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Biosynthesis of Natural Products

Problems of substrate nonpermeability and location of isotopic label are described.

A. Ian Scott

Natural products, for many years the touchstones of structural and synthetic organic chemists, continue to be discovered from various sources; and 1000 structures are added annually to a storehouse of some 12,000 described molecules whose molecular weights are between 20 and 2000. These compounds are usually referred to as "secondary" metabolites because their biochemical function in the host organism is in most cases obscure. Yet in the few cell-free systems that have been developed to study the biosynthesis of natural products, the enzymology appears to follow the operation of mechanisms identical or closely allied to "primary" metabolism. In view of the considerable practical difficulties in the preparation of the enzymes of secondary metabolism, knowledge of this field has mainly been derived from the results of administration of ^{14}C-, ^{2}H-, and ^{3}H-labeled substrates to the intact plant or organism, with subsequent appropriate degradative techniques to locate the label, and, more recently, from the use of isotope ratio techniques to ensure nonrandomization. Although great progress in experimental techniques has been made, it seems to be an intrinsic property of many higher plant systems that specific incorporations of between 0.01 and 0.1 percent represent an average level of success. The purpose of this article is to suggest that several techniques can be used to improve a given situation which at first sight might be indicative of negative incorporation of a suspected precursor. In addition, in systems where incorporation of 5 percent or more can be achieved (usually in fungi or bacteria) the use of ^{13}C as biosynthetic label offers unique advantages and can be combined very successfully with the results of other kinds of radioactive labeling experiments.

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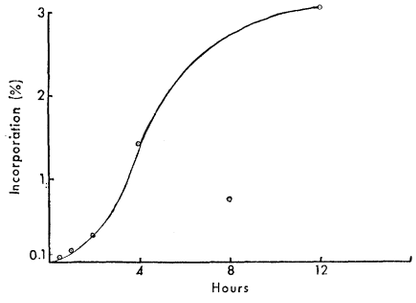
Identification of Intermediates by Short-Term Incubation in Vinca rosea

Of the plants in which indole alkaloids occur, perhaps none holds more interest for the chemist or the biochemist than *Vinca rosea* in that the periwinkle produces a profuse array of indole alkaloids including some 100 representatives of all the important structural classes (*1–3*). It is also an ideal plant with which to begin cell-free studies, for the viable seeds are readily available, easy to cultivate, and the seedlings possess a remarkable capacity for synthesizing alkaloids with a vigor that is perhaps matched only by the chemotaxonomists who have bestowed such complex names on these compounds.

Earlier biosynthetic studies (*2*) with *V. rosea* utilized either intact shoots or seeds. After some experimentation it was decided to use young seedlings (9 to 17 days from germination) grown from a mixed strain (Burpee) at 33°C in an environmental chamber with full light. Intact seedlings were removed and placed in groups of four (the average weight was 16 milligrams) in ¼-dram vials (1 dram = 1.8 grams). The seedlings remained healthy and continued to develop root growth for about 9 to 10 days in water at 33°C. The range of alkaloids present in the 9-day seedlings approximated to that of mature *V. rosea*, and the more predominant compounds were easily detectable by thin-layer chromatography (TLC) (*4*). These were vindoline (**13**), coronaridine (**12**), catharanthine (**11**), akuammicine (**5**), and vinervine (**6**) (see chart 1). One of the main difficulties in carrying out cell-free studies with *V. rosea* is the absence (or virtual absence) in the mature plant of many alkaloids which correspond to the intermediates of the various pathways. In practical terms this will probably require preparative scale cell-free incubations to isolate and characterize these dynamic compounds. However, with the use of short-term incubation techniques with the above seedlings, some progress can be made toward the solution of this problem.

The main events of indole alkaloid biosynthesis based on earlier incorporation data (*2*) are summarized in chart 1. Here the structures marked with an asterisk (*) correspond to alkaloids identified below. The remaining alkaloids have already been described from mature plants and germinating seeds of *V. rosea* (*2*).

Incorporation of tryptophan into the alkaloids takes place in two distinct phases. For the first 2 hours of incubation it is linear (Fig. 1), but after this interval a rapid increase in the rate of incorporation is observed. Between 12 and 48 hours, a maximum of 3 percent is reached and this level is maintained during the full time of the experiment



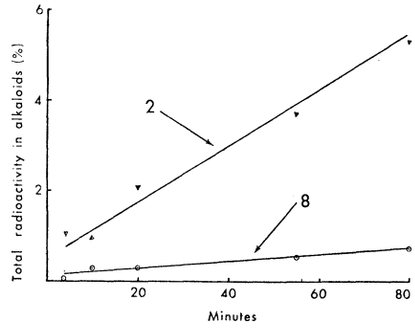


Fig. 1 (left). Incorporation (percent) of DL[^{14}C]tryptophan into alkaloids in *V. rosea* seedlings during 12 hours. Fig. 2 (right). Relative radioactivities of geissoschizine (**2**) (triangles) and tabersonine (**8**) (circles) during the first 80 minutes of DL[^{14}C]tryptophan feeding.

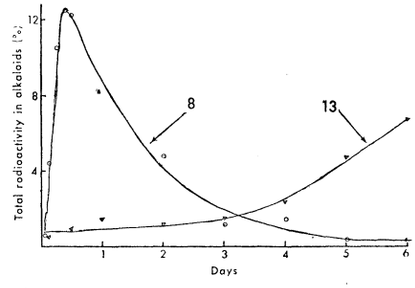


Fig. 3. Comparison of radioactivity in tabersonine (**8**) (circles) and vindoline (**13**) (triangles) in *V. rosea* seedlings after administration of DL[^{14}C]tryptophan during 6 days.

(up to 8 days). It is concluded from these results that no large pool of tryptophan is present and also that 8 days is insufficient time for the distribution of radioactivity to approximate to the "normal" percentage of alkaloid distribution in the seedlings since after 8 days vindoline (**13**) (a major alkaloid of *Vinca*) contains only 7 percent of all the activity. Shoots of mature *Vinca* which have been used for most of the available feeding data incorporate tryptophan but at a reduced initial rate, and the final (constant) percentage is about 1.0. The latter observation is of particular interest for comparative studies with diversely aged biological material.

As can be seen from Fig. 2, activity is being incorporated into geissoschizine (**2**) at eight times the rate of incorporation into tabersonine (**8**). This is consistent with geissoschizine being a precursor of tabersonine, and, although the experiment does not demonstrate this relationship, the necessary evidence for the sequence has been secured in another way (*2*). Further, the geissoschizine and tabersonine pools have not in 80 minutes reached their maximum value of radioactivity. In fact the tabersonine pool attains maximum radioactivity after 9 hours (Fig. 3). At this time [2-^{14}C]tryptophan with a specific activity of 52 millicuries per millimole gives rise to tabersonine of specific activity of 15.6 mc/mmole (30 percent specific incorporation), indicating the extremely small size of the tryptophan pool available for alkaloid synthesis and of tabersonine. Autoradiograms show that tabersonine is metabolized almost as rapidly as it is formed, and in fact its activity falls (Fig. 3) from 12 to 2 percent in 3 days. Refeeding ^{14}C-labeled tabersonine isolated after tryptophan had been fed for 9 hours gives a number of labeled metabolites. After 1 day when there is still a large amount of unchanged tabersonine (**8**), it was found that epoxytabersonine (**9**), methoxytabersonine (**10**), and coronaridine (**12**) are all labeled. After 6 days there is almost no tabersonine remaining while vindoline (**13**) has gained activity (Fig. 3).

The role of epoxytabersonine [(−)-lochnericine] (**9**) in the overall biosynthetic map is at present obscure. It is a minor constituent of the normal alkaloid pattern of *Vinca*, but after 9 hours it has 4.3 percent of all the activity and a specific activity of 10 mc/mmole (from tryptophan of specific activity 52 mc/mmole). It is formed from tabersonine (**8**) as shown from refeeding [^{14}C]tabersonine. It is not converted to tabersonine, nor is it transformed to vindoline.

Although vindoline can be detected on autoradiograms after 1 day, its activity does not begin to increase rapidly until after 3 days (Fig. 3). At this stage the amount of radioactivity in the alkaloids has reached a maximum, and the activity in vindoline will presumably increase rapidly to a maximum value that will not alter unless vindoline is broken down. In contrast, the amount of radioactivity in the *Strychnos* alkaloid akuammicine (**5**) (approximately 8 percent of the total) and its phenolic derivative vinervine (**6**) (14 percent!), remains unchanged from 9 hours to 6 days, a fact which suggests that these alkaloids are shunt

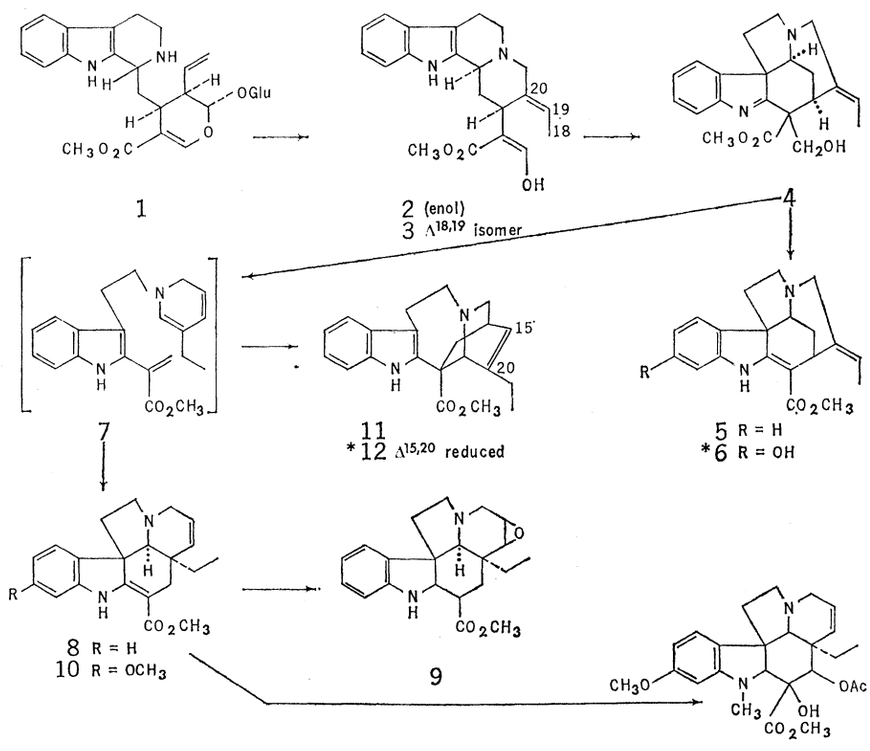


Chart 1. Structures of the monomeric alkaloids of *V. rosea*.

products formed from a rapidly metabolizing key intermediate postulated (*2*) to be preakuammicine (**4**) at a constant rate during the early part of the biosynthesis, that is, when the incorporation of label from tryptophan is still increasing.

The precursor activity of tabersonine (**8**) for both vindoline (**13**) (*Aspidosperma*) and coronaridine (**12**) (*Iboga*) emerges from the above experiments and confirms the earlier findings with seeds and shoots. The pathway from tabersonine to coronaridine (**12**) has until recently been presumably via catharanthine (**11**) and presents an intriguing problem of sequence, for the postulated acrylic ester (**7**) has been invoked at two stages in the biosynthesis (*2*), that is, after geissoschizine (**2**) and again after tabersonine (**8**). The obligatory role of latter alkaloid *Iboga* synthesis may require reassessment when the relative incorporation data is available at the enzyme level. However, the special place of tabersonine in the pattern of alkaloid synthesis in *Vinca* is demonstrated by the foregoing results because after 9 hours this alkaloid is the dominantly labeled species—yet it is almost undetectable on the sixth day

In summary the technique of short-term incubation has led to the discovery of both static and dynamic constituents of the maturing seedling. The specific incorporation of tryptophan into the alkaloids is approaching a more satisfactory value (25 to 30 percent), and the profiles revealed for tabersonine and geissoschizine are suggestive that true intermediates can be identified and subsequently confirmed by extension of this easily applied technique to other species.

Biosynthesis of Strychnine in Strychnos nux-vomica

In contrast to the relative ease of incorporation of the building blocks and indeed the larger intermediate substrates in *V. rosea*, species such as *Rauwolfia serpentina* and *Strychnos nux-vomica* have, from the outset of the biosynthetic studies, displayed most disappointing properties as far as precursor uptake has been concerned. Indeed if these had been the only plants available for study it might be suggested that our knowledge of the intricate mechanisms whereby mevalonate and tryptophan are cometabolized to the complex indole alkaloids of the major structural types (for example, the *Strychnos* alkaloids) would still be in its infancy. Thus the administration of geissoschizine (**2**) [a known precursor of the "*Strychnos*" alkaloid akuammicine (**5**) in *V. rosea*] to *S. nux-vomica* under "normal" feeding conditions (5 to 7 days) led to insignificant ($<2 \times 10^{-3}$ percent) incorporations into strychnine (**14**). More surprisingly the Wieland-Gumlich aldehyde (**15**) and diaboline (**16**), which seem almost certain candidates for intermediacy (near the end of the pathway), failed to incorporate over the same time scale. Reinvestigation of the problem involved the technique of replanting young seedlings after 5 days incubation with **2, 15, 16,** and **17** labeled with ^{3}H in the aryl group. After a further 100 days strychnine was isolated from each feeding, crystallized to constant radioactivity, and (in the case of positive incorporation) degraded to locate the position of the tritium label. Reference to Fig. 4 shows that indeed geissoschizine (**2**) fulfills the same pivotal role in *S. nux-vomica* as it does in *V. rosea*, but that the result would have been in doubt without resource to the "prolonged contact" technique. Similarly the anticipated but hitherto unrealized conversion of Wieland-Gumlich aldehyde (**15**) to strychnine (**14**) (1.6 percent) could be demonstrated (*5*). Finally the nonincorporation of diaboline (**16**) after 70 days (*6*) suggests strongly that the seventh ring of the complex strychnine molecule is built in a clockwise carbon-to-carbon addition of the acetic acid unit, rather than from the nitrogen-carbon condensation which would have required diaboline as an intermediate. The nonincorporation of geissoschizal (**17**) reveals that, by a hitherto undisclosed mechanism, a carbon atom is lost somewhere between geissoschizine and Wieland-Gumlich aldehyde but not by a simple decarbomethoxylation reaction (*5*).

These experiments do not begin to answer the question of genetic control and evolution in a plant such as *S. nux-vomica*, which, although producing quite massive concentrations of strychnine and brucine by a process now shown to emanate from geissoschizine, does not elaborate the variety of rearranged structures of the *Aspidosperma* and *Iboga* families engendered by *V. rosea* from the latter precursor.

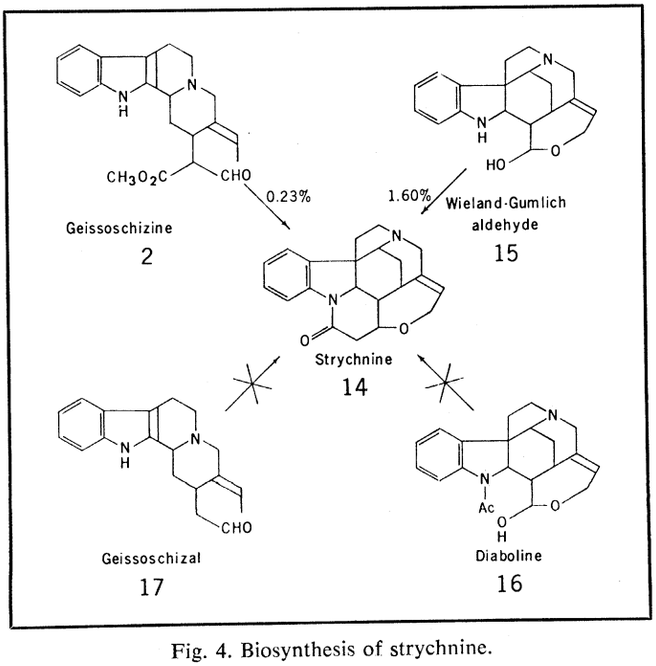


Fig. 4. Biosynthesis of strychnine.

Application of ^{13}C-Labeling in the Biosynthesis of Natural Products

Although ^{13}C was used in the early studies of steroid biosynthesis and its distribution was analyzed by mass spectrometry, it is only recently that the first reports of the application of continuous wave and Fourier transform ^{13}C nuclear magnetic resonance (NMR) spectroscopy to the solution (without recourse to degradation of the intact metabolite) of a precursor relationship have appeared. Several descriptions of the techniques are now available (*7*) and, provided that successful assignment of the natural abundance spectrum is made, enrichments above the natural abundance (1.1 percent) of ^{13}C can be used to delineate the pathway with 60 to 90 percent enriched substrates. A recent example from our own laboratory (*8, 9*) may be used to illustrate the power of this tool (along with ^{14}C labeling) in the study of the intermediary metabolism

of one of the most complex of natural products, vitamin B_{12} (**19**; cyanocobalamin). Several other recent biosynthetic studies with ^{13}C have been made (*7*).

Biosynthesis of Vitamin B_{12}

Early experiments with ^{14}C in *Propionibacterium shermanii* had revealed that the pathway succinate-glycine to δ-aminolevulinic acid (**20**) to cobyrinic acid (**21**) obtains in corrin biosynthesis. Thereafter by successive amidation and addition of the aminoisopropanol-phosphate-ribose-dimethylbenzimidazole side chain and finally the cobalt atom the full structure of the vitamin and coenzyme are reached. First, Fourier transform ^{13}C NMR was used to confirm that eight molecules of aminolevulinic acid were utilized, and this was borne out by feeding [2-^{13}C]aminolevulinic acid (240 mg) to *P. shermanii* (500 grams of wet cells) for 70 hours. The resultant cyanocobalamin showed enrichment of seven methylene and one methyl signal (Fig. 5a), a result in full accord with this concept (*9*). Similarly the intact incorporation of 4 moles of [8-^{13}C]porphobilinogen (**22**) is revealed in Fig. 5b, where dicyanocobalamin was used to separate the otherwise convergent signals in cyanocobalamin (the *mono*cyano form of the vitamin). It has always seemed an attractive possibility by analogy with the magnesium (chlorophyll) and iron (heme) pathways that the cobalt pathway forms B_{12} from uroporphyrinogen III (**23**) by a methylative ring-contraction sequence. This has now been shown (*8*) to be correct by obtaining the same pattern (Fig. 5) of labeling as in the [8-^{13}C]porphobilinogen experiment by feeding **23** prepared in turn from the [8-^{13}C]porphobilinogen specimen used to obtain the data in Fig. 5b. All that remains is to account for the so-called "extra" methyl groups in B_{12}, of which there seemed to be either six or seven. The latter number is shown to be correct by feeding experiments with [5-^{13}C]aminolevulinic acid in which only seven enrichments were obtained at sp^2 carbons but none in the methyl region of corrin; and ^{13}C-enriched methionine which, in the derived dicyanocobalamin (Fig. 5d), shows the insertion of seven methionine-derived methyl groups (*9*). Furthermore, comparison of the Fourier transform ^{13}C NMR spectra of cobinamide and neocobinamide enriched

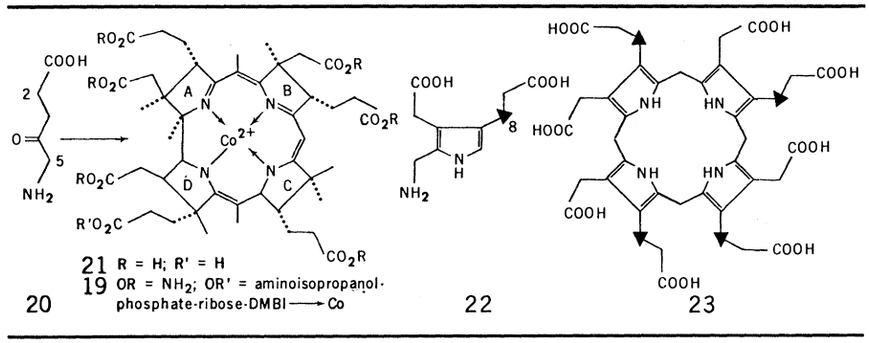


Chart 2. Biosynthesis of B_{12}.

with [^{13}C]methionine reveals (*10*) a downfield shift of its enriched methyl group in ring C corresponding to a deshielding of 11.7 parts per million (Fig. 6). Hence the stereochemistry of methylation in ring C must be in the α-configuration (*10*). The second methyl group of the gem-dimethyl array in ring C comes from decarboxylation of an acetate side chain of porphobilinogen; that is, it is derived from carbon 2 of aminolevulinic acid (see Fig. 5a). Thus the pathway porphobilinogen to uroporphyrinogen III, and the gain of seven methionine-derived methyl groups can be used to construct a mechanism for the process.

Such a mechanism embodying these results has been suggested (*10*) (Fig. 7). The stage is now set for the approach to the enzymology of the various steps in corrin formation between uroporphyrinogen III and cobyrinic acid. To this end a cell-free system has been developed (*11*) which converts aminolevulinic acid to cobyrinic acid,

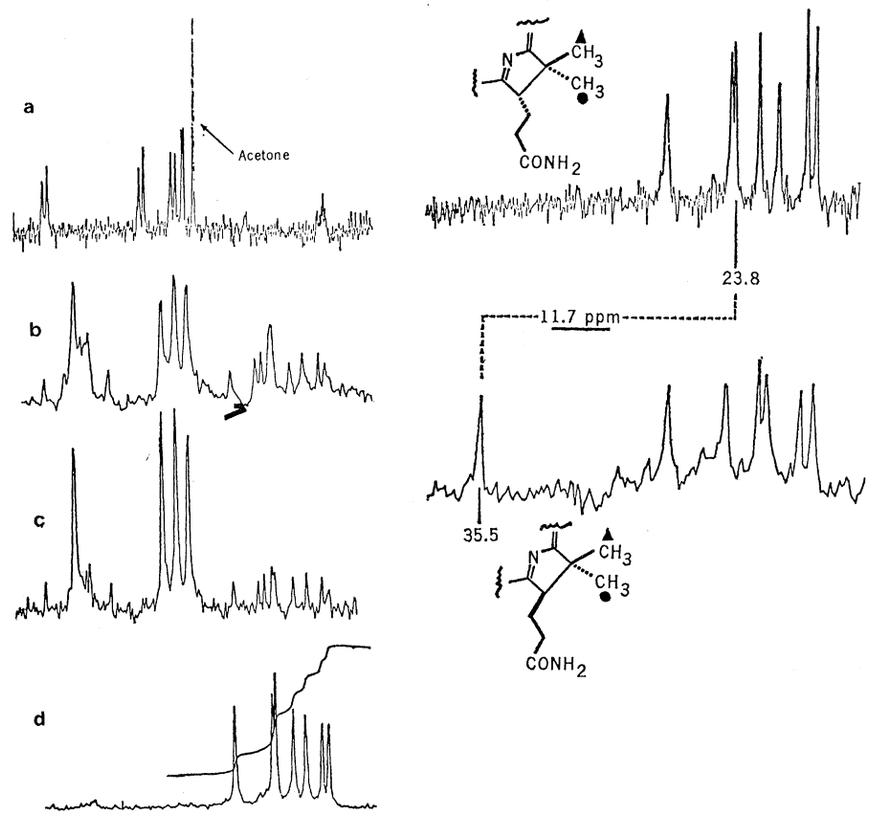


Fig. 5 (left). Proton-noise decoupled Fourier transform ^{13}C NMR spectra of vitamin B_{12} as (a) cyanocobalamin enriched with [2-^{13}C]aminolevulinic acid; (b) and as dicyanocobalamin enriched with [8-^{13}C]porphobilinogen; (c) uroporphyrinogen III; and (d) [methyl-^{13}C]methionine. Details of chemical shift data and experimental conditions are described in (*9*). Fig. 6 (right). Comparison of the proton-noise decoupled Fourier transform ^{13}C NMR spectra of dicyanocobinamide (top) and dicyanoneocobinamide (bottom). Chemical shifts are downfield from hexamethyldisilazine.

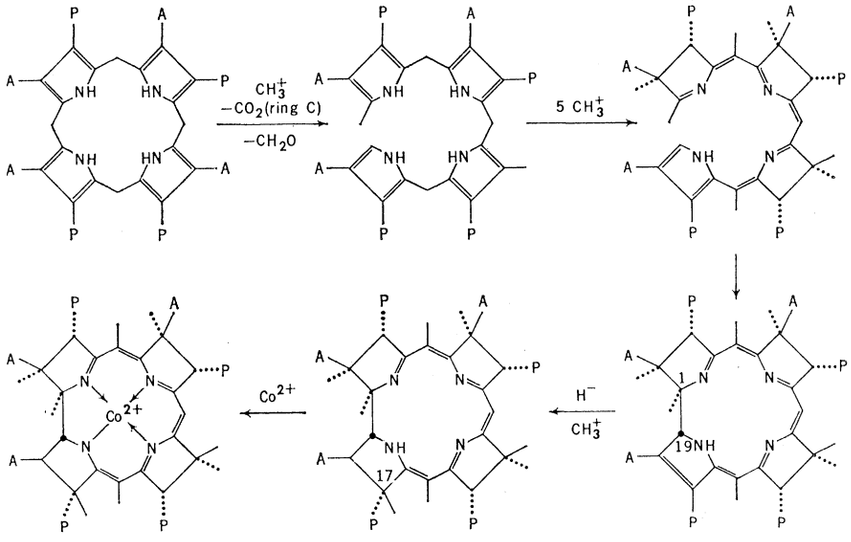


Fig. 7. Proposed mechanism for the biosynthesis of cobyrinic acid from uroporphyrinogen III.

and in addition utilizes uroporphyrinogen III as a precursor, thereby removing the permeability problems associated with larger precursors. Fractionation of the crude homogenate on a large scale to permit ^{13}C analysis of the interactions between enzyme and substrate is in progress.

Summary and Conclusions

The experimental verification of a proposed biosynthetic pathway for a given natural product is often difficult to obtain with the use of the whole organism (permeability factors) or, in the case of higher plants, a cell-free system. Until the purified enzyme for each step of biosynthesis is available, biosynthetic studies can, however, be carried out, albeit with modest incorporation values, by means of either hydroponic or injection methodology. Where viable seed sources are available it is suggested that improvements of several orders of magnitude in incorporation can be achieved by a short-term incubation (small pool size of precursors; trapping of reactive intermediates) or a long-term feeding (equilibration of precursor with the compartmentalized or induced synthetases). In bacterial, fungal, mammalian, and plant systems, where incorporation efficiencies provide the opportunity to study ^{13}C enrichment (at least equal to natural abundance of the isotope), we can expect a rapid expansion since the method removes the tedium of carbon-by-carbon degradation. For the next few years, however, the prognosis would seem to favor parallel studies of ^{13}C and ^{2}H, and of $^{14}C/^{3}H$ ratio techniques since the last-mentioned method provides more information concerning the stereoselectivity of labeling processes on the microgram scale.

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