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METHANE INCORPORATION BY PROKARYOTIC  
PHOTOSYNTHETIC MICROORGANISMS

Charles J. Norton, Martha Kirk, and Melvin Calvin

August 1970

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METHANE INCORPORATION BY PROCARYOTIC  
PHOTOSYNTHETIC MICROORGANISMS

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ABSTRACT

The procaryotic photosynthetic microorganisms Anacystis nidulans, Nostoc and Rhodospirillum rubrum have cell walls and membranes that are resistant to the solution of methane in their lipid components and intracellular fluids. But Anacystis nidulans possesses a limited bioxidant system, a portion of which may be extracellularly secreted, which rapidly oxidizes methane to carbon dioxide. Small C<sup>14</sup> activities derived from CH<sub>4</sub> in excess of experimental error are detected in all the major biochemical fractions of Anacystis nidulans and Nostoc. This limited capacity to metabolize methane appears to be a vestigial potentiality that originated over two billion years ago in the early evolution of photosynthetic bacteria and blue-green algae.



## I. INTRODUCTION

Silverman<sup>1</sup> reviewed and pointed out that in general very little is known about the abilities of microorganisms to utilize methane:

"The predominant methane-oxidizing species reported to date are either pseudomonads or mycobacteria. It is important to discover whether so widespread a phenomenon as methane oxidation, in contrast to the oxidation of other hydrocarbons, is restricted to these two general groups of bacteria. It would be interesting to see if there are other methane-oxidizing microorganisms. ... Do other microorganisms exist in nature, incapable of growth at the expense of methane alone, but able to oxidize it when other organic compounds furnish the necessary carbon and energy for growth?"

Virtually nothing is known about the ability of the lower photosynthetic organisms, especially the prokaryotes, which appear to be transitional between nonphotosynthetic bacteria and the higher photosynthetic eucaryotic green algae, to utilize methane.

We have carried out preliminary investigations with C<sup>14</sup>-labelled methane to investigate the abilities of Rhodospirillum rubrum, Nostoc, and Anacystis nidulans to incorporate and possibly assimilate methane. The idea was entertained that photobacteria which do not liberate oxygen from water during photosynthesis might use methane as an alternative electron source, similar to the way photosynthetic anaerobic Thiorhodaceae bacteria use hydrogen sulfide.

The two major questions asked are, "Do prokaryotic photosynthetic organisms have any ability to oxidize methane? If so, by what mechanisms may this oxidation be effected?"

## II. EXPERIMENTAL

### A. The Microorganisms

The photosynthetic prokaryotes investigated were the photobacterium Rhodospirillum rubrum, and the blue-green algae Anacystis nidulans and Nostoc. These are illustrated in Figures 1, 2, and 3<sup>2,3</sup>. These microorganisms were cultured in the microbiology laboratory from type cultures on standard growth media (Tables 1-A, 2-A, and 3-A) and monitored for culture purity.

#### 1. Semicontinuous culture

The blue-green algae Anacystis nidulans and Nostoc were cultured under semicontinuous routine microbiological laboratory conditions in blue-green algae media (Tables 1-A and 2-A) and harvested at convenient bi-weekly intervals for the shake-flask and steady-state experiments.

Rhodospirillum rubrum was cultured under routine microbiological laboratory conditions and a 10.0 ml volume taken for inoculation of sterilized standard medium (Table 3-A) in a 25 ml rubber-septum capped serum bottle.

#### 2. Algae harvesting

The algae were harvested fresh immediately before each experiment by centrifugation at 20°C in 250 ml centrifuge bottles in an International Centrifuge operated at 1600 rpm for 10 minutes. The supernatant fluid was decanted off and the packed cells were redispersed and rinsed into

calibrated 12.0 ml centrifuge tubes with medium diluted to 20% of the original concentration and centrifuged at 2800 rpm for 10 minutes. The wet, packed cell volume was read and a calculated volume of 20% diluted media used to quantitatively transfer and dilute the resuspended cells to give a 1.0% suspension of cells in the final 44 ml shake flask or 80 ml steady-state apparatus.

3. Adaption with methane

In subsequent experiments, the semicontinuous culture was modified, replacing the normal 5% CO<sub>2</sub>-containing air line with a gas stream of 1% cold methane, 4% CO<sub>2</sub> and 95% air. In a few experiments this line was changed to introduce 1% cold methane in 99% nitrogen freed of oxygen by bubbling through chromous chloride reagent<sup>4</sup>. The copresence of CH<sub>4</sub> in CO<sub>2</sub>-containing air streams had little affect on the densities of cells harvested, but prolonged exposure to CH<sub>4</sub> in the absence of CO<sub>2</sub> eventually killed the Anacystis nidulans culture.

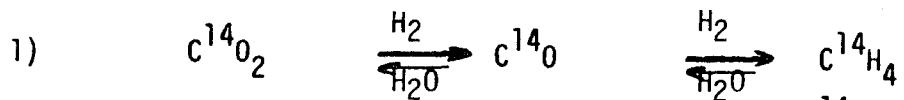
4. C<sup>14</sup>H<sub>4</sub>

The undiluted C<sup>14</sup>H<sub>4</sub> was obtained from the New England Nuclear Corporation (NEN) in 38 ml (including the external vial stem) breakseal flasks labelled NEC-060 Methane-C<sup>14</sup> 0.50 millicurie/1.5 milligrams. The specific activity was 5.3<sub>4</sub> mC/m mole.

5. Impurities in C<sup>14</sup>H<sub>4</sub>

The New England Nuclear Corporation was unable to detect any significant labelled impurities ( $\geq 0.1\%$ ) by gas-liquid chromatography. We also were unable to detect labelled impurities  $\geq 0.1\%$  with a gas-liquid

chromatographic apparatus equipped with a proportional counter. But extraction of the breakseal contents with 1.00 ml of aq. 2N NaOH indicated the presence of  $0.075 \pm 0.002\%$  ( $0.375 \mu\text{C}$ ) base-soluble gaseous impurity, presumably mostly  $\text{C}^{14}\text{O}_2$ , but perhaps including a little  $\text{C}^{14}\text{O}$  also. Subsequent treatment of an aliquot of this base-treated  $\text{C}^{14}$  methane with 0.25 ml of Arapahoe Chemical Company 6M phenyl Grignard reagent<sup>5</sup> in 2 ml anhydrous ether under nitrogen, decomposition with dilute aq. HCl, ether extraction, careful evaporation, and solution in 18.0 ml of scintillation fluid indicated the residual presence of 0.0073% ( $0.037 \mu\text{C}$ ) of  $\text{C}^{14}\text{O}$ . The presence of  $\text{C}^{14}\text{O}$  was also confirmed by its removal by treatment with ammoniacal cuprous chloride. These are reasonable impurity reagent levels to be anticipated from the incomplete catalytic reduction<sup>6</sup> and purification of  $\text{C}^{14}\text{H}_4$  prepared from  $\text{C}^{14}\text{O}_2$ :



After these discoveries, it is obvious that  $\text{C}^{14}\text{H}_4$  free of  $\text{C}^{14}\text{O}_2$  and  $\text{C}^{14}\text{O}$  could be best prepared from  $\text{C}^{14}\text{H}_3\text{I}$  by the Grignard reaction and subsequent decomposition. Unfortunately, the available International Chemical and Nuclear (Cat. No. 0747)  $\text{C}^{14}\text{H}_3\text{I}$  specific activity 53 mC/m mole, was exhausted in early experiments and more was not immediately available at the close of the first author's postdoctoral research stay at Berkeley.

#### 6. Culture apparatus

Small-scale culture studies were made in a flattened 44 ml flask stoppered with a rubber septum in the illuminated apparatus illustrated in Figure 4.<sup>7</sup> Samples of the culture and gas were taken with time with small syringes.

The steady-state apparatus (Figure 5) which was used in several larger-scale experiments has been well described in the literature.<sup>8</sup> Convenient 2.0 ml samples were taken with time and quenched with aq. 80% ethanol. In one experiment the gas phase at the end of the experiment was circulated for 30 minutes through two traps containing aq. 2N NaOH to absorb CO<sub>2</sub> and one containing dimedone reagent<sup>9</sup> to detect formaldehyde.

#### 7. Dilution of C<sup>14</sup>H<sub>4</sub> with cold methane

For the small-scale shake-flask experiments, the C<sup>14</sup>H<sub>4</sub> in the breakseal was diluted directly with 99% research grade cold methane. The dilution was effected at laboratory pressure and temperature by inverting the breakseal flask, adding a cylindric magnetic iron bar (1.1 ml volume), capping the stem with a small rubber septum (secured with copper wire), evacuating and flushing the volume between the glass breakseal tip and the septum with dry N<sub>2</sub> several times with the aid of a syringe needle on the end of a house vacuum line, after final evacuation inverting the breakseal quickly to break the seal, and slowly adding cold methane at low pressure until the total pressure in the breakseal flask slightly exceeded atmospheric pressure as indicated by a small bubbler lead under soap solution communicating to the breakseal by a small syringe needle tip (Figure 6). This procedure was found to be very simple, practical, and more convenient than a cumbersome classic gas buret system first tried on the C<sup>14</sup>H<sub>4</sub> prepared from C<sup>14</sup>H<sub>3</sub>I by the Grignard reaction. In several steady-state experiments the C<sup>14</sup>H<sub>4</sub> was transferred directly into the recycle gas line without dilution with cold methane.

8. Removal of C<sup>14</sup> Impurities

A 1.00 ml volume of aq. 2N NaOH was added to the breakseal flask and carefully shaken to absorb the C<sup>14</sup>O<sub>2</sub> impurity. The breakseal flask was stored upside down to reduce contact and absorption of C<sup>14</sup>H<sub>4</sub> by the rubber septum. The specific activity after dilution was 0.020 mC/mg of methane. As each 1.00 ml volume (activity of 2.97  $\times$  10<sup>6</sup> dpm) was withdrawn with a volumetric gas syringe from the breakseal for analysis or shake-flask experiments, an additional 1.00 ml of aq. 2N NaOH was added to maintain the original atmospheric pressure. In several shake-flask experiments, the diluted C<sup>14</sup>H<sub>4</sub> was further treated with ammoniacal cuprous chloride reagent<sup>10</sup> to remove the C<sup>14</sup>O trace impurity.

Grignard-generated C<sup>14</sup>H<sub>4</sub> was prepared from 1CN C<sup>14</sup>H<sub>3</sub>I for the first two steady-state experiments. In the last steady-state experiment the C<sup>14</sup>O<sub>2</sub> impurity was frozen out of the undiluted C<sup>14</sup>H<sub>4</sub> with a liquid nitrogen-isopentane bath before breaking the breakseal and allowing the C<sup>14</sup>H<sub>4</sub> to enter the circulating steady-state gas system.

9. Assay of C<sup>14</sup>H<sub>4</sub>

Rough assays of the C<sup>14</sup>H<sub>4</sub> activity with poor reproducibilities were obtained by dissolving 0.10 ml aliquots taken with a Hamilton gas syringe and injected directly below the surface of 18.0 ml of scintillation fluid (Table 4-A). Accurate and reproducible assays were obtained by combusting 0.10 ml aliquots in a helium gas train over cupric oxide followed by direct absorption of the resultant C<sup>14</sup>O<sub>2</sub> by slow bubbling into scintillation fluid containing quarternary ammonium base (NCS).

10. Assay of C<sup>14</sup>O<sub>2</sub> and C<sup>14</sup>O

C<sup>14</sup>O<sub>2</sub> in the methane or gas phases over cultures was assayed by taking a 0.10 or 0.25 ml aliquot with a 1.00 ml gas syringe and injecting

this into a 10 ml serum vial capped with a rubber septum and containing 1.0 ml of aq. 1 N NaOH. The contents of the vial were thoroughly mixed with an electrical vibrator, decapped, thoroughly purged of  $\text{C}^{14}\text{H}_4$  with dry  $\text{N}_2$  for 20 minutes, quantitatively transferred to a 5.00 ml volumetric flask, diluted to the mark, and a 0.10 or 0.20 ml aliquot dispersed in 18.0 ml of scintillation fluid for counting. An equal volume of Cab-O-Sil improved the counting results.

$\text{C}^{14}\text{O}_2$  and/or  $\text{C}^{14}\text{O}$  were assayed by taking a 0.25 ml aliquot of gas with a 1.00 ml gas syringe and injecting this into a 10 ml serum vial capped with a rubber septum and containing 0.25 ml 6M phenyl Grignard reagent in 2 ml of diethyl ether. The workup and counting procedure is described above.

#### 11. Assay of culture suspensions

Culture suspension levels of activity were determined by dispersing 0.10 ml aliquots taken with a Hamilton syringe directly into 18.0 ml of scintillation fluid in a counting vial. The addition of approximately an equal volume of Cab-O-Sil followed by 15 minutes dispersion in a Cole-Palmer Ultrasonic sonication apparatus improved the counting.

The culture suspension  $\text{C}^{14}$ -activity levels, largely reflecting the solution of  $\text{C}^{14}\text{H}_4$  in water, quickly approach equilibrium and on shaking change very little with larger changes in  $\text{C}^{14}\text{H}_4$  in the gas phase. The suspension activity levels agree very well with the levels and constancy predicted by Dalton's Law<sup>11</sup>:

2) Solubility of methane in water at 1 atm

$$= n_{\text{CH}_4} = \frac{(760)(1000)}{31.4 \times 10^6 (18.02)} = 1.34 \times 10^{-3} \text{ mole CH}_4/\text{liter}$$

A 3.0% methane atmosphere existed initially above the 10.0 ml of aq. culture suspension in the shake-flask experiment, so the predicted activity level is,

$$3) (0.030) \left( \frac{1.34 \times 10^{-3} \text{ mole CH}_4}{1000 \text{ ml}} \right) (10 \text{ ml}) \left( \frac{0.356 \mu\text{C}}{\mu \text{ mole}} \right) \left( \frac{10^6 \mu \text{ mole}}{\text{mole}} \right) \left( 2.2 \times 10^6 \frac{\text{dpm}}{\mu\text{C}} \right)$$

=  $3.1 \times 10^5$  dpm in the total 10.0 ml of culture suspension.

This corresponds to about  $2.5 \times 10^5$  cpm at a typical counting efficiency of 80% and agrees very well with experimental values.

#### 12. Assay of filtered cells

The activities of cells were determined by taking a convenient aliquot of 0.25 ml with a syringe, ejecting this carefully onto a pre-soaked and partially dried 0.45  $\mu$  Millipore filter membrane mounted in a vacuum filter, rinsing the syringe twice onto the filter with 0.5 ml volumes of water, partially drying the filter with several minutes of suction, removing the filter with tweezers, and directly dissolving it with sonication for 15 minutes in about 18 ml of Cab-O-Sil and 18.0 ml of scintillation fluid in a counting vial.

#### 13. Attempted paper chromatographs of quenched cells

The standard radioautograph chromatography procedure<sup>12</sup> used for quenched green algae was used for blue-green algae grown in the steady-state apparatus. But the levels of specific activity were too low to detect any eluted biochemicals when the film was developed for one week. Hand counting of the origin point of concentrate application showed about 60% of the original activity remained at the origin after vertical and

horizontal elution with the solvents -- the eluted 40% activity was too thinly spread and low to indicate any soluble  $C^{14}$ -labelled biochemicals.

14. Biochemical fractionation

A standard biochemical procedure developed for Escherichia coli<sup>13</sup> was applicable to the photosynthetic prokaryotes which have many cell wall properties in common with bacteria<sup>14,15</sup>. Only traces of activity were detected in the slow-growing small shake-flask culture biochemical fractions over 2-hour periods of exposure to  $C^{14}H_4$ , but larger amounts were detected on allowing a culture to stand 3 days in the laboratory. Appreciable biochemical incorporation is obtained in rapidly growing blue-green algae in the steady-state apparatus and is indicated by results in Tables 5-A and 6-A.

15. Scintillation counting

Gas, suspension, filtered cells, and biochemical fraction aliquots were counted for time intervals to give less than 1% counting error in scintillation fluid vials in a Tri-Carb scintillation counter. For some purposes, counts per minute (cpm) were adequate for experimental comparisons, in other cases corrections were made for the counting efficiency and disintegrations per minute (dpm) calculated.

Aliquot sample volumes and timings were chosen in the shake-flask experiments to maintain the starting ratios of the suspension and the gas phases. Corrections were made for previous aliquots in calculating the assays for the total culture system with time.

16. Material balances

The material balance of  $C^{14}$  activity in the small shake-flask system is about 70% due to appreciable absorption of  $C^{14}H_4$  into the rubber

septum. The inadequacies in material balances in the shake-flask experiments are circumvented by comparing results obtained at comparable levels of total suspension C<sup>14</sup> activity and using counting blanks identical in all chemical and physical characteristics excepting exposure to C<sup>14</sup>H<sub>4</sub>.

The percent C<sup>14</sup> activity lost to the numerous Tygon connections (about 30) and the greased joints and stopcocks (about 20) in the steady-state system is about 87%. Obviously, to achieve good material balances in future experiments with C<sup>14</sup>H<sub>4</sub>, special attention must be devoted to the experimental design of the apparatus to minimize C<sup>14</sup>H<sub>4</sub> absorption losses. A pre-culture was run in the steady-state apparatus to establish a maximum level of C<sup>14</sup>-contamination in this system.

### III. RESULTS AND DISCUSSION

#### A. General Considerations

Abiotic protobiochemical evolution<sup>16</sup> and subsequent eobiological evolution is generally believed to have started under an initially reductive terrestrial atmosphere containing hydrogen, methane, carbon monoxide, carbon dioxide, and ammonia.<sup>17,18,19</sup> The thermodynamic equilibria of carbon compounds derived from varying elemental proportions of oxygen, carbon, and hydrogen are illustrated in Figure 7.<sup>20</sup>

The highlights of geologic, atmospheric and biological evolutions are graphically summarized in Figure 8.<sup>21</sup> Early abiotic protobiochemical evolution proceeded substantially in reductive atmospheric and marine

environments. Terrestrial uplift and orogenesis with mineral eutrophication in rivers, lakes, estuaries, and sedimentary basins. A cogent summary of the more salient Pre-Paleozoic biological evolutionary events is given in Figure 9<sup>22</sup>.

Since both hydrogen and methane are hypothesized to be significant components of the early reductive terrestrial atmosphere that existed before and during the emergenic photosynthetic prokaryotes, it is reasonable to look among extant species for evidence of vestigial capacity to utilize methane. This search is encouraged by the fact that the higher green algae Scenedesmus exhibits hydrogenase activity<sup>23</sup> for cleaving the hydrogen-hydrogen bond (104.2 kcal/mole). The methane carbon-hydrogen bond (102 kcal/mole) is slightly weaker and lies between the known autotropically utilized extremes of the low-energy sulfur-hydrogen bond (83 kcal/mole), medium-energy nitrogen-hydrogen bond (93.4 kcal/mole), and the high-energy oxygen-hydrogen bond (110.6 kcal/mole).<sup>24,25</sup> Hence, it appears worthwhile to look for coeval methanase activity among the transitional photosynthetic prokaryotic organisms.

#### B. Phylogenetic Relationships of Photosynthetic Organisms

The phylogenetic relationships of extant lower microorganisms are outlined in Figure 10.<sup>26</sup> Prokaryotic microorganisms lack membrane-bound subcellular organelles such as a nuclei, chloroplasts, and mitochondria.<sup>15, 27</sup> The photosynthetic biochemical systems of the photosynthetic bacteria and cyanophytes (blue-green algae)<sup>28</sup> are consequently deployed throughout the cytoplasms bound on reticulate plasma membranes.<sup>3,14,15</sup>

Another common feature of both of these lower photosynthetic protists is their comparatively primitive photosynthetic systems. Photosynthetic bacteria fix carbon dioxide without the concomitant liberation of oxygen from water. Their alternative hydrogen-donors and hydrogen transport systems<sup>29</sup> are not explicitly known, but probably involve carbon-hydrogen bond breaking.

The microorganisms with which we are experimentally concerned are members of the kingdom Plantae, Division I. **Protophyta**, Class I. Schizophyceae (the blue-green algae containing the photosynthetic pigment phycocyanin<sup>30</sup> in addition to chlorophyll), and Class II. Shizomycetes (all bacteria, including a few species which contain photosynthetic pigments and several species that oxidize methane and higher hydrocarbon homologs).

The distinctions between Schizophyceae and the higher algae of Division II. Thallophyta are summarized in Table I.<sup>2</sup>

TABLE I

Major Groups of Algae<sup>2</sup>

Group	Size, Structure, Etc.	Reproduction	Habitat
Euglenophyta (Euglena)	Microscopic, unicellular; store fat and paramylum	Longitudinal fission; simple sex cells	Fresh water
Cyanophyta (Blue-green algae)	Usually microscopic; multicellular or unicellular; store glycogen	Asexual fission	Fresh water and soil
Chlorophyta (Green algae)	Microscopic, a few macroscopic (e.g. a few inches); unicellular or multicellular; store starch	Asexual fission and zoospores; primitive sexual fusion	Fresh water, soil, tree bark
Chrysophyta (Diatoms, etc.)	Microscopic, mostly unicellular; store oils	Usually asexual	Fresh and salt water (some in arctic), soil, higher plants
Phaeophyta (Brown algae; seaweeds, kelp, etc.)	Multicellular, large (up to several hundred feet); store manitol, laminarin (a polysaccharide)	Sexual; some asexual zoospores	Salt water (cool)
Rhodophyta (Red algae; seaweeds)	Multicellular, macroscopic (up to 4 feet); store flurodean starch	Sexual by well differentiated male and female germ cells; asexual by spores	Salt water (warm)

The Schizomycetea contains ten orders with 72 genera of which only Order I. Pseudomonadales is of immediate interest to us. This order is regarded by some investigators as the most primitive bacteria.<sup>31</sup> The large family Pseudomonadaceae comprises 12 genera, including the genus Pseudomonas which includes 150 species, several of which are uniquely interesting for their metabolic abilities.<sup>32,33</sup>

Pseudomonas aeruginosa is an animal pathogen, often associated with human infections, which metabolizes hydrocarbons. (The ability to utilize hydrocarbons is found sporadically throughout the animal and plant kingdoms in bacteria, Fungi imperfecti, and even in higher animal organs, such as the liver.) Pseudomonas methanica, uniquely obligate for methane substrate,<sup>34</sup> possesses complex membranes and is often closely associated with parasitic microorganisms from which it is very difficult to isolate.<sup>35</sup> Pseudomonas methanitrifacans has been reported to oxidize methane as well as fix atmospheric nitrogen.<sup>36</sup>

### C. Experimental Studies

#### 1. Shake-Flask Studies

The results of preliminary shake-flask studies are summarized in Figures 11 through 28. In the experiments on Figures 11 through 23, the system was vigorously shaken for 20 seconds after the methane injection.

Figure 11 shows that a substantial fraction of the C<sup>14</sup> activity in the 1.00 ml of injected C<sup>14</sup>-labelled methane is lost over a period of 40 minutes in the shake flask. Most of the loss is due to absorption in the rubber septum cap. This was demonstrated by soaking the septum in scintillation fluid and counting. Fortunately, the Dalton's Law-predicted

level of solution activity is rapidly attained and fairly insensitive to the  $C^{14}H_4$  loss, making shake-flask comparisons valid.

Figure 12 shows that killed cells (autoclaved) in the shake flask slowly absorb a little  $C^{14}$  activity, but much less (0.2%) than their proportion (1.0%) of the suspension. Obviously, the cell walls and membranes actually pose barriers to the permeation of  $C^{14}H_4$ . This is a reasonable observation because the cell walls and membranes are faced by highly polar protein and lipid functional groups.<sup>37</sup> Apparently, there is no active transport of methane; its slight absorption is purely physical.

Figures 13 and 14 indicate that there is little difference in  $C^{14}$  incorporation after a one-day adaption of Anacystis nidulans with a gas stream containing 1%  $CH_4$ , 4%  $CO_2$ , and 95% air. A slight relative increase in incorporation of  $C^{14}H_4$  appears to be effective by the presence of 0.5 ml of 0.24 M  $NaHCO_3$  (120  $\mu$  moles).

But the sodium bicarbonate effect on promoting  $C^{14}H_4$  incorporation is very well manifest after a three-day adaption (Figure 15) and is consistently increased thereafter. The mechanism by which sodium carbonate effects this increased  $C^{14}H_4$  incorporation is unknown. Perhaps it makes a molecular passage in the cell wall and membrane for the methane to follow.

Continued mild adaption of the Anacystis nidulans culture with the 1%  $CH_4$ , 4%  $CO_2$ , and 95% air for eight days increases  $C^{14}H_4$  incorporated and increased the sodium bicarbonate facilitation of this incorporation (Figure 16).

Eight-day adaption with four hours of rigorous adaption with 1%  $CH_4$  and 99%  $N_2$  (oxygen free) does not enhance either the  $C^{14}H_4$  incorporation

nor the sodium bicarbonate facilitation of this incorporation (Figure 17).

After a four-day rigorous adaption within a gas stream of 1%  $\text{CH}_4$  and 99%  $\text{N}_2$  (oxygen free), the level of  $\text{C}^{14}\text{H}_4$  incorporation increased greatly, the sodium bicarbonate enhancement persisted, but the cells were found to be dead (Figure 18).

The pretreatment of the  $\text{C}^{14}$ -labelled  $\text{CH}_4$  with 1.00 ml of ammoniacal cuprous chloride reagent slightly reduced the level of  $\text{C}^{14}$  incorporation from about 0.11% (Figure 13) of the suspension level to about 0.10% (Figure 19) indicating that the removal of a possible trace of  $\text{C}^{14}\text{O}$  impurity has little significant affect on the  $\text{C}^{14}$  incorporation. Therefore, since the  $\text{C}^{14}\text{O}_2$  impurity (and almost all the  $\text{C}^{14}\text{O}$  impurity) has been previously removed from the  $\text{C}^{14}$ -labelled methane in all these experiments, the  $\text{C}^{14}$  incorporation observed must be due to  $\text{C}^{14}\text{H}_4$ . The fact that comparable  $\text{C}^{14}$  incorporation obtains with autoclaved cells indicates it is due to physical solution of  $\text{C}^{14}\text{H}_4$  in the cell membrane lipids.

Nostoc is found to incorporate  $\text{C}^{14}\text{H}_4$  much more slowly than Anacystis nidulans under comparable conditions (Figure 20). The facilitation of sodium carbonate was also indicated but diminished with time. This observation is consistent with the slower rate of  $\text{C}^{14}$  incorporation for Nostoc versus Anacystis nidulans observed in the steady-state experiments below.

A rubber-capped 25 ml serum vial filled with sterilized medium was inoculated with 10.0 ml of cultured Rhodospirillum rubrum to give about a 1% cell suspension as indicated by centrifugation (Figure 21). The  $\text{C}^{14}\text{H}_4$  loss to the rubber septum is obviously more rapid than in the

shake-flask system where the ratio of the gas to suspension and the  $C^{14}H_4$  dilution are much greater. The  $C^{14}$  activity in the centrifuged and rinsed cells counted is also more variable but increases even as the solution activity slowly falls. The early cell  $C^{14}$  activity is 0.08% of the initial suspension level, again suggesting that the cell walls and membranes are resistant to permeation by  $C^{14}H_4$  from the solutions. The positive slope of the cell incorporation curve, despite the falling total suspension level of  $C^{14}$  activity, indicates some slow incorporation of a small amount of  $C^{14}H_4$  into the membrane lipids and possibly the biochemical pools of the microorganisms.

The various results above obtained with Anacystis nidulans are summarized in Figure 22, whereon are plotted the levels of suspension  $C^{14}$  activities against the levels of cell  $C^{14}$  activities at 30 minutes. In most cases (except with freshly autoclaved cells and with ammoniacal cuprous chloride reagent treated methane), sodium bicarbonate facilitates  $C^{14}H_4$  incorporation into Anacystis nidulans. Also in most cases, prolonged adaptions with methane increases the level of  $C^{14}H_4$  incorporation (Curves A and B). Apparently, some methane adaption is effected. Higher levels of incorporation obtain with dead cells, either killed by autoclaving or prolonged anaerobic treatment with 1%  $CH_4$  and 99%  $N_2$ . The fact that ammoniacal cuprous chloride reagent treatment to remove  $C^{14}O$  traces has slight effect on the amount of  $C^{14}$  incorporated indicates the incorporation is largely due to  $C^{14}H_4$ . A major part of the absorption in live and dead cells is physical solution in cell membrane lipids, which are good aliphatic solvents for hydrocarbons.

On Figure 23 are plotted the results of sampling the gas phases above shake-flask cultures and various media (filtered through 0.45  $\mu$  Millipore filters). Ten milliliters of these liquids were placed in the shake flask with 1.00 ml of diluted, base-treated  $\text{C}^{14}\text{H}_4$  and vigorously shaken for 20 seconds. Aliquot samples from the equilibrated gas phase were treated with 0.25 ml of 6 M phenyl Grignard reagent in 2 ml of dry ether. The Grignard-fixed  $\text{C}^{14}$  activity (presumably  $\text{C}^{14}\text{O}$  and/or  $\text{C}^{14}\text{O}_2$ ) was subsequently counted and found to be very much above the Grignard reagent blanks ( $38.5 \pm 1.5$  cpm) for the base-treated  $\text{C}^{14}\text{H}_4$  before contact with the fluids. The highest gas-phase aliquot  $\text{C}^{14}$  activity obtained with the 1% Anacystis nidulans culture gave a net value of 436.6 cpm at 0.5 minute at a counting efficiency of 0.792. Hence the total activity in the shake flask is:

$$\left(\frac{34.0 \text{ ml}}{0.25 \text{ ml}}\right) \left(\frac{436.6 \text{ cpm}}{0.792}\right) = 75,000 \text{ dpm}$$

or

$$\frac{75,000 \text{ dpm reacted with Grignard reagent}}{3.0 \times 10^7 \text{ dpm total injected } \text{C}^{14} \text{ activity}} \times 100\% = 0.24\% \text{ of total injected } \text{C}^{14} \text{ activity}$$

This value is well in excess of the original levels of  $\text{C}^{14}\text{O}_2$  and  $\text{C}^{14}\text{O}$  impurities (0.07%) even before the base treatment and definitely indicates the bioxidation of methane. Furthermore, some of this bioxidation appears to be effected by extra-cellular bioxidants secreted into the media and passing through 0.45  $\mu$  Millipore filters.

The shake-flask culture of Anacystis nidulans was also carried out under a more slowly equilibrating condition—the system was not vigorously shaken at  $t = 0$  on the addition of the  $\text{C}^{14}$  labelled methane but allowed to mix slowly in the swirling apparatus. The results are presented in

Figure 24. Total C<sup>14</sup> activity in the gas phase, largely C<sup>14</sup>H<sub>4</sub>, was incompletely determined by direct injection of a 0.10 ml aliquot below the surface of the scintillation fluid. But this inadequate procedure accounted for less than 30% of the original injected activity:

$$4) \frac{0.50 \text{ mC} \times 1.00 \text{ ml injected}}{36.9 \text{ ml breakseal volume -} 1.0 \text{ ml magnetic bar volume}} \times \frac{2.22 \times 10^9 \text{ dpm}}{1 \text{ mC}} = \frac{3.0 \times 10^7 \text{ dpm,}}{\text{C}^{14} \text{ activity of}} \\ \text{CH}_4 \text{ injected into the shake flask}$$

The results are interpreted as follows: During the first 15 minutes a significant and increasing portion of the gas-phase aliquot activity is dissolved in the 4.0 ml of aq. 2 M NaOH and C<sup>14</sup>O<sub>2</sub> is formed from the biochemical oxidation of C<sup>14</sup>H<sub>4</sub> by Anacystis nidulans since this C<sup>14</sup> activity is several orders of magnitude (10<sup>4</sup>-10<sup>5</sup>) in excess of the original C<sup>14</sup>O impurity (0.007%) remaining in the diluted C<sup>14</sup>-labelled methane after aq. 2 M NaOH treatment. (Carbon monoxide is soluble to the extent of 0.02404 volumes per volume of water at 25°C<sup>38</sup> and reacts with aq. NaOH to form formate.<sup>39</sup>) The level of total C<sup>14</sup> activity in the suspension approaches the level predicted by Dalton's Law but falls off from this value after about 10 minutes as dissolved C<sup>14</sup>H<sub>4</sub> is oxidized to C<sup>14</sup>O<sub>2</sub> and exchanged to the gas phase faster than it is equilibrated with the bicarbonate in the suspension. As the gaseous C<sup>14</sup>O<sub>2</sub> is equilibrated slowly back into solution with the bicarbonate, the level of suspension C<sup>14</sup> activity again rises and a little C<sup>14</sup>H<sub>4</sub> and some C<sup>14</sup>O<sub>3</sub><sup>-</sup> is very slowly incorporated into the cells.

The production of C<sup>14</sup>O<sub>2</sub> is further qualitatively confirmed in repeat Anacystis nidulans experiments in the initial presence and absence of light (Figure 25). The exact levels of C<sup>14</sup> activity are not the same because during the week that elapsed the breakseal lost some C<sup>14</sup> activity.

But the two points on curve "A.n.-3" qualitatively confirm "A.n.-1" presented previously (both experiments without initial shaking), and compare interestingly with run "A.n.-2," where the  $C^{14}H_4$  was added in the dark at  $t = -3$  minutes with moderate initial shaking. As on Figure 24, further swirling in the apparatus effects rapid oxidation of the methane. Shaking in the dark appears to reduce the rate of oxidation and exchange to the gas phase initially, but this rate increases after 14 minutes. After a period of illumination, the rate of oxidation exceeds the rate of removal from the gas phase for a short period of time in all three experiments.

The fact that a significant part of the solution  $C^{14}$  activity is  $C^{14}O_2$  from bioxidation of  $C^{14}H_4$ —directly oxidized by extra- and possibly by intracellular oxidase system(s) or indirectly by biogenerated singlet oxygen—is indicated in Figure 26. The addition of the quarternary ammonium hydroxide reagent (NCS) to the scintillation fluid increases the counting level of the suspension 10 to 20% by reducing  $C^{14}O_2$  loss on transfer and standing.

The level of  $C^{14}$  activity in the shake-flask cellular material was too low for chromatographic and radioautographic analyses. The biochemical fractionation procedure developed for Escherichia coli indicated only traces of  $C^{14}$  activity in the biochemical fractions after a 1 to 2 hour exposure to  $C^{14}H_4$ ; a culture allowed to stand on the shelf for several days indicated some incorporation (Table II). Higher levels of biochemical incorporation well in excess of experimental errors are reported for the more rapidly photosynthesized steady-state cultures.

TABLE II

Biochemical Incorporation of C<sup>14</sup> Activity  
by Anacystis nidulans in Shake-Flask  
Experiment Increases with Time

Time of Contact with C <sup>14</sup> H <sub>4</sub> :	30 minutes <sup>1</sup> :	3 days <sup>2</sup> :
<u>Fraction/C<sup>14</sup> Activity</u>	<u>μC</u>	<u>μC</u>
Supernatant solution after centrifugation	0.0034	0.0035
Cold TCA Extract	0.00035	0.0020
Alcohol-Ether Extract	0.00010	0.00012
Hot TCA Extract	0.00004	0.0012
Insoluble in Hot TCA	0.00000	0.00059

1. Swirled in shake-flask apparatus at 25°C

2. Standing at laboratory illumination at about 25°C

2) Steady-State Experiments

In the steady-state apparatus, the culture of the blue-green algae Anacystis nidulans and Nostoc, was followed more closely on a larger scale. The apparatus and general procedures are well described in the literature.<sup>8,12</sup> Efficient illumination and rapid gas-phase recycle through the aqueous phase promote rapid photosynthesis and growth of the algae under controlled, reproducible, near-constant conditions of illumination, suspension density, pH, and temperature. Rapid rate of photosynthesis is indicated by the disappearance of carbon dioxide with concomitant oxygen production, both of which are continuously analyzed and recorded on a strip chart along with pH, temperature, and C<sup>14</sup> activity added as C<sup>14</sup>H<sub>4</sub>. The results of the steady-state experiments with the blue-green algae are summarized in Table III.

Steady-state Experiments 1 and 2 with Nostoc and Anacystis nidulans, respectively, were made with C<sup>14</sup>H<sub>4</sub> generated by the Grignard reaction from C<sup>14</sup> methyl iodide. The concentraton of methane in the recycle gas phase at the start of these experiments was 3.1%. The initial gas-phase C<sup>14</sup> activities were very low—5.1 and 2.3 microcuries initial ( $\mu\text{C}_0$ ). Final C<sup>14</sup> activities in the recycle gas phase at the end of the experiments were 3.0 and 0.21  $\mu\text{C}_f$ . Hand counting of evaporated aliquots of the cell suspensions accounted for 273% and 30% respectively of the C<sup>14</sup> activity loss from the gas phase and indicates serious contamination errors in these early experiments.

Experiments 3 and 4 were carried out with Anacystis nidulans cultures preconditioned for 7-1/2 to 16 days in the presence of 1% methane, 4% carbon dioxide, and 95% air. The C<sup>14</sup>-labelled methane was obtained directly from breakseals and had a labelled specific activity of 5.34  $\mu\text{C}/\mu\text{ mole}$ .

TABLE III

Steady-State Culture of Blue-Green Algae with  $C^{14}H_4$ 

Exp. No.	Algae	Steady-State Culture Conditions				$C^{14}H_4$			$C^{14}$ Activity in Gas Phase			$C^{14}$ Activity in Cells	
		Methane Induction Time	T, °C	pH	t, min.	$[CH_4]_0$	$[CO_2]_0$	Spec. act. $\mu C/\mu$ mole	$\mu C_0$	$\mu C_f$	$\Delta \mu C^{3,8}$	Incorporated $\mu C_i$	% of $\Delta \mu C$
1	<u>Nostoc</u>	17 min.	25	6.0	132	3.1	4.0	3.58X10 <sup>-3</sup>	5.1	4.0	1.1	3.0 <sup>4</sup>	273 <sup>7</sup>
2	<u>Anacystis nidulans</u>	60 min.	25	6.3	40	3.1	2.0	3.58X10 <sup>-3</sup>	2.3	1.6	0.7	0.21 <sup>4</sup>	30 <sup>7</sup>
3	"	7.5 days	25	6.3	30	0.64	2.0	5.34	644	402	242	0.61 <sup>5</sup>	0.25
4	"	16 days	20	6.4	110	0.51	2.0	5.34	463	210	253	0.61 <sup>5</sup>	0.24
5	<u>Nostoc</u>	0	22	6.4-8.0	126	0.73	2.0	5.34	653	541	112	0.74 <sup>5</sup>	0.66
6	"	0	20	6.2	{20 in dark 47 in light}	0.58	2.0	5.34	527	449	76	3.75 <sup>6</sup>	4.9
7	"	0	20	6.2	110	0.58	2.0	5.34	544	522	22	0.82 <sup>6</sup>	3.7

1. Induced before experiment in steady-state apparatus with recycle atmosphere of 0.5% cold methane, 2.0% cold carbon dioxide and 97.5% air under illumination.  $C^{14}H_4$  generated from Grignard reaction of  $C^{14}H_4I$ .

- Labelled value corrected for any dilution with cold methane.
- Determined from ionization chamber reading on calibrated strip-chart record.
- Hand counting of chromatogram origin spot.
- Scintillation count of final cell suspension.
- Summation of nonvolatile biochemical fractions counted by scintillation counting in the apparatus.
- In error due to very low values of  $\Delta \mu C$  in same magnitude as levels of  $C^{14}O_2$ .
- Most of the  $\Delta \mu C$  loss in gas phase  $C^{14}$  activity is due to absorption of  $C^{14}H_4$  in the Tygon and greased valves and stopcocks in the apparatus.

The concentrations of the methane gas in the recycle gas system were 0.64% and 0.51%, respectively. The initial  $C^{14}$  activities indicated by the ionization chamber were 644 and  $463 \mu C_0$ , respectively. At the end of the experiment, the  $C^{14}$  activity in the recycle gas phases were 0.73 and  $0.61 \mu C_f$ . Cell suspensions were counted in the scintillation counter and accounted for 0.30% and 0.24% of the  $C^{14}$  activity lost from the gas phase.

Figure 27 presents the results of hand counting paper chromatogram origins before and after solvent elutions of evaporated cell suspension aliquots from Experiment 3 at various times.

The rates of incorporation of  $C^{14}$  activity into the cells are compared for Experiments 4 with Anacystis nidulans and for Experiment 5 with Nostoc in Figures 28 and 29. In both experiments, comparable levels of cell saturations are rapidly approached at about 20 minutes with 400-420 cpm of  $C^{14}$  activity in  $100\lambda$  aliquots of cell suspension. Anacystis nidulans incorporates 0.25% of the total added  $C^{14}$  activity, whereas Nostoc incorporates 0.30% of the added  $C^{14}$  activity. The  $C^{14}$  methane used in these experiments was not pretreated with base to remove the 0.075% ( $0.375 \mu C$ )  $C^{14}O_2$  impurity, so about half of this activity is due to  $C^{14}O_2$  impurity in the breakseal methane. No correction is made for possible  $C^{14}O_2$  contamination in the steady-state apparatus either.

Experiments 5 and 6 were carried out with non-induced Nostoc cultures and were cultured for 126 minutes in Experiment 5, and a combination of 18 minutes in the dark and 47 minutes in the light in Experiment 6. Breakseal methane of  $5.34 \mu C/\mu$  mole specific activity was used, giving methane concentrations in the recycle gas phases of 0.73%

and 0.58%. The initial  $C^{14}$  activities indicated by the ionization chamber were 653 and  $527 \mu C_0$ . The  $C^{14}$  activity in the recycle gas phase at the end of the experiments was 0.74 and  $3.75 \mu C_f$ . Scintillation counting of biochemical fractions from the ethanol quenched cell suspensions from Experiment 6 accounted for 3.7% of the  $C^{14}$  activity lost from the gas phase.

The blue-green algae from Experiments 6 and 7 were biochemically fractionated by the procedure developed for Escherichia coli; results are summarized in Figures 30 and 31. The biochemical fractionation procedure proved to be very adaptable and indicates differences both in the rates of bioincorporation and in the profile patterns between these two blue-green algae.

Experiment 7 with Nostoc cultured for 110 minutes was performed using breakseal methane which was prechilled with isopentane-dry ice to remove  $C^{14}O_2$  impurity before admission into the steady-state apparatus. The concentration of the methane in the gas phase was 0.58%. During the culture period, 22  $\mu C$  of activity were lost from the gas phase, corresponding to 4% of the introduced  $C^{14}$  activity. Twelve percent of this lost activity was accounted for in the cell suspension after quenching in alcohol and degassing.

A detailed summary analysis of Experiment 7 is given in Table 4. A blank culture was run for 15 minutes and indicated the presence of 0.20  $\mu C$  contamination in the apparatus. The actual blank for the experiment itself probably was less than this value. Twenty-two  $\mu C$  of  $C^{14}$  activity were lost from the gas phase. After 30 minutes of culture, the gas phase was recycled for 15 minutes through a series of two caustic traps containing

TABLE IV  
Incorporation of  $C^{14}H_4$ <sup>a</sup> by Nostoc in the  
Steady-State Culture<sup>b</sup> Apparatus

	$\mu C$
<u>Blank Culture</u> (15 min. culture before addition of $C^{14}H_4$ )	0.20 <sup>c</sup>
<u>Radioactivity Introduced</u> ( $C^{14}H_4$ disappearance from the gas phase in 30 min. of culture)	22
<u>Radioactivity Accounted for after 30 min. of Culture<sup>d</sup></u>	
A. In gas phase:	
HCHO in the gas phase absorbed in dimedone trap	nil
$C^{14}O_2$ in the gas phase absorbed in caustic trap	0.27
B. In liquid phase (80 ml) syringe sampled and injected below surface of scintillation fluid	2.64
Dissolved gases lost on methanol quenching of the culture (mostly $C^{14}H_4$ )	1.82
Nonvolatiles remaining in suspension (80 ml)	0.82
Biochemical fractions	
Supernatant soln. after centrifugation	0.11
Cold TCA-sol. metabolites	0.10
Alc.-ether-sol. lipids	0.10
Hot TCA-sol. nucleic acid hydrolyzate	0.20
Insol. proteins and pigments	<u>0.07</u>
Total (75% of original nonvolatiles)	0.59

- a.  $544\mu C C^{14}H_4$  freed of 0.075%  $C^{14}O_2$  ( $0.375\mu C$ ) impurity by fractional distillation from the breakseal sample holder chilled in isopentane-liquid  $N_2$ ; still contained about 0.0073%  $C^{14}O$  ( $0.037\mu C$ ).
- b. Eighty ml of 1% wet cells in normal media diluted to 20% with a circulating 335 ml gas atmosphere initially containing 2.0%  $CO_2$ , 0.58%  $C^{14}H_4$ , 97.4% air, at 20°C and 1 atm.
- c. This value is due to  $C^{14}O_2$  background from previous experiments in the apparatus, and probably is in excess of the maximum contamination in the subsequent experiment. The blank culture was removed and the apparatus again rinsed with water before starting the labelled culture experiment.
- d. Estimated loss in 20 Tygon connections is 2.5  $\mu C$  and in 30 greased joints and stopcocks is 10  $\mu C$ .

10 ml of aq. 2 M NaOH each and one trap containing dimedone reagent to detect formaldehyde. The formaldehyde test was nil. The  $C^{14}O_2$  caught in the caustic traps was 0.27  $\mu$ C—more than the 0.20  $\mu$ C value obtained in the culture blank before the experiment, thus indicating some production of  $C^{14}O_2$  from  $C^{14}H_4$  by Nostoc during the experiment.

The cell suspension lost 1.82  $\mu$ C, 69% of the original value of 2.64  $\mu$ C on quenching and degassing in four volumes of methanol. The nonvolatile components were determined by the biochemical fractionation procedure and accounted for 72% of the nonvolatile activity. The total  $C^{14}$  activity (0.82  $\mu$ C) incorporated into nonvolatile biochemicals over the culture period is four times greater than the level of  $C^{14}$  contamination.

Twelve percent (2.64  $\mu$ C) of the  $C^{14}$  activity which disappeared from the gas phase (22  $\mu$ C) was accounted for in the cell suspension. Most of the  $C^{14}H_4$  was lost by adsorption in the Tygon connections and in the greased joints and stopcocks. These losses and their magnitude were confirmed by rinsing a stopcock and soaking a Tygon connection in scintillation fluid.

The  $C^{14}$  activity in the culture suspension rose steadily over the 30 minutes from 4808 to 5831 cpm for a 100 $\lambda$  aliquot as the  $C^{14}$  activity in the gas phase steadily decreased, indicating incorporation beyond simple  $C^{14}H_4$  solution in the aqueous phase.

#### IV. CONCLUSIONS

The conclusions from the shake-flask studies are:

1. The solution level of  $C^{14}H_4$  agrees well with the calculated value from Dalton's Law, and equilibrium is approached in

less than 10 minutes in the shaking apparatus. Nearly constant levels obtained over the course of the blue-green algae experiments, and these levels are little affected by the presence or absence of added sodium bicarbonate.

2. The relative  $C^{14}$  activity in rinsed cells is much lower than  $C^{14}$  activity in the suspension, indicating that the intact cell walls and membranes pose barriers to  $C^{14}$  transport and diffusion into the cellular fluid.
3. Killed cells appear to take longer times to approach saturation but give comparable, and in some cases higher, levels of cellular  $C^{14}$  activity, suggesting that the disrupted cellular membrane facilitates  $C^{14}H_4$  solution into the lipid fractions.
4. Cells with one-day adaption take a little longer for solution saturation equilibrium, but the levels of  $C^{14}$  activity saturation are very similar to the nonadapted culture.
5. Sodium bicarbonate consistently appears to facilitate  $C^{14}$  incorporation into the cells.
6. After several days of adaption, the solution equilibrium is more rapid and sodium bicarbonate facilitation increases. After eight-day adaption, the sodium bicarbonate facilitation is even greater.

7. Eight-day adaption followed by four hours with 1% methane and 99% nitrogen (oxygen excluded) gives little difference from eight-day adaption alone, and the bicarbonate effect is about the same.
8. Four-day rigorous treatment with 1% methane and 99% nitrogen (oxygen excluded) increases the  $C^{14}$  absorption. The cells died but the sodium bicarbonate facilitation persisted.
9. Since the methane has been treated with concentrated base to remove carbon dioxide and a major portion of the carbon monoxide, the  $C^{14}$  incorporation into the live and dead cells is largely as dissolved  $C^{14}H_4$ . Furthermore, ammoniacal cuprous chloride reagent changes slightly the level of  $C^{14}$  incorporation.
10. Some  $C^{14}O_2$  is produced from  $C^{14}H_4$  by bioxidation and detected in the gas phase in fair amounts; this  $C^{14}O_2$  is more slowly equilibrated with solution bicarbonate and very slowly incorporated into the cells.

The following conclusions are drawn from the steady-state experiments:

1. Very small amounts of  $C^{14}$  activity, derived from  $C^{14}$  methane in excess of the apparatus contamination level or impurities in the labelled methane, are incorporated into blue-green algae: Nostoc and Anacystis nidulans.
2.  $C^{14}$  activity is incorporated rapidly into all the biochemicals within the blue-green algae culture in the steady-state apparatus.

The apparatus used for these preliminary shake-flask and steady-state cultures are inadequate, as indicated by their significant adsorption of labelled methane in rubber septums, Tygon tubing, and greased connections. In order to do quantitative experiments, attention needs to be given to the design of the apparatus.

Although the  $C^{14}O_2$  and  $C^{14}O$  impurities can be removed from methane received in breakseals, the genesis of labelled methane from  $C^{14}$  methyl iodide by the Grignard reaction would be more practical. It would also be more convenient to work with methane having a much higher level of specific activity. With higher activities, analysis for the biosynthetic pathways by radioautographic techniques would be feasible, as well as by the biochemical fractionation scheme described above. This would allow one to investigate the biosynthetic mechanisms and pathways of methane bioxidation in blue-green algae.

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38. Mark, H.F., et al., eds., Kirk-Othmer Encyclopedia of Chemical Technology, 2nd Ed, Vol 4, p. 427, John Wiley & Sons, New York (1964).
39. Latimer, W.M., and Hildebrand, J.H., Reference Book of Inorganic Chemistry, Revised Edition, p. 271, The Macmillan Co., New York (1940).

TABLE 1-A  
Blue-Green Algae Culture Medium for Nostoc (BGM)<sup>a</sup>

<u>Ingredients</u>	<u>for</u>	<u>1 liter solution</u>
1 M Mg SO <sub>4</sub> · 7H <sub>2</sub> O		0.9 ml
1 M K <sub>2</sub> HPO <sub>4</sub>		5.0 ml
1 M KNO <sub>3</sub>		5.0 ml
1 M Ca(NO <sub>3</sub> ) <sub>2</sub> · 4H <sub>2</sub> O		0.09 ml
1 M NaNO <sub>3</sub>		5.0 ml
Fe Versenol <sup>b</sup>		1.6 ml
Trace elements (A-4) <sup>c</sup>		0.9 ml
EDTA soln (50 g/liter)		1.0 ml
Add aq. H <sub>2</sub> SO <sub>4</sub> to adjust to pH = 7.4 and dilute with distilled water to 1 liter.		

- a. Allen, M. B. and Arnon, D. I., *Plant Physiol.* 30, 4, 1955.
- b. Preparation of Fe Versenol 120 solution for media
  1. Dissolve 43 ml Versenol 120 in 500 ml H<sub>2</sub>O
  2. Dissolve 14.3 gms FeSO<sub>4</sub> · 7H<sub>2</sub>O in 500 ml H<sub>2</sub>O. FeSO<sub>4</sub> · 7H<sub>2</sub>O must be pure. The presence of white crystals indicates Fe(OH)<sub>2</sub> is present. Fe(OH)<sub>2</sub> will not be chelated as readily by the Versenol 120.
  3. Using separatory funnel, add the dissolved FeSO<sub>4</sub> drop by drop into the Versenol 120 solution with continuous stirring.
  4. Aerate overnight. Protect from light. pH about 9.7 (after aeration).
  5. Wrap bottle in aluminum foil as chelate is light sensitive.
  6. Store bottle in cool place.
- USE: 1.74 ml to 1 liter of nutrient solution.

NOTE: This recipe can be substituted for the Fe Versenol recipe if the proportionate increase is made in the amount used. Developed at LCB by unknown.

Versenol-120 = Sodium Tri, N-Hydroxyethyl-ethylenediamine Triacetate  
Fisher Scientific Co., Fair Lawn, N.J.

- c. Trace elements: Arnon's A-4 + Cobalt + Molybdate

	<u>gm/liter in stock solution</u>
CoCl <sub>2</sub> · 6H <sub>2</sub> O	0.040
H <sub>3</sub> Bo <sub>3</sub>	2.86
MnCl <sub>2</sub> · 4H <sub>2</sub> O	1.81
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	0.222

TABLE 1-A (continued)

gm/liter in stock solution

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.079
$\text{MoO}_3$ (99.5%)	0.015
Co + Mo conc. taken from Arnon's B-7	

Arnon, D. I., Microelements in Culture Solution Experiments with  
Higher Plants; Am. J. Bot., 25, p. 325-332, 1938.

TABLE 2-A

Blue-Green Algae Culture Medium for  
Anacystis Nidulans (MKC)<sup>a</sup>

<u>Ingredient</u>	<u>1 liter solution</u>
1 M MgSO <sub>4</sub>	1 ml
1 M K <sub>2</sub> HPO <sub>4</sub>	6 ml (add last)
1 M Ca(NO <sub>3</sub> ) <sub>2</sub>	0.106 ml
1 M KNO <sub>3</sub>	9.9 ml
100 g/l Na Citrate	1.65 ml
50 mg/100 ml FeSO <sub>4</sub> ·7H <sub>2</sub> O	8.1 ml
Arnon's A-4 + Mo + Co <sup>b</sup>	1 ml

a. Kratz and Myers, Nutrition and Growth of Several Blue-Green Algae; Am. J. of Bot, 42, p. 282-284, 1955.

b. See Arnon's formula in Table 1-A.

TABLE 3-A  
Modified Hutner's Medium for  
Rhodopseudomonas and Rhodospirillum<sup>a</sup>

Stock solutions:

1. Potassium phosphate, pH 6.8	1.0 M
2. Ammonium DL-malate, pH 6.8	1.0 M
3. Metals "44" per 100 ml:	
Fe Versen-01	20 ml (100 mg Fe)
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	1095.0 mg (250 mg Zn)
(MnCl <sub>2</sub> · 4H <sub>2</sub> O)	(150.0 mg)(50 mg Mn)
CuSO <sub>4</sub> · 5H <sub>2</sub> O	39.2 mg (10 mg Cu)
(CoCl <sub>2</sub> · 6H <sub>2</sub> O)	(20.0 mg)(5 mg Co)
(H <sub>3</sub> Bo <sub>3</sub> )	(11.4 mg)(2 mg B)

Add a few drops of sulfuric acid to retard precipitation.

4. Concentrated Base per liter:

Nitrilotriacetic acid	10.0 g
MgSO <sub>4</sub>	14.45 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	3.335 g
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> · 4H <sub>2</sub> O	9.25 mg
FeSO <sub>4</sub> · 7H <sub>2</sub> O	99.0 mg
Nicotinic acid	50.0 mg
Thiamin · HCl	25.0 mg
Biotin	0.5 mg
Metals "44"	50.0 ml

Dissolve NTA separately and neutralize with KOH (about 7.3 g); add the rest of the ingredients and adjust to pH 6.6 to 6.8 before making to volume.

5. For each liter of complete medium: take 20 ml each of solutions 1, 2 and 4, make to one liter with distilled water and add 1.0 g of casein hydrolysate. The precipitate which forms during autoclaving will redissolve on cooling. Final pH 6.8 to 7.2.

NOTE: The casein hydrolysate may be replaced by a mixture of 0.1% L-glutamic acid and 0.1% sodium acetate · 3H<sub>2</sub>O.

If the medium becomes alkaline during growth, magnesium ammonium phosphate is deposited. This may be prevented by aerating with a gas mixture containing 5% CO<sub>2</sub> or by reducing the ammonium concentration from 0.04 M to around 0.01 M.

Slant Medium: Yeast extract - 0.3 gm, agar - 2 gms, H<sub>2</sub>O - 100 ml, casamine acids - 0.2 gm.

a. Cohen-Bazire, Sistrom, and Stanier; Journal of Cellular and Comparative Physiology, 49, p. 29, 1957.

TABLE 4-A  
Scintillation Fluid Formula

500 ml NEC Fluor Concentrate II (RSL)

2500 ml absolute ethanol

2700 ml reagent grade toluene

500 g reagent grade naphthalene

Stirred to effect complete solution.

18.0 ml of this fluid was used routinely in standard, capped scintillation counting vials. Some improvement in counting efficiency was achieved by improved dispersion in the presence of an approximately equal volume of Cab-O-Sil. The absorption of  $C^{14}O_2$  in combustion assays was improved by the addition of excess quarternary ammonium base (NCS).

TABLE 5-A  
Biochemical Fractions into Which Methane is  
Incorporated by Anacystis Nidulans<sup>a</sup>

Time of contact with C <sup>14</sup> H <sub>4</sub>	30 minutes	
<u>Fraction</u>	<u>CPM/g cells</u>	<u>%</u>
1. Cold TCA Extract (sol. metabolites)	102,397.4	24.6
2. Alc. sol. } Lipids	58,893.3 }	16.0
3. Ether sol. }	7,747.4 }	
4. Hot TCA Extract	197,640.0	47.5
5. Acidic alc. rinse	6,480.0	1.6
6. Ether rinse	106.7	0.0
7. Insol. in Hot TCA ("nucleic acid hydrolyzate")	43,186.0	10.4
Total	416,450.8	100.1

a. 1% wet cells from steady-state apparatus at 20°C.

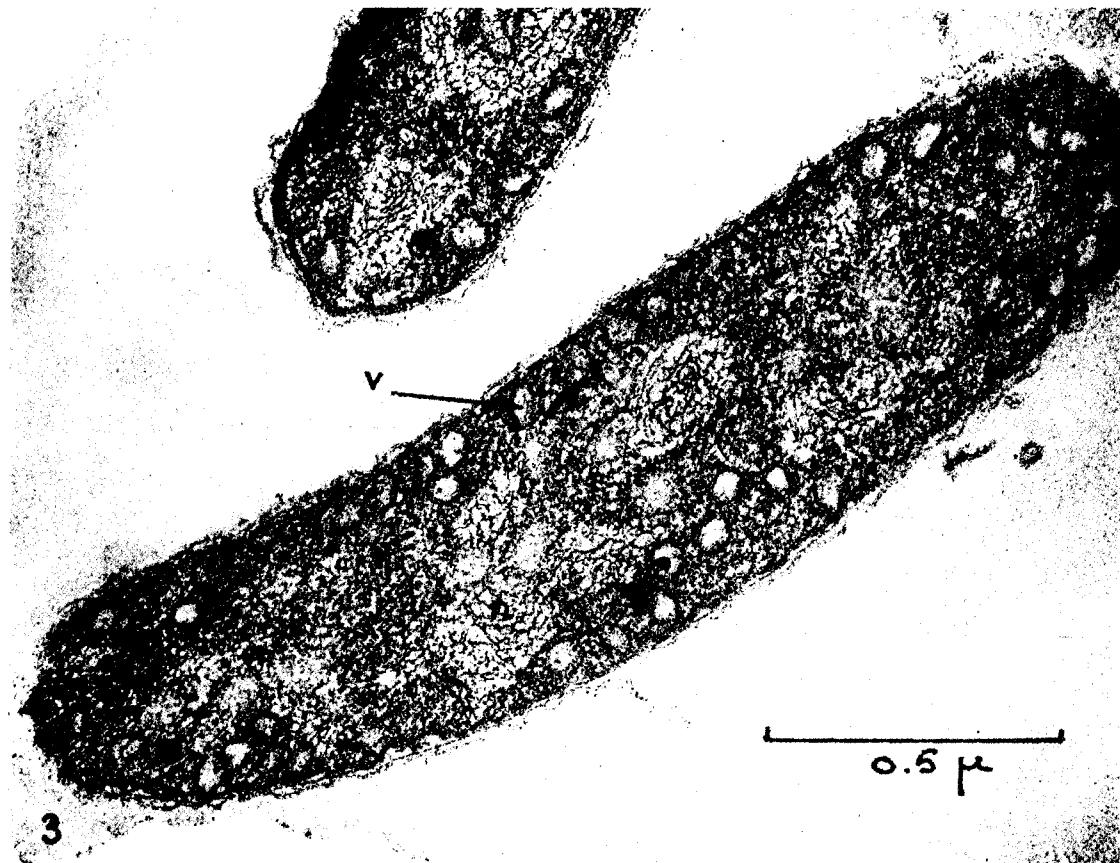
TABLE 6-A

Biochemical Fractions into Which Methane

is Incorporated by *Nostoc*<sup>a</sup>

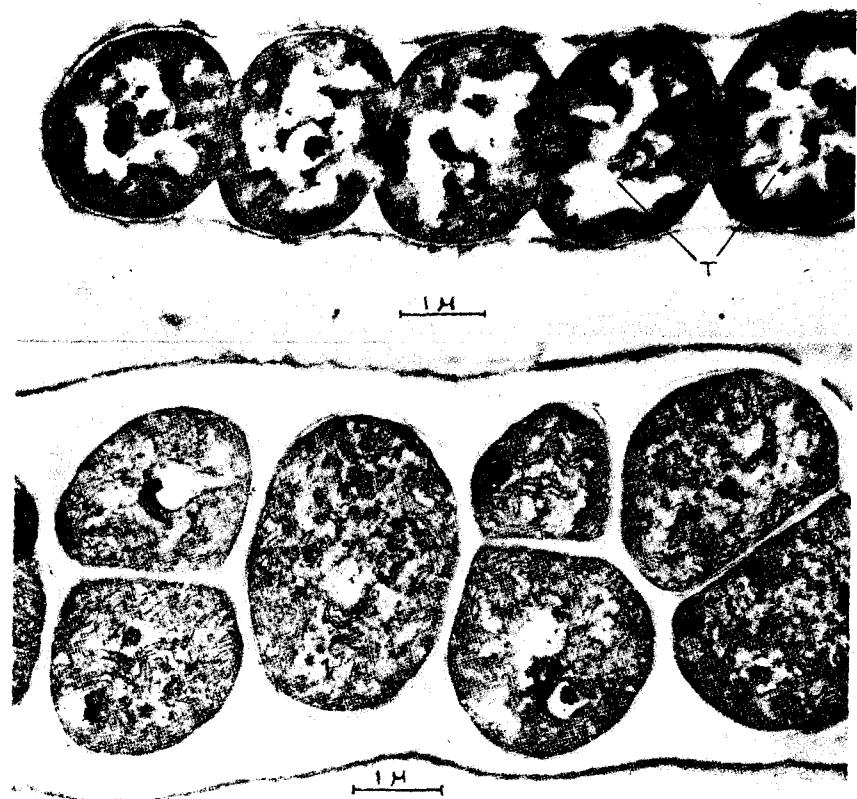
Time of contact with C <sup>14</sup> H <sub>4</sub>	68 min.	121 min.
Fraction	CPM/g cells, %	CPM/g cells, %
1. Cold TCA Extract (sol. metabolites)	74,300	21.8
2. Alc. sol.	30,200	63,100
3. Ether sol.	2,400	3,700
4. Hot TCA Extract	161,000	47.3
5. Insol. in Hot TCA	72,800	21.4
Total	340,700	100.0

a. 1% wet cells from steady-state apparatus at 22°C.



XBB7012-5384

FIGURE 1. Section of Rhodospirillum rubum grown photosynthetically at moderate light intensity (1000 ftc).<sup>30</sup>



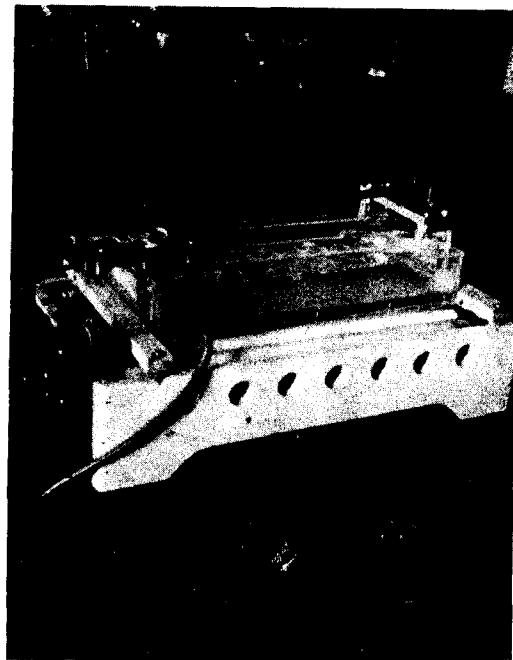
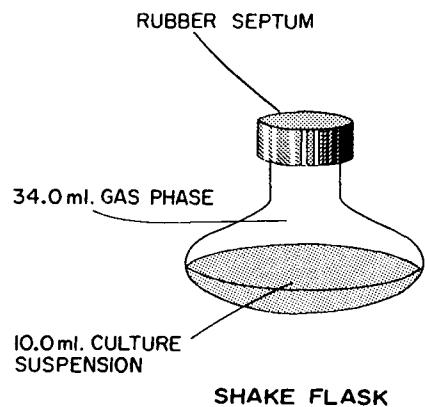
XBB7012-5385

FIGURE 2. *Nostoc*, a blue-green alga. Low-density nuclear material is abundant in the upper picture, and threadlike structures (T) are clearly shown in the nucleoplasm of the cells at the right. Membranous chloroplast equivalents almost fill the cells in the lower picture. (Electron photomicrographs by G. B. Chapman.)<sup>2</sup>



XBB7012-5383

FIGURE 3. Ultrastructure of *Anacystis nidulans* showing continuity of lamellae with the cytoplasmic membrane. Cytoplasmic membrane invaginates to form both part of the double membrane.  $KMnO_4$  fixation  $\times 60,000$ . Marker indicates  $0.1 \mu m$ .<sup>3</sup>



XBB7012-5382

FIGURE 4. Shake-flask vessel and illuminated swirling, thermostated apparatus.

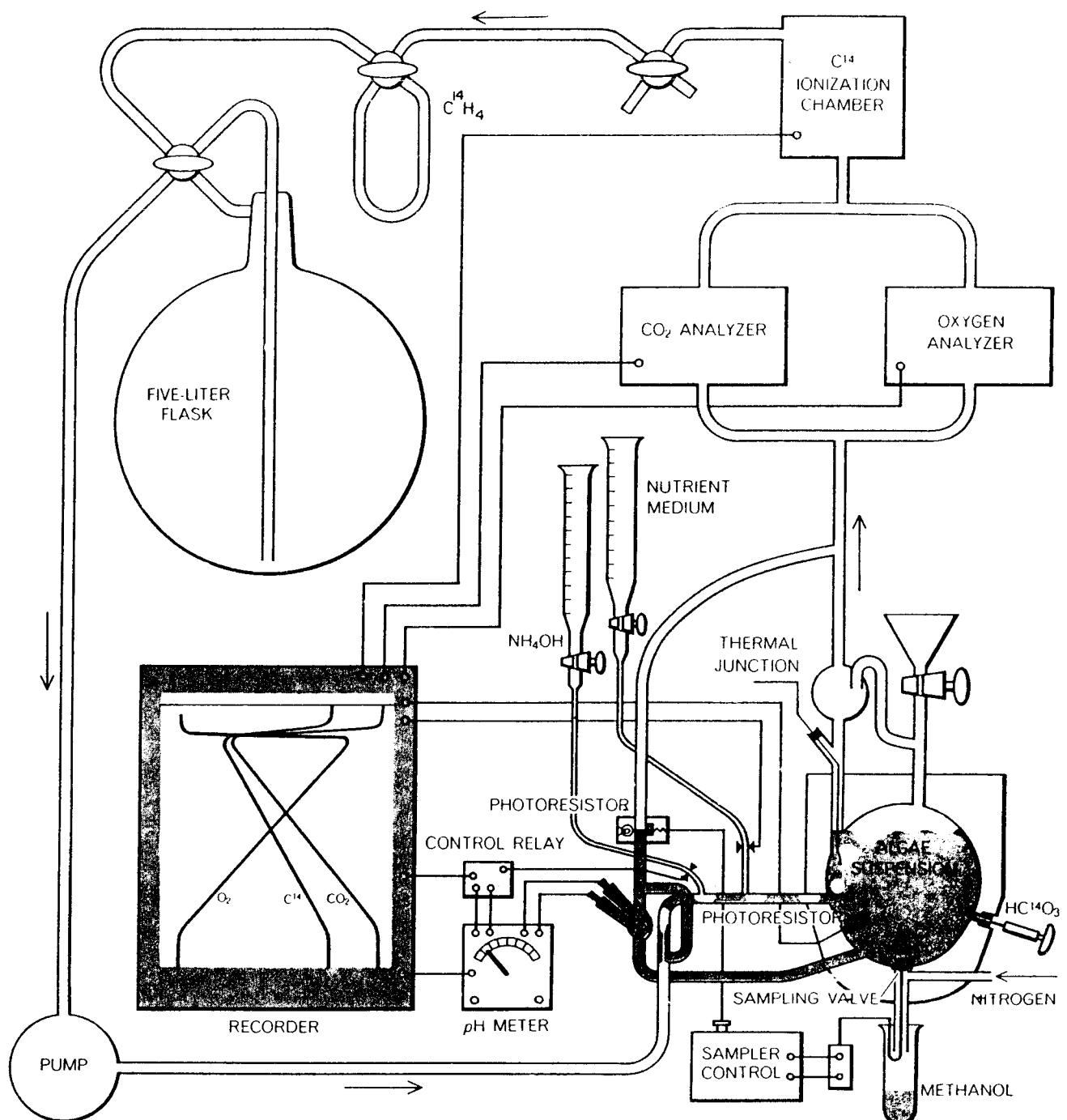


FIGURE 5. Steady-state apparatus permits experimental control and study of photosynthesis. The algae are suspended in nutrient in a transparent vessel (lower right). A gas pump circulates a mixture of air and ordinary carbon dioxide to the vessel, where it bubbles through the suspensions. Labeled carbon is added in the form of  $\text{C}^{14}\text{H}_4$ . Measurements of the oxygen, carbon dioxide, and labeled carbon levels in the gas are recorded continuously. The pH is maintained at a constant value by means of the pH meter. The sampler control allows removal of samples into the test tube.<sup>12</sup>

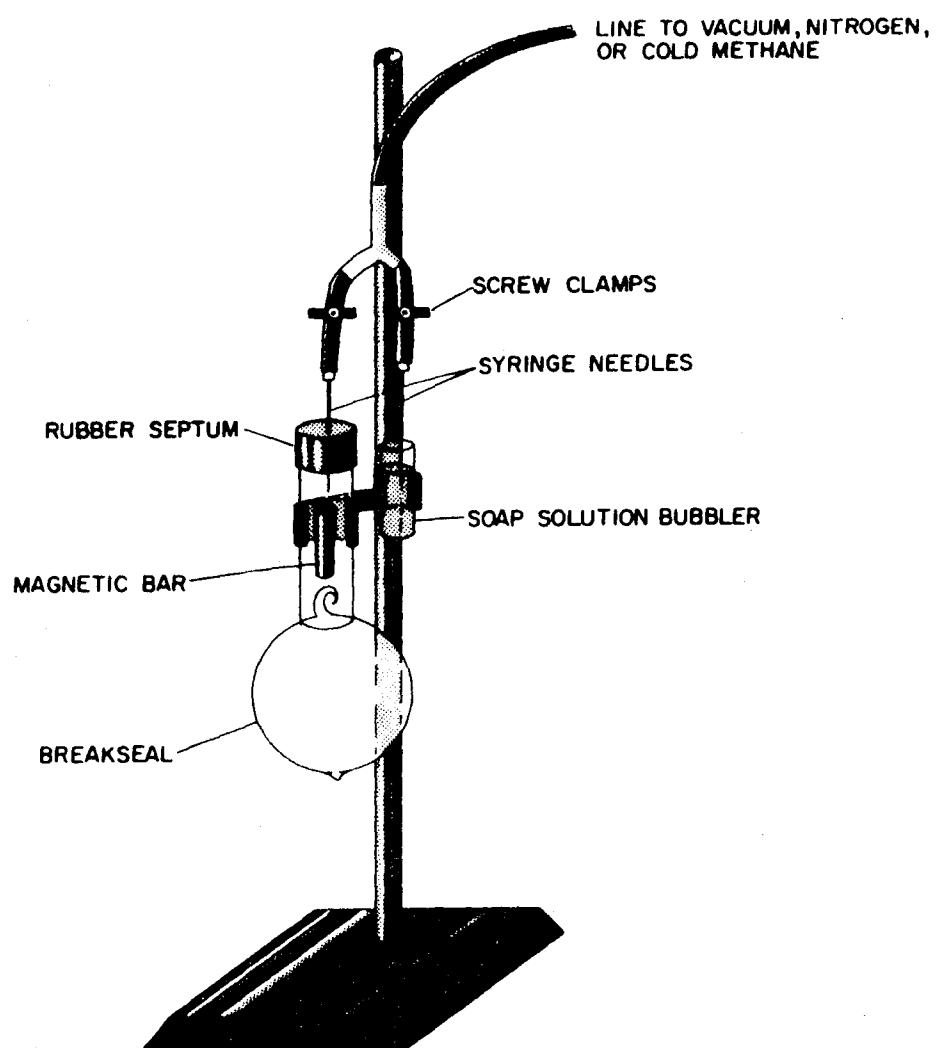


FIGURE 6. Apparatus for dilution of C<sup>14</sup> methane with cold methane.

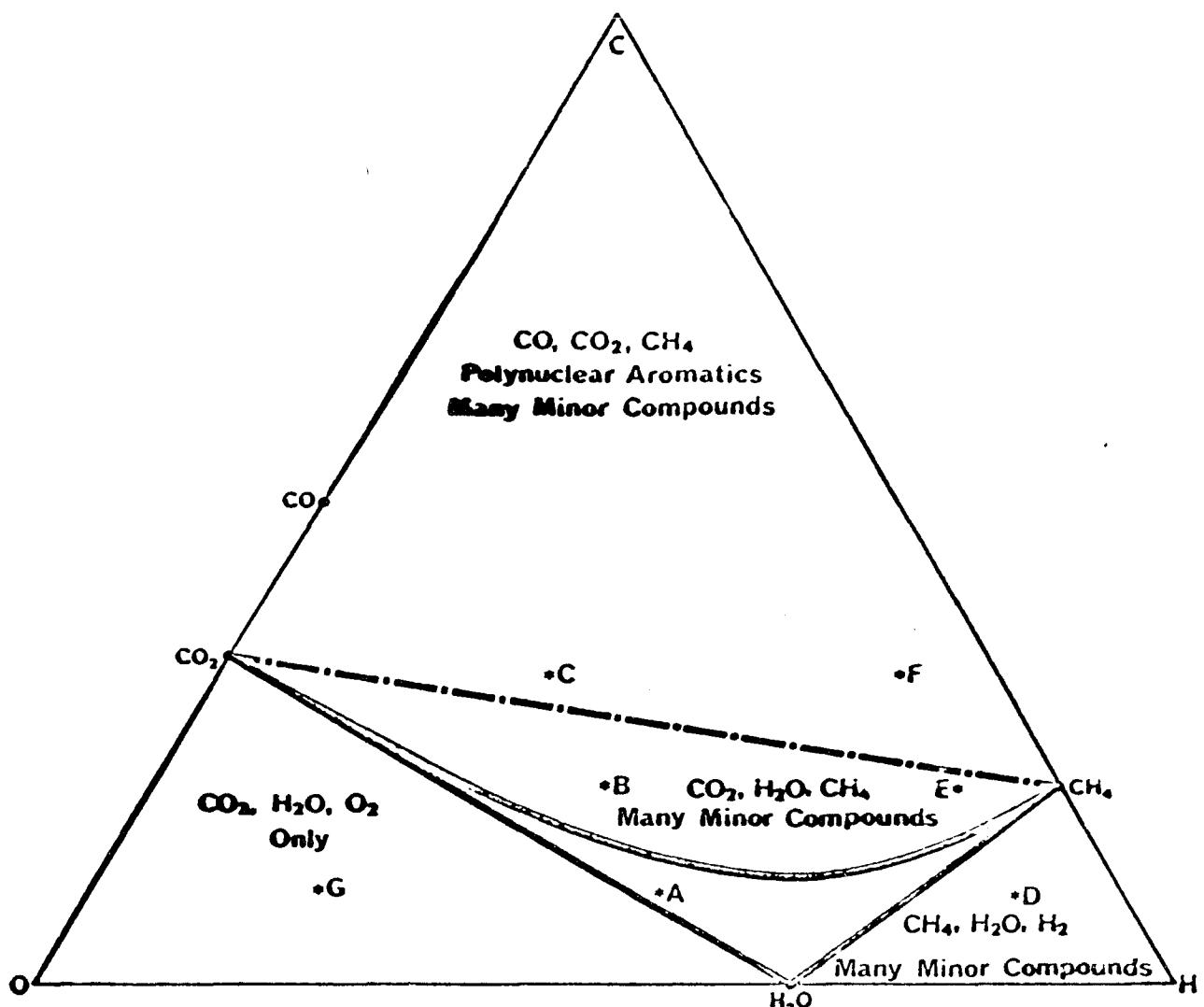


FIGURE 7. Thermodynamic equilibria in atmospheres of varying elemental proportions. The ternary diagram provides a display of systems of all possible relative proportions of C, H, and O. The points corresponding to atmospheres of pure gases of the major compounds are indicated and regions where different compounds are important are shown. The solid curve indicates the phase boundary along which graphite becomes stable at 1 atm. pressure and 500°K. The activation energy for this reaction is so high that under many conditions it does not occur and gaseous equilibria above this line are observed. Above the line  $\text{CH}_4\text{-CO}_2$ , equilibrium favors the formation of large proportions of polycyclic aromatic compounds or asphalts and a lesser increase in most of the other families of compounds. The graphite and asphalt lines are always present, but their position varies with temperature and pressure. A represents a system with C:H:O ratio 10:50:40; B, 20:40:40; C, 30:30:40; D, 10:80:10; E, 20:70:10; F, 30:60:10; and G, 10:20:70.<sup>18</sup>

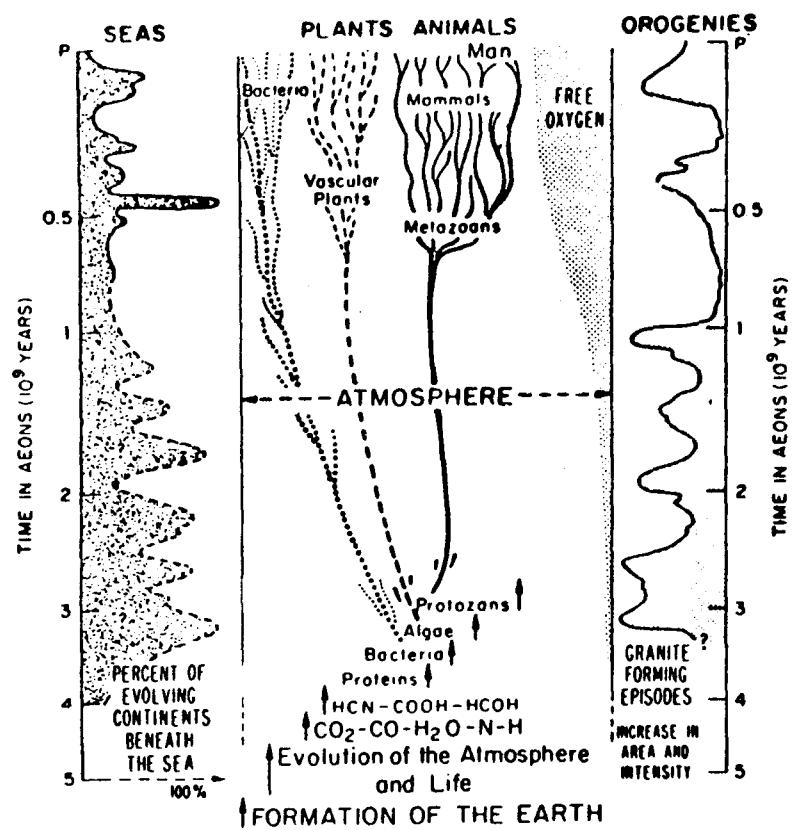


FIGURE 8. Interrelations, with geological time, of the fossil record of life, the evolution of the atmosphere, mountain-building, granite-forming events (orogenies), and the advance and retreat of seas across the continents. Modified from Cloud, 1968; Engel, 1964; Fisher, 1965; Holland, 1965; and many others.<sup>20</sup>

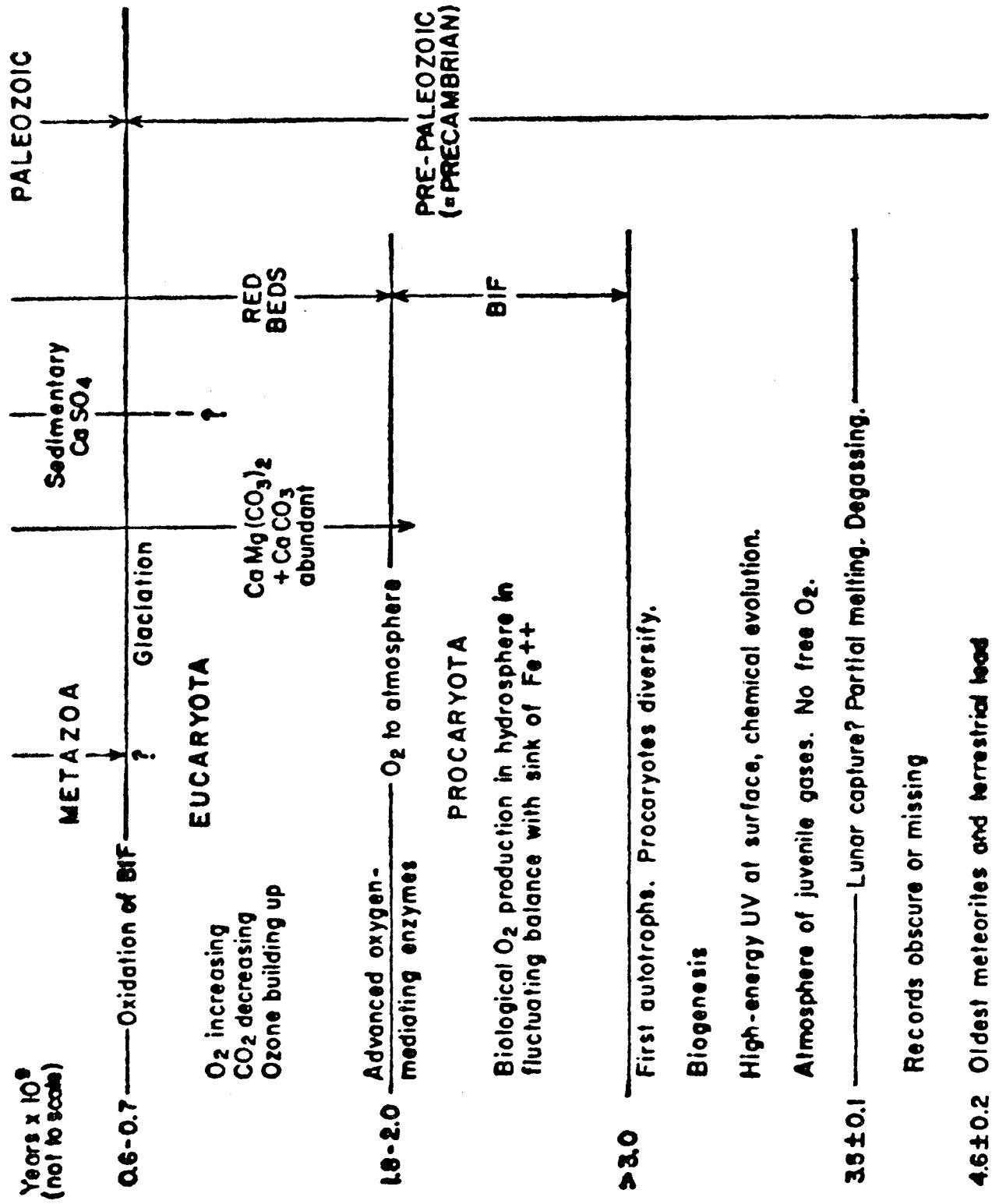


FIGURE 9. Postulated main features of interacting biospheric, lithospheric, and atmospheric evolution on the primitive earth.<sup>21</sup>

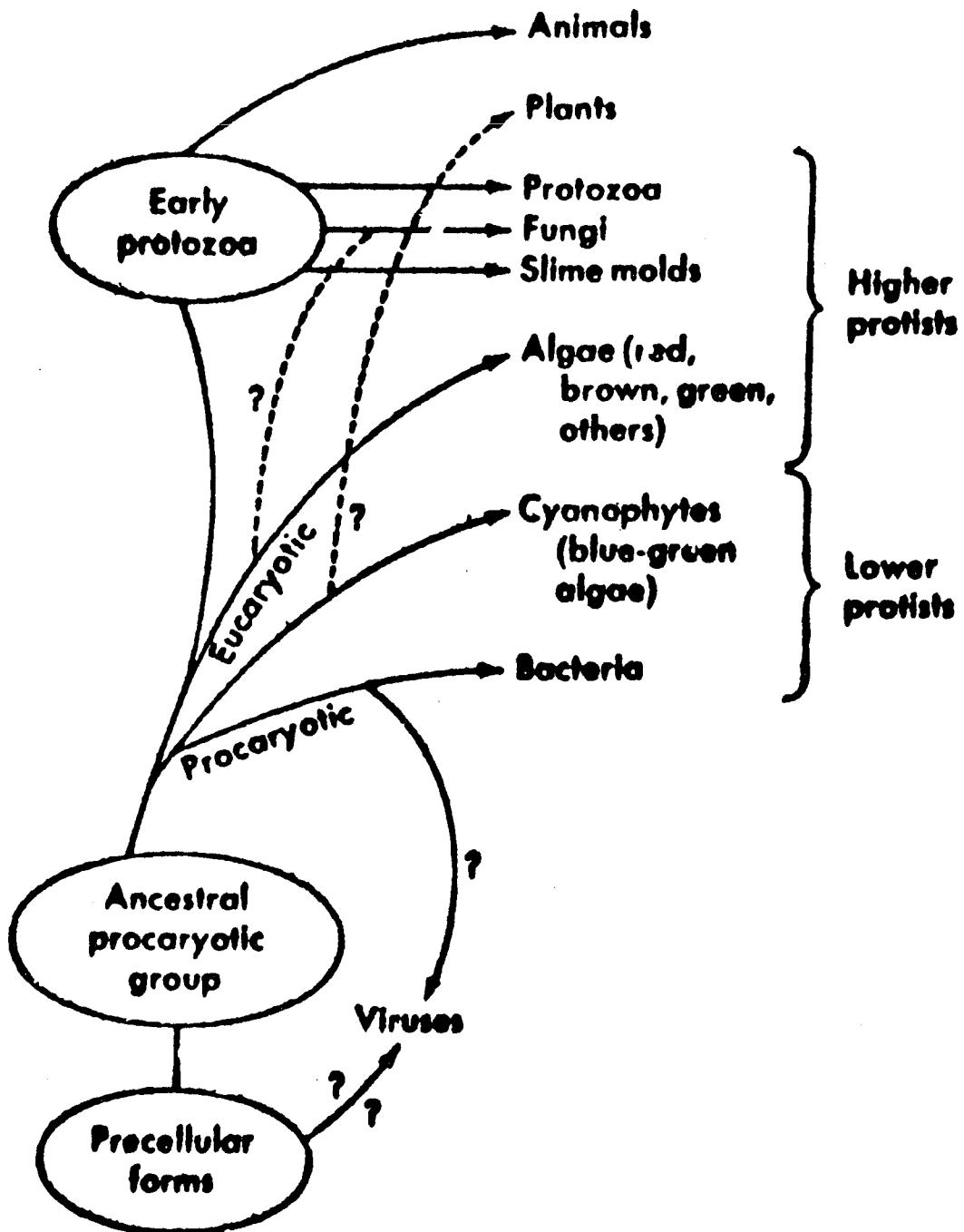


FIGURE 10. Biological Evolution.<sup>26</sup>

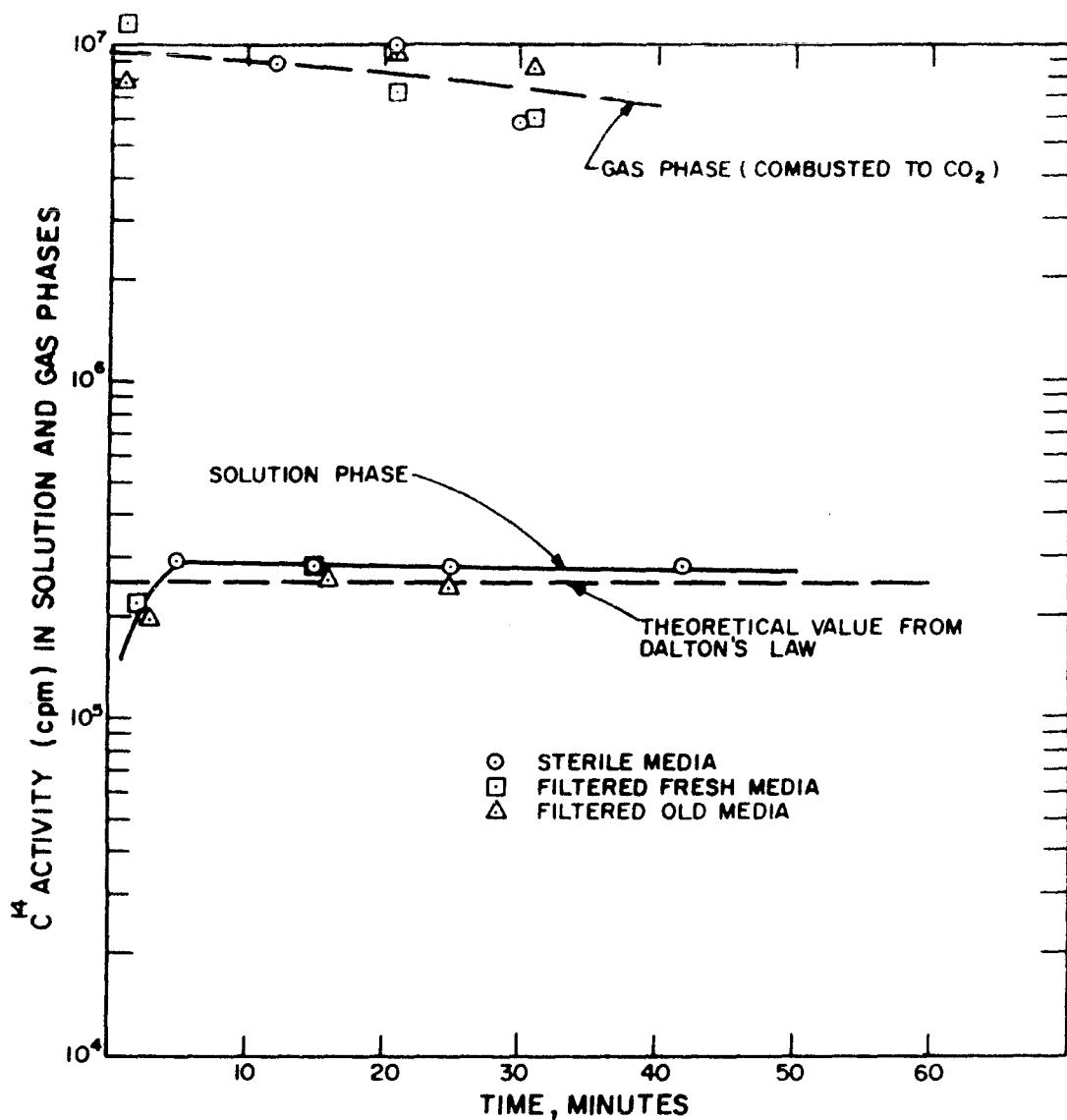


FIGURE 11. As predicted from Dalton's law,  $C^{14}$  activities in solution are fairly constant and independent of changes in methane partial pressures in the gas phase.

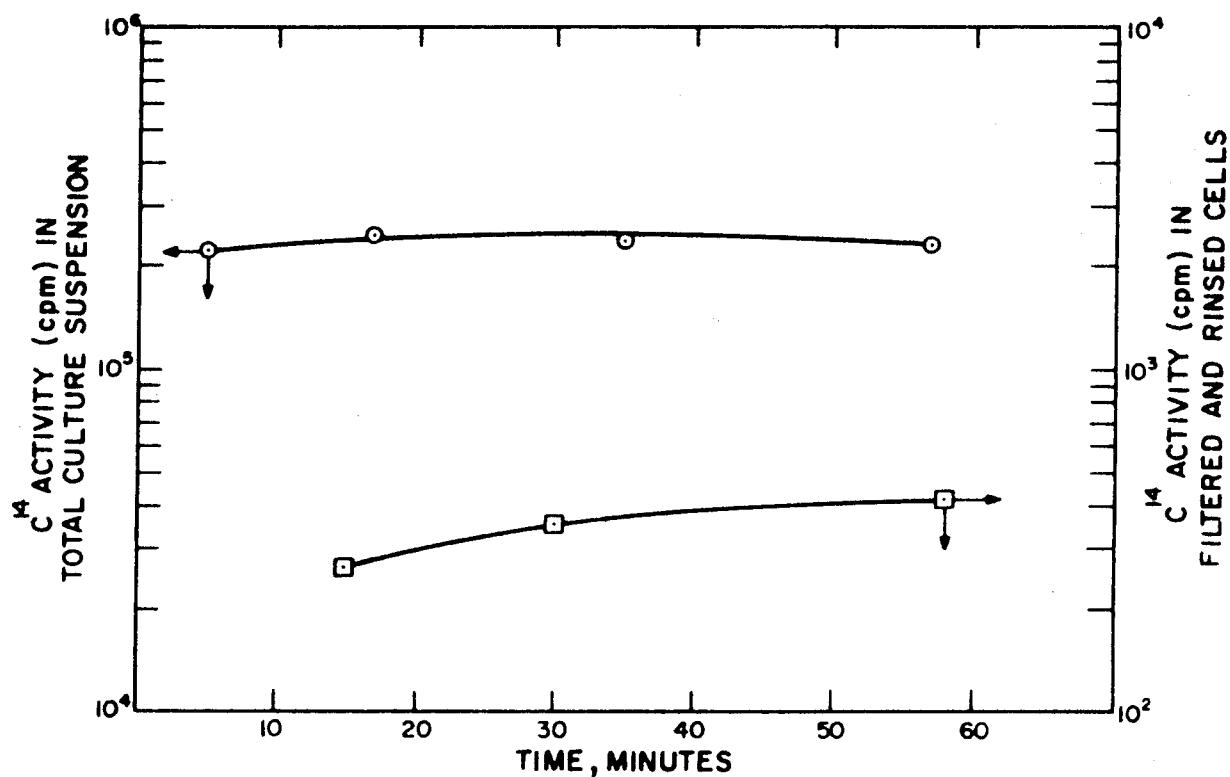


FIGURE 12. Cell walls and membranes pose a barrier to  $\text{C}^{14}\text{H}_4$  absorption. Only about 0.2% of the solution  $\text{C}^{14}$  activity gets into the 1.0% suspension of killed cells by physical transport.

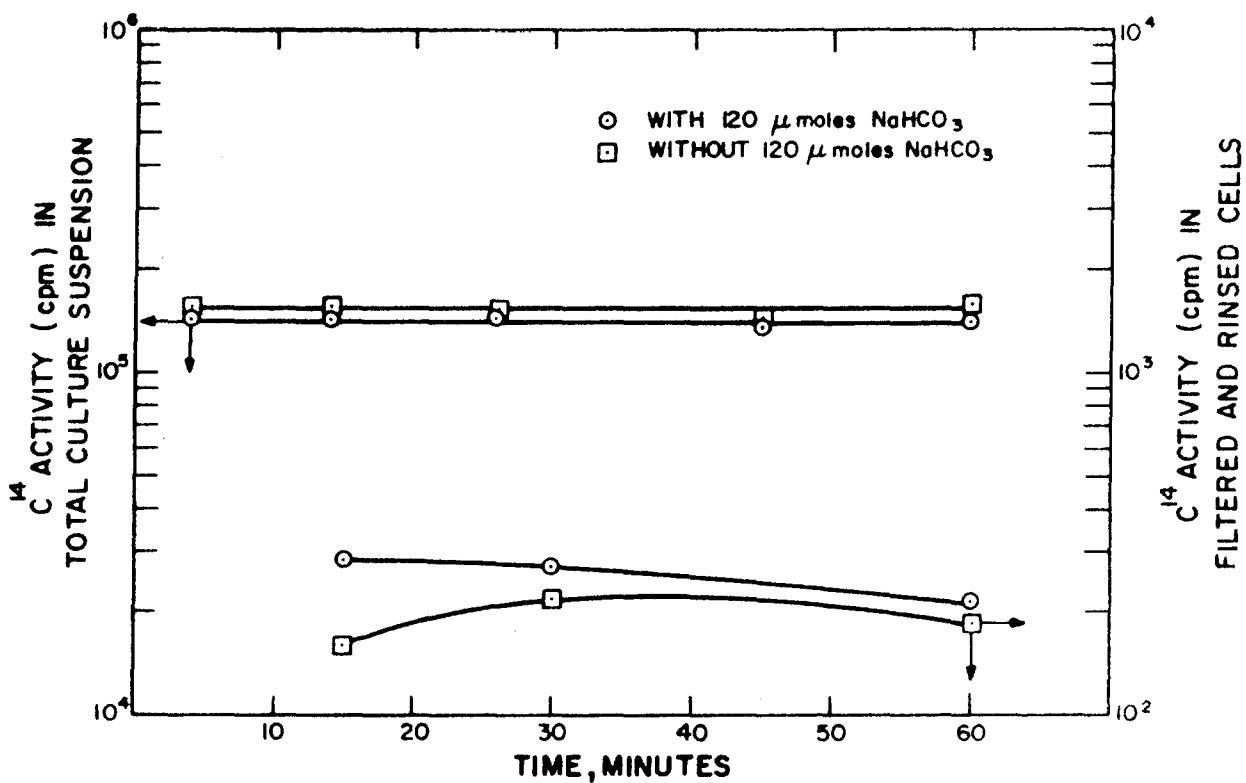


FIGURE 13. Unadapted culture differs little from one-day methane-adapted culture and shows little sodium bicarbonate effect.

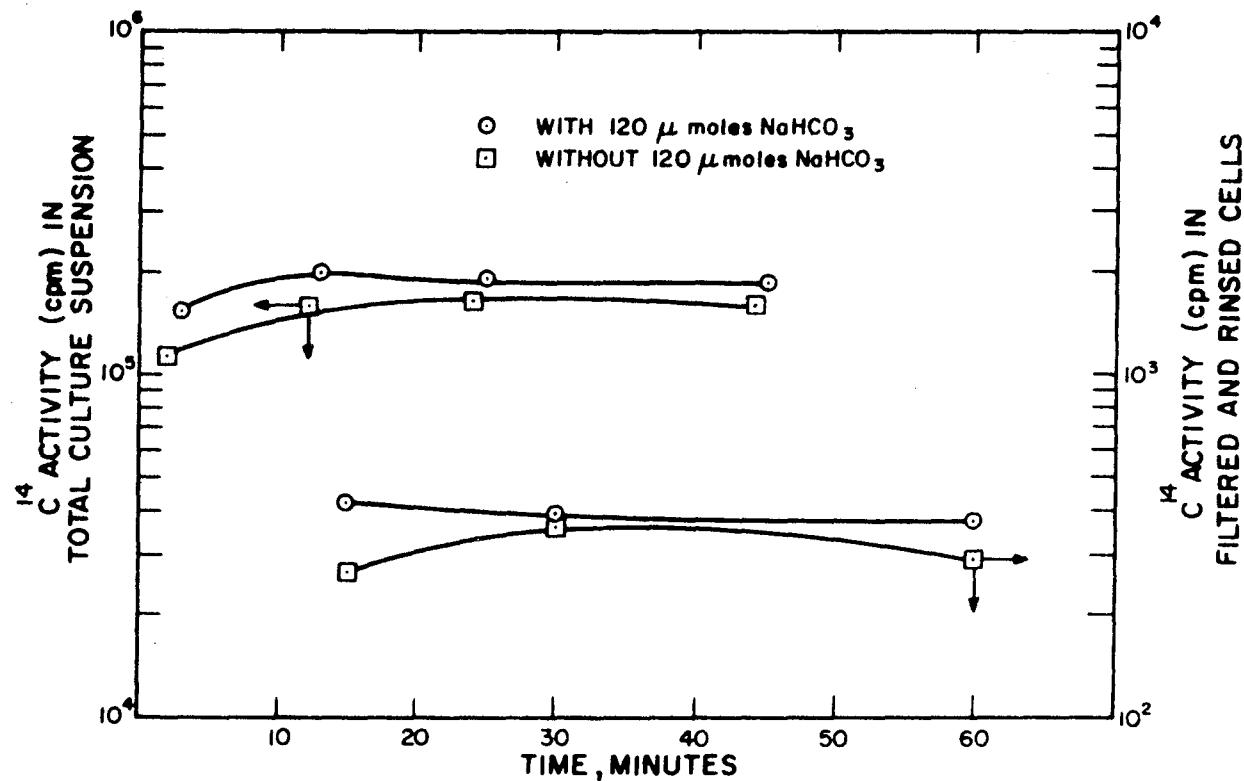


FIGURE 14. After one-day methane adaption, slightly more  $\text{C}^{14}$  activity is incorporated into the cells in the presence of sodium bicarbonate than in the absence of sodium bicarbonate.

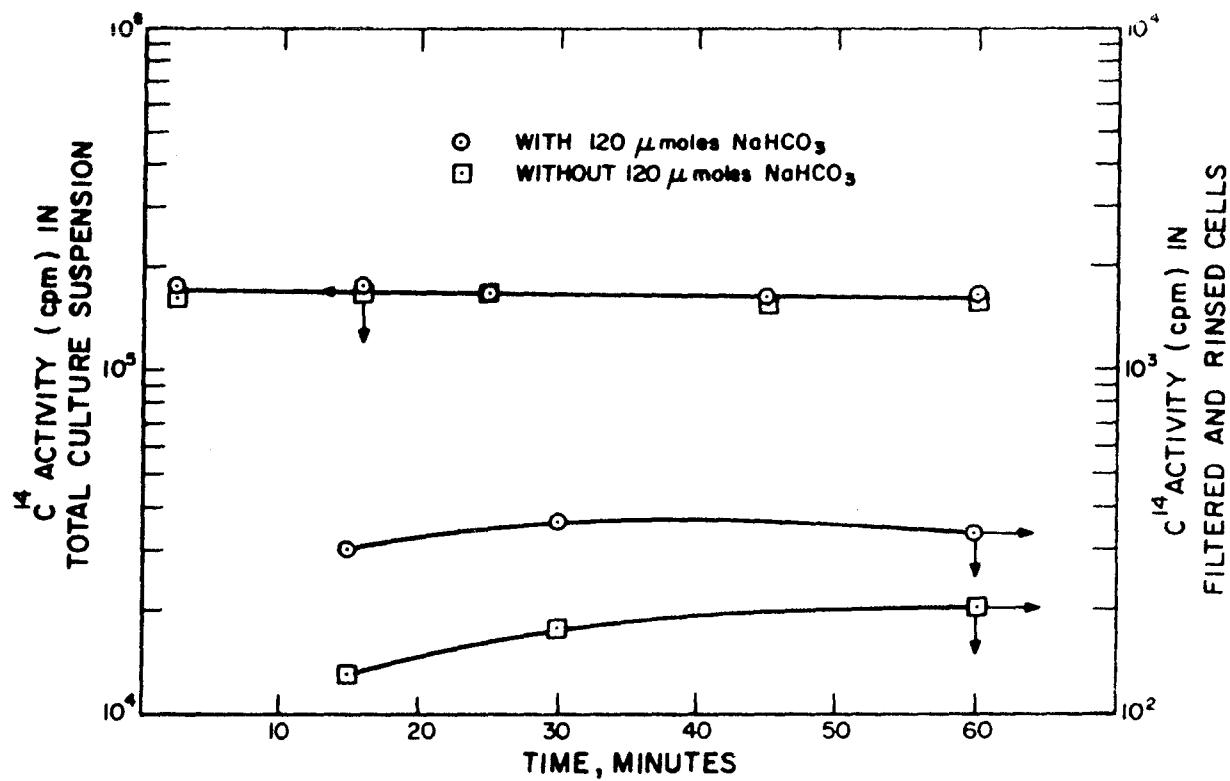


FIGURE 15. After three-day methane adaption, the sodium bicarbonate effect is very pronounced.

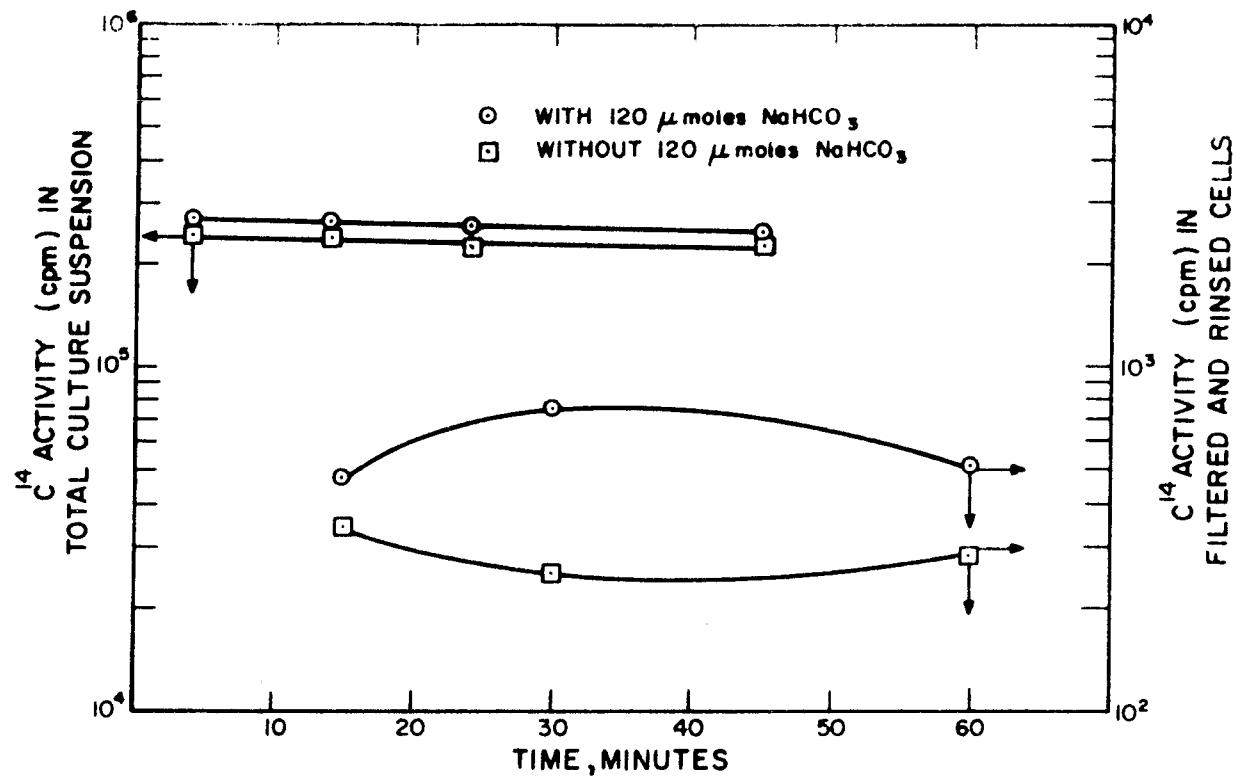


FIGURE 16. After eight-day methane adaption, the sodium bicarbonate effect continues to increase.

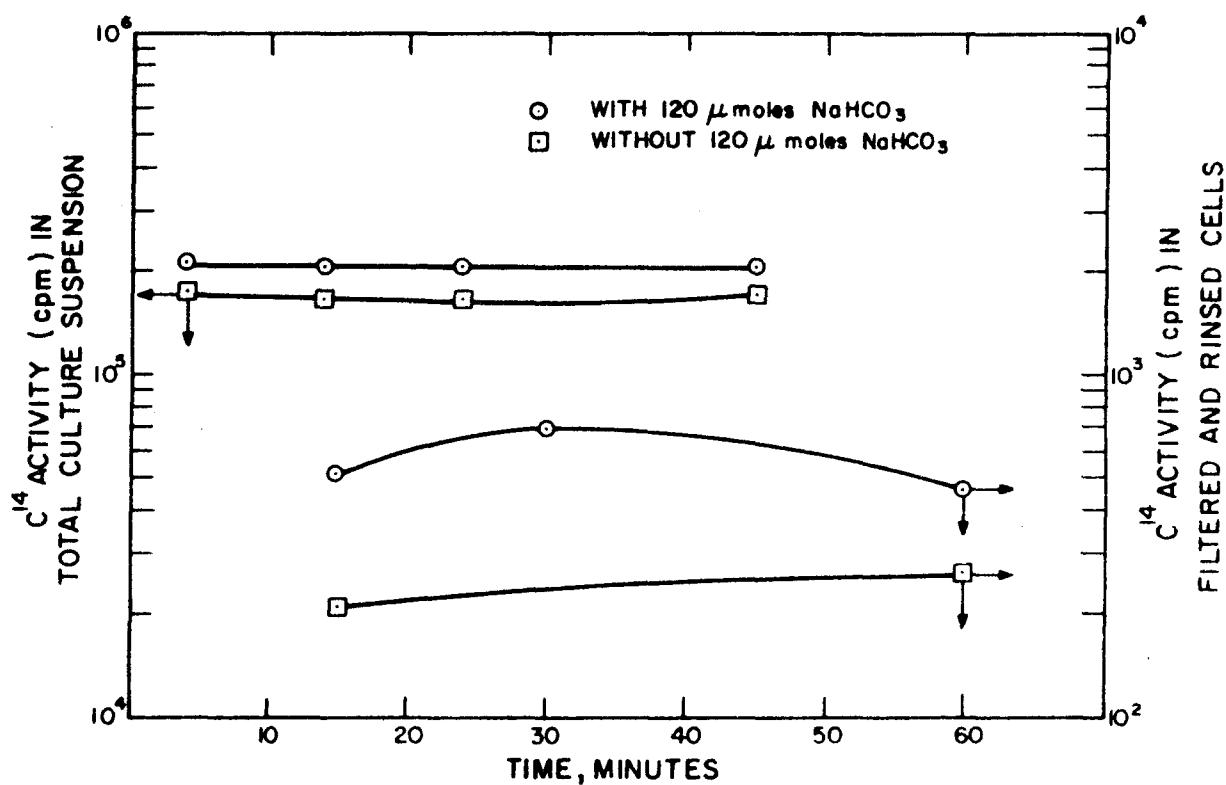


FIGURE 17. Eight-day adaption followed by four hours of 1%  $\text{CH}_4$  - 99%  $\text{N}_2$  (without  $\text{O}_2$ ) does not enhance the sodium bicarbonate effect.

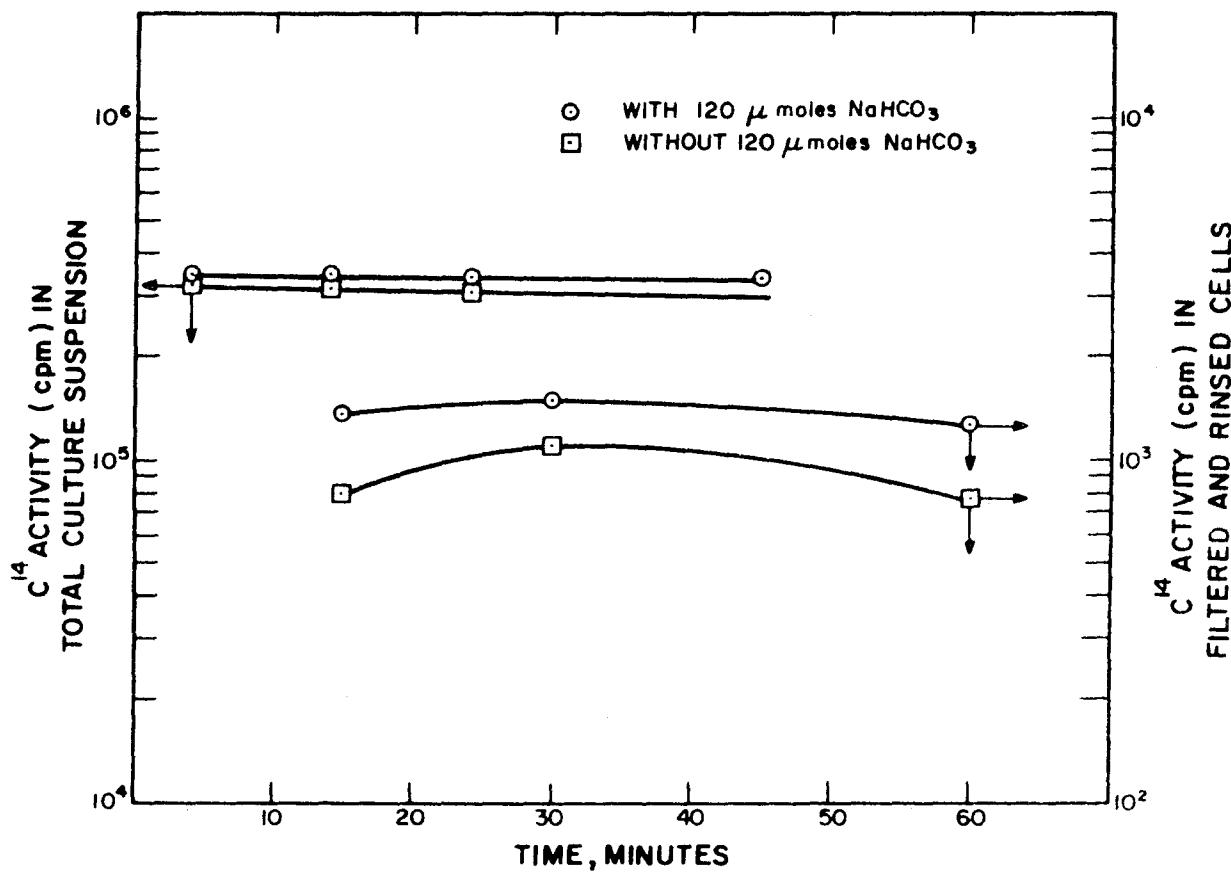


FIGURE 18. Four-day rigorous adaption with 1% CH<sub>4</sub> -99% N<sub>2</sub> (without O<sub>2</sub>) increases cell C<sup>14</sup> activities but is fatal.

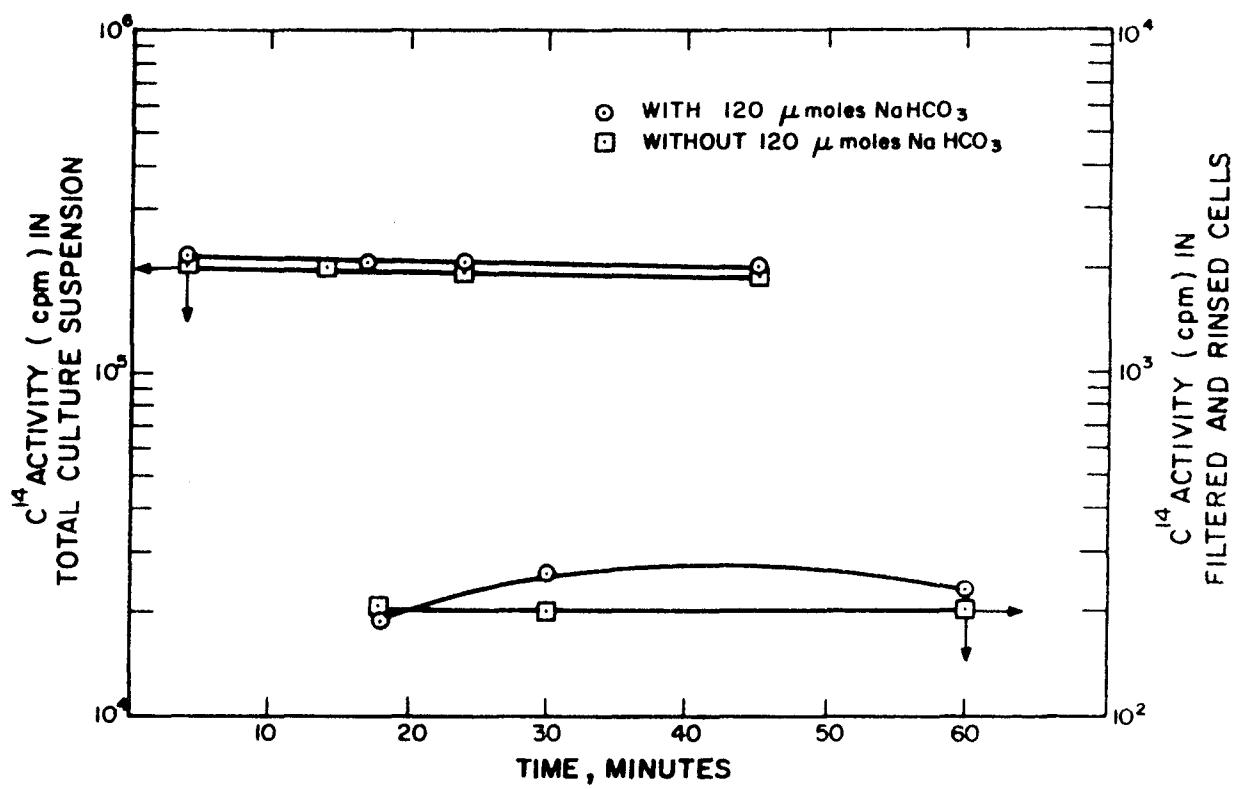


FIGURE 19. Ammoniacal cuprous chloride reagent treatment to remove  $\text{C}^{14}\text{O}$  slightly reduces the  $\text{C}^{14}$  activity level of unadapted cells. Sodium bicarbonate enhancement persists.

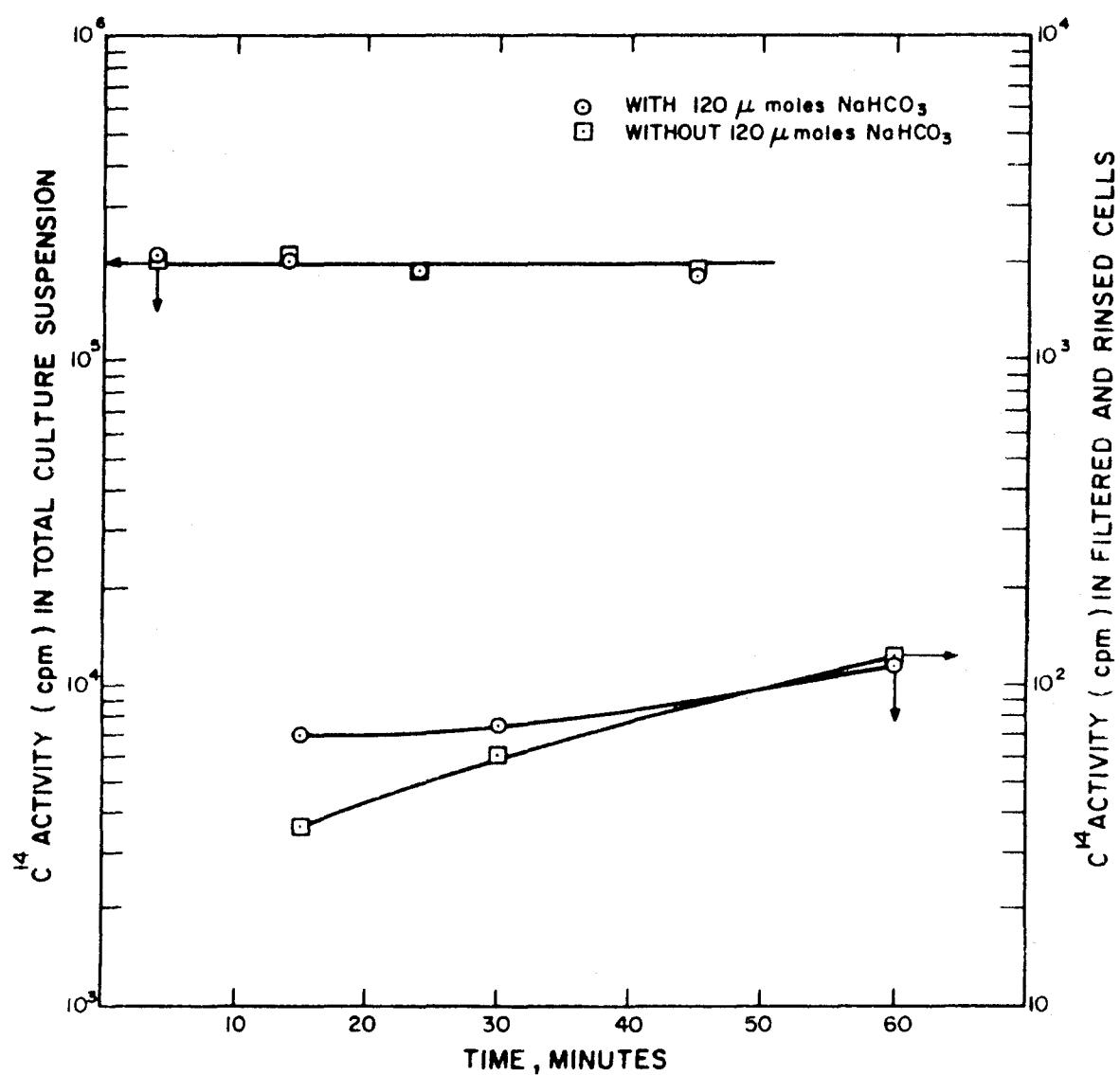


FIGURE 20. Nostoc incorporates  $\text{C}^{14}\text{H}_4$  more slowly and to a lesser extent than Anacystis nidulans. Sodium bicarbonate facilitates early absorption.

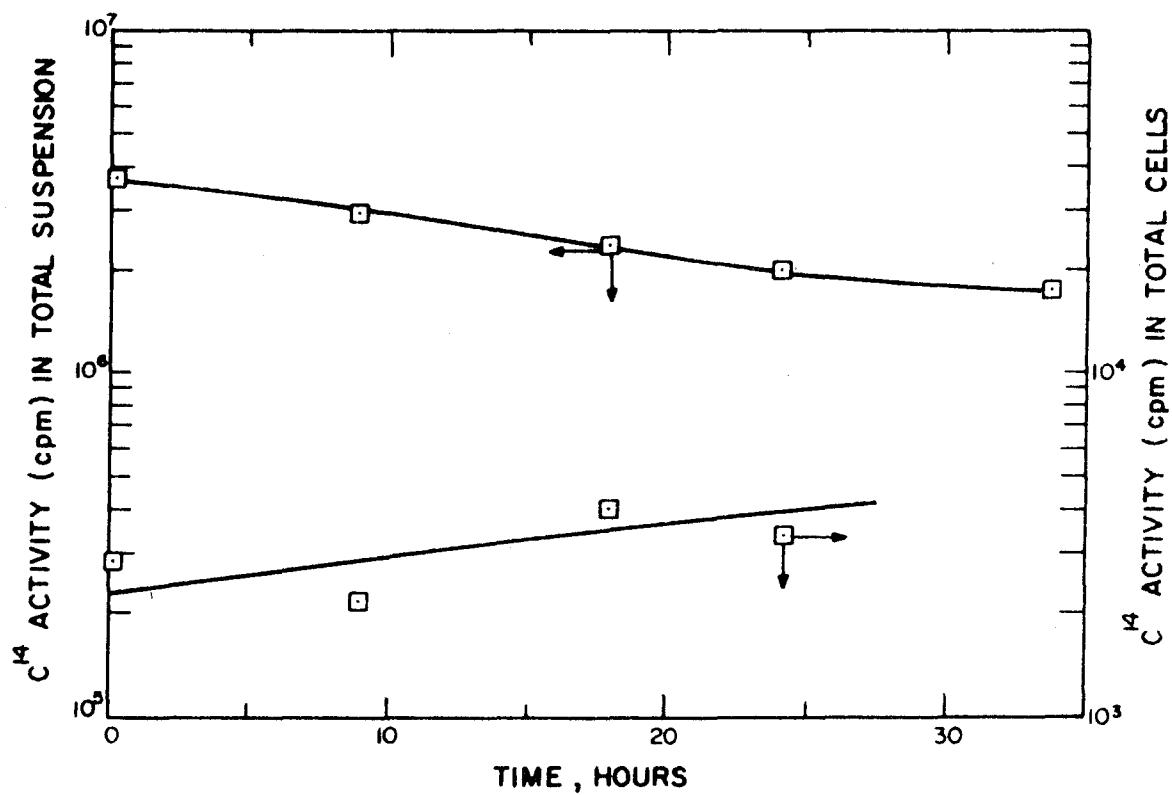


FIGURE 21. *Rhodospirillum rubrum* absorbs little  $\text{C}^{14}\text{H}_4$ , grows slowly.

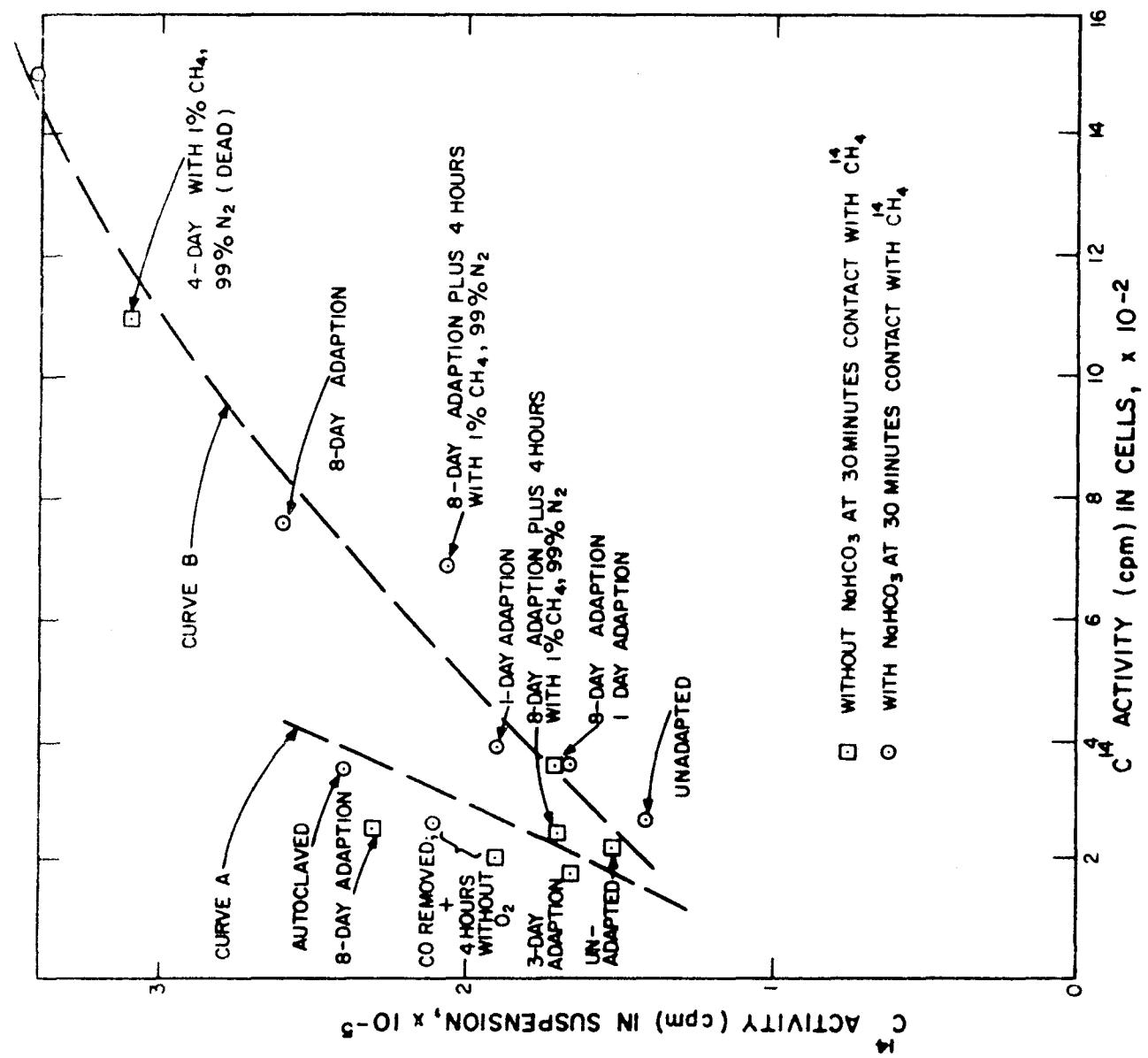


FIGURE 22. Methane adaptation is effected in *Anacystis nidulans*. Sodium bicarbonate facilitates incorporation of  $\text{C}^{14}\text{H}_4$ .

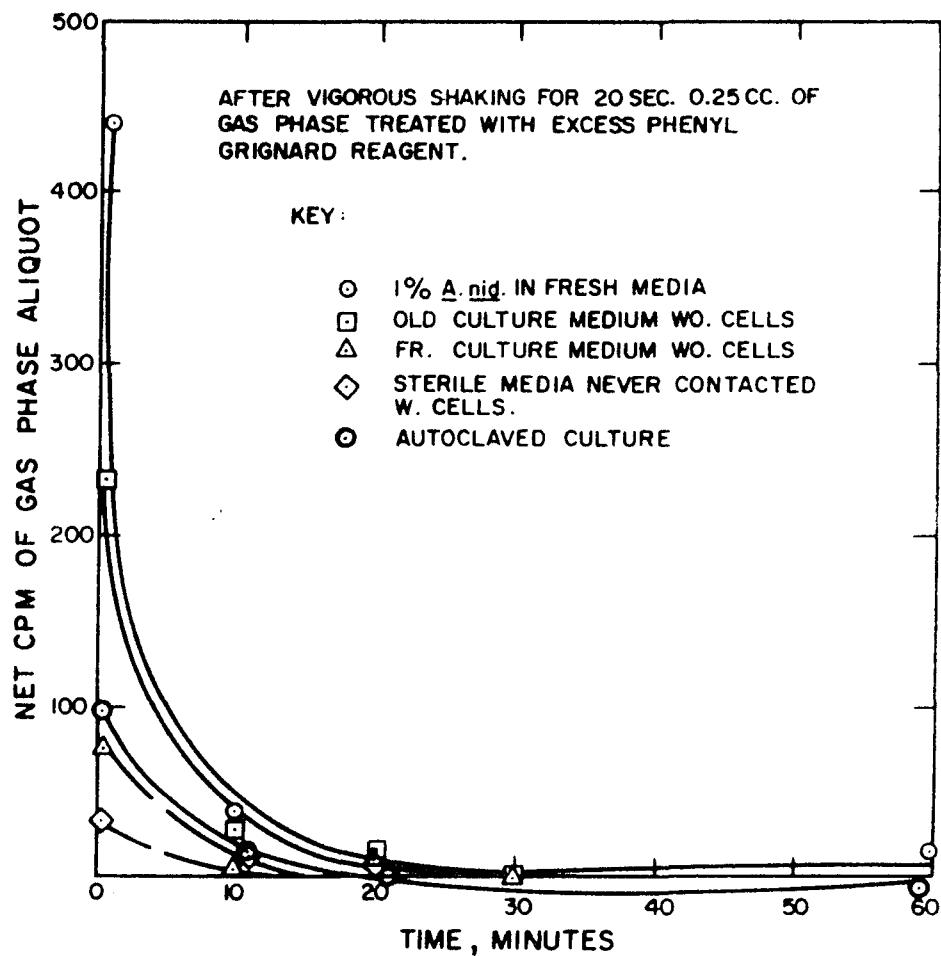


FIGURE 23. *Anacystis nidulans* cells and extracellular secretions rapidly oxidize  $\text{C}^{14}\text{H}_4$  to  $\text{C}^{14}\text{O}$  which is slowly equilibrated with the aqueous  $\text{NaHCO}_3$ .

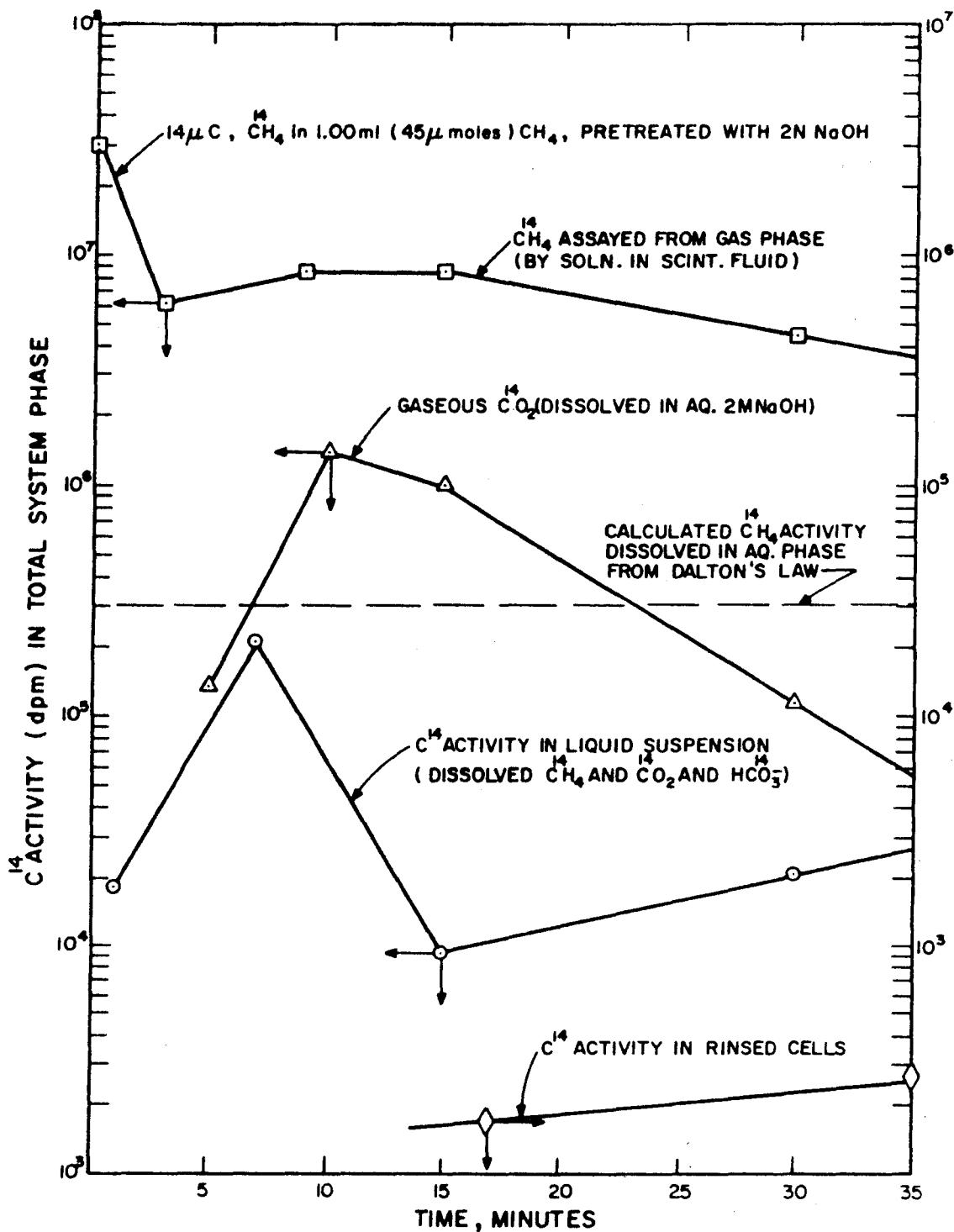


FIGURE 24. C<sup>14</sup> incorporation by Anacystis nidulans in the shake flask under slow initial equilibration (A.N.-1).

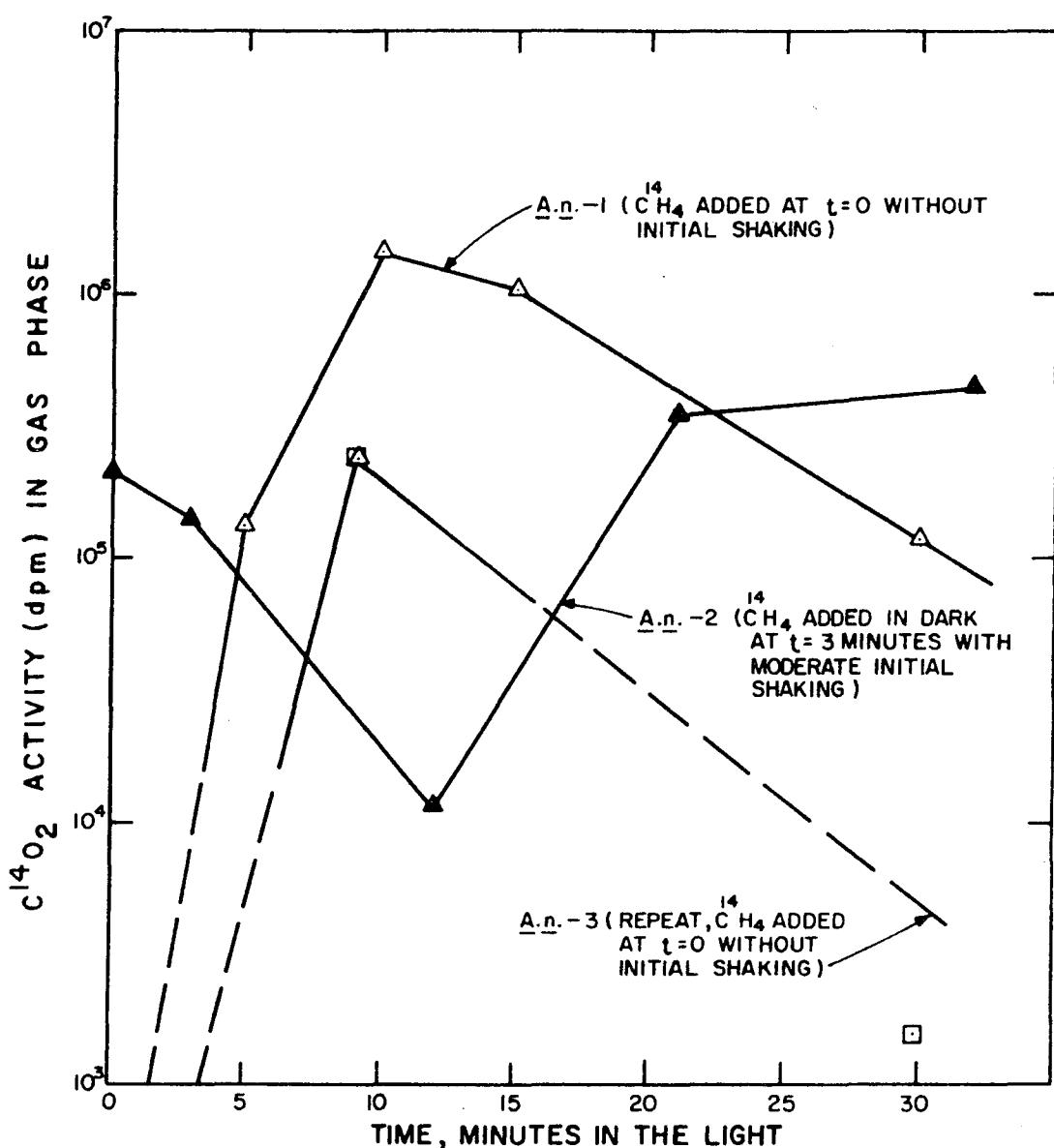


FIGURE 25.  $C^{14}$ -methane oxidation to  $C^{14}O_2$  by Anacystis nidulans is confirmed in the presence and absence of light.

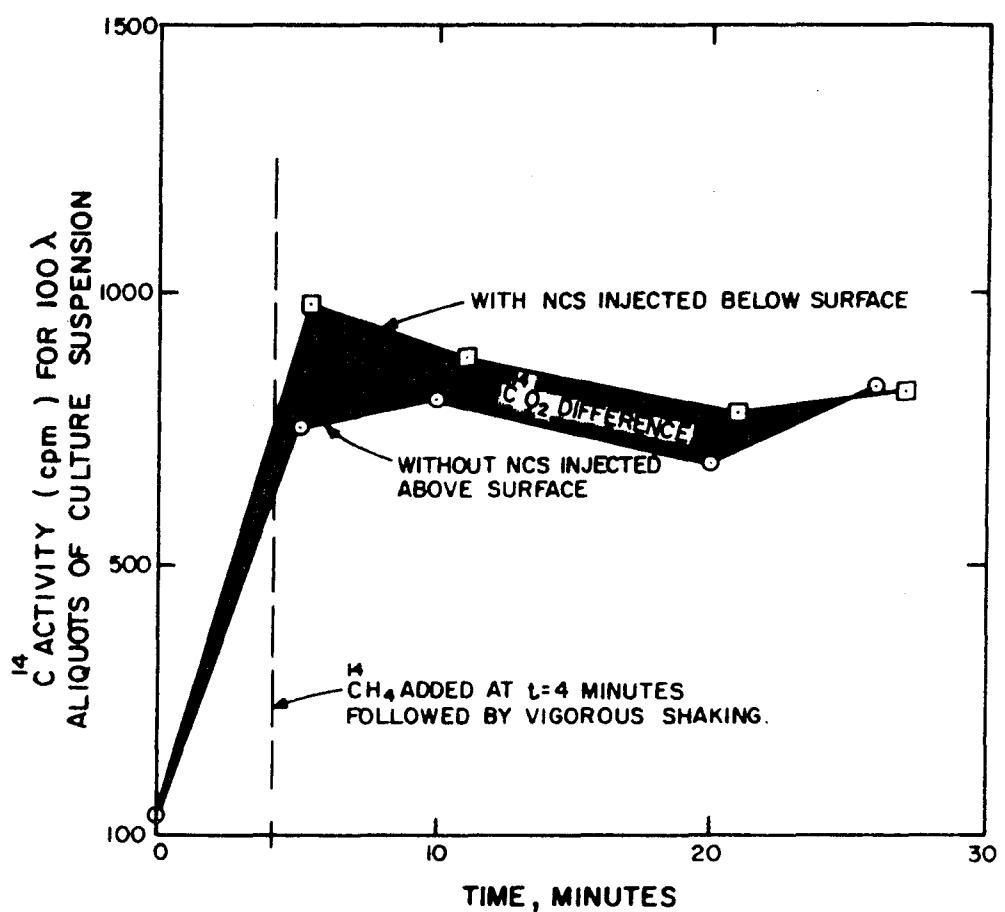


FIGURE 26. A 10 to 20% increase in  $\text{C}^{14}$  activity is obtained with NCS and subsurface injection of liquid suspension aliquots into the scintillation fluid.

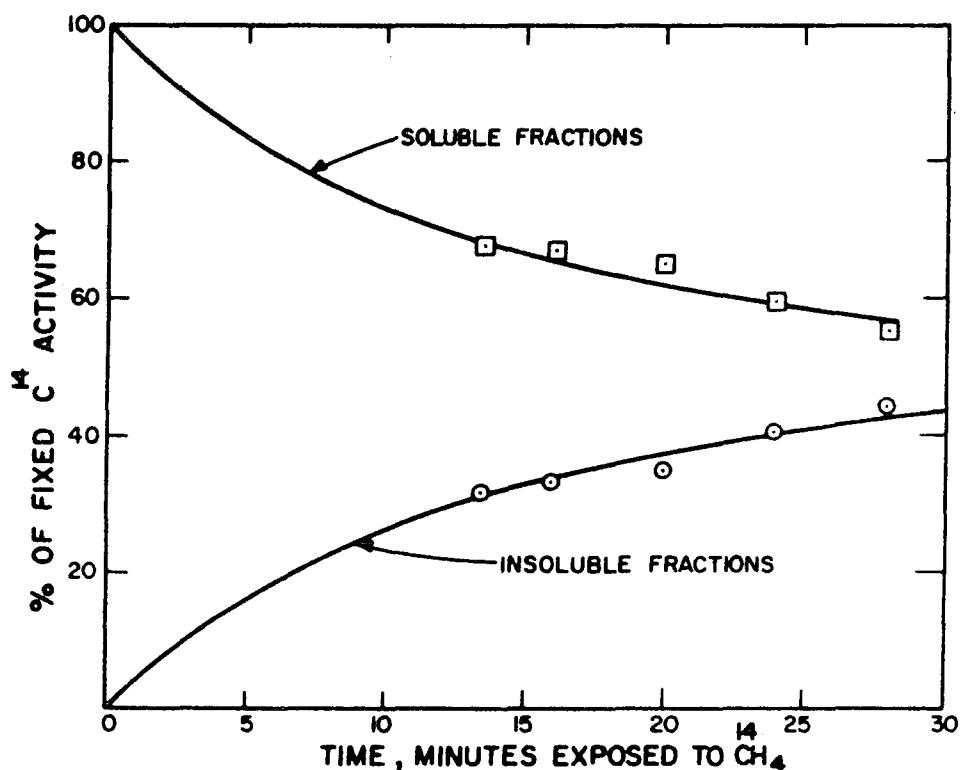


FIGURE 27. Anacystis nidulans incorporates  $\text{C}^{14}$  into both soluble and insoluble components in the chromatographic development solvents.

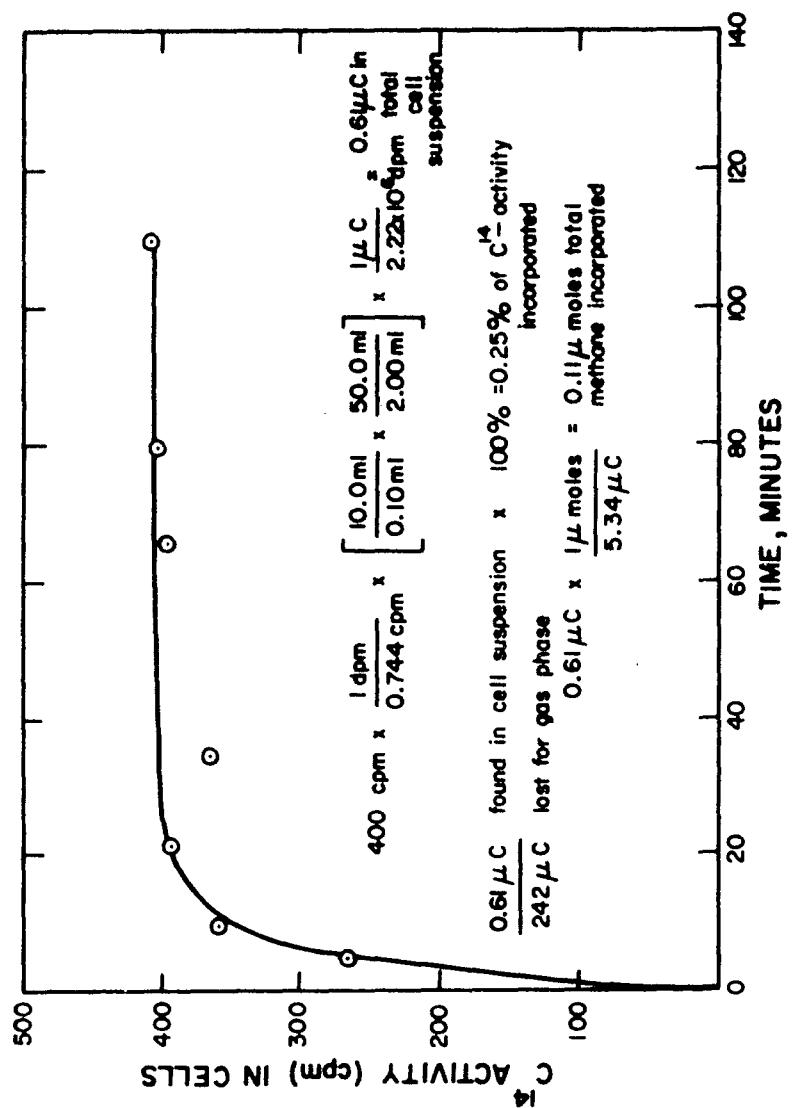


FIGURE 28. Anacystis nidulans cells are saturated in C<sup>14</sup> in about 20 minutes in steady-state apparatus.

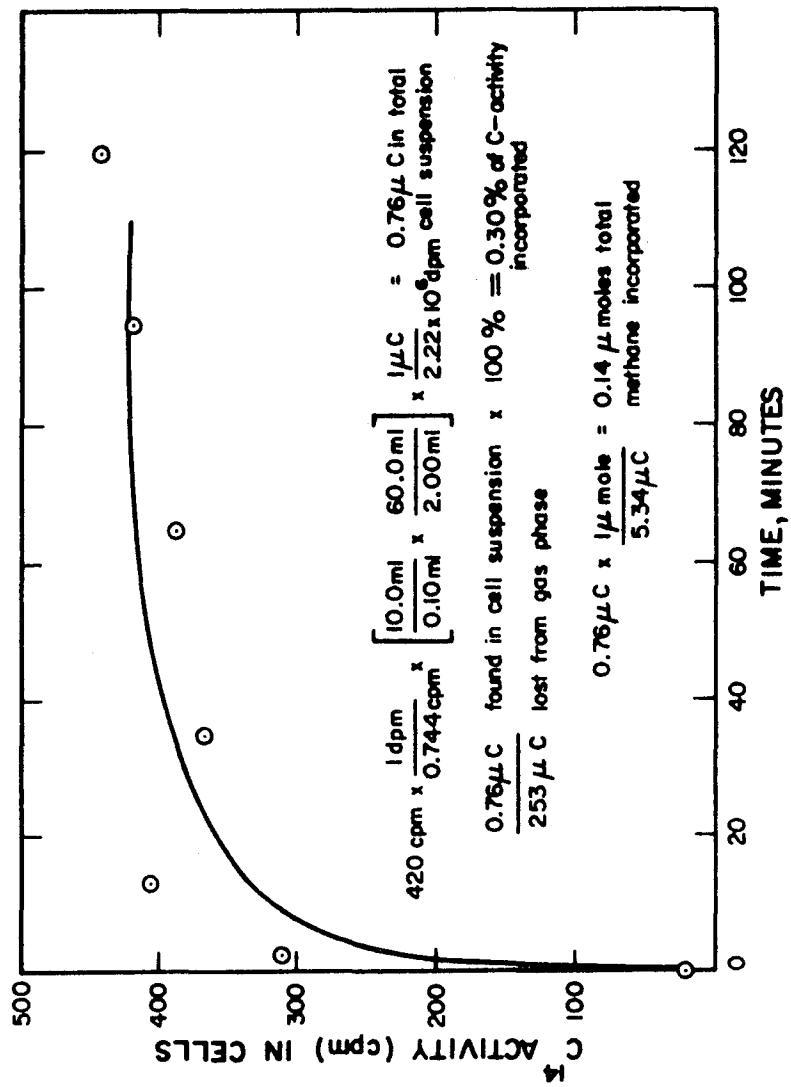


FIGURE 29. Nostoc cells are saturated in  $\text{C}^{14}$  in about 20 minutes in steady-state apparatus.

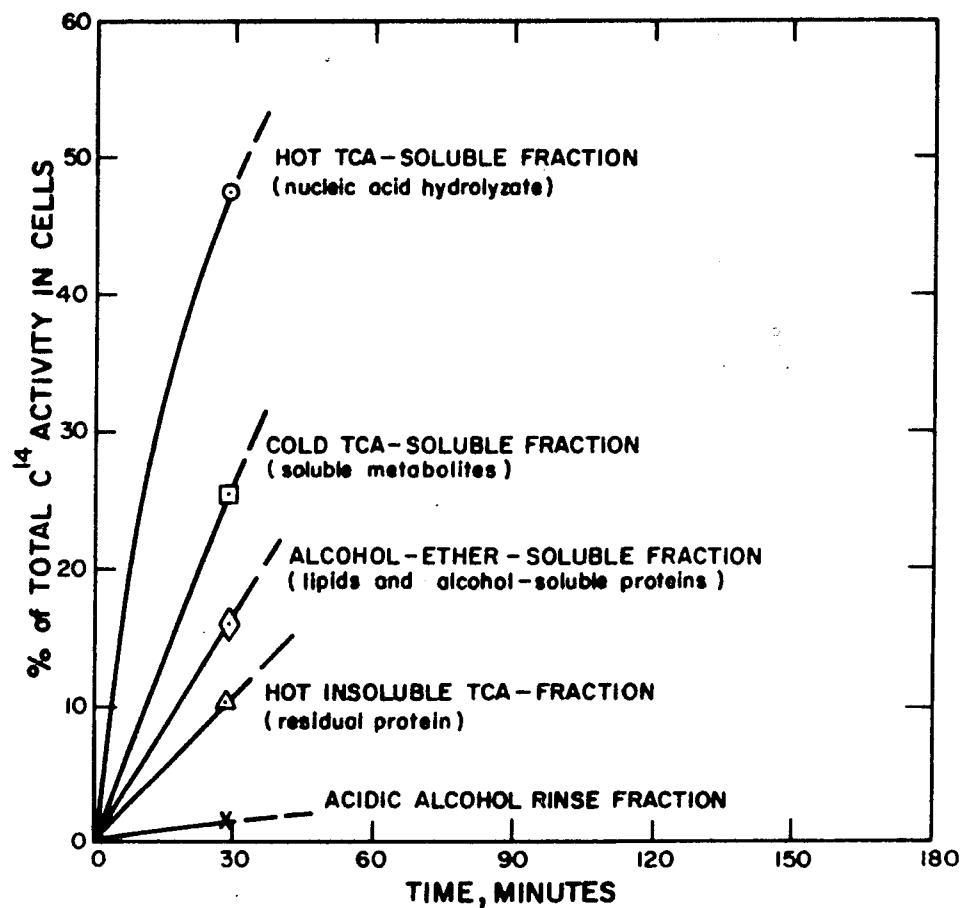


FIGURE 30. *Anacystis nidulans* incorporates  $\text{C}^{14}$  more rapidly than *Nostoc* into a distinctive biochemical profile.

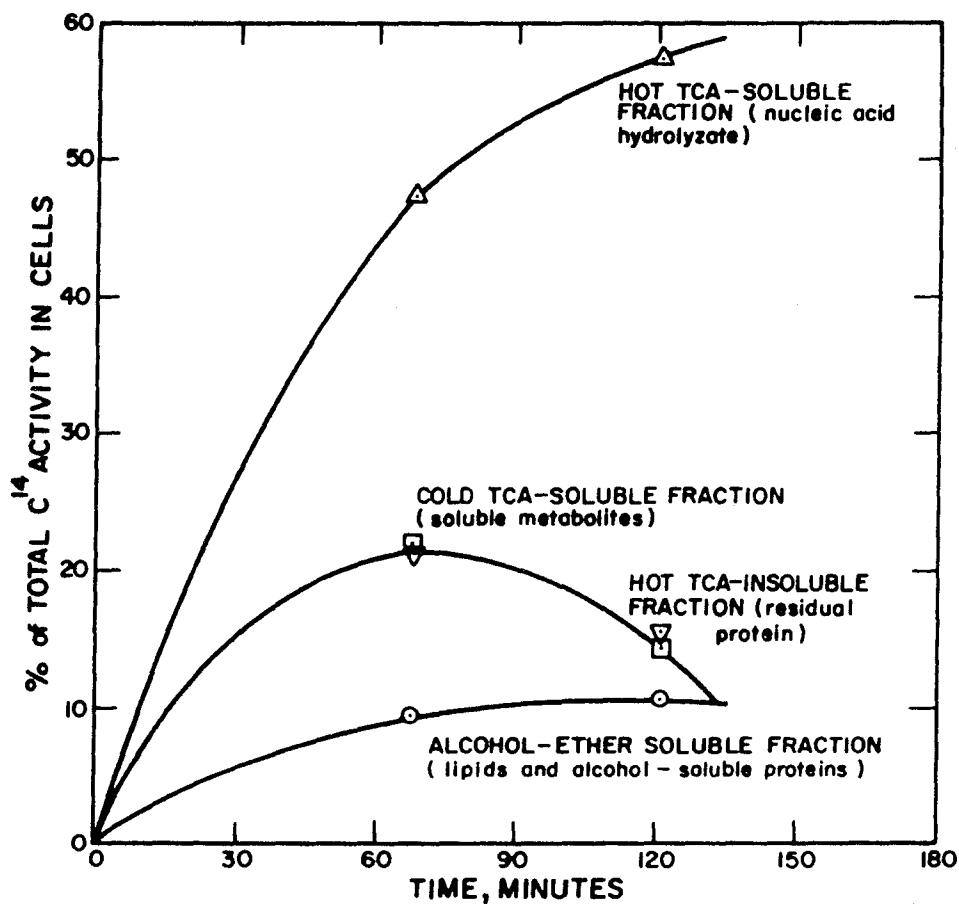


FIGURE 31. Nostoc incorporates  $\text{C}^{14}$  more slowly than Anacystis nidulans into a distinctive biochemical profile.



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