Some Applications of Tritium Labeling for the Exploration of Biochemical Mechanisms

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The organic chemist interested in living systems is certain to have his imagination caught by the amazing speed, control, and specificity of enzymatic processes. The fact that his admiration is mixed with some envy is healthy for future research.

A full understanding of how the enzymes achieve what they do will undoubtedly depend on a combination of results from many research areas. One essential contribution to this pool of fundamental information is the elucidation, in precise chemical and stereochemical terms, of the changes which occur when one intermediate is converted into the next along a biosynthetic or degradative pathway. Such knowledge will interlock, sooner or later, with research on enzyme kinetics and X-ray analysis of crystalline enzymes, for example.

Many biochemical reactions involve the addition of hydrogen to or the removal of hydrogen from a carbon atom or the transfer of hydrogen intramolecularly from one carbon atom to another. In principle, these processes are susceptible to study with the two heavy hydrogen isotopes. Deuterium (^{2}H), discovered in 1932,[1] has been used with conspicuous success for research on enzyme-mediated reactions. A classical example is the proof[2] that yeast alcohol dehydrogenase works in a stereospecific way (Figure 1).

Deuterium labeling has its own particular advantages. When used at virtually complete enrichment (>98% ^{2}H) or at least with moderate enrichment, the label can be followed by mass spectrometric and nmr techniques and often the *site* as well as the presence of ^{2}H is revealed by these methods. Further, the sense of chirality and the enantiomeric purity of the system RCHDR can be determined by ord measurements in favorable cases.[3]

The attractions of deuterium diminish, however, when heavy dilution with unlabeled material occurs in the biochemical system; this often happens when whole plants are studied. In these circumstances and in some others mentioned later, tritium (^{3}H, half-life 12.26 years) becomes the label of choice. Further, its readily availability has led to the use of tritium in many recent studies where deuterium would formerly have been employed.

Each isotope has advantages and disadvantages; those of tritium will be illustrated in the sequel. The examples given are drawn largely from our own research, that being the character of these Accounts. However, the points of general importance arise as a distillate from our own experiences and from those of others, especially from the work of Cornforth[4] and Arigoni[5] and their colleagues.

To limit the area of discussion, we shall not be directly concerned with kinetic measurements of proton exchange between a substrate and the aqueous medium, catalyzed by an enzyme. The power of this approach has been brought out by Rose.[7] Kinetic isotope effects are, however, of fundamental importance to our discussion. We shall start with a substrate specifically labeled with ^{3}H and follow the fate of this label over one or more enzyme-mediated reaction(s).

Labeling Methods

Specific ^{3}H labeling of a substrate, or of an intermediate on the synthetic route to it, can be achieved in a variety of ways. The most commonly used methods are: (a) proton exchange at activated positions; (b) quench of carbanions; (c) reductive methods; (d) synthesis from commercially available ^{3}H-labeled intermediates. Tritiated water and tritium gas are the cheapest starting materials. For most applications, the isotope is used at tracer levels.

It is essential for many of our later arguments that the ^{3}H label(s) introduced by any of these methods should have been proved to be entirely at the expected site(s) by degradation (*e.g.*, ref 8) or by a strictly parallel sequence in the deuterium series (*e.g.*, ref 9).

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(1) H. C. Urey, F. Brickwedde, and G. M. Murphy, *Phys. Rev.*, [2] **39**, 164 (1932).

(2) F. A. Loewus, F. H. Westheimer, and B. Vennesland, *J. Amer. Chem. Soc.*, **75**, 5018 (1953).

(3) *E.g.*, J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Ryback, and G. J. Schroepfer, Jr., *Proc. Roy. Soc., Ser. B*, **163**, 436 (1966).

(4) Reviewed by J. W. Cornforth, *Quart. Rev., Chem. Soc.*, **23**, 125 (1969); J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature (London)*, **221**, 1212 (1969).

(5) *Inter alia*, J. Lüthy, J. Rétey, and D. Arigoni, *ibid.*, **221**, 1213 (1969); D. Arigoni, F. Lynen, and J. Rétey, *Helv. Chim. Acta*, **49**, 311 (1966); L. Bücklers, A. Umani-Ronchi, J. Rétey, and D. Arigoni, *Experientia*, **26**, 931 (1970), and unpublished work quoted in ref 6.

(6) The problems are discussed by D. Arigoni and E. L. Eliel, *Top. Stereochem.*, **4**, 127 (1969).

(7) I. A. Rose in "The Enzymes," P. D. Boyer, Ed., Vol. 2, 3rd ed, Academic Press, New York, N. Y., 1970, p 281.

(8) A. R. Battersby, S. H. Brown, and T. G. Payne, *Chem. Commun.*, 827 (1970).

(9) A. R. Battersby and R. J. Parry, *ibid.*, 31 (1971).

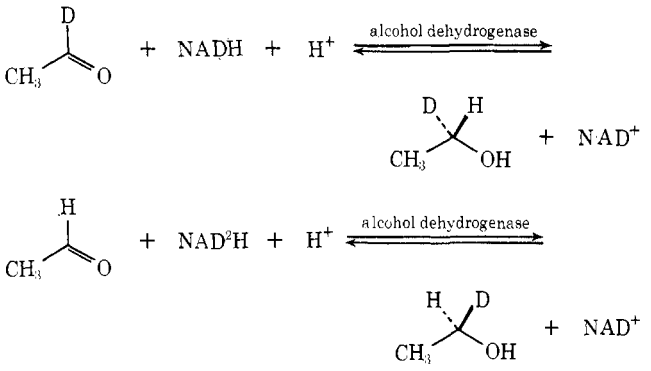

Figure 1. Stereospecificity of alcohol dehydrogenase.

Phenylalanine Ammonia-lyase

Several points of general importance are illustrated by the stereochemical research[10] on phenylalanine ammonia-lyase. This enzyme catalyzes the elimination of a proton and ammonia from L-phenylalanine (**1**) to give *trans*-cinnamate (**2**). (3*R*)-L-[3-$^3H_1$,1-^{14}C]Phenylalanine and (3*S*)-L-[3-$^3H_1$,1-^{14}C]phenylalanine (see **1**) were synthesized. The former contains **1**, **3**, and **4** with **1** being by far the major component. Let us assume that the (3*S*)-L and (3*R*)-L samples are enantiomerically pure at the C-3-labeled site, that the H–C-3 bond is broken in the rate-limiting step (evidence for this will be presented later), and that labeled and unlabeled molecules of substrate in solution are accepted equally well at the enzymatic active site by a rapidly reversible process.[11] We can now consider three possibilities for enzymatic attack, as follows.

(a) The enzyme stereospecifically removes the *pro-S* hydrogen atom from C-3 in the process **1** → **2**. If we neglect secondary isotope effects,[12] the rates at which the three species **3**, **4**, and **1** are converted into cinnamic acid will be governed by their proportions in the mixture. Thus, at all stages of the conversion **1** → **2**, both the cinnamic acid and the unchanged phenylalanine will show a ^3H:^{14}C ratio equal to that of the starting material (100% ^3H retention).

(b) The enzyme stereospecifically removes the *pro-R* hydrogen atom from C-3. In this case, **3** will be converted into cinnamic acid more slowly than **1** and **4**, so that ^3H-labeled species **3** will build up in the unconverted phenylalanine. This will be observed as a significant rise in ^3H:^{14}C ratio in the recovered phenylalanine at, say, 50% conversion. The cinnamic acid formed throughout will contain only ^{14}C-labeled material (0% ^3H retention).

(c) The enzyme is nonstereospecific. This situation is best discussed[13] by considering the nonstereospecific (random) oxidation of stereospecifically ^2H,^{14}C-labeled ethanol (a mixture of species **5**, **6**, and **7**); the same assumptions are made as for the phenylalanine case. Species **6** and **7** will now undergo conversion into acetaldehyde at almost twice the rate of **5**, so that product will be produced initially showing somewhat less than 50% of the original ^3H:^{14}C ratio. Later however, the content of ^3H-labeled species in the residual ethanol will have risen and the acetaldehyde being produced from it will also show a greater ^3H:^{14}C ratio. At *complete conversion*, the total acetaldehyde could show a ^3H:^{14}C ratio equal to 90% of the original value; at this point, k_H/k_T = % ^3H retained/% ^3H lost.

If (3*RS*)-L-[3-$^3H_2$,1-^{14}C]phenylalanine had been the substrate in examples a and b and (1*RS*)-[1-$^3H_2$,2-^{14}C]-ethanol for example c, the same arguments show that the following retention values should be observed for the products: in cases a and b, 50% ^3H retention at all stages of the reaction; in case c, initially *ca.* 50% retention, rising to 90% retention at complete conversion (assuming k_H/k_T = 9), *i.e.*, the same result as for c above.

We can now consider the results found experimentally when the labeled phenylalanines were treated with the enzyme. It was known[10] that the (3*R*)-L-[3-$^3H_1$,1-^{14}C]phenylalanine contained *ca.* 10% of the (3*S*)-L isomer and the ^3H retention in cinnamic acid produced from this sample was 88% (relative to ^{14}C). Similarly, the (3*S*)-L-[3-$^3H_1$,1-^{14}C]phenylalanine (containing *ca.* 10% of the (3*R*)-L isomer) afforded cinnamic acid with 11% ^3H retention. The phenylalanine recovered at 45% conversion in the latter case showed a ^3H:^{14}C ratio of 6.97 compared with the starting value of 5.90. These results prove that the enzyme stereospecifically removes the *pro-S* hydrogen atom from C-3 of L-phenylalanine.

It is important to emphasize that these experiments lead to a rigorous conclusion without there being a requirement for enantiomeric purity at C-3. Also, by using both 3*R* and 3*S* labels, there was no necessity to ensure complete conversion of substrate into product. In contrast, great care must be taken when deuterium labeling is used;[6] it should be remembered that high enrichments are being used with deuterium in contrast to tracer levels with tritium. The danger can readily be seen if the ammonia-lyase enzyme is considered to act on 3-$^2H_1$-labeled phenylalanines of still lower enantiomeric purity[14] at C-3, *e.g.*, a sample containing 70% of (3*S*)-L-[3-$^2H_1$]phenylalanine and 30% of the (3*R*)-L-[3-$^2H_1$]isomer. If k_H/k_D is assumed to be *ca.* 4, then the cinnamic acid formed at 35% conversion would be rich in deuterium because it would have been derived largely from the 3*R* species present. The false conclusion might then be drawn that the enzyme specifically attacks the *pro-R* hydrogen atom at C-3 of phenylalanine.

Hydroxylation at Saturated Carbon

This important reaction is generally carried out in

(10) K. R. Hanson, R. H. Wightman, J. Staunton, and A. R. Battersby, *Chem. Commun.*, 185 (1971).

(11) The last assumption is usually acceptable but not always so; see ref 12.

(12) J. H. Richards in ref 7, p 321.

(13) It is unlikely that a fully random process at C-3 could be observed with L-phenylalanine because of the asymmetry at C-2.

(14) It will be shown later that unambiguous results are obtained in the $^3H_1$ series at these levels of enantiomeric purity.

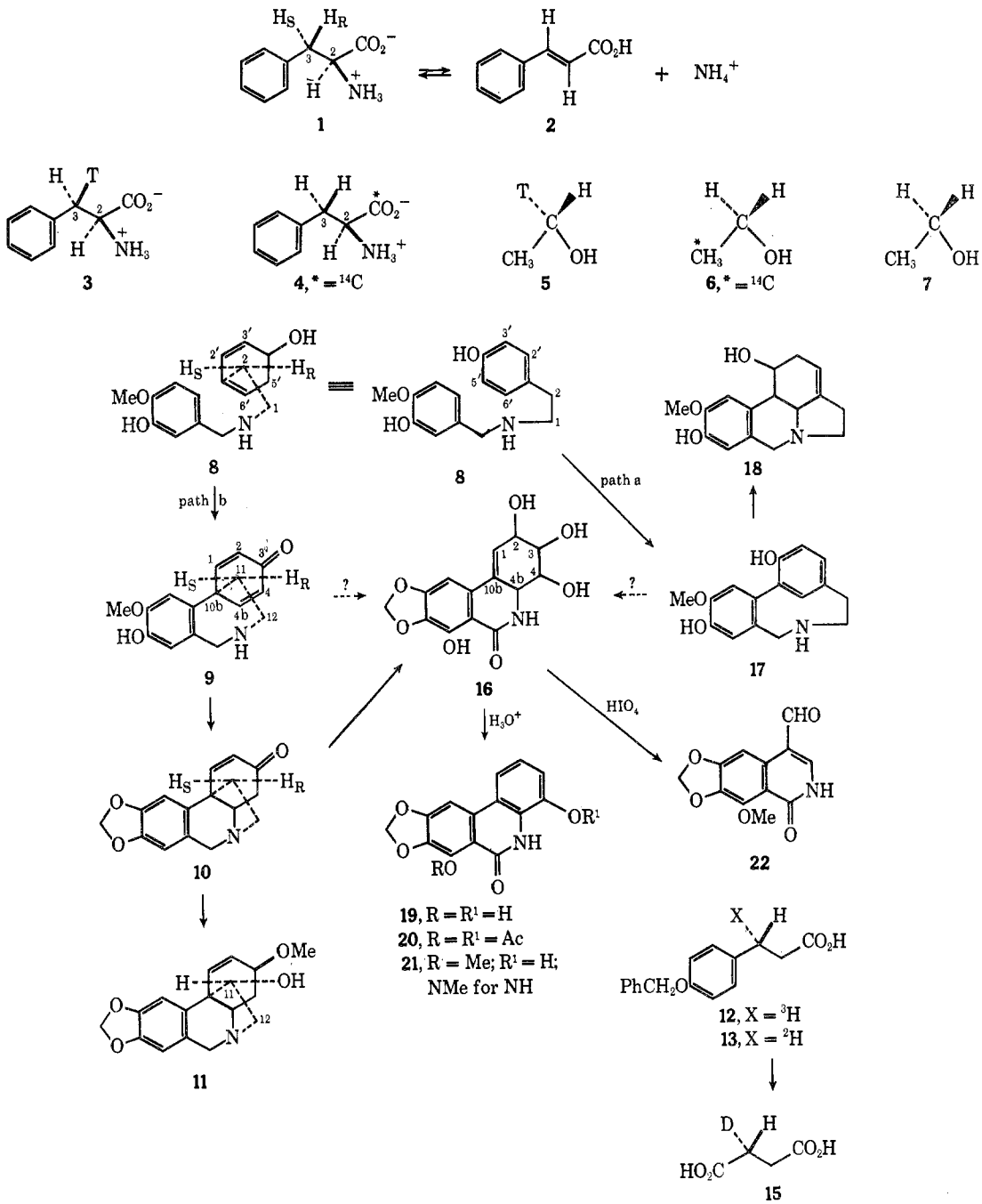

Figure 2. Biosynthesis of haemanthamine and narciclasine.

living systems by a mixed function oxidase working in conjunction with oxygen and a reducing agent.[15] For the cases studied so far, mainly in the aliphatic and steroid series, hydroxylation occurred with retention of configuration. Work on the examples below was undertaken as part of a wider study of hydroxylations at sites of greatly differing chemical and steric environment.

Haemanthamine (**11**) is biosynthesized in *Amaryllidaceae* plants from *O*-methylnorbelladine[16] (**8**) *via* path b in Figure 2, and the hydroxyl group is introduced after the oxocrinine skeleton (**10**) has been built.[17] The stereochemical course of this reaction can thus be determined[18] using **8** labeled stereospecifically with tritium at C-2. The synthetic route to one enantiomer of ^{3}H-labeled **8** involved **12** chosen so that an exactly parallel sequence with deuterium labeling **13** would allow the sense of chirality and enantiomeric purity at

(15) O. Hayaishi, *Annu. Rev. Biochem.*, **38**, 21 (1969); G. A. Hamilton, *Advan. Enzymol.*, **32**, 55 (1969).

(16) W. C. Wildman, H. M. Fales, R. J. Highet, S. W. Breuer, and A. R. Battersby, *Proc. Chem. Soc. London*, 180 (1962); D. H. R. Barton, G. W. Kirby, J. B. Taylor, and G. M. Thomas, *J. Chem. Soc.*, 4545 (1963).

(17) A. R. Battersby, C. Fuganti, and J. Staunton, unpublished work.

(18) A. R. Battersby, J. E. Kelsey, and J. Staunton, *Chem. Commun.*, 183 (1971); *cf.* related work by G. W. Kirby and J. Michael, *ibid.*, 187 (1971).

the labeled site to be determined by degradation to succinic acid (**15**). The ^3H-labeled **8** was thus shown to contain *ca.* 70% of *O*-methyl-(2*S*)-[2-$^3H_1$]norbelladine and *ca.* 30% of the 2*R* enantiomer (sample A). A second sample, B, in which the proportions of the two enantiomers were reversed, was similarly synthesized. When sample A in admixture with ^{14}C-labeled **8** was fed to the living system, the ^3H retention in haemanthamine (**11**) was 66 ± 4% (^3H was shown to be solely at C-11. In contrast, sample B of **8** gave 31 ± 2% retention of ^3H in **11**, while *O*-methyl-(2*RS*)-[2-$^3H_2$,1-^{14}C]norbelladine (**8**) led to **11** with 50 ± 2% ^3H retention. The obvious ^3H,^{14}C-labeling experiment at C-1 of **8** showed that C-12 of the haemanthamine skeleton is not involved in the hydroxylation process. Thus, the combined evidence proves that hydroxylation occurs with retention of configuration at C-11, presumably by a mechanism involving direct insertion of oxygen into the C–H bond.[15]

^3H Labeling of Aromatic Systems

Aryl-^3H labels have been widely used to determine whether substance A can be converted into substance B in a biological system, but we shall not be further concerned with this obvious application here; much more information can be gained in appropriate cases as outlined below.

Several *Amaryllidaceae* plants have been shown to contain the lactam narciclasine **16**,[19] a substance of marked antimitotic activity, in addition to haemanthamine (**11**) and norpluviine (**18**). The previous section discussed the biosynthesis of haemanthamine by phenol coupling from *O*-methylnorbelladine (**8**) *via* path b; the same precursor leads to norpluviine (**18**), but here path a is followed. It seemed probable that narciclasine (**16**) could be formed by a variant of these same biosynthetic processes. Thus, degradation at a late stage of the skeleton produced by either path a or path b could generate **16**. The information which allowed a decision in favor of path b for narciclasine biosynthesis came initially from work based on aryl-^3H labeling.[20]

[3′,5′-$^3H_2$]Tyramine was prepared from tyramine by base-catalyzed exchange and was used for the synthesis of *O*-methyl-[3′,5′-$^3H_2$,*methyl*-^{14}C]norbelladine[21] (**8**). When this precursor (expt 1 in Table I) was fed to daffodil plants, it was incorporated into the alkaloids **11** and **18** and also into narciclasine (**16**). Table I collects the ^3H:^{14}C ratios observed for these three substances and for the degradation products **19** and **22**

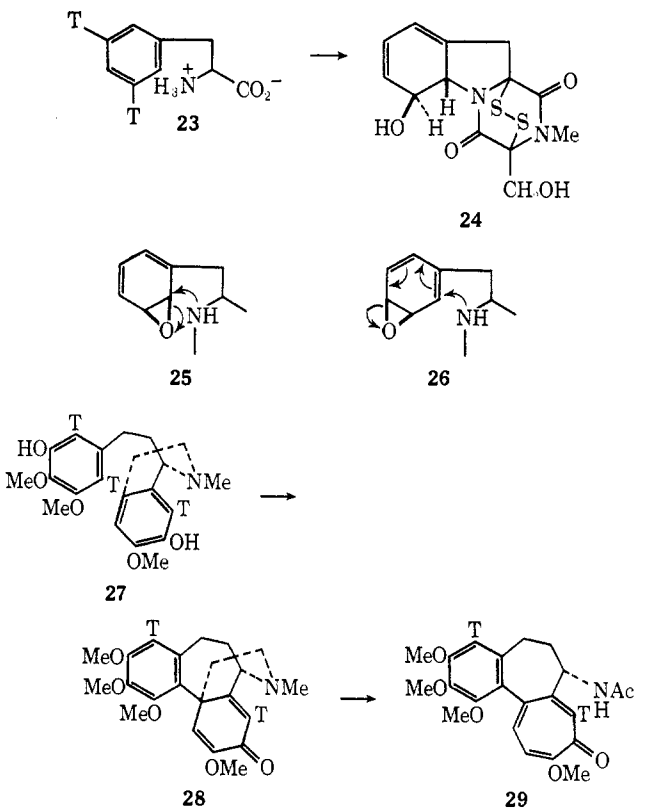

Figure 3. Biosynthesis of gliotoxin and colchicine.

Table I

Substance	Expt 1 with 3,′5′-^3H (**8**); % ^3H retained	Expt 2 with 2′,6′-^3H (**8**); % ^3H retained
Precursor **8**	100	100
Narciclasine **16**	75 ± 4	100 ± 5
Norpluviine **17**	49 ± 2	
Aldehyde **22**	<5	100 ± 5
Lactam **20**	50 ± 2	
Lactam **19**		50 ± 2

from narciclasine. The values in the narciclasine series are those expected for initial coupling *via* path b to label C-2 and C-4 of **16** apart from the partial loss of ^3H from C-4 of narciclasine itself. This feature requires further study, but it does not affect the main argument.

A parallel synthesis[20] afforded **8** ^3H-labeled at positions 2′ and 6′. 4-Benzyloxylphenol was exchanged in mild base with tritiated water, and the phenolic hydroxyl group was then reductively removed to give [3,5-$^3H_2$]phenol. This was converted into the corresponding tyramine from which *O*-methyl-[2′,6′-$^3H_2$,-*methyl*-^{14}C]norbelladine (**8**) was synthesized. Incorporation into narciclasine (**16**) as before was expected to label C-1 and C-4b, and the results in Table I were obtained (expt 2); again the values for the degradation products are included. When the phenol **21** derived from **19** in expt 2 was exchanged in D_2O–triethylamine,[22] almost quantitative conversion to dideuterio-

(19) F. Piozzi, C. Fuganti, R. Mondelli, and G. Ceriotti, *Tetrahedron*, **24**, 1119 (1968); T. Okamoto, Y. Torii, and Y. Isogai, *Chem. Pharm. Bull., Jap.*, **16**, 1860 (1968); G. Savona, F. Piozzi, and M. L. Marino, *Chem. Commun.*, 1006 (1970); A. Mondon and K. Krohn, *Tetrahedron Lett.*, 2123 (1970); *Chem. Ber.*, **103**, 2729 (1970).

(20) A. R. Battersby, C. Fuganti, and J. Staunton, *Chem. Commun.*, 1154 (1971).

(21) The way the labeling patterns are indicated in the brackets must be regarded as being a "shorthand" system. Virtually all the labeled molecules carry only *one tritium atom*, but because a mixture of labeled species is present, a large assembly of molecules will have in effect the stated patterns.

(22) This technique of demonstrating complete exchange by replacement with deuterium may cause problems if the sample is of low activity because D_2O can contain appreciable ^3H activity (personal communication from Professor D. Arigoni).

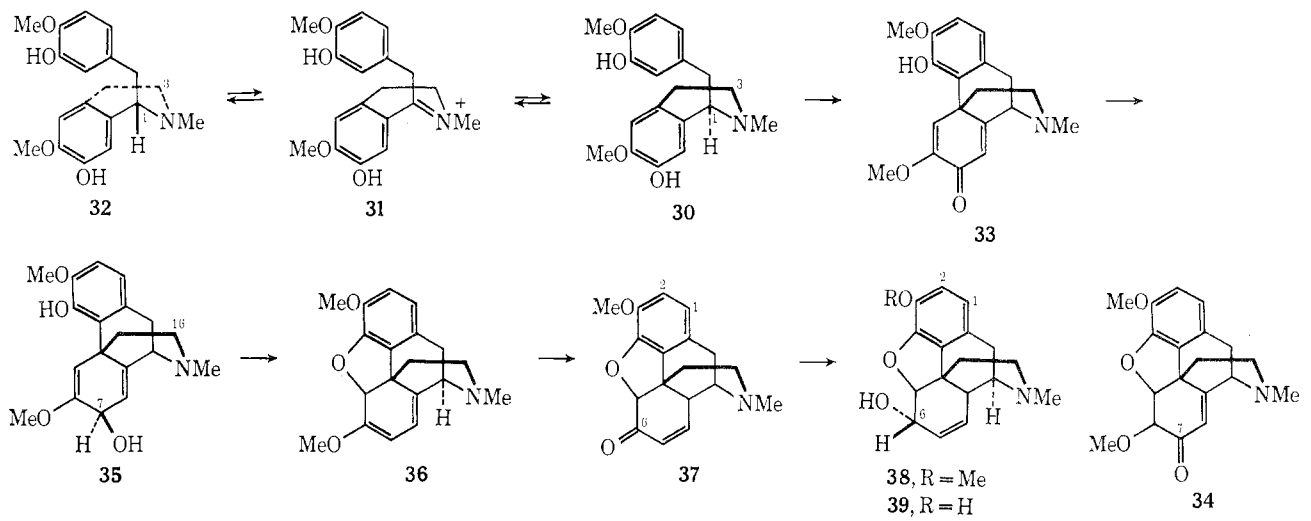

Figure 4. Some stages of the biosynthetic pathway to morphine.

21 occurred (91% D_2, 8% D_1 species); this product contained <5% of the original ^3H activity.

The two foregoing sets of results clearly show that path b is followed to narciclasine (**16**); they also pointed to the next experiment. This was to feed ^3H-labeled oxocrinine (**10**) and the good incorporation of this precursor into narciclasine interlocks with the aryl-^3H-labeling work.

Three other applications of aryl-^3H labels can be mentioned (Figure 3). Powerful use has been made of this approach in studying the "NIH shift" in which aryl-^3H labels were unexpectedly transferred rather than eliminated; the story has been fully reviewed.[23] A different case of ^3H retention allowed conclusions to be drawn about the biosynthesis of gliotoxin (**24**). [3′,5′-$^3H_2$]-Phenylalanine (**23**) was incorporated[24] without ^3H loss into **24**, a result which indicates that an arene oxide (**25** or **26**) is an intermediate. Finally, ^3H labeling has been used to confirm that the amount of aryl-^3H retained over a biochemical sequence agrees with that expected for the favored biosynthetic scheme. For example, colchicine (**29**) has been shown[25] to be biosynthesized from autumnaline (**27**) *via* the dienone **28**. Loss of one-third of the ^3H activity is expected over these steps, and, in practice, 30% of the original tritium was eliminated from **27** to **29**.

Biosynthesis of the Morphine Group of Alkaloids

In this section and the next, we shall be examining the oxidation level at those sites in biosynthetic intermediates which are of decisive mechanistic importance. Figure 4 shows part of the biosynthetic pathway to the morphine alkaloids as it is known today. Feeding experiments with (±)-reticuline (**30** and **32**) had demonstrated specific incorporation of activity from this substrate into morphine (**39**), codeine (**38**), and thebaine[26,27] (**36**). A stereochemical test could then be superimposed on the tracer studies since it was expected from structural relations that only (−)-reticuline (**30**) would be a direct precursor of the alkaloids. The required substrates were (−)-[1-^3H,3-^{14}C]reticuline (**30**) and the (+)-enantiomer **32**. Surprisingly, (+)- and (−)-reticuline were both incorporated with equal efficiency into the morphine alkaloids as measured by ^{14}C. However, (−)-reticuline (**30**) was built into morphine with *ca.* 60% *retention* of ^3H whereas (+)-reticuline (**32**) *lost* 82% of its ^3H on conversion into morphine.[28] This result[29] immediately pointed to the illustrated oxidation reduction system where (+)- and (−)-reticulines undergo interconversion by way of 1,2-dehydroreticuline (**31**); the appropriate incorporation experiment with ^{14}C-labeled (**31**) confirmed this deduction.[29]

Biochemical oxidative coupling of (−)-reticuline (**30**) yields (+)-salutaridine (**33**), and there was great interest in how the dienone is converted into thebaine (**36**). This could involve (a) ring closure to the benzofuran (**34**) with subsequent removal of the C-7 oxygen function or (b) initial reduction to a dienol (**35**), so that closure of the ether bridge is prompted by loss from C-7 of some good leaving group OX^- (a phosphate anion, perhaps). The decisive experiment required tritium labeling at C-7 of **35** to be measured against an internal ^{14}C label at C-16. Only in this way could one be sure that the observed good conversion of salutaridinol-I (**35**) into thebaine (**36**) in poppy plants had not occurred by oxidation *in vivo* to the dienone **33** followed by incorporation *via* path a. Since **35** yielded thebaine without significant loss of ^3H from C-7, path a was excluded and b was confirmed.[30]

(23) G. Guroff, J. W. Daly, D. M. Jerina, T. Renson, B. Witkop, and S. Udenfriend, *Science*, **157**, 1524 (1967); D. W. Russell, E. E. Conn, A. Sutter, and H. Grisebach, *Biochim. Biophys. Acta*, **170**, 210 (1968); W. R. Bowman, I. T. Bruce, and G. W. Kirby, *Chem. Commun.*, 1075 (1969).

(24) N. Johns and G. W. Kirby, *ibid.*, 163 (1971); *cf.* J. D. Bu'Lock and A. P. Ryles, *ibid.*, 1404 (1970), where ^2H labeling was used.

(25) A. R. Battersby, *Pure Appl. Chem.*, **14**, 117 (1967).

(26) A. R. Battersby, *Proc. Chem. Soc. London*, 189 (1963).

(27) D. H. R. Barton, *ibid.*, 293 (1963).

(28) Complete loss of ^3H from (+)-reticuline would be expected on simple grounds, and the small retention probably arises by transfer of ^3H from (+)-reticuline (**32**) *via* a cofactor to **31**, yielding a little (−)-[1-^3H]reticuline (**30**), which is then incorporated into the alkaloids.

(29) A. R. Battersby, D. M. Foulkes, and R. Binks, *J. Chem. Soc.*, 3323 (1965).

(30) D. H. R. Barton, G. W. Kirby, W. Steglich, G. M. Thomas, A. R. Battersby, T. A. Dobson, and H. Ramuz, *ibid.*, 2423 (1965).

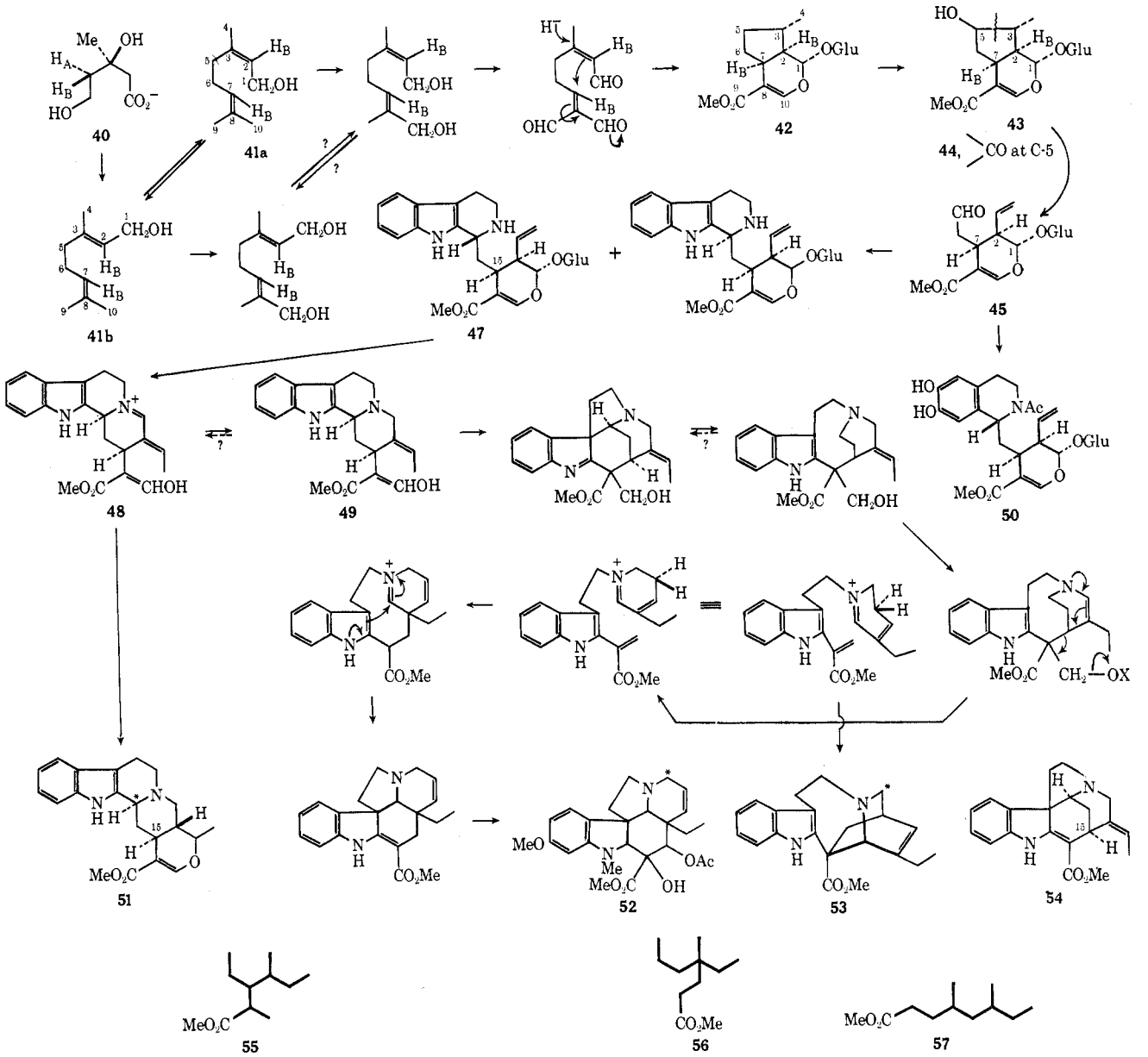

Figure 5. Biosynthesis of cyclopentane monoterpenes and of the indole alkaloids.

Biosynthesis of the Terpenoid Indole Alkaloids

The fascinating biosynthetic problems presented by the large group of indole alkaloids have largely been solved over the past seven years and a full review is available.[31] Despite their bewildering structural variety, three main groups of alkaloids were recognized: (a) the *Corynanthe–Strychnos* type, *e.g.*, ajmalicine (**51**) and akuammicine (**54**), which possess the nontryptamine unit **55**, (b) the *Aspidosperma* type, *e.g.*,vindoline (**52**), in which the nontryptamine unit appears as **56**, and (c) the *Iboga* type, *e.g.*, catharanthine (**53**), having a third arrangement of this unit (**57**). The two major puzzles at the outset of this work were, first, what is the origin of the nontryptamine units, and, second, by what mechanism are the three different skeletal types constructed.

The story of how these questions were answered is complex, with evidence coming from several directions;[31] for our purpose it will suffice to set out the biosynthetic sequence in Figure 5 for discussion of the contributions made by ^3H labeling. We will start at the stage when it was known that the three nontryptamine units are all derivable from mevalonate (**40**) by way of geraniol–nerol (**41a** and **b**) and, further, that loganin (**43**) is the key cyclopentanoid intermediate. This leads to secologanin (**45**) ready for introduction of the tryptamine residue. It was clear then that by labeling mevalonate, geraniol–nerol, and loganin with tritium at the various sites, one could obtain *direct* evidence about the following: (a) formation of the cyclopentanoid skeleton (**42** and **43**), (b) the cleavage process **43** → **45**, (c) the rearrangement steps over the late stages of alkaloid modification. This approach has great strength. The results,[32] in the form of tritium loss or retention (Table II), provide fundamental facts

(31) A. R. Battersby, *Chem. Soc. Spec. Period. Rep., The Alkaloids*, 1, 31 (1971).

Table II

Expt no.	Precursor	Site of label in loganin	% retention skeletal ^3H Loganin	51	52	53
1	[1-$^3H_2$,2-^{14}C]Geraniol	C-1	45	45	47	48
2	[2-^3H,2-^{14}C]Geraniol	C-2	95	<5	<5	<5
3	[2-^3H,2-^{14}C]Nerol	C-2	101	<5	<5	<5
4	Sodium (3*R*,4*R*)-[4-$^3H_1$,2-^{14}C]-mevalonate	C-2 and 7	103	49	52	49
5	Sodium (3*R*,4*S*)-[4-$^3H_1$,2-^{14}C]-mevalonate		10 ± 5	<5	<5	<5
6	[5-^3H,O-*methyl*-^3H]-Loganin	C-5		96	99	102

about a complex set of biochemical reactions. Possible biosynthetic schemes can then be rejected or retained depending on their ability to account for these facts.

By the arguments used earlier, it follows from expt 1 that the oxidative step involved in the conversion of C-1 of geraniol–nerol into C-1 of loganin (**43**) is a stereospecific process. Further, the hydrogen atom at this center is unaffected throughout all the remaining stages to the three final alkaloids. In contrast, hydrogen at C-2 of geraniol–nerol is retained as far as formation of loganin (expt 2), and a parallel research proved that this label is still present in secologanin[33] (**45**). However, none of the three types of indole alkaloid (**51**, **52**, and **53**) retained the label from C-2 of geraniol, which pointed to early isomerization of the vinyl residue present in vincoside (**47**) to an ethylidene group, presumably at stage **48**. Formation of ajmalicine (**51**) from **48** is then chemically straightforward. The ethylidene isomerization also pointed to geissoschizine (**49**) as a probable intermediate on the pathway to the *Aspidosperma* (**52**) and *Iboga* (**53**) series. It was satisfying to find that intact incorporations of [*O-methyl*-^3H,*aryl*-^3H]geissoschizine occurred into all three types of indole alkaloid (**51**, **52**, and **53**) and also into the *Strychnos* alkaloid akuammicine[36] (**54**).

Long before the pathway shown in Figure 5 had been elucidated, it had been suggested[37] that there must be biosynthetic significance in the almost complete stereochemical constancy observed at C-15 in the *Corynanthe–Strychnos* group of alkaloids (see **51** and **54**); there was thus special interest in C-7 of loganin. Advantage was taken here of stereospecifically labeled mevalonate (**40**) since it was known in other biological systems[4] that both 4-H_B protons in the two mevalonate units are *retained* to geraniol (**41b**), whereas the two 4-H_A protons are *lost*. Experiments 4 and 5 showed that there is retention of both 4-H_B protons of mevalonate through to loganin (**43**) to label C-2 and C-7. Only one of these two labels in loganin is then lost on formation of the three final alkaloids (**51**, **52**, and **53**), and this is already known by expt 2 to be that at C-2. Therefore, that at C-7 is *retained* throughout, in agreement with the stereochemistry at C-15 of the large *Corynanthe–Strychnos* group being dictated by that at C-7 of loganin.

[5-^3H,*O-methyl*-^3H]Loganin was prepared[38] from dehydrologanin (**44**), and when converted into the alkaloids in *Vinca rosea*, its label was retained at the starred position in each case[39] (**51**, **52**, and **53**).

These experiments put strict limitations on possible mechanisms for the biosynthesis of cyclopentane monoterpenes and for the indole alkaloids. Current work on positions 3 and 6 of loganin will tighten the experimental test still further. The reader should follow the ^3H labels discussed above through the biosynthetic scheme in Figure 5 to confirm that this scheme and the findings in Table II are in accord. In concentrating attention here on tritium labeling, perhaps a reminder is needed that a large amount of evidence is available from other approaches[31,40] which supports the illustrated pathway.

Prospect

The power of tritium labeling as a tool for the exploration of enzyme-mediated reactions will be evident from the foregoing examples and from those in the leading references. This approach will undoubtedly lead to an upsurge of knowledge about enzymatic reactions which change or produce prochiral centers. A network of substrates and enzymes is available now in which the members have been interrelated in stereochemical terms; the future membership will certainly become vast. Increasingly, one enzyme or substrate in the network will be used as part of the attack on another problem. There will be similar intense interest in reactions which produce or destroy methyl groups[4,5] and in the stereochemistry of reactions involving the transfer of methyl groups, particularly from sulfur to carbon, oxygen, and nitrogen. An exciting future prospect is the interlock of this assembly of stereochemical and mechanistic information with knowledge of the enzymatic active site structures from X-ray analysis.

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(33) *Cephaelis ipecacuanha* plants use loganin (**43**) for the biosynthesis of ipecoside[34] (**50**), and this amide is a convenient substance which, in effect, allows study of the labeling in secologanin (**45**). When [2-^3H,1-^{14}C]geraniol was fed to *C. ipecacuanha* plants,[35] there was no loss of tritium over the stages of biological conversion into ipecoside.

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