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STUDIES ON THE KINETICS OF GLYCOSIDASES FROM CHEMICALLY-INDUCED RAT COLONIC TUMOURS AND NORMAL RAT COLON

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Summary

K_m values of β-*N*-acetylglucosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase EC 3.2.1.30), β-*N*-acetylgalactosaminidase (EC 3.2.1.53), β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) and α-L-fucosidase (α-L-fucoside fucohydrolase EC 3.2.1.51) of distal colonic tumours, induced in rats by 1,2-dimethylhydrazine, were found to be significantly different compared with the values for the enzymes of the colonic mucosa of the control and tumour-bearing animals and of the proximal colonic tumours. The inhibition kinetics data also showed a significant difference between the enzymes of the distal colon tumours and of other experimental tissues. The data on the effect of pH on enzyme kinetics (p*K* values) showed no significant difference in the catalytic groups of the active centres of enzymes from tumours and from the control colonic mucosa. Tumour β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase compared with the enzymes from other experimental tissues were found to be different in their thermal inactivation kinetics. K_m values of 14 days old foetal intestinal β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase were significantly different from the values obtained for the adult mucosal enzymes but were similar to those of the distal colonic tumour enzymes.

Introduction

Evidence that the levels of hydrolytic enzymes increase in tumours and in transformed cells in culture has been accumulating [1]. It has been reported that colonic tumours, induced in rats by 1,2-dimethylhydrazine, and the colonic mucosa of tumour-bearing animals, have elevated levels of glycosidase activi-

ties compared with the mucosa of control animals [2]. A more detailed study has shown that the increase in the tumour enzyme levels and the frequency of tumour incidence were greater in the distal than in the proximal colon [3]. Histochemically the distal colonic tumours were non-mucinous whereas tumours arising in the proximal colon were usually mucinous [4]. Because of the role of glycosidases in mucin metabolism, the present work was carried out to compare the kinetics of glycosidases of tumours and of the colonic mucosa in order to assess the enzyme increase in the neoplastic transformation.

Materials and Methods

Animals and tissues

Wistar male rats, 10—12 weeks old, were injected subcutaneously once weekly for a period of 18—22 weeks with 1,2-dimethylhydrazine dihydrochloride (DMH, 20 mg/kg body weight) in 4.0% di-sodium EDTA while the control animals were given di-sodium EDTA solution. Colonic mucosa and tumours were collected. The tissues were homogenized in ice cold saline containing 0.1% Triton X-100, by 40 strokes with a Teflon pestle in a Potter-Elvehjem homogenizer. The detailed procedure has been described previously [2].

Foetal tissue

Wistar foetuses and neonates were used. The foetal age was determined by counting the day of the finding of the vaginal plug as D_0 of pregnancy. The neonates were less than 16 h old. From foetuses the whole length of intestine and from neonates colons were removed using microdissection techniques. Tissue homogenates were prepared as described previously [2].

Estimation of glycosidases

The activities of β-*N*-acetylglucosaminidase (EC 3.2.1.30), β-*N*-acetylgalactosaminidase (EC 3.2.1.53), β-D-galactosidase (EC 3.2.1.23) and α-L-fucosidase (EC 3.2.1.51) were measured using the *p*-nitrophenyl derivatives of β-*N*-acetylglucosaminide, β-*N*-acetylgalactosaminide, β-D-galactoside and α-L-fucoside (Sigma, London) respectively. Except where indicated, 0.1 ml of tissue homogenate was incubated with 0.2 μmol of substrate and 50 μmol of citrate buffer (Sorensen citrate), pH 4.2, in a final volume of 1.0 ml. After 1 h of incubation at 37° C the reaction was terminated by the addition of 1.0 ml of ice cold 0.4 M glycine: NaOH buffer pH 10.5. The mixture was centrifuged at 5000 × *g* for 10 min and the absorbance of the released *p*-nitrophenol in the supernatant was measured at 400 nm. Controls were run with water replacing the enzyme or other reagents. The enzyme activities were calculated as μmol of *p*-nitrophenol released/h/mg of protein. *p*-Nitrophenol was used as standard.

Kinetics studies

In enzyme kinetics experiments, substrate concentrations in the final assay mixture ranged from 0.2 to 1.25 mM. In inhibition studied, aliquots of tissue homogenate were mixed with different concentrations of the inhibitors prior to the addition of the substrate solution. *N*-acetylglucosamine, *N*-acetyl-

galactosamine, *N*-acetylmannosamine, D-mannosamine, D-glucosamine, D-galactosamine, L-fucose, D-fucose, D-galactose, D-glucose (Sigma, London), *N*-acetylglucosaminolactone, *N*-acetylgalactosaminolactone, galactonolactone and fuconolactone (Koch-Light Laboratories) were used in the enzyme-inhibition studies. Aldonolactones dissolved in water by making the solution pH 2.0 with HCl were heated at 100°C for 7.5 min. The solutions were then cooled and their pH adjusted to 4.2 [5]. These solutions were prepared freshly at relatively high concentrations and diluted immediately before use.

Analysis of kinetic data

Initial-velocity data were first inspected in the form of double-reciprocal plots to determine the appropriate rate law. The corresponding rate equation was then fitted to the data according to the method described by Cleland [6] using computer programs written in Algol 60 for use in the ICL 1906A computer installation at Leeds University. Initial velocity data for varying concentrations of the substrate and inhibitor were fitted by Eqn 1 if competitive inhibition was evident, or by Eqn 2 if noncompetitive inhibition was more appropriate.

$$v = VA/[K_m(1 + I/K_{is}) + A] \quad (1)$$

$$v = VA/[K_m(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (2)$$

where S and I are the substrate and inhibitor concentrations, K_m is the Michaelis constant for S, K_{is} and K_{ii} are inhibitor constants respectively affecting the slope and intercept of double-reciprocal plots, and V is the maximum velocity. When there was doubt upon the visual inspection as to the nature of

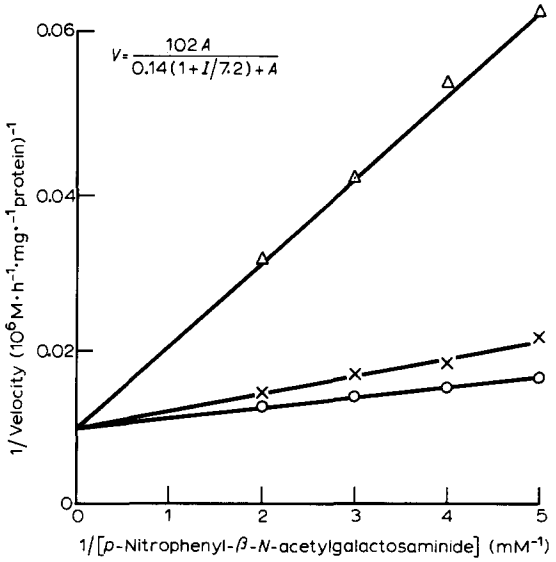

Fig. 1. Lineweaver-Burk Plots of β-*N*-acetylgalactosaminidase from colonic mucosa of tumour-bearing rats. *p*-Nitrophenyl-β-*N*-acetylgalactosaminide was used as a substrate and enzyme assays were carried out at pH 4.5 with no inhibitor (○———○) and with 5.0 μM (X———X) and 50.0 μM (△———△) of *N*-acetylgalactosaminolactone.

the inhibition, a choice was made between Eqn 1 and Eqn 2 by selecting the fit with the lower statistical variance. A diagram showing typical Lineweaver-Burk plots of a single experiment is given in Fig. 1.

Effect of pH

The effect of pH on the kinetics of glycosidases was studied using citrate buffer ranging from pH 3.5 to 5.0 except in the case of α-L-fucosidase where buffers up to pH 7.0 were used.

The dependence on pH of the values of the kinetics parameters was analysed according to Eqn 3.

$$y = \frac{Y}{1 + H/K_1 + K_2/H} \tag{3}$$

At a hydrogen ion concentration, H, the parameter has a value y, and the unknowns in the equation are Y, the pH-independent parameter, and K_1 and K_2 which are interpreted as the dissociation constants of groups in the active site of the enzyme which control the dependence of rate on pH. In the case of y being the maximum velocity, K_1 and K_2 refer to groups in the enzyme-substrate complex [7].

The computer program used in fitting the data to Eqn 3 incorporated a subroutine contained in the Nottingham Algorithms Group Library, No. E04GAA. This library is a collection of subroutines for numerical work with ICL 1906A computers, and E04GAA is based on the least-squares method of Marquardt [8]. It was necessary to develop this new program because the method of Cleland [6] proved to be unsatisfactory. A diagram showing the pH effect on the kinetics of tumour β-D-galactosidase is given in Fig. 2.

Thermostability of enzymes

Aliquots of tissue homogenates were heated at temperatures between 50 and 60° C for different time intervals and cooled prior to the enzyme assays.

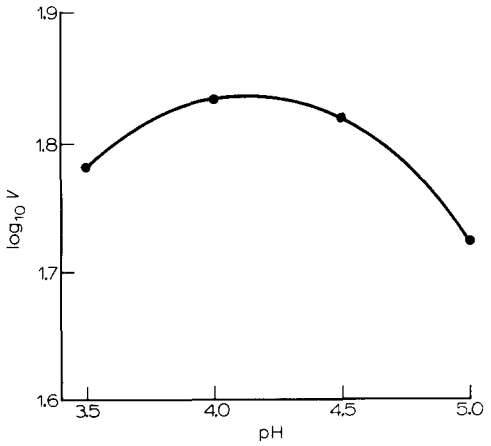

Fig. 2. Effect of pH on the kinetics of tumour β-D-galactosidase. The maximum velocity was determined as a function of pH in the presence of the competitive inhibitor galactonolactone. The solid line is a graph of Eqn 3 with the pH-independent maximum velocity 77.0 and $pK_1 = 2.91$, $pK_2 = 5.35$.

Protein determinations were made by the biuret reaction method [9]. The significance of the values was determined using Student's *t*-test.

Results

Michaelis constants (K_m) of glycosidases

The K_m values of four glycosidases for their corresponding *p*-nitrophenyl glycoside substrates (Table I) indicated no significant difference in the kinetic constants of the enzymes from the proximal colonic tumours and from the mucosa of normal and tumour-bearing animals. On the other hand, K_m values of distal colonic tumour β-*N*-acetylglucosaminidase, β-D-galactosidase and α-L-fucosidase were significantly lower ($P < 0.005$) and of β-*N*-acetylgalactosaminidase were significantly higher ($P < 0.005$) compared with the enzymes from other control and experimental tissues.

Inhibition kinetics of glycosidases

Among the inhibitors used, *N*-acetylgalactosamine, *N*-acetylglucosamine, D-mannosamine, *N*-acetylgalactosaminolactone and *N*-acetylglucosaminolactone were competitive inhibitors with respect to both *p*-nitrophenyl-*N*-acetylglucosaminide and *p*-nitrophenyl-*N*-acetylgalactosaminide as substrates. Hexosaminolactones were more effective competitive inhibitors than hexosamines and D-mannosamine (Tables II and III). β-D-Galactose and galactonolactone were competitive with respect to *p*-nitrophenyl-β-D-galactoside (Table IV). L-fucose acted competitively with respect to *p*-nitrophenyl-α-L-fucoside (Table IV) whereas fuconolactone was found to be ineffective even at very high concentrations (1.0—2.5 mM).

A comparison of K_i values for different inhibitors of β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities (Tables II and III) indicated no significant difference between the enzymes from the proximal colonic

TABLE I

K_m VALUES OF GLYCOSIDASES

Values are given as means ±S.E. of 12 experiments.

Tissues	(Substrates (mM)			
	p-Nitrophenyl-β-*N*-acetyl-glucosaminide	*p*-Nitrophenyl-β-*N*-acetyl-galactosaminide	*p*-Nitrophenyl-β-D-galactoside	*p*-Nitrophenyl-α-L-fucoside
Colonic mucosa of control rats	0.998 ±0.077	0.201 ±0.001	0.212 ±0.044	0.689 ±0.098
Colonic mucosa of tumour-bearing rats	0.910 ±0.122	0.220 ±0.031	0.212 ±0.026	0.535 ±0.110
Proximal colonic tumors	0.780 ±0.092	0.331 ±0.076	0.204 ±0.052	0.321 ±0.063
Distal colonic tumors	0.408* ±0.052	0.571* ±0.064	0.121* ±0.040	0.185* ±0.042

* Significantly different from the preceding values in the same column ($P < 0.005$).

TABLE II

K_i VALUES OF INHIBITORS FOR β-*N*-ACETYLGLUCOSAMINIDASE

Values are given as means ±S.E. of 6 experiments.

Tissues	Inhibitors				
	N-acetyl-galacto-samine (mM)	*N*-acetyl gluco-samine (mM)	Manno-samine (mM)	*N*-acetyl-gluco-samino-lactone (μM)	*N*-acetyl-galactosamino-lactone (μM)
Colonic mucosa of control rats	0.500 ±0.079	4.383 ±0.522	2.392 ±0.450	2.400 ±0.126	0.898 ±0.085
Colonic mucosa of tumour-bearing rats	0.504 ±0.135	5.260 ±0.782	2.542 ±0.594	2.387 ±0.510	0.929 ±0.115
Proximal colonic tumors	0.495 ±0.022	4.399 ±0.453	2.418 ±0.137	3.205 ±0.295	0.944 ±0.025
Distal colonic tumors	0.307* ±0.017	1.681* ±0.604	2.261 ±0.327	1.503* ±0.124	0.358* ±0.017

* Significantly different from the preceding values in the same column ($P < 0.005$).

tumours and from the colonic mucosa of the normal and the tumour-bearing animals. However, the K_i values for *N*-acetylhexosamines and *N*-acetylhexosaminolactones of distal colonic tumour β-*N*-acetylglucosaminidase were significantly lower ($P < 0.005$) and of β-*N*-acetylgalactosaminidase were significantly higher ($P < 0.005$) compared with the values obtained for enzymes from other test materials (Tables II and III). The data on D-mannosamine showed no statistical differences in the K_i values when enzymes from any of these tissues were used (Tables II and III). The K_i values of distal colonic tumour β-D-galac-

TABLE III

K_i VALUES OF INHIBITORS FOR β-*N*-ACETYLGALACTOSAMINIDASE

Values are given as means ±S.E. of 6 experiments.

Tissues	Inhibitors				
	N-acetyl-galacto-samine (mM)	*N*-acetyl gluco-samine (mM)	Manno-samine (mM)	*N*-acetyl-gluco-samino-lactone (μM)	*N*-acetyl-galactosamino-lactone (μM)
Colonic mucosa of control rats	0.659 ±0.010	6.238 ±0.119	6.401 ±0.396	7.635 ±2.488	4.043 ±0.823
Colonic mucosa of tumour-bearing rats	0.595 ±0.036	6.583 ±0.147	6.609 ±0.442	6.543 ±2.503	5.595 ±1.767
Proximal colonic tumours	0.768 ±0.139	6.527 ±0.138	5.078 ±0.749	5.776 ±1.516	3.474 ±0.035
Distal colonic tumours	1.286* ±0.276	6.812 ±0.263	6.127 ±1.951	9.232* ±1.263	7.026* ±0.936

* Significantly different from the proceding values in the same column ($P < 0.005$).

TABLE IV

K_i VALUES OF INHIBITORS FOR β-D-GALACTOSIDASE AND α-L-FUCOSIDASE

Values are given as means ±S.E. of 6 experiments.

Tissues	β-D-Galactosidase inhibitors		α-L-Fucosidase Inhibitors (α-L-fucose) (mM)
	β-D-galactose (mM)	Galactosono-lactone (mM)	
Colonic mucosa of control rats	1.478 ±0.416	0.127 ±0.024	3.171 ±0.301
Colonic mucosa of tumour-bearing rats	1.636 ±0.497	0.144 ±0.041	2.789 ±0.062
Proximal colonic tumours	1.887 ±0.476	0.153 ±0.020	2.768 ±0.321
Distal colonic tumours	0.657* ±0.123	0.081* ±0.012	0.898* ±0.160

* Significantly different from the preceding values in the same column ($P < 0.005$).

tosidase for β-D-galactose and galactonolactone and of α-L-fucosidase for α-L-fucose were also significantly lower ($P < 0.005$) compared with the enzymes from other experimental and control tissues (Table IV).

Effect of pH

The data on the pK values determining the pH dependence of the maximum velocity for glycosidases showed no significant difference among the enzymes from the tumours and the colonic mucosa of the normal tumour bearing animal (Table V).

TABLE V

pK VALUES DETERMINING THE pH DEPENDENCE OF THE MAXIMUM VELOCITY FOR GLYCOSIDASES

Values are given as means ±S.E. of 5 experiments.

Enzymes	Tissues			
		Colonic mucosa of control rats	Colonic mucosa of tumour-bearing rats	Tumours
β-*N*-Acetylglucosaminidase	pK_1	4.1 ± 0.05	4.1 ± 0.10	4.1 ± 0.05
	pK_2	4.9 ± 0.05	4.6 ± 0.10	4.8 ± 0.05
β-*N*-Acetylgalactosaminidase	pK_1	4.1 ± 0.10	4.1 ± 0.10	6.1 ± 0.15
	pK_2	4.8 ± 0.10	5.2 ± 0.15	2.9 ± 0.15
β-D-Galactosidase	pK_1	3.0 ± 0.0	3.1 ± 0.10	2.9 ± 0.10
	pK_2	5.2 ± 0.0	5.1 ± 0.05	5.4 ± 0.10
α-L-Fucosidase	pK_1	5.9 ± 0.2	5.5 ± 0.05	6.5 ± 0.20
	pK_2	4.6 ± 0.1	4.9 ± 0.05	4.9 ± 0.10

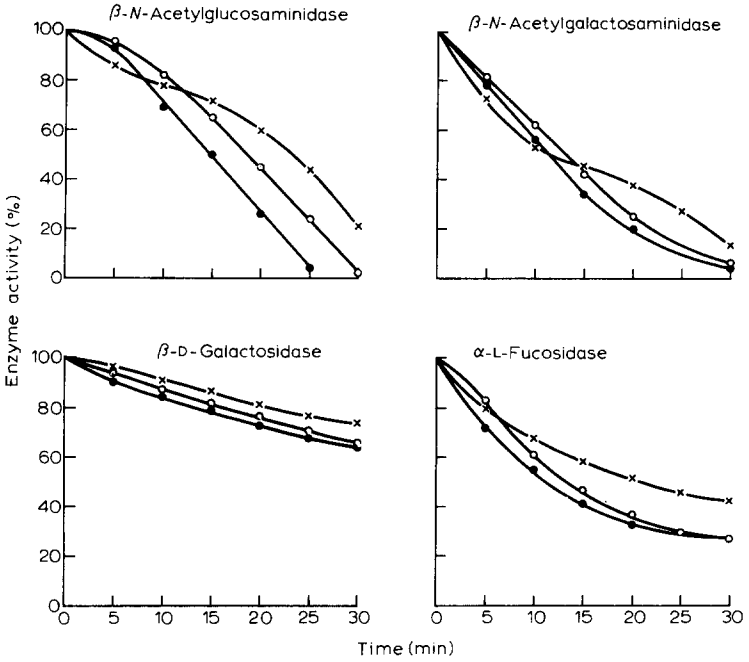

Fig. 3. Thermal inactivation of glycosidases at 55° C. Homogenates of colonic mucosa from control (●———●) and tumour-bearing rats (○———○) and from the tumours (X———X) were heated at 55° C for different time intervals prior to the assays at 37° C.

Thermal inactivation

The kinetics of thermal inactivation were studied at 50, 55 and 60° C. Heating of the tissue homogenates at 50° C even for 60 min did not produce more than 20% inactivation of any of the enzymes. On the other hand, heating at 60° C for 5—10 min inactivated the enzymes by about 80%. However, when

TABLE VI

K_m VALUES OF FOETAL INTESTINAL GLYCOSIDASES

Values are given as means ±S.E. of 5 experiments on 14 days old foetuses, 3 experiments on 18 days old foetuses, 4 experiments on neonates and of 12 experiments on adult rat colons.

Tissues	*p*-Nitrophenyl-β-*N*-acetyl-glucosaminide (mM)	*p*-Nitrophenyl-β-*N*-acetyl-galactosaminide (mM)	*p*-Nitrophenyl-β-D-galactoside (mM)	*p*-Nitrophenyl-α-L-fucoside (mM)
14 days old foetuses	0.494* ±0.031	0.517* ±0.137	0.257 ±0.001	0.387 ±0.273
18 days old foetuses	1.049 ±0.273	0.242 ±0.001	0.182 ±0.001	0.308 ±0.031
16 h old neonates	1.528 ±0.298	0.158 ±0.010	0.243 ±0.044	0.347 ±0.054
Adult animals	0.998 ±0.077	0.201 ±0.001	0.212 ±0.044	0.689 ±0.098

* Significantly different from other values in the same colum ($P < 0.005$).

the homogenates were heated at 55°C the enzymes showed different inactivation profiles (Fig. 3). A difference in the thermal inactivation of the tumour β-*N*-acetylhexosaminidases compared with the enzymes from other tissues could also be seen in Fig. 3.

Michaelis constants (K_m) of foetal intestinal glycosidases

The K_m values of glycosidases from 14 and 18 days old foetuses and of neonates (about 16 h old) are given in Table VI. A comparison of the data with those on the adult animal enzymes showed that the K_m values of two *N*-acetylhexosaminidases of 14 days old foetuses were significantly different from the K_m values obtained for those enzymes from 18 days old foetuses, neonates and the adult animals. However, there was no significant difference in the K_m values of β-D-galactosidase and α-L-fucosidase of the foetuses' neonates and adult animals.

Discussion

The kinetics of colonic β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, β-D-galactosidase and α-L-fucosidase were studied using tissue homogenates in 0.1% Triton X-100 and *p*-nitrophenyl glycoside substrates. The K_m values obtained were in good agreement with the values published for these enzymes from various other mammalian tissues [10,11]. Each enzyme appeared to act on its own specific substrate as shown by the fact that the double-reciprocal plots were linear. When two or more enzymes are acting on the same substrate, a downward curvature can be expected in such plots [12]. Colonic glycosidases were competitively inhibited by glycosides and aldonolactones of the corresponding configuration. Aldonolactones were more effective inhibitors than the glycosides except that fuconolactone failed to inhibit α-L-fucosidase activity. Our data on inhibition kinetics of colon enzymes confirm the results obtained for glycosidases from other mammalian tissues [11,13,14].

A comparison of the inhibition kinetics of β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase is of some interest since the dissimilarity in the K_i values for different inhibitors did indicate the presence of two separate enzymes rather than of a single enzyme hydrolysing both substrates as suggested for the rat testis enzyme previously [10]. Nevertheless, the pK values data for the two *N*-acetylhexosaminidases showed similarity in the catalytic groups of their active centres. The inhibition of these enzymes not only by their corresponding sugar products and aldonolactones, but also by other monosaccharides and aldonolactones, suggests that the nature of the aglycone in the complex natural substrates, particularly the penultimate sugar residues, may play an important role in regulating the catalytic activity of these enzymes.

The kinetics data also indicated that β-*N*-acetylglucosaminidase, β-D-galactosidase and α-L-fucosidase of the distal colonic tumours had significantly lower K_m values, and β-*N*-acetylgalactosaminidase a significantly higher K_m value compared with those obtained for the proximal colonic tumours and for the colonic mucosa of the control and the tumour-bearing animals. These observations were reinforced by the data from the inhibition studies. Thermal

inactivation data also indicated some difference between the tumour and the normal β-*N*-acetylhexosaminidases. The pattern of conformational changes at 55° C in the tumour enzymes was sigmoidal whereas in the normal enzymes it was linear. Our data on the pH effect suggested that the catalytic groups of the active centres of the glycosidases from the mucosa of the control and tumour bearing animals and from the colonic tumours were probably identical. These observations suggest that the difference in the kinetic behaviour of the distal tumour enzymes must be due to some structural changes in the non-catalytic groups of the enzyme active centres.

Although no kinetic studies were carried out on mixtures of the enzymes from different tissues, the amounts of the hydrolysis products, after mixing different quantities of homogenates from cancerous, experimental and control tissues, were found to be true arithmatical means of the enzyme activities in every case.

The K_m values of 14 days old foetal β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase were similar to the K_m values obtained for these enzymes from the distal colonic tumours. Similarly, an electrophoretically fast-moving form of hexosaminidase was present in rat foetal liver and hepatoma. It was replaced in adult liver by a slow-moving form [15].

Further kinetic studies and polyacrylamide-gel electrophoresis on the purified enzyme fractions prepared by ammonium sulphate precipitation and Sephadex and DEAE-cellulose chromatography are under progress. These results and the amino acid and carbohydrate analyses of the purified enzymes will be reported shortly.

Acknowledgements

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