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THE STEREOCHEMICAL FATE OF CHIRAL-METHYL VALINES IN CEPHALOSPORIN C BIOSYNTHESIS[1]

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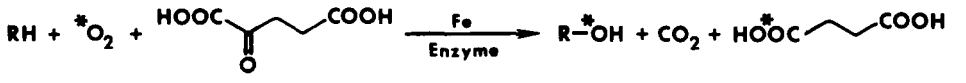
ABSTRACT.—Samples of D,L-valine bearing chiral-methyl groups in the 3-*pro(R)* and 3-*pro(S)* positions have been prepared to investigate the stereochemical course of the α-ketoglutarate-dependent dioxygenase-catalyzed ring expansion of pencillin N to deacetoxycephalosporin C and the 3′-hydroxylation of the latter to deacetylcephalosporin C, respectively. The orientation of tritium at the diastereotopic C-2 methylene positions of cephalosporin C has been determined in a kinetic assay involving its conversion to *N-t*-butoxycarbonyldeacetylcephalosporin C-1β-oxide and monitoring the loss of tritium at constant pH, ionic strength, and temperature. Control experiments are described that demonstrate the validity of treating tritium losses from the methylene as parallel pseudo-first-order processes. An equal distribution to tritium between the two C-2 loci was observed accompanied by a large intrinsic isotope effect. It was concluded that the ring expansion takes place with complete epimerization. Parallel stereochemical examination of the cephem 3′-hydroxylation was carried out by oxidative degradation of cephalosporin C to obtain samples of acetylglycolate. After saponification to glycolate, these specimens were assayed with glycolate oxidase to reveal the overall stereochemical course of hydroxylation as retention.

The origin of the penicillin and cephalosporin antibiotics is a classic problem in natural product biosynthesis. In recent years, great experimental effort has been brought to bear on the remarkable double oxidative closure that carries the Arnstein tripeptide (**2**) to isopenicillin N (**3**) (1). This reaction involves the loss of four hydrogen equivalents but requires only one molecule of dioxygen (2). This stoichiometry, in which presumably both atoms of oxygen are reduced to water, is a singularly important feature of this conversion. No intermediates have been isolated in the cyclization process (3), but kinetic studies indicate that β-lactam formation precedes generation of the thiazolidine ring (4). The enzyme responsible, isopenicillin N synthetase, MW ca. 38,000, has recently been purified to homogeneity and requires ferrous ions for activity (5). Prior to further modification of the penam nucleus, a small stereochemical adjustment occurs in the normal course of reaction in Cephalosporia (6) and streptomycetes (7) whereby the adipyl side chain is epimerized to form penicillin N (**4**). The next two transformations in the pathway are chemically quite distinct, the oxidative ring expansion of **4** to deacetoxycephalosporin C (**5**) and 3′-hydroxylation of the latter to deacetylcephalosporin C (**6**). Evidence from cell-free studies suggests strongly, however, that both reactions are carried out by α-ketoglutarate-dependent dioxygenases (8,9). The generalized course for hydroxylations of this type is illustrated in Scheme 1 where enzymes of this class activate oxygen in the decomposition of α-ketoglutarate to form carbon dioxide



SCHEME 1

and succinate. The latter bears one of the atoms of molecular oxygen (*) and the other appears in the oxidized substrate (*). It would seem (9) that the cephem 3′-hydroxylation of **5** to **6** fits the pattern illustrated in Scheme 1 while the relation to the penam/

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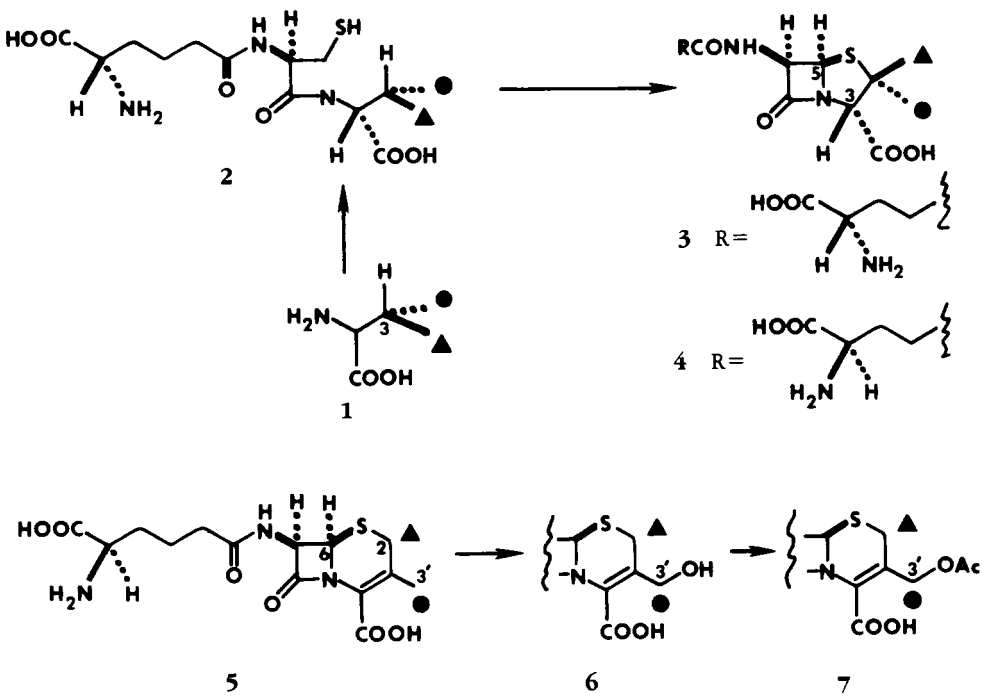
[2]This lecture is dedicated to my father in his 75th year.

cephem ring expansion of **4** to **5** is less obvious. Clearly, the formation of isopenicillin N (**3**) from tripeptide **2** is an altogether different, possibly unique, transformation. This lecture will describe experiments designed to map the stereochemical fate of valine (**1**) having chiral-methyl groups specifically at the 3-*pro(R)* (▲) and 3-*pro(S)* (●) positions into cephalosporin C (**7**) to investigate the oxidative ring expansion of pencillin N (**4**) to deacetoxycephalosporin C (**5**) and its 3′-hydroxylation to deacetylcephalosporin C (**6**).

PENAM/CEPHEM RING EXPANSION.—Cephalosporin C (**7**) is derived in vivo from simple acylation of deacetyl cephalosporin C (**6**) by acetylCoA (10). It is the major cephalosporin product of the organism used in the present studies, *Acremonium strictum* (ATCC 36225), and co-occurs with lesser amounts of **5** and **6**. At the outset of our work, it was known that the 3-*pro(R)* methyl group (▲) of valine specifically labeled the β-methyl of penicillin and the C-2 methylene of cephalosporin while the 3-*pro(S)* methyl (●) correspondingly labeled the α-methyl group of penicillins and the 3′-acetoxymethylene of cephalosporin C (**7**) (11). It was subsequently shown concurrent with the present work that, while the valine α-position is believed to undergo inversion in the course of formation of the Arnstein tripeptide (**2**), the stereochemical identity of the β-carbon remains unchanged and, hence, the C-S bond formation implicit in isopenicillin N (**3**) takes place with overall retention of configuration (12).

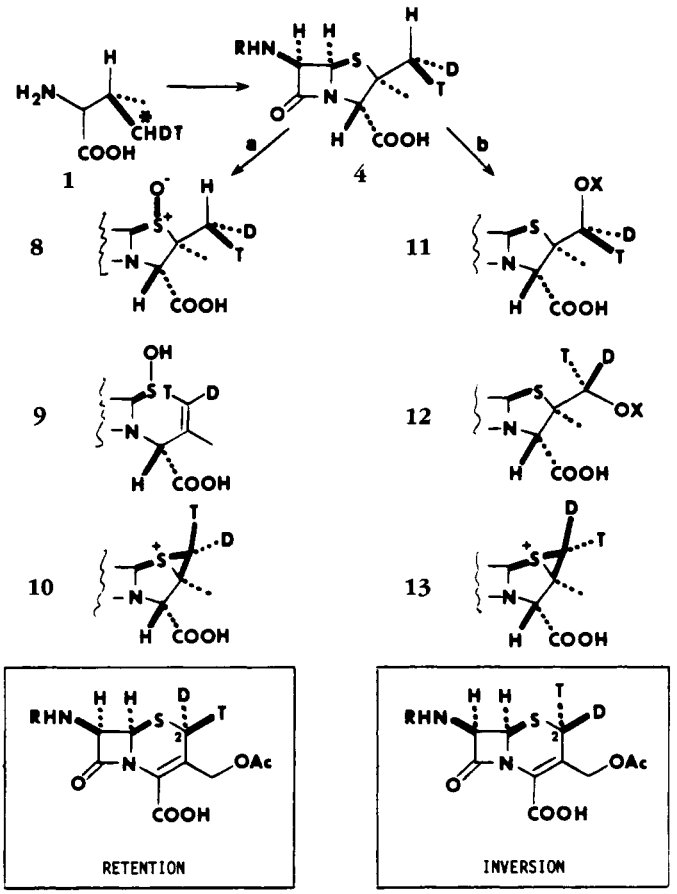
Our initial consideration of the possible stereochemical course of the penam/cephem ring expansion turned on analogies to precedented in vitro chemistry. Depicted in Scheme 2 are the most important stereochemical details of this analysis for an (*R*)-methyl group (a) through the well-studied thermal rearrangement reaction discovered by Morin (13). Sigmatropic reorganization of the pencillin sulfoxide (**8**) would be expected (14) to proceed with a normal isotope effect. For simplicity, only removal of protium from the chiral-methyl group is shown to give the reactive sulfenic acid (**9**). Nucleophilic attack of the *Z*-olefin of **9** from inspection of models would be anticipated to take place as shown to generate the thiiranium species (**10**). Deprotonation and open-



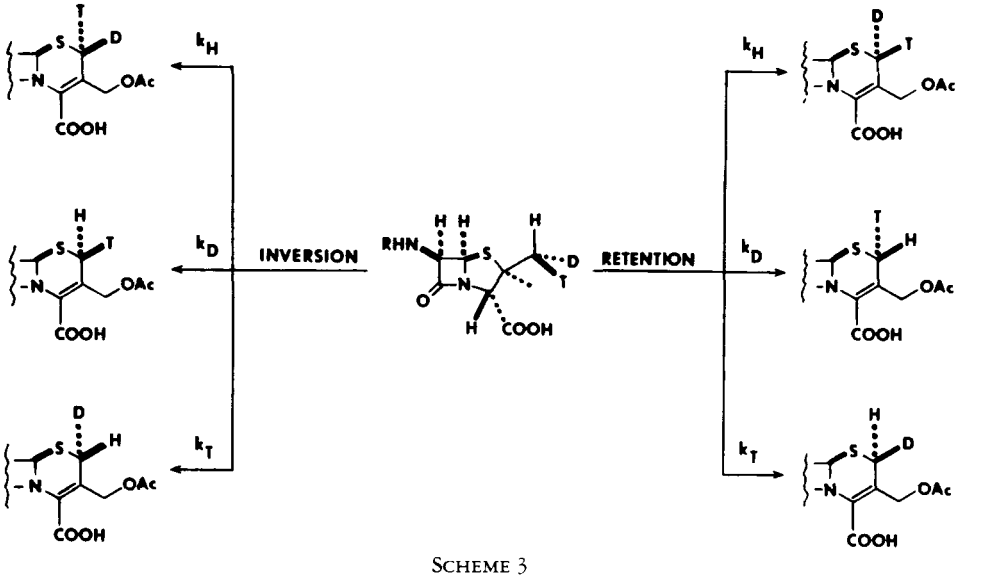
ing of the strained 3-membered ring would then give deacetoxycephalosporin C where the new S—C-2 bond would be formed with overall *retention* of configuration.

Alternatively, it is known that the reactive thiiranium intermediate may be generated as illustrated in path (b) where some leaving group, X, for example, a halogen as chlorine or bromine (15), can be displaced by sulfur. Assuming, in a biochemical analogue of such a process, hydroxylation with retention of stereochemistry and proceeding with a significant kinetic isotope effect, as has been consistently observed in hydroxylations at unactivated methyl carbons (16,17), an intermediate as **11** (X=OH) could be proposed. If this hydroxyl were rendered a leaving group by, e.g., protonation, phosphorylation, etc., its displacement would occur with inversion of configuration to the thiiranium ion (**13**). Upon deprotonation and opening, the cephalosporin S—C-2 bond would be formed with net *inversion* of configuration.

For simplicity, only ^{1}H-removal was shown in Scheme 2. The intrinsic isotope effect for the ring expansion process would, of course, not be infinite, although, as noted above, normal positive isotope effects might reasonably be expected a priori for both the electrocyclic process (path a) (14) and the hydroxylation of an unactivated methyl group (16,17). For a given stereochemical outcome, retention or inversion, the actual distribution of labeled species will partition according to the magnitude of the intrinsic isotope effect as represented by Scheme 3. Methyl groups can be made chiral by virtue of substitution with protium, deuterium, and tritium. But as the radioisotope tritium is only present in tracer amounts, only one in several million molecules will actually be



SCHEME 2

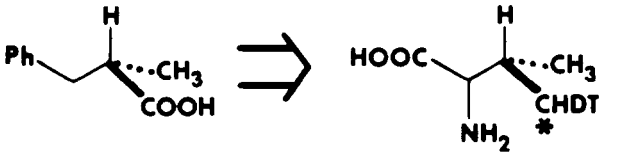


SCHEME 3

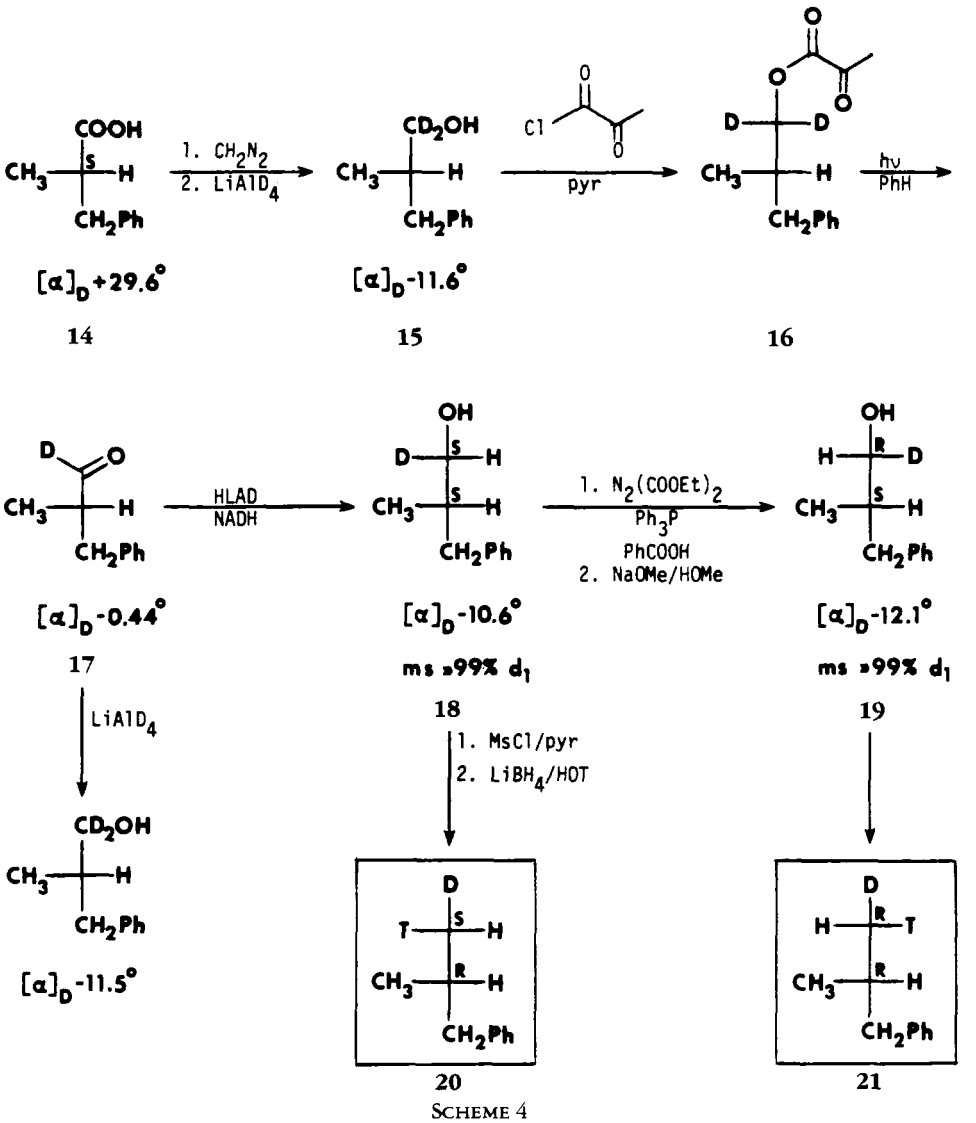
chiral. Stereochemical assay of the fate of these groups must be radiochemical. As a consequence, those species represented in Scheme 3 by the rate process k_T lose radiolabel and will drop out of any configurational assay. The ratio of labeled products, therefore, will depend on k_H/k_D, the intrinsic deuterium isotope effect.

Having in hand a microbiological system to produce cephalosporin C (**7**), two requirements had to be met in order to solve the problem of determining the stereochemical course of the ring expansion and the 3′-hydroxylation reactions. The first was the development of a synthesis of chiral-methyl valines whose methyl groups were both of defined configuration and adjacent to an asymmetric center whose optical purity had likewise to be controlled. The second, more difficult but potentially more interesting task was the development of an analytical method to determine the orientation of tritium label at the cephem C-2 methylene. Solutions to these problems will be taken in order.

Preparation of chiral-methyl valine.—A number of syntheses of chiral-methyl groups have been described (18). The present requirement that such groups reside in valine specifically at either the 3-*pro*(*R*)- or the 3-*pro*(*S*)-locus imposed a previously unaddressed synthetic problem that was approached as shown conceptually below. α-Methylhydrocinnamic acid had been resolved into its optical antipodes and unambiguously correlated by classical means (19). The (*S*)-(+)-enantiomer (**14**) was chosen to establish the correct absolute configuration at what would become the valine β-carbon. The plan was to convert the carboxyl function in a stereochemically defined manner to (*R*)- and (*S*)-methyl groups and then to oxidatively degrade the phenyl ring to a carboxylic acid and, lastly, to introduce the α-amino function. Owing to the ready intervention of amino acid racemases in the fermentation, the configuration of the α-carbon was unimportant. The route taken is shown in Scheme 4. (*S*)-(+)-α-Methylhydrocinnamic acid (**14**) was treated with CH_2N_2 and reduced with lithium aluminum



14 **1**



SCHEME 4

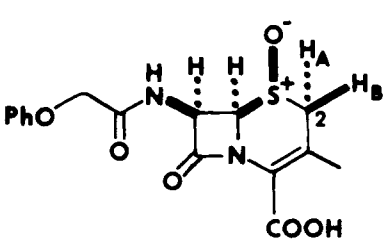
deuteride to (*S*)-(−)-[1-$^2H_2$]-2-methyl-3-phenylpropanol (**15**). Oxidation of this deuterated alcohol to the d_1-aldehyde (**17**) was plagued by disproportionation and partial racemization under a variety of reaction conditions (20). These difficulties were circumvented by conversion to the pyruvyl ester (**16**) and photolysis of the latter in C_6H_6 under neutral conditions (21) to afford the desired aldehyde (**17**). Re-reduction of this material with lithium aluminum deuteride gave a sample of the d_1-alcohol (**15**) having, within experimental error, the same specific rotation attesting to the maintenance of optical integrity at the aldehyde α-center. The purified aldehyde (**17**) was immediately and readily reduced to the (1*S*,2*S*)-alcohol (**18**) using horse liver alcohol dehydrogenase (HLAD) in dioxane/phosphate buffer in the presence of NAD^+ and EtOH as hydride donor. Complete stereochemical inversion at the carbinol center was achieved using the Mitsunobu reaction. The presence of diastereomeric impurities in each of the d_1-alcohols, **18** and **19**, was not detectable with the nmr shift reagent Eu(hfc)$_3$ (20) under conditions where all four stereoisomers were discernable in control experiments with stereorandom material. Similarly, HLAD can be expected to deliver hydride to the *re*-face of aldehyde **17** on the basis of extensive literature precedent (22). However, this

point was demonstrated beyond doubt for **18** as described elsewhere (20,23). It is noteworthy that alcohols **18** and **19**, diastereomeric at C-1 by virtue of hydrogen substitution, had specific rotations which bracket that of the d_2-alcohol (**15**) having a single chiral center (see Scheme 4).

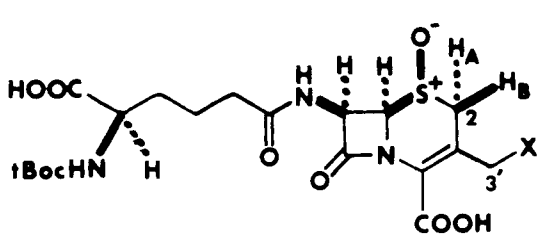
The optically pure alcohols **18** and **19** were separately converted to their methanesulfonates and displaced with lithium borotritide generated in tetrahydrofuran according to the method of R. Cornforth (24). The chiral-methyl-containing isobutylbenzenes **20** and **21** were adsorbed on silica gel and ozonolyzed at −78° (25) to afford, after elution, ca. 75% yields of the corresponding pure isovaleric acids. These were converted by literature procedures (20) to samples of D,L-(3*R*,4*S*)- and (3*R*,4*R*)-[4-^{2}H,^{3}H]valine (**1**), (▲=CHDT), respectively.

Development of the stereochemical assay.—Development of an assay to establish tritium orientation at C-2 in cephalosporin C (**7**) was the most difficult technical, if not intellectual, problem to be overcome in this investigation. Orthodoxy would require that stereochemical analysis at this methylene position be achieved by its reconversion to a chiral-methyl group by a sequence of reactions taking place with a known or provable stereochemical outcome. Excision of the methyl group, typically by Kuhn-Roth oxidation, would allow configurational assay of chiral acetic acid by the Cornforth-Arigoni protocol (26). The chemical lability and density of functionality make this approach a daunting one for the case of cephalosporin C. Alkylation of sulfur with the intention of displacing the resulting sulfonium species (1,2-cleavage to generate a chiral-methyl group presumably with inversion of configuration) would certainly compete with exchange at C-2 and 1,6-cleavage of the cephem nucleus (stable acylimminium ion). Other approaches can be visualized, but clearly substantial technical problems would be encountered in the chemical and stereochemical characterization of the intermediates. Alternatively, provided chiral-methyl valines of sufficiently high specific radioactivity could be prepared, stereochemical analysis by tritium nmr would become trivial as the diastereotopic C-2 methylene hydrogens appear as an AB-quartet and have been securely assigned. We will return to this option later.

Both of these approaches to the analytical problem involved obvious experimental difficulties. Wary in particular of the stereochemical uncertainties promised by degradation, we sought to investigate base-catalyzed exchange adjacent to sulfur bearing a formal positive charge, but in a fashion controlled by stereoelectronic effects, as a third, perhaps heretical, solution to this problem. It had been known for sometime that penicillins and cephalosporins having a free amide NH oxidize selectively to form the more hindered β-sulfoxides (27). Experiments with confirmationally-constrained sulfoxides show that deprotonation with hydroxide and alkoxide bases takes place with a marked preference for sulfinyl anion formation *anti* to the S-O bond (28,29). As a preliminary test, 7-phenoxyacetyldeacetoxycephalosporin C-1β-oxide (**22**) and *N*-*t*-Boc-cephalosporin C-1β-oxide (**23**) were prepared (30) and the disappearance of the C-2 methylene



22



23 X=OAc

24 X=OH

hydrogens was monitored by ^1H nmr in buffered D_2O (30). H_A and H_B give rise to a distinctive AB-quartet whose integrated intensities were treated as parallel pseudo first-order processes as a function of time. It was found that H_A exchanged on the order of ten times faster than H_B as anticipated from the precedents cited above.

Heartened by the initial success of this experimental approach, we sought to extend it to a radiochemical assay of cephalosporin C labeled from chiral-methyl valine. Sulfoxides **22** and **23** were exhaustively exchanged at C-2 in D_2O, crystallized, dried, and exchanged again in D_2O containing a small amount of high specific activity tritiated water. Early in the exchange, the amounts of radiolabel in positions A and B, T_A and T_B, would not be the same, but at *equilibrium* they would effectively become so, the absolute levels of T_A and T_B being dependent only on the specific activity of the medium and the solvent isotope effect (31). After treatment of the labeled sulfoxides at pH 6.1, a pH where tritium was removed from NH and OH functions but not detectably from C-2, **22** and **23** were crystallized to constant activity.

If one considers the general case of tritium exchange from the C-2 methylene of **22** and **23**, the kinetic scheme shown below in equation 1 is obtained where T_A and T_B represent the tritium concentration at C-2α and C-2β, respectively; k_A and k_B are their first-order rates of loss. A further process, k_i, can be visualized where exchange (all exchange assays were conducted in D_2O) of the companion isotope at C-2, in this case, deuterium, occurs with stereochemical inversion to carry tritium label from the α-position to the β-position or vice versa. However, assuming that the relative energies of radioisotope at C-2α and C-2β are the same, then the rate constant for sulfinyl anion inversion, k_i, is the *same* in both directions. While for diastereomers this equality cannot be strictly correct, it may be justly taken to be very nearly so (31).

$$\overset{k_A}{\longleftarrow} T_A \overset{k_i}{\rightleftharpoons} T_B \overset{k_B}{\longrightarrow} \qquad \text{(Eq. 1)}$$

Two limiting cases may be discussed qualitatively. First, if the barrier to sulfinyl anion inversion were very low ($k_i >> k_A > k_B$), tritium equilibration between loci A and B would be rapid relative to the rates of loss. If the disappearance of tritium were plotted (semilog) as a function of time, a straight line would result whose slope would give the sum of k_A and k_B. This rapid interchange of T_A and T_B would defeat the envisioned assay, and the T_A/T_B ratio at $t=0$ would be inaccessible. This situation is, however, contrary to one's chemical intuition as tritium loss and sulfinyl anion inversion through deuturium exchange both involve the same set of ionizations distinguished only by a kinetic isotope effect (k_D/k_T). It will be seen that this effect is small and does not affect the assay in a significant way.

Second, if the anion inversion barrier, k_i, were comparatively high ($k_A > k_B >> k_i$), tritium in position A would neither cross over to B, nor the reverse. The loss of radioisotope from T_A and T_B would now take place as parallel, but independent, first-order processes. The intercepts at $t=0$ would give the initial T_A/T_B ratio—precisely the result desired from the radiochemical assay.

Third, if k_i were of similar or intermediate magnitude to k_A and k_B ($k_A > k_i > k_B$), a significantly more difficult case would arise. From equation 1, complete expressions for the loss of T_A and T_B are:

$$\frac{dT_A}{dt} = -(k_A + k_i)\,T_A + k_i\,T_B \qquad \text{(Eq. 2)}$$

$$\frac{dT_B}{dt} = k_i T_A - (k_B + k_i)\,T_B$$

These coupled equations have a general solution of the form:

$$T_A(t)=ae^{-\lambda_1 t}+be^{-\lambda_2 t} \qquad \text{(Eq. 3)}$$

$$T_B(t)=Ae^{-\lambda_1 t}+Be^{-\lambda_2 t}$$

However, if one considers the special case where at $t=0$, $T_B=T_B$ (the condition achieved by chemical exchange to equilibrium as noted above), several useful observations may be made with regard to the envisioned stereochemical assay. We have shown from first principles elsewhere (30) that for $\lambda_1>\lambda_2$ and for times $t>>\lambda_1$, the following approximation can be made:

$$T_A+T_B=(b+B)e^{-\lambda_2 t} \qquad \text{(Eq. 4)}$$

Taking the logarithm of equation 4 defines a line having a slope of λ_2 and y-intercept of $\ln(b+B)$. If it were found that this intercept were equal to one-half of the total amount of tritium present at C-2 of the cephalosporin in question, $b+B=1/2$, then it can be further shown (30) that the sulfinyl anion inversion process characterizerd by k_i must be very low or effectively zero. That is, if the inversion barrier is high relative to k_A and k_B, the equations in 2 simplify to:

$$\frac{dT_A}{dt}=-k_A T_A \qquad \frac{dT_B}{dt}=-k_B T_B \qquad \text{(Eq. 5)}$$

The simple uncoupled first-order equations in 5, of course, define the second case treated qualitatively above for parallel first-order losses of tritium from T_A and T_B. Qualitatively, one may visualize that as the inversion barrier is lowered, one would expect the experimentally determined T_A/T_B ratio to deviate from 50:50 toward k_A:k_B. That is, fractions of total tritium activity at C-2 greater than 50% at locus A represent increasing contributions of the inversion process.

The radiochemical exchange assays were carried out over greater than two half-lives for the slower exchanging site (>6,000 min). Cephalosporin C derivative **23** was converted to its more hydrolytically stable (32) deacetyl compound **24** by treatment of **23** at pH 6.4-6.6 (33) with citrus acetylesterase (34). In separate experiments under carefully controlled conditions of pH, temperature, and ionic strength, sulfoxides **22** and **24** gave tritium loss data as a function of time in D_2O medium, which, when treated as parallel first-order processes gave $t=0$ intercepts shown as the observed T_A/T_B ratios in Table 1. For the samples of **22** and **24** having theoretical T_A/T_B ratios of 50:50 from chemical exchange *in* of tritium of equilibrium, for sulfoxide **22** the deviation experimentally is small (experiment 1) and for **22** it is undetectable (experiment 4). This important finding established the general validity of the assay to determine the initial distribution of tritium at C-2α and C-2β by monitoring the kinetics of its loss as parallel but essentially first-order processes.

Stereochemical course of the ring expansion.—Having secured an assay for the distribution of tritium at the C-2 methylene of cephalosporin C (**7**), samples of (3*R*,4*R*)- and (3*R*,4*S*)-[4-^{2}H,3H] valine (**1**) were incorporated into the antibiotic in growing cultures of *A. strictum* (30). The cephalosporin C produced was isolated by chromatography on carbon and preparative hplc, diluted about fivefold with radioinactive material and converted to its *N*-*t*-Boc-cephalosporin C-1β-oxide (**23**) (30). After crystallization to constant specific activity, specimens of **23** were treated with citrus acetylesterase and submitted to the exchange assay. The averaged results of duplicate trials are summarized in Table 1 (experiments 5 and 6). We were surprised and for a time troubled to discover that the distribution of tritium at the cephalosporin methylene of biosynthetic origin was *equal* from valine possessing both (*R*)- and (*S*)-methyl groups.

TABLE 1. Tritium Distributions in Positions A and B for Sulfoxides **22** and **24** as Determined in the Exchange Assay

Experiment	Substrate (source)	Theoretical T_A:T_B	Experimental T_A:T_B
1	**22** (chemical exch.)	50:50	54:46±2
2	**22** (10 min exch.)	89:11	85:15±2
3	**22** (120 min exch.)	86:14	76:24±2
4	**24** (chemical exch.)	50:50	50:50±2
5	**24** (from (*R*)-valine)	——	50:50±2
6	**24** (from (*S*)-valine)	——	48:52±2

The unexpected observation of an apparent total absence of stereoselectivity in the penam/cephem ring expansion in vivo demanded an independent demonstration that the exchanged assay was indeed valid. This was provided by recognizing that if sulfinyl anion inversion, represented by k_i in equation 1, were a minor process during the exchange of radioactivity *out* of a cephalosporin, as demonstrated for **22** and **24**, then exchanged *in* of tritium could be treated similarly as uncoupled processes and T_A/T_B estimated at any time t. The uptake of tritium by a cephalosporin sulfoxide from a pool of effectively constant specific activity, therefore, can be expressed as:

$$\frac{dT_A}{dt} = -k_A T_A + G_A \qquad \frac{dT_B}{dt} = -k_B T_B + G_B \qquad \text{(Eq. 6)}$$

where k_A and k_B are the first-order rates of loss from the cephem sulfoxide once label has been incorporated and G_A and G_B represent the zero-order rates of gain of radioisotope. It can be shown that if one wished to know the absolute amounts of tritium at A and B, the gain terms G_A and G_B would have to be evaluated. However, we are interested only in the *ratio* T_A/T_B, in which case a great simplification emerges where it can be further shown that (30):

$$\frac{T_A}{T_B} = \frac{(1-e^{-k_A t})}{(1-e^{-k_B t})} \qquad \text{(Eq. 7)}$$

This is a well-behaved function which depends only upon the experimentally determined values of k_A and k_B and time. Taken at its limits: $t \to 0$, $T_A/T_B = k_A/k_B$ and $t \to \infty$, $T_A/T_B = 1$, the case which was demonstrated earlier in experiments 1 and 4 (Table 1).

Using the much more readily handled sulfoxide **22**, exhaustive deuteration as before followed by short-term exposure to D_2O buffer containing tritiated H_2O for 10 and 120 min gave two samples of **22** unequally labeled at C-2. After removal of tritium from NH and OH sites, crystallization to constant specific activity and assay, agreement with theory was very good for the 10 min sample (experiment 2, Table 1) and somewhat less so for the 120 min specimen (experiment 3) owing to small amounts of sulfinyl inversion in both the exchange *in* and *out* (cf. experiment 1). In any event, it is clear that the assay is capable of distinguishing both equal and unequal distributions of radiolabel C-2α and C-2β, slightly underestimating it is the case of sulfoxide **22**. The exchange assay may be presumed to be more accurate for cephalosporin derivative **24** where sulfinyl anion inversion is evidently a less significant process (cf. experiments 1 and 4).

Magnitude of the isotope effect in the ring expansion.—At this point, the observed equal distribution of tritium between the two diastereotopic C-2 methylene positions in cephalosporin C(**7**) labeled by incorporation of chiral-methyl valine could be interpre-

ted at extremes: (a) the intrinsic isotope effect (k_H/k_D, cf. Scheme 3) for functionalization of the penicillin N (**4**) β-methyl group and ring expansion is unity, and the assay as constituted is unable to extract any stereochemical information that may have in fact been contained in the experiment or, (b) $k_H/k_D \neq 1$ and complete stereochemical scrambling at the methyl center has occurred.

That the isotope effect is normal and, indeed, substantial was demonstrated in a double-label experiment where D,L-(3*R*,4*R*)-[4-^{2}H,^{3}H]valine (**1**) was mixed with D,L-[4-^{14}C]valine and incorporated into cephalosporin C. The kinetic isotope effect from tritium and deuterium at the 3-*pro*(*R*)-methyl group will be expressed (primary) only in the penam/cephem ring expansion itself, apart from a small β-secondary isotope effect possible during the sulfur insertion step at C-3 of valine in the formation of penicillin. If the intrinsic isotope effect for methyl functionalization were unity, the doubly radiolabeled sample of valine would be expected to lose one third of its tritium activity on incorporation into cephalosporin C relative to the ^{14}C-internal standard. Two experiments were carried out whose results are summarized in Table 2.

TABLE 2. Incorporation of D,L-(3*R*,4*R*)-[4-^{2}H,^{3}H],[4-^{14}C] valine into Cephalosporin C

Experiment	valine fed (mg/160 ml)	cephalosporin C day isolated	^{3}H/^{14}C	
			valine	**24** (% ^{3}H retained)
1	40	7	4.53	4.70(104)
2	20	6.5	4.67	5.25(112)
	20	8	4.67	4.96(106)

Experiment 1 was run according to the procedures used in experiments 5 and 6, Table 1. A very slight, 4% increase in the ^{3}H/^{14}C-ratio was observed owing to minor experimental error in scintillation counting. An alternative explanation that degradation of the cephalosporin C in the fermentation might artifactually raise this isotope ratio in a step sensitive to tritium substitution at C-2 was eliminated in experiment 2, Table 2, where samples collected at different times gave, within experimental error, an essentially unchanged ^{3}H/^{14}C-ratio with respect to the valine administered. The intrinsic isotope effect, therefore, must be significantly greater than unity, and, hence, the stereochemical outcome of the penam/cephem ring expansion is complete epimerization.

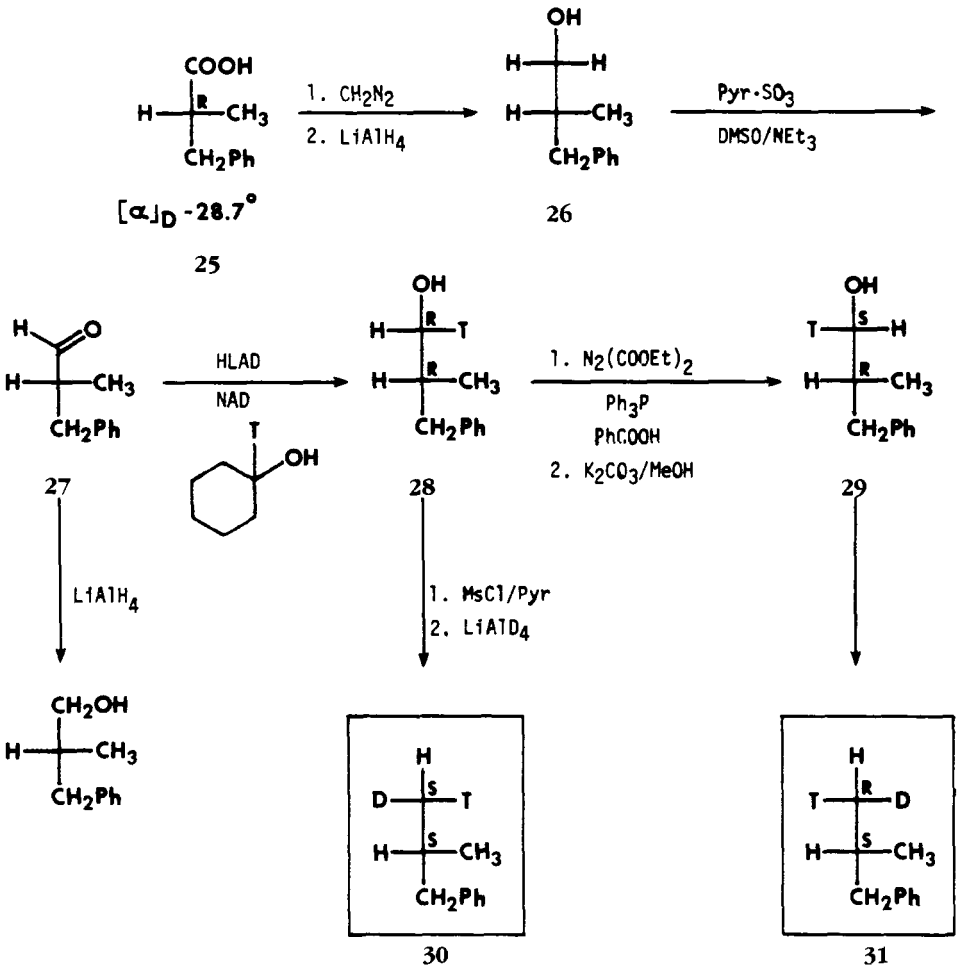
A second, independent line of evidence which indicates that the isotope effect expressed in the ring expansion is quite large may be found in the recent tritium nmr results of Crout (35,36). In these studies, incorporations of very high specific activity chiral-methyl valines into cephalosporin C were carried out. Initial reports claimed a cephalosporin T_A/T_B ratio of 2:1 from *both* (*R*)- and (*S*)-methyl groups; that is, a non-complimentary result (35). This quite unprecedented report, which we attribute to relaxation effects (30), has been revised in more recent experiments where the integrated intensities of tritium reasonances assignable to C-2α and C-2β were more nearly equal (36). However, with regard to the isotope effect in question, the ^{3}H-nmr spectra of these cephalosporin C samples, with *and without* broad band proton decoupling, showed unchanged multiplicity (singlets), indicating that tritium at positions C-2α and C-2β was always paired with deuterium and not detectably with protium.

In conclusion, we are led inescapably to the position that the oxidative ring expansion of pencillin N (**4**) to deacetoxycephalosporin C (**5**) takes place with a substantial, normal primary isotope effect but with net loss of stereochemical integrity in the formation of the new cephem S-C-2 bond.

CEPHEM 3′-HYDROXYLATION.—At the time the first preliminary results were becoming available (37) at Hopkins on the stereochemical fate of chiral-methyl valines in the ring expansion, reports from other laboratories had recently appeared indicating that both the penam-cephem transformation (8) of **4** to **5** and the 3′-hydroxylation (9) of the latter to **6** were catalyzed by α-ketoglutarate-dependent dioxgenases. The observed complete loss of stereochemical integrity was unexpected and represented the first stereochemical investigation of a sulfur insertion reaction at a methyl center. In an attempt to determine whether configurational scrambling was a function uniquely associated with the sulfur insertion reaction or a stereochemical feature common to hydroxylation as well, and perhaps linked mechanistically to oxygen activation and a mode of action for this class of enzymes, the 3′-hydroxylation of **5** to **6** was investigated. For comparison, other classes of heme-iron and nonheme-iron oxygenases are known to carry out hydroxylations at unactivated methyl carbons with retention of configuration (16).

Synthesis of chiral-methyl valine.—Required now were samples of valine having chiral-methyl groups in the 3-*pro(S)* position. Their synthesis was patterned after that outlined earlier in Scheme 4 for the diastereomeric 3-*pro(R)*-labeled valines. Technical improvements were made in the order and means by which hydrogen isotopes were introduced as detailed in Scheme 5. The antipodal acid **25** was esterified and reduced to the undeuterated alcohol **26**. Moffat oxidation (38) as described by Evans (39) gave the aldehyde **27** in 87% yield with minimal racemization as assayed previously. Tritium was introduced from [1-^{3}H]-cyclohexanol in the HLAD-catalyzed reduction essentially according to the procedure of Battersby (40). Parallel experiments with monodeuterated materials were correlated with their enantiomers in Scheme 4 to unambigiously establish the absolute configurations of alcohols **28** and **29**. Deuterium was introduced finally in the reduction of the corresponding mesylates with lithium aluminum deuteride. Elaboration of the resulting isobutylbenzenes **30** and **31** to D,L-(3*S*,4*S*)- and (3*S*,4*R*)-[4-^{2}H,^{3}H]valine (**32**) was carried out as before.

Stereochemical analysis at C-3′.—Examination of the literature on chemical modification of the cephalosporin 3′-position and the well-known facility of lactonization did not auger well for stereospecific conversion of the acetoxymethylene of cephalosporin C (**3**) into a chiral methyl group for configurational assay (26) (as acetic acid from oxidative degradation). However, anticipating a significant, normal kinetic isotope effect in the hydroxylation at C-3′ on the basis of literature precedent (16,17) and our experience with the ring expansion, we chose to ozonolyze the labeled cephalosporin **33** with the hope of obtaining acetyl glycolate (**36**) as shown in Scheme 6. A priori one might expect oxidation to take place initially at sulfur followed by slower cleavage of the ceph-3-em bond to generate an intermediate as **34**. Presuming further that the preferred direction of enolization would be toward the sulfoxide/sulfone as in **35**, a final cleavage would afford acetylglycolate (**36**) which in turn could be saponified to glycolic acid (**37**). However, while the literature is not extensive on this point, it in fact suggests that the double bond is more reactive than the sulfide with ozone (41). In the event, an experiment in D_2O supported these hopes giving about a 20% yield of glycolate whose *p*-bromophenacyl ester showed a 4% incorporation of deuterium (by ms), implying little enolization toward the acetate (and hence little racemization) in a hypothetical intermediate as **35**. Radiolabeled glycolates so obtained, after addition of ^{14}C-internal standard, could be analyzed for the distribution of tritium between the enantiotopic hydroxymethylene positions with glycolate oxidase, an enzyme known to remove stereospecifically the *pro(R)*-hydrogen, H_B, in the formation of glyoxylate **38** (42). Determination of the overall stereochemical course of hydroxylation could, therefore, be made.

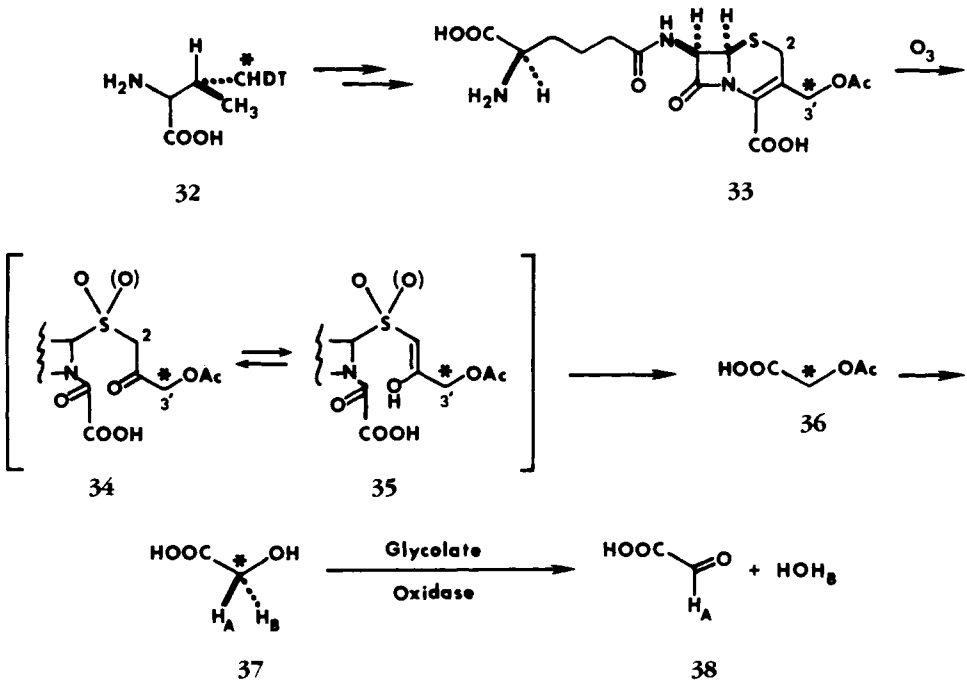


SCHEME 5

Stereochemical course of the 3′-hydroxylation.—A sample of racemic [2-^{3}H]-glycolate was synthesized by the method of Stubbe and Abeles (43) and combined with commercially available [2-^{14}C]-glycolate. Enzymic assay under standard conditions (44), chromatography of the incubation mixture on Dowex-1 (OAc form), and derivatization of the purified glyoxylate as its oxime showed a 54% retention of tritium. As noted in Table 3, that fraction of the total tritium activity presented to the assay that was released to the medium was 45%, a value complementary to that observed in the glyoxylate itself (45).

The samples of valine containing chiral-methyl groups in the 3-*pro*(*S*)-position were fed to growing cultures of *A. strictum*, essentially as before, and the cephalosporin produced was isolated by chromatography on carbon and preparative hplc (>95% pure). These were ozonolyzed, treated with base to saponify the acetylglycolate present, and the glycolate was purified by ion exchange chromotography (44). [2-^{14}C]Glycolate was added, a portion converted to its *p*-bromophenacyl ester to provide a measure of the starting ^{3}H/^{14}C-ratio, and the rest was assayed as above with glycolate oxidase. The data obtained for the (*R*)- and (*S*)-methyl substrates are summarized in Table 3.

For cephalosporin C derived from valine containing an (*S*)-methyl group, tritium isotope was largely lost while the complementary result was obtained from (*R*)-methyl-containing material. The corresponding appearance of tritium as tritiated water in the incubation medium was of an internally consistent magnitude as shown in Table 3 for



SCHEME 6

each case. Therefore, the cephem 3′-hydroxylation occurs with net stereochemical *retention*. This finding correlates with the ^{3}H-nmr results of Crout (35,36) where this allylic hydroxylation was seen to be at least stereoselective and accompanied by a substantial intrinsic kinetic isotope effect. However, as nmr spectral assignments of the acetoxymethylene hydrogens in **33** are not known, no determination of the absolute stereochemical sense of the 3′-hydroxylation was possible.

CONCLUSION.—The full analytic power of chiral-methyl groups as stereochemical and mechanistic probes can be appreciated in the fact that the three isotopes of hydrogen are effectively homotopic and hence essentially indistinguishable, except for the intrinsic isotope effect involved in their removal during the course of enzymatic reactions converting them to methylenes. Whether an intermediate formed is a cation, radical,

TABLE 3. Enzymic Assay of Tritium Distribution in Glycolates from Chiral-Methyl Valine Incorporation into Cephalosporin C

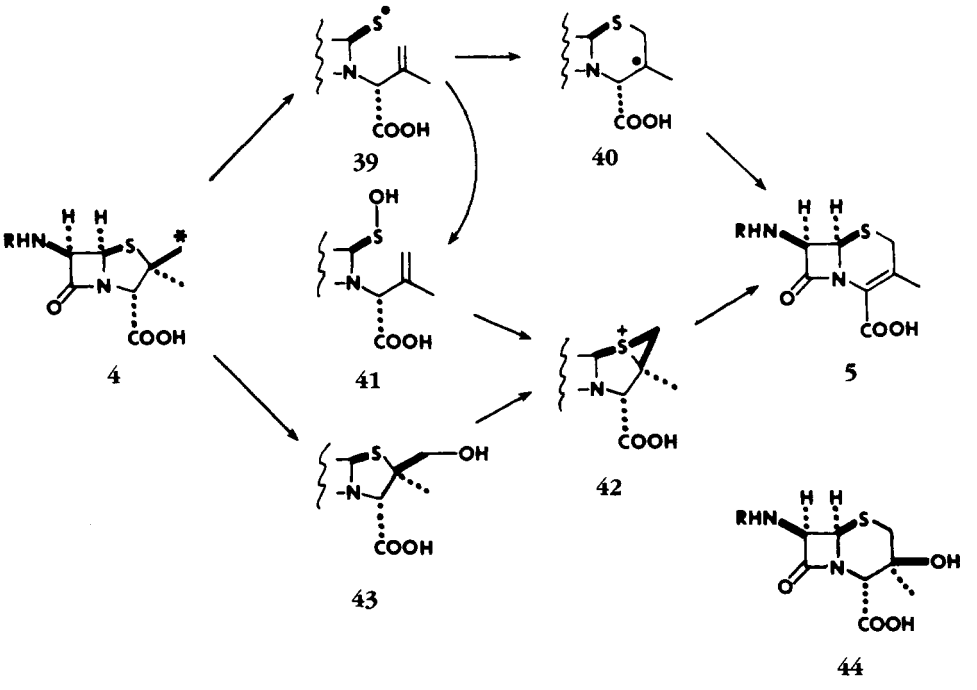
Experiment	Glycolate origin	^{3}H/^{14}C		^{3}H retained (%)	^{3}H in H_2O (%)
		Glycolate[a]	Glyoxylate		
1	racemic	3.80	2.08[b]	54	45
2	(3*S*,4*S*)valine				
	A	1.5	0.40[b]	27	—
	B	2.14	0.69[c]	32	82
3	(3*S*,4*R*)valine				
	A	4.74	3.57[c]	75	28
	B	4.65	3.43[c]	74	31

[a]Determined as the *p*-bromophenacyl ester.
[b]Determined as the oxime.
[c]Determined as the bisulfite addition product.

or carbanion, the faces of this reactive species remain sterically indistinguishable as a free intermediate. An important exception is when such a species is generated adjacent to a conjugating functional group as a carbonyl or carbon-carbon double bond. In this instance, rotation may be disfavored energetically and make the faces of the intermediate distinguishable in the chiral environment of an enzyme active site. In contrast (with the special exception of carbon substituted as Caabb), methylene hydrogens will be enantiotopic or diasteriotopic and hence fundamentally distinguishable in an enzymic reaction.

As noted at the outset of this lecture, both the penam/cephem ring expansion and the cephem 3′-hydroxylation appear from cell-free studies (8,9) to be catalyzed by α-ketoglutarate-dependent dioxygenases. A particularly interesting recent study of an enzyme of this class, δ-butyrobetaine hydroxylase, has been reported by Blanchard (46). The oxidation to carnitine by this calf liver enzyme takes place with retention of configuration (methylene center) and occurs with large primary and α- and β-secondary isotope effects. These observations indicate changes in hybridization during the course of hydroxylation consistent with radical or cationic intermediates and were interpreted as supporting the proposed mechanism of Siegel (47). The cephem 3′-hydroxylation proceeds similarly with a high intrinsic isotope effect and with overall retention of stereochemistry. By analogy to Blanchard's work (46), hydrogen abstraction from C-3′ of deacetoxycephalosporin C5 would generate presumably an allylic radical or cation—both known to have substantial barriers to rotation in simple systems (48). The more complex substitution pattern of the present cephalosporin case will almost certainly exhibit a lower barrier to rotation than the classic literature examples (48), but some residual barrier would be expected to exist. Therefore, unlike the stereochemical studies of hydroxylations at unactivated methyl centers (16) with other classes of oxidases, the observation of retention in the cephem 3′-hydroxylation may (or may not) reflect a mechanistic imperative of the enzyme but may only result from the hindered rotation of a hypothetical allylic intermediate. Similarly, as a corollary, it would not be correct to infer in the absence of allylic stabilization that racemization would necessarily be observed in an α-ketoglutarate-dioxygenase-catalyzed oxidation at an unactivated methyl center.

However, in contrast to this state of affairs, the β-methyl group of pencillin N 4 is unactivated, and its observed complete epimerization enroute to becoming the C-2 methylene of deacetoxycephalosporin C (**5**), while initially unexpected, is the most interesting and mechanistically revealing of stereochemical outcomes. Our present thinking on the oxidative ring expansion process is summarized in Scheme 7. Abstraction of hydrogen isotope from **4** bearing a chiral methyl group (*=CHDT) accompanied by a high intrinsic isotope effect, as experimentally observed, would afford most probably a radical (**4**, *=CDT$^{\bullet}$). Important physical-organic studies of Kampmeier suggest that this abstraction would take place without participation of the adjacent sulfur (49) to allow C-C bond rotation presumably reversible but rapid cleavage (49) to a thiyl radical as **39**. This radical would proceed directly to cephem products by chemistry well-precedented in vitro(50), that is **39** to **40** to **5**. Alternatively, thiyl radical **39** may consumate a hydroxylation reaction to generate sulfenic acid **41**, which would proceed in thoroughly precedented fashion by ionic routes via **40** to **5** (13). In this connection, it is interesting to note that the 3-β-hydroxycepham (**44**), which could be visualized to arise from analogous scavenging of radical **40**, has been observed as a minor component in commercial fermentations (51). Some evidence exists to suggest that a third route to **5** via the hydroxylated intermediate **43** is unlikely (1). Lastly, consummation of an enzymic hydroxylation by one-electron transfer **4** (*=CH$^{+}_{2}$) may be proposed to lead directly to thiiranium species **42** and deprotonation to **5**.



SCHEME 7

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I am deeply grateful to Alan B. Theis, A. Scott Neese, and Ellen B. Barrabee, whose skill and patience have seen this problem through to completion over several years. I am pleased to acknowledge and thank Professor D. Poland of this Department for providing a rigorous treatment for the cephalosporin sulfoxide exchange assay whose behavior I was only able to intuit. Finally, the National Institutes of Health (AI 14937 and RR 07041), the A.P. Sloan Foundation, the Camille and Henry Dreyfus Foundation, and Merck Research Laboratories, are thanked for financial support.

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