JOURNAL OF Pharmaceutical Sciences

October 1968 volume 57, number 10

Review Article

Biosynthesis of the Tetracycline Antibiotics

By L. A. MITSCHER

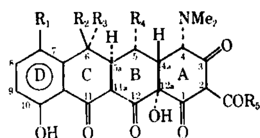
INVESTIGATION of the metabolic products of biological systems, accelerated in recent years by the discovery and application of chromatographic and spectroscopic methods, has resulted in the structural elucidation of an enormous number of natural substances. The endeavor to bring classifying order into this accumulation of data has resulted over the years in various unifying structural theories (1–5). These have often been put forth with the stated or implied assumption that the structures of the molecules reflect the underlying biological processes by which they are formed. Classic examples of such systems would include the isoprene rule, the polyketide theory, and the biogenesis of alkaloids from amino acids. Among the most exciting discoveries of recent years has been the finding of experimental evidence indicating that many of these systems do indeed have a real biological basis. As a result we now recognize great families of natural products derived from the ubiquitous primary metabolites: acetate-malonate, shikimate-prephenate, and acetate-mevalonate. An accelerating effort in recent years has resulted in the gathering of numerous examples of members of each of these great classes, along with a number of examples of natural products containing structural features derived from more than one class of precursor. Even while this great unifying effort has gone forth, examples have begun to appear of studies involving the next logical phase of study, elucidation of the detailed biosynthetic pathways linking the primary metabolites with their derived natural products. The morphine alkaloids (6, 7), the steroids (8–10), and the tetracycline antibiotics (11–14) constitute the systems best studied to date. These systems are of outstanding medical and scientific importance and, interestingly, each belongs to a different precursor-product class and they may well serve as prototypes for the many studies to come. It is the purpose of this review to describe the present state of our knowledge of the biosynthesis of the tetracycline antibiotics and their relationship to the mainstream of acetate–malonate-derived microbial products. A number of practical applications has been accomplished in this area making this study especially instructive for those interested in the preparation of new antibiotics.

The tetracyclines are highly oxygenated hydronaphthacene derivatives produced by fermentation of various *Streptomyces* species, by structural modification of fermentation tetracyclines, and, in certain cases, by total synthesis. The formulas of those tetracyclines which are produced wholly or primarily by fermentative processes are given in Table I, which also contains the numbering and lettering system used for these substances.

Many of the compounds that comprise the domain of the natural products chemist are classified as secondary metabolites. A secondary metabolite may be defined as a substance with no presently apparent utility (such as structure, energy, reproduction, growth, *etc.*) to the producing organism and which is elaborated, usually, when the primary growth and replication

Received from the College of Pharmacy, The Ohio State University, Columbus, OH 43210

TABLE I—TETRACYCLINES PRODUCED BY FERMENTATION



No.	Compd.	R_1	R_2	R_3	R_4	R_5
I	Chlortetracycline	Cl	OH	Me	H	NH_2
II	Oxytetracycline	H	OH	Me	OH	NH_2
III	Tetracycline	H	OH	Me	H	NH_2
IV	6-Demethylchlortetracycline	Cl	OH	H	H	NH_2
V	Oxychlortetracycline	Cl	OH	Me	OH	NH_2
VI	2-Acetyldecarboxamidooxytetracycline	H	OH	Me	OH	CH_3
VII	2-Acetyldecarboxamidotetracycline	H	OH	Me	H	CH_3
VIII	2-Acetyldecarboxamidochlortetracycline	Cl	OH	Me	H	CH_3

phase of colony or plant development has ceased. These substances are often produced in relatively large quantites, are frequently of complex structure, and occasionally have useful physiological properties in human and veterinary practice. The tetracycline antibiotics are excellent examples of such metabolites.

An excellent review of the chemistry and general history of the tetracyclines up to about 1962 has been published (15), and the synthesis of tetracycline analogs has been reviewed (16). A number of reviews of the biosynthesis of the tetracyclines from various viewpoints has also appeared in recent years (11–14).

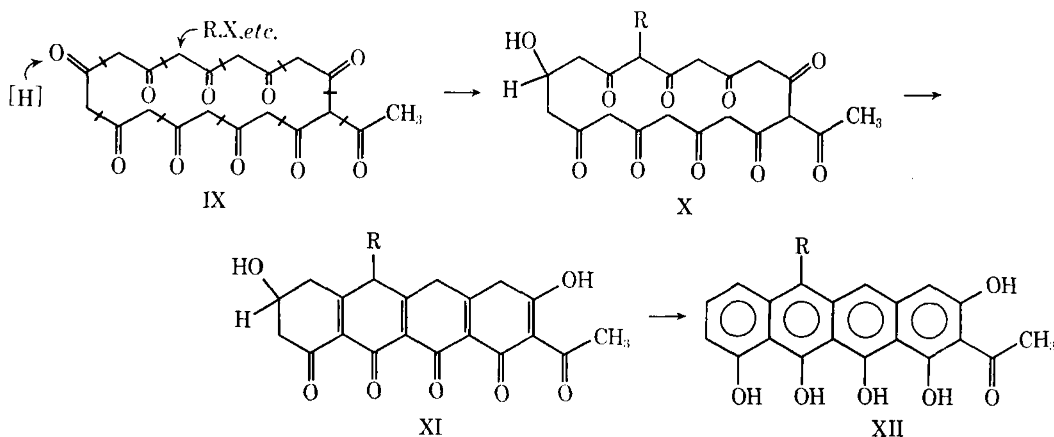
FUNDAMENTAL UNITS

Before any experimental evidence was available, Birch (17), Robinson (3), and Woodward (18) predicted on theoretical grounds that the carbon skeleton of the tetracyclines is constructed from "acetate" units linked together head-to-tail. At about the same time Snell (19, 20) was able to show that sodium acetate-2-^{14}C was incorporated into oxytetracycline (II) to the extent of 5–10%. The same result was obtained independently by Miller *et al.* (21). Birch, Snell, and Thompson (22, 23) and Gatenbeck (24) subsequently were able to show by degradation studies that the carbon atoms were indeed derived from acetate carboxyl and methyl in an alternating sequence although, because of experimental difficulties, complete degradation of the tetracyclines to isolate every carbon atom for absolutely rigorous proof has still not been accomplished. The C- and N-methyl groups are derived from methionine (21, 22, 25) as expected, and the nitrogen atom at C_4 likely comes from glutamic acid. Certain tetracyclines (I, IV, V, VIII) have a chlorine atom at C_7. In chlortetracycline (I) biosynthesis, this atom comes from chloride ion in the medium and is not a necessary feature for overall tetracycline formation as the final concentration of tetracyclines produced by a given mutant appears to be independent of the chloride ion concentration in the medium (27). When care is taken to exclude chloride ion from the medium, normally halogenating strains produce unhalogenated tetracyclines in undiminished quantity. Some strains will accept bromide ion as a substitute and thus produce bromotetracycline ($I, R_1 = Br$) (26–29). This substance is quite active biologically though it has not found clinical application. A number of compounds, some of which are more readily recognized as antithyroid drugs, inhibit chlorination (30, 31).

POLYKETIDE THEORY AND TETRACYCLINE BIOSYNTHESIS

The manner in which these fundamental building blocks are assembled can be predicted using the general tenets of the polyketide theory as elaborated in a masterful series of experiments by Birch and his school (32–34).

According to this theory the majority of the phenolic secondary metabolites produced by microorganisms (particularly the fungi) can be considered to be polymers derived from various metabolic acids, with "acetate" forming the most common component. For simplicity of exposition the basic tenets of polyketide theory can be illustrated through the hypothetical tetracene derivative (XII) which can be supposed to have been formed from 10 "acetate-equivalents" linked head-to-tail in a regular manner to form hypothetical intermediate IX, which is called a polyketide because of the alternation of carbonyl and methylene groups in a large ring. This substance can be likened to a zipper which is ready to close. The carbon skeleton of the aromatic rings is formed by a series of transannular carbonyl-methylene condensations followed by enolizations. A large open-ring system such as X is capable of assuming many shapes by simple folding. The shape assumed during cyclization will determine the aromatic ring system formed. Control of the specificity of the process is considered to be one of the functions of the enzymes involved. The majority of those oxygens not involved in ring closures are retained in the product as, indeed, it was this circumstance that



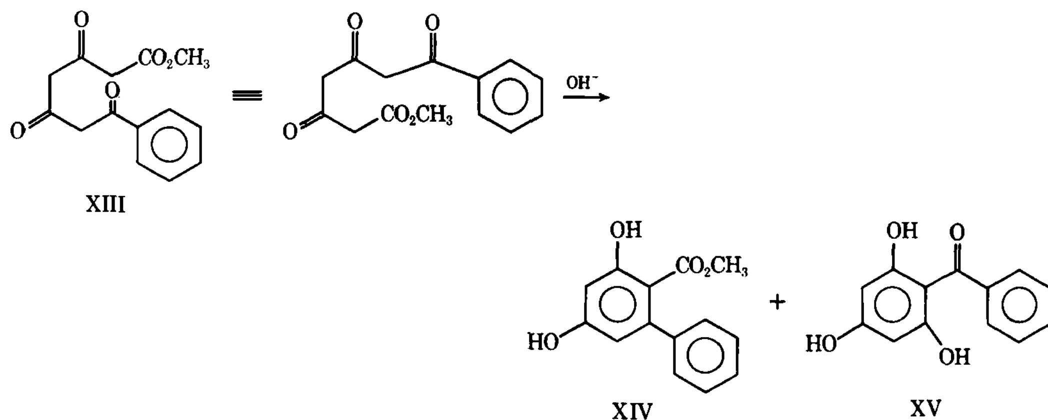
Scheme I

led Birch to propose polyketide involvement in natural processes in the first place. A number of secondary reactions are also frequently encountered. The different oxygenation patterns of the two terminal rings in XII are particularly illustrative of one of the proposed ways in which the basic oxygenation pattern can be altered. Direct removal of a phenolic hydroxyl group as such, in the laboratory, is a high-energy process. A process involving much lower energy requirements and with ample precedent in fatty acid biosynthesis is simple reduction to a β-hydroxyketone followed by dehydration. One presumes a biological parallel. These reactions presumably occur before cyclization to hypothetical XII and are illustrated by formulas X and XI (Scheme I). It is interesting to note that oxygen atoms are frequently "missing" in analogous positions from a variety of polyketide-derived aromatic products and it is tempting to suggest that this oxygen has no essential function in the cyclization or activation process as it has been formulated. This observation has recently been emphasized in the elaboration of an ingenious theory which attempts to explain the steric control of cyclization on the basis of chelation with metal ions on an enzyme surface. Examination of this hypothesis is highly recommended to those with a special interest in this topic (35). Additional functions, such as halogens, alkyl groups, isoprene units, extra oxygen functions, *etc.*, can readily be visualized as being introduced into the activated methylene groups of the polyketides by electrophilic-type reactions. In fact, in microbial products thought to be elaborated from polyketides, such "extra" functions are almost invariably found on methyl-derived carbons—almost never occurring on carboxyl-derived carbons. It must be emphasized, however, that these "extra" functions may also be introduced after the fully aromatic stage is achieved. This will be clearly apparent later when the sequential aspects of tetracycline biosynthesis are discussed. These few general considerations, as simple as they are, are sufficient to rationalize the formation of the vast majority of polyketide-derived substances. The enormous utility of polyketide theory, not only in rationalizing biogenesis but also in predicting structures when a series of otherwise equivalent expressions is available, has led to its virtually general acceptance despite the fact that no one has yet succeeded in isolating a polyketide as such from either chemical or biological sources. Some interesting beginnings have been made in mimicking these processes by syntheses of poly-β-carbonyl derivatives and examining their rearrangement products (36–41) and some suggestive structures have been isolated from unnatural, or blocked, biological systems (42–48).

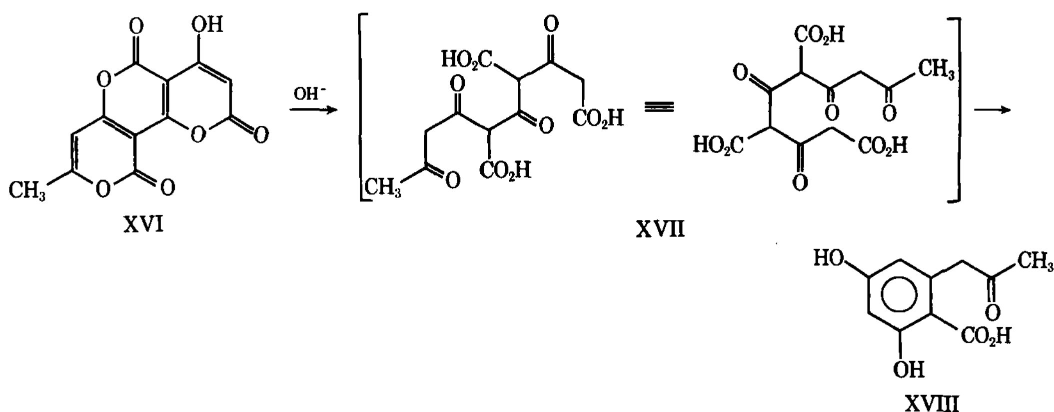
For example, synthetic triketoester (XIII) undergoes cyclization in alkaline solution to give a β-resorcylic ester (XIV) and/or an acylphloroglucinol (XV) (39) (Scheme II). The product composition varied widely depending upon the reaction conditions. These products are precisely those predicted by the original polyketide theory (17) and naturally occurring examples of both types of products are numerous (the phenyl substituent, however, is not usual).

Synthetic pyrones (XVI) behave in a similar manner and presumably do so by the intermediacy of poly-β-ketones (XVII) (36). The product in this case (XVIII) has been isolated from natural sources as well (Scheme III).

When tropolone (XIX) biosynthesis is interrupted by the use of enzymatic blocking agents, "tetraacetic lactone" (XXI) can be isolated (48) (Scheme IV). This material clearly looks like a rejected or incomplete polyketide (48).



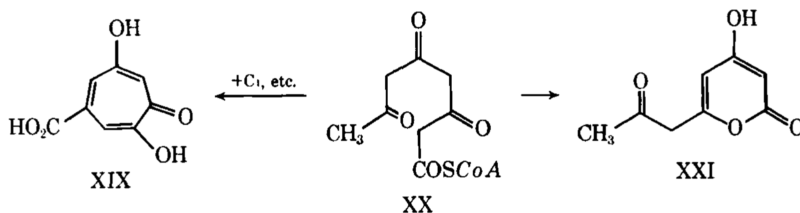
Scheme II



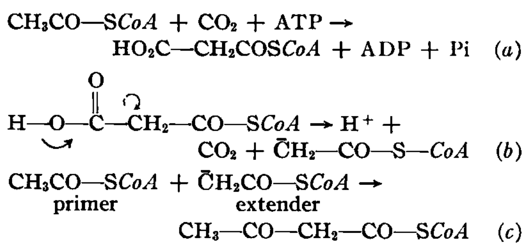
Scheme III

Many other examples could be quoted (36–48) but these are sufficient to illustrate the point that materials resembling polyketides can be isolated from natural systems and that these materials have the general properties required of them by polyketide theory. Ultimate success in this difficult area will undoubtedly depend on development of suitable means of stabilizing the highly reactive polyketides once they are freed from their enzymatic supports.

This exposition is, however, like most appealingly simple approximations, now known to be an understatement. Polyketide formation may be viewed in a practical sense as an aberration in fatty acid biosynthesis in which most of the same apparatus is utilized but to a different end. The growing chain is not reduced, dehydrated, and reduced, successively, as in typical fatty acid biosynthesis. The polyketocarbonyl structure persists in the growing chain. The polyene macrolides would appear on this basis to be intermediate forms biosynthetically between the fatty acids and the aromatic metabolites. It has now become apparent that acetyl coenzyme A and malonyl coezyme A are the main fatty acid and polyketide precursors (thus the term "acetate-

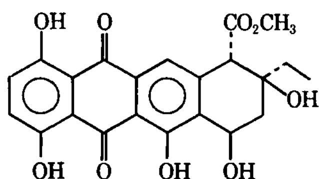


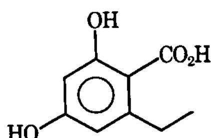
Scheme IV



Scheme V

equivalent" used in early work is amply justified!) (24, 49–51). In polymer terms acetyl *CoA* is thought to serve as the primer with the thiol ester grouping being easily displaced (a good





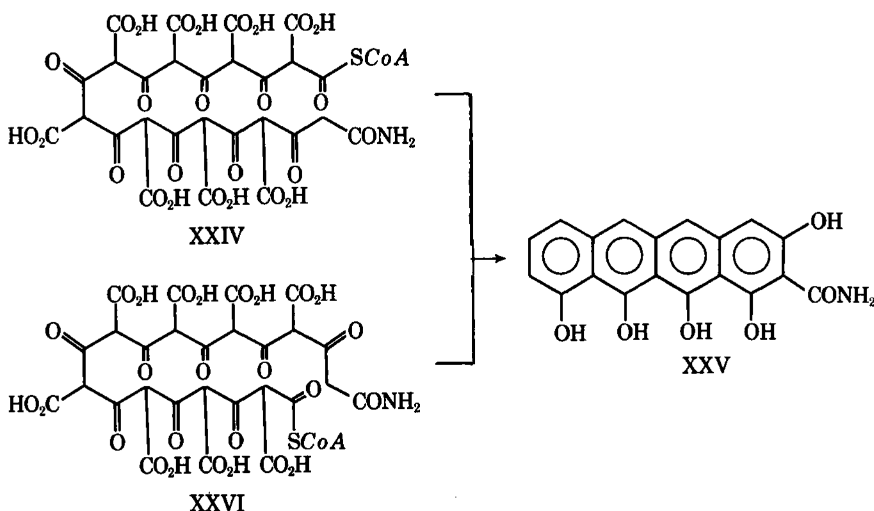
"leaving group") facilitating coupling through nucleophilic reaction. Carboxylation of acetyl *CoA* to form malonyl *CoA* provides the extender, facilitating the reaction by increasing the ease of development of a negative charge on the acetate methyl carbon. These ideas are illustrated in Scheme V, *a*, *b*, and *c*.

It must be emphasized that these expressions are expository and do not exclude the possibility that the sequence involved is concerted and, therefore, that the carbon dioxide may be lost subsequent to or during the coupling rather than, as illustrated above, before. As the role of carbon dioxide is essentially catalytic, the involvement of malonate was missed in early work and the fairly recent appreciation of its central role in these reactions is of outstanding importance. Reduction of the growing chain would lead to fatty acid biosynthesis, whereas persistance would lead to aromatic products. This suggests that one factor controlling the amount of metabolism down these two branches may be the amount of reducing capacity (NADPH) available to the organism at the time.

A number of other common metabolic acids, particularly propionate [either naturally as in the case of ε-pyrromycinone (XXII) (52) or by forcing as with homoorsellenic acid (XXIII) (53)], are known to serve as primers, and chain extension can occur *via* other acids, such as methylmalonate, as is the case in most nonpolyene macrolide antibiotics (54).

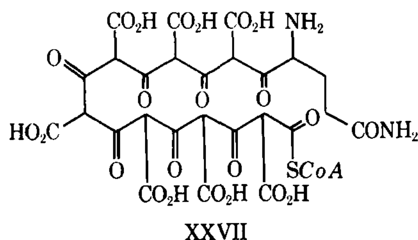
TETRACYCLINES AS POLYKETIDES

In the case of the tetracyclines specifically, it is now generally, though not entirely without reservation (14, 55), accepted that malonamyl *CoA* serves as the primer and that extension occurs *via* eight malonate units so that more sophisticated versions of polyketide IX can be put forth X(XIV and XXVI) (Scheme VI). As there is no convenient terminal methyl group to act as a marker, the exact folding is not obvious and both



Scheme VI

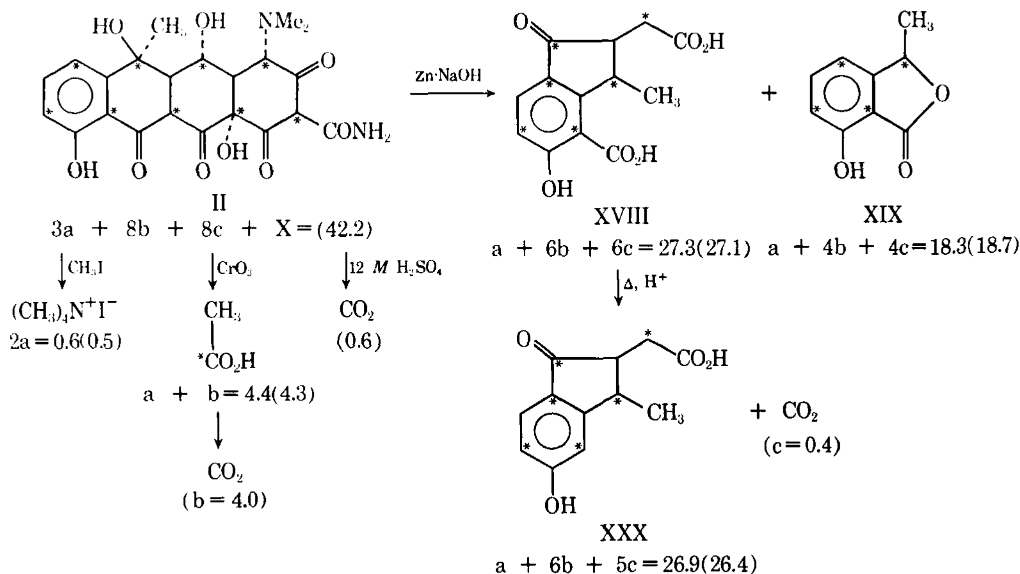
XXIV and XXVI equally satisfy the available evidence. There is no direct evidence for the involvement of malonamyl *CoA*. An interesting theory which has not been entirely abandoned (14) suggested that the majority of Ring A of oxytetracycline was derived from a glutamine unit. This ingenious idea would require the involvement of hypothetical polyketide XXVII, and was advanced prior to the recognition of the role of malonate in polyketide biosynthesis (22, 23). Having noticed that the amount of



XXVII

radioactivity incorporated into Ring A was often lower than anticipated when radioactive acetate was fed, an alternate primer unit was sought. It was found that glutamic acid-2-^{14}C did lead to oxytetracycline with most of the radioactivity in Ring A. Unfortunately Ring A is very difficult to degrade cleanly and in good yield to well-defined products, so that most of the work on the primer unit still depends on obtaining the extent of labeling by difference and the definitive experiment involving intact incorporation of a doubly labeled starter has yet to be reported.

After observing that the extent of labeling of the carboxamide carbon was higher than seemed likely from metabolic scrambling of acetate, an effect which Snell and Birch themselves questioned (23), Gatenbeck obtained results (24) which suggest strongly that malonate, thought to be in the form of malonamyl *CoA*, is the starter unit and that the higher than expected labeling of the carboxamide function resulted from metabolic oxidation of acetate to carbon dioxide followed by fixation into malonate *via* the tricarboxylic acid cycle. The earlier result with glutamate could be explained in the same way—that is, that glutamate acts as a metabolic source of carbon dioxide. The timing of the fixation and the time of incorporation of the starting unit could explain the variable radioactivity in the primer unit. It will be noted from inspection of formulas XXIV and XXVI that all of the carbon dioxide molecules fixed into the hypothetical polyketide are subsequently lost *with the single exception of the one in the starter unit.* Thus this theory predicts a specific enrichment of Ring A by carbon dioxide, an effect Gatenbeck was able to achieve using radioactive sodium bicarbonate. Glutamate had no specific promoting effect over the other Krebs cycle acids tested. Using the data of Birch and Snell (22, 23) and Gatenbeck's hypothesis (24), it is possible to achieve an excellent fit for the experimental data (Scheme VII).



Scheme VII—Incorporation of radioactivity into oxytetracycline from $^{14}CH_3CO_2H$. Calculated figures are followed by normalized experimental values in parentheses.

Radioactivity in Scheme VII is expressed as relative molar activities (R.M.A.) $\times$ 10^{-3}. R.M.A. is defined as the number of counts/

100 sec. for an infinitely thick sample of 1 $cm.^2$ cross-sectional area. Thus it is directly proportional to the molar specific activity. Using methyl-labeled acetate the molecule as a whole had R.M.A. = 42.2×10^3 in this particular experiment and the parameters involved are fourfold:

a = contribution due to the one-carbon pool derived *via* oxidation of the acetic acid and subsequent general metabolic incorporation of the labeled carbon dioxide. This could be measured directly by isolation of the amino methyls, which are methionine derived, and from the activity of the C_6 methyl determined by Kuhn-Roth oxidation and subsequent degradation. a = 0.3.

b = contribution of the methyl of acetic acid. This is measurable from the intensity of the activity of the C_6 carbon, isolated as the carboxyl end of the acetic acid from the Kuhn-Roth oxidation. b = 4.1.

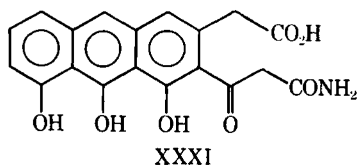
c = contribution of the carboxyl of acetic acid. The radioactivity of this carbon is due to metabolic scrambling and experience has indicated that a value of 5–10% is appropriate. This could, however, be measured directly as the activity of the carboxyl in terracinoic acid (XVIII). c = 0.4.

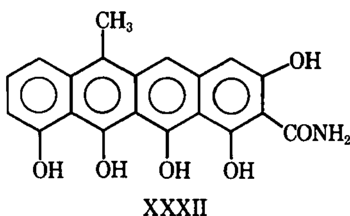
x = activity in the primer unit can be calculated using the values for a, b, and c. If the molecule is wholly acetate derived, then its makeup will be $3a + 8b + 8c + x = 42.2$ and therefore $x = 5.3$. This would give an incorporation level of about 12.5% into the primer unit which is equivalent to that which Gatenbeck found using radio-labeled malonate (10–20%). Birch determined the level in the carboxamide carbon to be 0.6. Thus the starter would be $b + c + 0.6 = 5.1$ which is reasonably close to the expected value ($b + c = 4.5$). As the acetate involved in the starter unit must undergo at least one additional reaction compared with those used in extension, the agreement should not be expected to be exact. The variability observed in this unit in various experiments (14, 24) might reflect the relative efficiencies of these two processes and the extent to which the organism uses these building blocks for other purposes between the time when they are assembled and when they are incorporated into tetracyclines.

While it is true that specific degradation of Ring A is still to be achieved and double-labeling studies with malonic acid semiamide have either failed (55) or have not yet been reported, the balance of evidence currently favors Gatenbeck's modification of Birch and Snell's hypothesis. One additional bit of circumstantial evidence favors this theory. The production of 2-acetyl-2-decarboxamidotetracycline derivatives (VI, VII, VIII) by certain mutant strains (56–58) is most readily rationalized by presuming a block in malonamyl *CoA* formation and the subsequent utilization of acetoacetyl *CoA* in its place. This would implicate polyketides XXIV or XXVI rather than XXVII. No experimental confirmation of this inference by labeling studies has yet appeared. Another circumstantial bit of supporting evidence derives from experiments indicating (*vide infra*) that the C_4 nitrogen is introduced at a later stage and does not therefore come as part of the primer unit. Thus the evidence available at present is in favor of the whole carbon skeleton being acetate-malonate derived and so the tetracyclines appear to be typical, although unusually complex, polyketide-derived molecules. No other known polyketide product has malonic acid semiamide as the presumed starter unit.

PRETETRAMIDS

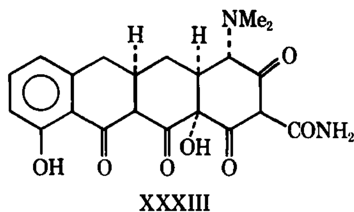
Having concluded that hypothetical polyketides XXIV and XXVI are satisfactory rationalizations for the experimental evidence, it is immensely gratifying to find that certain aromatic degradation products of the tetracyclines which closely resemble XXV are in fact excellent biological precursors of the tetracyclines. The simplest of these, pretetramid (XXV) is very close indeed (59, 60). The biotransformation yields of XXV to finished antibiotic ranged from 3–75% depending upon the substrate and the mutant used. Pretetramid is the earliest recognizable and well-characterized specific tetracycline precursor known at present. Protetrone (XXXI), isolated from a blocked mutant culture, appears to be a shunt product resulting from an imperfection in the cyclization process (11). Synthesis of aromatic pretetramid derivatives and subsequent bioconversion is a tool that should allow the preparation of novel tetracyclines. This approach depends upon the subsequent enzymes being relatively insensitive to the presence of unusual structural features and it is disappointing that, so far, no really unusual tetracyclines have reportedly been made in this way. Still, work with the various pretetramids has given considerable insight into the biological reactions involved. Pretetramids lacking a C_6 methyl group do not give rise to 6-methylated tetracyclines, so methylation may be presumed to be a very early step. Synthetic 6-methyl pretetramid (XXXII) is readily bioconverted to 6-methylated tetracyclines as theory would demand. Obviously then, the subsequent en-





zymes in the two series are insensitive to the presence or absence of the C_6 methyl and the biosynthesis follows a parallel course from here on.

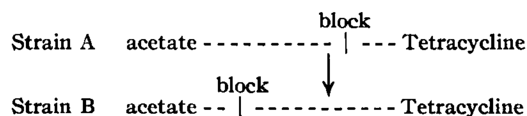
These inferences are quite consistent with the observation that a number of mutant cultures produce 6-demethyltetracycline derivatives (61, 62) and, under certain conditions, normal strains can be induced to form 6-demethyl derivatives by the use of enzymatic-blocking agents such as various sulfonamides, aminopterin, and ethionine (63–66). These appear to operate by inhibiting one-carbon transfer reactions and methionine is usually able to overcome these inhibitions. Ethionine dramatically reduces the antibiotic-producing potential of some *S. aureofaciens* strains. More will be made of this observation later. Under certain conditions, ethionine can substitute for methionine, so ethionine can to a certain extent both repress methionine utilization-formation or substitute for it in biosynthesis in some strains, and ethylmethylaminotetracycline was prepared in this way (67). The practical importance of the 6-demethyltetracyclines lies in their greater acid stability as compared with the normal tetracyclines which contain a tertiary-benzylic hydroxyl at C_6 which is readily lost to form the anhydrotetracyclines (XLII). These later substances are strongly bound to serum proteins and are not clinically useful as antibiotics. The tendency to form anhydrotetracyclines is diminished by removal of the C_6 methyl. A chemically derived product of the 6-demethyltetracyclines, 6-demethyl-6-deoxytetracycline (XXXIII), is the simplest known tetracycline possessing essentially full antibiotic activity and has been used to prepare a variety of new tetracyclines by electrophilic reactions under conditions which normal tetracyclines would not survive (68). 7-Chloro-6-demethyltetracycline (IV) was produced from blocked mutants and is an article of commerce, representing the first economic fruit of these fundamental



studies. In contrast to the C_6 methylation, C_7 chlorination of various pretetramids does take place using chlorinating *S. aureofaciens* strains so this is a later step (53).

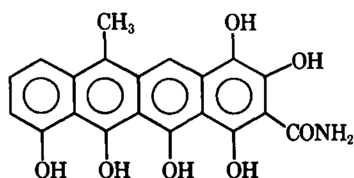
With the presumptive role of these partially and wholly synthetic derivatives established, the isolation of 4-hydroxy-6-methylpretetramid (XXXIV) from a blocked mutant was a very significant advance indeed (69, 70). The methodology involved should be of general utility in similar sequence problems and bears some comment. In contrast to mutations involving pathways in primary metabolism which are relatively easy to detect for they often lead to death or nutritional dependence on media factors, mutations deleting enzymes in secondary metabolism are apparently not usually lethal. The cell can survive quite nicely whether it produces tetracyline or not. To make the detection of productive mutants easier, the phenomenon of cosynthesis was used in a brilliant series of investigations by J. R. D. McCormick and his group at Lederle. This approach has recently been described in detail (11, 71) and can be synopsized figuratively as shown in Scheme VIII.

Cosynthesis depends upon the use of two nutritionally-intact blocked mutants, neither of which is able alone to synthesize active antibiotic. If certain conditions are met, however, growth of the two strains in mixed culture can produce tetracyclines. Thus consider Mutants A and B—one blocked (lacking at least one important enzyme or cofactor for a given step in the sequence) early and the other blocked late in the sequence (Scheme VIII). If a stable intermediate is

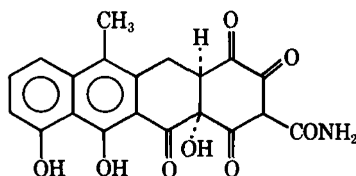


Scheme VIII—A simplified illustration of cosynthesis.

assembled by Mutant A, is excreted into the medium, and is taken up by Mutant B, Mutant B has the necessary enzymes to finish the synthesis. This ingenious method allows one not only to detect the presence of a stable intermediate but also to demonstrate its biological capabilities. Barring coincidence, positive results in



XXXIV



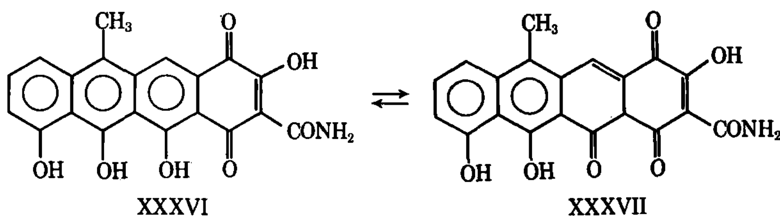
XXXV

this whole cell system are quite meaningful whereas negative results may not be (due to permeability problems, instability, *etc.*). Given enough mutants, the position of the break in the biosynthetic path can be worked out with some certainty as has been done with at least 16 mutant strains (11). The use of these strains obviates the necessity of using radio-labeled precursors as net synthesis is detected by biological activity (antibiosis).

Individually nonproductive strains V655 and and ED1369, in mixed fermentation, produced 7-chlortetracycline (60). Strain ED1369 was also able to form chlortetracycline when various fractions from V655 mash were added. Using this as an assay method, McCormick was able to isolate and characterize the elaborated agent as 4-hydroxy-6-methyl pretetramid (XXXIV) and to show that the pure product was bioconvertable to chlortetracycline in up to 75% yield. These findings strongly support the polyketide-pretetramid hypothesis and suggest that 4-hydroxylation is the next step in the sequence and that chlorination occurs subsequently. Transformation of 6-methylpretetramid to 4-hydroxy-6-methylpretetramid has been accomplished recently by purely chemical means though under fairly drastic conditions (72). As the 4-hydroxypretetramids are now fairly readily prepared by partial synthesis, and are at least one enzymatic stage further down the pathway, once again one can hope that new tetracyclines will result from mixed chemical-biological studies. The experiments reported to date, however, have not achieved this goal (12, 73, 74).

STEPWISE DEAROMATIZATION—THE 4-KETOANHYDROTETRACYCLINES

The next derivatives for which precursor activity has been demonstrated are the 4-ketoanhydrotetracyclines (XXXV). These substances have been prepared synthetically in a variety of ways (75, 76) and preliminary accounts of successful bioconversion have appeared using both whole cell (12) and cell-free systems (13). The details of the path between XXXIV and XXXV are obscure but one can propose, for the sake of argument, oxidation to quinone XXXVI which formally accepts a mole of water stereospecifically or (when represented by its enolic form XXXVII) (Scheme IX), more likely, by analogy with later

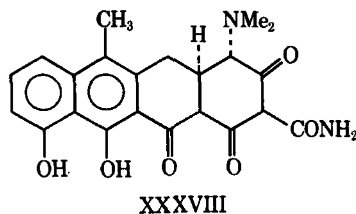


XXXVI XXXVII

Scheme IX

steps, undergoes oxygenation of the β-diketo-system followed by reduction of the 4*a*, 5-double bond. Once again whole cell systems are able to halogenate these substances. Cell-free systems have not exhibited chlorinating ability here, perhaps due to the lack of necessary cofactors or instability of the responsible enzymes (13).

The biological 12*a*-hydroxylation of certain synthetic anhydrotetracycline (XXXVIII) derivatives has been accomplished but not by tetracycline-producing strains, and these reactions

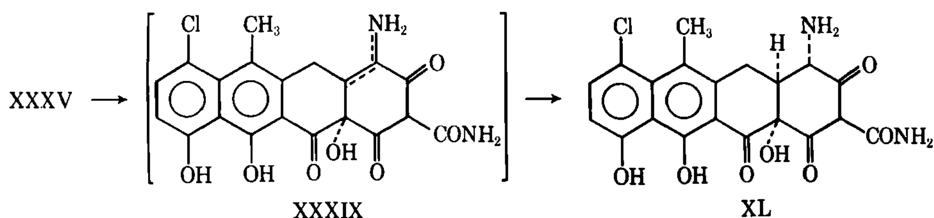


XXXVIII

may be taken, for our purposes, as being outside the normal pathway (77). Chemical hydroxylation of the same type of derivative has also been carried out (78, 79).

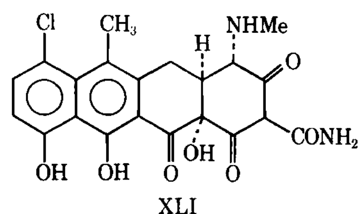
4-AMINOANHYDROTETRACYCLINES

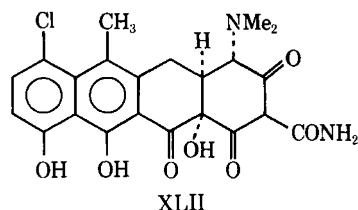
The next piece of evidence bearing on the proposed sequence was provided by experiments involving genetically complete strains. Addition



Scheme X

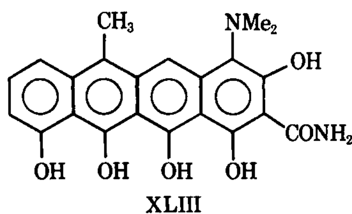
of ethionine at a suitable stage apparently represses methionine synthesis and, in the presence of an insufficient supply of methionine, tetracycline biosynthesis is shut off at an intermediate stage. The presence of precursors could be detected conveniently and quantitatively by assaying cellular extracts in the presence of methionine-$^{14}CH_3$ in a cell-free enzyme preparation made by sonification of suitable *Streptomycete* strains. The preparation of cell-free enzyme systems capable of elaborating secondary metabolites has usually been unsuccessful, thus it was most gratifying to find conditions whereby this could be accomplished with tetracycline-producing strains and, further, to find that they were capable of transforming even fairly early precursors (all the way back to the 4-ketoderivatives) (13). The competence of these soluble enzyme systems is an especially powerful tool because diffusion and cellular permeability are no longer factors of concern. Thus, providing the appropriate cofactors are supplied, the results obtainable take on a different order of reliability when using this sort of system. With this experimental approach it was possible to find conditions whereby treatment of whole cell cultures of *S. aureofaciens* and *S. rimosus* with ethionine resulted in high yields of these blocked intermediates and the products were isolated and characterized (Scheme X). These materials were shown to be 4-aminoanhydrotetracyclines (represented by XL) and, further, it was shown that they were past the point of halogenation. This narrowly restricts the position in the sequence where this step occurs and from this point onward, a parallel biosynthesis is utilized for halo and nonhalo-tetracyclines. Various degrees of methylation are present in these substances providing additional evidence that the three carbons of the tetracyclines which are methionine derived are sensitive to different extents to the action of ethionine. It could be shown by addition of limiting amounts of *S*-adenosylmethionine that the amino derivatives were stepwise alkylated to the monomethylamino (XLI) and then the dimethylaminoanhydrotetracyclines (XLII). The 4-β-epimers are not biologically convertable by these systems indicating something of the steric requirements of the enzymes involved (80). Discovery of the introduction of



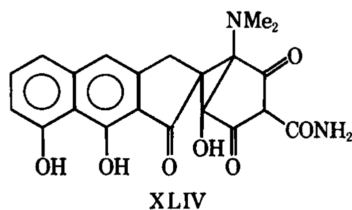


nitrogen at this stage is added circumstantial evidence against glutamate as a direct precursor of substantial portions of Ring A.

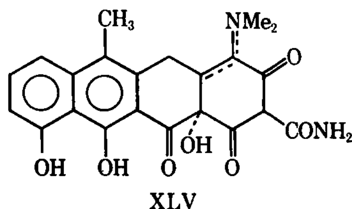
One problem of concern in all sequence work involving mutants or chemical blocking agents is the possibility that some of the substances discovered may have good precursor activity but not be in fact actual intermediates. Such stabilized shunt products or extraneous materials could be converted to mainstream intermediates by enzymes not ordinarily involved in the normal biosynthetic sequence. A wonderfully instructive example of this pitfall comes from work with 4-dimethylaminopretetramids (XLIII) which are available by degradation of tetracyclines (77). These compounds serve as reasonably efficient precursors in whole-cell systems (59). However, in the presence of methionine-$^{14}CH_3$ the dimethylamino group of the finished tetracyclines became radioactive and one is forced to conclude that the cell must remove the dimethylamino function, perhaps producing a 4-hydroxypretetramid or its corresponding quinone, and then reintroduce this function later in its normal time. The alternate possibility, stepwise dealkylation followed by realkylation, seems less likely although must still be allowed as a possibility. This serves as a warning in drawing hasty conclusions.



XLIII



XLIV

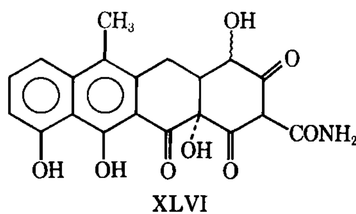


XLV

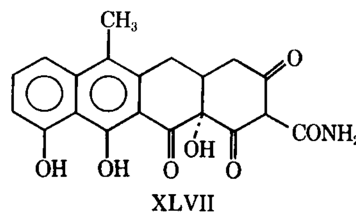
In this context the aminoanhydrotetracyclines, although highly active as precursors, were at first thought by some investigators to be stabilized shunt products (12). Thus at least two alternate sequences may be put forth between the 4-ketoanhydrotetracyclines and the anhydrotetracyclines. The evidence for the published sequence (12) was based, in part, on the structure of metatetrene, which shunt product has been isolated from mutant strain T219 and reported to have novel structure XLIV. This structure is now considered to be erroneous and the intermediacy of XLV is now doubted although not disproven (82, 104).[1] Such evidence as is now available favors pathway XXXV → XXXIX → XL → XLII and this sequence is generally accepted. Thus the C_4 nitrogen appears to be introduced into the sequence by reductive amination at the 4-ketoanhydrotetracycline stage and the products are stepwise methylated to the anhydrotetracyclines. Interestingly, the closely related 4-hydroxy (XLVI) and 4-desoxyanhydrotetracyclines (XLVII) are inert (12).

It has been demonstrated for one group of mutants (Class I) that the rate of chloride utilization for tetracycline biosynthesis is independent of the chloride concentration (26) so the overall rate-limiting (slow) step must be prior to the aminoanhydro stage for halogenation precedes the formation of XL and probably XXXIX.

[1] Subsequent to the submission of this review, an article appeared describing isolation of 4-aminoanhydrodemethylchlortetracycline (the 6-demethyl analog of XL) from a blocked mutant (104). This discovery is strong confirmatory evidence for the sequence related herein.



XLVI



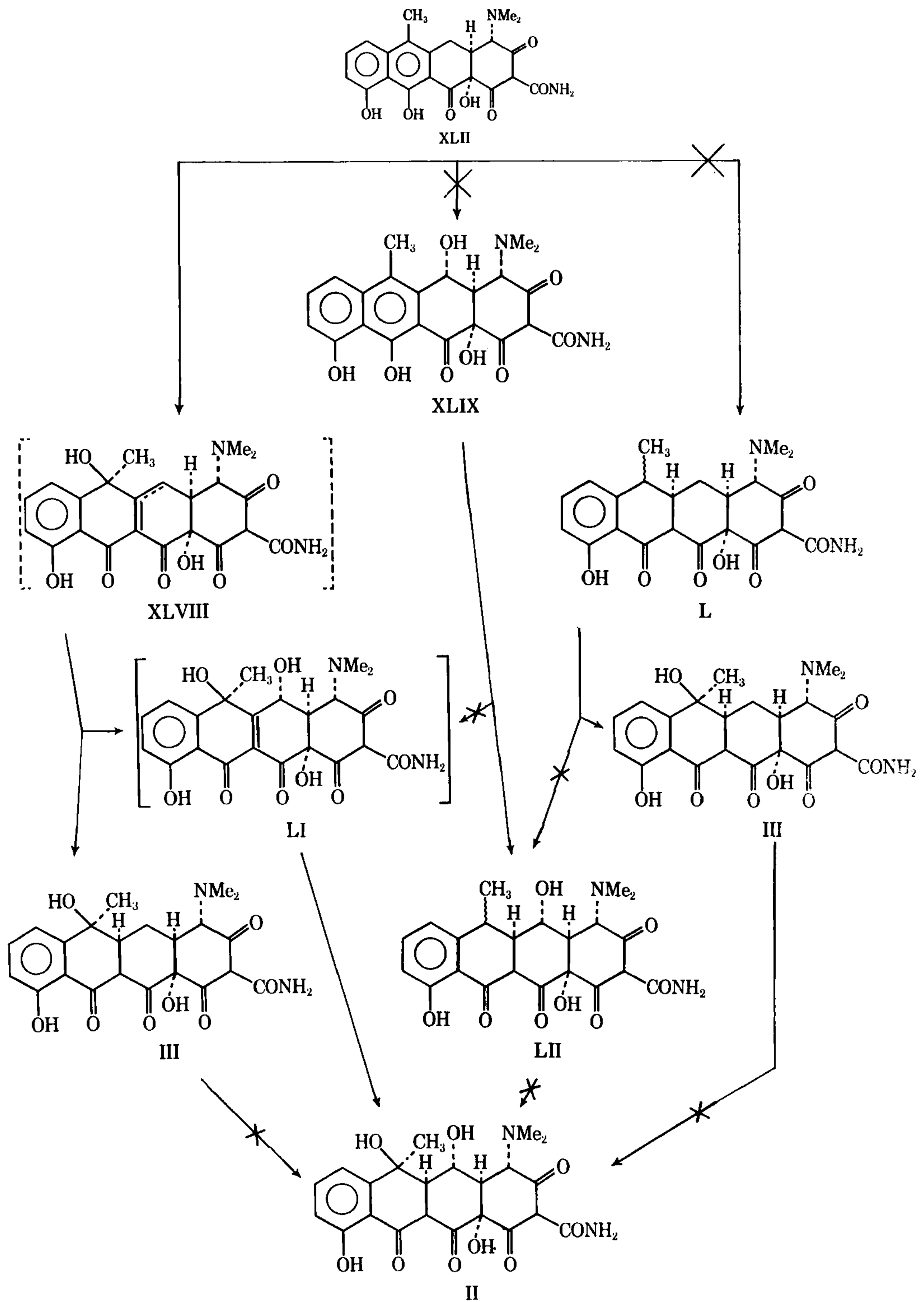
XLVII

ANHYDROTETRACYCLINES

The products of the methylation, the anhydrotetracyclines, are very readily formed by dehydration of tetracyclines carrying an oxygen at C_6 and have been available by partial synthesis for a long time. They are readily transformed to finished tetracyclines by a number of whole-cell and cell-free systems (11, 14), but have not yet been reported as having been isolated from normal, blocked, or mutant cultures. This is surprising for they are stable chemically and their *in vitro* antibiotic properties should make them readily detectable. It has been surmised that they may be toxic to the producing organism so that inability to carry out the next step may cause them to accumulate to a toxic level and such mutations might then become self-limiting (12).

TERMINAL REACTIONS

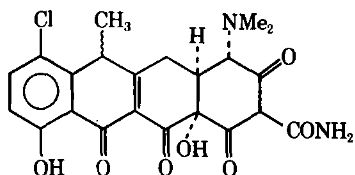
The remaining reactions in the sequence are clear. Because the number of possible intermediates in the sequence between anhydrotetracycline (XXXIII) and oxytetracycline (II) are limited, the method of approach used was to obtain as many as possible of the plausible intermediates and test them one after another in a cell-free system (83). The results are given in Scheme XI. Those reactions which could not be demonstrated in cell-free enzyme preparations have been blocked with an X. Note that there is only one contiguous sequence between anhydrotetracycline (XLII) and oxytetracycline (II). Compound XLVIII is encased in dotted brackets as it has been prepared in microgram quantities only. Compound LI is encased in solid brackets as it has only been detected on paper chromatograms and has not been isolated as a single chemical entity. The other substances have been fully characterized.



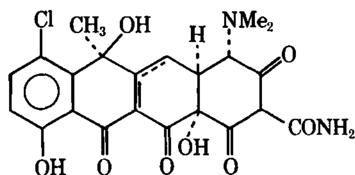
Scheme XI—Terminal reactions in tetracycline biosynthesis.

Certain strains utilize air as an oxidizing agent and transform anhydrotetracycline (XLII) to 5a(11a)-dehydrotetracycline (XLVIII). The 7-chloro analog of XLVIII (LV) was one of the first tetracycline intermediates to be isolated from a blocked mutant and to be converted biologically to a finished antibiotic (71, 83–85). It is interesting to note that the latter transforma-

tion has also been achieved in the laboratory by photochemical oxygenation of anhydrotetracyclines (86, 87) but also that no evidence is available for a free hydroperoxide intermediate in cell-free studies (13). The anhydrotetracyclines can be drawn in their ketonic tautomeric state (LIV) in which the C_6 hydrogen is both benzylic and allylic and should be very susceptible to free-radical oxygenation processes. Interestingly, the *in vitro* photooxygenation appears to fail unless a C_7 chlorine atom is present (86, 87). A biological feature of interest here is that *S. rimosus* strains are not able to carry out a C_6 oxygenation reaction on anhydrotetracyclines bearing a C_7 chlorine (84). This presents an interesting contrast in structural requirements between the biological and photochemical process. As the aminoanhydrotetracyclines do not serve as substrates for the C_6 oxygenating system in either *S. aureofaciens* or *S. rimosus* cell-free systems, it is easy to see why they accumulate under ethionine blockade and to explain the otherwise puzzling observation that no amino or methylaminotetracyclines have been isolated from biological systems, particularly those leading to 6-demethylchlortetracycline under ethionine blockade (13).



LIV



LV

5*a*(11*a*)-Dehydrotetracycline has proven to be too unstable for successful isolation in more than microgram amounts; however, sufficient material was isolated by elution from paper chromatograms that its biological role could be evaluated in whole-cell systems. This substance is a key intermediate, serving as the branch point at which the biosynthesis of oxytetracycline (II) and tetracycline (III) diverge. Once again a point is reached where parallel pathways develop after the introduction of one of the "extra" features of the tetracyclines. As had been predicted (84, 88), reduction with NaDPH under aerobic conditions with *S. aureofaciens* enzymes leads to tetracycline whereas 5-oxygenation under aerobic conditions (with *S. rimosus* enzymes) leads to oxytetracycline.[2] When 5*a*(11*a*)-dehydrochlortetracycline is used, biological reduction produces chlortetracycline. It is possible to convert 6-deoxytetracycline to tetracycline in cell-free systems, but the anticedent reaction, conversion of anhydrotetracycline to 6-deoxytetracycline, has not been observed despite careful attempts. If this failure is fundamental rather than tactical, then the oxygenation of L is an incidental reaction and does not represent a valid *in vivo* pathway. Thus all the available evidence at present supports paths XLII → XLVIII → LI → II and XLII → XLVIII → III for the terminal sequences.

It is interesting to note that cultures producing mixtures of either oxytetracycline and tetracycline or chlortetracycline and tetracycline are not uncommon. However, no reports have appeared of cultures producing mixtures of chlortetracycline and oxytetracycline. This interesting fact bore on a problem of longstanding interest, namely: why was oxychlortetracycline (V) not known in nature, and how could this "hybrid" be prepared? It is possible to hypothesize that each sequence of reactions leading to the formation of a secondary metabolite has as its ultimate aim the elaboration of a "target substance" (89). This material may be considered to be the most highly elaborated (in terms of the extra functions added to the basic polyketide chain) metabolite that the culture was able to produce in its evolutionary history. In these terms oxychlortetracycline (V) might serve as the target substance that all tetracycline-producing *Streptomycetes* once made. In the course of time, mutations, in the light of this theory, conceivably caused the deletion of one or more enzymes and all strains eventually lost the ability to produce this material. This is entertaining, though purely speculative, but it heightens interest in the recent successful preparation of oxychlortetracycline through use of biosynthetic theory and methodology (90–92).

Knowing that *S. rimosus* was unable to handle anhydrochlortetracycline (84), and having the terminal sequence for tetracycline and oxytetracycline in hand, examination of the chlorinated analogs of XLVIII and LI in *S. rimosus* systems seemed called for in the hope that the nonconversion of anhydrochlortetracycline was due to the C_7-chlorofunction being inhibitory for the

[2] J. R. D. McCormick, (87) *Reference 12*, p. 82, reports that 5*a*(11*a*)-dehydrotetracycline undergoes a facile allylic rearrangement to 5*β*-hydroxyanhydrooxytetracycline. Analogous reactions in a similar series have been reported (88).

early enzymes and that subsequent enzymes would be less specific in their structural requirements.

It was indeed found to be possible to transform $5a(11a)$-dehydrochlortetracycline (LV) to oxychlortetracycline (V) in excellent yield using either cell-free or whole-cell *S. rimosus* preparations. It is apparent that *S. rimosus* lacks the enzymes or cofactors necessary for halogenation of tetracycline precursors and, even if it did not, it would be unable to carry the precursor past the anhydrochlortetracycline stage, for the 7-halogen apparently inhibits the C_6-oxygenase of *S. rimosus*. This, then, explains on an enzymatic level why oxychlortetracycline remained so elusive for so long. It also suggests that natural strains producing LV may yet be found and, indeed, the pronounced instability of LV may be a partial explanation for this lack (in addition to the enzymatic factors discussed above).

SUMMARY

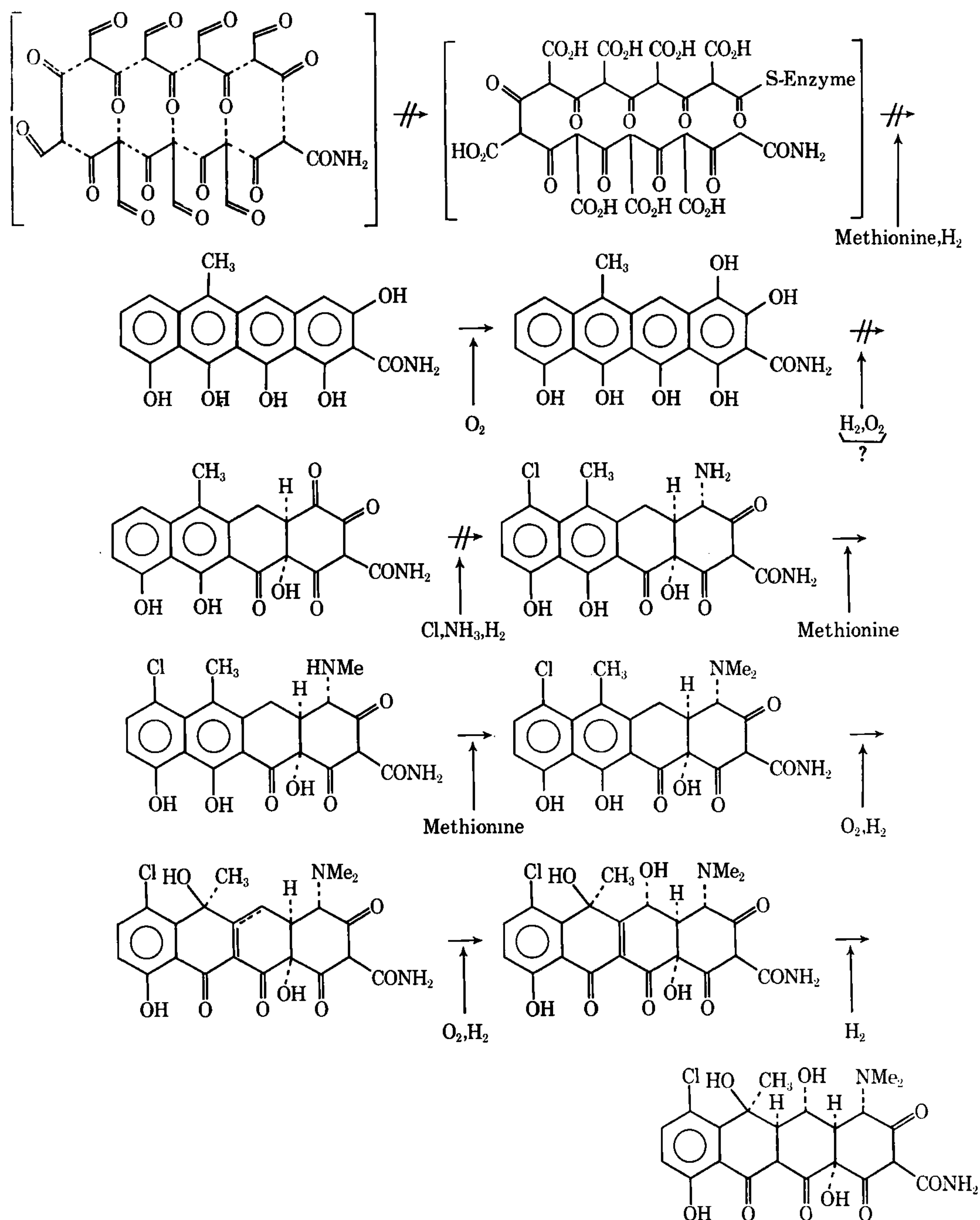
Our current understanding of the biosynthetic scheme by which the tetracycline antibiotics are synthesized in nature is set forth in Scheme XII. Oxychlortetracycline is the most highly elaborated tetracycline presently known, embodying the structural elements of chlortetracycline, tetracycline, and oxytetracycline, so it was chosen to exemplify the sequence. Lack of enzymes or cofactors causes the deletion of one or more steps, several of which serve as branch points. Omission of halogen, for example, leads to oxytetracycline and omission of the final hydroxylation step leads to chlortetracycline, and so on.

FUTURE GOALS

It is possible, indeed one of the hopes of this review, that successful preparation of new antibiotics making use of biosynthetic theory either to suggest new structures or new means of preparation, as in several cases mentioned herein, will stimulate an increasing number of other workers to take a similar approach. This is particularly important at a time when empirical screening methods for new antibiotics have clearly reached the point of diminishing returns considering the effort expended. Even in fields as thoroughly studied as the tetracyclines there are goals left to achieve. By no means the only remaining lack, but illustrative of the point, is the preparation of the still missing 8-hydroxytetracyclines. Polyketide theory suggests that they should exist, and experience with oxychlortetracycline suggests that enzymatic factors may in part be at fault. Miller (13) has discussed the theoretical parameters involved in preparing 6-demethyloxytetracycline. There appears at present to be no theoretical reason why this compound should not be prepared by biochemical manipulation of a suitable *S. rimosus* system. On the other hand the reaction sequences in Scheme XI suggest that a 6-deoxytetracycline antibiotic would be unlikely as a natural fermentation product (13), just as V was not a natural product in a strict sense. Very little work has been done on the enzymes themselves involved in secondary metabolism and many fascinating questions remain to be solved here.

Certainly the conclusion is warranted that a great deal of sequential information, even though some of the pieces are still largely circumstantial, is now available dealing with the intimate steps by which various *Streptomyces* species elaborate the tetracycline antibiotics starting with very simple primary metabolites. It is also apparent that there are no real surprises in terms of the types of reactions involved. The major unique characteristic of secondary metabolism is the substrates themselves. It is fascinating to consider that, using a relatively limited number of reactions over and over again in various sequences, microbes are capable of elaborating such a multitude of compounds. The complexity of these substances, including the tetracyclines, has taxed the ingenuity and been the joy of structural, synthetic, and biological chemists for many years. Despite their central role in mankind's attack against disease, it is curious that there is little real evidence that secondary metabolites are of much importance to the organism itself. Darwinian arguments can be advanced in the case of the tetracyclines and other antibiotics but for the vast majority of secondary metabolites such arguments are not supportable. Either we have not yet detected a function for the majority of these materials or they are themselves metabolic "flotsam."

Still, it is possible to rationalize the value of secondary metabolism to the organism (bearing in mind that a more direct, but currently obscure, primary function may someday be discovered). The important factor may not be the compounds themselves but rather the process whereby they are formed. Secondary metabolism is often considered to begin when some essential media constituent has become exhausted thus subjecting the culture to metabolic stress. The organism has the choice of death or adaptation to the new circumstances of its environment. A whole sequence of enzymes may be induced (89) pro-



Scheme XII—A summary of the biosynthetic pathway of the tetracycline antibiotics as currently understood. These reactions which appear to involve more than one step and intermediates which have not yet been well characterized are signified by hatched arrows (-/+>).

ducing substances not normally present during primary metabolism. It is alternatively possible that shutting off the normal reactions of primary metabolism may permit the diversion of newly excess raw materials into pathways which are present in primary metabolism but not operating except to a minor extent. It is also possible that a few bridging enzymes are induced by the sudden piling up of a metabolite normally rapidly turning over and not present in large amount. This would lead down new paths of metabolism involving enzymes which normally have some other catalytic function, or perhaps only a structural function. The answer as to which if any of these possible interpretations is correct must await the next logical phase of inquiry—study of

the enzymes themselves; a few pioneering papers have appeared in this area (93–102). This would bear on the question of whether the function of secondary metabolism in the microorganism is to maintain intact for as long as possible the largest number of enzymes and cofactors. This would have survival value by permitting continued operation, even at the expense of a great deal of cellular energy, on the chance that environmental conditions would change and allow the cells to resume primary metabolism (103). The future promises to provide answers to this and many other intriguing questions raised by the phenomenon of secondary metabolism.

REFERENCES

(1) "Biogenesis of Natural Compounds," Bernfeld, P., Ed., Pergamon Press, Oxford, England, 1963.
(2) Richards, J. H., and Hendrickson, J. B., "Biosynthesis of Steroids, Terpenes, and Acetogenins," W. A. Benjamin, New York, N. Y., 1964.
(3) Robinson, R., "Structural Relations of Natural Products," Clarendon Press, Oxford, England, 1955.
(4) Hendrickson, J. B., "The Molecules of Nature," W. A. Benjamin, New York, N. Y., 1965.
(5) Bu'Lock, J. D., "The Biosynthesis of Natural Products," McGraw-Hill, New York, N. Y., 1965.
(6) Barton, D. H. R., *Chem. Brit.*, **3**, 330(1967).
(7) Kirby, G. W., *Science*, **155**, 170(1967).
(8) Clayton, R. B., *Quart. Rev.*, **19**, 168, 201(1965).
(9) Lynen, F., *Angew. Chem.*, **77**, 929(1965).
(10) Bloch, K., *ibid.*, **77**, 944(1965).
(11) McCormick, J. R. D., in "Antibiotics, Advances in Research, Production, and Clinical Use," Herold, M., and Gabriel, Z., Eds., Butterworths, London, England, 1966, pp. 556–565.
(12) McCormick, J. R. D., in "Biogenesis of Antibiotic Substances," Vanek, Z., and Hostalek, Z., Eds., Academic Press, New York, N. Y., 1965, chap. 8.
(13) Miller, P. A., in "Developments in Industrial Microbiology," Vol. 8, American Institute of Biological Sciences, Washington, D. C., 1967, chap. 11.
(14) Turley, R. H., and Snell, J. F., in "Biosynthesis of Antibiotics," Vol. I, Snell, J. F., Ed., Academic Press, New York, N. Y., 1966, chap. 3.
(15) Muxfeldt, H., and Bangert, R., *Fortschr. Chem. Org. Naturstoffe*, **21**, 80(1963).
(16) Barrett, G. C., *J. Pharm. Sci.*, **52**, 309(1963).
(17) Birch, A. J., *Fortschr. Chem. Org. Naturstoffe*, **14**, 186(1957).
(18) Woodward, R. B., *Angew. Chem.*, **68**, 13(1956).
(19) Snell, J. F., Wagner, R., and Hochstein, F. A., *Proc. Intern. Conf. Peaceful Uses At. Energy*, **12**, 431(1955).
(20) Snell, J. F., *Atompraxis*, **7**, 460(1961).
(21) Miller, P. A., McCormick, J. R. D., and Doerschuk, A. P., *Science*, **123**, 1030(1956).
(22) Snell, J. F., Birch, A. J., and Thompson, P. J., *J. Am. Chem. Soc.*, **82**, 2402(1960).
(23) Birch, A. J., Snell, J. F., and Thompson, P. J., *J. Chem. Soc.*, **1962**, 425.
(24) Gatenbeck, S., *Biochem. Biophys. Res. Commun.*, **6**, 422(1962).
(25) Miller, P. A., Saturnelli, A., Martin, J. H., Mitscher, L. A., and Bohonos, N., *ibid.*, **16**, 285(1964).
(26) Doerschuk, A. P., McCormick, J. R. D., Goodman, J. J., Szumski, S. A., Growich, J. A., Miller, P. A., Bitler, B. A., Jensen, E. R., Matrishin, M., Petty, M. A., and Phelps, A. S., *J. Am. Chem. Soc.*, **81**, 3069(1959).
(27) Doerschuk, A. P., McCormick, J. R. D., Goodman, J. J., Szumski, S. A., Growich, J. A., Miller, P. A., Bitler, B. A., Jensen, E. R., Petty, M. A., and Phelps, A. S., *ibid.*, **78**, 1508(1956).
(28) Gourevitch, A., Misick, M., and Lein, J., *Antibiot. Chemotherapy*, **5**, 448(1955).
(29) Kollár, J., and Járai, M., *Acta Microbiol. Acad. Sci. Hung.*, **9**, 145(1962).
(30) Goodman, J. J., Matrishin, M., Young, R. W., and McCormick, J. R. D., *J. Microbiol.*, **78**, 492(1959).
(31) Sensi, P., *Il. Farmaco Sci. Ed.*, **10**, 346(1955).
(32) Hendrickson, J. B., *op. cit.* p. 22.
(33) Birch, A. J., *Proc. Chem. Soc.*, **1962**, 3.
(34) Birch, A. J., *Science*, **156**, 202(1967).
(35) Bu'Lock, J. D., "Essays in Biosynthesis and Microbial Development," Wiley, New York, N. Y., 1967.
(36) Money, T., Qureshi, I. H., Webster, G. B., and Scott, A. I., *J. Am. Chem. Soc.*, **87**, 3004(1965).
(37) Money, T., Douglas, J. L., and Scott, A. I., *ibid.*, **88**, 624(1966).
(38) Comer, F. W., Money, T., and Scott, A. I., *Chem. Commun.*, **1967**, 231.
(39) Harris, T. M., and Carney, R. L., *ibid.*, **88**, 2053 (1966); *J. Am. Chem. Soc.*, **89**, 6734 (1967).
(40) Combie, L., Games, E. D. E., and Knight, M. H., *Chem. Commun.*, **1966**, 355.
(41) James, A. W. G., *ibid.*, **1966**, 357.
(42) Brodie, J. D., Wasson, G., and Porter, J. W., *J. Biol. Chem.*, **239**, 1346(1964).
(43) Brock, D. J. H., and Bloch, E. K., *Biochem. Biophys. Res. Commun.*, **23**, 775(1966).
(44) Brenneisen, P. E., Acker, T. E., and Tanenbaum, S. W., *J. Am. Chem. Soc.*, **86**, 1264(1964).
(45) Acker, T. E., Brenneisen, P. E., and Tanenbaum, S. W., *ibid.*, **88**, 834(1966).
(46) Harris, T. M., Harris, C. M., and Light, R. J., *Biochim. Biophys. Acta*, **120**, 420(1966).
(47) Bentley, R., Ghaperhy, J. S., and Keil, J. G., *Arch. Biochem. Biophys.*, **111**, 80(1965).
(48) Bentley, R., and Zwitkowits, P. M., *J. Am. Chem. Soc.*, **89**, 676, 681 (1967).
(49) Wakil, S. J., *ibid.*, **80**, 6465(1958).
(50) Lynen, F., and Tada, M., *Angew. Chem.*, **73**, 513 (1961).
(51) Mosbach, K., *Naturwissenschaften*, **48**, 525(1961).
(52) Ollis, W. D., Sutherland, I. O., Codner, R. C., Gordon, J. J., and Miller, G. A., *Proc. Chem. Soc.*, **1960**, 347.
(53) Mosbach, K., *Acta Chem. Scand.*, **18**, 1591(1964).
(54) Grisebach, H., "Biosynthetic Patterns in Microorganisms and Higher Plants," Wiley, New York, N. Y., 1967, chap. 2.
(55) *Reference 12*, p. 74.
(56) Hochstein, F. A., Schach von Wittenau, M., Tanner, F. W., and Murai, K., *J. Am. Chem. Soc.*, **82**, 5934(1960).
(57) Miller, M. W., and Hochstein, F. A., *J. Org. Chem.*, **27**, 2525(1962).
(58) Lancini, G. C., and Sensi, P., *Experientia*, **20**, 83 (1964).
(59) McCormick, J. R. D., Johnson, S., and Sjolander, N. O., *J. Am. Chem. Soc.*, **85**, 1692(1963).
(60) McCormick, J. R. D., Reichenthal, J., Johnson, S., and Sjolander, N. O., *ibid.*, **85**, 1694(1963).
(61) McCormick, J. R. D., Sjolander, N. O., Hirsch, U., Jensen, E. R., and Doerschuk, A. P., *ibid.*, **79**, 4561(1957).
(62) Hendlin, D., Dulaney, E. L., Drescher, D., Cook, F., and Chaiet, L., *Biochim. Biophys. Acta*, **58**, 635(1962).
(63) Goodman, J. J., and Miller, P. A., *Biotechnol. Bioeng.*, **4**, 391(1962).
(64) Perlman, D., Henser, L. J., Semar, J. B., Frazier, W. R., and Boska, J. S., *J. Am. Chem. Soc.*, **83**, 4481(1961).
(65) Neidleman, S. L., Albu, E., and Bienstock, E., *Biotechnol. Bioeng.*, **5**, 87(1963).
(66) Neidleman, S. L., Bienstock, E., and Bennett, R. E., *Biochim. Biophys. Acta*, **71**, 199(1963).
(67) Neidleman, S. L., Weisenborn, F. L., and Bouchard, J., *Biotechnol. Bioeng.*, **5**, 83(1963).
(68) Martell, M. J., Jr., Ross, A. S., and Boothe, J. H., *J. Med. Chem.*, **10**, 359(1966).
(69) McCormick, J. R. D., Joachim, U. H., Jensen, E. R., Johnson, S., and Sjolander, N. O., *J. Am. Chem. Soc.*, **87**, 1793(1965).
(70) McCormick, J. R. D., and Jensen, E. R., *ibid.*, **87**, 1794(1965).
(71) McCormick, J. R. D., Hirsch, U., Sjolander, N. O., and Doerschuk, A. P., *ibid.*, **82**, 5006(1960).
(72) Hassall, C. H., and Winters, T. E., *Chem. Commun.*, **1967**, 77; Baldwin, J. E., Barton, D. H. R., Bould, L., and Magnus, P. D., *ibid.*, **1967**, 319.
(73) Hlavka, J. J., Bitha, P., and Boothe, J. H., *J. Am. Chem. Soc.*, **87**, 1795(1965).
(74) Hlavka, J. J., Bitha, P., and Boothe, J. H., *Tetrahedron Letters*, **1967**, 1139.
(75) Blackwood, R. K., and Stephens, C. R., *Can. J. Chem.*, **43**, 1382(1965).
(76) Blackwood, R. K., and Stephens, C. R., *J. Am. Chem. Soc.*, **86**, 2736(1964).
(77) Holmlund, C. E., Andres, W. W., and Shay, A. J., *ibid.*, **81**, 4748, 4750(1959).
(78) Muxfeldt, H., and Kuretzer, A., *Naturwissenschaften*, **46**, 204(1959).
(79) Muxfeldt, H., Buhr, G., and Bangert, L., *Angew. Chem. Intern. Ed.*, **1**, 157(1962).
(80) Barbatschi, F., Dann, M., Martin, J. H., Miller, P. A., Mitscher, L. A., and Bohonos, N., *Experientia*, **21**, 162(1965).
(81) Blackwood, R. K., Renhard, H. H., and Stephens, C. R., *J. Am. Chem. Soc.*, **82**, 745(1960).
(82) McCormick, J. R. D., lecture before the 4th International Congress of Chemotherapy, Washington, D. C., October 20, 1965. Also see *Reference 13*, p. 103.
(83) Miller, P. A., Hash, J. H., Lincks, M., and Bohonos, N., *Biochem. Biophys. Res. Commun.*, **18**, 325(1965).
(84) McCormick, J. R. D., Miller, P. A., Johnson, S., Arnold, N. H., and Sjolander, N. O., *J. Am. Chem. Soc.*, **84**, 3023(1962).
(85) McCormick, J. R. D., Sjolander, N. O., Miller, P. A., Hirsch, U., Arnold, N. H., and Doerschuk, A. P., *ibid.*, **80**, 6460(1958).
(86) Scott, A. I., and Bedford, C. T., *ibid.*, **84**, 2271(1962).
(87) Schach von Wittenau, M., *J. Org. Chem.*, **29**, 2746 (1964).
(88) Schach von Wittenau, M., Hochstein, F. A., and Stephens, C. R., *J. Org. Chem.*, **28**, 2454 (1963).
(89) Bu'Lock, J. D., and Powell, A. J., *Experientia*, **21**, 55(1965).

(90) Mitscher, L. A., Martin, J. H., Miller, P. A., Shu, P., and Bohonos, N., *J. Am. Chem. Soc.*, **88**, 3647(1966).
(91) Martin, J. H., Mitscher, L. A., Miller, P. A., Shu, P., and Bohonos, N., *Antimicrobial Agents Chemotherapy*, **1966**, 563.
(92) Mitscher, L. A., Martin, J. H., Dornbush, A., Leeson, L., and Redin, G., *ibid.*, **1966**, 568.
(93) Pettersson, G., *Acta Chem. Scand.*, **20**, 657(1966).
(94) Shaw, P. D., and Hager, L. P., *J. Biol. Chem.*, **236**, 1626(1961).
(95) *Ibid.*, **234**, 2565(1959).
(96) Shaw, P. D. and Hager, L. P., *J. Am. Chem. Soc.*, **81**, 6527(1959).
(97) Sjöland, S., and Gattenbeck, S., *Acta Chem. Scand.*, **20**, 1053(1966).
(98) Light, R. J., *J. Biol. Chem.*, **242**, 1880(1967).
(99) Otani, S., Yamanoi, Y., Saito, Y., and Otani, S., *Biochem. Biophys. Res. Commun.*, **25**, 590(1966).
(100) Dennis, D. T., and West, C. A., *J. Biol. Chem.*, **242**, 3293(1967).
(101) Upper, C. D., and West, C. A., *ibid.*, **242**, 3285(1967).
(102) Shaw, P. D., *Biochem.*, **6**, 2253(1967).
(103) Bu'Lock, J. D., *Advances Appl. Microbiol.*, **3**, 293 (1961).
(104) McCormick, J. R. D., Jensen, E. R., Johnson, S., and Sjolander, N. O., *J. Am. Chem. Soc.*, **90**, 2201(1968).

Keyphrases

Tetracycline antibiotics—biosynthesis review
Biosynthetic processes—tetracyclines
Polyketide theory—tetracycline biosynthesis
Structure, basic—tetracyclines
Pretetramids—tetracycline precursors
4-Ketoanhydrotetracyclines—dearomatization
4-Aminoanhydrotetracyclines—tetracycline intermediates
Oxidation, tetracyclines—final biosynthetic step

Research Articles

Nuclear *In Vitro* Method of Continuously Evaluating Release Rates of Solid Dosage Forms

By WILLIAM J. McCLINTOCK*, DANE O. KILDSIG, WAYNE V. KESSLER, and GILBERT S. BANKER

A nuclear *in vitro* continuous release rate measuring method has been developed and evaluated which permits determination of the release rates of ^{14}C-labeled materials from solid dosage forms. The nuclear apparatus consisted of a scintillation flow cell and a liquid scintillation spectrometer which recorded count rates automatically. The nuclear method was compared with a spectral method in measuring the release rates of ^{14}C-labeled caffeine and carrier caffeine from three different tablet systems. The two methods were equally precise and there was no statistical difference in the release rates determined by the two methods of analysis for any of the tablet systems studied. The nuclear method may be more convenient and less sensitive to the presence of interfering substances than a spectral method. The nuclear method can be employed as a continuous and automated methodology to reflect the effect of formulation or physical changes on the release rate of dosage forms.

Concern with maximum drug availability and effectiveness has resulted in the establishment of various dissolution tests as *in vitro* indicators of availability. Often the comparison of the apparatus used in one laboratory and that used in another yields significant differences in the results of dissolution tests on the same dosage forms and drugs. The rotating-bottle method (1–3), the modified USP disintegration testing basket attached to a Gershberg-Stoll apparatus (4, 5), and the rotating disk (6–8), involve the periodic removal of samples followed by some form of quantitative analysis. Automated methods of evaluating *in vitro* release rates have been developed which employ continuous spectrophotometric analysis (9, 10). However, the use of radionuclides as labels on drug molecules affords a direct method of determining dissolution rates regardless of the drug's spectral characteristics.

Several investigators have substituted radionuclide detection for chemical analysis in the mea-

Received May 22, 1968, from the Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Science, Purdue University, Lafayette, IN 47907
Accepted for publication July 12, 1968.
This investigation was supported by fellowship 1-F1-GM-21,146 from the Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
* Present address: Vick Divisions Research and Development, Mount Vernon, N. Y.