Biochemical Pharmacology, Vol. 19, pp. 71–89. Pergamon Press. 1970. Printed in Great Britain.

ACTION OF TUMOUR INHIBITORY PYRAZOLOTRIAZINES ON *KLEBSIELLA AEROGENES*—II

INHIBITION BY 6-HALOGENOACETYL-3-METHYL-4-METHYLENEPYRAZOLO[3,2-*c*]-*as*-TRIAZINE AND ITS ANTAGONISM

GILLIAN M. ARDEN, D. J. W. GRANT and M. W. PARTRIDGE

Department of Pharmacy, The University, Nottingham, NG7 2RD U.K.

(*Received* 6 *January* 1969; *accepted* 27 *June* 1969)

Abstract—The action of the following tumour inhibitory 6-acyl-3-methyl-4-methylenepyrazolo[3,2-*c*]-*as*-triazines (6-acyl-DMPT) against the growth of *Klebsiella aerogenes* NCTC 418 and its specific protein synthesis has been studied at concentrations up to their rather low solubility limits:- 6-acetyl-, 6-fluoroacetyl-, 6-chloroacetyl-, 6-iodoacetyl- and 6-dichloroacetyl-DMPT. The solubilities of the compounds and the specific rates of hydrolysis of their 6-acyl bonds have been determined.

6-Iodoacetyl-DMPT inhibited growth and the synthesis of β-galactosidase and histidine ammonia-lyase. In the absence of growth 6-iodoacetyl- and 6-chloroacetyl-DMPT inhibited the induction of β-galactosidase synthesis without delay; cysteine, glutathione and 6-mercaptopurine antagonized these effects, which suggests that the inhibitors alkylate thiol groups. The specific rate of alkylation of cysteine by 6-iodoacetyl-DMPT was 120 times that by iodoacetic acid and 1500 times the specific rate of hydrolysis of 6-iodoacetyl-DMPT to iodoacetic acid. The intact 6-acyl bond was essential for the high biological and chemical activity of 6-iodoacetyl-DMPT.

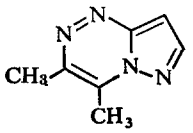
6-Dichloroacetyl-DMPT had a low activity and was rapidly hydrolysed. 6-Fluoroacetyl-DMPT and 6-acetyl-DMPT were inactive. The above 6-halogenoacetyl-DMPT derivatives did not act as concurrent blocking agents. No alteration in the levels of DNA, RNA and total protein in growing or non-dividing bacteria actively synthesizing β-galactosidase were observed in their presence.

3,4-DIMETHYLPYRAZOLO[3,2-*c*]-*as*-TRIAZINE (DMPT) and certain of its 6-acyl derivatives (Table 1) inhibit the growth of mouse sarcoma S180 and both the Walker tumour and a methylcholanthrene-induced tumour in rats.[1] With the exception of 6-chloroacetyl-DMPT, which produced excessive loss of body weight in the test animals, these compounds had low toxicity at tumour inhibitory concentrations.

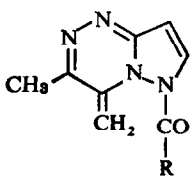
The relationship between the structure (Table 1) and activity[1] of the 6-acyl-DMPT derivatives suggests the following hypotheses (a–d) for the mode of action of the compounds.

(a) All but one of the inhibitors investigated contain halogenoacetyl groups and could be hydrolysed in the cell to the halogenoacetic acid and DMPT. One or both of these products could be active; if both have the same order of inhibitory activity, a concurrent blocking process may occur. DMPT would interfere with the metabolism or function of histidine (Part 1) and the halogenoacetic acids could act as enzyme and metabolic inhibitors.[2,3] Iodoacetic acid (IAA) and iodoacetamide (IAM) alkylate biological materials including proteins; in the physiological pH range they react

TABLE 1. STRUCTURE OF DIMETHYLPYRAZOLOTRIAZINE AND SOME OF ITS 6-ACYL DERIVATIVES[1]



3,4-Dimethylpyrazolo[3,2-*c*]-*as*-triazine (DMPT)



6-Acyl derivative	R
6-Acetyl-DMPT	CH_3
6-Fluoroacetyl-DMPT	$F.CH_2$
6-Chloroacetyl-DMPT	$Cl.CH_2$
6-Iodoacetyl-DMPT	$I.CH_2$
6-Dichloroacetyl-DMPT	Cl_2CH

6-Acyl-3-methyl-4-methylenepyrazolo[3,2-*c*]-*as*-triazine (6-Acyl-DMPT)

most readily with these groups and less readily with other alkylatable groups, e.g. amino groups.[2] IAA has been successfully used clinically as a palliative treatment for some human neoplasms[4,5] and inhibits the growth of various bacterial species.[2] Chloroacetic acid reacts comparatively slowly with thiol groups and has little activity either as a biological alkylating agent or as a metabolic inhibitor.[2,6,7] Fluoroacetic acid does not act as an alkylating agent but owes its toxicity to its conversion to fluorocitric acid which inhibits aconitate hydratase in the tricarboxylic acid cycle.[8]

(b) The conversion of an inhibitory halogenoacetic acid to its 6-halogenoacetyl-DMPT derivatives or the conversion of the inhibitory DMPT to a 6-acyl-DMPT may increase the penetration of the inhibitor through cell membranes and into tissues. For example, IAA ($pK = 3{\cdot}12$) in the physiological pH range exists mostly in the ionized form, which is less able to penetrate cellular permeability barriers than the unionized acid,[2,9] whereas the suppression of the ionization of IAA by its conversion to IAM tends to facilitate cell penetration.[10] Similarly, certain *N*-iodoacetyl derivatives of amino acids, particularly *N*-iodoacetylphenylalanine, inhibit mouse Sarcoma S180 more than IAA does.[11] The amino acid residue is believed to act as a carrier of the iodoacetyl group.

(c) 6-Iodacetyl-DMPT, and possibly 6-chloroacetyl-DMPT, may act as biological alkylating agents in their own right. For example, some iodoacetyl derivatives of a series of amides and esters are potent alkylating agents[12] and ethylene-bis-iodoacetate inhibits some human tumours.

(d) The 6-acyl-DMPT derivatives could act as biological acylating agents.

Several of the above mechanisms could operate together. Since IAA and IAM are important examples in the above hypotheses, they were used as reference compounds. The stages by which the action of the tumour inhibitors was studied using bacteria have been outlined in Part I.

MATERIALS AND METHODS

Inhibitors and antagonists. The 6-acyl-3-methyl-4-methylenepyrazolo[3,2-*c*]-*as*-triazines (Table 1) were prepared by the methods of Partridge and Stevens.[13] Solutions of inhibitors and antagonists were sterilized, when necessary, by membrane filtration at room temperature.

Properties of the inhibitors in aqueous solution. Ultraviolet and visible absorption spectrograms of aqueous solutions of pyrazolotriazines were recorded, and closely resemble those of ethanolic solutions.[13] Table 2 shows the positions (λ_{max}) and molar

TABLE 2. ULTRAVIOLET ABSORPTION PEAKS AND SOLUBILITY IN WATER AND SPECIFIC RATES OF HYDROLYSIS OF 6-ACYL-DMPT DERIVATIVES

Compound	λmax mμ	Solubility at 40° μg/ml	Solubility at 40° mM	Hydrolysis at 40° Time for 50% hydrolysis (hr)	Hydrolysis at 40° Specific rate (min^{-1})	Dissociation constant*
DMPT	(225 41000) (285 1910) (361 2700)	$7{\cdot}5 \times 10^4$	506			
6-Acetyl-DMPT	(226 12100) (265† 14000) (289 15200)	218	1·15	1240	$3{\cdot}11 \times 10^{-5}$	$1{\cdot}86 \times 10^{-5}$
6-Fluoroacetyl-DMPT	(228 9050) (266† 12300) (286 13500)	110	0·528	276	$1{\cdot}39 \times 10^{-4}$	$2{\cdot}19 \times 10^{-3}$
6-Chloroacetyl-DMPT	(227 9530) (266† 13000) (294 15600)	26	0·116	63	$6{\cdot}14 \times 10^{-4}$	$1{\cdot}55 \times 10^{-3}$
6-Iodoacetyl-DMPT	(224 10700) (301 15700)	30	0·095	93	$4{\cdot}14 \times 10^{-4}$	$7{\cdot}59 \times 10^{-4}$
6-Dichloroacetyl-DMPT	(227 12500) (296 18500)	14‡	0·054‡	3·5	$1{\cdot}10 \times 10^{-2}$	$5{\cdot}50 \times 10^{-2}$

* Dissociation constant at 37° and at an ionic strength of 0·15 of the acid liberated in the hydrolysis of the compound.[2]
† Inflexion.
‡ Solubility corrected for the hydrolysis of the compound to DMPT; this correction was negligible for the other compounds.

extinction coefficients (ϵ) of the absorption peaks. The solutions obeyed the Beer–Lambert law at the wavelengths of maximum absorption and so the concentrations of the 6-acyl-DMPTs in solution were determined spectrophotometrically.

Saturated aqueous solutions were prepared by shaking excess of the finely divided solid compounds with water at 40·0° and filtering. The low solubilities of the 6-acyl-DMPTs were an important factor in the present work.

Saturated aqueous solutions of the 6-acyl-DMPTs at 40·0° showed a gradual increase in extinction at 361 mμ due to the appearance of DMPT formed by hydrolysis. From measurements of the extinction at 361 mμ at various times, the hydrolyses were found to be of the first order and their specific rates (Table 2) were found to be related to the dissociation constant of the acid liberated.[2]

Determination of thiol groups in bacteria. The bacteria in suspension (M = 3000) in phosphate buffer (pH 7·12) were disintegrated ultrasonically for 4 min as described in Part I and the thiol content was determined by the iodide persulphate reaction.[14]

Other details. The remainder of the Materials and Methods section is identical with that in Part I. In this report the term "induction" refers to induced enzyme synthesis.

RESULTS

Effect of the inhibitors on the rate of growth

The time (T) taken for a glucose-adapted strain to grow from its inoculum size, M = 3, to M = 200 (Part I) is plotted against the concentration of the inhibitor

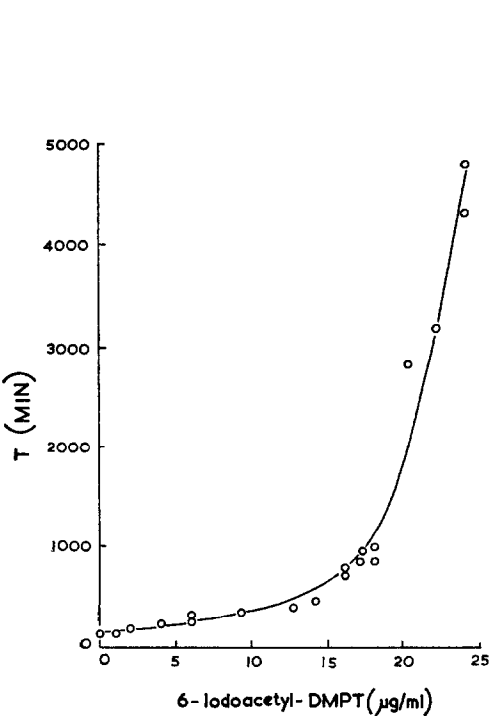


FIG. 1a

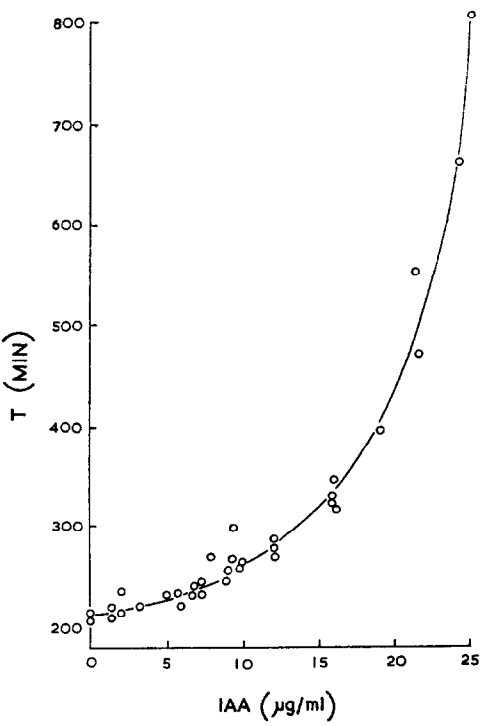


FIG. 1b

FIG. 1. Effect of various concentrations of 6-iodoacetyl-DMPT (a) and IAA (b) on the growth of *K. aerogenes* in glucose medium. T is the time in min taken for the bacterial concentration M to increase from 3·0 to 200 in glucose medium.

present in the glucose medium as in Fig. 1. 6-Acetyl-, 6-dichloroacetyl- and 6-fluoroacetyl-DMPT did not affect T (220 min) up to their solubility limits. 6-Chloroacetyl-DMPT at 0 to 17 μg/ml did not affect T (220 min) and thereafter T increased slightly, reaching 250 min at the solubility limit of 26 μg/ml. 6-Iodoacetyl-DMPT and IAA were the strongest inhibitors of growth (Fig. 1). 6-Iodoacetyl-DMPT completely hydrolysed by boiling the solution for 5 min, and equimolecular mixtures of IAA

and DMPT produced the same degree of inhibition as the equivalent molar concentration of IAA (Fig. 2) and were less active than unhydrolysed 6-iodoacetyl-DMPT. DMPT was active only at much greater concentrations (Part I). Thus, for the greatest activity the molecule of 6-iodoacetyl-DMPT must be intact and the effect of the iodoacetyl group overwhelms that of the DMPT moiety.

Effect of the inhibitors on DNA, RNA, total protein and β-galactosidase activity during growth

The lactose-adapted strain was grown in lactose medium in the presence and absence of the following inhibitors: 6-chloroacetyl-DMPT, 19·4 μg/ml; 6-iodoacetyl-DMPT, 6·7 and 7·0 μg/ml; IAA, 8·2 and 14 μg/ml; equimolecular mixtures of DMPT

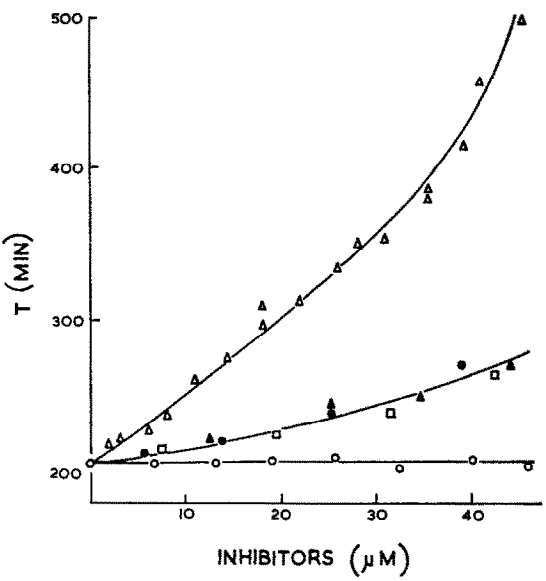


FIG. 2. Comparison of the effects of various molar concentrations of 6-iodoacetyl-DMPT (△), hydrolysed 6-iodoacetyl-DMPT (▲), an equimolecular mixture of IAA and DMPT (□), IAA alone (●) and DMPT alone (○) on the growth of *K. aerogenes* in glucose medium. T is the time in min taken for the bacterial concentration M to increase from 3·0 to 200.

and IAA equivalent to 6·7 and 13·4 μg of 6-iodoacetyl-DMPT/ml. In the presence of the above inhibitors the bacteria had the same DNA, RNA, and total protein levels, indicated in Part I, and β-galactosidase activity as the controls at the same point in the growth cycle, with the exception that 6-iodoacetyl-DMPT and IAA (alone) reduced the β-galactosidase activity (Fig. 3). Since β-galactosidase is required for growth in lactose medium,[15,16] its reduced activity might merely reflect a lower growth rate in this medium. As DMPT at 1000 μg/ml increased the mean generation time from 40 to 49 min but did not affect the β-galactosidase activity (Part I), the reduction in activity caused by 6-iodoacetyl-DMPT and by IAA does not reflect the respective mean generation times of 49 and 46–61 min but must have other causes.

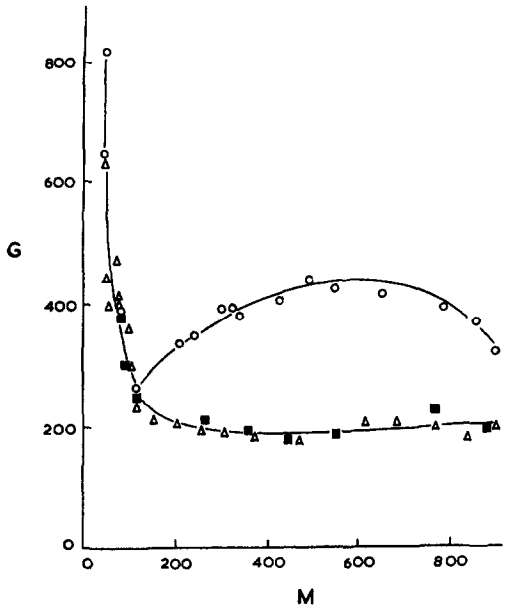


FIG. 3. β-Galactosidase activity, G, of *K. aerogenes* during growth in lactose medium in the presence of 6-iodoacetyl-DMPT at 6·7 μg/ml (△) or IAA at 14 μg/ml (■) and in the absence of inhibitor as control (○). M is the bacterial concentration.

Effect of the inhibitors on the β-galactosidase activity of non-dividing bacteria

Lactose trained bacteria were grown in limiting lactose medium, washed and resuspended in phosphate buffer (pH 7·12) to give M = 2500. Samples were incubated at 40·0° in the presence and absence of the following inhibitors:—6-acetyl-DMPT, 169 μg/ml; 6-chloroacetyl-DMPT, 24 μg/ml: 6-dichloroacetyl-DMPT, 14 μg/ml; 6-iodoacetyl-DMPT, 10 μg/ml; IAA, 40 μg/ml; IAM, 30 μg/ml. The β-galactosidase activity was determined after centrifuging and washing the bacteria. None of the inhibitors affected the β-galactosidase activity within 60 min of incubation. 6-Iodoacetyl-DMPT, IAA and IAM after 120 min incubation reduced the β-galactosidase activity by 1·5, 10 and 11·5 per cent respectively and after 180 min incubation by 7, 17 and 20 per cent respectively. The other inhibitors produced no effect at 0–210 min. None of the inhibitors affected the β-galactosidase activity when present in the assay solution only or when present in the solution during both the ultrasonic disintegration and the assay.

Effect of the inhibitors when added initially on induced enzyme synthesis by non-dividing bacteria

The inhibitor was added to samples of lactose induction medium before inoculation. Increasing concentrations of 6-iodoacetyl-DMPT, 6-chloroacetyl-DMPT, IAA or IAM increasingly inhibited the induction of β-galactosidase by non-dividing cells (Figs. 4 and 5). Lower concentrations of 6-iodoacetyl-DMPT (0–5 μg/ml) or of 6-chloroacetyl-DMPT (0–12 μg/ml) tended merely to delay the commencement of induction, which indicates that these inhibitors exerted an immediate effect which

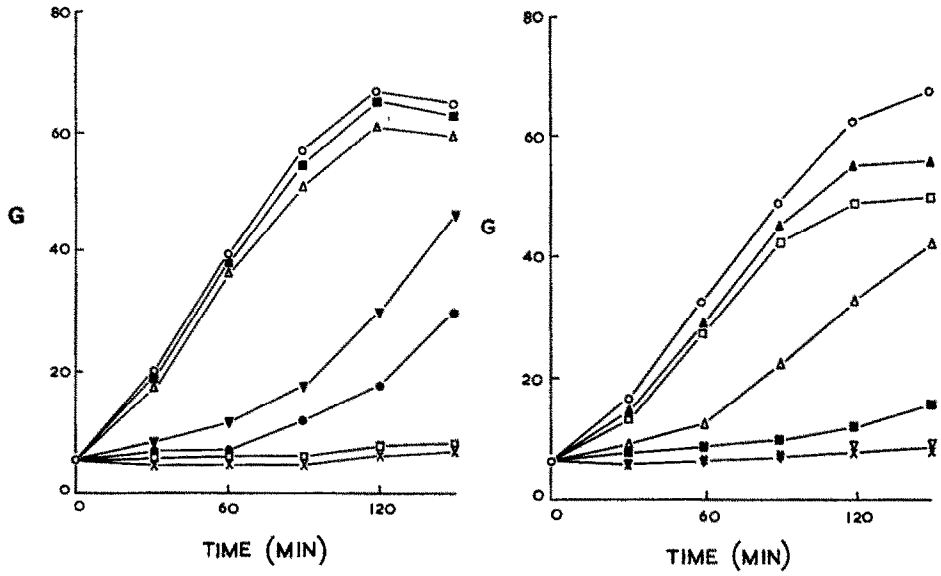


FIG. 4a FIG. 4b

FIG. 4. Effect of various concentrations of 6-iodoacetyl-DMPT (a) or 6-chloroacetyl-DMPT (b) present before inoculation at zero time on the induction of β-galactosidase synthesis. G is the β-galactosidase activity. (a) 6-Iodoacetyl-DMPT concentrations: 0 (○), 1·5 (■), 3·8 (△), 5·2 (▼), 7·5 (●), 10·0 (□), 15·4 μg/ml (×). (b) 6-Chloroacetyl-DMPT concentrations: 0 (○), 5·9 (▲), 10·8 (□), 14·2 (△), 18·4 μg/ml (■) and with excess of solid inhibitor (▽, ×).

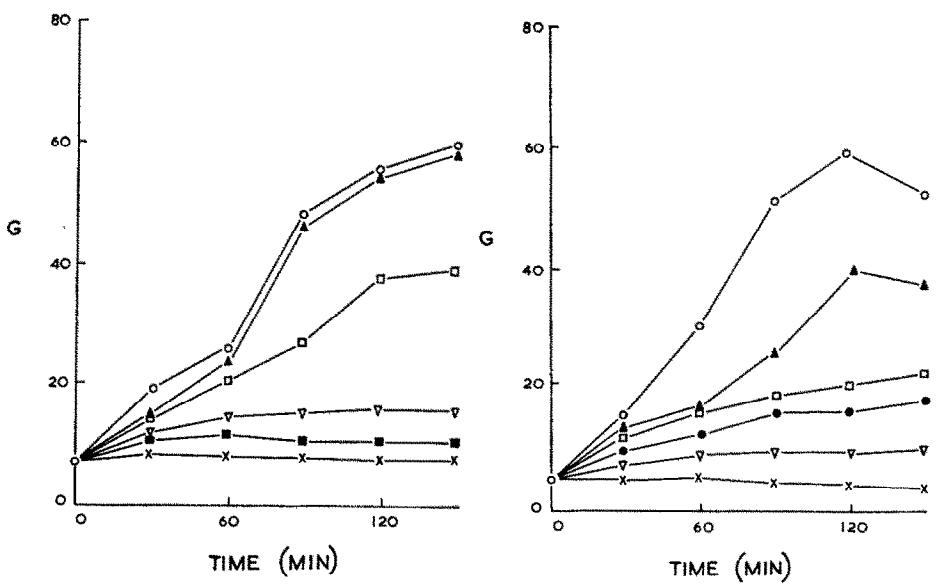


FIG. 5a FIG. 5b

FIG. 5. Effect of various concentrations of IAA (a) or IAM (b) present before inoculation at zero time on the induction of β-galactosidase synthesis. G is the β-galactosidase activity. (a) IAA concentrations: 0 (○), 5·9 (▲), 11·8 (□), 23·7 (▽), 35·5 (■), 47·4 μg/ml (×). (b) IAM concentrations: 0 (○), 5 (▲), 9 (□), 13 (●), 20 (▽), 40 μg/ml (×).

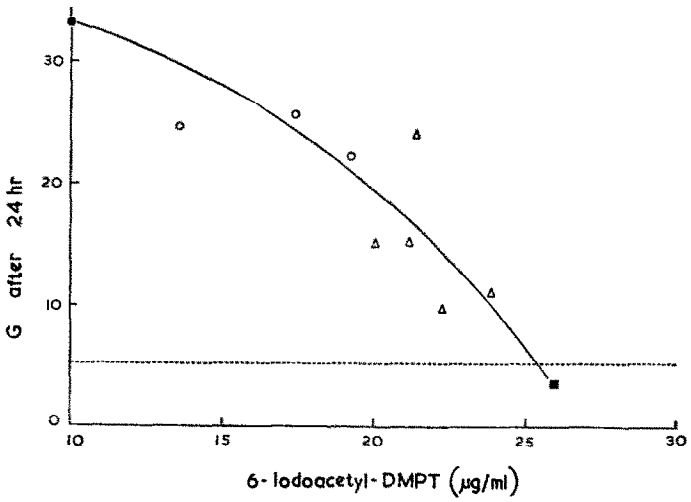


FIG. 6. Effect of various concentrations of 6-iodoacetyl-DMPT present in the lactose induction medium before inoculation at zero time on the β-galactosidase activity, G, attained in the induction medium 24 hr after inoculation. G of uninhibited control ●. G of inoculum ..... Growth in induction medium: appreciable ○, slight △, none ■.

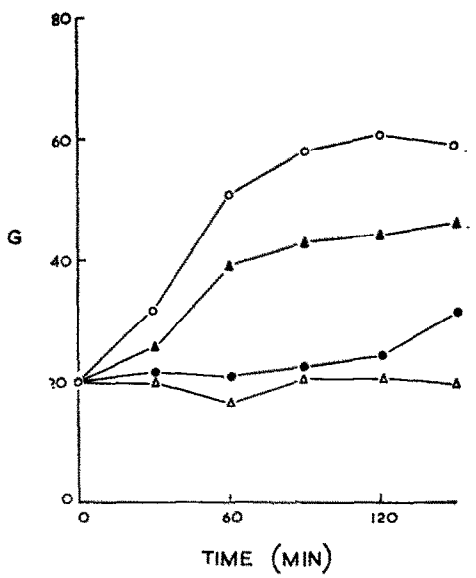


FIG. 7. Effect of the following concentrations of 6-iodoacetyl-DMPT present before inoculation at zero time on the induction of histidine ammonia-lyase synthesis: 0 (○), 5 (▲), 7·5 (●), 10 μg/ml (△). H is the histidine ammonia-lyase activity.

the bacteria later overcame; higher concentrations reduced the rate of induction and the bacteria recovered more slowly. Inhibition of β-galactosidase synthesis by IAA and to a lesser extent by IAM increased with time (Fig. 5).

For complete inhibition of β-galactosidase induction the following minimum concentration of inhibitor was required: 6-iodoacetyl-DMPT 10 μg/ml (31·6 μM); 6-chloroacetyl-DMPT 18 μg/ml (80·1 μM); IAM 30 μg/ml (162 μM); IAA 40 μg/ml (215 μM). 6-Iodoacetyl-DMPT at 26 μg/ml (82·2 μM) completely inhibited β-galactosidase induction for at least 24 hr as well as inhibiting growth in the induction medium

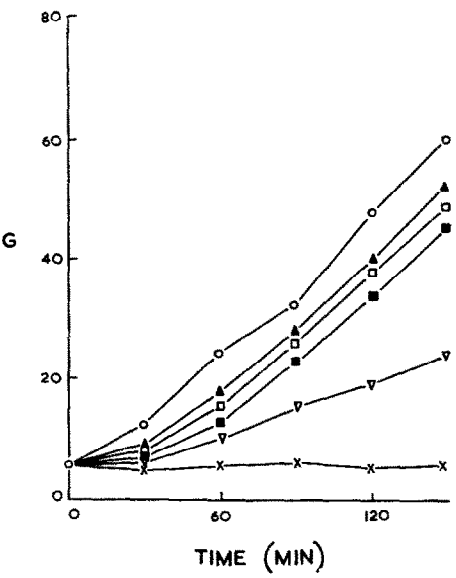


FIG. 8

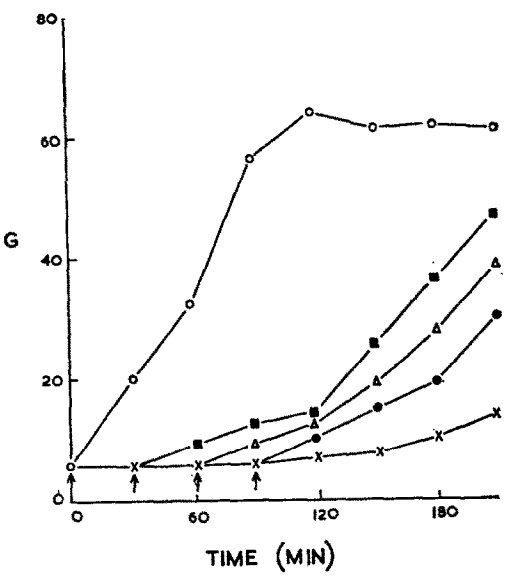


FIG. 9

FIG. 8. Effect of the following times of preincubation with 6-iodoacetyl-DMPT at 10 μg/ml in "salts" medium on the induction of β-galactosidase synthesis in *K. aerogenes* following transference to induction medium free of inhibitor: 0 min, control (○); 10 min (▲); 20 min (□); 30 min (■), 60 min (▽). × represents a control culture containing 6-iodoacetyl-DMPT at 10 μg/ml. G is the β-galactosidase activity.

FIG. 9. Effect of the following times of preincubation with 6-iodoacetyl-DMPT at 10 μg/ml in lactose induction medium on the induction of β-galactosidase synthesis in *K. aerogenes* following transference to fresh induction medium free of inhibitor: 0 min, control (○); 30 min (■); 60 min (△); 90 min (●). × represents a control culture containing 6-iodoacetyl-DMPT at 10 μg/ml. Zero time is the moment of inoculation into the original medium containing the inhibitor. Each arrow represents the point of resuspension in the medium free of inhibitor. G is the β-galactosidase activity.

(Fig. 6), but 6-chloroacetyl-DMPT even at its solubility limit (26 μg/ml, 116 μM) did neither. 6-Iodoacetyl-DMPT at 26 μg/ml (82·2 μM) and IAM at 30 μg/ml (162 μM) actually reduced the activity of existing enzyme (Figs. 5 and 6). Thus, the above inhibitors fell into the following order in their degree and speed in inhibiting the induction of β-galactosidase: 6-iodoacetyl-DMPT > 6-chloroacetyl-DMPT > IAM > IAA.

6-Fluoroacetyl-DMPT at 74 μg/ml or 6-acetyl-DMPT at 93 μg/ml had no effect on the induction of β-galactosidase by non-dividing bacteria; 6-dichloroacetyl-DMPT at 14 μg/ml slightly reduced the rate of enzyme synthesis for the first 30 min, but afterwards it continued at the same rate as that of the control

The presence of 6-iodoacetyl-DMPT at 5 μg/ml or 10 μg/ml did not affect the fairly constant levels of DNA (0·24 μg/M), RNA (0·16 μg/M) and total protein (0·37 μg/M) during β-galactosidase induction by non-dividing cells.

When the induction system contained both methyl-β-D-thiogalactopyranoside (0·196 g/l.) and maltose (4 g/l.) in place of lactose, the kinetics of induction and the effects of 6-iodoacetyl-DMPT thereon were almost the same as in the lactose system.

Increasing concentrations of 6-iodoacetyl-DMPT increasingly inhibited the induction of histidine ammonia-lyase (Fig. 7) by non-dividing cells and at 10 μg/ml inhibition was complete.

Effect of preincubation of the bacteria in the presence of 6-iodoacetyl-DMPT on the induction of β-galactosidase synthesis

The bacteria were incubated with 6-iodoacetyl-DMPT at 10 μg/ml either in "salts" solution (Fig. 8) or in lactose induction medium (Fig. 9). After suitable times the bacteria were harvested by centrifugation, resuspended in "salts" solution free of inhibitor and incubated in lactose induction medium free of inhibitor. After short times of previous incubation with 6-iodoacetyl-DMPT β-galactosidase induction was first delayed and then proceeded at the same rate as that of the control without inhibitor, (Figs. 8 and 9). Previous incubation with the inhibitor in "salts" medium

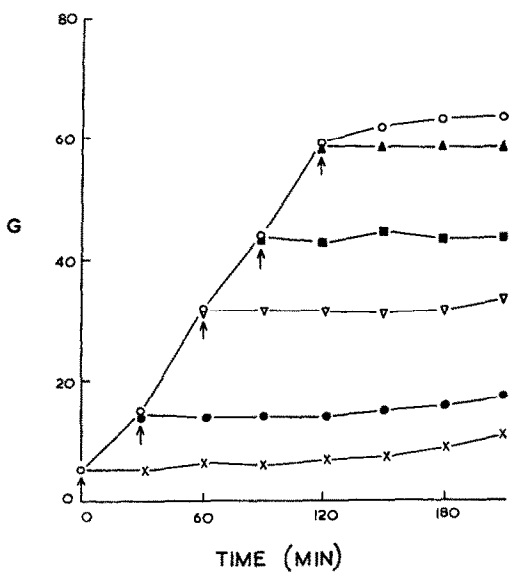


FIG. 10. Effect of the addition of 6-iodoacetyl-DMPT at 10 μg/ml at various times indicated by the arrows on the induction of β-galactosidase synthesis. The lactose induction medium was inoculated at zero time. The points represent the β-galactosidase activity, G, of the uninhibited control ○, and after addition of 6-iodoacetyl-DMPT at zero time ×, at 30 min, ●, at 60 min ▽, at 90 min ■, and at 120 min ▲.

for 10, 20 or 30 min delayed induction for an approximately equal time, but incubation for 60 min also reduced the rate of enzyme synthesis (Fig. 8). Thus, the continued presence of 6-iodoacetyl-DMPT was not essential for inhibition but exerted the greatest effect. The damage to the induction mechanism increased as the period of pre-incubation with the inhibitor increased.

Effect of addition of 6-iodoacetyl-DMPT to bacterial systems synthesizing β-galactosidase

To uninhibited lactose induction systems in which induction has been occurring for various times samples of fresh induction medium containing 6-iodoacetyl-DMPT were added to give 10 μg of inhibitor/ml of medium. After addition of 6-iodoacetyl-DMPT the β-galactosidase activity remained constant for some hours (Fig. 10); this shows that the substance immediately and completely inhibited induction of the enzyme. After induction for 60 min, addition of IAM at 30 μg/ml or IAA at 40 μg/ml in this way gave a similar response (cf. Fig. 5). The bacteria are therefore more rapidly inhibited by IAM and IAA when enzyme induction is under way than at its commencement.

To an inducing system which contained 6-iodoacetyl-DMPT at 7·5 μg/ml and in which induction had begun, fresh induction medium containing inhibitor was added to give 7·5 μg of inhibitor/ml of the whole system, any 6-iodoacetyl-DMPT already present being neglected. When induction had recommenced, addition of fresh induction medium containing 6-iodoacetyl-DMPT was repeated, and when induction had again begun, a further addition was made. After each successive addition of 6-iodoacetyl-DMPT, β-galactosidase induction was increasingly delayed and slowed down (Fig. 11). The final addition of inhibitor at 600 min delayed induction for a further 720 min. Each addition of 6-iodoacetyl-DMPT caused an immediate decrease in the thiol concentration of the bacteria (Fig. 11). Each time induction of β-galactosidase restarted, the thiol concentration began to increase but at a faster rate, which was about the same during each successive induction. Thus, the recovery of the bacteria was accompanied by the production of thiol groups but the inhibition was not completely reversible.

Antagonism of the action of 6-iodoacetyl-DMPT, 6-chloroacetyl-DMPT, iodoacetic acid and iodoacetamide by thiols

A lactose induction medium containing 6-iodoacetyl-DMPT or 6-chloroacetyl-DMPT was inoculated with bacteria and the β-galactosidase activity was determined at various times during incubation. The potential antagonist was added 30 min before, during or 30 min after inoculation. In all cases the controls were (i) the system without inhibitor but with potential antagonist, (ii) the system with inhibitor but without potential antagonist and (iii) the system without either. Controls (ii) and (iii) yielded the expected results hitherto described. With control (i) 2,3-dimercaptopropanol and thioglycollic acid actually reduced the β-galactosidase activity, but the other potential antagonists did not. The results (Table 3) were similar for 6-iodoacetyl-DMPT and 6-chloroacetyl-DMPT and indicate a rapid reaction between thiol compounds and the inhibitor. The most effective antagonist was cysteine, followed by glutathione and 6-mercaptopurine.

As the time of contact of the organism with 6-iodoacetyl-DMPT (10 μg/ml) in the lactose induction medium increased (Fig. 12), the degree of antagonism produced by a given concentration of added cysteine or glutathione decreased. The inhibition

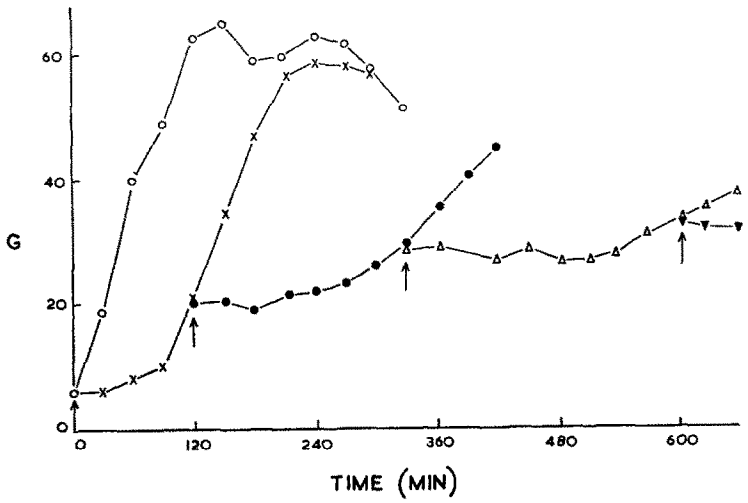


FIG. 11a

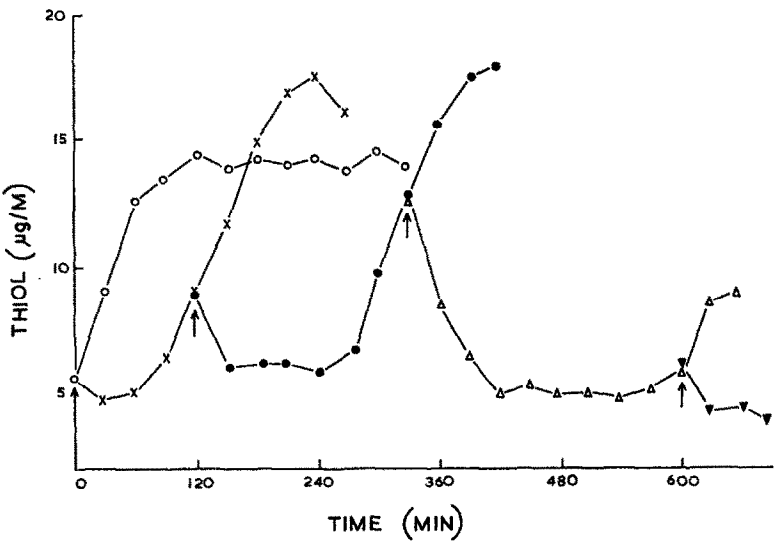


FIG. 11b

FIG. 11. The induction of β-galactosidase synthesis (upper curve) and the production of thiol groups (lower curve) in *K. aerogenes* before and after the serial addition of 6-iodoacetyl-DMPT. The arrows indicate the addition of 6-iodoacetyl-DMPT solution at the following times to give 7·5 μg/ml in the lactose induction medium: zero time (×), 120 min (●), 330 min (△), 600 min (▼). The control culture free of inhibitor (○) and the inhibited culture (×) were inoculated at zero time. G is the β-galactosidase activity.

TABLE 3. SEARCH FOR ANTAGONISTS OF THE ACTION OF 6-IODOACETYL-DMPT AND 6-CHLOROACETYL-DMPT IN INHIBITING THE INDUCTION OF β-GALACTOSIDASE SYNTHESIS

Time of addition of potential antagonist at 100 μg/ml	Percentage antagonism* of 6-iodoacetyl-DMPT at 10 μg/ml			
	Complete (100%)	Partial (%)		None (0%)
30 min before inoculation	L-cysteine HCl	adenine	40	albumin
	glutathione	guanine	38	DL-methionine
	6-mercaptopurine	xanthine	35	phloroglucinol
		methyl-thiouracil	75	†thioglycollic acid
		thiosemicarbazide	25	cyanocobalamin
		†2,3-dimercaptopropanol	52	
		folic acid	35	
		L-tyrosine	15	
		L-histidine HCl	20	
		L-glutamine	12	
		DL-aspartic acid	20	
At inoculation	L-cysteine HCl			
	6-mercaptopurine			
	glutathione			
30 min after inoculation	L-cysteine HCl	glutathione	40	methylthiouracil
		6-mercaptopurine	45	adenine
		L-histidine HCl	10	guanine
				xanthine
				thiosemicarbazide
				folic acid
				DL-aspartic acid
				2,3-dimercapto-propanol

Time of addition of potential antagonist at 200 μg/ml	Percentage antagonism* of 6-chloroacetyl-DMPT at 18 μg/ml			
	Complete (100%)	Partial (%)		None (0%)
30 min before inoculation	L-cysteine HCl	adenine	38	albumin
	glutathione	guanine	32	DL-methionine
	6-mercaptopurine	xanthine	35	phloroglucinol
		folic acid	30	†thioglycollic acid
		L-tyrosine	28	L-histidine HCl
		cyanocobalamin	40	DL-aspartic acid
At inoculation	L-cysteine HCl			
	glutathione			
	6-mercaptopurine			
30 min after inoculation		L-cysteine HCl	62	adenine
		glutathione	60	cyanocobalamin
				6-mercaptopurine
				L-tyrosine
				folic acid
				xanthine
				guanine

* Percentage antagonism = $\frac{\text{Rate of increase in } \beta\text{-galactosidase activity in the presence of inhibitor and antagonist} \times 100}{\text{Rate of increase in } \beta\text{-galactosidase activity in the absence of inhibitor and antagonist}}$

† β-galactosidase activity reduced

The potential antagonist was added to the lactose induction medium containing the inhibitor. Temperature: before inoculation 20°; after inoculation 40°.

became insensitive to glutathione (200 μg/ml, 651 μM) more rapidly than to cysteine (100 μg/ml, 635 μM), which indicates that the glutathione molecule is a less effective antagonist of 6-iodoacetyl-DMPT than the cysteine molecule. As the time of contact of the organism with 6-iodoacetyl-DMPT or with 6-chloroacetyl-DMPT increased (Table 4), the number of equivalents of cysteine required to antagonize its action also increased. A higher concentration of 6-iodoacetyl-DMPT required a disproportionately higher concentration of cysteine for complete antagonism. Thus, irreversible damage to the induction mechanism increased with increasing time of contact with the inhibitor and with increasing concentration of the inhibitor.

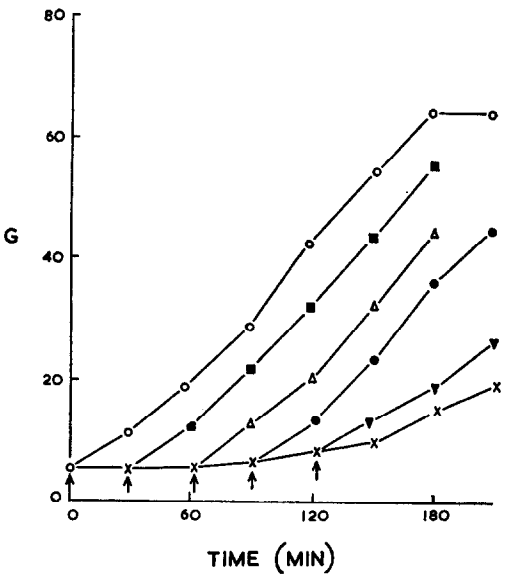


FIG. 12a

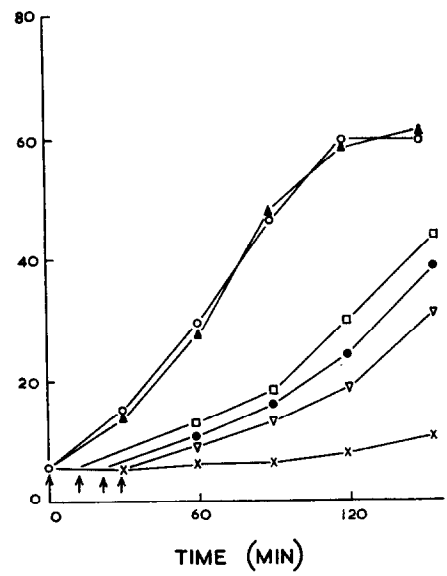


FIG. 12b

FIG. 12. Effect of addition of cysteine at 100 μg/ml (a) or glutathione at 200 μg/ml (b) at various times indicated by the arrows to cultures of *K. aerogenes* in which induction of β-galactosidase synthesis is being inhibited by 6-iodoacetyl-DMPT at 10 μg/ml. G is the β-galactosidase activity. ○ represents the control culture free of inhibitor and antagonist and × represents the inhibited culture, both of which were inoculated at zero time. (a) Addition of cysteine at the following times: zero time (○), 30 min (■), 60 min (△), 90 min (●), 120 min (▼). (b) Addition of glutathione at the following times: zero time (▲), 10 min (□), 20 min (●), 30 min (▽).

Cysteine exerted no antagonism to the action of IAA, and antagonized IAM less effectively than 6-iodoacetyl-DMPT (Table 4). Thus the iodoacetyl derivatives were antagonized by cysteine in the following order:—6-iodoacetyl-DMPT > IAM > IAA.

Studies of the rate of alkylation of antagonists by iodoacetyl derivatives

Certain compounds antagonize iodoacetyl derivatives by being alkylated by them. The alkylation is accompanied by the formation of iodide ions. Aqueous solutions of the iodoacetyl derivative at 10 μg/ml and antagonist at 100 μg/ml were mixed and

TABLE 4. ANTAGONISM BY CYSTEINE OF THE ACTION OF HALOGENOACETYL DERIVATIVES IN INHIBITING THE INDUCTION OF β-GALACTOSIDASE SYNTHESIS IN *K. AEROGENES*

Time in min of addition of antagonist after inoculation	Percentage antagonism* with cysteine as antagonist					
	Molar ratio antagonist: inhibitor					
	0·2:1	1:1	2:1	5:1	10:1	20:1
Inhibitor: 6-iodoacetyl-DMPT at 10 μg/ml						
0	24	100	100		100	
30		7	14	34		100
60		2	4	8		100
90						100
Inhibitor: 6-iodoacetyl-DMPT at 20 μg/ml						
0	21	61	83	100		100
10						46
20						25
30		0	0	0		15
45						0
Inhibitor: 6-chloroacetyl-DMPT at 18 μg/ml						
0		58	78	98	100	
30		33	56	60	72	100
60					44	100
90					7	58
Inhibitor: IAA at 40 μg/ml						
0		0			0	
30		0			0	
Inhibitor: IAM at 30 μg/ml						
0		11			65	
30		1			5	

$$\text{* Percentage antagonism} = \frac{\text{Rate of increase in } \beta\text{-galactosidase activity in the presence of inhibitor and antagonist} \times 100}{\text{Rate of increase in } \beta\text{-galactosidase activity in the absence of inhibitor and antagonist}}$$

TABLE 5. SPECIFIC RATES OF IODIDE ION FORMATION DURING THE REACTION BETWEEN IODOACETYL DERIVATIVES AND AQUEOUS SOLUTIONS OF THEIR ANTAGONISTS

Antagonist at 100 μg/ml	First order specific reaction rates in min^{-1} at 20°		
	6-iodoacetyl-DMPT at 10 μg/ml	IAM at 10 μg/ml	IAA at 10 μg/ml
cysteine	$6{\cdot}2 \times 10^{-1}$	$4{\cdot}0 \times 10^{-2}$	$5{\cdot}0 \times 10^{-3}$
glutathione	$5{\cdot}8 \times 10^{-1}$	$8{\cdot}2 \times 10^{-3}$	$9{\cdot}6 \times 10^{-4}$
6-mercaptopurine	$7{\cdot}7 \times 10^{-1}$	$3{\cdot}9 \times 10^{-3}$	$4{\cdot}2 \times 10^{-4}$
2,3-dimercaptopropanol	$5{\cdot}1 \times 10^{-1}$	$3{\cdot}8 \times 10^{-3}$	$2{\cdot}2 \times 10^{-4}$
methylthiouracil	$4{\cdot}8 \times 10^{-1}$	$2{\cdot}2 \times 10^{-3}$	$3{\cdot}4 \times 10^{-4}$
xanthine	$4{\cdot}6 \times 10^{-4}$	$2{\cdot}2 \times 10^{-4}$	$1{\cdot}7 \times 10^{-4}$
guanine	$3{\cdot}0 \times 10^{-4}$	$1{\cdot}9 \times 10^{-4}$	$1{\cdot}3 \times 10^{-4}$
adenine	$1{\cdot}8 \times 10^{-4}$	$2{\cdot}6 \times 10^{-4}$	$9{\cdot}9 \times 10^{-5}$
folic acid	$2{\cdot}4 \times 10^{-4}$	$6{\cdot}0 \times 10^{-5}$	$5{\cdot}3 \times 10^{-5}$
histidine	$3{\cdot}6 \times 10^{-4}$	$5{\cdot}6 \times 10^{-5}$	$1{\cdot}1 \times 10^{-4}$
tyrosine	$2{\cdot}0 \times 10^{-4}$	$7{\cdot}8 \times 10^{-5}$	$2{\cdot}4 \times 10^{-5}$
no antagonist	$3{\cdot}8 \times 10^{-4}$	$1{\cdot}9 \times 10^{-5}$	$2{\cdot}3 \times 10^{-5}$

kept at 20°. At 1-min intervals a 1 ml sample was determined for iodide ion by a sensitive catalytic method.[17] Since 6-iodoacetyl-DMPT interfered with the iodide determination, it was removed by shaking a 2 ml sample of the aqueous reaction mixture with 5 ml of chloroform.

Since the antagonist was in large excess, the reaction was of the first order (Table 5). The specific rates of liberation of iodide ion from the iodoacetyl derivatives with compounds which did not contain a thiol group were very similar to the rates in the absence of antagonists, i.e. for hydrolysis at the iodine atom. The specific rates of iodide ion formation with thiols were much greater than the above rates and therefore represent the true specific rates of alkylation of these antagonists; the rates were in the order: 6-iodoacetyl-DMPT > IAM > IAA.

Uptake of cysteine and glutathione by the bacterial cells

Bacteria were grown in nutrient broth, centrifuged, washed and rapidly resuspended in "salts" solution (to give M = 2500) containing cysteine at 20 μg/ml or glutathione at 40 μg/ml. At 1-min intervals after mixing, the concentration of thiol groups in a 1 ml sample was determined.[14] The equilibrium concentration of thiol groups (Table 6) in the suspension was obtained 1 min after mixing and remained unchanged for at least 20 min thereafter. Cysteine was more readily taken up than glutathione.

TABLE 6. UPTAKE OF CYSTEINE AND GLUTATHIONE BY *K. AEROGENES*

	Thiol concentration μg/ml	
Source of thiol groups	Cysteine	Glutathione
(a) Thiol compound alone	4·5	4·9
(b) Bacteria alone	1·0	1·0
a + b, assuming no uptake	5·5	5·9
Mixture of thiol compound and bacteria, assuming uptake was complete	1·8	4·8

Uptake is shown by the decrease in thiol concentration on mixing at 20° a suspension of bacteria at a concentration M = 2500 in "salts" solution with cysteine at 20 μg/ml or glutathione at 40 μg/ml.

DISCUSSION

None of the 6-acyl-DMPTs tested noticeably affected the DNA, RNA or total protein levels during growth or during induction of β-galactosidase by non-dividing bacteria. Furthermore, since none of the compounds tested inactivated β-galactosidase during the assay or within one hour of incubation with non-dividing bacteria, any observed effects refer to the induction of the synthesis of β-galactosidase. The importance of these considerations is discussed in Part I. β-Galactosidase synthesis was less inhibited in growing than in non-dividing bacteria. Thus, the bacteria were better able to antagonize the inhibition of β-galactosidase synthesis when growing in a lactose medium, in which the enzyme was required for growth,[15,16] than when not dividing in a lactose medium. In the following discussion "bacterial metabolism" refers to the growth of *Klebsiella aerogenes* NCTC 418 and the induction of β-galactosidase in non-dividing cells. The water solubility of DMPT was greatly reduced by 6-acylation and was still further reduced on replacement of acetyl hydrogen atoms by

halogen atoms (Table 2). This suggests the the distribution of the compounds might differ in the animal body and in the bacterial cell. Differences in distribution and dose may explain why 6-dichloroacetyl-DMPT and 6-acetyl-DMPT inhibited tumour growth[1] appreciably but bacterial metabolism only slightly or not at all.

6-Iodoacetyl-DMPT and 6-chloroacetyl-DMPT were the most active of the derivatives against both tumour growth[1] and bacterial metabolism and were the only ones capable of acting as or releasing biological alkylating agents.

The most effective antagonists of the action of 6-iodoacetyl-DMPT and 6-chloroacetyl-DMPT to be tested were the thiols, cysteine, glutathione and 6-mercaptopurine, which react rapidly with 6-iodoacetyl-DMPT as follows:—

$$R—SH + I—CH_2—CO— = R—S—CH_2—CO— + H^+ + I^-.$$

This suggests that these inhibitors act by alkylating the thiol groups of enzymes necessary for the synthesis or de-repression of β-galactosidase. The inhibition of β-galactosidase synthesis by suitable concentrations of 6-iodoacetyl-DMPT can be overcome by the organism, perhaps by (1) resynthesis of the damaged enzymes during the lag which precedes a fresh increase in β-galactosidase activity (Fig. 11) or (2) by chemical reactivation of the blocked thiol groups. The addition of each dose of 6-iodoacetyl-DMPT caused an immediate decrease in the thiol concentration of the organisms. The recovery of the bacteria was accompanied by the production of thiol groups, but the inhibition of β-galactosidase synthesis by 6-iodoacetyl-DMPT was not completely reversible (Fig. 11). Furthermore, increasing time of contact with the inhibitor (Figs. 8, 9 and 12) and increasing concentration of the inhibitor (Fig. 4 and Table 4) caused increasing damage and required a higher proportion of thiol for complete antagonism; the damage eventually became irreversible. Only two molecules of antagonist R—SH, are required for reactivation of each thiol group of an enzyme, E—SH, inhibited by an iodoacetyl derivative, $E—S—CH_2—CO— + R—SH = E—S—S—R + CH_3—CO—E—S—S—R + R—SH = E—SH + R—S—S—R$, whereas the actual number of molecular proportions of thiol required to restore β-galactosidase synthesis varied from 1 to 20 or more. On the whole, the first explanation is favoured for the recovery of the organism.

Since the molecule of 6-iodoacetyl-DMPT must remain intact to exert the greatest inhibition, and since the effect of the iodoacetyl group overwhelms that of the DMPT moiety, 6-iodoacetyl-DMPT probably does not act as a concurrent blocking agent. Studies of the chemical, toxic and tumour inhibitory properties of other N-substituted haloacetamides and haloacetate esters also indicate that the intact molecule is necessary for the greatest biological activity.[12]

The iodoacetyl derivatives fell into the following graded series in their degree and speed of inhibition of the induction of β-galactosidase, their antagonism by cysteine and their rates of reaction with thiol compounds: 6-iodoacetyl-DMPT > IAM > IAA. The speed with which the compounds inhibited the induction of β-galactosidase might be linked with their rate of penetration into the cell or with their rate of reaction with enzymes, both of which might also fall into this order. IAA (pH = 3·12) is considered to act slowly in penetrating cells[2,3] and in inactivating enzymes[18,19] because it is highly ionized at the physiological pH. The specific rate of alkylation of cysteine by 6-iodoacetyl-DMPT ($6·2 \times 10^{-2}$ min^{-1} at 20°) was much greater than the specific rate of spontaneous hydrolysis of 6-iodoacetyl-DMPT ($4·1 \times 10^{-4}$ min^{-1}

at 40°) and the specific rate of alkylation of cysteine by IAA ($5{\cdot}0 \times 10^{-3}$ min^{-1} at 20°). Therefore, even if hydrolysis of 6-iodoacetyl-DMPT were enzymatic and instantaneous, the liberated IAA would not react with cysteine at the rate observed for 6-iodoacetyl-DMPT. Thus, when the acyl linkage was intact, 6-iodoacetyl-DMPT was a more reactive alkylating agent than when this linkage had been hydrolysed, and the observed inhibition by 6-iodoacetyl-DMPT and its antagonism cannot be explained in terms of hydrolysis of this bond. Thus, 6-iodoacetyl-DMPT inhibited by acting as an alkylating agent in its own right and was more effective than IAM or IAA. The DMPT part of the 6-iodoacetyl-DMPT molecule had therefore increased the reactivity of the iodoacetyl group with thiol compounds and with cellular materials, while probably also acting as a carrier and thereby increasing the ability of the iodoacetyl group to penetrate the cell.

6-Chloroacetyl-DMPT probably acts as a biological alkylating agent like 6-iodoacetyl-DMPT, since its action was antagonized by a very similar range of compounds as the action of 6-iodoacetyl-DMPT (Table 3).

6-Chloroacetyl-DMPT was in all respects a less potent inhibitor and alkylating agent than 6-iodoacetyl-DMPT and was less readily antagonized by cysteine, which indicates that 6-chloroacetyl-DMPT reacts more slowly with thiols. Chloroacetic acid is itself a less potent inhibitor and alkylating agent than IAA.[2,6,7] 6-Dichloroacetyl-DMPT was rapidly destroyed by hydrolysis and is unlikely to act as an alkylating agent. 6-Acetyl-DMPT and 6-fluoroacetyl-DMPT cannot act as alkylating agents. 6-Fluoroacetyl-DMPT was inactive against tumours and bacterial metabolism, presumably because the acetyl bond is too stable to release fluoroacetic acid by hydrolysis and suggests that enzymatic hydrolysis does not occur with any of the 6-acyl-DMPT derivatives.

The following conclusions may be drawn concerning the validity of the original hypotheses for the mode of action of the 6-acyl-DMPTs. Hypothesis (a) is incorrect; the 6-halogenoacetyl-DMPT derivatives do not act as concurrent blocking agents and their action cannot be explained in terms of their hydrolysis in the cell. Hypothesis (b) is probably correct; the conversion of iodoacetic acid to 6-iodoacetyl-DMPT appears to increase the rate and degree of penetration into the cell. Hypothesis (c) is correct; 6-iodoacetyl-DMPT and 6-chloroacetyl-DMPT act as biological alkylating agents in their own right. No evidence was forthcoming for or against the ability of the compounds investigated to act as biological acylating agents according to hypothesis (d). Alkylation, where it takes place, is so powerful that acylation, if it occurs, must play a minor role.

Acknowledgements—The authors wish to thank Mr. A. A. Alvey and Mr. P. J. Wragg for technical assistance, the Medical Research Council for a research and training grant (for G.M.A.) and to the British Empire Cancer Campaign for financial support.

REFERENCES

1. R. W. BALDWIN, M. W. PARTRIDGE and M. G. F. STEVENS, *J. Pharm. Pharmac.* **18**, 1S (1966).
2. J. L. WEBB, *Enzyme and Metabolic Inhibitors*, vol. 3, pp. 13, 168, 270, 18, 274, 9. Academic Press, New York (1966).
3. E. RACKER and I. KRIMSKY, *Fedn Proc.* **17**, 1135 (1958).
4. M. M. BLACK and I. S. KLEINER, *Cancer Res.* **7**, 717 (1947).
5. M. M. BLACK, I. S. KLEINER and H. BOLKER, *Cancer Res.* **7**, 818 (1947).
6. E. S. G. BARRON, *Advanc. Enzymol.* **11**, 201 (1951).

7. S. DALGAARD-MIKKELSEN, S. A. KVORNING and K. O. MØLLER, *Acta Pharmac. Tox.* **11**, 13 (1955).
8. R. A. PETERS, *Disc. Faraday Soc.* No. 20, *The Physical Chemistry of Enzymes*, p. 189 (1955).
9. R. A. LEWIN and R. H. MINTZ, *Archs Biochem. Biophys.* **54**, 246 (1955).
10. J. N. STANNARD, *Am. J. Physiol.* **122**, 379 (1938).
11. O. M. FRIEDMAN, S. FENIG and H. SOMER, *Proc. Am. Assoc. Cancer. Res.* **2**, 299 (1958).
12. S. P. KRAMER *et al. J. Natl. Cancer Inst.* **31**, 297 (1963).
13. M. W. PARTRIDGE and M. G. F. STEVENS, *J. Chem. Soc. C*, 1127 (1966).
14. Y. N. IL'INA, B. A. TALMUD and P. V. AFANAS'EV, *Prikl. Biochim i Mikrobiol.* **1**, 352 (1965) *Chem. Abstracts* **63**, 11985d (1965).
15. N. RICHARDS and SIR CYRIL HINSHELWOOD, *Proc. Roy. Soc.* **B, 154**, 463 (1961).
16. N. RICHARDS and SIR CYRIL HINSHELWOOD, *Proc. Roy. Soc.* **B, 156**, 20 (1962).
17. E. B. SANDELL and J. M. KOLTHOFF, *Mikrochim. Acta* **1**, 9 (1937).
18. J. L. STILL, *Biochem. J.* **34**, 1374 (1940).
19. A. A. APP and A. N. MEISS, *Archs Biochem. Biophys.* **77**, 181 (1958).