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5-MERCAPTODEOXYURIDINE—ITS ENZYMATIC SYNTHESIS AND MODE OF ACTION IN MICROBIOLOGICAL SYSTEMS*

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Abstract—5-Mercapto-2′-deoxyuridine (MUdR) was synthesized by enzymatic transfer of the deoxyribosyl group of thymidine (TdR) to 5-mercaptouracil (MU), by using the *trans-N*-deoxyribosylase from *Lactobacillus helveticus*. The acceptor and donor activities of MU, MUdR and their respective disulfides were studied in this enzyme system; the disulfides were found inactive as substrates, but they showed the same growth inhibitory activities as the corresponding thiols in the microbiological assays. MUdR was nearly as active as 5-fluoro-2′-deoxyuridine (FUdR) in the *Lactobacillus leichmannii* assay, but was considerably less active than FUdR in the *Lactobacillus arabinosus* and *Streptococcus faecalis* systems. Inhibition analysis studies indicated that MUdR, like FUdR, acts via inhibition of thymidylate synthetase. The significant differences found in the spectra of activities of MU and MUdR, as compared to those of 5-fluorouracil (FU) and FUdR, are related to differences in the routes and efficiency of their metabolic activation in which the mercapto analogs, due to the size of their 5-S^- group, are restricted to the pathways available for the metabolic transformations of thymine and TdR, while the fluoro analogs may be converted to the nucleotide via the uracil pathway. Some synergism and cross-resistance studies are also reported.

THE SYNTHESIS of 5-mercaptouracil (MU), a structural analog of thymine, was reported several years ago.[1, 2] This compound and some of its S-substituted derivatives, including MU-disulfide, were found to act as competitive antagonists of thymine in *Lactobacillus leichmannii*[3] and to potentiate, in several experimental neoplasms,[4–6] the antitumor activities of 5-fluorouracil (FU), 5-fluorodeoxyuridine (FUdR) and the folic acid inhibitors, 2,4-diamino-6,7-dimethyl-2,4-dideoxyalloxazine[7] and methotrexate. It was proposed that MU may block a secondary pathway of the biosynthesis of DNA-thymidine, one which would involve the utilization of thymine. It appeared of interest to synthesize the deoxyriboside of MU, i.e. 5-mercapto-2′-deoxyuridine (MUdR), which was expected to act as an antimetabolite of thymidine (TdR). A chemical synthesis of both MUdR and its α-anomer was recently reported.[8] This paper describes the enzymatic synthesis of MUdR (disulfide), which was achieved prior to the chemical synthesis and which supplied the material used for the microbiological studies.

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Preliminary experiments in our laboratory indicated that a "*trans-N*-deoxyribosylase" (nucleoside: pyrimidine (purine) deoxyribosyltransferase, EC 2.4.2.6) present in *Lactobacillus helveticus* extracts,[9] which was partially purified and studied by Roush and Betz,[10] was capable of transferring the deoxyribosyl group from deoxyinosine to MU.[11] However, the new deoxyriboside was obtained in extremely poor yield and could not be purified satisfactorily for chemical analysis and biological studies. Somewhat better results were obtained when the deoxyinosine was replaced with TdR as the deoxyribosyl donor, and the yield was considerably increased when conditions were so modified as to protect MU from oxidation before and during the enzymatic reaction. By this procedure, described below, several hundred milligrams of MUdR were prepared and isolated in the form of analytically pure MUdR-disulfide. During this work it became apparent that the difficulties encountered in the synthesis of MUdR were, in part, due to some of the characteristic chemical properties of the compound. Subsequent studies using spectrophotometric techniques established that in aqueous buffer solutions both MU and MUdR undergo unusually rapid autoxidation[12, 13] to the corresponding disulfides, but the latter can be readily reconverted to the free thiols by reduction with dithiothreitol (DTT),[12] glutathione or other aliphatic sulfhydryl compounds.[14]

Further studies relating to the enzymatic synthesis of MUdR and to the mode of action of this compound in various microbiological assay systems are presented in this paper.

MATERIALS AND METHODS

Preparation of the enzyme. The original procedure of Roush and Betz[10] was modified in the following manner. The growth medium (10 l.) was inoculated with a culture of *L. helveticus* (ATCC 8018) grown in two tubes, each containing 10 ml of stock medium. After incubation at 37° for 72 hr, the cells were harvested with a Sharples centrifuge and a yield of approximately 100 g of packed cells was obtained. All subsequent operations were performed below 4°. The cells were extracted by grinding with 300 g of fine sand (Fisher S-25) moistened with 150 ml of 0·1 M citrate–phosphate buffer, pH 5·7. The mixture was centrifuged at 39,000 *g* for 45 min and the nucleic acids were precipitated from the supernatant by the addition of 1/20 vol. of a 1 M $MnCl_2$ solution. After 1 hr of standing, the mixture was centrifuged for 1 hr at 39,000 *g*, and the supernatant was brought to 40 per cent saturation by the addition of solid $(NH_4)_2SO_4$. After centrifuging for 1 hr at 39,000 *g*, more solid $(NH_4)_2SO_4$ was added to the supernatant to 70 per cent saturation and, after 2 hr of standing, the suspension was centrifuged again at 39,000 *g* for 90 min. The precipitate was dissolved in 22 ml cold distilled H_2O and stored in aliquots at −10 to −20° to give the enzyme solution used for the preparation of MUdR.

The *trans-N*-deoxyribosylase activity of the preparations was in the range of 1–5 μmole TdR converted/hr/mg protein, as determined by the procedure described below. Protein content was determined by the method of Lowry *et al.*[15]

Enzymatic synthesis of MUdR; isolation of MUdR-disulfide. 5-Mercaptouracil (MU), 30 mg, and TdR, 90 mg, were dissolved in 6·0 ml of 0·1 M citrate–phosphate buffer, pH 5·9, from which oxygen had been previously removed by bubbling through nitrogen for 2 hr. The enzyme solution, 2·0 ml, was added and the reaction mixture was incubated with shaking for 2·5 hr at 37° under N_2 atmosphere.

The reaction was stopped by heating the mixture in a boiling water bath for 5 min. The precipitated proteins were removed by centrifugation and an aliquot of the supernatant solution was spotted on Whatman No. 1 paper and chromatographed for 17 hr (descending chromatography) with H_2O-saturated butanol as the solvent. The chromatogram was compared with that of a "control" mixture from which MU was omitted. Two fluorescent spots were observed under u.v. light for the reaction mixture with R_f values of 0·36 and 0·60, whereas the "control" yielded only one spot with an R_f of 0·60 (TdR and thymine). Both chromatographic spots of the reaction mixture and the one of the "control" gave the positive test for deoxyribose (pink color upon treatment with the cysteine–sulfuric acid reagent[16]).

The remaining supernatant was lyophilized and the lyophilized reaction mixture was extracted with 5 × 15 ml of hot absolute ethanol. The extracts were combined and evaporated to dryness under reduced pressure, yielding 143 mg of "alcohol-soluble fraction." Paper chromatography (as above) gave two fluorescent spots; one of them was identified as TdR and thymine (R_f 0·60) while the other spot (R_f 0·36) gave positive tests for both sulfhydryl and deoxyribose, suggesting the presence of MUdR. The alcohol-insoluble part was shown by paper chromatography to contain some TdR and thymine.

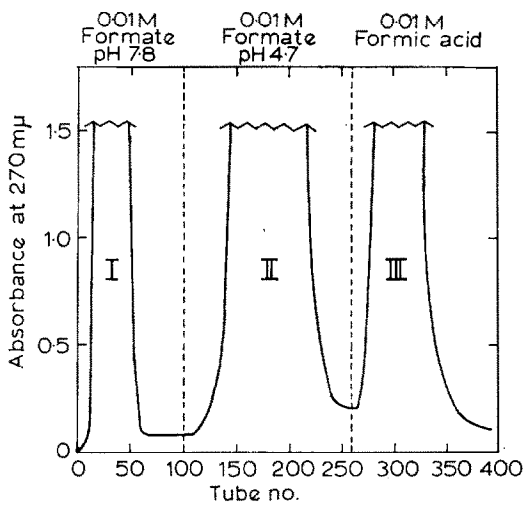

FIG. 1. Chromatography of the enzymatic reaction mixture on a Dowex formate column. The elution of the 50–100 mesh Dowex 21K formate column, 2·2 × 50 cm, with the eluants indicated, was conducted at a flow rate of 3·5 ml/min. In each tube, 10-ml fractions of the eluate were collected. Other details are given under Materials and Methods.

Several batches were combined to give a total of 750 mg of the alcohol-soluble fraction, which was dissolved in 15 ml of 0·01 M ammonium formate buffer, pH 7·8, and applied to a Dowex 21K (50–100 mesh) formate column, previously equilibrated with the same buffer. The elution of the material from the column was monitored with a Beckman DB spectrophotometer at 270 mμ; the elution pattern is shown in Fig. 1. Fraction I was eluted with 0·01 M ammonium formate buffer, pH 7·8. After

changing the pH to 4·7, a second u.v. absorbing material was eluted (Fraction II, 182 mg). A third u.v.-absorbing fraction was obtained upon elution with 0·1 M formic acid (Fraction III, 60 mg).

Fraction I was identified as a mixture of thymine and TdR by paper chromatography as described above. Fraction II had the same u.v.-absorption maximum (270 mμ) in both acid and alkaline solution, whereas the spectra of Fraction III showed an acid–base shift (λ_{max} = 270 mμ at acid or neutral pH and 285–290 mμ at alkaline pH) characteristic for MU (disulfide) and other 2,4-dioxopyridimidine bases. On the basis of dry weight, u.v. spectra and comparison of microbiological activities, Fraction II was estimated to contain about 85 per cent of the total yield of the desired product in the form of MUdR-disulfide, with none or very little contamination with the free base; Fraction III was estimated to contain the remaining 15 per cent (probably as unoxidized MUdR) and most of the unreacted MU.

Fraction II was lyophilized and the residue was taken up in H_2O and relyophilized; this was repeated four times. The crude material was dissolved in 15 ml of hot absolute ethanol, filtered, partially concentrated *in vacuo* and allowed to crystallize for 3 days at − 10°. Recrystallization from a methanol–ether mixture yielded 49 mg of analytically pure MUdR-disulfide (m.p. 210·5 to 211·0°).

Anal. Calcd. for $C_{18}H_{22}N_4O_{10}S_2$: C, 41·69; H, 4·27; N, 10·80; 0, 30·85; S, 12·36. Found: C, 41·64; H, 4·50; N, 10·74; 0, 30·81; S, 12·41. The u.v. spectra,[12] pK_a values[12] and some other characteristic physical and chemical properties[13, 17] of this compound have been reported.

Trans-N-*deoxyribosylase assay.* Deoxyribosyl transfer was assayed by a modification of the spectrophotometric method introduced by Friedkin and Roberts for the determination of TdR phosphorylase activity.[18] The total molar absorbancy change associated with the complete transfer of the deoxyribosyl moiety was determined for each donor–acceptor pair at the wavelength of maximum absorbancy change. The ultraviolet absorption of thymine plus deoxycytidine vs. TdR plus cytosine yielded a ΔE value at 300 mμ (ΔE_{300}) of $2{\cdot}5 \times 10^3$ at pH 13. The differential spectrum of MUdR and MU at pH 5·7 (Fig. 2) shows a peak at 348 mμ ($\Delta E_{348} = 1{\cdot}2 \times 10^3$). Since none of the natural pyrimidine bases and nucleosides show any absorbance above 320 mμ at the 5·7 pH, the absorbancy change in the 350 mμ region provides a convenient way of measuring the substrate activity of MU or MUdR in the presence of any of the other deoxyribosyl donors or acceptors.

Unless otherwise stated, the reaction mixture contained the following: 180 μmole citrate–phosphate buffer, pH 5·7; 3·0 μmole pyrimidine deoxyriboside (donor); 30 μmole pyrimidine base (acceptor); 7·5 μmole DTT (where indicated); 1·32 mg (as protein) of the *L. helveticus* enzyme preparation, in a total volume of 3·0 ml. The reaction mixtures were divided into equal volumes, one of which was incubated at 37° for 90 min ("test"); the other was kept at 0° ("blank"). At the end of the incubation period, the protein was precipitated in both the "test" and the "blank" by heat denaturation (5 min in a boiling water bath) and removed by centrifugation. The supernatants were diluted with equal volumes of 0·3 N NaOH for the measurement of TdR–thymine interconversion or with 0·1 M citrate–phosphate buffer, pH 5·7, containing 0·05 M DTT whenever the interconversion of MU and MUdR was to be measured. The absorbancy of the "test" was read against the "blank" at the given wavelength (see above) in a Beckman DU spectrophotometer and, from the measured

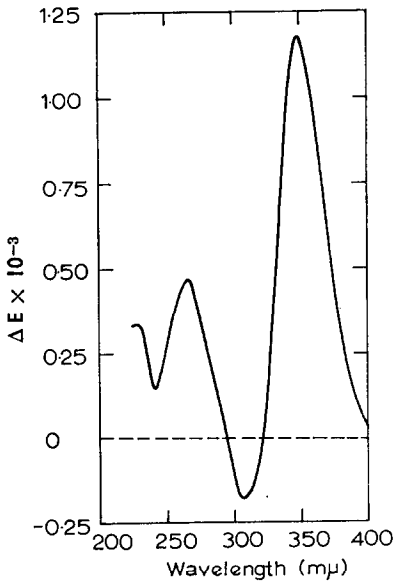

FIG. 2. Differential ultraviolet absorption spectrum of 5-mercaptodeoxyuridine (MUdR) and 5-mercaptouracil (MU) in 0·1 M citrate–phosphate buffer, pH 5·7, containing $2{\cdot}5 \times 10^{-3}$ M dithiothreitol (DTT).

absorbancy change, the per cent conversion (deoxyribosyl transfer) was calculated on the basis of the ΔE values for 100 per cent conversion.

Microbiological assay systems. The microbiological procedures previously described for *L. leichmannii* (ATCC 7830),[19] *Lactobacillus arabinosus* (ATCC 8014)[3] and *Streptococcus faecalis* (ATCC 8043)[20] were used for assaying the potency of MU and MUdR and for carrying out the inhibition analyses and combination studies. The FUdR-resistant mutant, SF/FUdR, was selected from *S. faecalis* 8043 by the serial transfer technique, as described previously.[21]

RESULTS

Table 1 shows the substrate activities as deoxyribosyl "acceptors" or "donors" of MU, MU-disulfide, MUdR and MUdR-disulfide in comparison with those of cytosine, thymine or TdR in the *L. helveticus* enzyme system. When MU or MU-disulfide was the "acceptor" and TdR the "donor," a 1:1·8 molar ratio was chosen in order to approximate the conditions used in the preparative procedure (see Materials and Methods). Similarly, for the study of the reverse reaction, in one experiment (No. 9), 0·8 mole equivalent of TdR was added (to an equimolar ratio of MUdR and thymine), which slowed down the rate of the reverse reaction, as expected.

The results in Table 1 show that both disulfides are entirely inactive as substrates for this enzyme, unless dithiothreitol (DTT) is added to the system. As previously demonstrated,[12] DTT almost instantly reduces the disulfides of MU and MUdR to the corresponding free thiols. In the presence of DTT, however, the disulfides as well as the free thiols (MU and MUdR) show greater substrate activities than the free thiols in the absence of DTT. This difference may be due to the gradual oxidation of the thiols to the disulfides, which may have an inhibitory effect[14] on the enzyme;

this process is reversed by DTT. DTT itself shows no effect on the rate of the enzymatic reaction (TdR → cytosine system, Nos. 1, 2).

It appears that MUdR (or MUdR-disulfide plus DTT) is a considerably better substrate than MU (or MU-disulfide plus DTT) as compared to TdR and cytosine, respectively, as "donor" and "acceptor", This is analogous to the reported greater

TABLE 1. SUBSTRATE SPECIFICITY OF *L. HELVETICUS TRANS-N*-DEOXYRIBOSYLASE

Experiment No.	Donor*		Acceptor*		Addition*		Per cent conversion* (Deoxyribosyl transfer)
	Compound	(mM)	Compound	(mM)	Compound	(mM)	
1	TdR	1·8	Cytosine	1·0			37·5
2	TdR	1·8	Cytosine	1·0	DTT	2·5	36·0
3	TdR	1·8	MU	1·0			9·0
4	TdR	1·8	MU	1·0	DTT	2·5	20·0
5	TdR	1·8	MU-DS	0·5			0·0
6	TdR	1·8	MU-DS	0·5	DTT	2·5	19·7
7	MUdR	1·0	Thymine	1·0			6·3
8	MUdR	1·0	Thymine	1·0	DTT	2·5	28·5
9	MUdR	1·0	Thymine	1·0	DTT	2·5	
					TdR	0·8	24·4
10	MUdR	1·0	Cytosine	1·0	DTT	2·5	32·4
11	MUdR-DS	0·5	Thymine	1·0			0·0
12	MUdR-DS	0·5	Thymine	1·0	DTT	2·5	29·0

* Abbreviations: TdR = thymidine; DTT = dithiothreitol; MU = 5-mercaptouracil; MU-DS = 5-mercaptouracil disulfide; MUdR = 5-mercaptodeoxyuridine; MUdR-DS = 5-mercaptodeoxyuridine disulfide.

† In each experiment, a reaction mixture containing the dexoyribosyl donor and acceptor at the given concentration (with the addition of DTT and TdR if indicated) was incubated with 0·44 mg/ml of the enzyme for 90 min at 37°, and the extent of the transfer of the deoxyribosyl group from the donor to the acceptor was determined from the measured absorbancy changes as described under Materials and Methods.

substrate activities of several deoxyribonucleoside "donors" as compared to the corresponding pyrimidine "acceptors" in the *Escherichia coli* TdR phosphorylase system.[22] Thus, the reaction equilibrium in the enzymatic synthesis of MUdR is unfavorable and the estimated theoretical (maximum) yield in the case of a 1:1·8 molar ratio of MU and TdR is less than 32 per cent (see Fig. 3).

In microbiological assay systems, the disulfides showed the same inhibitory activities as the corresponding free thiols, MU and MUdR respectively. Replacement of the cysteine present in the growth media with cystine or, conversely, addition of DTT or glutathione to the growth media, was without any observable effect and the disulfides remained indistinguishable from the corresponding free thiols. Since the disulfides were inactive as substrates for the *trans-N*-deoxyribosylase (see above) and the reduced form of the MUdR was also shown to be required for the activity of this compound in other enzyme systems,[23, 24] it appears likely that the bacterial cells are capable of reducing the disulfides to the thiols.

In Table 2, the activities of the 5-mercaptopyrimidines, MU and MUdR, are compared with those of the analogous 5-fluoropyrimidines, FU and FUdR, in four different microbiological assay systems. In making such a comparison, the difference in the composition of the growth media has to be considered. The media used for the growth of *L. leichmannii* and *L. arabinosus* contain uracil, which is capable of reversing

to a certain extent the growth inhibitions caused by these 5-substituted uracil analogs and their nucleosides. In spite of the presence of uracil in its growth medium, *L. leichmannii* is the most sensitive of the three organisms to inhibition by MUdR.

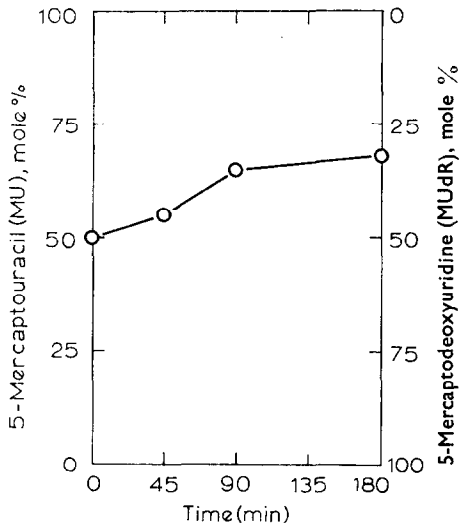

FIG. 3. Change of the relative molar concentrations of 5-mercaptouracil (MU) and 5-mercaptodeoxyuridine (MUdR), expressed as mole percentages of the total concentration of MU and MUdR, during incubation with *L. helveticus trans-N*-deoxyribosylase in the presence of thymine and thymidine (TdR). The reaction mixtures contained an equimolar concentration of MU and MUdR (5×10^{-4} M), 5×10^{-4} M thymine, $1{\cdot}3 \times 10^{-3}$ M TdR, $2{\cdot}5 \times 10^{-3}$ M dithiothreitol (DTT), 0·44 mg/ml of *L. helveticus* enzyme preparation, and 0·1 M citrate–phosphate buffer, pH 5·7. The reactions were terminated after 45, 90 and 180 min of incubation (37°), respectively, and the concentrations of MU and MUdR in the reaction mixtures were determined according to the procedure described under Materials and Methods.

TABLE 2. COMPARISON OF THE GROWTH INHIBITORY ACTIVITIES OF 5-MERCAPTO- AND 5-FLUOROPYRIMIDINES

Compound	Concentration (M) for 50 per cent growth inhibition of*			
	L. leichmannii	*L. arabinosus*	*S. faecalis*	SF/FUdR
MU†	3×10^{-5}	7×10^{-4}	3×10^{-6}	1×10^{-3}
MUdR	6×10^{-8}	4×10^{-6}	1×10^{-7}	8×10^{-5}
FU	4×10^{-8}	2×10^{-7}	5×10^{-11}	7×10^{-5}
FUdR	3×10^{-8}	1×10^{-9}	1×10^{-11}	6×10^{-5}

* *L. leichmannii* (ATCC 7830) and *L. arabinosus* (ATCC 8014) were grown in media containing adenine, guanine and uracil. The medium used for the growth of *S. faecalis* (ATCC 8043) and SF/FUdR (an FUdR-resistant mutant of *S. faecalis*) was devoid of purines and pyrimidines (see Materials and Methods).

† MU = 5-mercaptouracil; MUdR = 5-mercaptodeoxyuridine; FU = 5-fluorouracil; FUdR = 5-fluorodeoxyuridine.

MUdR is approximately as effective as FU and FUdR in preventing the growth of *L. leichmannii*, while MU is about 500-fold less active. On the other hand, as assayed with *L. arabinosus*, both mercaptopyrimidines are less active than the corresponding fluoropyridimidines by approximately three orders of magnitude. Furthermore, in

this system both deoxyribonucleosides are about 200 times more active than the corresponding bases. Finally, in *S. faecalis* (the parent strain), grown in a medium devoid of uracil, the difference in the activities of the mercapto and fluoro analogs is the largest among the three organisms, but the difference between the deoxyribosides and the corresponding free bases is only 3-fold. However, the differences between the mercapto and fluoro analogs almost disappear in the FUdR-resistant mutant of *S. faecalis* (SF/FUdR), which was grown in the same medium as the parent strain.

In order to determine the possible sites of action of MUdR, an inhibition analysis[25] was carried out with two of the test systems. In *L. leichmannii*, the growth inhibition by MUdR was prevented competitively by 2′-deoxyuridine (UdR) over a 1000-fold range of inhibitor concentrations (from 5×10^{-7} to 5×10^{-4} M). Above this concentration range, the amount of UdR required for reversal did not increase proportionately with the inhibitor concentration. On the other hand, thymine and TdR prevented the inhibition competitively only at very low inhibitor concentrations (10^{-7} to 10^{-6} M), whereas above this range the reversal by either thymine or TdR was clearly noncompetitive. In the presence of 4×10^{-5} M thymine, MUdR was as effective as TdR, UdR or other deoxyribosides in replacing vitamin B_{12} (i.e. in supporting growth in the absence of vitamin B_{12}). This is in line with the observed deoxyribosyl "donor" activity of MUdR in the enzymatic study.

In contrast, the inhibition analysis with *S. faecalis* (the parent strain) showed a clearly competitive relationship with all pyrimidines tested. Table 3 shows the inhibition indices obtained with MU and MUdR in comparison with those for FU

TABLE 3. REVERSAL OF THE 5-MERCAPTO- AND 5-FLUOROPYRIMIDINE INHIBITION OF *S. FAECALIS* BY VARIOUS PYRIMIDINE BASES AND NUCLEOSIDES

Reversing agent	Inhibition index* obtained with			
	MU	FU ($\times 10^3$)	MUdR	FUdR ($\times 10^3$)
Thymidine	9	10	0·9	7
Thymine	1	1	0·7	1
2′-Deoxyuridine	1	1	0·8	1
Uridine	0·9	0·2	0·08	0·1
2′-Deoxycytidine	0·7	0·03	0·07	0·08
Uracil	0·2	0·03	0·03	0·04
Cytidine	0·1	0·01	0·03	0·01
Cytosine	0·001	0·008	0·004	0·004

* Inhibition index = [I]/[S] for 50 per cent growth inhibition, where [I] is the concentration of the inhibitor (MU, FU, MUdR or FUdR respectively) and [S] is the corresponding concentration of the reversing agent required for half-maximal growth. The inhibition indices were determined at least over a 1000-fold concentration range (for [S] = 10^{-3} to 10^{-6} M). See Table 2 for abbreviations.

and FUdR. While in the case of MU and FU the inhibition indices differ by a factor of 10^3, the relative reversing potencies of the natural pyrimidines are the same. In this system, both thymine and TdR prevent the inhibition competitively. In the case of MUdR and FUdR a similar relationship holds, except that TdR was only as effective as thymine or UdR in reversing the inhibition by MUdR, while it was 7–10 times more effective than thymine or UdR in reversing the inhibitions by FUdR, FU or MU.

In view of the general similarity of the inhibition analysis for MUdR and FUdR in *S. faecalis*, it was assumed that the inhibition of the growth of this organism by these two compounds was effected at the same biochemical site. A combination experiment[26] shown in Fig. 4 indicated, however, that the two compounds are synergistic

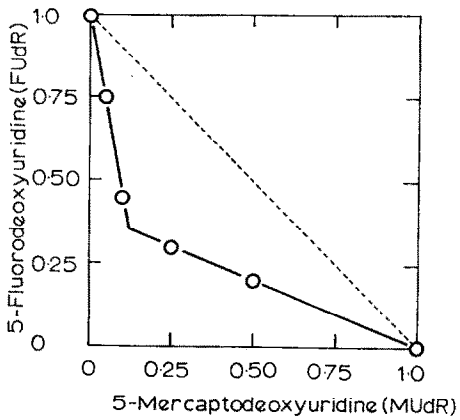

FIG. 4. Synergism between 5-mercaptodeoxyuridine (MUdR) and 5-fluorodeoxyuridine (FUdR) in the growth inhibition of *S. faecalis*. The points of the curve represent various combinations of the two drugs required for half-maximal growth inhibition, expressed as fractions of the half-maximal inhibitory concentration of each drug when used alone. (The theoretical case of an additive effect is represented by the dotted line.)

in their inhibitory action, implying that, in addition to the same site, one or more different but metabolically interrelated sites are inhibited by the two agents. The latter interpretation appeared to be further supported by the results obtained with the FUdR-resistant strain derived from *S. faecalis* (SF/FUdR), in which the potency of MUdR decreased by only 3 orders of magnitude, while that of FUdR was by 7 orders of magnitude less than in the parent strain (Table 2).

In contrast, a combination experiment conducted with *L. leichmannii* showed MUdR and FUdR having only additive effects.

DISCUSSION

Trans-N-deoxyribosylase, found to be present in various lactobacilli and other microorganisms that utilize deoxyribonucleosides for growth,[9, 27–29] was previously shown to have a low order of substrate specificity.[10, 29] In our studies, MUdR was shown to be an effective deoxyribosyl donor and MU a somewhat poorer acceptor; however, the corresponding disulfides were entirely inactive as substrates for the *L. helveticus* enzyme. Thus, it is necessary to prevent the rapid autoxidation[12] of MU in solution before and during the enzymatic synthesis of MUdR. This was achieved in the preparative procedure by keeping the solutions under nitrogen atmosphere and by employing a citrate buffer which decreased the rate of autoxidation by chelating the catalytic trace metal ions.[13] Although MUdR-disulfide was the product eventually isolated from the enzymatic reaction, most of the oxidation of the MUdR to the disulfide occurred during the isolation. The large increase in the substrate activities

of MU and MUdR upon addition of DTT suggests that small amounts of disulfides formed during the reaction may act as inhibitors of the enzyme; this inhibition is prevented by DTT, which reduces the disulfides as soon as they are formed. On the basis of this observation, the preparative method may be substantially improved by the addition of DTT to the enzymatic reaction mixture.

From structural considerations, it was anticipated that MUdR would act as an antimetabolite of TdR. At physiologic pH, the sulfhydryl group of MUdR is fully ionized (pK_a 5·0)[12] and, since its ionic radius[30] is 2·19 Å, it resembles the van der Waals radius of the CH_3 group (2·00 Å) of TdR. Moreover, MU had been found to be a competitive antagonist of thymine in *L. leichmannii*.[3] However, the inhibition analysis of MUdR in *L. leichmannii* (as described above) showed that the inhibitory effect of this compound is reversed by TdR in an essentially noncompetitive manner. This indicates[25] that in this organism MUdR interferes with the synthesis rather than the utilization of TdR. Since UdR reverses the inhibition competitively, the primary metabolic site of the growth inhibition appears to be the thymidylate (TdRP) synthetase. Preliminary studies, using enzyme preparations from *E. coli*, have shown that MUdR (but not MUdR-disulfide) is phosphorylated by TdR kinase[23] to the corresponding 5′-monophosphate (MUdRP) which, in turn, is a potent inhibitor of TdRP synthetase ($K_i = 4 \times 10^{-8}$ M).[24] Thus, MUdRP closely resembles the 5′-monophosphate of FUdR (FUdRP), which is the most potent and specific inhibitor known among the substrate-analogs of TdRP synthetase.[31]

The similarity of action between MUdR and FUdR is shown by the results of the inhibition analysis carried out in *S. faecalis* (Table 3). Whereas in this organism the inhibition indices obtained with the two analogs differ by a factor of 10^3–10^4, the relative reversing abilities of the various natural metabolites are essentially the same. However, it is surprising that in this organism thymine and TdR reverse the inhibition by both MUdR and FUdR in a competitive manner. If the TdRP synthetase were the only site of inhibition, then thymine and TdR, supplying the product of the inhibited reaction via a route circumventing the TdRP synthetase step, would be expected to reverse the inhibition noncompetitively,[25] as we have seen is the case in the *L. leichmannii* system. Further, since the 5′-monophosphate derivatives of the two analogs possess comparable affinities for TdRP synthestase,[14] one would expect that MUdR should inhibit the growth of *S. faecalis* at nearly equivalent concentrations as they indeed do in *L. leichmannii* (Table 2). In order to explain the results with *S. faecalis* (i.e. the competitiveness of thymine and TdR with both MUdR and FUdR as well as the large difference in potency between the two nucleoside analogs), one must assume that in this system the inhibitory activities of MUdR and FUdR and their reversal by TdR depend on the relative rates (or extent) of conversion of each of the three deoxyribosides to the corresponding 5′-monophosphates, the synthesis of TdRP (either by TdRP synthetase or by TdR kinase) being the overall rate-limiting condition of growth. Thus in *S. faecalis* FUdR appears to be much more effectively activated via phosphorylation by the TdR and UdR kinases than MUdR and in the reversal study the two analogs appear to inhibit the phosphorylation of TdR in a competitive manner. The difference in the potencies of FUdR and MUdR disappears in the FUdR resistant mutant in which the two analogs are approximately equally inhibitory at 10^{-4} M concentration, suggesting that there is no difference in their degree of activation. However, at such a high concentration, both FUdR and

MUdR may inhibit TdRP synthetase directly without being converted to the 5'-monophosphates.[32]

Thus, the results of the inhibition analysis both with *L. leichmannii* and *S. faecalis* are consistent with the suggestion that the principal metabolic site of the growth inhibitory action of MUdR, like that of FUdR,[31, 33] is the TdRP synthetase catalyzed reaction. The synergism between MUdR and FUdR observed in the *S. faecalis* system (Fig. 4). indicates, however, that additional metabolic sites are affected. It is possible that at the relatively high MUdR/FUdR concentration ratios required in this system the synergism is due to the interference of MUdR with the breakdown of FUdR by, e.g. nucleoside phosphorylase. It should be recalled that in the case of *L. leichmannii*, where the concentrations of MUdR and FUdR required for inhibition are approximately equal, no such synergism could be observed between the two nucleoside analogs.

The apparent discrepancy that in *L. leichmannii* the inhibition of growth by MU was competitively reversed by thymine and noncompetitively by TdR,[3] while the inhibition by MUdR is reversed noncompetitively by both thymine and TdR, can be explained by the competition between MU and thymine for the *trans-N*-deoxyribosylase; this enzyme was demonstrated to be present in *L. leichmannii*[29] and was shown, in this paper, to utilize both MU and thymine as deoxyribosyl "acceptors". On the other hand, MUdR was shown to be a good deoxyribosyl "donor" in the *trans-N*-deoxyribosylase system and its support of the growth of *L. leichmannii* in the absence of vitamin B_{12} and presence of thymine (see above) indicates that it can serve as a source of the deoxyribosyl group in this organism, thereby actually promoting the conversion of thymine to TdR. The large difference in potency between MU and MUdR in the *L. leichmannii* system (Table 2) parallels the difference in the potencies of 5-bromouracil (BrU) and 5-bromodeoxyuridine (BrUdR)[3] and also the difference in the growth-promoting activities of thymine and TdR,[3] but is in contrast to the equal activities of FU and FUdR. This supports the suggestion that MU, like BrU and thymine, is activated via deoxyribosylation rather than by conversion to the deoxyribotide via the pathway followed by FU and uracil.[31, 34] Thus, MU and MUdR, as postulated, may be considered as analogs of thymine and TdR respectively; it is of marked interest, therefore, that MUdRP is a potent inhibitor of TdRP synthetase,[24] competitive with the substrate, deoxyuridylate.

From a chemotherapeutic point of view, the differences in selectivity between the mercapto and fluoro analogs (apparent from their different spectra of activity shown in Table 2) are of potential interest. It should be kept in mind that these differences in selectivity were related to the differences (discussed above) in the routes and efficiency of metabolic activation, since the ultimate metabolic site of action of these analogs appeared to be the same. Thus, FU and FUdR should be very efficiently activated even in cells with relatively low kinase activity, while the activation of MU and MUdR appears to be more selectively dependent on the level of TdR kinase activity and that of MU depends also on the *trans-N*-deoxyribosylase activity (thymine pathway) of the various cells. In addition, due to the ready oxidizability of MU and MUdR and in view of the inability of the corresponding disulfides to serve as substrates for the *trans-N*-deoxyribosylase (Table 1) and for the TdR kinase,[23] respectively, the selectivities of the two mercapto analogs may be modified by possible differences in the capacities of the various cells to reduce the disulfides to the free thiols. It is conceivable that

the different selectivities of MU and MUdR as compared to FU and FUdR will find useful applications in chemotherapy.

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