in complete incubation medium. Eight cases of fibrocystic disease exhibited nuclear reaction product (see arrows). The nuclear product was usually less dense and less frequently observed compared to malignant mammary tissues.

E.M. mag. = 5000X

Neg. mag. = 2X

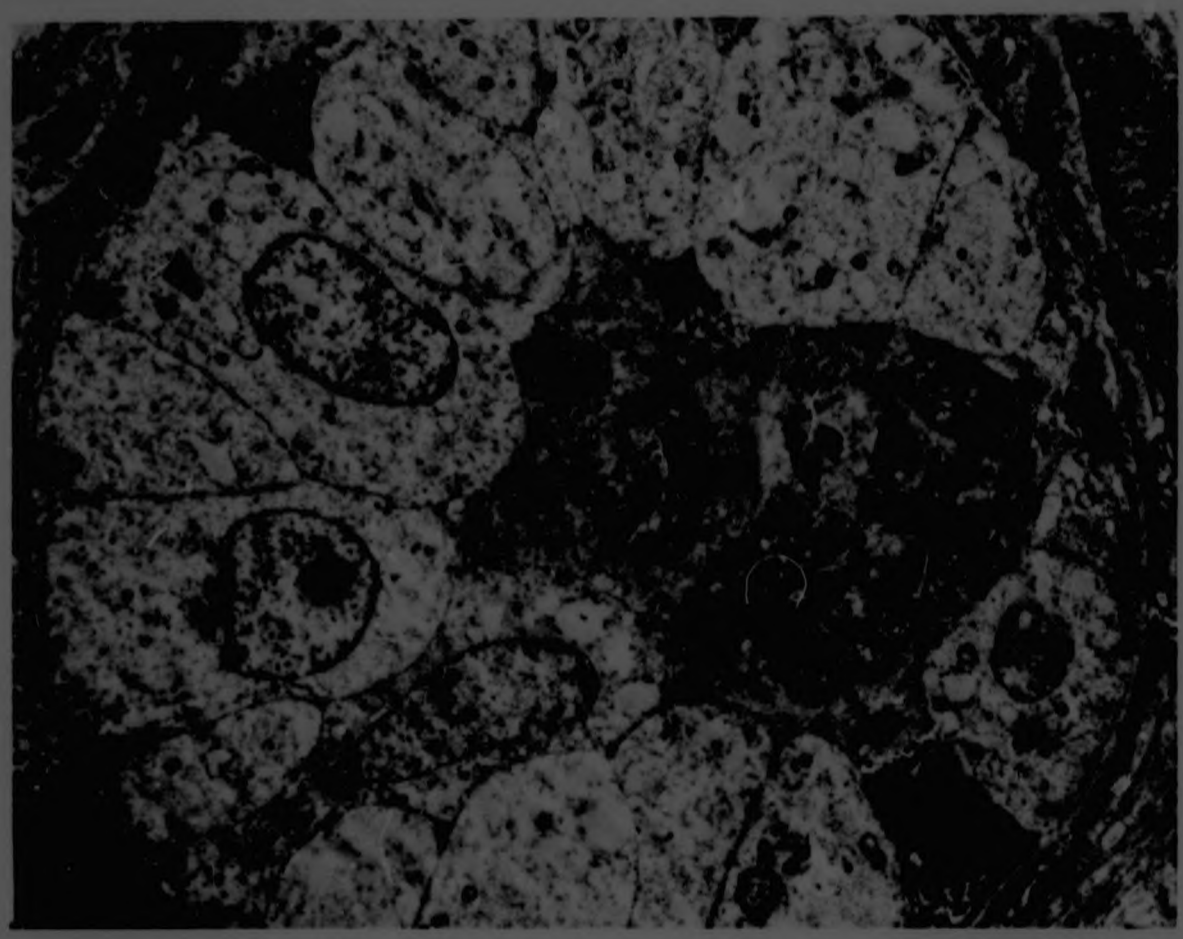


Plate 3:5. Normal human breast tissue in complete incubation medium. In the four cases studied, cytochemical localization of cPDE activity was not observed in the nuclei and nucleoli of normal breast cells.

E.M. mag. = 4000X

Neg. mag. = 2X

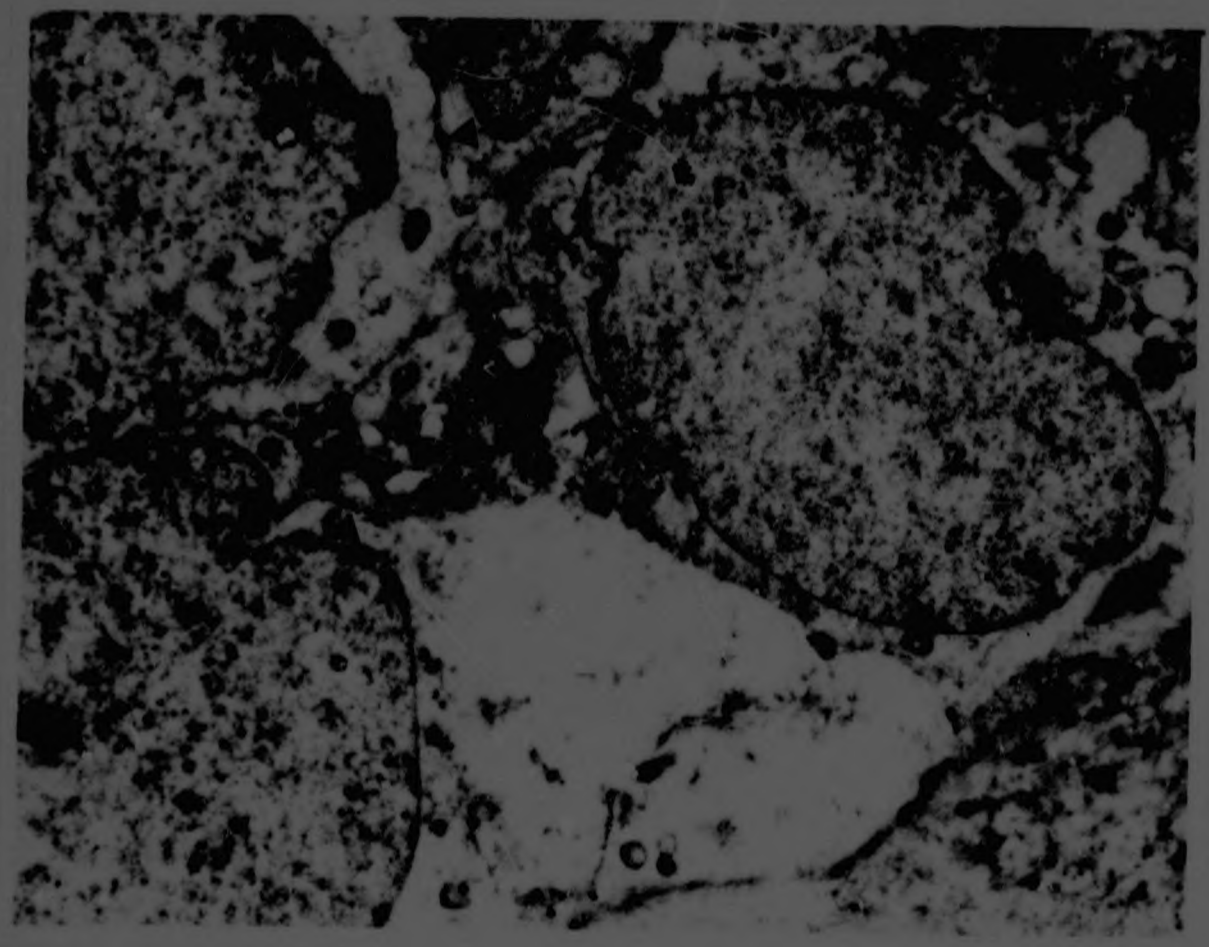


Plate 3:6. Normal human mammary tissue in control incubation medium with 10 mM theophylline added. The addition of 10 mM theopnylline did not affect the cPDE activity patterns in any human breast tissue. Cytoplasmic staining was frequently observed in normal breast tissue (4 cases studied) (see arrows).

E.M. mag. = 4000X

Neg. mag. = 2X

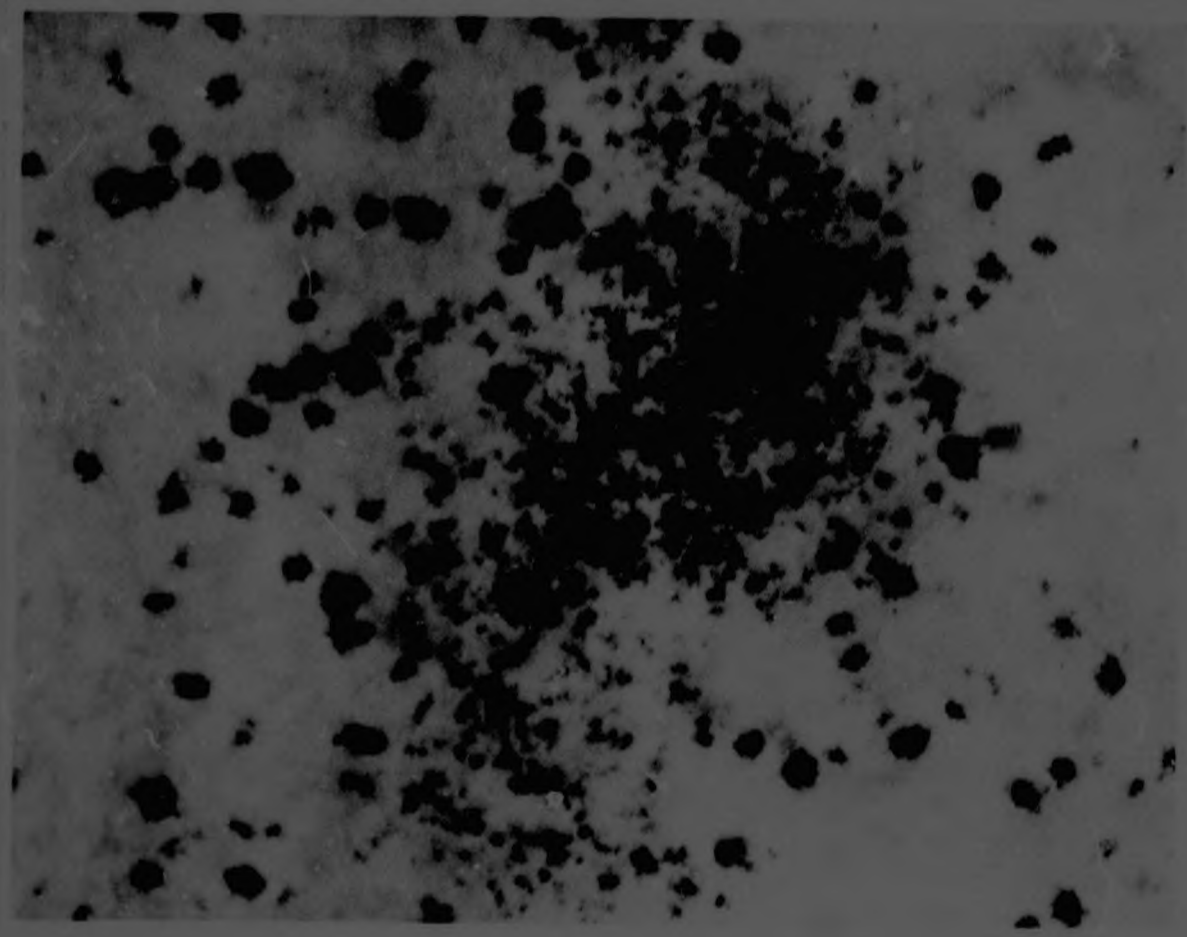


Plate 3:7. High magnification view of lead deposit (cPDE activity) in nucleolus of human mammary carcinoma in complete incubation medium.

E.M. mag. = 50000X

Neg. mag. = 2X

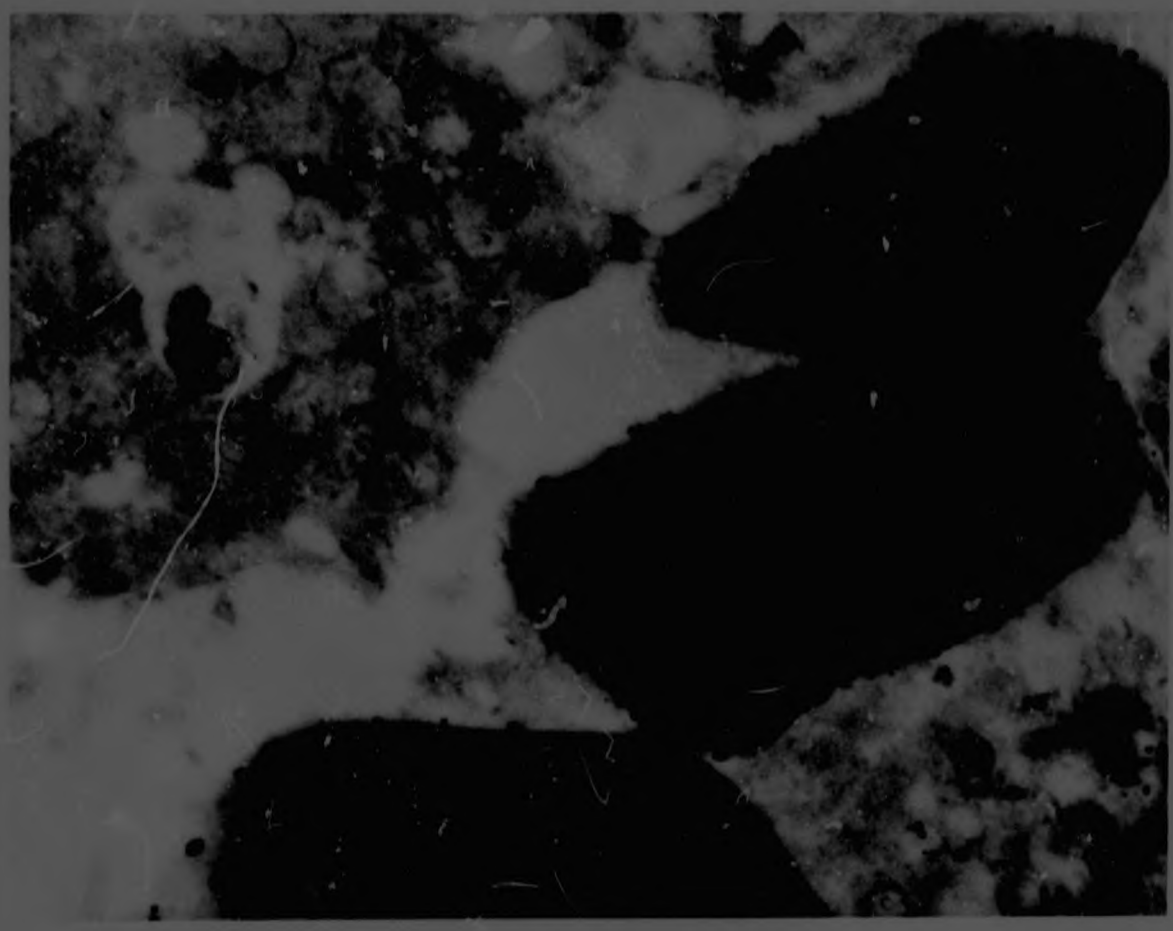


Plate 3:8. View of human red blood cells (rbc) of human malignant mammary tissue in control incubation medium with 10 mM theophylline. Cytoplasmic lead deposit (cPDE activity) was observed in rbc's in all mammary tissues incubated in complete medium. The addition, however, of 10 mM theophylline had no effect on the rbc cytoplasmic cPDE activity (see arrows).

E.M. mag. = 6000X

Neg. mag. = 2X

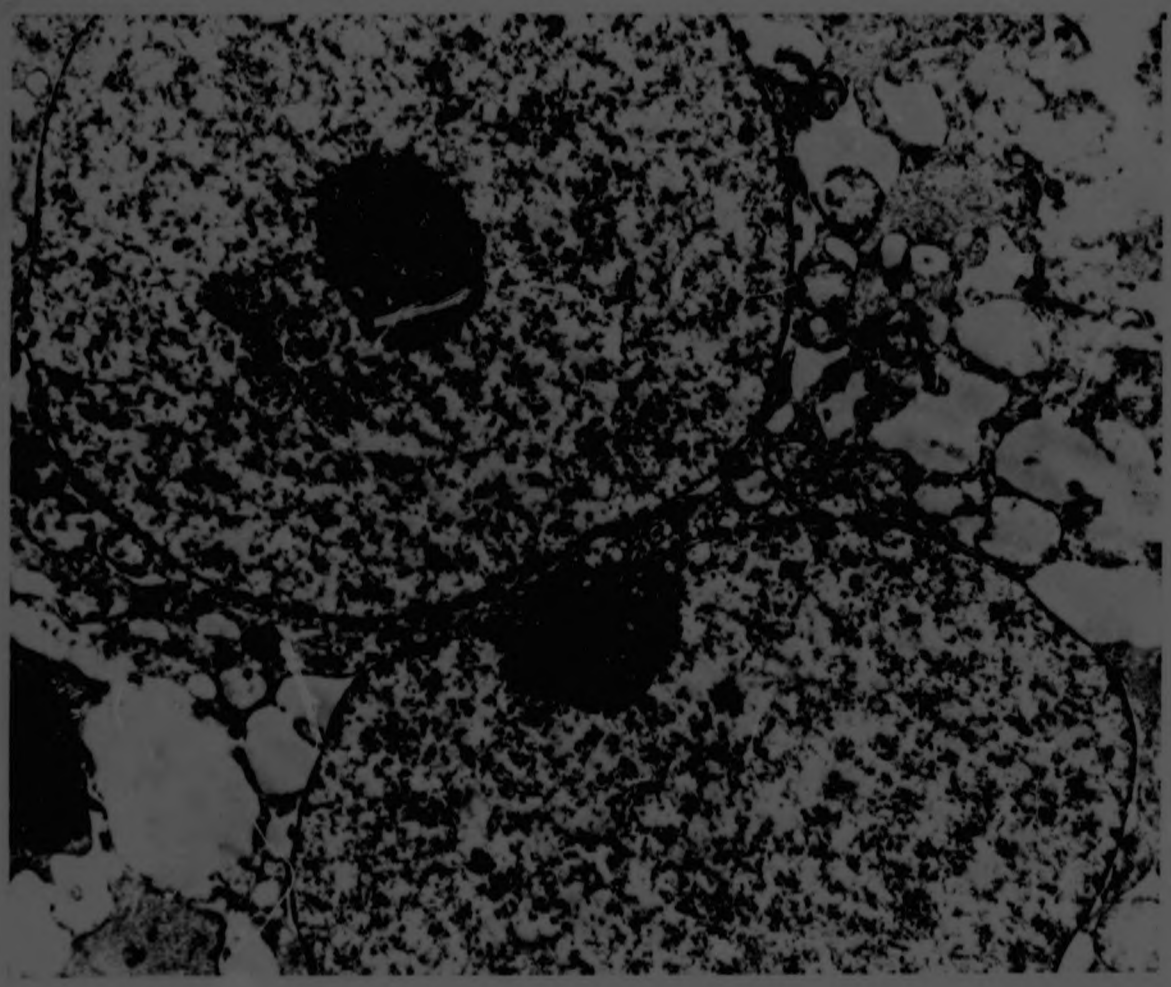


Plate 3:9. Human mammary cancer tissue in control incubation medium without cAMP. No nuclear or cytoplasmic cPDE activity was observed.

E.M. mag. = 4000X

Neg. mag. = 2X

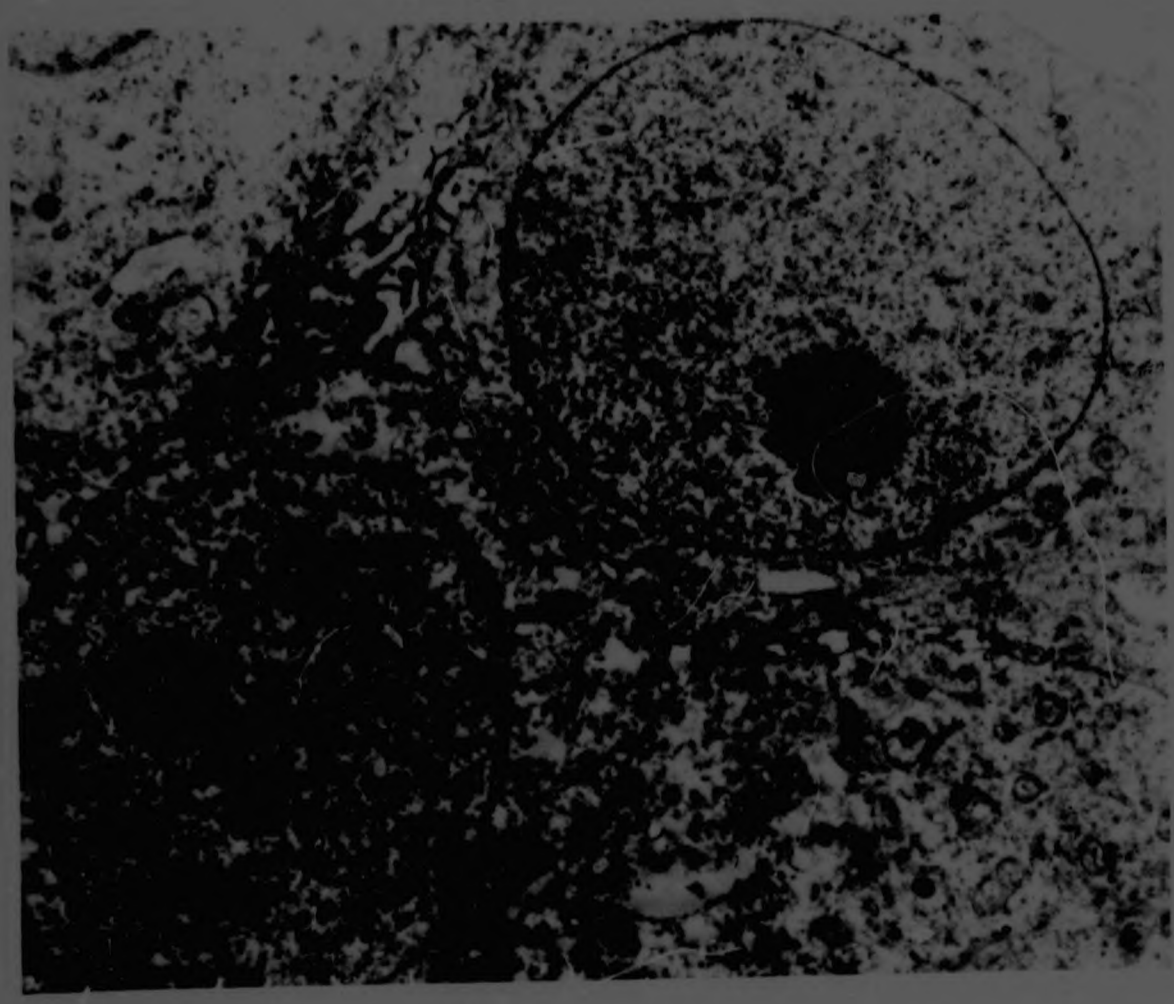


Plate 3:10. Human mammary cancer tissue in control incubation medium with 15 mM papaverine. No nuclear or cytoplasmic cPDE activity was observed.

E.M. mag. = 4000X

Neg. mag. = 2X

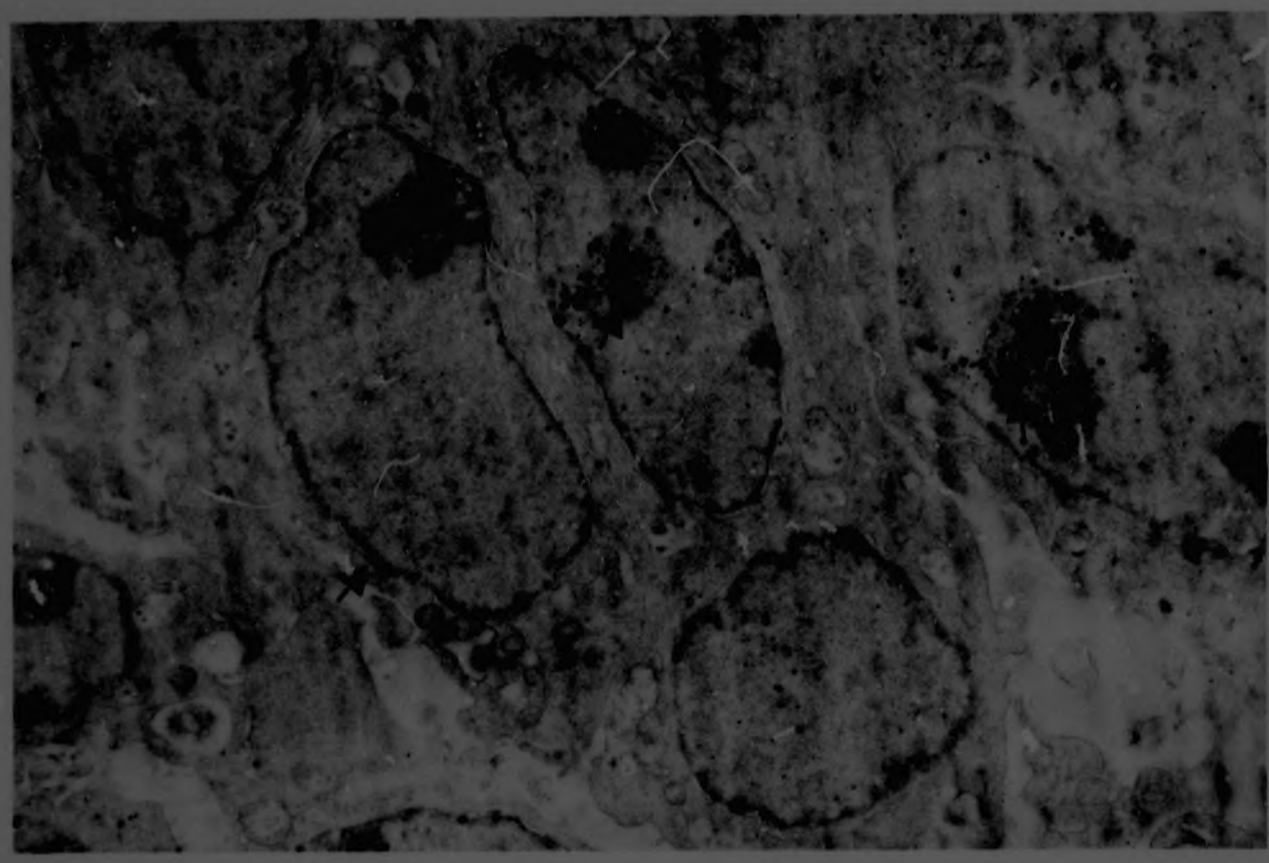


Plate 3:11. Murine mammary cancer tissue (F1 hybrid described in Chapter 2) in complete incubation medium. Cytochemical localization of cPDE activity particularly concentrated in the nucleioli of murine malignant carcinoma (see arrows).

E.M. mag. = 4000X

Neg. mag. = 2X

Discussion

The inference from these findings is that the detectable cPDE, possibly a high affinity form, associated with the nucleus is active either during cell proliferation or during increased metabolic activity or perhaps apparent at a less differentiated state of cell development. The findings in other tissues reported in the literature tend to favour the metabolic state theory and will be discussed later.

In my study, the epithelial cells of normal tissues did not show nuclear associated activity. Fibrocystic disease of the breast exhibited nuclear cPDE activity although less strikingly than the malignant tissues. These results imply that the state of differentiation, in addition to either proliferation or metabolic activity, is an important factor in the expression of the nuclear cPDE to such an extent that it is detectable by ultracytochemical techniques. Parallel biochemical studies in these human mammary tissues are required to confirm these findings. Of interest, an Honours Research Project by Ms. K. Silberman under the supervision of Professor Savage and myself showed cPDE activity to be associated with isolated nuclei of the murine malignant mammary tissue (F1 hybrid).

Ultracytochemical evidence for cPDE nuclear activity was shown for the murine mammary tumour tissue (Plate 3:11). A study based on the same ultracytochemical technique in rat cerebellum reported only one membrane-bound cPDE form which was confined to the post-synapses (Sugimura and Mizutani 1978).

In the literature, direct biochemical evidence for a nuclear associated cPDE has been shown in buffalo sperm (Bhatnager and Anand 1982; 1983). These workers were the first to report on a cPDE firmly attached to the chromatin although the enzyme is still to be solubilized. Work on membrane associated cPDE in bovine cardiac ventricles has revealed that about 60% of the total activity was present in the nuclear fraction (Ahluwalia and Rhoads, 1982). They were able to solubilize the nuclear fraction by sonication and found that the enzyme had lost differential inhibition effects to calmodulin inhibitors but they did not characterize it further. In addition, they reported that the nuclear cPDE was inhibited by 80% in the presence of 50 uM papaverine. A later report by these authors showed that they had been able to purify partially and characterize the cardiac nuclear cPDE enzymes (Ahluwalia *et al.* 1984). Two nuclear cPDE forms were solubilized by sonication of purified cardiac nuclear extracts (Ahluwalia *et al.* 1984). Most of the nuclear cPDE activity was associated with a 6.6 S form which appeared to be calcium-sensitive and cGMP-specific. For cGMP the reported Km was 4.8 uM with a Vmax of 440 units/mg protein while the Km for cAMP was 1.1 uM with a Vmax of 53 units/mg protein (Ahluwalia *et al.* 1984). The second cPDE nuclear activity was associated with a 4.4 S form and this lower mass enzyme was found to be calcium/calmodulin independent and cAMP-specific. The reported Km for cAMP was 20 uM with a Vmax of 450 units/mg protein (Ahluwalia *et al.* 1984). Finally, Ahluwalia and associates (1984) noted that the

specific activity of the 6.6 S cardiac nuclear cPDE (solubilized) was much greater than the same form in purified liver nuclei (actual values not given). These findings are significant in that cardiac cells do not divide, unlike the cells I studied. It is interesting to note that, despite the differences in proliferative activity between cardiac and tumour cells, both cell types are very active metabolically and have a large supply of oxygenated blood. It appears, therefore, that the detection of significant nuclear associated membrane-bound cPDE activity may be a function of the metabolic state of the cell rather than either the proliferative index or state of differentiation. Furthermore in support of this, Ahluwalia and associates (1984) stated that the nuclear cPDE activity was much higher in the cardiac cells in comparison to liver cells which do divide but posses a lower metabolic index. There is one early report of histochemically localized cPDE activity associated with the nuclear membrane of the unstimulated human small lymphocyte (Coulson and Kennedy 1971). A later report on the ultracytochemically localized cPDE forms in rat submaxillary gland has briefly mentioned occasional nuclear membrane staining of acinar and endothelial cells (Kalderon *et al.* 1977).

The hypothesis that the nuclear cPDE might be a low Km, high affinity form, is derived from reported biochemical data on membrane-bound (other than nuclear) cPDE enzymes which tend to fall into the type IV cPDE category and these forms usually exhibit high affinities for cAMP (see Introduction).

The reason why I did not observe cPDE nuclear activity in human normal mammary tissue using the ultracytochemical technique might be due to reasons other than increased metabolic activity in malignant versus benign mammary tumours.

It is important to consider the effect of extracellular cAMP, present in the incubation medium, on the tissues studied. Exogenous cAMP could exert influence by; (a) diffusion across plasma membrane, (b) induction of a specific cPDE, (c) activation of an ectophosphodiesterase, (d) other means, such as induction of a cPDE inhibitor although this is still to be clearly elucidated (Niles and Loewy 1981). It is known that cAMP is a normal constituent of extracellular fluids *in vivo* and there is considerable indirect evidence that cAMP crosses cell membranes (Szabo and Burke 1972). Work on amoebae, which had been starved, showed induction of both intra and extracellular cPDE activities prior to differentiation of the amoebae, (Klein and Darmon 1975) and there is evidence for a hormonally sensitive cAMP-specific PDE (type IV cAMP PDE) situated on the external plasma membranes of liver cells - an ectoenzyme (Smoake *et al.* 1981(b)). Any one of these above possibilities, or combinations thereof, might be differently expressed in malignant and benign cells compared with normal tissues studied. Hence cAMP might diffuse more rapidly across the malignant cell membranes enabling the nuclear cPDE to be histochemically detected.

Difficulties associated with histochemical methods utilizing lead ion should not be overlooked and these have been

thoroughly reviewed with respect to cPDE by Kalderon (1977). In the primary fixation stage where 2.5% glutaraldehyde is invariably used for cPDE histochemistry, it was shown by De Jong (1967) that in a tobacco cell line this fixative specifically activated a nuclear associated acid phosphatase which was most dense, especially in nucleoli, when the cells were at their maximum growth phase. The mode of action of this effect was not ascertained. In our experiments, however, malignant tissue controls with no exogenous 5'-nucleotidase, showed very slight or no staining in the nuclear processes of the cells, where this activity was normally observed. This indicated that glutaraldehyde was not responsible for nuclear lead deposition in my experiments. The very slight activity seen might have been due to the presence of endogenous 5'-nucleotidase. The latter enzyme is usually accepted as a plasma membrane marker (Chuang *et al.* 1984). Also of significance, is the fact that the normal mammary tissue did not exhibit nuclear cPDE activity despite the identical treatment conditions with glutaraldehyde. Moreover, Kalderon (1977) reported satisfactory preservation of cPDE activity in dissociated thyroid cells in the presence of glutaraldehyde and also there was no nuclear associated cPDE activity.

The 1.5 mM concentration of lead ions used in the media is apparently sufficient to cause the non-enzymatic hydrolysis of ATP (Rosenthal *et al.* 1966), while the hydrolysis of cAMP is reported to show no appreciable increase in dephosphorylation (Moses and Rosenthal 1968) even at a 4.0 mM lead ion

concentration (Kalderon *et al.* 1977).

In addition, my findings based on the controls, where lead alone or lead plus cAMP were present, little or no nuclear activity stain was observed in either normal or malignant tissue. It is possible, if unlikely, that the combination of exogenous cAMP and 5'-nucleotidase might form lead complexes in the incubation media with selective affinity for certain tissue reactive groups present in the malignant cells of the breast and to a lesser extent in fibrocystic tissue. The only other staining similar to our observations was reported on the cytochemical demonstrations of NAD-pyrophosphorylase (NMN+=enzyme=NAD +PP) in liver cells demonstrating enzyme activity strongly associated with the nucleolus, interchromatin granules and coiled bodies which largely consist of ribonucleoprotein RNP (Unger and Buchwalow 1975). The authors used both a manganese and lead method with a good selection of controls.

Of concern, is the fact that controls with 10 mM theopnylline, a fairly potent inhibitor of cPDE *in vitro* did not inhibit the staining of the nuclear areas in the cells where it was observed. Papaverine (15 mM), however, completely inhibited any form of either nuclear or cytoplasmic cPDE staining. In the literature, there are conflicting histochemical results as to the inhibitory effects of theophylline. Lack of total inhibition by 50 mM theophylline on locust plasma membrane was reported (Benedeczky and Rozsa 1981) whereas in other tissues work on *Dicytostelium* amoebae showed no inhibition of the cPDE

on the outer plasma membrane using 50 mM theophylline (Farnham 1975). In other tissues, sucessful inhibition was observed (Florendo *et al.* 1971). In my study, erythrocytes, either present in vasculature or displaced in the above tissues, often exhibited a specific cPDE activity stain associated with the plasma membrane which has also been observed in murine red blood cells (Singer and Ariano 1981). Since the activity stain on the human erythrocytes was also observed in the presence of 10 mM theophylline (Plate 3:8) the implication is, that the cell membranes of the breast tissues are not very permeable to theophylline at a 10 mM concentration. This finding in conjunction with the previous points raised suggests that theophylline is not a suitable inhibitor under the experimental conditions and tissues studied in this report.

It may be argued that the cytochemical staining observed in this study could be explained by a non-enzymatic redistribution of the lead phosphate deposited. This phenomenon does not explain why the normal cells did not exhibit the staining. Only additional biochemical studies will resolve this point.

CHAPTER FOUR

SOLUBLE cPDE FORMS IN HUMAN MAMMARY TISSUES

Objectives

The aims of this research project were to characterize the soluble cPDE enzymes in human mammary cancer tissues and normal breast tissues. Usually, the normal mammary tissues were not taken from the same patient, in contrast to my subsequent work in uterine tissues, because most of the breast cancer cases were biopsies rather than total mastectomies. I was unable to find a correlation between oestrogen receptor content and cPDE levels in tumour tissues. Nonetheless, the activity staining for cPDE forms on non-denaturing polyacrylamide gels were remarkably consisten . I was able to demonstrate at least six cPDE activity forms in breast cancer cytosols and four in normal mammary cytosols at low acrylamide concentrations. By applying Ferguson plot analysis, I was able to calculate the molecular masses, not previously reported to my knowledge, of the observed bands in both normal and neoplastic mammary cells. I also characterized the kinetic parameters of some of the tumour cPDE activity forms by eluting the band of interest from preparative non-denaturing polyacrylamide gels and applying kinetic analysis to the cPDE enzymes over a range of cAMP

concentrations. Finally, the effect of preincubating the mammary cytosols with EGTA, to interfere with calmodulin binding by removal of calcium ions, was studied.

Introduction

Some of the cPDE enzymes have recently been purified to homogeneity in human tissues and other mammalian species (see Chapter 1). There appear to be at least four distinct classes of cyclic nucleotide phosphodiesterases (cPDE's) which have been tentatively classified as types I, II, III and IV (Appleman *et al.* 1984). Within these subgroups, there are often marked differences, either between species or different tissues within the same species, with respect to one or more of the following; substrate preferences, kinetic parameters, molecular masses and inhibitor sensitivities. Whether all the variations reported reflect true *in vivo* differences or result from differences in methodology remains equivocal. This implies caution when attempting to relate the effect of therapeutic drugs on cPDE enzymes from animal studies to man.

Early reports based on measurements of cAMP levels in crude mammary tumours indicated that the substrate levels were raised in these tissues in comparison to normal mammary tissues (Minton *et al.* 1974; Guerinot *et al.* 1977). Work on rat mammary tissues indicated the same pattern of cAMP levels but the authors reported that the mammary epithelial cell content (the

cells of interest) were markedly reduced in normal mammary tissue compared to malignant tissue (Cohen and Chan 1974). Similarly, the soluble cPDE levels (only cAMP tested as the substrate) were found to be raised in human mammary tumours compared to the normal tissue counterparts (Singer *et al.* 1976; Kung *et al.* 1977) provided that the levels were expressed on a per protein basis as averse to DNA content (Kung *et al.* 1977). A different finding appeared to be true in rat DMBA-induced mammary tumours versus normal tissue (Rillema *et al.* 1978). When the data were based on either RNA content or wet tissue mass the cPDE levels were higher in the tumours but on the basis of either soluble protein or DNA the cPDE levels were the same or less than those found in normal mammary glands from virgin or mid-pregnant rats (Rillema *et al.* 1978). Clearly, the heterogeneous nature of breast cancer and the different epithelial cell content of normal mammary tissues during pregnancy makes meaningful comparisons of cPDE levels and their respective substrates very difficult. There have also been conflicting findings in mammary cells in culture with respect to cPDE levels and their substrates. For example, rat mammary normal cells have been shown to have higher cPDE levels compared to tumour cells but the reverse was shown at low cAMP concentrations (0.01 uM cAMP) (Cohen *et al.* 1976).
Of interest, though, there is evidence that exogenous cAMP or analogues stimulate the growth of cultured human mammary cells (Taylor-Papadimitriou *et al.* 1980) and rat mammary tumour implants (Klein and Loizzi 1977). Others, using a collagen gel

system, have demonstrated that cholera toxin (an irreversible stimulator of adenyl cyclase leading to raised cAMP levels) stimulates the growth of murine mammary epithelial cells in culture (Yang *et al.* 1979). These findings suggest that in mammary epithelial cells cAMP acts as a positive stimulus for cell proliferation. *In vivo* evidence for this has been shown in rat mammary glands where cAMP levels rise until the end of pregnancy (Sapag-Hagar and Greenbaum 1974(a)). The latter authors have shown that cPDE levels parallel the rise of cAMP but not the fall and thus the cPDE levels (cAMP only) also remain high during lactation (Sapag-Hagar and Greenbaum 1974(b); Rillema 1976). Nonetheless, the above findings do not seem to apply when tumour cells exhibit high levels of oestrogen and progesterone receptors indicative of hormonally dependent cells such as is the case in DMBA-induced rat tumours and MCF7 human breast cancer cells. In these cell types Cho-Chung and associates have demonstrated that dibutyryl cAMP caused *in vivo* regression of DMBA tumours and *in vitro* suppression of MCF7 cells (Cho-Chung *et al.* 1980; Cho-Chung 1986). The latter authors found that both cAMP and cPDE were greatest in regressing DMBA tumours of ovarectomized rats (Matusik and Hilf 1976; Cho-Chung *et al.* 1978). It should be noted, however, that there is one report, based on the same tumour model, where the opposite was demonstrated (Ip and Dao 1980). My studies based on the oestrogen receptor content of human mammary tumour cytosols with respect to levels of cPDE activities did not yield correlative findings.

There has been relatively little work concerning even the partial purification and characterization of the soluble cPDE forms in normal, benign and neoplastic mammary tissues (Chatterjee and Kim 1975; Cho-Chung and Newcomer 1977; Mullaney and Clegg 1984) particularly in humans (Singer *et al.* 1976; Larner and Rutherford 1982). The latter teams have partially characterized three or more soluble mammary cPDE forms by anionic exchange chromatography. A more thorough characterization of three soluble cPDE enzymes in rat mammary tissues, using similar techniques, has also been given (Mullaney and Clegg 1984). Significantly, Mullaney and Clegg (1984) ascribed three functional characteristics of two of the cPDE forms, resembling type I and type IV classification, that appeared unique to rat mammary tissues. There has also been a report on the partial purification of a membrane-bound mammary cPDE (type IV) (Aitchison *et al.* 1984).

Utilizing a different approach, I characterized the molecular sizes, not hitherto reported to my knowledge, and kinetic properties of one of the soluble forms of the cPDE enzymes in normal and malignant human mammary tissue. This was achieved by adapting a native polyacrylamide electrophoretic disc gel system to slab gels with modifications to improve resolution of different enzyme forms in both normal and malignant mammary tissue. Ferguson plot analysis of electrophoresis gels requires a minimum of tissue which is a necessary prerequisite in any human studies. The characterization of the different enzyme forms and the effect of EGTA are discussed.

Materials and Methods

Materials.

Human mammary tissue (1-3 g) was obtained through biopsy from patients during routine medical operative procedures. A section from each biopsy was submitted for microscopic analysis. The remaining tissue was immediately placed in liquid nitrogen (-196^{o} C) and stored for no longer than three weeks. Cyclic (8-^{3}H) AMP (sp. radioactivity 26 Ci/mmol) was purchased from the Radiochemical Centre (Amersham,Bucks, UK); *Crotalus atrox* 5'-nucleotidase (Sigma grade IV), molecular mass markers for non-denaturing polyacrylamide-gel electrophoresis, Jack Bean urease tetramer and dimer, bovine serum albumin dimer and monomer and bovine heart calmodulin-stimulated phosphodiesterase were obtained from Sigma (Sigma, St Louis, MO, USA); cAMP, alkaline phosphatase, 5'-AMP, adenosine and calmodulin were purchased from Boehringer (Boehringer, Lewes, East Sussex, UK); molecular mass markers, thyroglobulin and ovalbumin for non-denaturing electrophoresis were obtained from Pharmacia (Pharmacia, Uppsala, Sweden); Dowex AG 1-X2 (200-400) and the Bio-Rad protein kit were from Bio-Rad (Bio-Rad Laboratories, Richmond CA, USA). All other chemicals, of analytical grade, were purchased from Merck (Merck, Darmstadt, Germany) or BDH (BDH Chemicals, Poole, Dorset, UK).

Tissue cytosol preparation.

For the biochemical assays the tissues were pulverized in liquid nitrogen and homogenized in 4 parts (w/v) of either of two ice cold buffers which were A, 0.01 M Tris/HCl, pH 7.5, 0.3 mM PMSF, 3.75 mM mercaptoethanol, 1.1 M glycerol, 1 mM $CaCl_2$, 5 mM $MgCl_2$; B, 0.062 M Tris/HCl, pH 6.8, 0.3 mM PMSF, 3.75 mM mercaptoethhanol and 1.1 M glycerol.

Oestrogen receptor determination.

These assays were routinely performed in the Department of Nuclear Medicine by myself with technical assistance according to previously published methods (Collings *et al.* 1980; Robinson *et al.* 1985). Essentially the method involved a multiple point dextran-coated charcoal radioassay technique followed by Scatchard plot analysis (Scatchard 1949) for quantification and characterization of the steroid receptors.

Cyclic nucleotide phosphodiesterase assay.

This was a two-step radioassay of Thompson and Appleman (1971) and further modified by Azhar and Menon (1977). For routine assays, a 200 ul final incubation mixture included: buffer A, ^3H cAMP (200000 dpm), unlabelled cAMP at 1 and 100 uM concentrations and suitably diluted cytosols. After incubation for 10 minutes at 30^{C} C, the tubes were placed for 2 minutes in a boiling water bath and then allowed to cool. 5'-Nucleotidase

was added to each tube (1 unit/tube) in a 50 ul aliquot and the reaction mixture incubated for 10 minutes at 37° C. The reaction was terminated by addition of 750 ul of a slurry (1:3) of Bio-Rad AG 1-X2 resin and 3 mM acetic acid. After vortexing, the tubes were centrifuged at 5000xg in a Benchtop centrifuge for 10 minutes and the radioactivity in the supernatant was measured by liquid scintillation. For kinetic analyses the substrate concentration range was from 0.5 uM to 100 uM and the enzymes were diluted to provide linearity in the assays performed. No more than 25% of the substrate was consumed in each assay. The unit of phosphodiesterase activity was either in pmole or nmole cAMP hydrolyzed per minute per milligram cytosol protein.

Protein Determination.

Protein was measured using the Bio-Rad protein assay kit modified from the method of Bradford (1976) using bovine serum albumin as a standard.

Non-denaturing polyacrylamide-gel electrophoresis and enzyme activity stain.

Crude mammary cytosols were routinely run on 7.5% native polyacrylamide-gel electrophoresis slabs using an LKB Vertical Electrophoresis system according to the method of Laemmli (1970) with the following modifications; no SDS was used, 3.75 mM mercaptoethanol instead of 0.72 M and the tank or electrode

buffer (pH 8.3) contained 2.5 mM Tris and 19.2 mM glycine. Crude tissue cytosols were loaded in 50 ul aliquots (<5 mg protein /ml) and run at 20 mA per slab (constant current) for 5 hours at 4° C. Volumes of cytosol, if not homogenized nor equilibrated in buffer B, had an equal volume of gel sample buffer added (0.5 M Tris-HCl, pH 6.8, 1.1 M glycerol, 0.025% bromophenol blue). Gels were stained for enzyme activity from the method of Goren and Rosen (1971), with the following modifications. After electrophoresis, the gels were placed in filtered (Whatman NO.2) buffer (300 ml) of 0.1 M Tris-maleate pH 7.0 and gently rinsed 2 or 3 times in a clean plastic container. The final reaction mixture was then added to the slabs and contained 0.1 M Tris /maleate, pH 7.0, 2.5 mM magnesium sulphate, 1.5 mM lead nitrate, alkaline phosphatase (1unit/ml) and cAMP or cGMP at 300 uM concentration unless otherwise stated. The slabs were incubated for 30 minutes to 1 hour at 37° C or overnight at room temperature with gentle agitation in a waterbath shaker. A lid or cover, to prevent evaporation of the incubation mixture, was necessary. Then the gels were washed with constant agitation in several changes of distilled deionized water for 2 hours and developed for 2 minutes in 0.01 M ammonium sulphide solution. The gels were stored in distilled water until photographed or analyzed by densitometry scanning using a Beckman Densitometer CDS 200.

Partial purification of cPDE.

Preparative gels, used for isolation of enzyme for kinetic analysis of cPDE (only cAMP tested), were prepared as above except that a blank comb was used and a 2 cm wide vertical strip of the central portion of the gel was developed for activity in the minimum time (2 hours) while the rest of the gel was kept at 0-4° C. When the bands in the central portion were developed the area of undeveloped gel corresponding with the same Rf as that of the aligned activity bands was excised. The strips (2-3 mm) were placed in labelled glass test tubes and homogenized with a glass homogenizer and eluted for 24 hours at 4° C in 0.25-0.5 ml of buffer A. The mixture was filtered through a Millipore 0.45 micron pore filter under pressure and stored at -70° C until a sufficient quantity had been accumulated for enzyme kinetic analysis.

Molecular mass analysis of soluble mammary cPDE by Ferguson plot analysis of non-denaturing polyacrylamide-gel electrophoresis.

This was performed by measuring Rf values of activity bands for either cAMP or cGMP at different gel concentrations, with a range from 5% to 8.5%, and constructing a Ferguson plot of log Rf versus gel concentrations (Ferguson 1964; Hedrick and Smith 1968; Bryan 1977). Corrections were made for shrinkage of the gel, occurring during protein staining. The standards used were: Thyroglobulin, 669000; Jack Bean urease tetramer and

dimer, 480000 and 240000; bovine serum albumin dimer and monomer, 133200 and 66000; chicken ovalbumin 45000.

Incubation of human mammary cytosols of normal and malignant tissues with EGTA.

The effect of incubation of human mammary cytosols of normal and malignant tissues with EGTA, an inhibitor of calmodulin-sensitive cPDE classified as type I cPDE, was studied. Aliquots of 500 ul of cyotsol (<5 mg protein/ml), were incubated in the presence of 1 mM or 2 mM EGTA. Incubation was carried out for fifteen minutes and parallel control samples, without EGTA, were also run. After completion of the incubation the cytosols were subjected to non-denaturing polyacrylamide slab-gel electrophoresis as previously outlined. The theory behind this rationale being that the EGTA removes calcium ions which are a necessary prerequisite for the functional conformation of type I cPDE (see section on calmodulin in the introduction). Since the activity incubation steps contain no calcium ions, the results would be the same as if EGTA had been added to the activity incubation mixture. The advantage of my technique was that I could run EGTA cytosols and control cytosols on the same slab under identical incubation conditions.

Results

No correlation between cPDE levels at 1 uM and 100 uM cAMP concentrations and oestrogen receptor content in 42 human breast tumour cytosols was found ($r<0.5$). The range of cPDE activity at 1uM cAMP was 0.018-0.197 nmol/min/mg protein and at 100 uM cAMP was 0.551-4.667 nmol/min/mg protein. The tumour cytosols were classified as hormone independent if there were less than 10 fmol/mg protein of unbound oestrogen receptors present and receptor rich (that is hormone dependent) if there were more than 10 fmol/mg protein present.

Non-denaturing polyacrylamide-gel electrophoresis and activity staining of cPDE forms of human normal and malignant cytosols were run on 5-8.5% gels as described. Three forms were revealed in normal cytosols (lane 1 Fig. 4:1a) and six bands resolved in malignant cytosols (lane 2 Fig. 4:1a). The normal tissue sometimes showed a fourth lightly staining band. At higher gel concentrations (>7%) fewer forms could be resolved (Fig. 4:1a). Invariably, malignant tissue cytosols exibited overall denser staining activity compared to normal mammary cytosols run at equal soluble protein concentrations under identical experimental conditions. In addition, either 300 uM cAMP or cGMP showed the same activity pattern as analyzed by densitometry scanning. A densitometry scan of the cPDE activity profile of human mammary tumour cytosol at 300 uM cAMP is shown (Fig. 4:1b). Control experiments performed in the absence of cAMP, cGMP or alkaline phosphatase resulted in blank gels (as

previously shown in the murine tissues in Chapter 2). Incubation of the gels in the presence of cCMP, 5'AMP, adenosine or ATP did not produce any activity bands. Incubation of the cytosols with isobutylmethylxanthine (100 uM) after electrophoresis showed complete inhibition of activity (results same as for uterine tissues in Chapter Five). Ferguson plots for the activity bands 1,3,4 and 6 and the individual data for each band are shown (Fig. 4:2 and Tables 4:1 and 4:2).

Table 4:1. Molecular masses (Mr) and charge densities (Yo) obtained from Ferguson plot analysis of cPDE activity bands in cytosols from human malignant and normal breast tissues. Gels were run and activity bands developed as described in Materials and Methods. Each result is the mean of three experiments.

Band No.	Mr	SD	Yo	SD
	Malignant			
1	168000	5000	2.37	0.05
2	158000	6300	2.59	0.08
3	160000	7600	2.71	0.11
4	161000	8000	2.81	0.09
5	163000	6500	2.95	0.09
6	160000	6200	3.21	0.09
	Normal			
1	167000	5400	2.39	0.06
2	160000	6300	2.62	0.09
3	162000	5800	2.73	0.09
4	161000	7500	2.84	0.10

Table 4:2. Data of the molecular masses of cPDE bands in cytosols of human malignant and normal breast tissue observed following preincubation of the cytosols with 1 mM EGTA. Each result is the mean of three experiments.

Band No. Fig 4:1b	Mr	± SD	Yo	± SD
1E, lane 2	171000	7800	2.42	0.08
2E, lane 2	134000	7500	2.27	0.09
1E, lane 4	181000	8100	2.38	0.09
2E, lane 4	136000	7600	2.24	0.11

Fig. 4:1a. The soluble cPDE activity profiles of human malignant and normal breast tissue run on non-denaturing polyacrylamide slab gels. a) normal cytosol (lane 1) and malignant cytosol (lane 3) run on 5% acrylamide gel, b) normal cytosol (lane 1) and malignant cytosol (lane 3) run on a 7% acrylamide gel, c) normal cytosol (lane 1) and malignant cytosol (lane 3) run on a 7.5% gel. Lanes 2 and 4 in a), b) and c) show normal and malignant cytosols after pretreatment with 2 mM, 1 mM and 2mM EGTA respectively. Soluble protein loaded was 5 mg/ml in 50 ul aliquots.

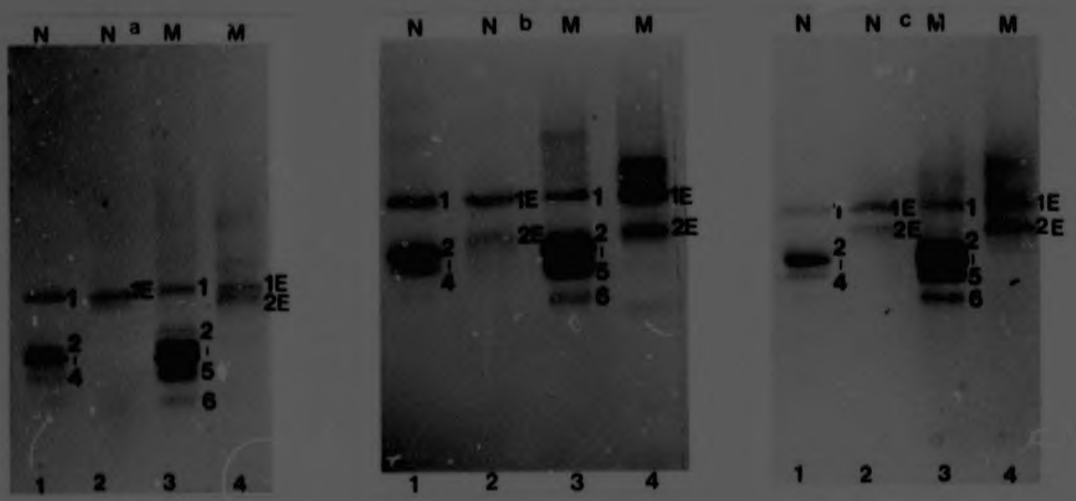


Fig. 4:1b. Densitometry scan of the soluble cPDE activity profile of human malignant mammary cytosol run on nd PAGE (5 mg protein/ml in a 50 ul aliquot) coupled to the cPDE activity stain. Sensitivity setting = 3. The substrate concentration was 100 uM cAMP. The relative cPDE activity in each peak as a % of the total tumour cPDE activity was: background of tumour 9%; band 1 of tumour 17%; bands 2 to 5 of tumour 64%; band 6 of tumour 11%.

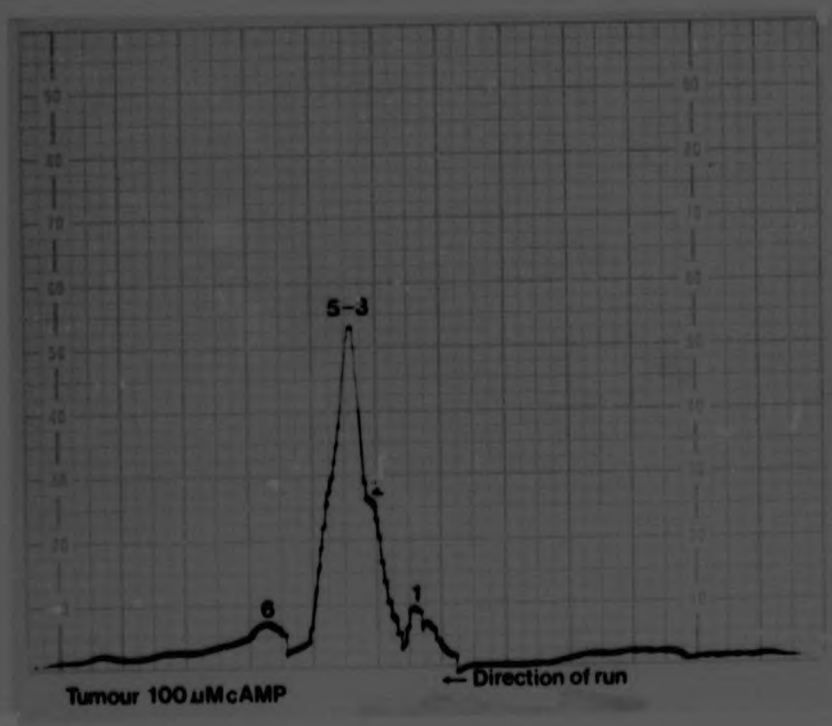
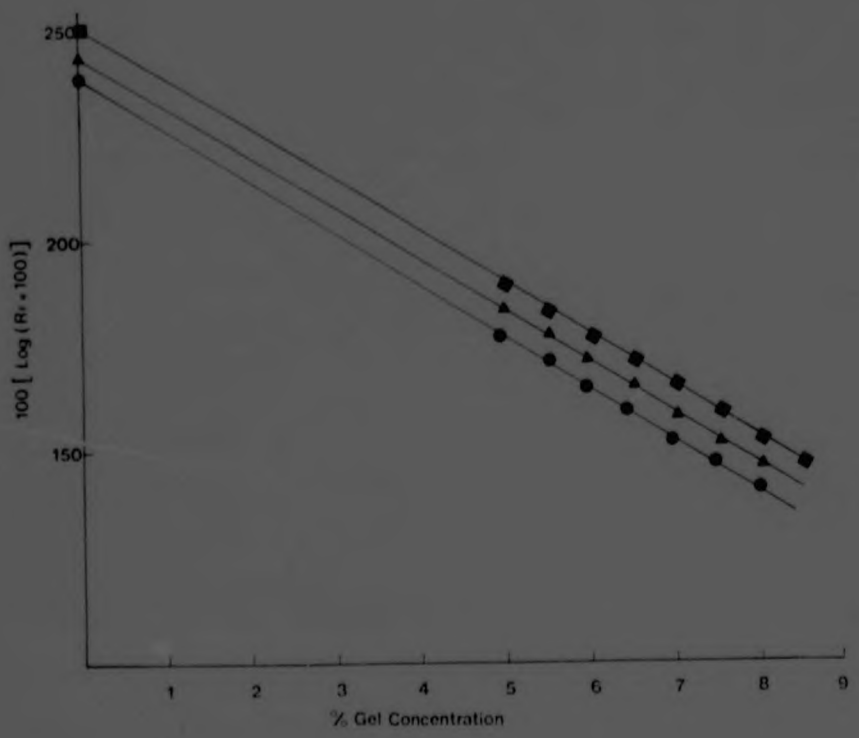
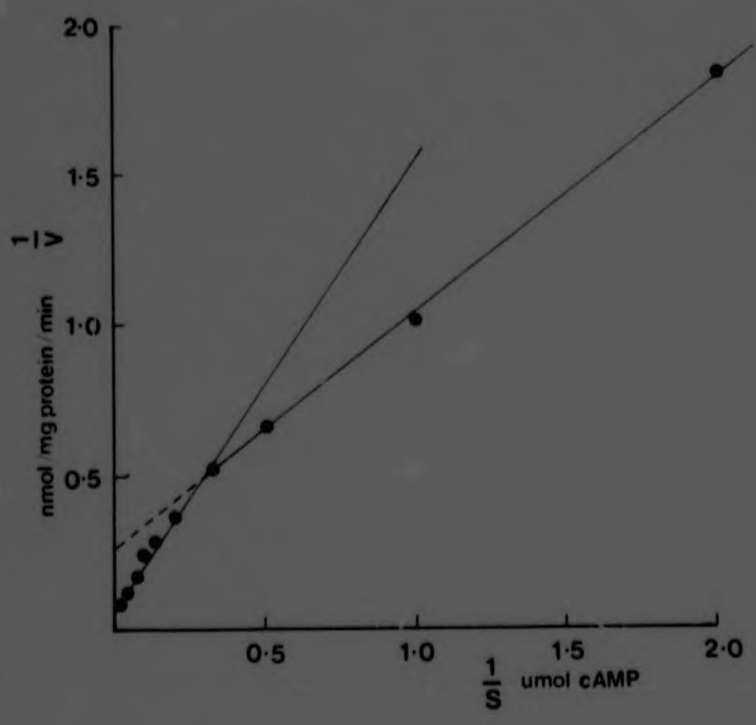


Fig. 4:2. Ferguson plot of cPDE enzymes from crude cytosols of human normal and malignant breast tissue. Samples were run at different gel concentrations from 5-8%. For each gel concentration, the plot of Rf (logarithmic scale) of the activity bands versus gel concentration (%) was constructed from the results (mean s.d.) of three separate runs, by using least-squares linear regression. The plots represent: band 1 (●), band 2 (▶) and band 4 (■)



In malignant breast tissue there appear to be two different molecular mass forms namely band 1 of approximately Mr 169000 and bands 2-6 of Mr 160000 ±7000 with different charges. Enzyme kinetic analysis of the malignant band 1 form, extracted as described in the methods, gave a Lineweaver-Burk plot as shown in Fig. 4:3. The plot for the band 1 form was non-linear and the data indicated either two or more enzyme forms or an allosteric enzyme exhibiting negative co-operativity. The apparent Km values were 3.8 and 50 um with respective Vmax values of 4.5 and 25 nmol/mg protein /min. Preincubation of human mammary cytosols with EGTA showed lack of inhibition of band 1 and appearance of anomalous forms of lower molecular mass and charge increase in both normal and malignant tissue (Fig. 4:1c). The apparent molecular masses in daltons and charge densities are summarized in Table 4:2.

Fig. 4:3. Kinetics of cPDE activity of the band 1 form from malignant breast tissue. Assays were done between 0.5 uM and 100 uM cAMP concentrations. Results of a representative experiment are shown as a Lineweaver-Burk plot (Lineweaver and Burk 1934). Two kinetically distinct forms are apparent with Km values of 3.8 uM and 50 uM.



Discussion

The fact that no correlation between cPDE activity and oestrogen receptor content was apparent in human mammary cancer cytosols may have been due to the heterogeneous nature of the different tumour tissues. Also it is well established that patients who are oestrogen receptor positive do not all respond to hormone ablation therapy (Robinson *et al.* 1985) which implies either non-functional receptors or mixed populations of receptor rich and poor cells with the latter being selected during therapy. In short, the large number of variables summarized below could well explain why no correlation was found.

1. Epithelial content of the different tumours was not established.
2. The tumours may have contained both oestrogen-dependent and oestrogen-independent cells.
3. The unbound oestrogen receptors measured may not have been functional, that is, the tumour growth was hormone-independent.
4. Since only unbound receptors are measured using the standard methodology, those receptors saturated with endogenous oestrogen would not be detected. This would imply variation in receptor content during the female cycle.

Clearly, for such comparative measurements to be meaningful the tissues studied should be within the same animal at a specified day of the cycle for *in vivo* assessments and in different cell lines treated under identical conditions for *in vitro* studies.

Cytosol from human mammary tissue could, however, be resolved into six bands of enzyme activity by non-denaturing electrophoresis on slabs of lower gel concentrations (<7%). Furthermore, this pattern was consistent in all the different mammary tumours studied. Normal mammary tissue appeared to show only 4 bands (Fig. 4:1a) of activity. Invariably, malignant tissue cytosols exhibited overall denser staining activity compared to normal mammary cytosols at equal soluble protein concentrations. This was evident in over six separate experiments. This finding is in agreement with Kung and associates (1977) who found that cPDE activity was markedly raised in human mammary carcinomas provided the results were compared per unit mass of tissue or per unit mass of cellular protein. When the data was expressed as enzyme activity per cell by these workers, however, the enzyme activities were reversed between the cell types. Since I was unable to ascertain the epithelial content of the mammary tissues I studied no definitive observations can be made regarding my findings. Measuring the DNA content would not have circumvented the problem because it is well established that malignant mammary cells often exhibit polyploidy (Prasad 1980).

The majority of the staining activity appeared to be associated with activity band 4 in malignant breast cytosol and band 2 in normal tissue which had the same Rf as that of band 4 of malignant tissue. The activity pattern of this band, with the same Rf in both normal and malignant tissues, would suggest that this was a common form of cPDE hydrolyzing both cAMP and

cGMP. There was also the presence of additional forms associated with breast cancer. Whether these forms are unique to malignant human mammary tissue remains to be fully determined since it is possible that the gel technique used might not have been sufficiently sensitive to detect those bands at the soluble protein concentration of normal mammary tissue used which was the same as that for the malignant tissues (5 mg soluble protein per ml).

Larner and Rutherford (1982) reported on cPDE in individual human mammary ductal carcinomas and fibroadenomas. They observed at least three forms in both tissue types that were separable with an ionic gradient on DEAE-Sephacel. Of interest, was their finding that the enzymes of crude malignant cytosols exhibited non-linear kinetics, as would be expected for more than one form, but that overall they exhibited higher affinity than the enzymes from benign tumours.

At least three distinct forms of cPDE have been reported in normal rat mammary tissues, using a similar means of biochemical separation to that of Larner and Rutherford (1982), which exhibit unique properties compared to the same cPDE types in other tissues (Mullaney and Clegg 1984).

Ferguson plot analysis of the activity bands observed in the present study revealed two different molecular mass species and a cluster of five charge isomers (Table 4:1) in malignant cytosols. Band 1 appeared to be a distinct enzyme not only in having a slightly higher molecular mass of about 169000 but also in being completely uninhibited by preincubation with EGTA

at concentrations as high as 2 mM (Fig. 4:1b). This would suggest that band 1 is a unique enzyme that is calmodulin independent. Calmodulin-independent enzymes have been described in the literature and are encompassed in the classified type II, type III and type IV enzymes. Since this activity band was always present in either incubation with cAMP or cGMP this would preclude type III forms which preferentially hydrolyze cGMP and the type IV enzymes that are specific for cAMP. The type II cPDE enzymes are interesting in that at certain concentrations of cAMP, micromolar concentrations of cGMP actually stimulate the hydrolysis of cAMP.

Kinetic analysis of band 1, using cAMP as substrate, indicated non-linear kinetics on double reciprocal plots showing apparent negative cooperativity. This could either suggest the presence of more than one enzyme, not sufficiently separated as a result of the method described here, or one enzyme exhibiting allosteric properties. The latter being a feature of regulatory enzymes, although speculative, is favoured by myself. The calculated Km values of approximately 3.8 uM and 50.1 uM were not dissimilar from those previously reported for crude human mammary cytosols (Larner *et al.* 1982) and rat mammary cytosols (Chatterjee 1975). No comment can be made on the molecular mass of 169000 as this is the first report, to my knowledge, on the enzyme in human breast tissue.

The five other more anionic bands observed in malignant cytosols exhibited identical approximate molecular masses of 160000 daltons but with different charge densities. These forms

were inhibited by 2 mM EGTA and could therefore be regarded as calcium/calmodulin-dependent. Bands 3 to 5 were observed in the normal cytosols but not 2 and 6. Whether these forms represent isoenzymes of different subunit structure or distinct enzymes is still to be elucidated. Recent reports by other workers (Sharma *et al.* 1984) using monoclonal antibodies have shown in bovine brain a calmodulin-dependent cPDE (type I) composed of two different subunits. This gives rise to three isoenzymes, two heterodimers and one homodimer.

Ferguson plot analysis of the anomalously migrating form seen in both normal and malignant tissue incubated in the presence of EGTA (arrowed band in Fig. 4:1 a,b, and c) revealed an apparent Mr of 135000. This would fit in well with the hypothesis that this band represents cPDE activity of normally calmodulin-dependent form (type I) with two molecules of calmodulin removed, but still exhibiting basal activity.

The results described here emphasizes the advantage of slab gel electrophoresis over disc gels when comparing the different electrophoretic forms of cPDE forms present in various human tissues. The method is also useful for the separation and hence kinetic characterization of the individual forms using small amounts of crude cytosol. The technique should prove useful for the analysis of the enzyme forms in other human tissues particularly with respect to the effect of inhibitors under different incubation conditions.

CHAPTER FIVE

SOLUBLE cPDE FORMS IN HUMAN LEIOMYOMA OF THE UTERUS

Objectives

The aims of this study were to characterize the soluble cPDE enzymes from cytosols of human leiomyoma of the uterus and the corresponding normal human myometrium tissue and also to purify the leiomyoma forms where possible. Since uterine leiomyoma are large benign tumours it was possible to collect sufficient quantities of the tissue for purification studies. The characterization of the leiomyoma and myometrium cPDE enzymes was achieved by non-denaturing polyacrylamide-gel electrophoresis followed by a specific cPDE-activity stain and analysis of the observed forms by Ferguson plot (Ferguson 1964). I was able to establish the presence of at least seven forms of soluble cPDE in the uterine tissues. Partial purification of leiomyoma cPDE bands 3 to 6 was attained through batch anionic-exchange chromatography of cytosolic supernatant followed by affinity chromatography, native and SDS polyacrylamide-gel electrophoresis. The possible relationship of cPDE enzymes and oestrogen-receptor content in the two uterine tissues was also investigated.

Introduction

The role of cyclic nucleotides as "second messengers" and hence intracellular mediators of many physiological, biochemical and pharmacological actions is well established. Indeed, cGMP is implicated in the inositol-lipid pathway (Berridge and Irvine 1984(a)) (see Chapter 1 in this thesis). At present, there are few reports in the literature on the purification and characterization of the cPDE enzymes found in human tissues. This has been done in: human platelets (Hidaka and Asano 1976; Grant and Colman 1984; Umekawa *et al.* 1984); in leukaemic cells (Onali *et al.* 1985); in human fat cells (Kuribayashi *et al.* 1987); human pregnant and nonpregnant myometrium (Kofinas *et al.* 1987); and in human cardiac ventricle (Reeves *et al.* 1987). The latter three publications, in fact, have only reported on the partial purification and characterization of some of the human cPDE forms.

The uterus is composed of two discrete target tissues; the endometrium which is of epithelial origin; and secondly the myometrium which, although composed of several layers of cells, is essentially derived from smooth muscle. In this thesis, comparisons of cPDE forms were therefore made between the human leiomyoma tumour and adjacent human myometrium from the same patient undergoing hysterectomy, since both tissues are of smooth mucle origin. I have also studied the content of oestrogen and progesterone receptors in these two tissues (Sadan *et al.* 1987) as others have shown that progesterone and

oestrogen, in addition to other steroid hormones and gonadotropins, are implicated in cyclic nucleotide metabolism (Etingof *et al.* 1984; Vallet-Strouve *et al.* 1984). Early, it was demonstrated that oestrogen hormone administration to ovariectomized rats was associated with an initial rapid increase in the uterine concentration of cAMP (Szego and Davis 1967). Another group reported that at least part of this acute response was the result of increased production of cAMP by adenyl cyclase (Rosenfeld and O'Malley 1970). It has also been suggested that cAMP is a mediator in the action of lutenizing hormone (LH) and follicle-stimulating hormone (FSH)(Goff and Armstrong 1977). Since the mid-Seventies, there appear to have been at least five different groups who have studied cPDE activities in uterine tissues of humans, monkeys and rats, although complete purification and extensive characterization of any of the different forms of cPDE have not been attained until now. The respective findings of these different groups will be discussed and their results compared to the results obtained on human leiomyoma and myometrium tissues in this thesis.

Materials and Methods

Materials.

Cyclic (8-^{3}H) AMP (specific radioactivity 26 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, Bucks., UK); *Crotalus atrox* 5'-nucleotidase (Sigma grade IV), molecular mass markers Jack Bean urease tetramer and dimer and bovine serum albumin dimer and monomer markers for non-denaturing polyacrylamide-gel electrophoresis and bovine heart calmodulin stimulated phosphodiesterase were obtained from Sigma (Sigma, St.Louis, MO, USA). cAMP, cGMP, alkaline phosphatase, 5'-AMP, adenosine and calmodulin were purchased from Boehringer (Boehringer, Lewes, East Sussex, UK); molecular mass markers for SDS electrophoresis, non-denaturing electrophoresis and sucrose gradients, and Cyanogen Bromide activated Sepharose were obtained from Pharmacia (Pharmacia, Uppsala, Sweden); Dowex AG 1-X2(200-400) and the Bio-Rad protein kit were from Bio-Rad (Bio-Rad Laboratories, Richmond, CA, USA); DEAE-52 was obtained from BDH (BDH Chemicals, Poole, Dorset, UK); The theophylline-Sepharose affinity matrix was prepared by the method of Marchmont *et al.* (1981(a)); All other chemicals of analytical grade were purchased from Merck (Merck, Darmstadt, Germany) or BDH.

Tissue cytosol preparation.

Leiomyoma, myometrium and uterine tissue specimens were

obtained from patients, during total hysterectomy for routine medical procedures, through biopsy (2-6 g uterine tissue and 10-30 g leiomyoma). The remaining tissue was submitted for histological analysis. The specimens were frozen immediately in liquid nitrogen and stored at -196° C for no more than two weeks. For the various biochemical assays, the tissues were pulverized in liquid nitrogen and homogenized in 4 parts (w/v) of either of three ice cold buffers namely: A 0.01 M Tris/HCl, pH 7.5, 0.3 mM PMSF, 3.75 mM mercaptoethanol, 1.1 M glycerol, 1 mM $CaCl_2$, 5 mM $MgCl_2$; B 0.025 M Imidazole/HCl, pH 7.5, 0.3 mM PMSF, 3.75 mM mercaptoethanol, 1.1 M glycerol; C 0.0625 M Tris/HCl, pH 6.8, 0.3 mM PMSF, 3.75 mM mercaptoethanol and 1.1 M glycerol. After homogenization, extracts were spun at 150000xg for 60 minutes and the supernatants were stored at -70° C until used for electrophoresis or enzyme activity determinations.

Purification of soluble cPDE in human leiomyoma by affinity chromatography.

Pulverized leiomyoma (30 g) was homogenized at 4° C in buffer B and centrifuged at 150000xg for 60 minutes. About 150 ml of clear supernatant was adjusted to 0.1 M with NaCl and added to 50 ml DE-52 resin previously equilibrated with buffer B. This was washed in the adjusted buffer until no more protein was detected. The 0.1 M NaCl fraction was pelleted with 70% ammonium sulphate and stored at -70° C. The DE-52 resin was then

eluted with about 50 ml of buffer B adjusted to 0.4 M with NaCl. This 0.1-0.4 M NaCl fraction was added to 20 ml of theophylline-Sepharose at 4° C with gentle agitation for 16 hours. The affinity resin was then repeatedly washed with buffer B/0.4 M NaCl until no more protein was eluted. The affinity resin was eluted with about 20 ml 0.4 M NaCl/buffer B containing 500 uM isobutylmethylxanthine (MIX) and gently agitated for 2 hours. The resin was filtered through a porous filter and the filtrate was brought up to 70% with solid ammonium sulphate, allowed to stand on ice, and then spun at 40000xg at 4° C for 30 minutes. The pellet was stored at -70° C before further purification. Further purification was either by preparative non-denaturing polyacrylamide-gel electrophoresis and extraction of the enzyme from the activity band, which was then subjected to SDS polyacrylamide-gel electrophoresis, or by sucrose gradient ultracentrifugation.

Cyclic nucleotide phosphodiesterase assay.

This was a two-step radioassay of (Thompson and Appleman 1971; Thompson *et al.* 1974) and further modified by Boudreau and Drummond (1975) and Azhar and Menon (1977). For routine assays, a 200 ul final incubation mixture included: buffer A, ^{3}H-cAMP (200000 dpm), unlabelled cAMP at 1 and 100 uM concentrations and suitably diluted cytosols. After incubation for 10 minutes at 30° C, the tubes were placed for 2 minutes in a boiling water bath and then allowed to cool. 5'-Nucleotidase was added to

each tube (1 unit/tube) in a 50 ul aliquot and the reaction mixture incubated for 10 minutes at 37° C. The reaction was terminated by addition of 750 ul of a slurry (1:3) of Bio-Rad AG 1-X2 resin in 3 mM acetic acid. After vortexing, the tubes were centrifuged at 5000xg for 10 minutes and the radioactivity in the supernatant was measured by liquid scintillation. For kinetic analyses the substrate concentration range was from 0.5 uM to 100 uM and the enzymes were diluted to provide linearity in the assays performed. No more than 25% of the substrate was consumed in each assay. The unit of phosphodiesterase activity was either in pmole or nmole cAMP hydrolyzed per minute per milligram cytosol protein.

Protein Determination.

Protein was measured using the Bio-Rad protein assay kit modified from the method of Bradford (1976) using bovine serum albumin as a standard.

Non-denaturing polyacrylamide-gel electrophoresis and enzyme activity stain.

Cyclic nucleotide phosphodiesterases were routinely run on 7.5% native polyacrylamide-gel electrophoresis slabs using an LKB Vertical Electrophoresis system according to the method of Laemmli (1970) with the following modifications; no SDS was used, 3.75 mM mercaptoethanol instead of 0.72 M and the tank or electrode buffer (pH 8.3) contained 2.5 mM Tris and 19.2 mM

glycine. Crude tissue cytosols or purified extracts were loaded in 50 ul aliquots (5 mg protein/ml) and run at 20 mA per slab (constant current) for 5 hours at 4° C. Volumes of cytosol, if not homogenized or equilibrated in buffer C, had an equal volume of gel sample buffer added (0.5 M Tris-HCl, pH 6.8, 1.1 M glycerol, 0.025% bromophenol blue). Gels were stained for enzyme activity from the method of Goren *et al.* (1971), with the following modifications. The gels were placed in filtered (Whatman No.2) buffer (300 ml) of 0.1 M Tris-maleate, pH 7.0, and gently rinsed 2 or 3 times. The final reaction mixture was then added to the slabs and contained 0.1 M Tris/maleate, pH 7.0, 2.5 mM magnesium sulphate, 1.5 mM lead nitrate, alkaline phosphatase (1 unit/ml) and cAMP or cGMP at 300 uM concentration unless otherwise stated. The slabs were incubated for 30 minutes to 1 hour at 37° C or overnight at room temperature with gentle agitation in a waterbath shaker. Then the gels were washed with constant agitation in several changes of distilled deionized water for 2 hours and developed for 2 minutes in 0.01 M ammonium sulphide solution. The gels were stored in distilled water until photographed or analyzed by densitometry scanning using a Beckman Densitometer CDS 200. Preparative gels used for isolation of enzyme for kinetic analysis or SDS polyacrylamide-gel electrophoresis were prepared as above except that a blank comb was used and a 2 cm vertical strip of the central portion of the gel was developed for activity in the minimum time (2 hours) while the rest of the gel was kept at 0-4° C. When the bands in the central

portion were developed the area of undeveloped gel corresponding with the same Rf as that of the aligned activity bands was excised. The strips (2-3 mm) were placed in labelled glass test tubes and homogenized with a glass homogenizer and eluted for 24 hours at 4° C in 0.25-0.5 ml buffer A. The mixture was filtered through a Millipore 0.45 um pore filter under pressure and stored at -70° C until a sufficient quantity had been accumulated for enzyme kinetic analysis. The above method was also used as the final purification step of the enzyme from leiomyoma except that the filtered eluent was precipitated with ice cold ethanol and the protein run on SDS polyacrylamide-gel electrophoresis as described by Laemmli (1970).

Molecular mass analysis of cyclic nucleotide phosphodiesterase by non-denaturing polyacrylamide-gel electrophoresis.

This was performed by measuring Rf values of activity bands at different gel concentrations, from a range of 5% to 8.5%, and constructing a Ferguson plot of log Rf versus gel concentration (Ferguson 1964; Rodbard and Chrambach 1971; Bryan 1977). Shrinkage of the gel occurring during protein staining of the standards was corrected for, when estimating the electrophoretic mobility. The following standards (obtained from Sigma) were used: Thyroglobulin, 669000; Jack Bean urease tetramer and dimer, 480000 and 240000; bovine serum albumin dimer and monomer, 132000 and 66000; and chicken ovalbumin, 45000.

Molecular mass determination of cPDE forms by sucrose gradient ultracentrifugation.

Continuous 10 ml linear sucrose gradients of 10-30% (w/w) containing 0.05 M Tris/HCl, pH 7.5, 0.05 M NaCl were prepared according to the method of Martin and Ames (1961). An aliquot (200 ul) of clear supernatant of either crude leiomyoma cytosol (5 mg protein/ml) or purified leiomyoma extract, which had been equilibrated in buffer A either by dialysis or exchange by passing through a small G-25 column, was added to the sucrose gradient and centrifuged for 16 hours using a Beckman SW 41 Ti rotor in a Beckman L8-70 Ultracentrifuge at 40000 rpm (w^2t = 1.01×10^{12} sec) at 5^{o} C. Fractions (0.5 ml) were collected from the top downwards and each aliquot was assayed for cPDE activity at two substrate concentrations of 1 and 100 uM cAMP. Catalase, Mr 232000, 11.6 S; aldolase, Mr 156000, 7.8 S; albumin, Mr 67000, 4.4 S; ovalbumin, Mr 43000, 3.5 S and chymotrypsinogen A, Mr 25000, 2.5 S were used as sedimentation standards.

Results

Non-denaturing polyacrylamide-gel electrophoresis results.

Non-denaturing polyacrylamide-gel electrophoresis and activity staining of cPDE forms on 7.5% gels of leiomyoma cytosols revealed three activity bands for cAMP and cGMP, as shown (Fig. 5:1b). Reducing the gel concentration to 6% or less resolved seven activity bands for leiomyoma, myometrium and uterus (Fig. 5:1a). When the same soluble protein concentrations of leiomyoma (5 mg protein/ml) and myometrium (5 mg protein/ml) from the same patient were loaded on gels it was always noted that the leiomyoma cPDE activity bands were less dense (see Figs. 5:1a, 5:2b, 5:2c). Control experiments performed in the absence of cAMP, cGMP or alkaline phosphatase, showed no activity (as seen for murine mammary tumour cytosols in Chapter 2). Incubation in the presence of cCMP, 5'-AMP or adenosine did not produce any activity staining (Fig. 5:2a).

Post-electrophoresis incubation of gels with MIX (100 uM) gave complete inhibition (Fig. 5:2a). These control results were the same as those found in cytosols of murine tissues (Chapter 2) and human mammary tissues (Chapter 4). Densitometry scans of human leiomyoma and myometrium cytosols showed increased soluble cPDE activity in the myometrium compared to leiomyoma (Figs. 5:2b, 5:2c).

Fig. 5:1. Soluble cPDE activities of uterine tissues on native polyacrylamide gel-electrophoresis. Soluble protein loaded was 5 mg/ml in 50 ul aliquots. Samples (a) of crude cytosols of human leiomyoma (L), myometrium (M), and uterus (U) were run, as described in the experimental section and stained for cPDE activity at 6% gel concentration depicting seven bands, 1-7. Samples (b) of crude leiomyoma were run at 7.5% gel concentration and stained for cPDE with both cAMP and cGMP. Samples (c) and (d) of crude leiomyoma cytosols, lane on the right of each picture, samples were run in the presence of 1 mM EGTA.

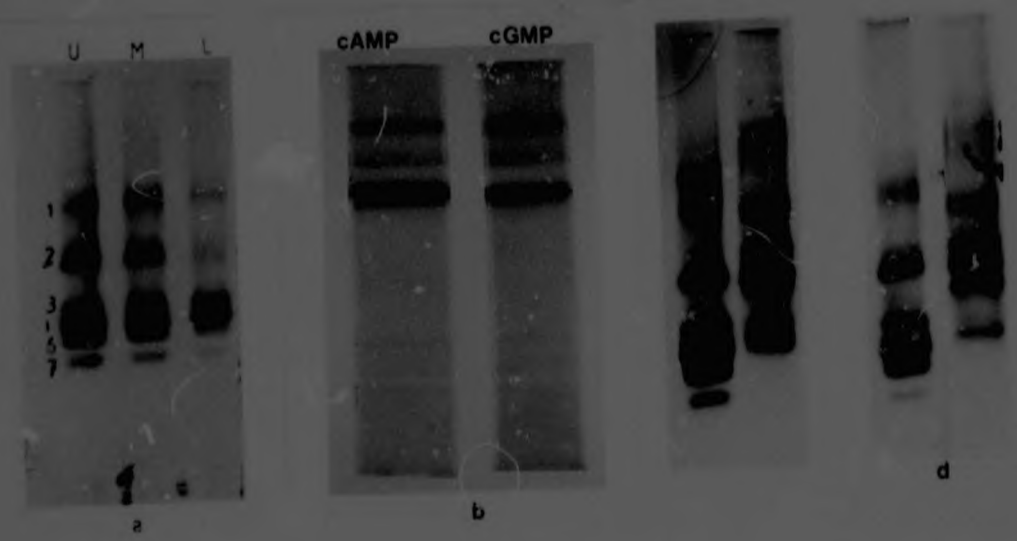


Fig. 5:1. Soluble cPDE activities of uterine tissues on native polyacrylamide gel-electrophoresis. Soluble protein loaded was 5 mg/ml in 50 ul aliquots. Samples (a) of crude cytosols of human leiomyoma (L), myometrium (M), and uterus (U) were run, as described in the experimental section and stained for cPDE activity at 6% gel concentration depicting seven bands, 1-7. Samples (b) of crude leiomyoma were run at 7.5% gel concentration and stained for cPDE with both cAMP and cGMP. Samples (c) and (d) of crude leiomyoma cytosols, lane on the right of each picture, samples were run in the presence of 1 mM EGTA.

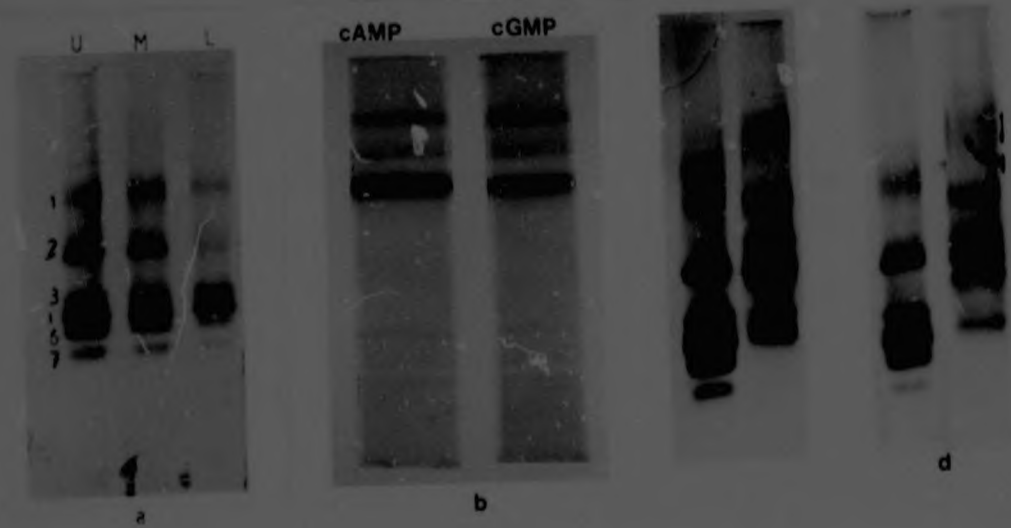


Fig. 5:2a. Control gels with human leiomyoma cytosols coupled to the specific cPDE activity stain. Soluble protein loaded was 5 mg/ml in 50 ul aliquots.
Control (1) represents an incubation in the presence of cGMP. Control (2) is incubation in the presence of 5'-AMP and control (3) with adenosine. In control (4) the incubation was performed in the presence of MIX (100 uM).

Fig. 5:2b. Densitometry scans of the cPDE activity profile of human leiomyoma cytosol (5 mg/ml protein in a 50 ul aliquot) when run on nd PAGE coupled to the cPDE activity stain at 100 uM cAMP (left) and 100 uM cGMP (right). Sensitivity setting = 2. The relative cPDE activity in each peak(s) as a % of the total cPDE activity was: background of leiomyoma 12% at 100 uM cAMP and 1% at 100 uM cGMP; band 1 of leiomyoma 22% at 100 uM cAMP and 23% at 100 uM cGMP; band 2 of leiomyoma 16% at 100 uM cAMP and 21% at 100 uM cGMP; bands 3-6 of leiomyoma 50% at 100 uM cAMP and 55% at 100 uM cGMP.

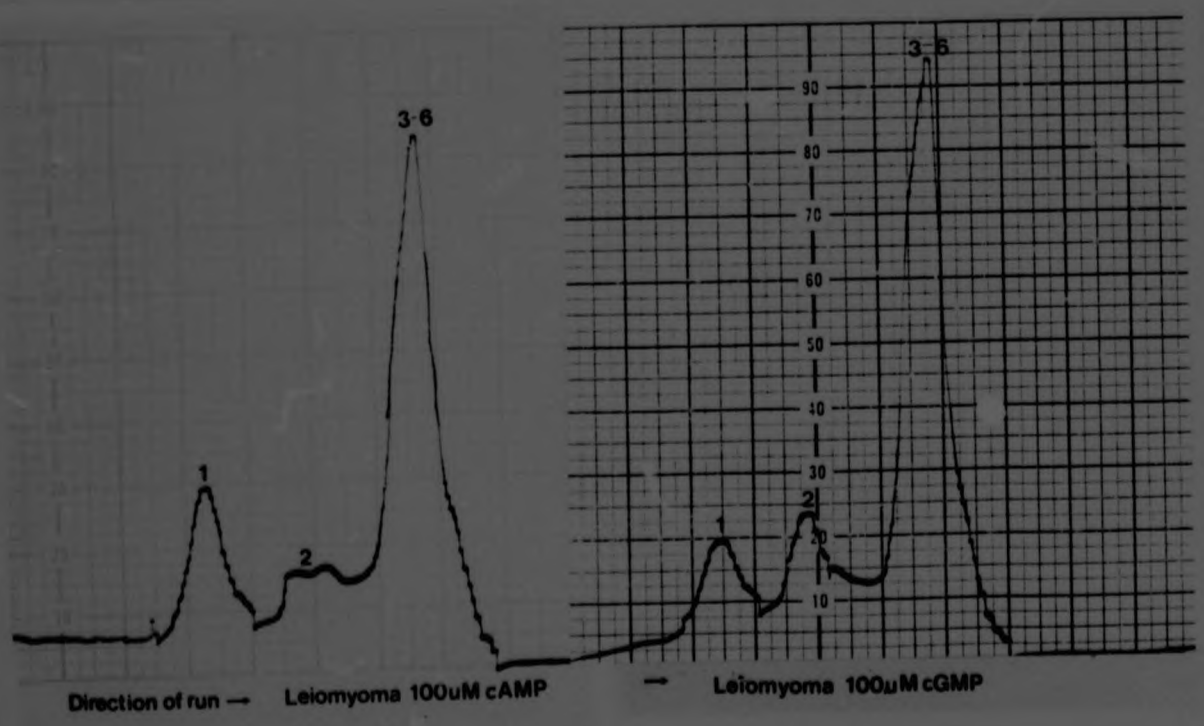
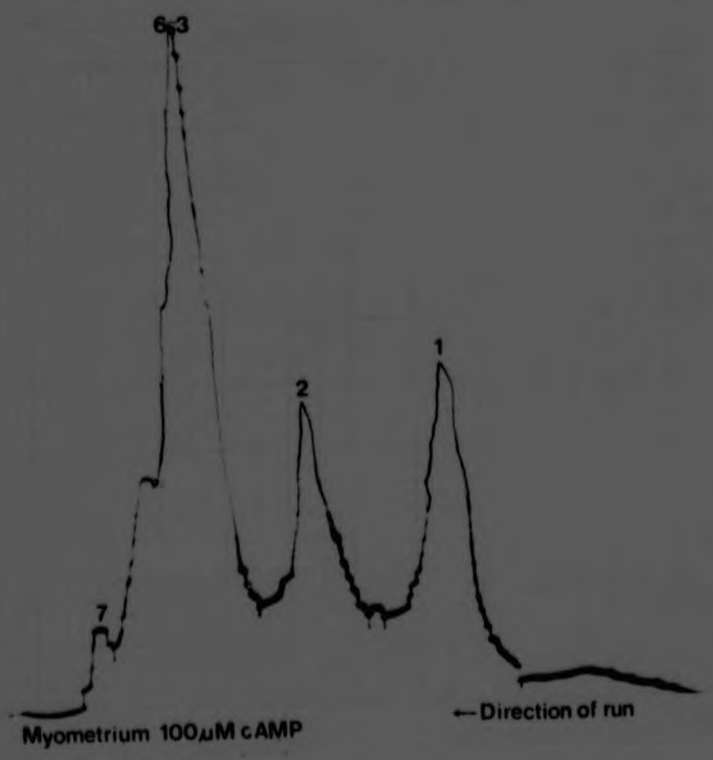


Fig. 5:2c. Densitometry scan of the cPDE activity profile of human myometrium cytosol (5 mg/ml protein in a 100 ul aliquot) when run on nd PAGE coupled to the cPDE activity stain at 100 uM cAMP. Sensitivity setting = 2. The relative cPDE activity in each peak(s) as a % of the total cPDE in the myometrium scan was: background of myometrium 3%; band 1 of myometrium 23%; band 2 of myometrium 22%; bands 3-6 of myometrium 50%; band 7 of myometrium 3%. Note, that the sensitivity setting and soluble protein loaded were identical to (Figs.5:2b and 5:2c), and yet the area under the peaks in the myometrium profile were clearly greater than those in the leiomyoma profile. This suggests that there is greater soluble cPDE activity in human myometrium cytosols compared to human leiomyoma cytosol.



Ferguson plots for the activity bands 1,2,3 and 6 and the individual data for each band are shown (Fig. 5:3 and Table 5:1).

Table 5:1. Characterization of cPDE activity in human leiomyoma, myometrium and uterus using Ferguson plot analysis. All gels were incubated in the presence of 300 uM cAMP.

Band No.	Mr	S.D.	Yo charge density	No. of points	r2
1	229000	4000	2.49	8	.996
2	186000	3000	2.64	7	.995
3	175000	3000	2.98	4	.980
4	175000	3000	3.08	6	.990
5	173000	3000	3.16	8	.993
6	173000	3000	3.36	7	.996
7	162000	4000	3.39	7	.994

It would appear that there are four forms of different Mr namely 229000 (band 1), 186000 (band 2), 173000-175000 (bands 3-6) and 162000 (band 7). Bands 3-6 represent forms of similar molecular mass but different charge densities.