THE PRODUCTION AND FURTHER METABOLISM OF CYANIDE BY THE SNOW MOULD BASIDIOMYCETE.

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by

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To my parents,

who have made it possible for my studies to reach this stage ,because of their encouragement and support,for which I will always be extremely grateful.

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ABSTRACT

Growth and cyanide production by the snow mould basidiomycete was observed in plate, still and shake cultures. It was shown that growth in shake cultures was the most suitable for further investigations of growth and cyanide production by this fungus. Measurable growth and cyanide production was obtained in shake cultures with glucose, acetate or succinate as the carbon source.

Cyanide production was shown to have many of the usual characteristics of secondary metabolite production. Cyanogenesis was stimulated by including glycine or serine in the growth medium during growth on glucose. Glycine was not used as either a sole carbon or nitrogen source by this fungus. No further stimulation of cyanide production during growth on glucose plus glycine was obtained by supplementing the medium with methionine. However, supplementation of media containing glucose and glycine with N,N-dimethylglycine or betaine caused greater stimulation of cyanide production. Interpretation of this stimulation was complicated by the effects of betaine and N,N-dimethylglycine on growth and cultural appearance. Inorganic phosphate, Fe^{2+} and Zn^{2+}, common effectors of secondary metabolism, did not inhibit or stimulate cyanide production by the snow mould basidiomycete at the concentrations tested.

During growth of the snow mould in media containing glucose and either [1-^{14}C] or [2-^{14}C] glycine, the carbon-1 atom of glycine was converted principally to CO_2 and the carbon-2 atom was largely converted to cyanide. No intermediates of the cyanogenic pathway were isolated, but the possibility that glyoxylic acid or pyruvic acid cyanohydrins are intermediates was shown to be unlikely. No build-up of glycine occurred prior to cyanogenesis by cultures incubated in media containing [U-^{14}C] glucose.

Glycine stimulated cyanide production by cultures of the snow mould basidiomycete grown with acetate, where cyanide production was always associated with growth. Methionine also slightly stimulated cyanide production when added to media containing both acetate and glycine. In contrast to glucose-containing cultures during growth in media containing acetate and either [1-^{14}C] or [2-^{14}C] glycine, both carbon atoms of glycine were catabolised to an equal extent to cyanide, but more $^{14}CO_2$ was produced from the [1-^{14}C] glycine than [2-^{14}C] glycine.

A role for cyanide production by this fungus is proposed.

Cyanide was catabolised by the snow mould basidiomycete mainly to CO_2 during growth on either glucose or acetate. The maximal rate of CO_2 production from

cyanide during growth on glucose occurred at the time when cyanogenesis was maximal. However, CO_2 production from cyanide during growth on acetate was maximal before cyanogenesis reached its maximum rate. The conversion of cyanide to CO_2 by cell-free extracts has also been observed.

Incubation of cultures of the snow mould basidiomycete with $K^{14}CN$ resulted in only low amounts of radioactivity becoming associated with alanine, glutamate, formamide and β-cyanoalanine. The enzymes for alanine biosynthesis from NH_3, HCN and succinic semialdehyde were not shown to be present at detectable levels throughout growth in glucose as reported by Strobel (1966, 1967). Formamide hydrolyase activity in cell-free extracts was also absent. A pathway for the conversion of cyanide to CO_2 is proposed.

The possibility that cyanogenesis by the snow mould basidiomycete could be used as a model system to elucidate the major role of secondary metabolism (which includes the production of antibiotics and other medically or industrially important compounds) is discussed.

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CHAPTER ONE

(INTRODUCTION)

The occurrence of cyanide production by living organisms

Members of the plant, animal and microbial kingdoms have been shown to produce cyanide (Knowles, 1976; Conn, 1979). Many of these cyanogenic organisms are higher plants (nearly 1000 species from 70 to 80 families; Eyjolfsson, 1970), a large number of which serve as food for man and other animals. It is because of the possible correlation between several tropical human illnesses and the poisoning of animals, with diets of cyanogenic plants (Montgomery, 1969) that there has been widespread research into the mechanism of plant cyanide production. The importance of this research is highlighted by the high level of cyanide formation by Cassava (Manihot esculata) which is the basic source of carbohydrate for 300 million people in tropical areas of the world. (Conn, 1973, 1979). Roots of cassava contain between 50 and 250 mg. HCN $(1\text{oog.tissue})^{-1}$, most of which is retained after harvesting, unless they are properly prepared for consumption. The diet, in some tropical regions, therefore leads to the daily consumption of half the lethal dose of cyanide (Conn, 1973; Knowles, 1976).

Fungi form the second largest group of organisms known to produce cyanide. It has been claimed (Locquin, 1944) that 300 species of basidiomycetes from 52 genera and several ascomycetes are cyanogenic. More

recently, low levels of cyanide production by Phycomycetes have been reported (Russo et al, 1977). Despite the abundance of cyanide-producing fungi, very little research into the mechanism of cyanide production by these organisms has been carried out. This possibly reflects the difficulties experienced in growing these fungi in 'shake' culture and the slow growth rate of those cyanogenic fungi that have been successfully cultured (Ward and Lebeau, 1962).

Other groups of cyanide producers are much smaller in terms of the number of individual organisms forming cyanide (Knowles, 1976). Cyanide is formed by the non-photosynthetic bacterium (Chromobacterium violaceum. (Michaels and Corpe, 1965) and several Pseudomonas species (Knowles, 1976). The photosynthetic cyanobacterium Anocystis nidulans also produces cyanide (Pistorius et al.,1979), as does the alga Chlorella vulgaris (Gewitz et al., 1974).

Finally, several millipedes (Eisner et al.,1962) and insects (Moore, 1967; Jones, Parsons and Rothschild, 1962) produce cyanide. Although, in one case (Jacobson, 1966), the cyanide has been shown not to be derived directly from the diet, the possibility of microbial production within the insects has not been ruled out.

Secondary Metabolism

Cyanide formation by several plants and micro-organisms has been described as being a product of secondary metabolism (Conn, 1973; Castric, 1975). Before a discussion of the mechanism of cyanide production, an account of this aspect of metabolism is desirable, particularly with respect to microbial secondary metabolism, to enable some of the observations recorded in later sections to be put into perspective. In addition, this discussion may help us to clarify the role of cyanide formation by the producer organisms.

The metabolism of a large number of organisms may be empirically separated into two parts. As an organism grows, it utilises the carbon and nitrogen sources it is provided with to generate metabolites essential for its development. These activities constitute what is termed the primary metabolism of the organism, and the compounds generated are called primary metabolites.

Secondary metabolism, however, produces components (secondary metabolites) whose functions in the maintenance, development or reproduction of the producing organism are not readily appreciable (Drew and Demain, 1976), but whose ubiquitous distribution, suggests that they are of great importance.

Many primary metabolites can serve as precursors for secondary metabolism, examples are acetyl CoA (the most important single precursor of fungal secondary metabolism; Turner, 1971), fatty acids, amino acids or sugars. These produce many classes of organic compounds (Table1.1)a significant number of which have members that are, or can be, of industrial and medical importance. The penicillins and cephalosporins (β-lactams) are probably the most medically important of the secondary metabolites that have been isolated from fungi, especially now that chemical modifications have extended their range of activity (Snow and Franklin, 1975). Other secondary metabolite antibiotics such as the tetracyclines are also medically important, in this case as broad specificity antibiotics, but they are more frequently of restricted use.

Several secondary metabolites are reknown for their toxic rather than beneficial effects on man and other animals. Amanitin, which is produced by Amanita phalloids and is associated with mushroom poisoning represents one of the many poisons that are manufactured by fungi. Also in this group are the ergot alkaloids, which have been the cause of many social disasters, due to the producer fungus (Claviceps purpurea) infecting rye, which is then made into bread and results in the large scale poisoning of the

Table 1.1

Examples of the types of products obtained from secondary metabolism

Amino sugars	Lactones	Pyridines
Anthocyanins	Macrolides	Pyrones
Anthraquinones	Naphthalenes	Pyrroles
Aziridines	Naphthaquinones	Pyrrolidones
Benzoquinones	Nitriles	Pyrrolines
Coumarins	Nucleosides	Pyrrolizines
Diazines	Oligopeptides	Quinolines
Epoxides	Perylenes	Quinolinols
Ergoline alkaloids	Phenazines	Quinones
Flavonoids	Phenoxazinones	Salicylates
Furans	Phthaldehydes	Terpenoids
Glutaramides	Piperazines	Tetracyclines
Glycopeptides	Polyacetylenes	Tetronic acids
Glycosides	Polyenes	Triazines
Hydroxyamines	Polypeptides	Tropolones
Indole derivatives	Pyrazines	

consumers. Ergot alkaloid production has also been associated with cattle poisoning (Bacon, Porter and Robbins, 1979).

With the availability of this enormous range of compounds produced by secondary metabolism, future uses, especially for industrial purposes, will almost certainly be found as the present sources of chemicals, such as oil, become less available. Added to this, many secondary metabolites are finding uses as specific inhibitors of metabolic processes and are thereby helping to elucidate many biochemical problems.

What then is the reason that so many organisms produce secondary metabolites, especially as in some cases very large quantities of them are formed? Many suggestions have been made about the reasons for secondary metabolite synthesis (Turner, 1971; Weinberg, 1978). These may be summarised, as follows:-

(1) The process of secondary metabolism in an evolutionary relic.

(2) Secondary metabolites serve as food-storage materials.

(3) Secondary metabolites are artifacts released from dead cells.

(4) Secondary metabolites confer a selective advantage on producer organisms because of their antibiotic properties.

(5) **The actual process of secondary metabolism rather than the secondary metabolite is important, as it helps to regulate the metabolism of the producer organism.**

The first four proposals are unlikely. To propose that secondary metabolism is an evolutionary relic, assumes that the many thousands of known secondary metabolites had roles to play in the past but have now become obsolete, yet the secondary metabolites continue to be formed. It would be highly improbable that this is so, as redundant metabolic processes would be expected to be disadvantageous to the survival of the producer organisms. Proposals 2, 3 and 4 may explain the occurrence of some secondary metabolites, but cannot be generally applicable to account for all secondary metabolite production. For example, many secondary metabolites are excreted showing that they cannot be used as carbon or nitrogen sources. Secondly, it is commonly observed that secondary metabolites are produced by living organisms, rather than non-viable cells. Finally, the vast majority of secondary metabolites do not have antibiotic properties.

Recent reviewers of this topic (Weinberg, 1978; Demain, Kennel and Aharonowitz, 1979) favour proposal five as its most likely role. It is now considered

that secondary metabolism acts as an alternative regulatory process to prevent excessive build-up of a specific primary metabolite. At this stage, it should be mentioned that the strains of organisms usually investigated are specially selected for their ability to produce copious amounts of a secondary metabolite.This must be bourne in mind when considering the significance of secondary metabolite production by them as a means of regulation (or with respect to Proposal 4). Even so, there are several links between general metabolite production (Neijessel and Tempest, 1979) and secondary metabolism, which shows that this process may be important to the producer organism, even where the amount of a secondary metabolite produced is low. For instance Escherichia coli, grown aerobically in batch culture in a defined medium containing glucose (as the only carbon source), will produce CO_2 and new cell material as the only major products. Conversely, if E. coli is grown anaerobically, in this same medium, fewer cells are produced and ethanol, succinate, lactate, hydrogen and CO_2 are found as end products (Blackwood, Neish and Ledingham, 1956). This shows that metabolism can be altered in response to oxygen limitation, by avoiding the build-up of reduced pyridine nucleotides using metabolic products as hydrogen acceptors.

Although this may be a rather trite example, it does illustrate how different metabolites may be produced as the conditions of growth change. It is possible to suggest that secondary metabolite production could represent a similar way of coping with changes in the environment of the producer organism, for example caused by nutrient depletion or changes of temperature and pH. (Tempest and Hunter, 1965; Gale and Epps, 1942).

The characteristics of secondary metabolism by batch cultures.

These have been summarised by Castric (1975) and are as follows:-

(i) Production of a particular secondary metabolite has a limited taxonomic distribution.

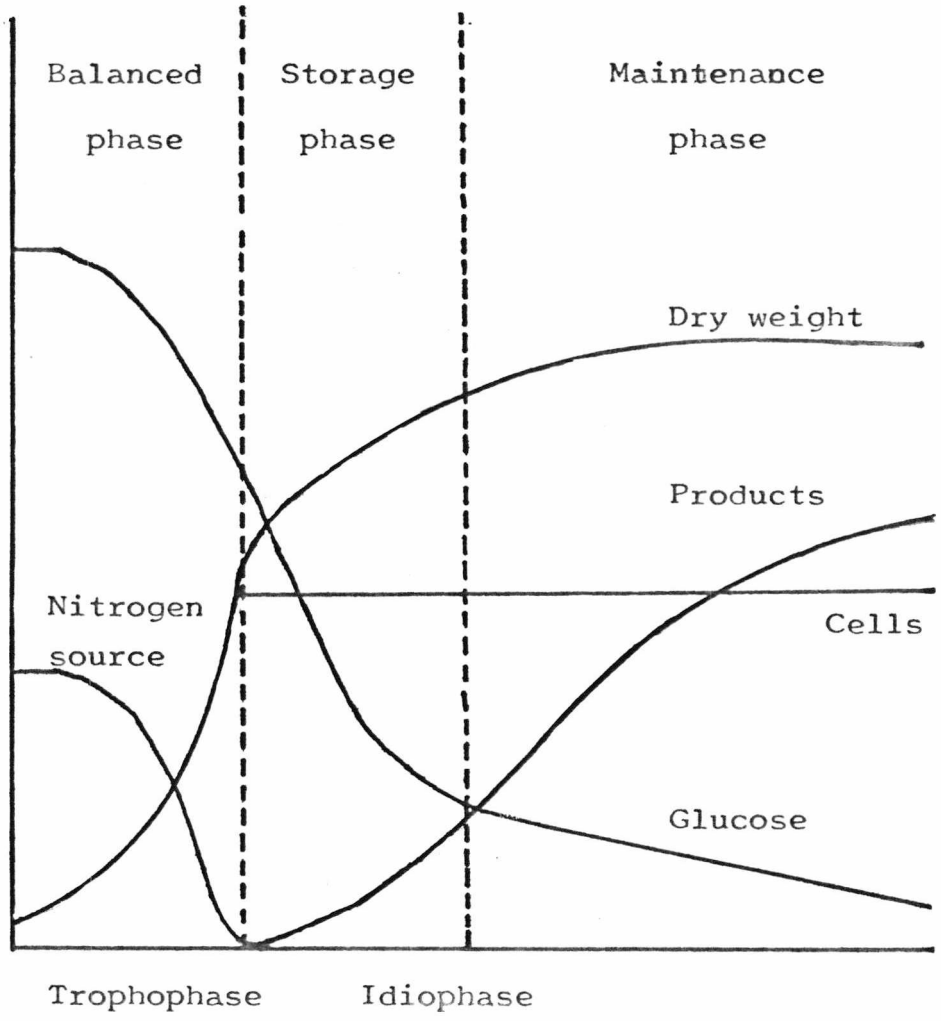
(ii) Synthesis from its primary metabolite precursor usually occurs after the period of active cell growth.

(iii) Synthesis is preceded by transcription and translation, resulting in the production of the relevant synthases.

(iv) Synthesis occurs within ranges of temperature, oxygen tension, specific metal ion and phosphate concentrations that are narrower than those over which growth occurs.

Figure 1.1

Nitrogen limited fermentation of submerged cultures of fungi



This suggests that, in batch cultures, the onset of secondary metabolite production usually occurs after a growth limiting nutrient has been exhausted from the culture medium. The limited taxonomic distribution and widely differing secondary metabolites produced suggests that individual groups of organisms have developed different systems for the solution of similar problems.

Before considering the regulation and effectors of secondary metabolism, the terminology and description of growth phases in batch cultures needs to be introduced. There is ample evidence (Barrow *et al.*, 1961; Bu'lock, 1967) that growth and metabolism of batch cultures pass through several distinct phases. The example of nitrogen limited fermentation of submerged ('shake') cultures of fungi is shown in figure 1.1. Alternative nomenclature is recorded in this figure, but throughout this Thesis the terminology of Bu'lock (1967) will be used whenever possible.

The period of incubation during which the organism grows in an exponential manner with the uptake of nutrients in a constant ratio is described as the trophophase. Production of secondary metabolites rarely occurs during this period of incubation, which usually ends with exhaustion of a nutrient (in this case the nitrogen source). At this point, the cell

number (but not necessarily the dry weight) remains nearly constant, but several metabolic changes presumably begin to occur. This period of metabolism is termed the idiophase, during which idiolites (secondary metabolites) are produced.

Regulation and effectors of Secondary Metabolism

How is secondary metabolism regulated and what role do the many effectors of this process have? The details of the regulation of secondary metabolism will depend largely on the individual pathways concerned. As the substrates for these processes are produced by primary metabolism, the two systems (if they can in reality be separated) must be under similar methods of control (Bezborodov, 1978). The mechanisms of regulation are, therefore, likely to involve induction and repression of the enzymes required and also allosteric and metabolic regulation of enzyme activities. An example of how more than one type of regulation affects secondary metabolite production is the production of patulin by Penicillium urticae (Bullock, Sherperd and Winstanley, 1967). By adding protein synthesis inhibitors at different stages of the fermentation, it was revealed that 6-methylsalicylic (an intermediate in the process) synthase is a metabolically stable enzyme produced during the trophophase and activated during the idiophase. Conversely, the conversion of

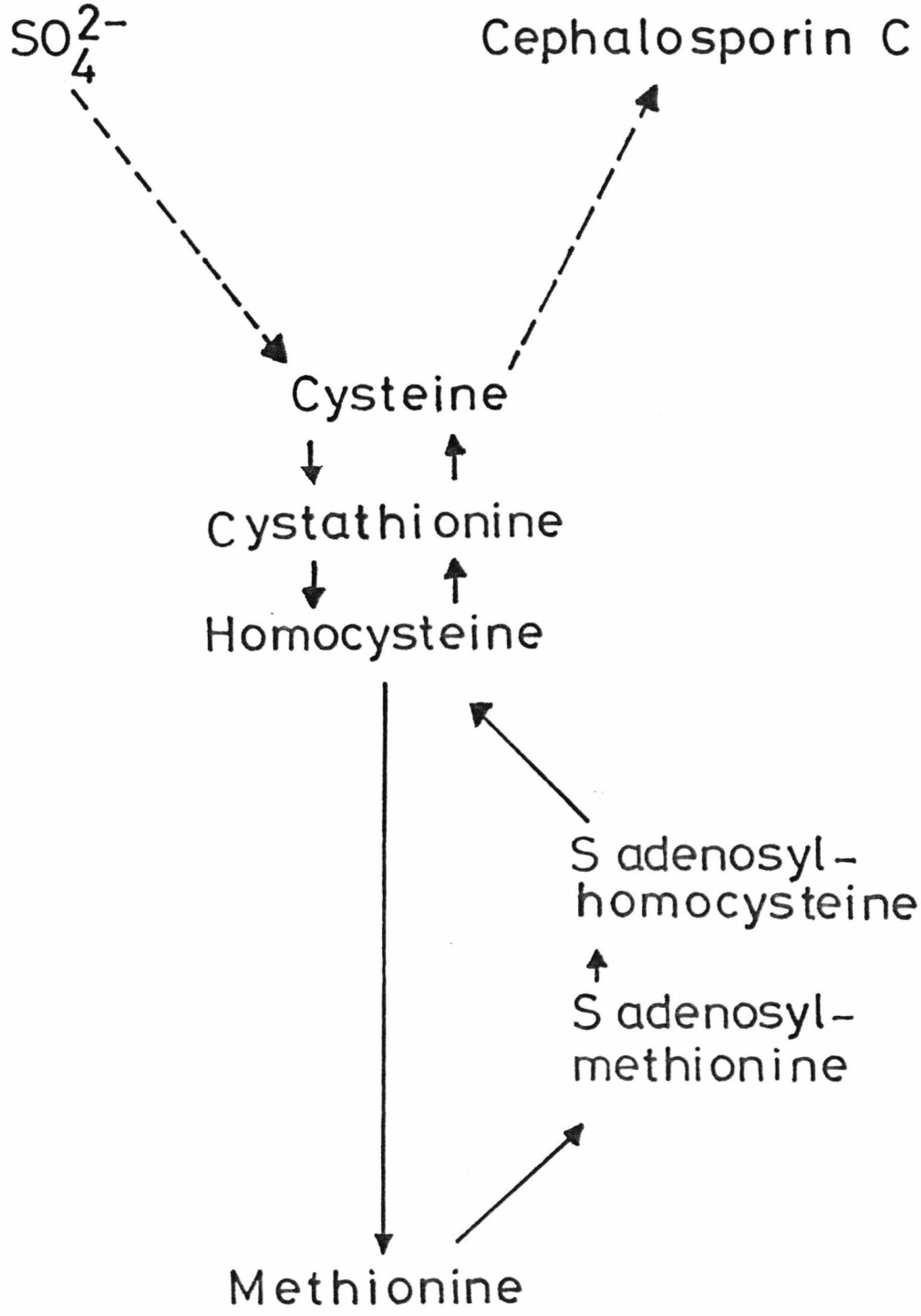
6-methylsalicylic acid to gentisyl derivatives and then to patulin was mediated by metabolically labile enzymes whose formation was induced during the trophophase.

Detailed knowledge of many pathways of secondary metabolite production is often lacking, but two systems where a large amount of data has accumulated are those for cephalosporin and penicillin production. These will be used to illustrate the complex interelationship between primary and secondary metabolism. (Drew and Demain, 1975; Masurekar and Demain, 1974; Figures 1.2 and 1.3).

Methionine acted as an inducer of the cephalosporin 'C' producing pathway in Cephalosporium acremonium (Demain and Inamine, 1970) when added during growth (before antibiotic synthesis). Addition to the cultures after growth has stopped had little affect and did not stimulate antibiotic production. Surprisingly, no other sulphur compounds (such as cysteine) stimulated antibiotic production to the same extent. The requirement for methionine was further substantiated by the isolation of mutants blocked in the interconversion of methionine and cysteine. When just enough methionine was present to allow growth of the mutants in the presence of excess cysteine, little or no antibiotic was produced, unless excess methionine was added (Demain, 1976). Interestingly, the non-sulphur containing analogue, norleucine, was able to replace

Figure 1.2

Probable pathway of sulphur metabolism in Cephalosporium sp.



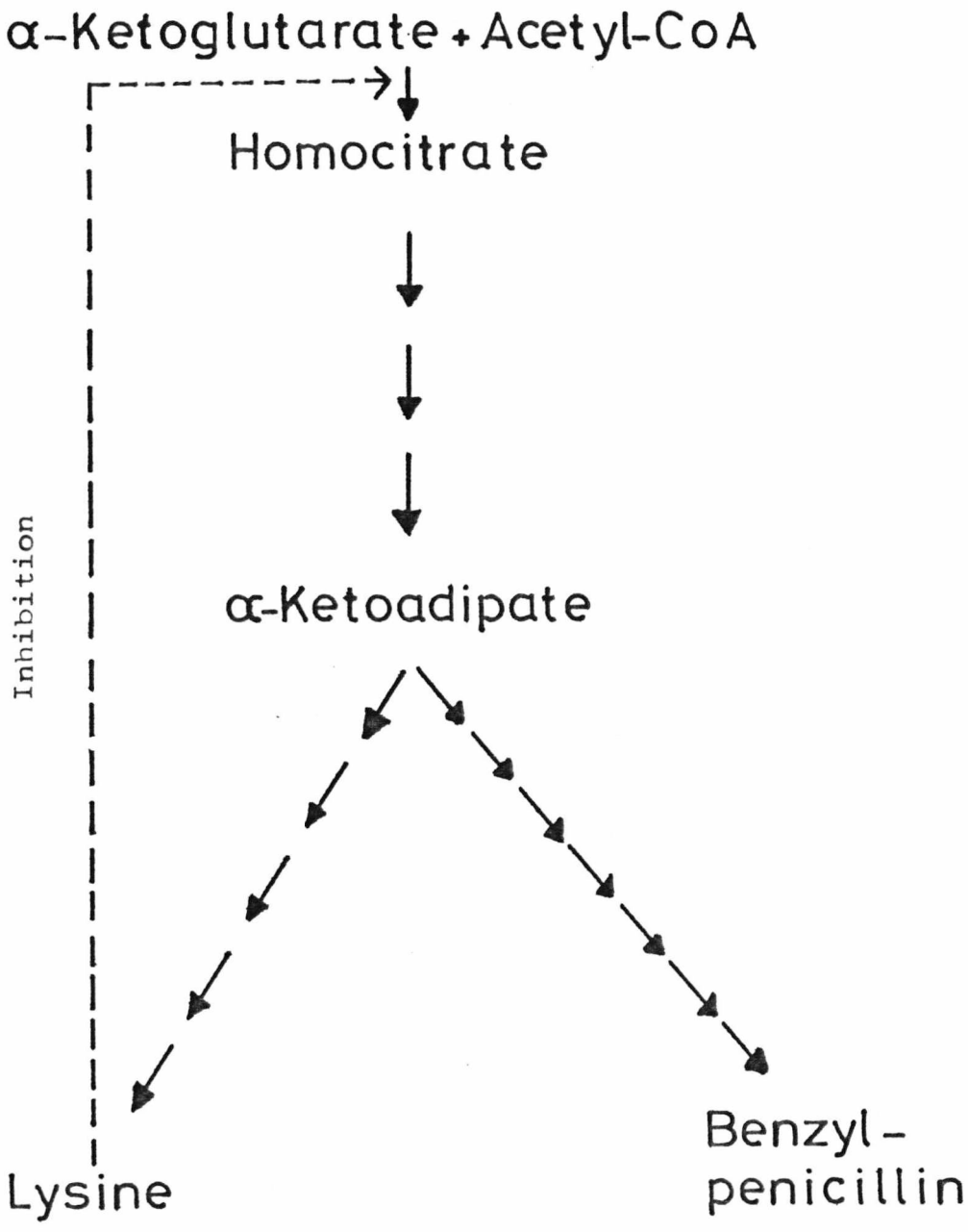
methionine as an inducer.

The roles that norleucine and methionine play in cephalosporin production are complicated by the fact that they both affect the morphology of the mycelia. When the fungus was grown in a medium containing sulphate, the mycelia were filamentous, but in a medium containing either methionine or norleucine (Drew, Winstanely and Demain, 1976) the mycelia were swollen, irregular and highly fragmented into athrospores. Whether this effect is related to antibiotic production was not investigated.

Before an account of other effectors of secondary metabolism, the difficulty of determining whether a particular effector is active because of its relationship to secondary metabolism or because of indirect action needs to be mentioned. The best example of this is the production of penicillins by Penicillium chryogenum. This involves part of the pathway for lysine biosynthesis. Lysine can apparently act as a negative effector of homocitrate synthase (Demain and Masurekar, 1974). It should, therefore, not only inhibit its own synthesis but also the synthesis of penicillin (Figure 1.3). Mutants that had an increased ability to produce lysine showed a proportional decrease of penicillin formation (Masurekor and Demain, 1974). It is, therefore, difficult to appreciate the importance of lysine biosynthesis to penicillin production and vice-versa.

Figure 1.3

Probable pathway of penicillin biosynthesis in Penicillium chryogenum



Other more general effectors or regulators of secondary metabolism include carbon or nitrogen sources, inorganic phosphate and trace elements. (Weinberg, 1977, 1978; Demain *et al.*, 1979).

The possibility of repression or inhibition of enzymes for secondary metabolism by the catabolism of rapidly utilised carbon sources has been proposed by Demain (1979 *et al.*) and may be important, since many secondary metabolites can inhibit the growth of the producer organism. Nitrogen catabolite repression has been less extensively investigated, but has also been implicated in the production of certain secondary metabolites. (Dulaney, 1948). Before a general hypothesis for catabolite regulation of secondary metabolism can be put forward, however, it is important that more information is obtained from other pathways of secondary metabolite production. Nevertheless, for β-lactam biosynthesis there is good evidence that the time of appearance and the amount produced during growth (Demain *et al.*, 1979) reflect catabolite repression.

The relationship between the inorganic phosphate concentration of the culture medium and secondary metabolite synthesis varies between different organisms. Moreover, even micro-organisms that produce the same secondary metabolite are affected differently by inorganic phosphate. For instance, cyanide production by a pseudomonad (Meganathen and Castric, 1977) requires

a particular inorganic phosphate concentration for maximal cyanide biosynthesis. Above and below this concentration of inorganic phosphate cyanide production is lower. On the other hand, cyanogenesis by C. violaceum (Rodgers and Knowles, 1978) was stimulated as the inorganic phosphate concentration was increased from 10 to 100 mM, but higher concentrations of phosphate resulted in no further production of cyanide.

Conversely, production of candicidin (Martin and Demain, 1977) and prodigiosin (Witney, Failla and Weinberg, 1977) showed the reverse of this situation, where progressive inhibition of their biosynthesis occurred as the concentration of inorganic phosphate was increased.

The reason behind this effect of phosphate on secondary metabolism is unknown, but several ideas have been put forward. Gersh, Skurk and Romer (1979) have noted that phosphate inhibition of turimyan biosynthesis can be reversed by cyclic AMP and positive effectors of the adenyl cyclase system. They concluded that both phosphate and cyclic AMP are involved in transcription, thereby causing regulation of the types and amounts of proteins synthesised. The effect of phosphate in this process and cyanide production by the pseudomonad (Meganathen and Castric, 1977) requires de-novo protein biosynthesis.

Others (Drew and Demain, 1976) suggest that the effect of changing the exogenous phosphate level may be due to it causing a shift in the ratio of adenine-nucteotides (AMP, ADP and ATP) in the cell. It has been suggested that a high phosphate concentration stimulates ATP formation leading to a high energy charge (Atkinson, 1969; Knowles, 1977) which may inhibit or stimulate secondary metabolism. Solomonson and Spehar (1979) suggest that for Chlorella to form cyanide it is the ATP to ADP ratio that is important. However, there have so far been no studies on the relationship between phosphate level, energy charge and secondary metabolite synthesis.

The effect of trace elements in this process is also hard to discern. There are, nonetheless, several generalisations that can be made (Weinberg, 1978). The quantity of zinc in a culture medium is frequently critical for secondary metabolic processes by fungi and actinomycetes, whereas in Bacillus species the critical metal ion is often manganese and, in other bacterial species, it is usually iron.

No site of action for these metals has been recorded, although several proposals have been made. For example, it has been suggested that iron derepression of diphtheria toxin production is due to the production of an iron-binding protein (Murphy, Skiver and McBride, 1976). Alternatively, it has been proposed that phosphoglycerate phosphomutase is activated by manganese,

explaining the effect that this metal ion has in the Bacillus species (Oh and Freeze, 1976).

It is likely that the actions of phosphate and trace elements differ in different organisms and that they affect processes in such a way as to generate the need for secondary metabolite production, to help restore metabolism to its preferred state. Zinc, for instance, is sometimes associated with the activity of aldolases (Kowal, Cremona and Horecker, 1966), dehydrogenases (Hoch and Vallee, 1955), polymerases (Lattke and Weser, 1976) or carboxylases (Scrutton, Young and Litter, 1970) in fungal metabolism. The one thing that all these effectors (Phosphate and metal ions) have in common is that their time of addition to batch cultures is important, which supports the idea that it is the state of metabolism which is critical for secondary metabolite production.

The role of cyanide production in cyanogenic organisms

Having introduced secondary metabolism in general, it is possible to discuss the potential functions of cyanide production by cyanogenic microorganisms. Figure 1.4 records seven proposals for cyanide production. These are in essence similar to those put forward to explain secondary metabolism in general and are open to the same comments.

Proposals (i) and (iv) may explain the continued selection of some cyanide producers, but many cyano-

Fig. 1.4

Proposals for cyanide production by living organisms

(i) To increase competitiveness.

(ii) To prevent predatory activity.

(iii) To enable parasitism.

(iv) To act as a carbon and/or nitrogen source.

(v) To regulate the levels of a particular metabolite(s) within the cell (below potentially toxic or lethal levels).

(vi) Cyanide production has no role, but is an evolutionary relic.

(vii) That cyanide is produced only during autolysis of dead cells.

genic microorganisms in their normal environments must produce very low amounts of cyanide. It is unlikely that this is an important factor.

Proposal (ii) would also be of significance for only a limited number of cyanide producers. Proposal (iii) is likewise of importance to an equally small number of cyanogenic organisms of which some isolates of the snow mould basidiomycete are examples (see later section).

The concept that cyanide production is an evolutionary relic (proposal vi) is doubtful for the same reasons as previously stated for secondary metabolism in general and proposal (vii) has been shown not to be true for at least one organism (Ward and Lebeau, 1962).

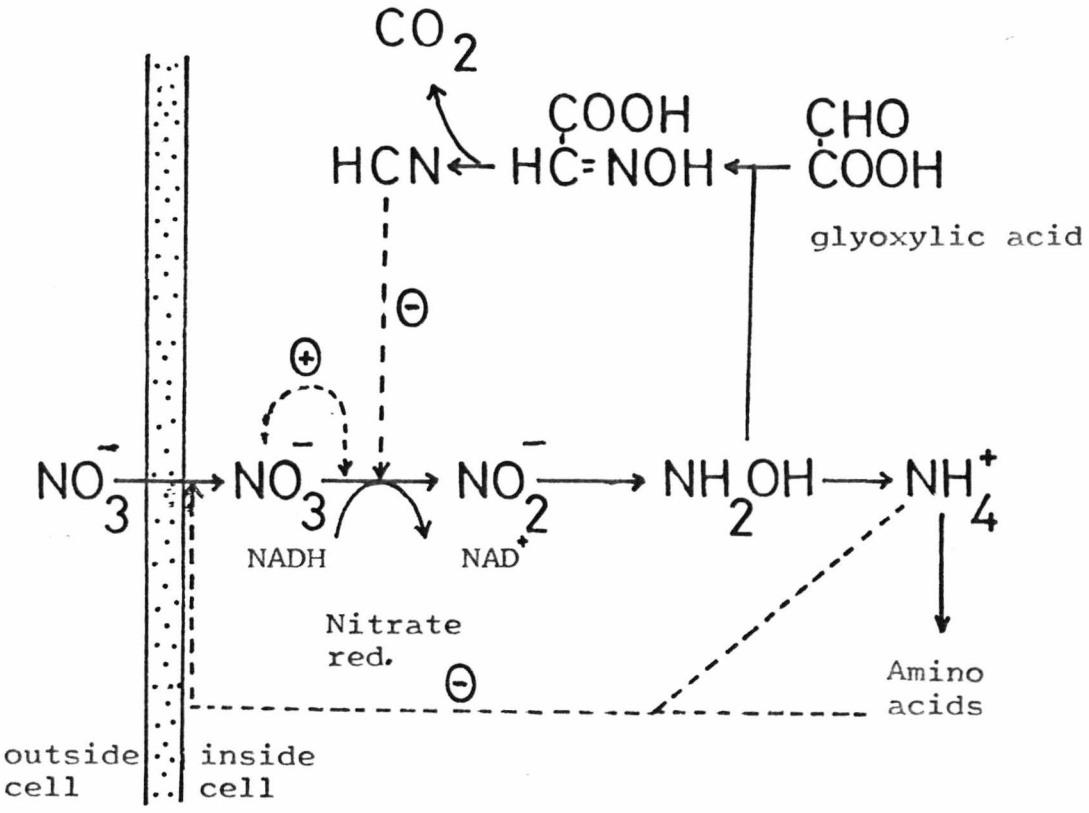
Therefore, we are left with proposal (v) or some other unforeseen reason. Proposal (v) would concern cyanide production in the regulation of metabolism. However, what are the areas of metabolism likely to be involved?

Amino acids (see next section) are the direct precursors of cyanide production in all the cyanogenic organisms so far investigated. The likelyhood that cyanide production could be a means of regulating intracellular amino acid pools must be considered, but why produce cyanide (or cyanohydrins)? To answer this question requires much more information than is

Figure 1.5

Regulation of nitrate reductase

by cyanide



available to date. In the meantime, one can suggest several possibilities. Bacterial cyanogenesis, as will be seen, is dramatically affected by compounds that are capable of donating methyl groups. (e.g. methionine). It is thus possible that cyanide production from glycine may be involved with regulation of the size, composition or turnover of the carbon-one pool of these organisms.

In the algae, a different role has been proposed (Solomonson and Spehar, 1977, 1979). Here cyanide is thought to be involved in the regulation of nitrate reductase activity which is specifically inactivated by minute quantities of cyanide (Figure1.5). The significance of this in the algae is complicated by the many possible precursors for cyanide (Solomonson and Speher, 1979; Pistorius *et al.*, 1977).

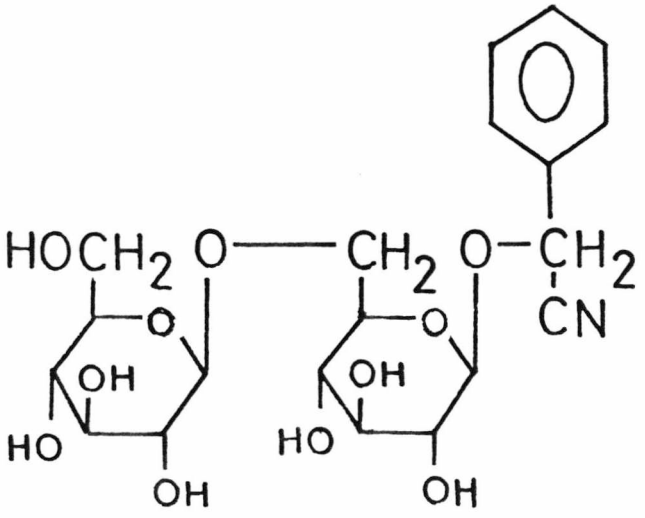
Cyanide production by higher plants

As mentioned previously, many plants produce cyanide, of which several are of agricultural importance. (e.g. alfalfa, peaches, almonds, beans, sorghum and flax).

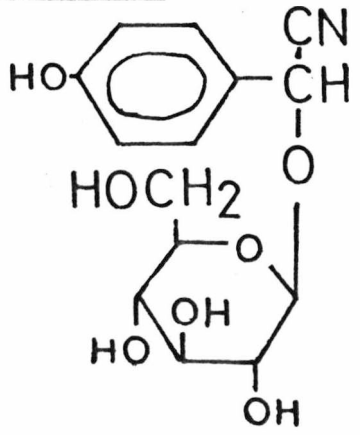
The biosynthesis of cyanide is via a multistep process and begins in most cases from an amino-acid (Conn, 1979) which is catabolised to either a cyanogenic glucoside (for example, Fig.1.6) of which 23 are known (Seigler, 1977; Eljolfssen, 1970) or, less commonly, to a cyanogenic lipid (Mikolajczak, 1971)

Figure 1.6

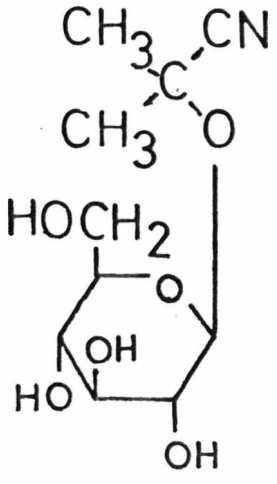
Structures of some common cyanogenic glycosides



Amygdalin



Dhurrin



Linamarin

from which cyanide is released.

Details of this pathway have been gathered for many years, which was initially based on studies involving radioactive amino acids. These showed that the carboxyl group of the amino acid was lost as CO_2 and that the α-carbon plus the amino-nitrogen were used to produce the nitrile group of the cyanogenic glucoside (Butler and Conn, 1964), without breakage of the carbon-nitrogen bond. This indicated that all the intermediates in the pathway must contain nitrogen.

The nature of these intermediates was revealed by Tapper and Butler (1971) to include aldoximes, nitriles and α-hydroxynitriles. However, when [^{14}C-] labelled L-tyrosine was administered to sorghum seedlings little of the radioactivity became associated with these types of compound and it required the use of trapping experiments to substantiate their role as intermediates.

An example of this can be taken from the data of Tapper and Butler (1971). To shoots of flax seedlings they administered isobutyraldoxime and isobutyronitrile, simultaneously with [^{14}C] valine. After incubation, the plant tissues were extracted and the intermediates administered were isolated, purified and examined for radioactivity. The aldoxime and the nitrile had acquired [^{14}C] from the [^{14}C] valine. These results were used to propose the pathway in Figure 1.7 (Hahlbrock et al.,1968; Conn, 1979). Further important proof

of this sequence has been provided by studies with cell-free enzyme systems.

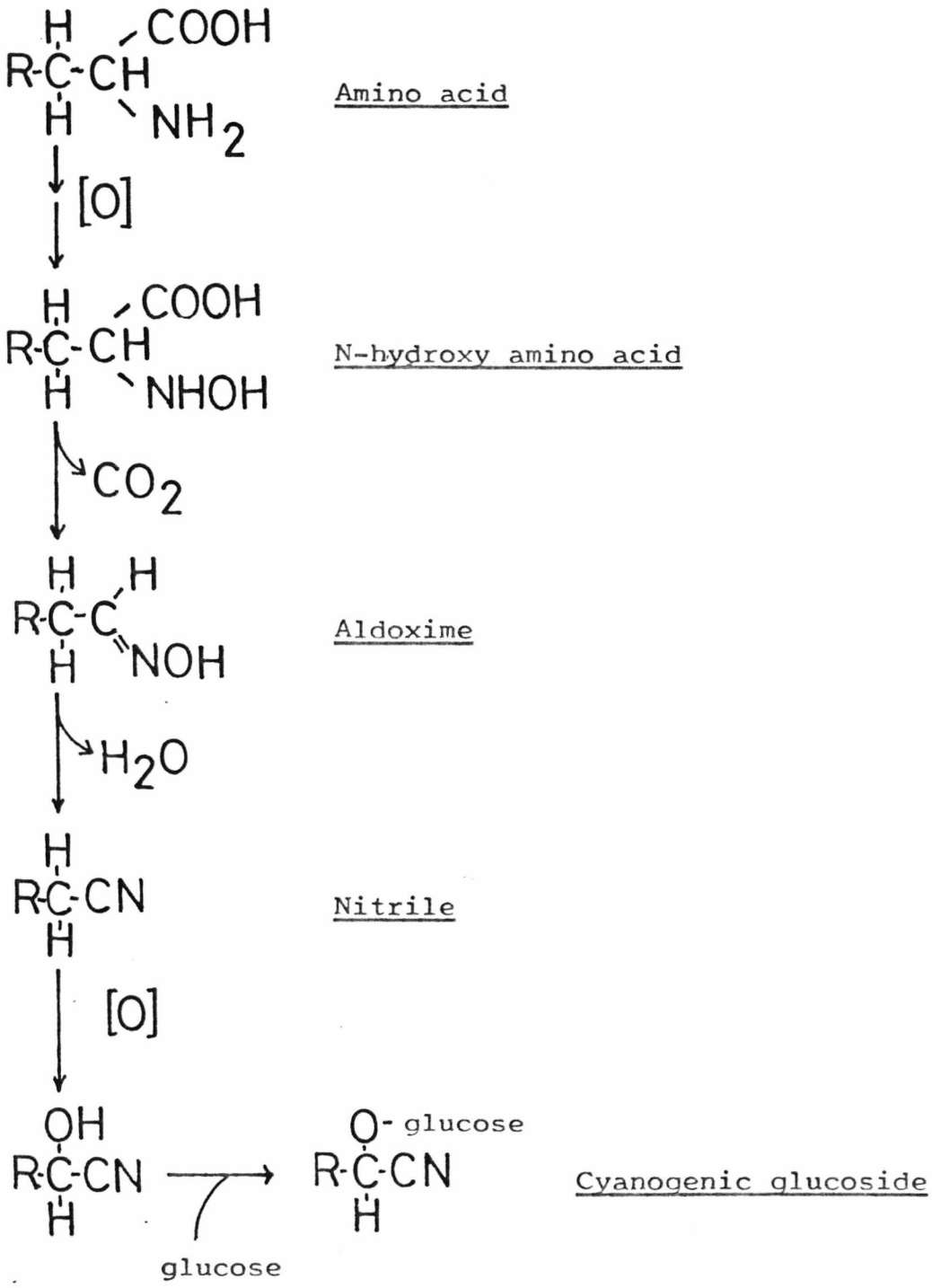
The first step in this procedure has been confirmed in the case of production of dhurrin, where [^{14}C]-N hydroxytyrosine was produced from L- [U-^{14}C] tyrosine in the presence of NADPH, O_2 and N-hydroxytyrosine as a trap. In addition, sorghum particles will utilise N-hydroxytyrosine, converting it to the aldoxime nitrile and p-hydroxymandelonitrile (Moller and Conn, 1978).

Production of an aldoxine from a hydroxy-amino acid (step 2) has been obtained by soluble enzyme systems in three crucifers (Kindl and Underhill, 1968). The existence and order of the third and fourth steps has been revealed from investigations involving sorghum particles. (Shimada and Conn, 1977). Finally, step five (glycosylation of the α-hydroxynitriles) occurs in flax and sorghum. Glycosyl transferases from these organisms catalyse the transfer of glucose from UDP-glucose to the appropriate α-hydroxynitriles, but show differing degrees of substrate specificity. (Hahlbrook and Conn, 1970; Reay and Conn, 1974).

Use of particulate enzyme systems from sorghum have revealed that the biosynthesis of dhurrin in this plant is a highly channelled process. Thus, several biosynthetic enzymes found in preparations

Figure 1.7

The biosynthetic pathway for cyanogenic glucosides in higher plants



appear to be organised such that they can accept the substrate formed from the preceeding enzyme in the pathway, in preference to the same compound when added externally.

Catabolism of cyanogenic glycosides to produce the appropriate aldehyde and cyanide (Figure 1.8) occurs in two steps. A glucosidase removes glucose to produce the α-hydroxynitrile from which an oxynitrilase acts to release cyanide. In certain cases cyanide release may be by enzymes derived from organisms that parasitise the cyanogenic plant. For example, pathogenic types of basidiomycetes (such as isolates of the snow mould basidiomycete; (Coletelo and Ward, 1961) have been shown to produce extracellular β-glucosidases. The release of cyanide from the α-hydroxynitrile may then be by a fungal oxnitrilase or nonenzymatically.

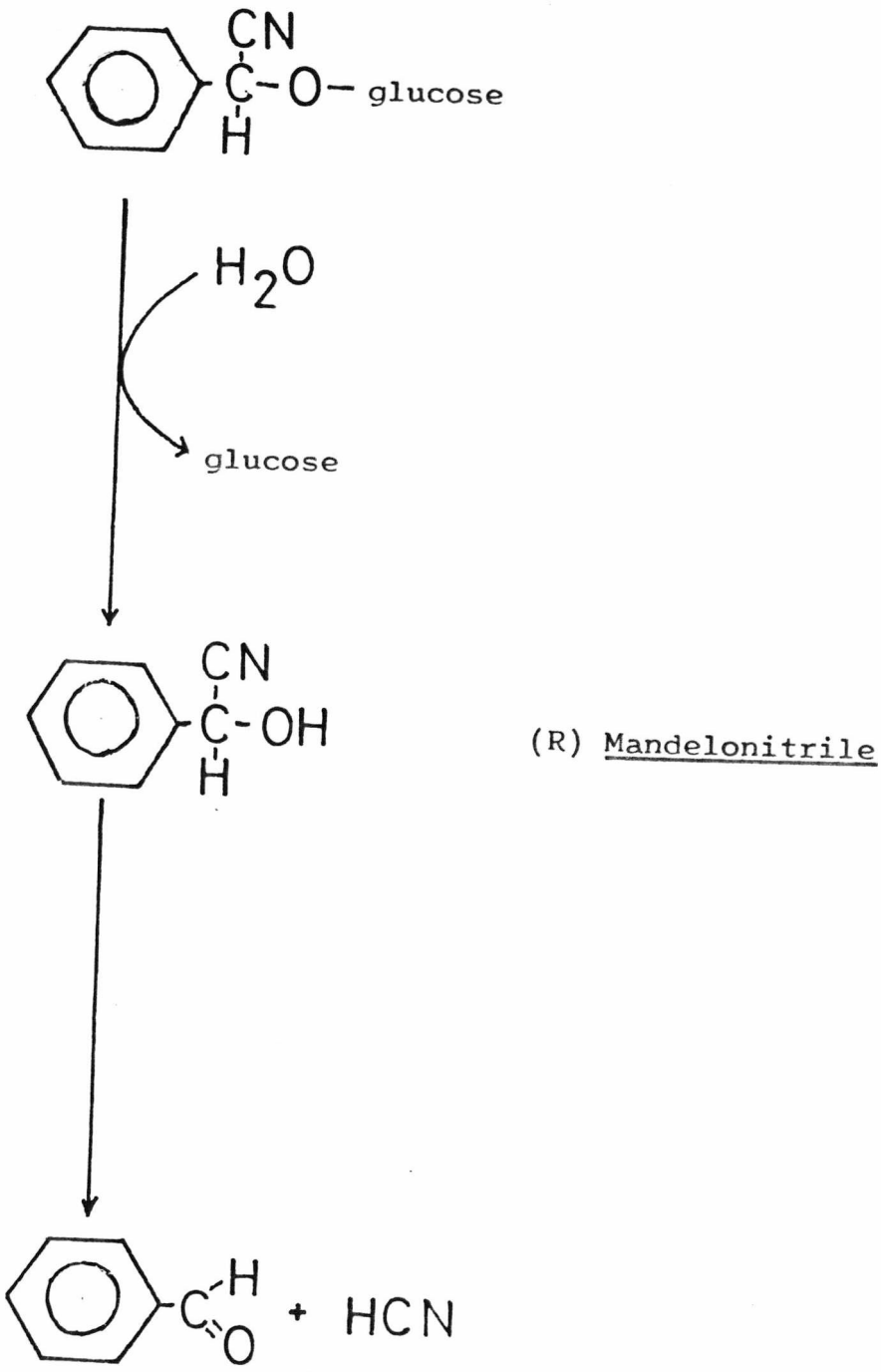
Cyanide production by photosynthetic microorganisms

Cyanogenesis by photosynthetic microorganisms has been reported for only the alga Chlorella vulgaris and the cyanobacterum Anacystis nidulans (Gewitz et al., 1974; Pistorious et al., 1979). Little is known of the pathway of cyanide production in these microorganisms but in both amino acid oxidase activity is important.

For example, when amino-acid oxidases were supplemented with a particulate preparation derived from

Figure 1.8

Cyanide production from prunasin



C. vulgaris, peroxidase or a redox metal ion, cyanide could be produced from several amino acids (Pistorius et al., 1977). The C. vulgaris particulate fraction was considered to act more like a metal ion than as a peroxidase. D-histidine was the best substrate for cyanide production by both organisms.

Other workers (Solomonson and Spehar, 1979) have shown that cell-free extracts of C. vulgaris can produce cyanide from glyoxylic acid oxime. It remains to be seen whether cyanide production by algae occurs via a similar pathway to those found in higher plants. Moreover, since glyoxylic acid oxime could be an intermediate in cyanide production from glycine by bacteria and fungi (see later) this could be a very important observation.

Bacterial cyanogenesis

Cyanide production by bacteria has been identified in only a few species (Knowles, 1976). It is likely, though, with the information now available about culture medium composition for optimal cyanide production and the availability of recently-developed sensitive assays for cyanide, that many more cyanogenic bacteria will soon be identified.

Glycine is the only amino-acid that is known to be directly catabolised to cyanide by bacteria (Lorck, 1948) and its inclusion in the growth medium is required for maximal cyanide production (Brysk, Corpe and Hankes,

1969; Brysk, Lauinger and Rissler, 1969; Castric, 1975, 1977; Michaels and Corpe, 1965; Wissing, 1974).

Incubation of non-proliferating cells of *Chromabacterium violaceum* with $[1\text{-}^{14}C]$ or $[2\text{-}^{14}C]$ glycine (Michaels, Hankes and Corpe, 1965) revealed that about 10% of the $[1\text{-}^{14}C]$ glycine was converted to CO_2 and 11% of the $[2\text{-}^{14}C]$ glycine to cyanide. The cyanide produced had a specific activity of 94% of the added $[2\text{-}^{14}C]$ glycine, whereas with $[1\text{-}^{14}C]$ glycine the specific activity of the cyanide produced was below 0.1% of the initial value. It has been shown in a *Pseudomonas species* (Wissing, 1974) that the molar ratio of cyanide produced to the total glycine consumed was 1:1·1. Therefore, it seems likely that the pathway of cyanide production in these bacteria can be represented by:-

$$NH_2\overset{+}{C}H_2\text{-}\overset{O}{C}OOH \longrightarrow \overset{+}{H}CN + \overset{O}{C}O_2 + 4H$$

However, in *Pseudomonas aeruginosa* (Castic, 1977) $H^{14}CN$ was produced to an equal extent from either $[1\text{-}^{14}C]$ glycine or $[2\text{-}^{14}C]$ glycine. Equal amounts of $^{14}CO_2$ and $H^{14}CN$ were obtained from $[1,2\text{-}^{14}C]$ glycine. This would imply that the stoichiometry of the process is the same but that the carbon atoms of glycine became scrambled prior to the conversion to cyanide. Whether this was due to primary or secondary metabolism of

glycine was not determined.

The production of cyanide by bacteria has also been shown to be stimulated by methionine. Stimulation of cyanogenesis in C. violaceum could also be caused by betaine, N,N,-dimethylglycine and choline (Michaels and Corpe, 1965). It was subsequently shown that methionine did not act as a precursor of cyanide (Michaels et al., 1965). This effect of methionine also occurs in a cyanogenic Pseudomonas species (Wissing, 1968; Castric, 1977), but its role in cyanide production remains unknown, as do the effects of inorganic phosphate and iron (as mentioned in a previous section).

To conclude, the effectors of bacterial cyanogenesis demonstrated in the studies by Wissing(1974) must be mentioned. He has shown that, in a Pseudomonas species cyanide production was low under anaerobic conditions. Oxygen and artificial electron acceptors (phenazine methosulphate, methylene blue, 2, 6-dichlorophenol indophenol and ferricyanide) could somewhat stimulate cyanide production. The stimulation of cyanide production that occurred in the presence of oxygen could be reduced by inhibitors of flavin-linked enzymes, but not by amytal or rotenone. However, it is not yet possible to state whether, in fact, the process is respiratory linked, peroxidative or due to an oxygenase.

Unlike both algal and plant cyanide production, there has been only one report of the production of cyanide by bacterial cell-free extracts (Wissing, 1975). No compounds similar to the plants cyanogens have been identified in cyanide-producing bacteria. It should be mentioned that the enzyme system for cyanide production by C. violaceum (Rodgers and Knowles, 1978) are induced during the late exponential phase of growth. Both glycine and methionine act as inducers for this system. This is an important observation that must be considered in future attempts to investigate cyanide production by cell-free extracts of this bacterium and presumably others.

Fungal cyanogenesis

Despite this being the second largest group of cyanide producing organisms, they remain only scantily studied in comparison to plant and bacterial cyanide producers. Of the 300 or more species of fungi that have been reported to be cyanogenic, only two basidiomycetes, Marasmius oreades and the snow mould fungus have been studied further. Both of these fungi are associated with plant diseases.

Snow mould disease, or winter crown rot, is associated with forage plants, such as alfalfa. The fungus that causes this disease is a psychrophilic cyanide-producing basidiomycete, which is particularly

Table 1.2

Characteristics of three types of isolates of a psychrophilic basidiomycete

Characteristics	Isolate A	Isolate B	Isolate C
Colony characteristics	Relatively slow growing	Fairly rapidly growing	Very rapidly growing
Cyanide formation in culture	None	Prolific	None
Cyanide formation on alfalfa in the field (*Medicago sativa*)	Prolific (600-655 $\mu g.ml.^{-1}$ in crown buds)	Moderate (175-240 $\mu g.ml.^{-1}$ in crown buds	None
Pathogenicity to *M. sativa* in the field	(a) 3-18% survival (b) 0-3% survival	(a) 85-95% survival (b) 47-60% survival	(a)) (b)) 100 % survival
Pathogenicity to *M. sativa* in a controlled environment	28-38% survival	68-81% survival	98% survival
Pathogenicity to a grass (*Dactylis glomerata*) in the field	62-70% survival	33-42% survival	100% survival
Cyanide tolerance in culture	Low (up to 50 µg. of HCN per ml.)	High (up to 250 µg of HCN per ml.)	Low (up to 50 µg. of HCN per ml.)

widespread in Western Canada. Normally, the fungus must become associated with the host plant (several plants can serve in this role, Cormack, 1948), during the autumn or early winter.

The disease probably develops, as follows. During the following Spring, when the snow covering the infected plants begins to thaw, the fungus produces a β -glucosidase (Coletelo and Ward, 1961), which causes cyanide production in the crown buds (by catabolising the plant cyanogens present) before invasion of the host tissues begins. (Lebeau and Dickson, 1953; Lebeau, Cormack and Moffat, 1959). The disintegration of the crown bud tissue then ensues.

Three isolates of the psychrophilic basidiomycete have been obtained (Ward, Lebeau and Cormack, 1961). Their characteristics are summarised in Table 1.2. Type A isolates cause production of cyanide in crown buds, are good pathogens, but produce low quantities of cyanide in culture. Type C isolates are poor pathogens and produce no cyanide in culture. Type B isolates are moderate pathogens, but are prolific cyanide producers in culture.

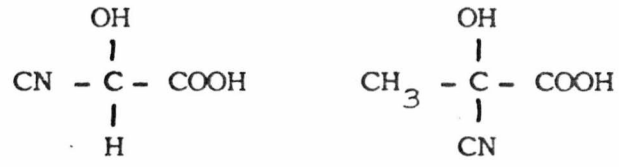
Cyanide production in culture by isolates of this fungus was subsequently studied using one of the type B group organisms, isolate W_2 (Ward and Lebeau, 1962). These workers claimed that cyanide was released into the

growth medium at the beginning of autolysis. In a later paper, it was reported that the production of cyanide was probably from a cyanogenic compound, which was unstable above pH6 and temperatures above 40°C, and which accumulated in the mycelia during growth (Ward, 1964).

Research was then directed to determine the nature of this cyanogenic compound, to see whether it was similar to any of the known plant cyanogens. Ward and Thorn (1966) showed that glycine was the best precursor of cyanide in the snow mould fungus, that the carbon-2 atom was converted to cyanide and the carbon-nitrogen bond of glycine was not broken during the synthesis (Ward, Strarrat and Robinson, 1977). It was also shown that the unidentified cyanogenic compound was produced in equal amounts in media supplemented with either $[1\text{-}^{14}C]$ or $[2\text{-}^{14}C]$ glycine, but that it became labelled only when $[2\text{-}^{14}C]$ glycine was present (Ward and Thorn, 1966). N- $[2\text{-}^{14}C]$ hydroxyglycine and $[2\text{-}^{14}C]$ glyoxylic acid oxime (possible intermediates of cyanide production from glycine) were very poor precursors of cyanide but, as this work was done using whole cells, the possibility that these compounds were not taken up by the fungal cells could be an explanation for these results. Furthermore, if the cyanide-producing

pathway was related to that observed for amino acid catabolism to cyanide in plants, the channelling of intermediates (mentioned previously) could also account for this phenomenom. The claim that other amino acids are converted to cyanogenic glucosides by this fungus (Stevens and Strobel, 1968) has been clearly shown to be erroneous (Ward, Thorn and Starratt, 1971).

More recently, the cyanogenic compounds produced by this fungus have been shown to be mainly the cyanohydrins of glyoxylic acid and pyruvic acid (Tapper and MacDonald, 1974):-



glyoxylic acid cyanohydrin

pyruvic acid cyanohydrin

In addition, these workers could find no evidence for cyanogenic glucoside production.

In contrast to cyanide production by bacteria, methionine, betaine and N,N-dimethylglycine were shown to inhibit cyanide production by the snow mould (Ward and Thorn, 1966). Formaldehyde, but not formic acid, had a similar effect. Further investigations of these effects were not recorded.

Marasmius oreades, associated with the fairy ring disease of grass lands, lawns, pastures and parks (Lebeau and Hawn, 1963) has been little studied. However, the close similarities between winter crown rot and the fairy ring disease suggests that the process of cyanide production by this fungus could be similar to that occurring in the snow mould basidiomycete. Examination of still cultures of M. oreades (Ward and Thorn, 1965) has shown that cyanohydrins of similar properties to those isolated from the snow mould basidiomycete were produced, but that no cyanogenic glycosides were formed.

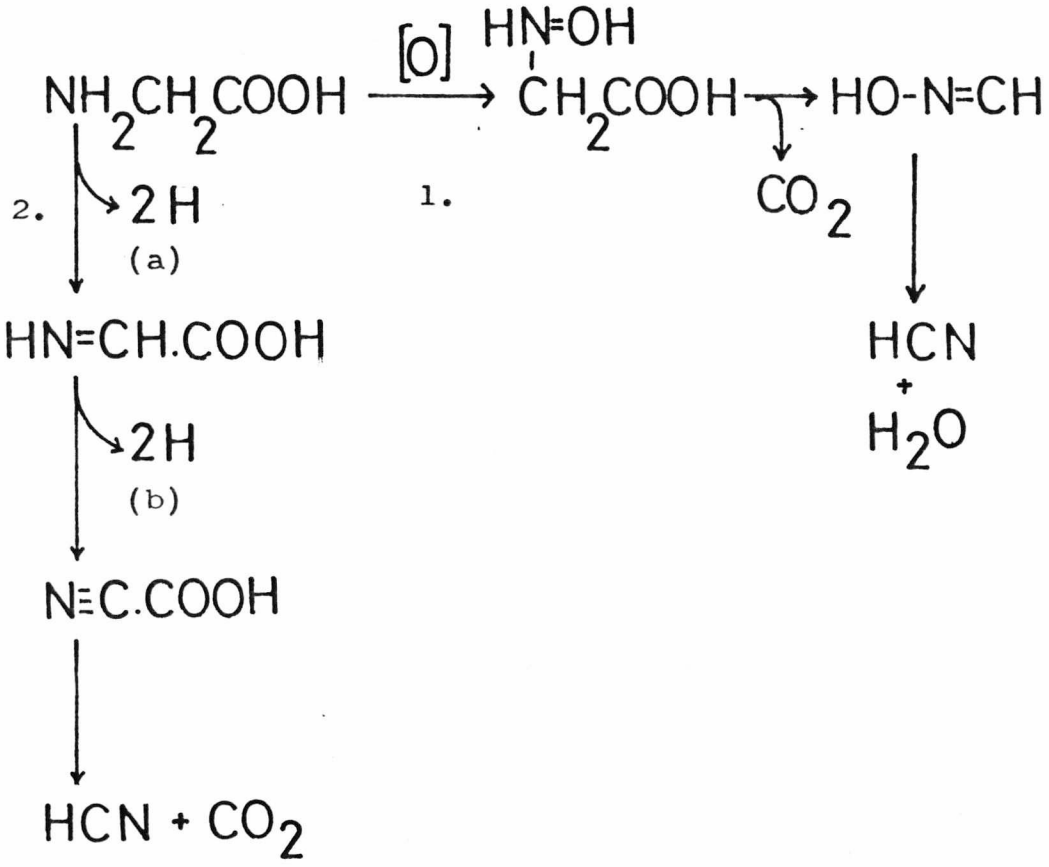
The mechanism of cyanide production by microbes

Conn (1973) has speculated that bacterial and fungal cyanide production from glycine proceeds via an analogous pathway to that observed in plants (Figure1.9 pathway (i)). Little evidence for this has been reported, although the absence of cyanogens produced from glycine would not be unexpected if this was the case.

Wissing (1974) produced an alternative route to explain the involvement of flavoproteins, suggested by the effects of inhibitors of these compounds on cyanide production by a Pseudomonas species (pathway(ii)). It is interesting to note that step 'a' could be peroxidative and flavin-linked and that the action of the enzyme could be related to that shown by glycine oxidase.

Figure 1.9

Proposed pathways for cyanide production from glycine by microorganisms



This would have special relevance to cyanide production by photosynthetic microorganisms (see earlier section) and it is further revealing to note that an imine has been postulated as an intermediate of glycine oxidation by glycine oxidase. (Hafner and Wellner, 1971, 1979).

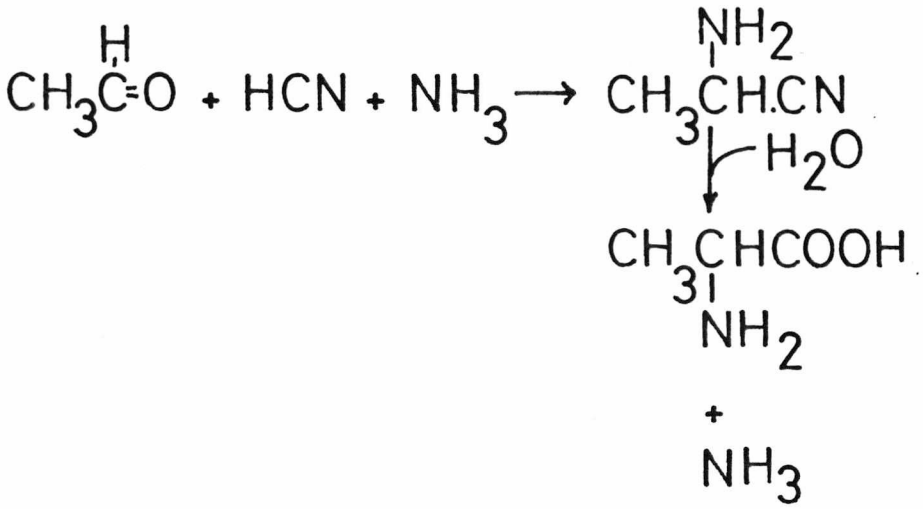
The resistance of microorganisms to inorganic cyanide: Catabolism or assimilation of cyanide and nitriles by fungi

It is hard to discern whether any of the pathways involved in the catabolism of cyanide have an assimilating or detoxifying function. Because of the reactivity of cyanide with aldehydes and ketones, the first step of many of these pathways presumably proceeds non-enzymically to an appreciable extent (Strobel, 1966).

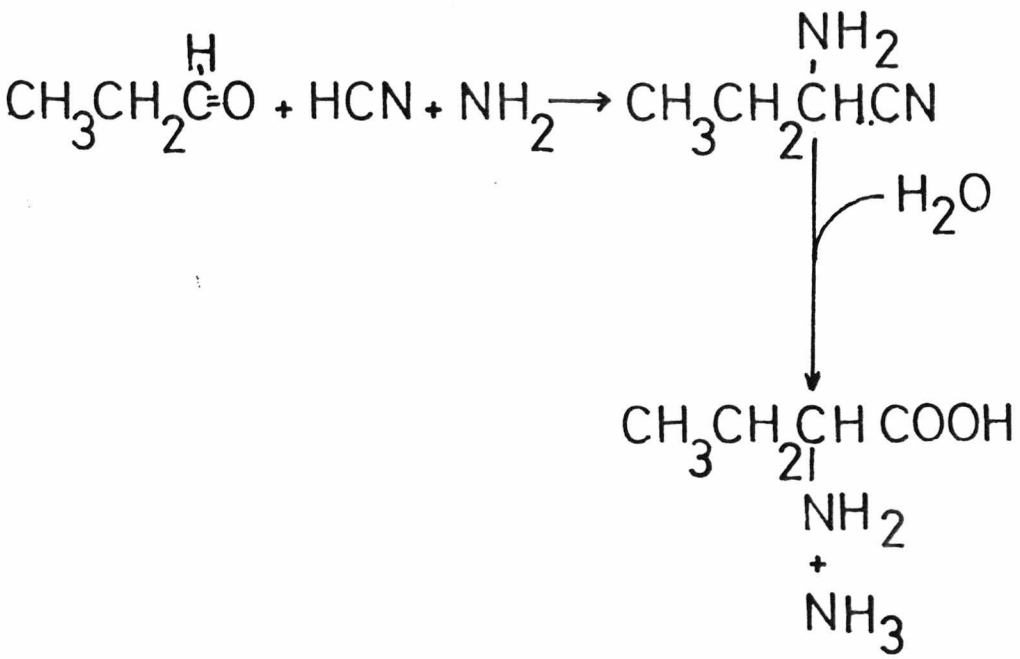
Allen and Strobel (1966) have shown that M. oreades Pholiota adiposa, Pholiota aurviella, Pholiota praecox and Rhizopus nigricans incorporate $H^{14}CN$ into alanine and, to a lesser extent, into other amino acids. They also showed that Fusarium nivale incorporated cyanide into asparagine only and that no amino-acids became labelled during incubation of Clitocybe illudens, Rhizoctonia solani, Aspergillus flavus and Fusarium solani, with $H^{14}CN$. In addition, the snow mould basidiomycete was shown to incorporate cyanide into alanine and glutamate (Strobel, 1964). Incubation of mycelia with $H^{14}CN$ resulted in the incorporation of

Figure 1.10

(a) Alanine production by the snow mould basidiomycete from acetaldehyde, ammonia and cyanide



(b) α-Aminobutyric acid fromation by Rhizoctonia solani from propionaldehyde, ammonia and cyanide



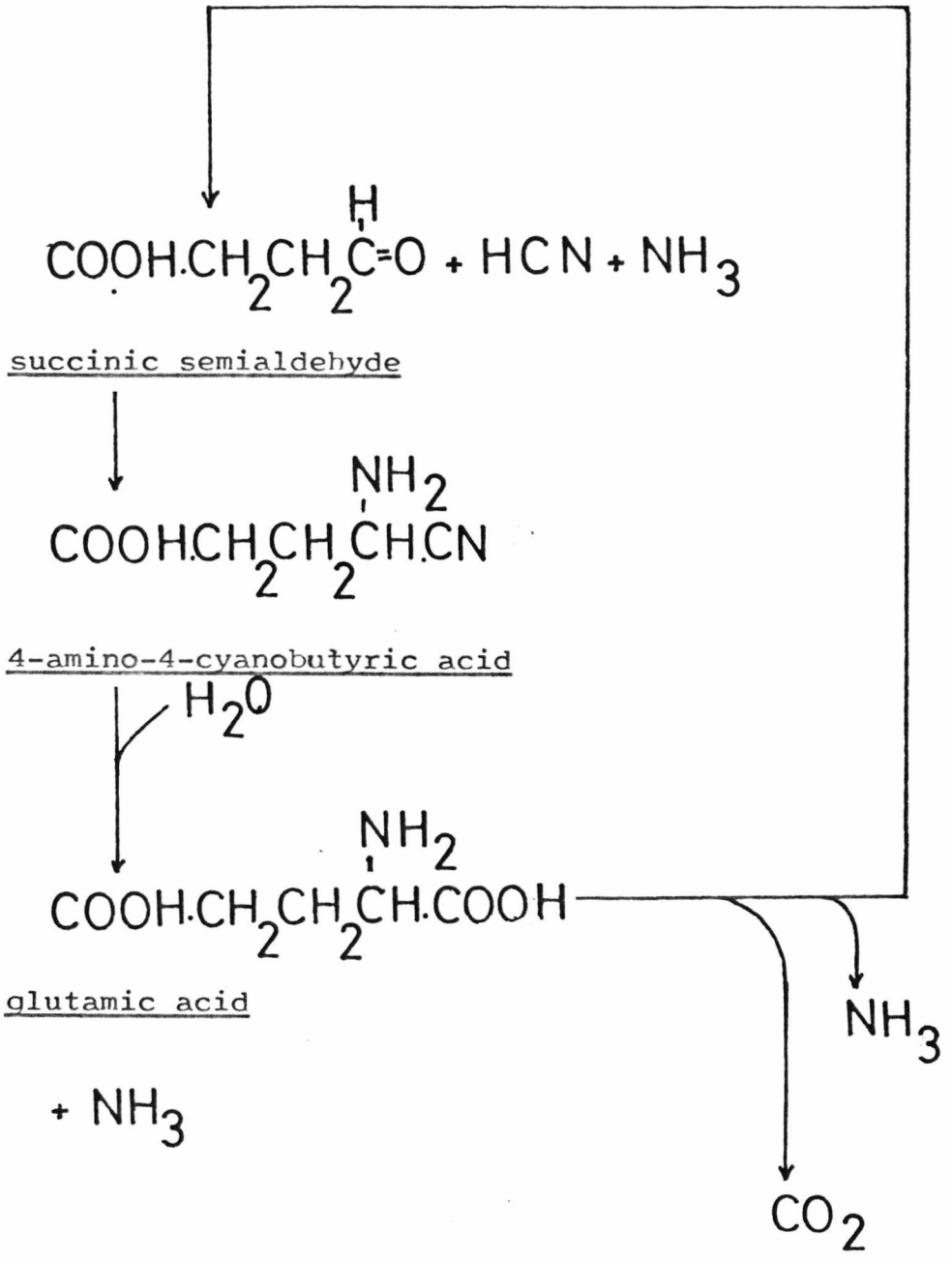
label during the first 12h. mainly into α-amino-propionitrile. As the amount of radioactivity associated with this compound fell throughout the next 12h. of incubation, labelled alanine was steadily produced. Using $H^{14}C^{15}N$, it was shown that the carbon atom of cyanide appeared exclusively in the C_1 atom of both α-aminopropionitrile and alanine. The nitrogen atom first appeared in the cyano group of the nitrile but was not present in alanine. These results suggest the pathway for alanine synthesis given in Figure 1.10. Production of α-aminopropionitrile by extracts prepared from the fungus supports this proposal, but the conversion of the nitrile to alanine by extracts was not recorded.

A similar type of pathway has been proposed for the synthesis of glutamate (Fig.1.11). This involves succinic semialdehyde, which could combine with HCN and NH_3 to produce 4 amino-4 cyanobutyric acid. Little activity in cell-free extracts was found for this step, although 4-amino4-cyanobutyrate nitrilase activity was observed. In addition, glutamate decarboxylase, succinic semialdehyde dehydrogenase and 3-aminobutyrate glutamate transaminase activities were also present and a cyclic pathway for conversion of HCN to CO_2 was proposed by Strobel (1967).

The amounts of alanine and glutamic acid that became labelled during incubation with $H^{14}CN$ were

Figure 1.11

Cyanide detoxication by the snow mould basidiomycete with glutamic acid as an intermediate



extremely small and the role that these pathways have in the assimilation or detoxification of cyanide would appear to be of little significance. Even so, it would be interesting to see if the nitrilase activities in this fungus are of broad specificity This may reveal whether these enzymes are used to catabolise any nitriles produced (due to the reactivity of cyanide), to give an aminoacid as the product since this type of pathway has also been reported to occur in at least one other fungus (Allen and Strobel, 1966).

Other pathways of cyanide catabolism by fungi involve its conversion to CO_2 and at least one fungus can degrade aromatic nitriles (Harper, 1977) by a nitrilase of broad specificity to produce a carboxylic acid with the release of ammonia.

Work in this area originally began by an investigation of the mechanism by which pathogenic fungi that release cyanide from their host cyanogens are able to grow in the presence of relatively high concentrations of cyanide (Fry and Millar, 1971a).

An example is the work on Stemphylium loti, which is associated with the copperspot disease of birds foot trefoil (Lotus corniculatus). It was shown that this fungus induced, on exposure to cyanide, a formamide hydrolyase, which converts cyanide to formamide

(Fry and Millar, 1971a, 1971b, 1972). Subsequent work has revealed that formamide hydrolyase activity is associated with the synthesis of an alternate electron transport chain, both of which are sensitive to salicyl hydroxamic acid. (Rissler and Millar, 1977). The existence of this enzyme in other fungi has recently been revealed (Fry and Evans, 1977). It was recorded that formamide hydrolyase was produced by only one out of six fungi not pathogenic to plants, nine out of fourteen pathogens of non-cyanogenic plants and all eleven pathogens of cyanogenic plants. To conclude this section, many methylotrophic bacteria can grow on formamide as a carbon and nitrogen source (Putilina, 1961). It may, therefore, be possible to isolate organisms that can use cyanide for growth.

Catabolism and assimilation of cyanide and nitriles by bacteria and plants

To date, no pathways involving nitrile formation from aldehydes, HCN and NH_3 have been reported. Nevertheless, several observations of nitrile metabolism by bacteria have recently been recorded (Harper, 1977; Digeronimo and Antoine, 1976; Firmin and Gray, 1976). The number of nitriles that could be metabolised by Nocardia rhodochrous LL100-21 is impressive. Aceto - nitrile, hydroacrylonitrile and propionitrile could all act as both carbon and nitrogen sources for growth

and, although less favourable, butenitrile, succinonitrile or acetolanitrile could also be used. It was shown that the enzyme system for hydrolysis of acetonitrile was both intracellular and inducible. Furthermore, the hydrolysis was shown to be a two-step process with acetamide as an intermediate. The final products were ammonia and acetic acid.

It is revealing to compare fungal cyanide catabolism with this process, as the pathways would appear to be similar and can be summarised as:-

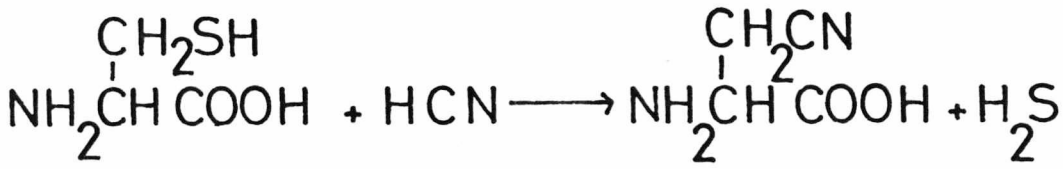
$$R\text{-}CN \longrightarrow R\text{-}CONH_2 \xrightarrow{NH_3} R\text{-}CO_2H \quad (\longrightarrow CO_2)$$

Presumably the production of CO_2 and NH_3 from cyanide by *Bacillus pumilus* could also proceed by this route but this interpretation is complicated by the observation that $^{14}CO_2$ appeared in the medium before $^{15}NH_3$ when cells were incubated with $K^{14}CN$ or $KC^{15}N$ (Skowronski and Strobel, 1969). Radioactive CO_2 production, from $K^{14}CN$ has also been observed in sewage cultures (Raef, Characklis, Kessick and Ward, 1975).

Probably the most common pathway for cyanide catabolism in plants and bacteria is the production of β-cyanoalanine, which can then be further metabolised to asparagine, aspartic acid or the dipeptide γ-glutamyl-β-cyanoalanine. The synthesis of β-cyanoalanine in

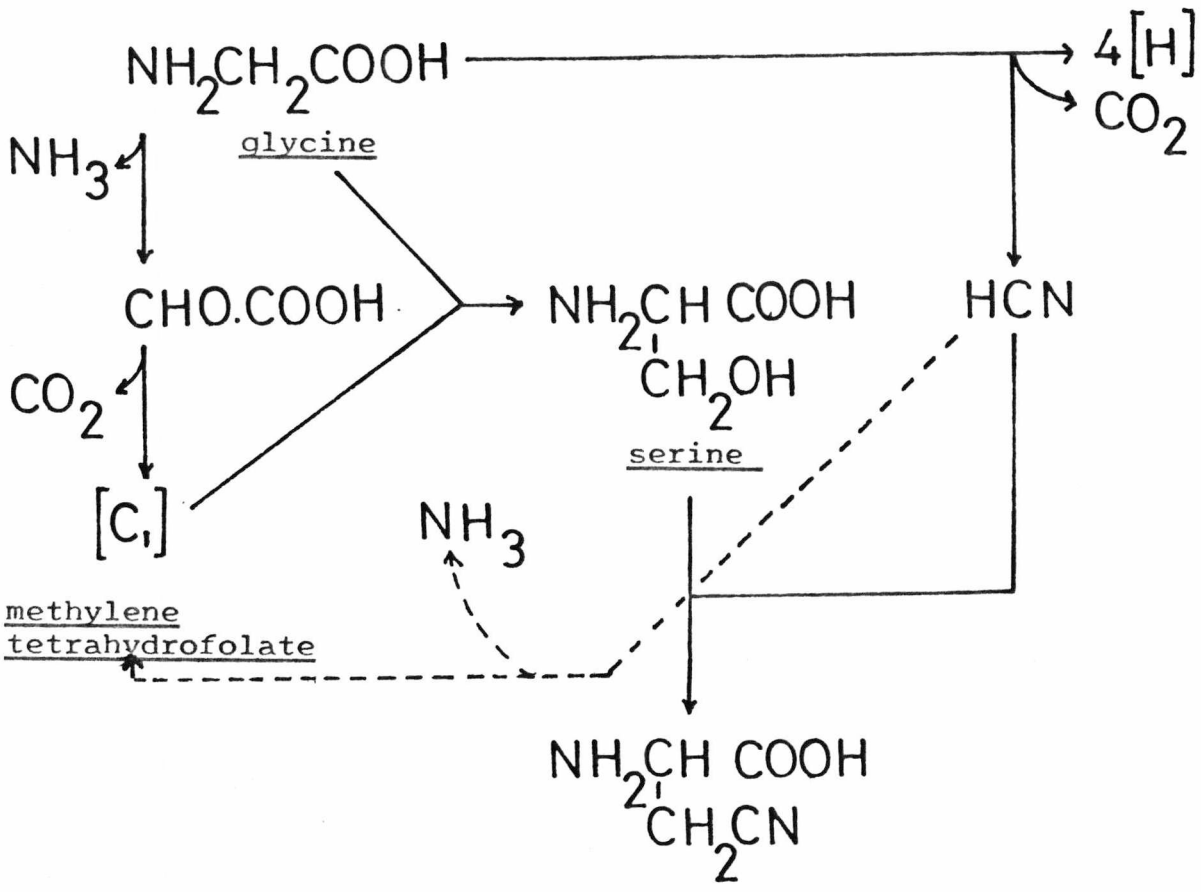
Figure 1.12

(a) β-cyanoalanine biosynthesis in plants



cysteine

(b) β-cyanoalanine biosynthesis in Chromobacterium violaceum

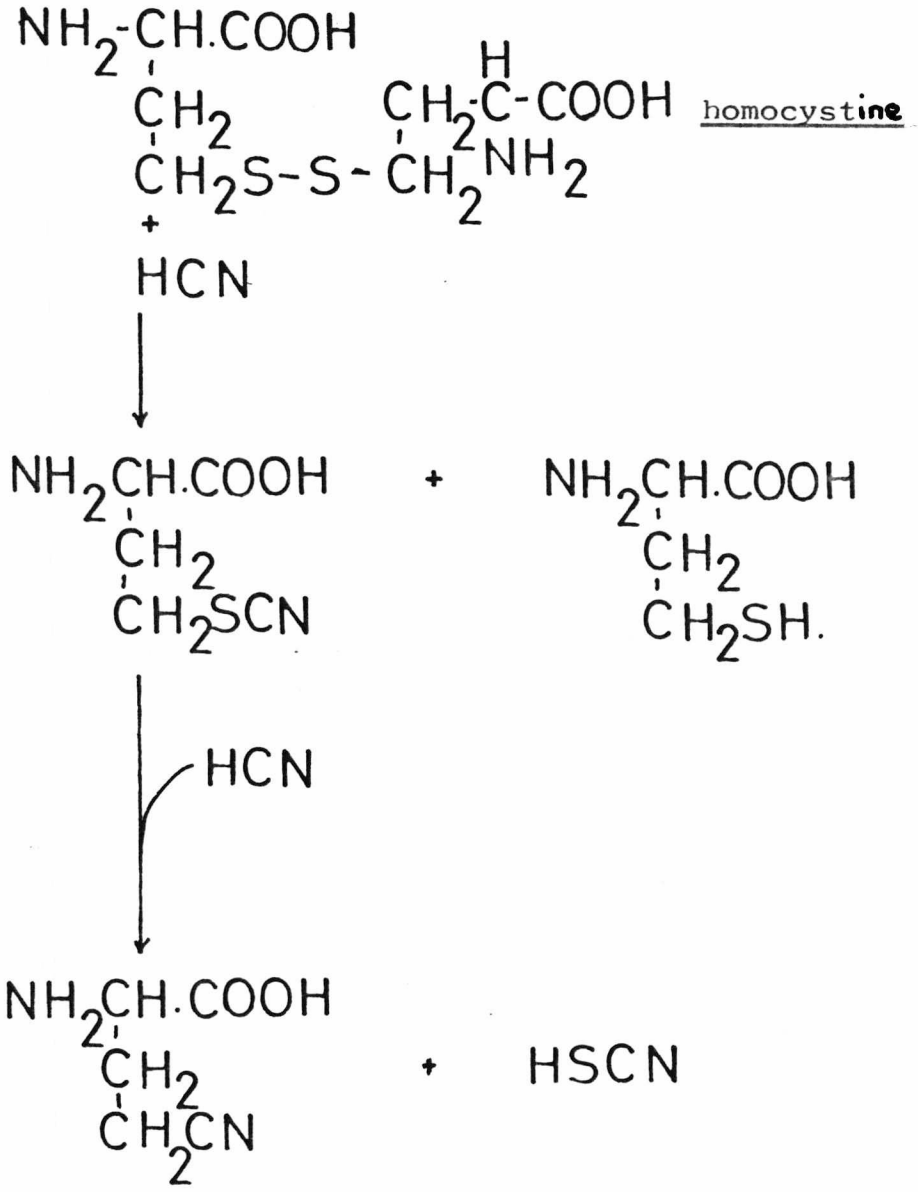


plants is from cysteine and HCN (Fig.1.12a Akopyan, Braunstein and Goryachenkova , 1975 ; Blumentahl, Hendrickson, Abrol and Conn, 1968; Hendrickson and Conn, 1969), although o-acetyl serine may also be used. β-cyanoalanine production by bacteria uses serine and HCN as precursors (Brysk Corpe and Links, 1969). Brysk et al. (1969) have shown, using radioactive glycine and formaldehyde, that serine can be produced from these compounds by C. violaceum and then subsequently converted to asparagine via β-cyanoalanine. Their data have been summarised in Figure 1.12b. Not mentioned in their report, but considering the preceeding discussion of CO_2 production from cyanide, it is also possible that a carbon-one unit was required for the synthesis of serine, which could have been provided by catabolism of cyanide. This would result in the removal of three molecules of glycine for every molecule of β-cyanoalanine synthesised. Other pathways of cyanide assimilation have also been identified in C. violaceum (Rodgers and Knowles, 1978; Brysk and Ressler, 1970). It has been shown, for example, that γ-cyano-α-aminobutyric acid may be synthesised from homocystine and cyanide by a purified enzyme. The pathway proposed is shown in Figure 1.13.

In addition to this pathway for cyanide catabolism C. violaceum, E. coli and P. aeruginosa have been shown to possess the enzyme rhodenese (Rodgers and

Figure 1.13

γ-Cyano-α-aminobutyric acid synthesis
by Chromobacterium violaceum



Knowles, 1978; Lang, 1933; Ryan, Gourlie and Tilton, 1979). This enzyme catalyses the following reaction:-

$$S_2O_3^{2-} + CN^- \longrightarrow SO_3^{2-} + SCN$$

and has been shown to be located in the periplasmic space in P. aeuroginosa (Ryan et al., 1979).

Recently, it has been shown (Rodgers and Knowles, 1978) that these enzymes are induced by C. violaceum only after cyanide production has occurred, and cyanide presumably acted as an inducer for their production. The effect of glycine and methionine on the induction and activity of these enzymes was complicated. Studies on the enzymology of these enzymes have shown that β-cyanoalanine synthase activity is about 60% inhibited by 2.0 mM methionine and higher concentrations caused little further inhibition. Glycine does not affect the activity of this enzyme. Rhodenese and γ-cyano-α-aminobutyric acid synthase were unaffected by glycine or methionine.

Cyanide resistant respiration

Examples of cyanide resistant respiration are numerous in the plant, animal and microbial kingdoms. (Henry and Nyns, 1975). The majority of these organisms, of course, do not produce cyanide.

Alternative electron transport systems in bacteria are cytochrome-linked (Niven, Collins and Knowles, 1975; Ashcroft and Haddock, 1975; Arima and Oka, 1965;

Mizushinia and Arima, 1960), whereas in various eukaryotic microorganisms they are independant of the cytochrome-linked respiratory system (Henry and Nyns, 1975; Lloyd, 1974). In the latter organisms, the so-called salicylhydroxamic acid (SHAM) - sensitive pathway is involved, the components of which are still uncertain.

It is interesting that the proposed roles for these cyanide-resistant respiratory pathways are similar to those put forward for secondary metabolism. Their induction is often associated with batch culture age or the ageing of cells (Marsh and Goddard, 1939; Matsunaka, Morita and Conti, 1966; Henry, Hamaide-Delplus and Nyns, 1974). It is particularly interesting that, in some batch cultures, the induction takes place during the early idiophase (e.g. in Candida lipolytica; Henry et al., 1974). Observations that the alternative SHAM-sensitive pathway does not couple electron transport to the phosphorylation of ADP (Bonner and Bendall, 1968; Henry and Nyns, 1975) lead to one particularly interesting possibility for the ubiquitous distribution of cyanide-insensitive respiration. This is that it is used to eliminate toxic substances or unnecessary metabolites in micro-organisms which have reached the stationary phase of growth, under conditions where ATP synthesis is not

required or is impossible, as in tightly-coupled mitochondria that have been depleted of their ADP content (Henry and Nyns, 1975). This is very similar to the proposed function of secondary metabolism and may represent yet another way of regulating the balance of metabolism.

The fact that these alternative electron transport pathways are insensitive to cyanide is probably coincidental. It would be extremely surprising if they were evolutionary relics left over from the days when cyanide was a common metabolite in the environment. (Oró and Kiball, 1962). Nevertheless, their existence may be important when considering the role that they play in enabling organisms to compete in an environment such as the root-rhizosphere of cyanogenic plants, that contains appreciable quantities of cyanide.

From the preceeding sections, it is apparent that cyanide could play a small, but very significant role, in the carbon and nitrogen cycles in the soil. Many plants, fungi and bacteria can both produce and utilise cyanide. There could, therefore, be a 'cyanide cycle' acting as part of the carbon and nitrogen cycles.

The activity of at least one cyanide detoxifying enzyme in the soil as a stable entity has been observed by Singh and Tabatabai (1978). Inevitably, the

use of this and other cyanide-catabolising enzymes have been investigated for use in detoxifying industrial cyanide waste (Svenson and Andersson, 1977) a process that is currently expensive due to the requirements for bleach or other chemical detoxicants. (Green and Smith, 1972).

Cyanide production and further metabolism could, therefore, yield information of academic, ecological and industrial importance.

Aims of this study

It is apparent that the processes of cyanide production and catabolism by microorganisms has received relatively little attention (Knowles, 1976) despite the widespread occurrence of these phenomena. Furthermore, even allowing for the scant attention paid to this area of metabolism, studies on fungi have received a disproportionally small amount of interest, even though a wide range of fungi, especially basidiomycetes are apparently prolifically cyanogenic.

The objective of the present study has, therefore, been to expand and clarify some of the previously reported work on fungal cyanogenesis and detoxification. Particular attention needs to be paid to the relationship between cyanide production and the carbon source used, and to determine the effectors of the cyanogenic system. However, this investigation would not be complete

without corresponding studies of the significance of cyanide detoxification by cyanogenic fungi.

Finally, using the results obtained from the above studies, it is hoped to be able to resolve the role that cyanide production has in the metabolism of fungi, or at least permit a working hypothesis to be adduced. In this manner, the results might also lead to a greater understanding of the fundamental role of secondary metabolism by microorganisms.

With these objectives in mind, the most suitable organism for this study is undoubtedly the W_2-isolate of the snow mould basidiomycete.

CHAPTER TWO

(MATERIALS AND METHODS)

Cultivation of Fungi

Slant, plate (when used for preparation of still culture inocula) and still cultures employed a medium containing 15 $gl.^{-1}$ glucose, 5$gl.^{-1}$ malt extract and 5$gl.^{-1}$ yeast extract. This medium was solidified as required by addition of 15$gl.^{-1}$ Bacto-agar. Slant and plate cultures were grown on 15 to 20 ml. solidified medium; still cultures were grown in 250 ml. conical flasks with latex foam stoppers, containing 50 ml. medium. In all cases incubation was at 15°C.

Shake cultures used the defined medium described by Ward and Coletelo (1960). The components of this medium were 2.36 $gl.^{-1}$ asparagine, 1 $gl.^{-1}$ KH_2PO_4, 0.5$gl.^{-1}$ $MgSO_4$, trace elements and vitamins. These latter two components were stored as separate stock solutions at 10^3 - times the final concentration required and 1 ml. of each was added to each litre of medium. The final concentrations of trace elements in the medium were 4 $mgl.^{-1}$ $CaCl_2$, 0.715 $mgl.^{-1}$ $Fe_2(SO_4)_3$, 0.54 $mgl.^{-1}$ $ZnSO_4 . H_2O$, 0.245 $mgl.^{-1}$ $Co(NO_3)_2 6H_2O$, 0.194 $mgl.^{-1}$ $CuSO_4 . 5H_2O$, 0.135 $mgl.^{-1}$ $MnCl_2 4H_2O$ and 0.085 $mgl.^{-1}$ $H_2MoO_4 . H_2O$. Vitamins were added to give 5 $mgl.^{-1}$ i-inositol, 100 $\mu gl.^{-1}$ thiamine HCl, 100 $\mu gl.^{-1}$ calcium pantothenate, 100 $\mu gl.^{-1}$ pyridoxine HCl and 10 $\mu gl.^{-1}$ d-biotin. When the carbon source was either glucose or acetate, they were added prior to pH adjustment and sterilisation of the above components (minus vitamins). Other carbon sources

or additions were dissolved in a $lgl.^{-1}KH_2PO_4$ solution, pH adjustment and sterilisation were independent of the other components of the medium. Allowances were made such that the concentration of KH_2PO_4 in the completed medium was $lgl.^{-1}$. All solutions were adjusted to pH6 with 1MKOH and made up to final volumes, prior to sterilisation, ensuring that, after aseptic mixing of separately sterilised components, the final concentrations were correct. Vitamins, carbon sources, other than glucose or acetate and other additions, were filter sterilised through a 0.45 µm. membrane filter. The other components with glucose or acetate were autoclaved for 15 min. at 15 lbf. in^{-2} after distribution to culture vessels. Where conical flasks of 2l. or 250 ml. (plus or minus centre wells of height 4.4 cm. and diameter 2.4 cm.) capacity were employed, the final volume of medium added was 500 ml. and 50 ml. respectively. Latex foam stoppers were used and incubation was at 15°C in a Gallenkamp gyratory shaker at 275 rev. $min.^{-1}$. Shake culture media were solidified with 15 $gl.^{-1}$ Bactoagar when used in experiments where growth in shake or plate cultures were compared.

Preparation of inocula and measurement of growth

The W_2 - isolate of the snow mould basidiomycete and Marasmius oreades were kindly provided by Dr. E.W.B. Ward (Research Institute Agriculture, University Sub Post

Office, London, Ontario N6A 5B7). Both organisms were maintained on agar slants, subculturing every 3 to 4 months. The inocula for still cultures were obtained from 1 to 2 week old agar plate cultures. A 7 mm. diameter plug was removed from peripheral cells of the plate culture and transferred by aseptic means to a culture flask which was incubated under stationary conditions. Shake flask inocula of the snow mould basidiomycete were prepared from still cultures grown for 24 to 28d. (Ward and Coletelo,1960). Whole cultures were harvested by centrifugation at 1500g. for 15 min. in sterilised 50 ml. capped centrifuge tubes. Using sterile distilled water, cells were washed once, recentrifuged and resuspended to a final volume of 150 ml. Strict aseptic procedures were used throughout. ; The resuspended cells were homogenised with an Ultra-Turex homogeniser (Jonke u Kunkel, KG. Staufen i Breisgau), fitted with a pre-sterilised probe. Growth yields from shake cultures were independant of homogenisation time if longer than 1 min., but at least 5 min. was used as this gave more dispersed growth during the following incubation.

The concentration of the fungus in homogenates or growth in still or shake culture flasks have been expressed as the dry weight or changes of dry weight. Determination of dry weights was by collection of samples of homogenates

or whole cultures, onto previously dried and weighed filter papers. After drying at 110°C for 24h. the filter paper was transferred to a desiccator and allowed to cool to room temperature. The dry weight of the sample was determined from the difference in weight of a filter paper dried with fungus and one without added fungus. This procedure gave highly reproducible results. Shake cultures were inoculated with 1 ml. of homogenate adjusted with sterile distilled water to 3 $mgml^{-1}$ dry weight of fungus.

Measurement of cyanide production

The culture medium cyanide concentration was assayed periodically during incubation of the fungi using Epstein's (1947) method, after removal of fungal cells by centrifugation at 1500g for 15 min. Because cyanide is volatile, loss from the culture medium during growth was measured to determine the true total cyanide production. Cyanide loss was estimated using an alkaline 5% ($^{w}/v$) Na_2CO_3: 0.5% ($^{w}/v$) picric acid reagent. This was modified from that described by Skowronski and Strobel (1969) whose reagent in addition contained 0.1M NaOH, which caused some precipitation when used at 15°C. Shake cultures were incubated in 250 ml conical flasks fitted with centre wells. Filter sterilised reagent (1 ml) was added to each centre well, including a control flask containing 50 ml. uninoculated medium. Pre-sterilised

rubber stoppers were used to replace the original latex foam stoppers and the flasks were incubated for a further 1-5h. The reagent in each centre well was then transferred to a small test tube containing 0.5 ml. distilled water. The contents of each tube were mixed thoroughly and left for 30 min. at room temperature. Absorbance of the sample at 475nm. was measured against the control in a Unicam sp500 spectrophotometer. Potassium cyanide solutions (0-1mM) were used as standards, obtained by mixing 0.5 ml. of each solution with 1 ml. reagent. The cyanide trapped is expressed as μmoles (cyanide trapped) h^{-1}.

Measurement of other metabolites in the culture medium

In all cases the culture medium was assayed periodically for selected metabolites that accumulated or disappeared during incubation. Fungal cells were removed by centrifugation at 1500g. for 15 min.

Glucose was assayed by the method of Niven, Collins and Knowles (1977) and acetate by the method of Holt and Bergmeyer (1974). Alcohol was determined by the Bernt and Gutmann (1974) procedure and production of ammonia by the method of Fawcett and Scott (1960). Formamide production was assayed by the Fry and Millar (1972) procedure.

Breakage of fungal cells and protein measurement

Cultures were harvested and washed twice in fresh medium minus carbon and nitrogen sources by centrifugation at 1500g. for 15 min. Final resuspension was in the

buffer specified for each particular experiment and were to final volumes of 5,10, or 20 ml. depending on the quantity of material. Disruption was usually by two passages at 8000 lbf. in.$^{-2}$ through a French pressure cell. For enzyme assays one passage at 12000 lbf. in.$^{-2}$ was preferred, particularly when several samples were processed consecutively. Although breakage by one passage through the French press was not as efficient, samples could be processed more rapidly, thereby avoiding excessive heating of the pressure cell.

Protein concentrations of samples, after disruption, were measured following the centrifugation specified in each experiment. The modified Biuret procedure of Gornall Bardawill and David (1949) was used for solutions containing greater than 500 µg. (protein) ml.$^{-1}$ and the Lowry et al.,(1951) procedure for more dilute samples. Bovine serum albumin (B.S.A.) solutions were used as standards.

Glycine metabolism

The incorporation of the carbon atoms of glycine into selected metabolites during incubation of fungi was followed by measuring the amount of radioactivity incorporated into each metabolite when the fungus was incubated in media containing $[1\text{-}^{14}C]$ or $[2\text{-}^{14}C]$ glycine (specific activity 56 µCi. µmol.$^{-1}$ and 54 µCi. µmol.$^{-1}$, respectively). The final concentration of glycine in each experiment was 10mM.with radioactive glycine added to give a specific

activity of 20 nCiμmol.$^{-1}$. Incubation was in 250 ml centre well flasks.

After various periods of incubation the culture medium and fungal cells were separated by centrifugation at 1500g. for 15 min. (at 4°C). Disruption of the fungus by two passages through a French pressure cell at 8000 lbf.in.$^{-2}$ was followed by centrifugation as described above. Non-volatile metabolites were separated from each other by thin layer chromatography (t.l.c.) of 5μl cell free extract or culture medium. The chromatogram was developed in a butan-1-ol/acetone/water/dimethylamine (10:10:5:2) solvent system. Standard solutions of the selected metabolites were chromatographed on the same plates as the samples and their positions were determined after the development of the plate, using methods on page 66. The position of each metabolite in the sample was marked and this area of the t.l.c. plate removed and placed in a 5 ml capacity scintillation vial containing 0.5 ml distilled water. After thorough mixing, 4 ml phase combining system (P.C.S) was added, the vial stoppered and the contents re-mixed. The radioactivity in each vial was measured in a scintillation counter. To determine the total amount of radioactivity added to a t.l.c. plate, 5 μl. of sample were applied to, then immediately removed from the t.l.c. plate and treated as described above.

Radioactivity associated with volatile substances in samples was determined as follows. The radioactive content of 5µl. of the sample was measured and, from this value, the amount of radioactivity remaining after the application of 5 µl. sample to a t.l.c. plate, was subtracted. Any difference was due to radioactive volatile substances present in the sample (allowances were made for any quenching by the absorbant from the t.l.c. plate).

The conversion of glycine to CO_2 and HCN during incubation was determined as follows. During the 24h. before a culture was examined for non-volatile products of glycine metabolism, the CO_2 and HCN produced were trapped in 1 ml. sterile 4MKOH added aseptically to the centre well. The original latex foam stoppers were replaced with sterilised rubber stoppers after the KOH had been added. After 24h. further incubation of the culture, the KOH was removed from the centre well and the centre well washed with a further 1 ml. aliquot of 4MKOH. The two cyanide/CO_2 - containing KOH solutions were combined and mixed with 0.1 ml. of 10% ($^{w}/v$) Na_2CO_3 and 1 ml. saturated barium hydroxide solution. Collection of the precipitate formed was on a 0.45 µm. membrane filter. An 0.25 ml. aliquot of the filtrate was neutralised with 4MHCl,

keeping the solution cool and the radioactivity in the sample measured, after addition of 4 ml·P.C.S., in a scintillation counter. The precipitate was washed twice with 10 ml. volumes of distilled water, removed and left to dry at room temperature for 12-16h. Both filter and precipitate were added to the outer part of a 50 ml· conical flask fitted with a centre well 1 cm. diameter, 4 cm. height). After addition of 0.5 ml. phenethylamine and a 1 x 4 cm. filter paper strip to the centre well, the flask was stoppered with a serum cap. CO_2 was released from the precipitate by injection of 2 ml. 6MHNO_3 into the outer part of the conical flask, followed by agitation for 45 min. at room temperature. Longer agitation times gave little increase in the amount of CO_2 recovered. The contents of the centre well were transferred to a 5 ml scintillation vial and 4 ml. of 0.4% ($^{w}/v$) 2,5 diphenyl oxozole (PPO)/ 0.0001% ($^{w}/v$) 1,4-di 2-(5-phenyl-oxozoyl) (POPOP) in toulene added. The centre well was rinsed several times with some of the scintillation vial contents, which were finally recombined in the vial. After stoppering and mixing the contents of the vial the radioactivity was measured in a scintillation counter. The percentage recovery of the procedure was determined using radioactive $Na_2{}^{14}CO_3$ (specific activity 58.2 mCi. mmol.$^{-1}$)

and was shown to be 46-58%.

Cyanide metabolism

After incubating the fungus for various periods of time in 250 ml conical flasks, cultures were transferred aseptically to sterile 250 ml centre well conical flasks fitted with rubber stoppers. To the centre well was added sterile 4MKOH (1 ml) and to the culture, 2μCi $K^{14}CN$ (specific activity 59 mCi. $mmol^{-1}$). The flasks were further incubated for 24h. or longer. Radioactive $^{14}CO_2$ and $H^{14}CN$ in the centre well and culture medium were assayed as previously described. Several potential non-volatile products of cyanide metabolism were assayed in the culture medium and cells after their separation by centrifugation for 15 min. at 1500g (4°C). Fungal cells were disrupted and cell free extracts obtained by methods described in a previous section. Samples of culture medium and cell free extracts (5-15μl.) were applied to a cellulose t.l.c. plate along with selected authentic compounds. The plate was developed in a butan-1-ol/water/acetic acid/pyridine (100:30:20:10) solvent system. Radioactivity present in each sample, corresponding to a particular marker and the total radioactivity added to the plate were measured as previously described.

The amount of radioactivity associated with the protein of cell free extracts was measured after the protein had

been precipitated with 6% perchloric acid, after being separated from the remainder of the cell free extract by centrifugation, and redissolved in 2N KOH plus 0.5N triethanolamine buffer (pH 7.0). Cell debris and unbroken cells were solubilized with N.C.S. (Amersham) tissue solubiliser (½ ml.) after removal of the cell free supernatent. Incubation of the samples with N.C.S. tissue solubiliser was for 24h. at 30°C after which the radioactivity in the samples was measured following the addition of P.C.S. scintillation fluid.

Identification of compounds on t.l.c. plates

The methods used for identifying compounds were unaffected by the type of the t.l.c. plate or solvent system used. Amino acids were detected by spraying t.l.c. plates with 0.5% ninhydrin in butan-1-ol followed by drying with hot air until the characteristic purple-blue colours appeared. Cyanide and cyanohydrins were located by spraying t.l.c. plates with the picric acid reagent previously described, followed by incubation of the t.l.c. plates at room temperature until the brick red colour developed. Very low amounts of cyanide were detected using a 0.1% ($^{w}/_{v}$) K_2MnO_4 reagent. After spraying onto the t.l.c. plate, the area where cyanide was present became colourless against a pink background.

Organic acids were visualised with a 0.025% ($^{w}/v$) bromocresol green reagent made up in acetone/ distilled water (80:20); the acids showed yellow against a blue background, after spraying with the reagent, followed by exposure of the t.l.c. plate to NH_3 vapour.

Formamide and formadine were detected with a 10% ($^{w}/v$) NaOH, 10% ($^{w}/v$)Na nitroprusside and 10% ($^{w}/v$) K ferricyanide reagent diluted 3 times with distilled water. After spraying this reagent on to a plate, a pink colour was obtained for formamide and formadine.

Formate was visualised after spraying the t.l.c. plate with 1% ($^{w}/v$) $AgNO_3$ in 1M NH_3. A brown/black colour against a grey background was obtained after heating the plate for 10 min. at 110°C.

Cultures in which protein synthesis was inhibited

Cultures were incubated in 2l conical flasks containing glucose with or without glycine, both at final concentrations of 10mM. Using strict aseptic procedures, fungal cells were separated from the culture medium by centrifugation at 5000g for 5 min. (at 15°C) in sterile capped 250 ml. centrifuge bottles. The cells were washed twice by centrifugation, as described above, with fresh sterile culture medium (minus carbon and nitrogen sources) which was adjusted before sterilisation

to the pH of the culture medium at the time of harvesting. Final resuspension, with this fresh medium was in a sterile 2l. conical flask to a volume of 400 ml. and the contents mixed thoroughly until homogeneous. This preparation was then distributed into sterile 250 ml. centre well conical flasks by pipetting 40 ml into each flask. Cycloheximide and/or Chloramphenicol were added to give final concentrations of 50 $\mu g ml.^{-1}$ and 250 $\mu g ml.^{-1}$ respectively.

In all experiments glucose was added to give a final concentration of 10 mM and asparagine to give a final concentration of 15 mM. After any other additions, the final volume in each flask was adjusted to 50 ml. All additions to the flasks were filter sterilised through a 0.45 μm. millipore filter.

Cyanohydrin production

Cultures were grown in 2l flasks for 5,7,10 or 15 d. with glucose and glycine, both at initial concentrations of 10mM. After the required period of incubation, cultures were harvested by centrifugation at 5000g. for 5 min. and 15^{0}C. The culture medium was removed and several 1 ml. aliquots of it were placed in test tubes and cooled in an ice bucket. These aliquots were made alkaline to

liberate cyanide from any cyanohydrins present (they break down to cyanide and the ketone above pH6) by the addition of 0.1M KOH and adjusted to pH 7.0 with 0.1MHCl. The cyanide released by this treatment was measured using Epstein's method (1947).

Cyanohydrins in the cells were identified and assayed by using the first half of the procedure for cyanohydrin isolation described by Tapper and McDonald (1974). The cells were resuspended, without prior washing, in 50 ml 95% ($^{v}/v$) ethanol acidified with glacial acetic acid, (20:1) Constant stirring for 1 h. at room temperature was followed by centrifugation at 1500g. for 15 min., in capped 50 ml. centrifuge tubes. The supernatent was reduced in volume, using a Buchii rotary evaporator until 0.5 to 1 ml. remained. Chromatography of 15µl. concentrated extract on a cellulose t.l.c. plate was in a butan-1-ol/water/acetic acid/pyridine (100:30:20:10) solvent system. Cyanohydrins were detected on the t.l.c. plates as previously described.

The total cyanohydrin content of the extract was measured by mixing 0.25 ml. of extract with 0.25 ml. distilled water, followed by the addition of 1 ml. alkaline picrate reagent. After thorough mixing,

incubation was for 30 min. at room temperature, after which the absorbance was determined at 475 nm. against a distilled water control treated similarly. Glyoxylic acid cyanohydrin was prepared by the method of Tapper and McDonald (1974) and used to determine the percentage recovery of the procedure by adding a known quantity to 50 ml extraction solvent and treating as previously described. The recovery of cyanohydrin was between 55% to 65% and positive identification was always achieved after chromatography on the t.l.c. plates.

Conversion of KCN to CO_2 by cell free extracts

Cultures were grown in 2l. flasks with a medium containing glucose and glycine, both at initial concentrations of 10mM. After incubation, as required, cultures were harvested and washed once by centrifugation at 5000g for 5 min. (at $4^{o}C$), using a 5 mMKH_2PO_4 buffer adjusted to pH 7.0 with 1MKOH. The final resuspension volume was 20 ml. and disruption of the cells was by one passage at 12000 lbf. $in.^{-2}$, through a French pressure cell. Cell free extracts were prepared by centrifugation at 2000g, $4^{o}C$ for 15 min.

The extract was assayed for the ability to convert cyanide to CO_2 as follows:-

Into a 5 ml capacity scintillation vial were placed 100 µl·of 25 mM.Hepes - 25 mM.KH_2PO_4 - 25 mM.

triethanolamine buffer adjusted to the required pH, as specified for each experiment, with 4MKOH. The vials were equillabrated to 15°C and the substrate, 1 µl.K^{14}CN (specific activity 59 mCi.mmol.$^{-1}$), plus any other required supplements were added and the contents mixed. Finally 100 µl cell free extract, also equilibrated to 15°C, was added. After stoppering the vial with a serum cap and mixing of the contents, the vials were incubated for 5h. at 15°C. The reaction was stopped by addition of 50 µl. 4MKOH, 100 µl. 1% (^{w}/v) Na_2CO_3 and 100 µl. saturated Ba $(OH)_2$ solution, by injection through the serum cap. These vials were left to stand, after mixing the contents, for 12-16h. at room temperature. Separation of the precipitate from the assay mixture was by centrifugation at 12000g. in an Eppendorf 3200 centrifuge for 1 min. The supernatant was removed and an 0.25 ml. aliquot neutralised, after cooling to 4°C, with 0.25 ml. 4NHCl. Following the addition of 4 ml. P.C.S., the radioactivity was measured in a scintillation counter.

Measurement of the $^{14}CO_2$ trapped in the precipitate was as follows. The precipitate was resuspended in 100 µl. distilled water and placed in a plastic 500 µl. capacity cup which was fixed to a serum cap. Phenethylamine (0.5 ml) and a 1 x 4cm. filter paper

strip were placed in a 5 ml. capacity vial. The plastic cup attached to the serum cap was fixed inside the vial and CO_2 released from the precipitate by injection of 200 µl. 6M HNO_3 into the centre well, followed by 45 min. agitation of the vial. Longer agitation times gave little increase in the amount of $^{14}CO_2$ trapped. After removal of the plastic cup-serum cap assembly, 4 ml. of PPO - POPOP scintillation fluid were added to the vial. After the vial was stoppered and the contents mixed, the radioactivity in the vial was determined using a scintillation counter.

Preparation of dinitrofluorobenzene (DNFB) derivatives of amino acids

In experiments where amino acids were concentrated, which mainly involved radioactive substrates, the reaction of DNFB with amino acids was employed. This avoided the simultaneous concentration of salts in a sample so reducing the disadvantageous effects that this sometimes causes during t.l.c. of concentrated extracts. To 1ml capacity freeze-drier ampoules were added 100 µl. of sample followed by 10 µl. 1M K_2CO_3, pH 8.8 and 15 µl. 10% ($^{w}/v$) DNFB. After mixing the contents, the ampoules were wrapped in aluminium foil and incubated for 1h. at 60°C, in a water bath. The foil was removed and 30 µl. 11.6M

HCl added to the ampoules. After mixing the contents of the ampoules they were left to cool to room temperature. The DNFB.- amino acid derivatives were extracted from the reaction mixture using diethyl ether followed by ethylacetate. Several aliquots of either solvent were used to extract the DNFB derivatives. Each aliquot was added to the ampoule, the contents mixed for about 1 min. and the two phases allowed to separate out. The top phase was transferred to a clean 1 ml. capacity freeze drier ampoule taking care not to remove any of the aqueous layer. This procedure was repeated until the top phase remained colourless, all aliquots were bulked into one ampoule. The volume of the extracts were reduced by evaporation in a dessicator connected to a vacuum line. When the volume had decreased to around 50 µl., the remaining solvent was removed, after transfer of the ampoules to an Edwards B5A freeze -drier. The samples were removed, when the vacuum had reached 0.05 torr or less. Freeze dried samples were dissolved in 25 µl. methanol and 5, 10, 15 µl. applied to a silica-gel t.l.c. plate. Authentic aminoacid derivatives were also prepared as described above from 1mM stock solutions and applied to the plate. The chromatogram plate was developed in a chloroform/ methanol/acetic acid (85:13:2) solvent system, positions

of aminoacid derivatives, after chromatography, were detected by their characteristic yellow colour. The derivatives in the sample corresponding to the standards were scraped from the plate and, where appropriate, placed in 5 ml. capacity scintillation vials. After adding 0.5 ml. distilled water and 4 ml. P.C.S., the vials were stoppered, the contents mixed, and the radioactivity in each vial measured in a scintillation counter. Total radioactivity in the sample and the proportion of the radioactivity lost during application of the sample to the t.l.c. plate were measured as follows:-

Firstly, the radioactivity in 5 µl. of sample was measured. Secondly, 5 µl. of sample were applied to a piece of t.l.c. plate, immediately removed, and the radioactivity in the scrapings measured.

The proportion of radioactivity in samples lost during application to the t.l.c. plate could then be calculated. Silica scrapings did not cause significant quenching (less than 1 - 2%) but DNFB-derivatives did cause quenching, therefore necessitating construction of a quench curve as follows:-

Distilled water (0.5 ml.) containing 0.01 µCi. [1-^{14}C] glycine and 4 ml. P.C.S. was added to a 5 ml. capacity scintillation vial. The vial was stoppered and the

contents mixed thoroughly. Radioactivity in the vial was measured in a scintillation counter, recording the counts per minute in both the tritium (^{3}H) and Carbon - 14 (^{14}C) channels. The vial was unstoppered, 1 µl. DNFB. - glycine added and the radioactivity was re-recorded in each channel. This procedure was repeated to obtain other values for the quench curve.

DNFB. derivatives could also be quantified by removing them from the t.l.c. plate and eluting them from the silica-gel with 4M$NaHCO_3$. After removing the silica-gel by centrifugation, the absorbance of the DNFB. derivatives was measured at 345nm.

Alanine synthesis from acetaldehyde NH_3 and HCN

The procedure used was modified from that described by Strobel (1966). Cultures grown in media containing glucose as the carbon source were harvested, when required, and washed in 50 mM. K_2HPO4 (pH 7.8). After final resuspension to a volume of 5 or 10 ml. in this buffer, the fungal cells were disrupted by two passages through a French pressure cell. The resulting homogenate was centrifuged for 15 min. at 1500g. (4°C). After separation of the supernatant from the pellet, the two fractions were assayed as follows:-

Each fraction was adjusted with 50mM K_2HPO_4 (ph 7.8) to give a protein content of 3 $mgml.^{-1}$, and then

separated into several 1 ml. aliquots which were equilibrated to 15°C. After the pH of the separate components of the reaction mixture had been adjusted to pH 7.8, to each aliquot was added 30 µl. 1M $K^{14}CN$ ($2\mu Ci.mmol.^{-1}$), 30 µl. 1M. acetaldehyde and 30 µl. 1M. NH_4Cl. Following mixing, the samples were stoppered and incubated for up to 5h. at 15°C. The reaction was stopped with 6% PCA, and the protein removed by centrifugation at 1500g. DNFB. derivatives were prepared from the protein-free supernatent, after a sample had been removed and its radioactive content measured, as previously described. Following chromatography of the DNFB. derivatives, the radioactivity associated with alanine and other amines could be determined. From these measurements, the residual cyanide could also be estimated.

Glutamate synthesis from succinic semialdehyde, NH_3 and HCN

The fungal cells were grown and disrupted as described in the previous section. After adjusting the protein concentration of the samples to $3mg.ml.^{-1}$, glutamate synthesis was assayed, as follows (a modification of Strobel's method 1967):-

After the pH of the separate components of the reaction mixture had been adjusted to pH 7.8, to each 1 ml. aliquot of sample was added 50 µl. 15% ($^{w}/v$)

succinic semialdehyde, 30 µl. 1M NH_4Cl and 30 µl. 1M $K^{14}CN$ ($2\mu Ci.mmol.^{-1}$). DNFB. derivatives were prepared and the radioactivity associated with glutamate, other amines plus the residual cyanide, measured.

Determination of cyanide hydratase activity

The procedure used for the assay of cyanide hydratase was that described by Rissler and Millar (1977). Samples were prepared for assay, as previously described.

Whole cell respiration

Cultures were incubated in 250 ml. conical flasks and harvested by centrifugation at 1500g., 15°C for 15 min. The culture medium was removed and its pH measured. It was then boiled for 15 to 20 min., followed by cooling to 15°C. Measurement of the pH of this boiled medium was followed by adjustment, if necessary, to the pH value recorded after harvesting, with either 0.1M. NaOH or 0.1M. HCl.

The harvested fungal cells were washed once, with this boiled medium, by centrifugation and, finally, resuspended in the boiled medium to give a total volume of 25 ml. It was kept at 15°C. An homogeneous suspension of cells was obtained by using a Potter-Elvejham homogeniser, which caused little breakage of the fungal cells. Oxygen uptake by the fungal cell suspension was measured, using a Rank oxygen electrode connected to a W + W electronics chart recorder.

The fungal cell suspension was aerated for 1 min., after which 4 ml. were transferred to the chamber of an oxygen electrode. These fungal cells were magnetically stirred in the oxygen electrode chamber. The top of the chamber was fitted and the cells were incubated until a steady rate of oxygen uptake was recorded. Cyanide (0 to 1mM. final concentration) and/or salicyl hydroxamic acid (2.5 mM. final concentration) were added to the chamber. The effect on oxygen uptake by the fungal cells was then recorded. Solutions of either compound were freshly prepared before each experiment, salicyl hydroxamic acid was neutralised with 0.2M KOH and KCN was neutralised with 0.1M H_2SO_4.

Cytochrome spectra

Fungal cells were grown in batch culture in a medium containing acetate as the carbon source. The culture vessel was either a 5l. L.H. engineering fermenter pot containing 4.5l. medium, or a 2l. conical flask with the usual 500 ml. medium. The contents of 5l. fermenter pots were aerated at a rate of 5l. $min.^{-1}$ and agitated with a central stirrer at 500 rev. $min.^{-1}$. Cultures were harvested and washed once by centrifugation at 2000g, 4°C for 15 min., then resuspended to a final volume of 20 ml. The washing and resuspension medium contained 0.44M sucrose, 0.04M KCl, 0.01M Na_2PO_4, 0.01M.

Tris, 0.002M.EDTA, 0.2% BSA (added after the protein content of the extract had been measured) and 0.01M. Mg_2SO_4. This medium was adjusted to pH7 with 1MKOH. Two passages at 8000 lbf. $in.^{-2}$ through a French pressure cell was used to disrupt the fungal cells, after which whole cells were removed by centrifugation at 1500g, 4°C for 15 min. Fractionation of the cell-free extract was by centrifuging at 8000g. 4°C for 15 min. followed by centrifugation of the resulting supernatant at 150,000g, 4°C for 1h. The pellets, after resuspension in the previously described medium, and supernatents were examined after each centrifugation. Cytochromes were determined, using a Perkin-Elmer 356 spectro-photometer in the split-beam mode connected to a Perkin-Elmer 56 recorder. Division of 2 ml. sample equally between two cuvettes was followed by addition of a few grains of sodium dithionite to one cuvette and a few grains of sodium metabisulphate to the other. The contents of each cuvette were mixed thoroughly and the reduced minus oxidised spectrum of the sample recorded. H_2O_2 was sometimes used to replace the sodium metabisulphite used in this procedure.

Preparation of samples for the electron microscope

Samples were obtained by removing 5 ml. aliquots from cultures incubated in selected media. To these

5 ml aliquots were added 5 ml. 5% ($^{w}/v$) glutaraldehyde in cacodilate buffer (3.64% Na cocodilate adjusted to pH 7.4 with 1M HCl, 100 ml. and 2ml. 1M $CaCl_2$) and, after mixing, the samples were incubated for 2h. at 15°C. The fungal cells were then washed twice in culture medium, (removed from the remainder of the culture), by centrifugation at 1500g. for 15 min. After removal of the final supernatant, the fungal cells were resuspended in 1% ($^{w}/v$) osmium tetroxide in cacodilate buffer. Incubation of the samples for a further 1h. at 15°C was followed by 15 min. in 60% and 15 min. in 90% alcohol. This procedure was repeated with 30%, 60% and 90% Spurr's resin (Spurr, 1969), incubation in each concentration of resin was for 1h. Final incubation in 100% resin was for 20h., the fungal cells were resuspended twice in fresh resin during this incubation.

The fungal cells were fixed in the resin by heating the mixture for 12h. at 80°C in a mould. Sections were cut from the hardened resin/fungal cell block by using a Reichert OMR3 ultramicrotome. The sections were stained by incubation for 30 min. at 80°C in 5% ($^{w}/v$)Ur acetate in 1% acetic acid, followed by 10 min. at room temperature in 1.33% Pb $(NO_3)_2$ in 1.76% Na_3citrate $2H_2O$. An AE1801A electron microscope with an accelerating voltage of 60 kV. was used to examine the stained sections.

Chemicals

Cyclic AMP, cycloheximide, chloramphenicol were obtained from Sigma; $K^{14}CN$, [1-^{14}C] glycine, [2-^{14}C] glycine, [U-^{14}C] glucose from the Radiochemical Centre, Amersham; P.C.S. from Hopkins and Williams. Whenever possible, all other reagents were of analytical grade; glass distilled water was used throughout.

CHAPTER THREE

(RESULTS)

Dry weight values given in Figures 3.1 - 3.41 refer to the dry weight of whole 50 ml. cultures grown in 250 ml. conical flasks.

Growth and cyanide production in 'still' culture

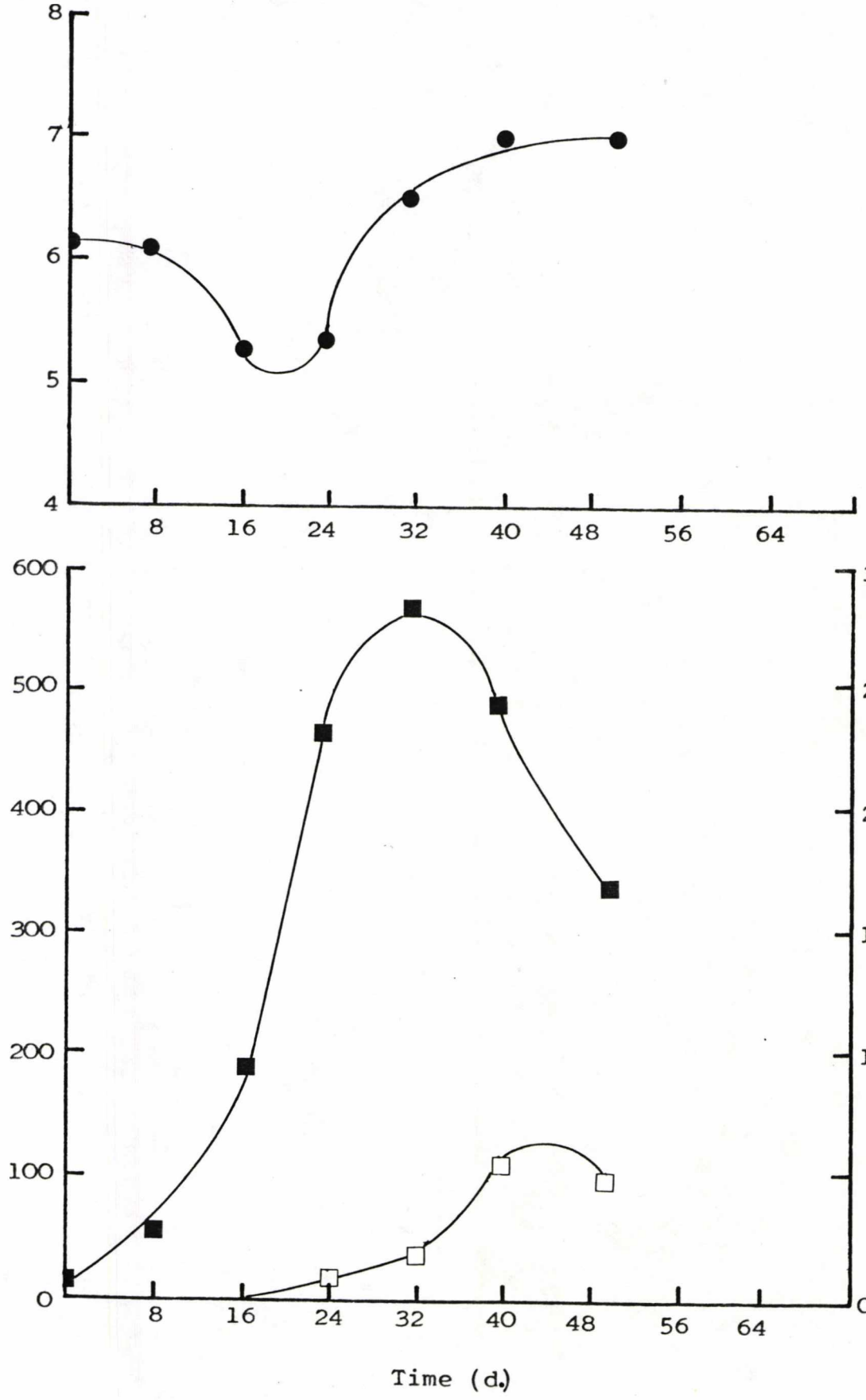
Figure 3.1(a and b) record pH changes of the culture medium, growth and cyanide production during incubation of the snow mould basidiomycete in 'still' culture with a complex medium. The fungal cell dry weight of cultures began to increase after 8d. incubation, reaching a maximum of 560 mg, 24d. later. During the initial period of growth, the inoculum formed a large aggregate of cells which then fragments into smaller aggregates as the more rapid increase of fungal cell dry weight occurs.

Cyanide first appeared in the culture medium towards the end of the period of rapid fungal cell dry weight increase. The maximum concentration of cyanide produced by cultures was between 50 and 60 μM., attained after 40d. incubation. No further major changes of cyanide concentration were observed in the following 8d.

The pH of the culture medium underwent several large changes during the 48d. incubation. Throughout the period of early growth, the pH fell from pH 6.2 to pH 5.3. The pH then began to rise slowly, followed by a period of rapid pH increase, coincidental with the appearance of cyanide in the culture medium. Very

Figure 3.1a - Culture medium pH changes (●) during growth of the snow mould basidiomycete in still culture, in a medium containing 5g$l.^{-1}$ malt extract, 5g$l.^{-1}$ yeast extract and 15 g$l.^{-1}$ glucose.

Figure 3.1b - Changes in the dry weight of cultures (■) and the culture medium cyanide concentration (□) during growth of the snow mould basidiomycete in still culture, in a medium containing 5gl^{-1} malt extract, 5g$l.^{-1}$ yeast extract and 15g$l.^{-1}$ glucose.


Culture medium pH.
8
7
6
5
4
8
16
24
32
40
48
56
64
Dry weight of culture (mg.)
600
500
400
300
200
100
0
Cyanide in culture medium (µM.)
300
250
200
150
100
50
0
Time (d.)

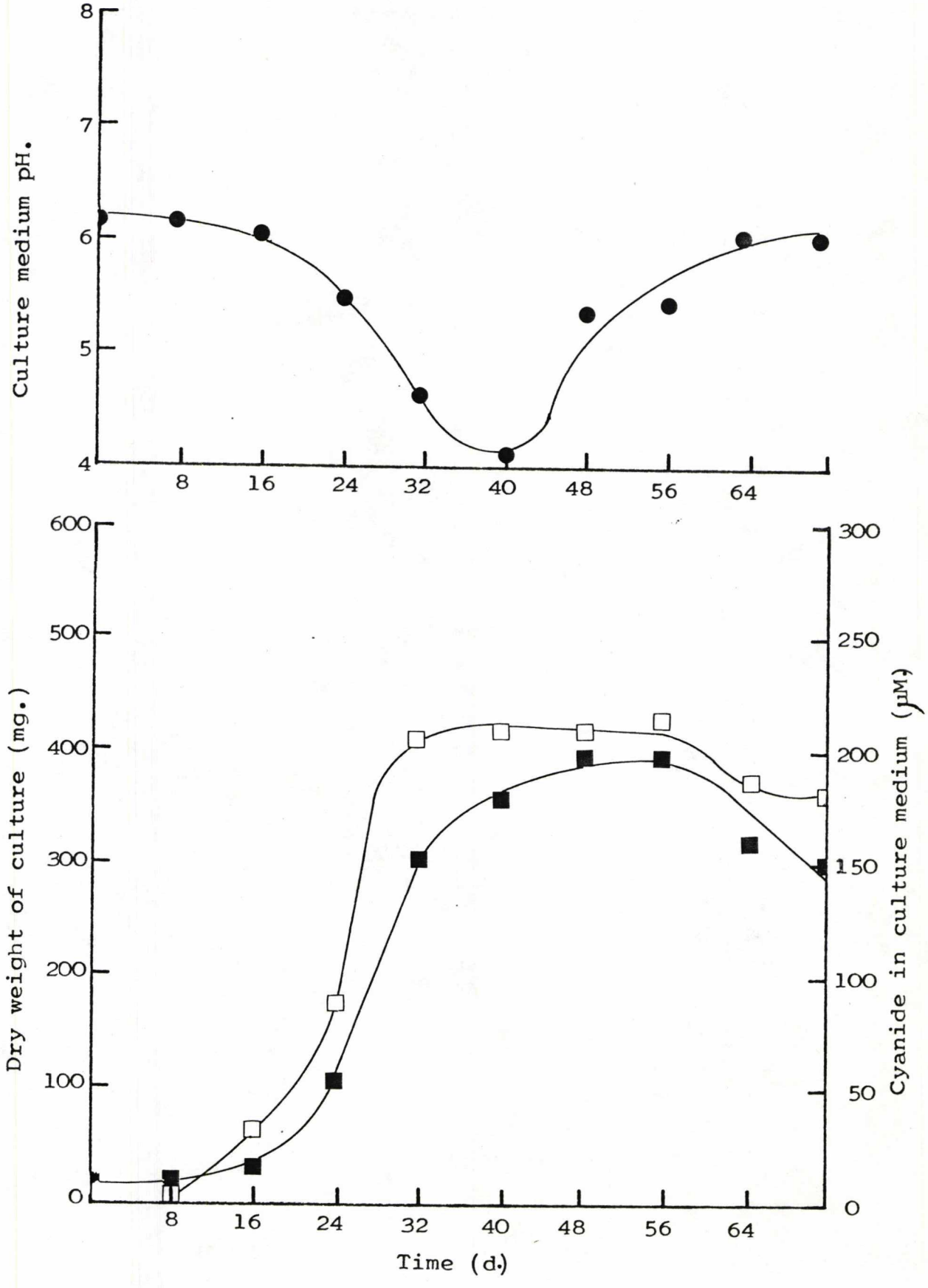
little change of the culture medium pH occurred during the last stages of incubation. Pigment production by these cultures was observed and began after 40 to 45d. No attempt was made to determine the nature or amount of the purple pigment produced.

Growth, pH changes of the culture medium and cyanide production during the incubation of Marasmius oreades in 'still' culture are recorded in figure 3.2 (a and b). A comparison of figures 3.1 and 3.2 reveal several differences between still cultures of M.oreades and the snow mould basidiomycete. The dry weight of cultures of M.oreades began to increase after 16d. incubation reaching a maximum of 390 mg., 40 to 48d. later. The appearance of the culture was that of a single aggregate of fungal cells, that developed from the initial inoculum and which showed little tendency to fragment. Cyanide appeared in the culture medium after 16d. incubation and was produced throughout the whole period of rapid increase in fungal cell dry weight. The maximum concentration of cyanide in the culture medium during incubation (200µM.) was attained after 32d. and no large changes in the cyanide concentration occurred in the following 40d.

During incubation, the culture medium pH changed in a similar manner to that observed in cultures of the

Figure 3.2a - Culture medium pH changes (●) during growth of Marasmius oreades in still culture, in a medium containing 5g$l.^{-1}$ malt extract, 5g$l.^{-1}$ yeast extract and 15 g$l.^{-1}$ glucose.

Figure 3.2b - Changes in the dry weight of cultures (■) and the culture medium cyanide concentration (□) during growth of Marasmius oreades in still culture, in a medium containing 5g$l.^{-1}$ malt extract, 5g$l.^{-1}$ yeast extract and 15g$l.^{-1}$ glucose.


Culture medium pH.
8
7
6
5
4
8
16
24
32
40
48
56
64
Dry weight of culture (mg.)
600
500
400
300
200
100
0
Cyanide in culture medium (μM)
300
250
200
150
100
50
0
Time (d.)

snow mould basidiomycete. However, the appearance of cyanide did not seem to be related to the changes in pH of the culture medium.

Pigment production, by cultures of M.oreades, began 40 to 50d. after inoculation and was often produced in such large amounts that it crystallised out on the surface of the mycelial mat. The brown pigment produced had an absorbance maximum at 340 nm but quantitative estimation of the pigment was not possible, because of the high and variable background absorbance of the culture medium.

Growth of the snow mould basidiomycete on plate and in 'shake' culture with selected carbon sources

The correlation between growth on plates and in shake culture was not a good one (Table 3.1). In many cases excellent growth on plates, as was observed with citrate, malate and lactate as the carbon source, produced poor results in 'shake' cultures. The best carbon source in shake culture was glucose, which produced not only the best fungal cell dry weight yield, but also cultures in which the fungal cells showed little tendency to clump during growth.

Measurable growth in shake cultures was achieved with only two of the other carbon sources tested, acetate and succinate, but with these carbon sources the

Table 3.1

Growth of the snow mould basidiomycete with different carbon sources

Carbon source	Agar plate culture. Diameter of culture after 20 days (mm.)		Shake culture. Maximum dry weight of fungal cells produced (mg.)	
Acetate (30 mM.)	21	a	41	d
Citrate (10 mM.)	32	a	13	p
Glycine (30 mM.)	18	a	10	
Galactose (10 mM.)	-		23	p
Glucose (10 mM.)	20	b	65	d
Glucose & Acetate (10 mM.) & (30 mM.)	-		87	d
Lactate (20 mM.)	28	a	10	p
Malate (15 mM.)	28	a	5	
Succinate (15 mM.)	24	a	40	d
No carbon source	21	c	15	p

Shake culture media were solidified with $15gl^{-1}$ Bacto-agar for use in agar plate cultures. Carbon sources were added to the liquid agar medium after sterilisation. All solutions were adjusted to pH 6.0. The letters a, b and c in the left hand column describe the appearance of the hyphae as white and fluffy, white but not fluffy and poorly developed, respectively. The letters p and d in the right hand column describe the type of growth that occurred, pelleted growth and dispersed growth respectively. Asparagine was always present as the nitrogen source.

cultures took longer to reach the dry weight maximum. Asparagine was routinely used as the nitrogen source, but when no additional carbon source was present, the fungus could use it as both the carbon and nitrogen source on plate and in 'shake' culture; growth was not good in either case and in shake cultures the fungal cells tended to form pellets. No growth was recorded with glycine as the carbon source.

Growth of the snow mould basidiomycete on plate and in 'shake' culture with selected nitrogen sources

Alanine provided the best growth in shake cultures (Table 3.2) but the increase in cyanide production observed could be accounted for by the increase of growth.

Ammonia also supported growth in shake cultures and enabled an estimate of how much of the culture dry weight was derived from nitrogen sources other than ammonia.

Glycine was not a good nitrogen source for shake cultures.

In all the following experiments, asparagine was used as a nitrogen source in preference to ammonia, due to the interference of ammonia with the Epstein cyanide assay (Epstein, 1947).

Growth and cyanide production in culture media containing different initial concentrations of glucose

The maximum fungal cell dry weight yield of 'shake' cultures was proportional to the initial concentration

Table 3.2

Growth of the snow mould basidiomycete with different nitrogen sources

Carbon source	Agar plate culture. Diameter of culture after 20 days (mm.)	Shake culture Maximum dry weight of fungal cells produced (mg.)
Glutamic acid (15 mM.)	13.90	-
Ammonium chloride (15 mM.)	11.49	54
Asparagine (15 mM.)	10.25	65
No nitrogen source	9.90	5
Alanine (15 mM.)	9.20	82
Glycine (15 mM.)	8.90	10
Methionine (15 mM.)	8.50	-
Serine (15 mM.)	6.20	-
Tryptophan (15 mM.)	6.20	-
Threonine (15 mM.)	1.80	-
Histidine (15 mM.)	1.80	-

(Glucose was used, in all cases, as the carbon source.)

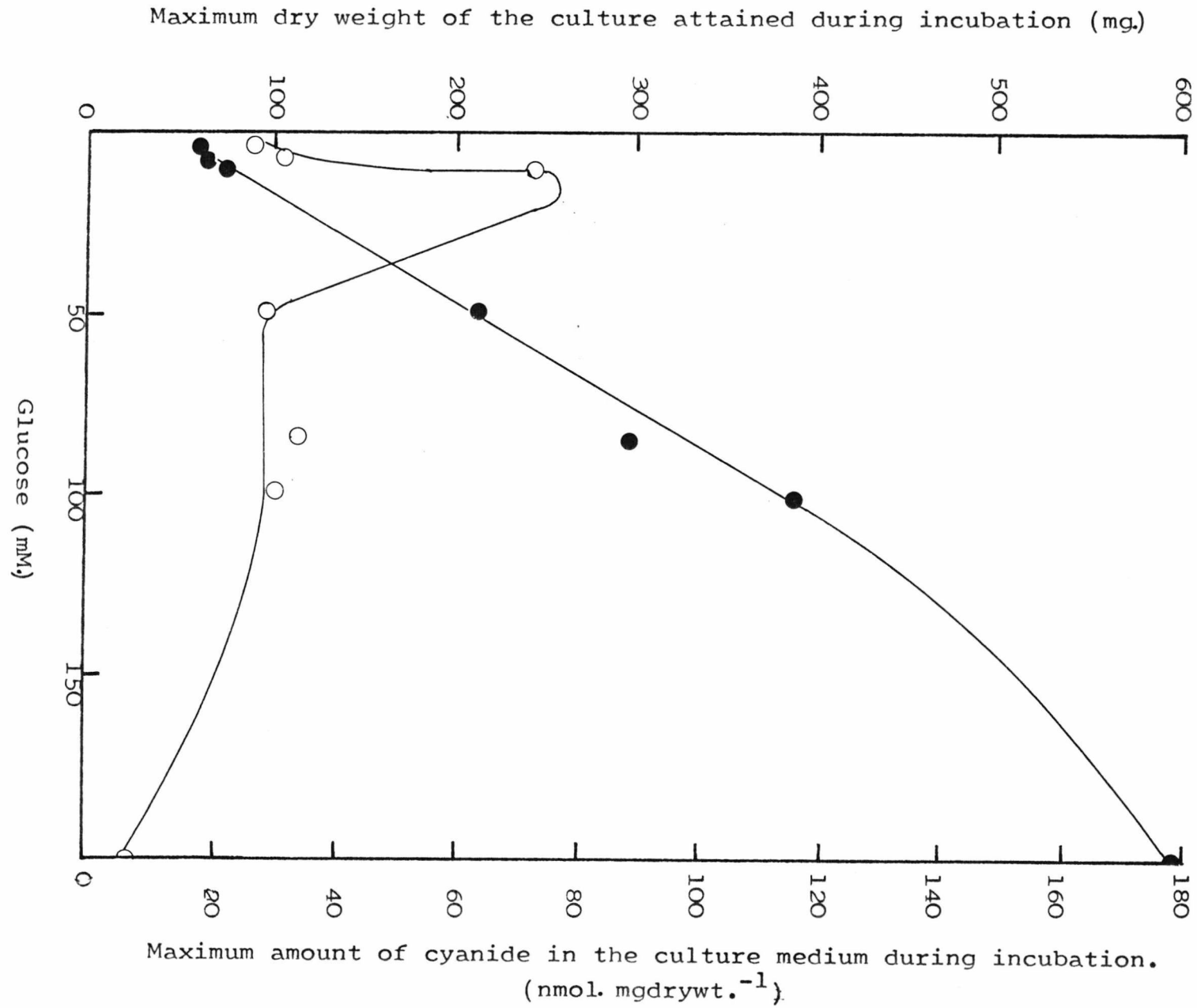
of glucose present in the culture medium (Figure 3.3) up to a maximum of 100 mM. Higher initial glucose concentrations did not produce proportionally higher maximum fungal cell dry weight yields. In cultures where the initial glucose concentration was higher than 50 mM, glucose remained in the culture medium (5 to 10 mM) after the maximum fungal cell dry weight in the cultures had been attained.

However, the most interesting result was the dependency of cyanide production by cultures on the initial glucose concentration of the culture medium. The amount of cyanide produced by cultures has been recorded as the cyanide present in the culture medium for each milligram dry weight of fungal cells present at the time when the concentration of cyanide in the culture medium was at its maximum (Figure 3.3)

This value reached a maximum at an initial glucose concentration of 10 mM. Above and below this concentration the maximum amount of cyanide produced for each milligram dry weight of fungal cells was lower, but quite similar, except for very high initial culture medium glucose concentrations, where the value obtained was very low.

Cultures grown in media with a high initial glucose concentration (83 mM. or higher) showed a tendency for

Figure 3.3 - Maximum dry weight (●) and maximum culture medium cyanide content (per mg.dry weight of the culture at this time, ○) during growth in shake culture in media containing different concentrations of glucose.


Maximum dry weight of the culture attained during incubation (mg.)
0
100
200
300
400
500
600
Glucose (mM.)
0
50
100
150
0
20
40
60
80
100
120
140
160
180
Maximum amount of cyanide in the culture medium during incubation.
(nmol. mgdrywt.$^{-1}$)

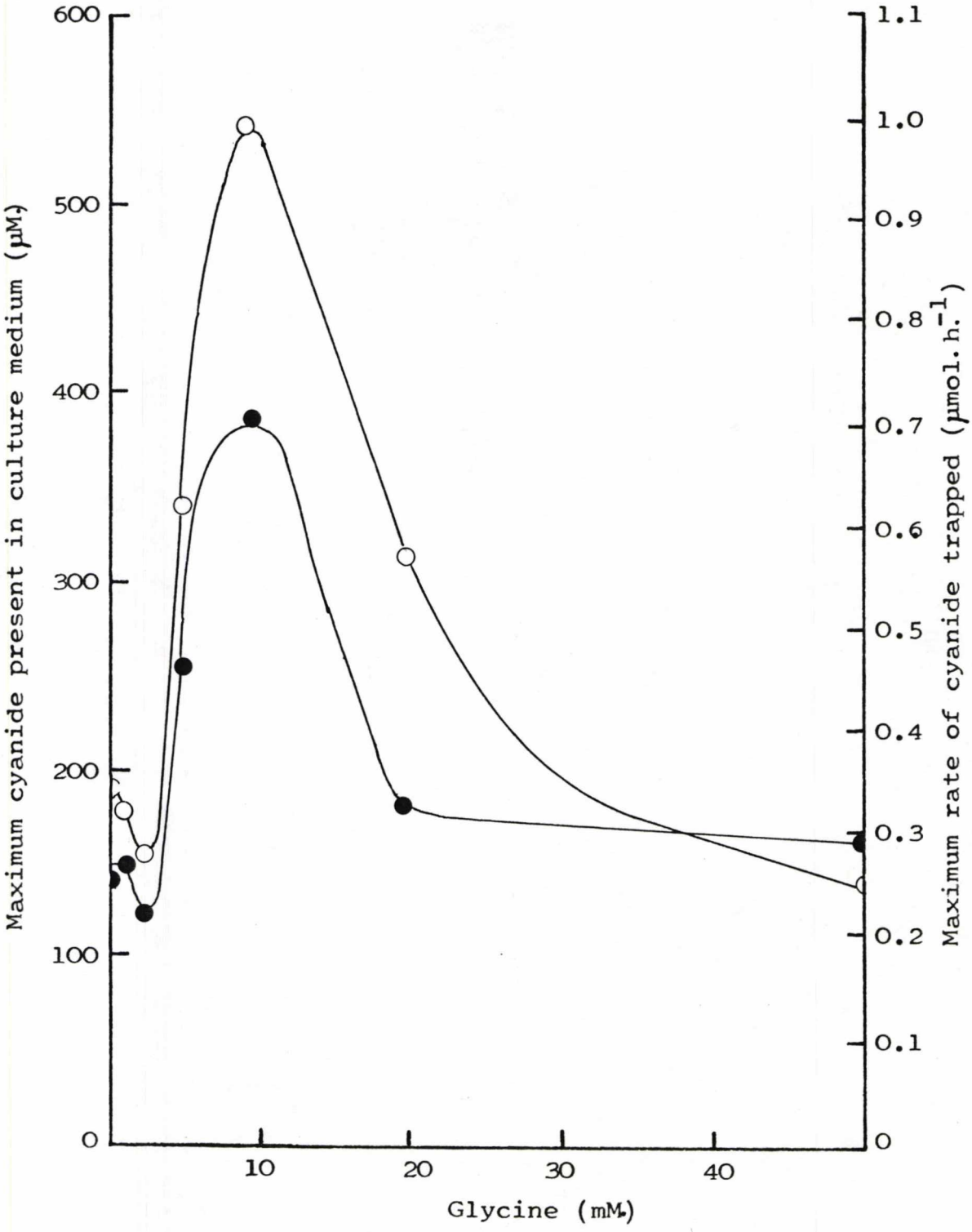
their fungal cells to clump. The degree of clumping appeared to be related to the production of an extracellular gum by these cultures. The production of cyanide also appeared to be associated with growth when the initial concentration of glucose was below 10 mM.

The relationship between the culture medium glycine concentration and cyanide production

Cyanide production by 'shake' cultures of the snow mould basidiomycete grown in media containing 10 mM glucose was stimulated over a narrow range of glycine concentrations (Figure 3.4). The maximum stimulation of cyanide production occurred when the culture medium was supplemented with 10 mM glycine, at concentrations of glycine greater than this, cyanide production decreased until little stimulation by the added glycine was observed. With glycine concentrations below 5 mM, cyanide production appeared to be inhibited, but measurements in this range proved to be too variable for this to be accurately assessed. Little glycine remained after incubation, when concentrations of glycine below 10 mM were used, but up to 40% remained in cultures incubated with 10 mM glycine. No stimulation of growth by glycine was recorded.

The way in which glycine stimulates cyanide production is recorded in Figure 3.5. Growth without glycine in the culture medium began after 4d. incubation and was associated with the decrease of glucose

Figure 3.4 - The maximum concentration of cyanide in the culture medium (●) and the maximum rate of cyanide trapped(○) during incubation of the snow mould basidiomycete in shake culture, in media containing different concentrations of glycine. Glucose (10 mM.) was used as the carbon source for all cultures.


Maximum cyanide present in culture medium (µM)
Maximum rate of cyanide trapped (µmol.h.$^{-1}$)
Glycine (mM)
600
500
400
300
200
100
0
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40

and asparagine concentrations. The maximum fungal cell dry weight achieved during incubation of the cultures occurred 3 to 4d. later and coincided with complete removal of glucose from the culture medium; 3 to 4 mM asparagine still remained. The pH of the culture medium then began to rise, as did the ammonium ion concentration (not shown), which reached nearly 5 mM on day 10.

At this stage of the incubation, cyanide first appeared in the culture medium, reaching a maximum concentration of 80 to 90 μM., 2d. later. By day 20, the medium concentration of cyanide had fallen to about 40 μM.

Growth, glucose, asparagine, NH_4 and pH changes during incubation in media containing glucose plus glycine did not vary much from cultures incubated with glucose alone, but glycine greatly affected cyanide production. In the presence of glycine, the appearance of cyanide in the culture medium began after 6 - 7d. incubation and reached a maximum concentration of nearly 300 μM., after 8 to 9d. The culture medium cyanide concentration then fell until after 20d. incubation it was just under 50 μM. By comparing cyanide loss from the culture medium of 8d. old cultures incubated with glucose plus glycine and the loss of cyanide from uninoculated culture media

Figure 3.5a - Changes of culture dry weight (●, without glycine: ○, with glycine) and culture medium cyanide content (■, without glycine: □, with glycine) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM glucose with or without 10 mM. glycine.

Figure 3.5b - Culture medium glucose content (●, without glycine: ○, with glycine) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM. glucose with or without 10 mM. glycine. Culture medium asparagine content during growth (■, without glycine: □ with glycine), was measured after the dinitrofluoro-benzene derivatives (DNFB) of the culture medium were prepared.

Figure 3.5c - Culture medium glycine content (●) and pH changes (○, without glycine: , □with glycine) during growth of the snow mould basiodiomycete in shake culture. Glycine concentrations were measured after producing the DNFB derivatives of the culture medium.

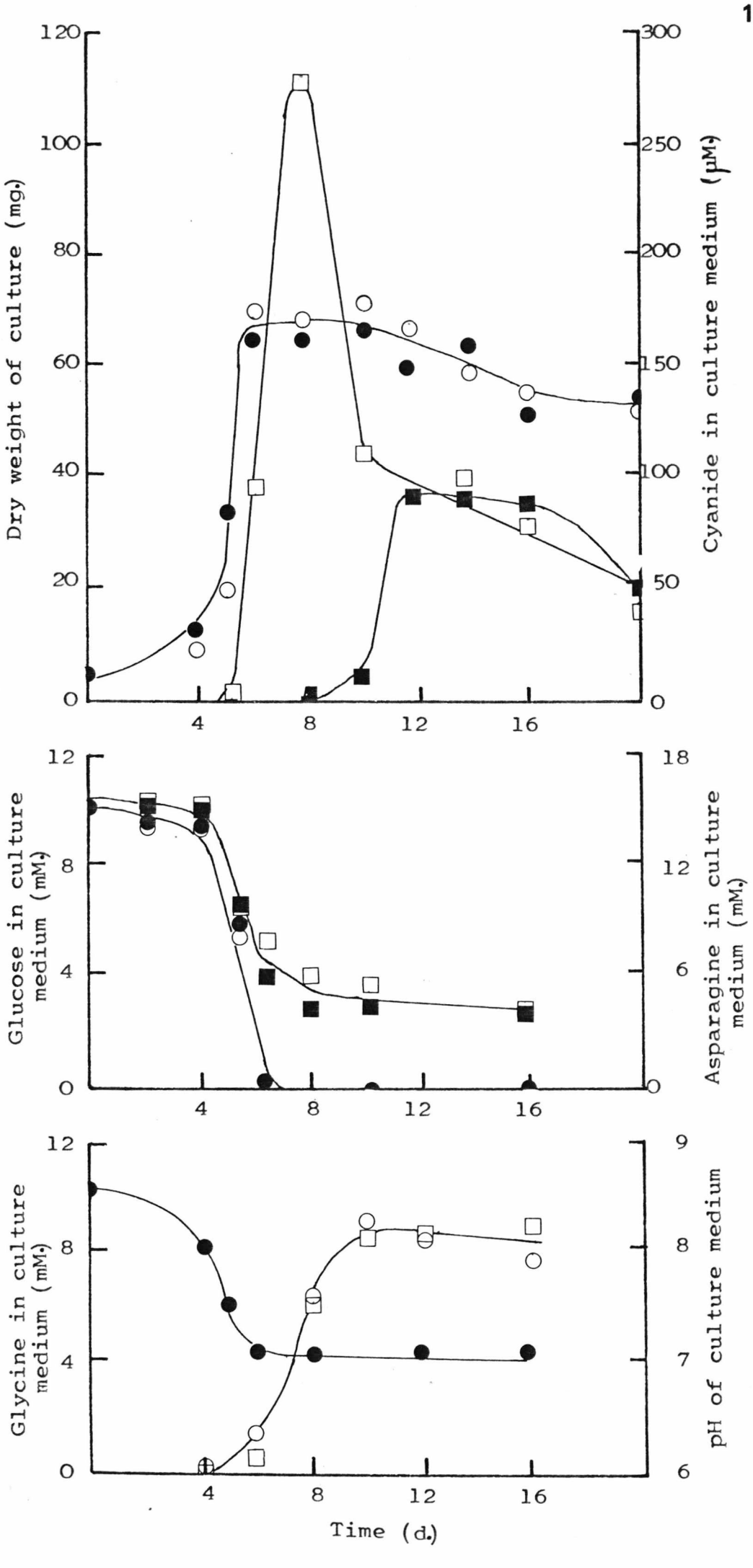

Dry weight of culture (mg.)
Cyanide in culture medium (μM.)
Glucose in culture medium (mM.)
Asparagine in culture medium (mM.)
Glycine in culture medium (mM.)
pH of culture medium
Time (d.)
0
20
40
60
80
100
120
50
100
150
200
250
300
4
8
12
16
6
7
9
18

Figure 3.6 - Loss of cyanide from culture media, uninoculated (♦) and inoculated (◇, 8 day old cultures) with the snow mould basidiomycete.

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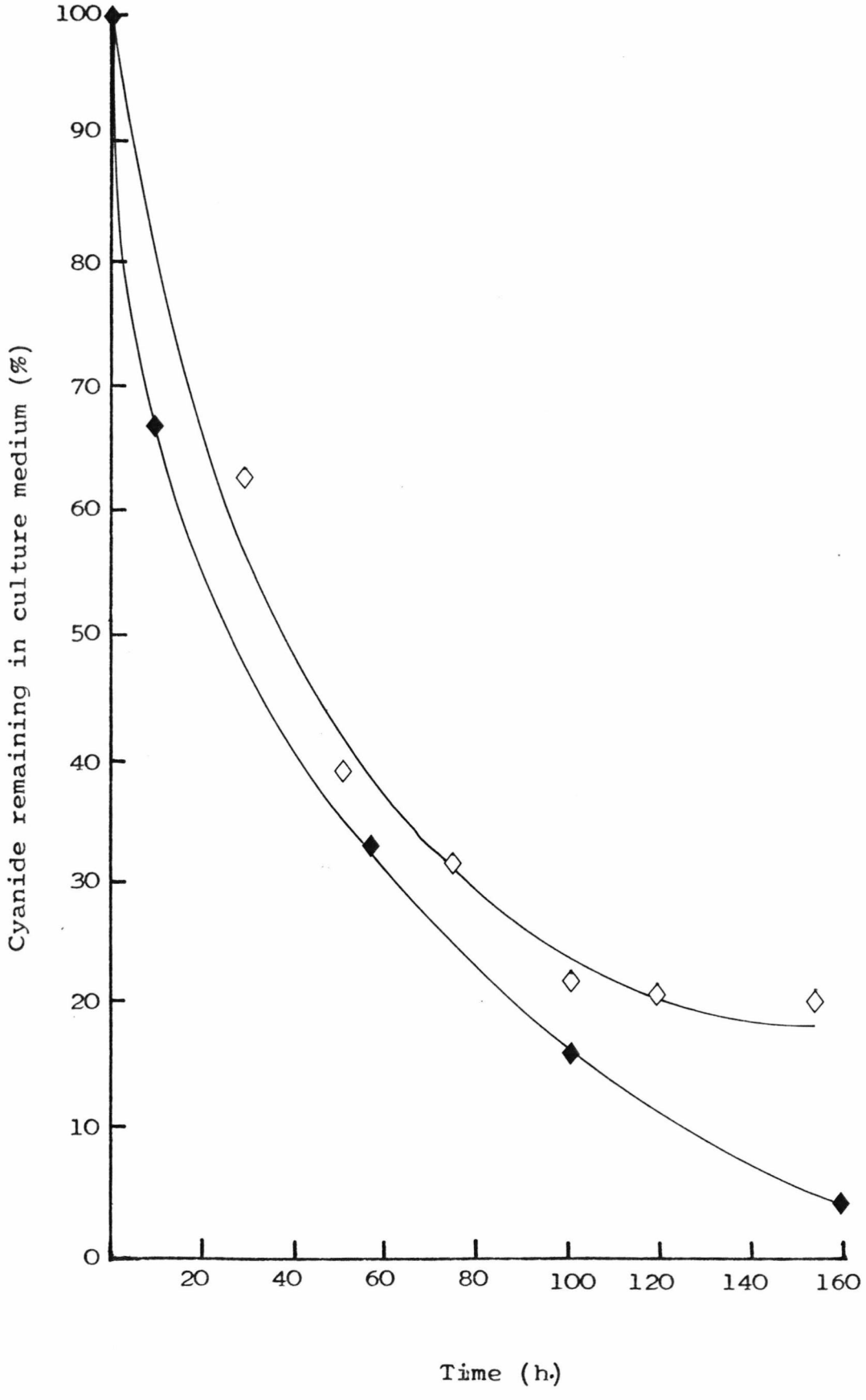

Cyanide remaining in culture medium (%)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
Time (h.)

adjusted to the cyanide concentration and pH of the inoculated culture, it appears that cyanide production in the inoculated cultures could be biphasic (Figure 3.6) The loss from either flask was initially the same, but on the equivalent of day 12 of incubation the loss of cyanide from the inoculated flask stops, whereas in the uninoculated flask the cyanide concentration continued to fall.

Glycine was removed from the culture medium during the period of active growth, but little was subsequently taken up during the 6 to 20 d. incubation period. (Figure 3.5).

Glycine stimulation of cyanide production and its dependence on the time of glycine addition to cultures

The time of glycine addition to cultures of the snow mould basidiomycete was critical for an increase of cyanide production (Figure 3.7). Glycine, if added to cultures before 8d. of incubation, stimulated cyanide production to an equal extent. No greater yields of cyanide were recorded for cultures where glycine had been added at the start of the incubation, compared to those where glycine addition was after 7d. incubation.

Addition of glycine to 12d. old cultures resulted in no stimulation of cyanide production.

The appearance of the fungal cells during incubation changed significantly (Figure 3.8). Young cultures

Figure 3.7 - The relationship between the time of 10 mM. glycine addition to the culture medium (at the time of inoculation ■, after 5d. growth □, 6d. growth ▲, 7d. growth △, and 12d. growth ◆,) of cultures of the snow mould basidiomycete and the amount of cyanide released into the culture medium.

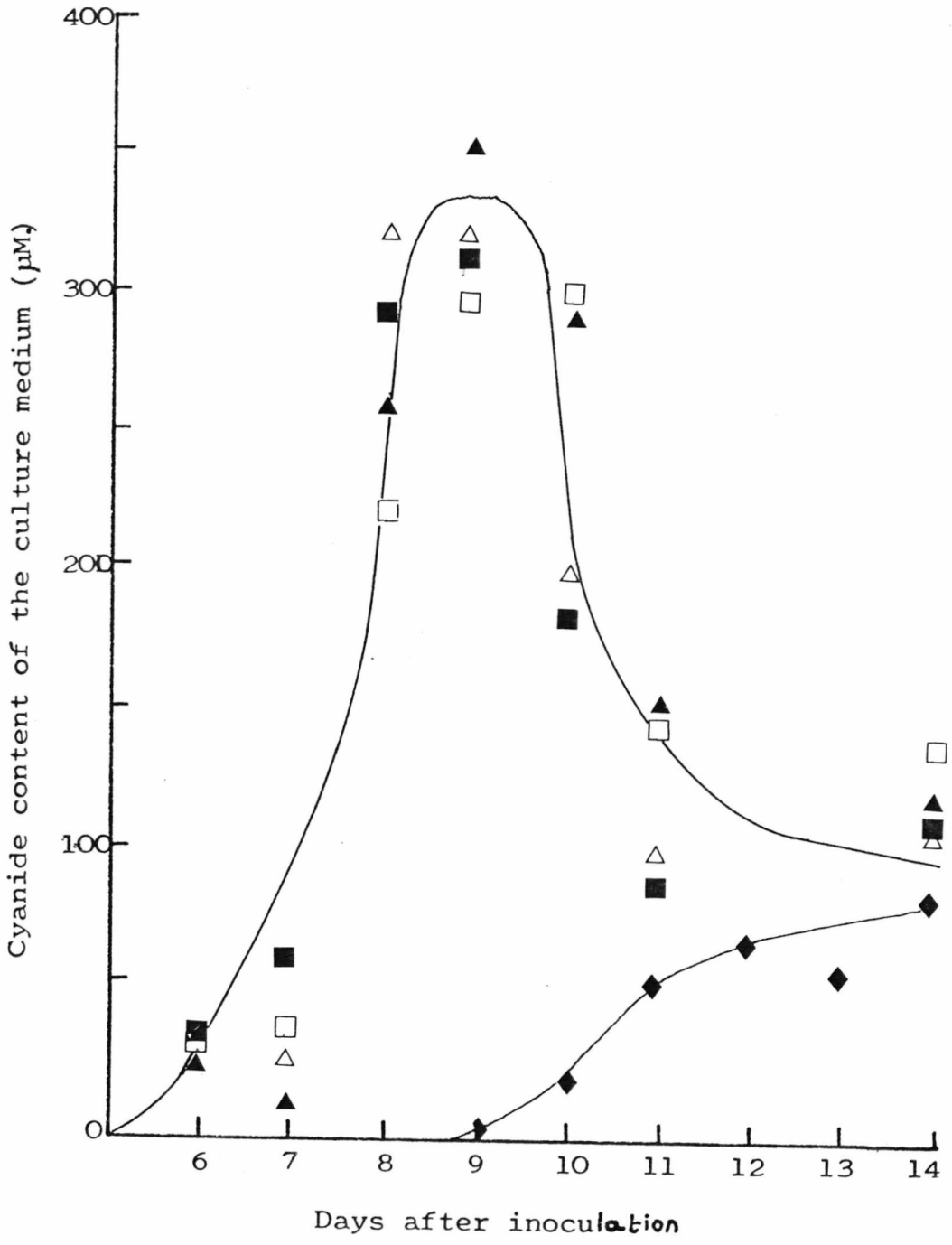
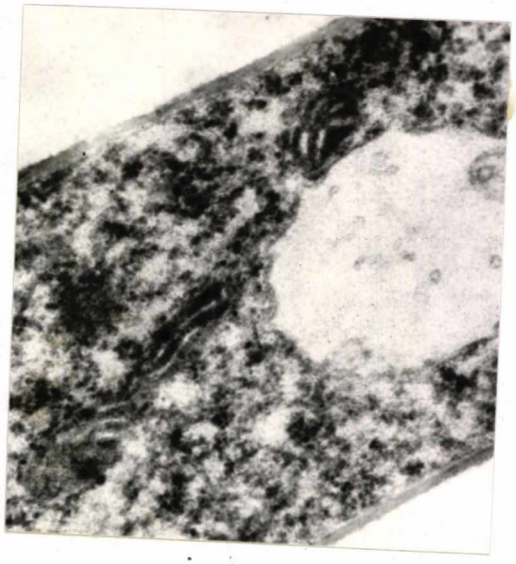

400
300
200
100
0
Cyanide content of the culture medium (µM)
6
7
8
9
10
11
12
13
14
Days after inoculation

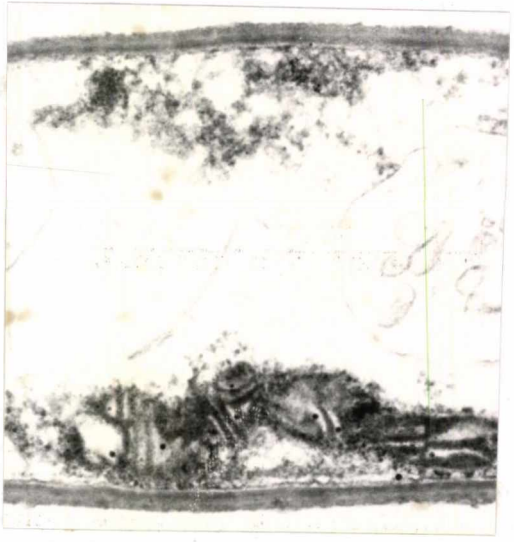
Figure 3.8 - The appearance of snow mould basidiomycete cells during growth in shake culture, in media containing 10 mM glucose.

Fungal cell from a 10d. old culture



Magnification x 25,000.

Fungal cell from a 31d. old culture



Magnification x 25,000

(less than 6d. old) had cells which contained very few large vacuoles. As the age of the cultures increased the occurrence of large vacuoles, in the cells was more often recorded, until after 31d. incubation, the lack of large vacoules was extremely rare. Protein synthesis inhibited cultures grown in 21 flasks proved to be too variable for studies to investigate the time at which the cyanide producing system was synthesised.

The effect of selected metabolites on the production of cyanide by the snow mould basidiomycete

One of the outstanding differences between bacterial and fungal cyanogenesis has been the role of methionine in these organisms. In bacteria, cyanogenesis is greatly stimulated by methionine (Knowles, 1976), whereas, in the snow mould basidiomycete, an apparent inhibition of cyanide production occurs (Ward and Thorn, 1966).

To evaluate the effect of methionine, a comparison of total cyanide production during incubation, for up to 28d. was made using cultures grown with glucose (Figure 3.9), glucose plus glycine (Figure 3.10) and glucose plus glycine plus methionine (Figure 3.11). Several interesting points were revealed by this comparison. Firstly, in all three cases between

Figure 3.9a - Changes in dry weight of cultures (■) and the culture medium cyanide content (□) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM. glucose.

Figure 3.9b - Rate at which cyanide was trapped in the centre well (▲), total cyanide trapped in the centre well (△) and total cyanide produced (◆) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM.glucose.

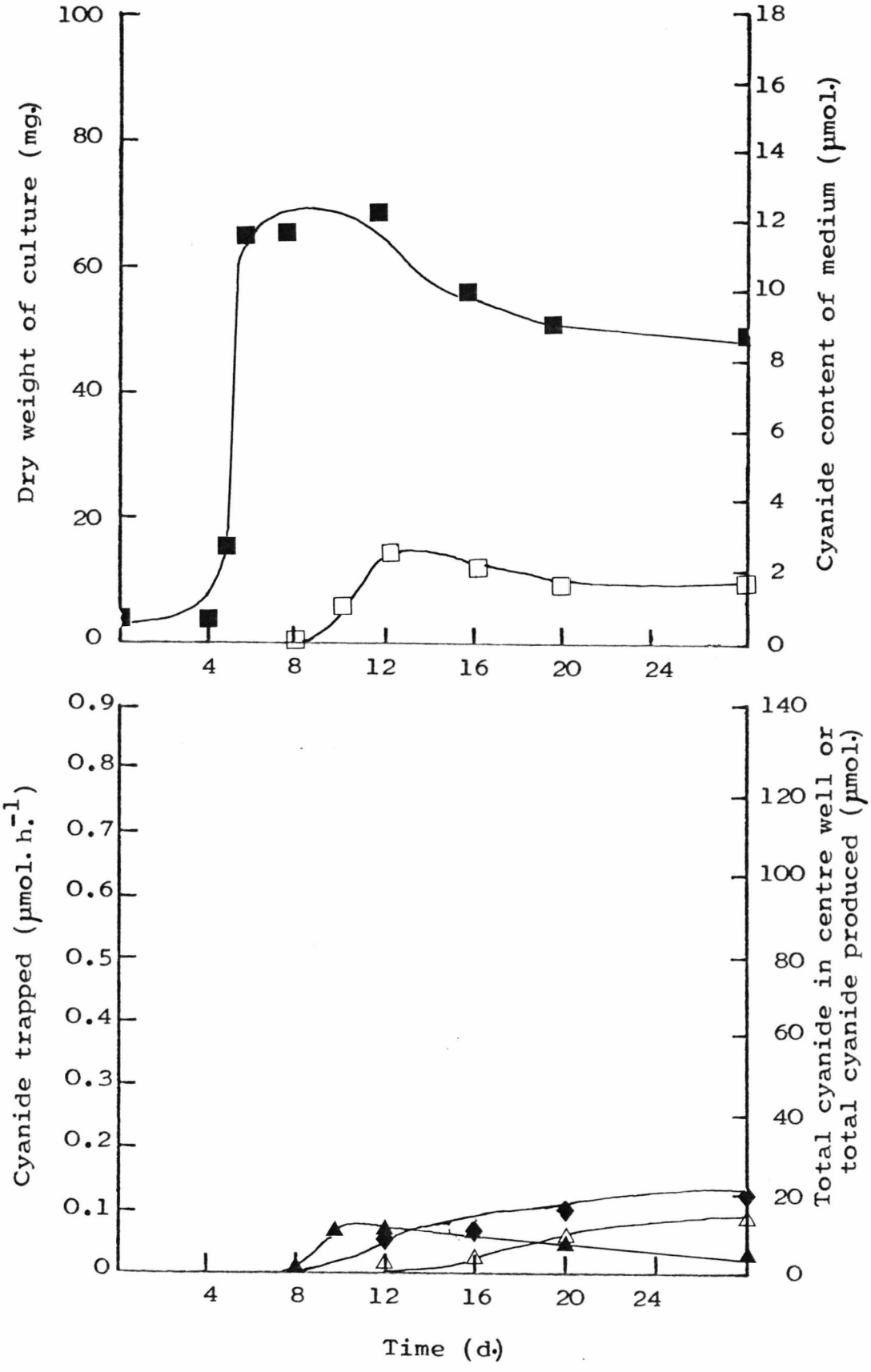

Dry weight of culture (mg.)
Cyanide content of medium (µmol.)
Cyanide trapped (µmol. h.$^{-1}$)
Total cyanide in centre well or total cyanide produced (µmol.)
Time (d.)
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
120
140
4
8
12
16
20
24

Figure 3.10a - Changes in dry weight of cultures (■) and the culture medium cyanide content (□) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM. glucose plus 10 mM. glycine.

Figure 3.10b - Rate at which cyanide was trapped in the centre well (▲), total cyanide trapped in the centre well (△) and total cyanide produced (◆) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM. glucose plus 10 mM. glycine.

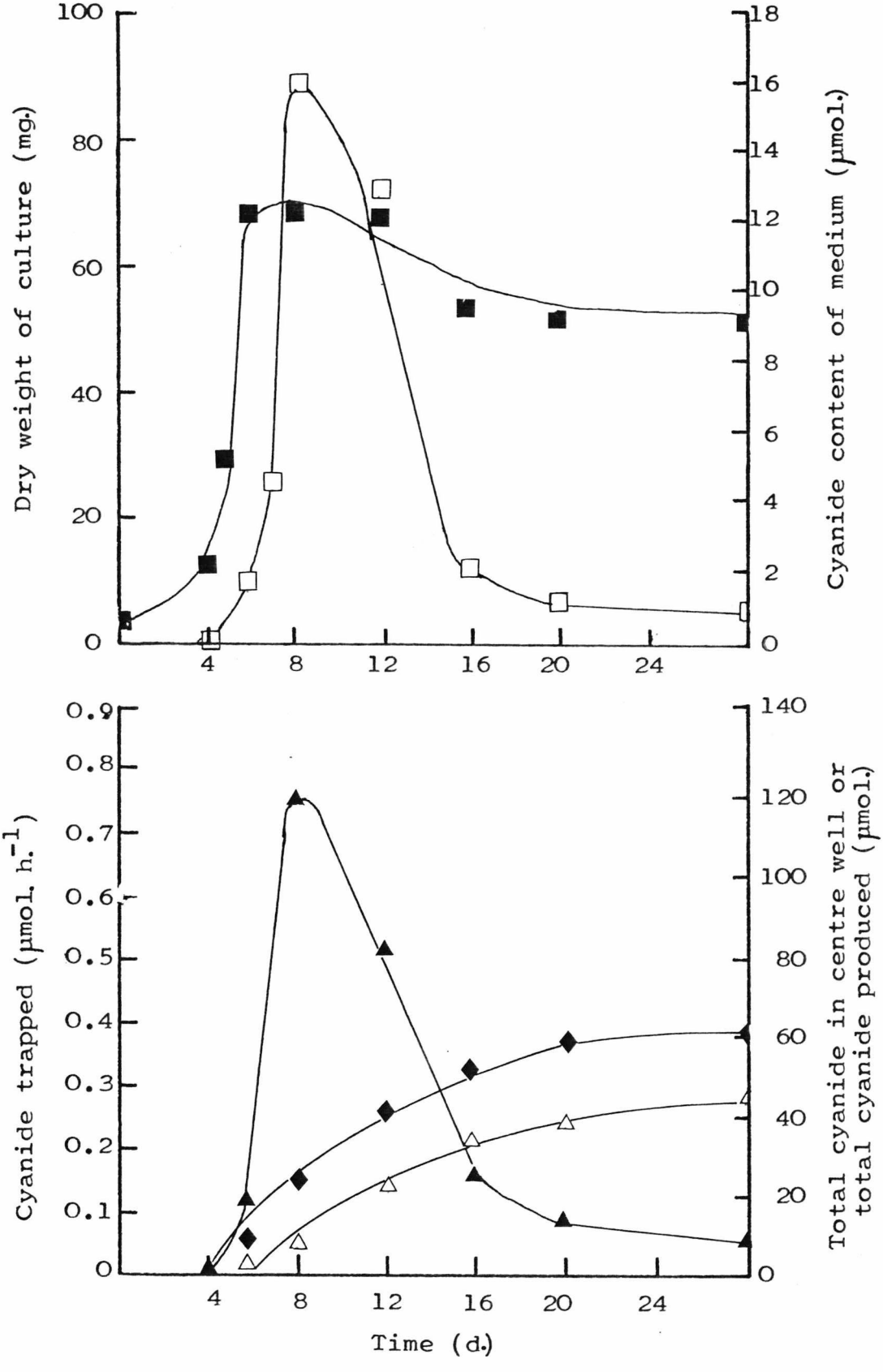
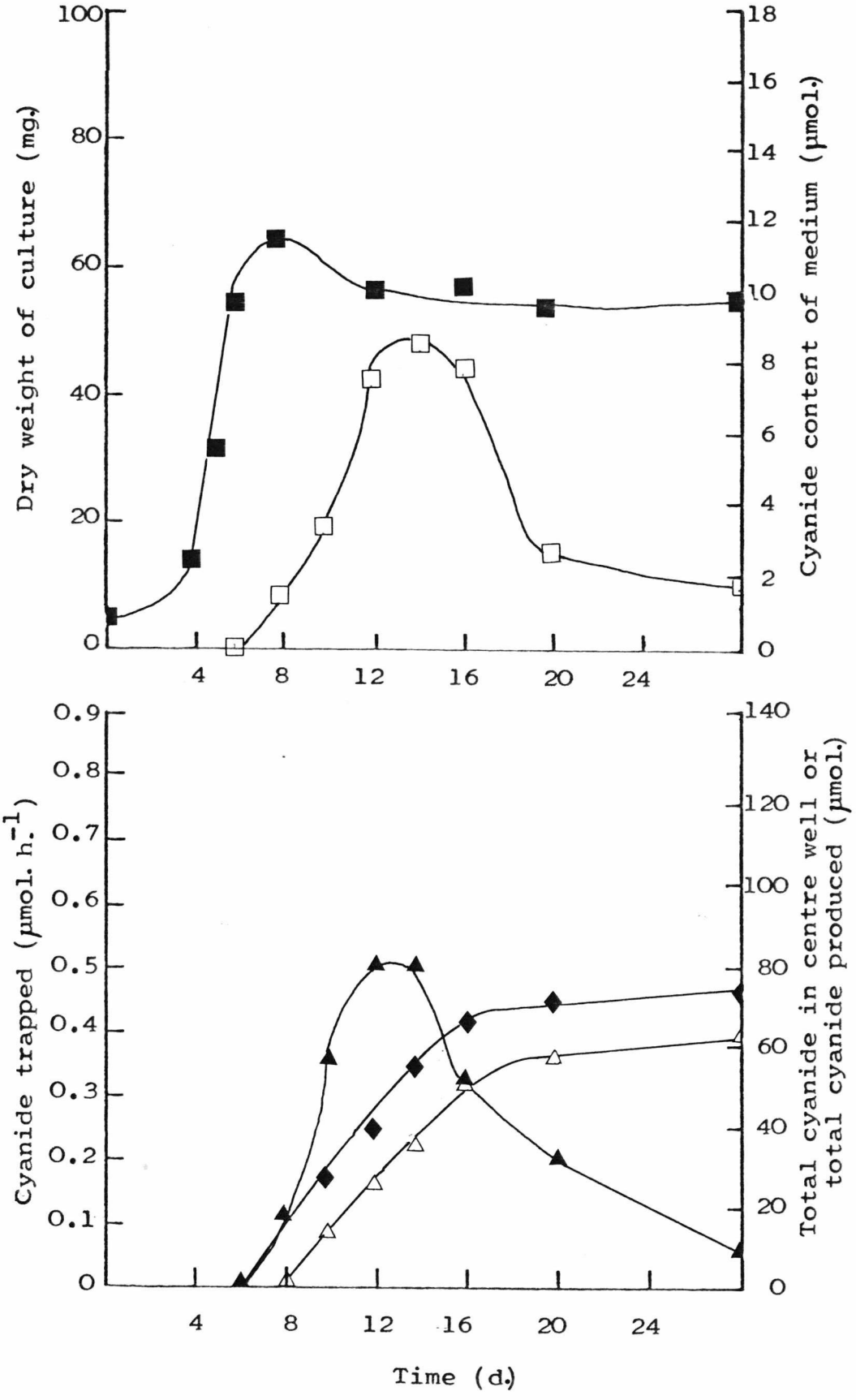

Dry weight of culture (mg.)
Cyanide content of medium (µmol.)
Cyanide trapped (µmol. h.$^{-1}$)
Total cyanide in centre well or total cyanide produced (µmol.)
Time (d.)
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
120
140
4
8
12
16
20
24

Figure 3.11a - Changes in dry weight of cultures (■) and the culture medium cyanide content (□) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM.glucose plus 10 mM.glycine and 10 mM.methionine.

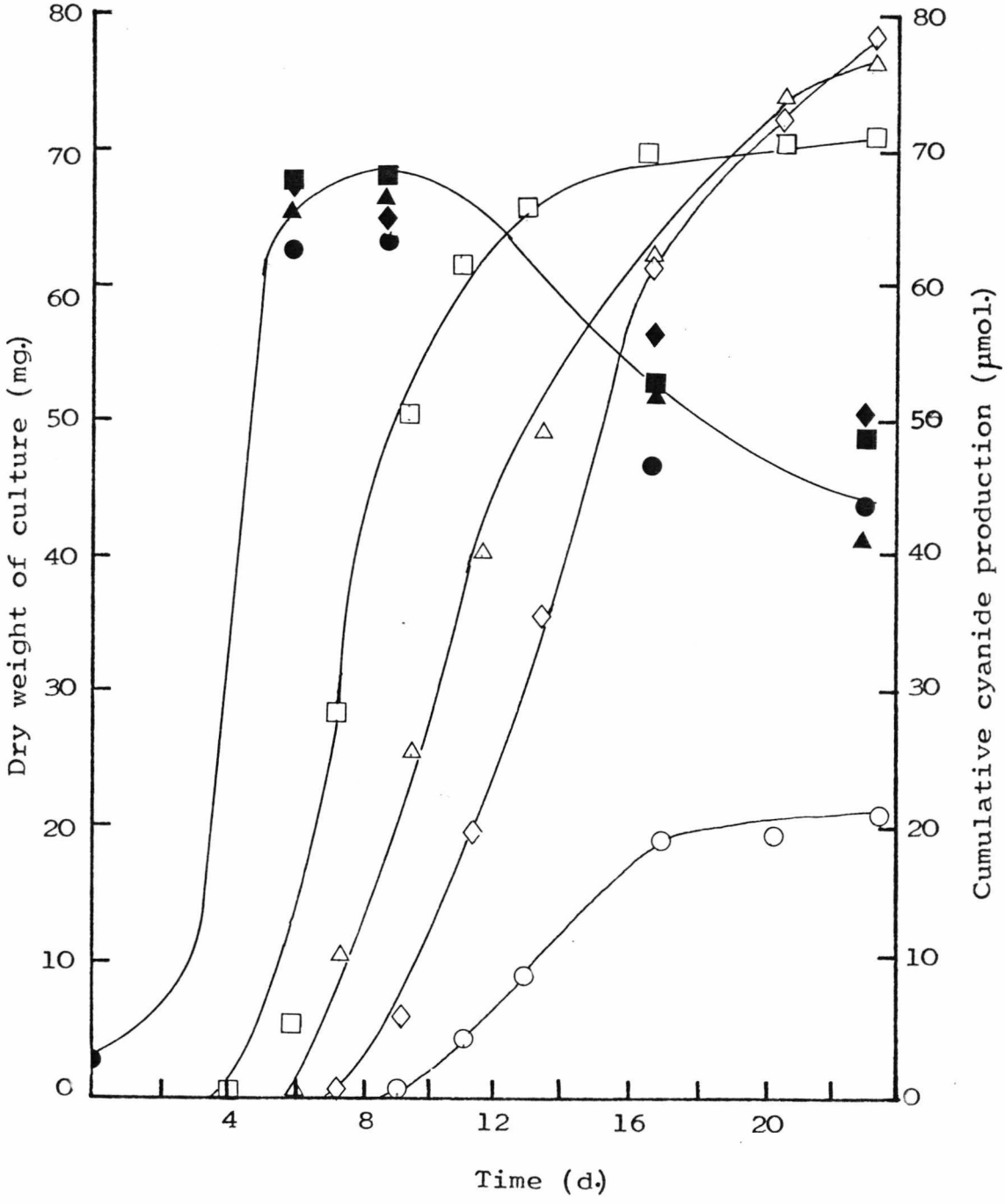
Figure 3.11b - Rate at which cyanide was trapped in the centre well (▲), total cyanide trapped in the centre well (△) and total cyanide produced (◆) during growth of the snow mould basidiomycete in shake culture, in media containing 10 mM. glucose plus 10 mM.glycine and 10 mM.methionine.


Dry weight of culture (mg.)
Cyanide content of medium (µmol.)
Cyanide trapped (µmol. h.$^{-1}$)
Total cyanide in centre well or total cyanide produced (µmol.)
Time (d.)

70 and 90% of the cyanide produced was volatilised from the cultures. Secondly 10 mM glycine trebled the amount of cyanide produced during the 28d. period of incubation from 20 µmol., without glycine, to 61 µmol. with glycine. Thirdly, 10 mM. methionine does not affect the total amount of cyanide produced, but does delay the appearance of cyanide by cultures incubated with glucose plus glycine. A similar effect was noted when cultures were grown with 83 mM glucose, where addition of 100 mM. methionine delayed cyanide production by several weeks. There was also a slight slowing down of growth under these conditions of incubation. In bacterial cyanogenesis, the concentration of methionine that maximally stimulates cyanide production is lower than the optimal concentration of glycine (Rodgers and Knowles, 1978). Lowering the concentration of the methionine added to cultures of the snow mould basidiomycete had no effect on the total cyanide produced (Figure 3.12) it only lessened the delay of cyanide production that was observed with the higher methionine concentrations. Methionine did not affect cyanide production by cultures incubated in the absence of glycine (Figure 3.13b).

The effect of a few other amino acids on cyanide production was also tested. Threonine and alanine had no effect that could not be correlated to extra

Figure 3.12 - The effect of methionine on growth (plus OmM ●, 2.5 mM.■, 5 mM.▲, or 10 mM.◆, methionine), and cyanide production (plus OmM.○, 2.5 mM.□, 5 mM. △, or 10 mM. methionine◇ by shake cultures of the snow mould basiodiomycete, when supplemented to media containing 10 mM. glucose plus 10 mM. glycine.


Dry weight of culture (mg.)
Cumulative cyanide production (µmol.)
Time (d.)
0
10
20
30
40
50
60
70
80
4
8
12
16
20

growth with these amino acids.

Serine was the only common amino acid tested that had an effect on cyanide production (Fig.3.13a). During incubation, cultures produced cyanide in two distinct phases. The first phase corresponded to the time of maximum cyanide production in cultures containing glycine and the second phase to the period of maximum cyanide production by cultures with only glucose and asparagine present in the medium. This second phase of cyanide production was nearly double that observed in cultures incubated without serine (Fig. 3.13a).

Other less common amino acids, betaine, sarcosine and N,N - dimethyl glycine were added to culture media with glycine to evaluate their possible roles as inhibitors of cyanide production in the snow mould basidiomycete, as suggested by Ward and Thorn (1966). Sarcosine had no effect on cyanide production, the results were nearly identical to those recorded in Figure 3.14a. N,N - dimethyl glycine and betaine both affected cyanide production and growth noticeably (Figure 3.14b and c). Growth with both amino acids present was slower and very much reduced the maximum fungal cell dry weights recorded. Cyanide was produced continually during growth and, if allowances are made for the dry weight differences between cultures, more cyanide was produced (2 to 3 times as much) than

Figure 3.13a - Growth (●) and cyanide production (○) by shake cultures of the snow mould basidiomycete in media containing 10 mM.glucose.

Figure 3.13b - Growth (■) and cyanide production (□) by shake cultures of the snow mould basidiomycete in media containing 10 mM.glucose plus 10 mM.methionine.

Figure 3.13c - Growth (▲) and cyanide production (△) by shake cultures of the snow mould basidiomycete in media containing 10 mM.glucose plus 10 mM.serine.

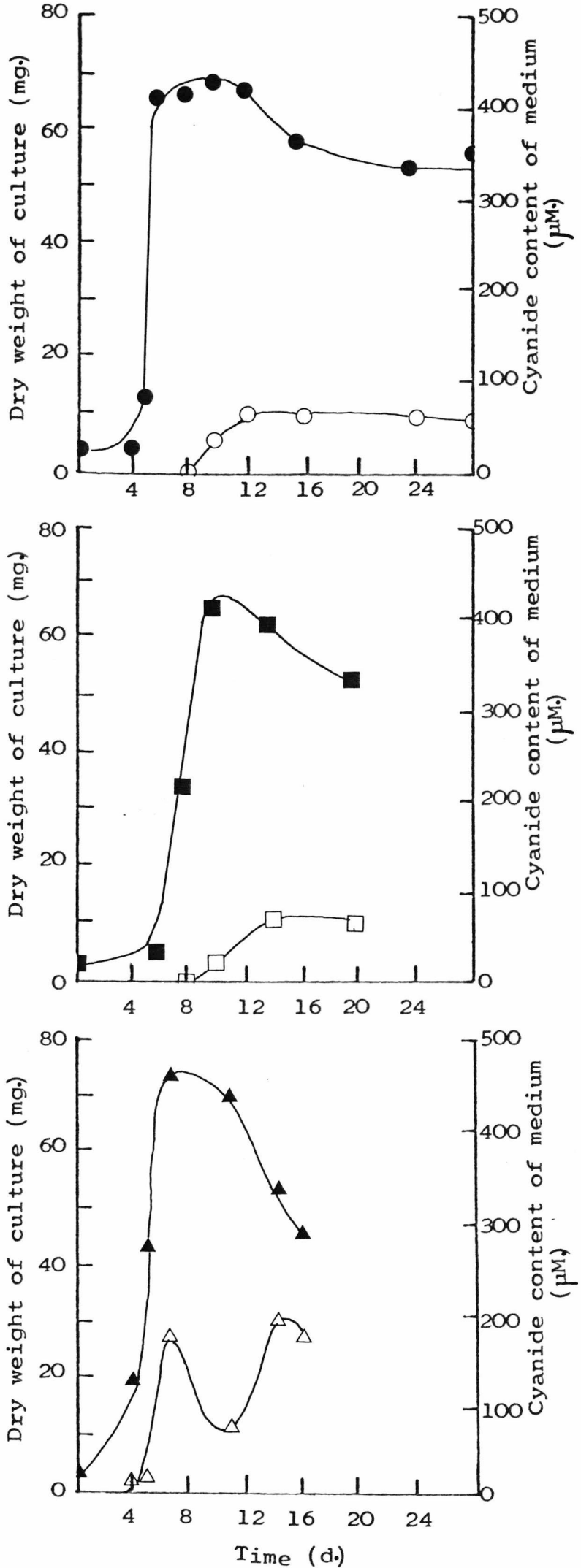
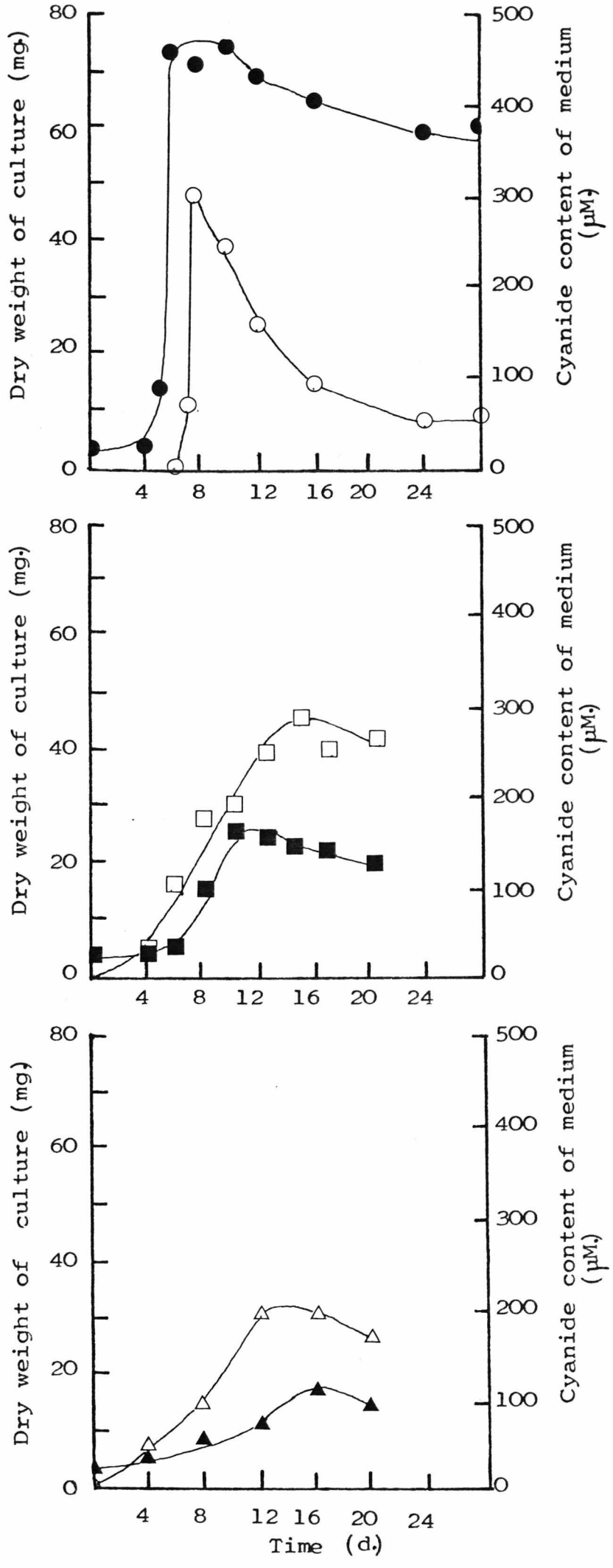

Dry weight of culture (mg.)
Cyanide content of medium (µM.)
0
20
40
60
80
100
200
300
400
500
4
8
12
16
20
24
Time (d.)

Figure 3.14a - Growth (●) and cyanide production (○) by shake cultures of the snow mould basidiomycete in media containing 10 mM.glucose plus 10 mM.glycine.

Figure 3.14b - Growth (■) and cyanide production (□) by shake cultures of the snow mould basidiomycete in media containing 10 mM.glucose plus 10 mM.glycine and 10 mM. N,N-dimethylglycine.

Figure 3.14c - Growth (▲) and cyanide production (△) by shake cultures of the snow mould basidiomycete in media containing 10 mM.glucose plus 10 mM.glycine and 10 mM. betaine.

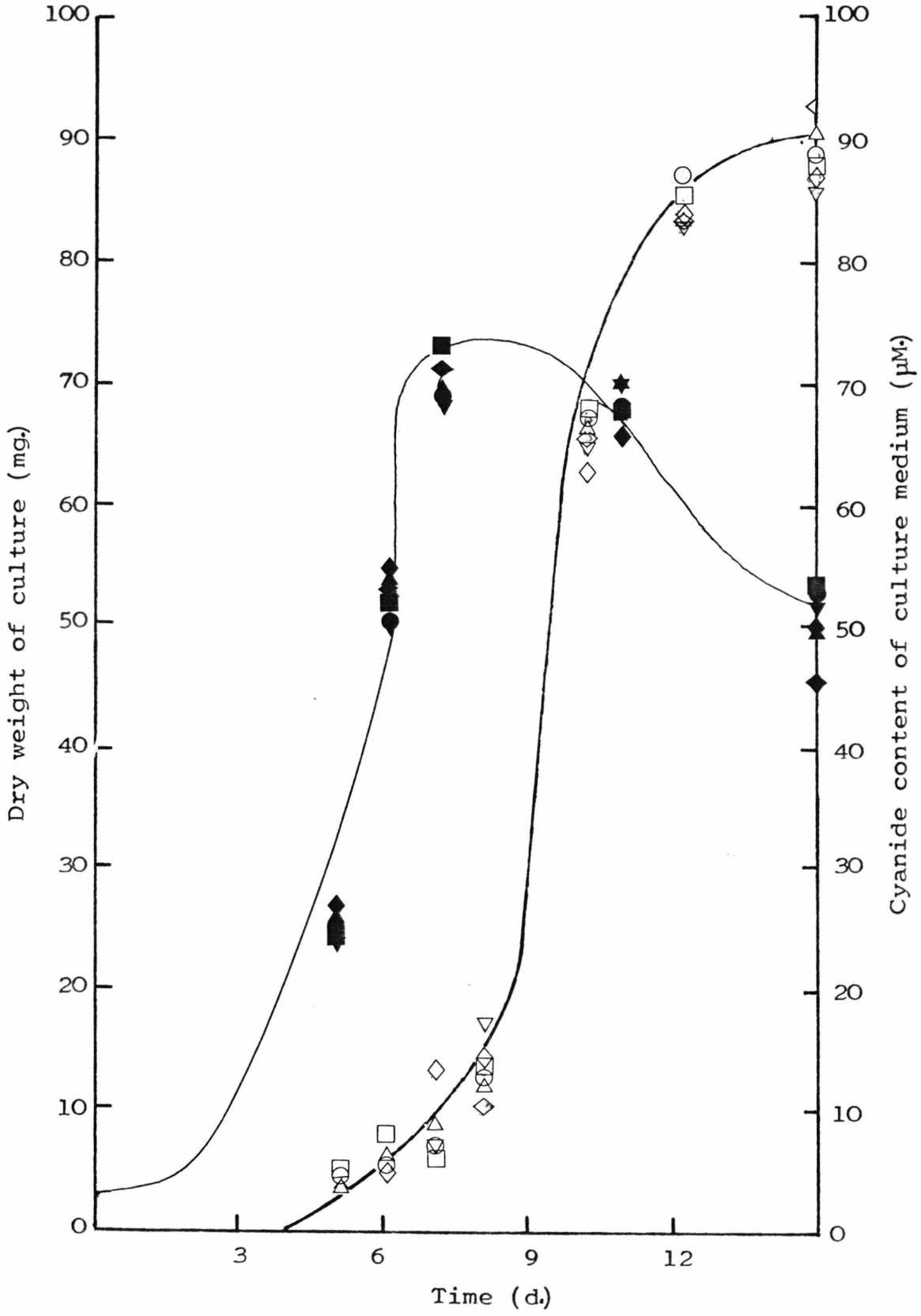

Dry weight of culture (mg.)
Cyanide content of medium (μM.)
0
20
40
60
80
100
200
300
400
500
4
8
12
16
20
24
Time (d.)

observed by cultures to which neither betaine or N,N -dimethyl glycine had been added (Figure 3.14a) Betaine delayed growth and stimulated cyanide production more than N,N - dimethyl glycine.

It was observed (Figure 3.6b) that cyanide production began in cultures incubated with 10 mM, glucose after most of the glucose had been removed from the culture medium. The possibility of catabolite repression by glucose was investigated by addition of exogenous cyclic AMP to the medium. No effect on cyanide production was recorded when cyclic AMP was added to the culture medium either at the beginning of, or during incubation (Figure 3.15). The effect of other derivatives of cyclic AMP, such as N,N - dibutyrl- cyclic AMP, on cyanide production were not investigated.

Two other metabolites, phosphate and Fe^{2+} have been reported to effect cyanide production in bacteria (Rodgers and Knowles, 1978; Castric 1975). Phosphate had only a small effect on cyanide production by the snow mould basidiomycete (Figure 3.16). However, the effect of Fe^{2+} was more noticeable, as its concentration in the culture medium was increased from 8 to 50 μM. a slight inhibition of cyanide production resulted. No further inhibition was obtained by increasing the concentration of Fe^{2+} above 50 μM.

Figure 3.15 - The effect of cyclic AMP on growth (without cyclic AMP ●, with cyclic AMP added at the start of the incubation ■, after 5d. ▲, 6d. ◆, 7d. ▼, or 8d. ◆ and cyanide production (without cyclic AMP ○, with cyclic AMP added at the start of the incubation □, after 5d. △, 6d. ◇, 7d. ▽, or 8d. ◇ by shake cultures of the snow mould basidiomycete, in media containing 10 mM. glucose.


Dry weight of culture (mg.)
Cyanide content of culture medium (µM.)
Time (d.)
0
10
20
30
40
50
60
70
80
90
100
3
6
9
12

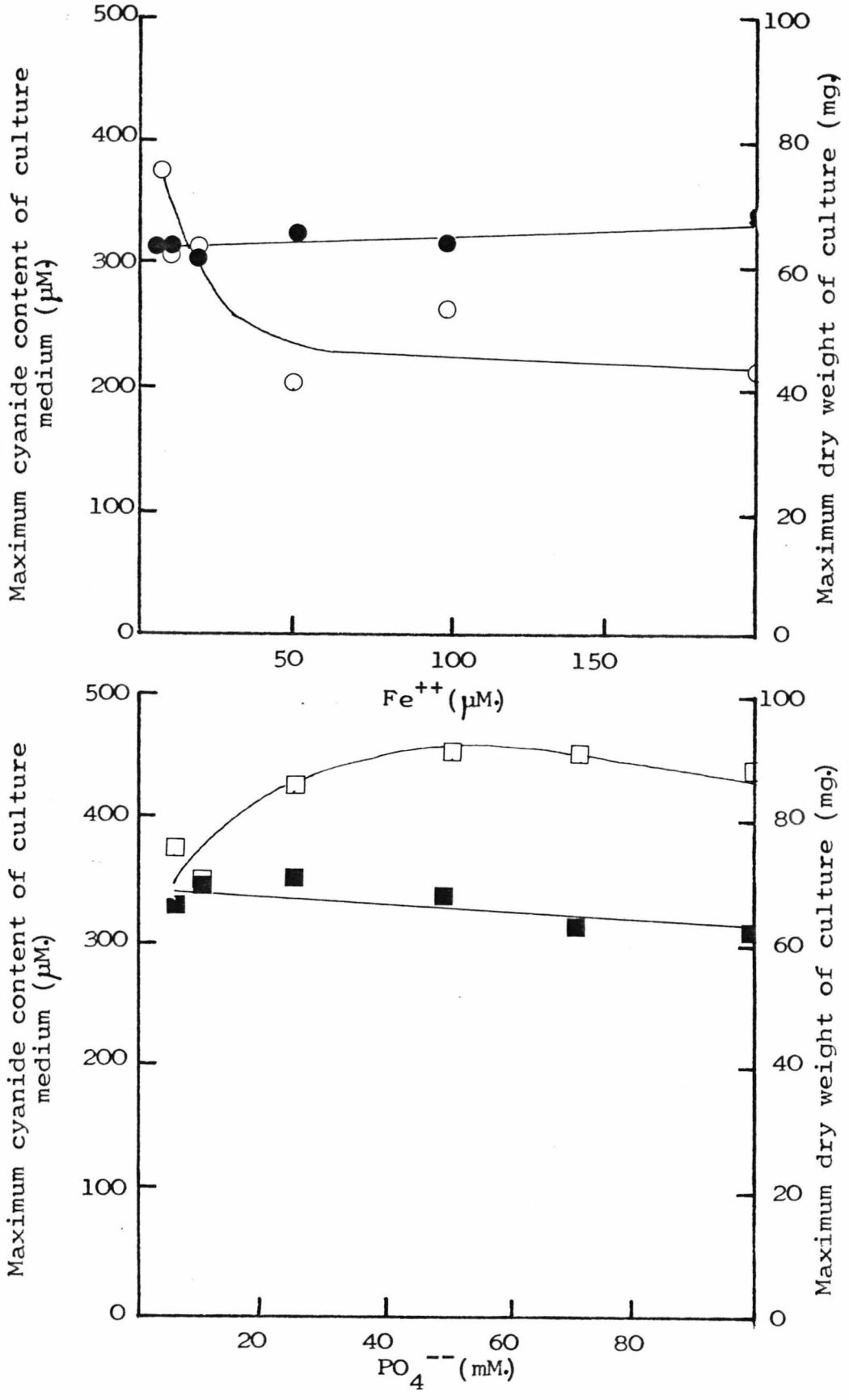
Zinc, a more potent effector of fungal secondary metabolism than Fe^{2+} (Weinberg 1978) had little effect on cyanide production. All three metabolites Fe^{2+}, Zn^{2+} and inorganic phosphate had no effect on growth and the final yield of fungal cell dry weight, but phosphate did cause clumping of the fungal cells, when present at a concentration above 50 mM.

Addition of amino acids to protein synthesis inhibited cultures produced some very interesting observations. Threonine, serine, glycine, betaine, N,N - dimethyl glycine and sarcosine, when added to 7d. old protein synthesis inhibited cultures caused no cyanide production. If, however, glycine was added with betaine or N,N - dimethyl glycine, cyanide production occurred (Figure 3.18). No other combination of these amino acids produced this effect. Cyanide production by these cultures became very much reduced after 3d. incubation, coinciding with a marked change of cultural appearance. Severe clumping of the fungal cells in the cultures took place, which was not observed in cultures to which other amino acids had been added.

Although the effect of these amino acids was did not vary, the extent of these effects varied a lot and so a comparison of cultures grown with or without glycine was difficult and inconclusive.

Figure 3.16 - The effect of varying the Fe^{2+} concentration in the culture medium on the maximum fungal cell dry weight yield (●) and maximum cyanide content (○) of the culture medium, during growth of shake cultures of the snow mould basidiomycete, in media containing 10 mM. glucose plus 10 mM. glycine.

Figure 3.17 - The effect of varying the PO_4^{--} concentration in the culture medium on the maximum fungal cell dry weight yield (■) and maximum cyanide content (□) of the culture medium, during growth of shake cultures of the snow mould basidiomycete, in media containing 10 mM. glucose plus 10 mM. glycine.


Maximum cyanide content of culture medium (μM.)
Maximum dry weight of culture (mg.)
500
400
300
200
100
0
100
80
60
40
20
0
50
100
150
Fe++ (μM.)
Maximum cyanide content of culture medium (μM.)
Maximum dry weight of culture (mg.)
500
400
300
200
100
0
100
80
60
40
20
0
20
40
60
80
PO4-- (mM.)

Chloramphenicol had little effect on cyanide production or growth at the concentration used.

Disruption of snow mould basidiomycete mycelia.

Several methods of cell disruption were attempted. Glass beads, except when used in the Braun disintegrator, caused little breakage when shaken with mycelia. Unfortunately, Braun disintegration proved to be unreliable due to the difficulty of temperature regulation during operation.

Freezing the cells in liquid nitrogen, followed by grinding in a pessel and mortar also gave poor results. The only mechanical procedure that produced reproducible efficiencies of breakage was the use of a French press. Breakage of the mycelia was estimated by protein release and visual examination. It was found that 2 passages through a French pressure cell at 8000 lbf. $in.^{-2}$ was the lowest number of passages and pressure, to provide the maximum visual disruption (Figures 3.19a and b) or protein release from mycelia (Figure 3.20). Higher pressures and number of passages produced no visual differences or little extra release of protein. Lower pressures produced less visual breakage and lower, more variable protein release. One passage through the French pressure cell at 12000 lbf. $in.^{-2}$ was roughly comparable to 2 passages at 8000 lbf. $in.^{-2}$ (about 80% of this value).

Figure 3.18 - The effect of 10 mM. glycine (●), 5mM. N,N dimethylglycine (■), 5mM. betaine (▲), 10 mM. glycine plus 5 mM. N,N dimethylglycine (◆), or 10 mM. glycine plus 5 mM. betaine (▼), on cyanide production by protein synthesis inhibited shake cultures prepared from 7d. old shake cultures of the snow mould basidiomycete grown in media containing 10 mM glucose. Protein synthesis was inhibited by 250 μg. ml.$^{-1}$ chloramphenicol and 50 μg. ml.$^{-1}$ cycloheximide.

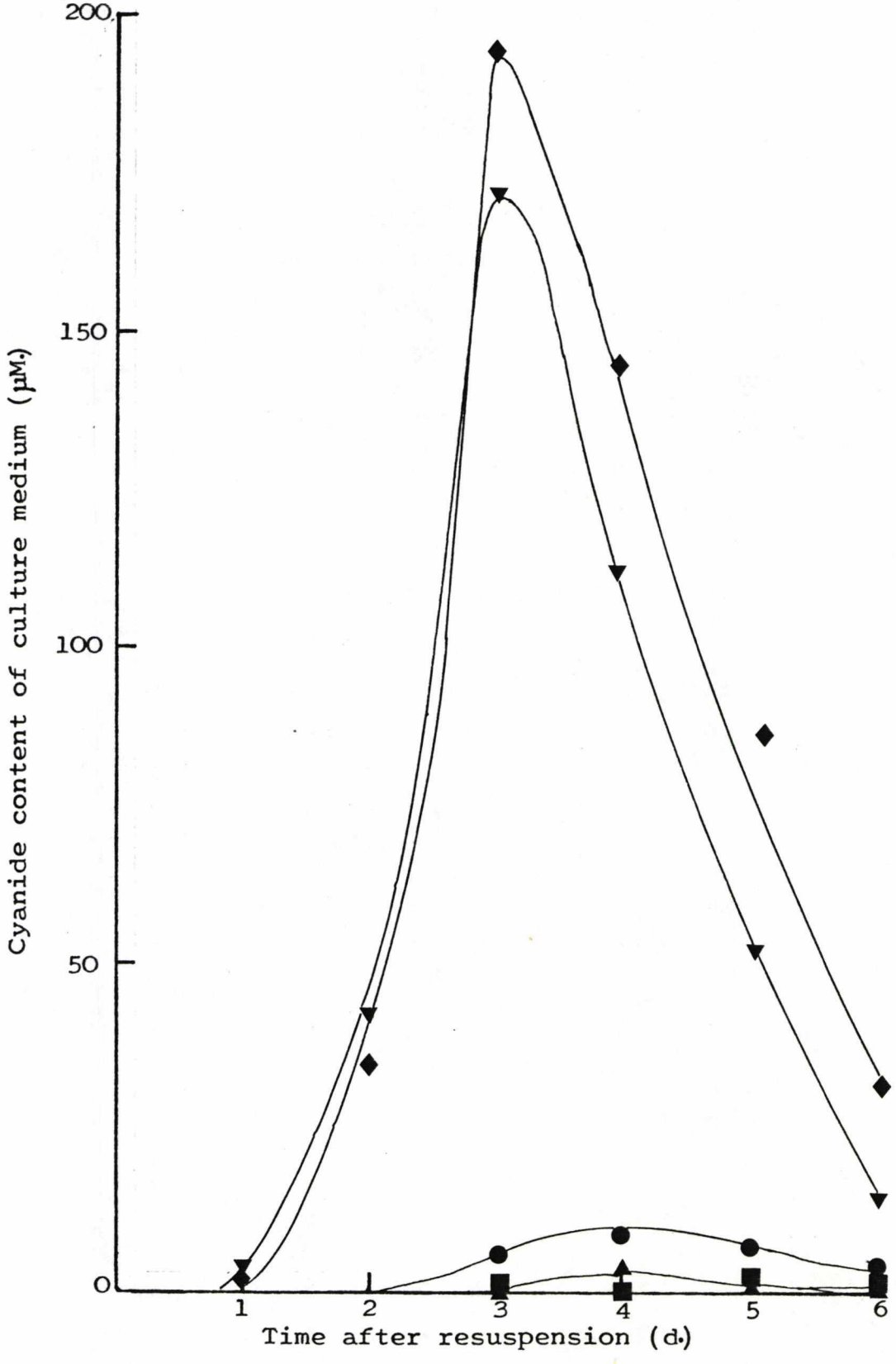

200
150
100
50
0
Cyanide content of culture medium (µM.)
1
2
3
4
5
6
Time after resuspension (d.)

Figure 3.19a - Mycelial appearance before two passages at 8000 lbf. $in.^{-2}$ through a French pressure cell:

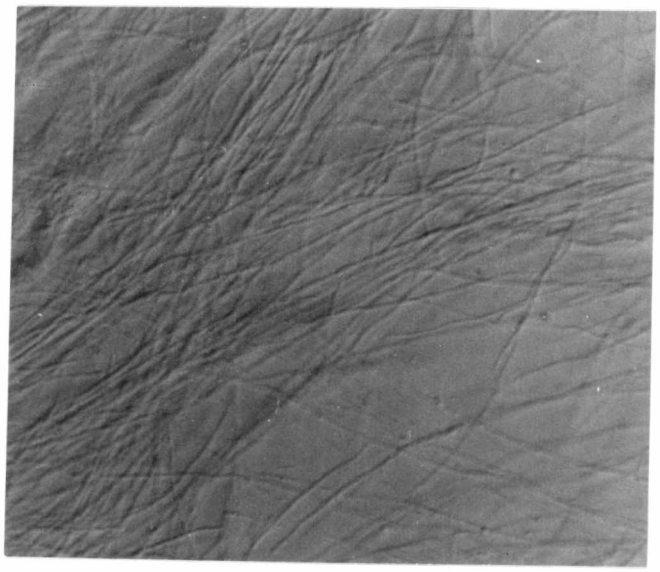
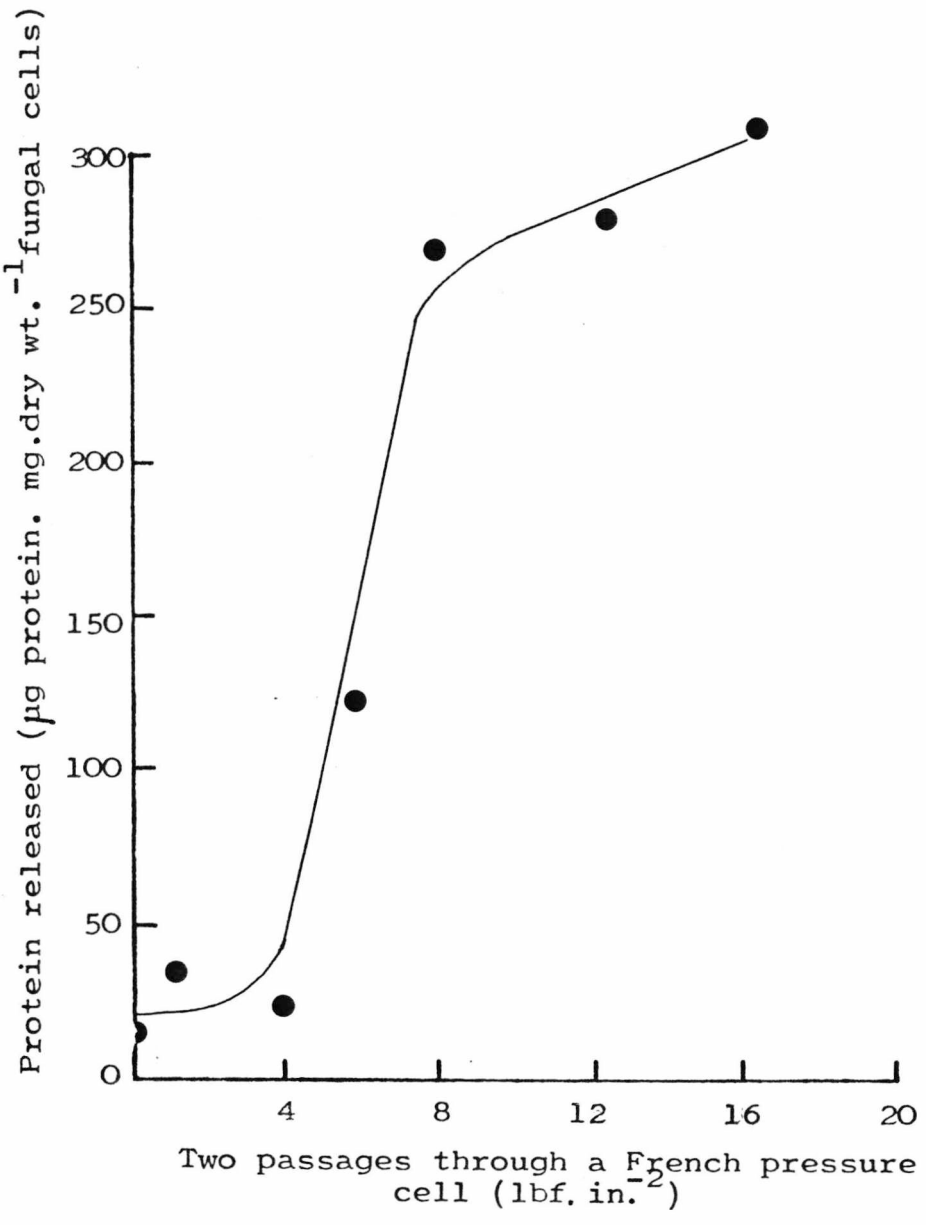


Figure 3.19b - Mycelial appearance after two passages at 8000 lbf. $in.^{-2}$ through a French pressure cell:

Figure 3.20 - Protein released from fungal cells ● harvested from 7d. old shake cultures of the snow mould basidiomycete, grown in media containing 10 mM. glucose.


Protein released (µg protein. mg.dry wt.$^{-1}$fungal cells)
300
250
200
150
100
50
0
4
8
12
16
20
Two passages through a French pressure cell (lbf. in.$^{-2}$)

Several attempts were made to induce lytic enzymes towards the snow mould basidiomycete using Trichoderma viride and Athrobacter luteus but no success was had with either of these organisms. Sonication of the mycelia was also ineffectual.

The efficiency of breakage was calculated by reference to an average total protein estimation of 280 µg. protein $mg.dry.wt^{-1}$ obtained by digestion of mycelia in 1M NaOH, followed by the measurement of protein by the Biuret procedure (Gornall, Bardawill and David (1949). This figure did not fluctuate much between mycelia of different ages except when very old or very young mycelia were used, where generally lower values were obtained.

The metabolism of $[U^{-14}C]$ glucose by cultures of the snow mould basidiomycete

Removal of glucose and radioactivity from the culture medium did not occur simultaneously during incubation (Figure 3.21b). At the end of 18d., nearly 25% of the original label remained in the culture medium, but no glucose could be detected at this time. The production of cyanide from $[U^{14}C]$ glucose during incubation also produced no noticeable change of the radioactive content of the culture medium.

Chromatography of the culture medium showed that the radioactivity remaining after glucose depletion was not associated with glyoxylic acid or pyruvic acid

Figure 3.21a - Growth (●) and culture medium cyanide content (○) during growth of shake cultures of the snow mould basidiomycete in media containing 10 mM. [U-^{14}C] glucose (6nCi.μmol.$^{-1}$).

Figure 3.21b - Changes of the glucose (■) and radioactive content of the culture medium (□) during growth of shake cultures of the snow mould basidiomycete, in media containing 10 mM. [U-^{14}C] glucose (6nCi μmol.$^{-1}$).

Figure 3.21c - $^{14}CO_2$ production (▲) and changes of the radioactive content (△) of cell-free extracts prepared from shake cultures of the snow mould basidiomycete grown in media containing 10 mM [U-^{14}C] glucose (6nCi.μmol.$^{-1}$).

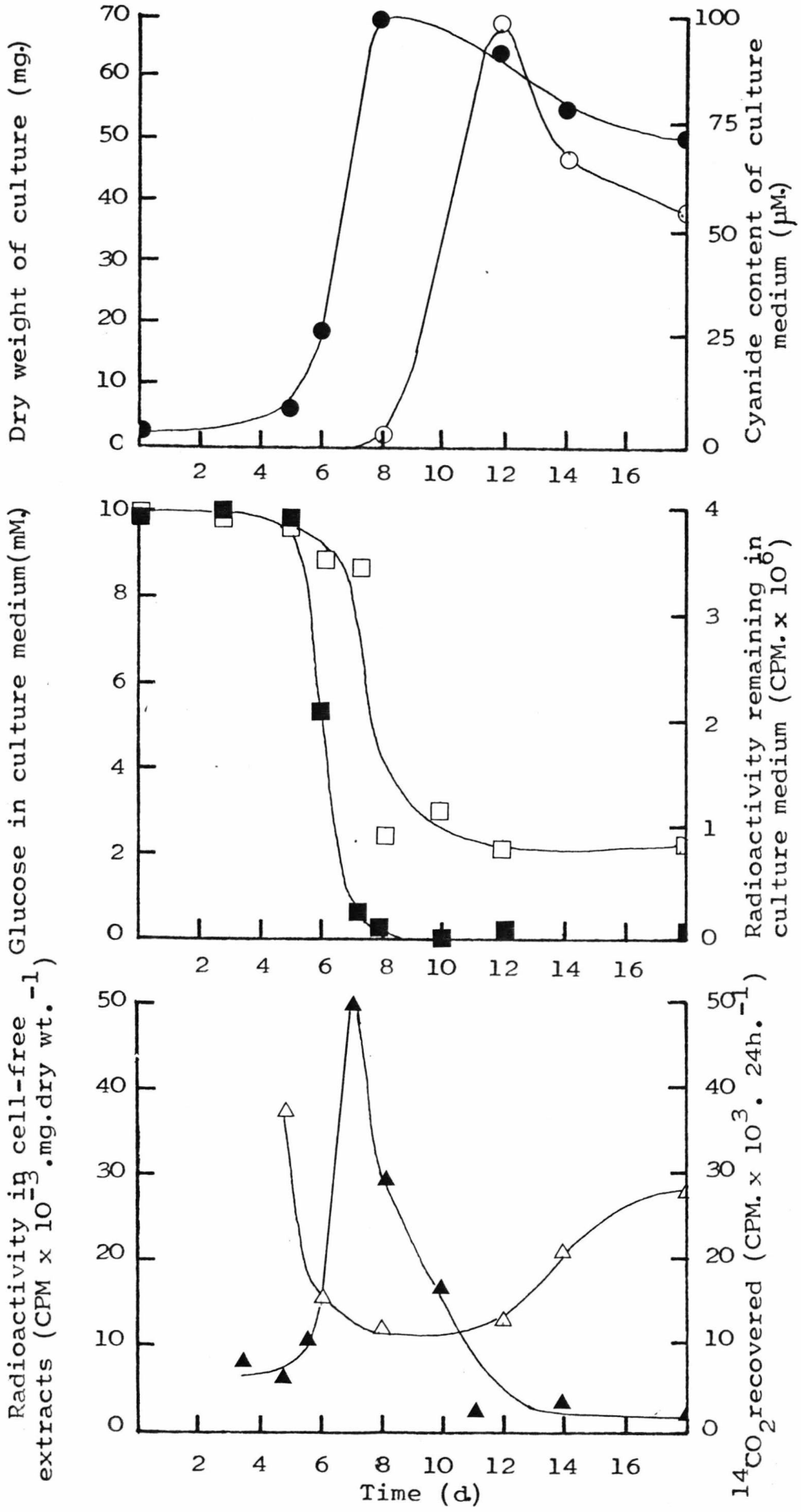
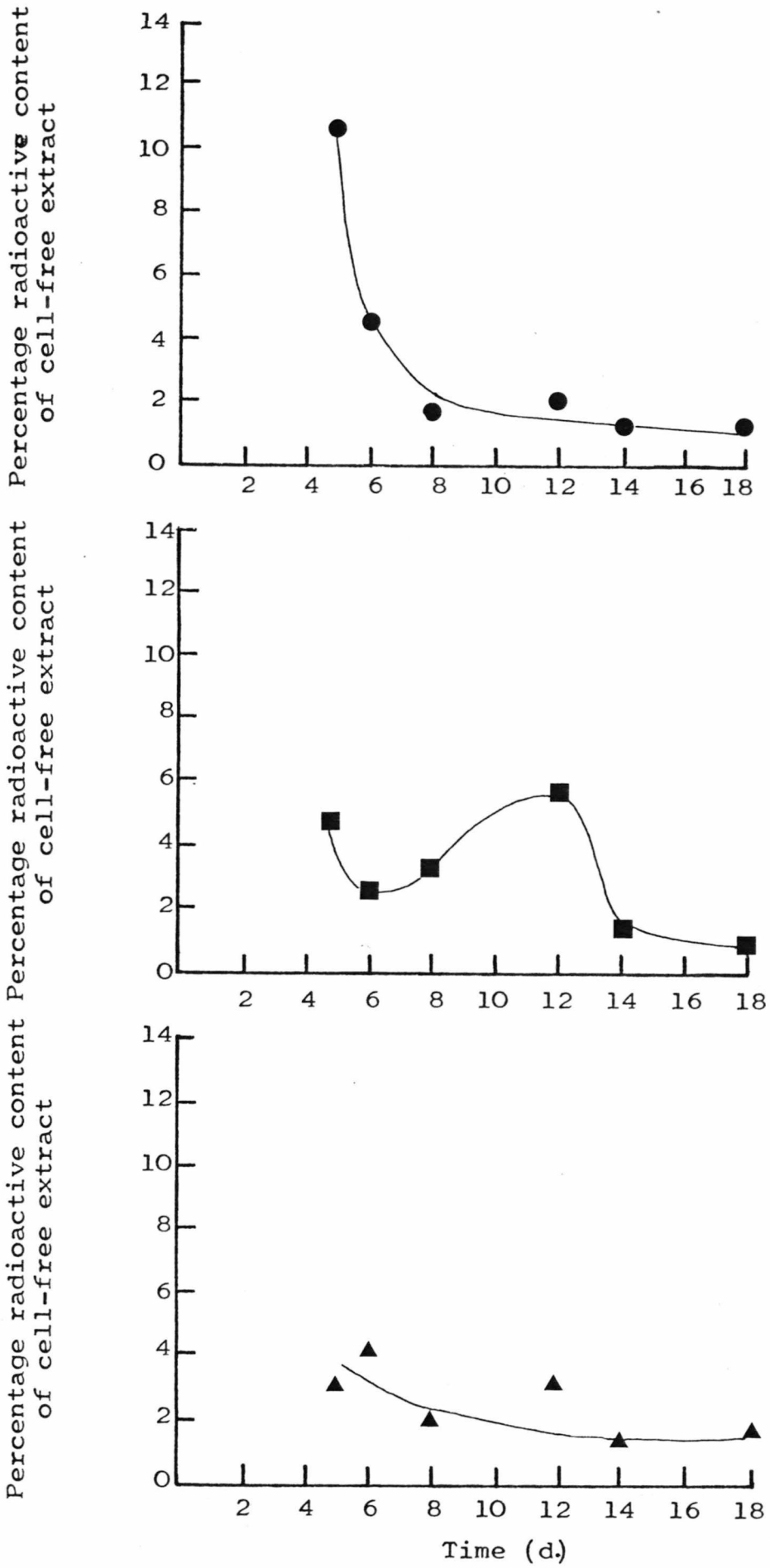

Dry weight of culture (mg.)
Cyanide content of culture medium (µM.)
Glucose in culture medium (mM.)
Radioactivity remaining in culture medium (CPM. x 10^6)
Radioactivity in cell-free extracts (CPM x 10^-3 .mg.dry wt.^-1)
14CO2 recovered (CPM. x 10^3 . 24h.^-1)
Time (d)

Figure 3.22a - Changes of the radioactivity associated with the glycine (●) present in cell-free extracts prepared from shake cultures of the snow mould basidiomycete grown in media containing 10 mM. [U-^{14}C] glucose (6nCi μmol.$^{-1}$).

Figure 3.22b - Changes of the radioactivity associated with alanine (■) present in cell-free extracts prepared from shake cultures of the snow mould basidiomycete grown in media containing 10 mM. [U-^{14}C]glucose (6nCi μmol.$^{-1}$).

Figure 3.22c - Changes of the radioactivity associated with aspartic acid (▲) present in cell-free extracts prepared from shake cultures of the snow mould basidiomycete grown in media containing 10 mM. [U-^{14}] glucose (6nCi μmol.$^{-1}$).


Percentage radioactive content of cell-free extract
Percentage radioactive content of cell-free extract
Percentage radioactive content of cell-free extract
0
2
4
6
8
10
12
14
16
18
Time (d.)

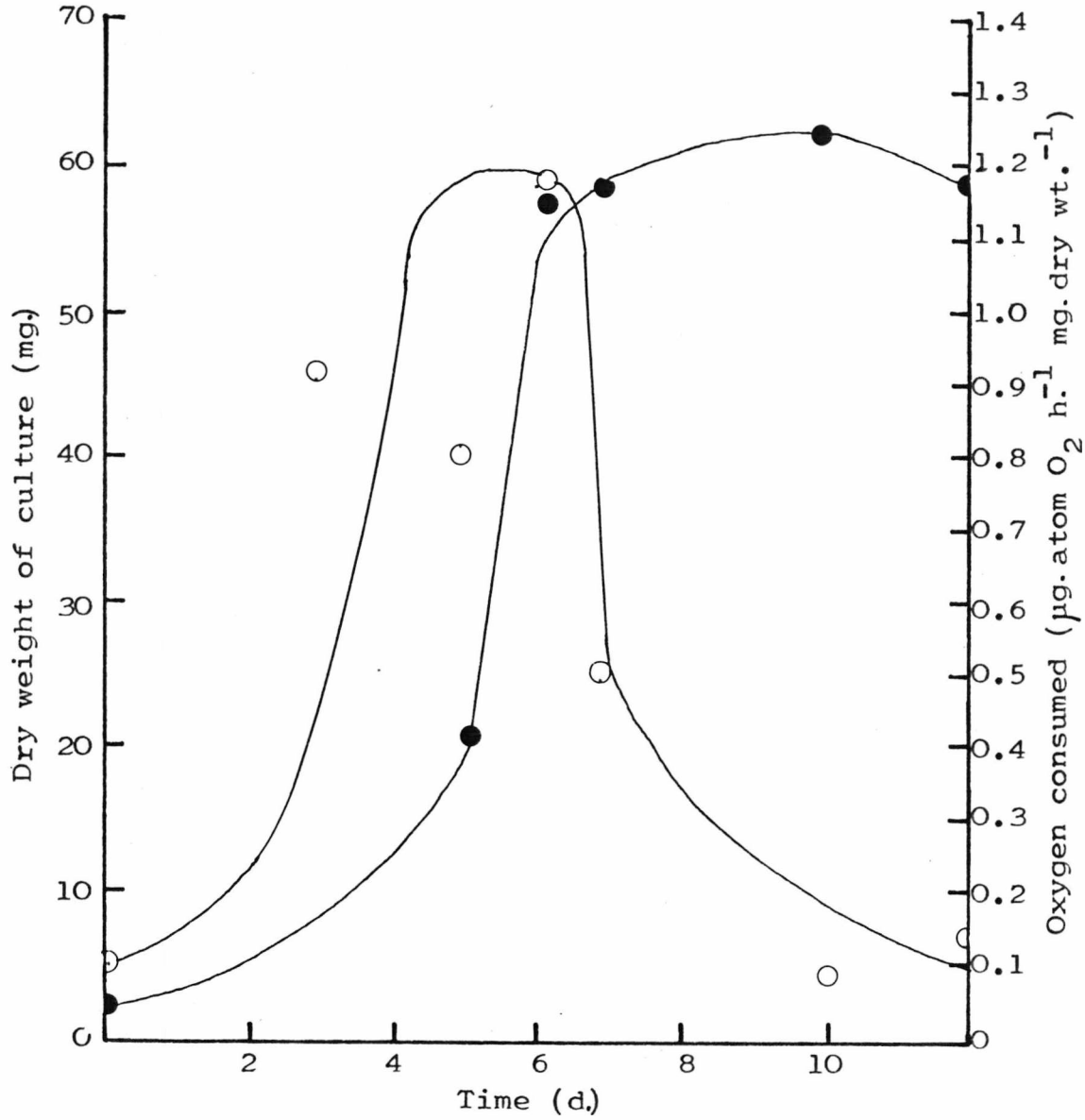
cyanohydrin. The nature of the product with which the radioactivity was associated remains unknown.

During incubation, the radioactive content of the cell free supernatents prepared by French pressing varied greatly (Figure 3.21c) After 5d. incubation, which was just before the rapid increases of fungal cell dry weight (Figure 3.21a) the radioactive content of the cell free supernatents was high. During the subsequent 7d. the radioactive content fell to a relatively constant level after which it slowly began to rise, coincidental with the autolysis of the cultures, marked by the steady decrease of fungal cell dry weight. The production of $^{14}CO_2$ (Figure 3.21c) and the increase of whole cell respiration (Figure 3.23) were maximal at the same stage of culture development, namely when the increase of fungal cell dry weight was most rapid. Inhibition of whole cell respiration (97%) was observed with 1 mM cyanide (41% inhibited with 200 μM. KCN).

No inhibition of whole cell respiration was obtained with 2.5 mM. salicyl hydroxamic acid. The effect of cyanide on whole cell respiration was similar throughout the incubation period and did not alter during the time of cyanide production.

Chromatography of the cell free supernatents revealed that the concentration of amino acids varies little during incubation (Figure 3.22). The concentration

Figure 3.23 - Whole cell respiration (○) during growth (●) of shake cultures of the snow mould basidiomycete, in media containing 10 mM. glucose.


Dry weight of culture (mg.)
Oxygen consumed (µg.atom O_2 $h.^{-1}$ mg.dry $wt.^{-1}$)
Time (d.)
0
10
20
30
40
50
60
70
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
2
4
6
8
10

of glycine was high before rapid growth began, but throughout the remaining incubation period it changes very slowly and it is almost constant. Of the other amino acids investigated, alanine and aspartic acid were typical (Figure 3.22b and c). Some showed a few variations during growth, as seen with alanine (Figure 3.22b) but the changes were not dramatic and most of the amino acid concentrations did not fluctuate during incubation as, for example, aspartic acid (Figure 3.22c). When due account has been taken for the different number of carbon atoms derived from [U-^{14}C] glucose, the concentrations of free glycine, or alanine present on day 8 of the incubation period were 198 nM and 132 nM respectively, assuming that 90% of the wet weight of a fungal cell is water. This also assumes that the asparagine used was converted to these amino acids in the same proportions as glucose.

The metabolism of [1-^{14}C] or [2-^{14}C] glycine by the snow mould basidiomycete

Figure 3.24b shows $^{14}CO_2$ and H^{14}CN production from [1-^{14}C] glycine during incubation in media containing 10 mM. glucose and 10 mM glycine (20 nCi. μmol.$^{-1}$). very little H^{14}CN was produced from [1-^{14}C] glycine, as revealed by the small amount of radioactive cyanide trapped during incubation. The production of $^{14}CO_2$ from [1-^{14}C] glycine was very much more significant

and was maximally produced on day 6 of incubation, coinciding with the period of most rapid glycine removal from the culture medium and just before cyanide production (Figure 3.25a). At the time of maximum cyanide production, the amount of $^{14}CO_2$ produced from [1-^{14}C] glycine was much greater than H^{14}CN production from [2-^{14}C] glycine (combination of the H^{14}CN trapped with an estimate of the H^{14}CN remaining in the culture medium Figure 3.25a.

The residual radioactivity in the culture medium during incubation could be fully accounted for, as unconverted glycine.

Production of $^{14}CO_2$ and H^{14}CN from [2-^{14}C] glycine is shown in Figure 3.25b. Very little $^{14}CO_2$ was recovered during the incubation but a large amount of H^{14}CN was produced from [2-^{14}C] glycine and coincided with the time of maximum cyanide production recorded in Figure 3.25a. In contrast to the results obtained with [1-^{14}C] glycine, the amount of residual radioactivity in the growth medium was greater than the residual [2-^{14}C] glycine.

Cultures were harvested after 7d. and 12d. incubation in media containing [2-^{14}C] glycine and the distribution of radioactivity in the cell free supernatents after chromatography on t.l.c. plates recorded (Figure 3.26). The majority of the radioactivity was,

Figure 3.24a - Growth (●) and culture medium cyanide content (○) during growth of shake cultures of the snow mould basidiomycete in media containing 10 mM glucose plus 10 mM. [1-^{14}C] glycine (20nCi.μmol.$^{-1}$).

Figure 3.24b - $^{14}CO_2$ (■) and H^{14}CN (□) production during growth of shake cultures of the snow mould basidiomycete in media containing 10 mM. glucose plus 10 mM [1-^{14}C] glycine (20nCi.μmol.$^{-1}$).

Figure 3.24c - Changes of the glycine (▲) and radioactive content of the culture medium (△) during growth of shake cultures of the snow mould basidiomycete, in media containing 10 mM. glucose plus 10 mM. [1-^{14}C] glycine (20nCi.μmol.$^{-1}$).

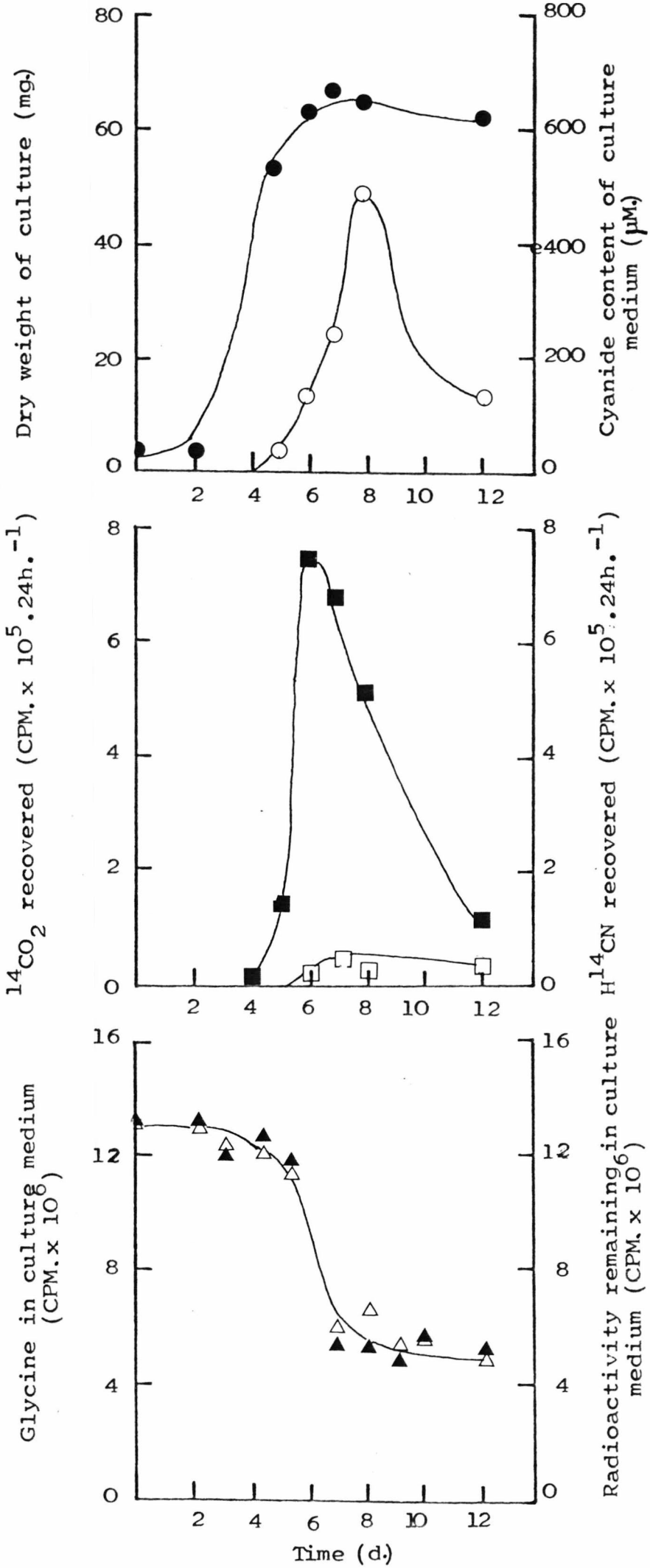
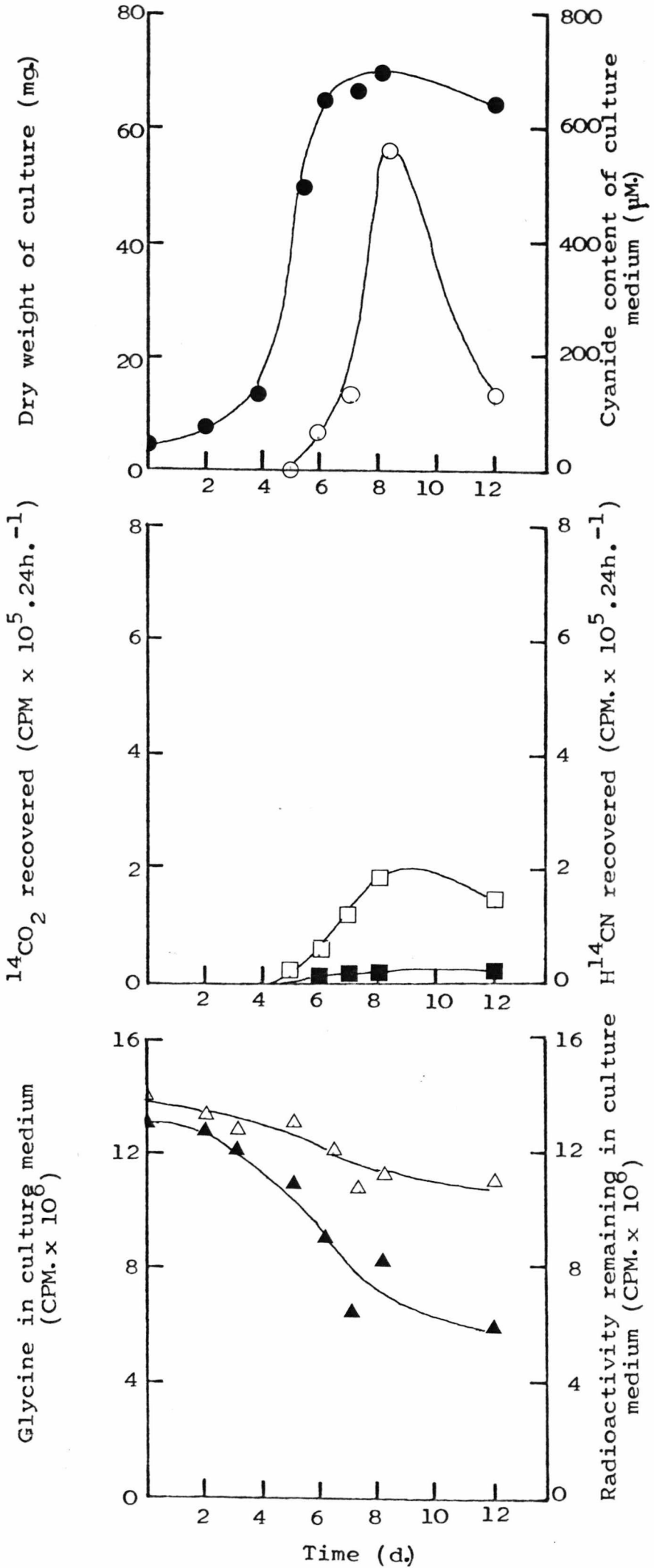

Dry weight of culture (mg.)
Cyanide content of culture medium (µM.)
$^{14}CO_2$ recovered (CPM. x 10^5.24h.$^{-1}$)
$H^{14}CN$ recovered (CPM. x 10^5.24h.$^{-1}$)
Glycine in culture medium (CPM. x 10^6)
Radioactivity remaining in culture medium (CPM. x 10^6)
Time (d.)

Figure 3.25a - Growth (●) and culture medium cyanide content (○) during growth of shake cultures of the snow mould basidiomycete in media containing 10 mM. glucose plus 10 mM. [2-^{14}C] glycine (20nCi. μmol.$^{-1}$).

Figure 3.25b - $^{14}CO_2$ (■) and H^{14}CN (□) production during growth of shake cultures of the snow mould basidiomycete in media containing 10 mM. glucose plus 10 mM. [2-^{14}C] glycine (20nCi. μmol.$^{-1}$).

Figure 3.25c - Changes of the glycine (▲) and radioactive content of the culture medium (△) during growth of shake cultures of the snow mould basidiomycete, in media containing 10 mM. glucose plus 10 mM. [2-^{14}C] glycine (20nCi. μmol.$^{-1}$).


Dry weight of culture (mg.)
Cyanide content of culture medium (μM.)
$^{14}CO_2$ recovered (CPM x 10^5.24h.$^{-1}$)
$H^{14}CN$ recovered (CPM. x 10^5.24h.$^{-1}$)
Glycine in culture medium (CPM. x 10^6)
Radioactivity remaining in culture medium (CPM. x 10^6)
Time (d.)

in both cases, associated with glycine. Peaks of radioactivity associated with serine, threonine and glyoxylic acid were not obtained. Only small changes of the distribution of radioactivity on the plates in the areas with which these compounds are associated was observed between 7 and 12d. old cultures. Similarly little radioactivity was associated with glyoxylic acid cyanohydrin. The only other peaks of radioactivity recorded on the t.l.c. plates were at the origin and at the solvent front.

The identity of the compound associated with the origin remains unknown, but the compound at the solvent front is almost certainly cyanide, the amount of which drops between 7. and 12d. incubation. Between day 7 and day 12, the concentration of glycine in the cells also drops from 400 μM. to 80 μM.

Figure 27 shows the distribution of radioactivity on t.l.c. plates after chromatography of 12d. old cell free supernatents prepared from cultures incubated with [2-^{14}C] glycine and 10 mM. methionine (the period of maximum cyanide production by these cultures).

A comparison between Figures 3.26b and 3.27 shows that a new peak of radioactivity was present in Figure 3. 27 (Rf. 0.1 to 0.17), and that the radioactivity associated with the origin decreased. More radio-activity was now associated with glycine, but

Figure 3.26

Distribution of radioactivity after thin layer chromatography of cell-free extracts prepared from 7d. (a) or 12d. (b) old shake cultures of the snow mould basidiomycete grown in media containing 10 mM. glucose plus 10 mM [2-^{14}C] glycine (20nCi. μmol.$^{-1}$)

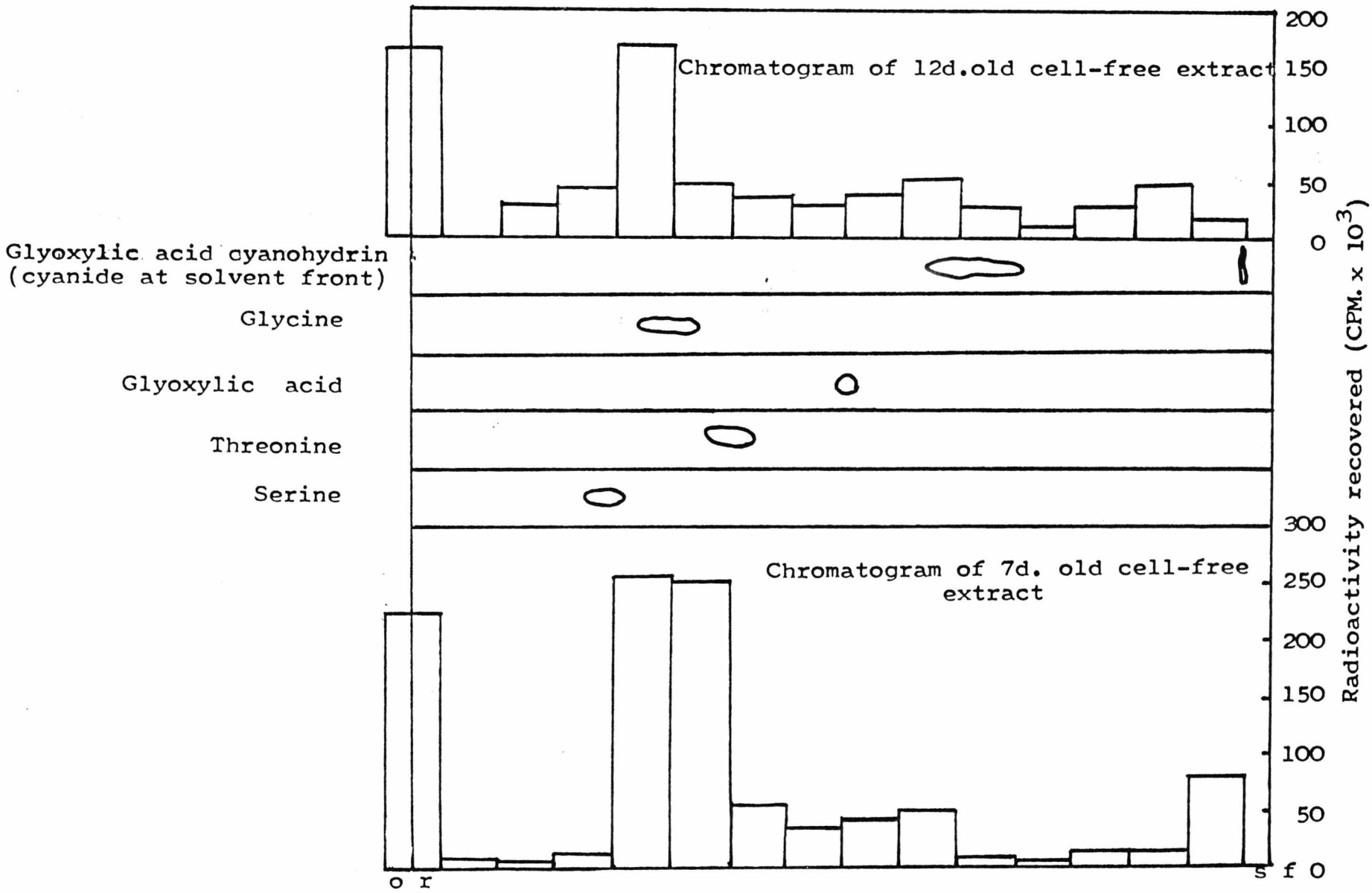
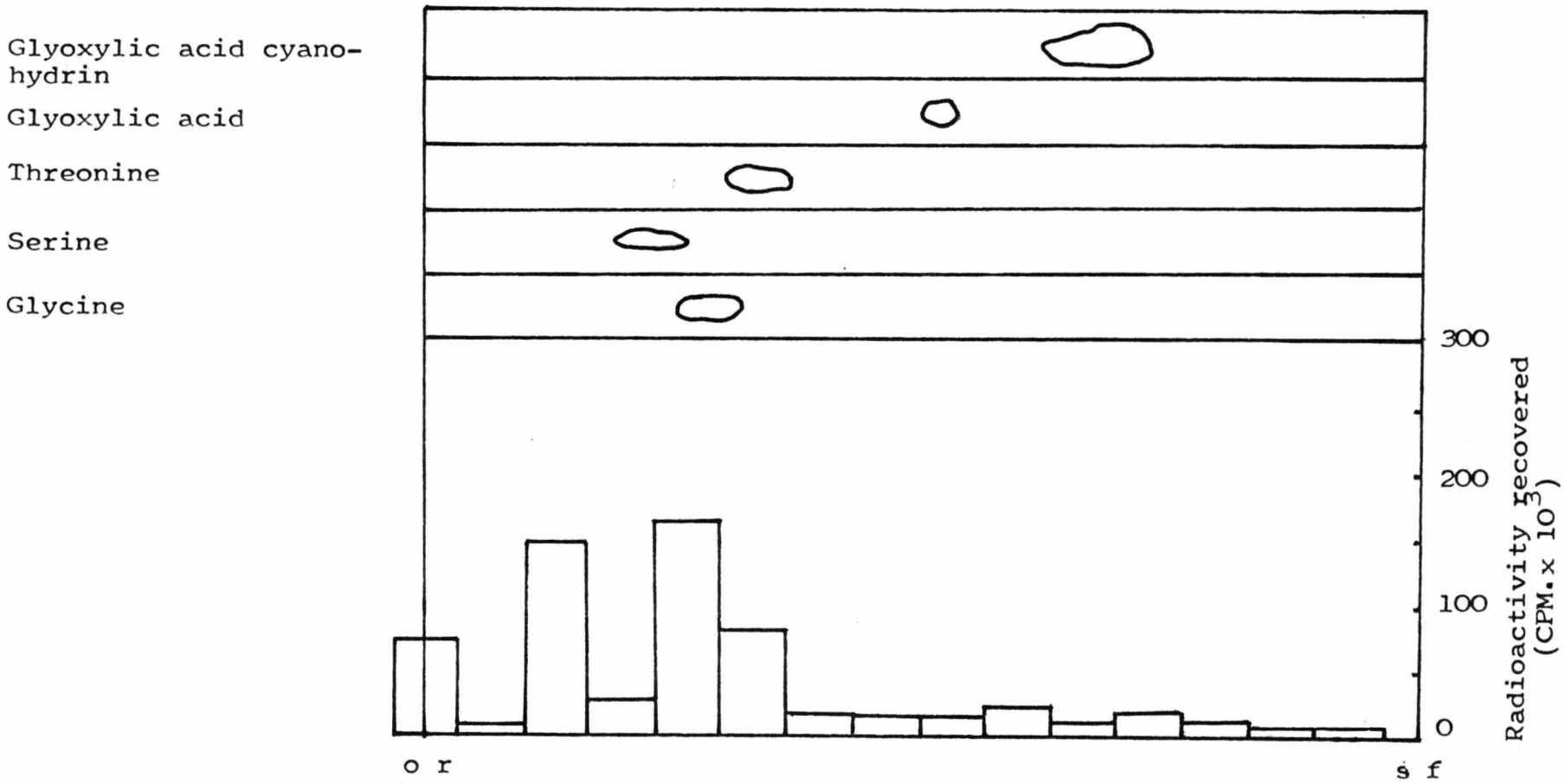

Chromatogram of 12d.old cell-free extract
200
150
100
50
0
Glyoxylic acid cyanohydrin
(cyanide at solvent front)
Glycine
Glyoxylic acid
Threonine
Serine
Chromatogram of 7d. old cell-free extract
300
250
200
150
100
50
0
Radioactivity recovered (CPM. x 10^3)
o r
s f

Figure 3.27

Distribution of radioactivity after thin layer chromatography of cell-free extracts prepared from 12d. old shake cultures of the snow mould basidiomycete grown in media containing 10 mM. glucose plus 10 mM $[2\text{-}^{14}C]$ glycine ($20 nCi.\mu mol.^{-1}$) and 10 mM. methionine.


Glyoxylic acid cyano-hydrin
Glyoxylic acid
Threonine
Serine
Glycine
300
200
100
0
Radioactivity recovered (CPM. x 10^3)
o r
s f

surprisingly little radioactivity appeared to be associated with cyanide. (There would be heavy losses due to the volatility of cyanide).

The fate of glycine during growth is summarised in Table 3.3 -of the original 500 µmol. present at the beginning of incubation half remains after 12d. The total glycine converted to cyanide (30 µmol.) is half that converted to CO_2 (64 µmol.). Nearly 200 µmol. were metabolised to unknown compounds, 85% of which was present in the culture medium.

This unknown compound(s) was not a cyanohydrin. Cultures were incubated (21) for 5, 7, 9 and 14d. in a medium containing glucose plus glycine. Extraction of the fungal cells and examination of the culture medium never revealed the presence of glyoxylic or pyruvic acid cyanohydrin. These compounds were not excreted in large amounts before, during, or after cyanide production by shake cultures of the snow mould basidiomycete.

Further metabolism of cyanide by cultures of the snow mould basidiomycete

It is necessary during any investigation on the metabolism of $K^{14}CN$ to assess the amount of breakdown that occurs normally in solutions of cyanide (Oró and Kimball, 1962). Chromatography of $K^{14}CN$ (Figure 3.28) revealed that 1.3% of the cyanide hydrolysed in 2.5 months (at 4°C) to

Table 3.3

The fate of glycine after 12d. growth of shake cultures of the snow mould basidiomycete in media containing 10 mM. glucose and 10 mM. glycine

Total glycine present at start	500 µmoles
Total glycine remaining in growth medium at end	251 µmoles
Total glycine remaining free in fungal cells	5 µmoles
Total converted to unknown substances in cell-free extract	23 µmoles
Total converted to unknown substances in medium	171 µmoles
Total converted to CO_2 and lost from the culture	64 µmoles
Total converted to HCN and lost from the culture was	15 µmoles
Total converted to HCN remaining in the culture medium	15 µmoles
Total glycine recovered	544 µmoles

(Data recorded from experiments using [1 or 2-^{14}C] glycine)

i.e. using results from 3 experiments with [1-^{14}C]glycine and 5 experiments with [2-^{14}C] glycine to determine the fate of both atoms of glycine.

formate. Little formamide or formadine were observed, but 1.1% of the cyanide had been converted to another product, thought to be a polymerisation product, with an Rf in the chromatographic system employed of 0.68. Therefore, a total of nearly 3% of the cyanide had been converted to other compounds during the 2.5 months. Solutions of $K^{14}CN$ were never kept longer than this period of time as cyanide was continually lost by volatisation and, after 3 months, very little remains. The process of volatilisation was increased by freezing and thawing $K^{14}CN$ solutions.

Figure 3.29 shows the relationship between the cyanide content of cell free extracts prepared from cultures at different stages of incubation, and the cyanide concentration of the culture medium. Radioactive cyanide (63 $\mu Ci.\mu mol.^{-1}$) was added to cultures and its distribution after 24h. or longer determined. Little of the radioactive cyanide became associated with protein or the cell walls (Figure 3.29b). It can be seen that the maximum cyanide content of the cell free extracts occurs at the time of maximum culture medium cyanide concentration.

Chromatography of cell free extracts prepared from 12d. old cultures revealed that a large proportion of the radioactivity remained as cyanide, but that 25% was present as several compounds, the majority of

Figure 3.28 - Distribution of radioactivity after thin layer chromatography of 3μCi. $K^{14}CN$ ($59mCi.mmol.^{-1}$) stored for 65d. in 100 mM. K_2HPO_4.

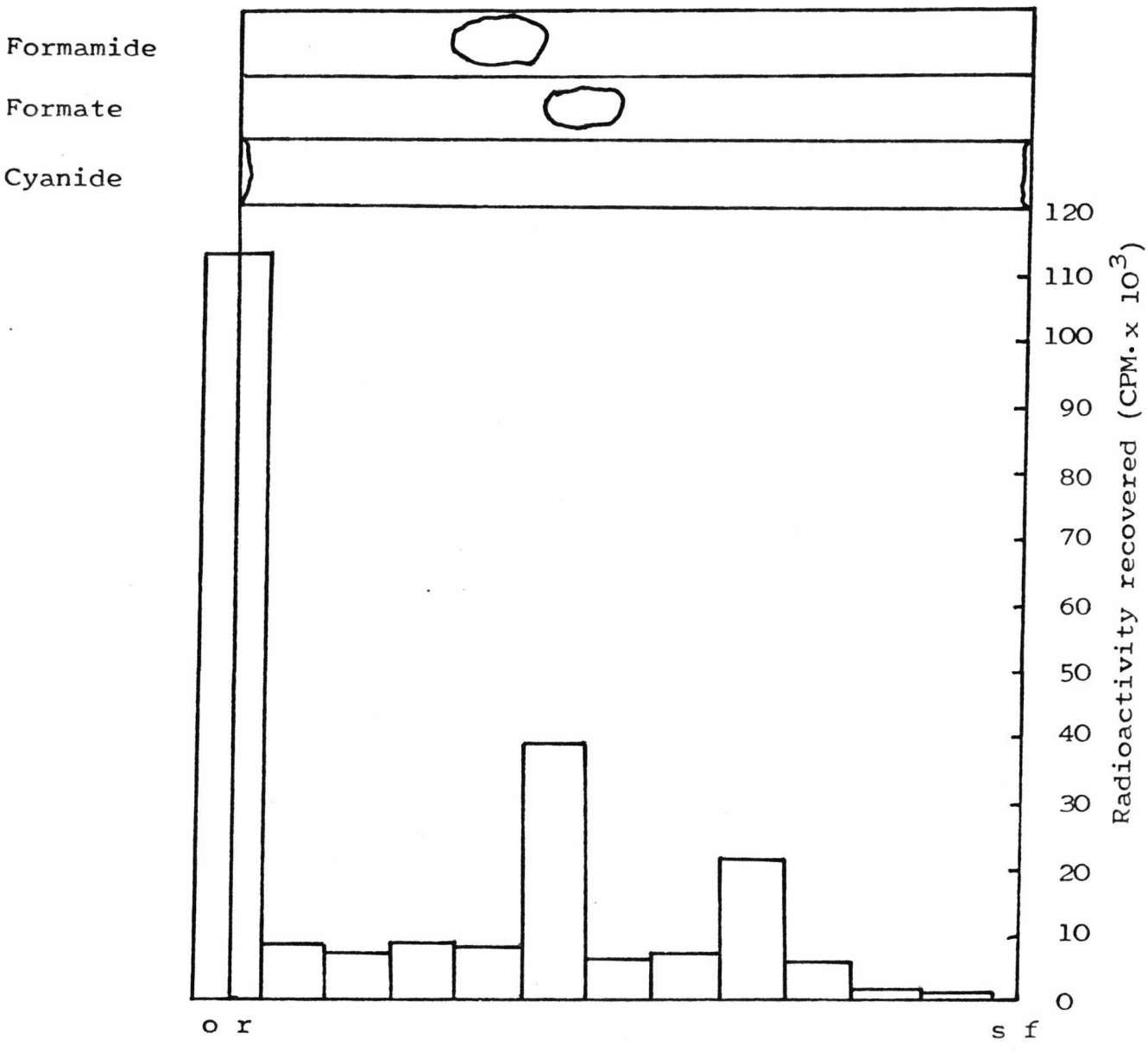
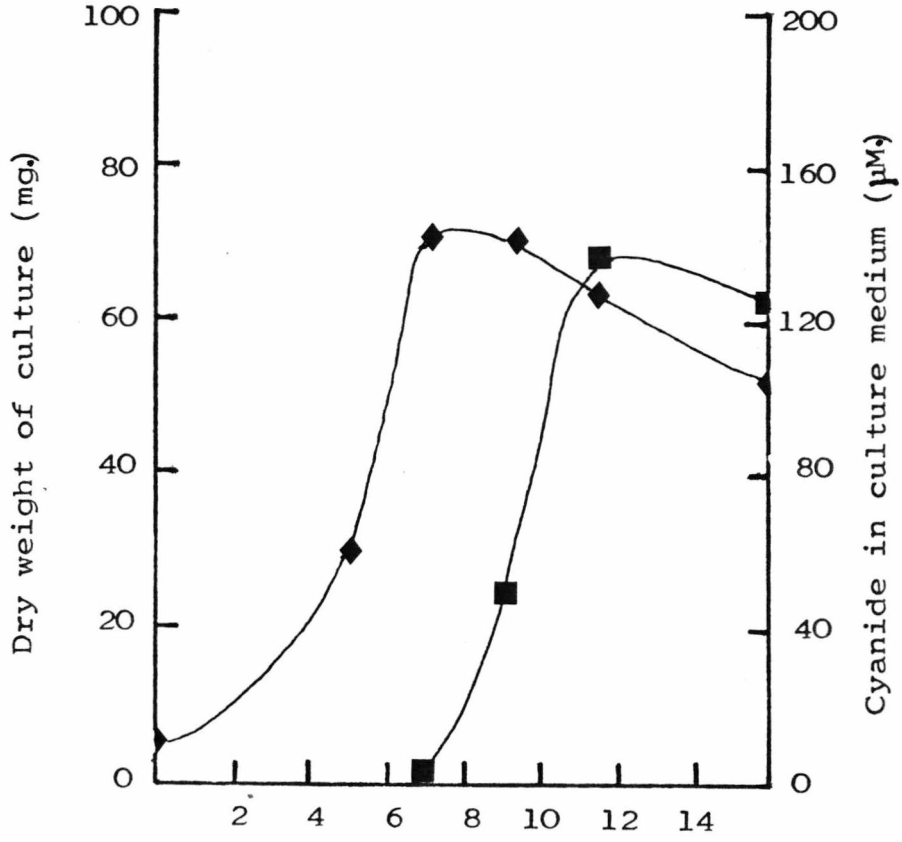
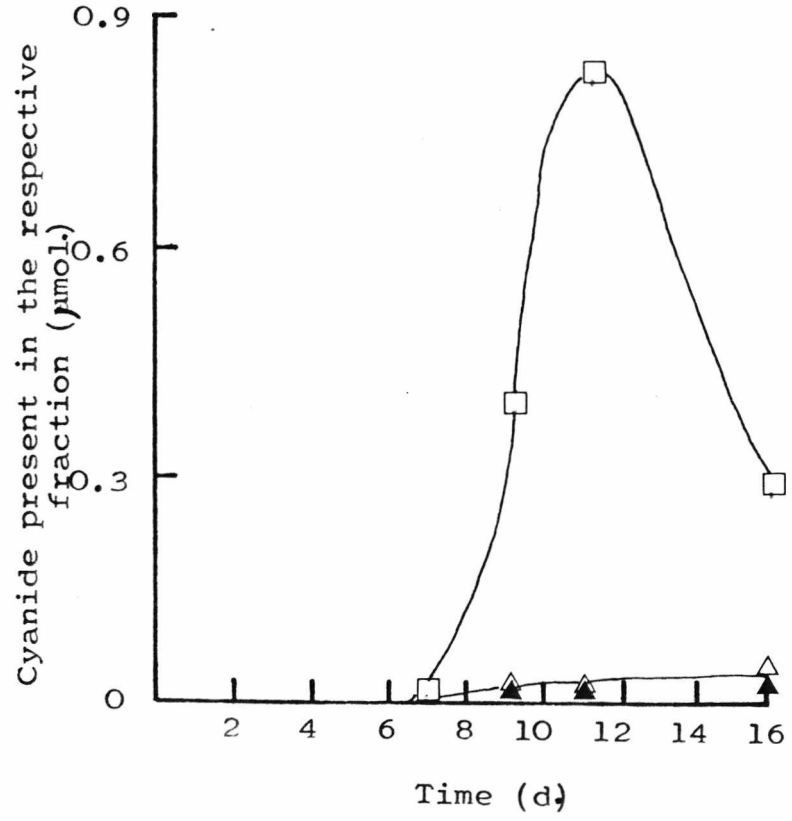

Formamide
Formate
Cyanide
120
110
100
90
80
70
60
50
40
30
20
10
0
Radioactivity recovered (CPM. x 10^3)
o r
s f

Figure 3.29 - The distribution of cyanide in the culture medium (■), cell-free extracts (□), protein of cell-free extracts▲and cell walls plus unbroken cells (△), prepared from shake cultures of the snow mould basidiomycete, following growth◆in media containing 10 mM glucose. Data was obtained by adding 2μCi.$K^{14}CN$ (59mCi.mmol.$^{-1}$) to cultures 24h. prior to harvesting. The fungal cells were disrupted by 2 passages at 8000 lbf. in.$^{-2}$ through a French pressure cell.

a.


Dry weight of culture (mg)
Cyanide in culture medium (µM)
100
80
60
40
20
0
200
160
120
80
40
0
2
4
6
8
10
12
14

b.


Cyanide present in the respective fraction (µmol)
0.9
0.6
0.3
0
2
4
6
8
10
12
14
16
Time (d)

which were associated with the positions of glyoxylic acid and pyruvic acid cyanohydrins on the t.l.c. plate. (Figure 3.30). No peaks of radioactivity were associated with glutamate or alanine. Chromatography of the culture medium removed from 12d. old cultures showed that again the majority of the radioactivity was still associated with cyanide, but now 30% of the radioactivity was present between Rf. 0.09 and Rf. 0.36, not between Rf. 0.54 to Rf 0.9.

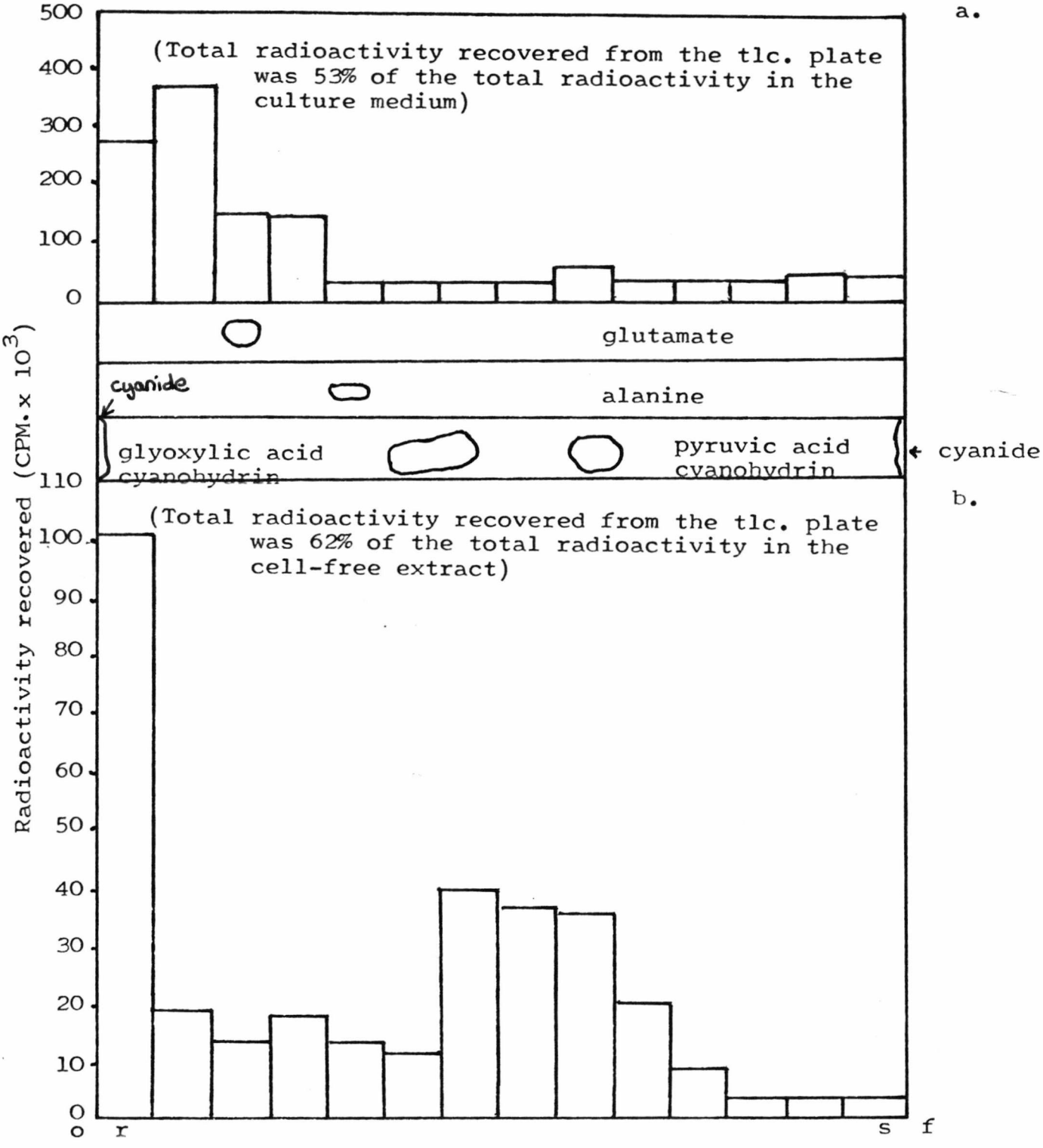
An investigation of radioactivity volatilised from cultures and trapped in KOH revealed that 15 to 19% of the total cyanide added was converted to CO_2 during the first 24h. incubation. More CO_2 was produced if longer incubation times were used, but 48h. incubation produced only a 60% increase in the amount of CO_2 trapped. Table 3.4 summarises the distribution of added $K^{14}CN$ after 24h. incubation with 12d. old cultures. The largest proportion of the radioactivity remains as $K^{14}CN$. Of the several possible products of cyanide metabolism investigated carbon dioxide was the only one produced in significant amounts. Very low levels of radioactivity (less than 0.001%) were associated with alanine, glutamate, β-cyanoalanine and aspartic acid. Formamide, although detected by both chromatography of DNFB. derivatives prepared from the culture medium and by the assay

Table 3.4 - Distribution of ^{14}C in products formed from $K^{14}CN$ by the snow mould fungus

	Percentage of ^{14}C recovered
Residual cyanide in growth medium	44-51
Trapped cyanide in centre well	25-31
Taken up by fungus	6-9
Protein etc.	2-4
Carbon dioxide	15-19
Alanine	0
Glutamate	0
β-Cyanoalanine	0
Asparagine/aspartate	0
Formamide	0

$K^{14}CN$ was added to a culture which had been grown in medium containing 10 mM-glucose for 12h., and the distribution of ^{14}C was measured 24h. later. Values are the ranges of results from five independent growth flasks. The total percentage recovery of counts was 81 to 93%.

Figure 3.30 - Distribution of radioactivity after thin layer chromatography (t.l.c.) of the culture medium (a) and cell-free extracts (b) from 12d. old shake cultures of the snow mould basidiomycete grown in media containing 10 mM glucose, after 24h. incubation with 2μCi. $K^{14}CN$. (59mCi. $mmol.^{-1}$).

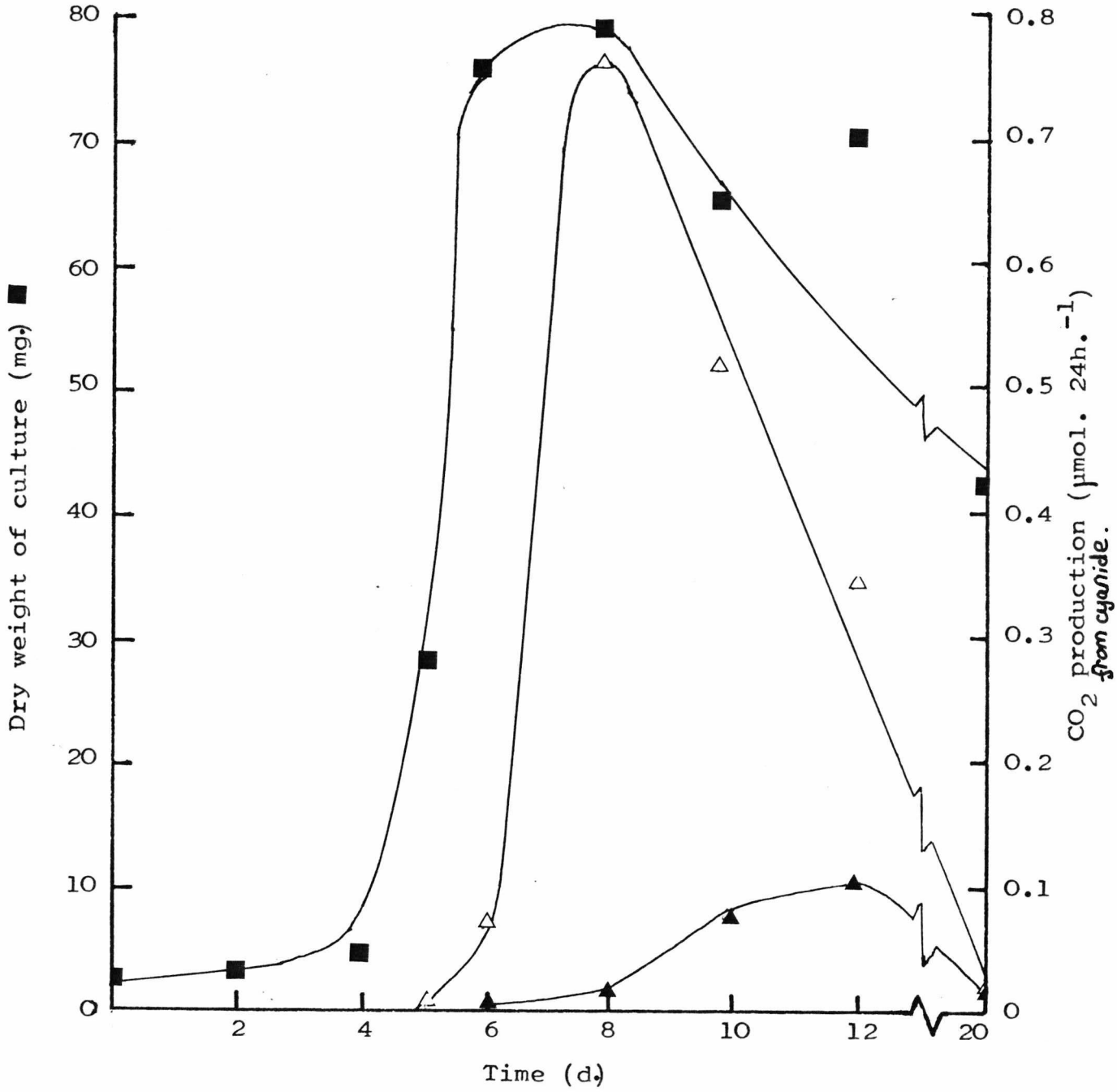

a.
(Total radioactivity recovered from the tlc. plate was 53% of the total radioactivity in the culture medium)
glutamate
alanine
cyanide
glyoxylic acid cyanohydrin
pyruvic acid cyanohydrin
cyanide
b.
(Total radioactivity recovered from the tlc. plate was 62% of the total radioactivity in the cell-free extract)
Radioactivity recovered (CPM. x 10^3)
500
400
300
200
100
0
110
100
90
80
70
60
50
40
30
20
10
0
o
r
s
f

described by Fry and Millar (1972), it had little or no radioactivity associated with it. The enzymes of cyanide metabolism described by Strobel (1966, 1968) that produce alanine and glutamate from the respective aldehydes (acetaldehyde and succinic semialdehyde), cyanide plus ammonia were not detected in 5, 8, 12 and 20d. old cultures. Formamide hydrolyase activity (Rissler and Millar, 1977) was also absent in these cultures.

Carbon dioxide production from cyanide was determined for cultures at different stages of incubation grown in media with or without glycine (Figure 3.31). The maximum amount of cyanide converted to CO_2 coincided in both cases with the time of maximum cyanide production.

Production of CO_2 from KCN was observed in cell free extracts prepared from 8d. old cultures (21) incubated in media containing glucose plus glycine. The activities recorded varied between different cultures and, in some cases, little or no activity was present. Reducing the number of passages through the French pressure cell from 2 at 8000 lbf.in.$^{-2}$ to 1 at 12000 lbf.in.$^{-2}$ only had a slight effect on the final activities observed, but did prevent excessive heating of the pressure cell, when several samples were processed during a period of 0.5 to 1h. The relationship between the protein concentration of cell-free extracts and the amount of $^{14}CO_2$ produced, (Fig. 3.32a) showed that production of $^{14}CO_2$ was dependent

Figure 3.31 - The conversion of cyanide to CO_2 by shake cultures of the snow mould basidiomycete grown ■ in media containing 10 mM. glucose (▲) or 10 mM. glucose plus 10 mM. glycine (△). Data was obtained by adding 2μCi. $K^{14}CN$ (59mCi.mmol.$^{-1}$) and trapping the $^{14}CO_2$ produced during the 24h. prior to harvesting.


Dry weight of culture (mg.) ■
CO_2 production (µmol. 24h.$^{-1}$) from cyanide.
Time (d.)
0
10
20
30
40
50
60
70
80
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
2
4
6
8
10
12
20

on the amount of protein present. Time courses of the reaction were difficult as the variability between samples was too great for significant differences to be observed for short incubation times. Long incubations (greater than 5h.) resulted in large losses of cyanide, which also caused variability.

The relationship between the activity of the extracts and pH is shown in Figure 3.32b. This pH effect was reproducible but it is difficult to determine the pH optimum for this enzyme, given the variability between samples and the effect of pH on cyanide.

Formate did not interfere with the conversion of cyanide to CO_2.

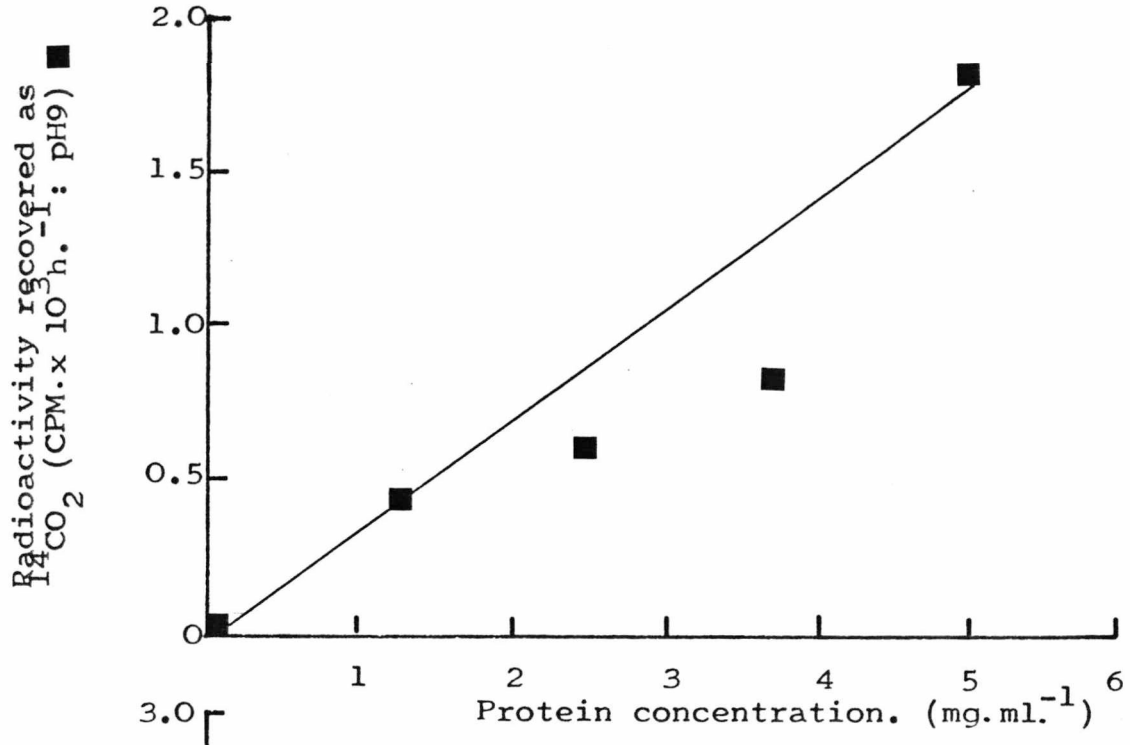
Growth and cyanide production by cultures of the snow mould basidiomycete incubated with acetate

As mentioned previously, of the many non-fermentable carbon sources tested (Table3.1)only acetate or succinate enabled measurable growth when present as the major carbon source. Similar patterns of cyanide production during incubation were obtained for both acetate and succinate, but growth was almost twice as fast with acetate. For this reason acetate was used for a comparison with glucose. The ability of the fungus to utilise various nitrogen sources in media containing acetate was almost identical to the results obtained in Table 3.2. Once again glycine was a very poor nitrogen

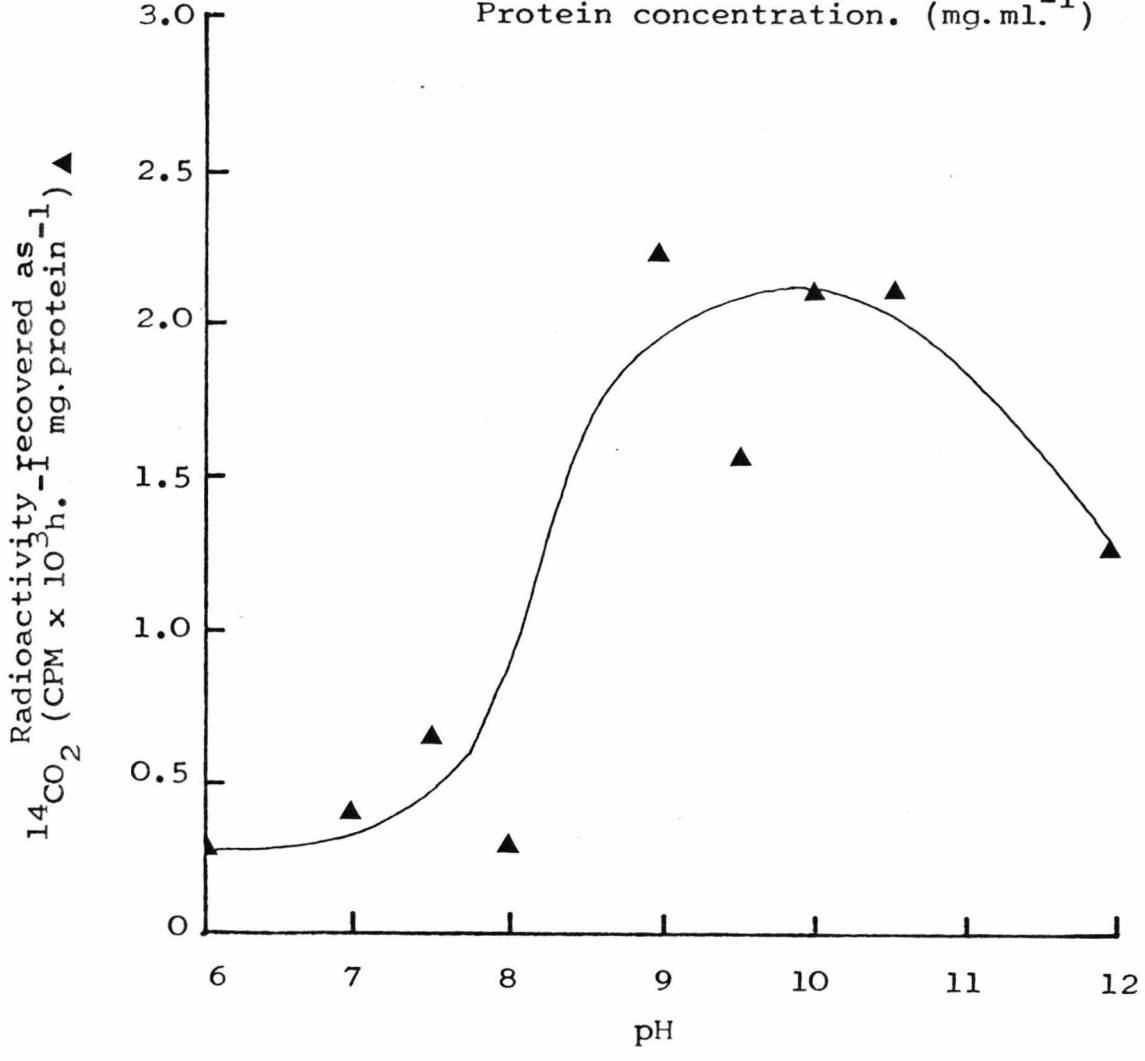
Figure 3.32a - The effect of varying the pH of the assay mixture on the rate of $^{14}CO_2$ production from $H^{14}CN$, by cell-free extracts prepared from 8d. old shake cultures of the snow mould basidiomycete grown in media containing 10 mM glucose, plus 10 mM glycine. The reaction mixture consisted of 100 µl. cell-free extract (5 mg.protein $ml.^{-1}$) in 25 mM. Hepes - 25 mM KH_2PO_4 - 25 mM. triethanolamine buffer and 1 µl $K^{14}CN$ (59 $mCi.mmol.^{-1}$).

Figure 3.32b - The effect of varying the protein content of the assay mixture on the rate of $^{14}CO_2$ production from $H^{14}CN$, by cell-free extracts prepared from 8d. old shake cultures of the snow mould basidiomycete grown in media containing 10 mM glucose, plus 10 mM glycine. The reaction mixture consisted of 100 µl. cell-free extract in 25 mM. Hepes - 25 mM KH_2PO_4 - 25 mM. triethanolamine buffer (pH 9) and 1 µl $K^{14}CN$ (59 $mCi.mmol.^{-1}$).

σ.


Radioactivity recovered as $^{14}CO_2$ (CPM.x 10^3h.$^{-1}$: pH9) ■
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
Protein concentration. (mg.ml.$^{-1}$)

ρ.


Radioactivity recovered as $^{14}CO_2$ (CPM x 10^3h.$^{-1}$ mg.protein^{-1}) ▲
3.0
2.5
2.0
1.5
1.0
0.5
0
6
7
8
9
10
11
12
pH

source for the fungus. Variation of the initial acetate concentration from 20 mM. to 60 mM. had little effect on cyanide production and the maximum fungal cell dry weight yield. An initial acetate concentration of 30 mM. was chosen for future experiments so that the molarity of the carbon present in the medium was the same as that in experiments where the medium contained 10 mM. glucose.

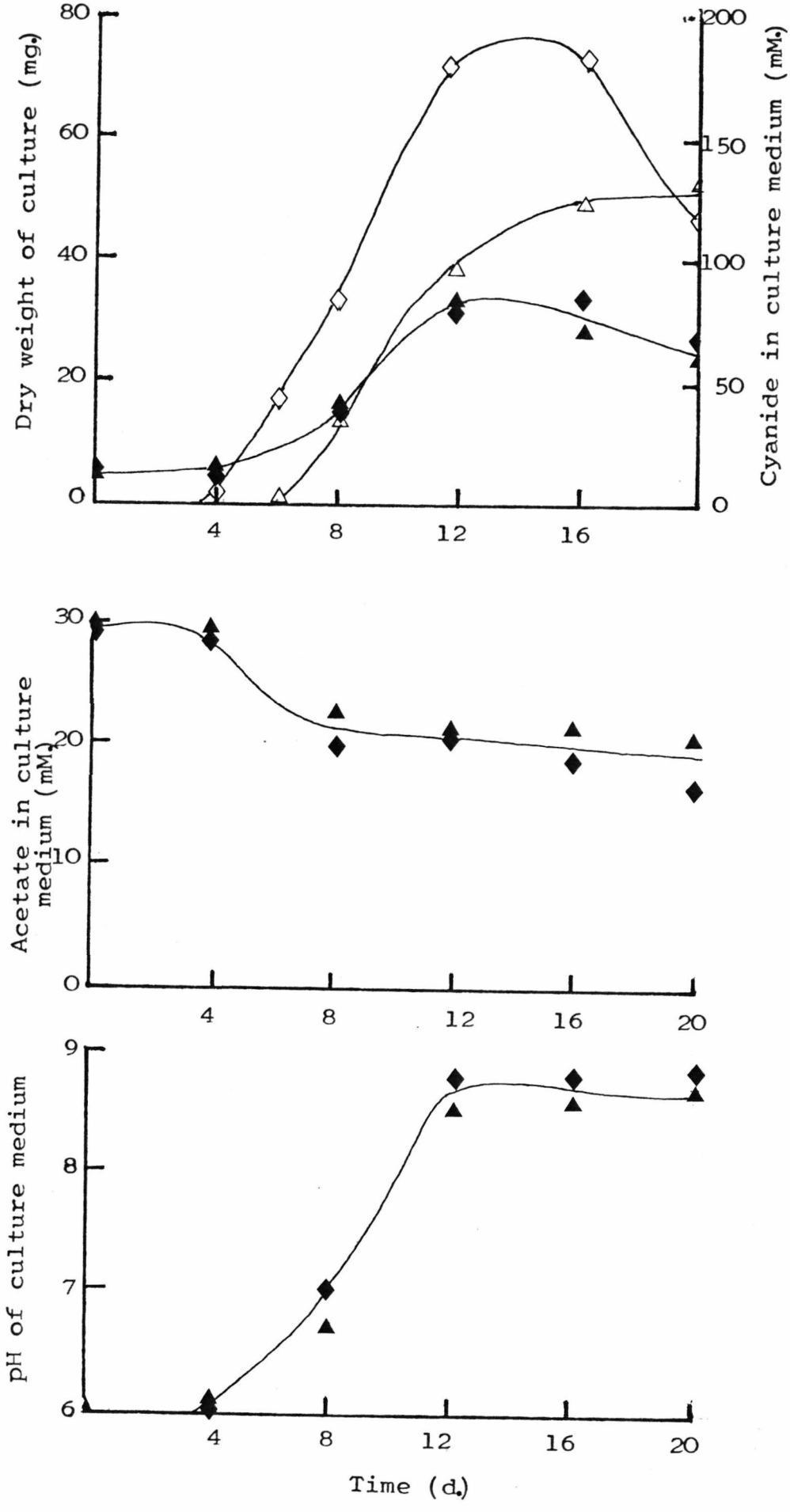
Figure 3.33 records growth, cyanide production, acetate concentration in the culture medium and pH changes that occurred during incubation of the snow mould basidiomycete in media containing acetate with or without glycine. The fungal cell dry weight, culture medium pH and acetate concentration changes were very similar in the presence or absence of glycine. Growth began after 6d. incubation, reaching a maximum fungal cell dry weight yield of 35 mg, 6d. later. During this period of incubation, the concentration of acetate falls from 30 to 18 mM. and the pH of the culture medium rose from pH 6.0 to pH 8.6. The pH and acetate concentration of the culture medium did not alter significantly during the final 12d. to 20d. incubation period, but the fungal cell dry weight decreased to 25 mg.

In contrast to glucose plus or minus glycine, cyanide was produced throughout the incubation period,

Figure 3.33a - Changes of culture dry weight (▲, without glycine: , ◆ with glycine) and culture medium cyanide content (△, without glycine: ◇, with glycine) during growth of the snow mould basidiomycete in shake culture, in media containing 30 mM. acetate with or without 10 mM. glycine.

Figure 3.33b- Culture medium acetate content (▲, without glycine: ◆, with glycine) during growth of the snow mould basidiomycete in shake culture, in media containing 30 mM. acetate with △ or without ◇ 10 mM. glycine.

Figure 3.33c - Culture medium pH changes (▲, without glycine: ◆, with glycine) during growth of the snow mould basiodiocycete in shake culture, in media containing 30 mM. acetate with or without 10 mM. glycine.


Dry weight of culture (mg.)
Cyanide in culture medium (mM.)
Acetate in culture medium (mM.)
pH of culture medium
Time (d.)
0
20
40
60
80
50
100
150
200
10
30
6
7
8
9
4
8
12
16
20

reaching a maximum concentration of 140 µM in the absence of glycine and 175 µM. when glycine was added at the time of inoculation. This stimulation of cyanide production by glycine was estimated by comparing the total cyanide produced by cultures with glycine (Figure 3.35 and without glycine (Figure 3.34). Again it was seen that 80 to 90% of the total cyanide produced was volatilised from cultures and the total cyanide production by cultures incubated without glycine (20 µmol.) was increased 2.5 times by the presence of 10 mM. glycine (47 µmol.). The inclusion of 10 mM methionine in media containing acetate plus glycine had two effects. Firstly growth was slowed down but the final maximum dry weight yield (Figure 3.36a) remained similar. Secondly, cyanide production was slightly stimulated where nearly 70 µmol. (Figure 3.36b) were produced during the 28d. incubation period.

Incubation of the snow mould basidiomycete in media that contained 10 mM. glucose plus 30 mM. acetate (Figure 3.37)shows that both the maximum fungal cell dry weight and cyanide concentration were increased (Figure 3.37a). The pH of the culture medium changed from pH 6.0 to pH 9.0 during incubation. It is noteworthy that glucose was removed completely from the culture medium before acetate began to disappear (Figure 3.37b).No diauxic growth was apparent.

Figure 3.34a - Changes in dry weight of cultures (■) and the culture medium cyanide content (□) during growth of the snow mould basidiomycete in shake culture, in media containing 30 mM. acetate.

Figure 3.34b - Rate at which cyanide was trapped in the centre well (▲), total cyanide trapped in the centre well (△) and total cyanide produced (◆) during growth of the snow mould basidiomycete in shake culture, in media containing 30 mM. acetate.

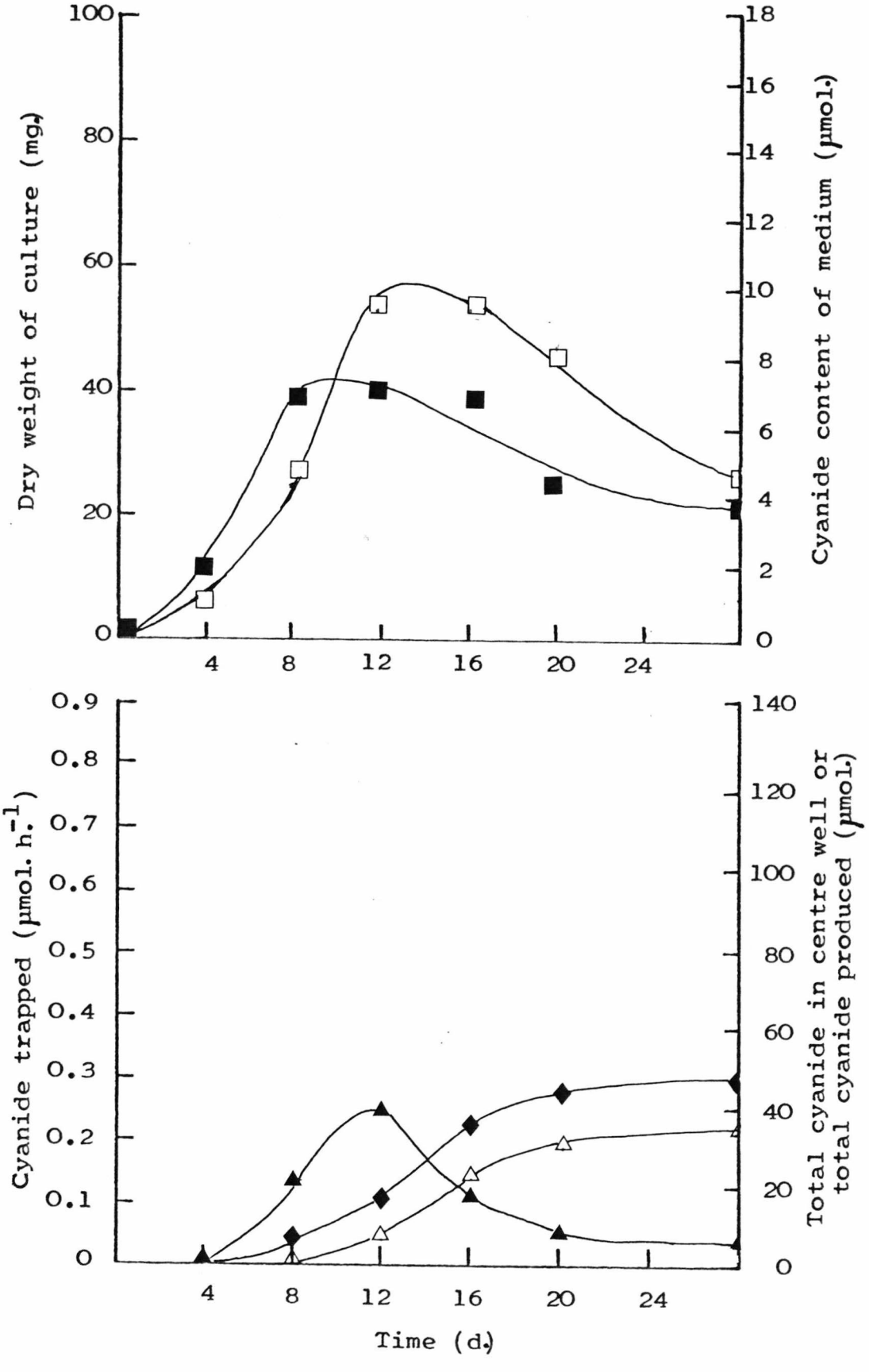

Dry weight of culture (mg.)
Cyanide content of medium (µmol.)
Cyanide trapped (µmol. h.$^{-1}$)
Total cyanide in centre well or total cyanide produced (µmol.)
Time (d.)
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
120
140
4
8
12
16
20
24

Figure 3.35a - Changes in dry weight of cultures (■) and the culture medium cyanide content (□) during growth of the snow mould basidiomycete in shake culture in media containing 30 mM.acetate plus 10 mM. glycine.

Figure 3.35b - Rate at which cyanide was trapped in the centre well (▲), total cyanide trapped in the centre well (△) and total cyanide produced (◆) during growth of the snow mould basidiomycete in shake culture, in media containing 30 mM.acetate plus 10 mM. glycine.

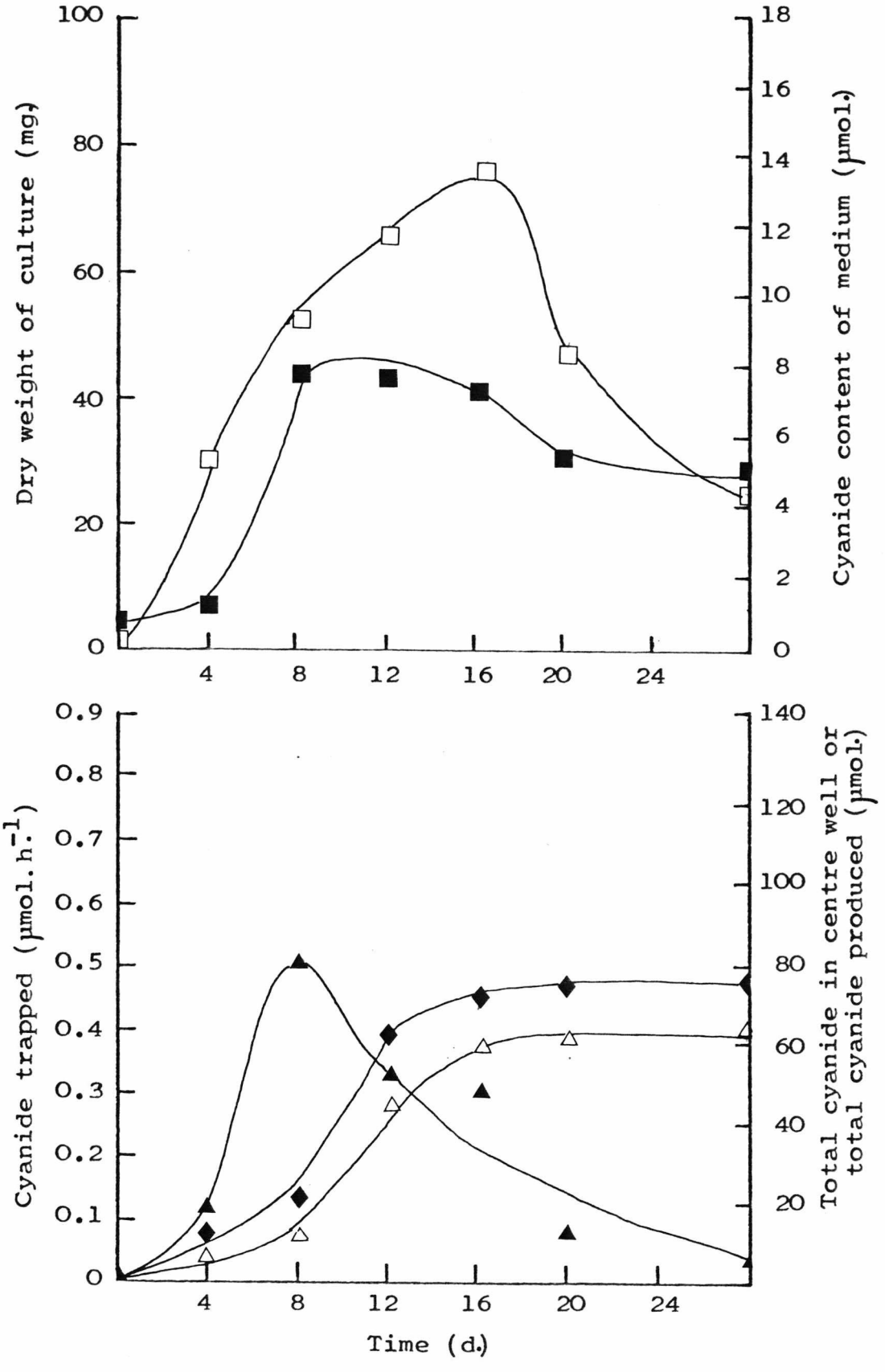
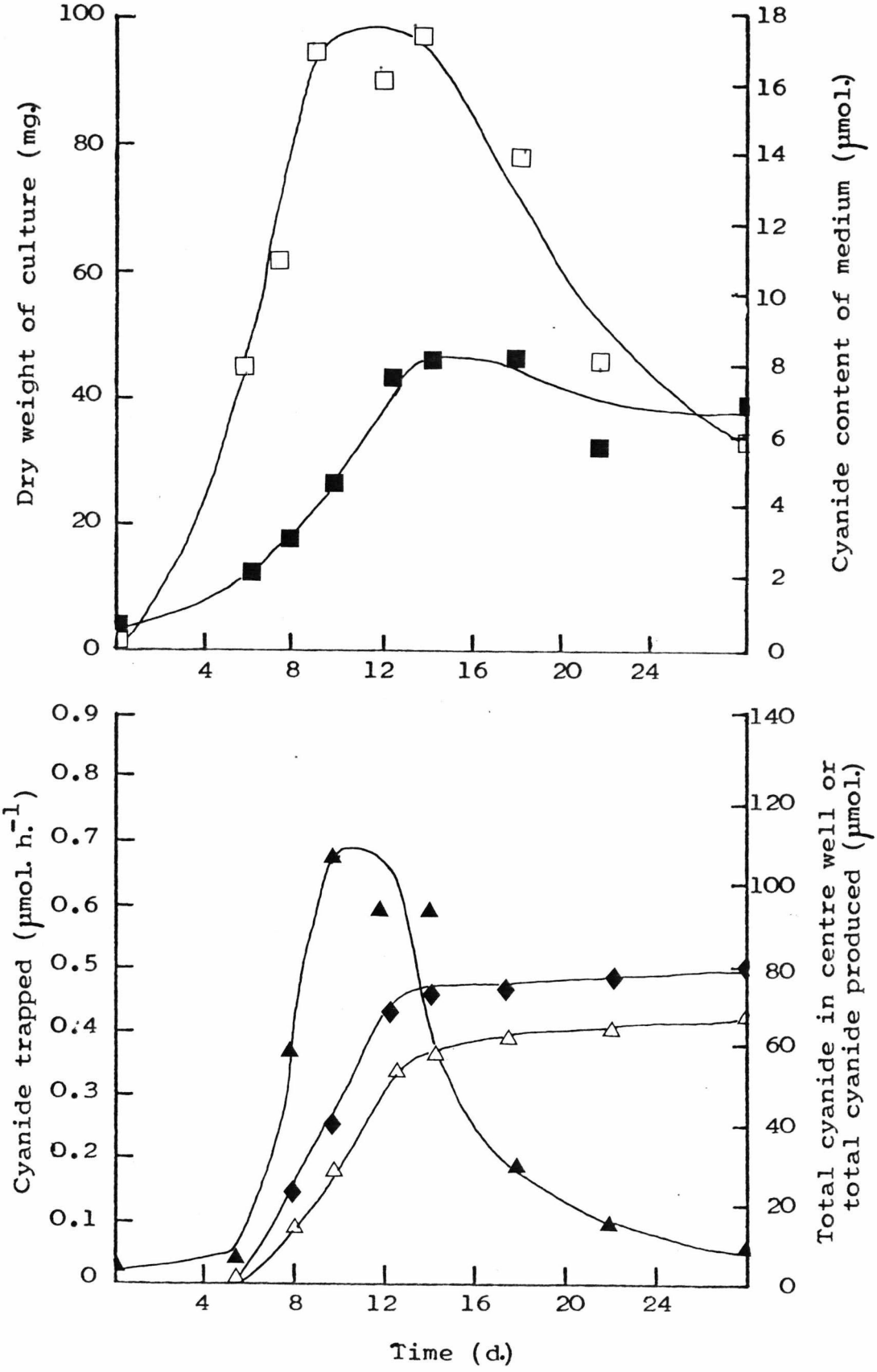

Dry weight of culture (mg)
Cyanide content of medium (µmol.)
Cyanide trapped (µmol.h.-1)
Total cyanide in centre well or total cyanide produced (µmol.)
Time (d.)
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
120
140
4
8
12
16
20
24

Figure 3.36a - Changes in dry weight of cultures (■) and the culture medium cyanide content (□) during growth of the snow mould basidiomycete in shake culture, in media containing 30 mM. acetate plus 10 mM. glycine and 10 mM. methionine.

Figure 3.36b - Rate at which cyanide was trapped in the centre well (▲), total cyanide trapped in the centre well (△) and total cyanide produced (◆) during growth of the snow mould basidiomycete in shake culture, in media containing 30 mM. acetate plus 10 mM. glycine and 10 mM. methionine.


Dry weight of culture (mg.)
Cyanide content of medium (µmol.)
Cyanide trapped (µmol. h.$^{-1}$)
Total cyanide in centre well or total cyanide produced (µmol.)
Time (d.)
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
120
140
4
8
12
16
20
24

Finally whole cell respiration by fungal cells incubated in acetate containing media was generally low (never greater than 0.3 $\mu g.O_2 h^{-1} mg dry wt^{-1}$) and was sensitive to cyanide (96% inhibition with 1mM. cyanide).

The metabolism of $[1\text{-}^{14}C]$ and $[2\text{-}^{14}C]$ glycine during incubation of the snow mould basidiomycete in media containing acetate as the major carbon source

As expected, growth and cyanide production were similar during incubation with either $[1\text{-}^{14}C]$ or $[2\text{-}^{14}C]$ glycine (20 $nCi.\mu mol.^{-1}$; Figures 3.38a and 3.39a). Surprisingly, the production of $H^{14}CN$ was also similar (Figures 3.38b and 3.39b) although nearly 3 times as much $^{14}CO_2$ was produced from $[1\text{-}^{14}C]$ glycine than from $[2\text{-}^{14}C]$ glycine. The amount of radioactivity in the culture medium did not change much during the 14d. incubation period, whereas the concentration of glycine fell continuously.

The nature of the compound to which the remaining radioactivity was associated remains unknown (obtained by subtracting the radioactivity present as glycine and an estimate of the $H^{14}CN$ content of the culture medium from the total radioactivity observed). Examination of metabolites produced from glycine, in cell free extracts, even when the specific activity of the $[2\text{-}^{14}C]$ glycine was increased, proved to be difficult because of the small amounts of radioactivity taken up.

Figure 3.37a - Growth (▲) and culture medium cyanide content (△) during growth of shake cultures of the snow mould basidiomycete in media containing 10 mM. glucose plus 30 mM. acetate.

Figure 3.37b - Changes of the glucose (◆) and acetate (◆) content of the culture medium and pH (□) changes during growth of shake cultures of the snow mould basidiomycete in media containing 10 mM. glucose plus 30 mM. acetate.

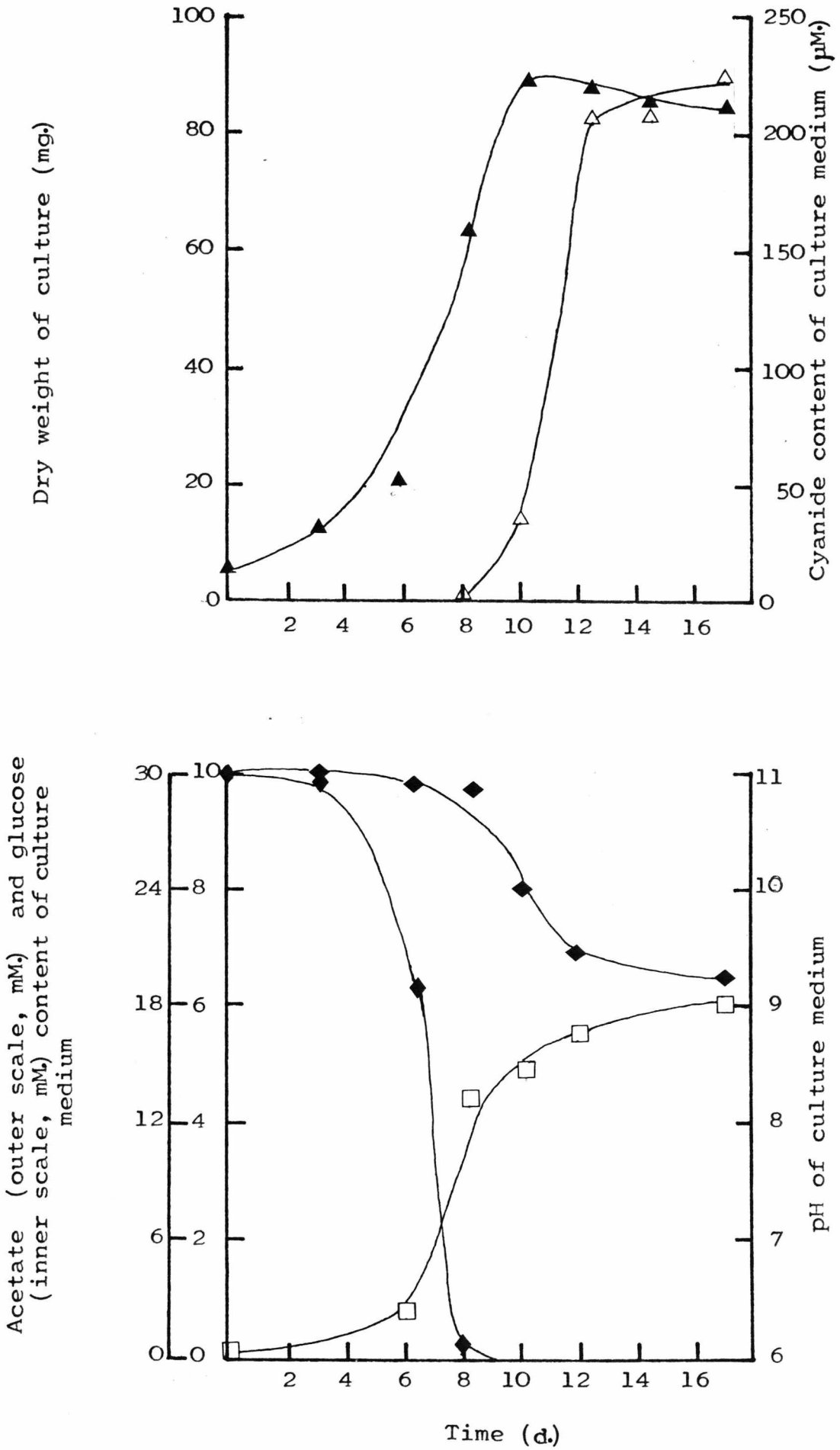

Dry weight of culture (mg.)
Cyanide content of culture medium (μM.)
Acetate (outer scale, mM.) and glucose (inner scale, mM.) content of culture medium
pH of culture medium
Time (d.)
0
20
40
60
80
100
50
150
200
250
6
7
8
9
10
11
12
18
24
30
2
4
6
8
10
12
14
16

Figure 3.38a - Growth (▲) and culture medium cyanide content (△) during growth of shake cultures of the snow mould basidiomycete in media containing 30 mM. acetate plus 10 mM. [1-^{14}C] glycine (20nCi.μmol.$^{-1}$).

Figure 3.38b - $^{14}CO_2$ (◆) and H^{14}CN (◇) production during growth of shake cultures of the snow mould basidiomycete in media containing 30 mM. acetate plus 10 mM. [1-^{14}C] glycine (20nCi.μmol.$^{-1}$).

Figure 3.38c - Changes of the glycine (◆) and radioactive content of the culture medium (◇) during growth of shake cultures of the snow mould basidiomycete, in media containing 30 mM. acetate plus 10 mM. [1-^{14}C] glycine (20nCi.μmol.$^{-1}$).

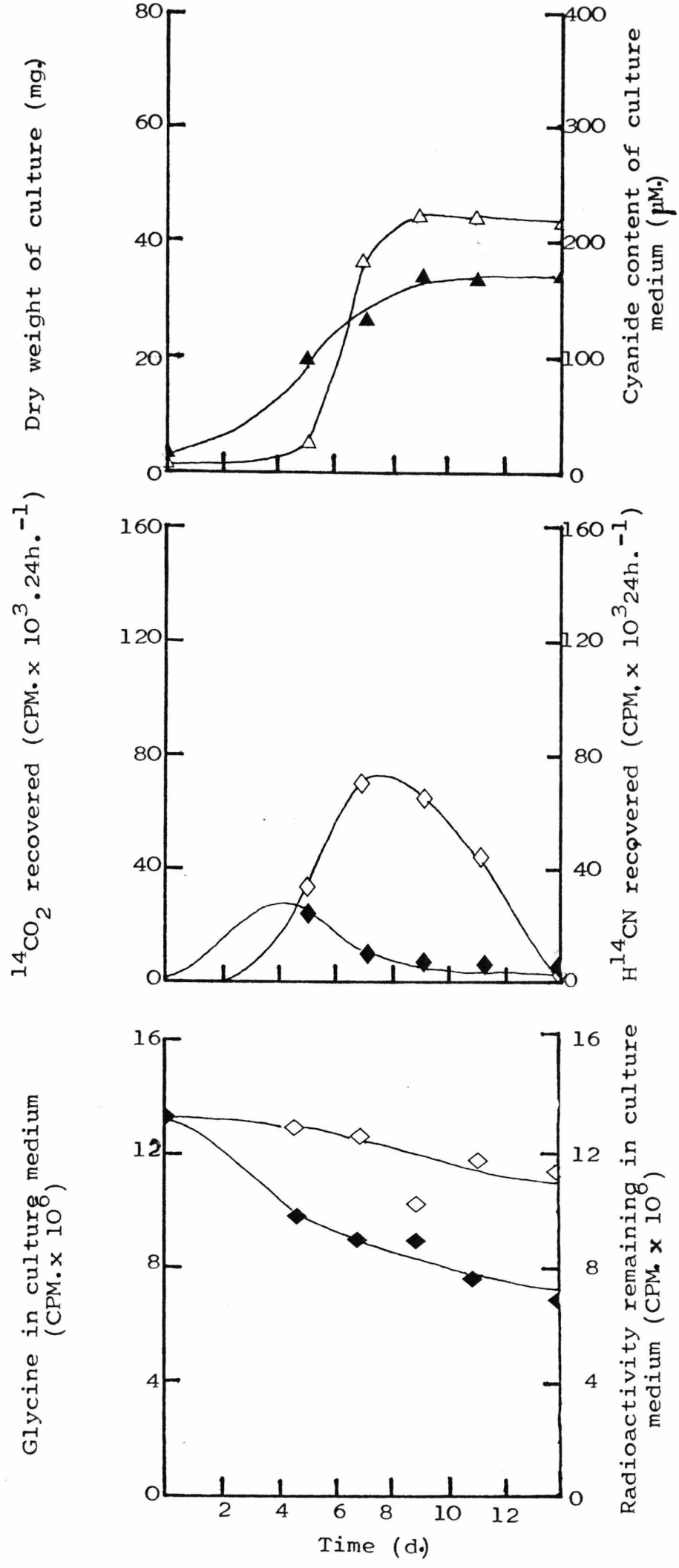
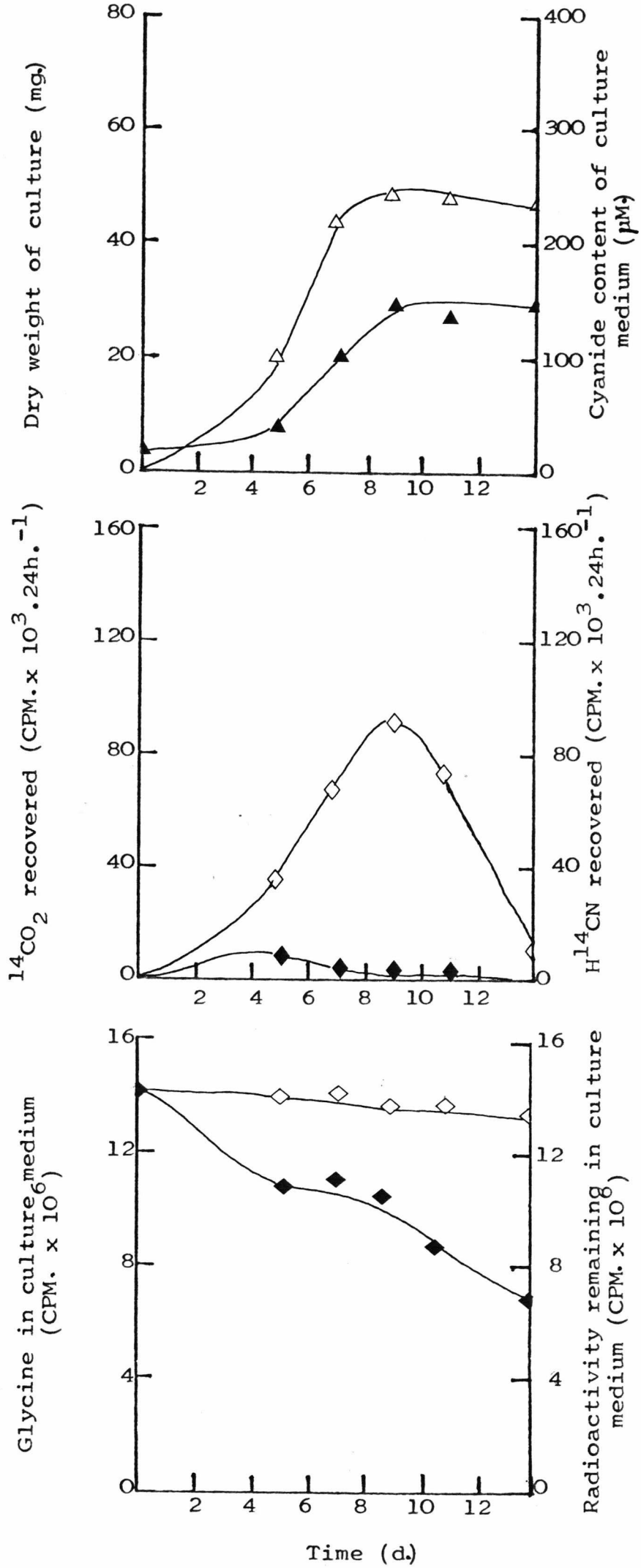

Dry weight of culture (mg)
Cyanide content of culture medium (μM.)
$^{14}CO_2$ recovered (CPM.x 10^3.24h.$^{-1}$)
$H^{14}CN$ recovered (CPM.x $10^3$24h.$^{-1}$)
Glycine in culture medium (CPM.x 10^6)
Radioactivity remaining in culture medium (CPM.x 10^6)
Time (d.)

Figure 3.39a - Growth (▲) and culture medium cyanide content (△) during growth of shake cultures of the snow mould basidiomycete in media containing 30 mM. acetate plus 10 mM. [2-^{14}C] glycine (20nCi.μmol.$^{-1}$).

Figure 3.39b - $^{14}CO_2$ (◆) and H^{14}CN (◇) production during growth of shake cultures of the snow mould basidiomycete in media containing 30 mM.acetate plus 10 mM.[2-^{14}C] glycine (20nCi.μmol.$^{-1}$)

Figure 3.39c - Changes of the glycine (◆) and radioactive content of the culture medium (◇) during growth of shake cultures of the snow mould basidiomycete, in media containing 30 mM.acetate plus 10 mM.[2-^{14}C]glycine (20nCi.μmol.$^{-1}$).


Dry weight of culture (mg.)
Cyanide content of culture medium (μM.)
$^{14}CO_2$ recovered (CPM.x 10^3.24h.$^{-1}$)
$H^{14}CN$ recovered (CPM.x 10^3.24h.$^{-1}$)
Glycine in culture medium (CPM. x 10^6)
Radioactivity remaining in culture medium (CPM.x 10^8)
Time (d.)

Conversion of [2-^{14}C] glycine to [2-^{14}C] glyoxylic acid could not be shown to occur during incubation.

Metabolism of cyanide by snow mould basidiomycete cultures incubated in media containing acetate

Cultures incubated in media containing acetate possess the ability to convert cyanide to CO_2 (Figure 3.40) In contrast to the results obtained from glucose grown cultures the maximum conversion of cyanide to CO_2 occurs before cyanide has reached its maximum concentration during incubation in the culture medium. At the time of maximum cyanide production (12 to 14d. after inoculation) the rate of CO_2 production from cyanide was 0.15 μmol.24h.$^{-1}$, compared 5.28 μmol. 24h.$^{-1}$ cyanide produced.

The amount of radioactivity present in the fungal cells during incubation (Figure 3.41) followed closely by the amount of cyanide present in the culture medium. Little of the radioactivity originally added as cyanide was converted to alanine, glutamate, β-cyanoalanine or formamide (less than 1%). Most of the radioactivity (80%) in both the fungal cells and the culture medium remained as cyanide. The remaining radioactivity was associated with unidentified compounds, which had similar chromatographic properties to those obtained from cultures incubated with glucose (Figure 3.30.)The distribution of radioactivity in cell free extracts differed only slightly between 5, 7 and 11d. old cultures.

Figure 3.40 - The conversion of cyanide to CO_2 (▲) and cyanide production (○) during growth (●) of shake cultures of the snow mould basidiomycete in media containing 30 mM. acetate. Data was obtained by adding 2μCi. $K^{14}CN$ (59mCi. $mmol^{-1}$) and trapping the $^{14}CO_2$ produced during the 24h. prior to harvesting.

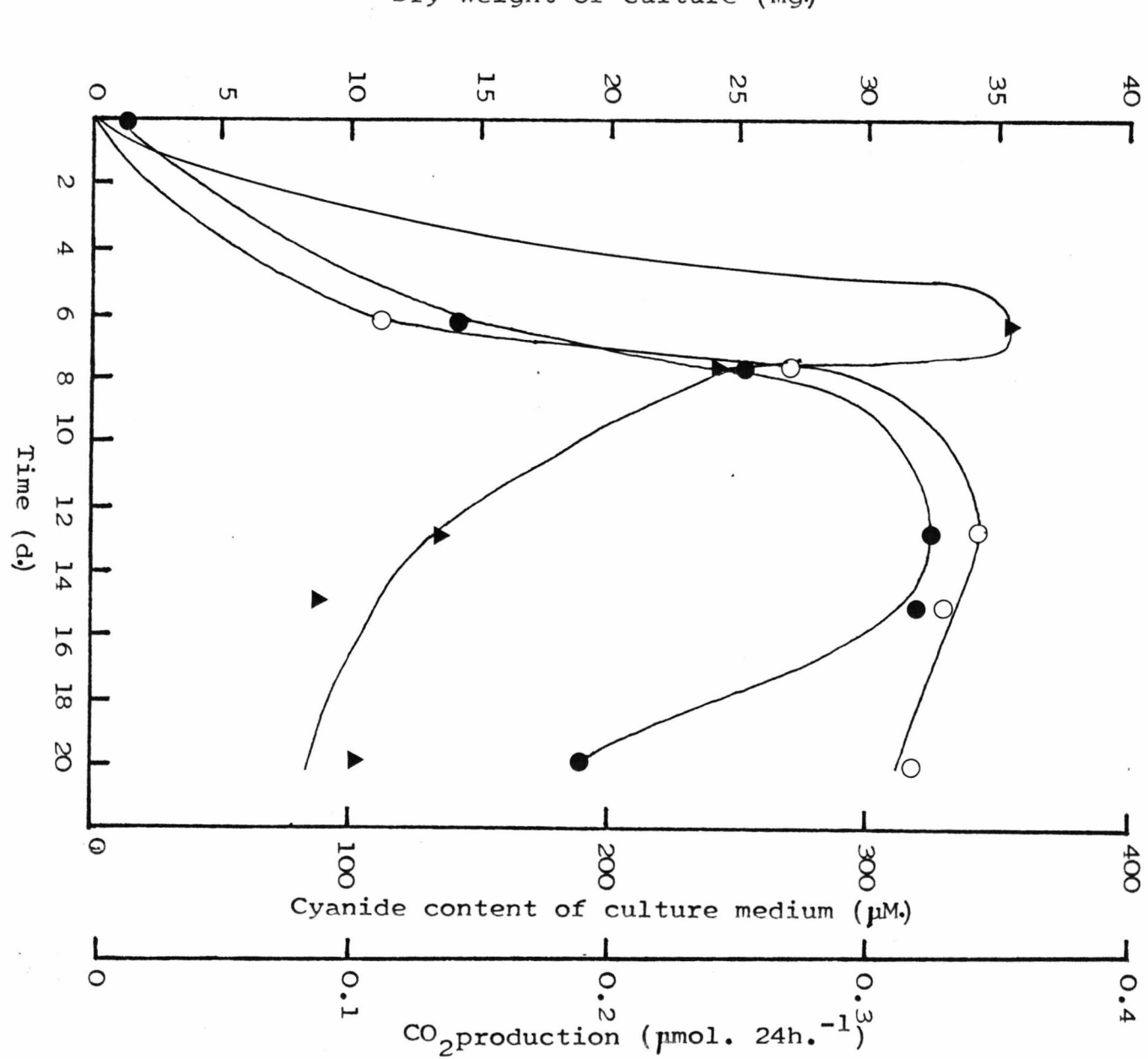
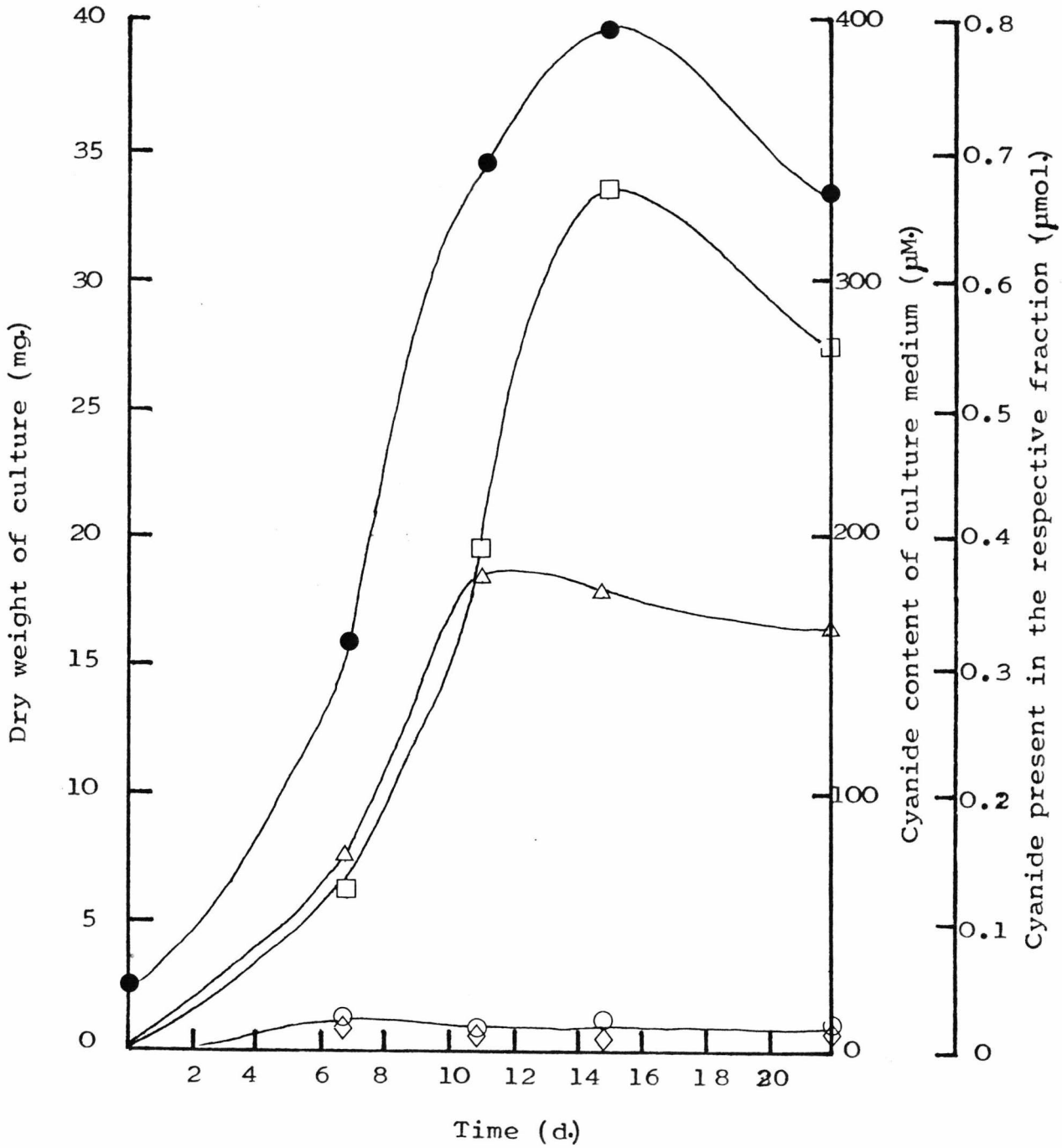

Dry weight of culture (mg.)
0
5
10
15
20
25
30
35
40
Time (d.)
2
4
6
8
10
12
14
16
18
20
0
100
200
300
400
Cyanide content of culture medium (µM.)
0
0.1
0.2
0.3
0.4
CO_2production (µmol. 24h.$^{-1}$)

Figure 3.41 - The distribution of cyanide in the culture medium (□), cell-free extracts (△), protein of cell-free ○ extracts and cell walls plus unbroken cells (◇), prepared from shake cultures of the snow mould basidiomycete, following growth (●) and cyanide production in media containing 30 mM. acetate. Data was obtained by adding 2μCi.$K^{14}CN$ (59mCi.$mmol.^{-1}$) to cultures 24h. prior to harvesting. The fungal cells were disrupted by 2 passages at 8000 lbf. $in.^{-2}$ through a French pressure cell.


Dry weight of culture (mg.)
Cyanide content of culture medium (µM)
Cyanide present in the respective fraction (µmol.)
Time (d.)
0
5
10
15
20
25
30
35
40
100
200
300
400
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
2
4
6
8
10
12
14
16
18
20

CHAPTER FOUR

(DISCUSSION)

Growth and cyanide production in 'still' cultures

The results obtained from 'still' cultures of Marasmius oreades and the snow mould basidiomycete reveal several differences between growth and cyanide production by these two organisms. Interpretation of these differences is difficult as 'still' cultures are poor systems for comparing the metabolism of two different fungi. Indeed, the production of cyanide is a good example of this difficulty. Cyanide appeared during growth of M. oreades cultures, but only at the end of the active growth phase and during the early stationary phase of cultures of the snow mould basidiomycete. Therefore, are these differences due to a specific dissimilarity of metabolism, or the way the two fungi develop in 'still' culture?

Cultures of M. oreades grow as a single mass of cells produced from the initial inoculum. This produces a colony of fungal cells that contain mycelia exposed to different physiological conditions. The result of this is that, even though the dry weight of the culture is low, a few fungal cells may have been exposed to environmental conditions, such that they must produce cyanide. As the fungal cell dry weight of the culture increases, so does the number of mycelia that produce cyanide. Unfortunately, this means that it is extremely difficult to determine the type of cells that are cyanogenic.

Still cultures of the snow mould basidiomycete develop very differently to those of M. oreades. The aggregate of cells produced from the initial inoculum fragments as the dry weight of the culture increases. This presumably results in the majority of cells being under similar physiological conditions. The result of this is that cyanide production would occur (if it is the physiological state of the mycelia that is important for this process) over a narrower period of time. As cyanide is produced at the end of growth of the snow mould basidiomycete, it is possible that it is the 'old' cells that have ceased to divide due to nutrient starvation, enhanced by their position in the centre of the fungal cell aggregates that produce cyanide.

It seems possible, therefore, that the difference in cyanide production between M. oreades and the snow mould basidiomycete in 'still' culture is due to the way the cultures of either organism develops rather than it being due to a dissimilarity of metabolism.

It has been shown that solvent extraction of still or shake cultures of M. oreades and the snow mould basidiomycete yields a mixture of cyanohydrins that are alkali-labile (Tapper and MacDonald, 1974; Ward and Thorn, 1964). These cyanohydrins have been postulated to be intermediates of cyanide production

by these fungi. The apparent correlation between the rise of the culture medium pH and the appearance of cyanide in cultures of the snow mould basidiomycete suggested the possibility that the cyanohydrins were excreted into the culture medium during growth and were then converted to HCN plus an aldehyde as the pH of the culture medium rose.

In contrast, cultures of M. oreades produced cyanide as the pH of the culture medium fell. Even at the end of growth the pH did not rise above pH 6.0. It therefore seems likely that the pH of the culture medium does not affect cyanide production, assuming that the mechanism of cyanide production is similar in both fungi and that cyanohydrins were not excreted into the culture medium during growth. However, it is not known whether a pH difference exists between fungal cells at the outside and inside of the aggregates in 'still' cultures of M. oreades.

The production of pigments as secondary metabolites by both these organisms is interesting. However, this is not unusual for fungi (Cochrane 1958). Unfortunately, it would not be worthwhile for a study of the relationship between pigment and cyanide production to be made until more information about the metabolism of these fungi has been obtained. Moreover, a comparison of cyanogenesis between these fungi will

have to await the successful cultivation of cyanogenic strains of M. oreades in 'shake' cultures, in a defined medium, to reduce artifacts caused by the different culture development.

Growth with selected carbon and nitrogen sources

Experiments performed with the snow mould basidiomycete were designed to compare cyanide production between cultures grown on fermentable or non-fermentable carbon sources and between cultures grown with different nitrogen sources. Due to the complexities involved and the slow growth of the organism, no investigation of the effect of pH, buffering capacity and temperature variation of the culture medium was attempted. Therefore a complete answer to the question of the ability of the snow mould basidiomycete to utilise a particular carbon or nitrogen source is not possible. Nevertheless, from the data collected, several important facts have been revealed.

Firstly, glycine could not be used as either a carbon or nitrogen source for the fungus. This enables an easier interpretation of experimental results involving glycine supplementation of culture medium.

Secondly, these studies have shown a poor correlation between the ability of this fungus to grow in 'shake' cultures and on agar plates. This means that a large range of carbon and nitrogen sources cannot be examined

for use in shake cultures, as determined by the more time and space efficient procedure of agar plate culturing.

The effect of varying the initial glucose concentration of culture media on cyanide production by shake cultures of the snow mould basidiomycete (Figure 3.3) is difficult to interpret, but could provide valuable evidence for the role of cyanogenesis in this fungus. Of several possible explanations for the results obtained, the relationship between nitrogen and carbon availability could be of most significance. With a high initial concentration of glucose in the culture medium, maximum growth will tend to be determined by nitrogen availability. Therefore, it may be expected that less nitrogen will be available for the production of cyanide at the end of growth. Conversely, with a high nitrogen and relatively low glucose content in the growth medium, cyanide production could be limited by the availability of carbon. Glucose (10 mM) and asparagine (15 mM) in the culture medium represents the ratio of carbon to nitrogen that was found to be optimal for cyanide production. The relationship between the carbon/nitrogen balance and cyanide production will be discussed further in a later section.

Other explanations for this result seem less plausible. For example, it could be postulated that at initial concentrations of glucose in the medium

above 10 mM some other factor becomes limiting for cyanide production, such as the oxygen tension of the culture medium. It has been shown that cyanogenesis by a Pseudomonas species has a requirement for oxygen (Wissing, 1972). However, cyanide production by the snow mould basidiomycete is relatively constant, even in media containing a very high initial glucose concentration.

The effect of glycine on cyanide production

It appears that the relationship between the initial concentration of glycine in the culture media and cyanide production is a complex one (Figure 3.4). In cyanide producing bacteria, no decrease of cyanide production was observed when the concentration of glycine was increased above the level that caused maximum production of cyanide (Rodgers and Knowles, 1978, Castric, 1975). A possible explanation of this relationship in the fungus is that at high glycine concentrations alternative routes of glycine catabolism predominate. If this is so, these routes would have to generate metabolites, which could not be utilised to provide extra growth of the fungus, which was observed not to occur. Of the several routes available for glycine catabolism, the one least likely to provide extra growth would be the glycine cleavage pathway (glycine $\longrightarrow$ $CO_2 + NH_3 + C_1$). The operation of this pathway

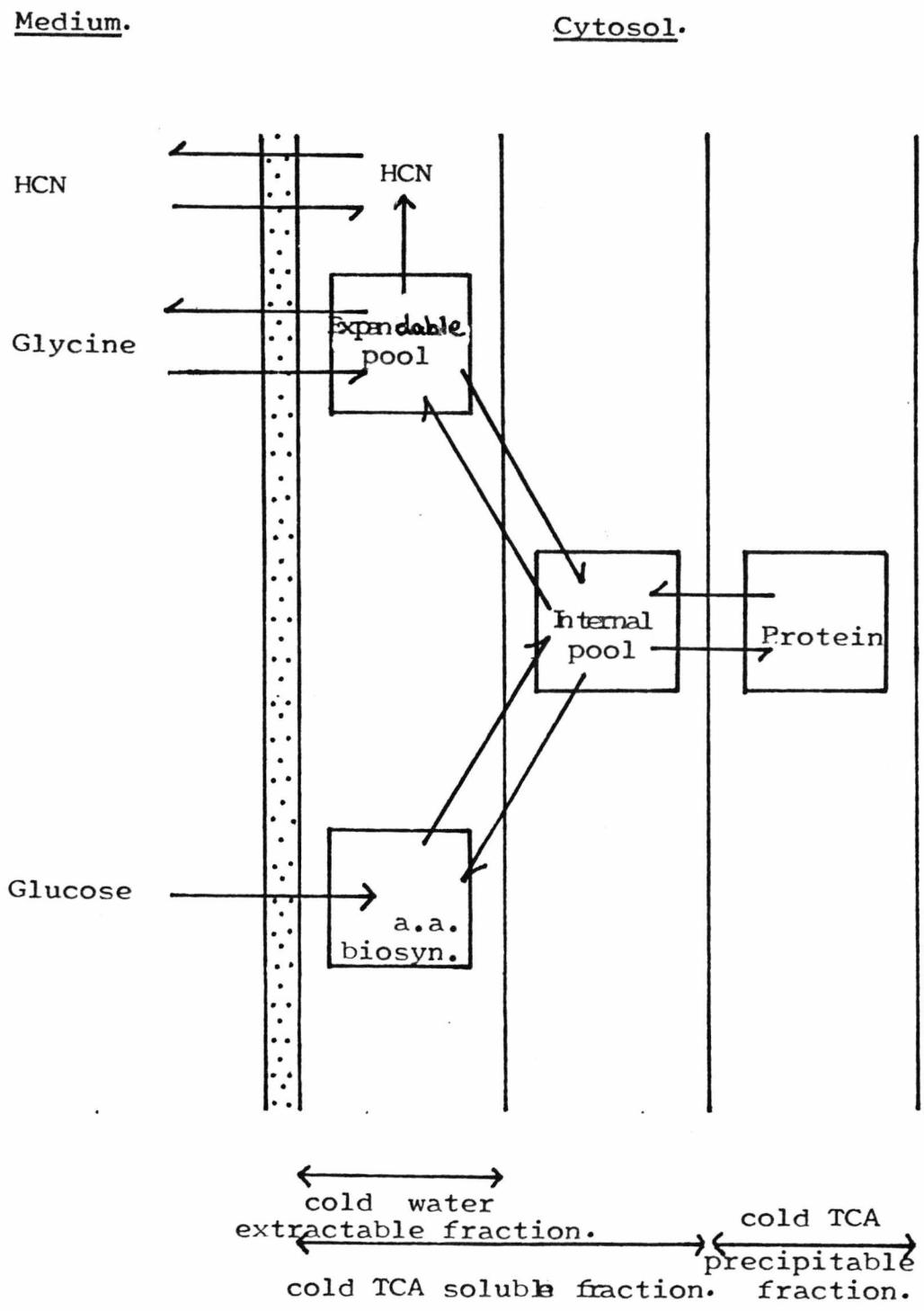
could cause an alteration of the carbon/nitrogen balance, making cyanide production less favourable.

Cyanide formation, when glycine was absent from the culture medium, occurred several days after growth had stopped, as a single production phase. With 10 mM glycine present, cyanide production was biphasic (Figures 3.5 and 3.6)The first phase of cyanide production began towards the end of the growth phase and the start of the idiophase. This period accounted for nearly all the additional cyanide production caused by the presence of exogenous glycine. The second phase of cyanide production coincides with that found in cultures incubated without glycine; the cyanide produced during this phase was nearly the same in both cases.

It has previously been reported in yeast and fungi that two distinct amino acid pools exist, one variable in size and the other relatively constant (Figure 4.1; Halverson and Cowie, 1961). The location of each of these pools is not known with certainty. In the snow mould basidiomycete cyanide production may be involved in regulation of the glycine concentration and hence of the overall size or composition of either or both of the amino acid pools.

During growth of the fungus in media without added glycine, the concentration of glycine in the cell would be dictated by its rate of synthesis in comparison to

Figure 4.1 Amino acid pools in fungal cells



its rate of utilisation for metabolism (protein synthesis, purine synthesis, etc.). When growth stops, the rate of utilisation of glycine would diminish. Control of the rate of supply of glycine would be required at this stage by allosteric regulation or by repression of the enzymes for its biosynthesis. Alternatively, some mechanism for its removal would have to be activated or induced. Thus, the internal pool of glycine, at this stage of growth, may be too large and could be reduced by increasing the size of the expandible pool from which the excess glycine would be catabolised to HCN. Presumably the enzymes for cyanide production could be synthesised either before they are actively required or induced by the increase in size of the glycine pool. Evidence supporting this latter hypothesis will be presented later.

Addition of glycine to culture media means that excess glycine is present at an earlier stage of development of the culture, resulting in the extra phase of cyanide production. Glycine is not removed from the culture medium after 6 or 7 days, so this first phase of cyanide production lasts only as long as it is required for removal of the excess glycine built up during the first 6 or 7 days of incubation. Treatment of the cultures to increase the permeability of the cells at this stage (Basabe, Lee and Weiss, 1978) may result in a continuation of the increased cyanide production.

Most of the variables measured during growth of the snow mould basidiomycete were similar with and without added glycine. In particular no differences were noted in the pH changes of the culture medium. A relationship between cyanide appearance and culture pH is, therefore, unlikely.

Time of glycine addition to cultures, mycelial appearance and stimulation of cyanide production

From the data recorded in Figure 3.7 it may be concluded that the fungus produces an active system for cyanide production after 7 or 8 days of growth in media containing 10 mM. glucose. Whether this represents the time of induction or activation of the cyanide producing system is unclear. However, the induction or activation of this system would appear not to depend on the concentration of glycine in the culture medium. No more cyanide was produced by cultures incubated for 7days in the presence of glycine, than from cultures incubated from the beginning with glycine (from the time of inoculation).

The lack of stimulation of cyanide production by glycine added to 12d. old cultures was, therefore, considered not to be due to the lower induction of the cyanogenic system (Although this could still be the case) but, more probably, it was due to some other factor, for example, an inability of glycine to enter the fungal cells.

It has previously been recorded (Cochrane, 1958) that vacuolated cells are less permeable to amino acids than fungal cells that are not highly vacuolated The cells of 12d. old cultures contain many large vacuoles (from visual estimation, which is only a qualitative method of examining the formation of subcellular structures by cells). Cultures incubated for 12d. could, therefore, contain a majority of fungal cells that are less permeable to glycine than fungal cells from younger cultures. This suggestion is supported by the fact that no change in the glycine content of the culture medium (even though up to 4 mM glycine remains) occurs after 6 to 7 days of incubation (Fig.3.5c).

The effect of selected metabolites on cyanide production by the snow mould basidiomycete

Ward and Thorn (1966) have previously shown that addition of methionine and other methylated compounds such as betaine, sarcosine and N,N-dimethylglycine, inhibited the stimulation of cyanide production resulting from the addition of glycine to cultures. This effect of methionine is opposite to that observed in bacteria (Castric, 1975; Rodgers and Knowles, 1978).

From Figures 3.9, 3.10 and 3.11 it is apparent that methionine has no effect on the total amount of cyanide produced, but only on the rate of cyanide production.

This would explain the apparent inhibition of cyanide production observed by Ward and Thorn (1966) who added glycine plus methionine to cultures and incubated them for only two days before measuring the effect of the additions; longer incubation times would presumably have given results similar to those recorded here.

Addition of other amino acids to cultures produced varying effects. In agreement with Ward and Thorn (1966), threonine did not affect growth or cyanide production and, as the cells are probably able to transport threonine into the cytosol, it would appear that this organism does not possess the ability to convert threonine to glycine.

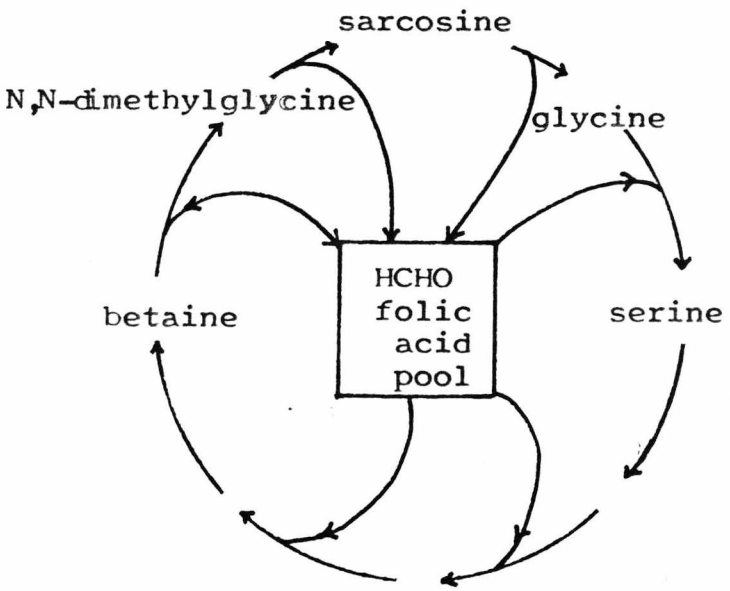
Serine affected cyanide production, but did not affect growth. This would be expected in cultures where asparagine was the nitrogen source. In this situation the fungus would preferentially convert serine to glycine (which cannot be used as either a carbon or a nitrogen source) in order to provide one-Carbon units, rather than convert the serine to pyruvate. Therefore, if serine-to-glycine is the preferred metabolic route, intracellular accumulation of glycine would result. The production of cyanide could then be used to lower the glycine concentration in the cell.

More cyanide was produced in the second phase of cyanide production by cultures incubated with serine (Figure 3.13c) than occurred with cultures incubated without added serine (Figure 3.3a). This could reflect the requirement for a second phase of serine metabolism by cultures of this age and suggests that the peak of cyanide production by cultures after 12d. incubation may partly be accounted for by the conversion of serine to glycine. However, without further information about the metabolism of the snow mould basidiomycete during growth, the causes and reasons for the two phases of cyanide production remain unclear.

The effect of betaine and N,N-dimethylglycine and the lack of effect of sarcosine on cyanide production and growth may be more revealing. These amino acids are N-methylated derivatives of glycine and may be used in this fungus and other organisms as major methyl group donors or acceptors, (Figure 4.2) in the place of methionine. It could be that sarcosine does not affect cyanide production because of the requirement for two methyl groups for its conversion to betaine or because it is not involved as a precursor for N,N-dimethylglycine and betaine.

If betaine and N,N-dimethylglycine do act as methyl group donors and acceptors, then their roles in cyanide production could be the same as the role of

Figure 4.2 - Inter-relationships of methylated glycine and one-carbon metabolism



methionine in bacterial cyanogenesis (Knowles, 1976). One function of these compounds in cyanogenic organisms may involve the regulation of the Carbon-one pool (Harvey and Dev, 1975; Blakley, 1969). This possibility will be discussed further in a later section.

Another possible reason for the effect of betaine and N,N-dimethylglycine is that these compounds could specifically inhibit one or more pathways of glycine metabolism. These non-cyanogenic pathways of glycine metabolism are required by the fungus to enable growth to proceed normally and their inhibition may adversely affect growth. Alternatively, blocking these pathways may result in the fungus using the cyanogenic pathway more, to regulate the excess glycine present. Inhibition of growth could then be due to cyanide rather than the blocking of other pathways of glycine metabolism.

The difference between the effects of these amino acids (N,N-dimethylglycine and betaine), as recorded here and as noted by Ward and Thorn (1966) are unknown but could be due to the much higher concentration of glucose (83 mM) they used. Alternatively, as suggested earlier for methionine addition, their incubation time of 2 days after the addition of glycine and either of these amino acids could have been too short for any effect to be seen.

No derepression of cyanide production was caused by cyclic AMP addition to cultures incubated with glucose. It is possible that other derivatives of cyclic AMP (e.g. N,N-dibutyryl cyclic AMP) would have had an effect. However, experimental results obtained with Neurospora crassa indicate that, at the concentration of cyclic AMP used in these experiments (5 mM), there is little difference between the effects of these derivatives and cyclic AMP (Feldman and Thayer, 1974)

Cyanide production by the snow mould basidiomycete was unaffected by Zn^{2+}, Fe^{2+} or phosphate, compounds that have previously been shown to affect secondary metabolite production by certain fungi and bacteria (Weinberg, 1978). As the role of these metabolites in secondary metabolism is unknown, the examination of other heavy metals on cyanide production by this fungus was not thought worthwhile, until more information is available about the cyanogenic system.

The interesting effect of amino acid addition to protein synthesis-inhibited cultures should also be considered. In these cultures, although the system for cyanide production is present (Figure 3.18) glycine, serine, N,N-dimethylglycine, betaine and theonine did not appear to function as precursors for cyanide production on their own. When glycine was added to these cultures in the presence of N,N-dimethylglycine or

betaine, cyanide was immediately produced. A further complication is that N,N-dimethylglycine and betaine caused massive clumping of the fungal cells. Therefore, differentiation between the effect of these morphological changes and any metabolic changes caused by the addition of either of these two amino acids, together with glycine, is not possible. It is tempting to speculate that betaine and N,N-dimethylglycine act as activators of the system.

The combination of results recorded previously, high glucose concentrations inhibitory to cyanide production, inability of glycine to stimulate cyanogenesis in protein-synthesis inhibited cultures (with 10 mM. glucose as an energy source) and continual cyanide production by cultures containing glucose, glycine and N,N-dimethylglycine or betaine, suggests that betaine and N,N-dimethylglycine could act by derepressing the repression of cyanogenesis caused by the presence of glucose. Whether it is in fact derepression or activation remains to be determined.

<u>The metabolism of [U-^{14}C] glucose by cultures of the snow mould Basidiomycete</u>

Studies with [U-^{14}C] glucose enable an estimate of amino acid concentrations in fungal cells at different times during incubation. The results assume that the proportion of glycine produced from the asparagine metabolised is in the same proportion to that produced

from the metabolism of glucose. It is also assumed that the majority of free amino acids present are released by cellular breakage using a French press. Cellular concentrations of free amino acids vary very little during growth and into the stationary phase. As no large increases occur on cessation of growth (when the demand for amino acids, particularly glycine, can reasonably be assumed to drop dramatically) the concentrations of free amino acids must be well regulated. The small changes that were observed in the radioactive content of cell-free supernatents (Figure 3.21c) may be due to volume changes of the fungal cells during incubation. Alternatively, the dry weight changes could represent deposition of polysaccaride or lipid as well as increases in cell numbers.

However, these changes may also be accounted for by the presence of large metabolite pools of biosynthetic precursors at the beginning of growth, which are subsequently reduced during active growth. The production and usage of these metabolites being carefully controlled throughout.

During autolysis the breakdown of fungal cell components may cause the radioactive content of the cytoplasm to rise again. The difficulty in perceiving the correct explanation for these changes is due to there being available only an inadequate method for

determining the true changes in cell numbers that occur during growth. However, it is clear that no very large increase in the glycine content of the cells occurs immediately before or on day 12 of incubation (when cyanogenesis occurs).

Whole cell respiration and $^{14}CO_2$ production both reach a maximum at the same stage of growth, when the fungal cell dry weight increases are most rapid. This is in common with other fungi and the subsequent decrease of whole cell respiration and $^{14}CO_2$ production has been suggested as being associated with the decrease in permeability of fungal cells at the end of growth (Cochrane, 1958). The permeability of cells of the snow mould basidiomycete would also appear to change during incubation (glycine, asparagine and methionine are no longer removed from the culture medium when growth ceases), but whether this is the explanation of these observations remains speculative.

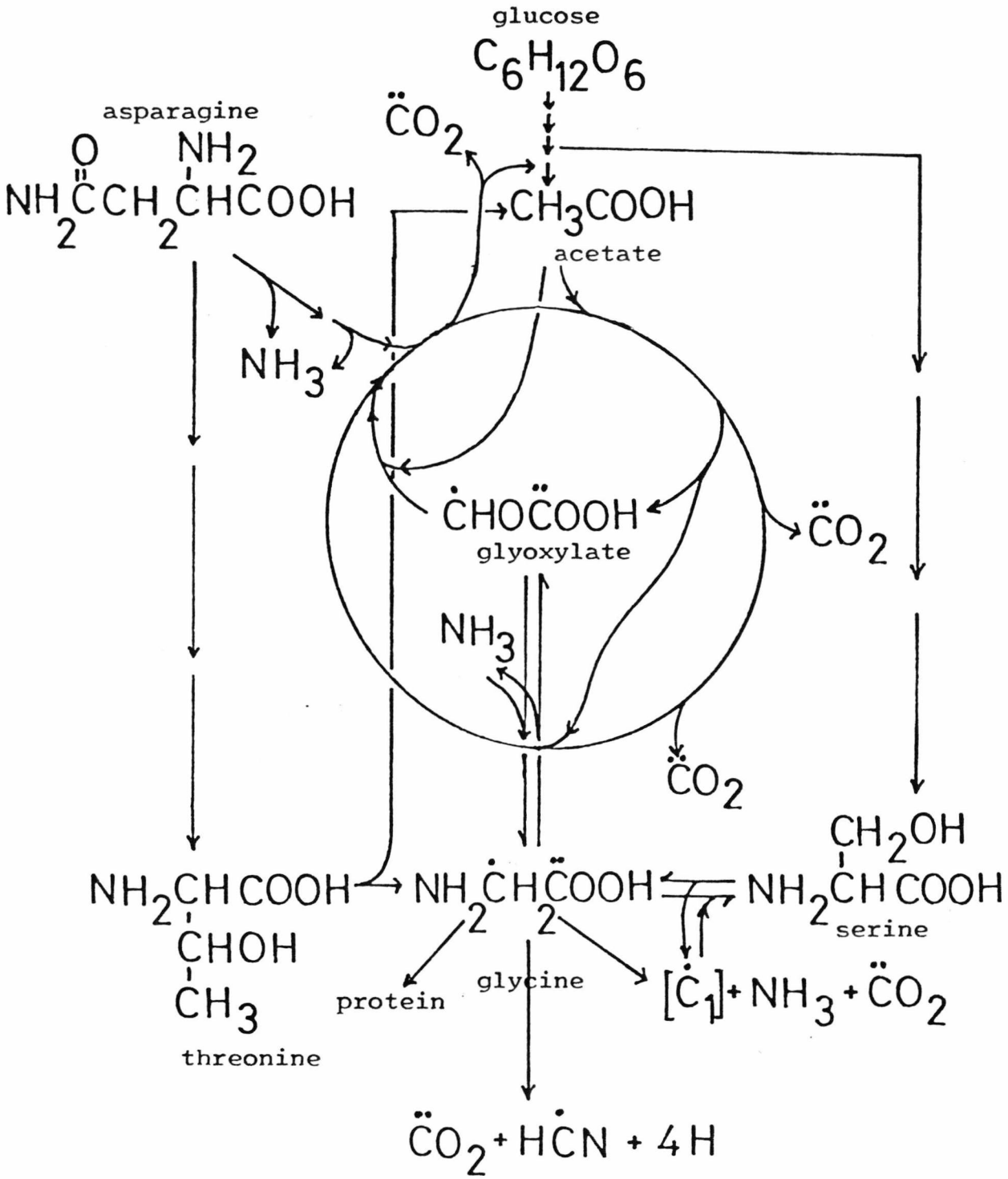
Whole cell respiration was not affected by salicyl hydroxamic acid, an inhibitor of alternative pathways of electron transport in many fungi and plants (Henry and Nyns, 1975). Until a successful mitochondrial preparation is obtained from the snow mould basidiomycete (see appendix) the existence or absence of a salicyl hydroxamic acid sensitive alternative electron transport chain cannot positively be stated.

Cyanide inhibited whole cell respiration in this fungus, but nearly 1 mM. KCN was required for total inhibition. This is a very high cyanide concentration indeed, 100 to 200 μM. cyanide is enough to totally inhibit respiration in most organisms. (Knowles, 1976; Henry and Nyns, 1975). This concentration (100-200 μM.) produced less than 50% inhibition in this fungus. Either the fungus possesses a cyanide resistant respiratory pathway or the cells have the capacity to detoxify cyanide, thus requiring more cyanide for 100% inhibition than would otherwise be necessary. As shown in the results section, and will be discussed below, pathways for cyanide detoxification do in fact exist in this fungus.

Glycine metabolism by the snow mould basidiomycete during growth on glucose

By using $[1\text{-}^{14}C]$ and $[2\text{-}^{14}C]$ glycine, it has been possible to postulate the importance of the many possible pathways of glycine metabolism in the snow mould basidiomycete (Figure 4.3). Its conversion to threonine and serine (even though serine hydroxylmethyl transferase is probably present (Figure 3.13c) seems unlikely from the recovery of radioactivity associated with these amino acids following incubation with radioactive glycine. If glycine were metabolised by any other route (Figure 4.3)

Figure 4.3 - Pathways of glycine metabolism

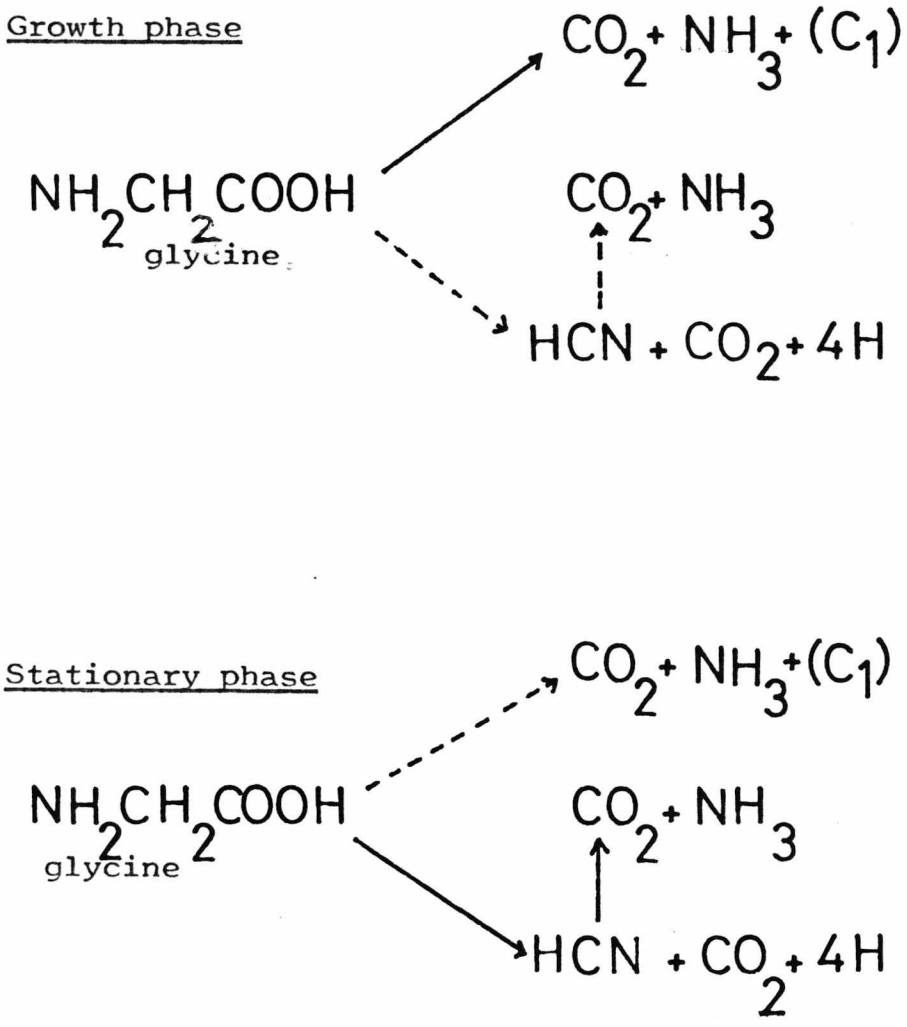


carbon dioxide would be produced initially, mainly from the carbon-one atom. From the data presented, it is not possible to say whether either of these routes (glycine cleavage or glyoxylate production) is the major pathway of glycine catabolism. However, for reasons stated earlier, it is most likely that glycine cleavage is the main pathway as glyoxylic acid, if further metabolised, would be expected to promote extra growth and, if not, to build up in the cell. Neither of these situations occurred. That glyoxylate was converted to oxalate and then to CO_2 is also unlikely during growth on glucose, as this would result in production of $^{14}CO_2$ from both carbon atoms, as oxalate is a symmetrical molecule.

If glycine is mainly catabolised via the glycine cleavage pathway, it could be that this causes an overproduction of carbon-one units (as methylene THF). Cyanide production from glycine may thus become favoured when growth stops (i.e. as shown by the decrease of $^{14}CO_2$ production from $\left[1\text{-}^{14}C\right]$ glycine) to catabolise glycine without further generation of carbon-one units. This is, indeed, found to be the case, as summarised in Figure4.4 and will be elaborated on in a later section.

The unknown products derived from the carbon-2 atom of glycine could represent products of carbon-one metabolism. It is likely, however, that these products

Figure 4.4 - Glycine catabolism by the snow mould fungus during growth on 10 mM. glucose plus 10 mM. glycine.



are mainly produced from the reaction of the highly reactive $H^{14}CN$ (produced from the carbon-2 atom of glycine) with other compounds which contain, for example, keto groups (to form cyanohydrins). It is, of course, because of this reactivity and volatibility of cyanide, that exact figures for total cyanide production are difficult to obtain.

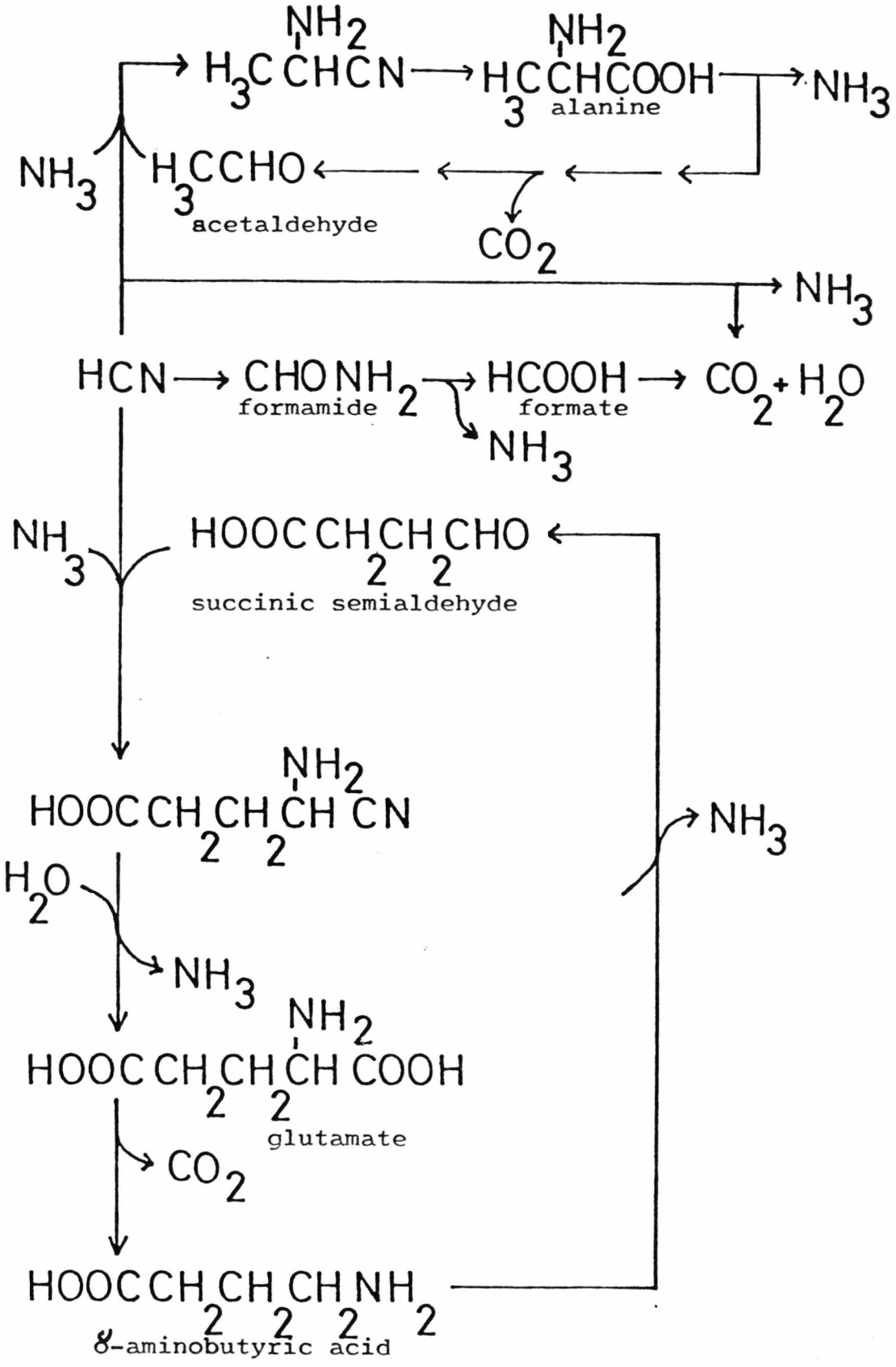
No evidence was obtained for glyoxylic acid or pyruvic acid cyanohydrin production from glycine. It is likely that the observation of these compounds by Tapper and MacDonald (1974) and the cyanide liberating compounds reported by Ward (1964) is a result of the chemical reactivity of cyanide rather than their being formed as intermediates of cyanide production.

Catabolism of cyanide by snow mould basiodiomycete cultures grown with glucose

The major pathway for the catabolism of cyanide by the snow mould basidiomycete grown in a medium containing glucose as the carbon source has CO_2 as the final product. Because the rate of conversion of cyanide to formate by non enzymatic routes is extremely slow, it could only account for a very small proportion of this CO_2 production.

There are four possible pathways of CO_2 formation from cyanide (Figure 4.5). The pathways that involve amino acid intermediates (Strobel, 1966, 1968) are

Figure 4.5 - Pathways of cyanide catabolism that could yield CO_2



considered unlikely to be the major route of cyanide catabolism. In agreement with Strobel (1964) only very small amounts of radioactivity were found associated with glutamate or alanine during incubation of cultures with $H^{14}CN$. As the intracellular pools of these amino-acids are likely to be quite large (Watson, 1976; Pillai and Srinivasan, 1956; Pyle, 1954) the turnover of the pools would have to be uniquely rapid, to explain these results. Hence the importance of these pathways must be very limited.

The other pathways postulated involve either direct production of CO_2 from cyanide or via a pathway involving formamide and formate as intermediates. Evidence for this has been provided by the observation of formamide hydrolyase activity in several fungi (Cyanide to formamide; Fry and Miller, 1972). Cell-free extracts did not contain formamide hydrolyase activity nor did added formate affect CO_2 production from cyanide by whole cells or cell-free extracts. Therefore, the direct conversion of cyanide to CO_2 is probably the most likely route.

Incubation of cell-free extracts at pH values greater than 8.0 resulted in stimulation of CO_2 production from cyanide In this region, cyanide (pka 9.1) would be present partially as CN^- rather than as totally undissociated HCN. This may explain the pH profile recorded in Figure 3.32b.; If it is assumed that CN^- is the substrate for the enzyme as the pH of the assay mixture is

increased, the amount of substrate available to the enzyme would also increase. This may cause a partial nullifying of an unfavourably high pH.

The reason for the variable activities of CO_2 production from cyanide by cell free extracts remains unclear, and may be a result of growth in 21 conical flasks. As mentioned previously, protein synthesis-inhibited cultures, prepared from 21 conical flask cultures, also gave very variable results.

In all the experiments with whole cells it was assumed that there was a rapid equilibration of the added $K^{14}CN$ with unlabelled cyanide in the culture medium and within the fungal cells. Although this is a reasonable assumption to make concerning the culture medium cyanide, equilibration of $K^{14}CN$ with the unlabelled cyanide within the fungal cell may not be as valid. Investigations of the capacity of cells to produce CO_2 from cyanide may, therefore, give results that are lower than the correct values in whole cell studies. The incubation of $H^{14}CN$ with whole cells that are still producing cyanide could result in the progressive dilution of label, so accounting for the fact that only 60% more $^{14}CO_2$ was produced during the second 24h. of a 48h. incubation period.

The use of cell free extracts for studying cyanide catabolism to CO_2 is, therefore preferable, but better assay and cultivation procedures will have

to be developed before studies on this system can proceed. A major drawback would still be the volatility and similar properties of CO_2 and HCN (both are trapped by KOH solutions). It would be interesting to investigate the specificity of this enzyme(s) to determine whether a non-volatile nitrile could be used as the substrate, enabling the development of an easier assay.

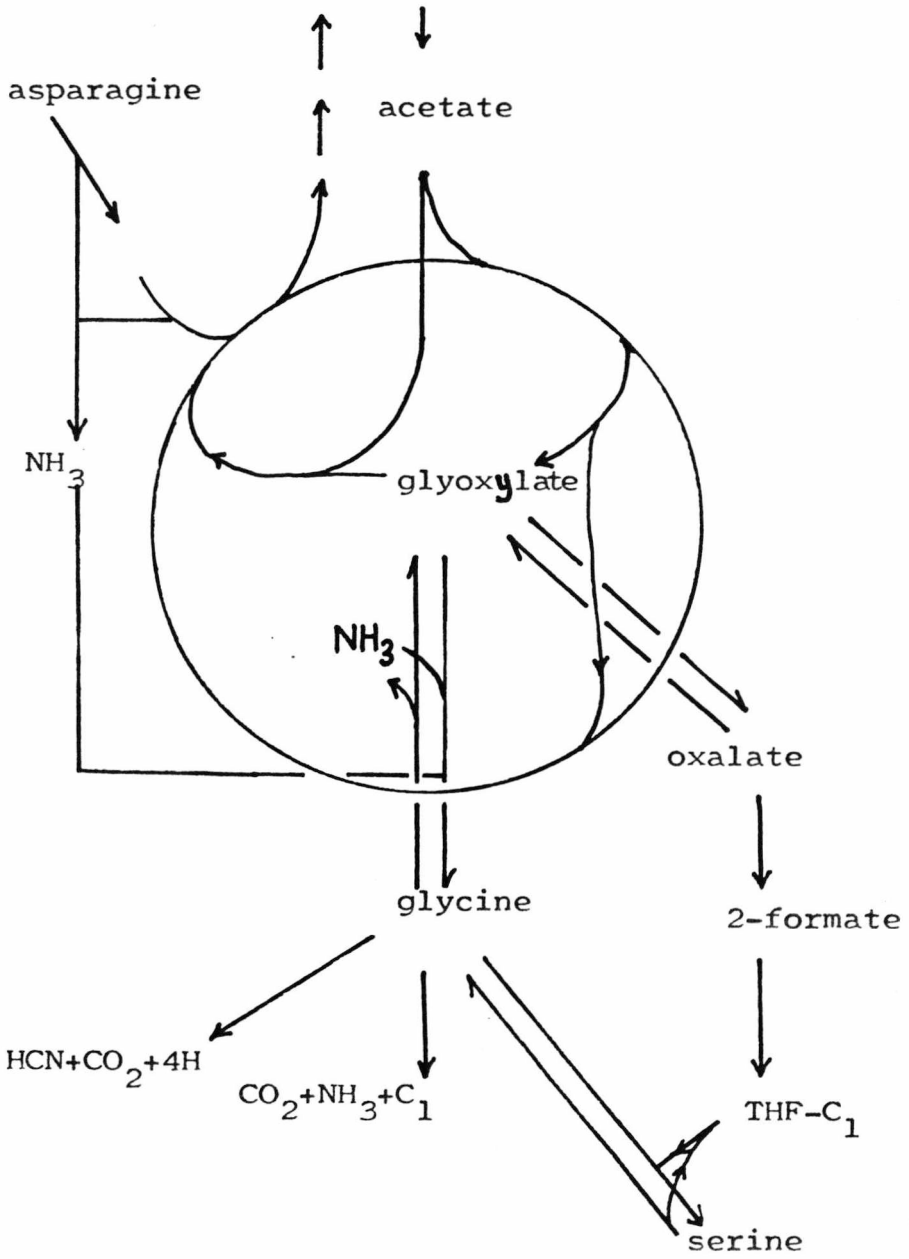
Growth, cyanide production and glycine metabolism by cultures of the snow mould basidiomycete grown in media containing acetate

Cyanide production by cultures grown in an acetate containing medium in the presence or absence of glycine occurred both during and after growth. This implies that the cyanogenic system is present in an active form throughout, and explains the additive effect of exogenous glycine on total cyanide production.

Both carbon atoms of glycine were converted to cyanide (to an almost equal extent), but more CO_2 was produced from the carbon-one atom than from the carbon-two atom. To explain these results, I have assumed that growth of the snow mould basidiomycete on acetate involves the glyoxylate cyde, in common with other microorganisms (Kornberg and Elsden, 1961) when grown in media containing acetate as the major carbon source.

Figure4.6 shows the metabolic relationships, between acetate, asparagine, glycine and glyoxylic acid, and illustrates the important role of asparagine as the nitrogen source for these cultures. The metabolism of asparagine and acetate have a feature in common:

Figure 4.6 - Relationships between glycine and acetate during growth in acetate containing media



(THF-tetrahydrofolic acid)

both cause generation of oxaloacetic acid as a crucial intermediate. During growth, the fungus presumably catabolises asparagine to aspartic acid and then to oxaloacetate, releasing two molecules of ammonia. Acetate metabolism via the glyoxylic acid cycle also produces oxaloacetate (Figure 4.6). To hypothesise why cyanide is continually biosynthesised during growth on acetate and asparagine it is proposed that the preferred route of oxaloacetate production is from asparagine and that glyoxylate produced from the metabolism of acetate is transaminated to glycine. This avoids build-up of oxaloacetate and excess glycine will be catabolised to cyanide.

Because production of cyanide occurs from either carbon atom of glycine in acetate grown cultures, an additional refinement of this scheme is necessary. To scramble the carbon atoms of glycine, it must be reversibly converted to a symmetrical metabolite. As glyoxylate production has been suggested to occur during growth of this fungus on acetate, the most likely symmetrical metabolite to be formed is oxalate. The utilisation of oxalate as a sole source of carbon has been demonstrated for several Pseudomonads (Quayle, 1963) and the existence of the step that converts oxalate to glyoxylate (and vice-versa) can reasonably be postulated. Interchange between the

glyoxylate, glycine and oxalate pools would have to be extensive to explain the data recorded. Nevertheless this could explain the scrambling of the atoms of glycine during growth of the snow mould basidiomycete on acetate and also the similar results for cyanide production from glycine recorded for a Pseudomonad grown on glutamate (Castric, 1975).

If oxalate is produced during growth on acetate, its conversion to formate could be an important source of carbon-one units for this fungus, especially as the provision of carbon-one units from the conversion of serine to glycine would be unfavourable due to the high intra-cellular glycine concentration (Blakley, 1969).

This explanation of the data is speculative but provides a basis for future experimentation. What would be the effect, for example, of varying the nitrogen source and its concentration on the total cyanide produced by acetate grown cultures? Are those enzymes necessary for the proposed inter-conversions present in sufficient quantities? The answers to these questions may reveal much information about why cyanide is produced by this fungus and indirectly why secondary metabolism in general is so ubiquitous throughout the plant, microbial and animal kingdoms.

Further questions that must be resolved are, why was growth with acetate so poor and why was only 30% of the acetate provided used? The answers to these questions could involve two separate factors. Firstly the uptake of acetate (pKa 4.75) is possibly pH dependant and, as the pH of the medium rises, the utilisation of acetate may become increasingly difficult. It would be interesting to try the effect of a more highly buffered culture medium and lower initial pH values. Secondly, the continued production of cyanide may eventually inhibit growth. Indeed it is perhaps surprising in this context that growth occurred, as the cyanide levels formed were sufficient to inhibit whole cell respiration of these cultures.

The fact that acetate also caused the earlier appearance of cyanide in a culture medium containing glucose plus acetate shows that the acetate produced cyanide during its metabolism. Very little lag was required (no diauxic growth was observed) before acetate utilisation. It may be that the enzymes of the glyoxalate cycle were already present during the first stage of growth with glucose. The reasons for this are currently unclear.

Cyanide catabolism by cultures grown in media containing acetate

There are few differences between the catabolism of cyanide by cultures grown on glucose or acetate. The major route of cyanide catabolism in both cases was

the production of CO_2, little or no alanine and glutamate became labelled during incubation with $K^{14}CN$. Also, in both cases, the cyanide taken up by the fungal cells was greatest during the period when the cyanide concentration in the culture medium was at its maximum.

The difference between glucose and acetate grown cultures was that CO_2 production from cyanide was maximal, in the acetate containing cultures, before the largest amounts of cyanide were produced. Possibly the appearance of cyanide is due to the inability of the cyanide catabolising enzyme(s), to cope with the amount of cyanide synthesised or that their activity diminishes as growth proceeds. It is also a distinct possibility that the $K^{14}CN$ added to cultures does not fully equilibrate with the intracellular cyanide. Therefore, a true idea of the capacity of these cultures to catabolise cyanide will have to await a successful assay for the enzyme(s) required to convert cyanide to CO_2.

Cyanide production as a secondary metabolite by the snow mould basidiomycete

This study has shown that production of cyanide by this fungus is influenced by the carbon source on which it is grown. A similar result was obtained from studies on cephalosporin biosynthesis by Streptomycetes clavuligerous (Demain, Kennel and Aharonowitz, 1979).

Growth of this organism was best with glycerol as the carbon source. However, although the amount of growth increased as the glycerol content of the culture medium was raised, above a particular initial concentration of glycerol, cepholosporin production actually fell. Growth of S. clavuligerous on low concentrations of glycerol or starch (a poorer carbon source than glycerol) caused cephalosporin production to become more closely associated with growth. The proposal by Demain et al. (1979) that these effects are caused by carbon catabolite repression therefore seems very plausible.

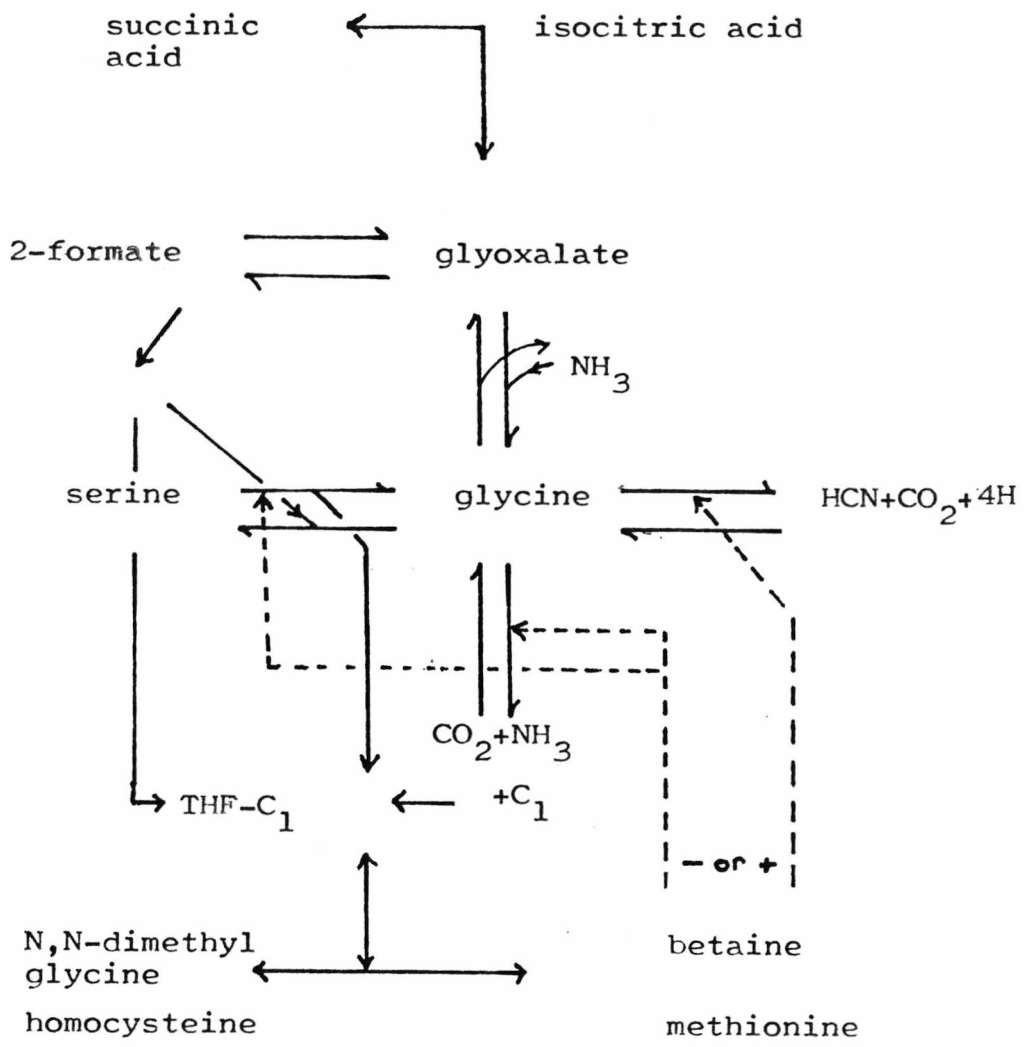
There is, however, an alternative explanation for this, if it is assumed that secondary metabolism is involved with changes of primary metabolism. During growth on a 'good' carbon source (one that is favourable in the sense that it permits rapid growth) metabolism is well balanced and the need to produce secondary metabolites does not occur. It is only when the carbon source has been used up that metabolism will become unbalanced, resulting in the need for removal of excess quantities of certain metabolites and hence secondary metabolite syntesis. For example, this could explain why betaine and N,N-dimethyl glycine caused apparent derepression of cyanide production by the snow mould fungus, and why cyclic AMP had no

effect. If this view is correct, then it is not the carbon source that represses secondary metabolism, but the type of growth which it produces that is important. The addition of N,N-dimethylglycine or betaine and the metabolism of acetate (affected by the presence of asparagine as the nitrogen source) could therefore be postulated to produce unbalanced metabolism that necessitates the production of cyanide.

What would be the likely nature of this inbalance? Bacterial cyanogenesis is stimulated by methionine (Michaels and Corpe, 1965; Wissing, 1968; Castric, 1977). On the other hand, cyanogenesis by the snow mould basidiomycete is not stimulated by methionine, but it is stimulated by betaine and N,N-di methylglycine As the cyanogenic systems of these organisms appear to be closely related (they require the same precursor glycine), it may be proposed that the effects caused by methionine and betaine or N,N-dimethylglycine are also similar. As shown in Figure 4.7 these compounds are probably involved in carbon-one metabolism as methyl-group donors (methionine and betaine) or acceptors (N,N-dimethylglycine).

Production of carbon-one units during growth of the snow mould basidiomycete on glucose could be via the action of serine hydroxy methyl transferase and the glycine cleavage pathway. With acetate as the

Figure 4.7 - The possible relationship of glycine to carbon-1 metabolism in the snow mould basidiomycete



(THF-tetrahydrofolic acid, - - - potential regulatory step)

carbon source, the pathway for conversion of glyoxylate (produced from the glyoxylate cycle that must be formed to enable the utilisation of acetate. Kornberg, 1965) to formate and the glycine cleavage pathway may be the preferred routes. When growth stops or if excessive amounts of glycine or glyoxylate are produced, the fungus must prevent excess production of carbon-one units and it may convert glyoxylate to glycine and then glycine to cyanide. Some of the cyanide, which is volatile, would be lost from the cultures and any remaining in the culture would be catabolised to CO_2. The fungus would thus avoid the disadvantageous effects of cyanide production whilst benefiting, by maintaining the correct balance between the supply of glycine and serine, to the demand for carbon-one units. Therefore, glycine is not likely to be involved with activation or induction of the cyanogenic system, but rather in synthesis of intermediates of carbon-one metabolism.

Concluding Remarks

To conclude, this study has provided good evidence, for the first time, for the function of production of a secondary metabolite. In addition, it has been shown that the different effects of various carbon sources on secondary metabolism could be for reasons

other than those already proposed (Drew and Demain, 1976; Demain *et al.* 1979). The effectors of cyanogenesis in the snow mould basidiomycete (particularly betaine, N,N- dimethylglycine and glycine) have highlighted the fact that future investigations on secondary metabolite production will require an equally intense study of primary metabolism (in this case glycine and carbon-one metabolism).

The pathway of cyanide production from glycine by this fungus remains unknown, but on the basis of the evidence presented in this Thesis, the involvement of glyoxylic acid or pyruvic acid cyanohydrins as intermediates can be considered extremely unlikely.

Although it has not been possible to quantify the significance of cyanide catabolism by the snow mould basidiomycete, the conversion of cyanide to CO_2 is clearly the most important pathway. It would be interesting to investigate this process further, especially as it could provide a cheap method for cyanide detoxification of industrial wastes. On the other hand, production of amino-acids from cyanide, NH_3 and an aldehyde, as proposed by Strobel (1966, 1967), was shown to be of little significance.

The data recorded in this Thesis have, therefore, laid a firm basis for future investigations on cyanide matabolism by the snow mould basidiomycete. It has

shown that, despite the difficulties of working with fungi (slow growth, toughness of the fungal cells, culturing difficulties, etc.) much useful and important information can be obtained and shows that the lack of attention that cyanogenic fungi have received is unmerited.

APPENDIX

Electron microscopy of whole cells taken from cultures of the snow mould basidiomycete

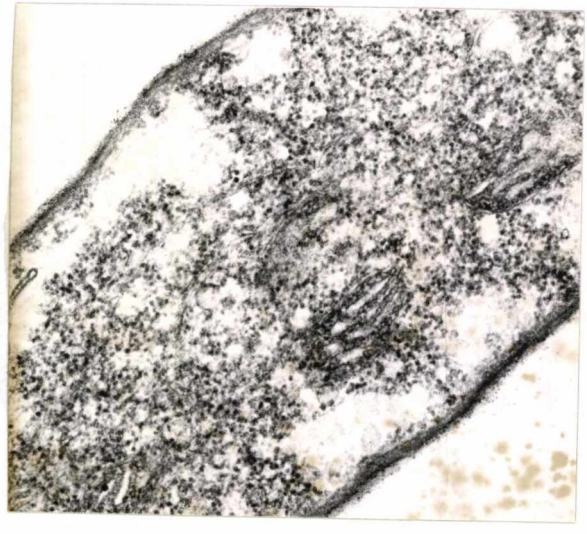
Shake cultures of the snow mould basidiomycete were harvested at different times during incubation in media containing 10 mM glucose, 10 mM glucose plus 10 mM glycine, 30 mM acetate or 30 mM acetate plus 10 mM glycine. Examination of fungal cells revealed that mitochondria were present throughout growth in all four types of media and that the presence of glycine did not affect the appearance of these mitochondria.

However, mitochondria in glucose-grown cells did appear to be more vacuolated than the mitochondria in acetate-grown cells (Figures A.I and A.2), and the numbers of mitochondria present in glucose-grown cells were lower than those observed in the acetate-grown cells (although a quantitative estimation of the number of mitochondria in the fungal cells was not attempted.

Disruption of cells of the snow mould basidiomycete and isolation of mitochondria

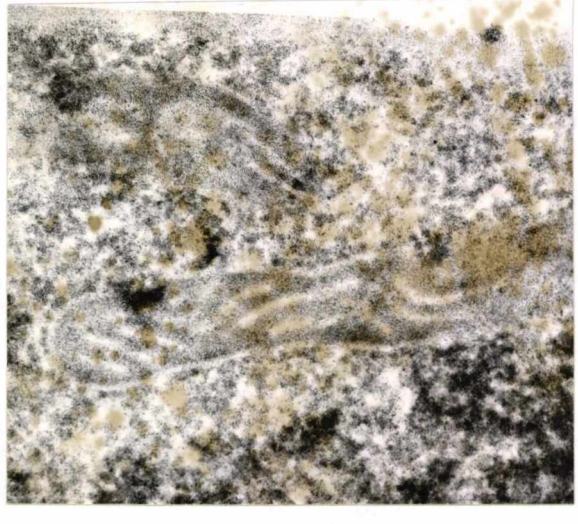
Cultures were grown in media containing either 30 mM acetate or 10 mM glucose for 7d. and 5d. respectively in a 5l fermentation vessel containing 4.5l of medium or a 2l conical flask containing 500 ml. of medium. At least 2.5g. of cells (dry weight) were grown in each case and stored frozen, if necessary, before use. The fungal cells were harvested, disrupted

Figure A.1 - Electron micrograph of a fungal cell from a 5d. old shake culture grown in media containing 10 mM glucose



Magnification x 25,000

Figure A.2 - Electron micrograph of a fungal cell from a 7d. old shake culture grown in media containing 30 mM acetate



Magnification x 25,000

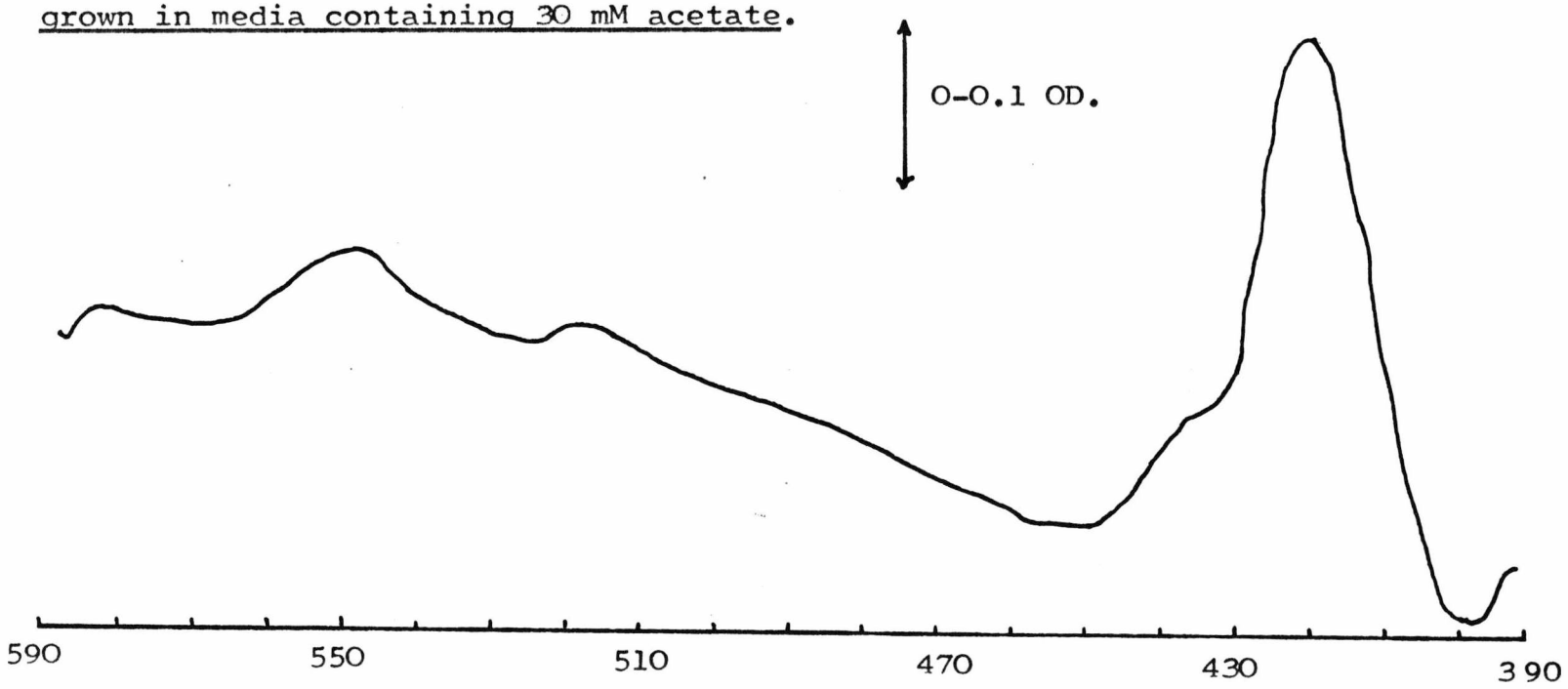
by two passages at 8000 lbf. in^{-2} through a French pressure cell and fractionated by centrifugation. Pellets obtained by centrifugation at 1500g., 8000g. and then 150,000g. and the 150,000g. supernatant were assayed for cytochromes, rotenone/cyanide-sensitive NADH oxidase, cyanide-sensitive ascorbate/N,N,N,N, tetra methyl-p-phenylenediamine (TMPD) oxidase activity. The pellets were also examined under the electron microscope.

Very little oxidase activity or the presence of any cytochromes were detected in preparations from glucose-grown cultures. Higher oxidase activity and the presence of higher levels of cytochromes were observed in preparations from acetate-grown 2l. or 5l. cultures, (which both gave similar results). Better activities were obtained when a protease inhibitor, phenylmethylsulfonyl-fluoride (PMSF), was included in the disruption medium.

Cytochromes were detected in the 8000g. and 150,000g. supernatants or pellets from preparations of acetate-grown cultures. The amounts recorded were always very low (Figure A3) and there was consistently never enough present to be able to resolve the cytochromes present.

Electron micrographs of 8000g. and 150,000g. pellets obtained from acetate-grown fungal-cell preparations revealed that they consisted mainly of vesicles with no obvious internal structure.

Figure A.3 - Cytochrome spectra dithionite reduced - H_2O_2 oxidised of 8000g. supernatant, obtained from 7d. old 2l, shake cultures of the snow mould basidiomycete grown in media containing 30 mM acetate.



(Protein content of sample 3.4 mg ml^{-1})

These results suggest that successful mitochondrial preparations from this fungus will require larger quantities of cells and an alternative method of disruption.

Attempts to obtain lytic enzymes to break down the cell wall of the snow mould basidiomycete from either Trichoderma viride or Athrobacter luteus were unsuccessful. Disruption, by shaking the cells vigorously in buffer containing glass beads of either 0.11 mm. or 0.44 mm. diameter, also produced poor results, both with respect to the oxidase activities of the preparations and the efficiency with which the cells were broken.

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