BIOCHEMICAL AND MORPHOLOGICAL CHANGES IN *STREPTOMYCES RIMOSUS* ON MUTATION TO HIGHER YIELDS OF OXYTETRACYCLINE

BY

MONIS AL-JAWADI

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TO MY MOTHER AND FATHER

MONIS AL-JAWADI

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ABSTRACT

The effect of mutation to higher yields of wild and mutant strains of S.rimosus producers of oxytetracycline (OTC) was investigated. The main areas of study were: fermentation studies in shaken and stirred cultures, paper chromatography of the broth produced by the cultures, study of the effect of culture conditions on the production, biochemical studies of the levels of enzymes and adenylates, cosynthesis studies using blocked mutants, morphological studies and some investigation of the taxonomy of the strains and mutants, using classical and a recently developed computer method. The results showed that the best producing mutants, of different origins, resemble S.rimosus, while the wild strains varied between S.rimosus and S.aureofaciens. The mutants grew steadily, were resistant to OTC, and had a long producing period. The wild strains grew erratically and inefficiently and were therefore difficult to control. They were sensitive to OTC and had a short production span. Biochemically, the mutants showed increases in the enzyme levels compared to the wild strains, but there seemed to be no relation between this and productivity. Characteristically, the mutants produced a brown pigment chemically related to OTC. Its rate of production was however, not directly linked with the formation of OTC, and it must be regarded as a drain on resources which causes a loss of OTC production. The main difference between the wild strains and the mutants were that the mutants are better controlled and more efficient and can be manipulated fairly easily. The wild strains differ among themselves and behave in an uncontrolled manner, so that it would be difficult to develop efficient processes with them. The results differed from those obtained by Czechoslovakian workers using S.aureofaciens producing of chlortetracycline, however their strains differed considerably from ours and the details of their work were also different.

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D E C L A R A T I O N

I wish to declare that while registered as a candidate for the award of Doctor of Philosophy by the C.N.A.A. I was not a registered candidate for any other higher award.

1. INTRODUCTION

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1.8 OBJECTIVES OF THE PRESENT PROGRAMME

There has been a general interest for a long time in the factors involved in the production of secondary metabolic products, especially in the biochemistry and physiology of these processes, recently increased by the possibility of applying direct genetic approaches to strain development. Of particular interest in this connection has been the investigation by a group of Czech. workers such as Hostalek et al (1976), Behal et al (1969a) and Vanek et al (1978), into the changes which took place in a wild strain of S.aureofaciens during mutation to higher level of chlortetracycline production. In general their improved mutant showed less active enzyme metabolism and growth activity. On the other hand, work by Calam and Smith (1981), with penicillin production by P.chrysogenum generally showed increased growth and enzyme activity suggesting that different principles were involved in this case.

The availability of a wild strain producing oxytetracycline (OTC), 72T1, and its mutant, Y20 giving several times as much OTC, (obtained from I.C.I. Ltd., Pharmaceutical Divisions) made possible the present programme in which a wide range of studies were applied on the different strains to compare the behaviour of S.rimosus with the results obtained by the Czech. workers with S.aureofaciens, and to obtain new information on changes associated with strain improvement.

Another factor was that there has been continuous interest in the genetics of oxytetracycline production, for example the recent paper by Rhodes et al (1981).

1.1 MEANING OF SECONDARY METABOLISM

The term secondary metabolic product was in the past applied to the production by plants of minor products such as colours, perfumes, alkaloids, resins etc. Although reluctantly at first, it is now recognised that these substances have a strong ecological significance. In fungi and streptomycetes, secondary metabolism was also recognised, but there has been a greater degree of argument as to whether it is significant or not. Some degree of ecological significance is now beginning to be recognised (Calam 1982; Demain and Piret (1981).

The areas of metabolism involved in the production of secondary metabolic products are shown in Tables 1 and 2 (Nüesch 1981).

Table 1. The general areas of primary and secondary metabolism

Primary metabolism	Area of Intermediate metabolism	Secondary metabolic Products
Oxygen → Carbo-hydrate → Fat → Nitrogen	Catabolism and Processes aimed at growth and energy production →	Supply of intermediates: Acetyl → Malonyl Amino acids Methyl groups Mevalonate etc. → "Starters" supplementary ↑ reactions	Colours Odours (terpenes) Antibiotics → Alkaloids etc. By-Products →

Primary metabolism (Table 1) is concerned with the processes of growth and energy production, leading, via intermediate metabolism, to various types of secondary metabolic products. In between is the important area providing intermediate substances which act as building blocks as well as the primers which act as starters of chain formation in polyketide and other products.

The differences between primary and secondary metabolism are summarised in Table 2.

Table 2. The differences between primary and secondary metabolic products

Secondary	Primary
A very heterogenous group of complex compounds, of low M.W. Individual products often restricted to particular isolates. Not essential for growth.	A group of universally occurring compounds. Essential for growth.

The biosynthesis of secondary metabolic products involves the following steps:-

(1) Initiation:- Leading to production of precursors, their transportation and activation.

(2) Synthesis of skeleton:- Polymerisation and chain elongation.

(3) Terminal reactions:- Oxidation, transformations, substitution of the skeleton leading to the final product.

(4) Excretion

These processes are complex and as much as 10% of the cell's genes may be involved in them. (cf. Vanek *et al* 1971).

Hostalek *et al* (1969a) showed that a chlortetracycline mutant giving higher yields showed reduced growth. Other workers think that secondary metabolism is linked with differentiation and accompanies particular events such as the onset of sporulation (Clark *et al* 1963).

Another aspect is the question of "over production", it is considered that cultures e.g. *P.chrysogenum* normally grow in nature in very restricted conditions and in competition with other soil inhabitants. Its physiology therefore reflects the requirement of the situation, and its secondary metabolism may form part of its life system. The mechanism triggering secondary metabolism will form part of this system (Calam 1982). The quantities of penicillin produced are small, experience suggests the equivalent of 0.01-0.05g/l in culture. The amount of material necessary to withdraw from the growth process for this purpose is very little. The productivity of such strains can be increased in suitable media. For example, in the present study, the wild strain (72T1), produced little OTC in media, like nutrient agar, but this can be increased to 1.0g/l in complex industrial media. By mutation and selection an output of 25g/l was obtained twenty years ago, and industrial production levels are probably now twice this value, even the mutant, (Y20), in the present study, which was derived from the wild strain 72T1 produces about half as much oxytetracycline as cells during the production period. This is clearly "over production". Where over

production starts though is hard to determine. Hostalek's wild strain produced about 0.2g/l and his mutants about 1.2g/l. However our own strains produced very little oxytetracycline when grown in his media.

It is under such low yielding conditions that most of the published work on the changes on mutation to higher yield have been conducted. While such experiments demonstrate factors associated with increased production, it is difficult to say whether these conclusions also apply to industrial conditions.

Secondary metabolism often appears late, when the cells are ageing and nutrients become exhausted, or when the oxygen supply becomes limiting in the submerged culture. This led to the view that reduced growth rate is necessary for it to occur. Some workers have linked this with the phosphate concentration, as a factor for controlling growth rate (Hostalek 1964; Biffi et al 1954).

1.2 PROTECTION SYSTEM OF STREPTOMYCETES AGAINST THEIR OWN ANTIBIOTICS

The mechanisms by which the microorganisms become able to resist their own antibiotics have been studied by many workers, and was frequently associated with ribosomes. For example Sagiyama et al (1980) stated that a neomycin sensitive strain of S.fradiae producing that antibiotic, showed ribosomes sensitive to neomycin. Mikulik et al (1971) showed that in S.aureofaciens there was accumulation of chlortetracycline in the cultivation medium, which resulted in the formation of a tetracycline-ribosome aggregate, and they concluded that the protein synthesizing system of tetracycline producing microorganisms was more resistant to the antibiotic effect than the similar system isolated from drug-sensitive bacteria. Skinner and Cundliffe (1982) established a connection between the presence of dimethyladenine in S.erythraeus and the

resistance of its ribosomes to antibiotics.

1.3 GENERAL MODES OF TETRACYCLINE BIOSYNTHESIS

The importance of the starter-chain formation system in OTC biosynthesis was pointed out by Gatenbeck (1961) who observed that the starter was monoamide of malonyl-CoA, on which acetyl-CoA units were built up head to tail to form the tetracycline structure leading to OTC. This process is

Starter ⟶ malonyl-CoA units ⟶ chain formation (polymerisasion) ⟶ cyclisiation ⟶ substitution ⟶ etc ⟶ tetracycline

Gatenbeck also noted that the acetoacetyl-CoA could act as a starter if malonamide was not in sufficient amount. It is also known that fatty acid biosynthesis starts from acetyl-CoA, malonyl units being added one by one which are then decarboxylated and reduced. These reactions involved the attachement of the units to protein particles which act as supports during reduction and chain extension. Thus fatty acid biosynthesis is said to occur on a template. Such templates are regarded as being an essential feature of antibiotic production. In the case of the tetracyclines, the amido group is essential for high anti-biotic activity.

The biosynthetic route to oxytetracycline has been recently re-investigated by Thomas and Williams (1983 a,b), using ^{13}C labelled acetate or malonate. The exclusive polyketide origin of the tetracycline nucleus was established as well as the direction of folding of a hypothetical linear intermediate. The direct incorporation of malonate into the carboxamide group was also demonstrated, with amidation by ammonia, as originally proposed by Gatenbeck (1961). The failure of some of the precursors, tested by Behal *et al* (1974) may have been due to wall impermeability.

Neüsch (1981) commented on the production of tetracycline, in which the main structure and the biosynthesis have been known for many years (Hochstein et al 1960), but very little is known about the synthesis of the polyketide structure, except that malonyl is involved. It is not known whether the enzymes involved in the production of intermediates are the same as the ones in the primary area or whether a separate group are required. The subject of the initiation and regulation of the production of secondary metabolic products and their over production has been the topic of considerable discussion (FEMS sympcsium 1981).

1.4 CZECHOSLOVAKIAN WORK ON THE BIOSYNTHESIS OF CHLORTETRACYCLINE (CTC)

Studies by Behal et al (1969a) showed that the lipid content was roughly the same in five strains of S.aureofaciens, and it did not vary greatly during the fermentation, being independent of the production of chlortetracycline (CTC), Behal et al (1969b) using labelled acetate, found that all strains of S.aureofaciens produced labelled fatty acids in small amounts especially during the period 12-24 hours. After this time labelled units of acetate contributed to CTC production especially with the highyielding mutants.

The enzyme for the synthesis of fatty acids, acetyl-CoA carboxylase, was found to be twice as high in the production strain (Behal et al 1970). Hostalek et al (1969a) studied the rate of production of CTC with relation to the TCA-cycle enzymes and found that in the production phase the activity of the enzymes in the low production strain was 2 to 5 times higher than the production strain. The participation of supplementary pathways, especially for the formationof malonyl from oxalacetic acid (O.A.A.) arising from PEP by carboxylation via PEP-carboxylase was discussed and described by Hostalek et al (1976). This author also found that malic enzyme and the pentose cycle enzymes were active

during the production phase and could contribute to the supply of NADPH, which is required for CTC production.

These and other results supported the view that supplementary pathways were providing acetyl and malonyl units for the biosynthesis of CTC. Hostalek and colleagues consider that, in their mutant CTC was mainly formed from malonyl derived from PEP rather than from acetyl units.

Investigation by Behal et la (1974) suggested that the mono-malonamide unit, which acts as a starter for the biosynthesis and which they referred to as the terminal group, is derived by different route. This conclusion was reached because, except for asparagine, a series of precursors tested were not incorporated into the terminal group, suggesting a different route for the synthesis of malonamide, e.g. by decomposition of a branched fatty acid (Mikulik et al, 1969).

Anhydrotetracycline oxygenase which converts anhydrotetracycline into dehydrotetracycline in the final stages of CTC biosynthesis has been examined by Behal et al (1979). It was found that CTC biosynthesis was parallel to the activity of that enzyme. They concluded it is possible that the activity of that enzyme is an indicator of the total activity of the enzyme system/sequence which is involved CTC formation and that this represen a limiting factor in the process of secondary biosynthesis in S.aureofaciens.

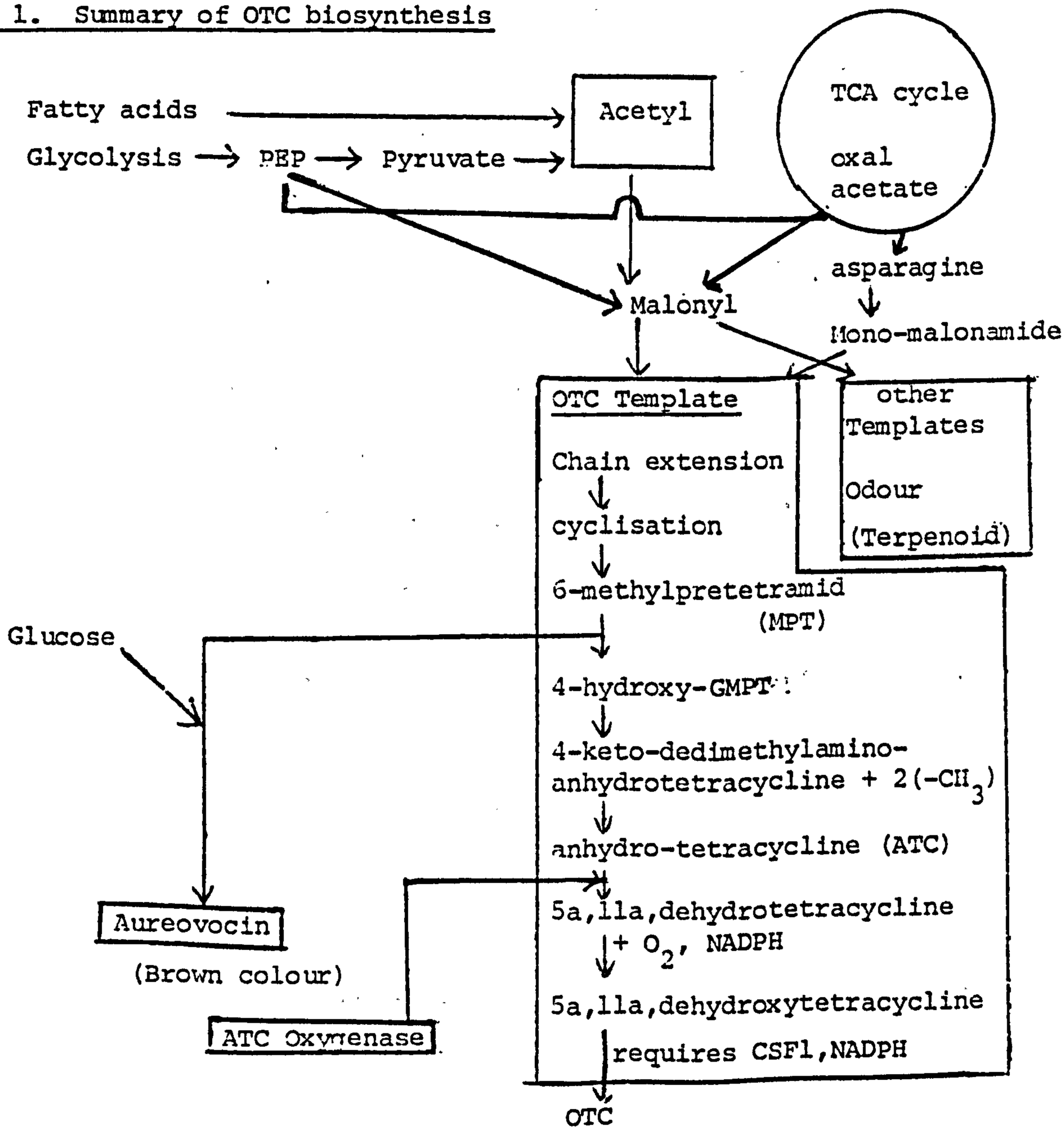
Podojil et al (1970) have isolated a brown compound, aureovocin, a glycoside of methylpretetramid, from cultures of S.aureofaciens, and stated that it occurs in an amount equal to chlortetracycline. This

must therefore draw away appreciable quantities of material that could be converted to CTC. In other work Mikulik et al (1969) have shown that CTC attaches itself to the ribosomes of S.aureofaciens with a possible harmful effect.

Hovorkova et al (1974) showed that S.aureofaciens has a strong glucosidase ability, this meant it could produce glucosides when monohydroxyanthraquinones were added to the medium. This is related to the formation of aureovocin. It is possible that this avoids the accumulation of chlortetracycline which would be toxic towards the producing organism..

A general reaction diagram for OTC biosynthesis summarised in Fig. 1

Fig 1. Summary of OTC biosynthesis



1.5 GENERAL METABOLISM AND MORPHOLOGY OF S.aureofaciens STRAINS

The general metabolism of the behaviour of the Czech. main strains has been given by Hostalek et al (1976). The strains concerned were Bg, the wild parental strain (also referred to as RA 37) and the mutant 8425 and blocked (CTC^-) mutants from both strains. The colonies of the substrate mycelium and the different pigments in the agar medium were yellow-orange-brownish, 8425 being rather darker. Both gave grey spores. The blocked mutants often showed darker shades with tendency to reddish or greenish shades.

The wild strains and 8425 gave five main products when the cultures were examined chromatographically, chlortetracycline (CTC) tetracycline (TC) aureovocin (AVC) and in addition, two unidentified compounds, B and C were also formed. In blocked strains one or more of these disappeared, except for C, which was always produced, aureovocin was nearly always lost. Two of the blocked mutants from 8425 produced novel compounds, either F and G together with demethyltetracycline (DTC) or compounds J & K (F,G,J and K were also unidentified). The ultra structure of the strains was examined and structures typical for streptomycetes were observed (Williams et al 1973). Mesosomes of all three types were observed, lamellar, tubular and vesicular. On the other hand a fibrous sheath was not observed.

The wild strain showed relatively uniform ultra structure, morphologically undifferentiated filamentous cells were found to contain homogeneous cytoplasm without vacuolation for as long as 72 hours. On the other hand, from the beginning 8425 showed large numbers of mesosomes. Vacuolation began after 12 hours, accompanied by the formation of membranous, multi-lamellar bodies. After 72 hours vacuolation of

cytoplasm reached the stage of almost total cell lysis. Electron dense ribosome-like bodies in cytoplasm survived in the cells, even after autolysis. Strain 8425 was characterised by extreme polymorphism, but did not form the bulges, described by Kurylowicz (1972), considered to be organelles in which the antibiotic synthesis takes place.

The blocked mutants also showed a variety of morphological changes.

It was noted that point mutations can cause wide effects on biosynthesis, growth pattern and product formation.

1.6 CHANGES ON MUTATION TO HIGHER YIELDS WITH OTHER ANTIBIOTIC PRODUCERS

Not much has been reported on this aspect of strain improvement. But some information is available in a few cases and this is summarised to provide a comparison.

I. Penicillin

The biosynthesis of penicillin involves condensation of three amino acids namely L-α-amino adipic acid, cystein and valine to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. This tripeptide forms two rings giving rise to the penicillin nucleus.

Martin et al (1979) has drawn attention to suppression of penicillin by lysine and penicillin itself.

On mutation to higher yield the following changes have been reported (Calam 1982)

(1) Relaxation of feed-back regulation of the production of valine and cysteine.

(2) Increased biosynthetic activity per mg cells.

(3) Increased activity of enzymes (aldolase, G6PDH & ICDH) of the central metabolic system.

(4) Increased resistance to penicillin.

(5) Improved growth-pattern in stirred culture with reduced effect of transitions during growth.

(6) Lower maintenance requirement (Mou 1979).

II Griseofulvin

Griseofulvin is produced from acetyl units via malonyl and therefore may provide some comparison with OTC. The industrial process is similar to that originally described by Glaxo (1954). A biochemical model describing a number of features of the metabolic system has been published (Calam et al 1971). The biosynthesis has also been described (Hockenhull 1963). A comparison of an early production mutant and a later mutant giving over twice the yield showed the following characters:-

(1) A thinner type of growth probably allowing better oxygen transfer.

(2) A better growth pattern allowing increased growth during the production phase and a more reactive system, with longer production.

(3) Increased production per mg of cells.

A characteristic feature of griseofulvin fermentation was a tendency toward too rapid growth, with a high rate of respiration which led to low or very low yields.

III. Chlortetracycline

Some of the main differences observed by the Czech workers were:-

(1) Lower extent of cell growth with the production mutant.

(2) Increased uptake of acetate, higher level of acetyl carboxylase; lower levels of citrate synthase, aconitic hydrase, ICDH, fumarate DH and MDH than in the wild strain.

(3) Increase in PEP-carboxylase activity in mutant.

(4) Adenylate 10 times higher in the wild strain but energy charge about the same; however the energy charge shows fluctuation values and are often low, around 0.5.

They concluded that in the mutant the primary precursors were mainly provided by a supplementary pathway via PEP carboxylase and that CTC is formed during conditions unfavourable to growth induced by phosphate limitation, so that production was greater (Behal et al 1981).

1.7 INVESTIGATION INTO THE GENETICS OF TETRACYCLINE PRODUCERS

As mentioned before, most of the work on the biosynthesis of tetracycline was done with S.aureofaciens. Vanek et al (1971) asked the question, "how many genes are required for the biosynthesis of chlortetracycline?" and he concluded that well over a hundred enzymes (and hence genes) participated in the OTC biosynthesis.

Genetical work has been done mainly with S.rimosus. The first genes which were located with S.rimosus gave a map like that of S.coelicolor (Friend and Hopwood, 1971; Alacevic, 1976). Friend et al (1978) found some evidence for a plasmid controlling fertility. Some of the genes associated with biosynthesis of OTC have been located by Pigac et al (1974). More recently a more detailed study on S.rimosus was completed by Rhodes et al (1981). In this work a number of OTC^- mutants were obtained and cosynthesis experiments used to locate the blocks in the biosynthetic route, although not all the mutations could be located precisely. All the mutations after ATC and the CSFI mutation were located at around 9 o'clock on the circular chromosome, while all the mutation before ATC were located near 3 o'clock. The existance of two clusters at 180° is thought to have a possible evolutionary significance. No evidence of plasmid born OTC genes was obtained.

A plasmid controlling fertility in S.rimosus has been described by Friend et al (1978), and the use of protoplasts in recombination has also been described by Hranueli et al (1983). The interaction of plasmids with the nuclei of other streptomycetes has been reported, for example the transfer of structural genes and resistance in S.fradiae producing tylosin (Baltz et al 1980). The use of protoplast fusion and transformation in Streptomyces in the developments of high yielding strains has been reported (Baltz and Matsushima 1983). These developments give the possibility of the production of high yielding strains by cloning.

1.8 OBJECTIVES OF THE PRESENT PROGRAMME

1) The initial object of the presentprogrammewas to repeat some of the Czech work on the growth pattern and enzyme concentrations in S.aureofaciens, and to see whether the changes occurring in the wild strains of S.rimosus on mutation to higher yields were similar to those observed by them.

2) To grow,the wild strains and the mutants in submerged culture (shaken and stirred) to observe the changes between the systems in the general physiology, growth, respiration and OTC production of the strains.

3) To compare the general morphology of the wild and mutant strains and its relation to OTC production and to compare it with the results of other workers.

4) To make experiments with blocked mutants (OTC^-) and cosynthesis.

5) To look at the identity of the strains and to compare the strains by different systems such as the older ISP system and a more recent method (Williams et al 1983b) and to study the effect of mutation on the characteristics of wild, mutant and blocked mutant strains.

2. MATERIAL AND METHODS

2.1 CULTURES

2.2 STORAGE OF THE STRAINS

2.3 MEDIA USED

2.4 FERMENTATION METHODS

2.5 ANALYTICAL METHODS

2.6 ENZYMES AND ADENYLATE: EXTRACTION AND ASSAY

2.7 MORPHOLOGICAL STUDIES

2. MATERIAL AND METHODS

2.1 CULTURES. Streptomyces are gram positive filamentous prokaryotic microorganisms which undergo cellular differentiation and produce antibiotics and other substances of diverse chemical structure and mode of action. (Hopwood and Merrick 1977).

The following cultures were used throughout the present study:

1) 72T1, a soil isolate from I.C.I. Laboratories, obtained in about 1960, identified by paper chromatography and biological tests as a producer of OTC, because of a white spore mass it was referred to as S.rimosus.

2) Y20, a mutant derived from 72T1 obtained in the I.C.I. Laboratories. This occurred in two forms, the original Y20, (inactive), and the reselected active strain Y20.

3) FF
4) A1
5) B3 — All the I.C.I. mutants which have been used previously
6) D9 for OTC production.

7) D9D7 mutant obtained from D9 in our Laboratories, yielding slightly more OTC than D9.

8) S.rimosus NRRL 2234 from Peoria, the original Pfizer strain.

9) S.capuensis originally described by Pierrels and stated to give 10g/l OTC (Pierrels. Pat.). Two cultures were obtained, the first donated by Pfizer which gave low yields, and NRRL 3501 obtained from Peoria which gave 5g/l in the shaken flasks.

10) Mutant A
11) Mutant B
12) Mutant D — Blocked mutants of Y20
13) Mutant 1
14) Mutant 3
15) Mutant 4

16) Mutant 5
17) Mutant 6
18) Mutant 7
19) Mutant 8
20) Mutant 9
21) NTG 1

— Blocked mutants of Y20

22) S.aureofaciens (A196) obtained from the collection of Dr. Sharples, biology department, Liverpool Polytechnic.

23) G65, donated by Pfizer as a wild strain of S.rimosus, and said to be mutated easily to give higher yields.

2.2 STORAGE OF THE STRAINS:- S.capuensis NRRL 3501 and S.rimosus NRRL 2234 were obtained as freeze-dried cultures. Agar slopes were made from them. All the I.C.I. strains were obtained as soil cultures, from which isolates were obtained and from which agar slopes were made.

A set of agar slopes were stored under oil at $4^{\circ}C$, and another set stored at $-70^{\circ}C$.

2.3 MEDIA USED

1) MEDIA FOR SLOPE CULTURES (ALL I.C.I. RECIPES)

Casein-yeast extract agar

Casein hydrolysate	2g/L
Yeast extract	2g/L
Starch (soluble)	10g/L
Lab Lemco	1g/L
$CaCl_2$	0.01g/L
Agar (Davis)	15g/L

No pH adjustment

XXVII 25 medium

Dextrin	10g/L
Casiton (Difco)	2g/L
Beef extract	1g/L
Yeast extract	1g/L
$CaCl_2$	20g/L
Agar (Oxoid No 3)	15g/L

No pH adjustment

RC medium (XVII25)

Sucrose	3g/L
Dextrin	15g/L
NaCl	0.05g/L
Urea A.A.	0.01g/L
KH_2PO_4	0.01g/L
Yeast extract	1g/L
Ferrous Sulphate	0.01g/L
Peptone	5g/L
Agar	20g/L

No pH adjustment

It was found that the best medium for agar cultures was RC medium: The type of the tube used for slopes has some effect on the type of sporulation, as it was much better to grow the strains on slopes in 6 X 1 in. (15 X 2.5 cm.) plugged test tubes than ordinary universal bottles.

2) MEDIA FOR SUBMERGED CULTURES

I. Inoculum media

KL medium

Sucrose	10g/l
C.S.L.*	10g/l
$CaCO_3$	1g/l
Ammonium sulphate	2g/l
KH_2PO_4	2g/l
$MgSO_4$	0.25g/l
Trace elements	10mls

Trace elements consist of

$MnSO_4$	5g/l
$CoCl_2$	0.5g/l

Second stage inoculum medium

Starch	30g/l
C.S.L.	20g/l
$CaCO_3$	10g/l
Ammonium sulphate	2.5g/l
P.P.G.**	0.25ml

*Corn steep liquor

**Polypropylene glycol 2025

II. Production media for shaken flasks and stirred cultures

Six media were tested, based on the Literature or I.C.I. information These included media used by the Czech. workers.

a) Media from the literature;

Medium (I) Jechova et al 1969	
Glucose	10g/l
Starch	19g/l
C.S.L.	10g/l
Ammonium sulphate	6g/l
$CaCO_3$	8g/l
Sodium chloride	5g/l
Arachis oil	2ml
(No pH adjustment)	

Medium (II) Herald et al 1956	
Sucrose	30g/l
Soya meal	20g/l
Sodium chloride	5g/l
Molasses	2g/l
$CaCO_3$	4g/l
Ammonium sulphate	2g/l
C.S.L.	5g/l
(No pH adjustment)	

Medium (III) Biffi et al 1954	
Sucrose	30g/l
C.S.L.	10g/l
$CaCO_3$	4g/l
Molasses	1g/l

Ammonium sulphate	20g/l
NaCl	0.5g/l

(No pH adjustment)

b) I.C.I media

Medium (IV)

Starch	55g/l
Ammonium sulphate	7g/l
$CaCO_3$	19g/l
C.S.L.	12g/l
Ammonium chloride	1.7g/l
P.P.G.	0.2ml
Arachis oil	20ml

(No pH adjustment)

Medium (V)

Starch	63g/l
Soya flour	7.5g/l
C.S.L.	12g/l
$CaCO_3$	22.5g/l
Ammonium sulphate	5g/l
Ammonium chloride	2g/l
KH_2PO_4	2g/l
$MnSO_4$	0.05g/l
$CaCl_2$	0.005g/l
Arachis oil	25ml

(No pH adjustment)

$CaCO_3$	16g/l
Starch	55g/l
C.S.L.	25g/l
Ammonium sulphate	7g/l
Ammonium chloride	1.7g/l
$CaCl_2$	0.005g/l
$MnSO_4$	0.05g/l
Arachis oil	20ml
P.P.G.	0.25ml

Of these media, I, II and III gave negligible quantities of OTC, and of the others (VI) was best. However, none of these media was very satisfactory, and investigation to find the most satisfactory medium for the mutants is described later. The type of chalk used was of great importance.

2.4 FERMENTATION METHODS

1) SHAKEN FLASK CULTURES

I. Fermentation apparatus

All cultures were grown out in 250 ml Erlenmeyer flasks incubated at 25°C on an orbital shaker giving 50 mm orbits at 220 rpm, at 25°C.

II. Fermentation procedures

a) Two stage-fermentation process

Flasks containing 50ml inoculum medium were seeded with spore suspensions, obtained by rubbing up slopes of the strains with 9ml water, using 10^7 spores/ml with Y20 and 10^6/ml with 72T1. The inoculum culture was then incubated for the required time on the shaker.

When substantial growth of mycelium had taken place 5ml of the inoculum was transferred to the production flasks which contain 30 ml of the production medium, which was then incubated as above for 4 to 5 days.

b) Three stage fermentation process

Flasks containing 50 ml KL medium were seeded with the spore suspension and then incubated for the required time (usually 48 hours) on the shaker. 5Ml of the growing mycelium were then transferred to flasks containing 50ml of inoculum medium and incubated as above for the required time (usually 30 hours). 5Ml of the resulting culture was transferred to the production flasks, which were incubated as above for four or five days.

2) STIRRED FERMENTATION

I. Fermenter apparatus

The 5-litre stainless steel fermenters, were 15 cm in diameter, 30 cm in height. On the upper edge was a rim with equally spaced screws coinciding with holes in the lid.
The lid was bolted to the cylinder with a rubber gasket, so as to ensure a tight seal.
Air was passed through an air-filter filled with cotton wool to a sparger at the bottom of the cylinder. Mounted immediately above the aparger on the stirring shaft was a 4 bladed stirrer 10 cm diameter, two blades were set at an angle of 45^{o} to the horizontal facing each other, the other two were set vertically so that the medium was thrown upwards; vortex formation in the fermenter was prevented by a baffle plate.

A sampling device connected to the fermenter by withdrawing samples aseptically by vacuum, and was also used for blowing the inoculum itself into the fermenter.

The air was blown into the fermenter using a small air compressor (Charles Austen Pumps Ltd).

The aeration rate was measured using specially calibrated rotameters. The fermenter has a working volume of 3-4 litres culture and was stirred at 520 rpm and aerated at 1.5 litres/minute. The temperature of the fermenter was controlled at 25-26^{o}C with a water bath.

II. Feed pumps:-

The feed pump used was a Watson Marlow flow inducer, MHRE 7 (Watson Marlow Ltd, Falmouth)

III. Batch operation

Production and inoculum medium used for the stirred culture were the same as those for the shaken flasks, but as the use of starch made it viscous, corn flour was used after (ABM Ltd Stockport, U.K.) Bacterase treatment. Amisol 07005 was also used as this source of starch gave a very thin solution after dissolving.

Fermenters were sterilized by autoclaving at 15 p.s.i. pressure, (121^{o}C) for 35 minutes, and then cooled. KL medium in the shaken flasks was seeded with spores and incubated for 48 hours, and then used to inoculate the inoculum fermenter. After 30 hours, 300 mls of the inoculum fermenter culture was used to inoculate the production stage.

Cultures were tested for contamination periodically by inoculation of an agar plate with some of the culture, additionally, direct examination of samples was made with a microscope, this was most useful as a rapid test for heavy contamination. However there was no problem with contamination throughout the study.

3) SAMPLING PROGRAMME OF THE STIRRED FERMENTERS

The following tests were carried out at daily intervals:

I. Protein concentration.

II. OTC production.

III. Carbon dioxide production % in exit gas.

IV. Oxygen uptake % in exit gas.

V. RQ measurement.

VI. pH pattern.

The last two were considered to be of prime importance in providing infomation as to the state and progress to the fermentation.

2.5 ANALYTICAL METHODS

Samples of about 20 mls volume were removed aseptically at intervals, for analysis.

I. Protein determination

1 ml of the sample in a centrifuge test tube was centrifuged and washed 3-4 times with distilled water followed each time by centrifugation. To the washed mycelium 3 mls of 4.0 N NaOH was added, followed by heating for three minutes in a boiling water-bath, then after cooling rapidly in an ice-bath, 2mls of 4.0 N HCl was added to the sample (Ecker et al 1961).

the method of Lowry et al (1951) was used for the protein determination, using crystalline bovine albumin as standard (BDH, Chemical.Pool. England).

II. Oxytetracycline determination

OTC was determined by two methods.

(a) Biological assay using the agar diffusion method with OTC dihydrate (Sigma Chemical. Co. London) as standard, against diluted filtrate of the tested sample and S.aureus as test organism.

(b) Chemical method using the ferric chloride method (Monastero et al 1951).

III. Carbon dioxide production % in exit gas.

Carbon dioxide in the effluent gas was measured by a LIRA Gas Analyser type 303 (Mine Safety Appliances Ltd. Shawhead, Coatbridge, Scotland). The effluent air from the fermenter was dried by passing it through a flask and condenser before entering the analyser, giving the additional advantage of avoiding the risk of the foam from the fermenter entering the analyser.

Analysis of carbon dioxide was also carried out by hand using a portable Haldane Apparatus.

IV. Oxygen uptake % in exit gas.

This was measured with Haldane Apparatus. The difference from the standard value for air, 20.9%, was taken.

V. RQ measurement

From the data obtained by the Haldane Apparatus of the % of

CO_2 and O_2, the RQ was calculated.

VI. pH measurement

The pH of the sample was taken using pH meter 7010 (Electronic Instrument Ltd. England)..

2.6 ENZYMES AND ADENYLATES: EXTRACTION AND ASSAY

1) DETERMINATION OF THE ENZYME ISOCITRATE DEHYDROGENASE (ICDH) AND MALIC DEHYDROGENASE (MDH).

1ml sample was taken from the test culture at suitable intervals, transferred into a centrifuge test tube which was held in an ice-bath to cool, the samples were centrifuged and washed 5 times with 5 ml Tris-buffer (0.1 M, pH 7.4), followed by centrifugation for 5 minutes at 10,000 rpm. The washed mycelium was next transferred to Mikle disintegration tube which contained 5 ml ice-cooled Tris-buffer and 4 g of Ballotini beads. The samples were homogenised by placing the tube on Mikle disintegrater (Mikle Laboratory Engineering Ltd., Surrey, England) which was placed in a cold room at $4^{o}C$. Samples were left in the disintegrator for 15 minutes. The homogenised suspension was then transferred to a centrifuge tube and centrifuged for 5 minutes at 10,000 rpm.

The supernatant was transferred to a clean test tube using Pasteur pipettes. Samples of this supernatant were held at $4^{o}C$ in an ice-bath until used for assay.

I. Enzyme assay for ISOCITRATE DEHYDROGENASE AND MALIC DEHYDROGENASE

The assay for ICDH was carried out using a Kit (Boehringer Mannheim). The kits were designed for clinical use but were easily adapted for our own purposes.

The assay for MDH was carried out using a method based on a Sigma kit (Sigma Co. London).

II. Assay of ICDH : (Biochemica Test Combination, Cat. No. 125 989).

0.5 Ml of the supernatant was added to 2.5 mls reagent one (trithanolamine 100 mmol/l, pH 7.5, DL-isocitrate 4.6 mmol/l, NaCl 52 mmol/l) and the mixture was incubated at 25°C in a water bath for 5 minutes; 0.1 ml of reagent two (1 mmol/l NADP + 124 mmol/l $MnSO_4$) was added and the mixed solution poured into a cuvette. The absorbance was measured after 1, 2 and 3, minutes at 340 nm wave length.

A blank reading was taken against air. The mean absorbance change per minute (ΔA/min) was determined and used for the calculation.

III. Calculation of ICDH :- The ΔA/min obtained from the increase in the optical density after the addition of NADP/$MnSO_4$ solution was recorded and the activity of ICDH in the sample calculated as follows

U/1 (25°C) = 984 X ΔA at 340 nm.

Ref: Biochemica Sheet, Cat. No. 125 989

IV. Assay of MDH :- Solutions of 0.1 mg/ml of oxalacetic acid (O.A.A), and of 0.2 mg/ml of NADH in potassium phosphate buffer (0.1M KH_2PO_4,K_2HPO_4, pH 7.1) were made freshly for every test.

To a cuvette the following were added, 2.5 ml of the buffer (Tris 0.1M pH 7.4), 0.2 ml of NADH solution and 0.1 ml of the enzyme extract and left in water bath at 25°C for 20 minutes, the reaction was started by the addition of 0.1 ml O.A.A. solutio

Reading was taken against water. The absorbance change was measured after 1, 2 and 3 minutes at 340 nm wave length. The mean absorbance change per minute (ΔA/min) was determined and used for the calculations.

V. Calculation of MDH :- After the addition of O.A.A. to the cuvette the decrease of the optical density was recorded at 340 nm. The enzyme activity was calculated as follows

U/ml = ΔA/min X 1000 X TCF

TCF is the temperature correction factor, and it is 1.0 at 25°C. Ref. Sigma technical bulletin number 340-UV.

4) DETERMINATION AND PARTIAL PURIFICATION OF CITRATE SYNTHASE

The extraction was the same as that carried out for ICDH and MDH but here 10 mls of the test culture was harvested, centrifuged, washed with Tris-buffer pH 8.1. 0.1 M. After extraction with the Mikle disintegrator the cell debris were removed by centrifugation (21,000 rpm for 15 minutes at 4°C), the supernatant was fractioned with solid $(NH_4)_2SO_4$, the fraction obtained by saturation to 50% was first removed by centrifugation (21,000 rpm for 15 minutes), the supernatant was further saturated to 70% $(NH_4)_2SO_4$ and centrifuged at 21,000 rpm for 15 minutes, the precipitate was then dissolved in 10 ml cold distilled water which was then used for the assay.

I. Assay of Citrate synthase. The enzyme activity was determined by the method of Srere et al (1963) which was also used by Hostalek et al (1969b). The reaction mixture (total volume 3 mls) contained 150 μ mole Tris-HCl buffer

pH 7.4, 150 n mole acetyl CoA 690 n mole oxaloacetate, 300 n mole DTNB [5,5' -Dithiobis-(2 nitrobenzoic Acid)] and 0.2 ml enzyme preparation, oxaloacetate and nucleotide were neutralised and used as Na salts, all the reagents (except DTNB) were prepared just before starting the test.

II. Calculation of citrate synthase :- In the reaction mixture there was 3 mls reaction volume in which there was 0.2 ml enzyme sample. The increase in the optical density was measured after the addition of the enzyme extract.

In a typical example the mean of the optical density change ΔA/minute was 0.04 so $\frac{0.04}{13600}$ = mole of DTNB/minute X 10^6 = 2.0 mole/minute of citric acid formed which is the strength in units/litre of mu/ml [The molar absorbancy index for the mercaptide at 412 nm is 13600 (Srere et al 1963)].

As the enzyme sample has been diluted 15 times, the concentration is 2.9 X 15 = 43.5 mu/ml, and the protein in this case was 3.9 mg/ml; mu/mg protein = $\frac{43.5}{3.9}$ = 11.1.

5) DETERMINATION OF ACETYL-COA-CARBOXYLASE AND PEP-CARBOXYLASE

I. Extraction of acetyl-CoA carboxylase

5 mls of the culture were placed in a centrifuge tube and subjected to centrifugation. The precipitated mycelium was then washed with cold imidazole buffer (0.1M at pH 6.5) followed by centrifugation. To the precipitate 5ml of the buffer was added and the mycelium disintegrated by Mickle disintegrator for 15 minutes followed by centrifugation at 10,000 rpm and 4°C for 10 minutes without further treatment and the supernatant was used for the assay.

II. Assay of acetyl-CoA-carboxylase

To a test tube the following were added, 3 µ mole of ATP, 1 µ moles $MnCl_2$, 0.05 µ moles acetyl-CoA, 0.05 mls of $NaH^{14}CO_3$ (specific activity 0.1mCi/mmol and 50 µ Ci/ml radioactive concentration) (Martin et al 1962). The reaction was started by the addition of 0.1 ml enzyme extract and incubated at 30°C for one hour. The reaction was stopped by the addition of Dowex 50-H resin until the pH fell to 2. The Dowex and the protein was sedimented by centrifugation and 0.05 ml of the supernatant solution was suspended in 4.95 ml of PCS Tm liquid scintillation counting solution (Amersham Internation, Amersham) and counted.

III. Acetyl-CoA-carboxylase calculation:

A 0.05 ml of the sample was taken from the centrifuge tube and added to 4.95 ml/PCS Tm liquid scintilation solution. The sample was then placed in the Packard counter and the count per minitues recorded. The enzyme activity was determined as follows:-

In a typical example we had 33,000 CPM, so the enzyme activity can be calculated by the following equation

$$\frac{RN}{2.22 \times 10\ 000 \times E \times SA \times T} = U$$

RN = CPM, E = % efficiency, SA = µCi/µmol, T = reaction time

2.22 x 10 000 X conversion factor

$$\frac{33\ 000}{2.22 \times 10\ 000 \times 58 \times 0.1 \times 30} = 0.00085 \text{ U/in the counter cell}$$

0.85 mU in the cell which contain 4.95 mls 0.05 ml sample

$$\frac{0.85 \times 5.0}{0.05} \times \frac{1}{5} = 17 \text{ mU/ml}$$

and if we have 4.5 mg/ml protein

$\frac{17}{4.5}$ = 3.78 mU/mg protein

IV. Extraction of the enzyme PEP-carboxylase

5 ML of the culture were placed in centrifuge tube followed by centrifugation and the precipitated mycelium then washed 3-4 times with ice-cold phosphate buffer, 0.05 M pH 7.2, followed each time by centrifugation. To the precipitated mycelium 5 ml of the buffer was added (Vorisek et al 1969) and suspension placed in the Mickle disintegrator, in the usual way, for extraction. The crude extract was then centrifuged at 10,000 rpm and 4°C for 10 minutes; the supernatant was used for the assay without further treatment.

V. Assay of PEP-carboxylase

The reaction mixture in a test tube contained in micromoles, PEP 4; $MnCl_2$ 1.4; Tris-HCl pH 7.4, 70; (de Villis et al 1963). 0.2 ml of the enzyme extract was added with 0.05 ml of $NaH^{14}CO_3$ (specific activity 0.1 mCi/mmol and radioactive concentration of 50 μ Ci/ml)-

The test tube was then placed in a water bath at 30°C for 30 minutes and the reaction was then stopped by the addition of Dowex-H resin until the pH fell to 2.

The mixture was then centrifuged to precipitate the protein and 0.5 ml of the supernatant was added to one ml of a concentrated solution of 2-4-dinitrophenyl hydrazine (DNP) in 2 N HCl as stabiliser; 0.05 ml of this solution was then added to the PSC Tm counting liquid (Amersham International, Amersham) and the cpm was measured.

VI. Calculation of PEP carboxylase

PEP carboxylase calculation was done as acetyl-CoA carboxylase taking into account the different dilution factors.

7) DETERMINATION OF ANHYDROTETRACYCLINE OXYGENASE

Anhydrotetracycline oxygenase was determined spectrophotometrically by measuring the decrease in the absorbance level at 440nm, over 3 minutes, which is directly proportional to the amount of anhydrotetracycline (ATC) transformed to 5a, 11a-dehydrotetracycline in the presence of NADPH and atmospheric oxygen (Behal et al 1979).

I. Preparation of mycelium and cell free extract:-

The mycelium was separated from the fermentation broth by centrifugation at 5,000 rpm for 3 minutes, washed three times with distilled water followed each time by centrifugation.

The mycelium was disintegrated with Mickle disintegrator at 4°C with 4 g Ballotini Beads and 5 ml of buffer (0.2M Tris-HCl pH 7.4; 2nM EDTA; 15% V/V glycerol; 1 nM meraptoethanol). After 15 minutes extraction, the homogenate was centrifuged at 14 000 rpm and 4°C for 20 minutes, the low molecular substances were removed with a 1 x 10 cm column of Sepahdex G25 (medium); (Pharmacea; Sweden). The enzyme was collected from the column at 3 ml intervals, the second 3 ml was used for the enzyme assay.

II. Enzyme assay

NADPH, glucose 6-phosphate dehydrogenase, 2 meraptoethanol, was obtained from Sigma, London. ATC was prepared according to Schlecht and Frank (1975). 3 ml reaction mixture contained the following: 0.015 ml (15 µM) glucose 6-phosphate dehydrogenase; 1.0 ml (0.9 µM) glucose 6-phosphate; 0.6ml (600 µg), NADPH; 1.0 ml of the enzyme extract, and 1.5 ml Tris-HCl buffer pH 7.4.

The reaction started by the addition of 0.3 ml of ATC (175 nM), the change in optical density over 3 minutes was recorded and used to determine the enzyme activity.

8) ADENYLATE DETERMINATION

The adenylate level was measured using Packard scintillation counter, which is the most sensitve method for this determination, by measuring the light output in the presence of Luciferin-Luciferase.

I. Extraction of the mycelium

3 mls of the culture were drawn from the culture and put into a centrifuge tube, which was placed in an ice-bath. It was then washed 4-5 times with 3 mls distilled water followed by centrifugation at 10,000 rpm for 3 minutes. After washing, another 3 mls of water were added to the tube and the tube was put in boiling water for 10 minutes followed by immediate cooling to $2^{\circ}C$ and kept at this temperature until assayed (Strehler et al 1957).

II. Preparation of Luciferase

Firefly Lantern was obtained from Sigma Ltd., London. 8 Lanterns were gound in a Griffith tube with 3 mls ice-cold 0.1 M sodium arsenate pH 7.4 for 5 minutes (Beutler & Baluda 1964). The extract was then transferred to a centrifuge test tube, the Griffith tube was then washed with another 1.0 ml buffer, which was added to the extract, giving a total volume of 4 mls of extract which was then centrifuged at 10,000 for 3 minutes. The tube was then kept in an ice-bath until used for the assay.

III. Glycylglycine buffer (25nM glyclglycine; 0.1M $MgSO_4$ pH 7.5)

1.65g of glycylglycine plus 2.3 g $MgSO_4.7H_2O$ were dissolved in 400 ml distilled water. pH was then adjusted with 1.0 N NaOH and water added to a final volume of 500 mls.

IV. Setting the scintilation counter

The scintilation counter was set to measure at optimum tritium setting (gain 52%, discrimination 50-1000) with repeat counting Dikstein et al (1964) and Addaki et al (1966)

V. Determination of ATP

1.7 mls glycylglycine buffer and 0.2 ml of lantern extract were kept in a clean counting glass vial, then 0.1 ml of the test sample was added to the vial at the time when it was entering the counter (to allow the shortest time possible between the addition of the sample and the counting, which was 6 sec.), so that the observations were obtained 7 sec. after the addition of the sample.

Each subsequent observation was obtained at 3 sec. intervals (1 sec. counting and 2 sec. printing).

VI. Determination of ADP

ADP was determined after the conversion to ATP by pyruvate kinase in the presence of Mg^{2+} (Chapman et al 1971). The reaction mixture in the vial was as follows: 0.05 ml K_2HPO_4 solution (7.5 nM pH 7.3), 0.0t ml phosphoenolpyruvate (0.5 nM), 0.05 ml pyruvate kinase solution (20 mg), 0.5 ml $MgCl_2$ solution (80 nM). After incubation at 30°C for 15 minutes the vials were transferred to an ice-bath until assayed. The assay of the sample was done in the same way as ATP, the resulting value was corrected for the content of ATP in the sample. (phosphoenolpyruvate and pyruvate kinase were obtained from Sigma, London).

VII. Determination of total adenylate and AMP

Total adenylate was determined as ATP after the conversion of AMP and ADP to ATP by incubation of the sample with pyruvate kinase and myokinase in the presence of Mg^{+2} (Curdova et al 1976). The reaction mixture was the same as ADP determination except that an addition of 5 µg myokinase (Sigma, London) followed by incubation at 30°C for 215 minutes. The sample was then transferred to an ice-bath until assayed. The total adenylate as ATP-ATP-ADP = AMP

VIII.. Calculation of ATP, ADP, AMP:

2.2 mg of standard ATP was dissolved in 25 mls of cooled distilled water, 5mls of that solution was diluted to a final volume of 500 mls with distilled water. 0.05 ml of the standard was then added to a vial which contained 2.0mls of glycylglycine buffer plus 0.1 ml of lantern extract.

The resulting solution contained 37 pmol/ml of ATP.

CPM were recorded in the usual way and by putting different volumes of the standard ATP into the vial, and a standard curve was prepared, from which the value of our mycelium extract was read in pmole/ml which was then converted to pmole/mg protein. A new standard curve was made every day.

2.7 MORPHOLOGICAL STUDIES

1) METHODS OF GROWTH

a) Solid medium

A sterilized cover-slip was carefully inserted at an angle of about 45° into an Oat-meal agar medium in a Petri dish until half the cover slip was in the medium, (see Williams et al 1968), strains Y20 and 72T1 were inoculated along the line where the medium met the upper surface of the cover slips, and inoculated for 10 days at 25°C. The cover slips were removed from the agar and examined by light and scanning electron microscopy.

b) Submerged medium

Inoculum medium flasks were seeded with spore suspension (10^6/ml) of the strain 72T1 and S.rimosus NRRL 2234, and 10^7ml for the strain Y20 and D9. Flasks were then incubated on the shaker at 25°C. After 48 hours 5 mls of the inoculum was used to added to the production flasks.

2) METHOD OF PREPARATION OF SAMPLES FOR USE IN SCANNING ELECTRON MICROSCOPY

Samples of mycelium from different aged cultures were washed three times by centrifugation and resuspended in N/100 HCl. Samples were then pipetted onto rounded cover slips (10mm diameter) and air dried. The cover slips were attached to the specimen support stub with "Durofix" adhesive (Rawplug Company Ltd., London, England) and coated with gold-palladium alloy. Specimens were examined with a "Stereoscan" S4 Scanning electron microscope (Cambridge Scientific Instruments) operated at an accelerating voltage of 30kV.

3. RESULTS

3.1 INTRODUCTORY STUDIES

1) IDENTIFICATION OF STRAINS USED

2) ESTABLISHMENT OF MUTANT STRAINS AND PRELIMINARY TESTS.

3) METHOD OF THE RESELECTION OF THE MUTANTS FOR THE MASTER CULTURES.

4) ESTABLISHMENT OF FERMENTATION METHODS

5) EFFECT OF OTC ON THE GROWTH OF Y2O AND 72T1

6) INDIRECT ESTIMATION OF CELL CONCENTRATION BY PROTEIN ASSAY.

1) IDENTIFICATION OF STRAINS USED

It was considered important to identify the strains used, in particular 72T1 and Y20. Historically, there has been much interest in this aspect, and more than one legal case has arisen over the use of cultures and mutants, said to differ from those covered by patents, especially with S.aureofaciens, in which the morphology of the spore chains can differ. With S.rimosus, new isolates have been patented, such as S.alboflavus (Villax, 1963) and S.capuensis (Pierrels 1963) isolated from soil, which look like mutants of S.rimosus. As a result of this confusion, it has become a custom to assign producers of tetracycline and chlortetracycline to S.aureofaciens and of oxytetracycline to S.rimosus. This was done with 72T1, which was a white, OTC producer.

Identification was carried out using the I.S.P. system as given in the 8th edition of Bergey's Mannual (1974), to which the criteria given by Pridhamet al (1974) were applied. In addition all the strains were grown on RC medium, and the colours that developed noted. The results are summarised in Table 3.

Table 3. Summary of ISP tests

Test	Strains:- S.aureofaciens (Bergey)	S.aureofaciens (GPS)	72T1	S.rimosus (Bergey)	S.rimosus 2234[1]	Y20	D9
colony	grey	grey	grey[2]	white	white	white	white
spore chains	spirals	spirals	F,H[3]	spirals	spirals	spirals	spirals
spore surface	All smooth						
Growth on carbohydrates:							
Glucose	+	ND[4]	+	+	+	+	+
Xylose	W[5]	-	W	-	trace	W	trace
Arabinose	+	ND	+	+	W	+	+
Rhamnose	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+
Galactose	+	ND	+	+	ND	ND	ND
Raffinose	-	-	trace	+	+	W	W
Mannitol	-	-	trace	+	+	+	+
Inositol	-	-	-	+	+	+	+
Sucrose	+	+	+	ND	-	-	-
Colours on RC medium;							
aerial	ND	white	white	ND	white	white	white
substrate	ND	MB	Pale	ND	MB	MB	MB
reverse	ND	Pale	MB	ND	MB	DB	DB

Notes:-

1. Pfizer strain

2- Grey on 3 media; glucose slopes, oatmeal & starch + salts.

3. F,H; flexuous with some hooks.

4. ND, not done

5. w, weak but positive growth.

Colours with RC medium: Pale = pale brown, MB = medium brown,
DB = dark brown (burnt sienna)

GPS, Dr. Sharples.

The results in Table 3 show that 72T1 agrees well with

S.aureofaciens; its spore chains were flexuous rather than spiral,

but Bergey's Mannual allows that this variation occurs in some

strains. The Pfizer strain of S.rimosus, and the mutants are

similar and agree with S.rimosus. The mutants differ, however,

when grown on RC medium (and on other rich medium, such as

Emerson's) in the production of a brown colour in the substrate

and a very dark brown colour in the reverse of the colony.

2) ESTABLISHMENT OF MUTANT STRAINS AND PRELIMINARY TESTS:-

Most of the mutants gave disappointing results when tested, so it

was necessary to reselect active isolates from the original slopes,

using shaken flask tests. It was also necessary to investigate

the shaken flask system so as to obtain optimal results.

72T1 appeared to be in the original state giving 0.5-1.0 g/l of OTC,

and it was used without reselection.

Y20 appeared to give OTC according to the chemical test, this was

not confirmed by the S.aureus plate test. A spore suspension was

plated and colonies tested for OTC production. The best colonies

gave 2-3 g/l OTC.

The other I.C.I. strains gave small quantities of OTC, the best was

D9, so it was decided to reselect it, in the same way as Y20.

To test the possibility of improvement of the Y20 and D9 isolates by mutation, they were mutated with NTG, a few colonies from D9 showed some improvement, the best was D9D7, none of Y20 colonies showed any improvement.

S.rimosus NRRL 2234 was used without reselection.

S.capuensis This culture was originally isolated by Pierrels and a slope was donated by Pfizer. It gave very dark brown colonies and a low yield of OTC (1.5 g/l). A second culture was obtained from Peoria (NRRL-B-350I) and seemed closer to the original high yielding strain and gave 5/1 OTC in the preliminary shaken flasks tests. In stirred culture there was excessive foaming and work with it was discontinued due to lack of time.

3) METHOD OF THE RESELECTION OF THE MUTANTS FOR THE MASTER CULTURES

The mutants obtained from I.C.I. Laboratories required reselection, as loss of productivity had occurred after long storage. Agar slopes of RC medium were sown with spores of each mutant and incubated for 10 days at 25°C. A spore suspension from the slopes was made with sterile water and after dilution it was plated on plates of RC medium, to give 20-50 colonies per plate. More than 1000 plates were incubated with the spores of the different mutants. After 10 days the plates were examined for the right type of colonies. With D9 and Y20 it was relatively easy, as the description of the right colonies was obtained from I.C.I. experts. A coloured photograph of FF colonies was also used as a key.

In the case of other mutants, random selections were made. A large number of colonies from the plates, about 200 altogether, were

picked off from the agar plates; they were suspended in 1 ml of sterile water and used to inoculate 3 slopes of RC medium. After incubation at 25°C for 10 days the slopes were examined for their uniformity, and about one in eight were discarded at this stage.

Slopes were tested for their production ability of OTC by seeding into inoculum flasks. The cultures from them were then added to production flasks, and after 4 days on the shaker the cultures were acidified, filtered and tested for the presence of OTC by measuring the U.V. spectrum. OTC was identified by a peak at 353 nm as shown in Figs. 2, 3; and 4 which compare the absorption spectra of OTC and the mutants.

I. Details of the test were as follows:-

The flasks were acidified by the addition of concentrated HCl until the pH fell to 1.5-2.0, then they were left on the shaker for 20 minutes, followed by filtration, the filtrate was then diluted to 1/200 with N/100 HCl and tested for absorption on the spectrophotometer at 353 nm. Table 4 shows the absorption at 353 nm of the original mutants before reselection; all gave low absorption and no peak at 353 nm.

Table 4. The absorption at 353 nm of the original mutants, before reselection diluted 1/200

Mutant	D9	B3	A1	E4	FF	Y20
Absorption at 353 nm	.11	.02	.07	.06	.08	.1

Table 5 shows the number of colonies tested for all mutants and the absorption they gave at 353 nm.

Table 5 The number of colonies tested and the absorption they gave at 353 nm

absorption	.04	.06	.08	.1	.12	.14	.16	.18	.2	.22	.24	.26	.28	.3	.32	.34
number of colonies from all the mutants	5	18	24	24	18	21	10	9	13	17	15	4	6	3	3	2

The two colonies which showed the highest absorption (i.e. .34) were from D9; the three with .32 absorption were from Y20; one colony of FF showed absorption at .26. The best colonies were retested, to select the best of the promising colonies.

Fig.2 U.V absorption of the mutants

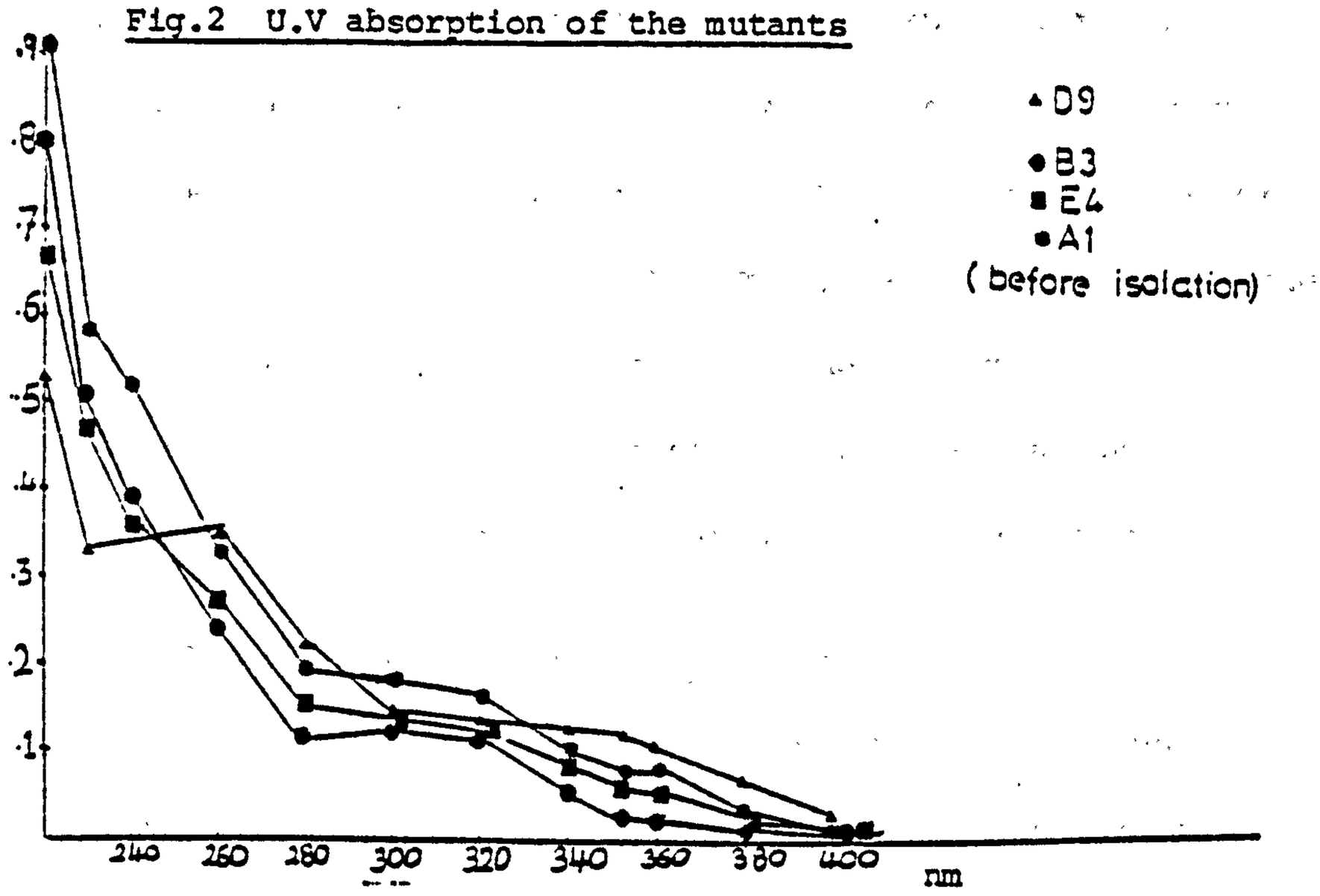
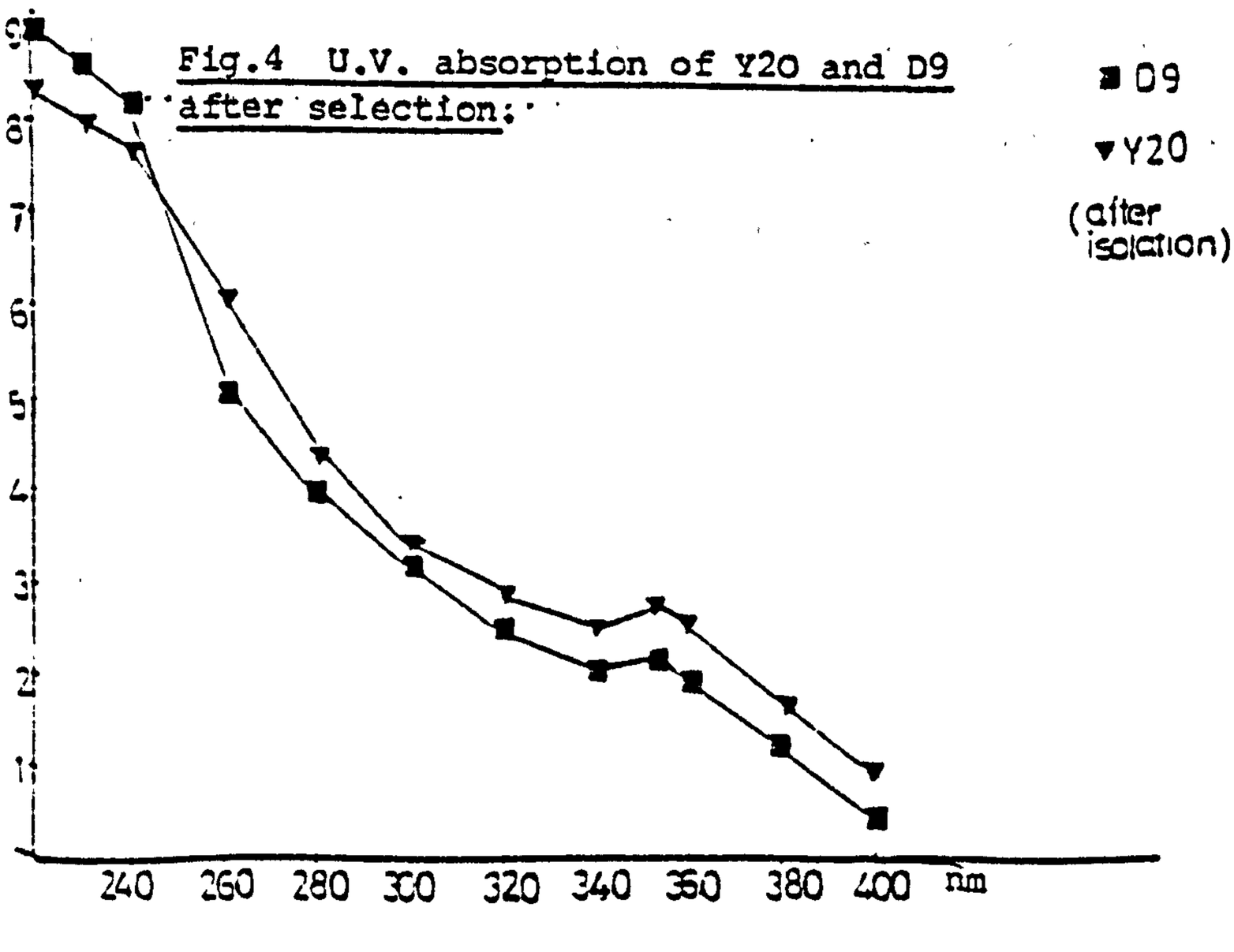


Fig.3 U.V absorption of Y20 and OTC

Fig.4 U.V. absorption of Y20 and D9 after selection:



II. <u>The shape and colour of the colonies which produced OTC</u>

All the productive mutants had rounded substrate colonies with a crenate edge, the colour was brown to dark brown in the centre, and waxy at the edges (Very light colonies or very dark ones gave negligible OTC). With Y20, the colonies showed some cracks in the centre. Aerial growth was off-white in colour. A very important feature was that the colonies gave an obvious pigmentation of the medium.

III. <u>Establishment of the master cultures</u>

After the reselection of the strains D9, Y20, FF, master cultures were made of each by filling one slope of each strain with sterilized liquid paraffin and kept at $4^{o}C$, another set of slopes were kept in the deep freeze at $-70^{o}C$.

A first generation slope for each strain was made every month from the master slope by transferring a loopfull to a slope of RC medium which was incubated at $25^{o}C$ for 10 days; from these "second generation" slopes were made in suitable numbers after they had grown as before, they were held in the refrigerator and used as required, the maximum storage life being twenty one days. For the inoculation of flask cultures, slope suspensions were made by rubbing off the spores with a small amount of sterile water which was then used for seeding the inoculum medium.

4) ESTABLISHMENT OF FERMENTATION METHODS

I. Establishment of the shaken flask process:

The original two and three stage procedures which were described in the methods section were found to give lower quantities of OTC than expected, it was suspected that this was due to the type of chalk used and the amount of C.S.L. on the medium. Tests were carried out using different types of chalk, different proportions of chalk and C.S.L. in the medium, and also on the quantities of the medium in the production flasks. Tables 6 and 7 give the results of two trials.

Table 6. Medium IV with different concentrations (average of three runs) of $CaCo_3$ and C.S.L. (Y2O) (BDH AR chalk)

	12 g/l C.S.L.	15 g/l C.S.L.	18 g/l C.S.L.
19g/l $CaCO_3$	2.7g/l OTC	2.9g/l OTC	2.4g/l OTC
23g/l $CaCO_3$	2.9g/l OTC	3.4g/l OTC	2.8g/l OTC
27g/l $CaCO_3$	2.6g/l 2 OTC	3.0g/l OTC	3.1g/l OTC

Table 7. Medium IV with different volumes of medium per flask

ml per flask	g/l OTC produced		
	Run 1	Run 2	Run 3
30	4.2	3.8	4.0
40	3.9	3.5	3.8
50	3.7	3.5	3.8
60	3.5	3.1	3.2

After the trial with different $CaCO_3$/C.S.L. proportions in the medium and the volume of it in the flasks, a trial was made to see the effect of the number of spores used to seed the KL or inoculum medium, and it was found that the best results could be obtained when the highest number of spores were used for the seeding, this was 10^7/ml in the case of Y20, and 10^6/ml in the case of 72T1, when the slopes were rubbed with 9 ml sterilized distilled water. Higher spore levels of that seem to have no effect on the amount of OTC formed or the type of growth.

Two other types of chalk, obtained from I.C.I. Pharmaceutical Division were also tested, one being Britomaya Violet Label (Melbourne Whiting Co., Royston, Herts). Neither gave good results in shaken flasks. After these tests all the experimental work in shaken flasks used modified medium IV for the production stage with the following composition:-

Starch	55g/l
Ammonium sulphate	7g/l
$CaCO_3$ (BDH,AR)	23g/l
C.S.L.	15g/l
Ammonium chloride	1.7g/l
P.PG	0.2 ml/l
Arachis oil	20 mls/l

30 mls of the medium were used per flask.

II. Establishment of the stirred culture process

(a) Inoculum preparation

Two stage inoculum was used with stirred culture. KL medium was inoculated with the spore suspension and incubated on the shaker for 48 hours at 25°C, this was used to inoculate the inoculum medium, which was used to inoculate the production stage, after 30 hours incubation in the case of the mutants, and for 48 hours for the wild strains, this was when the volume of the cells in the medium occupied more than 80% of a universal bottle if the culture was left to stand for 30 minutes.

(b) Main fermentation

In the first runs $CaCO_3$ was added to the production fermenter after the second day, 5 g/l per day till the fourth day, also, the stirring speed was 650 rpm. Later it was found that this speed is not the ideal one for the mutants, so the speed was reduced to 520 rpm which proved to be more useful with the mutants, aeration was initially one volume air to one volume medium, this also

proved not to be successful for the mutants and the flow was reduced to 0.5 volume to one volume medium. The oil tended to disappear from the production fermenter with the mutants after the second day so 20 mls of sterilized oil was added to the medium every day untill the end of the fermentation. P.P.G. was added to the wild strain fermenter, 1.5ml/day, as the wild strain shows high tendency to foam.

The type of $CaCO_3$ proved to be very important with the fermenters as BDH (Analar) which was used successfully with the shake flasks, proved unsuitable, but Britomaya Violet Label was very successful.

5) <u>EFFECT OF OTC ON THE GROWTH OF Y2O AND 72T1</u>

Several slopes of molten RC media were taken, and different amounts of predissolved OTC added to them, after cooling they were inoculated with the two strains Y2O and 72T1 and incubated at 25°C. It was noted that Y2O can grow on slopes containing high concentration of OTC, while 72T1 showed no growth except at the lowest concentration of OTC as shown in Table 8.

Table 8 Resistance of the strains to OTC

U/ml OTC in slope 960U = 1mg OTC	Strain	
	Y20	72T1
2000	growth	growth
3000	growth	poor growth
4000	growth	no growth
7000	growth	no growth
8000	growth	

6) INDIRECT EXTIMATION OF CELL CONCENTRATION BY PROTEIN ASSAYS

With the media used, it was difficult to measure cell concentrations accurately, on the account of the large amount of insoluble matter present. Measurements of the cells were essential to monitor growth. In former I.C.I. work, cells were measured by filtering a measured sample of culture, drying in vacuo, extraction of fat and then ashing to allow for inorganic matter. The result gave cells plus OTC, the latter being allowed for after assay. Even so, this method was unsatisfactory in the early stages (up to 48 hours) owing to the insolubility of some of the starch, values at "0" hours being around 11 g/l of dry matter.

In the present work, such a procedure would have been too laborious, and it was decided to use the protein analysis as a measure of cell growth, using a factor to give an estimate of the cells present.

These protein assays were being made in any case, in connection with

the estimation of enzyme concentrations.

Cell preparations, made by filtering and washing with acid, and then with ether, followed by drying, showed nitrogen contents of 6.0-6.5%, corresponding with a protein concentration of 38% on multiplication by 6.25. This estimate would include nitrogen in cell walls etc, so it might be too high. Hilliger and Nitzsche (1974) found a value of 31% with synthetic medium, or 43% with complex medium. Our medium is in the semi-synthetic category, containing 6 g/l of corn-steep solids, the rest being inorganic salts, starch and oil.

Further evidence was obtained by an experiment by Wilkinson, R. (unpubl.) in which several actinomycetes were grown in shaken flasks, using a semi-synthetic medium using malt-extract instead of starch and only 1% of chalk. Dry weight and protein measurements were made. Average results showed that 10 g/l protein corresponds to 30 g/l of cells, with a protein/cell ration of 0.33, though there was a rather high level of error. The estimates by all these methods was about 33%.

ON these grounds a factor of 3.0 was chosen which gave results in line with I.C.I. data, i.e. the production of 25/30 g/l of cells in 4-5 days.

It may give rather lower results at 24 hours, than should be the case. It was considered adequate for the purpose for which it was to be used.

3.2 FERMENTATION STUDIES

1) GROWTH AND METABOLISM OF WILD AND MUTANT STRAINS PRODUCING OTC.

2) RESULTS IN SHAKEN FLASKS.

3) GROWTH AND METABOLISM OF MUTANTS PRODUCTING LITTLE OR NO OTC ON AGAR MEDIUM.

4) DISCUSSION OF THE FERMENTATION RESULTS.

1) GROWTH AND METABOLISM OF WILD AND MUTANT STRAINS PRODUCING OTC

I. Stirred culture:

The cultures were grown in 5 litre fermenters inoculated with 300 mls inoculum. The inoculum were grown in similar fermenters, which were inoculated with 50 mls of culture in KL medium, giving a total volume of 3 litres. The results are given in the Appendix and a summary is given in Table 9.

(a) Mutants Y20 and D9D7:

These mutants gave the simplest type of growth and production curves, with deep brown colours and strong actinomycete odour. The results in Fig. 5 were taken from runs which provided full data including gas analysis i.e. runs 9 and 11 for Y20 and 17 and 18 for D9D7. The growth curves showed rapid growth up to 80-90 hours, after which growth became negative. OTC first appeared at about 30 hours and production continued until about 100-120 hours. The main differences between the strains were that D9D7 produced OTC for a longer period than Y20 (24 hours more), giving 7.5 g/l against 6.0 g/l with Y20.

Y20 and D9 were fairly similar although there was a more obvious decline in respiration with Y20 after 60 hours than with D9D7. The RQ values for both of the strains fell below 1.0 after 24 hours and remained around 0.8 during the production phase, after which they fell to 0.65-0.7 at the end of the fermentation.

Values were:-

RQ value during growth and production

Mutant	Age hours 24	48	72	96	120
Y20	1.05	0.82	0.77	0.88	0.71
D9D7	-	0.81	0.85	0.80	0.65

Gas analysis was not performed with D9D7 at 24 hours

Fig. 6 shows the growth rate, uptake of oxygen, output of CO_2 and OTC production rate, based on the data of Y20 (run 9 and 11).

The growth rate was higher up to 72 hours (when OTC reached 5g/l) then slowed down rapidly and became negative. Prior to the decline, respiration had also declined (this is not uncommon in the anti-biotic production, when growth and respiration are often relatively rapid at the first). When OTC production ceased (about 96 hours) the growth rate had already fallen, but respiration continued normally, although growth had become negative.

(b) Wild strain 72T1:

This strain showed an entirely different growth pattern. There was very rapid growth up to 24 hours followed by steady concentration of cells (Fig. 7), OTC production started about the same time as the cells stopped growing. As regards respiration, oxygen input was higher than Y20, the concentration in the effluent gas being 1.4% (48-84 hours) against 1.1% for Y20, although no growth was occurring.

With 72T1 only a slight brown colour (pale) was formed with a weak odour, suggesting a much weaker type of secondary metabolism. The

data used for Fig. 7 were based on runs 13 and 14, using inoculum grown for 48 hours. The other runs 6 and 10 with inoculum grown for 30 hours (as Y20) gave much weaker growth and OTC production, but almost equal consumption of oxygen and CO_2 production. This suggested that 72T1 is much less efficient than Y20 and that its metabolism is less well controlled.

Table 9. Stirred culture results

Strain	72T1					Y20			
Run No.	6*	10*	13	14	22	7**	8**	9	11
Age, hrs			Protein, g/l						
24	1.4	2.4	4.6	4.4	3.3	2.2	2.8	2.1	2.4
48	2.4	2.6	5.1	4.4	3.9	6.1	5.9	4.2	4.5
72	2.6	2.6	4.7	4.6	4.4	7.5	8.4	6.8	7.2
96	2.4	2.6	5.0	4.8	4.7	9.0	9.8	7.9	10.2
120	2.4							8.1	9.2
			OTC g/l						
48	0	0	0	0	0	-	1.9	1.9	1.4
72	0.5	0.1	0.9	0.8	0.4	2.0	3.1	2.8	4.2
96	0.5	0.3	1.0	0.9	0.8	4.4	6.3	5.7	6.6
120								5.9	6.0
			CO_2, % in effluent (v/v = 0.5)						
24	2.65	1.45	0.80	1.5		0.9	1.8	1.4	0.58
48	1.68	1.30	1.70	1.40		1.25	1.5	0.8	0.9
72	-	0.80	0.50	1.10		1.50	2.40	0.32	0.80
96	0.46	1.15	0.30	0.31		0.97	1.20	0.31	0.70
120								0.70	0.49
			O_2, % in effluent						
24	17.6	19.8	20.2	19.6			19.4	19.7	19.7
48	18.5	19.6	19.1	19.4				20.0	19.7
72	-	19.6	19.5	19.8				20.5	20.0
96	20.6	19.7	19.6	19.4					20.2
120								20.1	20.2

Strain	Y20 continued			D9D7			Y20 Inact.	Mutant 6
Run No.	12	20	25	5	17	18	19	
Age,hrs				Protein, g/l				
242	2.1	3.1	2.4	-	-	-	1.9	2.0
48	4.6	5.1	4.8	4.1	4.5	4.9	4.6	3.6
72	8.4	7.9	7.7	7.0	8.0	8.5	7.5	7.6
96	9.8	9.6	9.2	7.9	9.1	9.9	8.2	9.0
120	9.0				8.6	9.1	-	-
				OTC g/l				
48	1.3	2.3	1.9	1.1	1.7	1.9	0.9	0.3
72	3.4	5.8	5.3	2.4	3.5	3.9	3.4	2.1
96	5.6	6.8	7.1	4.3	6.0	6.6	4.0	3.9
120	5.7				6.9	7.9		
144					6.6	7.0		
				CO_2, % in effluent (V/V = 0.5)				
24	1.10			-	-	-		2.1
48	1.00			-	0.73	0.75		1.20
72	0.90			0.97	0.73	0.63		0.53
96	0.70			1.70	0.74	1.10		0.32
120	0.50				0.48	0.50		
				O_2, % in effluent				
24	19.9			-	-	-		18.4
48	19.8			-	20.0	20.0		19.8
72	19.6			19.8	20.0	19.4		20.1
96	20.0			18.2	19.8	18.8		20.2
120	20.2				20.3	20.0		

*Poor inoculum (30hr only)

**No oil addition at 40,72 hrs.

Fig. 5. Growth, respiration and OTC curves in stirred cultures

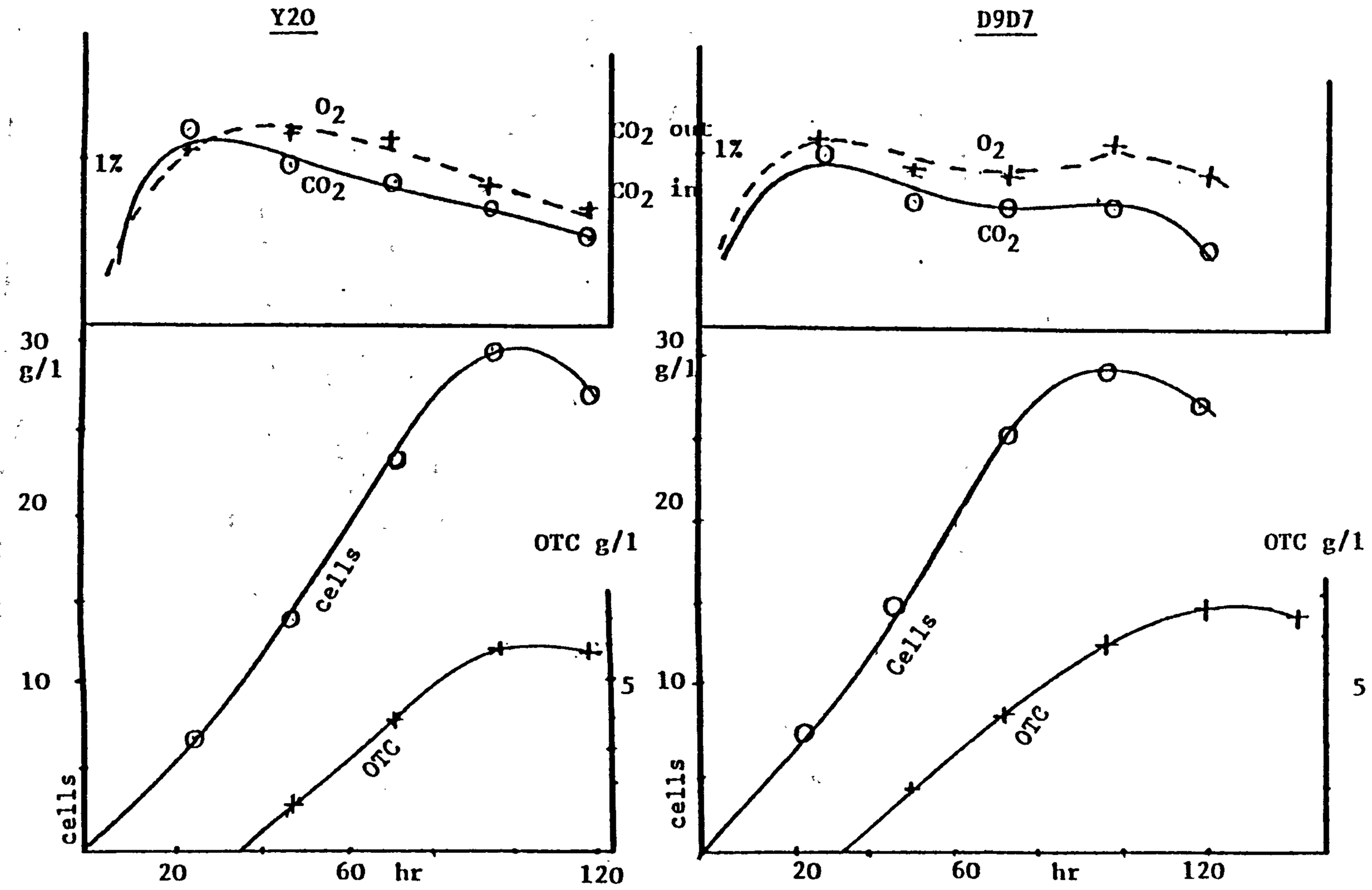


Fig 6

GROWTH RATE; RESPIRATION & OTC PRODUCTION, Y20

(Stirred Culture)

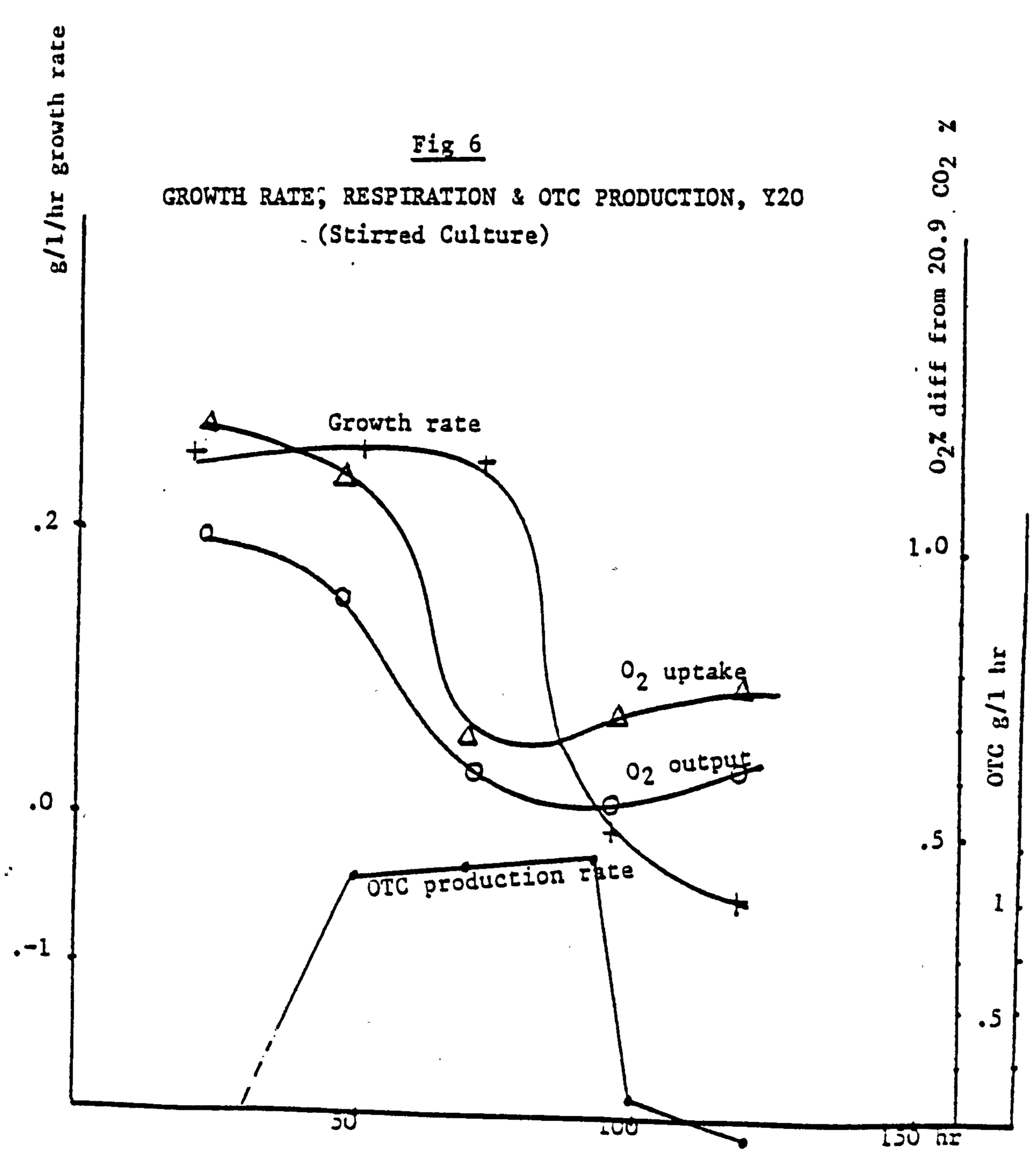
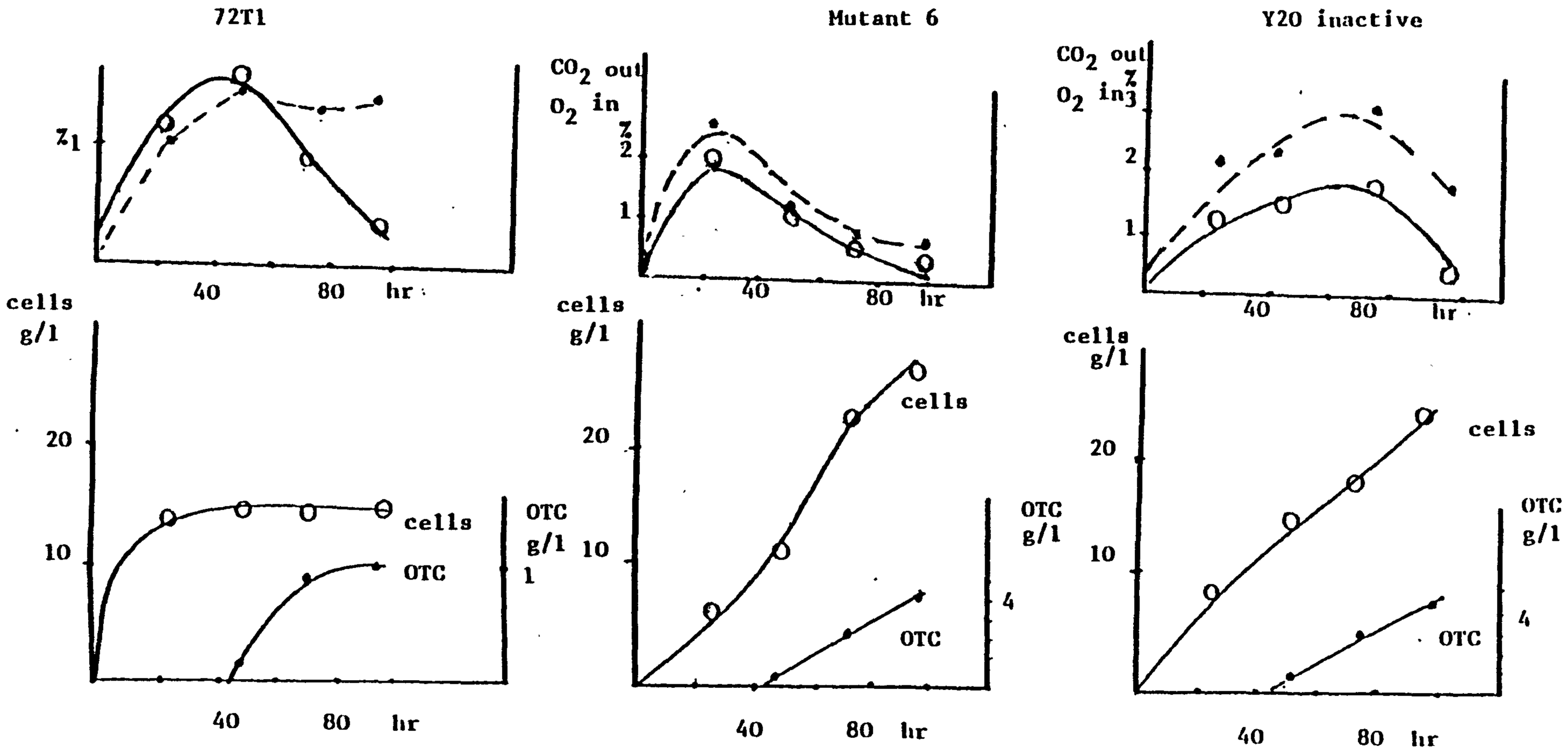


Fig. 7. Growth, Respiration and OTC stirred culture



2) RESULTS IN SHAKEN FLASKS

I. Y2O and 72T1

Figure 8 shows representative results in shaken flasks. The medium was the same as that used in stirred culture. Growth and OTC production are shown in the lower section, respiration above. Respiration was measured by passing air through the flasks and then closing the flasks with a rubber bung. The air in the flasks was analysed after 40 minutes incubation on the shaker. The flasks contained 30 ml of culture and 270 ml air. Uptake of oxygen and production of CO_2 could be readily calculated.

With Y2O, growth was rapid, but the final cell concentration was slightly lower than in stirred culture. OTC production was also lower, 3.5-3.9 g/l compared to 5.5 - 6.0 g/l. As will be shown later; this was due to inefficient metabolism with the formation of acetyl-OTC. The respiration rate was higher than in stirred culture (per gram cells), with RQ just below 1.0. The cultures became dark brown and the odour was strong. Tested with iodine, starch disappeared by 72 hours, and oily drops after 48 hours.

With 72T1, the metabolic pattern was different. Initial rapid growth ceased after 24 hours, and the cell concentration remained fairly constant at only 18 g/l. OTC reached ca 0.9 g/l after 96 hours. The respiration rate was lower. Owing to low lipase activity, and as with this strain in stirred culture, the oil was little used, and droplets remained present even after 96 hours. The much lighter colour of 72T1 cultures, is shown in the photograph.

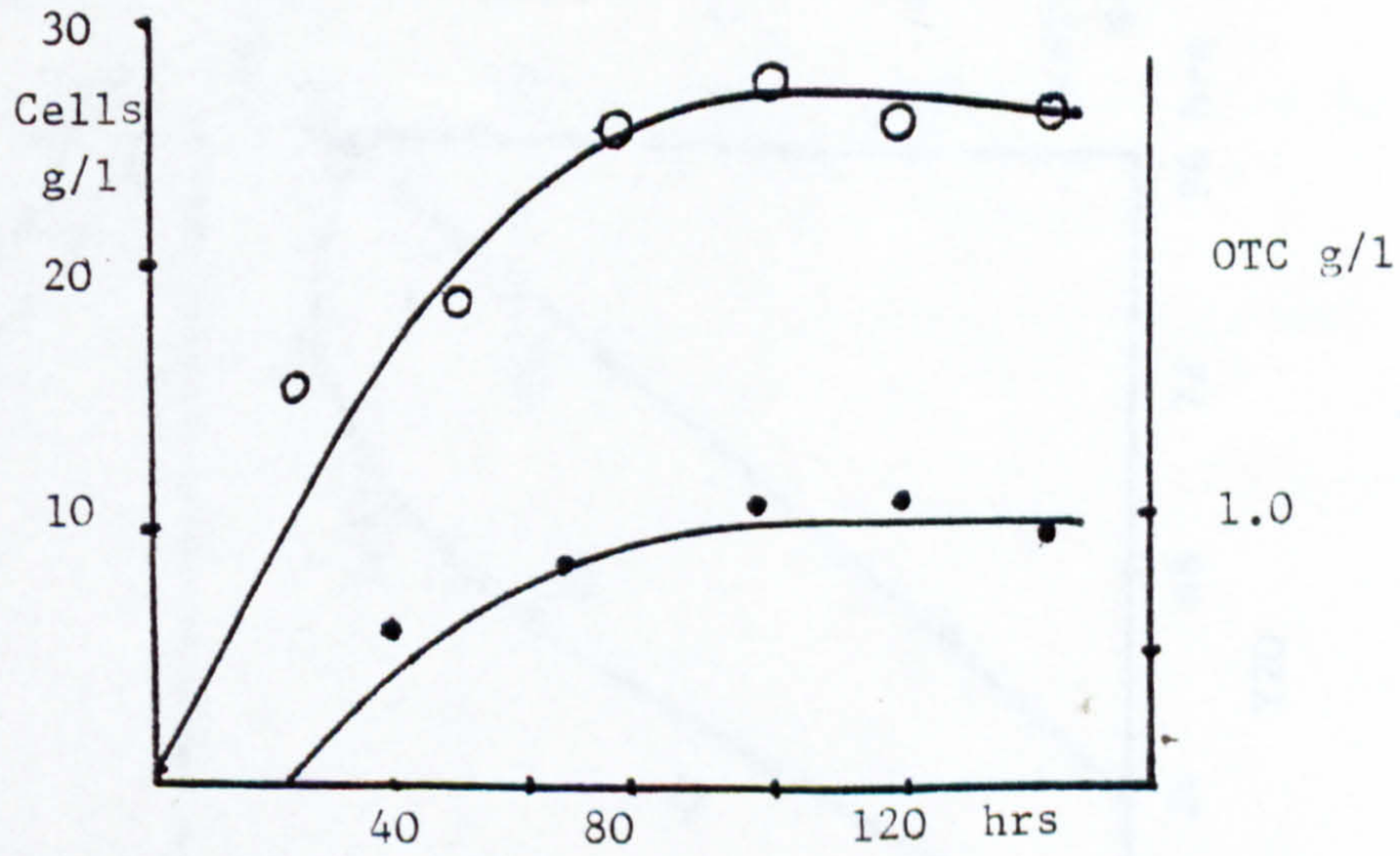
Y20

72T1

II. S.rimosus NRRL2234

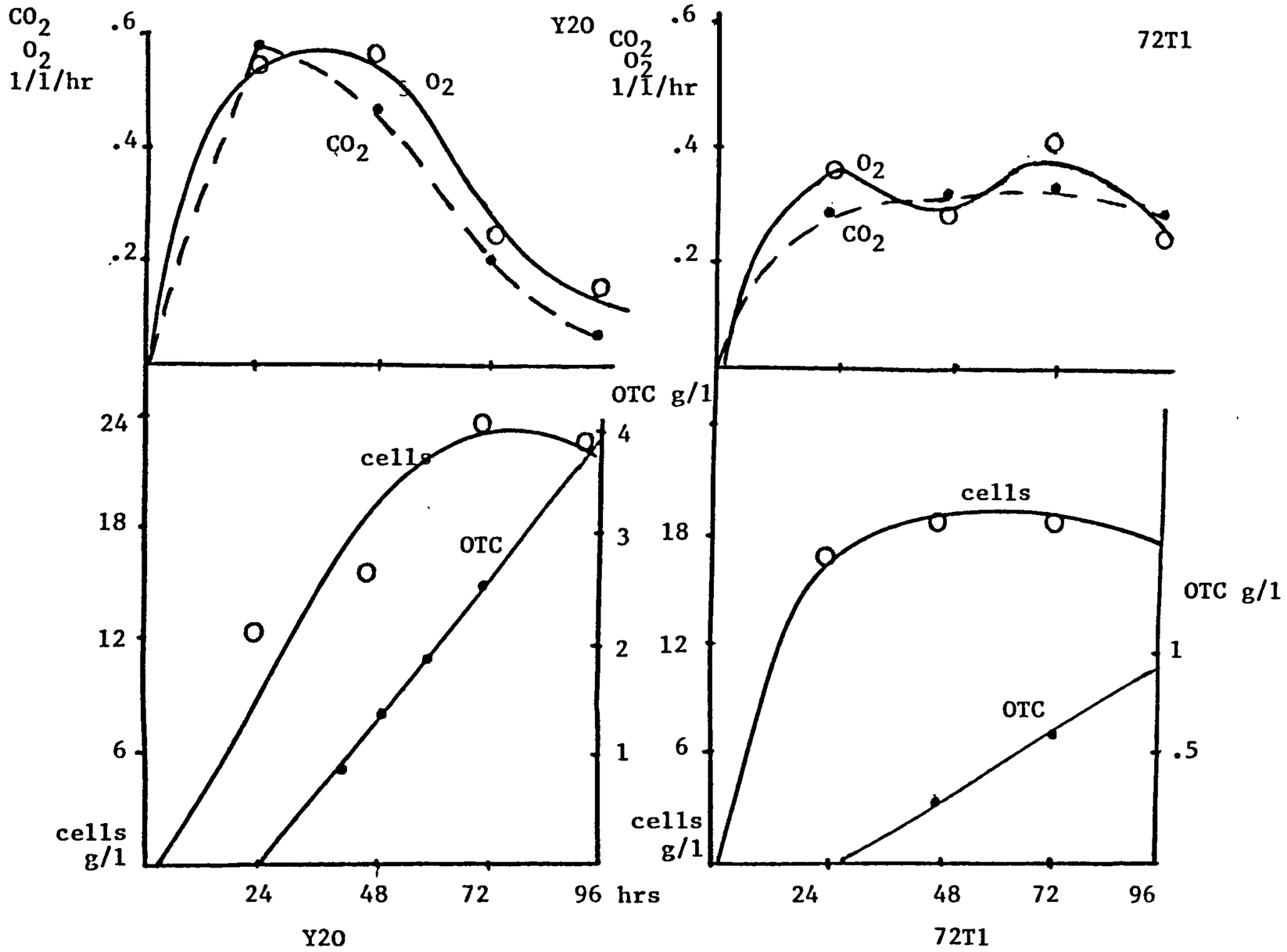
The results obtained with this strain (NRRL 2234) are illustrated in Fig. 9 based on two runs, the OTC assays were by the ferric chloride method, and are probably about one third too high. The growth curve shows rather rapid growth at first, but growth continued for a long time,

Fig. 9 S.rimosus in shaken flasks



resembling Y20, and there was also some development of a brown colouration. OTC production commenced at an earlier age.

Fig. 8. Growth Respiration and OTC curves (shaken culture)



3) GROWTH AND METABOLISM OF MUTANTS PRODUCING LITTLE OR NO OTC ON AGAR MEDIUM

I. Mutant 6:

This mutant of Y20 produced little or no OTC on agar or the shaken flasks, but produced OTC in the stirred culture. The metabolic and growth pattern shown in Fig. 7. The growth pattern was similar to Y20 but the ratio of OTC produced/cells was much lower, while respiration differs from Y20 by being higher at the beginning, after 48 hours the respiration was like that of Y20.

II. Y20 inactive:

This was the original I.C.I. mutant after storage in soil for 15 years. Like mutant 6 this mutant gave no OTC on agar or shaken flasks, but produced OTC in stirred culture. The growth pattern was like that of Y20, but the respiration rate differed from Y20, as CO_2 output showed slight increase with time upto 72 hours then it fell off sharply.(Fig.71. The RQ value remained around 0.6 for the first 3 days before it decreased to around 0.5 after 96 hours, indicating significant change in metabolism.

4) DISCUSSION OF THE FERMENTATION RESULTS

It is interesting to compare 72T1 and Y20 under the two standard conditions of stirred and shaken cultures as shown in Fig. 10. The main features were that in both stirred and shaken cultures, 72T1 gave faster initial growth, but for only a short time. In particular in stirred culture, growth ceased often after only 30 hours, probably before OTC production commenced. This rapid growth was accompanied by high CO_2 production, which fell sharply after 48 hours. This might be due to the fact that 72T1 was more sensitive to OTC than Y20, or it could be caused by lack of nutrients due to lower breakdown of fatty acids, as most of the oil added to 72T1 medium was still present at the end of the fermentation. In shaken flasks 72T1 grows rather better i.e. steady growth and more cell formation, with extensive CO_2 production. Y20 behaves less smoothly in shaken flasks than in stirred culture, with quite rapid growth, but with declining CO_2 production from as early as 48 hours, at which time the rate was particularly high. As high production needs steady production of cells and respiration, this occurs best with Y20 in stirred culture. 72T1 seemed to react poorly to stirred conditions while Y20 does not respond well to conditions in shaken flasks, as shown by the rapid fall in CO_2 production. Righelato *et al* (1968) stated that for a good production, steady respiration was needed to provide maintenance energy.

It was notable that all the OTC producing mutants gave much better growth than the wild strain especially in stirred culture.

In the case of Y20 and D9D7 attempts were made to prolong the

growth by the addition of starch, chalk, ammonium sulphate and oil during the late part of the fermentation, but these had no effect on growth or respiration. It was also observed that D O T was always around 50% of saturation, so there should be no lack of oxygen. It therefore appeared that the fermentation may be brought to an end due to the toxic effect of OTC which appeared to have a detrimental effect on the system.

Attempts were made to increase the OTC production, in stirred cultures with D9D7 and Y20 by the addition of malonamide to the culture (runs 17 & 12), but no increase in the level of OTC were recorded.

The effect of the stirred culture was more obvious with mutant 6 and Y20 inactive, as the stirred culture conditions forced these strains to produce OTC. With mutant 6 a high respiration rate was also observed, as with 72T1. This indicated that mutation not only altered the pattern of OTC production, it has made changes in the early stages of the metabolism.

The results showed that the process was very sensitive to growth conditions, for example:-

1) Stirred culture gave more OTC than shaken flasks, mainly because of the formation of OTC rather than acetyl-OTC and OTC.
2) The process was sensitive to the age of the inoculum as the mutants needed about 30 hours growth before they could be used to inoculate the production stage, this time increased to 48 hours for the wild strains.

3) The inoculum and production stages were sensitive to the stirring speed. Fast stirring caused the culture to be thrown into a fast growing state with rapid respiration so that it failed to produce a brown colour, odour and OTC, probably due to the failure of the metabolism to follow the correct course.

4) In the present study the process was found to be very sensitive to the type of chalk used, an effect which has been recognised in industrial OTC production. Chalk is known to effect the growth pattern of cultures, presumably by providing some support for the mycelium development. Also because OTC is a chelating agent and it forms an insoluble compound with $CaCO_3$ which removes most of the OTC from the solution (Riviere 1977). Chalk was also added to the medium to maintain a pH (6.5-7.0) suitable for the growth of the producing organism. At this pH the presence of chalk would tend to remove phosphate and metals which might have some negative effect on the growth.

5) If a culture passed into the correct pattern of metabolism this was usually held, and OTC production was good. The importance of the pattern of growth, in fermentations is recognized especially in industry.

6) The condition of the cells during the fermentation determined the amount of OTC formed, and production may continue after growth stops. According to I.C.I. data for 30 liter and large fermenters, production continued either with growth (production slowing) or without growth (production continuing) as shown in Fig. 11.

7) In spite of apparently poorer conditions of aeration and agitation, growth in shaken flasks was much faster during the

first 24 hours than in stirred culture. There was however a tendency to form acetyl-OTC instead of OTC.

8) All the signs were that growth and production were severely limited by the toxicity of the product. This is consistent with the general effect of antibiotics on the producing organism as mentioned above.

Fig. 10. Comparison between Y20 and 72T1 in shaken and stirred culture

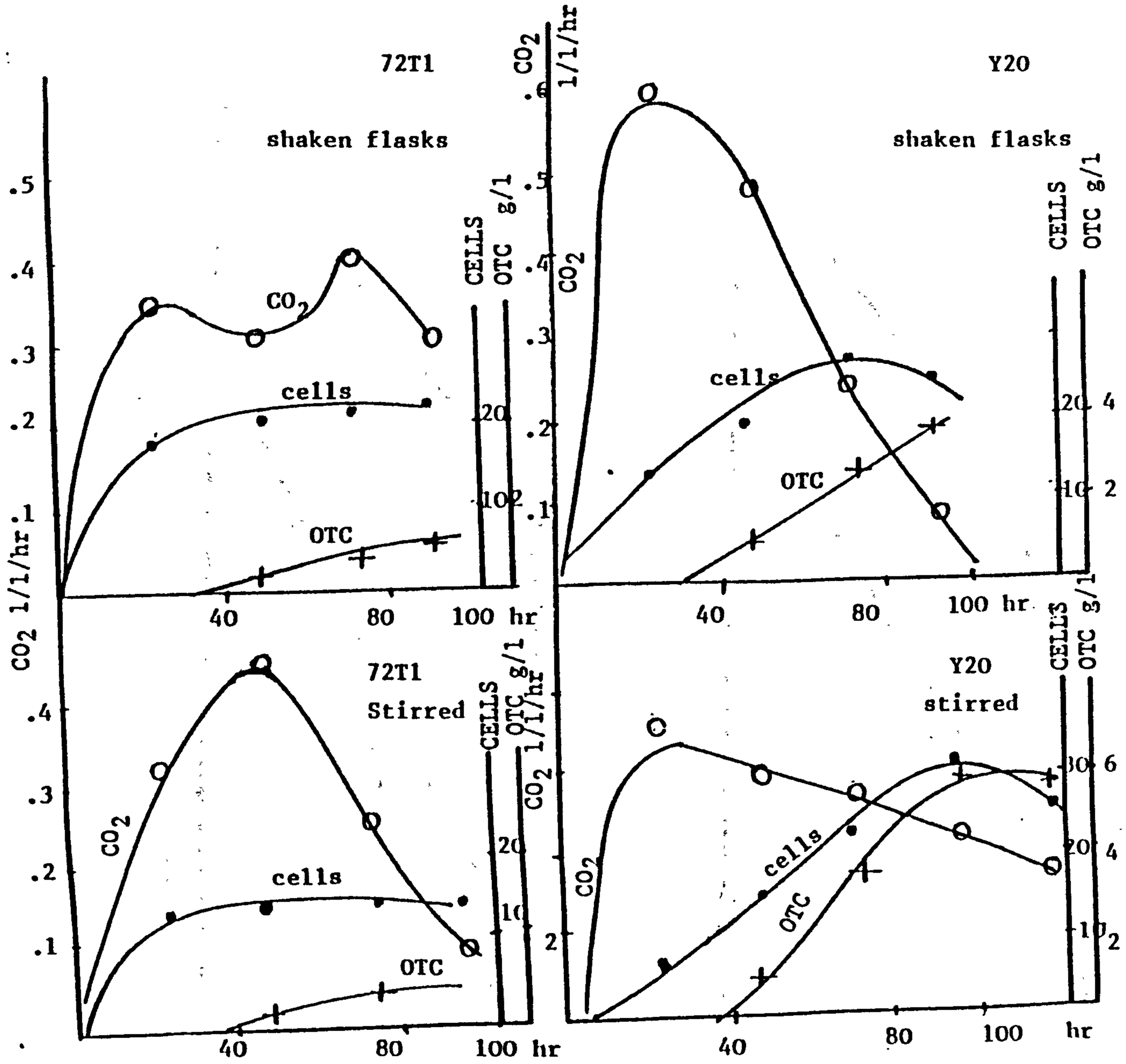
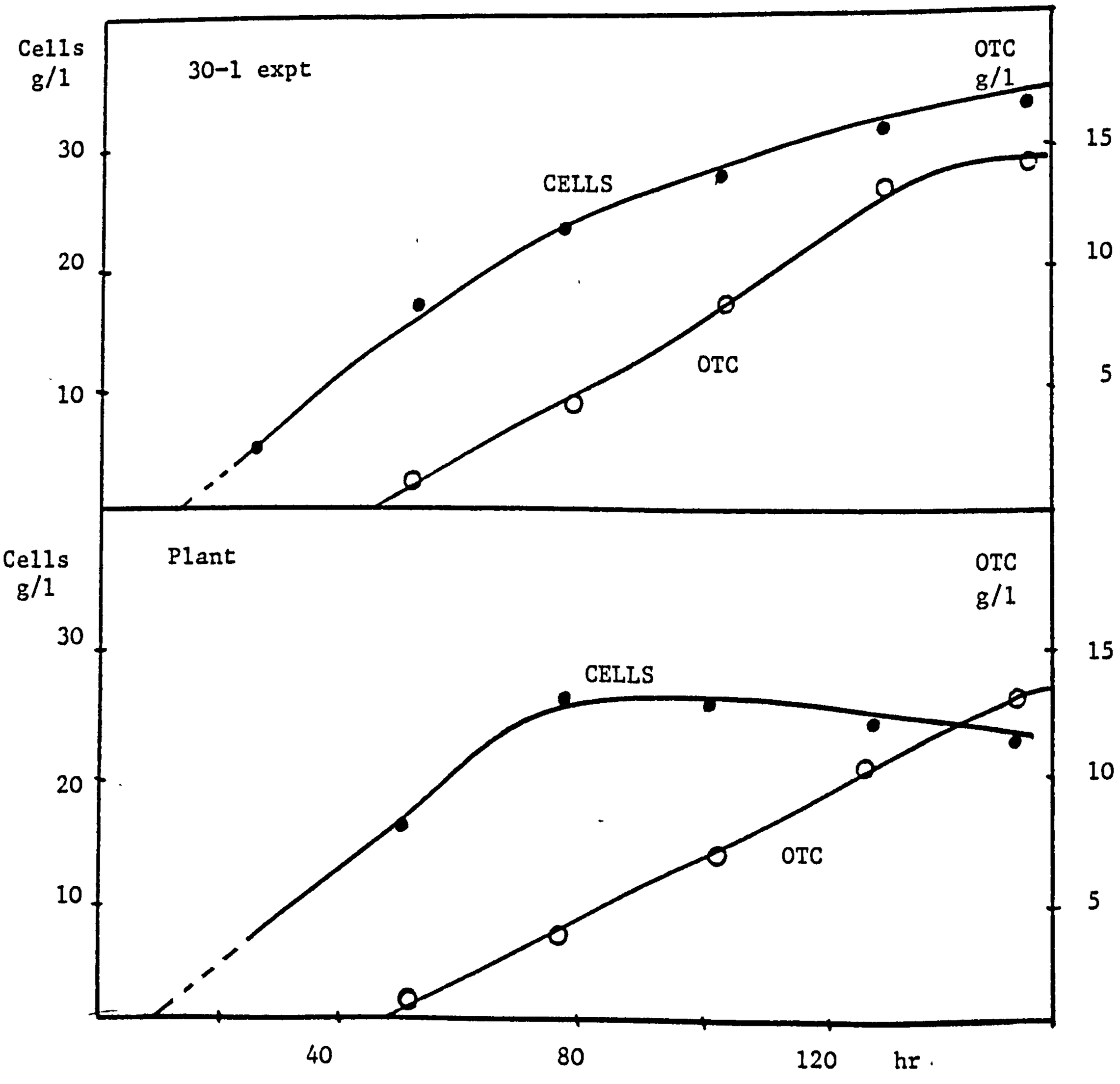


Fig. 11. Industrial data for OTC production



3.3 CHROMATOGRAPHY

1) PAPER CHROMATOGRAPHY OF TETRACYCLINE SOLUTION.

2) CHROMATOGRAPHY OF DIFFERENT TYPES OF BROTH.

3) RESULTS OF THE CHROMATOGRAPHY.

1) PAPER CHROMATOGRAPHY OF TETRACYCLINE SOLUTION

Broth extracts from the strains were tested for their OTC contents and other products, by three methods of paper chromatography.

I) The main method used was with Whatman P81 cellulose-phosphate paper developed with 0.1% NH_4Cl as described by Addison and Clark (1963), the spots were viewed under a U.V. lamp (Woods Glass) and patches were cut out and eluted with 10% NH_4Cl and the absorption spectrum examined.

II) Other resolving systems used were:

(a) butanol:acetic acid:water, 4:1:5 (Kelly et al 1960)

(b) chloroform:nitromethane:pyridine, 10:20:3 (Selzer et al 1957).

The Rf values of these systems were

	P81	butanol	nitromethane
OTC	.61	.59	.31
CTC	.61	.76	.50
TC	.59	.65	.28

(TC = tetracycline, CTC = chlortetracycline, OTC = oxytetracycline

The tests showed that in all cases the main spot of antibiotic was in the OTC position.

2) CHROMATOGRAPHY OF DIFFERENT TYPES OF BROTH

When the strains were grown in shaken flask culture, OTC production (g/l) was lower than in stirred culture. In addition, when the ferric chloride method for OTC assay was used a greenish colour was developed rather than the reddish brown of the stirred culture. It was clear that a mixed product were being obtained in the shaken cultures.

The fermentation broth were therefore tested by P81 paper chromatography and developed with 0.1% NH_4Cl. The fermentation broth after extraction with HCl was adjusted to pH 5 with sodium bicarbonate, after which 0.05-0.1ml was applied to the paper. The chromatograms were allowed to develop, then they were dried in air and examined under the U.V. light. Bands with different Rf values were observed. The bands were cut into small pieces, and placed in a minimal volume of 10% ammonium chloride solution for 20 minutes to dissolve, and examined under the uv-spectrophotometer using 1cm quartz cells. Typical results are shown in Fig. 12 which shows the chromatograms of Y20 and 72T1 in shaken cultures and Y20 in stirred culture. The bands are numbered 1,2,3, and 4 and the U.V. absorption spectra of selected bands are given, with the absorption spectrum of OTC shown separately. The position of OTC spots on parallel chromatograms are also shown.

The chromatograms of stirred cultures for D9D7 and Y20 are shown in Fig. 13.

The results can be summarised as follows

(I) Stirred culture:

0.1 ml was used. A single large spot brown flouresent appeared, at Rf 0.6, with strains Y20 or D9.. There was a weak blue flouresence at the front, but OTC was clearly the main product.

(II) Shaken culture:

In this case, more complex chromatograms developed, illustrated in Fig. 13.

There were three or four main bands:

(1) Blue flourescent band at the front (Rf .8-.9)

(2) Often a weak band, showing non specific absorption (Rf .7)

(3) A brown, golden or yellow florescent band, Rf .55-.6 due to OTC

(4) A second brown or yellow band, Rf ca .3 with corresponds to acetyl-OTC.

There was thus a considerable difference between the metabolic patterns in stirred cultures and shaken cultures, which explains the different results in density and colour produced with ferric chloride. Adding together the quantities of OTC and acetyl-OTC produced, total OTC formation was roughly equal by the two fermentation methods. The results obtained presumably arise because of the non-availability of mono-malonamide as reported by Gatanbeck (1961). The production of a mixture of substances by S.aureofaciens has been reported by Hostalek et al (1976) and mentioned before.

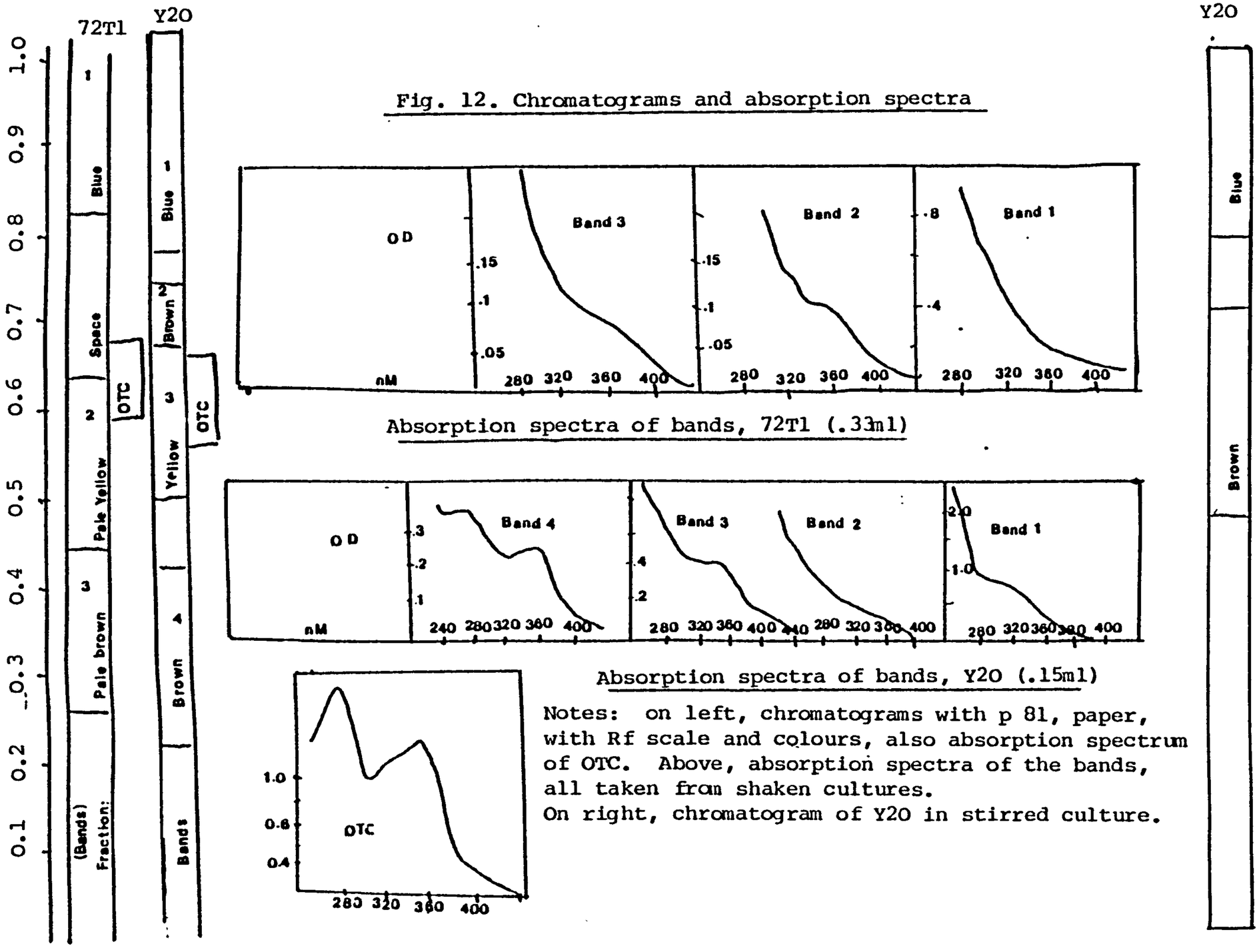


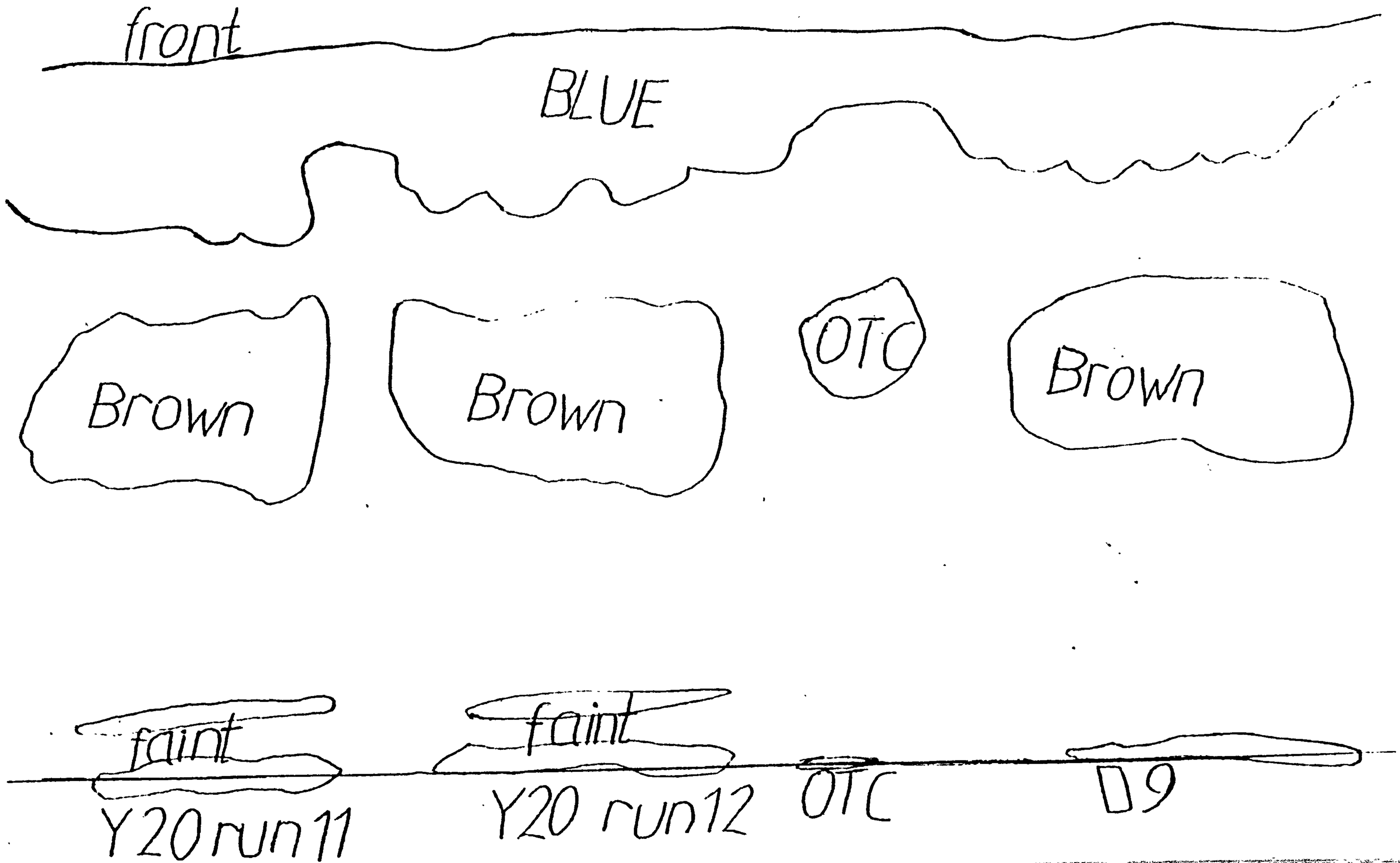
Fig. 12. Chromatograms and absorption spectra

Absorption spectra of bands, 72T1 (.33ml)

Absorption spectra of bands, Y20 (.15ml)

Notes: on left, chromatograms with p 81, paper, with Rf scale and colours, also absorption spectrum of OTC. Above, absorption spectra of the bands, all taken from shaken cultures.
On right, chromatogram of Y20 in stirred culture.

FIG. 13 Chromatograms of Stirred Culture Broths; 0.05 Ml.



3.4 EFFECT OF CULTURE CONDITIONS ON SECONDARY METABOLISM

As has already been mentioned, it was found that in stirred culture the main component obtained, after acid extraction, was OTC, while in shaken culture the OTC was contaminated with acetyl-OTC and other substances (cf. Fig 13). The pattern of these chromatograms has been summarised in Section 3.2.2, which describes four main fluorescent bands with P81 paper:-

(1)	Blue fluorescent band,	Rf 0.8-0.9
(2)	Weak band	0.7
(3)	Golden brown or yellow band	0.55-0.6 (OTC)
(4)	Golden brown or yellow band	0.3 (Acetyl-OTC).

Several other weak bands may also be seen.

It seemed possible that the difference between shaken culture and stirred culture might be due to the better stirring and aeration in stirred conditions, but the indications are that respiration rates are at least as high in the shaken flasks (Figs. 5,7 & 8). The possibility of the effect being due to the inoculum was therefore considered. In the first experiments, stirred culture inoculum was added to shaken-flask production medium, when stirred-culture type extracts were obtained, with only OTC and the blue band. This was confirmed in several repeat tests. It was also found that if stirred production fermenters were inoculated from shaken flasks, the normal stirred pattern of production occurred.

A comparative experiment was planned in which shaken culture inoculum was added in parallel to the shaken and stirred culture production stage. At the time in question it happened that supplies of BDH AR chalk (used in shaken flasks) and of Violet Label chalk (used in

stirred fermenters) had temporarily run out, and chalk obtained from Oakes Eddon Ltd., Liverpool, was used for both of the production stages. In both cases, OTC was the main product. All these results are summarised in Table 10.

These results were obtained at a late stage in the experimental programme, and further confirmation has not been possible, but the behaviour of the shaken and stirred cultures has been consistent over a long period with the types of chalk formerly used.

Table 10. Effect of fermentation conditions and chalk on metabolism

Fermentation Conditions and chalk		OTC g/l	Colour of culture 2 days old	Chromatogram Bands:			
Inoculum	Production			I	II	III	IV
Shaken BDH, AR	Shaken BDH, AR	3.5	pale	+	+	+	+
Stirred Violet Label	Stirred Violet Label	6.5	medium brown	+		+	
Stirred Violet Label	Shaken BDH, AR	5.1	medium brown	+		+	
Shaken Oakes Eddon	Shaken Oakes Eddon	4.7	light brown	+		+	

Experience with other fermentations has shown that inoculum can have a considerable effect on production, and this is recognised in the literature (Foster, 1949, Calam 1976). Meyrath et al. (1963) showed that inoculum size affected both pattern and efficiency of growth of

Aspergillus oryzae, while spore concentration has an important effect on the productivity of citric production with Aspergillus niger (Chaturvedi et al 1978). Spore concentration also had an important effect on the efficiency of inocula used for penicillin production (Smith and Calam, 1980). Chalk is well known, industrially, to affect the metabolic pattern of S. rimosus cultures, producing tetracyclines (cf. Riviere, 1977), and selection is needed to choose brands which give optimal yields. The main point of importance is the major effect produced on the metabolic pattern by such a minor factor. It is possible that only one or two genes are being affected in producing such a large effect.

3.5 BIOCHEMISTRY

1) ENZYME ASSAYS RESULTS

2) DISCUSSION OF ENZYMES RESULTS

3) ADENYLATE ASSAYS RESULTS

4) DISCUSSION OF ADENYLATE RESULTS

1) ENZYMES RESULTS

The results of the enzyme assays are given in Figs 14-21 which show the results in stirred and shaken flasks.

I) Enzymes in stirred cultures

ICDH	Fig. 14
MDH	Fig. 15
Acetyl-CoA carboxylase	Fig. 16
Citrate synthase	Fig. 17

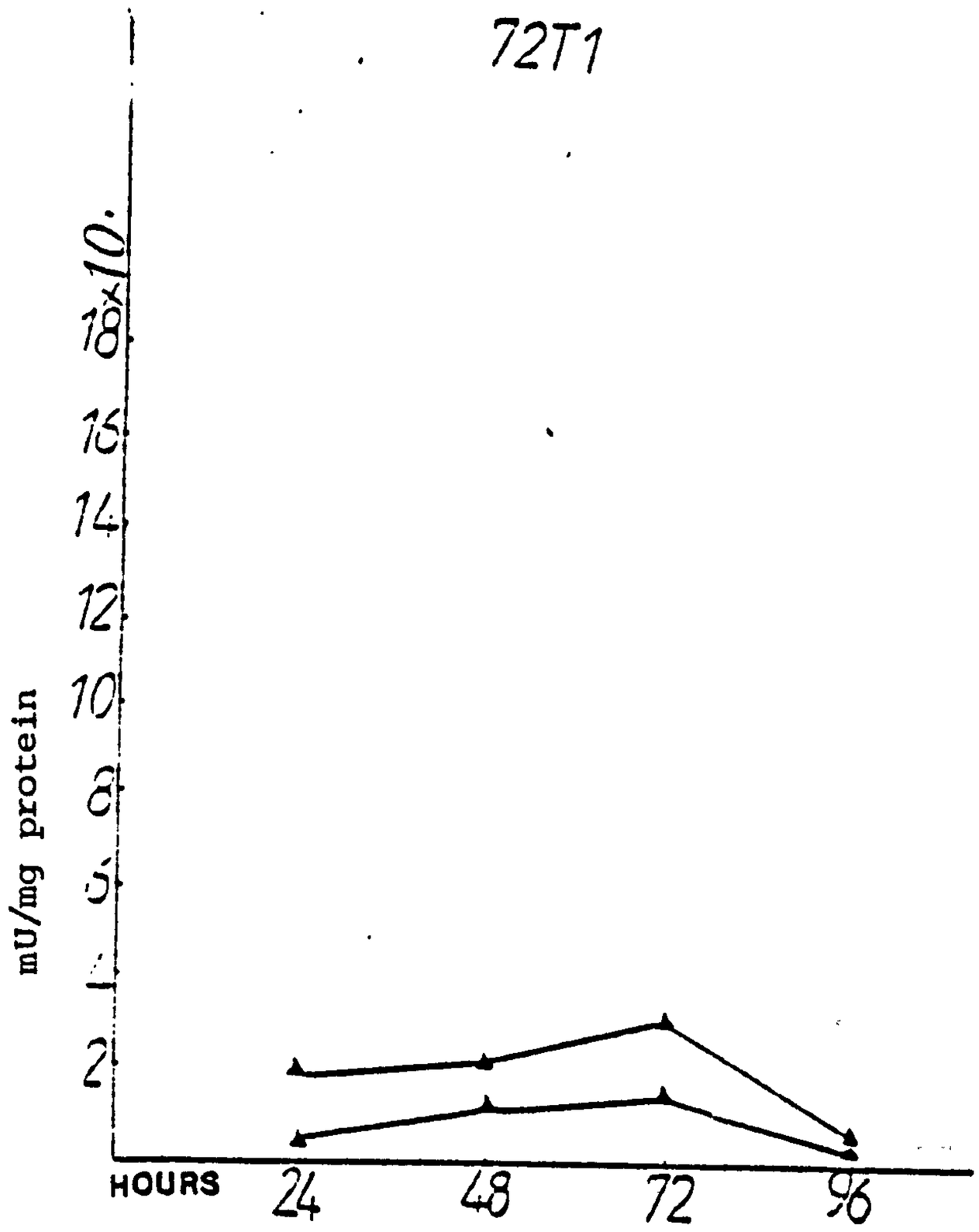
II) Enzymes of shaken flask cultures

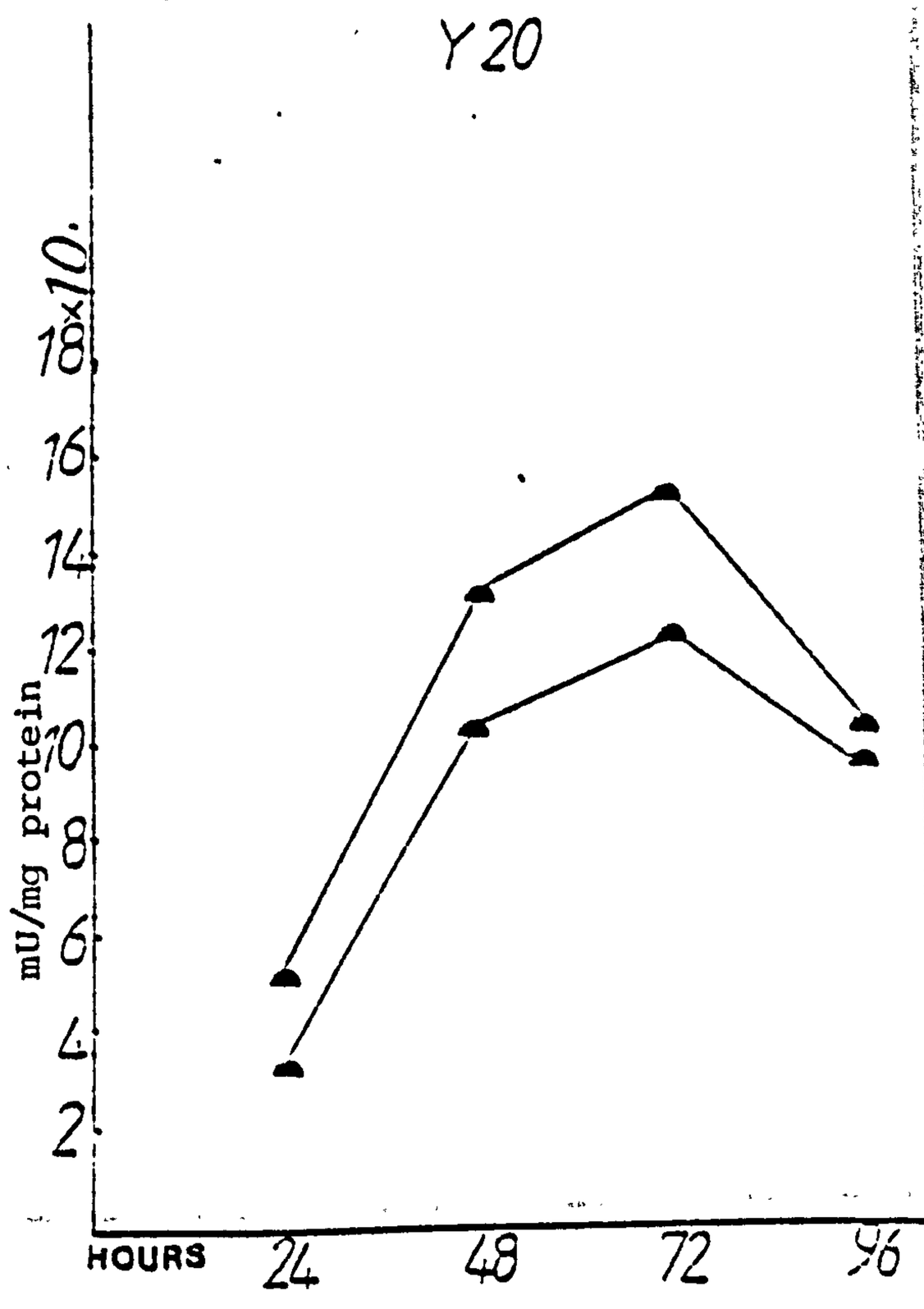
ICDH	Fig. 18
MDH	Fig. 19
PEP carboxylase	Fig. 20
Acetyl-CoA carboxylase	Fig. 21

With stirred culture the results of two batches are given, and the pairs of curves show good agreement.

With shaken cultures, more than two sets of flasks were tested, and the results showed quite a variation, which suggested great differences in the behaviour of this system. With each enzyme the lines show the standard deviation (±) on either side of the means, thus indicating general behaviour pattern and the degree of error. The biggest variation was with Acetyl-CoA carboxylase. With ATC-oxygenase the change in absorbance was recorded. Owing to the complexity of the purification procedure this was not converted to enzyme units.

Fig. 14 ICDH stirred culture (results of two tests)





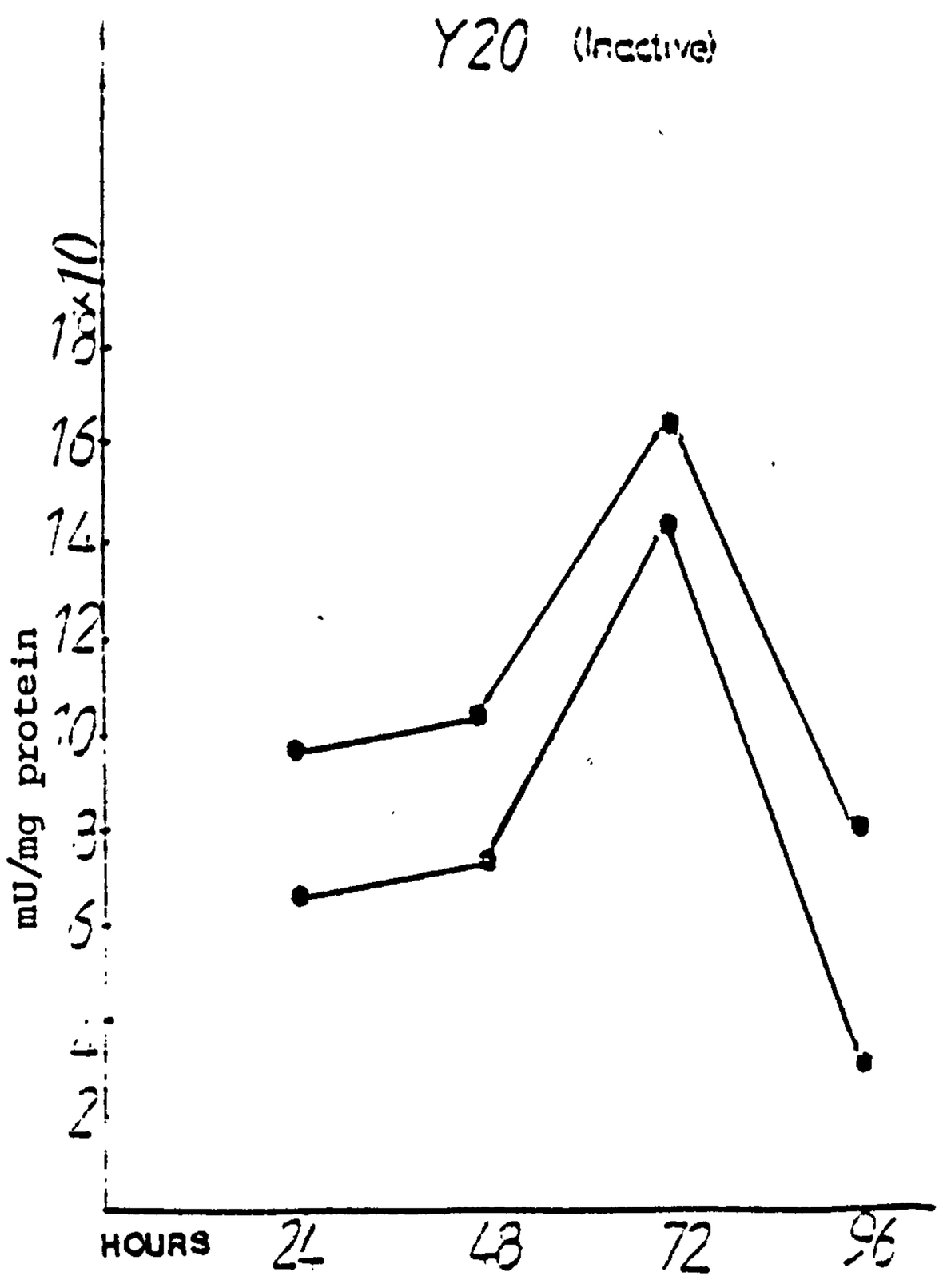
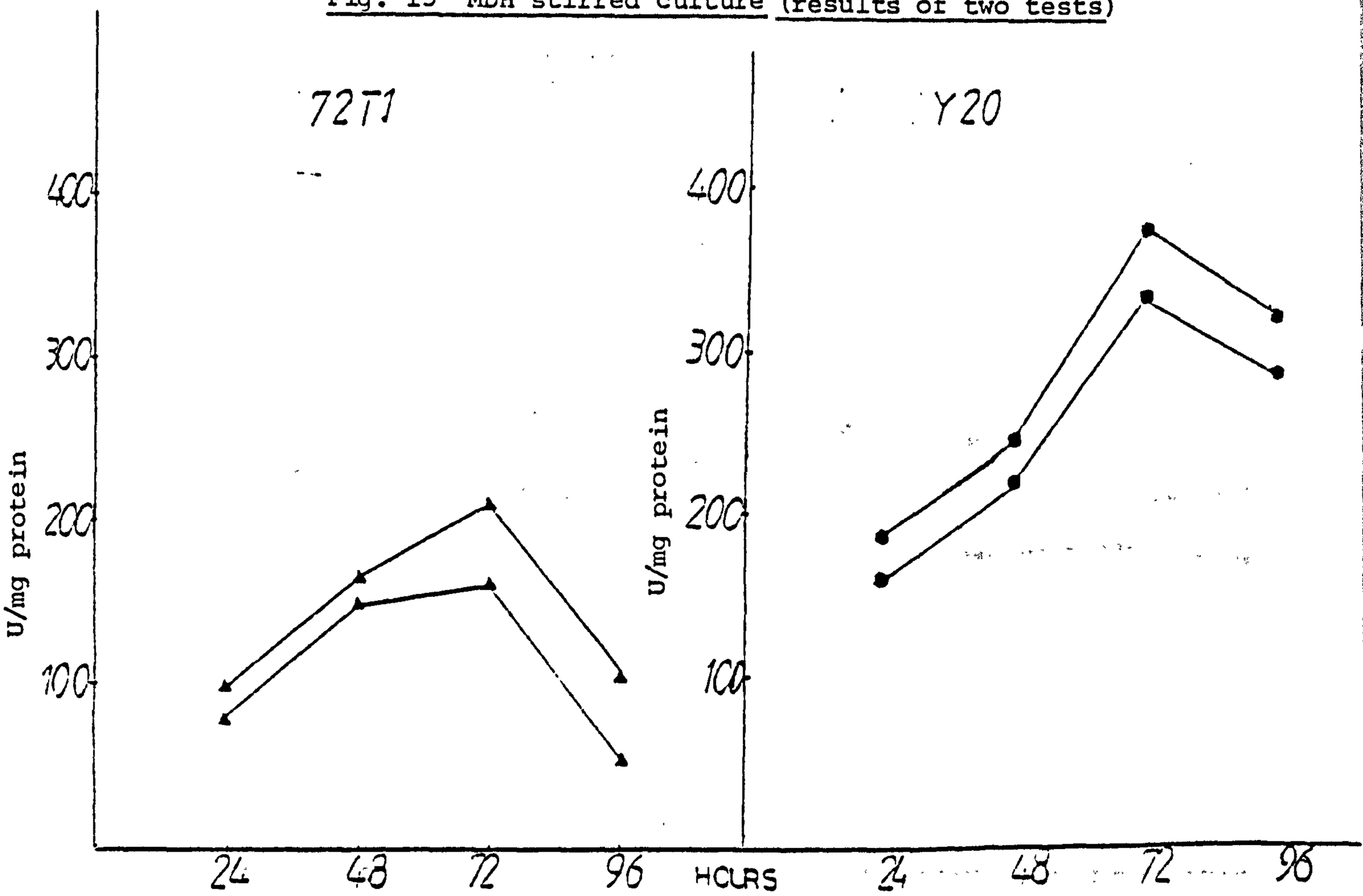


Fig. 15 MDH stirred culture (results of two tests)



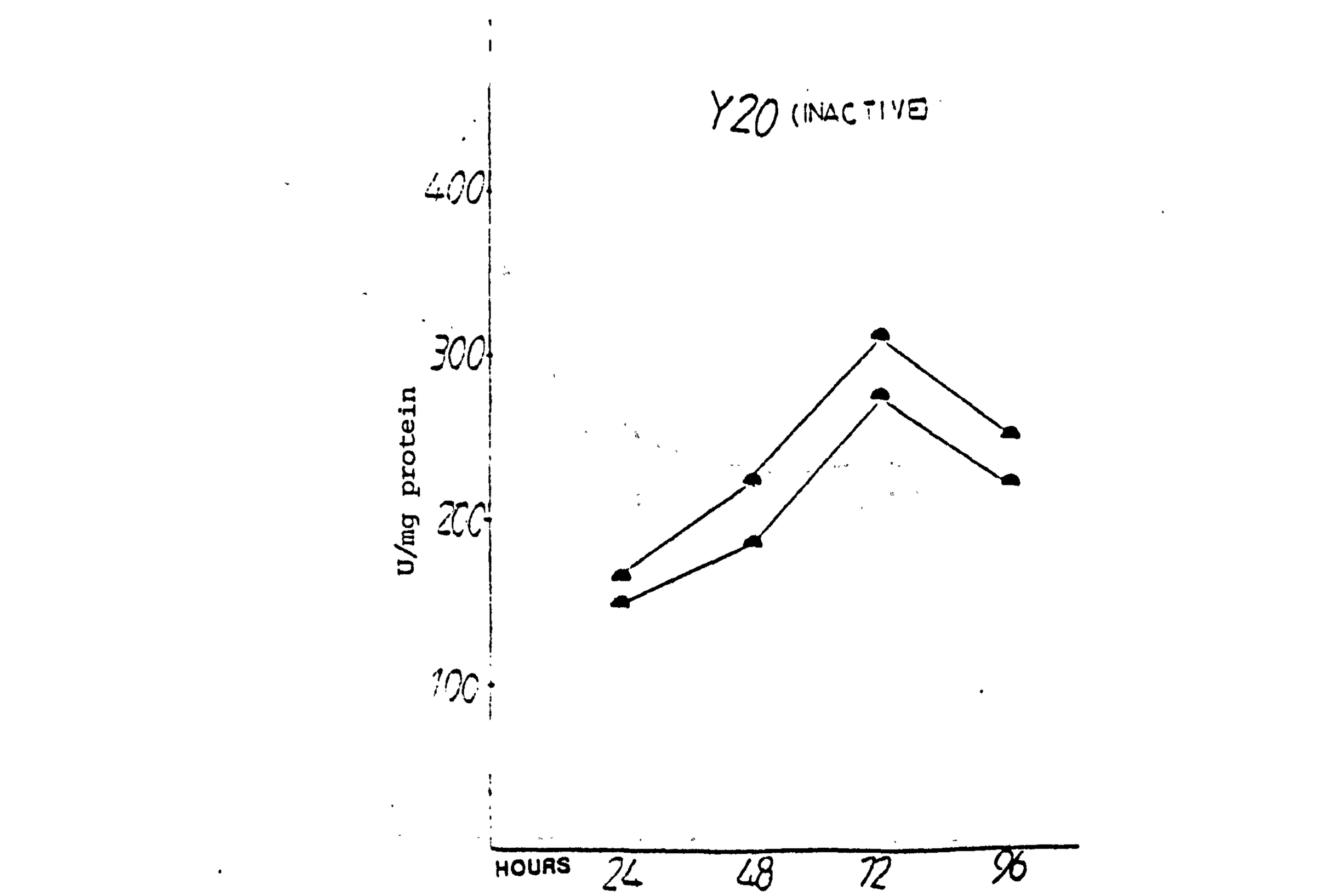
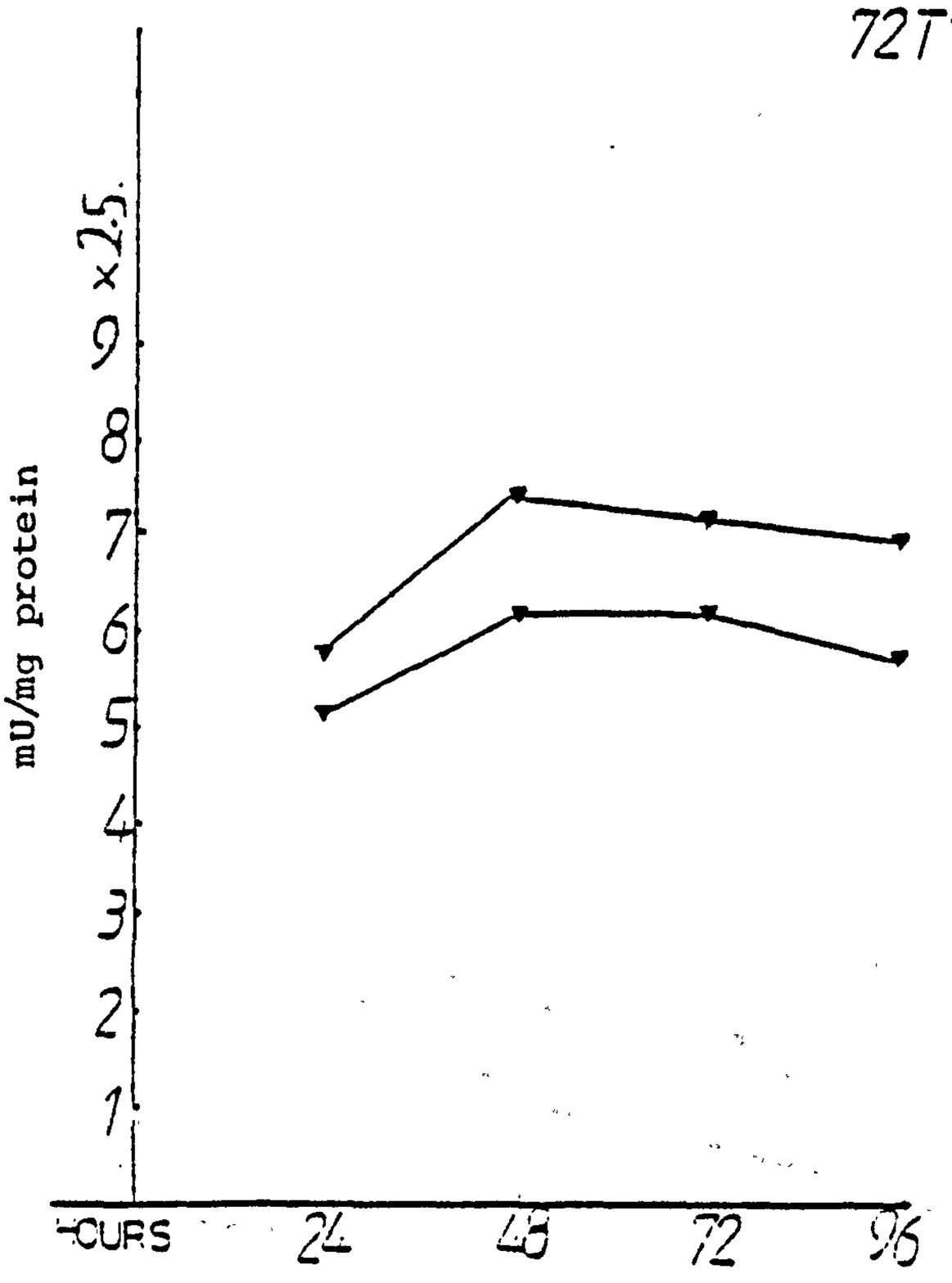
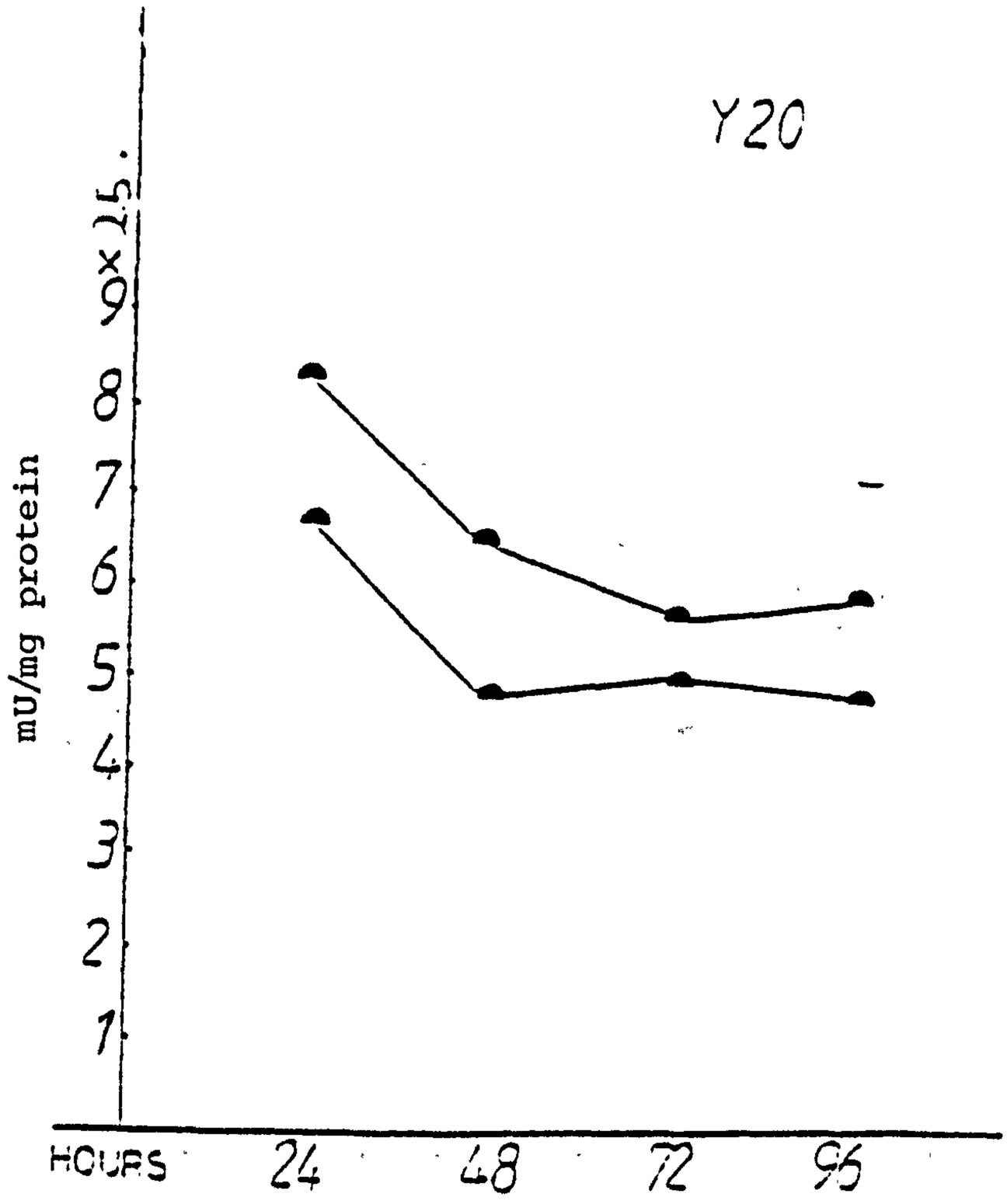


Fig. 16 Acetyl CoA carboxylae stirred culture (results of two tests)





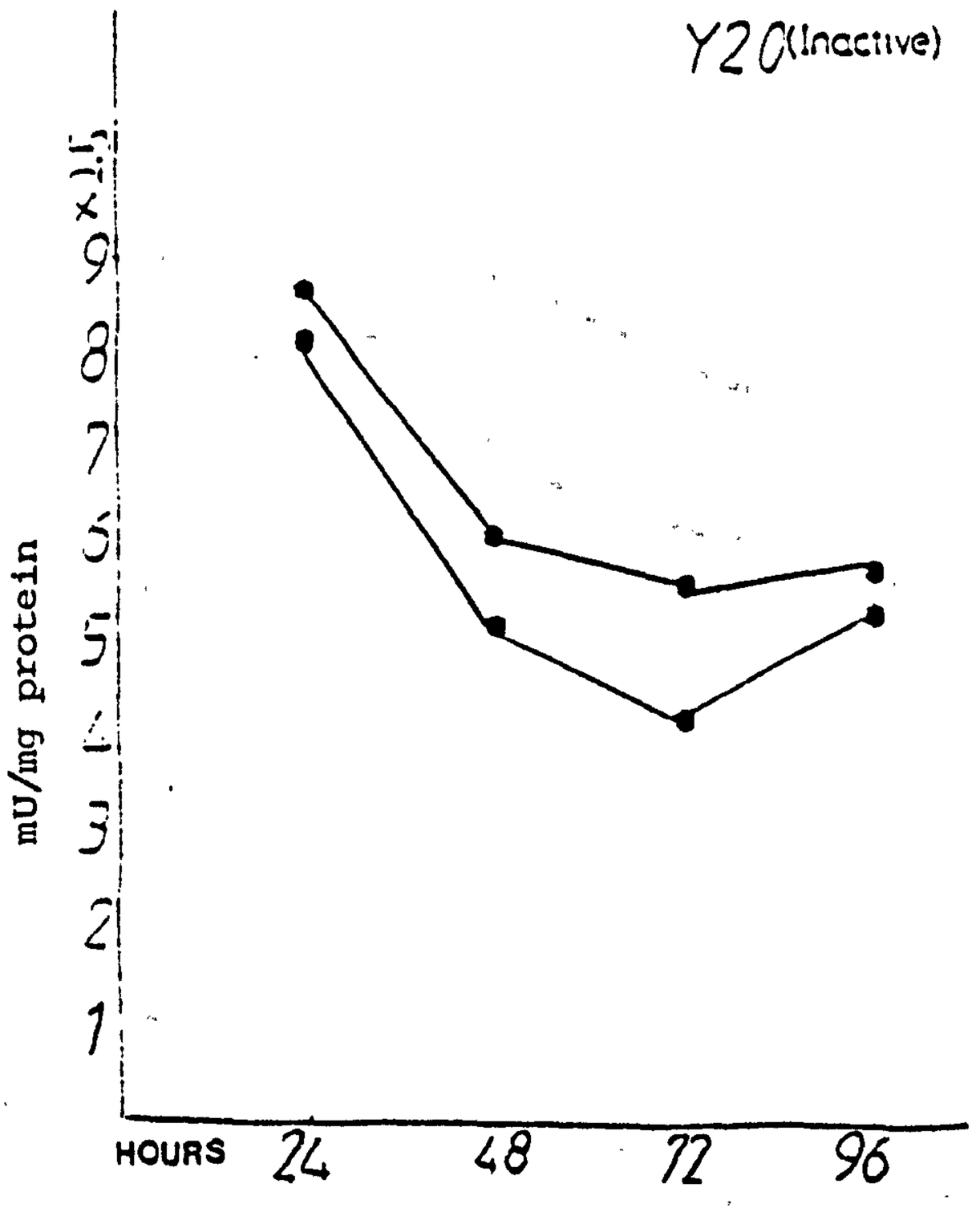


Fig. 17 Citrate synthase stirred culture (results of two tests)

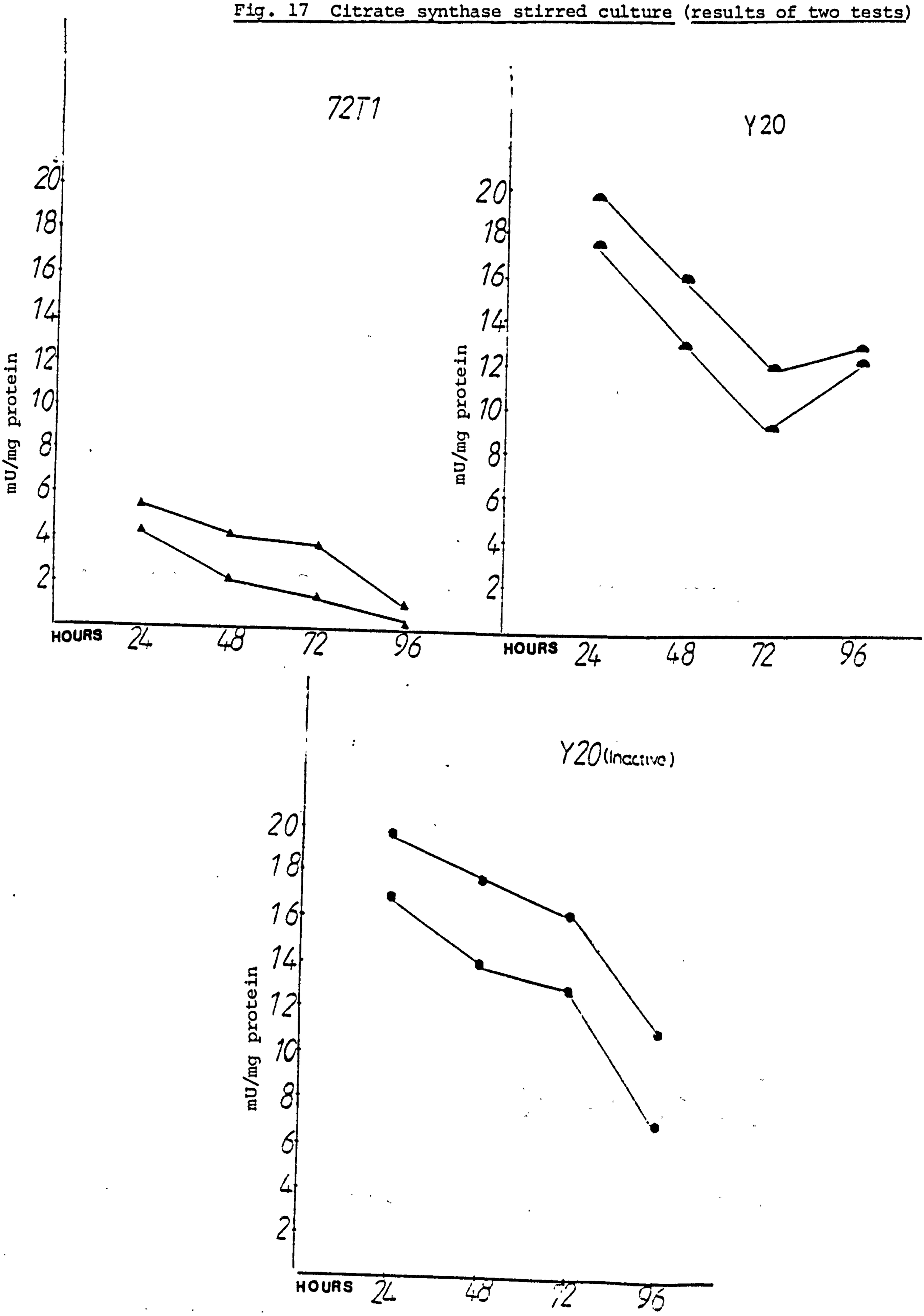


Fig. 18 ICDH shaken culture (lines show the mean ± S.E.)

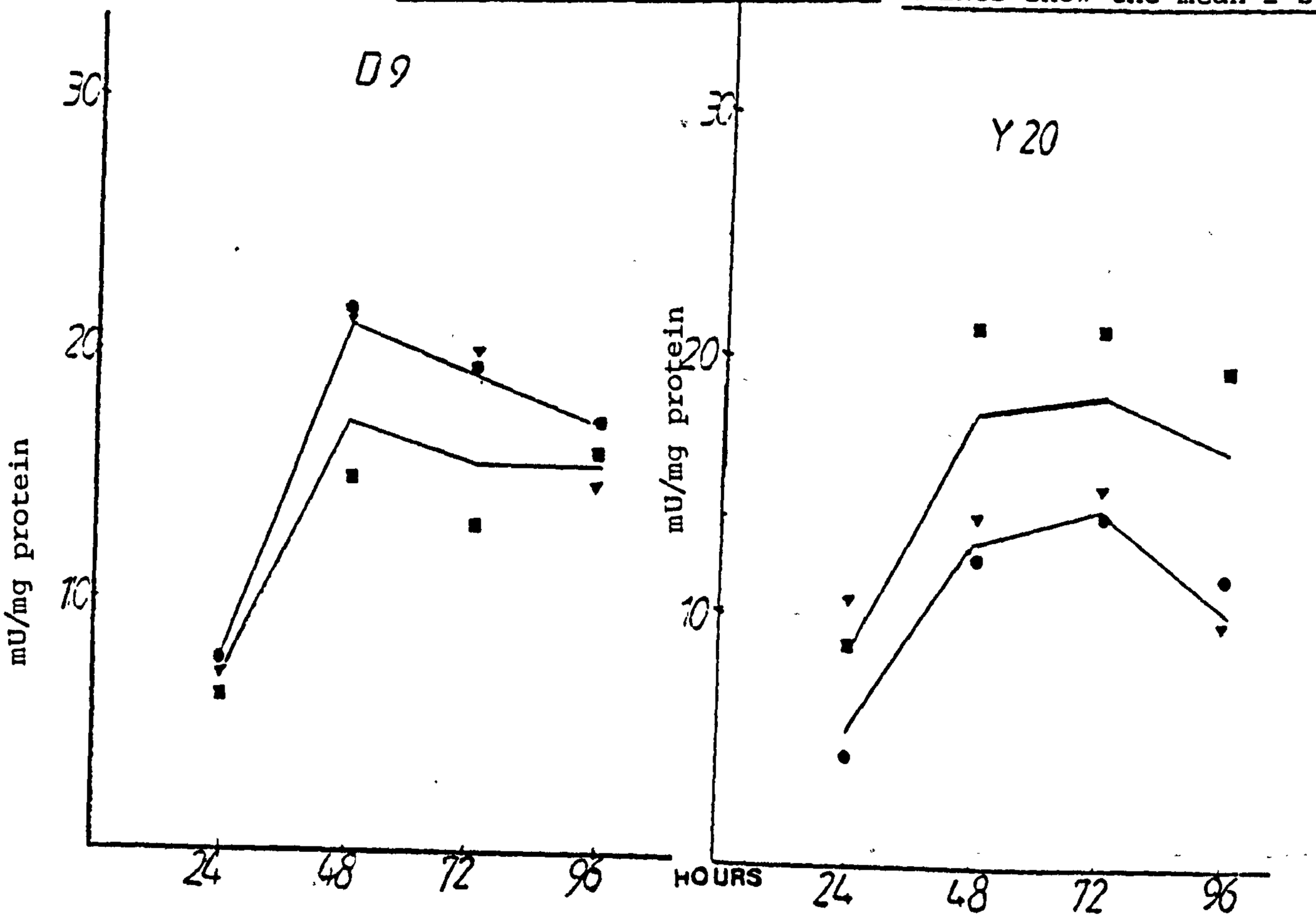


Fig 19. MDH shaken culture (lines show the mean ± S.E.)

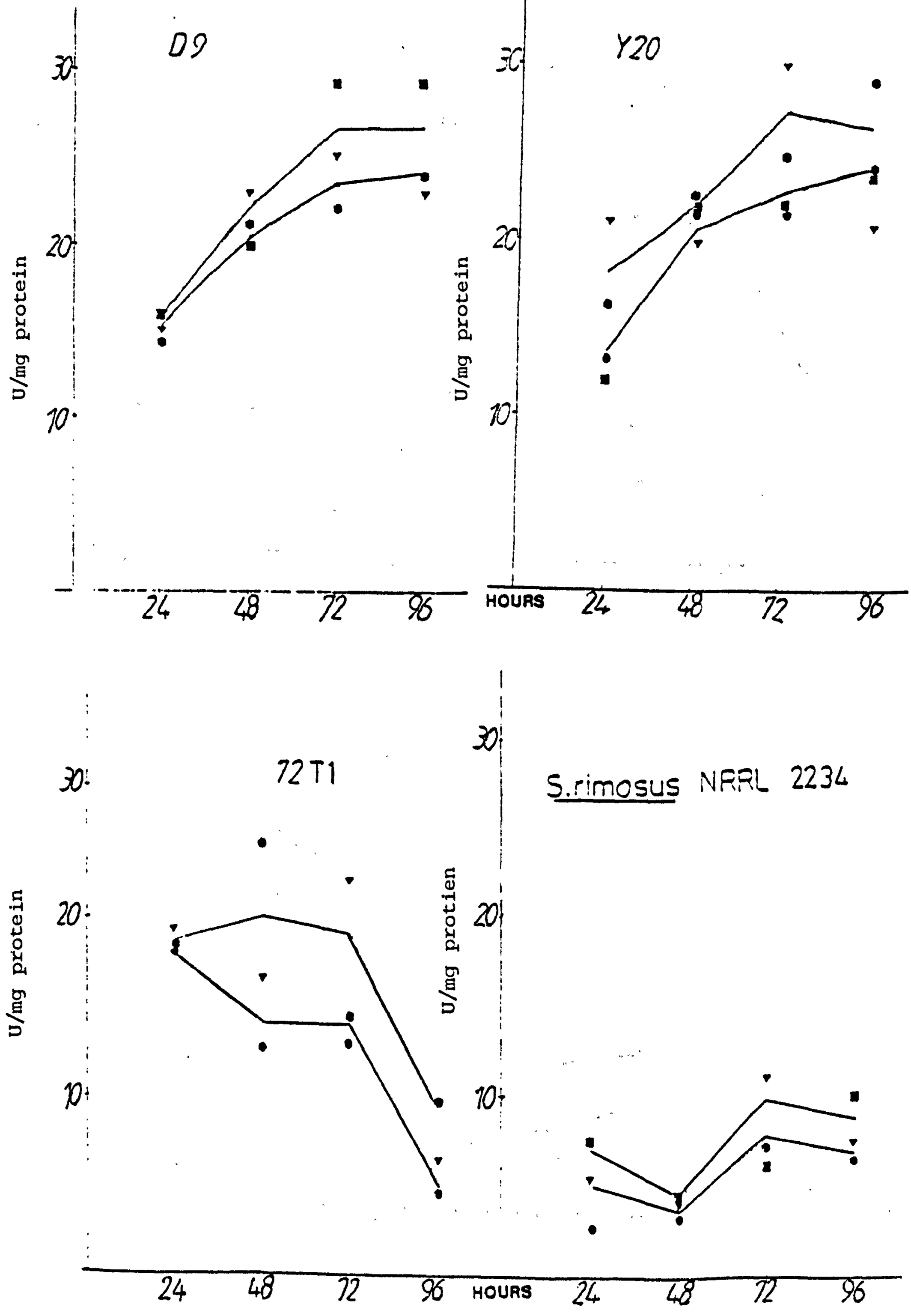
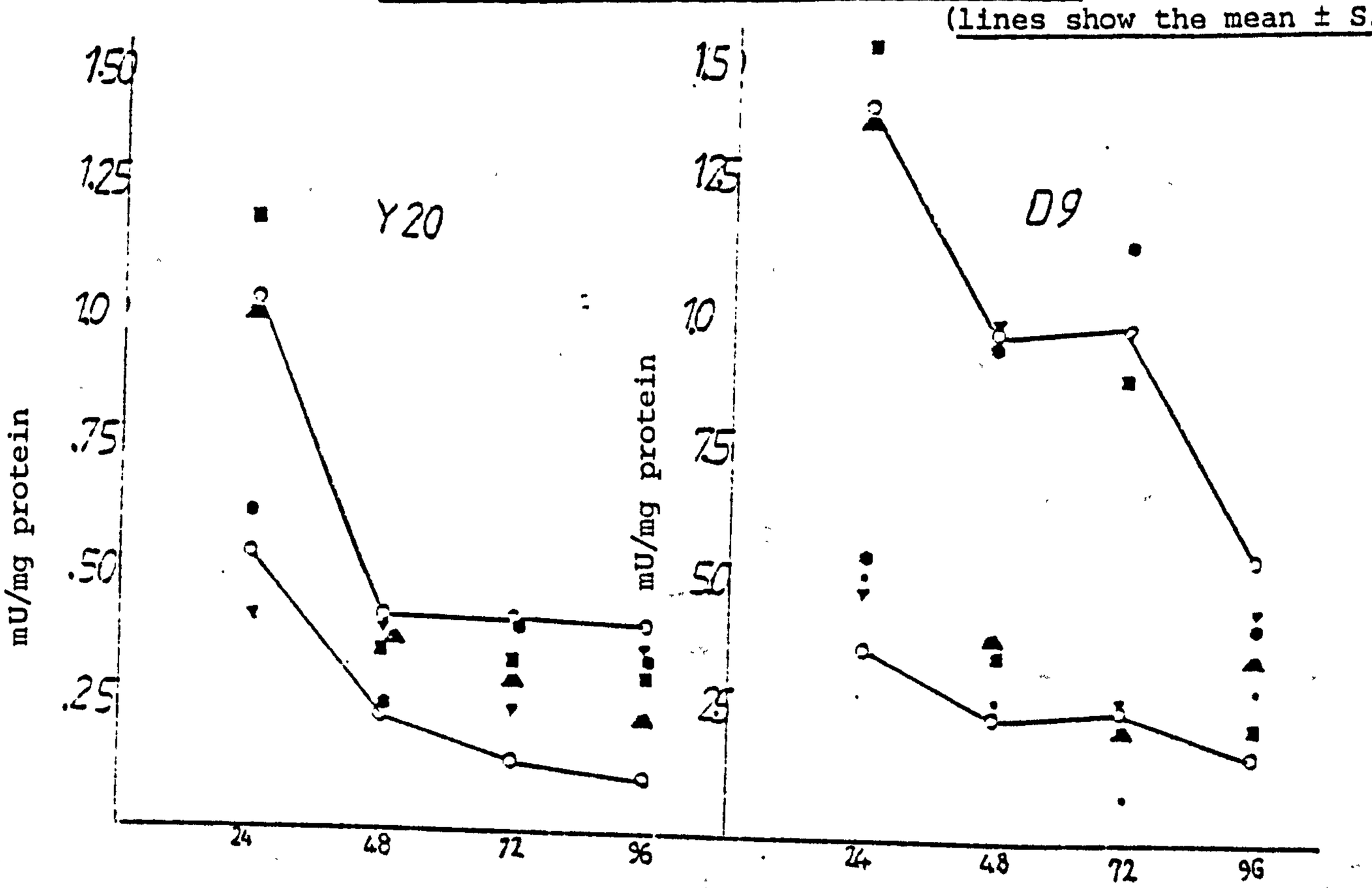


Fig. 20 PEP carboxylase shaken culture

(lines show the mean ± S.E.)



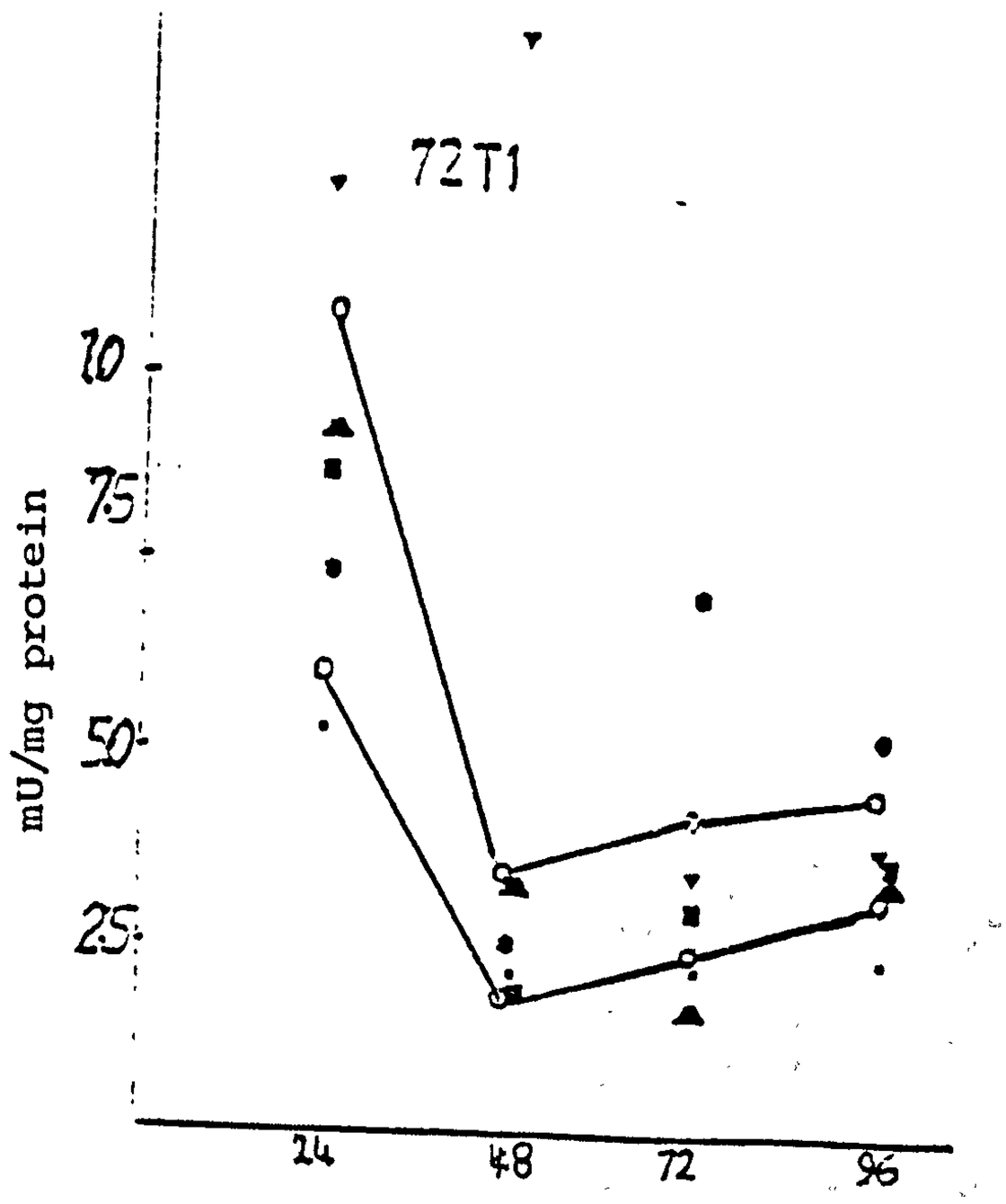
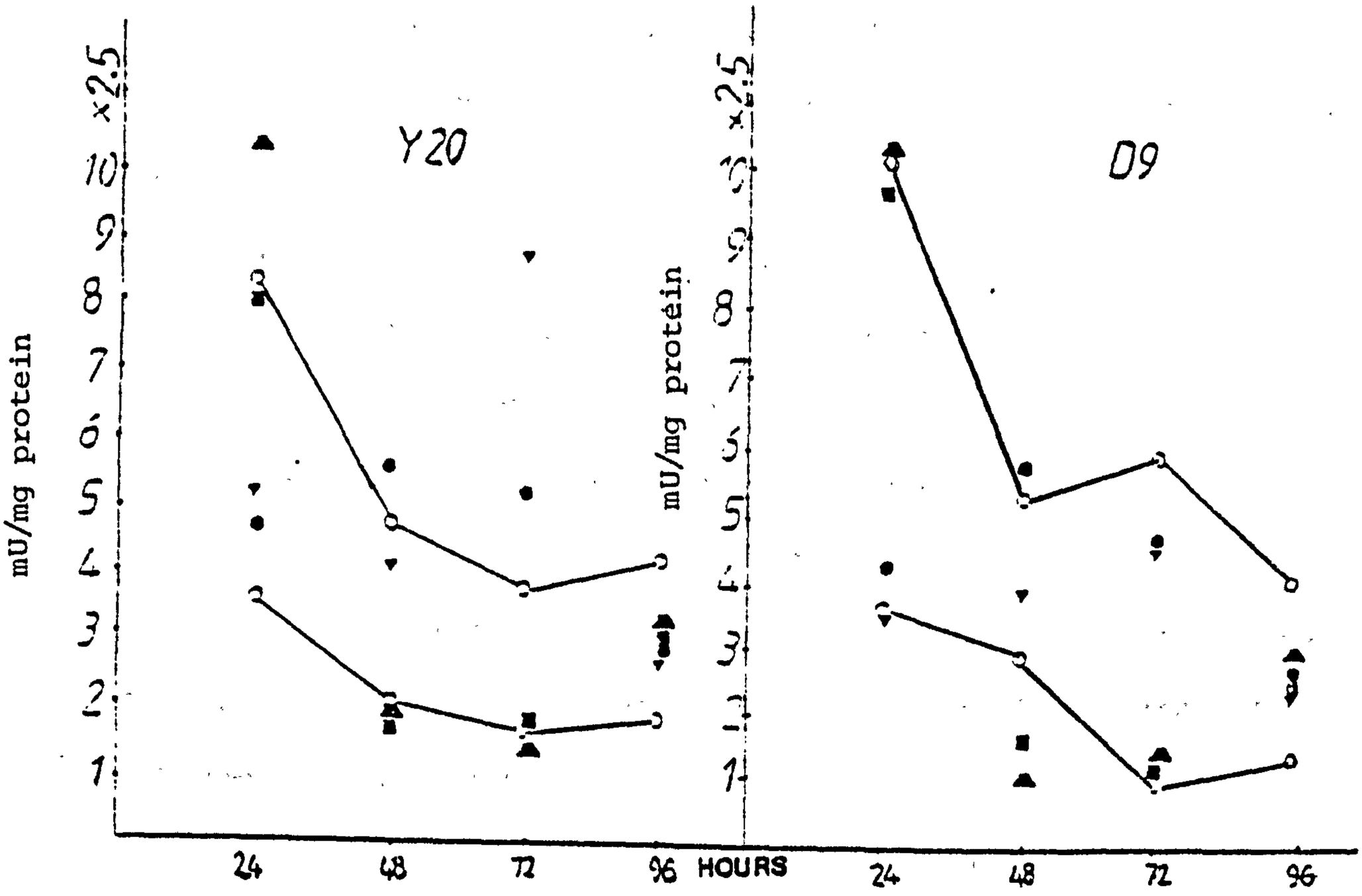
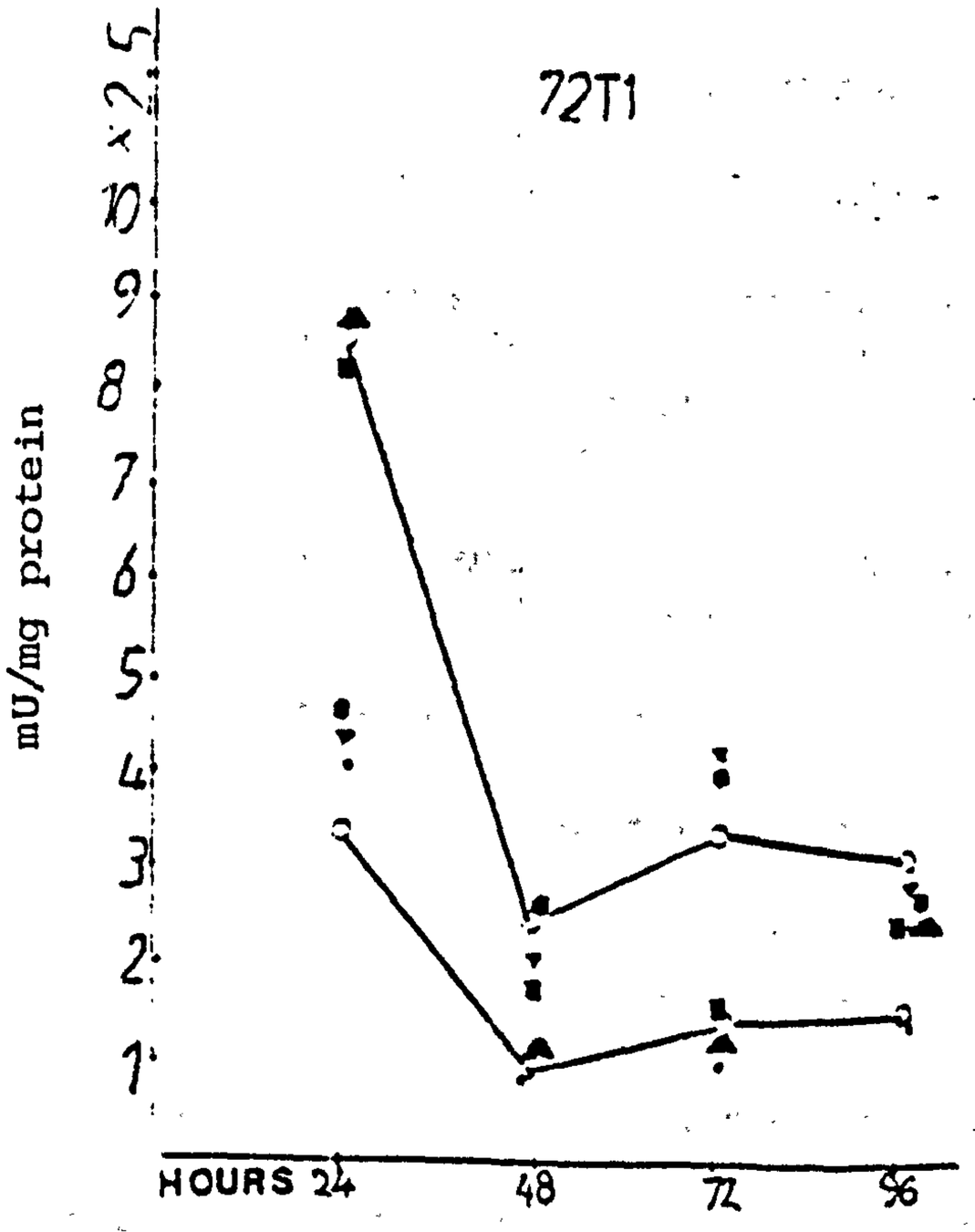


Fig. 21. Acetyl CoA carboxylase shaken culture
(lines show the mean ± S.E.)





2) DISCUSSION OF RESULTS

Experiments were carried out, mainly with 72T1 and Y20, in stirred culture to determine the activity of certain enzymes in the cells. Some tests were also made with S.rimosus and the mutants D9 and Y20 (Inactive). Points for consideration were:-

(1) to consider whether the enzyme concentrations are related to growth and antibiotic production,

(2) to determine the effect produced by mutation to higher yields,

(3) to provide a comparison with the results obtained by the Czech workers and the conclusions they have drawn from them.

The enzymes concerned related generally with different areas of cell metabolism. They were:-

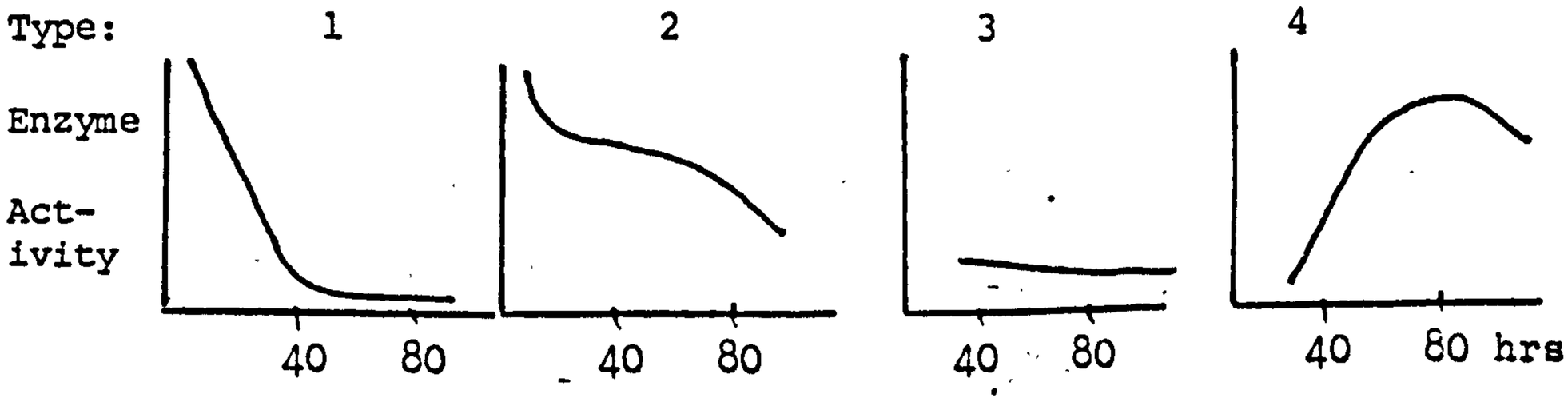
Enzyme	Area of metabolism
Citrate synthase	Start of TCA-cycle, with a possible influence on level of citrate in the cells
ICDH	Central portion of TCA-cycle, important in connection with the supply of $NADH_2$ and biosynthetic activity
MDH	Later stages of the TCA-cysle. Important in the synthesis of oxalacetate, an important intermediate in biosynthesis and glucogenesis.
PEP carboxylase	Important in gluccgenesis, or as a source of oxalacetate and malonate
Acetyl-CoA carboxylase	Important in the formation of malonate
ATC-oxygenase	An enzyme in the central area of OTC biosynthesis, regarded by the Czechs as an indicator of the cell's capacity to produce tetracyclines.

The meaning of the results of assays of enzyme activities, in cell-free extracts, is uncertain since there is no evidence that the result is representative of the enzyme in the cell. However,

large changes (x5 or x10) are an indication of increased conversion capacity in the cells.

I) <u>Enzyme activities: patterns of activity</u>

Enzyme activity might be expected to be related to the general activity of the cells, in terms of specific growth rate (μ) and OTC production (Q_{OTC}). Fig. 22 shows curves for μ and Q_{OTC} for 72T1 and Y20. For μ, values are higher at first (0.1-0.25) falling fairly rapidly to about 0.05 to 30-40 hours, continuing to fall more or less slowly. With 72T1 the fall is extremely rapid, to .001 by 40-60 hours. Q_{OTC} is highest between 30-80 hours, but with 72T1 the values are so low that they would probably not affect the general picture. These results suggest two patterns of behaviour (1 & 2), with two others as possibilities (3 & 4). These are illustrated in the sketches:



The activities indicated are (1) rapid at first, then low; (2) rapid at first falling away in two stages; (3) fairly constant throughout; (4) low at first, increasing during the production phase at 40-80 hours.

The effects shown may be summarised:-

	Type observed	
Enzyme	72T1	Y20
Cit. synthase	low, 3	2
ICDH	low, 3	4
MDH	4	4
PEP-carboxylase	1	1
Acetyl-CoA carboxylase	high, 3	high, 3

These results may be further broken down:

	72T1				Y20			
Type	1	2	3	4	1	2	3	4
Numbers	1	0	3	1	1	1	1	2

While not much significance can be attached to these figures, it does seem that 72T1 tends to show low activity during most stages of growth, while with Y20 activity is stronger. This is in line with the observation that growth continues longer with Y20. In general values do not parallel the growth rate μ.

In the case of ATC-oxygenase (see section III) the pattern of activity was unexpected, ie. a continuous level of activity from 24 hours onward. This is much the same in 72T1 and Y20. The figures are given in Table 11. The implication is that OTC-synthetase is always available, and that the rate of OTC production depends on the supply of intermediates to the system.

II) Enzyme activities: quantitative aspects

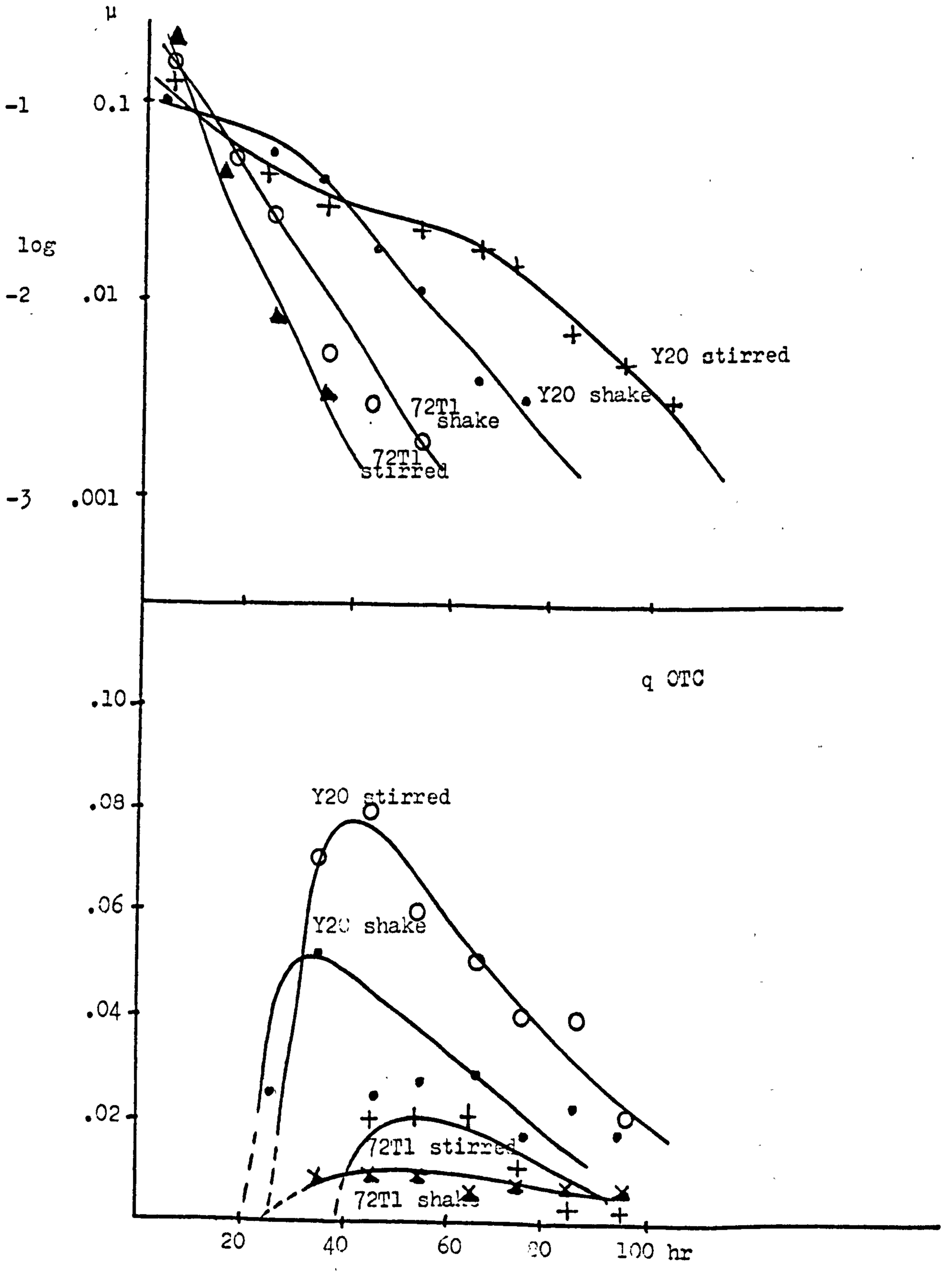
The table gives averaged results for the main enzymes:

Enzyme	Y20			72T1			Av. stirred / Shake
	stirred	Shake	Ratio	stirred	Shake	Ratio	
ICDH	150	20	7.5	15	6	2.5	5
Cit.synth	1.5	-	-	0.2	-	-	-
MDH	220	42	5.2	160	17	9.4	7.3
Ac.carb.	15	10	1.5	15	7.5	2	1.7

	Stirred culture			Shaken culture			Av. Y20 / 72T1
	Y20	72T1	ratio	Y20	72T1	ratio	
ICDH	150	10	15	20	6	3.3	9.1
Cit.synth	1.5	0.2	7.5	-	-	-	7.5
MDH	220	160	1.4	42	17	2.4	1.9
Ac carb.	15	15	1	10	7.5	1.3	1.1

There are differences between strains and between culture conditions, as has previously been described, but generally, enzyme activity is higher with Y20 or in stirred culture. Acetyl-CoA carboxylase, however, was high at the beginning, especially when high OTC produced in shaken culture with Y20 and D9. MDH is relatively similar between the strains, but is increased in stirred culture.

Fig, 22 Growth rate and q OTC for comparison with enzyme data



III. Acetyl-OTC oxygenase

Results of acetyl-OTC oxygenase based on shaken flasks cultures are summarised in Table 11. The values represent the absorbance difference after 3 minutes. A value of 0.05 corresponds to 7 U/mg protein approx.

Table 11. Results of the decline in O.D for acetyl-OTC oxygenase

culture age	Strain	Experiment No. 1	2	3	Average
DAYS	Y2O	-	-	.055	-
1	72T1	-	-	.10	-
	D9	-	-	-	-
	D9 D7	-	-	-	-
	Y2O	.015	.04	.065	.04
2	72T1	.017	.04	.03	.03
	D9	.02	.05	.02	.03
	D9 D7	.03	.04	-	.035
	Y2O	.02	.05	.04	.037
3	72T1	.04	-	.06	.05
	D9	.03	.05	.03	.037
	D9 D7	.08	.04	-	.06
	Y2O	.03	.04	-	.035
4	72T1	.02	.05	-	.035
	D9	.02	.03	-	.025
	D9 D7	.1	.04	-	.07
	Y2O	2.8	3.6	3.5	
OTC g/l at	72T1	v.low	1.2	-	
4 days	D9	2.1	2.4	2.3	
	D9 D7	3.1	3.5	-	

3) ADENYLATE RESULTS

The adenylate assays were made for both stirred and shaken cultures. The stirred culture results were based on standard runs with 72T1 and Y20, the latter giving 6.0 g/l of OTC (Figs 23 & 24).

The shaken cultures of Y20 were based on two standard runs, Y20 gave 3.8 g/l OTC (Figs 23 & 24).

The energy charge in a culture of the low production strain (Fig 23) was almost identical with that of the production strain. After 48 hours both strains showed a drop in the energy value but after 72 hours it started to rise in the wild strain, whilst it continued to fall in value in the mutant as shown in Fig 23 for stirred and shaken culture.

I. ATP level

With 72T1 ATP reached a maximum level at 24 hours in both the stirred and shaken culture. Y20 was about ten times less at the same stage. ATP fell sharply after 24 hours, but with Y20 the highestpeak was about 48 hours, after this it declined. Fig. 24 shows the level of ATP in Y20 and 72T1 in shaken and stirred cultures.

II. ADP level

The value was low in both of Y20 and 72T1, though with 72T1 it was a little higher at the beginning of the fermentation, as shown in Fig 24.

III. AMP level

Here the value in both of the strains were fairly steady at about 100 pM/mg protein, as shown in Fig 23.

4) DISCUSSION

The general levels of the adenylate was only about 400 pM/mg protein in Y20, and 800 pM/mg protein for 72T1. These levels seemed to be below the level likely to influence any effect as a repressor to the enzymic system namely isocitrate synthase (Boulton and Ratledge 1980).

The results indicated that intracellular adenylate level in S.rimosus is inversly proportional to the rate of biosynthesis and the yield of OTC. The time course of energy charge during cultivation is practically the same in both strains. A similar effect was described by Curdova et al (1976) with S.aureofaciens, who stated that a drop in the level of adenylate did not affect the value of energy charge.

The rise in AMP level after 48 hours can be accounted for in two ways: it can be ascribed to the existance of a certain regulatory effect, or to the inhomogeneity of the population, since the culture especially later stages of cultivation comprises cells of different ages. The observed increase in the level of AMP and ADP toward 72 hours of cultivation may be at least partially due to higher proportion of inactive dying cells.

Fig. 23. AMP and energy charge in stirred and shaken culture

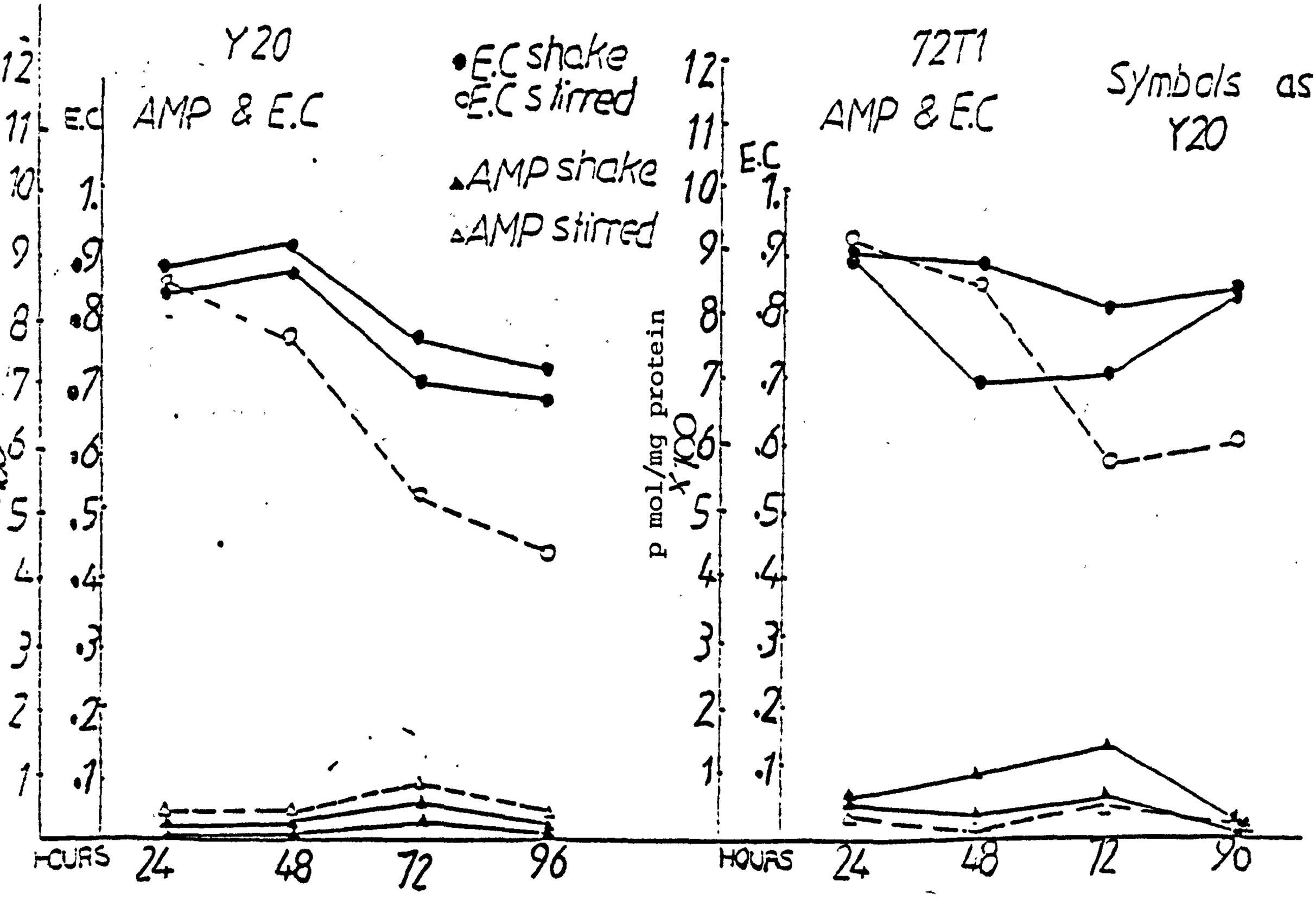
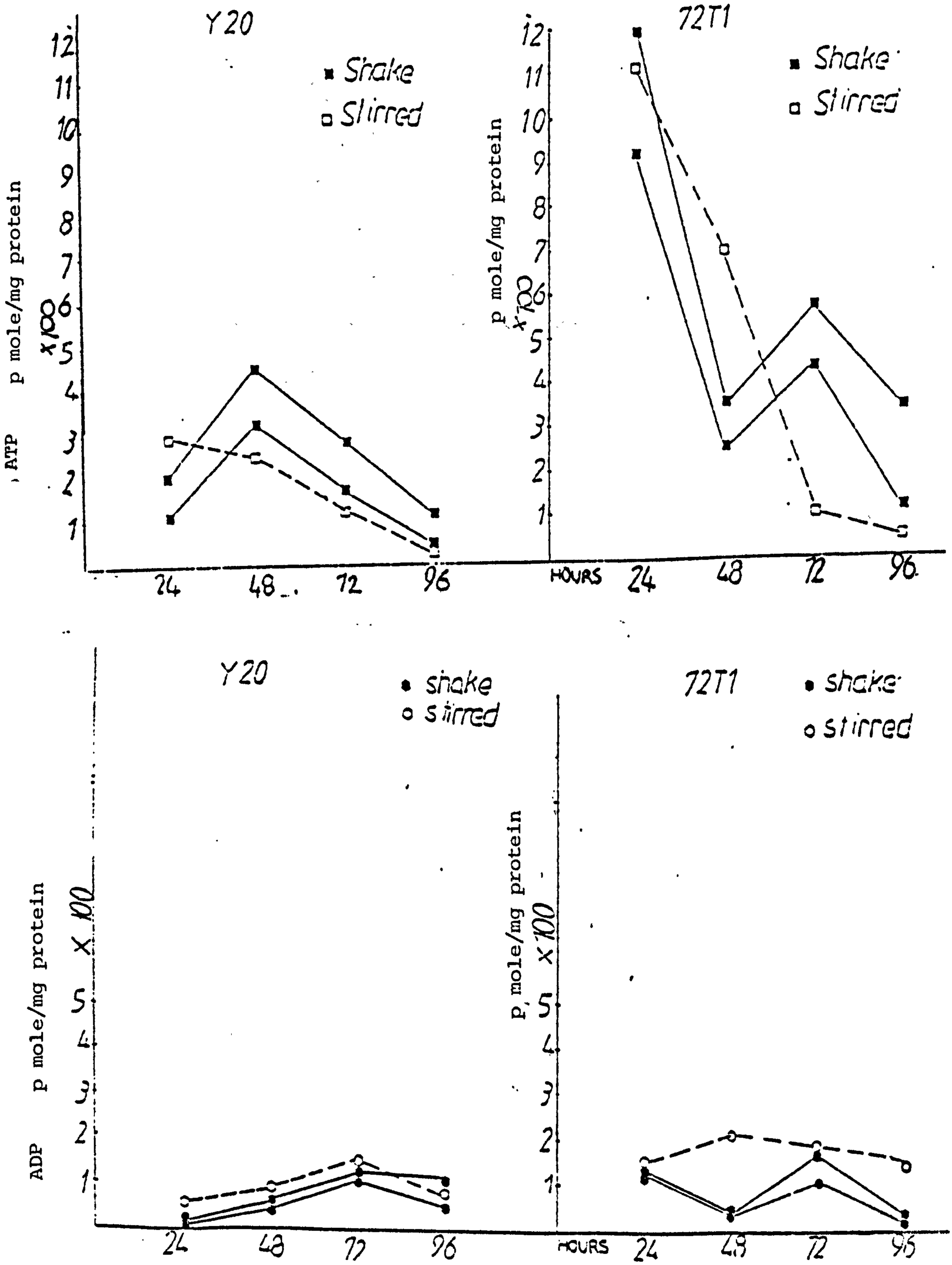


Fig. 24. ATP and ADP levels in stirred and shaken culture



3.6 COSYNTHESIS

1) OBTAINING BLOCKED MUTANTS.

2) TESTS FOR COSYNTHESIS.

3) RESULTS.

4) COLOUR TRANSFER IN AGAR TEST.

5) SHAKEN FLASKS TESTS OF OTC PRODUCTION.

6) DISCUSSION.

The type of changes in metabolism caused by the OTC^- mutation such as the presence or absence of brown colour, the changes in the pattern of chromatograms in the blocked mutants, the interaction between different types of mutants which might be blocked in different parts of biosynthetic chains, and the comparison between the blocked mutants with the wild strains were the main points of interest in the present study.

Work in this area was reported by Delić et al (1969), McCormick et al (1960, 1966) and Rhodes et al (1981), the outline of Rhodes work and the genes which were identified and the main stages in OTC biosynthesis were mentioned in the introduction.

1) OBTAINING BLOCKED MUTANTS

Blocked mutants were obtained using two types of mutagens:-

I) Gamma rays:- A spore suspension of Y20 was placed in sterilized tubes and exposed to 60,000 and 120,000 rad (the killing effect with 60,000 was about 90% and that of 120,000 about 98%). The spores were then plated on Emerson's agar plates and incubated for 10 days at 25°C. 440 colonies were picked off and transferred on agar blocks to another agar plate seeded with B.subtilus spores and then incubated for 24 hours at 37°C. Colonies which showed none or very small inhibition zones were picked up and tested for OTC production in shaken flasks. Out of the 440 colonies only 13 showed none or very little OTC production two of them considered to be leaky, leaving 11 blocked mutants.

They were A,B,D, 1,3,4,5,6,7,8 & 9.

II) N-methyl-N-Nitro-N-Nitrosoguanidine (NTG):-

1 Mg of the mutagen was dissolved in 1 ml phosphate buffer and then 1 ml of Y20 spore suspension added to it. After different time intervals (i.e. 10, 15 and 20 minutes) the spores were diluted with distilled water so as to provide the appropriate number of spores in the .1 ml suspension per plate of Emerson's agar medium (the killing effect was about 60% with 20 minutes treatment), 280 colonies were tested for their antibiotic production, as above. One showed no OTC production which was NTG 1.

2) TESTS FOR COSYNTHESIS

I) Test on agar medium:-

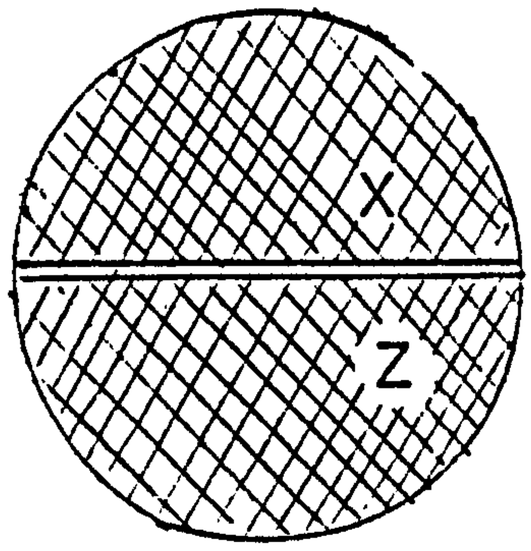
The mutants were grown in pairs and production sought.

Several methods were used as described by Rhodes et al (1981) and Delič et al (1969) they were:-

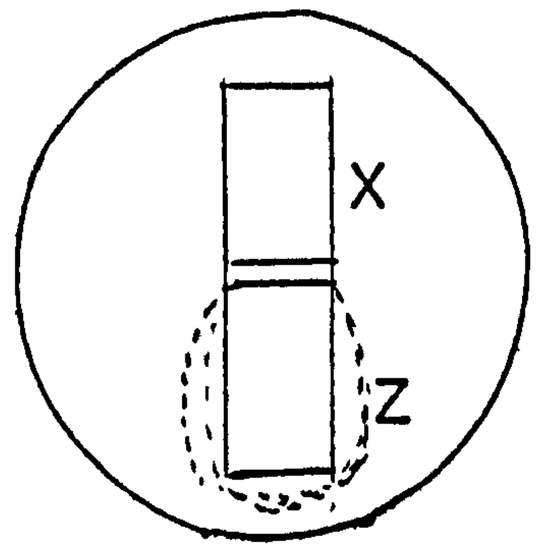
(a) The two non producing mutants were grown together on a small agar block and OTC production tested by placing the blocks on agar seeded with B.subtilis. Only a few tests were done by this method, as it was found to give about the same results as agar strip method.

(b) The two mutants were grown together on either side of an agar plate with a space of about 3 mm between them (Delič et al 1969). A strip was cut out across the gap, and was placed on agar seeded with B.subtilis and the appearence of OTC noted after the incubation (ie. if cosynthesis occurs, OTC will be produced either in the middle of the growth zone or on the side of one mutant or on both of the mutants).

The following diagram illustrates cosynthesis



Agar plate with the two mutants, X & Z growing on each side of the plate and a space between them.



Agar strip of the two mutants on seeded agar with inhibition zone around mutant Z.

The results showed that mutant Z has converted into OTC an intermediate produced by mutant X. In this way mutant X is a "convertor" or "driver" producing intermediates only, and mutant Z is a "secretor" which changed the intermediate into OTC.

II) Cosynthesis in shaken flasks

The two mutants were grown together in shaken flask culture and OTC production was tested.

3) RESULTS

I) Agar strip tests:- (see plates 1, 2 and 3)

These tests showed a variety of results, characterised by the A,B,C classification (Tables 12 and 13). These suggested a rather complicated series of biochemical relationships during metabolism. Each mutant when crossed with the others showed one of three patterns of behaviour:-

(1) crossed with mutants 6,B, and D there were many A-type results ie. mutants 6,B, and D did not produce OTC, but the other mutants were caused to produce OTC. Mutant 6 and D

when grown together gave only little OTC, though with 6 and B, B produced quite well. Mutant 6 produced OTC only with NTGI.

(2) Mutants such as 7 and 8 and A, usually gave cosynthesis with the others.

(3) Other mutants, like 4, were sometimes acceptors, otherwise showed cosynthesis.

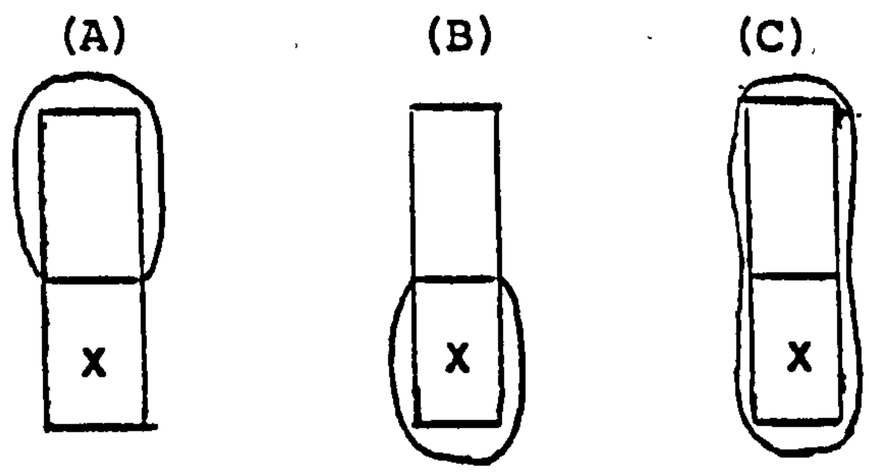
Table 12. Patern of cosynthesis between mutants in agar strips

Mutant - upper

Mutant - lower

	1	3	4	5	6	7	8	9	A	B	D
1		C	C	C	B	Aw	C	B	BC	B	B
3	C		C	AC	B	B	C	C	C	B	CorB+
4	C	C		C	B	Nil	B+	BC	BC	B	B
5	C	B	C		B	Var	C	C	A+	Var	B
6	A	A	A	A		A	A	A		A	Cw
7	B	C	Var	Var	B		C	C	C	BCw	CorA+
8	C	C	C	C	B	C		CA	Var	BC	Cw
9	BC	C	C	A	B	C	AC		A	B	B
A	AC	BC	C	Var	B	C	Var	Var		B	Bw
B	A	A	A	C	C	C	A	A	A		B+
D	A	A+	A+	A+	BCw	A+	Cw	A+	Aw	A+	

Key:-



Patterns of cosynthesis, with respect to mutant X:- Showing zone of inhibition of B.subtilis.

AC, BC, ... etc either or. slight inhibition at other end of the strip.

Var = Variable patterns of co-synthesis

II) Interaction of mutants with wild strains

The two wild strains were tested, 72T1 and S.rimosus NRRL 2234, also the mutant Y20 inactive which had lost its activity. All were tested against the blocked mutants on agar strips. The patterns of cosynthesis observed were compared with those given by the mutants. There was a considerable agreement between 72T1 with mutant A, supported by the signs of tests (P = 0.01), Y20 inactive showed some resemblance to mutant 6 though the value of P was only 0.25, NRRL 2234 did not agree well with any of the mutants, though mutant B was closest to it (P = 0.45).

Table 13. Comparison of cosynthesis patterns in wild strains and mutants crossed with mutant

	1	3	4	5	6	7	8	9	A	B	D
72T1	A	B	C	C	C	C	C	C	B	B	B
Mutant A	AC	BC	C	C	B	C	C	C	-	B	B
NRRL 2235	A	A	C	C	C	C	C	C	-	A	A
Mutant B	A	A	A	C	C	C	A	A	A	-	B
Y20 "inactive"	A	C	A	A	A	AC	C	A	Nil	Nil	Nil
Mutant 6	A	A	A	A	-	A	A	A	-	A	Weak

Probability of agreement due to chance	P
Mutant A: agrees with 72T1(9), disagrees (1)	0.01
Mutant B: agrees with NRRL 2234 (5), disagrees (4)	0.45
Mutant 6: agrees with Y20 inactive (6), disagrees (3)	0.25

Note:- Key as that of table (2) - means that no test was made, Var = C

4) COLOUR TRANSFER IN AGAR TEST

Previous work showed that the production of OTC, at increased yields, was associated with the production of a brown colour, which is probably aureovocin, a glycoside of MPT (Podijil et al 1970), as seen in Y20 and D9D7. Examination of the cosynthesis results with agar strips, showed that the production of OTC was not always associated with the production of this pigment alongside the OTC. Examination of data from the plates, and other records, gave the information shown in Table 14.

Table 14 Colour and OTC formation in agar strips

(see plates 1, 2 and 3)

Driver (No OTC production)				Acceptor (All show a zone of inhibition)	
Colour of driver strip		Mutant	X	Mutant	Colour of acceptor strip
Brown	3		X	72T1	cream
	5			7	cream
	6			72T1	cream
	7			72T1	cream
Light Brown	6			5	tan
	7			5	tan
Cream or off white	3			5	white
	5			A	cream
	6			1	light brown
	6			4	tan
	6			7	tan
	8			D	off white
	9			A	white
	9			7	very light brown (cream)
	A			72T1	cream
	D			7	tan
	D			B	light brown
	NTG1			6	light brown
Totals for groups:-					
Brown	4			4	cream
Light Brown	2			2	tan
Cream	12			6	cream
				3	light brown
				3	tan
Total	18			18	

Brown, or tan, goes with OTC 5
Light brown goes with OTC 3
Does not go 10

With Mutant 6 as Driver:
Number, 5:
Acceptors, cream 1, light brown 1
tan 3.

Summarising the results, OTC production is not particularly associated with the formation of a brown colour. When the driver was brown, the acceptor was always cream, and when the driver was cream only about half the cases showed a tan or light brown acceptor. This is contrary to expectations, but is readily apparent on the Plates.

5) SHAKEN FLASKS TESTS OF OTC PRODUCTION BY COSYNTHESIS

A summary of the results is shown in Table 15.

I. Mutants 6 & 9:- The two mutants were tested by themselves and also with 6 + 9 together. Mutant 6 resembles Y20 but with less material in band 3, see section 3.3(2) (Rf, .6, OTC, in the chromatogram) and mutant 9 was rather similar. When the two were grown together, band 3 became more prominent, and bands 1 and 4 (Rf .9 & .3) became less obvious. This result was reproducible, which suggests that material has been converted to OTC rather than acetyl-OTC.

II. Mutant B:- This mutant also showed a weak band 4 (Rf .3) and strong band 1 (Rf .9) and showed cosynthesis when crossed with mutant D.

III. Mutants 5,7,8 and A:- Mutant 5,7 and 8 gave rather pale cultures in the shaken flasks and the chromatograms showed little or no OTC. Mutant A was even more striking, showing only two blue flouresent bands. The cross, 7 + 5 showed little sign of cosynthesis but 7 + A and 8 + A showed apparent cosynthesis and the presence of OTC by assay.

IV. Mutant D:- This mutant showed only two blue bands, it cosynthesised weakly with mutant 6.

Summarising the results, the mutants showed very different characteristics. 6 and 9 produced OTC-like materials with a change in distribution when grown together, while other mutants produce little or no OTC-like material, one mutant, D, produced only two blue bands, though giving OTC in crosses. The results present a complicated picture, suggesting that several mechanisms were involved.

Table 15 Results of the shaken flasks mutants, with two crosses which showed cosynthesis

		OTC g/L	Brown colour in flasks	310 nM compound	Acetyl-OTC	OTC synthesis
Mutants	1	0.4	Br + +	Nil	Slight	
	3	Nil	Br +	+	Nil	
	4	0.4	LBr +	Nil	Nil	
	5	0.3	LBr +	Nil	Slight	
	6	0.9	Br + +	+	+ +	
	7	Nil	LBr +	Slight	Slight	
	8	0.4	Br + +	+ +	Slight	
	9	0.9	Br	+	+	
	A	Nil	LBr	Slight	Nil	
	B	Nil.	Br +	+ +	Slight	
	D	Nil	White	Slight	Nil	
crosses	6 + 9	3.5	Br + +	Slight	- -	+
	7 + 8	Nil	Br + + +	Nil	+	-
	7 + A	1.0	Br + +	+ +	+ +	+
	8 + A	1.0	Br + + +	+ +	+ +	+
	3 + A	1.0	LBr +	Nil	+	+
	3 + 6	0.8	Br +	Slight	Nil	+
	D + A	.4	Pale	+ +	Nil	+
	72T1+6	1.3	Br	Slight	+	+
	72T1+A	1.2	LBr	Slight	+	+
Strains	Y20	3.5	Br + + +	Slight	+ +	
	72T1	0.8	Pale		+	
	S.Capuensis	4.1	Br + + +	+	Band at Rf 0.2	

Br = brown, LBr = Light brown, + = appreciable, + + = medium, + + + = strong

V. Colour and OTC production in shaken flasks

Signs of cosynthesis, measured by OTC production, were usually accompanied by the formation of the brown colour, though pale colours did occur in a few cases(Table 15) as with D + A.

6) DISCUSSION

The number of mutants obtained were relatively small, and as all of them were capable of producing OTC with one or more of the others, they probably have been blocked in the area of intermediate metabolism, or more probably early in the OTC template, rather than late in the template.

Agar strips generally showed OTC production from pairs, but the driver effect was striking. For example mutants 6, D and B were notable for inducing biosynthesis in other blocked mutants, without producing themselves, while mutants 7, 8 and A usually gave cosynthesis with other mutants. These different patterns of activity reflected variations on the biochemistry of the mutants in the cosynthesis effect.

It is possible that in some cases, cosynthesis may arise from complementary blockage, which could be expected to give production as in wild strains or production mutants. Another possibility is differences in the affinitives of the key enzymes, so that production of an intermediate enables production in one but not the other.

In the case of mutants 6 and 9, with shaken cultures, when grown alone, they gave a brown colour and formed the acetyl-OTC band in the chromatogram, with other bands, but there was little or no OTC band. On growing together these bands were reduced and the OTC band appeared. This seems possibly to be due to an affinity effect. However, on agar their behaviour was different, as mutant 6 gave only a pale colour, which illustrates different effects of metabolism in the two systems of growth.

The mutants could be sub-divided in terms of the degree of brown colour produced. This varied from white to full dark brown shade. An important factor is probably the mode of formation of the pigments, which in agar cultures take place differently than in submerged cultures. It is generally recognized that the physiology of the submerged mycelium differs from the aerial type, and the condition probably differs from those associated with submerged growth. It is therefore difficult to interpret the differences between the results in shaken flasks and on agar medium, but it is probable that different kinds of interaction occurred.

The production of brown material is common in tetracycline production, it seems that it has some relation to productivity i.e. it is a feature used by operators to judge the progress and favourability of the metabolism in the producing cultures.

The brown colour, which is probably aureovocin, is an indicator of the OTC biosynthesis, which means that when an organism produces the brown colour, the metabolic system which feeds the OTC template must be very active, on the other hand it indicates a leakage from the template, which diverts intermediates from OTC formation to aureovocin. This explains why experienced selectors always selected brown colour producers and rejected the colonies which they thought were too brown, which could be caused by excessive diversion of most of the material in the OTC template, to form aureovocin rather than OTC.

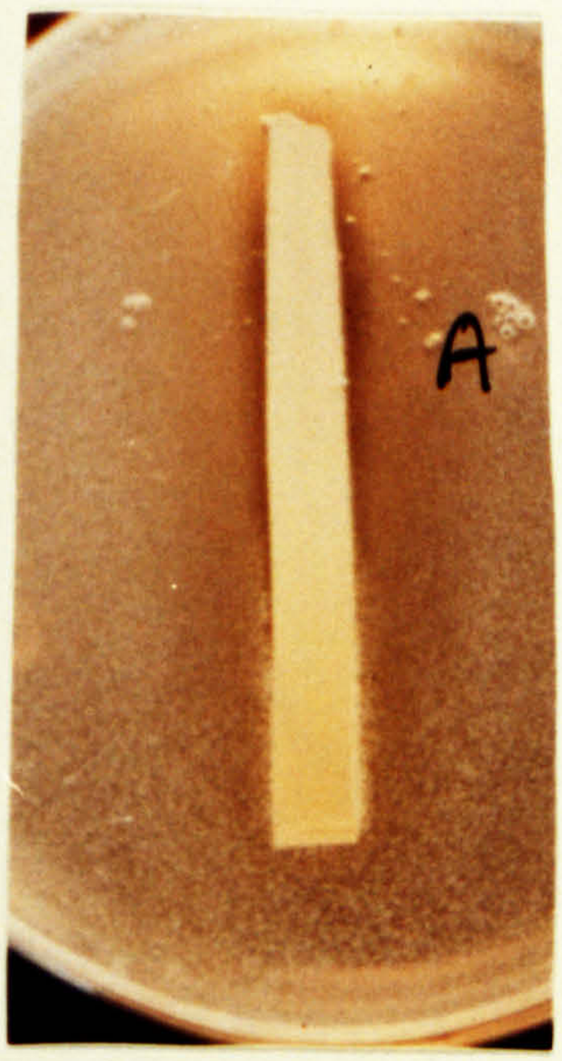
The indications that the formation of the brown colour is unnecessary for OTC production, are supported by the agar experiments. Often the cosynthesing mutants showed only a pale colour although more OTC was formed than is produced by 72T1, which is the typical pale, low yielder. With the larger amount of OTC some coloration might be expected.

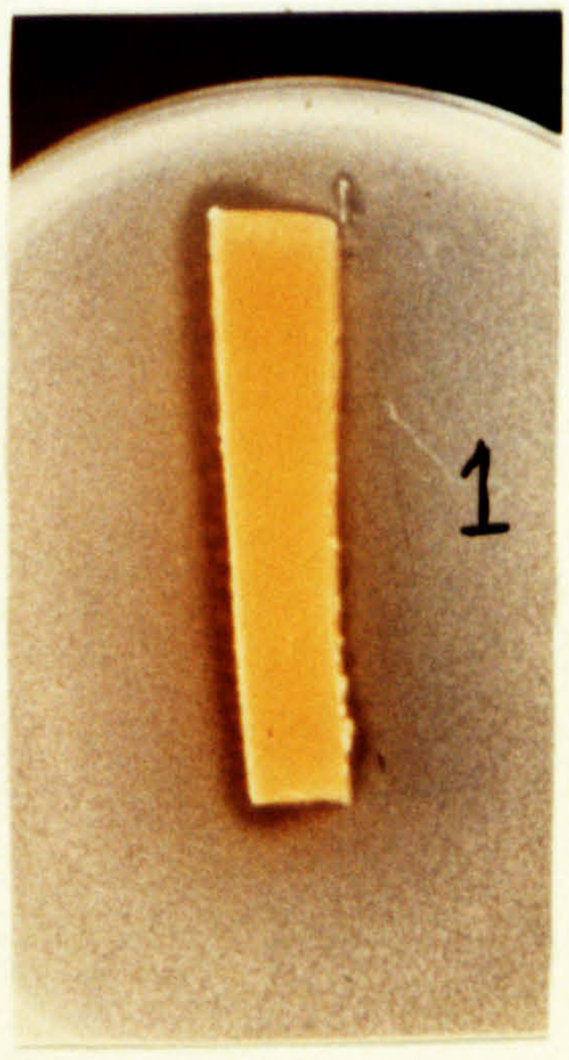
PLATE 1

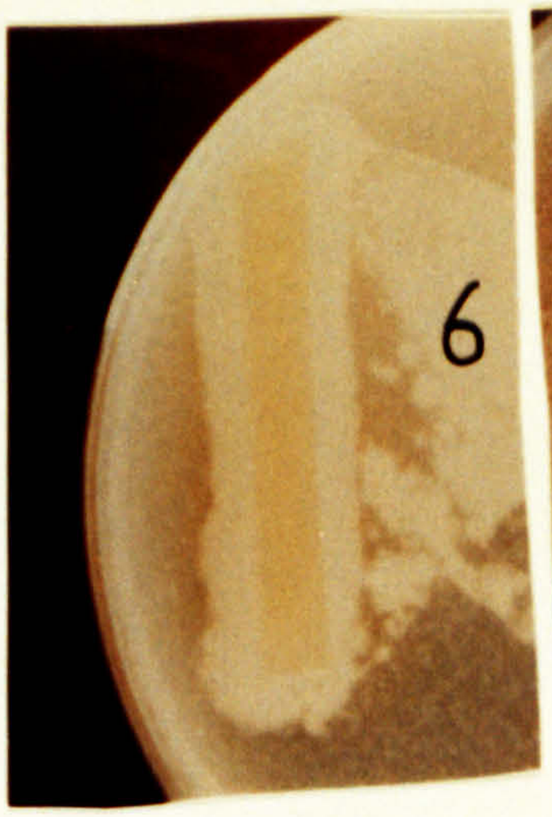
Blocked mutants, grown on Emerson"s agar and plated on agar seeded with B.subtilis, showing little or no antibiotic activity.

Mutants: A, 1, 7, 6 and D.

PLATE 1







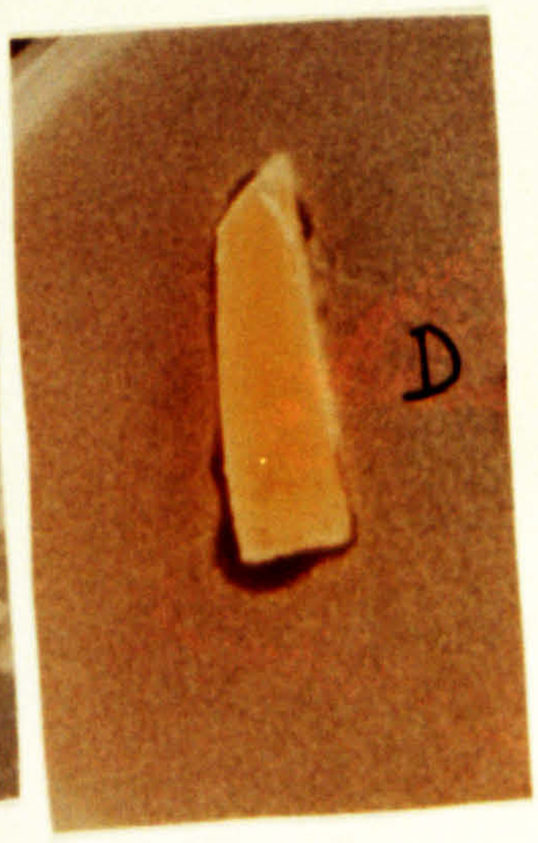


PLATE 2

Agar strips, bearing pairs of blocked mutants, showing different types of cosynthetic activity.

Pairs: 1 x 7

4 x 7

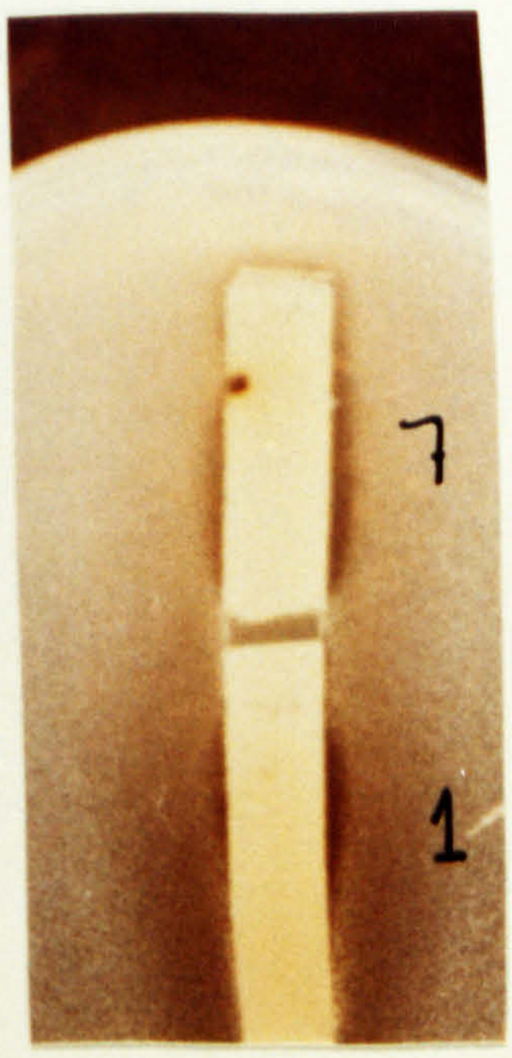
5 x 7

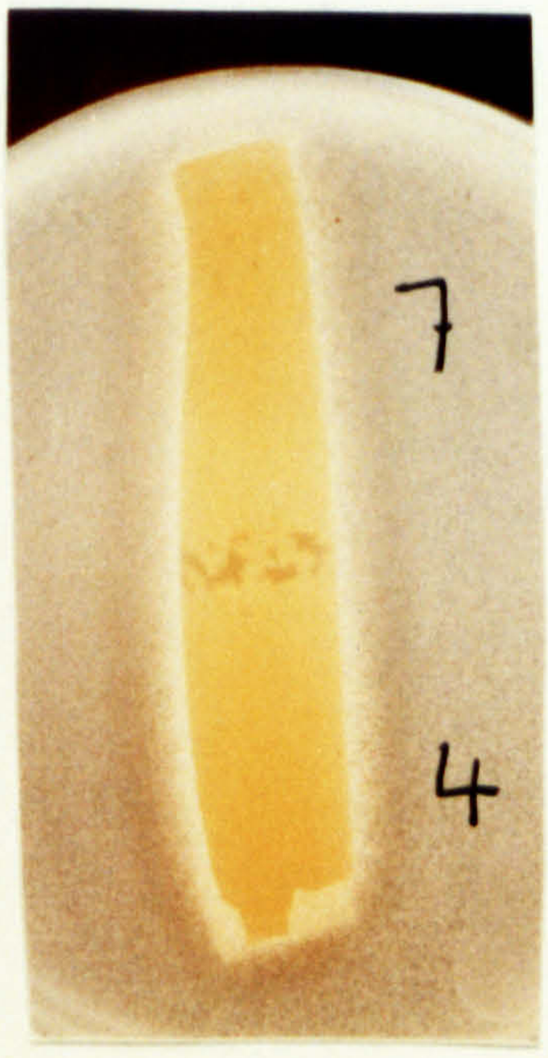
9 x 7

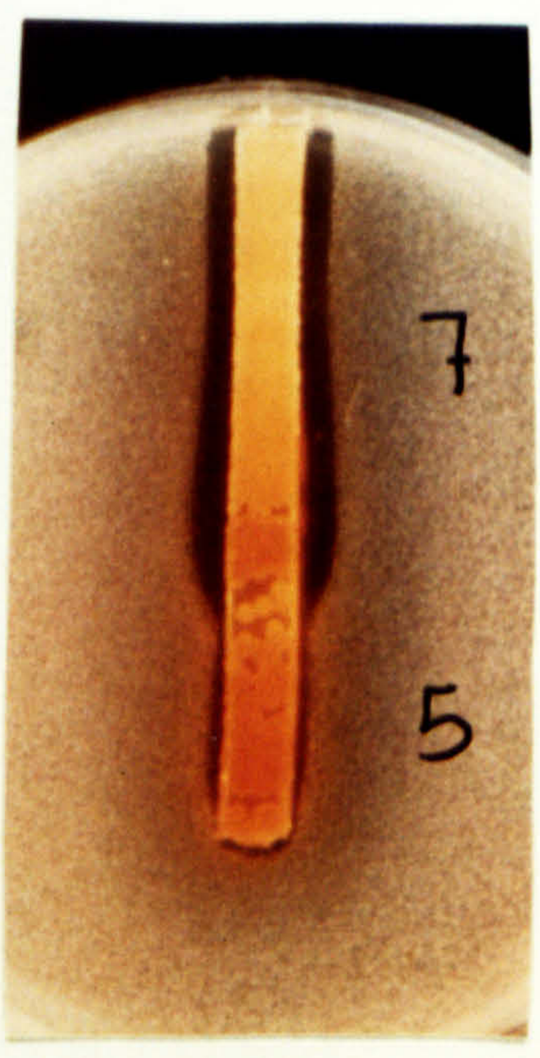
B x 7

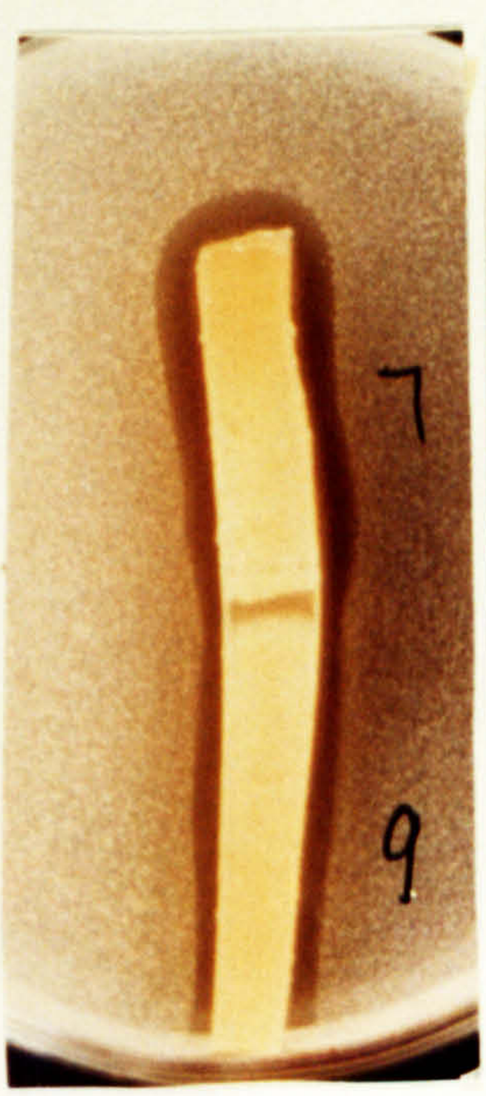
D x 7

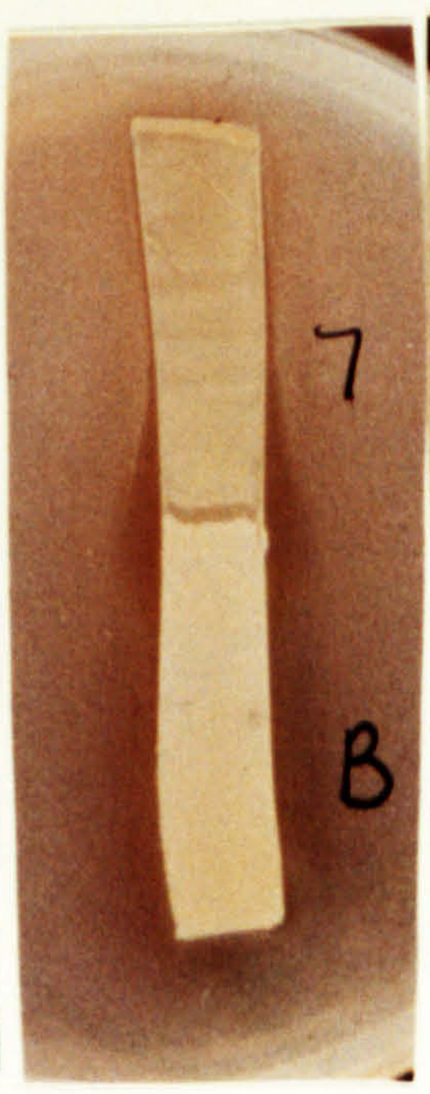
PLATE 2

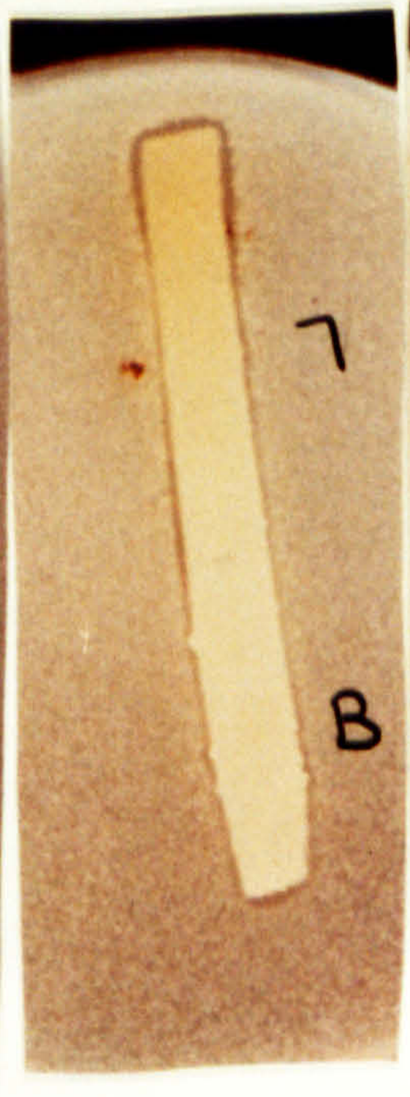












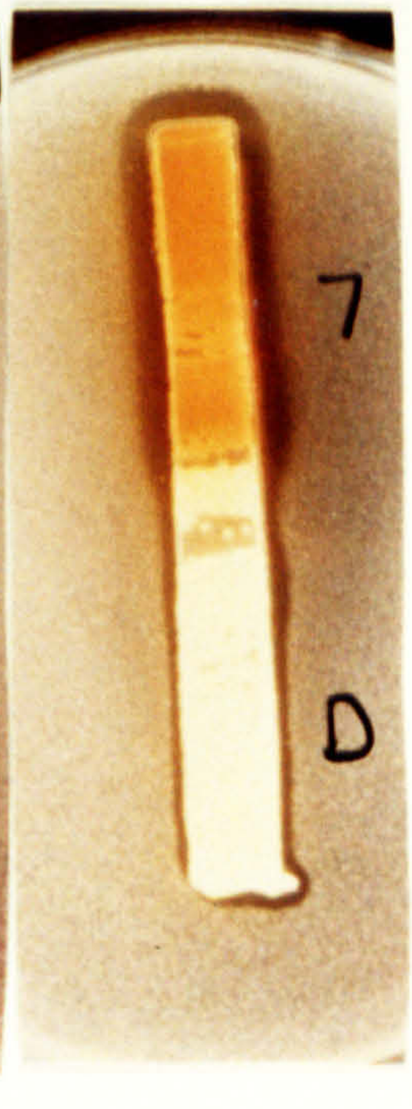


PLATE 3

Agar strip, bearing pairs of blocked mutants, showing different types of cosynthetic activity.

Mutants: 1 x 6

4 x 6

5 x 6

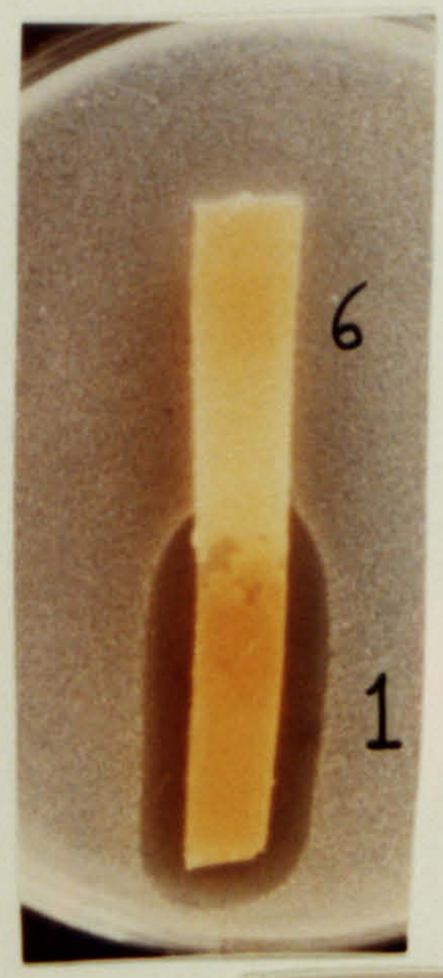
D x 6

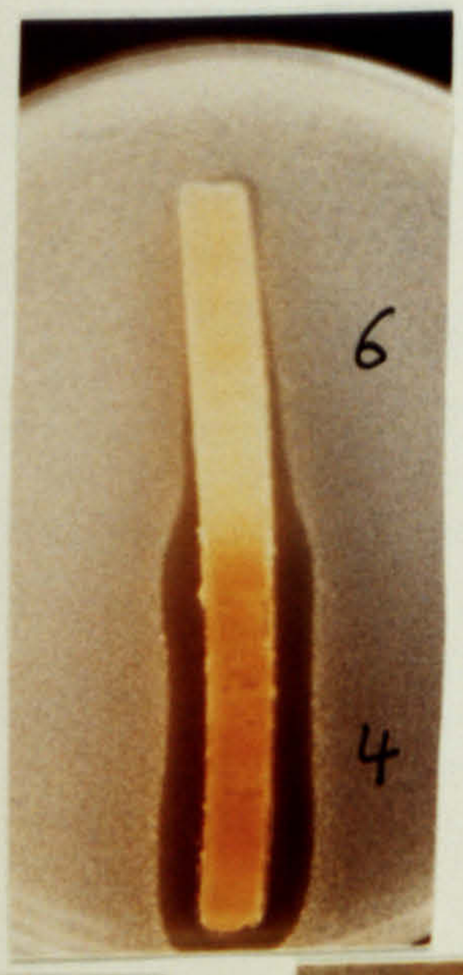
A x D

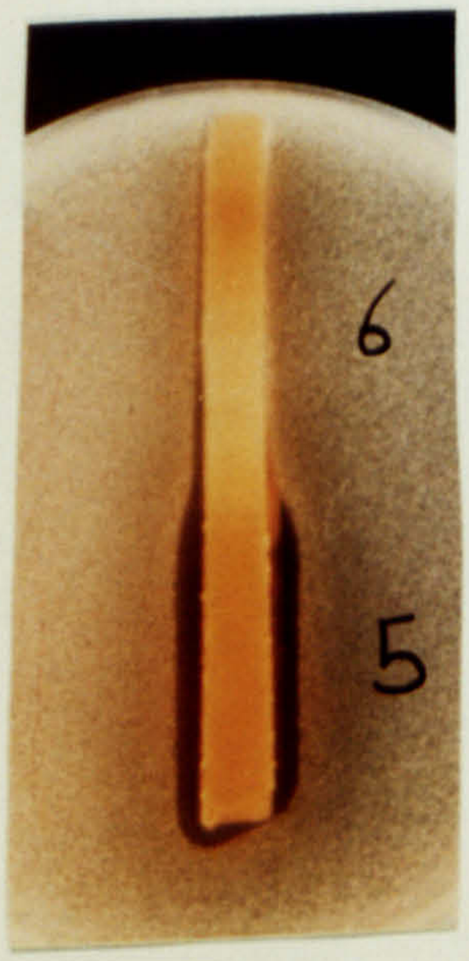
B x D

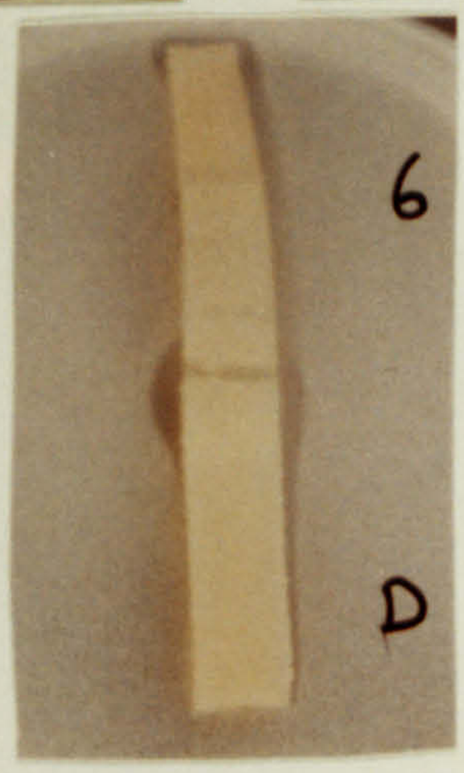
A x 9

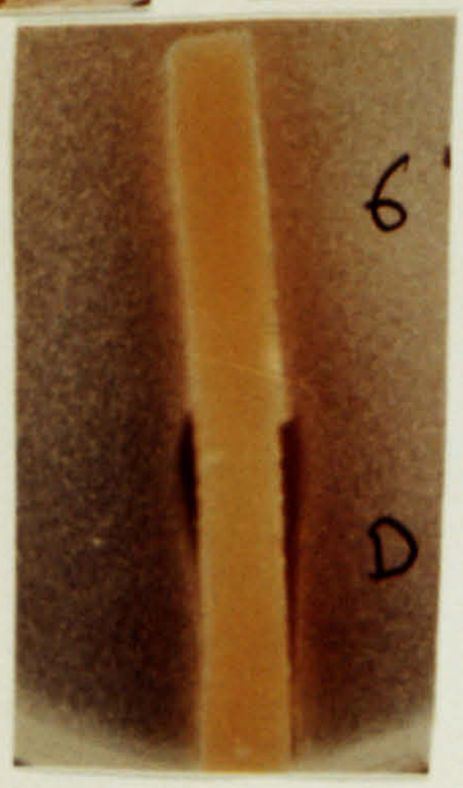
PLATE

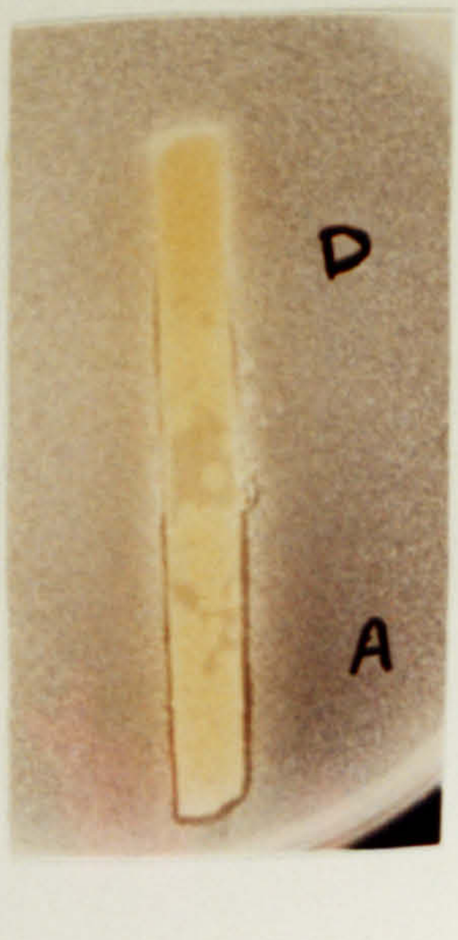


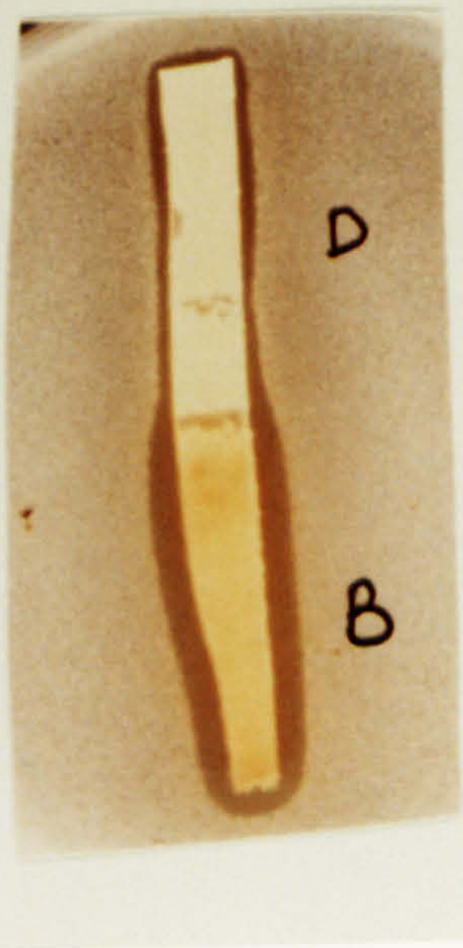


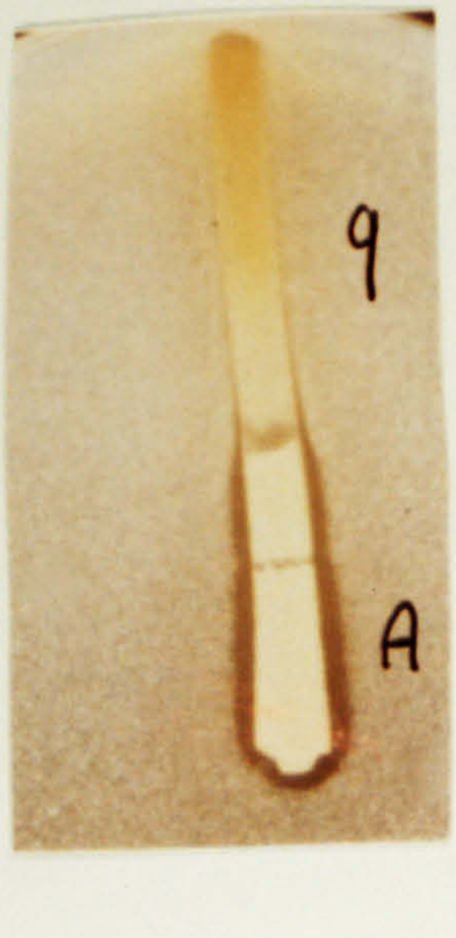












3.7 TAXONOMY

1) SUMMARY OF METHODS FOR CLASSIFICATION OF STREPTOMYCETES BY THE I.S.P. AND COMPUTER METHODS

2) COMPUTER IDENTIFICATION METHOD

3) CLUSTERING OF THE STRAINS USING WILLIAMS 41 TESTS

4) CLUSTERING USING DATA FROM THE EXTENDED I.S.P. METHOD

5) DISCUSSION

The main strains and mutants were tested in the first place using Bergeys (1974) Manual, which is based on the I.S.P. system, and this showed 72T1 resembled S.aureofaciens, while all the other strains identified with S.rimosus (Section 3.1.1). It was considered useful to extend the taxonomic studies to other mutants, especially the blocked mutants, by using the recently developed method of Williams et al (1983 b). The object was to check the behaviour of the mutants and blocked mutants in relation to their original species.

There were three sets of tests:-

1. The strains were submitted to Williams identification analysis using 41 tests (1983 b) and Sneath's (1979) MATIDEN programme which was adapted to the DEC-20 computer at the Polytechnic.
2. The results of the 41 tests were analysed into clusters using the simple matching coefficient (Sokal & Michener, 1958) and Jaccard coeffiecnt (Sneath 1957) included in the CLUSTAN programme used with DEC-20 computer (Fig 26). This method was repeated using data for a number of type strains, notably S.griseus, S.albus and Nocardia Mediterranea (Fig 27).

1) SUMMARY OF METHODS FOR CLASSIFICATION OF STREPTOMYCETES BY THE I.S.P. AND COMPUTER METHODS

Very many cultures of streptomycetes have been isolated and named, many hundreds of isolates having been given species names on the basis of relatively few characteristics, leading to great uncertainty as to their real taxonomic significance.

A major attempt was made to deal with this situation by the International Streptomyces Project (I.S.P.). In the course of this study 450 species were examined (Shirling & Gottlieb 1966, 1968a, b, 1969, 1972).

The criteria used in the I.S.P. system were:-

1) Micromorphology of spore-bearing hyphae

2) Surface ornamentation of spores

3) Colour of substrate mycelium

4) Colour of aerial mycelium after sporing

5) Colour of soluble pigments

6) Melanin production

7) Utilization of 9 carbon sources

The classification system described in the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons 1974) used data derived mainly from the I.S.P. system, the first 4 criteria were used to distinguish groups of species sharing the same morphological and pigmentation characters, which were later sub-divided by biochemical tests.

Although the I.S.P. project provided a standard system of tests and gave a workable system for the identification of streptomycetes, species were still described on the basis of a few critical tests, and there was no reduction in the number of species.

It seemed that reclassification was desirable, as it was likely that many of these species could be reduced to synonymy.

Many workers considered a wider range of characters could be used for classification [Gilardi et al (1960), Hill et al (1961), Hill & Silvestri (1962)] adopted a numerical taxonomic approach following the principles and methods of Sneath (1957). Sneath stated that methods of classification should involve the estimation of overall similarity and were based on the following principles:-

1) All features are of equal importance in creating taxa.

2) Organisms were classified upon an estimate of their overall similarity to one another.

3) The proportion of features common to two organisms represents their overall similarity (expressed as % S value)

4) Divisions into taxa are made on the basis of correlated features.

Williams et al (1983a) classified 475 species using 139 characters, employing S_{sm} and S_J coefficient and average linkage for clustering thereby reduced the different species to 61 clusters.

The major streptomycetes clusters defined by Williams et al (1983a) formed the basis for the development of a computer assisted identification scheme (Williams et al 1983b). This scheme involved the use of a probability matrix containing percent positive values for selected criteria. A total of 41 characters were selected, out of the 139 originally used, as the most diagnostic for the clusters using Sneath's CHARSEP and DIACHAR computer programmes. A total of 23 clusters were used in this identification method, as the minor clusters were not included in the probability matrix. As a result of that, what they called minor clusters such as cluster 14, containing S.aureofaciens, were not included as such. This meant that S.aureofaciens would be identified with cluster 29 (S.lydicus), which shared many S.aureofaciens characteristics. S.rimosus, which showed clear individual characteristics was the species name of cluster 42.

Additional techniques based on analytical chemistry and molecular biology have in the past 15 years, yielded considerable information on the relationships within the actinomycetes. With the development

of cell wall chemistry, the actinomycetes were divided into several chemotypes on the basis of their wall sugar and amino acids (Lechevalier and Lechevalier 1970). Williams et al data considered that all the true streptomycetes fall into the chemical type one group, containing LL-Diaminopimelic acid and glycine as diagnostic amino acids.

2) COMPUTER IDENTIFICATION METHOD

In the present study strains were submitted to the 41 tests described by Williams et al (1983b) (Table 16). Three ID coefficients were included in the programme which calculated (a) Willcox probability (Willcox et al 1973), (b) taxonomic distance, and (c) the standard error of the taxonomic distance.

For good identification Williams et al required a Willcox probability above .85, taxonomic distance less than 0.4 and standard error between 2.0 - 3.0, and preferably less than 2.0.

Table 16 The 41 test unit characters used by Williams et al (1983b)

Carbohydrate	Morphology & pigmentation	others:
Fructose	Melanin production	growth in NaCl 7%
Inulin	Pigmentation of substrate mycelium:	growth in phenol 0.1%
Rhamnose		growth at 45°C
Raffinose	1) Red/orange	growth in Na azide 0.01%
Inositol	2) Yellow/brown	Resistance to:
Manitol	Spore mass:	1) Rifampicin
Xylose	1) Red	2) Neomycin
Arbutin	2) Grey	Antimicrobial activity against:
Allantoin	3) Green	1) B.subtilis
Adonitol	Spore surface Smooth	2) A.niger
Cellobiose	Spore surface Rugose	3) S.murinus
	Spore chain:	Enzyme activity:
	1) Biv	1) Lacithinase 2) Nitrate reduction 3) Pectin hydrolysis
	2) Spir	4) H_2S production
	3) RA	Growth on nitrogen source:
	4) RF	1) L-Hydroxyproline
	Fragmentation	2) L-Histidine
		3) DL-α-Amino-n-butyric acid

The results obtained from the 41 test system (results and computer print out in the Appendix) were processed on the DEC 20 computer. The identification results obtained are shown in Table 17.

Table 17. Identification results with Williams method

Strain	Willcox probability	Cluster number	Cluster name
D9	.95	42	S.rimosus
Y2O	.95	42	S.rimosus
D9D7	.98	42	S.rimosus
FF	.95	42	S.rimosus
S.capuensis	.78	42	S.rimosus
S.rimosus NRRL 2234	.99	42	S.rimosus
S.aureofaciens	.96	29	S.lydicus
72T1	.99	29	S.lydicus
G65	.99	29	S.lydicus
Mutant 6	.61	16	S.albus
Mutant 9	.57	1	S.griseus
Mutant A	.78	1	S.griseus

It is interesting to look at the identification in more detail, using the information given in the computer printouts, which are shown in Table 18 sections (a),(b),(c) and (d). In (a) the three strains concerned 72T1, S.aureofaciens (A196)and G65 fall within cluster 29, denoted by Williams as S.lydicus, but there are signs of a large taxonomic distance and standard errors, i.e.

	Taxonomic distance	standard error	disagreement out of 41
72T1	.47	4.8	8
S.aureofaciens (A196)	.45	4.3	7
G65	.3	O.2	2

Both of the first two i.e. 72T1 and S.aurofaciens exceeded the recommended taxonomic distance of 0.4 and standard error of 2-3. Of the eight mismatches, six are common between 72T1 and S.aureofaciens, probably suggesting 72T1 is similar to S.aureofaciens but differs from S.lydicus.

Sections (b) and (c) give the identification of the S.rimosus strains, in which the identification is clear in all cases, although Y20 and FF are of different origins.

Section (d) deals with the blocked mutants, none of which are clearly identified, although mutant A seems very close to S.griseus, while mutant 6, based on taxonomic distance, has a resemblance to S.albus, originally thought by Pfizer to be a close relation to S.rimosus.

Note: The Coefficients 1, 2 and 3 in table 18 represents:

(1) = Willcox probability

(2) = taxonomic distance

(3) = standard error

Also the species identity is the cluster number.

Table 18. Results of computer identifications
(a) S.aureofaciens types

72T1 BEST IDENTIFICATION IS S.lydicus 29

SCORES TO ON COEFFICIENTS:

		1	2	3
S.LYDICUS	29	0.9999942	0.4681964	4.784666
STV.CINNAMONEUM	55	5.292452E-06	0.4886071	7.368281
S.LAVENDULAE	61	2.405795E-07	0.5447997	6.081887

IF ADDITIONAL TAXA ARE NEEDED? ENTER A POSITIVE INTEGER, ELSE ENTER ZERO

CHARACTERS AGAINST S.LYDICUS 29

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
ADONITOL	127	81.8	-
RAFFINOSE	122	81.8	-
INOSITOL	118	90.9	-
NA.AZIDE.0.01%	105	18.2	+
MANNITOL	119	90.9	-
NEOMYCIN	86	18.2	+
ARBUTIN	84	99	-
ALLANTOIN	81	18.2	+

S.aureofaciens BEST IDENTIFICATION IS S.plantensis 29

SCORES TO ON COEFFICIENTS:

		1	2	3
S.LYDICUS	29	0.9664607	0.4507858	4.272063
S.DIASTATOCHROM	19	0.01243921	0.5258649	4.065611
S.GRISEUS	1	0.01025504	0.5122533	4.616032

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO

CHARACTERS AGAINST S.LYDICUS 29

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
ADONITOL	127	81.8	-
RAFFINOSE	122	81.8	-
INOSITOL	118	90.9	-
MANNITOL	119	90.9	-
ARBUTIN	84	90	-
ALLANTOIN	81	18.2	+
S.MURINUS.INHIB	60	99	-

G65 BEST IDENTIFICATION IS S.plantensis 29

SCORES TO ON COEFFICIENTS:

		1	2	3
S.LYDICUS	29	0.9998614	0.2981865	-0.2207763
S.RIMOSUS	42	0.0001368728	0.3465126	3.231777
S.GRISEUS	1	1.018261E-06	0.4644048	3.344186

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO

CHARACTERS AGAINST S.LYDICUS 29

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
ALLONTOIN	81	18.2	+
SPORE.MASS.GREY	14	90.9	-

Table 18(b)

S.rimosus NRRL 2234 BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO ON COEFFICIENTS:

		1	2	3
S.RIMOSUS	42	0.9997158	0.25582	0.01976915
S.GRISODVIRIDIS	17	0.0002716561	0.3530515	1.783811
S.ROCHEI	12	7.996114E-06	0.4192548	2.21645

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
NEOMYCIN	86	99	-
ALLANTOIN	81	85.7	-

Y2O BEST IDENTIFICATION IS S.rimosus 42

SCORES TO ON COEFFICIENTS:

		1	2	3
S.RIMOSUS	42	0.9502475	0.3211606	2.33686
S.LYDICUS	29	0.04779387	0.4080149	3.012799
S.GRISEUS	1	0.001188076	0.4381932	2.647464

IF ADDITONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	39	99	-
NEOMYCIN	86	99	-
XANTHINE	72	85.7	-
LECITHINASE	47	85.7	-

S.capuensis BEST IDENTIFICATION IS S.rimosus 42

SCORES TO ON COEFFICIENTS:

		1	2	3
S.RIMOSUS	42	0.7863554	0.345808	3.206905
S.LYDICUS	29	0.0939298	0.441492	3.998434
S.ROCHEI	12	0.09250796	0.4452667	2.912353

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
?O

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99	-
NEOMYCIN	86	99	-
BSUBTILIS.INHI	54	99	-
INULIN	126	1	+

Table 18(c)

FF BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO ON COEFFICIENTS:

		1	2	3
S.RIMOSUS	42	0.956022	0.292796	1.335599
S.LYDICUS	29	0.03606122	0.3885408	2.439441
S.GRISEUS	1	0.006923709	0.4110825	1.926846

IF ADDITIONAL TAXA ARE NEEDED? ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99	-
NEOMYCIN	86	99	-
LECITHINASE	47	85.7	-

D9 BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO ON COEFFICIENTS:

		1	2	3
S.RIMOSUS	42	0.9502475	0.3211606	2.33686
S.LYDICUS	29	0.04779387	0.4080149	3.012799
S.GRISEUS	1	0.001188076	0.4381932	2.647464

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99	-
NEOMYCIN	86	99	-
XANTHINE	72	85.7	-
LECITHINASE	47	85.7	-

D9D7 BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO ON COEFFICIENTS:

		1	2	3
S.RIMOSUS	42	0.9889707	0.2613711	0.2263117
S.LYDICUS	29	0.01087594	0.3799076	2.185263
S.GRISEUS	1	7.089662E-05	0.4366319	2.605964

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99	-
NEOMYCIN	86	99	-

Table 18(d)

IDENTIFICATION OF OTC MUTANTS

Mutant A BEST IDENTIFICATION IS S.GRISEUS 1

SCORES TO ON COEFFICIENTS:

		1	2	3
S.GRISEUS	1	0.787627	0.4238771	2.266933
S.VENEZUELAE	6	0.1588763	0.4267253	2.306266
S.VIRIDOSPORUS	15	0.01759961	0.4596489	3.448579

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO

? 0

CHARACTERS AGAINST S.GRISEUS 1

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RAFFINOSE	122	16.9	+
H2S.PROD	53	91.5	-
LECITHINASE	47	5.6	+
L-HYDROXYPROUN	46	22.5	+
SPORE.CH.SPIR	4	8.6	+
SPORE.CH.RF	2	82.9	-

Mutant 9 BEST IDENTIFICATION IS S.GRUSEUS 1

SCORES TO ON COEFFICIENTS:

		1	2	3
S.GRISEUS	1	0.5712524	0.4378591	2.630584
S.DIASTATOCHROM	19	0.427865	0.4681538	2.631726
S.EXFOLIATUS	5	0.000398313	0.4870132	3.671047

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO

? 0

CHARACTERS AGAINST S.GRISEUS 1

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RAFFINOSE	122	16.9	+
XYLOSE	117	93	-
XANTHINE	72	97.2	-
H2S.PROD	53	91.5	-
L-HYDROXYPROUN	46	22.5	+

Mutant 6 BEST IDENTIFICATION IS S.ALBUS 16

SCORES TO ON COEFFICIENTS:

		1	2	3
S.ALBUS	16	0.6164403	0.3276667	3.044028
S.VIRIDOSPORUS	15	0.8800425	0.3830035	1.372809
S.ROCHEI	12	0.001901959	0.4125104	2.086015

CHARACTERS AGAINST S.ALBUS 16

CHARACTE	PERCENT	IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99	-
NO3.RED	52	1	+
H2S.PROD	53	83	-

3) CLUSTERING OF THE STRAINS USING WILLIAMS 41 TESTS

The strains were compared using the 41 test unit characters (Table 16), and the data was analysed using CLUSTAN 1A programme on the DEC-20 computer. Similarity was developed using both the simple matching coefficient (S_{sm}) which includes both positive and negative matches, and the Jaccard coefficient (S_J) which includes positive matches only. The strains were clustered by Ward's method (Ward 1963) and the group average methods.

The dendrograms in Fig 25 showed the results obtained by S_{sm} and S_J methods. The dendrograms with the additional data from selected type strains are shown in Fig 26.

In Fig 25(a) taking the coefficient 0.75 as significant, three main clusters were obtained:-

(1) All the producing mutants and G65

(2) The blocked mutants

(3) 72T1 and S.aureofaciens

Note the shift of G65 from the S.lydicus cluster to S.rimosus. Based on Jaccard analysis, (Fig 25b) a rather similar pattern was obtained, though the blocked mutant 9 appeared to be separated from the others. 72T1 and S.aureofaciens, are separated from the others, but there seems to be suggestion of differences between them i.e. fork at 0.625.

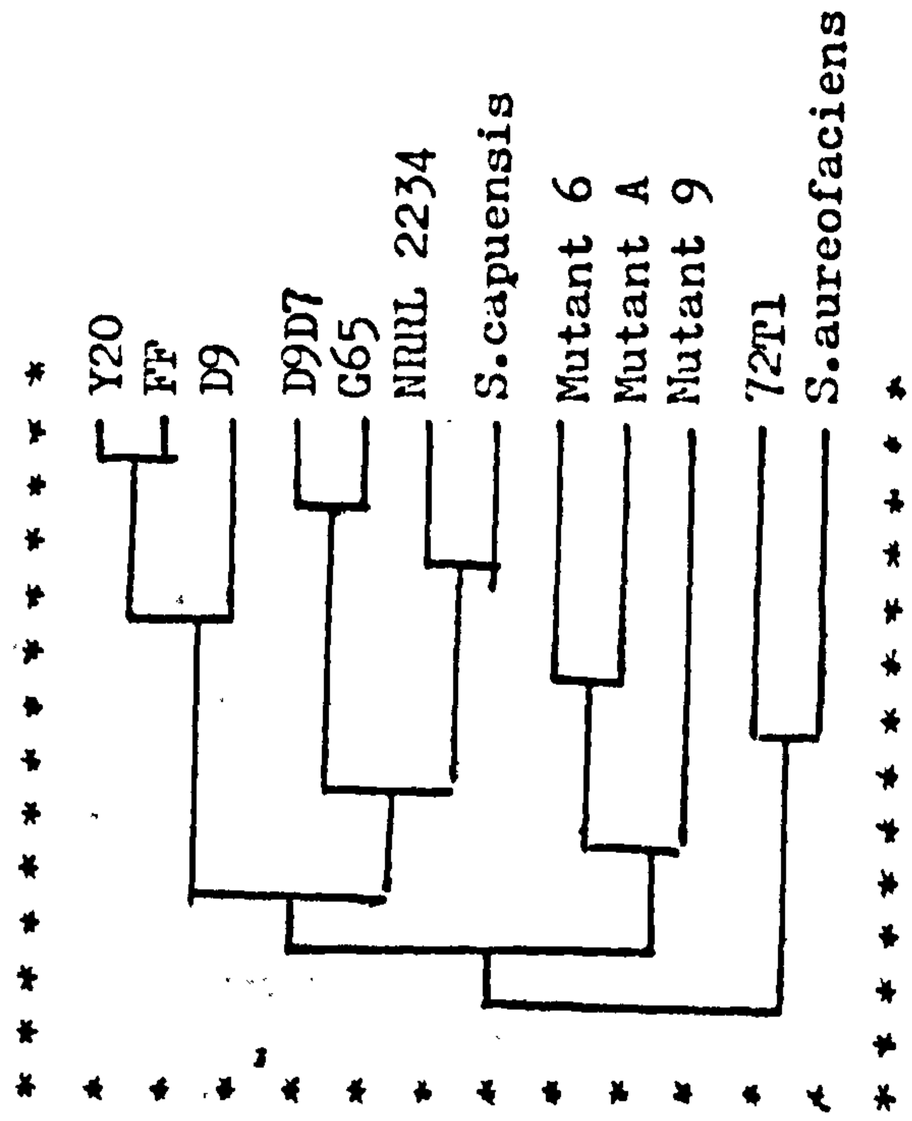
Fig 26 showed a good separation of the three added species, S.albus, S.griseus and Nocardia Mediteranea at coefficients 0.733 and 0.625 respectively. The clusters were similar to the previous classification.

Fig. 25 clustering using Williams 41 tests

Items grouped

Cycle	I	J	Coeff
1	1	4	0.950
2	3	6	0.950
3	7	8	0.950
4	1	2	0.917
5	9	11	0.875
6	5	12	0.850
7	3	7	0.825
8	9	10	0.792
9	1	3	0.751
10	1	9	0.600
11	1	5	0.115

Procedure complete

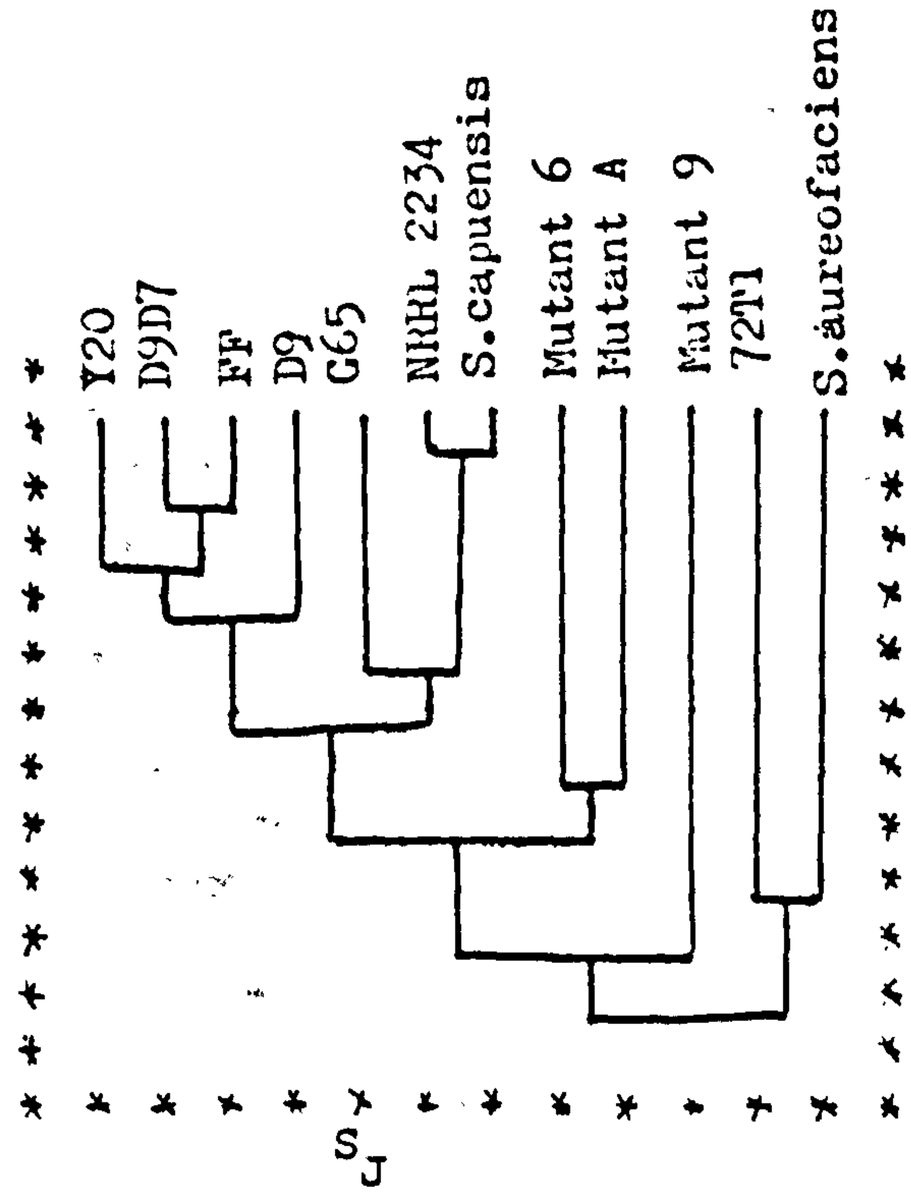


a) Simple matching coefficient
Ward's method

Items grouped

Cycle	I	J	Coeff
1	7	8	0.917
2	3	4	0.909
3	1	3	0.861
4	1	2	0.834
5	6	7	0.788
6	1	6	0.785
7	9	11	0.773
8	1	9	0.723
9	5	12	0.625
10	1	10	0.584
11	1	5	0.382

Procedure complete



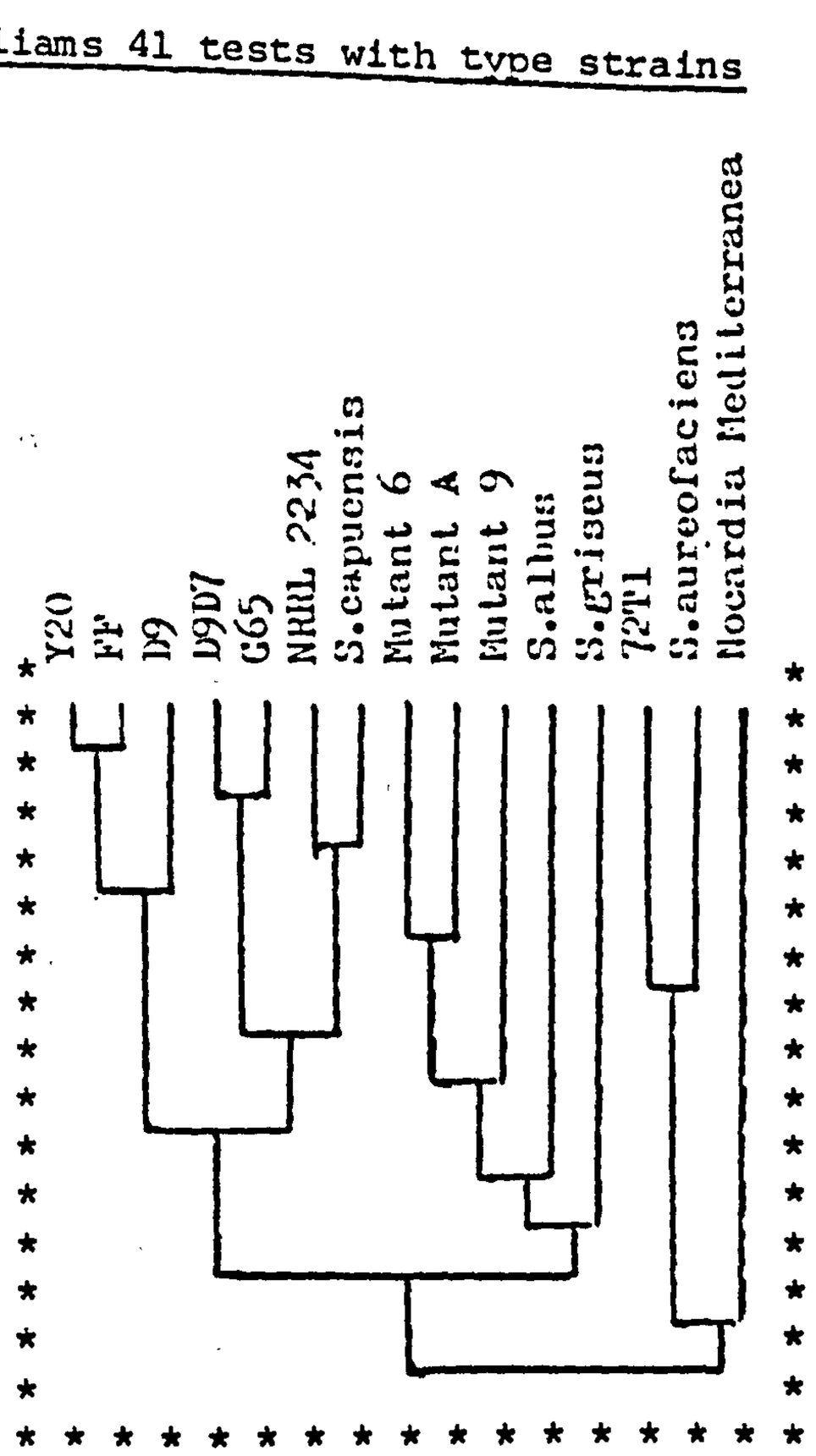
b) Group average

Fig 26. clustering using Williams 41 tests with type strains

Dendrogram

Items grouped

Cycle	I	J	Coeff
1	1	4	0.950
2	3	6	0.950
3	7	8	0.950
4	1	2	0.917
5	9	11	0.875
6	5	12	0.850
7	3	7	0.825
8	9	10	0.792
9	1	3	0.751
10	9	14	0.733
11	9	13	0.600
12	1	9	0.465
13	5	15	0.350
14	1	5	0.112



Procedure complete

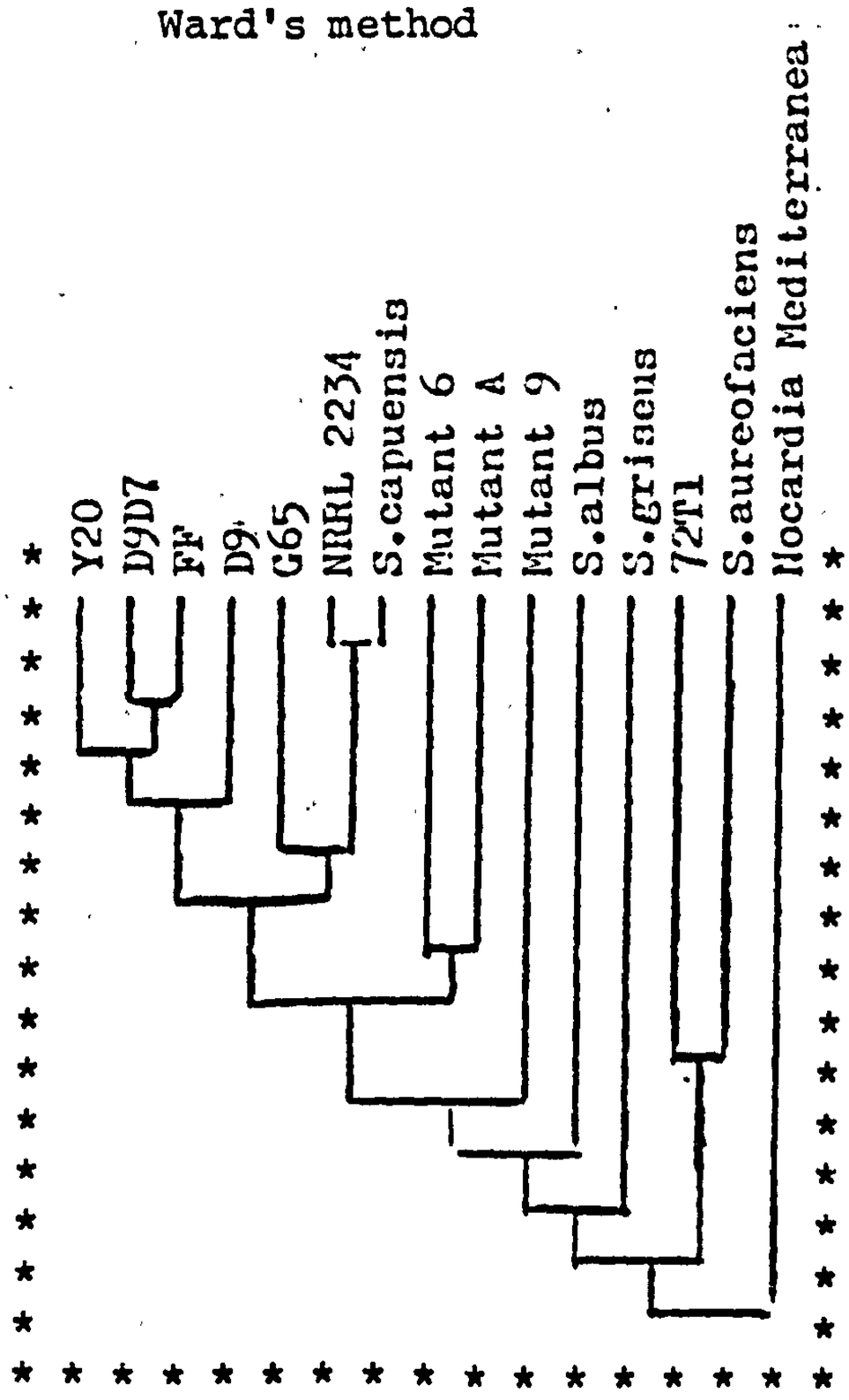
a) Simple matching coefficient

Ward's method

Dendrogram

Items grouped

Cycle	I	J	Coeff
1	7	8	0.917
2	3	4	0.909
3	1	3	0.861
4	1	2	0.834
5	6	7	0.788
6	1	6	0.785
7	9	11	0.773
8	1	9	0.723
9	5	12	0.625
10	1	10	0.584
11	1	14	0.561
12	1	13	0.536
13	1	5	0.378
14	1	15	0.274



Procedure complete

b) S_J

Group average

4) CLUSTERING USING DATA FROM THE EXTENDED I.S.P. METHOD

The strains were compared using the I.S.P. test results plus additional tests (tests and results in Table 19) the data was analysed with CLUSTAN as described before.

With the extended I.S.P. data 72T1 differs from S.rimosus NRRL 2234 and from the mutants Y20 and Y20 inactive. A feature of Y20 and other high yielders is that they grow on dextrin, and with yeast extract media gave a brown substrate mycelium with brown reverse (often very dark). This was also shown in S.capuensis. S.rimosus NRRL 2234 was reported by Pfizer to resemble S.albus but to be rather browner and the colonies show cracking. The OTC^- mutants (6,9 and A) besides losing OTC production or OTC and acetyl-OTC, showed loss of brown colour but generally all the mutants resembled Y20. S.rimosus NRRL 2234 and 72T1 differ from each other and the mutants, as the colour played an important role in the extended I.S.P. system. Fig 27 shows the dendrogram of the tests using CLUSTAN on the DEC-20 computer.

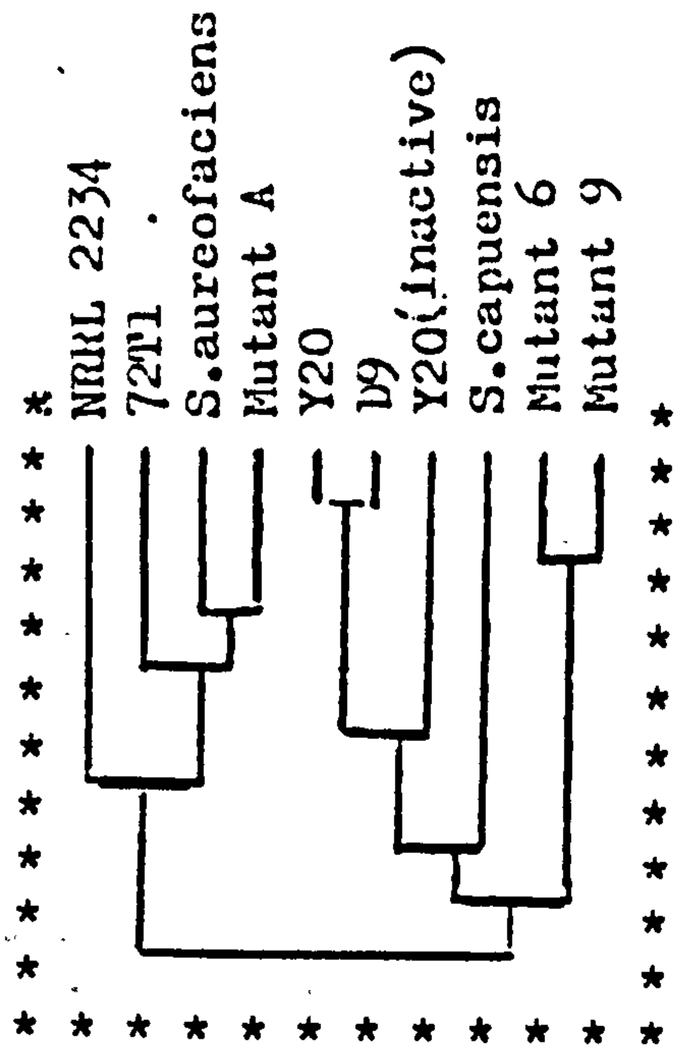
With this system the cluster break down was very different from those previously experienced. Expecially with the Jaccard system, only Y20 and D9 showed any similarity. This probably arose because tests used were likely to give sharp differences between the strains, a procedure probably unsuitable for the clustering techniques, and the number of test strains were also small, which tends to give unreliable clustering.

Fig. 27 clustering using Extended I.S.P. tests

Dendrogram

Items grouped

Cycle	I	J	Coeff
1	3	5	0.925
2	7	8	0.811
3	6	9	0.811
4	2	6	0.761
5	3	4	0.723
6	1	2	0.682
7	3	10	0.654
8	3	7	0.591
9	1	3	0.184



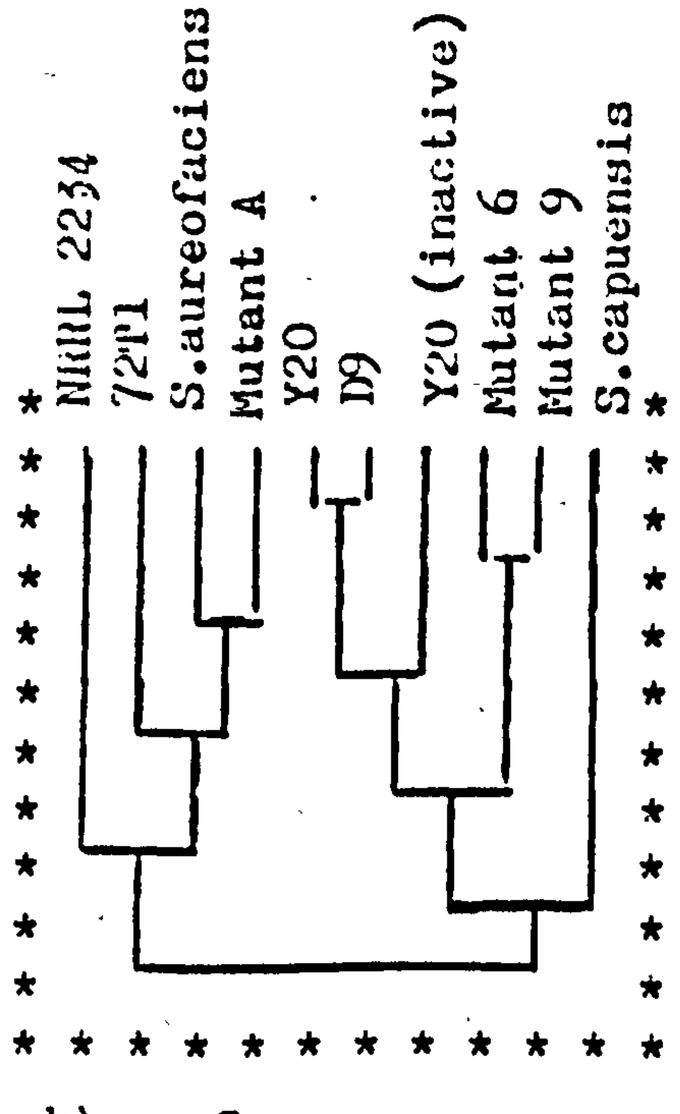
Procedure complete

a) Simple matching coefficient
Ward's method

Dendrogram

Items grouped

Cycle	I	J	Coeff
1	3	5	0.818
2	7	8	0.565
3	6	9	0.545
4	3	4	0.511
5	2	6	0.455
6	3	7	0.433
7	1	2	0.393
8	3	10	0.381
9	1	3	0.287



Procedure complete

b) S_J

Group average

Table 19 Results of the extended I.S.P. method

Strain	Emerson medium: Melanin / Aerial	Emerson medium: substrate 1 2 3	Emerson medium: Reverse 1 2 3 4	Emerson medium: cracking	Emerson medium: spiral	Carbohydrate: glucose	Arabinose	sucrose	xylose	inositol	mannitol	fructose	Rhamnose	Raffinose	OTc	ISP2: Aerial 1 2	ISP2: substrate 1 2 3	ISP2: Reverse 1 2 3 4
NRRL 2234	- -	+ - -	- + - -	+	+	+	+	-	+	+	+	+	-	+	1.0	-	+ - -	- + - -
72T1	- -	+ - -	+ - - -	-	-	+	+	+	-	-	-	+	-	-	.5	-	+ - -	- + - -
Y20	+ +	- - -	- - - +	-	+	+	+	-	+	+	+	-	-	+	3.5	+	- - +	- - + -
Y20 inactive	+ +	- - +	- - - +	-	+	+	+	-	-	+	-	-	-	-	0	-	+ - -	- + - -
D9	+ +	- - +	- - - +	-	+	+	+	-	+	+	+	-	-	+	4.5	+	- - +	- - + -
S.aureofaciens	- -	- + -	- + - -	-	+	+	+	+	-	-	+	-	-	-	Nil	+	+ - -	+ - - -
6	- -	- + -	- - + -	-	+	+	+	-	-	+	+	-	-	+	Nil	+	+ - -	- + - -
9	- -	- + -	- - + -	-	+	+	+	-	-	+	+	-	-	+	Nil	+	- + -	- - + -
A	- -	+ - -	+ - - -	-	+	+	+	-	+	+	+	-	-	+	Nil	-	+ - -	+ - - -
S.capuensis	+ -	- + -	- + - -	-	+	+	+	-	+	+	+	-	-	+	3.7	+	- + -	- + -

Strain	ISP3: Aerial 1 2	ISP3: substrate 1 2 3	ISP3: Reverse 1 2 3 4	ISP4: Aerial 1 2	ISP4: substrate 1 2 3	ISP4: Reverse 1 2 3 4	RC medium: Aerial 1	RC medium: substrate 1 2 3	RC medium: Reverse 1 2 3 4	Potato: Aerial 1	Potato: substrate 1 2 3	Potato: Reverse 1 2 3 4
NRRL 2234	+	+ - -	- + - -	-	+ - -	- + - -	-	- + -	- + - -	-	+ - -	+ - - -
72T1	+	+ - -	+ - - -	-	+ - -	+ - - -	-	+ - -	- + - -	-	+ - -	+ - - -
Y20	-	+ - -	+ - - -	-	- + -	+ - - -	-	- + -	- - - +	+	- - -	- - + -
Y20 inactive	-	+ - -	+ - - -	-	- + -	- - - -	-	- + -	- - - +	+	- + -	- - + -
D9	-	+ - -	+ - - -	-	- + -	- + - -	-	- + -	- - - +	+	- + -	- - - +
S.aureofaciens	+	+ - -	+ - - -	+	+ - -	+ - - -	+	- + -	+ - - -	-	- - -	- - + -
6	-	+ - -	+ - - -	+	+ - -	- + - -	+	- + -	+ - - -	+	- - -	- - - +
9	-	+ - -	+ - - -	+	+ - -	- - + -	+	+ - -	- + - -	+	- - +	- - - -
A	-	+ - -	+ - - -	-	+ - -	+ - - -	+	+ - -	+ - - -	-	+ - -	+ - - -
S.capuensis	-	+ - -	+ - - -	-	- + -	- + - -	-	+ - -	+ - - -	+	- + -	- + - -

Aerial 1 = off white
2 = pale grey

carbohydrate + = good growth
- = Nil or trace growth

Reverse 1 = pale
2 = light brown
3 = mid brown
4 = very dark brown

substrate 1 = pale
2 = brown
3 = dark brown

medium + = brown colour
- = pale colour

5) DISCUSSION

The results using Williams identification method of 72T1 (Willcox probability 0.99) and G65 (Willcox probability 0.95) indicated that they fell within the S.lydicus cluster and therefore showed a similarity to S.aureofaciens. The results also showed that all the OTC producing mutants identified well with S.rimosus. These strains consisted of Y20, FF, D9, and D9D7, the latter three are known to have originated from an S.rimosus ancestor. The Y20 group was derived from 72T1. The change from S.aureofaciens to S.rimosus, during this process was surprising. On the other hand, in one sense, S.aureofaciens and S.rimosus are very similar in that they produce tetracycline antibiotics. It is also known however that during the development of Y20 some hybridisation experiments were carried out, and mutants of S.rimosus NRRL 2234 may have been used, and it is therefore possible that the species change may have arisen in this way. In fact, 72T1 was originally named S.rimosus because it was a white Streptomyces producing oxytetracycline.

A study of the index to Chemical Abstracts (1947-1981), showed that the members of cluster 29 (S.lydicus) produced many types of antibiotics. Interestingly S.platensis produces oxytetracycline. In cluster 42 (S.rimosus) two members produced oxytetracycline, while the five others produced other antibiotics.

It is a particular interest that when OTC production was lost, in the blocked mutants, their identity could no longer be established. The resemblance suggested for mutant 6 and 9 were S.albus and S.griseus but with Willcox probability were only 0.61 and 0.57,

which is not very meaningful. With mutant A however, there was some resemblance to S.griseus (Willcox probability .78 and taxonomic distance .42). Table 18 also showed a number of changes of the test characteristics such as growth in xylose, lecithinase etc. This effect of a point mutation on many areas of the genomes has been noted by other workers, for example Hostalek et al (1976). Using the same data, obtained by Williams 41 tests, with the CLUSTAN package, a slightly different clustering was obtained. The OTC producing mutants were grouped under one cluster, with S.rimosus in a separate nearby cluster (Fig 26), on the other hand G65, which was identified as S.lydicus, cluster 29, by Williams method, was placed near S.rimosus with a distance from 72T1 and S.aureofaciens. The dendrograms also showed 72T1 and S.aureofaciens lying together in the tree, but separated by a good distance. The blocked mutants also grouped separately by this method.

These results are of interest as this clustering method separated the group of mutants, and compared the strains with each other, while with the Williams identification method comparisons were with data from type strains of different species located in the matrix.

The dendrogram with type strains added gave some clustering, with the added S.albus and S.griseus in the same area of the tree near the blocked mutants but with a good separation distance.

The extended I.S.P. test method and CLUSTAN package, involving the colours of the pigements produced by the test organism, showed slight differences between the OTC producing mutants and S.rimosus

NRRL 2234, while with the carbohydrate tests they resembled each other. As a result of this the OTC producing mutants were separated from S.rimosus NRRL 2234 in a different cluster. On the other hand 72T1 was nearer to S.aureofaciens than S.rimosus NRRL 2234.

The blocked mutants were clustered in one cluster separated from the others by a good margin.

While the simplification of the classification of the streptomycetes produced by Williams et al (1983a) and the corresponding identification system (Williams et al 1983b) represent an advance which should be valuable, these trials brought out certain problems with it. Some of them were:-

1. The absence of S.aureofaciens from the system means it was placed with S.Lydicus where the fit was not very good. The disappearance of such a well established species itself causes a problem among practical workers especially the strain used by Williams et al did not fit with the description of S.aureofaciens in Bergey's Manual.

2. The systems were sensitive to minor variations. Thus strain G65 appears as S.lydicus in the identification test, but as S.rimosus if the same results are clustered, the difference depending only two test results i.e. sensitivity tc rifampicin and neomycin.

3. The non-identification of the blocked mutants is also important. As a rule mutants are named after the parent culture. In this case the appearance of the unidentifiable culture was unexpected.

No doubt these and other problems, will be dealt with as more experience is gained.

3.8 MORPHOLOGY OF THE STRAINS Y20, D9, NRRL 2234 AND 72T1 IN AGAR AND SHAKEN FLASKS CULTURES UNDER SCANNING MICROSCOPY

1) STRUCTURE OF THE MYCELIUM IN AGAR MEDIUM FOR Y20 AND 72T1

2) STRUCTURE OF THE MYCELIUM IN SHAKEN CULTURES

3) DISCUSSION

3.8 MORPHOLOGY OF THE STRAINS Y2O, D9, NRRL 2234 AND 72T1 ON AGAR AND IN SHAKEN FLASKS CULTURES, BY SCANNING ELECTRON MICROSCOPY

1) STRUCTURE OF THE MYCELIUM ON AGAR MEDIUM WITH Y2O AND 72T1

The culture of 72T1 produced "long" hyphae with small branches ending in straight or flexuous sporophores (Plate 4, Fig.1). On the other hand the Y2O culture produced long coiled sporophores (Plate 4, Fig 2). Plate 4 Fig 3 & 4 shows the aerial spores.

2) STRUCTURE OF THE MYCELIUM IN SHAKEN CULTURES

I) 72T1:-

In the inoculum medium 72T1 showed "pellet" type colonies of 60µm diameter with an amorphous central mass (Plate 5 Fig.1.). In production medium, the 24 hours sample showed similar "pellet" type colonies, but the mycelium was more "open" towards the edges and the colony size was larger, 90µm, (Plate 5 Fig.2). Some of the hyphae also became septate, often with a narrowing towards the tips (Plate 5,Fig.3). After 24 hours, samples of the production medium, showed that the colonies became more "open" and the mycelium showed more septation (Plate 5 Fig.4). Such septation tended to lead to fragmentation of the hyphae into coccoid or bacillary units after 72 hours. (Plate 5 Figs 4,5 & 6). Structures similar in appearance to the "entomoform" structures as described by Tresner et al (1967) have been observed at 72 hours (Plate 6 Fig.4). After 96 hours most of the mycelium in the medium tended to consist of coccoid units (Plate 6 Figs.2 & 3), Plate 6 Fig 1 appeared to show the formation of a germ tube from these units.

II) Y20:-

In the case of Y20 the inoculum flasks showed colonies of the "open" type (Plate 6 Fig.5). This type of colony was also observed in the 24 hours production culture, and some of the mycelium also appeared to clump together within a mucilaginous "layer" (Plate 6 Fig.6), which has also been seen with 24 hours samples and more obvious after 72 hours. (Plate 7 Figs. 1 & 2). After 96 hours the mucilaginous layer appeared to be less and disappeared completely by 120 hours (Plate 7 Figs. 3 & 4).

Globular bodies similar to those reported by Williams et al (1974) were also observed with the 120 hours sample (Plate 7 Fig.5).

Fragmentation of the hyphae was not observed even after 120 hours (Plate 7 Fig.6).

III) D9:-

The inoculum mycelium of D9 consisted of hyphae which grew together and appeared to be covered by a layer of material similar to that noticed with Y20 production mycelium (Plate 8 Fig.1). In the production medium the mycelium was of the "open" type similar to that of Y20 but here the mucilaginous "layer" was more obvious at 24 hours (Plate 8 Fig.2). This "layer" decreased with time and by 120 hours it seemed to disappear (Plate 8 Figs 3,4,5 & 6). Fragmentation of the hyphae was not observed.

IV) S.rimosus NRRL 2234:-

The inoculum mycelium of NRRL 2234 showed no "pellet" type colonies but it was of the "open" type with mucilaginous material causing the hyphae to adhere together (Plate 9, Fig.1).

In the production flasks, the 24 hour sample showed similar type of colonies to the inoculum, with the hyphae adhering together within a mucilaginous "layer", (Plate 9 Fig.2). This layer disappeared after 48 hours and only "open" mycelium could be seen (Plate 9 Fig. 3). After 72 hours, the mycelium produced small fragile branches (Plate 9 Fig.4), which appeared to separate from the mycelium after 96 hours, to produce rod-shaped units. (Plate 9 Fig.5) and (Plate 9 Fig.6 under light microscopy).

Observation of the morphology of the strains in shaken cultures are summarised in Table 20.

Table 20 Summary of the morphology of the strains

STRAIN	OTC PRODUCED IN SHAKEN FLASKS	GENERAL GROWTH FORM	SPECIAL FEATURES (PRODUCTION STAGE)
72T1 Plate 5 Fig 1-6 Plate 6 Fig 1-4	0.5-0.8 g/l	grow very fast in the first 24 hours then very slowly	a) Forms pellet-like colonies with dense central region. b) After 24 hours the hypha tended to form segments and swelling. c) Formation of cocoid unit d) Formation of entomoform structure after 72 hours
Y20 Plate 6 Fig 5-6 Plate 7 Fig 1-6	2.5-3.5 g/l	forms a fairly continuous growth curve.	a) Open mycelium throughout the culture with mucilaginous layer around it. b) Rarely globular like material similar to those reported by Williams et al (1974).
D9 Plate 8 Fig 1-6	2.5-4.0 g/l	forms a continuous growth curve like Y20	a) Inoculum mycelium tended to clump together within a mucilaginous layer. b) In the production stage the mucilaginous layer tended to disappear after 72 hours.
NRRL 2234 Plate 9 Fig 1-6	0.8-1.0 g/l	fast growth until 24 hours then slower growth	Open mycelium, which formed rod shaped units in the culture after 96 hours.

3) DISCUSSION

The evidence for the correlation between structure and antibiotic production is, of course, circumstantial, yet great differences in morphology were observed in the present study between low and high antibiotic producers. Such differences may be related to the amount of antibiotic formed or the growth patterns of the strains.

In the present study the morphology of the strains in the submerged culture divided into three types of growth.

I) 72T1, the I.C.I. wild strain.

II) NRRL 2234 S.rimosus the original Pfizer strain.

III) D9 and Y20 the high yielding mutants.

The wild strain 72T1 showed pellet-like colonies in the production medium, which disappeared when the organism started to form OTC, leading to fragmented mycelium. These fragments formed coccoid units which resemble aerial spores, some of which started to form germ tubes (Plate 6 Fig.2).

Although the formation of spore chains by streptomycetes in submerged culture closely resembling aerial spores has been reported, (Gottlieb and Legator 1953, and Wilkin and Rhodes 1955), these results could not be repeated by other workers. These units are not uncommon during the growth of streptomycetes in submerged culture and are frequently referred to as spores (Zhuravleva et al 1982, Stastna et al 1976, Statstna et al 1979). Whether they are spores or not is still undecided as they showed lower resistance to heat and enzyme attack than do aerial spores, also thin sections under electron microscopy showed some

differences between the aerial spores and these structures (Haxell 1983).

Swollen branched structures were observed (Plate 6 Fig.4) similar to those termed "entomoform" bodies reported for S.aureofaciens by Williams et al (1974) and Tresner et al (1967) who suggested that they may have a taxonomic value.

It seems that in the case of mutants giving increased OTC production certain features were shared. Firstly "open" mycelium in the culture and secondly the formation of a mucilaginous layer in or around the mycelium and thirdly the ability of the mycelium to grow in the presence of OTC (low producers showed no growth on agar medium containing 400 U/ml OTC, while the high producers grow above that level).

The growth in liquid media for the low producers showed that they stopped growing after 48 hours, at the time when OTC was first detected. Finally all high yielding mutants possess a mycelium throughout the life of the culture i.e. without forming spore-like bodies or fragmented mycelium.

S.rimosus NRRL 2234 morphology was intermediate between 72T1 and the mutants, as the young culture showed open mycelial colonies, which broke down at the end of the fermentation giving mycelial fragments.

These observations agree with those of Hostalek et al (1976), in showing the absence of swelling of the mycelium which were described by Kurylowicz (1972).

PLATE (4)

Fig.(1) 10 days old aerial hyphe showing long coiled sporophores of Y20

Fig.(2) 10 days old aerial hyphe showing long branches ending with straight or flexous sporophores of 72T1.

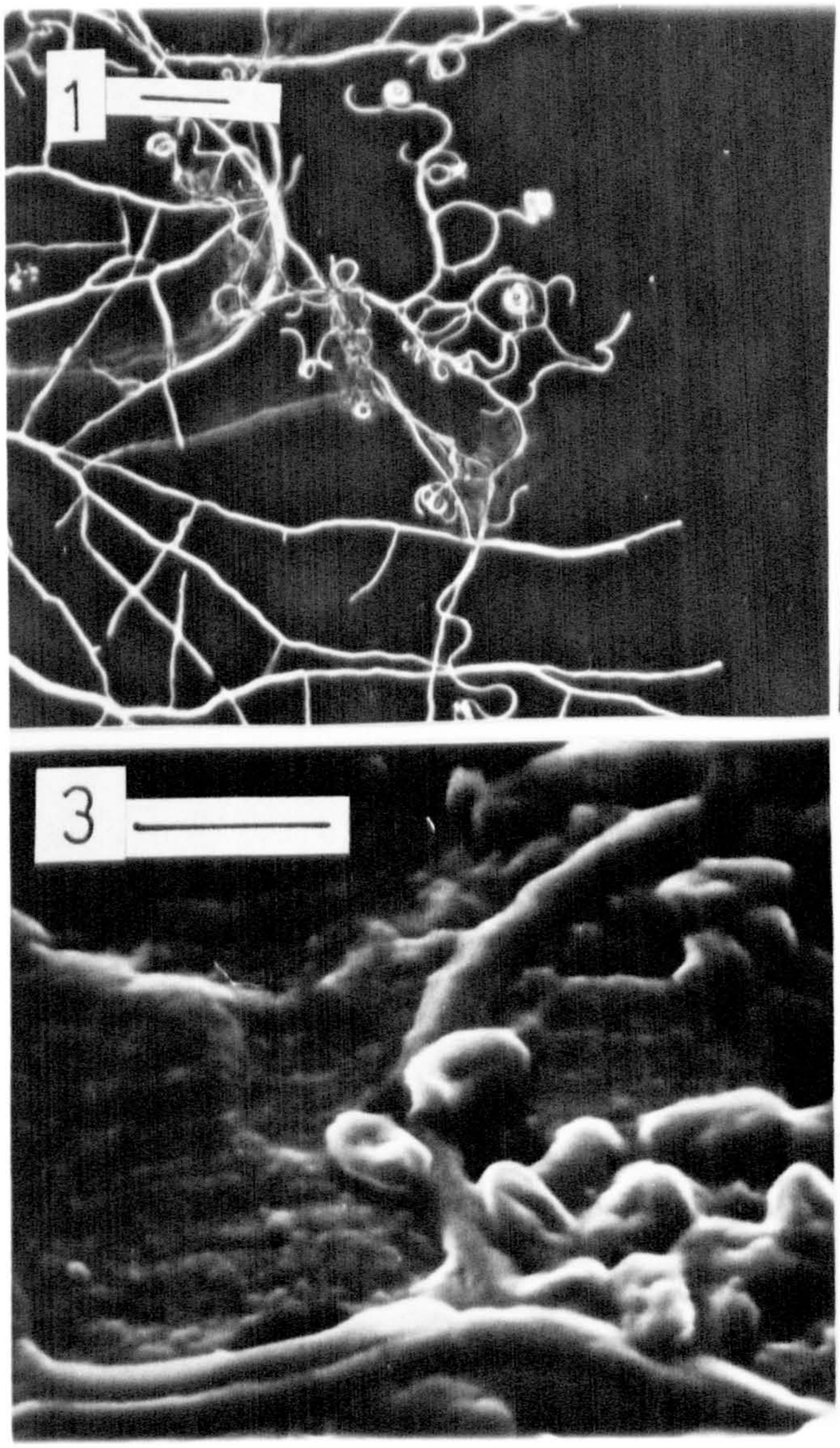
Fig.(3) Spores of Y20

Fig.(4) Spores of 72T1

bar in figs 1 & 2 represents 10 micron.

bar in figs 3 & 4 represents 2.5 micron.

PLATE 4



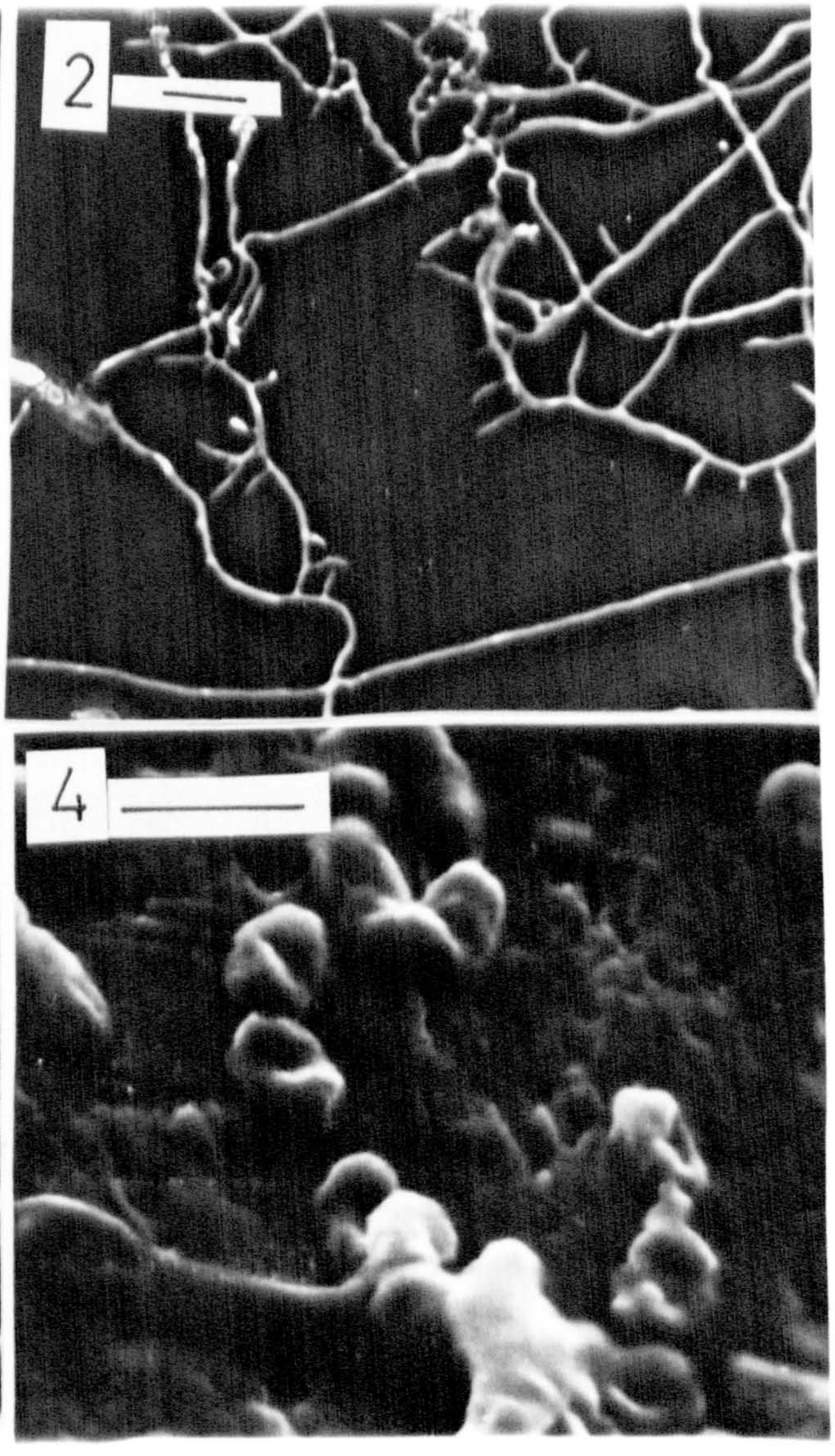


PLATE (5)

Fig.(1) Compact colony of 72T1 in inoculum medium 24 hours old 62 micron diameter.

Fig.(2) Compact colony of 72T1 in production medium 24 hours old 90 micron diameter.

Fig.(3) 24 hours mycelium of 72T1 from the edge of the colony of the production medium showing fragementation with a narrow end.

Fig.(4) 48 hours fragmented mycelium of 72T1 in production medium.

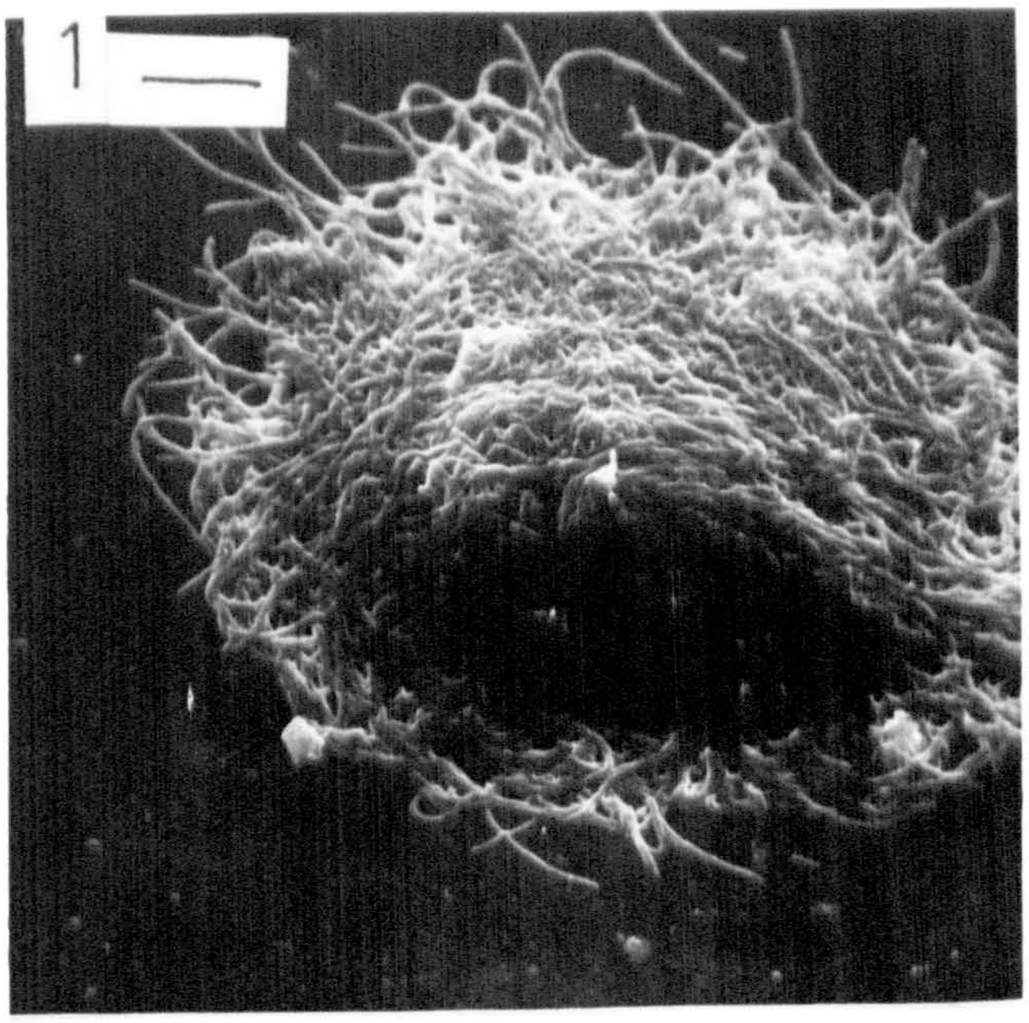
Figs.(5&6) Three and four days old mycelium of 72T1 in production medium showing spore-like structures.

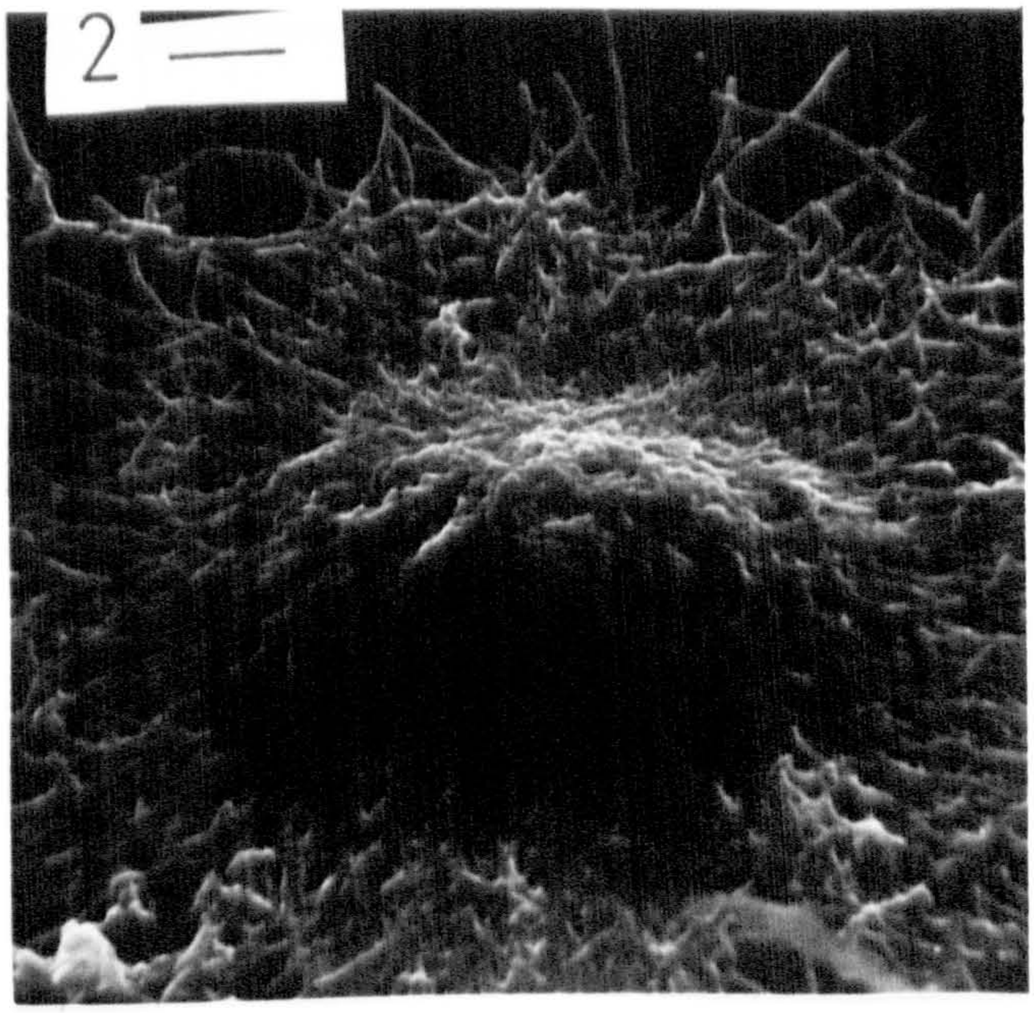
bar in figs. 1 & 2 represents 10 micron.

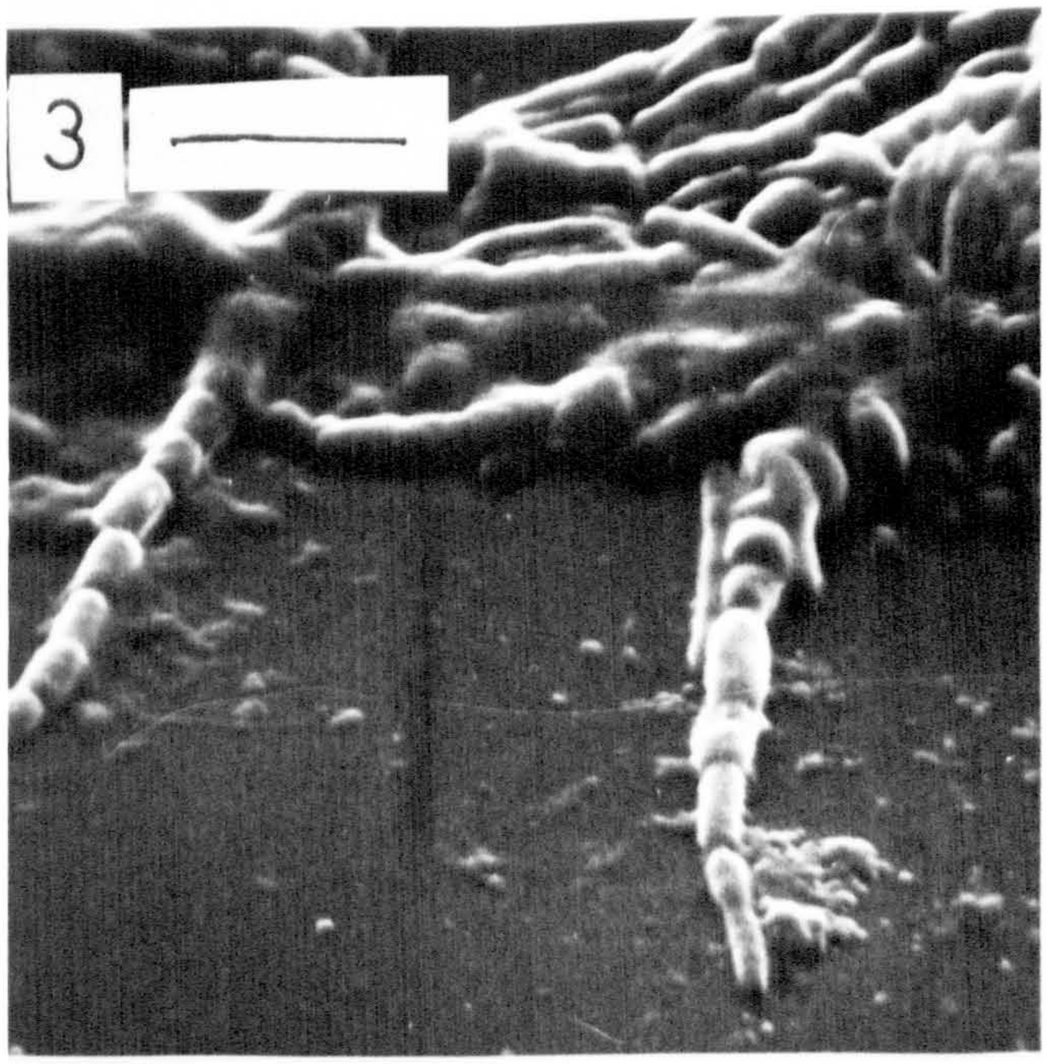
bar in figs. 3,4 & 5 represent 2.7 micron.

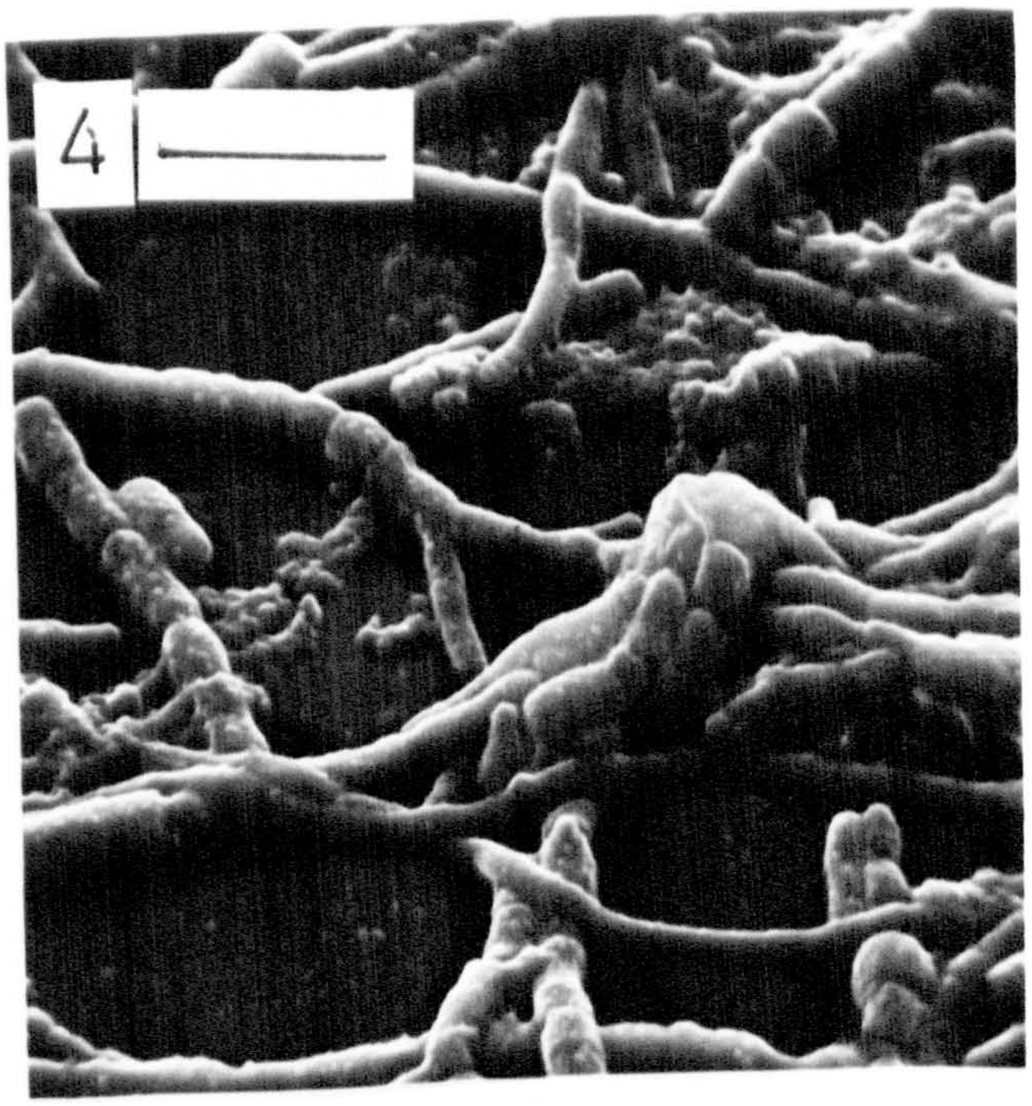
bar in fig 6 represents 1.2 micron.

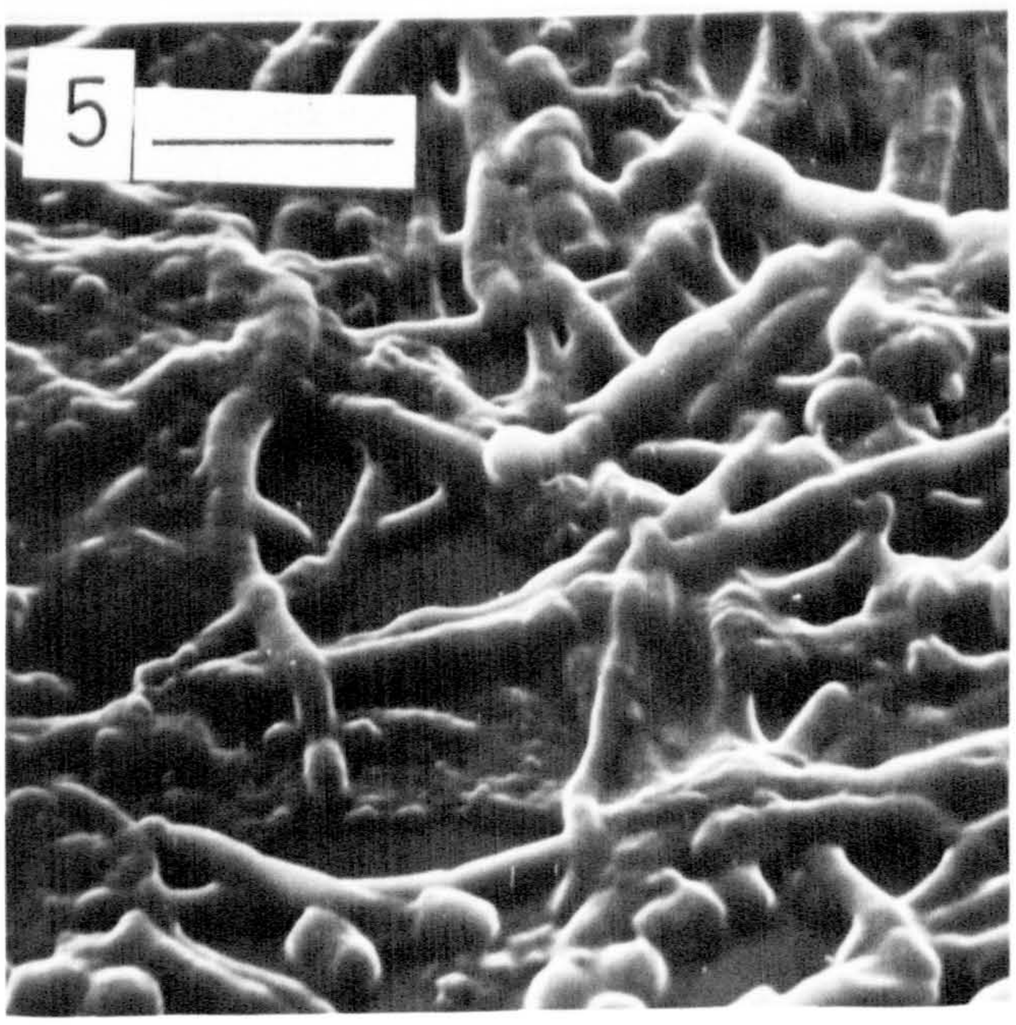
PLATE 5











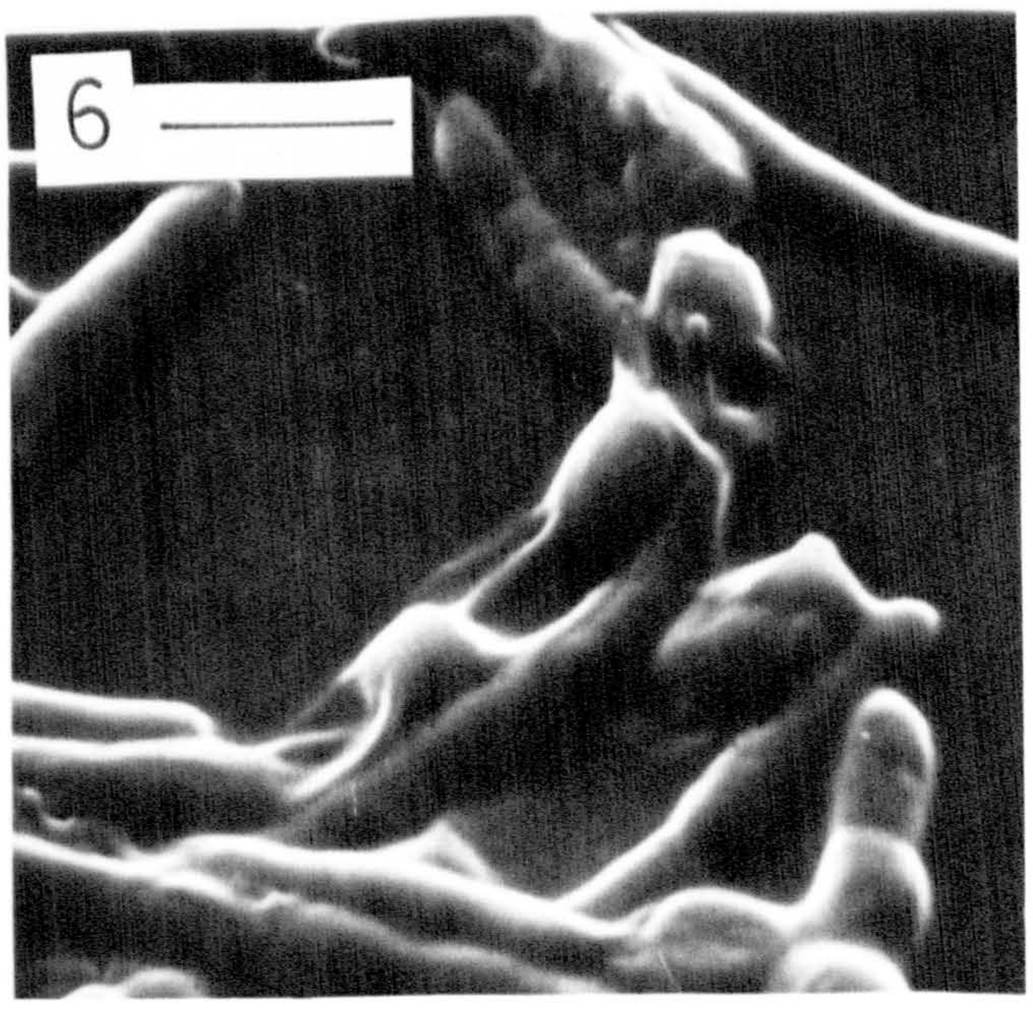


PLATE (6)

Fig.(1) Three days old production mycelium of 72T1 showing spore-like structure start to germinate

Figs.(2&3) Four days old production mycelium of 72T1 showing spore-like structure.

Fig.(4) Four days old production mycelium of 72T1 showing entomoform bodies.

Fig.(5) 24 hours old mycelium of Y20 in inoculum medium.

Fig.(6) 24 hours old mycelium of Y20 in production medium showing the gel-like material (arrow).

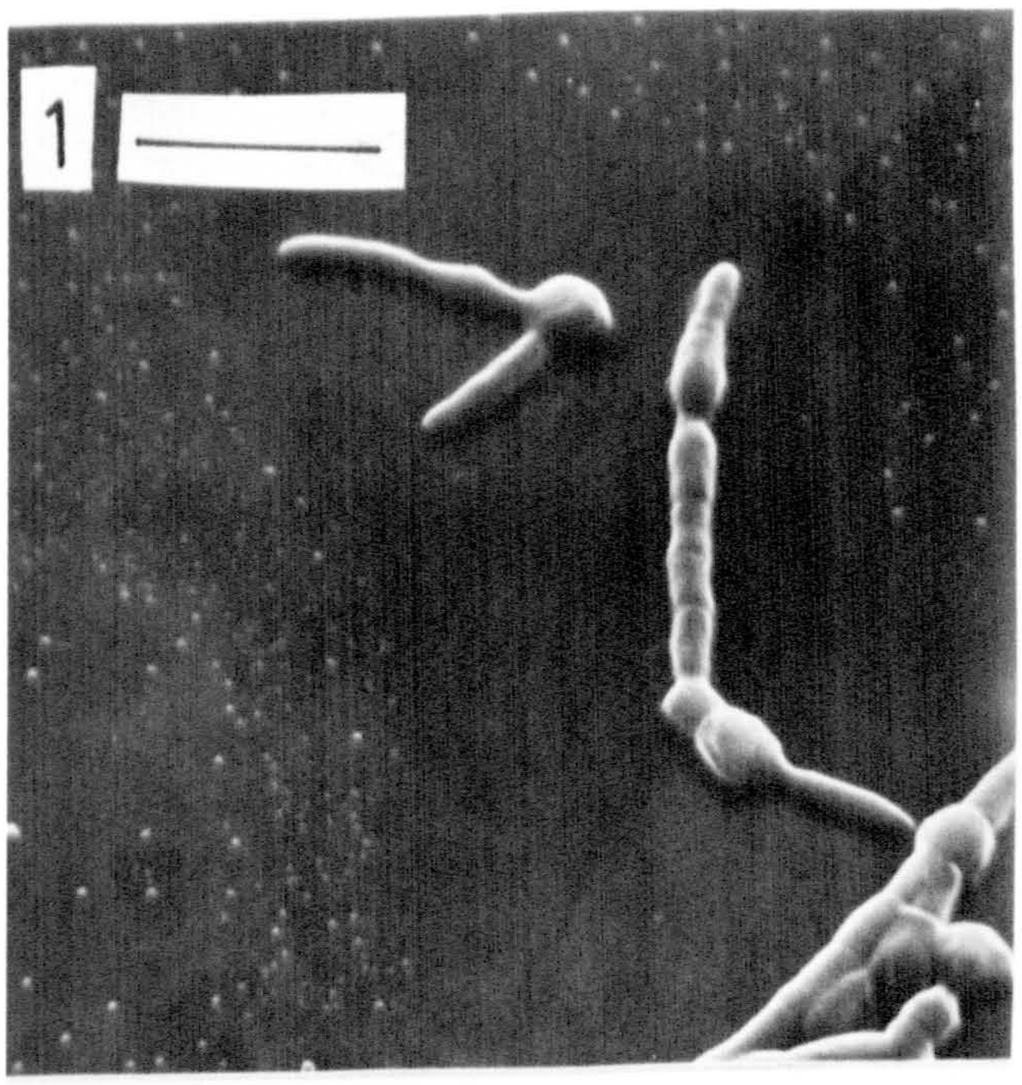
bar in Fig.1 represents 2.5 micron.

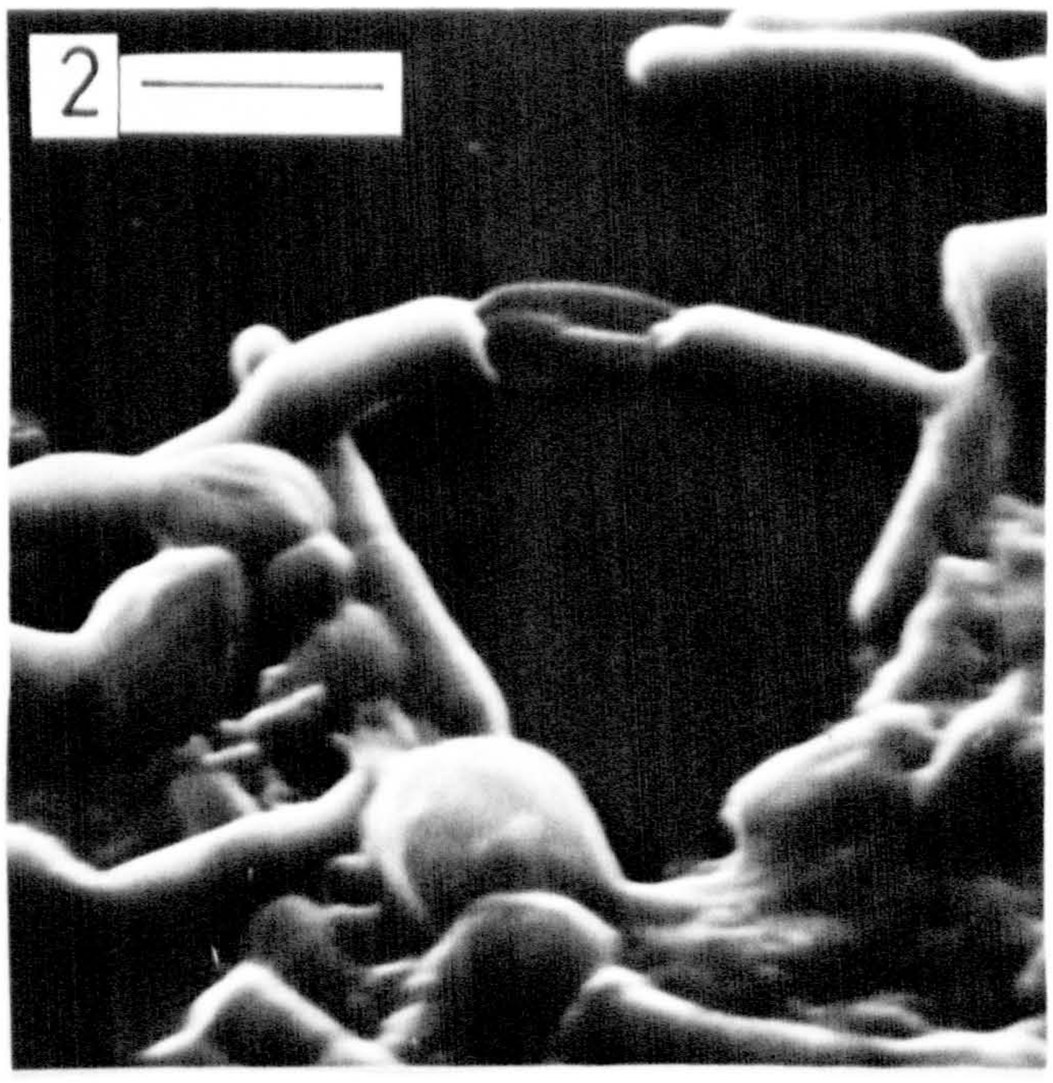
bar in figs 2,3 & 4 represent 12 micron.

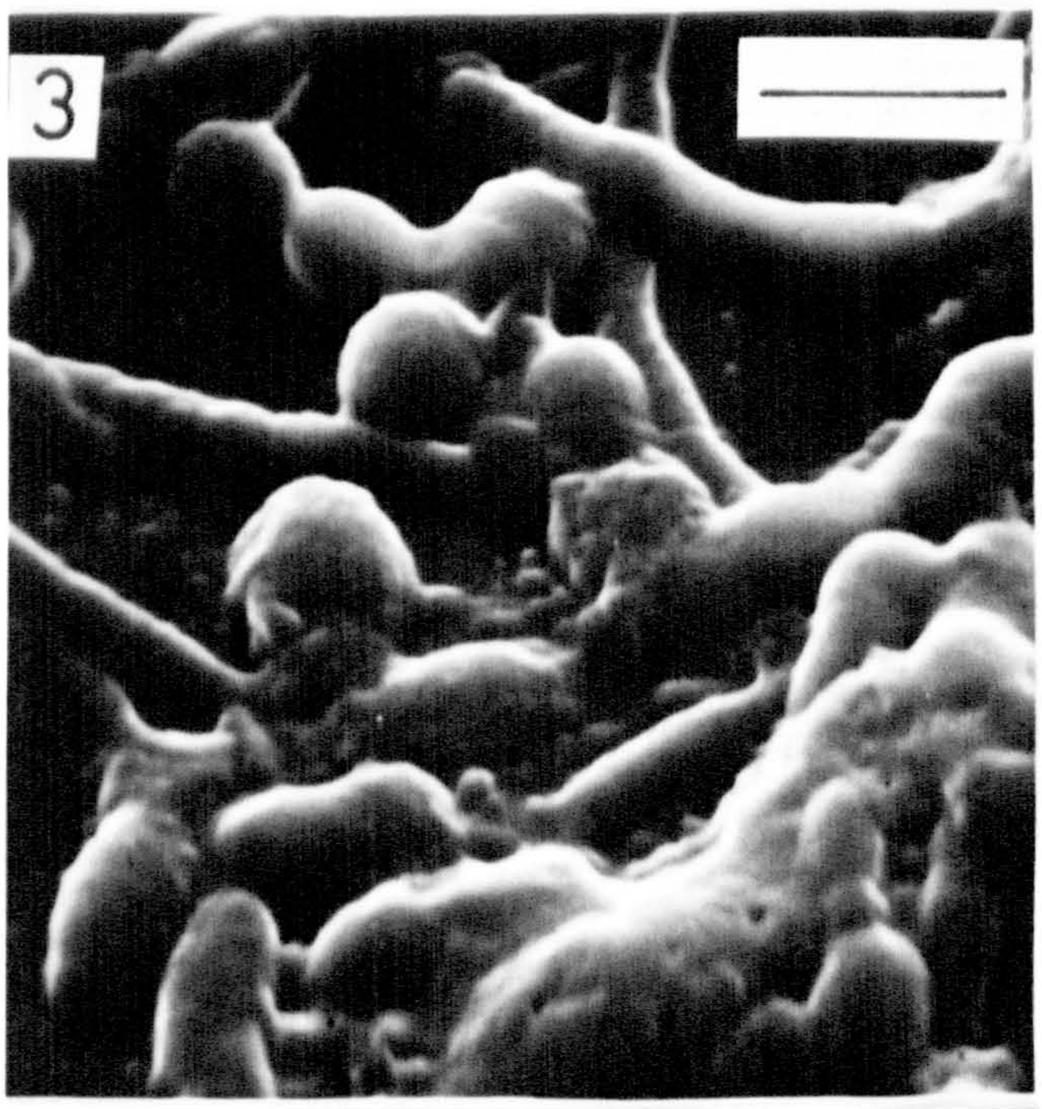
bar in fig.5 represents 1.8 micron.

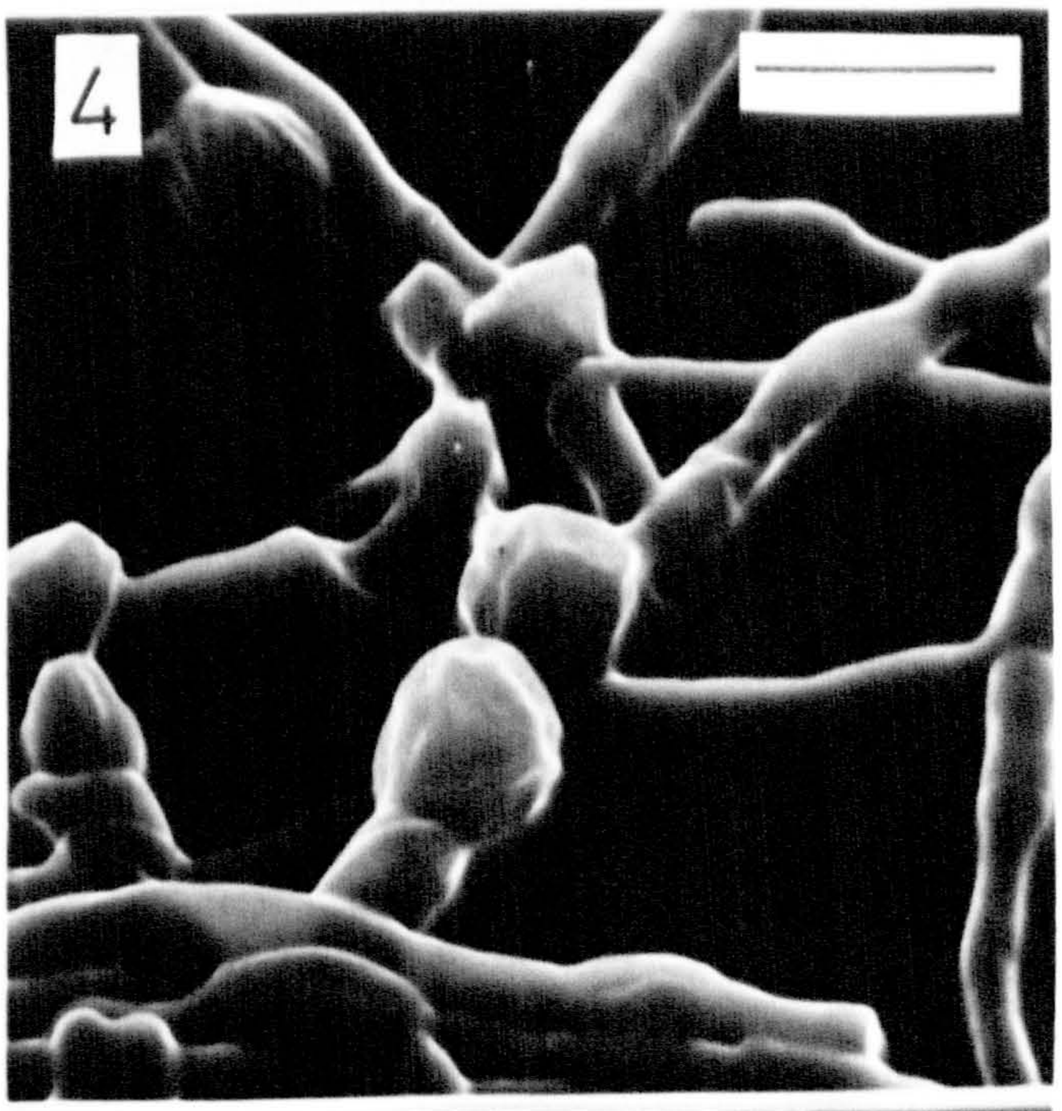
bar in fig 6 represents 20 micron.

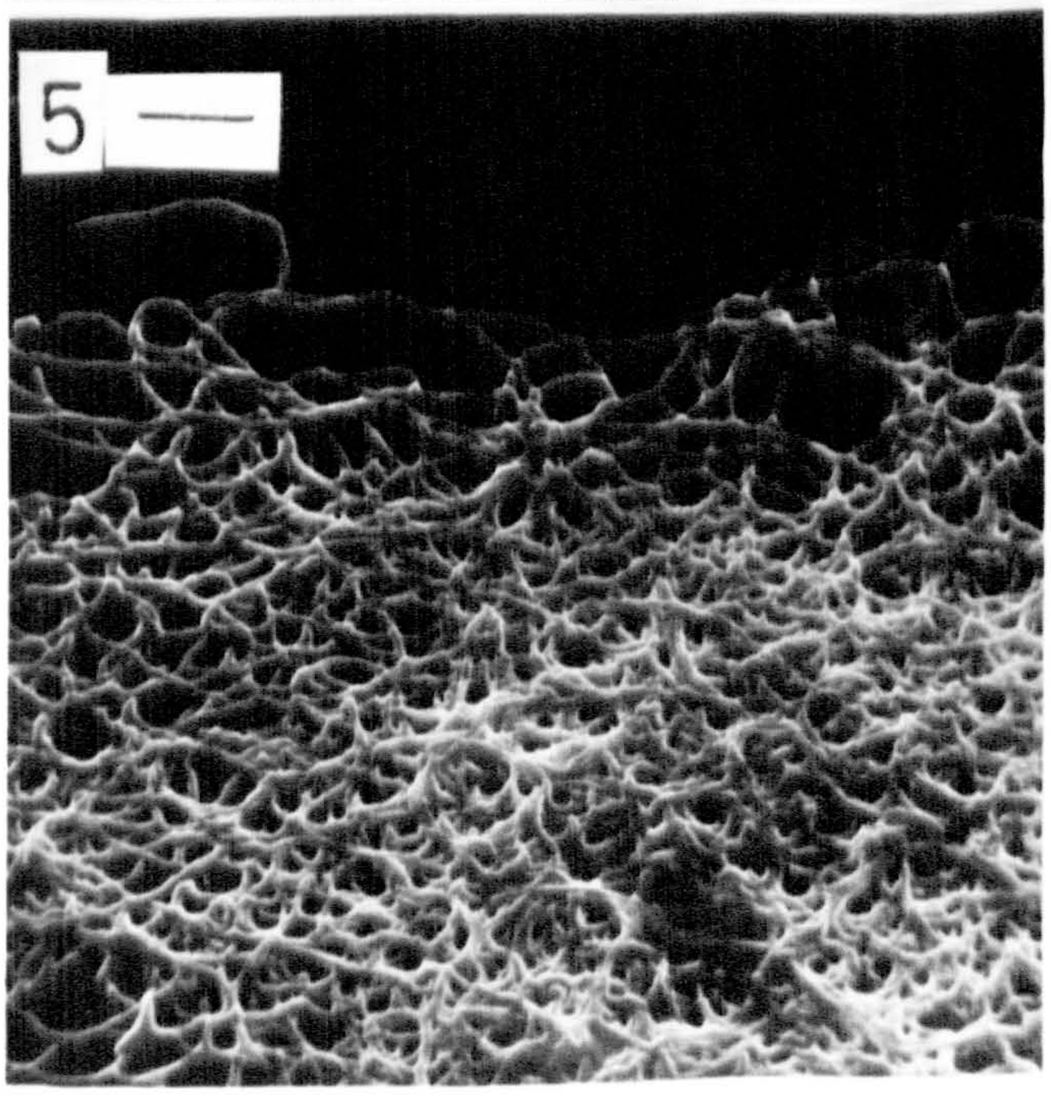
PLATE 6











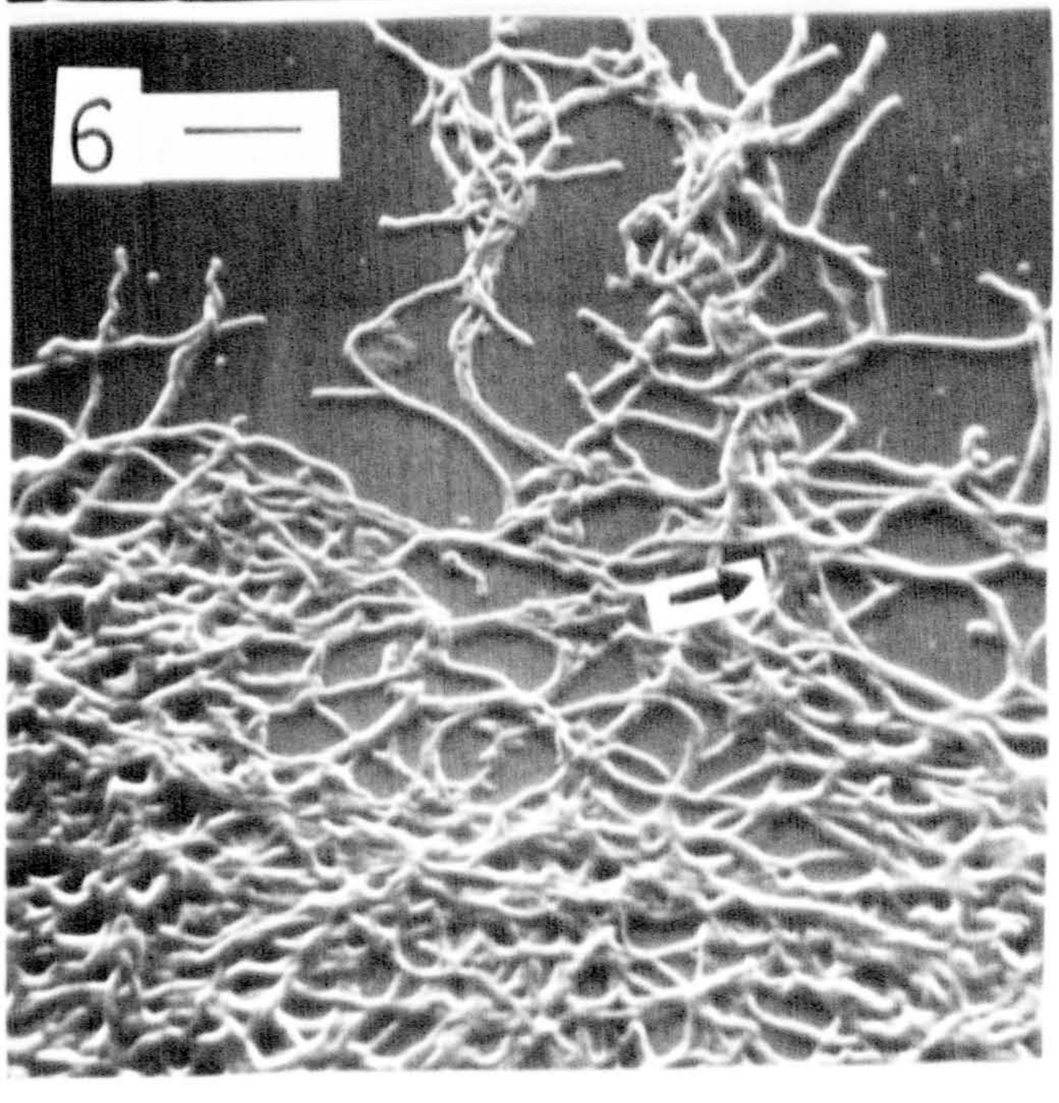


PLATE (7)

Fig.(1) 48 hours production mycelium of Y20 covered by gel-like materials.

Fig.(2) Three days production mycelium of Y20 showing the lessen of the gel material.

Fig.(3) Four days production mycelium of Y20

Fig.(4) Four days production mycelium of Y20

Figs.(5&6) Five days old mycelium of Y20 in production medium. Fig. 5 showed globular bodies.

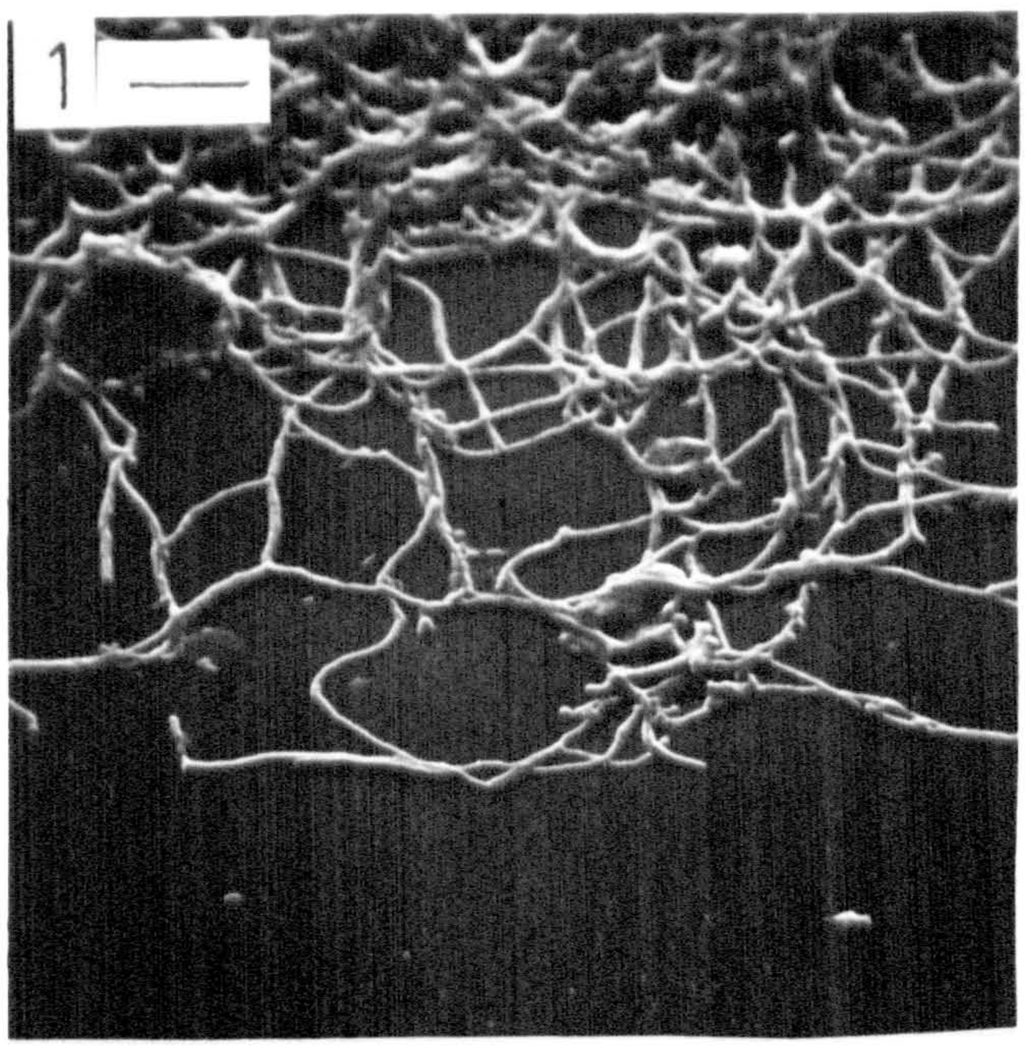
bar in Figs 1 & 2 represents 2 micron.

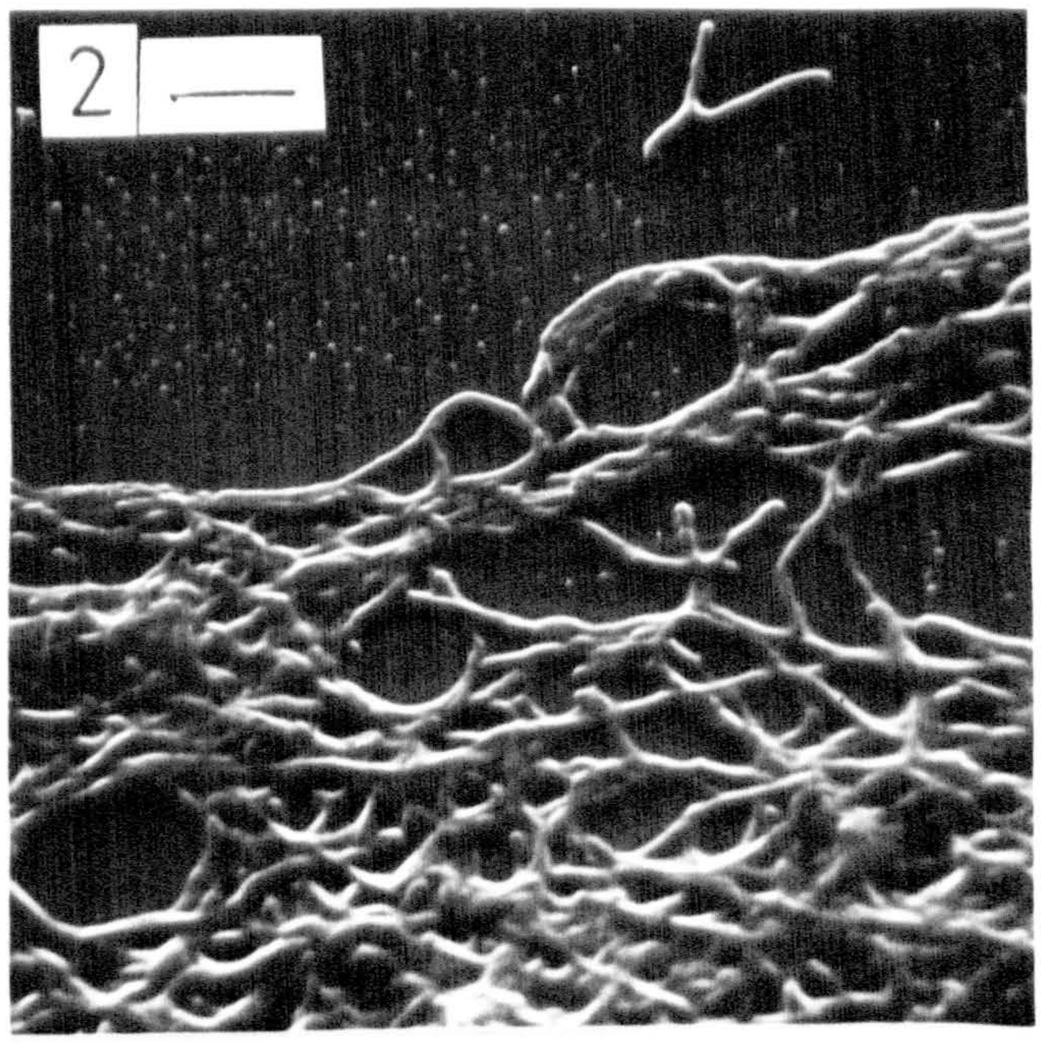
bar in fig 3 represents 10 micron.

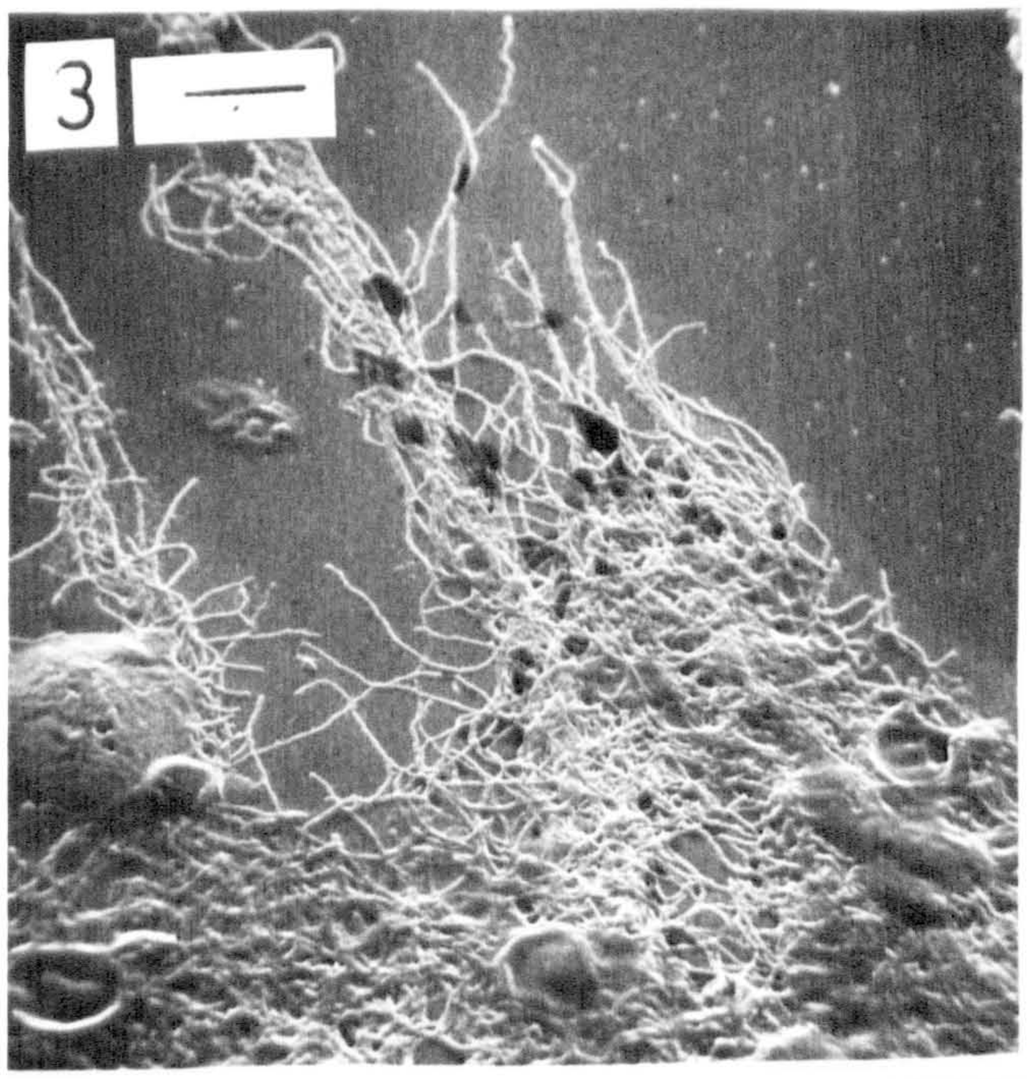
bar in figs 4 & 6 represents 1.2 micron.

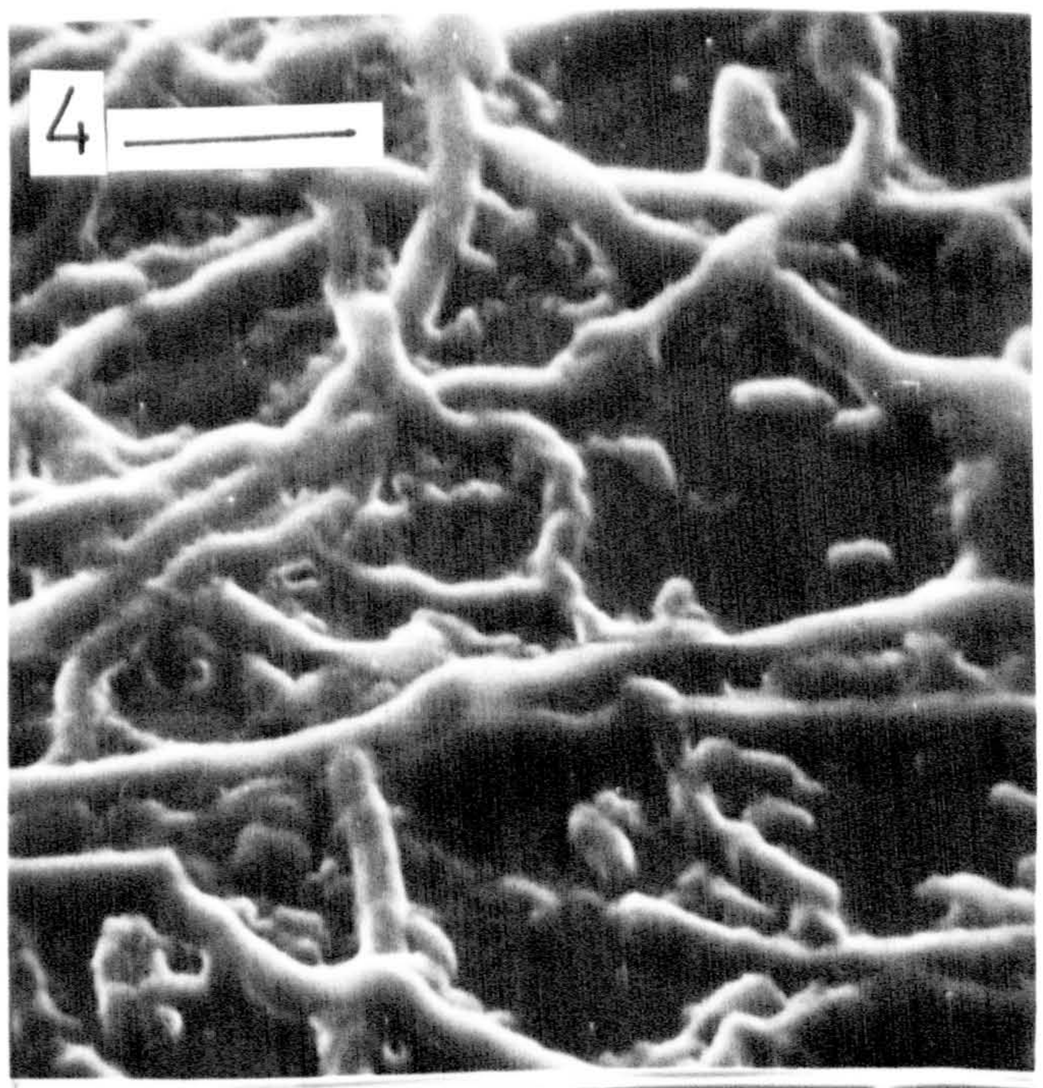
bar in fig 5 represents 6 micron.

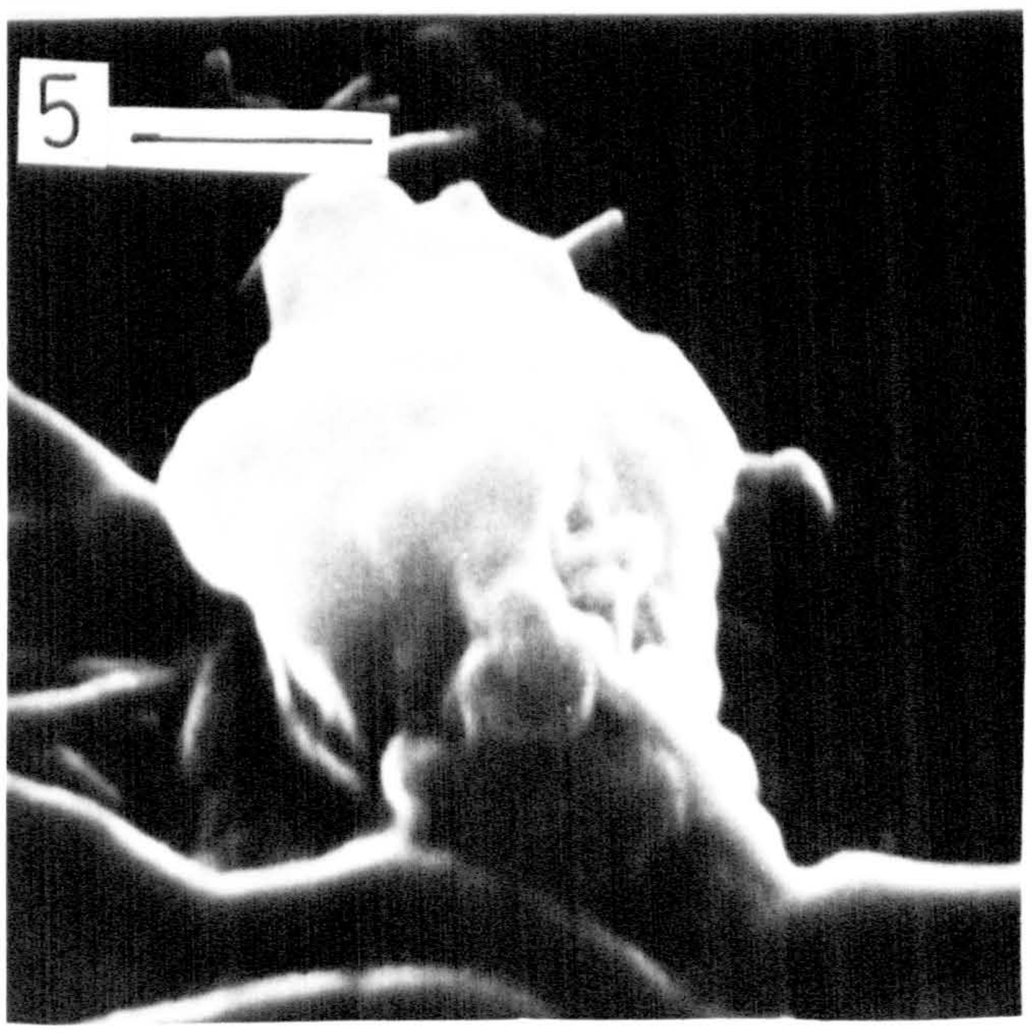
PLATE 7











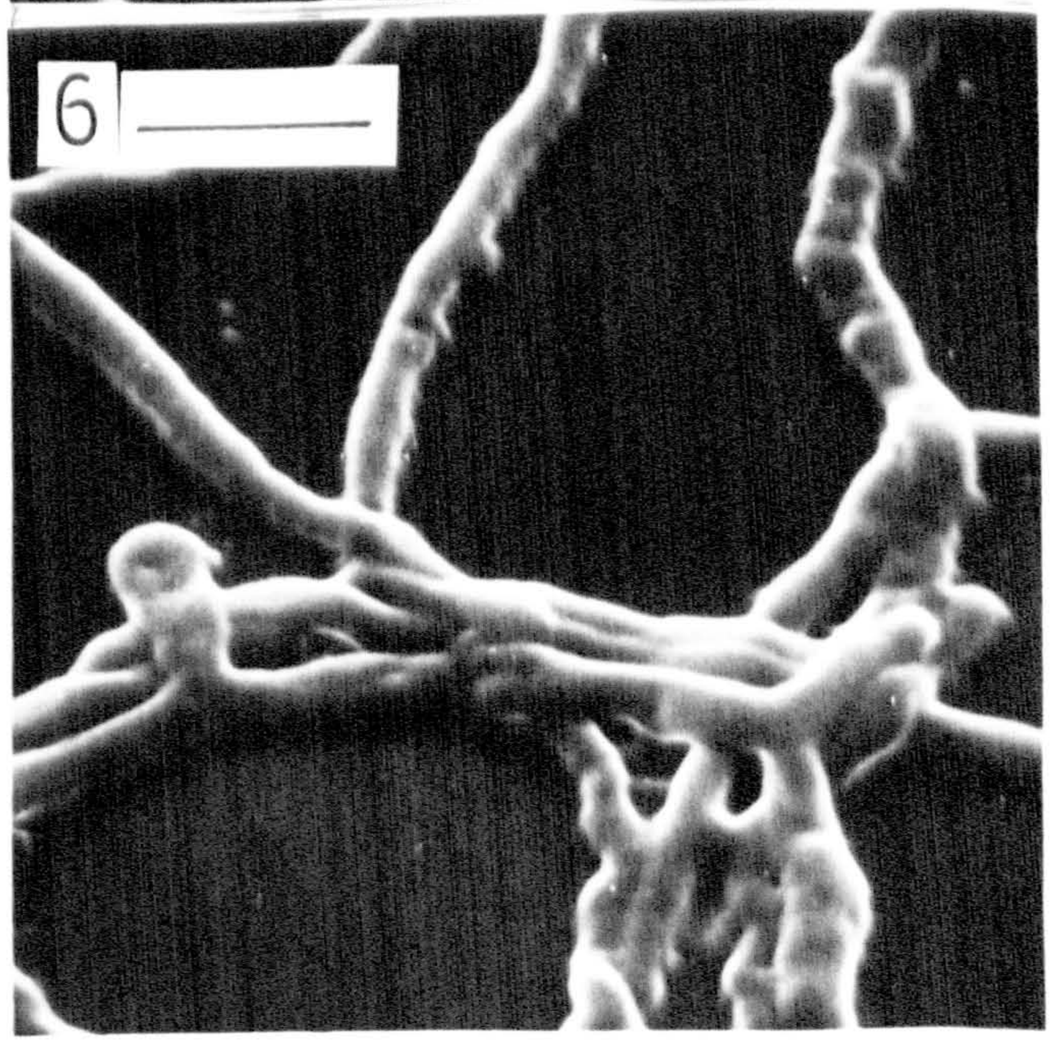


PLATE (8)

Fig.(1) 24 hours old inoculum mycelium of D9 covered by the gel material.

Fig.(2) One day old production mycelium of D9 showing the lessen of the gel material.

Fig.(3) Two days old mycelium of D9 in production medium showing the gel-like material around the mycelium.

Fig.(4) Three day old production mycelium of D9 showing the lessen of the gel-like material.

Figs.(5&6) Four days old mycelium of D9 showing the dissappearence of the gel-like material.

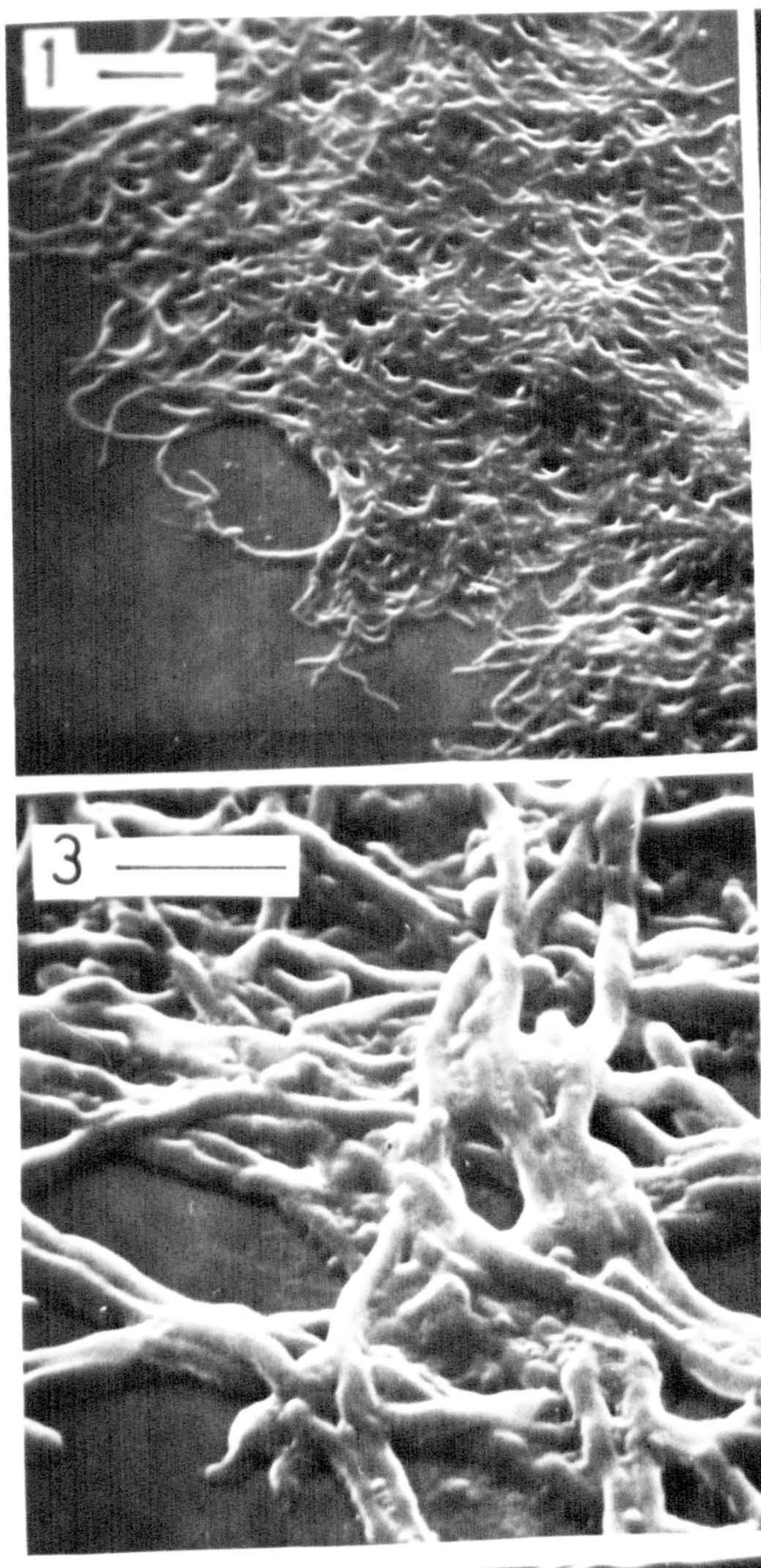
bar in figs 1 represents10 micron.

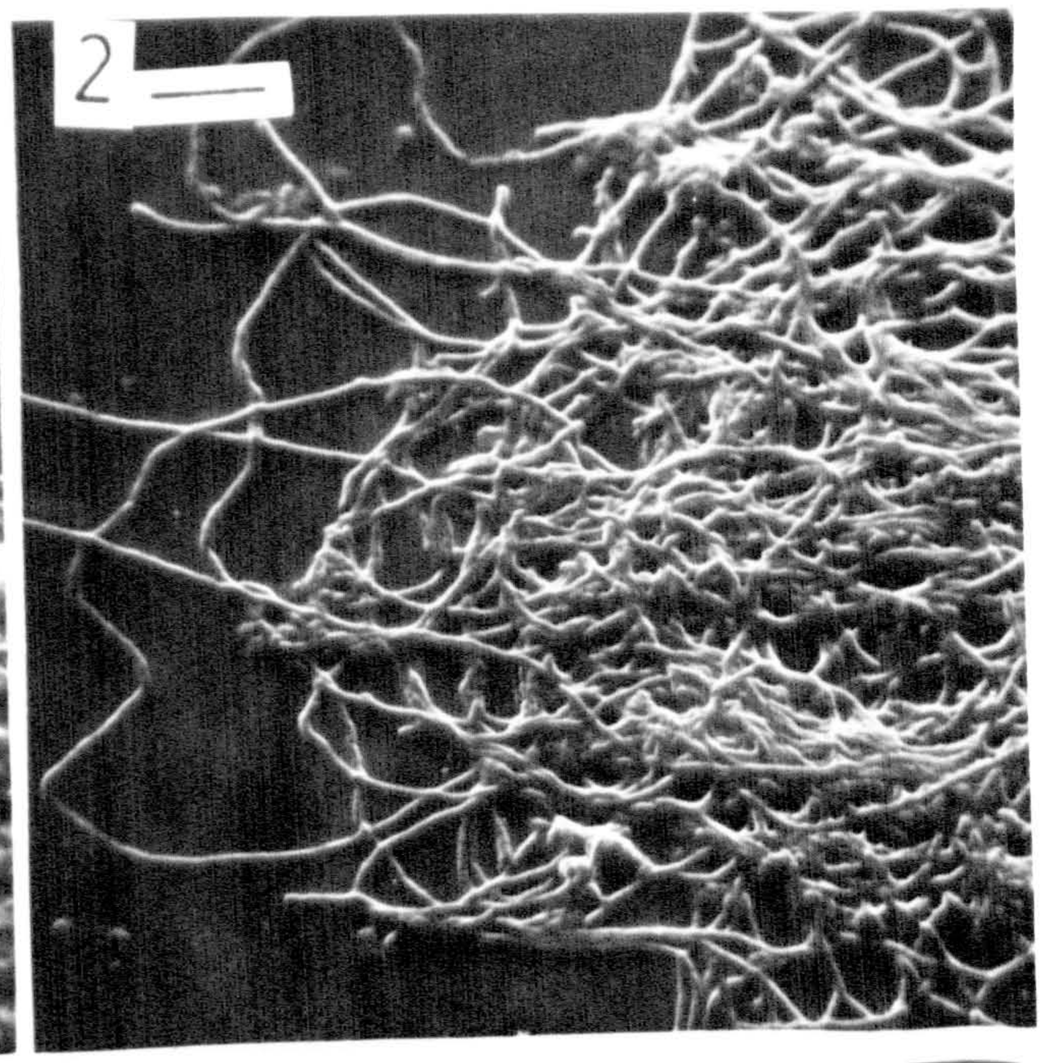
bar in fig 2 represents 20 micron.

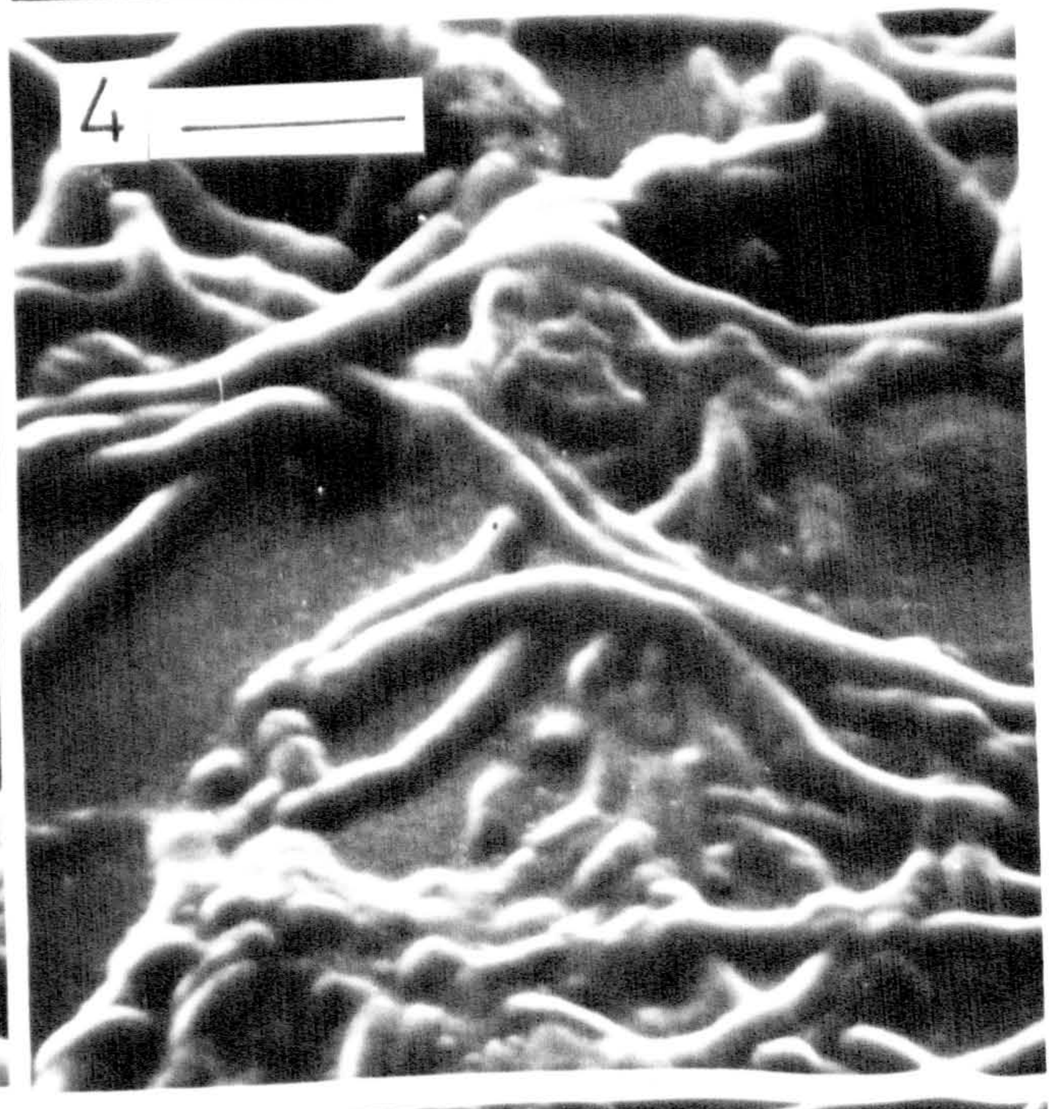
bar in fig. 3&4 represents 1.2 micron.

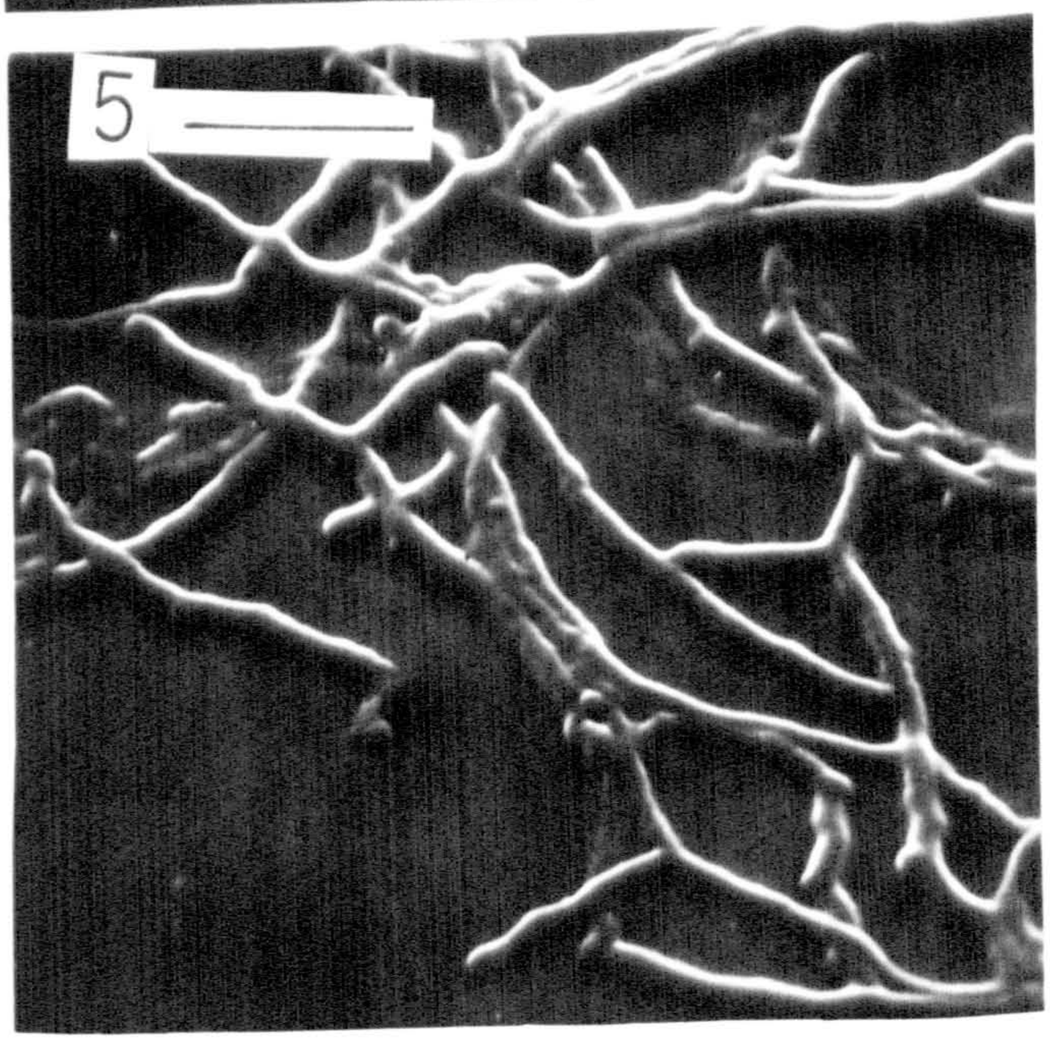
bar in fig 5 &6 represents 2.5 micron.

PLATE 8









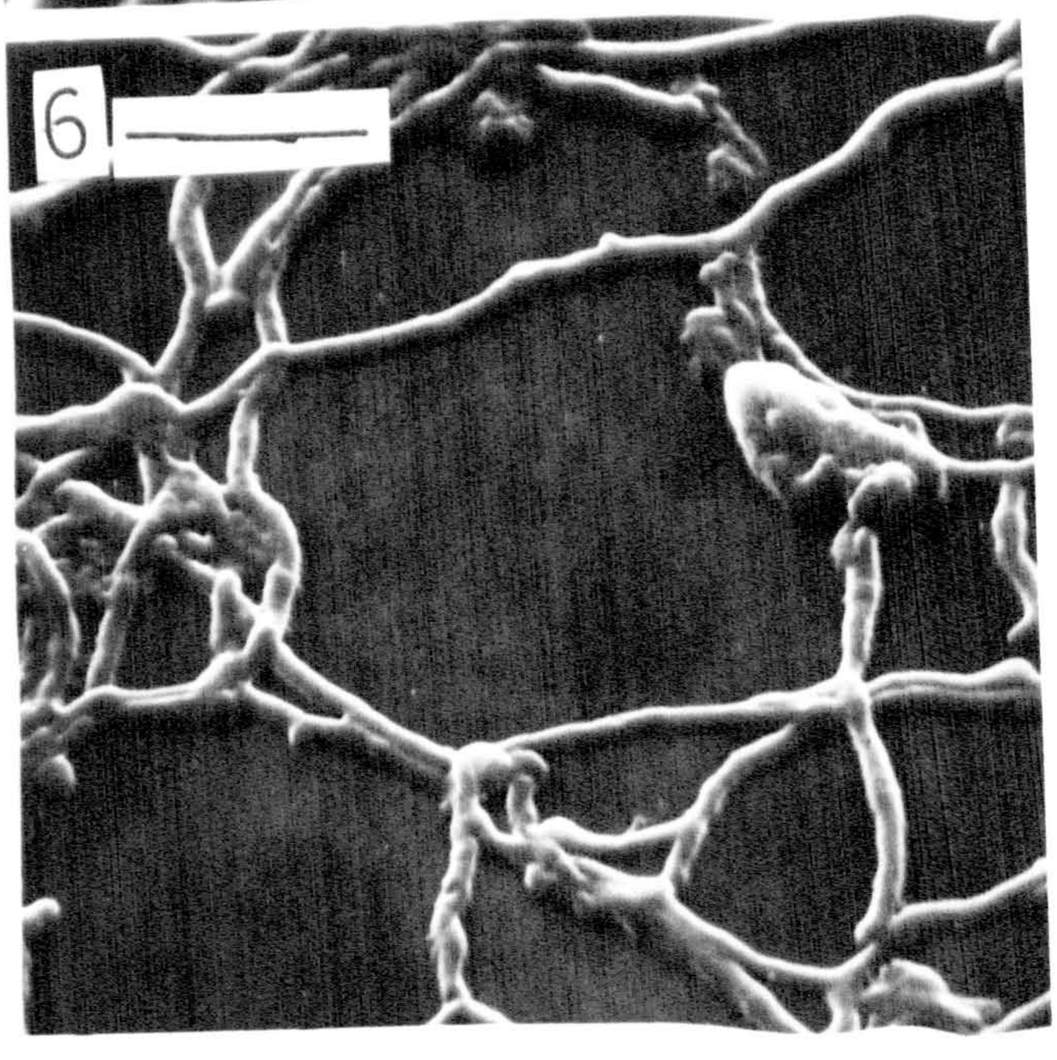


PLATE (9)

Fig.(1) 24 hours old inoculum mycelium of S.rimosus NRRL 2234 showing gel-like material and crystals.

Fig.(2) One day old mycelium of S.rimosus in production medium showing some gel-like material.

Fig.(3) Two days old production mycelium of S.rimosus

Fig.(4) Three days old production mycelium of S.rimosus showing small branches.

Fig.(5) Four day old production mycelium of S.rimosus showing bacterial-like structures.

Fig.(6) Light microscope of four days old production mycelium showing bacterial-like structures.

bar in figs 1 & 3 represents 20 micron.

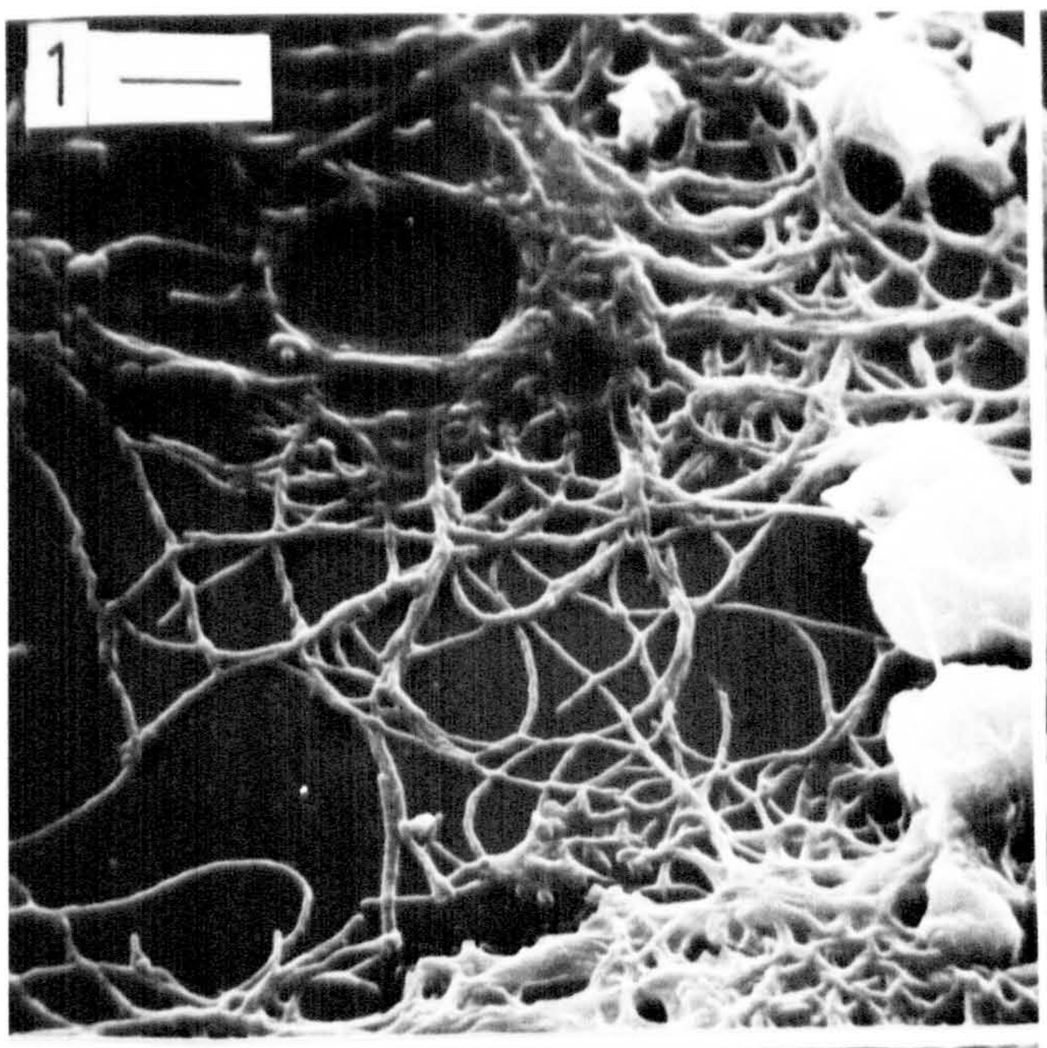
bar in fig 5 represents 20 micron.

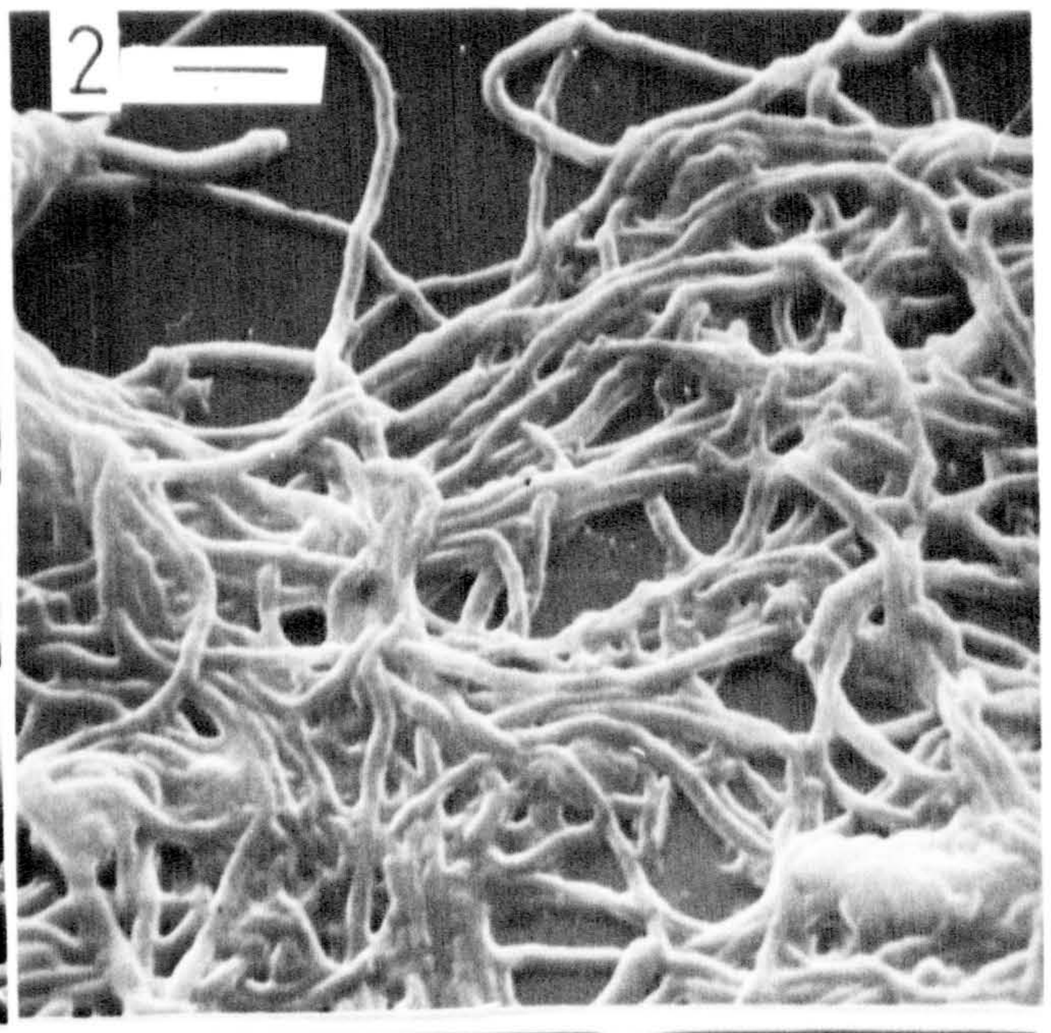
bar in fig 4 represents 6 micron.

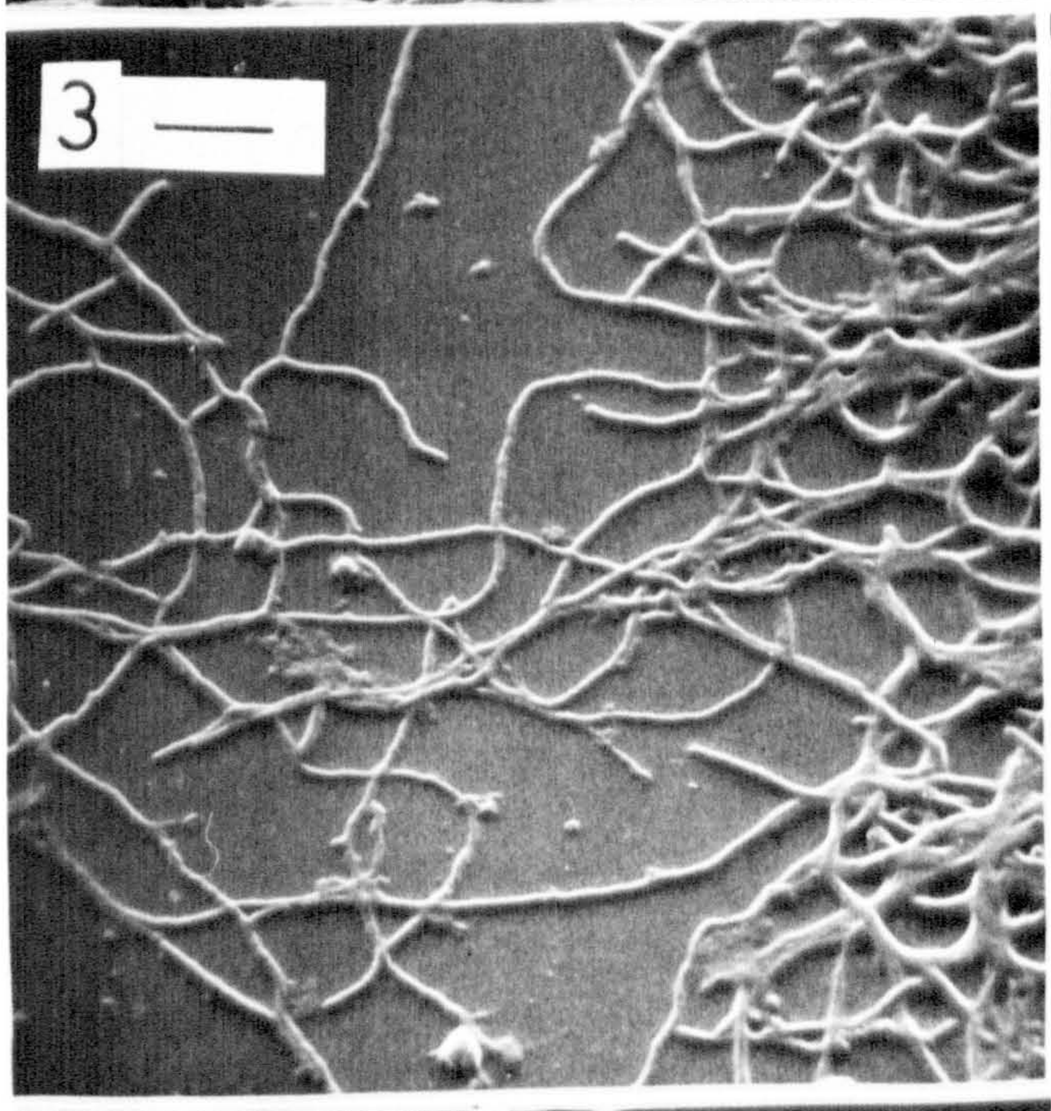
bar in fig 2 represents 8 micron.

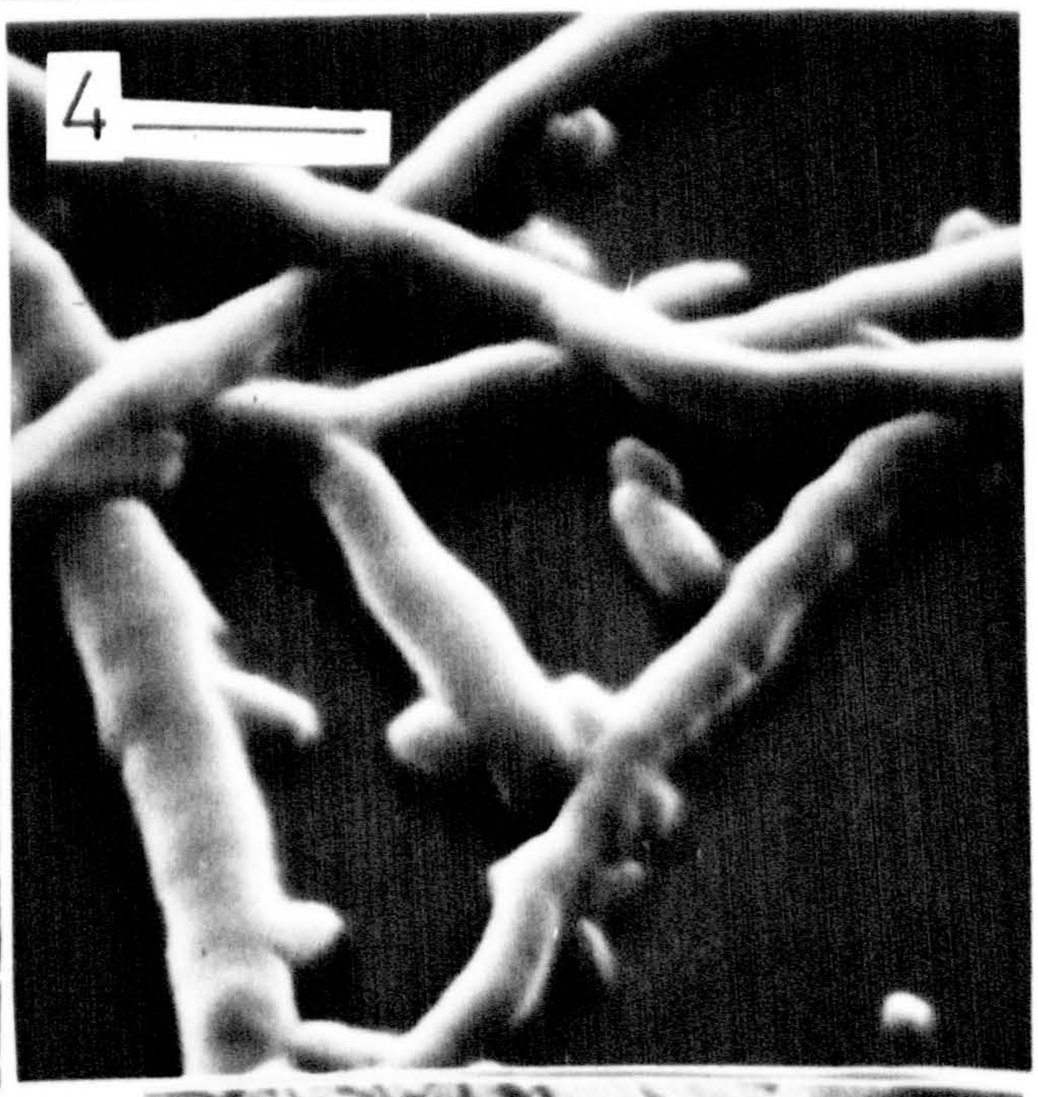
bar in fig 6 represents 10 micron.

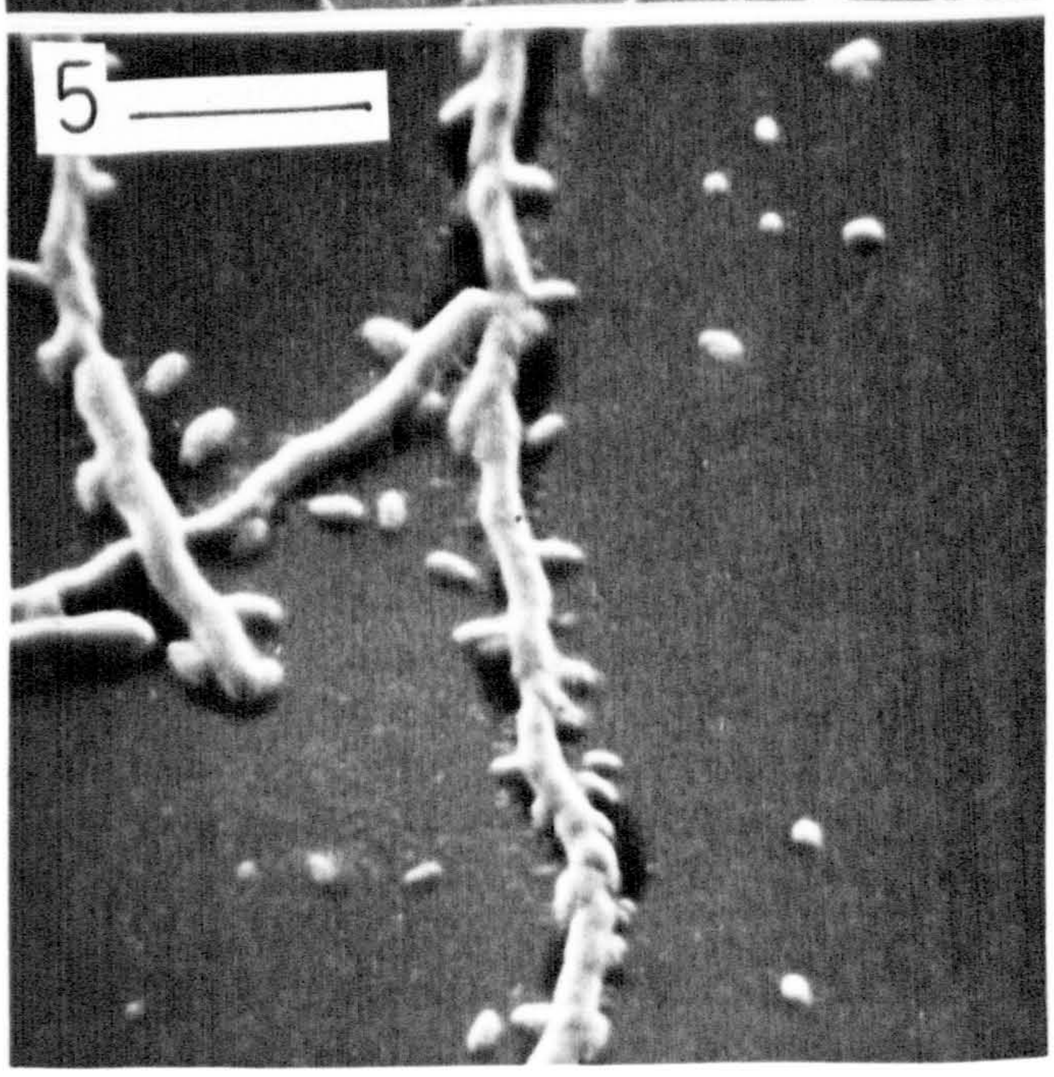
PLATE 9

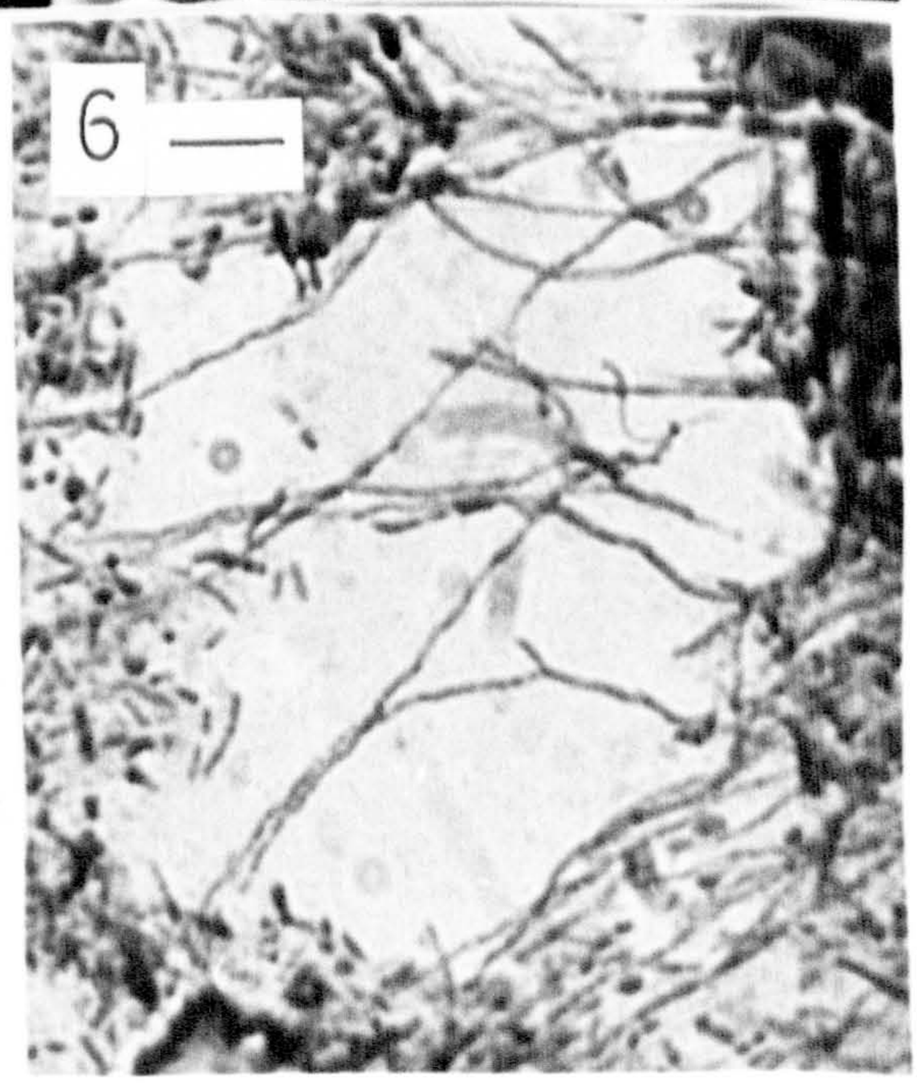












4. DISCUSSION

In the present study the results obtained showed significant changes had occurred between low producers i.e. wild strains and blocked mutants, and the producer mutant strains. The most important feature was the growth pattern in submerged culture. The wild strains showed a faster initial growth, which slows down often after the first day of incubation, especially in stirred culture. This rapid growth was accompanied by a high CO_2 production, followed by a decline after 48 hours. The production mutants on the other hand produced more cells than the wild strains and produced more OTC. They also showed steady respiration. In this connection, with Y2O, less cells were produced in shaken culture and the respiration rate was less smooth than in stirred culture. Also less OTC and more acetyl-OTC were produced in shaken cultures, than stirred cultures.

For a good yield, a good and steady growth is required. The results showed that the mutants were better adapted to stirred culture conditions as they grow better there than in shaken culture.

In general the wild strains grow in an uncontrolled manner, while the mutants grew steadily and efficiently in both stirred and shaken culture.

One feature in which the wild and mutant strains differ was the greater sensitivity of the wild strain to OTC. Antibiotic producing microorganisms are usually resistant to their own antibiotic (Demain 1974; Vining 1979). A number of mechanisms are responsible for this resistance, in particular the presence of a system which operates on the ribosomes or other areas (Mikulik et al 1971; Sugiyama et al 1980; Crameri et al 1983). It is considered that in S.rimosus mutants

resistance to OTC was due to a similar kind of modification, to make the target area in the cells resistant to OTC. The effect of resistance showed up particularly in high yielding industrial strains.

The important point brought in the present study was the different degrees of sensitivity of the strains to OTC. To obtain a high yield of OTC it is essential to obtain highly resistant strains. This can be done by mutation and selection, as with Y20, which was obtained from a very sensitive strain (i.e. 72T1), but it seems to be difficult to increase resistance of the mutants by this method beyond a certain point.

The best approach is the introduction of resistance by cloning, especially as it is known that this can be done practically. The resistance of a strain to antibiotic is due to particular enzymic functions. Thus a measure of resistance to neomycin or viomycin in the producer strains is conferred by enzymes which phosphorylate or acetylate the antibiotics (Davies and Smith 1978). In the case of S.aureofaciens, chlortetracycline is known to accumulate in the ribosomes, and interferes with protein synthesis (Mikulik et al 1971). The enzyme responsible for the dimethylation of adenine and the resistance of S. erythraeus to erythromycin has been isolated (Skinner and Cundliffe 1982). Thompson et al (1980, 1982, a,b,c) have cloned genes of this type into Streptomyces using plasmid vectors (thereby demonstrating rigorously their involvement in conferring drug resistance), and a start has been made with their use as probes to detect the location of the genes in various fractions of the DNA of the organism from which the genes were originally isolated. (For a detailed account of the technique and application of DNA cloning in Streptomyces see Hopwood and Chater, 1982). The antibiotic inactivating enzymes in Streptomyces are not necessarily confined to the producers of the relevant antibiotics, as shown by Shaw and Hopwood (1976), in the case of chloramphinicol producing strain

S. venezualae. Because resistance is associated with single, known, location, cloning is particularly suitable for strain improvement.

Methods of gene cloning systems have now been developed for Streptomyces, using both phage (Suarez and Chater 1980) and plasmid vectors (Bibb et al 1980, Hopwood et al 1981) and it has been established that interspecific cloning is possible. The normal procedure involves the use of plasmids as vectors to transfer doner DNA to the recipient strain in the form of protoplasts, Baltz (1981). The chromosome map is known (Friend and Hopwood 1971; Rhodes et al 1981) and the method of protoplast fusion in S. rimosus has been described by Hranueli et al (1983).

Rhodes et al (1984) have recently shown that neither of the endogenous sex-factor plasmids SRP1 and SRP2 in S.rimosus, proved amenable to physical isolation or analysis, so a restricted varient of plasmid pIJ 303 (i.e. pIJ 303/Z12), which carried the S.azureus gene for thiostrepton resistance were used and transformed to S.rimosus. The plasmid pIJ 303 was one of a series of broad host range plasmids recently described by Kieser et al (1982). This plasmid integrated with the section of the chromosome of S.rimosus responsible for intermediate metabolism, and it also transferred resistance to OTC from industrial strain to non resistant mutant showing that the resistance gene must lie in the same area. A practical problem might be the availability of genes with conferring a sufficiently high level of resistance; these might be found by testing mutants for resistance which were not themselves highly productive, or, some highly resistant strains of S.aureofaciens might be available, another possibility is the introduction of a second resistance gene, by repeated cloning. A gene giving resistance to OTC might also be found in another species of Streptomyces (cf. Shaw and Hopwood, 1976).

Hopwood (1981) has pointed out that from an industrial standpoint, gene

cloning within Streptomyces offers the most direct route to assorting parts of antibiotics into new combination, thus potentiating the development of new products, it should also allow the introduction of genes allowing growth to cheap growth media, and the application of increases for existing products.

In the present study enzymic activity such as citrate synthase and MDH were found to be more active biosynthetically, and therefore the level of citrate and oxalacetate might be at higher levels in the mutants. Acetyl-CoA carboxylase, which is the key enzyme for fatty acids synthesis and the formation of malonyl units, was found to be most active in shaken cultures when mutants gave the best OTC production. Runs repeated with these mutants sometimes showed low OTC production and these also had low Acetyl-CoA carboxylase activity corresponding to the pattern shown by the wild strain. This suggested that malonate formation by the organism at the beginning of the cultivation could be essential to initiate the mechanism for OTC production.

ATC oxygenase, which acts in the central area of OTC biosynthesis (template), was found to be about the same in both of the wild and mutant strains. The cosynthesis results with blocked mutants showed that the production of OTC was independent of the formation of the brown colour, which suggested that the mutants were blocked in the area of intermediate metabolism or early in the OTC template prior to methyl pretetramid. This also suggests that the formation of the brown colour (probably aureovocin) represents a loss of material diverted from OTC formation. It is of interest that the genes involved in this area of metabolism were found by Rhodes et al (1981) to be in a single area of around 3 o'clock on the circular chromosome and that they thus seem relatively susceptible to mutation.

The morphology of the strains in submerged culture showed that certain features were common in the high producers, such as the formation of "open" mycelium throughout the cultivation. Some other features were common in the wild strains such as the formation of spore like structures or coccoid units suggesting a more primitive pattern of growth.

The taxonomic studies brought out several points of interest. Using the identification test system, the wild strain 72T1 was placed in a different cluster from the producing mutants, i.e. cluster 29 S.lydicus, though with a high taxonomic distance. This agrees with studies of morphology which showed that 72T1 was more like S.aureofaciens than S.rimosus, while all the producing mutants were in the S.rimosus cluster(42).

The blocked mutants were unidentified in the Williams programme and occurred separately from Y20 when the clustering technique was applied. These results stress the extreme effect of what appeared to be point mutations.

Although the results were generally in line with expectations, they showed a number of loose ends, especially in the placing of some strains eg. G65. in different places in different tests, and the inability of the computer system to identify three blocked mutants. These results suggested that the situation was still incomplete, but it is likely that these differences will be resolved by further research.

Considerable differences were obtained in the present study compared to the Czech workers these were summarised in Table 21.

Table 21 Comparison between the present mutants and the Czech high yielding mutant

Factor	Czech work	present work
1) Growth pattern	growth repression	grow better than the wild strain
2) Acetyl-CoA	Lower	Higher, especially at the beginning of high producers runs in shaken cultures.
3) PEP-carboxylase	Higher	About the same as the wild strain
4) ICDH, MDH Citrate synthase etc.	Lower	Higher
5) ATC-oxygenase	Higher	About the same as the wild strain
6) Level of adenylate	Higher, which is alleged to inhibit citrate synthase	Ditto, though the total level was too low to cause enzyme inhibition

The main difference observed is that while the results obtained by the Czechoslovakians showed an apparent correlation between the patterns of increased enzyme activities and probable biosynthetic routes, no such correlations occurred in the present work. This parallels the results of Whitworth and Ratledge (1975 a,b), who studied fat production in yeasts and fungi. With the first organism tests, Candida sp. 107, high enzyme activities were observed, associated with pathways likely to be involved with the production of large quantities of fat. With other organisms, however, no such correlations were observed, and in some it was unclear where the necessary NADPH was coming from. In fact, the enzymes measured in connection with the production of chlor- and oxytetracycline were non-specific, and there is no reason why a correlation should be expected. Regulatory systems are more likely to be the key factor in the complex biosynthetic network that must exist.

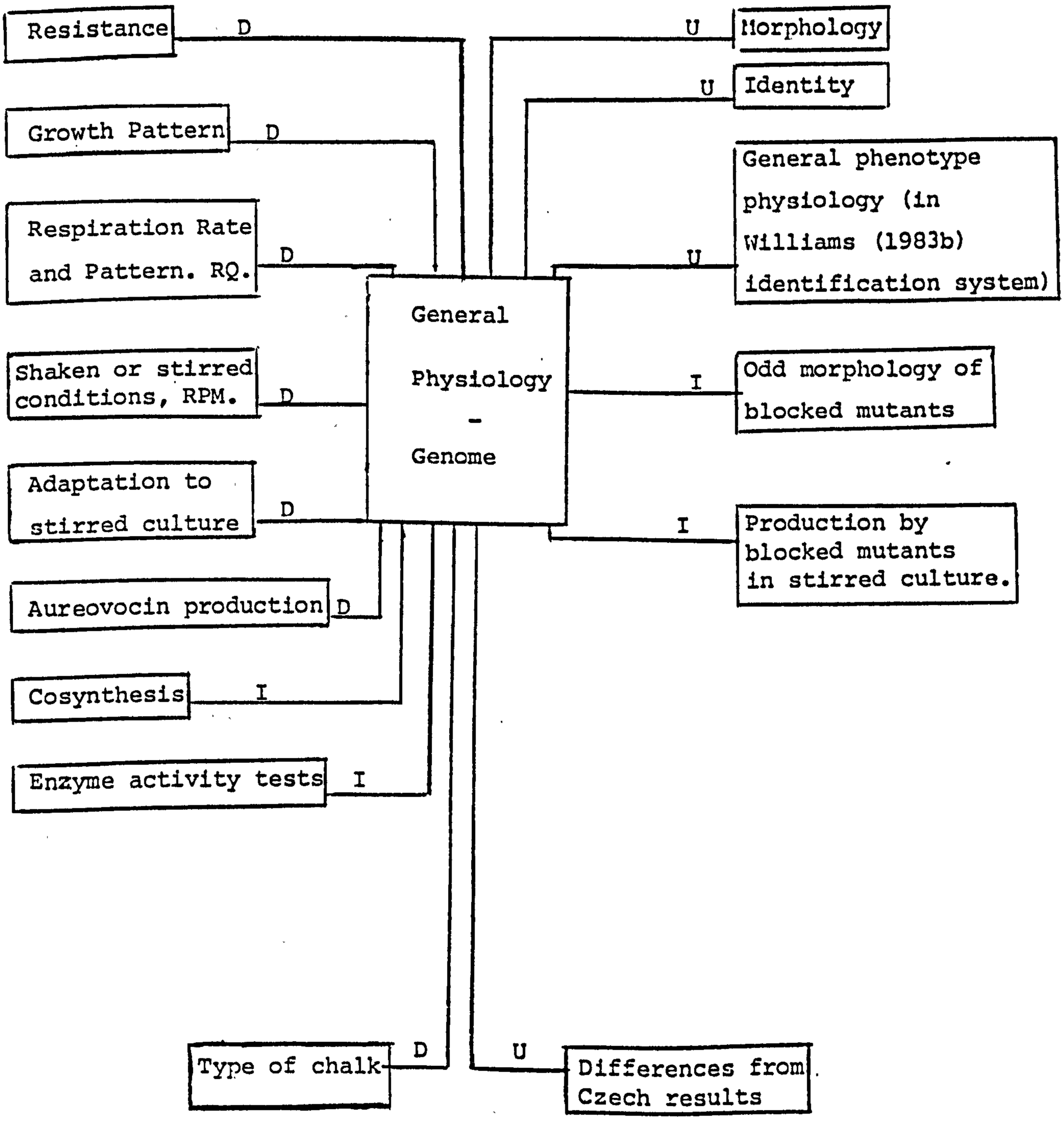
Observed results do point to the existence of poor regulation of the wild strains. Both the Czech mutants and the mutants in the present study gave more controlled growth and metabolism. For example in the Czech work, reduced enxymic activity in the mutants controlled growth and led to increased production in the same way that addition of benzylthiocyanate limited glycolysis and produced the same effect (Hostalek 1964). With the present mutants, growth was increased and the enzymic activity was higher, but there was also a change to a better pattern of growth, implying better overall regulation of the system so as to give increased production.

A point emerging from the present study is that the metabolic system is a complex one as shown, for example, by its sensitivity to the quantity and quality of the chalk used, and the speed of stirring, also by the way the blocked mutants produced OTC in stirred culture, but not in shaken cultures or on agar.

To try to draw together the information obtained a diagram has been prepared (Fig 28) showing all the aspects studied, which were all linked to the total physiology of the cells. Righelato et al (1968) have shown the high proportion of energy required by cells, and production of OTC must occur against this background of central activity.

Indications are given against the links to central physiology of each aspect of cell behaviour studied, seven were marked as directly involved, four as relatively unimportant and two as interesting but their significance was unclear. All those which were considred to be directly involved are related to growth and respiration and stress that high yields of OTC are only obvious when optimal conditions are met, in this respect the effect of chalk was unexplained, but it is known to be important (Riviere 1977). Presumably, when growth and respiration conditions are good, intermediates are in excess and available for the biosynthesis. Resistance to OTC acts across this pattern and is clearly necessary for efficient growth to continue for any length of time.

Fig. (28) Digagram indicating activities and links with productivity



D = Directly involved

I = Interesting observation, but their significance unclear

U = Relatively unimportant

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A P P E N D I X 1

Batch data: growth, production, enzyme tests

RUN 3

STRAIN Y20

STIRRED CULTURE

AGE HOURS	pH	CO_2	O_2	RQ	protein	OTC g/l
24	7.0				1.3	
48	6.6					
72	6.4	.47	20.0	.52	5.6	
96	6.4	.7	19.5	.5		
120						5 g/l

This run was the 1st which has been done with ICI chalk and with low speed

RUN 4

STRAIN Y20

STIRRED CULTURE

AGE HOURS	pH	PROTEIN g/l	OTC g/l
24			
48	6.2		
72	6.4		4.1
96	6.6		

RUN 5

STRAIN D7

STIRRED CULTURE

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24						
48	6.1				4.1	1.1
72	6.7	0.97	19.85	0.92	7.0	2.4
96	6.4	1.7	18.2	0.63	7.9	4.3
120	6.4				7.5	5.1

RUN 6

STRAIN 72T1

STIRRED CULTURE

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	5.8	2.65	17.6	0.8	1.4	-
48	6.5	1.68	18.5	0.7	2.4	Nil
72	6.8	-	-	-	2.6	Nil
96	6.9	0.46	20.6	1.53	2.4	0.5
120	6.8	-	-	-	2.4	0.5

on this run 23 hours inoculum have been used to inoculate the production medium and as a result of that very poor growth took place and very low OTC which appeared on the broth after 72 hours.

RUN 7

STRAIN Y20

STIRRED CULTURE

ICI 1964 production media have been used in this run, but the amount of C.S.L. have been reduced to 20 g/l and the amount of $CaCo_3$ have been increased to 18.5 g/l.

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	6.6	0.9 0.87	20.1	1.08	2.25	Nil
48	6.8	1.25	-	-	6.15	Nil
72	6.1	1.5	-	-	7.5	1.5 2.0
96	6.1	0.97	-	-	9.0	4.4

As a trial run for the test of the effect of the amount of C.S.L. and $CaCo_3$ has on the amount of OTC produced by Y20 and to compare it with run 8 which has lower C.S.L./1 and higher CaCo3/1.

RUN 8

STRAIN Y20

STIRRED CULTURE

In this run the amount of C.S.L. in ICI (1964) production media have been reduced to 15 g/1 and the amount of $CaCo_3$ have been increased to 23.3 g/1.

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/1	OTC g/1
24	6.8	1.78	19.4	1.18	2.85	Nil
48	6.8	1.52	-	-	5.9	1.9
72	6.2	2.4	-	-	8.45	3.1
96	6.2	1.2	-	-	9.8	6.3

RUN 9

STRAIN Y20

STIRRED CULTURE

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/1	OTC g/1
24	6.8	1.4	19.7	1.16	2.1	-
48	6.8	0.8	20.0	0.8	4.2	1.9
72	6.2	0.32	20.5	0.8	6.8	2.8
96	6.2	0.31	-	-	7.9	5.7
120	6.4	0.7	20.1	0.87	8.1	5.9
144	6.4	-	-	-	8.0	5.7

RUN 10

STRAIN 72T1

STIRRED CULTURE

Standard production media have been used in this run, the inoculum media used to inoculate the production fermenter was 30 hour old.

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	6.8	1.45	19.8	1.31	2.4	Nil
48	6.7	1.26	19.6	0.96	2.6	Nil
72	6.5	0.87	19.6	0.66	2.6	0.19
96	6.4	1.15	19.7	0.95	2.6	0.30

This run had been stopped after the 4th day, as there was large tendency of the media to form and as a result of that most of the mycelium had been thrown out the fermenter, and also it has been noticed that the mycelium have not grown more than the 2.6g/l in protein, also the volume of the cells in a universal which had been standing for 24 hours in the cold room did not exceed 2.0 mls out of total volume of ten.

STRAIN Y2O
STIRRED CULTURE

Amisol has been used in this run and ammonium sulphate added 48 and 72 hours 29/L

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	7.0	0.58%	19.69%	0.47	2.4	Nil
48	6.8	0.86%	19.7%	0.7	4.5	1.4
72	6.8	0.82%	20.3%	1.3	7.2	4.2 4.6
96	6.4	0.51%	20.2%	0.72	10.2	6.6 6.6
120	6.3	0.49%	20.2%	0.7	9.6	6.0 6.7

upper value of the OTC has been done with bioassay method using S.aureus as test organism, and the lower value in the square is for the OTC by $FeCl_2$ method. Oil has been added to the media 20 ml a day.

RUN 12

STRAIN Y2O

STIRRED CULTURE

corn flour has been used in this run as a carbon source

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	7.0	0.5%	19.9%	0.52	2.1	Nil
48	6.8	0.59%	19.8%	0.53	4.6	1.3
72	7.0	0.96%	19.6%	0.73	8.4	3.4 3.7
96	6.5	0.70%	20.09%	0.86	9.8	5.6 5.9
120	6.5	0.43%	20.2%	0.61	9.0	5.7 6.6

values of OTC in the table as that of run 11.

Oil has been added 20ml per day. Malonamide was added after 48 hours.

RUN 13

STRAIN 72T1

STIRRED CULTURE

In this run 50 grams of $CaCo_3$ plus 60 grams C.S.L. in 3 litre production media, KL inoculum media have been inoculated by the spore suspension in shaken flask and left in the shaker for 48 hours, inoculum fermenter have been inoculated by the KL inoculum, after 24 hours they have only occupied a quarter of a universal bottle so it has been decided to let it grow for another 24 hours after which the cells have been doubled, the production media have been inoculated by 10% of the inoculum.

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	6.5	.8	20.2	1.14	4.8	Nil
48	6.0	1.58	19.1	0.3	5.1	Nil
72	6.8	0.5	19.5	0.37	4.7	0.9
96	7.5	0.3	19.6	.30	5.0	1.0
120	7.8	0.09	20.7	.45	4.9	1.5

Colour of the culture mycelium was savannah, also the culture shows high tendency of foaming, as a result of that 0.75 of P.P.G have been added to the media in the 2nd, 3rd and 4th days.

RUN 14

STRAIN 72T1

STIRRED CULTURE

In this run the same conditions have been applied to those of run 13, but here the amount of $CaCo_3$, has been increased to 23.3 g/l and C.S.L. have decreased to 15 g/l.

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	6.8	1.27	19.6	0:97	4.4	Nil
48	6.4	1.33	19.4	0:88	4.4	Nil
72	6.6	0.9	19.6	0.69	4.6	0.8
96	7.4	0.48	19.4	0.32	4.8	0.9
120	7.4	0.18	20.1	0.22	4.8	1.2

RUN 16

STRAIN blocked mutant of Y20 (7)
STIRRED CULTURE

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	6.8	1.4	19.3	0.875		NIL
48	6.4	1.28	19.75	1.1		NIL
72	6.4	1.4	19.85	1.3		NIL
96	6.4	1.45	19.4	0.96		NIL
120						

with this run we tried the additon of malanamite to the fermenter to see if this will stimulate OTC production by this mutant as initial trials with all the blocked mutant on the shaken flasks showed that this mutant formed brown colour after the addition of the malonamite to the medium which we consider as a good sign for OTC production, although the test on the shaken flask broth gave no OTC, it is thought that it is a good idea to try it on the stirred culture.

RUN 17

STRAIN D9 D7

STIRRED CULTURE

Standard inoculum and production medium have been used, as the conditon as that of Y20

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24						
48	6.8	.73	20.0	.72	4.5	1.7
72	6.8	.73	20.0	.77	8.0	3.5
96	6.2	.74	19.8	.67	9.1	6.0
120	6.1	.48	20.3	.8	8.6	6.9
144						6.6

24 hours results have not been recorded due to industrial action by the unions. Malonide was added after 48 hours.

RUN 18

STRAIN D9D7

STIRRED CULTURE

AGE HOURS	pH	CO_2%	O_2%	RQ	PROTEIN g/1	OTC g/1
24	-	-	-	-	-	-
48	6.9	.75	20.0	.93	4.9	1.9
72	7.4	.63	19.4	.42	8.5	3.9
96	6.9	1.1	18.87	.53	9.9	6.6
120	6.6	.5	20.0	.55	9.1	7.9
144						7.0

As run 17 the results for the 24 hours have not recorded.

RUN 19

STRAIN Y20 (inactive)

STIRRED CULTURE

AGE HOURS	MDH U/mg prot	ICDH mU/mg prot	Citrate synthase mU/mg prot	PROTEIN g/l	OTC g/l
24	171	62	16.4	1.9	
48	213	104	14.2	4.6	.9
72	305	142	16.8	7.5	3.4
96	219	82	11.6	8.2	4.1

RUN 20

STRAIN Y20

STIRRED CULTURE

AGE HOURS	MDH U/mg prot	ICDH mU/mg prot	Citrate synthase mU/mg prot	PROTEIN g/l	OTC g/l
24	162	55	19	3.1	-
48	201	139	13	5.1	2.3
72	330	158	9	7.9	5.8
96	290	95	13	9.6	6.8

RUN 22

STRAIN 72T1

AGE HOURS	MDH U/mg prot	ICDH mU/mg prot	Citrate synthase mU/mg prot	PROTEIN g/l	OTC g/l
24	111	19	5.5	3.3	-
48	162	14.9	2.2	3.9	0 .4
72	212	15.0	1.6	4.4	0 .8
96	109	4.2	1.5	4.7	1.3

RUN 23

STRAIN 72T1

AGE HOURS	MDH U/mg prot	ICDH mU/mg prot	Citrate synthase mU/mg prot	Acety-CoA carboxylase mU/mg prot	PROTEIN g/l	OTC g/l
24	92	4.9	4.3	12.75	2.9	-
48	145	9.2	4.1	14.75	3.5	0 .2
72	162	29.5	3.8	17.3	4.3	0 .7
96	46	2.0	0.8	14.3	4.6	1.1

RUN 25

STRAIN Y20

AGE HOURS	MDH U/mg prot	ICDH mU/mg prot	Citrate synthase mU/mg prot	Acetyl-CoA carbosylase mU/mg prot	PROTEIN g/l	OTC g/l
24	184	32.4	17.9	17.3	2.4	-
48	232	108	16.5	15.8	4.8	1.9
72	386	121	13.3	13.3	7.7	5.3
96	323	115	12.3	14.6	9.7	7.1

RUN 24

STRAIN Y20 (inactive)

AGE HOURS	MDH U/mg prot	ICDH mU/mg prot	Citrate synthase mU/mg prot	Acetyl-CoA carboxylase mU/mg prot	PROTEIN g/l	OTC g/l
24	168	97.5	19.8	20.3	2.1	-
48	181	75.7	18.6	15.4	4.4	1.4
72	278	167	13.8	14.5	7.1	4.0
96	251	32.4	7.6	14.8	8.2	3.7

Run 28

Strain Y20

Stirred culture

AGE HOURS	ATP p mole/ mg prot.	ADP p mole/ mg prot.	AMP p mole/ mg prot.	E.C.	PROTEIN g/l	OTC g/l
24	290	61	32	0.83	3.1	0.3
48	256	91	42	0.77	5.1	1.8
72	120	150	95	0.53	6.8	4.8
96	26	69	39	0.44	8.9	7.1

Run 28

Strain Y20

AGE HOURS	MDH Umg/prot	Acetyl-CoA carboxy-lase mU/ mg prot.	PROTEIN g/l	OTC g/l
24		21.7	3.1	0.3
48		12.2	5.1	1.8
72		12.7	6.8	4.8
96		10.9	8.9	7.1

RUN 29

STRAIN 72T1

STIRRED CULTURE

AGE HOURS	ATP p mole/mg prot.	ADP p mole/mg prot.	AMP p mole/mg prot.	E.C.	PROTEIN g/l	OTC g/l
24	1135	158	49	.9	3.1	-
48	695	213	29	.85	3.9	.3
72	112	298	52	.58	5.2	.9
96	59	242	11	.62	5.6	1.2

RUN 29

STRAIN 72T1

AGE HOURS	Acetyl-CoA mU/mg prot	PROTEIN g/l	OTC g/l
24	14.5	3.1	-
48	18.5	3.9	.3
72	15.3	5.2	.9
96	17.3	5.6	1.2

RUN 30

STRAIN Y20 (inactive)

STIRRED CULTURE

AGE HOURS	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	1.2	18.8	.57	3.2	
48	1.38	18.5	.57	4.8	1.2
72	1.8	17.9	.60	6.0	3.4
96	0.9	19.0	.47	8.8	4.6

RUN 30

STRAIN Y20 (inactive)

AGE HOURS	Acetyl-CoA carboxylase mU/mg prot	PROTEIN g/l	OTC g/l
24	21.8	3.2	-
48	12.9	4.8	1.2
72	10.5	5.9	3.4
96	13.2	8.8	4.6

RUN 33

STRAIN MUTANT 6

AGE HOURS	CO_2%	O_2%	RQ	PROTEIN g/l	OTC g/l
24	2.1	18.4	.84	2.0	Nil
48	1.2	19.8	1.09	3.6	.3
72	.53	20.1	.66	7.6	2.1
96	.32	20.2	.45	9.0	3.9

STRAIN Y20

SHAKEN FLASKS

AGE HOURS	pH	CO_2%	O_2%	RQ
24		4.28	16.7	1.01
48		3.7	16.7	0.88
72		1.5	19.2	0.88
96		.45	20.2	0.64

STRAIN 72T1

SHAKEN FLASKS

AGE HOURS	pH	CO_2%	O_2%	RQ
24		2.7	18.8	1.28
48		2.2	18.7	1.0
72		3.1	14.8	0.5
96		1.9	18.8	0.9

S.rimosus NRRL 2234 ICDH mU/mg protein (Shaken flasks)

Age hours	Run 1	Run 2	Run 3
24	3.1	4.7	2.66
48	3.76	3.2	3.6
72	7.87	7.8	9.0
96	12.3	10.8	8.7

S.rimosus NRRL 2234 MDH U/mg protein (shaken flasks)

Age hours	Run 1	Run 2	Run 3
24	7.3	5.5	2.6
48	4.5	4.3	3.3
72	6.7	11.0	7.4
96	10.2	7.3	6.3

D9 ICDH mU/mg protein (Shaken flasks)

Age hours	Run 1	Run 2	Run 3	Run 4
24	7.3	7.4	5.9	-
48	21.4	21.8	14.5	-
72	20	20	13	-
96	14.5	17.2	16.4	-

D9 MDH U/mg protein (Shaken flasks)

Age hours	Run 1	Run 2	Run 3
24	15.5	14.6	15.2
48	23.8	21.7	20.0
72	24.8	22.7	28.8
96	22.9	23.5	28.5

72T1 ICDH mU/mg protein (shaken flasks)

Age hours	Run 1	Run 2	Run 3
24	5.0	6.8	7.7
48	4.8	3.9	6.1
72	15.0	5.2	6.2
96	10.1	4.8	3.7

72T1 MDH U/mg protein (Shaken flasks)

Age hours	Run 1	Run 2	Run 3
24	18.9	20.1	19.1
48	24.3	11.4	16.9
72	14.5	12.8	22.5
96	9.8	10.1	6.3

Y2O ICDH mU/mg protein (Shaken flasks)

Age hours	Run 1	Run 2	Run 3
24	7.7	4.2	10.1
48	21.4	11.8	13.1
72	21.6	13.2	14.4
96	19.6	11.3	8.2

Y2O MDH U/mg protein (Shaken flasks)

Age hours	Run 1	Run 2	Run 3	Run 4
24	12.1	13.5	16.7	21.4
48	22.2	22.2	22.7	20.0
72	22.7	22.4	25.2	31.8
96	23.9	24.0	29.0	21.4

Protein g/1 (Y20)

Age hours	Run 1	Run 2	Run 3	Run 4
24	4.5	3.75	4.2	6.8
48	5.3	5.3	4.68	8.8
72	6.76	7.8	5.28	7.7
96	7.8	7.5	4.95	9.7

OTC g/1 (Y20)

Age hours	Run 1	Run 2	Run 3	Run 4
24	-	-	-	-
48	0.04	0.05	0.08	0.05
72	2.1	1.9	2.3	2.5
96	3.7	3.9	3.1	2.8

Protein g/l Strain 72T1 Shaken flasks

Age hours	Run 1	Run 2	Run 3	Run 4
24	4.9	4.8	4.8	7.5
48	4.9	4.9	6.9	8.1
72	5.7	4.8	7.8	8.8
96	7.5	6.0	7.5	9.0

OTC g/l Strain 72T1 Shaken flasks

Age hours	Run 1	Run 2	Run 3	Run 4
24	-	-	-	-
48	0.2	0.1	0.3	0.4
72	0.6	0.7	0.5	0.6
96	0.9	1.0	0.9	0.8

pH

Strains 72T1 and Y20

Shaken flasks

Age hours		Run 1	Run 2	Run 3	Run 4
24	72T1	5.2	4.8	4.9	5.2
	Y20	6.4	5.4	5.4	5.0
48	72T1	5.0	4.8	4.4	4.7
	Y20	4.9	4.8	5.1	5.2
72	72T1	4.9	5.2	5.0	5.1
	Y20	4.2	5.0	6.7	5.9
96	72T1	5.2	5.2	5.3	5.1
	Y20	4.9	5.2	5.8	5.2

Acetyl-CoA carboxylase mU/mg protein

strain 72T1

shaken flasks

Age	run 5	run 6	run 7	run 8	run 9
24	10.2	11.3	20.6	22.1	9.9
38	5.4	6.27	2.57	3.4	2.3
72	10.5	10.4	3.4	2.8	3.1
96	7.0	5.0	6.3	6.5	4.0

OTC production

strain 72T1

shaken flasks

Age	run 5	run 6	run 7	run 8	run 9
24	-	-	-	-	-
48	0.1	0.3	0.15	0.2	0.1
72	0.4	0.39	0.42	0.44	0.3
96	0.6	0.8	0.73	0.82	0.6

PEP Carboxylase

Y20

Shaken flasks

Age	Run 5	Run 6	Run 7	Run 8
24	11.5	12.0	6.0	4.1
48	3.5	3.4	2.5	3.9
72	2.9	2.9	4.0	2.6
96	2.5	2.7	3.3	3.4

PEP Carboxylase

72T1

Shaken flasks

Age	Run 5	Run 6	Run 7	Run 8	Run 9
24	12.4	7.4	9.3	8.7	5.4
48	18.2	2.5	3.5	2.0	2.2
72	2.9	6.9	1.9	2.6	2.1
96	3.2	5.5	3.0	3.1	2.3

PEP Carboxylase

D9

Shaken flasks

Age	Run 5	Run 6	Run 7	Run 8	Run 9
24	4.9	5.1	5.8	15.6	13.6
48	9.6	9.0	2.6	3.4	3.7
72	2.5	11.9	1.0	8.2	2.2
96	4.5	4.2	3.3	2.3	3.4

Acetyl-CoA Carboxylase mU/mg protein

Strain D9

Shaken flasks

Age hours	Run 5	Run 6	Run 7	Run 8
24	8.8	10.5	24.5	25.5
48	10.1	14.8	4.5	2.5
72	11.5	12	4.3	4.9
96	6.3	6.8	6.7	6.9

OTC Production

Strain D9

Shaken flasks

Age hours	Run 5	Run 6	Run 7	Run 8	Run 9
24	-	-	-	-	-
48	0.6	0.7	0.9	0.9	0.6
72	1.3	2.5	3.2	3.1	2.1
96	2.1	2.8	4.5	4.1	2.3

Acetyl-CoA carboxylase mU/mg protein

Strain Y20

Shaken flasks

Age	Run 5	Run 6	Run 7	Run 8
24	11.6	20.1	25.6	13.2
28	14.4	3.9	5.0	11.8
72	12.9	4.8	4.2	22
96	7.3	7.5	7.9	7.1

OTC production

Strain Y20

Shaken flasks g/l

Age	Run 5	Run 6	Run 7	Run 8
24	0.4	0.45	-	-
48	1.5	1.4	0.8	0.9
72	2.5	2.6	1.9	2.1
96	3.8	4.8	4.3	3.4

shaken flasks

Run 9

Y20

ATP, ADP & AMP p mole/mg protein

AGE HOURS	ATP	ADP	AMP	E.C.
24	204	21	16	0.88
48	451	37	23	0.91
72	169	114	0.37	0.7
96	116	108	12	0.72

All units P mole/mg protein

Shaken flasks

Run 10

Y20

ATP, ADP & AMP p mole/mg protein

AGE HOURS	ATP	ADP	AMP	E.C.
24	113	15	14	0.84
48	325	46	28	0.87
72	276	133	59	0.73
96	48	35	14	0.67

Run 10

72T1

Shaken Flasks

ATP, ADP & AMP p mole/mg protein

AGE HOURS	ATP	ADP	AMP	E.C.	PROTEIN g/l	OTC g/l
24	916	124	49	0.89		
48	248	37	96	0.69		
72	439	114	140	0.71		
96	347	108	21	0.84		

Run 11

72T1

Shaken Flasks

ATP, ADP & AMP p mol/mg protein

AGE HOURS	ATP	ADP	AMP	E.C.	PROTEIN g/l	OTC g/l
24	1197	134	68	0.9		
48	353	34	33	0.88		
72	571	169	62	0.81		
96	210	120	4	0.84		

A P P E N D I X 2

Results sheets for computer identification tests

72T1

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	-	81.8	11.1
CELLOBIOSE	133	-	72.7	1
RHAMNOSE	121	-	18.2	33.3
RAFFINOSE	122	-	81.8	11.1
INOSITOL	118	-	90.9	77.8
NA.AZIDE.0.01%	105	+	18.2	11.1
MANNITOL	119	-	90.9	11.1
XYLOSE	117	-	27.3	11.1
NACL.7%	102	+	54.5	1
PHENOL 0.1%	109	-	9.1	1
GROWTH.45C	99	-	1	11.1
RIFAMPICIN	89	-	9.1	33.3
NEOMYCIN	86	+	18.2	99
ARBUTIN	84	-	99	1
XANTHINE	72	+	81.8	55.6
ALLANTOIN	81	+	18.2	1
A.NIGER.INHIB	61	+	99	99
S.MURINUS.INHIB	60	+	99	88.9
NO3.RED	52	-	9.1	1
H2S.PROD	53	-	1	22.2
PECTIN.HYD	50	-	1	1
LECITHINASE	47	-	63.6	99
L-HYDROXYPROLN	46	+	54.5	77.8
L-HISTIDINE	44	+	36.4	99
DL-AMINO-BUTYR	36	-	9.1	11.1
FRAGMENTATION	34	-	1	1
MELANIN.PYIA	32	-	1	77.8
SOBSTR.PIG.RED	20	-	1	1
BSUBTILIS.INHI	54	+	72.7	77.8
SPORE.MASS.GREY	14	+	90.9	11.1
SPORE.MASS.RED	12	-	9.1	66.7
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	63.6	99
SPORE.CH.BTV	5	-	1	99
SPORE.CH.SPIR	4	+	99	1
SPORE.CH.RA	3	-	1	1
SPORE.CH.RF	2	-	1	1
INULIN	126	-	18	13
FRUCTOSE	120	+	90.9	99
SUBSTR.PIG.YBR	19	+	99	99
SPORE.MAS.GREEN	15	-	1	1

72T1 BEST IDENTIFICATION IS S.lydicus 29

SCORES TO ON COEFFICIENTS: 1 2
3

S.lydicus	29	0.9999942	0.4681964	4.784566
STV.CINNAMONEUM	55	5.292452E-06	0.4886071	7.368281
S.LAVENDULAE	61	2.405795E-07	0.5447997	6.081897

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.lydicus 29

CHARACTE		PERCENT IN TAXON	VALUE IN UNKNOWN
ADONITOL	127	81.8	-
RAFFINOSE	122	81.8	-
INOSITOL	118	90.9	-
NA.AZIDE.0.01%	105	18.2	+
MANNITOL	119	90.9	-
NEOMYCIN	86	18.2	+
ARBUTIN	84	99	-
ALLANTOIN	81	18.2	+

S.aureofaciens

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	-	81.8	15.8
CELLOBIOSE	133	+	72.7	99
RHAMNOSE	121	-	18.2	94.7
RAFFINOSE	122	-	91.8	84.2
INOSITOL	118	-	90.9	84.2
NA.AZIDE.0.01%	105	-	18.2	5.3
MANNITOL	119	-	90.9	89.5
XYLOSE	117	-	27.3	89.5
NACL.7%	102	-	54.5	31.6
PHENOL 0.1%	109	-	9.1	94.7
GROWTH.45C	99	-	1	15.8
RIFAMPICIN	89	-	9.1	68.4
NEOMYCIN	86	-	18.2	1
ARBUTIN	84	-	99	52.6
XANTHINE	72	+	81.8	52.6
ALLANTOIN	81	+	18.2	31.6
A.NIGER.INHIB	61	+	99	10.5
S.MURINUS.INHIB	60	-	99	5.3
NO3.RED	52	-	9.1	47.4
H2S.PROD	53	-	1	78.9
PECTIN.HYD	50	-	1	68.4
LECITHINASE	47	-	63.6	1
L-HYDROXYPROLN	46	+	54.5	21.1
L-HISTIDINE	44	+	36.4	68.4
DL-AMINO-BUTYR	36	-	9.1	31.6
FRAGMENTATION	34	-	1	5.3
MELANIN.PYIA	32	-	1	47.4
SOBSTR.PIG.RED	29	-	1	15.8
BSUBTILIS.INHI	54	+	72.7	21.1
SPORE.MASS.GREY	14	+	90.9	52.9
SPORE.MASS.RED	12	-	9.1	17.6
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	63.6	99
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	99	57.9
SPORE.CH.RA	3	-	1	10.5
SPORE.CH.RF	2	-	1	42.1
INULIN	126	-	18	63
FRUCTOSE	120	+	90.9	99
SUBSTR.PIG.YBR	19	+	99	78.9
SPORE.MAS.GREEN	15	-	1	1

S.aureofaciens BEST IDENTIFICATION IS S. lydicus 29

SCORES TO CN COEFFICIENTS: 1 2 3

S.lydicus	29	0.9664607	0.4507858	4.272063
S.DIASTATOCHROM	19	0.91243921	0.5258649	4.065511
S.GRISEUS	1	0.01025504	0.5122533	4.516032

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.lydicus 29

CHARACTE		PERCENT IN TAXON	VALUE IN UNKNOWN
ADONITOL	127	81.8	-
RAFFINOSE	122	81.8	-
INOSITOL	118	90.9	-
MANNITOL	119	90.9	-
ARBUTIN	84	99	-
ALLANTOIN	81	18.2	+
S.MURINUS.INHIB	60	99	-

D9

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	+	99	81.8
CELLOBIOSE	133	+	99	72.7
RHAMNOSE	121.	-	1	18.2
RAFFINOSE	122	+	85.7	81.8
INOSITOL	118	+	99	90.9
NA.AZIDE.0.01%	105	+	99	18.2
MANNITOL	119	+	99	90.9
XYLOSE	117	+	85.7	27.3
NACL.7%	102	+	99	54.5
PHENOL 0.1%	109	+	71.4	9.1
GROWTH.45C	99	-	42.9	1
RIFAMPICIN	89	-	99	9.1
NEOMYCIN	86	-	99	18.2
ARBUTIN	84	+	71.4	99
XANTHINE	72	-	85.7	81.8
ALLANTOIN	81	+	85.7	18.2
A.NIGER.INHIB	51	+	99	99
S.MURINUS.INHIB	50	+	99	99
NO3.RED	52	+	85.7	9.1
H2S.PROD	53	-	14.3	1
PECTIN.HYD	50	-	14.3	1
LECITHINASE	47	-	85.7	63.6
L-HYDROXYPROLN	46	-	28.6	54.5
L-HISTIDINE	44	+	99	36.4
DL-AMINO-BUTYR	36	-	1	9.1
FRAGMENTATION	34	-	1	1
MELANIN.PYIA	32	-	1	1
SOBSTR.PIG.RED	20	-	1	1
BSUBTILIS.INHI	54	+	99	72.7
SPORE.MASS.GREY	14	-	1	90.9
SPORE.MASS.RED	12	-	1	9.1
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	85.7	63.6
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	57.1	99
SPORE.CH.RA	3	-	1	1
SPORE.CH.RF	2	-	14.3	1
INULIN	125	-	1	18
FRUCTOSE	120	+	85.7	90.9
SUBSTR.PIG.YBR	19	+	99	99
SPORE.MAS.GREEN	15	-	1	1

D9 BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO 3	ON COEFFICIENTS:	1	2
S.RIMOSUS	42	0.9502475	0.3211606	2.33686
S.lydicus	29	0.04779387	0.4080149	3.312799
S.GRISEUS	1	0.001198075	0.4381932	2.647464

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO
? 1

S.GRISEOVIRIDIS	17	0.0004630349	0.4260557	4.013692

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99	-
NEOMYCIN	86	99	-
XANTHINE	72	85.7	-
LECITHINASE	47	85.7	-

MUTANT 6

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:
BEST TAXON	NEXT BEST TAXON		
ADONITOL	127	+ 99	22.2
CELLOBIOSE	133	+ 99	99
RHAMNOSE	121	- 17	55.7
RAFFINOSE	122	+ 33	22.2
INOSITOL	118	+ 33	38.9
NA.AZIDE.0.01%	105	+ 99	55.6
MANNITOL	119	+ 99	99
XYLOSE	117	+ 67	99
NACL.7%	102	+ 99	44.4
PHENOL 0.1%	109	- 17	22.2
GROWTH.45C	99	+ 99	55.7
RIFAMPICIN	39	- 99	33.3
NEOMYCIN	35	- 1	1
ARBUTIN	84	+ 99	99
XANTHINE	72	+ 83	22.2
ALLANTOIN	81	+ 99	33.3
A.NIGER.INHIB	61	- 17	1
S.MURINUS.INHIB	60	- 17	22.2
NO3.RED	52	+ 1	22.2
H2S.PROD	53	- 83	88.9
PECTIN.HYD	50	- 1	22.2
LECITHINASE	47	- 1	11.1
L-HYDROXYPROUN	46	- 67	1
L-HISTIDINE	44	+ 99	77.8
DL-AMINO-BUTYR	35	- 1	55.7
FRAGMENTATION	34	- 1	1
MELANIN.PYIA	32	- 1	33.3
SOBSTR.PIG.RED	20	- 1	1
BSUBTILIS.INHI	54	- 17	11.1
SPORE.MASS.GREY	14	- 1	42.9
SPORE.MASS.RED	12	- 1	1
SPORE.SURF.RUGO	10	- 1	1
SPORE.SURF.SM	6	+ 99	55.7
SPORE.CH.BIV	5	- 1	1
SPORE.CH.SPIR	4	+ 99	77.8
SPORE.CH.RA	3	- 1	1
SPORE.CH.RF	2	- 1	22.2
INULIN	125	- 1	1
FRUCTOSE	120	+ 67	99
SUBSTR.PIG.YBR	19	+ 85.7	99
SPORE.MAS.GREEN	15	- 1	1

MUTANT 6 BEST IDENTIFICATION IS S.ALBUS 15

SCORES TO ON COEFFICIENTS: 1 2 3

S.ALBUS	16	0.6164403	0.3279567	3.044023
S.VIRIDOSPORUS	15	0.3800425	0.3820035	1.372809
S.ROCHEI	12	0.001901959	0.4125104	2.036115

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO

CHARACTERS AGAINST S.ALBUS 15

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	39	99 -
NO3.RED	52	1 +
H2S.PROD	53	83 -

S.rimosus NRRL 2234

CHARACTER	VALUE IN UNKNOWN	PERCENT IN:	
BEST TAXON	NEXT BEST TAXON		
ADONITOL	127	+	99	66.7
CELLOBIOSE	133	+	99	99
RHAMNOSE	121	-	1	33.3
RAFFINOSE	122	+	85.7	50.5
INOSITOL	118	+	99	66.7
NA.AZIDE.0.01%	105	+	99	99
MANNITOL	119	+	99	99
XYLOSE	117	+	85.7	83.3
NACL.7%	102	+	99	83.3
PHENOL 0.1%	109	+	71.4	83.3
GROWTH.45C	99	+	42.9	66.7
RIFAMPICIN	89	+	99	83.3
NEOMYCIN	86	-	99	1
ARBUTIN	84	+	71.4	99
XANTHINE	72	+	85.7	83.3
ALLANTOIN	81	-	85.7	16.7
A.NIGER.INHIB	61	+	99	33.3
S.MURINUS.INHIB	60	+	99	80
NO3.RED	52	+	85.7	33.3
H2S.PROD	53	-	14.3	99
PECTIN.HYD	50	-	14.3	66.7
LECITHINASE	47	+	85.7	50
L-HYDROXYPROLN	46	-	28.6	16.7
L-HISTIDINE	44	+	99	83.3
DL-AMINO-BUTYR	36	-	1	1
FRAGMENTATION	34	-	1	1
MELANIN.PYIA	32	-	1	1
SOBSTR.PIG.RED	20	-	1	1
BSUBTILIS.INHI	54	+	99	99
SPORE.MASS.GREY	14	-	1	1
SPORE.MASS.RED	12	-	1	99
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	85.7	99
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	57.1	99
SPORE.CH.RA	3	-	1	1
SPORE.CH.RF	2	-	14.3	1
INULIN	126	-	1	17
FRUCTOSE	120	+	85.7	99
SUBSTR.PIG.YBR	19	+	99	99
SPORE.MAS.GREEN	15	-	1	1

S.rimosus NRRL 2234 BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO 3 CN COEFFICIENTS:

		1	2
S.RIMOSUS	42	0.9997158	0.25552	0.01976915
S.GRISEDVIRIDIS	17	0.0002716561	0.3530515	1.783811
S.ROCHEI	12	7.996114E-05	0.4192548	2.21645

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
NEOMYCIN	35	99	-
ALLANTOIN	31	85.7	-

G65

CHARACTER	VALUE IN UNKNOWN	PERCENT IN:	
BEST TAXON	NEXT BEST TAXON		
ADONITOL	127	+	81.8	99
CELLOBIOSE	133	+	72.7	99
RHAMNOSE	121	-	18.2	1
RAFFINOSE	122	+	81.8	85.7
INOSITOL	118	+	90.9	99
NA.AZIDE.0.01%	105	-	18.2	99
MANNITOL	119	+	90.9	99
XYLOSE	117	+	27.3	85.7
NACL.7%	102	+	54.5	99
PHENOL 0.1%	109	-	9.1	71.4
GROWTH.45C	99	-	1	42.9
RIFAMPICIN	89	-	9.1	99
NEOMYCIN	86	-	18.2	99
ARBUTIN	84	+	99	71.4
XANTHINE	72	+	81.8	85.7
ALLANTOIN	81	+	18.2	85.7
A.NIGER.INHIB	61	+	99	99
S.MURINUS.INHIB	60	+	99	99
NO3.RED	52	-	9.1	85.7
H2S.PROD	53	-	1	14.3
PECTIN.HYD	50	-	1	14.3
LECITHINASE	47	+	63.6	85.7
L-HYDROXYPROUN	46	-	54.5	28.6
L-HISTIDINE	44	+	36.4	99
DL-AMINO-BUTYR	36	-	9.1	1
FRAGMENTATION	34	-	1	1
MELANIN.PYIA	32	-	1	1
SOBSTR.PIG.RED	20	-	1	1
BSUBTILIS.INHI	54	+	72.7	99
SPORE.MASS.GREY	14	-	90.9	1
SPORE.MASS.RED	12	-	9.1	1
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	63.6	85.7
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	99	57.1
SPORE.CH.RA	3	-	1	1
SPORE.CH.RF	2	-	1	14.3
INULIN	126	-	18	1
FRUCTOSE	120	+	90.9	85.7
SUBSTR.PIG.YBR	19	+	99	99
SPORE.MAS.GREEN	15	-	1	1

G65 BEST IDENTIFICATION IS S.lydicus 29

SCORES TO 3 ON COEFFICIENTS:		1	2
S.lydicus	29	0.9998614	0.2981865	-0.2207763
S.RIMOSUS	42	0.0001368728	0.3465126	3.231777
S.GRISEUS	1	1.018251E-06	0.4644048	3.344186

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.lydicus 29

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
ALLANTOIN	31	18.2	+
SPORE.MASS.GREY	14	90.9	-

Mutant A

CHARACTER		VALUE IN UNKNOWN	PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	+	50.7	12.5
CELLOBIOSE	133	+	99	99
RHAMNOSE	121	-	62	37.5
RAFFINOSE	122	+	16.9	62.5
INOSITOL	118	+	36.6	62.5
NA.AZIDE.0.01%	105	+	56.3	62.5
MANNITOL	119	+	94.4	37.5
XYLOSE	117	+	93	99
NACL.7%	102	+	83.1	37.5
PHENOL 0.1%	109	+	90.1	99
GROWTH.45C	99	-	7	1
RIFAMPICIN	89	-	53.5	99
NEOMYCIN	86	-	1.4	25
ARBUTIN	94	+	98.6	99
XANTHINE	72	+	97.2	87.5
ALLANTOIN	81	+	83.1	75
A.NIGER.INHIB	61	-	32.4	25
S.MURINUS.INHIB	60	-	39.4	87.5
NO3.RED	52	+	54.9	99
H2S.PROD	53	-	91.5	62.5
PECTIN.HYD	50	-	45.1	12.5
LECITHINASE	47	+	5.6	75
L-HYDROXYPROUN	46	+	22.5	87.5
L-HISTIDINE	44	+	64.3	25
DL-AMINO-BUTYR	36	-	53.5	87.5
FRAGMENTATION	34	-	1.4	1
MELANIN.PYIA	32	-	7	87.5
SOBSTR.PIG.RED	20	-	1.4	1
BSUBTILIS.INHI	54	-	28.2	50
SPORE.MASS.GREY	14	-	20	12.5
SPORE.MASS.RED	12	-	3.1	12.5
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	99	62.5
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	8.6	50
SPORE.CH.RA	3	-	12.9	12.5
SPORE.CH.RF	2	-	92.9	37.5
INULIN	126	-	10	1
FRUCTOSE	120	+	93	99
SUBSTR.PIG.YER	19	+	95.8	99
SPORE.MAS.GREEN	15	-	1	1

Mutant A BEST IDENTIFICATION IS S.GRISEUS 1

SCORES TO ON COEFFICIENTS:		1	2
3			
S.GRISEUS	1	0.787627	0.4238771	2.266933
S.VENEZUELAE	6	0.1588763	0.4257253	2.306256
S.VIRIDOSPORUS	15	0.01759961	0.4596489	3.448579

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.GRISEUS 1

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
RAFFINOSE	122	16.9	+
H2S.PROD	53	91.5	-
LECITHINASE	47	5.6	+
L-HYDROXYPROUN	46	22.5	+
SPORE.CH.SPIR	4	8.6	+
SPORE.CH.RF	2	92.9	-

Mutant 9

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	+	50.7	15.8
CELLOBIOSE	133	+	99	99
RHAMNOSE	121	-	62	94.7
RAFFINOSE	122	+	15.9	84.2
INOSITOL	118	+	36.6	84.2
NA.AZIDE.0.01%	105	-	56.3	5.3
MANNITOL	119	+	94.4	89.5
XYLOSE	117	-	93	89.5
NACL.7%	102	+	33.1	31.6
PHENOL 0.1%	109	+	90.1	94.7
GROWTH.45C	99	-	7	15.8
RIFAMPICIN	89	-	53.5	68.4
NEOMYCIN	86	-	1.4	1
ARBUTIN	84	+	98.6	52.6
XANTHINE	72	-	97.2	52.6
ALLANTOIN	81	+	83.1	31.6
A.NIGER.INHIB	61	-	32.4	10.5
S.MURINUS.INHIB	60	-	39.4	5.3
NO3.RED	52	+	54.9	47.4
H2S.PROD	53	-	91.5	78.9
PECTIN.HYD	50	-	45.1	68.4
LECITHINASE	47	-	5.6	1
L-HYDROXYPROUN	46	+	22.5	21.1
L-HISTIDINE	44	+	64.8	68.4
DL-AMINO-BUTYR	36	-	53.5	31.6
FRAGMENTATION	34	-	1.4	5.3
MELANIN.PYIA	32	-	7	47.4
SOBSTR.PIG.RED	20	-	1.4	15.8
BSUBTILIS.INHI	54	-	28.2	21.1
SPORE.MASS.GREY	14	-	20	52.9
SPORE.MASS.RED	12	-	3.1	17.6
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	99	99
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	-	8.6	57.9
SPORE.CH.RA	3	-	12.9	10.5
SPORE.CH.RF	2	+	82.9	42.1
INULIN	125	-	10	63
FRUCTOSE	120	+	93	99
SUBSTR.PIG.YBR	19	+	95.8	78.9
SPORE.MAS.GREEN	15	-	1	1

Mutant 9 BEST IDENTIFICATION IS S.GRISEUS 1

SCORES TO 3	ON COEFFICIENTS:	1	2
S.GRISEUS	1	0.5712524	0.4378591	2.638584
S.DIASTATOCHROM	19	0.427865	0.4681538	2.631725
S.EXFOLIATUS	5	0.000398313	0.4870132	3.671047

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.GRISEUS 1

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
RAFFINOSE	122	15.9	+
XYLOSE	117	93	-
XANTHINE	72	97.2	-
H2S.PROD	53	91.5	-
L-HYDROXYPROUN	46	22.5	+

FF

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:
BEST TAXON	NEXT BEST TAXON		
ADONITOL	127	+	99 81.8
CELLOBIOSE	133	+	99 72.7
RHAMNOSE	121	-	1 18.2
R-FFINOSE	122	+	85.7 81.8
I:OSITOL	118	+	99 90.9
NA.AZIDE.0.01%	105	+	99 18.2
MANNITOL	119	+	99 90.9
XYLOSE	117	+	85.7 27.3
NACL.7%	102	+	99 54.5
PHENCL 0.1%	109	+	71.4 9.1
GROWTH.45C	99	-	42.9 1
RIFAMPICIN	89	-	99 9.1
NEOMYCIN	86	-	99 18.2
ARBUTIN	84	+	71.4 99
XANTHINE	72	+	85.7 81.8
ALLANTOIN	91	+	85.7 18.2
A.NIGER.INHIB	61	+	99 99
S.MURINUS.INHIB	60	+	99 99
NO3.RED	52	+	85.7 9.1
H2S.PROD	53	-	14.3 1
PECTIN.HYD	50	-	14.3 1
LECITHINASE	47	-	85.7 63.6
L-HYDROXYPROLN	46	-	28.6 54.5
L-HISTIDINE	44	+	99 36.4
DL-AMINO-BUTYR	36	-	1 9.1
FRAGMENTATION	34	-	1 1
MELANIN.PYIA	32	-	1 1
SOBSTR.PIG.RED	20	-	1 1
BSUBTILIS.INHI	54	+	99 72.7
SPORE.MASS.GREY	14	-	1 90.9
SPORE.MASS.RED	12	-	1 9.1
SPORE.SURF.RUGO	10	-	1 1
SPORE.SURF.SM	6	+	85.7 63.6
SPORE.CH.BIV	5	-	1 1
SPORE.CH.SPIR	4	+	57.1 99
SPORE.CH.RA	3	-	1 1
SPORE.CH.RF	2	-	14.3 1
INULIN	126	-	1 18
FRUCTOSE	120	+	85.7 90.9
SUBSTR.PIG.YBR	19	+	99 99
SPORE.MAS.GREEN	15	-	1 1

FF BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO 3	ON COEFFICIENTS:	1	2
S.RIMOSUS	42	0.956022	0.292796 1.335599
S.lydicus	29	0.03606122	0.3885408 2.439441
S.GRISEUS	1	0.006923709	0.4110825 1.926846

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST C4ARACTE	PERCENT IN	S.RIMOSUS 42 TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99	-
NEOMYCIN	86	99	-
LECITHINASE	47	85.7	-

S.capuensis

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	+	99	81.8
CELLOBIOSE	133	+	99	72.7
RHAMNOSE	121	-	1	18.2
RAFFINOSE	122	+	85.7	81.8
INOSITOL	118	+	99	90.9
NA.AZIDE.0.01%	105	+	99	18.2
MANNITOL	119	+	99	90.9
XYLOSE	117	+	85.7	27.3
NACL.7%	102	+	99	54.5
PHENOL 0.1%	109	+	71.4	9.1
GROWTH.45C	99	+	42.9	1
RIFAMPICIN	89	-	99	9.1
NEOMYCIN	86	-	99	18.2
ARBUTIN	94	+	71.4	99
XANTHINE	72	+	85.7	81.8
ALLANTOIN	81	+	85.7	18.2
A.NIGER.INHIB	61	+	99	99
S.MURINUS.INHIB	60	+	99	99
NO3.RED	52	+	85.7	9.1
H2S.PROD	53	-	14.3	1
PECTIN.HYD	50	-	14.3	1
LECITHINASE	47	+	85.7	63.6
L-HYDROXYPROLN	46	-	28.6	54.5
L-HISTIDINE	44	+	99	36.4
DL-AMINO-BUTYR	36	-	1	9.1
FRAGMENTATION	34	-	1	1
MELANIN.PYIA	32	-	1	1
SOBSTR.PIG.RED	20	-	1	1
BSUBTILIS.INHI	54	-	99	72.7
SPORE.MASS.GREY	14	-	1	90.9
SPORE.MASS.RED	12	-	1	9.1
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	85.7	63.6
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	57.1	99
SPORE.CH.RA	3	-	1	1
SPORE.CH.RF	2	-	14.3	1
INULIN	126	+	1	18
FRUCTOSE	120	+	85.7	90.9
SUBSTR.PIG.YBR	19	+	99	99
SPORE.MAS.GREEN	15	-	1	1

s.capuensis BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO ON COEFFICIENTS: 1 2
3

S.RIMOSUS	42	0.7863554	0.345808	3.206905
S.lydicus	29	0.0939298	0.441492	3.998434
S.ROCHEI	12	0.09250796	0.4452567	2.912352

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99 -
NEOMYCIN	86	99 -
BSUBTILIS.INHI	54	99 -
INULIN	126	1 +

D9D7

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	+	99	81.8
CELLOBIOSE	133	+	99	72.7
RHAMNOSE	121	-	1	18.2
RAFFINOSE	122	+	85.7	81.8
INOSITOL	118	+	99	90.9
NA.AZIDE.0.01%	105	+	99	18.2
MANNITOL	119	+	99	90.9
XYLOSE	117	+	85.7	27.3
NACL.7%	102	+	99	54.5
PHENOL 0.1%	109	+	71.4	9.1
GROWTH.45C	99	-	42.9	1
RIFAMPICIN	89	-	99	9.1
NEOMYCIN	86	-	99	18.2
ARBUTIN	84	+	71.4	99
XANTHINE	72	+	85.7	81.8
ALLANTOIN	81	+	85.7	18.2
A.NIGER.INHIB	61	+	99	99
S.MURINUS.INHIB	60	+	99	99
NO3.RED	52	+	85.7	9.1
H2S.PROD	53	-	14.3	1
PECTIN.HYD	50	-	14.3	1
LECITHINASE	47	+	85.7	63.6
L-HYDROXYPROLN	46	-	28.6	54.5
L-HISTIDINE	44	+	99	36.4
DL-AMINO-BUTYR	36	-	1	9.1
FRAGMENTATION	34	-	1	1
MELANIN.PYIA	32	-	1	1
SOBSTR.PIG.RED	20	-	1	1
BSUBTILIS.INHI	54	+	99	72.7
SPORE.MASS.GREY	14	-	1	90.9
SPORE.MASS.RED	12	-	1	9.1
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	85.7	63.6
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	57.1	99
SPORE.CH.RA	3	-	1	1
SPORE.CH.RF	2	-	14.3	1
INULIN	126	-	1	18
FRUCTOSE	120	+	85.7	90.9
SUBSTR.PIG.YBR	19	+	99	99
SPORE.MAS.GREEN	15	-	1	1

D9D7 BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO ON COEFFICIENTS: 1 2
3

S.RIMOSUS	42	0.9889707	0.2613711	0.2263117
S.lydicus	29	0.01097594	0.3799076	2.185263
S.GRISEUS	1	7.089652E-05	0.4366319	2.605964

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 0

CHARACTERS AGAINST • S.RIMOSUS 42

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	89	99 -
NEOMYCIN	86	99 -

Y20

CHARACTER	VALUE IN UNKNOWN		PERCENT IN:	
BEST TAXON	NEXT BEST TAXON			
ADONITOL	127	+	99	31.3
CELLOBIOSE	133	+	99	72.7
RHAMNOSE	121	-	1	18.2
RAFFINOSE	122	+	85.7	81.8
INOSITOL	118	+	99	90.9
NA.AZIDE.0.01%	105	+	99	18.2
MANNITOL	119	+	99	90.9
XYLOSE	117	+	85.7	27.3
NACL.7%	102	+	99	54.5
PHENOL 0.1%	109	+	71.4	9.1
GROWTH.45C	99	-	42.9	1
RIFAMPICIN	39	-	99	9.1
NEOMYCIN	86	-	99	18.2
ARBUTIN	34	+	71.4	99
XANTHINE	72	-	85.7	81.8
ALLANTOIN	81	+	85.7	18.2
A.NIGER.INHIB	61	+	99	99
S.MURINUS.INHIB	60	+	99	99
NO3.RED	52	+	85.7	9.1
H2S.PROD	53	-	14.3	1
PECTIN.HYD	50	-	14.3	1
LECITHINASE	47	-	35.7	63.6
L-HYDROXYPROLN	46	-	28.6	54.5
L-HISTIDINE	44	+	99	36.4
DL-AMINO-BUTYR	36	-	1	9.1
FRAGMENTATION	34	-	1	1
MELANIN.PYIA	32	-	1	1
SOBSTR.PIG.RED	28	-	1	1
BSUBTILIS.INHI	54	+	99	72.7
SPORE.MASS.GREY	14	-	1	90.9
SPORE.MASS.RED	12	-	1	9.1
SPORE.SURF.RUGO	10	-	1	1
SPORE.SURF.SM	6	+	85.7	63.6
SPORE.CH.BIV	5	-	1	1
SPORE.CH.SPIR	4	+	57.1	99
SPORE.CH.RA	3	-	1	1
SPORE.CH.RF	2	-	14.3	1
INULIN	125	-	1	18
FRUCTOSE	120	+	85.7	90.9
SUBSTR.PIG.YBR	19	+	99	99
SPORE.MAS.GREEN	15	-	1	1

Y20 BEST IDENTIFICATION IS S.RIMOSUS 42

SCORES TO ON COEFFICIENTS: 1 2
3

S.RIMOSUS	42	0.9502475	0.3211606	2.33686
S.lydicus	29	0.04779387	0.4080149	3.012799
S.GRISEUS	1	0.001188076	0.4381932	2.647464

IF ADDITIONAL TAXA ARE NEEDED, ENTER A POSITIVE
INTEGER, ELSE ENTER ZERO
? 3

CHARACTERS AGAINST S.RIMOSUS 42

CHARACTE	PERCENT IN TAXON	VALUE IN UNKNOWN
RIFAMPICIN	39	99	-
NEOMYCIN	86	99	-
XANTHINE	72	85.7	-
LECITHINASE	47	35.7	-