A New Method for the Preparation of Uniformly ^{14}C -Labelled Compounds by Using *Anacystis nidulans*

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1. Growth conditions were devised which enabled *Anacystis nidulans* to be grown on high specific radioactivity $^{14}\text{CO}_2$ (at over 98% isotopic abundance of ^{14}C) in a closed system with greater than 85% substrate utilization. 2. The DNA and RNA content of *A. nidulans* was estimated by various methods and compared with that of *Chlorella pyrenoidosa*. 3. A procedure was developed for the quantitative extraction and separation of the DNA and RNA from *A. nidulans*. 4. The fractionation and analysis of the DNA and RNA includes methods for the preparation of the uniformly ^{14}C -labelled deoxyribo-nucleoside monophosphates and the uniformly ^{14}C -labelled ribonucleoside monophosphates. 5. The base 6-methylaminopurine was identified as a component of the DNA. 6. The preparation and total yield of uniformly ^{14}C -labelled amino acids from *A. nidulans* are also briefly described. The results are discussed in relation to the use of other micro-organisms for the preparation of compounds labelled with ^{14}C .

Amino acids and nucleotides uniformly labelled with ^{14}C have traditionally been obtained from the photosynthetic organism *Chlorella pyrenoidosa* grown on $^{14}\text{CO}_2$ (Nejedly *et al.*, 1968) or from yeast grown on uniformly ^{14}C -labelled invert sugar (Laufer *et al.*, 1964). The use of photosynthetic organisms has been preferred because $^{14}\text{CO}_2$ is a simple and convenient starting material. In addition such organisms generally incorporate about 90% of the supplied substrate into their cellular material.

With increasing need for high specific radioactivity ^{14}C -labelled compounds and in particular labelled nucleotides the choice of *C. pyrenoidosa* for their preparation has been subject to critical re-evaluation. Analysis has shown that *C. pyrenoidosa* has a satisfactory RNA content (about 3% of the dry weight) but a less satisfactory DNA content (about 0.2%). Prokaryotic organisms such as bacteria often have a higher nucleic acid content with higher DNA/RNA ratios. However, these organisms are generally unsuitable for the preparation of labelled compounds as only 30% of the carbon source in the medium of a heterotroph is incorporated into the cells (Roberts *et al.*, 1955). The ideal organism for this work should be a radiation-resistant photoautotroph with a high nucleic acid content.

Blue-green algae are similar to bacteria in having a prokaryotic cellular organization but differ in that they are usually obligate photoautotrophs (Holm-Hansen, 1968). Evidence of the resistance of blue-

green algae to external radiation was obtained by Krauss (1966) who showed that viable bacteria-free cultures could be obtained after exposure to a ^{60}Co source. Craig *et al.* (1969) determined the amount of DNA present in seven species of blue-green algae. Expressed as a percentage of the dry weight the DNA content of these algae ranged from 0.43 to 1.7. The DNA content of *A. nidulans*, however, at 1.7% was double that of any other species examined.

Results are presented here which show that *A. nidulans* can be grown photoautotrophically on $^{14}\text{CO}_2$ at specific radioactivities that are close to 100% isotopic abundance. Approximately 85% of the $^{14}\text{CO}_2$ supplied is incorporated into the cells. Procedures are described for the isolation of ^{14}C -labelled nucleotides and amino acids from this organism. This is the first report of the use of a blue-green alga for the preparation of ^{14}C -labelled compounds at high molar specific radioactivity (British Patent no. 1 342 098).

Materials and Methods

Organism

Anacystis nidulans no. 625 was obtained from The Culture Collection of Algae, Department of Botany, Indiana University, Ind., U.S.A. *Chlorella pyrenoidosa* was originally supplied in 1953 by Professor G. E. Fogg (Department of Botany, University College, London, U.K.).

Chemicals

Salmon sperm DNA, 6-methylaminopurine-9-ribofuranoside, DNAase* I (deoxyribonuclease, EC 3.1.4.5), phosphodiesterase I (oligonucleate nucleotidohydrolase, EC 3.1.4.1) and 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) were obtained from Sigma (London) Chemical Company Ltd. (Kingston-upon-Thames, Surrey, U.K.). Purified yeast RNA was from British Drug Houses Ltd. (Poole, Dorset, U.K.) and Triton X-100 detergent from Lening Chemicals Ltd. (Jarrow, Durham, U.K.). PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene] were obtained from Nuclear Enterprises (Edinburgh, U.K.).

Methods

Medium and growth conditions. *C. pyrenoidosa* was grown at 28°C on $^{14}\text{CO}_2$, in the same apparatus as was used for *A. nidulans*, on the medium described by Myers *et al.* (1951). The culture was illuminated by twelve 40W daylight fluorescent tubes arranged around the growth vessel at a distance of 12cm.

A. nidulans was grown as follows. Stock cultures were maintained on 1% (w/v) agar slopes by the method of Allen (1968). Other cultures were grown at 37°C in modifications of the medium C and medium D of Kratz & Myers (1955a). Medium C, supplemented with 0.05% (w/v) NaHCO_3 , was used for all open systems, i.e. cultures continuously gassed with 5% CO_2 in air. Medium D, modified by the addition of 10g of $\text{K}_2\text{HPO}_4/\text{l}$ and adjusted to pH 9.6 with NaOH, was used for the large-scale growth of the organism on $^{14}\text{CO}_2$ in a closed system.

Modified medium D (5litres) was contained in a cylindrical vessel which has a total volume of 25litres. The vessel had a centrally placed port sealed by a rubber membrane through which a metal agitation rod was inserted. A side port, protected by a sterile filter, provided means for the admission of $^{14}\text{CO}_2$, sampling the culture medium and the addition of HCl. After addition of a 5% (v/v) inoculum the growth vessel was evacuated to approx. 40kPa below atmospheric pressure. $^{14}\text{CO}_2$ (100mmol; specific radioactivity 60Ci/mol) was generated from $\text{Ba}^{14}\text{CO}_3$ by using HClO_4 and then swept into the growth vessel with CO_2 -free air until the pressure had risen to approx. 13kPa below atmospheric pressure. The culture was illuminated by sixteen 40W daylight fluorescent tubes arranged around the growth vessel at a distance of 12cm and the culture was gently agitated. After 24h growth under the conditions described the culture had an E_{380} of 3.

Monitoring growth and control of pH. At suitable intervals, 10ml portions of the culture were removed from the growth vessel through a capillary sampling tube. The extinction, radioactivity and pH of the solution were measured.

(a) Extinction. The extinction of the suspension was measured at 380nm with Unicam SP.800 spectrophotometer.

(b) Radioactivity. At all stages throughout the growth, radioactive samples (0.1ml) were measured in a Nuclear-Chicago Mark I liquid-scintillation counter by using 10ml of Triton X-100-PPO-toluene scintillant (2 parts of toluene containing 5.285g of PPO/l and 1.12g of POPOP/l + 1 part of Triton X-100) containing 10% (v/v) water. Radioactivity in the supernatant (from a sample of the culture) was determined after centrifugation. The radioactivity incorporated into the cells was measured after passing the samples (0.1ml) through Millipore filters (0.48 μm). The latter were dried and counted for radioactivity in the same scintillant as above with the omission of the 10% water.

(c) Control of pH. During growth on $^{14}\text{CO}_2$ the pH of *A. nidulans* cultures was maintained between 7.5 and 8.5 by the addition of HCl.

(d) Harvesting. *C. pyrenoidosa* was harvested after 50h and *A. nidulans* after 28h. The cells were collected by centrifuging the culture at 2°C and 20000g by using an MSE High-Speed 18 refrigerated centrifuge fitted with a flow-through rotor.

Estimation of nucleic acids. Nucleic acid was extracted from approx. 100mg dry wt. as described by Smillie & Krotkov (1960). Total nucleic acid present in the HClO_4 extract was calculated from the $E_{268.5}$ by using $\epsilon = 9850$ as reported by Logan *et al.* (1952). DNA was estimated by using the modified diphenylamine reaction of Burton (1956). Highly polymerized salmon DNA was used as a standard. RNA was estimated by the orcinol procedure of Markham (1955) and from the u.v. absorption of a sample hydrolysed in 0.5M- HClO_4 . Purified yeast RNA was used as a standard. The standard RNA contained 7% (w/v) phosphorus and the ϵ_p calculated after hydrolysis in 0.5M- HClO_4 was 9957.

Preparative extraction and separation of nucleic acid from *A. nidulans*. Lipids were removed by extraction with ethanol and ethanol-chloroform (9:1, v/v). The cells (9.8g wet wt.) were suspended in 60ml of ethanol by stirring with a glass rod. The suspension was centrifuged for 10min at 10000g after being heated in a water bath at 70°C for 15min. The supernatant was removed by decantation and the residue treated as above with a further 60ml of ethanol and finally heated with 60ml of 10% (v/v) chloroform in ethanol at 70°C for 10min.

* Abbreviation: DNAase, deoxyribonuclease.

Water-soluble material was extracted from the lipid-free residue by sonication in 80ml of distilled water for 10min. The suspension was centrifuged, the supernatant decanted and the process repeated.

To extract the DNA, the pellet was suspended by sonication in 80ml of 0.02M-Tris-HCl buffer, pH 7.5, containing 0.01 M-MgCl₂. Ribonuclease-free DNAase I (5mg, 2000 Kunitz units/mg) was added to the suspension which was incubated at 30°C with magnetic stirring. The reaction was followed by measuring the increase in E_{260} of the supernatant. When the reaction was complete (about 2h) the entire suspension was centrifuged and the supernatant decanted, the residue was washed by suspension in a further 50ml of 0.02M-Tris-HCl buffer, pH 7.5. After centrifugation the supernatant was combined with that from the previous incubation.

To extract the RNA, the residue was transferred to a round-bottomed flask with 90ml of aq. 10% (w/v) NaCl and the mixture heated to 100°C under reflux by an oil bath. The progress of the extraction was followed by measuring the E_{260} of samples of the supernatant until there was no further increase in the extinction (5h). The suspension was then poured into a centrifuge bottle which has been preheated by immersion in a boiling-water bath. The suspension was centrifuged without delay at 10000g for 20min at ambient temperature. The supernatant containing the RNA was decanted and the residue extracted a second time with 10% (w/v) NaCl for 5h as described above.

Ethanol (3 vol.) was added to the combined NaCl extracts and the solution allowed to stand at 0–5°C for at least 4h. The precipitated high-molecular-weight RNA was then collected by centrifuging the solution at 15000g for 20min at 5°C. The precipitate was dissolved in 0.02M-Tris-HCl buffer, pH 9.0, containing 0.01 M-MgCl₂.

Phosphodiesterase I degradation of RNA. Snake venom phosphodiesterase was further purified by ion-exchange column chromatography (Keller, 1964) and stored at –20°C before use. Commercial phosphodiesterase I [10units (where 1 unit is 1 $\mu\text{mol/min}$)] was purified by this method (recovery of E_{280} units was 41%) and added to a solution containing about 60mg of RNA. The solution was incubated at 30°C and the reaction monitored by following the increase in E_{260} of the supernatant obtained after precipitating the remaining high-molecular-weight RNA with ethanol. When the reaction appeared complete (about 18h), ethanol (3 vol.) was added to the reaction mixture and the solution kept at 4°C for 2h. The precipitate was removed by centrifuging at 15000g for 20min at 5°C and dissolved in 50ml of 0.02M-Tris-HCl buffer, pH 9, containing 0.01 M-MgCl₂ and incubated again with phosphodiesterase I as described above.

The ethanol supernatants from both phosphodiesterase I treatments were combined, the ethanol was removed by rotary evaporation at 30°C, and the residue redissolved in water. The nucleotide composition of the solution was determined by paper chromatography in solvent A.

Phosphodiesterase degradation of deoxyoligonucleotides. DNA extracted as deoxyoligonucleotides (about 20mg) was degraded to 5'-monophosphates by incubation with purified phosphodiesterase I [10units of commercial phosphodiesterase purified by the method of Keller (1964); recovery of E_{280} units was 38%]. The solution was incubated at 30°C and the reaction monitored by following the increase in E_{260} of the solution. When the reaction appeared complete (about 1h), ethanol (2 vol.) was added and the reaction mixture centrifuged at 15000g for 20min at 5°C. The supernatant was decanted and the ethanol removed by rotary evaporation at 30°C. The residue was redissolved in water and its nucleotide composition determined by paper chromatography in solvent A.

Column chromatography of mononucleotides. The crude mixture of deoxyribonucleoside monophosphates was chromatographed on a column (84cm $\times$ 2.8cm diam.) of DEAE-Sephadex A25 (bicarbonate form). The column was developed with a linear gradient consisting of 6litres of 0.25M-NH₄HCO₃ and 6litres of water; 25ml fractions were collected.

The crude mixture of ribonucleoside monophosphates was chromatographed on a column (125cm $\times$ 5cm diam.) of DEAE-Sephadex A25 (bicarbonate form). The column was developed with a linear gradient consisting of 10 litres of 0.35M-NH₄HCO₃ and 10 litres of water. Fractions (50ml) were collected.

The eluates from both columns were automatically monitored for radioactivity (Bremsstrahlung) and also for u.v. absorption at 254nm by using a LKB Uvicord I. A sample of each fraction was analysed for nucleotide composition by paper chromatography in solvent A. In addition, the dTMP- and dAMP-containing fractions eluted from the deoxyribonucleotide column were analysed in solvent B (24h elution).

NH₄HCO₃ was removed from each of the combined column fractions by rotary evaporation at 30°C. Further purification of the individual column fractions was accomplished by paper chromatography in the solvent systems listed below.

Acid hydrolysis of 6-methylaminopurine deoxyriboside 5'-monophosphate. Approximately 4 μCi of 6-methylaminopurine deoxyriboside 5'-monophosphate contained in 100 μl of water was mixed with an equal volume of 0.2M-formic acid and incubated at 60°C. Samples were removed at hourly intervals and analysed by paper chromatography with solvent F.

Incubation of 6-methylaminopurine deoxyriboside 5'-monophosphate with 5'-nucleotidase. Approximately 4 μ Ci of 6-methylaminopurine deoxyriboside 5'-monophosphate was incubated at 20°C for 2.5 h with 2.5 μ mol of MgCl₂, 10 μ mol of Tris-HCl buffer, pH 8.0, and an excess (2 units) of 5'-nucleotidase. The final volume of the solution was 120 μ l. At the end of the incubation samples were analysed by paper chromatography in solvents F and G.

Preparation of amino acids. The insoluble residue remaining after extraction with 10% NaCl was heated with 4M-HCl at 110°C for 16 h in a sealed tube. The solution was filtered through a Millipore filter (10 μ m) and the filtrate applied to a column (1.5 m $\times$ 5 cm diam.) of Dowex 50 (H⁺ form). The amino acids were eluted with a linear gradient of HCl with 20 litres of distilled water in the mixing chamber and 20 litres of 3M-HCl in the reservoir. After column chromatography the amino acid fractions were further purified by paper chromatography in solvent B and finally by crystallization from ethanol-water (4:1, v/v).

Paper chromatography. Analytical and preparative paper chromatography were carried out on Whatman no. 1 and 3 MM paper respectively. Chromatograms were developed by descending elution for 16 h unless otherwise stated. After chromatography radioactive areas were located by preparing radioautograms with 'Kodirex' X-ray film.

Non-volatile components of the various solvent systems were removed from the preparative paper chromatograms by chromatographic elution with solvent E before elution of the required compounds with distilled water.

Solvent systems. The solvent systems were as follows: A, isobutyric acid-1M-NH₃ (5:3, v/v); B, butan-1-ol-water-acetic acid (12:5:3, by vol.); C, ethanol-satd. sodium tetraborate-5M-ammonium acetate-0.5M-EDTA (440:160:40:1, by vol.); D, ethanol-1M-ammonium acetate, pH 7.5 (5:2, v/v); E, propan-1-ol-water (9:1, v/v); F, propan-2-ol-NH₃ (sp.gr. 0.88)-water (7:1:2, by vol.); G, butan-1-ol-ethanol-water (105:64:31, by vol.); H, 0.2M-NH₄HCO₃.

Thin-layer chromatography. This was carried out on Merck glass plates (20 cm $\times$ 20 cm) precoated with polyethyleneimine-cellulose F. The normal elution distance was 15 cm.

Results

Growth conditions

Preliminary experiments were carried out to establish the conditions which would ensure maximum incorporation of ¹⁴CO₂ into the cells. *A. nidulans* has an optimum pH range for growth of 7.5-9.0 (Kratz & Myers, 1955a). When this organism was

grown in a closed system, it was found necessary to supplement medium D of Kratz & Myers (1955a) with 10 g of K₂HPO₄/l to keep the pH within these limits. The modified medium now had sufficient buffering capacity to prevent the pH falling below 7.5 after the addition of 100 mmol of CO₂. Fig. 1 shows the carbon balance and the variation in pH for a typical experiment where *A. nidulans* was grown on low specific radioactivity ¹⁴CO₂ (50 mCi/mol) in a closed system. During growth, as CO₂ was absorbed from the medium, the pH increased and if unchecked rose above pH 10. Preliminary experiments showed that considerable cell lysis occurred at the higher pH values as indicated by the appearance of a yellow-green supernatant and by a fall in the extinction of the suspension. When the pH was kept between 8.0 and 8.5 by the addition of HCl (see Fig. 1) fixation of ¹⁴CO₂ reached 95%.

After the preliminary tracer experiments to establish the optimum growth conditions, *A. nidulans* was successfully grown on high specific radioactivity ¹⁴CO₂ (6 Ci; 100 mmol) with 87% fixation of ¹⁴CO₂ into the cells. It was observed that the growth rates of cultures grown at high specific radioactivity did not differ from those of duplicate cultures, grown under the same conditions, on non-radioactive CO₂.

Extraction of nucleic acid

Extraction for analytical estimation. It was found that the nucleic acid content of *A. nidulans* was the same whether the cells were grown on high specific radioactivity ¹⁴CO₂ (60 Ci/mol) or low specific radioactivity ¹⁴CO₂ (50 mCi/mol). The DNA content of *A. nidulans* was 1.7%, approx. 8.5 times that of *C. pyrenoidosa* (grown on high specific radioactivity ¹⁴CO₂); the RNA content, however, at 4.8% was only 1.4 times that of *C. pyrenoidosa*. These analytical values for nucleic acid obtained after extraction of the cells under vigorous conditions, were used to measure the efficacy of the preparative extractions described below.

Preparative extraction. Methods for quantitative extraction of total nucleic acid from *A. nidulans* and separation of RNA and DNA were examined. The separation of RNA and DNA as completely as possible at the first stage of the extraction was preferred and this would simplify the subsequent purification of the mononucleotides and decrease losses. The results of several extractions, carried out on cells grown at low specific radioactivity, are shown in Table 1. In the first extraction the lipid-free residue was heated to 100°C for 7 h in 10% (w/v) NaCl. This procedure removed 89% of the total nucleic acid but only 50% of the DNA. The remaining DNA associated with the insoluble residue was resistant to further extraction with 10% (w/v) NaCl at 100°C.

Fig. 1. Growth of *A. nidulans* in a closed vessel

¹⁴CO₂ (5 mCi; 100 mmol) was generated immediately after inoculation of 5l of culture medium with a 3.6% (v/v) inoculum. Further details are given in the Materials and Methods section. During growth, samples of the suspension were removed and monitored for pH (○), E₃₈₀ (●), and radioactivity in the cells (△) and supernatant (□). The pH during the latter stages of growth was kept below 8.5 by the addition of 10ml of 1M-HCl as indicated by the arrows. About 4.72 mCi of radioactivity (94.4%) was recovered in the harvested cells.

Table 1. Extraction of DNA and RNA from *A. nidulans* with deoxyribonuclease I and 10% NaCl

In extraction procedure 1, *A. nidulans* cells were harvested and lipids were removed by extraction with hot ethanol and ethanol-chloroform (9:1, v/v). Nucleic acid was extracted from the lipid-free residue with 10% (w/v) NaCl at 100°C. After centrifugation the insoluble residue was incubated with DNAase I at pH 7.5. In extraction procedure 2, DNA was extracted from the lipid-free residue by incubation with DNAase I at pH 7.5. After washing with buffer, RNA was removed from the insoluble residue by heating with 10% (w/v) NaCl at 100°C. Nucleic acid content is given as % dry wt.

Extraction procedure	DNA by diphenyl-amine method (%)	RNA content by difference (%)		Total nucleic acid content by u.v. absorption (%)
Procedure 1				
7h with NaCl	0.85	5.15		6.0
DNAase I, pH 7.5	0.55	0.29		0.84
Total	1.40	5.44		6.84
		<div>By u.v. absorption By orcinol method</div>		
Procedure 2				
DNAase I, pH 7.5	1.81	—	—	2.2
Resuspension of residue in buffer	0.14	—	—	0.25
5h with NaCl	0.02	5.4	6.4	5.4
4h with NaCl	—	0.4	0.6	0.4
Total	1.97	5.8	7.0	8.25

Table 2. Summary of preliminary fractionation of *A. nidulans* grown on high-specific-radioactivity $^{14}\text{CO}_2$

A. nidulans was grown under continuous illumination in 5 litres of culture medium in the presence of 100 mmol of $^{14}\text{CO}_2$ (60Ci/mol). After 28 h the cells (9.8 g wet wt. = 0.98 g dry wt.) were harvested and fractionated as described in the Materials and Methods section.

Fraction	Radioactivity (mCi)	Radioactivity (% of total in cells)
Whole cells	5200	100
Lipids	655	12.6
Water-soluble extract	394	7.6
DNAase I extract	112	2.2
First NaCl extract	240	4.6
Second NaCl extract	65	1.3
Insoluble residue (protein with some carbohydrate)	3729	71.7

However, incubation of the residue with DNAase I removed most of the remaining DNA as oligonucleotides. In the second extraction the order of the enzyme incubation and NaCl extraction was reversed. The lipid-free residue was first incubated with DNAase I and after 2 h 92% of the total DNA had been removed by the enzyme. The remainder was removed by washing the residue once in Tris-HCl buffer, pH 7.5. The amount of DNA extracted by hot 10% (w/v) NaCl, during the first RNA extraction, was only about 1% of the total DNA obtained. This was a considerable improvement over the first extraction procedure and was subsequently adopted as the standard method.

If the 10% (w/v) NaCl suspension was allowed to cool to room temperature before centrifugation, extinction measurements showed that up to 20% of the RNA originally present in the supernatant (determined after rapid centrifugation of a small sample) was re-adsorbed on to the insoluble residue. Negligible losses were encountered if the 10% NaCl suspension was centrifuged immediately, without cooling, after the initial extraction period.

Table 2 summarizes the initial fractionation of *A. nidulans* grown on 6Ci of high specific radioactivity $^{14}\text{CO}_2$.

Degradation of the nucleic acids and purification of the mononucleotides. Phosphodiesterase I degradation of the oligodeoxyribonucleotides from the DNAase extract was essentially complete within 30 min. Analysis of the reaction mixture by paper chromatography showed it to consist of approx. 54% deoxyribomononucleotides. This corresponds to about 0.83% of the total radioactivity incorporated into the cells or 49% of the DNA estimated to be present by analytical methods described above. Approximately 56% (170 mCi) of the radioactivity in the combined NaCl extracts was precipitated by

ethanol. After degradation with phosphodiesterase I, 155 mCi (i.e. >90% of the ethanol-precipitable material) was recovered showing virtually complete degradation of the RNA by this enzyme. Analysis of the solubilized fraction by paper chromatography showed that 70% of the radioactivity was present as ribonucleotides. This corresponds to about 2.2% of the total radioactivity incorporated into the cells or 46% of the RNA estimated to be present by analytical methods. Preliminary fractionation of the crude mixture of ribonucleoside monophosphates (155 mCi containing approximately 70% nucleotides) into four main fractions was achieved by chromatography on a column of DEAE-Sephadex as described in the Materials and Methods section. The results of the analysis on the individual fractions are given in Table 3. The radiochemical purity of the major nucleotide in each fraction was low, i.e. 80–87%. This is a typical feature observed when purifying crude mixtures of compounds from uniformly labelled organisms where all the cellular constituents are labelled. The deoxyribonucleotide contamination of the fractions was very low and amounted to only 1–2%. The individual ribonucleotides were separated from the corresponding deoxyribonucleotides and other minor impurities by paper chromatography in solvent A.

The deoxyribonucleotides (80 mCi containing approximately 54% nucleotides) were also fractionated on a column of DEAE-Sephadex (see the Materials and Methods section). A separation of dCMP and dTMP was not achieved even though a shallow linear gradient was used. The separate column fractions were analysed by paper chromatography (Table 4). Contamination by ribonucleotides ranged from 4 to 7% which was higher than the corresponding deoxynucleotide contamination of the ribonucleotide fractions even though the DNA was extracted by using a specific electrophoretically purified ribonuclease-free enzyme. Since enzymic degradation of the RNA was unlikely to be from this source, the contamination probably resulted from non-specific solubilization or from traces of *A. nidulans* ribonuclease which had survived the lipid extraction procedure.

Further purification of the deoxyribonucleoside monophosphates was accomplished by paper chromatography. Fraction 1 (see Table 4) was chromatographed in solvent A. Two major radioactive bands separated, the one with the higher mobility was dCMP and the other was TMP contaminated with CMP. After elution from the chromatogram TMP was separated from CMP by further paper chromatography in solvent B. Fraction 2 (89% dAMP) was purified by chromatography in solvent B and fraction 3 (93% dGMP) in solvent A.

The guanine-cytosine (G+C) base composition of the *A. nidulans* DNA, calculated from the total

Table 3. Fractionation and analysis of $[\text{U-}^{14}\text{C}]$ ribonucleoside monophosphates

$[\text{U-}^{14}\text{C}]$ RNA (170 mCi) was degraded with phosphodiesterase I and the reaction mixture chromatographed on a column of DEAE-Sephadex (bicarbonate form) and 50 ml fractions were collected. Further details are given in the Materials and Methods section.

Fraction (numbered in order of elution)	Major nucleotide present	Total radioactivity (mCi)	Radiochemical purity (%)	Contaminating deoxyribonucleo- tide (%)	Total radioactivity as ribonucleotide (mCi)	Amount of nucleotide present in RNA (%)
1	CMP	30.0	79.4	2.2	23.8	23.1
2	UMP	21.6	87.5	—	18.9	18.3
3	AMP	34.0	85.7	1.5	29.8	28.8
4	GMP	35.5	86.5	1.3	30.7	29.8

Table 4. Fractionation and analysis of $[\text{U-}^{14}\text{C}]$ deoxyribonucleoside monophosphates

$[\text{U-}^{14}\text{C}]$ DNA (in the lipid-free residue) was degraded with DNAase I followed by phosphodiesterase I and the reaction mixture chromatographed on a column of DEAE-Sephadex (bicarbonate form); 50 ml fractions were collected. Further details are given in the Materials and Methods section.

Fraction (numbered in order of elution)	Total radioactivity (mCi)	Radioactivity as nucleotides (%)	Nucleotides present	Amount of each nucleotide (% of total)	Radioactivity in each nucleotide (mCi)	Amount of nucleotide present in DNA (%)
1	23.5	97	dTMP	45	10.3	24.5
			dCMP	51	11.6	27.6
			CMP	4	0.9	—
			dAMP	89	9.8	23.3
2	12.0	92	AMP	6	0.7	—
			6-methylaminopurine deoxyribonucleoside monophosphate	5	0.6	1.4
3	11.8	88	dGMP	93	9.7	23.1
			GMP	7	0.7	—

radioactivity in the respective deoxynucleotide was 51%. This compared favourably with a G+C content of 53% determined from the DNA 'melting' temperature (Craig *et al.*, 1969) and indicated that there was no selective loss of any deoxynucleotide during processing.

The radiochemical purity of the ribonucleoside and deoxyribonucleoside monophosphates was in the range 97–99% when analysed by paper chromatography in solvent systems A–D, and by t.l.c. on polyethyleneimine-cellulose in system H. The average specific radioactivity was 57 ± 2 Ci/atom of ^{14}C which was consistent with the calculated dilution expected from the non-radioactive inoculum and the high fixation of the $^{14}\text{CO}_2$ into the cells. This value also agrees with the specific radioactivities of several amino acids purified from the protein hydrolysate and thus indicates uniformity of labelling.

Characterization of impurity associated with dAMP

When the dAMP fraction (fraction 2, Table 4) was purified by paper chromatography in solvent B, a significant impurity with a higher R_F value clearly

separated from the main radioactive band. On the basis of the evidence given below this compound was identified as 6-methylaminopurine deoxyriboside-5'-monophosphate.

(1) The u.v.-light-absorption spectra of the compound at pH 7.0 and 13.0 were similar with an absorption maximum at 265–266 nm. At pH 2.4 the compound had a higher ϵ with the absorption maximum shifted to 262 nm (Fig. 2). The spectrum was very similar to that of compounds which contain an 'adenine-type' base and the spectral changes in acid and alkali correspond to those reported for the riboside of 6-methylaminopurine (N^6 -methyladenine) by Jones & Robins (1963). Adenine bases methylated in other positions have different spectral characteristics.

(2) After incubation with 5'-nucleotidase, chromatograms developed with either solvent F or G showed the only major radioactive spot to have a higher R_F value than the starting material and also a higher R_F value than a 6-methylaminopurine riboside marker. This is direct evidence that the compound is a 5'-nucleotide, but is not 6-methylaminopurine riboside monophosphate.

Fig. 2. U.v.-absorption spectrum of 6-methylaminopurine deoxyriboside 5'-monophosphate at pH 7.0 and 2.4

The absorption spectrum was measured in 0.1M-sodium phosphate buffer, pH 7 (—) and in 0.2M-formic acid, pH 2.4 (---) by using a Unicam SP.800 spectrophotometer.

(3) After incubating with formic acid at pH 2.0 and 60°C, chromatograms developed in solvent F showed that 50% hydrolysis had occurred after only 1 h and that almost all the original compound had been hydrolysed after 7 h. Quantitative analysis of the chromatogram of the 7 h sample, showed that 44% of the radioactivity moved as a single spot (inferred to be deoxyribose 5'-phosphate) having a lower R_F value than the starting material and 54% of the radioactivity co-chromatographed with a 6-methylaminopurine marker. This distribution of radioactivity is consistent with the original compound containing a C_6 base and a C_5 sugar. Evidence for the sugar being deoxyribose comes first from the fact that the compound was extracted from *A. nidulans* by using DNAase and secondly, the compound produced after incubation with 5'-nucleotidase had a higher R_F value than the 6-methylaminopurine riboside marker and thirdly, the ease of hydrolysis by acid. The *N*-glycoside bond of deoxyribonucleotides is known to be far more labile than the corresponding bond of ribonucleotides (Monks *et al.*, 1972). We concluded therefore that the compound was 6-methylaminopurine deoxyriboside-5'-monophosphate. This compound represented 1.4% of the DNA and 5.7% of the dAMP fraction.

No 5-methylcytosine was found on analysis of the dCMP fraction of *A. nidulans*.

Processing of the protein fraction (insoluble residue)

The insoluble residue containing 3.7 Ci of radioactivity and approximately 60% of protein was

hydrolysed to the constituent amino acids by heating with 4M-HCl in a sealed tube. After filtration of the residue 3.3 Ci of radioactivity was fractionated on an ion-exchange column as described in the Materials and Methods section. After the final purification by recrystallization a total of 2.4 Ci of radioactivity was obtained as radiochemically pure (>98%) amino acids.

Discussion

Growth conditions

To obtain maximum fixation of $^{14}CO_2$ the growth conditions for *A. nidulans* were investigated in some detail. The most versatile basal media for the growth of a range of blue-green algae were originally developed by Kratz & Myers (1955a). Their media C and D were designed for the provision of CO_2 for growth by gassing with air or up to 4% CO_2 in air. The pH of medium C in equilibrium with air was 7.8 but in equilibrium with 4% CO_2 the value fell to 6.8. This was below the optimum pH range for growth of *A. nidulans* and a modification of medium C was necessary. Carr (1969) reported that growth in open systems was erratic when cultures of this organism were gassed with air. We found that the success of growth with media gassed with air depended on the rate of aeration; with rates of 10–30 litres/min a healthy culture with an E_{380} of 3 could be obtained in 24 h (initial inoculum 5%) by using standard conditions but at rates of 1–3 litres/min growth was normally very slow and cultures deficient in pigment were obtained. It was inadvisable to use these cultures to inoculate other vessels as very long lag periods (up to 24 h) often occurred even when the culture was supplied with adequate CO_2 and therefore for reproducible work the cultures were gassed with air + CO_2 mixtures.

Since the maximum fixation of a given amount of radioactive CO_2 was required a closed system was used. All the gas was generated at the start of the experiment and resulted in 10% CO_2 in the gas phase. A modification of medium D was used to ensure that the pH values of the medium in equilibrium with the gas was within the optimum range for growth of the organism. Difficulties with the control of pH value in the growth of algae on CO_2 in closed systems are increased when the organism has an alkaline pH growth optimum. An organism which grows well below pH 7, e.g. *Chlorella* (Kratz & Myers, 1955b), does not usually require modified media or the adjustment of pH during growth.

Analytical procedures for DNA and RNA estimation

Estimations of total DNA and RNA content of *A. nidulans* were done after extraction of the lipid-free cells with hot $HClO_4$. The values obtained were

used to compare the efficiency of the preparative extractions carried out later.

The estimation of DNA by determination of deoxypentose content with diphenylamine was not subject to serious interference from protein and other compounds which may be present in the extract (for a review see Munro & Fleck, 1966). Thus the value of 1.7% reported by Craig *et al.* (1969), and confirmed during the present work is probably a reliable estimate of the absolute amount of DNA in *A. nidulans*, assuming that hot HClO_4 gives a quantitative extraction. Moreover, this value agrees well with the amount of DNA determined after extraction by an enzymic method (see Table 2).

The values obtained for the RNA content are probably less reliable. The orcinol procedure for ribose determination is known to be subject to interference from other carbohydrates. The values for RNA content of *A. nidulans* determined by u.v. absorption were consistently lower than the orcinol estimations and were considered to be closer to the true values.

Preparative extractions

Recent preparative methods for the extraction of nucleic acids are designed to ensure that their native structure remains intact but contaminants such as protein and polysaccharide are removed as completely as possible. Residual protein is usually decreased to a minimum by several treatments with detergents, chloroform-octanol or phenol (for a review see Kirby, 1964), final purity being of primary importance, not yield. In the present work yield was of major importance and retention of macromolecular structure was only required to allow precipitation of nucleic acid from solution, in certain procedures.

Strong salt solutions can be used to extract nucleic acids from micro-organisms without extensive degradation but with various yields. Smillie & Krotkov (1960) extracted samples of *Euglena* with 10% (w/v) NaCl at 100°C and removed over 90% of the nucleic acid. However, when the same method was applied to *Chlorella* the best extraction contained 50% of the nucleic acid with only 16% of the total DNA. We have shown that extraction of a lipid-free sample from *A. nidulans* with 10% (w/v) NaCl at 100°C will remove about 89% of the total nucleic acid (containing virtually 100% of the RNA) but only 50% of the DNA. The residual DNA could not be extracted by further treatment with NaCl but was removed by treatment with DNAase I. In further experiments the lipid-free residue of *A. nidulans* was first treated with DNAase I and the entire DNA fraction (contaminated with only 5–6% RNA) was removed by this method. The RNA (contaminated with only 1–2% DNA) was subse-

quently extracted as a separate fraction with hot 10% (w/v) NaCl.

There have been few reports of the use of DNAase as a primary method to remove DNA from tissues. Kirk (1963) has shown that digestion with DNAase I removed only 42% of the DNA from a lipid-free chloroplast preparation. The amount extracted by DNAase I could be increased to 60% if the chloroplast preparation was partially deproteinized by the method of Marmur (1961). The ease of extraction of the DNA from tissue residues by enzymic methods is probably related to the degree of its association with other cellular constituents, and especially the type and amount of protein in the deoxyribonucleoprotein complex. It is noteworthy that a report by Makino & Tsuzuki (1971) suggests the absence of the strongly basic histones in the blue-green algae *Anabaena cylindrica*. An amino acid analysis showed that the acid-extractable protein of the deoxyribonucleoprotein complex was in fact neutral.

Kirby (1964) stated that all procedures which liberate DNA from tissues also liberate RNA. However, with enzymic methods for removal of DNA concomitant extraction of RNA will probably be lower than with any other technique. Thus by choosing a suitable sequence of extraction methods, DNA and RNA can be quantitatively removed from *A. nidulans* into almost completely separate fractions.

Identification of 6-methylaminopurine in *A. nidulans* DNA

It is well known that 6-methylaminopurine and 5-methylcytosine occur as minor bases in bacterial and phage DNA (Dunn & Smith, 1958; Nikolskaya *et al.*, 1968; Vanyushin *et al.*, 1968). Our evidence indicates that 6-methylaminopurine is a component of *A. nidulans* DNA. The isolation of 6-methylaminopurine from the DNA of the blue-green alga *Plectonema boryanum* has also been reported (Kaye *et al.*, 1967). The isolation from *A. nidulans* and *P. boryanum* of 6-methylaminopurine, a base which has not been found in higher plants, is noteworthy from the phylogenetic point of view as it furnishes additional biochemical evidence for the close relationship between bacteria and blue-green algae.

Comparison of *A. nidulans*, as a source of uniformly ^{14}C -labelled compounds with other organisms

A. nidulans was able to fix into the cells up to 95% of the supplied $^{14}\text{CO}_2$. This carbon balance is ideal for the preparation of labelled compounds and is similar to that found for *Chlorella* grown on $^{14}\text{CO}_2$ at low specific radioactivity (K. G. Oldham & N. Lucas, unpublished work) but contrasts sharply with that found in bacteria. Values have been published (Roberts *et al.*, 1955) for *Escherichia coli* which show

that of the radioactivity supplied from [^{14}C]glucose only 30% was fixed into the cells, 48% was evolved as CO_2 and 22% was excreted into the medium chiefly as acetic acid. *A. nidulans* whether grown on either high or low specific radioactivity $^{14}\text{CO}_2$ had a similar nucleic acid content and contained about 8 times as much DNA and 1.4 times as much RNA as that found in *Chlorella*. Although bacteria often have a higher content of nucleic acid (Vendrelly & Lehault, 1946) than *A. nidulans* their unfavourable carbon balance must be allowed for when calculating theoretical recoveries of labelled nucleic acid and other compounds. The yield of uniformly ^{14}C -labelled amino acids from *A. nidulans* was 48% of the total radioactivity in the cells, which is approximately twice the yield obtained from *C. pyrenoidosa* (K. G. Oldham & N. Lucas, unpublished work).

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