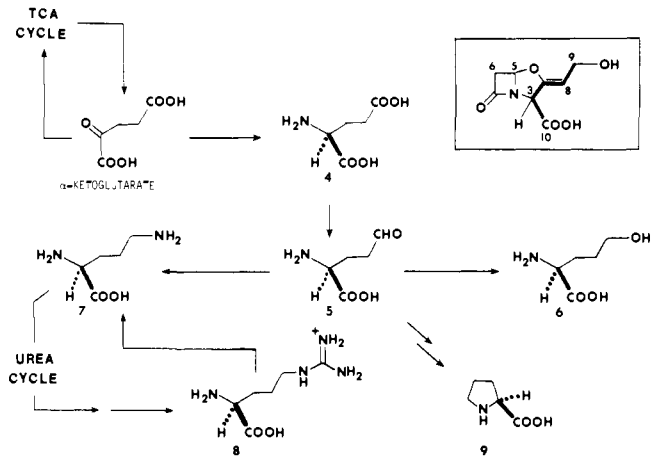
Table I. Incorporation of Radiolabeled Substrates into *p*-Bromobenzyl Clavulanate (**1**, R = PBB)

expt	amino acid	amt,[a] mmol	% ^{14}C spec incorp	% ^{3}H retained	% ^{14}C in C_3 unit	medium[b]
1	L-[U-^{14}C]Glu	0.25	0.24			G
2	L-[U-^{14}C]Glu	0.50	0.32		23.1	T
3	L-[U-^{14}C]HNV[c]	0.25	0.13			G
4	L-[5-^{3}H,U-^{14}C]-HNV[c]	0.40	0.17	15		G
5	L-[5-^{3}H,U-^{14}C]-HNV[c]	0.60	0.54	42		T
6	D,L-[U-^{14}C]-HNV[c]	0.50	0.56		6.0	T
7	L-[U-^{14}C]Pro	0.25	0.11			G
8	L-[U-^{14}C]Arg	0.25	1.61			G
9	L-[U-^{14}C]Orn	0.25	2.11			G
10	L-[U-^{14}C]Orn	0.50	4.86			G
11	L-[U-^{14}C]Orn	0.50	4.01		1.5	T
12	L-[U-^{14}C]Orn	1.00	9.49			G
13	D,L-[5-^{13}H,U,^{14}C]-Orn[c]	0.50	3.69	47		G

[a] Amount of labeled substrate fed (mmol) to 1.5-L total fermentation volume. [b] Principal carbon sources used for two fermentation media: glycerol (G), triglyceride (T), see text. [c] HNV = δ-hydroxynorvaline. Tritium label is stereorandom at C-5.

Scheme I



acid precursors are all readily derived in conventional fashion from L-glutamic acid (**4**) as shown in Scheme I. By administration of 0.25 mmol of **4** and **6**–**9** to fermentations in the glycerol medium (experiments 1, 3, 7, 8, and 9), it can be seen that δ-hydroxynorvaline (**6**, HNV)[8] and proline (**9**) gave similarly low but weakly positive incorporations of radioactivity, glutamic acid (**4**) was about 2-fold higher, and arginine (**8**) and ornithine (**7**) were 15–20 times higher. Comparing relative levels of incorporation in glycerol- and triglyceride-based media for **4**, **6**, and **7** (experiments 1/2, 4/5, and 10/11), with the exception of HNV (**6**), shows that they are quite comparable. Experiments 9, 10, and 12 reveal a nearly linear response of incorporation rate to amount of radiolabeled L-ornithine supplied. In sum these data suggest that among the potential candidates, the urea cycle amino acids, particularly ornithine, are the most efficiently utilized.

The important corollary issue of specificity of labeling may be addressed in several ways. First, **4**, **6**, and **7**, 0.50 mmol of each, were incubated in the triglyceride medium (experiments 2, 6, and 11). As noted above, gluconeogenesis is enhanced under these conditions, and if secondary incorporation were to take place, e.g., by reversion of the C_5 precursor to α-ketoglutarate, circulation of radiolabel around the TCA cycle would result in the appearance of radioactivity in the β-lactam carbons. Degradations of the clavulanate (**1**, R = PBB) isolated in these experiments to (*E*)-methyl 3-(dibenzylamino)acrylate (**3**) afforded the requisite measure of this possibility. For glutamic acid (**4**), HNV (**6**), and ornithine (**7**), the extent of randomization of label into carbons 5–7 was 23.1%, 6.0%, and 1.5%, respectively.[9] Second, the fate of (5*R*, *S*)-[5-^{3}H,U-^{14}C]HNV[6] and -ornithine[10] (experiments 4, 5, and 13) was examined, and notably both of these amino acids lost approximately one-half of their tritium label on incorporation into clavulanate.

At first sight the loss of roughly one-half of the 5-^{3}H label from ornithine and HNV might be interpreted to suggest that glutamate semialdehyde (**5**) provides the logical nexus of the incorporation data, against one's initial structural bias favoring HNV (**6**).[11] While this possibility cannot be strictly excluded, the low specific incorporations of **4**, **6**, and **9** as well as observed randomization of label into carbons 5–7 of **1** in the triglyceride medium disfavor this hypothesis. The urea cycle amino acids are far better utilized, particularly ornithine,[12] with minimum randomization into the C_3 unit of clavulanate. The terminal amino function could be visualized to provide an intermediate binding site for later transamination and reduction.

Acknowledgment. We are deeply indebted to E. B. Barrabee and W. C. Synder of these laboratories for many preliminary experiments, to Drs. I. D. Fleming, C. A. Jones, and P. C. Cherry (Glaxo) for a sample of lithium clavulanate and helpful advice on its production and isolation, and lastly to The National Institutes of Health (AI 14937 and RR 07041), the A. P. Sloan Foundation, and Merck Laboratories for financial support.

Registry No. 1 (R = H), 58001-44-8; **1** (R = PBB), 60297-60-1; **3**, 14376-86-4; **4**, 56-86-0; **5**, 2886-91-1; **6**, 533-88-0; **7**, 70-26-8; **8**, 74-79-3; **9**, 147-85-3.

(8) Murphy, W. H.; Gottschalk, A. *Biochim. Biophys. Acta* **1961**, *52*, 349–360.

(9) An analogous result has been shown for L-glutamate by another approach. See ref 4.

(10) Prepared by considerable modification of the chemistry of: Moe, O. A.; Warner, D. T. *J. Am. Chem. Soc.* **1948**, *70*, 2763–2767. Albertson, N. F.; Archer, S. *Ibid.* **1945**, *67*, 2043–2044.

(11) For comparison a documented role for HNV in polyoxin biosynthesis has been established: Funayama, S.; Isono, K. *Biochemistry* **1977**, *16*, 3121–3127.

(12) The comparatively efficient utilization of ornithine has also been observed at Glaxo (Dr. I. D. Fleming, personal communication).

Biosynthesis of Clavulanic Acid: Origin of the C_3 Unit

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Derivation of the oxazolidine portion of clavulanic acid (**1**, R = H) from a C_5 amino acid, most probably ornithine,[1] maintains a pattern founded in classic experiments of penicillin and cephalosporin biosynthesis[2] and extended in recent studies of nocardicin A[3] and sulfazecin/SQ26180[4] that these β-lactam antibiotics are

† Research Fellow of the Alfred P. Sloan Foundation 1982–1986, Camille and Henry Dreyfus Teacher-Scholar, 1983–1988.

(1) Townsend, C. A.; Ho, M.-F. *J. Am. Chem. soc.*, preceding paper in this issue.

(2) For an excellent review, see: Queener, S. W.; Neuss, N. In "Chemistry and Biology of β-Lactam Antibiotics"; Morin, R. B., Gorman, M., Eds.; Academic Press: New York, 1982; Vol. 3 pp 1–81.

(3) Townsend, C. A.; Brown, A. M. *J. Am. Chem. Soc.* **1983**, *105*, 913–918. Townsend, C. A.; Brown, A. M.; Nguyen, L. T. *Ibid.* **1983**, *105*, 919–927.

(4) Imada, A., paper presented at the 21st Interscience Conference on Antimicrobial Agents for Chemotherapy, American Society for Microbiology, Chicago, IL, Nov 1981. O'Sullivan, J.; Gillum, A. M.; Aklonis, C. A.; Souser, M. L.; Sykes, R. B. *Antimicrob. Agents Chemother.* **1982**, *21*, 558–564.

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Table I. Incorporation of Radiolabeled Substrates into *p*-Bromobenzyl Clavulanate

			1		C_3 unit (2)	
expt	precursor	amt, mmol[a]	^{14}C spec incorp	% ^3H retained	% ^{14}C (% ^3H ret)[b]	medium[c]
1	[1,3-^{14}C]glycerol[d]		225		44	G
2	[2-^{3}H,1,3-^{14}C]glycerol[d]		235	0.1		G
3	[1,3-^{3}H,1,3-^{14}C]glycerol[d]		222	19	44 (24)	G
4	[1-^{14}C]pyruvate	5 × 1.5	0.09			G
5	[3-^{14}C]pyruvate	5 × 1.5	4.74		0.2	G
6	[1-^{14}C]pyruvate	0.50	0.43		92	T
7	[3-^{14}C]pyruvate	0.50	0.55		75	T
8	L-[2-^{3}H,1-^{14}C]Ser[e]	0.50	0.20	9.3	87	T
9		2.0	0.63	1.5	87	T
10	D-[2-^{3}H,1-^{14}C]Ser[f]	0.30	0.24	<0.5		T
11	D-[2-^{3}H,1-^{14}C]glycerate[g]	0.50	0.28	11	101	T
12		1.5	1.6	11		T
13		2.0	2.2	4.5	95	T
14	L-[2-^{3}H,1-^{14}C]glycerate[h]	0.9	1.2	<1.0		T

[a] Amount of labeled substrate fed (mmol) to 1.5-L total fermentation volume. [b] The ratio of tritium retained in **2** with respect to $^3H/^{14}C$ ratio of substrate administered. [c] Glycerol (G) and triglyceride (T) media, see text. [d] See ref 8. Glycerol specific activity was determined for its tris(*p*-nitrobenzoate). [e] 95% ee; experiment 9, 98% ee. Serine was counted as its *N*-tosyl derivative. [f] 88% ee. [g] 88% ee; experiment 13, 72% ee. Glycerate was counted as its calcium salt dihydrate. [h] 95% ee.

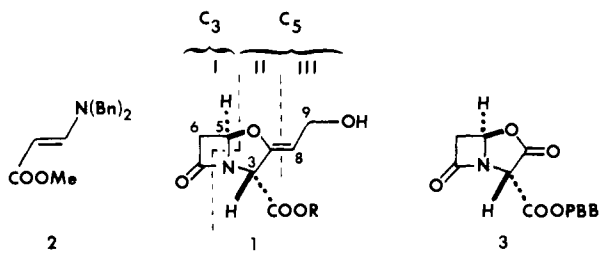
Table II. Incorporation and Distribution of Radiolabel in **1** (R = PBB) from C_3 Intermediates

			1		distribution of label in 1					
					^{14}C			^3H		
expt	precursor	amt, mmol[a]	^{14}C spec incorp	% H ret	I	II	III	H-5	H-6	H-8
15	L-[2-^{3}H,1-^{14}C]Ser	0.50[c]	0.20	9.3	87 ± 1	13 ± 2	0 ± 1			*d*
16		2.0[e]	0.64	1.5	87 ± 1	12 ± 2	1 ± 1	15 ± 6	11 ± 16	74 ± 10
17	D-[2-^{3}H,1-^{14}C]glycerate	0.50[f]	0.28	11	100 ± 1	0 ± 2	0 ± 1			*d*
18		2.0[g]	2.2	4.5	95 ± 2	5 ± 4	1 ± 2	3 ± 3	87 ± 7	10 ± 4
19	D,L-[2-^{3}H]glycerate	0.50						4 ± 1	77 ± 2	19 ± 1
20	[2-^{3}H,1-^{14}C]Gly	2.0	0.43	19.3	73 ± 2	26 ± 6	11 ± 4	26 ± 1	3 ± 2	74 ± 1
21	[1,3-^{3}H,1,3-^{14}C]glycerol[h]		204	21	45 ± 1	33 ± 2	22 ± 1	53 ± 1	0 ± 2	47 ± 1

[a] Amount administered to a total of 1.5 h of the triglyceride medium. [b] See ref 9, 14, and 15. [c] 95% ee. [d] Tritium activity too low to obtain meaningful data; see ref 14. [e] 98% ee. [f] 88% ee. [g] 72% ee. [h] See ref 8.

entirely amino acid derived. In marked contrast, however, we demonstrate herein derivation of the β-lactam carbons of clavulanate from D-glycerate—a case of mixed biogenesis.

Incorporations of radiolabeled potential precursors and isolation of clavulanic acid as its *p*-bromobenzyl (PBB) ester (**1**, R = PBB) were carried out as described in the previous paper[1] and degradations to **2** and **3** essentially as reported.[5] Glycerol- and tri-



glyceride-based fermentation media (designated G and T, respectively, in Table I) were employed to treat severe technical problems of high flux through and rapid equilibria among related primary metabolites in the producing organism, *Streptomyces clavuligerus* (ATCC 27064).[1,5,6]

Labeling patterns in clavulanic acid from incorporation of [1,2-$^{13}C_2$]acetate, [^{13}C]carbonate, and [1,3-$^{13}C_2$]glycerol have indicated derivation of the β-lactam carbons from a glycolytic intermediate (pyruvate was suggested[6]). Therefore, first to test whether glycerol was metabolized in the expected fashion, or by some aberant pathway,[7] incorporations of doubly labeled glycerol were carried out (experiments 1–3).[8] Consistent with the normal pathway to G3P, effectively all of the tritium from [2-^{3}H,1,3-^{14}C]glycerol was lost on incorporation into **1** while 19% was retained from [1,3-^{3}H,1,3-^{14}C]glycerol. In the latter case degradation of **1** (R = PBB) to obtain carbons 5–7 as **2** yielded a $^3H/^{14}C$ ratio consistent with one of the four glycerol tritia appearing in clavulanate at C-5.[9]

At the level of 0.25 mmol/1.5 L used previously[1] to obtain good incorporations of C_5 amino acids in the glycerol medium,[6] cysteine, serine, and glycerate gave disappointingly low incorporations of radioactivity into **1** (<0.1%). Suspecting that these low incorporation rates were the function of a massive flux of glycerol through the intermediates of glycolysis, attention was turned to the triglyceride medium[6] to generate these intermediates indirectly via gluconeogenesis rather than by the direct fermentation of carbohydrate. Experiments with [1-^{14}C]- and [3-^{14}C]pyruvate in both of these media revealed (even in pulsed feedings of five 1.5-mmol doses) that no label appeared in the β-lactam carbons of **1** in the glycerol medium (experiments 4 and 5), while significant levels were attained in the triglyceride medium (experiments 6 and 7).

Heartened by this outcome, the separate incorporations of L- and D-[2-^{3}H,1-^{14}C]serine[12] and [2-^{3}H, 1-^{14}C]glycerate[13] were examined (experiments 8–14). Both the L and D antipodes of these

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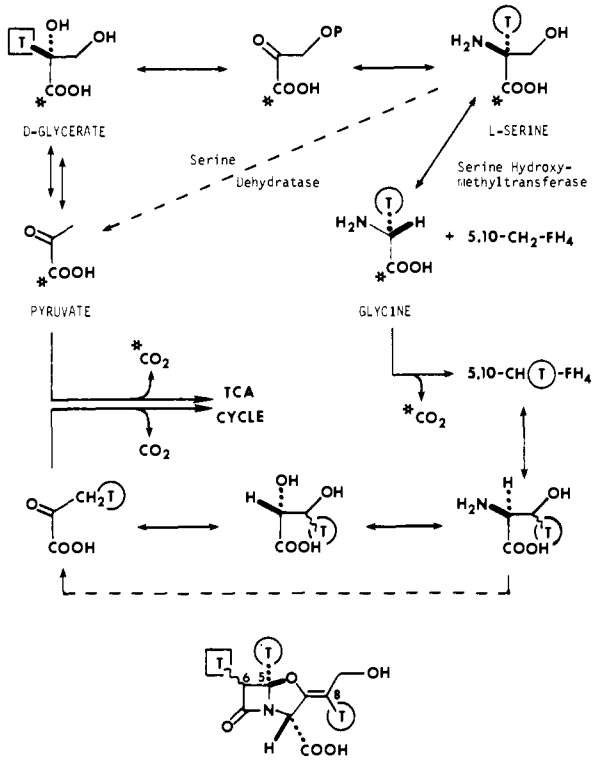
(6) Elson, S. W.; Oliver, R. S. *J. Antibiot.* **1978**, *31*, 586–592. Elson, S. W. In "Recent Advances in the Chemistry of β-Lactam Antibiotics"; Gregory, G. I., Ed.; Royal Society of Chemistry: London, 1981; pp 142–150.
(7) For example, a rearrangement of the sort catalyzed by dioldehydrase. See also a case of unusual glycerol metabolism in aplasmomycin: Chen, T. S. S.; Chang, C.-J.; Floss, H. G. *J. Am. Chem. Soc.* **1981**, *103*, 4565–4568.
(8) The high ^{14}C-specific incorporation ratios shown in Tables I and II for glycerol arise from the fact that the initial glycerol specific activities were based on the total amount of glycerol present in the medium.
(9) Control experiments clearly showed that tritium label present at C-6 in **1** (R = PBB) is lost during formation of **3**.
(10) A modification of that described in: Brewer, S. J.; Taylor, P. M.; Turner, M. K. *Biochem. J.* **1980**, *185*, 555–564.
(11) Beecham, Group, Ltd., Ger. Offen. 2517316.
(12) Synthesis was based on the following procedures: Meils, E. W.; McPhie, P. *J. Biol. Chem.* **1974**, *249*, 2852–2857. Greenstein, J. P.; Winitz, M. "Chemistry of the Amino Acids"; Wiley: New York, 1961; pp 2229–2234.
(13) Fischer, E.; Jacobs, W. A. *Chem. Ber.* **1907**, *40*, 1057–1070.

Scheme I



substrates gave comparable levels of ^{14}C incorporation, but only L-serine and D-glycerate gave positive incorporations of tritium. ^{14}C incorporations were somewhat more responsive to increasing amounts of D-glycerate supplied than L-serine (experiments 8 and 9 vs. 11-13), but relative tritium retentions were remarkably similar, albeit low (ca. 10%, cf. experiments 8 and 11), presumably owing to racemization and redox rates competitive with incorporation into clavulanate. The important point, however, is that the direct precursor of the β-lactam carbons would be anticipated to specifically label C-6 of clavulanate with tritium from its α-position, a condition that cannot be met by both D-glycerate and L-serine (see Scheme I). In experimental design, therefore, the low absolute tritium incorporation could be counterbalanced by locating the ^{14}C internal standard at C-1 to be lost when C_3 intermediate (intact incorporation) was metabolized to acetyl-CoA and entered the TCA cycle (secondary incorporation into the C-5 unit).

Careful degradations[14] of **1** (R = PBB), derived from administration of variously labeled substrates, to **2** and **3** allowed determination of the ^{14}C distribution in segments I (C-5-7), II (C-2,3,10), and III (C-8,9) as well as tritium activity at H-5, H-6,[9] and H-8[15] (Table II). A dichotomy emerges in these data that α-tritium from L-serine resides principally at C-8 and C-5 with significantly lesser amounts at C-6 (experiment 16), whereas analogously labeled D-glycerate suffers the opposite fate labeling C-6 most highly with substantially smaller amounts at C-5 and C-8 (experiments 18 and 19). These data imply, beyond the largely intact incorporation of D-glycerate, that L-serine α-tritium label finds its way to the β-carbon of the true C_3 intermediate. Illustrated in Scheme I, this behavior can be readily accounted for by the agency of serine hydroxymethylase[3,16] to afford L-[3-^3H]serine and hence D-[3-^3H]glycerate and, like [1,3-^3H,1,3-^{14}C]glycerol (experiment 21), result in the appearance of tritium activity at C-5 and C-8. To establish firmly this rationale, [2-^3H,1-^{14}C]glycine was examined (experiment 20). Unlike the serine experiment above.[17] high levels of ^3H incorporation were observed and more precise estimation of tritium distribution was possible. As expected, no label was found at C-6 with carbons 5 and 8 bearing all the activity in a ratio of 1:3.

Therefore, of the possible C_3-glycolytic intermediates, it may be concluded that D-glycerate is utilized most directly to become the β-lactam carbons of **1** in union with a C_5 amino acid, ornithine, to generate ultimately clavulanic acid.

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Registry No. 1 (R = H), 58001-44-8; **1** (R = PBB), 60297-60-1; **2**, 14376-86-4; **3**, 63837-22-9; D-glyceric acid, 6000-40-4.

(14) Spillover of ^{14}C disintegrations introduced comparatively larger errors in computing ^3H activities which have been forthrightly recorded in Table II.

(15) Derivation of the C-9 methylene from the carboxylate (C-5) of α-ketoglutarate prohibits tritium activity in the TCA cycle from appearing at C-9 (except by hydride delivery from, e.g., reduced pyridine nucleotide).

(16) Wasserman, H. H.; Sykes, R. J.; Peverada, P.; Shaw, C. K.; Cushley, R. J.; Lipsky, S. R. *J. Am. Chem. Soc.* **1973**, *95*, 6874-6875 and references cited therein.

(17) Considerable loss of serine 2-^3H label occurs in rapid, reversible reaction to 3-phosphohydroxypyruvate.

Detailed Rate Studies on the Wittig Reaction of Nonstabilized Phosphorus Ylides via ^{31}P, ^1H, and ^{13}C NMR Spectroscopy. Insight into Kinetic vs. Thermodynamic Control of Stereochemistry

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Stereochemistry is an interesting, if not important, aspect of the Wittig olefination reaction, but it has generally been viewed only from the isomeric alkene reaction products.[1] However, our observation of both cis and trans oxaphosphetanes, by high-field ^{31}P NMR spectroscopy below -20 °C, has opened a new avenue to stereochemical studies.[2] Indeed, our initial work revealed that alkene Z/E ratios may not correspond with oxaphosphetane cis/trans ratios.[2] For example, condensation of benzaldehyde with ylide **1a** [from the phosphonium bromide and LiHMDS (lithium hexamethyldisilazide)] in tetrahydrofuran (THF) below -30 °C gave a 78:22 mixture of cis/trans oxaphosphetanes **2a**/**3a** but a 60:40 mixture of *Z*-/*E*-*n*-propylstyrenes (Scheme I). Since such "stereochemical drift" has profound significance relative to the validity of using alkene isomer ratios for mechanistic interpretation concerning carbon-carbon bond formation, we pursued the matter further. We report herein the first detailed rate measurements and kinetic analysis on intermediates (oxaphosphetanes) and products (alkenes) in the Wittig reaction. Our results disclose some intimate features of the Wittig reaction heretofore not fully appreciated. In particular, rate studies on the (lithium salt) reaction of **1a** with benzaldehyde pinpoint bias in oxaphosphetane equilibration as the key source of "stereochemical drift". Also, we found that reactions of salt-free ylide **1b** with benzaldehyde or pivaldehyde show dramatic "stereochemical drift", which partly accounts for the unusually high *E* stereoselectivity observed with such trialkylphosphorus ylides compared to the corresponding triphenylphosphorus ylides.[1c,3]

(1) For a variety of examples, see: (a) Schlosser, M. *Top. Stereochem.* **1970**, *5*, 1. (b) Gosney, I.; Rowley, A. G. In "Organophosphorus Reagents in Organic Synthesis"; Cadogan, J. I. G., Ed.; Academic Press: New York, 1979; pp 17-153. (c) Schlosser, M.; Schaub, B. *J. Am. Chem. Soc.* **1982**, *104*, 5821. (d) Vedejs, E.; Meier, G. P.; Snoble, K. A. J. *Ibid.* **1981**, *103*, 2823. (e) McEwen, W. E.; Cooney, J. V. *J. Org. Chem.* **1983**, *48*, 983. (f) Bestmann, H. J. *Pure Appl. Chem.* **1980**, *52*, 771.

(2) Reitz, A. B.; Mutter, M. S.; Maryanoff, B. E. *J. Am. Chem. Soc.* **1984**, *106*, 1873.

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