Biochimica et Biophysica Acta, 397 (1975) 134—143

BBA 67546

CHARACTERISATION OF CYCLIC ADENOSINE 3′:5′-MONOPHOSPHATE PHOSPHODIESTERASE FROM WALKER CARCINOMA SENSITIVE AND RESISTANT TO BIFUNCTIONAL ALKYLATING AGENTS

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(Received January 27th, 1975)

Summary

Walker carcinoma cell lines sensitive or resistant to bifunctional alkylating agents have been found to contain multiple forms of cyclic AMP phosphodiesterase (3′:5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17). These activities have been resolved using Sepharose 6B gel filtration and their apparent molecular weights have been estimated.

The enzyme appears to occur in four active forms of apparent mol. wts of >1 000 000, 430 000, 350 000 and 225 000, when assayed at low substrate concentrations. Evidence has been obtained which suggests that all four forms of the enzyme are composed of subunits of mol. wt of approximately 15 000 and are interconvertible.

While the ionic strength of the buffer affected the predominance of the different forms, the presence of cyclic AMP at 10^{-6} M had no effect on aggregation or dissociation of the enzyme.

An activity shift from high molecular weight forms of the enzyme to low molecular weight forms has been found in the resistant tumour at low substrate concentration. No change in elution profile between sensitive and resistant tumours was observed for the low affinity form of the enzyme.

The pH optima of the enzymes with both high and low affinity for the substrate was found to be pH 8.0 in the sensitive line. In the resistant tumour the pH optima of the high affinity form is shifted to pH 8.4 while the low affinity form remains at pH 8.0.

The high affinity forms of the phosphodiesterase in the sensitive and resistant tumour also differed in their inhibition by theophylline. In both cases inhibition was of the competitive type with K_i values for the sensitive and resistant lines being 2.35 and 0.32 mM, respectively. There was no significant difference in the inhibition of the low affinity form between the sensitive and resistant tumour.

Introduction

The intracellular concentration of adenosine 3′,5′-monophosphate (cyclic AMP) depends on the balance between its rate of synthesis by adenylate cyclase and the rate of breakdown by cyclic AMP phosphodiesterase. Many investigators have shown that the phosphodiesterase exists in multiple forms [1—3] which can be separated by starch gel electrophoresis [4,5], gel filtration [1—3] and polyacrylamide gel electrophoresis [6,7]. In many tissues both a high affinity form (possessing a low K_m value for the substrate), and a low affinity form (possessing a high K_m value for the substrate) have been found [3,8,9,10]. This enzyme has been considered as a potential target for the manifestation of the anti-tumour properties of bifunctional alkylating agents [11,12]. It has been found that such agents only cause an elevation of intracellular cyclic AMP in Walker carcinoma cells sensitive to their cytotoxic action. This effect is produced by an inhibition of the low K_m form of the phosphodiesterase. In tumours sensitive to the inhibitory action of bifunctional alkylating agents it has been found that the form of the phosphodiesterase with a low K_m value for the substrate contributes a higher percentage to the total activity than in lines resistant to such inhibitory action. Thus in sensitive Walker carcinoma the apparent V of the low K_m form is 38% of the total activity, whereas this is reduced to 15% in a cell line with a 10-fold resistance to the bifunctional alkylating agent chlorambucil, and 9% in a 70-fold resistant line [11].

This paper describes a detailed study of the cyclic AMP phosphodiesterase from Walker carcinoma, which is either sensitive or resistant, to the cytotoxic effects of bifunctional alkylating agents.

Materials and Methods

Chemicals

The following materials and reagents have been used with the source indicated in parentheses. [8-^{3}H]Adenosine 3′,5′-cyclic phosphate, ammonium salt, 27.5 Ci/mmol (The Radiochemical Centre, Amersham). 5′-Ribonucleotide phosphohydrolase, EC 3.1.3.5, from *Ophiophagus hannah* and Sepharose 6B (Sigma Chemical Co., St. Louis Mo., U.S.A.). Sephadex G 150 (Pharmacia, Uppsala, Sweden). Adenosine 3′,5′-cyclic phosphoric acid, Folin and Ciocalteu's reagent, Amberlite CG 400 Type 1, 100—200 mesh resin and theophylline (British Drug Houses Ltd., Poole, Dorset). NE 233 Liquid Scintillator (Nuclear Enterprises Ltd., Edinburgh). Kieselgel GF_{254} nach Stahl, Type 60, (Anderman and Co. Ltd., Surrey). PCS Solubiliser (Hopkin and Williams, Essex).

All the other reagents used were commercially available. Before use the anion exchange resin was washed with 1 M NaOH, 1 M HCl and water until neutral.

Preparation of cyclic 3′,5′-nucleotide phosphodiesterase

Walker carcinoma ascites cells, sensitive and resistant to alkylating agents were used as a source of enzyme. WR was a cell line with induced resistance to the monofunctional alkylating agent, 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), and WM was resistant to the difunctional alkylating agent melphalan.

Both tumours showed a degree of cross-resistance. Wistar Chester Beatty male rats were killed by cervical dislocation seven days after intraperitoneal tumour implantation. The tumour cells were removed in isotonic saline and washed three times with three volumes of 160 mM Tris · HCl (pH 7.2) containing 7.5 g of NH_4Cl/l [13] to lyse red cells, followed by two washings with 10 ml of isotonic saline, centrifuging between each wash. The cells were resuspended in 0.25 M sucrose and sonicated (1 s/ml) with an MSE sonic oscillator. The sonicated suspension was centrifuged at 100 000 × *g* for 1 h at 4°C and dialysed for 20 h at 4°C against 50 mM Tris · HCl, pH 7.5, containing 10 mM $MgCl_2$. It was then stored in aliquots at −20°C or used immediately. Protein was determined by the method of Lowry et al. [14], using bovine serum albumin as standard. Cell number was determined with a Coulter counter, model FN.

Assay of phosphodiesterase activity

Two assay methods were used for the determination of phosphodiesterase activity. The first method involves a thin-layer chromatographic separation of cyclic [8-^{3}H]AMP from 5′-[8-^{3}H]AMP as previously described [11]. Enzyme samples (100 μl) were incubated at 35°C with an equal volume of 100 mM Tris · HCl, pH 8.1, containing 10 mM $MgSO_4$ and the appropriate concentration of labelled cyclic AMP, for a time interval which gave less than 10% hydrolysis of the substrate. The reaction was terminated by immersion in boiling water for 3 min and the cooled, centrifuged reaction mixture was co-chromatographed on a silicic acid thin-layer chromatographic plate with 5′-AMP in a solvent system consisting of propan-2-ol, ethyl acetate, 13 M ammonia (59 : 25 : 16, by vol.). Radioactivity was determined in NE 233 scintillation fluid using a Tracerlab liquid scintillation spectrometer. Under these conditions the rate of hydrolysis of cyclic AMP was proportional to the amount of enzyme and to elapsed time.

In the second method activity was measured according to the method of Brooker et al. [15] as modified by Thompson and Appleman [1]. An aliquot of the enzyme solution (200 μl) was mixed with the substrate (100 μl) containing cyclic [8-^{3}H]AMP to give a final substrate concentration of 1000 or 5 μM. Incubation was carried out for 10 min at 35°C, and the reaction was terminated by boiling for 1 min. Snake venom (0.1 mg) was then added in a volume of 100 μl of the Tris · HCl buffer, and incubation was continued for a further 10 min at 35°C. At the end of this period 1 ml of 1 : 2 (v/v in water) slurry of Amberlite CG 400 Type 1, 100—200 mesh ion exchange resin was added. After 30 min of contact with the resin, the mixtures were centrifuged and the radioactivity in 0.5 ml of the supernatant fluid was determined by liquid scintillation counting in PCS scintillation fluid.

Sepharose 6B gel filtration

The void volumes of the columns was determined using a 0.2% solution of blue dextran. The following proteins were used for calibration of the column with respect to molecular weight: myoglobin, chymotrypsinogen, ovalbumin, albumin, γ-globulin and apoferritin. All chromatographic separations were carried out at 4°C.

Results

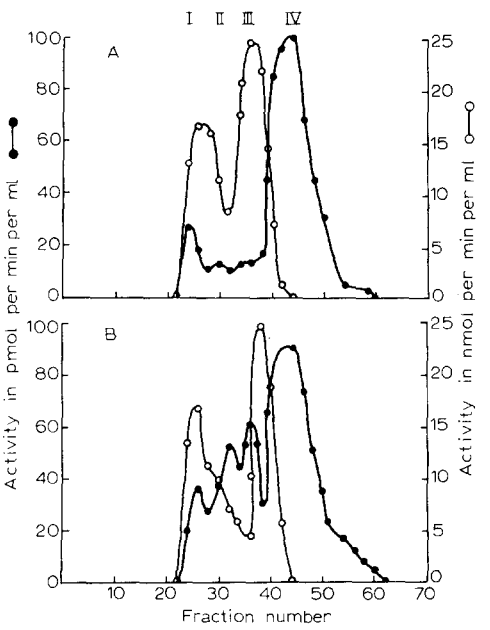
Gel filtration on Sepharose 6B

A detailed study of the cyclic nucleotide phosphodiesterase from the 100 000 × *g* supernatant fraction of Walker carcinoma has previously been reported [11]. This analysis indicates the presence of two enzyme activities with apparent K_m values of 82.5 ± 7.0 μM and 1.1 ± 0.3 μM. To distinguish between these two 'forms' the activity was measured at substrate concentrations of 1000 μM (which was considered to measure mainly the high K_m form) and 5 μM (for the low K_m form of the enzyme). Knowing the K_m values of the two enzyme forms the activities at low substrate concentration can be corrected for the high K_m enzyme and vice versa. At a substrate concentration of 1000 μM the contribution of the low K_m activity is 29 per cent, whilst at 5 μM cyclic AMP the contribution of the high K_m activity to that observed is 15 per cent. Both sensitive and resistant forms of the tumour were shown to contain similar amounts of protein per cell (250 μg and 244 μg/10^6 cells, respectively) and therefore equal amounts of protein of the two cell lines were used for gel filtration studies and the activity per ml was compared directly. The activity profiles of phosphodiesterase, measured at low and high substrate concentration, in the supernatant fractions of resistant (WM) and sensitive Walker carcinoma after Sepharose gel filtration are shown in Figs 1A and 1B, respectively. When measured at low substrate concentration four distinct peaks of phosphodiesterase activity (I, II, III, IV) were found in the sensitive tumour, while the activity of two of these (II, III) were found to be greatly reduced in the resistant line. Peak I was eluted with the void volume of the column indicating a molecular weight greater than 1 000 000. The elution of peaks II, III and IV corresponded to their having apparent molecular weights of approximately 430 000, 350 000 and 225 000, respectively. These values of molecular weight are based on columns standardized with globular proteins, but if the phosphodiesterases are nonglobular or conjugated proteins their molecular weights are likely to be quite different. In contrast to the activity profile observed at low substrate concentrations, that measured at high substrate concentration, is similar in both sensitive and resistant tumours. In this case only two peaks of phosphodiesterase activity were observed corresponding approximately in molecular weight to peaks I and III as measured at low substrate concentration.

When frozen rather than fresh samples of the 100 000 × *g* supernatant fraction of the resistant Walker tumour were applied to Sepharose columns, it was noticed that the activity of peaks II and III was increased compared with the profile shown in Fig. 1A, while the activity of peak IV was decreased. This suggests that an equilibrium exists between the enzyme species II, III and IV. To investigate this possibility the fractions corresponding to each of the three peaks were pooled, dialysed overnight against a 1 : 20 dilution of the Tris · HCl buffer pH 7.5, containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol as used in the filtration, and then concentrated 20-fold. When each of these concentrates was subjected to Sepharose gel chromatography an activity profile similar to that shown in Fig. 2B was obtained, and this suggests that there had been a re-equilibrium between the three forms of the enzyme. If, however, the fractions corresponding to the individual peaks were concentrated without pre

dialysis and re-applied to a Sepharose column only a single peak of activity was observed as shown for peak IV in Fig. 2A. The slight broadening of this peak probably represents some dissociation to lower molecular weight forms. This suggests that the equilibrium between the forms II, III and IV is dependent on the salt concentration, and that a reduction in ionic strength for a given amount of enzyme protein causes a re-aggregation as shown in Fig. 2B.

To investigate more fully the effect of ionic strength on the dissociation of the enzyme the material corresponding to peak IV was subjected to Sepharose gel filtration in 50 mM Tris · HCl, pH 7.5, containing 0.6 M NaCl. The activity profile shown in Fig. 2C shows no high molecular weight forms, but



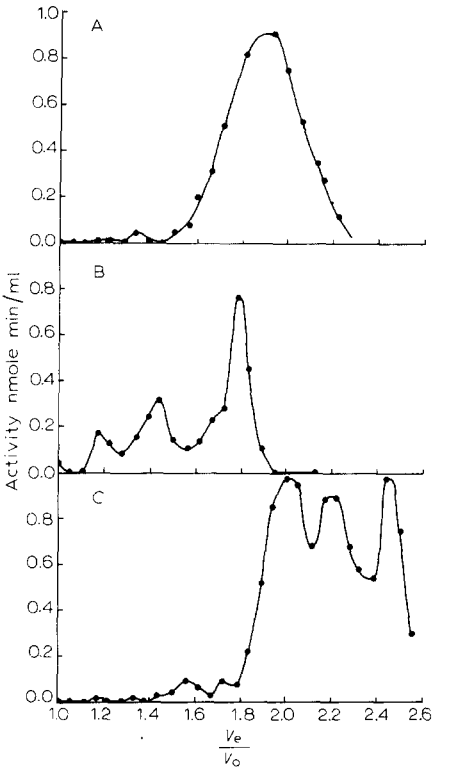


Fig. 1. Sepharose 6B gel filtration of resistant (WM) Walker carcinoma 100 000 × *g* supernatant fraction (A) and sensitive (B) in 50 mM Tris · HCl, pH 7.5, containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. A volume of 2.5 ml of total supernatant containing 27.5 mg of protein was applied to a column (110 cm × 1.8 cm) and elution was performed with Tris/$MgCl_2$/mercaptoethanol buffer. Flow rate was 0.5 ml/min. Fractions of 3 ml were collected and phosphodiesterase activity was estimated at a cyclic AMP concentration of either 5 μM (●——●) or 1000 μM (○——○) using the second phosphodiesterase assay method.

Fig. 2. Interconversion between the three forms of cyclic AMP phosphodiesterase. Activity was determined by the second phosphodiesterase assay method at a substrate concentration of 5 μM. A. The fractions corresponding to peak IV from the separation shown in Fig. 1B were pooled and concentrated by vacuum dialysis and a portion (588 μg of protein in 300 μl of buffer) was reapplied to a Sepharose 6B column (35 cm × 1.5 cm) in Tris/$MgCl_2$/mercaptoethanol buffer and 1 ml fractions were collected. Flow rate was 20 ml/h. B. The fractions corresponding to peak IV were pooled, dialysed against 2.5 mM Tris · HCl, pH 7.5, containing 0.5 mM $MgCl_2$, concentrated 20-fold by vacuum dialysis, and a portion (700 μg of protein in 300 μl of buffer) was reapplied to the same Sepharose 6B column as in A. C. As in A except that the buffer contained 0.6 M NaCl.

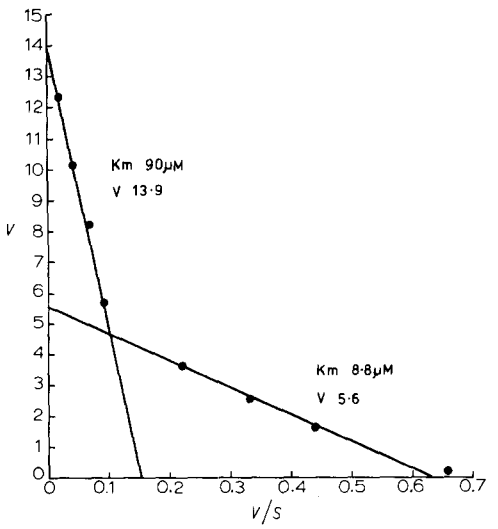


Fig. 3. Hofstee plot for the hydrolysis of cyclic AMP by fraction IV (Fig. 1B), which was concentrated without pre-dialysis. Samples were analysed as in method 1 and the initial velocities were determined at substrate concentrations ranging from 0.25 to 1000 μM. V = initial velocity (nmol of cyclic AMP hydrolysed/min/mg protein); S = substrate concentration (μM).

three new forms with apparent molecular weights of 120 000, 90 000 and 60 000 were detected. Increasing the salt concentration further to 1.0 M NaCl, and using Sephadex G 150 gel chromatography led to the appearance of three peaks of activity, measured at low substrate concentration, with apparent molecular weights, estimated by the method of Kulkerni and Mehrotra [16], of approximately 60 000, 30 000 and 15 000. This suggests that each of the three forms II, III and IV consist of subunits of apparent molecular weight 15 000.

The kinetics of hydrolysis of cyclic AMP by the enzyme corresponding to activity peak IV, which was concentrated without prior dialysis, is expressed by means of a Hofstee plot in Fig. 3. In this plot the slope is the negative value of the apparent K_m value and the intercept on the ordinate is the value of the apparent V. This plot suggests the presence of two activity forms similar to that observed in the crude 100 000 × g supernatant fraction. Whilst the K_m values of both low and high affinity forms (90 and 8.8 μM) are higher than those found in the supernatant fraction, the percentage of the high affinity form of the total enzyme (39%) is similar to that found originally (38%). Non-linear kinetics on hydrolysis of cyclic AMP by the fractions corresponding to peak IV does provide some evidence for negative cooperativity [17], that is, in a single protein species the binding of a ligand decreases the affinity of binding sites of neighbouring subunits. This cannot be investigated at the moment, however, since at the present stage of purification no definite conclusion can be drawn regarding the protein homogenety and state of aggregation of peak IV.

The interconvertibility of the enzymic material giving rise to peaks II, III and IV suggested that binding of the substrate, cyclic AMP, may lead to a change in the equilibrium ratio of the various forms of the enzyme. This possibility was investigated by chromatography on Sepharose 6B in the presence of low physiological concentrations of cyclic AMP (1 μM). No change in elution pattern or activity ratio was obtained (results not shown).

Activity-pH profiles

The pH-activity profile of phosphodiesterase from sensitive and resistant Walker carcinoma (WR) is shown in Figs 4A and 4B, respectively. There is a shift in pH optimum of the high affinity form of the enzyme from pH 8.0 in the sensitive tumour to pH 8.4 in the resistant tumour. The pH optimum of the low affinity form of the enzyme is pH 8.0 in both tumours. A similar shift in pH optimum of the high affinity form of the phosphodiesterase to pH 8.5 is also observed in Walker carcinoma with a 70-fold resistance to chlorambucil (data not shown). These results provide additional evidence for a change in the high affinity form of the phosphodiesterase in resistant tumours.

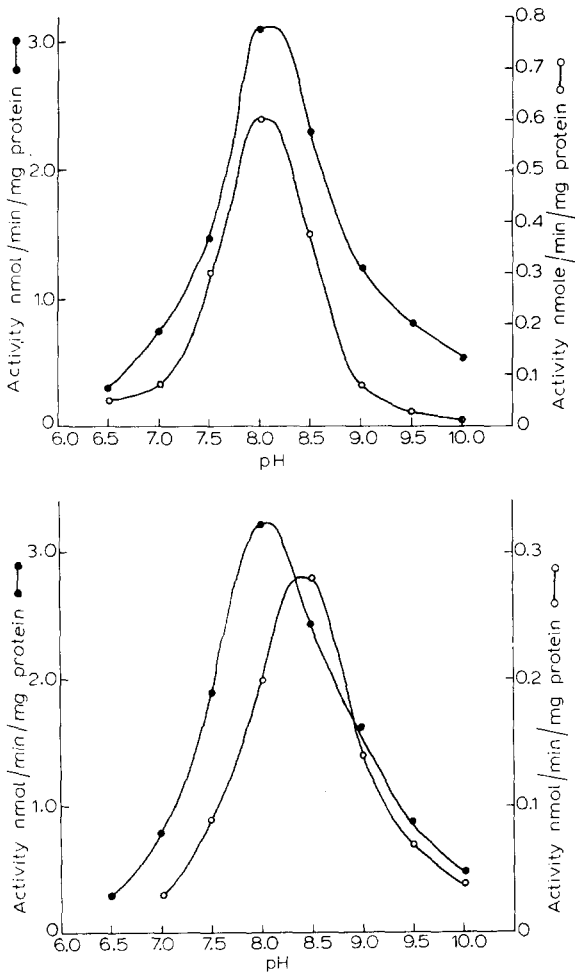


Fig. 4. Effect of pH on the activity of sensitive (A) and resistant (B) (WR) Walker carcinoma. Phosphodiesterase activity was assayed at 1000 μM (●——●) or 2.5 μM (○——○) cyclic AMP by the first phosphodiesterase assay method, except that 50 mM Tris · maleate buffer was substituted for 100 mM Tris · HCl.

Inhibition by theophylline

The methylxanthines, theophylline and caffeine, are known inhibitors of cyclic AMP phosphodiesterase activity. The low K_m forms of the phosphodiesterases from sensitive and resistant Walker carcinoma (WR) can be distinguished further by their response to inhibition by theophylline. In both cases inhibition is of the competitive type, but K_i values were calculated to be 2.35 and 0.32 mM for the sensitive and resistant tumours, respectively. The lower inhibition constant for the resistant tumour correlates with its greater sensitivity ($1D_{50}$ 150 μg/ml) to theophylline compared with the sensitive line ($1D_{50}$ 250 μg/ml). Since the intracellular concentration of cyclic AMP in Walker carcinoma is in the range 1—2 μM this form of the enzyme is likely to be more important in regulating levels of the cyclic nucleotide under physiological conditions. Inhibition of the high K_m phosphodiesterase is also of the competitive type, but in this case the K_i values for sensitive and resistant tumour (1.2 and 3.6 mM, respectively) are much closer.

Discussion

The anomalous kinetic behaviour observed with cyclic nucleotide phosphodiesterase can be explained either by multiple binding sites on one enzyme with negative cooperativity [17] or distinct forms of the enzyme with differing affinities for cyclic AMP. Using Sepharose 6B gel filtration Klotz et al. [18] resolved the cyclic nucleotide phosphodiesterase of rat adipose tissue into three different forms with molecular weights of >1 000 000, 400 000—1 000 000 and <400 000. Thompson and Appleman [3] have reported values of approximately 400 000 for the high K_m form of the enzyme and approximately 200 000 for the low K_m form from the mammalian tissues tested. Slightly lower molecular weights have been reported for the enzyme from frog erythrocytes [19] and much lower estimates for the molecular weights of the rat kidney enzyme have been reported [20].

The results presented indicate that multiple cyclic nucleotide phosphodiesterase activities also exist for Walker carcinoma. Four peaks of enzyme activity corresponding to molecular weights of approximately >1 000 000, 430 000, 350 000 and 225 000 were obtained when a crude 100 000 × *g* supernatant fraction was submitted to Sepharose gel filtration and the fractions were assayed at a physiological concentration of cyclic AMP, namely 5 μM. When the fractions were assayed at 1 mM cyclic AMP, apart from the peak in the column void volume the major activity fraction corresponds to an apparent molecular weight of approximately 350 000. In contrast the 100 000 × *g* supernatant fraction from Walker carcinoma resistant to bifunctional alkylating agents showed a much reduced activity of the fractions corresponding to molecular weights of 430 000 and 350 000, when assayed at low substrate concentration. No difference in elution profile between sensitive and resistant tumour was observed at high substrate concentration.

This latter result is interesting in view of the greater sensitivity of the low K_m form of the phosphodiesterase from Walker carcinoma to the inhibitory action of a bifunctional alkylating agent [12]. This form is important in regulating intracellular levels of cyclic AMP under physiological conditions. Further

evidence for a change of the form of the low K_m phosphodiesterase on the acquisition of resistance to bifunctional alkylating agents is provided by the shift in pH optimum from pH 8.0 to pH 8.4 in the resistant tumour, and the different inhibition constants for the competitive inhibitor theophylline towards the low K_m form of the enzyme from sensitive and resistant tumours. These results suggest a change in the primary structure of the catalytic site of the enzyme. It has been shown previously [11] that although the K_m values of both low and high affinity forms of the enzyme do not differ appreciably in sensitive and resistant tumours there is a decrease in the V of the low K_m form with increasing resistance, while the V of the high K_m form remains constant. Using combinations of the enzyme from sensitive and resistant tumours it was found that the specific activity of the combination did not deviate significantly from what would be expected by simple additivity indicating that no excess cytoplasmic enzyme inhibitor was present in the resistant cells, or activator in the sensitive cells. This indicates a loss of high affinity enzyme protein with increased resistance to bifunctional alkylating agents, which in WM appears to be due to the loss of two specific enzyme fractions.

The interrelationship between the three peaks of phosphodiesterase activity measured at low substrate concentration is shown by the reaggregation of the enzymic form IV to form also forms II and III when the salt concentration is reduced by dialysis (Fig. 2B) and its dissociation to forms with apparent molecular weights of 120 000, 90 000 and 60 000 when gel filtration is carried out in the presence of 0.6 M NaCl (Fig. 2C). Sheppard and Tsien [21] reported an increased activity of the high affinity cyclic AMP phosphodiesterase from rat erythrocytes on exposure to 2 M NaCl at 4—5° C. This effect probably arises from a dissociation of a multiaggregate form. A similar two fold increase in activity of peak IV was observed when chromatography was carried out in the presence of 0.6 M NaCl. This shows that the subunits of lower molecular weight have a higher enzyme activity than the multiaggregate forms. Since the fraction corresponding in molecular weight to 90 000 can not be an integral subunit of any of the higher molecular weight forms, the monomer is probably much smaller with a molecular weights of either 30 000 or 15 000. Chromatography on Sephadex G 150 in the presence of 1 M NaCl led to the appearance of activity forms of apparent molecular weights of 60 000, 30 000 and 15 000, all of which are catalytically active. Thus the monomer would appear to have an apparent molecular weight of 15 000. Although Schroder and Rickenberg [2] reported that 10^{-7} M cyclic AMP caused a dissociation of bovine liver phosphodiesterase no effect of 10^{-6} M cyclic AMP on the interconversion of the various forms of the tumour enzyme was observed in the present experiments.

Evidence for the tissue specificity of cyclic nucleotide phosphodiesterase has been provided by the differential sensitivity of the enzyme from several tissues to a variety of reversible inhibitors [22] and by the differing electrophoretic patterns produced by a variety of rat tissues [4]. Since neoplastic cells have been shown to have a defect in their cyclic AMP metabolism [23—25] resulting in lower intracellular levels of the cyclic nucleotide [26,27] this tissue specificity of the phosphodiesterase may provide an important new target for anti-tumour chemotherapy, by the administration of agents which specifically affect the phosphodiesterase of the neoplastic tissue.

Acknowledgements

The author wishes to thank Professor L. Young for his interest and Dr T.A. Connors and Mr K. Merai for supplying the Walker tumours. This work has been supported by a grant from the Cancer Research Campaign.

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