CYANIDE PRODUCTION AND DEGRADATION BY *CHROMOBACTERIUM VIOLACEUM*

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by

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to my parents,
for the help and encouragement
that enabled my studies to reach this stage.

Poul Anderson 1926 -

> *I have yet to see any problem, however complicated, which when you looked at it in the right way, did not become still more complicated.*

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ABSTRACT

Chromobacterium violaceum is one of a small number of bacteria that have previously been found to produce inorganic cyanide. Glycine is known to be the substrate for cyanide formation by these organisms but the mechanism by which cyanide is formed from glycine is unknown. Methionine has been found to stimulate cyanogenesis by bacteria; again the mechanism is unknown. *C.violaceum* also possesses the ability to metabolise the cyanide produced, and the formation of the amino acids β-cyanoalanine, asparagine, aspartate and γ-cyano-α-aminobutyric acid from cyanide by non-proliferating cells has previously been demonstrated.

This study is an attempt to investigate further the conditions of cyanide production by *C.violaceum*, when grown on L-glutamate as carbon and nitrogen source, and the relationship between the formation of cyanide and its utilisation or degradation.

Although glycine is the substrate for cyanide production the relationship between the glycine concentration of the growth medium, and the amount of cyanide produced was found to be more complex than a simple precursor-product relationship. At low glycine or methionine concentrations glycine was found to inhibit cyano-genesis but this inhibitory effect was overcome at high glycine or methionine concentrations. Glycine is postulated to partially repress synthesis of the cyanide producing system. Cyanide is produced during the transition from exponential growth to the stationary phase of growth, and work with an inhibitor of protein synthesis suggests that the cyanide producing system is induced during exponential growth. Increases in the ferrous ion and phosphate concentrations of the growth medium were found to stimulate cyanide production but variations in growth

temperature and initial pH value of the growth medium, over ranges which supported growth, had no effect on cyanide production. The conditions of cyanide production are typical of those of microbial secondary metabolites and it is suggested that cyanide is such a metabolite.

C.violaceum has previously been found to contain two cyanide utilising enzymes, β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase. The presence of these two activities is confirmed, and a third cyanide utilising enzyme, rhodanese, was also detected. The three cyanide utilising enzymes are found to be induced towards the end of exponential growth in cells growing under high cyanide - (i.e. grown on glutamate supplemented with glycine and methionine) or low cyanide - (i.e. grown on glutamate alone) producing conditions. This occurs at a time when the cyanide content of the medium is increasing and cyanide is postulated to induce the synthesis of the cyanide utilising enzymes. Inclusion of glycine in the growth medium partially represses the induction of β-cyanoalanine synthase and γ-cyano-α-amino butyric acid synthase, and methionine is shown to inhibit β-cyanoalanine synthase activity. β-cyanoalanine was found to accumulate in the medium of stationary phase cultures but neither γ-cyano-α-amino-butyric acid nor thiocyanate could be detected, suggesting that β-cyano-alanine synthase is the major enzyme involved in further metabolism of cyanide. No evidence could be found for the conversion of β-cyanoalanine to asparagine and aspartate. Addition of chloramphenicol to low- and high-cyanide evolving cultures was found to have a marked effect on the medium cyanide levels. These observations are shown to have been caused by chlor-amphenicol blocking the induction of the cyanide utilising enzymes and the resulting inhibition of β-cyanoalanine formation from cyanide.

A scheme (Fig.41) is put forward to explain the effects of glycine and methionine on the cyanide content of growing cultures of *C.violaceum*.

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CHAPTER THREE - RESULTS

CHAPTER ONE

(INTRODUCTION)

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The Occurrence of Cyanide in Nature.

Inorganic cyanide production by living organisms is a widespread phenomenon, occurring in both the prokaryotic and eukaryotic kingdoms. Many cyanogenic organisms also possess the ability to utilise or degrade the cyanide produced.

Approximately one thousand species of higher plants, many of which are of economic importance, have been reported to be cyanogenic (Conn, 1969). Many eukaryotic microorganisms, including a wide range of fungi (Knowles, 1976) and the alga *Chlorella vulgaris* (Gewitz et al., 1976) produce cyanide. Although cyanide production is widespread in eukaryotic microorganisms, certain Pseudomonads and *Chromobacterium violaceum* are the only bacterial species known to produce cyanide (Knowles, 1976).

Virtually all the organisms so far found to be cyanogenic exist in soil which makes cyanide an important constituent of the soil environment. An investigation of the various mechanisms of cyanide production, utilisation and resistance is therefore necessary for a complete understanding of the ecology of the root rhizosphere.

Although the cyanide produced by all cyanogenic organisms has an amino-acid as a precursor, cyanide producers can be divided into two main groups on the basis of the intermediates involved in cyanide formation. Cyanide production by the higher plants involves the formation of a stable intermediate, a cyanogenic glycoside, from which cyanide is liberated only by the action of a series of enzymes (Conn, 1969). In contrast, cyanogenic microorganisms produce cyanide either directly from an amino acid or via unstable intermediates which have yet to be identified but which may be glyoxylic acid cyanohydrin or another cyanohydrin (Knowles, 1976).

Cyanide Production by Higher Plants.

Cyanogenic glycosides, the stable intermediates in plant cyanogenesis,

are compounds which liberate HCN, one or more molecules of sugar and an aldehyde or ketone on treatment with dilute acid or the appropriate hydrolytic enzymes (see Fig.1). The majority of cyanogenic glycosides are formed from one of four amino acids, valine, isoleucine, phenylalanine and tyrosine (Conn, 1973). Experiments, involving feeding radiolabelled amino acids to cyanogenic plants, have shown that the α-carbon atom and the amino nitrogen of the amino acid are used to form the cyanide group of cyanogenic glycosides, apparently without cleavage of the covalent CN linkage. The biosynthetic pathway of cyanogenic glycosides (Fig.1) is thought to involve aldoximes and nitriles as intermediates (MacFarlane et al., 1975; Shimada and Conn, 1977), resulting in the formation of an unstable cyanohydrin which is glucosylated to form a stable cyanogenic glycoside.

Catabolism of plant cyanogenic glycosides to release hydrogen cyanide occurs in two steps. Initially a glucosidase (EC 3.2.1.21, β-D-glucose glucohydrolase) acts to remove glucose and release the cyanohydrin. Although the cyanohydrin is unstable hydrogen cyanide release is facilitated by the action of an oxynitrilase (Fig.1). In healthy, undisturbed plant tissue the cyanogenic glycoside and the enzymes for its catabolism are compartmentalised so hydrogen cyanide release does not occur. On injury to the tissue the compartments are broken and cyanide liberated (Conn, 1969).

Cyanide Production by Microorganisms.

In contrast to higher plant cyanogenesis, microbial cyanide production does not involve the formation of a stable cyanogenic intermediate which can be enzymically degraded to release free cyanide; instead cyanide formation proceeds from the precursor amino acid either directly or via unstable intermediates.

(a) *Chlorella*. The alga *Chlorella vulgaris* is the only eukaryotic microorganism, other than the fungi, in which cyanide production has been

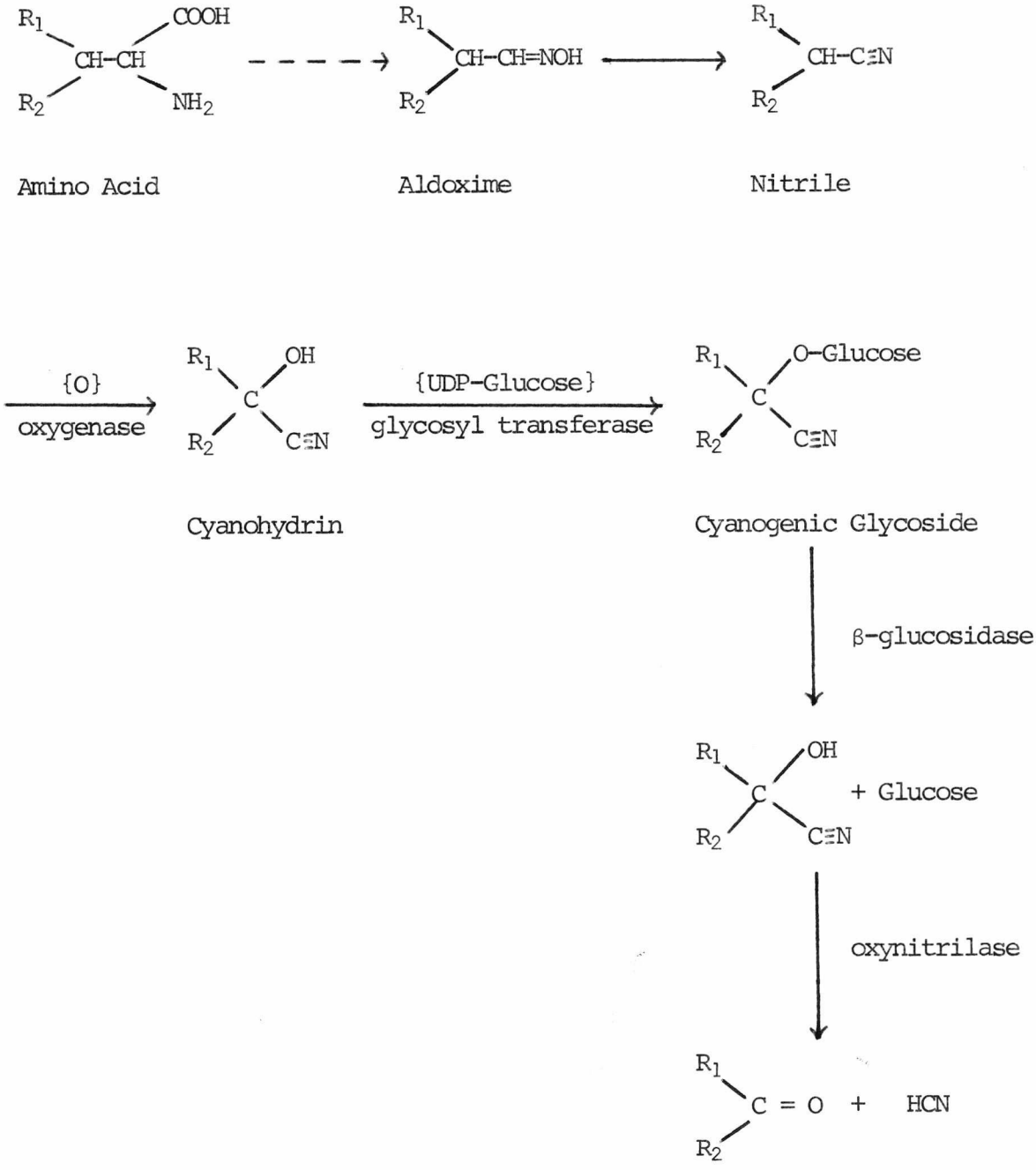


FIGURE 1 - Pathway of cyanide formation in higher plants.

(from Knowles, 1976)

reported. A reversible inactivation of nitrate reductase in this organism, which appeared to be related to its ability to produce cyanide, was observed (Pistorius et al., 1976). Cyanide production by *Chlorella* is stimulated by light and oxygen (Gewitz et al., 1976). Extracts have been obtained from this organism which catalyse the formation of cyanide from the naturally occurring aromatic amino acids (viz. histidine, tyrosine, phenylalanine and tryptophan) with D-histidine being the best substrate for cyanide production (Pistorius et al., 1977). The formation of HCN from D-histidine by these extracts was shown to be due to the combined action of a soluble protein and a particulate component. Either horse radish peroxidase (EC 1.11.1.7) or a metal ion with redox properties (e.g. ions of manganese or vanadium) could be substituted for the particulate component. The D-amino acid oxidase (EC 1.4.3.3) from kidney and the L-amino acid oxidase (EC 1.4.3.2) from snake venom were found to cause HCN production from histidine when supplemented with the particulate preparation from *Chlorella* or with peroxidase or with a redox metal ion.

Subsequently, the soluble protein was partially purified (Pistorius and Voss, 1977). It catalysed the formation of HCN from D-histidine when supplemented with peroxidase or with a redox metal ion, and was characterised as a D-amino acid oxidase. Only about 20% of the D-histidine is converted to HCN, the rest undergoes the normal oxidative deamination process. Cyanogenesis is therefore a side-reaction of the D-amino acid oxidase. The mechanism of HCN production from histidine by the D-amino acid oxidase has yet to be elucidated.

(b) Fungi. Cyanide production has been observed in a wide range of fungi; it has been suggested that cyanide may be a normal fungal metabolite (Hutchinson, 1973). Most of the studies done on fungal cyanogenesis have been made using a psychrophilic basidiomycete which causes snow-mold

(winter crown rot) in alfalfa and other forage plants. The pathology of the disease is related to the ability of the basidiomycete to produce cyanide and to release a β-glucosidase which breaks down the host plant's cyanogenic glucosides, thus causing more release of cyanide (Colotelo and Ward, 1961).

Cyanogenesis of fungi was first studied in liquid culture by Lebeau and Dickson (1953). They showed that the snow-mold basidiomycete grew as mycelia with clamp connections in stationary liquid culture in a basal salts medium, in soybean meal soil, or ground alfalfa crown tissues. The optimum temperature for both growth and cyanide production was 12°C. Cyanide production occurred during active growth on all media but was greatest on the complex media. Organic nitrogen sources were found to be more favourable for cyanide synthesis than inorganic ones (Lebeau and Dickson, 1955).

Growing in shake flasks, on complex and synthetic media, the snow-mold basidiomycete produces cyanide only during the autolytic phase, several days after growth had reached a maximum (Ward and Lebeau, 1962). This would seem to contradict the earlier observations on cyanide production during active growth of mycelial surface colonies. However, in mycelia the growing outer edge is followed by an inner area of autolysing organisms which may be responsible for cyanide production.

The snow-mold basidiomycete produces an unstable cyanogenic compound in shake cultures (Ward, 1964). Free cyanide could not be detected in cultures until the start of stationary phase but an increasing rate of synthesis of the cyanogen occurred during active growth, and at all times the concentration of the cyanogen was much greater than that of free cyanide. The major cyanogen present in extracts of snow-mold basidiomycete has been shown to be glyoxylic acid cyanohydrin (Tapper and MacDonald, 1974).

Lower concentrations of pyruvic acid cyanohydrin were also formed. Glyoxylic acid cyanohydrin has properties similar to the cyanogen isolated by Ward (1964) and is presumably the same compound. Why cyanide is released from the cyanogen during autolysis is not known; possibly an oxynitrilase is induced during this phase.

Glycine has been established as the precursor for cyanide in the snow-mold basidiomycete. Inclusion of glycine in a glucose-basal salts growth medium stimulated cyanide production by the snow-mold basidiomycete growing in shake flasks (Ward and Thorn, 1966). Labelling studies confirmed that cyanide is produced from glycine and that it is derived from the methylene carbon of glycine and not the carboxyl carbon (Ward and Thorn, 1966). Serine was also able to act as a cyanide precursor although not as efficiently as glycine; presumably serine was being converted to glycine by serine hydroxymethyltransferase (EC 2.1.2.1). These results have been confirmed (Ward et al., 1971) and extended to show that the carbon-nitrogen bond of glycine remains intact during the conversion to cyanide (Ward et al., 1977). However, the exact pathway of cyanide formation from glycine by the snow-mold basidiomycete remains to be established. In particular, it is not known if glyoxylic acid cyanohydrin is an intermediate in cyanide formation.

Cyanide production by *Marasmius oreades* has been demonstrated, both in natural conditions (Filer, 1965) and in shake flask cultures during the autolytic phase of mycelial growth (Lebeau and Hawn, 1963). *M.oreades* forms a cyanogen of similar properties to the glyoxylic acid cyanohydrin produced by the snow-mold basidiomycete (Ward and Thorn, 1965) but no studies have been made to determine if glycine is the cyanide precursor in this organism.

(c) Bacteria. In contrast to its widespread distribution among the Fungi, cyanogenesis by bacteria is limited to certain pseudomonads and *Chromobacterium violaceum*. *C.violaceum* was the first bacterium from which cyanide production was reported (Clawson and Young, 1913). Subsequently, *Bacillus pyocyaneus (Pseudomonas aeruginosa)* was found to produce cyanide by Patty (1921) who also attempted to quantitate the amount of cyanide formed. *C.violaceum* was found to be a more vigorous cyanide producer than *Ps.aeruginosa* by Sneath et al., (1953) and cyanide production has been used as a diagnostic test for *C.violaceum* (Sneath, 1956). Other bacteria which have been demonstrated to be cyanogenic are: *Pseudomonas chlorophis* (Michaels and Corpe, 1965); *Pseudomonas aureofaciens* (Michaels and Corpe, 1965); an unidentified *Pseudomonas* species (Wissing, 1968); *Pseudomonas fluorescens* (Freeman et al., 1975); *Pseudomonas polycolor* (Castric, 1975).

Lorck (1948) was the first person to investigate the conditions of cyanide production. In strains of *Ps.aeruginosa* the cyanide yield of cultures incubated at 26°C was three times that of cultures grown at 37°C but no mention is made of the relative growth rates at either temperature. Experiments with synthetic media showed that cyanide could be formed with glycine as the only nitrogen source whereas cultures with glutamic acid or asparagine as nitrogen source did not form cyanide. Further experiments in synthetic glycine media, with the addition of small amounts of nutrient broth or yeast extract, suggested that these substances contain factors which accelerate cyanide production.

Michaels and Corpe (1965) confirmed that cyanide production is dependent on the inclusion of glycine in the growth medium. *C.violaceum*, which grew well on peptone or on a minimal salts medium containing L-glutamate, DL-alanine, L-histidine or glucose plus ammonia, produced a larger amount of cyanide (630 μM) when grown on peptone than on the other substrates.

Addition of guanine, alanine, glycine or methionine, but not any of forty-five other amino acids and nitrogenous compounds, to stationary phase cultures grown on glutamate stimulated cyanide production without further growth. A mixture of glycine and methionine had a synergistic effect, stimulating cyanide production to a level similar to that found for growth on peptone. Glycine could be replaced by several related compounds, and glycine methyl ester gave an even greater cyanide yield.

Growing *C.violaceum* to stationary phase on glutamate-basal salts medium, harvesting, washing and resuspending the cells in basal salts, results in cyanide formation on addition of glycine and methionine (Michaels and Corpe, 1965). Addition of glycine and methionine to the growth medium two hours before harvesting substantially increased the cyanide yield. Addition of succinate, malate or fumarate to the washed cell suspension in basal salts containing glycine and methionine doubled the cyanide yield without further growth. This stimulating effect was inhibited by both azide and dinitrophenol, suggesting that succinate, malate or fumarate may be acting as respiratory energy sources.

These experiments established glycine as the substrate for cyanide production although the role of methionine is equivocal. Methionine can be replaced by betaine, dimethylglycine or choline, suggesting that it could be acting as a methyl donor (Michaels and Corpe, 1965).

In all cyanogenic bacterial species studied so far, maximal cyanide production in growing cultures occurs during the transition from exponential growth to the stationary phase of growth. This has been observed in an unidentified *Pseudomonas* species (Wissing, 1968), *Ps.aeruginosa* (Castric, 1975) and *C.violaceum* (Michaels and Corpe, 1965; Niven et al., 1975). However, the cause of cessation of growth was not clear in any of these studies. If growth ceased, due to oxygen limitation rather than depletion

of a carbon or nitrogen source, it is possible that maximal cyanide production was due to conditions of low aeration rather than cessation of growth. This seems unlikely in the light of the observation that *Ps.aeruginosa* when grown anaerobically with nitrate as electron acceptor produces very little cyanide yet when anaerobically grown cells are aerated cyanide production occurs (Castric, 1975). Also, it has been demonstrated that cyanide production by cells of a *Pseudomonas* species suspended in phosphate buffer plus glycine is low under anaerobic conditions and that oxygen or artificial electron acceptors (e.g. phenazine methosulphate, methylene blue, 2,6-dichlorophenol indophenol and ferricyanide) can stimulate cyanide production (Wissing, 1974).

Production of a compound at the transition between the tropho- and idiophases of growth is typical of microbial secondary metabolites (Drew and Demain, 1977) suggesting that cyanide is such a product. Microbial secondary metabolism may be characterised as follows: (a) there is a limited taxonomic distribution of a given secondary metabolite; (b) synthesis from primary metabolite precursors occurs after the period of active cell growth, at which time limited amounts of the compound are produced over a relatively brief period of time; (c) synthesis is preceded by transcription and translation, resulting in the secondary metabolite synthetases; (d) synthesis occurs within ranges of temperature, oxygen, specific metal ions and phosphate concentration which are narrower than those over which growth occurs (Weinberg, 1971). Certainly there is a limited taxonomic distribution of cyanide production (Castric, 1975) and cyanogenesis occurs after the period of active cell growth in *Ps.aeruginosa*, a *Pseudomonas* species and *C.violaceum*. Also cyanogenesis in *Ps.aeruginosa* occurs within ranges of temperature (Castric, 1975) and phosphate concentration (Megathanan and Castric, 1977) narrower than those over which growth occurs. Cyanide

production by *Ps.aeruginosa* is dependent on the iron concentration (in the form of $FeCl_3$) in the growth medium (Castric, 1975). Finally, the addition of chloramphenicol during growth of *Ps.aeruginosa* inhibited cyanogenesis, indicating the need for protein synthesis before cyanide production occurs (Castric, 1975). All these observations lend weight to the suggestion that cyanide is a bacterial secondary metabolite.

There have been no reports of the formation of any cyanogenic compounds by bacteria.

The mechanism of cyanide formation from glycine remains unclear, although radioisotope experiments have been used to establish which amino acids can act as cyanide precursors and to determine the origin of the carbon and nitrogen atoms of cyanide. In experiments using *C.violaceum*, cells incubated in a chemically defined medium containing methionine and {^{14}C} labelled glycine, {^{14}C}labelled cyanide was formed (Michaels et al., 1965). When {2-^{14}C} glycine was used a significant fraction of the cyanide formed was labelled, but when {1-^{14}C} glycine or {1-*methyl*-^{14}C}methionine was added instead, the specific activity of the liberated cyanide was very low. Carbon dioxide was evolved by the incubation mixture and this was found to be labelled when {1-^{14}C} glycine but not {2-^{14}C}glycine or {1-*methyl*-^{14}C} methionine was added. The fraction of {1-^{14}C}glycine in the evolved carbon dioxide was found to be almost equivalent to the cyanide arising from {2-^{14}C} glycine. These results suggest that cyanide and carbon dioxide are formed from glycine in equimolar amounts and that the carbon atom of cyanide is derived from the methylene group of glycine whilst the carbon atom of carbon dioxide is derived from the carboxyl group of glycine.

Brysk et al., (1969b) attempted to estimate how much exogenously added glycine is converted to cyanide by *C.violaceum* growing on a glutamate-basal salts medium. After seven hours growth the medium was supplemented with

methionine plus {2-$^{14}C^{15}N$}glycine and growth was continued for a further eighteen hours. Under these conditions further metabolism of cyanide to β-cyanoalanine occurs. Taking into account the amount of cyanide metabolised to β-cyanoalanine, at least 32% of the glycine utilised by *C.violaceum* was converted to cyanide. Under these conditions cyanide would appear to be an important pathway of glycine metabolism. The $^{15}N/^{14}C$ ratios of the administered glycine, unconsumed glycine, and cyanide and β-cyanoalanine formed were essentially the same, indicating that cyanide is produced from glycine without cleavage of the carbon-nitrogen bond.

Using washed cells of a *Pseudomonas* species resuspended in phosphate buffer, in the absence of methyl donors or energy sources, the molar ratio of cyanide produced to glycine consumed has been measured (Wissing, 1974). A ratio of one was obtained suggesting that no further metabolism of cyanide occurred under these conditions. An involvement of electron transfer mediated by a flavoprotein is indicated by the observation that cyanide production by resuspended cells was stimulated by oxygen and various artificial electron acceptors but inhibited by a number of flavin enzyme inhibitors. Further evidence for the involvement of flavin enzyme(s) in cyanide production from glycine comes from cell-free extracts of the *Pseudomonas* species (Wissing, 1975). With these extracts cyanide production was stimulated by the inclusion of flavin adenine dinucleotide in the incubation mixture. Sucrose density gradient fractionation of the cell-free extracts showed that the cyanide-producing activity is associated with particulate, rather than soluble, material.

The radioisotope experiments with the snow-mold basidiomycete and *C.violaceum* suggest that cyanide production from glycine proceeds by the same pathway in fungi and bacteria. On the basis of these results and his own work on the *Pseudomonas* species, Wissing (1974) has proposed the

mechanism of cyanide production depicted in Fig.2(a). This involves two flavoprotein oxidation steps and an unstable imino acid as an intermediate; this is consistent with the available data but there is as yet no evidence for the involvement of an imino acid. Knowles (1976) has suggested that this is a simplification and that an extra step may be involved (see Fig.2(b)). It is speculated that the first step may involve a peroxidative, flavin linked enzyme related to glycine oxidase, an enzyme for which an imino acid has been implicated as an intermediate in its reaction mechanism (Hafner and Wellner, 1971).

Radiolabelling experiments on cyanide production by *Ps.aeruginosa* have indicated that the pathway of cyanogenesis from glycine by this organism may differ from that of the organisms mentioned above (Castric, 1977). Glycine, threonine and serine were found to be good precursors of cyanide whilst methionine was not. Presumably threonine is converted to glycine by threonine aldolase whilst serine is converted to glycine through the action of serine hydroxymethyltransferase. A significant difference in cyanogenesis by *Ps.aeruginosa* compared with *C.violaceum* is that {^{14}C}cyanide is produced with low dilution of label from either {1-^{14}C}glycine or {2-^{14}C}glycine by *Ps.aeruginosa* whereas only {2-^{14}C}glycine can serve as a precursor for {^{14}C} cyanide in *C.violaceum*. When whole cells of *Ps.aeruginosa* were fed {1,2-^{14}C}glycine, cyanide and bicarbonate were the only radioactive extracellular products observed.

The difference in the labelling pattern of cyanide produced by *Ps.aeruginosa* may be accounted for in one of two ways (Castric, 1977). Glycine may be metabolised via a primary metabolic pathway that results in a randomisation of label before conversion to the cyanide precursor. Alternatively, randomisation may occur during secondary metabolism with the conversion of the immediate precursor to cyanide. No evidence was presented

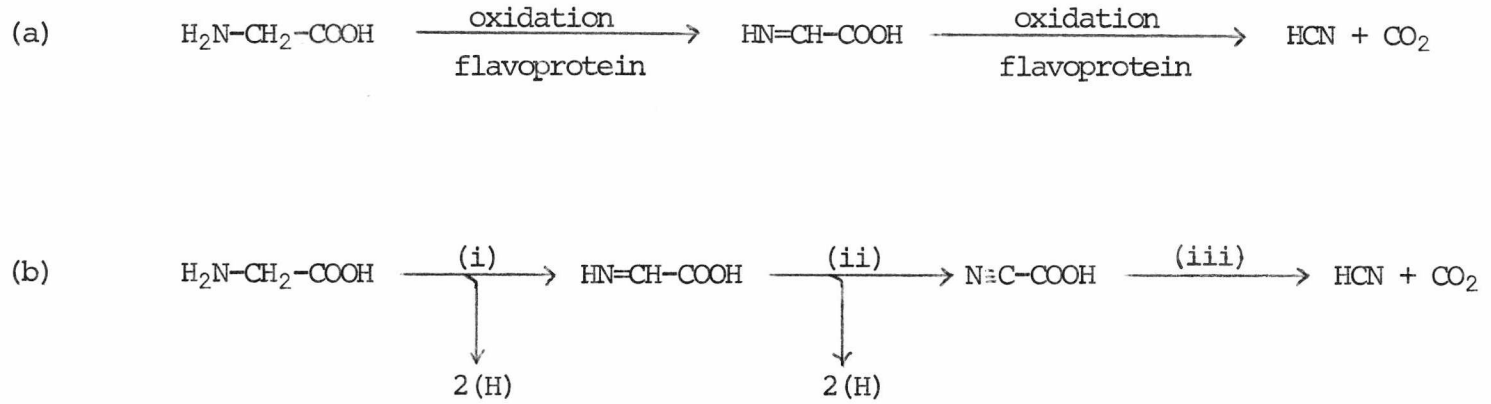


FIGURE 2 - Possible pathways of cyanide formation from glycine by microorganisms.

for either pathway or for any intermediates on the pathways. If the first possibility occurs, i.e. randomisation of label in glycine before its conversion to cyanide, it is possible that the mechanism of cyanide formation from glycine is the same in both *Ps.aeruginosa* and *C.violaceum*.

The role of methionine in cyanide production from glycine is not clear. Although methionine stimulates cyanogenesis by bacteria it does not do so in the snow-mold basidiomycete (A.W. Bunch, personal communication). Betaine, dimethyl glycine and choline can replace methionine in stimulating cyanide production by *C.violaceum* suggesting that methionine is functioning as a methyl donor. Fig.3 gives an outline of the inter-related system for production of serine, glycine and methionine by bacteria. In *Escherichia coli*, addition of methionine to the growth medium represses synthesis of serine hydroxymethyl-transferase, 5,10-methylene tetrahydrofolate reductase and homocysteine methyltransferase (Mansouri et al., 1972). The action of methionine in stimulating cyanogenesis in bacteria may well be related to its ability to regulate one-carbon metabolism and the interconversion of serine and glycine.

Cyanide Degradation and Assimilation.

Many cyanogenic organisms possess metabolic pathways of cyanide degradation and assimilation but as far as it is known, are unable to grow on cyanide as a carbon and/or nitrogen source. There are also examples of microorganisms that, although unable to produce cyanide, have the ability to degrade or assimilate cyanide. In addition, a few bacteria have been found to be capable of growth on cyanide as the sole carbon and nitrogen source which raises the interesting possibility of various bacteria utilizing the cyanide excreted by other organisms as a growth substrate.

The metabolic pathways of cyanide degradation and assimilation so far investigated are outlined below.

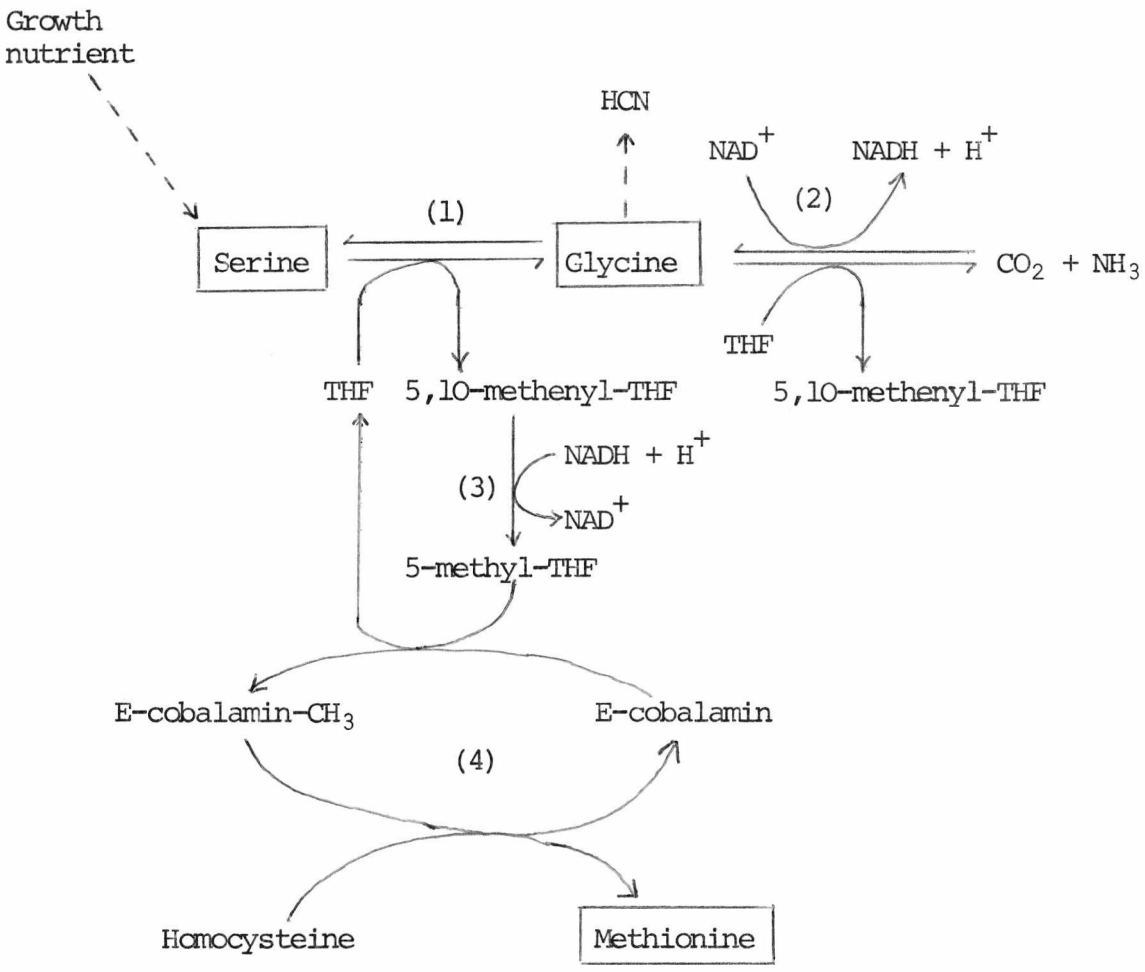


FIGURE 3 - Basic reactions of the one-carbon pathway and the inter-related metabolism of serine, glycine and methionine.

Enzymes (1) serine hydroxymethyltransferase

(2) glycine cleavage enzyme

(3) 5,10-methyl-tetrahydrofolate reductase

(4) homocysteine methyltransferase

THF; tetrahydrofolic acid.

(a) Higher Plants. Numerous higher plants can assimilate cyanide, the initial product being β-cyanoalanine (Floss et al., 1965). β-cyanoalanine synthase (EC 4.4.1.9, L-cysteine hydrogen sulphide-lyase {adding HCN}) has been purified from the mitochondria of *Lupinus angustifolia* (blue lupine). It catalyses β-cyanoalanine formation from cysteine and HCN and contains pyridoxal phosphate as a cofactor (Hendrickson and Conn, 1969; Akopyan and Goryachenkova, 1976). β-cyanoalanine can be converted to asparagine (Blumenthal-Goldschmidt et al., 1963) or the dipeptide γ-glutamyl-β-cyanoalanine (Fowden and Bell, 1965) which is a normal constituent of *Vicia* species but only occurs in other species when they are fed cyanide (Ressler et al., 1963). In those plants that form asparagine from cyanide, feeding with {^{14}C}cyanide results in label entering the amide carbon of asparagine (Blumenthal-Goldschmidt et al., 1963).

No further metabolism of γ-glutamyl-β-cyanoalanine has been demonstrated suggesting that this pathway may merely be a means of detoxifying cyanide whereas cyanide incorporation into asparagine is a means of assimilating cyanide into amino acids as further metabolism of asparagine into aspartate is to be expected.

A cyanide metabolising activity has been partially purified from the mesocarp of Loquat (Shirai et al., 1977). Stoichiometric liberation of ammonia from cyanide was observed and the cyanide metabolising activity is thought to consist of three enzymes catalysing the following reactions (Shirai, 1977):

$$HCN + H_2O \longrightarrow HCONH_2 \longrightarrow (H_2C = NOH) \xrightarrow{H_2O} HCOOH + NH_3$$

The initial reaction of the sequence may be catalysed by formamide hydro-lyase, an enzyme, formed by spores of the fungus *Stemphylium loti*, which converts cyanide to formamide (Fry and Miller, 1972).

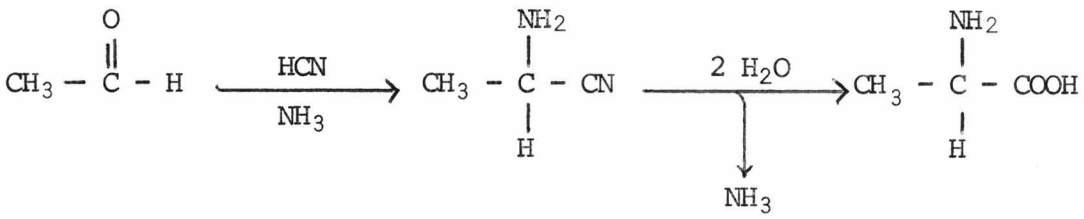
(b) Eukaryotic Microorganisms. A number of eukaryotic microorganisms have been shown to assimilate cyanide into a range of compounds such as β-cyanoalanine, asparagine, alanine, glutamic acid, α-aminobutyric acid and formamide. The only metabolic pathway characterised are those for the formation of alanine, glutamic acid and α-aminobutyric acid; there is an underlying similarity between these incorporation routes.

Several fungi have been tested for their ability to assimilate {^{14}C} cyanide and of these the only one to incorporate cyanide into asparagine was *Fusarium nivale* (Allen and Strobel, 1966). The pathway of asparagine formation in this fungus has not been investigated but it may involve β-cyanoalanine as an intermediate. There is a precedent for β-cyanoalanine formation in eukaryotic microorganisms as the alga *Chlorella pyrenoidosa* forms β-cyanoalanine and γ-glutamyl-β-cyanoalanine when fed cyanide (Fowden and Bell, 1965).

Of the fungi examined for their ability to assimilate {^{14}C}cyanide, the snow mold basidiomycete plus *M.oreades*, *Pholiota adiposa*, *Pholiota aurivella*, *Pholiota praecox* and *Rhizopus nigricans* accumulated labelled alanine and, to a lesser extent, glutamate (Allen and Strobel, 1966). In shorter-term experiments with the snow mold basidiomycete alanine was the only labelled amino acid. All these species are cyanogenic except for *R.nigricans* (Allen and Strobel, 1966).

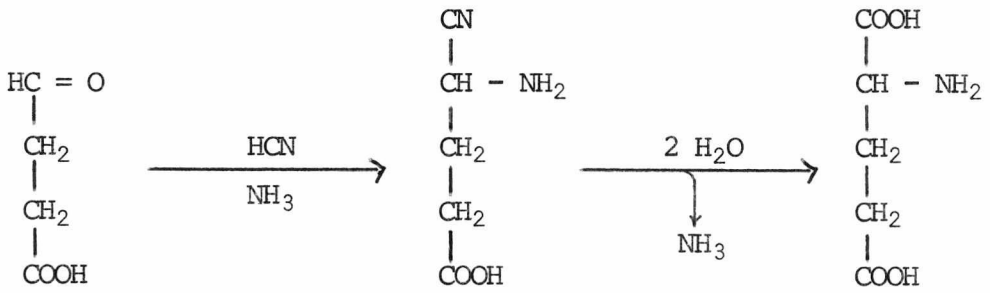
On incubation with {^{14}C}cyanide mycelia of the snow mold basidiomycete formed labelled α-aminopropionitrile and L-alanine (Strobel, 1966). The label appeared first in α-aminopropionitrile and then decreased whilst that in alanine increased steadily. When {^{14}C^{15}N}cyanide was used, carbon labelling appeared in the Cl position exclusively of both products whilst nitrogen labelling appeared only in the cyano group of α-aminopropionitrile. These results are consistent with the pathway depicted in Fig.4(a). This

(a) Alanine formation



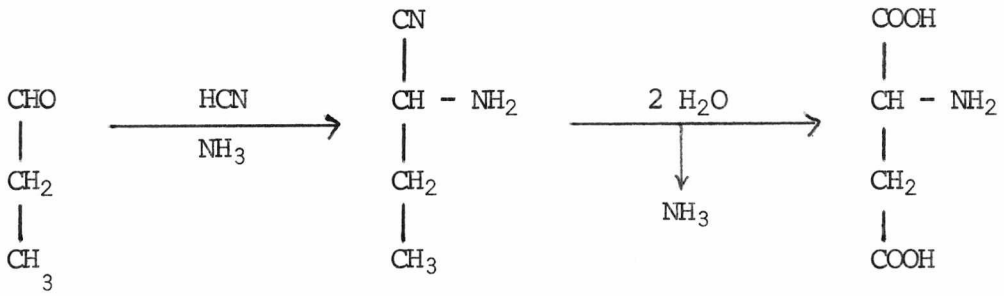
acetaldehyde α-aminopropionitrile L-alanine

(b) Glutamic acid formation.



Succinic semialdehyde 4-amino-4-cyanobutyric acid glutamic acid

(c) α-aminobutyric acid formation.



propionaldehyde α-aminobutyronitrile α-aminobutyric acid

FIGURE 4 - Pathways of cyanide assimilation in Fungi.

hypothesis is further supported by the demonstration that incubation of a crude extract from the fungus with KCN and acetaldehyde results in the formation of α-aminopropionitrile (Strobel, 1966). The breakdown of the nitrile may be catalysed by a nitrilase (Robinson and Hook, 1964; Thimann and Mahadevan, 1964).

The observation that labelled glutamate is formed when the snow mold basidiomycete is fed {^{14}C}cyanide has been confirmed and the pathway of glutamate formation from cyanide elucidated (Strobel, 1967). 4-amino-4-cyano-butyric acid was isolated from cultures of the fungus; labelling experiments and the use of cell-free extracts demonstrated that this compound was synthesised from cyanide, ammonia and succinic semialdehyde. Similar experiments established that 4-amino-4-cyanobutyric acid can act as a precursor of glutamate. A nitrilase activity, which may catalyse glutamate formation from 4-amino-4-cyanobutyric acid, was observed. A pathway analogous to that for alanine formation has been proposed for glutamate synthesis from cyanide (see Fig.4b).

Although it is not cyanogenic, the fungus *Rhizoctonia solani* incorporates cyanide into α-aminobutyric acid (Mundy et al., 1973). On incubation with {^{14}C}cyanide, ammonia and propionaldehyde, the fungus accumulates {^{14}C}α-aminobutyronitrile followed by {^{14}C}α-aminobutyric acid. An α-amino-butyronitrilase activity was detected. Presumably the pathway of α-amino-butyric acid formation is similar to that for alanine and glutamic acid formation from cyanide (see Fig.4(c)).

The pathway for synthesis of alanine, glutamic acid and α-aminobutyric acid from cyanide, in a variety of fungi, have much in common. All involve condensation of cyanide and ammonia with an aldehyde leading to formation of a nitrile. The nitrile is then acted on by a nitrilase giving rise to ammonia and an amino acid. Consequently, these pathways result in the

incorporation of the carbon atom of cyanide into an amino acid but the nitrogen atom is released as ammonia which may or may not be used by the fungus for the synthesis of amino acids. No further metabolism of α-amino-butyric acid has been detected so this pathway could be regarded as one of cyanide detoxication rather than assimilation.

As mentioned above, the fungus *S. loti* contains formamide hydrolyase, an enzyme which converts cyanide to formamide. This activity has also been found in *Gloeocerspora sorghi* (Fry and Munch, 1974). Both these organisms are pathogenic fungi causing lesions on cyanogenic plants by secreting a β-glucosidase which facilitates release of cyanide from the host plant cyanogens (Millar and Higgins, 1970). They appear to possess formamide hydrolyase as a cyanide detoxication mechanism. Knowles (1976) has suggested that the simplest method of detoxifying cyanide is to convert it to formate and then to carbon dioxide by formate dehydrogenase (EC 1.2.1.2). Conversion of cyanide to formate could be <u>via</u> formamide, catalysed by formamide hydro-lyase and formamide amidohydrolyase (EC 3.5.1.4). This may be the pathway operating in *S. loti* and *G. sorghi* and could be confirmed by observing {^{14}C}carbon dioxide formation from {^{14}C}cyanide fed to these organisms.

(c) <u>Bacteria</u>. Bacterial cyanide degradation and utilisation has not been as extensively studied as fungal cyanide metabolism. A number of cyanide utilising bacteria have been reported but of the known cyanogenic bacteria it is only in *C. violaceum* that cyanide utilisation has been investigated. Reports of bacteria able to use cyanide as carbon and/or nitrogen source for growth have been made but the pathways of cyanide metabolism in these organisms have not been characterised.

In contrast to cyanide production the routes of cyanide assimilation

in bacteria have more in common with those in higher plants than those in eukaryotic microorganisms. *Escherichia coli* possesses a β-cyanoalanine synthase activity (Dunnill and Fowden, 1965). Extracts of *E.coli* converted {^{14}C}serine and cyanide to {^{14}C}β-cyanoalanine in a reaction that was stimulated four-fold by 10mM ATP. Cysteine could substitute for serine as a substrate for β-cyanoalanine synthesis and it was suggested that the *E.coli* β-cyanoalanine synthase activity represents a secondary function of an indispensable but non specific enzyme, possibly serine sulphydrase (EC 4.2.1.22, L-serine hydro-lyase).

A strain of *Bacillus megaterium* which metabolises cyanide has been isolated from soil (Castric and Strobel, 1969). Incubation of whole cells of this organism with {^{14}C}cyanide results in the formation of {^{14}C}-aspartic acid and {^{14}C}carbon dioxide. Besides cyanide, serine and β-cyano-alanine were effectively converted by whole cells to asparagine. Analysis of asparagine isolated in an experiment in which whole cells were administered {^{13}C^{15}N}cyanide and {^{14}C}serine showed that the nitrogen and carbon of the amide group were derived from cyanide; {^{14}C}β-cyanoalanine was isolated as a product of this experiment. It was hypothesised that the metabolic pathway in this organism begins with the condensation of serine and cyanide to form β-cyanoalanine which is in turn hydrolysed by nitrilase to asparagine and then to aspartate in a reaction catalysed by asparagine synthase (see Fig.5). Furthermore, cell-free extracts of *B.megaterium* catalysed the conversion of {^{14}C}cyanide and serine to {^{14}C}asparagine and {^{14}C}aspartic acid. β-cyanoalanine could not be detected during conversion of serine to asparagine by cell-free extracts; possibly the nitrilase enzyme was more active than β-cyanoalanine synthase under the conditions used and β-cyanoalanine did not accumulate.

In a further series of experiments with cell-free extracts of the cyanide-metabolising strain of *B.megaterium* it was found that cysteine and

o-acetyl serine were seventeen to eighteen times more effective than serine as substrates for β-cyanoalanine synthesis (Castric and Conn, 1971). This suggested that one of the enzymes of cysteine biosynthesis had a β-cyanoalanine synthase activity as a secondary function. Growth of the bacterium in the presence of cyanide did not induce β-cyanoalanine synthase, indicating that this activity is not a physiological function of the enzyme. In contrast, growth of the bacterium with cysteine, rather than sulphate, as sulphur source, equally repressed β-cyanoalanine (from cyanide and cysteine) and cysteine (from o-acetyl serine and sulphide) synthesis. A fortyfold purification of o-acetyl serine sulphydrase resulted in the co-purification of β-cyanoalanine synthase and the two activities co-chromatographed on diethylaminoethyl cellulose and Sephadex G-100.

β-cyanoalanine was found to accumulate in the culture medium when washed cells of *C.violaceum*, grown on glutamate-basal salts medium, were incubated with glycine, methionine and succinate (Brysk et al., 1969a). β-cyanoalanine accumulated in the culture fluid to a maximal concentration in five hours and with further incubation began to disappear from the supernatant fluid. Cells incubated with {^{14}C}cyanide incorporated radioactivity in β-cyanoalanine and asparagine with the greatest amount of radioactivity being incorporated by cells incubated with {^{14}C}cyanide, serine and succinate. The radioactivity from {^{14}C}cyanide was found to be incorporated into the cyano group of β-cyanoalanine and the amide group of asparagine. The presence of a methyl donor such as methionine or N,N-dimethyl glycine enhanced the synthesis of β-cyanoalanine when glycine was used as substrate.

The incorporation of {^{14}C}substrate into β-cyanoalanine by *C.violaceum* cells incubated with glycine, methionine and succinate was studied (Brysk et al., 1969a). When {2-^{14}C}glycine was used radioactivity was found mainly

in the C2, C3 and C4 atoms of β-cyanoalanine. Incubation with {^{14}C}-succinate did not produce labelled β-cyanoalanine; presumably succinate was oxidised to produce energy for ATP formation. The use of {^{14}C}-formaldehyde, but not {^{14}C}formate, gave rise to labelled β-cyanoalanine. Despite β-cyanoalanine formation being stimulated by methionine, incubation with {1-methyl-^{14}C}methionine did not result in labelled β-cyanoalanine.

C.violaceum cells incubated with {^{14}C}β-cyanoalanine produced {^{14}C}-aspartic acid in cell hydrolysates indicating that the asparagine produced from β-cyanoalanine is further metabolised to aspartic acid by this organism.

The incorporation of label from radioactive glycine into β-cyano-alanine has led to the proposal that glycine can act not only as a precursor of cyanide but also to supply serine (Brysk et al., 1969a). A possible pathway of glycine and cyanide metabolism by *C.violaceum* is depicted in Figure 5. Utilisation of three molecules of glycine is required for formation of one molecule of β-cyanoalanine. One molecule is converted to cyanide and carbon dioxide, one to a one-carbon unit (via glyoxylate and formaldehyde) and one links up with the one-carbon unit to form serine, which then condenses with cyanide to form β-cyanoalanine. *C.violaceum* has been shown to oxidise glycine with formation of glyoxylate (Brysk, 1967), which has been reported to be oxidised to formaldehyde (Paretsky and Werkman, 1950). One-carbon compounds such as formaldehyde have been shown to be involved in the tetrahydrofolate system which participates in the serine hydroxymethyltransferase reaction (Kornberg and Elsden, 1961). This would explain labelling of β-cyanoalanine arising from {^{14}C}formaldehyde; presumably methionine is not involved in the generation of one-carbon units for the serine hydroxymethyltransferase reaction in this organism.

The properties of the β-cyanoalanine synthase enzyme in *C.violaceum*

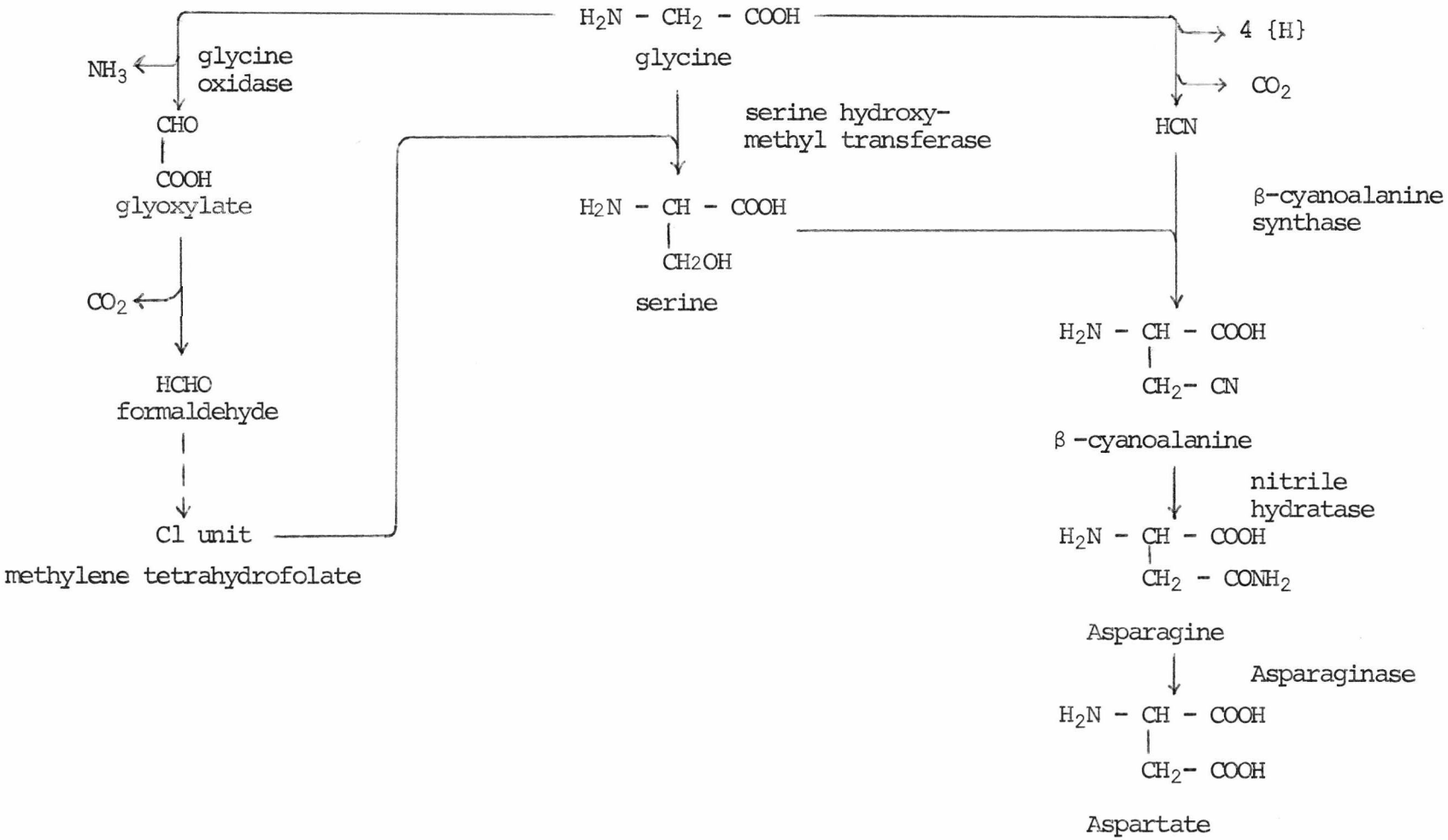


FIGURE 5 - Possible pathway of glycine and cyanide metabolism in *C.violaceum*

(from Knowles, 1976)

have not been studied so it is not known whether this is the physiological function of the enzyme or a secondary function of one of the enzymes of cysteine biosynthesis which appears to be the case in *E.coli* and *B.megaterium*. The fact that serine can stimulate β-cyanoalanine formation in all three organisms leads one to ask how the conversion from serine occurs. Knowles (1976) has suggested three possibilities:-

(i) serine acts as a direct substrate for β-cyanoalanine formation by a serine-linked β-cyanoalanine synthase.

(ii) serine sulphydrylase converts serine to cysteine which is then converted to β-cyanoalanine by a cysteine-linked β-cyanoalanine synthase (cf. higher plants) or by a secondary function of o-acetyl serine sulphydrylase.

(iii) serine is converted to o-acetyl serine by serine acetyltransferase (EC 2.1.3.3), (acetyl-coenzymeA : L-serine o-acetyl transferase) and then to β-cyanoalanine by o-acetyl serine sulphydrylase.

In *E.coli* and *B.megaterium* probably pathway (ii) occurs. Neither organism is cyanogenic and presumably has not needed to evolve a separate cyanide-utilising enzyme. In contrast *C.violaceum* produces cyanide and it seems likely that it has evolved a physiologically functional β-cyano-alanine synthase rather than this activity being a secondary function of another enzyme.

Another product of cyanide fixation by *C.violaceum* which has been identified is γ-cyano-α-aminobutyric acid (Brysk and Ressler, 1970). The capacity of washed, resuspended cells to produce γ-cyano-α-aminobutyric acid was greatest in cells harvested at the start of exponential growth. Incubation of washed, early-exponential phase cells with cyanide plus glutamate, serine or threonine, resulted in the formation of γ-cyano-α-aminobutyric acid and β-cyanoalanine but if aspartate or asparagine were

used instead only γ-cyano-α-aminobutyric acid was formed. Aspartate was thought to be acting as a feedback inhibitor of β-cyanoalanine synthase. Labelling experiments showed that the cyano group of γ-cyano-α-aminobutyric acid stemmed from cyanide and all the other carbon atoms from aspartate. Either aspartate acted as a direct precursor of the nitrile, or a substance derived from it without degradation of the carbon chain is the precursor. No evidence was found for further metabolism of γ-cyano-α-aminobutyric acid suggesting that it is a detoxication product of cyanide rather than the first metabolite of a pathway of cyanide assimilation.

An enzyme has been purified from *C.violaceum* that catalyses γ-cyano-α-aminobutyric acid synthesis from homocystine and cyanide (Ressler et al., 1973). The first step of the reaction is the non-enzymatic formation of γ-thiocyano-α-aminobutyric acid and homocysteine from cyanide and homocystine (see Fig.6). The enzyme γ-cyano-α-aminobutyric acid synthase (EC 4.4.1, γ-thiocyano-α-aminobutyric acid thiocyano-lyase : adding cyanide) then catalyses the formation of γ-cyano-α-aminobutyric acid and thiocyanate. Pyridoxal phosphate is required as a cofactor. Washed cells of *C.violaceum* probably convert aspartate to homocystine before addition of cyanide and γ-thiocyano-α-aminobutyric acid formation.

Another enzyme that appears to be involved in cyanide detoxication is rhodanese (EC 2.8.1.1, thiosulphate : cyanide sulphur-transferase) which catalyses the formation of thiocyanate from cyanide and thiosulphate. Rhodanese has been discovered in a wide range of animals and bacteria (Westley, 1973). Its role in mammalian tissue appears to be simply that of detoxifying cyanide as indicated by its location in the mitochondria, (Sorbo, 1953) and by the observation that cyanide-inhibited cytochrome oxidase is reactivated by addition of rhodanese and thiosulphate (Sorbo, 1957). However, rhodanese has been shown to catalyse a wide range of reactions

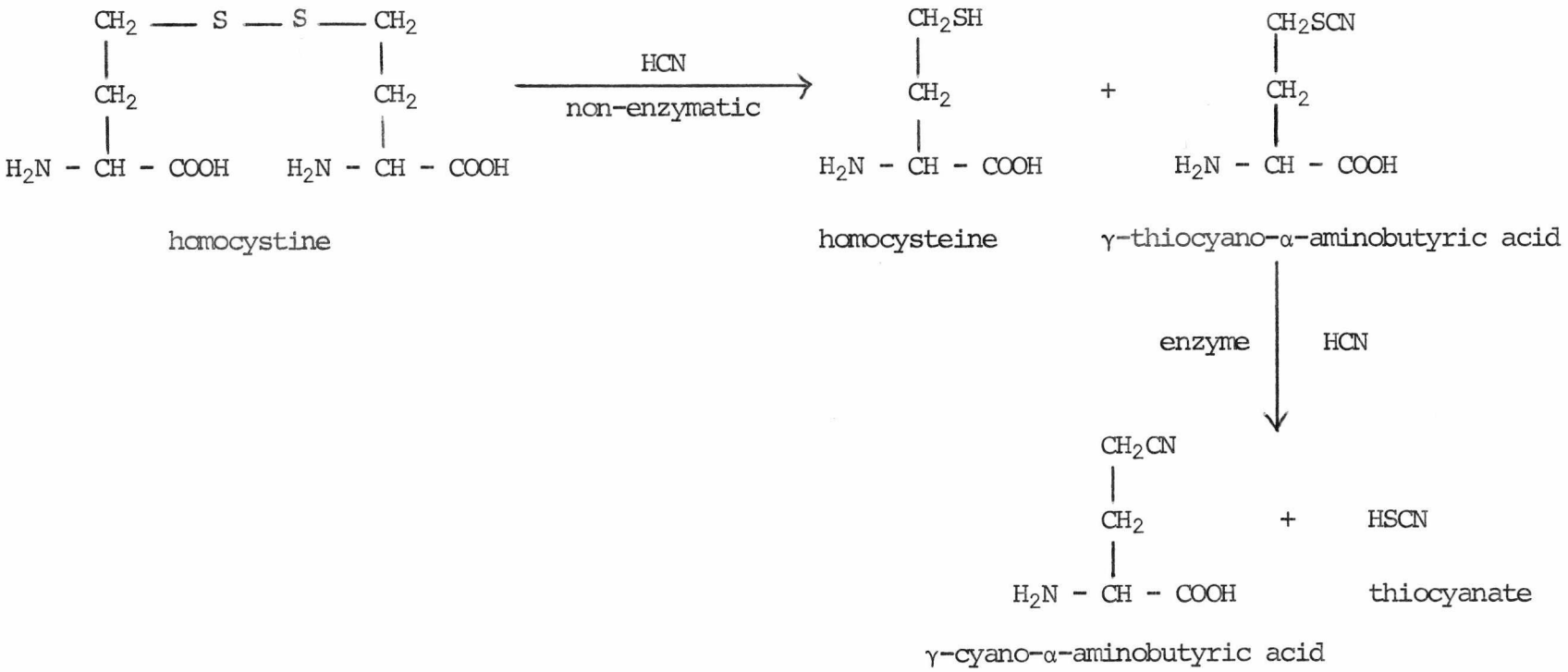


FIGURE 6 - γ-cyano-α-aminobutyric acid formation in *C.violaceum*.

involving transformations of sulphur-containing molecules and the numerous observations of rhodanese in *Thiobacillus* species and photosynthetic bacteria suggest that in these organisms it has a role in sulphur metabolism rather than in cyanide detoxication (Westley, 1973). In a number of bacteria rhodanese activity does not correlate with thiosulphate metabolism and Westley (1973) has pointed out that nonspecific reactions can be mistaken for rhodanese activity. However, growth of *Thiobacillus denitrificans*, *Rhodopseudomonas palustris* and a *Streptomyces* species in cyanide containing media resulted in the induction of rhodanese activity (Bowen et al., 1965; Yoch and Lindstrom, 1971; Oi and Yamamoto, 1977). It seems likely that rhodanese has a role in cyanide detoxication by some bacteria although there have been no reports of its presence in cyanogenic bacteria.

There have been a number of reports of bacteria that not only degrade cyanide but utilise that cyanide as a carbon and/or nitrogen source for growth. A strain of *Bacillus pumilus* that degrades cyanide to carbon dioxide and ammonia has been isolated from soil (Skowronski and Strobel, 1969). This bacterium is able to grow in basal salts medium with cyanide as the only carbon and nitrogen source. If $\{^{14}C^{15}N\}$cyanide is used, $\{^{14}C\}$-carbon dioxide and $\{^{15}N\}$ammonia accumulate in the medium. Nothing is known of the pathways of cyanide metabolism in this bacterium. A number of other bacteria that can utilise cyanide as the sole carbon and/or nitrogen source for growth have been isolated (Ware and Painter, 1955; Winter, 1963; Furuki et al., 1972) but the pathways of cyanide utilisation have yet to be elucidated. Knowles (1976) has suggested that formamide hydrolyase may be involved as many methylotrophic bacteria can grow on formamide as a carbon and nitrogen source.

Cyanide Resistance in Living Organisms.

The toxicity of cyanide is well known, although the thought that it is a specific inhibitor of cytochrome oxidase is a common misconception. Cyanide is a very reactive molecule, it forms stable complexes with metals, reacts with keto groups to form cyanohydrins and reduces thiol groups (Rappoport, 1969). Dixon and Webb (1964) have listed some forty enzymes for which inhibition by cyanide has been demonstrated. Many of these enzymes require 10^{-4} to 10^{-2}M cyanide for significant inhibition compared to less than 10^{-4}M cyanide for cytochrome oxidase inhibition. This list contains a number of haemoproteins, copper containing enzymes (e.g. cytochrome oxidase) and enzymes containing zinc and other metals, but in addition it contains many enzymes depending on pyridoxal phosphate or on disulphide groups for their activity. Thus, cyanide may inhibit enzymes by several different mechanisms. It may combine with an essential metal in the enzyme; it may remove a metal from the enzyme as an inactive complex; it may combine with a carbonyl group in the enzyme itself, in a cofactor or prosthetic group (e.g. pyridoxal) or even in the substrate; it can act as a reducing agent to break essential disulphide links in the enzyme. The inhibition of some pyridoxal phosphate enzymes by cyanide is particularly interesting in view of the fact that the only two cyanide-utilising enzymes purified (β-cyanoalanine synthase from blue lupine and γ-cyano-α-aminobutyric acid synthase from *C.violaceum*)both contain pyridoxal phosphate as prosthetic group.

This lack of specificity of cyanide raises the question as to how organisms adapt to growth whilst producing cyanide or in the presence of exogenously added cyanide. They can either induce enzymes for degradation and detoxication of cyanide, as described earlier, or produce cyanide-resistant enzymes. There have been many investigations of cyanide-resistant

respiration in both eukaryotic and prokaryotic organisms but there appears to have been no research on the adaptation of other cyanide-sensitive enzymes to cyanide-resistance. Cyanide-resistant respiration falls neatly into two types: eukaryotic organisms produce cyanide-resistant alternate oxidases which are not cytochromes whilst prokaryotes produce cyanide-resistant alternate oxidases which are cytochromes. Both types will be discussed later.

A third method by which organisms might exhibit cyanide resistance is by exclusion of cyanide from the cytoplasm or mitochondrial matrix by the plasma or mitochondrial membrane. If the cytochrome oxidase was located on the inner side of the membrane and the membrane was cyanide-impermeable, then exogenously added cyanide would be prevented from inhibiting cytochrome oxidase or the other cyanide-sensitive enzymes. In the case of cyanogenic bacteria it is interesting that the cyanide producing system appears to be membrane bound (Wissing, 1975); possibly the cyanide produced is excreted directly into the medium. However, hydrogen cyanide has a pKa of 9.1 and at physiological pH values is essentially undissociated. As a small uncharged molecule it probably permeates membranes rapidly (Alemohammed and Knowles, 1974). In addition, Wissing and Knowles (see Knowles, 1976) have shown that the cyanogenic *Pseudomonas* species is rapidly permeated by cyanide and so it is unlikely that cyanide resistance can be explained by exclusion of cyanide from the cytoplasm or mitochondrial matrix.

Cyanide-Resistant Respiration in Eukaryotes. Many higher plants and eukaryotic microorganisms possess, in addition to cytochrome oxidase, an alternative pathway to molecular oxygen which is insensitive to inhibition by cyanide (Palmer, 1976; Henry and Nyns, 1975). Cytochromes are not components of this alternate oxidase system in which non-haem iron proteins are thought to be involved (Bendall and Bonner, 1971). In both plants and

eukaryotic microorganisms the alternative cyanide-insensitive respiratory pathway has been shown not to be involved in the conservation of energy through adenosine triphosphate generation (Moore et al., 1978; De Troostembergh and Nyns, 1978). Although the cyanide-insensitive respiratory pathway is induced under conditions which lead to a decrease in electron flux along the main respiratory pathway, its function is unclear (Nesvera, 1977; Medenstev and Akimento, 1977). There would seem to have been no studies made of cyanide-insensitive respiration in cyanogenic eukaryotes. In the fungus *S.loti*, induction of formamide hydrolyase is correlated with increases in the activity of the cyanide-insensitive respiratory pathway, giving this organism two methods of cyanide resistance (Rissler and Millar, 1977).

Cyanide-Resistant Respiration in Bacteria. Although many bacteria possess membrane-bound electron-transport chains that are very similar, in general terms, to their counterparts in mitochondria, some bacteria can form a respiratory system considerably more complex than that of mitochondria (White and Sinclair,1971). They contain multiple primary dehydrogenases and oxidases which are linked by overlapping and interconnecting pathways, resulting in 'branching' of the electron transport system.

Often one of the cytochrome oxidases of the electron transport system is more cyanide-resistant than the others. Two of the most common bacterial cytochrome oxidases are cytochrome o and cytochrome d. The cytochrome d from *Achromobacter* and *E.coli* is considerably more cyanide-resistant than the cytochrome o from the same organism (Oka and Arima, 1965; Pudek and Bragg, 1975).

The cyanide-resistant oxidase of *Achromobacter* and *E.coli* is induced by growth of the bacteria in the presence of cyanide (Arima and Oka, 1965; Pudek and Bragg, 1975). Induction of the cyanide-resistant oxidase of

E.coli and *Pseudomonas putida* also occurs in stationary phase cells grown in the absence of cyanide (Pudek and Bragg, 1975; Sweet and Peterson, 1978). Oxygen or carbon limitation, or the presence of cyanide in the growth medium, would result in a limited supply of energy to the cell and possibly this general condition triggers induction of the cyanide-resistant electron transport pathway.

In contrast to the induction of cyanide-insensitive respiration in response to changes in the environment, the cyanide-resistant branch of the electron transport chain of *Azotobacter vinelandii* and *Beneckea natriegens* is constitutive (Jones and Redfearn, 1966; Ackrell and Jones, 1971; Weston et al., 1974).

Of particular interest is the respiratory system of *C.violaceum* which has been investigated in cells grown under conditions of high cyanide evolution and conditions where little cyanide is evolved. The maximum cyanide produced in each case was 1.8mM and 30μM respectively (Niven et al., 1975). It seems likely that *C.violaceum* produces a linear respiratory pathway with cyanide-resistant oxidases when grown under high-cyanide evolving conditions and when grown under low-cyanide producing conditions, synthesises an additional respiratory branch with cyanide-sensitive oxidases. The cyanide-resistant oxidase is not known. Cells grown under high-cyanide evolving conditions contain cytochromes $\underline{a_1}$, $\underline{d}$, $\underline{o}$ and $\underline{c}$ which bind carbon monoxide, an indication of their ability to function as an oxidase, whereas cells grown under low-cyanide producing conditions contain only cytochromes $\underline{o}$ and $\underline{c}$ as the carbon-monoxide binding cytochromes. Cytochromes $\underline{a_1}$, $\underline{d}$ and $\underline{o}$ are known to function as bacterial oxidases but carbon-monoxide-binding cytochromes $\underline{c}$ are rare and it is not known if they function as oxidases (Weston et al., 1974). On the evidence of other cyanide-resistant bacteria it seems probable that cytochrome $\underline{d}$ is the cyanide-insensitive oxidase in *C.violaceum*.

The Genus *Chromobacterium*.

The genus *Chromobacterium* is composed of non-sporulating, Gram negative, heterotrophic, rod-shaped bacteria which produce a violet, water insoluble pigment and possess both polar monotrichous and peritrichous flagella (Bergey, 1974). The organisms are ubiquitous in nature and some types cause disease in animals and man.

At present the genus consists of two species, *Chromobacterium violaceum* and *Chromobacterium lividum*, although a third member, *Chromobacterium fluviatile* sp.nov. has been proposed (Moss et al., 1978). Strains of *C.violaceum* are mesophilic, facultative aerobes which produce cyanide whilst strains of *C.lividum* are psychrophilic, strict aerobes and do not produce cyanide. The systematic position of the genus is uncertain; it possesses many of the characteristics of the *Pseudomonadaceae* and some of the characteristics of the *Enterobacteriaceae*. It may be closely allied to the *Vibrionaceae* (Bergey, 1974).

Strains of *Chromobacterium* are widely distributed in soil and water; it seems that *C.violaceum* is more commonly found in soil and *C.lividum* in water (Corpe, 1951; Ryall and Moss, 1975).

The violet pigment violacein is identical in both species and is an indole derivative (Ballantine et al., 1958) whose formation is dependent upon the inclusion of tryptophan in the growth medium (De Moss and Evans, 1957).

Aim of Project.

A wide range of organisms have been found to either produce cyanide or metabolise it, and in a number of cases, to be capable of both. That some cyanogenic organisms have not yet been reported to utilise or degrade cyanide is probably due to this aspect of their metabolism not having been

investigated rather than their lacking this ability; presumably all cyanogenic organisms possess degradative pathways for cyanide. Almost all the organisms involved in cyanide production or utilisation are found in soil and this has led to the proposal of a 'cyanide microcycle' operating in the soil (Allen and Strobel, 1966). In the microatmosphere of the soil molecules of cyanide could be directly transferred from plants to microorganisms, and vice versa, as sources of nitrogen and/or carbon without prior conversion to carbon dioxide and ammonia. Carbon-nitrogen cycling through microbial formamide metabolism has also been proposed,(Thatcher and Weaver, 1976). The cycle involves cyanogenic plants, phytopathogenic fungi which convert cyanogenic glycosides to formamide, methylotrophic bacteria which can utilise formamide as a growth substrate, and nitrifying bacteria. So, in addition to their intrinsic interest, an understanding of bacterial mechanisms of cyanide production and utilisation is of considerable importance for a deeper comprehension of soil ecology and its agricultural implications.

It has been suggested that cyanide is a bacterial secondary metabolite (Castric, 1975). Most of the evidence for this claim has come from studies on cyanogenesis by *Ps.aeruginosa* and further investigations of the conditions for cyanide production by other bacteria are required for this claim to be substantiated. Many microbial secondary metabolites are of industrial importance because of their antibiotic (e.g. penicillin) or pharmacologic (e.g. aflatoxin) effects but the regulation of secondary metabolism is poorly understood (Demain, 1968). If cyanide is a secondary metabolite it would make an ideal model system for studying the regulation of secondary metabolism due to its relatively simple biosynthetic pathway and the ease with which it can be assayed.

Although glycine has been shown to be the precursor of cyanide in bacteria and methionine shown to stimulate cyanogenesis, these observations

have been made using non-proliferating cells or cultures to which glycine and/or methionine were added towards the end of exponential growth (Michaels and Corpe, 1965; Michaels et al., 1965; Brysk et al., 1969b). There have been no investigations of the time-course of cyanogenesis by bacterial cultures growing on a basal-salts medium supplemented with carbon and nitrogen sources plus glycine and/or methionine. The influence of culture parameters such as temperature, ferrous ion concentration and phosphate ion concentration, on cyanide production has been studied in *Ps.aeruginosa* (Castric, 1975) but not in *C.violaceum*.

Non-proliferating cells of *C.violaceum* have been shown to accumulate β-cyanoalanine and γ-cyano-α-aminobutyric acid as cyanide-fixation products in the medium (Brysk et al., 1969a; Brysk and Ressler, 1970) but the production of these compounds by growing cultures of *C.violaceum* has not been investigated. There have been no studies on the levels of β-cyano-alanine synthase and γ-cyano-α-aminobutyric acid synthase in cultures of *C.violaceum* growing under low - or high-cyanide producing conditions. Formamide hydrolyase has been found to be involved in fungal cyanide-fixation (Fry and Millar, 1972) and rhodanese implicated in bacterial cyanide detoxication but *C.violaceum* has not been examined for either enzyme.

It is the aim of this project to investigate the conditions of cyanide production by growing cultures of *C.violaceum*, with reference to the properties of microbial secondary metabolism, and to establish which enzymes *C.violaceum* possesses for cyanide utilisation or degradation. The levels of such cyanide-fixing enzymes in *C.violaceum* will be measured and the relationship between the formation and utilisation or degradation of cyanide by growing cultures of this organism will be investigated.

CHAPTER TWO

(MATERIALS AND METHODS)

Organism and Growth Conditions

Chromobacterium violaceum (NCIB 9131, D252, supplied by Dr. Dorothy Jones of the University of Leicester) was maintained on nutrient agar slopes at room temperature and subcultured once a month.

C.violaceum was grown in a minimal medium consisting of M-9 salts (Miller, 1972) with ammonium salts omitted, 1ml trace metals l^{-1} (Bauchop and Elsden, 1960) with the ferrous ion concentration raised to 30μM, and 10mM L-glutamic acid as sole carbon and nitrogen source. Where stated the medium was supplemented with 2mM glycine and/or 0.5mM L-methionine. A 1% inoculum of a 20-24h stationary phase culture grown on 10mM glutamate was used to inoculate experimental flasks.

In cultures from which violacein was extracted, *C.violaceum* was grown on a complex medium consisting of 100mM glycerol in nutrient broth (25g Oxoid dehydrated medium l^{-1}).

In experiments where the effect of phosphate on cyanide production was studied, Tris (hydroxymethyl)aminomethane (50mM Tris-HCl, pH 7.5) replaced phosphate as the buffer in M-9 salts. The use of Tris-HCl as a buffer increased the lag phase of growth but had no effect on the rate of growth or the growth yield. A starter flask containing 4mM phosphate was used to minimise phosphate transfer to the experimental flasks. The phosphate added to each flask consisted of equimolar concentrations of Na_2HPO_4 and KH_2PO_4.

Cultures from which extracts were prepared for assaying cyanide producing activity, were grown on a complex medium consisting of 8 g l^{-1}. Bacto-nutrient and 5 g l^{-1} NaCl, made up to 1 l with tapwater. The pH of the medium was adjusted to 7.5 using 1M NaOH.

In experiments where the cyanide concentration in the medium was measured, growth took place in 100ml of medium in 250ml conical flasks whereas in

experiments where cell-free extracts were prepared for enzyme assays, growth took place in 500ml of medium in 2 l conical flasks; incubation was at 30°C, in a gyrotatory shaker (250 rev.min^{-1}). Cyanide production and growth were not affected by the size of the conical flask used.

Bacterial growth was measured turbidimetrically at 750nm, using 10mm cuvettes, in a SP500 spectrophotometer (Pye-Unicam Ltd., Cambridge).

Cyanide Determination.

The medium cyanide concentration in cultures of cyanogenic bacteria is a balance between the rate of cyanide synthesis by the organism, and its rate of utilization or degradation by the organism plus, since it is volatile, the rate of loss of cyanide to the environment. Preliminary experiments with cotton wool plugged, uninoculated shake-flasks to which 150-250μM potassium cyanide had been added to the growth medium showed that approximately 0.9μmole h^{-1} of cyanide was lost; this figure was considered negligible in comparison to the rates of cyanide formation and utilisation by cultures of *C.violaceum* growing under cyanogenic conditions. Castric (1975) has devised a rubber stopper for shake-flasks with a glass tube insert containing a NaOH wick to trap cyanide lost from the culture. Preliminary experiments using this type of stopper compared to cotton wool plugs confirmed that only a low level of cyanide loss occurs from the medium. Cotton wool plugs were therefore used in all further experiments.

The cyanide content of the growth medium was assayed by the method of Epstein (1947). This assay is interfered with by a number of volatile acids, including acetic acid, (Boxer and Rickards, 1951) consequently during the cyanohydrin extraction procedure a modification of the method of Guilbault and Kramer (1966) was used to assay for cyanide. This method has been modified by Gewitz et al. (1976) and the version used here is a combination

of the two.

The reagent used consisted of 1.0ml 0.1M o-dinitrobenzene in ethylene glycol monomethylether, 1.0ml 0.1M p-nitrobenzaldehyde in ethyleneglycol monomethylether and 0.1ml 0.5M NaOH. These solutions were equilibrated separately at 25°C before mixing, followed by addition of 0.1ml cyanide solution. The formation of o-nitrophenyl hydroxylamine dianion at 25°C was followed continuously by monitoring the absorbance at 560nm on a SP1800 recording spectrophotometer (Pye-Unicam Ltd., Cambridge). A standard curve of initial rate of dye formation against cyanide concentration was constructed.

Preparation of cell-free extracts.

Extracts for the measurement of cyanide-utilising enzyme activities were prepared by centrifuging bacteria at 23000 g (MSE HS18 centrifuge) for 10 min, washing twice with 200mM Tris-HCl buffer (pH 8.5) and resuspending in 5ml fresh buffer. Bacteria were disrupted by sonication (MSE sonicator, 150 W, 6 x 15s, 0°C) and centrifuged at 10000 g for 10 min at 4°C to give a cell-free extract.

Extracts for the measurement of cyanide-producing activity were prepared by centrifuging bacteria at 14600 g (Sorvall Superspeed RC-2 centrifuge) for 10 min, washing twice with 100mM potassium phosphate buffer (pH 8.0) and resuspending in 30ml fresh buffer. Bacteria were disrupted by sonication (MSE sonicator, 150 W, 4 x 60s, 0°C) and centrifuged at 100000 g (Beckman Model L ultra-centrifuge) for 45 min to give sediment and supernatant fractions. The sediment was resuspended in 1.1ml fresh buffer.

Extracts were not dialysed.

Spectrum of violacein.

A stationary phase culture of *C.violaceum*, grown on a complex medium,

was centrifuged at 23000 g (MSE HS18 centrifuge) for 10 min to remove bacteria; violacein remained in suspension. The supernatant was centrifuged at 23000 g for a further 15 min to sediment the violacein particles. Violacein was dissolved in 95% ethanol and its spectrum determined against an ethanol blank in a Hitachi-Perkin Elmer 356 spectrophotometer.

Determination of enzyme activities.

β-cyanoalanine synthase was assayed by the method of Hendrickson and Conn (1969) and γ-cyano-α-aminobutyric acid synthase by the method of Ressler et al. (1973) except that a time course determination was used in both cases. The β-cyanoalanine synthase reaction mixture was buffered at pH 9.0 whilst that for γ-cyano-α-aminobutyric acid synthase was buffered at pH 9.1.

Rhodanese was assayed as described by Smith and Lascelles (1966).

Formamide hydro-lyase was assayed by the method of Fry and Millar(1972) except that a time course determination was used.

Cyanide-producing enzyme activity was assayed as detailed by Wissing, (1974).

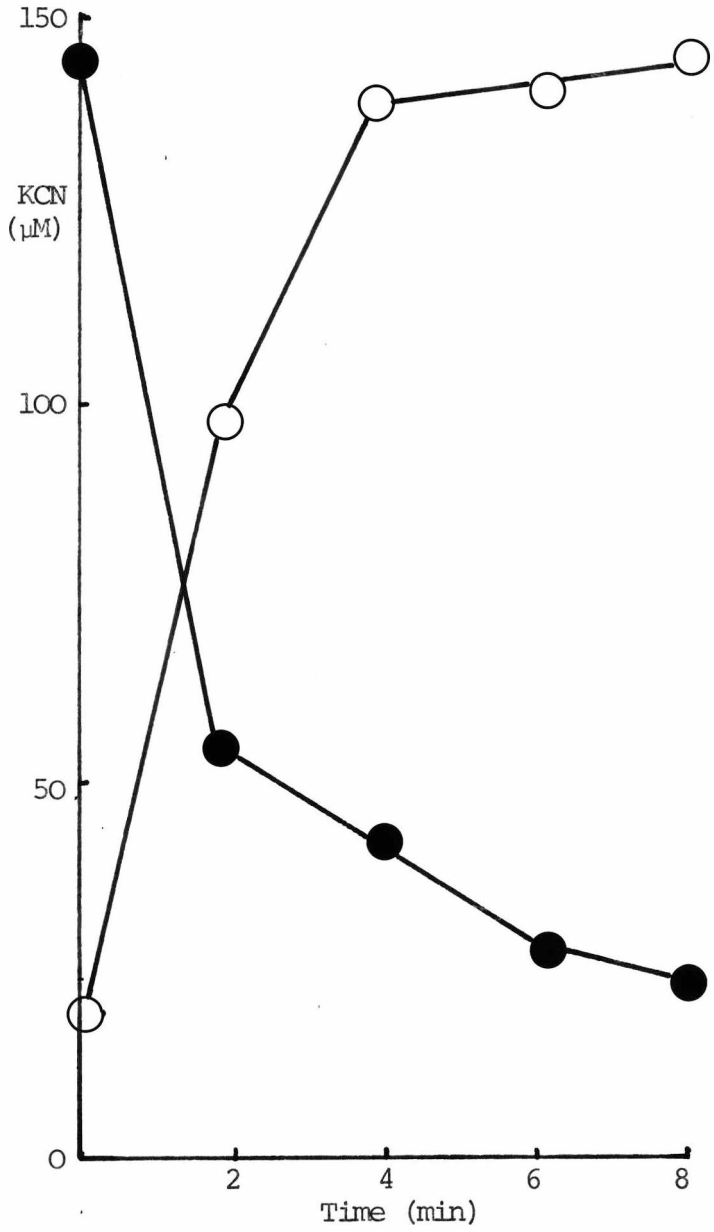
Distribution of radioactivity from {^{14}C}KCN added to *C.violaceum* cultures.

4μCi of {^{14}C}KCN (specific activity 1μCi μmole^{-1}) were added to 100ml cultures of *C.violaceum* in the late exponential phase of growth. At intervals, 3ml samples of the culture were removed and 1ml of this filtered using 0.45μm Millipore filters (Millipore S.A., Molsheim, France) to remove bacteria. The filtrate was washed with 15ml M-9 salts (ammonium salts omitted) and the filter paper placed in a scintillation vial for determination of radioactivity. A control experiment showed that the filter paper did not affect the efficiency of counting due to ^{14}C.

The other 2ml of the culture sample was centrifuged on a bench centrifuge to remove bacteria. 0.5ml of the supernatant was placed in a scintillation vial in order to measure the total amount of radioactivity in the culture medium. A further 1.0ml of the supernatant was placed in a glass vessel and a continuous stream of air blown through the liquid for 10 min whilst stirring with a magnetic stirrer. The cyanide, and presumably any CO_2 dissolved in the medium, blown off by this method were trapped in 1.0ml 1M NaOH. A preliminary experiment, using 1.0ml 150μM KCN, showed that after 6 min of aeration little of the cyanide remained in the vessel, and that all the cyanide blown off was trapped in the NaOH (Fig.7). To determine the amount of radioactivity present in non-volatile derivatives of cyanide, 0.5ml of the culture medium from which cyanide had been blown off, was placed in a scintillation vial. 0.5ml of the NaOH trap was neutralised with 0.5ml of 1M HCl and 0.5ml of this taken for counting. If significant amounts of cyanide were being converted to CO_2 one would expect little change in the amount of radioactivity in the NaOH trap, but as the radioactivity levels dropped, as predicted from the medium cyanide concentration in all experiments, it was concluded that no significant conversion of cyanide to CO_2 was taking place.

To those vials not containing 0.5ml liquid, 0.5ml water was added, and 5ml of PCS scintillation fluid (Phase Combining System, Amersham-Searle) was added to all samples. Counting took place in a Packard Tri-Carb scintillation counter. The NaOH trap samples were neutralised before addition of PCS as PCS is not miscible with aqueous solutions at alkaline pH values.

FIGURE 7 - Rate of KCN blow-off from culture medium samples. The cyanide concentrations in the medium sample (—●—) and in the alkali trap (—○—) were followed.



Rate of incorporation of radioactivity from {^{14}C}KCN into β-cyanoalanine.

8μCi of {^{14}C}KCN (specific activity 1μCi μmole^{-1}) were added to 100ml cultures of *C.violaceum* in the late exponential phase of growth. The total amount of radioactivity in the medium, and the distribution of this between the volatile and non-volatile fractions of the medium were determined as described above.

The radioactivity present as β-cyanoalanine was determined by preparing dinitrophenyl (DNP) derivatives of the medium amino acids, separating these by silica gel thin layer chromatography, and counting the radioactivity in each amino acid derivative.

DNP-derivatives of the medium amino acids were prepared by mixing 100μl of cyanide-free medium with 10μl 1M K_2CO_3 and 15μl of a 10% w/v solution of 1-fluoro-2-dinitrobenzene (DNFB) in absolute ethanol. The mixture was incubated for 1 hour in the dark at 70°C. Preliminary experiments showed that using 15μl DNFB and incubating for 1 hour was sufficient for total reaction with the concentrations of amino acids found in the growth medium. After cooling, the mixture was acidified with 50μl of concentrated HCl and the DNP-amino acids extracted with diethyl ether, thus separating the sample into organic and aqueous phases. 25μl of the aqueous phase were made up to 0.5ml with water for counting the radioactivity using PCS scintillation fluid.

Ether was blown-off from the organic phase by aeration, and the sample freeze-dried using an Edwards freeze-drier. The freeze-dried sample was resuspended in 25μl methanol. Three 5μl aliquots were taken; one was made up to 0.5ml with water and counted for radioactivity using PCS whereas the other two were spotted on silica gel thin layer chromatography plates. One spot was scraped off immediately and counted for radioactivity in the presence of 0.5ml water and 5ml PCS; this allowed an estimation of the

amount of sample lost during the process of spotting to be made. The other spot was subjected to chromatography, after which 1cm squares were scraped off along the length of the chromatogram and the amount of radioactivity in each square counted as above. The amount of radioactivity in β-cyanoalanine and other amino acids was calculated by comparing those squares containing radioactivity with the position of DNP-amino acid standards run concurrently with the growth medium samples.

Standards of DNP-derivatives of glutamate, glycine, methionine, β-cyano-alanine, asparagine and aspartate were prepared by treating 50μmoles of each amino acid as detailed above.

Due to their bright yellow colour, solutions of DNP-amino acids cause quenching of scintillation when mixed with radioactive compounds and scintillation fluid. A quench curve was constructed by adding increasing amounts of a DNP-glutamate solution to a vial containing a known amount of radioactivity and plotting the ratio of counts in the ^{14}C channel to counts in the ^{3}H channel against the percentage of the original counts obtained at a given channel ratio. Use of this quench curve corrected for any quenching of scintillation caused by DNP-amino acids.

Extraction of cyanohydrins.

Cyanohydrins were extracted by a modification of the method of Tapper and MacDonald (1974). A 100ml culture of *C.violaceum* was blended with 400ml of 95% ethanol acidified with 20ml of glacial acetic acid. This was left to stand for 30 min then, after filtering through a 0.45μm Millipore filter, the bulked filtrate was concentrated to about 100ml by boiling off the ethanol.

The solution was applied to a 10ml column of Dowex 50W X8 cation exchange resin in H^{+} form (20-50 mesh) and washed with water until most of

the cyanide reacting substance had passed through. 10ml fractions were collected.

Fractions 3-9 of the eluate were combined and applied to a 50ml column of Dowex 2 X8 anion exchange resin in the acetate form (50-100 mesh) followed by 100ml of water. The principal cyanogens were then eluted with 1M HCl and 20ml fractions collected and assayed for cyanide using the modified method of Guilbault and Kramer (1966).

Cyanohydrins decompose into cyanide and the parental carbonyl compound at alkali pH values. By assaying the fractions for cyanide at acid and alkali pH values it was possible to determine the amount of cyanohydrin they contained.

Using a pure solution of glyoxylic acid cyanohydrin it was found that the recovery of cyanohydrin from the Dowex 50W X8 column was 76% and from the Dowex 2 X8 column, 34%.

Thin Layer Chromatography.

Thin layer chromatography of amino acids present in the growth medium of *C.violaceum* cultures was performed using cellulose thin layer plates (Eastman Chromagram). Samples of *C.violaceum* cultures grown to stationary phase (15h) were centrifuged in a Beckman Microfuge to remove bacteria. 10µl samples of media were spotted along with 5µl aliquots of amino acid standards; glutamate, glycine, methionine, β-cyanoalanine, aspartate, asparagine and alanine. The concentration of amino acid standard solutions was 1mM, except for alanine which was 5mM. The solvent used for chromatography was methanol-pyridine-water (20:1:5 v/v) and the chromatogram was developed by spraying with 0.25% ninhydrin in acetone and heating at 110°C for twenty minutes. All amino acids gave a purple colour on development, except asparagine (blue-grey) and β-cyanoalanine (blue-green).

Silica gel plates (Eastman Chromagram) were used for thin layer chromatography of DNP-amino acids. 5µl samples of growth medium DNP-derivatives and of DNP-amino acid standards were used. The solvent for chromatography was chloroform-methanol-glacial acetic acid (85:13:2 v/v). The DNP-amino acids could be detected visually due to their yellow colour.

Cellulose thin layer plates were used for chromatography of cyanohydrins. In the experiment where a culture extract was prepared, 5µl samples of extract, glyoxylic acid cyanohydrin and pyruvic acid cyanohydrin were run in a solvent consisting of butan-1-ol-acetic acid-pyridine-water (10:2:1:3 v/v) and the chromatogram developed by dipping in an acetone solution of $AgNO_3$ (0.1ml of saturated aqueous $AgNO_3$ in 20ml acetone) followed by dipping in 0.5M NaOH in ethanol. This gave dark spots on a tan background immediately.

In the experiment where untreated growth medium was used, 5µl samples of growth media and glyoxylic acid cyanohydrin were run in the same solvent as above. The plates were developed by spraying with a 1:1 mixture of a 1% picric acid solution and a 10% Na_2CO_3 solution and leaving in a damp atmosphere for several hours. This method gave brown spots on a yellow background.

Chloramphenicol

Chloramphenicol was dissolved in water to a concentration of 250µg ml^{-1} and filter sterilised using 0.45µm Millipore filters.

Thiocyanate assays.

Thiocyanate was assayed by two methods, those of Epstein (1947) and Sorbo (1953).

Protein assay.

Protein was assayed by the modified Biuret method of Gornall et al. (1949) using bovine serum albumin as standard.

Chemicals.

Chloramphenicol was obtained from Sigma; {^{14}C}KCN from The Radiochemical Centre, Amersham; PCS from Hopkin and Williams. Whenever possible, all other reagents were of analytical grade; glass-distilled water was used throughout.

CHAPTER THREE

(RESULTS)

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Preliminary Experiments.

(a) Spectrum of violacein: *C.violaceum* produces a violet pigment, violacein, when grown on media containing tryptophan (DeMoss and Evans, 1957). The absorption of light by this pigment could interfere with measurements of bacterial growth by turbidometric methods. In order to establish a wavelength at which violacein does not absorb light the pigment was extracted from stationary phase cells grown on 100mM glycerol plus nutrient broth. The absorption spectrum of a solution of violacein in ethanol is shown in Fig.8. Violacein does not absorb light at wavelengths greater than 750nm. All measurements of bacterial growth have therefore been made using the scattering of light at a wavelength of 750nm.

(b) Growth curves: L-glutamic acid was chosen as the combined carbon and nitrogen source for growth of *C.violaceum* in common with all previous investigations of cyanide metabolism in *C.violaceum* because this presented the opportunity for comparisons to be made between the various studies.

The growth of *C.violaceum* on L-glutamate as sole carbon and nitrogen source, over a range of concentrations from 6mM to 18mM, is shown in Fig.9. At concentrations of glutamate above 12mM the transition from exponential growth to the stationary phase of growth was gradual, rather than immediate, indicating that oxygen limitation, rather than carbon or nitrogen depletion, was probably the cause of cessation of growth. In order to ensure that carbon or nitrogen depletion is the cause of growth cessation in experimental cultures, 10mM glutamate was chosen as the carbon and nitrogen source for growth.

A 1% inoculum of a stationary phase culture of *C.violaceum* was used to inoculate flasks of medium containing M9 salts plus 10mM glycine and/or 10mM L-methionine as carbon and/or nitrogen source. No growth was observed

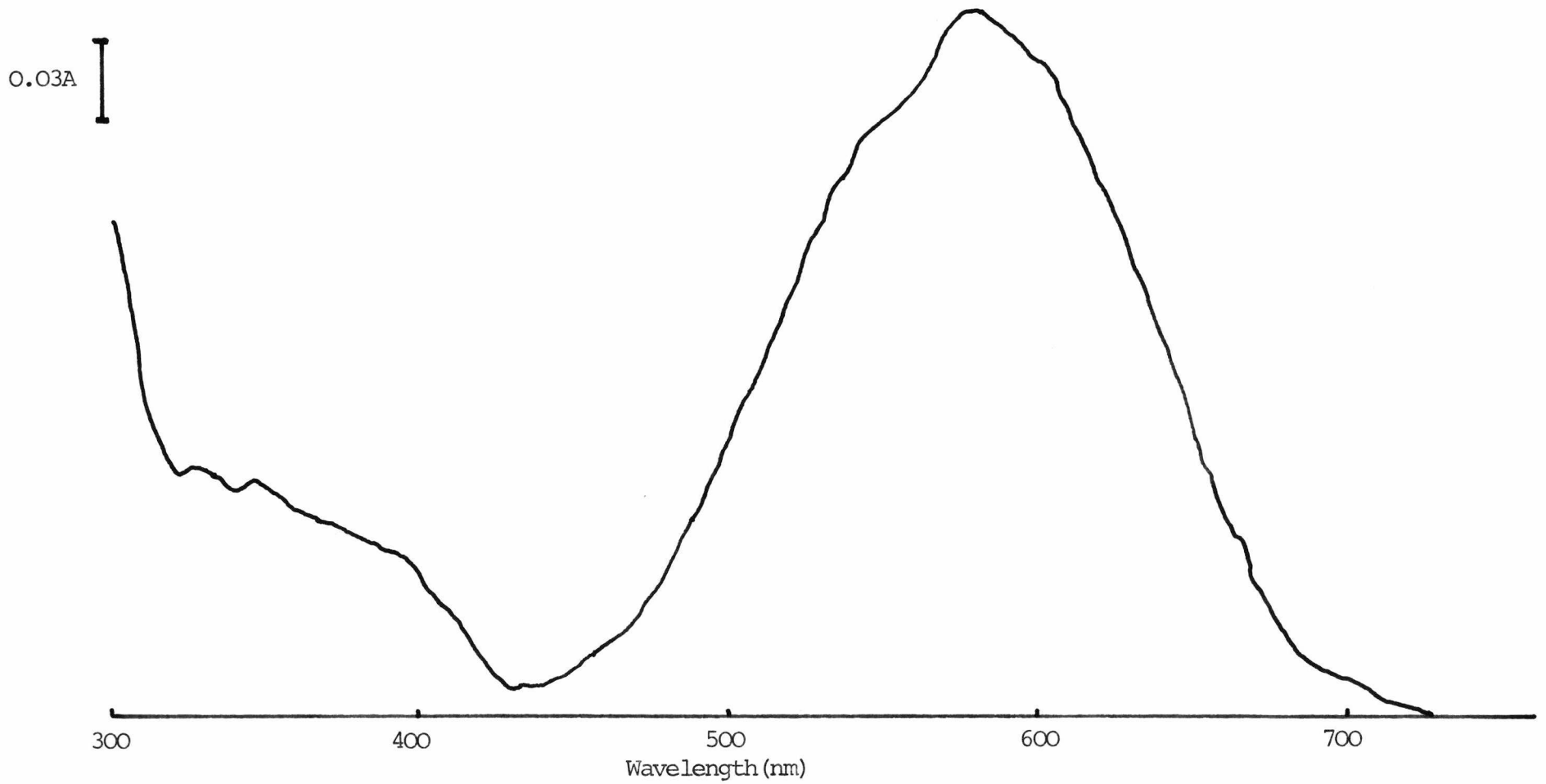
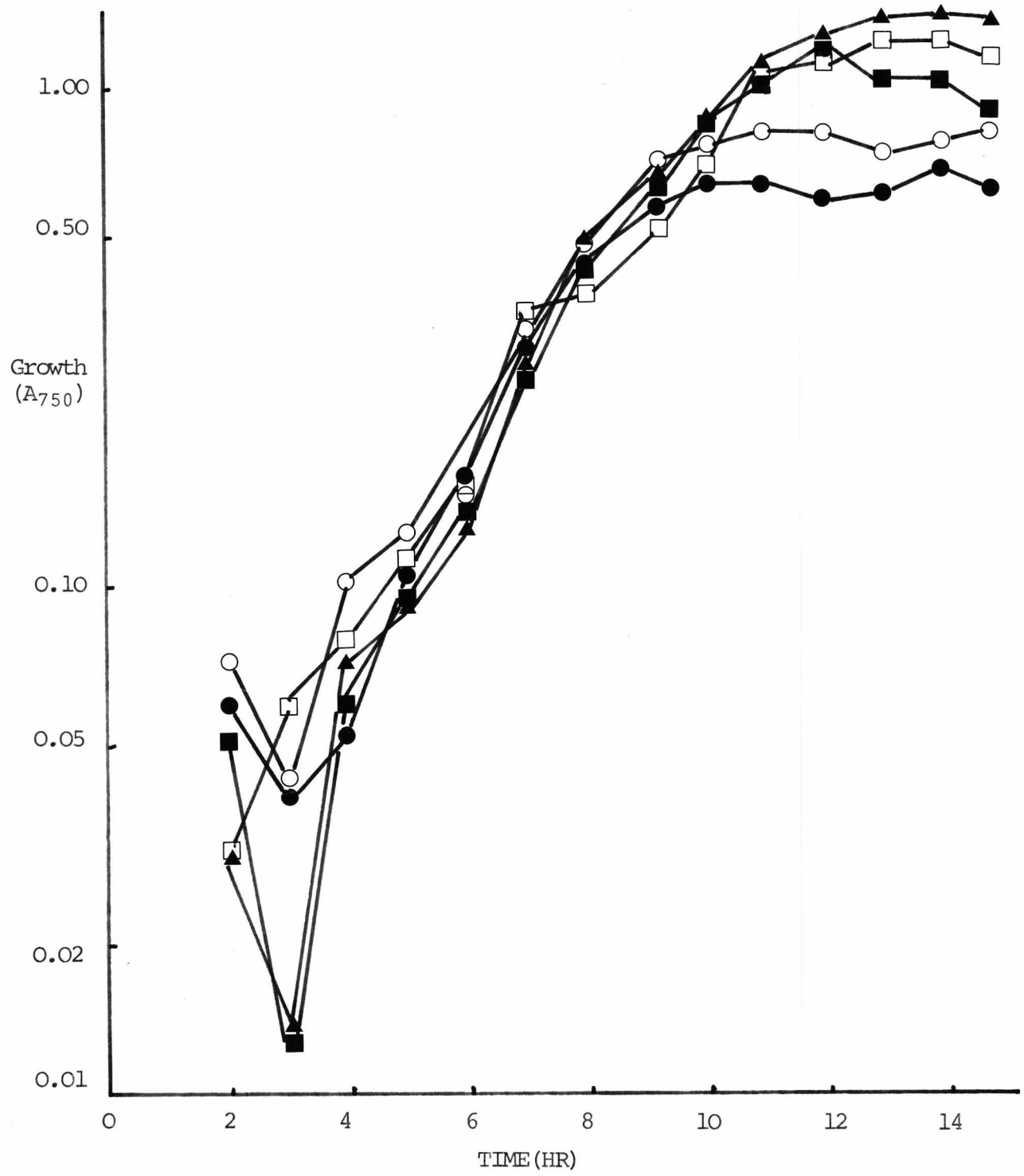


FIGURE 8 - Spectrum of violacein in ethanol

FIGURE 9 - Growth of *C.violaceum* on various concentrations of L-glutamate. 6(-●-), 9(-o-), 12(-■-), 15(-□-) and 18mM(-▲-) glutamate was used.



over a period of 96 hours indicating that *C.violaceum* is unable to use these two amino acids, either alone or in combination, as sole carbon and/or nitrogen sources for growth.

(c) Time course of cyanide production: The time course of cyanide production was followed in cultures of *C.violaceum* growing on 10mM glutamate supplemented with either 6mM glycine alone or in combination with 6mM methionine (Fig.10). The concentrations of glycine and methionine were chosen arbitrarily. The cyanide concentration of the growth medium, in both cases, reached a maximum at around 14 hours incubation.

(d) Optimum concentrations of glycine and methionine for maximum cyanide production: The production of cyanide was followed when *C.violaceum* was grown on 10mM glutamate supplemented with varying concentrations of glycine and methionine. Figs.11 and 12 show the variation in the maximum cyanide content of the growth medium with glycine and methionine concentrations. At methionine concentrations of 1.0mM or less, increasing glycine concentrations of up to 1.0mM result in a decrease in the maximum cyanide level of the medium but this inhibitory effect of glycine is overcome at glycine concentrations above 1.0mM. At methionine concentrations above 1.0mM all concentrations of glycine stimulate cyanide production. In both cases, glycine concentrations above 2.0mM have little further effect on the cyanide content of the medium. At any given glycine concentration, the maximal cyanide content of the medium increases with the methionine concentration up to values of 0.5mM, above which further increases in the methionine concentration have little effect on the cyanide level. These concentrations of glycine and methionine are lower than previously reported for maximal cyanogenesis by *C.violaceum* (Michaels and Corpe, 1965) and *Ps.aeruginosa* (Castric, 1977). The previously reported concentrations of

FIGURE 10 - Time course of Cyanide Production by *C.violaceum*
Growth in media containing 10mM glutamate, supplemented with 6mM glycine alone (—●—), or with 6mM glycine plus 6mM methionine (—○—) is shown. Cyanide production was followed during growth on glutamate plus glycine (—▲—) and on glutamate plus glycine and methionine (—△—).

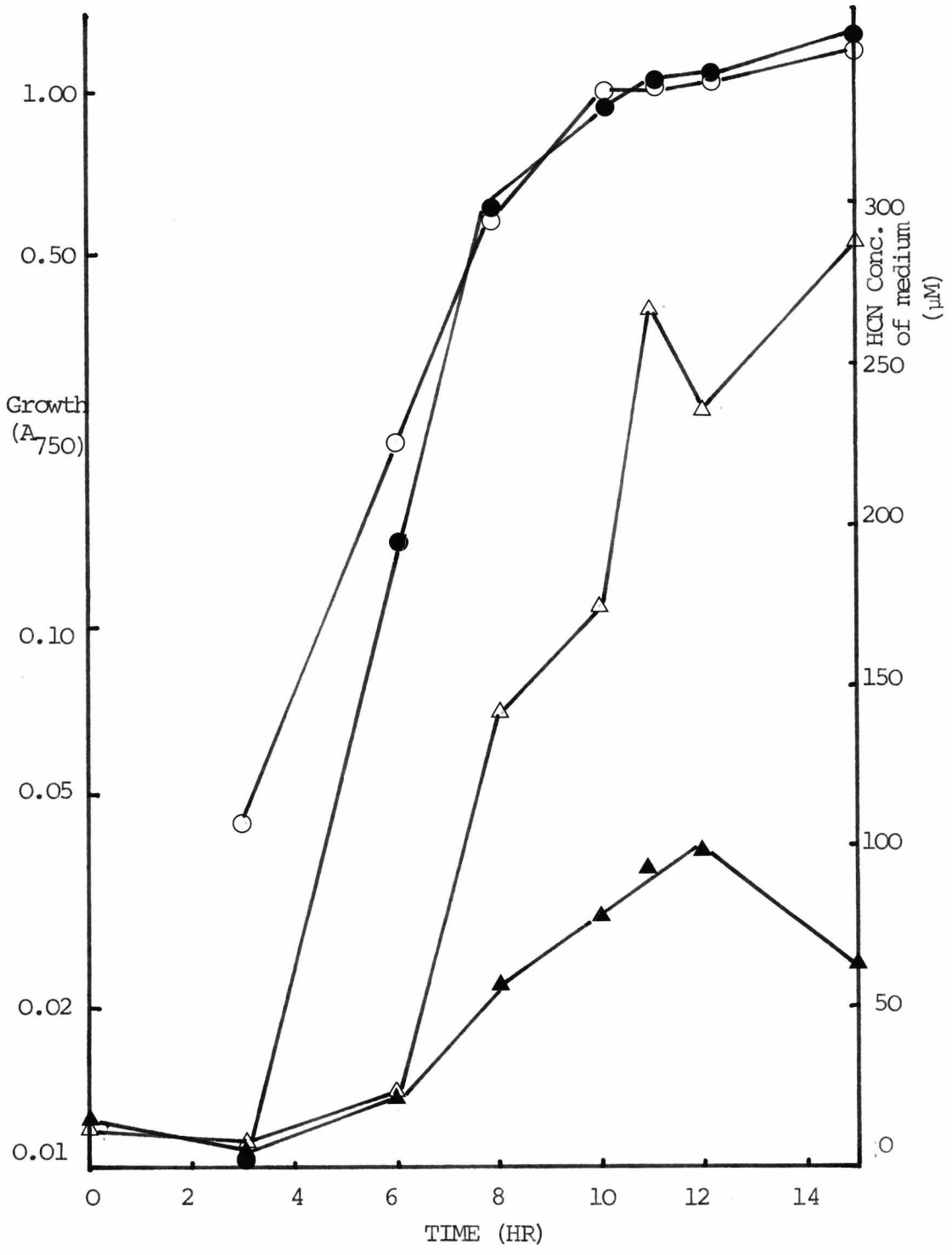


FIGURE 11 - Effect upon cyanide production by *C.violaceum* of varying the glycine concentration of the medium over a range of constant methionine concentrations. 0(—●—), 0.1(—○—), 0.5(—▲—), 1.0(—△—), and 5.0mM (—■—) concentrations of methionine were used.

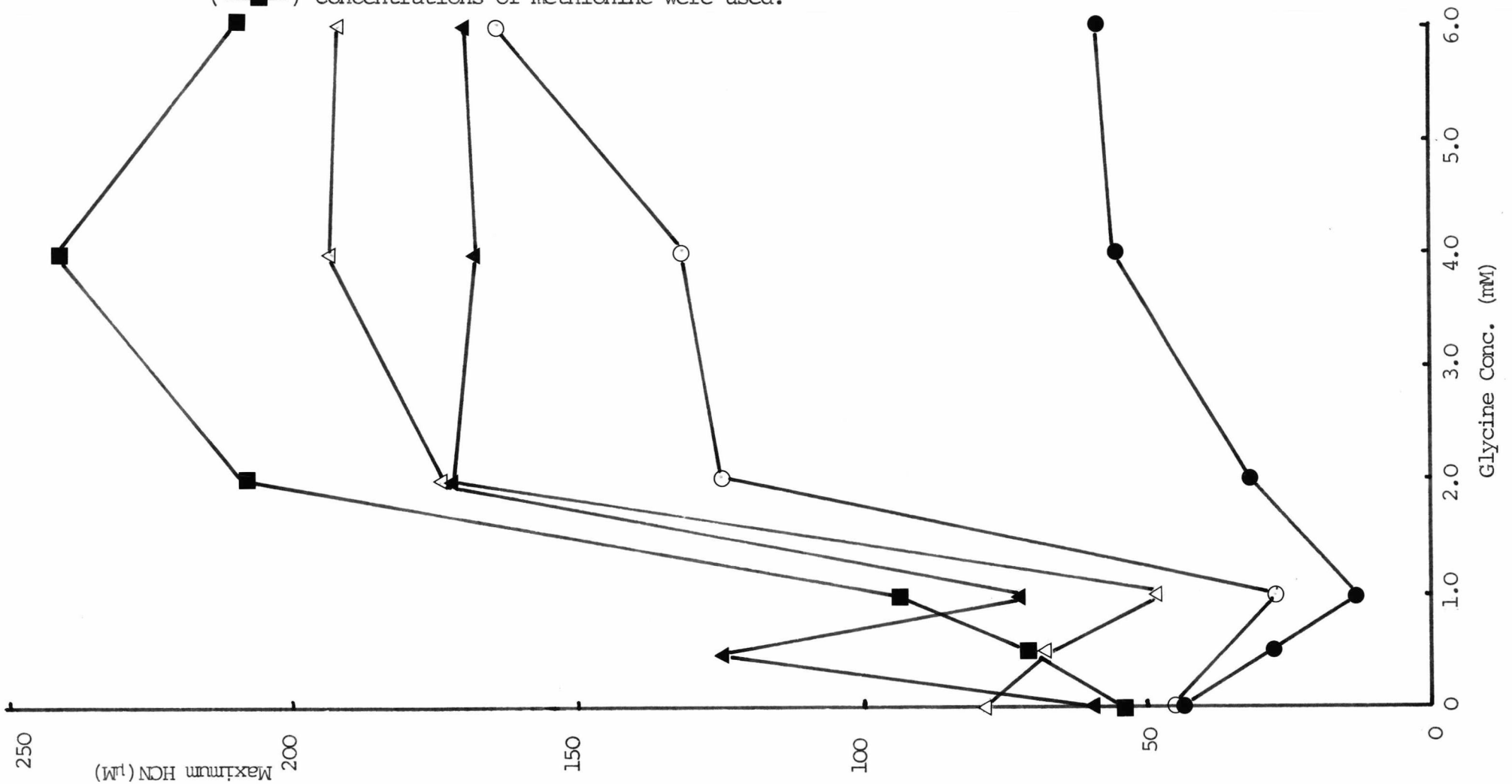
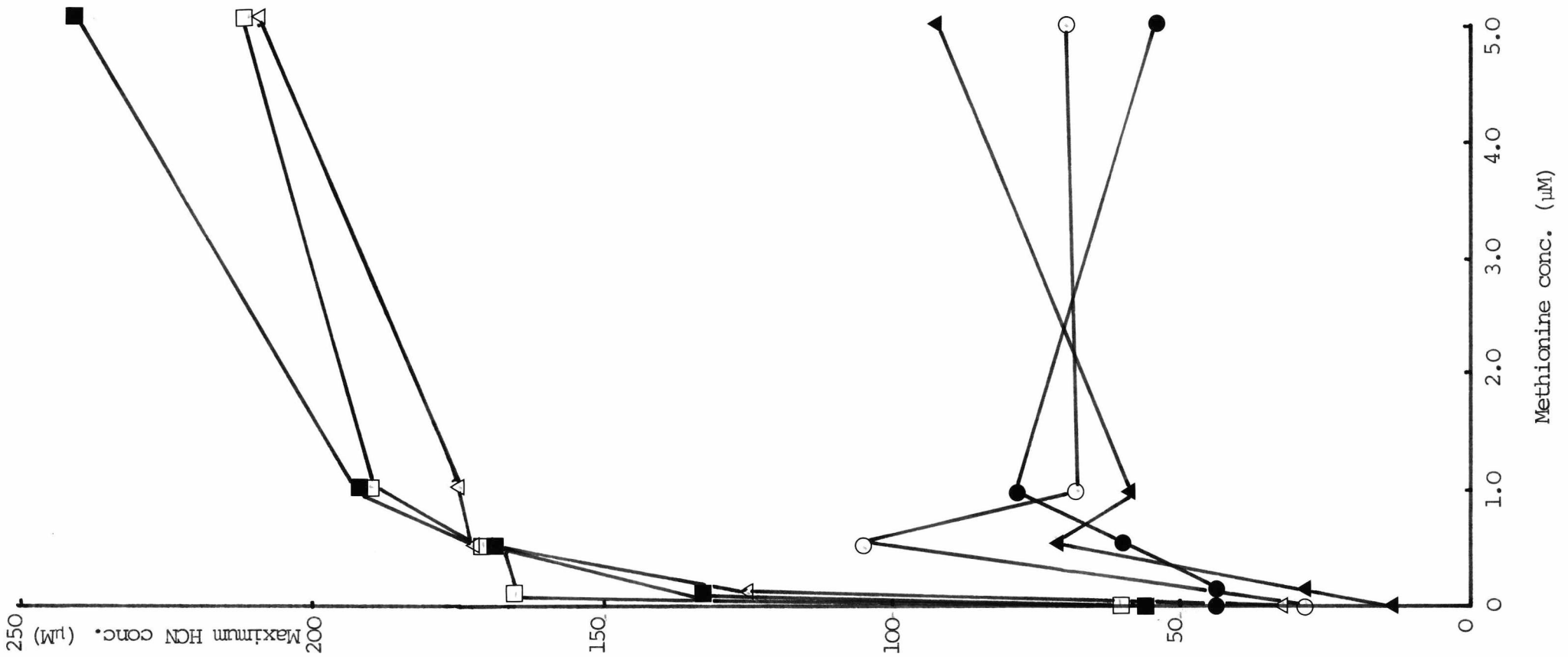


FIGURE 12 - Effect upon cyanide production by *C.violaceum* of varying the methionine concentration of the medium over a range of constant glycine concentrations. 0(—●—), 0.5(—○—), 1.0(—▲—) 2.0(—△—), 4.0(—■—), and 6.0mM(—□—) concentrations of glycine were used.



glycine and methionine at which maximal cyanide production by *C.violaceum* occurred were 5mM and 1mM respectively whilst for *Ps.aeruginosa*, 10mM glycine and 5mM methionine were the concentrations reported.

For studies of cyanide production by *C.violaceum* growing on 10mM glutamate as carbon and nitrogen source, 2.0mM glycine and 0.5mM methionine were chosen as supplements to the growth medium.

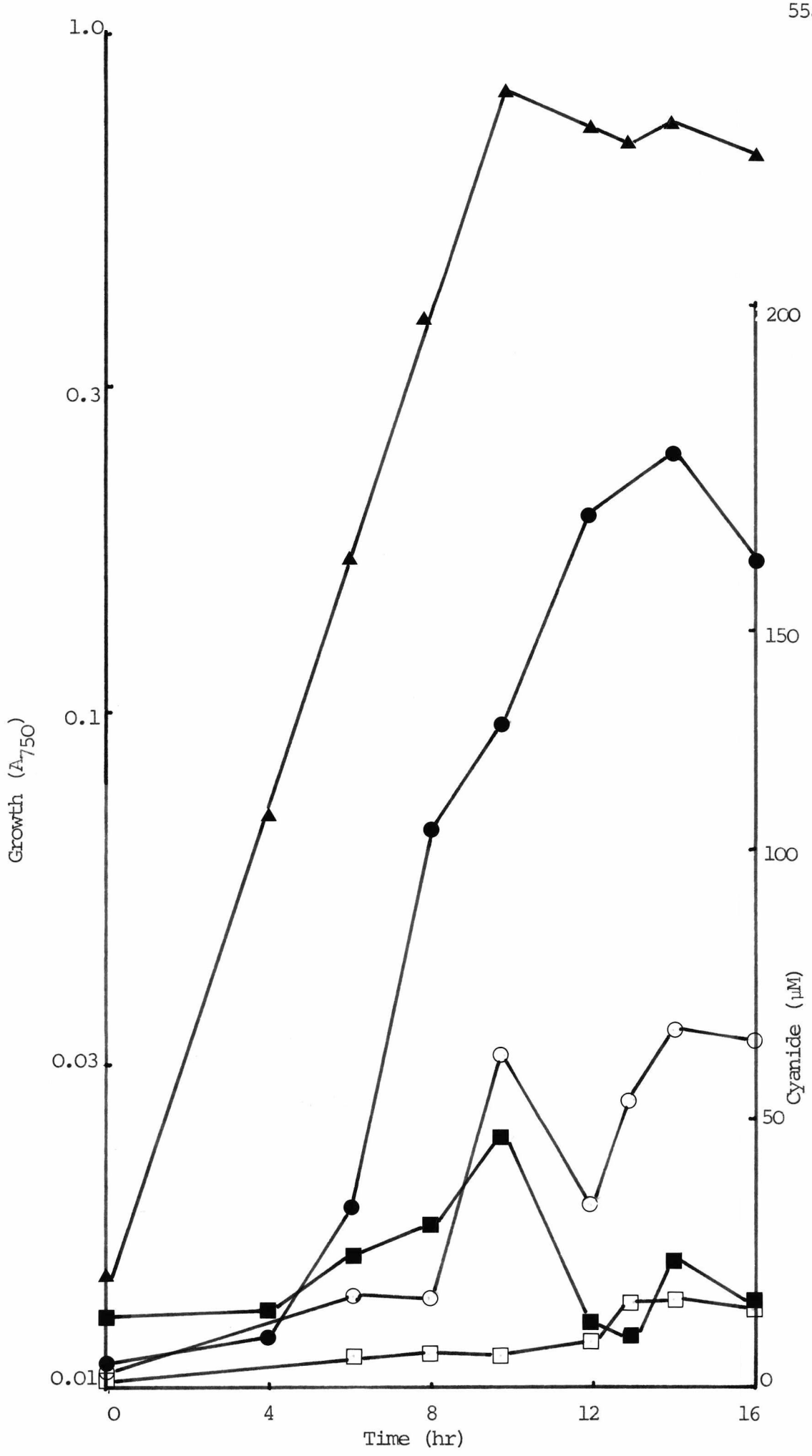
Growth Conditions and Cyanide Production.

Figure 13 shows cyanide production by *C.violaceum* growing on 10mM glutamate as a combined carbon and nitrogen source, in the absence and presence of 2.0mM glycine and 0.5mM methionine. For clarity, only the growth curve for glutamate alone has been shown. Inclusion of glycine and/or methionine in the medium had little effect on the growth rate, although occasionally some reduction in the final yield has been observed. In confirmation of the data of Michaels and Corpe (1965), little cyanide was produced during growth on glutamate alone. Supplementation of the growth medium with glycine or methionine alone led to a small increase in the cyanide content of the medium but in combination they caused a massive increase in the medium cyanide level. Cyanide production occurred towards the end of exponential growth and during the early stationary phase. Similar results have been obtained in an unidentified *Pseudomonas* species (Wissing, 1968) and in *Ps.aeruginosa* (Castric, 1975).

The pH of the growth medium was monitored throughout growth. It rose from a value of 6.70 at the time of inoculation to one of 7.00 in the stationary phase of growth.

It was not necessary for methionine to be present in the medium throughout exponential growth for stimulation of cyanogenesis to occur. Addition of 0.5mM methionine to late exponential phase cells growing on glutamate

FIGURE 13 - Growth and cyanide evolution by *C.violaceum*, for reasons of clarity only growth in media containing 10mM glutamate is shown (—▲—). Cyanide evolution was followed during growth on 10mM glutamate (—□—), supplemented with 2mM glycine (—■—), 0.5mM methionine (—○—) and 2mM glycine plus 0.5mM methionine (—●—).


1.0
0.3
0.1
0.03
0.01
Growth (A_{750})
200
150
100
50
0
Cyanide (μM)
0
4
8
12
16
Time (hr)

alone (Fig.14) or on glutamate plus glycine (Fig.15) was sufficient to increase the cyanide content of the medium to that seen when methionine was present throughout growth (Fig.13).

The effects of a number of culture parameters on growth and cyanide production were investigated.

(a) Growth temperature: Although the growth rate increased with temperature over the range tested, 25 - 35^{o}C, growth yield and cyanide production were essentially unaffected by the growth temperature.

(b) Growth medium pH: Cyanide production, growth rate and final yield were unaffected by growth media with initial pH values between 6.0 and 8.0.

(c) Ferrous ion concentration: As in *Ps.aeruginosa* (Castric, 1975), cyanogenesis by *C.violaceum* is stimulated by the ferrous ion content of the growth medium (Fig.16). This stimulation is linear with the logarithm of the ferrous ion concentration which is characteristic of a metal effect on secondary metabolism (Weinberg, 1970). Although higher concentrations of ferrous ion gave greater yields of cyanide, 30μM $FeSO_4$ was routinely used in growth media as precipitation problems were experienced with higher levels. No effect of ferrous ion concentration on growth yield was observed.

(d) Phosphate concentration : Increasing the medium, inorganic phosphate concentration also caused an increase in cyanogenesis (Fig.16). Above 100mM phosphate the growth yield declined somewhat and 68mM phosphate was routinely used in the growth medium. *C.violaceum* appears to be different to *Ps.aeruginosa* in its response to phosphate as Megathanan and Castric (1977) have reported that cyanogenesis is inhibited in the latter organism by phosphate concentrations greater than 10mM. The production of many secondary metabolites is inhibited at higher phosphate concentrations (Weinberg, 1974).

FIGURE 14 - Addition of methionine to late-exponential phase cultures growing on glutamate. *C.violaceum* was grown on 10mM glutamate (—●—), and 0.5mM methionine (—○—) added at the time indicated by the arrow. Cyanide production was followed in the absence (—▲—) and presence (—△—) of methionine.

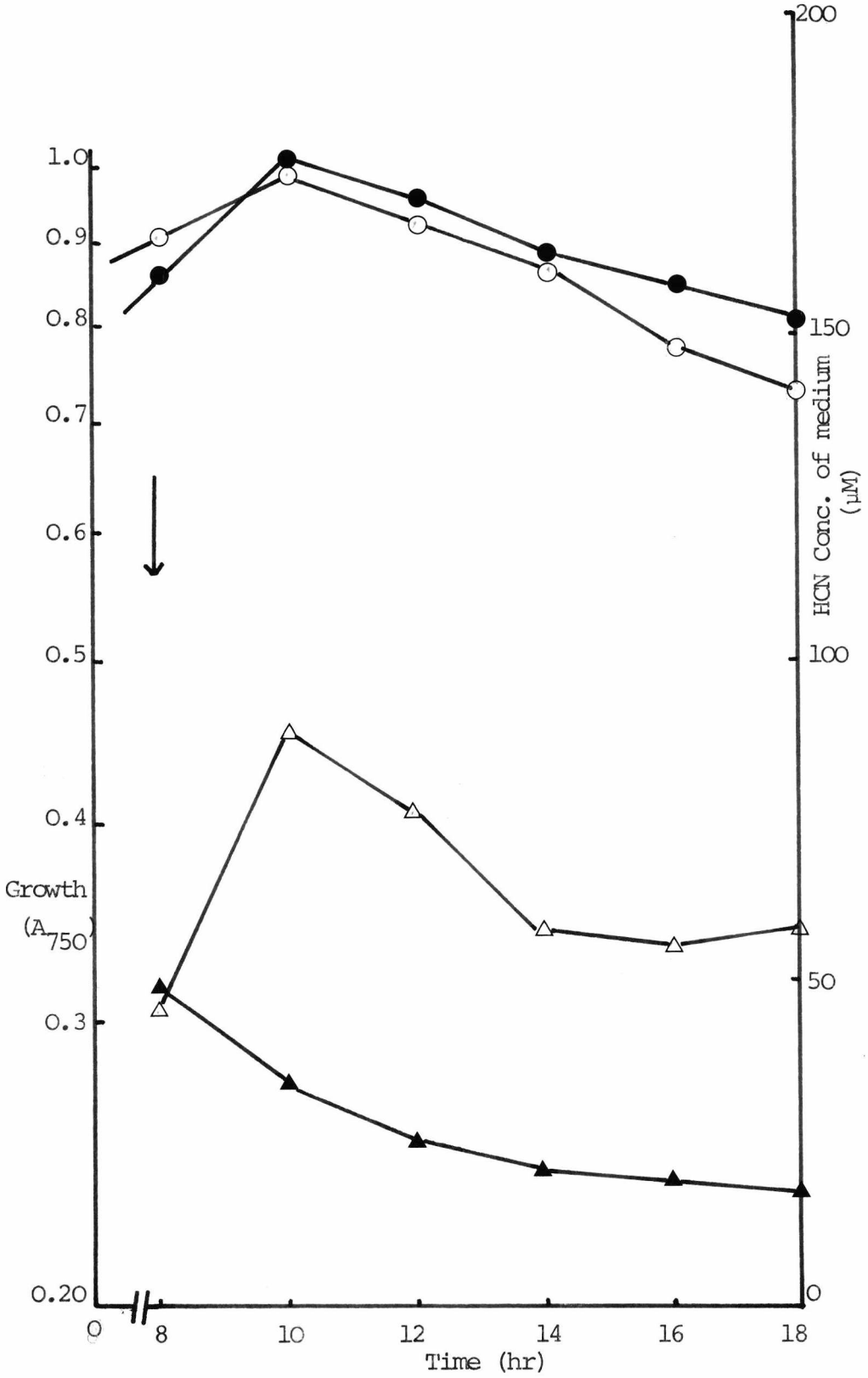


FIGURE 15 - Addition of methionine to late-exponential phase cultures growing on glutamate supplemented with glycine. *C.violaceum* was grown on 10mM glutamate plus 2.0mM glycine (—●—), and 0.5mM methionine (—○—) added at the time indicated by the arrow. Cyanide production was followed in the absence (—▲—) and presence (—△—) of methionine.

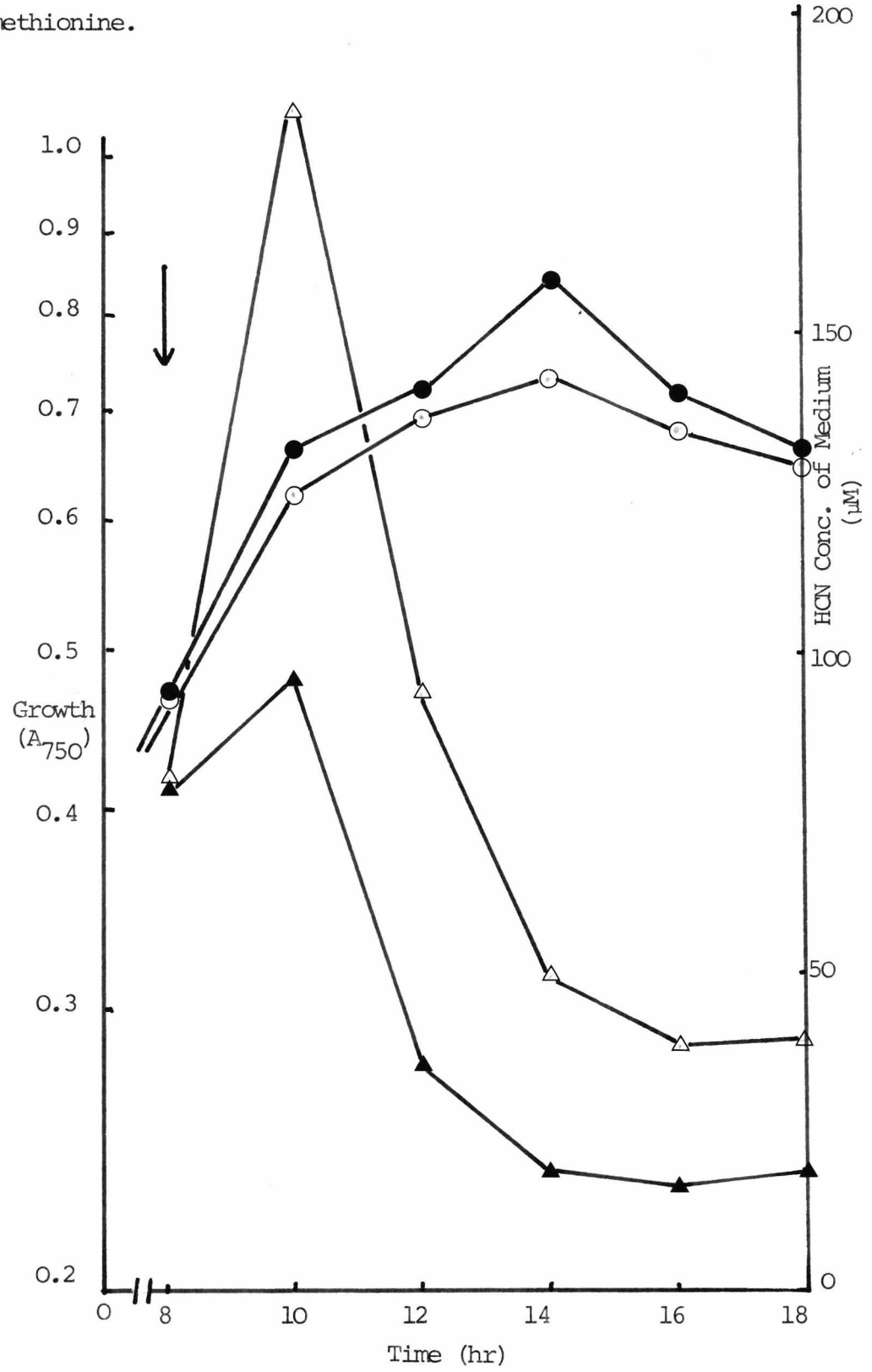
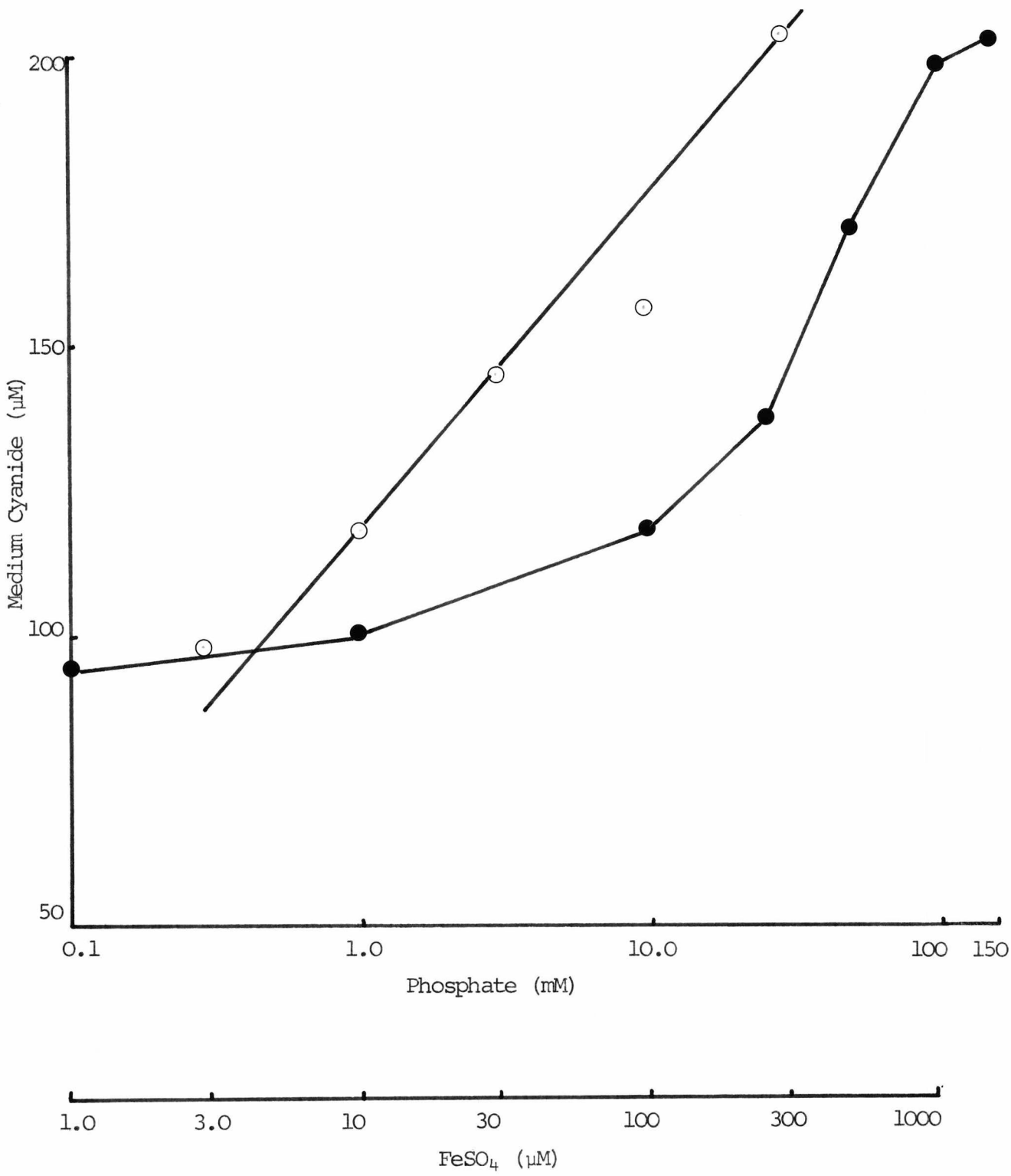


FIGURE 16 - The effect of ferrous ion (as $FeSO_4$) concentration (—○—) and phosphate concentration (—●—) on the maximum level of cyanide evolved by cultures of *C.violaceum* growing on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine. In the experiment where $FeSO_4$ concentration was varied the phosphate content was maintained at 68mM, and where the phosphate content was varied the $FeSO_4$ concentration was 30μM



Cyanide Production by Cell-Free Extracts.

(The work in this part of the thesis was carried out during a short stay at the laboratory of Dr. F. Wissing, Department of Biochemistry, The Royal Dental College, Arhus, Denmark).

Cyanide production was investigated using resuspended whole cells or extracts prepared from stationary phase cells grown on a Bacto-nutrient medium. The incubation mixture contained phosphate buffer and, where stated, 1.0mM methionione. The mixture was incubated for 20 minutes in the case of resuspended whole cells, and 8 minutes in the case of extracts, to obtain a value for the endogenous rate of cyanide production, before the addition of 20mM glycine.

With unwashed whole cells, the rate of endogenous cyanide production was essentially the same in the presence or absence of methionine and addition of glycine did not stimulate the rate of cyanogenesis. Washed, resuspended whole cells, an unfractionated sonicate and the supernatant fraction obtained upon centrifugation of the sonicate all gave similar endogenous rates of cyanide production although these rates were much slower (approximately 100-fold less) than the endogenous rate obtained with unwashed whole cells. Again the endogenous rates were the same in the presence or absence of methionine and glycine did not stimulate cyanide production.

With a resuspended pellet, obtained by centrifuging the sonicate, the endogenous rates of cyanide formation in the absence and presence of methionine were similar to those of the supernatant fraction but glycine addition stimulated cyanide production 5-fold in the presence and absence of methionine. However, glycine stimulation of cyanogenesis could not be repeated using extracts prepared on other days.

In studies of cyanide production by extracts of a *Pseudomonas* species, Wissing (personal communication) found that addition of sodium deoxycholate to the sonicate before centrifugation, and the inclusion of 5mM dithiothreitol and/or 20mM formic acid in the incubation mixture increased the rate of cyanide formation. These additives were tried with *C.violaceum* extracts but the rate of endogenous cyanogenesis was not increased and the extracts were not made susceptible to glycine-stimulation of cyanide production.

Cyanide-utilising Enzymes.

(a) Induction of the cyanide-utilising enzymes: Three cyanide-utilising enzyme activities, β-cyanoalanine synthase, γ-cyano-α-aminobutyric acid synthase and rhodanese, were found in extracts of *C.violaceum*. A fourth enzyme, formamide hydro-lyase, could not be detected in extracts of stationary phase cells grown under low- (i.e. grown on glutamate alone) or high- (i.e. grown on glutamate plus glycine and methionine) cyanide producing conditions.

In cultures growing on glutamate alone, or on glutamate plus glycine and methionine, induction of β-cyanoalanine synthase occurs during the late exponential and early stationary phases of growth (Fig.17). The concentration of the enzyme increased after about 8 hours growth, to a maximal value about 2 hours after the stationary phase had been attained (12 hours growth).

Essentially the same picture is seen for γ-cyano-α-aminobutyric acid synthase, except that the concentration of the enzyme reached a maximal value after about 14 hours growth (Fig.18).

Inclusion of glycine plus methionine in the medium caused a significant reduction of the induction of β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase during the exponential-stationary phase transition period. The extent of the reduction in the degree of induction of these

FIGURE 17 - Growth (—●— , —○—) and β-cyanoalanine synthase activity (—■— , —□—) in extracts of *C.violaceum* grown on 10mM glutamate (—●— , —■—) and on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine (—○— , —□—). Specific activity units are nmoles H_2S evolved/min/mg.protein).

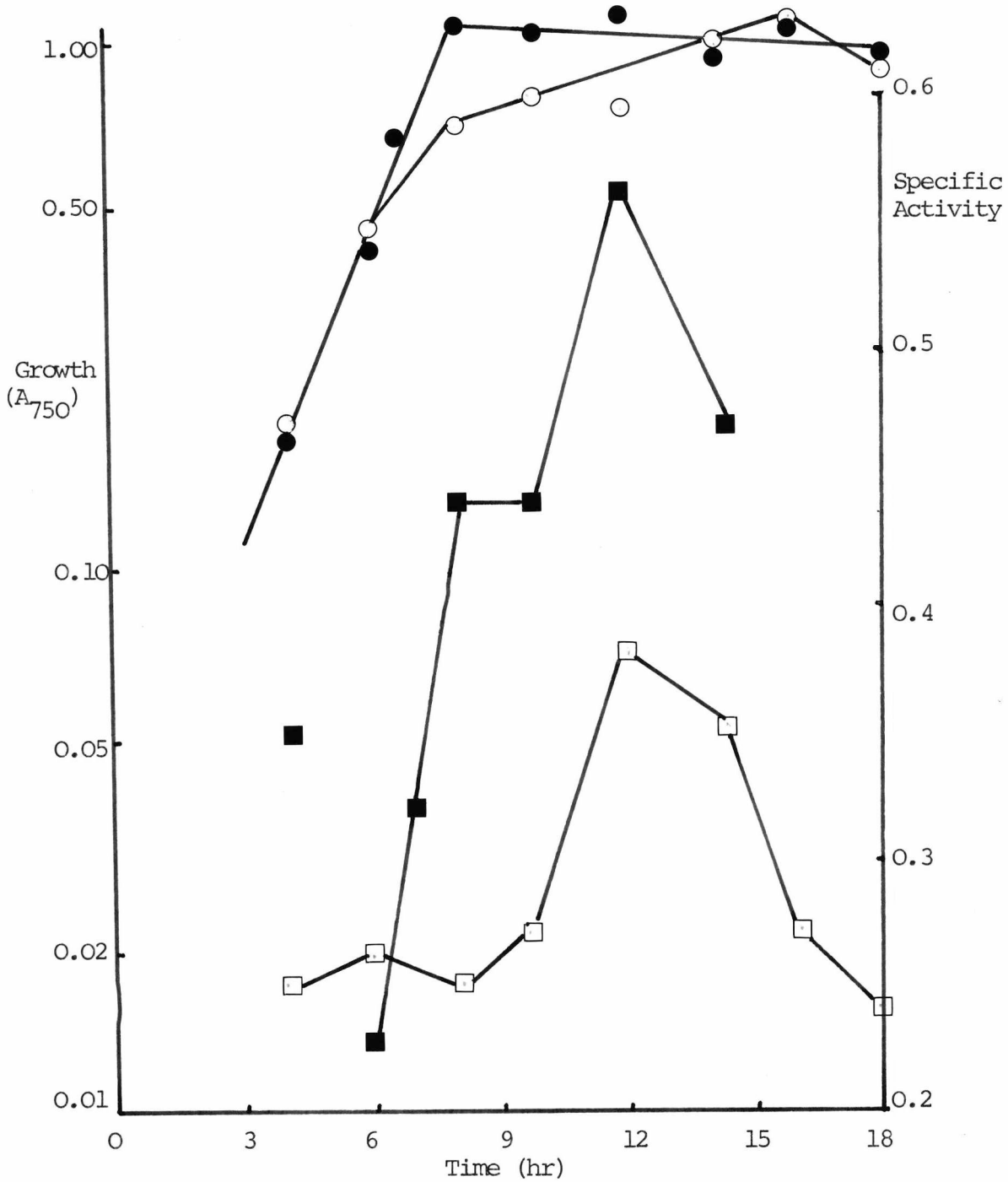
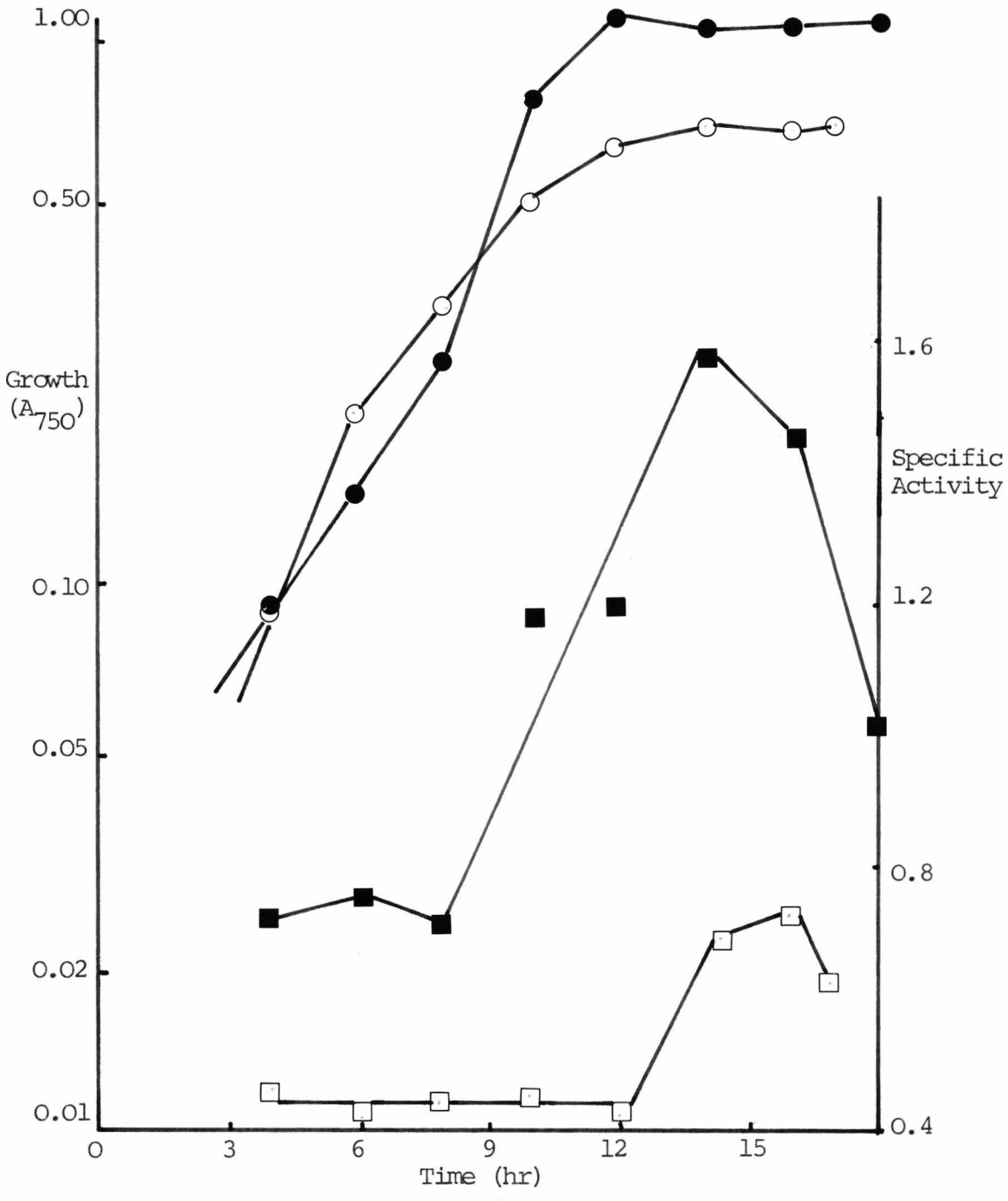


FIGURE 18 - Growth (—●— , —○—) and γ-cyano-α-aminobutyric acid synthase activity (—■— , —□—) in extracts of *C.violaceum* grown on 10mM glutamate (—●— , —■—) and on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine (—○— , —□—). Specific activity units are nmoles SCN^- produced/min/mg.protein.



two enzymes was similar when glycine alone was included as when glycine and methionine were included in the medium (Table 1).

Rhodanese is also induced during the late exponential and early stationary phases of cultures growing on glutamate alone or on glutamate plus glycine and methionine (Fig.19). Again the enzyme concentration increased after about 8 hours growth and reached a maximum about 2 hours into the stationary phase. Rhodanese differs from β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase in that the extent of its induction is greater in cultures growing on glutamate plus glycine and methionine than in cultures growing on glutamate alone or on glutamate plus glycine (Table 1).

(b) Inhibition of β-cyanoalanine synthase activity by methionine: The β-cyanoalanine synthase activity of extracts of stationary phase cells grown on glutamate alone was inhibited by methionine but not by glycine (Fig.20). β-cyanoalanine synthase is about 60% inhibited by 2.0mM methionine yet higher concentrations of methionine cause little further inhibition. Glycine, in concentrations of up to 6.0mM, caused virtually no inhibition of β-cyanoalanine synthase and combinations of glycine and methionine had no greater inhibitory effect than methionine alone.

Concentrations of methionine and glycine, of up to 5.0mM and 6.0mM respectively, either alone or in combination, displayed no significant inhibition of the γ-cyano-α-aminobutyric acid synthase and rhodanese activities of extracts of stationary phase cells grown on glutamate alone.

FIGURE 19 - Growth (—●— , —○—) and rhodanese activity (—■— , —□—) in extracts of *C.violaceum* grown on 10mM glutamate (—●— , —■—) and on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine (—○— , —□—). Specific activity units are nmoles, 2,6-dichlorophenolindophenol reduced/min/mg protein.

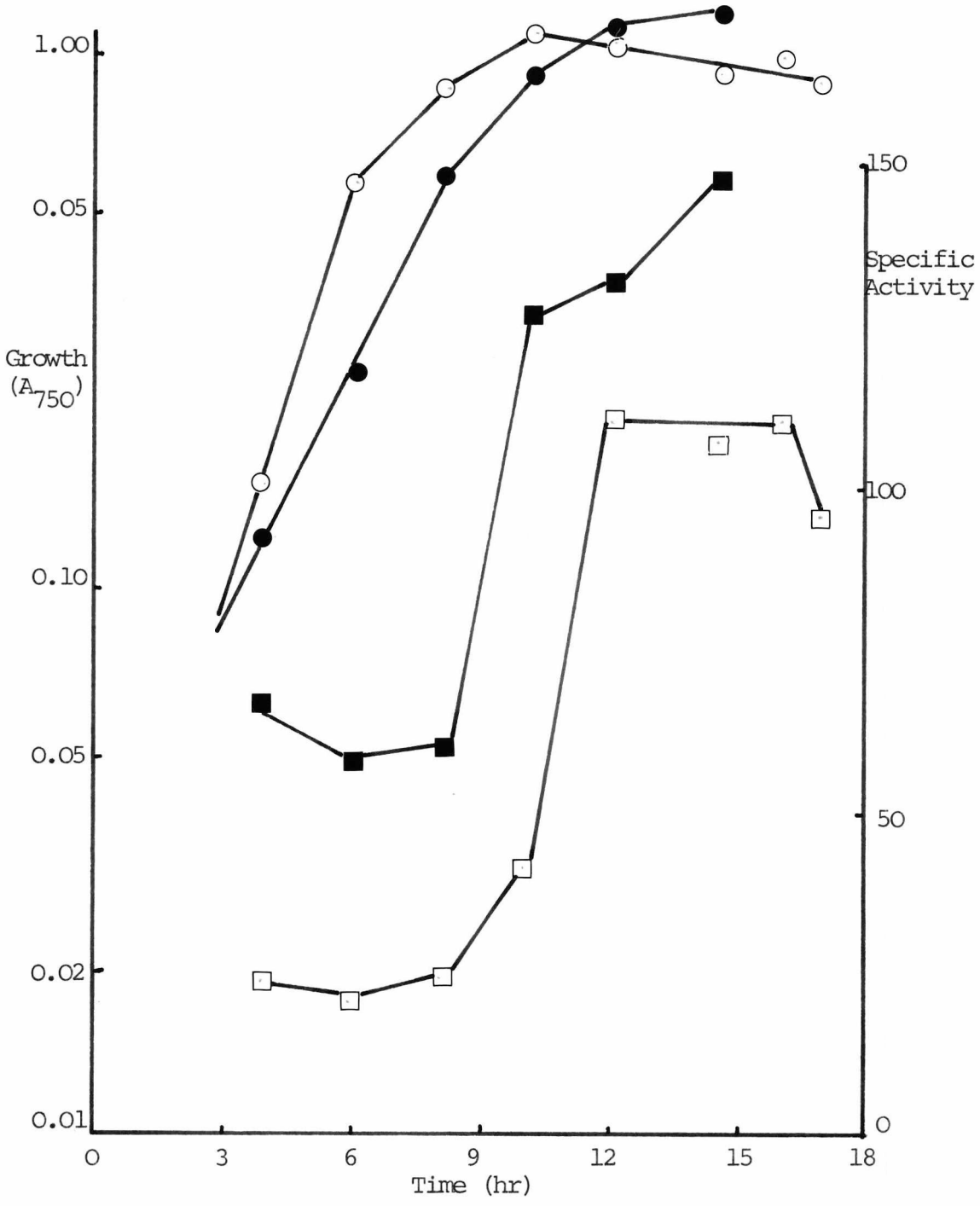


TABLE 1. Specific activities of the cyanide-utilising enzymes in cell-free extracts of cultures grown on glutamate and on glutamate supplemented with glycine or glycine plus methionine

	Time (h)	Glu	Medium	Glu, gly + met	Ratio of specific activities after 8h and 12h or 14h growth		
			Glu + gly		Glu	Glu + gly	Glu,gly + met
β-cyano alanine synthase	8	0.25	0.32	0.25			
	12	0.56	0.39	0.38	2.24	1.22	1.52
γ-cyano-α-amino butyric acid synthase	8	0.73	0.36	0.43			
	14	1.58	0.56	0.68	2.16	1.57	1.58
Rhodanese	8	60	41	25			
	12	132	85	110	2.20	2.13	4.40

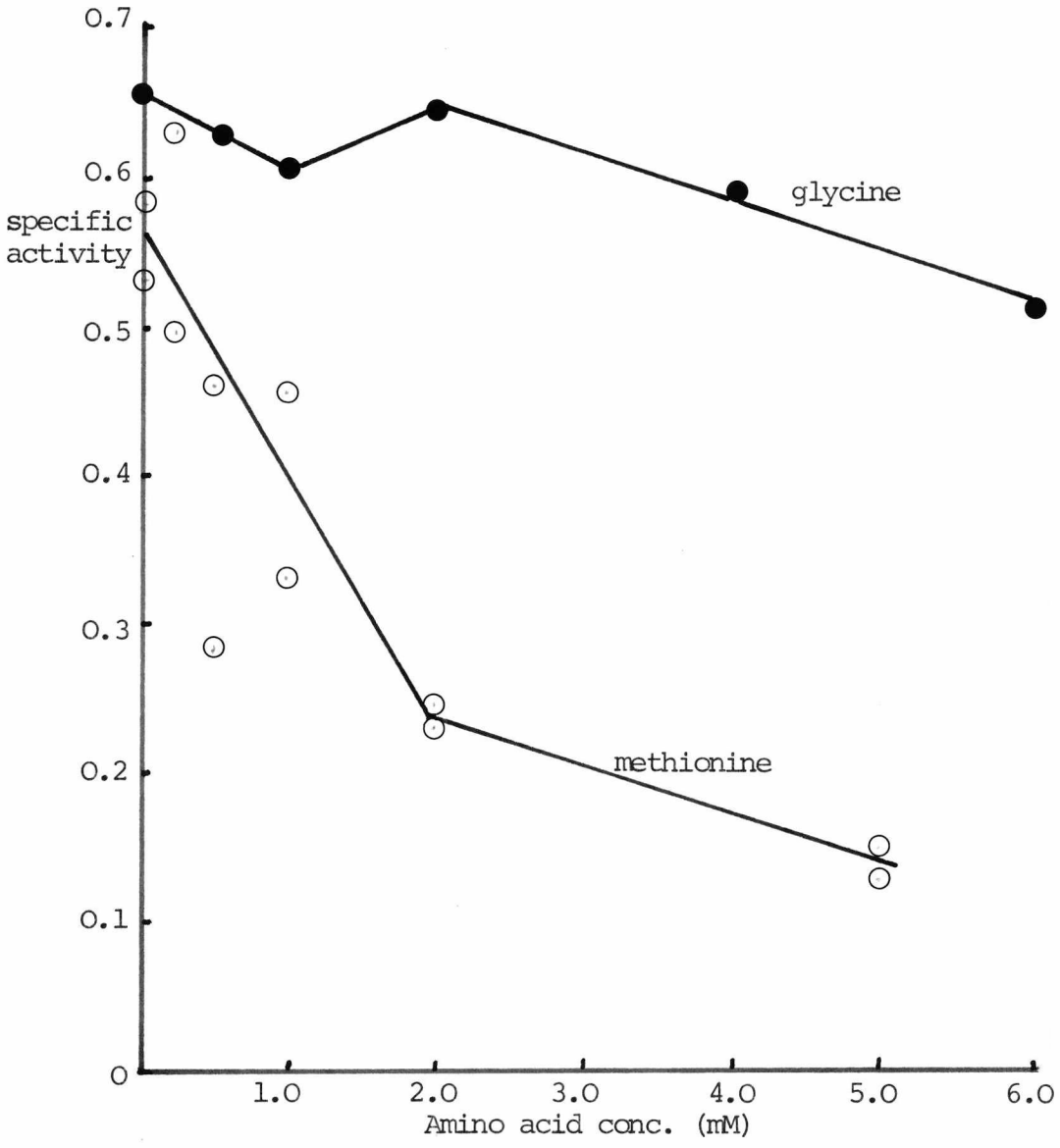
Specific activity units; β-cyanoalanine synthase, nmoles H_2S evolved/min/mg protein; γ-cyano-α-amino-butyric acid synthase, nmoles SCN^- produced/min/mg protein; rhodanese, nmoles DCIP reduced/min/mg protein

Where added, the concentrations of the amino acids in the growth medium were; glutamate, 10mM; glycine, 2mM; methionine, 0.5mM

FIGURE 20 - Effect of glycine and methionine on β-cyanoalanine synthase activity of *C.violaceum* extracts.

Extracts were prepared from stationary phase cells grown on 10mM glutamate alone.

Specific activity units - nmoles H_2S produced/min/mg protein.



The Effect of Chloramphenicol Addition to Growing Cultures upon the Cyanide Content of the Growth Medium.

In the following series of experiments involving addition of the protein synthesis inhibitor chloramphenicol, to growing cultures of *C.violaceum*, three concentrations of chloramphenicol, 5, 25 and 50µg/ml, were used, except where stated. For reasons of clarity only the effects of 5 and 50µg/ml are shown in the diagrams.

(a) Chloramphenicol addition to mid-exponential phase cultures: Chloramphenicol was added to the medium of mid-exponential phase cultures (6 hours growth), i.e. prior to the onset of cyanogenesis.

Fig.21 shows the effect of adding chloramphenicol to mid-exponential phase cultures growing on glutamate alone. Growth was inhibited within an hour of chloramphenicol addition; the extent of inhibition increasing with the chloramphenicol concentration. The low cyanide content of the medium was decreased somewhat by chloramphenicol addition but the inhibitory effect was not enhanced by higher chloramphenicol concentrations.

Addition of chloramphenicol to mid-exponential phase cultures (6 hours growth) growing on glutamate plus glycine and methionine inhibited growth almost immediately and considerably inhibited cyanogenesis (Fig.22). Again the inhibitory effect on growth increased with chloramphenicol concentration whilst the degree of inhibition of cyanogenesis was similar at all chloramphenicol concentrations.

In the absence of chloramphenicol, the cyanide content of the medium containing glutamate plus glycine and methionione decreased after around 12 hours growth, the time by which the cyanide-utilising enzymes have been induced. Presumably, this decrease in cyanide concentration was due to assimilation or detoxication of cyanide.

FIGURE 21 - Effect of chloramphenicol (CAP) addition to mid-exponential phase cultures growing on 10mM glutamate. Growth (—●— , —■— , —▲—) and cyanide production (—○— , —□— , —△—) were followed in the absence (—●— , —○—) and presence of 5µg/ml (—■— , —□—) and 50µg/ml (—▲— , —△—) CAP. CAP was added at the time indicated by arrow.

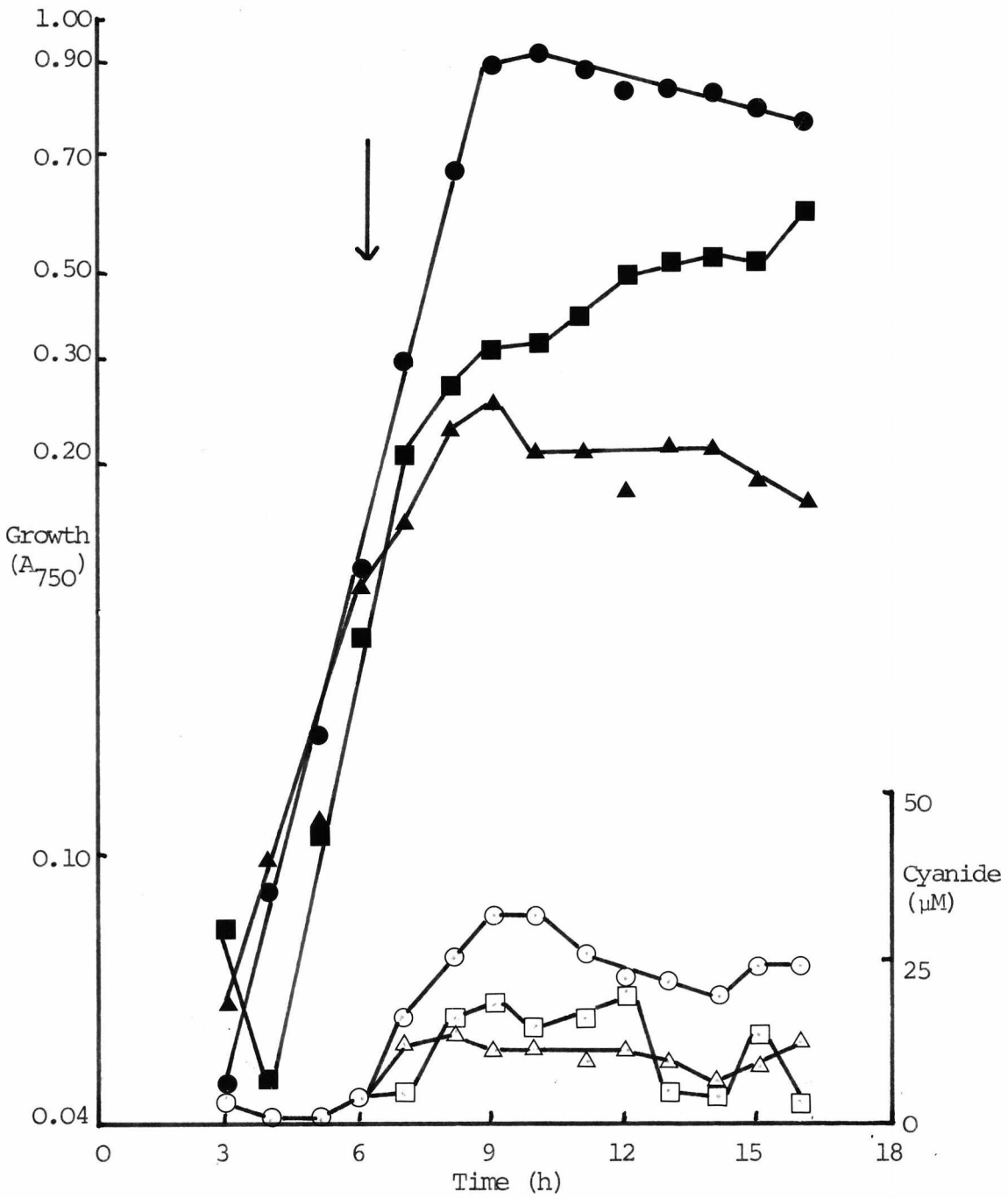
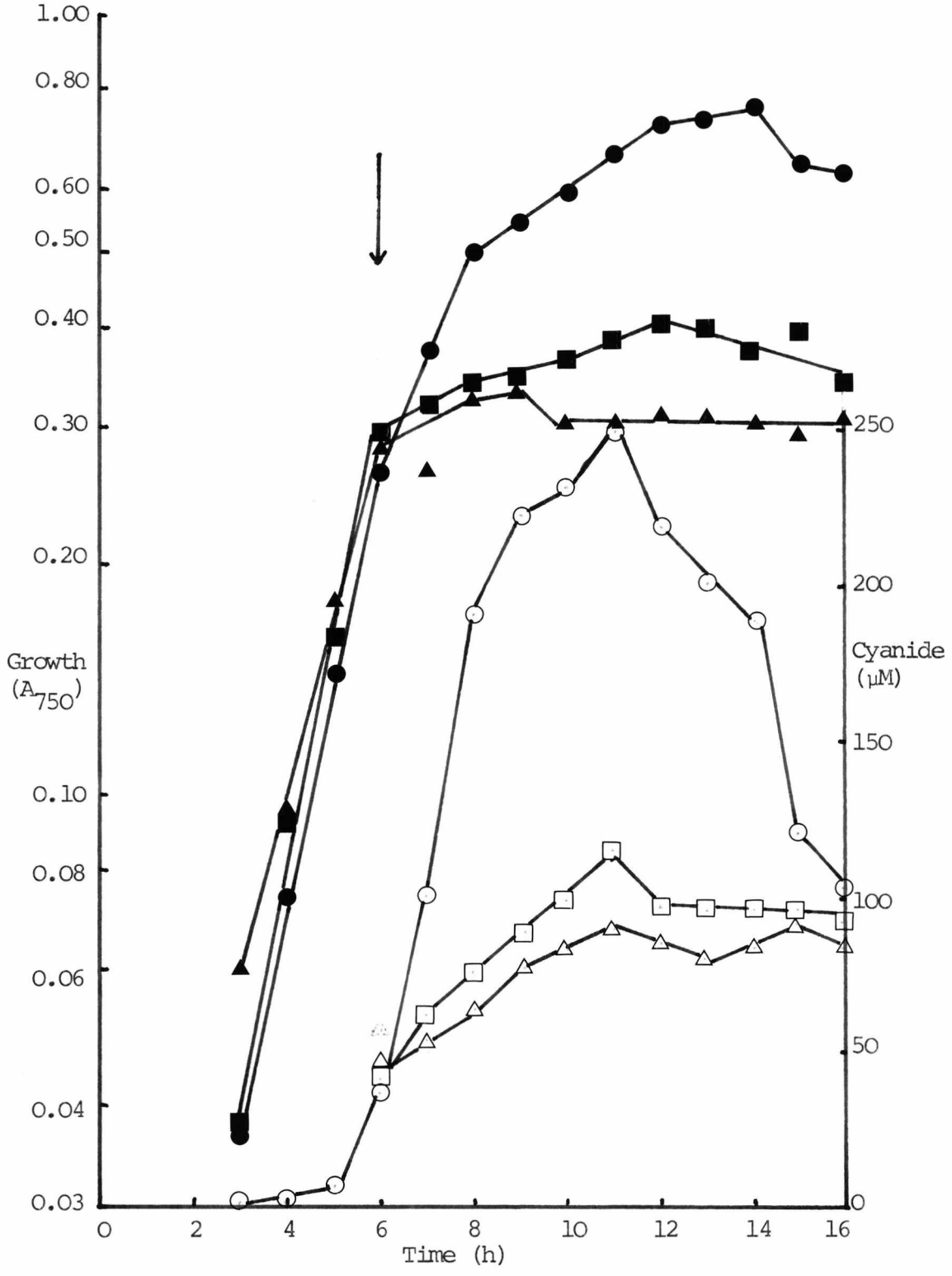


FIGURE 22 - Effect of chloramphenicol (CAP) addition to mid-exponential phase cultures growing on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine. Growth (—●— , —■— , —▲—) and cyanide production (—○— , —□— , —△—) were followed in the absence (—●— , —○—) and presence of 5µg/ml (—■— , —□—) and 50µg/ml (—▲— , —△—) CAP. CAP was added at time indicated by arrow.



(b) Chloramphenicol addition to late-exponential phase cultures: The addition of chloramphenicol to mid-exponential phase cultures, prior to cyanogenesis, inhibited the increase in cyanide content of the medium seen towards the end of exponential growth. This suggests that the cyanide-producing system is synthesised during exponential growth but, in view of the difficulties encountered in obtaining a cyanide-forming activity in cell extracts, this cannot be confirmed. However, following the cyanide content of the growth medium after chloramphenicol addition towards the end of exponential growth, by which time the cyanide producing system has been induced, may provide information on how the induction of the cyanide-utilising enzymes influences the medium cyanide concentration. Fig.23 demonstrates that addition of chloramphenicol to late-exponential phase cultures (9.5 hours growth), growing on glutamate alone, results in some inhibition of growth but the cyanide concentration of the medium is increased 2-3 fold. Both the inhibition of growth and the enhancement of the medium cyanide level increase with chloramphenicol concentration.

In contrast, addition of chloramphenicol to late-exponential phase cultures (9.5 hours growth), growing on glutamate plus glycine and methionine, had little effect on the maximum cyanide concentration, a slight lowering resulted, but the rate of decrease in the cyanide concentration was inhibited (Fig.24). Growth was inhibited almost immediately by higher levels of chloramphenicol although a concentration of 5µg/ml was not inhibitory.

Hydrogen cyanide is a volatile molecule and to ensure that the decrease in cyanide concentration was due to its assimilation, rather than blow-off from the medium, a control experiment was designed. Cyanide solutions were incubated in identical conditions to those used for growth of *C.violaceum*. Three concentrations, 150, 200 and 250µM, of cyanide were used, in the

FIGURE 23 - Effect of chloramphenicol (CAP) addition to late-exponential phase cultures growing on 10mM glutamate. Growth (—●—, —■—, —▲—) and cyanide production (—○—, —□—, —△—) were followed in the absence (—●—, —○—) and presence of 5µg/ml (—■—, —□—) and 50µg/ml (—▲—, —△—) CAP. CAP was added at the time indicated by arrow.

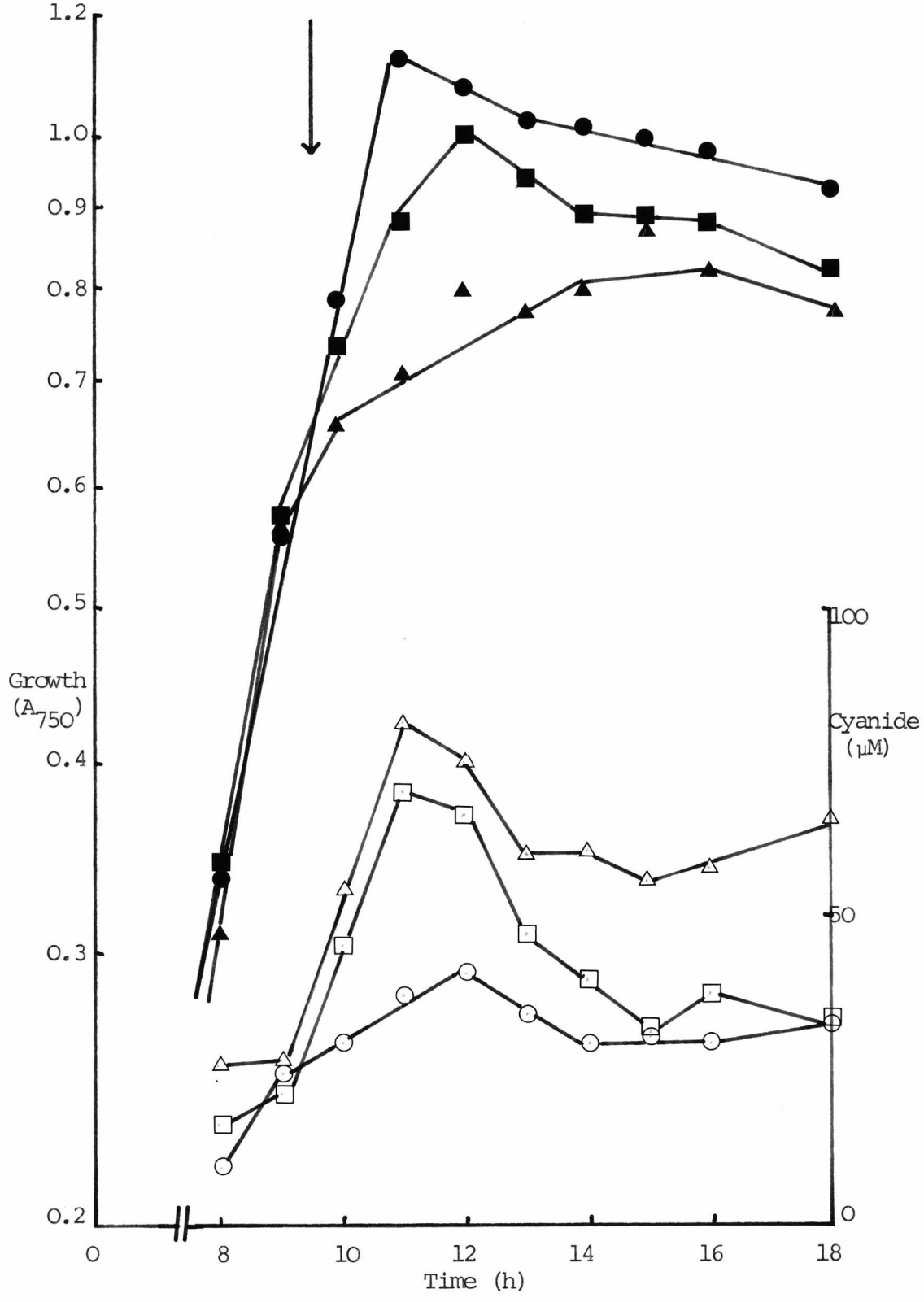
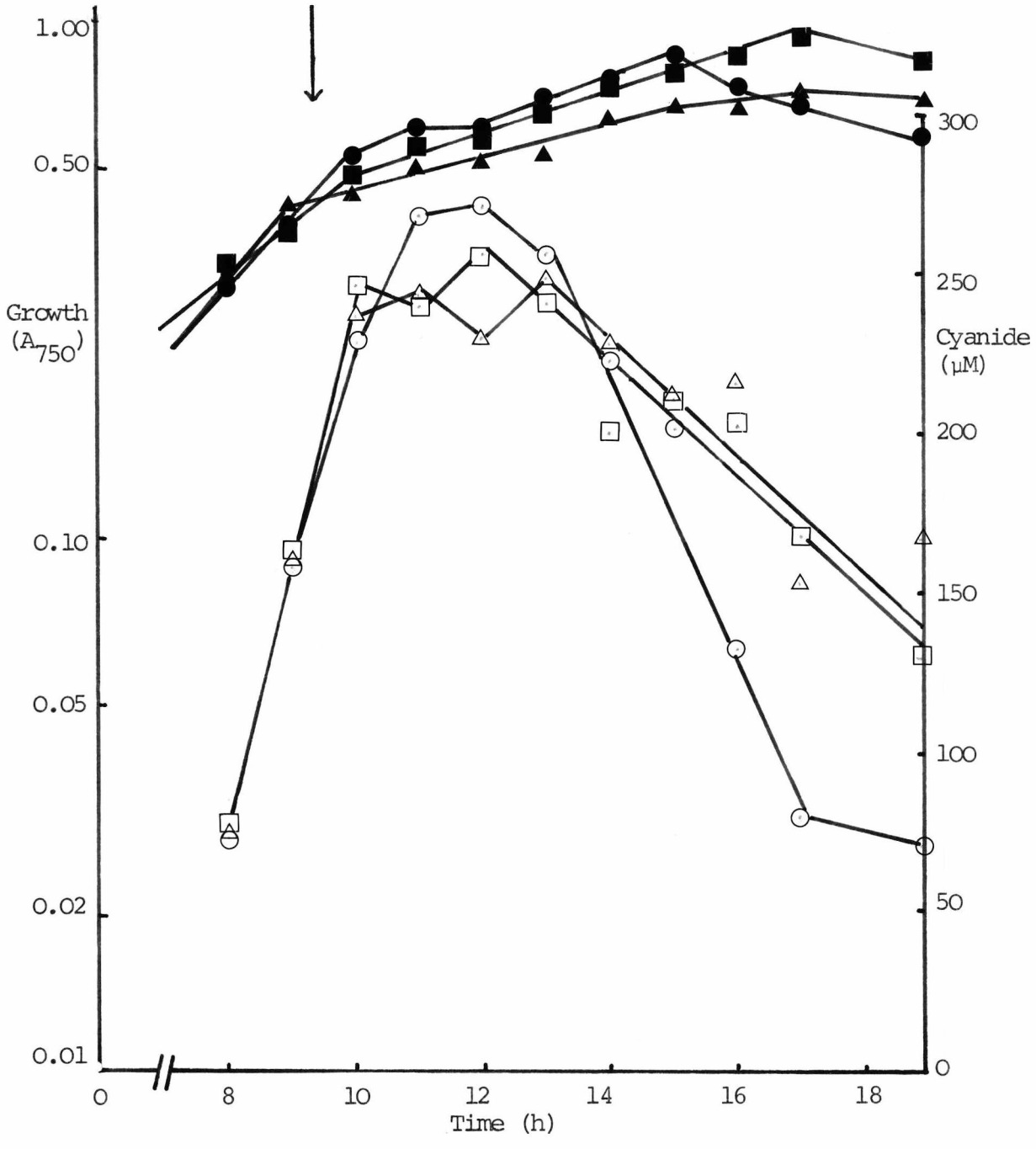


FIGURE 24 - Effect of chloramphenicol (CAP) addition to late-exponential phase cultures growing on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine. Growth (—●— , —■— , —▲—) and cyanide production (—○— , —□— , —△—) were followed in the absence (—●— ,—○—) and presence of 5µg/ml (—■— , —□—) and 50µg/ml (—▲— , —△—) CAP. CAP was added at the time indicated by arrow.



presence and absence of 50μg/ml chloramphenicol. The cyanide concentration was measured at two hour intervals for eight hours and the blow-off rates were found to be similar in each case. An average value of 0.85μmoles/hour was obtained for the cyanide blow-off rate, approximately 2% of the maximum rate of cyanide disappearance observed in the bacterial cultures. A table of cyanide disapperance rates, at the various chloramphenicol concentrations, in bacterial cultures is presented (Table 2). Addition of chloramphenicol at all concentrations used results in an approximate halving of the rate of cyanide disappearance.

Addition of chloramphenicol to late-exponential phase cultures (8.5 hours growth) growing on glutamate supplemented with glycine had the same effect as adding chloramphenicol to cultures growing on glutamate plus glycine and methionine (Fig.25; c.f. Fig.24). Growth was inhibited immediately on addition of chloramphenicol; the extent of inhibition of growth increased with chloramphenicol concentration. The maximum concentration of cyanide attained by the cultures was virtually unaffected by chloramphenicol addition but the rate of cyanide disappearance from the medium was inhibited at all chloramphenicol concentrations to a similar degree.

In contrast, the addition of chloramphenicol to late exponential phase cultures (8 hours growth) growing on glutamate supplemented with methionine had the same effect as adding chloramphenicol to cultures growing on glutamate alone (Fig.26,; cf. Fig.23). In this experiment the concentrations of chloramphenicol used were 12.5, 25 and 50μg/ml. Only the effect of 12.5 and 50μg/ml chloramphenicol are shown in the diagram. An inhibition of growth increasing with chloramphenicol concentration, and an enhancement of the medium cyanide level were observed. The maximum cyanide level of the medium was increased by a similar extent with all concentrations of chloram-

TABLE 2.

Rates of cyanide disappearance from the medium of *C.violaceum* growing on 10mM glutamate plus 2mM glycine and 0.5mM methionine

CAP concentration (μg/ml)	Rate of cyanide disappearance (μmoles/hr)
0	42
5	27
25	15
50	22

Chloramphenicol (CAP) was added at the concentration shown to late-exponential phase cultures (9.5h growth). The rates of cyanide disappearance have been corrected for the rate of cyanide blow-off in the absence of bacteria.

FIGURE 25 - Effect of chloramphenicol (CAP) addition to late-exponential phase cultures growing on 10mM glutamate supplemented with 2mM glycine. Growth (●, ■, ▲) and cyanide production (○, □, △) were followed in the absence (●, ○) and presence of 5μg/ml CAP (■, □) and 50μg/ml CAP (▲, △). CAP was added at the time indicated by arrow.

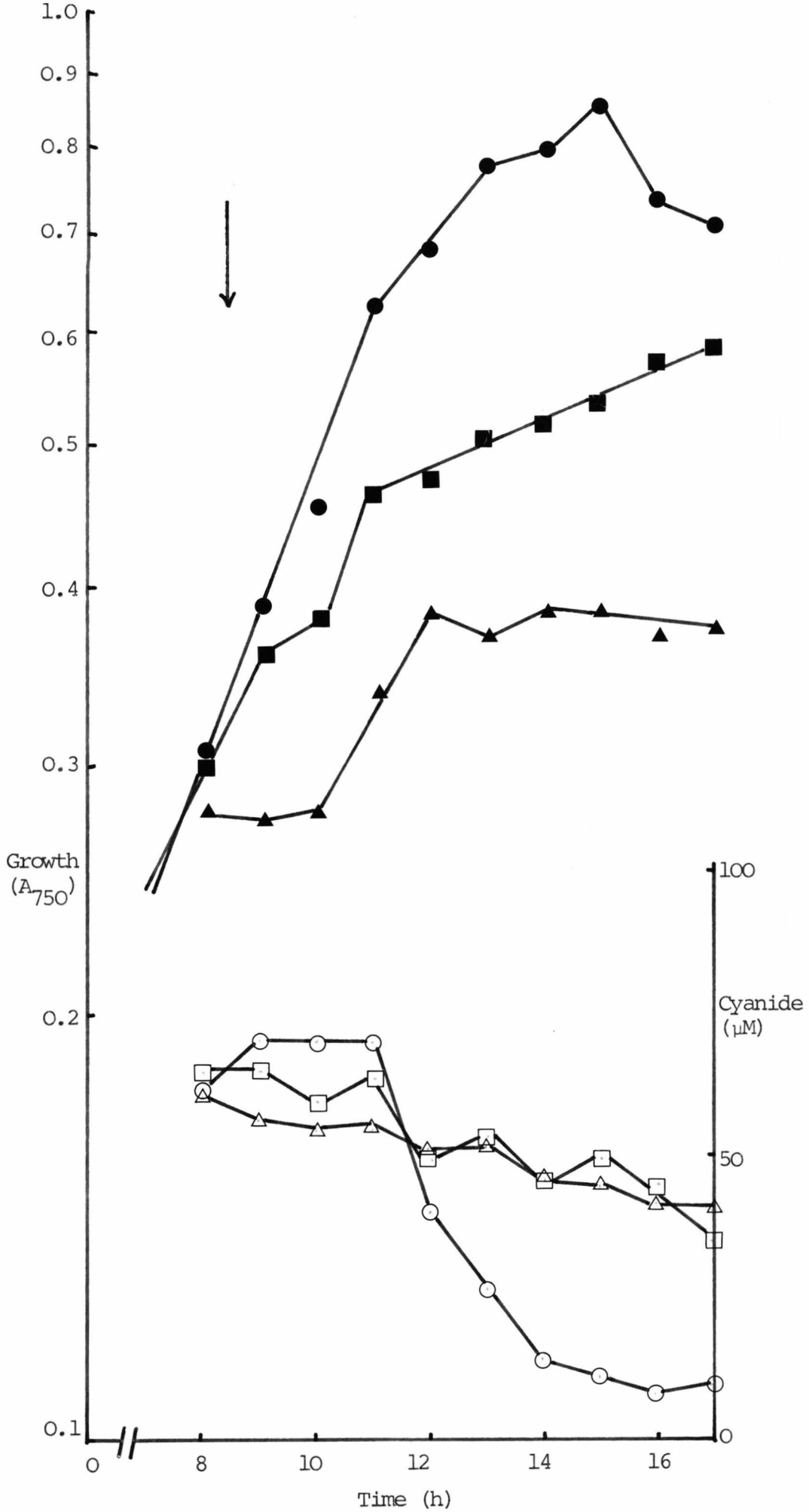
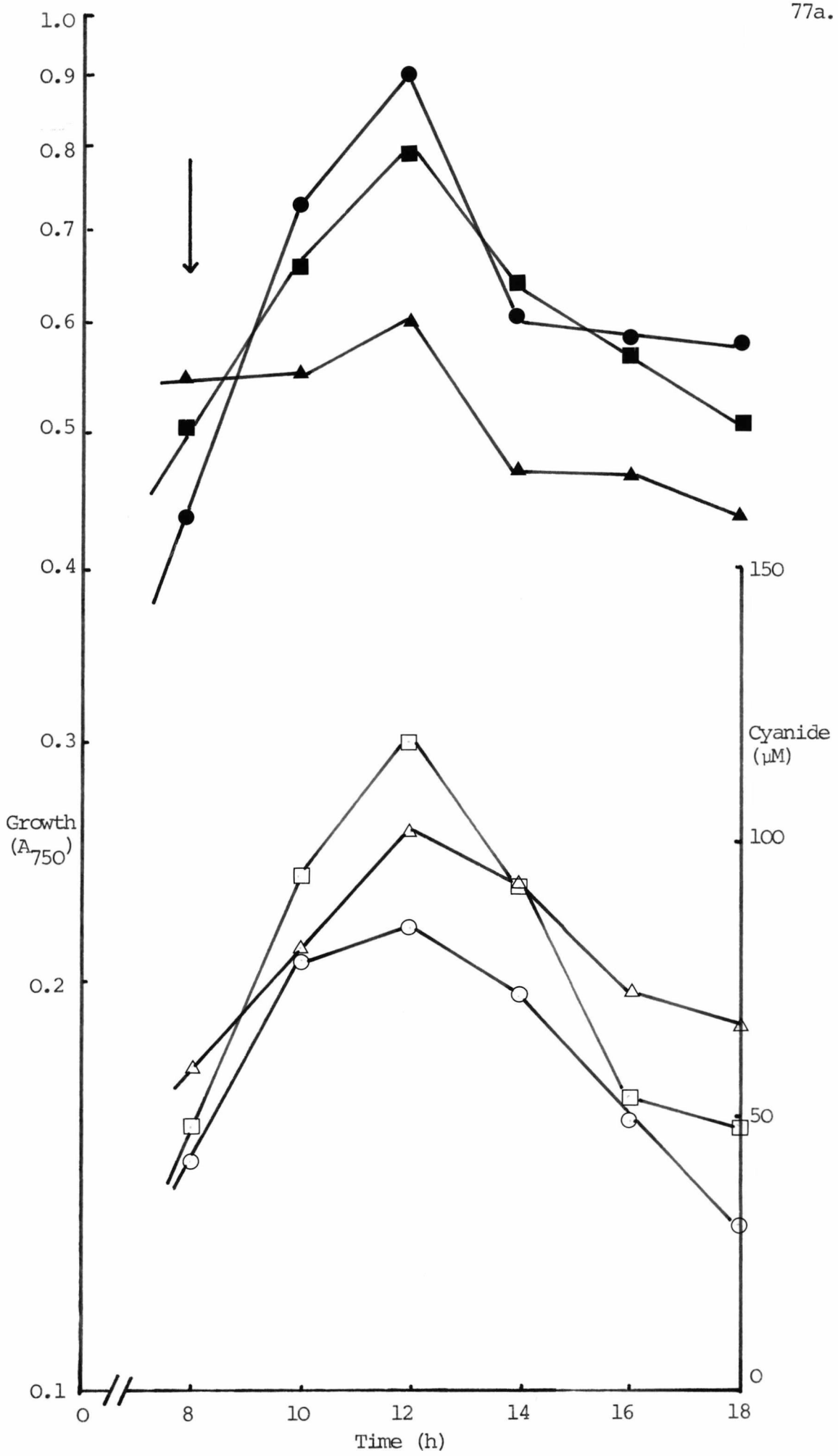

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Growth
(A_{750})
100
50
0
Cyanide
(μM)
0
8
10
12
14
16
Time (h)

FIGURE 26 - Effect of chloramphenicol (CAP) addition to late-exponential phase cultures growing on 10mM glutamate supplemented with 0.5 mM methionine. Growth (—●—, —■—, —▲—) and cyanide production (—○—, —□—, —△—) were followed in the absence (—●—, —○—) and presence of 12.5μg/ml CAP (—■—, —□—) and 50μg/ml CAP (—▲—, —△—). CAP was added at the time indicated by arrow.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Growth
(A_{750})
150
100
50
0
Cyanide
(μM)
0
8
10
12
14
16
18
Time (h)

phenicol used. After the stationary phase had been attained a considerable drop in the turbidity of the cultures was observed in the presence and absence of chloramphenicol. This was the only occasion on which a drop of this magnitude was seen; the causes of this phenomenon are not known.

(c) Chloramphenicol addition to stationary phase cultures: If chloramphenicol is added at a time later than 9.5 hours growth to cultures growing on glutamate alone (Fig.27) or on glutamate plus glycine and methionine (Fig.28) the medium cyanide content is not affected showing that chloramphenicol does not inhibit the activity of the cyanide-utilising enzymes.

(d) Chloramphenicol addition and induction of the cyanide-utilising enzymes: Addition of chloramphenicol in the late-exponential phase of growth (9.5 hours growth) to cultures growing on glutamate alone or on glutamate plus glycine and methionine prevented the induction of all three cyanide-utilising enzymes (Table 3). In the presence of chloramphenicol there was either no increase in the specific activity of the enzyme in the stationary phase of growth or this increase was a fraction of that observed in the absence of chloramphenicol.

Cyanide Assimilation.

In cultures of *C.violaceum* growing on glutamate plus glycine and methionine the medium cyanide concentration begins to fall from around the time of full induction of the cyanide-utilising enzymes. That the rate of cyanide disappearance from the medium is inhibited when induction of the cyanide-utilising enzymes is prevented suggests that the drop in cyanide concentration is due to cyanide assimilation or detoxication.

(a) Location of the products of cyanide fixation: By adding small concentrations of radiolabelled cyanide to cultures of *C.violaceum*, taking

FIGURE 27 - Effect of chloramphenicol (CAP) addition during stationary phase of growth of *C.violaceum* growing on 10mM glutamate. Growth (-●-,-■-) and cyanide evolution (-○-, -□-) were followed in the absence (-●-, -○-) and presence (-■-, -□-) of 25µg/ml CAP. CAP was added at time indicated by arrow.

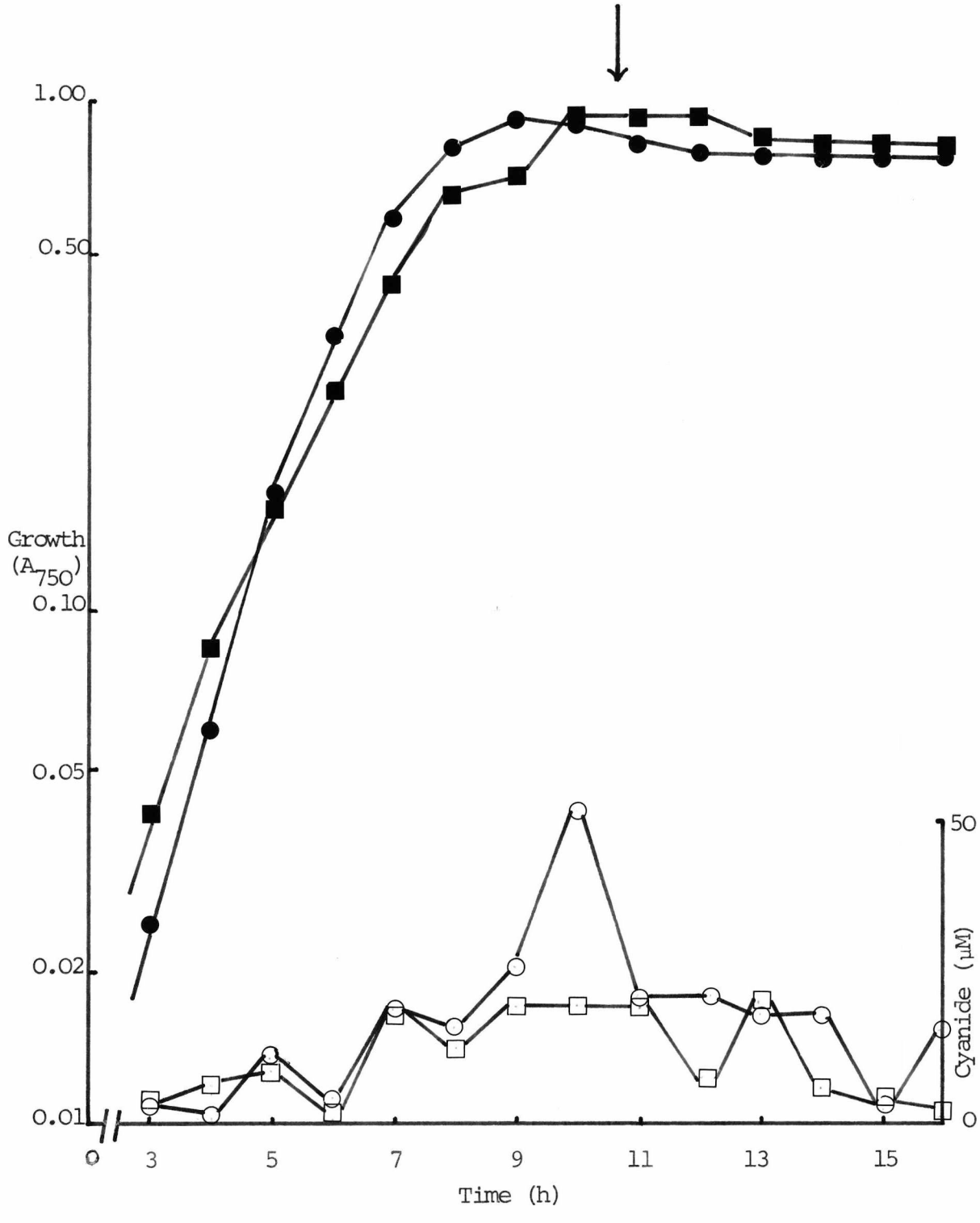


FIGURE 28 - Effect of chloramphenicol (CAP) addition during stationary phase of growth of *C.violaceum* growing on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine. Growth (—●— , —■—) and cyanide production (—○—, —□—) were followed in the absence (—●— , —○—) and presence (—■—, —□—) of 25µg/ml CAP. CAP was added at time indicated by arrow.

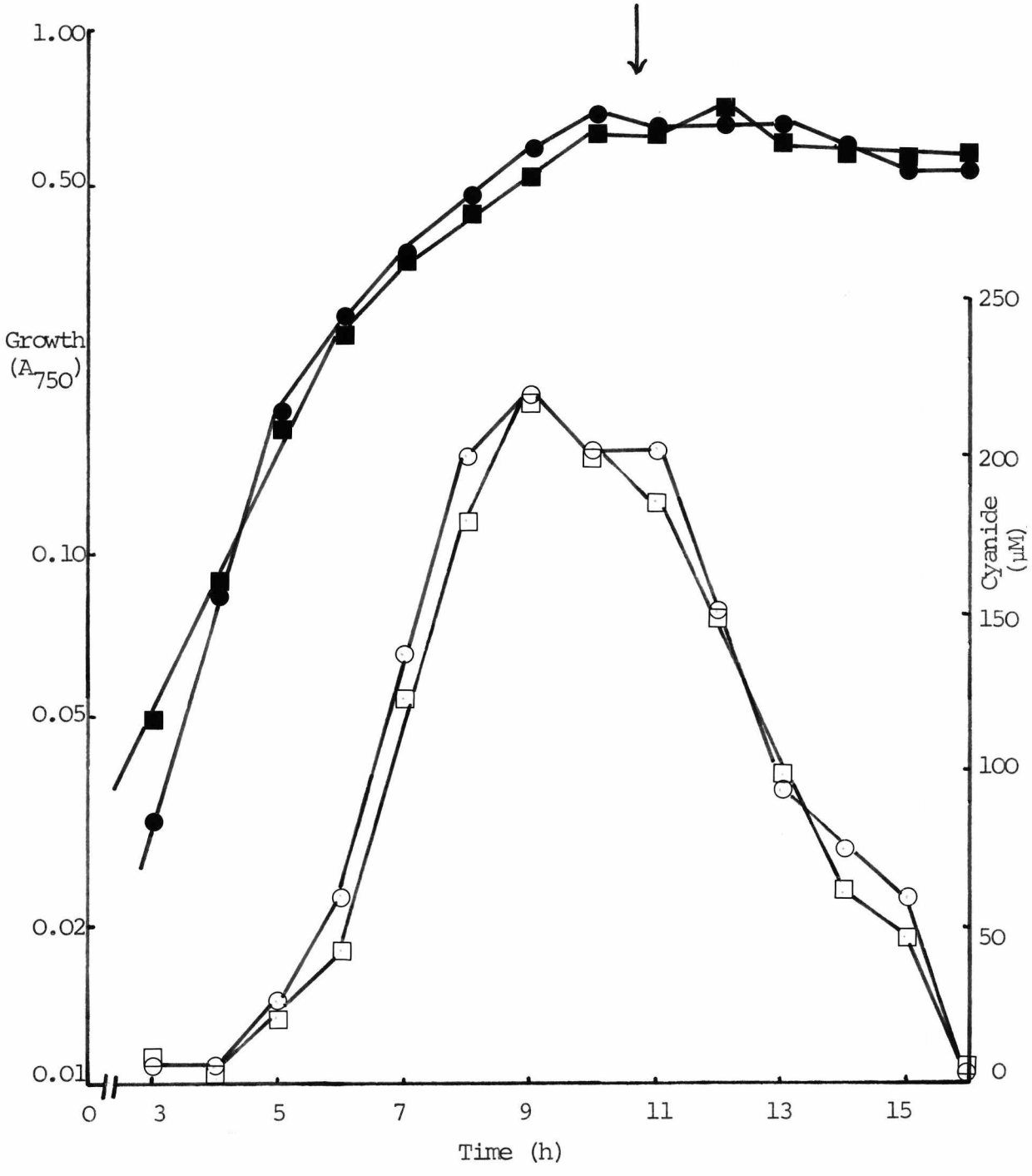


TABLE 3

Effect of chloramphenicol addition to late logarithmic phase cultures of *C.violaceum* on the induction of the cyanide-utilising enzymes.

	% increase in specific activity			
	Medium			
	10mM glutamate		10mM glutamate, 2mM glycine, 0.5mM methionine	
Chloramphenicol	-	+	-	+
β-cyanoalanine synthase	124	28	52	0
γ-cyano-α-amino-butyric acid synthase	116	0	58	0
rhodanese	120	15	340	71

Chloramphenicol was added to a final concentration of 50μg/ml after 9.5 hours growth. The percentage increase in specific activity is the change between activity measured at 8 hours and 12 hours (14 hours for γ-cyano-α-aminobutyric acid synthase) of growth.

samples at time intervals and separating the cells from the growth medium, the radioactivity incorporated into the cells and that remaining in the growth medium can be measured. As cyanide is a volatile molecule at neutral and acidic pH values it can be blown off from the growth medium by aeration and trapped in sodium hydroxide. By measuring the radioactivity in the alkali trap and that remaining in the growth medium, estimates can be obtained for the amount of unconverted cyanide and the amount of cyanide converted into non-volatile derivatives in the growth medium.

Fig.29 shows the change in the distribution of radioactivity in the various fractions of the culture with time, after addition of 4μCi of {^{14}C} KCN(4μmoles) to cells of *C.violaceum* grown on glutamate plus glycine and methionine. Radiolabelled cyanide was added at 12 hours growth, around the time at which the cyanide content of the culture reaches a maximum. The results are expressed as a percentage of the total radioactivity in a culture at a given time, thus taking into account the rate of loss of radioactive cyanide to the environment. The amount of labelled cyanide in the medium decreased over an eight hour period whilst the amount of radioactivty in non-volatile derivatives of cyanide in the medium increased with time, reaching a level of 85% of the total radioactivity in the culture. Approximately 40% of the added labelled cyanide was converted to derivatives within the time taken (about five minutes) to collect and separate the first sample. Although the amount of radioactivity in the cells increased slowly with time it did not rise above 2% of the total label in the culture.

Adding chloramphenicol to late-exponential phase cultures growing on glutamate plus glycine and methionine, two hours before addition of {^{14}C}KCN, had no effect on the rate of conversion of cyanide to non-volatile derivatives in the medium (Fig.30). The amount of radioactivity in derivatives of cyanide increased from an initial value of 10% to reach a plateau of approx-

KCN

FIGURE 29 - Addition of 4μCi {^{14}C}/to early stationary phase cultures of *C.violaceum* grown on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine. Growth (●) and the percentage of the total radioactivity in the culture present as cyanide in the medium (■), as non-volatile derivatives of cyanide in the medium (▲), and as unknown compounds in the bacterial cells (△). {^{14}C} KCN was added at the time indicated by arrow.

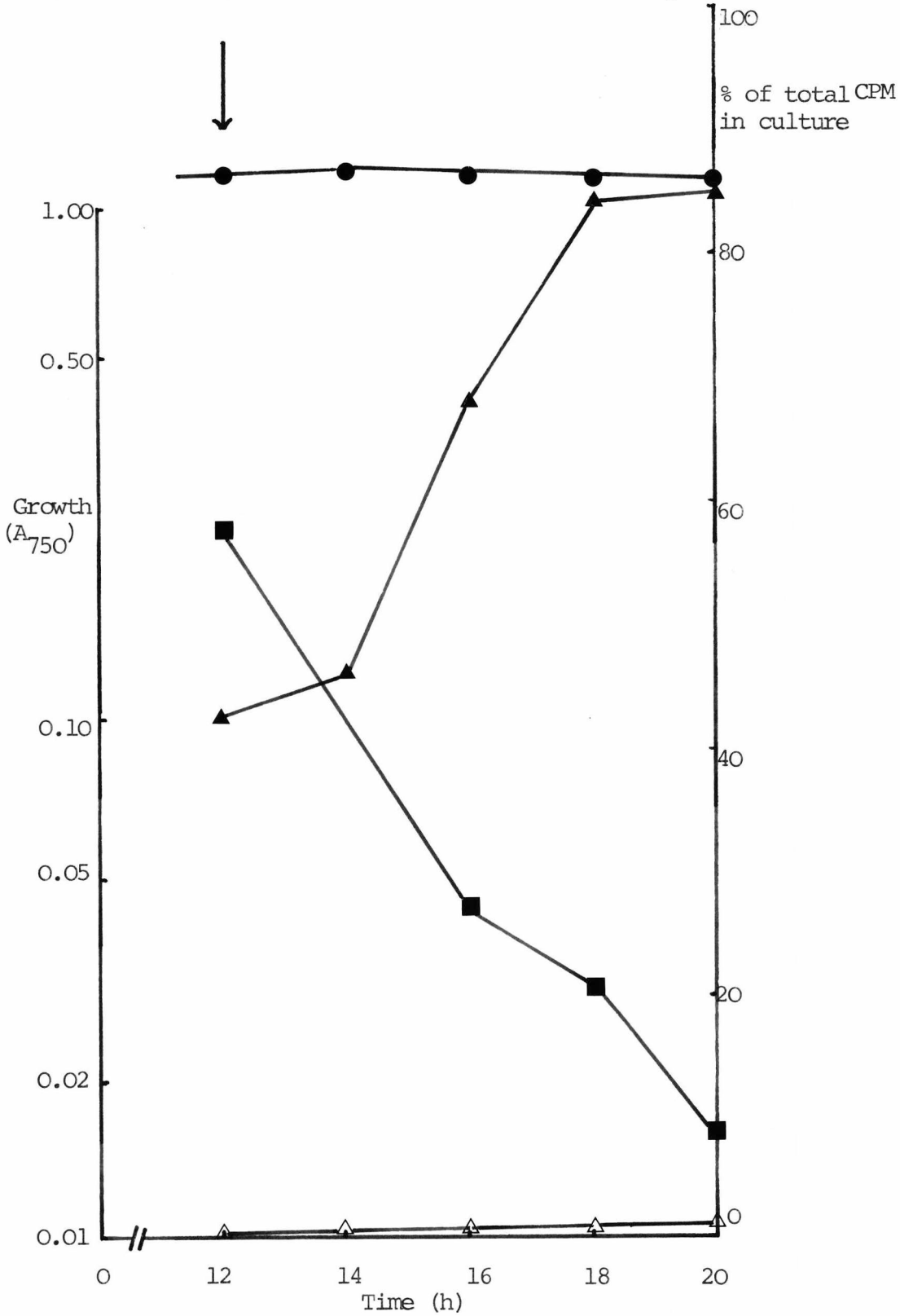
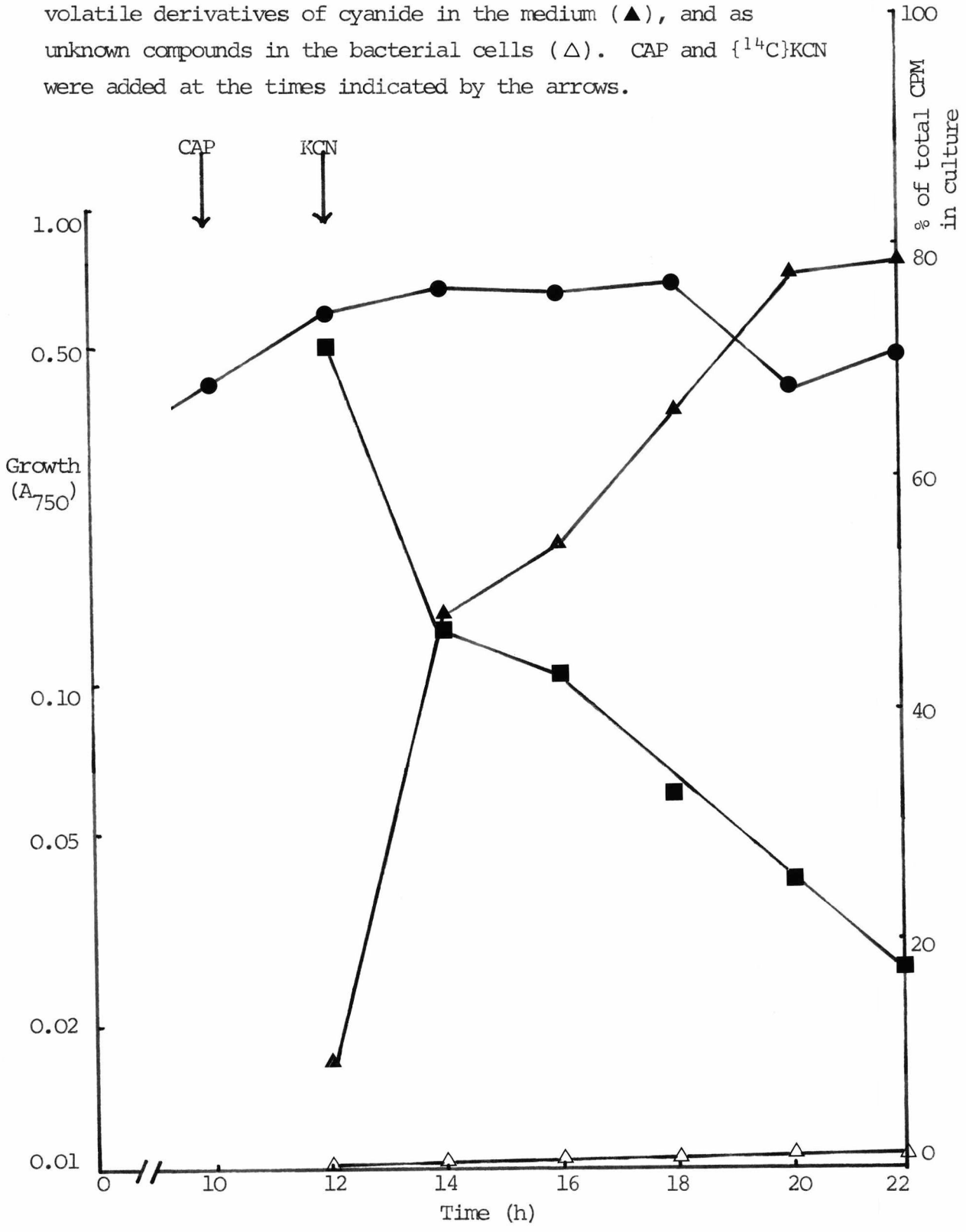


FIGURE 30 - Effect of addition of 50µg/ml chloramphenicol (CAP) upon the distribution of radioactivity from 4µCi {^{14}C}KCN added to early stationary phase cultures of *C.violaceum* grown on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine. Growth (●) and the percentage of the total radioactivity in the culture present as cyanide in the medium (■), as non-volatile derivatives of cyanide in the medium (▲), and as unknown compounds in the bacterial cells (△). CAP and {^{14}C}KCN were added at the times indicated by the arrows.



imately 80% eight hours after addition of {^{14}C}KCN. Variations in the initial value with experiment probably reflect differences in the speed of sampling. Again, the amount of radioactivity in cells did not rise above 2%

Although addition of {^{14}C}KCN to 12 hour cultures growing on glutamate alone presented a similar picture to that seen for cultures growing on glutamate plus glycine and methionine, the extent of conversion of cyanide to non-volatile derivatives was somewhat lower in the former growth medium (Fig.31). The amount of labelled cyanide decreased from an initial value of around 70% to one of under 40% over eight hours whilst the level of radioactivity in cyanide derivatives in the medium increased from 20% to 60% over the same period. The radioactivity in cells did not rise above 3% of the total. Again, the addition of chloramphenicol two hours before adding {^{14}C}KCN had no effect on the cyanide conversion rate (Fig.32). Over eight hours, the amount of radioactive cyanide dropped from around 65% to approximately 30% whilst the level of radioactive cyanide derivatives rose from 30% to 63%. Radioactivity in cells was a small fraction of the total,never rising above 3%.

Cyanide may have been converted to non-volatile derivatives through non-enzymatic reactions with the components of the growth medium. Fig.33 shows the results of a control experiment in which 4μCi{^{14}C}KCN were incubated in 250ml flasks containing growth medium, including glutamate, glycine and methionine plus 200μM unlabelled KCN, under the usual experimental conditions except for the absence of bacteria. Flasks containing 50μg/ml chloramphenicol in addition to the other components were also incubated. At no time was more than 10% of the radioactivity in a non-volatile form. In the technique used to blow-off cyanide up to 10% of the original cyanide concentration may remain after aeration (Fig.6). The amount of radioactivity

FIGURE 31 - Addition of 4μCi {^{14}C}KCN to early stationary phase cultures of *C.violaceum* grown on 10mM glutamate. Growth (●) and the percentage of the total radioactivity in the culture present as cyanide in the medium (■), as non-volatile derivatives of cyanide in the medium (▲), and as unknown compounds in the bacterial cells (△). {^{14}C}KCN was added at the time indicated by arrow.

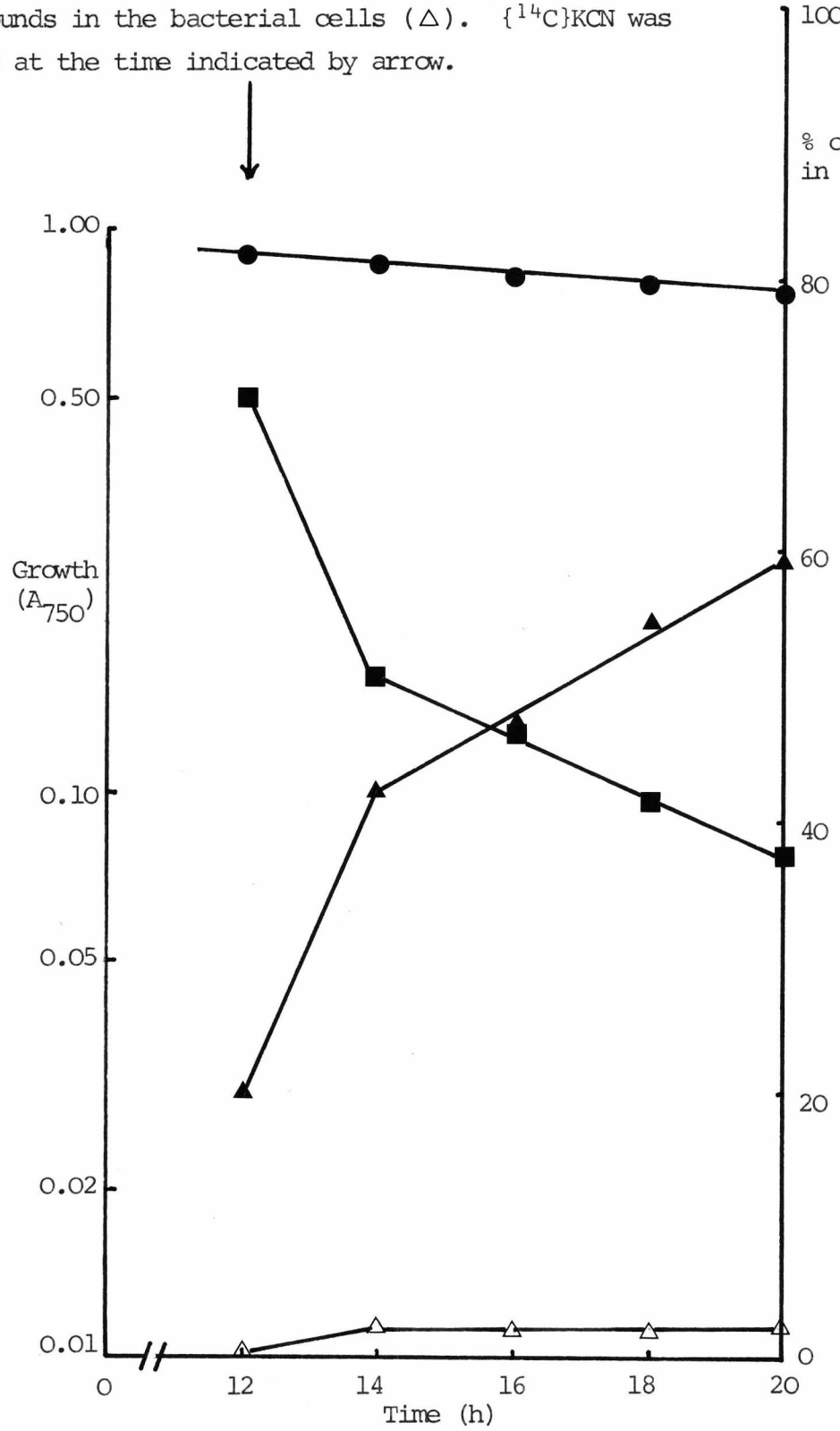


FIGURE 32 - Effect of addition of 50µg/ml chloramphenicol (CAP) upon the distribution of radioactivity from 4µCi{^{14}C}KCN added to early stationary phase cultures of *C.violaceum* grown on 10mM glutamate. Growth (●) and the percentage of the total radioactivity in the culture present as cyanide in the medium (■), as non-volatile derivatives of cyanide in the medium (▲), and as unknown compounds in the bacterial cells (△). CAP and {^{14}C} KCN were added at the times indicated by arrows.

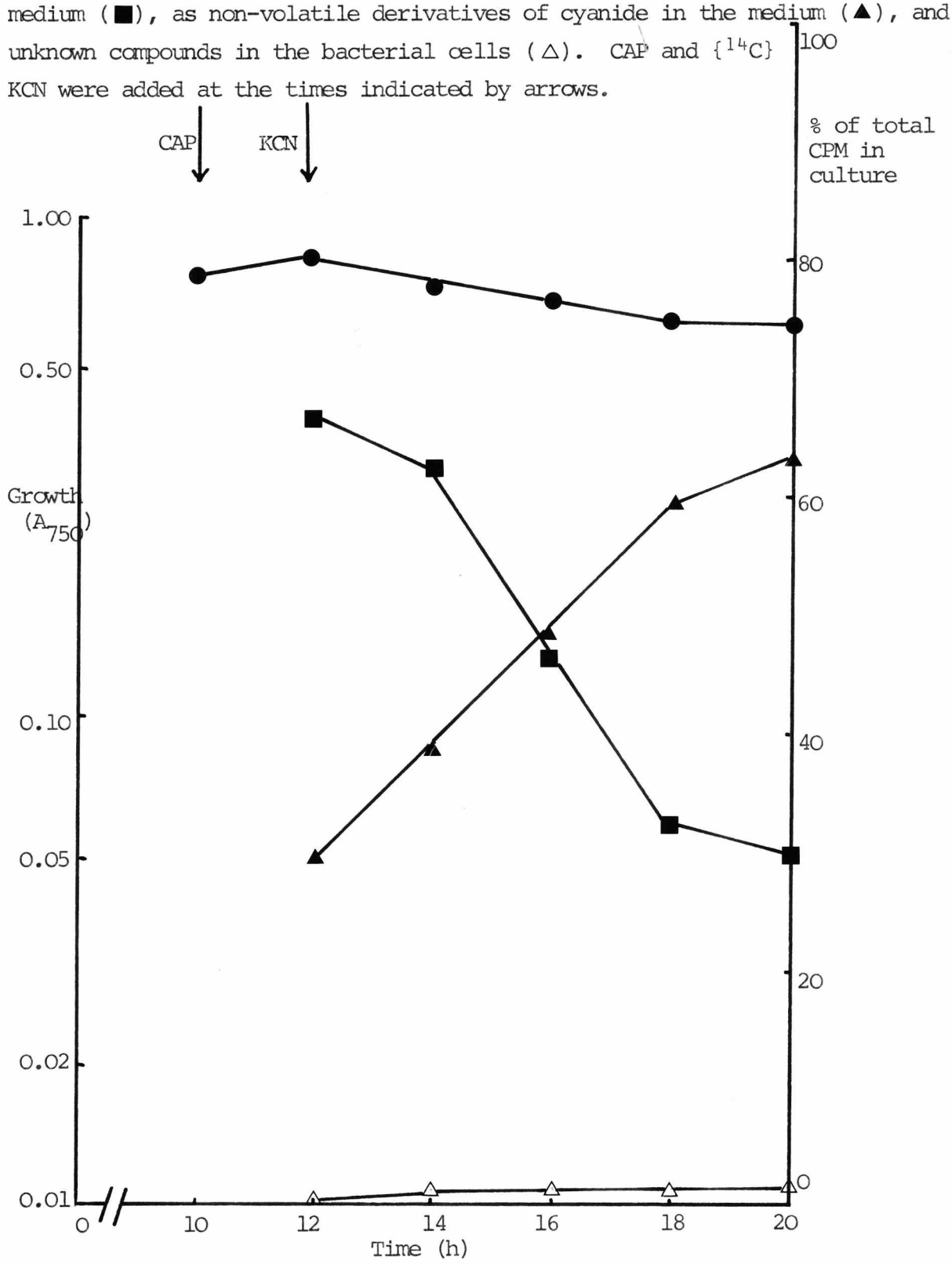
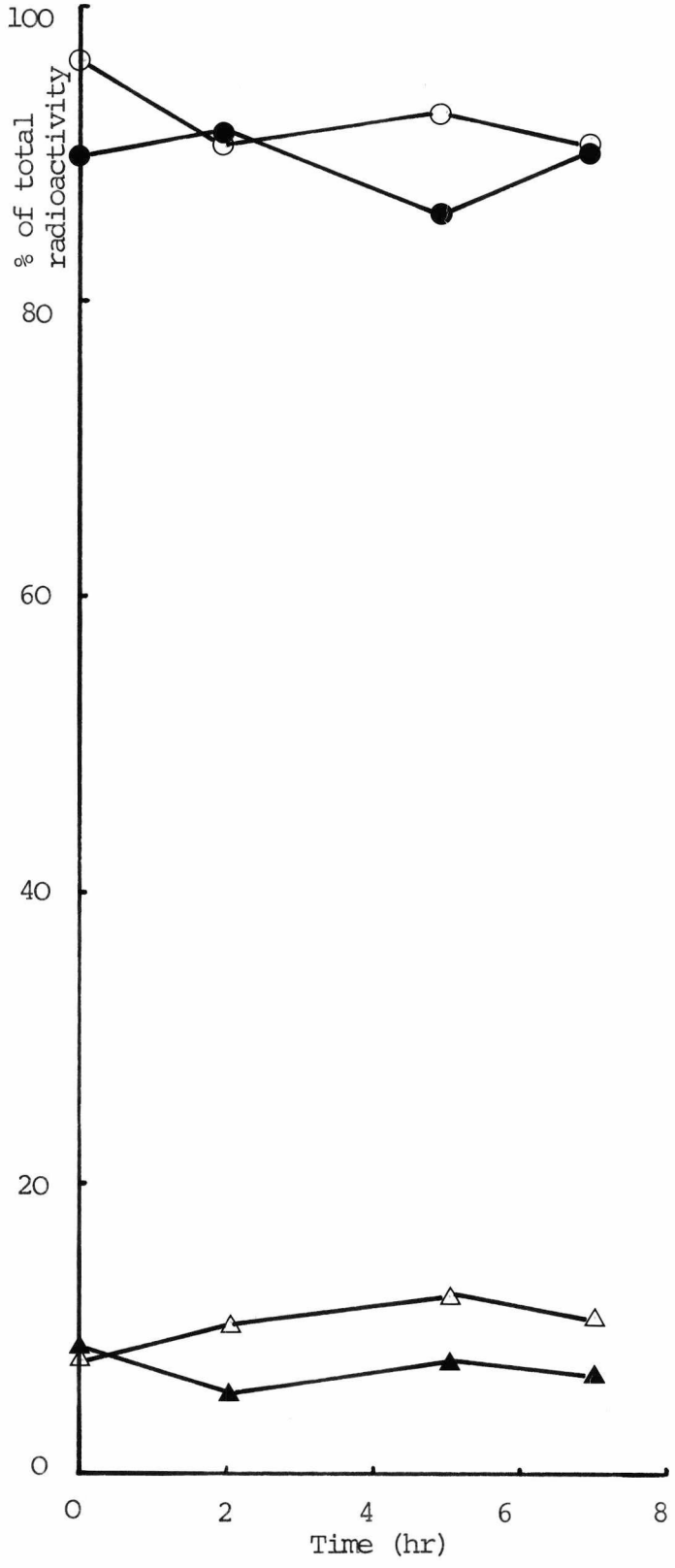


FIGURE 33 - Control experiment. Flasks containing M9 salts plus 10mM glutamate, 2mM glycine and 0.5mM glycine were incubated with 200μM KCN plus 4μCi {^{14}C} KCN. The amount of radioactivity present as free cyanide (—●—, —○—) and as 'bound' cyanide (—▲—, —△—) was followed in the absence (—●—, —▲—) and presence (—○—, —△—) of 50μg/ml chloramphenicol.



remaining after blowing off cyanide is within the experimental limits of removal and therefore non-enzymatic reactions of cyanide with components of the medium can be eliminated as factors in these experiments.

(b) Identification of the amino acids present in the growth medium: Brysk et al. (1969b) have suggested that the major pathway of cyanide assimilation in *C.violaceum* is formation of asparagine and aspartate via β-cyanoalanine, which accumulates in the growth medium. Addition of labelled cyanide to stationary phase cultures of *C.violaceum* results in the accummulation of radioactive derivatives of cyanide in the medium, suggesting that labelled compounds, possibly aminoacids, are being formed from the added cyanide and accumulating in the growth medium.

Thin layer chromatography was used to analyse the aminoacid composition of the growth medium of a *C.violaceum* culture grown to the stationary phase (14 hours) on glutamate plus glycine and methionine. On developing the chromatogram for amino acids, three spots could be seen in the medium sample (Table 4). Two spots corresponded to the glycine and methionine standards whilst the third, although it was the same unusual blue-green colour, had a lower R_f value than the β-cyanoalanine standard. The experiment was repeated using another stationary phase culture (12 hours) grown on glutamate plus glycine and methionine (Table 5). This time a mixture of growth medium and β-cyanoalanine standard was applied. The β-cyanoalanine spot was retarded compared to a standard run in the absence of growth medium, confirming that the blue-green spot observed in the previous experiment was β-cyanoalanine. Again glycine and methionine were detected in the growth medium.

C.violaceum was grown to stationary phase (15 hours) on four media containing glutamate, either alone or in combination with glycine and/or methionine, and samples of the growth medium chromatographed (Table 6). On

TABLE 4

Cellulose thin layer chromatography of the medium of *C.violaceum* grown to the stationary phase (14h) on 10mM glutamate plus 2mM glycine and 0.5mM methionine.

Sample	Colour	R_f value
Growth medium - Spot 1	Purple	0.35
Spot 2	Faint blue-green	0.45
Spot 3	Purple	0.62
Aspartate	Purple	0.67
Asparagine	Blue-grey	0.20
Glycine	Purple	0.36
Glutamate	Purple	0.73
β-Cyanoalanine	Blue-green	0.52
Methionine	Purple	0.62
Alanine	Purple	0.56

10µl sample of growth medium and 5µl samples of amino acid standards were applied. All standard solutions were 1mM except alanine (5mM). The spots were developed by spraying with 0.25% w/v ninhydrin in acetone and heating at 110°C for 20 minutes.

TABLE 5

Cellulose thin layer chromatography of the medium of *C.violaceum* grown to the stationary phase (12h) on 10mM glutamate plus 2mM glycine and 0.5mM methionine.

Sample	Colour	R_f value
Growth medium plus		
β-cyanoalanine - Spot 1	Purple	0.36
Spot 2	Blue-green	0.46
Spot 3	Purple	0.64
Tryptophan	Purple	0.40
Glutamate	Purple	0.73
Methionine	Purple	0.62
Glycine	Purple	0.37
β-cyanoalanine	Blue-green	0.52
Alanine	Purple	0.57

5µl sample of growth medium (plus 5µl of β-cyanoalanine) and 5µl samples of amino acid standards were applied. All standard solutions were 1mM except alanine (5mM). The spots were developed by spraying with 0.25% w/v ninhydrin in acetone and heating at 110°C for 20 minutes.

TABLE 6

Cellulose thin layer chromatography of the medium of *C.violaceum* grown to the stationary phase (15h). Four growth media were used; 10mM glutamate alone (G); 10mM glutamate plus 2mM glycine (GG); 10mM glutamate plus 0.5mM methionine (GM); 10mM glutamate plus 2mM glycine and 0.5mM methionine (GGM).

Sample	Colour	R_f value
Growth medium G - Spot 1	Purple	0.35
Spot 2	Faint Purple	0.63
Growth medium GG - Spot 1	Purple	0.34
Spot 2	Blue-green	0.46
Spot 3	Faint Purple	0.62
Growth medium GM - Spot 1	Faint Purple	0.35
Spot 2	Purple	0.62
Growth medium GGM - Spot 1	Purple	0.35
Spot 2	Blue-green	0.46
Spot 3	Purple	0.63
Glutamate	Purple	0.76
Glycine	Purple	0.37
Methionine	Purple	0.63
β-Cyanoalanine	Blue-green	0.53
Aspartate	Purple	0.69
Asparagine	Blue-grey	0.19

10µl samples of growth medium and 5µl samples of amino acid standards were applied. All standard solutions were 1mM. The spots were developed by spraying with 0.25% w/v ninhydrin in acetone and heating at 110°C for 20 minutes.

development of the plate, all media contained spots corresponding to glycine and methionine although these spots were larger in those media to which the amino acids had been added. Only the cultures grown on glutamate supplemented with glycine alone or with glycine and methionione, contained β-cyanoalanine.

(c) Incorporation of label from {^{14}C}KCN into β-cyanoalanine: The labelling of compounds in the growth medium of *C.violaceum* after addition of {^{14}C}KCN was studied. At time intervals after addition of {^{14}C}KCN, dinitrophenyl derivatives of the growth medium amino acids were prepared and extracted into organic solvent. These derivatives were separated by thin layer chromatography and the amount of radioactivity in each compound measured. Of the amino acids in the growth medium significant labelling appeared only in β-cyanoalanine.

Fig.34 shows the flow of label in β-cyanoalanine after addition of {^{14}C}KCN to late exponential phase cultures of *C.violaceum* growing on glutamate plus glycine and methionine. Over eight hours the fraction of radioactivity in the medium present as cyanide dropped from an initial value of around 60% to one of approximately 20%. This is in good agreement with earlier results (see Fig.29). The amount of label present in β-cyanoalanine rose from 5% to 32%, at six hours, and then began to drop, presumably due to further metabolism although no labelling of asparagine or aspartate could be detected in the medium. These observations compare well with those made by Brysk et al. (1969) using resuspended cells. Another compound, which could not be identified, was found to contain radioactivity. The label in this compound dropped from an initial value of 8% to 1% over eight hours. The unknown compound had a R_f value of 0.86 on thin layer chromatography and therefore is not an amino acid. It could not be detected visually but registered as a peak of radioactivity.

FIGURE 34 - Distribution of label from 8μCi {^{14}C}KCN added to late exponential phase (10h) cultures of *C.violaceum* growing on 10mM glutamate plus 2mM glycine and 0.5mM methionine. Growth (●) and percentage of total radioactivity in cyanide (■). β-cyanoalanine (▲) and an unidentified compound (□) were followed. {^{14}C}KCN was added at time indicated by arrow.

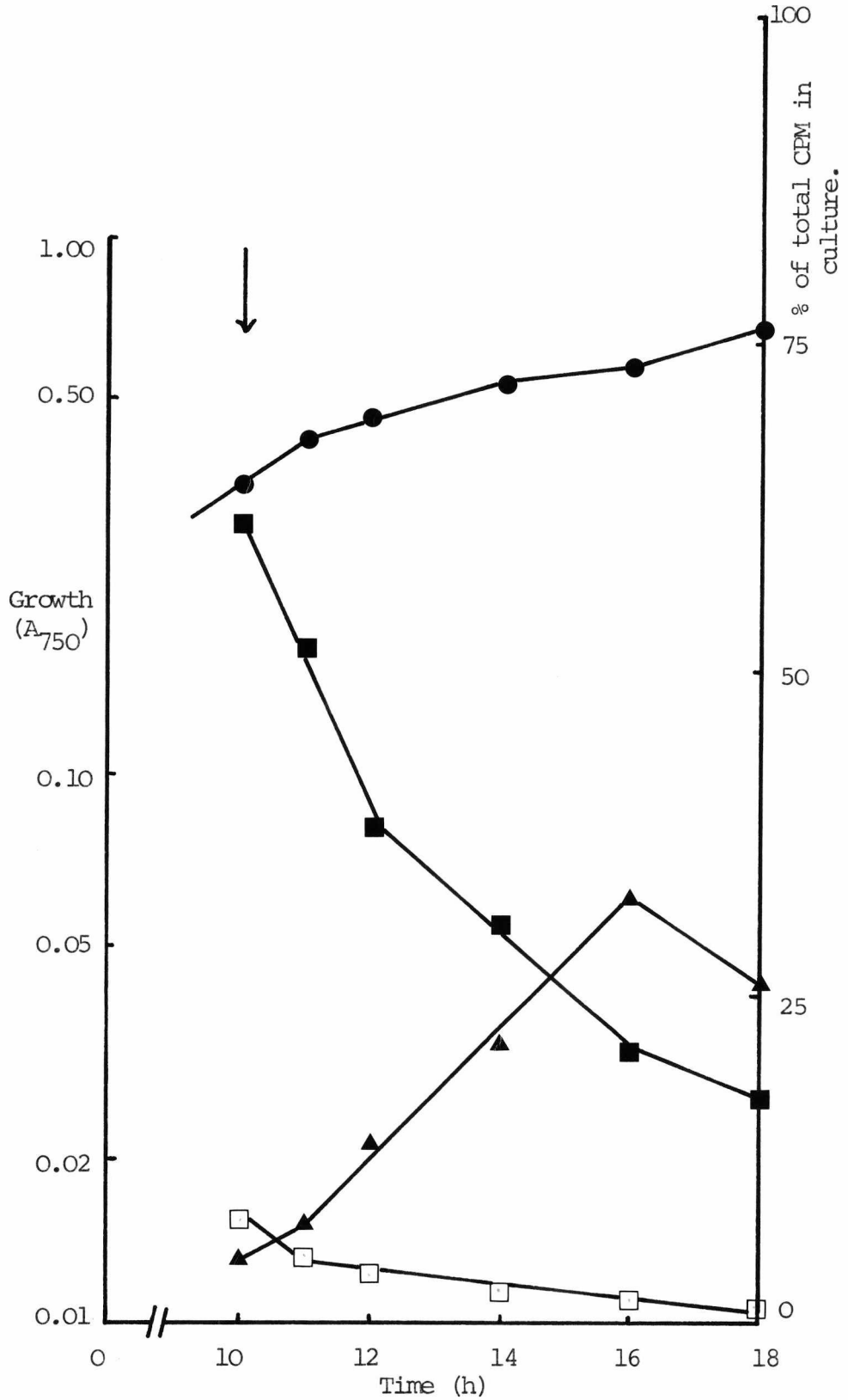


TABLE 7

Addition of {^{14}C}KCN to late exponential phase (10h) cultures of *C.violaceum* growing on 10mM glutamate plus 2mM glycine and 0.5mM methionine. Distribution of radioactivity in organic and aqueous phases of dinitrophenyl-amino acid preparation and final recovery of added radioactivity

Time (HR)	Percentate of Radioactivity in		Total Percentage Recovery
	Organic Phase	Aqueous Phase	
10	65	35	40
11	61	39	73
12	67	33	57
14	78	22	74
16	71	29	79
18	57	43	80

As part of the preparation of dinitrophenyl derivatives of the growth medium amino acids, the dinitrophenyl amino acids were extracted into ether, thus splitting the medium into organic-soluble and aqueous-soluble fractions. Table 7 shows the distribution of radioactivity between the organic and aqueous phases, and the recovery of radioactivity at the end of the separation procedure, in the above experiment. The distribution of radioactivity remained fairly constant throughout with 60-70% of the activity present in the organic phase. The recovery of radioactivity tended to vary from samples in this and subsequent experiments. Virtually all the losses in radioactivity were sustained when applying the samples of organic phase to the chromatograms. The amount lost was estimated in each case by applying a second sample, scraping the spot off immediately and counting the radioactivity. The quantities lost were allowed for when calculating the radioactivity in β-cyanoalanine and the unknown compound.

Adding chloramphenicol to late exponential phase cells growing on glutamate plus glycine and methionine, one hour before addition of {^{14}C}KCN, inhibited the accumulation of radioactivity in β-cyanoalanine (Fig.35). Less than 15% of the total radioactivity was incorporated into β-cyanoalanine compared to more than 30% in the absence of chloramphenicol. Although the initial fraction of radioactivity present as cyanide was less in the presence than in the absence of chloramphenicol, the final values were similar in both cases, agreeing with the earlier experiments (Figs.29,30). Chloramphenicol addition had little effect on the percentage of radioactivity incorporated into the unknown compound. The distribution of radioactivity between the organic and aqueous phases of the preparation was affected by the addition of chloramphenicol; 60%-70% of the radioactivity was in the aqueous phase compared to 30-40% in the absence of chloramphenicol (Table 8).

Addition of {^{14}C}KCN to late exponential phase cultures growing on

FIGURE 35 - Effect of chloramphenicol (CAP) addition to late-exponential phase cultures of *C.violaceum* growing on 10mM glutamate supplemented with 2mM glycine and 0.5mM methionine, one hour before addition of {^{14}C}KCN, on distribution of label. Growth (●) and percentage of radioactivity in cyanide (■), β-cyanoalanine (▲) and an unidentified compound (□) were followed. 50μg/ml CAP and 8μCi{^{14}C}KCN were added at times indicated by arrows.

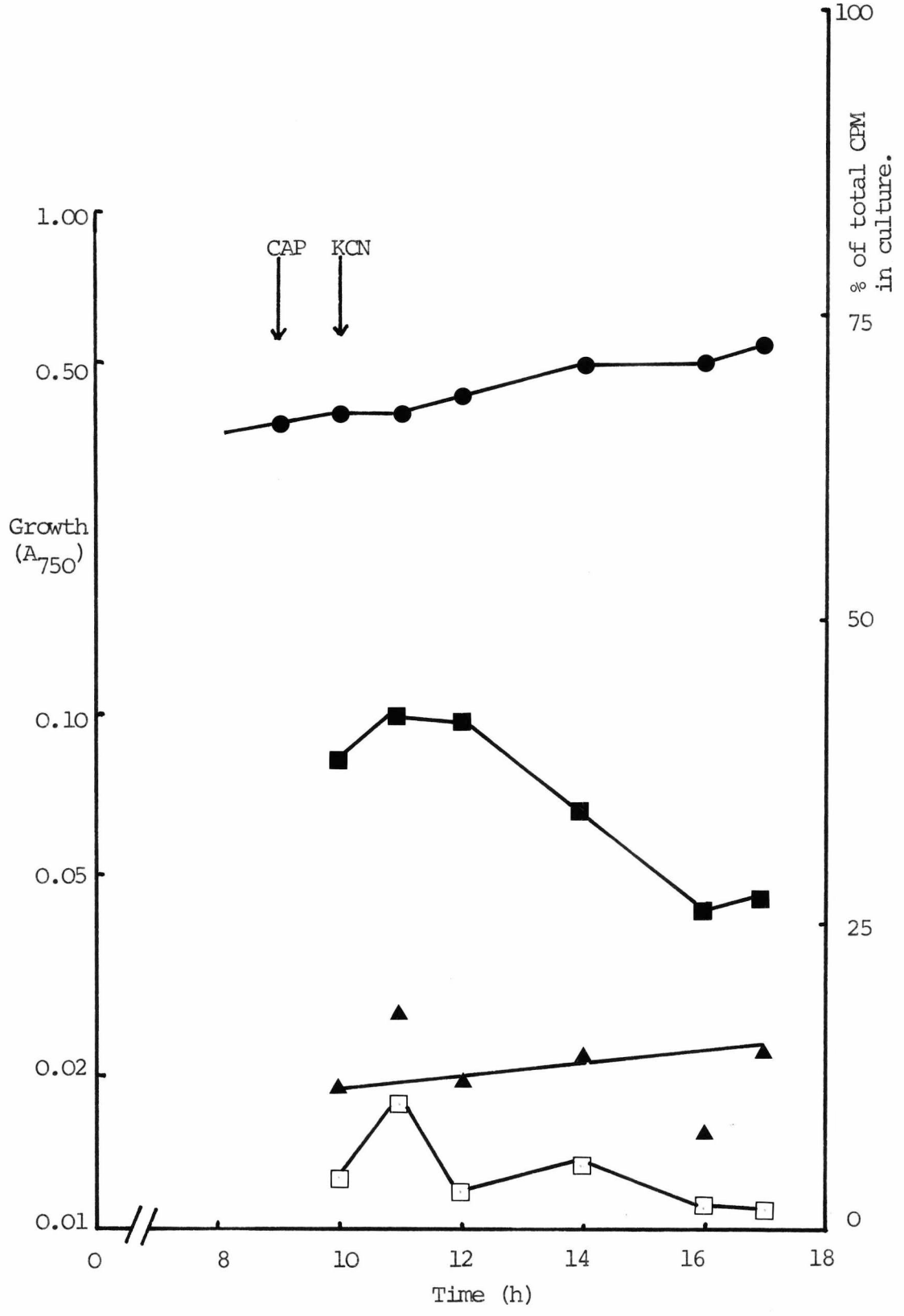


TABLE 8

Addition of 50μg/ml chloramphenicol (9h) and {^{14}C}KCN (10h) to late exponential phase cultures of *C.violaceum* grown on 10mM glutamate plus 2mM glycine and 0.5mM methionine. Distribution of radioactivity in organic and aqueous phases of dinitrophenylamino acid preparation and final recovery of added radioactivity.

Time (hr)	Percentage of Radioactivity in		Total Percentage Recovery
	Organic Phase	Aqueous Phase	
10	31	69	42
11	35	65	104
12	48	52	104
14	48	52	134
16	25	75	124
17	36	64	78

glutamate resulted in approximately 15% of the cyanide being converted to β-cyanoalanine after two hours incubation, falling to 4% over the following six hours (Fig.36). The radioactivity present as cyanide declined in a similar fashion to cultures grown on glutamate, glycine and methionine (cf. Fig.34). No radioactivity could be detected in the unknown compound. The distribution of radioactivity between the organic and aqueous phases altered during the experiment (Table 9). For two hours approximately 60% of the radioactivity was present in the organic phase but this decreased to 20% on further incubation. This is in contrast to cultures grown on glutamate plus glycine and methionione, in which the distribution remained constant (Table 7).

In glutamate cultures, to which chloramphenicol was added one hour before {^{14}C}KCN addition, the incorporation of radioactivity into β-cyanoalanine was inhibited (Fig.37). Only 7% of the total radioactivity accumulated in β-cyanoalanine compared to 15% in the absence of chloramphenicol, and a decrease in the value was not observed. Although the initial fraction of radioactivity present as cyanide was low compared with cultures to which chloramphenicol had not been added, the final value was similar in both cultures. The distribution of radioactivity between the organic and aqueous phases was altered by chloramphenicol addition (Table 10). Initially 18% of the radioactivity was present in the organic phase in the presence of chloramphenicol, rising to 30% over five hours, compared to approximately 60% decreasing to 20% in the absence of chloramphenicol.

The labelled compounds present in the aqueous phase of the dinitrophenylamino acid preparation have not been identified. One possibility is thiocyanate which is a product of both the rhodanese and γ-cyano-α-aminobutyric acid synthase reactions. Thiocyanate production was assayed for in the medium of *C.violaceum*, grown on glutamate alone or in combination with glycine and/or methionine, by two methods with sensitivities of around

FIGURE 36 - Distribution of label from 8μCi{^{14}C}KCN added to late exponential phase (9.5h) cultures of *C.violaceum* growing on 10mM glutamate. Growth (●) and percentage of total radioactivity in cyanide (■) and β-cyanoalanine (▲) were followed. {^{14}C}KCN was added at time indicated by arrow.

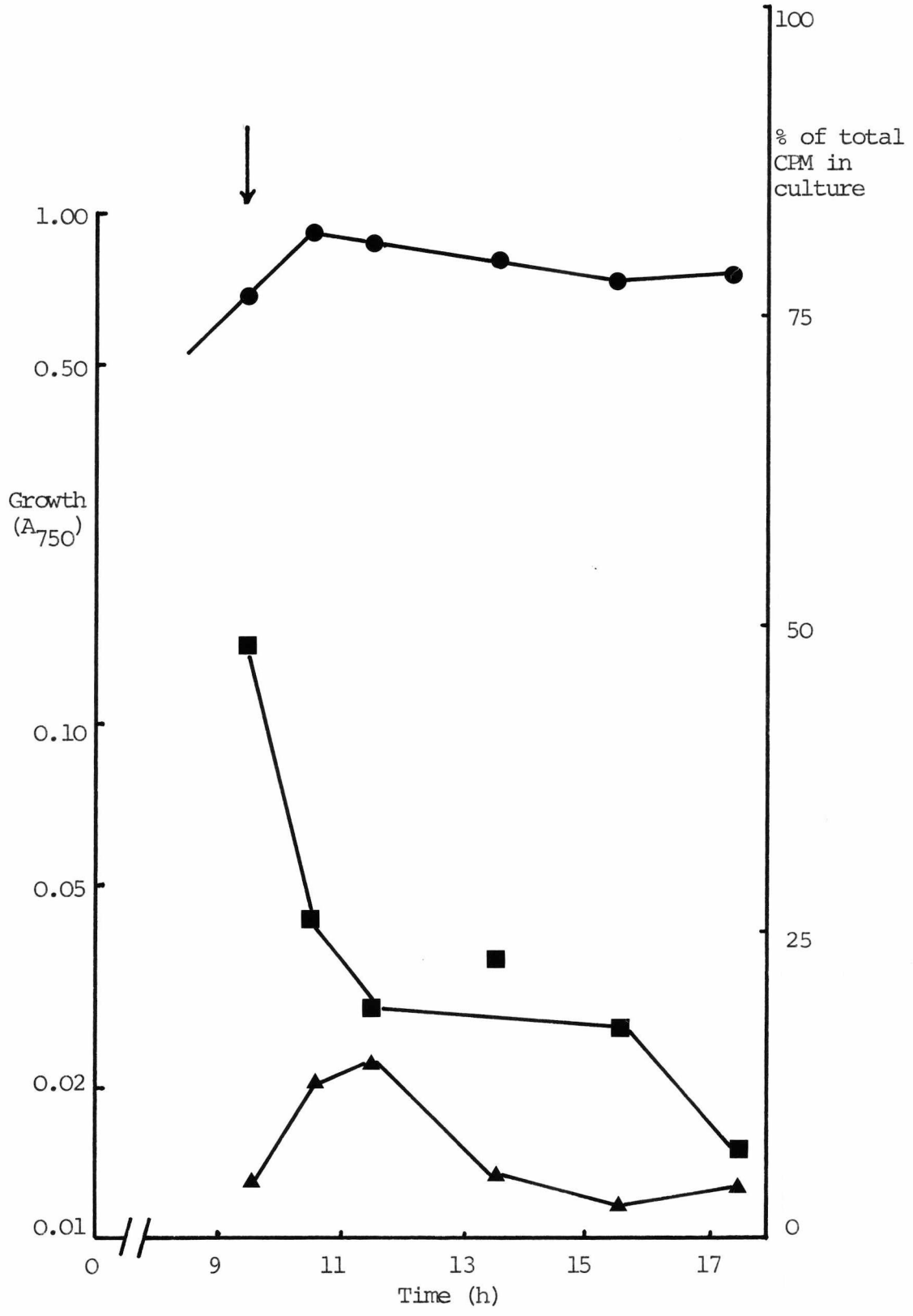


TABLE 9

Addition of {^{14}C}KCN to late exponential phase (9.5h) cultures of *C.violaceum* growing on 10mM glutamate. Distribution of radioactivity in organic and aqueous phases of dinitrophenyl-amino acid preparation and final recovery of added radioactivity.

Time (hr)	Percentage of Radioactivity in		Total Percentage recovery
	Organic Phase	Aqueous Phase	
9.5	46	54	97
10.5	59	41	67
11.5	61	39	56
13.5	28	72	256
15.5	20	80	123
17.5	22	78	112

FIGURE 37 - Effect of chloramphenicol (CAP) addition to late exponential phase cultures of *C.violaceum* growing on 10mM glutamate, one hour before addition of {^{14}C}KCN, on distribution of label. Growth (●) and percentage of total radioactivity in cyanide (■) and β-cyanoalanine (▲) were followed. 50μg/ml CAP and 8μCi{^{14}C}KCN were added at times indicated by arrows.

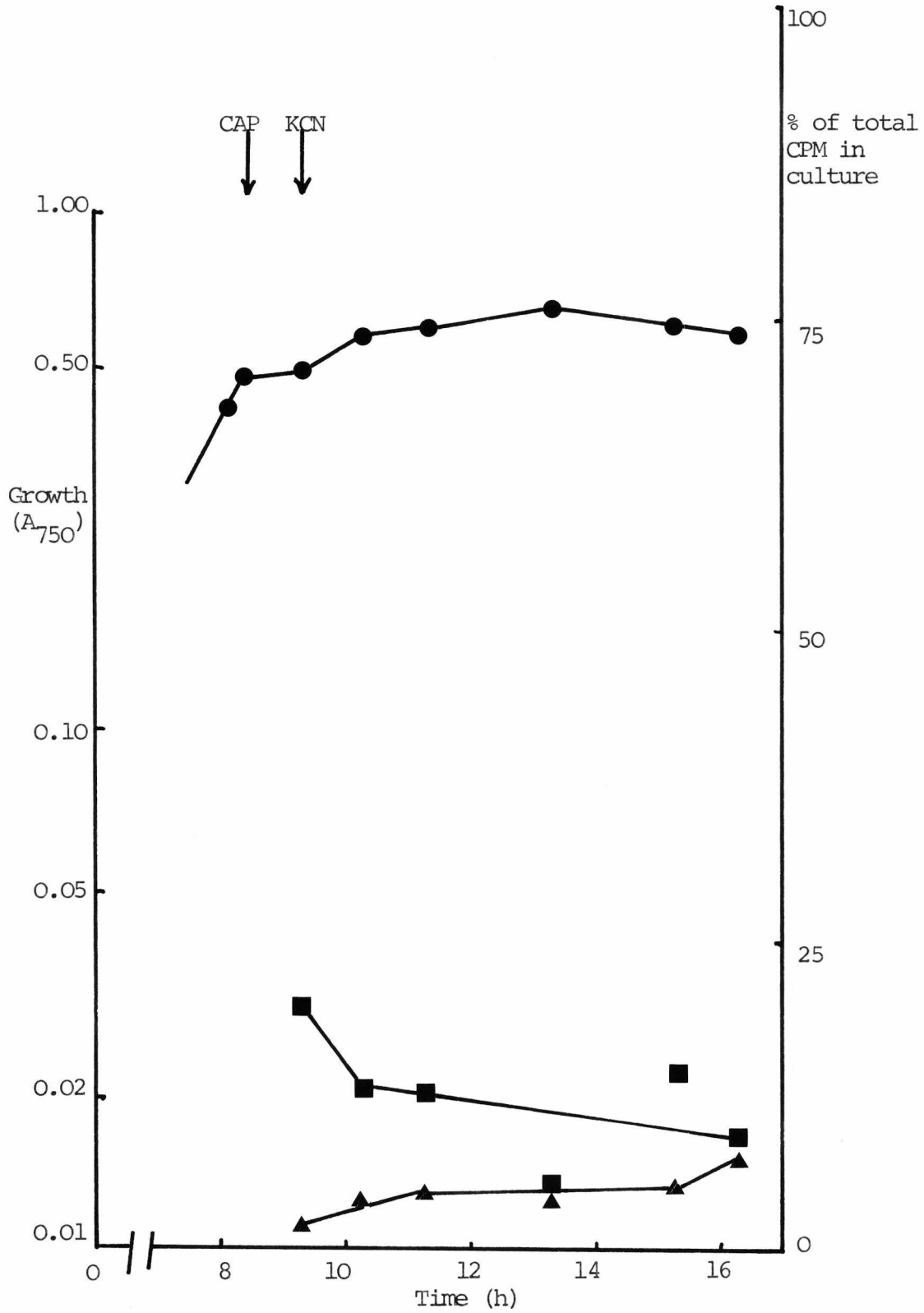


TABLE 10

Addition of 50µg/ml chloramphenicol (8.25h) and {^{14}C}KCN (9.25h) to late exponential phase cultures of *C.violaceum* growing on 10mM glutamate. Distribution of radioactivity in organic and aqueous phases of dinitrophenyl amino acid preparation and final recovery of added radioactivity

Time (hr)	Percentage of Radioactivity in		Total Percentage Recovery
	Organic Phase	Aqueous Phase	
9.25	18	82	30
10.25	22	78	81
11.25	23	77	81
13.25	26	74	75
15.25	25	75	65
16.25	30	70	71

1nmole of thiocyanate. Thiocyanate could not be detected in any of the growth media with up to 21 hours growth.

Cyanohydrins are other compounds which may become labelled after addition of {^{14}C}KCN to *C.violaceum* cultures. Glyoxylic acid cyanohydrin was included in the dinitrophenylamino acid preparation and found to be distributed between both the organic and aqueous phases. However, under the conditions used for thin layer chromatography of dinitrophenylamino-acids, glyoxylic acid cyanohydrin remained at the origin. As there was no accumulation of radioactivity at the origin of chromatograms of growth medium derivatives this compound can be eliminated as a labelled component of the aqueous phase. Glyoxylic acid cyanohydrin can also be eliminated as a possibility for the unknown labelled compound observed in the medium of cultures grown on glutamate plus glycine and methionine because of the differences in R_f values.

Cyanohydrins.

Although two cyanohydrins, derivatives of glyoxylic acid and pyruvic acid, have been detected in a snow mold basidiomycete (Tapper and MacDonald, 1974) none have been found in bacteria. An attempt to detect cyanohydrins in *C.violaceum* cultures is outlined below.

Cyanogenic fractions were isolated from a stationary phase culture, grown on glutamate plus glycine and methionine, by ion exchange chromatography on a cation exchange resin in the H^+ form followed by an anion exchange resin in the acetate form, eluted with dilute hydrochloric acid. Cyanohydrins decompose into cyanide and the parent carbonyl compound at alkali pH values. By assaying the fractions coming off the ion exchange columns for cyanide at acid and alkali pH it was possible to determine those fractions containing cyanohydrins. Fig.38 shows the elution profile for a culture extract applied to a cation exchange column. Two peaks of cyanide-reacting material were observed. Fractions 3-9 were combined and applied to an anion exchange column from which they were eluted with dilute hydrochloric acid (Fig.39). Again, two peaks of cyanide reacting material were observed. The recovery of cyanide reacting material from the anion exchange column was 34%.

Cellulose thin layer chromatography of fraction 10 eluted from the anion exchange column resolved two components, both of which overlapped the glyoxylic acid cyanohydrin standard (R_f value 0.26) (Fig.40). Nothing in the fraction ran with the pyruvic acid cyanohydrin standard (R_f value 0.55).

No cyanogenic fractions could be detected in extracts of a stationary phase culture grown on glutamate alone.

Ward (1964) studied the properties of a cyanogenic fraction extracted from a snow-mold basidiomycete which was subsequently identified as glyoxylic

acid cyanohydrin and pyruvic acid cyanohydrin (Tapper and MacDonald, 1974). Breakdown of the cyanohydrins is appreciable at 40°C and becomes rapid above 60°C. The procedure used for extraction of cyanogenic compounds from *C.violaceum* included a step that involved heating the extract to temperatures of at least 60°C. Thin layer chromatography of untreated growth medium from a culture grown to stationary phase on glutamate plus glycine and methionine was employed as an alternative to the extraction procedure. A faint streak in the region of the glyoxylic acid cyanohydrin standard could be seen (Fig.40). The method for development of the plate includes spraying a cyanide-detecting reagent dissolved in alkali, consequently the compound observed in the medium may be a cyanohydrin or the spot is possibly due to cyanide present in the medium.

No cyanogenic compounds could be detected in the medium of cultures grown on glutamate alone.

FIGURE 38 - Extraction of cyanohydrins from a stationary phase culture of *C.violaceum* grown on 10mM glutamate plus 2mM glycine and 0.5mM methionine. Elution of cyanide-reacting material from a Dowex 50W-X8 cation exchange column in the H^+ form.

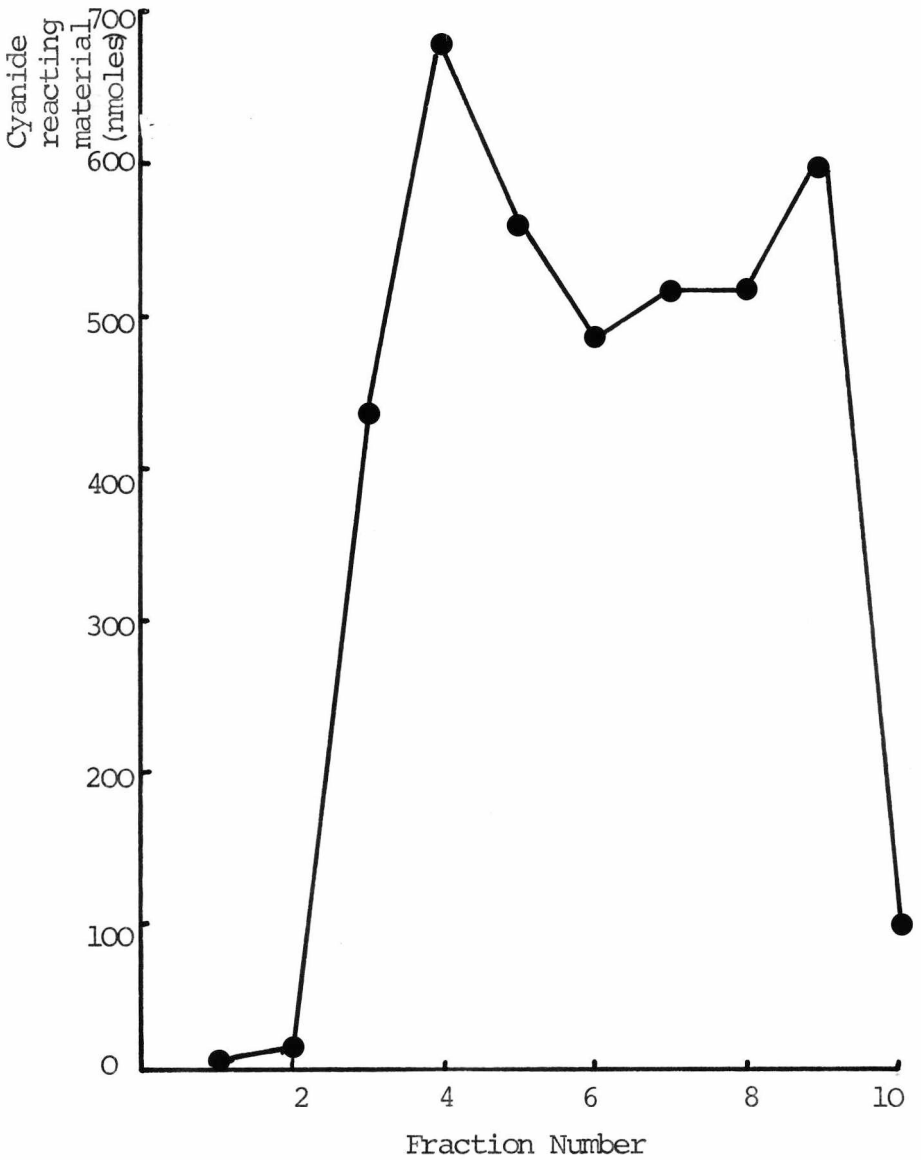


Figure 39 - Extraction of cyanohydrins from a stationary phase culture of *C.violaceum* grown on 10mM glutamate plus 2mM glycine and 0.5mM methionine. Elution of cyanide reacting material from a Dowex 2-X8 anion exchange column in the acetate form.

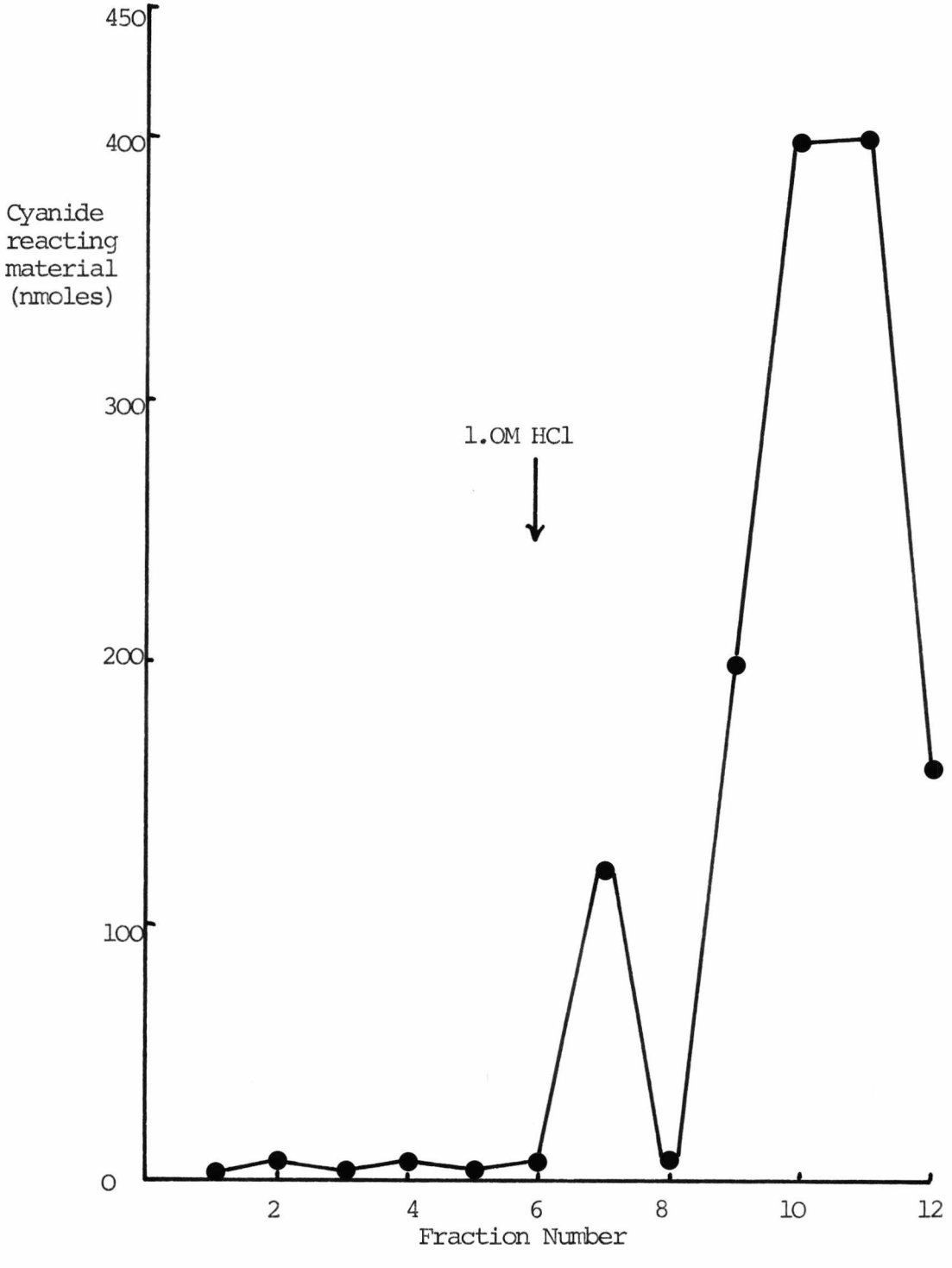
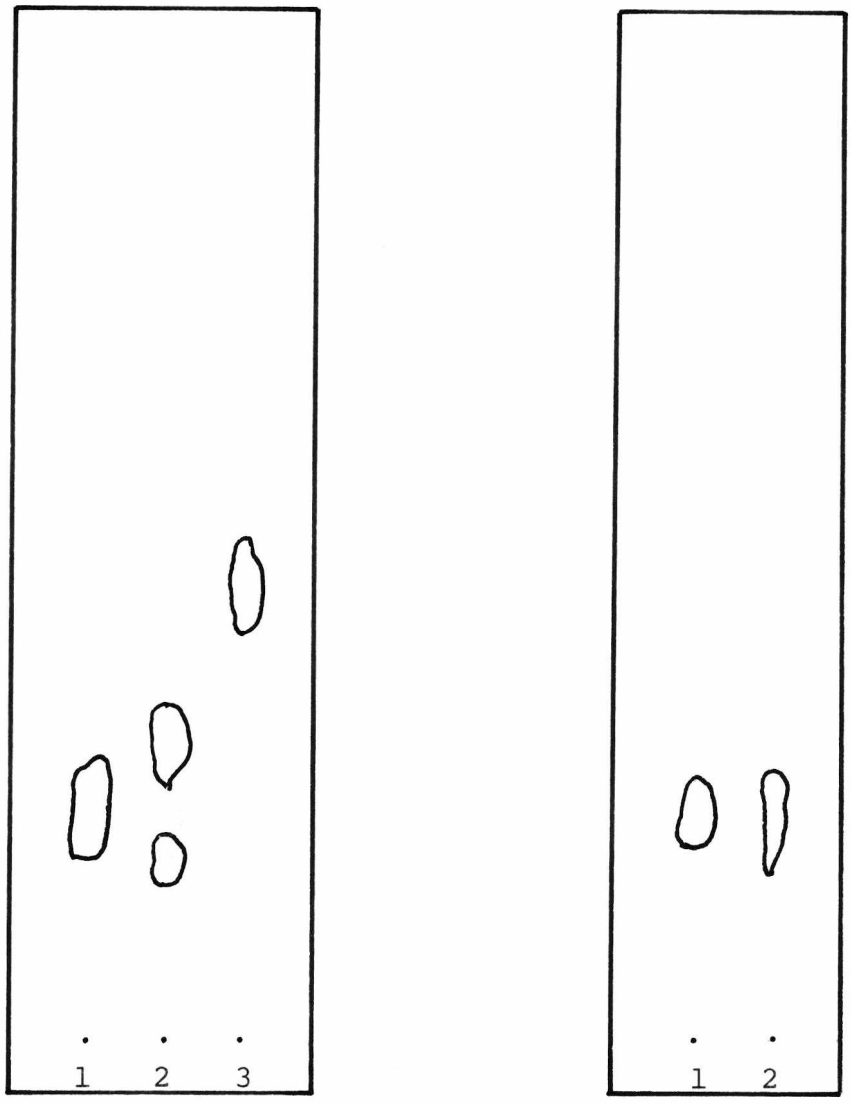


FIGURE 40 - Cellulose thin layer chromatography of extracts from stationary phase cultures of *C.violaceum* grown on 10mM glutamate plus 2mM glycine and 0.5mM methionine. (A) Chromatography of fraction 10 from Dowex2-X8 anion exchange column(2). Glyoxylic acid cyanohydrin(1) and pyruvic acid cyanohydrin(3) were run as standards. (B) Chromatography of growth medium(2). Glyoxylic acid cyanohydrin was run as standard(1).



(A) (B)

CHAPTER FOUR

(DISCUSSION)

Although Wissing (1975) has obtained a cell-free extract of an unidentified pseudomonad capable of producing cyanide from glycine, attempts to prepare extracts of *C.violaceum* with the same ability have so far failed. Consequently, little is known about the enzymic step(s) involved in the conversion, although speculative pathways have been proposed (see Knowles, 1976). On the other hand, several enzymes involved in cyanide assimilation and detoxication by cyanogenic microorganisms have been identified, yet little is known about their properties and whether, for example, their activities are regulated by the cellular or medium cyanide, glycine or methionine levels (Knowles, 1976). Thus, it is not possible to quantify the levels of enzyme(s) involved in cyanide production during growth of *C.violaceum* and,whereas the activities of the assimilatory or detoxifying enzymes can be assayed in vitro, their in vivo activities cannot be estimated. Despite these limitations, an assessment can be made, from measurements of the medium cyanide level, concerning the balance between cyanide production and degradation in growing cultures of *C.violaceum*.

Experiments in which glycine and/or methionine were included in the growth medium (Fig.13) confirm, with growing cultures, earlier observations, made with nonproliferating cells (Michaels and Corpe, 1965), that cyanogenesis by *C.violaceum* is stimulated by glycine and methionine. Radioactive studies have established glycine as the substrate for cyanide production (Michaels et al., 1965) and as such it would be expected to stimulate cyanide formation. However, the situation is more complicated than a simple relationship between substrate concentration and amount of product formed. At low medium concentrations of glycine and methionine (both less than 1.0mM), glycine inhibits cyanide production (Fig.11). This suggests

that glycine is partially repressing synthesis of the cyanide producing system but at higher concentrations of glycine the effect on cyanide production is overcome by virtue of the greater concentration of substrate available for cyanide formation. The evidence for this hypothesis is strengthened by a number of observations made during the period this thesis was being written (Collins and Knowles, unpublished observations). *C.violaceum* was grown in media containing glutamate alone or glutamate supplemented with glycine and/or methionine. Cells were harvested in the late exponential phase of growth, washed,resuspended in media containing chloramphenicol and all possible combinations of glutamate, glycine and methionine, and assayed for their ability to produce cyanide. In all cases, whatever the content of the resuspension medium, cells that had been grown in media supplemented with glycine produced less cyanide than those cells grown in the absence of glycine. On the other hand, the presence or absence of methionine in the growth medium had no effect on the amount of cyanide produced after resuspension. This again suggests that glycine is partially repressing the synthesis of the cyanide producing system.

Methionine has been known to stimulate cyanogenesis by *C.violaceum* for some time but the mechanism through which it exerts this effect remains to be elucidated. Radiolabelling experiments established that the methyl group of methionine does not act as a precursor for cyanide (Michaels et al., 1965) and the ability of the methyl donors, betaine, dimethylglycine and choline to substitute for methionine in stimulating cyanogenesis (Michaels and Corpe, 1965) suggests that the action of methionine is connected with one-carbon metabolism. Fig.3 shows an outline of the interrelated system for the production of serine, glycine and methionine in *E.coli*, the only bacterium in which the regulation of these pathways has been studied in

detail. It should be noted that the system does not simply provide glycine and methionine for incorporation into proteins but is also the major source of supply of one-carbon units for the synthesis of purine bases, thymidine and histidine, and of glycine for incorporation into purines and pyrimidines (Taylor et al., 1966). Regulation of the system must be extremely flexible, depending on the supply of glycine, methionine and other sources of one-carbon units, as well as the demand for protein synthesis and one-carbon units.

Under normal physiological conditions serine serves as the metabolic precursor to glycine via the serine transhydroxymethylase reaction, although the reaction is reversible and under certain conditions serine can be synthesised from glycine(Pizer, 1965). Another enzyme involved in glycine metabolism in *E.coli* is the glycine cleavage enzyme which is also reversible and can function either in the synthesis of glycine or the catabolism of serine and glycine (Kikuchi, 1973). When acting in the direction of glycine cleavage this enzyme generates 5,10-methylene tetrahydrofolate. *E.coli* has been found to be capable of generating one-carbon units from glycine (Newman and Magasanik, 1963) and the glycine cleavage enzyme is thought to be involved (Csonka, 1977). Inclusion of methionine in the growth medium of *E.coli* represses the synthesis of homocysteine methyltransferase, 5,10-methylene tetrahydrofolate reductase and serine transhydroxymethylase (Mansouri et al., 1972) and this repression of one-carbon metabolism is thought to cause a build up of intracellular glycine (Miller and Newman, 1974). Exogenous glycine appears to induce the glycine cleavage system in *E.coli* (Meedel and Pizer, 1974) and this sytem may be a means of regulating the levels of glycine as well as providing one-carbon units for biosynthetic purposes. In *C.violaceum* inclusion of glycine and/or methionine in the growth medium might be expected to lead to an increase

in the intracellular glycine pool. Cyanide production from glycine may well be a means of regulating glycine levels, either as a complement to, or in place of, the glycine cleavage system. Clearly a great deal of work remains to be done on clarification of the pathways and regulation of one-carbon metabolism in *C.violaceum* before this hypothesis can be tested.

It is interesting that the biosynthesis of the antibiotic cephalosporin C by a *Cephalosporium* sp. is stimulated by the inclusion of methionione in the growth medium (Demain and Newkirk, 1962). Methionine is an efficient precursor of the sulphur atom of cephalosporin C but it also seems to be exerting a regulatory effect, through an unknown mechanism,on synthesis of the antibiotic (Drew and Demain, 1977). Betaine and choline can substitute for methionine in this respect (Ott et al., 1962) and it may be that the regulation, by methionine, of secondary metabolite production by *Cephalosporium* is mediated through a similar mechanism as methionine stimulation of cyanide production by *C.violaceum*.

Cyanide production by *C.violaceum* occurs during the transition from exponential growth to the stationary phase of growth (Fig.13) and the observation that the addition of chloramphenicol to early exponential phase cultures growing on glutamate supplemented with glycine and methionine inhibits cyanide production (Fig.22), suggests that the cyanide producing system is induced during exponential growth. This also appears to be the case in *Ps.aeruginosa*. The cyanide producing capacity of this organism was measured by harvesting cells at various points throughout the growth curve, washing, resuspending in a medium containing glycine and methionine, and measuring the cyanide produced. An increase in the cyanide producing capacity of cells was observed in the later stages of exponential growth, followed by a decrease in this capacity during the stationary phase of

growth (P.A. Castric, personal communication).

Production of a compound at the transition between the tropho- and idiophases of growth is typical of microbial secondary metabolism (Drew and Demain, 1977). Cyanide production by *C.violaceum* also displays a number of additional characteristics of secondary metabolism as given by Weinberg (1971). There is a limited taxonomic distribution of cyanogenesis in the genus *Chromobacterium; C.violaceum*, but not *C.lividium* (Bergey, 1974) or *C.fluviatile* sp.nov. (Moss et al., 1978), produces cyanide. Synthesis of cyanide would seem to be proceeded by transcription and translation, resulting in secondary metabolite synthesis. The level of cyanide production by *C.violaceum* is influenced by the ferrous ion and phosphate ion concentrations of the growth medium (Fig.16), although no effect of growth temperature or initial pH of the medium on cyanogenesis could be observed. The conditions of cyanide production by *C.violaceum* can be seen to contain a number of features consistent with the conclusion that cyanide is a secondary metabolite of this bacterium.

In *Ps.aeruginosa* (Castric, 1975) and *C.violaceum*, the stimulation of cyanogenesis by ferrous ion is linear with the logarithm of the metal ion concentration, which is characteristic of a metal effect on secondary metabolism (Weinberg, 1970). The role of metals in secondary metabolism is unknown (Weinberg, 1970) and the mechanism through which iron exerts its effect on cyanogenesis remains an open question.

The response of cyanide production to phosphate concentration differs between *Ps.aeruginosa* and *C.violaceum*. Optimum cyanogenesis by *Ps.aeruginosa* occurs within a narrow range of phosphate concentration of 1-10mM (Megathanan and Castric, 1977). Above and below these concentrations there is a marked decrease in cyanide production and little cyanide is produced at

0.1 or 100mM phosphate concentrations. In contrast, cyanide production by *C.violaceum* increases with phosphate concentration over the range of 1.0 to 100mM phosphate and there is appreciable cyanogenesis at 0.1mM and 150mM phosphate. The phosphate concentrations at which cyanide production is stimulated in *C.violaceum* tend to be inhibitory for the production of most secondary metabolites (Weinberg, 1974) and in this respect cyanogenesis by *Ps.aeruginosa*, rather than *C.violaceum*, is typical of the majority of secondary metabolites.

Many reasons have been advanced to explain the effect of phosphate on secondary metabolism but none have yet been verified (Weinberg, 1974). Of relevance may be the observations that in *E.coli* phosphate concentration influences the presence of iso accepting tRNA species (Mann and Huang, 1974), while in *Ps.aeruginosa* ribosome content decreases during periods of phosphate limitation (Hou et al., 1966). These factors may selectively inhibit the synthesis of proteins involved in secondary metabolism. Also of importance may be the observations of Erecinska et al., (1977) that the concentration of intracellular phosphate in the yeast *Candida utilis* was dependent on the phosphate concentration in the growth medium, and their finding that the concentration of intracellular phosphate was implicated as an important contributor to the regulatory mechanisms of cellular metabolism. Alternatively, phosphate may be having a direct effect on the cyanide producing enzyme(s) but direct evidence of this must await characterisation of these enzymes.

It is not immediately clear why some microorganisms produce cyanide although it is possible at this stage to speculate on the reasons. Cyanide can be considered to be a microbial secondary metabolite and it may therefore be helpful to look at the proposed functions of secondary metabolism.

It has been suggested that the process of secondary metabolism is far more important to the producer cell than the final products themselves (Weinberg, 1971). As suggested above, cyanide production by *C.violaceum* may function to remove glycine. The occurrence of cyanogenesis towards the end of the exponential phase of growth correlates with the suggestion of Drew and Demain (1977) that the pool sizes of primary metabolites (such as glycine) may increase at the end of exponential growth, as the demand for these metabolites for biosynthetic purposes decreases. That the glycine pool is large in stationary phase *C.violaceum* cells is suggested by the observation that stationary phase cultures grown on media not supplemented with glycine have excreted small amounts of glycine into the medium (Table 6). In addition, the stimulatory effect of methionine on cyanogenesis may possibly be explained by its ability to regulate the intracellular glycine level (see above). Glycine can cause bacterial cell lysis, as a result of inhibition of cell wall synthesising enzymes(Hammes et al., 1973), and cyanogenesis may serve to prevent the build up of this potentially toxic molecule at the end of exponential growth. With this in mind it would be interesting to examine the effect of cyanide production on the viability of *C.violaceum*.

Another explanation of the function of cyanogenesis is that cyanide excretion may be used to destroy or inhibit the growth of neighbouring organisms, thus increasing the competetiveness of the excreting organism. However, this explanation seems unlikely in view of the fact that the cyanide producing organism would require concentrations of glycine, sufficient for the synthesis of inhibitory amounts of cyanide, which can be expected to occur very infrequently outside the laboratory. In this respect, it would be interesting to study the competetiveness of cyanide producing and non-

cyanide producing mutants of *C.violaceum*, if such mutants were to become available, in mixed bacterial populations in a chemostat.

Whatever the function of cyanogenesis, it would seem that the value of the process to *C.violaceum* is increased by the ability of the organism to utilize the cyanide produced in the formation of amino acids.

The cyanide content of the medium of *C.violaceum* cultures, grown under high cyanide producing conditions, starts to decrease a few hours into the stationary phase of growth (Fig.22). The rate of cyanide blow-off from the medium is very small compared to the rate of decrease in cyanide content of cultures, suggesting that further metabolism is occurring. Two cyanide-utilising activities, β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase, have previously been reported in *C.violaceum* and their presence is confirmed by this work. Two other cyanide-utilising enzymes, rhodanese and formamide hydrolyase, were assayed for, but only rhodanese was detected.

All three cyanide-utilising enzymes are induced towards the end of exponential growth (Figs.17, 18, 19) but they differ in their patterns of induction. The cyanide-utilising enzymes are induced at a time when the cyanide content of the medium is increasing, and it may be that even the low levels of cyanide formed by cultures growing on glutamate alone are sufficient to cause induction. There was a greater extent of induction of β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase in cells grown under low cyanide producing conditions than in cells grown under high cyanide producing conditions. This partial repression of the two enzymes in cultures grown under high cyanide producing conditions was found to be caused by the presence of glycine in the medium, rather than by the high levels of cyanide formed during growth under these conditions (Table 1). A

rationale for the partial repression of these two enzymes by glycine remains to be established. In contrast, the rhodanese content of cells showed a greater increase in cultures grown under high cyanide producing conditions than under low cyanide producing conditions. Rhodanese is induced by the same amount in cells growing on glutamate, either alone or supplemented with glycine, but shows increased induction in cells grown on glutamate supplemented with glycine and methionine (Table 1). Methionine, or more likely the higher levels of cyanide resulting from the inclusion of methionine in the medium, could be the cause of this increased induction.

A β-cyanoalanine synthase activity has been found in one other cyanogenic bacteria, an unidentified pseudomonad (F. Wissing, personal communication). In two non-cyanogenic bacteria, *E.coli* and *B.megaterium*, for which β-cyanoalanine synthase activities were reported, this activity was found to be a secondary activity of an enzyme involved in cysteine biosynthesis (Dunnill and Fowden, 1965; Castric and Conn, 1971). The nature of the β-cyanoalanine synthase activity in *C.violaceum* is unknown, but studies on the effect of inducers and repressors of cysteine biosynthesis on β-cyanoalanine synthase levels, and purification of the enzyme could clarify the situation. Methionine is synthesised from cysteine by microorganisms (Flavin, 1975), and the inhibition of β-cyanoalanine synthase by methionine (Fig.20) could be interpreted as feedback inhibition if β-cyanoalanine synthase activity is a secondary function of an enzyme involved in cysteine biosynthesis. Feedback inhibition of cysteine synthase by methionine has been observed in *Neurospora crassa* (Wiebers and Garner, 1967).

The γ-cyano-α-aminobutyric acid synthase activity of *C.violaceum* was investigated using cell-free extracts by Brysk and Ressler (1970) who found

that early exponential phase cells exhibited maximal activity of the enzyme. This is in conflict with the data obtained here but an explanation may lie in the different strains of *C.violaceum* used in the two studies.

Rhodanese has been found in a wide range of bacteria, and two cyanogenic bacteria, *C.violaceum* and an unidentified pseudomonad (F. Wissing, personal communication), are known to possess the enzyme. However, rhodanese can catalyse a large number of reactions involving sulphane sulphur atoms (Westley, 1973) and it is possible that the physiological role of the enzyme is concerned with sulphur metabolism rather than cyanide metabolism. It cannot be stated with any certainty that cyanide detoxication is the physiological function of rhodanese in *C.violaceum*.

The demonstration of in vitro enzyme activities does not necessarily mean that these enzymes are functional in vivo, but the occurrence of β-cyanoalanine in the medium of *C.violaceum* cultures (Table 6) indicates that β-cyanoalanine synthase is a physiologically functional enzyme. Thiocyanate, a product of both the γ-cyano-α-aminobutyric acid synthase and rhodanese reactions, could not be detected in the growth medium, leaving the in vivo activities of these enzymes open to doubt.

The low level of cyanide found in the medium of cultures grown on glutamate alone is presumably due to the relatively low rate of its production, in the absence of exogenously added substrate, in comparison to its removal by the cyanide degrading enzymes. Inhibition of cyanide degradation, through the prevention of induction of the cyanide-utilizing enzymes might be expected to result in an increase in cyanide concentration in the medium. This is observed on addition of chloramphenicol to late-exponential phase cultures, by which time the cyanide-producing system is fully induced (Fig.23). Chloramphenicol addition at this point in the growth curve inhibits

the induction of cyanide-utilising enzymes in cells growing under low and high cyanide producing conditions (Table 3).

Chloramphenicol addition to late exponential phase cultures growing on glutamate supplemeted with glycine and methionine, has little effect on the level of cyanide production but the following decrease in the cyanide concentration of the medium is inhibited (Fig.24). The effect of adding chloramphenicol, at the same point in the growth curve, on the cyanide content of cultures grown on glutamate supplemented with glycine is the same as that observed on addition of chloramphenicol to cultures growing on glutamate supplemented with glycine and methionine (Fig.25). In contrast, chloramphenicol addition, in the late exponential phase of growth, to cultures growing on glutamate supplemented with methionine has the same effect on the cyanide content of the medium as chloramphenicol addition to cultures growing on glutamate alone (Fig.26).

The inhibition of induction of the cyanide-utilising enzymes, caused by chloramphenicol addition to late exponential phase cultures, suggests that these observations can be interpreted in terms of the inhibition of cyanide utilisation and degradation. Cells growing on media not containing glycine are producing cyanide at a low rate compared to its removal by the cyanide utilising enzymes, and inhibition of cyanide removal results in an increase in the cyanide content of these cultures. Cells growing on media containing glycine are producing cyanide at a higher rate than those growing in the absence of glycine (at the concentration of glycine used in these experiments the inhibitory effect of glycine on cyanogenesis is overcome) but the activities of the cyanide utilising enzymes are lower in the presence of glycine than in its absence. Inhibition of cyanide removal has only a slight effect on the amount of cyanide produced by these cultures but reduces the rate

at which cyanide is removed from the medium.

This interpretation is based on in vitro studies of the effect of chloramphenicol addition on induction of the cyanide utilising enzymes. An increase in the intracellular glycine pool, as a result of protein synthesis inhibition by chloramphenicol, would manifest itself in an increased rate of cyanide synthesis, and this could account for some of the observations. Consequently, the effect of chloramphenicol addition on the in vivo utilisation of cyanide was studied in order to establish that chloramphenicol was inhibiting cyanide degradation by whole cells.

Addition of {^{14}C}KCN to early stationary phase cultures of *C.violaceum* grown on glutamate supplemented with glycine and methionine, resulted in an accumulation of radioactivity in non-volatile derivatives of cyanide in the medium, and a concomitant decrease in the fraction of radioactivity present in the medium as cyanide (Fig.29). There was little accumulation of radioactivity in the bacterial cells, and an attempt to determine which compounds contained this radioactivity was not made. The addition of chloramphenicol in the late exponential phase of growth, before addition of {^{14}C}KCN, had no effect on the conversion of cyanide to non-volatile derivatives, or on the amount of radioactivity accumulating in bacterial cells (Fig.30). A similar picture could be seen for cultures grown on glutamate alone although the extent of conversion of cyanide to non-volatile derivatives was not as great (Fig.31); again, the addition of chloramphenicol before {^{14}C}KCN addition had no effect on the conversion of cyanide to non-volatile derivatives (Fig.32).

The failure of chloramphenicol to inhibit the conversion of cyanide to non-volatile derivatives is puzzling. An explanation may lie in non-specific reactions of cyanide with components of the medium. Although a control experiment ruled out the possibility of a considerable fraction of

the cyanide reacting with those components of the medium present at the time of inoculation, it is possible that cyanide is reacting with compounds excreted by cells in the stationary phase. Cyanide may react with some of these compounds to form cyanohydrins, and at the pH value of the growth medium there will be an equilibrium between free and 'bound' cyanide with the free cyanide being available for further metabolism *via* the cyanide utilising enzymes. With this in mind it is interesting that there is some evidence for the presence of one, or possibly two, cyanohydrins in the medium of stationary phase cultures grown in high cyanide producing conditions (Fig.40), although the evidence is by no means conclusive. Chloramphenicol would not be expected to have any effect on non-enzymatic reactions of cyanide. A full explanation of these observations will probably be aided by characterisation of the composition of the stationary phase culture medium.

Previous workers have shown that resuspended cells of *C.violaceum* can convert cyanide to β-cyanoalanine (Brysk *et al.*, 1969a) and γ-cyano-α-aminobutyric acid (Brysk and Ressler, 1970), both of which accumulated in the culture fluid. Further metabolism of β-cyanoalanine to asparagine and aspartate was also observed. In order to determine if any of these amino acids are produced by growing cultures of *C.violaceum* the amino acid composition of stationary phase cultures was investigated. β-cyanoalanine could be detected in the media of cultures grown on glutamate supplemented with glycine, either alone or in combination with methionine, but not in cultures grown on glutamate, either alone or supplemented with methionine (Table 6). Asparagine, aspartate and γ-cyano-α-aminobutyric acid could not be detected in any media, suggesting that β-cyanoalanine is the major product of cyanide metabolism under these growth conditions.

The addition of {^{14}C}KCN to stationary phase cultures grown on glutamate, either alone or supplemented with glycine and methionine, results in the accumulation of radioactivity in β-cyanoalanine present in the medium, but not in any other amino acid, confirming that β-cyanoalanine is the major product of cyanide metabolism (Figs.34, 36). The addition of chloramphenicol before {^{14}C}KCN addition, although it does not inhibit the conversion of cyanide to all non-volatile derivatives, inhibits the conversion of cyanide to β-cyanoalanine (Figs.35, 37) confirming that the effect of chloramphenicol on the cyanide content of the growth medium is mediated through an inhibition of cyanide utilisation.

Small amounts of radioactivity could be detected in an unknown compound in cultures grown under high cyanide producing conditions (Fig.34). The amount of radioactivity in this compound declined over the period of incubation suggesting that it is an intermediate in the further metabolism of cyanide but an elucidation of the role this compound plays in cyanide metabolism must await its characterisation.

A greater fraction of the exogenously added {^{14}C}KCN was converted to β-cyanoalanine by cultures growing on glutamate supplemented with glycine and methionine, than by cultures growing on glutamate alone,despite the specific activity of β-cyanoalanine synthase being higher in the latter cultures. Nothing is known of the properties or the in vivo regulation of β-cyanoalanine synthase in *C.violaceum* and these two, apparently conflicting sets of data, may be reconciled by purification of the enzyme and an investigation of its regulation. For instance, if β-cyanoalanine synthase activity is a secondary function of another enzyme, competition between various substrates for the catalytic site of the enzyme may be a factor influencing the nature of the results obtained.

Of the {^{14}C}KCN added to cultures growing on glutamate supplemented with glycine and methionine, some 35% was converted to β-cyanoalanine and 20% remained as free cyanide, leaving some 45% to be accounted for. For cultures growing on glutamate alone, the equivalent figures are β-cyanoalanine 15% and free cyanide 20%, leaving some 65% to be accounted for. Radioactivity could not be detected in any other amino acids. The method employed for the separation of β-cyanoalanine involved treatment of the growth medium with DNFB and subsequent collection of the DNP-amino acids so formed by extraction into organic solvent. Most of the unaccounted radioactivity resided in the aqueous layer formed as a result of this separation procedure. Presumably the compounds present in the aqueous layer are hydrophilic in nature. Thiocyanate, a product of both the γ-cyano-α-aminobutyric acid synthase and rhodanese reactions, could be one of the compounds present in the aqueous layer. However, thiocyanate could not be detected in the growth medium of stationary phase cultures, eliminating it as a component of the aqueous layer and suggesting that the in vivo activities of γ-cyano-α-aminobutyric acid synthase and rhodanese are low. It is possible that the radioactivity in the aqueous layer resides in products formed by non-enzymatic reactions of cyanide with compounds excreted by stationary phase cells but confirmation of this must await determination of the components of the aqueous layer.

The relative amounts of radioactivity in the organic and aqueous phases differs between preparations from cultures grown on glutamate supplemented with glycine and methionine, and cultures grown on glutamate alone (Tables 7, 9). Most of the radioactivity resides in the organic layer of preparations from cultures grown on glutamate supplemented with glycine and methionine whereas in preparations from cultures grown on

glutamate alone most of the radioactivity resides in the aqueous layer. This probably reflects the observation that a greater fraction of radioactivity is incorporated into β-cyanoalanine by high cyanide producing cultures. Chloramphenicol addition causes the proportion of radioactivity in the aqueous layer to increase (Tables 8, 10) thus reinforcing the hypothesis that the less cyanide incorporated into β-cyanoalanine the more radioactivity remains in the aqueous layer of DNP-amino acid preparations.

The cyanide content of the growth medium of *C.violaceum* is clearly a reflection of the relative rates of cyanide production and utilisation. Factors which affect either of these processes will have an influence on the cyanide level of the medium, and compounds which stimulate the cyanide producing system, such as glycine and methionine, appear to enhance their effects by inhibiting the synthesis or action of the cyanide utilising enzymes. A scheme is put forward as a tentative, working hypothesis, to explain the effects of glycine and methionine in enhancing the cyanide content of *C.violaceum* cultures (Fig.41).

Glycine is the substrate for cyanide production, although the enzyme system catalysing the reaction awaits characterisation, and as such would be expected to stimulate cyanide formation. However, glycine surprisingly also appears to partially repress synthesis of the cyanide producing system, and only at high glycine or methionine concentrations does glycine have a stimulatory effect on cyanide production. This stimulatory effect of glycine is enhanced by its partially repressive effect on synthesis of two of the cyanide-utilising enzymes, β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase. Cyanide may act as an inducer of synthesis of the cyanide utilising enzymes but this remains to be confirmed. Methionine stimulates cyanogenesis by an unknown mechanism, which may be related to its ability

to increase the intracellular glycine levels. It enhances this effect by inhibiting activity of β-cyanoalanine synthase, which is the major route for further metabolism of cyanide. No conversion of β-cyanoalanine to asparagine or aspartate could be detected under the conditions used in this investigation.

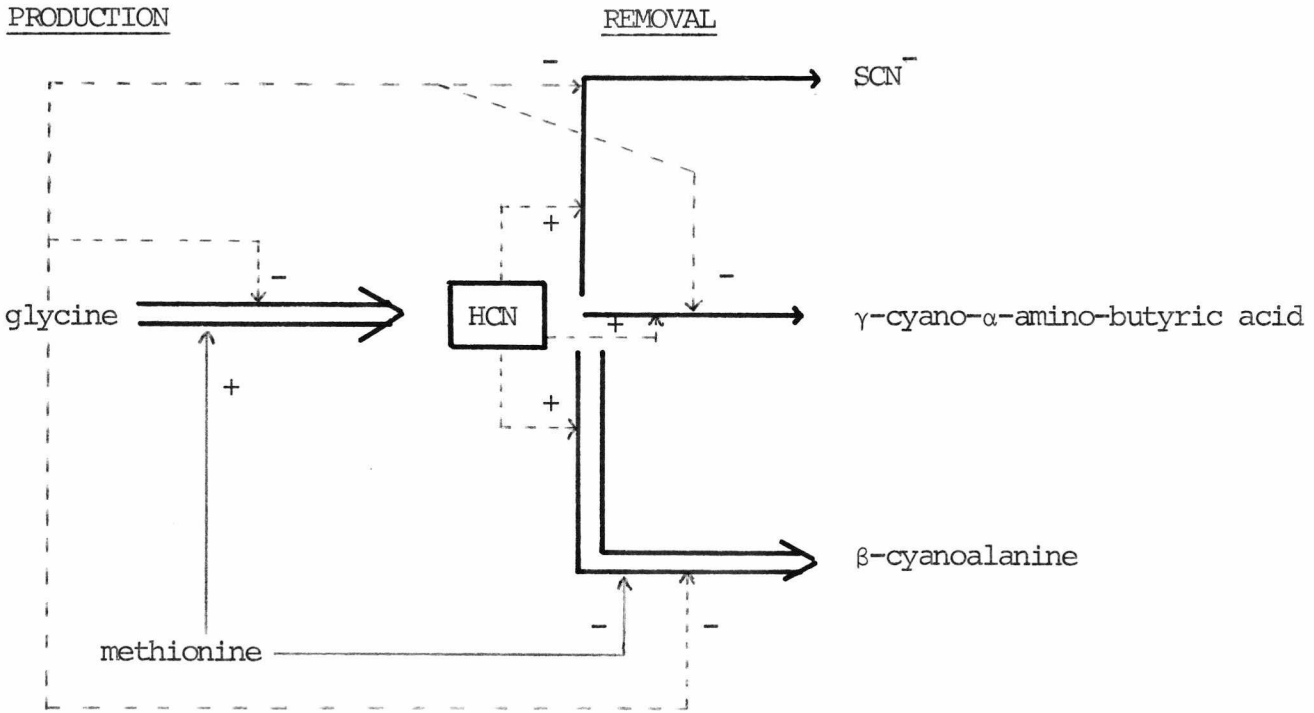


FIGURE 41 - Scheme showing how glycine and methionine stimulate the cyanide content of *C.violaceum* cultures.

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