or more of the ionic components of the reaction system we are now engaged in a study of the effects produced by other added salts not only of the type MX but also MX_2 and M_2X.

Experimental

Enzyme Experiments.—The chloroacetyl-L-tyrosinamide preparation was that which was described previously.[4] The α-chymotrypsin preparation was an Armour product, lot no. 90402. All experiments were conducted in aqueous solutions at 25° and *p*H 7.75. The reaction mixtures were 0.02 *M* in the THAM component and 0.01 *M* in the HCl component of a THAM–HCl buffer and of varying molarity with respect to added sodium or potassium chloride. The extent of reaction was determined titrimetrically by the method of Iselin and Niemann[13] as modified by Huang and Niemann.[14]

(13) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

(14) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

Evaluation of Experimental Data.—The data obtained from each experiment were presented in the form of $([S]_0 - [S]_t)$ *vs.* *t* and $\ln [S]_0/[S]_t$ *vs.* *t* plots and the corrected values of v_0 which were obtained from each of these plots by the method of Jennings and Niemann[10] were averaged to give the mean values of v_0 which are summarized in Tables I–III, inclusive. It was found that any given value of v_0 obtained from a corrected $([S]_0 - [S]_t)$ *vs.* *t* plot was in good agreement with the corresponding value of v_0 obtained from a $\ln [S]_0/[S]_t$ *vs.* *t* plot, in general the difference between the two values being less than ±2%. It also should be noted that all of the experiments described in this study were conducted under conditions which were compatible with the methods which were used for the evaluation of the experimental data, *i.e.*, $[E] = 1.92 \times 10^{-5}$ *M*, $E_S' = [E]/K_S = 0.07 \times 10^{-2}$ and $S_S' = [S]/K_S = 0.4$–1.2.

Acknowledgments.—The authors wish to express their indebtedness to Dr. R. Lutwack and Mr. R. A. Bernhard for their assistance in the evaluation of the experimental data.

PASADENA 4, CALIFORNIA

[CONTRIBUTION FROM THE RESEARCH DEPARTMENT, THE ARMOUR LABORATORIES]

Synthesis of Compounds Related to Thymine. II. Effect of Thymine Antagonists on the Biosynthesis of DNA

BY THOMAS J. BARDOS, GEORGIA M. LEVIN, ROSS R. HERR AND HARRY L. GORDON

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The patterns of deoxyribonucleic acid biosynthesis have been studied in *Lactobacillus leichmannii* and *Lactobacillus arabinosus*. The modes of action of various metabolic antagonists, particularly 5-bromouracil and its nucleosides, are discussed. The systems described are used to study the biological action of three new thymine antagonists: 5-sulfur-substituted uracils.

In the course of a synthetic program designed to obtain compounds which would inhibit specific steps of nucleic acid biosynthesis, we have prepared several new structural analogs of thymine. The synthesis of three of these compounds, 5-mercaptouracil, 5-uracilyl disulfide and uracil-5-isothiouronium chloride was recently reported.[1]

For a systematic evaluation of the biological actions of our compounds, variations of the "inhibition analysis"[2] technique were used. Two microbiological systems were selected to represent two different patterns of nucleic acid biosynthesis. The two species, *Lactobacillus leichmannii* 313 and *Lactobacillus arabinosus*, were grown under standard conditions which were chosen to make specific steps of nucleic acid biosynthesis the "growth limiting reactions" of the systems. The new compounds were tested in both systems for their ability to inhibit the growth of these organisms and also to determine the reversibility of their growth inhibitory action by a number of metabolites, intermediates, and catalytic factors presumably involved in the biosynthesis of nucleic acids. However, to interpret our results, it was necessary to obtain additional information on the metabolic patterns of these organisms by studying the action of known inhibitors under the defined conditions of our systems.[3]

(1) T. J. Bardos, R. R. Herr and T. Enkoji, THIS JOURNAL, **77**, 960 (1955).

(2) W. Shive, *Ann. N. Y. Acad.*, **52**, 1212 (1950).

(3) For comparison with earlier work in this field, the authors wish to refer the reader especially to the papers of Hitchings and his collaborators on their "*Lactobacillus casei* model"; G. M. Hitchings, G. B. Elion, E. A. Falco, P. B. Russell, M. S. Sherwood and H. Vanderwerff, *J. Biol. Chem.*, **183**, 1 (1950); *Ann. N. Y. Acad.*, **52**, 1318 (1950).

Results and Discussion

Use of the technique of "inhibition analysis" can provide information useful in the understanding of biochemical interrelationships in given microbiological systems. However, evidence provided by this method cannot be considered as unequivocal. With this reservation in mind, and after comparing our results with those of other investigators using various lactobacilli, we wish to propose the hypothetical scheme presented in Fig. 1 for the biosynthesis of deoxyribosides, and use this as a basis for the subsequent discussion of our experimental results.

The medium used in our *L. leichmannii* experiments is composed so that the folic acid and vitamin B_{12} concentrations are fixed at levels just sufficient for maximal growth in 16 hours. The other metabolites, including the purines and uracil, are present in excess. Consequently, folic acid and vitamin B_{12} are the limiting growth factors in this system and, under the given conditions, the biosynthesis of the thymidine component of DNA appears to be the growth-rate limiting reaction.[4] In this biosynthesis both folic acid and vitamin B_{12} are involved as catalytic factors.[5] We found that folic acid can be replaced by folinic acid; the synthetic

(4) The terms "limiting reaction," "competitive," and "noncompetitive" inhibition, "product-effect," "inhibition index," etc., used by us in the discussion are defined by Shive in ref. 2 and in R. J. Williams, R. E. Eakin, E. Beerstecher and W. Shive, "The Biochemistry of B-Vitamins," Reinhold Publ. Corp., New York, N. Y., 1950, pp. 443–480.

(5) W. Shive, J. M. Ravel and W. M. Hardin, *J. Biol. Chem.*, **176**, 991 (1948).

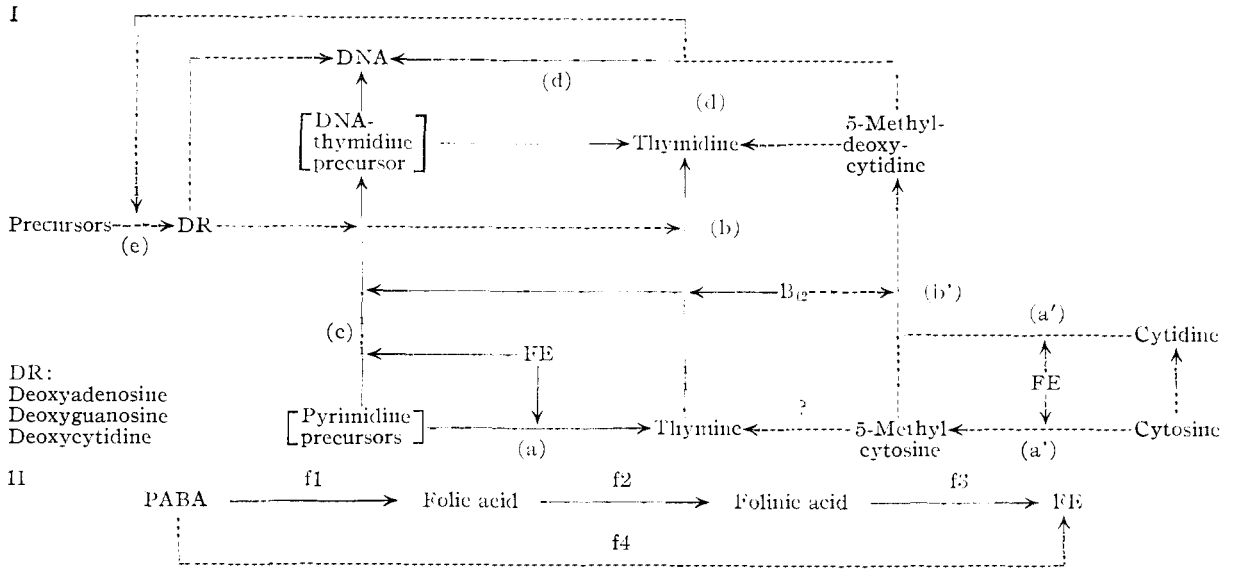

Fig. 1.—Proposed scheme of DNA–thymidine biosynthesis in Lactobacilli.

"(*dl*)" folinic acid- SF[6] (or leucovorin[7]) is about half as active as folic acid in maintaining growth of this organism. Furthermore, the two factors, folic (or folinic) acid and vitamin B_{12} appear to act independently of each other in this system, catalyzing two distinct reaction-steps. Folic acid can be replaced (almost completely) by thymine, while vitamin B_{12} cannot be replaced by thymine but can be replaced by thymidine and other deoxyribosides.[8] Thymidine replaces both factors.[5] Therefore, we assume, in agreement with earlier investigators[5,9] that folic (or folinic) acid is required in the synthesis of the thymine moiety, along route (a) of our scheme (see Fig. 1), while vitamin B_{12} catalyzes the conversion of thymine into thymidine (route (b)). Since thymine is not necessarily an intermediate in all organisms,[10] we must assume the existence of an alternative route (c), leading through another, unknown intermediate by reactions catalyzed subsequently or simultaneously by folic acid and vitamin B_{12}. A third alternative route may lead through 5-methylcytosine: (a') and (b'), as evidenced by the thymine-like action of 5-methyl-cytosine in reversing the various inhibitors (see Table II). However, it is possible that 5-methylcytosine is either converted to thymine or is able to replace thymine in its own enzyme system.[3]

Since, in the absence of exogenous thymine, folic (or folinic) acid is required for routes (a), (a') and (c), antagonists of folic acid will be expected to block all routes to the synthesis of thymidine and therefore inhibit the growth of the organism. Three different types of folic acid antagonists were used: (1) a close structural analog of folic acid, represented by 4-aminopteroylglutamic acid (aminopterin), (2) a "synthetic pterin"[11] represented by 2,4-diamino-6,7-diphenylpteridine,[12] and (3) a 2,4,-

TABLE I

REVERSAL OF GROWTH INHIBITION BY FOLIC ACID ANTAGONISTS IN *L. Leichmannii*

Inhibitor	Concn., μg./tube	For ½-maximal reversal required, μg./tube: Thymine	Thymidine	Folic acid	Folinic acid
Aminopterin	0.3	..	..	1.25	1.5
Aminopterin	0.6	..	..	2.4	5.2
Aminopterin	1.2	1.5	0.5	4.5	10.2
Aminopterin	1.8	..	..	7.5	16.5
Aminopterin	2.5	1.4	0.4	12.5	22.5
Aminopterin	5.0	1.2	0.4	35.0	...
Aminopterin	25	1.2	0.3	...	...
Aminopterin	200	1.1	0.3	...	..
Pteridine[d]	5	0.8	0.3	[a]	0.008
Pteridine	10	..	..	...	.012
Pteridine	25	1.0	0.3	...	.040
Pteridine	50	..	..	...	.090
Pteridine	100	1.1	0.4	...	.600
Pteridine	200	1.8	0.4	...	.075
Pteridine	400	..	..	...	.075
Pyrimidine[e]	1	..	..	0.0005	.0008
Pyrimidine	2.5	1.5	0.4	0.025	.003
Pyrimidine	5	1.9	0.5	0.350	.006
Pyrimidine	10	1.9	0.5	[b]	.018
Pyrimidine	25	2.4	0.6	[a]	.050
Pyrimidine	50	..	..	...	.500
Pyrimidine	75	..	..	...	5.000
Pyrimidine	100	2.6	1.0	...	[b]
Pyrimidine	250	2.6[c]	1.0[c]	...	[a]

[a] No reversal. [b] Partial reversal, but ½-maximal growth not obtained. [c] Half maximal growth obtained, but maximal growth could not be restored even with high concentrations of the reversing agent. [d] 2,4-Diamino-6,7-diphenylpteridine. [e] 2,4-Diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine.[14]

(6) E. H. Flynn, T. J. Bond, T. J. Bardos and W. Shive, THIS JOURNAL, **73**, 1979 (1951).

(7) We are indebted to Dr. Jukes of Lederle Laboratories for a sample of this substance.

(8) E. Kitay, W. S. McNutt and E. E. Snell, *J. Biol. Chem.*, **177**, 993 (1949).

(9) L. D. Wright, H. R. Skeggs and J. W. Huff, *ibid.*, **175**, 655 (1948).

(10) See ref. 1, Williams, *et al.*, p. 474.

(11) L. J. Daniel, L. C. Norris, M. L. Scott and G. F. Heuser, *J. Biol. Chem.*, **169**, 689 (1947).

(12) Prepared by D. B. Olsen according to the procedure described by M. E. Mallette, E. C. Taylor and C. K. Cain, THIS JOURNAL, **69**, 1814 (1947).

diaminopyrimidine,[13] represented by 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine.[14] We found that all three of these compounds inhibited the growth of *L. leichmannii*. The reversal of their inhibitory effect is shown in Table I.

If thymine, the product of reaction (a), is added to the system, the inhibition by any concentration of folic acid antagonist is reversed non-competitively since reaction (b) may proceed without folic acid catalysis to produce thymidine. Similarly, thymidine, the "final" product of the inhibited reactions, restores growth non-competitively. This we found true for all three of the folic acid antagonists used. However, there are important differences in the relative activities of folic acid and folinic acid in the reversal of the action of the three anti-folic compounds (Table I).

The inhibition of *L. leichmannii* by aminopterin may be competitively reversed with either folic or folinic acid. Folic acid is slightly more active than synthetic folinic acid. In contrast to this, the inhibition by 2,4-diamino-6,7-diphenylpteridine is insensitive to folic acid but is readily reversible with folinic acid. This reversal appears to be competitive at low levels of the inhibitor, but becomes non-competitive at high levels. The 2,4-diaminopyrimidine inhibition is reversible with folic acid only at low levels, with the effectiveness of folic acid decreasing rapidly with the increasing concentration of the pyrimidine. Folinic acid remains effective as a reversing agent through a broader range, but also becomes less effective as the inhibitor concentration increases.[15]

These differences can be explained[13] by the differences in the degree of the specificities of the three types of antagonists, which may allow them to interfere at one or more points in the synthesis of the "folic acid enzyme" (FE). Folic acid antagonists less specific than aminopterin, such as the pterin and the diaminopyrimidine, may interfere with the conversion of folic acid to folinic acid and thereby differentiate between these two forms of the vitamin. A somewhat similar situation involving the greater capacity of folinic acid to reverse the inhibition of *L. casei* by methylfolic acid led to the discovery of the "folinic acid group" of these vitamins.[16]

In order to obtain more information regarding the substrates and intermediates leading to thymidine in this system, we studied the action of several structural analogs of thymine. Among these, 5-nitrouracil has been characterized by Hitchings, *et al.*,[17] as a folic acid antagonist and by Shive[2] as a uracil antagonist. In our *L. leichmannii* system, uracil has relatively little, and folic acid has no reversing action on the inhibitory effect of 5-nitrouracil. On the other hand, thymine, 5-methylcytosine and thymidine completely and non-competitively reverse this inhibition (see Table II). The 5-nitrouracil, therefore, appears to interfere with routes (a), (a′) and (c), that is, with those reactions which require the folic acid enzyme (FE). In these reactions, 5-nitrouracil acts as a substrate-analog. Whether the substrate with which it directly competes for this enzyme is uracil or some other thymine-precursor cannot be decided but, in any case, 5-nitrouracil has a greater affinity for FE than either uracil or cytosine, as evident from Table II.

It has been reported by Weygand and Wacker[18] that 5-bromouracil inhibition of *L. leichmannii* is competitively reversed by both thymine and thymidine. Our data summarized in Table II confirm this and, in addition, we found that cytosine is about as active as thymine in competitively reversing this inhibition. Cytidine is much more active, especially at low levels of the inhibitor. Deoxycytidine is essentially inactive, but 5-methylcytosine is about $1/4$ as active as thymine and uracil also has some reversing activity at low levels of the inhibitor.

It has also been reported by Hitchings, *et al.*,[17] and by Weygand, *et al.*,[19,20] that in *L. casei* as well as in *S. faecalis*, 5-bromouracil is inhibitory only if these organisms are grown in a medium in which the folic acid has been replaced by thymine. In the presence of folic acid, there is not only no inhibition, but 5-bromouracil is even stimulatory.[17] In our *L. leichmannii* system, 5-bromouracil inhibits growth in the presence of folic acid (as well as in a folic acid-free thymine medium); it becomes stimulatory only (at low levels) if 5-nitrouracil is present in the medium (Table III). This could be explained by assuming that in *L. leichmannii*, route (a)–(b) is the main pathway of the thymidine synthesis involving a thymine intermediate. The 5-bromouracil, bearing a close steric similarity to thymine, competes with thymine for the B_{12} enzyme along route (b), and apparently is itself converted to the corresponding deoxyriboside.[21] This, in turn, competes with thymidine in the synthesis of DNA.[20] As a consequence, 5-bromouracil inhibits both routes (b) and (d).

Conversely, we must assume that in *L. casei* and *S. faecalis*, route (c) is the predominant one if folic acid is available, and route (b) becomes operative only if these bacteria have to use pre-formed thymine in the absence of folic acid. In the sequence of reactions along route (c), 5-bromouracil may be incorporated into DNA without interfering with thymine and thymidine. If we assume that, up to a certain thymidine/5-bromodeoxyuridine ratio, the incorporation of the abnormal deoxyriboside in place of thymidine is of no immediate harm to the organism, the stimulatory action of 5-bromouracil in the presence of folic acid could be interpreted as a kind of "sparing action" caused by supplementing the amount of DNA-thymidine produced by route (c). In the *L. leichmannii* system, such "sparing action" comes into effect only when routes (a) and

(13) G. H. Hitchings, E. A. Falco, H. Vanderwerff, P. B. Russell and G. B. Elion, *J. Biol. Chem.*, **199**, 43 (1952).

(14) We are grateful to Dr. Hitchings for a sample of this compound.

(15) This behavior of the diaminopyrimidine is similar to the reported action of the same compound in *S. faecalis*[13] and dissimilar to its action in *L. casei*.[13]

(16) T. J. Bond, T. J. Bardos, M. E. Sibley and W. Shive, THIS JOURNAL, **71**, 3852 (1949).

(17) G. H. Hitchings, G. B. Elion and E. A. Falco, *J. Biol. Chem.*, **185**, 643 (1950).

(18) F. Weygand and A. Wacker, *Z. Naturforschung*, **5b**, 46 (1950).

(19) F. Weygand, A. Wacker and H. Grisbach, *ibid.*, **6b**, 177 (1951).

(20) F. Weygand, A. Wacker and H. Dellweg, *ibid.*, **7b**, 19 (1952).

(21) W. H. Prusoff, *Proc. Soc. Exp. Biol. Med.*, **85**, 564 (1954).

TABLE II

REVERSAL OF GROWTH INHIBITION BY STRUCTURAL ANALOGS OF THYMINE AND THYMIDINE IN *L. Leichmannii*

Inhibitor	Concn., μg./tube	For 1/2-maximal reversal required, μg./tube: Thymine	Thymidine	Uracil	Uridine	Cytosine	Cytidine	5-Methylcytosine
5-Nitrouracil	100	1.1	0.3	..	..	600	6	..
5-Nitrouracil	300	1.2	0.3	1000	[a]	[b]	3000	18
5-Nitrouracil	1000	1.4	0.4	4500	..	[b]	[a]	18
5-Nitrouracil	3000	1.4	0.4	[b]	..	[a]	[a]	20
5-Bromouracil	250	150	1.5	2000	..	90	3	600
5-Bromouracil	500	300	2.5	..	..	230	12	1200
5-Bromouracil	1000	750	5.0	[a]	..	550	20	2600
5-Bromouracil	2500	1800	12	[a]	..	2800	50	..
5-Bromodeoxyuridine	25	..	3	..	..	..	..	..
5-Bromodeoxyuridine	50	35	6	..	..	..	..	80
5-Bromodeoxyuridine	100	60	12	..	..	..	..	..
5-Bromodeoxyuridine	250	120	30	..	..	..	..	180
5-Bromodeoxyuridine	500	150	75	..	..	..	..	300
5-Mercaptouracil	25	4	0.25	..	..	..	..	..
5-Mercaptouracil	50	6	0.30	..	..	..	..	..
5-Mercaptouracil	100	11	0.35	..	..	..	..	..
5-Uracilyl disulfide	25	3	0.35	180	90	2000[c]	200	16
5-Uracilyl disulfide	50	5	0.45	240	170	4000[c]	450[c]	25
5-Uracilyl disulfide	100	10	0.60	400[c]	550	..	2000[c]	38
5-Uracilyl disulfide	250	25	0.90	900[c]	[b]	[a]	[a]	70
5-Uracilyl disulfide	500	50	1.3	[b]	..	..	..	..
4-Uracilyl disulfide	1000	80	2.2	..	..	..	..	..
5-Uracilyl disulfide	2500	200[c]	3.3[c]	..	..	..	..	..
Uracil-5-isothiouronium chloride	250	8	0.3	..	..	..	..	..
Uracil-5-isothiouronium chloride	1000	20	0.5	..	..	..	..	..
Uracil-5-isothiouronium chloride	2500	75	0.9	..	..	..	..	..

[a] No reversal. [b] Partial reversal, but 1/2-maximal growth was not obtained. [c] Half-maximal reversal was obtained, but maximal growth could not be restored even with high levels of the reversing agent.

(a′) are blocked by 5-nitrouracil at a level of this inhibitor which is insufficient for completely blocking route (c). As long as DNA-thymidine is being formed by route (c), 5-bromouracil would supplement the amount of DNA-thymidine formed, thereby giving a stimulatory effect.

Further evidence concerning this hypothesis was obtained from analogous inhibition studies using 5-bromodeoxyuridine. This compound was prepared by the bromination of deoxyuridine in water,[22] and was found to be highly inhibitory in the *L. leichmannii* system. Concentrations of 1γ/5 ml. gave half-maximal growth and 2γ/5 ml. gave almost complete growth inhibition. This inhibition was competitively reversible with either thymine or thymidine (see Table II), as was 5-bromouracil. However, while in the case of inhibition by 5-bromouracil thymidine was more than 100 times as effective as thymine as a reversing agent, thymidine was only 2–5 times as effective as thymine in reversing the inhibition by 5-bromodeoxyuridine. In this latter case, the ratio between the reversing activities of thymine and thymidine corresponds to their relative activities as growth factors in this organism (see Table VI) as well as to their relative activities as non-competitive reversing agents of folic acid antagonists (see Table I).[23] This indicates that in contrast to 5-bromouracil, 5-bromodeoxyuridine does not inhibit route (b), that is, the conversion of thymine to thymidine. It acts as an inhibitor of the utilization of thymidine *via* route (d), and thymine acts as a reversing agent of this compound by first being converted to thymidine.

In addition, Table II shows that one molar equivalent of thymidine gives half-maximal growth in the presence of eight molar equivalents of 5-bromodeoxyuridine, or in the presence of two hundred molar equivalents of 5-bromouracil. This could indicate that about one out of twenty-five molecules of 5-bromouracil was converted to its deoxyriboside by route (b). Also, in the presence of 5-bromouracil, the thymine–thymidine conversion ratio dropped from 1:5 to less than 1:100. This can be considered a measure of the competitive blocking by 5-bromouracil of thymidine synthesis *via* route (b).

Thus the quantitative data on 5-bromodeoxyuridine inhibition are at least compatible with the assumption that 5-bromouracil acts as a competitive inhibitor of thymidine by being first converted to 5-bromodeoxyuridine, and that it is the latter compound which competes with thymidine. However, the stimulatory effect of 5-bromouracil cannot be attributed directly to its deoxyriboside, as 5-bromodeoxyuridine shows a much weaker reversing action on 5-nitrouracil inhibition than does 5-bromouracil (Table III). It appears that 5-bromouracil,

(22) During the time this paper was being prepared, the preparation of 5-bromodeoxyuridine was reported by R. E. Beltz and D. W. Visser, THIS JOURNAL, **77**, 736 (1955). The method used by us was similar and is described in the Experimental Section.

(23) With increasing concentration of thymine, this thymine/thymidine activity ratio increases from 1:5 to about 1:2, indicating an acceleration of the conversion of thymine to thymidine in accordance with the law of mass action.

TABLE III

REVERSAL OF 5-NITROURACIL INHIBITION OF *L. Leichmannii* WITH 5-BROMOURACIL AND ITS NUCLEOSIDES

Reversing agent[a]	μg./tube	Turbidity[b]
5-Bromouracil	0	92
5-Bromouracil	1	63
5-Bromouracil	2.5	60
5-Bromouracil	5	57
5-Bromouracil	10	56
5-Bromouracil	25	44
5-Bromouracil	50	46
5-Bromouracil	100	62
5-Bromouracil	250	85
5-Bromouridine	0	91
5-Bromouridine	1	62
5-Bromouridine	2.5	63
5-Bromouridine	5	67
5-Bromouridine	10	63
5-Bromouridine	25	60
5-Bromouridine	50	68
5-Bromouridine	100	66
5-Bromouridine	250	58
5-Bromodeoxyuridine	0	94
5-Bromodeoxyuridine	.01	89
5-Bromodeoxyuridine	.025	88
5-Bromodeoxyuridine	.05	86
5-Bromodeoxyuridine	.075	88
5-Bromodeoxyuridine	.10	92
5-Bromodeoxyuridine	.25	94
5-Bromodeoxyuridine	.50	97
5-Bromodeoxyuridine	1.0	97

[a] Medium contains 100 μg./tube 5-nitrouracil. [b] Turbidometer readings, % transmission. No growth = 100, maximum growth = 12.

exerting its "thymidine-sparing action" through route (c), does not go through 5-bromodeoxyuridine as such, but perhaps goes through a conjugate of this compound or is "built in"[19] directly into the DNA. Consequently, it seems necessary to assume two routes of 5-bromouracil conversion: (1) by the enzymes of route (b) to 5-bromodeoxyuridine, and (2) by the enzymes of route (c) to "DNA-5-bromouracil."

The riboside of 5-bromouracil, 5-bromouridine, was synthesized according to the procedure of Fukuhara and Visser.[24] This compound was found relatively inactive as an inhibitor in this system, and the slight inhibition obtained at high levels was reversible with thymidine. On the other hand, in reversing 5-nitrouracil inhibition, 5-bromouridine is, on a molar basis, about as active as 5-bromouracil.

In this *L. leichmannii* system, all three 5-sulfur substituted uracil derivatives tested were found to be inhibitory at relatively low concentrations. On a molar basis, 5-uracilyl isothiouronium chloride is about equally as active as 5-mercaptouracil and 5-uracilyl disulfide is twice as active. This may indicate interconversion of the three compounds in the organism, all yielding ultimately the same compound.

All three compounds behave as competitive antagonists of thymine. In this they differ from 5-bromouracil by their apparent inability of being converted to their deoxyribosides. This is indicated by the essentially non-competitive reversal of the inhibition by thymidine, and by the lack of any such stimulatory effects as we observed in the case of 5-bromouracil.

At the present, time only the disulfide has been studied extensively. The results which are given in Table II show that 5-methylcytosine is about one-fourth as active as thymine in competitively reversing the inhibiting effect of the disulfide. Uracil and uridine are somewhat effective at low concentrations of the inhibitor. Cytosine and cytidine show even less effect than uracil and uridine on 5-uracilyl disulfide inhibition, in contrast to their significant reversing action on 5-bromouracil. However, here, too, the riboside is much more active than the pyrimidine.

Our results with *L. arabinosus* indicate that in this organism thymine is not a normal intermediate of thymidine synthesis. It is known that this organism does not require external supply of folic (or folinic) acid nor of vitamin B_{12} for normal growth.[25] Apparently, it is able to synthesize its "folic acid enzyme" (FE) from *p*-aminobenzoic acid, perhaps without the intermediate formation of folic acid and folinic acid (route f-4 of Scheme II) (see Fig. 1). Although the three folic acid antagonists, aminopterin, 2,4-diamino-6,7-diphenylpteridine and the 2,4-diaminopyrimidine are just as effective as inhibitors in this system as in *L. leichmannii*, both folic acid and folinic acid are inactive as reversing agents (Table IV). They are less effective the less the antagonist resembles the structure of folic–folinic acid. Only aminopterin can be reversed, apparently competitively, at low levels of the inhibitor, and this requires massive doses of folic or folinic acid. Synthetic folinic acid is about twice as effective as folic acid. The pterin and the diaminopyrimidine are essentially irreversible with either folic or folinic acid.

On the other hand, the reversal of these folic acid antagonists with thymidine is somewhat of the competitive type, *i.e.*, the amount of thymidine required increases with the concentration of the inhibitor, indicating that thymidine, in this case, is not only a product of the inhibited reaction, but is also directly involved with the action of the inhibited enzyme (FE). It has been reported[26] that relatively small amounts of thymidine potentiate the activity of folinic acid in *L. citrovorum* and a synergism between these two compounds has been suggested. More recently, Weaver and Shive[27] reported that the utilization, by a cell-free extract of *L. arabinosus*, of 5-amino-4-imidazolcarboxamide (a purine precursor) was inhibited by aminopterin. This inhibition could be reversed by thymidine while folinic acid acted synergistically with thymidine. These authors suggested that thymidine may act by forming an intermediate conjugate with the purine precursor. In our Scheme I, the arrow indicating an action of thymidine at reaction step (e) was, in the case of *L. leichmannii*, meant to represent a possible deoxyribose transfer from thymi-

(24) T. K. Fukuhara and D. W. Visser, *J. Biol. Chem.*, **190**, 95 (1951).

(25) J. O. Lampen and M. J. Jones, *ibid.*, **170**, 133 (1947).

(26) T. J. Bardos, T. J. Bond, J. Humphreys and W. Shive, THIS JOURNAL, **71**, 3852 (1949).

(27) J. M. Weaver and W. Shive, *ibid.*, **75**, 4628 (1953).

TABLE IV

REVERSAL OF GROWTH INHIBITION BY FOLIC ACID ANTAGONISTS IN *L. Arabinosus*

Inhibitor	Concn., μg./tube	For ½-maximal reversal required, μg./tube: Folic acid	Folinic acid	Thymine	Thymidine	5-Bromouracil	5-Bromouridine
Aminopterin	0.8	75	30	400[c]	2.5[c]	1000[c]	..
Aminopterin	1.0	90	50	..	3.5[c]	..	..
Aminopterin	2.0	90	..	..	5.0[c]	..	..
Aminopterin	4.0	250	..	..	..	..	..
2,4-Diamino-6,7-diphenylpteridine	2	[b]	[b]	900	0.1	35[c]	[b]
2,4-Diamino-6,7-diphenylpteridine	4	[b]	..	3000	0.4	200[c]	[a]
2,4-Diamino-6,7-diphenylpteridine	6	[a]	..	..	0.6	..	..
2,4-Diamino-6,7-diphenylpteridine	10	..	..	..	0.8	..	..
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-methylpyrimidine[14]	2.0	[a]	[a]	750[c]	0.02	8	3
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-methylpyrimidine	2.5	..	..	..	0.06	14[c]	5
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-methylpyrimidine	5	..	..	..	0.20	[b]	[b]
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-methylpyrimidine	10	..	..	..	0.40	[a]	[a]
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-methylpyrimidine	25	..	..	..	1.20	..	..
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-methylpyrimidine	50	..	..	..	500[c]	..	..

[a] No reversal. [b] Partial reversal, but ½-maximal growth was not obtained. [c] Half-maximal reversal was obtained, but maximal growth could not be restored even with high concentrations of the reversing agent.

dine to the purines[28] which were present in the medium. In the case of *L. arabinosus*, the action of thymidine might be a catalytic action on the formation of purine nucleosides. It is interesting to note that our results from the growth inhibition experiments with *L. arabinosus* agreed, in a quantitative manner, with the results of the cell-free enzyme experiments referred to above.

Evidence against thymine being an intermediate in this organism is indicated by the inactivity of 5-bromouracil as inhibitor. If thymidine synthesis proceeds entirely by route (c) then, in the sense of the above discussion, 5-bromouracil is expected to be stimulatory. Indeed, this is the case in *L. arabinosus*, evident in the competitive reversal of 5-nitrouracil inhibition (Table V) and even in the partial reversal of the folic acid antagonists in this system (Table IV).

Although 5-bromouracil has little inhibitory activity and its riboside is essentially inactive in *L. arabinosus*, 5-bromodeoxyuridine is highly active as an inhibitor (see Tables V and VII). It is competitively reversible with thymidine as it was in *L. leichmannii*, but thymine is much less active here as a reversing agent. If we can apply to this system our observation on *L. leichmannii*, that 5-bromodeoxyuridine does not inhibit the thymine–thymidine conversion (route b), then the relative activities of thymine and thymidine in reversing 5-bromodeoxyuridine inhibition could be considered a measure of the ability of *L. arabinosus* to convert thymine into thymidine. This thymine–thymidine activity ratio increases[23] from 1:450, at lower levels, to 1:120, at high levels of thymine (Table V). In contrast to the high stimulatory activity of 5-bromouracil in this organism, 5-bromodeoxyuridine has, here too, very little stimulatory action at its sub-inhibitory levels. It appears, therefore, that in *L. arabinosus*, 5-bromouracil is largely converted to "DNA-5-bromouracil" and only to a small extent to 5-bromodeoxyuridine, while the reverse was found in *L. leichmannii*. This is compatible with the relative inefficiency of route (b) in *L. arabinosus*. Again, as a reversing agent, 5-bromouridine is more active, on a molar basis than 5-bromouracil. Moreover, 5-bromouridine reverses the inhibitory action of 5-bromodeoxyuridine, at low levels of the inhibitor (Table V).

Thymine itself has relatively little activity in reversal of the inhibition by 5-nitrouracil or the three folic acid antagonists, and its action is competitive with 5-nitrouracil (Table V). This indicates that in this system thymine is not the product of the reaction blocked by these agents. Thymine in this case appears to be a "substitute" metabolite, and a less satisfactory one than the abnormal metabolite, 5-bromouracil. The "normal" thymidine-precursor in this system remains unknown.

In the *L. arabinosus* system, the 5-mercaptouracil compounds had relatively low inhibitory activity (Table VIII). Since they behave as thymine antagonists, this agrees with the non-essential role of thymine in this system. The sulfur compounds are active only at such high concentrations that it is doubtful that we can still consider their action in this case as specific. At the lowest inhibitory levels, thymine can reverse the inhibition by these compounds, but at higher concentrations the inhibition becomes irreversible with thymine. None of the other metabolites tested have any reversing action.[29] Even thymidine produces only 10–20% reversal. This inability of thymidine to completely reverse the inhibition indicates a non-specific toxic action of these compounds at these high concentrations.

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Experimental

5-Bromodeoxyuridine.[22]—The procedure described was used by Fukuhara and Visser[24] for the preparation of 5-bromouridine.

Deoxyuridine (2.28 g., 0.01 mole) was dissolved in 50 ml. of water and saturated bromine water was added until

(28) W. S. MacNutt, *Biochem. J.*, **50**, 384 (1952).

(29) These include uracil, uridine, cytosine, cytidine, 5-methylcytosine, adenine, guanine, adenosine, guanosine, deoxycytidine, deoxyadenosine, deoxyguanosine, *p*-aminobenzoic acid, folic acid and folinic acid.

TABLE V

REVERSAL OF GROWTH INHIBITION BY STRUCTURAL ANALOGS OF THYMINE AND THYMIDINE IN *L. Arabinosus*

Inhibitor	Concn., μg./tube	For ½-maximal reversal required, μg./tube: Thymine	Thymidine	Uracil	Cytosine	Cytidine	5-Bromo-uracil	5-Bromo-uridine
5-Nitrouracil	500	150	1.0	500	80	25	2.0	3
5-Nitrouracil	750	200	1.2	700	..	..	..	..
5-Nitrouracil	1000	300	1.2	1000	..	80	3.4	4
5-Nitrouracil	1500	..	1.2	..	..	..	..	..
5-Nitrouracil	2000	400	1.3	..	..	[a]	8	7
5-Nitrouracil	4000	850	1.5	..	..	..	15	10
5-Nitrouracil	6000	1300	1.5	..	..	..	25	25
5-Bromodeoxyuridine	1	..	<0.1	..	..	..	[a]	20
5-Bromodeoxyuridine	2.5	..	0.15	..	..	..	[a]	150
5-Bromodeoxyuridine	5	150	0.3	..	..	..	[a]	500
5-Bromodeoxyuridine	10	250	0.7	..	..	..	..	[b]
5-Bromodeoxyuridine	25	400	1.8	..	..	..	..	[a]
5-Bromodeoxyuridine	50	600	5	..	..	..	..	..
5-Bromodeoxyuridine	100	..	10	..	..	..	..	..
5-Bromodeoxyuridine	250	..	30	..	..	..	..	..
5-Uracilyl disulfide	600	500	[b]	[a]	[a]	[a]	[a]	[a]
5-Uracilyl disulfide	700	800[c]	[b]	..	..	..	..	..
5-Uracilyl disulfide	1000	[a]	[a]	..	..	..	..	..
Uracil-5-isothiouronium chloride	3500	3000[c]	[b]	[a]	[a]	[a]	..	..

[a] No reversal. [b] Partial reversal, but ½-maximal growth was not obtained. [c] Half-maximal reversal was obtained, but maximal growth could not be restored even with high levels of the reversing agent.

the yellow color persisted. Air was then bubbled in until the solution was clear, and the clear solution was lyophilized. The residue was taken up in 250 ml. of absolute alcohol, refluxed for 15 minutes, and concentrated to 30 ml. under reduced pressure. Crystals formed slowly overnight. The solid was filtered and recrystallized from absolute alcohol, treating with charcoal while hot. The yield was 0.82 g. (27%) of 5-bromodeoxyuridine, m.p. 181–183°.

Anal. Calcd. for $C_9H_{11}O_6N_2Br$: C, 35.21; H, 3.61; N, 9.13; Br, 26.0. Found: C, 35.36; H, 3.78; N, 9.04; Br, 23.8.

Microbiological Methods. *Lactobacillus leichmannii 313.*—The composition of the basal medium and the techniques used in this assay were recently reported in detail.[30] In the present experiments, however, the assay tubes were incubated for 16 hours. Furthermore, the folic acid concentration of the basal medium was changed to 0.080 μg. per 100 ml., and the vitamin B_{12} concentration to 0.003 μg. per 100 ml. In one experiment, where the growth promoting activities of folic acid, folinic acid, thymine and thymidine were compared (Table VI), the vitamin B_{12} concentration was raised to 0.006 μg. per 100 ml., while the folic acid was omitted from the basal medium.

TABLE VI

COMPARATIVE GROWTH FACTOR ACTIVITIES OF FOLIC ACID, FOLINIC ACID, THYMINE AND THYMIDINE IN *L. Leichmannii*

Growth factors[a]—Turbidometer readings[b]: μg./tube	Folic acid	Folinic acid	μg./tube	Thymine	Thymidine
0[a]	90[b]	90[b]	0[a]	83[b]	83[b]
.0001	85	93	.05	83	60
.00025	76	89	.10	80	58
.00050	57	75	.25	72	50
.00075	30	67	.50	61	42
.0010	20	56	1.0	44	31
.0015	13	38	2.5	30	30
.002	12	31	5	27	27
.004	11	16	10	27	27
.005	10	12	25	20	21

[a] Medium contains 0.3 mμg./tube vitamin B_{12}, but no folic acid. [b] Turbidometer readings: % transmission. No growth = 100.

(30) T. J. Bardos, H. L. Gordon and E. F. Heenan, THIS JOURNAL, **77**, 3115 (1955).

Lactobacillus arabinosus.—The organism was maintained on a stock medium containing 2.5% yeast extract, 0.5% dextrose, 0.5% anhydrous sodium acetate and 1.5% agar. The basal growth medium used in the inhibition and reversal experiments was a modification of the one described by Skeggs and Wright.[31] In our basal medium, cystine was

TABLE VII

INHIBITORY EFFECT OF 5-BROMOURACIL AND ITS NUCLEOSIDES IN *L. Leichmannii* AND *L. Arabinosus*

Inhibitors—Turbidometer readings[a]: μg./tube	In *L. leichmannii* 5-Br-U[b]	R[c]	DR[d]	In *L. arabinosus* 5-Br-U[b]	R[c]	DR[d]
0	12[a]	13[a]	13[a]	31[a]	22[a]	20[a]
0.5	..	..	38	..	..	65
0.75	..	..	..	..	..	92
1	..	..	53	..	..	94
2.5	..	..	85	..	..	98
25	20	26	100	..	25	100
100	35	38	..	37	25	..
250	85	46	..	40	25	..
500	91	46	..	48	..	..
1000	93	44	..	52	26	..

[a] Turbidometer readings: % transmission. No growth = 100. [b] 5-Bromouracil. [c] 5-Bromouridine. [d] 5-Bromodeoxyuridine.

TABLE VIII

INHIBITORY EFFECT OF 5-MERCAPTOURACIL AND ITS DERIVATIVES IN *L. Leichmannii* AND *L. Arabinosus*

Inhibitors—Turbidometer readings[a]: μg./tube	In *L. leichmannii* 5-MU[b]	UDS[c]	UTH[d]	μg./tube	In *L. arabinosus* 5-MU[b]	UDS[c]	UTH[d]
0	39[a]	39[a]	38[a]	0	33[a]	34[a]	34[a]
2.5	49	49	43	100	36	37	..
5.0	62	67	53	250	42	45	..
7.5	81	83	63	500	60	74	..
10	87	89	77	750	85	90	..
15	88	92	85	1000	90	92	45
25	93	94	91	2500	..	..	98

[a] Turbidometer readings: % transmission. No growth = 100. [b] 5-Mercaptouracil. [c] 5-Uracilyldisulfide. [d] Uracil-5-isothiouronium chloride.

(31) H. R. Skeggs and L. D. Wright, *J. Biol. Chem.*, **156**, 21 (1944).

replaced by cysteine hydrochloride, 10 mg. per 100 ml., and the vitamin concentrations were changed to the following values (per 100 ml.): thiamine hydrochloride, 50 μg.; pyridoxine hydrochloride, 100 μg.; pyridoxal hydrochloride, 30 μg.; pyridoxamine dihydrochloride, 30 μg.; calcium pantothenate, 50 μg.; nicotinic acid, 100 μg.; riboflavin, 50 μg.; biotin, 0.10 μg.; *p*-aminobenzoic acid, 10 μg.

The techniques used were the same as in the *L. leichmannii* assay, except that the tubes were incubated 20 hours, which incubation time was necessary for obtaining maximal growth in the control tubes.

Inhibition Experiments.—The inhibitors were dissolved and diluted to the required concentrations. One milliliter of each of these dilutions was added to four milliliters of 1.25-fold basal medium, and the inhibitor concentrations were expressed as μg. per (5 ml.) tube.

Reversal Experiments.—The inhibitor was added, in dry form or from stock solution, to the 1.25-fold basal medium. Four milliliters of this medium was added to each assay tube, and one milliliter of a series of dilutions of the reversing agent was added to it.

The turbidity reading corresponding to half-maximal growth was determined from the standard curve. This value was usually between 55 and 65. For every inhibitor concentration in the medium, the turbidity readings were plotted against the concentrations of the reversing agent in the corresponding assay tubes. The concentration of the reversing agent required for half-maximal growth (*i.e.*, half-maximal reversal) was obtained by interpolation to the half-maximal turbidity reading.

These values were tabulated in Tables I, II, IV and V.

CHICAGO 9, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

The Reactivation of Acetylcholinesterase Inhibited by Tetraethyl Pyrophosphate and Diisopropyl Fluorophosphate[1]

BY IRWIN B. WILSON, S. GINSBERG AND E. K. MEISLICH

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The reactivation of TEPP and DFP inhibited acetylcholinesterase has been studied with several reactivators and under various conditions with the aim of elucidating the mechanism of the reaction. It is hoped that once the principles are clarified it will be possible to synthesize compounds of practical value in overcoming the toxic effects of these inhibitors. It has been shown that the hydroxamic acids form complexes with the inhibited enzyme analogous to the complexes formed between active enzymes and their substrates. It has been shown that the hydroxamate ions are the reactivators. The shape of the *p*H dependence of reactivation has been interpreted in terms of the theory of inhibition and reactivation which had been previously developed. The reactivation is a combined acid–base attack with the solvent supplying the proton in most cases, but in the case of choline the latter makes both attacks. The anionic site is still functional in TEPP inhibited enzyme, though less so than in the normal enzyme, and serves to promote the activity of certain reactivators. The anionic site does not appear to be functional in DFP inhibited enzyme. The reactivation of DFP inhibited enzyme depends upon the conditions of inhibitions and storage and some of the difficulty in restoring the enzyme can be attributed to secondary effects arising from unfavorable conditions of inhibition and storage. Of the reactivators considered in this paper nicotinhydroxamic acid methiodide is the best for overcoming TEPP inhibition and roughly the same as nicotinhydroxamic acid in overcoming DFP inhibition.

Certain phosphate esters such as tetraalkyl pyrophosphates, dialkyl *p*-nitrophenyl phosphates and dialkylfluorophosphates are potent irreversible inhibitors of acetylcholinesterase and of esterases in general. These compounds are of general interest because the most potent chemical warfare gases and some powerful insecticides belong to this class and owe their lethality to the inhibition of acetylcholinesterase.

The development of the theory of enzymatic hydrolysis[2,3] clarified the mechanism of irreversible inhibition and suggested the means whereby reactivation might be achieved.[4,5] The active site of this enzyme may be conveniently considered as consisting of two subsites: (a) an anionic site which contributes to the catalytic activity by binding and orienting substituted ammonium structures such as that which occurs in acetylcholine; (b) an ester interacting or esteratic site which contains an acidic and a basic group, both of which are necessary for activity. During enzymatic hydrolysis the esteratic site makes a combined acid base attack upon esters and the basic group is thereby acylated. This acyl enzyme reacts rapidly with water to yield a carboxylic acid and free enzyme.

The alkyl phosphates function as inhibitors by interacting with the enzyme in much the same way as substrates to form in this case a dialkylphosphoryl enzyme, but unlike the analogous acyl enzyme the phosphoryl enzyme does not readily react with water and so the free and active enzyme is not rapidly restored.

The equation for the inhibitory reaction (here illustrated with a fluorophosphate) is

$$\mathrm{H{-}\ddot{G}} + (\mathrm{RO})_2{-}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}{-}\mathrm{F} \longrightarrow \mathrm{G}{-}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}(\mathrm{OR})_2 + \mathrm{HF}$$

Here H-G is the esteratic site of the enzyme: H represents the acidic group and the electron pair (. .) represents the basic group.

It would appear from the theory that the enzyme could be dephosphorylated by means of bimolecular nucleophilic displacement reactions and its activity thus be restored. And in fact, when R = ethyl (inhibitor = tetraethyl pyrophosphate (TEPP) or diethyl fluorophosphate) reactivation is readily accomplished with nucleophilic reagents such as hydroxylamine, but when R = isopropyl (inhibi-

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(2) (a) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950); (b) I. B. Wilson, F. Bergmann and D. Nachmansohn, *ibid.*, **186**, 781 (1950).

(3) I. B. Wilson, in "The Mechanism of Enzyme Action," Ed. by W. D. McElroy and B. Glass, The John Hopkins Press, Baltimore, Md., 1954, p. 642.

(4) I. B. Wilson, *J. Biol. Chem.*, **190**, 111 (1951).

(5) I. B. Wilson, *ibid.*, **199**, 113 (1952).