required that the methylenedioxy group be at positions 7, 8 and, thus, the structure II may be written for lunine.

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Biosynthesis of Tetracyclines. I. The Halide Metabolism of *Streptomyces aureofaciens* Mutants. The Preparation and Characterization of Tetracycline, 7-Chloro^{36}-tetracycline and 7-Bromotetracycline

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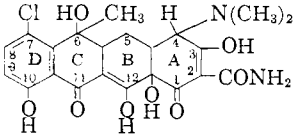
Quantitative measurements have been made of the chloride and bromide metabolism, as regards incorporation into the tetracyclines, of three *Streptomyces aureofaciens* mutants. These three are closely related genetically, but differ from each other in the quantitative features of their halide metabolism. They represent two metabolic classes. The classification is based on the independence or dependence on concentration of chloride of the rate of conversion of chloride to 7-chlorotetracycline.[1] The mutants differ substantially in their susceptibility to inhibition by bromide of the incorporation of chloride into 7-chlorotetracycline. This inhibition has been shown reversible by excess chloride. Halogenation in all three mutants is substantially inhibited by thiocyanate; the degree of reversibility of this inhibition varies among the mutants. The total terminal tetracyclines concentration of these systems is independent of chloride and bromide concentration from values near zero to values ten times the stoichiometric equivalent of the total tetracyclines potential of the system. The total tetracyclines potential is the sum of the millimoles per liter of 7-chlorotetracycline, 7-bromotetracycline and tetracycline present at the end of the fermentation cycle. Withholding halide or inhibiting halide utilization results in biosynthesis of equimolar amounts of the unsubstituted product, tetracycline. The fermentation end products from these mutants do not contain any significant quantities of chlorinated organic materials except 7-chlorotetracycline. Tetracycline, 7-chloro^{36}-tetracycline and 7-bromotetracycline have been prepared biosynthetically through the use of *Streptomyces aureofaciens* Duggar. Some of their properties have been determined. The preparation of 7-chloro^{36}-tetracycline started with HCl^{36} and proceeded in good yield in a manner involving substantially no isotopic dilution.[2]

Introduction and Discussion

The abilities of a variety of biological systems to incorporate the four halides into halogenated organic compounds of many types are well known. Metabolic experiments with *Streptomyces aureofaciens* Duggar grown in media containing chloride^{36} and in media containing no available halogen except chloride ion have shown that chloride can serve the organism efficiently as the sole source of the chlorine in the broad-spectrum antibiotic, 7-chlorotetracycline (CTC), resulting from the mold's metabolic processes.[3] Similar experiments



CTC

using media containing no available halogen except bromide ion have shown that bromide can serve the organism efficiently as the sole source of the bromine in the broad spectrum antibiotic, 7-bromotetracycline (BTC).[4] Witholding chloride and bromide[5] or inhibiting halide utilization[6] prevents the formation of CTC and BTC and results in the formation of equivalent amounts of tetracycline (TC). In the mutants described here, bromination is accomplished at a lesser rate than chlorination; for bromide levels not toxic to the organism, this reduction in halogenation rate is accompanied by the formation of more TC. The total terminal tetracyclines concentration of these fermentation systems is independent of the chloride and bromide concentration from values near zero to values ten times the stoichiometric equivalent of the total tetracyclines potential of the particular system. (Total tetracyclines potential is the sum of the millimoles per liter of 7-chlorotetracycline, 7-bromotetracycline and tetracycline present at the end of the fermentation cycle.)

The three mutants described here are coded BC-41, S-1055 and S-580. Mutants S-1055 and S-580 are direct isolates from cultures of BC-41. All are descendants of the original *S. aureofaciens* A-377 soil isolate of Duggar. They resemble each other in the over-all aspects of their halide metabolism but differ in the quantitative features of their halide metabolism, falling into two classes. Class I contains those mutants whose rate of chloride utilization for CTC is independent of chloride ion concentration; Class II includes those whose rate of chloride utilization depends on

(1) The trademarks of the American Cyanamid Company for tetracycline and 7-chlorotetracycline are Achromycin and Aureomycin, respectively.

(2) A summary of the material presented here has previously been published: A. P. Doerschuk, J. R. D. McCormick, J. J. Goodman, S. A. Szumski, J. A. Growich, P. A. Miller, B. A. Bitler, E. R. Jensen, M. A. Petty and A. S. Phelps, THIS JOURNAL, **78**, 1508 (1956).

(3) M. A. Petty and M. Matrishin, Abst. of Papers, 118th Meeting, American Chemical Society, 1950.

(4) P. Sensi, *Il. Farmaco Sci. Ed.*, **10**, 346 (1955).

(5) G. Rolland and P. Sensi, *Farm. sci. e. tec. (Pavia)*, **10**, No. 1, 37 (1955).

(6) A. Gourevitch, M. Misiek and J. Lein, *Antibiotics and Chemotherapy, V*, No. 8, 448 (1955).

TABLE I

RATE OF PRODUCTION (MEQ./L./HR.) OF CTC AND TC AT FERMENTATION AGE 32 HR. BY THREE MUTANTS AT SEVERAL CHLORIDE CONCENTRATIONS

Chloride concn. (meq./l.)	Mutant BC-41			Mutant S-1055			Mutant S-580		
	CTC	TC	Total	CTC	TC	Total	CTC	TC	Total
0.0	0.00	0.17	0.17	0.00	0.074	0.074	0.00	0.10	0.10
2.8	.15	.016	.17	.025	.065	.090	.0025	.080	.083
8.5	.16	.014	.17	.043	.048	.091			
11.3	.15	.016	.17	.053	.038	.091	.031	.054	.085
16.9	.16	.016	.18	.062	.027	.089	.048	.047	.095

chloride ion concentration. The experimental data used for classification are presented in Table I.

Mutant BC-41 is a member of Class I. As shown in Table I, the rate of appearance of CTC is independent of chloride concentration. At 32 hr., the rate of accumulation is 0.16 meq./l./hr. This rate was found to be independent of time from 24 to 96 hr. while chloride remained. The data of Table I show that, while chloride is present, TC is produced at 0.016 meq./l./hr. This rate also was found independent of time from 24 to 96 hr., so long as chloride was present. From the relative magnitudes of these rates and their independence of chloride concentration and time, it is apparent that chloride in quantities up to 91% (100 × 0.16/(0.16 + 0.016)) of that stoichiometrically equivalent to the total tetracyclines potential of the system at any particular time will be completely utilized for CTC and that chloride exhaustion is possible. This is presented experimentally in Fig. 1. Quantities of chloride in excess of this amount should have no effect on the fermentation. This has been observed for quantities of chloride threefold (Fig. 1) and tenfold in excess of that theoretically utilizable. After chloride exhaustion, the TC production rate increased to 0.17 meq./l./hr., the sum of the rates of production of TC and CTC before chloride exhaustion.

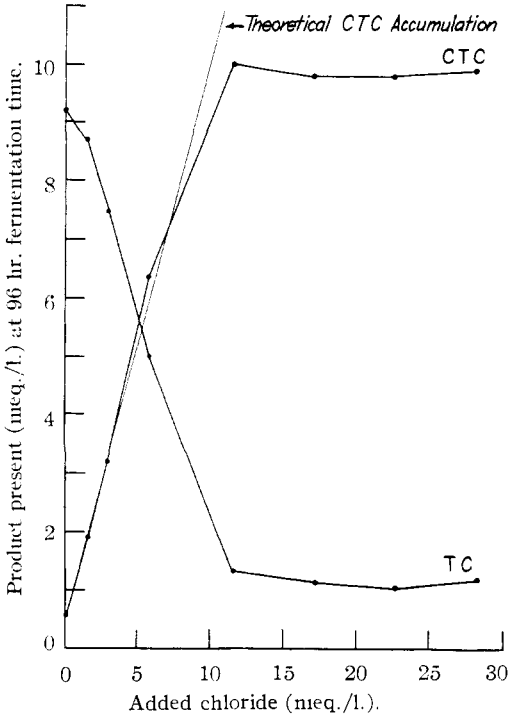


Fig. 1.—Mutant BC-41. Demonstration of the complete utilization of added chloride and of the complementary relationship of CTC and TC.

Bromide, when present as the only halide, is utilized for BTC biosynthesis at the rate of 0.05 meq./l./hr. (Table II). TC synthesis occurred simultaneously at the rate of 0.11 meq./l./hr. All these rates were found to be independent of bromide concentration and independent of time from 24 to 96 hr. From this it follows that a BC-41 fermentation containing only bromide should produce a mixture of tetracyclines containing about 30% BTC and 70% TC. Examination of Fig. 2 shows that this proportion does occur. For bromide concentrations up to about 2.5 meq./l., these relationships permit complete utilization of bromide for BTC (Fig. 2). Significant additional BTC is not formed at bromide levels above 2.5 meq./l., even though such levels are well below the stoichiometric equivalence of the total tetracyclines potential. BTC has not, over a wide range of bromide concentrations, been found to approach the 92% of the total final tetracyclines found for CTC.

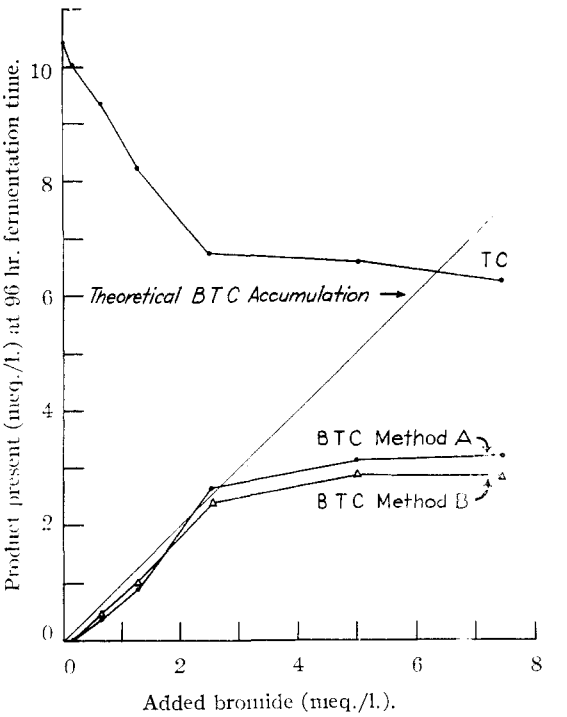


Fig. 2.—Mutant BC-41. Demonstration of the complete utilization of added bromide and of the complementary relationship of BTC and TC.

TABLE II

RATE OF PRODUCTION (MEQ./L./HR.) OF BTC AND TC AT FERMENTATION AGE 32 HR. BY THREE MUTANTS[a] AT SEVERAL BROMIDE CONCENTRATIONS IN THE ABSENCE OF CHLORIDE

Bromide concn. (meq./l.)	Mutant BC-41 BTC	Mutant BC-41 TC	Mutant BC-41 Total
0.0	0.000	0.16	0.16
1.25	.050	.11	.16
2.50	.052	.11	.16
5.00	.047	.11	.16

[a] No BTC could be detected with S-1055 and S-580 at any bromide level.

Bromide, when present with excess chloride, results in relatively small inhibition of chlorination and a correspondingly small increase in formation of TC (Table III). Small amounts of BTC are probably formed but could not be detected. In general, under these conditions chlorination is the preferred process. Chlorination by BC-41 is substantially inhibited by thiocyanate (Table IV). This inhibition is not reversible by excess chloride (compare the CTC accumulation at 5.8 and 28 meq./l. of chloride at both levels of thiocyanate in Table IV). The decrease in CTC in the inhibited system is reflected by an approximately equivalent increase in TC.[7]

TABLE III

INHIBITION OF CHLORINATION IN THREE MUTANTS[a] BY BROMIDE AS SHOWN BY ANTIBIOTIC ACCUMULATION AT 96 HR. (MEQ./L.)

Bromide concn. (meq./l.)	Mutant BC-41 35 meq. Cl^-/L. CTC	Mutant BC-41 35 meq. Cl^-/L. TC	Mutant S-580 5.8 meq. Cl^-/L. CTC	Mutant S-580 5.8 meq. Cl^-/L. TC	Mutant S-580 35 meq. Cl^-/L. CTC	Mutant S-580 35 meq. Cl^-/L. TC
0.00	7.76	0.82	1.98	4.89	4.22	2.07
.125	7.85	0.86	1.19	5.94	3.50	2.72
.312			0.87	6.16	2.44	3.78
.625	7.85	1.02	.58	6.18	1.82	3.86
1.25	7.56	1.17	.41	6.08	1.38	4.49
2.50	7.56	1.45	.29	6.24	1.04	4.79
3.75			.29	5.90	0.78	5.21
5.00	7.47	1.76				
7.50	7.17	1.75				

[a] The behavior of S-1055 is qualitatively similar to that of S-580.

TABLE IV

INHIBITION OF CHLORINATION IN THREE MUTANTS[a] BY THIOCYANATE AS SHOWN BY ANTIBIOTIC ACCUMULATION AT 96 HR. (MEQ./L.)

Thiocyanate concn. (meq./l.)	Mutant BC-41 5.8 meq. SCN^-/L. CTC	Mutant BC-41 5.8 meq. SCN^-/L. TC	Mutant BC-41 28.2 meq. SCN^-/L. CTC	Mutant BC-41 28.2 meq. SCN^-/L. TC	Mutant S-580 5.8 meq. SCN^-/L. CTC	Mutant S-580 5.8 meq. SCN^-/L. TC	Mutant S-580 28.2 meq. SCN^-/L. CTC	Mutant S-580 28.2 meq. SCN^-/L. TC
0.00	5.34	5.21	11.4	1.10	2.07	3.51	3.73	1.17
.172					2.16	3.76	3.90	1.53
.431					1.92	4.03	3.78	1.79
.862					1.77	3.97	3.64	2.09
1.72					1.49	4.10	3.06	2.80
3.50	4.61	5.47	4.46	6.38	1.24	4.36	2.42	3.42
17.5	1.79	8.71	1.61	8.78				

[a] The behavior of S-1055 is qualitatively similar to that of S-580.

(7) No 7-thiocyanotetracycline has been detected in any of the systems described here. Paper chromatography of thiocyanate-containing fermented mashes showed a new spot. Thiocyanate addition at the end of the fermentation resulted in a similar finding, as did also the addition of thiocyanate to crystalline TC. Fermentation of $S^{35}CN^-$-containing mashes followed by silver precipitation of $S^{35}CN^-$ and paper chromatographic examination of the supernatant showed only the expected CTC and TC and no more than traces of radioactivity. Paper chomatography prior to silver precipitation showed strong radioactivity at the position of the new spot. It was therefore concluded that the new chromatographic spot was an artifact, resulting from the action of TC as a "carrier" for thiocyanate.

Mutants S-580 and S-1055 are members of Class II. Mutant S-580, selected from a culture of BC-41, possesses, under the uniform fermentation conditions described here, a total tetracyclines potential approximately 55% that of BC-41 (Fig. 1 and 3). Table I shows that for S-580 the rate of

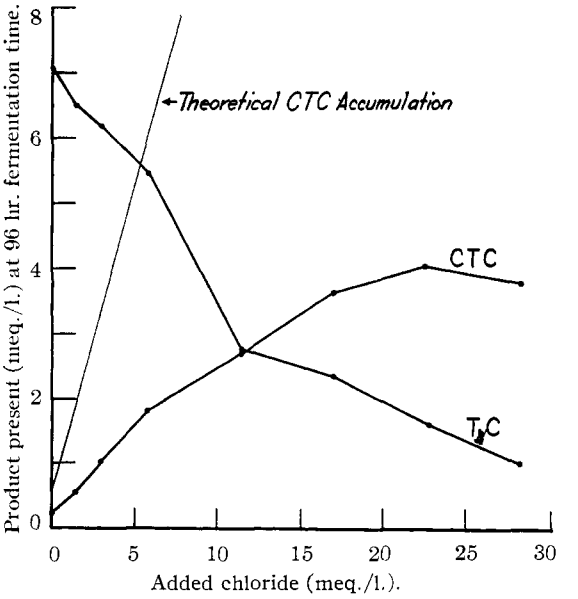


Fig. 3.—Mutant S-580. Demonstration of the incomplete utilization of added chloride and of the complementary relationship of CTC and TC.

appearance of CTC is a function of chloride concentration over the entire range from minimal to at least two times the stoichiometric equivalent of the total tetracyclines potential. Time studies have shown the CTC and TC to accumulate simultaneously during the entire fermentation period over the entire range of chloride studied. In S-580 complete utilization of chloride for CTC is not observed (Fig. 3). The total tetracyclines potential of the system is unchanged by the chloride level (Fig. 3). This observation, and the direct rate measurements of Table I, show that the rate of TC production is inversely related to the chloride concentration. All rates were observed to be approximately constant over the time interval from 24 to 72 hr., the expected changes in rates due to utilization of chloride being smaller than readily detectable. Bromide, when present as the only halide, was not detectably incorporated into BTC. Bromide, in the presence of chloride, markedly represses chlorination, with attendant production of additional TC; within limits, this inhibition of chlorination by bromide can be reversed through the addition of more chloride (Table III). Chlorination by S-580 is substantially inhibited by thiocyanate; within limits, this inhibition is reversible by excess chloride (Table IV).

Mutant S-1055, selected from a culture of BC-41, is a member of Class II and possesses, under the uniform fermentation conditions described here, a total tetracyclines potential approximately 70%

that of BC-41 (Figs. 1 and 4). Table I shows that for S-1055 the rate of appearance of CTC is a function of chloride concentration over the entire range from minimal to two times the stoichiometric equivalent of the total tetracyclines potential. Chloride concentrations up to 13 equivalents have not depressed total tetracyclines. Time studies have shown that CTC and TC accumulate simultaneously during the entire fermentation period over the entire range of chloride studied. For S-1055, the rates of chloride utilization for CTC are high enough to permit chloride concentrations from minimal to about 2 meq./l. (about 25% of the total tetracyclines potential) to be utilized completely for CTC (Fig. 4). All rates were observed to be approximately constant over the time interval from 24 to 72 hr., the expected changes in rates due to utilization of chloride being smaller than readily detectable. The behavior toward bromide, with and without chloride, is similar to that of S-580. Chlorination by S-1055 is substantially inhibited by thiocyanate; within limits, this inhibition is reversible by excess chloride.

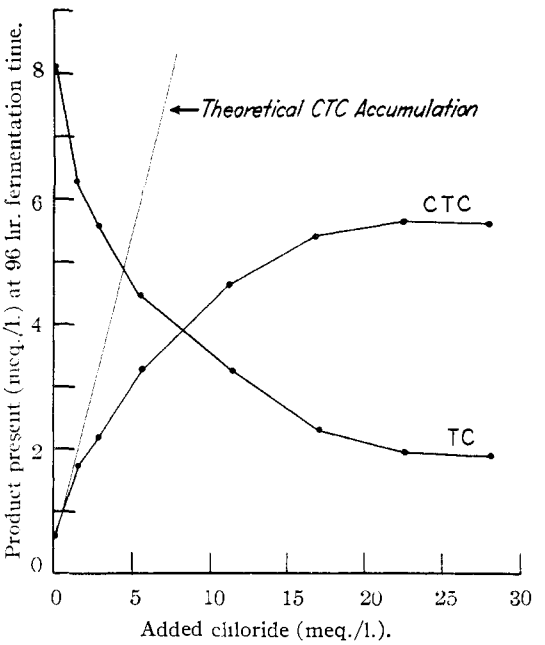


Fig. 4.—Mutant S-1055. Demonstration of the effect of chloride level on the extent of utilization of chloride and of the complementary relationship of CTC and TC.

It should be noted that many rates of chloride utilization, with attendant complete or incomplete utilization of chloride for CTC, are possible in both Class I and Class II, and that the classification is based not on the completeness or incompleteness of chloride utilization for CTC but rather on the dependence or independence on chloride concentration of the rate of chloride utilization for CTC.

For all fermentations described here, substantially all of the chlorine not present as CTC was in the form of chloride ion, as shown by silver chloride precipitation and paper chromatography. Some of the cell material exhibits a small chloride-binding property, but for the cases examined this chloride was completely exchangeable with chloride^{36}, within the limits of experimental error.

The foregoing information has been used for the purpose of preparing tetracycline, 7-chloro^{36}-tetracycline and 7-bromotetracycline. Mutant BC-41, the most effective utilizer of chloride, has been grown in quantity in the absence of chloride, and the TC produced has been isolated as the pure material. A comparison between it and TC produced by the catalytic hydrogenation of CTC[8] showed that they were identical. Behavior toward assay procedures, partition chromatographic data and behavior to paper chromatographic systems indicate that the material produced in the other fermentation systems and here described as TC is also identical with TC prepared by catalytic hydrogenation of CTC.

Mutant BC-41 has been used to prepare 7-chloro^{36}-tetracycline with a specific molar radioactivity substantially equal to that of the HCl^{36} obtainable from the United States Atomic Energy Commission. Fermenting a medium containing no deliberately added halogen but chloride^{36} equivalent to 1600 μg./ml. of CTC resulted in a 98% utilization of chloride for CTC. The material was separated by partition chromatography from the TC also produced. Isolation without non-radioactive dilution from the column eluate gave pure, crystalline 7-chloro^{36}-tetracycline hydrochloride in an over-all yield from chloride^{36} of 56%. The specific radioactivity was 8.4 μCuries/mM.; the value predicted from the radioactivity of the chloride was 10 μCuries/mM.

The preparation of crystalline BTC has been accomplished through the use of mutant BC-41. A fermentation carried out on a medium containing 400 p.p.m. of bromide and only traces of chloride resulted in a product containing 850 μg./ml. of BTC and 1750 μg./ml. of TC. They were separated on a partition chromatographic column. Isolation from the column eluate gave crystalline BTC hydrochloride. BTC has been catalytically hydrogenated to TC under conditions hydrogenating CTC to TC.[8]

Methods

All experiments were carried out under constant fermentation conditions, only the halides being varied. The nutrient medium contained starch, lard oil, corn-steep liquor, water and mineral salts, with or without added halides.[9] These materials, except for corn steep liquor, are all readily obtained essentially free of available halogen. Untreated corn steep liquor, at the concentrations used, contributed on the average about 100 p.p.m. of chloride to the medium, theoretically sufficient for about 1400 μg./ml. of CTC. Many experiments required a basal medium essentially halide-free which could be enriched in measured amounts with chloride and bromide. Several methods were devised for removing chloride from corn steep liquor. One was precipitation by Ag^+ or Hg_2^{++} of the chloride in a diluted steep acidified to *p*H 2.0 with sulfuric acid, followed by precipitation of excess Ag^+ or Hg^{++} with H_2S, removal of excess H_2S *in vacuo*, and adjustment of the *p*H to 4.0 with ammonia water. A variety of anion-exchange columns were also used, particularly sulfuric acid-exhausted Amberlite IR-4B. Supplementation with phosphate completely re-

(8) J. H. Booth, J. Morton, J. P. Petisi, R. G. Wilkinson and J. H. Williams, THIS JOURNAL, **75**, 4621 (1953); L. H. Conover, W. T. Moreland, A. R. English, C. R. Stephens and F. J. Pilgrim, *ibid.*, **75**, 4623 (1953).

(9) J. J. Goodman, Canadian Patent 499,649 (Feb. 2, 1954).

stored these treated corn steep liquors to their original tetracyclines potential.

Three types of assays were used: (1) a fluorometric assay depending on a rearrangement at *p*H 10 and 100° to fluorescent products and a measurement of the fluorescence.[10] BTC yields 27% the response of CTC, while TC yields no response. (2) A spectrophotometric assay (Hiscox) depending on a preliminary alkaline treatment for the destruction of CTC and BTC followed by an acid dehydration of TC to anhydrotetracycline, measured spectrophotometrically.[11] TC responds, while CTC and BTC give no response. (3) A turbidimetric assay depending on antibiotic inhibition of the growth of *Staphylococcus aureus* (F.D.A. 209P). BTC and TC yield, respectively, 90 and 25% the response of CTC.

For fermentations involving bromide addition, parallel runs without bromide were made. The BTC was calculated in two ways, both on the assumption that the CTC formed was constant for all bromide levels: (A) BTC = (fluoro. assay expressed as CTC—fluoro. assay in zero bromide run)/0.27; (B) BTC = (turb. assay expressed as CTC—(TC by spec. assay/4)—fluoro. assay in zero bromide run)/0.9.

For fermentations with Mutant BC-41, the results of the two different calculations for BTC were in agreement (see Fig. 2); in general, the data presented throughout this paper represents method A. The agreement between these two methods verifies the assumption that in BC-41 the CTC level formed is independent of the bromide level. Qualitatively, this assumption is of secondary importance in the chloride-free, bromide-containing cases because the residual chloride concentration is small compared to the BTC concentration formed. Further, for fermentations containing high concentrations of both chloride and bromide, it was observed that, in BC-41, the addition of high bromide concentrations to fermentations containing high chloride levels had little effect on the fluorometric assay. Also, microanalysis of CTC isolated by column chromatography from these fermentations showed no bromine, CTC and BTC being non-separable in the chromatographic system used. A very different situation exists in the cases of Mutants S-580 and S-1055. Here the results of the two methods of calculation for BTC fail to agree, indicating that the assumption that the CTC formed is independent of the bromide concentration is not valid. The situation for both S-580 and S-1055 is, however, very much simplified by the fact that neither mutant forms appreciable BTC, as was demonstrated in the chloride-free case by no increase in fluorometric assay on bromide addition and in the chloride-high, bromide-high case by no bromine in the CTC isolated by chromatography as above.

Interpretation

The facts presented in the foregoing that are the most useful for the purpose of interpreting the nature of the route of tetracyclines biosynthesis are as follows.

1. The concentration of total tetracyclines accumulated by any given mutant is independent of chloride or bromide concentration.

2. For some mutants, the concentration of halogenated tetracyclines obtained is a function of halide concentration; for other mutants, it is not.

3. For all mutants, the concentration of halogenated tetracyclines obtained is susceptible to the effects of specific inhibitors; for some cases, the inhibitions are reversible by excess halide.

These facts lead to a representation for the biosynthetic pathway of the type shown here.

The independence of total tetracyclines and halide composition requires that the over-all rate limiting step lie before halogenation. R_{X^-} and R_2 are the over-all rates for the conversion of A into XB and C, respectively, these being the first intermediates not reversibly reconvertible to A on the pathways from A to CTC and to TC, respectively. Note that C may or may not be the unhalogenated form of XB. Thus, the ratio R_{X^-}/R_2, is the ratio of halogenated tetracycline to tetracycline in the product. In mutants showing a strong dependency of halogenation rate on halide ion concentration, such as S-580, the rate for the conversion of A to XB can be expressed

$$R_{X^-} = f(X^-)$$

For mutants incorporating halide at rates independent of halide ion concentration, such as BC-41, it follows that R_{X^-} is not a function of halide concentration. For BC-41, when chloride is the halide the value of R_{Cl^-}/R_2 is approximately 11, while when bromide is the halide the value of R_{Br^-}/R_2 is approximately 0.45. Assuming that halide ion does not affect the rate in the non-halogenating branch, R_2, it then follows that the ratio, R_{Cl^-}/R_{Br^-}, is 11/0.45, or approximately 25. Hence, for BC-41, the addition of bromide to a chloride-rich system would be expected to result in a product containing no more than 4% BTC, a concentration below the limits of sensitivity of the analytical methods. In actual fact, no BTC was detected, in agreement with the prediction.

For the case of S-580, at a chloride concentration of 35 meq./l., R_{Cl^-}/R_2 is about 2.0, a value much smaller than for the case of BC-41. If R_{Cl^-}/R_{Br^-} for S-580 is approximately 25, as with BC-41, then R_{Br^-}/R_2 for S-580 should be about 0.08, a value so small that bromide should not be utilized effectively even in the absence of chloride. This has, in fact, been observed to be the case (Table II).

A further extension of this reasoning can serve to interpret, through a single scheme, the apparently contrasting susceptibility of BC-41 and S-580 to inhibition by bromide of chloride utilization. If the slowest step in the over-all enzymatic bromination reaction (E = enzyme) is

$$A + E \rightleftarrows AE \quad (1)$$

$$AE + Br^- \rightleftarrows AEBr^- \quad (2)$$

$$AEBr^- \longrightarrow BrB + E + e^- \quad (3)$$

step 3, then bromide addition has the effect of tieing up some of E as $AEBr^-$. For BC-41, where R_{Cl^-}/R_2 is 11, the capacity of the halogenating branch must be at least 11 times that required to handle all of A, since the capacity of the non-halogenating branch is at least sufficient to handle all of A, as it does in the absence of halide. Even if bromide ties up half the halogenating capacity, the halogenating capacity remaining will be sufficient to chlorinate at least 5.5 times as much A as is formed. The expected product under these conditions would then have a CTC/TC ratio of 5.5/1 or 85% CTC, as opposed to a CTC/TC ratio

(10) D. H. Feldman, H. S. Kelsey and J. C. Cavagnol, *Anal. Chem.*, **29**, 1697 (1957).

(11) D. Hiscox, *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 237 (1951).

(12) P. A. Miller, J. R. D. McCormick and A. P. Doerschuk, *Science*, **123**, 1030 (1956).

of 11/1 or 92% CTC in the non-bromide case. That is, bromide would here exert little apparent inhibitory action; in actual fact, only a slight inhibition was observed (Table III).

For S-580, the capacity of the non-halogenating branch is still at least enough to handle all of A, since in the absence of halide there is no reduction in total tetracyclines formation; but, in contrast to BC-41, the halogenating capacity of S-580 may be as low as two thirds that required to chlorinate all of $A(R_{Cl^-}/R_2$ at 35 meq./l. of chloride is 2.0). If one-half of this lesser halogenating capacity is withdrawn by formation of $AEBr^-$, the expected product under bromide-containing conditions would have a CTC/TC ratio of 0.33/1 or 25% CTC, as opposed to a CTC/TC ratio of 0.67/1 or 40% CTC in the non-bromide case. Hence, bromide in the S-580 case appears as an effective chlorination inhibitor. This is in agreement with observation, the interpretation being in terms of a reduced quantity of halogenating enzyme in S-580. The reversibility of bromide inhibition in S-580 by excess chloride is interpretable as a competition between Br^- and Cl^- for formation of AEX.[7]

Acknowledgment.—The authors wish to acknowledge, with appreciation, the aid of Mr. H. S. Kelsey under whose direction the antibiotic assays were done, of Mr. L. Brancone under whose direction the microanalyses were done, and of Mr. W. Muller under whose direction the ultraviolet absorption and antibiotic stability determinations were done.

Experimental

Chloride Removal from Corn Steep Liquor. A. Silver Nitrate Method.—Corn steep liquor was diluted with two volumes of water, filtered and acidified to *p*H 2 with 10% sulfuric acid. Aqueous silver nitrate was added to complete precipitation. The mixture was filtered, contacted with hydrogen sulfide, filtered, vacuum concentrated to three-quarters volume and adjusted to *p*H 4.0 with ammonia water.

B. **Amberlite IR-4B (Sulfate) Method.**—Amberlite IR-4B resin was slurried with 1 *N* sulfuric acid and washed with distilled water. One hundred grams of corn steep liquor was diluted with an equal volume of water, filtered and passed through a resin-packed column 3 cm. in diameter and 25 cm. high. The column was regenerated for further use with 1 *N* sulfuric acid.

Fermentation Conditions.—All fermentations were conducted at 26.5-27.5° on a Gump rotary shaker (2.25" diameter stroke, 185 r.p.m.) and were divided into two stages: an initial 24 hr. inoculum stage starting with spores and yielding a vegetative inoculum and a final 96 hr. stage starting with the vegetative inoculum and yielding the final fermentation products. Spores were added to 100-ml. quantities of sterile medium contained in 500-ml. cotton-plugged erlenmeyer flasks and composed of (g./l.): sucrose, 30; corn steep liquor, 20; $(NH_4)_2SO_4$, 2; $CaCO_3$, 7. The second stage was carried out in 250-ml. erlenmeyer flasks containing 25 ml. of medium composed of (g./l.)[9]: corn starch (low chloride), 55; silver dehalogenated corn steep liquor, 30 (original weight basis); $CaCO_3$, 9; $(NH_4)_2SO_4$, 5; 85% H_3PO_4, 0.04; $FeSO_4 \cdot 7H_2O$, 0.060; $MnSO_4 \cdot 4H_2O$, 0.050; $ZnSO_4 \cdot 7H_2O$, 0.100; lard oil, 20 ml. The residual chloride content of this medium was about 10–15 p.p.m. Each flask received one ml. of vegetative inoculum. Use of Amberlite IR-4B (sulfate) treated corn steep liquor increased the corn steep liquor requirement to 40 g./l. (original weight basis) and the 85% H_3PO_4 requirement to 0.425 g./l. Those experiments involving chloride levels above that contributed by untreated corn steep liquor were run with untreated corn steep liquor without phosphate supplementation. Chloride, bromide and thiocyanate were added as the ammonium, potassium or magnesium salts. Three to six replicates for each set of fermentation conditions were pooled and from the pool duplicate samples were submitted to the various assays.

Isolation from Fermentation Products and Characterization of Tetracycline.—Isolation and purification of TC from *S. aureofaciens* fermentation products was accomplished as described previously.[5] Comparison of fermentation-produced TC and TC produced by the catalytic hydrogenation of CTC showed no differences in ultraviolet and infrared spectra, decomposition rates in acid and in alkali, color tests, elemental analysis, optical rotation, *in vitro* antibiotic activity over a series of microorganisms and paper chromatographic R_F values.

7-Chloro^{36}-tetracycline.—A 1-l. fermentation with Mutant BC-41 was carried out on a medium containing minimal available halogen except for 34 μC. (121 mg.) of chloride^{36} added as 0.37 *N* hydrochloric acid. This chloride was stoichiometrically equivalent to 1600 μg./ml. of CTC·HCl; assay of the fermented material showed 98% utilization of chloride for CTC. The *p*H of the material was adjusted to 7.5 with sodium hydroxide and the mixture was filtered. The filter cake was extracted five times with 250-ml. portions of wet butanol at *p*H 1.2. To the combined extracts were added 25 g. of sodium chloride and 1.0 g. of Darco G-60. The mixture was stirred one hour, after which the butanol phase was separated, filtered, concentrated *in vacuo* at 30° to approximately 35 ml., and acidified to *p*H 1.2 (HCl). Four ml. of Cellosolve was added. The mixture was seeded with CTC·HCl, aged for 17 hours and filtered. The CTC·HCl–TC·HCl crystals obtained were washed with small amounts of butanol and acetone, dried *in vacuo* at 35°, washed with water and redried at 35°. They weighed 3.48 g., assayed 47.7% CTC·HCl and 20.0% TC·HCl, and counted 11.87 counts/minute/μg., corresponding to a radioactivity recovery from mash of 75%. Further work-up of the mother liquor yielded a second crystal crop.

A mixture of 80% *n*-butyl alcohol and 20% chloroform was equilibrated with an equal volume of water, the *p*H of the system having been adjusted to 2.0 with concentrated hydrochloric acid. Celite 545 was washed with hydrochloric acid, then with water and dried. To 1000 g. of this Celite was added 500 ml. of the aqueous phase of the above mixture. A three inch diameter column was packed to a height of 35 cm., using 675 g. of the wet Celite. 3.38 g. of the CTC·HCl–TC·HCl crude crystals was dissolved at 75° in a mixture of 57 ml. of *n*-butyl alcohol and 24 ml. of water, at *p*H 2.0, and cooled. Thirty-four g. of Celite was added, and the mixture was packed on top of the column. The column was developed at the rate of about 400 ml./hr. with the equilibrated organic phase. The first nine 100-ml. cuts contained essentially all the radioactivity. Cuts two through nine were pooled and the butanol replaced with 800 ml. of water by distillation *in vacuo* at *p*H 2.5 (HCl). The *p*H was adjusted to 2.0 (HCl) and the solution washed twice with 10% volumes of chloroform. The washed aqueous solution was concentrated *in vacuo* to 10 ml. (its *p*H changed to 1.0), crystals forming as the concentration proceeded. The slurry was aged two hours at room temperature and filtered. The crystals were washed with small quantities of 0.1 *N* hydrochloric acid, cellosolve and acetone, and dried *in vacuo*. The 7-chloro^{36}-tetracycline hydrochloride crystals weighed 1.19 g., assayed 97% CTC·HCl, and had an observed specific radioactivity of 8.4 μC./m*M*., representing a radioactivity recovery of 74% from the crude crystals, or 56% from mash. The original HCl^{36} had a specific radioactivity of 10 μC./m*M*. The counting was done under conditions resulting in negligible self-absorption in a gas flow counter with 50% geometry.

Paper chromatographic inspection of the product showed homogeneity of radioactivity and no TC.

7-Bromotetracycline.—A 40-l. fermentation was carried out with Mutant BC-41 on a medium containing minimal available halogen and KBr at 6.0 g./l. Chloride low water was prepared by passage through an Amberlite Resin IRA-400 (OH) column. The Amberlite Resin IR-4B (sulfate) treatment of the corn steep liquor was done twice. The calcium carbonate was thoroughly washed with chloride-low water. Starch was replaced as a carbohydrate source by dextrin freed of chloride by dissolving to a 10% aqueous solution at 100°, cooling to 35–40°, and slurrying 1–2 hours with 10%, of the weight of dextrin, of Amberlite Resin MB-1, a mixed cation–anion exchanger. Filtration through

cheese cloth removed the resin particles at the end of the slurry period. The final fermentation product assayed 850 μg./ml. BTC and 1700 μg./ml. TC. The initial isolation steps through the concentrated butanol extract were as for 7-chloro[36]-tetracycline, yielding a concentrated butanol extract containing approximately 40,000 μg./ml. total antibiotic. This was back extracted at *p*H 2.0 four times with equal volumes of water and the pooled water extracts were washed two times with 10% volumes of chloroform. The washed water extract was concentrated to 50,000 μg./ml., adjusted to *p*H 5.6 with sodium hydroxide and aged several hours at *p*H 5.6 and 5°. The resulting crystalline product was filtered cold, washed with water and dried *in vacuo*. The product weighed 8.90 g. and assayed 38.8% BTC by fluorometric analysis and 38.0% BTC by bromine content. Much of the remainder was TC.

The BTC was separated from the TC by the partition chromatographic system described previously, the BTC running ahead of the TC. The BTC yield from the crystalline BTC–TC mixture to chromatographed, crystalline BTC·HCl was 61%, not including rechromatography of BTC–TC intermediate column cuts. The crystals were dissolved in 5.5 volumes of an 8:2 Cellosolve–water mixture by the use of triethylamine to form the triethylamine salt. The solution was treated with Darco G-60, filtered, acidified to *p*H 2.0 with concentrated HCl and aged with stirring for 22 hr. The yield from crude hydrochloride to recrystallized BTC·HCl was 69%. Small, variable quantities of CTC, not separated by the chromatographic operation, were present in the BTC preparations, arising from the efficient utilization by BC-41 of traces of chloride for CTC even in the presence of large excesses of bromide. A variety of countercurrent extraction, partition column and paper chromatographic systems useful in separating CTC, TC, 5-hydroxytetracycline[13] and their C.4-epimers[14] failed to separate effectively mixtures of varying ratios of CTC and BTC. Corrections of the properties of a number of batches of BTC, with varying amounts of CTC present, have yielded consistently these properties of CTC free BTC-HCl $[\alpha]^{25}D$ (0.5% in 0.03 *N* aq. HCl): BTC·HCl, −205°; CTC·HCl, −235°. Solubility in water (25°): BTC·HCl, 1.36%; CTC·HCl, 1.40%. Solubility in dry *n*-butyl alcohol (25°): BTC·HCl, 0.038%; CTC·HCl, 0.013%. Response on a weight basis of BTC·HCl in terms of CTC·HCl to analytical procedures: turbidimetric (*S. aureus*), 95%; turbidimetric (*E. coli*), 90%; fluorometric, 27%; Hiscox, 45.0%; spectrophotometric (368 mμ, 0.1 *N* H_2SO_4), 77%.

(13) The trademark of Charles Pfizer and Co. for 5-hydroxytetracycline is Terramycin.

(14) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom and A. P. Doerschuk, THIS JOURNAL, **78**, 3547 (1956).

ULTRAVIOLET SPECTRA IN 0.1 *N* H_2SO_4

BTC·HCl		CTC·HCl		TC·HCl	
λ_{max}, mμ	ϵ_{max} × 10^{-3}	λ_{max}, mμ	ϵ_{max} × 10^{-3}	λ_{max}, mμ	ϵ_{max} × 10^{-3}
368	9.15	368	10.7	355	14.6
260	18.9	265	18.3	268	18.9
228	17.2	228	17.6	218	13.9

HALF-LIVES (MINUTES) IN ACID AND ALKALI

Conditions	BTC	CTC	TC
*p*H 10.0 carbonate buffer (aq.), 22°	10.9	18.6	>600
0.2 *N* H_2SO_4 (aq.), 100°	18.8	8.2	< 2

Tetracycline from 7-Bromotetracycline.—Reduction of BTC under conditions described previously for CTC[8] yielded TC, identified by ultraviolet and infrared absorption spectra, paper chromatographic behavior and acid destruction rate.

Non-accumulation during the Fermentation of Chlorine-containing Substances other than 7-Chlorotetracycline.—A 25-ml. fermentation was carried out with Mutant BC-41 in the presence of no available halogen except 44 mg. of chloride[36] (150 μC./g.) (equivalent to 25,000 μg. CTC/ml.). At harvest the fermented mash contained 4750 μg. CTC·HCl/ml. An acid extract of the mash was treated with silver nitrate, filtered, treated with hydrogen sulfide and filtered. A paper chromatogram of the resulting solution showed by radioautography a major zone corresponding to the CTC region and only traces of radioactivity at the origin.

Non-bromination by Mutants BC-41, S-580 and S-1055 in Fermentation Systems Containing Bromide and Chloride.—Fermentations containing both chloride and bromide were carried out with mutants BC-41, S-580 and S-1055, respectively. CTC was isolated from the fermentation product by essentially the chromatographic method described previously for 7-chloro[36]-tetracycline and for BTC. This isolation procedure does not separate CTC and BTC; hence any BTC formed would be in the isolated CTC in a proportion essentially unaltered from that of the crude fermentation product itself. For none of the mutants did the isolated CTC contain detectable quantities of bromine. Hence it was concluded that bromination had not occurred to an appreciable extent in any of the chloride–bromide cases.

Non-bromination by Mutants S-580 and S-1055 in Fermentation Systems Containing Bromide without Chloride.—For mutants S-580 and S-1055, fermentations containing no chloride and a variety of bromide levels all gave essentially zero fluorometric assays, as did fermentations containing neither chloride nor bromide. This behavior contrasts with that found for Mutant BC-41, where the fluorometric assay for the no-chloride case was found to vary with bromide level.

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