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PORPHYRIN FORMATION AND ITS REGULATION IN *ARTHROBACTER*

Porphyrines in Arthrobacter: synthese en regulatie

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INTRODUCTION

Two prominent pigments which may be found in higher organisms as well as in microbes are the green compound chlorophyll and the red pigment heme or iron protoporphyrin. The first indication that these two pigments are structurally related was obtained by HOPPE-SEYLER (cf. GRANICK and MAUZERALL, 1961). He found that drastic treatment of chlorophyll with alkali converted the green pigment to a red compound. The absorption spectrum of this red pigment was found to resemble that of the red compound derived from hemoglobin by treatment with concentrated sulphuric acid (later found to be protoporphyrin).

After 1880 a series of basic studies on the chemistry of these pigments culminated in the establishment of the structure of heme in 1928 and of chlorophyll in 1934. WILLSTÄTTER showed that heme and chlorophyll are built upon the same tetrapyrrole plan. He also found that chlorophyll was a magnesium-containing complex and heme an iron complex. Especially FISCHER and CONANT determined the composition and position of the side chains attached to the pyrroles, and they elucidated the differences in structure of the two pigments (cf. GRANICK and MAUZERALL, 1961).

The attention of the investigators after 1935 was mainly focused on the biosynthesis of the above-mentioned pigments. Work originating from the laboratories of SHEMIN, NEUBERGER, RIMINGTON and GRANICK has considerably contributed to the elucidation of the biosynthetic pathway of heme and chlorophyll. The suggested scheme of the formation of chlorophyll (GRANICK, 1954) based on experiments with a micro-organism and the biosynthesis of heme in erythrocytes (SHEMIN, 1955) have much in common. The investigations made after 1955 with different kinds of organisms provide considerable evidence for one biosynthetic pathway of chlorophyll and of heme in all organisms.

When the biosynthesis of these pigments had been established, LASCELLES (1956) started investigations on the regulation of the synthesis of heme and chlorophyll in *Rhodopseudomonas spheroides*, a photosynthetic micro-organism. She showed that in addition to the requirement of iron for the synthesis of heme, iron was required by this bacterium for the biosynthesis of chlorophyll. Inadequate amounts of iron in the nutrient solution of the above-mentioned microbe caused chlorophyll deficiency, which was accompanied by accumulation of precursors of chlorophyll in the culture fluid. This phenomenon was first observed by VAN NIEL (1944).

Accumulation of precursors of heme caused by iron deficiency has been observed in Vertebrata. A diminished synthesis of heme is called anemia. In normal men or animals only traces of these precursors can be detected. In some pathological states, however, e.g. the porphyria diseases, copious amounts of these compounds are excreted in the urine. Curing of patients of the disease cannot be achieved by addition of iron to the diet. This disease is brought about by genetic deviations. Another form of anemia is characterized by a diminished synthesis of heme even though men or animals are provided with sufficient iron.

In this case, enzyme deficiences might be responsible for the anemia (SCHRETLEN, 1965).

So far only few investigations have dealt with the function of iron in the biosynthesis of heme in aerobic micro-organisms. Coryneform bacteria, particularly those of the genus *Arthrobacter* are involved in this study.

Arthrobacters are coryneform bacteria with an irregular cell morphology. These organisms are generally aerobic and can oxidize a wide variety of organic compounds. They are mostly fully devoid of fermentative capacities. Arthrobacters occur in large number in soil, in waste water and on the surface of particularly soft cheese. Since 1958 considerable attention has been given to the morphology, taxonomy, ecology and physiology of these bacteria in the present laboratory (MULDER et al., 1962; MULDER and ANTHEUNISSE, 1963; MULDER et al., 1966; ADAMSE, 1966; ZEVENHUIZEN, 1966).

Investigating the physiology of arthrobacters, the occurrence of a red-brown pigment in the culture filtrate of one strain was one of the first observations concerning porphyrin accumulation (ANTHEUNISSE, unpublished results). Later it was shown (see Chapter III) that this pigment was a porphyrin.

CHAPTER I

LITERATURE AND PURPOSE OF THE PRESENT INVESTIGATION

1. LITERATURE

1.1. *Introduction*

At least four reviews came out concerning the biosynthesis of porphyrins in the years 1961–1968 (GRANICK and MAUZERALL, 1961; LASCELLES, 1962, 1963, 1964). In addition FALK (1964) reported a profound analysis of the chemistry of porphyrins and metallo-porphyrins. In the following pages a general review will be given of the structure and nomenclature of porphyrins as an introduction to the present investigation. The discussion of the biosynthesis of porphyrins will be chiefly confined to the investigations of LASCELLES (1956, 1960, 1963) who studied *Rhodopseudomomas spheroides*, strain NCIB 8253. The biosynthesis and the regulation of the biosynthesis of vitamin B_{12} or vitamin B_{12} derivatives have not been included in this survey.

1.2. *The structure and nomenclature of porphyrins*

Porphyrins are derivatives of porphin (Fig. I-1) which consists of four (A, B, C and D) monopyrroles linked by methene bridges (α, β, γ and δ). The following modifications are possible.

a. Substitution of the C-atoms 1–8. The substituent can be an alkyl, an acetic acid, a propionic acid group or an H-atom.
b. Incorporation of metals. Some tetrapyrroles are devoid of metals. The physiologically active porphyrins contain magnesium, iron or cobalt.

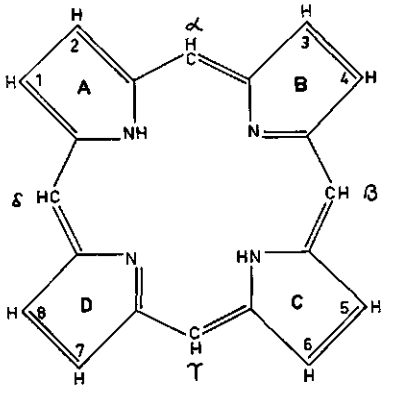


FIG. I-1. Porphin.

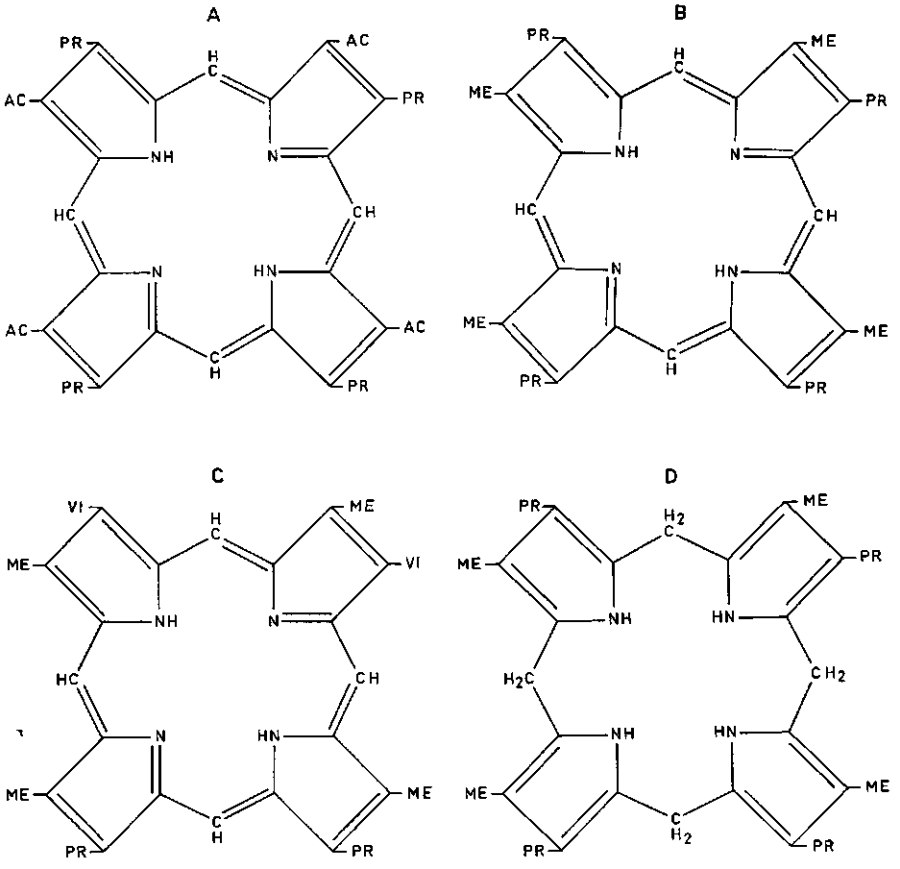


FIG. I-2. Uroporphyrin III (A), coproporphyrin III (B), protoporphyrin IX (C) and coproporphyrinogen III (D). AC: acetic acid, PR: propionic acid, ME: methyl, VI: vinyl group.

c. Substitution or replacement of the methene bridge carbons. Substituents may be hydrogen atoms as in porphyrinogens, or the cyclopentanone group as in the chlorophylls. The corrin structure of vitamin B_{12} or vitamin B_{12} derivatives lost the δ methene bridge carbon.

Besides heme and chlorophyll, uroporphyrin III, coproporphyrin III and protoporphyrin IX are the porphyrins commonly found in nature (Fig. I-2). Uroporphyrin has 4 acetic acid and 4 propionic acid side chains and coproporphyrin 4 methyl and 4 propionic acid side chains. The various groups can be arranged in 4 ways. Therefore, uroporphyrin and coproporphyrin have 4 isomeric forms: I, II, III and IV.

Protoporphyrin has 2 methyl, 2 propionic acid and 2 vinyl groups and therefore has 15 isomeric forms.

A porphyrin is an oxidized porphyrinogen (Fig. I-2). Porphyrinogens are the

true intermediates in the biosynthesis of protoporphyrin IX. Therefore, an accumulation of porphyrinogens is not visible under anaerobic conditions, whereas under aerobic conditions the cells or the environment of the cells producing these compounds have a red-brown colour. Porphyrins can be reduced to the corresponding porphyrinogens with sodium amalgam.

Complexes of uroporphyrin and coproporphyrin with metals have been found in the urine of men and animals. It seems highly probable that these complexes are formed spontaneously because porphyrins tend to form chelates. The structure of the physiologically active metallo-porphyrins heme and bacteriochlorophyll is shown in Figure I-3.

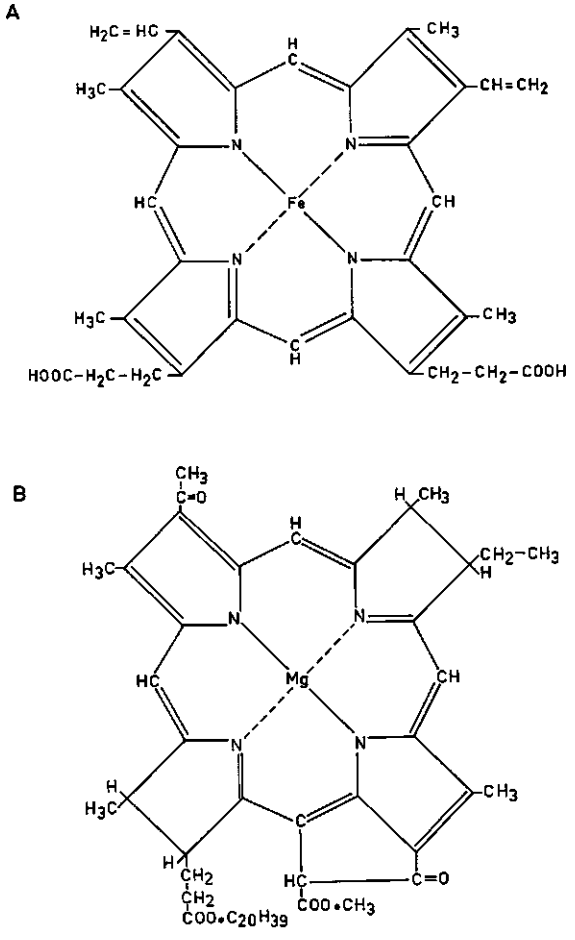


FIG. I-3. Heme (A) and bacteriochlorophyll (B).

1.3. *The function and distribution of tetrapyrroles*

Heme and chlorophyll are intracellularly bound to specific proteins. In this form they play a dominant role in the energy metabolism as carriers of oxygen (hemoglobins), as carriers of electrons (cytochromes) or as a trap for radiant energy (chlorophylls).

VIRTANEN and LAINE (1946) and subsequently SMITH (1949) found hemoglobin, known as leghemoglobin or legoglobin, in nitrogen-fixing root nodules of leguminous plants. Ineffective root nodules contain no hemoglobin. This may depend on the type of *Rhizobium*, but also on molybdenum deficiency (MULDER, 1954) or CO_2 deficiency of the root system (MULDER and VAN VEEN, 1960). These observations suggest that hemoglobin is involved in the process of nitrogen fixation. However, in experiments with nitrogen-fixing bacteroid suspensions, BERGERSEN and TURNER (1967) have shown that nitrogen fixation by bacteroids required no hemoglobin. Since the latter compound is apparently an essential component in nitrogen fixation in intact nodules, BERGERSEN and TURNER (1967) suggested that it is required for the oxygen transport to the bacteroids in the interior parts of the nodules where the free oxygen tension in view of nitrogen fixation has to be low.

The biological function of free porphyrins is unknown. They are regarded as the oxidized form of porphyrinogens, the true intermediates in the biosynthesis of heme and chlorophyll. The occurrence of free porphyrins is presumably the result of some metabolic block in the biosynthetic pathway leading to heme or chlorophyll. Therefore free porphyrins are relatively rare in nature. In higher plants and mammalia only traces of these tetrapyrroles are found. When, however, the synthesis of the functional tetrapyrroles heme or chlorophyll is inhibited, large quantities of free porphyrins may be accumulated. This is the case in the porphyria disease with respect to the synthesis of heme. The 3 dominant forms of this disease are the following.

a. Congenital porphyria. Accumulation of porphyrins occurs in the skin, and the urine is rich in uroporphyrin.
b. Acute porphyria. Large quantities of δ-aminolevulinic acid and porphobilinogen are found in the urine. These compounds are precursors of porphyrins.
c. Porphyria cutanea tarda. The urine is red-brown coloured due to a mixture of uroporphyrin and coproporphyrin.

Acute porphyria is also induced by certain drugs, e.g. 3,5-dicarbethoxy-1,4-dihydrocollidine and acetyl-isopropylacetamide.

The accumulation of free porphyrins in micro-organisms was first observed in the culture fluids of several *Rhodospeudomonas* species (VAN NIEL, 1944). Particularly members of the Athiorhodaceae may excrete considerable amounts of coproporphyrin III.

The accumulation of coproporphyrin III in non-photosynthetic micro-organisms is also rather common. The following micro-organisms are capable of producing predominantly coproporphyrin III: *Corynebacterium diphtheriae*, *C. erythrogenes*, *Arthrobacter globiformis*, *Arthrobacter* JG-9, *Propionibacterium shermanii*, *Micrococcus lysodeikticus*, *Bacillus cereus*, *Staphylococcus aureus*,

Mycobacterium karlinski and other mycobacteria, *Saccharomyces anamensis* and *S. cerevisiae* (LASCELLES, 1962).

1.4. *The biosynthesis of tetrapyrroles*

With the establishment of the structure of heme and chlorophyll it became apparent that the biosynthetic pathways must have much in common. Work on the biosynthesis of chlorophyll with mutants of *Chlorella* (GRANICK, 1954) and of heme with erythrocytes (SHEMIN, 1955) supported that idea.

The scheme of the biosynthesis of heme is shown in Figure I-4A. It will be seen that the initial reaction in the biosynthetic chain is the enzymatic condensation of glycine and succinyl-CoA leading to δ-aminolevulinic acid. Later it was shown that pyridoxal phosphate is the coenzyme of δ-aminolevulinic acid synthetase. This enzyme is considered the first enzyme of the tetrapyrrole synthesis. Subsequently, two molecules of δ-aminolevulinic acid condense by the removal of two molecules of H_2O to form the monopyrrole porphobilinogen (enzyme: δ-aminolevulinic acid dehydratase). Four molecules of porphobilino-

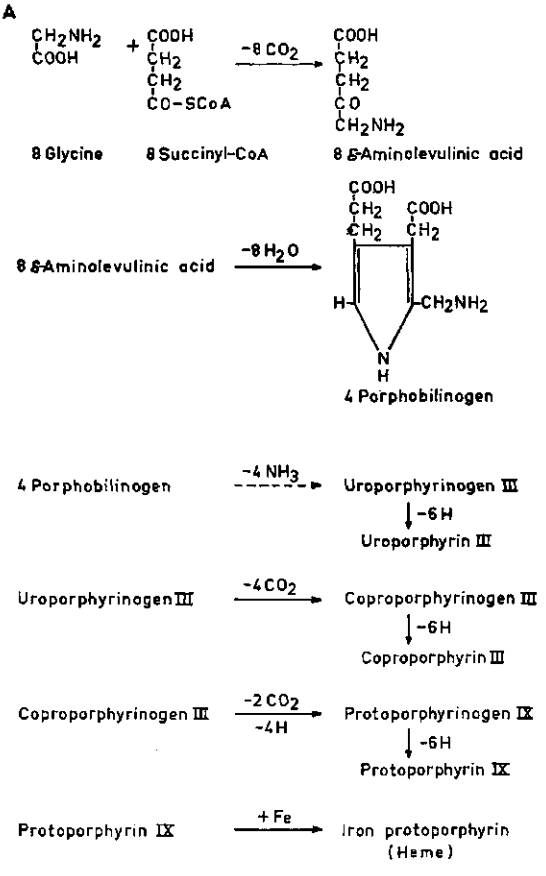


FIG. I-4A. The biosynthesis of heme.

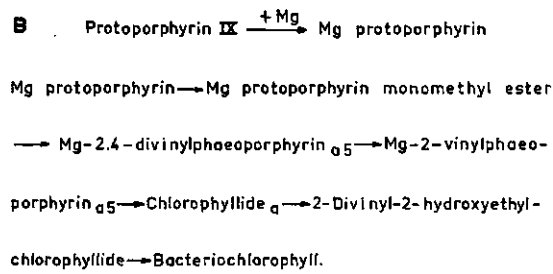


FIG. I-4B. A tentative scheme of the biosynthesis of bacteriochlorophyll.

gen condense to form uroporphyrinogen III with the elimination of four NH_3 groups (enzyme: porphobilinogen deaminase). So far it is unknown whether or not a dipyrrole is an intermediate in this reaction (BOGORAD, 1958). Decarboxylation of the acetic acid side chains leads to coproporphyrinogen III (enzyme: urogenase). The next step is the oxidation of two specific propionic acid groups to vinyl groups and subsequently the pyrrole ring is oxidized to yield protoporphyrin IX (enzyme:coproporphyrinogen oxidase). Incorporation of iron forms iron protoporphyrin or heme (enzyme: heme synthetase or protoporphyrin ferrochelatase). The enzymes urogenase, coproporphyrinogen III oxidase and heme synthetase have a strong substrate specificity. Only type III isomers of uroporphyrinogen and coproporphyrinogen are involved in the biosynthesis of heme and chlorophyll.

The biosynthesis of protoporphyrin IX has also been studied in micro-organisms other than *Chlorella*. Even before the biosynthesis had been disclosed, it was found that N^{15}-containing glycine was incorporated into the extracellular coproporphyrinogen III and the intracellular heme formed by *Corynebacterium diphtheriae*. In the culture filtrate of the photosynthetic bacteria *Rhodopseudomonas spheroides* (LASCELLES, 1956), *R. capsulatus* (COOPER, 1963) and *Rhodospirillum rubrum*, large quantities of porphyrins were found after addition of δ-aminolevulinic acid. In addition the enzymes ALA synthetase, ALA dehydratase and heme synthetase have been demonstrated (LASCELLES, 1964). TOWNSLEY and NEILANDS (1957) observed the conversion of δ-aminolevulinic acid to porphobilinogen, uroporphyrinogen III and coproporphyrinogen III by *Micrococcus lysodeikticus*. LASCELLES (1962) showed that incubation of iron-deficient cells of the ciliate *Tetrahymena vorax* with glycine and succinic acid resulted in the accumulation of uroporphyrin III, coproporphyrin III and protoporphyrin IX. Recently KONOVA and RYBAKOVA (1967) found that the amount of porphyrins produced by many actinomycetes considerably increased by the addition of δ-aminolevulinic acid. A strain of *Bacterium ruminicola*, isolated from the stomach of cows by CALDWELL et al. (1965) had a heme requirement which could also be fulfilled by uroporphyrin III and coproporphyrin III.

All the above-mentioned observations confirm the occurrence of the biosynthetic chain of heme in micro-organisms as found by SHEMIN (1955) in erythrocytes (Fig. I-4A).

The scheme of the biosynthesis of bacteriochlorophyll from protoporphyrin

IX in *Rhodopseudomonas spheroides* proposed by LASCELLES (1966) is given in Figure I-4B. It closely resembles the scheme of *Chlorella*, suggested by GRANICK (1950). It is a tentative scheme as many enzymes of this chain have still to be demonstrated. Only the synthesis of Mg protoporphyrin monomethyl ester in *R. spheroides* has been investigated at an enzymic level. GIBSON et al. (1962) detected the following reactions:

$$\text{Protoporphyrin IX} \xrightarrow{\text{Mg}} \text{Mg protoporphyrin}$$

$$\text{Mg protoporphyrin} + \text{S-adenosylmethionine} \longrightarrow$$
$$\text{Mg protoporphyrin monomethyl ester} + \text{S-adenosylhomocysteine}$$

In addition to investigations concerning the synthesis of heme and chlorophyll, attention has been paid to the localization of the enzymes of the tetrapyrrole pathway. From studies of GRANICK and MAUZERALL (1961) and LASCELLES (1964) it may be concluded that in all cells the enzymes catalysing the conversion of coproporphyrinogen III to heme or chlorophyll are integrated with membranous structures, the so-called organelles. These may be mitochondria, chloroplasts, chromatophores and cytoplasmic membranes. Purified preparations of these structures consisting of about 50% protein and 50% lipid (mainly phospholipid) may contain cytochromes, carotenoids, quinones and chlorophylls. In bacteria belonging to the Athiorhodaceae the enzymes catalysing the conversion of glycine and succinyl-CoA to coproporphyrinogen III are localized in the soluble fraction of the cell. In non-photosynthetic micro-organisms only δ-aminolevulinic acid dehydratase is detectable in this fraction. In liver cells all the enzymes up to coproporphyrinogen III except δ-aminolevulinic acid synthetase are found in the soluble fraction.

1.5. *The regulation of the biosynthesis of tetrapyrroles*

PAPPENHEIMER (1947) was the first to observe the effect of iron on the synthesis of heme and the accumulation of coproporphyrin III in *Corynebacterium diphtheriae*. During the subsequent twenty years many papers appeared dealing with the regulation of tetrapyrrole biosynthesis. In the following review of these reports the influence of B vitamins, oxygen tension and iron will be discussed.

1.5.1. B Vitamins and the biosynthesis of tetrapyrroles

The vitamin pyridoxal phosphate is the coenzyme of δ-aminolevulinic acid synthetase. A deficiency of this vitamin in chickens causes anemia. The importance of the vitamin has been confirmed in experiments with micro-organisms. Cells of *Tetrahymena vorax* deficient in pyridoxal phosphate had a strongly reduced ability to convert glycine and succinic acid to porphyrins. The conversion of δ-aminolevulinic acid to porphyrins was unaffected by this deficiency (LASCELLES, 1962).

The influence of pantothenic acid on the synthesis of porphyrins by *Tetrahymena vorax* was also studied by LASCELLES (1962). The amount of porphyrin formed from glycine and succinic acid by cells grown with suboptimal amounts

of pantothenate was only 20% of that synthesized by normally grown cells. Pantothenic acid is required for a normal synthesis of porphyrins because of its participation in the formation of succinyl-CoA. Pantothenate deficiency did not reduce the conversion of δ-aminolevulinic acid to porphyrins. However, the composition of the porphyrin mixture was modified. The quantity of protoporphyrin formed was considerably reduced. This might be an indication that pantothenic acid is either directly or indirectly involved in the conversion of coproporphyrinogen III to protoporphyrin IX. The underlying mechanism is not yet clear. The same is true of the observation of STICH and EISGRUBER (1951) who found that unlike normal cells, riboflavin-deficient cells of *Saccharomyces anamensis* accumulated coproporphyrin.

Cells of *Rhodopseudomonas spheroides*, deficient in biotin, thiamine, or nicotinic acid, had a reduced ability to convert glycine and α-ketoglutarate to porphyrins. The conversion of δ-aminolevulinic acid to porphyrins was not affected by these vitamins (LASCELLES, 1956). It is thought (LASCELLES, 1962) that thiamine and nicotinic acid are required for the conversion of α-ketoglutarate to succinyl-CoA while biotin might be directly concerned with the activity of δ-aminolevulinic acid synthetase (as its coenzyme).

A further group of growth factors which may have some importance in the synthesis of heme are the siderochroms (ferrichrome, coprogen, terregens factor). These organic compounds contain iron, are water-soluble and have a red-brown colour with a maximum absorbancy between 420 and 440 mμ. NEILANDS and BURNHAM (1961) studied a ferrichrome-requiring *Arthrobacter* (strain JG-9) which could use heme instead of ferrichrome. Subsequently it was shown (BURNHAM, 1963) that ferrichrome may be used as iron source for the synthesis of heme from protoporphyrin IX. Similar results were obtained with *R. spheroides* and with a strain of *Staphylococcus aureus* (KNÜSEL et al., 1967).

1.5.2. Oxygen and the biosynthesis of tetrapyrroles

It is a well-known fact that men and animals form more hemoglobin at a reduced oxygen tension. This effect is not restricted to mammalia as the oxygen tension also plays a dominant role in the biosynthesis of heme and chlorophyll by micro-organisms. LENHOFF et al. (1956) found that cells of *Pseudomonas fluorescens* grown in an atmosphere with 1% oxygen contained 13–14 times more cytochrome c than those grown in an atmosphere containing 20% oxygen.

In contrast to this unfavourable effect of oxygen on the synthesis of heme, SCHAEFFER (1952) found that in the facultatively anaerobic micro-organisms *Saccharomyces cerevisiae* and *Bacillus cereus*, cells grown under moderately aerobic conditions contained more heme than those grown under anaerobic conditions. In the case of *B. cereus* this amounted to a 5 times higher heme content.

LASCELLES (1960) has made many attempts to understand the mechanism by which oxygen represses the synthesis of bacteriochlorophyll in *Rhodopseudomonas spheroides*. She observed that in chlorophyll-synthesizing cells grown at a low oxygen tension the activities of δ-aminolevulinic acid synthetase and de-

hydratase were 2–4 times higher than in cells exposed to a high oxygen tension which did not synthesize chlorophyll. The repression of δ-aminolevulinic acid synthetase might be responsible for the effect of oxygen on chlorophyll formation. This explanation, however, does not fully account for the oxygen effect as the enzyme was still readily detectable in cells which did not form chlorophyll. Recently DREWS (1965) came to the same conclusion in a study of the formation of chlorophyll in *Rhodospirillum rubrum*.

A further objection against the foregoing explanation of the oxygen effect on chlorophyll formation is that introduction of oxygen into cultures of chlorophyll-synthesizing cells of *R. spheroides* immediately stopped the synthesis of chlorophyll. If a repressed synthesis of δ-aminolevulinic acid synthetase would be responsible for this effect, the formation of chlorophyll would only gradually diminish since the enzyme which was originally present in the cells would only gradually disappear by dilution.

A different hypothesis is that oxygen exerts its effect on the synthesis of chlorophyll by inhibiting the conversion of Mg protoporphyrin to its methyl ester (see Fig. I-4B). GIBSON et al. (1962) failed to detect the enzyme in aerobically grown cells of *R. spheroides*. Moreover the observation does not account for the oxygen effect since it would involve accumulation of precursor(s) of Mg protoporphyrin monomethyl ester, and this has never been observed. Oxygen reduced the accumulation of porphyrins in *R. spheroides* (LASCELLES, 1956). Therefore, it is likely that oxygen directly inhibits an early stage in the biosynthesis of chlorophyll.

The absence of the enzyme methylating Mg protoporphyrin in aerobically grown cells of *R. spheroides* was correlated with the absence of chromatophores to which this enzyme is attached (GIBSON et al., 1962). The absence of these organelles, which are invaginations of the cytoplasmic membrane, in aerobically grown cells of Athiorhodaceae was first observed by DREWS (1960) and GIESBRECHT and DREWS (1962) in *Rhodospirillum rubrum*. A similar observation was made in *R. sphaeroides* (COHEN-BAZIRE and KUNISAWA, 1963). The amount of chlorophyll in these bacteria is proportional to the number of chromatophores. LASCELLES (1965) analysed the membrane fraction of anaerobically and aerobically grown cells of *R. spheroides*. The former cells contained 160 times more chlorophyll, about 16 times more carotenoids, 3 times more heme, about 2 times more phospholipid and 30 times more ribulose diphosphate carboxylase than the aerobically grown cells. CARR (1964) found 2 times more ubiquinone in pigmented cells of Athiorhodaceae.

Recently MATHEWS (1966) published a paper dealing with the relation between the synthesis of phospholipids and that of carotenoids in non-photosynthetic bacteria. He could not detect any difference in phospholipid formation between cells of *Mycobacterium* and *Sarcina* containing carotenoids and cells devoid of these pigments. The carotenoids in these bacteria were associated with the cytoplasmic membrane.

1.5.3. Iron and the biosynthesis of tetrapyrroles

PAPPENHEIMER (1947) found that addition of iron to the medium of *C. diphtheriae* suppressed the accumulation of coproporphyrin III and enhanced the amount of heme formed by growing cells of this organism. His interpretation of this iron effect was that iron together with a porphyrin is required for the synthesis of heme. This view was supported by the observation that in cultures of *C. diphtheriae* there was a mole-for-mole relation between the amount of iron added and the amount of coproporphyrin III which failed to appear. More recent work with a different strain of *C. diphtheriae* did not give such clear-cut results (CLARKE, 1958).

After 1947 the eliminating effect of iron on the accumulation of porphyrins has been repeatedly confirmed with different types of micro-organisms (see section 1.3). The most striking effect of iron deficiency is that in nearly all organisms studied so far, coproporphyrin is accumulated. The accumulation of protoporphyrin IX in bacteria has relatively seldom been observed.

The theory of PAPPENHEIMER (1947) is not tenable. LASCELLES (1956) incubated iron-deficient, aerobically grown cells of *R. spheroides* under semi-anaerobic conditions in the light with glycine and α-ketoglutarate. Coproporphyrin III was the dominant porphyrin formed within 24 hours. Addition of iron reduced the accumulation of coproporphyrin III and enhanced the production of chlorophyll and heme. δ-Aminolevulinic acid was able to replace glycine and α-ketoglutarate as substrate for coproporphyrin III formation under conditions of iron deficiency. Incubation of the bacteria with both δ-aminolevulinic acid and iron resulted in the formation of coproporphyrin III and protoporphyrin IX. This is an indication that iron is required for the conversion of coproporphyrin III to protoporphyrin IX.

In *S. anamensis* (STICH and EISGRUBER, 1951) and *R. spheroides* (LASCELLES, 1956) the amount of coproporphyrin III, accumulated under conditions of riboflavin and iron deficiency, respectively, was at least 10 times higher than the amount of heme and chlorophyll formed when no porphyrin accumulated. These reactions imply some kind of regulatory mechanism. BURNHAM (1962) found that heme suppressed the formation of coproporphyrin III from glycine and α-ketoglutarate. The conversion of δ-aminolevulinic acid to porphyrins was not affected by heme. Later BURNHAM and LASCELLES (1963) showed that heme repressed the synthesis of δ-aminolevulinic acid synthetase (enzyme repression) and inhibited its activity (enzyme inhibition). However, it was found that the presence of heme in growing cultures of *R. spheroides* did not inhibit the formation of chlorophyll.

Chlorophyll or derivatives of chlorophyll exerted no effect on δ-aminolevulinic acid synthetase. This was considered to be due to the insolubility of these compounds.

1.5.4. Exceptions to the general relation between iron and coproporphyrin III accumulation

There are a few exceptions to the general rule that iron decreases the accu mulation of coproporphyrin III.

STICH and EISGRUBER (1951) persisted in their opinion that the quantity of coproporphyrin produced by *S. anamensis* was not decreased by the addition of iron to the nutrient solution. According to these authors riboflavin was the only compound decreasing the porphyrin accumulation and increasing the amount of heme formed. However, an explanation of this phenomenon was not presented.

A second exception seems to be the porphyrin accumulation by the bacterium *Arthrobacter* JG-9. NEILANDS and BURNHAM (1961) found that this microbe produced copious amounts of porphyrin although 5 μg/ml Fe was present in the nutrient solution. Since the amount of iron required to prevent coproporphyrin III accumulation by several other micro-organisms is less, iron apparently did not reduce the amount of porphyrin formed by this coryneform microbe. The effect of iron on the accumulation of coproporphyrin III in *Arthrobacter globiformis* is also less clear. MORRIS (1960) stated that particularly iron deficiency gave rise to porphyrin accumulation in this bacterium. However, no data were presented by this author.

The third exception is the porphyrin accumulation by *R. spheroides*. GIBSON et al. (1962) observed a complete inhibition of chlorophyll formation by ethionine, the ethyl analogue of methionine. This inhibition was accompanied with coproporphyrin III accumulation. The amount of porphyrin produced under these particular conditions was unaffected by iron. Methionine was found to be the only compound eliminating this accumulation and restoring the bacteriochlorophyll formation. The inhibition of the synthesis of chlorophyll was due to the formation of S-adenosylethionine which competitively inhibited S-adenosylmethionine formation. The latter compound (see Fig. I-4B) serves as donor of methyl groups in the synthesis of Mg protoporphyrin monomethyl ester. The authors did not explain the coproporphyrin III accumulation caused by ethionine.

1.6. *Summary*

The literature concerning the biosynthesis of heme provides strong evidence for the existence of one biosynthetic pathway for heme in all kinds of organisms.

Pyridoxal phosphate has a specific function in the synthesis of tetrapyrroles; this vitamin is the coenzyme of δ-aminolevulinic acid synthetase.

The effect of oxygen on the synthesis of porphyrins has so far seldom been studied in micro-organisms synthesizing heme only. Nor has the regulation of the synthesis of porphyrins by these organisms been investigated.

Iron probably influences the conversion of coproporhyrinogen III to protoporphyrin IX. This might explain the coproporphyrin accumulation in microorganisms grown in iron-deficient media.

It is unknown how pantothenic acid, riboflavin and ethionine affect the conversion of coproporphyrinogen III to protoporphyrin IX.

2. The purpose of the present investigation

In an experiment with approximately 100 coryneform bacteria isolated from soil and cheese it was found that under certain nutritional conditions a red-brown colour was produced, which appeared to be due to the presence of coproporphyrin III. This pigment was not detected in the culture fluid of some other bacteria including *Aerobacter aerogenes*, *Escherichia coli*, *Pseudomonas fluorescens*, *Azotobacter vinelandii* and *Agrobacterium tumefaciens*. The formation of the red colour was apparently typical for coryneform organisms, and it was thought that this character could be used as an identification test for coryneform bacteria. However, during a more thorough investigation it was found that mycobacteria, some micrococci species and some bacilli were also able to produce coproporphyrin III. These observations are in agreement with those found by Bariéty et al. (1957), Schaeffer (1952) and Townsley and Neilands (1957), who detected coproporphyrin III in the cultures of mycobacteria, bacilli and micrococci, respectively. Recently the formation of porphyrins in *Achromobacter metalcaligenes* (Doss and Mannheim, 1967) and in many actinomycetes (Konova and Rybakova, 1967) has been described. Therefore the original idea to use red pigment formation as a specific property for the identification of coryneform bacteria had to be given up.

In the course of the above-mentioned study the suppression of coproporphyrin III accumulation by added iron, as described by Lascelles (1956), was observed in only a few *Arthrobacter* strains. Therefore it was decided to start a detailed investigation to explain the accumulation of coproporphyrin III by two *Arthrobacter* strains growing in media containing relatively large amounts of iron.

Attention has been given to the following items:

a. the type of porphyrin formed by the two *Arthrobacter* strains,
b. the conditions under which accumulation of porphyrins is optimal and,
c. the function of iron in the conversion of coproporphyrinogen III to heme.

CHAPTER II

MATERIAL AND METHODS

1. *Micro-organisms, media and cultivation*

The micro-organisms included in this investigation have been obtained from the collection of the Laboratory of Microbiology at Wageningen.

The *Arthrobacter* strains 223 and 144, used in most experiments, had been isolated from soil. They were kept on slants containing 1% (w/v) glucose; 0.1% (w/v) K_2HPO_4; 0.25% (w/v) NH_4Cl; 0.03% (w/v) $MgSO_4.7H_2O$; 0.25% (w/v) $CaCO_3$, 10^{-4} μg/ml biotin and 1.2% (w/v) agar.

Two-days-old cultures of these strains were inoculated into 40 ml nutrient solution (100 ml flask) with the composition as used in the agar medium. After 48 hours incubation, 1 ml of this subculture was used to inoculate 200 ml solution (500 ml flask) which had the same composition as far as the macro-elements are concerned. The following trace elements were added: Fe (1 μg/ml) and 0.5 ml of the mixture containing 50 mg $ZnSO_4.7H_2O$, 50 mg $CuSO_4.5H_2O$, 500 mg $MnSO_4.2H_2O$, 10 mg $Na_2MoO_4.2H_2O$ and 10 mg $CoCl_2.6H_2O$ per 1 (Medium A).

For studying the decomposition of choline both glucose and NH_4Cl in medium A were replaced by 0.2% (w/v) choline. All experiments were carried out at 30°C.

2. *Sterilization of media and cleaning of flasks*

All compounds present in agar media were sterilized together. Some compounds of medium A (glucose, iron and biotin) were sterilized separately. Heat-labile nutrients which were sometimes added to medium A were sterilized by Seitz filtration.

The components of media in which choline, betaine, N.N-dimethylglycine, sarcosine, glycine and serine served as the only C and N-source were sterilized separately to prevent precipitation of magnesium salts which would have interfered with turbidity measurements. Choline and related compounds were sterilized by Seitz filtration.

All flasks were washed with concentrated sulphuric acid and subsequently filled with boiling water. When the water had cooled down, the flasks were rinsed with tap water and several times with glass-distilled water.

3. *Aeration level*

The degree of aeration of the cultures is indicated as the rate at which sulphite is oxidized (cf. VAN GILS, 1966); it will be mentioned for each experiment. The sulphite oxidation value in the subculture amounted to 15 mMol O_2/liter hr.

4. *Removal of iron from the nutrient solution*

Iron, present as contamination in some compounds of nutrient solution A,

was removed with 8-hydroxyquinoline according to the method of WARING and WERKMAN (1942). For glucose the procedure was as follows: 10 ml chloroform containing 5 mg 8-hydroxyquinoline was added to 100 ml glucose solution (25%, w/v). After vigorous shaking, another 10 ml chloroform was added and shaken again. The two layers were separated by centrifugation (during 5 min. at $2{,}000 \times g$) and the chloroform containing the iron chelate was discarded. After repeating this procedure the glucose solution was boiled to remove the dissolved chloroform. The removal of iron from K_2HPO_4 and NH_4Cl was carried out in the same way. Iron-free $CaCO_3$ was obtained by adding a purified solution of Na_2CO_3 to a purified $CaCl_2$ solution. The precipitated $CaCO_3$ was washed several times with glass-distilled water and dried. Other compounds of medium A were not treated with 8–hydroxyquinoline because they were used either in small concentrations or they contained negligible amounts of iron like vitamin-free Casamino acids applied in media for toxin-producing micro-organisms.

5. *Determination and uptake of iron*

Iron was chemically estimated with o-phenantroline according to CLARKE (1958) or microbiologically using the fungus *Aspergillus niger*. This fungus reached its maximum cell yield in medium A when 9 μg Fe per 200 ml was present. The quantity of iron present in or on *Arthrobacter* cells was estimated by adding a suitable amount of hydrolysed bacterial cells to medium A and inoculating the solution with *A. niger*. The rate of iron uptake by the arthrobacters was determined by sampling the bacterial culture at fixed times. At the same time the amount of iron left in the medium was determined. The values obtained for iron recovery varied between 93 and 106%.

6. *Determination of cell yield*

The quantity of cell material was determined by harvesting the cells after $CaCO_3$ had been removed by centrifugation ($2{,}000 \times g$ for 5 min.). The cell material was washed twice with water and dried at 100 °C.

7. *Determination of carbohydrates*

The anthrone method of TREVELYAN and HARRISON (1952) was used. The wet bacteria were first extracted with acetone to obtain a clear solution after hydrolysis.

8. *Determination of nitrogen*

The bacteria in 10 ml culture solution were harvested, washed twice with glass-distilled water and hydrolysed with 5 ml 10 N H_2SO_4 (containing 0.2 g/l $CuSeO_3$). After boiling for two hours a few drops of H_2O_2 were added and the sample heated for a further 30 minutes. Subsequently KOH (40%, w/v) was added and ammonia was distilled into H_3BO_3 solution (2%, w/v) and estimated by titration with 0.05 N H_2SO_4.

9. *Isolation of porphyrins* (FALK, 1964)

When the bacteria were grown in the above-mentioned media, the porphyrins produced are adsorbed by the $CaCO_3$. In addition they have a great affinity towards proteins (FALK, 1964). To liberate the porphyrins glacial acetic acid was added to 25 ml of the culture until pH 4 and subsequently 25 ml ethyl acetate. After vigorous shaking the porphyrins dissolved quantitatively in the ethyl acetate and could easily be collected.

10. *Identification of porphyrins* (FALK, 1964)

The ethyl acetate layer was washed with 0.6 M Na-acetate and water. The porphyrins were extracted from the ethyl acetate layer with 3.0 N HCl. The pH of this acid solution was brought to 4 with saturated Na-acetate and extracted with ether. Washing of the etherial solution with water was followed by the extraction from this solution of coproporphyrin III (CP III) with 0.3 N HCl and of protoporphyrin IX (PP IX) with 1.2 N HCl.

The following characteristics of porphyrins were determined for their identification:

a. the HCl fraction in which the porphyrin was present,
b. the spectrum of the porphyrin in acid and alkaline solution,
c. the spectrum of the methyl ester of the porphyrin in chloroform (the conversion of porphyrins to their methyl esters was performed according to FALK(1964)),
d. the influence of the HCl concentration on the spectrum of the porphyrin,
e. the Rf-value in the solvent system lutidine: water (1 :1, v/v) in NH_3 atmosphere.

Arthrobacter porphyrin was compared with authentic CP III derived from culture filtrates of *Corynebacterium diphtheriae*. The sample was a gift of Dr. June Lascelles, Department of Bacteriology, Los Angeles, California. The other porphyrins and δ-aminolevulinic acid were obtained from Light and Co.

11. *Determination of porphyrins* (FALK, 1964)

The porphyrin content of 1 ml ethyl acetate was estimated by comparing the absorbancy at 408 mμ with that of a known solution. Any coproporphyrinogen III (CPG III) formed was estimated by converting it to CP III by keeping the ethyl acetate at room temperature for 24 hours and shaking it regularly. The rate of this conversion was sometimes increased by addition of a few drops of an I_2 solution (0.05 %, w/v).

12. *Isolation and determination of heme*

Heme was extracted in the way indicated by FALK (1964). It was extracted with acetone containing 1 % (w/v) 2 N HCl. After evaporating the acetone, the residue was extracted with ether. This ether extract was evaporated and heme was dissolved in 0.01 N NaOH. The amount of heme was determined as the pyridine hemochrogen. Cytochrome c heme, which remained in the cells after the extraction with acid acetone, was isolated by incubating the cells overnight

with an $HgSO_4$ solution (0.06%, w/v), containing 0.4 ml glacial acetic acid per 100 ml. This treatment eliminated the association between the heme and protein moiety of cytochrome c.

13. *Determination of δ-aminolevulinic acid (ALA)*

Some acetylacetone (0.5 ml) was added to 5 ml culture filtrate. The pH was adjusted to 4–5 and the mixture during 10 minutes heated in a boiling water-bath. After cooling, 2 ml of the mixture was added to 2 ml Ehrlich's reagent. The absorbancy at 553 mμ was determined after 15 minutes. The reagent of Ehrlich was prepared by dissolving 500 mg paradimethylaminobenzaldehyde in 100 ml HCl (5%, w/v), FALK, 1964).

14. *Determination of porphobilinogen (PBG)*

A volume of the reagent of Mauzerall and Granick (FALK, 1964) was added to an equal volume of the culture filtrate. After 10 minutes the absorbancy (555 mμ) was determined. The reagent was prepared by dissolving 1 g paradimethylaminobenzaldehyde in 30 ml glacial acetic acid to which 8 ml 7% (w/v) perchloric acid was added. This mixture was filled up with acetic acid to 50 ml.

15. *Extraction of phospholipids*

Before harvesting the bacteria, the cultures were kept in a boiling water-bath for about 5 minutes in order to destroy phospholipases (HAVERKATE, 1965). These enzymes catalyse the hydrolysis of phospholipids (see Chapter V). The collected bacteria were extracted with a mixture of chloroform: methanol (1:2, v/v). For the extraction of about 10 g cell material (dry weight) 300 ml of this mixture was used. After homogenizing, this mixture was heated for 10 minutes. Another volume of chloroform was added and the extraction continued overnight at room temperature. The mixture was then filtered, the filtrate supplied with 2 volumes of water, and after shaking the 2 layers were separated by centrifugation (5 min. at 2,000 × *g*). The lower layer (chloroform) was removed by means of a pipette. A further volume of chloroform was added to the methanol: water phase and the procedure repeated. The combined chloroform extracts were transferred to a rotary evaporator and the chloroform evaporated at a temperature not exceeding 45 °C. The residue containing still some protein was redissolved in 10 ml of a mixture of chloroform: methanol (1:1, v/v) and then one volume of water was added. After separating the layers the chloroform layer was stored at 4 °C. This extract contained the phospholipids and no protein was left.

16. *Identification of phospholipids* (HOUTSMULLER, 1966)

Thin-layer chromatography was applied to analyse the mixture of phospholipids. The plates were covered with about 1 mm silica gel G (Merck) with the aid of a microslide. After drying at room temperature, the plates were activated by heating at 110 °C for 1 hour, and after this treatment they were immediately

used. Plates older than 4 days were discarded. Different solvent systems were used to separate the phospholipids, of which spots were made visible in the following way:

a. spraying with 50% H_2SO_4 (w/v): organic substances in the chloroform appear as brown-black spots after heating the plate at 110°C for 5 minutes;
b. spraying with ninhydrin: only phospholipids containing NH_2 groups are stained after heating the plate for 5 minutes at 110°C,
c. spraying with ammoniummolybdate reagent: all phospholipids give a blue colour,
d. spraying with Dragendorff's reagent: only phospholipids with a methyl group containing N-base give a red colour.

For a more detailed analysis of the phospholipid mixture mild alkaline hydrolysis was carried out according to the method of HAVERKATE (1965). The free fatty acids resulting from this hydrolysis were extracted with a mixture of butanol: chloroform (1:1, v/v). The hydrolysis products soluble in water were identified by means of thin-layer chromatography. A chloroform extract of *Bacillus megaterium* which had been shown to contain phosphatidylglycerol and phosphatidylethanolamine (OP DEN KAMP, 1967) served as reference. This extract was a gift of Dr. J. A. F. Op den Kamp, Department of Biochemistry, University of Utrecht. In order to find out whether phosphatidylcholine (lecithin) is present in cells of *Arthrobacter* (strain 223 and 144) the phospholipids of 3 strains of *Agrobacterium tumefaciens* were analysed for comparison. It has been shown several times that this bacterium contains lecithin (SHERR and LAW, 1965). The strains of *Agrobacterium tumefaciens* were grown in a nutrient solution with the following composition: 0.5% (w/v) glucose; 0.5% (w/v) Casamino acids (Difco); 0.1 (w/v) yeast extract (Difco); 0.1% (w/v) K_2HPO_4; 0.03% (w/v) $MgSO_4.7H_2O$ and trace elements (see 1), at a temperature of 30°C.

17. *Determination of phospholipids*

The silica gel containing the spot of the phospholipid was removed from the plate and mixed with 2 ml glass-distilled water. After addition of 0.5 ml 10 N H_2SO_4 the mixture was heated for 6 hours at 110°C. A few drops of 30% H_2O_2 were added and the heating was continued for another 4 hours. Subsequently 4.6 ml 0.22% (w/v) ammoniummolybdate and 0.2 ml Fiske-Subbaroff reagent were added. This mixture was vigorously shaken, boiled for 7 minutes, cooled and diluted. The absorbancy was determined at 830 mμ. The reagent was prepared by dissolving 30 g $NaHSO_3$, 1 g Na_2SO_4 and 0.5 g 1-amino-2-napthyl-4-sulphonic acid in 100 ml glass-distilled water (BARTLETT, 1959).

18. *Identification of amino acids and amino alcohols*

To analyse the decomposition products of choline, the culture filtrates of choline, betaine, N.N-dimethylglycine, sarcosine and glycine-grown cells were concentrated by evaporation. The amino acids present in these filtrates were identified by means of thin-layer chromatography. The plates were made as indicated under 16 and different solvent systems were used (STAHL, 1962).

Ninhydrin was used as spray reagent for amino acids and a benzoquinone solution (1 %, w/v) in alcohol for amino alcohols (SHIEH, 1966).

19. *Identification and determination of formaldehyde*

Chromotropic acid (1,8-dihydroxy-napthyl-3,6-disulphonic acid) was dissolved in glass-distilled water (1 %, w/v). After filtration the solution was filled up to 500 ml with a mixture of conc. H_2SO_4-water (2:1, v/v). The reagent was stored in the dark and weekly refreshed. To detect formaldehyde in the culture filtrate, 6 ml of the chromotropic acid reagent was added to 1 ml culture filtrate. The mixture was heated in boiling water for 30 minutes and the colour produced compared with that of authentic formaldehyde. The amount of formaldehyde was determined by comparison the absorbancy at 570 mμ with that of a known solution (SHIEH, 1966). Formic acid was estimated in the same way after reduction (cf. FEIGL, 1960).

20. *Identification of pyruvic acid*

To filtrates of choline-grown cultures (0.5 %, w/v) 2,4 dinitrophenylhydrazine in 6 N HCl was added. The yellow-orange precipitate was collected by centrifugation, subsequently washed with glass-distilled water and dissolved in 0.5 N NaOH. The spectrum was compared with the phenylhydrazone of authentic pyruvic acid. In addition the isolated product was run in the solvent system butanol: acetic acid: water (60:38:2, v/v/v) to compare the Rf-value with that of the authentic reaction product.

CHAPTER III

THE FORMATION OF PORPHYRINS BY IRON-SUFFICIENT CELLS OF *ARTHROBACTER*, STRAIN 223, GROWING IN A MINERAL MEDIUM WITH GLUCOSE AS THE C-SOURCE

1. Introduction

The blocking of the biosynthesis of a physiologically important compound is often accompanied with the accumulation of one or more precursors. The study of the conditions under which the precursor is accumulated may contribute to the knowledge of the regulation of the biosynthesis of the compound concerned.

The red-brown colour which sometimes appears in the culture fluid of *Arthrobacter* strains is caused by porphyrins, the precursors of heme. Apparently the synthesis of heme is inhibited in cells which produce porphyrins. Experiments were carried out to trace the factors which influence porphyrin formation by growing cells of *Arthrobacter*, strain 223. Nutrient solution A was used because yeast extract or a mixture of amino acids (Casamino acids) introduced compounds which seemed to have a specific function in the accumulation of CP III in *Arthrobacter*, as appeared from the results of several preliminary experiments.

2. Results and discussion

2.1. *The kind of porphyrins formed by different Arthrobacter strains*

The strains isolated from the soil viz. 1, 4, 144, 166, 223 and the cheese strains 269 and 293, grown on agar slants containing 0.7% (w/v) yeast extract and 1% (w/v) glucose, were inoculated into nutrient solution A enriched with 0.25% (w/v) Casamino acids. After incubation for 72 hours all the cultures had turned red-brown. The pigment was isolated and identified as indicated in chapter II.

Arthrobacter, strain 223, formed a porphyrin whose properties were identical with those of authentic CP III. This was true of the absorption spectra in 0.15 N HCl (Fig. III–1A) and 0.02 N NaOH (Fig. III–1B), the spectrum of the methyl ester (Fig. III–1C) and the influence of the HCl concentration on the absorption spectrum (Fig. III–1D). The isolated porphyrin was extracted from the ether fraction with the same HCl concentration as authentic CP III and it had the same Rf-value (Table III–1). In addition to a large amount of CP III (porphyrin a, Table III–1) strain 223 formed a small quantity of PP IX (porphyrin b, Table III–1).

The isolated porphyrins of strain 1, 4, 144, 166, 269 and 293 proved to be indentical with CP III.

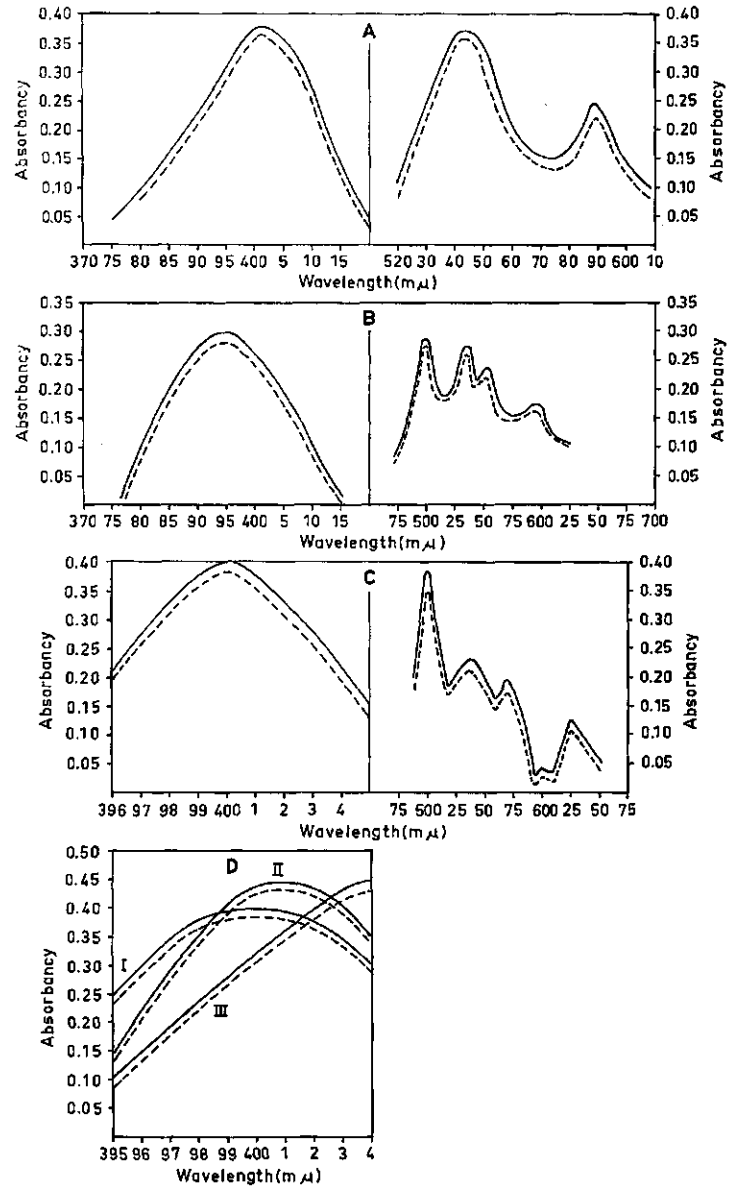


Fig. III-1. A comparison of some characteristics of authentic CP III (———) and the product isolated from the culture filtrate of *Arthrobacter*, strain 223 (- - - -). Spectra in acid (A) and alkaline (B) solution; spectra of the methyl ester (C); effect of HCl concentration on maximum absorbancy (D); D I: 0.05 N HCl, D II: 0.15 N HCl, D III: 0.3 N HCl.

TABLE III-1. HCl-fraction and Rf-value of porphyrins isolated from cultures of a number of *Arthrobacter* strains. Solvent system 2,6 lutidine:water (1:1, v/v) in NH_3 atmosphere.

Porphyrin		HCl-fraction	Rf-value
Authentic CP III		0.3 N	0.51
PP IX		1.4	0.86
Methyl ester of authentic CP III		–	1.00
PP IX		–	1.00
Porphyrin in culture of strain	1	0.3 N	0.49
	4	0.3	0.50
	144	0.3	0.49
	166	0.3	0.53
	269	0.3	0.51
	223 (a)	0.3	0.50
	223 (b)	1.4	0.84
	293	0.3	0.51
Methyl ester of porphyrin strain	223 (a)	–	1.00
	223 (b)	–	1.00

2.2. *The reproducibility of the accumulation of CP III by using complex media*

By using complex media for studying the accumulation of CP III, the results obtained were hard to reproduce (Table III-2). The composition of the nutrient solution used in experiments 1 and 2 was the same while in both cases two-

TABLE III-2. The accumulation of CP III in cultures of different *Arthrobacter* strains (isolated from soil) in medium A containing 0.25% (w/v) Casamino acids with and without iron and additional suphur-containing amino acids (configuration in small type). In both series of experiments 2-days-old cultures grown on slants containing yeast extract (0.7%, w/v) and glucose (1.0% w/v) were used for inoculation. From 0–30 hr, cultures agitated, sulphite oxidation value: 15 mMol O_2/liter hr; from 30–96 hr, cultures stationary, sulphite oxidation value: 7 mMol O_2/liter hr.

Strain	Experiment	CP III (mμMol/ml culture)		
		$-Fe^{+++}$	+ 0.01% (w/v) l-Cysteine	+ 0.02% (w/v) dl-Homocysteine
1	1	75	61	13
	2	112	20	141
4	1	14	67	112
	2	79	18	25
29	1	7	61	59
	2	68	0	0
144	1	67	0	0
	2	125	77	14
158	1	61	72	91
	2	92	11	135
159	1	81	88	11
	2	3	45	76
223	1	40	0	16
	2	0	112	62

days-old cultures were used for inoculation. These cultures were transferred every two days. The second experiment was started six days after the first. So the inoculum of the second experiment consisted of cells, resulting from three transfers of the culture used for the first experiment.

Furthermore Table III-2 shows that iron did not suppress CP III accumulation in a number of cultures and that S-containing amino acids may have a marked influence on the amount of CP III accumulated in cultures of these strains. As the results of the two experiments do not agree, it is impossible to draw any definite conclusion from this experiment.

The importance of sulphur-containing amino acids and iron in the porphyrin metabolism of *Arthrobacter* will be discussed in detail in the following chapters.

2.3. *The biotin requirement of Arthrobacter, strain 223*

To improve the reproducibility of the results, *Arthrobacter*, strain 223, was cultivated in nutrient solution A. After several transfers the culture was plated out on a medium with the same composition. Subsequently a single colony was transferred to an agar slant containing medium A. This culture was denoted as *Arthrobacter*, strain 223, and used for further study. The same procedure was followed for *Arthrobacter*, strain 144.

The response to biotin of *Arthrobacter*, strain 223, was shown in an experiment with agitated media supplied with different amounts of the vitamin (Table III-3). The maximum cell yield was obtained at about 5×10^{-4} μg/ml in the medium with 0.25% (w/v) $CaCO_3$ and at about 10^{-3} μg /ml biotin in the medium with 1.0% (w/v) $CaCO_3$. In the latter case, however, the yield was somewhat larger.

A biotin requirement of 10^{-3} μg/ml is in agreement with that of the strain of *Arthrobacter globiformis* studied by VELDKAMP et al. (1963), but it largely exceeds that found by CHAN and STEPHENSON (1961). Since nutrient solutions of different composition have been used by these authors, an accurate comparison of the biotin requirement of the different *Arthrobacter* strains is impossible.

TABLE III-3. The biotin requirement of *Arthrobacter*, strain 223, in medium A with two different amounts of $CaCO_3$. Suphite oxidation value: 11 mMol O_2/liter hr.

Biotin (μg/ml)	Cell yield (dry mg weight cells/ml culture)	
	0.25% (w/v) $CaCO_3$	1.0% (w/v) $CaCO_3$
–	0	0
10^{-5}	0.9	0.2
5×10^{-5}	2.4	0.9
10^{-4}	2.9	2.4
3×10^{-4}	3.2	–
5×10^{-4}	3.3	3.7
10^{-3}	3.2	4.0
10^{-2}	3.3	4.1

CHAN and STEPHENSON (1961) observed biotin transfer in these coryneform micro-organisms. Cells used as inoculum might contain such a large amount of biotin that normal growth takes place in biotin-free media. This phenomenon was not observed in *Arthrobacter*, strain 223.

Many soil arthrobacters require biotin as the only growth factor. Of the strains isolated by MULDER and ANTHEUNISSE (1963) approximately 50% grew in a mineral salts medium with glucose as the C-source when biotin was present.

2.4. *Biotin and the accumulation of CP III*

Cells of *Arthrobacter*, strain 223, accumulated CP III only when more biotin was present in the nutrient solution than was required for obtaining a maximum cell yield (Table III-4). Detectable amounts of CP III accumulated at 3×10^{-4} μg/ml biotin, but the maximum quantity was found at 10^{-3} μg/ml. More biotin did not further increase the CP III concentration. Similar results were obtained with other biotin-dependent *Arthrobacter* strains (e.g. 219 and 224).

LASCELLES (1956) found a similar effect of biotin on the accumulation of CP III by iron-deficient cells of *Rhodopseudomonas spheroides*. Thiamine and nicotinic acid, which were also required by this organism, affected CP III accumulation in the same way. In spite of these observations, LASCELLES (1962) assumed that biotin, and not thiamine or nicotinic acid, forms part of the enzyme ALA synthetase.

To test this assumption for *Arthrobacter*, an experiment was carried out with *Arthrobacter*, strain 257, which required both thiamine and biotin when growing in nutrient solution A. The effect of biotin was determined at an optimal thiamine level and the effect of thiamine at an optimal biotin content of the medium. Figure III-2 shows that CP III accumulated in the nutrient solution only when biotin as well as thiamine were present in excessive amounts. Biotin and thiamine have the same effect on CP III accumulation, and this does not support the idea that biotin participates in ALA synthetase. Biotin will be dealt with further in chapter VII.

TABLE III-4. The effect of biotin on cell yield and accumulation of CP III in cultures of *Arthrobacter*, strain 223. Medium A. Sulphite oxidation value: 18 mMol O_2/liter hr. Incubation for 96 hours.

Biotin (μg/ml)	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)
–	0.05	0
10^{-5}	0.8	0
5×10^{-5}	2.5	0
10^{-4}	3.0	0
3×10^{-4}	3.1	2
5×10^{-4}	3.2	13
10^{-3}	3.2	21
10^{-2}	3.2	22

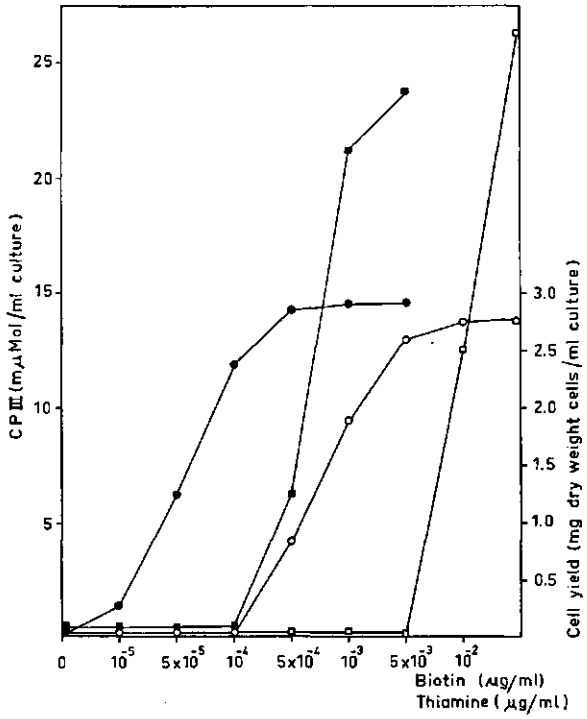


FIG. III-2. The effect of biotin (closed symbols) and thiamine (open symbols) on cell yield (circles) and CP III accumulation (rectangulars) in cultures of *Arthrobacter*, strain 257 (isolated from cheese). Medium A enriched with glycine and l-glutamic acid (each 50 μg/ml), and dl-homocysteine (25 μg/ml). Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 96 hours.

2.5. *The effect of iron and the uptake of this metal*

One of the most remarkable observations during the present investigation was the accumulation of CP III in nutrient solutions containing 1 μg/ml Fe^{+++}. This result confirmed the observation of NEILANDS and BURNHAM (1961) that *Arthrobacter* JG-9 produced copious amounts of CP III in spite of the large amounts of iron in the nutrient solution. After completion of this investigation a paper of MIDDLETON and GUNNER (1968) appeared dealing with the coproporphyrin III accumulation by *Arthrobacter globiformis*. These authors also observed CP III accumulation in media containing large amounts of iron.

The accumulation of CP III by *Arthrobacter* cells under these conditions might be the result of a reduced uptake of iron. To test this hypothesis, two experiments were carried out. In the first experiment the influence of increasing amounts of iron on the accumulation of CP III was studied. The results of this experiment (Table III-5) show that at least part of the added iron must have been taken up by the bacteria, as small amounts of this metal affected both cell yield and CP III accumulation. In order to test the possibility that large amounts

TABLE III-5. The effect of iron on cell yield and CP III accumulation in cultures of *Arthrobacter*, strain 223. Medium A. Sulphite oxidation value: 11 mMol O_2/liter hr.

Iron added (μg Fe^{+++}/ml)	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)
–	1.3	0
0.075	3.1	17
0.15	3.2	26
0.225	3.2	27
0.30	3.2	26
0.375	3.2	25
0.45	3.1	27
0.75	3.1	28
1.05	3.2	27
1.35	3.1	27
1.65	3.2	28
1.95	3.1	27
2.25	3.1	25
2.55	3.2	26

of iron, which did not reduce CP III accumulation, had not been taken up by the bacteria, a second experiment was carried out. *Arthrobacter* cultures supplied with 5, 10, 20, 50 and 300 μg Fe^{+++} per 200 ml nutrient solution, respectively, were analysed for iron at several times after starting the experiment. Figure III-3 shows that iron rapidly disappeared from the medium. When half the glucose added had been consumed practically all the iron had disappeared from the medium. The results obtained with *Arthrobacter*, strain 223, are in good agreement with those of PAPPENHEIMER (1947) in *Corynebacterium diphtheriae* as far as the removel of iron from the medium by the bacteria is concerned, however, the results with *Arthrobacter* did not agree with those of PAPPENHEIMER (1947) as regards the suppressing effect of large amounts of iron on CP III accumulation.

Although the results with *Arthrobacter*, strain 223, provide no conclusive evidence concerning the uptake of large amounts of iron, it was concluded that CP III accumulation in cultures of *Arthrobacter*, strain 223, may also be caused by other factors.

2.6. *The effect of other trace elements*

Omitting the mixture of trace elements from the nutrient solution, except iron, resulted in the elimination of CP III accumulation. Systematic investigations revealed (Table III-6a) that this was due to the zinc supply of the cells. In contrast to the cell yield, the formation of heme and the accumulation of CP III were markedly influenced by the absence of zinc. Apparently the zinc content of the medium was large enough for normal growth, but not for the accumulation of CP III. None of the other trace elements tested had an effect similar to that of zinc.

As may be seen from Table III-6b, increasing concentrations of zinc hardly influenced cell yield, but they reduced the heme content of the cells and con-

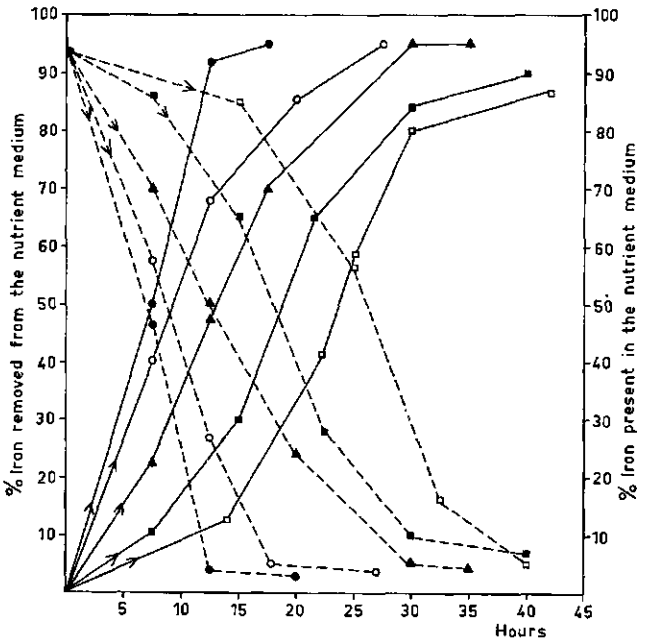


FIG. III-3. The removal of different amounts of iron from medium A by growing cells of *Arthrobacter*, strain 223. Sulphite oxidation value: 18 mMol O_2/liter hr. To 200 ml medium 5 (●), 10 (○), 20 (▲), 50 (■) and 300 (□) μg Fe^{+++} (as $FeCl_3$) were added. Samples were withdrawn at intervals to determine the iron (%) removed from the medium (———) and iron (%) left in the medium (- - - -).

TABLE III-6. a. The effect of trace elements, except iron, on cell yield and formation of porphyrins in cultures of *Arthrobacter*, strain 223. Medium A. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation time 96 hours. b. The effect of zinc. For incubation conditions see a.

TABLE III-6a

Trace element omitted from medium A	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
–	3.0	27	0.3
Zn, Cu, Mn, Mo, B, Co	2,8	0	1.8
Zn	2.9	0	2.0
Cu	3.1	24	0.2
Mn	3.1	26	0.4
Mo	3.2	29	0.3
B	3.0	27	0.3
Co	3.1	26	0.4

comitantly enhanced the accumulation of CP III. This enhancing effect of zinc on CP III accumulation was also observed in *Arthrobacter globiformis* by MIDDLETON and GUNNER (1968).

TABLE III-6b.

Zinc added (10^{-2} μg/ml)	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
–	3.0	0	2.4
0.05	3.2	3	1.1
0.15	3.2	9	0.3
0.25	3.1	15	0.4
0.35	3.2	22	0.3
0.45	3.2	26	0.4
0.6	3.1	25	0.2
1.2	3.1	27	0.3
2.4	3.3	26	0.4

WEGENER and ROMANO (1964) found that especially zinc stimulated the synthesis of the enzyme isocitratase in *Rhizopus nigricans*, growing with glucose as the C-source. The effect of zinc on the formation of this enzyme was interpreted as a stimulation of the synthesis of RNA, which is responsible for the formation of the enzyme.

Another effect of zinc is its specific stimulation of the activity of the enzyme phospholipase C (HOUTSMULLER, 1966). Chapter V deals with the question to what extent the favourable action of zinc on the accumulation of CP III in *Arthrobacter*, strain 223, might be attributed to an increased phospholipase C activity.

2.7. *The effect of some macro-elements on the accumulation of CP III*

The influence of some macro-elements was studied in relation to the failing effect of large amounts of iron on the elimination of CP III accumulation.

Table III-7a gives the results of an experiment with increasing amounts of sulfate. Maximum cell yield was obtained at 7 μg sulfate per ml. The accumulation of CP III started at 10 μg/ml, reaching its maximum at about 18 μg/ml sulfate. The fact that maximum cell yield was obtained at 7 μg/ml sulfate probably means that the absence of CP III accumulation at this sulfate concentration was not due to a low ALA synthetase activity owing to a deficiency of cysteine.

With increasing amounts of phosphate and ammonium chloride different results were obtained. Low concentrations of both compounds, as with sulfate, resulted in a reduced cell yield without any CP III accumulation. However, excessive amounts of both nutrients, which did not inhibit bacterial growth also eliminated the accumulation of CP III.

The results obtained with increasing amounts of phosphate and ammonium chloride closely resemble those found by LASCELLES (1956) in *Rhodopseudomonas spheroides*. Absence of CP III accumulation at phosphate and ammonium concentrations already sufficient for optimal growth may be due to a relative shortage of the precursors of CPG III (glycine and succinic acid). However,

TABLE III-7. The effect of sulfate (a), ammonium (b) and phosphate (c) on cell yield and accumulation of CP III in cultures of *Arthrobacter*, strain 223. The nutrients of medium A had been purified only in the experiment with sulfate. Sulphite oxidation value: 11 mMol O_2/liter hr.

TABLE III-7a

Sulphate ($\mu g\ SO_4^{--}$/ml)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμMol/ml culture)
–	0.4	0
3.6	1.6	0
7.2	3.4	0
10.9	3.2	2
14.5	3.2	12
18.1	3.3	26
36.2	3.4	25
72.4	3.2	26
108.6	3.2	27
144.8	3.2	26
289.6	3.3	26
579.2	3.2	25
1,115.0	3.2	27

TABLE III-7 b

Ammonium salt ($\mu g\ NH_4Cl$/ml)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμMol/ml culture)
–	0.1	0
250	1.9	0
500	3.2	0
1,000	3.4	9
1,500	3.2	21
2,000	3.2	25
2,500	3.2	26
4,000	3.1	25
5,000	3.3	10
6,000	3.2	5
7,000	3.2	0

other explanations may exist as well because both nutrients influence a large number of reactions in the cell.

2.8. *The final pH of the nutrient solution and the formation of porphyrins*

In order to neutralize the medium during the growth of the bacteria, $CaCO_3$ was added. The occurring drop in pH had to be attributed to the consumption of ammonium ions only; no amino acids or organic acids were produced.

A higher cell yield, with heme as the dominant porphyrin and no CP III accumulation, was found at a higher pH (Table III-8). Apparently the conver-

TABLE III-7c.

Phosphate ($\mu g\ H_2PO_4^-$/ml)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμMol/ml culture)
-	0.2	0
12.5	1.0	0
25.0	2.1	0
50.0	3.2	6
100.0	3.2	13
200.0	3.3	27
400.0	3.1	21
500.0	3.1	19
1,000.0	3.3	4

TABLE III-8. Final pH, cell yield and synthesis of porphyrins in cultures of *Arthrobacter*, strain 223. Medium A. Sulphite oxidation value: 18 mMol O_2/liter hr.

$CaCO_3$ (%, w/v)	Final pH	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
1.0	7.0	4.2	0	1.2
0.8	6.8	4.1	3	1.0
0.6	6.7	3.9	7	0.7
0.4	6.4	3.5	13	0.4
0.2	5.9	3.2	29	0.3

sion of CPG III to PP IX was not inhibited under these conditions. A similar pH effect has been observed in *Arthrobacter* JG-9 (NEILANDS and BURNHAM, 1961).

So far no explanation is available for the effect of the pH on CP III accumulation. Although cell yield was favourably influenced by a higher pH, growth rate was considerably reduced. The maximum yield in a medium containing 0.25% (w/v) $CaCO_3$ was reached about 40 hours after inoculation in contrast to a medium with 1.0% (w/v) $CaCO_3$ where it was not obtained until 96 hours incubation. The difference in growth rate of the bacteria may also have affected the formation of porphyrins.

2.9. *The effect of the aeration rate on the formation of porphyrins*

Before studying the effect of the aeration rate on the formation of CPG III, an experiment was carried out to determine the relationship between the sulphite oxidation value on the one hand, and the way of aeration and the quantity of nutrient solution per 500 ml flask on the other hand (Fig. III-4).

In an agitated medium more oxygen was dissolved than in a stationary medium, the sulphite oxidation values being 11 and 4 mMol O_2/liter hr, respectively. Applying the same intensity of aeration, the quantity of dissolved oxygen

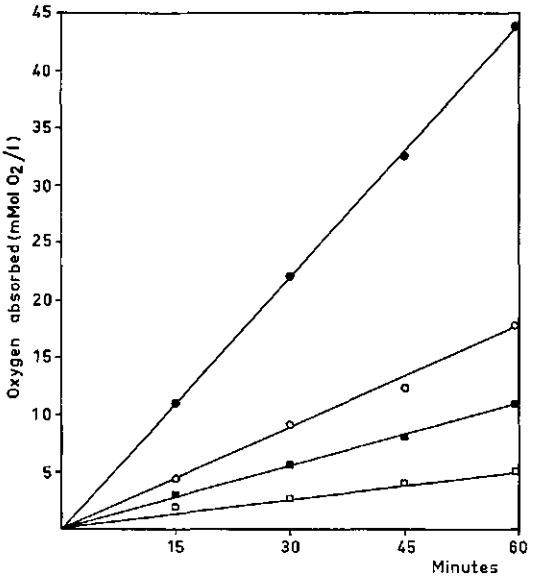


FIG. III-4. The influence of the aeration and the amount of nutrient solution on the quantity of oxygen absorbed. □: 200 ml medium per 500 ml flask (stationary); ■: 200 ml medium per 500 ml flask (moderate agitation); ○: 200 ml medium per 500 ml flask (vigorous agitation); ●: 100 ml medium per 500 ml flask (vigorous agitation).

was larger when the quantity of the medium was smaller. Sulphite oxidation values amounted to 44 and 18 mMol O_2/liter hr, being obtained with 100 and 200 ml of nutrient solution, respectively.

The effect of the aeration rate on growth and CP III accumulation was studied by inoculating 2 ml of the usual subculture (see Chapter II) of *Arthrobacter*, strain 223, into nutrient solutions submitted to the aeration rates 4, 11, and 18, and 1 ml into a medium submitted to 44 mMol O_2/liter hr. Throughout the entire incubation period the bacteria were grown at these aeration rates. Table III-9 shows that the degree of aeration influenced both cell yield and amount of

TABLE III-9. The effect of the aeration rate on cell yield and formation of porphyrins in cultures of *Arthrobacter*, strain 223. Medium A. Incubation time 96 hours.

Sulphite oxidation value (mMol O_2/ liter hr)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμMol/ ml culture)	Heme (mμMol/ ml culture)
4	1.1	0	0.1
11	3.2	29	0.4
18	3.1	20	0.5
44	3.2	2	0.5

CP III accumulated. Only 30% of the maximum cell yield was obtained at 4 mMol O_2/liter hr, no CP III being accumulated. Practically no differences in cell yield were obtained at 11, 18 and 44 mMol 0_2/liter hr, respectively. Large amounts of CP III accumulated at 11 and 18 mMol O_2/liter hr, but at 44 mMol O_2/liter hr practically no CP III was found. The quantity of heme formed at any aeration rate never exceeded 0.6 mμMol per ml, being many times smaller than the maximum quantity that could be synthesized (Chapter IV).

The results of this experiment confirm those obtained with *Arthrobacter globiformis* by MORRIS (1960). It is impossible to conclude from these data whether a low aeration inhibited the CP III accumulation directly or by way of reduced growth. More detailed information about this question was obtained in the following experiments.

2.9.1. Low aeration rate

In order to find out whether a low aeration rate has a direct effect on CP III accumulation, cultures forming CPG III at the aeration of 18 mMol O_2/liter hr were brought to 4 and 11 mMol O_2/liter hr, respectively. The incubation was continued and samples were withdrawn at intervals for determination of cell yield and CP III. Figure III-5 shows that the growth rate was clearly reduced but the final yield only slightly affected at 4 mMol O_2/liter hr. At this aeration rate

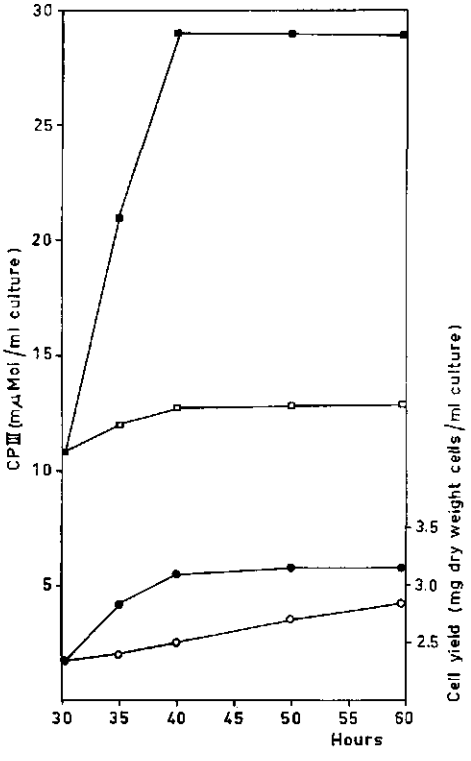


FIG. III-5. The effect of low and moderate aeration rates on cell yield (circles) and CP III accumulation (rectangulars) in cultures of *Arthrobacter*, strain 223. Medium A. The bacteria were grown at an aeration rate of 18 mMol O_2/liter hr for 30 hours before being transferred to 4 (open symbols) and 11 mMol O_2/liter hr (closed symbols).

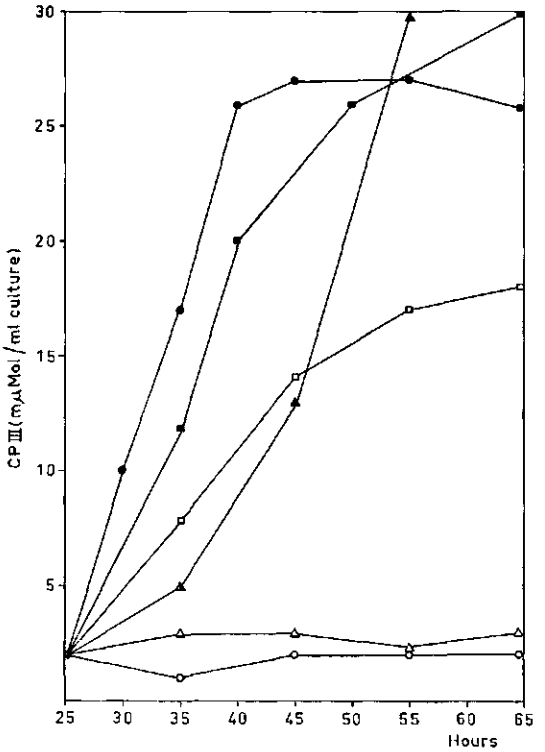


FIG. III-6. The effect of some precursors of CPG III on the accumulation of CP III in cultures of *Arthrobacter*, strain 223, submitted to an aeration rate of 4 mMol O_2/liter hr. Medium A. The bacteria were grown at an aeration rate of 18 mMol O_2/liter hr before being transferred to 4 mMol O_2/liter hr. The precursors were added at this time. ○: 4 mMol O_2/liter hr, no addition; △: 4 mMol O_2/liter hr, glycine (50 μg/ml) added; □: 4 mMol O_2/liter hr, succinic acid (50 μg/ml) added; ■: 4 mMol O_2/liter hr, l-glutamic acid (50 μg/ml) added; ▲: 4 mMol O_2/liter hr, ALA (200 μg/ml) added; ●: 11 mMol O_2/liter hr, no addition.

CP III accumulation was practically stopped. Instead of CP III, no accumulation of ALA, PBG or UP III was detected at this aeration rate. This might indicate that a low aeration rate inhibited the synthesis of ALA.

This result seems to be at variance with observations made in Athiorhodaceae (LASCELLES, 1960; DREWS, 1965). The synthesis of bacteriochlorophyll by these organisms, which requires a rapid formation of ALA, was found to occur only at a low aeration rate.

To find out whether the reduced formation of ALA was responsible for the absence of CP III accumulation in the above-mentioned experiment, glutamic acid, succinic acid, glycine and ALA were added to cultures submitted to a low aeration rate at the time the porphyrin accumulation had started.

As appears from Figure III-6, ALA, glutamic acid and succinic acid considerably increased the formation of CPG III, whereas glycine had no effect. At the end of the experiment, succinic acid appeared to be the only compound added which had not completely been taken up by the cells. The favourable effect of glutamic acid and succinic acid on CP III accumulation might indicate that the low aeration rate inhibited the synthesis of ALA and consequently that of CPG III as a result of a shortage of substrate (C_4). This observation is in good agreement with that of KREBS and LOWENSTEIN (1957) who observed a reduced formation of tricarboxylic acid cycle enzymes in different types of aerobic cells

exposed to a low degree of aeration. The effect of succinic acid on the accumulation of CP III was less pronounced than that of glutamic acid. This difference was probably due to the slower uptake of the former compound.

The results obtained were in good agreement with earlier observations concerning CP III accumulation in arthrobacters growing in a nutrient solution containing Casamino acids. When in these cultures the porphyrin accumulation had started, it was hardly affected by exposing the cultures to a low aeration rate. The fact that a low degree of aeration did not eliminate the accumulation of CP III in cultures of *Arthrobacter*, strain 223, supplied with glutamic acid, succinic acid or ALA, clearly demonstrates that the activity of the enzymes catalysing the conversion of glycine and succinyl-CoA to CPG III was not reduced by lowering the aeration rate. This conclusion is in good agreement with the observations of LASCELLES (1956) in *Rhodopseudomonas spheroides* and of DREWS (1965) in *Rhodospirillum rubrum* that a low oxygen tension does not exert an unfavourable action on the activity or synthesis of the enzymes ALA synthetase and ALA dehydratase.

2.9.2. High aeration rate

From Table III-9 it can be seen that cells of *Arthrobacter*, strain 223, growing at an aeration rate of 44 mMol O_2/liter hr accumulated practically no CP III. Although a shortage of succinic acid hardly seemed to be involved, a series of experiments was carried out to test this hypothesis. Cultures of *Arthrobacter*, strain 223, growing in medium A at an aeration rate of 18 mMol O_2/liter hr were submitted to 44 mMol O_2/liter hr and supplied with ALA, glutamic acid, glycine and succinic acid, respectively. A control culture without these substrates was submitted to 11 mMol O_2/liter hr.

As appears from Figure III-7, the accumulation of CP III in the culture exposed to 11 mMol O_2/liter hr proceeded rapidly. Addition of ALA to the culture exposed to 44 mMol O_2/liter hr also resulted in the formation of CPG III. Glycine, succinic acid and glutamic acid had no effect in spite of the complete uptake of these compounds by the cells. This result indicates that the enzyme ALA synthetase either does not function at a high degree of aeration or is not synthesized in sufficient amounts.

Since more CPG III was formed from ALA at a low aeration level (Fig. III-6) than at a high aeration level (Fig. III-7), it seems to be possible to assume that oxygen has some unfavourable effect either on the synthesis or on the activity of one or more enzymes, catalysing the conversion of ALA to CPG III.

In a subsequent experiment cells of *Arthrobacter*, strain 223, growing in medium A at 11 mMol O_2/liter hr were submitted to a high aeration rate different periods of time after CP III accumulation had started. Figure III-8 shows that the increased aeration rate immediately stopped the increase of the concentration of CP III. If this aeration rate would have inhibited merely the synthesis of the enzyme ALA synthetase, the increase of the porphyrin concentration would have been reduced gradually, as the enzyme already present would gradually have been diluted out. As this was not the case, the conclusion may be

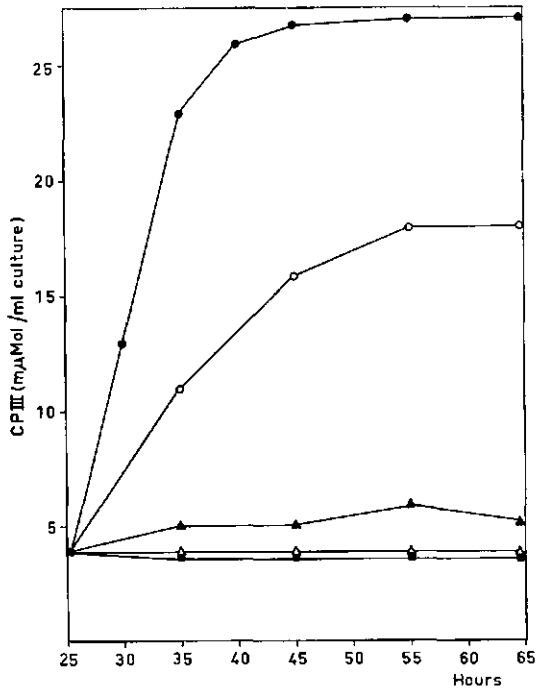


FIG. III-7. The effect of some precursors of CPG III on the accumulation of CP III in cultures of *Arthrobacter*, strain 223, submitted to an aeration rate of 44 mMol O_2/liter hr. Medium A. The bacteria were grown at an aeration rate of 18 mMol O_2/liter hr before being transferred to 44 mMol O_2/liter hr. The precursors were added at this time. ■: 44 mMol O_2/liter hr, no addition; △: 44 mMol O_2/liter hr, glycine (50 μg/ml) added; ▲: 44 mMol O_2/liter hr, succinic acid (50 μg/ml) added; ○: 44 mMol O_2/liter hr, ALA (200 μg/ml) added; ●: 11 mMol O_2/liter hr, no addition (control).

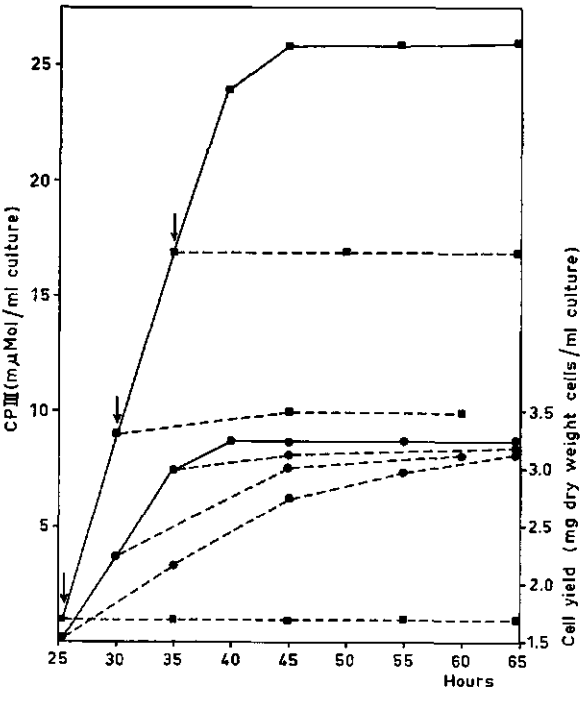


FIG. III-8. The effect of a high aeration rate on cell yield (●) and CP III accumulation (■) in cultures of *Arthrobacter*, strain 223. Medium A. The bacteria were grown at an aeration rate of 11 mMol O_2/liter hr (———). At different times (arrows) cultures were submitted to 44 mMol O_2/liter hr (- - - -).

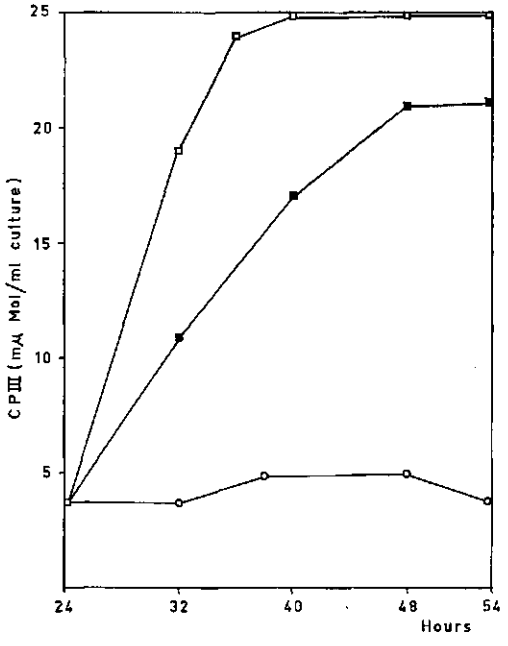


FIG. III-9. The rate of accumulation of CP III in cultures of *Arthrobacter*, strain 223 at different aeration rates. The bacteria were grown at an aeration rate of 11 mMol O_2/liter hr (□) for 24 hours before being transferred to 18 (■) and 4 (○) mMol O_2/liter hr. Medium A.

that the high degree of aeration has inhibited the activity of ALA synthetase.

To compare the effect of moderately high (18), moderate (11), and low (4 mMol O_2/liter hr) aeration rates on CP III accumulation of *Arthrobacter*, strain 223, was cultivated in medium A at 11 mMol O_2/liter hr for 24 hours. Subsequently the culture was submitted to an aeration rate of 18 mMol O_2/liter hr and the incubation was continued. At fixed times samples were taken to estimate CP III accumulated. From the data recorded in Figure III-9 it will be seen that the rate of the formation of CPG III at 18 mMol O_2/liter hr was considerably reduced as compared with that at 11 mMol O_2/liter hr. The ultimate amount of CP III, however, differed only slightly. From the results of this experiment it can not be concluded whether the decreased rate of CPG III formation under moderately high aeration was due to repression of the synthesis or to inhibition of the activity of the enzymes catalysing the formation of CPG III.

3. SUMMARY AND CONCLUSIONS

Different *Arthrobacter* strains growing in a nutrient solution with Casamino acids accumulated considerable amounts of coproporphyrin III (CP III). The reproducibility of the results obtained with complex media was inferior to that in a simple mineral medium with glucose as the C-source (medium A). Therefore the latter medium was used to study the effect of various factors on CP III accumulation. *Arthrobacter*, strain 223, was used as the test organism in these studies.

Arthrobacter, strain 223, grew well in medium A when the latter was supplied with biotin. Accumulation of CP III occurred at biotin levels exceeding those required for optimal growth. Thiamine reacted similarly when supplied in increasing concentrations to biotin-containing medium A inoculated with an *Arthrobacter* strain requiring biotin as well as thiamine.

Small amounts of iron were required for growth and enhanced CP III accumulation in medium A. Large amounts of iron in contrast to many literature recordings were found to be unable to prevent the accumulation of CP III. This discrepancy apparently did not depend on the inadequate absorption of iron by cells of *Arthrobacter*, strain 223.

Of a further number of trace elements tested (Zn, Cu, Mn, Co, Mo, B) zinc in excessive amounts inhibited heme formation and concomitantly stimulated CP III accumulation.

Ammonium chloride, phosphate and sulfate in amounts approximately three times higher than the minimum amounts required for optimal growth brought about accumulation of CP III. Excessive amounts of the former two nutrients suppressed CP III accumulation but this was not the case with sulfate.

No accumulation of CP III by *Arthrobacter*, strain 223, took place in medium A supplied with 1.0% $CaCO_3$ (pH 7). With decreasing amounts of $CaCO_3$ (correlated with decreasing pH values) CP III production increased.

The aeration rate of the culture had a pronounced effect on CP III accumulation by *Arthrobacter*, strain 223. Porphyrin formation reached its maximum at an aeration rate of 11 mMol O_2/liter hr. Doubling this aeration rate slightly decreased the amount of CP III but a four times higher rate (44 mMol O_2/liter hr) almost entirely eliminated CP III accumulation. The same was true of a low aeration rate (4 mMol O_2/liter hr).

The absence of CP III accumulation at a low degree of aeration was due to a reduced ALA formation. The latter depended on a diminished formation of the C_4-building block. Cells of *Arthrobacter*, strain 223, exposed to a low degree of aeration performed a ready CP III accumulation when ALA, succinic acid and glutamic acid were supplied. It was therefore concluded that the enzymes catalysing the conversion of glycine and succinyl-CoA to CPG III were not unfavourably affected by a low aeration rate.

The failure of CP III accumulation at a high aeration rate (44 mMol O_2/liter hr) was partly restored by supplying ALA but not by succinic acid or glutamic acid showing that the high aeration rate inhibited the synthesis or the activity of ALA synthetase. That the activity of ALA synthetase was involved was concluded from the fact that exposure of CP III-accumulating cells to a high aeration rate immediately stopped the accumulation of CP III. The enzymes catalysing the conversion of ALA to CPG III were also unfavourably affected by a high aeration rate, be it to a smaller extent than ALA synthetase.

The rate of the accumulation of CP III at a moderately high aeration rate (18 mMol O_2/liter hr) was considerably decreased compared with that at a moderate aeration rate (11 mMol O_2/liter hr); the ultimate amount of CP III accumulated was only slightly reduced by the higher aeration rate.

CHAPTER IV

THE INFLUENCE OF SULPHUR-CONTAINING AMINO ACIDS ON THE FORMATION OF PORPHYRINS IN CULTURES OF *ARTHROBACTER*, STRAIN 223

1. Introduction

As demonstrated in the previous chapter, sulfate, iron and zinc, in amounts larger than those required for maximum cell yield, are essential for the accumulation of CP III in *Arthrobacter*, strain 223. Theoretically, the action of these compounds might be due to the formation of a particular metabolite playing an important role in the synthesis of heme.

The function of sulfate in medium A is to supply the bacteria with sulphur for the synthesis of cysteine, methionine and other S-containing metabolites which occur in every bacterium. This fact and the observations made during preliminary experiments that methionine had a marked influence on the accumulation of CP III called for a study of the effect of S-containing amino acids on the formation of porphyrins.

2. Results and discussion

2.1. *The quantity of sulfate and the formation of porphyrins*

It is reasonable to assume that sulfate exerted its action on the synthesis of heme in *Arthrobacter*, strain 223, as a result of its conversion to S-containing amino acids (see 2.3). In order to study the relation between the quantity of sulfate in the nutrient medium, porphyrin formation and the activity of the enzymes ALA synthetase and ALA dehydratase which requires cysteine, *Arthrobacter*, strain 223, was grown in medium A supplied with increasing amounts of sulfate. The bacterium was subcultured in a medium containing 0.01% (w/v) $MgSO_4.7H_2O$. After incubation for two days, 1 ml of the subculture was transferred to portions (200 ml) of purified medium A supplied with different amounts of magnesium sulfate (the magnesium supply of these cultures was brought to the same level by adding different amounts of $MgCl_2$).

Figure IV-1 shows that the maximum cell yield and the maximum amount of heme were attained with about 10 μg/ml sulfate. Higher concentrations did not affect the cell yield but reduced the quantity of heme formed. This reduction was accompanied with the accumulation of CP III. From the results of this experiment it may be concluded that in cells grown with 10 as well as in those grown with 20 μg/ml sulfate or more, the enzymes catalysing the conversion of glycine and succinyl-CoA to CPG III are active because either heme or CPG III was found as the dominant porphyrin. It is clear that active enzymes are requisite for

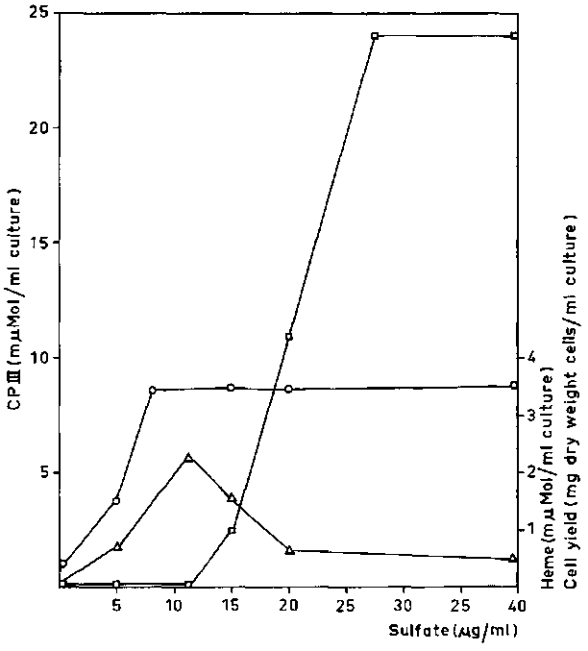


FIG. IV-1. The effect of different concentrations of magnesium sulfate on cell yield (○), formation of heme (△), and accumulation of CP III (□) in cultures of *Arthrobacter*, strain 223. Medium A. Sulphite oxidation value: 11 mMol O_2/liter hr.

the formation of these porphyrins. Another indication for the activity of the enzymes catalysing the conversion of ALA to CPG III in cells grown at 10 μg/ml sulfate was obtained by adding ALA (200 μg/ml). This resulted in the appearance of CP III in the culture (16 mμMol/ml). These results indicate that the beneficial effect of relatively high concentrations of sulfate on the accumulation of CP III was not due to an increased activity of ALA synthetase or ALA dehydratase resulting from an enhanced cysteine formation. So far it is unknown whether heme regulates the synthesis and the activity of ALA synthetase in non-photosynthetic micro-organisms. Experiments dealing with this phenomenon will be discussed in chapter VI.

2.2. *Sulphur-containing amino acids and the synthesis of porphyrins in Arthrobacter, strain 223*

Investigations on the synthesis of methionine in micro-organisms have resulted in the scheme as given in Figure IV-2. The enzymes catalysing the conversion of cysteine (formed from serine) to methionine have been demonstrated in *Escherichia coli* and in *Salmonella typhimurium* (ROWBURY and WOODS, 1966). In bacteria threonine and lysine, which have not been included in Figure IV-2, are also formed from aspartic β-semialdehyde. So, methionine, threonine and lysine have a common precursor (UMBARGER and DAVIS, 1962; MEISTER, 1965).

When *Arthrobacter*, strain 223, was cultivated in a purified nearly sulfate-free medium A containing l-methionine (40 μg/ml) as the only S-source, a

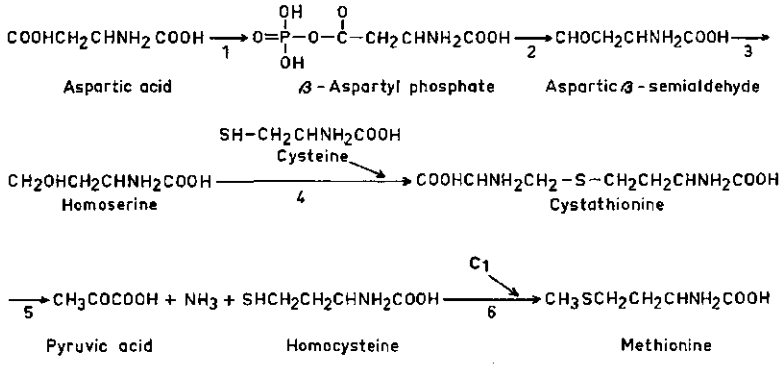


FIG. IV-2. The biosynthesis of methionine in micro-organisms (UMBARGER and DAVIS, 1962). C_1: one carbon unit.

strongly reduced cell yield was obtained. This was due to the fact that cysteine and other S-containing compounds were not derived from methionine. Homocysteine reacted similarly to methionine but with cysteine maximum cell yield was obtained. The fact that cysteine was used as the only S-source means that methionine and other cellular S-compounds were derived from it.

When unpurified chemicals were used and no sulfate was added, *Arthrobacter*, strain 223, grew well in medium A. The maximum cell yield was almost attained indicating that this medium contained sufficient amounts of sulfate as contamination.

To study the effect of sulphur-containing amino acids on the formation of porphyrins by *Arthrobacter*, strain 223, sulfate, cysteine, cystine, homocysteine and methionine were supplied to the above-mentioned unpurified medium A. The results of this experiment are shown in Table IV-1. No significant difference in cell yield was observed between the cultures with different S-sources. The addition of cysteine, cystine and homocysteine had the same effect on the accumulation of CP III as sulfate addition. Methionine, however, eliminated the CP III accumulation and considerably enhanced the formation of heme.

TABLE IV-1. Cell yield and synthesis of porphyrins in cultures of *Arthrobacter*, strain 223, growing in unpurified medium A supplied with different S-sources. Sulphite oxidation value: 11 mMol O_2/l/hr.

S-Source (100 μg/ml)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμMol/ ml culture)	Heme (mμMol/ ml culture)
–	2.7*	0	1.9
$(NH_4)_2SO_4$	3.2	25	0.4
l-Cysteine	3.3	27	0.5
l-Cystine	3.2	24	0.5
dl-Homocysteine	3.1	21	0.3
l-Methionine	3.3	0	5.7

* Sulfate present as contamination.

Methionine is required for the synthesis of bacteriochlorophyll because the methyl group of Mg protoporphyrin monomethyl ester is derived from methionine (see Fig. I-4B) as has been demonstrated by GIBSON et al. (1962). Since methionine is not directly involved in the synthesis of heme (iron protoporphyrin) it probably plays an important indirect role in the synthesis of this tetrapyrrole in *Arthrobacter*, strain 223, presumably in the conversion of CPG III to PP IX.

2.3. *Homocysteine and CP III accumulation*

For a better understanding of the effect of S-containing amino acids on the synthesis of heme by *Arthrobacter*, strain 223, it was thought to be essential to know which of the two amino acids cysteine or homocysteine is specifically involved in the accumulation of CP III.

The cultivation of the bacterium in an unpurified medium A supplied with increasing quantities of sulfate, cysteine, homocysteine and methionine, respectively, provided the first indication that the amino acid in question would be homocysteine. As appears from Table IV-2, large amounts of CP III accumulated when sulfate, cysteine or homocysteine were present in the nutrient medium, but not with methionine. Since sulfate, via cysteine can be converted to homocysteine and methionine, but the reverse reactions were not detectable, homocysteine rather than cysteine seems to be the compound responsible for the CP III accumulation in cultures growing in media supplied with sulfate, cysteine or homocysteine.

To obtain more evidence for the stimulation of CP III accumulation by homocysteine, use was made of mechanisms controlling the synthesis of homocysteine in bacteria. The formation of homocysteine depends on the amount of β-aspartyl phosphate (formed from aspartic acid; enzyme: aspartokinase; Fig. IV-2, reaction 1). In *Escherichia coli* at least two aspartokinases have been demonstrated (UMBARGER and DAVIS, 1962). Of one enzyme the synthesis and activity are inhibited by lysine while of the other only the activity is inhibited

TABLE IV-2. The accumulation of CP III in cultures of *Arthrobacter*, strain 223, growing in medium A (contaminated with sulfate) supplied with increasing amounts of sulfate, cysteine, homocysteine and methionine, respectively. Sulphite oxidation value: 11 mMol O_2/liter hr.

Amount of S-source added (μg/ml)	CP III (mμMol/ml culture)			
	$(NH_4)_2SO_4$	l-Cysteine	dl-Homocysteine	l-Methionine
–	0	0	0	0
10	1	2	5	0
20	15	8	11	3
30	26	26	17	5
40	25	24	1	0
50	–*	23	8	0
100	29	25	23	0

* Not determined.

TABLE IV-3. The effect of different amounts of threonine on the cell yield and the accumulation of CP III in cultures of *Arthrobacter*, strain 223, growing in medium A with sulfate, sulfate + cysteine, and sulfate + homocysteine as S-source. Sulphite oxidation value: 11 mMol O_2/ liter hr.

S-Source (μg/ml)	Amino acids added (μg/ml)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμMol/ ml culture)
$(NH_4)_2SO_4$(40)	–	3.1	27
	l-threonine (5)	3.0	21
	(10)	3.1	15
	(15)	3.0	4
	(20)	3.0	0
	l-threonine (20) + dl-homoserine (50)	3.1	25
	l-threonine (20) + dl-homocysteine (50)	3.1	24
$(NH_4)_2SO_4$(40) +l-cysteine (40)	–	3.2	29
	l-threonine (20)	3.1	0
	l-threonine (20) + dl-homoserine (50)	3.2	24
	l-threonine (20) + dl-homocysteine (50)	3.1	26
$(NH_4)_2SO_4$(40) + dl-homocysteine (100)	–	3.2	26
	l-threonine (20)	3.2	24
	(40)	3.1	29

by threonine. A similar effect of threonine has been found in *Rhodopseudomonas spheroides* and *Micrococcus glutamicus* (MEISTER, 1965).

The presence of threonine (50 μg/ml) in medium A prevented the growth of *Arthrobacter* strain 223 (incubation time 48 hours). A similar quantity of lysine had no effect. The growth-inhibiting action of threonine was eliminated by homoserine and homocysteine, thus providing evidence that threonine adversely affected the formation of homoserine and homocysteine, presumably as a result of inhibiting the conversion of aspartic acid to homoserine. ENSIGN and WOLFE (1964) obtained similar results with *Arthrobacter crystallopoietes.*

To provide more evidence that homocysteine is responsible for CP III accumulation in iron-sufficient cultures of *Arthrobacter*, strain 223, increasing quantities of threonine were added to nutrient solution A, containing different S-sources. One series of media contained sulfate only, the second sulfate + cysteine and the third sulfate + homocysteine. From the results of this experiment (Table IV-3) it will be seen that threonine (20 μg/ml) did not affect the cell yield significantly. However, in a sulfate and a sulfate + cysteine medium threonine ful-

TABLE IV-4. The accumulation of CP III in cultures of *Arthrobacter*, strain 223, growing in medium A with increasing amounts of $CaCO_3$ and different S-containing amino acids. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation time 96 hours.

S-Source (μg/ml)	$CaCO_3$ (%, w/v)	CP III (mμMol/ml culture)	Final pH
$(NH_4)_2SO_4$(100)	0.25	27	6.0
	0.50	11	6.4
	1.00	0	6.9
$(NH_4)_2SO_4$(100) + l-cysteine (50)	1.00	0	6.8
+ dl-homocysteine (100)	1.00	31	6.7
+ l-cysteine + dl-homocysteine	1.00	29	6.7
+ dl-homocysteine (100)	0.25	21	5.8
	0.50	24	6.1
	1.00	32	6.7

ly prevented CP III accumulation while the presence of homoserine or homocysteine again stimulated porphyrin formation. These results confirmed those reported in Table IV-2 and clearly indicate that the accumulation of CP III in iron-sufficient cultures of *Arthrobacter*, strain 223, would be due to homocysteine which is thought to prevent the formation of PP IX from CPG III.

Another indication for the unfavourable effect of homocysteine on the above-mentioned reaction was obtained by growing *Arthrobacter*, strain 223, in media with different concentrations of $CaCO_3$ and different S-containing amino acids. The data of this experiment show (Table IV-4) that porphyrin accumulation was prevented by the presence of 1 % (w/v) $CaCO_3$ in media with sulfate or sulfate + cysteine. The presence of homocysteine in these media restored the CP III accumulation. With sulfate + homocysteine as the S-source, $CaCO_3$ increased the quantity of CP III which accumulated in the culture.

The favourable effect of homocysteine on the accumulation of CP III was not observed in *Arthrobacter*, strain 223, only. Five other strains when growing in media containing 0.5% (w/v) $CaCO_3$ and sulfate (100 μg$(NH_4)_2SO_4$/ml), sulfate + cysteine, or sulfate + homocysteine accumulated the largest amounts of porphyrin in the last-mentioned medium (Table IV-5). The favourable effect of methionine on the accumulation of CP III found in strain 41 and 144 will be discussed in the next chapter.

The above experiments provide evidence that homocysteine adversely influences the formation of heme in *Arthrobacter*, strain 223, by unfavourably affecting the conversion of CPG III to PP IX, thus stimulating the accumulation of CPG III and consequently that of CP III.

2.4. *The specificity of the action of methionine*

To confirm the specificity of the beneficial action of methionine on heme

TABLE IV-5. The accumulation of CP III in cultures of 6 *Arthrobacter* strains isolated from soil, growing in medium A (containing sulfate) supplied with different S-containing amino acids. Sulphite oxidation value: 11 mMol O_2/liter hr. 0.5% (w/v) $CaCO_3$.

Strain	Experiment	CP III (mμMol/ml culture)			
		Medium A	Medium A + l-Cysteine (40μg/ml)	Medium A + dl-Homocysteine (80μg/ml)	Medium A + l-Methionine (50μg/ml)
29	1	0	0	14	0
	2	2	1	16	0
41	1	4	6	9	6
	2	0	1	15	9
144	1	0	0	23	8
	2	0	0	32	11
223	1	13	11	24	0
	2	9	14	23	0
224	1	12	10	21	0
	2	14	13	26	0
307	1	2	3	6	0
	2	4	1	9	2

formation, the effect of a large number of compounds on the accumulation of CP III was tested. Different quantities, depending upon the compound, were added to medium A containing sulfate (100 μg$(NH_4)_2SO_4$/ml). None of the amino acids tested had an effect similar to that of methionine (Table IV-6). Threonine was not included in this experiment because it had been tested earlier.

The results of other experiments showed that the B vitamins thiamine, nicotinic acid, pantothenic acid, riboflavin, pyridoxal phosphate, folic acid and

TABLE IV-6. The effect of a number of amino acids on the formation of porphyrins by *Arthrobacter*, strain 223, growing in medium A, containing large amounts of sulfate. Sulphite oxidation value: 11 mMol O_2/liter hr.

Amino acid added (50 μg/ml)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
—	26	0.6
l-Methionine	0	5.3
l-Valine	24	0.5
l-Leucine	26	0.3
l-Phenylalanine	21	0.6
l-Tyrosine	24	0.4
l-Alanine	29	0.3
l-Glutamic acid	31	0.5
l-Aspartic acid	29	0.4
l-Lysine	21	0.4

vitamin B_{12} used in the concentrations 5×10^{-5} to 10^{-1} μg/ml were unable to eliminate the accumulation of CP III. The presence of 10 μg/ml adenine, guanine, xanthine, hypoxanthine, orotic acid, thymidine, cytosine or uracil likewise did not reduce the accumulation of CP III.

These results suggest that methionine has a specific indirect action in the formation of heme by *Arthrobacter*, strain 223, by stimulating the conversion of CPG III to PP IX. A similar effect of methionine on this conversion has been observed in *Rhodopseudomonas spheroides* (GIBSON et al., 1962). In *Saccharomyces anamensis* riboflavin enhanced this conversion (STICH and EISGRUBER, 1951) and in *Tetrahymena vorax* pantothenate (LASCELLES, 1962).

2.5. *The effect of methionine*

The data of Table IV-6 show that the presence of 50μg/ml methionine in a complete medium A (containing a high amount of sulfate) prevented the accumulation of CP III. One of the reasons for this effect might be the inhibition of the synthesis of homocysteine, which adversely affected the conversion of CPG III to PP IX, by the existence of a feedback control mechanism. The synthesis of methionine (Fig. IV-2) has been extensively studied by ROWBURY (1964) in *Salmonella typhimurium* and in *Escherichia coli* by ROWBURY and WOODS (1966). They showed that methionine inhibited the synthesis and the activity of the enzymes catalysing the formation of cystathionine (Fig. IV-2, reaction 4) and homocysteine (reaction 5). It was supposed that in *Arthrobacter*, strain 223, the formation of methionine and its regulation proceed similarly.

To test the above-mentioned possibility, methionine was added to nutrient solutions with sulfate and sulfate + cysteine, respectively. The amount of methionine applied (50 μg/ml) caused maximum inhibition of homocysteine formation in *S. typhimurium* and *E. coli*. From the results obtained in the present study with *Arthrobacter*, strain 223, it appears that methionine prevented CP III accumulation in the sulfate medium, but not in the medium with sulfate + cysteine (Table IV-7).

The different response to methionine in both media could have been due to a reduced uptake of methionine in the medium with sulfate + cysteine. To test this hypothesis the following experiment was carried out. *Arthrobacter*,

TABLE IV-7. The effect of methionine on the formation of porphyrins by *Arthrobacter*, strain 223. Medium A. Sulphite oxidation value: 11 mMol O_2/liter hr.

S-Source (μg/ml)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
$(NH_4)_2SO_4$(100)	26	0.4
+ l-methionine (50)	0	5.2
$(NH_4)_2SO_4$(100)	28	0.5
$(NH_4)_2SO_4$(100) + l-cysteine (50)	31	0.4
$(NH_4)_2SO_4$(100) + l-cysteine (50) + l-methionine (50)	25	0.5

TABLE IV-8. Sulphur as sulfate, cysteine or methionine left in the culture medium A after the cultivation of *Arthrobacter*, strain 223, and subsequently used as S-source in the culture medium by a second culture of the same organism. Sulphite oxidation value: 11 mMol O_2/liter hr.

Experiment	S-Source of orginal medium (μg/ml)	Additional S-compound added to filtrate of the original culture (μg/ml)	Cell yield (mg dry weight cells/ml) of 2nd culture	CP III (mμMol/ml) of 2nd culture
A_1	$(NH_4)_2SO_4$(100) + l-methionine(80)	-	3.1	25
A_2		l-methionine(50)	3.0	0
B_1	$(NH_4)_2SO_4$(6) + l-cysteine(50) + l-methionine(80)	-	0.48	0
B_2		$(NH_4)_2SO_4$(100)	3.3	26
B_3		$(NH_4)_2SO_4$(100) + l-methionine(50)	3.2	0
B_4		l-cysteine(50) + l-methionine(50)	3.2	29

strain 223, was cultivated in purified nutrient solutions A containing sulfate (100 μg/ml) + methionine (80 μg/ml) and sulfate (6 μg/ml) + cysteine (50 μg/ml) + methionine (80 μg/ml), respectively. After incubation for 48 hours, $CaCO_3$ and bacteria were removed by centrifugation, and mineral salts except sulfate in a concentration as occurring in medium A were added to the culture filtrates. The S-containing compounds added to these filtrates are given in Table IV-8. After sterilization this mixture, separately sterilized glucose (1%, w/v), biotin (10^{-2} μg/ml) and iron (1 μg/ml) were added. Subsequently *Arthrobacter*, strain 223, was inoculated and after an incubation period of 60 hours cell yield and CP III content were determined.

As appears from Table IV-8 sufficient sulfate was left in the original medium to obtain maximum cell yield and CP III content in the second culture (A1). Methionine was largely taken up from the original culture because much CP III accumulated in the second culture, which was completely prevented by methionine (A2). Cysteine and methionine had almost entirely been taken up by the original culture (B1 and B2). The fact that addition of sulfate to the culture filtrate of B1 resulted in a maximum cell yield and a high amount of CP III in the second culture proves that the presence of cysteine had no adverse effect on the uptake of methionine by the cells of the first culture. In similar experiments, the data of which have not been reported in the present paper, it was found that methionine was taken up more rapidly than cysteine or homocysteine when both amino acids (methionine + cysteine or methionine + homocysteine) were added in equal amounts to medium A. The results obtained with

TABLE IV-9. The effect of threonine on the accumulation of CP III in *Arthrobacter*, strain 223, in the presence of large amounts of methionine in the nutrient (A) solution. Sulphite oxidation value: 11 mMol O_2/liter hr.

S-Source (μg/ml)	l-Methionine added (μg/ml)	Threonine and other amino acids added (μg/ml)	CP III (mμMol/ml culture)
$(NH_4)_2SO_4$(100) + l-Cysteine(50)	–	–	27
	50	–	24
	50	l-threonine(50)	0
	50	l-threonine(50) + dl-homoserine(50)	22
	50	l-threonine(50) + dl-homocysteine(80)	26
$(NH_4)_2SO_4$(100) + dl-Homocysteine (80)	–	–	21
	80	–	26
	80	l-threonine(60)	29

the addition of methionine to the culture filtrates of B3 and B4 confirm the results of the previous experiment.

In order to obtain a better insight into the accumulation of CP III in cultures of *Arthrobacter*, strain 223, in media containing high amounts of methionine +cysteine or methionine + homocysteine, an experiment was carried out with threonine. The results of this experiment (Table IV-9) show that threonine prevented the accumulation of CP III in the medium with cysteine + methionine, but not in the medium with homocysteine + methionine. In accordance with this, in the medium with cysteine the presence of homoserine as well as that of homocysteine restored the CP III accumulation. The results of this experiment indicate that homocysteine is an important compound in CP III accumulation. In addition the results provide evidence that the synthesis of homocysteine in cells growing in a medium with sulfate + cysteine + methionine was not suppressed to a large extent by methionine.

The results recorded in Tables IV-7, 8 and 9 suggest that in a medium with sulfate + cysteine the formation of homocysteine is not suppressed by methionine as a result of feedback control. This fact brought about accumulation of CP III. In view of this result it is questionable whether the elimination of CP III accumulation by methionine in a medium containing sulfate only was due to such a feedback control mechanism or to some other mechanism.

2.6. *The effect of different amounts of methionine on CP III accumulation*

To gain more information concerning the effect of methionine on CP III accumulation, *Arthrobacter*, strain 223, was grown in medium A containing

TABLE IV-10a. The effect of increasing amounts of methionine on cell yield and the synthesis of porphyrins in cultures of *Arthrobacter*, strain 223. Complete medium A supplied with l-cysteine (50 μg/ml). Sulphite oxidation value: 11 mMol O_2/liter hr.

l-Methionine (μg/ml)	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
–	3.0	29	0.5
10	2.8	12	1.1
20	3.0	0	4.2
30	3.3	5	2.1
40	3.3	11	0.7
50	3.2	19	0.7
60	3.1	27	0.5
70	3.2	24	0.5
80	3.2	26	0.4
90	3.2	25	0.6
100	3.1	28	0.6

TABLE IV-10b. The effect of increasing amounts of methionine on cell yield and the synthesis of porphyrins in cultures of *Arthrobacter*, strain 223. Medium A supplied with dl-homocysteine (80 μg/ml). Sulphite oxidation value: 11 mMol O_2/liter hr.

l-Methionine (μg/ml)	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
–	3.1	22	0.5
10	3.1	27	0.4
20	3.2	33	0.2
30	3.1	39	0.7
40	3.0	19	0.5
50	3.0	5	2.5
60	3.2	0	5.7
70	3.1	11	2.1
80	3.2	24	1.0
90	3.2	26	1.2
100	3.2	25	1.0

increasing amounts of this amino acid and 0.05% (w/v)$MgSO_4.7H_2O$. One set of these media in addition had received cysteine (50 μg/ml), a second homocysteine (80 μg/ml), whereas a control set was left without the latter amino acids.

The results of these experiments, recorded in Tables IV-10 a, b and c, show that without methionine large amounts of CP III accumulated. With increasing amounts of methionine added, CP III accumulation decreased until a zero level. In the cultures supplied with cysteine this level was attained with 20 μg/ml methionine, in those with homocysteine with 60 μg/ml and in the control media, with sulfate only, with about 50 μg/ml.

TABLE IV-10c. The effect of the amount of methionine on the cell yield and the synthesis of porphyrins in *Arthrobacter*, strain 223. Medium A. Sulphite oxidation value: 11 mMol O_2/liter hr.

l-Methionine (μg/ml)	Cell yield (mg dry weight cells/ml culture)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
–	3.1	26	0.4
10	3.2	24	0.4
20	3.2	19	0.6
30	3.3	9	1.0
40	3.3	3	1.1
50	3.1	1	2.5
60	3.1	0	3.8
70	3.1	0	5.7
80	3.2	0	6.0
90	3.0	0	5.9
100	3.2	0	6.0

However, a further increase of the methionine concentration recovered the CP III accumulation in the cultures with cysteine or homocysteine, but not in those with sulfate only. In all instances the heme content of the cultures varied in proportion to the CP III content.

The fact that the accumulation of CP III in a medium with homocysteine was completely suppressed by a distinct amount of methionine excludes the possibility that methionine exerted its favourable effect on heme formation and its unfavourable effect on CP III accumulation as a result of a feedback mechanism inhibiting the synthesis of homocysteine. It may be assumed that the inhibitory effect of distinct concentrations of methionine on CP III accumulation in cultures with sulfate + cysteine (Table IV-10 a) or sulfate only (Table IV-10 c) likewise were not due to this mechanism. This conclusion is in agreement with that drawn from the results reported in Table IV-7.

TABLE IV-11. The ratio between the amount of CP III accumulated under abnormal cultural conditions and the amount of heme (or bacteriochlorophyll), synthesized under normal conditions, in several micro-organisms.

Organism	Normal tetrapyrrole	Conditions for CP III accumulation	Ratio CP III/Heme (or b.chlorophyll)
Saccharomyces anamensis	heme	riboflavin deficiency	10
Rhodopseudomonas spheroides	b. chlorophyll	ethionine addition	4
		iron deficiency	10
Arthrobacter, strain 223	heme	high sulfate*	10
		high methionine**	5

* see Fig. IV-1
** see Table-10c

2.7. *The CP III: heme ratio in micro-organisms*

The CP III: heme ratio's of *Arthrobacter* cultures grown under conditions of heme formation and CP III accumulation have been given in Table IV-11. They are compared with that obtained with *Saccharomyces anamensis* (STICH and EISGRUBER, 1951) and with CP III: bacteriochlorophyll ratio's in *Rhodopseudomonas spheroides* (LASCELLES, 1956; GIBSON et al., 1962). In all cases considerably higher amounts of CP III accumulated than heme (or bacteriochlorophyll) was formed under normal conditions. This overproduction of CP III in *Arthrobacter* may be due to a feedback control mechanism, which failed to function.

3. SUMMARY AND CONCLUSIONS

The accumulation of CP III in cultures of *Arthrobacter*, strain 223, required the presence of sulfate in amounts considerably higher than those required for obtaining maximum cell yield. Similar results were obtained by supplying the sulphur as cysteine or homocysteine (to an unpurified medium A). Methionine stimulated the formation of heme and eliminated CP III accumulation.

Since approximately equal amounts of sulfate, cysteine and homocysteine, respectively, were required for CP III accumulation, one of the latter amino acids was thought to be responsible for this process. The fact that *Arthrobacter*, strain 223, easily converted cysteine to homocysteine, whereas the reverse process did not take place, is a strong indication that homocysteine is responsible for CP III accumulation.

More evidence as to the importance of homocysteine in CP III accumulation was provided by making use of the fact that threonine inhibits the formation of homoserine, one of the precursors of homocysteine. The addition of small amounts of threonine to the medium prevented CP III accumulation. When in addition to threonine, homoserine or homocysteine had been added, CP III accumulation proceeded normally.

Similar conclusions concerning the role of homocysteine in the accumulation of CP III were derived from experiments with $CaCO_3$. Relatively large amounts of $CaCO_3$ prevented the accumulation of CP III in cultures of *Arthrobacter*, strain 223, unless homocysteine was present.

The beneficial effect of homocysteine on CP III accumulation was also found with 5 other *Arthrobacter* strains.

Of a number of amino acids, purines, pyrimidines and B vitamins added to medium A containing sulfate as the sole S-source, methionine was the only compound which, when supplied in amounts of 50 μg/ml, eliminated the accumulation of CP III.

Since in *Escherichia coli* methionine inhibits the synthesis of homocysteine as a result of a feedback control mechanism, the possibility that the eliminating effect of methionine on CP III accumulation in *Arthrobacter*, strain 223, growing in medium A was due to this mechanism had not to be rejected. However, the validity of this hypothesis seems to be doubtful, because the same amount of

methionine when added to a medium with sulfate + cysteine did not eliminate CP III accumulation. More evidence against the latter hypothesis was provided by the fact that a distinct methionine concentration was able to suppress CP III accumulation completely in a medium containing homocysteine.

Experiments with increasing concentrations of methionine added to media containing sulfate, sulfate + cysteine, and sulfate + homocysteine, respectively, showed that at distinct concentrations of methionine CP III accumulation was eliminated. With larger methionine concentrations CP III accumulation was resumed in the media with cysteine and with homocysteine but not in that with sulfate only.

The results of the experiments with increasing amounts of methionine furthermore showed that under certain conditions homocysteine did not stimulate CP III accumulation. In these instances a high amount of heme was formed.

CHAPTER V

THE RELATION BETWEEN METHIONINE AND CHOLINE IN THE FORMATION OF PORPHYRINS IN CULTURES OF *ARTHROBACTER*, STRAIN 223

1. INTRODUCTION

To explain the influence of methionine on the accumulation of coproporphyrin III (CP III; see chapter IV) in cultures of *Arthrobacter*, strain 223, the possibility that methionine exerted its action by serving as donor for labile methyl groups had not to be excluded. The methyl group of methionine may be incorporated into, e.g., lecithin (phosphatidylcholine, PC, Fig. V-1) a phospholipid built up from glycerol, two fatty acids, phosphate and choline. The methyl groups of choline in all kinds of cells investigated so far, have been found to be derived from methionine (VAN DEENEN, 1965).

Lecithin (PC) often forms a considerable part of the phospholipids present in cells of animal origin. It has also been found in many photosynthetic bacteria such as *Chromatium* and several members of the genus *Rhodopseudomonas*

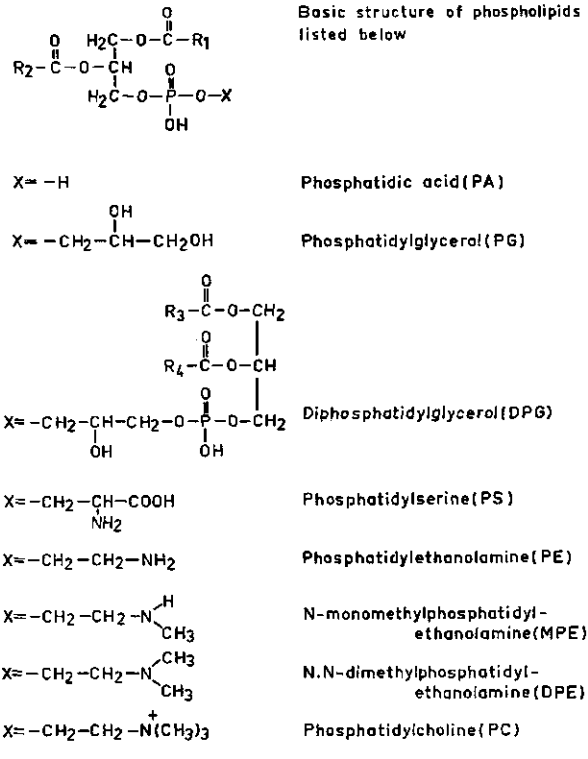
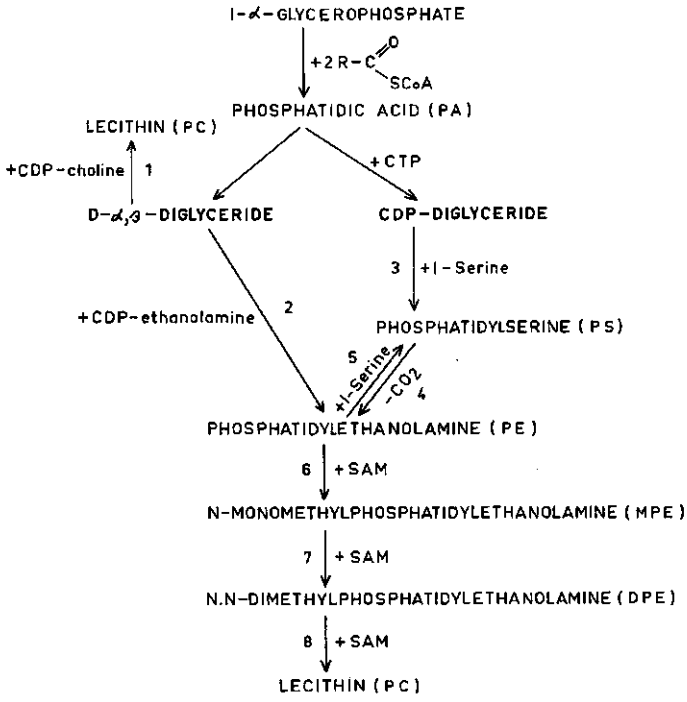
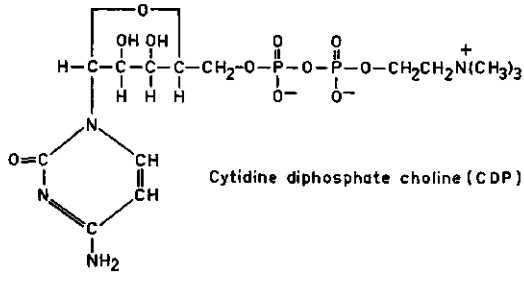


FIG. V-1. Some phospholipids occurring in bacteria. R_1, R_2, R_3, R_4: alkyl chains of fatty acids.





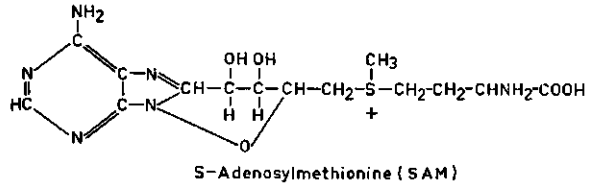


FIG. V-2. The biosynthesis of some nitrogen-containing phospholipids (VAN DEENEN, 1965).

(HAVERKATE, 1965). However, in non-photosynthetic bateria the presence of this phospholipid has been demonstrated without doubt so far only in *Agrobacterium tumefaciens*, in some other representatives of the genus *Agrobacterium* (cf. HOUTSMULLER, 1966), and in a few strains of *Brucella abortus* (THIELE et al., 1968).

The biosynthesis of the most common N-containing phospholipids (Fig. V-2) can be summarized as follows:

1. Lecithin (PC) can be formed from:
   a. cytidine diphosphate (CDP) choline and D-α, β-diglyceride (reaction 1; in mammalian tissues),
   b. phosphatidylethanolamine (PE) and S-adenosylmethionine (SAM) by a stepwise methylation (reactions 6, 7, 8; in mammalian tissues and bacteria).
2. Phosphatidylethanolamine (PE) can be formed from:
   a. cytidine diphosphate (CDP) ethanolamine and D-α, β-diglyceride (reaction 2; in mammalian tissues),
   b. phosphatidylserine (PS) by decarboxylation of serine at phospholipid level (reaction 4; in mammalian tissues and in bacteria).
3. Phosphatidylserine (PS) can be formed from:
   a. phosphatidylethanolamine (PE) and l-serine (reaction 5; in mammalian tissues),
   b. cytidine diphosphate (CDP) diglyceride and l-serine (reaction 3; in mammalian tissues and in bacteria).

The *de novo* synthesis of lecithin can competitively be inhibited by homocysteine and ethionine. These amino acids inhibit the methylation of PE and consequently inhibit MPE formation. Methionine is the only compound known which can eliminate the inhibitory effect of homocysteine and ethionine on the synthesis of MPE (KANESHIRO and LAW, 1964; MEISTER, 1965).

In the decomposition of choline in mammalia (Fig. V-3) first an oxidation reaction takes place resulting in the formation of betaine. Betaine aldehyde has occasionally been found as intermediate in this conversion. Betaine is subsequently demethylated to glycine under the formation of formaldehyde. Glycine may be converted to serine which may be used for the formation of phosphatidylserine (GREENBERG, 1961).

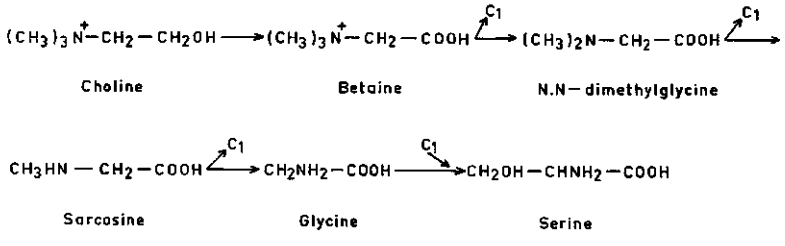


FIG. V-3. The demethylation of choline in mammalia (GREENBERG, 1961). C_1: one carbon unit.

To study whether methionine exerted its action on the accumulation of CP III (see chapter IV) by serving as donor for one carbon units, experiments were carried out with formaldehyde, choline and a few compounds formed from choline by *Arthrobacter*, strain 223.

2. Results and discussion

2.1. *The accumulation of CP III and the absence of phospholipids with labile methyl groups containing N-bases*

The results of the experiments reported in chapter IV for the following reasons do not support the hypothesis that the eliminating effect of methionine on the accumulation of CP III in cultures of *Arthrobacter*, strain 223, was due to the increased content of lecithin, the formation of which requires the methyl group of methionine.

a. The quantity of methionine required to prevent CP III accumulation (Table IV-10c) in medium A was much larger than the corresponding amount of choline found by Crocken and Nyc (1964) required for the synthesis of PC in *Neurospora crassa*. When DPE or MPE would be the hypothetical compound, the amount of methionine would still be lower.

b. The quantity of methionine needed for preventing CP III accumulation in medium A with sulfate as the only S-source (Table IV-10c) was much larger than that in medium A + cysteine (Table IV-10a). If lack of lecithin would have been responsible for CP III accumulation, the same amount of methionine with both S-cources should have been required for preventing the accumulation of the porphyrin.

c. Larger quantities of methionine brought about CP III accumulation in cultures of *Arthrobacter*, strain 223, growing in media with cysteine or homocysteine. This should have been caused by a decrease of the quantity of lecithin. However, so far high concentrations of methionine have never been found to exert an unfavourable effect on the formation of lecithin.

d. No lecithin (PC) or other phospholipids with methyl groups containing N-bases (MPE and DPE) were detected in cells of *Arthrobacter*, strain 223, grown in medium A + methionine. On the contrary PE was found in such cells.

When lecithin formation would not be responsible for the eliminating effect of methionine on CP III accumulation, the stimulating action of homocysteine on CP III accumulation can not be explained by the inhibiting effect of homocysteine on the synthesis of this phospholipid.

2.2. *The replacement of methionine by formaldehyde in CP III accumulation*

A different explanation of the methionine effect could be that the methyl group of this amino acid would be converted to formaldehyde (via lecithin) which would stimulate the synthesis of the compound required for the conversion of CPG III to PP IX. To decide whether the effect of methionine on CP III accumulation could be attributed to the conversion of the methyl group of this

amino acid to formaldehyde, different concentrations of the latter compound were added to nutrient solutions A containing 100 μg $(NH_4)_2SO_4$/ml, A + cysteine and A + homocysteine. As may be seen from Table V-1 the presence of approximately 5 μg/ml formaldehyde in the media A and A + homocysteine and of about 3 μg/ml in medium A + cysteine prevented the accumulation of CP III. Larger quantities of formaldehyde, however, again stimulated porphyrin accumulation in the three media used. Threonine prevented the accumulation of CP III in the media containing sulfate or sulfate + cysteine. The presence of 25 μg/ml formaldehyde or more again prevented CP III accumulation.

The smallest quantity of formaldehyde required to prevent CP III accumulation was about equal to the amount of formaldehyde that can theoretically be formed from the lowest amount of methionine required to prevent CP III accumulation. This might indicate that the effect of methionine on CP III accumulation was due to the improved one carbon unit supply of the bacteria.

More evidence concerning the relationship between CP III accumulation and the supply of one carbon units was gained by growing *Arthrobacter*, strain 223, at high and low zinc concentrations and at different biotin levels. Earlier experiments (see chapter III) had shown that a high zinc concentration favourably affected porphyrin accumulation. The same was true of a high biotin level in the nutrient solution. To see if the effect of the zinc supply and of the biotin supply of the bacteria were connected with the availability of one carbon units, experiments were carried out with washed *Arthrobacter* cells grown at a high and a low zinc concentration and at a high and low biotin level. Since in many living cells one carbon units are derived from glyoxylic acid, the above-mentioned cells were incubated for two hours with glyoxylic acid in a phosphate buffer of pH 7.0.

The results obtained show (Table V-2) that cells grown at a high zinc con-

TABLE V-1. The effect of increasing amounts of formaldehyde on the accumulation of CP III in cultures of *Arthrobacter*, strain 223, growing in medium A with different S-sources. Sulphite oxidation value: 11 mMol O_2/liter hr.

Formaldehyde (μg/ml)	CP III (mμMol/ml culture)		
	Medium A	Medium A + l-Cysteine (40μg/ml)	Medium A + dl-Homocysteine (80μg/ml)
-	26	29	24
1	24	20	23
2	26	7	21
3	19	0	10
4	12	5	1
5	0	17	0
6	6	21	7
7	9	19	18
8	13	24	19
9	14	17	12
10	16	12	9
15	1	0	0
25	0	0	0

TABLE V-2. The formation of formic acid from glyoxylic acid by washed cell suspensions of *Arthrobacter*, strain 223, grown under different conditions in medium A. The incubation mixture contained 2 mg cells (dry weight) and 2 mg glyoxylic acid per ml in a phosphate buffer of pH 7.0. After an incubation period of 2 hours formic acid was determined in the filtrate.

Conditions of growth	Dominant tetrapyrrole	Substrate for washed cell suspensions	Formic acid (μg/ml) produced
High zinc; high biotin	CP III	–	0
		glyoxylic acid	1.8
		glyoxylic acid + Na-acetate	0.2
Low zinc; high biotin	heme	–	0
		glyoxylic acid	5.7
		glyoxylic acid + Na-acetate	2.9
High zinc; low biotin	heme	–	0
		glyoxylic acid	6.1
		glyoxylic acid + Na-acetate	5.9

centration, which contained CP III as the dominant tetrapyrrole produced much less formic acid than the heme-containing cells grown at a low zinc concentration (cells were grown at a high biotin level). Na-acetate (2 mg/ml) reduced the amount of formic acid produced by high zinc cells to a much larger degree than that by low zinc cells. This indicates that in high zinc cells more glyoxylate may be utilized in the glyoxylic acid cycle than in low zinc cells, an observation which would be in agreement with a finding of WEGENER and ROMANO (1964).

Heme-containing cells grown at a high zinc level but at a low biotin level produced a high amount of formic acid, which was almost unaffected by Na-acetate. The latter may indicate a low activity of the glyoxylic acid cycle enzymes in biotin-deficient cells, an observation made earlier by KINOSHITA (1962).

No further study was made of the relation between one carbon unit metabolism on the one hand and zinc and biotin on the other hand.

2.3. *The action of formaldehyde*

To elucidate the effect of formaldehyde, porphyrin-accumulating cells were supplied with a number of amino acids which were thought to be involved in the processes leading to the accumulation of CP III. Cells of *Arthrobacter*, strain 223, grown in medium A, were collected by centrifugation, washed with glass-distilled water and incubated for two hours in a phosphate buffer of pH 7.0, supplied with threonine, serine, glycine and glycine + formaldehyde. The culture filtrates were desalted by ion exchange and subsequently analysed for amino acids by thin-layer chromatography after removal of the cells by centrifugation.

As appears from Figure V-4, glycine had been formed from threonine, as reported earlier for *Arthrobacter globiformis* by MCGILLAVRY and MORRIS (1966). No formation of glycine or formaldehyde could be detected when the

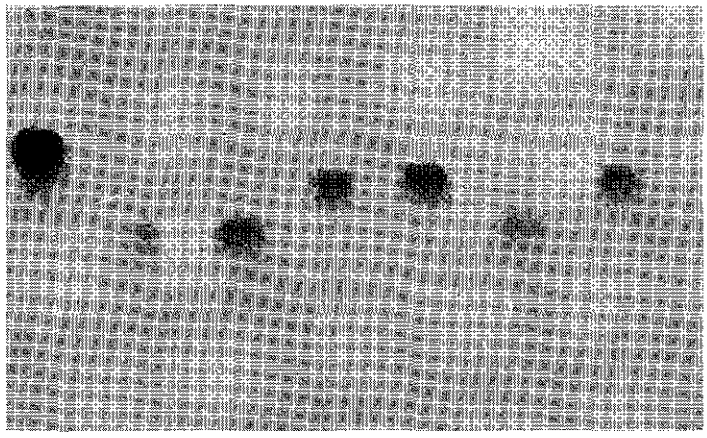


FIG. V-4. Thin-layer chromatogram of amino acids present in the culture filtrate of suspensions of CPG III-accumulating cells of *Arthrobacter*, strain 223, incubated for two hours with threonine (C), serine (E), glycine (F) and glycine + formaldehyde (G), respectively. Blanks, incubated without bacteria, were present with threonine (A), glycine (B) and serine (D). Chromatogram was run in the solvent system ethanol: water (70: 30, v/v).

cells were incubated with serine. However, serine was formed from glycine and formaldehyde (see also Table V-3). In *Mycobacterium smegmatis* the same conversion has been found to occur (TSUKAMURA et al., 1966).

The results of this experiment indicate that the suppressing effect of formaldehyde on CP III accumulation (Table V-1) may have been due to an increased formation of serine.

Another effect of formaldehyde might be the stimulation of the synthesis of methionine. FORSTER et al., (1963) found one carbon units to be required for the formation of methionine from homocysteine in *Escherichia coli*. Serine in that reaction served as donor for one carbon units. In order to find out whether

TABLE V-3. The effect of formaldehyde on the formation of serine and methionine by suspensions (2 mg dry weight cells) of CPG III-accumulating cells of *Arthrobacter*, strain 223, grown in medium A. Time of incubation 2 hours. Serine was estimated according to the method of SHIEH (1966) and methionine with *Arthrobacter*, strain 250, which requires methionine.

Substrate (μg/ml)	Amino acid formed (μg/ml)	
	Serine	Methionine
–	0	0
Glycine (100)	0	–*
Glycine (100) + formaldehyde (50)	18	–*
Homocysteine (100)	–*	0
Homocysteine (100) + formaldehyde (50)	–*	11

* Not determined.

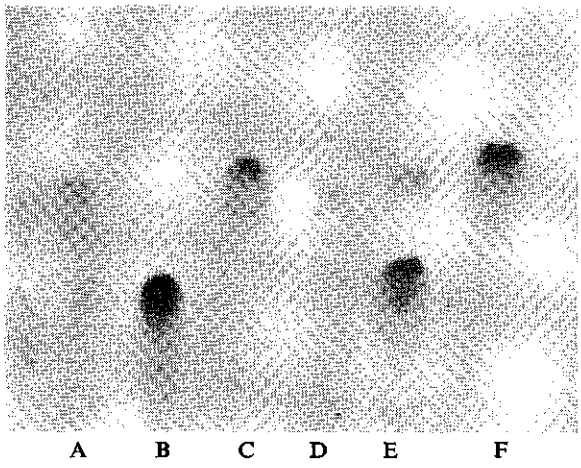


FIG. V-5. Thin-layer chromatogram of amino acids present in the culture filtrate of suspensions of CPG III-accumulating cells of *Arthrobacter*, strain 223, incubated for two hours with homocysteine (D), homocysteine + serine (E) and homocysteine + formaldehyde (F), respectively. Blanks, incubated without bacteria, were present with homocysteine (A), serine (B) and methionine (C). Chromatogram was run in the solvent system ethanol: water (70: 30, v/v).

formaldehyde influences the synthesis of methionine in *Arthrobacter*, strain 223, porphyrin-accumulating cells of this strain were incubated with homocysteine either with serine or formaldehyde. As shown in Figure V-5 methionine was only formed from homocysteine + formaldehyde and not from homocysteine + serine (see also Table V-3). In *Arthrobacter*, strain 223, in contrast to *Escherichia coli*, serine apparently does not serve as donor for one carbon units. This conclusion is in agreement with the results of the previous experiment (Fig. V-4).

The fact that formaldehyde is required for the formation of methionine means that methionine, in addition to the conversion of its methyl groups to formaldehyde, can contribute to a better supply of one carbon units as a result of its sparing action on the utilization of these compounds. The results of the experiment recorded in Figure V-5 might indicate that the unfavourable effect of larger quantities of formaldehyde on the conversion of CP III to PP IX is based on an enhanced methionine formation.

The difference in the smallest quantities of formaldehyde preventing the accumulation of CP III in *Arthrobacter*, strain 223, growing in medium A and medium A + cysteine, respectively, (Table V-1) might be attributed to the fact that in the former medium the bacterium itself had to synthesize its cysteine. This formation requires one carbon units because cysteine is presumably formed from glycine, formaldehyde and H_2S.

The results of the foregoing experiments provide evidence that the effect of added methionine on the accumulation of CP III depends on the improved supply of one carbon units. Since formaldehyde stimulated serine formation, the

compound having a favourable effect on the conversion of CPG III to PP IX was thought to have been derived from serine. Phosphatidylethanolamine (PE) was thought to be the compound in question. Phosphatidylserine (PS) seems more logical, but this phospholipid is seldom found in bacteria in substantial amounts; the PS formed is often converted to PE (KANFER and KENNEDY, 1963, 1964).

2.4. *The transfer of the methyl group of methionine in Arthrobacter, strain 223*

The hypothesis that the methyl group of methionine via choline would be converted to formaldehyde was discussed in section 2 of this chapter. If this supposition would be correct, choline could be expected to have the same effect on the accumulation of CP III as formaldehyde. To demonstrate this effect different concentrations of choline were added to medium A (containing 100 μg $(NH_4)_2SO_4$/ml) with different S-sources. The results of this experiment show (Table V-4) that the amount of choline required to prevent the accumulation of CP III was equal in medium A and A + homocysteine, amounting to about 12.5 μg/ml. In medium A + cysteine this quantity was about 5 μg/ml. The CP III accumulation occurring at larger choline concentrations in the media with sulfate or sulfate + cysteine was prevented by threonine.

Comparing the effect of choline on the accumulation of CP III with that of formaldehyde (Table V-1), it appears that the smallest quantity of formaldehyde preventing CP III accumulation is equal to the quantity which can be formed from the smallest quantity of choline preventing porphyrin accumulation. These results provide evidence that at least a part of the methyl groups of the methionine added to medium A or A + homocysteine via choline was converted to for-

TABLE V-4. The effect of increasing amounts of choline on cell yield and accumulation of CP III in cultures of *Arthrobacter*, strain 223, grown in medium A with different S-sources. Sulphite oxidation value: 11 mMol O_2/liter hr.

Choline (μg/ml)	Medium A		Medium A + l-Cysteine (40 μg/ml)		Medium A + dl-Homocysteine (80 μg/ml)	
	Cell yield (mg dry weight cells/ ml culture)	CP III (mμ Mol/ ml culture)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμ Mol/ ml culture)	Cell yield (mg dry weight cells/ ml culture)	CP III (mμ Mol/ ml culture)
–	3.1	28	3.1	31	3.1	22
2.5	3.0	27	3.3	19	3.1	27
5.0	3.0	25	3.1	2	3.0	21
7.5	3.1	12	3.0	9	3.2	16
10.0	3.0	1	3.0	21	3.0	5
12.5	3.0	0	3.2	27	2.9	0
15.0	3.0	7	3.0	28	3.1	9
17.5	3.1	19	3.0	25	3.1	23
25.0	3.2	25	3.1	24	3.1	22
30.0	3.1	27	3.2	27	3.2	27

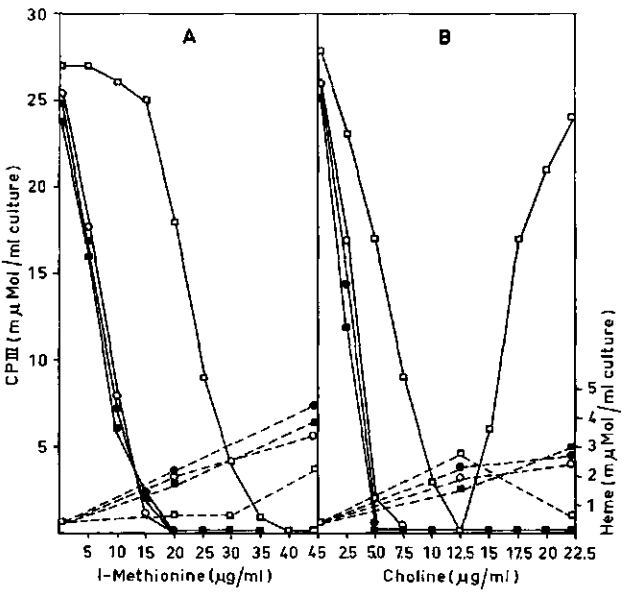


FIG. V-6. The effect of increasing amounts of methionine (A) and of choline (B), in the absence (□) and presence of N.N-dimethylglycine (○), serine (●) and ethanolamine (■), on the accumulation of CP III (solid lines) and the formation of heme (broken lines) in cultures of *Arthrobacter*, strain 223. Sulphite oxidation value: 18 mMol O_2/liter hr.

maldehyde. The utilization of choline by bacteria has been extensively studied by SHERR and LAW (1965). They found that choline is only taken up by bacteria which are able to synthesize and decompose choline. A number of bacteria but not *Arthrobacter* sp. were tested by these authors.

Another indication as to the conversion via choline of the methyl group of methionine to formaldehyde was obtained by the presence of N.N-dimethylglycine and serine (products derived from choline decomposition by *Arthrobacter*, strain 223) in media in which CP III accumulation was expected. The results of this experiment, plotted in Figure V-6, show that the presence of these degradation products reduced the amounts of methionine and choline required for eliminating CP III accumulation. Ethanolamine reacted similarly to N.N-dimethylglycine and serine while in a subsequent experiment sarcosine was found to have also the same effect.

So far no evidence has been obtained for the existence in bacteria of the enzyme catalysing the formation of phosphatidylethanolamine from D-α, β-diglyceride and CDP-ethanolamine (Fig. V-2, reaction 2). Therefore the beneficial effect of ethanolamine, similar to that of N.N-dimethylglycine and sarcosine on PP IX formation is probably due to its supply of one carbon units, required for the synthesis of serine. This amino acid is needed for the formation of phosphatidylethanolamine, the compound thought to be essential for the conversion of CPG III to PP IX.

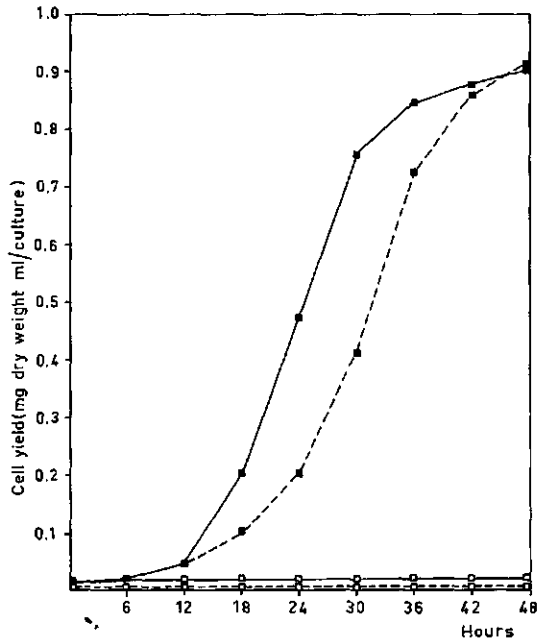


FIG. V-7. The growth of *Arthrobacter* strains 223 (broken lines) and 368 (solid lines) in a medium with (■) choline as the sole C and N-source; □: no choline added. Sulphite oxidation value: 11 mMol O_2/liter hr.

2.5. *Some decomposition products of choline*

To get acquainted with the decomposition of choline by *Arthrobacter*, strain 223, one ml of a culture of this strain grown in medium A was inoculated into a mineral medium (A without glucose and nitrogen) containing choline as the sole C and N-source. The growth was excellent as will be seen from the data given in Figure V-7. One ml of the latter culture was subsequently transferred to media with 0.2% (w/v) choline, 0.2% (w/v) betaine, 0.2% (w/v) N.N-dimethylglycine and 0.2% (w/v) sarcosine, respectively. All these substrates allowed a good growth of the organism. Samples were withdrawn at intervals for analysis of the culture filtrates. The bacteria were removed by centrifugation, the filtrates desalted by ion exchange, evaporated and analysed for amino acids by thin-layer chromatography.

Figure V-8 shows that sarcosine and serine had been formed from choline, betaine and N.N-dimethylglycine. Serine (which was converted to pyruvic acid) had also been formed by cells grown with sarcosine and glycine. Formaldehyde was formed from choline, betaine, N.N-dimethylglycine and sarcosine (Fig. V-9). No methionine was found in the filtrates of cultures grown with choline or betaine. Nor when homocysteine was added.

Similar results were obtained with *Arthrobacter*, strain 368, while experiments with *Achromobacter cholinophagum* (SHIEH, 1965) had given almost the same

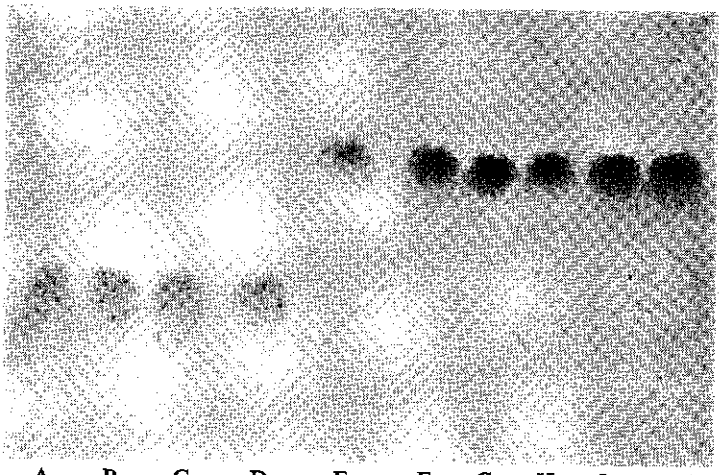


FIG. V-8. Thin-layer chromatogram of ninhydrin-positive substances in culture filtrates of choline (B, F), betaine (C, G), N.N-dimethylglycine (D, H), sarcosine (I) and glycine-grown (K) cells of *Arthrobacter*, strain 223. A: authentic sarcosine, E: authentic serine.

reaction products. These organisms thus decompose choline in the same way as mammalia. However, they do not require homocysteine for the conversion of betaine to N.N-dimethylglycine as it is the case with mammalia.

2.6. *The effect of homocysteine on the decomposition of choline by Arthrobacter, strain 223*

The effect of homocysteine on the degradation of choline was studied as follows: *Arthrobacter*, strain 223, was inoculated into mineral medium A (wit-

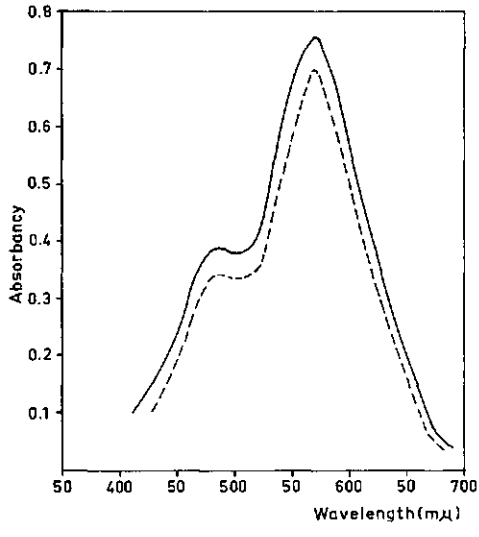


FIG. V-9. The spectrum of the reaction product of formaldehyde with chromotropic acid. Authentic formaldehyde (solid lines) and culture filtrate of choline-grown cells of *Arthrobacter*, strain 223 (broken lines).

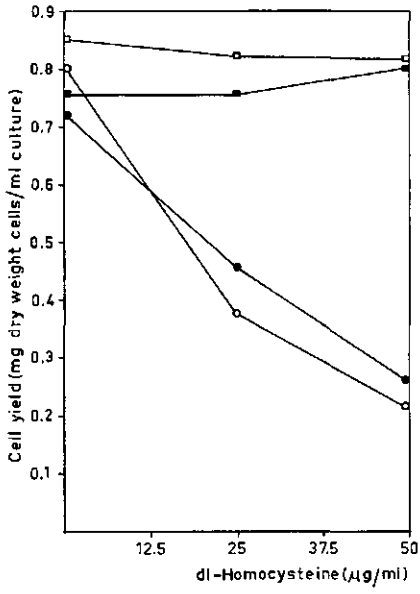


FIG. V-10. The effect of homocysteine on the growth of *Arthrobacter*, strain 223, in media with choline (●), betaine (○), N.N-dimethylglycine (□) and sarcosine (■) respectively, as the sole C and N-source. Incubation time 42 hours.

hout glucose and nitrogen) with 0.2% (w/v) choline, 0.2% (w/v) betaine, 0.2% (w/v) N.N-dimethylglycine and 0.2% (w/v) sarcosine, respectively, (40 ml medium in 100 ml flasks). After incubation for 48 hours, one ml of these cultures was transferred to 500 ml flasks, containing 200 ml of the same medium with different concentrations of homocysteine.

As shown in Figure V-10 homocysteine inhibited the growth of the bacteria with choline and betaine in contrast to that with N.N-dimethylglycine and sarcosine. However, after 72 hours of incubation the cell yield in all cultures was equal. These results indicate that the site of interference of homocysteine was between betaine and N.N-dimethylglycine. This conclusion was confirmed by incubating betaine-grown cells with betaine in phosphate buffer (pH 7.0). The incubation mixture contained per ml: 2 mg cells (dry weight) and 2 mg betaine. Formaldehyde was determined after two hours incubation. From Table V-5 it will be seen that homocysteine prevented the formation of formaldehyde from betaine. Similar results were obtained with other *Arthrobacter* strains, confirming the results obtained with *Achromobacter cholinophagum* (SHIEH, 1966).

TABLE V-5. The effect of homocysteine on the formation of formaldehyde from betaine by *Arthrobacter*, strain 223. Betaine-grown cells were incubated with betaine (2 mg/ml) in phosphate buffer (pH 7.0) during 2 hours.

Substrate	Formaldehyde (µg/ml)
–	0
Betaine	3.7
Betaine + dl-homocysteine (25 µg/ml)	0

2.7. *The effect of homocysteine on CP III accumulation*

The above-mentioned results might imply that in *Arthrobacter*, strain 223, and in a number of other *Arthrobacter* strains growing in a medium with large amounts of iron, CP III accumulation, for which homocysteine was found to be responsible, might be attributed to the inhibition of the formation of formaldehyde from choline. To provide evidence as to this hypothesis, the observation that accumulation of CP III also takes place in media with larger amounts of methionine (Table IV-10a and b) and choline (Table V-4) was considered. Such media were supplied with increasing amounts of N.N-dimethylglycine, ethanolamine and serine. The former two compounds may increase the amount of available one carbon units required for the synthesis of serine needed for phosphatidylethanolamine formation.

Figure V-11 shows that N.N-dimethylglycine, serine and ethanolamine were capable of preventing CP III accumulation. The effect of N.N-dimethylglycine and ethanolamine was due to a better supply of one carbon units stimulating serine formation. This is concluded from the fact that serine itself had a similar effect on CP III accumulation. That these one carbon units supplying compounds were able to suppress CP III accumulation, whereas large amounts of methionine or choline were unable to prevent this accumulation is thus considered to be due to an inhibited formation of formaldehyde from betaine.

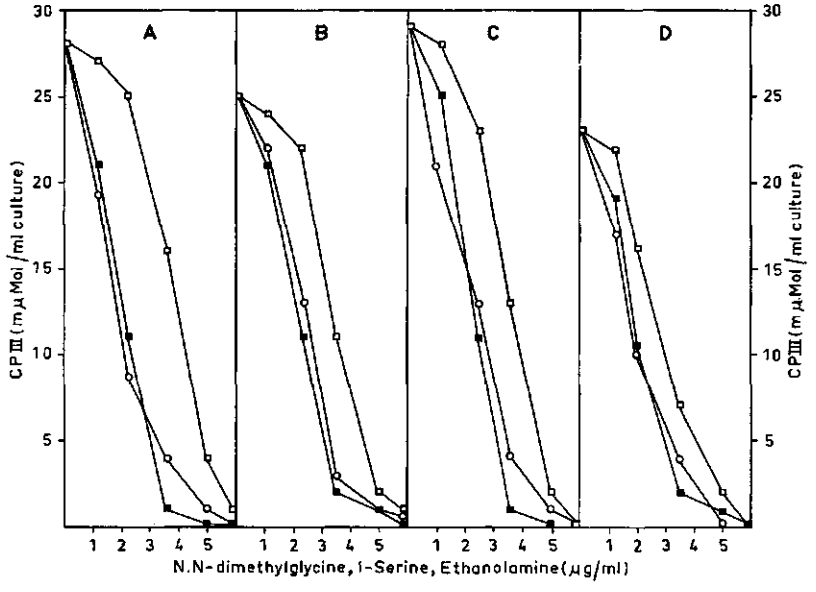


FIG. V-11. The effect of increasing amounts of N.N-dimethylglycine (□), serine (■) and ethanolamine (○) on the accumulation of CP III in cultures of *Arthrobacter*, strain 223, growing in media with large amounts of methionine and cysteine (A), methionine and homocysteine (B), choline and cysteine (C), and choline and homocysteine (D). Sulphite oxidation value: 11 mMol O_2/liter hr.

TABLE V-6. The effect of ethionine on the synthesis of porphyrins by *Arthrobacter*, strain 223, growing in medium A and in medium A supplied with cysteine and large amounts of methionine. Sulphite oxidation value: 11 mMol O_2/liter hr.

dl-Ethionine (μg/ml)	Medium A*		Medium A** + l-Cysteine (40μg/ml) + l-Methionine (50μg/ml)	
	CP III (mμMol/ ml culture)	Heme (mμMol/ ml culture)	CP III (mμMol/ ml culture)	Heme (mμMol/ ml culture)
–	27	0.4	29	0.5
2	25	0.5	26	0.4
4	20	0.8	27	0.5
6	10	1.0	25	0.4
8	1	2.0	19	0.7
10	0	3.1	12	0.9
20	0	2.1	6	1.3
40	5	1.2	0	2.2
80	9	0.3	0	2.9

* Ethionine added 24 hours after incubation
** Ethionine added at the time of inoculation

2.8. *The effect of ethionine*

In order to know whether a methylation reaction was involved in CP III accumulation, increasing concentrations of ethionine were added to medium A (containing 100 μg $(NH_4)_2SO_4$) and A + high amounts of cysteine and methionine. Ethionine inhibits methylation reactions as a result of its conversion to S-adenosylethionine and competitively inhibiting the formation of S-adenosylmethionine. In addition S-adenosylethionine may inhibit enzymes (cf. LASCELLES, 1964) catalysing methylations. Because very small amounts of ethionine already inhibited the growth in medium A when added at the time of inoculation, increasing amounts were added about 20 hours after inoculation. It appears from Table V-6 that ethionine reduced the accumulation of CP III and concomitantly enhanced heme formation. A similar effect of ethionine was found in media with large amounts of formaldehyde and choline. The amount of ethionine required to eliminate CP III accumulation in medium A without methionine was considerably lower than that in medium A supplied with large amounts of methionine (choline or formaldehyde), indicating that in the bacteria growing in the latter medium methylation was more pronounced. Since methylation reactions are involved in the conversion of PE to PC (via MPE and DPE), suppression of these reactions in *Arthrobacter*, strain 223, apparently brings about an increased concentration of PE, the compound thought to be essential for the formation of PP IX from CPG III.

Besides ethionine, homocysteine can also inhibit methylation reactions as a result of its conversion to S-adenosylhomocysteine. This means that S-adenosylhomocysteine might inhibit CP III accumulation and concomitantly enhance heme formation. It is therefore an attractive hypothesis that cells in which

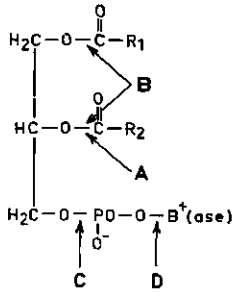


FIG. V-12. The enzymic hydrolysis of lecithin. The arrows indicate the site of attack of the phospholipases A, B, C and D. R_1, R_2: alkyl chains of fatty acids.

homocysteine formation is not inhibited (Table IV-10a at about 20 μg/ml methionine) or cells growing in media with large amounts of homocysteine (Table IV-10b, at about 50 μg/ml methionine) fail to accumulate CPG III because of the formation of S-adenosylhomocysteine. Attempts to support this hypothesis experimentally by estimating the amount of S-adenosylhomocysteine and S-adenosylmethionine were unsuccessful possibly because the amounts of these compounds were beyond the limit of detection of the method employed.

2.9. *Phospholipase activity of Arthrobacter, strain 223*

The products of hydrolysis of phospholipids resulting from phospholipase activity depend on the type of phospholipase (Fig. V-12).

To determine the type of phospholipase (cf. HOUTSMULLER, 1966) occurring in *Arthrobacter*, strain 223, washed cells cultivated in medium A (or A + cysteine + methionine) were incubated with egg phospholipids mainly containing PC and PE. The phospholipase was destroyed by heating the incubation mixture for 5 minutes in boiling water; phospholipids and neutral lipids were extracted with chloroform and subjected to thin-layer chromatography. The data of this analysis (Fig. V-13) show that only neutral lipids were formed from the phospholipids. The chromatographic behaviour of the main water-soluble

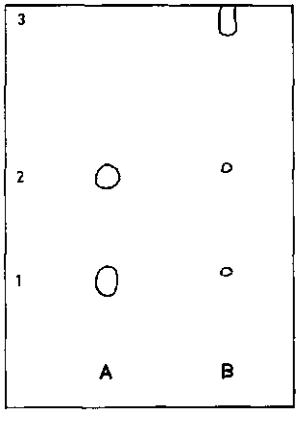


FIG. V-13. Thin-layer chromatogram of egg phospholipids before (A) and after (B) incubation with cells of *Arthrobacter*, strain 223, for 16 hours. 1: phosphatidylcholine, 2: phosphatidylethanolamine and 3: neutral lipids. Chromatogram was run in the solvent system chloroform: methanol: water (65: 30: 5, v/v/v).

TABLE V-7. The hydrolysis of phospholipids by cell suspensions of *Arthrobacter*, strain 223. Phospholipids dissolved in 0.5 ml ether were added to the incubation mixture (5 ml) containing 600 mg (dry weight) cells, 0.75% (w/v) NaCl and zinc (0.5 μg/ml). The ether was evaporated at 35 °C, the mixture homogenized and incubated overnight at 30 °C. Hydrolysis finished by heating during 5 minutes at 100 °C. No correction made for the phospholipid content of the cells.

Experiment	Phospholipids (mg) incubated	Unsaturated fatty acids in phospholipids	Hydrolysis (%)
1	20 Egg phospholipids (76% PC + 18% PE)	+	92
2	20 *Arthrobacter* phospholipids (PG, DPG and PA)	+	5
3	20 *Arthrobacter* phospholipids (PG, DPG, PA and PE)	+	8
4	10 (1) + 10 (2)		87
5	10 (1) + 10 (3)		94

TABLE V-8. The effect of zinc on the rate of hydrolysis of egg phospholipids, containing 76% PC and 18% PE by cell suspensions of *Arthrobacter*, strain 223. Bacteria were grown in medium A without zinc. Conditions of incubation as in table V-7.

Time of incubation (hours)	Hydrolysis of phospholipids (%)	
	+ Zinc	- Zinc
0	2	4
8	37	25
16	89	48

compound was similar to that of phosphorylcholine, showing that phospholipase C was involved in the breakdown of lecithin by *Arthrobacter*, strain 223.

In a subsequent experiment it was shown that almost no hydrolysis of phospholipids, including PA, PE, PG and DPG, took place, unless PC was present (Table V-7). This observation is in agreement with that made by VAN DEENEN et al. (1961) with *Clostridium welchii*. It strongly indicates that the favourable effect of large amounts of methionine on CP III accumulation in *Arthrobacter*, strain 223, is due to an enhanced phospholipase C activity, resulting in the hydrolysis of phospholipids, including PE.

The effect of zinc on phospholipase C activity (HOUTSMULLER, 1966) was tested with cells of *Arthrobacter*, strain 223, grown in medium A without added zinc (the medium contained still sufficient zinc as contamination to allow maximum cell yield). Washed cells were incubated with egg phospholipids with and without zinc. The data obtained (Table V-8) clearly demonstrate the beneficial effect of zinc on phospholipase C activity of *Arthrobacter*, strain 223.

3. SUMMARY AND CONCLUSIONS

To explain the beneficial effect of methionine on the conversion of CPG III to PP IX, a process which automatically eliminates CP III accumulation, much attention was paid to the formation of lecithin and related phospholipids, which depends on the presence of methionine.

The fact that no lecithin or other phospholipids with methyl groups containing N-bases were detected in *Arthrobacter* cells (strain 223) in which the presence of methionine had eliminated CP III accumulation, excluded the possibility that these phospholipids are directly involved in the formation of PP IX from CPG III by *Arthrobacter*, strain 223. However, in an indirect way they might play a part in the formation of PP IX. The observation that relatively large amounts of phosphatidylethanolamine (PE) occurred in the above-mentioned bacteria and the presumed relationship between this phospholipid and the supply of methionine prompted to a detailed study of the synthesis and breakdown of PE in connection with CP III accumulation and supply of sulphur-containning amino acids.

Since the methyl group of methionine may be converted to formaldehyde (via choline) the effect of formaldehyde and choline on CP III accumulation was studied. Addition of small amounts of formaldehyde to cultures of *Arthrobacter*, strain 223, prevented the accumulation of CP III. Larger amounts of this one carbon unit again brought about CP III accumulation. Very large amounts of formaldehyde resulted in heme-containing cells. The smallest amount of formaldehyde required to prevent CP III accumulation was equal to the amount of formaldehyde which could be derived from the smallest amount of methionine preventing this accumulation. More evidence concerning the connection between one carbon unit supply and CP III accumulation was provided by experiments with different amounts of zinc and biotin. Excessive amounts of zinc and of biotin required for CP III accumulation, brought about a strong reduction of the formation of one carbon units from glyoxylic acid.

The effect of different amounts of choline on CP III accumulation resembled that of formaldehyde, indicating that formaldehyde can be formed from choline. More evidence as to the conversion of the methyl group of methionine to formaldehyde (via choline) was provided by the fact that decomposition products of choline as N.N-dimethylglycine and sarcosine reduced the amount of methionine or choline required to eliminate CP III accumulation.

If phosphatidylethanolamine (PE) would be the compound required for the conversion of CPG III to PP IX, the beneficial effect of methionine, choline and formaldehyde on PE formation would depend on the reaction: glycine + formaldehyde $\rightarrow$ serine. The latter amino acid may react with CDP-diglyceride under the formation of PS, which is decarboxylated to PE. The validity of this hypothesis was shown by the rapid formation of serine from glycine and formaldehyde in washed cells of *Arthrobacter*, strain 223.

An additional beneficial effect of methionine on the formation of PE was due to the sparing action of methionine on the utilization of one carbon units,

which in *Arthrobacter* cells growing without added methionine are partly required for the synthesis of this amino acid. This was concluded from the fact that washed cells readily formed methionine from homocysteine and formaldehyde.

Experiments with *Arthobacter*, strain 223, with high amounts of methionine have shown a resumed accumulation of CP III, when homocysteine (or cysteine, which is converted to homocysteine) was present in the medium (see chapter IV). Similar results have been obtained with relatively high concentrations of formaldehyde and choline. However, with these two compounds stimulating PP IX formation, the presence of homocysteine (or cysteine) was not required to obtain resumed CP III accumulation, indicating that the bacteria under these conditions formed sufficient cysteine.

To explain the stimulating effect of large amounts of methionine, formaldehyde or choline on CP III accumulation, it should be noted that these compounds promote the methylation of PE, thus decreasing the concentration of the latter phospholipid. However, a far more serious result of the methylation of PE to PC may be the stimulation of the activity of phopholipase C, the enzyme responsible for the breakdown of several phospholipids, including PE. This effect was shown by incubating a number of phospholipids with cells of *Arthrobacter*, strain 223. These phospholipids were only hydrolysed when lecithin was present. Zinc exerted a stimulating action on phospholipipase C. During this process, lecithin itself was also hydrolysed.

Evidence as to the correctness of this explanation was provided by the results of experiments with ethionine (an inhibitor of methylation reactions) added to bacteria growing with high amounts of methionine, choline or formaldehyde. No CP III accumulation occurred in contrast to control cultures which had received no ethionine.

The resumed CP III accumulation with high concentrations of methionine, choline or formaldehyde required homocysteine. The blocking of the formation of this latter amino acid in cells growing in a cysteine-containing medium suppressed the CP III accumulation completely. Homocysteine was thought to inhibit the decomposition of choline, resulting in a reduced one carbon unit supply and consequently in a reduced formation of serine. To prove the validity of this hypothesis, the decomposition of choline was studied by analysing the culture filtrates of *Arthrobacter*, strain 223, grown with choline, betaine, N.N-dimethylglycine, sarcosine and glycine, respectively. Sarcosine was detected in media with the former three substrates, serine in all five media and formaldehyde with the former four. Subsequently increasing amounts of homocysteine were added to media with choline, betaine, N.N-dimethylglycine and sarcosine, respectively, as the only C and N-source. A pronounced inhibition of the initial growth was observed in the media with choline and betaine, but not with N.N-dimethylglycine and sarcosine, showing that the conversion of betaine to N.N-dimethylglycine was blocked by homocysteine. This conclusion was confirmed by the observation that the formation of formaldehyde from betaine by betaine-grown cells was inhibited by homocysteine. The fact that addition of N.N-

dimethylglycine or sarcosine to media with large amounts of methionine or choline prevented CP III accumulation provided evidence that the beneficial effect of homocysteine on CP III accumulation was brought about by the inhibition of the formation of formaldehyde from choline.

CHAPTER VI

THE FORMATION OF PORPHYRINS BY IRON-DEFICIENT *ARTHROBACTER*, STRAIN 223

1. INTRODUCTION

In this chapter a description is given of experiments carried out to study the accumulation of porphyrins under conditions of iron deficiency. Since heme contains iron, *Arthrobacter*, strain 223, growing in iron-deficient media might be expected to synthesize less heme than growing in a medium supplied with optimal amounts of iron. As heme might be expected to affect the synthesis of porphyrins by way of enzyme repression and feedback inhibition, the reduced heme content in iron-deficient cells might stimulate the formation of precursors of heme like CPG III. A number of experiments dealing with this mechanism are discussed in the first part of this chapter. In the second part experiments are described dealing with the kind of porphyrin accumulated under conditions of iron deficiency. Although under the latter conditions the accumulation of PP IX, the direct precursor of heme, might be expected, such an accumulation did not occur. Instead, CP III was found in excessive amounts, indicating that iron plays a role in the conversion of CPG III to PP IX. Because this conversion in *Arthrobacter*, strain 223, presumably depends on the presence of phosphatidylethanolamine (chapter V) iron was thought to be involved in the synthesis of PE. FULCO and BLOCH (1964) have shown that in liver, yeast, *Mycobacterium phlei* and *Corynebacterium diphtheriae*, iron participates (as cofactor) in the conversion of saturated fatty acids to the corresponding unsaturated ones. Since phosphatidylethanolamine isolated from a wide variety of cells has been found to contain unsaturated fatty acids (KANESHIRO and MARR, 1961; HAVERKATE, 1965; VAN DEENEN, 1965), CP III accumulation in iron-deficient cultures of *Arthrobacter*, strain 223, might be due to an inadequate formation of unsaturated fatty acids leading to a reduced formation of PE.

2. RESULTS AND DISCUSSION

2.1. *The effect of manganese, S-containing amino acids and ethanolamine on the accumulation of CP III*

For studying the formation of porphyrins under conditions of iron-deficiency, *Arthrobacter*, strain 223, was inoculated into purified medium A supplied with cysteine and excessive amounts of methionine, and in addition with N.N-dimethylglycine (medium B_1), serine (medium B_2) or ethanolamine (medium B_3), respectively. As shown in the previous chapter, no CP III accumulation occurred in these media, owing to a sufficient one carbon unit supply which presumably lead to PE formation.

TABLE VI-1. The effect of manganese on the accumulation of CP III in iron-deficient cultures of *Arthrobacter*, strain 223, growing in media B_1, B_2 and B_3. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 80 hours.

Manganese (10^{-2}µg/ml)	CP III (mµMol/ml culture)					
	B_1 N.N-Dimethylglycine (7.5µg/ml)		B_2 l-Serine (7.5µg/ml)		B_3 Ethanolamine (7.5µg/ml)	
	− Iron	+ Iron	− Iron	+ Iron	− Iron	+ Iron
–	31	0	27	0	7	0
0.5	16	0	19	0	13	0
1.0	4	0	6	0	19	0
5.0	0	0	0	0	23*	0
10.0	0	0	0	0	9	0

* Designated iron-deficient medium B_3.

When no iron was added to the B media, CP III accumulated. The amount of this porphyrin depended on the manganese supply (Table VI-1). However, iron eliminated the effect of manganese on CP III accumulation. Thus, CP III accumulation in iron-deficient cultures of *Arthrobacter*, strain 223, was affected by manganese, whereas in iron-sufficient cultures it was unaffected by manganese (medium A, Table III-6a). Similar results have been obtained in *Rhodopseudomonas spheroides*. CP III accumulation in suspensions of iron-deficient cells of this microbe was favoured by a distinct amount of manganese (LASCELLES, 1956). When relatively large amounts of ethionine had been added to cultures of *R. sphaeroides* CP III accumulation occurred independently of the iron or manganese supply of the bacteria (GIBSON et al., 1962).

Although the effect of manganese on CP III accumulation in *Arthrobacter*, strain 223, closely resembled the manganese effect found in *R. spheroides*, no detailed studies were carried out to elucidate the action of this metal because it seems to be involved in several reactions of different types, e.g. the formation of higher fatty acids (WAKIL et al., 1958, 1960).

In the experiments carried out in the present chapter, medium B_3 supplied with 0.05 µgMn/ml was used. Therefore, B_3 as used in the following pages always means the manganese-enriched B_3 medium.

In the course of the present investigation, several compounds have been shown to favour the formation of heme. The effect of all these compounds was ascribed to their ability to serve as donor of one carbon units, required for the synthesis of serine, which is needed for PE formation. The effect of a number of these compounds was studied in relation to the iron supply of the bacteria. The results of these experiments (Table VI-2a) show that omission of either cysteine, methionine, or ethanolamine prevented accumulation of CP III in iron-deficient cultures while the heme content of the cells was also low. The presence of iron under these circumstances in a number of cases brought about CP III accumulation. The latter results are in agreement with those obtained in chap-

TABLE VI-2. a. The formation of porphyrins by *Arthrobacter*, strain 223, as effected by iron. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 80 hours. b. The effect of modified concentrations of NH_4Cl and K_2HPO_4 on porphyrin formation as affected by the iron sypply of the bacteria.

a. Compound omitted from medium B_3	Porphyrin (mμMol/ml culture)			
	− Iron		+ Iron (0.5μg/ml)	
	CP III	Heme	CP III	Heme
—	28	0.1	0	3.8
Cysteine	0	0.3	10	1.0
Methionine	0	0.2	22	0.4
Ethanolamine	0	0.1	26	0.5
b. Nutrient salt (%, w/v)				
0.25 NH_4Cl + 0.1 K_2HPO_4	28	0.1	0	3.9
0.05 K_2HPO_4	40	0.1	0	3.8
0.4 NH_4Cl + 0.1 K_2HPO_4	31	0.2	0	4.2

ter IV and V. Table VI-2b gives the effect of different concentrations of NH_4Cl and K_2HPO_4 on porphyrin formation in *Arthrobacter* cultures with a different iron supply. The only effect observed was that reduction of the amount of K_2HPO_4 slightly stimulated CP III accumulation.

2.2. *The effect of increasing amounts of iron on cell yield and porphyrin formation*

In a subsequent experiment, the effect of the iron content of the medium on

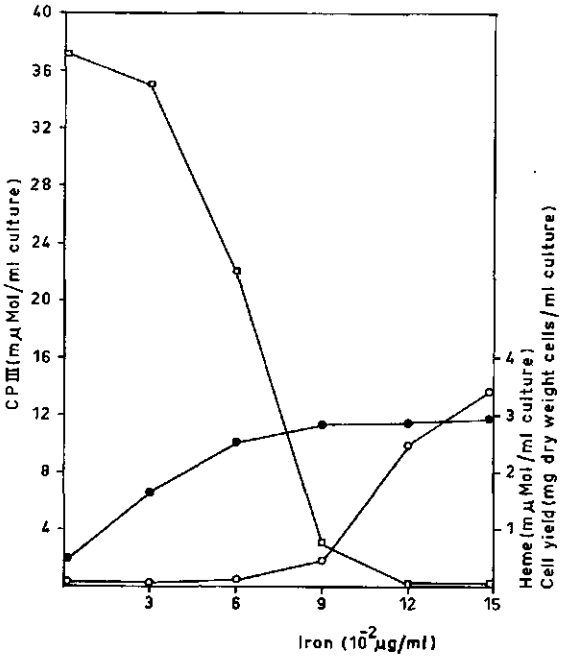


FIG. VI-1. The effect of different amounts of iron added to iron-deficient media B_3 on cell yield (●), accumulation of CP III (□) and heme formation (○) in cultures of *Arthrobacter*, strain 223. Sulphite oxidation rate: 11 mMol O_2/liter hr. Incubation for 96 hours.

cell yield, CP III accumulation and heme formation was studied. The results of this experiment, plotted in Figure VI-1, show that the growth of *Arthrobacter*, strain 223, was considerably reduced by iron deficiency. This observation corroborated the data recorded in Table III-5. An increased iron supply stimulated heme formation, whereas CP III accumulation dropped to zero. For maximum heme formation a larger amount of iron was required than for the elimination of porphyrin accumulation. The same has been found for the formation of bacteriochlorophyll in *Rhodopseudomonas spheroides*. The amount of iron required to prevent CP III accumulation in *Arthrobacter*, strain 223, was approximately equal to that found in *R. spheroides* (LASCELLES, 1956) but it was much smaller than that found in *R. capsulatus* (COOPER, 1963) and in some strains of *Corynebacterium diphtheriae* (CLARKE, 1958). The difference in the composition of the media used for cultivating these bacteria as well as the difference in uptake and assimilation of iron may be responsible for these discrepancies.

The quantity of heme formed by *Arthrobacter*, strain 223, in the medium with 0.12 μg/ml of iron was about 1/15 of the amount of CP III found in the culture without added iron.

2.3. *The effect of heme and PP IX on CP III accumulation*

The influence of heme and PP IX on CP III accumulation in cultures of *Arthrobacter*, strain 223, was studied by adding different amounts of these compounds to nutrient solutions in which CP III accumulation might be expected.

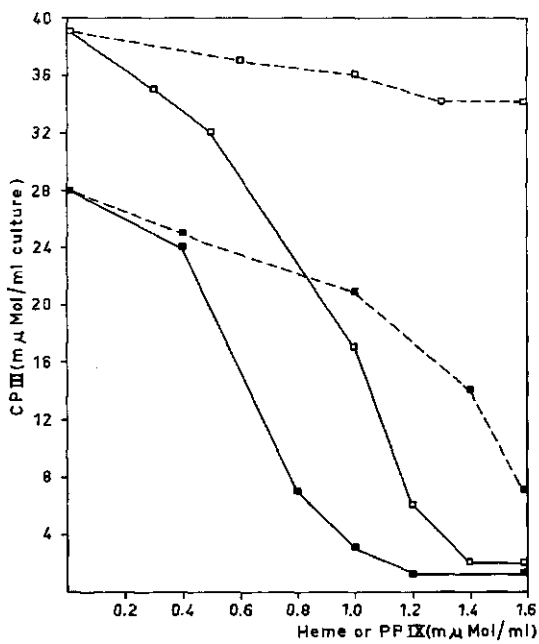


FIG. VI-2. The effect of heme (———) and PP IX (- - - -) on the accumulation of CP III in iron-deficient cultures (□) of *Arthrobacter*, strain 223, growing in medium B_3 and iron-sufficient cultures (■) growing medium A. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 96 hours.

In the case of iron-deficient cultures medium B_3 was used, in the case of iron-sufficient cultures medium A. The porphyrins were added at the time of inoculation and had completely been taken up by the bacteria at the end of the experiment.

From the data plotted in Figure VI-2 it will be seen that added heme practically entirely prevented CP III accumulation in both iron-deficient and iron-sufficient cultures. The amount of heme required to prevent the accumulation of CP III was of the same order as the amount formed by cells growing in media with 0.1 μgFe^{+++}/ml, the lowest iron level at which no accumulation of CP III occurred (Fig. VI-1). PP IX to a large extent eliminated the accumulation of CP III in iron-sufficient cultures, but not in iron-deficient cultures. The explanation for this different response is that in the former but not in the latter cells PP IX was rapidly converted to heme. Instead of CP III no precursors of CPG III accumulated in the cultures growing in the heme-containing media.

From these results it may be concluded that the amount of heme formed by cells of *Arthrobacter*, strain 223, is regulating the formation of CPG III. This could be due either to a reduced supply of substrates (glycine and succinyl-CoA) for ALA formation, or to a repressing or inhibiting effect on the enzymes catalysing the conversion of these substrates to CPG III. Since addition of glycine + glutamic acid to iron-deficient, heme-containing medium B_3 did not restore CP III accumulation, lack of substrate was not the cause of the strongly reduced CP III accumulation in the cultures growing in the heme-containing media.

2.4. *The main site of the regulation of porphyrin formation*

To study the controlling action of heme on CP III accumulation, an experiment was carried out to localize the main site for this control mechanism. Heme and ALA were added to media in which during the growth of *Arthrobacter*, strain 223, accumulation of CP III occurred. Both compounds were completely taken up by the cells. The results show (Fig. VI-3) that the addition of ALA restored CP III accumulation. Since glycine and succinyl-CoA served as substrates for CPG III formation in media without added ALA, the results point to ALA synthetase as the principal site for a control mechanism exerted by heme. Thus more glycine and succinyl-CoA are converted to ALA by cells with a low heme content than by cells with a high heme content. Similar results have been obtained in *Rhosopseudomonas spheroides* (BURNHAM and LASCELLES, 1963).

To obtain more detailed information about the inhibition of the activity of ALA synthetase, an experiment was carried out in which at different times a physiological amount of heme was given to cultures of *Arthrobacter*, strain 223. The increase of the amount of CP III was about halved by the heme added (Fig. VI-4) showing that the activity of ALA synthetase of *Arthrobacter* is not completely inhibited by heme as had been observed in *Rhodopseudomonas spheroides* (LASCELLES, 1964). If this were the case, no increase of the amount of CP III would have taken place after the addition of heme.

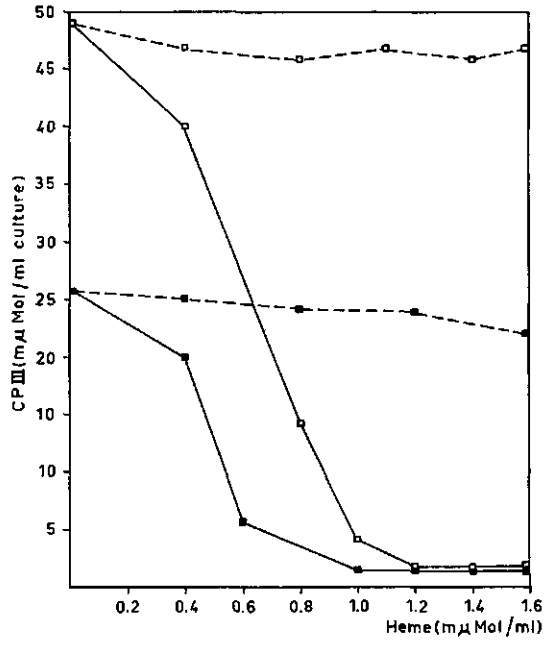


FIG. VI-3. The effect of heme on the accumulation of CP III in iron-deficient cultures (◧) of *Arthrobacter*, strain 223, growing in medium B_8 and in iron-sufficient cultures (■) growing in medium A. The cultures with (- - - -) and without (——) ALA (300 μg/ml) were incubated for 96 hours. Sulphite oxidation value: 11 mMol O_2/liter hr.

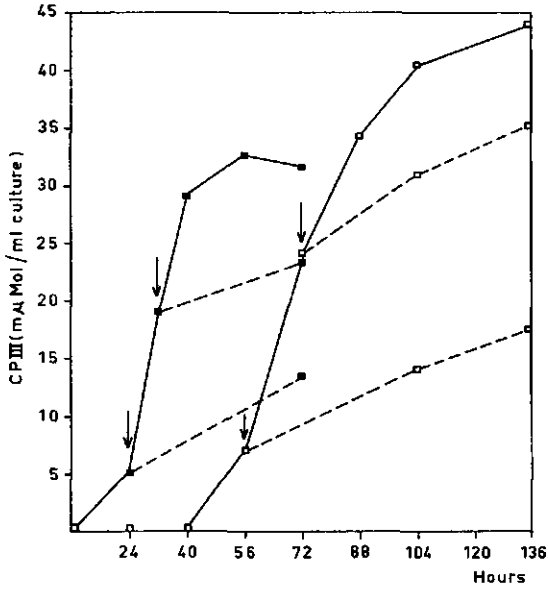


FIG. VI-4. The effect of heme (1.4 mμMol/ml) on the accumulation of CP III (- - - -) in iron-deficient (□) and iron-sufficient (■) cultures of *Arthrobacter*, strain 223, growing in medium B_8 and A, respectively. Heme was added to control cultures (———) at different times (arrows) and the incubation continued. Sulphite oxidation value: 11 mMol O_2/liter hr.

A comparison of the curves of Figures VI-2 and 4 shows that heme suppressed CP III accumulation completely when added at the beginning of the experiment (Fig. VI-2) but only partly when added to cultures accumulating CPG III (Fig. VI-4). This suggests that the complete inhibition of CP III accumulation by heme may be brought about partly by inhibition of ALA synthetase activity (feedbak control) and partly by repression of ALA synthetase formation.

2.5. *The effect of heme on the conversion of ALA to CPG III*

The results reported in Figure VI-3 might show that the enzymes catalysing the conversion of ALA to CPG III were not controlled by heme. To obtain more evidence as to this conclusion, *Arthrobacter*, strain 223, was inoculated into medium A and cultivated at an aeration rate of 11 mMol O_2/liter hr. When CP III accumulation had just started, the cultures were submitted to 4 mMol O_2/liter hr and supplied with ALA. As shown in chapter III, reduction of the aeration rate to this level immediately stopped CP III accumulation. This was found to be due to a curtailed supply of substrates for ALA formation. The addition of ALA to these cultures restored CP III accumulation. When in addition heme was added, the ultimate amount of CP III was only slightly lowered (Fig. VI-5). However, heme reduced the rate of CP III accumulation considerably. This could either be due to repression of the enzymes concerned or to feedback inhibition.

To distinguish between these two possibilities *Arthrobacter*, strain 233, was inoculated into medium A. When the CP III concentration had reached a value

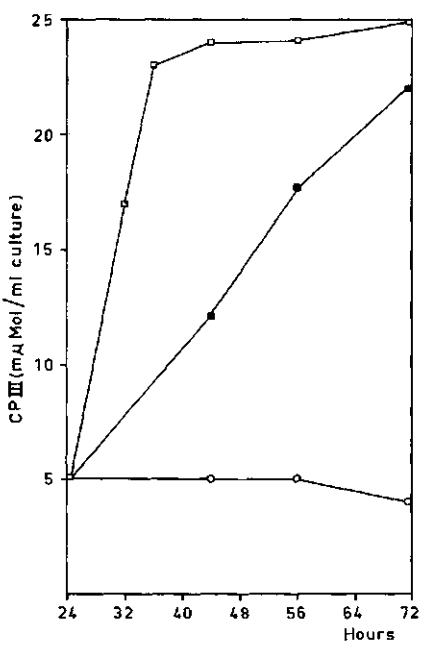


FIG. VI-5. The effect of heme (1.4 mμMol/ml) on the accumulation of CP III in iron-sufficient cultures of *Arthrobacter*, strain 223, (medium A) supplied with ALA (125 μg/ml). The bacteria were grown at an aeration rate of 11 mMol O_2/liter hr, for 24 hours. At that time the cultures were submitted to 4 mMol O_2/liter hr (○), supplied with ALA (□) and ALA + heme (■).

TABLE VI-3. The effect of heme on CPG III formation from ALA by cell suspensions of *Arthrobacter*, strain 223. Bacteria were grown during 40 hours in medium A, before being collected by centrifugation. The incubation mixture contained per ml: 3 mg cells (dry weight), phosphate buffer pH 7.0, $MgSO_4.7H_20$ (0.05%, w/v) and ALA (250μg/ml). Incubation time 24 hours.

Heme added (mμMol/ml)	CP III (mμMol/ml)
0	37
0.2	41
0.4	38
0.8	35
1.2	39
1.6	40
2.0	37
4.0	40

of 20 mμMol/ml, the bacteria were harvested by centrifugation, washed once with 0.75% (w/v) NaCl and suspended in a phosphate buffer of pH 7.5. ALA + heme were given to these suspensions. From the data recorded in Table VI-3 it will be seen that heme did not affect CPG III formation from ALA. Hence it is improbable that the activity of the enzymes catalysing the conversion of ALA to CPG III was inhibited by heme. The decreased rate of CPG III formation from ALA by growing cells when incubated with heme (Fig. VI-5) probably depended on the repression of the enzymes catalysing these reactions.

Summarizing the results with whole cells reported in the foregoing sections, it can be stated that the main effect of heme on tetrapyrrole synthesis in *Arthrobacter*, strain 223, was the reduction of the conversion of glycine and succinyl-CoA to ALA. ALA synthetase was repressed as well as inhibited by heme.

No evidence was found concerning an inhibitory effect of heme on the activity of the enzymes catalysing the conversion of ALA to CPG III. The effect of heme on the rate of these reactions presumably depended on repression of these enzymes.

2.6. *The effect of heme on the activity of the enzymes ALA synthetase and ALA dehydratase*

The preparation of cell-free extracts was as follows. Bacteria were collected by centrifugation, washed once with glass-distilled water and suspended in 20 ml glass-distilled water (5 mg dry weight/ml). When necessary these suspensions were stored at –20 °C before preparing the extracts. The cells were disrupted by shaking (4000/min.) twice with glass beads (Ø: 0.25–0.30 mm) for 1 minute after being cooled at 4 °C for 2 hours (Zellhomogenisator, Braun, Melsungen). Whole organisms and debris were removed by centrifugation at 5,000 × *g* for 20 minutes at 4 °C. This crude extract was divided into a soluble fraction and a particulate fraction by centrifugation at about 100,000 × *g* for 90 minutes. Temperature never exceeded 15 °C.

TABLE VI-4. The localization of the enzymes δ-aminolevulinic acid (ALA) synthetase and ALA dehydratase in *Arthrobacter*, strain 223, grown in medium A for 36 hours. Sulphite oxidation value: 11 mMol O_2/liter hr.

Fraction of cell-free extract	ALA synthetase (units/mg protein/hr)	ALA dehydratase (units/mg protein/hr)
Soluble	0	77
Particulate	56	0

The reaction mixture for the estimation of the activity of ALA synthetase (LASCELLES, 1959) contained: 0.2 ml extract of organism; phosphate buffer (pH 7.6), 0.1 Mol; $MgSO_4.7H_2O$, 50 μMol; cysteine, 2 μMol; coenzyme A, 0.2 μMol; pyridoxal phosphate, 0.2 μMol; ATP, 16 μMol; glycine, 200 mμMol; succinic acid, 200 mμMol; water to 2 ml. The reaction was carried out at 30 °C and stopped after 1 hour by adding 10 % (w/v) trichloroacetic acid.

The reaction mixture for the estimation (LASCELLES, 1959) of ALA dehydratase activity contained: 0.2 ml extract of the organism; phosphate buffer (pH 7.6) 0.1 M; $MgSO_4.7H_2O$, 50 μMol; cysteine, 2 μMol; δ-aminolevulinic acid (ALA), 200 mμMol; water to 2.0 ml. The reaction was stopped after 1 hour by adding 10 % (w/v) trichloroacetic acid.

One unit of ALA synthetase or ALA dehydratase is defined as the amount catalysing the formation of 1 mμMol of ALA or porphobilinogen (PBG), respectively. ALA and PBG were estimated as indicated in chapter II.

From Table VI-4 it will be seen that ALA synthetase activity was entirely localized in the crude particulate fraction, whereas ALA dehydratase activity was only present in the soluble fraction of *Arthrobacter*, strain 223. Similar results have been obtained by GRANICK (1966) who found that ALA synthetase of liver was located in the mitochondria.

Table VI-5 shows that ALA synthetase was moderately inhibited by heme in

TABLE VI-5. The inhibition of δ-aminolevulinic acid (ALA) synthetase and ALA dehydratase by heme (iron protoporphyrin). *Arthrobacter*, strain 223, was grown for 44 hours in medium A before being harvested. Sulphite oxidation value: 11 mMol O_2/liter hr.

Heme added (mμMol)	ALA synthetase (units/hr)*	ALA dehydratase (units/hr)**
–	48	83
0.1	49	80
0.2	37	87
0.4	33	86
0.8	29	80
1.2	25	84
1.6	22	89
2.0	24	85

* 1.4 mg protein
** 0.7 mg protein

contrast to ALA dehydratase which was unaffected. The latter fact accounts for the observation that porphyrin formation from ALA by cell suspensions (Table VI-3) was unaffected by heme. The same is true of the porphyrin formation from ALA by growing cells (Fig. VI-3). The partial inhibition of ALA synthetase by heme is in agreement with the results of experiments with growing cells, which show that feedback inhibition of ALA synthetase by heme might be involved in the elimination of CP III accumulation by heme.

2.7. *The effect of heme and iron on the synthesis of ALA synthetase and ALA dehydratase*

To confirm the results of the experiments with whole cells that the synthesis of ALA synthetase and ALA dehydratase was repressed by heme, experiments with cell-free extracts were carried out. *Arthrobacter*, strain 223, was inoculated into medium B_3 supplied either with heme (added as hemin) or iron. After 72 hours incubation the activity of the enzymes was estimated. Heme had considerably reduced the synthesis of both enzymes (Table VI-6). The presence of iron in the medium affected both enzymes in the same way as heme, added to the medium. It may therefore be concluded that iron exerted its action on the enzymes ALA synthetase and ALA dehydratase by way of its incorporation into heme. The results of this experiment together with those reported in Table VI-5 (inhibition of ALA synthetase) may account for the fact that heme (or iron) almost completely suppressed porphyrin formation from glycine and succinyl-CoA (Fig. VI-3). Heme inhibited ALA synthetase but not ALA dehydratase activity. Both enzymes were repressed to about the same extent while CPG III formation from ALA was not reduced by heme. It might therefore be concluded that the feedback inhibition of ALA synthetase is a more important process in the regulation of porphyrin formation in *Arthrobacter* than the repression of ALA synthetase and ALA dehydratase.

The results of experiments with cell-free extracts of *Arthrobacter*, strain 223, are in agreement with those of *Rhodopseudomonas spheroides*, except that in contrast to *Arthrobacter*, the enzyme ALA synthetase was found completely inhibited by heme (BURNHAM and LASCELLES, 1963). The variable values obtained for the activity of ALA synthetase in *Arthrobacter* are probably due to the instability of this enzyme. Storage for 48 hours at 4 °C of the crude membrane preparations resulted in the complete loss of the activity of this enzyme.

TABLE VI-6. The effect of iron and heme on the synthesis of δ-aminolevulinic acid (ALA) synthetase and ALA dehydratase. *Arthrobacter*, strain 223, was grown in medium B_3 for 72 hours. Sulphite oxidation value: 11 mMol O_2/liter hr.

Growth conditions	ALA synthetase (units/mg protein/hr)	ALA dehydratase (units/mg protein/hr)
Iron-deficient	61	68
Iron-deficient + heme (5mμMol/ml)	13	22
Iron-sufficient	18	14

2.8. *The effect of saturated fatty acids on the accumulation of CP III in iron-deficient cultures of Arthrobacter, strain 223*

Iron, in addition to its function as substrate for heme, catalyses the conversion of CPG III to PP IX, owing to its favourable effect on the formation of phosphatidylethanolamine (PE), the compound assumed to be responsible for the above reaction in *Arthrobacter*, strain 223. As a result of the latter function, iron deficiency of cultures of *Arthrobacter*, strain 223, gives rise to a reduced conversion of CPG III to PP IX, thus to a reduced heme formation. The heme-depending control mechanism fails to function and large amounts of CP III may accumulate (Fig. VI-1).

To test the effect of iron on PE formation (as a result of its stimulating action on the formation of unsaturated fatty acids; see introduction of this chapter) experiments with different types of fatty acids have been performed. The fatty acids were dissolved in a suitable amount of a methanol: acetone (1:1, v/v) mixture and sterilized together with iron-deficient medium B_3. Special attention was given to the unsaturated fatty acids palmitoleic acid and oleic acid, because these fatty acids are widely distributed in non-photosynthetic bacteria (KANESHIRO and MARR, 1961; FULCO and BLOCH, 1964).

The synthesis of heme was hardly enhanced by supplying several saturated fatty acids to the iron-deficient medium B_3 (Table VI-7). However, the following combinations of fatty acids were able to reduce the amount of CP III: myristic acid + palmitic acid, myristic acid + stearic acid and palmitic acid + stearic acid. The effect of these combinations of fatty acids on CP III accumulation might depend on their conversion to the corresponding unsaturated fatty acids by the bacteria, utilizing the iron present as an impurity in iron-deficient medium B_3. The fact that without added iron bacterial growth was possible supports this idea.

The growth of *Arthrobacter*, strain 223, in iron-deficient medium B_3 was only slightly affected by supplying several saturated fatty acids. The presence of

TABLE VI-7. The effect of saturated fatty acids in iron-deficient medium B_3 on the formation of porphyrins in cultures of *Arthrobacter*, strain 223. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 96 hours.

Fatty acid (3μg/ml) added	Porphyrins (mμMol/ml culture)	
	CP III	Heme
–	46	0.1
Acetic acid	38	0.2
Butyric acid	35	0.2
Caproic acid	30	0.1
Caprylic acid	36	0.0
Myristic acid	31	0.1
Palmitic acid	30	0.1
Stearic acid	37	0.2
Myristic acid + palmitic acid	21	0.3
Myristic acid + stearic acid	15	0.2
Palmitic acid + stearic acid	17	0.1

palmitoleic acid + stearic acid gave only 15% more cell yield providing evidence for the almost complete absence of iron in the amounts of these acids added.

2.9. *The effect of unsaturated fatty acids on the CP III accumulation in iron-deficient cultures of Arthrobacter, strain 223*

The unsaturated fatty acids oleic acid and palmitoleic acid when added separately did not prevent the accumulation of CP III (Fig. VI-6). Supplied along with myristic, palmitic or stearic acid, the accumulation of CP III was completely prevented. The elimination of CP III accumulation by these fatty acids was thought to be the result of an improved PE formation. This idea is supported by the observation of KANESHIRO and MARR (1961) that in bacteria myristic, palmitic, palmitoleic, oleic and stearic acids are usually esterified in phosphatidylethanolamine. It does, however, not necessarily mean that these fatty acids are also present in PE of *Arthrobacter*, strain 223. Before being incorporated into this phospholipid, conversion to closely related fatty acids might occur. It is probable that the addition of the above-mentioned mixture of fatty acids to an iron-deficient medium B_3 enhanced the formation of PE and consequently

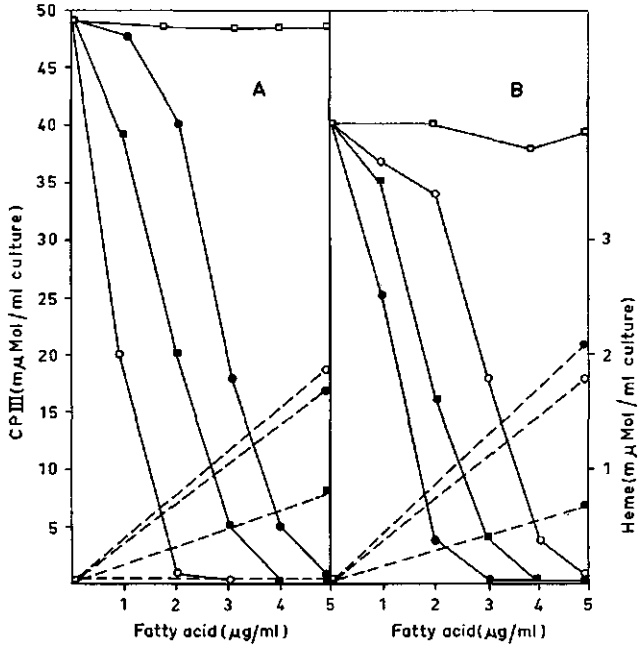


FIG. VI-6. The effect of unsaturated fatty acids on the formation of CPG III (———) and heme (- - - -) in iron-deficient cultures of *Arthrobacter*, strain 223. Medium B_3. Sulphite oxidation value: 11 mMol O_2/liter hr. Fatty acids added at time of inoculation. A. □: oleic acid; ●: oleic acid (3 μg/ml) + stearic acid; ■: oleic acid (3 μg/ml) + palmitic acid; ○: oleic acid (3 μg/ml) + myristic acid. B. □: palmitoleic acid; ○: palmitoleic acid (3 μg/ml) + stearic acid; ■: palmitoleic acid (3 μg/ml) + palmitic acid; ●: palmitoleic acid (3 μg/ml) + myristic acid.

the synthesis of heme which in turn completely prevented CP III accumulation. The fact that heme was formed implies that medium B_3 contained some iron as impurity, enabling the conversion of PP IX to heme.

The exceptions to the general rule that iron prevents the accumulation of CP III by micro-organisms may be explained by the theory of PE formation. FULCO and BLOCH (1964) found that the conversion of stearyl-CoA to oleic acid requires the following cofactors: iron, TPN, FAD and oxygen.

STICH and EISGRUBER (1951) demonstrated that the accumulation of porphyrin in the riboflavin-requiring yeast *Saccharomyces anamensis* could not be prevented by iron, but only by riboflavin. The yeast may synthesize insufficient amounts of unsaturated fatty acids under riboflavin-deficient conditions because this vitamin participates in the formation of FAD which is required for the synthesis of unsaturated fatty acids present in PE (and other phospholipids).

The function of pantothenate in the conversion of CPG III to PP IX in *Tetrahymena vorax* (LASCELLES, 1962) may also be based on the formation of unsaturated fatty acids since coenzyme A which contains pantothenate is required for the synthesis of unsaturated fatty acids.

Ethionine eliminated the effect of iron on the accumulation of CP III by iron-deficient cells of *Rhodopseudomonas spheroides* (GIBSON et al., 1962). This may have been due to the adverse effect of ethionine on the synthesis of phospholipids in this bacterium. ROBINSON and SEAKENS (1962) have found that the synthesis of phospholipids in livers of rats is markedly decreased by ethionine, resulting in the accumulation of triglycerides in these organs. The composition of the nitrogen-containing phospholipids of *R. spheroides* closely resembled that of liver (HAVERKATE, 1965).

2.10. *The accumulation of PP IX in an iron-deficient medium containing fatty acids*

The presence of small amounts of myristic acid (2 μg/ml) and oleic acid (2 μg/ml) in an iron-deficient nutrient solution B_3 resulted in a considerable porphyrin accumulation, particularly PP IX. The presence of iron in this medium resulted in an increased synthesis of heme (Fig. VI-7) which was attended with a reduction of the amount of PP IX formed. The quantity of iron to prevent this accumulation was approximately 0.7 μg/ml, i.e. seven times larger than the quantity of iron preventing CP III accumulation. This result closely resembled the results obtained by COOPER (1963) with *Rhodopseudomonas capsulatus*. In this photosynthetic micro-organism far more iron was required to prevent the accumulation of Mg protoporphyrin monomethyl ester than to prevent CP III accumulation. This chlorophyll intermediate was produced by incubating the cells in a medium containing Tween 80. Although there is a certain similarity between the media used for *Arthrobacter*, strain 223, and *R. capsulatus*, the requirement of these large quantities of iron is obscure. A different rate of uptake of iron under different conditions may be an explanation.

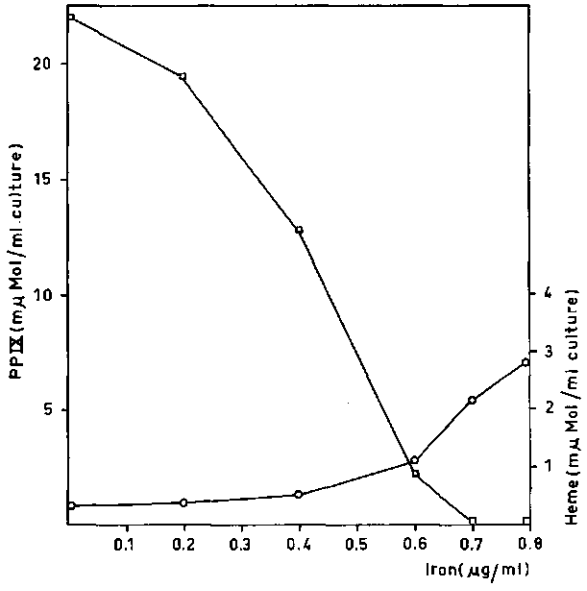


FIG. VI-7. The effect of iron on the accumulation of PP IX (□) and the formation of heme (○) in cultures of *Arthrobacter*, strain 223, growing in iron-deficient medium B_3 supplied with oleic acid (2 μg/ml) and myristic acid (2 μg/ml). Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 96 hours.

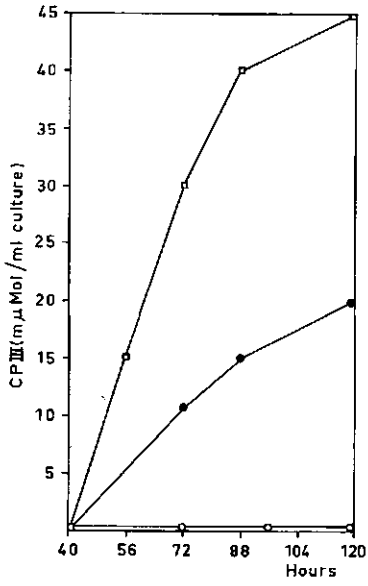
2.11. *The aeration rate and the accumulation of CP III in iron-deficient cultures of Arthrobacter, strain 223*

Experiments recorded in chapter III have shown the important effect of the aeration rate of cultures of *Arthrobacter*, strain 223, growing in iron-sufficient medium A, on the accumulation of CP III. Low and high aeration rates (4 and 44 mMol O_2/liter hr, respectively) prevented CP III accumulation, which was maximal at 11 mMol O_2/liter hr. The cause of the failing CP III accumulation at the low aeration level was found to depend on the shortage of C 4 substrate for ALA formation. This explanation did not hold in the case of a high aeration rate. Addition of ALA, but not that of glycine + glutamic acid, restored CP III accumulation in cultures with a high aeration rate, be it to a less extent than at a low aeration rate.

The results obtained with cultures of *Arthrobacter*, strain 223, growing in an iron-deficient medium B_3 were similar to those with iron-sufficient cultures (compare Figures III-6 and 7 with VI-8A and B).

2.12. *The effect of a high aeration rate on the synthesis of the enzymes ALA synthetase and ALA dehydratase*

To study in which way a high aeration rate exerted its action on the accumulation of CP III, *Arthrobacter*, strain 223, was grown at an aeration rate of 11 and 44 mMol O_2/liter hr, respectively, in medium A for 48 hours. The cells were collected by centrifugation, washed with distilled water, disrupted with glass beads, and the cell-free extracts obtained was used for enzyme estimation. From Table VI-8 it will be seen that at a high aeration rate of 44 mMol O_2/liter hr the synthesis of ALA synthetase and ALA dehydratase was repressed for approxi-



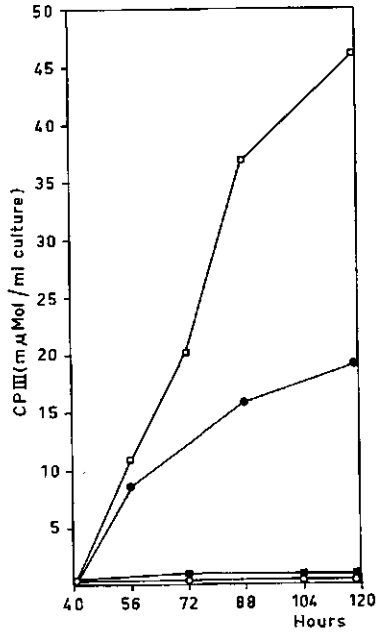


FIG. VI-8a. The effect of the aeration rate on the accumulation of CP III in cultures of *Arthrobacter*, strain 223, growing in iron-deficient medium B 3. The bacteria were grown at an aeration rate of 11 mMol O_2/liter hr (□) for 40 hours. At this time some cultures were transferred to 18 (●) and 44 (○) mMol O_2/liter hr.

FIG. VI-8b. The effect of ALA (250 μg/ml) and glutamic acid (200 μg/ml) + glycine (200 μg/ml) on the accumulation of CP III in cultures of *Arthrobacter*, strain 223, growing at 44 mMol O_2/liter hr in iron-deficient medium B 3. The precursors were added after 40 hours incubation. ○: no addition; ■: 1-glutamic acid and glycine added; ●: ALA added; □: ALA added and cultures submitted to 18 mMol O_2/liter hr.

mately 70%. This repression does not fully account for the effect of a high aeration rate on CP III accumulation because the aeration level of 44 mMol O_2/liter hr suppressed CP III accumulation completely. Therefore, it may be assumed that a high aeration rate in addition to the repression of ALA synthetase and ALA dehydratase inhibits the action of these enzymes. In *Arthrobacter*, strain 223, ALA synthetase inhibition at a high aeration rate apparently was much more pronounced than ALA dehydratase. The inhibition of the ac-

TABLE VI-8. The effect of a high aeration rate on the synthesis of the enzymes δ-aminolevulinic acid (ALA) synthetase and ALA dehydratase in *Arthrobacter*, strain 223, grown in medium A for 34 hours.

Aeration rate (mMol O_2/liter hr)	ALA synthetase (units/mg protein/hr)	ALA dehydratase (units/mg protein/hr)
11	40	96
44	12	27

tivity of these enzymes by a high oxygen tension has been clearly demonstrated by FALK et al. (1959).

3. SUMMARY AND CONCLUSIONS

The effect of iron on the formation of different types of porphyrin by *Arthrobacter*, strain 223, was studied in a medium with optimal amounts of one carbon units and manganese (medium B_3). As shown in the previous chapter no coproporphyrin III (CP III) accumulation occurred in this medium when adequate amounts of iron were present.

Reducing the iron concentration of that medium brought about a decreased heme formation, attended with an accumulation of CP III and ultimately a reduced cell yield. Under certain conditions no CP III accumulation occurred in the iron-deficient medium B_3 (omission of cysteine, methionine or ethanolamine). No study was made to explain the effect of these conditions.

It was thought that iron has at least two functions in porphyrin formation in *Arthrobacter*, viz. a. it is required for the synthesis of (iron-containing) heme from (iron-free) protoporphyrin IX (PP IX), and b. it may contribute (via its stimulating effect on the conversion of saturated to unsaturated fatty acids) to the synthesis of phosphatidylethanolamine (PE), which is required for the transformation of CPG III into PP IX, and ultimately heme. The latter compound (as a result of repression and feedback control) may regulate porphyrin formation.

The control of porphyrin synthesis in *Arthrobacter*, strain 223, was studied by adding increasing amounts of heme (added as hemin) to culture media in which CP III accumulation occurred either in the absence (medium B_3) or in the presence (medium A) of adequate amounts of iron. In both cases CP III accumulation was prevented. Porphyrin accumulation was restored by adding δ-aminolevulinic acid (ALA), but not by the addition of glycine + glutamic acid. This demonstrates that ALA synthetase, the enzyme catalysing the conversion of glycine and succinyl-CoA to ALA, is the main site for the regulation of porphyrin synthesis by heme. This enzyme was found to be localized in the crude membrane fraction.

Further experiments with whole cells of *Arthrobacter* showed that porphyrin formation is regulated by repression of the synthesis and by inhibition of the activity (feedback control) of ALA synthetase. This was confirmed by experiments with cell-free extracts.

The conversion of ALA to CPG III by growing cells of *Arthrobacter* was slightly depressed by added heme. The formation of porphyrins from ALA by cell suspensions, was not affected by heme indicating that the activity of the enzymes responsible for this conversion, was not involved. The observed effect of heme, therefore, had to be attributed to enzyme repression.

Heme added to cell-free extracts did not inhibit the activity of ALA dehydratase, one of the enzymes responsible for the conversion of ALA to CPG III.

Added to growing cells it considerably reduced the synthesis of ALA dehydratase, indicating that this enzyme was repressed by heme.

Iron, added to iron-deficient medium B_3 brought about the same reduction of ALA synthetase and ALA dehydratase synthesis as heme added to this medium, showing that iron affected porphyrin accumulation as a result of its incorporation into heme.

The second important function of iron in porphyrin metabolism was shown by supplying different types of fatty acids to iron-deficient medium B_3. Large amounts of CP III accumulated in this medium in the absence or presence of several saturated fatty acids, or of the unsaturated fatty acids palmitoleic acid or oleic acid. However, mixtures of palmitoleic acid or oleic acid with one of the following saturated fatty acids: myristic, palmitic or stearic acid, prevented CP III accumulation and enhanced heme formation.

The presence of small amounts of myristic acid and oleic acid in iron-deficient medium B_3 gave rise to the accumulation of PP IX. To prevent the accumulation of this porphyrin, iron in amounts many times higher than those required for preventing CP III accumulation had to be supplied.

The accumulation of porphyrins in both iron-sufficient (A medium) and iron-deficient (B_3 medium) cultures depended on the aeration rate of the culture medium. No CP III accumulation occurred at low and high aeration rates, respectively. Lack of substrate (C_4 compound) for ALA formation was the cause in the former case while in the latter case the synthesis of the enzymes ALA synthetase and ALA dehydratase was reduced for approximately 70%. In addition to the reduced synthesis of these enzymes, inhibition of their activity by high aeration rates was presumably involved.

CHAPTER VII

THE SYNTHESIS OF PORPHYRINS BY BIOTIN-DEFICIENT CELLS OF *ARTHROBACTER*, STRAIN 223

1. Introduction

Rhodopseudomonas spheroides grown in iron-deficient medium with sub-optimal amounts of biotin accumulated no porphyrin as contrasted with similar media containing adequate amounts of biotin. Lascelles (1960, 1962), who described this phenomenon, did not exclude the possibility that biotin as coenzyme participates in the enzyme ALA synthetase.

The aim of the experiments described in the present chapter was to test this biotin effect with *Arthrobacter*, strain 223. In this investigation use was made of an earlier observation with *Arthrobacter*, strain 252 (isolated from cheese) that the addition of large amounts of threonine to heme-forming cells resulted in the accumulation of CP III.

This effect seemed to be at variance with that reported in chapter IV where small quantities of threonine reduced the quantity of CP III when this amino acid was present in the nutrient solution at the beginning of the experiment. However, it should be kept in mind that in these two series of experiments a. the quantities of threonine used were quite different, b. the time of addition of threonine varied markedly and c. the amount of biotin in the nutrient solution was not the same.

2. Results and discussion

2.1. *The influence of the biotin level of the medium on the formation of porphyrins by Arthrobacter, strain 223*

In order to study the effect of biotin on the formation of porphyrins, 1 ml of the usual subculture of strain 223 was inoculated into medium A containing increasing concentrations of this vitamin. After incubation for 48 hours, CP III and heme were determined. Figure VII-1 shows that increased concentrations of biotin, up to 10^{-4} μg/ml, gave increased amounts of heme and cell material. However, with higher concentrations the heme content fell sharply, whereas CP III accumulated. The ratio CP III (biotin-rich cells) : heme (biotin-deficient cells) was 5:1. The results of this experiment show that in biotin-deficient cells the enzyme ALA synthetase must be active because heme was formed by these cells.

2.2. *The effect of threonine on porphyrin formation by biotin-deficient cultures of Arthrobacter, strain 223*

In a subsequent experiment biotin-deficient cells of *Arthrobacter*, strain 223,

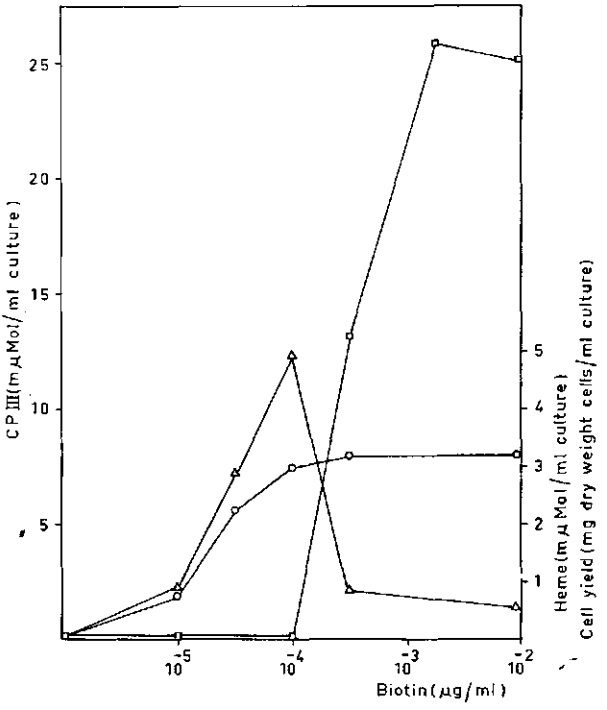


FIG. VII-1. Cell yield (○), CP III accumulation(□) and heme formation (△) in cultures of *Arthrobacter*, strain 223, grown in medium A containing different concentrations of biotin. Sulphite oxidation value: 11 mMol O_2/liter hr.

were tested for their ability to accumulate porphyrin by supplying the amino acid threonine. Since large amounts of this amino acid prevented the growth of this bacterium when added to the medium, increasing amounts were added 21 hours after inoculation when the growth had already started. Cell yield and heme were estimated after a total incubation period of 96 hours. Table VII-1 shows that the reduction of the heme formation which was attended with the accumulation of CP III increased with the amount of threonine added. The quantity of cell material was hardly influenced by threonine.

In another experiment where 62.5 μg/ml threonine was added, CP III accumulated at a considerably lower biotin concentration than without threonine (Fig. VII-2) Even when 2.5×10^{-5} μg/ml biotin was present in the nutrient solution, a large amount of CP III was found in the culture. The results of these two experiments clearly show that biotin-deficient cultures of *Arthrobacter*, strain 223, were capable of excreting large amounts of porphyrin which presumably must have been the result of the active enzyme ALA synthetase. In other words biotin does not participate as coenzyme in the enzyme ALA synthetase.

An enhancing effect of threonine on the accumulation of CP III has also been observed in *Rhodopseudomonas spheroides* (GIBSON et al., 1962). Iron in this bacterium eliminated the threonine-induced porphyrin formation. The effect

TABLE VII-1. The effect of threonine on cell yield and the synthesis of porphyrins in biotin-deficient cultures of *Arthrobacter*, strain 223. Sulphite oxidation value: 11 mMol O_2/liter hr. Medium A with 5×10^{-5} µg/ml biotin. Threonine was added 21 hours after inoculation. Incubation for 96 hours.

Threonine added (µg/ml)	Cell yield (mg dry weight cells/ml culture)	CP III (mµMol/ml culture)	Heme (mµMol/ml culture)
–	2.7*	0	3.6
12.5	2.6	0	2.4
25.0	2.5	3	1.9
37.5	2.6	9	1.0
50.0	2.6	16	0.8
62.5	2.7	20	0.5
75.0	2.6	24	0.5
87.5	2.7	22	0.6

* This amount is about 90% of that obtained with an optimal biotin supply.

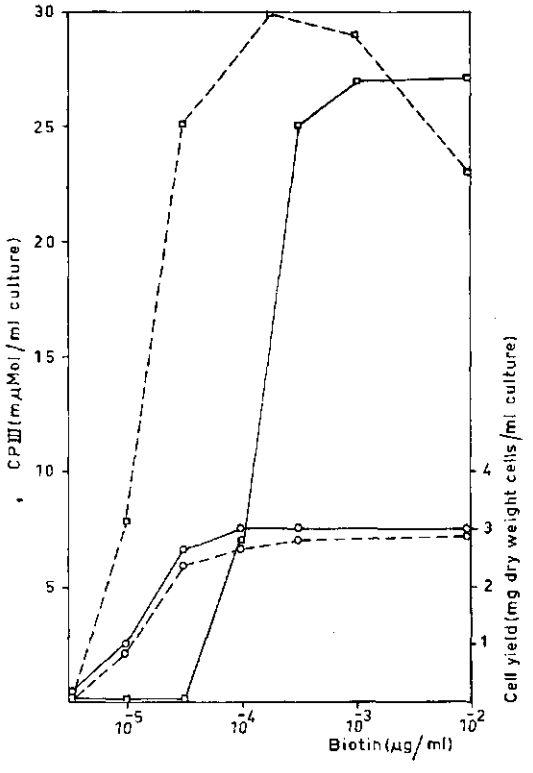


FIG. VII-2. Cell yield (○) and accumulation of CP III (□) in cultures of *Arthrobacter*, strain 223, grown in the absence (———) and presence (- - - -) of threonine with different concentrations of biotin. Threonine (62.5 µg/ml) was added to medium A 26 hours after inoculation. Sulphite oxidation value: 11 mMol O_2/liter hr.

TABLE VII-2. The effect of iron on the accumulation of CP III caused by the addition of threonine to biotin-deficient cultures of *Arthrobacter*, strain 223. Medium A with 5×10^{-5} μg/ml biotin. Sulphite oxidation value: 11 mMol O_2/liter hr. Threonine (75 μg/ml) added together with iron 26 hours after inoculation.

Iron added (μg Fe^{+++}/ml)	CP III (mμMol/ml culture)
0.1	8
0.2	25
0.3	27
0.4	24
0.5	29
0.7	21
0.9	25
1.0	26

of iron on CP III accumulation in biotin-deficient cultures supplied with threonine was also examined in *Arthrobacter*, strain 223, by incubating the bacteria in iron-free A medium containing 5×10^{-5} μg/ml biotin. After 24 hours incubation threonine (75 μg/ml) was added to the cultures along with increasing quantities of iron. Table VII-2 shows that iron had a stimulating effect on CP III accumulation while larger quantities of iron did not decrease the amount of CP III, indicating that the effect of threonine on porphyrin formation by *Arthrobacter*, strain 223, is not identical with that in *Rhodopseudomonas spheroides*, where iron did prevent the accumulation of CP III induced by threonine.

To obtain information concerning the specificity of the effect of threonine on the formation of porphyrins by biotin-deficient *Arthrobacter*, strain 223, a number of other amino acids were added to cultures, growing at a biotin level of 5×10^{-5} μg/ml. The following amino acids added in amounts of 75 μg/ml did not induce CP III accumulation: aspartic acid, glutamic acid, glycine, leucine, isoleucine, valine, lysine, phenylalanine, histidine, tyrosine and cysteine, indicating that threonine among the amino acids tested specifically influenced the synthesis of heme in *Arthrobacter*, strain 223, by inhibiting the conversion of CPG III to PP IX. (This threonine effect was also demonstrated in other *Arthrobacter* strains).

2.3. *The elimination of the threonine induced CP III accumulation by one carbon units supplying compounds and some amino acids*

The effect of threonine had presumably to be ascribed to a decreased formation of one carbon units which resulted in serine deficiency and consequently deficiency of phosphatidylethanolamine (PE), the compound apparently involved in the conversion of CPG III to PP IX. To test this hypothesis some compounds which may serve as donor for one carbon units were added along with threonine. The amino acids homoserine, homocysteine and methionine were also tested because threonine is known to inhibit their formation. CP III and heme were determined after an incubation period of 96 hours. From Table VII-3 it will be seen that the addition of choline, N.N-dimethylglycine, N.N-

TABLE VII-3. The elimination by several compounds of CP III-accumulation caused by added threonine in cultures of *Arthrobacter*, strain 223. Sulphite oxidation value: 11m Mol O_2/liter hr. Medium A with 5×10^{-5} µg/ml biotin. Threonine and other compounds were added 24 hours after inoculation. Incubation time 96 hours.

Threonine added (µg/ml)	Compound added along with threonine (µg/ml)	CP III (mµMol/ ml culture)	Heme (mµMol/ ml culture)
–	–	0	4.2
75	–	29	0.4
	choline (10)	0	2.3
	N.N-dimethylglycine (10)	0	2.7
	N.N-dimethylethanolamine (10)	0	3.1
	N-monomethylethanolamine (10)	0	2.8
	ethanolamine (10)	0	3.0
	dl-homoserine (30)	0	1.9
	dl-homocysteine (30)	0	2.3
	l-methionine (30)	0	3.0
	l-serine (15)	0	3.4

dimethylethanolamine, N-monomethylethanolamine, ethanolamine and serine, respectively, prevented the accumulation of CP III by biotin-deficient cells in cultures supplied with large amounts of threonine and had a favourable effect on the formation of heme. In a subsequent experiment betaine, sarcosine and formaldehyde were found to have the same effect. The effect of all these compounds, except that of serine, may be ascribed to their ability to serve as donor for one carbon units required for the synthesis of serine from glycine. It is reasonable to assume that the porphyrin-accumulating cells had sufficient glycine at their disposal as glycine is the precursor of porphyrins.

The eliminating effect of homoserine and homocysteine on porphyrin accumulation can less easily be explained as these amino acids do not act as donors for one carbon units. Since the accumulation of CP III caused by threonine did not start until 24 hours after the addition of this amino acid, a stimulated formation of methionine in these cells may be a possible explanation. Methionine may serve as donor for one carbon units (via choline).

The results of this experiment show that the addition of threonine reduced the supply of one carbon units which adversely affected the formation of serine and consequently that of PE. This prevented the conversion of CPG III to PP IX and subsequently the formation of heme. As the regulation mechanism failed to function, large amounts of CP III accumulated. Why addition of threonine resulted in a shortage of one carbon units has not fully been elucidated. Although threonine is the precursor of glycine (cf. chapter V), the latter amino acid had not the same effect as threonine on porphyrin formation by biotin-deficient *Arthrobacter*, strain 223. When cells grown in a threonine-containing medium were incubated with this amino acid, glycine only was detected in the culture filtrate. This result is at variance with that obtained by MCGILLAVRY and MORRIS (1966) who found glycine and acetyl-CoA as decomposition pro-

ducts of threonine in *Arthrobacter*. In view of these results acetyl-CoA could be responsible for the accumulation of CP III in biotin-deficient cells supplied with threonine. This idea is supported by the fact that acetate, as contrasted with glycine, added to biotin-deficient cultures of *Arthrobacter*, strain 223, stimulated the accumulation of CP III. This might be ascribed to the reaction of acetate with glyoxylic acid (glyoxylic acid cycle), thus reducing the production of one carbon units from glyoxylate.

2.4. *The effect of methionine on the formation of porphyrins by biotin-deficient Arthrobacter, strain 223*

To see if methionine in relatively large amounts was able to bring about CP III accumulation in biotin-deficient cultures of *Arthrobacter*, strain 223, 1 ml of the usual subculture was inoculated into medium A with 5×10^{-5} μg/ml biotin and different amounts of sulfate and S-containing amino acids.

The results of this experiment show (Table VII-4) that CP III accumulated when 40 μg/ml methionine was present. However, this accumulation required relatively large amounts of sulfate.

The presence of threonine in the latter medium (or in a medium with cysteine) completely prevented the accumulation of CP III but not in the medium with homocysteine. This result indicated that the accumulation of CP III in medium A containing suboptimal amounts of biotin and supplied with methionine would depend on the action of homocysteine. The results obtained with biotin-rich cells grown in cysteine-containing media with large amounts of methionine led to the same conclusion. Thus the accumulation of CP III in biotin-deficient as well as in biotin-sufficient cultures of *Arthrobacter*, grown in cysteine-containing media with relatively large amounts of methionine might partly be attributed to the formation of homocysteine, an inhibitor of the formation of one carbon units from choline.

TABLE VII-4. The effect of methionine on the accumulation of CP III in biotin-deficient cultures of *Arthrobacter*, strain 223. Medium A with 5×10^{-5} μg/ml biotin. Sulphite oxidation value: 11 mMol O_2/liter hr. Methionine and threonine were added at the time of inoculation. Incubation for about 60 hours.

S-source (μg/ml)	l-Methionine (μg/ml)	l-Threonine (μg/ml)	CP III (mμMol/ml culture)
$(NH_4)_2SO_4$ (30)	–	–	0
	40	–	0
(100)	40	–	26
	40	50	0
l-Cysteine (50)	–	–	0
	40	–	29
	40	50	0
$(NH_4)_2SO_4$ (30) + dl-homocysteine (40)	–	–	0
	40	–	22
	40	50	28

A further reason for the effect of methionine on CP III accumulation in biotin-deficient media might be that relatively large amounts of this amino acid as a result of methylation (ethionine prevented the porphyrin accumulation induced by methionine) are able to bring about formation of PC from PE, followed by decomposition of the phospholipids by phospholipase activity. The choline released by this hydrolysis could not serve as donor for one carbon units because the formation of the latter was inhibited by homocysteine.

2.5. *The elimination of CP III accumulation in methionine-containing biotin-deficient medium by several one carbon units supplying compounds, glycine and serine*

Evidence of the above-mentioned explanation was provided by the results of an experiment in which a number of compounds serving as donor for one carbon units, were added to biotin-deficient media containing methionine. As shown in Table VII-5 N.N-dimethylglycine, sarcosine, formaldehyde, N.N-dimethylethanolamine, N-monomethylethanolamine and ethanolamine stimulated the formation of heme, which was attended with a suppression of CP III accumulation. Glycine had no effect. When the beneficial effect of methionine on CP III accumulation, at least partly, had to be ascribed to the formation of homocysteine which inhibits the formation of formaldehyde from choline and thus the synthesis of serine, one of the direct precursors of PE, added serine should have the same effect on CP III accumulation as the donors for one carbon units. This appeared to be true (Table VII-5).

The apparently contradictory effect of threonine on CP III accumulation in biotin-deficient media without and with methionine (cf. sections 2.2 and 2.4) may be explained by the fact that this amino acid was added under different conditions. In the experiments described in section 2.2 it was added to one-day-old

TABLE VII-5. The elimination of the accumulation of CP III, owing to the presence of methionine in medium A with suboptimal amounts of biotin (5×10^{-5} μg/ml) and $(NH_4)_2SO_4$ (100 μg/ml) in cultures of *Arthrobacter*, strain 223. Sulphite oxidation value: 11 mMol O_2/liter hr. The compounds tested were added at the time of inoculation. Incubation for 60 hours.

l-Methionine (μg/ml)	Compound (15 μg/ml) added	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)
–	–	–	4.5
40	–	24	0.3
	N.N-dimethylglycine	0	3.1
	sarcosine	0	3.5
	glycine	29	0.2
	formaldehyde	0	2.8
	N.N-dimethylethanolamine	0	3.0
	N-monomethylethanolamine	0	3.1
	ethanolamine	0	2.9
	serine	0	3.1

TABLE VII-6. The effect of manganese on the accumulation of CP III by biotin-deficient cells of *Arthrobacter*, strain 223, caused by iron deficiency. Medium A with cysteine (50 μg/ml) and methionine (50 μg/ml) containing N.N-dimethylglycine, l-serine and ethanolamine, respectively. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 80 hours.

Mn (10^{-2}μg/ml)	CP III (mμMol/ml culture)					
	N.N-Dimethylglycine (20μg/ml)		l-Serine (20μg/ml)		Ethanolamine (20μg/ml)	
	− Iron	+ Iron (1μg/ml)	− Iron	+ Iron (1μg/ml)	− Iron	+ Iron (1μg/ml)
–	40	0	48	0	38	0
1	37	0	41	0	33	0
10	33	0	29	0	27	0

cultures; it reduced the supply of one carbon units and consequently it adversely affected PE formation. In the experiment described in section 2.4 threonine was added to culture media containing relatively large amounts of methionine + sulfate or methionine + cysteine. Its depressing effect on CP III accumulation may be explained by the inhibition of the formation of homoserine and consequently that of homocysteine, the amino acid inhibiting PE formation and thus stimulating CP III accumulation.

2.6. *The accumulation of CP III in biotin-deficient cultures of Arthrobacter, strain 223, owing to iron deficiency*

The accumulation of this porphyrin as a result of iron deficiency in biotin-deficient cells was studied by reducing the quantity of biotin in medium B_3 (chapter VI) to 5×10^{-5} μg/ml. From Table VII-6 it will be seen that CP III accumulated when no iron was added. The amount of CP III under these conditions was affected by manganese, be it to a lesser extent than with 10^{-2} μg/ml biotin (cf. Table VI-1).

The effect of heme, aeration rate and fatty acids on CP III accumulation in biotin-deficient cultures owing to iron deficiency was similar to that in biotin-rich cultures growing in iron-deficient medium B_3.

2.7. *The activity of δ-aminolevulinic acid (ALA) synthetase and ALA dehydratase in biotin-deficient Arthrobacter, strain 223*

That the absence of CP III accumulation in biotin-deficient cultures of *Arthrobacter*, strain 223 (growing in medium A), was not due to a strongly reduced activity of ALA synthetase was concluded from the following observations, recorded in the present chapter.

a. Biotin-deficient cells produced considerable amounts of heme (A medium).
b. One-day-old biotin-deficient cells produced large amounts of porphyrin when supplied with threonine (A medium).
c. Addition of methionine to biotin-deficient media brought about CP III accumulation (A medium).

TABLE VII-7. The activity of δ-aminolevulinic acid (ALA) synthetase and ALA dehydratase in *Arthrobacter*, strain 223, grown in media with different concentrations of biotin.

Medium	Biotin content (μg/ml)	Dominant tetrapyrrole	ALA synthetase (units/mg protein)	ALA dehydratase (units/mg protein)
A	10^{-2}	CP III	47	78
A	5×10^{-5}	heme	18	23
A + Threonine*	5×10^{-5}	CP III	59	91

* Added 24 hours after inoculation.

d. Large amounts of CP III accumulated in biotin-deficient cultures growing in media (B_3) containing inadequate amounts of iron.

To provide more direct evidence as to the effect of biotin on the activity of ALA synthetase and ALA dehydratase, experiments with cell-free extracts have been carried out. From the results of these experiments (Table VII-7) it will be seen that the activity of ALA synthetase and ALA dehydratase in biotin-deficient threonine-treated cells (low heme content) was about 3 times larger than in biotin-deficient cells grown without threonine (high heme content) and about similar to that in cells grown in media containing excessive amounts of biotin (low heme content). From these results it can be concluded that the activity of these enzymes was not defined by the biotin supply of the bacteria but by their heme content.

3. SUMMARY AND CONCLUSIONS

Cell yield of *Arthrobacter*, strain 223, grown in medium A with 10^{-4} μg/ml biotin was nearly maximal. No CP III accumulated at this biotin level, whereas the heme content was high. With increased amounts of biotin, the CP III content of the culture rose sharply while the heme content dropped to a low level. The ratio of CP III (biotin-rich cultures) to heme (biotin-deficient cultures) was approximately 5:1.

When precultivated biotin-deficient *Arthrobacter* cultures were supplied with a large amount of threonine, the heme content of the cells was strongly reduced, but CP III accumulated.

The CP III content of biotin-deficient cultures (A medium) supplied with threonine dropped to a low value when the iron content of the medium was reduced. However, increased amounts of iron did not reduce the CP III content; the same was true of cultures supplied with adequate amounts of biotin.

Of a series of other amino acids (including glycine, valine, leucine, isoleucine, lysine, glutamic acid, aspartic acid, cysteine, phenylalanine, tyrosine and histidine) none was able to bring about CP III accumulation in precultivated biotin-deficient *Arthrobacter* cultures.

The enhancing effect of threonine on CP III accumulation in biotin-deficient

Arthrobacter cultures was eliminated by a number of compounds capable of serving as donor for one carbon units (choline, N.N-dimethylethanolamine, N-monomethylethanolamine, ethanolamine, betaine, N.N-dimethylglycine and sarcosine). This shows that the threonine-induced CP III accumulation had to be attributed to a shortage of one carbon units, resulting in a reduced formation of serine and presumably of PE, the compound assumed to be required for the transformation of CPG III into PP IX. In agreement with this conclusion, added serine also eliminated the accumulation of CP III induced by threonine. Homoserine, homocysteine and methionine were also able to restore heme formation and to eliminate CP III accumulation when added along with threonine to one-day-old biotin-deficient cultures. No explanation of the effect of the former two amino acids can be given.

When large amounts of methionine were added to biotin-deficient media containing relatively large amounts of sulfate, sulfate + cysteine, and sulfate + homocysteine, respectively, heme formation was strongly reduced whereas CP III accumulated. Threonine added along with methionine eliminated CP III accumulation in the cultures with sulfate and cysteine, respectively, but had no effect when homocysteine was present. This indicates that the effect of methionine under the conditions of this experiment would depend on the action of homocysteine (threonine inhibits the formation of homoserine and consequently that of homocysteine). A further reason of the stimulatory effect of methionine on CP III accumulation might be the partial methylation of PE to PC followed by a rapid decomposition of phospholipids by phospholipase activity. Evidence of the above-mentioned explanation of the methionine effect was provided by adding to culture media compounds known to be able to supply one carbon units, or by adding serine. In all cases CP III accumulation was prevented.

The results reported in this chapter for the following reasons do not support the idea that the absence of CP III accumulation in biotin-deficient *Arthrobacter*, strain 223, was due to a reduced activity of ALA synthetase: a. heme production in biotin-deficient cells, b. CP III accumulation by supplying one-day-old biotin-deficient cultures with threonine, c. CP III accumulation in media containing methionine and d. in iron-deficient medium B_3. More direct evidence that biotin-deficient cells had an active ALA synthetase was provided by estimation of its activity in cell-free extracts of bacteria grown under different conditions. In biotin-deficient cells grown with threonine (low heme) both ALA synthetase and ALA dehydratase were about 4 times as active as in similar cells grown without threonine (high heme). Bacteria grown with excessive amounts of biotin (low heme) had about the same enzyme activity as biotin-deficient cells (low heme). This shows that heme rather than biotin was controlling ALA synthetase activity.

CHAPTER VIII

THE INTEGRATION OF PROTEIN SYNTHESIS AND HEME FORMATION IN *ARTHROBACTER*, STRAIN 223

1. Introduction

In the preceding chapters attention has mainly been focused on the regulation of heme formation in *Arthrobacter*, strain 223. In bacteria heme is partly bound to specific proteins, forming the cytochromes. It is reasonable to presume (Lascelles, 1964) that bacteria have the disposal of mechanisms controlling the synthesis of heme to match the supply of the specific protein. The same seems true of the formation of bacteriochlorophyll and that of the specific protein component to which chlorophyll is attached in photosynthetic bacteria. Several studies have shown that protein inhibitors as chloramphenicol and actinomycin D (messenger-RNA synthesis) inhibit bacteriochlorophyll formation in Athiorhodaceae. According to Lascelles (1964), these observations might be explained by inhibition of the synthesis of the protein component to which intermediates of chlorophyll are possibly bound. This protein could end up as the structural protein to which the completed bacteriochlorophyll is attached.

So far no attention has been paid to this aspect of the regulation of heme formation in non-photosynthetic bacteria. In the present chapter experiments are described dealing with the integration of protein synthesis and heme formation in *Arthrobacter*, strain 223. In addition a search was made for protein-bound intermediates in heme formation in this bacterium.

2. Results and discussion

2.1. *Inhibition of protein synthesis and CP III accumulation in Arthrobacter, strain 223*

Chloramphenicol (1 μg/ml), added to the nutrient solution before inoculation, completely inhibited the growth of *Arthrobacter*, strain 223. Therefore, in a subsequent experiment this protein inhibitor was added to growing cultures at a time that no CP III was detectable in the culture. The data of this experiment (Table VIII-1) show that protein synthesis as well as CP III accumulation were entirely stopped by chloramphenicol as contrasted to the control cultures without the antibiotic. This might indicate that in *Arthrobacter*, strain 223, one or more precursors of CPG III are bound to protein. However, no accumulation of such precursors in cultures with chloramphenicol was detected. A further possibility was that the inhibition of the accumulation of CP III by chloramphenicol depended on a complete suppression of the formation of ALA, pos-

TABLE VIII-1. The effect of chloramphenicol on the synthesis of protein and CPG III in cultures of *Arthrobacter*, strain 223. The inhibitor was added 20 hours after inoculation (no porphyrin formed at that time). Medium A. Sulphite oxidation value: 11 mMol O_2/liter hr.

Chloramphenicol added (μg/ml)	μg Bact. N/ml on time of addition	μg Bact. N/ml culture after 48 hours incubation	CP III (mμMol/ml culture)
–	–	159	27
10	92	101	2
50	92	96	0

sibly because the enzyme ALA synthetase was not or hardly present in the cells when the inhibitor was added.

To test this possibility, chloramphenicol was added to porphyrin-accumulating cultures i.e. to cells having the disposal of ALA synthetase and of the enzymes of subsequent steps of the biosynthetic chain up to CPG III. The addition of chloramphenicol entirely suppressed both protein synthesis and porphyrin accumulation (Fig. VIII-1). The latter could not be restored by supplying glycine, glutamic acid, glycine + glutamic acid or ALA, respectively. Nor was

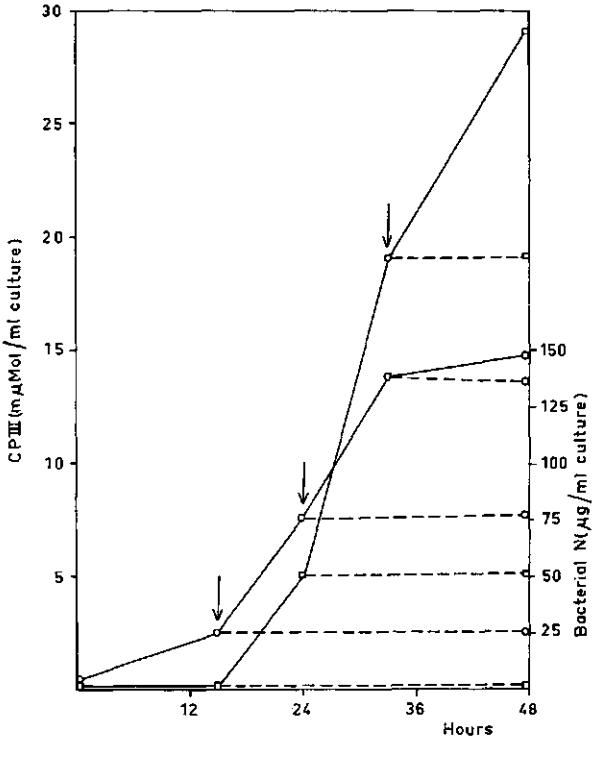


FIG. VIII-1. The effect of chloramphenicol (40 μg/ml) on CP III accumulation (□) and protein synthesis (○) during the growth of *Arthrobacter*, strain 223, in medium A. At different times (arrows) chloramphenicol was added to control cultures (———) and the incubation continued (- - - -). Sulphite oxidation value: 11 mMol O_2/liter hr.

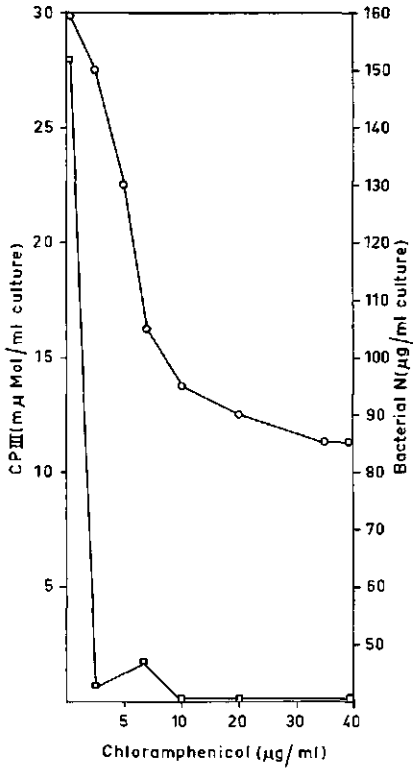


FIG. VIII-2. The effect of increasing amounts of chloramphenicol on CP III accumulation (□) and on protein synthesis (○) during the growth of *Arthrobacter*, strain 223, in medium A for 72 hours. Chloramphenicol added at the time that no CP III was detectable in the cultures. Sulphite oxidation value: 11 mMol O_2/liter hr.

it possible to detect precursors of CPG III in cultures containing chloramphenicol. The addition of small quantities of the antibiotic to cultures in which CP III accumulation had just started showed that CP III accumulation was more sensitive to chloramphenicol than protein synthesis (Fig. VIII-2). The results obtained with actinomycin D were similar to those obtained with chloramphenicol. It may therefore be concluded that the formation of CPG III is closely associated with the synthesis of protein.

To obtain more information about the effect of chloramphenicol on CP III accumulation, ALA dehydratase, one of the enzymes involved in CP III accumulation was examined. Two hours after the addition of chloramphenicol to porphyrin-accumulating cultures, the bacteria were collected by centrifugation and disrupted to prepare a cell-free extract. The activity of ALA dehydratase in this extract compared with that in extracts of control cultures which had received no chloramphenicol was decreased with 25 and 71 %, respectively (duplicates). The partial breakdown of this enzyme by added chloramphenicol did not account for the immediate complete inhibition of CP III accumulation after the addition of the antibiotic. In Athiorhodaceae no detectable breakdown of this enzyme has been found (LASCELLES, 1964) upon exposure to actinomycin D.

The results of the foregoing experiments support the hypothesis that in chloramphenicol-treated cells of *Arthrobacter*, strain 223, tetrapyrrole formation is completely blocked by the inhibition of the synthesis of some unknown protein involved in the action of the enzymes of the tetrapyrrole biosynthetic chain. This compound, apparently, is inhibiting the enzyme activity in vivo more effectively than in vitro.

2.2. *Inhibition of protein synthesis and heme formation in Arthrobacter, strain 223*

In order to investigate whether inhibition of the synthesis of the protein moieties of the cytochromes in *Arthrobacter* is accompanied with inhibition of the synthesis of heme, *Arthrobacter*, strain 223, was inoculated into medium A + methionine (60 μg/ml). At different periods of time after inoculation chloramphenicol (20 μg/ml) was added to the cultures. The results show (Fig. VIII-3) that heme formation took place until the moment chloramphenicol was added. Instead of heme no accumulation of one or more of its precursors was found. Addition of ALA did not restore heme formation in chloramphenicol-treated cells of *Arthrobacter*, strain 223.

The results of this experiment which are similar to those on CP III accumulation (Fig. VIII-1) clearly show that the synthesis of these porphyrins in *Arthrobacter* is associated with protein synthesis and consequently with the synthesis of the protein moieties of the cytochromes. After this work had been carried out, a paper of CLARK-WALKER et al. (1967) appeared dealing with the relation between protein synthesis and cytochrome formation in *Spirillum itersonii*.

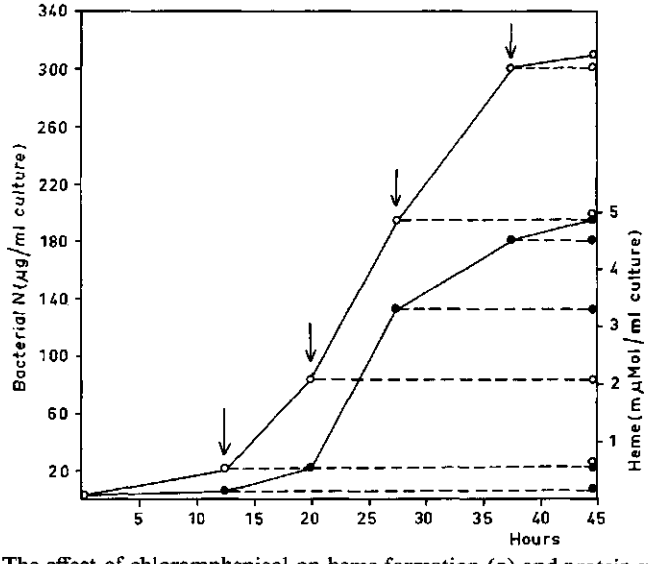


FIG. VIII-3. The effect of chloramphenicol on heme formation (●) and protein synthesis (○) during the growth of *Arthrobacter*, strain 223, in medium A containing methionine (60 μg/ml). At different times (arrows) chloramphenicol was added to control cultures (———) and the incubation continued (- - - -). Sulphite oxidation value: 11 mMol O_2/liter hr.

The observations with this micro-organism were similar to those reported above for *Arthrobacter*, strain 223. These authors concluded that the formation of the heme prosthetic group is closely integrated with the synthesis of the protein moiety.

2.3. *The occurrence of protein-bound porphyrins in Arthrobacter, strain 223*

To study whether in *Arthrobacter* protein-bound porphyrins other than heme would occur, a method had to be found which would enable reduction of the synthesis of protein (including that of the protein bound to porphyrins) without eliminating the activity of the enzymes catalysing the formation of porphyrins. In this connection a reduced formation of protein owing to nitrogen deficiency was considered to be a possible method.

Arthrobacter, strain 223, was inoculated into nutrient solution A containing 0.25 and 0.1 % (w/v)NH_4Cl, respectively. To some of the latter cultures 24 hours after inoculation ALA (225 μg/ml) was added. The organism was also inoculated into medium B_3 containing the compounds of medium A, in addition cysteine, methionine and ethanolamine, myristic acid (2 μg/ml), oleic acid (2 μg/ml) and a reduced amount of iron (0.1 μg Fe^{+++}/ml) still allowing maximum growth. This medium was applied with a normal (0.25 %) and a low content (0.1 %, w/v) of NH_4Cl. To part of the latter cultures 24 hours after inoculation ALA (225 μg/ml) was added. A third medium used in this experiment was medium A containing methionine (60 μg/ml). This medium was also applied with 0.25 and 0.1 % (w/v)NH_4Cl, while ALA (225 μg/ml) was added to some of the latter cultures 24 hours after inoculation.

From the results of this experiment (Table VIII-2) it will be seen that at the lowest concentration of NH_4Cl, at which about 50 % less protein calculated per ml bacterial culture was synthesized, the amount of porphyrins formed was considerably reduced. Addition of ALA, however, restored the formation of CPG III, PP IX and heme, showing that in spite of the reduced protein produc-

TABLE VIII-2. The effect of nitrogen deficiency on the formation of CPG III, PP IX and heme in cultures of *Arthrobacter*, strain 223, growing in media A, B_3 (modified, see text) + iron and A + methionine, respectively. Incubation time 96 hours. Sulphite oxidation value: 11 mMol O_2/liter hr.

Medium	NH_4Cl (%, w/v)	ALA (μg/ml) added 24 hours after inoculation	Porphyrin (mμMol/ml culture)		
			CP III	PP IX	Heme
A	0.25	0	27	–	–
	0.1	0	6	–	–
	0.1	225	31	–	–
B_3	0.25	0	–	23	–
	0.1	0	–	2	–
	0.1	225	–	19	–
A + l-Methionine (60 μg/ml)	0.25	0	–	–	5.4
	0.1	0	–	–	1.8
	0.1	225	–	–	4.9

tion, which would have adversely affected the formation of protein-bound precursors, conversion of the precursors proceeded readily. This is in agreement with the fact that no measurable amounts of ALA or PBG were detected in the nitrogen-deficient cultures. The results with nitrogen-deficient cells provide strong evidence of the absence of protein-bound intermediates in the synthesis of heme in *Arthrobacter*, strain 223. The porphyrins are not bound to protein before they have been converted to iron protoporphyrin (heme). This conclusion is in agreement with the results obtained by CLARK-WALKER et al. (1967) with *Spirillum itersonii*.

3. SUMMARY AND CONCLUSIONS

In living cells heme occurs in conjugation with specific proteins (hemoglobin, cytochromes). The regulation of the synthesis of these compounds not only involves the tetrapyrrole prosthetic group but also its protein moiety. To study the integration of heme formation and protein synthesis in *Arthrobacter*, strain 223, a number of experiments have been carried out with chloramphenicol.

Addition of chloramphenicol to porphyrin-accumulating cells immediately stopped protein synthesis and porphyrin formation (CP III accumulation). No precursors of CPG III were detected in the cultures incubated with the antibiotic, eliminating the hypothesis that protein-bound precursors of CPG III would exist and would be involved in the inhibiting effect of chloramphenicol on CPG III formation. Addition of ALA did not restore CP III accumulation in cultures incubated with the antibiotic; ALA dehydratase was only partly broken down by the addition of chloramphenicol.

The general conclusion drawn of these experiments with an inhibitor of protein synthesis was that the inhibition of the synthesis of the protein moiety of the cytochrome molecule is associated with the inhibition of the formation of heme.

Reduction of the protein synthesis of *Arthrobacter* cells by decreasing the nitrogen supply of the culture medium brought about a pronounced reduction of the amount of CP III, PP IX and heme. In contrast to the chloramphenicol-treated cells, addition of ALA restored the synthesis of these porphyrins indicating the absence of protein-bound intermediates of heme in cells of *Arthrobacter*, strain 223.

CHAPTER IX

PHOSPHOLIPIDS AND THE SYNTHESIS OF PORPHYRINS BY *ARTHROBACTER*, STRAIN 223

1. Introduction

In chapters V and VI it was shown that the synthesis of porphyrins by *Arthrobacter*, strain 223, may be affected to a large extent by N-bases of phospholipids and by fatty acids. The enzyme coproporphyrinogen III oxidase catalysing the conversion of CPG III to PP IX has been found to be localized in the organelles of all kinds of cells studied so far. Phospholipids represent an important part of these membranous structures. During the last 10 years attention has been focused on the activity of the enzymes localized in these structures. Although several reviews dealing with this subject have been published, some enzymic reactions, influenced by phospholipids, will be described in this chapter.

Clostridium welchii produces a toxin inhibiting the oxidation of succinic acid in mammalian cells. This toxin contains phospholipase C which catalyses the hydrolysis of a phospholipid into a diglyceride and a phosphomonoester. After it was known that the oxidation of succinic acid takes place in mitochondria, it became clear that the inhibition of the oxidation by the toxin depends on the presence of phospholipase C in this toxin. Not only phospholipase C but also other phospholipases were subsequently demonstrated to inhibit the action of succinic acid oxidase (VAN DEENEN, 1965). Recently ABDULLA and DAVISON (1965) showed that the oxidation of succinic acid in yeast occurred in a phospholipid-bound form.

A second example is the relation between phospholipids and the activity of β-hydroxybutyric acid dehydrogenase. JURTSHUCK et al. (1963) found that this mitochondrial enzyme was activated by a phospholipid mixture isolated from beef heart mitochondria. From a detailed analysis it appeared that lecithin specifically stimulated the activity of this enzyme. FLEISHER et al. (1966) recently confirmed this specificity.

The enzyme catalysing the transfer of glucose or galactose from uridine diphosphate to a lipopolysaccharide receptor was found to be activated by phosphatidylethanolamine (ROTHFIELD and HORECKER, 1964). In subsequent experiments it was shown (ROTHFIELD and TAKESHITA, 1966) that PE can be replaced by PS, PG, DPG and PA, but not by PC, indicating that the N-bases of phospholipids may have an important influence on the activity of enzymes. These investigations were carried out with the Gram-negative bacterium *Salmonella typhimurium* whose cell wall contains high amounts of lipopolysaccharides.

ANDERSON and STROMINGER (1965) found phospholipid-bound intermediates of cell wall glycopeptides in *Staphylococcus aureus* and *Micrococcus lysodeikticus*. These peptides were bound to glycerol through a pyrophosphate bridge.

It was supposed that this complex is formed to pass the cytoplasmic membrane to be used for the synthesis of an extracellular product, the cell wall.

Cyclopropane fatty acids are unsaturated fatty acids into which, at the place of the double bond, a one carbon unit has been incorporated. The analysis of the fatty acids isolated from a number of bacteria showed the widespread occurrence of cyclopropane fatty acids. It has been shown that for the synthesis of these fatty acids the corresponding unsaturated fatty acids had to be esterified with glycerylphosphorylethanolamine (PE without fatty acids). Although the activity of many particle-bound enzymes has found to be stimulated by phospholipids, the mechanism of this stimulation is still unknown (VAN DEENEN, 1965).

In this chapter experiments are described dealing with the phospholipid composition of *Arthrobacter*, strain 223, either producing large amounts of iron-free porphyrins or synthesizing the iron-containing heme as the dominant tetrapyrrole. In addition, the results of some experiments with cell-free extracts are reported to confirm the hypothesis put forward in chapter V that the accumulation of CP III in cultures of *Arthrobacter*, strain 223, in media with adequate amounts of iron was due to the absence of PE in the cells of these cultures. For the estimation of the phospholipid composition of *Arthrobacter*, strain 223, the bacteria were cultivated under the conditions as indicated in the foregoing chapters and harvested when the formation of the predominant porphyrin was in progress.

2. RESULTS AND DISCUSSION

2.1. *Phospholipids and lipid-like substances of Arthrobacter, strain 223, grown in medium A, rich in biotin and iron*

2.1.1. Phospholipids

Three phospholipids were detectable in *Arthrobacter*, strain 223, accumulating large amounts of CP III in media with excessive amounts of biotin and iron (Fig. IX-1, A, B, C, D, E). These phospholipids were chromatographed with the solvent systems chloroform:methanol:water (60:30:5, v/v/v) and diisobutylketone: acetic acid: water (40:20:8, v/v/v), respectively. The Rf-values of the 3 *Arthrobacter* phospholipids were identical with those of PG, DPG and PA. The water-soluble hydrolysis products (obtained by mild alkaline treatment) were chromatographed with the solvent systems propanol: ammonia: water (6:3:1, v/v/v) and butanol: acetic acid: water (4:1:5, v/v/v), respectively.

Glycerophosphorylglycerol was found to be the water-soluble hydrolysis product of PG, glycerophosphorylglycerophosphorylglycerol that of DPG, and glycerophosphate that of PA. From these results it was concluded that in *Arthrobacter*, strain 223, accumulating large amounts of CP III in media with both iron and biotin in excess, phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and phosphatidic acid (PA) were the major phospholipids.

Heme-forming cells (Fig. IX-2, F, G, H, I, K, L, M) of *Arthrobacter*, grown in

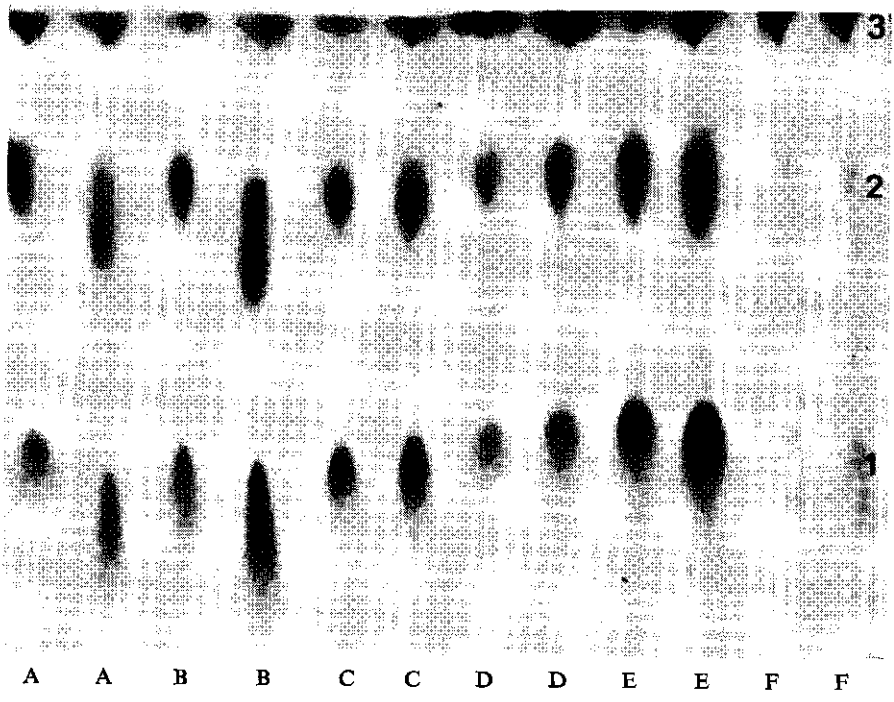


FIG. IX-1. Thin-layer chromatogram of phospholipids of CP III – accumulating iron-sufficient (A, B, C, D, E) and iron-deficient (F) *Arthrobacter*, strain 223. Solvent system chloroform: methanol: water (65: 30: 5, v/v/v). Sprayed with H_2SO_4. 1: phosphatidylglycerol; 2: diphosphatidylglycerol; 3: phosphatidic acid (+ neutral lipids).

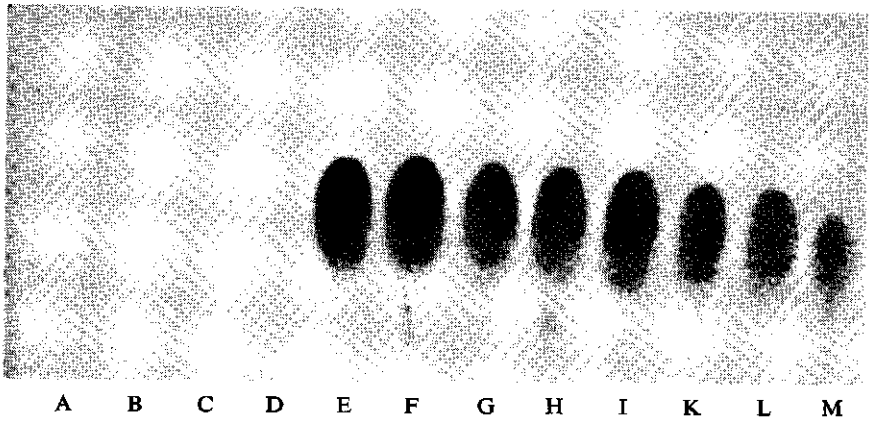


FIG. IX-2. Thin-layer chromatogram of phospholipids of biotin-sufficient, iron-sufficient *Arthrobacter*, strain 223, accumulating CP III (A, B, C, D) or forming heme (F, G, H, I, K, L). Solvent system chloroform: methanol: water (65:30:5, v/v/v). Sprayed with ninhydrin. E: phosphatidylethanolamine isolated from *Agrobacterium tumefaciens*.

medium A with biotin and iron in excess and one carbon unit compounds, in contrast to those accumulating CP III (Fig. IX-2, A, B, C, D), contained, in addition to PG, DPG, PA, the NH_2-containing phosphatidylethanolamine (PE). (This phospholipid was chromatographically identical with authentic PE and PE isolated from *Agrobacterium tumefaciens* while the water-soluble hydrolysis product was identical with authentic glycerophosphorylethanolamine). In contrast to *Agrobacterium tumefaciens* no phospholipids with labile methyl groups containing N-bases were detected in *Arthrobacter*, strain 223, grown in media with large amounts of methionine and choline. Nor when strain 223 was cultivated in media with choline as the only C and N-source. These results are in agreement with those of SHIEH and SPEARS (1967) obtained with *Achromobacter cholinophagum*, grown in a nutrient solution with choline as the only C and N-source.

Table IX-1 gives the composition of the phospholipids extracted from porphyrin-accumulating and heme-forming cells of *Arthrobacter*, strain 223. The most striking difference between these two types of cells was the absence of PE in CP III-accumulating and the presence of PE in the heme-forming cells. The latter cells were cultivated in medium A supplied with compounds capable of serving as donor for one carbon units required for the synthesis of serine. This confirms the hypothesis put forward in chapter V that the presence of PE (in the cells) prevented the accumulation of CP III in cultures of *Arthrobacter*, strain 223, grown in media with large amounts of iron.

Arthrobacter cells, cultivated in media (as indicated) with large amounts of ethionine in contrast to those with small amounts had a low phospholipid content, an observation which is in accordance with that in rats to which diets containing large amounts of this amino acid had been fed (ROBINSON and SEAKENS, 1962).

2.1.2. Lipid-like substances

In contrast to iron-sufficient cells of *Arthrobacter*, strain 223, producing heme, iron-sufficient cells which accumulated large amounts of porphyrin had a strongly increased content of lipid-like substance(s). These lipids were insoluble in ether, acetone or chloroform, but dissolved in a mixture of acetone: water (9:1, v/v) or of methanol: chloroform (9:1, v/v), indicating the rather polar character of these substance(s). Table IX-2 shows that CPG III-accumulating cells contained about 45% (of dry weight) of these lipids whereas heme-containing cells only 4–5%. Since the latter cells had been grown in a sulfate medium enriched with methionine, this amino acid apparently played a part in the elimination of these lipids. A similar effect of methionine and sometimes of choline has been found in livers of mammalia (ROBINSON and SEAKENS, 1962).

In order to determine the relationship between the accumulation of these lipid-like substances and CP III accumulation, *Arthrobacter*, strain 223, was inoculated into medium A. Samples were withdrawn at intervals to determine the content of the lipids (acetone: water, 9:1, v/v, used for extraction) and the amount of CP III. From Figure IX-3 it will be seen that the accumulation of the

TABLE IX-1. The relation between the type of phospholipid, and the type of porphyrin formed by *Arthrobacter*, strain 223, grown in biotin-rich medium A containing adequate amounts of iron.

Compound in medium A in addition to large amounts of sulfate	Details of exp.	Predominant tetrapyrrole (mμMol/ml culture)		Total phospholipids (% dry weight cells)	Composition, %			
					PG	DPG	PE	PA
–	Table IV-3	12	CP III	0.55	32	49	0	14
l-Methionine	Table IV-6	2.1	Heme	0.51	24	12	50	11
l-Cysteine	Table IV-3	15	CP III	0.51	37	48	0	10
l-Cysteine + l-Methionine (small)	Table IV-10a	3.0	Heme	0.48	28	10	46	12
l-Cysteine + l-Methionine (large)	Table IV-10a	17	CP III	0.42	29	59	0	9
l-Cysteine + l-Methionine (large) + N.N-Dimethylglycine	Figure V-11	1.9	Heme	0.57	21	12	47	17
dl-Homocysteine + l-Methionine	Table IV-10b	2.6	Heme	0.48	27	18	41	10
dl-Homocysteine + l-Methionine (large)	Table IV-10b	14	CP III	0.43	32	51	0	9
dl-Homocysteine + l-Methionine (large) + N.N-Dimethylglycine	Figure V-11	2.5	Heme	0.40	30	9	51	10
dl-Ethionine (small)	Table V-6	1.8	Heme	0.38	24	17	42	13
dl-Ethionine (large)	Table V-6	7	CP III	0.08	35	37	8	14

PG: phosphatidylglycerol; DPG: diphosphatidylglycerol; PE: phosphatidylethanolamine; PA: phosphatidic acid.

TABLE IX-2. The lipid content of iron-sufficient cells of *Arthrobacter*, strain 223, grown in medium A (containing sulfate) with different S-containing amino acids. Sulphite oxidation value: 11 mMol O_2/liter hr. Incubation for 48 hours.

S-Containing amino acid (μg/ml)	Porphyrin (mμMol/ml culture)		Lipids (% dry weight cells)
	CP III	Heme	
–	24	0.4	45
l-Cysteine (50μg/ml)	25	0.3	41
dl-Homocysteine (80μg/ml)	26	0.4	49
l-Methionine (60μg/ml)	0	5.1	4
l-Cysteine (50μg/ml) + l-Methionine (50μg/ml)	29	0.5	50

TABLE IX-3. The phospholipid composition of biotin-rich, iron-deficient cells of *Arthrobacter*, strain 223.

Compounds added to iron-deficient medium B_3	Predominant tetrapyrrole	Total phospholipids (% dry weight cells)	Composition, %			
			PG	DPG	PE	PA
–	CP III	0.03	35	45	0	18
Iron	Heme	0.55	22	13	48	14
Myristic acid + Oleic acid	Heme (low)	0.31	3	42	47	8
Palmitoleic acid + Oleic acid	Heme	0.33	2	43	47	10
Stearic acid + Oleic acid	Heme	0.41	3	36	40	15
Myristic acid + Palmitoleic acid	Heme (low)	0.39	24	2	61	7
Palmitic acid + Palmitoleic acid	Heme	0.37	31	5	49	13
Stearic acid + Palmitoleic acid	Heme	0.46	37	4	41	16
Myristic acid + Oleic acid (small amounts)	PP IX	0.23	1	2	94	1
Myristic acid + Oleic acid + Iron	Heme	0.36	2	2	79	16

lipids preceded the CP III accumulation. No further study was made of these substances.

2.2. *Phospholipids of Arthrobacter, strain 223, grown in biotin-rich medium B_3 without added iron*

Table IX-3 shows that iron-deficient, CP III-accumulating, *Arthrobacter* cells had a much lower phospholipid content than those well supplied with this metal.

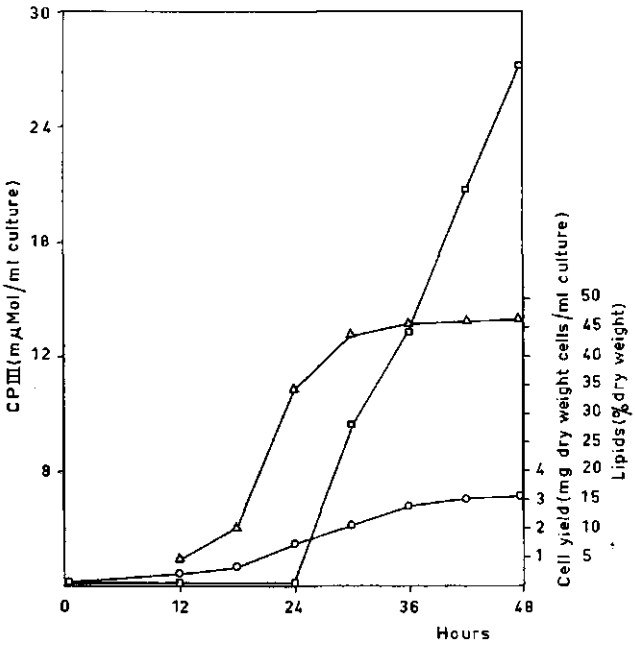


FIG. IX-3. The relation between the lipid content (△) of *Arthrobacter*, strain 223, and the accumulation of CP III (□) during the growth (○ = cell yield) in medium A. Sulphite oxidation value: 11 mMol O_2/liter hr.

PG and DPG were the major phospholipids in iron-deficient *Arthrobacter*, strain 223, indicating that not all 4 phospholipids detected in this strain were equally affected by iron (Fig. IX-1, F). When adequate amounts of iron had been supplied, all phospholipids were present and the total phospholipid content attained a normal level, while no CP III accumulation occurred.

Addition of long chain fatty acids to the iron-deficient medium B_3 which prevented CP III accumulation strongly stimulated the formation of PE, whereas the content of PG, DPG and PA increased less regularly.

The presence of smaller amounts of myristic acid and oleic acid in iron-deficient medium B_3 resulted in the formation of PP IX and that of PE, providing additional evidence that this phospholipid is required for the conversion of CPG III to PP IX. When iron was also present in large amounts in this medium, two phospholipids were found viz. PE and PA while heme was the dominant porphyrin. This indicates that PA may be involved in the conversion of PP IX to heme.

2.3. *The phospholipid composition of biotin-deficient Arthrobacter, strain 223*

Biotin-deficient cells of *Arthrobacter*, strain 223, grown in medium A supplied with adequate amounts of iron formed heme and contained PE. This phospholipid was not detectable in the cells when threonine was added to one-day-old cultures or when the biotin-deficient medium contained methionine. Com-

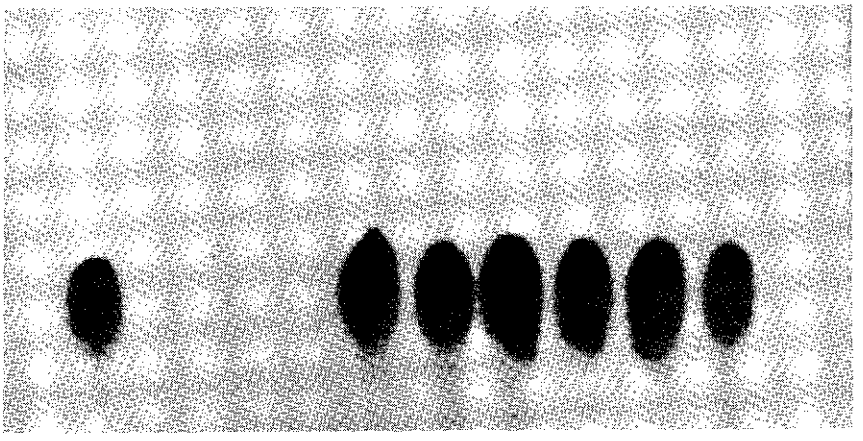


FIG. IX-4. Thin-layer chromatogram of phospholipids of biotin-deficient, iron-sufficient *Arthrobacter*, strain 223, accumulating CP III (B, C, D) or forming heme (A, F, G, H, I, K). Solvent system chloroform: methanol: water (65:30:5, v/v/v). Sprayed with ninhydrin. E: phosphatidylethanolamine (*Agrobacterium tumefaciens*).

pounds supplying one carbon units or serine in both cases restored heme formation and brought about a strongly increased PE content of the cells (Table IX-4, Fig. IX-4).

The results obtained with fatty acids added to biotin-deficient and iron-deficient medium B_3 (Table IX-5, Fig. IX-5) closely resembled those recorded in Table IX-3.

TABLE IX-4. The phospholipid composition of biotin-deficient, iron-sufficient cells of *Arthrobacter*, strain 223.

Compound present in biotin-deficient A medium	Details of exp.	Predominant tetrapyrrole	Total phospholipids (% dry weight cells)	Composition, %			
				PG	DPG	PE	PA
–	Table VII-1	Heme	0.42	5	19	63	11
*Threonine	Table VII-1	CP III	0.35	41	39	0	17
*Threonine + *Methionine	Table VII-3	Heme	0.37	17	34	36	9
*Threonine + *N.N-Dimethylglycine	Table VII-3	Heme	0.46	22	29	32	15
*Threonine + *Serine	Table VII-3	Heme	0.40	17	14	51	17
Methionine	Table VII-5	CP III	0.41	48	37	0	14
Methionine + N.N-Dimethylglycine	Table VII-5	Heme	0.49	12	36	40	7
Methionine + Serine	Table VII-5	Heme	0.38	9	34	45	12

* Added approximately 24 hours after inoculation.

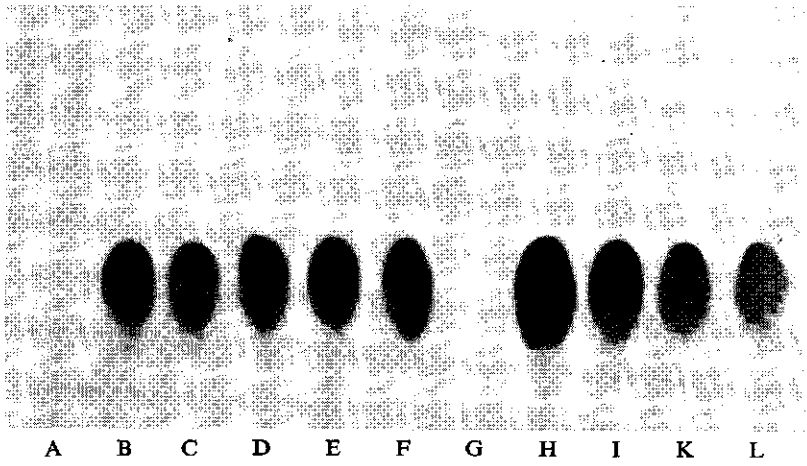


FIG. IX-5. Thin-layer chromatogram of phospholipids of biotin-deficient *Arthrobacter*, strain 223, grown in iron-deficient medium (CP III accumulation occurred) in the absence (A, G) and presence (B, C, D, E, F, I, K, L) of long chain fatty acids eleminating CP III accumulation. Solvent system chloroform: methanol: water (65:30:5, v/v/v). Sprayed with ninhydrin. H: phosphatidylethanolamine (*Agrobacterium tumefaciens*).

2.4. *Phospholipase C activity*

In order to determine whether the absence of phosphatidylethanolamine in CPG III-accumulating cells growing in medium A must be ascribed to phospholipase activity, the bacteria were harvested at different periods of time after inoculation to estimate their phospholipid content and the composition of the

TABLE IX-5. The phospholipid composition of biotin-deficient, iron-deficient cells of *Arthrobacter*, strain 223.

Compound present in iron-deficient B_3 medium	Predominant tetrapyrrole	Total phospholipids (% dry weight cells)	Composition, %			
			PG	DPG	PE	PA
–	CP III	0.05	5	65	0	28
Iron	Heme	0.47	21	15	46	14
Palmitic acid + Oleic acid	Heme	0.39	11	23	45	19
Stearic acid + Oleic acid	Heme	0.42	5	28	48	14
Palmitoleic acid + Palmitic acid	Heme	0.43	15	11	57	17
Palmitoleic acid + Stearic acid	Heme	0.47	18	7	61	9
Myristic acid + Oleic acid (small)	PP IX	0.27	2	0	95	0
Myristic acid + Oleic acid (small) + Iron	Heme	0.39	2	3	72	18

TABLE IX-6. The course of the phospholipid content and the composition of the phospholipids in *Arthrobacter*, strain 223, growing in medium A (a) and A supplied with cysteine and methionine (b).

	Time of harvesting after inoculation (hours)	CP III (mμMol/ml culture)	Heme (mμMol/ml culture)	Total phospholipids (% dry weight cells)	Composition, %			
					PG	DPG	PE	PA
a	17	0	0.4	0.41	16	34	39	9
	26	4	0.5	0.08	44	48	0	4
	34	16	0.4	0.45	35	45	0	15
b	15	0	0.2	0.28	37	6	47	13
	30	1	0.3	0.04	36	56	0	11
	39	14	0.2	0.38	48	37	0	14

phospholipids. The data in Table IX-6 clearly show that in the interval 17–26 hours after inoculation a rapid disappearance of all phospholipids occurred, the reduction being most pronounced with PE. From 26–34 hours the phospholipid content returned to a normal level. However, PE was not formed any more. The results obtained with *Arthrobacter*, strain 223, growing in medium A containing large amounts of cysteine and methionine (CP III accumulation occurred) were the same as those with cells growing in medium A.

2.5. *The effect of phospholipids on the formation of PP IX from ALA by cell-free extracts of Arthrobacter, strain 223*

To examine the effect of phospholipids on the synthesis of porphyrins an experiment has been performed with cell-free extracts of *Arthrobacter*, strain 223, grown in iron-deficient medium B_3 for 72 hours. These bacteria had a strongly reduced phospholipid content and PE could not be detected. At the time of harvesting, CP III accumulation was in progress.

The incubation mixture contained: cell-free extract; phosphate buffer, pH 7.8; ALA, 400 mμMol/ml; phospholipids (dissolved in peroxide-free ether)

TABLE IX-7. The effect of phospholipids on the conversion of δ-aminolevulinic acid (ALA) to porphyrins by a cell-free extract (1.4 mg protein/ml) of *Arthrobacter*, strain 223, grown in iron-deficient medium B_3. Incubation time 8 hours.

Compound added (2 mg/ml)	Porphyrin formed (mμMol/ml)	
	CP III	PP IX
—	24	1
PE (*Arthrobacter*, strain 223)	14	18
PE (mammalia)	12	15
PG + DPG + PA (*Arthrobacter*, strain 223)	28	3
PC + PE (egg)	24	7
Fe^{+++}	31	0
Fe^{++}	25	2

as indicated; total volume 5 ml. The incubation (at 30°C) was finished after 4 hours by adding trichloroacetic acid. Porphyrins were estimated as indicated in chapter II.

From Table IX-7 it will be seen that the formation of PP IX was strongly enhanced by the addition of phosphatidylethanolamine, either isolated from *Arthrobacter*, strain 223, or from mammalia. A mixture of PG, DPG and PA, isolated from *Arthrobacter*, strain 223, had practically no effect on the amount of PP IX formed. The same was true of iron. Purified egg phospholipids containing mainly PC and PE stimulated the formation of PP IX to some extent.

Prolonged incubation (10 hours) of the soluble fraction of the cell-free extract with ALA and PE did not bring about PP IX formation. CP III was present, be it in a small amount. This indicates that the enzyme coproporphyrinogen oxidase of *Arthrobacter*, strain 223, is located on the membranes.

From the fact that iron stimulated PE formation and eliminated CP III in cultures of *Arthrobacter*, strain 223, whereas it had no stimulating effect on the formation of PP IX from ALA by cell-free extracts in contrast to PE, it is concluded that in whole cells iron exerted its stimulating effect on the conversion of CPG III to PP IX by way of its stimulating effect on the formation of PE.

2.6. *The effect of phospholipids on the formation of heme from protoporphyrin IX and iron by cell-free extracts of Arthrobacter, strain 223*

The results of the experiments described in section 2.3. suggested that the phospholipid PA is involved in the formation of heme from protoporphyrin IX and iron. To obtain more information concerning this reaction, *Arthrobacter*, strain 223, was inoculated into iron-deficient medium B_3 supplied with small amounts of myristic acid (2 μg/ml) and oleic acid (2 μg/ml). Under these conditions PE is almost the only phospholipid. Accumulation of PP IX took place at the time of harvesting. The crude membrane fraction was obtained by centrifugating a cell-free extract at about 100,000 × *g* for 90 minutes. This fraction was incubated with phosphate buffer, pH 8.5; 40 mμMol protoporphyrin IX; 5 mMol Fe^{++} (as $FeSO_4$). 2 mMol cysteine and phospholipids (dissolved in peroxide-free ether) as indicated. The incubation (at 30°C) was finished after

TABLE IX-8. The effect of phospholipids on the conversion of PP IX and ferrous iron to heme by a membrane preparation (2.1 mg protein/ml) of *Arthrobacter*, strain 223, grown in iron-deficient medium B_3 supplied with myristic acid (2μg/ml) and oleic acid (2μg/ml).

Phospholipid added (2mg/ml)	Heme (mμMol/ml)
–	0.1
PA (containing unsaturated fatty acids)	8.9
PC + PE (egg)	0.2
PG + DPG + PA (*Arthrobacter*, strain 223)	2.1
PA (containing palmitic acid only)	3.1

1 hour by adding acetic acid to 10% (w/v). Porphyrins were extracted and estimated as indicated in chapter II.

From Table IX-8 it will be seen that phosphatidic acid containing unsaturated fatty acids strongly stimulated heme formation in contrast to a mixture of phosphatidylcholine (PC) and phosphatidylethanolamine(PE). A mixture of PG, DPG and PA isolated from *Arthrobacter*, strain 223, was moderately active. The amount of heme formed by addition of phosphatidic acid esterified with saturated fatty acids only was 30% of the amount formed by adding phosphatidic acid containing unsaturated fatty acids (prepared from egg lecithin).

The enzyme heme synthetase responsible for the transformation of PP IX and iron into heme is present in the membrane fraction of *Arthrobacter* cells. This was concluded from the fact that no heme had been synthesized when the soluble fraction was incubated with PA, PP IX and iron for several hours. Since in heme-forming cells PA is also located in the membranes, stimulation of the enzyme heme synthetase by this phospholipid seems obvious.

3. Summary and conclusions

In the preceding chapters evidence has been provided of the function of phospholipids in heme formation in *Arthrobacter*, strain 223. The central position of phosphatidylethanolamine (PE) in the conversion of CPG III to PP IX was discussed. In the present chapter a comparison was made of the phospholipid composition of *Arthrobacter* cells, grown under different conditions, and their ability to form different types of porphyrin.

Cultivated in medium A with excessive amounts of biotin and iron, the cells contained phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and phosphatidic acid (PA) but no measurable amount of PE. These cells accumulated large amounts of coproporphyrin III. Suppression of CP III accumulation (attended with heme formation) by supplying compounds able to serve as donor for one carbon units to the nutrient solution, was always accompanied with the occurrence in the bacteria of considerable amounts of PE, confirming the results of experiments described in the foregoing chapters concerning the important function of PE in heme formation.

CPG III-accumulating cells of *Arthrobacter*, strain 223, grown in medium A with excessive amounts of biotin and iron, in addition to forming PG, DPG and PA, produced large amounts of lipid-like substances which accumulated in the cells. Addition of methionine to this medium, which eliminated CP III accumulation and brought about heme formation, almost entirely eliminated the production of these lipid-like substances. No further study has been made of these substances.

When *Arthrobacter* was cultivated in biotin-rich medium B_8 containing inadequate amounts of iron, CP III accumulated whereas the heme content was low. In such cells the total phospholipid content was strongly reduced and PE was not detectable. When this nutrient medium had been supplied with mixtures

of long chain saturated + unsaturated fatty acids, the phospholipid content of the bacteria, in particular that of PE, was much higher. This was attended with a suppression of CP III accumulation and concomitantly with the formation of heme.

When small amounts of myristic acid and oleic acid were present in the biotin-rich, iron-deficient medium B_3, PE was practically the only phospholipid formed. Under these conditions PP IX was the porphyrin which accumulated. When the latter medium had been supplied with large amounts of iron, two phospholipids (PE and PA) were found in the cells, while heme became the predominant porphyrin. This indicated that PA was involved in the conversion of PP IX to heme.

Biotin-deficient cells of *Arthrobacter*, supplied with large amounts of iron (medium A), which formed heme as the dominant tetrapyrrole contained a large amount of PE. Threonine, added to one-day-old biotin-deficient cultures, and methionine, added to biotin-deficient media, brought about CP III accumulation which was attended with the absence of PE and an increased content of PG and DPG in the cells. The heme content dropped to a low level. One carbon units supplying compounds, or serine, added to these cultures resulted in a high PE content of the cells, a restored heme formation and an eliminated CP III accumulation.

Biotin-deficient cells supplied with inadequate amounts of iron (medium B_3) resembled biotin-sufficient cells grown under iron-deficient conditions viz. a low content of phospholipids and no detectable amount of PE while CP III was the predominant porphyrin. Addition of iron in both cases resulted in a normal content of phospholipids which to a large percentage consisted of PE. Instead of CP III, heme was the prevailing porphyrin. Mixtures of saturated and unsaturated long chain fatty acids, added to the biotin-deficient, iron-deficient medium B_3 had the same effect as in the case of biotin-sufficient, iron-deficient media viz. formation of PE as the most important phospholipid and heme as the predominant porphyrin.

When *Arthrobacter*, strain 223, growing in medium A with adequate amounts of biotin and iron or in medium A supplied with large amounts of cysteine and methionine, started to accumulate CP III, the phospholipid content dropped to a low level, apparently due to the activity of phospholipase C. With continued incubation, the phospholipid content showed a ready increase. The newly formed phospholipids contained no PE, as was the case with the phospholopid initially present in young cells, but large amounts of PG and DPG.

The results described so far have shown that PE is responsible for the conversion of CPG III to PP IX. To provide more evidence as to this conclusion, experiments with cell-free extracts of iron-deficient cells of *Arthrobacter*, strain 223, grown for 72 hours in medium B_3 were carried out. These cells had a low phospholipid content while PE was not detectable; CP III accumulation was in progress at the time of harvesting. PE from *Arthrobacter* (strain 223) or from mammalia was found to stimulate the conversion of ALA to PP IX, as contrasted to a mixture of PG + DPG + PA or iron. From the fact that iron (which sti-

mulated PE formation and eliminated CP III accumulation in living cells of *Arthrobacter*, strain 223, growing in medium B_3) did not favour the conversion of ALA to PP IX by cell-free extracts in contrast to PE, it was concluded that in whole cells iron exerted its favourable effect on the conversion of CPG III to PP IX by way of its stimulating effect on the formation of PE.

To study the effect of phospholipids on the conversion of PP IX to heme, the membrane fraction of *Arthrobacter* cells grown in the biotin-sufficient, iron-deficient medium B_3 supplied with small amounts of myristic acid and oleic acid was incubated with PP IX and ferrous iron and different phospholipids. A clear correlation was found between PA, heme formation. PC and PE did not affect this formation. PA containing unsaturated fatty acids was approximately 3 times as active as that containing saturated fatty acids only. A mixture of PG, DPG and PA isolated from *Arthrobacter*, strain 223, was moderately active.

SUMMARY

Porphyrins (tetrapyrroles) are the basic compounds of a number of substances functioning in living organisms as carriers of oxygen (hemoglobin), carriers of electrons (cytochromes) or as a trap for radiant energy (chlorophyll). In these active forms the tetrapyrroles contain a metal and are bound to a specific protein.

In addition to the protein-bound tetrapyrroles, free porphyrins are found in nature. These compounds may be considered as the oxidized form of porphyrinogens, the true intermediates in the biosynthesis of heme (one of the two basic compounds of hemoglobin and cytochromes) and chlorophyll. The free porphyrins have no known biological function; their accumulation in general indicates the presence of some metabolic block in the biosynthetic pathway leading to heme or chlorophyll. Therefore, the accumulation of free porphyrins may be used as a tool for studying the effect of various factors on tetrapyrrole synthesis.

So far the effect of B vitamins, oxygen and iron on the synthesis of heme and chlorophyll has been studied in different types of organisms including men, animals, plants, algae (particularly *Chlorella* species), photosynthetic bacteria and some heterotrophic bacteria (particularly *Corynebacterium diphtheriae* and *Micrococcus lysodeikticus*).

During the study of the physiology of a number of *Arthrobacter* strains by collaborators of the Laboratory of Microbiology, Wageningen, a red pigment was found to occur in certain strains and under certain conditions (Antheunisse, private communication). Continuing this investigation, the present author found the red pigment to be coproporphyrin III (CP III).

Since iron in relatively large amounts in a number of *Arthrobacter* strains did not eliminate CP III accumulation, as it had been found by LASCELLES in *Rhodopseudomonas spheroides*, the present investigation started with experiments to study this phenomenon. In subsequent experiments the regulation of the porphyrin synthesis in *Arthrobacter*, strain 223, was studied.

In Chapter III the results of experiments with this organism growing in a mineral salts medium with glucose as the carbon source (medium A) have been given. To obtain CP III accumulation, biotin, zinc, sulfate, phosphate and ammonium ions had to be present in the nutrient medium in considerably higher concentrations than those required for giving maximum cell yield. Iron was also required in small amounts for growth and CP III accumulation. Increasing amounts of Fe did not eliminate CP III accumulation as it has been described for a number of bacteria in the literature. Report of more detailed experiments concerning the effect of iron on porphyrin synthesis is given in chapters VI and IX. $CaCO_3$ supplied in amounts of 1%, although favourably affecting cell yield, entirely prevented CP III accumulation. With decreasing amounts of $CaCO_3$, CP III accumulation increased.

The aeration rate of the culture was found to have a pronounced effect on the synthesis of the porphyrin by *Arthrobacter*, strain 223. Maximum values for CP III were obtained at an aeration rate of 11 mMol O_2/liter hr. Doubling this aeration rate slightly decreased the amount of CP III accumulated, but a four times higher rate (44 mMol O_2/liter hr) although not affecting cell yield, almost eliminated CP III accumulation. Decreasing the aeration rate to 4 mMol O_2/liter hr, which considerably reduced cell yield, entirely eliminated CP III accumulation. The latter was restored by adding δ-aminolevulinic acid (ALA, a precursor of CPG III), succinic acid or glutamic acid, showing that lack of substrate, and not reduced activity or reduced formation of the enzymes involved in CPG III formation, was responsible for the reduced CP III accumulation at a low aeration rate.

The failing of the CP III accumulation at a high aeration rate (44 mMol O_2/liter hr) was partly restored by adding ALA but not by adding succinic acid or glutamic acid. That the eliminated activity of ALA synthetase at a high aeration was the cause of the eliminated CP III accumulation was concluded from the fact that CPG III – producing cells stopped their production upon transfer to a high aeration rate. The enzymes catalysing the conversion of ALA to CPG III were also unfavourably affected by a high aeration rate but to a smaller extent than ALA synthetase.

Chapter IV deals with the results of experiments concerning the influence of sulphur-containing amino acids on the formation of porphyrins in cultures of *Arthrobacter*, strain 223. They were done because in chapter III relatively large amounts of sulfate were shown to be required for the accumulation of CP III. In this process sulfate was assumed to exert its effect as a result of its conversion to homocysteine. This assumption was based on the following observations. (1) In CP III-accumulating *Arthrobacter* cultures sulfate could be replaced by cysteine or homocysteine (traces of sulfate as contamination being present in the nutrient medium). Since *Arthrobacter*, strain 223, easily converted cysteine to homocysteine but did not catalyse the reverse process, cysteine could not be formed from homocysteine and consequently could not be the cause of the CP III accumulation. (2) Small amounts of threonine, an inhibitor of the formation of homoserine, prevented the accumulation of CP III in cultures with sulfate or cysteine. The threonine effect could be eliminated by adding homocysteine or homoserine, a precursor of homocysteine. (3) Addition of 1% $CaCO_3$ to medium A prevented the CP III accumulation unless homocysteine was present.

In addition to threonine, methionine when present in sulfate-containing medium A in amounts of 50 μg/ml or more, prevented the accumulation of CP III. Of a number of other compounds including amino acids, purines, pyrimidines B vitamins, none was able to eliminate CP III accumulation. The eliminating effect of methionine on CP III accumulation, might be explained by assuming the functioning of a feedback mechanism controlling the synthesis of homocysteine. That this hypothesis, however, was not valid was concluded from the observations that (a) 50 μg/ml methionine which eliminated CP III accumulation

in a sulfate medium, did not prevent porphyrin accumulation when present in a medium with sulfate + cysteine, (b) the presence of homocysteine in an A medium containing approximately 50 μg/ml methionine did not give CP III accumulation. The elimination of CP III accumulation in medium A by methionine presumably depended on the synthesis of a compound which favoured the conversion of CPG III to PP IX and heme. Larger concentrations of methionine under certain conditions (A medium with cysteine or with homocysteine) brought about the decomposition of this compound and consequently enhanced CP III accumulation.

In Chapter V the effect of methionine and homocysteine on the formation of porphyrins by *Arthrobacter*, strain 223, was studied in more detail. Since the methyl group of methionine may be converted to formaldehyde (via choline) a comparison was made between the effect of increasing amounts of methionine and that of increasing amounts of formaldehyde on CP III accumulation in A media containing sulfate, sulfate + cysteine and sulfate + homocysteine, respectively. A far-going similarity between the effect of methionine and that of formaldehyde on CP III accumulation and heme formation was observed (the smallest amount of formaldehyde required to prevent CP III accumulation equalled the amount of formaldehyde which could have been derived from the smallest amount of methionine preventing this accumulation). From this similarity it was concluded that the effect of methionine on CP III accumulation had to be ascribed to the improved supply of one carbon units (via choline, N.N-dimethylglycine, sarcosine). Evidence as to this hypothesis was provided by the fact that the effect of different amounts of choline on CP III accumulation resembled that of formaldehyde and methionine. A second way in which methionine may increase the one carbon units supply of the cells is by its sparing action on the utilization of these units (no methionine has to be formed from homocysteine + one carbon units).

One of the most important reactions in *Arthrobacter*, strain 223, in which one carbon units are involved is the synthesis of serine from glycine. Since serine via phosphatidylserine (PS) is one of the precursors of phosphatidylethanolamine (PE), the latter compound was assumed to be required for the conversion of CPG III to PP IX. Decrease of the amount of PE in the cell would automatically increase the amount of CP III.

Large amounts of methionine (added to the A medium supplied with cysteine or homocysteine), formaldehyde or choline gave a resumed accumulation of CP III. This effect was thought to be due to the removal of PE from the cells, partly by methylation of PE to PC, partly by enhanced phospholipase C activity as a result of the formation of PC.

The favourable effect of large amounts of methionine, formaldehyde or choline on CP III accumulation required the presence of homocysteine. This amino acid was found to inhibit the formation of one carbon units from choline by inhibiting the conversion of betaine to N.N-dimethylglycine. This resulted in a reduced one carbon units supply and consequently in a reduced formation of

serine. More evidence as to this conclusion was obtained by the fact that N.N-dimethylglycine or sarcosine when added to media with large amounts of methionine or choline prevented CP III accumulation.

In Chapter VI a report is given of a number of experiments dealing with the effect of the iron supply of the bacteria on the formation of porphyrins. The preliminary experiments on this subject, reported in Chapter III, have shown that excessive amounts of iron which according to literature recordings concerning several types of microbes, should eliminate CP III accumulation, did not show this effect when applied to *Arthrobacter*, strain 223, growing in medium A.

The experiments described in the previous chapters have shown that CP III accumulation in *Arthrobacter*, strain 223, cultures can be prevented and concomitantly heme formation enhanced by improving the supply of one carbon units. Therefore, the resumed studies on iron supply were carried out with a modified nutrient medium (B_3) containing in addition to medium A, methionine, cysteine, ethanolamine and some additional manganese.

Reducing the amount of iron added to medium B_3 brought about a decreased formation of the iron-containing heme in *Arthrobacter*, strain 223, attended with the accumulation of CP III in amounts several times larger than the maximum amount of heme formed with large amounts of iron. This overproduction of CP III implied the presence of a regulatory mechanism depending on heme. The correctness of this hypothesis was shown by adding increasing amounts of heme to the culture medium in which CP III accumulation occurred and by estimating enzyme activity in cell-free extracts of *Arthrobacter*, strain 223. The main site of the regulating effect of heme on CP III accumulation was found to be localized in the enzyme converting glycine and succinyl-CoA to ALA (ALA synthetase). The synthesis of this enzyme was strongly repressed by heme while its activity was moderately inhibited (feedback control).

The activity of ALA dehydratase, one of the enzymes catalysing the conversion of ALA to CPG III, was not inhibited by heme; however, the synthesis of this enzyme was considerably repressed.

Iron added to iron-deficient medium B_3 (in which CP III accumulation occurred) affected the synthesis of ALA synthetase and that of ALA dehydratase similarly to added heme, showing that iron affected porphyrin accumulation as a result of its incorporation into heme.

In addition to its regulating effect on porphyrin accumulation via heme formation, iron may affect porphyrin synthesis by stimulating the conversion of CPG III to PP IX. This was found to be due to the favouring effect of iron on PE formation as a result of the stimulation of the formation of fatty acids, which are required for the synthesis of PE.

The presence of small amounts of myristic and oleic acid in iron-deficient medium B_3 gave rise to the accumulation of PP IX by *Arthrobacter*, strain 223. Large amounts of iron added to this medium prevented the accumulation of PP IX and gave heme formation.

The accumulation of CP III in iron-deficient cultures (B_3 medium) of *Arthrobacter*, strain 223, was affected by the aeration rate of the culture medium similarly to that in iron-sufficient cultures (A medium) as reported in Chapter III. A high aeration rate depressed the synthesis of ALA synthetase and that of ALA dehydratase.

In Chapter VII the effect of the biotin supply of *Arthrobacter*, strain 223, on the formation of porphyrins was studied in more detail. Earlier experiments, reported in chapter III, had shown that CP III accumulation in medium A required considerably larger amounts of biotin than those required for optimal growth. For the investigations discussed in chapter VII, A medium containing 5×10^{-5} μg/ml biotin was used. At this concentration of biotin no CP III accumulated and heme was formed.

When 1 day-old biotin-deficient cultures of *Arthrobacter*, strain 223, were supplied with a large amount of threonine, the heme content of the cells was strongly reduced and CP III accumulated. This accumulation was not reduced by increasing the supply of iron, but it was eliminated by a number of compounds serving as donor for one carbon units, showing that threonine is promoting CPG III formation as a result of inhibiting serine formation and thus formation of PE. This conclusion was confirmed by the fact that the addition of serine along with threonine prevented the accumulation of CP III.

A further possibility to restore the accumulation of CP III in biotin-deficient *Arthrobacter* cultures was to add a large amount of methionine to the nutrient solution. The CP III accumulation, similarly to that with threonine, was prevented by adding compounds serving as donor for one carbon units or by adding serine. This shows that methionine interfered with PE. (More details concerning this phenomenon in chapters V and IX).

The fact that in biotin-deficient *Arthrobacter* cultures a ready accumulation of CP III can be achieved (addition of threonine or methionine) shows that the enzymes catalysing the formation of CPG III, like ALA synthetase, are functioning normally. This conclusion is in disagreement with literature recordings claiming that a reduced synthesis of ALA synthetase and a reduced activity of this enzyme are responsible for the absence of CP III accumulation in biotin-deficient bacteria. More evidence against the latter explanation was obtained by the results of experiments with cell-free extracts of biotin-deficient CP III-accumulating arthrobacters grown in the presence of threonine. The ALA synthetase and ALA dehydratase activities in these cells were similar to those of biotin-sufficient cells, accumulating CP III.

In Chapter VIII the integration of protein synthesis and heme formation has been discussed. In cells heme is associated with specific proteins (hemoglobin, cytochromes). Therefore, the regulation of the synthesis of these compounds not only involves the tetrapyrrole part but also the protein moiety. To study the relationship of heme formation and protein synthesis in *Arthrobacter*, strain 223, experiments with chloramphenicol have been carried out.

Addition of chloramphenicol immediately stopped the synthesis of bacterial protein as well as the porphyrin synthesis (CP III accumulation and heme formation). No precursors of CPG III were detected in the cultures incubated with the antibiotic, eliminating the hypothesis that protein-bound precursors of CPG III would exist and would be involved in the inhibiting effect of chloramphenicol on porphyrin formation. Addition of ALA did not restore the porphyrin formation in *Arthrobacter* cultures incubated with the antibiotic.

Decrease of the amount of nitrogen in the medium reduced the amount of synthesized porphyrins to a larger degree than that of bacterial protein. Addition of ALA restored the synthesis of porphyrins, indicating the absence of protein-bound precursors of heme in *Arthrobacter*.

In Chapter IX a comparison was made of the phospholipid composition of *Arthrobacter* cells and the ability of these cells to form different types of porphyrin. Evidence of the functioning of phospholipids in heme formation in *Arthrobacter*, strain 223, has been obtained in chapters V, VI and VII. In these chapters the functioning of phosphatidylethanolamine (PE) in the conversion of CPG III to PP IX was emphasized.

To study the relationship between phospholipids and porphyrin synthesis, cells of *Arthrobacter*, strain 223, either producing large amounts of iron-free porphyrins or synthesizing the iron-containing heme as the predominant tetrapyrrole have been analysed for phospholipid composition.

To obtain cells accumulating CP III, PP IX, or forming heme, use was made of the experimental results recorded in chapters IV, V, VI and VIII.

1. CP III-accumulating cells, grown in medium A with excessive amounts of biotin and iron, contained phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), and phosphatidic acid (PA).

Suppression of the CP III accumulation (which was attended with heme formation) by supplying this medium with compounds serving as donor for one carbon units gave cells containing in addition to the three above-mentioned phospholipids, phosphatidylethanolamine (PE).

2. A different medium in which *Arthrobacter*, strain 223, accumulated large amounts of CP III, was the biotin-rich medium B_3 without added iron. The phospholipid content of those cells was very low, and no PE was detectable.

When this medium had been supplied with mixtures of long chain fatty acids (palmitoleic or oleic acid + a C_{14}, C_{16} or C_{18} saturated fatty acid), CP III accumulation was prevented whereas heme formation was strongly increased. The phospholipid content of these cells was much higher, with PE being the dominant phosphatide.

When the biotin-rich iron-deficient medium B_3 had been supplied with small amounts of myristic acid + oleic acid, PP IX was the porphyrin which accumulated in the culture medium, and PE practically the only phospholipid of the cells.

Supplied with excessive amounts of iron, no PP IX accumulated in the latter medium. Heme was the predominant porphyrin and PE and PA the most important phospholipids. The correlation between accumulation of PP IX and

absence of PA suggested that the latter phospholipid is involved in the conversion of PP IX to heme.

3. In biotin-deficient *Arthrobacter* cultures, grown in medium A with large amounts of iron, no CP III was found; heme was the dominant porphyrin formed and PE the prevailing phospholipid.

When large amounts of threonine were added to *Arthrobacter* cultures grown for 1 day in this medium, or methionine was added to this medium before inoculation, CP III accumulated while the heme content of the cells remained low; no PE was detected in these cells, but increased amounts of PG and DPG.

When the latter medium along with threonine was supplied with N,N-dimethylglycine or serine, CP III accumulation was prevented, heme was formed in a considerable amount while PE was the predominant phospholipid. The same results were obtained when the latter medium was supplied with methionine + N, N-dimethylglycine or serine.

4. Biotin-deficient, iron-deficient *Arthrobacter* cultures grown in medium B_3, accumulated CP III; the heme content as well as the phospholipid content of the cells was low.

Addition of long chain fatty acids to this medium had the same effect as when these acids had been added to the biotin-sufficient, iron-deficient medium (see this summary, 2).

When *Arthrobacter*, strain 223, in medium A with large amounts of biotin and iron (1), started to accumulate CP III, the phospholipid content dropped to a low level, apparently due to the activity of phospholipase C. With continued incubation, the phospholipid content returned to a normal level with PG and DPG, but not PE, as predominant phospholipids found.

To confirm the results obtained in the above-mentioned experiments with whole cells that PE favours the conversion of CPG III to PP IX, experiments were performed with cell-free extracts of *Arthrobacter*, strain 223, grown for 72 hours in iron-deficient B_3 medium. These bacteria had a low content of phospholipids, while no PE was detectable.

The conversion of ALA to PP IX by these extracts required the presence of PE isolated either from *Arthrobacter* or from mammalia. A mixture of PG + DPG + PA, or iron, had no effect. Since iron when supplied to *Arthrobacter* growing in the iron-deficient B_3 medium catalysed this reaction, it seemed to be obvious that in *Arthrobacter* cells this metal exerts its favourable effect as a result of its stimulating effect on PE formation.

To confirm the conclusion that in *Arthrobacter* PA would be involved in the conversion of PP IX to heme, experiments were carried out with the membrane fraction of *Arthrobacter*, strain 223, grown in biotin-sufficient, iron-deficient medium B_3 containing small amounts of myristic acid and oleic acid. The conversion of PP IX + ferrous iron to heme by this membrane fraction was favoured by PA. The highest amount of heme was found with PA containing unsaturated + saturated fatty acids. PA with saturated fatty acids only and a mixture of PA + PG + DPG, isolated from *Arthrobacter*, strain 223, were also active be it to a less extent. PC + PE had no activity.

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SAMENVATTING

Porphyrines (tetrapyrrolen) zijn basiscomponenten van een aantal stoffen, die in levende organismen dienen voor het transport van zuurstof (haemoglobine), electronen (cytochromen) of voor het opvangen van stralingsenergie (chlorophyl). De tetrapyrrolen in deze vorm bevatten een metaal en zijn gebonden aan een specifiek eiwit.

Behalve eiwit-gebonden tetrapyrrolen worden ook vrije porphyrines in de natuur aangetroffen. Deze kunnen beschouwd worden als geoxydeerde porphyrinogenen, de precursors van haem en chlorophyl. De biologische functie van deze vrije porphyrines is niet bekend; hun accumulatie wijst er in het algemeen op dat de vorming van haem of chlorophyl is geblokkeerd. Daarom kan de accumulatie van deze porphyrines als een middel gebruikt worden voor het bestuderen van het effect van verschillende factoren op de vorming van tetrapyrrolen.

Tot nu toe is het effect van B vitamines, zuurstof en ijzer op de vorming van haem en chlorophyl bestudeerd in verschillende organismen waaronder mensen, dieren, planten, algen (vooral *Chlorella* soorten), photosynthetische bacterien en enkele heterotrofe bacterien (vooral *Corynebacterium diphtheriae* en *Micrococcus lysodeikticus*).

Bij het bestuderen van de physiologie van *Arthrobacter* door medewerkers van het Laboratorium voor Microbiologie te Wageningen werd een rood pigment in de cultuurvloeistof gevonden bij een aantal stammen die gekweekt werden onder bepaalde condities (ANTHEUNISSE, persoonlijke mededeling). Bij het voortzetten van dit onderzoek bleek dit rode pigment coproporphyrine III (CP III) te zijn.

LASCELLES (1956) vond dat ijzer de CP III accumulatie elimineerde bij *Rhodopseudomonas speroides*. Dit bleek niet het geval bij een aantal stammen van *Arthrobacter*. Daarom werd dit onderzoek begonnen met experimenten om dit verschijnsel te bestuderen. In latere experimenten werd de regulatie van de porphyrinesynthese in *Arthrobacter*, stam 223, bestudeerd.

In Hoofdstuk III zijn de resultaten vermeld van de experimenten met groeiende cultures van dit organisme in een medium met anorganische zouten en met glucose als koolstofbron (medium A). Ter verkrijging van CP III accumulatie moesten biotine, zink, sulfaat, fosfaat en ammonium ionen in hoeveelheden aanwezig zijn, die aanzienlijk groter waren dan die welke nodig waren voor maximale celopbrengst. Ook waren kleine hoeveelheden ijzer nodig voor de groei en de accumulatie van CP III. Stijgende hoeveelheden ijzer elimineerden de CP III accumulatie niet zoals dat in de literatuur voor een aantal bacteriën is beschreven. (In hoofdstuk VI en IX zijn meer experimenten vermeld, die betrekking hebben op het effect van ijzer op de porphyrinevorming). De aanwezigheid van 1% $CaCO_3$ in het medium voorkwam CP III accumulatie hoewel het de celop-

brengst gunstig beinvloedde. Door de hoeveelheid $CaCO_3$ te verminderen steeg de hoeveelheid CP III.

De vorming van dit porphyrine door *Arthrobacter*, stam 223, bleek sterk te worden beinvloed door de aeratie van de cultuur. De maximale hoeveelheid CP III werd gevonden bij een aeratie, equivalent aan 11 mMol O_2/liter/uur. De hoeveelheid CP III werd maar weinig verlaagd als de aeratie verdubbeld werd. Bijna geen CP III werd gevonden bij 44 mMol O_2/liter/uur ofschoon de celopbrengst niet werd beinvloed. Het verlagen van de aeratie tot 4 mMol O_2/liter uur, verminderde de celopbrengst aanzienlijk en elimineerde de CP III accumulatie. Deze laatste kon hersteld worden door het toevoegen van δ-aminolevulinezuur (ALA, een precursor van CPG III), barnsteenzuur of glutaminezuur. Hieruit bleek dat een tekort aan substraat de oorzaak was van de verminderde CP III accumulatie bij een verlaagde aeratie en niet een verminderde activiteit of synthese van de enzymen die betrokken zijn bij de CPG III vorming.

Het niet geaccumuleerd worden van CP III bij een aeratie van 44 mMol O_2/liter/uur kon gedeeltelijk hersteld worden door toevoeging van ALA, maar niet door het toevoegen van barnsteenzuur of glutaminezuur. Dat een geëlimineerde ALA synthetase-activiteit de oorzaak was van het zich niet ophopen van CP III werd geconcludeerd uit het feit dat bij het verhogen van de aeratie van een CP III ophopende cultuur tot 44 mMol O_2/liter/uur de porphyrineproductie onmiddellijk stopte. De enzymen die de omzetting van ALA in CPG III katalyseren werden ook ongunstig beinvloed door een verhoogde aeratie, maar in mindere mate dan ALA synthetase.

In Hoofdstuk IV zijn de resultaten vermeld van de experimenten die betrekking hebben op de invloed van zwavelhoudende aminozuren op de vorming van porphyrines by cultures van *Arthrobacter*, stam 223. Deze werden gedaan omdat gevonden was dat relatief grote hoeveelheden sulfaat nodig waren voor de accumulatie van CP III zoals beschreven in hoofdstuk III. Er werd verondersteld dat sulfaat via de omzetting in homocysteïne zijn werking uitoefende. Dit was gebaseerd op de volgende waarnemingen: 1. Bij CP III accumulerende *Arthrobacter* cultures kon sulfaat vervangen worden door cysteïne of homocysteïne in een medium dat nog sulfaat als verontreiniging bevatte. Omdat *Arthrobacter*, stam 223, cysteïne gemakkelijk kan omzetten in homocysteïne, maar homocysteïne niet in cystenïe, kon cysteïne niet de oorzaak zijn van de CP III accumulatie. 2. Kleine hoeveelheden threonine, dat de vorming van homoserine remt, voorkwamen de accumulatie van CP III in cultures met sulfaat of cysteïne. Het effect van threonine kon worden geëlimineerd door toevoeging van homocysteïne, of van homoserine wat één van de precursors van homocysteïne is. 3. De aanwezigheid van 1 % $CaCO_3$ in medium A voorkwam de CP III accumulatie tenzij homocysteïne aanwezig was.

Behalve threonine voorkwam ook methionine (50 μgr/ml of meer) de CP III accumulatie in het sulfaatbevattende medium A. Een aantal andere aminozuren, purines, pyrimidines en B vitamines bleken niet in staat te zijn de CP III accumulatie te elimineren. Het elimineren van de CP III accumulatie door methio-

nine zou verklaard kunnen worden door een eindproductremming te veronderstellen die de synthese van homocysteïne reguleert. Dat deze hypothese niet juist was, werd geconcludeerd uit de waarnemingen dat: 1. methionine (50 μgr/ml) de CP III accumulatie voorkwam in een sulfaatbevattend medium, maar niet in een medium met sulfaat + cysteïne, 2. de aanwezigheid van homocysteïne in een medium met 50 μgr/ml methionine geen CP III accumulatie tot gevolg had.

Het elimineren van de CP III accumulatie in medium A door methionine berustte waarschijnlijk op de synthese van een stof die de omzetting van CPG III in protoporphyrine IX (PP IX) en haem bevorderde. Grotere hoeveelheden methionine veroorzaakten in bepaalde gevallen (A medium met cysteine of homocysteine) de afbraak van deze stof en bevorderden als een gevolg daarvan de CP III accumulatie.

In Hoofdstuk V werd de invloed van methionine en homocysteine op de CP III accumulatie meer gedetailleerd bestudeerd. Omdat de methylgroep van methionine in formaldehyde kan worden omgezet (via choline) werd een vergelijking gemaakt tussen het effect van opklimmende hoeveelheden methionine en opklimmende hoeveelheden formaldehyde op de CP III accumulatie in medium A met respectievelijk sulfaat, sulfaat + cysteine en sulfaat + homocysteine. Het effect van methionine op de CP III accumulatie en de haemvorming vertoonde grote gelijkenis met dat van formaldehyde (de kleinste hoeveelheid formaldehyde die nodig was om de CP III accumulatie te voorkomen was gelijk aan de hoeveelheid formaldehyde die kon worden betrokken van de kleinste hoeveelheid methionine die deze accumulatie voorkwam). Uit deze analogie werd de conclusie getrokken dat het effect van methionine op de CP III accumulatie moest worden toegeschreven aan de verbeterde voorziening met C_1 fragmenten (via choline, N,N-dimethylglycine, sarcosine). Bewijsmateriaal voor deze hypothese was het feit dat het effect van verschillende hoeveelheden choline op de CP III accumulatie bijna gelijk was aan dat van formaldehyde en methionine. Een tweede manier waarop methionine de C_1 fragment voorziening van de cellen kon verbeteren was het beperken van het verbruik van deze C_1 fragmenten (er hoeft geen methionine gevormd te worden uit homocysteine + C_1 fragment).

Eén van de belangrijkste reacties in *Arthrobacter*, stam 223, waarbij C_1 fragmenten betrokken zijn, is de synthese van serine uit glycine. Omdat serine via phosphatidylserine (PS) één van de precursors is van phosphatidylethanolamine (PE), werd de laatste component verondersteld nodig te zijn voor de omzetting van CPG III in PP IX. Een verlaging van de hoeveelheid PE in de cel zou automatisch de hoeveelheid CP III verhogen.

Grotere hoeveelheden methionine (toegevoegd aan medium A dat cysteïne of homocysteïne bevatte), formaldehyde of choline deden de CP III accumulatie weer stijgen. Dit effect werd verondersteld veroorzaakt te worden door het verdwijnen van PE uit de cellen, gedeeltelijk door methylering tot phosphatidylcholine (PC) en gedeeltelijk door een verhoogde phospholipase C activiteit tengevolge van de vorming van PC.

Het gunstige effect van grote hoeveelheden methionine, formaldehyde of choline op de CP III accumulatie vereiste de aanwezigheid van homocysteine. Dit aminozuur bleek de vorming van formaldehyde uit choline te verminderen door de omzetting van betaine in N,N-dimethylglycine te remmen. Dit resulteerde in een verminderde voorziening met C_1 fragmenten en als een gevolg daarvan in een verminderde vorming van serine. Meer bewijsmateriaal voor deze conclusie vormde het feit dat N,N-dimethylglycine en sarcosine de CP III accumulatie voorkwamen wanneer deze aan media werden toegevoegd die grote hoeveelheden methionine of choline bevatten.

In Hoofdstuk VI worden experimenten vermeld die handelen over het effect van de ijzervoorziening van de bacterien op de vorming van porphyrines. De voorafgaande experimenten inzake dit onderwerp, vermeld in hoofdstuk III, hebben aangetoond dat grote hoeveelheden ijzer, toegevoegd aan medium A, niet in staat bleken de CP III accumulatie te elimineren overeenkomstig literatuurgegevens betreffende verscheidene typen microben.

De experimenten beschreven in de voorafgaande hoofdstukken hebben aangetoond dat de CP III accumulatie kan worden voorkomen door de C_1 fragment voorziening te verbeteren. Daarom werd het effect van ijzer opnieuw onderzocht met een medium (B_3) dat behalve de componenten van medium A methionine, cysteine en ethanolamine bevatte en bovendien mangaan.

Het reduceren van de toegevoegde hoeveelheid ijzer aan medium B_3 veroorzaakte een verminderde vorming van het ijzerbevattende haem door *Arthrobacter*, strain 223, wat gepaard ging met de accumulatie van een hoeveelheid CP III, die verscheidene keren groter was dan de hoeveelheid haem die gevormd werd met grote hoeveelheden ijzer. Deze overproductie van CP III duidde indirect op de aanwezigheid van een reguleringsmechanisme dat afhankelijk is van haem. De juistheid van deze hypothese werd aangetoond door het toevoegen van stijgende hoeveelheden haem aan medium A of B_3 waarin CP III accumulatie plaats vond en door het bepalen van de activiteit van de enzymen in celvrije extracten van *Arthrobacter*, stam 223. De belangrijkste plaats van het regulerende effect van haem op de CP III accumulatie bleek de omzetting van glycine en barnsteenzuur-CoA in ALA te zijn (ALA synthetase). De synthese van dit enzym werd sterk gerepresseerd door haem, terwijl de activiteit er van matig werd geremd (eindproductremming).

De activiteit van ALA dehydratase, één van de enzymen die de omzetting van ALA in CPG III katalyseren, werd niet geïnhibiteerd door haem; de synthese van dit enzym echter werd sterk gerepresseerd.

IJzer toegevoegd aan het ijzerdeficiënte medium B_3 (waarin CP III accumulatie optrad) had hetzelfde effect op de synthese van ALA synthetase en ALA dehydratase als toegevoegd haem. Hieruit blijkt dat ijzer de accumulatie van porphyrines beinvloedde via de inbouw in haem.

Behalve het regulerende effect van ijzer op de porphyrine-accumulatie via de vorming van haem, kan het de porphyrinevorming ook beinvloeden door de omzetting van CPG III in PP IX te stimuleren. Er werd gevonden dat dit te

wijten was aan het gunstige effect van ijzer op de PE vorming als een gevolg van de stimulering van de synthese van vetzuren, die nodig zijn voor de vorming van PE.

De aanwezigheid van kleine hoeveelheden myristinezuur en oliezuur in het ijzerdeficiënte medium B_3 gaf aanleiding tot de accumulatie van PP IX bij *Arthrobacter*, stam 223. De toevoeging van grote hoeveelheden ijzer aan dit medium voorkwam de accumulatie van PP IX en had de vorming van haem tot gevolg.

De accumulatie van CP III door ijzerdeficiënte cultures van *Arthrobacter*, stam 223, (medium B_3) werd op dezelfde wijze beinvloed door de aeratie van het cultuurmedium als die door ijzerbevattende cultures (A medium) zoals vermeld in hoofdstuk III. Een hoge aeratie verminderde zowel de synthese van ALA synthetase als van ALA dehydratase.

In Hoofdstuk VII werd het effect van de biotinevoorziening van *Arthrobacter*, stam 223, op de vorming van porphyrines meer gedetailleerd bestudeerd. Voorafgaande experimenten, vermeld in hoofdstuk III, hadden aangetoond dat er voor CP III accumulatie aanzienlijk meer biotine in medium A aanwezig moest zijn dan nodig was om optimale groei te verkrijgen. In de experimenten besproken in hoofdstuk VII werd medium A gebruikt met 5.10^{-5} µgr/ml biotine. Bij deze concentratie hoopte zich geen CP III op en werd er haem gevormd.

Toevoeging van grote hoeveelheden threonine aan 1 dag oude cultures van *Arthrobacter*, stam 223, reduceerde het haemgehalte van de cellen sterk en CP III hoopte zich op. Deze accumulatie werd niet verminderd door het verhogen van de hoeveelheid ijzer in het medium, maar werd geëlimineerd door een aantal componenten die fungeren als donor voor C_1 fragmenten. Dit toont aan dat threonine de CPG III vorming bevorderde als een gevolg van het remmen van de vorming van serine en dus van de vorming van PE. Deze conclusie werd bevestigd door het feit dat toevoeging van serine tegelijk met threonine de CP III accumulatie voorkwam.

Nog een andere mogelijkheid om de CP III accumulatie in biotinedeficiënte *Arthrobacter* cultures te herstellen was het toevoegen van relatief vrij grote hoeveelheden methionine aan de voedingsoplossing. De CP III accumulatie werd voorkomen, zoals bij threonine, door toevoeging van componenten die fungeren als donor voor C_1 fragmenten of door serine. Dit toont aan dat methionine een storende invloed had op PE (meer details betreffende dit verschijnsel in de hoofdstukken V en IX). Dat biotinedeficiënte *Arthrobacter* cultures gemakkelijk tot CP III accumulatie gebracht konden worden, toont aan dat de enzymen die de vorming van CPG III katalyseren, zoals ALA synthetase, normaal functioneren. Deze conclusie is niet in overeenstemming met literatuurgegevens waarin beweerd wordt dat een verminderde vorming van ALA synthetase en een verminderde activiteit van dit enzym verantwoordelijk zijn voor het niet geaccumuleerd worden van CP III door biotinedeficiënte bacteriën. Meer bewijsmateriaal tegen deze laatste hypothese werd verkregen door experimenten met celvrije extracten van biotinedeficiënte CP III accumulerende

cellen van *Arthrobacter* die gekweekt waren in een medium met threonine. De activiteit van ALA synthetase en ALA dehydratase in deze cellen was gelijk aan die in biotinerijke cellen.

In Hoofdstuk VIII is de integratie van de synthese van eiwit met die van haem besproken. Haem is intracellulair gebonden aan specifieke eiwitten (haemoglobine, cytochromen). Daarom is bij de regulatie van de synthese van deze componenten niet alleen het tetrapyrrool deel betrokken, maar ook het eiwitgedeelte. Experimenten met chlooramphenicol werden uitgevoerd om de relatie tussen eiwitsynthese en haemvorming na te gaan.

Door toevoeging van chlooramphenicol werd zowel de eiwitsynthese van de bacteriën als de porphyrinesynthese gestopt (CP III accumulatie en haemvorming). Geen precursors van CPG III werden gevonden in de cultures met het antibioticum wat de hypothese elimineerde dat er eiwitgebonden precursors van CPG III of haem zouden bestaan en dat deze betrokken zouden zijn bij het remmende effect van chlooramphenicol op de porphyrinevorming. Toevoeging van ALA aan cultures met het antibioticum herstelde de porphyrinesynthese niet.

Een vermindering van de hoeveelheid stikstof in het medium reduceerde de hoeveelheid porphyrine veel meer dan de hoeveelheid bacterieeiwit. Dat toevoeging van ALA de porphyrinevorming herstelde, wijst op de afwezigheid van eiwitgebonden precursors van haem in *Arthrobacter*.

In Hoofdstuk IX werd een vergelijking gemaakt tussen de phospholipidesamenstelling van *Arthrobacter* en de mogelijkheid tot het vormen van verschillende soorten porphyrines. Bewijsmateriaal voor het functioneren van phospholipiden bij de vorming van haem was verkregen in de hoofdstukken V, VI en VII. Daarin werd de nadruk gelegd op de functie van phosphatidylethanolamine bij de omzetting van CPG III in PP IX.

Om de relatie te bestuderen tussen phospholipiden en porphyrinevorming werd de phospholipidesamenstelling geanalyseerd van cellen van *Arthrobacter*, stam 223, die ijzervrije porphyrines produceerden en van die welke het ijzerbevattende haem vormden.

Om cellen te verkrijgen die CP III of PP IX accumuleerden of die haem vormden werd gebruik gemaakt van de resultaten van de experimenten vermeld in de hoofdstukken IV, V, VI en VII.

1. CP III accumulerende cellen gegroeid in medium A met overmaat biotine en ijzer bleken phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) en phosphatidylzuur (PA) te bevatten.

Toevoeging van componenten die fungeerden als donor voor C_1 fragmenten en die de CP III accumulatie onderdrukten (wat gepaard ging met haemvorming) resulteerde in cellen die behalve de drie bovengenoemde phospholipiden ook nog phosphatidylethanolamine (PE) bezaten.

2. Een ander medium waarin *Arthrobacter*, stam 223, grote hoeveelheden CP III ophoopte was het biotinerijke medium B_3 zonder toegevoegd ijzer. Het

phospholipidegehalte van deze cellen was erg laag en er kon geen PE in aangetoond worden.

De accumulatie van CP III werd voorkomen (terwijl een sterke haemvorming plaats vond) door toevoeging van mengsels van hogere vetzuren (palmitoleinezuur of oliezuur + een C_{14}, C_{16} of C_{18} verzadigd vetzuur). Het phospholipidegehalte van deze cellen was veel hoger en PE was het dominerende phosphatide.

PP IX werd geaccumuleerd wanneer het biotinerijke, ijzerdeficiënte medium B_3 kleine hoeveelheden myristinezuur + oliezuur bevatte. PE was bijna het enige phospholipide in deze cellen.

Bij toevoeging van grote hoeveelheden ijzer aan dit laatste medium werd geen PP IX geaccumuleerd. Haem was het overheersende porphyrine en PE en PA waren de belangrijkste phospholipiden. De correlatie tussen PP IX accumulatie en afwezigheid van PA suggereerde dat dit laatste phospholipide is betrokken bij de omzetting van PP IX in haem.

3. In biotinedeficiënte *Arthrobacter* cultures die gekweekt werden in medium A met grote hoeveelheden ijzer werd geen CP III gevonden; haem was het overheersende porphyrine en PE het belangrijkste phospholipide. CP III accumuleerde en het haemgehalte was sterk verlaagd, wanneer threonine in grote hoeveelheden werd gegeven aan 1 dag oude cultures in dit medium of wanneer methionine voor het enten werd toegevoegd aan het medium. Er werd geen PE gevonden in de cellen, die verhoogde hoeveelheden PG en DPG bezaten.

Het toevoegen aan dit laatste medium van N,N-dimethylglycine of serine tegelijk met threonine voorkwam de CP III accumulatie; haem werd in aanzienlijke hoeveelheden gevormd terwijl PE het overheersende phospholipide was. Dezelde resultaten werden verkregen wanneer aan dit laatste medium methionine + N,N-dimethylglycine of serine werden toegevoegd.

4. Biotinedeficiënte, ijzerdeficiënte *Arthrobacter* cultures, gegroeid in medium B_3, accumuleerden CP III; zowel het haem- als het phospholipidegehalte van de cellen was laag.

Toevoeging van hogere vetzuren aan dit medium had hetzelfde effect als de toevoeging van deze zuren aan een biotinerijk, ijzerdeficiënt medium (zie deze samenvatting, 2).

Wanneer *Arthrobacter*, stam 223, in medium A met grote hoeveelheden biotine en ijzer (1) CP III begon te accumuleren daalde het phospholipidegehalte tot een laag niveau wat blijkbaar het gevolg was van phospholipase C activiteit. Bij voortzetting van de incubatie steeg het phospholipidegehalte weer tot een normaal niveau met PG en DPG, maar niet PE, als de overheersende phospholipiden.

In bovengenoemde experimenten met intacte cellen blijkt dat PE de omzetting van CPG III in PP IX bevordert. Om deze resultaten te bevestigen werden experimenten uitgevoerd met celvrije extracten van *Arthrobacter*, stam 223, die gedurende 72 uur gekweekt was in het ijzerdeficiënte medium B_3. Deze bacterien hadden een laag phospholipidegehalte en PE was niet aantoonbaar.

Voor de omzetting van ALA in PP IX door deze extracten was PE, geisoleerd van *Arthrobacter* of van zoogdieren, nodig. Een mengsel van PG + DPG + PA

of ijzer hadden geen effect. Omdat ijzer toegevoegd aan het ijzerdeficiënte medium B_3 bovengenoemde reactie bij *Arthrobacter* katalyseerde, leek het voor de hand te liggen dat in cellen van *Arthrobacter* dit metaal zijn gunstige werking uitoefent als een gevolg van de stimulering van de vorming van PE.

Om de conclusie te bevestigen dat in *Arthrobacter* PA betrokken zou zijn bij de omzetting van PP IX in haem werden experimenten uitgevoerd met de membraanfractie van *Arthrobacter*, stam 223, die gekweekt was in het biotinerijke, ijzerdeficiënte medium B_3 met kleine hoeveelheden myristinezuur en oliezuur. De omzetting van PP IX + ijzer (ferro)in haem door deze membraanfractie werd gestimuleerd door PA. De grootste hoeveelheid haem werd gevormd met PA dat onverzadigde + verzadigde vetzuren bevat. PA dat alleen maar verzadigde vetzuren bevat en een mengsel van PA + PG + DPG, geisoleerd van *Arthrobacter*, stam 223, hadden ook een stimulerend effect zij het in mindere mate. Een mengsel van PC + PE had geen effect.

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