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Incorporation of Methionine-Derived Methyl Groups into Sirohaem by *Escherichia coli*

By LEWIS M. SIEGEL, PATRICIA S. DAVIS and MATTHEW J. MURPHY*
The Veterans Administration Hospital and the Department of Biochemistry, Duke University School of Medicine, Durham, NC 27710, *U.S.A.*

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Sirohaem is a new type of haem that has been detected as a prosthetic group of several bacterial and plant enzymes that catalyse the six-electron reductions of sulphite to sulphide or of nitrite to NH_3. When a methionine-requiring mutant of *Escherichia coli* K12 was grown on a minimal medium supplemented with D-glucose and L-[*Me*-^{3}H]methionine, 2.4 methyl groups per spectrophotometrically detectable haem group were incorporated into the sirohaem prosthetic group of the NADPH–sulphite reductase isolated from the organism. When the same strain of cells was grown on minimal medium supplemented with D-[U-^{14}C]glucose and L-[*Me*-^{3}H]methionine, the sirohaem isolated was found to contain a ratio of glucose-derived carbon/methionine-derived methyl of 19.8. This ratio is in excellent agreement with the value of 20 predicted by the iron–dimethylurotetrahydroporphyrin structure for sirohaem proposed by Murphy, Siegel, Kamin & Rosenthal [(1973) *J. Biol. Chem.* **248**, 2801–2814]. It can be concluded that sirohaem is indeed methylated, with the methyl groups derived from methionine (rather than by modification of existing side chains, as in protohaem). The structure proposed by Murphy *et al.* (1973) is therefore probably correct in its essential features. A possible relationship between the pathway for biosynthesis of sirohaem and that for synthesis of vitamin B_{12} is discussed.

Escherichia coli NADPH–sulphite reductase [sulphite reductase (NADPH), EC 1.8.1.2] catalyses the reduction of sulphite to sulphide (Siegel *et al.*, 1974) and of nitrite to NH_3 (Lazzarini & Atkinson, 1961; Siegel *et al.*, 1974). Both reactions involve a transfer of six electrons from reduced nicotinamide to enzyme-bound substrate. The enzyme contains, as the probable site of sulphite and nitrite binding and reduction (Siegel *et al.*, 1974; Rueger & Siegel, 1976), a novel haem prosthetic group termed 'sirohaem' (Siegel *et al.*, 1973; Murphy & Siegel, 1973). Sirohaem has been identified as a prosthetic group of several bacterial sulphite reductases (Murphy & Siegel, 1973; Murphy *et al.*, 1973*a*; Siegel, 1975) as well as of plant and fungal nitrite reductases (Murphy *et al.*, 1974; Vega *et al.*, 1975). Murphy *et al.* (1973*b*) isolated sirohaem from *E. coli* sulphite reductase and have partially characterized its molecular structure. Their studies showed that sirohaem represents a new class of biologically active tetrapyrrole compound, with the following properties.

(*a*) Sirohaem contains two partially saturated pyrrole rings adjacent to one another in an otherwise conjugated porphyrin macrocycle; thus sirohaem is formally classed as an iron tetrahydroporphyrin of the isobacteriochlorin type (as opposed to tetrahydroporphyrins of the bacteriochlorin type, typified by demetallated bacteriochlorophyll, in which saturation occurs in diagonally opposite pyrrole rings in the porphyrin macrocycle).

(*b*) Sirohaem contains eight carboxylic acid groups, which on the basis of mass-spectroscopic cleavage patterns are probably associated with acetic acid and propionic acid side chains.

(*c*) The porphyrin macrocycle of sirohaem derivatives is stable to photo-oxidation under conditions in which analogous derivatives of tetrahydroporphyrins are readily oxidized to chlorins. Thus it appears that the partial saturation of two pyrrole rings in sirohaem is achieved not by simple reduction (i.e. addition of hydrogen atoms), but rather by addition of a group or groups that cannot be readily removed under oxidizing conditions.

(*d*) Sirohaem (in the absence of any fifth or sixth ligands) has the molecular formula $FeC_{42}H_{44}N_4O_{16}$. This formula corresponds to that of iron–uroporphyrin with addition of the elements $(CH_3+H)_2$. On the basis of these findings, Murphy *et al.* (1973*b*) proposed that sirohaem is in fact an iron–dimethylurotetrahydroporphyrin, with a structure like that

* Present address: Guinness Research Laboratory, St. James Gate, Dublin, Ireland.

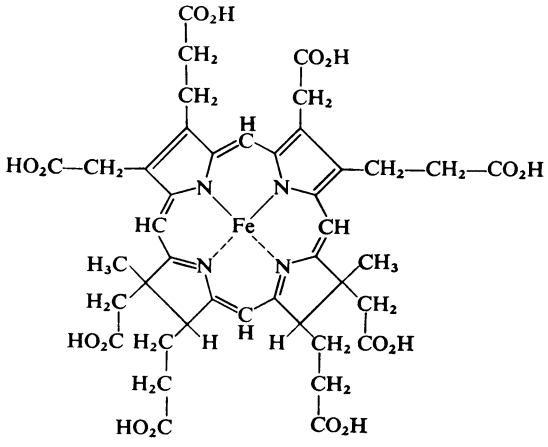

Fig. 1. *Iron–dimethylurotetrahydroporphyrin structure proposed for sirohaem by Murphy et al.* (1973*b*)
The exact positions of the methyl groups are not known (see the text).

shown in Fig. 1, but with the exact positions of the methyl groups with respect to the acetic acid and propionic acid side chains unspecified. They suggested that this type of structure could be derived metabolically by two successive methylations (presumably with *S*-adenosylmethionine as immediate methyl donor) of uroporphyrinogen III, an intermediate common to the biosynthesis of all known haem, chlorophyll and corrin compounds (Bogorad & Granick, 1953; Neve *et al.*, 1956; Scott *et al.*, 1975), followed by either spontaneous or enzyme-catalysed oxidation of the methylated uroporphyrinogen to the tetrahydroporphyrin state. The cyclically conjugated structure proposed for sirohaem would be both thermodynamically stable and resistant to further oxidation.

In the present paper we describe attempts to determine whether sirohaem is indeed methylated with methionine-derived methyl groups. To this end, a methionine-requiring mutant of *E. coli* K12 was grown on a minimal-salts medium supplemented with D-glucose (as carbon source) and L-methionine (as source of both sulphur and methyl groups). In Expt. A, the methionine was radioactively labelled with ^3H in its methyl group, and the glucose supplied in the medium was not radioactive. In Expt. B, both methionine and glucose were radioactively labelled, the former with ^3H in its methyl group and the latter with ^{14}C uniformly distributed in all the carbon atoms. NADPH–sulphite reductase was isolated from the radioactively labelled cells, sirohaem extracted from the enzyme and purified, and the specific radioactivity of the haem determined. The results show that, in agreement with the structure proposed by Murphy *et al.* (1973*b*), sirohaem contains approximately two methionine-derived methyl groups per iron atom and a ratio of 20 glucose-derived carbon atoms per methionine-derived methyl group.

Materials and Methods

Materials

L-[*Me*-^3H]Methionine (nominally 190 Ci/mol) and D-[U-^{14}C]glucose (nominally 196 Ci/mol) were obtained as solutions in 70% (v/v) ethanol and sterile water respectively from New England Nuclear Corp., Boston, MA, U.S.A. The radioactive materials were diluted with aqueous solutions of non-radioactive L-methionine or D-glucose, immediately before preparation of the growth media. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were of scintillation grade as supplied by New England Nuclear. Sephadex LH-20 was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of analytical-reagent grade and used as supplied unless otherwise specified. Strain RG350, a *met* F^- auxotroph of *E. coli* K12 (Greene & Radovich, 1975), was a gift of Dr. R. C. Greene, Department of Biochemistry, Duke University School of Medicine, Durham, NC, U.S.A.

Spectra

Absorption spectra were measured in silica cuvettes of 1 cm light-path at 23°C with a Cary model 14 spectrophotometer equipped with 0–1.0 and 1.0–2.0 absorbance slide wires. Fluorescence determinations were performed with a Turner Spectro 210 spectrophotofluorimeter.

Enzyme and protein assays

NADPH–sulphite reductase activity was determined spectrophotometrically at 340 nm by the method of Siegel *et al.* (1973). Protein concentrations were determined by the Zamenhof (1957) microadaptation of the biuret method, with bovine serum albumin as protein standard (Siegel *et al.*, 1973).

Growth of cells

E. coli RG350 was grown on Davis & Mingioli (1956) minimal-salts medium modified by omission of citrate and substitution of chloride salts for all sulphate salts and supplemented with D-glucose and L-methionine as follows. In Expt. A, the medium contained 27.8 mM-D-glucose (non-radioactive) and 0.05 mM-L-[*Me*-^3H]methionine of nominal specific radioactivity 9.0 Ci/mol. In Expt. B, the medium contained 5.55 mM-D-[U-^{14}C]glucose, of nominal specific radioactivity 0.015 Ci/mol, and 0.05 mM-L-[*Me*-^3H]methionine, of nominal specific radioactivity 1.75 Ci/mol. The stock solutions of radioactive

D-glucose (0.555M) and L-methionine (5mM) were filter-sterilized just before addition to the growth media.

Flasks (3-litre) containing 1-litre batches of growth medium were each inoculated with 1ml of a stationary-phase culture of *E. coli* RG350 cells which had been grown on an otherwise identical non-radioactive medium. Cultures were incubated on a rotary shaker at 37°C and the cells harvested by centrifugation at 10000*g* for 15min in the late-exponential phase of growth. In Expt. A, a total of 20 litres of culture medium was used; 28g wet wt. of cells was obtained, yielding 33 units* of NADPH–sulphite reductase activity (specific activity 0.015 unit/mg of protein) in the crude extract. In Expt. B, a total of 55 litres of culture medium was used: 64g wet wt. of cells was obtained, yielding 55 units of NADPH–sulphite reductase activity (specific activity 0.010 unit/mg of protein) in the crude extract.

Isolation of NADPH–sulphite reductase

Cells from all culture flasks for a given growth medium were combined and suspended in 2ml/g wet wt. of cells of cold 0.05M-potassium phosphate buffer, pH7.7, containing 0.1mM-EDTA. The cell suspension was placed in a Rosett cell and subjected to sonic disruption for 5min with a Branson model S-125 sonifier set for maximum power output. Temperature was maintained at or below 20°C during the sonication process. All subsequent steps were performed at 0–4°C. Unbroken cells and debris were removed by centrifugation for 30min at 15000rev./min in a Sorvall RC-2B centrifuge (9in rotor). NADPH–sulphite reductase was purified from the crude extracts by the procedure of Siegel *et al.* (1973). The yield of enzyme was 6.2mg (17.8 units) in Expt. A, and 6.8mg (19.2 units) in Expt. B. The enzyme in both preparations exhibited the absorption-spectral characteristics specified by Siegel *et al.* (1973) for homogeneous NADPH–sulphite reductase.

Preparation of sirohaem

The solutions of radioactive sulphite reductase were exhaustively dialysed against 0.05M-potassium phosphate buffer, pH7.7, containing 0.1mM-EDTA, and were then concentrated to approx. 0.4ml with a Schleicher and Schuell collodion-membrane ultrafiltration apparatus. Sirohaem was extracted by treatment of each vol. of the concentrated enzyme solution at 0°C with 9vol. of acetone containing 0.015M-HCl, and the haem subsequently purified by chromatography on columns of Sephadex LH-20 equilibrated with pyridine by the procedure of Murphy *et al.* (1973*b*). The absorption spectra of the purified sirohaem fractions obtained in Expts. A and B were identical with that reported for sirohaem in pyridine by Siegel *et al.* (1973). The purified haem contained no flavin detectable by fluorimetric analysis. Haem concentrations were determined from the $A_{557-700}$ of the pyridine solutions, with the use of the absorption coefficient, $\Delta\varepsilon_{557-700} = 1.57 \times 10^4 M^{-1} \cdot cm^{-1}$ determined by Siegel *et al.* (1973). A total of 14.5 and 23.6nmol of purified sirohaem were obtained in Expts. A and B respectively.

Measurement of radioactivity

Radioactivity was determined with a Packard model 3375 Tri-Carb liquid-scintillation spectrometer. Scintillation vials contained 1.0ml of aqueous sample containing the radioisotope and 10ml of Triton X-100/toluene (1:2, v/v) containing 4g of 2,5-diphenyloxazole/litre and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre (Patterson & Greene, 1965). Radioactivity was determined for a total of 20min for all samples. In Expt. A, conditions were chosen to provide good efficiency for determination of ^3H. In Expt. B, instrument channels were set at energies appropriate for a high degree of discrimination between ^3H and ^{14}C. The effects of quenching by solvent (in particular pyridine) on the interpretation of the results were minimized by ensuring that all samples measured for radioactivity contained identical amounts of all solvents used. There was no quenching of the radioactivity determined for L-[*Me*-^{3}H]methionine by glucose or sirohaem under the conditions used in these experiments. Similarly, the radioactivity of D-[U-^{14}C]glucose was not significantly altered by addition of methionine or haem to the glucose solutions. Finally, neither glucose nor methionine, at the concentrations used in these experiments, quenched the radioactivity of ^3H- or ($^3H+^{14}C$)-labelled sirohaem.

The specific radioactivities used in calculations are those determined by measurement of the radioactivities of the actual L-[*Me*-^{3}H]methionine and D-[U-^{14}C]glucose solutions used for preparation of the growth media. As a check on these specific radioactivities, amino acid analyses were performed on the radioactive protein residues remaining after extraction of sirohaem from NADPH–sulphite reductase with acetone/HCl. The residues were hydrolysed in 6M-HCl at 100°C for 26h. The resulting solutions were freeze-dried and the residues dissolved in 9mM-HCl containing 0.08mM-L-norleucine as a standard. Samples of this solution were applied to a Spinco model 120B automatic amino acid analyser equipped with a stream-splitting device, which directed 30% of the ion-exchange-column effluent to ninhydrin analysis of amino acid content (Spackman *et al.*, 1958) and the remaining 70% of the effluent to a frac-

* One unit of NADPH–sulphite reductase activity represents that amount of enzyme required to catalyse oxidation of one μmol of NADPH/min under the assay conditions defined by Siegel *et al.* (1973).

tion collector for determination of radioactivity in individual fractions. The total radioactivity of all fractions containing an individual amino acid (or a pair of amino acids, if these were not well resolved in the fractions obtained) divided by the total amount of that amino acid present (from the ninhydrin analysis) yielded the specific radioactivity of that amino acid. It was found in both Expts. A and B that the specific ^3H radioactivity of the methionine (measured as the sulphoxide) present in hydrolysates of NADPH–sulphite reductase differed from the specific radioactivity of the L-[*Me*-^{3}H]methionine added to the growth medium by less than 5%. There were negligible amounts of ^3H (less than 0.05% methionine-derived ^3H per amino acid carbon atom) in amino acids other than methionine. Similarly, in Expt. B, the specific radioactivities (measured as ^{14}C per carbon atom) of the amino acids aspartic acid, threonine plus serine, glutamic acid, proline, glycine, alanine, valine, isoleucine plus leucine, and tyrosine plus phenylalanine differed by an average of only 2% (with a standard deviation of ±5%) from the specific radioactivity of the D-[U-^{14}C]glucose added to the growth medium.

Results and Discussion

The results of Expt. A are shown in Table 1. Sirohaem isolated from *E. coli* cells grown in medium containing L-[*Me*-^{3}H]methionine was found to contain 2.4 [^{3}H]methyl groups per molecule of haem. The amount of sirohaem present in the sample was measured spectrophotometrically, by using the previously reported absorption coefficient for sirohaem in pyridine (Siegel *et al.*, 1973). That coefficient was based on chemical determination of the iron content of purified sirohaem samples. Siegel *et al.* (1973) have indicated that sirohaem tends to become partially demetallated during the process of its isolation and purification. It is likely that Sephadex LH-20 chromatography (which efficiently removes the flavin and non-haem iron prosthetic groups of NADPH–sulphite reductase from the sirohaem; Siegel *et al.*, 1973) used to purify the haem would not efficiently separate sirohaem from any demetallated derivatives. Contamination of the sirohaem preparation with such demetallated derivatives would, of course, result in overestimation of the [^3H_3C]/tetrapyrrole ratio.

For this reason, it was desirable to obtain an estimation of the [^3H_3C]/tetrapyrrole ratio of sirohaem by a method that would be independent of the relative amounts of metallated and demetallated porphyrin. Such an estimation is afforded by the results of Expt. B, in which sirohaem was isolated from *E. coli* cells grown in the presence of both L-[*Me*-^{3}H]methionine and D-[U-^{14}C]glucose. Table 2 shows that, although the [^3H_3C]/haem and [^{14}C]/haem ratios are again slightly higher than would be expected on the basis of the structure in Fig. 1, the ratio of glucose-derived carbon (^{14}C)/methionine-derived methyl groups (^3H_3C), 19.8, is in fact identical within experimental error with the value of 20 [(42 total carbon atoms minus 2 methyl groups)/(2 methyl groups)] predicted from the iron–dimethylurotetrahydroporphyrin structure of Fig. 1, if the methyl groups are derived from methionine.

Table 1. *Incorporation of ^3H from* L-[*Me*-^{3}*H*]*methionine into sirohaem (Expt. A)*

Growth of cells, isolation of NADPH–sulphite reductase, extraction and purification of sirohaem, and general methods for determination of radioactivity were as described in the Materials and Methods section. All samples were prepared in quadruplicate; the results presented are an average for four determinations of each sample. Samples for radioactivity measurement contained, in addition to 10ml of the Triton X-100/toluene-based counting mixture: sample 1, 0.05ml of pyridine+0.95ml of water; sample 2, 0.05ml of 0.014mM-sirohaem in pyridine+0.95ml of water; sample 3, 0.05ml of pyridine+0.01ml of 1.0mM-L-[*Me*-^{3}H]methionine in water+0.94ml of water; sample 4, 0.05ml of 0.014mM-sirohaem in pyridine+0.01ml of 1.0mM-L-[*Me*-^{3}H]methionine in water+0.94ml of water. The methionine solution used in samples 3 and 4 was a 1:5 dilution of the 5mM-L-[*Me*-^{3}H]methionine stock solution used for preparation of the growth medium in Expt. A.

Sample	Radioactivity (d.p.m.)
1. Solvent only	24
2. Sirohaem (0.70nmol)	6475
3. L-[*Me*-^{3}H]Methionine (10nmol)	38390
4. Sirohaem (0.70nmol)+L-[*Me*-^{3}H]-methionine (10nmol)	44275 (44841)*

Calculated [^3H_3C]/haem = 2.4

* Calculated (in parentheses) sum of radioactivities of samples 2+3 less sample 1.

Tables 1 and 2 include results that show there is negligible interference by haem, methionine or glucose in the determination of radioactivity of the other two components. As indicated in the Materials and Methods section, amino acid analyses of the protein residues remaining after extraction of sirohaem from the radioactive NADPH–sulphite reductase preparations showed that: (*a*) there was negligible synthesis of methionine from glucose by the methionine auxotroph used in these experiments and (*b*) there was negligible incorporation of ^3H derived from methionine into other amino acids. Since several of the amino acids tested would be expected to contribute to (or be in equilibrium with) pools of 3-carboxypropionyl-CoA and glycine, the precursors of uroporphyrinogen III *in vivo*, it is clear that one can effectively ignore any mixing of the L-[*Me*-^{3}H]-methionine and D-[U-^{14}C]glucose pools in interpreting the results of Expt. B.

The results thus provide strong support for the

Table 2. *Incorporation of ^3H from* L-[*Me*-^3H]*methionine and* ^{14}C *from* D-[*U*-^{14}C]*glucose into sirohaem (Expt. B)*

Growth of cells, isolation of NADPH–sulphite reductase, extraction and purification of sirohaem, and general methods for determination of radioactivity were as described in the Materials and Methods section. Samples 1–3 were prepared in quadruplicate; the results presented are the average for four determinations of each sample. These samples contained, in addition to 10ml of the Triton X-100/toluene-based counting mixture: sample 1, 0.05ml of pyridine+0.95ml of water; sample 2, 0.05ml of pyridine+0.03ml of 1.0mM-L-[*Me*-^{3}H]methionine in water+0.95ml of water; sample 3, 0.05ml of pyridine+0.03ml of 55.5mM-D-[U-^{14}C]glucose in water+0.95ml of water. For determination of the radioactivity of the sirohaem, the pyridine solution of the haem obtained after Sephadex LH-20 chromatography was divided into two equal portions that were then treated identically. Each portion of haem was placed in a scintillation vial and evaporated to dryness at ambient temperature under a stream of dry N_2. To the residues were added 0.05ml of pyridine, 0.95ml of water, and 10ml of the Triton X-100/toluene-based counting mixture. These solutions constituted sample 4. After determination of the radioactivity of the sample-4 solutions, 0.03ml of 1.0mM-L-[*Me*-^{3}H]methionine in water was added to each solution (sample 5), and the radioactivity again measured. Finally, 0.03ml of 55.5mM-D-[U-^{14}C]glucose in water was added to each solution of Sample 5 and the radioactivity again determined (sample 6). The results presented for samples 4–6 are the average of duplicate determinations. The methionine stock solution used in these samples was a 1:5 dilution of the 5mM-L-[*Me*-^{3}H]methionine stock solution used for preparation of the growth medium in Expt. B. The glucose stock solution used in these samples was a 1:10 dilution of the 0.555M-D-[U-^{14}C]glucose stock solution used for preparation of the growth medium of Expt. B.

Sample	Radioactivity (d.p.m.) ^{3}H channel	^{14}C channel
1. Solvent only	13	16
2. L-[*Me*-^{3}H]Methionine (30nmol)	16469	358
3. D-[U-^{14}C]Glucose (1665nmol)	5769	34611
4. Sirohaem (11.8nmol)	15746	2272
5. Sirohaem (11.8nmol)+L-[*Me*-^{3}H]methionine (30nmol)	32366	2539
	(32202)*	(2614)*
6. Sirohaem (11.8nmol)+L-[*Me*-^{3}H]methionine (30nmol)+D-[U-^{14}C]glucose (1665nmol)	37859	37355
	(37958)†	(37209)†

Calculated [^3H_3C]/haem = 2.4
Calculated [^{14}C]/haem = 47
Calculated [^{14}C]/[^3H_3C] = 19.8

* Calculated (in parentheses) sum of radioactivities of samples 2+4 less sample 1.
† Calculated (in parentheses) sum of radioactivities of samples 2+3+4 less twice sample 1.

proposal of Murphy *et al.* (1973*b*) that sirohaem is an iron–dimethylurotetrahydroporphyrin. The methyl groups are derived metabolically from methionine. The suggestion of Murphy *et al.* (1973*b*) that sirohydrochlorin (the term used for iron-free sirohaem) is synthesized by successive methylations of uroporphyrinogen III, followed by spontaneous or enzyme-catalysed oxidation of the tetrapyrrole macrocycle, becomes increasingly plausible. Sirohaem thus may be metabolically distinct from other haem or chlorophyll compounds with known biological function (e.g., haem A, haem C), that are considered to be derived biosynthetically by modifications of protoporphyrinogen, protoporphyrin or protohaem (Burnham, 1969). Murphy *et al.* (1973*b*) pointed out that a dimethyluroporphyrinogen III might serve as an intermediate in the biosyntheses of both sirohaem and vitamin B_{12}. Bykhovsky & Zaitseva (1976) have reported that *Propionobacterium shermanii* cell suspensions actively synthesizing vitamin B_{12} yield substantial amounts of a methylated porphyrin in the culture fluid. The spectrophotometric properties reported for this compound (which the authors termed 'corriphyrin') are similar to those of sirohydrochlorin cation. In collaboration with Professor A. I. Scott of the Department of Chemistry, Yale University, Dr. A. J. Irwin and one of us (L. M. S.) have found that a compound produced *in vitro* when *P. shermanii* extracts are incubated with porphyrin precursors and *S*-adenosylmethionine is indeed sirohydrochlorin, and that the methyl groups and tetrapyrrole macrocycle of sirohydrochlorin are incorporated largely intact into cobyrinic acid, a corrinoid precursor of vitamin B_{12} that is actively synthesized by these extracts. These results suggest then, that in addition to serving as prosthetic group for two distinct enzymes, each capable of catalysing a multielectron-transfer reaction, sirohaem and closely related derivatives may serve as a unique branch point in the metabolic (and possibly evolutionary?) divergence of the haem and corrinoid compounds.

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