INHIBITION OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE PHOSPHODIESTERASE FROM WALKER CARCINOMA BY ASCORBIC AND DEHYDROASCORBIC ACIDS

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SUMMARY

Both ascorbic and dehydroascorbic acids were found to be reversible inhibitors of both the high and low affinity forms of the cyclic adenosine 3',5'-monophosphate phosphodiesterase from Walker carcinoma. In contrast only the high affinity form of the enzyme from rat liver was inhibited by dehydroascorbic acid.

INTRODUCTION

Dehydroascorbic acid has been postulated as being involved in the modulation of mitotic activity in both plant and animal cells (1). The antitumour properties of dehydroascorbic acid (2) support its role as a growth inhibitor as do the observations that levels of ascorbate are depleted in neoplastic cells (3) and that the ascorbic acid requirements are abnormally high in cancer patients(4).

cAMP* is thought to be the intracellular mediator by which eukaryotic cells control their rate of growth. Mitotic cells have a very much reduced level of this nucleotide compared with the two G phases (5,6) and addition of cAMP causes growth inhibition both *in vitro* (7,8) and *in vivo* (9,10). The mechanism by which dehydroascorbic acid causes an inhibition of mitosis is unknown, although as an electron acceptor it accords with Szent-Gyorgyi's theory (11) of the control of cell division. Lewin has postulated that the ascorbate system is involved in the formation of cAMP through its promotion of noraderenaline biosynthesis (12).

A possibility thus exists that some of the biochemical effects of both

* Abbreviation for cyclic adenosine 3',5'-monophosphate.

ascorbic and dehydroascorbic acids are mediated through the cAMP system. The level of cAMP in a cell is regulated by its rate of synthesis by adenylate cyclase, its rate of breakdown by cyclic nucleotide phosphodiesterase and its rate of loss to the exterior. In the present communication the effect of ascorbate and dehydroascorbate on the cyclic adenosine 3',5'-monophosphate phosphodiesterase (adenosine 3',5'-monophosphate phosphohydrolase, EC 3.1.4.17) of Walker carcinoma and rat liver has been investigated.

MATERIALS

8- [^{3}H] cAMP (sp.act. 27.5 Ci/mmole) was purchased from the Radiochemical Centre, Amersham and unlabelled cAMP from BDH Chemicals Ltd., Poole, Dorset. Scintillation fluid NE 233 was obtained from Nuclear Enterprises Ltd., Edinburgh.

METHODS Preparation of adenosine 3',5'-monophosphate phosphodiesterase

Walker carcinoma was obtained 7 days after i.p. implantation into Wistar male rats, and liver was obtained from the same animals. Animals were killed by cervical dislocation and the tumour cells were removed in 0.9% NaCl, washed several times with 0.016M Tris-HCl, pH 7.2, containing 7.5g of NH_4Cl/l (13), to remove blood, and finally with saline. The cells were then suspended in cold 0.25M sucrose and treated with a 20-Kc MSE sonic oscillator.

Liver tissue was removed immediately after the death of the animal and rinsed in cold 0.25M sucrose. A portion (1g) was blotted, minced and homogenized in 9 vol of 0.25M sucrose in a glass-teflon homogenizer. Both tissues were centrifuged at 100,000 x g for 1 hr at 4°C and the clear supernatant liquid, excluding the lipid layer, was used in the assay of phosphodiesterase.

Enzyme assay

Phosphodiesterase activity was determined as described previously (14). Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as standard. All assays were carried out at 10% or less of the total reaction in order to be in the linear portion of the enzyme assay. Under these conditions the rate of cAMP hydrolysis was proportional to the amount of enzyme and to elapsed time. Data are expressed as nmoles of [^{3}H] cAMP hydrolysed/min/mg protein.

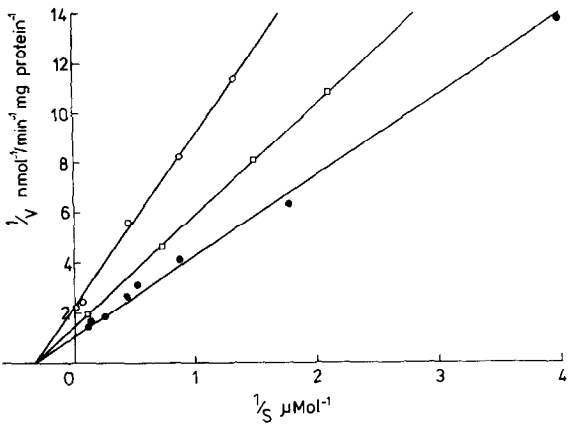

Fig. 1. Double reciprocal plot of the initial velocity of cAMP phosphodiesterase versus cAMP concentration at a constant ascorbate level. The ascorbic acid concentrations used were: ●—● none, ▽—▽ 4.6mM and ○—○ 9.75mM. The concentration of cAMP ranged from 0.2 to 18 uM.

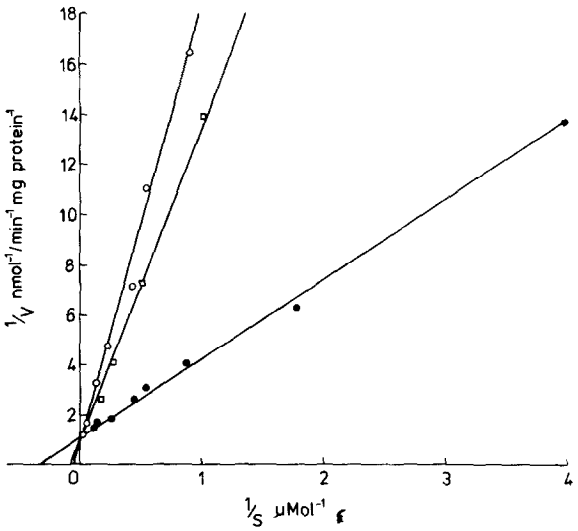

Fig. 2. Inhibition of cAMP hydrolysis by dehyroascorbic acid. The concentrations of dehydroascorbate were: ●—● none, ▽—▽ 6.71mM and ○—○ 9.94mM. Substrate concentration ranged from 0.2 to 18 uM.

RESULTS

The cAMP phosphodiesterase from Walker carcinoma behaves kinetically as if two separate activities exist, one with a low affinity for the substrate (Km 82.5µM) and the other with a high affinity (Km2.3µM). This suggests either

the presence of two enzyme activities or a negatively co-operative control system (16). Lineweaver - Burk plots of $^{1}/_{v}$ versus $^{1}/_{S}$ for inhibition of the low Km form of this enzyme by ascorbic and dehydroascorbic acid are shown in Figs 1 and 2 respectively. Thus for ascorbic acid the type of inhibition appears to be non-competitive with an apparent inhibitor constant Ki, 3.75×10^{-3}M, whilst for dehydroascorbic acid inhibition seems to be of the competitive type with Ki 1.95×10^{-3}M. These values of the inhibitor constant are comparable to that for theophylline (Ki 2.35×10^{-3}M). When inhibition of the high Km form of the enzyme was investigated, inhibition by both ascorbic and dehydroascorbic acid appeared to be of the competitive type with Ki values of 9×10^{-4}M and 1.43×10^{-3}M respectively.

In an experiment to determine if the inhibition of the cAMP phosphodiesterase by dehydroascorbic acid was reversible, a sample of the enzyme was divided into four parts. To two of these sufficient dehydroascorbic acid was added to achieve a final concentration of 10^{-2}M, and to each of the other two was added an equal volume of buffer. One half of both the treated and untreated enzymes was stored at 4^{o}C for 4 days, whereas the other half of each pair was dialysed, with frequent changes of buffer, for the same period. The enzyme solutions were then diluted to the appropriate protein concentrations and the enzyme activity was measured at 1,000μM cAMP for the high Km activity and 3.3μM for the low Km form. The nondialysed dehydroascorbic acid - treated enzyme was inhibited 30% at the high substrate concentration and 50% at the low substrate concentration. However, the corresponding dialysed sample was inhibited only 2% at the high substrate concentration and 5% at the low substrate concentration, thus showing that inhibition of cAMP phosphodiesterase by dehydroascorbic acid was reversible. Dialysis for the same period did not affect the untreated phosphodiesterase.

Rat liver also contains a high and a low Km cAMP phosphodiesterase activity. Only the low Km form (Km 5.56μM) was inhibited by treatment with dehydroascorbic acid. Inhibition appeared to be of the non-competitive type with Ki 4.44×10^{-3}M.

DISCUSSION

Both ascorbic and dehydroascorbic acids are reversible inhibitors of the cAMP phosphodiesterase from Walker carcinoma. Ascorbic acid differs from dehydroascorbic acid in being a non-competitive inhibitor of the low Km form of the enzyme and a competitive inhibitor of the high Km form, whilst dehydroascorbic acid is a competitive inhibitor of both forms of the enzyme. Theophylline has also been reported to show non-competitive inhibition at low substrate concentrations and competitive inhibition at high substrate concentrations of the phsophodiesterase from Novikoff rat hepatoma, mouse L and HeLa cells (17). The molecular basis for the different responses of the two enzymes to inhibitors is not known. The cAMP phosphodiesterase of rat liver differs from that of the Walker carcinoma in being inhibited by dehydroascorbic acid only at low substrate concentrations. Also the type of inhibition is non-competitive.

Although the inhibitor constants for both ascorbic and dehydroascorbic acids are high they are comparable to the values for theophylline. Furthermore the ascorbic acid contents of various tissues in human adults are of the same order of magnitude (1mM) (18). Thus one can speculate that ascorbic acid could participate in the regulation of intracellular cAMP levels through inhibitory action on phosphodiesterase.

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REFERENCES

1. Edgar, J.A. (1970) Nature 227, 24-26
2. Lührs, W. and Bacigalupo (1959) J. Egypt. Med. Ass. 42, 278-281
3. Waldo, A.L. and Zipf, R.E. (1955) Cancer 8, 187-190
4. Minor, A.H. and Ramirez, M.A. (1942) Cancer Res. 2, 509-513
5. Sheppard, J.R. and Prescott, D.M. (1972) Exptl. Cell Res. 75, 293-295
6. Millis, A.J.T., Forrest, G.A. and Pious, D.A. (1974) Exptl. Cell Res. 83, 335-343
7. Oler, A., Iannaccone, P.M. and Gordon, G.B. (1973) In vitro 9, 35-38
8. Heidrick, M.L. and Ryan, W.L. (1970) Cancer Res. 30, 376-378
9. Cho-Chung, Y.S. and Gullino, P.M. (1974) Science 183, 87-88
10. Keller, R. (1972) Life Sci. 11, 485-491

11 Szent Györgyi, A. (1968) Science 161, 988-990
12. Lewin, S. (1974) Biochem. Soc. Trans. 2, 400-403
13. Boyle, W. (1968) Transplantation 6, 761-764
14. Tisdale, M.J. and Phillips, B.J. (1974) Biochem. Pharmacol. in press.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
16. Levitzki, A. and Koshland, D.E. (1969) Proc. Nat. Acad. Sci. USA 62, 1121-1128
17. Schröder, J. and Plagemann, P.G.W. (1972) Cancer Res. 32, 1082-1087
18. Kirk, J.E. (1962) Vitams. Horm. 20, 67-139