AN INTRODUCTION TO THE ORIGIN AND BIOCHEMISTRY OF MICROBIAL HALOMETABOLITES

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I. Introduction

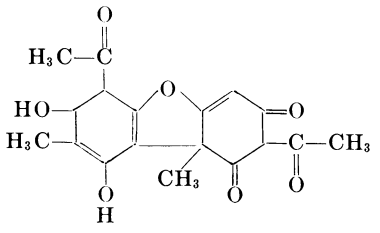
In the metabolism of fresh water or terrestrial bacteria and fungi, the halogens are not generally considered functional elements. Despite their estimated abundance, wherein chlorine is ranked as the eleventh most common element, and the relative aqueous solubility of halide salts, little is known of the metabolic processes involving these elements especially in the lower plants. Only in recent time has there been interest in halogen-containing metabolites and their biogenesis. The only western review on halometabolites is by Bracken (13) who discussed 12 of the presently known 29 "natural" chlorometabolites. It is the purpose of this review to consider the microbiology of the producers and the biosynthesis of halometabolites and their analogues.[1] As used here, the term halometabolite defines an organic halogen-containing molecule synthesized by a living organism from the metabolism of ionic halide.

Historically, the first record of a microbial halometabolite is given by Zopf (120) who isolated diploicin (Table 1: *9a*) from a lichen. A deshalo-analogue of diploicin has yet to be reported. The first record of a deshalo-analogue, atranorin (Table 1: *8b*), of a subsequently discovered halometabolite, chloroatranorin (Table 1: *8a*), is credited to Paterno and Oglialoro in 1877 (22). This history presents an anomaly, since the more common sequence of disclosure is for halometabolite discovery to precede that of deshalo-analogue. Of the halometabolites known, bromine derivatives are not produced with the same ease or abundance as their chloro-analogues. In fact, the brominating microorganisms produce more deshalo-analogue than bromometabolite in the presence of bromide. Since conventional nutrient media commonly contain chloride either as a result of using natural materials or through the addition of an inorganic chloride, *e.g.*, Czapek-Dox solution, opportunity for biosynthesis of chlorometabolites is commonly afforded. Even media of supposedly defined nature, such as Raulin-Thom which has no deliberately added chloride, have afforded sufficient chloride as an impurity to result in the biosynthesis of a halometabolite (89). Iodo- or fluorometabolites have not been reported despite attempts by several investigators to obtain them from microorganisms normally producing chlorometabolites.

[1] Literature published prior to February 1960 forms the basis for this review.

A. Incidence and Origin

One is led to speculate on the relative incidence and origin of halometabolites. In the case of a relatively common and simple nonhalo-organic metabolite, synthesized by a number of microorganisms and perhaps in several analogous forms, should one expect to find this to be produced as a halo-analogue by a few species? The example of atranorin and chloratranorin, and the depsidones (Table 1: *9*) is cited. If this be true, and since usnic acid:



has been reported as the most widely distributed acid in lichens, occurring in more than 90 different species, then one might expect to find chlorousnic acid in a few species of lichens. This has not been done. With the more complex but structurally related metabolites produced by only a few species, should one expect to find a culture capable of producing a halo-analogue? The facts do not seem to support this approach. For example, a number of strains of *Penicillium urticae* (*Penicillium griseofulvum*) produce griseofulvin (Table 1: *10d*) along with a smaller quantity of the deshalo-analogue (65), and only some of the griseofulvin-producing strains of *Penicillium nigricans* made small amounts of dechlorogriseofulvin (Table 1: *10e*). With *Streptomyces aureofaciens*, rarely does a natural isolant produce measurable amounts of tetracycline (Table 1: *12h*) in the presence of chloride, since the normal tendency is to produce chlortetracycline (Table 1: *12e*). There is no authenticated report of the isolation from nature of an *S. aureofaciens* strain incapable of producing chlortetracycline but producing instead "pure" tetracycline. All cultures capable of producing essentially "pure" tetracycline in the presence of available chloride are known to be laboratory-derived mutants and not isolants from nature. The halogenating mechanisms are therefore dominant in the wild forms of halometabolite producers.

Experiences with *S. aureofaciens* and *Streptomyces venezuelae* do not indicate that they are ubiquitous. Neither of these species was found in more than 0.03 per cent of soil samples from various countries (E. J. Backus, *personal communication*). Furthermore, out of 2475 cultures of *Streptomyces* species isolated from Korean soils, only 6 were found to produce a chloramphenicol-like activity (20). These facts point to either a relative rarity, or an area of scant knowledge concerning the incidence of most halometabolite producers in nature. Producers of corresponding deshalo-analogues seem to be even rarer, except for the one lichen metabolite, atranorin.

II. Relationships of Halometabolites

A. Chemical

Table 1 presents a total of 37 halometabolites of natural or laboratory origin, represented by 12 different structural groups in figure 1. The structure of 10 of the metabolites is yet to be determined. Excluded from this group are the penicillins which have been biosynthesized through the addition of chloro-precursors. Proposed structural configurations of the halometabolites are presented in figure 1. Of the 37 halometabolites, 29 are of natural or conventional culture origin. All of the 29 "natural" halometabolites are chlorine derivatives. Bromo-derivatives of three of the "natural" chlorometabolites have been biosynthesized and a fourth, bromo-demethyltetracycline (Table 1: *12a*) is described in patent literature. Of the 29 "natural" chlorometabolites, 7 have dechloro-analogues of biosynthetic origin (Table 1: *4b*, *4c*, *8b*, *9e*, *9f*, *10e*, *11b*, *12f*, *12h*).

There may be some merit in considering a distinction between chemically and biologically synthesized analogues. For example, neither rotiorin (VII) nor *N*-propionyl-*p*-nitrophenylserinol (III; $R^1{=}CH_3$, $R^2{=}H$) are the respective chemical deshalo-analogues of sclerotiorin (VI; R=Cl) and chloramphenicol (III; $R^1{=}R^2{=}Cl$), but they could well be considered bio-analogues.

Of the 29 "natural" halometabolites, with the exception of chloramphenicol, all have the halogen attached to a cyclic carbon of a 5- or 6-membered ring. However, the acetylserinol moiety of chloramphenicol might be an intermediate to the methyl-pyrrole ring type of moiety of pyoluteorin (IV) since a dehydration reaction could close type III structure into a type IV structure. Only caldariomycin (I) and chloramphenicol (Table 1: *4a*) have two chlorines attached to one carbon. It is not clear where the chlorines are attached in pyoluteorin (IV). The chlorine to carbon ratio is the greatest in drosophilin A (II), and it seems scarcely possible that this compound, *p*-methoxytetrachlorophenol, is a product of a fungus since the polychlorophenols are potent fungicides. Somewhat along the same line, mollisin (V) is closely related chemically to the fungicide dichlone, 2,3-dichloro-1,4-naphthoquinone. The structural homology of the depsides, depsidones, and spirans is interesting, with geodoxin (XIII) bridging the gap between the latter two groups.

One substance, pannarin (XI), appears to have escaped the attention of the better known abstracting journals.

The tetracyclines are the most complex of the chlorinated structures. Six analogues of chlortetracycline are known to be of biosynthetic origin. Oxytetracycline is not included here since, in a biological sense, it is not derived from chlortetracycline, but is included in the table because of chemical and certain biological relationships. Other tetracyclines, such as the epi-forms, are not included because they are not of biosynthetic origin.

Sclerotioramine has been reported to be a natural product (34) since it was isolated from cultures of *Penicillium multicolor* which had been incubated for a longer than normal time period. However, since sclerotiorin is known to be very susceptible to amination, and since *P. multicolor* is a notable producer of ammonia in older culture, sclerotioramine was more likely a result of the action of ammonia on sclerotiorin rather than a direct product of the organism. For this reason sclerotioramine is not included in table 1.

It is rather interesting that halogen-containing cyclic organic compounds are of more common occurrence, or knowledge, than aliphatic types. This may reflect a result of ease of detection since cyclic compounds are common organic pigments or have biological activity, thus facilitating isolation.

B. Biological

With the exception of some limited information concerning certain chlorinated anthraquinone pigments of flowers, apparently there is a general dearth of knowledge concerning halometabolites in the higher plants. Metabolism of chloride as measured by its uptake or effects on growth are noted, but what happens to the chloride is not disclosed. It seems unusual to find information on halometabolites limited to the Thallophyta. The limited number of genera and species which produce halometabolites is indicated in table 1. The taxonomic positions are scattered. In the Schizomycetes, the Eubacteriales are represented by the genus *Pseudomonas*, and the Actinomycetales by the genus *Streptomyces*. Three orders are represented in the Ascomycetes; the Lecanorales by species of *Buellia*, *Lecanora*, *Pannaria*, *Parmelia*, *Pertusaria*, and *Evernia*; the Pezizales by the genus *Mollisia*; and the Aspergillales by the species of *Aspergillus* and *Penicillium*. The genus *Psathyrella* represents the Basidiomycetes. Lastly, the genus *Caldariomyces* represents the Fungi Imperfecti. Other genera reported to metabolize chloride include *Absidia*, *Alternaria*, *Botrytis*, *Cephalosporium*, *Cladosporium*, *Clasterosporium*, *Helminthosporium*, *Mucor*, *Stemphylium*, *Stysanus*, *Syncephalastrum*, and *Trichoderma* (21). This metabolism was indicated by the uptake of chloride from the substrate, but no specific halometabolites were characterized.

Generally neglected in previous literature citations on fungus metabolites, the lichen metabolites are here considered to be true fungal products. This view is substantiated by the fact that the depside nuclei are known to be products of lichens (21) and of fungi (79); the depsidone nuclei are produced by lichens (80) and by cultured fungi (88). Halogenated depsidones are known to be products of both cultured fungi (27) and of lichens (5). Further, laboratory culture of the fungal component of a lichen, *Cladonia cristatella*, produced the same characteristic organic acids produced by the lichen in nature (17). All indications are that these characteristic organic compounds are the products of the fungal component of the lichen. Therefore, it seems reasonable to give Zopf (120) credit for finding the first fungal halometabolite, diploicin (IX), in the lichen *Buellia canescens*.

It is not uncommon to find reports of a common organic nucleus biosynthesized by a number of species in the achlorophyllous thallophytes. These common nuclei, however, are usually found only within certain taxonomic limitations. In considering the halometabolites (table 1 and figure 1), common molecular structures are produced by different genera in the case of the depsidones; spirans are produced by species of *Aspergillus* and *Penicillium*; the genus *Aspergillus* produces both depsidone and spiran derivatives. Specifically, groups 8, 9, and 10 of table 1 appear to be very common products of the ascomycetous fungi. In these cases, the microorganism had been described prior to the finding of the specific halometabolite concerned. Here reduction of the earlier used specific names to synonymy has often occurred. For example, *Penicillium janczewskii* has been reduced to synonymy with *P. nigricans*, and *P. griseofulvum* to *P. urticae*. Misidentification is not uncommon as, for example, the culture alleged to be *Aspergillus ustus*, the producer of

TABLE 1

Halometabolites, their analogues, and related compounds

Group, Name, and Formula	Structure: Refer to Figure 1	Disclosed Producers	References
ALIPHATICS			
1. Levulinic Acid			
a. δ-Chlorolevulinic acid, $C_5H_7O_3Cl$	$CH_2Cl—CO—CH_2—CH_2—COOH$	*Caldariomyces fumago Woron., strain* Ag92	Shaw *et al.* (97)
CYCLICS			
2. Cyclopentane			
a. Caldariomycin, $C_5H_8O_2Cl_2$	I	*C. fumago*, strains Ag92 and X13	Clutterbuck *et al.* (21)
3. Phenol			
a. Drosophilin A, $C_7H_4O_2Cl_4$	II	*Psathyrella subatrata* (*Drosophila subatrata* [Batsch. ex Fr.] Quel.)	Kavanagh *et al.* (53); Anchel (2)
4. Phenylserinols*			
a. Chloramphenicol, $C_{11}H_{12}O_5N_2Cl_2$	III; R^1=R^2=Cl	*Streptomyces venezuelae* Ehrlich *et al.*, strain A 65; *S. omiyaensis* Umezawa *et al.*, *Streptomyces* sp.	Ehrlich *et al.* (36, 37); Rebstock *et al.* (90); Umezawa *et al.* (109); Smith (101)
b. N-Acetyl-*p*-nitrophenylserinol, $C_{11}H_{14}O_5N_2$	III; R^1=R^2=H	*S. venezuelae*; *Streptomyces* sp.	Gottlieb *et al.* (43); Smith (101)
c. N-Butyryl-*p*-nitrophenylserinol, $C_{13}H_{18}O_5N_2$	III; R^1=CH_2—CH_3, R^2=H	*Streptomyces* sp.	Smith (101)
d. N-Dibromoacetyl-*p*-nitrophenylserinol, $C_{11}H_{12}O_5N_2Br_2$	III; R^1—R^2=Br	*Strepromyces* sp.	Smith (101)
e. N-Monobromomonochloro-acetyl-*p*-nitrophenylserinol, $C_{11}H_{12}O_5N_2BrCl$	III; R^1=Br, R^2=Cl	*Streptomyces* sp.	Smith (101)
f. N-Propionyl-*p*-nitrophenylserinol, $C_{12}H_{16}O_5N_2$	III; R^1=CH_3, R^2=H	*Streptomyces* sp.	Smith (101)
5. Phenyl pyrrolyl ketone			
a. Pyoluteorin, $C_{11}H_7O_3NCl_2$	IV	*Pseudomonas aeruginosa* culture T 359	Takeda (106); Takeda and Nakanishi (107)
6. Naphthoquinone			
a. Mollisin, $C_{14}H_{10}O_4Cl_2$	V	*Mollisia caesia* Sacc. sensu Sydow.; *M. fallens* Karst.	Gremmen (47); Van der Kerk and Overeen (111)
7. Azaphilones†			
a. Sclerotiorin, $C_{21}H_{23}O_5Cl$	VI	*Penicillium sclerotiorum* van Beyma; *P. multicolor* G.-M. and P.; *P. implicatum* Biourge	Curtin and Reilly (23); Fielding *et al.* (38); Birkinshaw (12); Yamamoto and Nishikawa (119)
b. Rotiorin, $C_{23}H_{24}O_5$	VII	*P. sclerotiorum*	Jackman *et al.* (52)

8. Depsides			
a. Chloroatranorin, $C_{19}H_{17}O_8Cl$	VIII; R^1=Cl	*Evernia prunastri* (L.) Ach., *Parmelia furfuracea* Ach., and other *Parmelia* spp.	Curd *et al.* (22); St. Pfau (104); Koller and Pöpl (57)
b. Atranorin, $C_{19}H_{18}O_8$	VIII; R=H	*Evernia prunastri*; *Parmelia* spp., widely distributed	Paternò and Oglialoro (Curd *et al*; (22))
9. Depsidones			
a. Diploicin, $C_{16}H_{10}O_5Cl_4$	IX	*Buellia* (*Diploicia* Dicks) *canescens* (Fries) deNot.	Zopf (120); Nolan (80); Spillane *et al.* (102)
b. Gangaleodin, $C_{18}H_{14}O_7Cl_2$	X	*Lecanora gangaleoides* Nyl.	Hardiman *et al.* (48); Nolan and Keane (81)
c. Pannarin, $C_{18}H_{15}O_6Cl$	XI	*Pannaria lanuginosa* Korb.	Yosioka (Asahina and Shibata (5))
d. Nidulin, $C_{20}H_{17}O_5Cl_3$	XII; R^1=Cl, R^2=CH_3	*Aspergillus nidulans* NRRL 2006	Dean *et al.* (26, 27)
e. Nornidulin (ustin), $C_{19}H_{15}O_5Cl_3$	XII; R^1=Cl, R^2=H	*A. nidulans* (*A. ustus*)	Kurung (60); Doering *et al.* (29); Dean *et al.* (26)
f. Dechloronornidulin, $C_{19}H_{17}O_6Cl_2$	XII; R^1=R^2=H	*A. nidulans* NRRL 2006	Dean *et al.* (25)
10. Spirans			
a. Geodoxin, $C_{17}H_{12}O_8Cl_2$	XIII	*A. terreus* Thom strain Ac 100	Hassall and McMorris (49)
b. Erdin, $C_{16}H_{10}O_7Cl_2$	XIV; R=H	*A. terreus* Thom strain no. 45	Raistrick and Smith (88); Barton and Scott (7)
c. Geodin, $C_{17}H_{12}O_7Cl_2$	XIV; R=CH_3	*A. terreus* Thom strain no. 45; *A. flavipes*	Raistrick and Smith (88); Barton and Scott (7); Delmotte *et al.* (28)
d. Griseofulvin, $C_{17}H_{17}O_6Cl$	XV; R=Cl	*P. urticae* Bain. (*P. griseofulvum* Dier.); *P. nigricans* (B.) Thom (*P. janczewskii* Zal.); *P.* (*Carpenteles*) *brefeldianum*	Oxford *et al.* (83); MacMillan (67); Brian *et al.* (15); Dean *et al.* (24)
e. Dechlorogriseofulvin, $C_{17}H_{18}O_6$	XV; R=H	*P. urticae; P. nigricans*	MacMillan (64, 65)
f. Bromo-dechlorogriseofulvin, $C_{17}H_{17}O_6Br$	XV; R=Br	*P. urticae; P. nigricans*	MacMillan (66)
11. Anthraquinones			
a. Nalgiolaxin, $C_{18}H_{15}O_6Cl$	XVI; R=Cl	*P. nalgiovensis* Laxa	Raistrick and Ziffer (89)
b. Nalgiovensin, $C_{18}H_{16}O_6$	XVI; R=H	*P. nalgiovensis*	Raistrick and Ziffer (89)
12. Tetracyclines			
a. 7-Bromo-6-demethyltetracycline, $C_{21}H_{21}N_2O_8Br$	XVII; R^1=Br, R^2=R^3=R^4=H, R^5=OH	*S. aureofaciens* Duggar	McCormick (70)
b. 7-Bromotetracycline, $C_{22}H_{23}N_2O_8Br$	XVII; R^1=Br, R_2=CH_3, R^3=R^4=H, R^5=OH	*S. aureofaciens* strains RP/911, BC-41	Sensi *et al.* (96); Doerschuk *et al.* (30)
c. 7-Chloro-5a(11a)-dehydrotetracycline, $C_{22}H_{21}N_2O_8Cl$	XVII; R^1=Cl, R^2=CH_3, R^3=none, R^4=H, R^5=0. Also double bond shifts from 11a(12) to 5a(11a)	*S. aureofaciens* strain S-1308	McCormick *et al.* (71)

TABLE 1.—*Continued*

Group, Name, and Formula	Structure: Refer to Figure 1	Disclosed Producers	References
d. 7-Chloro-6-demethyltetracycline, $C_{21}H_{21}N_2O_8Cl$	XVII; R^1=Cl, R^2=R^3=R^4=H, R^5=OH	*S. aureofaciens* strain S-640	McCormick *et al.* (72)
e. 7-Chlorotetracycline, $C_{22}H_{23}N_2O_8Cl$	XVII; R^1=Cl, R^2=CH_3, R^3=R^4=H, R^5=OH	*S. aureofaciens* ATCC 10762, 124162-d, 12551-4, 12748-51, NRRL-B1286-7 (*S. viridifaciens* ATCC 11989; *S. sayamaensis*)	Duggar (32); Stephens *et al.* (103) Gourevitch and Lein (45); Arishima *et al.* (3); Arishima and Sekizawa (4); author's data
f. 6-Demethyltetracycline, $C_{21}H_{22}N_2O_8$	XVII; R^1=R^2=R^3=R^4=H, R^5=OH	*S. aureofaciens* strain S-604	McCormick *et al.* (72)
g. 5-Oxytetracycline, $C_{22}H_{24}N_2O_9$	XVII; R^1=R^3=H, R^2=CH_3, R^4=R^5=OH	*S. rimosus*	Findlay *et al.* (39); Stephens *et al.* (103)
h. Tetracycline	XVII; R^1=R^3=R^4=H, R^2=CH_3, R^5=OH	*S. aureofaciens* ATCC nos. as listed *12e.* (*S. viridifaciens*, *S. sayamaensis*, *S. psammoticus* strain no. P19 and 4623/33; *S. fuscofaciens* ATTC 12061; *S. persimilis* PV 11640; *Streptomyces* sp. Culture nos. ATCC 11652, 11653, 11654, and 11834)	Minieri *et al.* (75, 76); Stephens *et al.* (103); Gourevitch and Lein (45); Lepetit (62, 63); Pfizer Corp. (85); Olin Mathieson (82)
13. Undetermined structure			
a. Actiduin, C, H, N, S, and Cl		*Streptomyces* sp.	Burton (16)
b. Concretin, $C_{14}H_7O_5Cl_3$	3 Hydroxyl groups	*Pertusaria concreta* Nyl. form *Westringii* Nyl.	Breen *et al.* (14)
c. Estin, $C_{16}H_{14}O_6Cl_2$‡	2 Methoxy groups	*Penicillium paxilli* var. *echinulatum*	Komatsu (58)
d. Exfoliatin, $C_{27}H_{40}O_{16}Cl \cdot H_2O$		*S. exfoliatus* Umezawa	Umezawa *et al.* (109)
e. Islanditoxin, $C_{25}H_{33}N_5O_8Cl_2$		*P. islandicum* Sopp.	Marumo (69)
f. Nordin, $C_{18}H_{16}O_8Cl_2$	3 Methoxy groups	*P. paxilli* var. *echinulatum*	Komatsu (58)
g. Sarcidin, C, H, N, S, and halogen		*S. achromogenes*	Takeuchi *et al.* (108)
h. Terrecin, C, H, N, and Cl		*A. terreus*	Iwata and Yosioka (51)
i. Unnamed product A, ($C_{18}H_{16}O_9$) and 6.9% chlorine		*Buellia canescens*	Nolan (80)
j. Unnamed, $C_{24}H_{20}O_9Cl_2$		*Lecanora sordida* Th. Fr.	Kennedy *et al.* (54)

* Chemical Abstracts suggests the name 2-amino-1,3-propanediols.

† As a convenience, this generic name is used. Robertson (34) indicates its general application should be restricted.

‡ Later found to be identical with geodin (Chem. Abst., **52,** 16473, 1958).

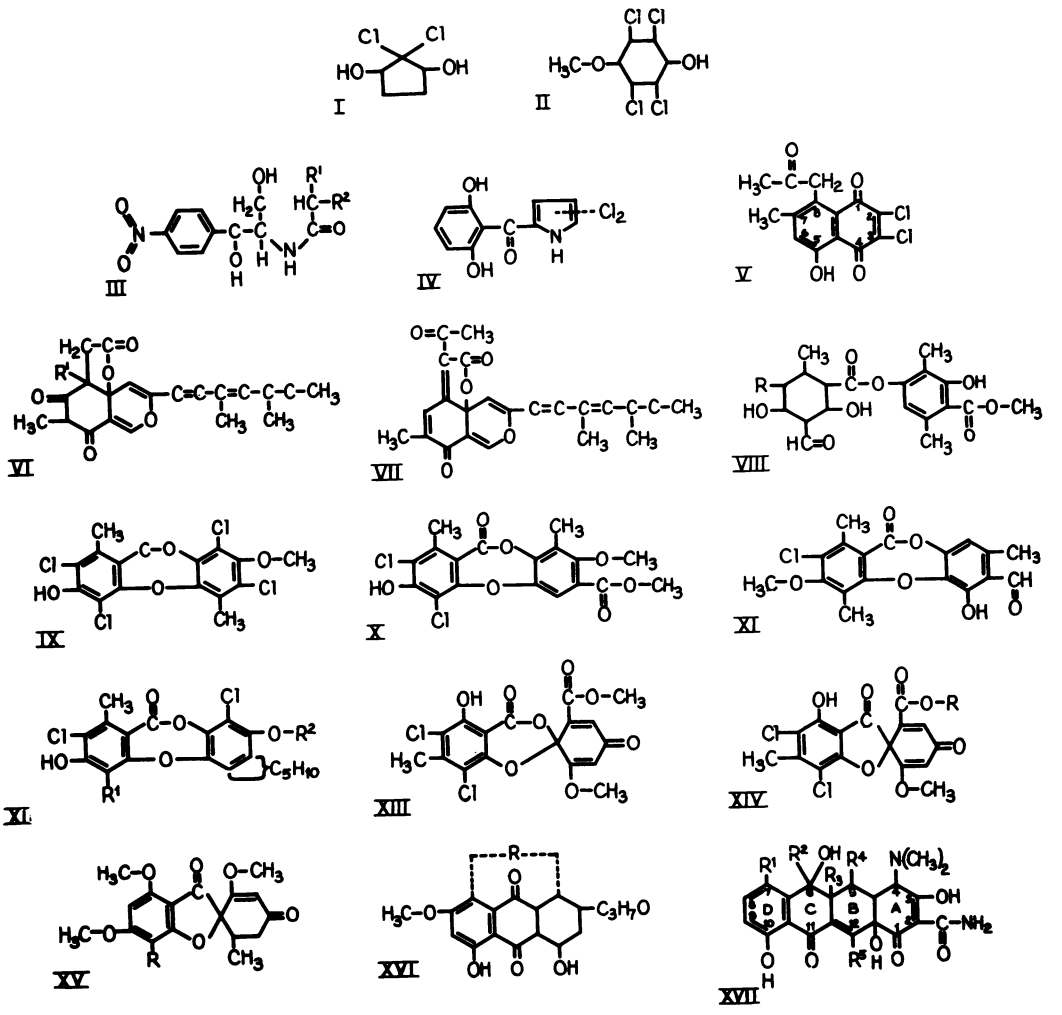


Figure 1. Structures and structural groups of halometabolites and related compounds

ustin, *i.e.*, nornidulin (table 1: *9e*), was later reidentified as *Aspergillus nidulans* (27).

A number of binomials are associated with specific halometabolites of *Streptomyces* origin. This may reflect among other things a failure to survey critically the known literature and to compare critically the new culture with related types (86). One cannot help having the impression that this is often applicable in patent documents, especially when the description of the alleged new species is excessively supplied with trivial information but inadequate with respect to presently accepted major taxonomic characteristics. The critical investigator is often at a loss with these proposed names, first through lack of essential information, and second, through lack of parallel laboratory comparisons when type-culture material is not publicly available.

Chloramphenicol (Table 1: *4a*) is produced by *S. venezuelae* (37). *Streptomyces omiyaensis* (110) was described as producing chloramphenicol, but no reference was given as to whether the type was compared with *S. venezuelae*, the distinction being made on the basis of lack of brown pigment in protein media and lack of curved sporophores; however, the sporophores were not described. They were later noted to be flexuous (86). At that time Umezawa *et al.* admitted the similarity of *Streptomyces phaeochromogenus* var. *chloromyceticus* to *S. venezuelae*, and the former was reduced to synonymy by Waksman and Lechevalier (114). The multiplicity of legitimate

species producing chloramphenicol has yet to be established.

Chlortetracycline (Table 1: *12e*) is produced by *S. aureofaciens* (32), but certain cultures of this species are not obligate halogenators of the tetracycline (Table 1: *12h*) nucleus (30, 59, 115) since isolants from nature as well as mutants from chloride-utilizing strains may produce tetracycline in addition to chlortetracycline even in the presence of excess chloride. It has not been our experience to find natural isolants of *Streptomyces* capable of producing tetracycline but incapable of producing chlortetracycline. Every natural isolant encountered here which was capable of producing tetracycline in any appreciable proportion could be classified as *S. aureofaciens* even when the property of antibiotic production was disregarded. Therefore, species alleged to produce tetracycline should be examined critically for identity with *S. aureofaciens*. *Streptomyces viridifaciens* ATCC 11,989 (45) is known from personal experience to make chlortetracycline in a conventional fermentation medium containing chloride, *e.g.*, a fermentation system such as described by Goodman *et al.* (41) containing 1000 ppm chloride. This culture yielded 1.06 g/L total tetracyclines with a chlortetracycline to tetracycline ratio of 8.9:1. In addition, *S. viridifaciens* conforms to accepted specific criteria of *S. aureofaciens* (E. J. Backus, *personal communication*; (6); author's experience) and in susceptibility to the monovalent *S. aureofaciens* actinophage (R. Weindling, *personal communication*). Therefore, specific rank is believed not justified for *S. viridifaciens*.

S. aureofaciens ATCC 11,926 is described as producing quinocycline, an antiboitic distinct from tetracycline and not containing halogen (18). *Streptomyces fuscofaciens* ATCC 12,061 (19) is alleged to produce quinocycline and tetracycline in media low in chloride. This British Patent states "... if the chloride ion content of the medium is reduced to a minimum, the proportion of tetracycline to other broad spectrum antibiotics is favorably affected." This indicates that *S. fuscofaciens* has characteristics in common with *S. aureofaciens*. Further, only one interpretation is known to be possible concerning reducing the proportion of "other broad spectrum antibiotics" in a tetracycline fermentation through the denial of chloride to that fermentation, namely, in the presence of chloride, chlortetracycline would be formed in greater proportions. The obvious conclusion is that culture ATCC 12,061 produces chlortetracycline! Lack of description of certain critical characters and lack of public availability of culture ATCC 12,061 make it impossible to confirm either the justification for the new epithet, or whether it does or does not produce a halometabolite.

Generally overlooked in the literature, *Streptomyces sayamaensis* (94, 95) is disclosed as producing chlortetracycline and tetracycline. These publications do not present a description of the culture, but this is only later found in a patent (4). A thorough study of this culture has shown a majority of specific and critical criteria in common with *S. aureofaciens* (E. J. Backus, *personal communication*; author's unpublished data), including sensitivity to the specifically monovalent *S. aureofaciens* actinophage (R. Weindling, *personal communication*). Therefore, it appears necessary to reduce *S. sayamaensis* to synonymy with *S. aureofaciens*.

Considering then whether chlortetracycline and tetracycline are the products of multiple species—and the evidence does not bear this out—and despite the fact that these four binomials have been proposed, although only two have been used by authorities in the scientific literature, only one of these two species appears to have met the criteria of valid publication, and that is *S. aureofaciens*. Other allegedly different taxons which have been proposed solely for the production of tetracycline will be noted below.

An interesting sidelight is the fact that although *Streptomyces rimosus* and *Streptomyces hygroscopicus* can produce oxytetracycline, neither of these is indicated as capable of producing a halogenated tetracycline.

III. Factors Influencing Biosynthesis of Halometabolites

A. Genetic

Strain improvement is in part a term borrowed from agricultural genetics. It denotes the obtaining of superior culturable stock. Of the known halometabolite producers, only *S. aureofaciens* has received much attention in the literature in this respect. The original strain of Duggar, A-377, produced 20 to 100 μg/ml of chlortetracycline. The first report of strain improvement with this microorganism recorded a high yield of 1300 μg/ml (113). In a series of mutation treatments, a strain has been derived by Growich (30)

which is said to produce a yield of 8.5 g/L of total tetracyclines (42). A comprehensive treatment of genetic manipulation of *S. aureofaciens* is presented by Alikhanian *et al.* (1). After a preliminary treatment of spores with ethyleneimine, ultraviolet irradiation gave a threefold increase over the frequency of morphological mutations achieved with ultraviolet light alone. An increased mutation rate coupled with an increased survival of spores resulted when irradiation with X-rays was followed 2 hr later by ultraviolet irradiation. The reverse sequence of irradiation or the sole use of one type of irradiation was not nearly as effective. In the selection of strains yielding high chlortetracycline to tetracycline ratios, a five-stage selection procedure gave strains of considerably increasing antibiotic-producing ability.

Strain-improvement procedures often have interesting and important side effects, such as rewarding the investigator with new analogues of the parent compound. Such was the case with the purely laboratory-derived demethyltetracyclines (72), and chlorodehydrotetracycline (71). Spontaneous variation in a strain of *Aspergillus terreus* producing geodin-erdin (XIV) resulted in the production of geodoxin (XIII) according to Hassal and McMorris (49).

Strain-improvement techniques have afforded production of deshalo-analogues even in the presence of available chloride ion. This is termed genetic control of halogenation, which here indicates the induction of variation in the ability of a culture to halogenate an organic metabolite. It has been known for some time that different strains of *A. terreus* differed in their ability to metabolize chloride as measured by the disappearance of chloride from the substrate (21), but there was no indication that any of them were or were not producing the same halometabolite or a deshalo-analogue. In fact, a deshalo-analogue of a halometabolite of *A. terreus* has yet to be reported. The first indication that different strains of the same species differed in their ability to produce the deshalo-analogue of a halometabolite was with *P. nigricans*, of which one culture produced no dechlorogriseofulvin, another culture produced dechlorogriseofulvin in a ratio range of about 1:20 to griseofulvin, and a third had a ratio range of about 1:4 (65). In the formation of chlortetracycline, *S. aureofaciens* is known to be a remarkable scavenger of chloride (31) even to the extent of its being suggested as a tool for the assay of chloride-containing materials (55). Sekizawa (93) reported an attempt to control halogenation of tetracycline genetically, but was unsuccessful. Shortly thereafter, Martin *et al.* (68) indicated that this could be done. They stated, "Both natural and induced mutants of *S. aureofaciens* may produce tetracycline in varying amounts in addition to other antibiotics. By selection of a natural or induced mutant which produces a comparatively high ratio of tetracycline to other antibiotics present, . . . and propagating the organism . . ., a fermentative mash results from which tetracycline may be economically recovered." In this patent are examples showing the tetracycline production in the presence of available chloride. These allegations were substantiated by Doerschuk *et al.* (30), who showed that the *S. aureofaciens* of Duggar and most of its progeny would halogenate the tetracycline molecule at a constant rate, independent of chloride concentration. These constant utilizers could be termed chloride scavengers, for at low chloride concentrations they could be used as analytical tools for the detection of traces of chloride. In addition, Doerschuk *et al.* described a second class of strains, originating from the first and halogenating the tetracycline molecule at a reduced and inconstant rate, which was dependent upon chloride concentration. The first and second classes differed in their behavior with respect to bromine and halogenation inhibitors. By experience, natural isolants of *S. aureofaciens* preferentially halogenate the tetracycline molecule, and only rare isolants produce any appreciable proportion of tetracycline in the presence of available chloride. With normal chloride-utilizing strains, tetracycline is synthesized at the expense of chlortetracycline.

More recently other workers have reported strains of *S. aureofaciens* which were not chloride scavengers. A natural isolant and induced mutants of *S. aureofaciens* were found to produce tetracycline in the presence of available chloride by Wang (115). From a normal chloride-scavenging strain, an inconstant utilizer was derived which produced a chlortetracycline to tetracycline ratio of 1:4 in a fermentation yielding a total potency of 1800 to 2000 μg/ml and containing an excess of available chloride ion (59). Mutants of *S. aureofaciens* "with considerably reduced chlorinating activity, insignificant in comparison with that of the parent strain," have been obtained by Kollár and Járai (56). The strains used by both Kotiuszko *et al.*, and Kollár and Járai

were of the same parental stock; *i.e.*, Soviet LS-536. That no absolute chloride-ignoring strain has been isolated from nature confirms our experience. However, a laboratory-derived mutant strain 4623/33, alleged to be that described in a British Patent Specification (62) appears to approach closely the point of completely lacking a functional chlorinating mechanism.

The ability of *S. aureofaciens* to halogenate a metabolite may thus be impaired by conventional laboratory mutagenic treatments, and genetic control of halogenation is thereby affirmed.

B. Chemical

In the formation of a halometabolite, available halide is obviously necessary. On the other hand, halide denial to a halometabolite producer does not assure the formation of the corresponding strict chemical deshalo-analogue (24, 52, 101). Dean *et al.* (24) reported that a halometabolite, griseofulvin, was obtained when *Penicillium brefeldianum* was grown on Czapek-Dox, a chloride-containing medium; but instead of griseofulvin an unrelated and nonhalogenated substance, fulvic acid, was obtained when this species was grown on a Raulin-Thom medium which does not contain deliberately added chloride. Jackman *et al.* (52) did not find deschlorosclerotiorin (VI, R=H) produced by *Penicillium sclerotiorum* in a Raulin-Thom medium, but rather a substance of altered chemical configuration which they termed rotiorin (VII). Rotiorin was produced in larger quantity along with sclerotiorin on Raulin-Thom medium than on Czapek-Dox medium. Smith (101) found an unexpected alteration of synthesis by a chloramphenicol-producing *Streptomyces* strain when halide was withheld. Instead of obtaining only the deshalo-analogue of chloramphenicol, in addition he obtained the *N*-propionyl- and *N*-butyryl-derivatives of *p*-nitrophenylserinol in amounts considerably in excess of what had been expected, based on the yield of chloramphenicol. Neither can it be said that the lack of halides deliberately added to conventional substrates precludes the formation of halometabolites. For example, Brian *et al.* (15) produced the curling factor, griseofulvin, on Raulin-Thom and corn steep media, neither of which had deliberately added halides; nalgiolaxin was encountered in cultures grown on Raulin-Thom solution (89); and chlortetracycline was first produced in a fermentation not further enriched with added halide (33). By definition, all microorganisms capable of synthesis of halometabolites must be able to metabolize ionic halide. Halopenicillins, which are achieved by feeding a halogenated precursor to the fermentation, are not halometabolites. These will not be considered here.

Only two substances, copper and fluoride, appear to be functional in promoting halogenation. In a patent (105) the ratio of chlortetracycline to tetracycline is said to be doubled in the presence of fluoride under certain conditions. Copper, which has long been recognized as functional in oxidative enzyme systems, is apparently functional in microbial halogenation (41, 93). The behavior of copper will be dealt with more fully under the section on Inhibition of Halogenation. Generalizations on the essentiality of copper for microbial halogenating enzymes become tenuous in view of the results of Gallicchio and Gottlieb (40) which indicate that biosynthesis of chloramphenicol-like activity was increased when copper was eliminated from a synthetic medium, and instead, that biosynthesis of such activity was stimulated by the presence of cations of magnesium, iron, and zinc.

There are no specific organic chemicals reported which favor halogenation. Specific organic compounds favoring synthesis of specific metabolites have been reported. For example, quinic acid and shikimic acid are reported as precursors in the biosynthesis of the tetracyclines (45). However, this precursor effect was not confirmed by independent investigators (74) who found only an insignificant amount of radioactive chlortetracycline after addition of shikimic-C^{14} acid to a fermentation which was rapidly synthesizing chlortetracycline. A number of organic compounds are reported by Vaněk (112) to stimulate the synthesis of chlortetracycline by a low yielding strain of *S. aureofaciens*. Among others, phenylacetic acid, β-indolylacetic acid, *p*-chlorophenoxyacetic acid, α-naphthylamine, α-naphthylacetic acid, and *p*-aminobenzoic acid were active. In our experience with better yielding strains, these or similar type compounds have not effected stimulation as reported by Vaněk. Certain chemicals have been reported as stimulating the biosynthesis of chlortetracycline. Benzyl thiocyanate at 0.5 mg/L stimulated the formation of as much as 800 mg/L chlortetracycline above a 1700 mg/L control yield (84). The mechanism of this action is not known, but it may be assumed to be in altering carbon metabolism (50). Egorov and

Baranova (35) reported stimulation by paradimethylaminobenzaldehyde, but from the data presented it is not possible to deduce whether this compound is acting as a stimulant or as a precursor for ring A (figure 1, *XVII*).

Salts of bivalent cations such as calcium, magnesium, and strontium, have a very desirable effect in the chlortetracycline fermentation system in that they precipitate or complex the antibiotic into an insoluble form and away from the presence of the formative microorganism (77). In fermentation mashes containing an alkali reserve of $CaCO_3$, less than 100 μg/ml of antibiotic is present in the mash filtrate, which is a level well tolerated by the *S. aureofaciens* strain used. When the concentration of antibiotic goes above 100 μg/ml in the filtrate, evidence of inhibition of the formative microorganism begins to appear. The highest publicly disclosed total tetracyclines yield is 8.5 g/L (42). On a material-balance basis, this indicates that at least 7 per cent of the total carbon of the nutrient medium has been converted into tetracyclines. If 80 to 90 per cent of the tetracycline carbon arises from the starch (74), then the carbon conversion is approximately 8 to 9 per cent; in these fermentations, the mycelial dry weights are in the range of 30 to 40 g/L, and this suggests that the mycelium is capable of the synthesis of a fifth to a fourth of its own weight of tetracyclines.

The concentration of phosphorus compounds in the fermentations by *S. aureofaciens* influences the amount of chlortetracycline formed. Biffi *et al.* (8), using a corn steep formula, showed that the addition of phosphate radically changed the metabolic behavior of *S. aureofaciens* in that ribonucleic acid content of the mycelium, sucrose and ammonia consumption, and pyruvic acid accumulation were increased, whereas chlortetracycline synthesis was sharply diminished. It seems that the carbon of the sucrose was being accumulated as pyruvic acid rather than being used in the synthesis of chlortetracycline. Other workers have confirmed these observations (87).

C. Inhibition of Halogenation

One of the most interesting developments concerning halometabolites is the field of chemical inhibition of halogenation. MacMillan (66) unintentionally presented the first data to this effect when he reported that strain no. 250 of *P. nigricans*, which normally produced no dechlorogriseofulvin, produced a mixture of bromoanalogue and dechlorogriseofulvin when grown in a bromide-rich, chloride-poor medium. The first purposeful disclosure was by Sekizawa (93), who found that bromine and four thio- or mercapto- compounds were effective in blocking the incorporation of chloride ions in a chlortetracycline fermentation. The inhibitory effect of bromide was confirmed (3, 46). Bromide ions act as a competitive inhibitor; *i.e.*, if one molecule of Br^- will block one molecule of Cl^-, or if the ratio of Br^- to Cl^- were 0.5, then the ratio of tetracycline to total tetracyclines would be 0.5. Thiocyanate, which is also classed as a competitive inhibitor, was not incorporated into the tetracycline molecule in contrast to bromide, which was incorporated to form bromotetracycline (Table 1: *12b*) (31). The action of competitive inhibitors may be reversed by excess of the normally utilized halide (31). These actions have an analogous parallel in the animal thyroid gland's uptake of iodine and its inhibition. Of even more interest were the enzyme inhibitors which were dealt with more fully by Lein *et al.* (61), and Goodman *et al.* (41). The best inhibitors in the system of Lein *et al.* were 2-benzoxazolethiol; 2,5-dimercapto-1,3,4-thiadiazole; and 2-mercaptobenzimidazole. Goodman *et al.* have confirmed the inhibitory effect of the second compound of Lein *et al.* and add 2-phenyl-5-mercapto-1,3,4-oxadiazole and 2-(2-furyl)-5-mercapto-oxadiazole to the list. One of the more remarkable aspects of these enzyme inhibitors is the minute amount required to block the chlorinating system. As little as 5 μg/ml of inhibitor was able to block the incorporation of 400 μg/ml of chloride into the tetracycline molecule. The data of Goodman *et al.* may be considered a rigorous test of inhibition since they were working with a high potency system which was enriched with chloride and with a known "chloride scavenging" strain. The action of these inhibitors appeared to be, at least in part, associated with a function of copper, probably a copper oxidase, since the addition of copper reversed the effect of the inhibition. The addition of excess chloride did not reverse the inhibition. Interestingly, silver ion was able partially to reverse the inhibition effect.

At present, data on the chemical inhibition of halogenation have not been extended to other halometabolites except chloramphenicol and caldariomycin. In chloramphenicol fermentation,

bromide was competitive with chloride (101), whereas fluoride or iodide had no effect. A number of chlorination inhibitors is indicated by Shaw and Hager (98) for *Caldariomyces fumago* in the synthesis of caldariomycin; sodium azide was the most potent, followed by thiourea, thiouracil, and 6-methylthiouracil. Inhibition to a lesser degree was indicated for bromide, iodide, and fluoride. Because of conflicting data on inhibitors, Shaw and Hager were unable to conclude that a copper or heme oxidase was implicated in this chlorination reaction. Their chlorination system was relatively insensitive to cyanide but highly sensitive to azide inhibitors. Copper oxidases should be affected by both of these two types of inhibitors. A stimulation of formation of tetracycline in a chlortetracycline fermentation has been attributed to increasing the concentration of corn steep liquor in the medium (78). However, from the data presented, it is difficult to interpret this effect as an inhibition of chlorination, or as an increase in the total potential of a system above its chlorinating capacity.

IV. Halogen Interchangeability

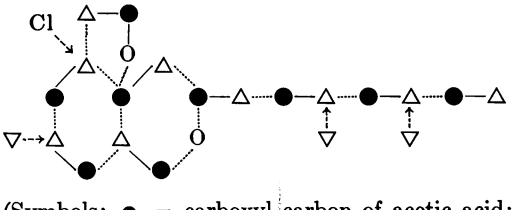
No workers have reported iodo- or fluorometabolites of microbial origin although attempts have been made to produce iodo- and/or fluorometabolites of *A. terreus* (88), of *P. sclerotiorum* (91), of *S. aureofaciens* (30), and of a chloramphenicol-producing Streptomyces (101). Only five bromo-derivatives are known: bromo-dechlorogriseofulvin (Table 1: *10f*) (66); bromotetracycline (Table 1: *12b*) (96); *N*-dibromoacetyl-*p*-nitrophenylserinol (Table 1: *4d*) and *N*-monobromomonochloroacetyl-*p*-nitrophenylserinol (Table 1: *4e*), the analogues of chloramphenicol (101); and bromo-demethyltetracycline (Table 1: *12a*) (70).

Although bromide interchanges with chloride, it is incorporated by the microorganisms involved at levels much lower than those recorded for chloride. For instance, *P. nigricans* strain no. 250, which did not produce dechlorogriseofulvin in chloride-rich media, when grown in a chloride-limited, bromide-rich medium, produced a mixture which, after extraction, yielded a ratio of 62:73 of bromo- and dechloro-analogues (66). *S. aureofaciens* of the type which normally produced chlortetracycline in chloride-rich media, when grown in a chloride-limited, bromide-rich medium produced a mixture which, after extraction, was composed of approximately nine parts of tetracycline to six parts of bromotetracycline as calculated from countercurrent distribution data of Sensi *et al.* (96). The high yielding mutant of *S. aureofaciens* (31), BC-41, brominated at only one-third of its chlorinating rate. This compares favorably with the calculations made from data of Sensi *et al.* Biologically, the bromo-analogues seem to offer no gross advantages over the chlorometabolites as antibiotics. Bromodechlorgriseofulvin is only one-seventh as active as griseofulvin (66). Bromotetracycline has essentially the same spectrum and level of activity as does chlortetracycline (96) and the bromo-analogues of chloramphenicol do not have a high order of activity against *Klebsiella pneumoniae* (101).

V. Biosynthesis and Mechanism of Halogenation

In the biosynthesis of penicillin, relatively large precursor fragments of the ultimate molecule are incorporated by Penicillium. This fact has misled many investigators in the search for analogous and economically attractive parallels in other valuable microbial metabolites. Birch *et al.* (9) have given evidence for the buildup of sclerotiorin from acetic acid units, and this theory of biosynthesis has support in studies on other microbial metabolites.

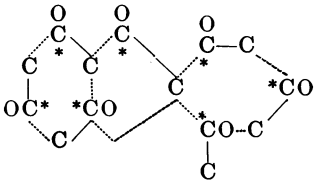
In separate experiments, by growing *P. multicolor* in media containing radioactive carbon-labeled acetic acid, both for the methyl and carbonyl carbon, and formic acid, Birch *et al.* (9) obtained different radioactive sclerotiorins. After degradation of these forms and measuring the radioactivity of different fragments, they conclude that the skeleton of sclerotiorin is formed from linkage of 9 molecules of acetic acid and 3 molecules of formic acid. They represent the origin of the molecule as:



(Symbols: ● = carboxyl carbon of acetic acid; △ = methyl carbon of acetic acid; ▽ = carbon of formic acid. Compare with formula VI of figure 1.) They also indicated through radio-

carbon studies that a possible precursor, a dienoic acid, was degraded to acetic acid by the growing culture prior to being incorporated into the sclerotiorin molecule.

Earlier Birch *et al.* (11) presented evidence, using carbonyl C^{14}-labeled acetic acid, for the biosynthesis of griseofulvin by a similar acetate linkage. They represent the origin of griseofulvin, arising from 7 molecules of carboxyl carbon-labeled *acetic acid, as:



(Compare with formula XV of figure 1.) Since the molecules of geodin and erdin (XIV) have the same skeleton as does griseofulvin, it may be assumed that their mechanism of origin is similar to that of griseofulvin. The same might be said for geodoxin (XIII), except that oxygen must be introduced between the carbons of one of the acetate groups in the linkage sequence postulated by Birch *et al.* for griseofulvin. It would be very interesting to see the same type of study on the products of *A. terreus* as has been done with those of *P. multicolor*. Since both of these species will produce these metabolites on media containing only glucose as an organic carbon source, they must be able to produce acetic acid and methyl donor compounds by their own metabolism.

The simplest halometabolite, caldariomycin, has received recent attention from Shaw, Beckwith, and Hager (97–100). They first demonstrated that β-ketoadipic acid and chloride were converted into chlorolevulinic acid by dried mycelial powders of *C. fumago*. The stoichiometry of this reaction was represented as follows:

$$\beta\text{-Ketoadipic acid} + Cl^- + \tfrac{1}{2}\,O_2 + 2H^+ \rightarrow CO_2 + H_2O + \text{chlorolevulinic acid.}$$

The pH optimum of the reaction was 4.8, consistent with the involvement of hydrogen ion. There was a high rate of oxygen uptake and coincident carbon dioxide evolution. Chlorination was not accomplished in the absence of oxygen. β-Ketoadipate was slowly oxidized in the absence of chloride, but without carbon dioxide production. The hydrolysis of a polysaccharide, present in considerable amount and resistant to attack by amylolytic enzymes, was deduced to be responsible for the high endogenous oxygen uptake by the reaction.

In later studies Shaw *et al.* separated two fractions from an extract of mycelial powders, (a) a heat-stable supernatant and (b) a heat-labile gel eluate, both of which were required for formation of chlorolevulinic acid. The heat-stable fraction contained a polysaccharide, and mild acid hydrolysis of this fraction enhanced its activity. Glucose and hydrogen peroxide would substitute for the heat-stable fraction. Further separation of the heat-labile fraction yielded two components, which were called: (c) a glucose oxidase fraction, and (d) a chloroperoxidase fraction. Both of these fractions were necessary for the synthesis of chlorolevulinic acid from chloride and β-ketoadipate in the presence of glucose. They formulated the reactions involved.

$$(\text{Glucose})_n + 3H_2O \xrightarrow{\text{hydrolase}} (\text{glucose})_{n-3} + 3\ \text{glucose}$$

$$3\ \text{Glucose} + 3O_2 + 3H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} 3\ \text{gluconic acid} + 3\,H_2O_2 + 3H^+$$

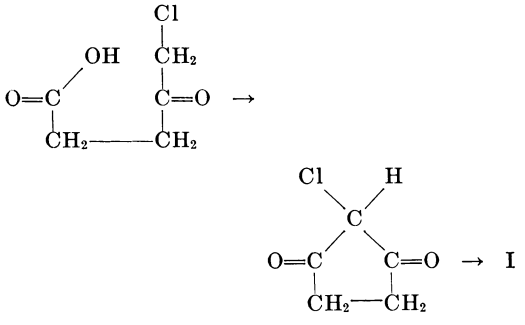
$$2\,H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2\,H_2O$$

$$H_2O_2 + Cl^- + {}^-OOC{-}CH_2{-}CO{-}CH_2{-}CH_2{-}COO^- + 2\,H^+ \xrightarrow{\text{chloroperoxidase}} CO_2 + ClCH_2{-}CO{-}CH_2{-}CH_2{-}COO^- + 2\,H_2O$$

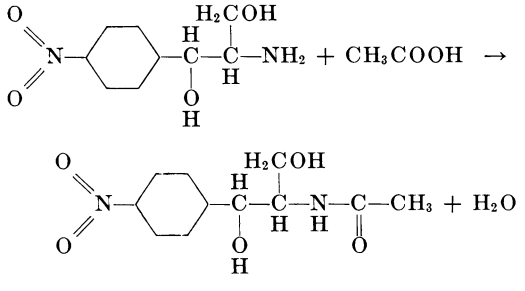
In the last reaction, they suggested that chloride ion was oxidized to a chlorinium ion with oxygen serving as an electron acceptor. They found no evidence to support the participation of an energy source such as adenosine triphosphate, except that there was a high rate of endogenous oxygen uptake. The fact that decarboxylation of β-ketoadipate did not proceed in the absence of chloride was evidence to them that an enzymatically generated free enol form of levulinic acid could not be the chlorine acceptor.

The isolated components of this system did not form caldariomycin. There was an 8 per cent conversion of the radioactive chlorine of γ-chlorolevulinic acid to caldariomycin by active cultures of *C. fumago* without evidence that

incorporation proceeded via hydrolysis of the substrate to yield chloride ion. It appears that the introduction of the second chlorine of caldariomycin is accomplished by a separate series of reactions and that the biogenesis of caldariomycin may be hypothesized to involve the cyclization of γ-chlorolevulinic acid.



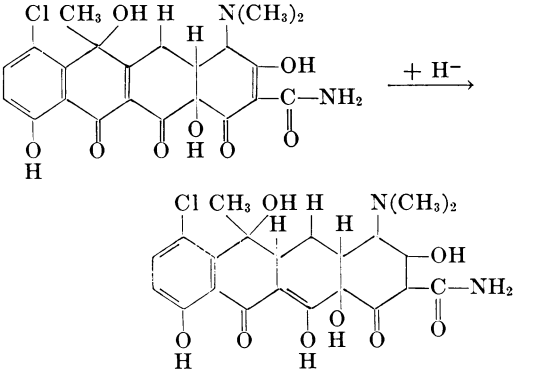
Knowledge of the biosynthesis of chloramphenicol is limited (43). *S. venezuelae* apparently does not chlorinate *N*-acetyl-*p*-nitrophenylserinol (44), for when *p*-nitrophenylserinol was added to cultures it was acetylated, but not acetylated and chlorinated.



The results of Wang *et al.* (118) led them to suggest that a possible precursor, dichloroacetic acid, is first converted into an intermediate prior to being incorporated into the chloramphenicol molecule. Their results did not indicate the prior conversion of organic chloride to ionic chloride except in the case of monochloroacetic acid.

In the biosynthesis of tetracyclines by *S. aureofaciens*, chlorination apparently did not occur in the later stages (46), but neither did it occur at the very beginning (31), because the over-all rate-limiting step preceded the halogenation step. Evidence that halogenation was not the final step is the fact that addition of tetracycline to a chlorinating system did not result in chlorination of the added tetracycline (115). Wang (116, 117) has isolated but not characterized organic chlorine-containing compounds from the young liquid cultures of *S. aureofaciens*. Three of these chlorine-containing organic fractions were tested for incorporation into chlortetracycline by radiochemical methods. Only one, called the "aqueous soluble chlorine compound," which was isolated from culture filtrate, was indicated to be incorporated. The other two were incorporated into the mycelium but not into chlortetracycline. It would appear from both the results of Wang and Doerschuk *et al.* (31) that the metabolized chloride is either incorporated into antibiotic or into mycelium because chlorine-containing organic compounds in the filtrate at the end of the fermentation were not observed.

The observation that the 7-chloro-5a(11a)-dehydrotetracycline can be biologically or chemically hydrogenated, thus yielding chlortetracycline (71, 73), led to the suggestion that this hydrogenation is the last step in the biosynthetic pathway.

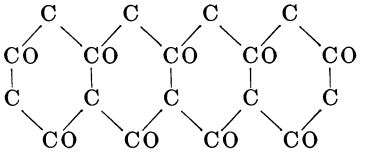


The dehydro compound, which had a low order of antibacterial activity *in vitro*, was produced by a mutant of *S. aureofaciens*. The presently favored proposal for the biogenesis of the basic naphthacene nucleus of chlortetracycline is by the "head to tail" linkages of molecules of acetic acid as first theorized for oxytetracycline by Robinson (92).

Apparently a remarkable series of enzymatic reactions is involved in the biosynthesis of the chlortetracycline molecule from simple beginnings. Radio-carbon was incorporated into the tetracycline skeleton by *S. aureofaciens* from 2-, 3-, 6- and poly-carbon nutrients (74). These authors show the specific incorporation of the 2-carbon of glycine, the methyl carbon of

methionine, the 1- and 2-carbons of acetate, and the 3-carbon of serine. The high percentage of incorporation of the 2-carbon of glycine and the methyl group of methionine indicated a rather direct route of incorporation. The almost equal incorporation of the 1- and 2-carbons of acetate indicated the use of the whole acetate group. The marked difference of incorporation of the carbons of glycine indicated that this was serving as a donor of 1-carbon groups. Degradation of chlortetracycline synthesized by a fermentation containing glycine 2-C^{14} indicated a concentration of radioactivity in the dimethylamino group of the molecule. Nonradioactive glycerol was said to furnish all the carbon required for the germination of spores, production of mycelium and formation of chlortetracycline by *S. aureofaciens* in a mineral salts medium.

If we postulate the origin of chlortetracycline from acetate linkages, it is more probable that the 7-carbon of chlortetracycline—that is, the carbon of the chlorine-carbon bond—originates from the methyl carbon of an acetate group. This has its parallel in other halometabolites (9–11). It is also reasonable to expect that the 1-, 10-, 11-, and 12-carbons of the molecule originate from carbonyl carbons of acetate groups. After linkage and cyclization of the acetate groups, a polyketone skeleton is organized.



At least 12 other reactions must be accomplished to complete the molecule: (a) addition of a $-CONH_2$ group at the 2-carbon; (b) hydroxylation of the 3-carbon; (c) amination of the 4-carbon with synchronous or subsequent methylation of the amine; (d) and (e), respective reduction of the 4a-, 5a-carbons and their hydrogenation; (f) and (g), respective hydroxylation and methylation of the 6-carbon; (h) chlorination of the 7-carbon; (i) reduction of the 8-carbon; (j) and (k), respective enolization of the 10- and 12-carbons; and (l), the hydroxylation of the 12a-carbon, which may be coupled with the reduction of the 4a-carbon. The sequence of these events is not known, except as mentioned earlier, reaction (d) may be one of the last to occur.

Thus a common basic mechanism of synthesis is indicated for a growing list of metabolites, but with infinite variations and terminations according to the species and its environment. There is insufficient evidence for generalizations concerning common mechanisms of halogenation, but instead the available evidence indicates differences in mechanisms.

VI. Function of Halometabolites

None of the halometabolites described has been assigned an essential function in the metabolism of the microorganism that produces it. Functionally, the antibiotic metabolites may serve to give the specific producer more *Lebensraum* than it would be afforded in ordinary commensal relations, but this effect has yet to be demonstrated in nature. The close similarity of certain halometabolites to certain antimetabolites; *i.e.*, vitamin K, its dichloro-antimetabolite, and diploicin- causes certain teleological speculations that these products are protective mechanisms. However, until more knowledge is amassed on the metabolism of chloride and of halometabolites, the attitude must be held that halometabolites are unusual and chance products of nature.

VII. References

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