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

INHIBITION BY 3,4-DIMETHYLPYRAZOLO[3,2-*c*]-*as*-TRIAZINE AND ITS ANTAGONISM BY HISTIDINE

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

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

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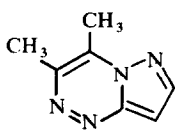
Abstract—The action of the tumour inhibitor, 3,4-dimethylpyrazolo[3,2-*c*]-*as*- triazine (DMPT), on the bacterium *Klebsiella aerogenes* NCTC 418 has been studied. DMPT at concentrations of the order 1 g/l. (6 mM) inhibited the growth of *K. aerogenes* and inhibited the induction of β-galactosidase in non-dividing cell suspensions, but did not affect the levels of DNA, RNA and total protein during growth. The purine analogues 6-mercaptopurine and 8-azaguanine gave other, different effects. The inhibition by DMPT of β-galactosidase induction was antagonized by histidine but not by other amino acids or by purines. DMPT also inhibited the induction of histidine ammonia-lyase and the inhibition was antagonized by histidine. The activity of histidine ammonia-lyase (K_m = 25 mM) was inhibited non-competitively by DMPT (K_i = 2·7 mM) and inhibition was total with DMPT at 3·4 mM. The effects of DMPT are discussed and it is concluded that DMPT does not act as a purine analogue but interferes with the metabolism of or with an essential function of histidine.

3,4-DIMETHYLPYRAZOLO[3,2-*c*]-*as*-TRIAZINE (DMPT) and certain of its derivatives inhibit the growth of mouse sarcoma S 180 and both the Walker tumour and a methylcholanthrene-induced tumour in rats.[1] DMPT has low-toxicity at tumour inhibitory concentrations. We wished to study the biochemistry of tumour inhibition by DMPT using, instead of tumours, *Klebsiella aerogenes* NCTC 418, a bacterium whose biochemistry is fairly well known and upon which the effect of inhibitors has been extensively studied.[2] Bacteria are useful systems for investigating the biochemistry of tumour inhibitors[3–5] and for screening potential tumour inhibitors.[6–8] The present studies proceeded through the following stages: (i) effect of the inhibitor on the rate of overall growth; (ii) effect on the levels of DNA, RNA and total protein; (iii) search for a cellular process sensitive to the inhibitor, such as an enzymic reaction or the induced synthesis (induction) of an enzyme, which are much more sensitive indicators of changes in metabolism than the levels of DNA, RNA and total protein; (iv) search for antagonists to the action of the inhibitor. The sensitive cellular processes used were the activity of L-histidine ammonia-lyase and the induction of this enzyme and of β-galactosidase. β-Galactosidase is induced by some β-D-galactopyranosides, e.g. lactose and methyl-β-D-thiogalactopyranoside, and is required by *K. aerogenes* for growth in chemically-defined medium containing lactose as sole source of carbon and energy.[9, 10] Enzyme induction is studied most easily in systems in which cell division and growth are not occurring. When *K. aerogenes* grown in nutrient broth is trans-

ferred to chemically-defined media of the type used, a lag of several hours usually precedes cell division and growth. If the new medium contains an inducer for β-galactosidase, the bacteria synthesize the enzyme at a more or less constant rate after a lag of about 3 min.[11] If the new medium contains L-histidine, the induction of L-histidine ammonia-lyase can be studied.

The structure of DMPT (I) resembles those of purines (II), e.g. adenine (R^1 = NH_2, R^2 = H), guanine (R^1 = OH, R^2 = NH_2), xanthine (R^1 = R^2 = OH), and hypoxanthine (R^1 = OH, R^2 = H); this suggests that DMPT might act as a purine analogue. The purine analogues, 6-mercaptopurine (6-MP; II, R^1 = SH, R^2 = H) and 8-azaguanine (8-AZG; III) which are known to inhibit the growth of tumours and of *Lactobacillus casei* by antagonism of natural purines,[3] were therefore used as reference compounds.



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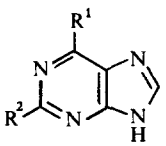

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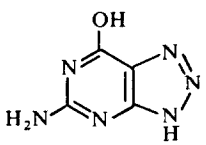


III

MATERIALS AND METHODS

Inhibitors and antagonists. DMPT was prepared by the method of Partridge and Stevens.[12] The term "histidine" refers to L-histidine monohydrochloride monohydrate, chromatographically homogeneous. Solutions of inhibitors and antagonists were sterilized, when necessary, by membrane filtration at room temperature.

The ultraviolet absorption spectra of DMPT in N/100 HCl and in distilled water each at 40° were unchanged after 8 days, whereas that of DMPT in N/100 NaOH at 40° changed slightly after 8 hr. DMPT was therefore stable to water and acid but was slowly decomposed by alkali.

Organism. The organism used was *Klebsiella aerogenes* NCTC 418, formerly known as *Aerobacter aerogenes*, *Bacterium lactis aerogenes* and *Bacterium aerogenes*, strain number 240.

Media. Nutrient broth media consisted of "Oxoid Nutrient Broth No. 2". Chemically defined media consisted of the following concentrations of analytical grade reagents in glass-distilled water: Na_2HPO_4, 2·41 g/l.; KH_2PO_4, 1·125 g/l.; $(NH_4)_2SO_4$, 0·950 g/l.; $MgSO_4 . 7H_2O$, 38·0 mg/l.; $FeSO_4 . 7H_2O$, 0·198 mg/l.; glucose, lactose or histidine as sole source of carbon and energy; pH 7·12. The "standard glucose (or lactose) medium" contained 3·85 g of glucose (or lactose)/1. The "limiting glucose (or lactose) medium" contained 1·54 g of glucose (or lactose)/1. The "histidine medium" contained 2·00 g of histidine monohydrochloride monohydrate/1. The mineral salts and the carbon source were sterilized separately before mixing under aseptic conditions.

Culture conditions. A stock strain of the organism, which has been fully conditioned to growth in standard glucose medium, was maintained by monthly subculture in nutrient broth, contained in airtight bottles at 15–20°. For the maintenance of other strains and for the determination of growth curves each culture was grown in 26·0 ml of sterile medium contained in a "Pyrex" 150 × 25 mm boiling tube sealed with a

sterile cotton-wool plug and maintained at 40·0° in a water bath. Through the cotton wool plug passed a sterile 1 ml graduated pipette for withdrawing samples of culture, if necessary, and a sterile Pasteur pipette through which filtered air was passed for aeration and agitation.

From the stock strain the following strains were prepared: a "nutrient broth strain" was subcultured daily in nutrient broth at 40·0° with aeration; a "glucose (or lactose) adapted strain" was subcultured daily for at least 15 days in standard glucose (or lactose) medium.

Large quantities of bacteria for chemical and enzyme assays and for experiments involving enzyme induction were grown in 1–7 l. quantities of medium contained in "Pyrex" flasks and maintained at 40·0° in a water bath. Each flask was strongly aerated through glass spargers in the medium and was fitted with a siphon tube for rapid sampling.

Growth was normally terminated by accumulation of toxic waste products, change in pH value and shortage of oxygen. When it was considered desirable that the lag on subsequent subculture should be as short as possible, exhaustion of the carbon source was arranged to limit growth; the parent was then cultured in limiting glucose (or lactose) medium.

Buffer solutions. "Salts solution": Na_2HPO_4, 3·91 g/l.; KH_2PO_4, 1·83 g/l.; $(NH_4)_2SO_4$, 1·54 g/l.; $MgSO_4 . 7H_2O$, 61·7 mg/l.; $FeSO_4 . 7H_2O$, 0·16 mg/l.; pH 7·12. "Phosphate buffer (pH 7·12)": Na_2HPO_4, 6·34 g/l.; KH_2PO_4, 2·96 g/l. "Pyrophosphate buffer (pH 8·9)": $Na_4P_2O_7 . 10H_2O$, 0·758 g/l., adjusted to pH 8·9 with HCl. "Phosphate–citrate buffer (pH 4·6)": citric acid, 6·49 g/l.; $Na_2HPO_4 . 12 H_2O$, 19·25 g/l.

Measurement of bacterial concentration. Bacterial concentration (M) was determined turbidimetrically.[13] The dry weight of bacteria in 1 ml of a suspension of unit bacterial concentration (M = 1·0) was 0·52 μg. All cultures were initiated with a standard inoculum, usually M = 3·0. Growth curves were determined by plotting log M against the time after inoculation.

Determination of DNA, RNA and total protein. Sufficient culture to satisfy the equation, ml of culture = 54,000/M of culture, was centrifuged. The bacterial pellet was washed in phosphate buffer (pH 7·12), recentrifuged and resuspended in sufficient phosphate buffer (about 18 ml) to give M = 3000. Ten ml of the suspension was treated[14] with $HClO_4$ to give a soluble extract of nucleic acids and a precipitate of protein. After centrifugation, the supernatant was assayed for DNA[15] by Burton's modification of Dische's diphenylamine method and for RNA[16] by Ceriotti's modification of Bial's orcinol method and the precipitate was assayed for total protein.[17] The respective assays were calibrated with the sodium salt of DNA from calf thymus (B.D.H.), yeast RNA and crystalline bovine plasma albumin Fraction V (Armour). The results were expressed in μg of DNA, RNA or protein divided by M of the final bacterial suspension.

Ultrasonic disintegration of bacteria. Bacteria in suspension were ultrasonically disrupted by applying for a suitable time the maximum ultrasonic power from a MSE 60 watt ultrasonic disintegrator through a $\frac{3}{4}$ in. (19mm) titanium probe to 6 ml of a suspension of bacteria in the appropriate buffer at 0°. The instrument was adjusted to give a meter reading of 1·5 amp with the probe just breaking the surface of the suspension.

Determination of β-galactosidase activity. The bacteria were suspended in phosphate

buffer (pH 7·12) to give M = 3000 and were disrupted ultrasonically for 4 min. The β-galactosidase activity was then determined by a modification[9, 10] of the method of Lederberg[18] and expressed in the units defined by Richards and Hinshelwood.[9, 10]

Induction of β-galactosidase synthesis in non-dividing bacteria. A modification of Creaser's method[19] was used in which β-galactosidase is synthesized preferentially to total bacterial substance. The nutrient broth strain was grown overnight in 1 l. of nutrient broth. The bacteria were harvested by centrifugation, washed with phosphate buffer (pH 7·12), centrifuged and resuspended in salts solution to give M = 12,000. twenty ml of this suspension was added to 80 ml of the following aerated induction medium at 40·0°: Na_2HPO_4, 1·95 g/l.; KH_2PO_4, 0·92 g/l.; $(NH_4)_2SO_4$, 0·77 g/l.; $MgSO_4 . 7H_2O$, 30·9 mg/l.; $FeSO_4 . 7H_2O$, 0·08 mg/l.; lactose, 25 g/l.; pH 7·12. Every 30 min, a 10 ml sample was pipetted into 25 ml of phosphate buffer (7·12) at room temperature, centrifuged and the bacteria were washed with phosphate buffer, centrifuged and suspended in 8 ml of phosphate buffer to give M = 3000. M and the β-galactosidase activity were determined and the β-galactosidase activity was plotted against the time after inoculation. All results for similarly treated cultures obtained on the same day were the same and any variation from day to day was small.

Determination of L-*histidine ammonia-lyase activity.* The bacteria were suspended in pyrophosphase buffer (pH 8·9) to give M= 3000 and were disrupted ultrasonically for 2 min. The histidine ammonia-lyase activity was then determined by a modification of the methods of Tabor and Meyler[20] and Magasanik.[21] one ml of disintegrated bacterial suspension and 0·5 ml of a solution of 3 g of reduced glutathione/l. of pyrophosphate buffer (pH 8·9) were mixed. One ml of a solution of 2·10 g of histidine/l. of water, adjusted to pH 9 with NaOH, was added. The increase in extinction at 277 mμ of the product, urocanic acid, was measured at intervals over 8 min at 20°. The histidine ammonia-lyase activity was expressed in units of mμ moles of urocanic acid produced per min divided by M of the added bacterial suspension.

Induction of L-*histidine ammonia-lyase synthesis in non-dividing bacteria.* Histidine ammonia-lyase was induced in the same manner as β-galactosidase, except that the induction medium contained histidine (0·2 g/l.) instead of lactose, and, after being washed, the bacterial samples from the induction medium were resuspended in pyrophosphate buffer (pH 8·9) to give M = 3000, and were then assayed for histidine ammonia-lyase.

RESULTS

Effect of DMPT on the rate of growth

Growth curves of the glucose-trained strain in standard glucose medium containing various concentrations of the inhibitors were determined; the inhibitor was omitted in the control. For each concentration of a given inhibitor duplicate cultures were grown; the experiment was repeated. The time (T) taken for the culture to grow from its inoculum size, M = 3, to M = 200 was found to be more reproducible than either the lag or the mean generation time and was plotted against the concentration of the inhibitors (Fig. 1). 6-MP from 0 to 100 μg/ml increased T from 220 min to 250 min but had no further effect up to its solubility limit of 375 μg/ml. 8-AZG from 0 to 35 μg/ml increased T from 220 min to 285 min, and thereafter increased T slightly to 315 min at the solubility limit of 155 μg/ml. DMPT at up to 100 μg/ml had a negligible

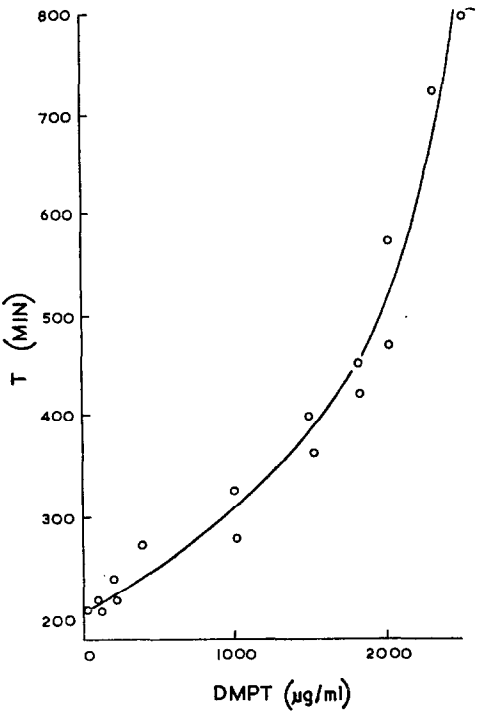


FIG. 1. Effect of various concentrations of DMPT on the growth of *K. aerogenes* in glucose medium. T is the time in min taken for the bacterial concentration, M, to increase from 3·0 to 200.

effect on T. With increasing concentration of DMPT, T increased steadily (Fig. 1). DMPT at 3000 μg/ml inhibited growth completely.

Effect of DMPT on the β-galactosidase activity of resting 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: DMPT, 1000 μg/ml; 6-MP, 200 μg/ml; 8-AZG, 101 μg/ml. The β-galactosidase activity was determined after centrifuging and washing the bacteria and was found to be unaffected by incubation with the inhibitors for up to 200 min. Similarly, 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 DMPT on DNA, RNA, total protein and β-galactosidase activity during growth

Lactose trained bacteria were grown in 7 l. of standard lactose medium containing the inhibitor at a partially inhibitory concentration and in 3·5 l. of similar medium free of inhibitor as a control. The inoculum size was adjusted to give M = 50 and at intervals of about 30 min the cultures were assayed. In the presence of DMPT at 1000 μg/ml, 6-MP at 200 μg/ml or 8-AZG at 101 μg/ml, the bacteria had the same DNA, RNA and total protein levels and β-galactosidase activity as the controls at the same, point in the growth cycle, with the exception that 8-AZG increased the DNA level and reduced the β-galactosidase activity (Fig. 2). Since β-galactosidase is required for growth in lactose medium,[9, 10] a reduced β-galactosidase activity might merely reflect

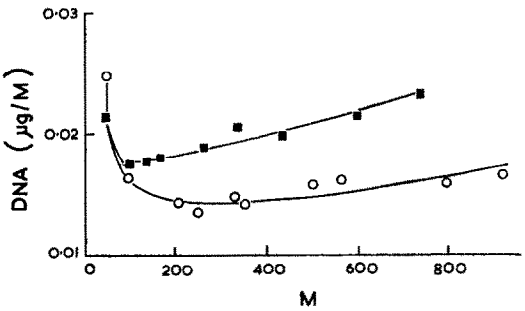


FIG. 2a

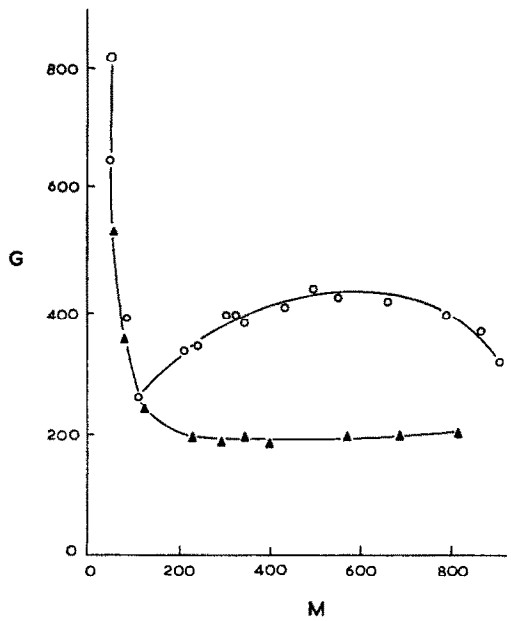


FIG. 2b

FIG. 2. DNA content and β-galactosidase activity, G, of *K. aerogenes* during growth in lactose medium in the presence of 8-AZG at 51 μg/ml (■) and 101 μg/ml (▲) and in the absence of the inhibitor as control (○). M is the bacterial concentration. The results with DMPT at 1000 μg/ml were the same as for the control in a separate experiment.

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, the reduction in β-galactosidase activity by 8-AZG at 101 μg/ml does not entirely reflect the mean generation time of 63 min but must partly result from other causes.

Since hydrogen sulphide gas was evolved from the cultures containing 6-MP at 200 μg/ml, 6-MP was metabolized by the bacteria.

Effect of DMPT added initially on the induction of β-galactosidase synthesis

Various concentrations of DMPT or 8-AZG were added to samples of the lactose induction medium before the bacteria were added. DMPT at concentrations which retarded growth (Fig. 1) also reduced the rate of synthesis of β-galactosidase but did

not delay the commencement of induction (Fig. 3). The rate of enzyme synthesis decreased with increasing concentration of DMPT; 750 μg/ml reduced the rate by 50 per cent and 2500 μg/ml reduced it to zero. 8-AZG at 100 μg/ml slightly reduced the rate of synthesis of β-galactosidase for the first hour, after which induction ceased. The presence of both 8-AZG at 100 μg/ml and guanine at 50 μg/ml gave the same kinetics of induction as the control culture free of inhibitor, which shows that guanine antagonizes the action of 8-AZG, as expected.

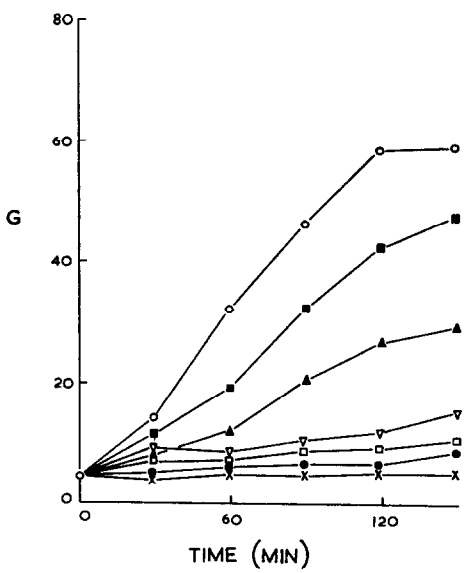


FIG. 3

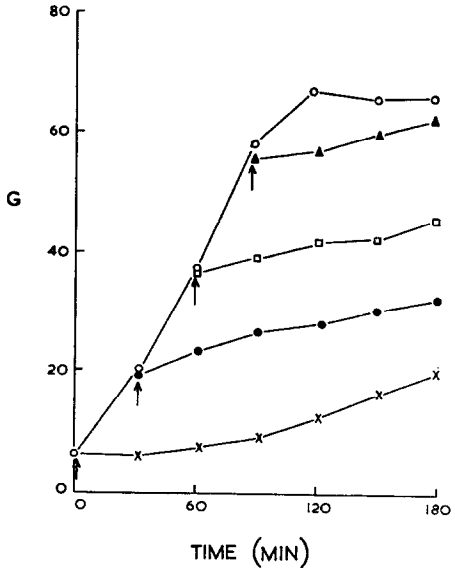


FIG. 4

FIG. 3. Effect of the following concentrations of DMPT present before inoculation at zero time on the induction of β-galactosidase synthesis: 0 (○), 500 (■), 750 (▲), 1000 (▽), 1500 (□), 2000 (●), 2500 μg/ml (×). G is the β-galactosidase activity.

FIG. 4. Effect of the addition of DMPT (1000 μg/ml) at various times (indicated by the arrows) after inoculation at zero time on the induction of β-galactosidase synthesis. The points represent the β-galactosidase activity, G, of the uninhibited control ○, and after addition of DMPT at zero time ×, at 30 min ●, at 60 min □, and at 90 min ▲.

The presence of DMPT at 1000 μg/ml or 1500 μg/ml did not affect the fairly constant levels of DNA (0·24 μg/M), RNA (0·16 μg/M) and total protein (0·38 μg/M) during the induction experiments described above.

Effect of addition of DMPT to a system actively synthesizing β-galactosidase

To uninhibited lactose systems in which induction had been occurring for 0, 30, 60 and 90 min (arrows in Fig. 4) DMPT was added to produce an overall concentration of 1000 μg/ml. Measurements of the β-galactosidase activity of the systems at intervals showed that, immediately after addition of DMPT, the rate of enzyme synthesis was reduced in comparison with the uninhibited control culture.

Antagonism of the action of DMPT in inhibiting the induction of β-galactosidase synthesis

To a lactose induction medium containing DMPT at 1000 μg/ml, the potential antagonist at 200 μg/ml was added, followed by the bacteria, and the β-galactosidase activity was determined at various times. Controls containing (i) DMPT but no antagonist, (ii) antagonist but no DMPT and (iii) neither DMPT nor antagonist were also inoculated. The inhibitory action of DMPT was antagonized by L-histidine monohydrochloride monohydrate (see below) and by ergothionine (15 per cent antagonism). The following compounds produced no antagonism:—(a) amino acids;-DL-α-alanine, L-arginine monohydrochloride, DL-aspartic acid, L-cysteine hydrochloride,L-cystine, L-glutamic acid, glycine, L-hydroxyproline, L-leucine, DL-*iso*-leucine, DL-*nor*-leucine, L-lysine monohydrochloride, DL-methionine, DL-ornithine hydrochloride, DL-β-phenylalanine, L-proline, DL-serine, DL-threonine, DL-trypophane, L-tyrosine, DL-valine; (b) purines;—adenine, guanine, xanthine; (c) other compounds;—folic acid, cyanocobalamin, glutathione, albumin, histamine acid phosphate, L-carnosine, allantoin, benzimidazole.

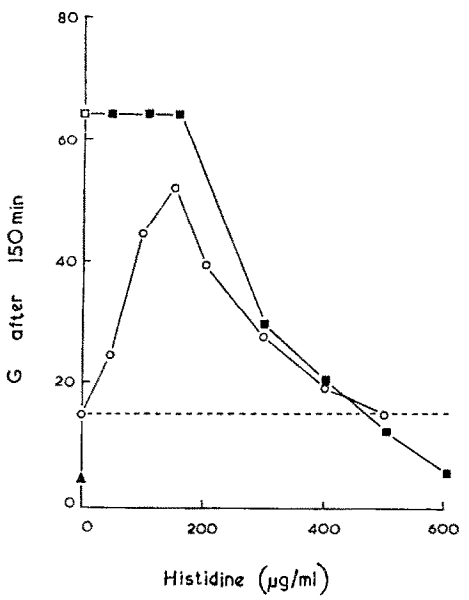


FIG. 5. Effect of L-histidine monohydrochloride monohydrate and DMPT on β-galactosidase induction as measured by the β-galactosidase activity, G, attained 150 min after inoculation of *K. aerogenes* into the induction medium. Various concentrations of histidine with DMPT at 1000 μg/ml (○) and without DMPT (■). Control without histidine and DMPT (□). DMPT at 1000 μg/ml without histidine (- - - - -). Inoculum (▲).

Lactose induction systems containing various concentrations of histidine with or without DMPT at 1000 μg/ml were inoculated and the β-galactosidase activity was determined 150 min later (Fig. 5). Histidine at 0–400 μg/ml reduced (i.e. antagonized) the inhibitory effect of DMPT on β-galactosidase induction, but at > 200 μg/ml itself inhibited induction. Histidine at 0–150 μg/ml did not inhibit induction but at > 200 μg/ml partially inhibited induction. Histidine at 300 μg/ml reduced the rate of enzyme

synthesis by about 50 per cent and 500 μg/ml reduced it to zero. Histidine at 150 μg/ml exhibited a high and maximal antagonism of the inhibition caused by DMPT.

Effect of DMPT on the activity of L-*histidine ammonia-lyase*

The Michaelis constant, K_m, of histidine ammonia-lyase was found to be 25 mM at 20° and was unaffected by the presence of DMPT (Fig. 6). The extrapolated value of the maximum rate of the enzyme reaction was reduced by the presence of DMPT at

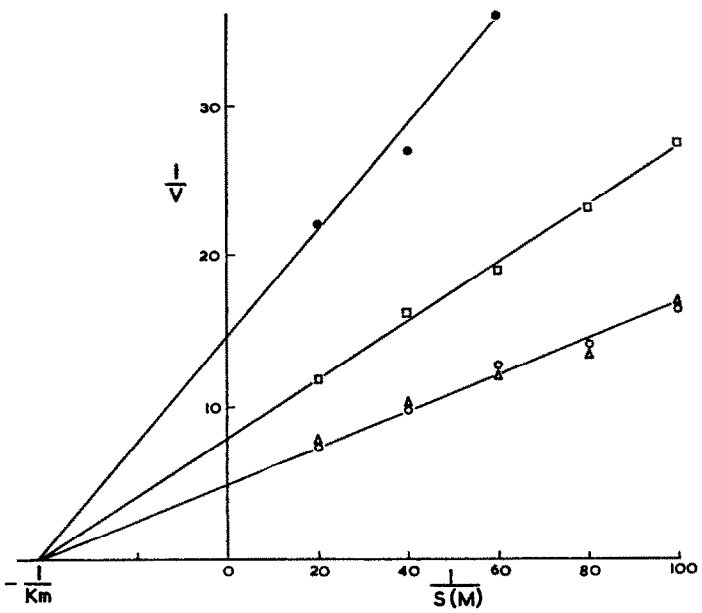


FIG. 6. Lineweaver–Burk[22] plot of L-histidine ammonia-lyase activity in the presence of DMPT at 0 (○), 250 (△), 350 (□) and 400 (●) μg/ml. S is the molar concentration of histidine. V is the rate of the reaction in units of 10^{-3} × activity defined in the Materials and Methods section.

concentrations greater than about 250 μg/ml and was negligible in the presence of DMPT at about 500 μg/ml. Thus DMPT inhibited the activity of histidine ammonia-lyase non-competitively. The inhibitor constant, K_i, of the inhibition was determined from the Lineweaver–Burk[22, 23] plot (Fig. 6) and was found to be 2·7 mM. Thus the affinity of the enzyme for DMPT was an order of magnitude greater than the affinity of the enzyme for the substrate.

Effect of DMPT on the induction of histidine ammonia-lyase synthesis

Various concentrations of DMPT were added to samples of the histidine induction medium before the bacteria were added. DMPT at concentrations which retarded growth (Fig. 1) also reduced the rate of induction of histidine ammonia-lyase, but did not delay the induction. The rate of enzyme synthesis decreased with increasing DMPT concentration (Fig. 7) but was not zero even in the presence of DMPT at 3000 μg/ml.

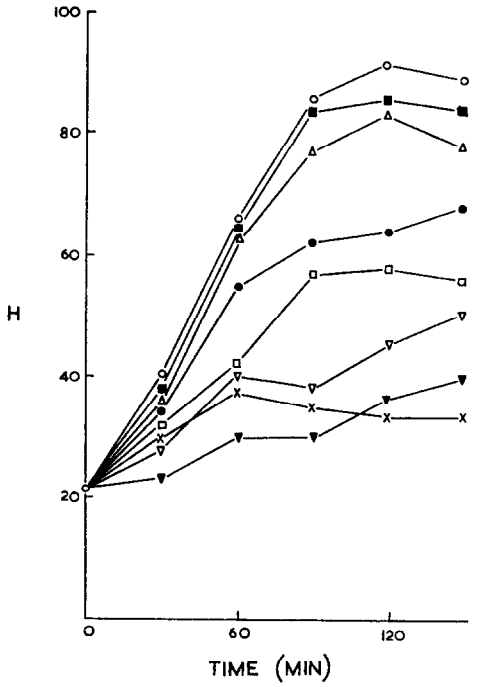


FIG. 7. Effect of the following concentrations of DMPT present before inoculation at zero time on the induction of L-histidine ammonia-lyase synthesis: 0 (○), 500 (■), 750 (△), 1000 (●), 1500 (□) 2000 (▽), 2500 (▼), 3000 μg/ml (×). H is the L-histidine ammonia-lyase activity.

Absence of chemical reaction between DMPT and histidine

Solutions containing DMPT (2000 μg/ml) or histidine (2000 μg/ml) or both were incubated at 40° for various times up to 24 hr and their ultraviolet absorption spectra were compared. The presence of histidine did not change the absorption spectrum of DMPT and vice versa. The above solutions were also shaken with chloroform to extract any possible DMPT–histidine complexes. The chloroform extracts were dried with anhydrous $MgSO_4$ and allowed to evaporate. The melting point and mixed melting point of the solid obtained were identical with that of DMPT. Thus chemical reaction between DMPT and histidine is unlikely and complex formation is probably weak or negligible.

DISCUSSION

The effects of the reference compounds, 6-MP and 8-AZG, on *Klebsiella aerogenes* NCTC 418 in the present work resemble in the following respects those previously reported with other bacteria. 6-MP (or 8-AZG) at increasing concentrations increasingly retarded growth but had no further effect above a certain concentration as with *Bacillus cereus*.[24, 25] 8-AZG retarded growth of *K. aerogenes*, increased the level of DNA and reduced the rate of synthesis of β-galactosidase during growth and completely inhibited the induction of β-galactosidase by non-dividing cells, although after a delay of 30 min; the latter inhibition was fully antagonized by guanine. These effects resemble those with *B. cereus*[26–28] and *Staphylococcus aureus*.[29, 30] The total protein levels in *K*

aerogenes were unaffected when growth was partially inhibited by 6-MP or 8-AZG as observed with *B. cereus*.[4, 31] 6-MP was only a feeble inhibitor of *K. aerogenes*, the only effects observed being a slight reduction in growth rate, although 6-MP was metabolized by this organism as observed with *B. cereus*.[24, 32]

In certain studies [29, 33, 34] of the effects of inhibitors on the induction of enzymes, such as β-galactosidase, by determination of enzymic activity, the effects of the inhibitor on activity have not been reported. Unless it has been shown that incubation of intact bacteria with the inhibitor over a reasonable time period, such as 1 hr, is without effect, the inhibitor could be inactivating a certain proportion of the enzyme as it was being synthesized or shortly afterwards. Similarly, unless it has been shown that the inhibitor is without effect on the enzyme assay, any inhibitor bound to the cells could be liberated during cellular disruption and could then inhibit the enzyme during its assay. In the present work 6-MP, 8-AZG and DMPT were without effect on the activity of β-galactosidase under the above conditions. This indicates that the observed inhibitory and non-inhibitory effects on the induction of β-galactosidase were real. In the present work DMPT inhibited the activity of histidine ammonia-lyase, which renders questionable the interpretation of the inhibitory effect of DMPT on the induction of this enzyme.

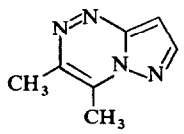
DMPT at concentrations of the order 1 g/l. (6 mM) which inhibited growth, did not inhibit the synthesis of β-galactosidase relative to growth during the bacterial growth cycle, but did reduce the rate of synthesis of β-galactosidase without a lag in an induction system in which cell division did not occur. The bacteria were able to antagonize the inhibition of β-galactosidase synthesis when growing in a lactose medium in which the enzyme was required for growth,[9, 10] than when resting in a lactose medium. Thus, the effects of DMPT on the organism were different from those of the purine analogues, 6-MP and 8-AZG.

The action of DMPT in inhibiting β-galactosidase induction was not antagonized by purines. The only effective antagonist among a range of amino acids and imidazole derivatives was histidine. The action of DMPT in inhibiting the induction of histidine ammonia-lyase was also antagonized by histidine, the inducer and substrate. DMPT inhibited β-galactosidase induction at about 500 to 2500 μg/ml (4–20 mM) and 750 μg/ml (5 mM) reduced the rate of induction by about 50 per cent. Histidine inhibited β-galactosidase induction at about 200 to 500 μg/ml (1–2·5 mM) and 300 μg/ml (1·5 mM) reduced the rate of induction by about 50 per cent. Histidine at 150 μg/ml (0·7 mM) also antagonized the inhibition by DMPT. These results suggest that DMPT and histidine inhibit at the same receptor site, that their effects are not additive and that the site has a higher affinity for histidine than for DMPT.

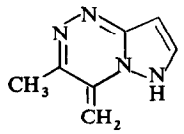
Histidine ammonia-lyase had a much higher affinity for the non-competitive inhibitor, DMPT (K_i = 2·7 mM) than for the substrate, histidine (K_m = 25 mM). Since inhibition was non-competitive, the substrate binding site and the inhibited site do not significantly influence each other. Since finite concentrations of the inhibitor (DMPT at 500 μg/ml, 3·4 mM) caused the rate of the enzyme reaction to fall to zero, the enzyme–substrate–inhibitor complex does not break down at all and so the inhibitor effectively reduces the amount of active enzyme (Dixon and Webb,[23] type IIa inhibition).

The above results suggest that DMPT does not act as a purine antagonist or analogue; it interferes with the metabolism of or with an essential function of histidine.

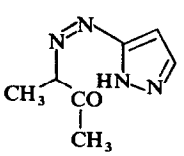
Complex formation between DMPT and histidine is probably weak or negligible. DMPT shows the reactions of two tautomers[12] (IV and V) either of which may undergo ring opening under basic conditions[35] to give one or both of two other tautomers (VI and VII). The observed inhibitions could result from the intervention of IV, V, VI or VII in the metabolism or function of histidine.



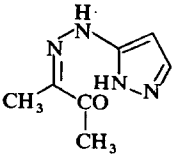
IV



V



VI



VII

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