J. gen. Microbiol. (1968), **53**, 147–162
Printed in Great Britain

The Growth of Purine Mutants of *Bacillus anthracis* in the Body of the Mouse

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(*Accepted for publication* 26 *March* 1968)

SUMMARY

Auxotrophic mutants of *Bacillus anthracis* with a specific requirement for adenine in their phenotype were isolated and the block in the purine pathway determined by enzymic activity of preparations obtained from the individual strains. The adenine-dependent mutants could be classed into two groups: one group was of strains devoid of any adenylosuccinate synthase (EC 6.3.4.4) activity; the presence of adenylosuccinate lyase (EC 4.3.2.2) activity was not detected in the strains of the other group. In these latter strains a single gene locus controls the two enzyme activities involved in the purine pathway, as established for other organisms.

None of the adenine-dependent strains was capable of killing mice although they were given a toxic dose of adenine at challenge. Reversion to prototrophy restored full virulence. The lack of proliferation of the adenine-dependent bacteria in the host could not be explained simply by a shortage of available adenine in the body of the mouse, since the adenine intake provided a certain concentration of this base at the inoculation site, in the peritoneal cavity, and in the blood stream. In spite of the fact that adenine-dependent mutants were producing both of the known aggressins, namely capsule and toxin, they were not capable of invading the blood stream or of multiplying there. It is assumed that something essential for invasion of the host was not produced by adenine-dependent mutants. The lack of this hypothetical factor rendered these organisms highly vulnerable to the defence mechanism of the host. In contrast to the adenine-dependent mutants, purine auxotrophs blocked either in the early part of the purine pathway or in the conversion of xanthylic acid to guanylic acid were found to grow readily in the body of the mouse, even in the absence of an exogeneous supply of purine bases. It is suggested that purine bases needed by these mutants for their growth might originate from an influx of purines from tissues damaged by the bacteria.

INTRODUCTION

Approximately 200 isolates with essential requirements either for nucleic acid bases or vitamins were obtained from a non-capsulated mutant of *Bacillus anthracis*, strain VOLLUM, in this laboratory (Ivánovics, Varga & Marjai, 1963). The reversion of the isolates to a capsulated form did not involve any change in the nutritional requirements of the mutants (Ivánovics & Marjai, 1964). When the capsulated forms of the auxotrophs were tested for their virulence in the mouse, all of the pyrimidine-dependent (uracil, thymine), or vitamin-dependent, strains were found to be fully virulent (Ivánovics & Marjai, 1964; and unpublished observations). Auxotrophic strains with a purine requirement showed a different pattern of pathogenicity, according to the deficiency in the *de novo* purine pathway. Strains which showed a block in the early

part of the purine pathway, where the requirement could be met by any of the purines hypoxanthine, xanthine, adenine or guanine, did not have a significant impairment of their virulence. Similarly, strains which required guanine specifically for growth also killed mice, although an exogeneous supply of guanine after infection of the animals enhanced the virulence of the bacteria. In contrast, auxotrophs which were adenine-dependent in their phenotype were not lethal to mice, even when a heavy inoculum (10^6 spores) was injected and supplementary adenine given after infection.

It was not possible to maintain a high concentration of adenine during the whole course of a conventional virulence test (2 weeks) because of the high turnover rate (Bennet, 1953; Schwarz & Rieke, 1963) and the toxicity of adenine (Raska, 1946; Brown, Roll & Plentl, 1947). The investigations presented in the present paper were made to devise short-term experiments during which as high a concentration as possible of adenine was maintained in the infected animals. It was noted by Philips, Thiersch & Bendich (1952) that death occurred 2–4 days after the administration of a lethal dose of adenine because of the deposition in the kidneys of 2,8-dioxy-adenine, a crystalline oxidation product of adenine produced *in vivo*. Thus, it was possible to follow the multiplication of the adenine-dependent bacteria in the peritoneal cavity during the interval of a few days between administration of the adenine and death of the animal.

An adenine-dependent mutant in our designation (Ivánovics & Marjai, 1964) means that the conversion of inosinic acid to adenylic acid was blocked in that strain. This conversion takes place by two enzymic reactions: in the first, inosinic acid is converted to the ribotide of adenylosuccinate; in the second, adenlyic acid is formed. Attempts were made to isolate and use both types of mutants, lacking either the first or the second enzymic reaction, in the virulence tests.

METHODS

Bacterial strains. The isolation and some characteristics of the purine auxotrophs of *Bacillus anthracis* were described earlier (Ivánovics *et al.* 1963). Several recently isolated adenine-dependent mutants were also included in this study. As a safety measure, non-capsulated (C^-) forms of auxotrophs were preferred to the capsulated (C^+) in certain experiments, such as the isolation of enzyme preparations. (see Table 1).

For the designation of individual purine auxotrophic strains, the nomenclature recommended by Demerec, Adelberg, Clark & Hartman (1966) could not be fully adopted since no genetic tool was available for recombination tests in *Bacillus anthracis*. Nevertheless, the use of this nomenclature was adopted as far as possible. The locus involved by mutation could be inferred either from the phenotype or from the altered enzymic activity of the mutants. Auxotrophs having a block before purine ring closure (inosinic acid) were symbolized by *pur*, whereas *ade* and *gua* mean that the conversion of inosinic acid either to adenylic acid or to guanylic acid was blocked. Biochemical investigations of *ade* mutants pointed to the site involved by mutation, and these mutants were divided accordingly into groups designated by roman numerals I and II. An arabic numeral affixed to the symbol with a hyphen indicated the individual origin of the mutants. Some of the prototrophic revertants of adenine auxotrophs were also included in this study, but whether their wild-type phenotype was due to back mutation of the affected nucleotides or to a suppressor mutation was not known.

Prototrophic revertants were isolated from the capsulogenic form of *ade* mutants. Thus, revertants were obtained from the following strains: *ade* II-3 C^+; *ade* II-4 C^+; *ade* I-1 C^+; and *ade* I-2 C^+.

Chemicals. Commercial preparations were used; purine bases and nucleotides were purchased from Reanal (Budapest) or Fluka AG (Buchs, Switzerland). Adenine was recrystallized and its purity checked by thin-layer chromatography. The ammonium salt of adenylosuccinic acid was a gift of Dr Haruo Momose (Central Research Laboratories, Ajinomoto Co., Ltd., Kawasaki, Japan).

Table 1. *Bacillus anthracis: list of strains used*

Serial no. of isolate (parent)	Designation of strain* (VC^+)	Phenotype, purine requirement (prototrophic)	Former designation‡ (strain VOLLUM)
SZ 61	*pur*-1	Any purine	61 C^-hy^-
SZ 30	*gua*-1†	Guanine	30 C^-gu^-
SZ 6	*ade* II-1	Adenine	6 C^-ad^-
SZ 20	*ade* II-2		20 C^-ad^-
SZ 23	*ade* II-3		23 C^-ad^-
SZ 214	*ade* II-4		.
SZ 341	*ade* I-1		.
SZ 344	*ade* I-2		.

* Both capsulogenic (C^+) and non-capsulogenic (C^-) forms of the strains were used in this study.
† Xanthine could not be substituted for guanine.
‡ See Ivánovics & Marjai (1964).

Media. A basal casein-hydrolysate medium (BCM) was used for growing auxotrophs under defined conditions; it contained thiamine 1 μg./ml. and was prepared as previously described (Ivánovics *et al.* 1963). In some cases BCM supplemented with additional metabolites (referred to as CCM) was also used (Ivánovics, 1964). A non-defined yeast-extract peptone medium (YP) was identical with that used in this laboratory (Cziszár & Ivánovics, 1965).

Cultivation of the bacteria. The bacteria were grown in liquid or on solid medium as previously described (Ivánovics *et al.* 1963; Ivánovics, 1964).

With few exceptions, standard spore suspensions were used for both virulence tests and as inoculum for obtaining cultures for enzyme preparation. Spore suspensions were made by inoculating several slopes of YP agar from a single colony, followed by incubation at 37° for 8 to 10 days. Each culture was washed off with phosphate buffer (0·01 M; pH 7) separately and centrifuged; the pellet was resuspended in buffer and digested in a water bath at 47° for 3 hr. Following this, the suspensions were centrifuged and the pellets taken up in distilled water. Each lot of spore material, corresponding to one agar slope, was tested for the presence of prototrophic revertants by inoculating about 10^7 spores on BCM agar. Those lots which yielded colonies were discarded; the remainder were pooled and stored at 4°.

Enzyme preparations and their assay. Two enzymes, adenylosuccinate synthase (EC 6.3.4.4), and adenylosuccinate lyase (EC 4.3.2.2), are involved in the conversion of inosinic acid to adenylic acid. To isolate these enzymes from prototrophs and various adenine-dependent mutants, a bacterial suspension was used, grown with vigorous shaking in CCM containing adenine 20 to 40 μg./ml. *Bacillus anthracis* is

known to produce extracellular proteolytic enzyme, the maximal release of which is at the end of the logarithmic growth phase. By harvesting the bacteria at early stationary phase and washing, one could rid the preparation of most of this proteolytic enzyme. One hundred ml. of culture yielded about 1 g. washed sediment.

Preparations with adenylosuccinate synthase activity were obtained by a slightly modified method, similar to that recommended by Lieberman (1956) for *Escherichia coli*. Washed bacteria (4 to 5 g. wet wt) were suspended in 25 ml. of 0·005 M-K_2HPO_4 which contained gelatin 1 % (w/v); the suspensions were treated ultrasonically in a MSE Ultrasonic Power unit at 2° to 4° for 15 min. The extract thus obtained was processed by the technique of Lieberman (1956). The gelatin, which protected adenylosuccinate synthase against proteolytic enzyme in the early phase of manipulation, was removed during the ammonium sulphate precipitations. The final product, termed 'low pH fraction' was used for estimating the enzyme activity of the bacteria, the assay of enzyme being done by Lieberman's 'Assay I' (1956). Assay systems containing various amounts of enzyme preparation were incubated at 37° for 30 min. The unit of activity (0·1 increase in extinction at 280 mμ) was calculated by interpolations of experimental data. The protein content of the enzyme preparations was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). The specific activity was then recorded as units/mg. protein.

To detect the presence of adenylosuccinate lyase in individual adenineless mutants, extracts were obtained by ultrasonic treatment from the bacteria as described above, except that no gelatin was added to the suspensions. The addition of streptomycin sulphate 1 % to the extract caused a heavy precipitate which was removed by centrifugation. The clear supernatant fluid, termed 'crude extract', was assayed for adenylosuccinate lyase, as recommended by Carter & Cohen (1956) for enzyme preparations from yeast. Activity was measured from the rate of spectral change induced by the enzyme in a solution of the ammonium salt of adenylosuccinate ribotide. The change in extinction at 280 mμ was constant in the first 2 min. The initial velocity was calculated, and 1 unit of activity defined as the amount of enzyme necessary to decrease the extinction by 1·0/min.

Accumulation of diazotable arylamines by non-proliferating suspensions of bacteria. In essence, the method of Gots & Love (1954) was followed. Bacteria were grown with vigorous shaking in BCM+adenine 5 μg./ml. Overnight cultures were centrifuged, the deposit washed with saline, and the bacteria resuspended in BCM diluted 1/10 with saline; the addition of 0·4 % glucose and thiamine 1 μg./ml. completed the system. Each ml. of suspension contained equiv. 0·5 mg. dry wt bacteria. After aeration for 3 hr at 37°, the bacteria were centrifuged down and the supernatant fluid tested for diazotable arylamines by the Bratton & Marshall (1939) procedure as recommended by Ravel, Eakin & Shive (1948). The diazonium salt of 5-amino-4-imidazole-N-succino-carboxamide ribonucleotide (SAICAR) is very unstable. Lukens & Buchanan (1959) recommended a procedure in which SAICAR gives a stable colour substance with the coupling agent. When parallel assays were made with the Bratton-Marshall. procedure and that recommended by Lukens & Buchanan (1959), the results of the two tests were a good indication for the presence of SAICAR.

Production and assay of the toxin. A basal medium made of Casamino acid as recommended by Professor H. Smith, (Birmingham, England; personal communication) was used for toxin production. The medium was buffered with 0·8% (w/v)

$NaHCO_3$ and 25 mg. charcoal was added to each 100 ml. In the case of an adenineless mutant, the medium contained adenine 20 μg./ml.

An inoculum of 10^7 colony-forming vegetative bacteria was added to 50 ml. medium in a 250 ml. Erlenmeyer flask and incubated statically at 37° for 20 to 22 hr. Horse serum (10%, v/v) was added to the supernatant fluid culture and the liquid sucked through a membrane filter (no. 4, Göttingen). Culture filtrates were assayed by intradermal inoculation into guinea-pigs (Smith, Keppie & Stanley, 1955) and by an agar diffusion method (Thorne & Belton, 1957; Sargeant, Stanley & Smith, 1960), with an antitoxic horse serum kindly supplied by Professor H. Smith.

Assay of multiplication of the bacteria in the peritoneal cavity of the mouse. Spores were pre-germinated in tryptone broth for 30 min. at 37° and firmly pelleted by centrifugation. The pellet was resuspended in saline containing the appropriate base at 2 mg./ml., the colony count of the suspension being 10^7/ml. Mice (25 to 30 g.) were inoculated intraperitoneally with 0·5 ml. of this material. Control groups were infected with pre-germinated spores suspended in saline. Because of the poor solubility of guanine, finely dispersed suspensions of this base in saline were used for preparing inocula.

When encapsulated bacilli were used for infection, a 20 hr agar culture, grown in the presence of 20% (v/v) CO_2 in air (Ivánovics, 1962), served as source of inoculum. Bacterial suspensions prepared from mucoid colonies were standardized by their turbidity. The chain-lengths of the bacteria and the number of colony-formers in the suspension were estimated simultaneously with the infection of mice.

Multiplication of the bacteria in the peritoneal cavity was followed by sacrificing two mice at intervals. The mice were killed by chloroform, the abdominal cavity rinsed with 5 ml. saline, and samples of pooled material plated in duplicate on YP agar. The animals kept for further observation were repeatedly injected intraperitoneally with 0·5 mg. of base. The total number of animals infected was 4 to 6 mice more than those sacrificed for assaying the colony counts; these extra animals served to estimate the survival period after infection. In some experiments, purine was given subcutaneously in an oily suspension 40 min. before infection.

The bacterial count of the blood was estimated on a freshly drawn and citrated sample of heart blood. Direct smears from the peritoneal surface served to ascertain the morphological and staining characteristics of the bacilli. The slides were stained with Giemsa solution after ether fixation.

Mice found dead were not assayed for colony count because of the fibrin clot which aggregated individual bacilli. However, in these cases, heart blood was plated on both YP and BCM agar to establish the cause of death and to exclude reversion of bacteria to prototrophy. Death of an animal with a sterile blood culture was considered to have been caused by the toxicity of adenine.

Detection of adenine and guanine in the body of mice injected with these purines. After the intraperitoneal injection of adenine, peritoneal washings of the mice were collected, adjusted to pH 4 with dilute acetic acid, and heated in a boiling water bath for 15 min. The centrifuged and neutralized extracts were assayed for adenine by the method of Ivánovics (1964).

The presence of adenine in blood plasma was detected as follows. After the subcutaneous injection of adenine in oil, heart blood was withdrawn from the mice under deep chloroform anaesthesia. The blood was added to a cooled centrifuge tube con-

taining heparin, and centrifuged. To a paraffin-coated test-tube (160 × 16 mm.) were added 0·4 ml. of plasma and 0·2 ml. of BCM, and the whole inoculated with a spore suspension of strain *ade* II-3 (inoculum size 2 to 4×10^4 spores/ml.). The tubes were rotated horizontally (1 revolution/10 min.) in an incubator at 37° for 24 hr, and a colony count then made by plating on YP agar. Because of chain formation, the actual number of bacilli was approximately 10 times greater than the colony count. A similar method served to determine guanine in blood plasma of mice not given guanine; strain *gua*-1 was then used as the assay organism.

RESULTS

Biochemical characteristics of adenine-dependent mutants of Bacillus anthracis

The adenine requirement of isolates SZ6, SZ20, SZ23, SZ214, SZ341 and SZ344 was highly specific; adenine could not be replaced by any of other purine. Growth of these strains in liquid BCM was initiated by a few μg. of adenine/ml.; the growth response reached its peak in a static culture with about 20 μg./ml. Adenosine could replace adenine, but the phosphatides of this nucleoside were only partly and slowly utilized. Strains of adenine-dependent mutants could be divided into two groups (I, II), according to their enzyme activity (see Table 2).

Table 2. *Bacillus anthracis: enzymic activity of preparations obtained from individual isolates of adenine-requiring auxotrophs*

No. of isolate	Specific enzymic activity*		Accumulation of arylamines‡	
	A. synthase	A. lyase	B.M.	L.B.
SZ6	28	0·00	−	+
SZ20	27	0·00	−	+
SZ23	38, 32	0·00	−	+
SZ214	23	0·00	−	+
SZ314	0, 0	0·27	−	−
SZ344	0	0·31	−	−
VC−†	31, 26, 28	0·35	−	−

* Adenylosuccinate synthase and adenylosuccinate lyase respectively. Numbers show the results of individual experiments.

† Non-capsulogenic form of wild type, VC^+.

‡ B.M. = Bratton-Marshall, L.B. = Lukens-Buchanan procedure. Extinction of colour substance at 560 mμ in 1 cm. cuvette amounted to 0·03 to 0·09 in individual experiments with different strains.

An effective enzyme preparation with adenylosuccinate synthase activity was obtained from strains belonging to group II (isolates SZ6, SZ23, SZ214). The specific activity of the enzyme preparations was considerably lower than that of the 'low pH fraction' obtained from *Escherichia coli* B by Lieberman (1956). For comparison *E. coli* B was also included in our study; the specific activity of 'low pH fraction' obtained from this strain was of the same order as those obtained from *Bacillus anthracis*.

In contrast, we did not find any adenylosuccinate lyase activity in extracts of *Bacillus anthracis* strains belonging to group II. Non-proliferating bacteria of these strains did not accumulate any substance giving a positive result with the original Bratton-Marshall test. On the other hand, an excretion of diazotable arylamine was detected by the procedure of Lukens & Buchanan (1959). This was a good indication for accumula-

tion of SAICAR. There is now strong evidence that the cleavage of both adenylosuccinate ribotide and 5-aminoimidazole-4-N-carboxamide ribotide (an early intermediate of purine synthesis) are catalysed by the same enzyme protein (EC 4.3.2.2) in mammalian tissues, in *Neurospora crassa* (Giles, Partridge & Nelson, 1957), and in some species of Enterobacteriaceae (Gots & Gollub, 1957; Gollub & Gots, 1959). This special case where a single gene locus controls two enzymic reactions is also true for *B. anthracis*.

The *Bacillus anthracis* isolates SZ341 and SZ344 formed another group (group I) of adenine-dependent mutants in which the enzyme adenylosuccinate synthase is missing in an effective form, whilst lyase activity can be detected in the crude extracts of bacteria. Supernatant fluids of non-proliferating bacterial suspensions of these strains contained a substance absorbing in the ultraviolet range with a peak at 248 mμ at pH 2; this maximum corresponds to that of inosinic acid. This accumulation of inosinic acid is in accordance with a block in the locus which controls adenylosuccinate synthase.

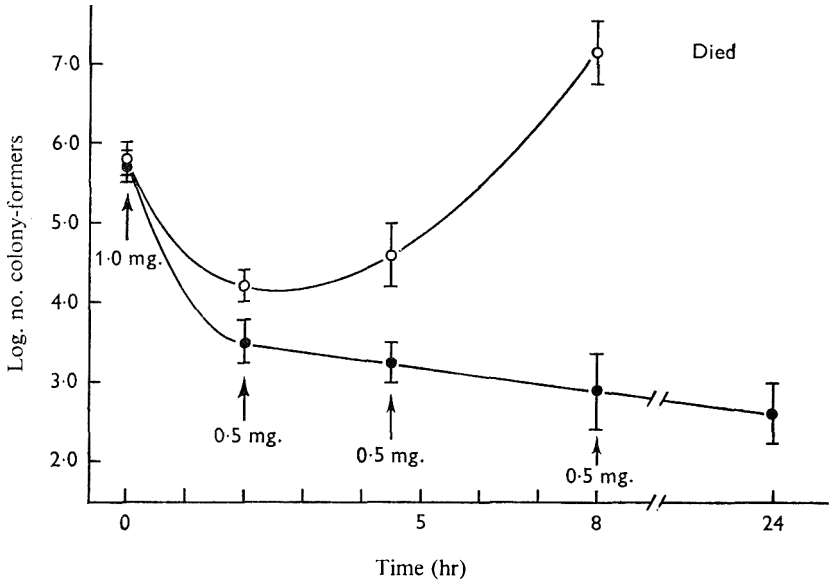

Fig. 1. *Bacillus anthracis*: number of colony-formers in the peritoneal cavity of mice infected with 5×10^6 pre-germinated spores of strain *ade* II-3 C^+ or of its prototrophic revertant. ●, Strain *ade* II-3 C^+; ○, prototrophic revertant. Arrows indicate intraperitoneal injection of adenine. Mean values and the standard deviation of three independent experiments.

In vivo growth of adenine auxotrophs

Recently isolated encapsulated strains of adenine-dependent mutants, i.e. *ade* II-4; *ade* I-1; *ade* I-2, were tested for virulence in the conventional way, as described for strains already studied (Ivánovics & Marjai, 1964). In spite of an adenine intake, animals infected with 10^6 spores either lived to the end of the experiment or, when they died, their heart blood did not yield colony-formers. By contrast, 20 spores of their prototrophic revertants killed mice regularly.

Figure 1 shows a short-term experiment with strain *ade* II-3 C^+ and its prototrophic

revertant, when the proliferation of bacteria was followed in the peritoneal cavity of the mouse. There was an initial decrease in colony count, even in the case of the revertant, which was, however, followed by rapid multiplication of the prototrophic bacteria. By comparison, the number of adenine-dependent bacilli gradually declined after the abrupt initial phase, in spite of the intensive adenine intake. In addition to the data shown by the curves, mice sacrificed at 48 to 72 hr after infection with *ade* II-3 C^+ still contained 10^2 to 10^3 colony-formers/ml. of peritoneal washings. Nevertheless, no deaths occurred which could be attributed to generalized infection. Animals in these experiments were killed by a delayed toxic effect of adenine, as indicated by a control group receiving adenine alone in which more than half of the mice died between the 3rd and 4th day.

It appeared reasonable to make similar experiments with adenine in an oily suspension (0·5 ml.) administered subcutaneously in a single dose, rather than in several doses given intraperitoneally. In a series of experiments, 2, 3 and 8 mg. adenine were given as single doses to mice infected with 5×10^6 pre-germinated spores. The initial colony count amounted to 2 to 3×10^5/ml. peritoneal washings, which declined considerably within 2 to 3 hr. This was followed by a gradual decline in the colony count by the 3rd to 4th day. It should, however, be stressed that a high proportion of mice given 8 mg. adenine died during the observation period without giving positive blood cultures.

There are two blocks in the biosynthetic pathway of adenine in strain *ade* II-3 C^+, both caused by the lack of the enzyme adenylosuccinate lyase. The early block in the pathway, when fumaric acid is not split from SAICAR, was overcome by any of the purines, such as hypoxanthine. Theoretically, this should have a sparing effect on adenine demand, since the synthesis of guanylic acid is brought about directly via inosinic acid instead of by the conversion of adenylic acid to gunylic acid. This assumption, however, could not be substantiated by an experiment in which infected mice were given hypoxanthine + adenine (5 mg. each); none of the infected mice died of anthrax. Similarly, no enhancing effect was found when histidine, a product of ATP-imidazole cycle, was given simultaneously with adenine + hypoxanthine.

In the experiments described so far, mice were infected by spores given a short germination period (30 min) in BCM + adenine. Only a small proportion of these spores showed any sign of germination when inoculated into mice. As it can be seen from Fig. 1, there was about a 1·5 log unit decrease in the colony count by the 3rd hour as compared with the initial value. In this early phase, before the germination of spores and encapsulation of the bacilli, the organisms appeared to be highly vulnerable to the defence mechanisms of the host. Indeed, only 2·5% of inoculated spores reached the exponential phase of multiplication.

This unfavourable lag in multiplication of bacteria suggested that it might be preferable to infect the mice with encapsulated vegetative bacilli instead of spores, and an experiment was made with encapsulated bacilli of strain *ade* II-3 C^+ (Table 3). The colony count in mice not given adenine gradually decreased to as little as 1 % of the initial value. By comparison, a high dose of adenine enhanced the multiplication of bacilli for 24 hr, although this high colony count in the peritoneal cavity was not associated with a bacteraemia. When blood samples (0·02 ml.) were taken at autopsy and plated on nutrient agar, either they did not yield any colonies, or in a few cases only one or two colonies.

Table 4 shows the results of another experiment in which mice were infected with

Table 3. *Bacillus anthracis: colony counts in the peritoneal washings of mice infected with* 4×10^5 *colony-formers of heavily encapsulated bacilli from strain ade II-3* C^+, *with and without adenine*

Because of chain formation, the actual number of bacilli in the inoculum was about 10 times greater than the colony count. In addition to the dead mice included in the Table, four mice died spontaneously on the 3rd to 5th day; their blood cultures were negative, and they had apparently died from the toxic effect of adenine. A control group of 7 mice given adenine alone died after the third day.

Time (hr)	Peritoneal washings: log. no. colony-formers/ml.	
	Without adenine	With 6 mg. adenine in oil subcutaneously
0	4·30	4·20
2	3·74	4·69
4	3·00	5·69
8	3·15	5·66
12	2·69	5·84
24	2·00	5·08
48	0·00	4·95

Table 4. *Bacillus anthracis: colony counts of peritoneal washings and the results of microscopic examination of peritoneal smears of mice infected with* 10^6 *colony-formers of heavily encapsulated bacilli of strain ade II-3* $C^+ + 1$ *mg. adenine*

Time (hr)	Further intraperitoneal injection of adenine (mg.)	Log. colony count/ml.	Microscopic observations*
0	1·0	5·09	Well-stained encapsulated bacilli
2	0·5	4·30	Mostly chains with 2–8 encapsulated bacilli; a proportion were poorly stained; some empty capsules
4·5	0·5	4·0	Some well-stained bacilli in short chains; many ghost-like forms
8	0·5	4·2	Proportion of poorly stained bacilli increased
12	0·5	4·3	Poorly stained and ghost-like bacilli increased in number
24	0·5	5·0	Beside well-stained bacilli disintegrated and ghost-like forms prevailed
36	Nil	2·9	Very few well-stained bacilli; many disintegrated and ghost-like forms
48	Nil	2·6	

* Faintly or unevenly stained shrunken bacilli are referred to as 'ghost-like forms'. Most of these structures were encapsulated; many empty capsules were also seen.

encapsulated bacilli. On this occasion, however, a heavier infecting dose consisting of 10^6 colony-formers + 1 mg. adenine was given intraperitoneally, and the intraperitoneal injection of adenine was continued for 24 hr. In addition to determination of colony counts, smears were taken from the peritoneal surface for microscopic examination.

A better insight was gained into the microscopic changes of inoculated bacilli

when an extremely high dose of bacilli (10^7 colony-formers = about 10^8 bacilli) was injected and the mice given adenine on a similar schedule (Table 4). In this experiment, most of the mice were alive at 32 hr in spite of the considerable colony count in the peritoneal cavity. Blood samples from mice which were sacrificed or died in this experiment contained none or only a few colony-formers.

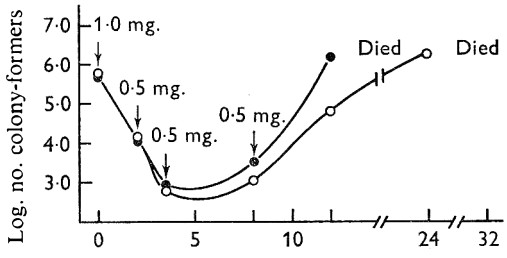

Fig. 2

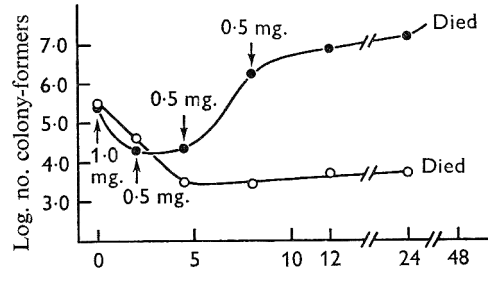

Fig. 3

Fig. 2. *Bacillus anthracis*: number of colony-formers in the peritoneal cavity of mice infected with 5×10^6 pre-germinated spores of strain *pur*-1. Geometric means of pooled data of three experiments. ○, No hypoxanthine; ●, hypoxanthine given. Arrows indicate the intraperitoneal injection of hypoxanthine.

Fig. 3. *Bacillus anthracis*: the number of colony-formers in the peritoneal cavity of mice infected with 5×10^6 pre-germinated spores of *gua*-1. Geometric means of pooled data of several experiments. ○, No guanine; ●, guanine given. Arrows indicate the intraperitoneal injection of guanine.

Microscopic observation of the peritoneal smears showed that the long chains broke into smaller units within a few hours after infection. This was associated with a diminished intensity of staining of many of the bacilli, which was soon followed by a gradual disintegration of the organisms. The capsule can withstand disintegration better than the bacillus itself; this resulted in many empty capsules. Finally, after 24 hr, the whole microscopic field was filled with empty capsules, ghost-like structures and disintegrated remnants, with only a few well-stained bacilli. The accumulation of host cells in the abdominal cavity of the infected mouse was slight or moderate as compared with the number of bacilli present. Besides small lymphocytes and cells with lobulated nucleus, giant cells, probably monocytes containing some engulfed disintegrated bacilli, were seen in the smears.

The experiments dealt with so far were exclusively made with strain *ade* II-3 C^+. The study of the growth of adenine-dependent strains *in vivo* was extended to other isolates belonging to group II; this resulted in identical observations about their virulence. None of the mice died of anthrax when injected with 5×10^6 spores intraperitoneally and with 5 mg. adenine in oil subcutaneously.

Isolates belonging to group I because of failure to form adenylosuccinate synthase were also found to be avirulent. Spores (5×10^6) of either of the strains *ade* I-1 C^+ or *ade* I-2 C^+ did not grow in the peritoneal cavity sufficiently to produce toxic death by anthrax bacteraemia, although the mice were given toxic doses of adenine. In comparison, spores of the prototrophic revertants of the above mentioned isolates grew rapidly in the body of the mouse, causing death.

In vivo growth of other purine auxotrophs

Both the strains *pur*-1 and *gua*-1 appeared to be highly virulent when examined by a conventional test for virulence (Ivánovics & Marjai, 1964). Their virulence could not be related to a 'leaky-ness' in their purine requirement, since their growth responses to the specific metabolites showed a similar intensity to those of the adenine-dependent mutants. *In vivo* multiplication of the above-mentioned strains was examined in the same way as the adenine-dependent mutants (Fig. 2, 3). The colony count of strain *pur*-1 showed a rapid decline in the initial phase of the experiment followed by an increase in number. All the mice died of generalized anthrax within 24 to 32 hr, even when the infection was not associated with administration of hypoxanthine. Only a moderate enhancing effect on the course of infection was given by hypoxanthine.

Table 5. *Bacillus anthracis: total number of colony-formers of strain gua-1 in the peritoneal cavity and the blood circulation of infected mice*

Mice were infected with 5×10^6 pre-germinated spores of strain *gua*-1; the mice were not given guanine. Total colony count in the blood circulation was calculated on the base of body weight of animals. Total blood: one-twelfth of body weight.

Time (hr)	No. of mice	Log values of geometric mean and its standard deviation	
		Peritoneal cavity	Blood circulation
5	2	$3{\cdot}84 \pm 0{\cdot}15$	$2{\cdot}77 \pm 0{\cdot}45$
7·5	1	3·50	2·62
20	2	$5{\cdot}01 \pm 0{\cdot}17$	$5{\cdot}29 \pm 0{\cdot}41$
24	5	$4{\cdot}52 \pm 0{\cdot}34$	$5{\cdot}89 \pm 0{\cdot}89$
32	3	$5{\cdot}04 \pm 0{\cdot}32$	$6{\cdot}74 \pm 0{\cdot}48$

Curves depicting the progress of infection with strain *gua*-1 showed a somewhat different pattern from that observed with the *pur*- auxotroph. The enhancing effect of guanine was very marked, although both groups of mice, whether or not treated with guanine, died 24 to 48 hr after infection. The marked enhancing effect of guanine indicated a shortage of this base in the peritoneal cavity. Nevertheless, the survival times of mice in the group without guanine were only a few hours longer than that of the group given guanine. Blood samples, taken a few hours after infection from mice not given guanine, yielded auxotrophic bacteria. A group of mice, each injected with 5×10^6 pre-germinated spores of strain *gua*-1, had the bacterial count determined in the peritoneal cavity as well as in the blood of individual mice at intervals (Table 5). Mice sacrificed as early as 5 hr after infection already had a bacteraemia. The total number of colony-formers in the whole blood circulation, however, was significantly lower than that in the peritoneal cavity. The colony counts were about the same by 7·5 hr after infection; bacilli in the blood finally outnumbered those in the peritoneal cavity. On the basis of this finding, one may suppose that the guanine supply in blood was ample to support growth of the bacilli to a degree fatal to the host. However, most of the mice died within 24 hr; survival after this period may have been due to individual variation in susceptibility of the mice. This may also account for the relatively low total number of bacilli circulating in the blood in the later part of the experiment.

Toxin production by adenine-dependent mutants

The complete lack of virulence of strains belonging to both groups of adenine-dependent mutants could not be related to any impairment in capsule formation (Table 4). Besides capsule production, another 'aggressin' produced by *Bacillus anthracis* is an oedema-producing toxin which later in the course of disease acts to kill the host (Smith, Keppie & Stanley, 1955). The question arose: could the lack of virulence of adenine-dependent strains be related to a loss of toxin production associated with mutation at the adenine locus? To examine this possibility toxin production *in vitro* by adenine-dependent strains belonging to both groups I and II was compared with that of the prototrophic strain VC^+. Several batches of culture filtrates were obtained from each strain and tested for ability to produce an oedematous lesion in guinea-pig skin and to give a precipitate in the agar diffusion method. All the filtrates appeared to be identical in potency either *in vivo* or *in vitro*. After 20 hr, the highest dilution of toxin preparation which just produced a detectable oedematous reaction was between 1/4 and 1/8, with the different filtrates. There was no significant difference in the potency of the filtrates from any of the adenine-dependent mutants and from the prototrophic parent stain; identical results were obtained with agar diffusion tests. The oedema-producing action of the filtrate was neutralized with horse hyperimmune serum effective in the agar diffusion test.

Table 6. *Bacillus anthracis: growth of strain ade II-3 in the presence of heparinized plasma obtained from mice injected subcutaneously with 5 mg. adenine in oil*

Colony counts: in BCM without plasma, $2{\cdot}6 \times 10^4$; in BCM + adenine, 10 μg./ml., $4{\cdot}7 \times 10^6$. The numbers in the Table represent the colony counts after an inoculum of $2{\cdot}5 \times 10^4$ colony-formers/ml. There was considerable chain formation in tubes at 2·5 to 24 hr, and in BCM + adenine 10 μg./ml.

Time of sampling (hr)	Number of colony-formers/ml.† after 24 hr incubation
0*	$2{\cdot}5 \times 10^4$
2·5	$3{\cdot}8 \times 10^6$
4·5	$7{\cdot}0 \times 10^5$
10	$2{\cdot}5 \times 10^5$
24	$1{\cdot}8 \times 10^5$

* Plasma from animals before inoculation of adenine.
† Colonies counted on BCM + adenine.

Availability of adenine in the body of mice given adenine

Even a generous supply of adenine to the mice did not support that degree of growth of the adenine-dependent bacilli necessary to kill the mice. Whether lack of adenine in the host or some other factor was involved in making the bacilli incapable of invading the blood stream was open to question. Attempts were therefore made to determine the adenine concentration in the bodies of mice given adenine. Apart from the difficulties of using chemical methods to determine adenine in the body fluids, preference was given to microbiological assay for two reasons: (i) it is simple to perform; (ii) the total utilizable metabolites are assayed, since the adenine-dependent strain can use, in addition to adenine itself, its nucleoside readily and its nucleotides

poorly. When mice, given 1 mg. adenine intraperitoneally, were sacrificed in pairs after 2 hr and the pooled washings of their peritoneal cavities assayed, a total of 30–35 μg. adenine was found. Thus, about 3% of the adenine injected was detected at this time. Repeated injections of 0·5 mg. adenine at intervals maintained a very low total amount (about 2 to 3 μg.) in the pooled washings. Nevertheless, the amount of adenine in the peritoneal fluid, the volume of which was not more than a few tenths of a millilitre, must have been enough to support growth of the bacilli. After injection of 5 mg. adenine, heparinized plasma samples were assayed for adenine, and the concentration of utilizable adenine metabolites detected with strain *ade* II-3. It can be seen from Table 6 that there was about the same growth of the inoculated bacilli in the sample taken 2 hr after the injection of adenine as was found in BCM+adenine 10 μg./ml. Substantial growth of bacilli was still supported by the plasma sample taken 10 hr after injection; there was also a slight growth of bacteria in the 24 hr sample.

It can be concluded from these results that there was rapid elimination of utilizable adenine from the body of the mouse, although its concentration appeared to be sufficient to support some growth of bacilli in the peritoneal cavity and in the blood stream, particularly when a heavy inoculum of encapsulated bacilli was used for infection. No substantial growth of *gua*-1 mutant was observed in cultures made with the plasma of normal mice. Heating the plasma samples (at 55° for 30 min.) before their addition to the assay system did not change the result observed with unheated plasma.

DISCUSSION

The literature on the virulence of auxotrophic mutants of Gram-negative pathogens has been reviewed by Burrows (1960), Panos & Ajl (1963) and Braun (1965); only relevant aspects will be discussed here. It can be stated that, according to the specific requirement of individual auxotrophs, they may either retain or lose their virulence. Availability of the required metabolite *in vivo* is apparently a crucial point in determining virulence. Thus, generally speaking, mutants which require either amino acids or vitamins, of which there is an ample source in the host, grow readily *in vivo*. There are, however, seemingly contradictory observations about the virulence of purine-requiring auxotrophs. Gowen, Stadler, Plough & Miller (1953) found that some of their adenine-dependent isolates of *Salmonella typhimurium* were of low virulence or avirulent, in contrast to others which displayed considerable virulence. Unfortunately, no data were presented about the precise block in the purine pathway of these auxotrophic strains; all their strains were referred to as 'adenine mutants'. The purine-requiring mutants of *Salmonella typhi* isolated and studied by Bacon, Burrows & Yates (1950*a*, *b*, 1951) were also only partially specified. It appears that some of these strains were defective either in the early part of purine synthesis or in the conversion of inosinic acid to guanylic acid. The low virulence of some of these auxotrophs was enhanced by injection of hypoxanthine simultaneously with the bacterium (Bacon *et al.* 1951). A similar observation was described by Formal, Baron & Spilman (1954) for a xanthine-dependent mutant of *S. typhi*. A purine auxotroph of *Pasteurella pestis* was studied by Burrows (1955), the growth requirement of which was met by hypoxanthine; it appeared to be of low virulence when bacteria only were injected, but its virulence was restored by administering hypoxanthine to the host when challenged with the mutant. So far as we know, no study has been made with an auxotrophic

mutant of any pathogenic bacterium with a specific requirement for adenine; that is, with strains in which the conversion of inosinic acid to adenylic acid is blocked.

It was reported from this laboratory (Iványovics & Marjai, 1964) that purine auxotrophs of *Bacillus anthracis* varied in virulence according to the site of the block in the purine pathway. In the present work we succeeded in isolating adenine-dependent mutants which could be grouped into two genotypes by their biochemical characteristics. Both groups of mutants, lacking either adenylosuccinate synthase or adenylosuccinate lyase, were unable to proliferate in the peritoneal cavity of the mouse, although the animals were injected with a large inoculum of spores and were given a toxic dose of adenine at the time of challenge. When a heavy inoculum of encapsulated bacilli was used, under similar experimental conditions as for the spore inoculum, a massive invasion of blood by the bacilli was not observed, although a certain number of viable organisms persisted in the peritoneal cavity. It seems that the limited number of bacilli which reached the blood stream, despite the presence of adenine in the blood which might support their growth, were hindered in proliferation by the defence mechanisms of the host. Apparently, adenine-dependent *B. anthracis* organisms are highly susceptible to these defence mechanisms, a supposition corroborated by their rapid disintegration in the peritoneal cavity.

In contrast to the adenine-dependent mutants of *Bacillus anthracis*, purine auxotrophs, either defective in the early part of the purine pathway or in the conversion of xanthylic acid to guanylic acid, grew readily in the mouse peritoneal cavity, leading to a generalized infection and death. A standard infecting dose of spores killed a mouse even without an exogeneous supply of purine; however, the course of infection was augmented by adequate administration of purine.

It is of interest that, although the multiplication of the guanine-dependent mutant in the peritoneal cavity was moderate, nevertheless the bacilli readily invaded the blood stream in the early period of infection. Bacilli which reached the blood stream multiplied freely and outnumbered the bacilli present at the site of infection. It is open to question how this mutant had access to the purine needed for its proliferation in the body. Formal *et al.* (1954) were not able to detect the presence of metabolites needed by their xanthine-dependant mutant of *Salmonella typhi* in the peritoneal fluid of mice. We also failed to demonstrate an ample availability of guanine in the blood of plasma this host. Nevertheless, a guanine-dependent strain of *Bacillus anthracis* grew readily both in the mouse peritoneal cavity and blood stream. A minimal amount of guanine and other purines in the peritoneal cavity may originate from injury caused by injection of the inoculum. When a small amount of sterile salt solution was injected intraperitoneally into a mouse, this evoked an accumulation of material absorbing at 260 mμ in the peritoneal washings (unpublished observation). Apparently, this minimal amount of purine was capable of initiating growth of germinated *B. anthracis* spores; the subsequent multiplication of the bacilli in the peritoneal cavity, and their invasion of the blood stream, may cause an influx of purine to the site of infection and to the blood stream from damaged host cells and tissues.

In view of the evidence presented above, it does not seem reasonable to ascribe the lack of virulence of the adenine-dependent mutants of *Bacillus anthracis* entirely to shortage of available adenine in the host. It may be that there is, in addition to capsule and toxin production, an intrinsic entity necessary for the proliferation *in vivo* of *B. anthracis*, and that this function is not present in the adenine-dependent mutants.

The authors are very much indebted to Professor H. Smith (Birmingham) for the unpublished formula of his medium and for a generous gift of anthrax antitoxic serum. We thank Dr Haruo Momose (Kawasaki, Japan) for the sample of adenylosuccinic acid he kindly gave us. Thanks are also due to Dr K. Csiszár for his participation in part of this investigation, and to Dr J. McGeachie (Royal Infirmary, Glasgow) for his help in preparing the manuscript.

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J. gen. Microbiol. (1968), **53**, 163–169
With 3 plates
Printed in Great Britain

Bud Formation in *Saccharomyces cerevisiae* and a Comparison with the Mechanism of Cell Division in Other Yeasts

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(*Accepted for publication 27 March 1968*)

SUMMARY

Bud formation in *Saccharomyces cerevisiae* occurs by an extension of the entire parent cell wall, as described previously. The dividing walls between the bud and the parent cell are laid down simultaneously, but the bud septum is completed more rapidly. The dividing walls separate before the bud is mature. Finally, the bud is released by the breakdown of the outer wall. A comparison between bud formation in *S. cerevisiae* and *Rhodotorula glutinis* reveals basic differences in the mechanisms which can be related to structures in mycelial fungi.

INTRODUCTION

There have been a number of investigations of the ultrastructure of *Saccharomyces cerevisiae*, commencing with the early studies of Agar & Douglas (1955) through to the freeze-etch study of Moor (1967). *S. cerevisiae* has proved a troublesome organism to study in the electron microscope as it is difficult to obtain satisfactory fixation using conventional techniques. This accounts for the confusion over certain aspects of yeast cytology and especially bud formation. Only recently (McClary & Bowers, 1965) has it been established unequivocally that the normal bud wall in *S. cerevisiae* is a continuation of the parent wall, and indeed that the bud is always surrounded by a wall layer. Although the relationship between the parent wall and the bud wall is now established there still remains the problem of how the bud is delimited and released from the parent cell. Marchant & Smith (1967) have shown that in *Rhodotorula glutinis* the bud wall is not formed from a direct continuation of the parent wall, but from a new wall layer formed within the parent cell. The different mode of formation of the bud in these two species immediately provokes a discussion on whether the two mechanisms have different origins or whether one is a simple modification of the other. In this paper we shall describe the budding process in *S. cerevisiae* in more detail than has been done previously and compare it with the already described process in *R. glutinis* and other yeasts. We shall also attempt to relate the differences to the existing phylogenetic schemes.

METHODS

The organism used for this study was *Saccharomyces cerevisiae* strain 21, kindly supplied by Dr D. Wilkie. This haploid strain, in common with most other haploid strains, tends to have several buds developing simultaneously. It was grown in a modified Wickerham medium (Wickerham, 1946) with 2% (w/v) melibiose as carbon source. This non-repressing fermentable substrate was selected as it was found to give

better preservation of cytological detail than glucose. The cells were harvested towards the end of the active growth phase, fixed and embedded according to Marchant & Smith (1967). Sections were cut with a diamond knife on an LKB ultratome, and post-stained with saturated alcoholic uranyl acetate (Gibbons & Grimstone, 1960) and lead citrate (Reynolds, 1963). The material was examined in a Siemens Elmiskop 1 electron microscope.

RESULTS

In the youngest bud stages (Pl. 1, fig. 1) the bud wall is obviously a continuation of the parent cell wall, as established by McClary & Bowers (1965). The young bud wall is, however, slightly thinner than the parent wall at this stage, and probably contains a certain amount of parent cell wall material as suggested by Chung, Hawirko & Isaac (1965). At the junction of the parent and bud cell walls, on the inner surface, can be seen an encircling layer of less electron-opaque wall material. The wall at this point is also thicker than at other parts of the parent cell, probably as a result of a modification of pre-existing wall material. Endoplasmic reticulum can often be observed in the young bud, as reported by Marchant & Smith (1967) and Moor (1967).

As the bud grows the electron-transparent region in the wall becomes more extensive and better defined (Pl. 1, fig. 2). At this stage organelles become evident in the bud, and the endoplasmic reticulum still appears to be active. Finally the bud reaches almost the same size as the parent cell and possesses a full complement of organelles, but there is no evidence of septum formation separating the bud from the parent cell (Pl. 1, fig. 3). Intermediate stages in septum formation are difficult to distinguish satisfactorily from oblique sections, but in our opinion the micrographs in Pl. 1, fig. 4 and Pl. 2, fig. 5 show the centripetal formation of the septa. The bud septum and the parent cell septum are formed simultaneously but it appears that the two septa are maintained as separate entities from the time of their formation; the parent cell septum is initially thinner and undergoes some modification in structure during development (Pl. 2, fig. 6, 7). The electron-transparent wall regions lose some of their integrity during the formation of the septa, but this is regained when the septa are mature.

The bud wall is initially continuous with the bud septum and with the parent cell wall (Pl. 2, fig. 8). Later the bud wall and the parent cell wall become separate (Pl. 3, fig. 9), possibly due to an outward increase in extent of the electron-transparent layer. The bud scar left on the parent cell after the release of the bud (Pl. 3, fig. 10) is essentially similar in appearance to that observed in *Rhodotorula glutinis* (Marchant & Smith, 1967). The concavity in the bud cell in the region of the birth scar gradually disappears until it is no longer visible in sections. The stages in bud formation in *Saccharomyces cerevisiae* are summarized in Fig. 1.

DISCUSSION

We have confirmed the assertion of McClary & Bowers (1965) that the bud wall in *Saccharomyces cerevisiae* is continuous with the existing parent cell wall. In addition we have demonstrated that the two wall layers dividing the parent cell and the bud are separate from each other long before the bud is mature. The bud is released from the parent cell by the cleavage of the outer wall where it is continuous with the parent cell wall. *Rhodotorula glutinis* is somewhat different in that the bud wall is not a continua-

tion of the major part of the parent cell wall. The dividing wall layers in this instance are not separate from each other during bud development, but cleave to release the bud (Fig. 2). In this respect a parallel can be drawn between the release of the daughter cell in *R. glutinis* and the separation of the fission cells in *Schizosaccharomyces octosporus* (Conti & Naylor, 1959).

Nečas & Svoboda (1967) in their study of regenerating protoplasts of *Saccharomyces cerevisiae* described a budding process similar to that described for *Rhodotorula glutinis* (Marchant & Smith, 1967). They do say, however, that the walls of buds formed from normal cells and from regenerating protoplasts may develop by different

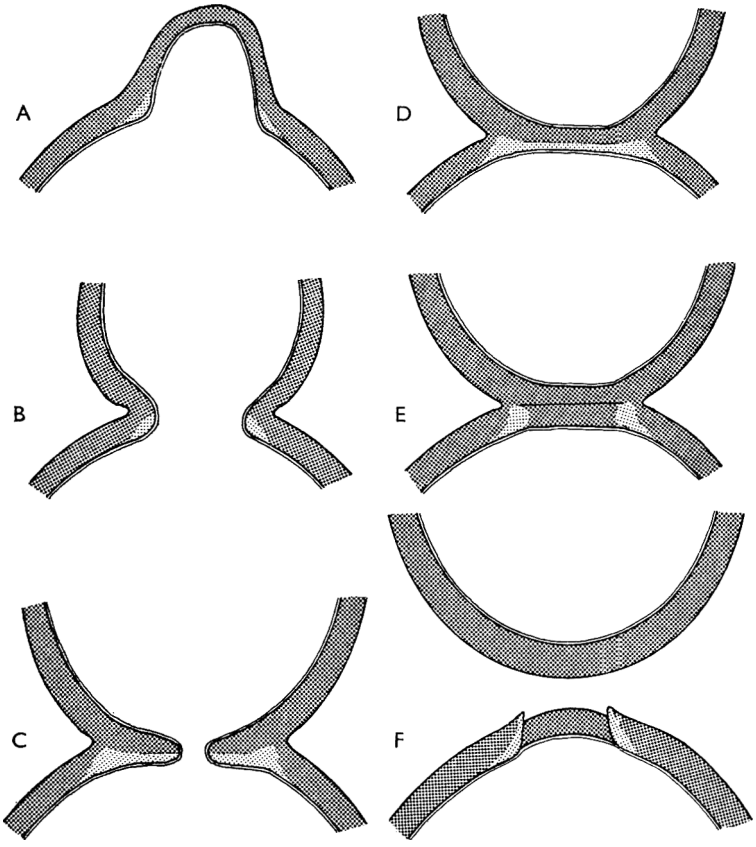

Fig. 1. Diagrammatic representation of the proposed stages of bud formation in *Saccharomyces cerevisiae*.

mechanisms. The scheme which they propose for budding in the regenerating protoplasts is obviously at variance with the mechanism in normal cells determined both from our observations and those of previous workers (McClary & Bowers, 1965; Hagedorn, 1964).

It is now certain that young buds in these yeasts are surrounded by a wall layer from their inception and do not result from a naked 'blow out' as suggested by Nickerson & Falcone (1959) and Nickerson (1963). Chung *et al.* (1965) have further shown that the bud contains little of the original parent cell-wall material and, in view of the fact that

the bud cell wall is always of a substantial thickness, this implies that cell wall synthesis in *Saccharomyces cerevisiae* must proceed rapidly to maintain bud growth. Moor (1967) noted that vesicles from the endoplasmic reticulum seemed to initiate bud formation in *S. cerevisiae*, and suggested that the vesicles carried the wall plasticizing enzymes to the site of incipient bud formation. Similar endoplasmic reticulum and vesicles were noted in developing buds of *Rhodotorula glutinis* by Marchant & Smith (1967), who proposed that wall material precursors might be transported in these vesicles. This proposal was based on the work of Marchant, Peat & Banbury (1967) on the growth of fungal hyphae. Obviously there must be some modification of the parent cell wall at the site of bud formation in yeasts and therefore the two proposals for the possible function of the endoplasmic reticulum and vesicles are not mutually exclusive.

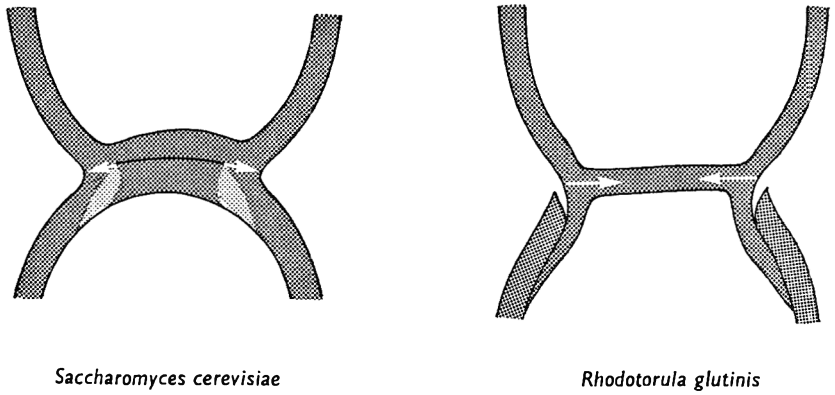

Fig. 2. Diagram illustrating the different modes of bud separation in *Saccharomyces cerevisiae* and *Rhodotorula glutinis*. The arrows indicate the direction of cleavage.

It appears that the derivation of the bud wall in *Saccharomyces cerevisiae* and *Rhodotorula glutinis* differs quite considerably. The bud wall of *S. cerevisiae* is a continuation of the whole of the parent cell wall rather than some of its constituent layers. Marchant & Smith (1967) stated that the bud wall in *R. glutinis* is formed from an entirely new wall layer produced inside the existing parent cell wall. Prusso & Wells (1967) in their study of *Sporobolomyces roseus* suggested that when the organism buds, produces a hyphal strand or gives rise to a sterigma, the outer wall layers are ruptured by the innermost pair of existing wall layers. They also stated that published micrographs of Rhodotorula species showed budding of this type. A similar controversy has arisen over spore germination in several filamentous fungi; Hawker & Abbot (1963) observed the formation of a new wall layer within the spores of Rhizopus species prior to germination. This interpretation was criticized by Tanaka (1966), who suggested that it might be simply a visualization of existing wall layers rather than *de novo* synthesis of a new layer. A similar mode of germination was, however, reported by Marchant (1966*a*, *b*) in *Fusarium culmorum* conidia. It is therefore feasible that the bud wall in *R. glutinis* may also be formed from a new wall layer produced within the parent cell rather than from existing wall layers.

When a comparison is made between budding and fission in the various yeasts the

similarities between all the processes become apparent. In all the budding systems examined new cell wall material is incorporated apically with respect to the plane of division. Therefore the new cell wall material is deposited in the bud wall, which is composed almost entirely of new material, although this deposition may not occur evenly round the bud (Chung *et al.* 1964). In the fission yeasts apical incorporation of wall material has also been observed using morphological features of the cells as markers (Mitchison, 1957) and by fluorescent antibody techniques (May, 1962). The portions of the daughter cells which have wall material synthesized after the initiation

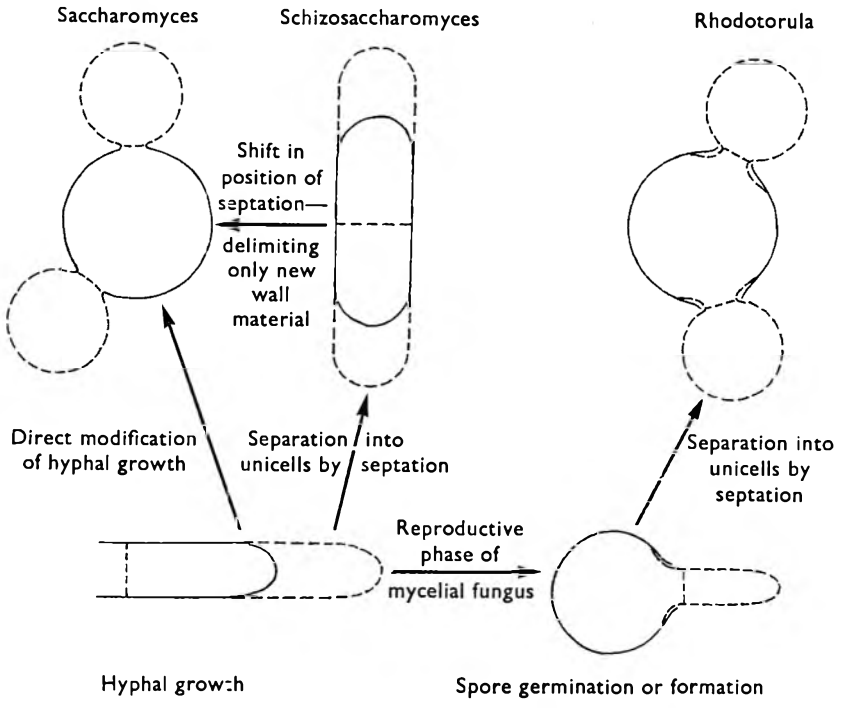

Fig. 3. Possible relationships between the growth forms of yeasts and mycelial fungi. Broken lines represent regions of new wall synthesis.

of division are summarized in Fig. 3. The budding species have entirely new cell walls around their daughter cells, while the fission species have some of the parent cell wall remaining in the daughter cells.

The yeasts represent an ill-defined group of morphologically similar species probably with polyphyletic origins. A discussion of the various modes of division, examined at the ultrastructural level, and comparisons with other fungi, may give a clue to possible lines of development of the yeast growth form. The apical addition of wall material can be compared directly with the growth of the fungal hyphae; in the unicellular condition some of the polarity of growth has necessarily been lost. In both *Saccharomyces cerevisiae* and Schizosaccharomyces species the growth of the daughter cell wall is similar to hyphal growth, producing a continuous extension of a single wall structure. In the yeasts septation gives rise to unicells rather than a septate mycelium;

some mycelial fungi, however, are capable of existing as yeast-like cells under certain conditions which substantiates the hypothesis that the yeast condition is not far removed from the mycelial condition. Daughter-cell formation in *S. cerevisiae* and Schizosaccharomyces species could therefore be a direct modification of hyphal growth. In *S. cerevisiae*, however, the position of septation has been moved so that only new wall material is cut-off in the bud, in contrast with the semiconservative fission of Schizosaccharomyces species. The system of budding in *S. cerevisiae* may have been derived from the fission mechanism or may have arisen independently from the hyphal form. Bud formation in *Rhodotorula glutinis*, in contrast, is comparable with the reproductive stages of certain mycelial fungi: spore formation (Trinci, Peat & Banbury, 1968; Lowry, Durkee & Susmann, 1967) and spore germination (Hawker & Abbott, 1963; Marchant, 1966*a*, *b*) (see Fig. 3). This suggests that the *R. glutinis* type of budding may have arisen from the reproductive phase of some mycelial fungi, a view to some extent supported by the fact that this mechanism can be traced in *Sporobolomyces roseus* (Prusso & Wells, 1967), which is thought to be a modified basidiospore system (Lodder, Slooff & Kreger-van Rij, 1958).

We would like to thank Dr D. Wilkie for kindly supplying *Saccharomyces cerevisiae* strain 21.

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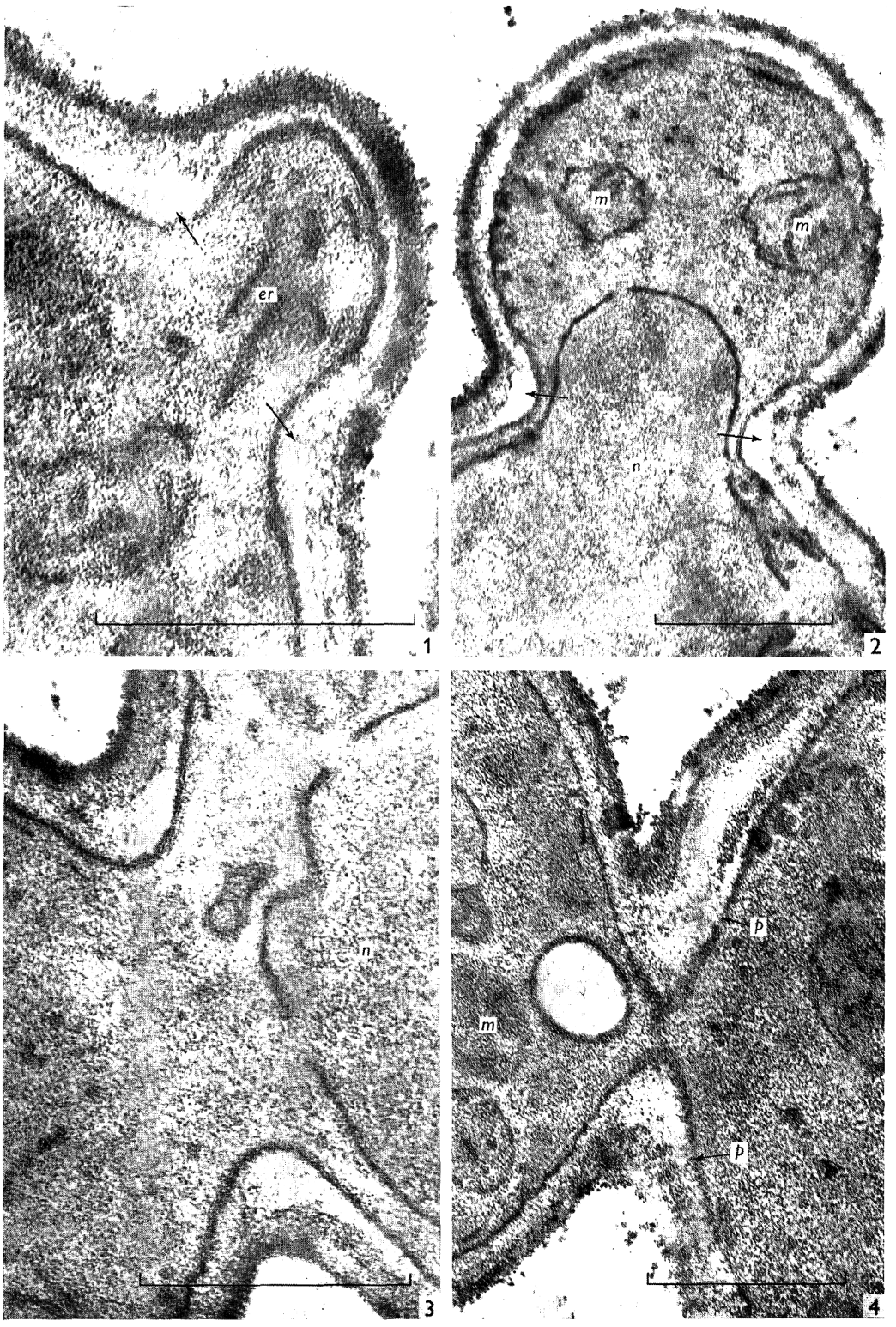

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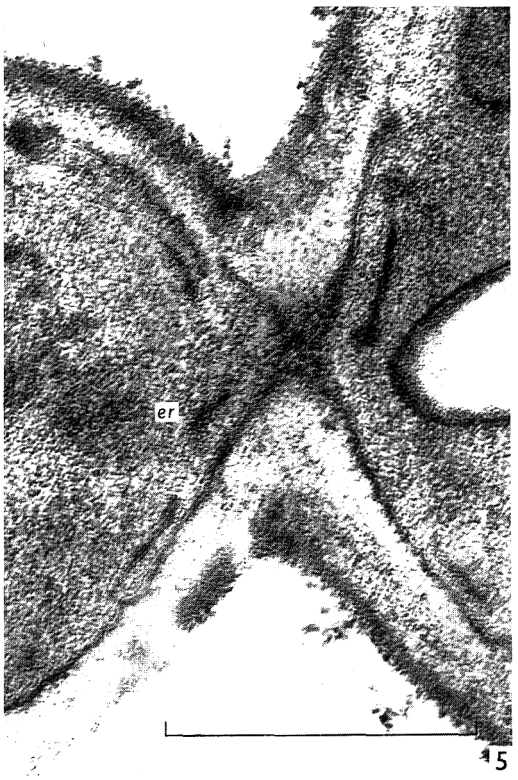

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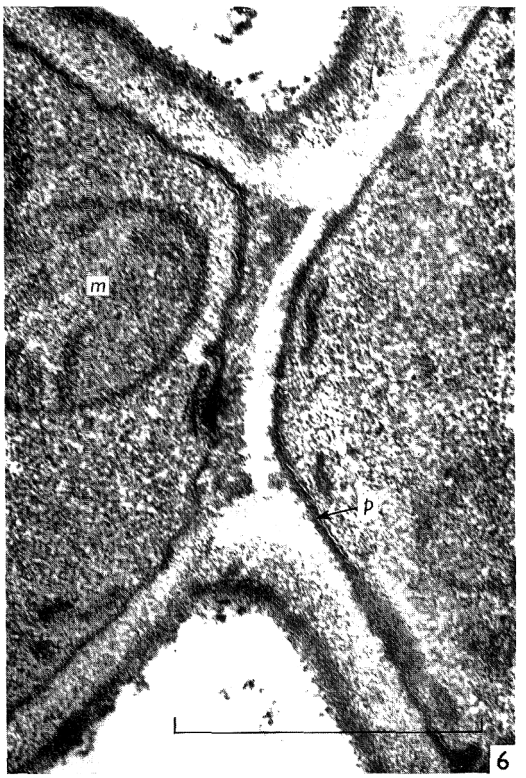

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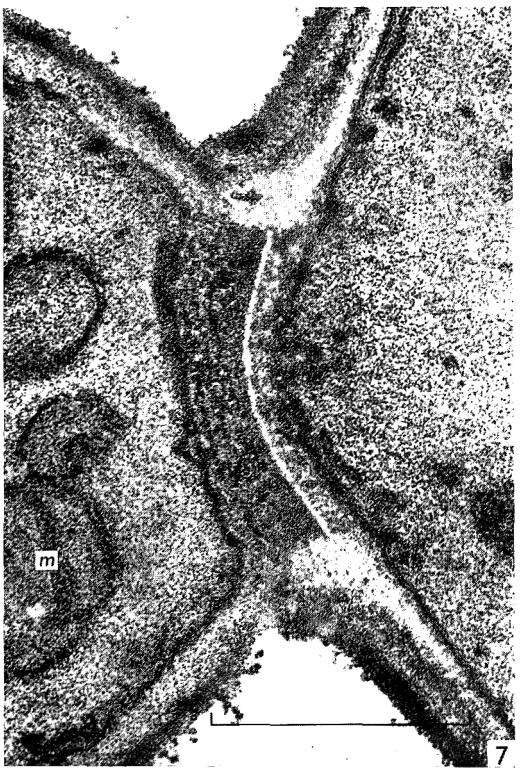

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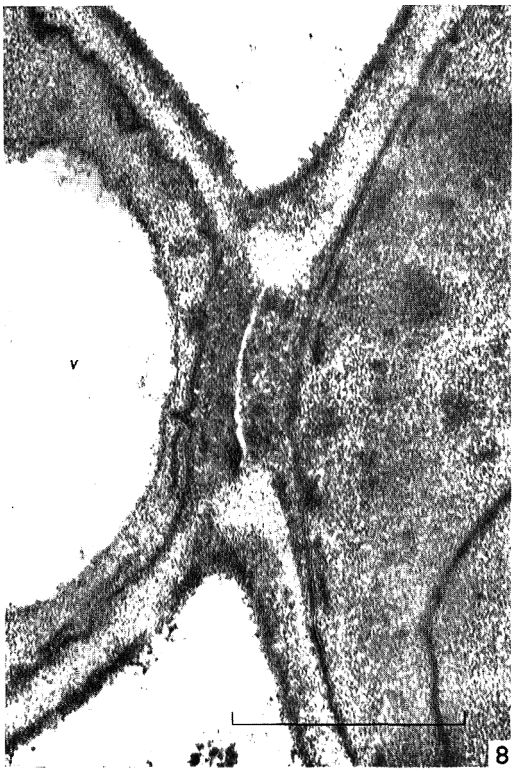

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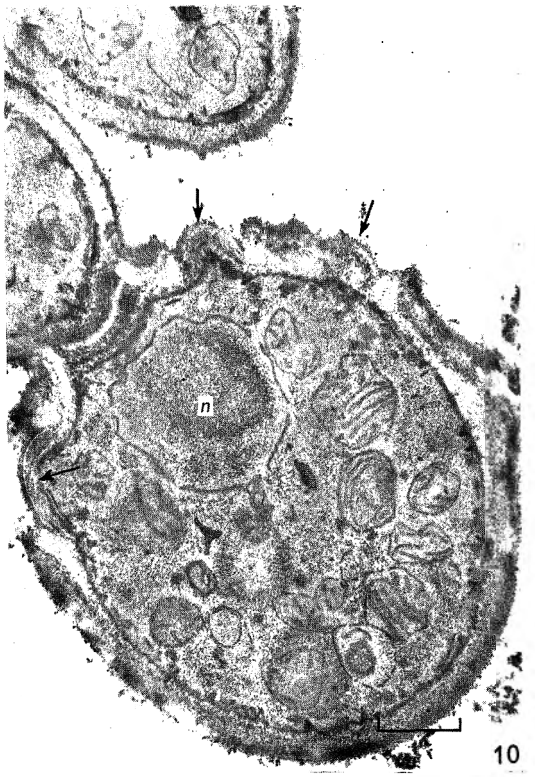

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EXPLANATION OF PLATES

Abbreviations: *er*, endoplasmic reticulum; *m*, mitochondrion; *n*, nucleus; *p*, plasmalemma; *v*, vacuole. Scale lines represent 0·5 μ. In figs. 3–10 the buds are situated to the left of the micrographs.

PLATE 1

Fig. 1. Early stage in bud formation, showing continuity of bud and parent walls and initial development of electron-transparent wall areas (arrowed).

Fig. 2. Small bud already possessing some organelles and with clearly defined electron-transparent wall areas (arrowed).

Fig. 3. Junction of parent and daughter cells immediately prior to septum formation.

Fig. 4. Initial stage in centripetal formation of cross septa.

PLATE 2

Fig. 5. Further stage in the development of transeptation.

Fig. 6. Junction of parent and daughter cells soon after completion of the septa, showing the very thin cell septum.

Fig. 7. Section showing the two septa separate and unequally developed.

Fig. 8. Septa fully developed and separated. Bud wall still continuous with parent cell wall.

PLATE 3

Fig. 9. Septa fully developed; bud wall beginning to become delimited from the parent wall.

Fig. 10. Old cell with several bud scars (arrowed), illustrating the permanence of the electron-transparent wall areas.

J. gen. Microbiol. (1968), **53**, 171–179
Printed in Great Britain

The Location of Nisin in the Producer Organism, *Streptococcus lactis*

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(*Accepted for publication* 28 *March* 1968)

SUMMARY

Streptococcus lactis organisms grown in a glucose-containing medium pH maintained at pH 6·8 (neutral cocci) contained three- to seven-fold more nisin/unit dry weight than cocci grown in the same medium without pH control (acid cocci, terminal pH 4·2). After chemical fractionation of acid cocci 57 % of the nisin was found in the fraction soluble in aqueous ethanol and 36 % in a trypsin-insoluble residue. After fractionation of broken cocci (acid and neutral) by differential centrifugation up to 60 % of the nisin was found in the 10,000 *g* sediment (walls). Nisin was also present in the 30,000 *g* sediment (membranes) and the 100,000 *g* sediment (ribosomes). The major difference between acid and neutral cocci was in the 100,000 *g* supernatant fluid (cell sap); cell sap from neutral cocci was 0·28 % nisin whereas that from acid cocci was 0·04 % nisin. Analysis of the cell wall indicated that it was composed of mucopeptide and polysaccharide. Teichoic acid could not be extracted. The polysaccharide was soluble in hot formamide and accounted for one-third of the dry weight of the wall; it contained rhamnose, galactose, glucose, glucosamine in the molar ratio of 5:1:1:1. Cell walls contributed 31 % to the dry weight of acid cocci and 42 % to the dry weight of neutral cocci.

INTRODUCTION

Many suggestions now exist in the literature that basic polypeptide antibiotics play a role in the bacterial differentiation which occurs at the time of sporulation (e.g. Schaeffer *et al.* 1963, Halvorson, 1965). Recently, further evidence supporting this notion has been obtained with *Bacillus polymixa* (Paulus, 1967). The role of basic proteins in the more overt differentiation processes of eukarytotic systems has been under discussion for several years (de Reuck & Knight, 1966). Recently, for example, Butler & Chipperfield (1967) showed that the inhibition of RNA polymerase by histones was a genuine effect and not an artefact due to decreased solubility. Certain strains of *Streptococcus lactis* produce an antibiotic called nisin, which is active against several Gram-positive bacteria (Mattick & Hirsch, 1947). This antibiotic is a small basic protein, and Hurst (1967) suggested that it may play a regulatory role in the producer organism connected with initiation and halting of growth. Thus the length of lag-phase of growth is directly related to the nisin content of the organism (Hurst & Dring, 1967). Recently Gross & Morell (1967) proposed that the antibiotic

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effect of nisin depends on interception of sulphydryl groups of metabolically important molecules such as co-enzyme A. Cheeseman & Berridge (1959) proposed that the molecular weight of nisin is 7,000, however Gross & Morell (1967) reported a value of 3,500; our own work (Ingram, Tombs & Hurst, 1967) has confirmed the original estimate of 7,000. Unlike other polypeptide antibiotics so far studied in detail, nisin appears to be synthesized ribosomally (Hurst, 1966*a*). In view of the possible function of nisin as a regulator in the producer organism we attempted in the present work to locate it within the cell by using chemical and physical procedures of cell fractionation.

METHODS

Organisms, media and culture conditions have already been described (Hurst, 1966*a*, *b*)

Nisin was estimated by bioassay against *Streptococcus cremoris* strain IP 5 using the standard of Hurst (1966*a*). Nisin bound to bacteria (cell nisin) was extracted and assayed after washing the cocci in fresh medium. Cell fractions for bioassay were adjusted with 10 N-HCl to give a final concentration of 0·05 N-HCl. HCl was not added to samples containing trichloroacetic acid (TCA). Before assay, samples were heated in a boiling water-bath for 5 min., centrifuged and the clear supernatant fluid used for assay.

Chemical fractionation of bacteria was as described by Park & Hancock (1960) except that the time of digestion with trypsin was increased to 3 hr. Briefly this method consisted of suspending a pellet containing 3 mg. dry wt in 3 ml. cold 5% TCA. The supernatant after centrifuging contained the low molecular weight TCA soluble substances. The residue was extracted at room temperature with 75% aqueous ethanol; the supernatant after centrifuging contained much of the lipid and other alcohol soluble cellular material. The residue was extracted with 5% TCA at 90° for 6 min. to dissolve nucleic acid and the residue was trypsin digested for 3 hr at 37° (1 mg. crystalline trypsin/ml. of 0·05 M-$(NH_4)HCO_3$ containing 0·005 N-NH_4OH). The final residue after trypsin extraction was resuspended in 3 ml. of 0·05 N-HCl, placed in a boiling water-bath for 5 min., centrifuged and the clear supernatant assayed.

Physical fractionation of bacteria. Washed suspensions of *Streptococcus lactis* strain in water were disrupted in a French pressure cell (American Instrument Co. Inc., Silver Spring, Md., U.S.A.) or a Braun homogenizer (Shandon Instrument Co., London), any intact cocci which survived being removed by twice centrifuging at 3000 *g* for 10 min., and discarding the sediment. The broken cocci were separated into four fractions by differential centrifugation: (i) 10,000 *g* sediment; (ii) 30,000 *g* sediment; (iii) 100,000 *g* sediment: (iv) 100,000 *g* supernatant fluid.

Cell walls (10,000 *g* sediment) were prepared by centrifuging broken cocci at 10,000 *g* for 10 min., washing the sediment twice with M-NaCl, and twice with distilled water. Cell walls were then digested with trypsin (100 μg./ml. at 37° in 0·01 M-sodium phosphate buffer pH 7·0 for 3 hr), after digestion the walls were washed and resuspended in distilled water.

Membranes (30,000 *g* sediment) were prepared by centrifuging the 10,000 *g* supernatant fluid at 30,000 *g* for 30 min. and washing the sediment twice with M-NaCl and twice with water. Although the 30,000 *g* sediment was designated membranes it undoubtedly contained other material as well, and was frequently contaminated with cell walls (as judged by amino sugar content); attempts to prepare membranes from spheroplasts of *Streptococcus lactis* were not successful.

Ribosomes (100,000 *g* sediment) were sedimented from the 30,000 *g* supernatant fluid by centrifuging at 100,000 *g* for 3 hr then washed and resuspended in the buffer described by Munro, Jackson & Korner (1964).

When appropriate, the nature of the fractions obtained by differential centrifuging was checked by examining with the electron microscope. Samples were most commonly shadowed, but stained thin sections were also examined.

Hydrolysis of cell walls. 10–20 mg. dry wt of cell walls were hydrolysed with 2 N-H_2SO_4 at 105° for 3 hr. After cooling, the hydrolysate was neutralized with saturated $Ba(OH)_2$ and solid $BaCO_3$ and clarified by centrifugation and filtration.

Neutral sugars were estimated by gas chromatography of their alditol acetate derivatives (Sawardeker, Sloneker & Jeanes, 1965), with mannose as an internal standard.

Amino sugar was estimated by the method of Levy & McAllan (1959). Where an estimate of the relative contributions of glucosamine and muramic acid was required, the methods of Rondle & Morgan (1955) and Cessi & Piliego (1960) were used, the former method giving a different spectrum for these amino sugars, whilst the latter measures glucosamine only.

Protein was determined by the biuret method (Gornall, Bardawill & David, 1957), with bovine plasma albumin as standard.

Reducing sugar was estimated by the method of Park & Johnson (1949), *phosphate* by the method of Allen (1940), and *nitrogen* by the method of Folin & Farmer (1912).

Ribosomal proteins were examined by polyacrylamide gel electrophoresis (Hurst, 1966*b*).

Reagents. A.R. grade reagents were used as far as possible. Mannose was from T. G. Gurr Ltd., London, muramic acid from Sigma Chemical Co., St Louis, U.S.A., glucosamine from British Drug Houses Ltd., Poole, England, trypsin from Seravac Laboratories (Pty) Ltd., Maidenhead, England, RNA-ase from Koch–Light and Co. Ltd., Colnbrook, England and lysozyme from Armour Pharmaceutical Co., Eastbourne, England.

RESULTS

Production of nisin by cultures of Streptococcus lactis

Analysis of stationary phase cultures of *Streptococcus lactis* shows that some of the nisin produced is excreted into the culture fluid (medium nisin) and the remainder bound to the cocci (cell nisin; Hurst & Dring, 1967). Table 1 shows the concentration and distribution of nisin in stationary phase cultures after growth with and without pH control. *S. lactis* is a vigorous acid producer and in an uncontrolled fermentation a limiting value of pH 4·2 may be reached (acid culture); when the culture is maintained at pH 6·8 (neutral culture) a higher yield of organism is obtained (2·6 mg. dry wt/ml. as compared with 0·8 mg. dry wt/ml.). It can be seen that not all the nisin originally present (i.e. cells + medium) can be accounted for after separation of culture fluid from cells (i.e. medium nisin and cell nisin). In these experiments 7 % of the nisin was cell bound in the acid culture and 61 % in the neutral culture. The nisin content of cocci was not diminished by prolonged washing with water or 0·01 M-sodium phosphate buffer (pH 6·8).

Chemical fractionation of Streptococcus lactis organisms

Cocci from an acid stationary-phase culture were fractionated by the method of Park & Hancock (1960) and the nisin content of the different fractions estimated; the results are shown in Table 2. Most of the nisin was recovered in the aqueous ethanol-soluble and trypsin-insoluble fractions. Only 72% of the nisin originally present in the whole cocci was recovered after this chemical fractionation. In one experiment the alcohol extraction step was repeated four times before passing on to the next extraction

Table 1. *Nisin yield and distribution in cultures of Streptococcus lactis grown with and without pH control*

Samples from stationary phase cultures were assayed for nisin content directly (complete culture), and after separation of cocci from culture fluid by centrifugation. The nisin content of cocci was estimated after washing in fresh medium and resuspending and extracting with an equal volume of hot 0·05 N-HCl.

	Nisin (μg./ml. culture)	
	Without pH control (terminal pH 4·2)	With pH control (controlled to pH 6·8)
Complete culture	30	194
Culture fluid	25	52
Cocci	2	82

Table 2. *Distribution of nisin after a chemical fractionation of Streptococcus lactis organisms*

Washed stationary-phase cocci (grown without pH control; 'acid' cocci) were fractionated by the method of Park & Hancock (1960), and the distribution of nisin estimated by bioassay.

Fraction	Nisin content (%) of total recovered
Cold trichloroacetic acid soluble	1
Aqueous ethanol soluble	57
Hot trichloroacetic acid soluble	3
Trypsin-soluble	2
Trypsin-insoluble residue	36

with hot trichloracetic acid. In this case the first alcohol extraction still contained 57% of the nisin and the subsequent extractions contained negligible amounts. The repeated alcohol extractions did not affect the amount of nisin recovered from the trypsin-insoluble residue which remained 36% as shown in Table 2. When whole cocci were incubated with trypsin (100 μg./ml. in 0·01 M-$NaHCO_3$ at 37° for 3 hr) there was no decrease in their nisin content.

Physical fractionation of Streptococcus lactis organisms; nature of the fractions

Broken cocci were fractionated by differential centrifugation as described. The 10,000 *g* pellet material was examined by electron microscopy and showed the classical appearance of cell walls (Salton, 1964). With increasing purification the walls became thinner and less easy to see but even after extraction with hot formamide (200° for 15 min) they retained their original shape.

The 30,000 *g* deposit was pale yellow and contained irregularly shaped particles, about one-tenth the size of cell walls. Chemical analysis supported the suggestion that the 30,000 *g* deposit was mostly membraneous material.

Table 3. *Distribution of nisin after physical fractionation of disrupted Streptococcus lactis organisms*

Cocci from stationary phase cultures ('acid' and 'neutral') were harvested, washed, disrupted and fractionated by differential centrifugation. The nisin content of the fractions was estimated by bioassay.

	Distribution of nisin			
	Acid culture		Neutral culture	
Fraction	% dry wt	% of total recovered	% dry wt	% of total recovered
Whole cocci	0·94	100	2·63	100
10,000 *g* deposit	1·98	65	2·70	43
30,000 *g* deposit	2·10	9	2·47	4
100,000 *g* deposit	1·66	18	2·42	7
100,000 *g* supernatant fluid	0·04	2	0·28	5
Recovery (%) of that in unbroken cells	—	94	—	59

Table 4. *Composition of Streptococcus lactis cell wall*

Cell walls were prepared by differential centrifugation of disrupted 'acid' stationary phase cocci. Intact cocci were removed by discarding the 3000 *g* deposit. Cell walls were deposited at 10,000 *g*, washed with 1 M-NaCl, digested with trypsin and washed with water. Neutral sugars, amino sugars and reducing substances were estimated after hydrolysis of 10 mg. of cell walls in 2 N-H_2SO_4 for 3 hr at 105°. Amino sugar is expressed as glucosamine equivalents and reducing substances as glucose equivalents. Protein was estimated by the biuret method, with bovine plasma albumin as standard.

	% dry wt of cell wall		% dry wt of cell wall
Amino sugar	9·0	Phosphate	0·6
Rhamnose	20·3	Nitrogen	7·0
Glucose	5·0	Protein	5·0
Galactose	3·3	Nisin	2·2
Ribose	1·7	Ash	9·5
Reducing substances	26·2		

Analysis of the 100,000 *g* deposit indicated that it was mostly ribosomes (61 % RNA, 39 % protein). Polyacrylamide gel electrophoresis of basic proteins from these ribosomes revealed about 20 distinct bands (see Traut, 1966; Moller & Castleman, 1967) including a band with the same mobility as nisin marker; the presence of nisin was confirmed by bioassay.

Distribution of nisin in physically separated fractions of broken organisms

Acid-grown and neutral-grown cocci were disrupted, fractionated by differential centrifugation and the nisin estimated in the various fractions; the results are given in Table 3.

Gravimetric determinations indicated that the cell wall contributed 30–40 % of

the dry wt of whole organisms, membranes 4%, ribosomes 10%, and supernatant fluid fraction (cell-sap) 46–56%. Complete recovery of fractions was not usually attempted. Although in the experiment quoted the yield of nisin was only threefold higher in the 'neutral' cocci, the results for 'acid' cocci were variable and on occasion they contained very little nisin (e.g. total nisin only 5 μg./ml. medium).

Analysis of cell walls

Table 4 summarizes the results for the composition of cell walls prepared from 'acid' stationary-phase cocci. The purification of cell walls involves a trypsin digestion (see Methods) and during this procedure the protein content decreased from 45% of the

Table 5. *Contribution of cell walls to dry weight of whole Streptococcus lactis organisms*

Cell walls were prepared from exponential (3 hr growth) and stationary phase (overnight growth) as described for Table 4. The amino sugar (as glucosamine equivalents) and rhamnose content of whole organisms and cell walls was estimated after hydrolysis in 2 N-H_2SO_4 for 3 hr at 105°. Calculation of the cell wall's contribution to the dry weight of the whole cocci is based on the assumption that rhamnose and amino sugars occur exclusively in the cell wall (this is supported by analysis of the other fractions).

	Rhamnose (% dry wt)	Amino sugar (% dry wt)	Cell wall as % dry wt of cocci: By Rhamnose	Cell wall as % dry wt of cocci: By amino sugar
Exponential cocci	7·9	2·8	29·2	31·2
'Exponential' walls	27·0	9·0		
Acid stationary cocci	6·8	2·6	29·6	31·6
'Acid stationary' walls	23·0	8·2		
Neutral stationary cocci	8·4	3·7	41·5	42·0
'Neutral stationary' walls	20·3	9·3		

Table 6. *Comparison of formamide-soluble fraction and formamide-insoluble fraction from cell wall of Streptococcus lactis*

Cell walls were prepared from 'acid' stationary phase cocci as described for Table 4 and heated in formamide at 200° for 15 min. The resultant formamide-insoluble fraction was deposited by centrifuging at 30,000 *g* for 15 min, and washed twice with water. Rhamnose and amino sugar (as glucosamine equivalents) were estimated after hydrolysis of the formamide soluble and insoluble fractions in 2 N-H_2SO_4 for 3 hr at 105°.

	Mole ratios: Amino sugar	Rhamnose	Glucose	Galactose
Formamide-soluble	1·1	5·2	1·0	0·8
Formamide-insoluble	1·0	0·1	0·1	0·1

dry wt of the walls to 5%. The nitrogen content suggested that the protein content of purified walls was somewhat higher. However, it should be remembered that the protein was expressed as bovine serum albumin and this might be the reason for the discrepancy. Nisin was not lost from walls during the trypsin digestion. Attempts to demonstrate the presence of teichoic acid in the walls proved negative, the phosphate content being unchanged after prolonged extraction with trichloroacetic acid (5% trichloroacetic acid at 0° for 72 hr). A comparison of the rhamnose and amino sugar

contents of whole organisms and of cell walls permitted an estimate of the contribution of walls to the dry wt of whole cocci (such a calculation assumes that these carbohydrates occur exclusively in the wall). Figures are given in Table 5 for exponential and stationary-phase organisms.

Formamide extraction of walls

Previous work with group A streptococci (Krause & McCarty, 1961) showed that Fuller's (1938) formamide extraction was an efficient means of separating polysaccharide from mucopeptide. Extraction of *Streptococcus lactis* cell walls with formamide at 200° for 15 min. yielded two fractions: formamide-soluble and formamide-insoluble. During the extraction procedure there was a dramatic decrease in turbidity. The ratio of amino sugar to rhamnose, glucose and galactose in acid hydrolysates of the formamide-soluble and formamide-insoluble fractions was markedly different (see Table 6). The *E* 505/530 mμ ratios in the Rondle & Morgan (1955) estimation for amino sugar in the two fractions were 0·88 for the formamide-soluble fraction and 1·35 for the formamide-insoluble fraction; in the same assay glucosamine alone gave a ratio of 0·85 whilst an equimolar mixture of muramic acid and glucosamine gave 1·33.

Sensitivity to lysozyme

Whole organisms of *Streptococcus lactis* and cell walls were incubated with lysozyme (100 μg./ml. in 0·1 M-sodium phosphate buffer pH 7·0 at 37°) and the extinction at 700 mμ followed. There was a very slow decrease in both cases (about 50 % in 24 hr). Extraction of cell walls with formamide (200° for 15 min) or hot 10 % trichloroacetic acid (6 hr at 60°) caused a slight increase in sensitivity to lysozyme (see Schleifer & Kandler, 1967, who solubilized *S. cremoris* by this treatment).

DISCUSSION

Nisin is a strongly basic surface-active molecule (Hirsch, 1954) so that its distribution within the producer organism cannot easily be established. For this reason two different methods of analysis of the *Streptococcus lactis* organisms were used here: chemical and physical. In the chemical separation 57 % of the nisin was recovered in the fraction soluble in aqueous ethanol. This fraction presumably contained most of the membrane material and also any ethanol-soluble protein present elsewhere in the cocci. Centrifugally prepared membranes contained less nisin than the aqueous ethanol soluble fraction (9 % of total as compared with 57 % of total). Thus much of the nisin in the alcohol soluble fraction did not come from membranes. However, in both fractionation procedures a comparable amount of nisin was found associated with the wall material (trypsin-insoluble residue and 10,000 *g* deposit). The higher nisin content of neutrally-grown cocci was partly due to the increased amount of wall material present and to the higher nisin content of the walls (Tables 3, 5). Although free nisin is ethanol soluble, nisin attached to the trypsin-insoluble residue was not removed by repeated extraction with ethanol or by repeated washing with M-NaCl. The nature of the bond between wall material and nisin remains to be determined. In its association with cell wall, stability to boiling with acid and solubility in ethanol, nisin resembles the M protein of the group A Streptococci (Krause & McCarty, 1961). Physical fractionation of neutrally-grown *S. lactis* organisms gave a poorer recovery of nisin than did acid-

grown cocci; more of the nisin in neutrally grown cocci may have been loosely bound and lost during washing. However, some of the extra nisin in the neutral cocci was found in the cell sap (Table 3). Differences in the concentration of nisin inside and outside the cocci may be caused by different pH values. During growth without pH control the lactic acid produced by fermentation may be causing an acid extraction of the 'acid' cocci. However, in a culture maintained at neutral pH, the inside of the cocci might be at a more acid pH value than that of the medium. Since nisin is more soluble at acid pH values it may distribute itself accordingly. Zarlengo & Abrams (1964) reported that the intracellular pH value of stationary-phase *S. faecalis* might be as low as pH 5·0.

The cell wall of *Streptococcus lactis* contains mucopeptide and a polysaccharide which can be separated by formamide extraction. However, even after 15 min. in boiling formamide, rhamnose was still present in the mucopeptide (insoluble residue). Residual rhamnose probably means incomplete removal of polysaccharide, and this may have caused the poor sensitivity to lysozyme. Although Krause & McCarty (1961) were unable to extract all the rhamnose from group A streptococcal cell walls with formamide, the mucopeptide obtained was completely solubilized by lysozyme. Table 6 suggests that the formamide-soluble polysaccharide of *S. lactis* contains rhamnose, glucose, galactose and glucosamine in the molar ratio of 5:1:1:1, the glucosamine in the polysaccharide being presumably *N*-acetylated, since partial acid-hydrolysates contained *N*-acetylamino sugar. Polysaccharide contributed about 30% of the dry wt of the *S. lactis* wall and mucopeptide about 10%. Schleifer & Kandler (1967) established the composition and amino acid sequence of mucopeptide from *S. lactis*. Little data is available about the ash content of cell walls but the value found here for *S. lactis* (about 10%) is similar to that quoted by Holdsworth (1952) for *Corynebacterium diphtheria*. Ribose was always present in the purified wall preparations of *S. lactis*, even after treatment with RNA-ase; the wall, membrane and other associated organelles may form a highly integrated structure in this organism.

We thank Dr R. Young for advice on chromatography, Mr M. Pugh for technical assistance and Mr M. Stubbs for electron microscopy.

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J. gen. Microbiol. (1968), **53**, 181–186
Printed in Great Britain

Plaque-size Mutants of the Cellular Slime Mould *Dictyostelium discoideum*

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(*Accepted for publication* 29 *March* 1968)

SUMMARY

Strains of *Dictyostelium discoideum* with stable hereditable alterations in growth rate have been isolated on the basis of plaque size following treatment of the myxamoebae with nitrosoguanidine. Myxamoebae of five independently isolated strains which form small plaques were mixed in all possible pairs and allowed to develop. Eight of the ten pairs gave strains with the ability to form large plaques. Several large-plaque strains isolated from such mixtures were found to segregate small-plaque strains during vegetative growth. The results are consistent with the hypothesis that these large-plaque strains result from recombination or complementation in a genome derived at least partially from two parental strains.

INTRODUCTION

The solitary vegetative myxamoebae of the cellular slime mould *Dictyostelium discoideum* aggregate forming assemblies of about 5×10^5 myxamoebae when their bacterial food supply is exhausted. These aggregated myxamoebae then proceed through a complex series of morphological steps and eventually differentiate into either the stalk or the spore cells of the sorus (fruiting body). In a suitable environment the spores germinate to form the vegetative myxamoebae. Treatment of the myxamoebae with a mutagen has led to the isolation of strains with stable hereditable aberrations in the developmental sequence and indicated that many events in this sequence are genetically controlled (Sussman & Sussman, 1953; Sussman, 1955; Yanagisawa, Loomis & Sussman, 1967). Further genetic analysis of this system has been hampered by the lack of a mating system and the inability to select for recombinant strains.

Both haploid and diploid strains have been described (Wilson & Ross, 1957; Sussman & Sussman, 1962). Sussman & Sussman (1963) have reported the isolation of a diploid strain, H-1, from a mixed culture of two strains, one of which forms a white, the other a brown sorus. This diploid strain forms sori with the wild type pigmentation (yellow). Strain H-1 was found to give rise at a low frequency to haploid progeny, most of which display one of the parental pigmentations, but a few of which have the pigmentation of an apparent recombinant. The rarity of formation of stable diploid strains, the low frequency of segregation of haploid strains from diploids and the inability to select or enrich for pigment markers has precluded further investigation of this system

We report the isolation of a novel class of mutants of *Dictyostelium discoideum* in which the growth rate is markedly decreased, giving rise to a characteristically small plaque-size. Under standard conditions, one wild-type cell in a population of 10^5 mutant cells can be readily detected and its progeny isolated, thus permitting selection procedures to be devised for recombinational events. We present evidence which suggests that genetic recombination and/or complementation can occur with a low frequency in *D. discoideum*.

METHODS

Chemicals. *N*-Methyl-N^1-nitroN^1-nitrosoguanidine (NTG) was obtained from the Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.). Actidione (cycloheximide) was the gift of the Upjohn Co. (Kalamazoo, Michigan, U.S.A.).

Organisms. Strain NC-4 is a haploid strain of *Dictyostelium discoideum* isolated by Raper (1935). An actidione-resistant strain which forms brown sori was isolated from NC-4 following treatment with NTG by Drs K. Yanagisawa & M. Sussman (personal communication); both strains were kindly given us by Dr M. Sussman.

Myxamoebae were grown on agar plates with *Aerobacter aerogenes* and allowed to develop at 22° on Millipore filter supports (AABPO 47) as described by Sussman (1966), with the modification that the filters were washed in boiling water for 15 min. and then stored in cold distilled water before use.

Genetic notation. No standard system of notation appears to exist for the various mutants of cellular slime moulds. In this paper we use the following symbols:

min	small (less than 1/10 wild-type) plaque size.
act	resistance to actidione.
agg	myxamoebae do not aggregate and hence do not form sori.
frt	myxamoebae aggregate but do not form a sorus.
spr	a sorus but no spores are formed.

It is not known whether the various morphology markers are allelic. The probability is that they are not.

Clonal analysis. When less than 10^4 wild-type myxamoebae or spores (or less than 10^5 *min* cells) are spread on medium in a 9 cm. diameter Petri dish the cells give definite plaques in the bacterial lawn (Sussman, 1951). To clone the myxamoebae a flamed platinum wire was touched to a plaque and then streaked on a growth plate previously spread with a heavy inoculum of *Aerobacter aerogenes*. After incubation for 2 days at 22° confluent clearing of the bacterial lawn occurred at the start of the streak and isolated plaques arose further along the streak.

Mutagenesis. Vegetative myxamoebae were treated with NTG and then plated immediately as described by Yanagisawa *et al.* (1967).

Sensitivity to actidione. To distinguish between wild-type and *act* phenotypes, cells were either streaked or spread on a growth plate containing 500 μg. actidione/ml. and which had been previously innoculated with about 10^8 *Aerobacter aerogenes*; *act* plaques were distinguishable after incubation for 3 days at 22°.

RESULTS

Isolation and characterization of min strains

Myxamoebae of the brown actidione-resistant haploid strain derived from NC-4 were treated with nitroguanidine and spread at a density of 10^3 myxamoeba per 9 cm. diameter plate (Yanagisawa *et al.* 1967). After incubation for 3 days at 22° there was a gradation in plaque size among the survivors. In a typical experiment with 91% killing, among 600 plaques measured, 30 had a diameter less than one-tenth that of an average wild-type plaque. Of these 30, 15 stably inherited the small plaque size character (*min*).

Table 1. *Dictyostelium discoideum: characteristics of strains used*

Strain	Relative size of plaque (arbitrary units)	Morphology	Drug resistance	Reversion rates to *min*$^+$	Reversion rates to *act*	Reversion rates to *frt*$^+$
min$^+$ stock	100	Wild type (*frt*$^+$)	*act*	—	—	—
min 1	4	Wild type (*frt*$^+$)	*act*$^+$	$< 5 \times 10^{-6}$	$1{\cdot}4 \times 10^{-5}$	—
min 2	4	No spores formed (*spr*)	*act*	5×10^{-6}	—	5×10^{-6}
min 3	10	Wild type (*frt*$^+$)	*act*	5×10^{-6}	—	—
min 4	10	Aggregateless (*agg*)	*act*	5×10^{-6}	—	2×10^{-2}
min 5	10	Aggregateless (*agg*)	*act*	5×10^{-6}	—	$< 10^{-6}$

When these 15 mutant strains were allowed to develop, it was noted that eight formed aberrant fruiting bodies. Reversion studies on five of these strains (Table 1) showed that the *min* mutant phenotypes reverted independently of, and with a frequency different from, the reversion frequencies of the other mutant phenotypes. It is possible that a second site mutation in the revertants resulted in alteration of only one expression of a pleiotrophic mutation. However, it is more probable that our conditions of mutagenesis often produced multiple mutational events in the same genome.

The genetic characteristics of the 5 *min* strains selected for further study are listed in Table 1. The yellow colour of the wild-type strain NC-4 resides, almost exclusively, in the spore mass of the sori and is probably due to a carotenoid derivative (Staples & Gregg, 1967). The vegetative myxamoebae never produced pigment and neither did the myxamoebae of any known *agg* derivatives of a yellow-pigmented strain, even when incubated in the absence of bacteria. The brown pigmented produced by our strain is of unknown chemical structure; it was produced by both *agg* and *frt*$^+$ strains when incubated more than 24 hr in the absence of bacteria. The pigment was excreted in large amounts and stained the agar a deep brown as well as causing the sori (when produced) to have a deep brown colour.

The reversion rates (Table 1) were determined by plating myxamoebae on 20 growth plates at 5×10^4 viable myxamoebae/plate and scoring plaque size after incubation for 2 days at 22°. Plaques were scored *min*$^+$ when, on subculturing, they gave rise to plaques which were more than twice the size of those of the parental strain. Frequently *min*$^+$ revertants were isolated which produced plaques of a size intermediate between the original *min* strain and the wild type. These partial revertants of the *min* characteristic may have resulted from second site mutational events or complete reversion of

one of several lesions which have a cumulative effect on the growth rate of the *min* strains. The reversion rates of the various markers were quite different and independent. No *agg*$^+$ revertants of *min* 5 were observed.

Isolation and characterization of min$^+$ progeny from min strains

The five *min* strains described in Table 1 were grown separately, washed free of bacteria, mixed in equal proportions in all possible pairs and allowed to develop on Millipore filter supports at 22°. Sori were formed in all cases after 22 hr with the exception of the pair *min* 4+*min* 5. Cells were washed off the Millipore supports at the time of deposition and after incubation for more than 22 hr were plated on at least three plates at 10^3 cells/plate. Viability was greater than 80 % initially but decreased to about 50 % after 22 hr.

Table 2. *Dictyostelium discoideum: the isolation of min$^+$ strains from mixtures of min mutants*

Parental strains	No. of experiments	No. of *min*$^+$ isolates	Frequency of *min*$^+$ isolates
min 1+*min* 2	3	5	$6{\cdot}2\times10^{-4}$
min 1+*min* 3	3	60	$1{\cdot}1\times10^{-2}$
min 1+*min* 4	2	2	$3{\cdot}8\times10^{-4}$
min 1+*min* 5	7	65	$3{\cdot}2\times10^{-3}$
min 2+*min* 3	1	2	$6{\cdot}7\times10^{-4}$
min 2+*min* 4	1	8	$3{\cdot}2\times10^{-3}$
min 3+*min* 4	2	0	$<2\times10^{-4}$
min 5+*min* 2	2	4	$7{\cdot}3\times10^{-4}$
min 5+*min* 3	1	2	$1{\cdot}3\times10^{-2}$
min 5+*min* 4	2	0	$<4\times10^{-4}$

Table 3. *Dictyostelium discoideum: phenotypes of min$^+$ progeny*

Parental strains	Experiment no.	Total no. of plaques	Frequency of *min*$^+$ plaques	Phenotypes of *min*$^+$ plaques	No. observed
min 1 *act*$^+$ *frt*$^+$+	1	5000	6×10^{-4}	*frt*$^+$ *act*$^+$	3
min 5 *act agg*	2	6000	7×10^{-4}	*agg act*	4
	3	800	5×10^{-3}	*agg act*	3
				frt$^+$ *act*$^+$	1
min 1 *act*$^+$ *frt*$^+$+	1	5000	4×10^{-4}	*frt*$^+$ *act*	2
min 2 *act spr*	2	2000	$1{\cdot}5\times10^{-3}$	*spr act*	3
min 1 *act*$^+$+ *min* 3 *act*	1	2500	2×10^{-3}	*act*	5

No large plaques were observed from cells of any of the pairs when plated out shortly after deposition on the Millipore supports, nor from any of the *min* strains allowed to develop separately; but cells from all pairs, except for two (*min* 4+*min* 5 and *min* 3+*min* 4), gave a few large plaques when plated after 22 hr of development. The frequency with which large plaques occurred varied from experiment to experiment (Table 3) and varied even more widely according to the pair under study (Table 2).

The apparent *min*$^+$ progeny from each pair were picked and streaked on fresh plates alongside both parental and wild-type strains. After incubation for 3 days, plaque size was measured and only those isolates which formed plaques at least twice the

size of the largest parental strain were scored as *min*$^+$. All *min*$^+$ isolated formed the dark-brown pigment. It was noticed that the majority of the original isolates gave many *min*$^+$ plaques when streaked, but also gave plaques the same size of the parental strain. These small plaques were unlikely to have arisen by contamination of the original isolate by parental cells and it seems likely that many *min*$^+$ progeny are unstable and segregate the parental *min* character (see below).

Where appropriate the *min*$^+$ progeny were also analysed for the unselected markers *act*$^+$, *agg* and *spr*$^+$; the results of these experiments are summarized in Table 3. The *min*$^+$ progeny from mixtures of *min* 1 and either *min* 5 or *min* 2 were of both parental phenotypes as regards drug resistance and morphological development. It can also be seen that the *act* and various morphological markers were not randomly distributed among the *min*$^+$ progeny, at least in the small number of isolates examined.

Table 4. *Dictyostelium discoideum: segregation of min*$^+$ *strains*

Parental strains	Phenotype of *min*$^+$ isolate	Phenotype of segregants observed
min 1 + *min* 3	*frt*$^+$ *act*$^+$	*min frt*$^+$ *act*$^+$
min 1 + *min* 5	*frt*$^+$ *act*$^+$	*min frt*$^+$ *act*$^+$ *min*$^+$ *frt act*
min 1 + *min* 3	*frt*$^+$ *act*	*min frt*$^+$ *act*

Segregation from min$^+$ *strains*

As mentioned previously, the first streak of many of the *min*$^+$ isolates gave both large and small plaques. When these plaques were in turn plated out, all the small-plaque isolates bred true as did some of the large-plaque isolates. However, many of the subcloned *min*$^+$ isolates continued, on further clonal analysis, to give both large-plaque and small-plaque strains. Several of these unstable *min*$^+$ isolates were analysed for segregation of the *min* character and, where appropriate, for the unselected characters. The results of these experiments are summarized in Table 4; *min* segregants were observed to occur at frequencies from 30 to 70% from different *min*$^+$ isolates. It is clear that many of the *min*$^+$ isolates segregated *min* progeny and the plaque-size phenotype segregated independently of drug resistance or morphological phenotype.

DISCUSSION

The biochemical mechanism(s) causing the slow growth rate and consequent *min* phenotype in *Dictyostelium discoideum* are unknown and likely to be complex. The myxamoebae are grown in a very rich medium containing bacteria as well as a high concentration of peptone and yeast extract. Thus, the growth rate is probably not restricted by limited availability of biosynthetic intermediates. The frequent occurrence of the *min* phenotype among mutagen-treated myxamoebae and the gradation of plaque sizes among separate *min* isolates suggest that any one of a number of mutations may cause the *min* character.

All the *min* strains described here are derivatives of the same strain. Since most of them will give *min*$^+$ descendants when allowed to develop in association, it is clear that recombinant progeny can arise in the absence of a clearly defined stable mating system. However, the frequency with which such *min*$^+$ isolates were produced varied

widely from experiment to experiment with the same pair and varied even more widely between different pairs, suggesting that some physiological state of competence or compatibility is involved.

One of the pairs which failed to give rise to observable *min*$^+$ isolates (*min* 3 + *min* 4) formed sori,but the ratio of parental phenotypes among spores of this pair was found to differ greatly from the ratio in which the myxamoebae were mixed. It appears that the association of cells of this pair is not an efficient or equal one. The other pair which failed to give rise to observable *min*$^+$ isolates (*min* 4 + *min* 5) did not form sori. It is possible that the varying frequencies reported in Table 3 reflect the efficiency with which the various strains form mixed sori.

Since random assortment of unselected characters was not observed among various *min*$^+$ isolates (Table 3), reciprocal recombination appears unlikely in the formation of *min*$^+$ progeny. Complementation of two complete genomes from *min* parental strains cannot account for the *min*$^+$ progeny since both *min*$^+$ *act* and *min*$^+$ *act*$^+$ strains may be obtained from the same pair of *min* parents (Table 3). However, recombination or complementation of partial genomes can account for our results. It is interesting that several of our *min*$^+$ isolates were found to form spores whose size distribution was closer to that reported by Sussman & Sussman (1962) for diploid spores than for haploid spores (W. F. Loomis, unpublished). The majority of the *min*$^+$ isolates, however, form spores with a haploid size distribution and these may be the result of recombinational events and haploidization.

We thank Dr M. Sussman for introducing us to the study of this organism. The expert technical assistance of Miss Karen Howard is gratefully acknowledged. One of us (J. M. A.) was a Harkness Fellow of the Commonwealth Fund during part of the work reported here. This study was supported by Grant GB-5830 from the National Science Foundation, U.S.A., to W.F.L. and by Grant B/SR/4260 from the Science Research Council, Great Britain to J.M.A.

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J. gen. Microbiol. (1968), **53**, 187–196
Printed in Great Britain

Observations on Intergeneric Transformation between Staphylococci and Streptococci

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SUMMARY

The occurrence of intergeneric transformation between staphylococci as DNA donors and Streptococcus strain CHALLIS as recipient was controlled. By various methods 126 DNA preparations were isolated from 21 staphylococcal strains resistant to different antibiotics (18 produced coagulase and DNase; three were white and did not produce coagulase or DNase). Eight of these preparations, originating from four strains of *Staphylococcus aureus* were active in intergeneric transformation. The streptomycin-resistance marker was transferred, the yield being 0·001–0·017 % of that of homospecific reaction. The authors did not succeed in transferring the penicillin-, novobiocin-, erythromycin- and oxytetracyclin-resistance markers. Repeated isolations were required to produce active DNA preparations and none of the various methods yielded active preparations consistently. The staphylococcal streptomycin-resistance marker re-isolated from the transformants of strain CHALLIS was transferred to CHALLIS with the same or even higher yield than the homospecific marker.

In the staphylococcal strains from which active preparations were obtained, the GC content was 32·7–37·6 % and was lower by 4·9–9 % from that in strain CHALLIS (41·8 %).

INTRODUCTION

The occurrence of interspecific or intergeneric transformation implies a certain degree of genetic homology between the donor and the recipient strain.

There are a considerable number of publications concerning interspecific transformations in several bacterial genera. They have been described in species of the genera *Rhizobium* (Balassa, 1957), *Hemophilus* (Leidy, Hahn & Alexander, 1959), *Bacillus* (Marmur, Seaman & Levine, 1963), and in pneumococci and streptococci (Bracco, Krauss, Roe & MacLeod, 1957; Pakula, Hulanicka & Walczak, 1958; Chen & Ravin, 1966).

Intergeneric transformations, on the other hand, have so far been demonstrated only in two cases—namely, between staphylococci and streptococci, and between members of the genera *Neisseria* and *Moraxella* (Catlin, 1964). A transformation between staphylococci and streptococci was reported by Pakula *et al.* (1958). It must be noted that their report was in the nature of a brief mention contained in publication dealing with interspecific transformations in streptococci. Pakula *et al.* used a transforming DNA isolated from only one staphylococcal strain, ST-70, a standard strain for phage-typing, resistant to streptomycin, novobiocin and erythromycin. As recipients, they employed streptococcal strains—CHALLIS, SANGUIS, MITIS and *Strepto-*

coccus pneumoniae sensitive to the three antibiotics mentioned above. Pakula *et al.* obtained transformants in the streptococcus CHALLIS and SANGUIS strains. The MITIS and *Streptococcus pneumoniae* strains did not give positive results. They succeeded in transferring only the streptomycin-resistance marker.

These observations were recently questioned by Marmur, Falkow & Mandel (1963) who wrote that the relation between *Staphylococcus* and *Streptococcus*, implied by transformation between these genera, awaited both further experimentation and evidence that the Staphylococcus species employed was properly identified.

In the case of the staphylococci, it has as yet been impossible to obtain homospecific transformations consistently. One of the basic obstacles in effecting repeated transformations in staphylococci may depend on difficulties in obtaining a high-molecular weight, biologically active DNA. This is, most probably, connected with the activity of staphylococcal nucleases. A method for isolating high-molecular weight DNA from staphylococci was described by Blobel (1961). This method is based on procedures for preparing DNA described earlier. However, no report has appeared to date on the biological activity of Blobel's preparations, although Blobel himself suggested such activity.

We undertook our studies on transformation between staphylococci and streptococci to find out, in view of the existing controversies, whether such transformations did in fact occur. We hoped to obtain additional information about the phenomenon, and also wanted to consider the possibility of utilizing such transformations as an auxiliary genetic method in studies on the antibiotic resistance of staphylococci. We decided to perform our studies using numerous staphylococcal strains; we also wanted to test the applicability of various methods of isolating DNA in order to obtain biologically active preparations. In the event of transformation occurring we decided further to examine the base composition of some of the strains used.

METHODS

Strains. A group H haemolytic streptococcus strain—CHALLIS—was employed as recipient. In homospecific transformation, a streptomycin-, novobiocin- and erythromycin-resistant mutant of strain CHALLIS served as DNA donor. The two strains originated from the collection of Professor R. Pakula.

For heterologous transformation, DNA was prepared from 21 different staphylococcal strains. These were resistant to 250 μg./ml. dihydrostreptomycin, and some of them were also resistant to penicillin, novobiocin, erythromycin and oxytetracycline. Eighteen of these strains, belonging to different phage types, produced coagulase and exocellular deoxyribonuclease (DNAse). They included strain 70 used by Pakula *et al.* and Morse's strain s-44 used by Blobel (1961). Three strains, 480, 13x, 1690, produced white pigment and did not produce coagulase or DNAse. All strains came either from the State Institute of Hygiene in Warsaw (Dr J. Jeliaszewicz's Laboratory) or from our own Department.

Transformation reaction. Transformations were performed in a dialysed medium prepared according to Dr R. Pakula (personal communication) 40 g. Neopeptone (Difco) were dissolved in 100 ml. distilled water, placed in a dialysing tube (Kalle Aktiengesellschaft-Wiesbaden, Biebrich) and dialysed at 4° in 1·5 l. distilled water. In the same manner, 40 g. Difco Yeast Extract were dialysed in 1·2 l. distilled water.

After dialysis for 48 hr, the diffusates were combined and made up to 2·5 l. with distilled water; 0·5 % NaCl, 0·17 % K_2HPO_4 and 0·02 % $CaCl_2$ were added; the solution was adjusted to pH 8·0, the medium warmed to 80° and filtered through filter paper. After the addition of 0·2 % glucose, the medium was adjusted to pH 7·6 and filtered through a Schott G-5 glass filter. This medium was supplemented with 0·2 % of bovine albumin (Sigma). The medium was inoculated with an overnight culture of the streptococcus strain CHALLIS in blood broth to obtained an initial bacterial population density of approximately 4×10^8 viable organisms/ml. and incubated in a water bath at 37° until maximum competence (usually 2 to 2·5 hr) was achieved. Subsequently, 0·2 ml. DNA isolated from the different staphylococcal strains was added to a 1·8 ml. sample of the culture. After 15 min. contact between the bacteria and DNA, the reaction was interrupted by the addition of 20 units DNAse (Distreptaza produced by Biomed, Poland). The cultures were further incubated for 105–115 min. to allow phenotypic expression of the antibiotic-resistance marker. The samples were then transferred to selective media. The transformants were isolated on blood agar plates containing 250 μg./ml. dihydrostreptomycin (Polfa, Poland), 0·02 or 0·03 units/ml. penicillin (Polfa), 15 or 25 μg./ml. novobiocin (Lepetit), 0·15 or 0·25 μg./ml. erythromycin and 4 or 8 μg./ml. oxytetracycline. The erythromycin and oxytetracycline used were standard preparations kindly supplied to us by the Institute of Antibiotics in Warsaw. The yield of transformation was calculated by comparing the number of transformants in 1 ml. of the culture with the number of viable bacteria in 1 ml. of the culture at the moment the DNA was added. The yield of intergeneric transformation is given as a percentage of that of the homospecific reaction. In preliminary trials, in determining the biological activity of the preparations isolated from staphylococci, the results of transformation were not measured quantitatively. In each experiment the occurrence of spontaneous antibiotic-resistant mutants was thoroughly controlled.

Determination of base composition. The base composition of DNA was estimated according to Marmur & Doty (1962). The DNA used was prepared as described by Marmur (1961). *Tm* was determined in a tenfold diluted solution of standard saline–citrate (SSC). In each trial, the *Tm* of strain CHALLIS DNA was examined simultaneously as a control. To determine the value of *Tm* in SSC, a calculation was made according to Silvestri & Hill (1965).

RESULTS

The applicability of various methods of isolating staphylococcal DNA for obtaining preparations active in intergeneric transformation

The study began with an attempt to obtain active DNA preparations from staphylococcus strain 70 used by Pakula *et al.* (1958). The strain was grown in nutrient broth or in Todd–Hewitt medium. The cocci were incubated at 37° for 24 hr with strong aeration or in stationary culture. To isolate the DNA, the method, described by Pakula & Tyc (1956), was used: the coccal sediment was suspended in a tenfold diluted solution of saline–citrate–EDTA (0·15 M-NaCl + 0·015 M-sodium citrate + 0·1 M-Na-EDTA) (SWC) and centrifuged. After washing, the cocci were suspended in a 1/10 dilution of SWC and warmed at 65° for 45 min. The cocci were centrifuged once more and suspended in the enzyme from *Streptomyces albus* supplemented with 0·015 M-sodium citrate. They were incubated with the enzyme in a water bath at 37° for 12 hr.

From the staphylococcal lysate, the DNA was precipitated with three volumes of ethyl alcohol (96°) and deproteinized by the chloroform method.

Using this method, several DNA preparations from strain 70 were obtained and examined for their ability to transfer streptomycin-resistance to strain CHALLIS;

Table 1. *Biological activity of staphylococcal DNAs isolated by various methods**

Method of DNA isolation	Symbols of strains	Number of preparations: Total obtained	Number of preparations: Active in transformation
Pakula & Tyc (1956)	7, 12, 13, 19, 22, 25, 27-SR, 30, 32, 44, 70, 71, 82, S-44, 27, 64, 209P–SR, 33,	42	3†
Catlin & Cunningham (1958)	13, 22, 27, 32, 44, 480, 13X, 1690	24	0
Marmur (1961)	13, 32,	4	0
Blobel (1961)	13, 32, S-44	4	0
Pakula & Tyc (1956) grown in presence of sodium citrate or Na-EDTA	13	2	0
Pakula & Tyc (1956) following substances added before isolation of DNA:	13, 27-SR	6	0
Penicillin (10 and 100 μg./ml.)	13, 27-SR	6	0
Streptomycin (500 μg./ml.)		4	2‡
Chloramphenicol (100 μg./ml.)		1	0
Hydroxylamine-HCl (60 μg./ml.)		1	0
Pakula & Tyc (1956) pH adjusted to:			
7·0	13, 19, 32	8	0
6·5	13, 32, S-44	5	0 §
6·0	7, 13, 19, 22, 32, 70, 209P-SR	13	2 ‖
5·5	13, 32,	4	0
5·0	13, 32	8	1¶

* Biological activity was measured in intergeneric transformation using *S. challis* as recipient of the streptomycin resistance markers.

† DNA derived from strains 13, 19 and 70.

‡ DNA derived from strain 13.

§ DNA deriving from strain s-44 showed very weak biological activity.

‖ DNA derived from strains 13 and 32.

¶ DNA derived from strain 32.

only one was active in heterologous transformation. Consequently, an effort was made to obtain active preparations from other staphylococcal strains by the same method. A total of 42 preparations from 18 different staphylococcal strains producing coagulase and DNAse (including those from strain 70) were obtained. Only three preparations isolated from strains 13, 19 and 70, were biologically active (see Table 1). Thus, despite careful control of the conditions of the procedure used and repeated isolation of DNA from some of the strains, it was not possible in these tests to get two biologically active preparations from any one strain.

Since difficulty in obtaining a biologically active DNA from staphylococci may be connected with the production of nucleases, attempts were made to modify the

method of Pakula & Tyc (1956). The modifications were designed to hinder the production of nucleases or inhibit their activity. In the first of these experiments DNA was isolated from cocci grown in the presence of 14·7 mg./ml. sodium citrate or 3 mg./ml. Na-EDTA. A second series of experiments was carried out in which the cocci, in the final stage of culture, were treated with 10 or 100 units/ml. penicillin, 500 μg./ml. dihydrostreptomycin, 100 μg./ml. chloramphenicol or 6 μg./ml. hydroxylamine-HCl. Finally DNA preparations were made at a low pH (5·0–7·0), making use of the observation by Osowiecki & Pakula (1962) that, as pH is lowered, the activity of staphylococcal DNAse decreases.

Furthermore, other methods—those of Catlin & Cunningham (1958), Marmur (1961) and Blobel (1961)—were employed in attempts to obtain from staphylococci DNA preparations active in heterologous transformation.

A total of 75 preparations were obtained in these trials from 11 different strains of *Staphylococcus aureus* (see Table 1). Only five of them were active. (In addition, one DNA preparation from strain s-44 displayed very weak—and irregular—activity. This was not used in further studies.)

From the three non-pigmented strains which did not produce coagulase or DNAse, nine DNA preparations were isolated by the Catlin & Cunningham method. None of them was active.

Yield of intergeneric transformation of streptomycin-resistance marker

All the active DNA preparations obtained during these experiments were used to study the transfer of the streptomycin-resistance marker to strain CHALLIS.

The results are presented in Table 2.

Table 2. *Yield of intergeneric transformation with the use of DNA from coagulase-positive staphylococci and of streptococcus strain CHALLIS as recipient**

DNA donor	Yield of heterologous transformation in percentage of homospecific transformation
Strain CHALLIS	100
Staphylococcus aureus 13	
Preparation no. 1	0·017
Preparation no. 2	0·005
Preparation no. 3	0·010
Preparation no. 4	0·003
S. aureus 19	0·014
S. aureus 32	
Preparation no. 1	0·001
Preparation no. 2	0·015
S. aureus 70	0·002

* The transformation reaction was performed as described in Materials and Methods. The streptomycin-resistance marker was transferred. For methods of obtaining DNA see Table 1.

As can be seen from Table 2, the yield of heterologous transformation was low compared with that of homospecific transformation and varied from 0·017 to 0·001 % of that obtained in homospecific transformation, depending on the preparation and on the strain used as donor.

The yield of heterologous transformation was measured repeatedly at intervals, using the same preparations isolated from strains 13 and 19. The results showed good agreement. The yield of transformation in individual experiments ranged from 0·013 to 0·017% with the preparation from strain 13, and from 0·011 to 0·014% with that from strain 19.

Time of phenotypic expression of streptomycin-resistance marker

The time of phenotypic expression of streptomycin resistance was determined as described by Pakula *et al.* (1962) who examined expression in strain CHALLIS using homospecific transformation.

In our experiments, the staphylococcal streptomycin-resistance marker was expressed phenotypically in the strain CHALLIS recipient *c.* 120 min. from the moment DNA has been added. Thus, the time of expression was identical with that in the case of a homospecific reaction.

Attempts to transfer various staphylooccal antibiotic-resistance markers to Streptococcus strain CHALLIS

Several attempts were made to transfer resistance to penicillin (0·02 and 0·03 units/ml.), novobiocin (15 and 25 μg./ml.), erythromycin (0·15 and 0·25 μg./ml.) and oxytetracycline (4 and 8 μg./ml.) to strain CHALLIS. The DNA preparations used in these experiments were those isolated from Staphylococcus strains 13, 19, 32 and 70 which had proved active in transferring streptomycin-resistance to this recipient.

The results were completely negative.

Integration of staphylococcal streptomycin-resistance marker in strain CHALLIS

In order to examine the degree of integration of the heterologous streptomycin-resistance marker into the genome of strain CHALLIS, three clones were isolated from the transformants obtained in transformations in which DNAs from staphylococcal strains 19 and 70 were used. DNA was isolated from these six transformants and subsequently used for homospecific transformation (Table 3).

The results in Table 3 show that the yield of homospecific transformation using DNA re-isolated from the transformants was even higher (20–100%) that in the case of DNA extracted from a streptomycin-resistant mutant of strain CHALLIS.

Comparison of base composition in DNA of strain CHALLIS and seven staphylococcal strains

The base composition of DNA in strain CHALLIS and seven staphylococcal strains used in these experiments was examined by *Tm* determination. Four staphylococcal strains which yielded preparations active in heterologous transformation were chosen, as well as three staphylococcal strains from which we did not succeed in obtaining active preparations. The results of these determinations are summarized in Table 4.

There were fairly large differences in the base composition of the various staphylococcal strains; GC content ranged from 31·9 to 37·6%. Three strains 64, 44 and 30 from which no active preparations were obtained had a somewhat lower GC content than those from which biologically active preparations were obtained. The GC content in staphylococcal DNA preparations active in intergeneric transformations was 4·9–9% lower than in strain CHALLIS.

Table 3. *Yield of homospecific transformation with the use of DNA re-isolated from strain* CHALLIS *transformants containing staphylococcal streptomycin-resistance marker**

	DNA from SM-R mutant of strain CHALLIS (control)	DNA re-isolated from strain CHALLIS transformant containing SM-R marker from *Staphylococcus aureus* 70			*S. aureus* 19		
	Clone –	Clone 1	Clone 2	Clone 3	Clone 1	Clone 2	Clone 3
No. of transformants/ml.	89×10^4	130×10^4	104×10^4	160×10^4	150×10^4	125×10^4	190×10^4
Yield (%)	3·87	5·6	4·5	6·9	6·5	5·4	8·3

* DNA was re-isolated from transformants by the Pakula & Tyc method (1956). Reaction was performed as described in Methods.

Table 4. *Comparison of base composition in DNA of seven staphylococcal strains and streptococcus strain* CHALLIS*

Source of DNA	GC content (%) calculated according to Silvestri & Hill (1965)
Staphylococcus aureus 70	37·6 (42·9)
19	36·8 (41·7)
13	35·1 (41·7)
	36·8 (42·4)
	35·6 (41·7)
32	32·7 (41·7)
64	31·9 (39·7)
44	32·5 (42·8)
30	31·9 (41·7)
Strain CHALLIS (mean value)	— (41·8)

* In each determination of the base content of staphylococcal DNA the GC content of strain CHALLIS DNA was determined as a control. The results of GC determinations of DNA in strain CHALLIS made according to Silvestri & Hill (1965) are given in parentheses. The result of 41·8 % for strain CHALLIS is a mean value of nine independent determinations. Biologically active preparations were obtained from staphylococcal strains 70, 19, 13 and 32; preparations from strains 64, 44 and 30 were inactive.

DISCUSSION

The studies reported above on intergeneric transformation between staphylococci as DNA donors and streptococcus strain CHALLIS as recipient confirm that transfer of the streptomycin-resistance marker does occur. Eight biologically active preparations were obtained from four different strains of *Staphylococcus aureus* producing coagulase and DNAse. We did not succeed in obtaining active preparations from 14 strains of *S. aureus* and three strains of *S. albus*.

Various methods were used to isolate staphylococcal DNA (Pakula & Tyc, 1956; Catlin & Cunningham, 1958; Marmur, 1961; Blobel, 1961). An attempt was also made to obtain active DNA preparations using various modifications of the Pakula & Tyc method designed, primarily, to inhibit the activity of staphylococcal nucleases. None of the methods yielded regularly staphylococcal DNAs active in intergeneric

transformation. Active preparations were obtained only by repeated isolations of DNA from some of the strains. Thus, for example, with the use of various methods four out of 50 preparations from strain 13, two out of 16 preparations from strain 32, and one out of eight preparations from strains 70 and 19 respectively proved to be biologically active.

The yield of transformants in intergeneric transformations was low (0·001–0·017 %) compared with that obtained in intraspecific transformation (Table 2). It was lower also than the yield of interspecific transformation in Streptococcus (Pakula, 1963), Bacillus (Marmur, Seaman & Levine, 1963) and Hemophilus (Schaeffer, 1958).

However, DNA isolated from transformants of strain CHALLIS into which a staphylococcal streptomycin-resistance marker was introduced gave a high yield of transformants in a homospecific reaction. The yield, in fact, was higher than that obtained in the control reaction (Table 3). This suggests that the staphylococcal streptomycin-resistance marker has become fully integrated into the chromosome of the recipient.

In the recipient strain CHALLIS, phenotypic expression of the staphylococcal streptomycin-resistance marker occurred after approx. 120 min.; thus, the period of expression was the same as in the case of the transformation of a homologous marker.

In the experiments described here, only the streptomycin resistance marker was transferred to strain CHALLIS. Despite several attempts, we did not succeed in transferring staphylococcal markers for penicillin, novobiocin, erythromycin and oxytetracycline resistance. As suggested by J. Marmur (private communication) for some species belonging to the genus *Bacillus* (Dubnau, Smith, Morell & Marmur, 1965), the streptomycin-resistance marker of Staphylococcus may also perhaps be located on a segment of the chromosome which, in the course of evolution, has retained the base sequence unchanged or almost unchanged, whereas in other chromosomal regions the sequence may have undergone more extensive alterations. If this is so, it may account for the fact that certain markers derived from related species or genera may be capable of integration while others may not.

The considerable difficulties involved in the isolation of active staphylococcal DNA, and also the negative result of the attempts to transfer antibiotic-resistance markers other than the streptomycin-resistance marker, make it impossible for the time being to use intergeneric transformation as an auxiliary method in studying the antibiotic-resistance of staphylococci.

In the study of base content, use was made of four staphylococcal strains from which biologically active preparations were isolated, and of three strains from which it was not possible to obtain such preparations. The GC content in strain CHALLIS was examined as well. In agreement with the reports in the literature (Hill, 1966), the differences in GC content in staphylococci proved to be quite considerable (31·9–37·6 %). The three staphylococcal strains from which no active preparations were obtained had a somewhat lower GC content than those from which such preparations were secured. However, this fact may be accidental and it is impossible to comment on it within the terms of reference of the present study. On the basis of several determinations it was found that the mean GC value in strain CHALLIS was 41·8 %. Bankowska (1963) reported that GC in strain CHALLIS amounted to 41 %, and Chen & Ravin (1966) determined the GC content in this strain to be 36·4 %. However, the latter authors performed their determination using the bromination method.

The differences in GC content in the DNA of strain CHALLIS and the staphylococcal

strains from which active preparations were obtained proved to be considerable and varied between 4·8 and 9%. The present study demonstrates the possibility of the occurrence of heterologous transformation (although of one marker only) in spite of rather large differences in the respective base content of the strains used in the reaction. In view of this, it seems advisable to elaborate with greater precision the opinion encountered in the literature (Marmur, Falkow & Mandel, 1963; Pakula, 1963) that similarity in base content between donor and recipient is an indispensable, though not the only, requirement for the occurrence of heterologous transformation in bacteria.

The authors are indebted to Miss Barbara Glinicka and Miss Alina Stępczyńska for their valuable technical assistance.

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J. gen. Microbiol. (1968), **53**, 197–204
With 4 plates
Printed in Great Britain

The Disruption of Lysosome-like Particles of *Solanum tuberosum* Cells during Infection by *Phytophthora erythroseptica* Pethybr.

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(*Accepted for publication 2 April* 1968)

SUMMARY

Histochemical methods showed that tuber tissue and tissue culture cells of *Solanum tuberosum* contained phase-dense particles which absorbed neutral red and fluorescent dye, and particles which contained acid phosphatase and a non-specific esterase. It is suggested that these structures may be comparable to the lysosomes of animal tissues and the lysosome-like structures of plant cells which possess similar and additional properties. During infection of *Solanum* tissues by *Phytophthora erythroseptica* there was swelling and disruption of these host cell particles, accompanied by the release of acid phosphatase and esterase. Biochemical assay for acid phosphatase confirmed that infection of host cells resulted in the liberation of acid phosphatase from a particulate to the supernatant fluid fraction of cell homogenates.

INTRODUCTION

Evidence from work on animal tissues indicates that lysosomes are involved in a variety of ways in cellular injury and in tissue regression (de Duve, 1963). It is now apparent that virus infection of cells (Allison & Mallucci, 1965; Mallucci & Allison, 1965) and actions of bacterial toxins (Bernheimer & Schwartz, 1964) may result in permeability changes in lysosomal membranes, causing the release into the cytoplasm of enzymes which initiate cytopathic effects that may lead ultimately to the death of the host cells. However, it is not certain whether the activities of these released lysosomal enzymes are a cause or a consequence of cell death. It has been shown (Gahan, 1965, 1967; Pitt & Walker, 1967; Pitt, 1968) that plant cells contain particles having affinities with the lysosomes of animal cells, but at present there is no information about the role of these structures in plant tissue pathology. The present work was designed to investigate the presence of such structures in tuber tissues of *Solanum tuberosum* and to examine their fate during infection with *Phytophthora erythroseptica*, the fungus responsible for pink rot disease of potatoes.

METHODS

Organisms. Phytophthora erythroseptica Pethybr. was isolated from a tuber of *Solanum tuberosum* cv. Majestic showing symptoms of pink rot disease. When re-inoculated into healthy tubers disease development was rapid.

Potato tubers cv. Majestic were grown in the Botanical Gardens of the University of Exeter and used throughout this investigation. Tubers were washed and surface sterilized in 70% (v/v) ethanol in water, wound-inoculated with discs of a malt-

extract agar culture of *Phytophthora erythroseptica*, and incubated for 4 to 7 days at 22°. Appropriate controls were inoculated with discs of sterile malt extract agar.

Callus cultures of tubers of *Solanum tuberosum* were obtained and grown by the method of Ingram & Robertson (1965) on media solidified with 0·5 % (w/v) Oxoid agar or as suspension cultures on liquid medium agitated at 150 rev./min. on a rotary mechanical shaker at 25°.

Inoculation of tissue cultures. Callus tissues grown for 4 to 6 weeks to a diameter of 3 to 4 cm. were divided into two portions and separated spatially on the medium. One portion was inoculated with a disc of the fungus culture and the other, serving as a control, was inoculated with a disc of sterile malt-extract agar. The tissues were then incubated for a further 1 to 4 days with intermittent examination. Suspension cultures were inoculated with discs of an agar culture of the fungus and shaken for a further period before use.

Microscopy. All observations were made with a Leitz 'Ortholux' microscope equipped with a transmitted light condenser, a Heine phase condenser and an incident light attachment permitting the use of a 250 W high-pressure mercury vapour lamp for fluorescence microscopy.

Histochemical methods. These were all established methods and were made on fresh hand-cut sections of infected and uninfected tuber tissues and on fresh callus tissue or free cells from suspension cultures. Tissue-culture material was fixed where necessary for 16 hr in cold Baker's formol-calcium or neutral formalin at 0 to 4°. Small blocks of tuber tissue were similarly fixed and sectioned on a freezing microtome at 25 to 50 μ.

Acid phosphatase. (*a*) The standard coupling azo dye method (Grogg & Pearse, 1952) was done on fresh and fixed sections and tissue culture material with sodium α-naphthyl phosphate as substrate (Koch–Light Laboratories Ltd.) and Fast garnet GBC salt (G. T. Gurr, Ltd.) with incubation for 15 to 60 min. (*b*) The post-coupling azo dye method (Rutenberg & Seligman, 1955) was used on similar tissues with sodium 6-benzoyl-2-naphthyl phosphate (Koch–Light, Ltd.) and incubation for 1 hr at 37° followed by coupling with Fast blue B salt (G. T. Gurr, Ltd.) at 0°. (*c*) The Gomori (1952) lead nitrate method was also used, but was found unsuitable for potato tuber tissue because of heavy non-specific lead deposits produced over a wide range of experimental conditions.

The specificity of these methods was checked by the use of 10^{-2} M-sodium fluoride in the incubation medium, by use of heated tissues and by omission of the substrates and the coupling dyes. In addition, tissues were pre-incubated in 0·25 % (w/v) Triton X-100 in 0·05 M-acetate buffer (pH 5·0) and frozen and thawed several times before staining for acid phosphatase in order to satisfy certain of the criteria proposed by Gahan (1967) for the identification of lysosome-like particles.

Esterases. The methods used included: the indoxyl procedure of Holt (1958) with *O*-acetyl-5-bromoindoxyl (K and K Laboratories Inc., New York) as substrate; the α-naphthyl acetate coupled with Fast blue B salt (G. T. Gurr, Ltd.) or hexazotised *p*-rosaniline method of Davis & Ornstein (1959); the Gomori (1952) procedure with Naphthol-AS acetate (G. T. Gurr, Ltd.) coupled with Fast red TR salt (G. T. Gurr, Ltd.); the thioacetic acid method (Wachstein, Meisel & Falcon, 1961) on fresh and fixed tissues.

Other hydrolases. Attempts to show activities of several other hydrolases included the use of the following methods: of Rutenberg *et al.* (1952) for arylsulphatase; of

Seligman *et al.* (1954) for β-D-glucuronidase; of Rutenberg *et al.* (1958) for β-D-galactosidase; of Vorbrodt (1961) for acid deoxyribonuclease II (DN-ase II).

Uptake of vital dyes and fluorochromes. Considerable evidence is now available that neutral red is taken up in non-toxic quantities and concentrated within the lysosomes of animal cells (Ogawa, Mizunu & Okamoto, 1961; Cohn & Wiener, 1963). The potato tuber tissue culture cells used in the present work accumulated this dye when incubated in culture media containing neutral red (G. T. Gurr, vital and fluorochrome) at 1/20,000 concentration for 2 hr in darkness. It was also found that cells stained for neutral red granules and fixed in 6% (w/v) glutaraldehyde in 0·1 M-cacodylate buffer (pH 7·4) and then stained for acid phosphatase by the standard coupling azo dye method contained a proportion of neutral red granules which also contained acid phosphatase.

Sections of healthy and uninfected potato tuber tissues were stained in neutral red and then fixed and stained for acid phosphatase by the above procedure.

Animal cell lysosomes accumulate acridine orange and other aminoacridines (Koenig, 1963; Robbins, Marcus & Gonatus, 1964) and fluoresce under appropriate conditions. In the present work euchrysin 3R (E. Gurr, Ltd.) was added to free cell suspensions at a final concentration of 1 in 5×10^4; the cells were incubated for 1 hr at 25° in darkness, washed, mounted in fresh medium containing fluorochrome. The preparations were examined by fluorescence microscopy with light of wavelength 400 to 500 mμ obtained by use of a BG 12 exciter filter and an appropriate suppressor filter. The cytoplasm fluoresced pale green with brighter green fluorescence in the nucleus and nucleolus. The cytoplasm contained numerous bright green or orange fluorescent particles, the colour apparently depending upon the amount of dye absorbed (Allison & Mallucci, 1965). Infected tuber tissue culture cells were examined in a similar manner, dye being added 1 hr before examination of samples. For comparison a sample of infected cells was mounted and incubated in a damp chamber and intermittent observations made on a single cell over 24 hr (Pl. 4, fig. 7, 8). Hand-cut sections of infected and uninfected tuber tissues of *Solanum tuberosum* were stained with similar dye concentrations and observed by similar methods.

Biochemical methods. Homogenization of infected callus tissue was done in such a way as to minimize disruption of the fungal component. Experiments showed that the addition of fungal samples to infected and uninfected callus cells followed by homogenization and centrifugation resulted in accumulation of the fungal component within the cell debris deposit; only a negligible amount was found in the supernatant fluid and particulate fractions used in subsequent work.

Infected and uninfected callus tissues were harvested and homogenized separately for 4 min. in 0·25 M-sucrose by using a Pyrex glass homogenizer rotating at about 200 rev./min. at 0 to 4°. The homogenate was then centrifuged for 10 min. at 2000 *g* at 0°, the deposit discarded, and the supernatant fluid further centrifuged for 20 min. at 35,000 *g* at 0°. The acid phosphatase within the supernatant fluid and particulate fractions was then determined by the method of Berthet & de Duve (1951) with β-glycerophosphate (British Drug Houses) as a substrate and a reaction time of 20 min. at 37°. Liberated phosphate was then determined by the method of Fiske & SubbaRow (1925) with spectrophotometric readings at 660 mμ taken 10 min. after addition of the molybdate. Appropriate blanks were determined along with readings made on reaction mixtures containing 0·01 M-NaF.

RESULTS

Staining reactions of uninfected tissues of Solanum

Living callus culture cells and suspensions of cells of tuber tissues of *Solanum tuberosum* contained phase-dense particles and structures which absorbed neutral red and euchrysin 3R (Pl. 4, fig. 9) in large quantities. Appropriate microscopy showed that the phase-dense particles were identical with those absorbing vital dyes and that these structures appeared as bright bodies when examined by dark-ground transmitted light microscopy. The particles observed by these methods were 0·2 to 2·0 μ in diameter. Similar observations were made by using fresh hand-cut sections of potato tuber tissue. However, in this tissue only a proportion of the cells contained particles which absorbed neutral red and euchrysin.

Tuber tissue sections and tissue culture cells, either fixed or unfixed, contained numerous particles which stained brown by the standard coupling azo dye method with incubation for 15 to 30 min. at 37° (Pl. 1, fig. 1; Pl. 3, fig. 5). Similar particles were shown by the post-coupling azo dye method, but localization was less well defined. These methods showed an even distribution of particulate staining throughout the tissues, with higher local concentrations in the xylem parenchyma and phloem parenchyma of the vascular elements of sections (Pl. 2, fig. 3). With incubation for less than 30 min. only slight diffuse staining of tissues was observed; longer periods of incubation resulted in a progressive increase in diffuse staining in all tissues, particularly in the vascular tissues.

Tissues subjected to freezing and thawing or incubation in Triton X-100 before incubation in the acid phosphatase test media showed slight diffuse staining and no particulate staining. Incubation of tissues in the presence of NaF or previous heating at 100° resulted in the absence of staining.

The particles revealed by the acid phosphatase methods appeared spherical and were 0·2 to 2·0 μ in diameter.

Histochemical methods for esterase including the indoxyl, α-naphthyl acetate coupled with hexazotized *p*-rosaniline and the thioacetic acid procedures showed particulate distribution of enzyme activities in the vascular tissues with some diffuse staining, but only slight particulate staining elsewhere in tuber sections. By using these methods with callus cells only slight diffuse staining was seen. However, fresh and fixed tuber sections and callus cells showed excellent particulate localization of esterase activity by using α-naphthyl acetate as substrate coupled with Fast blue B at 0°, and with the Gomori (1952) procedure with Naphthol-AS acetate as the substrate coupled with Fast red TR salt with 30 to 60 min. incubation at 22°. Both particulate and diffuse staining were resistant to the effects of the esterase inhibitor diethyl-*p*-nitrophenyl phosphate at 10^{-3} and 10^{-5} M. Particles stained by these methods had a similar size and distribution to those demonstrated by the standard coupling azo dye method for acid phosphatase.

Slight diffuse and particulate staining was shown in fresh and fixed tuber sections by using the method for β-D-galactosidase, but attempts to demonstrate activities of arylsulphatase, β-D-glucuronidase and DN-ase II were unsuccessful.

Staining reactions of tissues of Solanum infected by Phytophthora erythroseptica

Potato tubers that had been infected and incubated for 4 days at 22° and then examined for acid phosphatase activity by the coupling azo dye method, using fresh hand-cut sections and microtome sections of formalin-fixed material, showed fewer particles (Pl. 1, fig. 2) than uninfected tissues. Such particles as were visible were greatly enlarged and surrounded by areas of intense diffuse staining. Vital staining of these tissues with neutral red showed increased uptake of dye into swollen structures. By using the coupling azo dye method acid phosphatase activity was shown in these neutral-red granules after fixation in buffered glutaraldehyde. Tissues infected for a longer period, e.g. 7 days, still showed some enlarged particles containing acid phosphatase, but the staining was then generally diffuse (Pl. 2, fig. 4). At this stage of tissue infection neutral-red uptake was decreased and confined to a few swollen granules. The orange-fluorescing particles seen in healthy tissues were not observed in the infected tissues except in an occasional cell at the advancing edge of a disease lesion.

Table 1. *Potato callus tissue infected with Phytophthora erythroseptica*

Acid phosphatase activity in particulate (P) and supernatant fluid (S) fractions of potato callus tissue.

	Activity of fractions. Inorganic phosphate released (μg. P_i/20 min./g. tissue)		% total activity (P+S fractions) in fractions	
			P	S
Tissue	P	S		
Uninfected	142·5	79·1	64·3	35·7
4 days after infection	40·8	123·6	24·8	75·2

Tissue culture cells from infected callus cultures and from infected suspension cultures contained enlarged particles when stained by the coupling azo dye method for acid phosphatase (Pl. 3, fig. 6). Samples of cells taken from suspension cultures to which neutral red and euchrysin 3 R had been added showed slight enlargement, culminating in abrupt disruption of these particles over a period of 1 to 4 days (Pl. 4, fig. 7, 8). However, enlarged particles observed by vital staining never attained the size of those shown by the azo dye methods before disruption and liberation of their dye content into the cytoplasm. The appearance of infected tissues stained for non-specific esterase was basically similar to that shown by using acid phosphatase staining methods, but diffuse staining was much less intense and swollen structures were fewer.

Acid phosphatase assay of infected and uninfected cells of Solanum tuberosum

To test the possibility indicated by histochemical methods that acid phosphatase is liberated from cell particles of Solanum during infection by *Phytophthora erythroseptica*, the activities of this enzyme in the particulate and supernatant fluid fractions of appropriate cell homogenates were examined. Results of a typical experiment are given in Table 1.

DISCUSSION

There is convincing evidence for the existence in plant cells of structures which have attributes in common with the lysosomes of animal cells (Gahan, 1965, 1967; Pitt & Walker, 1967; Pitt, 1968). Although there are marked similarities between these structures there are also differences. In many cases only acid phosphatase or a limited number of other lysosomal hydrolases have been shown in these plant cell particles and this has often been taken as sufficient proof of their lysosomal nature. Furthermore, structures absorbing fluorochromes in plant cells have not yet been identified with certainty as those containing acid hydrolases. Although structure-linked latency of enzyme activity has not been shown here in the lysosome-like particles of tuber tissue of *Solanum tuberosum* several of the criteria for lysosomes listed by Gahan (1967) have been fulfilled: possession of acid phosphatase and esterase activity, uptake of vital dyes and fluorochromes and the Triton X-100 and freezing and thawing effects on membrane permeability. The biochemical evidence is also convincing; but since other hydrolases have not yet been found in some of the particles containing acid phosphatase it cannot be said with certainty that such structures are lysosomes or spherosomes. However, it was suggested by Frey-Wyssling & Mühlethaler (1965) that these two types of particle may be closely related, and Semadeni (1967) considers that the spherosomes may represent organelles equivalent to animal lysosomes.

During infection of tuber tissue of *Solanum tuberosum* by *Phytophthora erythroseptica* there is swelling and disruption of host cytoplasmic particles which have properties in common with lysosomes. This results in liberation of particulate acid phosphatase and esterase accompanied by rapid death of the host cells. It is possible that under appropriate conditions the activity of released acid phosphatase could account for some of the phosphate frequently reported to accumulate at sites of fungal infection. The precise factors involved in swelling and permeability changes of lysosomal membranes are not known, but the events observed in the present work bear a striking resemblance to those which follow virus infection of animal cells (Allison & Mallucci, 1965; Mallucci & Allison, 1965) and the effects produced by bacterial toxins on lysosomes of rabbit leucocytes (Bernheimer & Schwartz, 1964). The present studies, like those with animal tissues, do not establish whether disruption of such particles is a cause or a consequence of cell death. However, changes in lysosomes and in the structures examined in the present work precede any other histological or histochemical changes so far detected in the infected cells.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Uninfected potato tuber tissue section, fixed, stained for acid phosphatase by the coupling azo dye method. Note many small particles. × 733.

Fig. 2. Section of potato tuber tissue 4 days after infection with *Phytophthora erythroseptica*, treated as above. Fewer particles are visible, remaining particles may be greatly enlarged. Note diffuse stain. × 733.

PLATE 2

Fig. 3. Section of healthy potato tuber tissue stained for acid phosphatase by the standard coupling azo dye method after fixation. Note many small particles, particularly in the vascular tissues. × 358.

Fig. 4. Section of infected potato tuber tissue 7 days after inoculation, stained for acid phosphatase as in fig. 3. Note intense diffuse staining in vascular tissues. × 358.

PLATE 3

Fig. 5. Callus cells stained for acid phosphatase by the coupling azo dye method, post-fixed in cold neutral formalin. Note many discrete particles. × 500.

Fig. 6. Callus cell 48 hr after infection with discs of *Phytophthora erythroseptica*, fixed and stained as in fig. 5. Note enlarged particles some of which have coalesced, also intense diffuse staining. × 500.

PLATE 4

Fig. 7. Tissue culture cell from suspension culture to which euchrysin 3R had been added. Photographed by using incident fluorescence and low intensity dark-ground transmitted light microscopy 1 hr after addition of dye, and 3 hr after inoculation of the original culture with a mycelial suspension of *Phytophthora erythroseptica*. Note intense pinpoints of bright fluorescence (green and orange in original). × 266.

Fig. 8. Same cell as fig. 7 incubated in a damp chamber and photographed 24 hr after inoculation. Note many of original particles are no longer visible. × 266.

Fig. 9. Healthy tissue culture cell from suspension culture to which euchrysin 3R had been added. Photographed by fluorescence microscopy. Note many small brightly-fluorescing particles (green and orange in original). × 266.

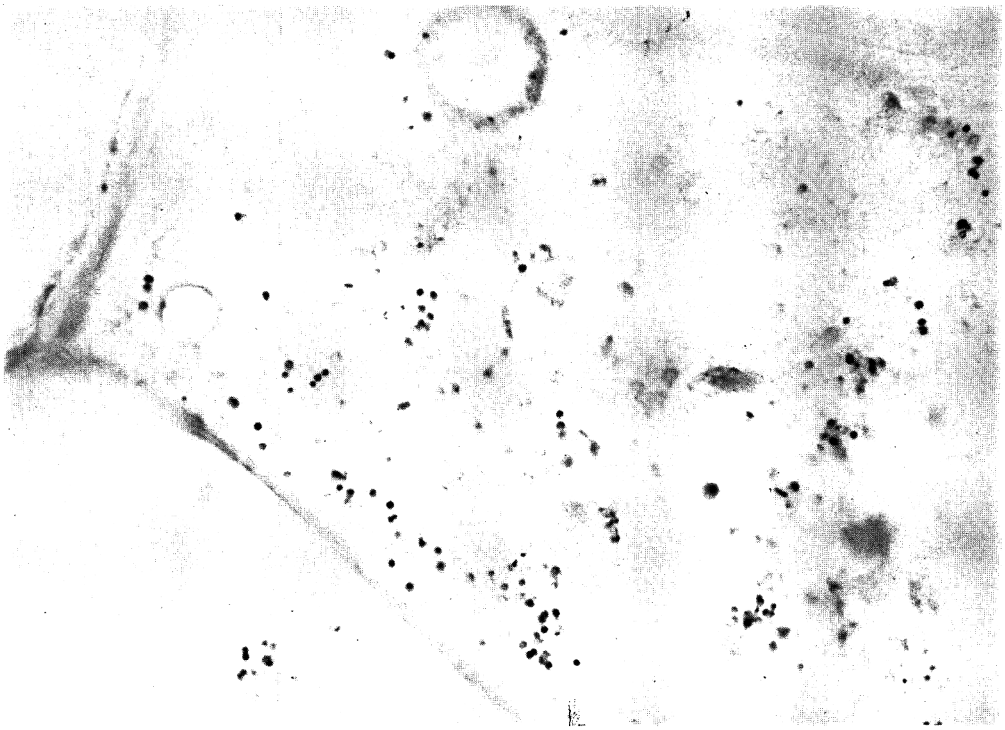


Fig. 1

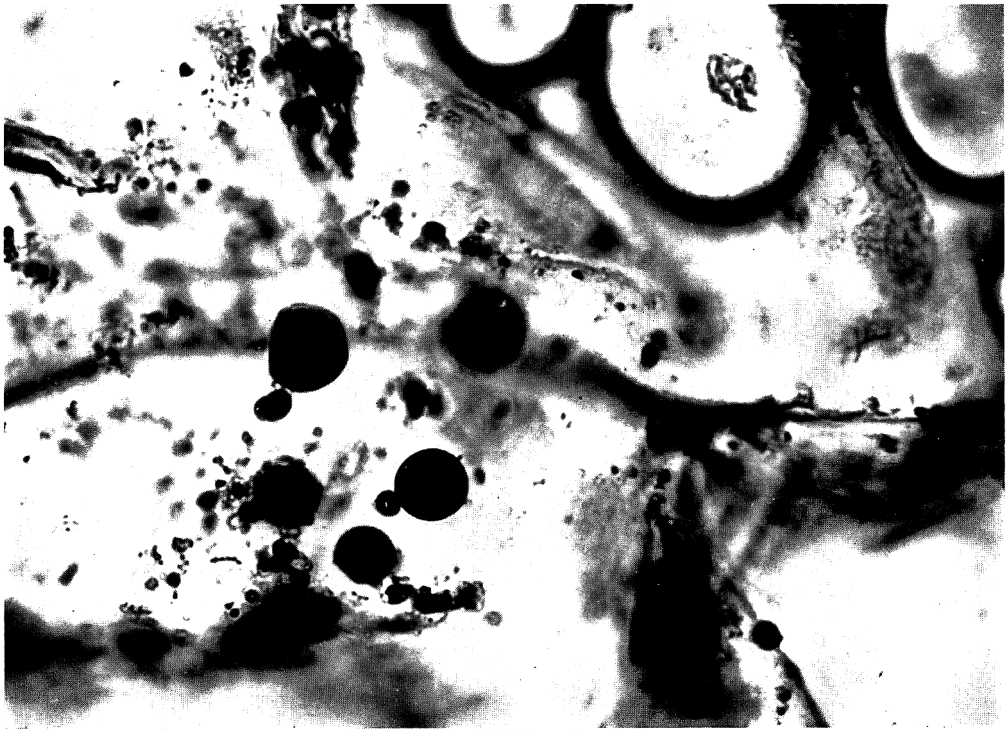


Fig. 2

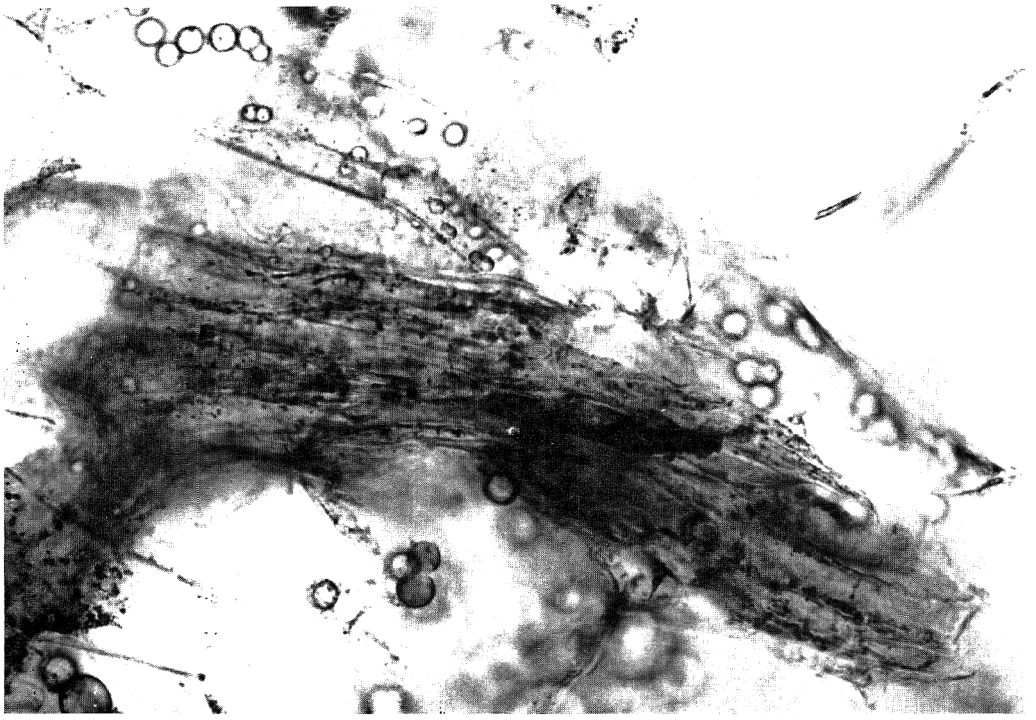
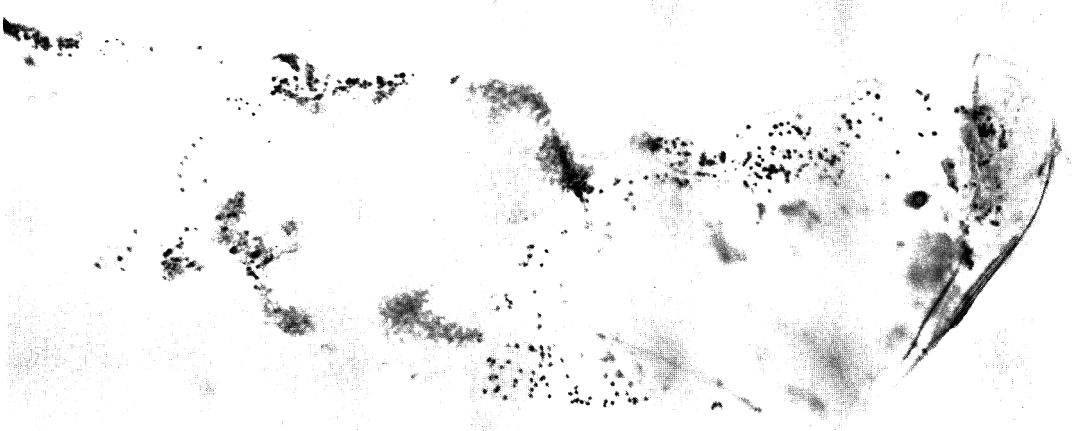


Fig. 3

Fig. 4



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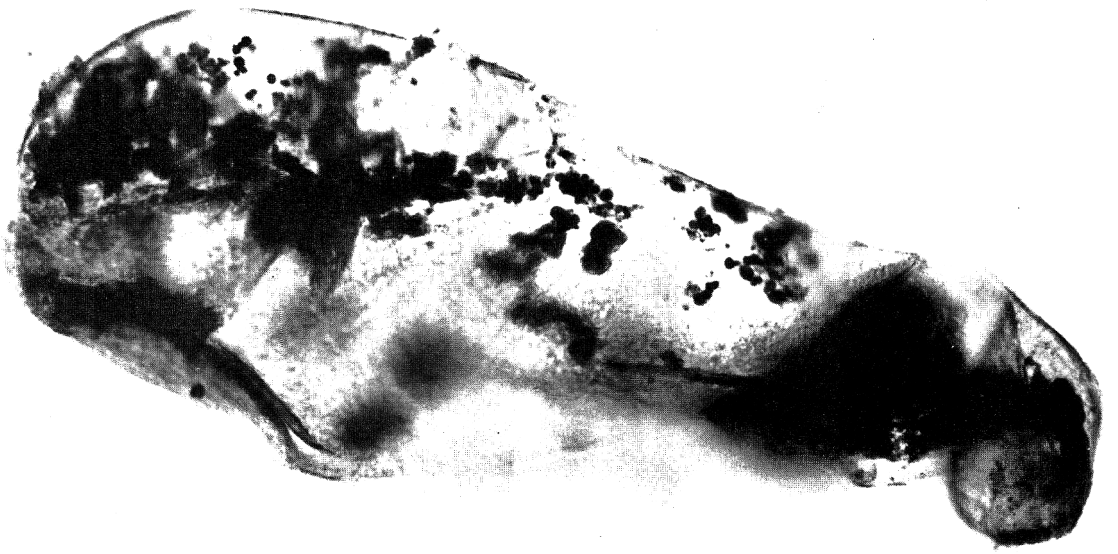

Fig. 6

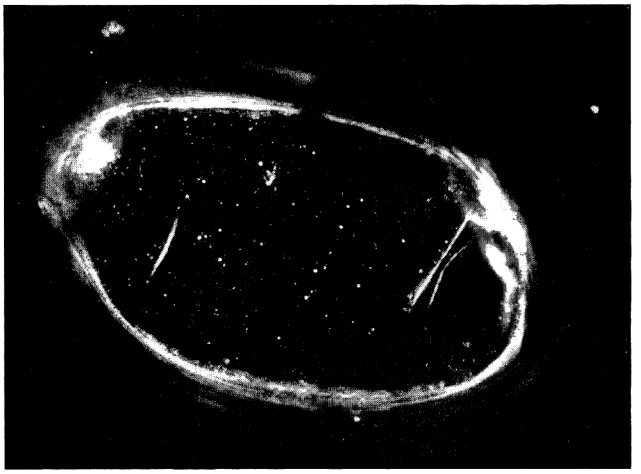

Fig. 7

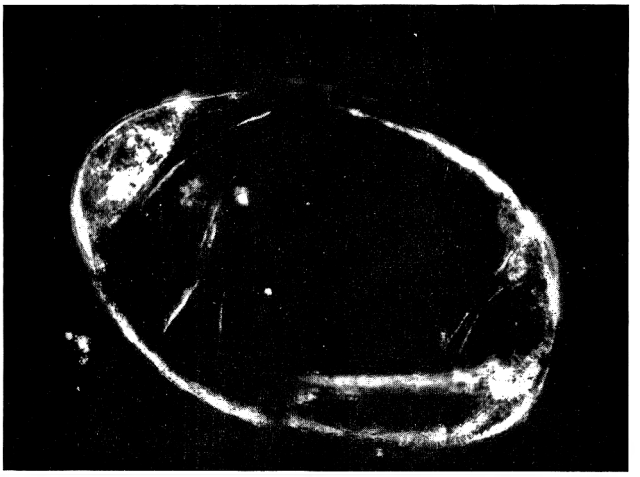

Fig. 8

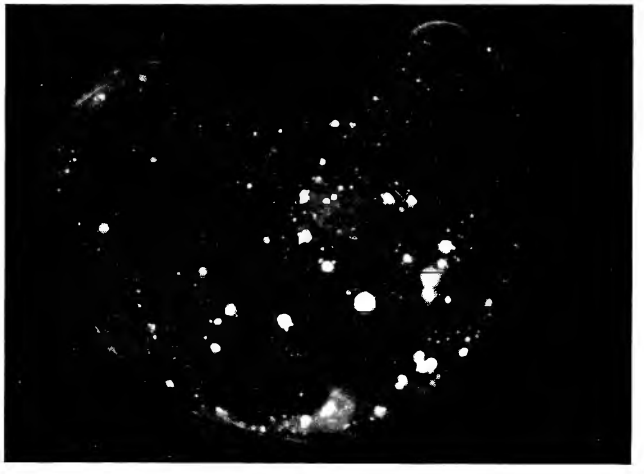

Fig. 9

J. gen. Microbiol. (1968), **53**, 205–220
Printed in Great Britain

Comparative Studies of Nitrogen Fixation by Soybean Root Nodules, Bacteroid Suspensions and Cell-free Extracts

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(*Accepted for publication* 3 *April* 1968)

SUMMARY

Rhizobium japonicum (CC711) was used to infect soybean seeds from which plants were grown. From the root nodules, bacteroid suspensions with initial rates of nitrogen fixation as high as those calculated for bacteroids in intact nodules were prepared. Oxygen, which was required for fixation by intact nodules and bacteroid suspensions, caused the eventual loss of bacteroid nitrogen-fixing ability, accompanied by an increase in O_2-uptake. In intact nodules and in bacteroid suspensions, increasing O_2 pressures resulted in higher values for K_m of nitrogen fixation. V_{max} also increased with increasing pO_2 and this was shown to be consistent with the characteristics of nitrogen fixation by anaerobic cell-free extracts of bacteroids which required an energy source (ATP), and a reductant (sodium dithionite).

Higher concentrations of carbon monoxide were required to inhibit nitrogen fixation by intact nodules than by bacteroid suspensions. Carbon monoxide was a competitive inhibitor of nitrogen fixation in bacteroid suspensions and K_i (CO) and K_m (N_2) values showed that the nitrogenase had about 30 times the apparent affinity for CO that it had for N_2.

In cell-free extracts of bacteroids, the nitrogen-fixing activity remained in the supernatant fluid after centrifugation at 100,000 *g* for 30 min. The extracts were inactivated at 0°. The K_m for nitrogen fixation by cell-free extracts was variable, 62–118 (N_2 concentration in mm. Hg. pressure), compared with 50–60 for intact nodules and 20 for intact bacteroids when K_m for these was measured in the range of pO_2 in which it was only slightly affected by O_2. The K_i for inhibition of nitrogen fixation by CO in extracts was similar to values obtained with intact bacteroids.

INTRODUCTION

Considerable understanding of the biochemistry of nitrogen fixation has developed since 1960 as a result of studies with cell-free extracts of free-living nitrogen-fixing bacteria (e.g. *Azotobacter vinelandii*, Bulen, Burns & LeComte, 1965; *Clostridium pasteurianum*, Mortenson, 1966). The symbiotic nitrogen-fixing system of legume nodules has been studied in intact detached nodules since the work of Aprison, Magee & Burris (1954), in nodule breis (Bergersen, 1966) and has been shown to be located wholly in the bacteroids (Bergersen & Turner, 1967). Koch, Evans & Russell (1967*a*, *b*) showed that the properties of cell-free extracts of Rhizobium bacteroids in many respects resemble those of extracts prepared from Azotobacter or Clostridium. The results to be given in the present paper are in substantial agreement with their results. In nature, it is the intact nodule tissue which accomplishes nitrogen fixation

and it is important to relate these new findings to the active component of the intact system, the bacteroids, which have developed the ability to perform this function only after growth within the complex structures of the nodule cells.

The effects of O_2 upon nitrogen fixation in detached intact nodules were studied by Bergersen (1962) but the inhibition of nitrogen fixation in this system by CO has only been studied in experiments with whole growing plants (Lind & Wilson, 1941). In the present paper, an account is given of experiments in which the effects of O_2 and CO upon intact nodules and Rhizobium bacteroid suspensions are compared and some properties of cell-free extracts of bacteroids are related to these effects.

METHODS

Nodules. Soybean plants (cv. Shelby) were grown in sand + vermiculite mixture in a glasshouse, as previously described (Bergersen, 1958), from seeds inoculated at sowing with strain CC711 of *Rhizobium japonicum.* Nodules were detached when they were aged about 35 days, and were used immediately.

Bacteroid suspensions. Breis were prepared under argon using a stainless-steel anaerobic press and washed bacteroids were prepared from the breis as previously described (Bergersen, 1966; Bergersen & Turner, 1967). In some experiments the suspending and washing medium contained 0·3 M-sucrose, 0·1 M-KH_2PO_4 (pH 7·0) and 1 mM-$MgSO_4$. Following the work of Koch *et al.* (1967*a*), in which polyvinyl-pyrrolidone and ascorbate were used to remove plant polyphenols, the method was modified and the initial brei was prepared in 0·1 M-KH_2PO_4 (pH 7·0) containing 1·5% (w/v) of a soluble polyvinyl-pyrrolidone (PVP) of average molecular weight 25,000 (Kollidon 25; B.A.S.F., Australia Ltd.) and 0·2 M-Na ascorbate. The bacteroids were then centrifuged down, and washed in buffered sucrose medium with the phosphate concentration decreased to 0·025 M. This modification followed the observation of inhibition of nitrogen fixation at higher phosphate concentrations (Bergersen, unpublished). The bacteroids prepared by the modified method were more active but their properties were otherwise unaltered.

Gas mixtures. These were prepared from good quality commercial gases and the composition is expressed as partial pressures measured in mm. Hg. $^{15}N_2$ was included in the gas mixtures or added to incubation vessels by syringe. Mixtures for anaerobic experiments were stored over a solution of chromous sulphate to remove traces of O_2. Mass spectrometer analysis was used to check gas composition in the vessels before and after incubation and also to measure respiration and evolution of H_2. In experiments with CO this inhibitor was injected into the reaction vessels by means of a gas chromatograph syringe.

Measurements of nitrogen fixation. Intact nodules (1·5 g. fresh wt) were incubated in 50 ml. Erlenmeyer flasks containing the desired gas mixture, the nodules then ground in 3 N-HCl and the soluble portion containing the non-protein nitrogen analysed for ^{15}N.

Washed bacteroid suspensions evolve NH_3-N from endogenous sources into the medium in addition to the accumulation of fixed NH_3-N. This is illustrated by the data from an experiment in which 3·75 μg. NH_3-N accumulated in the medium, while only 0·49 μg. was fixed. These effects, while of much smaller magnitude than reported for unfractionated nodule breis (Bergersen, 1966), are still sufficient to prevent the

use of direct nitrogen analysis for the measurement of nitrogen fixation; ^{15}N methods must therefore continue to be used. Fixation by bacteroid suspensions and by cell-free extracts was measured by using sidearm flasks with a gas volume of 30 or 70 ml. and containing 3 to 6 ml. liquid. The flasks were shaken at 25° and substrates, when used, were tipped from a sidearm. The fixed nitrogen accumulated as NH_3-N in the suspending medium (Bergersen & Turner, 1967). The ^{15}N content of this nitrogen was measured in the following manner in 1 ml. samples of suspension, withdrawn at intervals by hypodermic syringe from rubber-capped sidearms. The samples were centrifuged at 0 to 4° and the bacteroid pellet washed once with a further portion of the suspending medium. The combined supernatant fluids, with 50 or 100 μg. unlabelled NH_3-N as carrier, were distilled from a Markham still after adding an equal volume of saturated borate buffer (pH 10·5) and the distilled NH_3 trapped in 5 ml. 1 % (w/v) boric acid. Alternatively, the NH_3 in the samples was recovered by diffusion from a suspension with MgO in modified Conway dishes (Freney & Wetselaar, 1967). After titration of the NH_3-N, it was analysed for ^{15}N content and the μg. N fixed/vessel was calculated with regard to the initial liquid volume, the NH_3-N content of the samples (including the carrier NH_3-N), the atoms % ^{15}N excess of the N_2 in the gas mixture and the atoms % ^{15}N excess of the samples.

Protein determination. Because of the use of intact bacteroids it was necessary to use a protein-N method which was used throughout for whole nodules, bacteroids and extracts. Samples (0·5 ml.) of suspension or extract or a sample of ground dried nodules, were extracted with 4 ml. 0·1 N-H_2SO_4 containing 0·5 ml. Na tungstate (10 %, w/v). The precipitate, after overnight extraction and washing, was digested and the protein-N determined after distillation and titration. Protein (mg.) was given by 6·37 × mg. protein-N.

Bacteroid extracts. The bacteroids after washing were suspended in 15 ml. 0·025 M-KH_2PO_4 (pH 7·4) containing 2 mM-$MgSO_4$, under argon, and transferred to a previously argon-flushed pressure-release cell designed in the Division of Plant Industry, CSIRO, Canberra. Deoxyribonuclease (about 1 μg.; Mann Research Laboratories, New York) was added and the piston inserted under a stream of argon. The bacteroids were broken by using a pressure of 8 tons/sq. in. and the extract collected and centrifuged under argon. Extracts (total volume 3 to 5 ml.) were shaken with $^{15}N_2$ + argon mixtures at 25° with various additions. The ATP-generating system consisted of 6 μ-moles adenosine-5-triphosphate (Na salt; Nutritional Biochemicals Co., Cleveland, Ohio), 150 μmoles creatine phosphate and 1–2 mg. creatine phosphokinase (Sigma Chemical Co., St Louis, Mo.) all dissolved in 0·025 M-KH_2PO_4 and neutralized. The dithionite ($Na_2S_2O_4 . 2H_2O$, 24 μmoles/vessel), made up daily, was dissolved in argon-saturated phosphate buffer (0·1 M; pH 7·0) and stored under argon at 0° until used. The incubation vessels with the constituents of the ATP-generating system in the sidearms, were flushed three times with argon and then filled with gas mixture to a predetermined pressure and the extracts added by syringe through a rubber-capped port in one sidearm. The dithionite was added last, also by syringe. NH_3 was recovered from the flask contents by distillation from saturated borate (pH 10·5), titrated and analyzed for ^{15}N, and the amount of nitrogen fixed calculated as described above.

RESULTS

Comparison of bacteroid and nodule activity

Nitrogen fixation was greatest in bacteroid suspensions prepared from nodules disrupted in the presence of buffered PVP and ascorbate and washed and incubated in buffered sucrose containing 1 mM-Mg^{2+} and 16·7 mM-Na succinate. Initial fixation rates (0 to 20 min.) of 700 to 900 mμg. N/mg. protein/hr with pO_2 42 mm. Hg and pN_2 140 mm. Hg at 25°, was consistently obtained with this method. Bacteroids prepared from nodules disrupted in buffered sucrose (Bergersen & Turner, 1967) had initial fixation rates of 450–500 mμg. N/mg. protein/hr under the same conditions. To compare these rates of nitrogen fixation with the rates of fixation by bacteroids in intact nodule tissue, samples of nodules aged 35 days were incubated for 1 hr at 25° and the nitrogen-fixation rates and protein content of the nodules measured. These nodules contained 179 mg. protein and they fixed at a rate of 21 μg. N/hr at pO_2 140 mm. Hg, and at a rate of 60 μg. N/hr at pO_2 350 mm. Hg (both with pN_2 140 mm. Hg). In previous work (Bergersen & Turner, 1967, Table II), bacteroid protein accounted for about 56% of the nodule brei protein. Assuming the same proportion, the above rates of nitrogen fixation by intact nodules can be expressed as 210 and 599 mμg. N/mg. bacteroid protein/hr at pO_2 values of 140 and 350 mm. Hg, respectively. From these results it is concluded that bacteroids prepared anaerobically in the manner described are comparable in their initial nitrogen-fixing capacity with bacteroids in intact nodule tissue, although the high rates of fixation by suspensions are transitory (Fig. 1).

Effects of O_2 upon bacteroid suspensions

The results obtained were similar to those obtained with nodule breis (Bergersen, 1966); air had to be excluded during preparation of the bacteroids, but O_2 was required for nitrogen fixation. Table 1 shows the effect of vigorously aerating a bacteroid

Table 1. *Inactivation of bacteroids by aeration*

Inactivation was by bubbling air for 15 min. at 0°. Aerated treatments contained 53 mg. bacteroid protein/vessel and non-aerated treatments 48 mg. bacteroid protein/vessel. Incubated 30 min. at 23° with $^{15}N_2$ (168 mm. Hg) and O_2 (42 mm. Hg). Respiration and H_2 evolution measured mass-spectrometrically. Non-aerated bacteroids were also kept 15 min. at 0°. Bacteroids suspended in 0·1 M-phosphate (pH 7·0)+1 mM-Mg^{2+}.

Treatment	Succinate (100 μmoles)	N_2 fixed (mμg. N/mg. bacteroid protein)	H_2 evolution (ΔpH_2 atm.)	Respiration: ΔpO_2 (atm.)	Respiration: ΔpCO_2 (atm.)
Aerated	−	0	0	-46×10^{-4}	$+48\times10^{-4}$
Aerated	+	43	$1{\cdot}4\times10^{-4}$	-91×10^{-4}	$+86\times10^{-4}$
Non-aerated	−	383	$5{\cdot}5\times10^{-4}$	-90×10^{-4}	$+53\times10^{-4}$
Non-aerated	+	424	$6{\cdot}0\times10^{-4}$	-101×10^{-4}	$+86\times10^{-4}$

suspension in sucrose-free medium for 15 min. at 0°. First, this treatment decreased the endogenous O_2 uptake, presumably because of depletion of endogenous substrate resulting from the vigorous agitation in the sucrose-free suspending medium. The O_2 uptake was restored on adding succinate. These results may be compared with those obtained previously (Bergersen & Turner, 1967) in which it was found that anaerobic

washing of bacteroids in sucrose-free medium decreased the endogenous nitrogen-fixing activity to a very low value, the activity being restored by adding succinate or other substrates. Secondly, nitrogen fixation and hydrogen evolution were reduced by the aeration, and these activities were not restored by adding succinate.

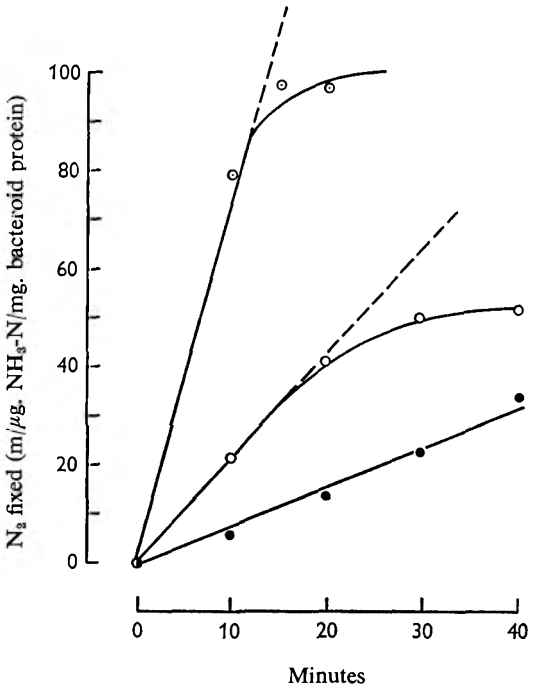

Fig. 1. The effect of O_2 concentration upon the time course of nitrogen fixation by washed bacteroids of *Rhizobium japonicum*. The dotted lines indicate the initial rates of fixation used for kinetic studies. The gas phase contained $^{15}N_2$ (70 mm. Hg) and pO_2 values (mm. Hg) of ●——● 21, ○——○ 42 and ⊙——⊙ 84; the balance to 700 mm. Hg was argon.

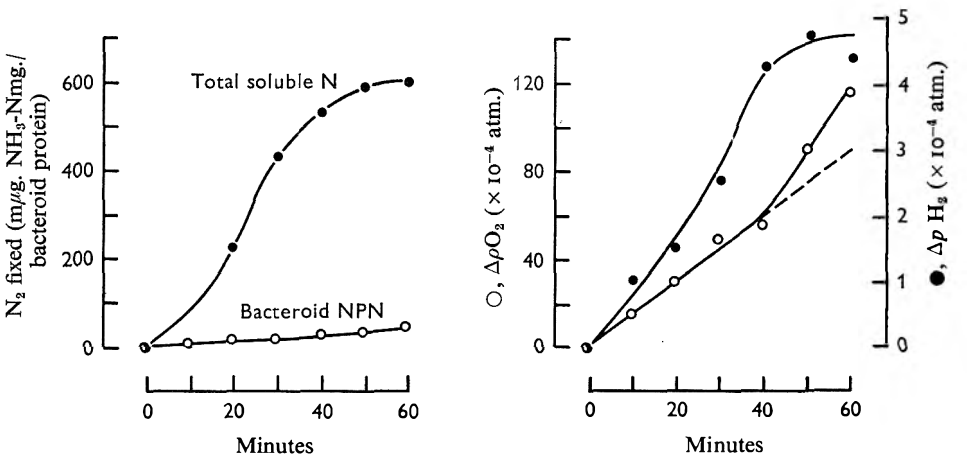

Fig. 2. The relationship between the time courses of nitrogen fixation, hydrogen evolution and respiration by washed bacteroids of *R. japonicum*. The distribution of the fixed N between non-protein-N (NPN) of bacteroids and suspending medium is also shown.

In Fig. 1, the effect of O_2 concentration upon the initial rates of nitrogen fixation and upon the time course are illustrated. Doubling the pO_2 value more than doubled the initial fixation rates but the course was much shortened. The inactivation of the nitrogen-fixing activity of bacteroids during incubation in the presence of O_2 may be due to the oxidation of labile-SH groups. Attempts to preserve the activity by the use of β-mercaptoethanol or thioglycollate (Fraenkel-Conrat, 1957) were unsuccessful; neither compound prolonged the activity and both were inhibitory to nitrogen fixation. The decline in fixation, which occurred after about 40 min. with pO_2 42 mm. Hg was accompanied by a decline in H_2 evolution and by an increase in respiration rate. This is illustrated in the data of Fig. 2, which presents the results from an experiment in which 6 vessels were incubated for the times shown, before the gas phase was sampled and analysed and the distribution of the newly fixed N in the bacteroid suspension determined. During nitrogen fixation, only about 3–5 % of the fixed N was found in the bacteroids after one washing, but after 60 min. when fixation had ceased, this increased slightly to 7 %. In other experiments, the soluble NH_3-N, which accounted for most of the fixed N in this experiment, declined after fixation ceased. These observations suggested that there may have been some assimilation of newly fixed soluble NH_3-N at this stage. This was been confirmed in experiments which showed slight labelling of α-amino-N of the bacteroids after 40 min.

Effects of O_2 upon reaction kinetic measurements with nodules and bacteroids

The characteristics of an enzymic reaction are most conveniently described in terms of the Michaelis equation:

$$v = V_{max}s/K_m + s$$

in which v is the reaction velocity, V_{max} the velocity with non-limiting substrate concentrations, s the substrate concentration and K_m the equilibrium constant of the reversible combination of enzyme and substrate (the Michaelis constant). In the work to be described, N_2 was the substrate and the values obtained were for the reduction of N_2 to NH_3.

In previous work with intact detached nodules (Bergersen, 1962) the apparent K_m for nitrogen fixation increased sharply at high values of pO_2 and was accompanied by inhibition of nitrogen fixation. Although V_{max} also increased in this range of pO_2 values, the increase was not significant and it was concluded that O_2 was a competitive inhibitor of nitrogen fixation in this system.

The effects of O_2 upon fixation by bacteroid suspensions appeared to be different: no inhibition was obtained at the highest concentration which permitted measurement of initial rates of nitrogen fixation. K_m and V_{max} for isolated bacteroids were therefore measured by using the reciprocal method of Dixon (1953). Six experiments were done, in each of which a pair of pO_2 values was compared. The data are shown in Fig. 3. In this work v was taken as the average rate of fixation over the first 10 or 20 min. (see Fig. 1) and s was measured in portions of an atmosphere of 700 mm. Hg. Statistical treatment was necessary in order to give confidence limits for the values obtained. A preliminary fit of $1/v$ upon $1/s$ was made, ignoring the increasing array dispersion with $1/s$. Deviates from the line were calculated and the absolute values of these plotted against estimated $1/v$. A quadratic trend was indicated and it was found that the array standard deviation was proportional to $(1/v)^2$. The values of $1/v$ and $1/s$

were then fitted with weights inversely proportional to the fourth power of the estimated $1/v$ of the first fitting, and the confidence limits of the intercepts giving $1/K_m$ and $1/V_{max}$ were calculated.

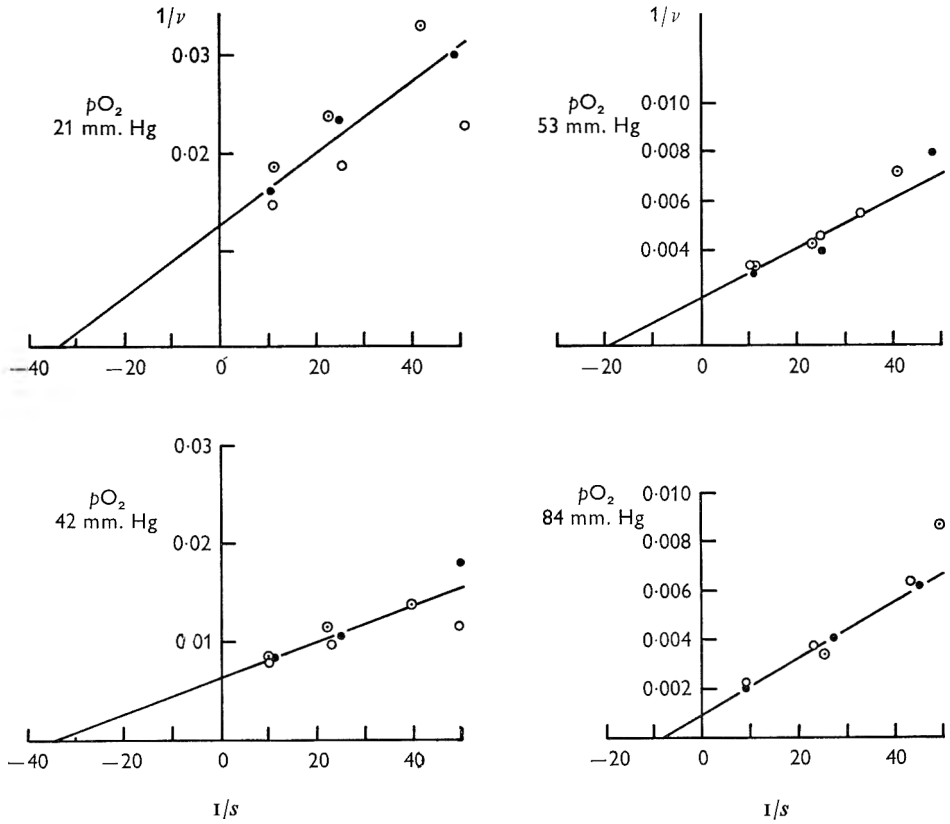

Fig. 3. Dixon–Lineweaver–Burk plots for nitrogen fixation by washed bacteroids of *R. japonicum* at pO_2 values of 21, 42, 53 and 84 mm. Hg. v measured as initial fixation rates in mμg. N/mg. protein/hr and s as portions of an atmosphere of 700 mm. Hg. The regression lines are fitted according to the weighted method described in the text. In each case, data for 3 experiments are shown.

Both K_m and V_{max} increased with increasing pO_2 values over the range 52 to 84 mm. Hg, while from 21 to 42 mm. Hg there was a significant increase in V_{max} but no change in K_m. In Fig. 4 these results are compared with data for intact nodules, used previously (Bergersen, 1962). Although no inhibition of nitrogen fixation by O_2 was obtained with bacteroids, in both intact nodules and bacteroids the kinetic effects of O_2 are comparable. A fourfold increase in pO_2 changed K_m and V_{max} by similar factors in each system, although with intact nodules the increase in V_{max} at high pO_2 was not significant, because of large experimental error. It is clear that increasing O_2 pressures decreased the apparent affinity of the nitrogenase for N_2 as shown by the increased values of K_m at higher pO_2 values. At the same time, increasing pO_2 values increased the velocity of the reaction. In these experiments it was observed that O_2-uptake was greatest at the lowest N_2 concentrations. This is in general agreement with results of

experiments such as that illustrated in Fig. 2, which suggests competition between O_2 and N_2 for reducing power.

The inhibition of nitrogen fixation by carbon monoxide

The effect of carbon monoxide upon nitrogen fixation by intact nodules is shown in Fig. 5, which presents data from a series of experiments, in which samples of 1·5 g. fresh weight of nodules were incubated for 1 hr at 25°, with pCO values of 0 to 8 mm.

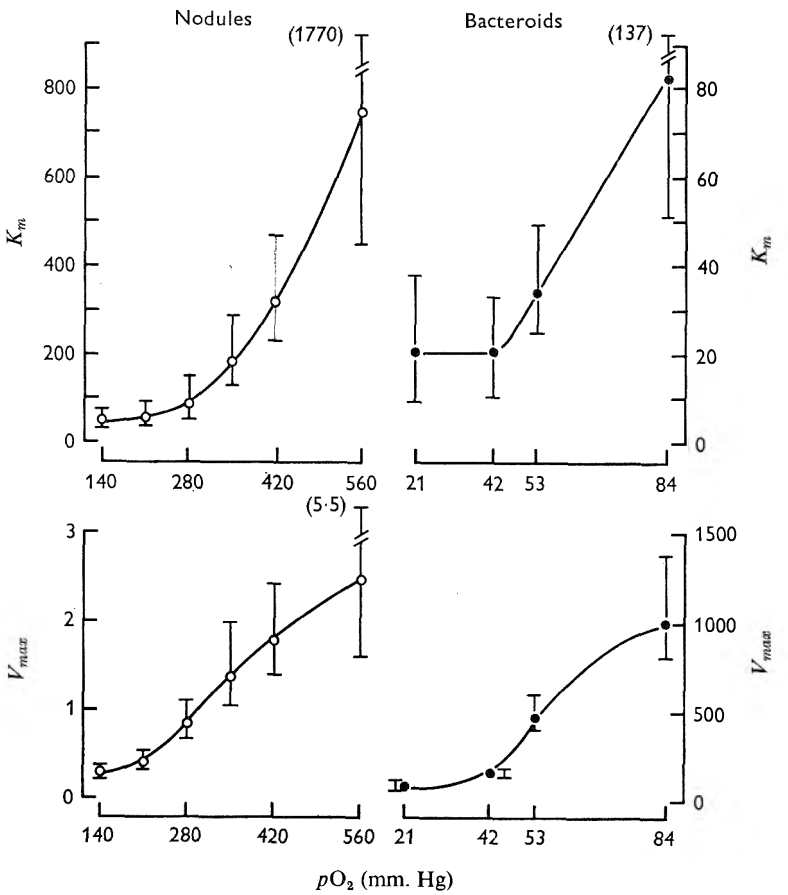

Fig. 4. Changes of K_m and V_{max} as a function of pO_2. Data for intact nodules from Bergersen (1962) O——O, and for bacteroid suspensions from the data of Fig. 3 ●——●. The values given are for s measured in mm. Hg: v for nodules was atoms % excess/hr and for bacteroids, v was mμg. N/mg. protein/hr. The vertical lines give the magnitude of the 95 % confidence limits.

Hg, a pO_2 of 140 mm. Hg, and pN_2 values of 56 to 140 mm. Hg. Although inhibition by CO was greater at lower pN_2 values, suggesting that inhibition was competitive, it was not possible to apply kinetic treatment because of the slightly stimulatory effect of low pressures of CO. Inhibition by 50 % occurred in the pCO range of 4 to 8 mm. Hg with a pN_2 range of 56 to 140 mm. Hg. There was no effect of CO upon respiration or H_2 evolution in these experiments.

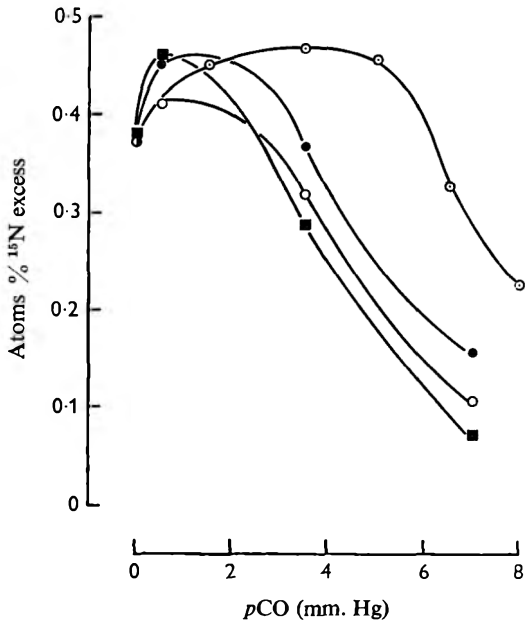

Fig. 5. Inhibition of nitrogen fixation in intact nodules by CO. Fixation measured as atoms % ^{15}N in nodule non-protein-N. pO_2 140 mm. Hg; pN_2 values (mm. Hg): ■——■ 55, ○——○ 99, ●——● 97 and ⊙——⊙ 140.

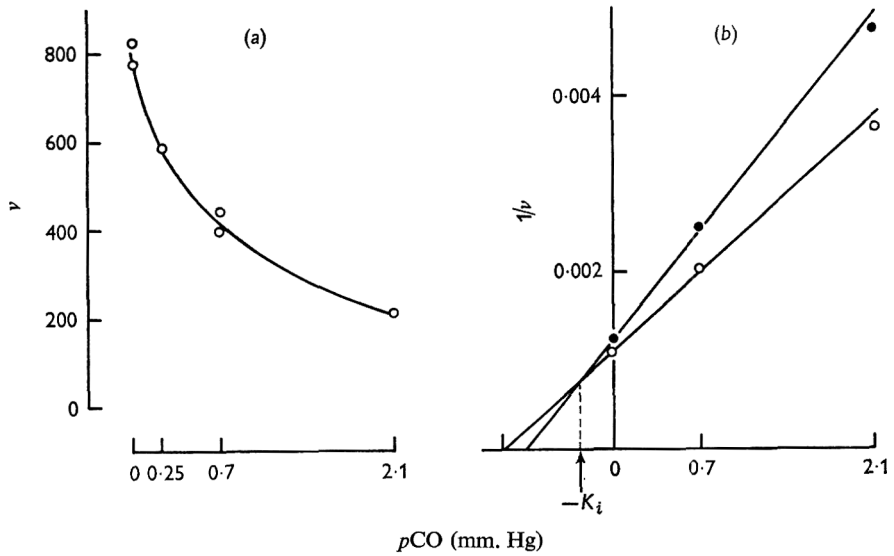

Fig. 6. Inhibition by CO of nitrogen fixation by *R. japonicum* bacteroid suspensions with a pO_2 42 mm. Hg. v = initial fixation rates in mμg. N/mg. protein/hr. (*a*) pN_2 = 70 mm. Hg; data for two experiments. (*b*) Competitive inhibition shown by the method of Dixon (1953). pN_2 (mm. Hg): ●——● 70 and ⊙——⊙ 140. Data for one experiment.

The inhibition of nitrogen fixation by CO in thrice-washed bacteroid suspensions was studied in experiments at pO_2 42 mm. Hg. The results are illustrated in Fig. 6, which shows that the inhibition was of standard form and that it was competitive (Dixon, 1953). The mean inhibitor constant (K_i, the equilibrium constant of the reversible combination of the nitrogenase with CO) was in these experiments $0{\cdot}43 \pm 0{\cdot}11$ with s and i measured in mm. Hg pressure, and the mean K_m was the same as that obtained in previous experiments with pO_2 42 mm. Hg (Fig. 4). There was no effect of CO upon respiration by bacteroids at concentrations which produced almost complete inhibition of nitrogen fixation. At higher CO concentrations there was partial inhibition of O_2-uptake which showed some evidence of light reversibility (Table 2).

Table 2. *Effects of CO upon nitrogen fixation and respiration of bacteroid suspensions*

Suspensions incubated for 30 min. at 25°. Gas phase contained O_2 (35 mm. Hg) and $^{15}N_2$ (140 mm. Hg). Nitrogen fixed measured as NH_3-N after addition of carrier and respiration measured mass-spectrometrically as changes in partial pressure. One atm. = 700 mm. Hg. Results of two experiments. Darkened flasks wrapped in foil; lighted flasks illuminated by Phillips Altrilux lamp (500 W) at a distance of 1 foot above the vessels.

		Respiration	
pCO (mm. Hg)	N fixed (μg.)	ΔpO_2 (atm.)	ΔpCO_2 (atm.)
0 (dark)	4·71	$-81{\cdot}4 \times 10^{-4}$	$+73{\cdot}2 \times 10^{-4}$
0·06 (dark)	2·88	$-80{\cdot}7 \times 10^{-4}$	$+77{\cdot}7 \times 10^{-4}$
6·0 (dark)	0·18	$-81{\cdot}8 \times 10^{-4}$	$+77{\cdot}8 \times 10^{-4}$
0 (light)	4·87	$-111{\cdot}9 \times 10^{-4}$	$+62{\cdot}0 \times 10^{-4}$
14 (dark)	0	$-69{\cdot}3 \times 10^{-4}$	$+54{\cdot}4 \times 10^{-4}$
14 (light)	0	$-84{\cdot}8 \times 10^{-4}$	$+59{\cdot}7 \times 10^{-4}$
300 (dark)	0	$-69{\cdot}0 \times 10^{-4}$	$+57{\cdot}3 \times 10^{-4}$
300 (light)	0	$-92{\cdot}0 \times 10^{-4}$	$+54{\cdot}3 \times 10^{-4}$

Experiments with cell-free extracts of bacteroids

Bacteroids broken under argon fixed N_2 into NH_3 under strictly anaerobic conditions when supplied with dithionite and an ATP-generating system; unbroken bacteroids did not fix N_2 under these conditions (Table 3). Omission of the dithionite from the broken-cell preparation decreased the activity by more than 90 %. In other experiments, omission of the creatine phosphate and creatine phosphokinase decreased nitrogen fixation by 30 to 60 % and omission of ATP decreased it to zero.

In some experiments (e.g. Table 3), centrifugation in the chilled rotor of the Spinco, under argon, to remove coarse debris and intact bacteroids, resulted in considerable loss of activity. In a later experiment (Table 4) storage of the broken bacteroids in ice, under argon, resulted in loss of half the activity in 30 min., as compared with the same preparation kept under argon at room temperature (20°). Centrifugation for 30 min. at 100,000 g in an unchilled rotor with the chamber at 20–25° and measurement of fixation by the pellet ($P_{100,000}$) and the supernatant fluid ($S_{100,000}$) showed that the highest specific activity resided in the latter fraction. This amount of fixation corresponded to 70 % of the activity of the unfractionated broken bacteroids, kept under argon at 20° for the same time (Table 4).

The time course of fixation was linear for the first 30 min. (Fig. 7a), and these initial rates were used for the calculation of K_m in experiments in which a range of N_2

pressures was used (Fig. 7*b*). Values of K_m with any one extract could be obtained within quite narrow confidence limits (Fig. 7*b*). However, there was considerable fluctuation between values obtained with extracts prepared on different days, even when rigidly standardized methods were used. In four experiments K_m was measured

Table 3. *Nitrogen fixation by cell-free extracts of bacteroids*

Vessels contained the preparations as listed in a total volume of 3·0 ml. Succinate, 100 μmoles/vessel. ATP-generating (ATP-gen.) system: ATP (Na salt) 6 μmoles; creatine phosphate 150 μmoles; creatine-creatine-phosphokinase 2 mg./vessel, $Na_2S_2O_4$, 24 μmoles/vessel. One atmosphere (700 mm. Hg) was made up of the gases shown and the balance with argon. Preparations were incubated for 1 hr at 25° with shaking.

Preparation	Additions	Atmosphere (mm. Hg) pO_2	Atmosphere (mm. Hg) $p^{15}N_2$	NH_3-N* vessel (μg.)	Protein vessel (mg.)	Atoms % ^{15}N excess	N fixed (mμg. N/mg. protein)
Whole bacteroids	Succinate	42	70	75	12·9	2·603	151·3
Whole bacteroids	ATP gen.+ $Na_2S_2O_4$	0	140	73	12·9	0·006	0·3
Broken bacteroids	ATP gen.	0	140	71	18·2	0·031	1·2
Broken bacteroids	ATP gen.+ $Na_2S_2O_4$	0	140	68	20·2	3·786	127·9
5000 g supernatant fluid	ATP gen.	0	140	75	21·0	0·039	1·4
5000 g supernatant fluid	ATP gen.+ $Na_2S_2O_4$	0	140	73	21·0	0·801	27·8

* Includes unlabelled carrier NH_3-N.

Table 4. *The distribution of activity in cell-free extracts of bacteroids and the effects of storage at 0°*

Vessels contained the extracts shown and, in a total volume of 3·0 ml., ATP (6 μmoles), creatine phosphate (150 μmoles), creatine phosphokinase (2 mg.), $Na_2S_2O_4$ (24 μmoles). Vessels were incubated with shaking at 25° for 1 hr with an atmosphere containing $p^{15}N_2$ 140 mm. Hg (balance was argon).

Treatment	Protein/vessel (mg.)	NH_3-N* vessel (μg.)	^{15}N atoms % excess	Nitrogen fixed (mμg.) per vessel	Nitrogen fixed (mμg.) per mg. protein	Nitrogen fixed (mμg.) Total preparation
Broken bacteroids 20°; 45 min.	13·8	65	1·435	933	67·8	12,126
Broken bacteroids 0°; 45 min.	13·8	61	0·721	440	32·0	5,718
$P_{100,000.\frac{1}{2}}$	7·7	38	0·192	73	9·5	711
$S_{100,000.\frac{1}{2}}$	7·1	69	1·156	798	113·1	8,425

* Includes carrier NH_3-N.

as 61·7, 64·5, 95·0 and 118·0 (N_2 pressure in mm. Hg). V_{max} varied from 1 to 2 mμmoles NH_3/min. mg. protein, higher values being obtained with experiments in which K_m values were low. The variation was too great to permit pooling of data to obtain confidence limits, as was done for bacteroid suspensions. These results show a three- to five-fold increase in K_m for extracts, as compared with the value for intact bacteriods measured in the range of pO_2 in which K_m was unaffected.

The effects of CO were measured in a manner similar to measurements made with

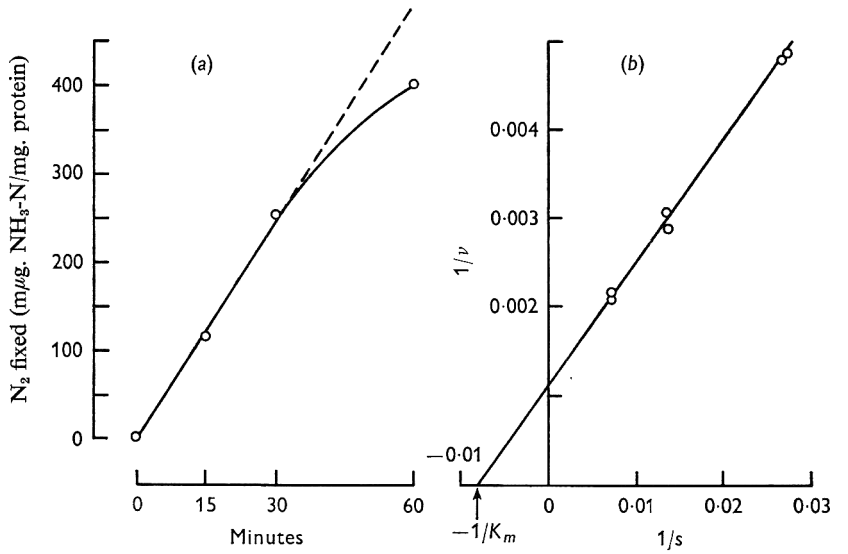

Fig. 7. (*a*) Nitrogen fixation by a cell-free extract of bacteroids of *R. japonicum*. Vessels contained 11·4 mg. $S_{100,000.\frac{1}{4}}$ protein, ATP (6 μmoles), creatine phosphate (150 μmoles), creatine phosphokinase (0·8 mg.), $Na_2S_2O_4$ (24 μmoles), in a total volume of 4 ml. Incubated with $^{15}N_2$ (119 mm. Hg) with shaking at 25°. (*b*) Determination of K_m for nitrogen fixation by $S_{100,000.\frac{1}{2}}$ protein. Details as in (*a*). *s* measured in mm. Hg and *v* measured as the initial velocity (0 to 30 min.) in mμg. N/mg. protein/hr. Data for one experiment.

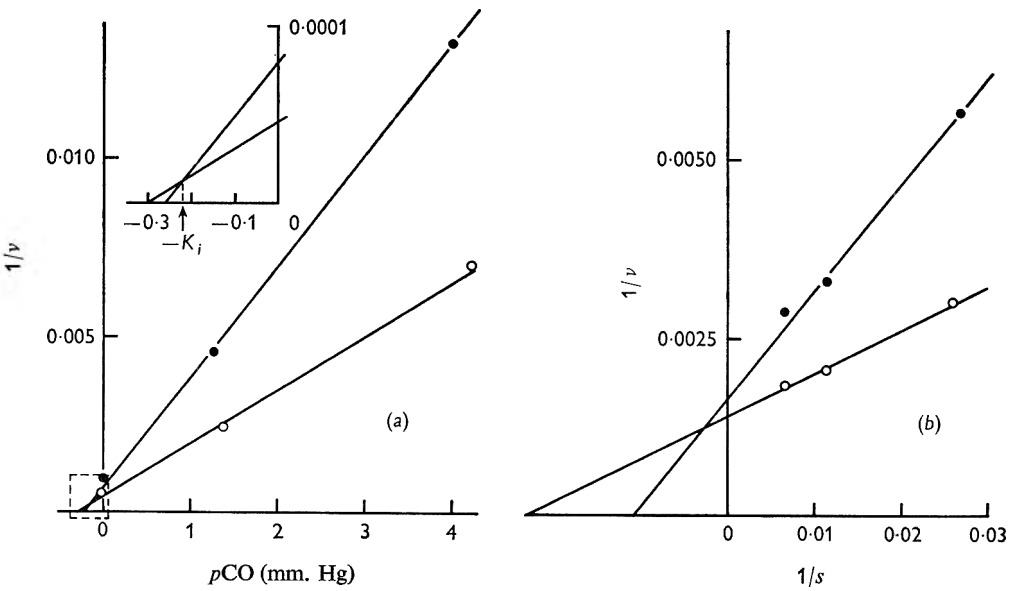

Fig. 8. Inhibition by CO of nitrogen fixation by cell-free extracts of bacteroids of *R. japonicum*. (*a*) Determination of K_i; data for one experiment. pN_2 (mm. Hg): ○——○, 140 and ●——● 35. (*b*) Plot of data confirming competitive inhibition. *i* and *s* measured in mg. Hg pressure. *v* measured in mμg. N/mg. protein/hr. Other details as described in the text. *p*CO (mm. Hg): ○——○ 0 and ●——● 1·3. The intercepts at $1/s = 0$ are not significantly different.

intact bacteroids. Carbon monoxide was a competitive inhibitor of nitrogen fixation in cell-free extracts with a K_i of $0{\cdot}23 \pm 0{\cdot}02$ (mm. Hg), but the lines for the two substrate concentrations intercepted at a low value of $1/v$ (Fig. 8*a*). A double reciprocal plot (Dixon, 1953) was therefore used to confirm the competitive nature of the inhibition (Fig. 8*b*). In all of these experiments, K_m varied between 62 and 77 mm. Hg.

DISCUSSION

The main findings of this work are summarized in Table 5 in which the effects of O_2 and CO upon the kinetics and other properties of the nitrogen-fixing system are compared for nodules, bacteroid suspensions and cell-free extracts of bacteroids. The results, which showed that the activity of cell-free extracts was decreased when stored at 0°, were similar to observations made with extracts of *Clostridium pasteurianum* and *Azotobacter vinelandii* (Dua & Burris 1965; R. C. Burns, personal communication). We have found no evidence of this effect before the disruption of the bacteroids.

Table 5. *Comparison of nitrogen fixation by intact nodules, bacteroid suspensions and cell-free extracts of bacteroids as influenced by oxygen and carbon monoxide*

	Intact nodules	Bacteroid suspensions	Extracts
Effects of O_2	Required; fixation linear with time at all pO_2 values. No fixation below pO_2 3·5 mm. Hg; maximum at 300 to 350 mm. Hg; inhibited at higher pO_2 values	Required; fixation linear with time below pO_2 14 mm. Hg; higher values increase initial rates up to 85 mm. Hg but the time course is shortened, due to inactivation	Causes inactivation; requirement replaced by use of ATP generating system + $Na_2S_2O_4$
K_m (N_2) (mm. Hg)	50·3 $\begin{cases}37{\cdot}0^* \\ 7{\cdot}8\end{cases}$ in air increases at pO_2 above 300 mm. Hg	20·4 $\begin{cases}10{\cdot}7^* \\ 33{\cdot}4\end{cases}$ at low pO_2 increases at pO_2 above 42 mm. Hg	Variable; 61 to 118 (compare 42·5 obtained by Koch *et al.* 1967*b*)
Effects of CO	Inhibitory; 50 % inhibited at pCO 4 to 8 mm. Hg. Less inhibition at higher pN_2 but data not suitable for kinetic analysis	Competitive inhibition: $K_i = 0{\cdot}43 \pm 0{\cdot}11$† mm. Hg	Competitive inhibition: $K_i = 0{\cdot}23 \pm 0{\cdot}02$† mm. Hg

* 95 % confidence limits. † Range of variation from mean value.

The effects of oxygen upon nitrogen fixation by bacteroids in suspension or in host tissue can be summarized as follows. (1) Oxygen is required, for nitrogen fixation by nodules and bacteroid suspensions, to provide available energy, as shown by the increases in v and V_{max} when the pO_2 value was increased. (2) The nitrogen-fixing system is inactivated by free oxygen. This is seen from the shortened time-course of fixation by isolated bacteroids with increasing pO_2 values and by deliberate aeration during preparation. (3) The apparent affinity of nitrogenase for N_2 is decreased in the presence of oxygen, as shown by the increased K_m with increasing pO_2. (4) The effects 2 and 3 must be separate, because the time-course of fixation by intact nodules is not shortened at high pO_2 values where K_m is sharply increased (Bergersen, 1962). This implies that the bacteroids are protected in the intact tissue from the inactivating effects of

high pO_2 but are exposed to its kinetic effects (see discussion in Bergersen & Turner 1967). (5) O_2 and N_2 may be regarded as being in competition for reducing power. This competition may not occur at the N_2-binding site but may be an expression of the presence of two divergent electron-transport pathways, one of which terminates with O_2 and the other with N_2. The increase in O_2-uptake which accompanied cessation of fixation by bacteroids (Fig. 2), and the kinetic experiments with bacteroids in which it was found that O_2-uptake was greatest at the lowest N_2 pressures, illustrate this. Because of the rapid inactivation of bacteroids at high pO_2 values it was not possible to reach concentrations of O_2 equivalent to those which produced inhibition in intact nodules. The nature of the apparent competition, therfore, remains in doubt because the cell-free system with dithionite is incompatible with the use of oxygen as an inhibitor.

Lind & Wilson (1941) showed that carbon monoxide was a specific but non-competitive inhibitor of nitrogen fixation by growing nodulated redclover plants. Except for some studies on the effects of CO upon the leghaemoglobin of nodules (Smith, 1949) and upon hydrogen metabolism (Hoch, Schneider & Burris, 1960; Bergersen, 1963), little work has been done with the legume system. Inhibition of nitrogen fixation by CO has been shown to be competititive with N_2 in cell-free extracts of *Clostridium pasteurianum*, with a K_i of about 0·3 mm. Hg (Lockshin & Burris, 1965). The form of the data for intact nodules shows why the competitive nature of inhibition of nitrogen fixation by CO was not shown earlier. The slightly stimulatory effect of low concentrations (2 to 6 mm. Hg) confounds kinetic treatment of the data. No explanation of this effect is offered; it may be related to binding of CO by the leghaemoglobin of the intact tissue. These effects were avoided when well-washed bacteroids were used and it was clearly shown that CO was a competitive inhibitor. The K_i value obtained ($0{\cdot}43 \pm 0{\cdot}11$) is very near to the value obtained by Lockshin & Burris (1965) with *C. pasteurianum* extracts; the value obtained with cell-free Rhizobium bacteroid extracts was slightly lower. When the solubilities in water at 25° of CO and N_2 are compared at the pressures corresponding to the K_i and K_m values for bacteroids (at pO_2 42 mm. Hg), concentrations of 0·54 μM for CO and 16·8 μM for N_2 are obtained. That is, the nitrogenase of intact bacteroids has about 30 times the apparent affinity for CO that it has for N_2.

Studies with intact growing *Azotobacter vinelandii* (Wilson, Burris & Lind, 1942) and *Clostridium pasteurianum* (Westlake & Wilson, 1959) have indicated a K_m for nitrogen fixation of 15 to 23 mm. Hg (0·02 to 0·03 atmosphere). These values are comparable with the value of 20 (Fig. 4) obtained for non-growing Rhizobium bacteroids in the range of pO_2 in which K_m was not affected. The higher value for K_m of 50 to 60, obtained at the lower pO_2 values with intact nodules, is presumed to arise as a consequence of a N_2 concentration gradient extending from the external atmosphere to the bacteroids within the host tissue.

After the work reported here was completed Koch *et al.* (1967*b*) reported a K_m of 0·056 atmosphere (42·6 mm. Hg) for nitrogen fixation by cell-free extracts of Rhizobium bacteroids. This value is less than that obtained by us, but it is substantially higher than the K_m for nitrogen fixation by intact bacteroids. The reported K_m values for cell-free extracts of Clostridium and Azotobacter are 4 to 10 times higher than values for intact nitrogen-fixing bacteria and in general have been found to be 0·16 to 0·20 atmosphere, i.e. 122–152 mm. Hg (e.g. Mortenson, 1964). Dilworth,

Subramanian, Munson & Burris (1965) showed that this increase in K_m might be due in part to the effects of H_2 when substantial evolution of H_2 occurs or when fixation is measured in the presence of H_2 and acetylphosphate. H_2 is a competitive inhibitor of nitrogen fixation and therefore tends to increase the apparent K_m. In the work with cell-free extracts of Rhizobium bacteroids described here, H_2 was always present in the gas phase but only to the extent of 0·1 to 0·2 mm. Hg., concentrations too small to affect K_m.

The nitrogen-fixation reaction may be expressed as follows:

$$E+N_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{:}N_2 \xrightarrow[k_3]{+6H} E+2NH_3,$$

where E is the nitrogenase complex and k_1, k_2 and k_3 are the respective rate constants. Strictly, $K_m = k_2/k_1$, but in the method used to measure K_m, the relationship between N_2 concentration and rate of NH_3 production is measured and it is assumed that k_3 is not rate-limiting. The following considerations suggest that k_3 cannot be ignored, especially when using cell-free extracts. Carbon monoxide is not reduced by nitrogenase to any significant extent; we have been unsuccessful in a search for any of the likely products of such a reaction. Therefore, in measuring K_i (CO), we have been dealing only with the affinity of the binding-site. This is also the site at which N_2 is bound, because CO is a competitive inhibitor of nitrogen fixation; it is also of the same mass and is iso-electronic with N_2. We have shown that the binding of CO to nitrogenase is relatively unaffected by disruption of the Rhizobium bacteroids because the K_i (CO) was 0·4 for bacteroids and 0·2 for extracts. It is therefore unlikely that the binding-site of the enzyme was affected by disruption of the bacteroids. It is thus possible that the apparent three- to five-fold increase in $K_m(N_2)$ which occurs when the bacteroids are disrupted is due to a decrease in k_3. That is, the substitution of dithionite and ATP for the natural reducing system leads to less rapid production of NH_3.

The authors express their appreciation to Mr G. A. McIntyre for the statistical treatment of the kinetic data and to Mrs A. Marcina and Mrs L. Hush for their part in the technical aspects of the experimental programme.

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J. gen. Microbiol. (1968), **53**, 221–229
Printed in Great Britain

Unexpected Serotypes of Mycoplasmas Isolated from Pigs

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(*Accepted for publication* 4 *April* 1968)

SUMMARY

Several mycoplasmas isolated from pigs and designated B strains were previously found to be different from *Mycoplasma hyorhinis* and *M. granularum*, and to comprise four distinct serotypes. In the present study three of these serotypes were identified as *M. laidlawii*, *M. gallinarum* and *M. iners*, respectively. One serotype represented by mycoplasma B3 could not be identified. The isolation of avian serotypes from pigs is discussed in the light of host specificity of mycoplasmas.

INTRODUCTION

Mycoplasma hyorhinis and *M. granularum* were first isolated some years ago from pigs (Switzer, 1955, 1964) and many times subsequently. Dinter, Danielsson & Bakos (1965) described several mycoplasmas isolated from pigs, some of which (SEP strains) were identified as *M. hyorhinis*. The remainder, designated B strains, were neither *M. hyorhinis* nor *M. granularum*; on the basis of agar gel diffusion and growth inhibition studies they comprised four distinct serotypes. In the present study most of the B strains have been identified as previously named mycoplasmas, by using metabolic-inhibition and growth-inhibition techniques. The specificity of mycoplasmas in tissue culture systems and *in vivo* is discussed in the light of the findings.

METHODS

Mycoplasma strains. The strains from pigs have been described previously (Dinter *et al.* 1965). In the present study two reference strains, *M. hyorhinis* F and *M. granularum*, and six B strains, B1 to B6, were compared with mycoplasmas isolated from sources other than the pig. The following serotypes were used: *M. anatis*, *M. gallinarum*, *M. gallisepticum*, *M. iners*, *M. meleagridis*, *M. synoviae*, IOWA 695, WRI, 186, 658, *M. bovigenitalium*, *M. mycoides*, DONETTA, D12, N29, 5M331, *M. canis*, *M. maculosum*, *M. spumans*, *M. agalactiae*, *M. mycoides* var. *capri*, *M. hominis*, *M. fermentans*, *M. orale* 1, *M. orale* 2, *M. pneumoniae*, *M. salivarium*, *M. arthritidis*, *M. histotropicus*, *M. neurolyticum*, *M. pulmonis*, PG5 and *M. laidlawii*. In addition, *M. hyorhinis* strains SEP SK (Dinter *et al.* 1965) and GDL (Purcell *et al.* 1966*a*) were used for antibody studies. Stock mycoplasma cultures were stored at $-70°$ in liquid medium and were titrated for viable organisms before use in the serological tests.

Mycoplasma media. Liquid medium consisted of Difco PPLO broth containing 20 % (v/v) unheated Burroughs Wellcome no. 6 horse serum, 10 % (v/v) of 25 % (w/v) extract of dried yeast (Distillers Co. Ltd.), 1/2000 thallium acetate, 1000 u. penicillin/ml. and 0·002 % phenol red. In addition, this medium contained either 0·1 % glucose or 0·1 % arginine. Solid medium consisted of the liquid medium with the addition of 1 % Oxoid Ionagar no. 2.

Titration of viable organisms. The principle of the method has been described before (Taylor-Robinson & Purcell, 1966). For those strains which metabolize glucose, 0·1 % glucose was incorporated in the medium which was adjusted to pH 7·8. For those strains which metabolize arginine, 0·1 % arginine was incorporated in the medium adjusted to pH 7·0. Serial tenfold dilutions of a mycoplasma suspension were made in liquid medium contained in screw-capped vials. The vials were incubated at 37° until the colour of the medium ceased to change. The highest dilution of the mycoplasma suspension which caused a colour change was regarded as the end-point of the titration. In addition, in some instances, 0·1 ml. amounts of the dilutions were inoculated on agar medium and these cultures incubated at 37° in an atmosphere of 95 % (v/v) nitrogen + 5 % (v/v) carbon dioxide. Colonies were counted when new colonies ceased to develop.

Rabbit antisera. Antisera to *Mycoplasma hyorhinis* strain F, *M. granularum* and to mycoplasma strains B1 to B6 were those described and used previously (Dinter *et al.* 1965). Antisera to other mycoplasma species were prepared in rabbits also. All sera were stored at −20° and were heated at 56° for 30 min. before use.

Growth inhibition on agar medium. The technique was that of Clyde (1964). Stock mycoplasma cultures, previously titrated, were diluted in medium so that 0·1 ml. contained about 10^5 colony-forming units. This was spread on the agar medium which had been dried for 30 min. at 37° before inoculation. Filter-paper discs (7 mm. diameter) were soaked with 0·025 ml. of undiluted antiserum and were placed on the agar. Zones of inhibition were recorded after incubation at 37° in 95 % (v/v) nitrogen + 5 % (v/v) carbon dioxide. Zones were measured from the edge of the disc to the edge of colony development.

Metabolic inhibition. The techniques previously described by Taylor-Robinson, Purcell, Wong & Chanock (1966), and by Purcell, Taylor-Robinson, Wong & Chanock (1966*b*) were used. In some tests, unheated guinea-pig serum was used at a final concentration of 3 % (v/v). Results were recorded when a colour change of about half a pH unit had occurred in the controls containing mycoplasma organisms but no antiserum.

RESULTS

Metabolism of mycoplasma strains isolated from pigs

In order to use the metabolic-inhibition test it was necessary to determine which substrate, glucose or arginine, was metabolized by each strain. *Mycoplasma hyorhinis*, *M. granularum* and strains B3 and B4 metabolized glucose but not arginine. Strains B1, B2 and B5 metabolized glucose poorly, and metabolized arginine also, while strain B6 metabolized arginine and not glucose. In subsequent metabolic-inhibition tests arginine was used for strains B1, B2 and B5.

Relationship between mycoplasma strains isolated from pigs

The porcine strains originally isolated by Bakos & Dinter (1963) were examined by the metabolic-inhibition technique. A rabbit antiserum prepared against each mycoplasma strain was tested against all the strains in the presence of unheated guinea-pig serum. Strictly, however, this was essential only in tests with B3 antiserum, the homologous titre of which was decreased at least 64-fold in the absence of guinea-pig serum. The results are shown in Table 1. *Mycoplasma hyorhinis*, *M. granularum* and strains B3, B4 and B6 were different from each other. Strains B1, B2 and B5 were closely

Table 1. *Relationship between mycoplasma strains isolated from pigs as shown by the metabolic-inhibition technique*

	Reciprocal of metabolic-inhibition titre with rabbit antisera to						
Mycoplasma	*M. hyorhinis* F	*M. granularum*	B1	B2	E3	B4	B6
M. hyorhinis F	**1280**	< 20	< 20	< 20	< 20	< 20	< 20
M. granularum	< 20	**320**	< 20	< 20	< 20	< 20	< 20
B1	< 20	< 20	**1280**	640	< 20	< 20	< 20
B2	nt	nt	640	**640**	nt	nt	nt
B5	nt	nt	640	320	nt	nt	nt
B3	< 20	< 20	< 20	< 20	**5120** or >	< 20	< 20
B4	< 20	< 20	< 20	< 20	< 20	**40**	< 20
B6	< 20	< 20	40	20	< 20	< 20	**1280**

nt = not tested.

Table 2. *Relationship of mycoplasma strains B1, B2 and B5 to Mycoplasma gallinarum*

	Reciprocal of metabolic-inhibition titre and zone of inhibition with rabbit antisera to		
Mycoplasma	B1	B2	*M. gallinarum*
B1	1280 (3·2)*	640 (1·6)	640 (2·6)
B2	640 (2·5)	640 (2·5)	320 (2·4)
B5	640 (2·8)	320 (2·6)	320 (4·0)
M. gallinarum	2560 (2·1)	640 (1·8)	2560 (2·8)

* In parentheses: zone of inhibition in mm.

related to each other but distinct from the other mycoplasmas. These results confirmed those previously recorded by Dinter *et al.* (1965) who used gel diffusion and growth-inhibition tests. However, none of the mycoplasmas, including *M. hyorhinis* and *M. granularum*, had previously been adequately examined for their relationship to other known mycoplasmas. Therefore, in the first instance, rabbit antiserum to each mycoplasma strain was tested against a variety of known mycoplasmas in the metabolic-inhibition test. When the results suggested a relationship with a known mycoplasma serotype, the rabbit antisera were examined in growth-inhibition tests, and, in addition, antiserum to the known species in question was tested also against the particular porcine mycoplasma strain.

Relationship of mycoplasma strains B1, B2 and B5 to Mycoplasma gallinarum

The results presented in Table 2 clearly show that strains B1, B2 and B5, known to be related to one another, are closely related to *Mycoplasma gallinarum* and may be regarded as strains of this known mycoplasma species. Biochemically, *M. gallinarum* was found to resemble strains B1, B2 and B5 in metabolizing both arginine and glucose, the latter poorly.

Relationship of mycoplasma strain B4 to Mycoplasma laidlawii

Antisera to *Mycoplasma laidlawii* inhibited this mycoplasma to low titre in metabolic-inhibition tests but gave wide zones of inhibition on agar. This was a finding not usually observed with other mycoplasmas. Since the B4 antiserum behaved likewise in tests with its homologous organism it raised the possibility that strain B4 was a strain of *M. laidlawii*. The results in Table 3 show that this is so. Metabolic-inhibition titres were low and were not enhanced by addition of unheated guinea-pig serum to the tests, whereas zones of inhibition on agar were readily observed. In addition, further evidence for the relationship was obtained in the following manner. When strain B4 was tested against its homologous rabbit antiserum by the disc inhibition method, several colonies were observed within the general zone of inhibition. Each of these 'within zone' colonies was cloned, propagated in liquid medium and then these materials were retested in both metabolic-inhibition and disc growth-inhibition tests against B4 and *M. laidlawii* antisera. Organisms grown from each 'within zone' colony were inhibited to low titre (20–40) in metabolic-inhibition tests with both B4 and *M. laidlawii* antisera; wide zones of inhibition on agar were observed with both antisera. Subsequent to these serological investigations, a culture of strain B4 was diluted in serial ten-fold steps in mycoplasma broth without horse serum and inoculated on agar medium without serum. On incubation at 37°, colonies developed as well on the serum-free medium as on medium with 20% (v/v) horse serum, indicating the biological similarity of strain B4 to *M. laidlawii*.

Table 3. *Relationship of mycoplasma strain B4 to Mycoplasma laidlawii*

	Reciprocal of metabolic-inhibition titre and zone of inhibition with rabbit antisera to	
Mycoplasma	B 4	*M. laidlawii*
B4	40 (4·6)*	80 (3·0)
M. laidlawii	40 (1·3)	80 (1·5)

* In parentheses: zone of inhibition in mm.

Relationship of mycoplasma strain B6 to Mycoplasma iners

The results in Table 4 show that strain B6 is closely related to *Mycoplasma iners*. B6 antiserum did not inhibit *M. iners* to as high a titre as it did its homologous organism in a metabolic-inhibition test. However, *M. iners* antiserum inhibited B6 and its homologous organism to an equal titre. Biochemically, *M. iners* was found to resemble strain B6 in metabolizing arginine and not glucose.

Serological examination of Mycoplasma hyorhinis, M. granularum and mycoplasma strain B3

Antisera to these three strains were examined in metabolic-inhibition tests against the mycoplasma serotypes listed in Methods. No cross-reactions were observed. In addition, antisera to some of the mycoplasmas listed in Methods were tested against strain B3 in metabolic-inhibition and growth-inhibition tests but no evidence of a relationship with a known mycoplasma species was obtained.

Metabolic-inhibition tests with randomly collected avian and porcine sera

No sera were available from the pigs from which the mycoplasma strains were isolated. However, the strains were tested against about 40 randomly collected pig sera and 30 randomly collected chicken sera. The occurrence of antibody is shown in Table 5. Antibody to *Mycoplasma hyorhinis* in the porcine sera was detected with strain GDL, but not with strain SEP SK. Variations in the ability of different strains of a serotype to detect antibody have been observed also with strains of *M. gallisepticum* (D. Taylor-Robinson & D. M. Berry, unpublished). Antibody to *M. granularum* in

Table 4. *Relationship of mycoplasma strain B6 to Mycoplasma iners*

	Reciprocal of metabolic-inhibition titre and zone of inhibition with rabbit antisera to	
Mycoplasma	B6	*M. iners*
B6	1280 (4·2)*	1280 (2·7)
M. iners	160 (2·8)	1280 (2·6)

* In parentheses: zone of inhibition in mm.

Table 5. *Antibody to various mycoplasmas in random porcine and avian sera measured by the metabolic-inhibition technique*

	Percentage of indicated sera containing antibody*	
Mycoplasma	Porcine	Avian
M. hyorhinis SEP SK	2	nt
M. hyorhinis GDL	76	nt
M. granularum	0	nt
B1	0	95
M. gallinarum	2	nt
B3	45	34
B6	14	27
M. iners	7	21

nt = not tested. * At a dilution of 1/2 or greater.

the porcine sera was not detected; this may have been due to the use of an antibody-insensitive strain. Antibody to strain B1 was not detected in porcine sera and antibody to the prototype *M. gallinarum* was rarely detected. However, antibody to strain B1 was detected in avian sera, suggesting the occurrence of this strain in birds rather than in pigs. Antibody to strain B3 was found in both porcine and avian sera which did not help, therefore, in assessing the natural host. Strain B6 and the proto-

type *M. iners* behaved similarly. The occurrence of antibody to *M. iners* in porcine sera may be indicative of occasional infection of pigs with this mycoplasma or, alternatively, it may be a heterotypic response due to a possible sharing of antigens between *M. iners* and other mycoplasmas infecting pigs.

DISCUSSION

The major point to be discussed concerns the host specificity of mycoplasmas. Specificity may be viewed at several levels. At the cellular level it would seem that there is little difficulty in infecting tissue culture cells derived from several avian or mammalian species with a mycoplasma isolated from one of them. Some examples of this are presented in Table 6. It is possible also experimentally to infect animals or birds of one species with a mycoplasma derived from another. Thus, infection of sheep and goats has been achieved by the subcutaneous inoculation of *Mycoplasma mycoides*

Table 6. *Examples of mycoplasma infection of tissue culture cells*

Mycoplasmas		Tissue culture cells		
Designation	Origin	Origin	CPE	Reference
M. pneumoniae	Man	Monkey kidney*	–	Chanock *et al.* (1960)
M. orale 1	Man	Chick embryo*	+	Somerson & Cook (1965)
M. hominis	Man	Hamster kidney (BHK 21)	+	Macpherson & Russell (1966)
M. pulmonis	Rodent	Man (HeLa)	+	Nelson (1960)
M. hyorhinis	Pig	Man (HEp-2)	+	Butler & Leach (1964)
M. bovigenitalium	Cattle	Pig kidney*	+	Afshar (1967)

* Primary or secondary cultures.

Table 7. *Some evidence against host specificity of mycoplasmas*

(i) Isolation studies
(*a*) *M. arthritidis* from human collagen disease (Jansson & Wager, 1967)
(*b*) *M. hyorhinis* from human bladder papilloma (Hayflick & Stanbridge, 1967)
(*c*) *M. agalactiae* affects sheep and goats (Hudson *et al.* 1967)
(*d*) *M. laidlawii* from various animals (see text)

(ii) Antibody studies
E.g. *M. hyorhinis* metabolic-inhibition antibody in human sera (D. Taylor-Robinson & R. H. Purcell, unpublished).

from cattle (Turner, Campbell & Dick, 1935) and infection of cotton rats and hamsters by the intranasal inoculation of *M. pneumoniae* from man (Eaton, Meiklejohn & van Herick, 1944; Dajani, Clyde & Denny, 1965). However, experimental inoculation may often be unsuccessful, probably because *in vivo* immune mechanisms play a part in preventing infection. For example, Switzer (1967) failed to infect mice, chickens, turkeys, calves, guinea pigs, hamsters, rabbits or sheep with *M. hyorhinis* isolated from pigs. Furthermore, under natural, as opposed to experimental, conditions there would seem to be an even greater degree of host specificity. In addition to immunological factors this may be due to the smaller number of mycoplasmas likely to be encountered under natural conditions. While in many instances there would appear to be a real host specificity, in others it is possible that this is more apparent than real. Apparent host specificity might arise from lack of opportunity to infect under natural conditions or because no attempts have been made to determine whether or not a

particular mycoplasma is capable of infecting another avian or mammalian species. From the foregoing it is clear that mycoplasma host specificity exists but that it is not likely to be absolute. In Table 7 some evidence is presented that under natural conditions specificity is not absolute. Whether the results of the present investigation represent further evidence against host specificity is open to question.

One of the strains, B4, was identified as *Mycoplasma laidlawii*. This is of interest serologically since in previous gel diffusion tests (Dinter *et al.* 1965) reciprocal cross-reactions of low order were noted between B4 and *M. granularum*, and Tully (1966) has reported a partial relationship of *M. granularum* strains with a *M. laidlawii* A strain (PG-8) in immunofluorescence tests. The finding of *M. laidlawii* in pigs would certainly not be inconsistent with the finding of this mycoplasma in other species by other workers. Thus, since its isolation from sewage (Laidlaw & Elford, 1936), it has been found in chickens (Adler, Shifrine & Ortmayer, 1961), man (Razin, Michmann & Shimshoni, 1964), cattle (Leach, 1967) and horses (D. Taylor-Robinson & S. P. Beveridge, unpublished); this, presumably, is reason enough for its presence in sewage.

The strains B1, B2 and B5 were identified as *Mycoplasma gallinarum* and B6 as *M. iners*, both these serotypes having been reported hitherto as occurring only in birds (Edward & Kanarek, 1960; Roberts, 1964; Dierks, Newman & Pomeroy, 1967). The isolation of avian serotypes from pigs raises the question of whether these mycoplasmas were, in fact, laboratory contaminants. Evidence against this idea is that all the isolations were made directly on mycoplasma medium and not through embryonated eggs and that strains B1, B2 and B5 were isolated at different times over a period of two years. It is of interest also that all B strains were isolated from pigs having pneumonia, with or without rhinitis. It is conceivable that damaged respiratory tract tissue might be susceptible to colonization by saprophytic or 'unrelated' mycoplasmas. On the other hand, it should be pointed out that specimens from birds were examined within the same laboratory, although stock cultures of *M. gallinarum* and *M. iners* were never handled. We thought that serological examination of random avian and porcine sera might help to determine the source of the mycoplasmas. However, the data are difficult to interpret. The occurrence of antibody to strain B1 in the sera of chickens but not pigs certainly suggests an avian host, although it might be possible for infection of pigs to occur occasionally without antibody development. The occurrence of antibody to strain B6 in pig sera might be due to specific infection or to infection with an antigenically related strain. Therefore, although we feel laboratory contamination is an unlikely explanation for our findings it is obviously not possible to resolve this point unequivocally. Nevertheless, our observations should at least make others aware that mycoplasmas generally associated with a particular species might infect another unrelated species and so stimulate further investigation. Our findings re-emphasize the need to test strains against all known mycoplasmas, and not merely against those commonly isolated from the same animal or bird, before regarding them as new species.

We thank Mrs Susan Beveridge for excellent technical assistance and Dr K. Bakos, National Veterinary Institute, Stockholm, for the collection of pig sera.

The M.R.C. Clinical Research Centre, Harvard Hospital, Salisbury, England, receives support, gratefully acknowleged, from the World Health Organization for reference work on mycoplasmas.

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J. gen. Microbiol. (1968), **53**, 231–236
With 2 plates
Printed in Great Britain

The Establishment of Hop Tissue Cultures and their Infection by Downy Mildew *Pseudoperonospora humuli* (Miy. & Tak.) Wilson under Aseptic Conditions

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(*Accepted for publication* 5 *April* 1968)

SUMMARY

Successful infection of hop tissue culture with *Pseudoperonospora humuli* was achieved. Infection was obtained from hyphae emerging from surface-sterilized systemically infected stems and petioles. Externally, infection was characterized by sterile aerial hyphae and sporangiophores, and internally by intercellular mycelium and intracellular haustoria. Although a considerable growth of mycelium into the agar medium was observed, it appeared to be dependent on the presence of host tissue. Attempts to establish axenic cultures of *P. humuli* were unsuccessful.

INTRODUCTION

Pseudoperonospora humuli is a member of the family Peronosporaceae and, as far as we are aware, has not previously been grown under aseptic conditions. All members of this family are obligate parasites and a few species have been grown on tissue cultures of their respective hosts. The growth of an obligate parasite in tissue culture was first achieved by Morel (1944, 1948) with vine downy mildew *Plasmopara viticola*. Since then at least two other downy mildews, *Peronospora parasitica* (Nakamura, 1965) and *Peronospora tabacina* (Izard, Lacharpagne & Schiltz, 1964) have been grown in tissue culture. A few rusts and powdery mildews, the other two main groups of obligate parasites, have also been grown in tissue culture (Brian, 1967). The present paper describes the establishment of hop tissue culture and its infection with *P. humuli* under aseptic conditions.

METHODS AND OBSERVATIONS

Tissue culture medium. Tissue cultures of *Humulus lupulus* (L.) var. Eastwell Golding were initiated and maintained on a solid medium based on Hildebrandt (1962) D-medium. The composition of the medium was: mineral salts: Na_2SO_4, 800 mg.; $Ca(NO_3)_2.4H_2O$, 400 mg.; $MgSO_4.7H_2O$, 180 mg.; KNO_3, 80 mg.; KCl, 65 mg.; $Na_2HPO_4.12H_2O$, 33 mg. trace elements: $ZnSO_4.7H_2O$, 6·0 mg.; $MnSO_4.4H_2O$, 4·5 mg.; KI, 3·0 mg.; H_3BO_3, 0·3 mg.; other additions: EDTA (Na/Fe complex; Na_2EDTA, 0·0372 g. + $FeSO_4.7H_2O$, 0·0278 g.) 65 mg.; glycine, 3·0 mg.; thiamine, 0·1 mg.; calcium pantothenate, 2·5 mg.; nicotinic acid, 0·5 mg.; pyridoxine, 0·8 mg.; α-naphthylacetic acid, 0·1 mg.; 2,4-dichlorophenoxyacetic acid, 6·0 mg.; sucrose, 20 g.; Difco yeast extract, 1 g.; coconut milk, 260 ml. The medium was made up to

1 l. with distilled water and solidified with 0·6% (w/v) Davis agar. Sterilization was by autoclaving for 15 min. at 121°.

Initiation of hop callus. Callus tissue was initiated from lengths of stem internode of Eastwell Golding. Lengths (3 cm.) of stem were dipped in 95% (v/v) ethanol in water for 30 sec., surface-sterilized in a saturated solution of calcium hypochlorite for 7 min. and finally washed 3 times with sterile distilled water. The ends of the stem were then cut off aseptically and a 1 to 1·5 cm. length placed on 20 ml. of the agar medium in a plastic Petri dish and incubated in the dark at 25°. Explants which showed no signs of bacterial contamination after one week (30 to 40% of total) were transferred to Pyrex boiling tubes containing 10 ml. agar medium (see Pl. 1, fig. 1 to 3). Tightly fitting cotton-wool plugs and aluminium foil caps decreased evaporation during the lengthy incubation period. Latent bacterial contamination occasionally appeared from some explants even after 4 weeks of incubation. Prolonging the immersion in calcium hypochlorite to 10 min. decreased the number of explants which were contaminated, but in most cases prevented callus formation.

Characteristics of callus growth

Callus formation was normally visible after 1 to 3 weeks and was followed by vigorous growth of white callus (Pl. 1, fig. 1). Several of the explants also produced adventitious roots and others died without forming a callus. In most cases the initial growth of white callus was normally followed by dehydration and eventual death over a period of 6 to 8 weeks. Callus death was not prevented by transferring the explant to fresh medium.

A few explants, however, produced sectors of brown callus amongst the original white callus (Pl. 1, fig. 2). The thicker woody explants from the base of the stem produced these brown sectors more readily. Growth of this brown tissue was very slow and no attempt was made to separate it from the original explant for 12 to 15 weeks. After separation, the sectors became darker brown and grew very slowly for 15 to 20 weeks. Lightish brown sectors of callus were then produced, with an improved growth rate. These sectors were selected out and placed on fresh medium and the improved growth rate has been maintained. Fifty boiling tubes of callus on solid medium (Pl. 1, fig. 3) were kept as a stock and subcultured every 8 weeks. No attempts to infect callus with *Pseudoperonospora humuli* were made until a stock of 50 callus cultures had been built up. Slight changes in the colour and in the rate of growth of the callus have occurred frequently.

Aseptic isolation of Pseudoperonospora humuli

The organism was isolated from systemically infected shoots of Eastwell Golding. Material was obtained from naturally-infected field plants and from artificially infected shoots. The latter can be produced by spraying young shoots (2 to 7 cm. high) emerging from a hop rootstock, with a sporangial or zoospore suspension of *P. humuli*, incubating in a polythene bag at 14° for 12 hr, then allowing the shoots to dry and grow in the normal manner.

The stems and petioles of systemically infected shoots were cut into 2 cm. lengths, dipped in 95% (v/v) ethanol in water for 30 sec., surface-sterilized in calcium hypochlorite for 10 min. and then washed 6 times with sterile distilled water. The sections of stem and petiole were then placed on 15 ml. of solid medium in plastic Petri dishes

and incubated at 25°. The incidence of bacterial contamination of surface-sterilized sections was usually less than 50%.

After 3 days, many of the petiole sections and a smaller proportion of stem sections were covered with sporangiophores bearing sporangia. Sterile hyphae were also produced, some aerial (Pl. 1, fig. 4) and some growing into the agar adjacent to the infected material. Removal of infected stems and petioles, after 7 days, revealed a considerable growth of mycelium of *P. humuli* in the agar (Pl. 1, fig. 5). However, no further mycelial growth occurred after removal of the host tissue.

Infection of tissue cultures

Infection of hop callus with *Pseudoperonospora humuli* was achieved by placing lengths of stem and petiole (sterilized 7 days previously) bearing sporangia and aerial hyphae on 10 ml. of the solid medium in boiling tubes. These were then surrounded or covered with hop callus tissue and incubated in a growth room at 14 to 15°, with a 16 hr photoperiod at 300 lux. After 1 week hyphae from infected stems and petioles were seen growing into the adjacent callus. The growth was very slow and, after 3 weeks, infection was only observed on callus adjacent to the infected material. The time taken for infection to spread over all or a high proportion of the uninfected callus varied from 6 to 20 weeks. External characteristics of infection were sterile aerial and surface hyphae, and sporangiophores bearing sporangia (Pl. 2, fig. 6, 7). The hyphae and sporangiophores varied markedly in density even over small areas of callus. The aerial and surface hyphae often had an irregular shape and branching habit, a feature characteristic of *P. humuli* in natural conditions (Coley-Smith, 1964). A considerable growth of mycelium was again observed in the agar medium, adjacent to the infected material. Hyphae emerged both from infected callus, and from infected stems and petioles, but rarely advanced more than 1 cm. into the agar. There was a marked decrease in density of mycelium with increase in distance from infected material. No fruiting structures were seen on the hyphae in the agar.

After 10 to 20 weeks callus on which *Pseudoperonospora humuli* had become established was removed from the boiling tube, subdivided in a sterile Petri dish and small infected sectors placed on 10 ml. of fresh agar. These were then covered with uninfected callus, on which infection usually became established within a week. Again, the rate of spread of infection varied markedly in different cultures. In this way *P. humuli* has so far been perpetuated in hop tissue for 7 months.

The presence of infected material in close proximity to uninfected callus did not appear to retard the growth rate of the latter. When sectors became heavily infected, however, growth appeared to cease. Attempts have been made to infect callus with sporangial and zoospore suspensions, obtained by washing sporangia from surface-sterilized systemically infected stems and petioles with sterile distilled water. So far infection has not been obtained.

Mycelium of Pseudoperonospora humuli in the agar

Attempts were made to grow *P. humuli* in axenic culture. Infected tissue cultures which supported a dense growth of mycelium into the agar were selected. The infected callus was carefully removed from the boiling tube, leaving some mycelium in the agar; but in the absence of host tissue no further mycelial growth took place. In another experiment small pieces of agar (0·5 to 1·0 cm. across) which contained myce-

lium were placed on fresh agar, and half of these were covered with uninfected callus. No further mycelial growth or infection of callus occurred.

Cytological investigation

Small sections of callus heavily infected with *Pseudoperonospora humuli* were selected for cytological examination. Material for permanent sections was fixed in formalin + acetic acid + ethanol (Johansen, 1940), dehydrated in ethanol amd embedded in fibrowax. Sections (10 μ thick) were stained with 1 % aqueous resorcin blue and dehydrated rapidly in absolute ethanol. Resorcin blue is a specific stain for callose which is deposited around haustoria and hyphae by host cells (Fraymouth, 1956).

Microscopical observation of stained sections showed that systemic infection was characterized by intercellular mycelium and intracellular haustoria. Haustoria and cell walls adjacent to intercellular mycelium were stained pale blue. The haustoria varied considerably in form but were usually branched or knob-like (Pl. 2, fig. 8). These observations were confirmed by squashing small pieces of fresh material and staining with resorcin blue. The haustoria were stained a much deeper blue in fresh material. Also small haustoria-like projections, which stained deeply, were seen at irregular intervals on some of the hyphae. No penetration of cells by such haustoria was observed. A similar phenomenon was observed with *Phytophthora infestans* on tissue aggregates of *Solanum tuberosum* (Ingram, 1967). No deeply staining structures were seen when uninfected callus was squashed and stained in the same way. The characteristics of haustoria and mycelium of *P. humuli* in hop tissue culture compare closely with those observed in naturally infected hop rootstocks (Coley-Smith, 1964).

DISCUSSION

The general pattern of growth of *Pseudoperonospora humuli* on hop tissue culture was similar to that described for *Plasmopara viticola* (Morel, 1944, 1948) and *Peronospora tabacina* (Izard, *et al.* 1964). However, with these two organisms no growth of hyphae from the infected callus into the agar medium was reported. Stow & Ihara (1962) claimed to have observed the emergence of mycelia of *P. humuli* from infected leaf tissue on to a modified White's medium, with consequent oospore production. In the present work no oospore production was observed. Cutter (1951, 1959, 1960) established axenic cultures of *Gymnosporangium juniper-virginianae* and *Uromyces ari-triphylli* on nutrient agar. From a small proportion of infected tissue cultures, hyphae grew into the agar and were then removed and grown in isolation on fresh agar. These axenic cultures retained their ability to parasitize host tissue. With *P. humuli* the case was very different. Hyphae always grew into the agar from adjacent infected tissue but when the host was removed their growth ceased and their ability to infect host material was lost.

It appeared that the growth into the agar of mycelium of *Pseudoperonospora humuli* was host-dependent; the host metabolites which must be responsible for this phenomenon are at present not known. The fact that there was no stimulatory effect of the solid or liquid medium on germ-tube growth provided further evidence for this dependence on host materials. The unknown growth principles are presumably translocated in the hyphae to the growing tip. If the hyphae are unable to translocate these over large distances, this might explain why growth in the agar is limited to 1 cm. and why

there is a marked decrease in density of mycelium with increasing distance from the infected host.

This work was initially done to investigate the possibility of producing sporangia aseptically in sufficient quantities for experiments involving the uptake of certain fungicides. However, the slow growth rate of the callus and the slow spread of infection have excluded this possibility. Surface-sterilized systemically infected stems and petioles offer more promising possibilities in this field.

Our thanks are due to Mr P. E. Smith for assistance with the photography and to the Science Research Council for the support of one of us (M.J.G.).

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EXPLANATION OF PLATES

PLATE I

Fig. 1 to 3. Stages in the initiation of tissue cultures of *Humulus lupulus* from lengths of stem internode. Grown on 10 ml. of solid medium in 6 × 1 in. Pyrex boiling tubes.

Fig. 1. White callus formation on stem explant. 30 days.

Fig. 2. Brown sectors amongst the white callus, on woody explant from the base of the plant. 10 weeks.

Fig. 3. Actively growing stock tissue culture completely separated from original explant.

Fig. 4, 5. Growth of *Pseudoperonospora humuli* from lengths of surface sterilized systemically infected stem and petiole, placed on solid medium and incubated at 25°.

Fig. 4. Sporangiophores and sterile aerial hyphae. 5 days.

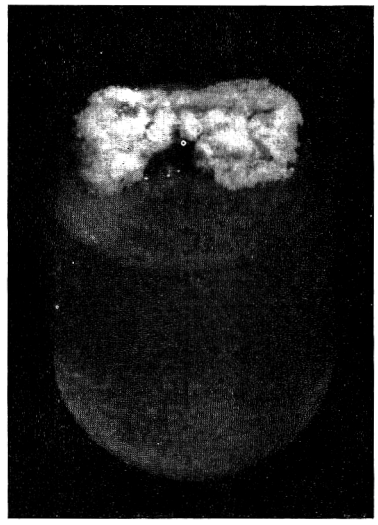
Fig. 5. Mycelium which had grown into the agar from a systemically infected stem. 7 days.

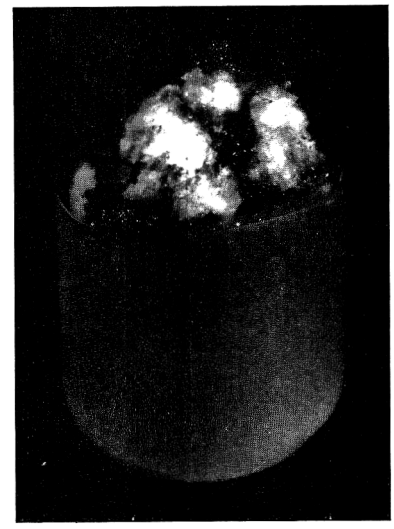
PLATE 2

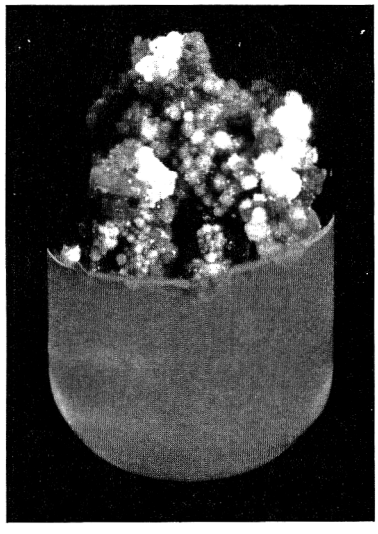
Fig. 6 to 8. Growth of *Pseudoperonospora humuli* on tissue cultures of *Humulus lupulus*.

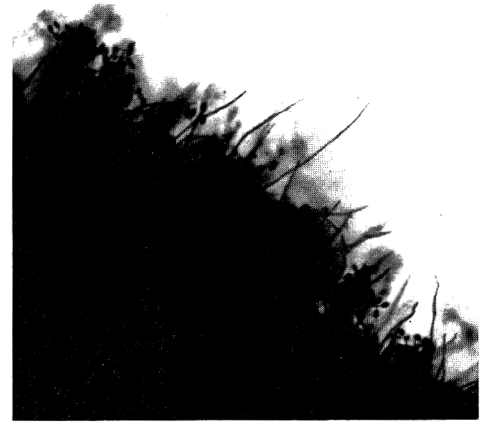
Fig. 6, 7. Sporangiophores bearing sporangia; and sterile aerial hyphae.

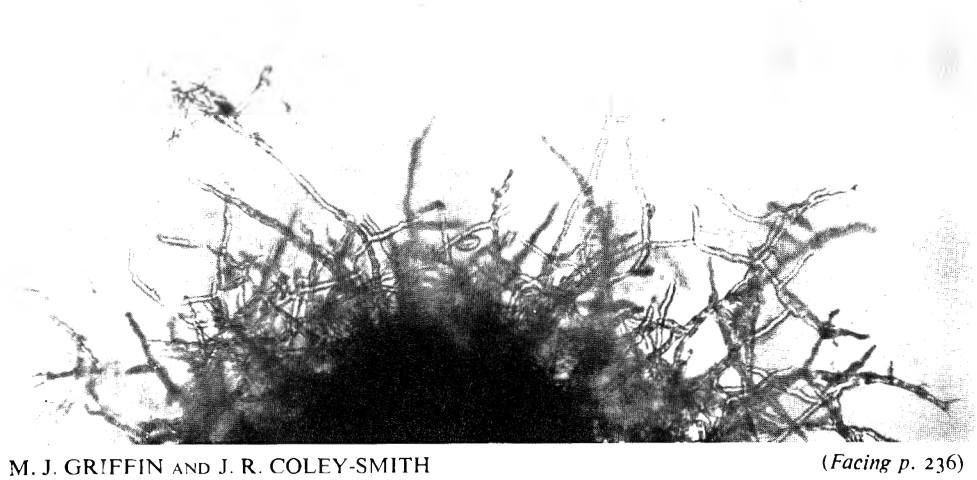
Fig. 8. Intracellular haustoria in section (10 μ thick) of hop callus. Stained with resorcin blue.











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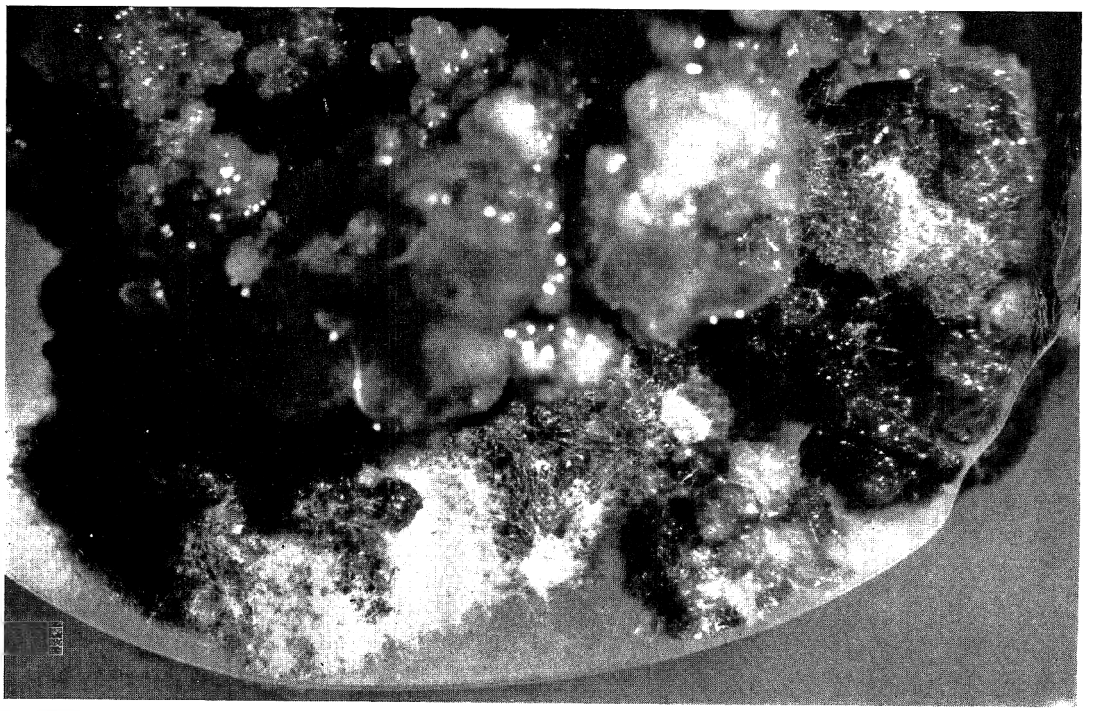

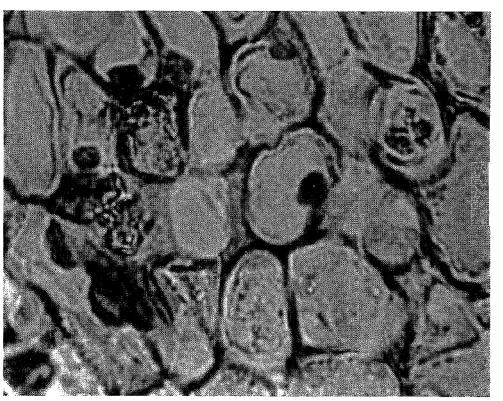

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J. gen. Microbiol. (1968), **53**, 237–251
Printed in Great Britain

Unbalanced Respiratory Growth of Euglena

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(*Accepted for publication* 6 *April* 1968)

SUMMARY

The respiratory physiology of *Euglena gracilis* grown heterotrophically on a defined medium was examined as a function of culture age when growth was supported by glucose, acetate or ethanol as sole carbon source. The endogenous rate of oxygen consumption in general paralleled that of total protein content, usually showing a steady decline through log. and stationary phases of growth. The rate of oxygen consumption stimulated by ethanol or acetate remained fairly constant during log. phase but. decreased sharply in early stationary phase. The specific activities of representative respiratory enzymes remained essentially proportional to one another and to total oxygen consumption, but the activities of malate synthase and the malic enzyme increased greatly in stationary phase glucose-grown organisms. In general, respiratory growth was out of balance with the rate of cell division but in essential balance with the rate of biosynthesis.

INTRODUCTION

In exponential increase of typical cell populations it is generally expected that the rate of DNA synthesis will equal that of cell division. Less stringent requirements exist for other biochemical fractions, however, and frequently the RNA or protein content of the 'average' cell decreases or sometimes increases over a generation, a situation referred to as 'unbalanced growth' (Barner & Cohen, 1956). It has been reported that some species, e.g. *Euglena gracilis*, may exhibit unbalanced growth on some carbon sources (e.g. acetate, succinate) but essentially balanced growth on others (e.g. ethanol (Buetow & Levedahl, 1962; Wilson & Levedahl, 1964). In unbalanced growth the average unicellular alga will be usually larger in stationary phase than during log. phase growth; presumably these patterns come full cycle during the lag period. It is of some interest to know whether higher orders of metabolic activity are subject to the same degree of control—or lack of it—in balanced or unbalanced growth. Tissue and organ differentiation, for example, must represent an extreme case of unbalanced growth at nearly all levels of cellular activity. The problem has received little attention at the cell level. The subject of the present report is a study of respiratory growth of *E. gracilis* as a function of culture age. Since oxygen consumption is the terminal step in a complex, but relatively well-known and easily monitored sequence of events, it was felt that such information might be conveniently compared with changes in the biochemical profile of Euglena, and at the same time referred to a somewhat higher degree of organization than 'total protein'. The oxygen requirements of *E. gracilis* are greatly dependent on the exogenous carbon source; some (acetate, ethanol) stimulate oxygen consumption considerably above the endogenous rate, while others (e.g. glucose) cause no stimulation (Cook & Heinrich, 1965; Danforth, 1953). We

have examined the problem of balanced versus unbalanced respiratory growth of *E. gracilis* grown on each of these three substrates. Particular attention was paid to enzymes of the glyoxylate cycle, since these are characteristically induced by the 2-C compounds and are also correlated with high oxygen consumption by Euglena (Cook & Carver, 1966). In general, it was observed that respiratory growth was in essential balance with biosynthesis of protein, but not with cell division; some exceptions were found.

METHODS

Euglena gracilis strain Z was used throughout. Axenic cultures were grown in the dark on a salt medium (Cramer & Myers, 1952) with carbon sources glucose, acetate or ethanol present initially at 0·03 M. Ethanol was aseptically added to the media after autoclaving; acetate and glucose were autoclaved as a part of the media. This medium is initially buffered with ammonium and potassium phosphates at pH 6·8; some caramelization of glucose occurred during autoclaving at this pH value, but control studies showed that this had no effect on growth rate. Ammonium phosphate also served as the sole nitrogen source. When required, the pH value was adjusted with H_2SO_4. The vitamins B_1 and B_{12}, necessary nutrients for *E. gracilis* Z, were autoclaved with the media.

Culture vessels were Pyrex cylinders of 1·5 or 3·0 l. capacity, fitted with a Pyrex jacket through which water at 30° was circulated. Cultures were stirred with a teflon-covered magnetic bar, and air sterilized by filtration through cotton-wool was bubbled from the bottom of the vessels. Samples were removed from the vessels by simple siphon devices. The culture volumes were sufficiently large to permit repeated analyses of a single culture over an entire growth curve. In one part of the work, a series of Erlenmeyer flasks containing acetate media were inoculated and grown in the dark at 30° without agitation or forced aeration; the organisms were then harvested at different times for analysis. While this approach offered less control than the larger cultures harvested sequentially, as shown below, both approaches yielded the same sort of data.

Counts of organisms were made with the Coulter cell counter. The pH values of the media and of samples of organism suspensions were measured with a Beckman Zeromatic pH meter.

Total cell protein was measured by a microbiuret method (Goa, 1953), with known numbers of euglenas (1 to 3×10^6) which had been previously washed and twice extracted with boiling ethanol. Ribonucleic acid (RNA) was estimated by the Ogur & Rosen method (1950), by using a known number of euglenas (1 to 3×10^6) extracted with lipid solvents and twice briefly with cold 5% (w/v) perchloric acid (PCA), followed by overnight digestion in 5% PCA in the refrigerator. Two washes with 5% PCA were added to this extract and the combined extracts, made up to volume, were read at 260 and 315 mμ with the Beckman DB spectrophotometer. The difference was used to calculate total RNA, using the equation $(E_{260} - E_{315}) \times 33{\cdot}2 = \mu$g. RNA/ml. In one experiment, DNA and RNA were estimated with the Schmidt & Thannhauser (1945) technique.

Dry mass was measured directly by drying a known number of washed euglenas in a tared aluminium dish at 80°, and weighing on a semimicro balance. Oxygen consumption was determined with a Clark electrode and graphically recorded. Organisms were

washed three times with water and suspended in the salt medium without substrate. This suspension was then placed in a water-jacketed reaction vessel and flushed with air. After equilibration to 30°, the Clark electrode was fitted into the suspension so as to make the vessel air-tight. The suspension was stirred with a magnetic bar. Oxygen consumption was recorded for a length of time adequate to ensure linearity (about 10 min.), to yield the endogenous rate of respiration. The substrate which had supported growth was then added, and respiration again recorded. At the end of such a run, a sample was removed for counting the organisms.

Respiration measurements were made in the dark, since it has been established that greening of Euglena can cause a significant decrease of oxygen consumption, at least when acetate is the carbon source (Cook, 1965). Organisms were briefly exposed to dim room light during washing; this degree of exposure was without effect on respiration. Also, suspension of the washed euglenas in fresh salt medium represented a change in pH value; the fresh medium was at pH 6·8, and the pH value of the growth medium varied with culture age (see below). However, it has been shown that pH changes in this range have no immediate effect on the oxygen consumption of Euglena grown with these substrates (Cook & Heinrich, 1965). The Clark electrode was calibrated with air-flushed and nitrogen-flushed water at 30° immediately before a run, to permit calculation of absolute amounts of oxygen consumed.

Organisms for enzyme analyses were washed three times with water and suspended in 4 ml. 0·02 M-tris buffer (pH 8·0), brought to 0°, and homogenized with the French press at 2000 lb./sq.in. at 0 to 4°. In some cases the crude homogenate was used directly, in other cases the supernatant fluid after centrifugation at 10,000 *g* at 0 to 2° was used for assay. In a given experiment, however, the method taken was consistent; for example, malate synthase activity over the growth curve in glucose-grown Euglena was always estimated in the supernatant fluid after centrifugation, while malate synthase activity over the growth curve of acetate-grown Euglena was always measured in crude homogenate. While this gave some absolute differences between experiments, the nature of the comparisons to be made did not make this important.

All enzyme assays were made spectrophotometrically at 25°. Enzymes studied included malate synthase (E.C. 4.1.3.2), malic enzyme (E.C. 1.1.1.40), isocitric dehydrogenase (E.C. 1.1.1.41), aconitase (E.C. 4.2.1.3), and malic dehydrogenase (E.C. 1.1.1.37). Details of the assays used were described previously (Heinrich & Cook, 1967). For malate synthase, one enzyme unit is that amount which converts 1 mμmole acetyl-CoA/min.; for the others, an enzyme unit is that amount causing an extinction change of 0·01/min. Specific activities are expressed as enzyme units/mg. protein. Protein content was estimated by the phenol method of Lowry, Rosebrough, Farr & Randall (1951), with either the crude homogenate or the supernatant fluid after centrifugation.

The population density of a culture was followed from inoculation. When the population had reached a density compatible with the analyses to be made (20,000–50,000 organisms/ml.), the culture was sampled for the first series of measurements. The count of organisms and the pH value of the sample were determined, and then the organisms centrifuged down, washed, and suspended in the salt medium for respiration measurement. The organisms were then washed again and divided into 3 or 4 portions, for enzyme analyses and protein, nucleic acids and dry mass determinations (the latter three measurements were done in triplicate at each sampling time). Usually

samples were taken at intervals of about one generation which was about 12 hr under the conditions used. About 2 hr were required in handling the organisms at sampling when all analyses were made.

RESULTS

Changes in pH value of cultures

Figure 1 shows typical growth and pH curves for *Euglena gracilis* Z under the conditions used here, with acetate, ethanol or glucose as sole carbon and energy source. Growth on glucose resulted in a lower pH value and growth on acetate a higher pH value; the reaction with growth on ethanol showed a very slight decrease of pH during log. phase, a little more pronounced decrease in early stationary phase, and with a pH increase as stationary phase progressed. It is difficult to assess the cause of these pH changes. In a phosphate-buffered medium, with ammonium phosphate as sole nitrogen source, removal of ammonia during growth would cause a net decrease in pH value if the carbon source were not charged. Growth with glucose resulted in a greater decrease in pH value than did growth with ethanol, suggesting that acidic by-products were formed with glucose. Acid production has been used to show whether micro-organisms metabolize carbohydrates fermentatively or oxidatively (Hugh & Leifson, 1953). However, no radioactive compounds other than glucose were detected by chromatography of the medium after long-term culture of Euglena with radioactive glucose (Belsky, 1955). The excretion of volatile acidic compounds was not excluded. Incorporation of acetate as the undissociated acid could account for the alkaline reaction occurring during growth on this substrate, assuming the incorporation occurred at a rate greater than the removal of ammonia. Pringsheim & Wiessner (1960) used this pH increase as a measure of acetate utilization by Euglena.

The final population density attained by *Euglena gracilis* Z was much lower when acetate was sole carbon source than with glucose (Cook & Heinrich, 1965) or ethanol, although ethanol-grown and acetate-grown Euglena have essentially the same physiological profile (Buetow & Padilla, 1963). Dr W. Danforth (personal communication) noted that acetate yielded higher population densities when growing cultures were periodically acidified. Our measurements confirmed this; the final population densities increased as the initial pH was decreased: (pH: population/ml.) 6·8, 250,000; 6·0, 300,000; 5·0, 400,000. pH 5·0 is the lower limit for growth of Euglena on acetate (Wilson, Buetow, Jahn & Levedahl, 1959; Cook & Heinrich, 1965).

Dean & Hinshelwood (1959) discussed the changes of pH value which occur during growth in batch culture. They concluded that enzyme content often appears to be affected not directly by the pH value of the medium, but by other factors which come into being concomitantly with the pH change. In the case of pH-limited growth on acetate, the simplest explanation would appear to be one of permeability (Wilson *et al.* 1959). In any event, the pH value of the medium need not reflect the pH value of the interior of the organism. As seen below, the specific activity (and total amount) of most of the enzymes studied here did not appear to be affected by the pH value of the medium. In the present context, the pH changes during the growth cycle apparently became limiting for growth with acetate but not with ethanol or glucose.

Endogenous respiration

A brief definition of endogenous respiration may be made here. The oxygen consumption of washed cells varies with the time after washing; most published studies with Euglena (including this paper) report this respiration measured immediately after washing. Wilson & Danforth (1958) argued that this endogenous oxygen consumption continues after addition of the exogenous carbon source (acetate or ethanol in their studies), and that the true oxygen consumption stimulated by the carbon source can be derived only by subtracting the endogenous value. This practice is followed here.

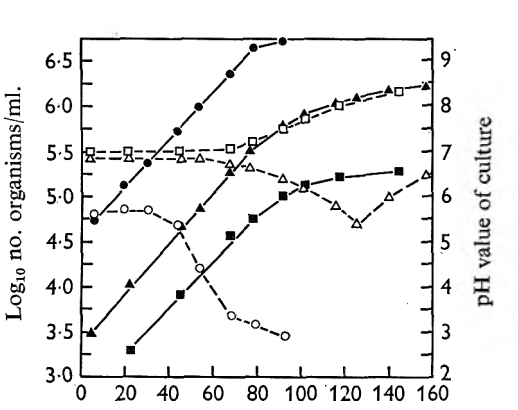

Fig. 1

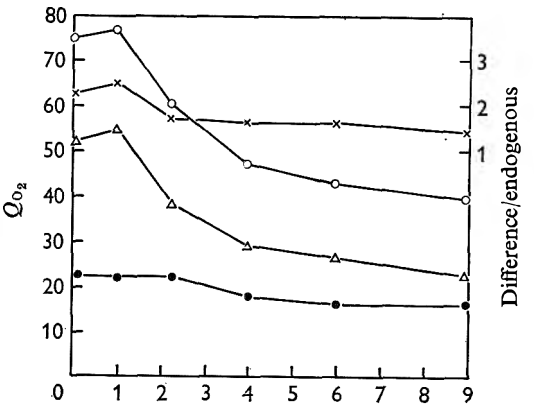

Fig. 2

Fig. 1. *Euglena gracilis* Z: change in culture pH as a function of culture age. Filled symbols, log. no. organisms/ml.; open symbols, culture pH. Carbon sources supporting growth were: glucose (circles), ethanol (triangles), acetate (squares).

Fig. 2. *E. gracilis* Z. Changes in the rate of oxygen consumption after washing organisms at time 0. The filled circles show the endogenous Q_{O_2}, and the open circles the Q_{O_2} after the addition of the growth substrate ethanol. The triangles show the differences between these two rates, i.e. the oxygen consumption stimulated by ethanol. Crosses show the ratio of respiration due to ethanol to the endogenous.

Figure 2 shows the change in the endogenous respiration value, the rate of oxygen consumption in presence of ethanol, and the difference between the two, as a function of time after washing. The *Euglena gracilis* Z organisms were grown with ethanol as sole carbon source, harvested in log. phase, washed, and incubated with forced aeration in the growth medium without ethanol. Samples of the suspension were removed periodically for measurement of endogenous respiration, after which ethanol was added to measure the degree of stimulation by substrate. The endogenous rate was unchanged for the first 2 hr. Stimulation of respiration by ethanol increased slightly after 1 hr, but the increase (2 to 3 %) was probably not significant. Since all other respiratory studies reported here were completed within 30 to 45 min. of harvest, they have been accepted as representative of oxygen consumption during growth.

Total oxygen consumption in presence of ethanol was 2·5 to 3·5 times greater than the endogenous value; the difference curve therefore has the same general shape as total respiration with ethanol (Fig. 2). Quantitative variations between the endogenous

and the difference curve during starvation are obvious; the endogenous rate was constant for the first 2 hr, while the difference rate decreased almost 30 % between the first and second hour of starvation. After 9 hr, the endogenous rate had decreased about 30 %, while the oxygen consumption stimulated by ethanol (the difference curve) had decreased about 55 %. This quantitative variation is perhaps best shown in the ratio [difference rate/endogenous rate], which is also plotted in Fig. 2. The rate of oxygen consumption stimulated by ethanol decayed much more rapidly than the endogenous rate. A similar phenomenon was observed when cultures were allowed to grow into stationary phase, probably for the reason to be discussed below.

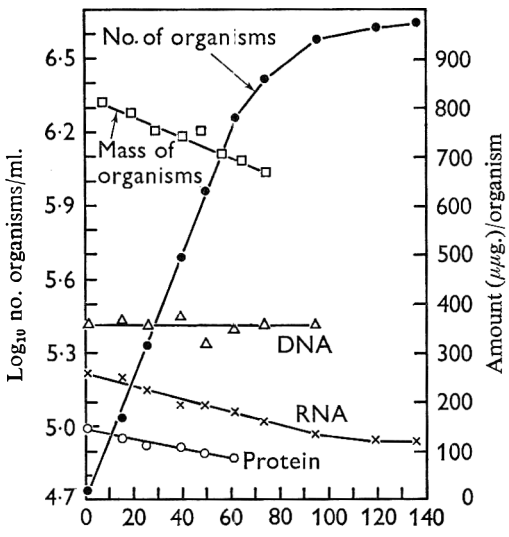

Fig. 3

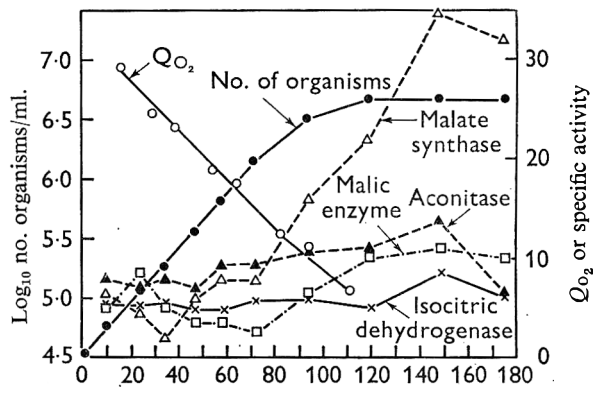

Fig. 4

Fig. 3. Biochemical profile of the average *Euglena gracilis* Z organism as a function of culture age; glucose as sole carbon source. To facilitate plotting, observed values for RNA were multiplied by 10 and those for DNA by 100.

Fig. 4. *E. gracilis* Z. Changes in respiration and the specific activity of representative respiratory enzymes as a function of culture age, with glucose as sole carbon source. The Q_{O_2} decreased steadily. Malate synthase and the malic enzyme showed signicant changes in activity.

Growth on glucose

The results with *Euglena gracilis* Z cultured with glucose as sole carbon source are summarized in Figs. 3 and 4. Figure 3 shows that protein, RNA, and total dry mass/organism decreased through log. phase and early stationary phase, at proportionate rates. The doubling time for number of organisms was 12·0 hr; in this time the average organism showed a decrease of about 10 % in mass, etc. The rate of cell division exceeded that of biosynthesis by about 10 %. The DNA content, within experimental error, was, however, constant. These data confirm and extend those obtained by Wilson & Levedahl (1964) with Euglena grown with ethanol, succinate, or acetate.

Figure 4 shows the rate of oxygen consumption (Q_{O_2}, μl. O_2/hr/10^6 organisms) and the specific activities of representative respiratory enzymes as a function of culture

age. The Q_{O_2}, which was about 30 in early log. phase, decreased continuously to nearly 5 in early stationary phase. The doubling time for organism numbers was 13·3 hr, and the Q_{O_2} decreased about 15 % in each generation. Glucose does not stimulate respiration significantly above the endogenous value (Cook & Heinrich, 1965), and these data, therefore, represent the respiratory rate both in absence and in presence of glucose.

Specific activities of the respiratory enzymes were essentially constant during log. phase growth. Since the activities are referred to protein content, this must mean that the total amount of these enzymes contained within the average organism must also have been decreasing with culture age. The specific activity of the two enzymes malate synthase and the 'malic enzyme' showed a marked increase beginning in early stationary phase. Since the total cell protein did not increase, it is inferred that the 'functional' concentration of these two enzymes was increased at this time. Whether this apparent increase was due to a true induction of enzyme synthesis or to a decreased degree of inhibition cannot be ascertained from these data. The data suggest a shift of metabolic emphasis, from a typical Krebs cycle oxidation of carbohydrates to utilization of fats. Metabolism of fat reserves in micro-organisms characteristically induces operation of the glyoxylate cycle (Kornberg, 1959). The supposition of a shift to fat metabolism is reinforced by the fact that the respiratory quotient of glucose-grown Euglena was about 1·0 during log.-phase growth, but decreased as low as 0·3 in stationary phase (Heinrich & Cook, 1967).

Growth on ethanol

With ethanol as sole carbon and energy source growth of *Euglena gracilis* Z was unbalanced in either a positive or negative direction. In one experiment the protein content of the average organism showed a slight increase during middle log. and early stationary phase (Fig. 5); the increase per organism amounted to about 7 % in a single generation. This experiment was terminated in very early stationary phase, but the average protein content tended to a constant value in late log. and early stationary phases. In two other experiments (also summarized in Fig. 5) the protein content of the average organism decreased during log. and early stationary phase, but remained constant throughout late stationary phase. While similar trends in protein content were found in two of these experiments, great variation existed in absolute amounts of protein per organism. The maximum amount found in any given experiment was 350, the second largest 310, and the largest amount found in the third experiment was 250 $\mu\mu$g./organism. This sort of discrepancy can probably be explained only by the past history of the organisms. In the present case, all the cultures had been previously adapted to growth on ethanol, and trends in protein content were most likely a result of culture age at inoculation, a condition which was not rigorously controlled in these experiments. The role of the lag period in balanced or unbalanced growth has not been adequately studied with any protozoan cell, although it is known that the length of the lag period increases with time spent in log. and stationary phases of growth (Prescott, 1957). In the present work, inoculations were always made with adapted organisms in log. or early stationary phase growth. In one of these experiments with ethanol-grown *Euglena gracilis* Z RNA values were followed and were essentially parallel to the protein content (Fig. 5).

Respiration as a function of culture age of *Euglena gracilis* Z grown with ethanol

was followed in two experiments (see Fig. 6). Under our cultural conditions, stationary phase growth with ethanol usually started at 0·5 to 1×10^6 organisms/ml. The endogenous respiration value of washed organisms showed a steady decrease throughout log. and stationary phase, from a value of about 25 μl./hr./10^6 organisms to a low value of 10 or less in stationary phase. Respiration values in presence of ethanol showed less tendency to decrease; indeed, the respiration stimulated by ethanol (i.e. above the endogenous value was very nearly constant during log.-phase growth, in one case showing an average decrease of only 2–3 % in one generation and in another case showing a slight increase during log. and early stationary phases of growth. The respiration stimulated by ethanol, however, decreased rapidly as the stationary phase progressed, much more rapidly than the decrease observed in the endogenous rate (Fig. 6).

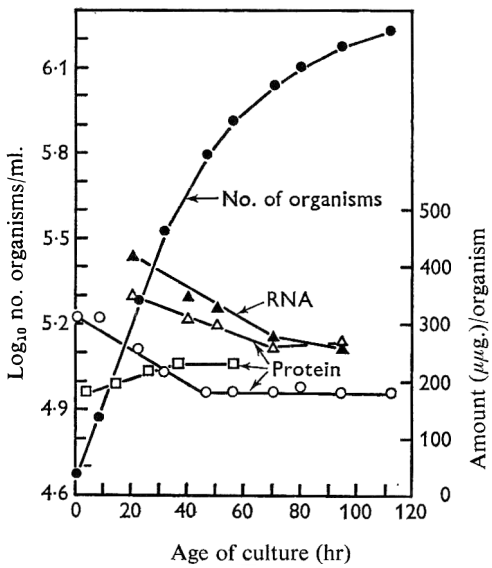

Fig. 5

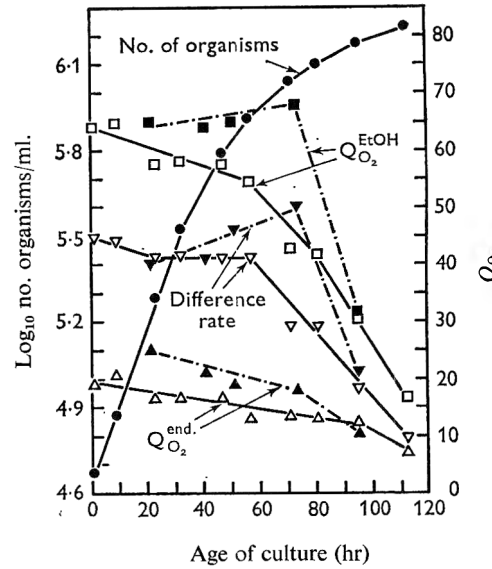

Fig. 6

Fig. 5. Changes in protein and RNA content of the average *Euglena gracilis* z organism as a function of culture age; ethanol as sole carbon source. A representative growth curve is shown; protein values were followed in three separate experiments. In one case, RNA levels (▲) were also followed.

Fig. 6. *E. gracilis* z. Rate of oxygen consumption as a function of culture age; ethanol as carbon source. Two experiments shown, as indicated by open or closed symbols, with a representative growth curve (●). Endogenous respiration rates are shown by △, ▲, respiration in the presence of ethanol by □, ■; and the difference between these two rates by ▽, ▼.

Activities of representative respiratory enzymes in ethanol-grown *Euglena gracilis* z are shown in Fig. 7. Isocitric dehydrogenase and malic dehydrogenase had constant specific activities during log. and early stationary phase, and the malic enzyme showed an increase in early stationary phase. These are patterns similar to those found with glucose-grown Euglena. Malate synthase, important in growth on C-2 compounds, showed constant high activities throughout log. phase. These assays were made on samples from the same culture that showed a steadily increasing protein content (Fig. 5); from this it may be assumed that at least a part of the total respiratory com-

plement was unbalanced with division in a positive direction. Unfortunately, our oxygen sensing equipment was inoperative during this run and no correlation could be made with total oxygen consumption.

Growth on acetate

According to Wilson & Levedahl (1964), culture of *Euglena gracilis* z on acetate gave 'more' unbalanced growth than was observed with ethanol, i.e. the average organism became smaller with each generation when acetate was sole carbon source. We confirmed this in the usual case; in three or four experiments, protein concentrations in the average organism decreased about 6% in a generation. A typical experiment is summarized in Fig. 8, which also shows how the rate of endogenous respiration decreased through log and early stationary phase. Oxygen consumption by the average

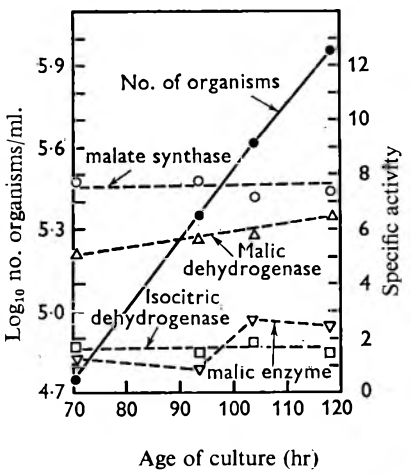

Fig. 7

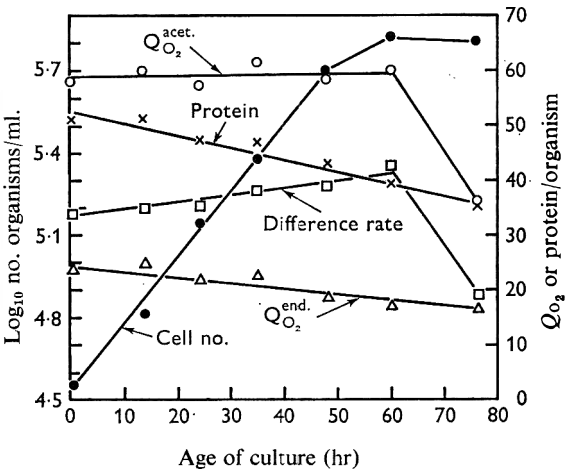

Fig. 8

Fig. 7. *Euglena gracilis* z. Specific activity of representative respiratory enzymes as a function of culture age; ethanol as carbon source. To facilitate graphing, activities for the malic enzyme and malate synthase were multiplied by 0·1 and those for malic dehydrogenase (MDH) by 0·01; those for isocitric dehydrogenase (IDH) are as indicated on the ordinate. Number of organisms (●) had reached early stationary phase by end of the experiment, and the malic enzyme activity showed an increase at this time; malate synthase activities were high over the entire growth curve, with no significant change at any time.

Fig. 8. *E. gracilis* z. Protein content and rate of oxygen consumption as a function of age of organisms; acetate as sole carbon source. Protein (×) and the endogenous respiration rate (△) decreased steadily throughout log. and stationary phases; oxygen consumption in the presence of acetate (○) constant during log. phase, but decreased sharply in stationary phase. The oxygen consumption actually stimulated by acetate (□) increased steadily during log. phase and decreased only in stationary phase. Observed values for protein content were 10 times greater than indicated on the ordinate.

organism in presence of acetate remained essentially constant, but with a precipitous decrease in early stationary phase. The rate of oxygen consumption actually stimulated by acetate (the 'difference' rate) increased slightly through log. phase growth, and showed a marked decrease coincident with the beginning of the stationary phase.

In one experiment with an acetate culture, a bathtub caulking compound was used to seal some of the supply lines. While none of the sealer came in direct contact with

the medium, some of its components were apparently volatile and inhibited division of *Euglena gracilis* Z; decrease of the growth rate began at populations of 40,000 to 50,000 organisms/ml., rather than at the usual 200,000 to 400,000 organisms/ml. (see Fig. 8). While the behaviour of *E. gracilis* Z in the presence of the inhibitor(s) is not to be regarded as typical, the experiment was instructive and is summarized in Fig. 9. The protein content of the average organism increased through the log. and early stationary phases to high values—2 to 3 times that of the 'typical' organism—and remained high into late stationary phase. The endogenous respiration rate, instead of decreasing, was constant or showed a slight increase over the whole period. Oxygen consumption stimulated by acetate increased steadily throughout log. phase and most of the stationary phase, showing the characteristic decrease only in very late stationary phase. The specific activity of the malic enzyme increased ten-fold over the whole period observed (cf. a comparable change in glucose-grown Euglena, Fig. 4), and remained high even in late stationary phase. The specific activity of malate synthase decreased slightly in early stationary phase, remained constant over most of stationary phase, and decreased rapidly in late stationary phase. An estimate of the total amount of these two enzymes in *E. gracilis* Z can be made by multiplying the specific activities by total cell protein. For clarity this curve is not shown in Fig. 9, but this estimate of the total amount of malate synthase, which catalyses an anaerobic reaction, paralleled total oxygen consumption stimulated by acetate.

In all the above experiments *Euglena gracilis* Z was grown in vigorously aerated vessels, and in general the rate of respiration decreased with culture age. In another series of experiments, in which the organism was grown in static Erlenmayer flasks with acetate medium having a surface:volume ratio adequate for optimal growth, the Q_{O_2} increased with culture age much like the experiment shown in Fig. 9, approaching 100 μl./hr./10^6 organisms in late log. phase before decreasing. This positive sort of unbalanced growth was qualitatively reproducible and appeared to be a result of the different growth conditions. Oxygen consumption by *E. gracilis* Z grown with acetate was high. It seems likely that a continuously decreasing oxygen tension in the medium as a result of increasing population density could cause a response toward higher respiratory capacity. Such adaptations were found by Gray, Wimpenny, Hughes & Mossman (1966) with *Escherichia coli* grown in different oxygen tensions. This sort of unbalanced growth of *E. gracilis* Z would appear to differ fundamentally from most of the results reported in this paper, in which oxygen was not a limiting factor.

Changes during the lag period

In most of the results described above, the average size of the organisms (based on protein, RNA, or mass) decreased during the log. phase; an exception was the one case where division but not growth was inhibited (see Fig. 9). The average size decreased less during growth with ethanol than with glucose or acetate, but on each substrate the decrease continued into stationary phase. It might be inferred that the lag phase must show an opposite trend, i.e. growth without division. The time course of these changes was followed in cultures of *Euglena gracilis* Z grown with ethanol or glucose, diluted from stationary phase to population densities compatible with the assays which were done. Figure 10 shows changes in the biochemical profile of *E. gracilis* Z following dilution of a stationary-phase glucose culture with fresh medium. There was a lag of about 20 hr before the rate of number increase became exponential, more than half of

this time with no significant increase in number. There was no evidence of any 'inoculation synchrony'. Upon dilution, however, there was an immediate increase in RNA content, followed shortly by an increase in total cell mass; protein content, on the other hand, remained static until division began. Thereafter, the average protein content steadily increased during early log. phase before a decline set in. This is one of the few circumstances where a strict correlation between the RNA and protein content of *E. gracilis* Z was not found. In normal log.-phase growth the ratio RNA:protein remains fairly constant under a wide variety of culture conditions (Cook, 1966). In the present case in lag phase, however, RNA synthesis clearly preceded the initiation of protein synthesis, and total cell mass approximately paralleled RNA rather than protein content during this transition period. In *E. gracilis* Z the major fractions of total mass are proteins, fats and the polysaccharide paramylum. It is inferred that in the lag period also there was an accumulation of fats or polysaccharides. It may

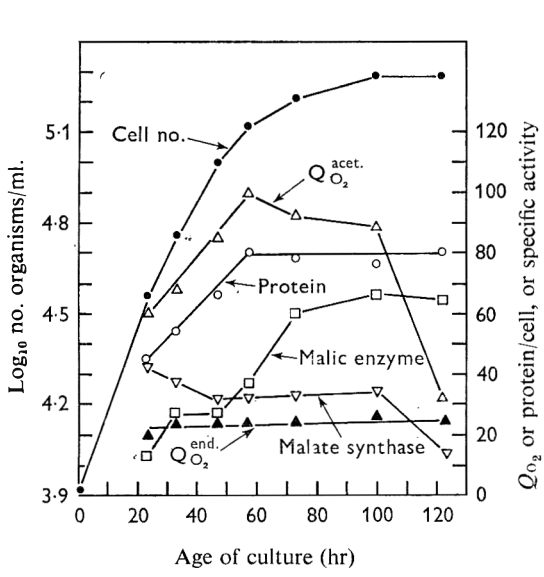

Fig. 9

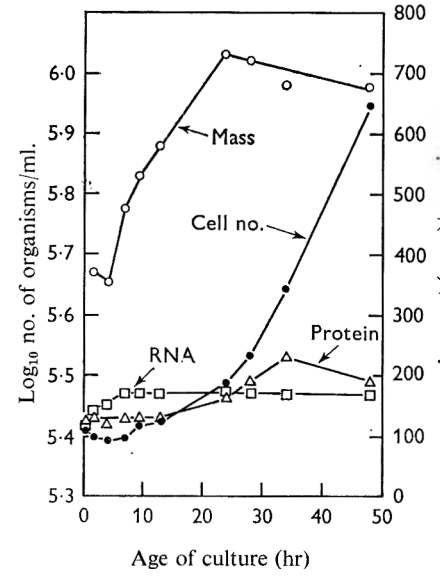

Fig. 10

Fig. 9. *Euglena gracilis* Z. Atypical experiment with acetate as sole carbon source. Division was inhibited very early by an unknown component of a sealing material, which entered the culture (the experiment shown in Fig. 8 can be taken as typical). The protein content of the average organism (○) almost doubled before inhibition was complete; the Q_{O_2} in the presence of acetate (△) showed a parallel increase, decreasing only in later stages of inhibition. The specific activity of malate synthase, referred to total protein, showed a slight early decrease, but was stable during most of the time, reflecting an increase in the total amount of this enzyme. The specific activity of the malic enzyme increased steadily except in the later stages of inhibition. Values for protein were 10 times greater than indicated on the ordinate.

Fig. 10. Changes in the biochemical profile of stationary-phase *E. gracilis* Z following dilution with fresh media; glucose as sole carbon source. Dilution at time 0, from 4×10^6 organisms/ml. RNA values (multiplied by 10 to facilitate graphing) increased well before those of protein.

be noted from Fig. 10 that the maximum amounts of RNA and protein attained in early log. phase glucose-grown *E. gracilis* Z were less than those shown in Fig. 3. The experiment described in Fig. 10 started with an initial population of 250,000 organisms/

ml.; that in Fig. 3 with less than 10,000 organisms/ml. A comparison of these two experiments indicates that positive unbalanced growth, i.e. increased average size of organisms with culture age, occurred in lag and very early log. phase, but that the size of the inoculum determined when this unbalanced growth became negative in sign, and average organism size started to decrease. Whether this was due strictly to population density or to ancillary conditions, e.g. a change to different pH values, cannot be determined from these data.

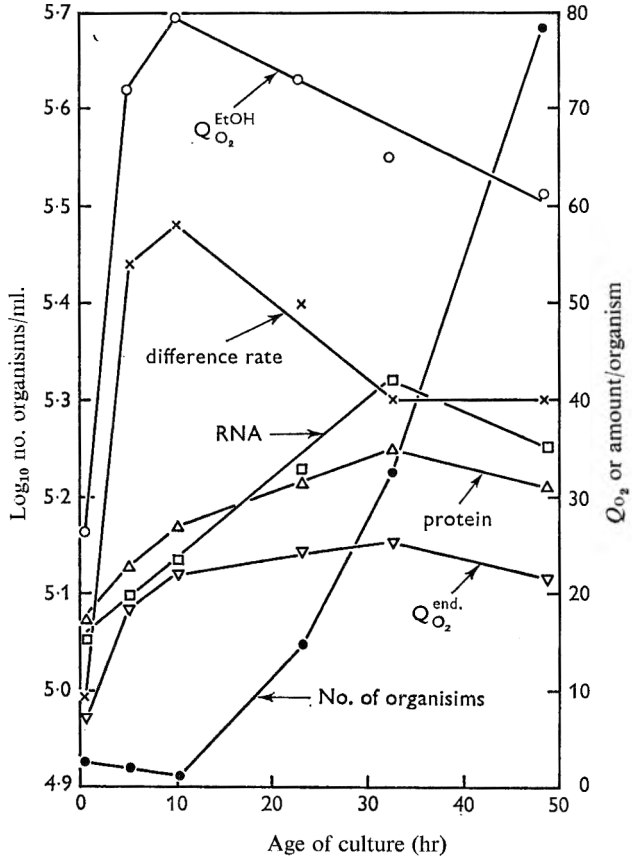

Fig. 11. Changes in the physiological profile of stationary phage *Euglena gracilis* z following dilution with fresh media; ethanol as sole carbon source. Dilution at time 0, from 3×10^6 organisms/ml. RNA and protein values were essentially parallel (protein values were 10 times those indicated on the ordinate). Both the endogenous rate of respiration (▽) and that in presence of ethanol (○) showed an early increase, in the lag period; the endogenous rate continued increasing into early log. phase, while the Q_{O_2} in the presence of ethanol began decreasing much earlier. The plot showing oxygen consumption actually stimulated by ethanol (×) accentuated this difference.

A comparable experiment with ethanol-grown *Euglena gracilis* z is shown in Fig. 11. A lag of 10 hr elapsed before division began, and protein and RNA synthesis began simultaneously immediately after dilution. (This culture had not been allowed to proceed as far into stationary phase as that described in Fig. 10, which may account for this difference.) Protein and RNA content were essentially parallel during lag and

early log. phases. The endogenous respiration, and that with ethanol, increased very rapidly, attaining near-optimum values after 5–10 hr and before any detectable increase in number of organisms. Figure 11 shows that the endogenous respiration rate went through a maximum only after the rate of division (and the protein content) had reached maximum values. The oxygen consumption stimulated by ethanol went through a maximum at about the end of lag phase, and by the time the endogenous respiration rate had reached its maximum, respiration stimulated by ethanol had already decreased some 35 %. This difference may be of no particular significance, but it does indicate that the endogenous respiration of *E. gracilis* Z can be separated from that stimulated by 2-C compounds in the lag period, perhaps even more clearly than during log.-phase growth.

DISCUSSION

Hutchens (1941) reported that the rate of oxygen consumption by *Chilomonas paramecium* decreased with culture age. This is in essential agreement with our findings with *Euglena gracilis* Z described here. Albergoni & Pranzetti (1963) found that the endogenous respiration of Euglena decreased during log. phase growth, and the oxidation of acetate decreased in parallel with the endogenous respiration; these organisms had been grown with glucose as principal carbon source, and the respiration stimulated by acetate was considerably less than that reported in the present work for organisms adapted to acetate. Qualitatively, however, the two sets of data are in essential agreement.

It has been recognized for some time that growth and division of cells are seldom balanced, so it is not surprising that the rate of oxygen consumption by *Euglena gracilis* Z was not constant during log. and early stationary phases of growth. Respiration paralleled increase of total cell protein; under conditions where one changed, the other changed in the same direction. It may be inferred that the enzyme complement of the respiratory apparatus (which must be an important fraction of the total cell protein) was synthesized at rates roughly proportional to the total rate of biosynthesis (or limited the latter). The specific activities of the respiratory enzymes, referred to protein, were constant over most of the incubation period studied, showing variation in some cases only in late stationary phase. The one exception which was observed to this trend was in late stationary phase acetate-grown or ethanol-grown *E. gracilis* Z, when cell protein changed little but the respiration stimulated by the substrate decreased to rates approaching the endogenous value. It was possible, during growth on these two substrates, to separate the endogenous respiration from that stimulated by the substrate in more than a quantitative manner. In our best experiments, the endogenous rate showed a steady decrease while the oxygen consumption due to substrate was essentially constant over most of the log. and early stationary phases of growth. While these trends did not differ greatly, they were consistently found in seven different experiments with organisms grown on these two substrates; the ratio between the 'difference' and the endogenous rates changed with culture age. The explanation for the change in this ratio is not clear. Perhaps the metabolism of acetate and ethanol involves oxidative pathways not utilized in the oxidation of endogenous reserves. The activity of malate synthase was always proportional to oxygen consumption stimulated by acetate or ethanol, in spite of the fact that the enzyme catalyses an anaerobic reaction. This correlation held even in late stationary phase, where these substrates

stimulated oxygen consumption very little. Possibly operation of the anaerobic glyoxylate by-pass in Euglena which is induced by 2-C compounds (Cook & Carver, 1966), co-ordinately induces an oxygen-requiring sequence of reactions different from those associated with the classic Krebs cycle. On the other hand, late stationary phase glucose-grown *E. gracilis* Z showed an increase in malate synthase without any concomitant increase in oxygen consumption. This change probably reflected a shift from utilization of carbohydrate to utilization of stored lipid. The respiratory quotient (CO_2:O_2) of glucose-grown Euglena decreased from 1·0 in log. phase to about 0·3 in late stationary phase in the experiments of Heinrich & Cook (1967).

It may be inferred from these studies that late log. phase *Euglena gracilis* Z organisms have less oxidative energy available for growth and division than do early log. phase organisms. This is reflected in the smaller size of the organisms in the later stages of the growth curve. It is not possible from these data to say that the rate of energy transformation limits biosynthesis, or vice versa. The decreased rates of energy transformation in late log. phase were without effect on the rate of multiplication, which remained constant for extended periods during growth with ethanol or glucose. Perhaps energy used in division processes, if this should prove to be considerable and constant in amount, is stored in compartments not available for general cell growth, much as proposed in Swann's 'energy reservoir' hypothesis (1957). Reference to Fig. 3 will show that total cell protein during growth on glucose was about twice as great in early log. phase as it was in late log. phase. It is difficult to imagine that the size of the mitotic apparatus, and the energy needed to activate it, could differ by this amount when the rate of mitosis itself was unchanging. However, the protein complement of the mitotic apparatus was about 10% of the total cell protein in sea-urchin eggs (Mazia, 1961), and might be even less in Euglena. Mazia (1961) emphasized that the energy required for cell division might be very small indeed. The magnitude of the changes in energy transformation studied here with Euglena, estimated by total oxygen consumption, may be several orders above that involved in mitosis and cytokinesis. Certainly the rate of respiration is more closely correlated with the rate of biosynthesis than with the rate of cell division.

This work was supported in part by N.I.H. Grant GN-12179 to the senior author and in part by a N.A.S.A. Grant (NsG-338) to the University of Maine, U.S.A.

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J. gen. Microbiol. (1968), **53**, 253–258
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Printed in Great Britain

Involvement of Autolysis of Cytoplasmic Membranes in the Process of Autolysis of *Bacillus cereus*

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(*Accepted for publication* 8 *April* 1968)

SUMMARY

When exponentially growing *Bacillus cereus* organisms were suspended in buffer or buffered hypertonic sucrose solution and incubated at 37°, a rapid decrease in turbidity of the suspension was observed (autolysis). During autolysis ultraviolet-absorbing substances leaked from the bacteria and over 50% of the total phospholipids was released. Of the amino sugars, main components of the cell walls of this organism, less than 20% was released. Phase-contrast microscopy showed the empty rod-shaped ghosts which increased in number, while the total counts were constant during autolysis.

Mg^{2+} and Mn^{2+} inhibited the autolysis; these cations either prevented leakage of ultraviolet absorbing substances or release of phospholipids. Isolated cytoplasmic membranes autolysed when the membranes were incubated at 37° in buffer solution (pH 7·0 to 7·5). It could be assumed, therefore, that the cytoplasmic membranes of *Bacillus cereus* were lysed faster than the cell walls during autolysis and that the autolysis of the membranes was inhibited by Mg^{2+} and Mn^{2+}.

INTRODUCTION

Bacterial cell-wall lytic enzymes (mureinases) are known to be associated closely with their own cell walls and work from several laboratories has suggested that bacterial autolysis was a lytic event caused by the action of these enzymes (Stolp & Starr, 1965). Autolysis is, however, a very complex event and decomposition of cellular components other than cell walls might be involved in the autolytic process. Norris (1957) showed that the lysis of *Bacillus cereus* resulted in loss of cell contents whilst at least part of the cell-wall structures remained intact. This would suggest that the cell membranes were lysed faster than the cell walls. The present paper gives some cytological and biochemical evidence for the involvement of lysis of cytoplasmic membranes in the autolysis of *B. cereus*.

METHODS

Organism. Bacillus cereus IAM 1656 was used throughout.

Medium. GYC medium (pH 7·0) contained the following nutrients: glucose, 10 g.; yeast extract (Difco), 5 g.; casein enzymic hydrolysate (Nutritional Biochemicals Corporation), 5 g.; 1000 ml. of water in which were dissolved: $MgSO_4.7H_2O$, 200 mg.; $MnSO_4.4H_2O$, 10 mg.; $FeSO_4.7H_2O$, 6 mg.; K_2HPO_4, 500 mg.; KH_2PO_4, 150 mg.; $CaCl_2$, 100 mg.; NaCl, 100 mg.

Culture conditions and handling of organisms. Fifty ml. of GYC medium in a 2 l. Erlenmeyer flask were inoculated with 0·25 ml. of *Bacillus cereus* culture grown in

brain heart infusion (Difco) at 37° for 15 hr on a rotary shaker. At exponential phase of growth, the bacteria were harvested by centrifugation and washed with 0·85 % (w/v) NaCl solution. Centrifugation was done (at 4°), the packed bacteria suspended in 3 ml. of tris HCl buffer (pH 7·5) and stored in an ice water bath. This suspension had to be used for autolysis experiments within about 30 min.

Measurement of autolysis. To 5 ml. of 0·05 M-tris-HCl buffer (pH 7·5) was added a small amount of the concentrated bacterial suspension to give an extinction of 0·3 at 650 mμ, autolysis being followed by the optical change in extinction at 650 mμ.

Preparation of protoplasts and cytoplasmic membranes of Bacillus cereus. For the preparation of protoplasts, the bacteria were suspended in 0·4 M-sucrose solution buffered with 0·1 M-phosphate (pH 6·8) containing 500 μg. lysozyme/ml. and incubated at 30°. Over 99 % of the bacteria were converted to protoplasts within 60 to 70 min. Protoplasts were centrifuged down and suspended in aqueous 5 mM-$MgCl_2$ (Mg water). Crude membrane fraction was obtained by centifugation of burst protoplast suspension at about 30,000 *g* for 15 min. The pellets were washed twice with Mg water and then dialysed against distilled water at 4° for 15 hr.

Chemical estimations. Hexosamine was determined by the method of Elson & Morgan modified by Svennerholm (1956). For the determination of lipid-phosphorus from material extractable by chloroform + methanol (2 + 1 by vol.), lyophylized samples of bacteria were digested with $HClO_4$ at 200° for 3 hr. Inorganic phosphorus was estimated by the method of Allen modified by Nakamura (1950).

Total bacterial counts were made by using a Petroff–Hausser counting chamber.

RESULTS

When exponentially growing *Bacillus cereus* organisms were suspended in tris HCl or phosphate buffer (pH 7·5) and incubated at 37°, a rapid decrease of the turbidity of the suspension was observed (Fig. 1). The optimum condition for the autolysis was 0·05 M-tris HCl buffer (pH 7·5) and 37°. The decrease in number of viable bacteria followed the decrease of turbidity (Fig. 1). On the other hand, total bacterial counts were constant during this time. Phase-contrast microscope observation showed that 'empty forms' appeared, in which the cell walls might be intact while the cytoplasmic contents were almost completely lost (Pl. 1, fig. 1–3). As shown in Fig. 2, the 'empty forms' increased very rapidly; almost all the bacteria were turned into the empty forms within 20 min.

Leakage of intracellular ultraviolet-absorbing substances during autolysis. During autolysis of *Bacillus cereus* in tris HCl buffer containing 0·4 M-sucrose, samples were taken at intervals, immediately chilled and filtered through Millipore Filter HA (Millipore Corporation, U.S.A.). The filtrates were measured for extinction at 260 mμ. As shown in Fig. 3 substances which absorbed at 260 mμ had leaked out of the bacteria. The decrease in turbidity of the suspension corresponded well with the leakage of the substances, which were composed mainly of partly degraded RNA and protein.

The effect of divalent cations on autolysis and leakage from the bacteria. Autolysis was markedly supressed when Mn^{2+} was supplied to the suspension; Mg^{2+} was less effective than Mn^{2+}; Ca^{2+} had no effect (Table 1). The leakage of ultraviolet-absorbing substances was markedly inhibited by Mn^{2+} or Mg^{2+} (Fig. 4).

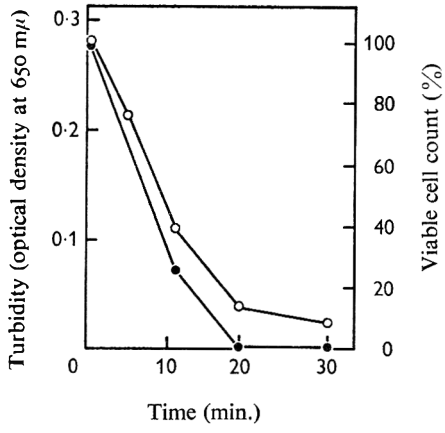

Fig. 1

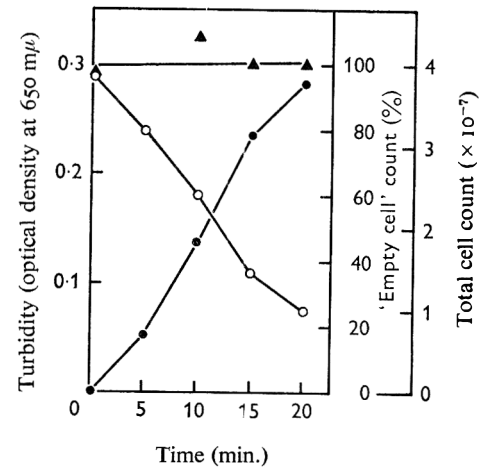

Fig. 2

Fig. 1. Changes in turbidity and viability during autolysis. ○, Turbidity; ●, viable counts.

Fig. 2. Changes in counts of total and empty forms during autolysis. ▲, Total cell counts; ●, empty form counts; ○, turbidity.

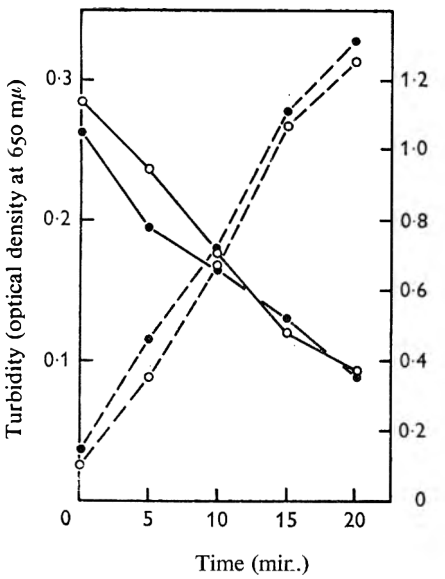

Fig. 3

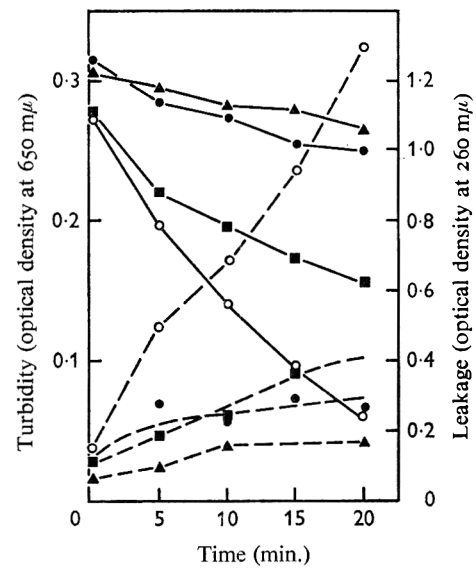

Fig. 4

Fig. 3. Leakage of intracellular ultraviolet-absorbing substances. Bacteria were suspended in tris-HCl buffer (○) or tris-buffered sucrose (●) and incubated at 37°. —, Turbidity; – – –, extinction at 260 mμ.

Fig. 4. Effect of divalent cations on the leakage. Bacteria were suspended in tris-buffered sucrose in the absence (○) or presence of 5 mM (▲) and 0·5 mM (■) of $MnCl_2$ or 5 mM of $MgCl_2$ (●). —, Turbidity; – – –, extinction at 260 mμ.

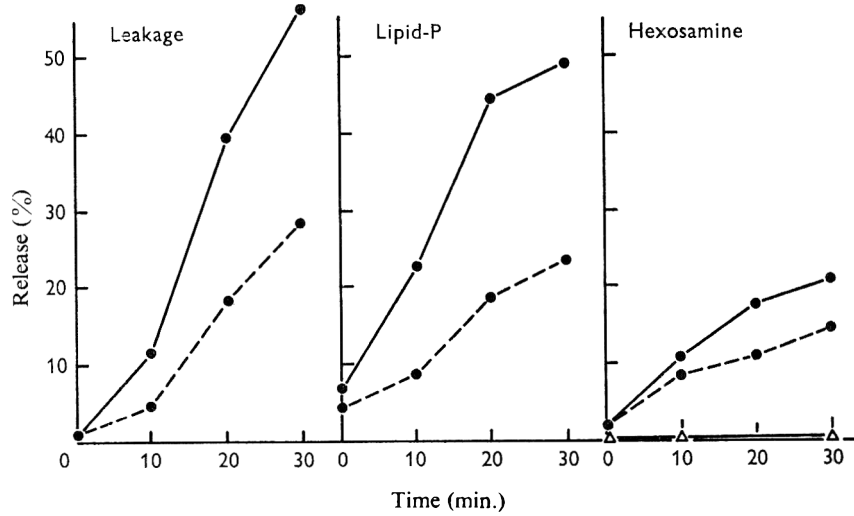

Fig. 5. Release of lipid-phosphorus and hexosamines during autolysis. Bacteria were suspended in tris-HCl buffer only (●—●) or tris+5 mM $MnCl_2$ (●- - -●). △——△, unhydrolysed supernatant fluid.

Table 1. *Effect of divalent cations on autolysis*

Additions	Autolysis*	% of control
None	43	100
$MnCl_2$, 5 mM	19	44
$MgCl_2$, 5 mM	29	68
$CaCl_2$, 5 mM	40	93

$$*\frac{E_{650}\text{ (0 min.)} - E_{650}\text{ (10 min.)}}{E_{650}\text{ (0 min.)}} \times 100.$$

Release of phospholipids and hexosamines during autolysis. Supernatant fluid of autolysed suspensions after centrifugation at 10,000 *g* contained phosphorus in a form extractable with chloroform + methanol (2 + 1 by vol.; lipid-P). The release of lipid-P increased during autolysis and about 50% of the total lipid-P was released within 30 min. (Fig. 5).

From whole organisms only 60% was extractable with extraction of chloroform + methanol; however, when the bacteria were autolysed for about 60 min. or treated ultrasonically at 10 kc for 10 min., lipid-P was easily extractable with this solvent mixture. The lipid-P gave the same value when extracted from bacteria disruption ultrasonically or autolysed; total lipid-P was taken as the value from ultrasonically treated suspensions.

The direct estimation of free hexosamines released from autolysed bacteria showed less than 0·6% of total hexosamines even after autolysis for 30 min. When such extracts were hydrolysed with 2 N-HCl at 105° for 16 hr, reactivity to the Elson–Morgan reagent was markedly increased. This meant that the hexosamines were released from the bacteria in the unhydrolysed state; calculation from the reactivity before and after acid hydrolysis showed that the hexosamines released by autolysis were polymers of at least 40 hexosamine units.

Estimation of hexosamines in the autolysis supernatant fluid after acid hydrolysis showed that the hexosamines released during autolysis were about 20% of the total. Mn^{2+} or Mg^{2+} inhibited the release of lipid-P but did not affect the release of hexosamine (Fig. 5).

Evidence for the autolysis of isolated membranes. When washed membranes, isolated by bursting protoplasts and centrifugations down, were suspended in tris-HCl or phosphate buffer (pH 7·5) and incubated at 37°, a rapid decrease of turbidity at 650 mμ was observed. The decrease of turbidity was completely inhibited by 1 mM-Mn^{2+} or Mg^{2+} (Fig. 6).

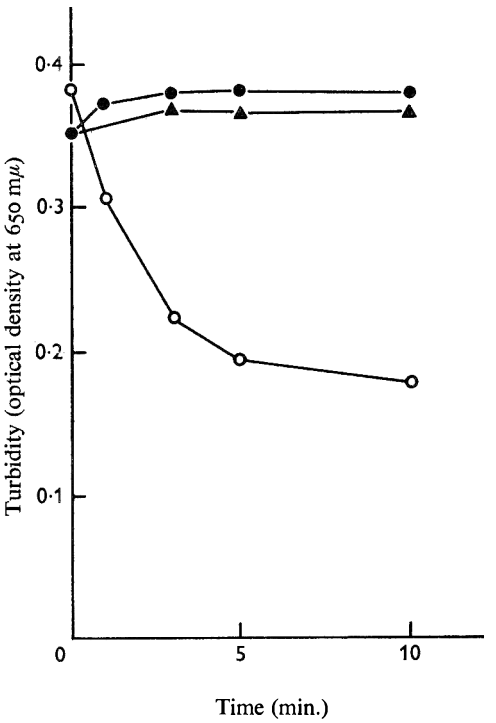

Fig. 6. Autolysis of isolated membranes of *Bacillus cereus* at 20° (pH 7·5). Washed and dialysed membranes obtained by bursting protoplasts of *B. cereus* were suspended in tris-HCl buffer in the absence (○) or presence of 5 mM-$MnCl_2$ (●) or 5 mM $MgCl_2$ (▲).

DISCUSSION

The involvement of changes of membraneous structures in autolysis of bacteria was suggested by Norris (1957) who isolated a lytic principle associated with culture of *Bacillus cereus*. He found loss of cell contents when the principle was added to bacterial suspensions. The results presented here show that the cytoplasmic membranes of *B. cereus* are digested in the process of autolysis. The leakage of ultraviolet-absorbing substances in hypertonic medium and the release of phospholipids (which are known to be one of the main constituents of bacterial membranes) from the bacteria were concomitant with the decrease of turbidity of the suspension. These observations show that decrease in turbidity paralleled the rate of loss of cell contents or the rate of damage of cell membranes. The cell walls after autolysis were comparatively intact.

Stolp & Starr (1965) proposed that bacterial autolysis might be defined as a lytic event caused by the action of the cell's own mureinases. However, the results described in this paper show that bacterial autolysis is a complex event and should not be defined as proposed by Stolp & Starr.

The isolated protoplast membranes of *Bacillus cereus* were autolysed when the membranes were incubated with buffer (pH 7·5) at 37°. The protoplast lytic activity is associated with the membranes of *B. cereus* (Kusaka, Tamaki & Koga, unpublished) and phospholipids are released from the membranes when they are incubated with buffer (pH 7·0 to 7·5) at 37° (Kusaka & Koga, unpublished). These facts suggest that certain factors which digest the membranes may be associated with the membranes themselves.

The authors are grateful to Dr S. Fukui for his interest and helpful suggestions.

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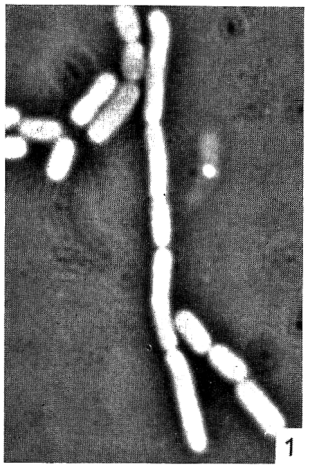
EXPLANATION OF PLATE

Magnification, × 2500

Fig. 1. Intact cells.

Fig. 2. A photograph of a partially lysed suspension which is a mixture of intact and completely lysed cells (autolysed for 10 min.)

Fig. 3. Completely lysed cells (autolysed for 25 min.) All photographs are taken through phase contrast microscope.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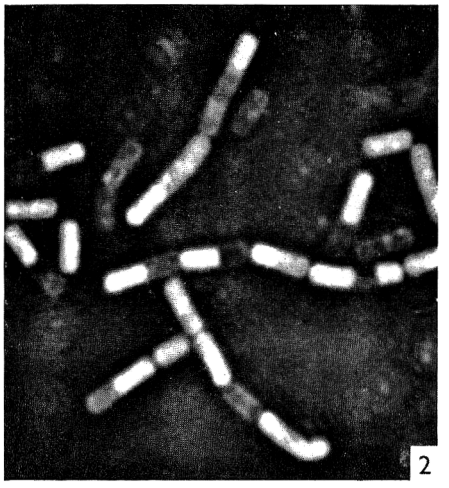
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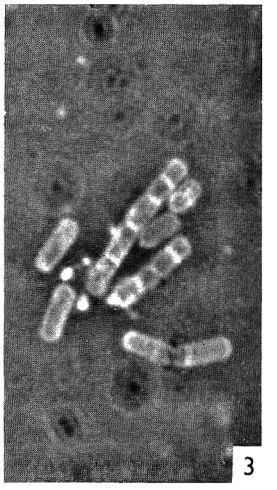
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J. gen. Microbiol. (1968), **53**, 259–273
Printed in Great Britain

Metabolism of Mandelate and Related Compounds by Bacterium NCIB 8250

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(*Accepted for publication* 9 *April* 1968)

SUMMARY

Bacterium NCIB 8250 was grown on mandelate, benzyl alcohol, benzoylformate, benzaldehyde or benzoate and also on 2-hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy derivatives of these compounds. Growth rates and yields of organism were measured for many of the substrates. The pathways of oxidation of mandelate and related compounds were investigated by the technique of simultaneous adaptation. Bacterium NCIB 8250 did not utilise D-mandelate; L-mandelate was oxidised via benzoylformate, benzaldehyde and benzoate to catechol. Benzyl alcohol was converted to catechol via benzaldehyde and benzoate. The 2-hydroxy-substituted compounds were oxidised to catechol by a parallel pathway. 4-Hydroxy-, 3,4-dihydroxy- and 4-hydroxy-3-methoxy-substituted compounds were all converted to 3,4-dihydroxybenzoate. Catechol and 3,4-dihydroxybenzoate underwent ring cleavage with the ultimate formation of β-oxoadipate.

INTRODUCTION

Mandelate can be utilized by some bacteria as sole source of carbon and energy for growth. The mechanism whereby *Pseudomonas putida* oxidizes this compound to intermediates of the tricarboxylic acid cycle has been thoroughly investigated by Stanier and his colleagues and is the original example of sequential induction (Stanier, 1947; Gunsalus, Stanier & Gunsalus, 1953; Gunsalus, Gunsalus & Stanier, 1953; Stanier, Gunsalus & Gunsalus, 1953; Hegeman, 1966*a*, *b*, *c*; Ornston & Stanier, 1966; Cánovas, Ornston & Stanier, 1967). Mandelate and a considerable number of related compounds also serve as growth substrates for bacterium NCIB 8250 (Fewson, 1967*a*). Bacterium NCIB 8250, which has generally been known as 'Vibrio C1', belongs to the Acinetobacter–Moraxella group of bacteria (Sebald & Véron, 1963; Véron, 1966; Fewson, 1967*b*). The members of this group have nutritional, and probably ecological, similarities to species of the genus *Pseudomonas*, but differ in important morphological respects and also possess a quite different DNA base composition (Cánovas *et al.* 1967) which suggests that the two groups are evolutionarily distinct. It therefore seemed of interest to investigate the way in which bacterium NCIB 8250 grows on a range of compounds related to mandelate and to compare the pathways with those operating in *P. putida*. It was also felt that the technique of simultaneous adaptation (Stanier, 1947) could be used to give information about the specificity of enzyme induction and activity. Thus it might be expected that if the organism were grown on as many of the likely intermediates and analogues as possible, and then

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exposed in the Warburg apparatus to a similar range of compounds, the resulting patterns of oxygen uptake would indicate the degree to which non-specificity of enzyme induction and activity contributed to the economy of the cell. Results of this type of experiment are reported in the present paper and suggest that relatively non-specific enzymes carry out the first stages in the oxidation of mandelate and a number of substituted derivatives. More specific enzymes are formed for the oxidation of benzoate and the substituted benzoates.

Some of the results given in this paper have been published in a preliminary form (Kennedy & Fewson, 1966).

METHODS

Organism. Bacterium NCIB 8250 was maintained, grown and harvested as described by Kennedy & Fewson (1968).

Mean generation times. Samples (8 ml.) of a 17 hr nutrient broth culture were inoculated into medium (800 ml.) contained in 1 l. flasks fitted with side arms to facilitate sampling. Cultures were grown under conditions of vigorous aeration at 30° in the apparatus described by Harvey, Fewson & Holms (1968). Growth was followed by taking samples (4 ml.) at appropriate times and measuring the extinction at 500 mμ in a Spectronic 20 colorimeter with standard tubes of internal diam. 11·7 mm. Growth rates were determined graphically by conventional means (Dawes, 1962).

Molar growth yields. The basal medium was $KH_2PO_4 + (NH_4)_2SO_4 + MgSO_4$ (Kennedy & Fewson, 1968) with various concentrations of carbon sources. Medium (50 ml.) contained in 250 ml. Erlenmeyer flasks plugged with cotton wool was inoculated (0·2 %, v/v) with a 17 hr nutrient broth culture. The cultures were incubated for 4 days at 30° on a rotary shaker (Mk V; L. H. Engineering Co., Bells Hill, Stoke Poges, Buckinghamshire) moving at about 180 oscillations/min. The extinctions of samples (4 ml.) were measured at 500 mμ as described above. Under these conditions the relationship between E_{500} and dry wt/ml. was constant up to E_{500} = approx. 0·4. Suspensions with extinctions greater than 0·3 were diluted with basal medium before reading. In this series of experiments, and for organisms grown in a range of media, a value of E_{500} = 1·0 was equivalent to 310 μg. dry wt/ml. Controls were included for growth in the basal medium without added carbon source, but such growth was barely detectable. Preliminary experiments showed that the medium after growth did not contain material which absorbed at 500 mμ. Most compounds showed a constant molar growth yield (μg. dry wt formed/μmole substrate) up to at least 2 mM but a few substrates, e.g. benzaldehyde, gave lower yields at concentrations above about 1 mM; in all cases, therefore, the molar growth yield was calculated from the initial slope of the graph of yield of organism plotted against substrate concentration.

Oxygen uptake by whole bacteria. The ability of cell suspensions of bacterium NCIB 8250 to oxidize a number of substrates was determined by conventional manometric techniques (Umbreit, Burris & Stauffer, 1957). Although it is not normal practice, respiration was measured in a medium potentially capable of supporting growth since it was anticipated that the patterns of enzyme induction would be of as much interest as the initial rates of oxygen uptake. In any case it is usually difficult to avoid some enzyme induction, even in so-called non-proliferating suspensions, unless inhibitors of protein synthesis are used. Suspensions of bacteria, washed once with ice-cold distilled water, were invariably used within 4 hr of harvesting. Each 15 ml. vessel

contained 0·1 ml. of 0·3 M-$(NH_4)_2SO_4$; 0·1 ml. of 0·03 M-$MgSO_4.7H_2O$; 0·1 ml. of 0·5 M-$KH_2PO_4 + K_2HPO_4$ buffer (pH 7·0); 0·8 ml. of aqueous bacterial suspension (10 mg. wet wt bacteria/ml.). The reaction was started by tipping 0·5 ml. of 7·2 mM-substrate (dissolved in 0·1 M-$KH_2PO_4 + K_2HPO_4$ and adjusted to pH 7·0 with 0·1 N-NaOH or 0·1 N-HCl) from the side arm. The centre well contained 0·2 ml. 20% (w/v) KOH and pleated filter paper (Whatman no. 52). The total liquid volume was 2·0 ml. The reaction was run at 30° in air. Preliminary experiments showed that the rate of oxygen uptake was proportional to amount of bacteria at least up to the concentration used.

Chromatographic separations. Hydroxybenzoic acids were separated by two-dimensional chromatography on Whatman no. 1 paper. The first solvent (ascending for 15 hr) was benzene + acetic acid + water (2 + 2 + 1, by vol., upper phase; Bray, Thorpe & White, 1950) and the second solvent (ascending for 5 hr) was butan-1-ol + pyridine + water (14 + 3 + 3, by vol.; Smith, 1960). Compounds were detected on the dried papers by absorption or fluorescence under ultraviolet radiation, and by spraying with diazotized *p*-nitroaniline, diazotized sulphanilic acid or ammoniacal silver nitrate (Smith, 1960).

Analytical methods. Values for the wet weights of bacteria were obtained by centrifuging appropriate volumes of suspension in tared centrifuge tubes, washing the precipitate by suspension in water and again centrifuging; excess moisture was removed with tissue and the tubes weighed. Dry weights were determined by adding suspensions of washed bacteria (washed with ice-cold distilled water) to tared 20 ml. sample bottles, and drying to constant weight at 105°.

The nitrogen content of bacteria was determined by digesting samples (about 0·5 mg. wet wt) at 180° with 0·25 ml. of 1% (w/v) selenium dioxide dissolved in 50% (v/v) H_2SO_4. Ammonia assays were made on the resulting digests by the Nessler method (Paul, 1958); A.R. $(NH_4)_2SO_4$ was used as standard.

Chemicals. Dr P. G. Heytler (Central Research Department, E. I. du Pont de Nemours and Co., Wilmington, Del., U.S.A.) kindly gave a sample of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. Actinomycin D was a gift from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.). 3-Hydroxybenzoic acid was bought from British Drug Houses Ltd. (Poole, Dorset) and recrystallized from hot water. β-Oxoadipic acid and puromycin dihydrochloride were bought from Sigma London Chemical Co. Ltd. (12 Lettice Street, London, S.W. 6). Chloramphenicol was from Parke, Davis and Co. Ltd. (Hounslow, Middlesex). The sources of other reagents are listed by Kennedy & Fewson (1968) or were the best quality which could be obtained commercially.

RESULTS

The growth parameters for batch cultures of bacterium NCIB 8250 grown on mandelate and related compounds are given in Tables 1 and 2. 4-Hydroxybenzoylformate, 3,4-dihydroxybenzoylformate, 4-hydroxy-3-methoxybenzoylformate and 3,4-dihydroxybenzyl alcohol were not available for testing. Growth rates with catechol and yields with catechol and 3,4-dihydroxybenzoate were not determined because of the brown colour produced in these media. Appreciable time lags (more than 10 min.) before the onset of exponential growth were observed with only a few substrates. Calculations of the length of these lags by conventional methods (e.g. Dawes, 1962)

Table 1. *Growth rates of bacterium NCIB 8250 with different substrates*

The basal medium contained $KH_2PO_4 + MgSO_4 + (NH_4)_2SO_4$. Concentrations of mandelate and the substituted mandelates refer to the L-isomers but all these compounds were added as double-strength D,L-mixtures. A 17 hr nutrient broth culture was used as inoculum (1·0 %, v/v) and growth at 30° was followed as described in Methods. Results are means of at least two experiments. Lags are described as present or absent since, as described in the text, their length tended to be difficult to estimate. n.g. = no growth.

	Substrate concentration (mM)				
	0·3	0·5	1·0	1·5	
Substrate	Mean generation times (min.)				Lag
D,L-Mandelate	95	87	73	71	−
Benzoylformate	.	82	83	84	−
Benzyl alcohol	.	51	52	56	−
Benzaldehyde	.	47	49	60	−
Benzoate	45	45	45	45	−
2-Hydroxy-D,L-mandelate	.	1893	1740	2280	+
2-Hydroxybenzoylformate	.	3696	2736	2352	+
2-Hydroxybenzyl alcohol	.	141	130	141	−
2-Hydroxybenzaldehyde	97	n.g.	n.g.	n.g.	−
2-Hydroxybenzoate	95	102	122	165	−
4-Hydroxy-D,L-mandelate	.	400	340	290	+
4-Hydroxybenzyl alcohol	.	83	76	76	−
4-Hydroxybenzaldehyde	.	87	99	112	−
4-Hydroxybenzoate	.	58	58	58	−
3,4-Dihydroxy-D,L-mandelate	.	+*	+*	+*	+
3,4-Dihydroxybenzaldehyde	85	101	173	303	+
3,4-Dihydroxybenzoate	.	56	57	57	−
4-Hydroxy-3-methoxy-D,L-mandelate	.	+*	+*	+*	+
4-Hydroxy-3-methoxybenzyl alcohol	.	230	220	220	+
4-Hydroxy-3-methoxybenzaldehyde	.	300	217	200	+
4-Hydroxy-3-methoxybenzoate	.	76	76	76	−
Succinate	.	45	45	45	−
Acetate	.	50	49	45	−

* These substrates supported growth but accurate estimates of mean generation times were not made.

Table 2. *Molar growth yield of bacterium NCIB 8250 on various substrates*

The basal medium contained $KH_2PO_4 + MgSO_4 + (NH_4)_2SO_4$. A 17 hr nutrient broth culture was used as inoculum (0·2 %, v/v) and the bacteria were grown at 30° for 4 days. Results are the means of at least five experiments.

Substrate	Molar growth yield (μg. dry wt/μmole substrate)
D,L-Mandelate	31·3
L-Mandelate	62·0
D-Mandelate	0
Benzoylformate	60·7
Benzyl alcohol	71·2
Benzaldehyde	62·1
Benzoate	55·1
2-Hydroxybenzoate	53·7
4-Hydroxybenzoate	53·8
4-Hydroxy-3-methoxybenzoate	53·4

gave values up to 2000 min. with 4-hydroxy-3-methoxy-D,L-mandelate, but in fact most of this was a period of acceleration in growth. Some compounds gave signs of depressed growth rates at higher concentrations. In the most extreme case 2-hydroxybenzaldehyde supported growth only at less than 0·5 mM: this type of effect was to some extent dependent on inoculum size. Rates of growth on 3,4-dihydroxy-D,L-mandelate and 4-hydroxy-3-methoxy-D,L-mandelate were variable, but of the same order as the rate with 2-hydroxy-D,L-mandelate. Examination of the residual media after cessation of growth on D,L-mandelate or 4-hydroxy-D,L-mandelate showed an optical rotation corresponding to the presence of the D(−) isomers.

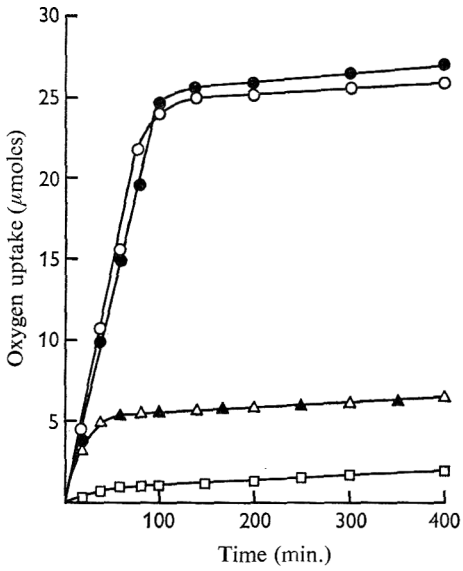

Fig. 1

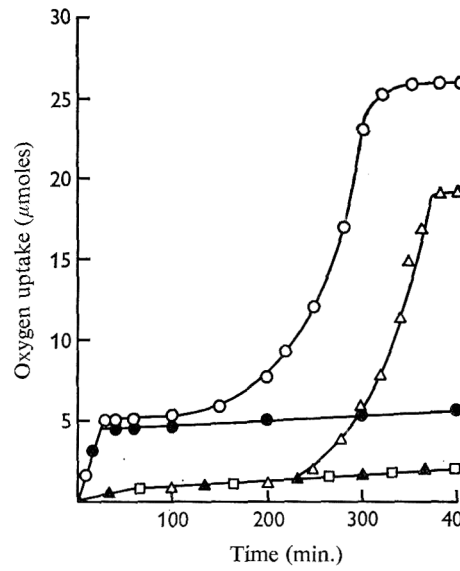

Fig. 2

Fig. 1. Oxygen uptake by a washed suspension of bacterium NCIB 8250 prepared after growth on 4-hydroxy-3-methoxybenzaldehyde. The bacteria were grown, harvested, washed and the rates of oxygen uptake measured manometrically. Each vessel contained 8·0 mg. wet wt bacteria and 3·6 μmoles substrate. ○, 4-Hydroxy-3-methoxybenzyl alcohol; △, 3-hydroxybenzyl alcohol; □, no added substrate; ●, 4-hydroxy-3-methoxybenzyl alcohol + 0·3 mM-puromycin; ▲, 3-hydroxybenzyl alcohol + 0·3 mM-puromycin.

Fig. 2. Oxygen uptake by a washed suspension of bacterium NCIB 8250 prepared after growth on 4-hydroxy-3-methoxybenzaldehyde. The bacteria were grown, harvested, washed and the rates of oxygen uptake measured manometrically. Each vessel contained 8·0 mg. wet wt bacteria and 3·6 μmoles substrate. ○, Benzyl alcohol; △, benzoate; □, no added substrate; ●, benzyl alcohol + 0·3 mM-puromycin; ▲, benzoate + 0·3 mM-puromycin.

For the experiments with washed suspensions the organism was grown on each available compound in turn and harvested in the late exponential phase of growth. The bacteria were washed and their ability to oxidize several substrates tested manometrically in a basal medium similar to that used for growth (Tables 3, 4). In all cases bacteria exposed to the substrate on which they had been grown gave an immediate oxygen uptake showing that the appropriate enzymes had survived the preparation procedure. Care was taken to harvest organisms during the penultimate generation and to test the oxygen uptake within 4 hr of harvesting since organisms grown into the

Table 3. *Patterns of oxygen utilization obtained with various substrates when incubated with washed suspensions of bacterium* NCIB *8250*

Bacterium NCIB 8250 was grown on each compound in turn, harvested, washed and the ability of the bacteria to oxidize the various challenge substrates estimated manometrically. Each vessel contained 8·0 mg. wet wt bacteria and 3·6 μmoles substrate. U, immediate and complete utilization of oxygen; L, lag followed by complete utilization; I-L-U, immediate incomplete oxygen uptake followed after an intermediate lag by complete oxygen utilization; n.t., not tested. The values in parentheses represent the initial rates of oxygen consumption (μmoles O_2/hr/mg.N) with the endogenous rates subtracted.

	Growth substrates											
Challenge substrate	D,L-Mandelate	Benzoylformate	Benzyl alcohol	Benzaldehyde	Benzoate	2-Hydroxy-D,L-mandelate	2-Hydroxybenzoylformate	2-Hydroxybenzyl alcohol	2-Hydroxybenzaldehyde	2-Hydroxybenzoate	Catechol	Succinate
D,L-Mandelate	U (175)	U (120)	L	L	L	U (108)	U (117)	L	L	L	L	L
Benzoylformate	U (172)	U (148)	L	L	L	U (76)	U (86)	L	L	L	L	L
Benzyl alcohol	U (43)	U (112)	U (186)	U (198)	L	n.t.	n.t.	U (194)	U (210)	L	L	L
Benzaldehyde	U (163)	U (150)	U (177)	U (212)	L	n.t.	n.t.	U (145)	U (175)	L	L	L
Benzoate	U (182)	U (134)	U (140)	U (188)	U (198)	U (40)	U (35)	U (145)	U (118)	U (130)	L	L
2-Hydroxy-D,L-mandelate	I (24)-L-U	n.t.	n.t.	n.t.	n.t.	U (50)	U (100)	n.t.	n.t.	n.t.	n.t.	L
2-Hydroxybenzoylformate	I (6)-L-U	n.t.	n.t.	n.t.	n.t.	U (32)	U (65)	n.t.	n.t.	n.t.	n.t.	n.t.
2-Hydroxybenzyl alochol	I (12)-L-U	I (29)-L-U	I (70)-L-U	I (55)-L-U	L	U (52)	U (88)	U (152)	U (116)	L	L	L
2-Hydroxybenzaldehyde	I (6)-L-U	I (11)-L-U	I (23)-L-U	I (31)-L-U	L	n.t.	n.t.	U (124)	U (81)	L	L	L
2-Hydroxybenzoate	L	L	L	L	L	U (43)	U (88)	U (124)	U (80)	U (166)	L	L
4-Hydroxy-D,L-mandelate	I (19)-L-U	I (13)-L-U	L	L	L	I (23)-L-U	I (54)-L-U	L	L	L	L	L
4-Hydroxybenzyl alcohol	I (19)-L-U	I (20)-L-U	I (25)-L-U	I (25)-L-U	L	n.t.	n.t.	I (39)-L-U	I (33)-L-U	L	L	L
4-Hydroxybenzaldehyde	I (3)-L-U	I (5)-L-U	I (7)-L-U	I (9)-L-U	L	n.t.	n.t.	I (17)-L-U	I (12)-L-U	L	L	L
4-Hydroxybenzoate	L	L	L	L	L	n.t.	n.t.	L	L	L	L	L
3,4-Dihydroxy-D,L-mandelate	I (25)-L-U	I (28)-L-U	L	n.t.	L	n.t.	I (54)-L-U	L	n.t.	L	L	L
3,4-Dihydroxybenzaldehyde	I (7)-L-U	I (3)-L-U	I (9)-L-U	I (18)-L-U	L	n.t.	n.t.	I (91)-L-U	I (14)-L-U	L	L	L
3,4-Dihydroxybenzoate	L	L	L	L	L	n.t.	n.t.	L	L	L	L	L
4-Hydroxy-3-methoxy-D,L-mandelate	I (15)-L-U	I (3)-L-U	L	L	L	n.t.	I (42)-L-U	L	L	L	L	L
4-Hydroxy-3-methoxybenzyl alcohol	I (9)-L-U	I (11)-L-U	I (18)-L-U	I (15)-L-U	L	n.t.	n.t.	I (19)-L-U	I (28)-L-U	L	L	L
4-Hydroxy-3-methoxybenzaldehyde	I (4)-L-U	I (2)-L-U	I (19)-L-U	I (9)-L-U	L	n.t.	n.t.	I (15)-L-U	I (16)-L-U	L	L	L
4-Hydroxy-3-methoxybenzoate	L	L	L	L	L	n.t.	n.t.	L	L	L	L	L
Catechol	U (174)	U (150)	U (160)	U (200)	U (195)	U (153)	U (282)	U (170)	U (150)	U (170)	U (205)	L
β-Oxoadipate	U (170)	U (100)	U (95)	U (105)	U (100)	U (59)	U (161)	U (160)	U (140)	U (170)	U (110)	L

Table 4. *Patterns of oxygen utilization obtained with a number of challenge substrates when incubated with washed suspensions of bacterium* NCIB *8250*

Bacterium NCIB 8250 was grown on each compound in turn, harvested, washed and the ability of the bacteria to oxidize various substrates estimated manometrically. Each vessel contained 8·0 mg. wet wt bacteria and 3·6 μmoles substrate. U, immediate and complete utilization of oxygen; L, lag followed by complete utilization; I-L-U, immediate incomplete oxygen uptake followed after an intermediate lag by complete oxygen utilization; n.t., not tested. The values in parentheses represent the initial rates of oxygen consumption (μmoles O_2/hr/mg.N.) with the endogenous rates subtracted.

Challenge substrate	Growth substrates: 4-Hydroxy-D,L-mandelate	4-Hydroxybenzyl alcohol	4-Hydroxybenzaldehyde	4-Hydroxybenzoate	3,4-Dihydroxy-D,L-mandelate	3,4-Dihydroxybenzaldehyde	3,4-Dihydroxybenzoate	4-Hydroxy-3-methoxy-D,L-mandelate	4-Hydroxy-3-methoxybenzyl alcohol	4-Hydroxy-3-methoxybenzaldehyde	4-Hydroxy-3-methoxybenzoate
D,L-Mandelate	I (48)-L-U	L	L	L	n.t.	L	L	I (38)-L-U	L	L	L
Benzoylformate	I (48)-L-U	L	L	L	n.t.	L	L	n.t.	L	L	L
Benzyl alcohol	I (82)-L-U	I (128)-L-U	I (128)-L-U	L	n.t.	I (101)-L-U	L	I (25)-L-U	I (117)-L-U	I (115)-L-U	L
Benzaldehyde	I (40)-L-U	I (34)-L-U	I (43)-L-U	L	n.t.	I (34)-L-U	L	I (48)-L-U	I (48)-L-U	I (43)-L-U	L
Benzoate	L	L	L	L	n.t.	L	L	L	L	L	L
2-Hydroxy-D,L-mandelate	I (40)-L-U	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	L
2-Hydroxybenzoylformate	I (20)-L-U	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2-Hydroxybenzyl alcohol	I (60)-L-U	I (128)-L-U	I (128)-L-U	L	n.t.	I (107)-L-U	L	n.t.	I (73)-L-U	I (95)-L-U	L
2-Hydroxybenzaldehyde	I (17)-L-U	I (34)-L-U	I (43)-L-U	L	n.t.	I (34)-L-U	L	n.t.	I (39)-L-U	I (33)-L-U	L
2-Hydroxybenzoate	L	L	L	L	n.t.	L	L	n.t.	L	L	L
4-Hydroxy-D,L-mandelate	U (187)	L	L	L	n.t.	L	L	I (31)-L-U	L	L	L
4-Hydroxybenzyl alcohol	U (187)	U (185)	U (210)	L	n.t.	I (60)-L-U	L	n.t.	I (125)-L-U	I (30)-L-U	L
4-Hydroxybenzaldehyde	U (152)	U (185)	U (210)	L	n.t.	I (19)-L-U	L	n.t.	I (108)-L-U	I (13)-L-U	L
4-Hydroxybenzoate	U (187)	U (185)	U (210)	U (180)	n.t.	L	L	n.t.	L	L	L
3,4-Dihydroxy-D,L-mandelate	U (93)	n.t.	n.t.	n.t.	U (34)	n.t.	n.t.	U (46)	n.t.	n.t.	n.t
3,4-Dihydroxybenzaldehyde	U (126)	U (147)	U (220)	L	U (34)	U (160)	L	U (130)	U (116)	U (185)	L
3,4-Dihydroxybenzoate	U (203)	U (147)	U (255)	U (196)	U (99)	U (160)	U (174)	U (194)	U (194)	U (54)	U (172)
4-Hydroxy-3-methoxy-D,L-mandelate	I (14)-L-U	L	L	L	n.t.	n.t.	n.t.	U (46)	L	L	n.t.
4-Hydroxy-3-methoxybenzyl alcohol	I (31)-L-U	I (62)-L-U	I (100)-L-U	L	I (13)-L-U	L	L	U (130)	U (116)	U (120)	L
4-Hydroxy-3-methoxybenzaldehyde	I (14)-L-U	I (23)-L-U	I (25)-L-U	L	I (7)-L-U	L	L	U (114)	U (100)	U (120)	L
4-Hydroxy-3-methoxybenzoate	L	L	L	L	L	L	L	U (96)	U (100)	U (148)	U (185)
Catechol	L	L	L	L	n.t.	L	L	n.t.	L	L	L
β-Oxoadipate	U (95)	U (130)	U (140)	U (120)	n.t.	U (180)	U (110)	n.t.	U (155)	U (200)	U (105)

stationary phase or stored (even at 4°) showed less reproducible results; the benzoate-oxidizing system especially appeared to be lost quite rapidly.

The types of oxygen uptake recorded in Tables 3 and 4 and Fig. 1 and 2 show three patterns. First, as exemplified by 4-hydroxy-3-methoxybenzyl alcohol in Fig. 1, an immediate and rapid uptake which came to completion at about 70% of the oxygen consumption required for complete oxidation of substrate. Presumably the balance of 30% represented oxidative assimilation (Clifton, 1946) since the presence of 0·5 mM-2,4-dinitrophenol increased the stoichiometry of oxygen uptake to 92%. Another uncoupling agent, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (Heytler & Prichard, 1962), had no effect at concentrations from 1 mμM to 0·1 μM; higher concentrations merely decreased the rate of oxidation. Presumably this compound did not penetrate the bacteria. The second pattern (as shown by benzoate in Fig. 2) was a lag followed by an increasing rate of oxygen uptake which again came to completion at about 70% of theoretical. The lag varied from a few minutes to several hours and presumably corresponded to *de novo* protein synthesis since the induced oxygen uptake was abolished by 0·3 mM-puromycin (Fig. 2). Chloramphenicol (20 μM) had the same effect, but actinomycin D (0·1 to 1 μg./ml.) was inactive, again probably because of failure to reach the active site. 2,4-Dinitrophenol (0·5 mM) increased the length of the lag. The third pattern of oxygen consumption was an immediate but limited uptake followed after an intermediate lag by complete oxygen utilization (e.g. benzyl alcohol in Fig. 2). The plateau corresponded to either 1μ atom or 1 μmole oxygen/μmole substrate and was always the amount of oxygen required for oxidation of the substrate to the benzoate level. The second part of the curve was inhibited by puromycin (Fig. 2) or chloramphenicol. The length of the plateau varied with the substrate and was generally longest for bacteria grown with a hydroxy-substituted compound and then exposed to the non-substituted analogue. Results with a few substrates (e.g. benzaldehyde, 2-hydroxybenzaldehyde) were sometimes more difficult to interpret because they distilled out of the side-arm before tipping. In all cases, however, the use of chloramphenicol allowed an unambiguous assignment of pattern of oxygen utilization, although the apparent stoichiometry was low. Values for oxygen uptake were corrected for the small amount of respiration in the absence of added substrate (about 6 μmoles O_2/mg. N/hr). The substrate(s) for the endogenous respiration is not known: preliminary experiments (Fewson, unpublished work) have failed to detect glycogen or poly-β-hydroxybutyrate.

The results given in Tables 3 and 4 show that bacterium NCIB 8250 grown on mandelate, benzoylformate or their analogues oxidized any compound which was more reduced than benzoate to at least the level of the corresponding benzoate. The oxygen uptake with D,L-mandelate or any substituted D,L-mandelate was only about half that for other substrates with the same number of carbon atoms. That this was due to the inactivity of the D-isomer was confirmed by the fact that L-mandelate was completely oxidized, whereas D-mandelate supported no oxygen uptake above the endogenous level. Bacteria grown with benzyl alcohol, benzaldehyde or their analogues oxidized any benzyl alcohol or benzaldehyde, but not mandelate or benzoylformate, to at least the benzoate level. Oxidation beyond the benzoate stage proceeded without a lag only when the challenge substrate had the same substituents on the benzene ring as had the growth substrate: the exceptions were that bacterium NCIB 8250 grown on any 2-hydroxy-substituted substrate totally oxidized the non-substituted compounds

and the organisms grown on 4-hydroxy- and 4-hydroxy-3-methoxy-substituted compounds immediately and totally oxidized the corresponding 3,4-dihydroxy analogues. Oxidation of the various benzoates by non-adapted bacteria proceeded only after a lag. All the bacteria grown with aromatic compounds immediately oxidized β-oxoadipate. Bacteria grown with succinate did not possess the enzymes required for the oxidation of the aromatic compounds or β-oxoadipate since none of these substances was oxidized until after a period of protein synthesis. In other experiments it was found that acetate was oxidized only after a very short lag by all batches of bacteria except those grown with acetate itself, which gave immediate oxidation. This lag was not changed by puromycin or chloramphenicol but was extended in the presence of 2,4-dinitrophenol, which may suggest that it was due to an ATP requirement for acetate activation or entry.

Table 5. *Oxidation of substrates which do not support growth by washed cell suspensions of bacterium* NCIB *8250*

Bacterium NCIB 8250 was grown on D,L-mandelate as sole source of carbon, harvested and washed. The ability of these bacteria to oxidize various substrates was estimated manometrically. Each vessel contained 8·0 mg. wet wt bacteria and 3·6 μmoles substrate.

(*a*) *Compounds which gave an immediate oxygen uptake corresponding to oxidation beyond the benzoate level:* 4-fluoro-D,L-mandelate, 4-fluorobenzyl alcohol, 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-fluorobenzoate, 3-fluorobenzoate, 4-fluorobenzoate.

(*b*) *Compounds which gave an immediate but limited oxygen uptake which corresponded stoichiometrically to oxidation to the corresponding benzoate:* 4-bromo-D,L-mandelate, 4-chloro-D,L-mandelate, 3-hydroxy-D,L-mandelate, 3-hydroxy-4-methoxy-D,L-mandelate, 3-chlorobenzyl alcohol, cinnamyl alcohol, 3,4-dimethoxybenzyl alcohol, 3-hydroxybenzyl alcohol, 3-methoxybenzyl alcohol, 4-methoxybenzyl alcohol, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde, cinnamaldehyde, 3,4-dichlorobenzaldehyde, 2,4-dihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 3-hydroxybenzaldehyde, 2-hydroxy-3-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-5-methoxybenzaldehyde, 3-methoxybenzaldehyde, 4-methoxybenzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde.

(*c*) *Compounds which gave no detectable oxygen utilization above the control:* 2,3,4,5,6-pentafluorobenzyl alcohol, 2-carboxybenzaldehyde, 2,6-dichlorobenzaldehyde, 2,4-dimethoxybenzaldehyde, 2-methoxybenzaldehyde, 2-nitrobenzaldehyde, 2,3,4,5,6-pentafluorobenzaldehyde, 3-aminobenzoate, 4-aminobenzoate, 2-bromobenzoate, 3-bromobenzoate, 4-bromobenzoate, 2-carboxybenzoate, 3-carboxybenzoate, 4-carboxybenzoate, 2-carboxy-3-nitrobenzoate, 2-carboxy-4-nitrobenzoate, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2,4-dichlorobenzoate, 2,6-dichlorobenzoate, 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate, 3,5-dihydroxybenzoate, 3,4-dimethoxybenzoate, 3,5-dinitrobenzoate, 2-hydroxy-4-aminobenzoate, 2-hydroxy-3-nitrobenzoate, 2-hydroxy-5-nitrobenzoate, 4-hydroxy-3-aminobenzoate, 3-hydroxybenzoate, 2-iodobenzoate, 3-iodobenzoate, 4-iodobenzoate, 2-mercaptobenzoate, 2-methoxybenzoate, 3-methoxybenzoate, 4-methoxybenzoate, 2-methylbenzoate, 3-methylbenzoate, 4-methylbenzoate, 2-nitrobenzoate, 3-nitrobenzoate, 4-nitrobenzoate, 3,4,5-trihydroxybenzoate, atrolactate, atropate, *p*-benzoquinone, cinnamate, 2-coumarate, 3-coumarate, 4-coumarate, cyclohexanecarboxylate, cyclohexanol, homocatechol, 2-hydroxycyclohexanone, phenoxyacetate, 2-phenylethanol, 3-phenylpropionate, phenylsuccinate, resorcinol, styrene glycol.

The experiments described in Tables 3 and 4 were all done with substrates which support growth and these results were therefore extended by using as 'challenge' substrates several compounds which do not give growth (Fewson, 1967*a*). Table 5 shows that bacterium NCIB 8250 grown with mandelate oxidized a number of fluorine-substituted compounds beyond the benzoate level. These bacteria metabolized a number of other substituted mandelates, benzyl alcohols and benzaldehydes with an oxygen uptake corresponding to the formation of the appropriate benzoate. The

exceptions were pentafluorobenzyl alcohol and a few benzaldehydes with large substituents at the 2-position which were not oxidized at an appreciable rate. None of the compounds tested showed an induced oxygen uptake, even after prolonged incubation. Similar results were obtained with bacteria grown on 2-hydroxybenzyl alcohol except that in this case none of the substituted mandelates was oxidised. Growth with 3,4-dihydroxybenzaldehyde or 4-hydroxy-3-methoxybenzyl alcohol gave the same results as were obtained by growth with 2-hydroxybenzyl alcohol, with the exception of the fluoro-compounds which were oxidized only to the fluorobenzoate level.

Table 6. *Stoichiometry of oxygen uptake by washed cell suspensions of bacterium NCIB 8250 when incubated with 3-hydroxy-substituted compounds*

Bacterium NCIB 8250 was grown with some of the aromatic compounds listed in Tables 3 and 4, harvested and washed. The final oxygen uptake obtained when the bacteria were incubated with 3-hydroxy-substituted compounds was measured manometrically. Each vessel contained 8·0 mg. wet wt bacteria and 3·6 μmoles substrate. Mean values ± S.E.M. are given with the number of determinations in parentheses. Values are corrected for the endogenous oxygen uptake.

Substrate	μmoles O_2/μmole substrate
3-Hydroxy-D,L-mandelate	0·49 ± 0·04 (4)
3-Hydroxybenzyl alcohol	0·98 ± 0·02 (12)
3-Hydroxybenzaldehyde	0·51 ± 0·02 (12)
3-Hydroxybenzoate	−0·03 ± 0·01 (17)

The partial oxidation of the 3-hydroxy-substituted compounds was examined in more detail. An example of the type of oxygen uptake observed is shown in Fig. 1. The stoichiometry of the reaction is given in Table 6 and corresponds closely to the oxidation of 3-hydroxy-L-mandelate, 3-hydroxybenzyl alcohol and 3-hydroxybenzaldehyde to 3-hydroxybenzoate. The formation of 3-hydroxybenzoate was confirmed as follows. Bacterium NCIB 8250 was grown into late exponential phase in 2 l. basal medium containing 10 mM-D,L-mandelate as carbon source. The bacteria were harvested by centrifugation, washed once with sterile basal medium and then resuspended in 2 l. sterile basal medium containing 2 g. 3-hydroxy-D,L-mandelate. The flask was stirred vigorously at 30° for 12 hr, 40 ml. 5 N-HCl then added and the bacteria removed by centrifugation. The supernatant fluid was extracted five times with 200 ml. quantities of ether. The aqueous residue had an ultraviolet absorption spectrum corresponding to 3-hydroxymandelic acid (extinction maximum at 274 mμ with a shoulder at 278 mμ, minimum at 240 mμ in 0·1 N-HCl; extinction maximum at 293 mμ and minimum at 264 mμ in 0·1 N-NaOH). The extinction at 274 mμ in 0·1 N-HCl (extinction coefficient of $1{\cdot}82 \cdot 10^6$ cm.^{2} mole^{-1}) corresponded to exactly half the amount of 3-hydroxy-D,L-mandelate originally present. The solution was laevorotary and presumably contained the residual 3-hydroxy-D-mandelate. The bulked ether extracts were washed twice with 100 ml. quantities of 0·1 N-HCl and evaporated to dryness at room temperature. The residue was dissolved in water and had an ultraviolet absorption spectrum corresponding to that of 3-hydroxybenzoate (absorption maxima: 296 mμ in 0·1 N-HCl, 314 mμ in 0·1 N-NaOH) except for a shoulder extending to about 350 mμ. Chromatographic examination as described in Methods revealed the presence of small amounts (less than 5%) of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid. These were removed by five recrystallizations from hot water. The final material (240 mg.)

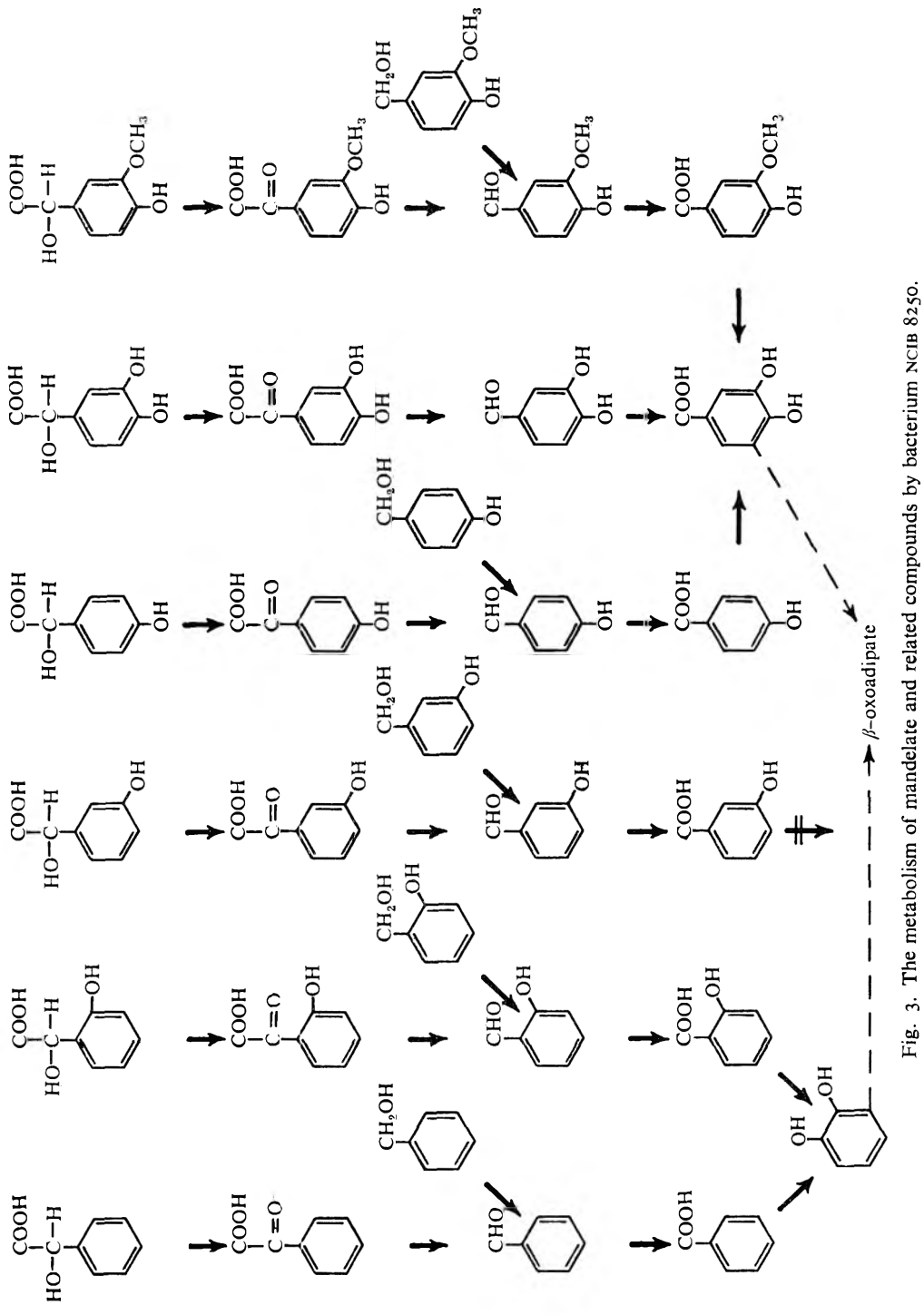

Fig. 3. The metabolism of mandelate and related compounds by bacterium NCIB 8250.

had an ultraviolet absorption spectrum identical with that of 3-hydroxybenzoic acid and m.p. 202–204°, unchanged by admixture with an authentic sample of 3-hydroxybenzoic acid.

DISCUSSION

The results reported in this paper suggest that bacterium NCIB 8250 oxidizes mandelate and related compounds by the converging pathway shown in Fig. 3. 4-Hydroxybenzoylformate, 3,4-dihydroxybenzoylformate and 4-hydroxy-3-methoxybenzoylformate are included by analogy with benzoylformate and 2-hydroxybenzoylformate. The inducible enzymes required for the ring cleavage of catechol and 3,4-dihydroxybenzoate have been found in this organism (Kennedy & Fewson, 1968). The aliphatic products presumably undergo rearrangement to give, ultimately, β-oxoadipate (e.g. Cánovas *et al.* 1967) which is oxidized by bacteria grown with aromatic compounds (Tables 3 and 4). Bacterium NCIB 8250 differs in this respect from *Moraxella calcoacetica* (strain 73) which is impermeable to β-oxoadipate (Cánovas *et al.* 1967). The system of oxidation of the non-substituted and 4-hydroxy-substituted compounds is the same as that in *Pseudomonas putida* (Stanier, 1947; Gunter, 1953) except that bacterium NCIB 8250 does not appear to possess the enzyme mandelate racemase (EC 5.1.2.2) since the organism can neither oxidize nor grow on D-mandelate. Stanier (1950) suggested that *P. putida* oxidized benzyl alcohol through benzaldehyde and benzoate, but no subsequent work appears to have been done on this branch of the pathway. There seems no reason to doubt that benzyl alcohol is oxidized in this way by bacterium NCIB 8250, although some workers (Claus & Walker, 1964; Hegeman, 1966*a*), working with a Pseudomonas species, have suggested that it is metabolized through some other unspecified pathway. The formal possibility that at least some proportion of mandelate might be metabolized by decarboxylation to benzyl alcohol does not appear to have been ruled out by workers using *P. putida*. A strong argument against this route in bacterium NCIB 8250 is the fact that benzyl alcohol supported a higher molar growth yield than did mandelate (Table 2): this probably means that the oxidation of benzyl alcohol to benzaldehyde yields biologically useful energy, whereas the oxidation of L-mandelate to benzaldehyde does not.

The oxidation of mandelates by bacterium NCIB 8250 grown on the benzoylformates, and the oxidation of the benzyl alcohols by organisms grown on mandelates, benzoylformates or benzaldehydes (Tables 3 and 4) suggests that the enzymes are controlled in coordinate groups. The regulons can apparently be de-repressed by the products as well as by the substrates of the first enzymes of the branches of the pathway. The control of these enzymes is considered more fully elsewhere (Kennedy & Fewson, 1968). Recent work has indicated that during mandelate oxidation by *Pseudomonas putida* also, coordinate groups of enzymes rather than individual enzymes, are induced sequentially (Stanier, Hegeman & Ornston, 1964; Hegeman, 1966*a–c*; Cánovas *et al.* 1967).

The patterns of oxygen uptake (Tables 3 to 5; Fig. 1, 2) suggest that the oxidation of compounds to the corresponding benzoates is carried out by enzymes which are, at least qualitatively, fairly non-specific both in their activity and induction. This is similar to the more restricted situation in *Pseudomonas putida* where the same series of enzymes catalyse the oxidation of mandelate and 4-hydroxymandelate to benzoate and 4-hydroxybenzoate respectively (e.g. Gunter, 1953). Substitution of the aromatic ring does lead to quantitative differences between the analogues as shown by the

variation in rates reported in Tables 3 and 4. This has been confirmed by studies with cell-free preparations (Kennedy & Fewson, 1968). In some cases substitution can completely block activity, for instance a large substituent in the 2-position (e.g. nitro, methoxy or carboxy) prevents oxidation of the benzaldehydes (Table 5). The quantitative differences in growth rates observed with the various substrates (Table 1) are frequently reflected in the rates of oxygen uptake with washed suspensions (Tables 3, 4). Presumably, however, growth rate is a function not only of enzyme activity but also of factors such as induction and permeability. Toxicity is clearly important in some cases.

Metabolism of the benzoates is brought about by specific enzymes which are generally produced only by growth on a homologous substrate. The exception to this appears to be benzoate oxidase which is induced under conditions where only 2-hydroxybenzoate hydroxylase would be expected (Kennedy & Fewson, 1968). This gave rise to the 'double simultaneous adaptation' of cells grown on 2-hydroxy-substituted compounds towards their non-substituted analogues (Table 3). The evidence presented in this paper points to the fact that the enzymes metabolising the various benzoates are much more specific than the enzymes higher up the pathway. This is not surprising since the benzoate enzymes all carry out reactions centred on the aromatic ring itself. Nevertheless, it must be borne in mind that much of the evidence for the benzoate specificity was obtained from manometric experiments in which some types of reaction, e.g. hydration, would not be detected. It is still possible, however, to use the data to make a number of deductions about the specificity of these enzymes. This can be illustrated by the benzoate oxidizing system. Cells possessing benzoate oxidase oxidized only benzoate and the monofluorobenzoates. This presumably reflects the similarities of the van der Waal's radii of the hydrogen and fluorine atoms (Pauling, 1960) since other substitutions, e.g. chloro or bromo, gave compounds which were inactive as substrates. Ali, Callely & Hayes (1962) have also shown that bacterium NCIB 8250 grown on benzoate can oxidize 3- and 4-fluorobenzoate. It must be noted that cells possessing benzoate oxidase did oxidize 3-hydroxybenzoate to the 2,3-dihydroxy and 2,5-dihydroxy derivatives to a very slight extent. Ichihara, Adachi, Hosokawa & Takeda (1962) found that benzoate oxidase from *Pseudomonas aeruginosa* and *Micrococcus ureae* (strain *Et*) had slight activity towards 3-hydroxybenzoate but did not determine the product of the reaction.

Cartwright & Smith (1967), in a paper published after the experimental work described here had been completed, described experiments on the demethylation of 4-hydroxy-3-methoxybenzoate by a Pseudomonas species isolated from coal tar. This organism was sequentially adapted to 4-hydroxybenzoate by growth on 4-hydroxy-3-methoxybenzoate so that these workers could not rule out the possibility that 3,4-dihydroxybenzoate arose by demethoxylation followed by hydroxylation, although other evidence strongly suggested a straightforward demethylation. No such ambiguity exists in bacterium NCIB 8250 since cells grown on 4-hydroxy-3-methoxybenzoate oxidized 4-hydroxybenzoate only after a lag (Table 4).

Attention has been drawn in recent years to the presence in micro-organisms of multi-enzyme sequences induced by, and active on, a range of related substrates. Examples of this are the enzymes involved in the degradation of camphor and related compounds (Gunsalus, Chapman & Kuo, 1965), perillyl alcohol (Balial, Bhattacharyya & Rangachari, 1966) as well as mandelate and 4-hydroxymandelate (Gunter,

1953; Stevenson & Mandelstam, 1965). The observations recorded in this paper have extended these findings to a quite different organism and to a larger number of compounds. In bacterium NCIB 8250 there is a considerable degree of economy of protein synthesis since just four enzymes mediate the conversion of almost twenty compounds to five benzoates. These key intermediates are subjected to a series of manipulations resulting in the convergent formation of catechol or 3,4-dihydroxybenzoate which then undergo ring cleavage with the subsequent degradation of the aliphatic products. This type of non-specificity has obvious advantages, especially in soil where a particular environment may well contain, for instance, a number of different analogues of benzaldehyde all formed by the degradation of lignin (see, for example, Stevenson, 1964). The possible ecological significance of partial degradations, such as the oxidation of 3-hydroxymandelate to 3-hydroxybenzoate should not be overlooked. Not only might this type of transformation be capable of providing energy, even if not serving as a carbon supply, but the product might be acted upon by other organisms.

We are grateful to Professor J. N. Davidson, F.R.S., for making available the facilities of this department and to Dr W. H. Holms for the use of apparatus purchased with a grant from the Royal Society. Mrs I. Gall has provided skilled technical assistance throughout this work.

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J. gen. Microbiol. (1968), **53**, 275–285
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Location and Activity of the Respiratory Enzymes of Baker's Yeast and Brewer's Bottom Yeast Grown under Anaerobic and Aerobic Conditions

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(*Accepted for publication* 10 *April* 1968)

SUMMARY

The activity of the electron-transport enzymes of baker's yeast or brewer's bottom yeast, grown under anaerobic conditions, was very low. When anaerobic baker's yeast was cultured aerobically to the mid-exponential phase with limited carbon source, the activity of the electron-transport enzymes increased 3- to 10-fold and, correspondingly, the activity in the stationary phase rose 10- to 50-fold. For brewer's bottom yeast the increase of activity induced by oxygen in the aerobic stationary phase was only about 3- to 4-fold and the activity was clearly lower than that of baker's yeast. The activity of the electron-transport enzymes accumulated in the 10,000 *g* sediment, which under aerobic conditions contained 60–80% of the total activity; the $NADPH_2$ oxidase system formed an exception. The activity of the enzymes of the citric acid cycle also increased under aerobic conditions but only 2- to 10-fold in baker's yeast of the aerobic stationary phase; in brewer's bottom yeast the increase during oxygen adaptation was proportionally greater. The bulk of the enzymes of the citric acid cycle were found in the postmitochondrial supernatant, while the 10,000 *g* sediment contained 20 to 40% of the total activity.

The 10,000 *g* sediment of anaerobically grown baker's yeast contained mitochondrial precursors, while the 10,000 *g* sediment from the aerobic exponential phase contained mitochondria with a more developed structure, showing a respiratory control ratio of 1·4–1·7 with several substrates. The internal structure of the mitochondria was not completely developed until the aerobic stationary phase, where the uptake of oxygen with several substrates also increased many fold.

INTRODUCTION

The existence of mitochondria in yeast cells has been clearly proved by electron microscopy (e.g. Vitols, North & Linnane, 1961) and by isolating mitochondria from yeast cells disrupted by mechanical means (e.g. Vitols & Linnane, 1961) or by enzymic methods (Duell, Inoue & Utter, 1964; Ohnishi, Kawaguchi & Hagihara, 1966). It is found that yeast grown anaerobically on glucose loses its capacity for oxidation (Tustanoff & Bartley, 1962). In the presence of oxygen and at a low glucose concentration respiration again takes place. Electron microscopy has revealed that besides the development of respiration, changes appear in the cell structure of the yeast, such as the formation of mitochondrial precursors and their further development to mitochondria (Yotsuyanagi, 1962; Wallace & Linnane, 1964; Polakis, Bartley & Meek, 1964). Alterations in respiratory particles and in various characteristic enzymic activities on release from glucose repression have been studied by Jayaraman, Cotman,

Mahler & Sharp (1966). We have investigated the activity of the electron-transport enzymes and the enzymes of the citric acid cycle in baker's yeast and brewer's bottom yeast grown under anaerobic and aerobic conditions, as well as their location in the yeast cell, using for cell fractionation the spheroplast method (Nurminen, Oura & Suomalainen, 1965; Suomalainen, Nurminen & Oura, 1967), by which it is possible to isolate subcellular particles in a much less damaged condition than when prepared by methods of mechanical disruption.

METHODS

Growth of yeast. Anaerobically cultured baker's yeast, stage R_3, produced at the Rajamäki Factories of the Finnish State Alcohol Monopoly and brewer's bottom yeast obtained from the brewery Sinebrychoff Ltd., Helsinki, were used as seed yeasts (0·3 g./l. of baker's yeast or 0·6 g./l. of brewer's bottom yeast). The growths were made in White medium (1954) at 30°. When the availability of the carbon source limited the growth, baker's yeast reached the mid-exponential phase after 10 hr, the yield being 6 g./l. under anaerobic and 10 g./l. under aerobic conditions. For the experiments on the aerobic stationary phase, baker's yeast was grown for 20 hr, the yield being 29 g./l. Brewer's bottom yeast took 17 hr to the exponential phase with a yield of 5 g./l., and 2 days to the aerobic stationary phase with a yield of 27 g./l. In aerobic growth 1 l. of air per minute and per litre was forced through the medium, and the anaerobic conditions during growth were obtained by passing a stream of oxygen-free nitrogen from which the traces of oxygen had been removed by alkaline pyrogallol through the growth medium. After growth, the yeast was centrifuged and washed several times with cold water, and used immediately for preparation of spheroplasts. From the anaerobic yeasts the spheroplasts were prepared by using the shortest possible time of digestion in a tightly closed vessel in order to avoid adaptation to respiration.

Preparation of spheroplasts. The yeast cells were suspended to a 10% suspension in a digestion medium containing 1·0 M-sorbitol, 0·01 M-$MgSO_4$, 0·01 M-tris-HCl, pH 7·5. 'Suc digestif d'*Helix pomatia*' (L'Industrie Biologique Française, Gennevilliers, Seine, France) was centrifuged at 3000 *g* for 15 min. and 5% (v/v) of this enzyme solution was added to the suspension, which was then incubated at 30° for the time reported. For yeast cells in the aerobic stationary phase 2-mercaptoethanol treatment was used before digestion. In this procedure the yeast was suspended to 10% in the digestion medium, 0·01 M-2-mercaptoethanol was added and the suspension incubated at room temperature for 30 min., after which the yeast was washed by centrifugation 3 times with cold digestion medium, and the digestion proceeded as above. The formation of spheroplasts was studied by phase-contrast microscopy and by measuring the extinction (Nurminen *et al.* 1965) as well as by determining, in connexion with fractionation, the amount of protein released into the 1000 *g* supernatant. Thus, the anaerobic and aerobic log.-phase yeast cells were digested for 60 and 90 minutes respectively and the yeast in the aerobic stationary phase, after pretreatment with 2-mercaptoethanol, for 3 hr. Instead of true protoplasts, spheroplasts, i.e. osmotically sensitive, digested cells with some cell wall remaining, were partly used in order to protect the inside of the cell against the digestion liquid.

Fractionation. The spheroplasts were centrifuged at 1000 *g* for 10 min. and washed twice by centrifugation with cold digestion medium. The fractionation was essentially

accomplished to the method of Duell *et al.* (1964). The spheroplasts were suspended in a hypotonic medium (medium A), containing 0·25 M-sucrose, 0·02 M-potassium phosphate buffer (pH 6·8) and 0·001 M-EDTA and, thus, lysis of the spheroplasts was caused by the osmotic shock. The suspension was homogenized for 30 sec. in a Potter–Elvehjem tissue homogenizer. The remaining whole cells, the nuclei and cell debris were removed by centrifugation at 1000 *g* for 10 min. It was possible to increase the yield in the 1000 *g* supernatant by repeated suspension of the 1000 *g* sediment in medium A, followed by slight homogenizing and centrifuging at 1000 *g* and, in some cases, this method was used. In order to prepare the mitochondrial fraction or the corresponding anaerobic sediment, the 1000 *g* supernatant was centrifuged at 10,000 *g* for 10 min. The 10,000 *g* sediment obtained was also washed by centrifugation, usually twice, by suspending in a medium, containing 20 % sucrose, 0·02 M-potassium phosphate buffer (pH 6·8) and 0·001 M-EDTA. When necessary on the basis of phase-contrast microscopy, the debris of the 1000 *g* sediment was removed by repeating the 1000 *g* centrifugation. The washings of sediments were combined with the corresponding supernatants.

Analytical methods. The protein was determined by the biuret method (Racusen & Johnstone, 1961) with crystalline serum albumin as reference. The dry matter of the yeast was determined gravimetrically.

Determination of the enzyme activities. The fractions isolated for enzyme determinations were kept overnight in 1 to 2 ml. lots (about 20 mg. protein per ml.) at $-20°$ and only thawed once. However, cytochrome *c* oxidase and succinate cytochrome *c* reductase were measured immediately after fractionation, since their activity was found to decrease during storage. The enzyme activities of the seed yeast were determined in the homogenate obtained by shaking in a Mickle disintegrator with Ballotini beads for 30 min. at 4°.

The activity of cytochrome *c* oxidase was measured by observing the rate of enzymatic oxidation for cytochrome *c* (Cooperstein & Lazarow, 1951; Yonetani & Ray, 1965) reduced by the method of Chantrenne (1955). $NADH_2$ cytochrome *c* reductase and succinate cytochrome *c* reductase were determined by registering the reduction of cytochrome *c* in a system where cytochrome *c* oxidase was inhibited with cyanide (Green & Ziegler, 1963). The $NADH_2$ oxidase system was examined by the method of Green & Ziegler (1963) and the $NADPH_2$ oxidase system correspondingly, with $NADPH_2$ as substrate. The activities of the primary $NADH_2$ dehydrogenase and succinate dehydrogenase were determined by using potassium ferricyanide as electron acceptor in the presence of cyanide (Rabinowitz & DeBernard, 1957). NAD-malic dehydrogenase was determined according to Ochoa (1955), NADP- and NAD-isocitrate dehydrogenase according to Kornberg (1955*a*, *b*), and fumarase (Racker, 1950; Massey, 1955) and aconitase (Racker, 1950; Anfinsen, 1955) according to Racker. The enzyme activities were determined spectrophotometrically at 25° by following the changes of extinction at 15-second intervals. The references were measured without the sample or without the substrate.

Measurement of oxygen consumption. The uptake of oxygen in some substrates was measured polarographically at 25°, using vibrating platinum electrode (Oxygraph, Gilson Medical Electronics, Middleton, Wisconsin, U.S.A.). The reaction medium contained 250 mM-sucrose, 20 mM-potassium phosphate buffer (pH 6·8), 1 mM-EDTA and 10 mM-KCl. Succinate, α-ketoglutarate, isocitrate, citrate, ethanol or lactate was

used as substrate in a concentration of 10 mM; for pyruvate and malate the concentration was 5 mM, and for $NADH_2$ 0·5 mM. The mitochondrial concentration in the assay was 0·14 to 1·25 mg. protein per ml. of reaction mixture on a total volume of 2·5 ml.

Electron microscopy. After fixation with osmium tetroxide the samples were dehydrated with successive alcohol concentrations and propylene oxide before being embedded in epoxy resin. Electron micrographs of thin sections were taken with a Siemens Elmiskop I or a Philips EM 200 electron microscope.

RESULTS AND DISCUSSION

Electron-transport enzymes. The specific activities of the electron-transport enzymes in homogenates and subcellular fractions of anaerobically and aerobically grown baker's yeast and brewer's bottom yeast are presented in Table 1. The activity of the electron-transport enzymes is very low under anaerobic conditions in both baker's yeast and brewer's bottom yeast. When baker's yeast had grown aerobically under conditions when the carbon source was limited until the middle exponential phase, the activity of the electron-transport enzymes in the cell homogenates had increased 3- to 10-fold, and reached its maximum, 10- to 50-fold, in the aerobic stationary phase. A similar, although clearly lower, oxygen-induced increase of activity was observed in brewer's bottom yeast, being in the aerobic stationary phase only 3- to 4-fold.

$NADH_2$ cytochrome *c* reductase was usually more active than succinate cytochrome *c* reductase. The same phenomenon was observed with the corresponding primary dehydrogenases when potassium ferricyanide was used as electron acceptor.

The activity of $NADPH_2$ oxidase in aerobic log.-phase cells of baker's yeast was only a fifth of that of $NADH_2$ oxidase, and the activities also differed in other ways. The $NADPH_2$ oxidase activity was essentially the same in baker's yeast grown to the exponential phase under both anaerobic and aerobic conditions and only 10 to 18% of the total activity was found in the 10,000 *g* sediment. According to Schatz & Klima (1964), the $NADPH_2$ oxidase system in yeast is associated with the microsomal fraction, but $NADPH_2$ oxidase activity is also found in the mitochondrial fraction (Schuurmans Stekhoven, 1966).

By electron microscopy it has been established that the presence of oxygen is necessary for the synthesis of mitochondria in yeast (Linnane, Vitols & Nowland, 1962; Wallace & Linnane, 1964), at least when glucose is used as carbon source. Studies on the enzyme activities in homogenates and electron micrographs of whole cells have revealed that glucose in high concentration represses the synthesis of respiratory enzymes and the development of mitochondria (Yotsuyanagi, 1962; Polakis, Bartley & Meek, 1964). This is in agreement with the observation that the activity of the electron-transport enzymes increased also in the log.-phase cells during aerobic growth on a medium containing a lowered concentration of glucose (Table 1). The greatest proportional increase occurred in the activity of succinate cytochrome *c* reductase which, according to Schatz (1963), is very sensitive to conditions inhibiting the development of mitochondria and is not to be found in the mitochondrial precursors.

In all the fractionations, the specific activity of the electron-transport enzymes was highest in the 10,000 *g* sediment (Table 1), and depended on the growth conditions in

Table 1. *Specific activities of some electron-transport enzymes in homogenates and subcellular fractions of baker's yeast and brewer's bottom yeast, grown under varying conditions*

	Cytochrome *c* oxidase	Succinate cytochrome *c* reductase	$NADH_2$ cytochrome *c* reductase	$NADH_2$ oxidase
Baker's yeast				
Seed yeast homogenate	0·003*	0·004	0·003	0·012
Anaerobic culture				
Homogenate	0·004	0·001	0·012	0·019
10,000 *g* sediment	0·014	0·005	0·046	0·104
10,000 *g* supernatant	0·000	0·001	0·006	0·011
Aerobic, middle exponential phase†				
Homogenate	0·042	0·005	0·038	0·066
10,000 *g* sediment	0·139	0·022	0·140	0·176
10,000 *g* supernatant	0·010	0·002	0·010	0·024
Aerobic, exponential phase‡				
Homogenate	0·042	0·025	0·054	0·054
10,000 *g* sediment	0·118	0·123	0·650	0·400
10,000 *g* supernatant	0·026	0·004	0·051	0·055
Aerobic, stationary phase				
Homogenate	0·205	0·028	0·226	0·174
10,000 *g* sediment	1·095	0·245	1·905	1·029
10,000 *g* supernatant	0·111	0·011	0·095	0·097
Brewer's bottom yeast				
Seed yeast homogenate	0·008	0·000	0·005	0·005
Anaerobic culture				
Homogenate	0·023	0·005	0·007	—
10,000 *g* sediment	0·067	0·017	0·011	—
10,000 *g* supernatant	0·002	0·000	0·004	—
Aerobic, exponential phase				
Homogenate	0·044	0·008	0·004	—
10,000 *g* sediment	0·229	0·085	0·008	—
10,000 *g* supernatant	0·028	0·000	0·006	—
Aerobic, stationary phase				
Homogenate	0·078	0·016	0·022	0·037
10,000 *g* sediment	0·100	0·018	0·051	0·051
10,000 *g* supernatant	0·058	0·014	0·021	0·024

* Specific activities expressed as μmoles of transformed substrate or electron acceptor per minute per mg. protein. 5 % (initial conc.) sucrose used as carbon source.

† Actual carbon source concentration about 2 %.

‡ Actual carbon source concentration about 0·6 %.

the same way as in the homogenates. Table 2 shows the distribution of the total activity in aerobically grown baker's yeast cells. The 10,000 *g* sediment contained 70–80 % of the total activity of cytochrome *c* oxidase, succinate cytochrome *c* reductase and $NADH_2$ cytochrome *c* reductase and about 60 % of the total activity of the $NADH_2$ oxidase. The latter could be inhibited by addition of antimycin A to 90 % in the homogenate and completely in the 10,000 *g* sediment of log.-phase cells. Hence 70 % of the $NADH_2$ oxidase activity, which was inhibited by antimycin A, was located in the 10,000 *g* sediment. The electron-transport enzymes investigated are found to be typical of electron-transport particles isolated from yeast by mechanical disintegration (Biggs & Linnane, 1963; Mackler *et al.* 1962; Mahler, Mackler, Grandchamp &

Slonimski, 1964) and thus also of mitochondria. By centrifugation of homogenate of mechanically disrupted baker's yeast cells at 26,300 *g* for 15 min., Schatz, Tuppy & Klima (1963) obtained a sediment from which they have isolated a mitochondrial fraction and which contained 89% of the total activity of $NADH_2$ oxidase and 88% of that of cytochrome *c* oxidase. Duell *et al.* (1964) have reported that the mitochondrial fraction separated from yeast by means of the spheroplast method (at a noticeably lower *g* value, 5000 *g* for 20 min.) contains almost all the cytochrome *c* oxidase activity. The conclusion can be drawn that the danger of damaging the mitochondria is markedly smaller when the spheroplast method is used than with methods of mechanical disintegration. Further, the recovery of the electron-transport enzymes, found in the fractions of the log.-phase cells, corresponded to 90 to 110% of the total activity of the homogenate before fractionation. Thus, the activity of the electron transport enzymes found in the post-mitochondrial supernatant apparently does not originate from mitochondria but from other parts of the cell, perhaps from mitochondrial precursors or other membraneous parts.

Table 2. *Distribution of total activities of some electron-transport enzymes and enzymes of the citric acid cycle in aerobically grown baker's yeast*

	Aerobic baker's yeast							
	Middle exponential phase 10,000 *g*				Stationary phase 10,000 *g*			
	Supernatant		Sediment		Supernatant		Sediment	
	Total activity*	%†	Total activity*	%†	Total activity*	%†	Total activity*	%†
Cytochrome *c* oxidase	5·5	24	17·4	76	12·3	27	32·4	73
Succinate cytochrome *c* reductase	0·7	18	3·1	82	1·6	30	3·7	70
$NADH_2$ cytochrome *c* reductase	5·2	27	13·8	73	12	26	35	74
$NADH_2$ oxidase	12·8	38	20·7	62	12	40	18	60
NAD-malic dehydrogenase	54	70	23	30	233	74	80	26
NADP-isocitrate dehydrogenase	2·6	53	2·3	47	7·0	79	1·9	21
Aconitase	5·8	62	3·6	38	26	76	8	24
Fumarase	9·6	60	6·3	40	—	—	—	—

* Specific activity multiplied with the amount of protein in the fraction.

† Distribution of total activity between the 10,000 *g* supernatant and 10,000 *g* sediment as a percentage of the sum.

Enzymes of the citric acid cycle. The specific activities of the enzymes of the citric acid cycle found in homogenates and fractions of anaerobically and aerobically grown baker's yeast and brewer's bottom yeast are shown in Table 3. The activities of these enzymes were also observed to increase on transfer from anaerobic growth conditions to the aerobic stationary phase, but here the differences relating to the growth conditions were smaller than those occurring in the electron-transport enzymes. Thus, NAD-malic dehydrogenase, NADP-isocitrate dehydrogenase, aconitase and fumarase were of the same order of magnitude in anaerobically as well as in aerobically grown log.-phase cells of baker's yeast. The reduction of the glucose concentration only partly increased the activities in the aerobic exponential phase. The specific activity of NAD-

malic dehydrogenase increased to 10-fold, while NADP-isocitrate dehydrogenase and aconitase rose only to 2-fold in aerobically grown baker's yeast in the stationary phase. In brewer's bottom yeast the corresponding increase of activity was proportionally larger because of the very low activity of the enzymes of the citric acid cycle in anaerobically grown brewer's bottom yeast.

Table 3. *Specific activities of some enzymes of the citric acid cycle in homogenates and subcellular fractions of baker's yeast and brewer's bottom yeast, grown under varying conditions*

	NAD-malic dehydrogenase	NADP-isocitrate dehydrogenase	Aconitase	Fumarase
Baker's yeast				
Seed yeast homogenate	0·167*	0·010	0·035	—
Anaerobic culture				
Homogenate	0·126	0·018	0·134	0·227
10,000 *g* sediment	0·852	0·096	0·140	0·454
10,000 *g* supernatant	0·063	0·013	0·072	0·089
Aerobic, middle exponential phase†				
Homogenate	0·141	0·018	0·078	0·197
10,000 *g* sediment	0·350	0·094	0·234	0·836
10,000 *g* supernatant	0·099	0·007	0·048	0·118
Aerobic, exponential phase‡				
Homogenate	0·994	0·036	0·085	—
10,000 *g* sediment	1·666	0·033	0·200	—
10,000 *g* supernatant	0·853	0·037	0·061	—
Aerobic, stationary phase				
Homogenate	1·424	0·039	0·327	—
10,000 *g* sediment	5·114	0·145	4·728	—
10,000 *g* supernatant	1·126	0·031	0·200	—
Brewer's bottom yeast				
Seed yeast homogenate	0·135	0·006	0·043	—
Anaerobic culture				
Homogenate	0·028	0·008	0·008	—
10,000 *g* sediment	0·033	0·013	0·045	—
Aerobic, exponential phase				
Homogenate	0·027	0·006	0·026	—
10,000 *g* sediment	0·015	0·015	0·050	—
Aerobic, stationary phase				
Homogenate	1·451	0·023	0·066	—
10,000 *g* sediment	2·560	0·042	0·125	—
10,000 *g* supernatant	0·468	0·007	0·014	—

* Specific activities expressed as μmoles of transformed substrate per minute per mg. protein. 5 % (initial conc.) sucrose used as carbon source.
† Actual carbon source concentration about 2 %.
‡ Actual carbon source concentration about 0·6 %.

It is interesting to note the considerable activity of the enzymes of the citric acid cycle in baker's yeast, occurring even under anaerobic growth conditions with low activity of electron-transport enzymes and no developed mitochondria. Similar conclusions have been reached by Jayaraman *et al.* (1966) from studies on the glucose repression. They found that the $NADH_2$ oxidase is subject to strong repression while

mitochondrial malic dehydrogenase appears less sensitive. Polakis *et al.* (1965) suggest as a possible explanation that enzymes of the citric acid cycle are necessary for amino acid and protein synthesis even under conditions where mitochondria and electron-transport enzymes are not needed for energy production because of the abundant energy supply from glycolysis.

NAD-malic dehydrogenase was observed to be the most active of the enzymes of the citric acid cycle, which apparently means participation in the regulatory mechanisms. NAD-isocitrate dehydrogenase was present in baker's yeast in smaller amounts than the NADP-linked enzyme; under anaerobic conditions the specific activity was 0·002 in the homogenate and 0·008 in the 10,000 *g* sediment. The specific activity of the enzymes of the citric acid cycle reached a maximum in the 10,000 *g* sediment and the activity depended on the growth conditions in the same way as in the homogenate. Contrary to the electron-transport enzymes, the bulk of the total activity of the enzymes of the citric acid cycle was found in the post-mitochondrial supernatant, while the 10,000 *g* sediment contained 20–40% (Table 2). Schatz *et al.* (1963) have reported that the sediment obtained by centrifugation of baker's yeast homogenate at 26,300 *g* for 15 min. contained 14% of the total fumarase activity, 13% of the aconitase and 8% of the NAD-malic dehydrogenase activities. A similar result was obtained by us as regards the distribution of the above enzymes in the mitochondrial and cytoplasmic fractions, although the portion of the 10,000 *g* sediment was clearly larger. Duell *et al.* (1964) have isolated the mitochondrial fraction of yeast by means of the spheroplast method. They found that the fraction contained about 26% of the NAD-malic dehydrogenase activity, while the bulk appeared in the post-mitochondrial supernatant, an observation that agrees well with our results. The location of isocitrate dehydrogenase in the yeast cell has not previously been investigated. In assays on animal cells it has been found that the soluble fraction contained 80% of the NADP-linked enzyme, while the mitochondrial fraction contains only 12% (Hogeboom & Schneider, 1950). In yeast, NADP-isocitrate dehydrogenase was likewise found mainly in the cytoplasmic fraction (Table 2). The enzymes of the citric acid cycle and the electron-transport enzymes had a different intracellular distribution and their activities depended on the growth conditions in different ways. The enzymes of the citric acid cycle have a synthetic function beside their role in energy production and are for the most part soluble. The synthesis of these enzymes is not as sensitive to glucose repression and oxygen induction as is the synthesis of the enzymes bound in the lipoprotein structures of mitochondria.

Oxidative properties and structure of the particles of the 10,000 *g sediment.* Particularly interesting are the properties of the particles of the 10,000 *g* sediment obtained from log.-phase yeast cells grown aerobically under glucose repression. The oxidative properties of the 10,000 *g* sediments prepared from middle log.-phase cells and from cells harvested during the stationary phase were compared (Table 4). As was previously observed in enzyme determinations, the uptake of oxygen when $NADH_2$ was used as substrate was markedly higher than when succinate was used. With $NADH_2$ the uptake of oxygen in the 10,000 *g* sediment obtained from the stationary phase was about 10-fold as compared with the uptake in the exponential phase. With succinate as substrate the corresponding increase was about 8-fold. The exogenous $NADH_2$ cannot permeate into the animal mitochondria if these are intact, while the yeast mitochondria are able to rapidly oxidize $NADH_2$, with a clear respiratory control

(Ohnishi *et al.* 1966). Even on ethanol or lactate as substrate, the uptake of oxygen in the stationary phase rose about 3-fold. However, it is to be noted that in several substrates the particles obtained at the exponential phase already had a respiratory control ratio of 1·4 to 1·7.

Table 4. *Oxidative properties of the 10,000 g sediment isolated from baker's yeast at different aerobic growth phases*

	Aerobic baker's yeast		
	Middle exponential phase 10,000 *g* sediment		Stationary phase 10,000 *g* sediment
Substrate	Oxidative rate*	RCR†	Oxidative rate
$NADH_2$	0·041	1·4	0·400
Succinate	0·008	1·4	0·062
Pyruvate + malate	0·034	1·4	0·040
Citrate	0·039	1·5	0·050
Isocitrate	0·013	1·7	0·014
α-Ketoglutarate	0·016	1·7	0·034
Lactate	0·064‡	1·4	0·208
Ethanol	0·033	1·0	0·101

* μatoms oxygen/min./mg. protein.

† Respiratory control ratio, rate with ADP/rate without ADP.

‡ When 5 μg./ml. antimycin A was added, the oxidative rate was 0·047. On the other hand, antimycin A completely inhibited oxygen uptake with $NADH_2$, succinate, pyruvate + malate or citrate as substrate, whether the sample was taken from the exponential or stationary phase.

As far as the mitochondria synthesis of *Saccharomyces cerevisiae* is known on the basis of electron micrographs Yotsuyanagi, 1962 and Wallace & Linnane, 1964 showed that the 10,000 *g* sediment isolated from baker's yeast cells by the spheroplast method contained mitochondrial precursors of different stages when the yeast had been grown under anaerobic conditions. At the aerobic exponential phase the cells contained, in addition to the precursors, further developed mitochondria (Pl. 1, fig. 1). The mitochondria were not completely developed until the cells had reached the aerobic stationary phase. So the structure of the isolated particles correlates with what has been observed as regards the activities of the respiratory enzymes during oxygen adaptation under carbon source limited growth.

This work was reported at the 4th meeting of the Federation of European Biochemical Societies, Oslo, 7 July 1967 (Nurminen & Suomalainen, 1967).

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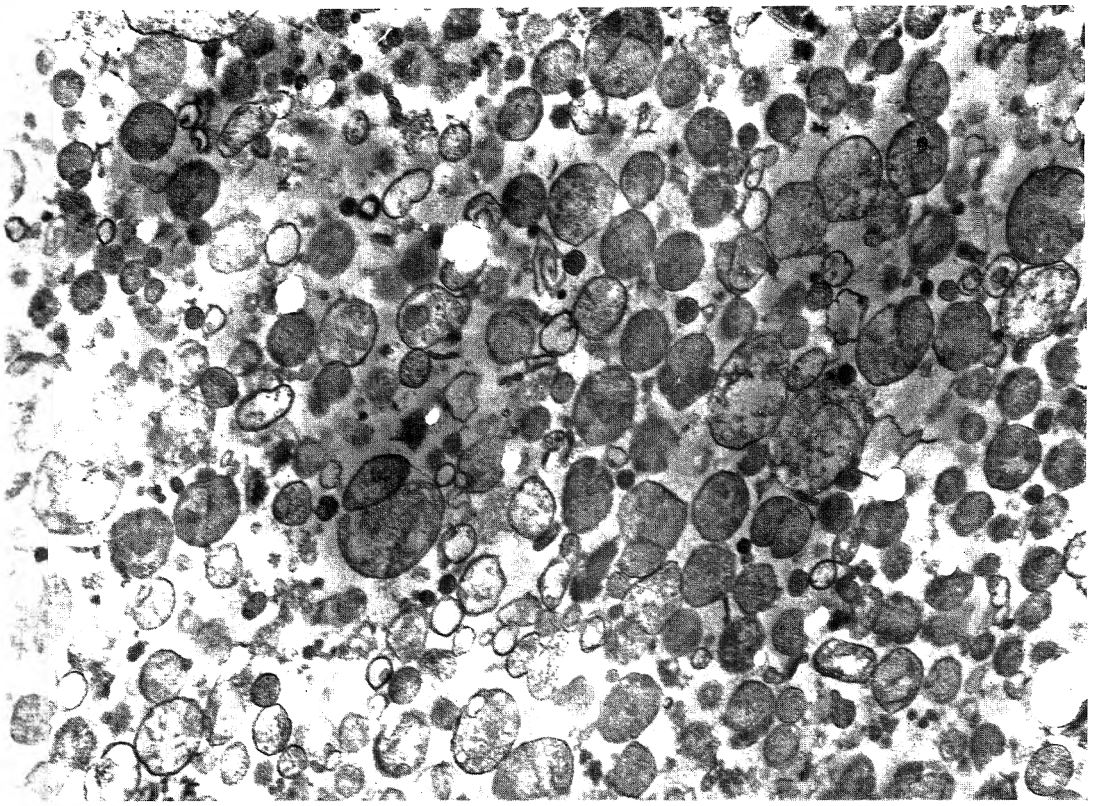

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EXPLANATION OF PLATE

Fig. 1. 10,000 *g* sediment isolated from baker's yeast cells grown aerobically to the mid-exponential phase. × 10,000.

J. gen. Microbiol. (1968), **53**, 287–289
With 2 plates
Printed in Great Britain

Electron Microscopic Observations of Dividing Somatic Nuclei in Saprolegnia

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(*Accepted for publication* 12 *April* 1968)

SUMMARY

An intranuclear spindle of about 40 microtubular fibrils, 200 Å in diameter, convergent to poles close to the inner membrane, with attendant centrioles external to the nuclear envelope, has been identified in sections of synchronously dividing nuclei in vegetative hyphae of *Saprolegnia ferax*.

INTRODUCTION

The mode of division of the somatic nuclei of Saprolegnia species has proved difficult to elucidate with the light microscope and has become the source of considerable argument since Hartog's investigations of 1895, which included *Saprolegnia ferax*, when he reported the duplication and partition of the chromosomes by an essentially mitotic process. Diverse and often inconclusive results have been obtained by subsequent investigators. Recently *S. ferax*, among other species, was examined again by Bakerspiegel (1960) and *S. delica* by Slifkin (1967), the latter including colchicine treatments. Both of these authors suggested that no nuclear spindle was formed and supported the view that the nuclear division is generally amitotic. Centrioles were reported in *S. ferax* by Gay & Greenwood (1966). Further investigations with the electron microscope now show that an intranuclear spindle accompanied by centrioles does exist in the dividing nuclei of *S. ferax*.

METHODS

The strain of *Saprolegnia ferax* (Gruithuisen) Thuret was obtained from vegetative subcultures originating from a single zoospore (Manton, Clarke & Greenwood, 1951). *S. furcata* Maurizia was obtained from Dr M. Dick (Reading University).

Petri dish cultures were made at 25° on a glucose, yeast-extract, peptone and salts agar (1 %) medium on which they remained indefinitely vegetative. Actively growing subcultures were fixed *in situ* with 5 % glutaraldehyde buffered at pH 7·0 with M/15 phosphate followed by similarily buffered 1 % osmic acid, dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were stained with 2 % aqueous uranyl acetate followed by Reynolds lead citrate.

RESULTS

In eight randomly selected young hyphae from the margin of a colony of about 2·5 cm. diam. all the nuclei were found to be in phases of division. This enabled a large number of nuclei to be examined in given states of division.

The spindle was small and at early metaphase, as seen in *Saprolegnia ferax* (Pl. 1, fig. 1), was situated eccentrically within the nuclear envelope which also enclosed a relatively large nucleolus, not included in the spindle but accounting for the greater part of the nuclear diameter at the equator of the mitotic figure.

The spindle consisted of about 40 microtubules of about 200 Å in diameter, spaced separately and fairly widely apart (Pl. 2, fig. 3) though convergent towards the poles. Some of the tubules ran from pole to pole whilst others terminated in the region of the equator on what appeared to be chromosomes (Pl. 1, fig. 1; Pl. 2, fig. 4). The terminations were less well defined than the kinetochores shown by Brinkley & Stubblefield (1966) in hamsters. The spindle tubules terminated abruptly at the poles where they approached close to the inner nuclear membrane but did not penetrate it. At each pole there was at this stage a centriole situated outside the membranes of the nucleus in a well-defined pocket of its envelope. The centrioles (Pl. 2, fig. 5) resembled in structure those described by Berlin & Bowen (1964) in *Albugo candida*; their mode of division is the subject of further work. From the centriolar region microtubules radiated into the cytoplasm and along the outside of the nuclear membranes. Pole to pole microtubules persisted during chromosomal separation and towards the completion of division (Pl. 2, fig. 2), the nucleus including the spindle became elongated. The nuclear envelope appeared to remain intact at all stages of division. Preliminary observations on similarly prepared material of *S. furcata* (not illustrated) showed centrioles and polar intranuclear microtubules comparable to those of *S. ferax* but the metaphase spindle was not observed.

DISCUSSION

The structural organization of the centrioles, microtubules and chromosome-like bodies in different stages of nuclear division of the Saprolegnia species here studied is consistent with the activity of a functional mitotic apparatus. The persistent nuclear envelope and small size of the spindle combined with the low number of widely separated microtubules in the latter and its eccentric position relative to the large nucleolus do not favour its detection with the light microscope. For these reasons Bakerspiegel (1960) and others may have found the critical feature of a spindle impossible to detect in *S. ferax* and other species. However, if a similar situation exists in *S. delica* the results of Slifkin (1967) with colchicine remain unexplained. In view of the present observations reports of amitotic divisions based on the absence of a spindle in Saprolegnia species and allied fungi, where information about their fine structure is lacking, should now be regarded as uncertain.

We would like to thank Dr M. Dick for supplying the culture of *S. furcata*, and Dr J. L. Gay for helpful discussions. I.B.H. held an S.R.C. studentship during the period of this work.

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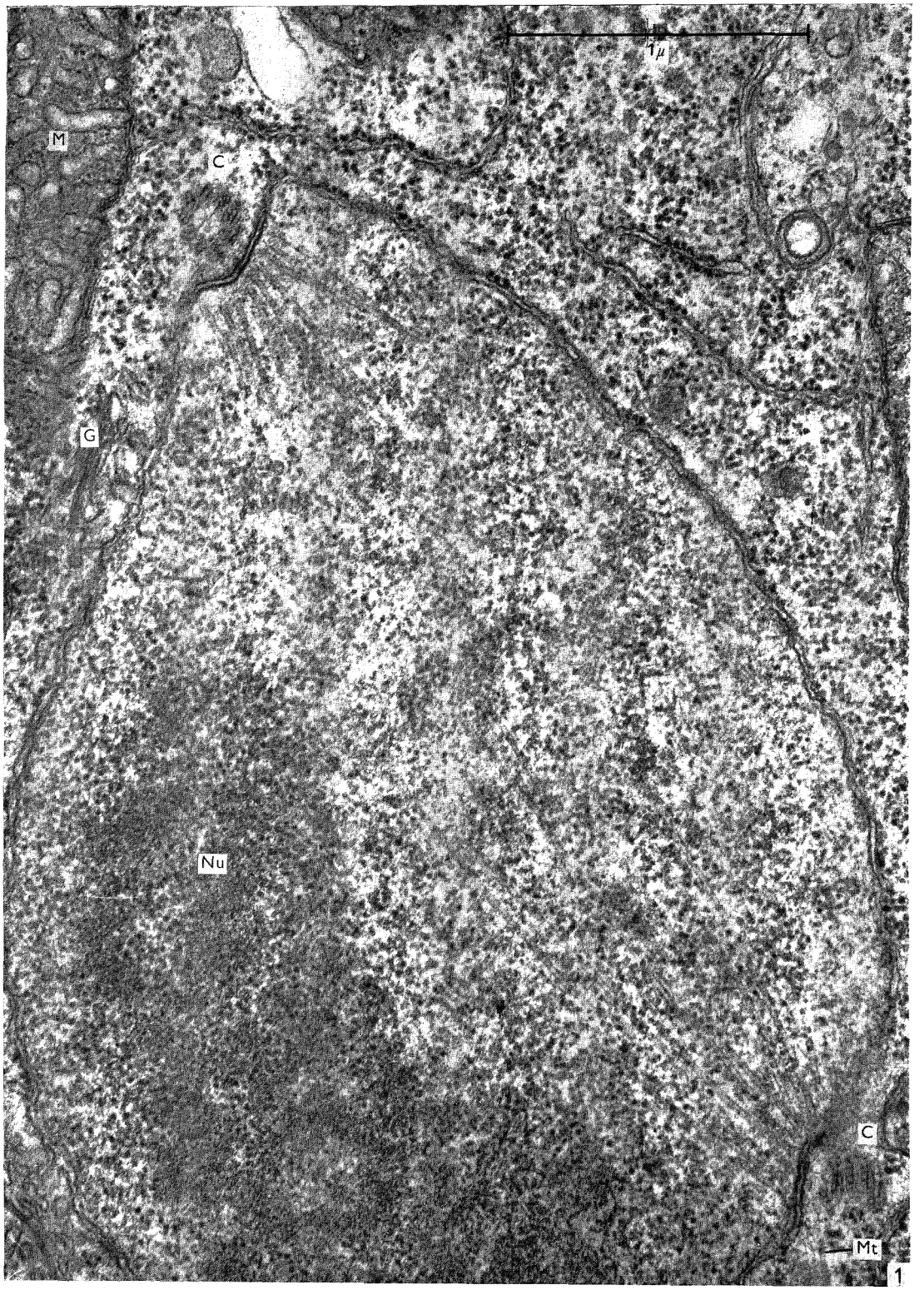
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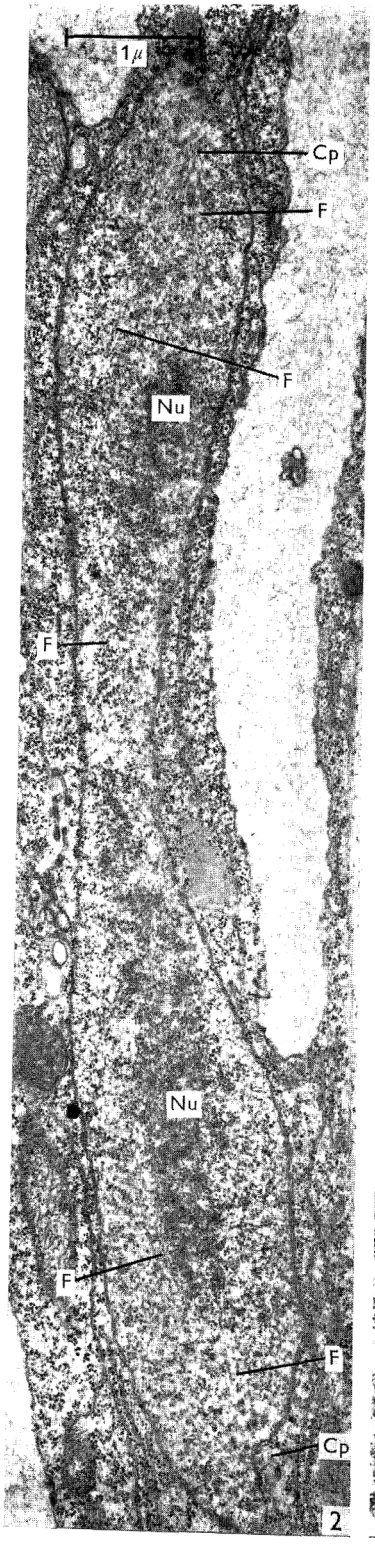
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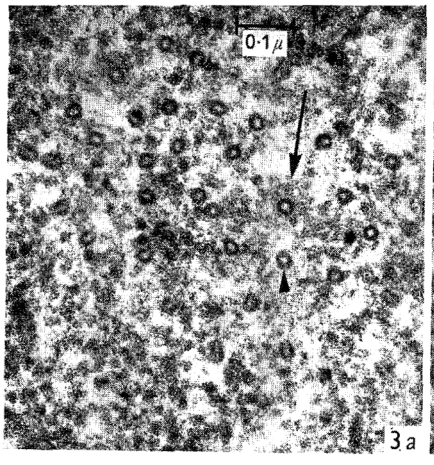
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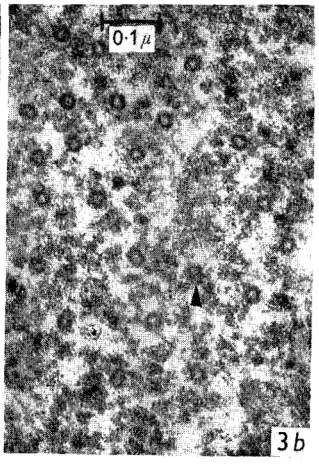
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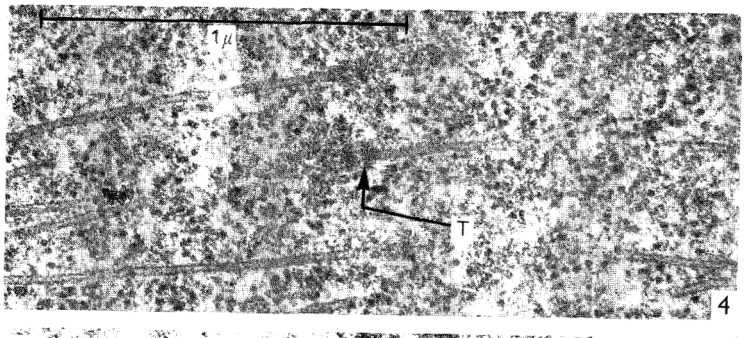
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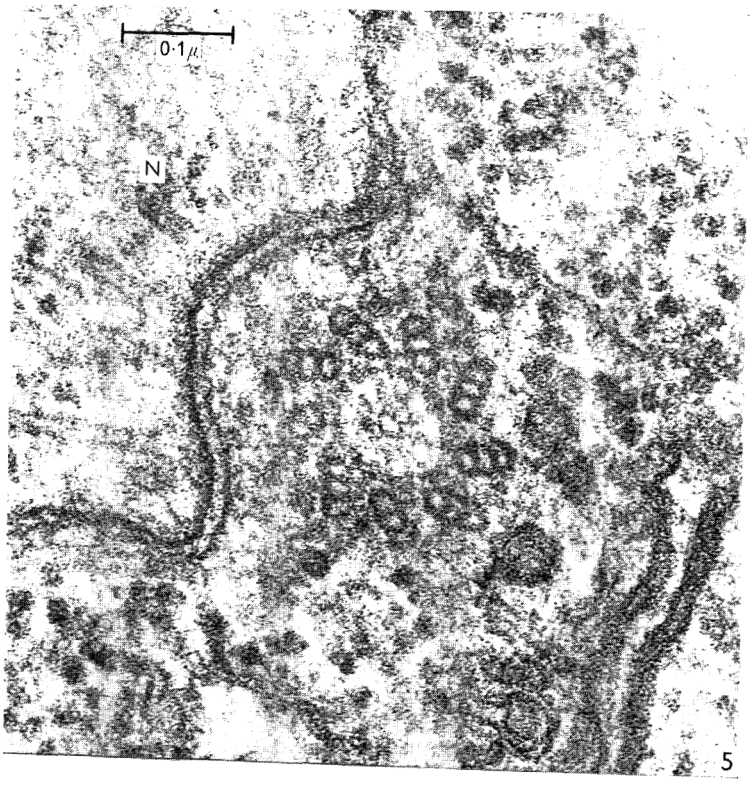
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EXPLANATION OF PLATES

Key to symbols: N = nucleus, Nu = nucleolus, C = centriole, G = Golgi body, M = mitochondrion, C *p* = centriolar position, F = spindle fibre, T = microtubular termination, Mt = cytoplasmic microtubule.

PLATE 1

Fig. 1. *Saprolegnia ferax*. Median longitudinal section through an early metaphase nucleus with eccentrically placed spindle and large nucleolus showing two centrioles (C) and spindle fibres some of which appear to terminate at chromosome like bodies in the equator of the figure. Note cytoplasmic microtubules (Mt). Neg. 8518.*

PLATE 2

Fig. 2. *S. ferax*. Longitudinal section of a telophase nucleus with 2 nucleoli (Nu) showing centriolar positions (C *p*) and spindle fibres (F). Neg. 8772.

Fig. 3. (*a*) *S. ferax*. A transverse section of spindle in the equatoral region showing the microtubular nature of the spindle fibres. Arrowed tubule represents a probable chromosomal termination in T.S. Neg. 8588. (*b*) The next section in the series showing continuity of the majority of tubules. Note absence of the tubule arrowed in (*a*). Neg. 8587.

Fig. 4. *S. ferax*. Longitudinal section of equatorial portion of spindle showing microtubular termination (T) resembling a kinetochore. *Cf.* fig. 3(*a*). Neg. 8516.

Fig. 5. *S. ferax*. Transverse section of a centriole, showing the nine triple fibres and central 'cartwheel', situated in a pocket of the nuclear envelope with polar terminations of the spindle fibres adjacent to its inner surface. Neg. 8592.

* Number of negative in collection of Botany Department, Imperial Collection.