cerium(IV), but gave a complex mixture of products. When treated with CAN, α-methyl- and α-*tert*-butylphenyldiazomethanes were converted to the corresponding ketones, the diazo groups of azabenzil, $PhC(N_2)COPh$, and ethyl diazoacetate were converted to the corresponding alkyl nitrates, and diphenyldiazomethane was converted to a mixture of benzophenone, benzhydrol, and benzhydryl nitrate. Some of the expected olefins were obtained from α- and β-naphthyldiazomethanes, but additional products were also formed in both cases. Ortho substituents, such as a methyl group or chlorine atom, also caused new products to appear in addition to the expected stilbenes.

Thus, the olefin-formation reaction seems to be very sensitive to substituents, a fact which can be easily rationalized by the above mechanism.

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(7) (a) Alfred P. Sloan Research Fellow, 1970–1972; (b) National Science Foundation (NSF) Trainee, 1967–1969; NSF Predoctoral Fellow, 1969–1971; (c) NSF High School Teacher Research Participant, Summer 1968.

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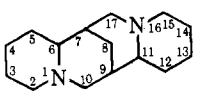
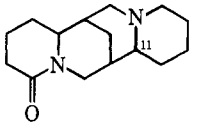
Biosynthesis of Lupin Alkaloids from $^{14}CO_2$. Evidence for the Independent Formation of Lupanine and Sparteine

Sir:

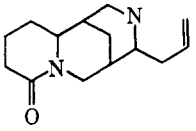
The plant *Lupinus arboreus* is reported to contain the bases sparteine (I) and lupanine (IIa)[1] while another species, *L. angustifolius*, is reported to contain lupanine, α-isolupanine (IIb), angustifoline (III), 13-hydroxylupanine (IV) (plus several of its esters), but not sparteine.[2,3] Sparteine is widely viewed[4,5] to play a primary role in the formation of this group of compounds by a series of successive oxidation reactions in the order



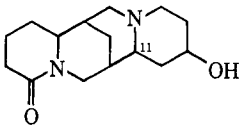
I



IIa (II*R*)
IIb (II*S*)



III



IV

$$\text{I} \longrightarrow \text{IIa} \longrightarrow \text{III} \longrightarrow \text{IV} \qquad (1)$$

A differing view, with the esters of hydroxylupanine playing a primary role, has been put forward on the basis of ontogenetic studies.[2] We now present kinetic evidence from exposure of plants of the above species and *L. polyphyllus* to $^{14}CO_2$ which indicate that sparteine and lupanine may be synthesized independently of one another.

It was anticipated that, following short periods of photosynthesis in an atmosphere of $^{14}CO_2$, early members of such a sequence of oxidations would be labeled more rapidly than latter ones and that their relative specific activities would lend further support to the preceding precursor–product relationships. Though data from this type of experiment cannot offer definite proof for such a sequence, it affords a unique opportunity in the case of CO_2 autotrophs to obtain information about *in vivo* rates of *de novo* synthesis, necessary for conferring on a compound the status of *intermediate* as opposed to that of *presursor*.[6,7] In addition, its potential for revealing kinetically interesting "secondary metabolites" has only begun to be exploited.[7,8]

Twenty-two *L. arboreus* and eight *L. angustifolius* plants, all 6 weeks old, were exposed separately to 3 and 1.2 mCi, respectively, of $^{14}CO_2$, in a flexible polyethylene chamber[7] for 2 hr at which time half of each group was immediately extracted for alkaloids as described previously.[6] The remaining plants were returned to normal air for 8 hr before being so extracted. Recovery of the above alkaloids was >90% by this procedure. The alkaloid extracts were analyzed by glpc-continuous combustion-flow counting[9] with all of the above compounds resolved on a 10% QF-1 column,[8] permitting their simultaneous specific activity determination. Tlc on three systems[8] followed by autoradiography confirmed the glpc labeling patterns. The identities of the sparteine and lupanine peaks eluted from glpc were confirmed by their mass spectra after collection from glpc.[8]

L. arboreus. In addition to the two major alkaloids, sparteine and lupanine, a third minor component eluting prior to lupanine from glpc was noted. Sparteine was the only labeled compound eluted from glpc in either time sample. Tlc–autoradiography confirmed sparteine as the only known alkaloid to be labeled, lupanine being completely inactive. About 25% of the total alkaloid ^{14}C was in several unidentified compounds (tlc–autoradiography) which were not eluted from glpc. By 8 hr, the specific activity of sparteine had nearly doubled yet lupanine was still inactive. Glpc data and specific activities for these two bases are given in Table I.

L. angustifolius. Lupanine, angustifoline, and hydroxylupanine were present in the ratio 5:7:2 as the major components on glpc, together with moderate amounts of two others, tentatively identified as hydroxylupanine ester(s) and α-isolupanine. Lupanine was the only labeled alkaloid detected by glpc of either time sample. Again about 25% of the ^{14}C was not eluted and was accounted for in several unidentified com-

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(3) Labeled sparteine was reported isolated by carrier trapping after feeding [2-^{14}C]lysine to *L. angustifolius* species: E. Nowacki, D. Nowacka, and R. U. Byerrum, *Bull. Acad. Polon. Sci., Ser. Sci. Biol.*, **14**, 97 (1966).
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pounds having the same behavior (tlc) as those in *L. arboreus*. No trace of sparteine was detected in either sample. By 8 hr, the lupanine specific activity had more than doubled, while the remaining alkaloids were still unlabeled. The glpc data and specific activities are tabulated in Table I.

Table I. Glc-Counting Data and Specific Activities of Individual Known Alkaloids of (A) *L. angustifolius* and (B) *L. arboreus*[a]

	Hr after exposure	Amount in μg: Sparteine	Lupanine	Angustifoline	Hydroxylupanine
A	0	*b*	46.4	69.6	24.9
	8		39.8	56.8	23.9
B	0	99.5	62.5		
	8	62.2	20.0		
		Specific Activity in dpm/μg			
A	0	*c*	66		
	8		146		
B	0	71,100			
	8	109,000			

[a] After exposure to $^{14}CO_2$ for 2 hr. [b] Not detected. [c] No activity detected.

Sparteine Carrier Trapping Experiment with *L. polyphyllus*. An experiment similar to the above was carried out with 30, 6-week-old plants of *L. polyphyllus* Russell, samples being taken at 0, 8, and 24 hr after $^{14}CO_2$ exposure. Just prior to maceration of each group of plants, 5 mg of sparteine (an amount close to that of lupanine normally present) was added. The recovered (>90%) sparteine was completely inactive in all times samples, lupanine being the only known alkaloid to be labeled. Angustifoline and hydroxylupanine (and its esters) were again unlabeled, even after 24 hr.

It is clear from these results that in *L. angustifolius* and *L. polyphyllus*, neither angustifoline nor hydroxylupanine is being synthesized at a measurable rate during a period of *de novo* synthesis of lupanine, so cannot be intermediate in the formation of the latter. This is consistent with eq 1 above and the observation that conversion of hydroxylupanine to lupanine is negligible in *L. angustifolius*.[10] It appears to rule out the possibility that lupanine may arise from angustifoline *via* hydroxylupanine;[11] however, the exact relation between angustifoline and hydroxylupanine cannot be settled, since neither was being formed at this stage of development. The inactivity of the hydroxylupanine ester(s) also excludes their serving as intermediates in the formation of lupanine.[2]

The complete absence of labeled sparteine during a period of *de novo* formation of lupanine in *L. angustifolius* and *L. polyphyllus* is in marked contrast to a previous report[3] and may indicate that lysine enters sparteine by a different (abberant) route than does CO_2, the plant's sole natural carbon source. It is however consistent with our observations that lupanine is the first known base to be synthesized in *Thermopsis rhombifolia* and *T. caroliniana*, which do not contain or form any detectable sparteine,[12] but was unexpected in view of the reported high (10%) conversion of sparteine to lupanine (not *vice versa*) by *L. angustifolius*.[10] The opposite finding of no lupanine synthesis while sparteine is being formed *de novo* in *L. arboreus* is surprising, since it is certain from the presence of lupanine in this plant that it can be formed at *some* stage of development. Since the synthesis of lupanine is not occurring *de novo* at this particular stage studied, it cannot be an intermediate in the formation of sparteine and indicates that the formation of each alkaloid may be independent of the other (in time), *viz.*

$$CO_2 \dashrightarrow X \xrightarrow{1} \text{sparteine} \xrightarrow{2} Y \xrightarrow{3} \text{lupanine} \qquad (2)$$

where the enzyme catalyzing reaction 1 is synthesized or activated at an earlier stage of development than that of 2 or 3. Equally compatible is a scheme whereby sparteine and lupanine are both formed from a common precursor Y, *viz.*

$$CO_2 \dashrightarrow Y \begin{cases} \xrightarrow{4} \text{sparteine} \\ \xrightarrow{5} \text{lupanine} \end{cases} \qquad (3a)$$

where the enzyme catalyzing reaction 4 is synthesized or activated at an earlier stage of development than that catalyzing reaction 5. A third possibility is that both are synthesized independently from separate precursors.

A choice between these alternatives may be possible if consideration is given to the following. (1) Sparteine and lupanine do not necessarily occur together in the same species.[2,3,8,13] (2) The oxidation of sparteine to lupanine could involve $\Delta^{1(2)}$-dehydrosparteine as an intermediate which would serve for the formation of either sparteine (by reduction) or lupanine (by oxidation). (3) An unidentified dehydrosparteine was formed when sparteine was administered to *L. luteus* (which contains sparteine but not lupanine).[14] Similarly, an unspecified dehydrosparteine was formed by *L. albus* (which contains sparteine and lupanine).[15,16] (4) Precursor feeding experiments are unable to distinguish an intermediate in a biosynthetic sequence from a side product with which it is in equilibrium.[17] (5) The reverse conversion of lupanine to sparteine has apparently only been examined in *L. angustifolius* where it is reported to be negligible.[10]

The simplest explanation encompassing the $^{14}CO_2$ data and the foregoing may be schematized (eq 3b)

$$\begin{array}{ccccc} & & \text{sparteine} & & \\ & & +2H \uparrow \quad \downarrow -2H & & \\ CO_2 & \dashrightarrow & \Delta^{1(2)}\text{-dehydrosparteine} & \xrightarrow{-2H} & \text{lupanine} \end{array} \qquad (3b)$$

with $\Delta^{1(2)}$-dehydrosparteine as the branching point in the synthesis of lupanine and sparteine. The ability of sparteine to serve as a precursor of lupanine would depend on the reversibility of reaction 4, eq 3a, or the presence, in some species, of a quinolizidine dehydrogenase such as has been demonstrated in *L. albus* and the nonalkaloid containing *Vicia faba* and *Phaseolus vulgaris*.[15,18] The position of unsaturation in the dehydrosparteines previously reported thus assumes a new importance. From the known behavior of $\Delta^{1(2)}$-

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dehydro-17-oxosparteine (lupanoline),[19] if $\Delta^{1(2)}$-dehydrosparteine were present, it should have been isolated by the procedures used here, but might not have been eluted during glc, because of the high polar (carbinolamine) character of the free base form of the compound. Clearly, additional studies on *L. arboreus* and other species known to produce both sparteine and lupanine are required.

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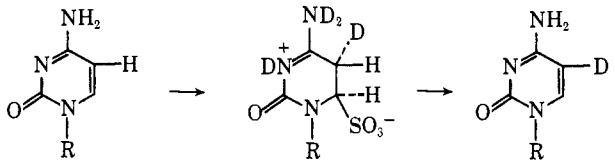
Bisulfite-Catalyzed Isotope Labeling of Cytidine 5′-Phosphate at Its 5 Position

Sir:

Bisulfite adds to the 5,6-double bond of pyrimidine nucleosides.[1-5] Cytidine undergoes a reversible addition of bisulfite to give 5,6-dihydrocytidine 6-sulfonate (**1**).[1,3] In a concentrated bisulfite solution the reaction rapidly reaches a point where cytidine and **1** are in an equilibrium. Regeneration of cytidine from **1** can be achieved either by removal of the bisulfite salt from the reaction mixture or by adjusting the pH of the mixture at a value higher than 8. A concomitant reaction that occurs in the equilibrium, cytidine ↔ **1**, is the deamination of **1** to give 5,6-dihydrouridine 6-sulfonate. We now wish to report that the bisulfite-catalyzed equilibrium between cytidine and **1** can be utilized for the isotope labeling of the 5 position of cytidine and cytidine 5′-phosphate. Conditions will be described with which practically no deamination of **1** takes place and yet a satisfactory incorporation of the isotope is obtained (see Scheme I).

Scheme I



1 in D_2O

Previous experiments have shown that more **1** is formed from cytidine in acidic bisulfite solutions than in a neutral bisulfite solution, and that the deamination of **1** is most pronounced at pH 4–6. Nmr studies have revealed that the preparation of **1** in 1 *M* $NaDSO_3$ at pD about 4, followed by the regeneration, in D_2O, of cytidine therefrom results in no incorporation of deuterium into the cytidine molecule.[3] It has now been found that when a D_2O solution of a mixture of cytidine 5′-phosphate and bisulfite is allowed to stand at pD 7.7, an exchange of deuterium with the hydrogen at C-5 of the cytosine ring takes place. The exchange was detected by directly measuring the nmr spectrum of the reaction mixture. Aside from signals at 3–6 ppm due to the adduct **1**,[3] signals of cytosine-ring protons were present at 6–8 ppm. As the exchange proceeded, the doublet signal centered at 8.01 ppm due to the 6-H of the cytosine ring changed into a triplet signal consisting of a singlet, due to the 6-H of the 5-D species, and a doublet, due to that of the 5-H species. This singlet was located at the middle of the doublet and the three peaks were well separated. Therefore the reaction extent can be accurately determined by measuring these signal strengths. As expected, the doublet at 6.13 ppm due to the 5-H decreased as a function of the time of treatment. However, this signal always remained a doublet, indicating no exchange of 6-H with deuterium. In order to minimize the hydrolysis of the 4-amino group during the reaction, we employed ammonium bisulfite as the source of the bisulfite salt, taking into account the recent report that an exchange amination of cytidine occurs in the presence of bisulfite.[6] The pH of the reaction mixture was fixed to the desired value by addition of sodium bisulfite. Extents of deuterium exchange and deamination were examined for cytidine 5′-phosphate using various reaction conditions. As Table I shows, both the incorporation of deuterium and the deamination proceeded slowly under the conditions employed. Both of these reactions were faster at a higher concentration of bisulfite and in more acidic solutions. However, it can clearly be seen that

Table I. Effect of Concentration and pH of Bisulfite Buffer on Deuterium Incorporation and Deamination of Cytidine 5′-Phosphate[a]

Expt no.	Concn of bisulfite (*M*)	pH[b]	pD[c]	Incorp of deuterium, % 24 hr	72 hr	Deamination, % 24 hr	72 hr
1	1.06	7.1		33.0		4.3	
2	0.96	7.5	7.7	14.2	24.6	0.6	2.1
3	1.88	7.2		53.6		6.4	
4	1.75	7.5	7.6	29.6	54.3		3.8
5	2.62	7.2		68.3		12.7	
6	2.48	7.5	7.6	46.2	68.7	2.3	8.5
7	2.40	7.7	7.9	29.4	54.6	1.5	3.1

[a] Disodium cytidine 5′-phosphate, 100 mg, was dissolved in 1 ml of D_2O. To this was added ammonium sulfite and sodium bisulfite in the amounts as listed below, and the resulting solution was allowed to stand at 37° in a tightly stoppered tube. Experiment number/$(NH_4)_2SO_3 \cdot H_2O$/$NaHSO_3$: 1/134 mg/20 mg; 2/134 mg/7.5 mg; 3/268 mg/30 mg; 4/268 mg/14 mg; 5/402 mg/40 mg; 6/402 mg/20 mg; 7/402 mg/10 mg. The extent of deamination was determined as follows. An aliquot (10 μl) was withdrawn and mixed with concentrated ammonia (10 μl) and the whole solution was subjected to two-dimensional cellulose thin-layer chromatography. The chromatographic solvents used were: first dimension, isobutyric acid–0.5 *N* NH_4OH (10:6, v/v); second dimension, isopropyl alcohol–concentrated HCl–water (75:17:8, by vol). Only two spots corresponding to cytidine 5′-phosphate and uridine 5′-phosphate were detected. Each of these compounds was eluted from the chromatogram with 0.01 *N* HCl and its quantity was determined by the uv spectrum. As a reference, a blank solution was prepared by eluting a non-uv absorbing zone of the same size on the thin-layer plate. [b] The pH values are those determined for corresponding H_2O solutions. [c] Reading on a pH meter.

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