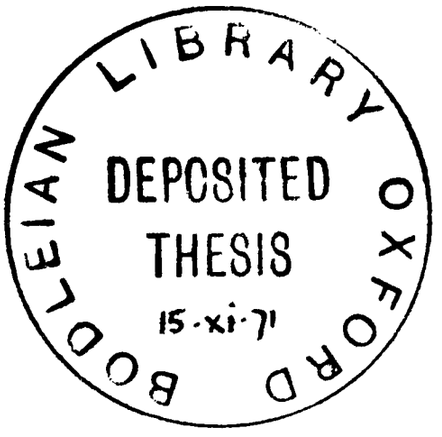

MICROBIOLOGICAL STUDIES OF SOME PEPTIDE ANTIBIOTICS

by

Margaret J. Duncan

(Linacre College)

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ABSTRACT

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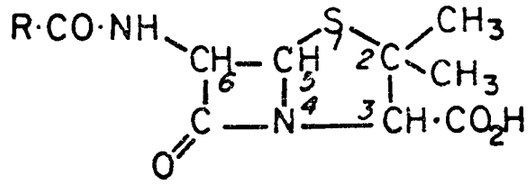
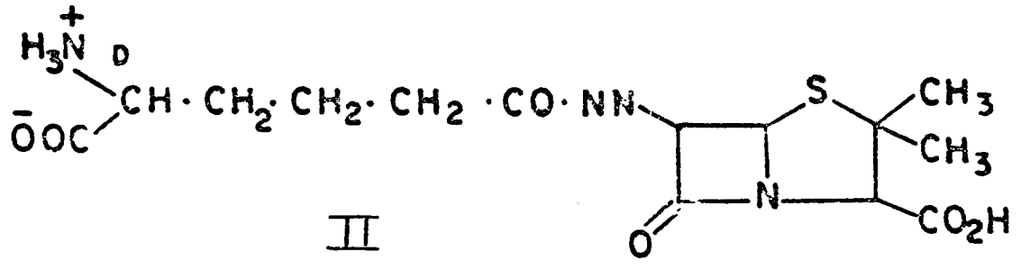
Introduction

Among the penicillin-producing organisms which are members of the Class Fungi Imperfecti are Penicillium chrysogenum and the Cephalosporium sp. C-91. The former can produce a series of extracellular penicillins (I) with non-polar side-chains (R.CO) which vary with the side-chain precursor added to the fermentation medium.



I

The Cephalosporium sp. produces only two extracellular antibiotics of this type and both have a δ-(D-α-aminoadipyl) side-chain. One is penicillin N (II) and the other cephalosporin C (III).



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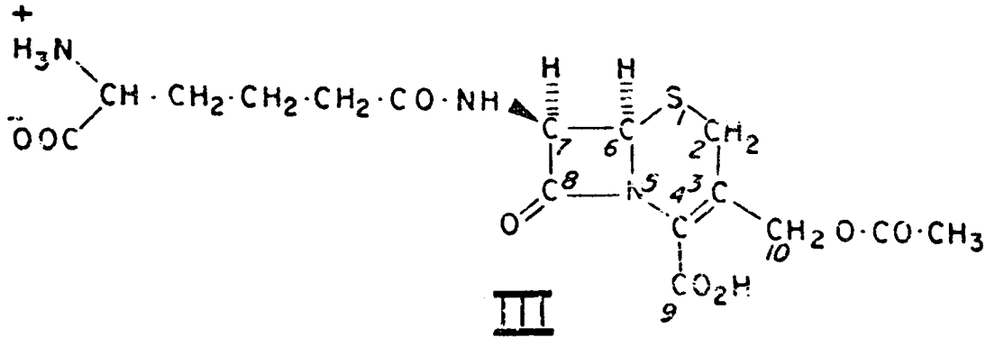
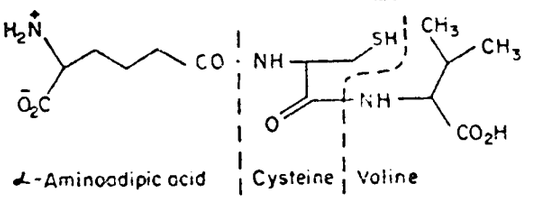


III

Early studies established that the biogenetic precursors of the ring systems of both benzylpenicillin and cephalosporin C were L-cysteine and L-valine, and that the side-chains were derived from phenylacetic acid and α-aminoadipic acid respectively. The tripeptide δ-(α-aminoadipyl)cysteinylvaline (ACV,IV) was isolated from the mycelium of P. chrysogenum (Arnstein & Morris, 1960) and subsequently detected in the Cephalosporium sp. also (Smith et al., 1967). This suggested that



IV

δ-(α-aminoadipyl)cysteinylvaline might be an intermediate in the biosynthesis of the penicillins and of cephalosporin C.

The difficulty of assessing the role of possible intermediates such as ACV in penicillin or cephalosporin biosynthesis has depended partly on the existence of permeability barriers. The development of broken-cell or cell-free systems in which these barriers have been removed and in which all or part of the biosynthetic pathways can be

followed is thus clearly important.

Cell-free or broken-cell systems have been used to study the biosynthesis of several peptide antibiotics. These systems have been prepared by mechanical rupture of the cells, or by enzymic digestion of the cell wall to form protoplasts, which were then easily disrupted by osmotic shock. Dissolution of the cell wall of B. licheniformis and of B. brevis by muramidase has led to cell-free systems capable of synthesising bacitracin and tyrocidine respectively (Ishihara, Sasaki & Shimura, 1968; Fujikawa, Suzuki & Kurahashi, 1966). However, protoplasts from fungi have proved more difficult to prepare (Villaneuva, 1965). Snail enzyme, an impure preparation containing approximately thirty enzymes, has commonly been used to prepare protoplasts from yeast cells. Cells from different strains and of different ages vary markedly in their susceptibility to snail enzyme, but young cells are in general more susceptible than older ones.

Aims and Scope of the Present Investigation

The main aim was to prepare protoplasts from the Cephalosporium sp. and to study their properties with the intention of using them as the starting material for the preparation of an active cell-free extract. Towards this end it was decided to find out which phase of a culture of

Cephalosporium sp. C-91 produced penicillin N and cephalosporin C at maximal rates, and the morphological appearance of the mycelium in this phase so that protoplasts could be prepared from this type of culture. Finally, it was hoped that a cell-free extract obtained from protoplasts would be able to carry out all or some of the steps associated with antibiotic biosynthesis.

Material and Methods

Cephalosporium sp. C-91 was grown in batch cultures in shake flasks in a chemically-defined medium. The cultures were in general seeded with a heterogeneous inoculum but in some experiments with a spore inoculum. The growth of the organism was plotted from dry weight measurements, antibiotic production was measured by bioassay and morphological changes were followed by phase-contrast microscopy and photo-micrography.

Protoplasts were prepared from mycelium harvested at 48 hours by resuspension in a solution of snail enzyme (40 mg/ml) and sodium chloride stabiliser (0.9M) after pretreatment with 2-mercaptoethanol or dithiothreitol. After incubation (3-4 hours) protoplasts were separated from intact mycelium and resuspended in stabiliser. The percentage yield of protoplasts was measured by comparing their content of ethanol-extractable nucleotide with that

obtained from a known weight of intact mycelium. Electron micrographs were made of mycelium and protoplasts after fixation in Kellenbergers buffer (Kellenberger, Ryter & Séchaud, 1958) and staining with osmium tetroxide.

For the measurement of [^{14}C]-valine uptake by mycelial preparations, samples from the resuspension fluids were withdrawn at intervals and centrifuged. A known volume of supernatant fluid was removed for radioactivity measurement.

Antibiotic production by the protoplasts was measured by bio-assay and by the incorporation of ^{14}C from DL-[1-^{14}C]-valine. Culture fluids of protoplasts and mycelium containing isotopically labelled antibiotics were desalted on Nuchar from which the antibiotics were eluted with ethanol.

Radioactive compounds in extracts of culture fluids were located after paper chromatography or electrophoresis by radioautography and counted on the paper; absolute radioactivities were calculated from the counts on paper. Small amounts of authentic cephalosporin C and penicillin N were added to samples containing radioactive antibiotics and their positions after analysis on paper were determined by scanning in ultraviolet light (Abraham & Newton, 1961).

Results and Discussion

Section I

The growth rate was divided into three phases. The

first began immediately after inoculation when the growth rate increased rapidly and was accompanied by the production of young, undifferentiated hyphae, and the germination of spores. Towards the end of this phase penicillin N production began, and, as in phase two, its production appeared to be associated with growing mycelium. During phase two the growth rate reached its maximum when cultures consisted of young hyphae in an extending and branching growth phase. Cephalosporin C production began later in phase two, while in phase three both antibiotics attained maximum production rates as the growth rate was falling. Thus, maximum antibiotic production was associated with the presence of non-growing, differentiated mycelium. Penicillin N appears to be synthesised by a pathway which was not restricted to non-growing cells, and which is different from, and initiated earlier than that which leads to cephalosporin C formation.

Section II

The cell walls of 48 hour cultures of the Cephalosporium sp. were not readily digested by snail enzyme. However, pretreatment for 1 hour with 0.02M-2-mercaptoethanol or 0.01M-dithiothreitol rendered it more susceptible to attack. In yeast cell walls 2-mercaptoethanol is thought to reduce disulphide bonds linking protein to mannan. Although there is no evidence to suggest that these linkages are

present in the Cephalosporium sp. cell walls, it is possible that a similar reaction occurs, after which the walls are more rapidly degraded by the snail enzyme complex.

The electron micrographs showed that the cell wall of Cephalosporium sp. C-91 was a four layered structure. After treatment with dithiothreitol the cytoplasm withdrew from the inner layer of the wall, while the walls themselves appeared unchanged. The protoplasts were completely spherical with no detectable vestiges of wall material at their surface. They possessed vacuoles, mitochondria and ribosomes in their cytoplasm, but the latter were more diffuse than in the mycelium. This and the loss of invagination in the protoplasmic membrane suggested that the stabilising solution had entered the protoplasts causing them to swell in a manner analogous to the swelling of damaged mammalian cells (Leaf, 1956).

Section III

Pretreatment of mycelium with dithiothreitol followed by incubation in sodium chloride stabiliser reduced penicillin N production by approximately 40% and that of cephalosporin C by 60-70%. Thus, it seemed that the cephalosporin C system was more sensitive to external changes in environment, possibly because certain enzymes or inter-

mediates involved in its formation are more labile than any of those involved in penicillin N production.

On the other hand, after treatment of mycelium with snail enzyme solution the production of cephalosporin C was inhibited less than that of penicillin N. Hence, it appears unlikely that cephalosporin C was formed from penicillin N. It is possible that co-factors or enzymes required for penicillin N synthesis were located outside the protoplast membrane and removed when the wall was dissolved. However, even when antibiotic synthesis could not be measured by bioassay, the formation of [^{14}C]-penicillin N as well as that of [^{14}C]-cephalosporin C could be detected after the addition of [^{14}C]-valine to the protoplast suspensions.

The protoplasts released cephalosporin C readily after agitation, as if it were only loosely bound to the cells. The total intracellular amino acid content of the protoplasts was estimated to be approximately one tenth of that of whole mycelium. In addition, it was found that potassium ions leaked out of the protoplasts during incubation, although enough appeared to be retained to maintain an energy-generating system.

The dipeptide δ-(DL- α-aminoadipyl)-L-cysteine was able to enter the protoplasts, although it did not enter the intact mycelial cell. But once inside it did not

appear to enhance antibiotic production, suggesting that the synthesis of δ-(α-aminoadipyl)cysteine was not a rate-limiting step in the biogenetic pathway in the protoplasts.

The uptake of DL-[1-^{14}C]-valine by suspensions of protoplasts was compared with that by starved mycelium in water and DTT-pretreated mycelium in 0.9M-NaCl. A similar overall pattern was obtained, which could be divided into three phases. In the initial uptake, lasting up to 10 minutes, as much as half of the total isotope was removed. In the next phase, from 10 to 30 minutes, there was a rise in the extracellular radioactivity which was assumed to be due to the release of [^{14}C]-valine from protoplasts and from mycelium. In the third phase, valine was taken up at a similar rate by protoplasts and mycelium, but more slowly than in the initial phase. Within this pattern, variations between suspensions were observed in the amounts of valine removed or released in each phase.

Section IV

The biosynthesis of glutathione, of δ-(α-aminoadipyl)cysteinylvaline and of penicillin N and cephalosporin C was studied in cell-free extracts prepared by grinding mycelium with sand or alumina, or from lysed protoplasts. In the presence of an ATP-generating system, [^{14}C]-gluta-

thione was formed from glutamic acid, cysteine and [^{14}C]-glycine. It was synthesised more readily in extracts from 72 hour mycelium than in those from 48 hour mycelium, and more readily in the supernatant than the particulate fractions of the extracts. In a protoplast extract, synthesis of δ-(α-aminoadipyl)cysteinylvaline (ACV) was detected only when δ-(L-α-aminoadipyl)-L-cysteine was added with ^{14}C-valine, which indicated that the formation of the dipeptide was rate-limiting in this cell-free system.

When α-aminoadipic acid, cysteine and [^{14}C]valine were added to protoplast lysates, small amounts of labelled antibiotics were formed. Hence, if ACV is an intermediate in antibiotic formation, the protoplast extracts must have synthesised it from its constituent amino acids. It is suggested that after being formed in minute amounts, ACV is rapidly transformed to penicillin N and cephalosporin C and thus escapes detection. Unlike the intact protoplasts, cell-free extracts derived from them synthesised more penicillin N than cephalosporin C. The formation of the latter may be more dependent on the structural integrity of the cell.

Conclusions

The work described in this thesis has shown that protoplasts can be made from Cephalosporium sp. which retain some of the ability of the intact mycelium to synthesise

penicillin N and cephalosporin C. Further, a cell-free system has been obtained by lysis of these protoplasts in which it has been possible to demonstrate the biosynthesis of δ-(α-aminoadipyl)cysteinylvaline from δ-(α-aminoadipyl)-cysteine and [^{14}C]-valine. Fractionation of this lysate has produced a 20,000 g supernatant, free of whole or broken protoplasts, which is able to synthesise small amounts of the antibiotics from α-aminoadipic acid, cysteine and [^{14}C]-valine.

It is concluded that more definitive information about biosynthesis precursors could be obtained by the use of isotopically labelled intermediates, such as δ-(α-aminoadipyl)cysteinylvaline, in the cell-free systems described here. From one of these systems it might be possible to prepare a purified enzyme complex capable of synthesising penicillin N and cephalosporin C.

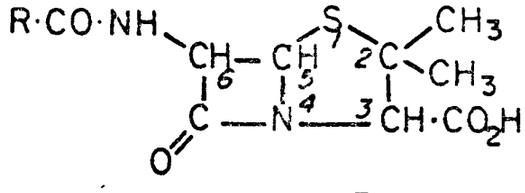
CONTENTS

INTRODUCTION

INTRODUCTION

This thesis describes studies relating to the formation of two peptide antibiotics, penicillin N and cephalosporin C, which are both produced by a species of Cephalosporium.

Penicillins (I) are N-acyl derivatives of 6-amino-penicillanic acid (6APA) (II)



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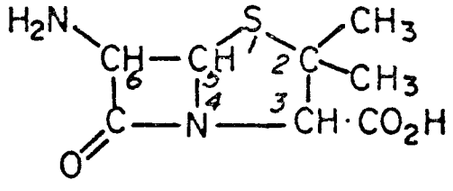


II

A series of penicillins with different non-polar side-chains can be obtained from Penicillium chrysogenum by the addition of appropriate side-chain precursors to the culture medium. Benzylpenicillin (I,R = $C_6H_5CH_2$) was the first member of the family to be used widely in medicine.

Microorganisms which produce penicillins and cephalosporins

Penicillins have been reported to be produced by several species of Aspergillus (Dulaney, 1947) and also by the thermophilic fungus, Malbranchia pulchella (Rode, Foster & Schuhardt, 1947), as well as by many Penicillia. An unusual type of penicillin, with a zwitterionic side-chain derived from D-α-aminoadipic acid, is produced by members of the genus Cephalosporium (Newton & Abraham, 1954; Abraham, 1962). This substance was first named cephalosporin N, but is now known as penicillin N. No other penicillins appear to be produced by the organisms concerned. Penicillin N was first characterized as a product of a Cephalosporium sp. resembling Cephalosporium acremonium which had been isolated by Brotzu in Sardinia in 1948 (Abraham, Newton, Crawford, Burton & Hale, 1953). Cephalosporium salmo-synnematum was reported in 1949 to produce an antibiotic named synnematin (Gottshall, Roberts & Portwood, 1949). Abraham, Newton, Crawford, Burton & Hale (1953) suggested that the major component of synnematin (Olson, Jennings, Pisano & Junek, 1954) was identical with cephalosporin N, and a comparison of the chemical and biological properties of the two products proved this to be so (Abraham et al., 1955).

In 1957 Grosklags & Swift reassigned Cephalosporium salmosynnematum to the genus Emericellopsis, as a new species, after the perfect stage of the organism had been found. Emericillopsis belongs to the Class Ascomycetes, and the Order of Eurotiales, but the conidia are like those of the Aspergilli and Penicillia. Confusion undoubtedly arose because there are no recognisable sex organs formed in Emericillopsis, and the peridium formed after reproduction is difficult to identify. In 1962 Pisano, Fleischman, Littman, Dutcher & Pansy reported that cephalosporin N was also produced by Paecilomyces persicinus. The Penicillium, Cephalosporium and Paecilomyces spp. are Fungi Imperfecti, since a sexually reproductive phase of their life cycle has not been found (Alexopoulos, 1962). They reproduce characteristically by means of conidia borne on conidiophores or by budding. Within the Form-Class Deuteromycetes all three genera belong to the Order Moniliales (Ainsworth & Bisby, 1962) and in this Order Paecilomyces shows a close structural resemblance to Penicillium.

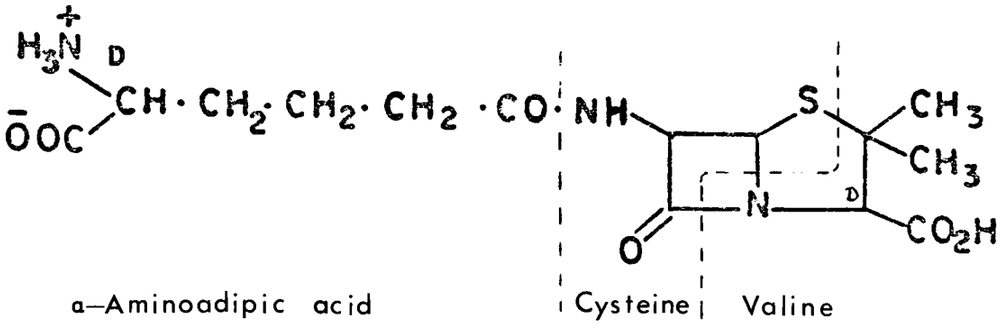
The Cephalosporium sp. isolated in Sardinia produces several antibiotics in addition to Penicillin N. The development of the work with this organism is described in the following section.

The cephalosporins

Brotzu found that the species of Cephalosporium which he had isolated in Sardinia inhibited the growth of several pathogenic bacteria on solid medium but concluded that he lacked resources to characterize the antibiotic concerned. He sent a culture of the Cephalosporium to Sir Howard Florey in Oxford where it was grown in the laboratory, and later on a larger scale at the Medical Research Council's Antibiotics Research Station, Clevedon. In the first experiments a series of related antibiotics were found in the culture fluid which were solvent - extractable and different from the active principle described by Brotzu. They were designated cephalosporins P_1 - P_5 (Burton and Abraham, 1951) since they were active only against gram-positive bacteria. These substances were shown to be acidic steroids.

In 1953, Abraham, Newton, Crawford, Burton & Hale reported that the substance whose action Brotzu had observed was "a new type of penicillin". This compound remained in the culture fluids after the extraction of cephalosporin P, and was called cephalosporin N because it showed anti-bacterial activity against gram-negative as well as gram-positive bacteria. The isolation of cephalosporin N was

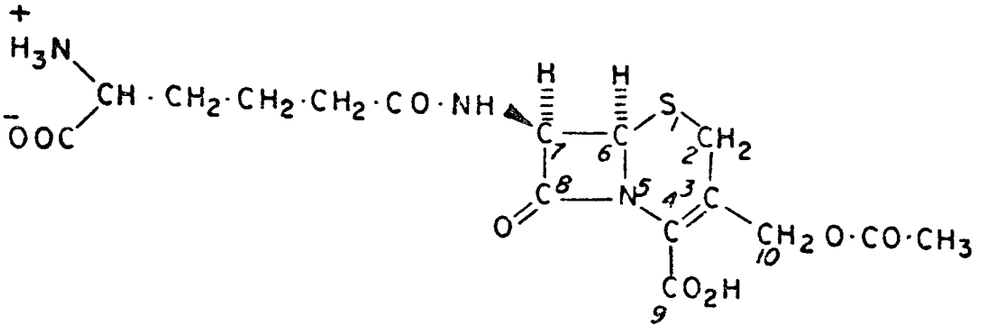
described by Abraham, Newton & Hale in 1954. The antibiotic was labile and hydrophilic. Chemical studies showed that it yielded penicillamine (β-thiolvaline) on acid hydrolysis and that it contained the typical penicillin nucleus. On vigorous hydrolysis with 6N-HCl, D-α-aminoadipic acid was liberated and the latter was shown to be linked to the rest of the molecule through its δ-carboxyl group. The D-isomer of this amino acid had not previously been found in Nature (Newton & Abraham, 1953). Cephalosporin N, later called penicillin N (Trown, Abraham, Newton, Hale & Miller, 1962) has the structure (III).



III

During the purification of penicillin N Newton & Abraham (1955) isolated a second antibiotic which was acid-stable at pH 3 and showed strong U.V. absorbence with λ max. at 260 nm. This substance was named cephalosporin C. Like penicillin N, it gave one mole of D-α-aminoadipic acid on hydrolysis, but it yielded no penicillamine and thus did not contain the characteristic penicillin nucleus (Abraham

& Newton, 1956). However, it showed an infra-red absorption band similar to that of the C = O bond in the lactam of the β-lactam-thiazolidine ring system of the penicillins and acted as a competitive inhibitor and inducer of the enzyme penicillinase. These properties suggested that cephalosporin C was structurally related to penicillin N. The results of further chemical work enabled structure IV to be proposed (Abraham & Newton, 1961) and this structure was confirmed by an X-ray crystallographic analysis (Hodgkin & Maslen, 1961).



IV

The antibacterial spectrum of cephalosporin C was similar to that of penicillin N, but its activity was about one tenth of that of the latter *in vitro* against a strain of *Staphylococcus aureus* which did not produce penicillinase and against *Salmonella typhi*. However, *Vibrio cholerae* and *Alkaligenes faecalis* A.T.C.C. 8750 (Claridge & Johnson, 1962), which were later used as test organisms, were about

ten times as sensitive to cephalosporin C as to penicillin N. Unlike penicillin N, cephalosporin C was resistant to penicillinase from Bacillus cereus and Staphylococcus aureus.

Brotzu's original organism did not produce as much cephalosporin C as penicillin N, but mutant 8650, obtained at the Antibiotics Research Station, Clevedon, by irradiating the original organism with U.V. light, produced more cephalosporin C and approximately equal amounts of both antibiotics.

Studies on the biosynthesis of penicillins and cephalosporins with whole cells

Relationship to the growth and life of the organism

In studies with P. chrysogenum Pirt & Righelato (1967) analysed growth and penicillin production curves from batch and continuous culture fermentations, and found that cell growth and antibiotic synthesis are not necessarily separated, as had previously been thought. They maintained the fungus on a minimal supply of glucose, which was just enough to keep it growing. When this ration was withdrawn, the rate of decay of penicillin synthetic activity was inversely related to the original growth rate, since the rate of antibiotic production decreased more slowly if the initial growth rate of the culture was high.

Amino acid analysis of cell fractions obtained after Cephalosporium sp. 8650 had been incubated in the presence

of [^{14}C]-α-aminoadipic acid showed that the labelled α-aminoadipic acid was only present in the intracellular pool, although lysine formed from [^{14}C]-α-aminoadipic acid was present in the cell wall (Smith, Warren, Newton & Abraham, 1967; Warren, Newton & Abraham, 1967a). From these analyses it seemed unlikely that penicillin N or cephalosporin C played a structural rôle in the organism. This is consistent with the results of studies with certain bacterial peptide antibiotics (Snoke, 1964; Brenner, Gray & Paulus, 1964; Bhattacharyya & Bose, 1967), which have disproved an earlier suggestion that such substances are incorporated into the spore coat of the producing organisms (Bernlohr & Novelli, 1963).

The reason for the apparent genetic linkage between antibiotic production and sporulation in B. subtilis remains obscure (Schaeffer, 1969).

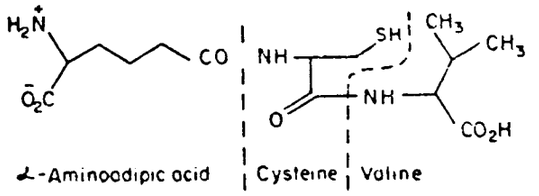
Precursors and intermediates

Penicillin N can be formally dissected into the amino acids D-α-aminoadipic acid, L-cysteine and D-valine (Structure III). In penicillin G (benzylpenicillin), which is produced by P. chrysogenum, the D-α-aminoadipic acid side-chain is replaced by phenylacetic acid. Arnstein & Grant (1954a) provided strong evidence that valine was the precursor of the penicillamine moiety of benzylpenicillin and that cystine was incorporated into the β-lactam ring.

Also (1954b) they showed that L-cystine was a more efficient precursor of penicillin than the D-isomer. Similarly, Arnstein & Clubb (1957) showed this to be true of L-valine also, and Arnstein & Margreiter (1958) reported the same finding even in strains which were more permeable than the original strain to D-valine. Inversion of L-valine to D-valine was assumed to occur during cyclisation to form the thiazolidine ring (Arnstein & Crawhall, 1957; Birch & Smith, 1958). Arnstein & Morris (1960ab) synthesised the dipeptides L-cystinyl-L-[^{14}C-carboxy]-valine and L-cystinyl-D-[^{14}C-carboxy]-valine and examined the efficiency with which these dipeptides acted as precursors of benzylpenicillin when added to suspensions of washed mycelium of P.chrysogenum in phosphate buffer. L-[U-^{14}C]-valine was added to a third suspension to compare its incorporation into antibiotic and protein with that of the peptides. There was no detectable uptake of the D-valine containing peptide and no radioactivity from it was incorporated into penicillin or mycelial protein. The mycelium appeared to incorporate more [^{14}C] from the LL-dipeptide into benzylpenicillin than into protein; and since the occurrence of cystinyl-valine bonds are rare in protein sequences, the radioactivity found in the protein was presumed to result from the hydrolysis of the peptide. On the other hand, more [^{14}C] from L-[U-^{14}C]-valine was found

in protein than in antibiotics. From these facts it was concluded that the LL-peptide was used selectively for antibiotic synthesis without previous hydrolysis. However, the specific radioactivity of the extracellular antibiotic was significantly lower than that of the extracellular dipeptide. Arnstein & Morris supposed that the specific radioactivity of the intracellular dipeptide would be equal to that outside the cell, because the amount of labelled dipeptide used could be assumed to be large in comparison with any unlabelled dipeptide inside the cell. Hence the relatively low specific radioactivity of the antibiotic was attributed to intracellular hydrolysis, resulting in the incorporation of free unlabelled and labelled valine into antibiotic. This suggested the existence of an alternative biosynthetic pathway which did not involve the dipeptide.

Following up these results, Arnstein, Artman, Morris & Toms (1960) began to look for L-cysteinyl-L-valine containing peptides in P. chrysogenum. Early work suggested that glutamylcysteinylvaline was present in the mycelium, but it was then found that glutamic acid was a contaminant of the tripeptide δ-(α-aminoadipyl)cysteinylvaline (V).



V

The latter was isolated in its sulphonic acid form, but the optical configurations of its amino acids were not determined. Arnstein et al. (1960) suggested that δ-(α-aminoadipyl)cysteinylvaline might be involved in penicillin N biosynthesis by Cephalosporium sp. because the amino acid sequence and δ-linkage of α-aminoadipic acid are similar in both compounds. They also suggested that penicillin N might be a precursor of the penicillins produced by P. chrysogenum.

The later discovery by Flynn et al. (1962) of isopenicillin N (containing an L-α-aminoadipyl side-chain) in P. chrysogenum fermentation broths and the finding of Warren, Newton & Abraham (1967a, b) that a peptide containing α-aminoadipic acid, cysteine and valine was present in the intracellular pool of the Cephalosporium sp. and was rapidly labelled with [^{14}C] from L-[^{14}C]-α-aminoadipic acid and L-[^{14}C]-valine were consistent with biosynthetic pathways of this kind in both organisms.

Arnstein & Morris (1960) suggested that the initial reaction in penicillin biosynthesis may be the condensation of α-aminoadipic acid and L-cysteine to form a dipeptide, with which valine would condense by a mechanism analogous to that in glutathione formation. In experiments with whole cells of the Cephalosporium sp. and with cell-extracts obtained by ultrasonic treatment and grinding with alumina,

Loder, Abraham & Newton (1969) studied the ability of δ-(α-aminoadipyl)-L-cysteine to act as a precursor of δ-(α-aminoadipyl)cysteinylvaline and of penicillin N and cephalosporin C.

δ-(L-α-aminoadipyl)-L-cysteine and the corresponding D- and DL-α-aminoadipyl isomers were synthesised. With whole cells δ-(L-α-aminoadipyl)-L-cysteine disappeared from the medium more rapidly than the D-isomer, but hydrolysis appeared to occur near the cell surface and little, if any, of the intact dipeptide entered the cell. When the dipeptides were incubated with the cell-extracts, hydrolysis, especially of the L-isomer, occurred. The hydrolysis of the dipeptides by whole cells made their rôle as precursors difficult to evaluate.

The possibility that penicillin N is a precursor of cephalosporin C has also been investigated. The conversion of phenoxymethylpenicillin sulphoxide methyl ester to cephalosporin derivatives has been achieved chemically (Morin et al., 1963, 1969). However, when [^{14}C]-penicillin N (labelled from [^{14}C]-valine) was added to a suspension of the Cephalosporium sp. it was not taken up to a detectable extent (Warren, 1964) and no evidence was obtained that any of it was converted to [^{14}C]-cephalosporin C.

Biosynthetic studies with whole cells have thus encountered permeability problems. These problems are not

only connected with low or insignificant rates of uptake of peptides, but also with the selective behaviour of cell walls and membranes for different optical isomers of single amino acids. For example, Warren, Newton & Abraham (1967a, b) found that the L-isomers of α-aminoadipic acid and valine were taken up much more rapidly than the D-isomers by washed suspensions of the Cephalosporium sp.. In cell-free systems such cell wall and membrane barriers do not exist and a more precise assessment of experimental results may be made.

Preparation of cell-free systems from micro-organisms

Disruption of microbial cells

Cell-free preparations have been obtained from various microorganisms which retain many of the biosynthetic abilities of the whole cell, and specific enzymes have been isolated from them without loss of activity. Having reviewed the methods available for obtaining such extracts, Gunsalus (1955) discussed their advantages and limitations.

Three ways of breaking microbial cells to obtain extracts and soluble enzymes are by damaging the cell membranes with certain organic solvents, by mechanical rupture and by specific enzymic digestion of the cell wall. During any of these procedures a certain amount of damage to the synthetic system is likely. For example, autolysis can lead to the destruction of a required fraction. Treatment

with organic solvents such as acetone, which damages permeability barriers and disrupts intracellular membranes, is often accompanied by the inactivation of certain enzymes. Important co-factors can also be reduced to an ineffective concentration during subsequent dilution with buffer.

Mechanical procedures have been very successful in the isolation of a number of soluble enzyme systems. The biosynthesis of several peptides, e.g. glutathione and gramicidin S, has been studied with soluble bacterial enzymes which were obtained by grinding damp-dry cells with abrasives such as sand, alumina and ballotini beads. Roberts, Strominger & Söll (1968) describe the preparation of a particulate enzyme fraction capable of incorporating amino acids into bacterial wall cell peptido-glycan. The extract was obtained by grinding bacteria with alumina, and the particulate matter washed from the alumina contained the active enzymes. Also included in this category are methods which depend on the mechanical breakage of cells by ice crystals as in the Hughes Press (1951). Shearing forces such as those produced by ultrasonic vibration can be effective in some cases, but in others the cells are very refractory and prolonged sonication can lead to the loss of enzyme activity.

For some enzyme systems (e.g. for those involved in the biosynthesis of bacitracin A) these methods proved too

drastic and a gentler procedure was used. This depended on the enzymic removal of the bacterial cell wall with muramidase (Ishihara, Sasaki & Shimura, 1968). Lysozyme (muramidase) was used by Fujikawa, Suzuki & Kurahashi (1966) to prepare protoplasts from B. brevis for studying the biosynthesis of tyrocidine. After the cell wall has been removed, the stabilised protoplasts which are left can be disrupted by osmotic shock or by abrasive methods. McQuillen (1960) has described both the preparation of bacterial protoplasts and some of their physiological properties.

Many biosynthetic systems have been studied successfully in cell-free systems from bacteria. In comparison, investigations with fungal cell-free extracts are scarce.

Fungal protoplasts

One method of obtaining cell-free extracts from fungi is via the preparation of protoplasts by enzymic digestion of the cell wall. The protoplasts can be disrupted under mild conditions to give particulate and supernatant fractions which can serve as starting materials for further fractionation.

Villanueva (1966) reviewed the scope of studies on fungal protoplasts. The cell wall of a filamentous fungus is a rigid tube composed of several layers. Kreger (1954) showed by X-ray diffraction that the cell wall of P. notatum contained chitin and glucan. The cell walls of P. digitatum

Sacc and P. italicum Whem were degraded chemically by Grisaro, Sharon & Barkai-Golan (1968). They showed that D-glucose contributed 50% of the total weight of the wall and that other major neutral sugars were D-galactose and mannose. Glucosamine was found after acid hydrolysis and was presumed to be a degradation product of chitin. After the chitin had been removed, the cell walls lost their gross structure and the implication was that chitin was responsible for cell wall rigidity. Protein accounted for only a small percentage of the total wall weight. Chitin accounted for 42% of the cell wall of P. chrysogenum, according to Troy & Koffler (1969), while galactose and mannose were present in amounts corresponding to 4 and 8% respectively.

Since the penicillia and Cephalosporium sp. belong to the same order of fungi, one would expect some similarity in cell wall composition. Lipid composed about 4% of the total cell wall weight of Penicillium sp. It was reported to be present in smaller quantities in the walls of Cephalosporium acremonium (Corda), which contained 1.7% lipid at 24 hours and 0.61% at 96 hours in shake flask cultures (Huber & Redstone, 1967). When cultures of Cephalosporium sp. mutant 8650 were stained for lipid with Sudan III, the cell wall appeared to be covered with a layer of stainable material which apparently increased with the

age of the culture (Smith, Warren, unpublished results). After high speed centrifugation of a preparation obtained by homogenisation and ultrasonic treatment of the organism, a white lipid layer is observed and this is a further indication of a mycelium with a high fat content (Duncan, unpublished results).

Protoplasts have been formed by the digestion of the fungal cell wall with an enzyme preparation obtained from the crop of the snail Helix pomatia (Villanueva, 1966). This extract is a complex mixture of over 30 enzymes (Holden & Tracey, 1950). These enzymes include cellulase, chitinase, laminarinase, glucosidases, galactosidases, lipase and glycerophosphatases. The complex was separated into three active fractions on Sephadex G-100 (Anderson & Millbank, 1966), and the activity of these fractions was assayed on isolated yeast cell walls and on intact cells. One fraction was responsible for liberating N-acetyl glucosamine, mannose and glucose; in addition, this fraction was also capable of solubilising the cell wall protein and effective in releasing protoplasts from whole cells. The other two fractions liberated glucose alone from isolated cell walls. It was implied that only when mannose was liberated from the cell wall would its rigidity be lost and protoplasts be formed; and it was suggested that in yeast cell walls which were resistant to dissolution the mannose fragment was situated

in the inner layers of the wall, and thus inaccessible to the enzyme.

According to Eddy & Williamson (1957), variability in resistance to the action of the enzyme mixture is also shown between yeast cells of the same strain, but of different age, and these workers have emphasised the importance of using young cells for protoplast formation. Davies & Elvin (1964) found that a pretreatment of yeast cells with 2-mercaptoethanol made the walls more susceptible to the action of the enzyme, and suggested that mercapto-ethanol may reduce disulphide bridges in protein complexes in the cell wall.

Protoplasts are osmotically sensitive, but are stabilised in an isotonic medium. However, osmotic sens-itivity does not exclude the possibility that some cell wall material is still present. Bacon, Jones & Ottolenghi (1969) have shown that, even on prolonged incubation in snail enzyme solution, yeast protoplasts still retain some cell wall material. The observation that yeast protoplasts are capable of regenerating their cell wall to a limited extent in liquid medium, and completely when embedded in a solid matrix like gelatin, indicates the persistence of material which later acts as a primer for cell wall resynthesis (Nečas & Svoboda, 1967).

Quantitative biochemical data on yeast and fungal

protoplasts are scarce. Longley, Rose & Knights (1968) studied the chemical composition of yeast protoplast membranes, and related their results to similar studies on other cell membranes. They found protein, lipid and sterol as major components, as well as traces of RNA and carbohydrate (glucan and mannan).

Proof that fungal protoplasts are capable of biosynthetic activity was provided by Trevithick and Metzenberg (1964). They showed that Neurospora crassa protoplasts secreted invertase over a 12 hour period of incubation, in amounts which roughly followed the amount of protein excreted over this time period. However, the amount of invertase found inside protoplasts during this time remained constant, showing that the enzyme was synthesised by the protoplasts and that its extracellular appearance was not due to net leakage from the cells.

Heick and Stewart (1965) have compared the metabolic properties of complete cells and protoplasts of Lipomyces lipofer. The latter were able to utilise mannitol as a substrate for respiration, which whole cells could not, but the authors suggested that this might be the result of prolonged incubation in mannitol stabiliser. Their results also showed that the sediment obtained from lysed protoplasts required extensive supplementation with co-factors to maintain respiration. In addition to ATP and Mg^{++}, NAD and

cytochrome C were needed, presumably because of the dilution of these factors below the optimal value in the reaction mixture.

Biosynthesis of peptide antibiotics in cell-free systems

General characteristics

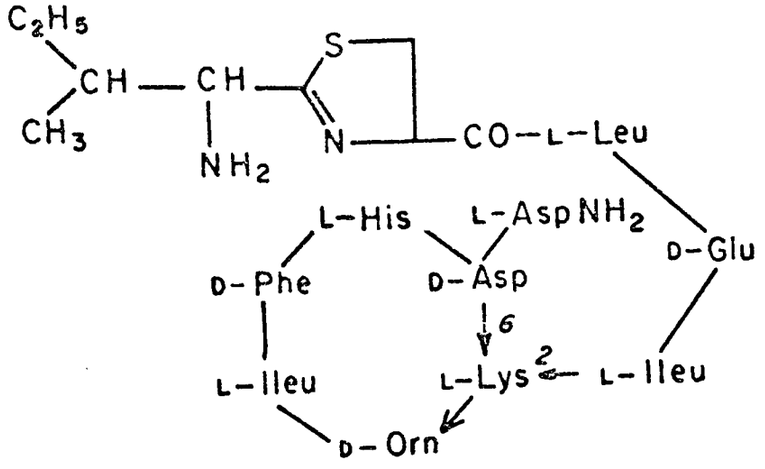
Studies of the biosynthesis of peptide antibiotics in bacterial cell-free extracts have encountered several common problems, some of which also arise in connection with the biosynthesis of the penicillins and cephalosporin C.

Peptide antibiotics usually contain one or more D-amino acid residues, but free D-amino acids are used less readily as precursors of these residues than are L-amino acids. The peptides often contain amino acids of a type which are not normally found in proteins (e.g. D-$\alpha\gamma$-diamino-butyric acid in polymyxin B) and sometimes a cyclic structure involving two or more amino acids. Their biosynthesis does not involve transfer RNA or ribosomes and thus differs in mechanism from that of proteins. There have been few reports of free intermediates isolated from cell-free extracts, and the relevance of those obtained is questionable. Biosynthesis may therefore occur on a specific enzyme complex before secretion of the complete antibiotic into the medium. Some of these findings are described below in more detail.

Bacitracin, Gramicidin S and Malformin

The bacitracins are a group of cyclic peptides, pro-

duced by B. licheniformis, of which bacitracin A (VI) is the main component.

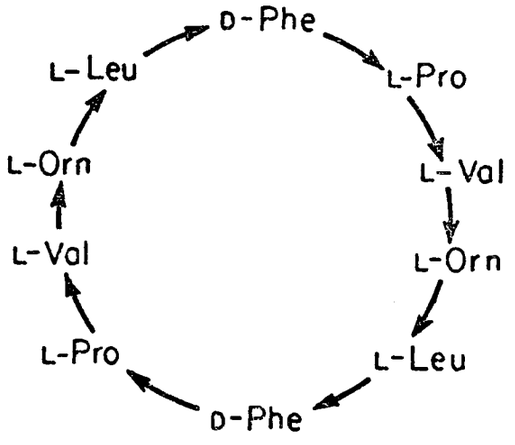


VI

The D-amino acids aspartic acid, phenylalanine and glutamic acid, which are present in bacitracin A, were inhibitory to antibiotic synthesis. Shimura, Sasaki & Sugawara (1964) demonstrated that cell-extracts from sonically disrupted cells could produce antibiotic and incorporate cysteine and isoleucine actively into the molecule. However, lysine was incorporated only very slightly, indicating that not all the biosynthetic mechanisms were operating efficiently. Ishihara, Sasaki & Shimura (1968) prepared an extract from protoplasts of B. licheniformis by use of muramidase and found that this preparation incorporated L-amino acids into bacitracin in

the presence of an ATP-generating system, whereas D-amino acids were inhibitory. RNAase, chloramphenicol or puromycin did not affect the biosynthesis of bacitracin A.

B. brevis produces gramicidin S (VII), a cyclic decapeptide consisting of two identical pentapeptide chains.

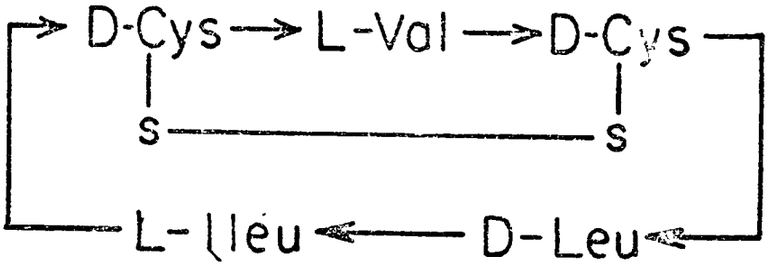


VII

Initial reports (Mach, Reich & Tatum, 1963; Eikhom, Jonson, Laland & Refsvik, 1963) showed that antibiotic synthesis by growing cells was insensitive to chloramphenicol, actinomycin D and puromycin. Experiments by Bodley, Uemura, Adiga, Okuda & Winnick (1964) and by Hall, Sedat, Adiga, Uemura & Winnick (1965) implicated a ribosomal system as a requirement for synthesis, but this claim was later withdrawn (Bhagavan et al. 1966). D-phenylalanine appears to be the starting point for synthesis (Kleinkauf, Gevers & Lipmann, 1968; Bredesen, Zimmer & Laland, 1969); L-phenylalanine is activated by ATP, attached to the enzyme system as an

adenylate, and racemised during the transfer of the amino acid adenylate to another site on the enzyme complex (Gevers, Kleinkauf & Lipmann, 1968). The other amino acids are activated and presumably transferred to adjacent sites in sequence, when peptide bond formation occurs. Tetrapeptides have been isolated from extracts by Holm, Frohølm & Laland (1966) and Tomino, Yamada, Itoh & Kurahashi (1967), but their role in the biogenesis of the decapeptide has not been defined.

The antibiotics discussed so far are all bacterial in origin but similar studies with cell-free extracts have been extended to peptides which are produced by fungi. Malformin, a cyclic pentapeptide antibiotic, is produced by Aspergillus niger; malformin A, the principal component, has the structure (VIII).



VIII

Yukioka & Winnick (1966) have demonstrated the biosynthesis of this compound in cell-free extracts obtained by breaking

the mycelium in a French press. They found that the active enzyme preparation synthesised antibiotic in the presence of inhibitors of protein synthesis. Labelled L- as well as D-[^{14}C]-leucine was used as a precursor of the D-valine residue of the peptide. Competition was demonstrated between L-[^{12}C]-leucine and D-[^{14}C]-leucine but it was also shown that excess D-[^{12}C]-leucine was unable to inhibit the incorporation of L-[^{14}C]-leucine. This led to the conclusion that the enzyme had a greater affinity for L-leucine than for the D-isomer. The inversion of free D- to L-leucine seemed unlikely, as this would have resulted in a dilution of L-[^{14}C]-leucine by the unlabelled L-leucine (from excess D-[^{12}C]-leucine).

Early studies with cephalosporins and penicillins

In 1963 Demain reported that cephalosporin C was synthesised in a broken cell system obtained by 60 minutes sonication of a washed suspension of Cephalosporium cells; after an initial 3 hour lag, the amount of antibiotic synthesised was comparable with that accumulated by ordinary washed cells. An attempt to fractionate the sonicate by centrifugation at 1000 g. for 5 min. revealed that both the pellet and the supernatant were required for efficient synthesis. Smith, Warren, Newton & Abraham (1967) attempted to repeat these results. They found that after 30 minutes sonication the synthesising system of the whole preparation

was greatly impaired and that antibiotic synthesis could only be detected after a 10 - 12 hour lag. After this time the rate of production increased rapidly, but this was accompanied by regrowth of surviving cells and the uptake of amino acids which had been liberated during sonication.

So far a system capable of the total biosynthesis of penicillins and cephalosporins has not been obtained; however, several groups of workers have made active cell-free extracts of P. chrysogenum from which enzymes have been isolated which may take part in the final stages of penicillin biosynthesis.

There have been many reports of enzymes capable of removing the side-chain from penicillins. Sakaguchi & Murao (1950) obtained 6-APA from benzylpenicillin by enzymic hydrolysis of the side-chain with an amidase from a strain of P. chrysogenum. Batchelor, Doyle, Nayler & Rolinson (1959) isolated 6-APA from P. chrysogenum fermentations to which side-chain precursors had not been added. However, the role of 6-APA in penicillin biosynthesis was not clear. With the subsequent isolation of isopenicillin N from P. chrysogenum (Flynn, McCormick, Stamper, De Valeria & Godzeski (1962) and Cole & Batchelor (1963)) it seemed that isopenicillin N might be a precursor of the benzylprnicillin produced by this organism and directly of

penicillin N in the Cephalosporium sp.. Sakaguchi & Murao's experiments thus suggested that 6-APA might represent the product of a later step in the reaction sequence, its formation depending on the susceptibility of its former side-chain to hydrolysis and its accumulation on the availability of side-chain precursors.

Vanderhaeghe, Claesen, Vleitinck & Parmentier (1968) isolated an acylase from acetone-dried P. chrysogenum. The enzyme was able to cleave several penicillins into 6-APA and the component aliphatic side-chain, the rate of cleavage increasing with the length of the side-chain. Two penicillins with polar side-chains, penicillin N and isopenicillin N, were not hydrolysed. The inactivity of the acylase with these substrates was attributed to the free carboxyl group of the side-chains since, after this carboxyl group had been esterified, cleavage of the side-chain occurred. If isopenicillin N is concerned in benzylpenicillin formation, its failure to be hydrolysed by the acylase of Vanderhaeghe et al. raises the question whether this enzyme is involved in the biosynthesis. Gatenbeck & Brunsberg (1968) suggested that the acylase has a broad specificity and that penicillins are not its main substrates. They reported that an acyltransferase was present in extracts prepared from sand-ground P. chrysogenum, and was responsible for the transfer of acyl side-chain pre-

cursors (as their Co-A derivatives) to 6-APA. Spencer & Maung (1970) were unable to separate the acylase and acyltransferase activities and suggested that they were both properties of a single thiol-dependent enzyme.

AIMS AND SCOPE OF THE PRESENT INVESTIGATION

AIMS AND SCOPE OF THE PRESENT INVESTIGATION

In the work described in this thesis the main objective was to obtain a cell-free extract from Cephalosporium sp. C-91 which would retain some of the antibiotic-synthesising ability of the intact mycelium. It was decided to approach this objective in the following manner:

1. To obtain information about the phase of the culture in which penicillin N and cephalosporin C were produced at maximal rates, and about the morphology of the mycelium in this phase.
2. To attempt to prepare protoplasts from this type of mycelium and study their ability to produce antibiotics in the presence and absence of possible precursors.
3. To determine whether cell-free extracts could be obtained from protoplasts which would carry out some or all of the steps involved in antibiotic synthesis.

MATERIALS AND METHODS

MATERIALS AND METHODS

General chemicals were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, and were of the "Analar" grade. 2-Mercaptoethanol was from Koch-Light Laboratories, Colnbrook, Bucks., and was stored under nitrogen while not in use; Cleland's Reagent (A grade) was received from Calbiochem, Los Angeles, California, and was kept at 4^{o}. Nuchar, an activated vegetable carbon, was obtained in granular form from Industrial Chemical Sales Division, West Virginia Pulp & Paper Company, 239 Park Avenue, New York City 17, N.Y., U.S.A. The cation exchange resin, Dowex 50 x 4 (H^{+} form) was manufactured by the Dow Chemical Company, Midland, Michigan, U.S.A.

Culture. Cephalosporium sp. strain C-91 (MF-4239) from Dr. A.L. Demain (Merck & Co., Inc., Rahway, N.J.), is a mutant of Cephalosporium sp. CMI49137 which is unable to utilize nitrate as a nitrogen source.

Maintenance of cultures. This was essentially the same as that described by Trown et al., 1962. The contents of a freeze-dried ampoule of a submaster culture of the Cephalosporium sp. CMI49137 mutant C-91 were mixed with a little nutrient broth. Loopfuls of this suspension were then used to inoculate 1 oz. Oxoid nutrient agar

slopes in 30 ml. screw cap bottles. These were incubated for 7 days at 25°C, and the mycelium which had grown used to inoculate slopes of 1/10th strength LePage & Campbell medium (1946), which was solidified with 2% (w/v) agar. The slopes were incubated for 14 days at 25°C and could be stored for up to a month at 4°C.

Growth of the fungus for antibiotic production. The mycelium, which was washed off the LePage slope with 5 ml. of water containing glass beads, was used to inoculate 100 ml. of Medium A in 500 ml. high-baffled Erlenmeyer flasks (Smith, Warren, Newton & Abraham, 1967). This medium consists of cornsteep liquor 27.2 g., ammonium acetate 4.4 g., sucrose 20 g., tap water *1* l. The pH was adjusted to 6.5 - 6.8 with NaOH before autoclaving at 15 p.s.i. for 20 min. The fungus was grown for 72 hr. in this medium, at 140 rev./min., on a reciprocating shaker with a 2" throw, 27°. The shaker was enclosed in a box through which was blown a slow stream of air. At this stage the inoculum consisted of mycelium, submerged spores and germinating spores. This heterogeneous culture was used to inoculate 100 ml. of fermentation Medium-19D (10% v/v). Medium 19-D is chemically defined (Demain, 1963) with the addition of L-asparagine in place of ammonium sulphate.

Medium 19-D has the composition

Salts 11A	KH_2PO_4	115 g.
	K_2HPO_4	156 g.
	Na_2SO_4	5.6 g.
	$MgSO_4.7H_2O$	1.3 g.
	$ZnSO_4.7H_2O$	220 mg.
	$MnSO_4.H_2O$	220 mg.
	$CuSO_4.H_2O$	55 mg.
	$CaCl_2$	425 mg.

Deionised water to *1* l.

Salts 10B	$Fe(NH_4)_2(SO_4).6H_2O$	20 g.

Deionised water to *1* l.

Medium	Salts 11A	135 ml.
	Salts 10B	7.5 ml.
	DL-methionine	5 g.
	Sucrose	36 g.
	Deionised water	758 ml.

pH adjusted to 7.3 - 7.5 with NaOH

	L-asparagine	7.5 g.

90 ml. of the medium were added to a 500 ml. Erlenmeyer flask with low baffles (Smith, Warren, Newton & Abraham, 1967) and 0.1 ml. oleic acid was added. Flasks containing

the medium were autoclaved at 15 p.s.i., for 20 min. and 10 ml. of a 27% (w/v) solution of glucose was added to each 90 ml. aseptically. The mycelium was incubated in this medium usually for 72 hr.; maximum titres of antibiotic were attained over a 72 - 96 hr. period.

pH measurements. All pH's were measured on an E.I.L. Model 23A Direct Reading pH meter (Electronic Instruments Limited, Richmond, Surrey). This was fitted with an E.I.L. GM23 glass electrode and a K130 Calomel electrode (Radiometer, Copenhagen).

Preparation of spore inocula. Two flasks of culture growing in Medium-A were taken off the shaker at 60 hr. Morphological examination showed that spores were either free in the medium or being formed at the end of sporangia. None of the spores observed appeared to be germinating. The culture was poured through a Millipore filter unit containing a circle of coarse stainless steel mesh (5 cms. diameter, grade 5.5, hole diameter 140 μ). The culture was stirred with a spatula to prevent the filter becoming blocked, and the rate of flow decreased until it became dropwise by adjusting the amount of air passing through a capillary tube attached to the side-arm of a Buchner receiving flask. When approximately 100 ml. of culture had

been passed through one filter unit, the rest was passed through another of the same type to ensure that spores were not lost due to the decreasing efficiency of a blocked filter.

The resulting filtrates were then passed through a finer stainless steel filter (85 μ mesh). The filtrate obtained was divided between 8 McCartney bottles and centrifuged on a Mistral 2L centrifuge with a swing-out head at 1,625 g for 10 min. at 4°C. The supernatants were discarded and the sediments were resuspended in approx. 10 ml. of Medium-19D. The suspensions were centrifuged as above and the washings discarded. The sediments were resuspended again in approx. 10 ml. of Medium-19D and the spores from 2 McCartney bottles used to inoculate 1 flask of Medium-19D. Therefore the spores from 2 flasks of Medium-A were used to seed 4 flasks of 19-D.

Measurements of dry weight. At different times during the growth of the fungus, samples (2.0 - 2.5 ml.) were withdrawn from the fermentation medium with a sterile Pasteur pipette. The volume was measured and the suspension was filtered on a weighed fritted glass sinter (No: 3 porosity). The mycelial mat was washed with an equal volume of dis-

tilled water, then heated at 110°C, 18 hrs. in an oven. After the sinter had cooled in a desiccator it was re-weighed and the dry weight in mg./ml. was calculated.

Morphological examination. Specimens of the fungus, mounted in water, were examined by phase-contact microscopy (Heineke system) at low magnification (×100), and high power (×400), on a Leitz-Wetzler Ortholux microscope and photographed with an automatic camera (Ernst Leitz, G.M.B.H., Wetzler, Germany).

Assay of antibiotics. A bioassay similar to that described by Brownlee et al. (1948) was used to measure the titres of penicillin N and cephalosporin C in culture fluids. The combined titres of penicillin N and cephalosporin C were assayed against Salmonella typhi (strain "Mrs. S"). Since this test organism is ten times as sensitive to penicillin N as to cephalosporin C the zone of inhibition measured is due, effectively, to penicillin N. The titre of cephalosporin C in culture fluids was measured against A. faecalis A.T.C.C. 8750 seeded in agar containing penicillinase (prepared in the laboratory according to the method given by Kuwabara, 1969), which inactivated the penicillin N in the culture fluids. Moreover, A. faecalis is ten times as sensitive to cephalosporin C as to penicillin N

Polypropylene petri dishes (9 cm. diam.) were filled

to a depth 3 - 6 mm. with phenol-red agar seeded with the test organism. The agar consisted of bacteriological peptone, Evans Medical Ltd., Speke, Liverpool (20 g.) NaCl (5 g.) distilled water to *1* l., Oxoid No. 3 agar (14 g.) was added to the solution and the pH adjusted to 7.4,5 ml. of 0.5% v/v phenol-red was added to *1* l. of agar.

Six equidistant holes, 8 mm. in diameter, were punched in the agar and filled with the solutions for assay.

The titres of penicillin N and cephalosporin C were estimated by comparison with a standard preparation of cephalosporin C (80% pure) which contained, by definition, 8 units of activity/mg. (Smith, Warren, Newton & Abraham, 1967).

Three holes in each plate were filled with different concentrations of the standard solution. For Salm. typhi concentrations of 4.0, 2.0 and 1.0 units/ml. were used and 0.5, 0.25, 0.125 units/ml. for A. faecalis. The three other holes were filled with the solutions containing the antibiotics whose titres were to be determined. Such solutions were usually assayed in triplicate at random positions through a series of assay plates. The plates were incubated at 37°C, for 18 hr., and the zones of

inhibition measured.

The log of the concentration of the known amount of antibiotic was plotted against average zone diameter, and the concentration of the antibiotic in the unknown solution read from this standard line.

The comparison of an unknown concentration of antibiotic in a solution against a standard solution eliminates errors which are inherent in the assay. These are: the age and density of the test organism used, the age of the assay plates, the depth of the agar and the rate of diffusion of the antibiotic. A limitation on the precision of the system is the assay of penicillin N/cephalosporin C mixtures against Salm. typhi with cephalosporin C as a standard, since there are no commercial preparations of penicillin N to replace this standard (see Smith, Warren, Newton & Abraham, 1967). This source of error becomes larger when small concentrations of antibiotic are assayed, but for titres of approximately 4.0 units/ml. the error was calculated to be less than 20%, and for assays carried out using the same batch of test plates less than 15% for penicillin N, and less than 10% for cephalosporin C.

Preparation and properties of protoplasts

Snail enzyme. The digestive juices of Helix pomatia was

from Industrie Biologique Française, Seine, France, and the liquid was stored at 4° while not in use. Aliquots (1/10th vol.) of a solution of cysteine hydrochloride (1% w/v) were added to 1.0 ml. portions of the enzyme solution to inactivate the merthiolate preservative (Marini, Arnow & Lampen, 1961). Approximately two volumes of water were added and the resulting solution was freeze-dried, giving 0.12-0.15 g. dry weight of material/ampoule of juice. The freeze-dried enzyme complex was stored at -20°.

Protoplast preparation. The fungus was grown as described previously and harvested after 48 hr. growth in Medium-19D. The mycelium was filtered from the culture fluid on Green's Hyduro 904 hardened filter paper. The cells were washed three times with equal volumes of distilled water, and then sucked dry, blotted with filter paper and weighed. The damp-dry mat was resuspended at a concentration of 1 g./5 ml. in 0.02M-2-mercaptoethanol (Davies & Elvin, 1964) or in 0.01M-dithiothreitol, made up in McIlvaine's buffer pH 7.6, and incubated on a rotary shaker (140 rev./min.) for 1 hr. at 30°C. The mycelium was washed with distilled water and re-suspended (10% w/v) in crude snail enzyme solution (40 mg./ml.) made up in McIlvaine's buffer pH 6.8 containing 0.9M-NaCl as stabiliser, and incubated at 30°C on a rotary shaker (120 rev./min.) in 50 ml. conical flasks for 3-4 hrs. The

resulting mixture of protoplasts and mycelium was centrifuged (5 min., 200 g) at room temp., and the supernatant fluid pipetted off. The sediment was washed twice with 5 ml. of 0.9M-NaCl. Protoplasts were separated from unattacked mycelium and cell wall debris by filtration through a No. 0 porosity glass sinter. The filtrate was examined microscopically and any hyphae present in the protoplast suspension were removed by filtration through a No. 1 porosity glass sinter. The protoplasts were concentrated by centrifugation (300 g, room temp., 5 min.).

Measurement of the percentage yield of protoplasts. Calculation of the yield of protoplasts from a known weight of mycelium was made from measurement of the amount of soluble nucleotides extracted with aqueous ethanol from the intracellular pool.

Protoplasts were resuspended in ice-cold aqueous ethanol (1 ml. 7:3 v/v), stored at 4°C for 5 min. and centrifuged (200 g, 5 min.) and the supernatant fluid removed.

A sample of mycelium (0.5 g. damp dry weight) which had first been preincubated in either of the reducing agents and then suspended in 0.9M-NaCl was also extracted with 70% ice-cold ethanol (1.5 ml.). The cells were kept at 4°C, centrifuged (300 g, 5 min.) and the supernatant

removed.

The absorption spectra of the ethanolic extracts obtained from protoplasts and mycelium were measured at 260 nm. The extinction at 260 nm given by the mycelial extract is directly proportional to the amount of nucleotide extracted from a known weight of cells. Hence measurement of the extinction of the extract from the protoplasts gives a measure of the percentage yield of protoplasts from a known weight of mycelium on the assumption the nucleotide content remains unchanged during protoplast formation. However, since the validity of this assumption is uncertain, the values given in this thesis for protoplast yields are likely to be low and may require future reassessment.

Sonication and osmotic lysis. Protoplasts in 2.0 ml. 0.8M-NaCl were sonicated for 30 sec., 1.0 A approx., 4°C on an M.S.E. 60 w. ultrasonic disintegrator. A titanium probe (1 cm. diam.) was used and its end was about 2 mm. below the surface of the suspension fluid.

Microscopic examination showed that the protoplasts had been disrupted. Cell membranes were separated from other particles by centrifugation (200 g, 5 min.). After the addition of amino acid precursors and an ATP-generating system, the extracts were incubated at 27°, 140 rev./min. for 2 hours.

Pellets of protoplasts were lysed osmotically by resuspension in 0.1M phosphate buffer (pH 7.0). Microscopic examination showed that none of the protoplasts had survived.

Other cell-free extracts

Preparation of alumina and sand ground cell extracts. The alumina (200-400 mesh) was obtained from Savory and Moore, London, W.1. It was washed with water and the pH of the suspension adjusted to neutrality. After filtration and washing with water, the alumina was dried at 37°C. Sand of 90-mesh (B.D.H. Ltd., Poole, Dorset) was washed with 3N-HCl, then with 2N-NaOH and finally water. It was filtered and dried at 37°C.

A known weight of 72 hr. damp-dry mycelium and an equal amount of abrasive were ground by hand in a mortar at 0°C, packed in ice. After 10 min. 1.0 ml. of 0.1M-tris buffer pH 7.0 was added. Grinding was continued for another 5 min. The paste was centrifuged at 4,500 g for 7 min., and the supernatant was removed. This supernatant was termed the crude extract. On occasion it was spun at 20,000 g and the resulting supernatant was used for experiments.

Supplements added to cell-free extracts

a) Glutathione biosynthesis. To 1.0 ml. extracts were added 20 μmole amounts of L-glutamic acid, L-cysteine.HCl

and glycine. The pH of the extracts was adjusted to 7.5-8.0.

b) ACV biosynthesis. DL-α-aminoadipic acid (11 μmoles), L-cysteine.HCl (5 μmoles) and DL-valine (17 μmoles) were added to 1 ml. samples of the sand extract of 72 hour mycelium which contained approximately 28 mg./ml. of protein. However, to the protoplast extracts DL-α-aminoadipylcysteine or the all L-isomer, DL- or L-α-aminoadipic acid, L-cysteine.HCl and valine were added at 2-4 μmoles/ml. concentrations. These precursors were added as neutralised solutions and the final pH of the extracts was adjusted to 7.0-7.5 when necessary.

δ-(DL-α-aminoadipyl)-L-cysteine, and the corresponding all L-isomer, were kindly provided by Dr. P.B.Loder. They were prepared from DL-α-aminoadipic acid or L-α-aminoadipic acid as described by Loder, Abraham & Newton (1969).

c) Antibiotic synthesis. DL-α-aminoadipic acid, L-cysteine.HCl and L-valine were added in approximately 10 μmolar amounts per ml. of extract. The pH's of the extracts were adjusted to 7.5-8.0 after the additions.

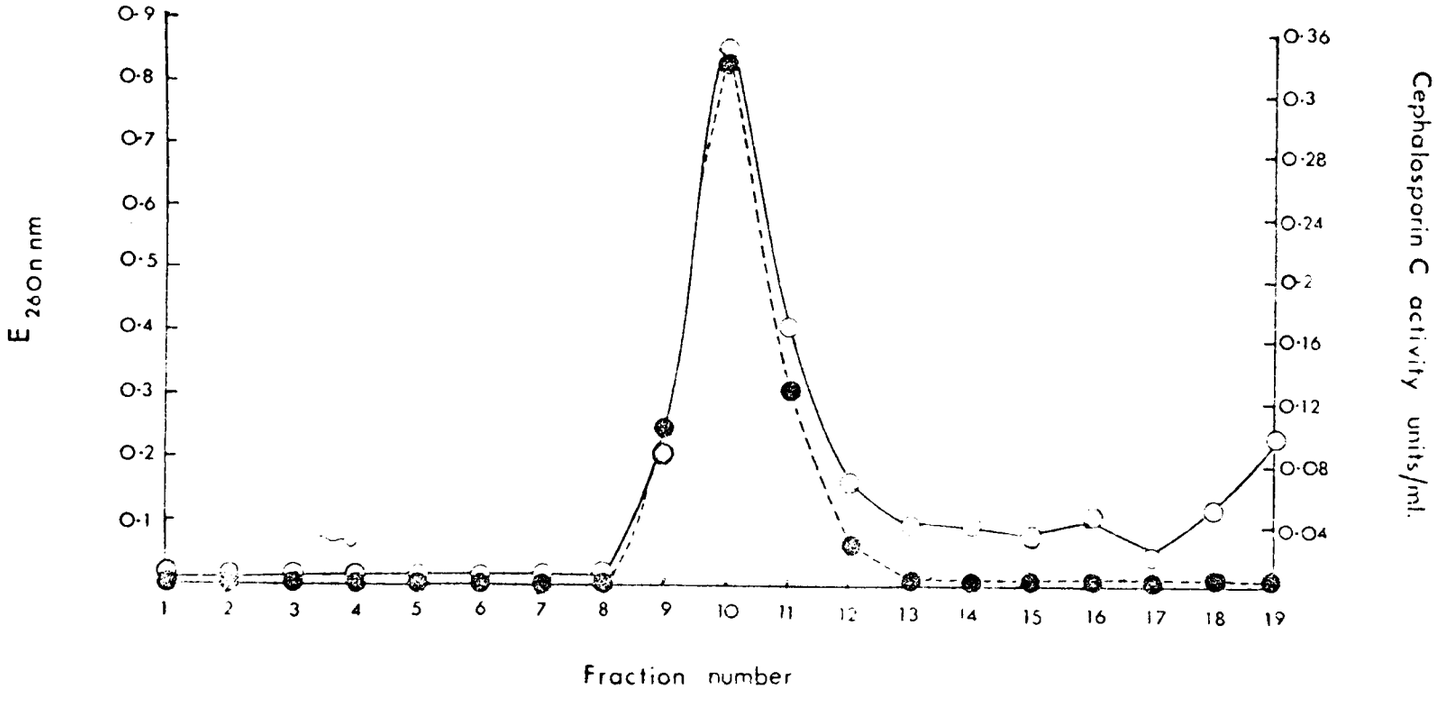
d) ATP-generating systems. To 1 ml. portions of extract were added ATP (10 μmoles), phosphenol pyruvate (15 μmoles), pyruvate kinase (100-200 μg.) and Mg^{++} (20 μmoles).

Analytical procedures

Protein estimation. The amount of protein in cell-free extracts was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of the extracellular and intracellular content of protoplasts. The concentration of potassium ions was measured with an E.E.L. flame photometer (Evans Electro-selenium Ltd., Halsted, Essex), Pat. No. 712700, Reg. Des. No. 866 150. The photometer was zeroed with distilled water and the 100% calibration was made with a solution containing potassium ions at a concentration of 2 mmolar. Samples were diluted similarly for measurement. The total intracellular potassium ion content was measured after ignition of the protoplast residue with concentrated sulphuric acid.

Fig.1 Elution Pattern of Cephalosporin C from Nuchar



●---● Antibacterial activity against A. faecalis

o——o $E_{260\ nm}$.

A sample of cephalosporin C (0.5 mg.) was dissolved in 5.0 ml. 0.9M-NaCl ($E_{260\ nm}$ 1.48; activity 0.8 units/ml.) and applied to a Nuchar column (approx. 1 x 4 cms.). The column was washed with water and percolate fractions 1-8 (2.0 ml.) were collected; cephalosporin C was eluted from the column with 50% (v/v) ethanol and fractions 9-19 (2.0 ml.) were collected. Chloride ions were present in fractions 1-8, and traces were found in fraction 9.

Desalting of culture fluids. Antibiotics in the protoplasts supernatants were separated from the NaCl stabiliser on Nuchar columns. These columns (3.5 x 1 cm. diam.) were washed with 50 ml. 50% v/v ethanol and then with 50 ml. water. The sample to be desalted was made up to 5.0 ml. with water and the extinction at 260 nm. measured before and after the addition of 0.5 mg. of cephalosporin C standard (8 units/mg. activity). Cephalosporin C was added to the sample as a marker to show when antibiotics were being eluted from the column.

The sample was applied to the column and the latter washed with 10 ml. of water. Two fractions, one of 10 ml. and the other 5 ml., were collected and their absorption spectra and total radioactivity measured. Antibiotics were eluted with 50% v/v ethanol. One 25 ml. and one 5 ml. fraction were collected and their absorption spectra and radioactivity similarly measured. The ethanol eluates were pooled and concentrated in vacuo to approx. 5.0 ml. and then freeze-dried. A typical pattern of an eluate containing cephalosporin C from Nuchar is shown in Fig.1.

Desalting of intermediates from broken-cell systems on Dowex 50 x 4 (H^+ form). The pH of cell-free extracts from protoplasts, or those obtained by grinding mycelium abrasives, was adjusted to 2 - 3 with HCl at the end of the re-

action. Precipitated protein and other insoluble materials were sedimented by centrifugation at 1,500g for 5 min. Amino acids and peptides in the supernatant (1 - 2.0 ml.) were freed from salt by absorbing them onto Dowex 50 x 4 (H^+ form) on a column (5 cm. x 1 cm. diam.). After the column had been washed with water, elution was carried out with M-pyridine. The freeze-dried eluate (1 - 5 mg.), which also included sulphur-containing amino acids and peptides, was oxidised in a 3% solution of performic acid in 98% (w/v) formic acid (0.1 - 0.5 ml.) (Hirs, 1956). The solution was kept at 0° for 6 hrs., then diluted with water (10 vol.) and freeze-dried. The residue was applied to a second Dowex 50 x 4 (H^+ form) column to separate amino-sulphonic acids and peptides from other amino acids. The percolate from this column was freeze-dried and kept for examination by paper electrophoresis and chromatography.

Examination of cell fractions. Mycelium and protoplasts were fractionated by the method described by Smith, Warren, Newton & Abraham (1967). This involved extraction of substances in the intracellular pool with 70% (v/v) ice-cold ethanol (3 ml./g. of mycelium) for 5 min. The aqueous alcohol was separated by filtration or centrifugation from the cells which were then extracted with ice-cold water

(3 ml./g. of cells) for 5 min. The cells were washed with a further 1.0 ml. of water. The ethanol and water extracts were combined and more water was added to reduce the ethanol concentration. The extracts were then reduced to a small volume by distillation in vacuo and freeze-dried.

Paper electrophoresis and chromatography. Portions of freeze-dried samples (one quarter of the total) containing antibiotics were subjected to electrophoresis on Whatman No. 1, or No. 4, paper at 70 v/cm. in an apparatus similar to that described by Katz, Dreyer & Anfinsen (1959) and in pyridine-acetate buffer (0.05 M to acetate) pH 4.5. They were chromatographed in butan-1-ol-acetic acid-water (4:1:5, by vol.) overnight, or for 6 hrs., at 0°.

After performic acid oxidation samples (one quarter or one fifth of the total) were subjected to electrophoresis on Whatman No. 1 paper in buffer at pH 1.8, 70 v/cm., for 2 hrs. The buffer consisted of a mixture of 20% (v/v) acetic acid and 2% (v/v) formic acid. Chromatograms of sulphonic acids were run in butan-1-ol-acetic acid-water (4:1:4 by vol.) for 16 hrs.

Amino acids and peptides were detected by colouration with ninhydrin solution, which contained ninhydrin and 2,4,6-collidine (each 0.16% (v/v)) in butan-1-ol (Woiwood

1949). Cephalosporin C and deacetylcephalosporin C were also detected by their absorption of ultra-violet light (Abraham & Newton, 1961). The presence of penicillin N and cephalosporin C in some samples was detected by antibacterial assay.

Radioactive compounds. L-[1-^{14}C]-glycine (specific activity 41.4 mCi/mmole), DL-[1-^{14}C]-valine (32.2 mCi/mmole), were obtained from the Radiochemical Centre, Amersham, Bucks.

Radioactivity measurements. Known volumes of solutions were counted as infinitely thin films spread on lens tissue held in aluminium planchets. When the liquid had dried, a few drops of a 0.1% solution of collodion in 1:1 mixture of ether and ethanol were applied, preventing the tissue from curling. The samples were counted in a Nuclear Chicago gas-flow detector with an automatic sample changer, decade scaler and printing timer (Models D47, 110B, 186, C111B, Nuclear Chicago Corporation, Les Plaines, Illinois, U.S.A.). Samples were counted in a gas mixture of argon (90%) and methane (10%). The counting tube was fitted with a thin mica end window and its efficiency calculated by counting a [^{14}C]-polymer reference source.

Radioactive spots on chromatograms were located by radioautography. X-ray films (Blue Brand, Kodak Ltd.) were exposed for 3 weeks. The spots were counted by a single-

end window, helium filled, Geiger-Muller detector. The counts were recorded on a linear decade scaler (Model 1800, Isotopes Development Limited, Reading).

Electron microscopy. A pellet of protoplasts or mycelium was fixed in 3.0 ml. of Kellenbergers sodium-veronal acetate (5.0 ml.), water (7.0 ml.), and 0.1 N-NaCl (7.0 ml.) (Kellenberger, Ryter & Séchaud, 1958). To the buffer were added 1.0 ml. osmium tetroxide (4% w/v), and NaCl (100 mg.). The pellet stood in the mixture for 3 hrs. at room temperature and was then washed in 0.8M-NaCl in McIlvaine's buffer pH 6.8.

Dr. D. Kay, Sir William Dunn School of Pathology, Oxford, prepared and sectioned the protoplasts for electron microscopy.

SECTION I

EXPERIMENTS RELATING TO THE GROWTH OF *CEPHALOSPORIUM* SP. C-91 IN BATCH CULTURES

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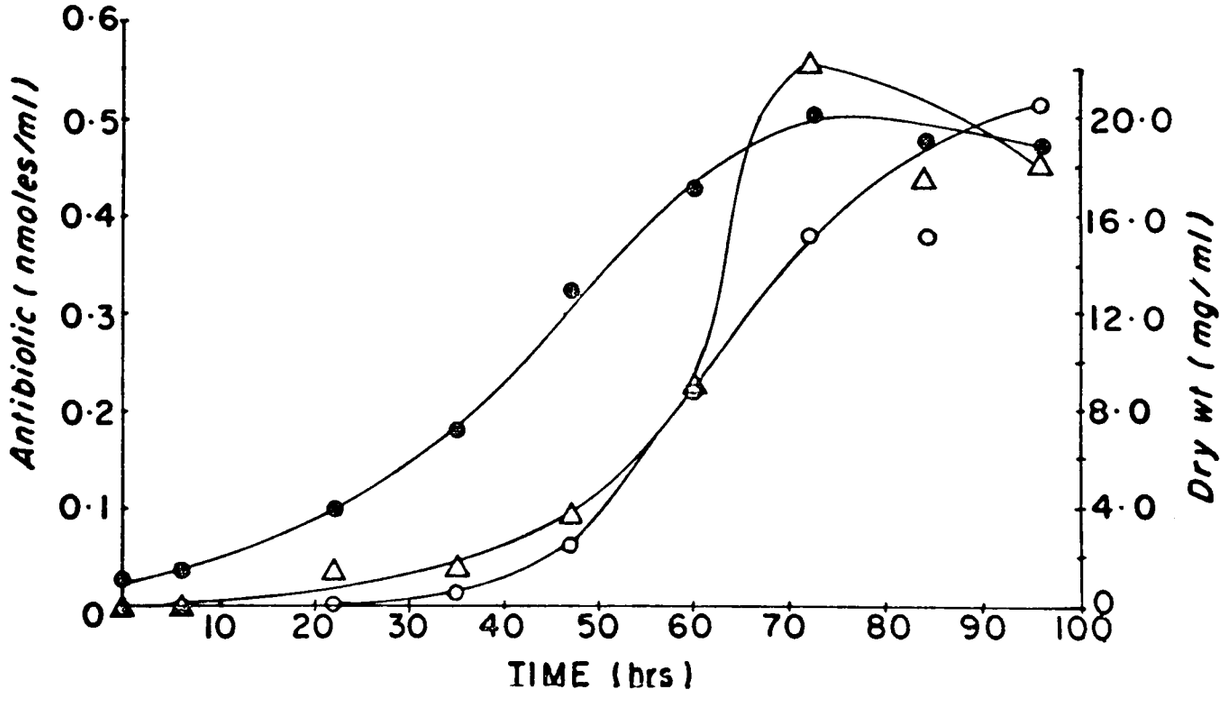
Previous experiments by Smith, Warren, Newton & Abraham (1967) provided evidence that both penicillin N and cephalosporin C were synthesised at maximal rates just before the mycelium became differentiated. These workers drew attention to the heterogeneity of the cell populations found in their fermentations, and emphasised that further investigations were necessary to establish which morphological type of mycelium produced the antibiotics most rapidly. As a preliminary step towards obtaining an active cell-free extract from *Cephalosporium* sp. growth and antibiotic production by the organism, in a chemically-defined medium, were reinvestigated. From the results of such experiments it was hoped that it would be possible to obtain a culture which was either producing antibiotics at maximal rates, or was about to do so. This culture could then be used as the starting material for the preparation of a cell-free system.

Fig. I. Antibiotic production and growth by Cephalosporium sp. C-91 from a heterogeneous inoculum during batch fermentation in Medium 19-D.



Δ——Δ Penicillin N (nmoles/ml.)
o——o Cephalosporin C (nmoles/ml.)
●——● Mycelial dry weight (mg./ml.)

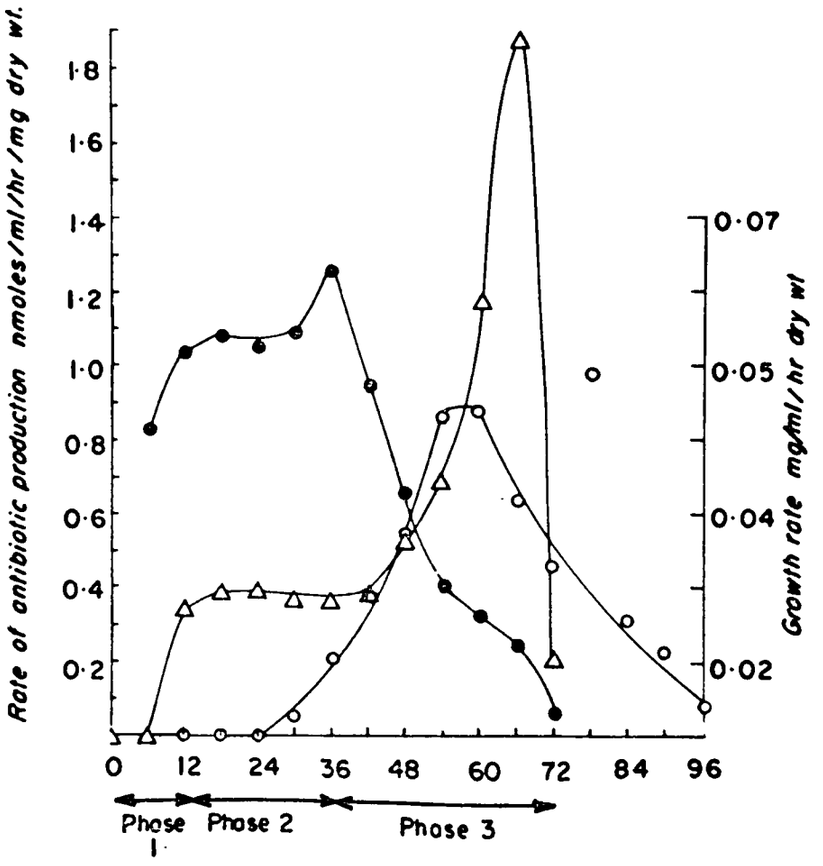
Results

Growth and antibiotic production in a synthetic medium during routine fermentation

A sample (10 ml.) of a 72 hour culture of Cephalosporium sp. C-91 which had been growing in the complex Medium-A, was added to 100 ml. of chemically-defined Medium 19-D. The inoculum was heterogeneous, consisting of spores, germinating spores, and undifferentiated septate hyphal strands (Plates I and II). After inoculation the cultures were incubated at 27°, 140 rev./min., on a rotary shaker as described in the Methods section. Samples (average volume 2.8 ml.) were withdrawn from the cultures at regular intervals for determinations of the amount of antibiotic produced and mycelial dry weight. These results are shown in Fig. 1. During the fermentation the morphological appearance of the fungus was examined by phase contrast microscopy. The descriptions are those of the general appearance of the organism at specified times. In addition, the results of this experiment were expressed as the rate of antibiotic production, i.e. nmoles/ml./hour/mg. dry weight of mycelium, while the dry weight data were analysed as the rate of growth in mg./ml./hour/mg. dry weight of mycelium. The results are presented in Fig. 2.

The growth rate curve was divided into three phases.

Fig. 2. Rate of Antibiotic production and growth by Cephalosporium sp., C-91 from a heterogeneous inoculum during batch culture fermentation in Medium 19-D.

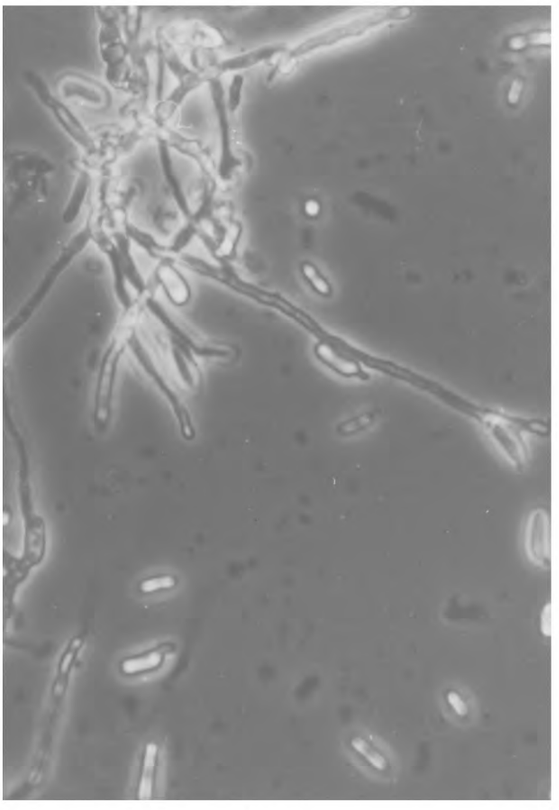


Δ——Δ Penicillin N (nmoles/ml./hr./dry weight)
o——o Cephalosporin C (nmoles/ml./hr./dry weight)
●——● Growth rate (mg./ml./hr./mg. dry weight)

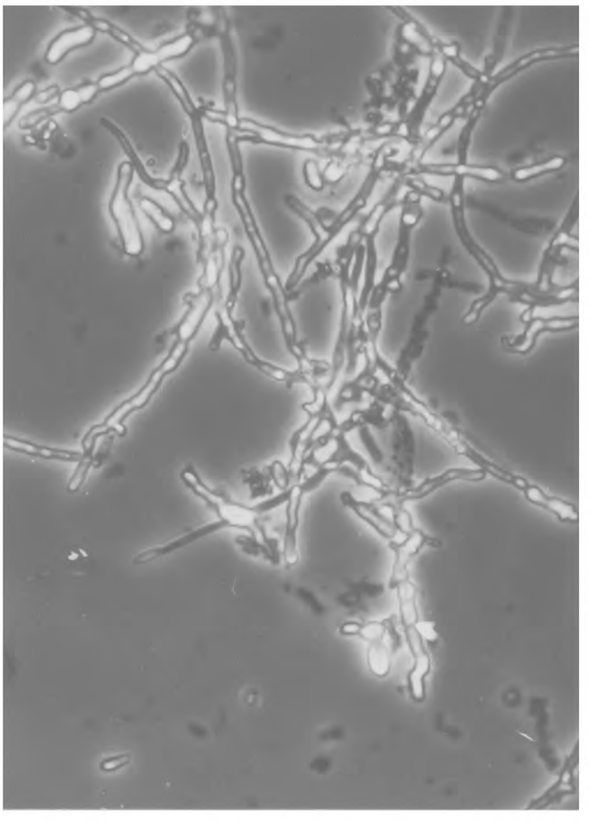
The first phase began immediately after inoculation, when the growth rate increased rapidly and reached 0.052 mg./ml./hour/mg. dry weight at 12 hours. However, although the growth rate per unit weight of mycelium had reached about 75% of its maximum value at 6 hours, it appeared that some undifferentiated, young hyphae, which may have been present in the inoculum, had autolysed (Plate III). The rate of production of penicillin N rose to 0.35 nmoles/ml./hour/mg. dry weight of mycelium at 12 hours, but cephalosporin C was not produced.

The second phase continued from 12 to 36 hours with the rate of penicillin N production remaining almost constant at 0.38 nmoles/ml./hour/mg. dry weight of mycelium, and this was paralleled up to 30 hours by a constant growth rate (0.053 mg./ml./hour/mg. dry weight). During this phase young, undifferentiated hyphae were growing out from centres of older cells (Plate IV shows mycelium at 22 hours). The growth rate reached a maximum value of 0.063 mg./ml./hour/mg. dry weight at 36 hours when the mycelium was in the extension growth phase (Plate V).

Thus, during the first two phases of the growth cycle, penicillin N production was associated with the presence of growing, undifferentiated cells, and was produced without cephalosporin C by the mycelium until the middle of the second



I



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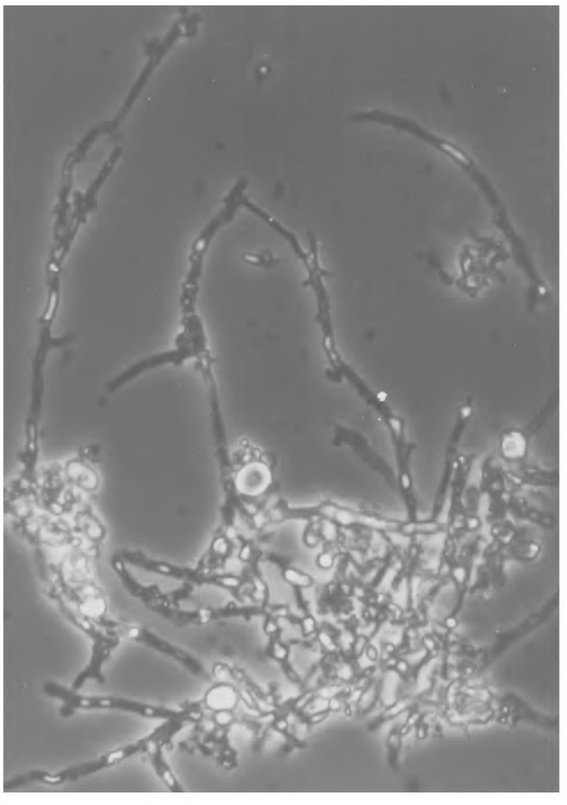

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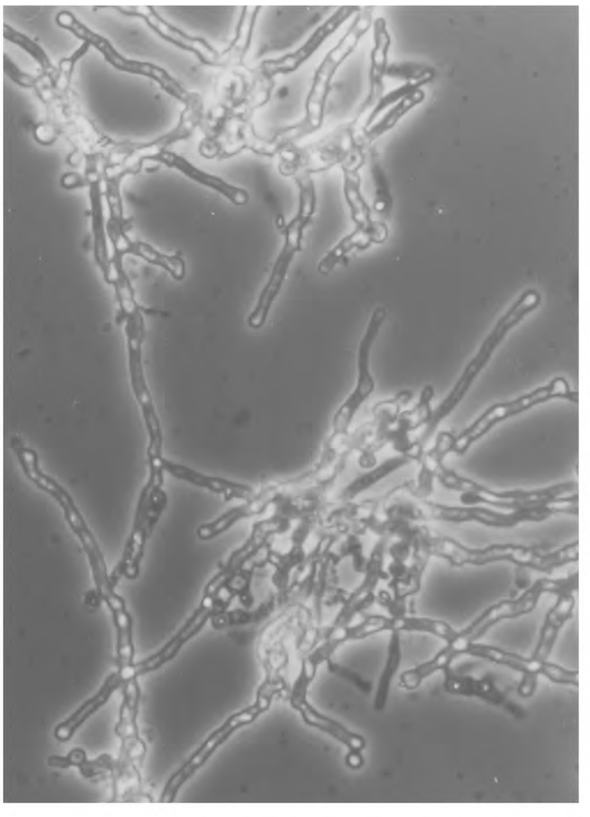


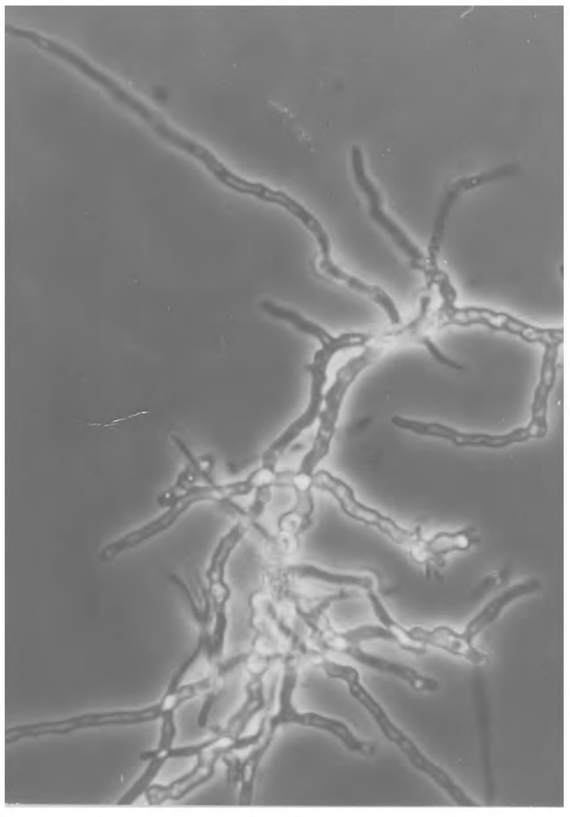
IV

Plate I. Culture of Cephalosporium sp. C-91 after 72 hours in Medium A. This culture was used to inoculate Medium 19-D (10% v/v) and consisted of spores and vegetative cells (Magnification x 400).

Plate II. Inoculum in Medium 19-D at 0 hours, showing heterogeneity of the cells (x 400).

Plate III. Culture after 6 hours incubation in 19-D. It appeared that some of the younger hyphae had lysed. (x 400).

Plate IV. After 22 hours incubation in 19-D, young hyphae can be seen growing from differentiated cells. (x 400).



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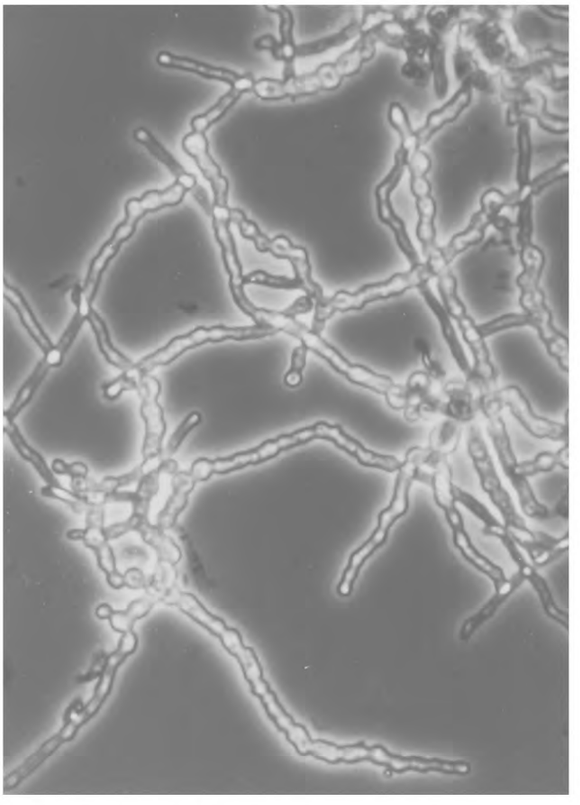
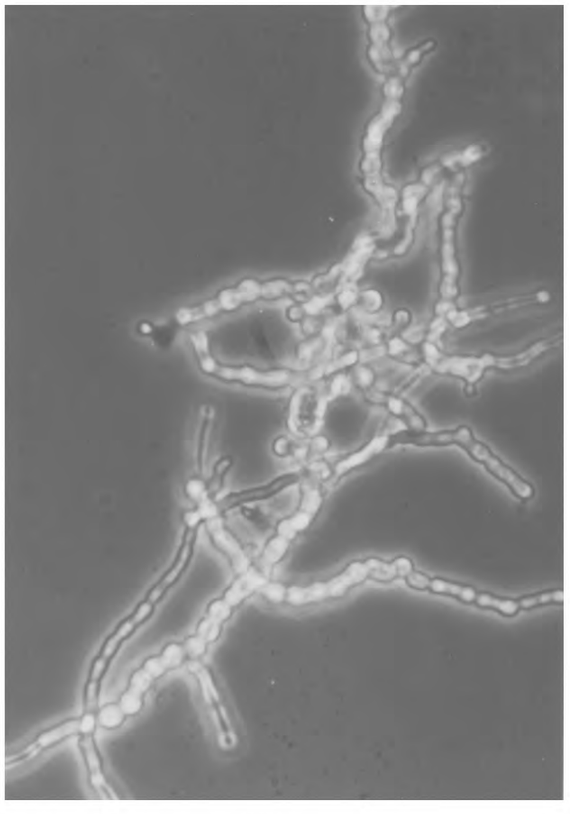
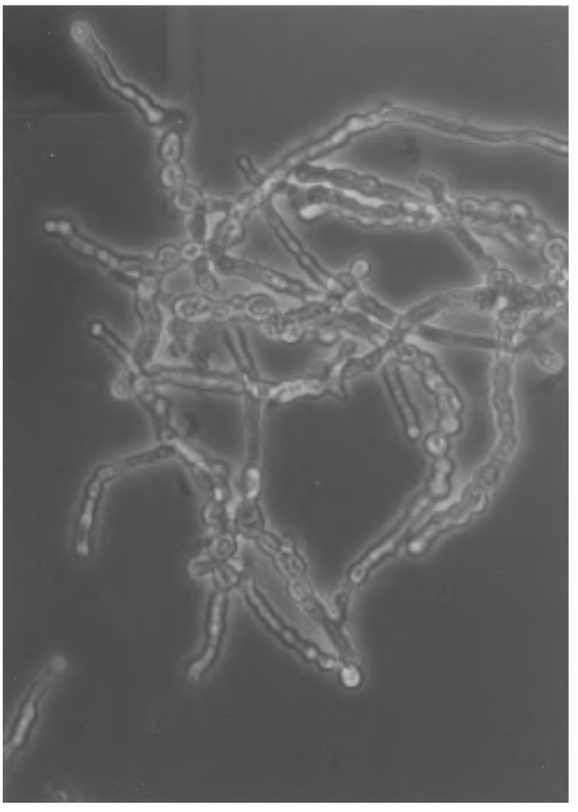


VI



VII



VIII

Plate V. The culture at 35 hours consisted predominantly of young, undifferentiated hyphae in the phase of extension growth. (x 400).

Plate VI. The mycelium had begun to differentiate by 47 hours when the hyphae were septated. (x 400).

Plate VII. After 60 hours the cells were becoming swollen with thickened cell walls. (x 400).

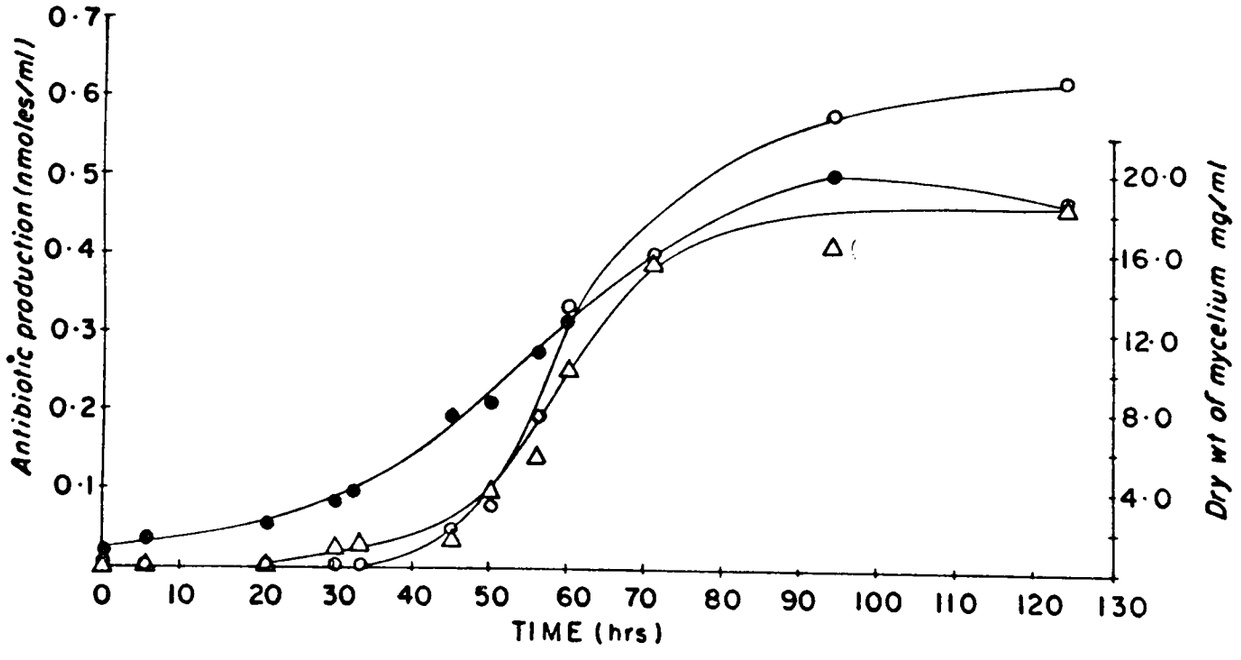
Plate VIII. The cells were becoming vacuolated by 72 hours, and the hyphae consisted of chains of yeast-like cells. (x 400).

phase. Cephalosporin C began to be produced at a detectable rate between 24 and 30 hours, towards the end of phase two.

Phase three (from 36 to 72 hours) began with the decline of the growth rate, which dropped abruptly between 36 and 54 hours from 0.063 to 0.02 mg./ml./hour/mg. dry weight and subsequently declined (54 to 66 hours) more slowly. At the beginning of this phase the rate of penicillin N production/unit weight of mycelium was still constant, but it rose to a maximum value of 1.87 nmoles/ml./hour/mg. dry weight at 66 hours, and then dropped sharply to about one tenth of this rate within the next 6 hours. In contrast, the rate of cephalosporin C production increased gradually from 24 hours until its maximum (0.9 nmoles/ml./hour/mg. dry weight of mycelium) was reached at 57 hours. The subsequent decrease in the rate of production of cephalosporin C was gradual and the rate was still falling at 96 hours.

After the growth rate had passed its maximum the mycelium became differentiated into hyphae consisting of chains of discrete cells. Differentiation was used in this context to denote the morphological change from the tubular, septate hyphal strands to those in which the cells were more easily discernible. Plate VI shows the morphological

Fig. 3. Antibiotic production and growth by Cephalosporium sp. C-91 from a spore inoculum during batch fermentation in Medium 19-D.



Δ——Δ Penicillin N (nmoles/ml.)
o——o Cephalosporin C (nmoles/ml.)
●——● Mycelial dry weight (mg./ml.)

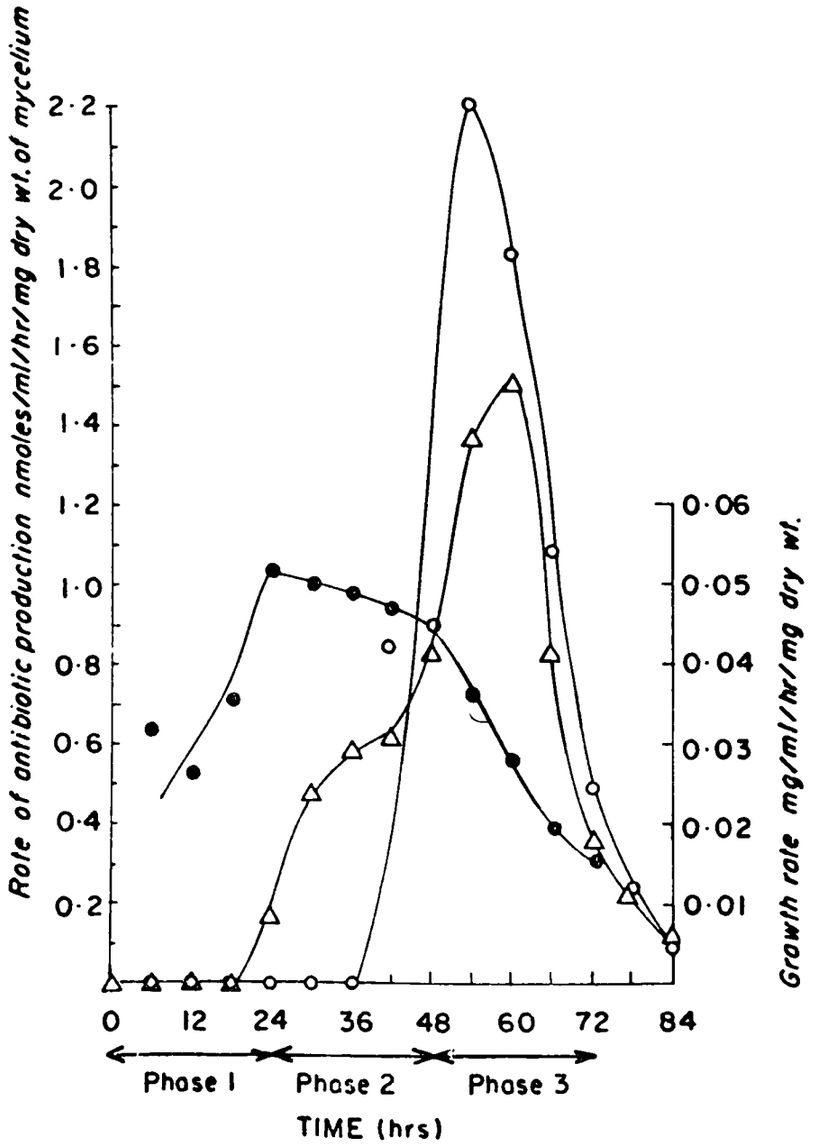
appearance of the mycelium at 47 hours. When antibiotics were produced maximally (54 to 66 hours) the hyphae were branching and contained swollen cells with thickened cell walls (Plate VII). A drop in the rate of antibiotic production was accompanied by the appearance of vacuolated yeast-like cells and the fragmentation of the hyphae (Plate VIII).

Growth and antibiotic production in a synthetic medium from a spore inoculum

Because of the heterogeneity of the starting inoculum in the previous experiment, it was difficult to specify, especially during the rapid growth phase, which morphological type of mycelium predominated and which types were present during the phase of antibiotic production. Therefore the preceding experiment was repeated with a spore suspension as an inoculum.

Preliminary experiments showed that after 60 hours incubation in the complex Medium-A, a culture of Cephalosporium sp. was heterogeneous but contained a large number of ungerminated spores (Plate IX). These spores were separated from the mycelium by filtration, concentrated by centrifugation (Plates X and XI) and used to inoculate Medium 19-D (as described in the Methods). The growth and antibiotic production curves obtained from this experiment are shown in

Fig. 4. Rate of antibiotic production and growth by Cephalosporium sp. C-91 from a spore inoculum during batch culture fermentation in Medium 19-D



Δ——Δ Penicillin N (nmoles/ml./hr./mg. dry weight of mycelium)

o——o Cephalosporin C (nmoles/ml./hr./mg. dry weight of mycelium)

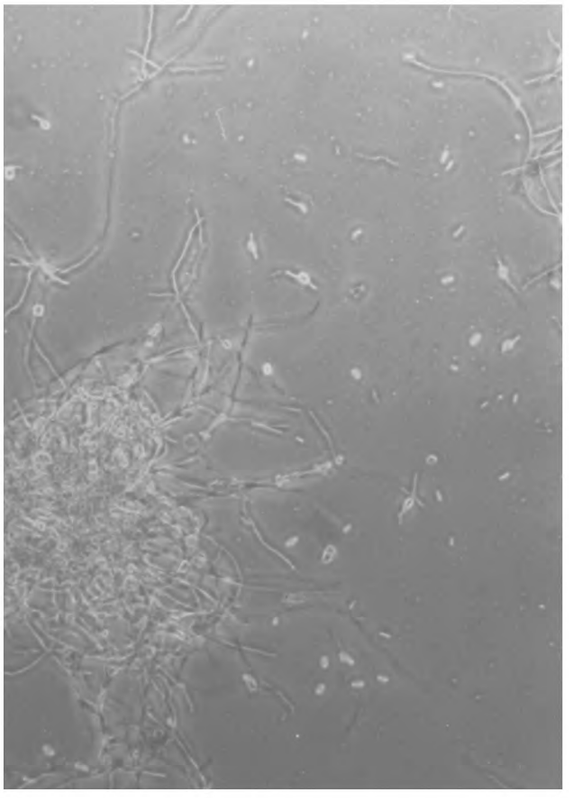
●——● Growth rate (mg./ml./hr./mg. dry weight)

Fig. 3 and the rates of growth and production per unit weight of mycelium in Fig. 4.

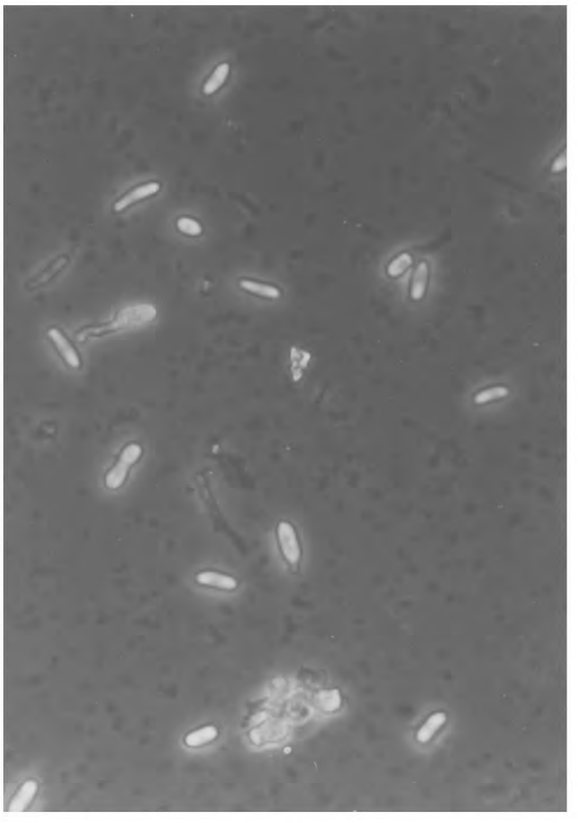
The growth rate curve of the mycelium growing from this type of inoculum can be analysed in the same way as the one obtained from the heterogeneous culture. The increase in the growth rate up to 24 hours corresponded to phase one and was associated with the germination of the spores and the growth of young hyphae (Plates XII and XIII). Shortly before this phase ended, between 18 and 24 hours, penicillin N began to be produced, but not cephalosporin C.

Phase two (24 to 48 hours) marks a very gradual decline in the growth rate from its maximum value of 0.052 to 0.045 mg./ml./hour/mg. dry weight over a period of 24 hours and corresponds with the virtually constant growth rate period of the former culture. Both cultures passed through similar morphological stages during this period with the hyphae extending in length and branching, as appears from a comparison of Plates IV and V, and XIV and XV from both fermentations. From 30 to 42 hours the initial acceleration in penicillin N production/unit weight of mycelium slowed down greatly, although a constant rate was not reached, and before the end of phase two the rate of production of penicillin N increased again.

The cephalosporin C production rate was only measurable at the end of the second phase, but the initial increase



IX



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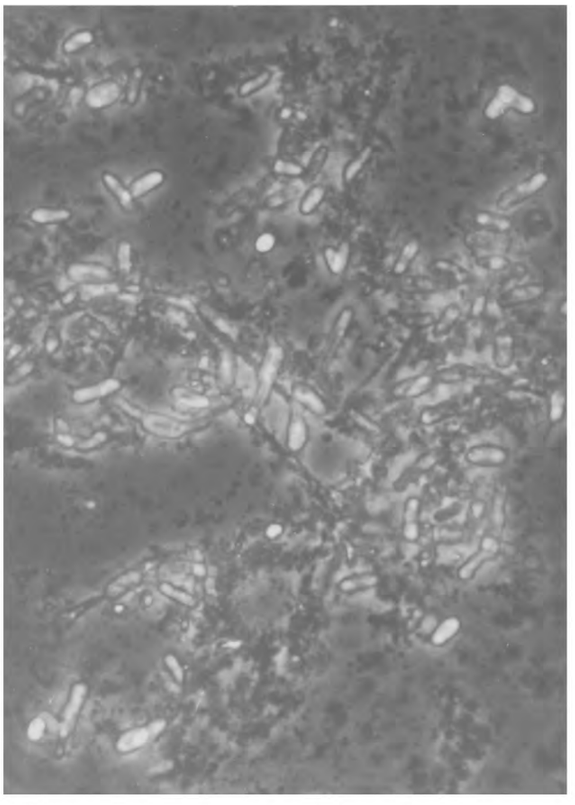

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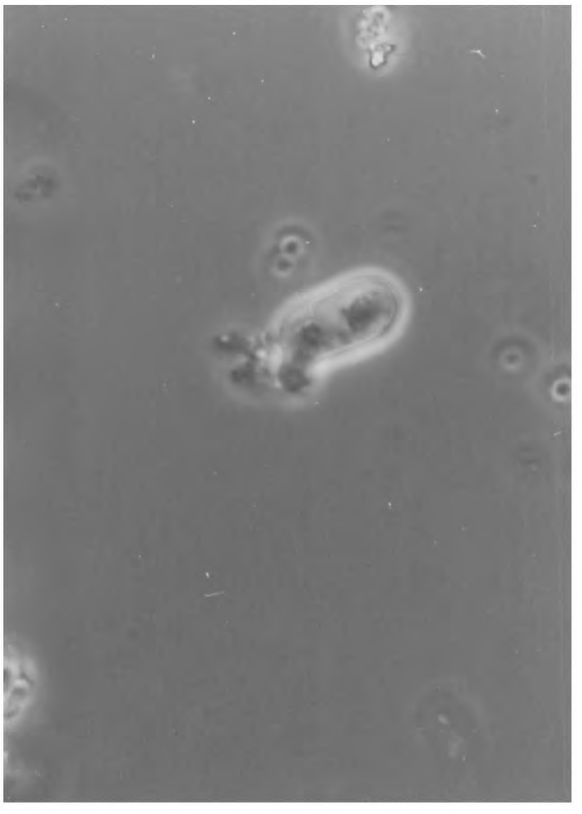


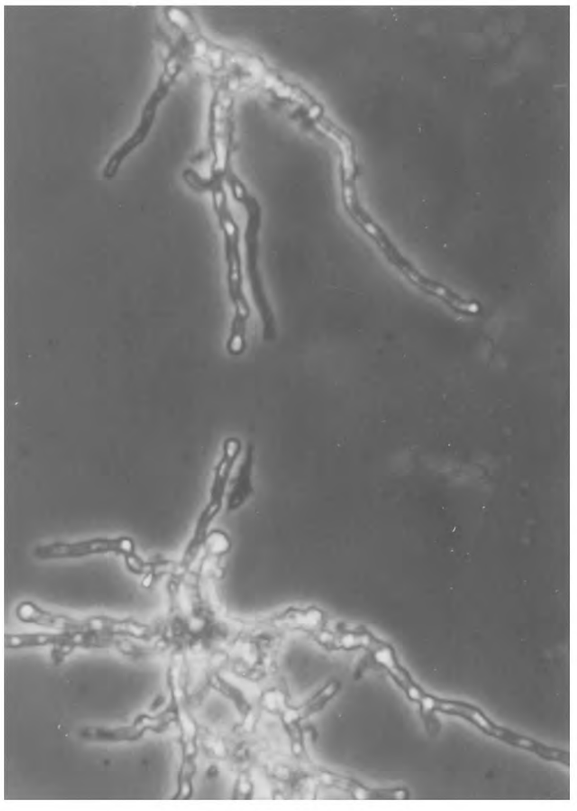
XII

Plate IX. Appearance of 60 hour culture of *Cephalosporium* sp. C-91 growing in complex Medium-A. (x 400).

Plate X. Spore filtrate obtained after passing the 60 hour culture through a stainless steel filter (hole diameter 140 μ). (x 400).

Plate XI. Concentrated spore suspension obtained after a second filtration through a mesh (85 μ hole diameter) followed by centrifugation (1,625 g). The debris which can be seen surrounding the spores is the sediment present in the Medium A which was also precipitated during centrifugation. (x 400).

Plate XII. After 5.5 hours the spores had begun to germinate. (x 400).



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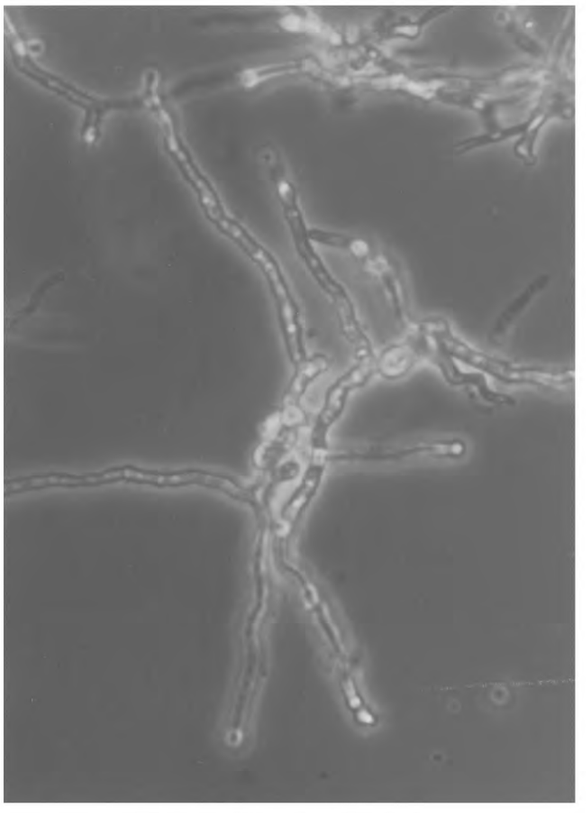

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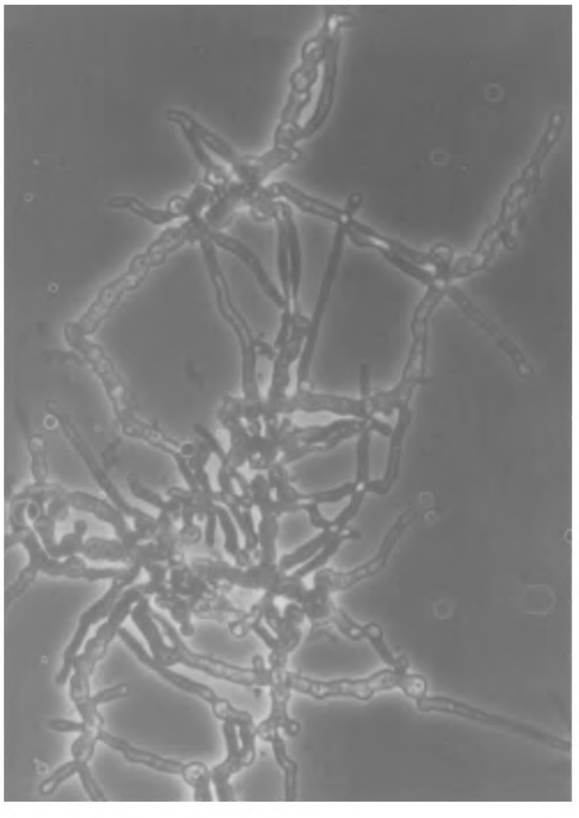

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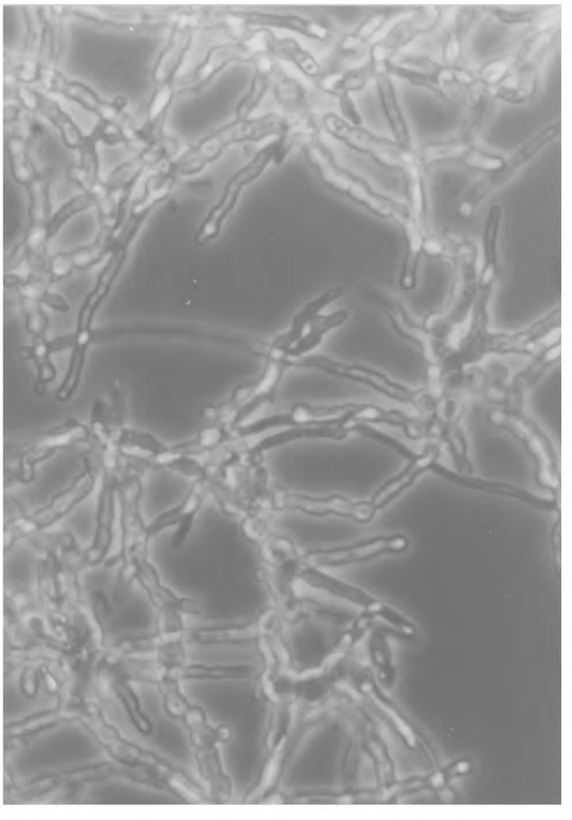


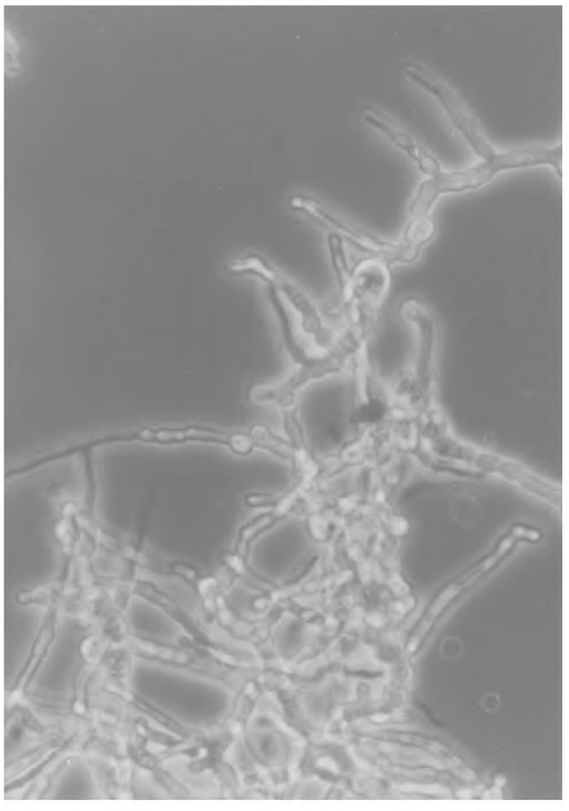
XVI

Plate XIII. Young hyphae are sent out from growth centres which consist of swollen cells after 21 hours in Medium 19-D. (x 400).

Plate XIV. By 33 hours the mycelium was branching and in the extension growth phase. (x 400).

Plate XV. Culture after 45 hours. Branched, but undifferentiated hyphae, are the main components of the culture at this age; the original growth centre have a swollen, yeast-like appearance. (x 400).

Plate XVI. After 56 hours growth in Medium 19-D, the mycelium was relatively homogeneous and differentiated with well-defined cells and cell walls. (x 400).



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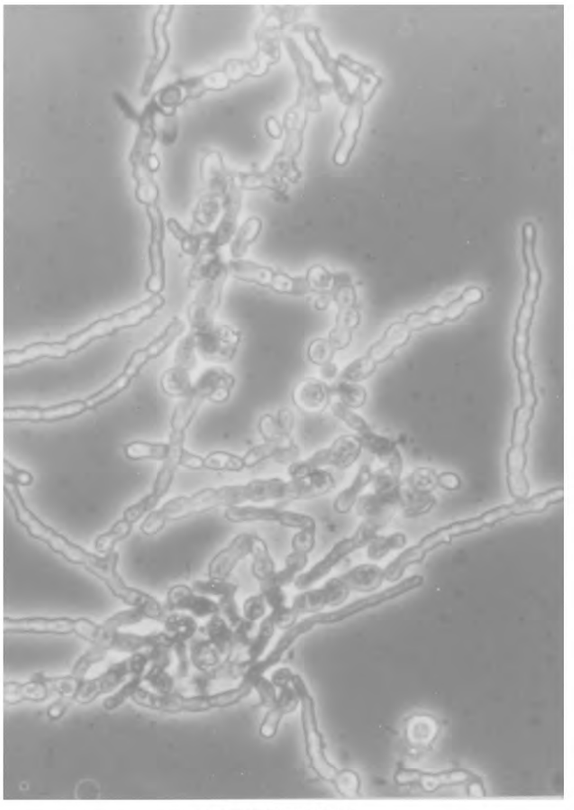
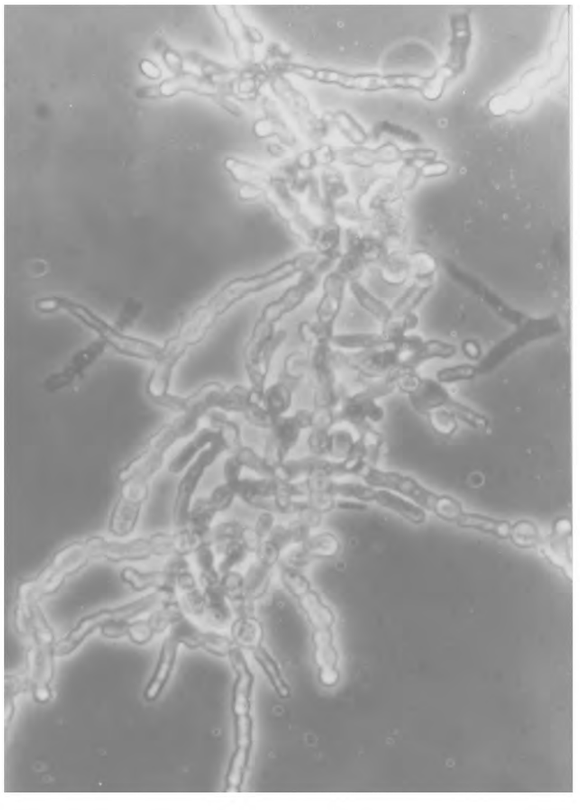
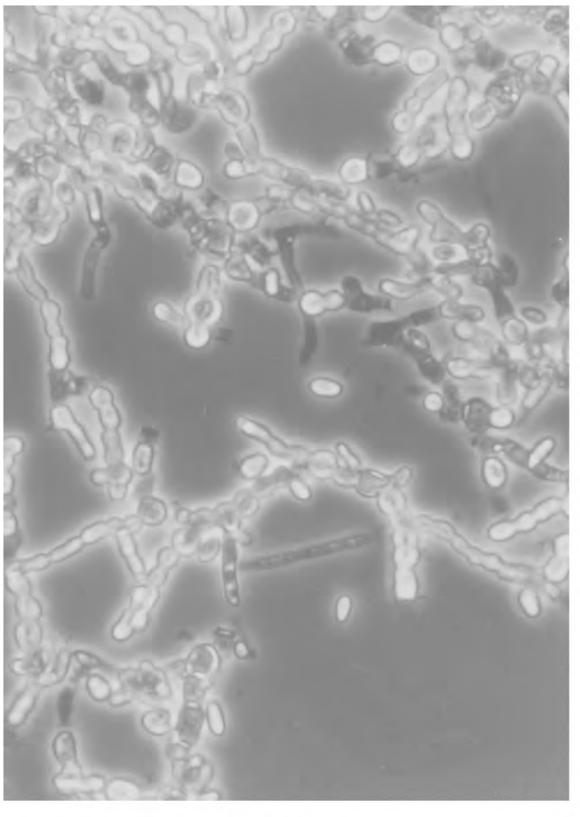


XVIII



XIX



XX

Plate XVII. Similarly at 60 hours the culture had a well-phased appearance. (x 400).

Plate XVIII. At 72 hours the cells were swollen and vacuolated and very refractile under the phase contrast microscope. (x 400).

Plate XIX. The mycelium at 90 hours was becoming more septate and fragmentation of the chains had begun. (x 400).

Plate XX. The culture at 124 hours was auto-lysing and fragmenting. Some empty cell walls could be seen and discrete unicells free in the medium. (x 400).

was so rapid that half of its maximum value was reached by the beginning of phase three (48 hours) and the maximum was attained at about 54 hours (2.2 nmoles/ml./hour/mg. dry weight of mycelium). Penicillin N appeared to be produced at its greatest rate shortly afterwards. The net production of both antibiotics then decreased at a similar rate up to 84 hours. Thus antibiotics were produced maximally in phase three by a differentiated and relatively homogeneous culture (Plates XVI and XVII) when the growth rate was falling. At 72 hours the hyphae were becoming septate and some yeast-like, granulated cells could be seen which were refractile under the phase contrast microscope (Plate XVIII). In the last stages of the fermentation (from 96 to 124 hours) the hyphae were segmented and lysis ensued with fragmentation of the hyphae (Plates XIX and XX).

Discussion

In batch fermentation under the conditions used in the present work, penicillin N and cephalosporin C reached their greatest concentration in the medium during the stationary growth phase of the organism. Further information about the sequence of events associated with growth and antibiotic production was obtained after analysis of the data in terms of rate of growth, and rate of antibiotic production.

As shown in Fig. 2, penicillin N production began after 6 to 12 hours in culture, but no cephalosporin C was synthesised up to 24 hours. An immediate conclusion to be drawn from these results is that the enzymes involved in the synthesis of penicillin N are elaborated quite early on in the growth cycle, before all of those required for cephalosporin C synthesis, although penicillin N synthesis is not restricted to (or apparently maximal in) rapidly growing cultures. The plateau region in phase two of the growth of the culture from a heterogeneous inoculum is formally analogous to the logarithmic growth phase of bacteria, in which every cell divides at a constant rate. However, a filamentous fungus normally divides near the growing tips so that cells are successively older the farther back they are from this location. In Plates IV and V it

can be seen that some hyphae are branching, and it is this mode of growth that may be represented by the plateau of the growth rate curve. As a consequence of branching older, yet undifferentiated cells removed from the hyphal tip may have reached the physiological age at which penicillin N synthesis can occur and may increase for a time at an almost exponential rate. It has been suggested that successive populations of cells could be reaching the same age and maintaining antibiotic production at a nearly constant rate for a longer period than would otherwise be the case (Newton, personal communication). The antibiotics were produced at their greatest rates in phase three, after the organism had passed its maximum growth rate. At this stage the mycelium consisted of branching chains of cells, but the cells near the tips of the hyphae were less differentiated than those further back along the hyphal strand (Plates VI and VII).

The results obtained when the mycelium was grown from a spore suspension substantiated the general conclusions drawn from the previous fermentation. Again it was found that penicillin N was synthesised by the culture when it was growing at a rapid rate, but the association between the rate of production and the growth rate was less prolonged, possibly because a significant number of cells

were in the same phase for a shorter period of time. This phase may have been prolonged in the heterogeneous fermentation because there were several cell populations present simultaneously.

No evidence was found from the production curves to suggest that penicillin N acted as a precursor of cephalosporin C. Experiments carried out by Warren (1964) also indicated that these antibiotics were formed by independent pathways; resting suspensions of 72 hr mycelium incorporated radioactivity from D-[1-^{14}C]-valine into cephalosporin C before penicillin N, although the antibiotics were being synthesised at equal rates. In addition, D-valine reduced the yield of penicillin N, but not that of cephalosporin C; therefore it seems that during the later stages of synthesis at least, they are produced independently of one another.

The results of the experiments described in this section do not indicate that penicillin N and cephalosporin C have no rôle in the growth of Cephalosporium sp., but, although penicillin N was produced while the organism was still growing, its maximum rate of production occurred when the growth rate was falling. Foster (1947), attempting to explain in physiological terms why micro-organisms produced antibiotics, suggested that as a result of cell

growth nutrients in the medium were depleted, and because of the subsequent growth limitations normal metabolites accumulate in the cells at abnormal concentrations. These metabolites direct synthesis towards unusual structures which are excreted into the medium by some organisms. This process he terms "shunt metabolism". Support for this hypothesis has been claimed in one case by Gatenbeck (1961-62) who postulated that accumulated malonamide acted as the trigger for the synthesis of the ring nucleus of the tetracyclines. With P. chrysogenum it was found that lysine inhibited benzylpenicillin production (Demain, 1957) but that α-aminoadipic acid (a lysine precursor in fungi) could reverse this inhibition. After the isolation of α-aminoadipylcysteinylvaline from P. chrysogenum mycelium (Arnstein & Morris, 1960) and after isopenicillin N (possessing the L-α-aminoadipic acid side-chain) had been found in P. chrysogenum culture fluids (Flynn et al., 1962), Somerson, Demain & Nunheimer (1961 and 1962) suggested that lysine inhibited the synthesis of its precursor α-aminoadipic acid by feed-back inhibition. These results could also be accounted for on the initiator hypothesis, if accumulated α-aminoadipic acid acted as the trigger for benzylpenicillin synthesis. In the case of Cephalorporium sp. there is no experimental evidence to show that a trigger mechanism of this type initiates the production of penicillin N or cephalosporin C.

SECTION II

STUDIES ON THE FORMATION AND PROPERTIES OF *CEPHALOSPORIUM* SP. C-91 PROTOPLASTS

SECTION II

STUDIES ON THE FORMATION AND PROPERTIES OF *CEPHALOSPORIUM* SP. C-91 PROTOPLASTS

Results

Preparation of protoplasts

Most workers (for example Holter & Ottolenghi, 1960; Garcia Mendoza & Villaneuva, 1962) have found that attempts to produce protoplasts from yeast cells were most successful when young cells in the logarithmic growth phase were used as the starting material. After 48 hours in normal batch culture, *Cephalosporium* sp. C-91 was at the beginning of the phase of maximum antibiotic production and in the middle of the growth phase as judged by the dry weight curve. Cultures of this age were used for preparing protoplasts.

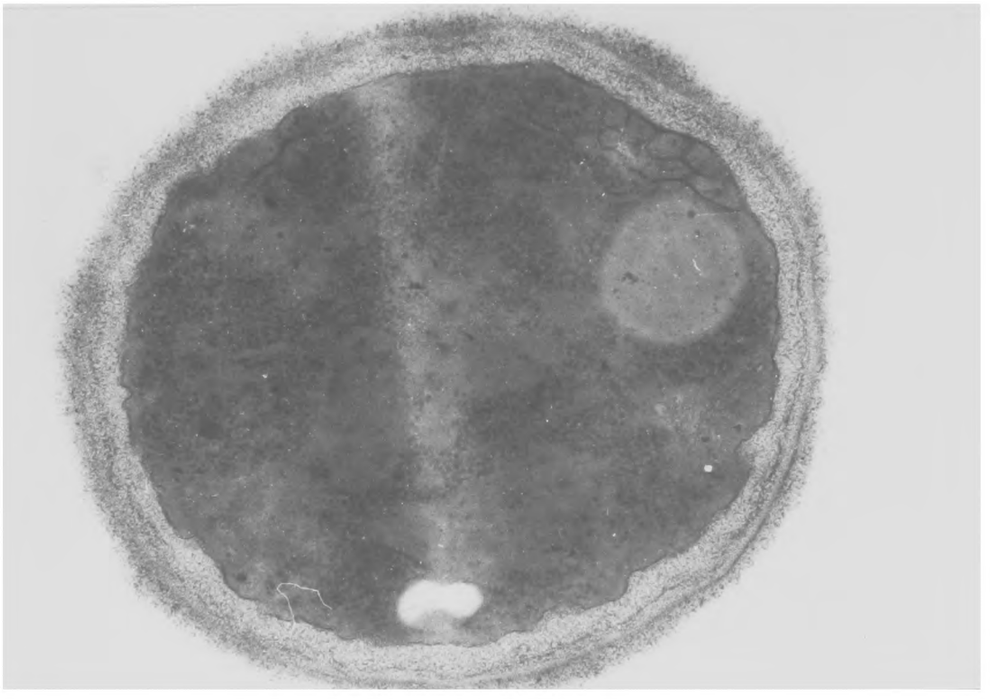
Preliminary experiments had indicated that the walls of 48 hour cells of the *Cephalosporium* sp. were not readily digested by snail enzyme. Prolonged incubation (24 to 48 hours) in solutions of enzyme gave rise to discrete fungal cells, which were spherical but which did not lyse when the tonicity of the medium was reduced and thus were not true protoplasts. Osmotic sensitivity was taken as a criterion of a true protoplast, since it implies the removal of the rigid protective barrier of the cell wall. It was found that when the *Cephalosporium* sp. was

pretreated with 0.02M-2-mercaptoethanol for 1 hour, and then incubated with snail enzyme for between 3 and 4 hours osmotically sensitive protoplasts were produced. However, since 2-mercaptoethanol had a deleterious effect on antibiotic production, the use of dithiothreitol (DTT), a similar but more effective reducing agent, was investigated. A preliminary experiment showed that pretreatment of mycelium with 0.01M-DTT in McIlvaine's buffer (pH 7.2) was as effective for the production of protoplasts as pretreatment with 0.02M-mercaptoethanol. Protoplasts were prepared routinely as described in the Methods Section using 0.9M-NaCl as a stabiliser. Mannitol was also tried as a stabiliser but the concentration required was so high that it was impossible to keep the solute in solution at room temperature. Attempts to stabilise protoplasts in sucrose (1.5M) resulted in their lysis for unknown reasons.

The fine structure of mycelium and protoplasts

Mycelium and protoplasts were prepared for electron microscopy as described in Methods.

The wall of a hyphal strand of the Cephalosporium sp. appears to be composed of four layers. The outer wall is approximately 500 Å thick and its outer surface is diffuse with no clearly defined limiting structure. The second layer is less electron-dense than the outer layer,



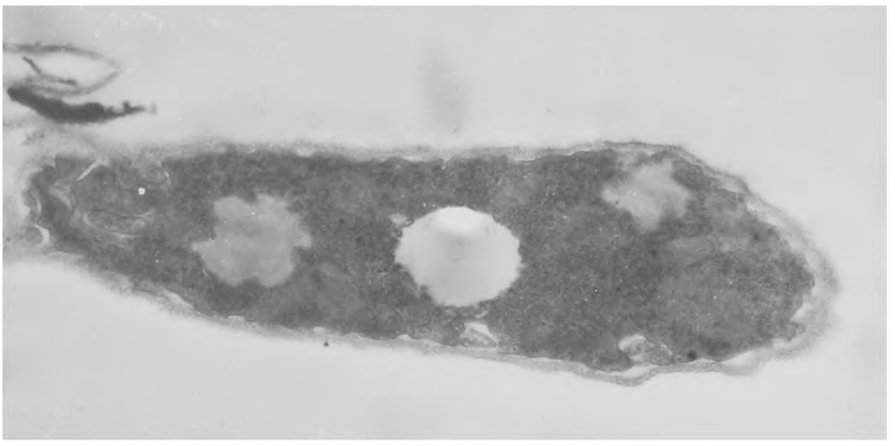
XXI (Magnification X 73,000)

XXII (X 73,000)

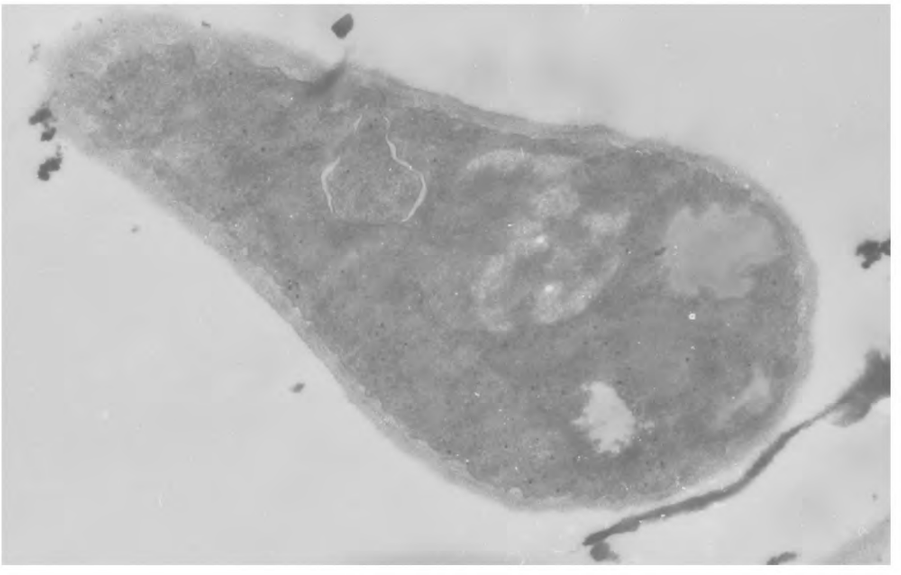
about 350 Å in width and quite clearly defined. The next layer is extremely electron-dense and thin (only 120 Å) while the fourth and innermost layer is the thickest (750 Å) and less dense than the third. This last layer is obviously in close contact with the cytoplasmic membrane surface (Plate XXI).

At the junction of two cells along a hyphal strand it appears that the two outer layers are shared by all the cells in that strand and that each cell is covered individually by the two inner layers of the wall (Plate XXII). The cytoplasm of the cell is densely packed with punctate ribosomes. Each cell contains several vacuoles, some of which are electron dense while others are opaque and possibly contain lipid. At the cytoplasmic membrane there are several invaginations and coiled tubular outgrowths of the cytoplasm.

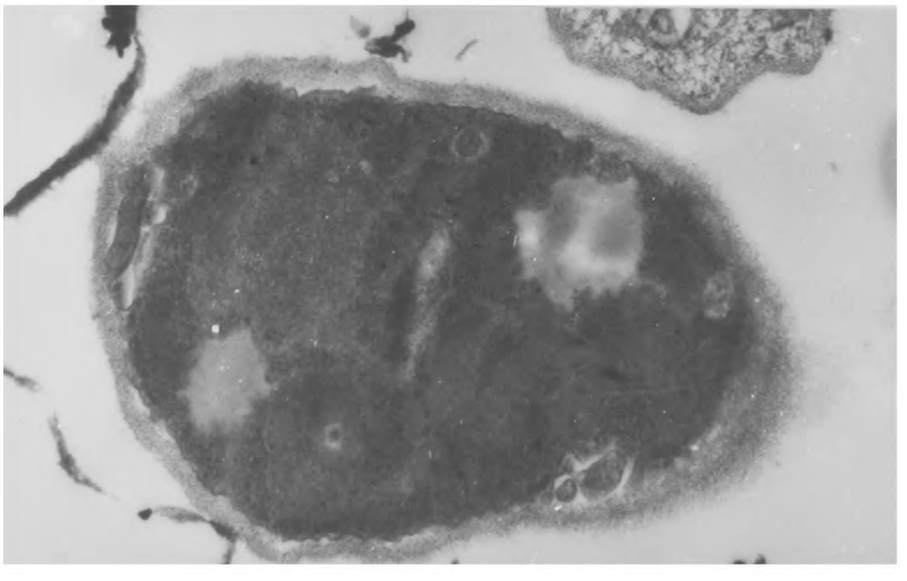
After pretreatment with 0.01M-DTT (pH 7.3) there was no measurable decrease in the thickness of the cell wall, and it thus seems that DTT does not remove a layer from the cell wall nor alter its shape visibly. However, the inner layer of the wall did not seem to be in such intimate contact with the cytoplasmic membrane. The cytoplasm of the cell appeared to have drawn away from the wall leaving a space between it and the membrane, particularly at points of invagination. Pretreatment with DTT did not appear to



XXIII a(x30,000)



XXIII b(x30,000)

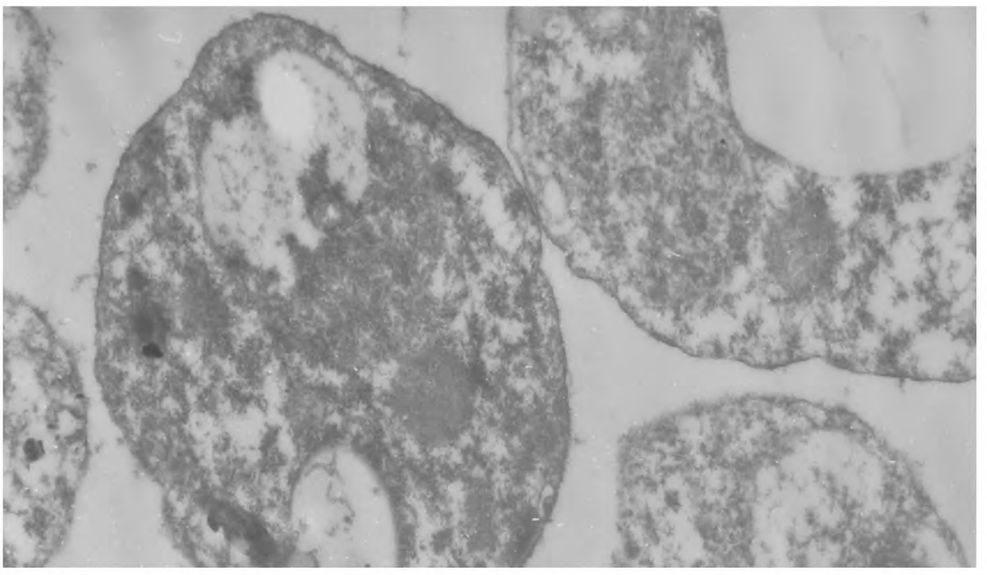


XXIII c(x40,000)

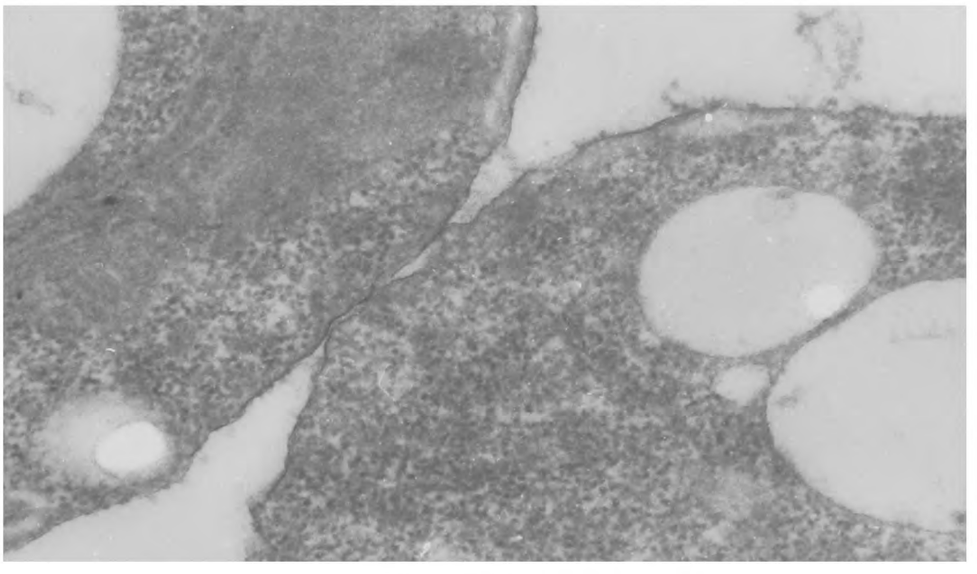
have any effect on the internal organisation of the cell (Plates XXIII a, b, c).

The most obvious features of the protoplasts are their spherical shape and their lack of the wall of the mycelial cell. The ribosomes are no longer tightly packed in the cytoplasm and the fact that they are more diffuse is an indication that the stabilising medium in which protoplasts were prepared was hyptonic to the cytoplasm of the original mycelial cells. This swelling is also suggested by the appearance of the membrane, which is no longer invaginated and in some cases is tightly stretched around the protoplast. In several photographs protoplasts can be seen touching and, at higher magnification, points of contact between the double-layered cytoplasmic membrane can be seen, providing proof that virtually all the cell wall material has been digested away during incubation with snail enzyme (Plates XXIV, XXV & XXVI).

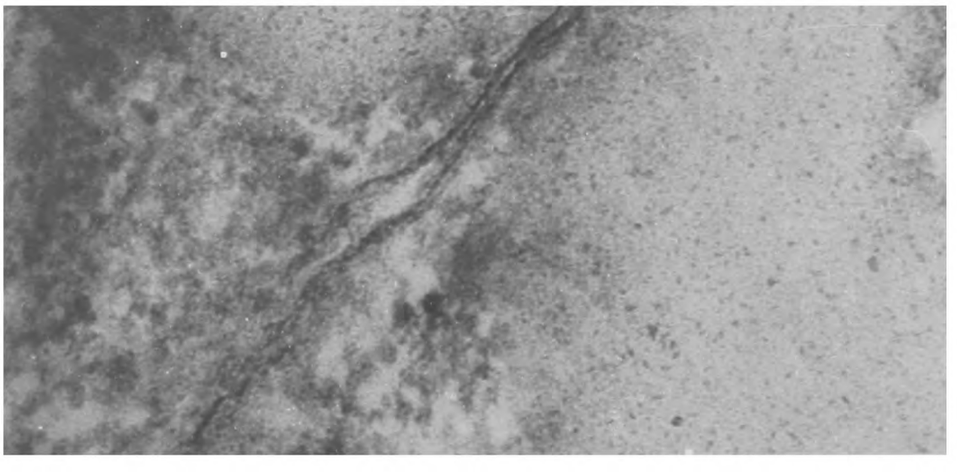
Apparently the protoplasts contain all the intracellular organelles of the normal cell. The internal structure can be discerned more easily because of the diffuseness of the cytoplasm. Between six and twelve mitochondria, oval in shape and with long, filiform cristae, can be seen in each protoplast (Plates XXVII & XXVIII).



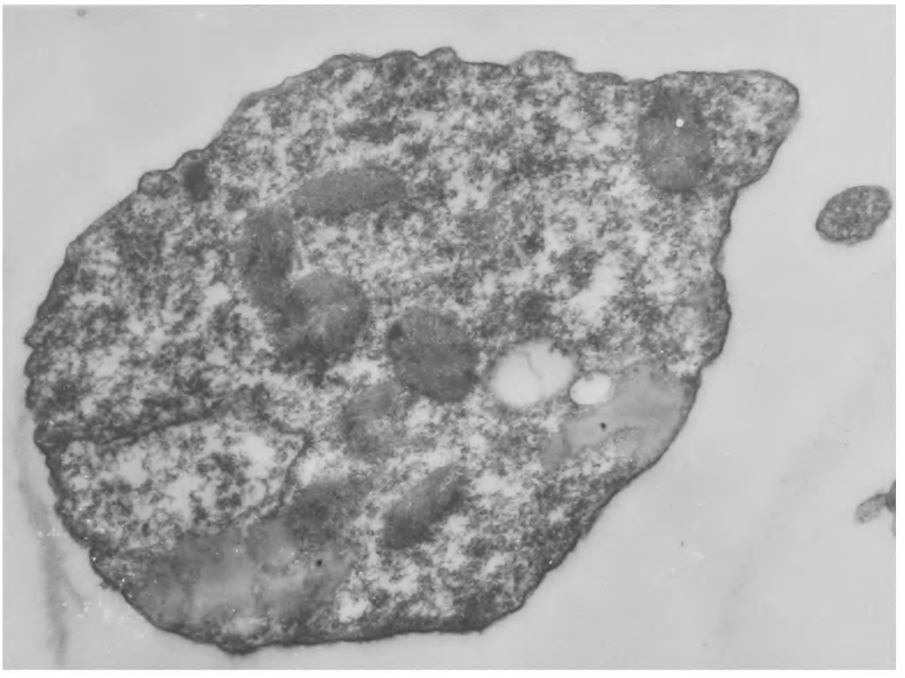
XXIV (x30,000)



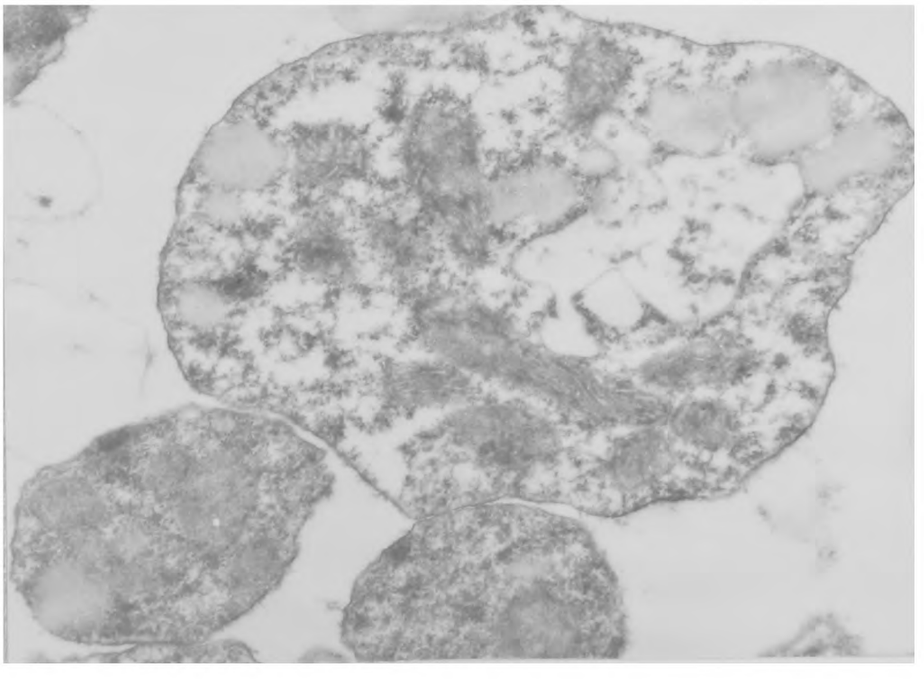
XXV (x100,000)



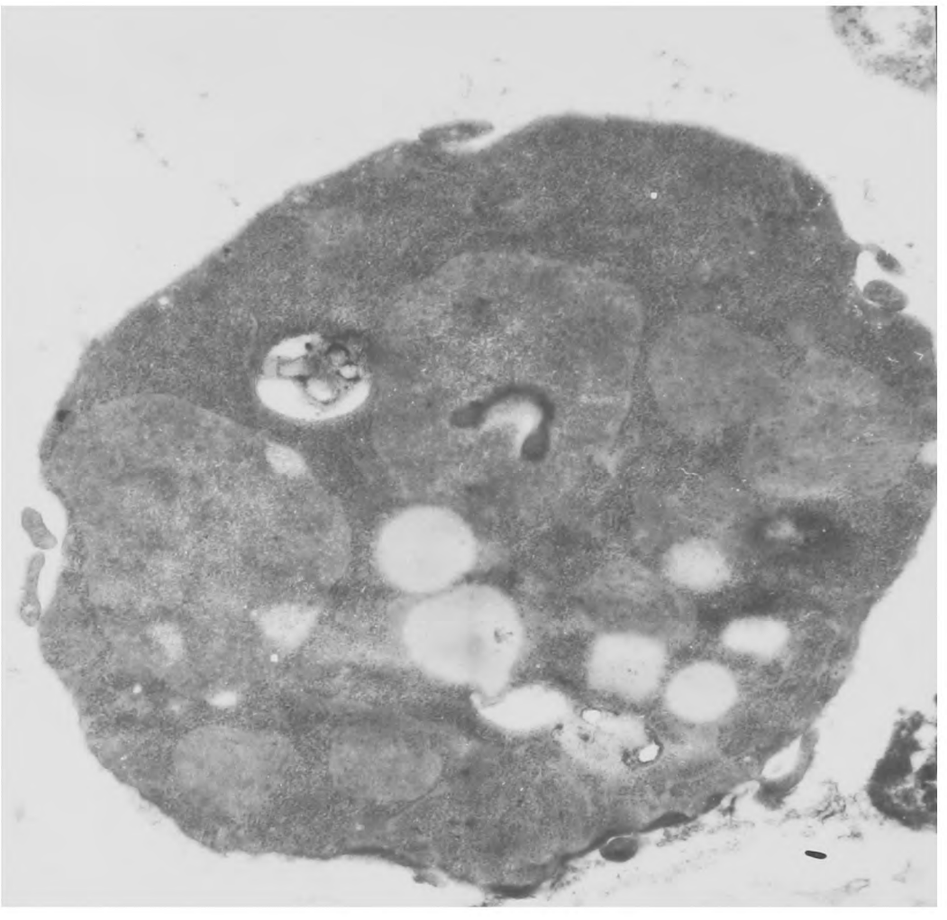
XXVI (x130,000)



XXVII (x30,000)



XXVIII (x30,000)



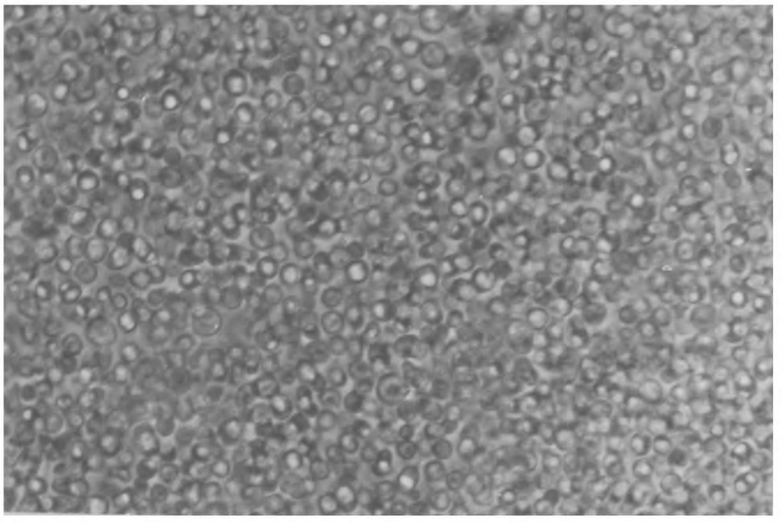
XXIX (x40,000)

There are several vacuoles in each protoplast, some of which are electron opaque and may be lipid-containing. Other vacuoles are bounded by a double-layered membrane and contain tubules which in some cases form a network throughout the lumen. In most protoplast sections no obvious nuclear structure could be identified, but in one an area could be determined which was lighter than the surrounding cytoplasm and contained a bolas-shaped pattern of high electron density suggestive of a chromosome (Plate XXIX).

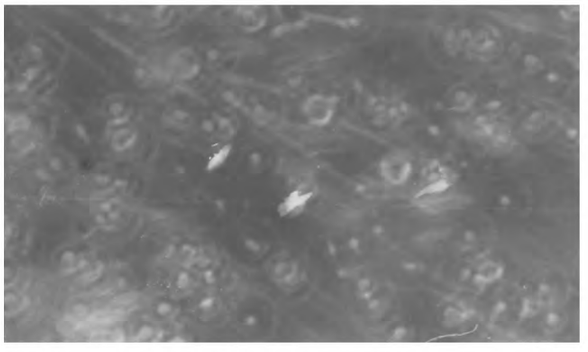
Osmotic sensitivity of protoplasts

Protoplasts were stabilised in a medium containing 0.9M-NaCl, which presumably had an osmotic pressure close to that of their cytoplasm. In this medium they were perfectly spherical. Under phase contrast they were refractile and several vacuoles could be seen in each protoplast (Plate XXX). When a drop of distilled water was allowed to diffuse under the cover slip of a slide preparation of protoplasts, there was a rapid streaming of the protoplasts due to the influx of water (Plate XXXI). The stages involved in the subsequent osmotic lysis are shown in Plates XXXII to XXXIV).

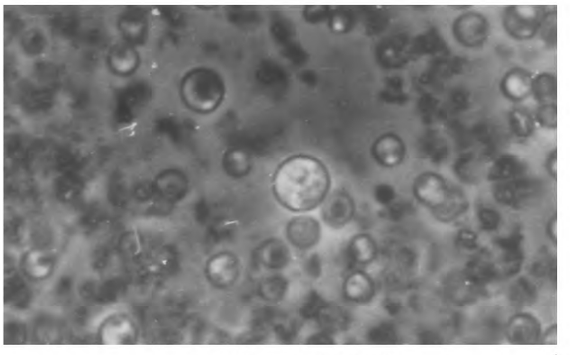
At first the protoplasts swelled, the protoplast membrane appearing to be tightly stretched around the



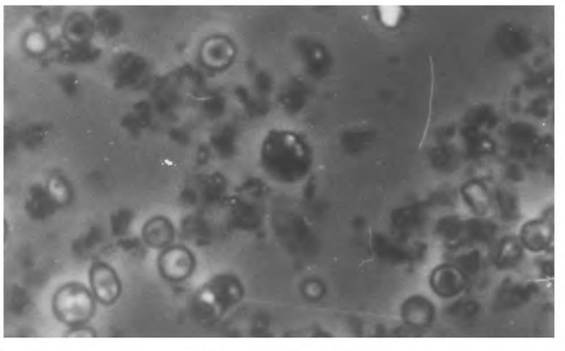
XXX



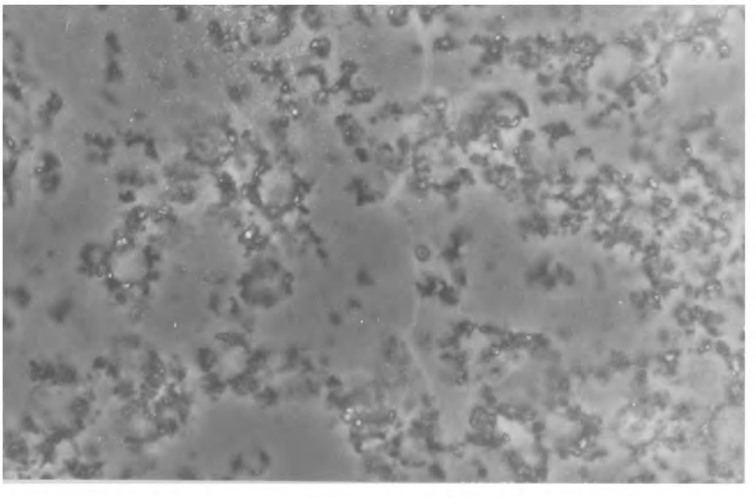
XXXI



XXXII



XXXIII



XXXVI

cytoplasm, and their internal structure became more clearly discernible. At this stage the refractility of the protoplasts under phase contrast increased. When the protoplasts lysed their membranes appeared to become perforated, although the spherical shape was still retained. The overall refractility of the intracellular contents was lost and this, presumably, was the reflection of a loss of cytoplasmic integrity, although some intracellular particles remained refractile even after they were released into the medium. Finally the whole protoplast structure disintegrated, leaving collapsed membranes, which appeared dark grey under phase contrast, in a fine dispersion of liberated particles. Some protoplasts lysed quicker than others, possibly because they had been rendered more fragile by the procedures employed in their preparation.

Discussion

Righelato, Trinci, Pirt & Peat (1968) reported that the cell walls of P. chrysogenum were composed of two ill-defined layers (in some sections only one layer was seen) and that around the outside of the wall there was a layer of diffuse, electron-dense material. The cell wall of the Cephalosporium sp. in cross section, is a four-layered structure. Seen in longitudinal section, two of these layers appeared to surround each cell, while the outer two layers covered the whole hyphal strand.

Treatment of the Cephalosporium sp. with 2-mercaptoethanol or DTT makes the cell wall more susceptible to attack by snail enzyme. It has been suggested that, in yeast cell walls at least, the reaction involves the reduction of disulphide bonds linking protein to mannan (Nickerson & Falcone, 1956). These disulphide linkages are thought to confer plasticity on the cell wall. At these sites after the reduction of the bonds, new material is probably incorporated into the wall, as, for example, during bud formation in yeasts. Evidence for the presence of sulphydryl groups in the walls of mycelial fungi has been provided by Pitt (1969). He showed by specific staining and radioautography that these groups were present at the tips of P. chrysogenum phialides where new wall material is

required after the cell divisions associated with sporulation. Although there is no evidence that disulphide bonds exist in Cephalosporium sp. cell walls, it is conceivable that they are present and that treatment with reducing agents splits these bonds and renders the wall more susceptible to removal by snail enzyme.

Aronson & Fitz-James (1968) reported that 50% of the spore coat protein of Bacillus subtilis was solubilised after the cells had been resuspended for 30 minutes in DTT solution; their electron-micrographs showed that the coat had effectively been removed from the spores after this treatment. The electron micrographs of the Cephalosporium sp. showed that after incubation with DTT the cell wall had not decreased in thickness, but that the cytoplasm appeared to have drawn away from the wall without losing the invaginations of the plasma membrane. This suggests that a chemical linkage may have existed originally between the two structures.

When distilled water was added to the protoplasts obtained after incubation with snail enzyme, they lysed almost immediately and the debris which remained was amorphous. Membrane ghosts which retained the shape of the protoplast could not be detected. This was evidence that no remnants of wall material persisted, and that true proto-

plasts had been formed.

The high density of ribosomes in the cytoplasm of the intact cells implies a concomitantly high level of metabolic activity, which would be normal in 48 hour mycelium since the growth rate was only just falling from its maximum value. The ribosomes in the cytoplasm of the protoplasts were in general more diffuse, suggesting that the cells may have taken up water from the surrounding medium. It is conceivable that the mycelial cytoplasm had a higher osmotic pressure than the suspending fluid, and that when the cell wall was removed water was taken up by the protoplasts. However, Leaf (1956) has shown that during the process of swelling in mammalian cells the fluid entering the cell is not water alone, but a solution approximately isotonic with the medium. He suggests that when these cells have a diminished metabolic activity the normal ionic gradients cannot be maintained. Thus sodium and chloride ions from the stabilising medium might have entered the protoplasts producing an increase in osmotic pressure which was followed by hydration and swelling.

SECTION III

BIOCHEMICAL PROPERTIES OF *CEPHALOSPORIUM* SP.C-91 PROTOPLASTS

SECTION III

BIOCHEMICAL PROPERTIES OF CEPHALOSPORIUM SP. C-91 PROTOPLASTS

Results

Effect of reagents used in protoplast formation on antibiotic production by intact mycelium

Figures 5 and 6 show the penicillin N and cephalosporin C content of the extracellular fluid at different times of suspensions of damp-dry 48 hour mycelium (1 g./6 ml.) which had been incubated for 5 hours in the reagents used for the preparation of protoplasts, as described in the Methods Section. Antibiotic titres were not measured at 0 hours because it was assumed that the mycelium would not produce a detectable amount of antibiotic between the time of washing and resuspension.

It appeared that 2-mercaptoethanol or dithiothreitol did not greatly affect penicillin N production, during incubation for 1 hour (Fig. 5). However, cephalosporin C production was reduced by approximately 60%. After resuspension in sodium chloride stabiliser cephalosporin C production was some 35% of the control value after 4 hours; production of penicillin N was 60% of the control at this time (Fig. 6).

During prolonged incubation in 2-mercaptoethanol

Effect of reagents used in protoplast preparation on antibiotic production by mycelium

Fig. 5

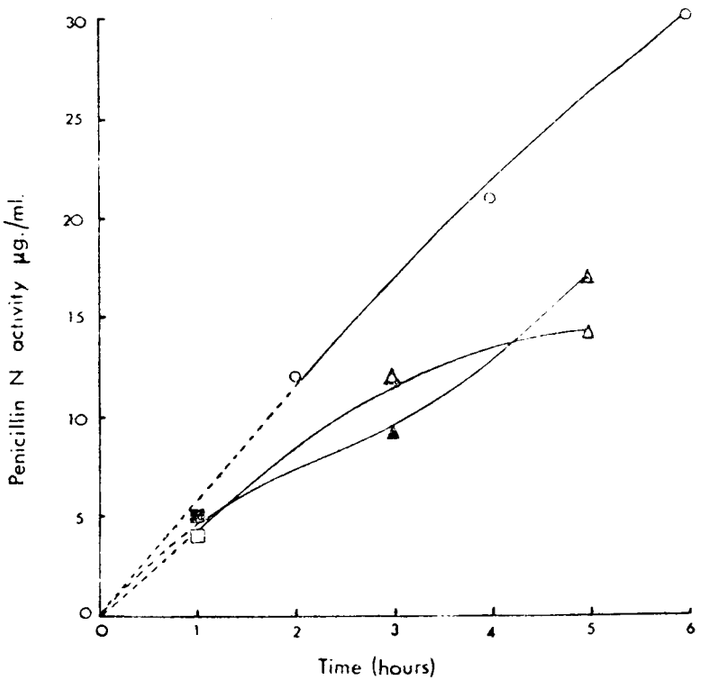
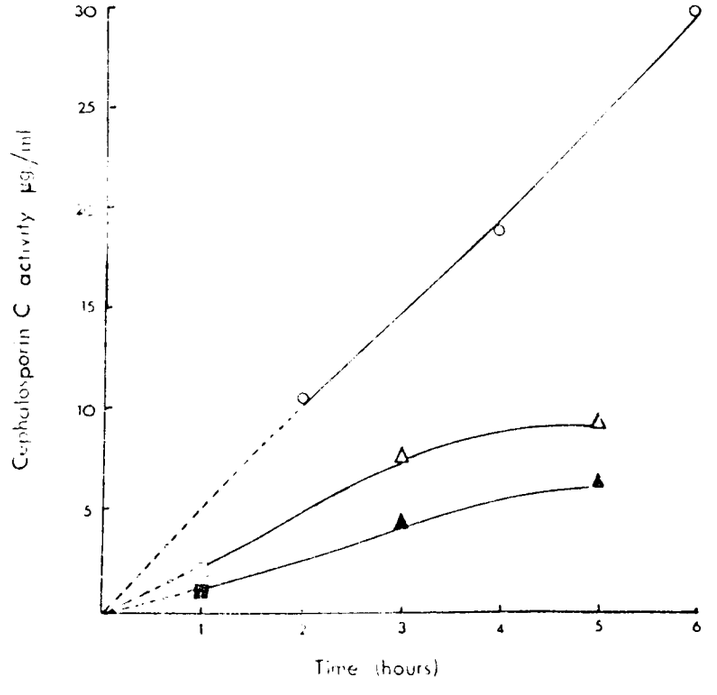


Fig. 6



o——o Mycelium in water

---□——Δ— Mycelium in 0.01M-DTT for 1 hour, washed and resuspended in 0.9M-NaCl for 4 hours

---■——▲— Mycelium in 0.02M-2-mercaptoethanol for 1 hour, washed and resuspended in 0.9M-NaCl for 4 hours.

To 1 g. samples of damp-dry 48 hour mycelium in 50 ml. shake flasks were added 6 ml. aliquots of reagents used in protoplast preparation. Samples were taken at the times shown above and the amounts of antibiotics in the culture fluids were assayed.

(8 hours) the mycelium had produced little more cephalosporin C than after 1 hour, but 50% of the penicillin N formed by the control mycelium in water. After 2 hours in the medium to which dithiothreitol had been added, however, cephalosporin C was restored to 50% of the control value, while the penicillin N synthetic system recovered completely and production was almost equal to that of the control mycelium.

Freshly prepared solutions of penicillin N and cephalosporin C in DTT or 2-mercaptoethanol showed activities by bioassay which were 25-40% and 15-30% lower respectively than solutions of the same concentration (mg./ml.) in water. This was possibly the result of a reaction between the antibiotics and the thiol groups of these reagents while the solutions diffused into the assay plates at 37° and/or of an effect on the sensitivity of the assay organism. Since incubation at 27° of these solutions for 8 hours produced only a slight further diminution of activity, it appeared unlikely that substantial inactivation of the antibiotics was taking place during the incubation of the mycelium with the reducing agents. However, antiobiotic production by the mycelium in DTT or 2-mercaptoethanol may well be some 15-40% higher than that suggested by the results of the hole-plate assays.

Antibiotic production by protoplasts and by control suspensions of starved mycelium and DTT-pretreated starved mycelium in 0.9M-NaCl.

Fig. 7

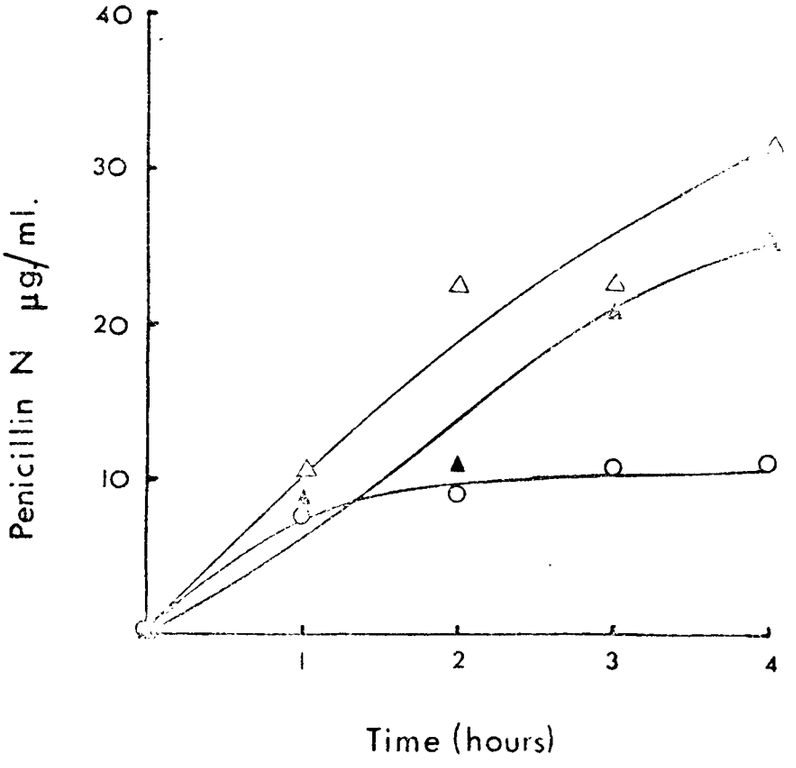
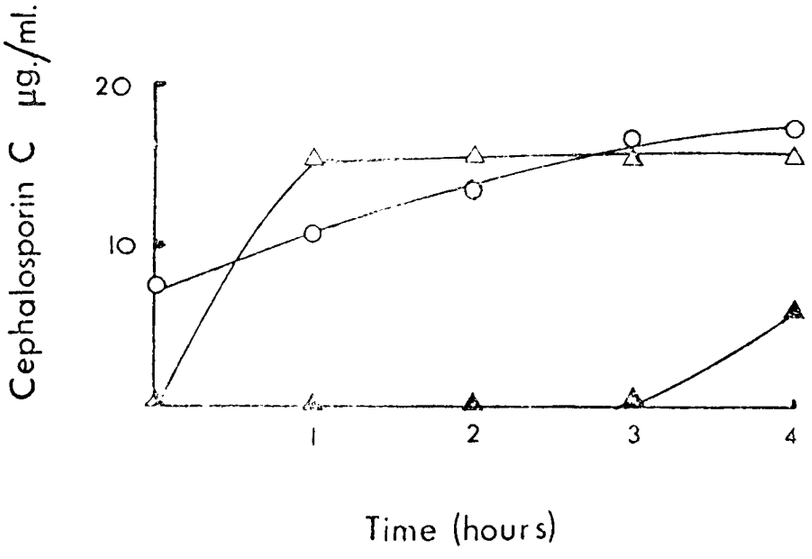


Fig. 8.



Mycelial suspensions were incubated at 200 rev./min., protoplast suspensions at 120 rev./min., at 27° for 4 hours.

Δ——Δ Mycelium (1 g./6 ml.) in water

▲——▲ Mycelium (1 g./6 ml.) in 0.9M-NaCl

o——o Protoplasts (0.3 g./2.5 ml.)* in 0.9M-NaCl

* This weight, and corresponding weights in subsequent Figures and Tables, refers to the weight of mycelium from which the protoplasts would have been derived if the yield were 100%.

Antibiotic production by protoplasts and by control suspensions of mycelium in water and in sodium chloride

A portion of damp-dry mycelium was resuspended in distilled water (1g/6ml) and incubated for the same length of time (3-4 hours) required to prepare protoplasts. This suspension was called "starved mycelium". Another portion of mycelium was pretreated with dithiothreitol for 1 hour, washed and resuspended (1g/10ml) in 0.9M-NaCl (pH 6.8) for 3-4 hours. Then both suspensions were filtered, washed with distilled water or 0.9M-NaCl and resuspended (1g/6ml) in fresh distilled water or sodium chloride. Samples of the suspensions were taken at intervals over 4 hours, the mycelium separated by filtration, and the amounts of antibiotics in the culture fluids determined. Similarly, samples of protoplast suspension were removed and, after centrifugation, the supernatant fluids assayed for antibacterial activity. The results are presented in Figs. 7 and 8.

Starved mycelium resuspended in fresh distilled water continued to produce penicillin N at the same rate as mycelium resuspended immediately after harvest (Figs. 5 and 7). For the first hour cephalosporin C also was produced at the same rate as in suspensions of fresh mycelium, but thereafter production apparently ceased and the titre remained constant for the rest of the incubation period

(Figs. 6 and 8).

In comparison with the suspension of starved mycelium in water, the starved mycelium resuspended in fresh sodium chloride solution (0.9M) produced 25% less penicillin N after 4 hours. Cephalosporin C was only produced after a lag period of 3 hours by this suspension and then at only 30% of the rate of production by the control mycelium.

After 4 hours incubation in snail enzyme solution the mycelial/protoplast preparation synthesised 6.2 µg./ml. of cephalosporin C, but penicillin N could not be detected in the snail enzyme supernatant. In addition, when the mycelial/protoplast sediment was washed with fresh stabiliser a further 6.2 µg./ml. of cephalosporin C was released, but no penicillin N.

Immediately after resuspension the pure preparation of protoplasts liberated 50% of the total amount of cephalosporin C found after 4 hours in their suspension fluids. Initially, the protoplasts synthesised penicillin N at a rate similar to that of the control suspension of mycelium. After 1 hour this rate decreased and after 4 hours the amount formed was 70% less than that produced by the mycelium. However, penicillin N production by the protoplasts was variable, and only two of the five preparations

Table 1. Incorporation of DL-[1-^{14}C]-valine into penicillin N and cephalosporin C

DL-[1-^{14}C]-valine (0.1 μmoles/ml.; 3.3 μCi/ml.) was added to the protoplast suspensions and (0.08 μmoles/ml.; 2.5 μCi/ml.) to the suspensions of mycelium. Both sets of suspensions were incubated at 27°, 140 rev./min. for 4 hours. The culture fluids were prepared for chromatography as described in the Methods Section.

Suspension	Penicillin N		Cephalosporin C	
	Total radioactivity nCi	units/ml.	Total radioactivity nCi	units/ml.
Protoplasts (0.6 g./ml.) in 0.9M-NaCl	2.0	<0.5	5.1	0.066
Mycelium (0.33 g./2 ml.) in 0.9-NaCl	1.3	<0.5	0.8	<0.06

tested produced enough penicillin N to be detected by bioassay.

Incorporation of DL-[1-^{14}C]-valine into penicillin N and cephalosporin C

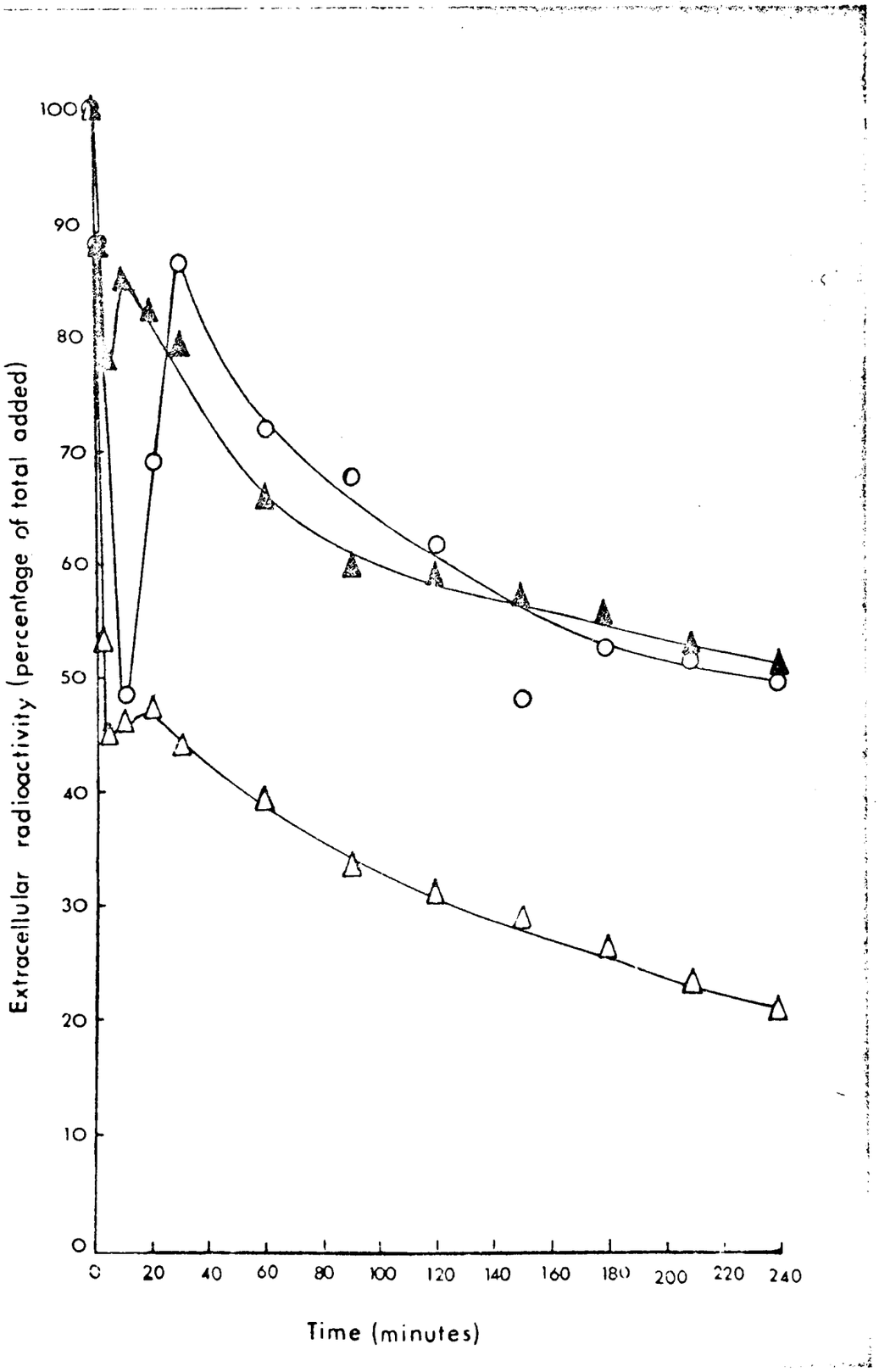
Table 1 shows the amounts of radioactivity which were incorporated into penicillin N and cephalosporin C after the addition of DL-[^{14}C]-valine to the extracellular fluids of suspensions of protoplasts and mycelium. In this experiment the mycelium was incubated at the same shaking speed as the protoplasts which proved too slow a rate to provide adequate aeration for mycelial suspensions of this concentration. However, it was possible to detect by radioautography amounts of antibiotics which were not detectable by bioassay.

Since the protoplasts contained approximately ten times less intracellular valine than the mycelium, [^{14}C]-valine of high specific activity taken up by the former would be diluted relatively less intracellularly and hence the specificity activity of the antibiotics would be higher than in the latter.

Uptake of DL-[1^{14}C]-valine by protoplasts and mycelium

In order to assess the significance of the results of studies on the incorporation of [^{14}C] from DL-[1-^{14}C]-valine into penicillin N and cephalosporin C by protoplasts, estimations were made of the ability of the protoplasts to

Fig. 9. Uptake of DL-[1-^{14}C]-valine by a preparation of protoplasts and by mycelium



Δ——Δ Mycelium (1g/6ml) in water

▲——▲ Mycelium (1g/6ml) in 0.9M-NaCl

o——o Protoplasts (0.4g/ml) in 0.9M-NaCl

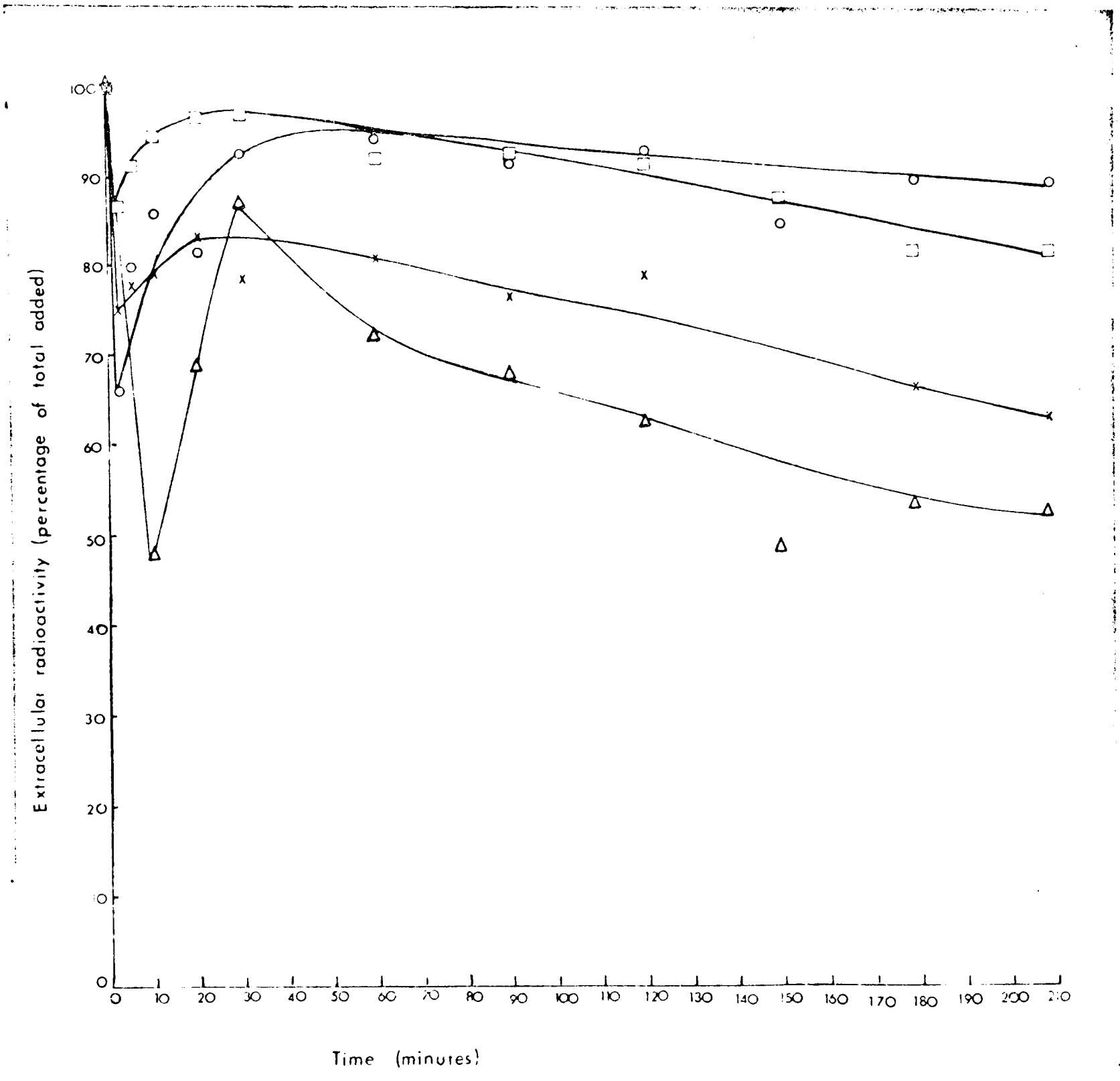
DL-[1-^{14}C]-valine (0.026 μmoles/ml; 0.83 μCi/ml) was added to suspensions of mycelium (1g/ml) in water, and of DTT-pretreated mycelium in 0.9M-NaCl in 50ml shake flasks. The suspensions were incubated at 200 rev/min., 27°, 4 hours. Samples (0.2ml) were removed and after centrifugation the amounts of radioactivity in the supernatant fluids were measured.

The protoplast suspensions (0.4g/ml) contained 0.078 μmoles/ml of DL-[1-^{14}C]-valine (2.5 μCi/ml) and was incubated at 120 rev/min., 27°, 4 hours. The sampling procedure was exactly the same as that of the mycelium except that 0.1 ml samples were taken.

take up [^{14}C]-valine from the suspension medium. The disappearance of ^{14}C from the medium was used as a measure of the uptake of valine, on the assumption that this represented the amount of added valine which became available for use inside the cell. Comparative experiments were carried out with suspensions of intact mycelium in water and in 0.9M-NaCl which had all originated from the same batch of 48 hour mycelium.

1. Uptake of low concentrations of DL-[1-^{14}C]-valine. The curve obtained by plotting the percentage of added ^{14}C which remained in the extracellular fluid against time could conveniently be divided into three sections (Fig. 9). The same general uptake pattern, which was obtained for each suspension, showed an initial phase of rapid uptake when as much as 50% of the isotope was removed from the medium over a period of 5 to 10 minutes. In the second phase, from about 5-10 until 30 minutes, there was a rise in the extracellular radioactivity. Thus, it seemed that some of the accumulated radioactivity was released by the cells into the suspension fluids. Subsequently, during the third phase, [^{14}C]-valine was taken up at a slower rate than before, and this rate continued during the rest of the incubation. However, the amounts of isotope taken up and released by each suspension in phases one and two differed considerably, while in phase three, the rates at which the

Fig. 10. Variations between different protoplast suspensions



Δ——Δ DL-[1-^{14}C]-valine (0.78 μmoles/ml.; 2.5 μCi/ml.) with protoplasts in 0.9-NaCl.

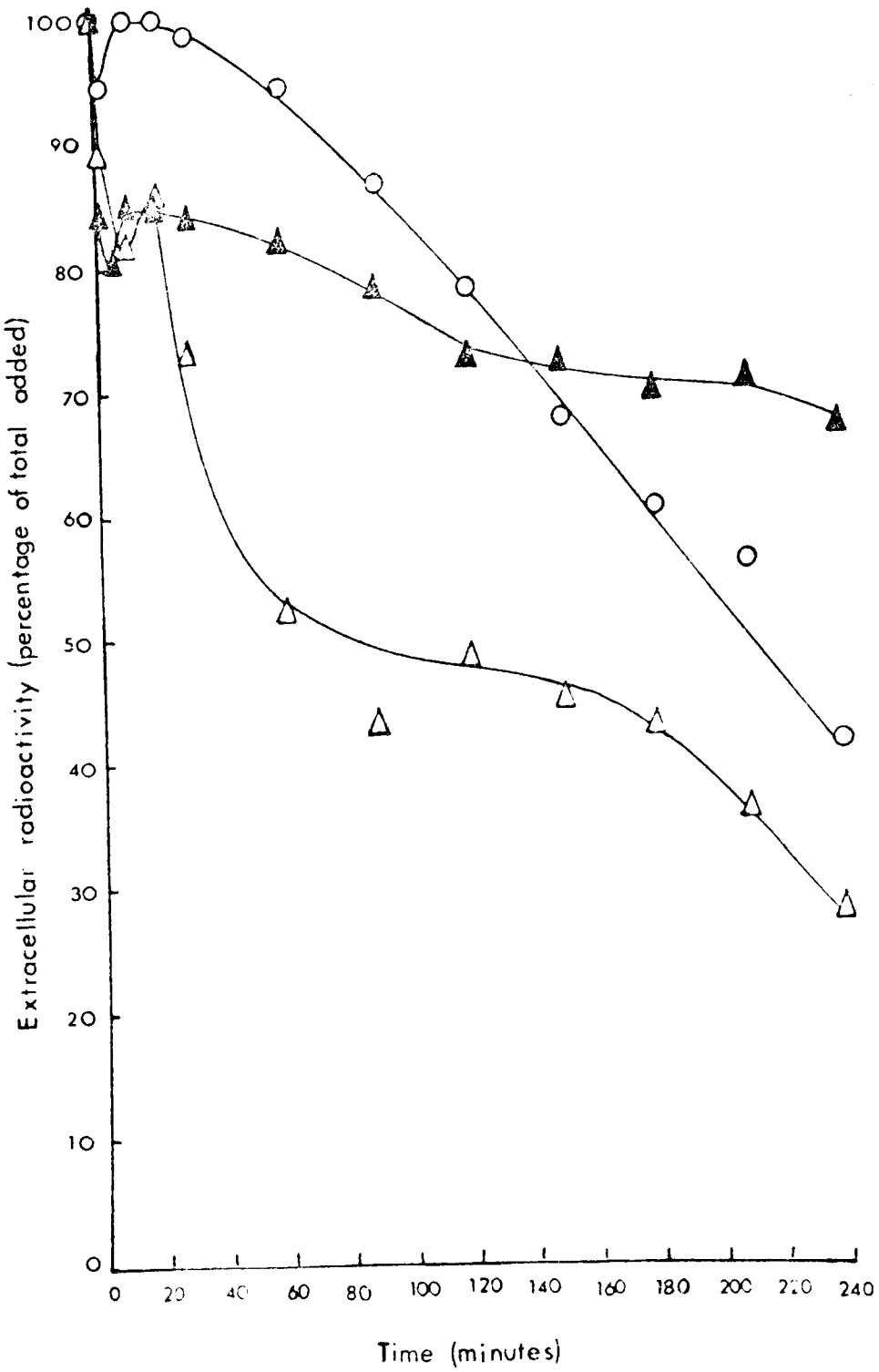
o——o DL-[1-^{14}C]-valine (0.078 μmoles/ml.; 2.5 μCi/ml) with protoplasts in 0.9M-NaCl (0.6g/ml).

¤——¤ DL-[1-^{14}C]-valine (0.04 μmoles/ml.; 1.42 μCi/ml) with protoplasts in 0.9M-NaCl (0.6g/ml).

x——x DL-[1-^{14}C]-valine (0.04 μmoles/ml.; 1.42 μCi/ml) with protoplasts in 0.9M-NaCl (0.5g/ml).

The sampling procedures are exactly as described in the legend to Fig. 9.

Fig. 11. Effect of increasing the concentration of DL-[1-^{14}C]-valine



Δ——Δ Mycelium (1g/6ml) in water

▲——▲ Mycelium (1g/6ml) in 0.9M-NaCl

o——o Protoplasts (0.4g/ml) in 0.9M-NaCl

DL-[1-^{14}C]-valine (1.7 µmoles/ml; 0.83 µCi/ml) was added to suspensions of mycelium (1g/6ml) in water, and pretreated mycelium in 0.9M-NaCl. To suspensions of protoplasts (0.4g/ml) was added DL-[1-^{14}C]-valine (1.7 µmoles/ml.; 2.5 µCi/ml).

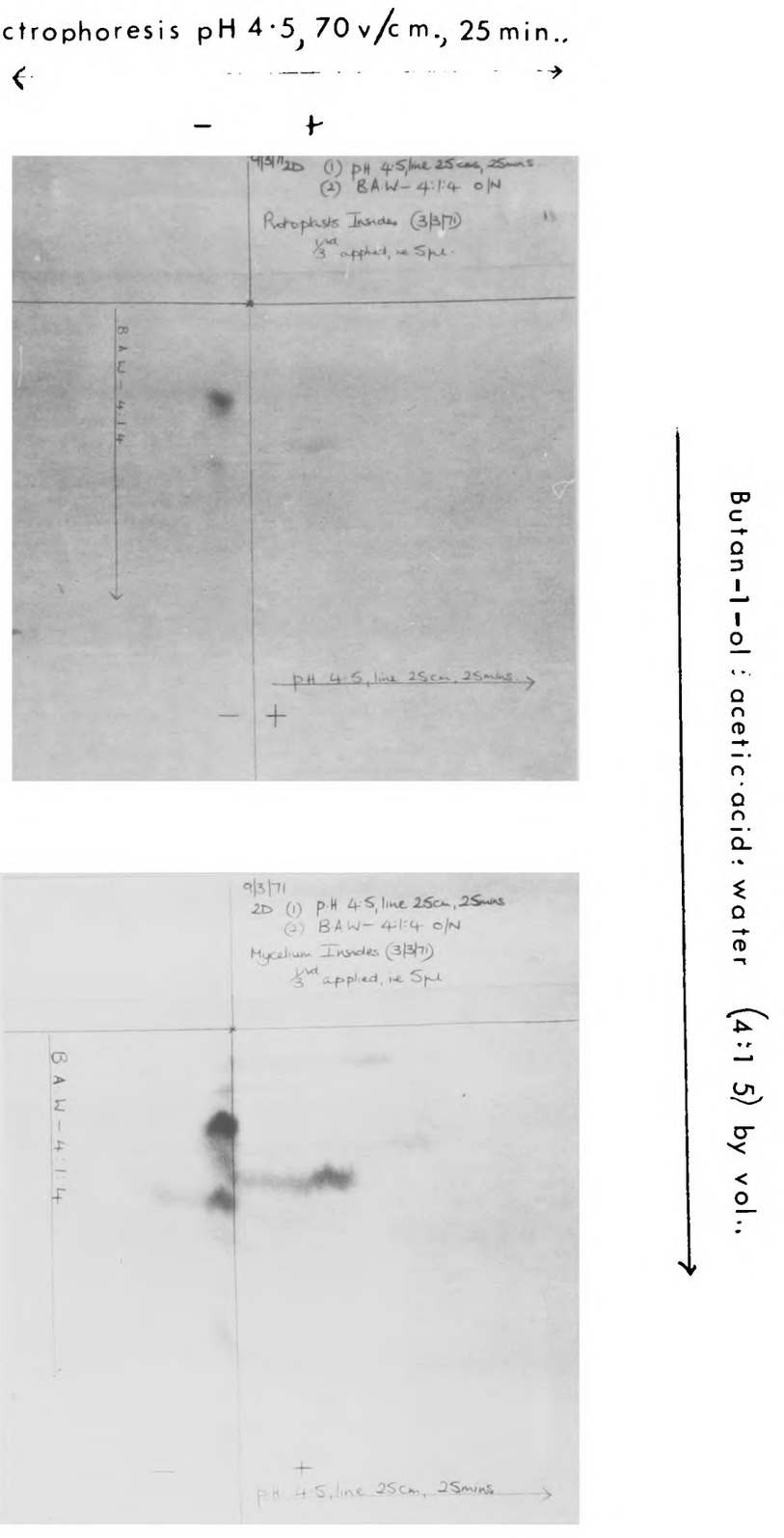
isotope disappeared from the suspension fluids were approximately the same with each suspension.

2. Variations between different protoplast suspensions. It can be seen in Fig. 10 that not only had individual suspensions taken up varying amounts of [^{14}C]-valine after 4 hours but that they also removed different amounts in the first phase, and released different amounts in the second. A higher initial rate of ^{14}C uptake was not necessarily associated with a greater release in the second phase, and the rate of removal of [^{14}C]-valine in the third phase did not appear to be correlated with previous transport activity.

3. Effect of increasing the concentration of DL-valine. Fig. 11 shows the percentage disappearance of radioactivity with time when the extracellular concentration of DL-[1-^{14}C]-valine was raised to 1.7 µmoles/ml by the addition of unlabelled valine. This concentration was 6.5 x 10 times greater than that used above (1, Fig. 9); nevertheless, the percentage of ^{14}C removed after 4 hours by each suspension was similar to that found with the lower concentration of valine. The overall uptake of ^{14}C was slower, however, 1 hour being required for the removal of 50% of the added ^{14}C from the extracellular fluid of the suspension of mycelium in water.

Other aspects of protoplast metabolism

In an attempt to find possible reasons for the



Figs. 12 & 13. Analysis by 2-dimensional paper electrophoresis and chromatography of the intracellular pools of equivalent amounts (on the basis of extractable nucleotide) of (12) protoplasts and (13) mycelium.

relatively poor ability of the protoplasts to synthesise antibiotics and to transport valine, other features of protoplast metabolism were examined.

The intracellular pools of protoplasts, and of mycelium in 0.9M-NaCl after incubation for 4 hours at 27°C were analysed by electrophoresis and chromatography on paper followed by coloration with ninhydrin (Figs. 12,13). Semi-quantitative visual estimations of the amounts of amino acids present indicated that the intracellular pools of the mycelial suspensions contained at least ten times as much total amino acid per cell as that of the protoplasts.

It was found that protoplasts released potassium ions into the stabilising solution (0.9M-NaCl) over the normal 4 hour incubation period. However, measurements of the potassium ion concentrations in the extracellular fluids and inside the protoplasts showed that, in spite of the efflux of K^+ ions, a more than adequate total internal amount (6.2 mmolar) remained after 4 hours to maintain an ATP-generating system.

δ-(DL-α-Aminoadipyl)-L-cysteine and antibiotic production by mycelium and protoplasts

It was thought that protoplasts might be more permeable to certain synthetic peptides than intact mycelium and that it might thus be possible to use protoplasts to study the involvement of these peptides in the biosynthesis of penicillin N and cephalosporin C.

To suspensions of mycelium in water and in 0.9M-NaCl

Table 2. δ-(DL-α-aminoadipyl)-L-cysteine and antibiotic production.

δ-(DL-α-aminoadipyl)-L-cysteine was added to each suspension to give a final concentration of 1.5 μmoles/ml. and L-valine to 2.1 μmoles/ml. DL-[1-^{14}C]-valine was added to the protoplast suspensions (5 μCi/ml.) and to the suspensions of mycelium (0.83 μCi/ml.) The protoplasts were incubated at 120 rev./min. and the mycelium at 200 rev./min., 27° for 4 hours. Antibiotics were extracted from the culture fluids and identified after paper electrophoresis and chromatography as described in the Methods Section.

Suspension	Addition	Total Radioactivity (nCi) Found in Penicillin N	Cephalosporin C
Mycelium in water (1 g./6 ml.)	DL-AC + L-Val	4.0	3.6
	L-Val	7.3	4.1
Mycelium in 0.9M-NaCl (1 g./6 ml.)	DL-AC + L-Val	2.2	0.3
	L-Val	6.2	1.3
Protoplasts in 0.9M-NaCl (0.4 g./ml.)	DL-AC + L-Val	0.4	0.7
	L-Val	0.8	1.1

and of protoplasts in 0.9M-NaCl were added δ-(DL-α- amino-adipyl)-L-cysteine and L-valine or L-valine alone. DL-[1-^{14}C]-Valine was added to all the suspensions. The amounts of antibiotic found in the extracellular fluids after 4 hours incubation at 27° are shown in Table 2.

It appeared that the presence of DL-AC had an inhibitory effect on antibiotic production by mycelium because these suspensions incorporated less total ^{14}C into antibiotics than the suspensions of mycelium which had been incubated with valine alone.

At the end of the incubation the extracellular fluid was removed by filtration and freeze-dried. The suspensions were each washed well with water, then extracted with aqueous ethanol to extract amino acids and other substances from the intracellular pool, and the extracts freeze-dried.

Analysis by 2-dimensional paper electrophoresis and chromatography revealed the presence of the disulphide of δ-(α-aminoadipyl)cysteine in the extracellular fluids from all the suspensions to which the dipeptide had been added. Approximately 10% of the added dipeptide was found in the intracellular pool of the protoplasts, but none was detected in the intracellular pools of the suspensions of intact mycelium. After oxidation, increased amounts of α-aminoadipic acid and cysteic acid were found in both the

intracellular pool and extracellular fluid from the suspension of mycelium in water. There was no evidence of hydrolysis of the dipeptide by the protoplasts or by the mycelium in sodium chloride.

Discussion

When 48 hour cultures of mycelium were incubated for 1 hour in 0.02M-2-mercaptoethanol or 0.01M-dithiothreitol there was at least an 80% decrease in cephalosporin C formation, but penicillin N production was relatively unaffected. It is known that when the rate of aeration of mycelial suspensions is reduced, a selective decrease in cephalosporin C production occurs (Warren, 1964). The effect of the thiol-containing compounds 2-mercaptoethanol and dithiothreitol may thus be analogous to that of a relatively low oxygen tension.

The overall results of the resuspension experiments indicated that the cephalosporin C-synthesising system in intact mycelium was more sensitive than that of penicillin N to external conditions. A possible explanation is that enzymes, or intermediates, involved specifically in cephalosporin C synthesis are more labile than those required for penicillin N production. Alternatively, the mycelium may be unable to transport small amounts of intracellular cephalosporin C to the outside of the cell in the presence of sodium chloride.

Whereas cephalosporin C synthesis was inhibited, but penicillin N synthesis was unaffected in this type of

mycelial suspension, the situation was reversed in the protoplast suspensions. These results militate against the hypothesis that penicillin N is a precursor of cephalosporin C. Cephalosporin C was always found in the protoplast suspension fluids immediately after resuspension, and the amounts present after 4 hours incubation were equivalent to those formed by control suspensions of mycelium in water. It seemed unlikely that all of the extracellular cephalosporin C present at the beginning of the incubation (sometimes 50% of the total produced) was synthesised de novo by the protoplasts. However, cephalosporin C leaked out of the protoplast/mycelial suspensions while they were incubating in snail enzyme solution and during the subsequent washing with stabiliser, indicating that after treatment with snail enzyme this antibiotic was loosely bound to cells, and thus easily removed by agitating the suspensions.

This finding raised the question why the protoplasts released cephalosporin C immediately after resuspension but not penicillin N. Treatment with snail enzyme may have depleted the mycelium of certain enzymes, co-factors or substrates involved in the later stages of penicillin N production, which possibly resided outside the protoplast membrane. The variability in penicillin N production by different preparations of protoplasts may reflect the extent

of this depletion and the ability of individual suspensions to replenish lost metabolites. Evidence that penicillin N and cephalosporin C were synthesised de novo by the protoplasts was provided by the observation that the latter incorporated ^{14}C from [^{14}C]-valine into both antibiotics, even when the amounts formed were not detectable by bioassay.

It was estimated that the total amount of free amino acids in the intracellular pool of the protoplasts was less than one tenth of that in the mycelial cells, relative to the amount of ethanol extractable material (presumably nucleotide) which showed a U.V. absorption at 260 nm. But before extraction of their amino acids the protoplasts had been centrifuged from their culture fluids and washed three times at the centrifuge. Since the protoplasts appear to have less retentive ability than the mycelial cells, it is possible that the intracellular amino acids leaked out into the stabiliser during washing.

Similarly, potassium ions leaked out of the protoplasts into the stabiliser during the 4 hour incubation period. Although an overall concentration of potassium adequate for an ATP-generating system was retained inside, it was not known whether this was readily available for the reactions involved in peptide bond formation.

One consequence of the enhanced permeability of the protoplasts was that the dipeptide δ-(DL-α-aminoadipyl)-L-cysteine was able to pass intact into the intracellular

pool. However, the protoplasts to which the dipeptide was added did not incorporate more radioactivity into antibiotics from DL-[^{14}C]-valine than control suspensions which had had valine alone added to them. A possible inference from this result is that, in contrast to the situation with broken cell systems of mycelium (Loder & Abraham, 1971), the synthesis of the dipeptide is not a rate limiting step in antibiotic formation by the protoplasts. But, it is also possible that δ-(α-aminoadipyl)-cysteine taken up by the protoplast is unable to exert a stimulatory effect because there are further permeability barriers which prevent it from reaching the sites at which antibiotic production occurs.

Further assessments of the metabolic activities of protoplasts and of mycelium were made from their abilities to take up DL-[1-^{14}C]-valine from their extracellular fluids.

Whether the transport of exogenous valine into the protoplast was energy-dependent and the extent to which this amino acid accumulated or existed in the free state in an intracellular compartment was not determined directly. However, earlier experiments by Warren, Newton & Abraham (1967b) had shown that L-[^{14}C]-valine was taken up very rapidly and almost completely by intact mycelium suspended in water, presumably by an active transport process. Nearly

50% of the ^{14}C was found as free valine in the intracellular pool within 10 minutes, but this valine was decreased by metabolism to 10% after one hour. In contrast, D-valine was taken up much more slowly and was converted to L-valine in the cell. It was thus reasonable to expect that a biphasic curve would be obtained for the uptake of DL-[1-^{14}C]-valine by protoplasts and would reflect, at least in part, differences in the behaviour of the D- and L-isomers of the amino acid. A transport system with a preference for L- rather than D-amino acids has also been reported in Botrytis fabae (Jones, 1963) and Neurospora crassa (Pall, 1969), although in the latter case a system which transported D- as well as L-amino acids was present in older cultures.

By analogy with the results of studies with intact mycelium, the initial and rapid disappearance of ^{14}C from the medium when DL-[1-^{14}C]-valine was added to a suspension of protoplasts in 0.9M-NaCl may be tentatively attributed to an uptake of L-valine. In one experiment this would have accounted for virtually all the L-valine added. With other preparations of protoplasts the uptake in the rapid phase was significantly lower, in parallel with the finding that less valine was taken up in this phase by intact mycelium suspended in 0.9M-NaCl than by mycelium suspended in water. The mechanism by which NaCl exerts

this effect is unknown. If a proportion of the surface acceptor sites involved in the transport of L-valine were inactive in a high concentration of sodium chloride the rate, rather than the extent, of uptake would be likely to be mainly affected. Moreover, it is clear that the transport mechanism was not saturated in the presence of NaCl by the lower of the two concentrations of valine used.

The onset of the second phase was characterised by a rapid increase in the amount of ^{14}C in the medium. A "huff and puff" mechanism has been suggested whereby a rapid uptake of solute by a cell is followed by an inflow of water which temporarily distends the cell membrane, and results in the release of some of the solute into the surrounding medium (McClure quoted by Britten, 1965). The protoplast membrane would be expected to be distended more easily than the corresponding membrane of the mycelial cell, and this might explain why the ^{14}C release was more striking with the protoplasts than with mycelium.

If the sequence of events suggested here were correct, the third phase, in which ^{14}C disappeared from the medium more slowly than in the first, could have been due to the uptake by the protoplasts of some, or most, of the remaining external L-valine, or to the uptake of both the L- and D-isomers. Experiments in which L- and D-valine were used separately would be required to throw further light on

this question. However, from the present experiments it could be concluded that the efficiency of incorporation of ^{14}C from added DL-[1-^{14}C]-valine into penicillin N and cephalosporin C by the protoplasts, would probably be lower than in corresponding experiments with intact mycelium.

SECTION IV

BIOSYNTHETIC ACTIVITY OF CELL-FREE EXTRACTS OF *CEPHALOSPORIUM* sp. C-91

SECTION IV

BIOSYNTHETIC ACTIVITY OF CELL-FREE EXTRACTS OF *CEPHALOSPORIUM* sp. C-91

Studies by Loder *et al.* (1969) showed that δ-(DL-α-aminoadipyl)-L-cysteine was not taken up by intact mycelium but was hydrolysed near the cell surface. Protoplasts did take up the dipeptide intact, but there were indications that its synthesis was not a rate-limiting step in the biogenesis of penicillin N and cephalosporin C in this system. Thus, cell-free extracts of *Cephalosporium* sp. C-91 were prepared to study the biosynthesis of antibiotics and their intermediates.

Results

Properties of cell-free extracts

Grinding the mycelium with sand or alumina gave rise to a very thick paste which after centrifugation at 4,500 g yielded a buff-coloured, opalescent supernatant containing small particles, broken cells and cell wall fragments. Centrifugation at 20,000 g precipitated the particulate matter as a brown sediment. The supernatant was clear and pale yellow in colour, and had a white lipid layer floating on top of it.

Suspensions of protoplasts appeared as fine dispersions of particles which were pale grey in colour.

Table 3. Properties of cell-free extracts obtained from *Cephalosporium* sp. C-91

The Extracts were prepared as described in the Methods Section and protein estimations were carried out on the whole extracts and/or their 20,000 g supernatants.

Extract	Mycelial weight (g.)	% yield protoplasts	Volume of extract (ml)	Protein (mg/ml)
Sand extract 72 hour mycelium				
20,000 supernatant	10		5.0	30
complete				27
Alumina extract 48 hour mycelium				
complete	4		1.0	
Alumina extract 72 hour mycelium	3.5		1.4	∿35*
complete				
Protoplast sonicate I	2.0	12		
20,000 supernatant			1.2	2.82
20,000 sediment			1.2	0.42
Protoplast sonicate II complete	10.5	12	1.0 ml	11
Protoplast lysate	9	12	4.0	6

*Loder *et al*. 1969.

After sonication less than 1% of the protoplasts remained intact, and no protoplasts survived osmotic lysis. Centrifugation of these extracts at 200 g sedimented membrane debris and intact protoplasts. The supernatant still contained numerous small particles and vacuoles, which were removed by centrifugation at 20,000 g and formed a pale buff sediment. The 20,000 g sediment was clear and colourless but still retained the lipid layer at its surface.

It was difficult to withdraw the high speed supernatants from either extract without introducing a small amount of lipid into the fraction as well. Some other properties of the extracts are shown in Table 3.

Biosynthesis of glutathione and the ACV complex*

Comparisons were made of the abilities of various cell-free extracts to bring about the synthesis of glutathione and the ACV complex from [^{14}C]-glycine and [^{14}C]-valine respectively in the presence of an ATP-generating system. The results of these experiments are shown in Table 4.

Glutathione was synthesised from L-glutamic acid, L-cysteine and [1-^{14}C]-glycine by all the extracts. The high speed supernatant fractions from the sand-ground mycelium

* The term "ACV complex" is used to describe a mixture of peptides containing α-aminoadipic acid, cysteine, valine and certain other amino acids.

Table 4. Biosynthesis of cysteine-containing peptides by cell-free extracts of Cephalosporium sp. C-91

Each experiment was performed with a different batch of mycelium. The amounts of amino acids added to the extracts are given in the Methods Section. Total volumes were 1.0 ml. The specific activity of the added [1-^{14}C]-glycine was 0.25 μCi/mmole in both the sand and protoplast sonicate extracts. The specific activity of added DL-[1-^{14}C]-valine in the sand extract was 0.125 μCi/mmole, and in the protoplast extracts 1.25 μCi/mmole. Incubations were for 2 hours at 27°, 140 rev./min. Abbreviations: AC, δ-(α-aminoadipyl)-L-cysteine; Aad, α-aminoadipic acid; ACV, δ-(α-aminoadipyl)-cysteinylvaline; P4 is described in the text.

	Extract	Precursors	Radioactivity (nCi) in Glutathione	ACV Complex	P4
	Alumina extract from 48 hour mycelium				
1	Complete extract	[^{14}C]-Gly+L-Glu+L-Cys	1.1		
	Alumina extract from 72 hour mycelium				
	Complete extract	"	7.9		
	Sand extract from 72 hour mycelium				
	Complete extract	"	6.9*		
	20000 g supernatant	"	45.6		
2	Complete extract	DL-[^{14}C]-Val+DL-Aad+L-Cys			0.6
	20000 g supernatant	"			0.6
	Protoplast sonicate from 48 hour mycelium				
3	20000 g sediment	[^{14}C]-Gly+L-Glu+L-Cys	0.3		
	20000 g supernatant	"	1.8		
	Protoplast sonicate				
4	Complete extract	DL-[^{14}C]-Val+DL-Aad+L-Cys		<0.1	
	Protoplast lysate	DL-[^{14}C]-Val+L-AC		0.4	
5	Complete extract	DL-[^{14}C]-Val+L-Aad+L-Cys		<0.1	
		DL-[^{14}C]-Val		<0.1	

* The low value obtained with the whole extract may be due to hydrolysis of γ-glutamylcysteine by particulate matter (see Loder et al., 1969).

and the protoplast sonicate incorporated approximately six times as much radioactivity from [^{14}C]-glycine into glutathione as the corresponding particular fractions. The supernatant from the sand-extract was twenty-five times as active in this respect as that from the protoplast sonicate, but the former contained ten times as much protein as the latter. It appeared that differences in the glutathione synthetic activity of these extracts may be dependent on the age of the starting mycelium since an alumina extract from 48 hour mycelium was eight times less efficient at synthesis than an extract from the same batch of mycelium harvested after 72 hours growth.

Unlike glutathione, which was synthesised from its component amino acids, the formation of the ACV complex from [^{14}C]-valine was detected only after the addition of the dipeptide δ-(L-α-aminoadipyl)-L-cysteine. The labelled ACV complex appeared as a single spot after electrophoresis at pH 1.8. Further analysis by chromatography in a second dimension in butan-1-ol:acetic acid:water resolved the complex into two close running spots. These were identified as the sulphonic acids of δ-(α-aminoadipyl)cysteinylvaline (P3) and of a peptide designated P2, which contains α-aminoadipic acid, cysteine, valine and glycine (Loder & Abraham, 1971).

After the addition of DL-α-aminoadipic acid, L-

Table 5. Incorporation of [^{14}C]-valine into antibiotics by cell-free extracts from protoplasts

DL-[1-^{14}C]-valine (sp. act 0.5 μCi/μmole) and an ATP-generating system was added to each extract. Incubations were for 2 hours at 140 rev./min., 27°. Antibiotics were separated from the extracts and identified as described in the Methods Section. Total volumes were 1.0 ml.

Extract	Protoplast concentration g./ml.	Protein concentration mg/ml.	Total Penicillin N nCi	Total Penicillin N nmoles	Total Cephalosporin C nCi	Total Cephalosporin C nmoles	N/C
Whole sonicate	0.4	3.5	1.4	5.6	0.2	0.8	7
200 g sediment			1.1	4.4	0.8	3.2	1.4
200 g supernatant			0.9	3.6	0.1	0.4	9
20000 g sediment			*		0.9	3.6	*
20000 g supernatant			*		0.1	0.4	*
Whole lysate	0.3	1.8	0.36	1.4	0.3	1.2	1.2
200 g supernatant			0.94	3.8	0.54	2.2	1.7
20000 g sediment			0.44	1.8	0.36	1.4	1.2
20000 g supernatant			1.0	4.0	0.54	2.2	1.9

* The amount of radioactivity incorporated into penicillin N by these fractions could not be measured because of contamination from [^{14}C]-valine after electrophoresis at pH 4.5 due to inefficient desalting.

cysteine and [^{14}C]-valine to complete sand-extracts and to the 20,000 g supernatants derived from them, labelled material was formed which migrated to almost the same position as the ACV complex when subjected to electrophoresis at pH 1.8. When chromatographed in butan-1-ol:acetic acid:water (4:1:4 by vol.) this material separated into two spots which showed R_{ACVSO_3H} values of 0.4 and 0.28, and was hereafter referred to as the P4 complex.

Biosynthesis of penicillin N and cephalosporin C by protoplasts disrupted by sonication or osmotic lysis

The antibiotics were synthesised in low yield by both types of extract from protoplasts, and fractions derived from them, from L-α-aminoadipic acid, L-cysteine and DL-[^{14}C]-valine, in the presence of an ATP-generating system (Table 5).

The experiments with extracts from sonicated protoplasts showed that the particulate fractions incorporated slightly more ^{14}C into penicillin N and cephalosporin C than the corresponding supernatant fractions. Conversely, it appeared that the supernatant fractions derived from osmotically lysed protoplasts were more active than the particulate fractions. The 20,000 g supernatant incorporated the most radioactivity into penicillin N, and was as efficient as the 200 g supernatant in bringing about the

synthesis of cephalosporin C. All the extracts incorporated more ^{14}C into penicillin N than into cephalosporin C. On the whole it appeared that the penicillin N:cephalosporin C ratio was higher in the sonic extracts than in those from the osmotically disrupted protoplasts.

Discussion

Cell-free extracts from 72 hour mycelium proved to be more efficient at synthesising glutathione than those derived from 48 hour cultures or those prepared from protoplasts. Most of the glutathione synthesising activity was found in the supernatant fractions of the sand extract and the protoplast sonicate. In the presence of an ATP-generating system these extracts possessed all the enzymes required for the synthesis of glutathione from its component amino acids.

In contrast, synthesis of the ACV complex was detected only after the addition of δ-(L-α-aminoadipyl)-L-cysteine to a suspension of osmotically lysed protoplasts. This finding suggests that not only is the dipeptide an intermediate in ACV formation, but also that, unlike the situation in intact protoplasts, its synthesis is a limiting factor in the cell-free extract.

It was found that protoplast sonicates and lysates were able to incorporate small amounts of ^{14}C-valine into antibiotics in the presence of α-aminoadipic acid, cysteine, and ^{14}C-valine. Thus, if δ-(α-aminoadipyl)-cysteinylvaline is an intermediate in antibiotic formation these extracts must have been able to synthesise the tripeptide from its component amino acids, but it may have undergone rapid turnover and hence been present in insufficient amount to be

detected.

So far, the synthesis of penicillin N and cephalosporin C in a cell-free system has only been detected in extracts prepared from protoplasts. After the protoplasts had been disrupted the penicillin N-forming biosynthetic system appeared to survive better than that forming cephalosporin C; while with the intact protoplasts the converse was true. It is possible that the synthesis of cephalosporin C is associated with an unstable complex which depends on the integrity of part of the protoplast for full activity.

On the assumption that the ^{14}C-labelled ACV complex had the same specific radioactivity as the added ^{14}C-L-valine and that the peptides were not further metabolised after they had been formed, the amounts synthesised can be calculated. From the results obtained in experiment 5 of Table 4, 2.2 nmoles of ACV complex were formed in two hours in a protoplast lysate derived from 1 g. of mycelium in 100% yield. In a broken cell system prepared from 72 hour mycelium by ultrasonic disintegration 30 nmoles of the ACV complex were formed in one hour in a preparation derived from 1 g. of mycelium and containing about 36 mg of protein (Loder & Abraham, 1971).

Similarly, the amounts of antibiotics formed can be calculated. The data in Table 5 indicates that 14 nmoles of

penicillin N and 2 nmoles of cephalosporin C were formed in four hours by a whole sonicate of protoplasts derived from 1 g. of mycelium. The amounts of extracellular penicillin N and cephalosporin C formed in this time by a suspension of 1 g. of washed mycelium are approximately 300 nmoles (Figures 5 and 6).

CONCLUSIONS

CONCLUSIONS

The present work has shown that osmotically-sensitive protoplasts can be made from the Cephalosporium sp. C-91 which retain 30-50% of the antibiotic synthesising ability of the whole mycelium. The process, however, is time-consuming and the protoplasts' relatively poor performance may be a reflection of their metabolic exhaustion. It might be possible to shorten the procedure by the use of purified enzyme preparations in place of crude snail enzyme and thereby produce metabolically more active protoplasts. Although mycelium harvested after 48 hours had been used because its wall is relatively thin, mycelium at a later period of growth, when antibiotic production is at its maximum, may yield protoplasts with a greater synthetic ability.

The uptake of [^{14}C]-valine by the protoplasts was not as efficient as that by intact mycelium. The initial rapid removal of ^{14}C after addition of DL-[^{14}C]-valine was followed by an equally rapid release of what was assumed to be [^{14}C]-valine, since it seemed unlikely that such a large increase in extracellular ^{14}C in such a short time could be due to the release of labelled antibiotics, especially from the protoplasts. More information could be obtained from analyses of the intracellular pool and extracellular fluids and the use of the individual D- and L-

isomers of [^{14}C]-valine to throw light on the fate of this amino acid when added to protoplast suspensions.

The increased permeability of the protoplasts was reflected in their ability to take up the dipeptide δ-(α-aminoadipyl)-cysteine intact, but also in the release of cephalosporin C on mechanical disturbance and in the depletion of the amino acid content of the intracellular pool. Although the dipeptide entered the protoplasts, it apparently had little effect on antibiotic synthesis, and it was not possible to draw any conclusions about its status as an intermediate in this system. But from experiments with cell-free systems it appeared that the dipeptide is a precursor of δ-(α-aminoadipyl)cysteinyl-valine and a related peptide.

In addition to members of the ACV complex, cell-free extracts from protoplasts synthesised both penicillin N and cephalosporin C, but with only 1% of the efficiency of the whole mycelium. Improvements in the efficiency of the cell-free systems would hence be desirable. The 20,000 g supernatant from the protoplast lysate, which contained neither intact protoplasts nor cell debris, could be the first step in the preparation of a purified enzyme complex able to synthesise the antibiotics.

Much of the work could be regarded as exploration: first, of the conditions required for making protoplasts

from the Cephalosporium sp., and thereafter of the potentialities of the protoplasts for the preparation of a cell-free extract in which to study the biosynthesis of penicillin N and cephalosporin C. It is evident that more information could be obtained from the systems described here: the possible involvement of δ-(α-amino-adipyl)cysteine, for instance, in antibiotic production by the protoplast extracts has not been explored; and the use of isotopically-labelled synthetic intermediates should throw further light on the biosynthetic pathway.

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