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ENZYME ACTIVATED ANTI-TUMOUR AGENTS—II. THE ROLE OF ALKALINE PHOSPHATASE IN THE RELEASE OF *p*-HYDROXYANILINE MUSTARD FROM ITS PHOSPHATE CONJUGATE IN CELLS IN CULTURE

P. WORKMAN, C. R. BALL and J. A. DOUBLE

Department of Experimental Pathology and Cancer Research, University of Leeds

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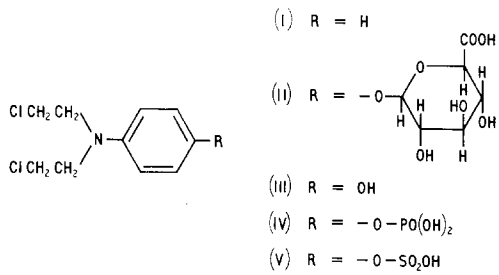
Abstract—The enzymes involved in the activation of the *O*-phosphate of *p*-hydroxyaniline mustard in cells in culture have been studied. The results are consistent with the view that the cytotoxic action of the drug against HeLa cells is dependent on the activity of alkaline phosphatase, of the carcinoplacental type, located in the cell membrane. The drug was shown to be a substrate for each of the isoenzymes of HeLa alkaline phosphatase. The importance of extracellular phosphatase in drug activation was also established.

The mouse plasma cell tumour Adj-PC5, normally resistant to alkylating agent therapy, was shown by Connors and Whisson [1, 2] to be extremely sensitive to aniline mustard (I). Complete regression of large tumours was obtained with a single treatment. A correlation was subsequently established between aniline



Scheme 1.

mustard sensitivity and tumour glucuronidase levels [3, 4] and it was suggested that the activity of the drug was due to metabolism to the *O*-glucuronide (AMGl, II) in the liver and subsequent hydrolysis in the high glucuronidase environment of the tumour liberating the potently cytotoxic *p*-hydroxyaniline mustard (AMOH, III) selectively in the tumour. Metabolism of AM to AMGl was later confirmed in the rat [5]. Preliminary clinical trials indicate that there may be a correlation between response to aniline mustard and glucuronidase activity in prostate tumours [6]. A logical extension of this work was the synthesis of AMGl and of the *O*-phosphate (AMPh, IV) and *O*-sulphate (AMSu, V) of AMOH as possible selective agents against tumours containing high levels of glucuronidase, phosphatase and sulphatase respectively [7]. Further work in this laboratory showed that AMPh and AMGl are deconjugated by the appropriate enzymes *in vitro* whereas AMSu is refractory to hydrolysis by arylsulphatases [8, 9].

AMPh, in common with the other conjugates, will be in the ionised form at physiological pH and consequently will be unable to penetrate cell membranes. Prior deconjugation of the drug by a phosphatase, located extracellularly or in the tumour, must occur in order to release the uncharged, chemically reactive and highly toxic AMOH. AMPh therefore possesses latent activity [10] and deconjugation results in drug activation. Therefore selectivity could be achieved by the preferential liberation of AMOH in tumours containing high phosphatase levels. If AMPh is to be used rationally to treat such tumours it is necessary to determine (1) the relative importance of acid and alkaline phosphatase activity and (2) the importance of particular isoenzymes, in drug activation. In this paper a more detailed investigation of the action of AMPh in HeLa cells in culture is described, which has been previously reported in abstract form [11].

HeLa cells contain alkaline phosphatase of the carcino-placental (or Regan) type [12, 13] and evidence was obtained that this enzyme was responsible for drug activation by the cells.

MATERIALS AND METHODS

Cell culture methods

HeLa cells were routinely cultured in spinner flasks at 0·5–2 × 10^5 cells/ml in Minimal essential Eagle's medium (MEM) for suspension culture, supplemented with 7% foetal bovine serum, penicillin and streptomycin (all reagents obtained from Flow Laboratories Ltd.). Monolayer cultures for colony survival assays were plated in MEM containing 15% foetal bovine serum and antibiotics. Cells were plated at suitable concentrations and treated in monolayer by replacing the medium with drug-containing medium. In certain experiments this medium had phosphatase activity destroyed by heat inactivation at 70 for 5 min and in others L-phenylalanine (10 mM) added as well. In all cases drug was added to the medium in dimethyl sulphoxide (1% v/v). After 1 hr at 37 the drug was removed by careful washing in normal medium, and normal medium then added for the final incubation at 37 to produce colonies. After an appropriate interval the colonies were fixed, stained and counted.

Drugs

The alkylating agents used in this study were a gift from the Chester Beatty Research Institute to whom we wish to express our sincere thanks. AMPh (*p*-di-2-chloroethylaminophenyl phosphate) was used as the dicyclohexylamine salt. AMOH (*p*-di-2-chloroethylaminophenol) was used as the hydrochloride. Drug solutions were used immediately to minimise mustard hydrolysis.

Enzyme methods

Enzyme preparation. Whole homogenates of suspension culture cells were prepared by centrifugation of cell suspensions (800 rev/min, 5 min), resuspending the cells and washing twice in NaCl solution (0·9%), resuspending in distilled water and homogenisation with an Ultra-Turrax homogeniser. Whole homogenates of monolayer cultured cells were prepared in the same way after removal of the cells from the monolayer by scraping.

For electrophoretic studies HeLa alkaline phosphatase was partially purified by a modification of the method of Morton [14]. Washed cells were suspended in Tris HCl buffer (0·2 M, pH 7·5) to give a 20% homogenate (wet wt/vol). The cells were broken by treatment with an ultrasonic disintegrator (Kerry's Ultrasonics Ltd., 3 × 10 sec treatments, 100 w). *N*-butanol was added (20% v/v), the homogenate was shaken thoroughly, centrifuged (2500 *g*, 45 min) and the lower aqueous layer removed.

Enzyme assays. Enzyme assays were normally carried out in a total volume of 0·6 ml containing equal parts of enzyme solution, buffer and substrate, at 37°. (i) Hydrolysis of the standard substrate *p*-nitrophenylphosphate (PNPP, Sigma Chemical Co. Ltd.) was measured by the release of *p*-nitrophenol. The reaction was stopped by the addition of 0·2% sodium dodecyl sulphate in 0·2 M glycine buffer, pH 12 (4·4 ml) and the extinction at 412 nm was determined against an appropriate blank with reference to a *p*-nitrophenol standard. (ii) Hydrolysis of AMPh was measured by the release of inorganic phosphate using a modification of the method of Fisk and Subba-Row [15]. The reaction was stopped by the addition of 5 N sulphuric acid (0·6 ml), followed by 2·5% ammonium molybdate solution (0·6 ml), reducing agent (0·1 ml), and water (5·0 ml). The reducing agent (100 ml) contained 1-amino-2-napthol-4-sulphonic acid (0·16 g), sodium bisulphate (0·8 g) and sodium sulphite (1·6 g). The extinction at 660 nm was determined against an appropriate blank and referred to a calibration curve of KH_2PO_4 in water. Incubation times were kept to a minimum to reduce complication by mustard hydrolysis.

The details of experimental conditions for assay of the various enzymes studied are given below.

1. *Acid phosphatase.* The acid phosphatase activity of HeLa cells, and foetal bovine serum was assayed in acetate buffer (66 mM, pH 4·9).

2. *Alkaline phosphatase.* Alkaline phosphatase activity was assayed in carbonate-bicarbonate buffer (33 mM); HeLa alkaline phosphatase at pH 10·6, foetal bovine serum at pH 10·1.

3. *'Neutral phosphatase'.* The term is used here to denote the activity observed when the assay was performed in Tris HCl buffer (66 mM) at pH 7·4, the pH of the cell-culture medium.

K_m *determination*

1. *Acid phosphatases.* The substrate concentration was varied over the range 0·125–2 mM for both PNPP and AMPh.

2. *Alkaline phosphatases.* The substrate concentration for PNPP was varied over the range 0·5–15 mM, that for AMPh over the range 0·125–2 mM, the upper limit of which was determined by the poor solubility of the drug in water.

K_m values were determined from double reciprocal plots of initial velocity against substrate concentration by the method of Lineweaver and Burk [16]. The line of best fit was computed by the method of least squares regression analysis using a Multi-8 computer.

Inhibition by L*-phenylalanine*

L-phenylalanine (LPA, Sigma Chemical Co. Ltd.) was dissolved in the appropriate buffer to give the required concentration and pH for the assay. Normal assay procedure was then carried out. The enzyme activities in the presence of LPA were compared to controls containing no inhibitor and the percentage activity remaining was determined.

The kinetic nature of the inhibition of LPA on HeLa alkaline phosphatase was studied by varying the substrate (PNPP) concentration (2–15 mM) at fixed inhibitor concentrations (0, 2·5, 5 mM). The results were analysed in the form of a double reciprocal plot of initial velocity against substrate concentration at the three inhibitor concentrations.

The inhibition data was also analysed by the method of Taketa and Pogell [17, 18]. A plot of log [control velocity − inhibited velocity/inhibited velocity] against log [LPA concentration] allows a calculation of the number of inhibitor molecules combining with one molecule of enzyme and of the dissociation constant of the inhibitor, K_i.

pH *Dependence of enzyme activity*

The effect of pH on the activity of HeLa alkaline phosphatase was studied over the pH range 9–12 in carbonate bicarbonate buffer at a PNPP concentration of 15 mM.

Enzyme specific activities

Total enzyme activity was determined, under the conditions described for particular enzymes, using PNPP (15 mM) as substrate. Protein concentration was estimated by the method of Lowry *et al.* [19], using bovine serum albumin (Sigma Chemical Co. Ltd.) as standard. Enzyme activities are expressed as μmoles/min/mg protein for HeLa enzymes and as μmoles/min/l for foetal bovine serum enzymes in cell culture medium.

Electrophoresis and isoenzyme preparation

The isoenzymes of HeLa cell alkaline phosphatase were separated by polyacrylamide gel disc electrophoresis based on the method of Davis [20], as used by Spencer [21–23]. Small-pore gels (105-mm long; 5 mM dia) were 5% polyacrylamide; large-pore (spacer) gels (10 mM; 5-mm dia) were 2·5% polyacrylamide. The sample (50–100 μl) was applied mixed with an equal volume of sucrose solution (40% w/v) and overlayered with buffer. The reservoir buffer was Tris–glycine (0·06% Tris, 0·29% glycine, pH 8·3) and

the upper solution contained 1 ml bromophenol blue (BDH Chemicals Ltd., 0·001%) as a marker. Electrophoresis was performed using an analytical polyacrylamide electrophoresis apparatus (Shandon Scientific Co. Ltd.), at a current of 5 mA per gel supplied by a Vokam power supply, for approximately 90 min according to the migration of the dye front, in a cool air stream or refrigerator.

Gels were stained for protein using 0·05% amido black (Edward Gurr Ltd.) in 7% acetic acid and stored in 7% acetic acid. A variety of techniques were used for the detection of alkaline phosphatase activity on the gels.

(i) *Diazo coupling technique.* Gels were incubated with a pre-filtered solution containing sodium β-naphthyl phosphate (Sigma Chemical Co. Ltd. 1 mg/ml) and Fast Blue B (tetrazotised *o*-dianisidine), Sigma Chemical Co. Ltd. 1 mg/ml) in 0·2 M Tris–HCl, pH 8·7. A red colour is produced in regions of enzyme activity. Gels were washed and stored in 7% acetic acid.

(ii) *Lead precipitation technique.* The method used was based on the modification of Gomori's [24] original procedure by Allen and Hyncik [25]. Gels were incubated with a solution containing 15 mM calcium chloride, 33 mM Tris, and either the normal substrate sodium β-glycerophosphate (Sigma Chemical Co. Ltd., 50 mM) or AMPh (saturated solution), adjusted to pH 9·5, for 15 min and 30 min respectively. Gels were then rapidly rinsed in distilled water and incubated for 30 min in a solution containing 3 mM lead nitrate, 80 mM Tris, adjusted to pH 7 with 1 N HNO_3. After thorough rinsing in distilled water the gels were incubated in 5% ammonium sulphide for 2 min, resulting in a precipitation of lead sulphide in regions of enzyme activity. All incubations were at room temperature. Gels were washed thoroughly and stored in 7% acetic acid.

(iii) p-*Nitrophenol phosphate method.* When a rapid non-permanent stain was required the gels were incubated in with 30 mM PNPP in 50 mM carbonate bicarbonate buffer pH 10·6.

The isoenzymes were prepared by slicing replicate gels with reference to a gel stained for enzyme activity with one of the above techniques. The gel slices were broken and the enzyme eluted with distilled water. The enzyme preparations were dialysed overnight against two changes of distilled water.

To investigate the effect of neuraminidase on the electrophoretic mobility of HeLa alkaline phosphatases 1 vol partially purified enzyme was incubated with 0·4 vol neuraminidase (*N*-acetylneuraminate glycohydrolase from *Vibrio cholerae*, BDH Chemical Ltd.; activity 500 units/ml), in 150 mM Tris–HCl buffer, pH 7·5, for 18 hr at 37°.

RESULTS

Cell survival experiments. The cytotoxicity of AMPh towards HeLa cells under various incubation conditions was investigated. Cell survival was determined by the ability of cells to form colonies at varying drug concentrations. The line of best fit was calculated by linear regression analysis. The results of the experiments are shown in Fig. 1 and the data is summarised in Table 1 in the form of the D_{37} (calculated dose required to reduce survival to 37 per cent of the control). The D_{37} for AMOH is included for comparison.

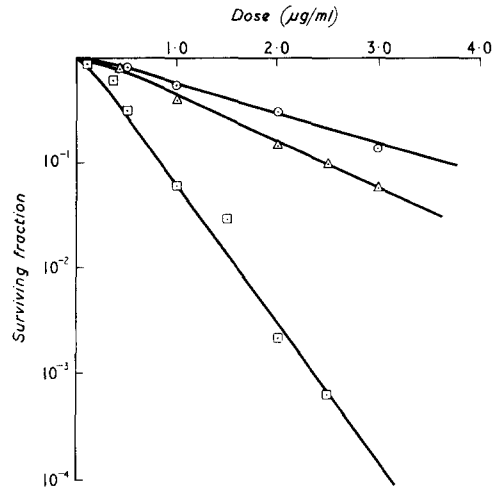


Fig. 1. HeLa cell survival curves. AMPh was incubated with cells in full medium (□), 'heat inactivated' medium (△), and 'heat inactivated' medium containing 10 mM LPA (○).

AMPh exhibits maximum effect when incubated with cells in normal medium, which contains considerable phosphatase activity (alkaline phosphatase 16·7 μmoles/min/l; 'neutral phosphatase' 0·8 μmoles/min/l; and acid phosphatase 1·2 μmoles/min/l). Kinetic experiments showed that AMPh is a good substrate for both acid and alkaline phosphatase (K_m = 0·15 mM, 0·50 mM respectively) from foetal bovine serum and it is clear that extracellular phosphatase activity is important in drug activation. Thus when this activity is destroyed prior to drug treatment, the observed D_{37} is much higher. Although the toxic effect of AMPh is much reduced in the absence of extracellular phosphatases cell killing is still observed, establishing the ability of the cells to activate the drug. This was to be expected from the finding that AMPh was a good substrate for both alkaline and acid phosphatase in whole homogenates of HeLa cells (see next section).

When the incubation was carried out in heat-inactivated medium containing LPA the D_{37} for the HeLa cell line was increased by about 50%. LPA is an inhibitor of HeLa alkaline phosphatase *in vitro* [12] but was found to have little effect on HeLa acid phosphatase.

AMPh as a substrate for HeLa phosphatases. Table 2 shows the K_m values for AMPh and the standard substrate PNPP for the acid and alkaline phosphatase of HeLa cell whole homogenates (monolayer cultured cells). AMPh is a good substrate for all the enzymes.

Table 1. Summary of the cell survival experiments

Treatment	D_{37} μg ml
AMOH in normal medium	0·20
AMPh in normal medium	0·43
AMPh in 'heat inactivated' medium	1·06
AMPh in 'heat inactivated' medium + LPA	1·65

Table 2. K_m values for acid and alkaline phosphatase of HeLa cell homogenate

	K_m AMPh (mM)	K_m PNPP (mM)
HeLa acid phosphatase	0·2	0·2
HeLa alkaline phosphatase	1·0	3·5

In order to elucidate the enzyme or enzymes responsible for drug activation by the cells the inhibitory properties of LPA toward the various phosphatases were studied.

Inhibition by LPA. The effect of LPA on the activity of alkaline, acid and 'neutral' phosphatases from HeLa cells is shown in Figure 2. Clearly alkaline phosphatase is inhibited quite strongly (80% inhibition), 'neutral phosphatase' less strongly (35% inhibition) and acid phosphatase not at all, by 10 mM LPA. LPA is known to inhibit several alkaline phosphatases, including HeLa, in a pH-dependent manner [26, 18], but it is not known to inhibit acid phosphatases. Kinetic analysis using double reciprocal plots confirmed that LPA was acting as an uncompetitive inhibitor of HeLa alkaline phosphatase (K_i = 2·5 mM). Treatment of the data by the method of Taketa and Pogell gave a similar K_i value and confirmed that one molecule of LPA binds per molecule of enzyme [18]. The inhibition of 'neutral' phosphatase activity is therefore likely to be due to inhibition of alkaline phosphatase activity, particularly in view of the far greater activity of this enzyme over acid phosphatase in the cell homogenate (Table 3).

Since alkaline phosphatase appeared to be important in the mechanism of action of AMPh it was necessary to establish if particular isoenzymes were involved. The results of this study are reported in the next section.

Isoenzyme studies. The electrophoretic variants (isoenzymes) of HeLa alkaline phosphatase were separated by polyacrylamide gel electrophoresis. The observed electrophoretic pattern is shown in Figure 3. The electrophoretogram from cells grown in suspension culture shows four well separated bands and differs from that obtained from cells in monolayer in which the fastest-running anodic band (isoenzyme 1) is absent. These results confirm those obtained by Spencer and Macrae [23]. An identical electrophoretic pattern to that shown in Figure 3(i) was obtained with the standard substrates, sodium β-naphthylphosphate (diazo coupling technique), sodium β-glycerophosphate (lead precipitation technique), PNPP, and also with AMPh. Thus AMPh is a substrate for all 4 isoenzymes. To confirm this the isoenzymes were purified by slicing the gels and eluting the enzyme. The contributions of the isoenzymes to the total activity were: 1: 3%, 2: 12%, 3: 39%, 4: 46%. Some kinetic properties were studied in an attempt to characterise the isoenzymes. The results are summarised in Table 4, together with data for whole homogenate. The kinetic properties observed for total alkaline phosphatase in whole homogenates of monolayer and suspension-cultured cells were identical.

AMPh is clearly a good substrate for all four isoenzymes. Since the loss of isoenzyme 1 from HeLa cells of monolayer and suspension-cultured cells were takes several days [23], each of these enzymes could be responsible for drug activation.

The overall electrophoretic pattern is of the carcino-placental type [27, 12] and the kinetic properties of (i) high pH optimum (10·6) and (ii) inhibition by LPA exhibited by each of the isoenzymes is consistent with the view that all four isoenzymes are of this type. The kinetic properties of the isoenzymes are similar which is in agreement with the general behaviour of alkaline phosphatase isoenzymes within other tis-

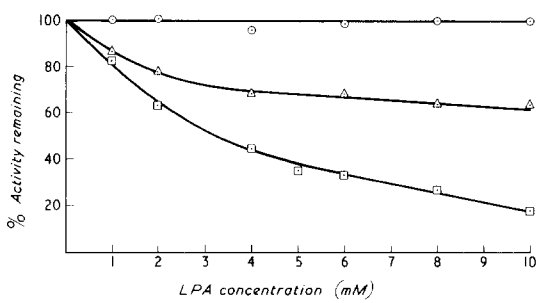


Fig. 2. Inhibition of phosphatase activity in HeLa cell homogenate by L-phenylalanine at pH 4·9 (○), pH 7·4 (△) and pH 10·6 (□).

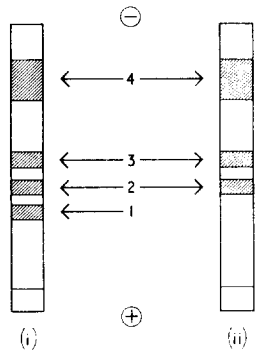


Fig. 3. Diagram of alkaline phosphatase activity on polyacrylamide gels of HeLa cell homogenate (i) suspension culture, (ii) monolayer culture.

Table 3. Specific activity of HeLa cell (monolayer) phosphatases

	Sp. act. (μmoles/min/mg ± S.E.M.)
Acid phosphatase	0·104 ± 0·007
Alkaline phosphatase	4·06 ± 0·18
'Neutral phosphatase'	0·091 ± 0·006

Table 4. Summary of the kinetic analysis of the isoenzymes of HeLa cell (suspension culture) alkaline phosphatase

	Enzyme				
	Isoenzyme 1	Isoenzyme 2	Isoenzyme 3	Isoenzyme 4	Whole homogenate
K_m PNPP (mM)	1·0	3·5	3·0	3·5	3·5
K_m AMPh (mM)	1·6	1·4	0·9	0·8	1·0
pH optimum	10·6	10·6	10·6	10·6	10·6
Inhibition by LPA (5 mM), %	60	65	65	65	65

sues [28]. Treatment with neuraminidase was found to reduce the mobility of the three faster running bands, and it is likely that the isoenzymes differ mainly in their sialic acid content [21]. Isoenzyme 1 was less sensitive than the other three to inhibition by LPA.

DISCUSSION

The primary object of this work was to determine the factors likely to be involved in the action of AMPh *in vivo*. AMPh is a strongly polar compound which would be fully ionised under physiological conditions and would therefore be unlikely to penetrate the cell membrane. In contrast the dephosphorylated compound, AMOH, would be taken up by the cell. It would seem a reasonable hypothesis that the observed cell killing effect of AMPh is due to the uptake of the potently cytotoxic AMOH produced by the action of a phosphatase located extracellularly or in the cell membrane. This is illustrated schematically in Figure 4. The results reported here support this view and suggest that the enzyme involved is alkaline phosphatase.

A number of observations are consistent with this hypothesis. AMPh is extremely toxic toward HeLa cells when phosphatase activity is present in the incubation medium and the toxicity is greatly reduced by the elimination of this activity by denaturation of the foetal bovine serum prior to treatment. This observation highlights two important facts. Firstly, that extracellular phosphatases may contribute considerably to deconjugation of the drug *in vivo* (both acid and alkaline phosphatases from foetal bovine serum accept the drug as a substrate). Secondly, that in the absence of extracellular phosphatase activity deconjugation is carried out by the cells. Acid and alkaline phosphatases from HeLa cells were able to deconjugate the drug *in vitro*. When LPA is included in the incubation medium the cytotoxicity of AMPh to HeLa cells is further reduced. LPA was shown to inhibit HeLa alkaline phosphatase exclusively and has no appreciable effect on HeLa acid phosphatase. These results clearly implicate alkaline phosphatase as the enzyme responsible for deconjugating the drug.

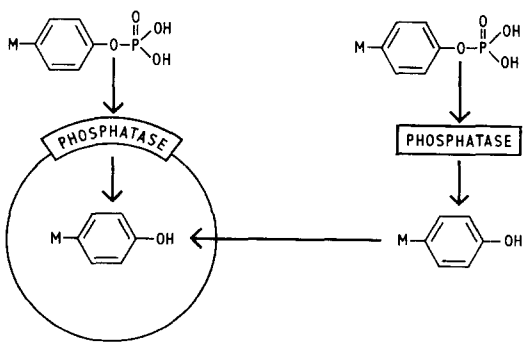


Fig. 4. Schematic representation of activation of AMPh.

This is in good agreement with the sub-cellular location of the phosphatases. Alkaline phosphatase is known to be associated, though not exclusively, with cell membranes, for example in the brush-borders of intestinal and kidney tubule epithelium where it may be involved in transport of metabolites [29]. In contrast acid phosphatase is mainly, though not exclusively, a lysosomal enzyme [30]. Cell fractionation studies have shown that a considerable proportion of HeLa cell alkaline phosphatase activity is associated with the plasma membrane [31]. Recently it was suggested that the uptake of Synkavit (a phosphorylated napthoquinol) and its tritiated analogues into cells in culture was dependent upon dephosphorylation by an alkaline phosphatase located in the plasma membrane [32, 33]. These authors showed that the uptake of the drug into HEp2 cells was reduced by inhibitors of alkaline phosphatase which was located mainly in the cell membrane. Unpublished work in this laboratory has shown that HEp2 alkaline phosphatase has similar kinetic and electrophoretic properties to the HeLa enzyme. It seems likely that a similar mechanism is operative in the activation of AMPh and Synkavit involving dephosphorylation by a carcino-placental type alkaline phosphatase at the cell membrane.

Our investigation of the kinetic properties of purified HeLa alkaline phosphatase isoenzymes showed that all the isoenzymes were of the carcinoplacental type and were able to deconjugate the drug *in vitro*. This is of possible clinical relevance in the light of recent reports of the occurrence of carcino-placental type alkaline phosphatase in neoplasms of various types [34, 35]. Tumours in which this enzyme or other membrane phosphatases are elevated, or those in which there are high levels of extracellular phosphatase in the tumour environment may be possible targets for AMPh. Tumours of the prostate, for example, often have elevated acid phosphatase. AMPh is known to be effective against the alkylating agent sensitive Adj-PC6A tumour [7] which contains elevated levels of both acid and alkaline phosphatase (unpublished results).

Several other studies have illustrated the importance of alkaline phosphatase in therapeutics. A correlation has been established between the alkaline phosphatase levels of various human cell lines and resistance of the glucose analogue 2-deoxyglucose [36]. It was suggested that the enzyme reduces the level of the active phosphorylated metabolite. A similar mechanism has been suggested for the aquirement of resistance of neoplastic cells to the 6-thiopurines and other compounds in which the phosphory-

lated metabolites are the active anti-tumour agents [37 42]. However Balogh and Szendröi [43] have reviewed the use of Honvan (diethylstilboestrol diphosphate), originally designed by Druckery and Raabe [44] as a specific agent for prostate tumours with high acid phosphatase, and concluded that prostatic phosphatase activity is probably not responssible for the clinical efficacy of this agent.

Work is in progress in this laboratory to determine the frequency with which acid and alkaline phosphatase (and also glucuronidase and sulphatases) are elevated in human tumours. Although the phosphatases are known to be elevated in a variety of human tumours [26] it remains to establish the levels required to produce a significant therapeutic advantage. Whether or not sensitivity to the drug can be correlated with total acid or alkaline phosphatase activity, or with particular isoenzymes, *in vitro*, will be investigated in a series of transplantable mouse colon tumours.

The effects of deconjugation of the drug by host phosphatases also remain to be established. The results described in this paper clearly suggest that extracellular phosphatase can contribute considerably to deconjugation. It has been suggested that Estracyt (a phosphorylated estradiol mustard, used clinically in the treatment of prostate tumours) is rapidly hydrolysed by serum phosphatases [45]. Furthermore serum phosphatases are elevated in a variety of malignant diseases (see review by Schwartz [46]). Extensive deconjugation of AMPh in the serum, or indeed in other host tissues, would obviously negate the potential selective effect of the drug. These problems will be investigated by analysis of the pharmacokinetics of AMPh and its metabolites.

The factors involved in activation of AMPh *in vitro* are clearly complex and of general relevance to chemotherapy involving phosphorylated agents. The cell culture model described in this paper has allowed the mechanisms involved at the cellular level to be determined.

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