Biochemical Pharmacology, Vol. 24, pp. 205–210. Pergamon Press, 1975. Printed in Great Britain.

ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE ACTIVITY IN EXPERIMENTAL ANIMAL TUMOURS WHICH ARE EITHER SENSITIVE OR RESISTANT TO BIFUNCTIONAL ALKYLATING AGENTS

MICHAEL J. TISDALE and BARRY J. PHILLIPS

Department of Biochemistry, St. Thomas's Hospital Medical School, London, SE1 7EH, England and Ludwig Institute for Cancer Research, Chester Beatty Research Institute, Fulham Road, London, SW3 6JB, England

(*Received* 14 *May* 1974; *accepted* 11 *July* 1974)

Abstract—The adenosine 3′,5′-monophosphate phosphodiesterase of Walker rat carcinoma 256, ADJ/PC6 plasma cell tumour, NK lymphoma, Sarcoma 180 and TLX5 lymphoma behaves kinetically as if two separate activities exist, one with a low affinity for the substrate and the other with a high affinity. The high K_m values are 82·5, 566, 590, 1975 and 1075 μM for the enzyme from each tumour respectively, and the low K_m values are 1·1, 17·7, 5·75, 7·1 and 4·4 μM. In the Walker carcinoma and PC6 plasma cytoma, tumours which are sensitive to alkylating agents, the apparent V_{max} of the low K_m forms are respectively 38 and 25 per cent of the total activity. In those tumours which are naturally resistant to the growth inhibitory properties of the alkylating agents, the apparent V_{max} of the low K_m form is less than 10 per cent of the total activity. Walker carcinoma showing a 20-fold resistance to chlorambucil[*p*-(di-2-chloroethylamino)phenylbutyric acid] has the V_{max} of the high affinity form decreased to 15 per cent of the total, while a 70-fold resistant line has the V_{max} of this form of the enzyme decreased to 9 per cent of the total. This decrease in the activity of the high affinity form of the enzyme in the resistant tumours does not appear to be due to the presence of an endogenous inhibitor. While the high K_m form of the enzyme in the Walker carcinoma is mainly confined to the cytosol, about half of the activity of the low K_m form appears to be associated with particulate fractions. This subcellular distribution does not differ appreciably in the sensitive and resistant tumours. The possible reasons for such an isoenzyme shift are discussed.

Adenosine 3′,5′-monophosphate (cyclic AMP) is known to function as a mediator of the action of a large number of hormones on a variety of target tissues [1]. Recent interest has focused on the relationship of cyclic AMP to the control of cell growth. The intracellular concentration of cyclic AMP increases at confluency in cells displaying contact inhibition of growth [2], and cell transformation with oncogenic viruses leads to lowered cyclic AMP levels [3, 4]. Cyclic AMP appears to qualify as a potential regulator of the cell cycle on the basis of its variable presence during the cell cycle [5, 6], its specific delay of mitosis [7], and the reversibility of its action. The growth inhibition appears to be caused by an inhibition of nucleic acid synthesis [8, 9], possibly by an inhibition of thymidine uptake by a decrease in thymidine kinase activity [10].

Cyclic 3′,5′-nucleotide phosphodiesterases (adenosine 3′,5′-monophosphate phosphohydrolase, EC 3.1.4.c) catalyse the hydrolysis of cyclic AMP and other 3′,5′-nucleotides to their respective 5′-nucleoside monophosphates. Since intracellular concentrations of cyclic AMP are in part determined by the rate of hydrolysis of the nucleotide, the characterization of the cyclic nucleotide phosphodiesterase from various tissues is important, as this may potentially be a target for antitumour chemotherapy. Thus inhibitors of this enzyme induce morphological differentiation of mouse neuroblastoma cells in culture [11], and inhibit the growth of experimental animal tumours *in vivo* [12, 13] as well as completely abolishing in hamsters the tumour-producing ability of chicken embryo lethal orphan (CELO) virus-transformed hamster skin cells [14].

The cyclic nucleotide phosphodiesterase from a number of tissues exists in multiple forms differing in their Michaelis–Menten constants (K_m values) [15, 16] as well as their electrophoretic and chromatographic behaviour [17, 18]. The functions of these multiple forms of the enzyme and their relative importance in the control of intracellular cyclic AMP levels are, however, not clear. The present paper is a report of an investigation of the multiple forms of the phosphodiesterase found in the experimental animal tumours which are sensitive and resistant to the effects of the antitumour alkylating agents.

MATERIALS AND METHODS

8-[^{3}H]-Cyclic AMP (sp. act. 20·7 Ci/m-mole) was purchased from the Radiochemical Centre, Amersham.

Scintillation fluid NE 233 was purchased from Nuclear Enterprises Ltd., Edinburgh.

Animal tumours and culture conditions. The Walker rat carcinoma 256 was obtained 7 days after i.p. implantation into Wistar Chester Beatty male rats. NK lymphoma was passaged subcutaneously in the inguinal region of female Balb/C$^-$ mice and material was obtained 25 days after tumour implantation. Sarcoma 180 was obtained 7 days after i.p. implantation into Balb/C$^-$ male mice. TLX5 lymphoma required 7 days to produce a tumour of sufficient size for use, after i.p. implantation in CBA/LAC female mice. The ADJ/PC6 plasma cell tumour was obtained 14 days after i.p. tumour implantation in female Balb/C$^-$ mice. All tumours were in the ascitic form.

A resistant line (WMI) was derived from sensitive Walker ascites carcinoma 256 (WS) by repeated treatment with increasing doses of melphalan [L-*p*-(di-2-chloroethylamino)phenylalanine] *in vivo*. This line has a degree of acquired resistance to other alkylating agents including chlorambucil. Both WS and WMI were adapted to culture, and a more resistant cell line (WMII) was developed *in vitro* by treating WMI cells every 10 days with progressively increasing doses of chlorambucil. Cells were grown in Dubecco's modified Eagle's medium supplemented with 10% foetal calf serum under an atmosphere of 10% CO_2 in air. The methods used to establish and maintain the Walker tumour cells in culture were as previously described [19].

Adenosine 3',5'-monophosphate phosphodiesterase preparation. All operations were carried out at 0–4°. All the cyclic 3',5'-nucleotide phosphodiesterases were prepared by essentially the same method.

Animals were killed by cervical dislocation and the ascites cells were removed in isotonic saline. If the cells were contaminated with blood they were washed as necessary with 0·016 M Tris–HCl, pH 7·2, containing 7·5 g of ammonium chloride/l. [20] and finally with saline, and centrifuged between each washing. Cultured cells were washed twice with saline. The cells were then suspended in cold 0·25 M sucrose at the following numbers of cells per ml: Walker carcinoma and ADJ/PC6 plasma cell tumour, 4×10^7; NK lymphoma and Sarcoma 180, 2×10^7, and TLX5 lymphoma, 5×10^7. The suspension was treated with a 20-Kc MSE sonic oscillator (1 sec/ml). The lysate was centrifuged at 100,000 *g* for 1 hr and the supernatant fluid was used for the assay of phosphodiesterase.

Assay of adenosine 3',5'-monophosphate phosphodiesterase. Assays were carried out in duplicate. The supernatant fluid was diluted to the appropriate protein concentration with the assay buffer which was 100 mM Tris–HCl, pH 8·1, containing 10 mM $MgSO_4$. Aliquots (100 μl) of the supernatant fluid were added to equal volumes of the reaction mixture containing 1000 to 0·2 μM [^{3}H]-cyclic AMP (100,000 cpm/assay). The reaction mixtures were incubated at 35°, 50 μl portions were removed at various times, and the enzyme activity was terminated by immersion in boiling water for 3 min. After cooling and centrifugation the aliquots were co-chromatographed on a silicic acid (Merck, Kieselgel GF_{254}) thin layer chromatography (t.l.c.) plate with 5'-AMP in a solvent system consisting of propan-2-ol, ethyl acetate, 13M ammonia (59:25:16, by vol) [21] and the spot corresponding to 5'-AMP was located under u.v. light (254 nm). Total [^{3}H]-cyclic AMP in the incubation mixture was determined under the same conditions, but with 100 μl of assay buffer or heat-inactivated enzyme added at zero time, and with marker cyclic AMP on the t.l.c. plate. In order to confirm that the position of the u.v. absorbance corresponded with the radioactivity the plates were also periodically scanned with a Berthold radiochromatogram scanner. Under these conditions cyclic AMP had an R_f value of approximately 0·4, while 5'-AMP remained at the starting point.

The silicic acid was removed from the plates and the radioactivity was estimated in 5 ml of scintillation fluid using a Tracer Lab liquid scintillation spectrometer.

Protein was estimated by the method of Lowry *et al.* [22] using bovine serum albumin as the standard.

For the standard assay the incubation time was adjusted to stop the reaction at less than 10 per cent cyclic AMP hydrolysed. Under these conditions the rate of cyclic AMP hydrolysis was proportional to the amount of enzyme and to elapsed time for all cell lines. Data are expressed as nmoles of [^{3}H]cyclic AMP hydrolysed per min per mg of protein.

RESULTS

Incubation conditions with respect to time and enzyme concentration were carefully chosen to ensure linearity of cyclic AMP hydrolysis. With 60–90 μg of protein per assay at the high substrate concentrations, the rate of production of 5'-AMP was linear with time up to 45 min. At the low substrate concentrations there was linearity up to 20 min at a protein concentration of 5–7 μg per assay. In both cases enzyme concentration and incubation time were chosen so that the amount of cyclic AMP hydrolysis did not exceed 10 per cent.

The rate of hydrolysis of cyclic AMP by sensitive Walker carcinoma 256 (WS) and the 10-fold (WMI) and 70-fold (WMII) resistant sublines is shown in Fig. 1. The data are expressed by Hofstee [23] plots in which the slope is the negative value for the apparent K_m and the intercept on the ordinate is the value for the apparent V_{max}. In each case the data may be fitted with two lines to a first approximation, which suggests either the presence of two enzyme activities for each tumour or a negatively co-operative control system [24]. Assuming the presence of two enzyme activities this analysis indicates that 38 per cent of the total phosphodiesterase activity in WS can be attributed to an enzyme with a low apparent K_m value (1·1 $\pm$ 0·3 μM). In both WMI and WMII the K_m values of this form of the enzyme (1·32 $\pm$ 0·05 and 0·9 $\pm$ 0·05 μM respectively) do not differ appreciably from that found

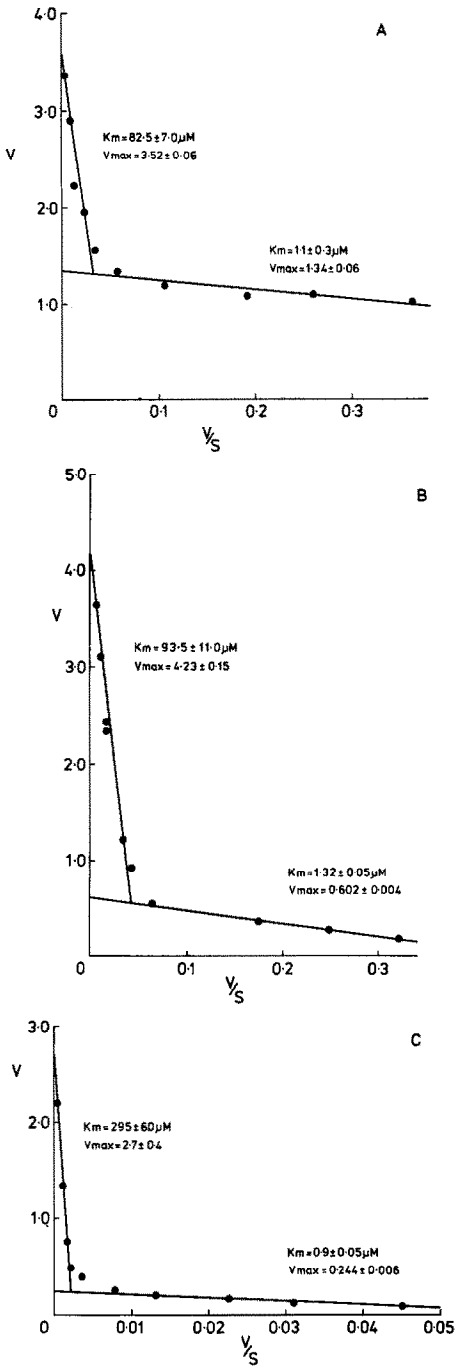

Fig. 1. Hofstee plots [23] for the hydrolysis of cyclic AMP by WS (A), WMI (B) and WMII (C) 100,000 *g* supernatant fractions. Samples were analysed as in Methods and the initial velocities were determined at substrate concentrations ranging from 0·2 to 1000 μM.

[v = initial velocity (nmoles of cyclic AMP hydrolysed/min/mg protein); S = substrate concentration (μM)]. The apparent K_m and V_{max} values and their variation was determined by the method of Wilkinson [25].

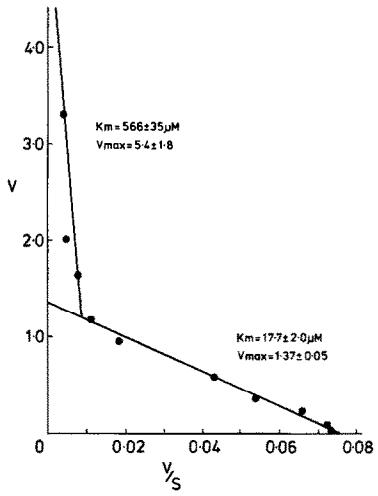

Fig. 2. Hofstee plot for the hydrolysis of cyclic AMP by ADJ/PC6 plasma cell tumour 100,000 *g* supernatant fraction. Conditions are as described in Fig. 1.

in WS. In contrast, only 14 per cent of the total phosphodiesterase activity in WMI and 9 per cent of the total activity in WMII is associated with this low K_m component. In both WS and WMI the K_m values of the low affinity form (82·5 ± 7·0 and 93·5 ± 11·0 μM respectively) and the V_{max} of this form (3·52 ± 0·06 and 4·23 ± 0·15 nmole/min/mg protein respectively) do not differ significantly. In WMII the K_m value of the low affinity form (295 ± 60 μM) is tripled, however, whilst the value of the V_{max} (2·7 ± 0·4 nmole/min/mg protein) is only 80 per cent of that found in WS. This suggests that the cyclic AMP phosphodiesterase in the 70-fold resistant tumour is radically different from that in the two related lines.

Figure 2 shows that a similar situation exists for the ADJ/PC6 plasmacytoma, which is also sensitive to bifunctional alkylating agents (Table 1). Again a Hofstee plot suggests that there are two forms of the

Table 1. Sensitivity of the various tumour lines to the difunctional alkylating agent Melphalan and an estimation of the doubling time of the ascites form *in vivo*

Tumour	T.I*	Doubling time (hr)	[Ref]
Sensitive-Walker carcinoma	25·7	17	26
Resistant-Walker carcinoma	—	17	26
ADJ/PC6 plasma cell tumour	165·5	48	27
NK lymphoma	1·0	48	27
Sarcoma 180	†	48	
TLX5 lymphoma	‡	12	28

* T.I. = therapeutic index = LD_{50}/ED_{90}.
† 75% inhibition at 20 mg/kg. LD_{50} 28 mg/kg.
‡ 18·8% increase in life span at 8 mg/kg.

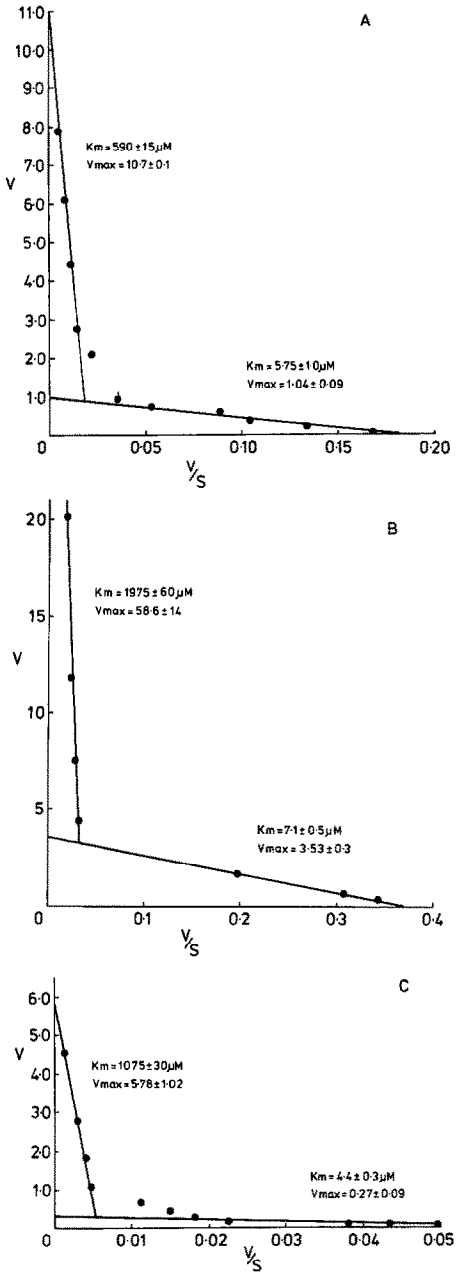

Fig. 3. Hofstee plots for the hydrolysis of cyclic AMP by 100,000 *g* supernatant fractions of NK lymphoma (A), Sarcoma 180 (B) and TLX5 lymphoma (C). Conditions are as described in Fig. 1.

enzyme. In this case also the low K_m form of the phosphodiesterase contributes a high percentage (25 per cent) to the total enzyme activity. The absolute values of the K_m of both forms of the enzyme are appreciably greater than was observed in WS, however, although the V_{max} of the low K_m form is almost identical to the value observed with the sensitive Walker carcinoma.

In contrast, the kinetics of the phosphodiesterase of the three experimental tumours shown in Fig. 3 (NK lymphoma, Sarcoma 180 and TLX5 lymphoma), which have a much reduced sensitivity to the antitumour effects of bifunctional alkylating agents (Table 1), resembles more closely the situation observed with the two resistant Walker sublines. In order of decreasing sensitivity to alkylating agents the contribution of the low K_m form of the phosphodiesterase to the total is 9 per cent in the NK lymphoma, 6 per cent in Sarcoma 180 and 5 per cent in TLX5 lymphoma. The K_m values for the low affinity form of the enzyme are also much higher than was the case for the Walker carcinoma, which would suggest that this form plays little part in the regulation of the cyclic AMP content of these tumours, which is usually in the range 1–2 μM.

Table 1 shows the sensitivity of the various tumour lines to the bifunctional alkylating agent melphalan, as well as an estimation of the doubling times of the ascites forms of the tumours *in vivo*. Although it has frequently been suggested that alkylating agents specifically inhibit rapidly growing tissues it can be seen that one of the most sensitive of the experimental tumours to the alkylating agents, the ADJ/PC6 plasma cell tumour, is in fact one of the slowest growing. On the other hand, one of the most resistant of the experimental tumours, the TLX5 lymphoma, has the fastest doubling time. There appears to be no difference in the growth rate between the sensitive and resistant Walker carcinoma even though there is a 20-fold difference in sensitivity to growth inhibition by melphalan.

In an attempt to discover if the lowering of the activity of the low K_m form of the phosphodiesterase is due to endogenous inhibitors, the effect of mixing total cell sonicated suspensions and heated suspensions of WMI and WMII with WS in equal proportions has been investigated (Table 2). The activity of the high K_m form of the phosphodiesterase was assayed at 1,000 μM cyclic AMP and the low K_m form of the enzyme at 3·3 μM cyclic AMP. The specific activity of the combination of the various fractions did not deviate significantly from what would be expected by simple additivity, indication that no excess cytoplasmic enzyme inhibitor was

Table 2. Effect on the specific activity of cyclic nucleotide phosphodiesterase by combination of enzyme from sensitive and resistant Walker carcinoma*

Treatment	Phosphodiesterase activity (nmoles/min/mg protein) Observed	Expected
WS + heated WMI	0·39	0·4
WS + heated WMII	0·4	0·4
WS + WMI (in equal proportions)	0·24	0·26
WS + WMII (in equal proportions)	0·22	0·23

* Results are means of two experiments with different concentrations of the enzyme from the two tumours.

Table 3. Distribution of high and low affinity cyclic nucleotide phosphodiesterase in sensitive and resistant Walker carcinoma

		WS		WMI		WMII	
Substrate concn (μM)		1000	3·3	1000	3·3	1000	3·3
Specific activity (nmole/min/mg protein)	Total	1·66	0·6	1·7	0·26	1·4	0·26
	100,000 g supernatant	3·32	0·46	3·93	0·4	2·3	0·18
	Cell pellet	—	0·24	—	0·23	—	0·28

present in the resistant cells, or activator in the sensitive cells.

An investigation of the relative proportion of the two forms of the enzyme found in broken cell preparations and in 100,000 g supernatant fractions of Walker tumour was carried out, in which the activity of the two forms of the enzyme was estimated at 1000 μM and 3·3 μM cyclic AMP as above. Table 3 shows that while all of the activity of the high K_m form of the phosphodiesterase found in total broken cell preparations was also found in the 100,000 g supernatant fraction, only 50 per cent of the low K_m form was associated with the soluble fraction. After centrifugation the sedimented pellet was resuspended in assay buffer and the activity of both forms of the phosphodiesterase was measured. While the high K_m form of the enzyme was found to be absent from this fraction, it was found to contain the remaining 50 per cent of the low K_m form. No difference in overall distribution was found between sensitive and resistant lines.

DISCUSSION

The kinetic evidence presented for the cyclic AMP phosphodiesterase activities in the various animal tumours is compatible with the suggestion that for each tumour there are two phosphodiesterase activities, one with a low apparent K_m and one with a high K_m for cyclic AMP. Two forms of the enzyme have been separated from rat brain [29]. The high K_m form displays normal Michaelis–Menten kinetics and probably represents a guanosine 3′,5′-phosphate phosphodiesterase, whilst the other form is specific for the hydrolysis of cyclic AMP and probably represents a negatively cooperative control system. Clark *et al.* [30] observed an increase in activity associated with the low K_m form of the enzyme from 4 per cent of the total activity in normal control liver to 25 per cent of the total activity in the rapidly growing hepatoma 3924A. They postulated that this shift in isoenzyme pattern was probably attributable to neoplastic transformation and not to growth rate. A change in the distribution of the two forms of the enzyme is also seen in other systems. Thus a two-fold increase in the V_{max} of the high K_m form of the phosphodiesterase is seen in adipocytes of genetically obese mice compared with adipocytes of normal mice [31]. When 3T3–4 cells reach confluency the specific activity of both forms of the phosphodiesterase rises from three- to five-fold [32].

The results presented here show a shift in isoenzyme pattern amongst the range of experimental tumours commonly employed for testing new antineoplastic drugs. This shift appears not to result from a difference in growth rate of the tumours. Instead the greatest contribution of the low K_m form of the phosphodiesterase to the total activity appears to be observed only in those tumours which are sensitive to the antitumour effects of the bifunctional alkylating agents. The induction of resistance in the Walker carcinoma causes a shift in isoenzyme pattern towards that seen in naturally insensitive tumours such as TLX5 lymphoma. This isoenzyme pattern is similar to that observed in normal liver [30], which may explain the insensitivity of this organ to the cell killing effects of the alkylating agents. The change in isoenzyme pattern is consistent with the low K_m phosphodiesterase being a target for the tumour inhibitory properties of these agents. Since there would appear to be no structural similarities between cyclic AMP and the majority of alkylating drugs commonly employed in antitumour therapy, the possibility that these agents react with a specific substrate site on the phosphodiesterase can probably be discounted. Assuming there is no selectivity of alkylation of any particular site in the cell, one way to overcome a lethal alkylation would be to reduce the number of such susceptible sites.

The decrease in enzymic activity of the low K_m phosphodiesterase observed in the resistant tumours could be due to a decreased synthesis of enzyme, an increase in degradation of enzyme or its conversion from an active to an inactive form. Any one of these mechanisms may operate in the present situation. Some evidence for a change in the tertiary structure of the enzyme in the resistant Walker carcinoma is presented in the following paper. The lowering of the activity of the low K_m forms of the phosphodiesterase in the resistant Walker tumours does not appear to be caused by the presence of inhibitors that can readily be dissociated from the enzyme, since mixing either heat inactivated, or non-heat inactivated, sonicated cell suspensions of either of the resistant sublines with sonicated cell suspensions of the sensitive line did not reduce the activity of either the high or the low affinity form of the enzyme.

The sub-cellular distribution of an enzyme may play a role in its inactivation by alkylating agents, particularly any association it may have with the plasma membrane. The chloroethyl group of aromatic bis-2-chloroethylamines binds to the surface of the cell [33] presumably by virtue of its fat solubility. Although the rate of alkylation by a compound reacting by an S_N1 mechanism would be expected to be reduced in such a medium of low dielectric constant, preferential concentration may outweigh such a reduced reactivity. The low K_m negatively cooperative phosphodiesterase in chicken embryo fibroblasts co-purifics with the plasma membrane [34]. In the present investigation while most of the high K_m phosphodiesterase was found in the 100,000 *g* supernatant fraction, only 50 per cent of the low K_m activity appeared to be associated with the cytosol. This does not rule out preferential adsorption of the low K_m form of the enzyme to the particulate fraction. Thompson and Appleman [29] also found that the low K_m form of the phosphodiesterase in rat brain tissue is particulate, whereas the high K_m form is not. This form in conjunction with adenyl cyclase could contribute to control of the availability of cyclic AMP necessary for the regulation of various metabolic functions. Although no detailed investigation of sub-cellular distribution of the enzyme has been attempted in the present investigation, there appeared to be no difference in distribution between supernatant and particulate fractions in the sensitive and resistant Walker carcinoma cells. Thus the shift in isoenzyme pattern in the resistant tumours is not due to decrease in the concentration of the low K_m form of the phosphodiesterase in the 100,000 *g* supernatant.

These results suggest that an investigation of the kinetics of the cyclic nucleotide phosphodiesterase in human cancers may show why some tumours are highly responsive to chemotherapy by alkylating agents while others are not.

Acknowledgements—The authors wish to thank Professor L. Young for his interest and Dr. B. Gillham for his assistance with the kinetic plots and Dr. T. A. Connors for providing the experimental animal tumours. M. J. Tisdale wishes to acknowledge the receipt of a research grant from the Cancer Research Campaign.

REFERENCES

1. G. A. Robison, R. W. Butcher and E. W. Sutherland, *Cyclic AMP*. Academic Press, New York (1971).
2. J. Otten, G. S. Johnson and I. Pastan, *Biochem. biophys. Res. Commun.* **44,** 1192 (1971).
3. A. Rein, R. A. Carchman, G. S. Johnson and I. Pastan, *Biochem. biophys. Res. Commun.* **52,** 899 (1973).
4. K. Raska, Jr. *Biochem. biophys. Res. Commun.* **50,** 35 (1973).
5. A. J. T. Millis, G. Forrest and D. A. Pious, *Biochem. biophys. Res. Commun.* **49,** 1645 (1972).
6. J. R. Sheppard and D. M. Prescott, *Expl Cell Res.* **75,** 293 (1972).
7. M. M. Burger, B. M. Bombik, B. McL. Breckenridge and J. R. Sheppard, *Nature New Biol.* **239,** 161 (1972).
8. R. Lim and K. Mitsunobu, *Life Sci.* **11,** 1063 (1972).
9. R. V. Wijk, W. D. Wicks, M. M. Bevers and J. V. Rijn, *Cancer Res.* **33,** 1331 (1973).
10. P. V. Hauschka, L. P. Everhart and R. W. Rubin, *Proc. natn. Acad. Sci. U.S.A.* **69,** 3542 (1972).
11. K. N. Prasad and J. R. Sheppard, *Expl Cell Res.* **73,** 436 (1972).
12. D. Webb, W. Braun and D. J. Plescia, *Cancer Res.* **32,** 1814 (1972).
13. R. Keller, *Life Sci.* **11,** 485 (1972).
14. P. K. Reddi and S. M. Constantinides, *Nature, Lond.* **238,** 286 (1972).
15. T. R. Russell, W. L. Terasaki and M. M. Appleman, *J. biol. Chem.* **248,** 1334 (1973).
16. M. S. Amer and R. F. Mayol, *Biochim. biophys. Acta* **309,** 149 (1973).
17. E. Monn and R. O. Christiansen, *Science, N.Y.* **173,** 540 (1971).
18. A. L. Pichard, J. Hanoune and J. C. Kaplin, *Biochim. biophys. Acta* **315,** 370 (1973).
19. B. J. Phillips, *Biochem. Pharmac.* **23,** 131 (1974).
20. W. Boyle, *Transplantation* **6,** 761 (1968).
21. W. J. Rutten, J. J. H. H. M. De Pont and S. L. Bonting, *Biochim. biophys. Acta* **274,** 201 (1972).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
23. B. H. J. Hofstee, *Science, N.Y.* **116,** 329 (1952).
24. A. Levitzki and D. E. Koshland Jr, *Proc. natn. Acad. Sci. U.S.A.* **62,** 1121 (1969).
25. G. N. Wilkinson, *Biochem. J.* **80,** 324 (1961).
26. D. H. Melzack, PhD Thesis Univ. of London pp 93 (1973).
27. J. A. Double, PhD Thesis Univ. of London pp 151 (1969).
28. J. R. Hare, Unpublished results.
29. W. J. Thompson and M. M. Appleman, *Biochemistry, N.Y.* **10,** 311 (1971).
30. J. F. Clark, H. P. Morris and G. Weber, *Cancer Res.* **33,** 356 (1973).
31. J. C. Kaplan, A. L. Pichard, M. H. Laudat and P. Laudat, *Biochem. biophys. Res. Commun.* **51,** 1008 (1973).
32. M. D'Armiento, G. S. Johnson and I. Pastan, *Proc. natn. Acad. Sci. U.S.A.* **69,** 459 (1972).
33. J. H. Linford, W. Hrynuik and L. G. Israels, *Biochem. Pharmac.* **18,** 2723 (1969).
34. T. Rusell and I. Pastan, *J. biol. Chem.* **248,** 5835 (1973).