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Purification and Characterization of the Lipase of *Pseudomonas fragi*

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SUMMARY

The lipase of *Pseudomonas fragi* (NRRL B-25) was purified 75- to 100-fold with an overall recovery of *ca.* 20 %.

The enzyme was found to exist in heavy and light forms exhibiting the same position specificity for triglycerides, Michaelis constants (*Km*), and apparent pH and temperature optima. The light form of the enzyme appeared to be present in the heavy one, probably in a complexed state.

The purified lipase was found to hydrolyze only glycerol esters of fatty acids; it required a water-fat interface and exhibited a 1,3-position specificity for triglycerides. The optimum pH for the purified enzyme with purified tributyrin as substrate was calculated to be 8·6 to 8·7 from initial velocity measurements with a pH-stat at 25°. The *Km* for the purified lipase with tributyrin was found to be $0{\cdot}9 \times 10^{-3}$ M at 25° and pH 7·2. Exposure of the purified enzyme to 40° for 10 min. caused a complete loss of activity. A 50 % loss of activity occurred after 10 min. exposure to about 35°. When exposed to pH values ranging from 5·3 to 9·5 for 1 hr at 2° and then assayed at pH 7·0 and 35°, the purified enzyme was found to be stable in the pH range 6·6–7·8.

INTRODUCTION

Although the existence of lipolytic bacteria has been recognized for many years, our understanding of bacterial lipolysis stems for the most part from studies with crude enzyme systems. Apart from the studies with a partially purified lipase (glycerol ester hydrolase, EC 3.1.1.3) of *Mycoplasma gallisepticum* (Rottem & Razin, 1964), and a report on the lipase of *Staphylococcus aureus* (Crenshaw & San Clemente, 1964), purified bacterial lipases have not been fully characterized. Therefore, as part of this laboratory's interest in lipolytic microorganisms, an intensive study of the lipase of *Pseudomonas fragi* was undertaken, not only to enhance our immediate understanding of this enzyme, but also to provide a foundation for future work into its structure, mechanism of action, and biosynthesis. This paper reports the purification and partial characterization of the enzyme.

METHODS

Microorganism and enzyme. Pseudomonas fragi (NRRL B-25) was chosen for this investigation because it produces an extracellular lipase (Mencher, Ng & Alford,

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1965) and because a synthetic medium was available for production of the enzyme (Alford & Pierce, 1963).

Materials. The sources of chemicals and reagents were as follows: tributyrin (Baker grade) from J. T. Baker Chemical Company; crystallized human albumin from Nutritional Biochemicals Corporation; casein (purified) from Difco; Stractan from Stein Hall and Company; Astec 4135 from American Lecithin Company, Inc.; ammonium sulphate from Mallinkrodt Chemical Works; DEAE-Sephadex from Pharmacia; Silica gel G from Research Specialities Company; Chromosorb P from Wilkins Instrument and Research Inc.; chromatographic alumina F-20 from Alcoa Chemicals; disc electrophoresis reagents from Canalco; and methyl esters of fatty acids from the Hormel Institute. Synthetic triglycerides (98 % pure) were kindly supplied by Dr R. G. Jensen of the University of Connecticut, Storrs.

Assays. Lipase activity was assayed by titrimetric measurement of fatty acids released from lard (Alford & Pierce, 1963) or tributyrin, or by a pH-stat determination employing a titrigraph-titrator (Radiometer, Copenhagen). Emulsions were prepared by six passes through a hand homogenizer of 5 % (w/v) tributyrin in water with 0·5 % (w/v) Astec 4135 as the emulsifier. The respective assays are described in full in the appropriate figure legends. A unit of lipase is defined as that amount of enzyme which catalyzes the release of 1 μmole of fatty acid from the substrate in 1 min. at pH 7·0 and 35°.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with human serum albumin as the standard.

Proteinase activity was estimated by McDonald's modification of Anson's technique (McDonald, 1964) with casein as the substrate.

Disc electrophoresis. Disc electrophoresis was performed by the procedure of Ornstein (1964) and Davis (1964) in 7·5 % acrylamide gel with a constant current of 5 mA at 2–3°. Samples (200 μg protein) were subjected to electrophoresis in duplicate; one of the gels was stained with Amido Schwartz and the other was sliced into 3 mm. sections which were pulverized and then assayed for lipase activity. In this way it was possible to relate the bands in the stained gel to lipase activity.

Sucrose density gradient centrifugation. Sucrose gradients were prepared in Beckman cellulose nitrate tubes, 2 × 0·5 in., by layering 1·0 ml. 40 % (w/v) sucrose in 0·02 M-sodium phosphate buffer, pH 6·6, on the bottom, 3·0 ml. 25 % (w/v) sucrose next, and finally 1·0 ml. 10 % (w/v) sucrose on the top. Samples, generally 0·2–0·3 ml., in the phosphate buffer, were layered on the 10 % sucrose. Centrifugation was conducted in a Beckman Model L-2 ultracentrifuge with an SW 39L rotor at 124,000*g* for 17·5 hr at 2–3°. After centrifugation, tubes were pierced at the bottom with a hypodermic needle and 0·5 ml. fractions were collected.

Lipolysis of synthetic triglycerides. Lipolysis of triglycerides was carried out as previously described (Alford, Pierce & Suggs, 1964), except that Stractan rather than Astec was used to emulsify the substrate. Enzyme was added to the reaction mixtures at a concentration sufficient to catalyse the release of 10 mg. or less of fatty acids from the triglyceride in 2 hr at 35°. After incubation, reaction mixtures were extracted with petroleum ether, evaporated to dryness under a stream of nitrogen, and taken up in 1·0 ml. or less of chloroform for subsequent thin-layer chromatography.

Thin-layer chromatography (*t.l.c.*). Lipolysis products (free fatty acids, diglycerides, and monoglycerides) and unreacted triglycerides were separated by thin-layer chroma-

tography as described by Clément, Clément & Bezard (1962) on glass plates, 20 × 20 cm, coated with a 250 μ layer of silica gel G. Samples (5–10 mg., in chloroform) were applied as drops along the origin, 40 mm from the bottom of the plates. Chromatograms were developed with petroleum ether + ethyl ether + acetic acid (90 + 30 + 1 by volume). When the solvent front had migrated 130 mm, chromatography was terminated. Zones were detected with iodine vapour and then scraped from the plates for analysis by gas-liquid chromatography.

Gas-liquid chromatography (g.l.c.). To convert fatty acids to methyl esters for analysis by g.l.c., the method of Kates (1964) was used. To the sample, still adsorbed on silica gel, were added 4·0 ml. of 0·7 N-anhydrous methanolic hydrochloric acid. The mixture was refluxed for 2 hr at 80–100° and then cooled. One ml. water was added, and the mixtures were extracted three times with 3 ml. of petroleum ether. The extracted samples were evaporated to 1·0 ml. or less under a stream of nitrogen for analysis by g.l.c. with an F and M Model 700 gas chromatograph equipped with a flame ionization detector. Samples containing 5–25 μg. of the fatty acid methyl esters were separated on Chromosorb P (45/60 mesh) coated with 20 % diethylene glycol succinate. The column temperature was 185°, and nitrogen was used as the carrier gas.

Production of crude lipase preparations. Pseudomonas fragi was grown in the synthetic medium of Alford & Pierce (1963) for 3 days at 20° under static conditions. The medium was separated from the cells by centrifugation and the supernatant culture fluid, which contained the lipolytic activity, was concentrated by ultrafiltration (Peterson & Sober, 1962). After concentration of the culture supernatant, insoluble material was removed by centrifuging and the supernatant fluid was dialysed against 20 vol. of distilled water for 3 hr and then lyophilized. The yield per litre of culture was 37 mg. of protein and 920 lipase units. The lyophilized material was stable for 4 to 5 months.

Preparation of DEAE-Sephadex for chromatography. DEAE-Sephadex (A-50, medium grade) was allowed to swell in an excess of water and, after fines had been removed by repeated decantations, was washed on a filter with 0·5 N-HCl, followed by water until the rinses were neutral. It was then washed with NaOH (0·5 N), followed by water until rinses were neutral. Next, the DEAE-Sephadex was washed with 0·02 M-NaH_2PO_4 until the pH of the rinse was 6·6, and then with 0·02 M-sodium phosphate buffer, pH 6·6. The anion exchanger was stored at 0–2° as a slurry in the phosphate buffer.

RESULTS

Purification of lipase

Purification was conducted at 0–2°. Crude, lyophilized lipase was dissolved in 0·02 N-sodium phosphate buffer, pH 6·6 (4 mg. protein/ml.) and solid ammonium sulphate was added with gentle stirring to 35% of saturation. The flask was allowed to stand for 15 min. and the material which precipitated was collected by centrifuging and dissolved in the phosphate buffer. The ammonium sulphate-fractionated material (3–5 mg. of protein in 3–4 ml. of buffer) was applied to a 1 × 20 cm. column of DEAE-Sephadex and eluted with buffer; 5 ml. fractions were collected and the flow rates varied from 20–30 ml./hr. After 30 ml. of eluent had passed through the column, a NaCl gradient was applied. The gradient was developed in a closed system with 2 M-NaCl in phosphate buffer in a reservoir leading to the mixing chamber which

contained 1 l. of buffer. In the first fractionations attempted, two peaks of proteolytic activity were eluted prior to elution of the lipase and some of this activity was detected in the lipase peak. To separate the lipolytic from the proteolytic activity, the NaCl gradient was interrupted at fraction 20, and at fraction 40, when all proteinase had eluted from the column, the gradient was reapplied and the lipase eluted. Fractions with high specific activity were pooled. The chromatographic separation is shown in Fig. 1. Table 1 showed that the lipase was purified 100-fold with an 18% yield of activity.

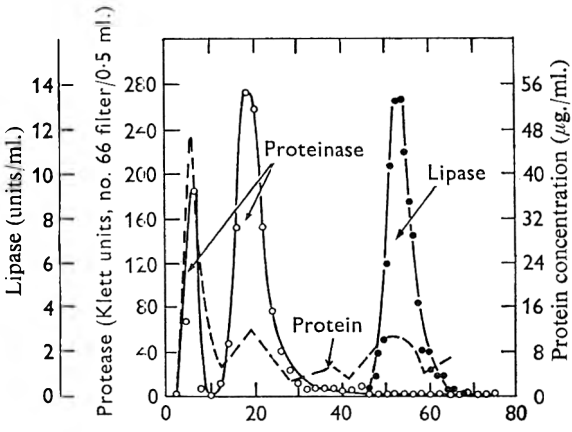

Fig. 1

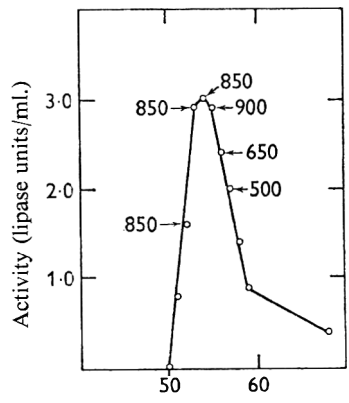

Fig. 2

Fig. 1. DEAE-Sephadex chromatography of *P. fragi* lipase. Chromatography was conducted as described in the text. ○, Proteinase activity; ●, lipase activity; and - - -, protein concentration.

Fig. 2. Specific activities of lipase in fractions from DEAE-Sephadex column. Conditions for chromatography as for Fig. 1, but peak taken from separate purification. Numbers with arrows designate specific activity.

Table 1. *Purification of Pseudomonas fragi lipase*

	Total protein (mg.)	Total lipase activity (units*)	Yield (% of original activity)	Sp. act. (units/mg. protein)	Purification
Culture supernatant	320	2890	100	9	1
Ultrafiltration, dialysis, lyophilization	63	1568	54	25	3
$(NH_4)_2SO_4$-fractionation	3·5	850	30	240	27
Chromatography on DEAE-Sephadex	0·55	510	18	925	103

* A unit of lipase is that amount of enzyme which liberated 1 μmole of fatty acid from a 20% lard suspension in 1 min. at 35°.

Purity of lipase

Specific activity. The specific activities of fractions within the lipase peak suggested homogeneity in the leading portion of the peak with slight contamination in the tail portion (Fig. 2). Figures 1 and 2 represent two separate purifications.

Disc electrophoresis. Purification of the lipase was monitored by disc electrophoresis.

Prior to column chromatography lipolytic activity resided in two faint bands and the bulk of the protein moved with the free dye front in a band which was not lipolytic. However, DEAE-Sephadex chromatography completely eliminated the band at the dye front and the column purified preparation was comprised mainly of the two bands of

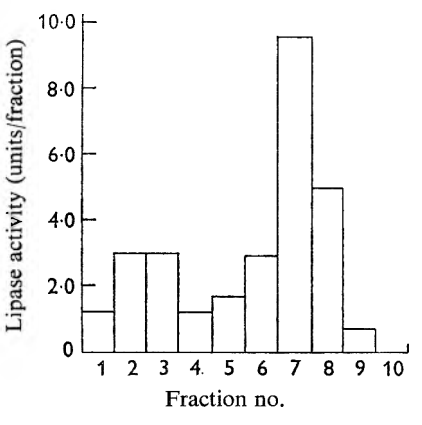

Fig. 3

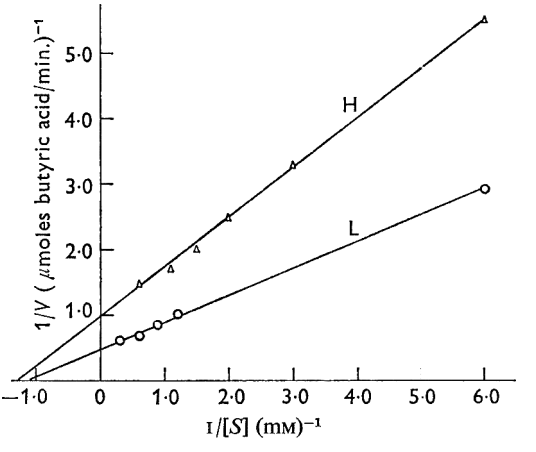

Fig. 4

Fig. 3. Sucrose density gradient centrifugation of ammonium sulphate-fractionated lipase. Procedures are described in the Methods Section. 'Heavy' material was obtained from fractions 2 and 3; 'light' from 6, 7 and 8.

Fig. 4. Double reciprocal plot for determination of K_m for lipases 'H' and 'L' with tributyrin. Initial velocity measurements were obtained with titrigraph-titrator by pH-stat determination (Desnuelle, Constantin & Baldy, 1955). Reaction vessels contained emulsified tributyrin at concentrations ranging from 0·16 to 3·30 mM, enzyme (constant: 'L', 21 μg. protein: 'H', 6 μg. protein) and H_2O to 10 ml. Assays were conducted at pH 7·2 and 25°. K_m was determined from the intercept with the x-axis.

Table 2. *Disc electrophoresis of 'H' and 'L' activities*

Distance from origin (mm.)	Lipase activity* 'H'	'L'	Purified lipase†
0–3	15·1	0·0	14·8
3–6	3·0	0·0	4·8
6–9	1·0	1·2	2·8
9–12	1·1	3·1	15·7
12–15	10·2	6·7	11·4
15–18	0·0	0·0	1·2
18–40	0·0	0·0	0·0

Disc electrophoresis of samples (200 μg. protein) was as described in Methods.

* Lipase activity expressed as ml. of 0·02 N base to neutralize the fatty acids released from lard substrate in 22 hr.

† Lipase purified by DEAE-Sephadex chromatography.

lipolytic activity. However, so little activity remained after electrophoresis that another method of separation was sought in order to ascertain whether these two components represented two lipases, two forms of the same enzyme, or an artifact of electrophoresis.

Density gradient centrifugation. Ammonium sulphate-fractionated lipase was

separated by sucrose density gradient centrifugation into a heavy component designated 'H' and a light component 'L' (Fig, 3). Ammonium sulphate-fractionated material was used rather than column purified lipase because of the excessive manipulations required to obtain sufficient quantities of column purified enzyme for the separation. Although the specific activities of the 'L' and 'H' components were about 235 and 250, respectively, as contrasted with that of about 900 for the column purified lipase, to avoid possible interconversion of the two components, no further purification was attempted. When these components were subjected to disc electrophoresis they were found to correspond to the two components formerly separated by electrophoresis (Table 2). Upon electrophoresis the 'H' component was resolved into two bands, one

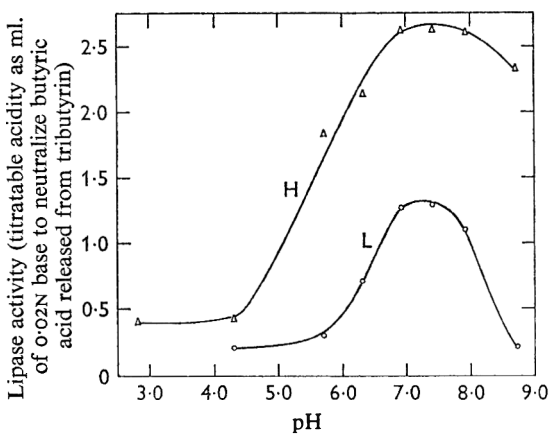

Fig. 5

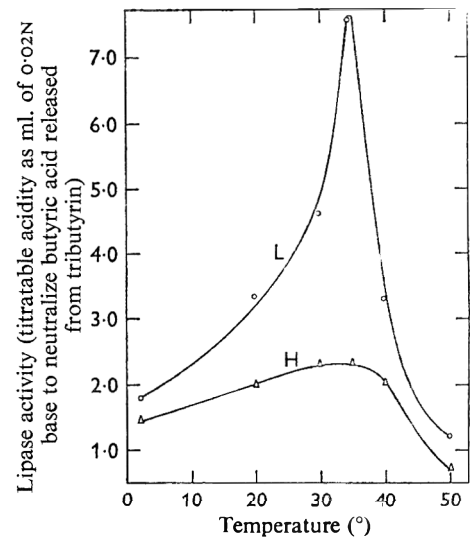

Fig. 6

Fig. 5. Effect of pH on activity of 'H' and 'L' lipases. Reaction mixtures contained 2 ml. of substrate (5%, w/v, tributyrin emulsion), 1 ml. of appropriate buffer (citrate-phosphate for pH 2·8, 4·3, 6·9; phosphate for pH 5·7, 6·3, 6·9, 7·4 and 7·9; tris for pH 8·7), 6 ml. of distilled H_2O, and 1 ml. of enzyme ('H', 4 μg. protein; 'L', 3 μg. protein). Mixtures were incubated at 35° for 20 min., 10 ml. of ethanol were added to them, and they were then titrated to pH 9·5 with 0·02 N-NaOH. Blanks containing water in lieu of enzyme were titrated and these values subtracted from those of the samples. The net values represent ml. of 0·02 N-NaOH required to neutralize the butyric acid released from the substrate.

Fig. 6. Effect of temperature on activity of 'H' and 'L' lipases. Lipase was assayed as described in legend for Fig. 5 at various temperatures at pH 7·0.

of which corresponded to that of the 'L' component, indicating that 'L' was present in the 'H' component. Further indication that the 'H' and 'L' activities represented two forms of the same enzyme was provided by the finding that they had similar *Km* values for tributyrin. The values for the 'H' and 'L' forms were calculated to be 0·8 and 0·9 mM, respectively. Benzonana & Desnuelle (1965), using a Coulter counter, demonstrated that the significant parameter in the determination of *Km* for insoluble substrates is interfacial area per unit volume. However, with the Coulter counter, the smallest particle that can be measured is 0·3 μ in diameter, and because 94 % by weight of the tributyrin emulsions used in our studies consisted of particles smaller than

that (as determined with the Coulter counter), determination of interfacial area with this apparatus was not possible. Nevertheless, repeated determinations of *Km* in terms of molar concentration with different batches of substrate yielded the same value. Had the interfacial area per unit volume varied from one emulsion to the next, this would not have been the case. Thus, the molar concentrations here are an indirect representation of the interfacial area per unit volume and have been reported as such in this paper in the interest of comparison of the 'H','L', and column purified preparations. Double reciprocal plots for the determination of the *Km*'s are shown in Fig. 4. It was also found that both components exhibited a 1,3-position specificity for the triglycerides tested (see below). Finally, an apparent range of pH 7–8 was optimal for both activities (Fig. 5), and the apparent temperature optima appeared to be the same, although the peak for the 'H' form was broad compared to that of the 'L' (Fig. 6).

Table 3. *Substrate specificity of purified Pseudomonas fragi lipase*

Hydrolysis of substrate was determined by pH-stat measurement with titrigraph-titrator at pH 7.0 and 25°. Reaction vessels contained substrate at concentration listed, purified enzyme (3 μg. protein), and H_2O to 10 ml. + Represents any activity above that of blank.

Substrate	Hydrolysis
Methyl butyrate: 90 mg./ml.	–
Methyl palmitate: 5 mg./ml.	–
Methyl oleate: 5 mg./ml.	–
Triacetin: 85 mg./ml.*	–
116 mg./ml.	+
Tripropionin: 2·5 mg./ml.	+
Tributyrin: 2·5 mg./ml.	+
Astec 4135: 2·5 mg./ml.	–
Lecithin (soy): 2·5 mg./ml.	–
Triglycerides of palmitic, oleic, and stearic acids	+
Diolein: 5 mg./ml.	+
Monoolein: 5 mg./ml.	+

* Soluble in water at this concentration.

Characterization of purified lipase

Substrate specificity. The *Commission on Enzymes* (1965) has recommended that enzymes which hydrolyse insoluble glycerol esters of fatty acids be classified as lipases. To test the conformity of the purified *Pseudomonas fragi* lipase to this definition, the following substrates were tested: methyl butyrate, methyl palmitate, methyl oleate, Astec 4135, lecithin (soy), triacetin, tripropionin, diolein, and monoolein (Table 3). To this list can be added the synthetic triglycerides of palmitic, stearic, and oleic acids used in the position specificity studies (see below). Only tri-, di-, and monoglycerides were hydrolysed by the lipase. Triacetin, which has limited solubility in water, was only hydrolysed at concentrations exceeding its solubility.

Position specificity for triglycerides. Crude preparations of *Pseudomonas fragi* exhibit a 1,3-position specificity for triglycerides (Alford, Pierce, & Suggs, 1964). In this respect the enzyme is similar to pancreatic lipase (Desnuelle & Savary, 1963). It was of interest to ascertain whether or not the bacterial enzyme would show a similar specificity after purification by DEAE-Sephadex chromatography or after density gradient fractionation. 2-Oleoyl palmito-stearin (POS), 1-palmitoyl diolein (POO),

2-palmitoyl diolein (OPO), and 2-stearyl-diolein (OSO) were hydrolysed by these enzyme preparations and the free fatty acids analysed. The specificity for the 1-3-position was still displayed by all three purified preparations (Table 4). That palmitic and stearic acids accounted for 6 and 7 % of the fatty acids released from OPO and OSO,

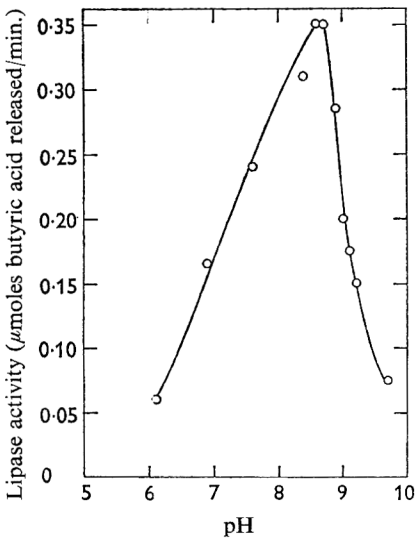

Fig. 7

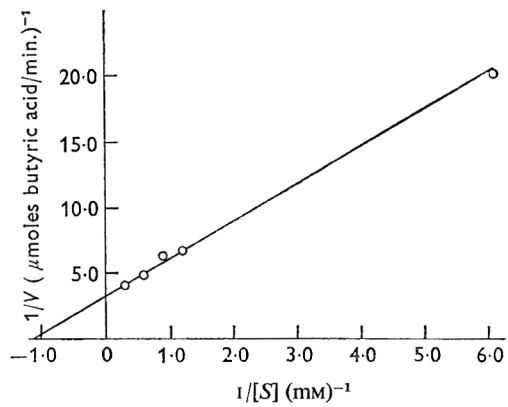

Fig. 8

Fig. 7. The pH optimum of purified *P. fragi* lipase. Initial velocity measurements were obtained with titrigraph-titrimeter by pH-stat determination. Reaction vessels contained 0·5 ml. of 5% tributyrin emulsion, 0·5 ml. purified lipase (6 μg. protein), and H_2O to 10 ml.

Fig. 8. Double reciprocal plot (1/v against 1/[S]) for determination of K_m of purified *P. fragi* lipase for tributyrin. Initial velocities were obtained as described in legend of Fig. 4. Reaction vessels contained emulsified tributyrin at varying concentrations (0·16–3·30 mM), 0·5 ml. of purified lipase (3·0 μg. protein), and distilled H_2O to 10 ml.

Table 4. *Hydrolysis of synthetic triglycerides by 'H', 'L' and (purified) 'P' lipases*

Triglyceride substrate	Weight of fatty acids released (%)								
	Oleic			Stearic			Palmitic		
	'P'	'H'	'L'	'P'	'H'	'L'	'P'	'H'	'L'
POS	2	3	2	49	49	50	49	47	48
POO	56	58	64	0	0	0	44	42	36
OPO	94	97	95	0	0	0	6	3	5
OSO	93	96	96	7	4	4	0	0	0

Enzyme was added in concentration sufficient to catalyse the release of 10 mg. or less of fatty acid from 50 mg. substrate in 2 hr. at 35° (approximately 2 units of enzyme based on assay with lard).

respectively, could be attributed to: (1) actual hydrolysis at the 2-position, (2) the presence of a small amount of triglyceride in which the fatty acid presumed to be in the 2-position was actually in the 1-position, (3) acyl migration, or (4) a combination of these possibilities.

pH optimum. To determine the pH optimum for the purified lipase, reaction mixtures were adjusted to the desired pH values with 0·01 N-HCl or NaOH and then assayed at those values with a pH-stat at 25°. An optimum of pH 8·6–8·7 with a rapid drop in activity above the optimum was found (Fig. 7).

Michaelis constant (K_m). The K_m for the purified lipase was found to be 0·9 mM with tributyrin substrate (Fig. 8).

Stability. Although crude preparations were relatively insensitive to freezing and thawing and to lyophilization, these procedures caused over 50 % inactivation of purified preparations. Further, the half-life of the enzyme decreased markedly with purification. Preparations with a specific activity of 700 displayed a half-life of 5 days at 2°, whereas those with a specific activity of 900 exhibited one of 15 hr.

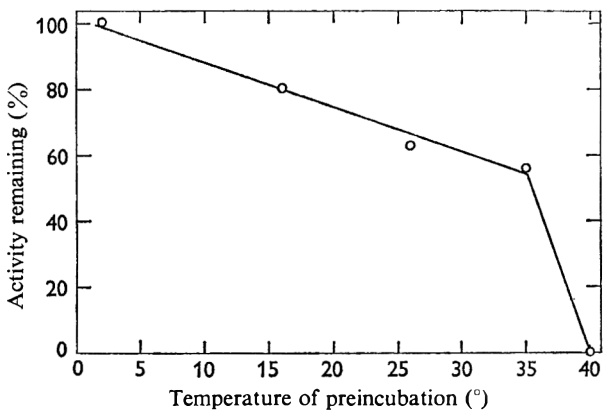

Fig. 9

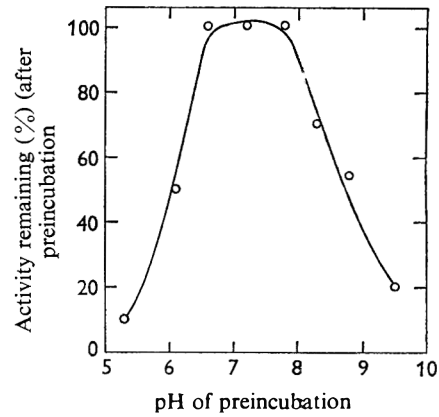

Fig. 10

Fig. 9. Effect of temperature on stability of purified *P. fragi* lipase. Samples of purified lipase (1 ml., 6 μg. protein) were pre-incubated for 10 min. at temperatures ranging from 3° to 40°. Nine ml. of substrate-buffer mixture (prewarmed to assay temperature) were then added and the flasks assayed for lipase activity (Alford & Pierce, 1963).

Fig. 10. Effect of pH on stability of purified lipase. Samples of purified *P. fragi* lipase were adjusted to various values in the range pH 5·4–9·6, preincubated for 1 hr at 2° and then assayed at pH 7·0 and 35° (Alford & Pierce, 1963).

With regard to heat stability, the purified enzyme was completely inactivated in 10 min. at 40°, and a 50 % loss of activity occurred after 10 min. at 35° (Fig. 9).

To determine the effect of pH on stability of the purified lipase, the lipase was preincubated at various pH values for 1 hr at 2° and then assayed at pH 7·0 and 35°. Figure 10 indicates destruction of the enzyme below pH 6·6 and above pH 7·9.

DISCUSSION

The borderline between true lipases and esterases is rather vague. Although the *Commission on Enzymes* (1965) has recommended that a lipase be termed a glycerol-ester hydrolase, reports of carboxylesterase activity in purified lipase preparations from porcine pancreas and fungi can be found in the literature (Desnuelle, 1961; Iwai, Tsujisaka & Fukumoto, 1964; Fukumoto, Iwai & Tsujisaka, 1964) and recently DeHaas, Sarda & Roger (1965) reported hydrolysis at the 1-position of phospholipids

by a highly purified preparation of porcine pancreatic lipase. While the possibility of contamination in the preparations cannot be ruled out altogether, it is quite possible that these enzymes represent lipases of a less specific nature and should be designated by a modified name. The lipase of *Pseudomonas fragi*, on the other hand, displays neither carboxylesterase nor phospholipase activity and thus it can be classified as a true glycerol-ester hydrolase. The requirement by this lipase for a glycerol moiety is of particular interest because it implies that the enzyme must bind to this portion of the substrate molecule in order to achieve a proper alignment of its catalytic groups, as suggested by Koshland (1963). That lecithin is not hydrolysed might be attributed to steric or electrostatic effects of the phosphorylcholine, preventing binding of the enzyme to the molecule or by preventing proper alignment of the catalytic groups. To test this possibility, purified lipase was preincubated with lecithin, and then triolein was added to the mixture. Hydrolysis of triolein proceeded at the same rate as when the enzyme had not been preincubated with lecithin, indicating that binding of lecithin by the enzyme had not occurred.

The pH optimum for activity of *Pseudomonas fragi* lipase has been reported to be pH 7·0 (Nashif & Nelson, 1953; Alford, Pierce & Sulzbacher, 1963). The present determination of pH 8·6–8·7 at first appears to be contradictory. However, these data can be reconciled by observing that the temperatures, substrates and methods used in the respective assays were different. The value of pH 8·6–8·7 was determined from initial velocity measurements, whereas that of pH 7·0 was not. Regarding the use of a pH-stat for determination of this optimum, Desnuelle, Constantin & Baldry (1955), using pancreatic lipase and olive oil, cautioned that such determinations are not valid because the fatty acids released from the substrate dissociate to a different degree depending upon the pH of the mixture. Thus, the observed reaction rate would actually reflect not only the rate of release of fatty acids from the substrate, but also the amount of dissociation of the acids. However, their statement is not applicable if pH $\gg pK_a$ for every species of acid produced. Furthermore, even this restriction is unnecessary if only a single acid is released. Knowing the pK_a, one can calculate the percentage ionization at each value from the Henderson–Hasselbalch equation and correct the initial velocity measurements accordingly. Such was the case with tributyrin.

The resolution of *Pseudomonas fragi* lipase preparations into two activities by electrophoresis and sucrose density gradient centrifugation, although not anticipated, is not strange. References in the literature to multi-molecular forms of proteins are commonplace and such techniques are often used to study these forms (Reithel, 1963). Regarding lipases specifically, Sarda, Maylié, Roger & Desnuelle (1964) reported the elution of a 'slow' and a 'fast' lipase peak during Sephadex chromatography of porcine pancreatic juice. The 'fast' lipase was converted to the 'slow' form by treatment with sodium deoxycholate and this led the investigators to speculate that the 'fast' component was a lipoprotein. Gelotte (1964) has reported the separation of pancreatic lipase into two enzymically active fractions by chromatography on Sephadex at various pH values and ionic strengths and has interpreted his data in terms of monomer and aggregate forms of the enzyme. Shahani & Chandan (1965) have used the analytical ultracentrifuge to study the association of milk lipase with other proteins. Depending upon the protein with which the enzyme was associated the activity was inhibited or enhanced by the formation of the complex. With respect to the lipase from *P. fragi*, the conclusion derived from the data in this investigation is that the enzyme exists in a

form which shall be designated 'L' for light and a heavy form 'L.x'. Disc electrophoresis of the two forms is pictured as follows:

$$\text{'L'} \xrightarrow{\text{Electrophoresis}} \text{'L' (fast-migrating)}$$

$$\text{'L.x'} \xrightarrow{\text{Electrophoresis}} \text{'L' (fast-migrating)} + \text{'x'}$$

and some undissociated 'L.x' (slow-migrating)

Pending structural analysis of the two forms, one can only speculate as to what 'x' is. The lipase could be complexed either with itself (dimerization), another protein, a lipid, or a carbohydrate. Conceivably, it could be complexed with some surface component of the cell. The enzyme was recently shown to be an extracellular one (Mencher *et al.* 1965), and although no significant cell-bound activity was detected in that study, this does not preclude association of the enzyme with the membrane or cell wall in an inactive form prior to its release into the medium. This possibility seems plausible in light of the report that the bound penicillinase of *Bacillus licheniformis* appears to be indistinguishable from the enzyme which is normally released into the medium, except for a difference in molecular weight (Pollock 1965). If the *Pseudomonas fragi* lipase is to be found associated with the cell wall or protoplast membrane in an inactive form, it would either have to be freed artifically in order to be identified by its activity, or be identified by immunochemical techniques or by structural analysis. The purified lipase should lend itself to such a study.

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Long-distance Spore Transport: Methods of Measurement, Vertical Spore Profiles and the Detection of Immigrant Spores

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SUMMARY

Airborne spores can be carried long distances, but little is known about the atmospheric transport processes involved or the rates at which spore clouds are depleted. Aircraft sampling is expensive and inevitably intermittent, and surface traps reveal only some of the processes involved. The best compromise is to combine surface and aircraft observations and to support both with detailed meteorological interpretation. Gravity slide traps exposed for 1 day indicate the arrival of spores less precisely than moving-slide impactors, which therefore provide a more accurate starting time for estimating the past track of spores from air trajectories. Catches of *Puccinia graminis* uredospores from continental European sources illustrated how immigration depends on the movement of atmospheric pressure systems and the gradients within them and suggested that in addition to surface air movement winds at the 700 and 500 mb. levels were important.

Aircraft of the Meteorological Research Flight, using suction impactors which operated approximately isokinetically, sampled air in the lower troposphere, both to ascertain vertical spore profiles over land and to intercept immigrant *Puccinia graminis* uredospores over the English Channel. The vertical distribution of spores seemed to be determined in the same way as that of other aerosol particles; atmospheric turbulence was a major factor and there were indications that wind shear, precipitation and surface deposition might be important. However, most spores are liberated periodically and so encounter different degrees of atmospheric turbulence depending on the diurnal periodicity of their concentration near the ground. Concentrations of 10^4 spores/m.^{3} occurred at heights up to 1000 m. and hundreds/m.^{3} at 3000 m. In unstable air spore concentrations often declined roughly logarithmically with height, but layers of stable air were often associated with abrupt changes of concentration. Details of vertical spore profiles also depended on the history of both the temperature profile and the spore cloud. Such factors tended to affect all spore types similarly: but occasionally some components, e.g. *P. graminis* uredospores, showed unique vertical profiles. One such profile, characterized by preferential 'erosion' of the spore cloud from air near the surface, may indicate travel remote from sources. Spores of plant pathogenic fungi were frequent in samples of air moving northward over the English Channel but their viability was not tested.

INTRODUCTION

Biological pollution of the atmosphere is chiefly by small organisms or propagules which can remain suspended in air, so most reports concern bacteria, insects or the spores of plants. This paper describes work done to study movements of plant pathogenic fungi, but pollen grains and the spores of saprophytic fungi are also mentioned.

The establishment of plant pathogens in areas previously free from them usually provokes discussion about their transport. There is often good reason to attribute introduction to increased travel or trade in plants, but there is also evidence that fungus spores can travel long distances in air, although the frequency, extent, range or direction of such transport cannot yet be predicted. Many factors, both physical and biological, influence the sequence of processes which biologists often include in the term 'dispersal', from the release of spores at one site to their establishment elsewhere. Our observations concern only atmospheric transport, and do not deal with measurements of source strength, spore viability, deposition or establishment. However, because knowledge of these processes is necessary to understand the mechanism of transport, some relevant literature must be mentioned.

Pollen from tall plants can fall directly into turbulent air, but prostrate plants and microfungi often require vectors or special discharge mechanisms to carry their pollen or spores across the thin 'boundary-layers' close to surfaces, where airflow is laminar (Ingold, 1953, 1965; Gregory, 1961; Meredith, 1963; Hirst & Stedman, 1963). Near the ground the concentration of airborne spores fluctuates greatly and rapidly because of changes in weather and in the rate of spore liberation (Hirst, 1953; Gregory & Hirst, 1957; Hamilton, 1959; Sreeramulu, 1959; Kramer, Pady & Wiley, 1963).

Attempts to predict the distance spores travel simply from their settling velocity and wind speed ceased when the role of turbulence in diffusion was accepted. Nevertheless, most spores are deposited closely around their sources. Conservative estimates suggest that in daytime < 1 to 25%, and at night 10–90% of spores (about 12 μ to 32 μ diam.) may be deposited within 40 m. of sources close to the ground (Chamberlain, 1956; Gregory, Longhurst & Sreeramulu, 1961; Sreeramulu & Ramalingam, 1961). In contrast, there is no agreed choice of the best parameters by which to predict the range and depletion of spore clouds, and contrasting theories are claimed to explain observed gradients (Schrödter, 1960; Gregory, 1961).

The 'velocity of spore deposition' (Chamberlain, 1956) from near-ground sources certainly depends on turbulence but seems greatest within a few metres of the source and then becomes constant at rather less than the terminal velocity of the spore (Chamberlain, 1956; Gregory, 1961). Beyond 100 m. from point sources, measurement of airborne spore concentration and deposition becomes difficult unless the source is prolific or a few particles are easy to detect. Thus there are very few measurements of the proportion of spores that escape deposition within 100 m. and so could travel far. However, Gregory (1962) calculated that this 'escape fraction' could commonly be as much as 10%. Many kilometres distant from sources the importance of gravitational settling and eddy-diffusion may change relative to other variables such as precipitation (May, 1958; Hirst, 1959; Gregory, 1961), vertical profiles of wind speed or temperature, and atmospheric pressure systems. Such factors are seldom included in existing dispersal theories but might have important and complex effects during prolonged travel. Until such effects are better understood, measured and included in dispersal theories there is little hope of accurately predicting economically significant transport of plant pathogens over long distances.

To measure how spore concentration varied with height to the limit of detectability, perhaps hundreds or thousands of miles downwind of large sources, as well as across the cloud to measure lateral dispersion, would be a prodigious task. Ideally such measurements should be repeated to show how changes in source strength, wind

direction and turbulence affect dispersal. To collect such complete information may never be feasible, so it is necessary to consider how to understand the principles from simpler measurements. Measurements of spore concentration on the ground and from aircraft can be made complementary, and the value of both is enhanced by detailed meteorological interpretation. Networks of spore traps operated continuously at ground level are not prohibitively expensive and show something of the extent and movement of spore clouds. In contrast, aircraft have a unique ability for sampling vertical or horizontal spore profiles quickly, but their short flight duration and cost prevent continuous sampling.

METHODS

Measurement of spore concentration near the earth's surface

Christensen (1942) and Gregory (1961) reviewed long-distance spore transport. None of the examples quoted has been more valuable to plant pathology than the discovery that epidemics of wheat stem rust in central North America were initiated by *Puccinia graminis tritici* uredospores blown from winter-sown crops in southern states (Stakman & Hamilton, 1939; Stakman & Harrar, 1957; Craigie, 1957). Recent pollen deposits have also yielded exotic grains presumed to have been deposited from the air (King & Kapp, 1963; Hafsten, 1951).

Spore concentrations have been measured on long surface transects by car (Craigie, 1945) or more often by ships (Sreeramulu, 1958; Gregory, 1961; Sack, 1949). Marine observations have the important advantage that it is not necessary to select identifiable, and usually rare, migrant spores from among the whole flora, because all terrestrial propagules must be of distant origin.

No existing spore trap allows the whole airborne flora to be identified, nor does any accurately assess spore deposition on host surfaces. Most of the catches we report came from traps used primarily to study spores as respiratory allergens and sited on buildings up to 30 m. above ground. Horizontal slides were exposed at Cardiff (1947 to 1956) and at the Bishop Rock Lighthouse (1953 to 1961). At Cardiff from April 1954 onwards moving slide impactors (Hirst, 1952) were used and sampled air continuously at 10 l./min., but the slides concerned were not scanned completely for *Puccinia graminis* uredospores until 1957. For the periods described in Figs. 7, 8 and 12 the entire catch was scanned for *P. graminis* uredospores, and this permitted a threshold concentration of 2 spores/m.^{3} air to be detected. For commoner spores only a fraction of the slide was examined, allowing 10–20 spores/m^3. to be detected.

When all types of spores were counted they were assigned to one of 35 categories (Gregory & Hirst, 1957), ranging from 'unclassified', through form groups or broad taxonomic divisions, to a few identified as species. In presenting catches, the total concentration of all spore types has sometimes been used, or smaller categories, such as 'Cladosporium' and 'pollens', selected to illustrate the behaviour of particles of different mass but similar average diurnal periodicity near the ground. Fungus spores are between 2 and 200 μ long (mostly 5 to 30 μ), they have settling velocities between 0·05 and 3·0 cm./sec. with a mode considerably less than 1 cm./sec., and different spores vary greatly in shape and ornamentation. Most pollen grains, like fungus spores, have a specific gravity close to 1·0 (Gregory, 1961) but are usually more nearly spherical, larger and, because of their greater mass, settle at 1 to 40 cm./sec., with a mode at about 3 cm./sec.

Meteorological interpretation

To explain variations in catches of spores from distant sources the past tracks of the air sampled had to be estimated. Movement of air within atmospheric pressure systems is governed by (1) the earth's rotation; (2) the gradients of atmospheric pressure; (3) the forces generated by flow within pressure systems; (4) the friction of air moving over the surface (*Meteorological Office*, 1960). The 'geostrophic trajectories' which we estimated from surface synoptic charts depended mainly on the first two, so strictly the 'surface' track applies to the movement of unretarded air, at 600–900 m., just above the friction layer over lowlands. Aerological charts, showing contours of the altitude of fixed pressure levels, were also used to estimate trajectories at the 700 and 500 mb. levels, roughly equivalent to heights of 3000 and 5600 m. respectively.

Table 1. *Standard vector errors of position* (*in km.*) *after stated periods* (*Hogg, 1961b*)

	6 hr	12 hr	24 hr	2 days	3 days	4 days	5 days
Surface	78	109	156	221	269	312	348
700 mb.	—	222	315	445	545	628	684
500 mb.	—	311	439	623	762	880	984

The process of estimating trajectories incurs several cumulative errors (Murray, 1954; Durst & Davies, 1957); Table 1 shows the radius of the circle around the true position of a parcel of air within which about two-thirds of the geostrophic trajectories would end. The same errors apply to trajectories drawn in the reverse direction, from source to trap, so they affect the time of arrival as well as the direction of travel and it is reasonable to accept that spores may be caught sooner or later than expected. Uncertain air movement in weak pressure gradients, for example in an anticyclone, a ridge or col, can introduce large errors, particularly in 'surface' trajectories. Sometimes the choice lies between paths that eventually result in entirely different gradients for the possible trajectories; see, for example, the trajectory relating 00.01 on 10 July 1959 in Fig. 12. Each surface trajectory was based on a series of synoptic charts, usually at 6 hr intervals. The successive past positions of a parcel of air that had reached a specified target at a given time (e.g. a spore trap when spores were first caught) were estimated by working backwards. The resulting trajectory was the best estimate for the path of the air. Surface trajectories were followed up to 5 days. Upper air trajectories were drawn from 12-hourly aerological charts and, because of the greater wind speed at these levels, the tracking was not usually continued beyond 2 or 3 days.

The vertical distribution of spores is often much affected by atmospheric stability. When the air is unstable, any vertical displacement (upward or downward) is likely to continue. When it is stable, such vertical displacement will not continue and the air will return to its original position. In general terms, therefore, air mixes more when it is unstable, and so has more effect on the distribution of airborne spores.

The stability of the air can be determined from a knowledge of the temperature lapse rate, which is the rate at which the temperature decreases with height. When the lapse rate of the air exceeds the dry adiabatic lapse rate 1° C./100 m. (3°/1000 ft.) it is said to be absolutely unstable; when it is less than the saturated adiabatic lapse rate (about

0·5°/100 m. or 1·5°/1000 ft. at low levels) it is absolutely stable. When it lies between these two it is said to be conditionally unstable, i.e. it is stable when unsaturated but becomes unstable when it is lifted enough to become saturated. On several diagrams, temperature–height diagrams indicate the stability of the air, in so far as it can be determined without a knowledge of its humidity, and the dry and saturated adiabatic lapse rates are given for reference.

Measurement of spore concentrations with aircraft

The first collections of fungus spores high above ground were made with the aid of kites and balloons (see Gregory, 1961); aeroplanes were first used in 1921 (Stakman, Henry, Curran & Christopher, 1923). Most workers have used the air speed to impact spores on sticky obstacles such as microscope slides (Stakman *et al.* 1923), small cylinders (Rempe, 1937), Petri dishes (Dillon Weston, 1929; Polunin & Kelly, 1952) or small square-section rods (Asai, 1960). These, and suction traps (which probably sampled at velocities very different from the forward speed of the aircraft), showed that pollens and fungus spores could be abundant in the lower troposphere and that some of them were alive.

All the collections we report were made by aircraft and crews of the Meteorological Research Flight, Farnborough. The Hastings or Varsity aircraft used carried a modified impactor of the type described by Pasquill (1955; see also Hirst & Hurst, 1967, Pl. 1). Samples were collected through a small circular sampling head, mounted horizontally and coaxial with the end of a long tube, which allowed the head to be projected 0·6 m. clear of the fuselage. This tube was mounted on a transverse carriage so that it could be retracted into the fuselage. The inboard end had a suction outlet, a vacuum gauge and a knob for selecting 120 positions. Each position was separated by 3°, or 1·62 mm., on the sampling drum, which was 6·15 cm. in diam. and 2·4 cm. wide and mounted centrally within the sampling head.

The sampling tube had a circular orifice 0·3 cm. diam. converting to a 1 cm. long slit 0·33 mm. wide, separated from the trapping surface by 0·5 mm. Pressure drop across the orifice was calibrated to give theoretically isokinetic sampling at all heights up to 3000 m. At 160 and 180 knots (1 knot = 1 nautical mile/hr = 1·853 km./hr) these were 152 and 178 mm. Hg, giving airflow rates of 35 and 39 l/min. Spores were impacted on an adhesive surface prepared and mounted as for ground-level traps (Hirst, 1953) except that a strip of transparent plastic film ('Melinex O', a clear polyethylene terephthalate film supplied by Imperial Chemical Industries Ltd., Plastics Division, Welwyn Garden City, Herfordshire., 0·127 mm. thick) was used instead of a glass slide. This film was evenly coated with melted petroleum jelly, containing 10% of paraffin wax (m.p. 54°). After gradual cooling the Melinex strip was wrapped tightly around the sampling drum to which the ends were fixed with double-sided adhesive plastic tape. The drum was then bolted into a tin and sealed to prevent contamination.

Close to the ground, in summer, there may often be several hundred thousand spores/m.^{3} air. Efficient spore traps then catch so many spores that occasional contaminants are seldom important, but at altitudes where spores are rare contaminants matter more. Although the Melinex strips were coated in a room with a filtered air supply, it would be naïve to suppose that contamination was eliminated or did not occur when drums were fitted and removed in the aircraft. Contamination, estimated by 'background counts' on areas where no sample trace was deposited, was rare and

restricted to occasional Cladosporium conidia, so little reliance should be placed in very small catches of these.

Because external air sampling was isokinetic with air speed, intake velocity was fast and even small particles were impacted efficiently by the orifice; traces contained much more fine dust than on the ground traps, which were designed to eliminate this. The dense deposits make the surface less adhesive and are difficult to count. Therefore, the duration of sampling was varied with height; usually 1 min. samples, which at 180 knots represent a core of air 3 mm. diameter and 3 nautical miles (5·56 km.) long, were taken below 1800 m. and 2 min. samples at greater heights. To decrease the detection threshold concentration and to allow sampling errors to be assessed, three consecutive samples were taken as replicates for each height when circling during ascents, or at each 'position' when making horizontal traverses. A single spore in a 1 min. sample represents approximately 30 spores/m.^{3} air, and scanning a triplet of traces provided approximately the same detection threshold as ground traps. Height and speed were usually constant during sampling, so a triplet of samples occupied at least 9 nautical miles of flight. The first sample of a triplet often contained more spores than the others; probably because some spores were impacted in the sampling orifice while flying between sampling positions with the impactor extruded but with suction off. From 1961 this error was avoided by retracting the sampler whenever possible and taking a dummy sample immediately before each triplet to be counted. In presenting results from earlier flights we have omitted the first sample of a triplet wherever there was a long interval between groups of samples.

Special precautions were required to identify the precise areas on which samples of spore-free air were impacted. The Melinex strip was marked in the impactor, at least once every 20 sample-positions, with a trace of Lycopodium powder dispersed in front of the orifice while the pump was switched on momentarily. Exposed Melinex strips were removed from their sealed tins and drums in the laboratory, then laid on a transparent scale above the stage of a microscope. The scale was marked to correspond with the 120 sample-positions, so that with the Lycopodium marker traces at known positions the strip could be cut accurately into lengths and mounted as permanent microscopical preparations (Hirst, 1953). The stage of the microscope used for counting was fitted with adjustable stops, set with the aid of the stage vernier and Lycopodium marker traces, to limit slide travel accurately to the areas where samples were deposited.

The presentation of results

Spore catches from aircraft have usually been expressed as the number deposited per unit area of the exposed sticky surface. Because so little is known of spore deposition and retention on sticky surfaces at aircraft speeds, the results are seldom comparable. Our sampling was volumetric and approximately isokinetic, so it was justifiable to convert the catches to concentrations in air.

Because vertical diffusion of spores depends much on thermal turbulence, easy comparison of the spore concentration and temperature profiles is desirable. Ideally, in the 'International Standard Atmosphere', temperature declines linearly at 6·5° C/kilometre up to 11 km., so it would be convenient if corresponding spore profiles could also be made linear. Johnson & Penman (1951) found that vertical density profiles of aphids up to 600 m. could often be straightened by plotting both height and concentration on log. scales. In Fig. 1*b* this method is compared with the linear scales of

Fig. 1 *a*, using pollen catches by Rempe (1937). In Fig. 1 *b* the bend in the curves above about 900 m. makes them insensitive to changes of concentration at greater heights. We adopted a linear height scale and a log. scale for spore concentration (Fig. 1 *c*), because this provides the best means of showing small changes in concentration and of comparing spore and temperature profiles.

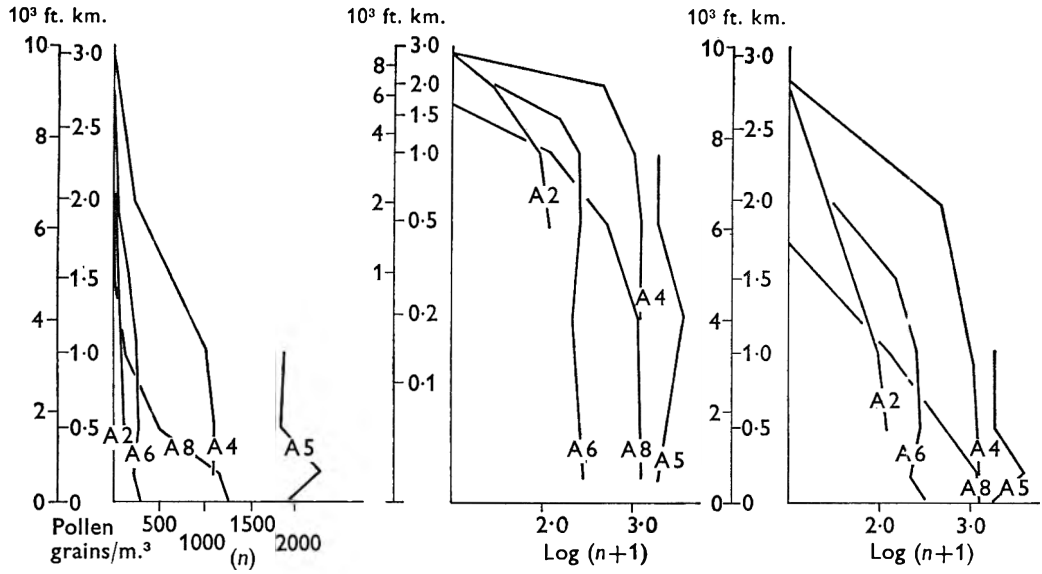

Fig. 1. Vertical pollen profiles measured by Rempe (1937) presented on three scales to illustrate the advantages of (*c*) linear height and log. spore concentration. Explanation in text.

Flight plans

Spore trapping had to be restricted to occasions when aircraft were engaged in other meteorological investigations, and flight plans had to be compatible both with with these and with air-traffic control regulations. Two types of flight are described in this paper: (1) spiral ascents or descents with straight and level portions during sampling; (2) flights, designed to intercept immigrant spore clouds, comprising a cross-wind traverse over the sea, of 100–300 nautical miles at 600 m., beneath civil airways (Fig. 2). (All 'miles' mentioned are nautical miles: 1 nautical mile = 1·85 km. All times are Greenwich Mean Time.) Near the centre of this traverse samples were collected during a spiral ascent from 300 to 3000 m. before resuming the traverse.

RESULTS

Vertical spore profiles

Previous work suggested that spore concentration usually decreased with height, and that this change was accentuated with ascent through temperature inversions; there was also evidence of wash-out or scrubbing by rain (see Gregory, 1961). Rempe (1937) found diurnal changes in the shape of vertical spore profiles. In unstable air during the day, pollens (presumably newly released) became almost uniformly mixed in the

lowest 1000 m. of the air, whereas at night there was some evidence that pollens became stratified according to size, and were most concentrated at a height well above ground. Although it is difficult to accept Rempe's explanation that pollen grains *collect* just above inversions, other workers have also reported a 'biological zone' several hundred metres above ground where spores are most prevalent (see Gregory, 1961).

At first air was sampled during all possible ascents, to improve methods and to test suggestions that temperature lapse rate affects spores in the same way as it affects other particles suspended in air. We could seldom measure ground-level concentrations beneath the ascents but this is less serious than it might seem because ground measurements are usually much affected by local sources of spores.

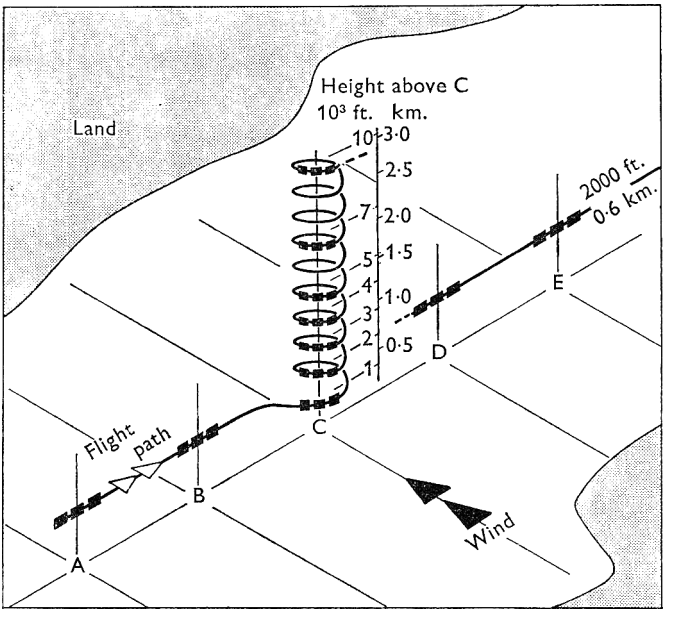

Fig. 2. Isometric diagram to illustrate flight plan used for intercepting immigrant uredospores over the English Channel. Not to scale, each black rectangle indicates an air sample.

Most spores fall slower than they move in eddies and convection, so in considering how temperature profiles are related to changes in concentration it is reasonable first to examine all spores (including pollen). Figure 3 shows profiles for six ascents partly through unstable air, the decrease in spore concentration with height is approximately logarithmic but within 900 m. of the surface spore concentration was often almost uniform, presumably as a result of very active mixing in the friction layer. There was no consistent inverse relation between lapse rates in unstable air and the rate spore concentration decreased, as Johnson (1957) found for aphids, perhaps because the spore profiles were not only fewer, but were measured at different seasons, times of day and over both land and sea. The considerable differences in spore profiles for the ascent and descent of Flight 13/57 are not explained by the rather small differences in temperature profiles. As divergences are large only at heights between stratocumulus cloud at 900 and 2000 m., they may reflect real heterogeneity. This possibility is supported by the closer agreement between ascent and descent profiles in Flights 9/57 and 10/57

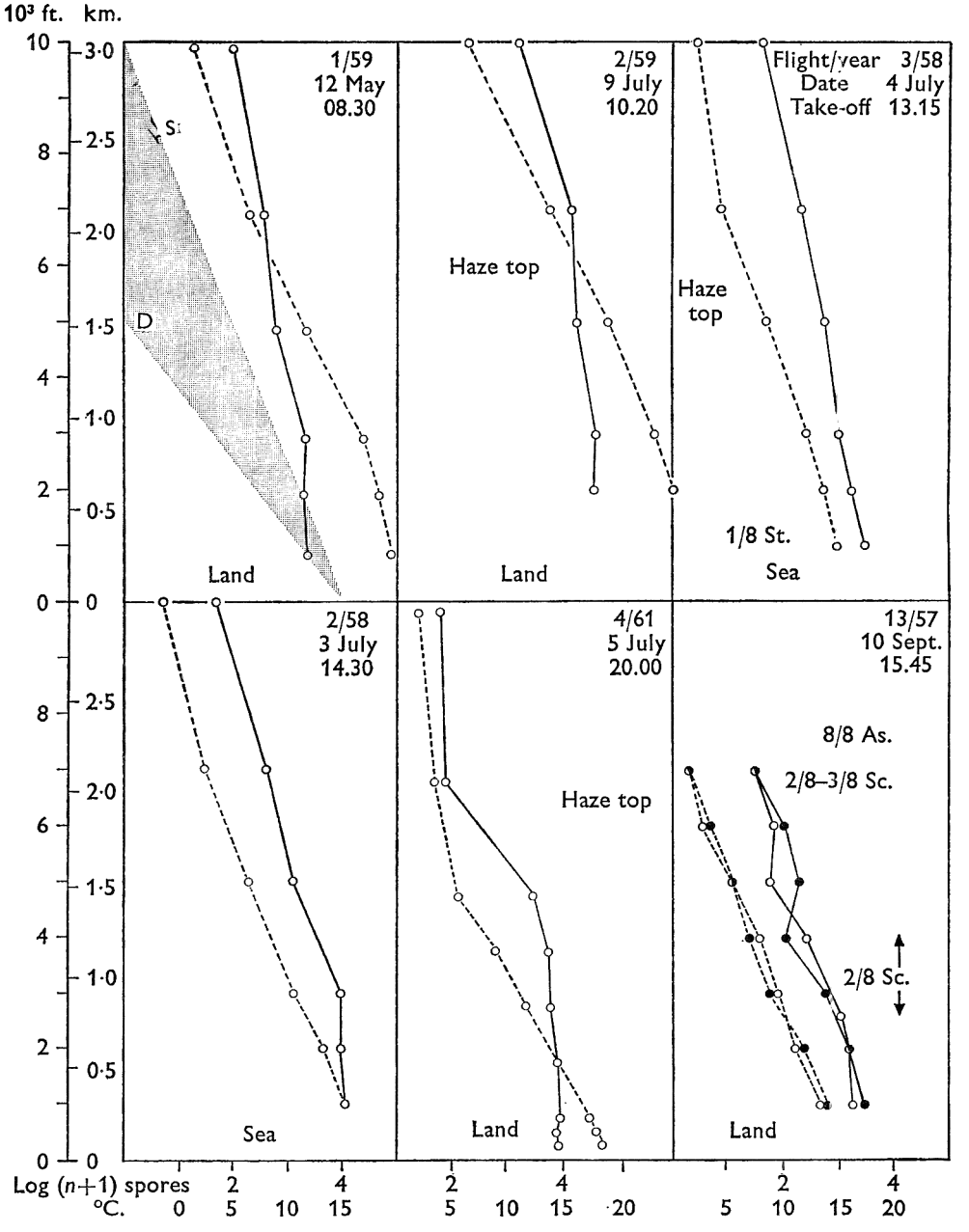

Fig. 3. Examples of vertical profiles of concentration of all spores (solid lines) in unstable or conditionally unstable air over land or sea as indicated beneath. Lower edge (D) of shaded wedge on diagram of Flight 1/59 indicates slope of dry adiabatic lapse rate and upper edge (S) the approximate saturated adiabatic lapse rate, temperature profiles dotted. Hollow dots indicate ascent and solid dots descent. Cloud layers indicated in 'oktas' (eighths) with standard abbreviations, arrows indicate thickness.

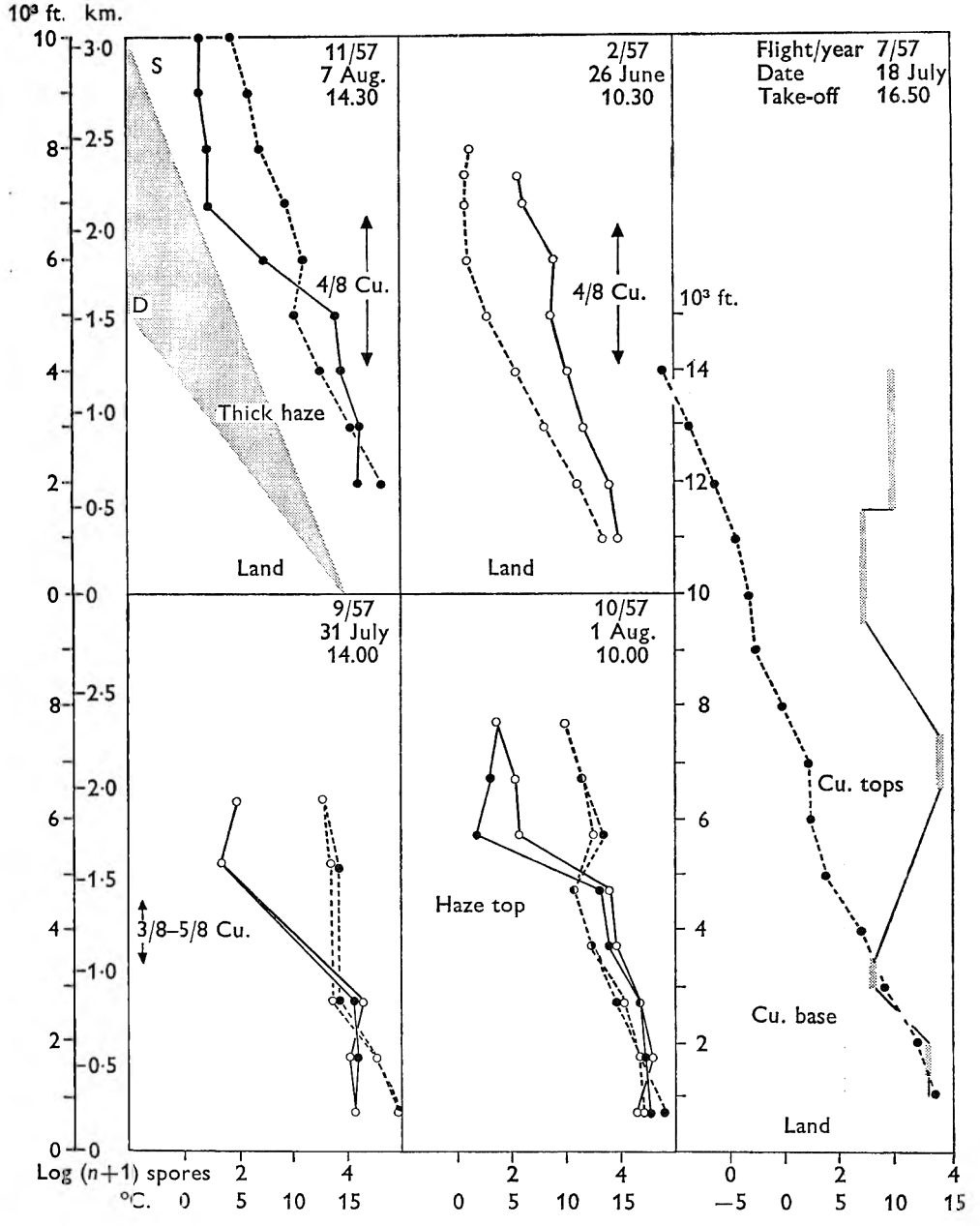

Fig. 4. Examples of vertical profiles of concentration of all spores through stable air layers. Other details as for Fig. 3, except dotted sections of profile for Flight 7/57 which show layers from which samples were taken during continuous descent, see text.

(Fig. 4), both of which measured changes in concentration through temperature inversions, as did Flight 11/57 when concentrations below the cumulus layer were more than 100 times greater than above it. Sharp decreases in spore concentration often coincided with visible 'haze tops', and concentrations often decreased at cloud heights,

presumably because there were inversions or isothermal layers. Although samples were rarely collected within clouds, Flights 2/57 and 7/57 (Fig. 4) may support the contention that there are more spores inside clouds than outside (Dillon Weston, 1929; Heise & Heise, 1928). Flight 7/57 was particularly interesting, although unfortunately another research programme dictated that the descent, over Hampshire, should be uninterrupted. Our samples were therefore unreplicated and were collected from layers of variable thickness rather than during level flight. The greatest catch (total of 6500 spores/m.^{3}) was at about 2000 m. near the cumulus tops. In contrast, only 500 spores/m.^{3} were caught just above cloud base at approximately 900 m. and 4000–5000 spores/m.^{3} below 600 m. Showers occurred in the area and the large differences may have been caused by local rain washing. Much larger concentrations (39,000–46,000 spores/m.^{3}) had been measured during the previous hour at 600 m. in very unstable air over the Midlands.

Contemporary temperature profiles cannot explain all features of spore profiles, for example, the increase in catch at 2100 m. in Flight 4/58 (Fig. 11*a* and p. 347) or why spore concentration did not decrease more in the stable isothermal layer above 1800 m. in Flight 2/57 (Fig. 4). Later observations suggest that explanations require knowledge of wind direction and speed at different heights, previous temperature profiles and the past movements of the air sampled.

Within one 24 hr period we were able to make several ascents, Fig. 5 shows results of Flights 2/61 to 7/61 with the take-off times from Farnborough, Hants. Triple samples (preceded by dummies) were collected over the same area at heights ranging from 75 to 3050 m. and spore concentration was measured near ground level throughout the period. Temperature profiles showed that, during most flights, the air was unstable up to about 1400 m. The exceptions were morning flights with inversions near the ground, slight in Flight 6/61 and strong in Flight 5/61. The spore trap placed on the roof of the control tower, 12 m. above ground, usually indicated smaller concentrations of Cladosporium conidia and pollen than those estimated from catches at the lowest height sampled by the aircraft. Had the locality been a prolific source, these day-liberated spores would have been expected to be most concentrated near the ground, however the immediate surroundings of the trap comprised much concrete runway, buildings and mown grass, with sandy heath-land beyond and probably contributed few spores. Alternatively, the smaller estimates of concentration from the surface trap may have resulted from imperfect matching of the calibration of aircraft and ground traps, errors difficult to overcome without experiments allowing both to be tested at their proper air speeds in spore clouds of equal density.

Day-time profiles of Cladosporium and pollen were alike in shape in the layers where lapse rate was enough to ensure instability and active mixing of the air. Comparison of temperature and spore profiles of Flights 6/61 and 7/61 suggests that when the height of the unstable layer increased the upper limit of dense concentrations was also raised. The earliest morning flight (5/61) preceded the daytime increase in spore concentrations and therefore should have shown effects of the night. Neither spore type had disappeared from the air, but maximum concentrations of Cladosporium and pollen, respectively, were only 1600 and 420/m.^{3} during Flight 5/61, whereas they had been 7000 and 850 in Flight 4/61, 12 hr earlier, and were 4000 and 245/m.^{3} in Flight 6/61, 3 hr later. There is a suggestion that the pollens fell farther during the night than the much lighter Cladosporium spores. *Ustilago* spp. (max. 1150/m.^{3}) were

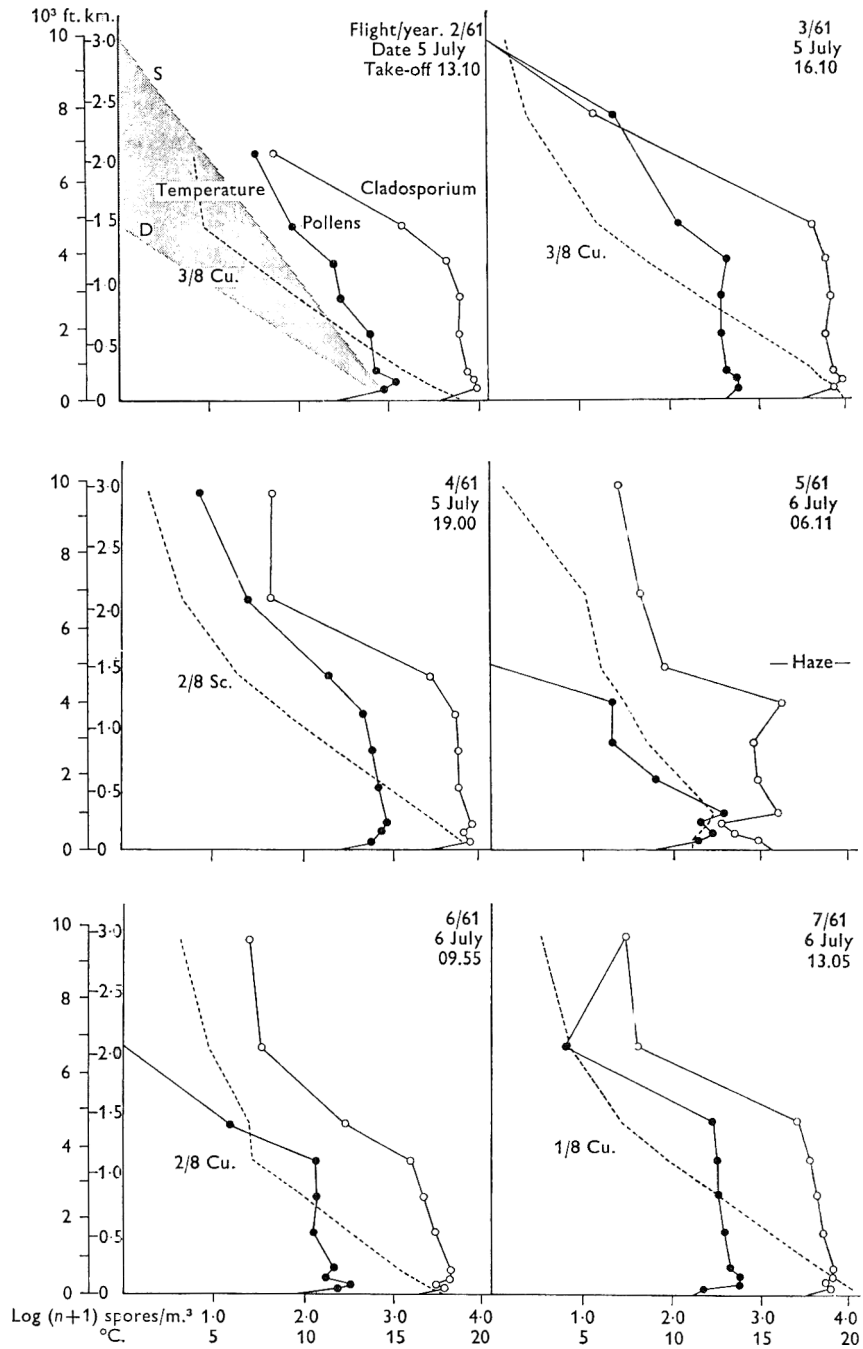

Fig. 5. Profiles of temperature (dotted), concentration of pollen (solid circles) and Cladosporium spores hollow circles) in a sequence of ascents over Farnborough, Hants. on 5 and 6 July 1961. Other details as in Fig. 3.)

the second most common fungus spores caught in the aircraft trap, followed by *Erysiphe* spp. (max. 230/m.^{3}), *Botrytis* spp. (max. 110/m.^{3}), *Alternaria* spp. (max. 90/m.^{3}), Epicoccum (max. 70/m.^{3}) and coloured basidiospores (max. 60/m.^{3}). *Polythrincium trifolii* (max. 35/m.^{3}) was almost limited to Flight 2/61. Helminthosporium, Torula and uredospores were caught on most flights but were always few. Of spores liberated in damp air only the composite 'Ascospore' group reached 90/m.^{3}, and Sporobolomyces, Tilletiopsis and hyaline basidiospores were rare.

Interception of immigrant spores

Distant seasonal movement of *Puccinia graminis* uredospores was demonstrated during the 1920's in North America (Stakman & Christensen, 1946; Johnson, 1961). Comparable movements have been reported in Russia (Shitikova-Roussakova, 1927), Finland (Kivi, 1953), Australia (Watson & Cass Smith, 1962), and in India by Mehta (1952), who first used routine air trajectories. Little was known of the spread of *P. graminis* to the British Isles until Hyde & Adams (1961*a*, *b*) related the presence of uredospores in catches at Cardiff with geostrophic trajectories drawn by Hogg (1961*a*, *b*; 1962) of air arriving at Plymouth.

Figure 6 shows the occurrence of possible or probable trajectories from Iberia and France, at the 'surface', 700 and 500 mb. levels for the years 1947–59. Mr H. A. Hyde and Mrs K. F. Adams (Asthma & Allergy Research Unit, St David's Hospital, Cardiff) have kindly allowed us to indicate days when one or more *Puccinia graminis* uredospores were caught, either on the Bishop Rock Lighthouse (49° 52′ N, 6° 27′ W) or on the roof of the National Museum of Wales, Cardiff. Horizontal ('gravity') sticky microscope slides were used at both stations, but in and after 1957 a Hirst spore trap was used at Cardiff. Early in the period May to July few north-bound tracks carried uredospores (Fig. 6) because the disease was not yet prevalent in southern Europe. When the stem rust disease was present in southern Europe but not in Britain there was a close relation between tracks and catches, but once the disease had developed in Britain, spores were not limited to air from the south. The importance of trajectories of European origin is therefore probably greater than a casual glance at Fig. 6 suggests.

In the years 1957–59 the Meteorological Research Flight were sometimes able to sample air masses of European origin during June and early July. When it was known that a flight had intercepted immigrant *Puccinia graminis* uredospores hourly estimates of uredospore concentration were made from catches of spore traps continuously measuring frequency of respiratory allergens in London or Cardiff.

Observations in 1957

Flight 4/57; 13.50–14.00, 4 July 1957. Results of this first interception flight are less accurate than later ones (samples were unreplicated, giving a detection threshold concentration 30 spores/m.^{3}) and incomplete because of impactor defects and the loss of some samples collected in rain. Nevertheless, the flight coincided with *Puccinia graminis* uredospore catches at Cardiff and on the Bishop Rock, and an ascent was completed about 30 nautical miles (55·59 km.) south of Portland Bill (50° 05′ N, 02° 30′ W). Air was unstable throughout the profile sampled, from 1060 to 2750 m. and single uredospores (about 30/m.^{3}) were caught at 1200, 2150, 2450 and 2750 m. Such small concentrations provide no information on the shape of the uredospore profile,

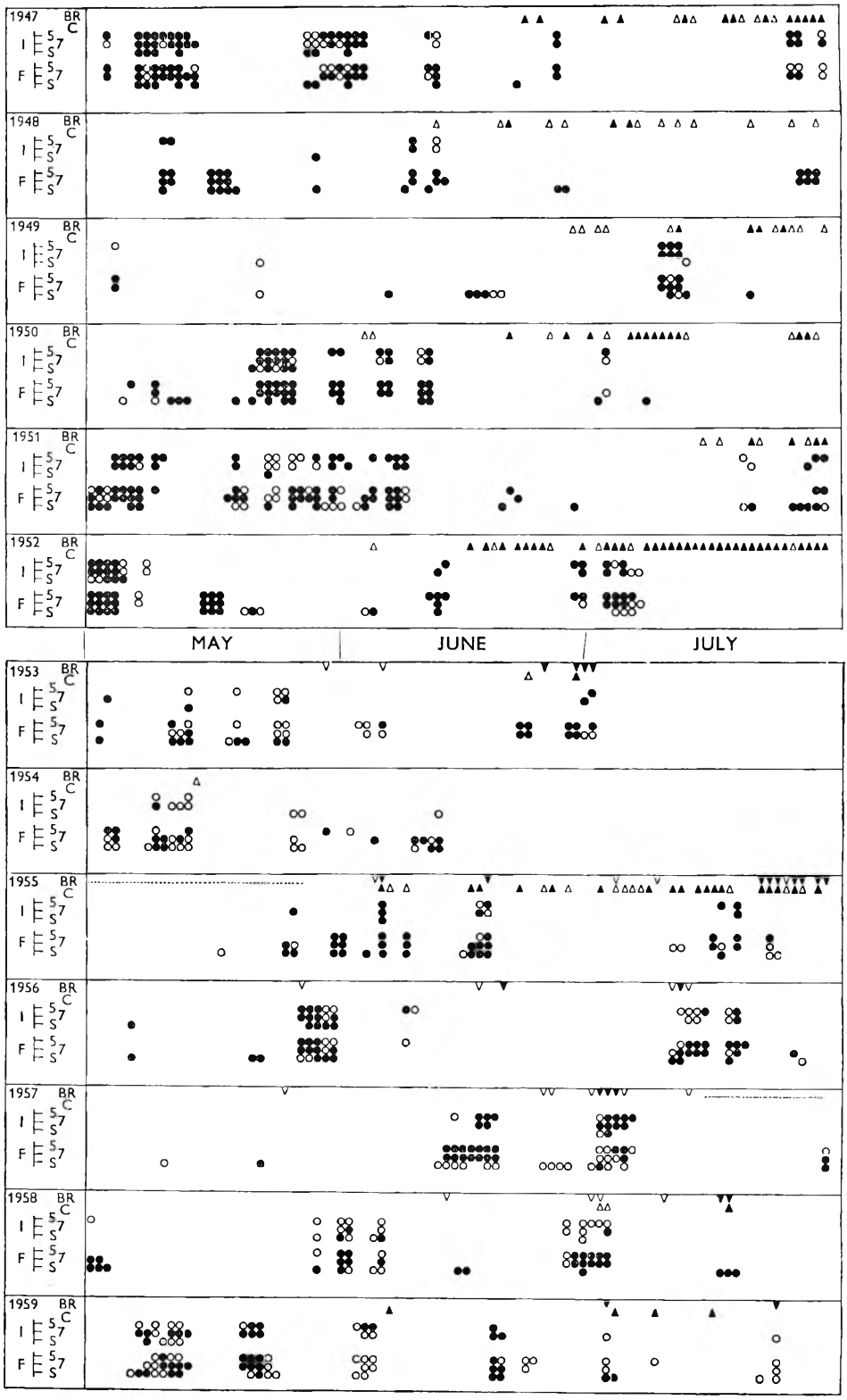

Fig. 6 For legend see opposite page.

but because they do show when uredospores occurred well above surface air movements are useful to the discussion below.

On 3 July a slow-moving depression west of Cape Finisterre and pressure high to the east on 3 July caused a slow northward air flow over Western Europe. During the period 4–7 July the depression filled, and over Britain surface air movement became light and variable until influenced by a small secondary depression on 6 July At the 700 and 500 mb. levels, however, the northward airflow persisted until 8 July (see Fig. 6).

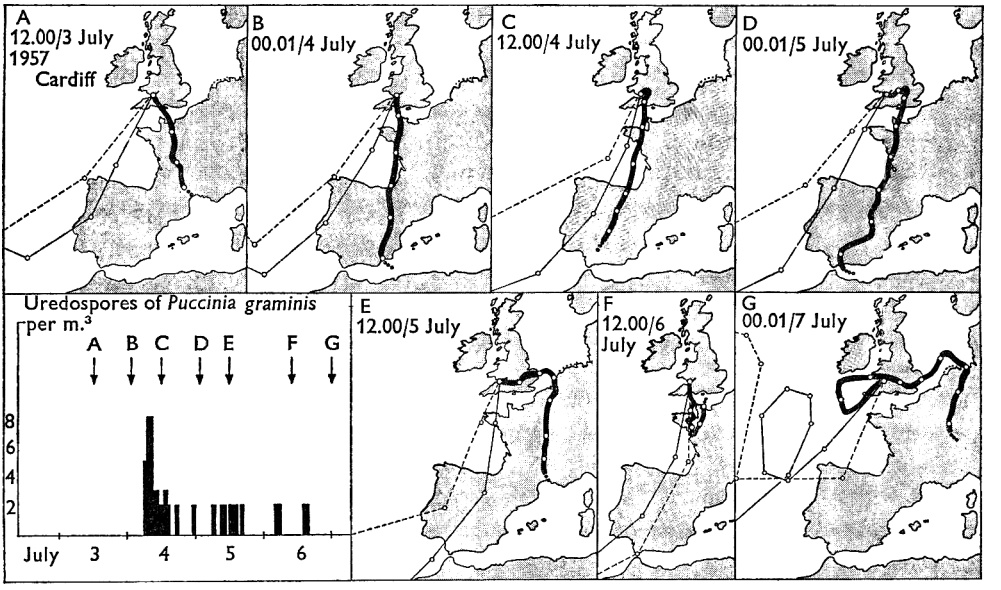

Fig. 7. Diagram showing concentration of *P. graminis* uredospores at Cardiff between 3 and 7 July 1957 and related trajectories of air arriving at Cardiff at the times indicated. Surface trajectories, thick lines; 700 mb. level, thin solid lines and 500 mb. dashed lines. Circles show estimated positions of the air sampled, at the time indicated, and at 12 hr intervals before arrival over Cardiff.

At Cardiff surface spore catches (Fig. 7) were small even during the maximum on 4 July, but single uredospores were caught through 5 and 6 July. In view of the synoptic situation it is tempting to wonder whether the peak concentration on 4 July indicated the arrival of spore-bearing air at all heights ('solid trajectories'), whereas

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Fig. 6. Upper two lines of each annual panel show *P. graminis* uredospore catches on the Bishop Rock Light House (inverted triangles) and at Cardiff (triangles with apex uppermost). At the former, 1953–59, all catches were with sticky horizontal microscope slides of which 5 cm² was scanned, similarly at Cardiff 1947–56. From 1957 to 1959 the Cardiff counts shown are from a single daily long axis traverse of a Hirst spore trap slide, having approximately the same sensitivity as the counts on horizontal slides. More detailed counts are available and some are used in Fig. 7. Hollow triangles indicate a single uredospore, solid triangles more than one.

Lower part of each panel indicates days when air trajectories at surface (s), 700 (7) or 500 mb. (5) possibly (hollow circle) or probably (solid circle) passed over France (F) or the Iberian peninsula (I). Slides missing where the line is dotted.

the later barely detectable concentrations represented uredospores reaching the slow-moving surface air from higher levels as a result of turbulence and gravitational settling. The evidence is insufficient to confirm or deny this hypothesis. The ascent over the English Channel, about 150 nautical miles south of Cardiff during the afternoon of 4 July, when surface catches suggested that the peak concentration had already passed, showed a few *Puccinia graminis* uredospores at heights up to 2750 m. This height is representative of the 700 mb. trajectories, but the vertical distribution might have resulted either from sedimentation and downward mixing of spores carried in upper winds, or from upward convective mixing of spores arriving in surface winds. In 1957, stem rust appeared in Portugal in April and later became severe. No record has been found of its prevalence in Spain, but it is usually common by early July, although by this date in 1957 it had not been reported in Central France or Belgium. Thus, prolific sources probably lay to the south of latitude 45° N. Surface trajectories could account for the greatest catch at Cardiff (Fig. 7) on 4 July when the air came over Spain. However, surface trajectories alone cannot explain the continued small catches at Cardiff on 6 July or those on the Bishop Rock on 2 and 3 July (Fig. 6), both of which could have resulted from spores which travelled along the faster 700 mb. trajectories.

Observations in 1958

Early in July 1958 a depression moving slowly eastwards over the Brest Peninsula into France brought air to southern England first from the south and then from the

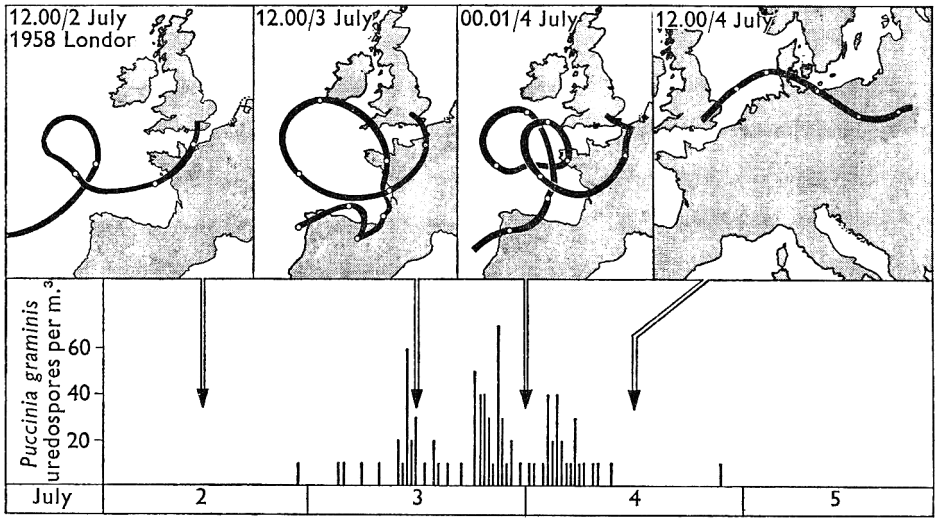

Fig. 8. Diagram showing concentrations of *P. graminis* uredospores at Paddington, London, between 2 and 5 July 1958 and related surface air trajectories. Circles show estimated positions of the air sampled at time indicated, and at 12 hr intervals before arrival.

east. Figure 8 shows the *Puccinia graminis* uredospore concentrations and past trajectories of surface air arriving in London during this period. Several trajectories suggested cycloidal paths representing air movement within the depression, showing the uncertainty of prediction in view of the probable errors quoted in Table 1. Uredospores

were caught only when trajectories passed over northern Spain and central France from midnight on 2–3 July until noon on 4 July (with a single spore later).

Flight 2/58; 12.47–14.37, 3 July 1958. Figure 9*a* shows the course of the horizontal traverse at 600 m. through positions 'A'–'F' with an ascent to 3050 m. at 'D', together with the concentrations of *Puccinia graminis* uredospores and of all other spores. The past trajectory of air sampled at 'A' (not shown) had a much shorter travel over France than that at 'F', but the total catches differed more than the ratio

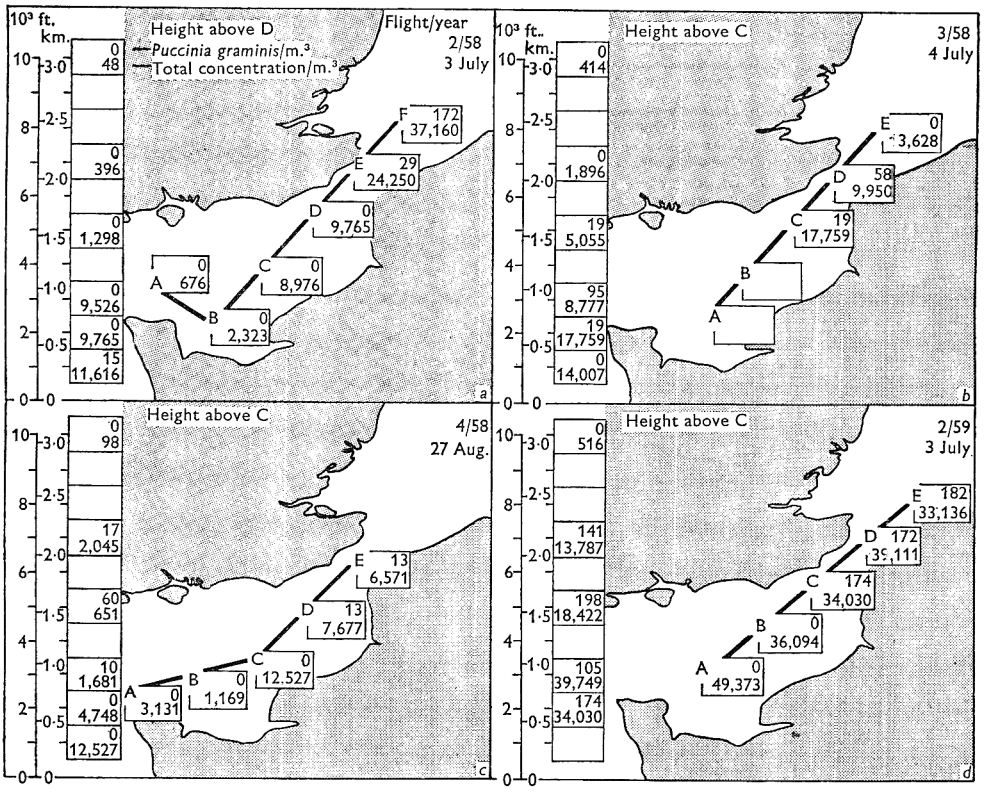

Fig. 9. Diagrams showing track of interception flights (see Fig. 2) (thick line) and sampling positions (lettered). At each position the box contains the estimated concentration of *P. graminis* uredospores (top) and all spores and pollen (bottom). For further details see text.

of distance travelled over land suggested. As most of the spores caught at 'F' were of types usually common close to the ground in daytime, the extent of daytime travel over land was examined. Air sampled at 'F' had been over France throughout 2 July, whereas air sampled at 'A' had crossed land only during the night of 2 July and briefly the following morning. Although few spore types are limited entirely to day or night liberation (Hirst, 1953), the ratios of catches at 'F:A' of the daytime Cladosporium, Ustilago and Alternaria were, respectively, 79:1, 35:1 and 94:1, whereas the predominantly nightime Sporobolomyces, Tilletiopsis and 'ascospore' groups were respectively 2:1, 1:1 and 2:1. This supports the idea that the time as well as the distance of air-flow over land may have been important in determining spore concentrations and, in the absence of other evidence, suggests a French source for the uredo-

spores which were commonest towards the east end of the traverse. The ascent over 'D' was too far west to intercept many uredospores, and although other spores were numerous up to 900 m., concentration decreased rapidly above this (Fig. 10). The temperature profile approximated to the saturated adiabatic lapse rate so there would have been little convection, and contemporary temperature profiles could not account for the height distribution of spores.

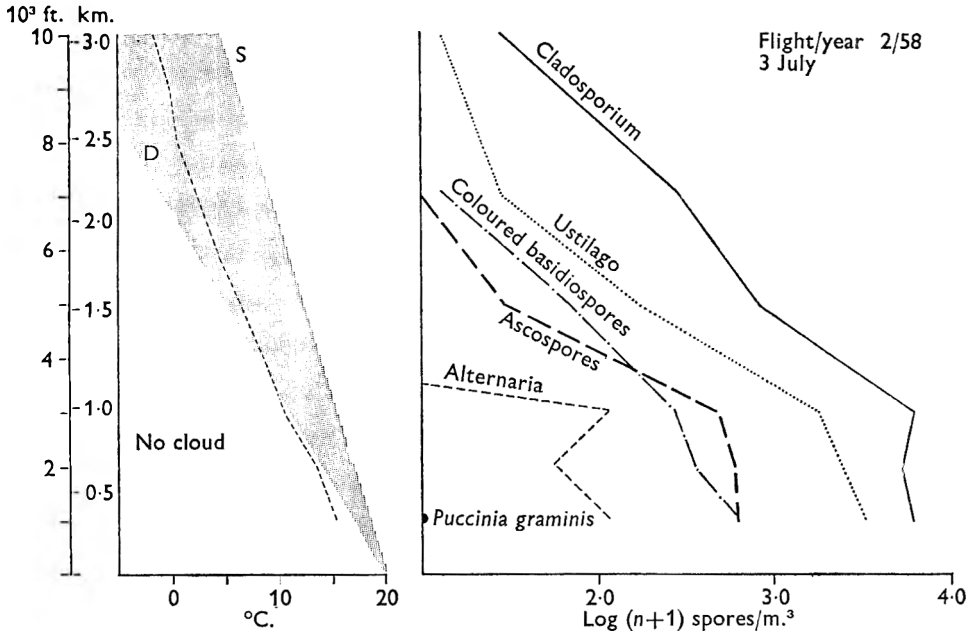

Fig. 10. Temperature profile (left) and profiles of concentrations of various spore types (right) in the ascent over D in Fig. 9*a*, Flight 2/58.

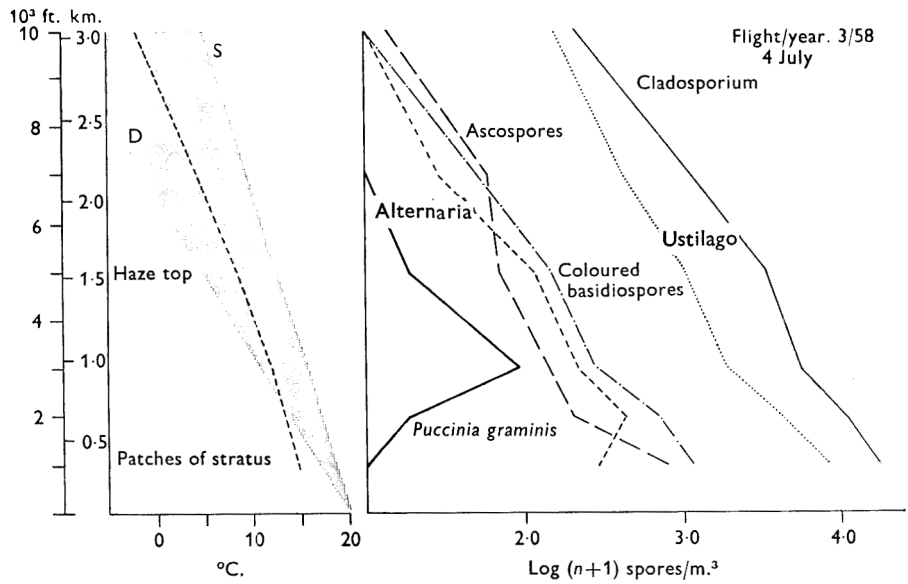

Fig. 11. Temperature profile (left) and profiles of concentrations of various spore types in the ascent over C in Fig. 9*b*, Flight 3/58.

Flight 3/58; 11.54–13.29, 4 July 1958. An interception flight was repeated next day but, despite modifications intended to avoid cloud, samples from 'A' and 'B' were lost because of rain (Fig. 9*b*). Fewer spores occurred in the eastern part of the traverse than on the previous day. However, the ascent above 'C' (Fig. 11) was more fortunately placed. There was some instability above 900 m. but the air was more stable beneath, with suggestions of an inversion near the surface. Above 600 m. most profiles of log. $(n-1)$ spores/m.^{3} of air decreased almost linearly with height. More *Puccinia graminis* uredospores were caught than the previous day but concentrations were still small compared to other types; most were at 900 m. Alternaria and other less common spore types were more frequent at 600 m. than at 300 m.

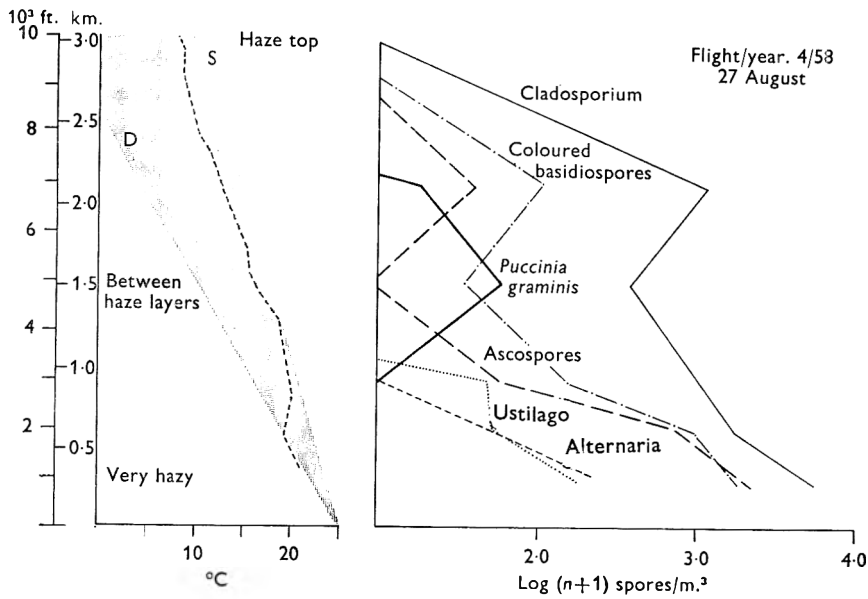

Fig. 12. Temperature profile (left) and profiles of concentrations of various spore types in the ascent over C in Fig. 9*c*, Flight 4/58.

Flight 4/58; 13.39–15.03, 27 August 1958. By 27 August 1958 *Puccinia graminis* was present on wheat crops in central and southern England, so further uredospore introductions were of more interest aerobiologically than agriculturally. The surface trajectory of the air sampled showed that it travelled north over France on 26 August, but earlier had been over England on the 25 August. At this time a small depression passed eastwards over southern England, and behind it air moved south into France, possibly almost to the Mediterranean in the evening of the 26th. Then an anticyclone developing over central Europe became dominant and by the time of the flight southerly winds were well established. Spore distribution was variable and again the ascent over 'C' (Fig. 9*c*) probably missed the greatest concentration of uredospores. Most spore profiles (Fig. 12) showed two dense strata at 300 and 2100 m., but *P. graminis* was unique in showing a single peak at 1500 m. Had the temperature inversion at 600 m. been widespread over Europe, it could explain the rapidly decreasing concentration in the lower half of the profile but not the increased frequency of spores at 2100 m. These spores seem to be survivors of a cloud raised by earlier convection, per-

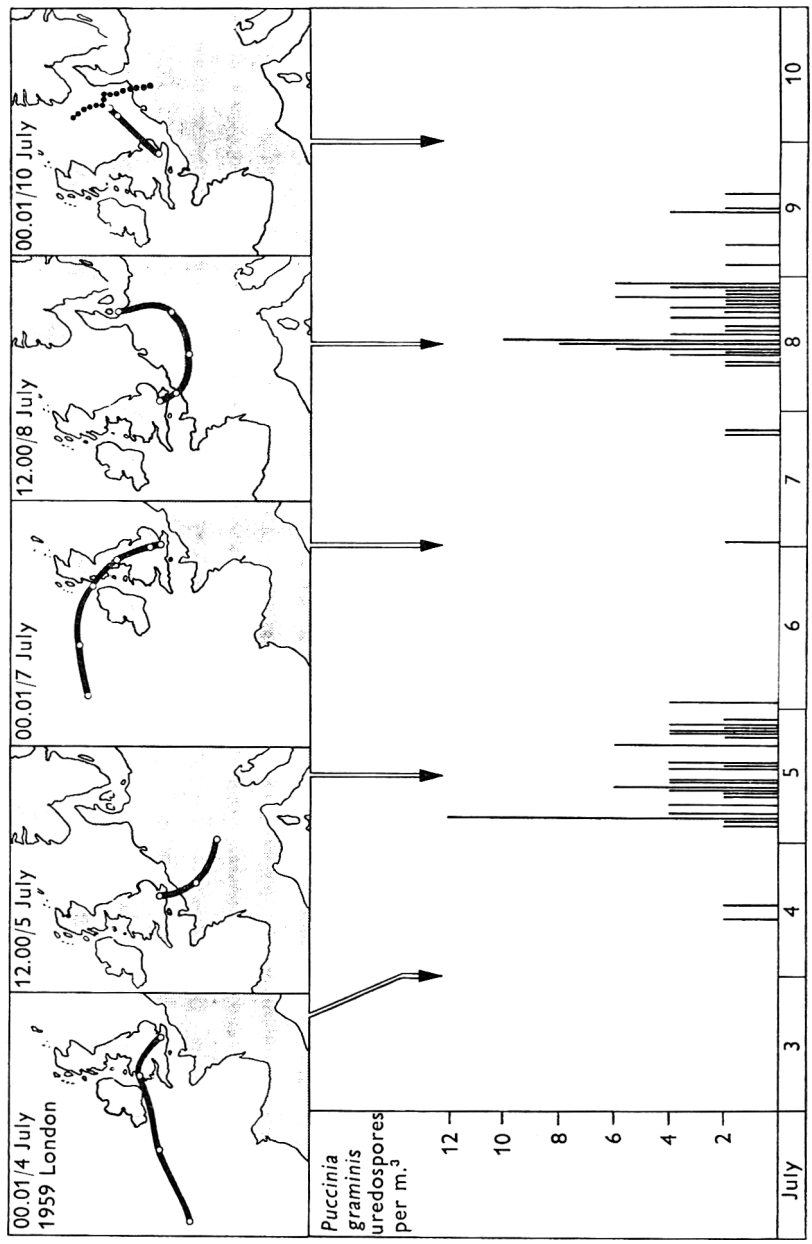

Fig. 13. Diagram showing concentrations of *P. graminis* uredospores over Paddington, London, between 3 and 10 July 1959 and related surface air trajectories. Circles show estimated positions of the air sampled, at time indicated, and at 12 hr intervals before arrival.

haps over France on 26 August or even over England on 25 August and retained in suspension by instability above the inversion. Even this theory fails to explain the contrasting *P. graminis* uredospore profile.

Observations in 1959

During the warm dry summer *Puccinia graminis* developed fast in Europe. The first record came in March from Andalusia, Spain; by early June it had spread to all except the north of the Iberian Peninsula and into south France, and by early July to north France. The uredospore introduction observed in July 1959 resulted from air movements around high-pressure systems rather than depressions which were important in 1957 and 1958. An anticyclone moved slowly east from the Bay of Biscay on 2 July, across north France by 4 July and weakened over the Baltic on 6 July. A second

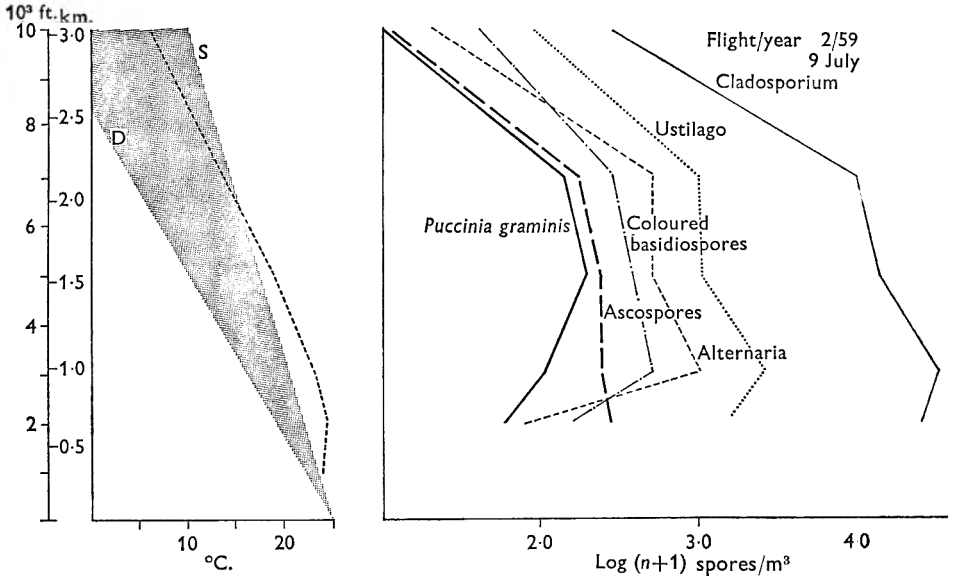

Fig. 14. Temperature profile (left) and profiles of concentrations of various spore types in the ascent over C in Fig. 9*d*.

anticyclone passed faster eastward over the south coast of England on 7 July and into Russia by 9 July. Figure 13 shows that northward air movement on the west side of each anticyclone brought uredospores to the trap operating in London, particularly on 5 and 8 July, whereas the trajectories which had passed over the Atlantic were free of all but occasional uredospores, these exceptions perhaps resulting from the admitted errors of time and position which trajectories imply.

Flight 2/59; 08.58–10.15, 9 July 1959. No flight could be made until almost the end of the second introduction. Most spores were common all along the traverse, but *Puccinia graminis* uredospores were limited to the eastern half (Fig. 9*d*). Hyde & Adams (Ogilvie & Thorpe, 1961) caught uredospores at the Bishop Rock on 4 July, and at Cardiff on 5 July during the first immigration, but only a few at Cardiff (10 July) during the second.

An inversion below 600 m. probably extended down to sea level, but above 900 m. there was conditional instability (Fig. 14), so that many of the spores raised by convection before the inversion formed could remain in suspension. By contrast, below the inversion gravitational settling and frictional turbulence would combine to decrease the number of airborne spores. The agreement between the profiles of various spore types and the weather situation both suggest that most spores collected come simultaneously from the same source area. The profiles for 'ascospores' and 'unclassified' types do not share the decrease in concentration below 900 m. and perhaps significantly both may be liberated at night.

DISCUSSION

Because of the difficulty of sampling from aircraft during rain, most flights were made during fine weather when airborne spores were probably commonest. Even allowing for this, the large concentrations found stress the frequency and potential of long-distance spore transport. Spores were caught on every occasion, 10^4 spores/m.^{3} were common at 600 m., usually decreasing to hundreds/m.^{3} at 3000 m. Samples from greater heights would have been interesting, particularly in jet streams, but the small concentrations at 3000 m. show that larger samples and prevention of contamination would be necessary to make them reliable. For the future we recognize the necessity of measuring spore viability and relating concentrations in vertical profiles to the strength of sources, ground level spore concentrations and to the number of spores deposited.

The vertical distribution of spores

The fungal flora of the atmosphere changes with time of day and the weather (Hirst, 1953), and we have selected Cladosporium to indicate convective activity because it is usually the dominant spore near the ground in daytime. Cladosporium conidia vary in length from 4 to 20 μ and probably in terminal velocity from 1 to 5 mm./sec. or, in still air, 90–450 m./day. Spores of many other fungi lie within this range. Pollens have been used as examples of larger particles, most of which fall at 2 cm./sec. or faster, equivalent at least to 1700 m./day.

Where convection was active, all day-liberated spore types showed similar profiles, presumably because velocities both in ascending and diffusing eddies are fast relative to settling velocities. This supports Gregory's (1961) contention that gravitational settling can reasonably be omitted from calculations of diffusion near sources in turbulent air. But, as Schrödter (1960) pointed out, spores continue falling in turbulent air, although when convection is active the net movement of the spore cloud may be upward. In calmer conditions air may subside, and the results from flights since those reported suggest stratification of the spore cloud according to particle size (Hirst, Stedman & Hurst, 1967; and see also Rempe, 1937).

Day-liberated spores were predominant in all vertical profiles, with Cladosporium (80 %) followed by pollens and Ustilago at their seasonal maxima (Gregory & Hirst, 1957). The following were the maximum concentrations of other important fungi crossing the English Channel from continental Europe: Ustilago (22,000/m.^{3}), Alternaria (1000/m.^{3}), unclassified uredospores (900/m.^{3}), Epicoccum (500/m.^{3}), *Polythrincium trifolii* (270/m^3), *Botrytis cinerea* (140/m.^{3}), Helminthosporium (40/m.^{3}), Erysiphe (30/m.^{3}) and Entomophthoraceae (10/m.^{3}). *Venturia inaequalis* conidia

(Hirst & Stedman, 1961) were often present (max. 90/m.^{3}) all along the transects of interception flights and once up to 2000 m. and conidia of pear scab (*V. pirina*) were also caught on one flight.

Marker spores to demonstrate transport processes at night are more difficult to choose, because types that are common enough are typical of damp air which may occur by day as well as at night. Of these types, ascospores (mostly coloured and multiseptate) were commonest (max. 4500/m.^{3}). Small concentrations of hyaline basidiospores and the ballistospores of epiphytic yeasts like Sporobolomyces and Tilletiopsis were caught occasionally, but were only included in the miscellaneous category 'other classified groups'.

The dependence of spore ascent on convection is demonstrated by the rapidly decreasing concentration with ascent through temperature inversions, often visible as 'haze tops' of which spores may be an important constituent. As any spore cloud derived from ground sources diminishes in concentration with height, it is interesting to see that thorough mixing in the frictional turbulence zone often gave an almost uniform concentration in the lowest kilometre. Spores were often more concentrated above temperature inversions than below them. Rempe (1937) attributed these dense concentrations to the accumulation of spores in or above the stable air stratum, and others (see Gregory, 1961) have discussed the existence of a 'biological zone' well above the earth's surface. Accumulation could occur if spores were captured within cloud droplets and released when the drops evaporated. Otherwise there is no force to carry the spores upwards or downwards through a stable layer in eddies, and equally none to prevent them falling through it at their terminal velocity.

These abnormal profiles are more probably vestigial rather than rudimentary, the result of inversions preventing upward diffusion but not downward settling. Thus when active convection raises spores through clear air to an inversion, the position of the inversion would be marked by a rapid decrease in concentration and probably a 'haze top'. An inversion developing near the ground after a day of active convection would not impede gravitational settling or diffusion by frictional turbulence. Above this layer of depletion a zone of relatively high concentration would remain where mixing by instability could continue and spores would be removed only by settling or by capture in droplets. Such theories could be tested by studying sequences of vertical spore profiles of night- and day-liberated spores, provided the complications of intense spore liberation sometimes associated with the onset of rain were excluded (Hirst & Stedman, 1963).

Aberrant spore profiles could result from factors other than temperature inversions, for example wash-out by rain, wind shear, or alternate travel over land and sea. More is known of the theory of spore capture by raindrops than of its effects. Drops larger than 2 mm. diameter are most efficient in trapping spores (Langmuir, 1948; see Gregory, 1961) and in depositing them, because they fall fast and evaporate slowly. Smaller droplets falling within clouds and then evaporating provide a possible, but unproven, mechanism of vertical spore transport and a unique means of concentrating spores in some strata. Dillon Weston (1929) reported significantly more fungal and bacterial colonies on Petri dishes exposed in cloud than in clear air; Heise & Heise (1948) also caught more ragweed pollen (Ambrosia species) and Alternaria species on sticky microscope slides exposed during flights through partial cloud cover. Unfortunately neither result proves that spores were more numerous in clouds, because the

efficiency with which free and droplet-borne spores are deposited on such trap surfaces at air speeds of about 90 knots (160 km./hr) is unknown. Also, more organisms may grow on Petri dishes exposed in cloud because they had been better protected against desiccation and radiation. Our trapping method was equally unreliable in cloud because even the 3 mm. orifice collected enough water to wash the trace and even to remove the adhesive layer. Consequently the aircraft avoided cloud when possible but most catches from within or between cloud were no larger than elsewhere. We have already suggested that rain modified the profile of Flight 7/57, but wind shear or the history of the air sampled was sometimes more important. During Flight 4/57 Cladosporium and Ustilago were the predominant fungi, at 2100 m. the concentration of all spores was 5080/m.^{3} compared with only 600/m.^{3} at 1200 m. The flight was made before the seasonal maximum of Ustilago in England and the large concentration in the upper part of the profile was therefore probably brought by the winds from the south at 700 and 500 mb. levels (see Fig. 7 and p. 344). The two strata of dense spore concentration shown in Flight 4/58 (Fig. 11*a*) probably reflected the previous movement of air nearer the surface.

These modifications would affect all spore types, but profiles recorded during interception flights show exceptions, particularly *Puccinia graminis* uredospores and Alternaria, of which there were fewer in the lowest zones sampled than in those immediately above. We wondered whether this shape could be associated with a distant origin. Thus profiles like that of *P. graminis* in Fig. 10*b* could be derived from the straight lines of most other spore types. When a spore cloud is blown from its source it is no longer replenished, but spores are still subject to deposition. Sedimentation and turbulent deposition will preferentially remove spores from the base of the profile, where despite their greater initial concentration 'erosion' should first be detectable by concentration decreasing. Spores of other fungi may have travelled as far as *P. graminis*, but while travelling over sources of new spores erosion would not be apparent and local and distant contributions to the profile would be indistinguishable. If this idea is correct, 'eroded' profiles of exotic spores might provide a useful means of studying the depletion of spore clouds during distant transport.

Horizontal travel of spores

Close to sources the limits of the 'dispersal cone' are reasonably well defined and determined by the ground, turbulence, and the speed and direction of the wind, but further from sources additional factors seem important. The direction of geostrophic winds approximates to the curvature of isobars; air movement is therefore seldom straight, so it is unwise to assume that spores have travelled far in the direction of the local wind. There are exceptions; for example, in the Northern Hemisphere a long and wide belt of southerly winds may occur between an anticyclone and a depression to the west of it, a situation Craigie (1945) found often responsible for the northward transport of *Puccinia graminis* uredospores to the mid-west and prairie wheat-growing areas of North America. This situation seemed less frequent in western Europe (see Fig. 6). Among the episodes we examined in detail, spores were carried within the circulation around both high- and low-pressure systems and successful transport demanded a delicate balance of time and motion relative to the positions of source and sink.

Sampling in Britain and over the English Channel, supported by meteorological analysis, produced convincing evidence of the immigration of *Puccinia graminis* uredo-

spores and probably other plant pathogens. However, estimating the probable trajectory of air sampled on the ground at any one point and time reveals little of the distant dispersal processes because it can indicate only the probable direction of the source, not its distance. It seemed possible that trajectories might be divided into day- and night-lengths to indicate corresponding sources of spores usually most common in the air by day or night. However, spore trapping among crops showed that many 'damp-air' types are also common in damp air by day, and even spores typical of dry air could be dispersed in great quantity at the start of heavy rain (Hirst & Stedman, 1963). Similarly, flights like those described provide only crude 'snapshots' of spore clouds during transport. Comprehensive sampling needs to be three-dimensional and repetitive. Expense prohibits such sampling, so the probable shape, size, development and depletion of spore clouds during distant dispersal will have to be inferred from orthodox meteorological data, and catches by ground traps whenever possible. Interpretation would be improved greatly by further information on the rate at which spore clouds develop above source areas and the speed at which they are depleted when moved away. These objectives seem to require more complex flights, particularly sequences of ascent during day and night, and during the travel of a spore cloud over the sea, the most convenient non-source area.

Many people have helped with this work at various times and we are grateful to all. The Director General of the Meteorological Office is specially thanked for the facilities and giving permission to publish. Nothing could have been accomplished without the willingness of the Meteorological Research Flight to add these observations to their normal duties and we are most grateful to Dr R. J. Murgatroyd, who was in charge, to Mr N. P. Garrod, Mr F. Singleton, Mr S. G. Cornford and to the air crews.

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Long-distance Spore Transport: Vertical Sections of Spore Clouds over the Sea

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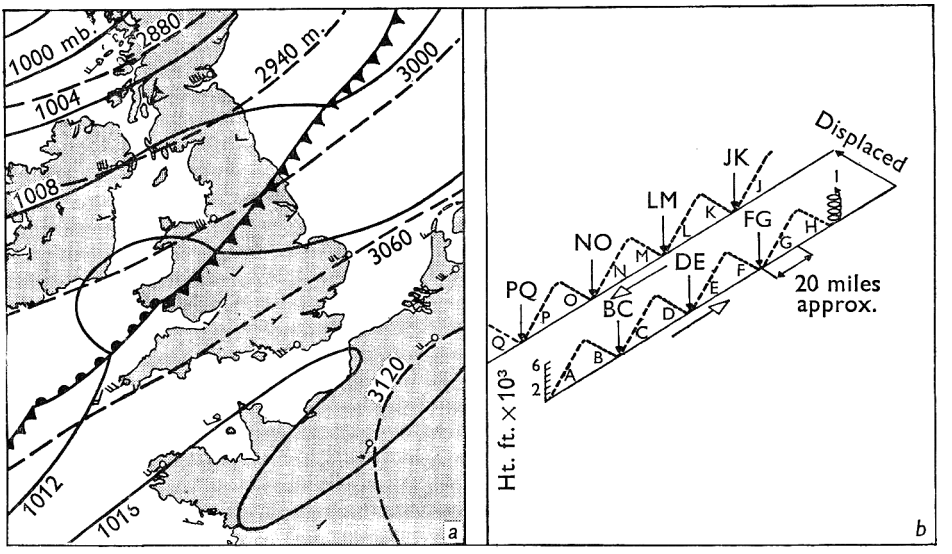
SUMMARY

To measure the rate at which spore clouds were depleted over the sea, spores were collected with isokinetic suction impactors mounted in aircraft of the Meteorological Research Flight, Farnborough. Remote from sources able to replenish spore clouds, preferential deposition from the lower layers caused 'erosion' of the base of vertical profiles of spore concentration leaving maxima at heights between 500 and 1500 m. To determine vertical spore distributions throughout the largest possible distance downwind of the English coast, a saw-tooth flight plan of alternating ascent and descent was used. Of four flights, two encountered favourable weather, rain interfered with sampling on one and unexpected winds across the track converted another into an unintended but interesting cross-wind section of spore distribution.

Pollens and Cladosporium spores were counted as examples of large and small spores liberated typically by day, and a composite group of spores liberated in damp air was chosen as an indicator of spores liberated mostly at night. In two flights in fine weather maximum spore concentrations occurred hundreds of miles off-shore. Diagrams showing height, distance from the coast and lines of equal spore concentration ('isospores') demonstrated discrete clouds of each marker spore type. Pollen and Cladosporium clouds were centred at approximately the same distances from the coast but with the pollen about 500 m. lower, probably because the pollen grains sedimented faster. Maximum concentrations of the damp-air group sometimes coincided and sometimes alternated with the day-liberated groups. Known periodicities of these spores over land, surface air trajectories and previous weather, suggested that the spore clouds which the aircraft overtook over the North Sea, were the residue of those produced from the British Isles on previous days or nights. Interpretation of the results was limited by meteorological uncertainties, the geographical complexity of probable source areas, and perhaps most by changes in the number of spores crossing the coast at different times of day, which prevented accurate measurement of rates of spore deposition.

Spores of many species were recognized over the North Sea. The plant pathogens included established distant migrants such as uredospores of *Puccinia graminis*, which apparently originated east of the Baltic. The viability of the spores was not tested, but it seems safe to assume that distant transport is both frequent and extensive and probably important in temperate latitudes in summer.



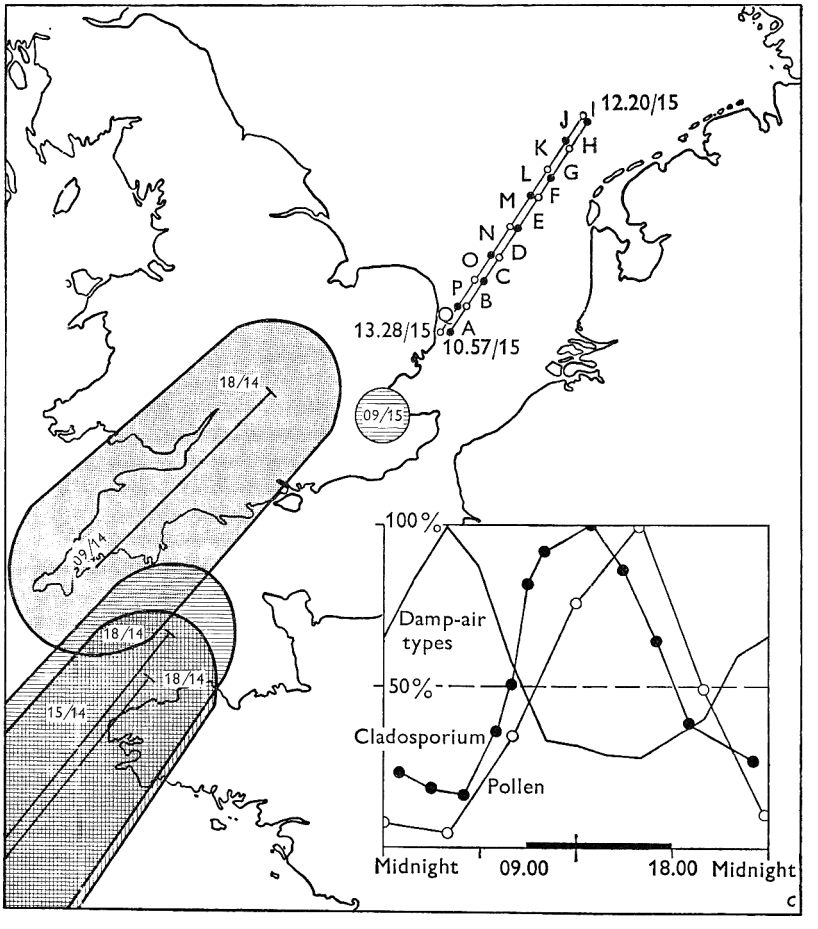


Fig. 1. For legend see opposite page.

INTRODUCTION

An earlier paper which described methods used to study vertical spore and temperature profiles and to intercept immigrant spores, showed that *Puccinia graminis* uredospores, the best examples of recognizable long-distance immigrants, were sometimes commonest at altitudes of 1–2 km. (Hirst, Stedman & Hogg, 1967). Such profiles may have resulted from preferential deposition from the base of the spore cloud and it seemed that 'eroded' profiles might be typical of spores that had travelled far from their source areas. It proved impracticable to test this idea directly by sampling over a source of uredospores and then at a series of points at increasing distances downwind. We therefore attempted to test whether progressive erosion of the bottom of profiles was a feature common to all spore types during travel over non-productive regions, by estimating changes in density of common constituents of the air-spora during transport over the North Sea downwind of sources in the British Isles.

METHODS

Although the spore-sampling methods and meteorological interpretation were similar to those already described (Hirst *et al.* 1967) a few modifications were necessary because the object was to measure several vertical profiles to the greatest practicable distance downwind from the coast. Instead of collecting during spiral ascents and descents, which occupies much flight time, a 'saw-tooth' plan of alternate ascent and descent on constant track was used. Air traffic control regulations often dictated height and track, but when possible the aircraft climbed above the convective layer far enough inland for the first profile to be sampled over land during descent at 300 m./min., ending at approximately 50 m. above sea level just clear of the coast. Instead of triplet samples preceded by a dummy (as in Hirst *et al.* 1967), collection was continuous with an instantaneous change-over to a new sampling drum position every minute. At the highest and lowest points a single sample was collected during level flight and a Lycopodium marker trace inserted between dummies. Further details are best introduced with the description of the flights to which they apply.

RESULTS

Flight 1/62; 10.57–13.28 G.M.T., 15 June 1962

On 14 June 1962 a complex depression north-west of Ireland and high pressure over the Mediterranean resulted in a broad band of south-westerly winds crossing south-

Fig. 1. Weather and plan of Flight 1/62. (*a*) Synoptic map for 00.01, 15 June 1962. Solid lines are surface isobars and corresponding winds are shown by arrows without circles (velocity, 5 knots (9·3 km. hr) per half feather). Dashed lines and arrows with circles show 700 mb. contours (m.) and winds at these heights. (*b*) Isometric plan of Flight 1/62. The outward track started with an ascent A then a descent B and so on. Each dash indicates a sample and an equal number were taken in the spiral ascent I. The diagram is not to scale and for clarity the return track is displaced as shown. (*c*) Map of flight track and times (A began at 10.00, 15 June). Inset shows that, with typical diurnal periodicities of concentration, Cladosporium and pollen are most common between 09.00 and 18.00 and damp-air types at night (Hirst, 1953). Shaded areas indicate probable source areas for the day-liberated spores estimated from surface trajectories of air sampled at A, vertical shading; at I, stippled and at Q, horizontal shading.

east England and the southern North Sea. In these areas lighter south-west winds continued during 15 June as the depression moved towards Iceland and a weak ridge moving from the West began to affect the southern half of the British Isles (Fig. 1*a*). A flight was planned with a starting-point just off East Anglia and a track of about 045° parallel to the wind.

Sampling began at 10.57 (all times are G.M.T.) at a height of 60 m. and a position north-east of Orfordness 52° 15′ N, 01° 48′ E (Fig. 1*c*). The first sample was taken during level flight followed by ascent A (Fig. 1*b*) to 1800 m., a sample in level flight and then descent B ending approximately 40 miles north-east of the starting-point (all distances are quoted in nautical miles: 1 nautical mile = 1·853 km.). The pattern was repeated to position 54° 15′ N, 05° 12′ E followed by a spiral ascent I, with continuous sampling. The saw-tooth pattern was reversed during the return flight on a reciprocal track, making a total of 126 samples.

Figure 1*b* distorts the length and height of the ascents and descents, in which the top and bottom of each sloping flight path were separated by 22 miles in length but by only 1800 m. in height. However, it was convenient to ignore this and to treat each sequence of samples as if it were a separate vertical profile. Samples taken in level flight at the top and bottom of each ascent or descent were common to two adjacent profiles but because most spores occurred at low altitudes, the profiles of a descent and an ascent were paired, sharing a common 30 m. sample, e.g. BC, DE, FG, etc. (Fig. 1*b*). We accepted the errors incurred as a result of displacing the small catches of each high-altitude sample by approximately 20 miles. The ascent A was unpaired and the descent H was paired with the spiral ascent I.

The temperature profiles were treated similarly and showed (Fig. 2) stable air near sea level. Above 300 m. there was a layer of conditionally unstable air, about 1000 m. deep at the north-east extremity of the flight but much shallower near the English coast.

Two spore types were used to indicate the distribution of spores liberated in dry daytime weather, total pollen (predominantly from grasses) and the lighter conidia of Cladosporium, a common saprophytic fungus. Concentrations of Sporobolomyces, Tilletiopsis, ascospores and hyaline basidiospores, were combined as an example of spores liberated in damp air, commonly at night.

Paired profiles are plotted in Fig. 2 on a linear height scale and a logarithmic scale for spore concentration (Hirst *et al.* 1967). To prevent confusion, the origin for each curve is displaced and lettered to correspond with those at the base of the profiles in order of increasing distance from the coast. Few of the profiles were as simple as those typical of unstable air (Hirst *et al.* 1967). In most, spores were fewer in the lowest samples than at 450 m. because they were deposited from the lower stable layer. This erosion was greater with pollen than with Cladosporium profiles, presumably because pollens are heavier and settled faster. The greatest spore concentration usually occurred between the inversions, at heights ranging from 450 to 1070 m., but consistently some Cladosporium and pollens also occurred above the upper inversion. Pollen grains in each of these strata were always most concentrated below the altitude of the greatest concentration of Cladosporium.

Further consideration of the profiles in Fig. 2 led to the surprising conclusions summarized in Fig. 3*a*, where the estimated concentrations in each profile were summed and expressed as a percentage of the greatest. For each spore group, the concentration

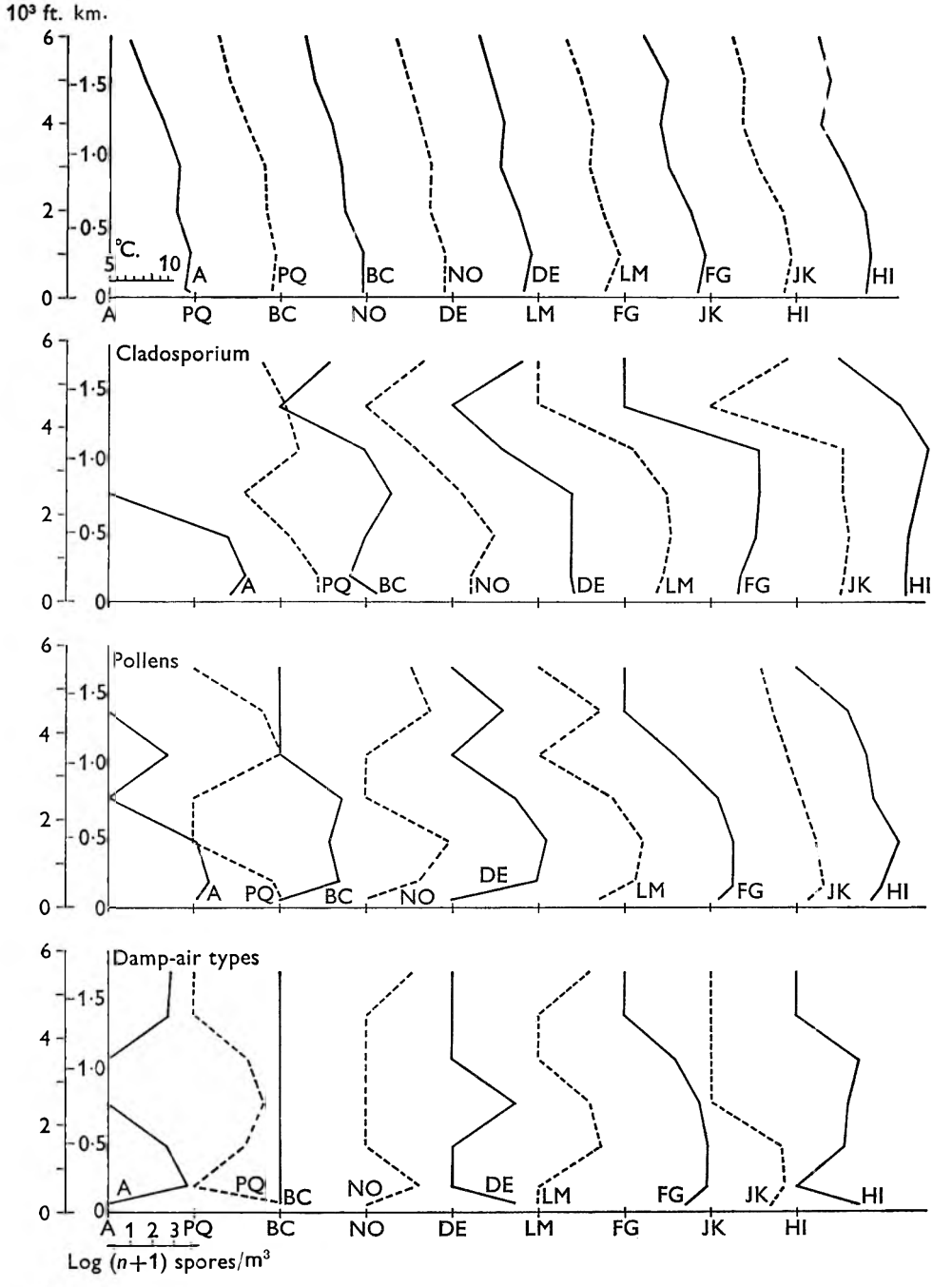


Fig. 2. Vertical profiles of temperature and concentration of Cladosporium, pollen and damp-air spores in Flight 1/62, 15 June 1962. Profiles (except A) are average in pairs as shown in Fig. 1 *b* and plotted with their origins (lettered below axis) displaced to the right approximately in proportion to distance of lowest sample from the coast. Profiles for the outward track in solid lines and for return track dotted lines.

decreased for the first 70 miles from the coast and then increased again to peaks about 170 miles out (Fig. 3*a*). The lower panel shows the height at which the maximum concentration occurred in the paired profiles at each distance from the coast. The height of the greatest Cladosporium concentrations increased as distance from the coast increased; pollen concentrations did not show a comparable trend. This spore distribution cannot be explained in terms of the typical decrease in concentration of spore clouds as they travel away from sources.

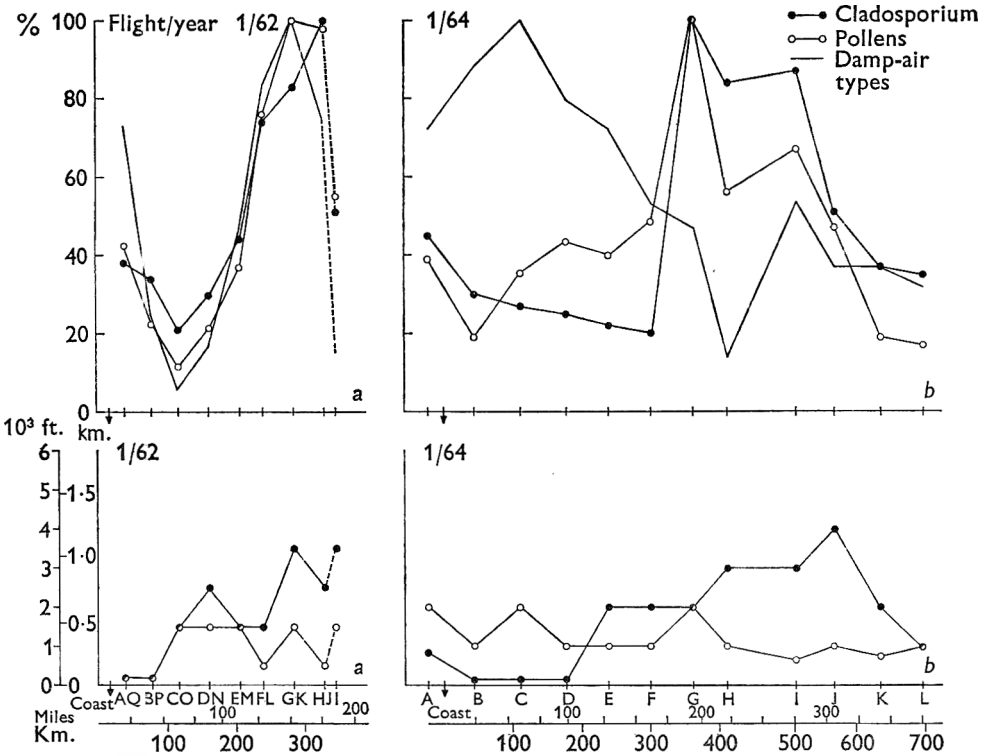


Fig. 3. Upper panels show sum of estimated concentrations within profiles (expressed as percentages of the greatest) and indicate that numbers of the three marker groups were greatest between 50 and 200 miles from the coast, both in Flight 1/62 (left) and Flight 1/64 (right). The lower panels show the heights at which the maximum concentration of Cladosporium and pollen occurred in relation to distance from the English coast.

Sequences of vertical profiles, like those in Fig. 2, give little impression of the form of the spore clouds they represent, so contoured diagrams of spore concentration were prepared. The estimated spore concentrations were plotted on a grid, showing height and distance within the vertical section and then lines ('isospores') were drawn through regions of equal spore concentration (Fig. 4*a*), and to accommodate the wide range of concentrations, the values of these lines were separated on a geometric progression increasing by a factor of two ($\log_2$ scale). In this diagram the outward and inward profiles at the same position were paired, not neighbouring profiles on the same flight path as in Fig. 2. Catches from ascent 'I' were therefore unpaired and as they suggest rather abrupt changes the isospores are shown dotted in this area. All three indicator groups were commoner near the coast than about 70 miles off-shore and all

were commonest about 150 miles out. Pollens were commonest at approximately 500 m. and Cladosporium at 1000 m. The similar distribution of pollens and 'damp-air types' was unexpected, because close to the ground they become common in quite different conditions.

The spores found concentrated at the north-east end of the flight could have come

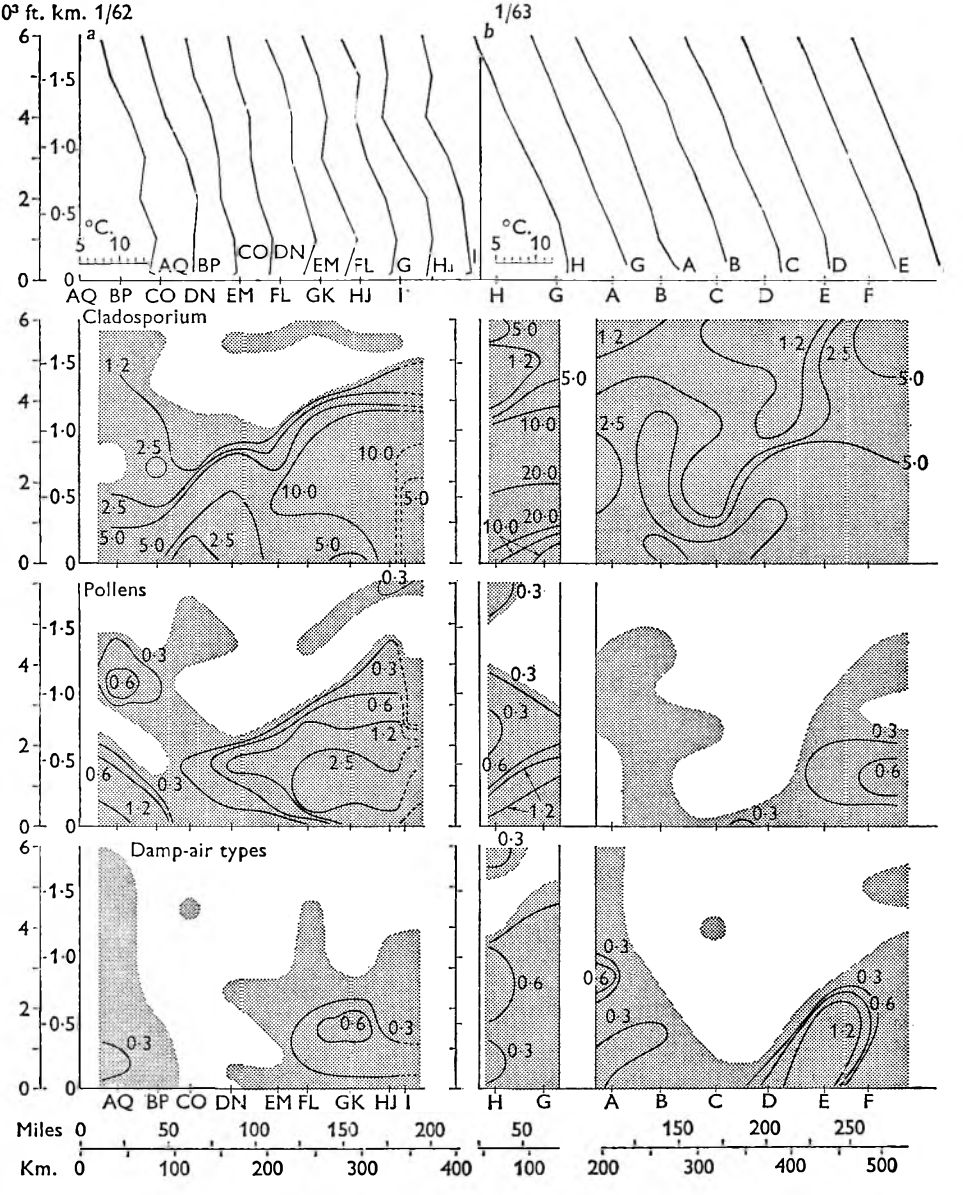


Fig. 4. Vertical temperature profiles (top), with the lettered origins displaced according to distance from the coast, Flight 1/62, left, and Flight 1/63, right. Below, isospore diagrams showing the distribution of marker spore types with height and distance. Shaded where any spores were detected, isospore lines separated on a geometric progression increasing by a factor of two, and marked in hundreds of spores/m.³, for explanation see text.

either from England or the Netherlands. Synoptic maps (Fig. 1*a*) suggested that the Netherlands was not a likely source and a surface air trajectory from I ran back to south-west England the previous day. Average diurnal periodicities of concentration of the three indicator groups near the ground (Fig. 1*c*, inset and Hirst, 1953) show that Cladosporium and pollen are commonest between 09.00 and 18.00 when convection is usually most active. From this knowledge, the probable source area of Cladosporium and pollens at the north-east extremity of the flight is indicated by the stippled land area within the zone of probable vector error in Fig. 1*c*, it comprised much of south-west and central England. Comparable back-tracking from the south-west extremity of the flight path at 10.00 and 12.00, 15 June, suggested small areas of north-west France and south-east England as the probable sources. Over south-west England on 14 June the air was probably unstable up to 1000 m., as a radiosonde ascent from Camborne, Cornwall, at 12.00, showed a dry adiabatic lapse rate from the surface to about 600 m. with maximum temperature of 18 °C on the coast and 23° inland. On 15 June the midday radiosonde from Hemsby, Norfolk, also revealed convection to about 1000 m. with a maximum temperature of 24°. The observed distribution of pollen and Cladosporium spores agreed well with this analysis and therefore supported the supposition that, near the coast, the aircraft sampled spores produced locally that morning (15 June) and perhaps in France the previous day, but that in the distant half of the track the aircraft moved into the spore cloud produced over south-west England on 14 June.

Had damp-air types followed their usual periodicity, they should have alternated, rather than coincided, with the dense portions of the pollen and Cladosporium spore clouds, provided that they remained suspended in air near the ground long enough to experience convection the following morning. On this occasion air sampled at 'I' should have almost cleared the Norfolk coast by 00.01, 15 June, before night-liberated spores would usually be prevalent. Air sampled slightly farther west at GK would probably have crossed the coast later and farther south and east; more spores would be expected here and more were found. At the south-west end of the flight path some spores were found and could have come from south-east England earlier that morning, but had they done so, even more damp-air spores would have been expected at points on Fig. 4 between CO and EM but there were none, a lack unexplained by the catches.

In this flight, spore concentrations were not large, with Cladosporium and pollens most common and next Ustilago (maximum 400/m.^{3} at 600 m. in ascent G), which was distributed similarly to Cladosporium. Other dry-air liberated spores included Alternaria, Botrytis, Epicoccum, Erysiphe, Polythrincium and Torula, but they were few. Ascospores were the commonest of the damp-air groups occurring up to 1800 m. in most profiles, and others were occasional.

Flight 1/63; 11.55–14.45 G.M.T., 17 July 1963

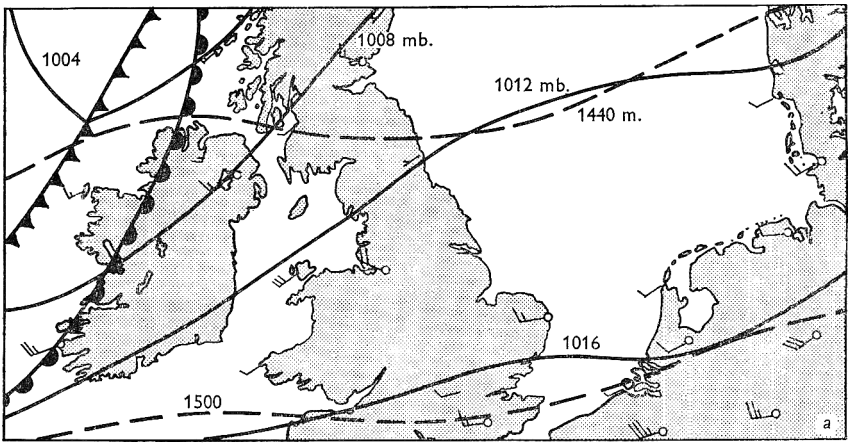
A flight twice as long as Flight 1/62 was planned in the hope it would pass through the spore clouds of more than one day, but the range of the aircraft was not enough to use the saw-tooth flight plan on both the outward and return legs. To keep well clear of the Dutch coast a downwind track (Fig. 5*b*) of 070° was flown from Flamborough Head, but unfortunately 8/8 stratocumulus cloud and rain prevented sampling for the first 100 miles; thereafter sampling was continuous to a position about 20 miles west of Esbjerg in Denmark. On the return leg towards Cromer, Norfolk, samples

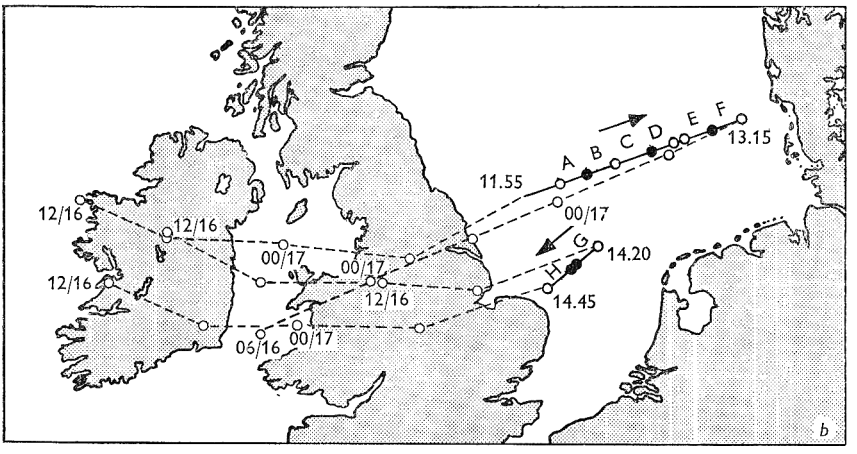
were taken only during a single descent and ascent within 100 miles of the coast, in an attempt to compensate for the loss of samples on the outward flight. The concentrations measured in profiles G and H did not match those in A but both are presented in Fig. 4*b* to illustrate differences existing only 100 miles and 3 hr apart. Temperature profiles (Fig. 4*b*) showed that air was unstable up to approximately 2000 m. above which there was stable air marked by a 'haze-top'. Spore concentrations were small on the outward leg, maxima 970/ m^3. for Cladosporium and 90/$m.^3$ for pollens in ascent F, and 200/$m.^3$ for the group of damp-air types in descent E, all beneath 760 m. More were caught on the return leg, with maximum concentrations in ascent H of respectively 3600, 170 and 110/$m.^3$ for the three groups.

The flight was made in a rather cloudy, maritime polar, west-south-westerly airstream, behind a cold front that had cleared England in the early hours and reached Denmark by midday on 16 July. No other fronts affected the British Isles until 06.00, 17 July, when rain reached much of Ireland and Scotland (Fig. 5*a*) but was too late to affect the air sampled during this flight. Air-flow was uniform in direction up to 2500 m. and saturated adiabatic lapse rate occurred up to inversions at 2500 m. at Liverpool and 1500 m. at Camborne at both 12.00 and 23.59, 16 July. During the flight, at 12.00, 17 July, there was a dry adiabatic lapse rate up to 1500 m and an isothermal layer above at Hemsby; temperatures taken over the sea in flight showed a stable layer just a little higher than the highest samples shown in Fig. 4*b*. Air trajectories were therefore estimated at 850 mb. (about 1500 m.) where winds were generally from the south-west at 20 knots (37·1 km./hr) or less (Fig. 5*b*).

Assuming, as in the analysis of Flight 1/62, that Cladosporium and pollens would most commonly be liberated and ascend between 09.00 and 18.00, an attempt was made in Fig. 5*c* to depict how the duration of daytime overland travel was related to the flight path. This track forms the lower margin of the hollow polygons and distance from it indicates the proportion of the period 09.00–18.00, 16 July, for which the air was estimated to have been over land. The small black triangle, displaced from the flight path, indicates the local contribution from Norfolk on the morning of 17 July. Thus more Cladosporium and pollen was expected near the west end and at the eastern extremity of the outward flight, and the trajectories suggest that spores represented by the left-hand polygon came from Ireland and by the right-hand polygon came from England and Wales. In a cool maritime polar air mass it is neither surprising that catches were rather small nor, in view of the instability up to 1800 m., that spore concentration varied so little with height. More spores were found in profiles G and H and they could have come from sources in Ireland during daytime on 16 July and eastern England early on 17 July. The greater catches in H than A perhaps resulted from longer travel over England and particularly over East Anglia on the morning of 17 July.

Except during or after rain, the damp-air types are liberated most frequently from 22.00 to 06.00. Thus, on the outward course, the number of damp-air spores derived from England on the night 16–17 July would be expected to increase betwen A and C and then to decrease to nothing at F, but most occurred at A, E and F. Spores at E could have come from Ireland on the night of 15–16 July, but their origin cannot be determined certainly and it is also unknown how much the concentration of these and other spores was decreased by rain wash near the English coast. Interpretation was also made difficult by Ireland and England behaving as tandem sources.





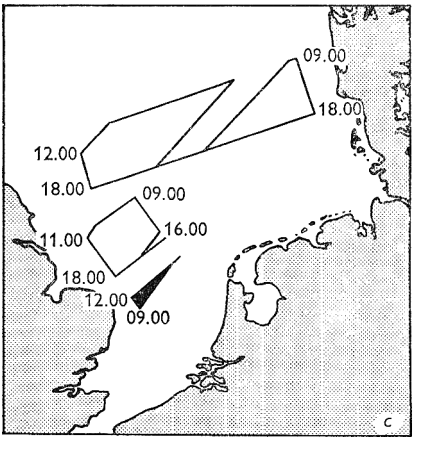


Fig. 5. For legend see opposite page.

Flight 2/63; 09.47–12.22 G.M.T., 31 July 1963

When this flight was planned on 30 July 1963 southerly winds were expected to continue over the sea north of the Wash. However, easterly winds extended farther north than expected and occupied the whole of the flight path for 360 miles north from King's Lynn to a position approximately midway between Wick and Stavanger (Fig. 6*c*, *d*). Thus the flight provided a cross-wind section up to 1800 m., instead of measuring depletion of spore clouds downwind. The return flight was on a reciprocal course at 1800 m.

Except near the coast (A and B, Fig. 7) temperature lapse rates were small or negative in the lowest 300 m. and also above 1500 m. in profiles A to E. Thereafter the base of the upper stable layer descended to mark a strong inversion at 900 m. (J, K) and finally rose to approximately 1500 m. at the northern extremity of the flight. The isospore diagram (Fig. 7) shows a considerable concentration of Cladosporium below 1000 m. near the Wash (A and B), and beyond this, concentrations were small until K to O. Smaller spore concentrations occurred at the temperature inversions of the upper stable layer, between L and O, than above or below. Pollen is not shown on Fig. 7 because few were caught and they were distributed similarly to Ustilago. Erysiphe, Epicoccum, Polythrincium, Entomophthoraceae, Helicospores and *Venturia inaequalis* occurred sporadically; Alternaria and Botrytis were more common (max. 160/m.^{3}). The damp-air group occurred widely but was common only in profiles B and L to Q. Ascospores (max. 580/m.^{3}) and hyaline basidiospores (max. 670/m.^{3}) were most common in ascent Q, whereas most Sporobolomyces (max. 670/m.^{3}) and Tilletiopsis (max. 250/m.^{3}) occurred at A, B and M. A few *Puccinia graminis* uredospores were caught in samples indicated by large dots on Fig. 7 (Cladosporium). On the outward flight 14 uredospores were caught in 111 samples, representing a mean concentration of 3/m.^{3} and including a maximum of 60/m.^{3} in one sample. During the return on the reciprocal course at 1800 m., 5 uredospores were caught in 20 samples representing an average concentration of about 6/m.^{3}.

Synoptic charts for the surface and 700 mb. levels are shown for 31 July (Fig. 6*a*, *b*) and examination of these and earlier charts was supplemented by information from radiosonde ascents and the occurrence of 'sferics' (long wave radio emissions from lightning flashes, see *Handbook of Aviation Meteorology*, 1960, p. 183). Pressure was high over north-west Russia with a ridge across Norway towards England and a shallow thundery trough over the Baltic. Less confidence can be placed in the trajectories drawn for this flight (Fig. 6*d*) than for others, because the flight path was close to the line of the ridge where wind speed and direction were difficult to determine. The alternative possibility that the track lay just to the west of the ridge line was investigated but the general pattern of movement from the Eastern Baltic during the night of

Fig. 5. Weather and flight details of Flight 1/63. (*a*) Weather situation at 00.01, 17 July 1963. Details as for Fig. 1*a* but dashed lines represent 850 mb. contours and winds at this height (approximately 1500 m.). (*b*) Flight track in solid lines with direction arrowed and four figure times. Letters indicate sloping profiles, hollow circles are high-points and solid circles are near the sea. Dashed lines show air trajectories at the 850 mb. level. Estimated positions at 6 hr intervals are shown but only 12 hr intervals are marked with hour/date. (*c*) Diagram showing the estimated proportion of the period 09.00–18.00 spent overland by the air sampled at various points along the flight track. For explanation see text.

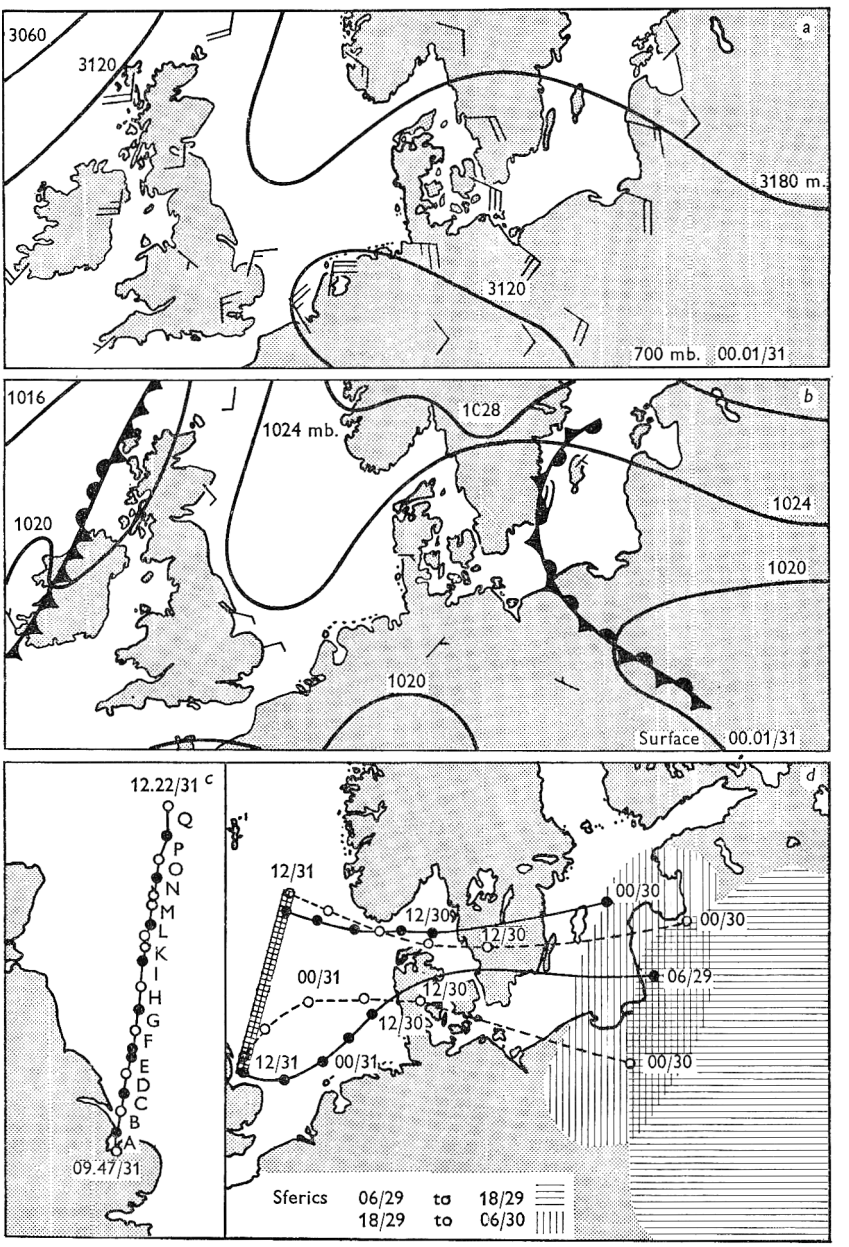


Fig. 6. Weather and details of Flight 2/63. (*a*) 700 mb. contours and winds at 00.01, 31 July 1963. (*b*) Surface weather map for 00.01, 31 July 1963. (*c*) Flight track with times and positions of profiles lettered, hollow circles were highest samples and solid circles were nearest the sea. (*d*) Flight track (shaded) and air trajectories to its extremities at 'surface' (approximately 500 m. in solid lines and circles) and at 700 mb. level (dashed line and open circles). Line shading indicates areas from which 'sferics' were detected at times and dates shown.

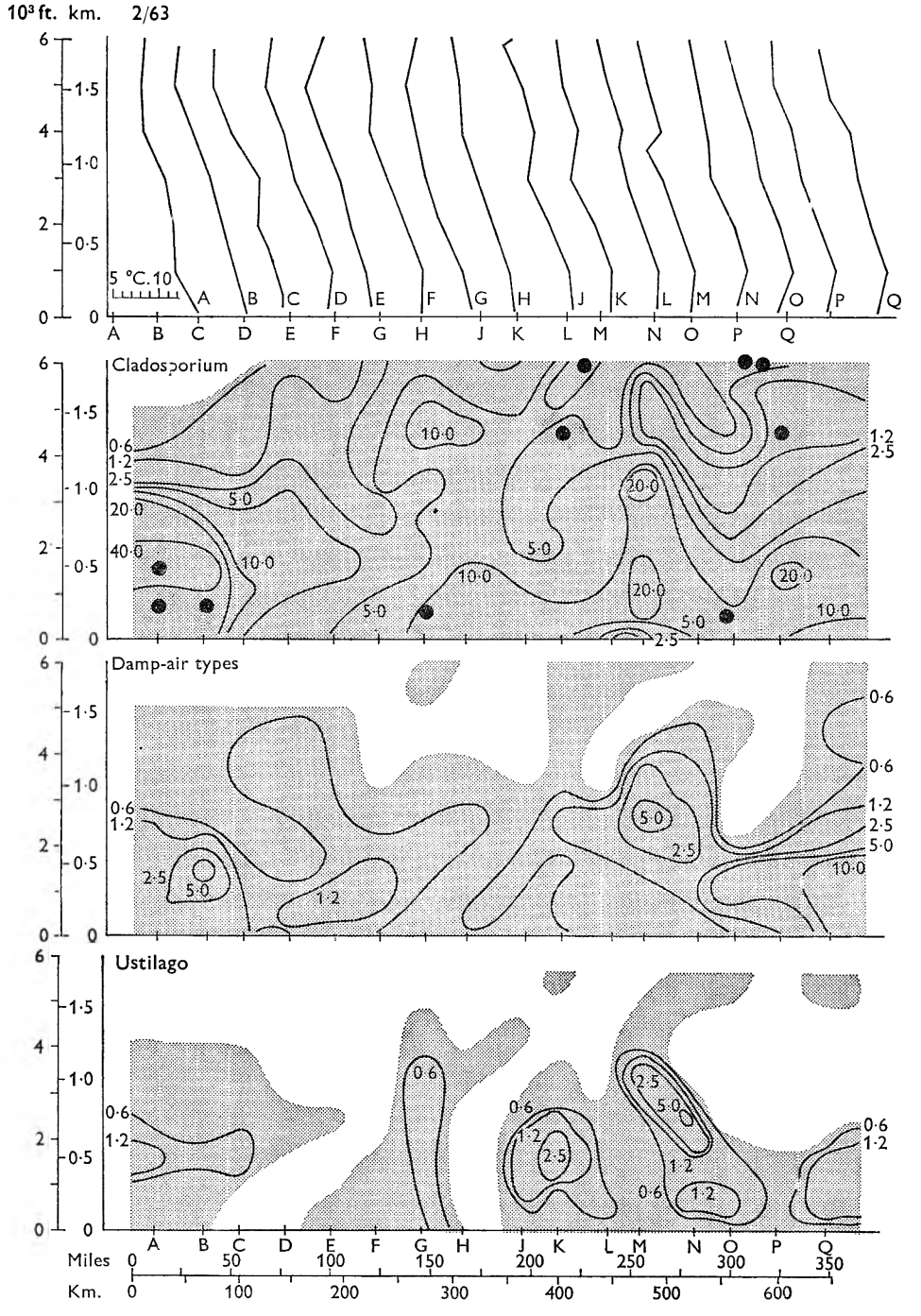


Fig. 7. Vertical temperature profiles (top) and isospore diagrams of Cladosporium, damp-air types and Ustilago in Flight 2/63 (details as Fig. 4). There were too few pollens and *Puccinia graminis* uredospores to show on separate diagrams but presence of the latter is indicated by black circles on the Cladosporium diagram.

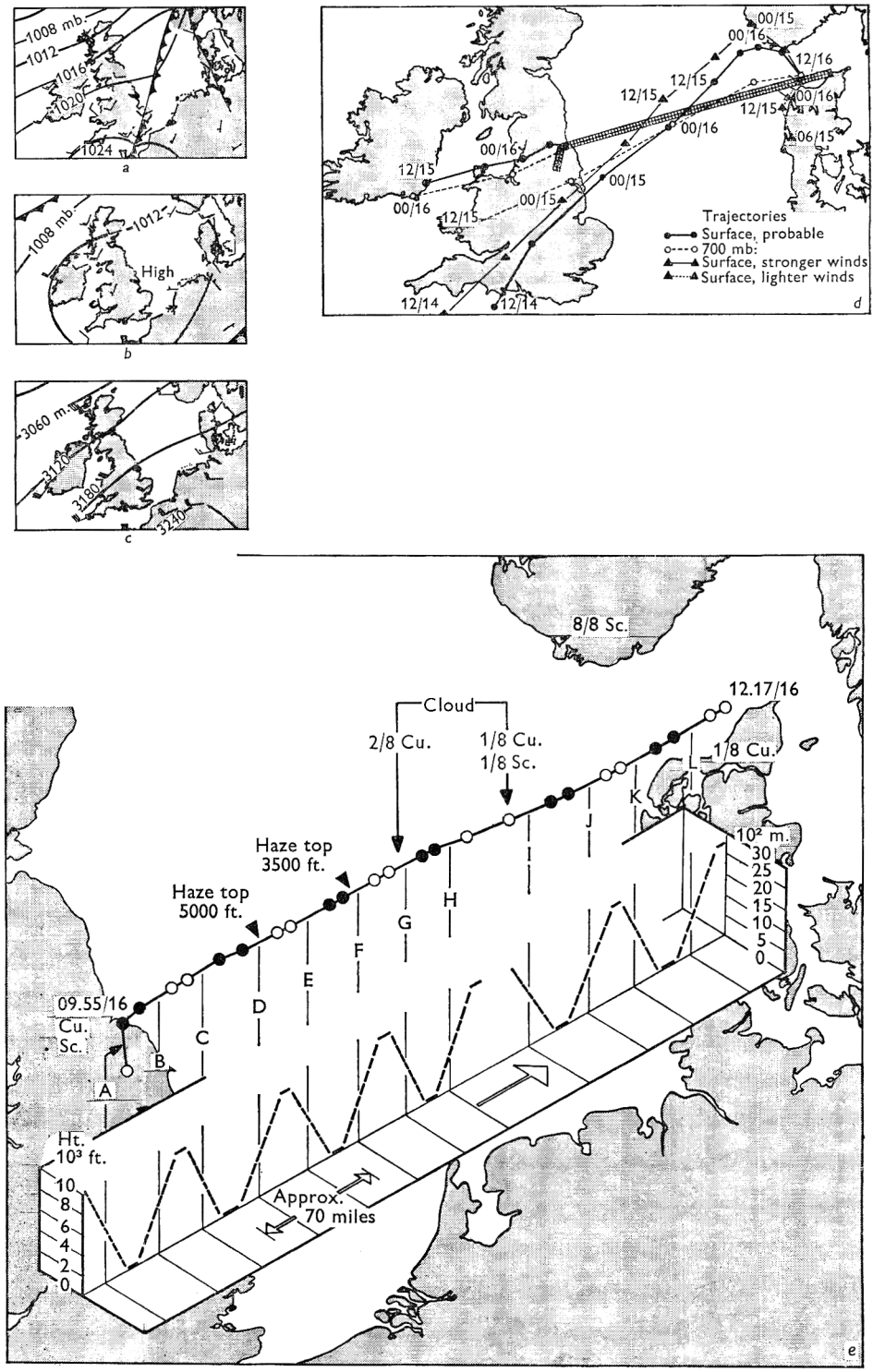


Fig. 8. For legend see opposite page.

29–30 July remained valid. In this area during the preceding 24 hr widespread sferic activity indicated vigorous convection and thunderstorms, which could have distributed spores favourably for distant transport.

The temperature profile measured from the aircraft during A was confirmed by the 12.00 radiosonde ascent from Hemsby, which revealed stability between 300 m. and 600 m. and above 1500 m. The temperature profile measured during Q also agreed closely with that measured upwind at Sola, Norway, where stable air below 300 m. gave place to unstable air up to about 3000 m.

Scarcity of information from possible source areas, the probability that spore concentrations may have been modified by precipitation, and doubts about trajectories combine to limit the conclusions that can be drawn from this flight. The distribution of Cladosporium and *Puccinia graminis* uredospores suggests they may have come from the same places but it is surprising that Ustilago, which is also commonly derived from grasses and cereals, was commonest beneath the inversion at 900–1200 m. between L and O. The scarcity of pollens is not surprising because little was probably liberated during the thundery weather in possible source areas. The reasons for the relative scarcity of spores in the southernmost third of the flight, the prominence of dry-air types above 600 m. in the centre and of damp-air types at the northern extremity, remain unexplained. The spore profiles and isospore diagrams seemed closely related to temperature distributions; the smallest concentrations occurred in the isothermal layer above 1500 m. at A and B, directly above the greatest concentration of Cladosporium. Proximity suggests these could have come from East Anglia but with stable air just above 300 m. both during the flight and at Hemsby it is difficult to see how so many could have risen so far so quickly; also the *P. graminis* uredospores accompanying them are most unlikely to have come from this area.

Flight 1/64: 09.45–12.15 G.M.T., 16 July 1964

During this flight sampling began with a descent, A, from near York to Redcar and then on a track of 060° for about 400 miles into the Skagerrak. The flight was again of 'saw-tooth' pattern but the ascents to 2700 m. were higher than previously (Fig. 8*e*). The pilot reported well broken cloud with a haze top at 1500 m. in the west and about 1000 m. farther east. These observations agreed with the irregular temperature profiles at the start of the flight but towards the centre the profiles suggested that the haze top was probably diffuse.

Few spores were caught above 1800 m. so the isospore diagram (Fig. 9) is limited to this height. It shows large Cladosporium concentrations near the start, mostly below 900 m., fewest about 150 miles out, followed by unusually large concentrations centred 1000 m. above sea level and 175–275 miles from the English coast (see also

Fig. 8. Weather and details of Flight 1/64. (*a*) Surface chart for 12.00, 15 July 1964. (*b*) Surface chart for 12.00, 16 July 1964. (*c*) 700 mb. chart for 12.00, 16 July 1964. (*d*) Flight track shaded and air trajectories to near each end at 'surface' and at 700 mb. level. Additional surface trajectories marked with triangles show the effect expected from winds both stronger and weaker than those assumed in the probable trajectory (see text). (*e*) Composite diagram showing, above, the track of Flight 1/64 and below, an isometric diagram of the saw-tooth flight plan. Open circles indicate high-points and solid circles near the sea, letters indicate ascents and descents and refer to the diagram below where each dash represents a sample.

Fig. 3*b*). Pollen was most concentrated 600 m. high and at 190 miles from the coast. By contrast, the damp-air types were most numerous in two discrete areas, on either side of the main Cladosporium cloud, respectively 50–125 and 300–350 miles from the coast.

From 14 July pressure was high from Spain to Central Europe and fronts that crossed the British Isles on 14 and early 15 July were followed by rising pressure (Fig. 8*a*).

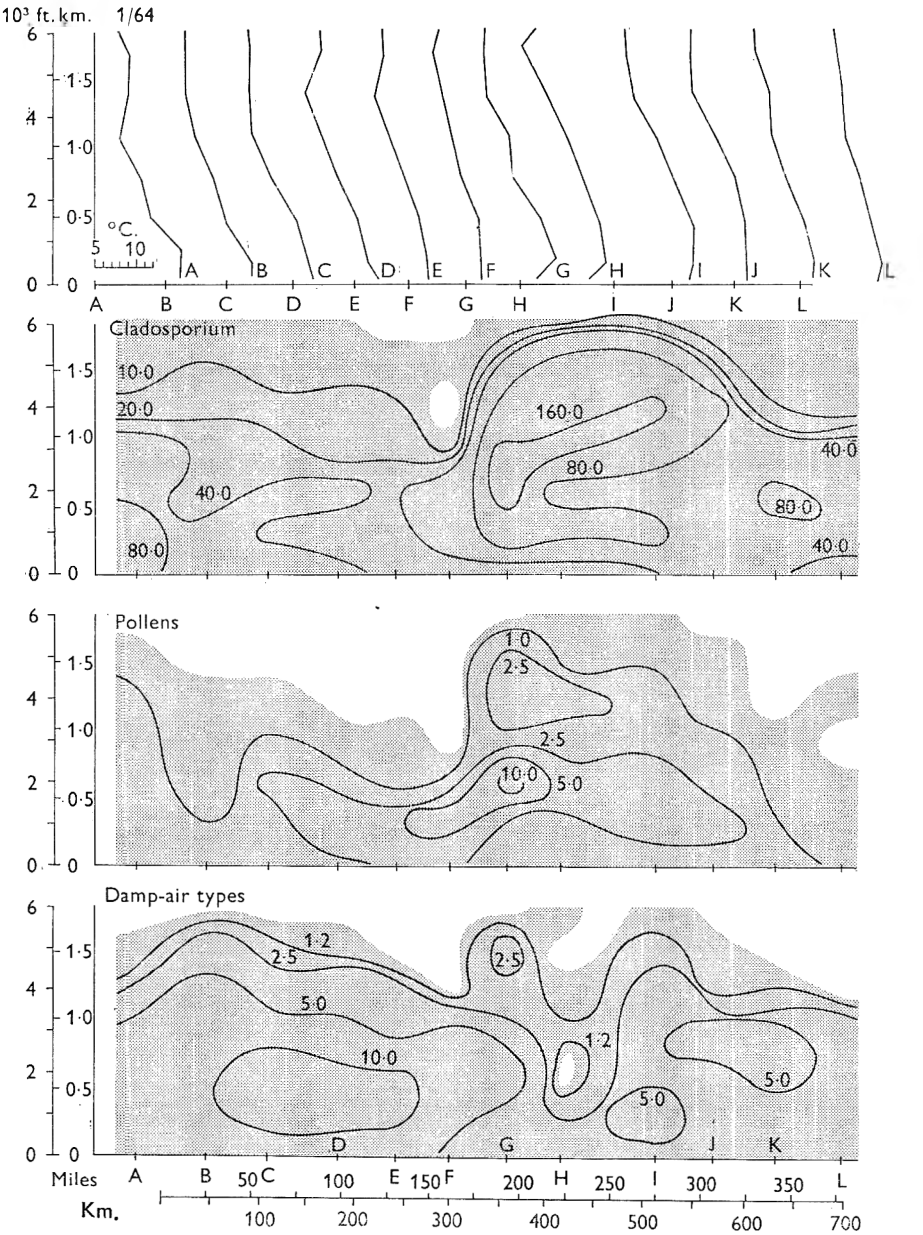


Fig. 9. Vertical temperature profiles (top) and isospore diagrams of Cladosporium, pollen and damp-air types in Flight 1/64 (details as Fig. 4).

A fairly definite south-westerly surface airstream on 15 July gave place by 16 July to lighter and more variable winds (Fig. 8*b*), which made back-tracking uncertain at low level although it could be done more reliably at 700 mb. (approximately 3000 m., Fig. 8*c*).

At first an attempt was made to explain the observed spore distribution by low-level winds. At the Yorkshire coast at 10.00 on 16 July, surface air would have crossed England after midnight (Fig. 8*d*), so the dense Cladosporium cloud low over the land near A could have been liberated east of the Pennines that morning. Spores liberated the previous day in North Wales or the south coast of Ireland may also have contributed to the cloud. Forced lifting by air flowing over the Pennines may have helped to disperse these spores upwards. Between C and E (Fig. 8*c*) trajectories suggested that surface air would have crossed England and Wales by night, and there were least dry-air types and most damp-air types in this region. Day-liberated spores would have been expected to be most common between E and G, and concentrations did increase in this region but much of each spore cloud was further east. Few damp-air types would be expected in the area just beyond the mid-point, and few were found. Again few night-liberated spores were expected or found at the eastern extremity L, suggesting that the South coast of Norway was not an important source. Some day-liberated spores released from southern England during the afternoon of 14 July might have reached L, but concentration did not increase there.

Spore distributions, particularly near the middle of the flight, were not adequately explained by low-level winds, so it was decided to consider higher winds. Upper air charts for 700 mb. showed that spores released from England and Wales the previous day would have reached the north-eastern end of the track by 12.00, 16 July (Fig. 8*d*). and air sampled at the mid-point would have crossed the land during the night. The discrepancies between the observed distribution and the distributions suggested by upper and surface winds were in different directions along the flight path, so it seemed possible that winds at an intermediate height might explain the observed distribution more accurately.

Over land the previous day, radiosonde ascents at Liverpool showed weak inversions between 900 and 1500 m., but these were too shallow to prevent penetration by active convective bubbles, so with maximum surface temperatures of 20 °C spores could easily have been lifted to 2000–2500 m. and some possibly even higher. At 3000 m. winds were often 3 to 4 times as strong as at the surface. The main wind shear occurred below 1500 m. and between 1500 and 3000 m. speeds differed by only 5–10 knots. It is therefore reasonable to assume that much of each spore cloud raised by convection on 15 July would have been carried east by winds intermediate in speed between those at the surface and those at 700 mb. Indeed the sloping axis of greatest concentration in the Cladosporium and pollen clouds is what would be expected from exposure to stronger winds at increasing height.

The flight was in a region of very light surface winds and much uncertainty existed, particularly at the eastern end, for the first few hours of back tracking. The probable surface track already mentioned assumed a moderate flow of about 6 knots, and winds of 8 knots would have made little difference (Fig. 8*d*) but very light winds (2–3 knots) would have altered the source area to West Denmark early the previous morning (Fig. 8*d*). There the weather was dull with light winds and little convection, so the scarcity of day-liberated spores at the east end of the flight path was not necessarily

evidence against an air trajectory from Denmark. Again assuming light winds, the air sampled at points farther west along the flight path would have come from Germany with less likelihood of high spore concentrations, but the diagrams show that day-liberated spores were more frequent here. Thus the distribution of spores in 'surface' air supports the theory that the British Isles were the source of the spores sampled even at L.

DISCUSSION

Many airborne fungal spores were detected hundreds of miles downwind from land in each of these flights. As we tried to select dry weather for flights we can claim no more than that long-distance transport occurs frequently in summer. Such observations will be of limited value to plant pathologists until we know how many of the spores are viable. At present there is no satisfactory routine method for growing cultures from trapped spores of saprophytes, or testing viability of obligate parasites. Until these deficiencies are remedied we may learn something from laboratory experiments which simulate the heights, temperatures and durations of flight of spores.

Hirst *et al.* (1967) found that, at 3000 m., spores were often too few to be detectable with 30–60 l. air samples. The saw-tooth flight plan therefore went high enough to detect most spores and provided a valuable compromise between range and flight duration. In interpreting the results we regarded the ascents and descents as though they were vertical profiles, but it must be remembered that in both the flight path was only 3° from horizontal and that the liberties we took in interpreting profiles would be permissible only in long flights over the sea. 'Contouring' the density of spore clouds, by constructing 'isospore' diagrams of vertical sections above the sea, simplified the demonstration of quite well-defined spore clouds, although it distorted their shape in the diagrams by contracting distance 140 times more than height.

In retrospect, it is not surprising that clouds of day- and night-liberated spores produced from the British Isles should be detectable, downwind over the sea, 1 or 2 days later. But when, in Flight 1/62, spores became more numerous as distance from the coast increased and, especially of Cladosporium, with increasing altitude, the reason was not immediately apparent. The explanation we propose is well supported by the meteorological analyses which assumed that spores were liberated at their usual time of day. In all flights attempts to track spore clouds back to their sources left some features unexplained; sometimes the trajectories were in doubt, distant sources did not always seem to have produced the expected number of spores at the usual times, or, in some flights, position, wind speed and direction made it difficult to identify source areas (for example, to discriminate between England and Ireland). Because of these difficulties many more flights would be required to prove the theory which now seems probably correct.

Most profiles showed sign of 'erosion' near the surface and the isospore diagrams showed maximum concentrations between 600 and 1200 m., which were 2 to 4 times greater than those of the surface air below. The flights did not test how quickly deposition eroded spore clouds. Presumably the process is continuous, but, above land sources, is often masked by preponderant spore liberation. Slight support for this idea was given in previous ascents (Hirst *et al.* 1967) when erosion of pollen and Cladosporium profiles, although usually detectable, was greatest at night. The shape of vertical profiles measured from aircraft must depend on the activity of vertical mixing during spore ascent and transport, in addition to the rate of deposition.

Thorough turbulent mixing did not occur during these flights, or spore concentrations would have been more nearly uniform with height.

The effects of temperature inversions seem complex, the simplest example is the limitation of upward spore transport by a pre-formed inversion. The development of inversions within existing spore clouds leads to more complicated profiles. Such inversions often develop close to the ground at night when convection is minimal, but spore deposition by settling or frictional turbulence may still proceed; thus the concentration of day-liberated spores is decreased, and most occur above the inversion, in the past erroneously interpreted as suggesting that spores accumulated above inversions (Rempe, 1937). Similar effects accompany inversions common near sea-level by day, because the stable air decreases turbulent diffusion of spores downward to replenish the concentration in surface air diminished by deposition. Spore concentration also decreased within stable air well above ground (see Figs. 4–8). Irregular profiles would be expected to result from shearing between air strata moving differently in speed or direction (see Flight 1/64).

Several flights provided evidence of gravitational stratification according to spore size. Pollen clouds were centred 300–600 m. lower than those of Cladosporium which is liberated at about the same time of day and ascends similarly in convection (Hirst *et al.* 1967) but has a much smaller terminal velocity. Presumably both types of spore would be equally diffused downward by eddies and deposited continuously but a greater proportion of the pollen cloud would be lost because these spores would fall faster into the air near the surface and be deposited from it. In addition to this separation of the main pollen and Cladosporium clouds, Flight 1/62 indicated a similar stratification in the few Cladosporium spores at 1650 m. and about 300 m. lower in the corresponding pollen profiles (Fig. 2).

The discovery of damp-air liberated spores at up to 1800 m. is of particular interest. Although these spores can be liberated on wet days, we attributed them to night-liberation in periods when little or no rain fell on the British Isles. Diffusion of night-liberated spores would seldom be helped by active convection during the period of release, and over England frictional turbulence seems unlikely to raise them to this height. An alternative explanation, proposed by Hirst (1953) to explain an abrupt decline of spore concentrations near the ground soon after dawn, is that, because of their small size, many remain suspended near the ground until dispersed upwards by convection soon after dawn. This possibility could be tested by sequences of ascents at different times of day and night, but we have been unable to repeat those made in 1961 (Hirst *et al.* 1967) when too few damp-air spores were caught to construct reliable profiles. Nor do we yet know enough of the biology of the organisms to say whether those disseminated are wasted or whether smallness is an advantage, which by delaying deposition allows distant transport of night-liberated spores.

Although we do not know whether spores can be carried across the Atlantic, it seems safe to assume that only a few would remain in trans-Atlantic air approaching the British Isles. To study depletion of spores over the North Sea we needed winds between south and west, and except for the British Isles, North-west France was probably the only potential European source of spores caught in Flights 1/62, 1/63 and 1/64.

Uniform spore concentrations would occur at the downwind coast of a great land mass liberating spores at a constant rate. However, Cladosporium and pollens are

liberated chiefly between 09.00 and 18.00 and our flights were made downwind of an island source which winds often crossed in 10 or 12 hr. Therefore the day-liberated spores would be carried clear of the downwind coast during the night to cross the sea as a discrete cloud, the origin of which could be determined. Spores released between 22.00 and 06.00 would alternate with but partly overlap the day-liberated spore clouds. Clouds released daily from larger sources or with slower winds would not be discrete, whereas smaller sources or faster winds would not allow such large concentrations of spores to accumulate and so would make the clouds less easy to recognize.

Though we were fortunate in working from an island source with size and winds apparently near the optimum for distinguishing daily spore clouds, the variability of spore concentrations leaving the coast seriously interfered with the original purpose of the flights, which was to measure the depletion of spore clouds. The proportion of spores deposited can only be measured when their initial concentration is determined by a sequence of ascents over the downwind coast. Without this information the rate of deposition cannot be measured by estimating the total spores represented by profiles at different distances from shore; neither can the shape of profiles, the maximum concentration nor the height at which this occurs be used, because these are continuously modified by erosion at the base and by turbulent mixing. Longer overland travel should make the spore clouds leaving the coast more uniform so that similar flights downwind of a continent might permit depletion to be measured accurately over greater distances.

Occasional eroded profiles have previously been reported, but our flights suggest they are typical remote from sources and also how they may arise. Depletion of spores in air near the surface has several important implications, because ground level spore traps must operate in a stratum partially depleted of far-travelled spores (see Hirst *et al.* 1967) and cannot be relied upon to measure accurately the concentrations passing overhead or even to show how this concentration varies with time. Until there are more measurements of atmospheric transport mechanisms, the relative contribution of distant and local sources to the catches of surface spore traps or to the diurnal periodicities of catches cannot be assessed. However, with westerly winds, the incursion of relatively spore-free Atlantic air is likely to influence diurnal periodicities of tropospheric spore concentrations considerably in the United Kingdom, and the effect may perhaps help to explain the difference among observations of Cladosporium periodicities in different parts of the world (see Hirst, 1953; Hamilton, 1959; Pady, Kramer & Wiley, 1962; Rich & Waggoner, 1962). Although we have rather few flights on which to report, the regularity with which many spores were detected far from land, together with previous interceptions (Hirst *et al.* 1967), suggests that long-distance transport is frequent during fine summer weather. The spores carried are so numerous that, if viable and effectively deposited by rain, even minor constituents of the cloud could have profound effects on crops.

We thank the staff of the Meteorological Office who have helped in this work; especially the Director General for permitting the flights and publication; Mr R. F. Zobel, then Head of the Meteorological Research Flight and his predecessor Dr R. J. Murgatroyd; also Mr J. F. Fisher and Mr S. G. Cornford. We are also grateful to Mrs Maureen Parker for technical assistance and for her care in preparing the diagrams.

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The Action of Light on Nitrate and Nitrite Assimilation by the Marine Chlorophyte, *Dunaliella tertiolecta* (Butcher)

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SUMMARY

Light caused up to a 20-fold increase in the rate of nitrate and nitrite assimilation by the marine chlorophyte *Dunaliella tertiolecta*. While higher rates of oxygen evolution were observed during both nitrate and nitrite assimilation, the extra oxygen released was not related to the amounts of nitrate or nitrite assimilated. Carbon dioxide was required for light to increase nitrate or nitrite assimilation; the addition of glucose did not overcome this requirement. The light intensities at which nitrate and nitrite assimilation reached their maximum rates were 300 and 1000 ft. candles respectively, while oxygen evolution and carbon fixation required 1800 ft. candles. In the presence of 3-(4-chlorophenyl)-1,1-dimethylurea (10^{-4} M), nitrate and nitrite assimilation were still increased by 30 % in light, although oxygen evolution decreased to 2 % of the control rate. Assimilation of nitrate and nitrite in light was decreased to 50 % by sodium azide or potassium cyanide at $1–5 \times 10^{-5}$ M; oxygen evolution was less sensitive, requiring more than 3×10^{-4} M to decrease the evolution to 50 %. Azide and cyanide both blocked dark nitrate and nitrite assimilation but at slightly higher concentrations than those required in the light. Iodoacetate (10^{-3} M) decreased dark nitrate and nitrite assimilation to 2 % and 38 % of control values, respectively; but, in the light, nitrate assimilation was not affected and nitrite assimilation was decreased by only 35 % at this concentration. It is concluded that the action of light in stimulating nitrate and nitrite assimilation cannot be explained on the basis of a single site of action as has been proposed for spinach chloroplasts and that two relatively independent sites exist in the living *D. tertiolecta* cell.

INTRODUCTION

That light stimulates nitrate and nitrite assimilation in unicellular algae (Syrett, 1962; Kessler, 1959) and in green leaves (Burstrom, 1943) has been well documented since the original discovery by Warburg & Negelein (1920). The explanation of this effect is not yet agreed upon. Warburg attributed the increase in assimilation rate as a permeability effect, and believed that in both light and dark, the reduction of nitrate to ammonia was coupled to respiration, and that the extra carbon dioxide production accompanying nitrate assimilation in the dark came from increased carbohydrate breakdown. The extra oxygen evolution observed in the light during nitrate assimilation was postulated to come from photolytic splitting of this carbon dioxide. With some minor modifications Warburg still adheres to this interpretation (Warburg, Krippahl & Jetschmann, 1965).

Evans & Nason (1953) demonstrated that the reduction of pyridine nucleotides by

chloroplasts could be coupled to the reduction of nitrate by the addition of nitrate reductase. More recent work by Paneque, Ramirez, Del Campo & Losada (1964) and Losada, Ramirez, Paneque & Del Campo (1965) showed that isolated spinach chloroplasts could reduce both nitrate and nitrite to ammonia in the light or, when a bacterial hydrogenase and hydrogen were added, in the dark (Del Campo, Paneque, Ramirez & Losada, 1965). This work strengthened an alternative theory of the action of light on nitrate assimilation proposed by van Niel, Allen & Wright (1953). This was that light increased the rate of nitrate reduction by providing directly increased amounts of photoreductant, rather than in the indirect manner proposed by Warburg. The extra oxygen production observed in the light was postulated to come from the water-splitting reaction and increased rates of electron flow resulted from the extra electrons going to reduce nitrate after the requirements of the carbon fixing system were saturated. Evidence for this was provided by the finding that nitrate assimilation inhibits carbon dioxide assimilation at low light intensities but not at high intensities. The work of Losada *et al.* (1965) and Paneque *et al.* (1964) indicated that a flavoprotein was the photoreductant for nitrate assimilation and that ferredoxin was the photoreductant in nitrite assimilation.

In spite of the apparently strong evidence in favour of the theory of direct action of light, there are still some experimental results which are difficult to explain by this theory. The most important is the requirement for carbon dioxide, if nitrate and nitrite assimilation in the light are to proceed at maximum rates (Davis, 1953; Kessler, 1964; Warburg *et al.* 1965). There is also a report by Bongers (1958) that nitrate and nitrite assimilation inhibit carbon fixation even at light intensities which are saturating for photosynthesis. Further, Hattori (1962) reported that nitrate and nitrite assimilation saturate at lower intensities than does oxygen evolution. Because of these anomalies it was felt that further experimental work on the action of light on nitrate and nitrite assimilation should be done. In the present paper, results obtained with the green unicellular alga *Dunaliella tertiolecta* are presented. This alga is an inhabitant of salt water estuaries and belongs to the order Volvocales; the green algae investigated to date belong to the order Chlorococcales. It was hoped that in addition to providing data on the fundamental problem of nitrate assimilation, some comparative data for other species of green algae might be obtained.

METHODS

Organism. A strain of *Dunaliella tertiolecta* (Butcher) obtained from Dr Mary Parke, Plymouth, U.K., was used in these experiments.

Culture methods. Organisms were grown in batch cultures of 2 l. contained in 5 l. Haffkine flasks. The composition of the medium was as follows: filtered sea water, salinity 28–30‰, 950 ml.; distilled water, 50 ml.; KNO_3, 75 mg.; $NaH_2PO_4.2H_2O$, 10 mg.; Fe citrate, 1·5 mg.; citric acid, 1·5 mg.; $MnSO_4.4H_2O$, 360 μg.; $ZnSO_4.7H_2O$, 44 μg.; $CoCl_2.6H_2O$, 20 μg.; $CoSO_4.5H_2O$, 19 μg.; thiamine hydrochloride, 200 μg; biotin, 1 μg.; vitamin B_{12} (cobalamin) 1 μg. The vitamins and phosphate were sterilized separately in the distilled water and added to the remainder of the media after cooling.

Growth conditions. Cultures were grown under illumination from fluorescent lamps which provided a light intensity of 400 ft. candles, and gassed with a slow stream of air +5 % (v/v) CO_2. Temperature was maintained at 22° ± 1°.

Preparation of experimental suspensions of organisms. Organisms were harvested whilst in their logarithmic growth phase (3–4 days) by centrifugation at 500 *g* for 20 min. at 5°. Packed organisms were resuspended in 100 ml. of 3 % (w/v) NaCl solution and centrifuged. This was repeated with 50 ml. of NaCl solution and after this organisms were resuspended in sufficient NaCl solution to provide a concentration of 0·4–0·5 mg. total chlorophyll/ml. The suspension was then held for 30–60 min. in the dark at room temperature before further use. The concentration of chlorophyll used was equivalent to $5–8 \times 10^7$ organisms/ml. suspension. (No damage to cells was observed by this preparation and cells were motile at time of each experiment.)

Measurements. Chlorophyll was determined by the method of Whatley & Arnon (1963). Total cell nitrogen was determined by Kjeldahl digestion followed by estimation of ammonia by the method of Newell & Dal Pont (1964). Numbers of organisms were determined by counting with a Petroff-Hauser slide after immobilizing with one drop of Bouins fluid/ml. diluted suspension. Nitrate and nitrite assimilation was determined by measurement of nitrate or nitrite present in the sample of reaction mixture from which the cells had been centrifuged before and after the experimental period. Nitrate was estimated by reduction to nitrite on a cadmium-mercury column (Morris & Riley, 1963) with the ammonia+ammonium chloride buffer of Grasshof (1964). Nitrite was estimated by pipetting the sample of reaction mixture directly into 3·5 ml. of 1 % (w/v) sulphanilic acid in 2·5 N-HCl, removing debris after 5 min. by centrifugation and adding a sample to a suitable volume of 0·02 % (w/v) *N*-1-naphthylethylenediamine and determining the extinction at 540 nm (mμ). Carbon assimilation was determined by adding 1 μmole $Na^{14}HCO_3$ ($4·2 \times 10^4$ dpm) to the reaction mixture at the beginning of the experiment, and then at its conclusion pipetting a sample into 5 ml. 5 % (v/v) 10 N-HCl in ethanol. The solution was warmed to expel dissolved CO_2 and a sample counted in a liquid scintillation counter, with the solvent described by Bray (1960).

Experimental conditions. Nitrate and nitrite assimilation measurements were done in a Warburg apparatus at 20°. Illumination was provided by incandescent lamps and was between 2500 and 3000 ft. candles intensity. Gas exchange was measured during the experiments. The complete reaction mixture in the flasks contained the following: suspension of organisms 1·0 ml.; tris buffer (pH 7·6), 125 μmoles; KNO_3 or KNO_2, 2·0 μmoles; $NaHCO_3$ 40 μmoles, and 3 % (w/v) NaCl, to total volume of 2·0 ml. Where carbon dioxide was omitted, $NaHCO_3$ was omitted and 0·15 ml. of 20 % (w/v) KOH added to centre well. Nitrate or nitrite was added to the reaction mixture at zero time by tipping from the flask side arm.

RESULTS

The effect of light on nitrate and nitrite assimilation by Dunaliella tertiolecta

Light increased the rate of nitrate and of nitrite assimilation by *Dunaliella tertiolecta*, although the increase varied from one batch of organisms to another. During the experimental period both dark and light assimilation were linear. Although the rates of both nitrate and nitrite assimilation varied between wide limits the rate of nitrite assimilation was generally higher. This agreed with the observation that nitrite did not accumulate in the suspending medium when nitrate was being assimilated.

Gas exchange accompanying nitrate and nitrite assimilation. Although in all but one of the experiments recorded in Tables 1, 2 and Fig. 1, nitrate or nitrite assimilation was accompanied by increased oxygen evolution, there was no consistency between the amount of extra oxygen evolved and the amount of nitrate or nitrite assimilated. For the purpose of discussion it is reasonable to assume that nitrate (or nitrite) assimilated was the equivalent of nitrate reduced, for the following reasons: (1) *Dunaliella tertiolecta* did not store either nitrate or nitrite; (2) excretion of nitrite or other products of incomplete nitrate reduction into the medium was not observed (B. Newell, G. Dal

Table 1. *Effect of light on nitrate assimilation by Dunaliella tertiolecta and the resultant oxygen evolution*

Each vessel contained in a total of 2 ml. the following: 0·5 mg. chlorophyll, 125 μmoles tris-HCl buffer (pH 7·6), 40 μmoles $NaHCO_3$, and 0·5 or 0·7 mmoles NaCl. Where nitrate was supplied it was as 2 μmoles KNO_3. The experimental period was 30 or 40 min. All values are expressed as μmoles/hr/mg. chlorophyll.

	NO_3 uptake		O_2 evolution		Extra O_2/NO_3
Expt.	Light	Dark	Control	$+NO_3$	uptake
32	2·18	0·92	38·4	39·2	0·6
53	1·62	0·14	51·6	58·2	4·5
55	3·34	1·30	31·2	30·4	−0·8
57	0·75	0·43	41·2	41·6	1·3
64	3·20	0·60	32·1	42·3	3·9
79	3·68	0·00	63·6	71·3	2·1
92	2·75	0·00	68·0	70·0	0·7
93	3·38	0·00	92·0	100·0	2·4
94	3·10	0·51	51·7	56·5	2·2
95	3·00	0·25	43·0	50·0	2·5

Table 2. *Effect of light on nitrite assimilation by Dunaliella tertiolecta and the resultant oxygen evolution*

Experimental conditions were as for Table 1 except that 2 μmoles KNO_2 were substituted for KNO_3. All values are expressed as μmoles/hr/mg. chlorophyll.

	NO_2 uptake		O_2 evolution		Extra O_2/NO_2
Expt.	Light	Dark	Control	$+NO_2$	uptake
71	2·00	0·60	38·0	40·0	1·4
79	8·00	3·85	38·5	50·0	2·7
80	6·80	3·66	55·8	61·0	1·6
88	2·30	0·00	118·0	125·0	3·2
99	2·62	0·86	33·5	37·2	2·1
100	5·25	1·59	59·3	79·5	5·5

Pont and B. R. Grant, unpublished observation). The gas evolution rates presented in these experiments were linear during the experimental periods; neither nitrate nor nitrite was exhausted in that time. Gas evolution has been corrected for gas uptake in dark controls in these experiments. Whilst it was not possible to test in each experiment that the gas evolved was oxygen, two separate experiments with acid chromous chloride in the side arm showed no gas evolution and the chromous chloride rapidly changed colour from blue to green.

Requirement for carbon dioxide. When bicarbonate was omitted from the reaction

mixture and potassium hydroxide was added to the centre well of the reaction flask neither nitrate nor nitrite assimilation was increased by light. Although in these experiments some gas evolution took place during the first 10 min. of illumination, it ceased abruptly at or before 10 min. Since the controls without nitrate or nitrite behaved in the same manner, this was not due to assimilation of nitrate in the absence of CO_2. Since the gas evolution and nitrate assimilation decreased with longer dark pre-incubation periods, it was probably due to small amounts of carbon dioxide in the system (Table 3). Glucose (10 μmoles/vessel) added to the reaction mixture did not overcome the requirement for carbon dioxide, although it increased nitrate assimilation and O_2 production (Table 4).

Table 3. *Effect of carbon dioxide on nitrate and nitrite assimilation by Dunaliella tertiolecta*

Each vessel contained in a total of 2 ml. the following: 0·5 mg. chlorophyll, 125 μmoles tris-HCl buffer (pH 7·6), 2 μmoles KNO_3 or KNO_2, 0·5 or 0·7 mmoles NaCl. Control vessels contained, in addition, 40 μmoles $NaHCO_3$ while CO_2-free vessels contained 0·15 ml. 20 % KOH in centre wells. Experimental period 40 mins. All values given as μmoles/hr/mg. chlorophyll.

	NO_3 assimilated	O_2 evolved	NO_2 assimilated	O_2 evolved
Air + CO_2 + light	2·50	68·0	4·00	48·2
Air − CO_2 + light	0·10	—*	1·00	—*
Air + CO_2 + dark	0·00	−6·4	1·20	−3·3
Air − CO_2 + dark	0·00	−7·4	1·20	−6·0

* A total of 1·5 μmoles O_2 was evolved in the experiment with NO_3 and 3·5 μmoles O_2 with NO_2; but evolution ceased after 5 and 10 mins., respectively, and was identical in amount with controls in which NO_3 and NO_2 were absent.

Table 4. *Effect of glucose on nitrate and nitrite assimilation by Dunaliella tertiolecta*

Each vessel contained in 2 ml., organisms containing 0·5 mg. chlorophyll, 125 μmoles tris-HCl buffer (pH 7·6), 0·5–0·7 mmoles NaCl, 40 μmoles $NaHCO_3$, and 2 μmoles of either KNO_3 or KNO_2; 10 μmoles D-glucose were added where indicated. Where no CO_2 was required $NaHCO_3$ was omitted and 0·15 ml. 20% (w/v) KOH was added to centre well. Gas phase air $\pm$ CO_2. All values in μmoles/hr/mg. chlorophyll.

Conditions	NO_3 assimilated	O_2 evolved	NO_2 assimilated	O_2 evolved
CO_2 + light	0·9	42·2	2·5	48·4
CO_2 + light + glucose	1·5	47·8	2·6	46·3
Light	0·2	3·1	0·2	2·6
Light + glucose	0·2	3·7	0·2	4·4
CO_2 + dark	0·0	−1·7	0·0	−2·2
CO_2 + dark + glucose	0·1	−1·9	0·0	−4·8
Dark	0·0	−0·6	0·0	−6·7
Dark + glucose	0·0	−2·2	0·0	−6·4

The effect of inhibitors

The inhibitor of photosynthesis 3-(4-chlorophenyl)-1, 1-dimethyl urea (CMU) did not decrease oxygen evolution and nitrate and nitrite assimilation equally. An inhibitor concentration at 10^{-4} M decreased oxygen evolution to 2 % of the control rate; but nitrate and nitrite assimilation continued at 25 and 41 % of the control rates. Lower

concentrations of CMU showed an even more marked difference in sensitivity between the two systems (Table 5). Potassium cyanide and sodium azide were even more effective in inhibiting nitrate and nitrite assimilation than they were in inhibiting photosynthetic

Table 5. *Effect of CMU on nitrate and nitrite assimilation by Dunaliella tertiolecta and oxygen evolution*

Experimental conditions as described under Table 1 except that 0·02 ml. ethanol or ethanol solution of 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) was added to each vessel. All values in μmoles/hr/mg. chlorophyll. Time of experiments: 30 mins.

	NO_3 assimilated	O_2 evolved	NO_2 assimilated	O_2 evolved
Control light	2·81	56·3	4·53	89·0
+CMU				
10^{-7}M	3·27	51·9	4·48	86·0
10^{-6}M	2·92	43·9	4·39	65·2
10^{-5}M	2·22	3·7	3·44	−1·4
10^{-4}M	1·69	−3·2	2·31	−6·9
Control dark	1·30	−4·2	1·47	−8·0
+CMU				
10^{-7}M	1·60	−4·1	1·38	−7·5
10^{-6}M	1·20	−4·1	1·38	−8·5
10^{-5}M	1·10	−4·4	1·38	−7·4
10^{-4}M	1·20	−4·4	1·38	−11·0

Table 6. *Effect of potassium cyanide and sodium azide on oxygen evolution and nitrate or nitrite assimilation*

Inhibitor	O_2 evolution or uptake	NO_3 assimilation	NO_2 assimilation
KCN light	3×10^{-4}*	1×10^{-5}	1×10^{-5}
KCN dark	1×10^{-3}	1×10^{-5}	5×10^{-4}
NaN_3 light	$> 1 \times 10^{-3}$	5×10^{-5}	5×10^{-5}
NaN_3 dark	$> 1 \times 10^{-3}$	1×10^{-4}	5×10^{-4}

* molar concentrations giving 50 % inhibition.

Table 7. *Effect of iodoacetate on nitrate and nitrite assimilation by Dunaliella tertiolecta*

Experimental conditions as for Table 1. All rates in μmoles/hr/mg. chlorophyll

	NO_3 assimilated	O_2 evolved	NO_2 assimilated	O_2 evolved
Light				
Control	3·20	36·2	5·25	87·0
+iodoacetate 10^{-4}M	3·80	65·0	5·25	80·0
+iodoacetate 10^{-3}M	3·30	52·0	3·45	58·3
Dark	0·73	−7·2	1·58	−7·7
Control				
+iodoacetate 10^{-4}M	0·01	−5·1	1·1	−7·2
+iodoacetate 10^{-3}M	0·02	−7·2	0·6	−5·1

oxygen evolution. At 10^{-3}M (the highest concentration tested) sodium azide decreased photosynthetic oxygen evolution by only 20 % (Table 6). Sodium iodoacetate inhibited nitrate and nitrite assimilation in the dark but not in the light. While it is not possible now to be certain that this effect was not due to destruction of the inhibitor in the light, there seems to be evidence for two separate paths of nitrate and nitrite assimilation

(Table 7). Sodium amytal, 2,4-dinitrophenol and *p*-chloromercuric-benzoate were without effect at the highest concentrations tested (5×10^{-4} M).

The effect of light intensity on nitrate and nitrite assimilation. The light intensity required to saturate nitrate and nitrite assimilation was much lower than that required to saturate oxygen evolution and carbon fixation. Since the light intensity was varied by using neutral density filters the effect was not due to a change in spectral characteristics of the light source. The actual maximum rates of the reactions shown in Fig. 2 were nitrate assimilation 2·5 μmoles/hr/mg. chlorophyll; nitrite, 5·5 μmoles/hr/mg. chlorophyll; oxygen, 40 μmoles/hr/mg. chlorophyll and 800 counts/min. per sample for cell carbon.

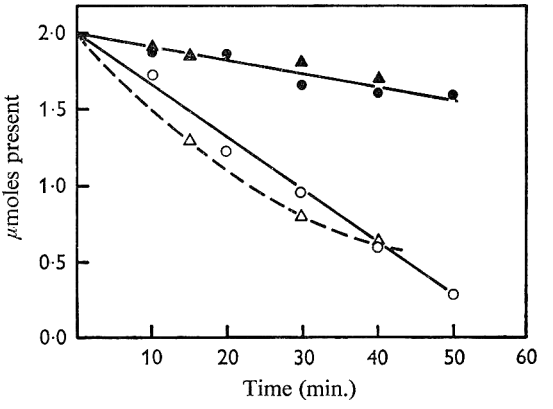

Fig. 1. *Dunaliella tertiolecta.* Rate of nitrate and nitrite assimilation in light and dark. ○, nitrate light; ●, nitrate dark; △, nitrite light; ▲, nitrite dark.

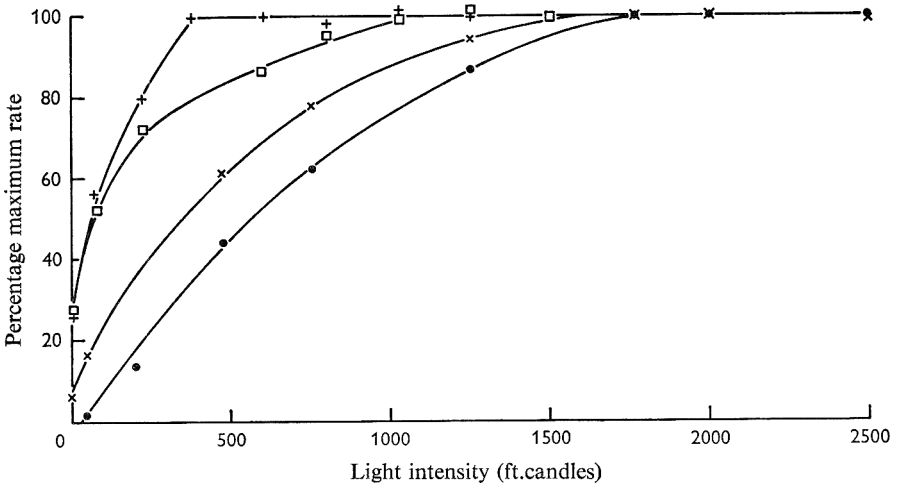

Fig. 2. *Dunaliella tertiolecta.* Effect of light intensity on nitrate assimilation, nitrite assimilation and oxygen evolution. +——+, NO_3 assimilation; □——□, NO_2 assimilation; ×——×, CO_2 assimilation; ●——●, O_2 production.

DISCUSSION

Before attempting to interpret these results it is important to determine to what extent they are supported by those of other workers. Bongers (1958) and Davis (1953) showed that 2·0 moles extra oxygen were produced per mole nitrate assimilated in the light. However, Warburg & Negelein (1920; see table XII, p. 90) found ratios between 3·7 and 6·0 O_2 per mole of NH_4 produced, though in a later publication (Warburg *et al.* 1965) it was claimed that the 2·0: 1 stoichiometry was established. Hattori (1962) did not find the 2: 1 stoichiometry value with nitrate, although he found the expected 1·5: 1 stoichiometry value with nitrite. However, in both this paper and in a later publication (Fujita & Hattori, 1963), it was stated that a 2·0: 1 ratio of O_2:NO_3 was established. Since Davis (1953) did not measure nitrate assimilated, Bongers (1958), remains the one unambiguous report in the literature with the theoretical stoichiometry, which the author could find. It is therefore perhaps not surprising that in the results presented in the present paper the O_2: NO_3 ratio varied from 0·6 to 4·5.

By contrast the 1·5: 1 ratio O_2: NO_2 is fairly well documented. In addition to Bonger's (1958) work, Hattori (1962) and Kessler (1964) both reported experimental confirmation of this figure. As *Dunaliella tertiolecta* did not give this stoichiometry it apparently behaves differently from the species of algae investigated by these workers. Where hydrogen uptake has been studied and related to nitrate and nitrite reduction, the expected stoichiometry of 3·0: 1 H_2: NO_2 was found (Kessler, 1957; Hattori, 1963; Stiller, 1966). However, in the investigation with nitrate Kessler reported low rates of H_2 uptake and difficulty in establishing the expected 4: 1 value.

The evidence for the requirement for CO_2 for nitrate and nitrite assimilation in the light was mentioned in the introduction to this paper. *Dunaliella tertiolecta* appears to be more strict in this requirement than Ankistrodesmus or Chlorella. The failure of glucose to replace CO_2 in *D. tertiolecta* in contrast to Davis's (1953) results with Chlorella may be due to the inability of *D. tertiolecta* to assimilate glucose at an appreciable rate. Preliminary experiments with ^{14}C-labelled glucose, whilst not definite, support this postulate. Reports of nitrate and nitrite assimilation in the absence of CO_2 are those of Kok (1951) and Bongers (1958); as Kessler (1964) points out, Bonger's results are ambiguous since under his experimental conditions rapid production of CO_2 by respiration was possible.

The chlorophenyl dimethylurea CMU has been shown to inhibit photosynthesis by blocking electron transport in the neighbourhood of the water-splitting reaction (Gingras & Lemasson, 1965). An inhibitory effect of CMU on nitrite assimilation was reported by Hattori (1962); however, since he used different systems for measuring the effect of CMU on nitrite assimilation and on photosynthesis his results are not comparable to those presented in the present paper. It was reported by Paasche (1965) that although coccolithophore formation in *Cyclotella nana* was dependent on light it was not as sensitive to CMU as was O_2 evolution, implying that light was affecting metabolism in ways other than simply by providing energy for photosynthesis. The effect of azide and KCN are consistent with the results obtained by Hattori (1962) and Losada *et al.* (1965), and are explicable on the basis of differential enzyme sensitivity. Both the results of Hattori (1962) and Bongers (1958) show that nitrate and nitrite assimilation saturate below the light intensity required to saturate photosynthtic oxygen evolution or carbon dioxide fixation. Thus with the single exception, that of a

failure to find the expected 1·5:1 O_2:NO_2 ratio in the light, the results presented in this paper agree with findings of most other workers who have investigated nitrate or nitrite assimilation by algae.

The question now arises whether these results can be explained by the scheme put forward by Losada *et al.* (1965) from their work on reconstituted spinach chloroplast systems. This scheme is itself a development of that originally proposed by van Niel *et al.* (1953). In its simplest form this system would show two experimentally demonstrable features. First, at saturating light intensities 2·0 moles extra O_2 would be produced per mole of NO_3 assimilated to NH_4 and 1·5 moles O_2 for every mole of NO_2. In the absence of CO_2 the stoichiometry would hold even at non-saturating light intensities. Secondly, any inhibition of photosynthetic electron flow should decrease oxygen evolution and nitrate and nitrite assimilation to the same degree. It is clear that the present results, together with most of those of other workers, do not support this simple model in whole organisms. By making several assumptions it is possible to reconcile some of these results with the Losada scheme. The requirement for CO_2 might be explained by the already known requirement of CO_2 for the Hill reaction (Ables, Brown & Mayne, 1961; Good, 1963). It needs to be shown, however, whether the CO_2 concentration required for nitrate and nitrite assimilation to reach saturation is the same as that required for the Hill reaction. The work of Davis (1953) suggests that substrate amounts of CO_2 are required. Losada *et al.* (1965) did not find any requirement for CO_2 in the spinach chloroplast system but they did indicate that it reduced nitrate very slowly when water was the electron donor. They explained this in terms of competition by cyclic electron flow catalysed by the relatively large amounts of flavine mononucleotide which they added.

The apparent failure of CMU to inhibit electron transport (as measured by O_2 evolution) and nitrate or nitrite reduction equally, indicates that the system supplying electrons to NADP is different from that supplying electrons to nitrate or nitrite, although in the case of nitrite, both would use ferredoxin as a primary electron acceptor. Although Losada spoke of a new type of non-cyclic photosynthetic phosphorylation it is doubtful if he intended this to be interpreted so broadly. There are two other possible explanations. Czygan (1963) reported that ascorbate might serve as an electron donor to reduce nitrate in *Ankistrodesmus braunii* in what he termed non-enzymic reduction. If this report is confirmed it would explain insensitivity to CMU as well as variable stoichiometry. Secondly, Grant & Whatley (1966) showed that CMU did not inhibit ATP production by cyclic photosynthetic phosphorylation, so that if light was required to produce extra ATP for assimilation of nitrate or nitrite, the electrons required being obtained from dark processes, this would explain that effect. The different light saturation requirement is, in a sense simply an extension of the CMU inhibition problem just discussed. Low light intensitites imply low electron flow rates, yet both nitrate and nitrite assimilating systems are saturated at rates well below the maximum, as measured by the carbon-fixing system. Since CO_2 was present in these experiments this again implies an electron transport system different in properties from that proposed for reduction of NADP. Alternatively, it implies that nitrate and nitrite reducing systems have a very much higher affinity for electrons, but a lower turnover number than the carbon-assimilating system. This needs to be investigated before it can be accepted as an explanation.

However, while by making these assumptions, it is possible to explain some of the

results contained in the present paper by Losada's scheme, the failure to observe stoichiometry as predicted by the theory for nitrate reduction remains a serious obstacle. The ranges of values for the $O_2 : NO_3$ and $O_2 : NO_2$ ratio are too great to be explained by experimental error. It is known from observations that cells low in total nitrogen give values closer to the expected 2·0 figure than those high in total nitrogen; but starving cells by resuspension in nitrate-free medium in the light for 4 to 24 h did not give any more reproducible results than these given here. It is concluded that the extra oxygen evolution is not perhaps exclusively related to nitrate and nitrite assimilation, at least in *Dunaliella tertiolecta*. In view of these difficulties a simpler hypothesis would be the assumption that there are two nitrate and nitrite assimilating systems in algae. One may be similar to that found by Losada for spinach chloroplasts and is the system directly responsive to light. A second system is a dark system dependent only upon light for its carbon source to supply energy. These two systems appear to be differentiated by the inhibitors CMU and iodoacetic acid; further separation might be possible by using the technique for whole chloroplast preparation recently worked out by Jeffrey, Ulrich & Allen (1966). Until these experiments have been done, preferably on several different species, the mode of action of light on nitrate and nitrite assimilation should remain open.

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Effects of Organic Cations on the Gram-negative Cell Wall and Their Bactericidal Activity with Ethylenediaminetetra-acetate and Surface Active Agents

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SUMMARY

When Gram-negative cell walls were damaged by lysozyme and ethylenediaminetetra-acetic acid (EDTA) in tris buffer, tris played an active role through its action as an organic cation. Other organic cations such as aliphatic amines and quaternary ammonium compounds were more effective than tris in modifying the cell wall and making it permeable to other solutes. The most effective organic cations had one C_{12} to C_{16} alkyl chain. At pH 10, EDTA 100 μg./ml and *N*,*N*-dimethyldodecylamine (DDA) 2·5 μg./ml. modified the cell wall of *Escherichia coli* so as to permit a more than 99·99 % kill by 20 μg./ml. of a zwitterionic surface active agent, 2-hydroxy-3-(dimethylhexadecylammonio) propane-1-sulphonate (HAPS), in 10 min. at 37°. At these concentrations, the individual compounds were bactericidally ineffective. Four other Gram-negative species were shown to be similarly susceptible to killing by this system. Together, EDTA and DDA appeared to remove surface components of the Gram-negative cell wall. Alone, DDA and other organic cations removed somatic antigens from the cell wall.

INTRODUCTION

Gram-negative bacteria are generally susceptible to the bactericidal action of cationic surface active agents, but are usually unaffected by those anionic and zwitterionic agents which are active against Gram-positive organisms. It was suggested by Voss (1963) that the greater resistance of Gram-negative species may be due to the greater complexity of their cell walls, which exclude the agent from the interior of the cell.

The Gram-negative cell wall may be so modified by treatment with EDTA in tris buffer at pH 8 as to permit conversion of the cell by lysozyme to osmotically fragile rods (Voss, 1964) or 'osmoplasts' (Asbell & Eagon, 1966*a*). In studies on *Escherichia coli* modified by treatment with EDTA and tris buffer, it was observed that such organisms may be killed by treatment with zwitterionic agents to which the organisms are normally resistant. As in the case of lysis by lysozyme, killing by the agent indicates changes induced in the cell wall by EDTA and tris buffer. Further study revealed that tris is not a physiologically inert buffer, but plays a specific role as an organic cation in modifying the permeability of the cell wall; other organic cations are much more effective than tris. Elucidation of the role of the organic cation forms the substance of this report.

METHODS

Organisms. The test organisms were *Escherichia coli* (ATCC 10536), *E. freundii*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Salmonella typhi*; *S. typhi* TY2V and 0901 and *E. freundii* (BALLERUP 107) were obtained through the courtesy of the late Dr P. R. Edwards (Communicable Disease Center, Public Health Service, Atlanta, Ga.), *S. typhi* 9992V and 12839 were obtained from the American Type Culture Collection. *Escherichia coli* was obtained as ATCC 10536 and used in the earlier part of this work; after the observation that this culture produced acid but no gas from glucose or lactose, it was replaced with a fresh, gas-forming culture of ATCC 10536 from the American Type Culture Collection with no discernible difference in results.

Test compounds. EDTA was used as the disodium salt; solutions were adjusted to pH 8·0 before sterilization. The zwitterionic agent 2-hydroxy-3-(dimethylhexadecylammonio)-propane-1-sulphonate (HAPS), was prepared and purified in our laboratories, and sterilized as a 0·1% solution in 0·85% NaCl; it is much less soluble in water alone. *N,N*-dimethyldodecylamine (DDA) was prepared by distillation of 'dimethyl coconut amine' (Armour). Some amines were derived from coconut or soybean oil, and thus contain a mixture of alkyl chains. Other compounds which were not available commercially were synthesized in our laboratories. Fatty amines were dissolved in ethanol, and diluted with water before use; at values up to about pH 10, they are present principally as cations.

Bactericidal tests. All organisms except *Proteus mirabilis* were grown in brain heart infusion broth (Difco) for 24 hr at 37°. Plate counts were made on brain heart infusion agar incubated at 37° for 24 hr; *P. mirabilis* was grown in nutrient broth, and plated on nutrient agar overlaid with plain agar to inhibit spreading. Exposure to bactericidal agents was done as described earlier (Voss, 1963) for 10 min. at 37° with suspensions of about 60–120 million washed organisms/ml.

Lysis. Changes in optical density of suspensions of organisms at room temperature were measured with a Coleman Junior spectrophotometer at 660 mμ.

Precipitin tests. Escherichia coli was harvested from ten 150 mm. plates, washed, and suspended in 400 ml. water. These suspensions contained 3–4 mg. dry wt. organisms/ml. Portions were treated for 10 min. at 37° with DDA 10 and 50 μg./ml. or with EDTA, HAPS, dodecyltrimethylammonium bromide (DTAB), or alkyldimethylbenzylammonium chloride (Roccal), all 50 μg./ml. in the presence or absence of Na_2CO_3 100 μg./ml. Organisms were removed by centrifugation for 20 min. at 5000 *g*. The supernatant fluids were tested for antigenic activity in double diffusion tests in agar, using as the source of antibody a 1/5 dilution of rabbit antiserum prepared against whole *E. coli* organisms.

RESULTS

Activity of cations other than tris

Tris was more effective than other buffers (e.g. phosphate buffer) in promoting the bactericidal activity of a mixture of EDTA and HAPS. The corresponding nitro compound, tris(hydroxymethyl) nitromethane, was inactive, indicating that it is the free amino group of tris buffer which affects the cell wall. Table 1 shows the results of a comparison of other amines and related compounds for activity in promoting the bactericidal action of EDTA and HAPS against *Escherichia coli* at concentrations at

Table 1. *Survival of Escherichia coli in systems containing EDTA (100 μg./ml.)+HAPS (20 μg./ml)+amines or other compounds*

Test compound	Concn. ($\times 10^{-4}$ M)	Survival (%) In complete systems	Survival (%) With test compound alone
Tris(2-Amino-2-hydroxymethyl-propane-1,3-diol)	500	3·0	.
	250	15	.
Tris(hydroxymethyl)nitromethane	500	74	.
Ammonia	500	71	.
Methylamine	500	16	.
Ethylamine	500	2·9	.
	250	17	.
Diethanolamine	250	0·72	.
	125	8·9	.
3-Aminopropanol	500	0·11	.
	250	5.4	.
	125	16	.
n-Propylamine	500	0·06	.
	250	0·40	.
	125	4·3	.
n-Butylamine	250	0·26	.
	125	0·82	.
sec-Butylamine	250	0·15	.
	125	0·87	.
Dibutylamine	125	~ 0·02	.
	62·5	4·0	.
n-Hexylamine	125	0·005	.
	62·5	0·007	.
	31·2	0·07	.
n-Octylamine	7·8	~ 0·001*	.
	3·9	~ 0·002*	.
n-Decylamine	15·6	0·001*	.
n-Dodecylamine	0·244	0·0028*	26
	0·122	0·021*	.
n-Hexadecylamine	0·244	0·037*	1·3*
	0·122	0·0049*	36*
N-Methyldodecylamine	0·244	0·0014*	16*
N,N-Dimethyldodecylamine (DDA)	0·244	~ 0·00006*	0·20*
	0·122	0·0042*	72*
	0·061	0·021*	.
N,N-Dimethylhexadecylamine	0·244	0·015*	2·2*
	0·122	> 0·4*	12*
Didecylamine	0·244	> 0·5*	70*
Dicoco amine†	0·244	0·055*	.
	0·122	0·089*	.
N-Methyl dicoco amine†	0·244	> 1*	80*
N-Methyl disoya amine‡	0·244	> 0·9*	.
Coco-1,3-propylene diamine†	0·244	0·00028*	5·1*
	0·122	0·0064*	40*
Dodecyl piperidine	0·244	~ 0·00002*	.
	0·122	0·00038*	4·8*
N,N-Dimethyldodecylamine oxide	0·244	> 0·9*	.
Coco ethanolamine†	0·244	24*	.
Coco amide†	0·244	18*	.

* In the presence of 100 μg./ml. sodium triphosphate.

† Derived from coconut oil fatty acids consisting of about 65% lauric acid, 25% myristic acid, and 10% palmitic acid.

‡ Derived from soybean oil fatty acids consisting of about 12% palmitic acid 25% oleic acid, and 53% linoleic acid.

which the latter two compounds together were ineffective. A number of amines of low molecular weight, more or less closely related to tris, were found to possess similar activity. As the length of the alkyl chain increased, the bactericidal activity of the mixture increased markedly. Concentrations of the amine buffers were decreased as activity increased; therefore sodium triphosphate ($Na_5P_3O_{10}$) 100 μg./ml. was added to maintain at pH 8·5–9·0. Maximum activity was reached with amines containing alkyl chains of 12–16 C atoms. The further substitution on the N atom of one or two methyl groups had little effect. Amines with two alkyl chains were less active. Such

Table 2. *Per cent survival of Escherichia coli in systems containing EDTA (100 μg./ml.) + Na_2CO_3 (100 μg./ml.) + HAPS (20 μg./ml.), and organic cations or related compounds*

Test compound	Concentration (μg./ml.)	Survival (%) Complete system	Survival (%) Test compound + Na_2CO_3 only
N,N-Dimethyldodecylamine (DDA)	2·5	0·0074	15
Decyl methyl sulphoxide	5	> 6	.
Hexadecyl methyl sulphoxide	5	> 6	.
Dodecyl methyl sulphone	5	> 6	.
Dodecyl methyl sulphide	5	> 6	.
Dodecyldimethylsulphoxonium methosulphate	2·5	0·0081	38
Dodecyldimethylsulphonium iodide	2·5	0·0033	68
3-Tridecylpyridine	2·5	> 6	.
Dodecyltrimethylammonium bromide	2·5	0·018	32
Cetyltrimethylammonium bromide	2·5	0·067	88
Cetylpyridinium chloride	2·5	0·019	78
Alkyldimethylbenzylammonium chloride (Roccal)	2·5	0·19	0·10
2-Dodecyl-1,3-bis-(trimethylammonio)-propane dibromide	2·5	0·00074	43
Dimethyldodecyl-3-(trimethylammonio)-propylammonium dibromide	2·5	0·00095	59
1-Dodecyl-2-imino-imidazolidine	2	0·135	30
1,2-Bis-(dimethylamino)-dodecane	2·5	0·0017	70
2-Chlorodimethyldodecylamine	2·5	0·11	54

related compounds as coconut alkyl ethanolamine or the amides of coconut fatty acids were ineffective, while other compounds such as coco-1,3-propylene diamine and dodecyl piperidine were highly active. The increasing activity of the aliphatic amines with EDTA and HAPS was not due to the increasing bactericidal activity of the amines alone as the length of the alkyl chain grew; the active amines effectively killed *E. coli*, in the presence of EDTA and HAPS, at concentrations at which the amines alone possessed only slight bactericidal activity.

A further series of organic cations and related compounds, including DDA, is compared in Table 2. In this case, the compounds were tested in the presence of EDTA 100 μg./ml., HAPS, 20 μg./ml. and Na_2CO_3 100 μg./ml. to buffer the system at pH 10. Na_2CO_3 was used instead of sodium triphosphate as a buffer to avoid combining the chelating effects of EDTA and triphosphate. Four non-ionic alkyl sulphur compounds were ineffective in increasing bactericidal activity, but two cationic sulphonium and sulphoxonium compounds were highly effective, as were a number of quaternary ammonium compounds and additional amines. It is evident that there was no specific requirement for a positively charged N atom. A compound with an alkyl chain of C_{12}

or longer and a positively charged group at one end was sufficient to give high bactericidal activity in the presence of EDTA, HAPS and an alkaline buffer.

Action of the bactericidal system

For further work, DDA was chosen as the organic cation; at a concentration of 2·5 μg./ml., it showed only very limited bactericidal activity against *Escherichia coli* at pH 10. EDTA was used at 100 μg./ml., at which concentration it was present in excess; decrease to 10 μg./ml. caused no significant loss of activity. At 20 μg./ml. and above, HAPS alone was not appreciably bactericidal for *E. coli*; however, increasing the concentration in the presence of the other components of the system increased killing, and decreasing the concentration decreased the killing. The concentration of Na_2CO_3 was not critical. A number of alkaline buffers gave similar results in the range pH 9–10.

EDTA could be replaced by a number of other chelating agents; however, only *N*-hydroxyethylethylenediaminetriacetic acid (Versenol), diethylenetriaminepentaacetic acid (Versenex 80), and 1,2-diaminocyclohexane-*N*,*N*′-tetra-acetic acid were as effective as EDTA.

Table 3. *Effect of omission of single components of the bactericidal system on kill of Escherichia coli*

EDTA (μg./ml.)	DDA (μg./ml.)	Na_2CO_3 (μg./ml.)	HAPS (μg./ml.)	Survival (%)
100	2·5	100	20	0·00040
100	0	100	20	42
0	2·5	100	20	18
100	2·5	0	20	0·012
100	2·5	100	0	34

HAPS, which is regarded as the actual lethal agent in the bactericidal system through its presumed effect on the cytoplasmic membrane (see Hotchkiss, 1946; Salton, 1951), could be replaced by other zwitterionic agents such as betaines, or by cationic quaternary ammonium compounds. Many of the latter are themselves highly bactericidal, and perhaps fulfil the function of organic cation and surface active agent simultaneously. Anionic and non-ionic surface active agents were ineffective as replacements for HAPS.

The fact that each component of the bactericidal system containing EDTA, DDA, HAPS and Na_2CO_3 was required for full activity is indicated by the data in Table 3. Omission of EDTA, DDA, or HAPS caused a sharp decrease in the killing of *Escherichia coli*; lack of Na_2CO_3 caused a much smaller decrease.

The bactericidal action of the components of the EDTA + DDA + Na_2CO_3 + HAPS system against *Escherichia coli* was studied in greater detail by exposing the organisms to one or two components under the conditions of the bactericidal test, removing the organisms by centrifugation, and then exposing them to the remainder of the system. The results of this study (Table 4) implied that EDTA and DDA acted jointly to exert a non-lethal effect on the cell wall, which thus became more permeable to HAPS. The surface active agent penetrates the wall and causes death of the organism, presumably by damaging the cytoplasmic membrane. The role of Na_2CO_3 was relatively non-specific, though it appeared to increase the effect of DDA more than that of EDTA or HAPS. Although killing might be influenced by carry-over of test compounds

from the initial to the final exposure, it seems clear that the initial effect of EDTA and especially of DDA was required *before* HAPS could kill the organism. This conclusion is supported by the survival curve shown in Fig. 1. It is assumed that the low rate of killing for the first minute or two represented the time required for EDTA and DDA to affect the cell wall.

Table 4. *Role of the components of the bactericidal system in killing Escherichia coli*

Concentrations of reagents: EDTA (100 μg./ml.), DDA (2·5 μg./ml.), Na_2CO_3 (100 μg./ml.), HAPS (20 μg./ml.).
Initial and final exposures: 10 min. at 37° C.

Initial exposure to	Final exposure to	Final pH value	Survival (%)
EDTA			114
	DDA + Na_2CO_3 + HAPS	9·9	49
DDA			80
	EDTA + Na_2CO_3 + HAPS	9·9	0·49
EDTA + DDA			118
	Na_2CO_3 + HAPS	9·9	0·015
EDTA + DDA + Na_2CO_3			34
	HAPS	8·0	0·0013
EDTA + Na_2CO_3			79
	DDA + HAPS	7·6	8·1
DDA + Na_2CO_3			54
	EDTA + HAPS	7·6	< 0·015
HAPS			87
	EDTA + DDA + Na_2CO_3	9·9	2·8
HAPS + Na_2CO_3			60
	EDTA + DDA	7·4	75
EDTA − HAPS			84
	DDA + Na_2CO_3	10·0	1·8
DDA + HAPS			92
	EDTA + Na_2CO_3	9·9	45
EDTA + Na_2CO_3 + HAPS			27
	DDA	7·9	9·2
DDA + Na_2CO_3 + HAPS			16
	EDTA	6·8	12

Six consecutive exposures of 12 isolates of *Escherichia coli* to the action of the EDTA + DDA + Na_2CO_3 + HAPS system did not produce any evidence that the survivors had mutated to a state of greater resistance to the bactericidal action.

Because of the great superiority of other alkyl cations over tris buffer in promoting the bactericidal activity of HAPS in the presence of EDTA, the efficiency of DDA in inducing lysis of *Escherichia coli* by lysozyme was compared with that of tris buffer. As shown in Table 5, 0·21 mg. DDA was more effective than 2·4 mg. tris, and about as effective as 12 mg. tris, in promoting lysis as measured by the decrease in optical density at 660 mμ.

A basic assumption throughout this work was that the surface active substance was the actual lethal agent, and that its action on the cytoplasmic membrane would be accompanied by a leakage of N and P from the cell. To test this, organisms at twice

the usual concentration were exposed to the EDTA + DDA + Na_2CO_3 + HAPS system for 10 min. at 37°. The N and P content of the supernatant fluid after centrifugation was compared with that of a control suspension of organisms. It was found that 4–5 μg.N/ml. and 0·8 μg.P/ml. were released on killing the organisms. Ultraviolet absorption curves showed a peak at 260 mμ in the supernatant fluid of killed organisms, with an optical density of 0·2–0·3. Calculations based on adenine ribonucleotide as a model system indicated that this quantity of nucleic acid would yield about 0·4–0·6 μg.P/ml. A major proportion of the P released may be accounted for as nucleic acid, as would

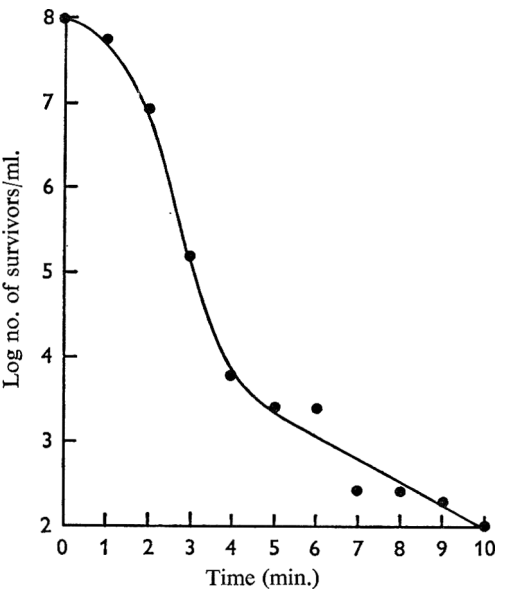

Fig. 1. Survival of *Escherichia coli* on exposure at 37° to EDTA (100 μg./ml.) + DDA (2·5 μg./ml.) + Na_2CO_3 (100 μg./ml.) + HAPS (20 μg./ml.).

Table 5. *Lysis of Escherichia coli by lysozyme, EDTA, and DDA or tris buffer**

EDTA (mg.)	DDA (mg.)	tris (mg.)	Na_2CO_3 (mg.)	lysozyme (mg.)	pH value	Decrease in special density (660 mμ) at 30 min.	Viscosity† at 30 min.
1·0	0·21	.	0·2	0·1	7·8	0·21	++
1·0	0·021	.	0·2	0·1	7·6	0·08	−
1·0	.	48	.	0·1	8·1	0·30	++++
1·0	.	12	.	0·1	8·1	0·20	++++
1·0	.	2·4	.	0·1	7·5	0·04	−
1·0	0·21	.	0·2	.	8·0	0·12	−
1·0	.	48	.	.	8·1	0·11	++

* 6 ml. washed bacterial suspension in 8 ml. total volume.
† −, no perceptible increase in viscosity; ++++, highly viscous.

be expected from the increased viscosity which appeared on lysis of the suspensions. This viscosity was decreased rapidly by adding a trace of deoxyribonuclease.

That the bactericidal activity of the EDTA + DDA + Na_2CO_3 + HAPS system was not restricted to *Escherichia coli* is shown by the data in Table 6. Four other Gram-

negative species were also killed by this system. At the concentrations used, none of the individual components were actively bactericidal. It is of interest that possession of an outermost layer of Vi antigen by some strains of *Salmonella typhi* and by the V dissociant of *E. freundii* appeared to confer some slight resistance to the killing effect.

Table 6. *Bactericidal activity of a mixture of EDTA (100 μg./ml.) + DDA (2·5 μg./ml) + Na_2CO_3 (100 μg./ml.) + HAPS (20 μg./ml.) against organisms other than Escherichia coli, and the effect of Vi antigen on survival*

Test organism	Vi antigen	% Survival
Proteus mirabilis		0·17
Pseudomonas aeruginosa		0·0048
Escherichia freundii (BALLERUP 107W)	−	0·0045
E. freundii (BALLERUP 107V)	+	0·034
Salmonella typhi 0901	−	0·005*
S. typhi TY2V	+	0·013*
S. typhi 9992V	+	0·0082*
S. typhi 12839	+	0·14*

* With DDA (1 μg./ml.), at which concentration DDA alone was not actively bactericidal against *S. typhi*.

Removal of somatic antigens by organic cations

It seemed probable that EDTA and DDA removed some surface components of the cell wall, thus rendering it more permeable to HAPS. To test this, washed suspensions of *Escherichia coli* were treated for 10 min. at 37° with dodecyltrimethylammonium bromide (DTAB), alkyldimethylbenzylammonium chloride (Roccal), DDA, EDTA or HAPS (at 10 or 50 μg./ml.), in the presence or absence of Na_2CO_3 100 μg./ml. The content of somatic antigens in these extracts was compared by the double diffusion method in agar, using rabbit antisera prepared against whole organisms. Comparison of the zones of precipitation showed that antigens were removed by DDA 50 μg./ml. and even more effectively by the other two cationic agents, DTAB and Roccal. Extraction by DDA 10 μg./ml. was less effective, but still evident. Evidence of reaction consisted primarily of a broad, diffuse zone of precipitate; in some cases minor bands of precipitate also indicated the presence of two slower-moving antigenic components. Na_2CO_3 had no perceptible effect on the extraction of antigens by the cations; Na_2CO_3, EDTA and HAPS were essentially ineffective in removing antigens from the cell wall.

DISCUSSION

Although Weidel, Frank & Martin (1960) concluded that the outermost layer of the cell wall of *Escherichia coli* is lipoprotein, the somatic antigens which are responsible for specific agglutination of Gram-negative bacteria are known to be polysaccharide or lipopolysaccharide. Shands (1965), by using ferritin-labelled antibody, demonstrated extension of somatic antigen for some distance beyond the cell wall of *E. coli* and *Salmonella typhimurium*. It is probably an over-simplification to regard the outermost layer of the multilayered cell envelope as consisting solely of lipoprotein or lipopolysaccharide. From biochemical and immunological evidence, both appear to be present.

Martin (1963) suggested that the complex wall of Gram-negative organisms affords more protection to the organism than does the simpler Gram-positive wall. Loss or decrease of somatic antigens at the cell surface decreases the resistance of Gram-

negative organisms to normal serum or to EDTA and lysozyme (Herzberg & Green, 1964; Osawa & Muschel, 1964; Wardlaw, 1963). Similarly, results given here indicate that absence of Vi antigen on *Escherichia freundii* and *Salmonella typhi* organisms is associated with greater susceptibility of the organisms. Thus, the demonstrated removal of somatic antigens from *E. coli* by DDA and other organic cations would be expected to increase the susceptibility of the organisms to deleterious agents.

Work with several species has shown that EDTA causes loss of lipid, lipoprotein or lipopolysaccharide from the cell wall (Colobert, 1958; Gray & Wilkinson, 1965; Leive, 1965). Repaske (1958) indicated that EDTA removed inorganic cations from cell walls. The importance of calcium and other divalent cations in the cell walls of various Gram-negative species (Humphrey & Vincent, 1962; Asbell & Eagon, 1966*a*, *b*) appears to be due to its function as a salt bridge in binding macromolecules on the surface of the cell wall.

Removal of surface components of the Gram-negative cell wall by organic cations and EDTA, or disorganization of the structure of the wall, may make it susceptible to the action of lysozyme and permeable to other solutes. When these solutes are surface active compounds which can now penetrate the cell wall, they may cause death of the cell by damaging the cytoplasmic membrane (Hotchkiss, 1946; Salton, 1951; Gilby & Few, 1960). In the system studied here, the lethal effect of HAPS on *Escherichia coli* is an indicator of changes induced by EDTA and DDA in the cell wall in the same manner as lysis by lysozyme indicates damage by EDTA and tris buffer (Repaske, 1958).

There is a growing realization that tris buffer actively modifies the effect of EDTA on the cell wall. Goldschmidt & Wyss (1966) showed that tris and other amines might assist the rupture of Azotobacter cysts by EDTA, but attributed the activity of the amines to the formation of complexes with EDTA. Asbell & Eagon (1966*a*, *b*) observed that tris buffer enhanced the lethal effect of EDTA on *Pseudomonas aeruginosa*. However, many workers have used EDTA in tris buffer without recognizing that the latter specifically affects the cell wall. For example, Nossal & Heppel (1966) exposed *Escherichia coli* to sucrose+EDTA in tris buffer preparatory to osmotic shock for the release of degradative enzymes, and Wolin (1966) lysed *Vibrio succinogenes* with EDTA in tris buffer. The work reported here suggests that a number of organic cations, including tris, specifically affect the Gram-negative cell wall by removing surface components.

It may be speculated that chelating agents and organic cations act together to cause non-lethal damage to the walls of Gram-negative organisms by breaking salt bridges and by salt formation with anionic polymers (lipoproteins, lipopolysaccharides) at the cell surface. Removal of these constituents of the cell wall renders the wall more permeable to other compounds, which can then penetrate to the interior of the cell. The penetration by a zwitterionic agent, such as HAPS, results in disorganization of the cytoplasmic membrane and death of the organism. The modified cell wall is still impermeable to some other compounds, such as anionic surfactants.

Organic cations containing an alkyl chain with about 12–16 C atoms may be used at low concentrations as a tool for dissection of the Gram-negative cell wall and for the preparation of partially purified extracts of somatic antigens. The O and Vi antigens of *Salmonella typhi* have been extracted by treatment of heavy suspensions of organisms with low concentrations of DDA (unpublished). With this organism, EDTA too is

effective in removing somatic antigens under similar conditions. Perhaps antigens suitable for use as vaccines could be prepared from a number of Gram-negative species in this fashion.

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The Production and Characterization of Lipases from a Micrococcus and a Pseudomonad

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SUMMARY

Extracellular lipase production was a constitutive property of the micrococcus and pseudomonad studied but was considerably influenced by nutritional and physical conditions. The lipase in culture supernatant fluids of the micrococcus was markedly heat resistant but became increasingly more thermolabile with the degree of purification obtained. The hydrolytic activity of partially purified extracellular lipase preparations from each organism was due to a single protein which was identical with a hydrolytic enzyme also found in cell-free extracts of each organism. The lipases from both organisms had general specificity towards ester linkages although the lipase from the micrococcus was markedly more active towards esters containing short chain fatty acids and comparatively less active towards trigylcerides containing long chain acids than was the pseudomonal lipase. The activity of both lipases showed an optimum for all substrates at pH 8·0–8·5 and did not decrease at higher pH values, indicating the involvement of an acidic group in the enzyme/substrate binding. The results of inhibition studies were consistent with the view that both lipases possess a serine-imidazole active centre and are therefore similar to esterolytic enzymes in mammalian systems.

INTRODUCTION

The properties of bacterial lipases have not yet been fully elucidated despite the vast literature that has accumulated. The diversity of the data seems to be partly due to the use of unpurified enzyme preparations and partly to the wide choice of substrates, assay conditions and methods used to determine lipase activity. Lipases form a rather indefinite section of the esterase group of enzymes, but it is useful to distinguish lipases from other esterases by the definition recommended by the *International Union of Biochemistry* (1961), namely that lipases hydrolyse emulsified esters of glycerol, whereas other esterases hydrolyse dissolved substrates. The choice of any substrate to detect bacterial lipases must be arbitrary, since nothing is known of their natural substrates or their physiological role in the metabolism of the bacteria. The technical difficulties involved in the quantitative estimation of lipase activity (Lawrence, 1967) and the absence of a method readily applicable to routine use have also contributed to the inconsistent results of different workers.

Of almost 100 bacteria, Gram-positive and Gram-negative, tested in two recent surveys (Fryer, Reiter & Lawrence, 1967; Fryer, Lawrence & Reiter, 1967) all produced diffusible lipases, although there was considerable variation in lipolytic activity

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between genera and even between strains of species in the same genus. A Gram-positive organism, *Micrococcus freudenreichii*, NCDO 1223, and a Gram-negative organism, *Fseudomonas fragi*, NCDO 752, which showed the highest lipase activity under the particular conditions of that survey were selected for more detailed study to determine whether gross differences existed between lipase production or the characteristics of the lipase formed by each organism. As it was found to be more difficult to detect lipase activity in liquid cultures than on solid media, a method of estimation was required which was more sensitive than those already in existence. A preliminary account of the use of the agar diffusion assay developed for this purpose has been reported (Lawrence, Fryer & Reiter, 1967).

METHODS

Media. The media listed in Table 1 were used, usually as 1 % (w/v) solutions. The organisms were subcultured twice in each of the media before the lipolytic activity was determined.

Chemicals. Trioctanoin, tridecanoin and trilaurin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; tributyrin, triolein and other chemicals from British Drug Houses Limited (BDH). Fresh butter was melted and filtered at 30° for use as an enzyme substrate. Chloramphenicol was obtained from Parke, Davis and Co., Detroit, Mich. Di-isopropylfluorophosphate (DFP) and diethyl *p*-nitrophenylphosphate (E600) were gifts from Dr G. C. Cheeseman and Dr P. Andrews, respectively.

Counts of organisms. Colony counts were made for both organisms on nutrient agar by the method of Miles & Misra (1938). In some experiments extinction measurements of suspensions were done at 610 mμ and those were converted into cell counts using predetermined graphs relating extinction to cell counts.

Cell-free extracts. The organisms were grown in Bactopeptone for 1–2 days, centrifuged down, washed in saline, re-centrifuged and re-suspended in 0·9 % NaCl solution. The majority of the pseudomonads were broken as shown by microscopic examination, after 3 min. ultrasonic treatment in a Branson Soniprobe Type 1130A (Dawe Instruments Ltd., London) used at maximum energy and the system kept cold with solid CO_2+ethanol. Ultrasonic treatment for 40 min. was necessary for comparable breakage of the micrococci.

Gel filtration. Sephadex G-100 gel filtration medium (140–400 mesh; Pharmacia, Uppsala, Sweden) was allowed to swell for 3 days, and G-200 (140–200 mesh) for at least 14 days, in 0·2 M-NaCl containing 0·025 M-$MgCl_2$ before use. Columns (55 cm. × 3 cm. diameter) were packed in the cold with the swollen gels and run at 0–5°.

Partial purification of lipases. The supernatant fluid of the bacterial cultures was two-thirds saturated with ammonium sulphate and the mixture left standing overnight at 4°. The precipitate was deposited by centrifugation, dissolved in a small volume of 0·2 M-NaCl containing 0·025 M-$MgCl_2$ and passed down a Sephadex G 100 column to remove low molecular weight material. The active fractions were pooled, the lipase again precipitated by saturation with ammonium sulphate, re-dissolved in a small volume of the above NaCl + $MgCl_2$ solution, added to a G 200 Sephadex column and eluted with the same salt solution. Five-ml. fractions were collected and monitored by determining absorption at 280 mμ or 230 mμ and also by testing for lipolytic activity either by the agar diffusion method or by potentiometric titration.

Gel electrophoresis. Esterases in the culture supernatant fluids and in freshly harvested bacteria which had been disintegrated by ultrasonic treatment were studied by electrophoresis in polyacrylamide gels at pH 7·3 and 8·7, essentially by the method described by Lund (1965). After separation of the proteins by electrophoresis, the gel slides were flooded with freshly prepared buffered solutions containing 0·04 % of either α-naphthyl acetate, butyrate or caprylate and 0·01 % (w/v) Fast blue B salt. Esterase activity was shown by the appearance of dark red bands which developed in 2 hr at room temperature.

Estimation of esterase activity. Hydrolysis of *o*-nitrophenylbutyrate (BDH) was measured by the spectrophotometric estimation of the liberated *o*-nitrophenol at 410 mμ in 1 cm. cuvettes and total volume of 3·2 ml. Assay mixtures contained 100 μmoles tris + HCl buffers (pH 8·0) and 7·5 μmoles of *o*-nitrophenylbutyrate (added as a solution in 0·2 ml. methanol). In preliminary experiments *p*-nitrophenylacetate was also used (Downey & Andrews, 1965*b*) but the low esterase activities of some cultures were of the same order as the rate of spontaneous hydrolysis of the acetate at pH 8, which made measurements less reliable than those with the more stable *o*-nitrophenylbutyrate.

Estimation of lipase activity

Potentiometric assay. The initial velocity of lipolysis was followed by continuous titration with 10^{-3} M-NaOH at a constant pH value at 25° by using a Radiometer titrator, type TTT 1c, coupled to a Radiometer titrigraph, type SBR 2C (Radiometer, Copenhagen, Denmark), as described by Downey & Andrews (1965*a*). The lipase solution, usually 2–3 ml., was brought to pH 8·0, at least 7 min. being required before steady conditions were attained (Fig. 1). This was noted both in the sample and in its control (boiled enzyme preparation); the reason why this occurred is not understood. The emulsified substrate (either 1 % triolein or 5 % tributyrin, stabilized with gum acacia as described by Downey & Andrews, 1965*a*) was then added, the titration being continued for a further 5 min. Controls were also done to check non-enzymic hydrolysis of each substrate. The relationship between an increase in enzyme concentration and lipase activity was slightly non-linear (Fig. 1), possibly because of adsorption of some lipase to the glass of the reaction vessel. The assay was found to be unsuitable for the estimation of very small amounts of acid below pH 7·0.

Agar diffusion assay. The thin layer agar diffusion method described earlier (Lawrence *et al.* 1967) was found to be most suitable for the routine estimation of lipase activity and was used for all determinations of lipase activity reported in this paper unless otherwise stated. The diameters of the zones of clearing (in mm.) of emulsions of various trigylcerides in agar, buffered with 0·05 M-phosphate, were measured after incubation at the pre-determined optimal temperature of 30°. The assay depends not only upon the solubility of di- and mono-glycerides but also upon the solubility of the calcium salts of the fatty acids produced by hydrolysis. For triglycerides containing fatty acids of twelve or fewer carbon atoms the hydrolysis is indicated by the clearing of the trigylyceride emulsion.

For triglycerides containing high molecular weight fatty acids the zones of hydrolysis show up as increased opaque zones against the emulsion background, presumably as a result of the precipitation of the calcium salts of the liberated fatty acids, since agar is known to contain exchangeable calcium (Cooper, 1963). The incorporation of

a fat-soluble dye into the medium, however, facilitated the detection of lipolytic activity against butterfat. The lipase was added to a phosphate buffered agar gel on which was then placed a lens tissue (Green's, 105), which had been painted with melted butterfat saturated with Victoria blue base. The hydrolysis of the thin uniform layer of butterfat was shown as a blue zone against the red background of unchanged dye.

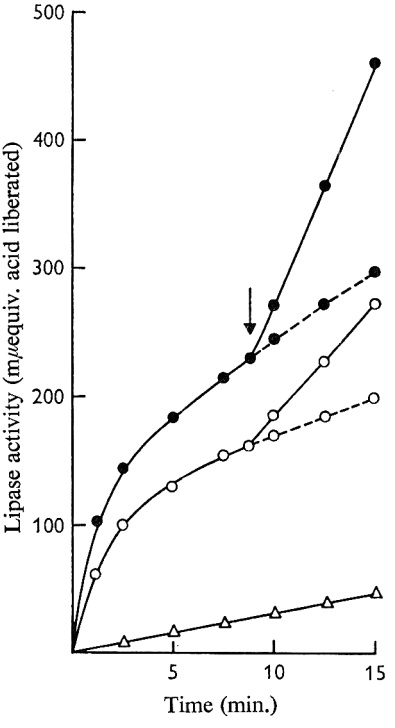

Fig. 1

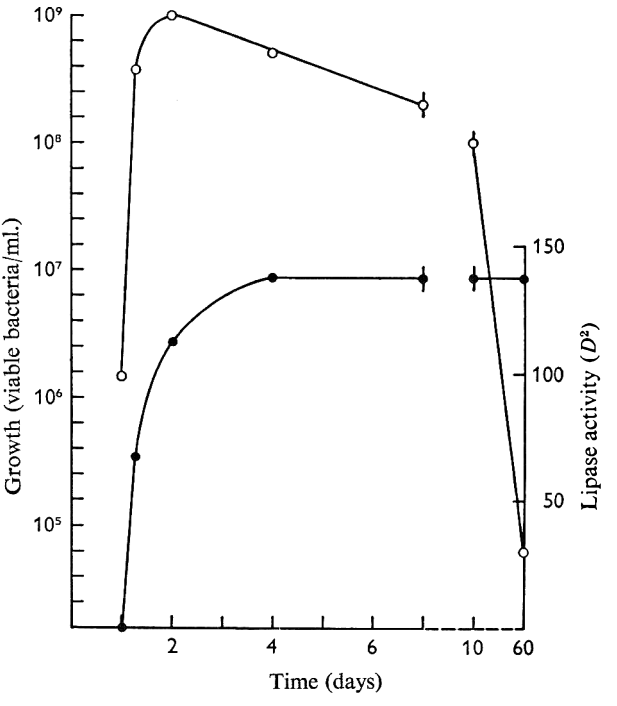

Fig. 2

Fig. 1. Effect of concentration of a partially purified lipase preparation from *Micrococcus freudenreichii* on the rate of hydrolysis of 5 % tributyrin (v/v), emulsified with 10 % (w/v) gum acacia in a pH stat. The substrate was added at ↓ to 0·05 ml. enzyme (○——○) and 0·1 ml. enzyme (●—●). Boiled enzyme controls are shown thus (○ - - - ○) and (● - - - ●). Substrate control (△——△).

Fig. 2. Effect of extended incubation upon the growth (○——○) and production of lipase (●——●) of static cultures of *Micrococcus freudenreichii* in 1 % (w/v) bactopeptone and 0·04 M-phosphate (pH 7·0) at 30°. Lipase activity was measured against emulsified tributyrin (0·1 %, v/v). D = diameter of zone of hydrolysis.

The correlation between the potentiometric assay and the tributyrin emulsion agar method was good (Fig. 5). The latter method, however, was able to detect lower levels of lipase activity, needed much smaller samples (0·004 ml.) and was considerably more simple to carry out than the potentiometric assay.

RESULTS

Factors affecting lipase production

Many of the conclusions drawn from previous studies on the effect of the growth medium and the conditions of growth on lipase production appeared to be contradictory (see review by Lawrence, 1967). The effect of such factors upon the two organisms used in this investigation was therefore determined in order to obtain optimal conditions for lipase formation.

Effect of the medium. The micrococcus and the pseudomonad were grown in different media containing peptones, Casamino acids and, in some cases, yeast extract. Most media supported good growth which, however, particularly in the case of the pseudomonad, resulted in no measurable lipase activity (Table 1). This confirmed a previous report (Nashif & Nelson, 1953) that some samples of peptone were less satisfactory than others as constituents of media for lipase production by bacteria. Lipase activity in the culture supernatant fluids was optimal for both organisms when

Table 1. *The effect of different media upon the growth and production of lipase by Pseudomonas fragi*

The organism was grown at 25° in 20 ml. lots of medium containing different peptones (1 %, w/v) adjusted to pH 7·0 with NaOH. The lipase activity in the culture supernatant fluids was tested after 16 hr and 40 hr incubation, against 0·1 % (v/v) tributyrin emulsion. D = diameter of zone of hydrolysis. + = zone of hydrolysis too small to measure.

	16 hr		40 hr	
Peptone component in Medium	Growth (E610)	Lipase activity (D^2)	Growth (E610)	Lipase activity (D^2)
Bacto peptone	0·74	16·0	0·95	4·0
Bacto proteose peptone	1·60	+	1·90	2·3
Bacto neopeptone	0·60	Nil	1·10	6·8
Bacto casitone	1·00	+	1·90	6·8
Bacto tryptone	1·30	+	2·00	3·6
Bacto tryptose	1·05	+	1·50	Nil
Bacto Casamino acids	0·29	Nil	1·00	Nil
Oxoid peptone	1·20	Nil	1·50	6·8
Oxoid tryptone	1·00	Nil	1·75	Nil
Oxoid tryptose	1·75	Nil	1·90	Nil
Evans peptone	1·60	+	2·00	+
Bacto peptone + 1 % Oxoid Lab Lemco	2·00	+	2·00	+
Bacto peptone + 0·2 % Bacto yeast extract	1·75	+	1·50	Nil

grown in the Bacto peptone medium, which was therefore used throughout this investigation. Growth and lipase production by the micrococcus was unaffected by the concentration of peptone between 0·5 and 2 % but growth and initial lipase production by the pseudomonad were considerably stimulated by an increase of peptone (Table 2). However this lipase initially formed was destroyed after 5–7 days at the higher peptone concentrations. It may be significant that the pseudomonad was strongly proteolytic for casein and gelatine, whereas proteolytic activity by the micrococcus was not detected.

While the two organisms produced lipase in media devoid of triglyceride substrates, it was possible that the addition of triglyceride might induce greater lipase production. However, low concentrations of tributyrin and trioctanoin (less than 10^{-3} M) had no significant effect on growth or lipase production although higher concentrations were inhibitory.

Table 2. *The effect of concentration of Bacto-peptone on the rate of destruction of the lipase formed by Pseudomonas fragi*

The organism was grown at 25° in Bacto peptone medium containing 0·04 M-phosphate buffer (pH 7·0). Lipase activity in the culture supernatant fluids after 1, 3, 7 and 14 days was tested against 0·1 % (v/v) tributyrin emulsion. D = diameter of zone of clearing of tributyrin emulsion. + = zone of hydrolysis too small to measure.

	Period of incubation							
	1 day		3 days		7 days		14 days	
% Bacto peptone	Growth (E610)	Lipase (D^2)	Growth (E610)	Lipase (D^2)	Growth (E610)	Lipase (D^2)	Growth (E610)	Lipase (D^2)
0·25	0·45	Nil	0·39	Nil	0·35	Nil	0·40	+
0·5	0·60	12·5	0·66	16·0	0·68	24·0	0·60	9·5
0·75	0·72	20·5	0·85	21·0	0·86	27·0	0·90	5·0
1·0	0·80	24·0	1·10	25·0	1·00	29·0	1·10	+
2·0	1·20	36·0	1·60	35·0	1·75	Nil	1·80	Nil

Table 3. *The effect of temperature upon the lipase activity in supernatant fluids of Pseudomonas fragi cultures*

The organism was grown at 25° in 40 ml. Bacto peptone (1 %, w/v) + 0·04 M-phosphate buffer (pH 7·0). Lipase activity in the supernatant fluids was tested after 1, 3 and 14 days against 0·1 % (v/v) tributyrin emulsion. D = diameter of zone of hydrolysis. + = zone of hydrolysis too small to measure.

	Time of incubation of cultures					
	1 day		3 days		14 days	
Temp.	Growth (E610)	Lipase (D^2)	Growth (E610)	Lipase (D^2)	Growth (E610)	Lipase (D^2)
22°	0·78	26	1·10	28	1·05	30·5
25°	0·86	21	1·10	21	1·10	Nil
30°	0·95	+	1·10	Nil	1·30	Nil

Effect of incubation temperature. The optimum temperature for lipase production by the pseudomonad was 22° and the lipase initially formed was unaffected by extended incubation at the temperature. The lipase present after 1 day at 25° was, however, completely destroyed after 14 days at that temperature and no lipase at all was detected at 30° (Table 3). Growth of the pseudomonad was not significantly affected by temperatures between 22° and 30° but was completely inhibited at 37°.

Incubation temperatures between 22° and 30° did not affect lipase production by the micrococcus, and the lipase initially formed was not destroyed by extended incubation between 22° and 37°. Both growth and lipase production, however, were lower when the micrococcus was grown at 37°.

Effect of aeration of growth medium. When the surface area/volume ratio of static cultures of the organisms was decreased there was a significant decrease in the lipase production per organism by the micrococcus but not by the pseudomonad (Table 4).

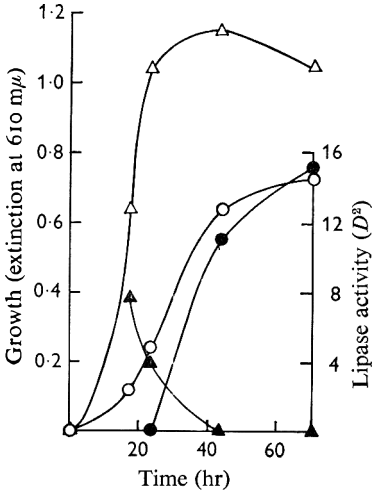

Fig. 3

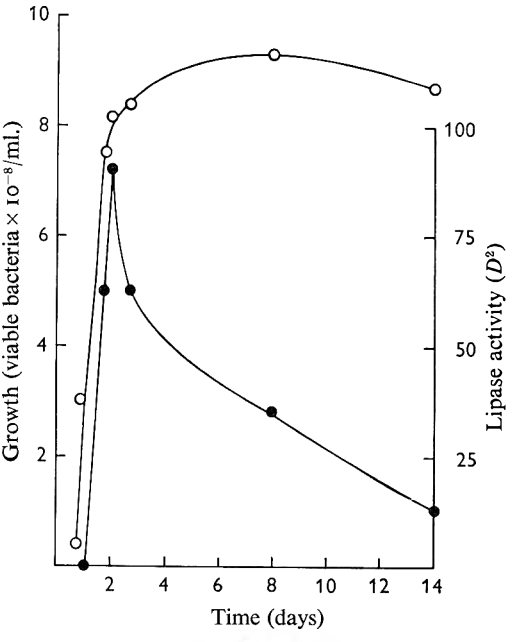

Fig. 4

Fig. 3. Effect of shaking upon the growth (△——△) and lipase production (▲—▲) of *Pseudomonas fragi* at 22° in bactopeptone (0·75 %, w/v). The growth (○——○) and lipase production (●——●) of static cultures were also recorded after the same time intervals. Lipase activity was measured against emulsified tributyrin (0·1 %, v/v). *D* = diameter of zone of hydrolysis.

Fig. 4. Effect of extended incubation upon the growth (○—○) and lipase production (●——●) of static cultures of *Pseudomonas fragi* in 1 % (w/v) bactopeptone and 0·04 M-phosphate, pH 7·0, at 22°. Lipase activity was measured against emulsified tributyrin (0·1 %, v/v). *D* = diameter of zone of hydrolysis.

Table 4. *The effect of degree of aeration upon the lipase production per organism of Micrococcus freudenreichii and Pseudomonas fragi*

The micrococcus was grown at 30° for 2 days in the specified volumes of Bacto peptone (1 %, w/v) medium in 250 ml. centrifuge bottles and the pseudomonad at 22° in Bacto peptone (1 %, w/v) + 0·05 M-phosphate buffer (pH 7·0). *D* is the diameter of zone of clearing of 0·1 % (v/v) tributyrin emulsion.

	Volume of medium		
	10 ml.	25 ml.	100 ml.
M. freudenreichii			
Lipase activity (D^2)	32·5	28·0	4·5
Count/ml. × 10^{-8}	1·2	0·8	0·8
Lipase/organism	4·9	6·0	1·0
Pseudomonas fragi			
Lipase activity (D^2)	30·5	11·0	5·0
Count/ml. × 10^{-8}	7·4	4·2	1·9
Lipase/organism	1·2	0·8	1·0

Lipase production by static cultures of the micrococcus increased to a maximum after incubation for 48–72 hr and did not decrease with further incubation (Fig. 2). Lipase production by static cultures of the pseudomonad increased gradually to a maximum after about 72 hr but decreased markedly on prolonged incubation (Figs. 3, 4).

Shaking the cultures to increase aeration resulted in increased growth and lipase activity in the first 24 hr with both organisms, but the lipase activity produced by the pseudomonad decreased rapidly on continued shaking. In a typical experiment, no activity was detected after shaking for 42 hr at 22° (Fig. 3).

Effect of initial pH value of growth medium. The lipase activity obtained from the pseudomonad grown in a medium initially at pH 6 was three to four times that at pH 8. The initial pH value of the medium was of little significance for lipase production from the micrococcus, although over extended periods an initial value between pH 7 and 8 gave slightly greater yields of lipase.

Extracellular nature of the lipases

Lipase activity was found in the supernatant fluids of young cultures during the logarithmic phase of growth of both organisms. Lipase production and numbers of organisms reached a maximum at almost the same time (Figs. 2–4). The finding of enzyme in the culture fluid may possibly result from the death or lysis of a small fraction of the organisms in a culture, but lipase activity did not increase on prolonged incubation of cultures of the micrococcus (Fig. 2) despite a great decrease in the number of viable organisms, and the activity actually decreased in cultures of the pseudomonad (Fig. 4). Similarly, the death of the micrococci occurred more rapidly when the pH value decreased below 7·0 but the lipase activity did not increase. The activity of a suspension of washed pseudomonads disrupted by ultrasonic treatment for 3 min. was at least sixty times less than that found in the supernatant fluid. The activity of lipase preparations from culture supernatant fluids of the pseudomonad was not affected by the same period of ultrasonic treatment.

Thick suspensions of whole organisms, the culture supernatant fluids and the cell-free extracts of organisms showed the same relative lipolytic activity towards the various substrates, indicating that the extracellular and intracellular enzymes were identical. Whole organisms which had been heated at 100° for 2 min. showed no activity.

Examination of partially purified lipase preparations

Behaviour on Sephadex columns. The elution of concentrated lipase preparations of both organisms on Sephadex G100 with salt solutions (0·02 M-NaCl + 0·025 M-$MgCl_2$) resulted in a single peak of lipase activity, the yield by this purification procedure being about 40 % of the original activity of the supernatant fluid. Two peaks were obtained, however, when the lipase preparations of both organisms were eluted on Sephadex G200 (Fig. 5). The fractions containing each peak showed the same relative activity towards different soluble and emulsified substrates, and the addition of inhibitors decreased the activity towards these substrates by the same proportion. It seems likely therefore that the activity obtained on Sephadex G200 was due to a single enzyme in each lipase preparation.

The rate of elution of the lipase activity from the Sephadex G200 columns also allowed an approximate molecular weight to be assigned to the lipases. The columns were calibrated with proteins of known molecular weight, according to the method

described by Andrews (1965). Thyroglobulin (mol. wt. 670,000), γ-globulin (mol. wt. 160,000), serum albumin (mol. wt. 67,000) and cytochrome *c* (mol. wt. 12,000) were used. The elution volumes of the peaks of activity off Sephadex G200 columns (Fig. 5) would indicate that the lipase activity was associated with proteins of molecular weights of about 25,000 and 250,000.

Thermostability of the lipases. The lipase in cultures of the micrococcus was extremely heat resistant, as were also the unpurified ammonium sulphate precipitates, which lost only 20 % of their lipase activity on boiling for 5 min. and could be stored for at least 6 months at 4° without loss of activity. The thermostability of the micrococcal lipase was dependent, however, upon its degree of purification. The lipase activity of

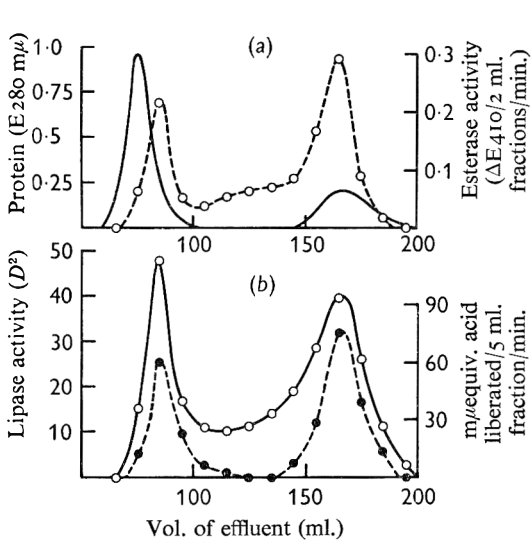

Fig. 5

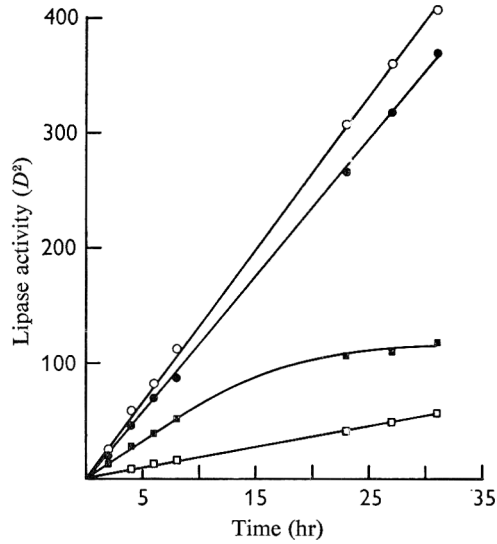

Fig. 6

Fig. 5. Gel filtration of a concentrated lipase preparation from *Micrococcus freudenreichii* on Sephadex G. 200 columns with 0·2 M-NaCl + 0·025 M-$MgCl_2$ as eluant; (*a*) material absorbing at 280 mμ (——) and esterase activity against o-nitrophenylbutyrate (○ - - - ○); (*b*) lipase activity against emulsified tributyrin (0·1 %, v/v) by the agar diffusion assay (○——○) and against emulsified tributyrin (5 %, v/v) using a pH stat (● - - - ●). *D* = diameter of zone of hydrolysis.

Fig. 6. The relationship between time of incubation and the zones of hydrolysis obtained on 0·1 % (v/v) tributyrin (○——○), trioctanoin (●——●), triolein (□——□) and the butterfat + Victoria Blue substrate (■——■) by a partially purified lipase of *Micrococcus freudenreichii*. *D* = diameter of zone of hydrolysis.

fractions from Sephadex G100 and G200 columns was completely destroyed by heating at 70° for 2 min. and lost 50 % of their activity in 48 hr at 4°. The major protective agent in impure preparations appeared to be the peptone of the growth medium, since ethanol precipitates of the enzyme, which were relatively uncontaminated with peptone, were considerably more heat labile than were more impure preparations precipitated by ammonium sulphate.

The lipase from the pseudomonad was relatively more heat sensitive than that from the micrococcus. A preparation of pseudomonal lipase obtained by precipitation with ammonium sulphate lost all activity at 100° for 3 min. and fractions from Sephadex columns lost 90 % of their activity in 72 hr at 4°.

Stability of the micrococcal lipase at various pH's. The partially purified micrococcal lipase was incubated at 30° for 24 hr with phosphate buffers at values between pH 4·8 and 8·0. The supernatant fluids from unshaken flasks showed significantly decreased lipase activity below pH 5·5 (Table 5) but this decrease did not take place in shaken flasks. Presumably the lipase was precipitated but not inactivated below pH 5·5. The loss of activity by precipitation of the lipase with decrease in pH value may explain the decrease upon prolonged incubation of the initial activity of supernatant fluids of micrococcal cultures grown in media containing glucose.

Table 5. *The effect of pH value upon the activity of partially purified lipase from Micrococcus freudenreichii*

Samples of the lipase solution were incubated at 30° for 24 hr in phosphate (0·1 M) buffers of different pH values. The lipase activity of the supernatant fluids and the shaken suspensions were tested against 0·1 % (v/v) tributyrin. D = the diameter of zone of hydrolysis. + = zone of hydrolysis too small to measure.

	Lipase activity (D^2)	
pH	Supernatant fluid	Suspension
4·8	+	115
5·3	75	130
6·0	160	155
6·8	140	125
8·0	170	165
Control (water)	160	155

Substrate specificity of lipases. Partially purified preparations of the extracellular lipases from the micrococcus and from the pseudomonad hydrolysed triglycerides containing short chain or long chain fatty aids. In the agar diffusion method of lipase assay the extent of hydrolysis was proportional to the area of the zone of activity. The extent of hydrolysis for each synthetic triglyceride was proportional to the period of incubation and remained linear for at least 30 hr (Fig. 6), although the hydrolysis of butterfat, measured by the indirect Victoria blue method, was linear for about 15 hr only.

The two lipases showed considerably less activity, as measured by the agar diffusion method, towards butterfat and triolein than towards synthetic triglycerides containing low molecular weight fatty acids. The inhibitory effect of the liberated oleic acid upon lipase activity (see below, section on inhibitors) over an extended incubation period may be significant, since the liberation of acid from triolein by the micrococcal lipase over an incubation period of 5 min. at controlled pH value was almost identical to that of tributyrin.

A linear relationship was also obtained when the logarithms of dilutions of the concentrated lipase preparations were plotted against the diameters of the zone of hydrolysis obtained with emulsions of various triglycerides (Fig. 7). The points where these straight lines cut the axis are the minimal lipase concentrations which give discernible zones with a particular substrate. These could be calculated and confirmed by experiment. A dilution of 1/17,800 of the partially purified micrococcal lipase was the least concentration that gave a zone with tributyrin emulsion, 1/3000 with tridecanoin emulsion and 1/205 with butterfat + Victoria blue (Fig. 7). The corresponding minimal

dilutions of the partially purified pseudomonad preparation were 1/360, 1/110 and 1/35, respectively (Fig. 8). Heating the partially purified lipase preparations at 80° for 2 min. completely destroyed their activity against all substrates, showing that non-enzymic hydrolysis was not responsible.

When the concentration of tributyrin was increased the zone of clearing obtained in a given time with a lipase preparation was decreased (Fig. 9). The sensitivity of the tributyrin emulsion assay was thus dependent upon the concentration of tributyrin used. A 1 % tributyrin emulsion gave zones that were approximately equivalent to those obtained with the butterfat+Victoria blue substrate under the assay conditions used.

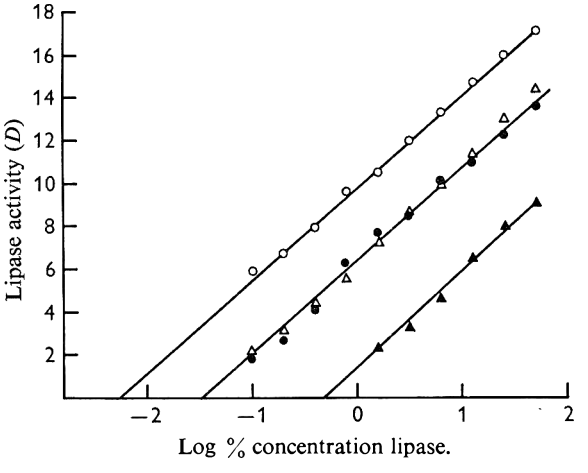

Fig. 7

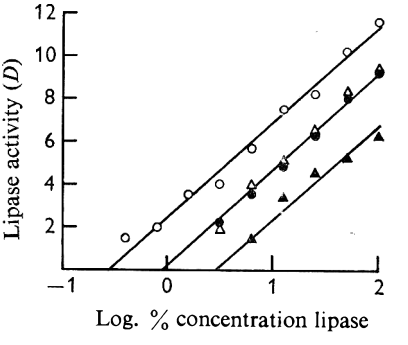

Fig. 8

Fig. 7. The relationship between the diameter of zones of hydrolysis of equimolecular concentrations of various triglycerides and logarithm percentage concentrations of the partially purified lipase of *Micrococcus feudenreichii* after incubation for 19 hr at 30°. 0·1 % (v/v) tributyrin (○——○), 0·17 % (v/v) trioctanoin (●——●), 0·2 % (v/v) tridecanoin (△——△) and butterfat-Victoria Blue (▲——▲). *D* = diameter of zone of hydrolysis.

Fig. 8. The relationship between the diameter of zones of hydrolysis of equimolecular concentrations of various triglycerides and logarithm percentage concentrations of the partially purified lipase of *Pseudomonas fragi* after incubation for 19 hr at 30°. 0·1 % (v/v) tributyrin (○——○), 0·17 % (v/v) trioctanoin (●——●), 0·2 % (v/v) tridecanoin (△——△) and butterfat+Victoria Blue (▲——▲). *D* = diameter of zone of hydrolysis.

Esterase activity. The micrococcal lipase preparations also showed marked activity towards simple esters such as *p*-nitrophenylacetate and *o*-nitrophenylbutyrate. When the rates of hydrolysis (change in extinction/min.) were appropriately corrected for spontaneous hydrolysis of the acetate, the esterase from the micrococcus appeared to be equally active against both esters. A Lineweaver–Burk plot of $1/v$ against $1/s$ and also a plot of v against v/s (Dowd & Riggs, 1965) were both linear, giving K_m values of 5×10^{-5} M and $2{\cdot}4 \times 10^{-5}$ M, respectively, for *o*-nitrophenylbutyrate. The linearity of these relationships suggests that a single esterase was attacking the substrate.

The similarity of the pH/activity plots for hydrolysis of *o*-nitrophenylbutyrate and emulsified tributyrin (Fig. 10) by the micrococcal lipase suggests that only one enzyme was involved in the hydrolysis of both soluble and emulsified substrates. The estimated pK values for the hydrolysis of *o*-nitrophenylbutyrate by the micrococcal lipase were

7·3 in phosphate and 7·1 in tris buffer. Similar pH/activity plots were obtained with the lipase preparation from the pseudomonad. The latter, however, showed almost no activity towards soluble aromatic esters, which was consistent with its comparatively greater activity towards emulsified triglycerides containing high molecular weight fatty acids than the micrococcal lipase.

Gel electrophoresis. Concentrated lipase preparations from the supernatant fluids of the micrococcal cultures were shown by gel electrophoresis at pH 7·3 and 8·7 to contain a single esterase which hydrolysed esters of α-naphthol. The acetate and butyrate were attacked more readily than the octanoate. Gel electrophoresis of cell-free extracts of the organism also showed only one esterase which was apparently identical with the enzyme found in the supernatant fluids.

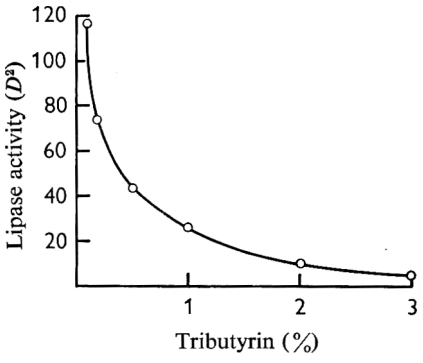

Fig. 9

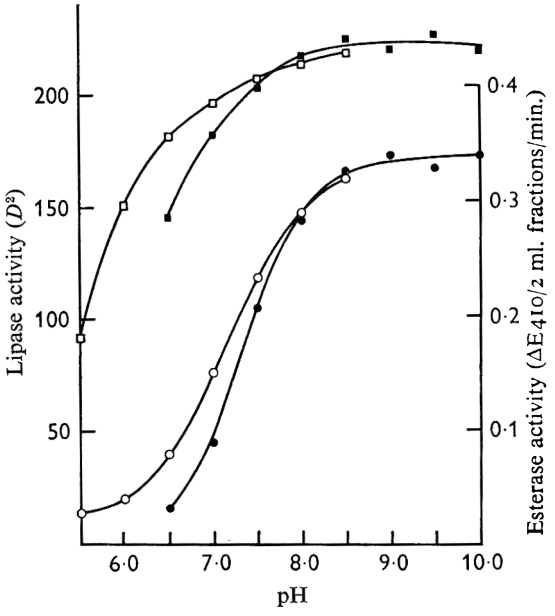

Fig. 10

Fig. 9. The relationship between time of incubation and the zones of hydrolysis obtained on emulsions of different concentrations of tributyrin by a partially purified lipase of *Micrococcus freudenreichii*. D = diameter of zone of hydrolysis.

Fig. 10. Effect of pH value and buffer system on the rate of hydrolysis of tributyrin (0·1 %, v/v) and *o*-nitrophenylbutyrate by a partially purified lipase from *Micrococcus freudenreichii*; *o*-nitrophenylbutyrate in phosphate (○——○) and tris HCl (●——●) buffers, tributyrin in phosphate (□——□) and tris HCl (■—■) buffers. D = diameter of zone of hydrolysis.

Lipase preparations from culture supernatant fluids of the pseudomonad showed only one weak band of hydrolytic activity on gel electrophoresis, whereas a second extremely weak band was also detected in cell-free extracts. These bands ran further than the band of hydrolytic activity associated with the lipase preparation from the micrococcus. The weak bands of activity obtained using esters of α-naphthol emphasized the relatively low activity towards soluble esters of the lipase preparation from the pseudomonad as compared to that from the micrococcus.

Dialysis of partially purified micrococcal lipase. Active fractions from Sephadex columns were pooled and the lipase concentrated by precipitation with ammonium sulphate. The precipitate was dissolved in water and dialysed against de-ionized water for 3 days, but the loss in activity was not greater than for non-dialysed controls, indicating the absence of a dialysable co-factor.

Table 6. *The inhibitory effect of metallic ions upon the activity of a partially purified preparation of lipase Micrococcus freudenreichii*

Lipase activity was determined at pH 8·0 against 0·1 % (v/v) tributyrin emulsion in tris and phosphate buffers and against *o*-nitrophenylbutyrate (*o*-NPB) in tris buffer. The metal ions were in contact with the enzyme for 60 min. before the assays were done. *Histidine and EDTA added to annul the inhibition, as described in text.

			Inhibition (%)		
				Tributyrin	
Metal	Concentration (M)	Re-activation* (10^{-3}M)	*o*-NPB (tris)	(tris)	(phosphate)
Zn	1×10^{-3}	—	100	93	78
	5×10^{-3}	—	100	54	44
	5×10^{-4}	—	90	44	20
	5×10^{-4}	Histidine	0	10	7
	—	Histidine	0	0	0
Zn	5×10^{-4}	EDTA	36	30	29
—	—	EDTA	35	40	0
Hg	1×10^{-3}	—	100	40	100
	5×10^{-3}	—	92	21	70
	5×10^{-4}	—	74	15	47
	5×10^{-4}	Histidine	14	0	0
	5×10^{-4}	EDTA	35	0	0

Inhibition of the lipases

Effect of metals. Zinc and mercuric ions were the most inhibitory metal ions of the wide range tested against both lipases, the inhibitory effect being annulled by histidine and less completely by EDTA (Table 6). Copper, nickel, cadmium and beryllium also partially inhibited the activity, in that order of decreasing effectiveness.

Effect of non-metallic inhibitors. The most effective inhibitor against the partially purified lipase of the micrococcus, and to a lesser extent of the pseudomonad lipase, was diethyl *p*-nitrophenylphosphate (E600). Di-isopropylfluorophosphate (DFP) was considerably less effective (Table 7). The degrees of inhibition of the activity of the lipase preparations against both emulsified tributyrin and soluble *o*-nitrophenylbutyrate were almost identical.

The specific reagent for thiol active centres, *p*-hydroxymercuribenzoate, was without effect and 2,4-dinitrofluorobenzene was inhibitory only in high concentrations (Table 7). Partially purified lipase preparations were inhibited by high concentrations of oleic acid, confirming similar findings for the lipases of *Pseudomonas ceruginosa* (Sierra, 1957), and *P. fragi* (Smith & Alford, 1966), and were partially inhibited (30–40 %) by EDTA in tris buffer (Table 7). The sensitivity of the lipases to both EDTA and oleic acid depended, however, upon their degree of purity, since unpurified preparations of lipase were unaffected.

Sodium fluoride was non-inhibitory for the lipases from both organisms, unlike its reported effect on the esterases of mycobacteria (Cohen, Kurshnick & Purdy, 1953)

Table 7. *The effect of inhibitors upon the activity of a partially purified lipase preparation from Micrococcus freudenreichii*

Activity was determined at pH 8·0 against 0·1 % (v/v) tributyrin emulsion (in both tris and phosphate buffer) and *o*-nitrophenylbutyrate (*o*-NPB) in tris buffer. The inhibitors were in contact with the enzyme for 60 min. before the assays were done.

		Inhibition (%)		
			Tributyrin	
Inhibitor	Concentration (M)	*o*-NPB (tris)	(tris)	(phosphate)
E600*	2×10^{-4}	100	100	100
DFP†	2×10^{-3}	80	91	90
	2×10^{-4}	17	11	20
EDTA	2×10^{-2}	35	37	0
	2×10^{-3}	40	38	0
	2×10^{-4}	0	0	0
2,4-DNFB‡	2×10^{-2}	100	90	94
	1×10^{-2}	48	60	57
Sodium oleate	2×10^{-3}	72	70	81
	2×10^{-4}	12	0	0

* E600, diethyl *p*-nitrophenylphosphate.
† DFP, di-isopropylfluorophosphate.
‡ 2,4-DNFB, 2,4-dinitrofluorobenzene.

Table 8. *The effect of organophosphorus compounds on the growth and lipase activity of cultures of Micrococcus freudenreichii and Pseudomonas fragi*

The micrococcus and the pseudomonand were grown at 30° and 22°, respectively, for 20 hr in 10 ml. Bacto peptone (0·75 %, w/v) medium containing 0·05 M-phosphate buffer (pH 7·0) and either the specified concentrations of inhibitors in ethanol or similar amounts of ethanol as controls. Lipase activity of the culture supernatant fluids was determined against 0·1 % (v/v) tributyrin emulsion. D = the diameter of the zone of hydrolysis. + = zone of hydrolysis too small to measure.

		Micrococcus		Pseudomonad	
		Growth (E610)	Lipase (D^2)	Growth (E610)	Lipase (D^2)
E600* (M)	10^{-4}	0·54	Nil	0·42	26·0
	10^{-5}	0·50	+	0·76	26·0
DFP† (M)	10^{-4}	0·58	18·5	0·47	20·5
	10^{-5}	0·58	22·0	0·78	26·0
Ethanol 1 %		0·56	18·5	0·56	30·5
0·1 %		0·54	20·5	0·74	30·5

* E600, diethyl p-nitrophenylphosphate.
† DFP, di-isopropylfluorophosphate.

and a staphylococcal lipase (Drummond & Tager, 1959). Smith, Worrell & Swanson (1949) reported that bacterial esterases were inhibited by chloramphenicol, but even high concentrations were found to be ineffective in the present work. Eserine between 10^{-5} and 10^{-2} M was also without effect upon the lipases from either organism.

Effect of organophosphorus compounds upon growth of the organisms. Growth of the micrococcus was unimpaired by the inclusion of low concentrations of E600 to the

medium although lipase activity in the culture supernatants was completely inhibited (Table 8). Low concentrations of DFP were without significant effect upon either growth of the micrococcus or its lipase activity. As the inhibitors were made up originally as 10^{-2} M solutions in anhydrous ethanol, controls with equivalent concentrations of ethanol+enzyme preparation were made. Neither of the above organophosphorus compounds was effective in inhibiting growth of the pseudomonad or its extracellular lipase activity. This is consistent with the finding (Mounter & Tuck, 1956) that Gram-negative bacteria hydrolyse organosphorous compounds more readily than do Gram-positive bacteria.

DISCUSSION

The extracellular nature of the lipases found in the supernatant fluids of young cultures of the micrococcus and the pseudomonad is in agreement with similar reports of extracellular lipase production by *Pseudomonas fragi* (Mencher, Ng & Alford, 1965) and by lactic acid bacteria (Fryer *et al.* 1967). The production of diffusible lipases by young cultures of over 100 strains of Gram-positive catalase-positive cocci, Gram-negative rods and lactic acid bacteria, taken at random (Fryer *et al.* 1967), is, in addition, tentative evidence that extracellular lipase production may be a general property of most, if not all, bacteria. The amounts of intracellular lipase detected may simply be a measure of the inefficiency of the process for the release of most of the new enzyme into the culture fluid and have no specific intracellular function (Lampen, 1965).

The extent to which extracellular lipase is formed, however, varies considerably, even between strains of the same species of organism and is markedly influenced by different nutritional and physical conditions. The lipase initially produced by the pseudomonad, but not that of the micrococcus, was destroyed by prolonged aeration, incubation at a temperature above 22° or by growing the organism in high concentrations of bactopeptone. It may be significant that the pseudomonad also produced an active extracellular proteinase, whereas the micrococcus did not. Preliminary investigations indicate that the proteinase activity of the pseudomonad is correlated with the destruction of the lipase initially produced.

Lipase formation by the two organisms studied in this paper was not enhanced by the addition of tributyrin or trioctanoin to the growth medium, which suggests that these bacterial lipases are not inducible in the generally accepted sense. The classification of enzymes as inducible or constitutive is, however, somewhat arbitrary, since constitutive enzyme formation is not itself a completely fixed property but varies depending on previous growth conditions (Davies, 1963). It seems unlikely that the formation of extracellular lipases plays an essential role in the metabolism of the micrococcus since its growth was unaffected by concentrations of the organophosphorus compound E600 which completely suppressed lipase activity in the culture supernatant fluids.

Lipase activity in the supernatant fluids of cultures of the micrococcus, and to a lesser extent in those of the pseudomonad, was markedly heat resistant. It is probable that the peptone of the growth medium stabilized the lipase, since the thermostability of the enzyme was dependent upon its degree of purification and other bacterial lipases have been shown to be stabilized by the presence of proteins (Virtanen, 1934).

The elution of concentrated preparations from both organisms on Sephadex G100 resulted in a single peak of lipase activity, but two peaks were obtained by elution on

Sephadex G200. The fractions containing each peak showed the same relative activity towards different soluble and emulsified substrates and these rates were decreased to the same extent when each peak was treated with inhibitors, suggesting that the activity was due to a single enzyme in each lipase preparation. It is reasonable to assume that the activity in the first peak was due to lipases either in a polymerized form or associated with inactive high molecular weight material, and that the later fractions which showed lipase activity contained lipase in a monomer form. The lipases from both organisms are therefore estimated to have molecular weights of about 25,000, which is consistent with the view that bacterial exo-enzymes are usually relatively small proteins (Pollock, 1962). Similar behaviour by pancreatic lipase when eluted from Sephadex gel columns has been reported (Gelotte, 1964).

It is generally accepted that although bacterial esterases show a very low degree of specificity towards ester linkages in general, there is a certain degree of specificity in regard to the chain length of the acid (Alford & Pierce, 1963). The results with the micrococcal lipase emphasize, however, the inadequate basis for a general distinction between esterases and lipases in micro-organisms, since both soluble esters and emulsified triglycerides containing short and long chain fatty acids were readily attacked. The optimal pH values for the hydrolysis of soluble *o*-nitrophenylbutyrate and emulsified triglycerides were identical, which indicates that the same enzyme was exerting a corresponding effect on each of the substrates. The relative rates of hydrolysis of both soluble and insoluble substrates by many different batches of the lipase preparations were very similar, even after considerable purification had been achieved, and were decreased to the same extent by inhibitors. The detection of a single hydrolytic enzyme only by gel electrophoresis of partially purified lipase preparations from culture supernatant fluids and cell-free extracts of the micrococcus provides additional, if less conclusive, support for the conclusion that some organisms produce a single esterase of general specificity rather than several esterases with different specificities. Similar views about other bacterial esterases have been put forward by Hugo & Beveridge (1962) and Rottem & Razin (1964).

Augustinsson (1961) proposed that the active centre of all esterases has a characteristic amino-acid sequence but that the rest of the molecule may vary. The latter might have little effect upon enzyme+substrate complex formation, which would account for the high degree of overlapping in hydrolysis of substrates by different esterases. Variations in physico-chemical properties of both the enzyme and the substrate molecules might explain, however, the differences in degree of specificity found with microbial esterases from different sources. Thus the micrococcal lipase preparation was about 50 times more active against tributyrin, 25 times against tridecanoin but only 6 times more active against butterfat than was the lipase from the pseudomonad. This greater specificity of the micrococcal lipase towards esters containing short chain fatty acids was particularly pronounced in the case of the soluble aromatic esters against which the pseudomonad lipase showed almost no activity.

The inhibition of the micrococcal lipase by organophosphorus compounds, by 2,4-dinitrofluorobenzene in alkaline solution and the annulment of metal-ion inhibition by histidine and the absence of inhibition by –SH reagents suggest that the active centre may contain both serine and imidazole groups (Barnard & Stein, 1958). The estimated pK values (between 6·2 and 7·2) for the hydrolysis of both *o*-nitrophenylbutyrate and emulsified tributyrin are also consistent with an active imidazole centre

(Gutfreund, 1965). The pH/activity curves for the hydrolysis of both *o*-nitrophenylbutyrate and emulsified tributyrin by the micrococcal lipase reached an optimum at pH 8·5 which did not decline with increasing alkalinity. This may be interpreted as evidence for the involvement of an acidic group in the binding of the substrates (Bergmann, Rimon & Segal, 1958). The low activity towards soluble esters of the pseudomonad lipase did not allow such clear-cut conclusions to be drawn about its active centre, but the data obtained by using the triglyceride emulsion diffusion assay indicates that a serine-imidazole active centre may also be involved.

Grateful acknowledgement is made to Mr T. Morichi for carrying out the gel electrophoresis studies.

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The Preparation and Biochemical Properties of Mitochondria from *Neurospora crassa*

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SUMMARY

Mitochondria were prepared in large quantities from *Neurospora crassa* by grinding hyphae with glass beads in an Eppenbach Micro-mill. Observation in the electron microscope indicated that some of the isolated mitochondria were probably damaged during preparation. Nevertheless, the crude mitochondrial fraction was capable of coupling phosphorylation to the oxidation of seven different substrates tested. The P:O ratio obtained with succinate ranged from 0·7 to 1·3 in different experiments with different mitochondrial preparations, but only low respiratory control ratios were observed. The effects of common inhibitors of oxidative phosphorylation were similar to those reported with mammalian mitochondria. In addition, ATPase and ATP-Pi exchange activities, as well as ion accumulation, were measured in the Neurospora mitochondria. The use of a 'preparation' medium containing sucrose, 0·25 M; EDTA, 0·005 M (pH 7·0); bovine serum albumin (0·15 %, w/v) was necessary to obtain these results.

INTRODUCTION

The filimentous fungi contain intracytoplasmic organelles which have the basic structural characteristics of mammalian mitochondria (Moore & McAlear, 1963; Tsuda, 1956; Shatkin & Tatum, 1959). In fact, the distinct mitochondria and nuclei in the 'higher' fungi cytologically distinguish these microbes from the more primitive protists (Moore & McAlear, 1963; Iterson, 1965). Only recently, however, have discrete subcellular particles capable of effecting oxidative phosphorylation been successfully isolated from these organisms (Vitols & Linnane, 1961; Hall & Greenawalt, 1964; Ohnishi, Kawaguchi & Hagihara, 1966). In order to isolate intact mitochondria from *Neurospora crassa* the thick resistant heteropolysaccharide-containing cell walls of this organism must be broken or removed without extensively damaging the mitochondria. Because of differences in morphology and chemical composition, not all forms of *N. crassa* are equally susceptible to disruption by a particular method, i.e. some procedures useful in breaking long vegetative hyphae are ineffective in disrupting the smaller more spherical conidia. Luck (1963) isolated mitochondria from hyphae by grinding with sand in a mortar and pestle, but only relatively small quantities of these vegetative cells can be disrupted easily by this procedure. The study of oxidative phosphorylation by mitochondria isolated from cells broken in this manner has not

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been reported. Mitochondria have been prepared from conidia and from hyphae of *Neurospora crassa* which were converted enzymically to 'protoplasts' and then lysed (Weiss, 1965). The long incubation time (10–15 hr) required for digestion of the cell wall makes this procedure unsuitable for many studies; mitochondria from a given form, e.g. conidia, may be structurally or functionally altered during long incubation periods. Similar procedures have been used, however, to produce spheroplasts of yeast cells (Duell, Inoue, & Utter, 1964) from which functional mitochondria have been obtained.

This paper describes a procedure for preparing mitochondria from *Neurospora crassa* which are capable of coupling phosphorylation to the oxidation of several substrates. The data reported here were obtained in studies utilizing mitochondria isolated in large quantities from 2-day hyphae broken by high-speed homogenization in the presence of glass beads. This procedure made it possible also to compare oxidative phosphorylation by mitochondria from conidia, germinating conidia and hyphae (these latter results will be reported elsewhere). A brief report of the present work has been communicated (Hall & Greenawalt, 1964).

METHODS

Growth of Neurospora crassa and production of conidia. Stock cultures of *Neurospora crassa*, wild-type strain SY 7A (obtained from the Fungal Genetic Stock Centre, Dartmouth College, Hanover, N.H., U.S.A.) were maintained on 2 % agar slopes of the minimal medium described by Wainwright (1959) and stored at 5–8° until used. It is essential for biochemical studies to have a supply of conidia available: (*a*) as a source from which mitochondria can be prepared directly, or (*b*) as inocula for producing germinating conidia or hyphae, but the formation of mature conidia by *N. crassa* requires 7–9 days. However, it was found in preliminary studies that conidia harvested and suspended in distilled water remained viable for at least 7 days with no change in the rate or percentage of germination when stored under aseptic conditions at 4°. Therefore, each week 20 agar slopes of Wainwright's conidiation medium (1959) were inoculated and grown at room temperature under fluorescent lamps to induce conidiation; conidia produced on these slopes were then used for the subsequent large-scale production of conidia. At the end of 4–5 days conidia from each of 16 slopes were scratched with a loop from the surface of the slopes into 10 ml. sterile distilled water. The conidial suspensions were combined and ultrasonically treated for 2 min. in a Di Sontegrator System 40 (Ultrasonic Industries, Albertson, L.I., New York, U.S.A.) to break up conidial clumps. The suspension was filtered through cheesecloth and 5 ml. was added to each of sixteen, 2·8 l. Fernbach flasks containing 500 ml. of Wainwright's solid medium (1959). The conidial inoculum was spread evenly over the agar surface, the flasks wrapped with aluminium foil to protect from light and the cultures incubated for 3 days at 30°. The wrappings were then removed and incubation continued at room temperature under continuous light for an additional 4–6 days. To allow adequate gaseous exchange for optimal conidiation and still maintain aseptic conditions, covers for these flasks were made of two layers of cheesecloth, one layer of non-absorbent cotton-wool, one layer of cheesecloth, another layer of cotton-wool and finally a layer of cheesecloth; these covers were fastened firmly in place with rubber bands.

Conidia were harvested by adding 10 sterile marbles (10–15 mm. diam.) and 200 ml. sterile distilled water containing 3 drops of silicone antifoam type B (Dow Corning Corp., Midland, Mich., U.S.A.) as a wetting agent, to each flask. The flasks were shaken by hand to remove the mat of growth from the agar surface. The suspensions were combined, filtered through four layers of cheesecloth to remove bits of hyphae and ultrasonically treated (as above) for 2 min. The dark orange conidial suspension was stored at 4° for no longer than 1 week before use. Sixteen Erlenmeyer flasks yielded 2·5 l. of conidial suspension containing about 2×10^8 conidia/ml.

Growth of hyphae. Hyphae were grown by aseptically transferring about 50 ml. of the conidial suspension containing about 10^{10} conidia (50 ml. $\times 2 \times 10^8$ conidia/ml.) into 500 ml. of Vogel's complete medium (1956) + 3 drops of silicone antifoam in a 2 l. Erlenmeyer flask. Cultures were incubated for 2 days at 30° on a rotary shaker (about 265 rev./min.).

Preparation of mitochondria. Quantities of mitochondria sufficient to do numerous biochemical assays were obtained from 1 l. of a 2-day culture of *Neurospora crassa*. The hyphae were collected (centrifugation at 500*g* for 5 min.), washed at 0–4° by suspension and centrifugation in distilled water, and finally suspended in 500 ml. of a 'preparation' medium which consisted of: sucrose, 0.25 M; EDTA, 0·005 M (pH 7·0); and bovine serum albumin (BSA), 0·15 % (w/v). All subsequent operations were done in the cold (0–4°). To a sample (250 ml.) of the hyphae suspended in preparation medium were added 500*g* acid-washed glass beads (0·2 mm. diam.) and 4 drops of silicone antifoam; the mixture of beads and organism were poured into a chilled Eppenbach Micro-mill, Model MV-6-3 (Gifford-Wood Co., Hudson, N.Y., U.S.A.) and ground at maximal speed for 1 min. at a gap setting of 1/30,000 in. The ratio of liquid volume to bead volume and the time of grinding was carefully standardized to obtain adequate cell breakage and subsequent separation of functional mitochondria. Under the conditions outlined here about 75 % of the hyphal cells were disrupted. Broken cells and beads were spun out of the mill, at low speed with the gap completely open, into a large beaker and the contents were allowed to sediment for a few min. The unsedimented material was decanted. The beads were washed twice with preparation medium to remove trapped cellular components and the washes were added to the decanted liquid. The mitochrondria were collected from the decanted liquid by differential centrifugation, as a fraction sedimenting between 1500*g* (10 min.) and 8000*g* (30 min.) A second 1500*g* centrifugation before the final sedimentation of the mitochondria at 8000*g* removed additional amounts of contaminating cell wall fragments. The final mitochondrial pellet was suspended in 3–5 ml. of preparation medium to give a final concentration of 20–30 mg. protein/ml. All suspensions were made with Ten-Broeck ground glass homogenizers. The complete preparative procedure required about 2·5 hr.

Electron microscopy. Samples of intact hyphae, of the crude mitochondrial fractions and of all fractions separated by density gradient centrifugation were fixed with 0·6 % aqueous $KMnO_4$ and 'post-fixed' with 5 % uranyl acetate (North, 1961). The fixed pellets were dehydrated by rapid passage through a cold (−10°) ethanol series and embedded in Epon 812 by the method of Luft (1961). Thin sections were cut on a Porter–Blum or an LKB microtome. Sections were not post-stained.

Samples of fractions obtained at different stages during the preparation of mitochondria were negatively stained with 1 % potassium phosphotungstate (PTA, pH 6·5–

6.8) to determine whether the mitochondria were grossly contaminated with other cellular components. Sections and negatively stained preparations were observed in Siemens Elmiskop 1 double-condenser electron microscope operated at 80 kV. with 50 μ objective apertures.

Biochemical assays. Oxidative phosphorylation was calculated by measuring the respiration manometrically at 25° (using 5 ml. or 15 ml. vessels) according to Slater & Holton (1954) and by determining the amount of inorganic phosphate (Pi) esterified (Gomori 1942). All reaction media used for biochemical assays were at pH 6·9.

Respiratory control at 25° was estimated by the method of Chance & Williams (1955) with a Clark oxygen electrode.

Difference spectra, from which the content of the cytochromes in the mitochondria was estimated, were obtained by the procedure of King, Nickel & Jensen (1964) by using a Beckman DK Recording Spectrophotometer.

Continuous sucrose density gradients (20–65 %, w/v; 0·58–1·9 M) were prepared with a Buchler Densigrad apparatus (Buchler Instruments, Fort Lee, New Jersey, U.S.A.) by the procedure of Luck (1963) following Britten & Roberts (1960). The same apparatus was used to collect fractions from the gradient. A sample of the mitochondrial preparation (0·5–0·9 ml.) was layered on the top of a 4·6 ml. gradient and centrifuged for 1 hr at 40,000 rev./min. in a Spinco centrifuge.

The mitochondrial adenosine triphosphatase (ATPase) activity was determined by the release of inorganic phosphate (Cooper & Lehninger, 1957); the adenosine triphosphate-inorganic phosphate (ATP-^{32}Pi) exchange reaction by the formation of AT^{32}P (Wadkins & Lehninger, 1963); calcium accumulation by the disappearance of $^{45}Ca^{2+}$ from the reaction medium (Rossi & Lehninger, 1963); the adenosine triphosphate-adenosine diphosphate (ATP–ADP) exchange by the incorporation of ADP-1-^{14}C into ATP (Wadkins & Lehninger, 1963). The uptake of leucine-1-^{14}C by mitochondria preparations was estimated by the method of Truman & Korner (1962).

Protein was estimated usually by the microbiuret method of Goa (1953), but when the high sucrose concentration in fractions from the density gradient was found to interfere, the method of Lowry, Rosebrough, Farr & Randall (1951) was used. Crystalline bovine serum albumin served as a standard in these determinations.

RESULTS

Electron microscopy

Mitochondria were easily recognized in thin sections of hyphae of *Neurospora crassa* fixed with $KMnO_4$ + uranyl acetate (Pl. 1, fig. 1); the cristae were distinct and the basic structural appearance was similar to that typical of mitochondria from other cells. The mitochondrial matrices appeared to have about the same opacity as the cytoplasm, and the cells in general had the diluted appearance characteristic of many cells and tissues fixed with $KMnO_4$. Ribosomes could not be distinguished, probably because the sections were not stained and the contrast was relatively low.

The mitochondrial preparation obtained by differential centrifugation between 1500*g* and 8000*g* contained some contaminating cellular structures but was primarily composed of mitochondria (Pl. 1, fig. 2). Much of the contamination was cell wall material which was readily identified in negatively stained preparations by its dis-

tinctive fibrous appearance (Pl. 2, fig. 3). Most major contamination detectable in the electron microscope, however, was removed by centrifugation on the sucrose density gradients; this purification of mitochondria was also indicated by the increased rate of oxidation of succinate (Fig. 1).

Three mitochondrial profiles were seen in about equal numbers in thin sections of the unfractionated mitochondrial preparation (Pl. 1, fig. 2, m1, m2, m3) and also in the purified mitochondrial fraction collected from sucrose gradients. This heterogeneity in mitochondrial appearance probably was due, in part, to damage incurred during preparation. This interpretation is supported by the relatively low respiratory control ratios (compared with intact rat liver mitochondria) and high Mg-ATPase activity of mitochondria from *Neurospora crassa* (Tables 3, 4). The structure of mitochondria from hyphal cells appeared indistinguishable from mitochondria isolated from conidia or germinating conidia by the grinding procedure used.

Isolated mitochondria of *Neurospora crassa*, negatively stained with PTA, contained small particles (80–90 Å diameter) apparently associated with the inner mitochondrial membranes; the outer membrane, at least in some profiles, appeared relatively smooth (Pl. 2, fig. 4). This observation agrees with that reported previously by Stoeckenius (1963). However, thin strands of membranes with attached particles were seen much less frequently than in negatively stained preparations of rat liver mitochondria (unpublished observations). It is possible that mitochondria of *N. crassa* are less susceptible to disruption during negative staining than are rat liver mitochondria; disruption of the membrane may be necessary for the particles to be visible in the electron microscope (Sjostrand, Andersson-Cedergren & Karlsson, 1964).

Table 1. *Requirements for oxidative phosphorylation**

The complete reaction mixture contained 10 mM-succinate, 125 mM-sucrose, 10 mM-$MgCl_2$, 10 mM-potassium phosphate (pH 6·9), 1·5 mM-EDTA, 1 mM-ADP, 20 mM-glucose, 150 K.M. units hexokinase/ml., 3·15 mg. mitochondrial protein. Final volume 2·0 ml. Omission of sucrose, bovine serum albumin or EDTA was not complete since concentrations of 25 mM, 0·15 mg./ml. and 0·5 mM, respectively, resulted from their addition with the mitochondrial preparation (0·2 ml.). Uptake of O_2 was measured manometrically at 25° for 20 min.

	Uptake/mg. protein/hr		
Assay system	μmoles Pi	μatoms O_2	P:O ratio
Complete	6·2	5·0	1·3
−sucrose	6·4	5·8	1·1
−BSA	6·5	7·2	0·9
−EDTA	7·9	6·8	1·2
$-Mg^{++}$	0·9	5·7	0·2
−ADP	0	4·4	c
−Pi	0	2·3	c
−hexokinase and glucose	2·2	6·9	0·3
−succinate	0	0	0
−mitochondria	0	0	0

* Succinate was used as substrate.

Biochemical properties of Neurospora mitochondria

Oxidative phosphorylation. Table 1 shows that mitochondria isolated from *Neurospora crassa* by the procedure outlined here coupled the phosphorylation of ADP to ATP to the oxidation of succinate. The data indicate that additions of substrate, Mg^{2+}

ADP, Pi, hexokinase and glucose to the reaction medium were essential for this activity. In respect of these requirements, mitochondria from *N. crassa* are similar to mitochondria from mammalian tissues. The authors discovered in preliminary experiments that sucrose – bovine serum albumin + EDTA in the preparation medium facilitated the isolation of biochemically active mitochondria from *N. crassa*. These compounds were therefore used in the medium in which the mitochondria were suspended and were added as part of the mitochondrial suspension to the oxidative phosphorylation reaction mixture. As a result, failure to make further additions of these compounds affected oxidative phosphorylation only slightly (see Table 1). The P:O ratios obtained in different experiments with succinate as substrate ranged from about 0·7 to 1·3.

Table 2. *Effect of respiratory inhibitors and uncouplers on oxidative phosphorylation**

Reaction mixture was that given in Table 1 but the final volume was 1 ml. and the following additions were made where indicated: DNP, 0·4 mM; antimycin A, 0·5 μg./ml.; oligomycin, 5 μg./ml.; KCN, 1 mM; malonate, 20 mM; oleate, 0·1 mM; atractylate, 0·1 mM; *p*-F_3MeOCCP, 1 μM; *m*-Cl-CCP, 2·5 μM; gramicidin, 6 μM. Between 3 and 4 mg. mitochondrial protein were added in each experiment, which were run for 20–40 min. at 25°, with 5 ml. volume manometric vessels.

	Uptake/mg. protein/hr		
Assay system	μmoles Pi	μatoms O_2	P:O ratio
Control—no additions	5·9	7·2	0·82
+ Antimycin A	0	0	0
+ KCN	0	0	0
+ Malonate	0	0	0
+ Oligomycin	0	5·8	0
+ DNP	0	2·4	0
+ Oleate	0	1·5	0
+ Atractylate	0	5·0	0
+ *p*-F_3O-CCP	1·8	4·8	0·37
+ *m*-Cl-CCP	0·5	2·4	0·21
+ Gramicidin	0·9	3·7	0·25

* Succinate was used as substrate.

A number of compounds, at concentrations which commonly uncouple or inhibit oxidative phosphorylation in mammalian mitochondria, also depressed the P:O ratios obtained with mitochondria from *Neurospora crassa* when succinate was the substrate (Table 2). In the presence of antimycin A, KCN or malonate, no oxygen uptake or phosphorylation was observed. Oligomycin, 2,4-dinitrophenol (DNP), oleate and atractylate were effective uncouplers of the phosphorylation of ADP to ATP and oxygen consumption was decreased by various degrees with these reagents. With gramicidin, carbonyl cyanide-*m*-chlorophenylhydrazone (*m*-Cl-CCP), and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (*p*-CF_3O-CCP) in the reaction medium, the P:O ratios were decreased.

Table 3 shows that mitochondria of *Neurospora crassa* isolated from 2-day hyphae by the grinding procedure outlined here, coupled phosphorylation to the oxidation of the seven substrates which were tested. In some cases, the P:O ratios were somewhat less than theoretical values; however, to our knowledge these data represent the first detailed study of mitochondria capable of carrying out oxidative phosphorylation from fungal cells other than yeasts. Table 3 shows also that some respiratory control was

observed with the isolated mitochondria of *N. crassa*. The respiratory control ratios were much lower than those reported for mammalian mitochondria where ratios of 4–10 are not uncommon. Also the respiratory control ratios obtained for mitochondria from *N. crassa* were in general somewhat lower than those reported for yeast mitochondria (Ohnishi, *et al.*, 1966). The mitochondria of *N. crassa* responded to the addition of the ADP to about the same extent as 'loosely coupled' mammalian mitochondria.

Cytochrome content. Figure 2 shows the difference spectrum (reduced-oxidized) of the cytochrome in mitochondria from 2-day hyphae. Characteristic peaks at 605, 445, 562 and a shoulder at 553 mμ indicated the presence of cytochromes a_1, a_3, *b* and *c*, respectively. Quantitatively these cytochromes were in the ratio about 1:1:1:1 although cytochrome *c* was slightly lower than the others.

Table 3. *Oxidative phosphorylation and respiratory control of Neurospora mitochondria using various substrates*

A. Oxidative phosphorylation. The reaction mixtures were as in Table 1 with the substrates varied as follows: 10 mM-succinate; 10 mM-citrate, -isocitrate, -α-ketoglutarate, -pyruvate + 2·5 mM-malate (all plus 1 mM-NAD); 10 mM-$NADH_2$; 25 mM-ascorbate + 0·3 mM-TMPD. Between 3 and 7 mg. mitochondrial protein were added in each experiment which was run for 10–80 min. (depending on substrate) at 25°.

B. Respiratory control. The reaction mixtures contained, in a total volume of 2 ml.: sucrose, 125 mM (or 106·3 mM); bovine serum albumin, 3·15 mg./ml. (or 3·04 mg./ml.); EDTA, 0·5 mM (or 0·13 mM); $MgCl_2$, 2 mM; ADP 0·1 mM; potassium phosphate (pH 6·9), 5 mM; succinate, 10 mM; citrate, isocitrate, α-ketoglutarate or pyruvate + 2·5 mM malate, 10 mM each, all + 1 mM-NAD; $NADH_2$, 10 mM; ascorbate, 25 mM. + TMPD, 0·3 mM. The figures in brackets reflect the lower quantities of mitochondria added in the case of ascorbate and $NADH_2$ oxidation. Between 1 and 8 mg. mitochondrial protein (depending on substrate) were added. Rate of O_2 uptake was measured polarographically (Clark oxygen electrode) and at 25° calculated as mμatoms O_2 taken up/mg. protein/min.

Substrate	Uptake per/mg. protein/hr: μmoles Pi	Uptake per/mg. protein/hr: μatoms O_2	P:O ratio	R.C. ratio*
Ascorbate+TMPD	5·2	8·4	0·6	1·36
$NADH_2$	5·8	6·9	0·8	1·15
Succinate	7·5	5·0	1·5	1·70
Pyruvate (malate)	0·8	0·4	1·9	2·30
Citrate	7·6	2·6	3·0	2·00
Isocitrate	2·4	1·5	1·6	1·70
α–Ketoglutarate	4·7	1·4	3·3	2·00

$$\text{*Respiratory control ratio} = \frac{\text{rate of } O_2 \text{ uptake in presence of added ADP}}{\text{rate of } O_2 \text{ uptake in absence of added ADP}}.$$

Activities related to oxidative phosphorylation. A number of enzymic activities associated with the oxidative phosphorylation reactions of mammalian mitochondria were assayed to determine whether these could be detected in mitochondria from *Neurospora crassa*. The 'partial reactions' of oxidative phosphorylation which were surveyed included Mg^{2+}- and DNP-stimulated ATPase, ADP-Pi exchange activity, and ion accumulation. Mitochondria isolated from 2-day hyphae were used in these studies. Table 4 shows that ATPase, ATP-Pi exchange and ion accumulation activities could be measured in mitochondria from *N. crassa*. The rather low ATPase activity was stimulated by Mg^{2+} but not by DNP, and was inhibited by oligomycin. Results from other

experiments showed that the Mg^{2+}-stimulated ATPase in mitochondria from conidia and germinating conidia was 4–5 times higher than that reported here. The ATP-Pi exchange activity was sensitive to DNP and to oligomicin (Table 4) suggesting that this activity was related to oxidative phosphorylation. Accumulation of $^{45}Ca^{2+}$ by *N. crassa* mitochondria was supported by substrate oxidation, but the rate of uptake was considerably slower than that observed with intact rat liver mitochondria (Lehninger, Rossi & Greenawalt, 1963; Rossi & Lehninger, 1963). This activity, as in rat liver mitochondria, appeared to be respiration-dependent since KCN inhibited the reaction (Table 4). A slow rate of Ca^{2+} accumulation was supported by ATP; this appeared to be insensitive to KCN and to oligomycin. The mitochondria from *N. crassa*

Table 4. *Activities related to oxidative phosphorylation*

A. ATPase activity of isolated mitochondria. The reaction mixtures contained, in a total volume of 1 ml., the following: 15 mM-ATP, (pH 7); 1·2–2·6 mg. mitochondrial protein; and, as indicated: 0·4 mM-DNP; 3 mM-$MgCl_2$; 10 μg. oligomycin/ml. The reactions were run for 15 min. (Mg^{2+}) or 30 min. (DNP) at 25°.

B. ATP-^{32}Pi exchange activity of isolated mitochondria. The reaction mixtures contained, in a final volume of 1 ml., the following: 15 mM-ATP, (pH 7); 1 mM-$MgCl_2$; 10 mM-potassium phosphate, ^{32}P (pH 6·9); 1·2–2·6 mg. mitochondrial protein; and, as indicated: 0·4 mM-DNP; 10 μg-oligomycin/ml. The reactions were run for 30 min. at 25°.

C. Uptake of Ca^{2+} by isolated mitochondria. The reaction mixtures contained, in a final volume of 5 ml., the following: 10 mM-tris-HCl (pH 7); 80 mM-NaCl; 10 mM-$MgCl_2$; 4 mM-potassium phosphate (pH 7); 3 mM-$^{45}CaCl_2$; 3 mM-ATP (succinate-supported uptake) or 15 mM-ATP (ATP-supported uptake); 4–8 mg. mitochondrial protein; and the following, where indicated: 10 mM-succinate; mM-KCN; 10 μg. oligomycin/5 ml. The reactions were run for 20 min. at 30°.

ATPase		ATP-^{32}Pi Exchange		Ca^{++} accumulation	
System	Spec. act.*	System	Spec. Act.†	System	Spec. Act.‡
1. Control	0·07	1. Complete	154	A. substrate-supported	
2. +DNP	0·05	2. +DNP	0	1. complete (succinate)	1·0
3. +DNP+oligomycin	0·02	3. +Oligomycin	0	2. +KCN	0·3
4. $+Mg^{2+}$	1·66			3. +Oligomycin	0·9
5. $+Mg^{2+}$+oligomycin	0·29			B. ATP-supported	
				1. Complete (ATP)	0·4
				2. +KCN	0·4
				3. +Oligomycin	0·4

* μmoles Pi/mg. protein/hr.
† μmoles AT^{32}P/mg. protein/hr.
‡ μmoles Ca^{2+}/mg. protein/hr.

showed no capacity to accumulate either Mg^{2+} or Sr^{2+}, which are readily taken up by mammalian mitochondria (Brierley, Bachmann & Green, 1962: Carafoli, Weiland & Lehninger, 1965). In addition to these reactions, some ATP-ADP exchange activity and a slow rate of incorporation of leucine-^{14}C by the mitochondria were detected.

Density gradient fractionation. It is clear that mitochondria isolated from 2-day hyphae of *Neurospora crassa* by the procedure described, were capable of carrying out biochemical reactions typical of mammalian mitochondria. However, the low respiratory control ratios, the presence of Mg^{2+}-ATPase and absence of DNP-ATPase, and the variation in mitochondrial profiles seen in the electron microscope (Pl. 1, fig. 2) suggested that the mitochondria were damaged to some extent during preparation. Furthermore, it was apparent from electron microscopic examination that the crude

mitochondrial preparation was contaminated with other subcellular material, especially cell wall fragments. It was of interest, therefore, to determine whether a more homogenous mitochondrial fraction might be separated on sucrose density gradients.

Centrifugation of the crude mitochondrial preparation on a linear sucrose density gradient produced four fractions. Two major bands were observed on the gradient, a distinct, orange 'mitochondrial' fraction and lighter, more diffuse 'submitochondrial' fraction. A third, very faint band which contained very little of the total protein placed on the gradient formed above the sub-mitochondrial fraction. The fourth fraction sedimented as a pellet at the bottom of the tube. The bands were collected dropwise from the tubes in 25-drop fractions and assayed for enzymic activities and for cytochromes. Difference spectra showed that both the mitochondrial and submitochondrial

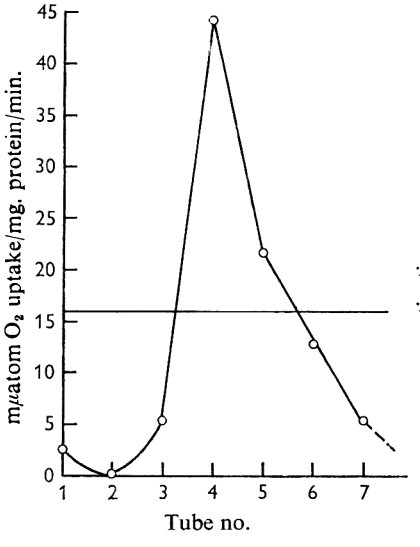

Fig. 1

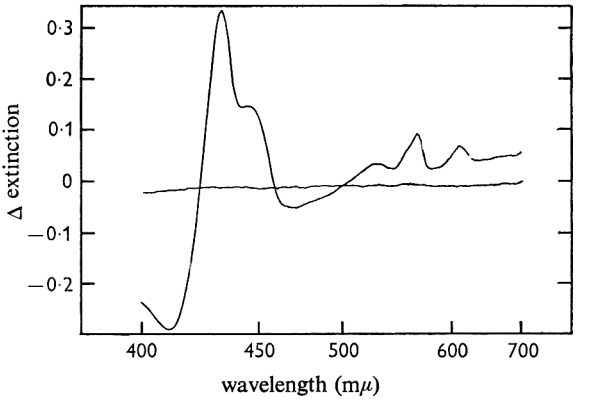

Fig. 2

Fig. 1. Distribution of the succinoxidase activity in the mitochondrial fractions obtained from the sucrose density gradients. Rate of O_2 uptake was determined polarographically as described in legend for Table 3. The rate of oxidation of succinate by the unfractionated mitochondrial preparation is indicated by the horizontal line.

Fig. 2. Difference spectra (reduced minus oxidized) of *Neurospora crassa* mitochondria. Mitochondria (27 mg. protein) were added to each of two cuvettes in a final volume of 3 ml. The reduced spectrum was obtained by adding excess $Na_2S_2O_4$ to one cuvette following the method of King *et al.* (1964).

fractions contained cytochromes and that both were capable of oxidizing succinate, NADH and ascorbate+TMPD. The submitochondrial fraction oxidized NADH more rapidly than the mitochondrial fraction but the reverse was true with succinate as substrate. The relative rates of succinate oxidation by the mitochondrial fraction (collected in tubes 3 and 4) and the submitochondrial fraction (tubes 5 and 6) are shown in Fig. 2. Ascorbate+TMPD was oxidized by both fractions at rates intermediate to the oxidation of NADH and succinate. Respiratory control, although low, was present only in the mitochondrial fraction.

The pellet, mitochondrial and submitochondrial fractions were negatively stained with PTA and examined in the electron microscope. The pellet contained large frag-

ments of cell wall, some mitochondria, and unidentified cellular material. Mostly intact mitochondria were present in the mitochondrial band and the submitochondrial fraction contained membranous profiles, many of which resembled mitochondrial fragments. Only limited material from the uppermost band was available for examination but this fraction appeared to contain primarily small membranous vesicles (possibly microsomal membranes) and very small pieces of cell wall.

DISCUSSION

Specific conditions were found to be required to isolate biochemically competent mitochondria from hyphae of *Neurospora crassa*. The inclusion of bovine serum albumin and the omision of phosphate (which may preclude mitochondrial swelling and the uncoupling of phosphorylation) in the isolation medium enabled the isolation of a mitochondrial fraction from *N. crassa* which was capable of performing oxidative phosphorylation. Bovine serum albumin was shown by Wojtczak & Wojtczak (1960) to be beneficial in the isolation of functional mitochondria from insects, and they showed that it removed fatty acids which uncoupled oxidative phosphorylation; it is possible that the albumin performs the same function in the preparations of mitochondria from *N. crassa* described here. During the present work it was observed that the first crude mitochondrial fraction, i.e. the mitochondria in the supernatant fluid of the first centrifugation at 1500*g*, was unable to form ATP although substrates were oxidized. However, when the mitochondria were subsequently centrifuged from the crude supernatant fluid (at 8000*g*) they did phosphorylate ADP to ATP, even when the supernatant fluid from the 8000*g* centrifugation was added back to the mitochondria. Apparently an uncoupling phenomenon occurred before the physical separation of the mitochondria from the supernatant fluid fraction. This uncoupling action of the crude supernatant fluid fraction was not investigated further, it may be related to the requirement for serum albumin in the preparation medium.

From Tables 1 and 2 it is evident that the oxidation of succinate by the mitochondrial fraction isolated from *Neurospora crassa* was linked to phosphorylation with characteristics similar to those shown by mitochondria isolated from many other organisms. The data in Tables 3 and 4 show that *N. crassa* mitochondria coupled phosphorylation to the oxidation of a number of substrates, exhibited some respiratory control, and carried out a number of enzymic activities linked energetically to oxidative phosphorylation.

Biochemical and ultrastructural evidence suggest that the mitochondria in the crude preparation were damaged to some extent in the isolation procedure. However, it is possible that the uncoupling phenomenon mentioned above is not completely reversible and affects the biochemical activities of the mitochondria in the crude preparation. The respiratory control ratios recorded for *Neurospora crassa* mitochondria were low as compared with those of mammalian mitochondria, but were comparable with those reported for yeast mitochondria (Ohnishi *et al.* 1966). Mitochondria have been isolated from *N. crassa* by milder treatment (Greenawalt, Hall, & Wallis, 1967) with only a slight increase in respiratory control ratios.

Fractionation of the mitochondrial preparation on sucrose density gradients enabled the collection of a purer mitochondrial fraction since cell wall contamination and fragmented mitochondria were separated from the major mitochondrial band. The presence of cytochromes $a+a_3$, *b* and *c* indicated that the submitochondrial fraction

was most likely derived from the mitochondria. Respiratory control ratios were recorded only with the mitochondrial fraction.

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EXPLANATION OF PLATES

PLATE 1

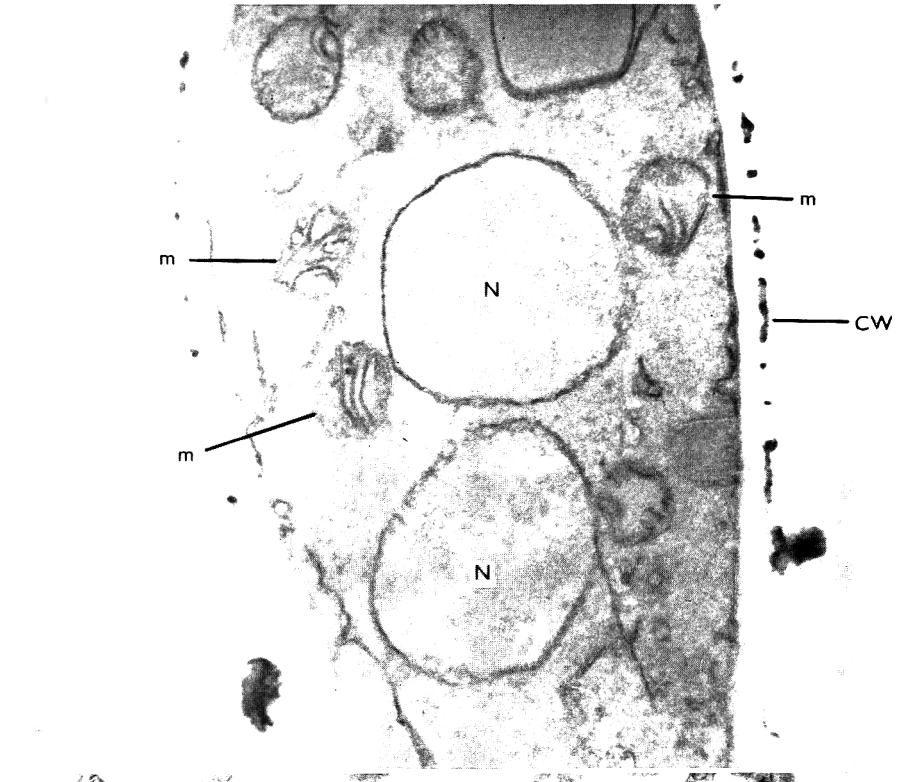
Fig. 1. Thin section of a 2-day hypha of *Neurospora crassa*. Nuclei (N) and mitochondria (m) including the cristae are clearly visible. The cell wall (CW) is only lightly stained. Fixed with $KMnO_4$ and uranyl acetate. Unstained. × 26,000.

Fig. 2. Mitochondria isolated from 2-day hyphae of *Neurospora crassa*. Three profiles (m_1, m_2, m_3) can be seen. Fixed with $KMnO_4$ + uranyl acetate. Unstained. × 20,000.

PLATE 2

Fig. 3. A large fragment of cell wall contaminating the crude mitochondrial fraction. The distinct fibrous appearance of this material can be seen. Negatively-stained with potassium phosphotungstate (PTA), pH 6·5. × 60,000.

Fig. 4. Isolated mitochondrion negatively stained with PTA, pH 6·5. The relatively smooth outer membrane (OM) is partly disrupted and torn away from the mitochondrion. The inner membrane (IM) protrudes (left-centre of the micrograph) and has small (90 Å) projecting particles associated with it. × 65,000. Inset: enlargement of portion of inner membrane outlined by inked lines. The 90 Å particles are clearly seen. × 140,000.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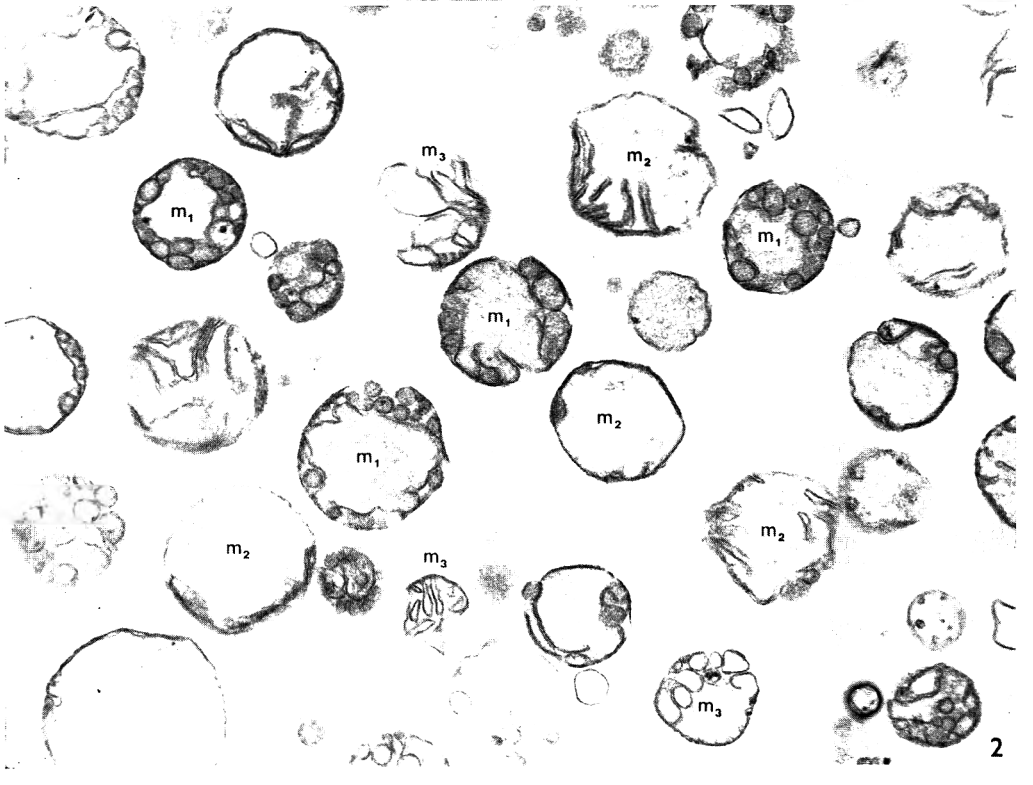
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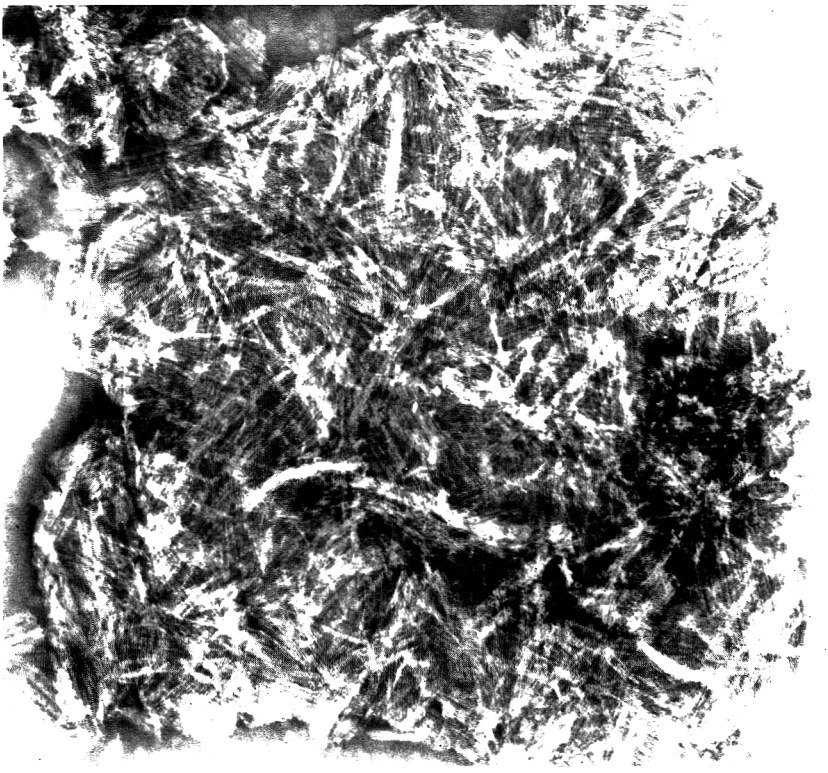

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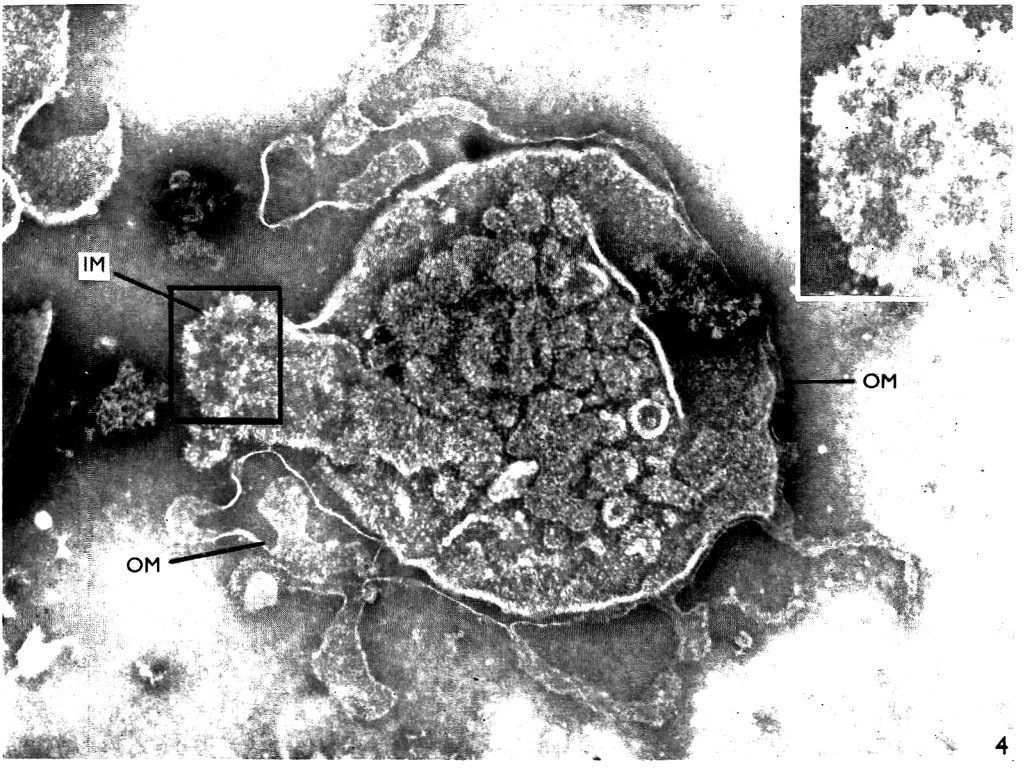

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Leuconostoc oenos sp.nov.

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SUMMARY

Nineteen strains of Gram-positive cocci isolated from wine and belonging to the genus *Leuconostoc* were examined and are considered to form a new species which is named *Leuconostoc oenos*. These strains differ from other leuconostocs because they grow better in a medium containing tomato juice than in yeast glucose citrate broth normally used in this laboratory for the genus. Furthermore, they grow well in media in which the pH value is too acid for other Leuconostoc species to initiate growth. The cultures of *L. oenos* do not form dextran from sucrose. They form acid from fructose, glucose, trehalose and aesculin, generally from melibiose and salicin. They may form acid from arabinose, xylose, galactose, mannose and cellobiose, but not from lactose, maltose, sucrose, raffinose, dextrin, glycerol, mannitol or sorbitol.

INTRODUCTION

Gram-positive cocci belonging to the genus *Leuconostoc* occur in wine as part of the normal bacteriological flora (Bidan, 1956; Radler, 1958; Ingraham, Vaughn & Cooke, 1960; Peynaud & Domercq, 1961), but none of these reports satisfactorily identifies the Leuconostoc species which they described. Cultures obtained from Drs Radler, Peynaud and Ingraham were therefore examined and compared with strains of other species of the genus *Leuconostoc* held in the National Collection of Dairy Organisms (Shinfield) and described by Garvie (1960).

METHODS

Cultures. The strains of wine leuconostocs examined are listed in Table 1. They were found to require conditions of growth different from those required by other species of the same genus. The media and methods used had therefore to be modified to suit the wine strains.

General media for, and cultivation of, wine leuconostocs. The cultures were grown in acidic tomato broth (ATB) consisting of (w/v): Evans peptone, 1·0 %; Yeastrel, 0·5 %; glucose, 1·0 %; $MgSO_4.7H_2O$, 0·02 %; $MnSO_4.4H_2O$, 0·005 %; and (v/v): tomato juice, 25 %; pH 4·8; autoclaved at 121° for 15 min.; before inoculation 0·5 ml. of 1·0 % (w/v) solution of cysteine hydrochloride sterilized by filtration was added to each 10 ml. of media. The cultures were incubated at 22° for 3–4 days.

Stock cultures. Agar stabs were prepared by adding 1·5 % agar to ATB. After inoculation these were incubated in anaerobic jars, evacuated then filled with H_2+CO_2 (90:10, v/v). The cultures were then kept in cold store for several months.

General media for, and cultivation of, non-wine leuconostocs. These strains were

grown in yeast glucose citrate broth (YGCB) consisting of (w/v): Evans peptone, 1·0 %; Lemco, 1·0 %; Yeastrel, 0·5 %; glucose, 1·0 %; triammonium citrate, 0·5 %; sodium acetate, 0·2 %; $MgSO_4.7H_2O$, 0·02 %; $MnSO_4.4H_2O$, 0·005 %; and (v/v): Tween 80, 0·1 %; pH 6·7; autoclaved 121° for 15 min.

Table 1. *Source of cultures of Leuconostoc examined*

NCDO no.	Designation when received	Donor
1668	Plince 3	E. Peynaud, Station Agronomique et Oenologique, Bordeaux, France
1669	St Caprais 105	
1670	Ducru Beaucaillon 6	
1671	Brane 33	
1672	Fourtet 1	
1673	Peyreau 1	
1674	Baudry 1	
1675	St Caprais 133	
1705	Fourtet 2	
1706	Grand Puy 3	
1707	Palmer 6	
1708	St Caprais 131	
1709	St Caprais 122	
1694	12 A	F. Radler, C.S.I.R.O., Merbein, Australia
1695	16 A	
1696	45 B	
1821	ML 25	R. E. Kunkee, University of California
1822	ML 27	
1823	ML 34	

Cysteine hydrochloride was added before inoculation with *Leuconostoc cremoris* (*Betacoccus cremoris*, Knudsen & Sørensen, 1929) and cultures of strains of this species were incubated at 22° for 48 hr. The same conditions of growth were used for a few group III strains (Garvie, 1960). All other strains were incubated at 30° for 24 hr (without cysteine hydrochloride).

The turbidity (extinction) of cultures was measured in ATB or YGCB with a Lumetron colorimeter model 400A (Photovolt Corporation, N.Y.) and an orange (580 mμ) filter.

Growth at pH 3·7 was tested in citric+malic acid broth (CMB) which consisted of (w/v): Evans peptone, 1·0 %; Yeastrel, 0·5 %; glucose, 1·0 %; citric acid, 0·25 %; DL-malic acid, 0·25%; KH_2PO_4, 0·25%; $MgSO_4.7H_2O$, 0·02%; $MnSO_4.4H_2O$, 0·005%; and (v/v): Tween 80, 0·1%; tomato juice, 5·0%. The medium was divided and portions adjusted to pH 6·7, 4·7 or 3·7, tubed in 5 ml. quantities, and sterilized by autoclaving at 121° for 15 min. (The pH value of the media did not change on sterilization.) Cysteine hydrochloride was added for the strains of *Leuconostoc oenos* and *L. cremoris* and tubes were inoculated with one drop of a culture in ATB or YGCB. All cultures were seeded into CMB at pH 3·7. Control cultures of *L. oenos* were grown in CMB at pH 4·7 and for control cultures of other strains in CMB at pH 6·7.

Growth in the presence of 10 % (v/v) ethanol. CMB was prepared at double strength and adjusted to pH 4·7 for *Leuconostoc oenos*, and pH 6·7 for other species. The medium was tubed in 2·5 ml. quantities and water added to bring the volume to 4·5 ml. (0·25 ml. less for those strains to which cysteine hydrochloride was added). The medium was autoclaved at 121° for 15 min., cooled and 0·5 ml. absolute ethanol

then added. Tubes were inoculated with a drop of culture, closed with a sterile rubber bung and incubated for 4 days for *L. oenos* or 1–2 days for other species.

Growth in litmus milk (LM) and yeast glucose litmus milk (YGLM). Cultures were incubated at 22° for 7 days.

Growth temperatures. Growth was observed in ATB (with cysteine hydrochloride added) after incubation for 3 days at 37·5° and 7 days at 10°.

Production of ammonia from arginine. Cultures were grown at 22° for 7 days in the medium used by Garvie (1960) and in MRSB (de Man, Rogosa & Sharpe, 1960) prepared without ammonium citrate but with 0·3 % arginine hydrochloride. Nessler's solution was used to test for the production of ammonia.

Production of dextran from sucrose. One ml. of a 50 % (w/v) solution of sucrose, sterilized by autoclaving at 121° for 15 min., was added to 10 ml. ATB agar and plates poured and streaked. Since the wine leuconostocs do not grow aerobically the plates were incubated as already described in an atmosphere of H_2+CO_2 for 14 days.

Utilization of citrate. One ml. of a 10 % (w/v) solution of triammonium citrate was added to 10 ml. ATB, cysteine added and the tubes inoculated. After 3 days' incubation at 22° the residual citrate was assayed by using *Streptococcus lactis* var. *diacetilactis* NCDO 1007 (Garvie, 1967*a*).

Type of lactic acid formed. The cultures were incubated for 3 days in dilute tomato broth (Garvie, 1967*b*). The type of lactic acid formed was estimated by using a DPN method for L(+)-lactate and a modification of the method of van den Hamer & Elias (1958) for D(−)-lactate (Garvie, 1967*b*).

*'Carbohydrate' fermentation.*The basal medium consisted of (w/v): Evans peptone, 1·5 %; Yeastrel, 0·6 %; NaCl, 0·5 %; agar, 0·5 %; bromcresol green, 0·004 %; pH 5·2. The medium was prepared in 5 ml. quantities in 6 in. × $\frac{3}{8}$ in. test tubes and autoclaved at 121° for 15 min. The tubes were placed in boiling water to melt the agar, cooled to 45° and 0·5 ml. of 2 % (w/v) Seitz filtered 'Carbohydrate' added. The tubes were inoculated with 0·2 ml. of a 3-day culture, allowed to set and incubated for 21 days at 22°.

The production of gas from glucose. The method of Abd-el-Malek & Gibson (1948) was used.

Catalase was detected by emulsifying the growth from agar in a drop of H_2O_2 (20 vol.) and observing gas formation.

RESULTS

All the nineteen strains of wine leuconstocs were Gram-positive, catalase negative, and formed pairs of chains of cocci. They formed gas from glucose, did not form ammonia from arginine and did not change litmus milk. They formed D(−)-lactic acid. These properties are characteristic of bacteria which belong to the genus *Leuconostoc.*

Growth conditions for wine leuconostoc

YGCB did not support the growth of five of the wine leuconostoc strains (Garvie & Mabbitt, 1967). All nineteen strains grew in ATB, but maximum turbidity was obtained only after several days of incubation. A comparison was made between the growth of the wine cocci and thirty-two other leuconostocs, in both YGCB and ATB. Both media were prepared at pH values of 6·7, 6·0, 5·5 and 4·8. (It was found, as with CMB, that the pH values of YGCB and ATB did not change on sterilization.)

The results are summarized in Table 2.

Of the non-wine leuconostocs (groups I–IV) only three strains (*Leuconostoc paramesenteroides*; Garvie, 1967*c*) grew well in YGCB starting at pH 4·8, and of these only two grew in ATB starting at the same pH value. The other twenty-nine cultures did not grow in media at pH 4·8 but they grew in both media starting at pH 6·7, giving a higher extinction in YGCB than ATB. Maximum turbidity was reached in 24 hr for most strains, but 48 hr were required for *L. cremoris* and other strains which grow best at 22°. None of the strains which grew in ATB at initial pH 4·8 grew in

Table 2. *Range of turbidity (extinction) readings obtained on cultures of Leuconostocs grown in yeast glucose citrate broth (YGCB) and acidic tomato broth (ATB)*

Species*	No. of strains examined	Medium: YGCB, Initial pH value: pH 6·7 (Lumetron readings)	Medium: YGCB, Initial pH value: pH 4·8 (Lumetron readings)	Medium: ATB, Initial pH value: pH 6·7 (Lumetron readings)	Medium: ATB, Initial pH value: pH 4·8 (Lumetron readings)
Group I. *L. cremoris*	6	3·1–1·6	0·6–0·0	2·0–1·15	0·0
Group II. *L. lactis*	3	3·5–2·2	0·65–0·4	2·7–1·4	1·1–0·0
Group III. *L. paramesenteroides*	6	5·1–3·2	5·1–0·0	5·8–0·7	3·7–0·5
Group IV } *L. dextranicum*	3	3·1–2·7	0·7–0·2	2·6–1·9	0·5–0·25
Group V } *L. dextranicum*	4	4·0–2·1	1·3–0·0	3·6–1·7	0·7–0·2
Group VI. *L. mesenteroides*	10	4·9–3·6	2·0–0·0	3·8–2·5	1·8–0·7
Group VII a } *L. oenos*	5	1·2–0·3	2·4–0·8	4·8–3·5	3·7–2·6
Group VII b } *L. oenos*	14	4·0–2·0	4·2–1·7	5·4–3·0	4.7–2·2

* Leuconostoc species named according to Garvie (1967*c*).

CMB at initial pH 4·2 or 3·7. On the other hand, the wine strains grew well in ATB (initial pH 4·8) and of these 14 also grew in YGCB starting at both pH 6·7 and 4·8 but not as well as in ATB. Growth was slow with all strains and incubation was therefore continued for 3 days. All strains grew in CMB at initial pH 3·7 but growth was not as good as at pH 4·2 or 4·7. A few strains gave slight growth in CMB adjusted to pH 3·2. The ability to grow well in CMB (initial pH 4·2) is used to separate *Leuconostoc oenos* from other species of the genus, for these do not grow in this medium. The addition of cysteine to the medium had a greater effect on those strains of *L. oenos* which did not grow in YGCB than on those which grew in this medium. The growth of most strains was improved by the addition of cysteine; for five strains it was essential.

Characteristics of the wine leuconostocs

Table 3 gives the results obtained with the wine leuconostocs and compares them with the characteristics of other species (Garvie, 1960).

In the earlier work the fermentative properties of strains were examined in a medium adjusted to pH 6·7 and with bromcresol purple (BCP) as indicator, but since the wine strains grew better in media adjusted to a more acid pH value they were examined in a different medium adjusted to pH 5·2 and with bromcresol green as indicator. However, 12 strains (two of each species) previously examined in BCP medium were tested in the low pH medium; the fermentation pattern was found to be

unaffected by the change of medium. The substances fermented are shown in Table 3. Although there was no tomato juice in the medium results were obtained with all strains of *Leuconostoc oenos* by using the techniques described. On a few occasions a smaller inoculum or washed organisms were tried but acid was not formed from any substrate.

Table 3. *The characters of the species of the genus Leuconostoc*

Figures in parentheses are the numbers of strains examined in that particular test. Where no figures in parentheses are given all strains were examined. Other figures give the number of strains giving a positive reaction.

	L. cremoris Group I	*L. lactis* Group II	*L. oenos* Group VII	*L. paramesenteroides* Group III	*L. dextranicum* Group IV	*L. dextranicum* Group V	*L. mesenteroides* Group VI
No. of strains examined . . .	10	9	19	17	5	16	31
Growth in YGCB*	+ (6)	+ (3)	14	+ (6)	+ (3)	+ (4)	+ (10)
Growth in ATB* better than YGCB	− (6)	− (3)	+	− (6)	− (3)	− (4)	− (10)
Growth in 10% (v/v) ethanol in CMB*	− (2)	− (2)	+	− (2)	− (2)	− (2)	− (2)
Growth at pH 4·8 in CMB	− (6)	− (3)	+	2 (6)	− (2)	− (4)	Slight (10)
Growth at pH 3·7 in CMB	− (2)	− (2)	+	− (2)†	− (2)	− (2)	− (2)
Growth at 37·5°	−	+	15	14	4	+	28
YGLM* reaction acid	+	+	10	17	+	+	+
clot	4	2	−	10	+	15	27
reduction	1	2 (slight)	2	2	+	12	26
gas	1	−	−	2	1	7	14
Dextran synthesis	−	−	−	−	+	+	+
Dissimilation of citrate	+ (6)	1 (5)	18	1 (8)	1 (13)		2 (11)
Acid from arabinose	−	1	9	16	−	−	+
xylose	−	−	3	5	−	+	24
fructose	−	7	+	16	+	14	30
glucose	+	+	+	+	+	+	+
galactose	+	+	4	+	3	13	29
mannose	−	8	11	+	+	15	30
cellobiose	−	−	13	7	−	3	19
lactose	+	+	−	5	2	10	16
maltose	−	+	−	16	3	+	29
sucrose	−	8	−	15	4	+	+
trehalose	−	1	+	+	+	+	+
melibiose	−	8	14	+	−	15	25
raffinose	−	3	−	7	−	9	16
dextrin	−	−	−	6	−	−	−
aesculin	−	−	+	7	−	8	29
salicin	−	−	14	−	−	3	24
mannitol	−	−	−	7	−	3	11

† The two strains which grow at pH 4·8.

* YGCB = yeast glucose citrate broth; ATB = acidic tomato broth; CMB = citric-malic acid broth; YGLM = yeast glucose litmus milk.

DISCUSSION

It is suggested that the wine leuconostocs should be separated from other species of the same genus because the wine strains grow in media with a low initial pH value (i.e. 3·7) and grow well at pH 4·2, while other leuconostocs will not grow in media with an initial pH value of 4·2 or less. Acid tolerance is not the only property which

separates the wine strains from all the other species. Failure to ferment sucrose and maltose excludes them from all species except *Leuconostoc cremoris* while failure to ferment lactose and (usually) galactose together with the ability to form acid from fructose, aesculin and (usually) salicin and melibiose excludes them from *L. cremoris*. For reasons given elsewhere (Garvie, 1967*c*) *L. dextranicum* and *L. mesenteroides* are considered to include only strains which form dextran from sucrose. The non-slime forming strains previously included in *L. mesenteroides* are placed in a new species *L. paramesenteroides*. The wine cultures do not form slime, and are therefore excluded from *L. dextranicum* and *L. mesenteroides*. *L. paramesenteroides* is most likely to be confused with the wine strains but acid production from maltose, sucrose, salicin and aesculin should put the majority of strains in the correct species.

Table 4. *The separation of six proposed species of Leuconostoc*

	L. mesenteroides	*L. dextranicum*	*L. paramesenteroides*	*L. lactis*	*L. cremoris*	*L. oenos*
Production of slime from sucrose	+	+	−	−	−	−
Acid formed from arabinose	+	−	u+	u−	−	±
lactose	± (slow)	± (slow)	± (slow)	+	+	−
maltose	u+	u+	u+	+	−	−
sucrose	+	u+	u+	u+	−	−
trehalose	+	+	+	u−	−	+
Hydrolysis of aesculin	u+	u−	±	−	−	+
salicin	u+	u−	−	−	−	u+
Growth at pH 4·8	− or slight	− or slight	±	−	−	+
4·2	−	−	−	−	−	+
3·7	−	−	−	−	−	+

u+ = most strains +, occasional strains only found to be −;
u− = most strains −, occasional strains only found to be +.
± = some strains +, some strains negative

When strains of *Leuconostocs oenos* were first cultured in this laboratory growth was poor. When strains of other Leuconostoc species had been difficult to grow the addition of cysteine hydrochloride to the medium greatly improved growth. This was therefore tried with *L. oenos* and was successful. The Leuconostoc strains encouraged by cysteine had also been found to grow well on agar media when incubated in anaerobic jars evacuated and filled with a mixture of hydrogen and carbon dioxide, while growth of these strains on nutrient agar when incubated aerobically is unreliable. Similarly, *L. oenos* does not grow aerobically on agar media. The addition of cysteine to broth media and incubation of plates in an atmosphere of hydrogen + carbon dioxide has not been found to inhibit the growth of any leuconostocs and is useful for growing all strains of *L. cremoris*, strains received from Dr Whittenbury as representative of his group 1 (Whittenbury, 1966) and also for a few other strains of *L. paramesenteroides* and a few of *L. dextranicum*.

Fornachon (1964) and Malan, Ozino & Gandini (1965) reported sucrose fermentation by strains of leuconostocs they isolated from wine. Although in both these papers the cultures were regarded as belonging either to *Leuconostoc mesenteroides* or *L. dextranicum* the pH value of the media used makes this classification appear unlikely and the cultures described appear to be *L. oenos*. It is possible that some strains or under some conditions acidophilic leuconostocs ferment sucrose (see below). As

might be expected wine leuconostocs are more tolerant to ethanol than are other leuconostocs. It is proposed to call the wine strains *L. oenos*. A suggested differential key is given in Table 4: other differences between the species are shown in Table 3.

Difficulties in naming leuconostocs found in wine have been reported previously. Bidan (1956) was unable to give a species name to one of his strains, the other he called *Leuconostoc gracile*. This name was chosen because Pedersen (*Bergey's Manual*, 1948) had found that one strain of '*Bacterium gracile*' (Muller-Thurgau & Osterwalder, 1913, 1918) which he received from Dr A. Osterwalder was probably a leuconostoc. Neither this strain nor any of the originals are now available and it is not possible to be sure that the original description of '*B. gracile*' referred to a leuconostoc. Since Bidan's culture fermented lactose and raffinose it is doubtful whether it was the same as the present wine strains. Radler (1958) concluded his cultures were like, but not identical with, *L. citrovorum*, and Pilone & Kunkee (1965) use the name *L. citrovorum* for ML 34 (NCDO 1823). Radler's strains and strain ML 34 are included in the present work and are typical of *L. oenos*. Fornachon (1964) described acidophilic cocci found in Australian wine and concluded that they were non-dextran forming varieties of *L. mesenteroides*. Two strains received from Dr J. C. M. Fornachon while this paper was in preparation were found to be *L. oenos*. Similarly, of six strains received from Professor C. E. Malan as *L. mesenteroides*, *L. dextranicum* and *L. citrovorum*, five are *L. oenos* and one has not been satisfactorily identified. Using the methods given none of these eight strains formed acid from sucrose.

The gas-forming cocci isolated from wine and gas-forming cocci isolated from other sources have not always been seen to be different, partly perhaps because leuconostocs have been confused amongst themselves and also with organisms of other genera. *Leuconostoc cremoris* seems to be found only in the field of dairy bacteriology, but other species are more widely distributed. At this time the true taxonomic significance of the ability to tolerate and grow at acid pH values cannot be assessed, but since this property is linked with differences which have classically been used as a means of separation among the lactic-acid bacteria, acid tolerance is an additional argument for defining a new species.

Leuconostoc oenos is not a homogenous group and some strains are considerably easier to grow than others. It is difficult to select a type strain, however, NCDO 1674 is proposed.

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The Growth Factor and Amino Acid Requirements of Species of the Genus *Leuconostoc*, including *Leuconostoc paramesenteroides* (sp.nov.) and *Leuconostoc oenos*

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SUMMARY

The vitamin requirements of 63 strains and the amino acid requirements of 40 strains belonging to the genus *Leuconostoc* have been determined. The amino acid requirements separate the genus into dextran and non-dextran formers and in particular indicated that non-dextran formers should be excluded from *Leuconostoc mesenteroides*. The growth factor requirements support the division of the leuconostocs by other methods into six species: *Leuconostoc mesenteroides*, *L. dextranicum*, *L. paramesenteroides*, *L. lactis*, *L. cremoris* and *L. oenos*.

INTRODUCTION

No previous studies of the vitamin and amino acid requirements of leuconostoc strains has included representatives from all the species of the genus, but reports have been confined to strains selected either by habitat or species. Dunn, Shankman, Camien & Block (1947) and Shankman *et al.* (1947) examined a variety of lactic acid bacteria which included eight leuconostocs, all dextran formers (although some were designated *Leuconostoc citrovorum*), and Whiteside-Carlson & Carlson (1949) and Whiteside-Carlson & Rosano (1951) worked only with strains which made dextran. In the dairy field, Prouty (1961) examined cultures of *L. dextranicum* and *L. citrovorum* isolated from starters. Radler (1958) studied three strains isolated from wine and Peynaud, Lafon-Lafourcade & Domercq (1965) included 37 heterofermentative cocci in their study of the nutrition of lactic acid bacteria from wine. The media and methods used in all these experiments differed considerably and it is not easy therefore to compare the requirements of the different species.

In the present work strains of all the known species of leuconostoc have been used, i.e. representatives from the six groups reported by Garvie (1960) and also from the acidophilic strains found in wine (Garvie, 1967).

METHODS

Strains. The strains were all from the National Collection of Dairy Organisms (NCDO), Shinfield, and the NCDO accession numbers of those used are given in Table 1. Many of the strains were included in earlier work (Garvie, 1960) but others have been deposited in the NCDO since the completion of that work, namely strains NCDO 1583–1598 by the National Collection of Industrial Bacteria (NCIB; Torry

Research Station, Aberdeen); NCDO 1563, 1568 and 1659 by Dr R. Whittenbury (East of Scotland Agricultural College, Edinburgh); NCDO 1656 by Dr C. J. A. van den Hamer (Utrecht, Holland); NCDO 1668–1675 by Dr E. Peynaud (Station Agronomique et Oenologique de Bordeaux); NCDO 1694–1696 by Dr F. Radler (Merbein, Victoria, Australia); and NCDO 1389 by Dr P. R. Elliker (Oregon, U.S.A.).

Table 1. *Leuconostoc strains examined for growth factor and amino acid requirement*

Group	Species	NCDO numbers of the strains examined for: Growth factor and amino acid requirement	Growth factor requirement only	Strains examined by other workers
I	*L. cremoris*	543, 705, 1033, 1071, 1389	828	
II	*L. lactis*	532, 533, 546	549, 956, 959	
III	*L. paramesenteroides*	803, 871, 1563, 1568, 1569, 1590	870, 883, 955, 957, 958, 1589, 1656	
IV	*L. dextranicum*	517, 824, 1591	529, 531, 862	824
V	*L. dextranicum*	183, 516, 550, 1582	537, 812, 861, 880, 1592, 1598	516
VI	*L. mesenteroides*	518, 519, 522, 523, 551, 768, 1583, 1584, 1585, 1587	527, 538, 541, 797, 807, 876, 1588, 1593, 1594	518, 519, 522, 551, 523
VII	*L. oenos*	1668, 1669, 1671, 1672, 1673, 1674, 1675, 1694, 1695	1696	1694, 1695, 1696

NCDO = National Collection of Dairy Organisms, NIRD, Shinfield, Berkshire.

Media for maintaining cultures. The cultures were grown in the media used by Garvie (1967), yeast glucose citrate broth (YGCB) for groups I–VI and acidic tomato broth (ATB) for group VII. A 1 % (w/v) solution of cysteine hydrochloride was sterilized by Seitz filtration and 0·5 ml. added/10 ml. media for strains of group I (*Leuconostoc cremoris*; *Betacoccus cremoris* Knudsen & Sørensen (1929)) and group VII (*L. oenos*). These species were incubated at 22°, most strains of other species were incubated at 30°, but a few strains of groups III and IV grew better at 22° and the growth of these was helped by the addition of cysteine hydrochloride. Incubation was generally for 24 hr but some cultures, particularly those at 22°, required 48 hr or longer.

Media for determining requirement for amino acids and vitamins. The amino acid requirements were estimated in medium A (Table 2) from which each amino acid was omitted in turn. This medium was similar to that used by Reiter & Oram (1962) and is based on the amino acid analysis of milk. It was used, because before devising YGCB it had been observed that some strains of *Leuconostoc cremoris* grew more readily in milk supplemented with Yeastrel and glucose than in the broth medium used at that time.

The vitamin requirements were assessed in medium B (Table 2) from which each growth factor was omitted in turn and in which vitamin-free acid-hydrolysed casein and vitamin-free enzymic digest of casein were substituted for the amino acid mix of

medium A. The acid-hydrolysed casein was prepared by the method of Ford, Perry & Briggs (1958) but with an additional 200 mg. cystine added to the filtrate from 50 g. casein. The enzymic digest of casein was prepared according to Roberts & Snell (1946). Media A and B were dispensed in 5 ml. quantities in 6 in. × $\frac{5}{8}$ in. test tubes, closed with metal caps and sterilized by autoclaving at 115° for 10 min. and cooled immediately in cold water.

Table 2. *Composition of media for examining amino acid and growth factor requirements of strains of leuconostocs*

All media were prepared at pH 5·0 for *Leuconostoc oenos* and pH 6·7 for other species.

In media A, B and C (basal components)		Medium A (amino acids)	
Glucose	10 g.	L-Alanine	300 mg.
Sodium acetate ($3H_2O$)	2 g.	L-Arginine	400 mg.
Triammonium citrate	2 g.	L-Aspartic acid	750 mg.
K_2HPO_4	2 g.	L-Cystine	200 mg.
KH_2PO_4	2 g.	L-Glutamic acid	750 mg.
$MgSO_4.7H_2O$	0·2 g.	Glycine	200 mg.
$MnSO_4.4H_2O$	0·05 g.	L-Histidine	500 mg.
$FeSO_4.7H_2O$	0·05 g.	DL-Isoleucine	200 mg.
Tween 80	1 ml.	L-Leucine	200 mg.
		L-Lysine	800 mg.
		DL-Methionine	150 mg.
Guanine	5 mg.	DL-Phenylalanine	200 mg.
Adenine	5 mg.	DProline	500 mg.
Uracil	5 mg.	DL-Serine	400 mg.
Xanthine	5 mg.	DL-Threonine	200 mg.
Pyridoxal	2 mg.	L-Tryptophan	200 mg.
Nicotinic acid	1 mg.	L-Tyrosine	100 mg.
Calcium-D-pantothenate	1 mg.	L-Valine	300 mg.
Riboflavin	1 mg.	or medium B, amino-acid sources, casein hydrolysate 100 ml. + enzymic digest of casein,	100 ml.
Thiamine	1 mg.		
Vitamin B_{12} (cobalamin)	1 μg.		
Biotin	10 μg.		
p-Amino benzoic acid	5 μg.	or medium C, amino-acid source, tryptone,	4 g.
Folic acid	10 μg.		
Total volume	1000 ml.		

Determination of amino acid requirements. Cultures were transferred from YGCB or ATB to a tryptone broth (medium C, Table 2), of the appropriate initial pH value and with the addition of cysteine hydrochloride for strains of groups I and VII. Cultures of strains from groups I and VII required several transfers through medium C before rapid growth was obtained. Strains of other groups were transferred twice. One drop of a culture in medium C was inoculated into each of the amino acid test media (media A) and the tubes incubated at the appropriate temperature. It was necessary to add cysteine to medium A when testing groups I and VII strains but not for strains of other groups. Cysteine was omitted when a requirement for cystine was being examined.

The time of incubation could not be standardized because the differences in growth rate between the cultures was even greater than in the general media. Incubation was continued until the complete medium showed good growth. With many strains 24 hr was adequate, but with others incubation was prolonged to 7–10 days. Growth was

estimated photometrically as the extinction at 580 mμ with a Hilger Biochem absorptiometer.

Determination of growth factor requirements. One drop of a culture in YGCB or ATB was inoculated into a series of tubes of medium B, each deficient in one growth factor and into the complete medium. The inoculated tubes were incubated at the appropriate temperature until good growth in the complete medium was observed. The cultures were transferred twice more through medium B of the same composition and the requirement for a particular growth factor assessed by the presence or absence of growth in the third subculture. As before, cysteine was added to cultures of groups I and VII.

Determination of a biotin requirement. A requirement for biotin was estimated in the same way as for the other vitamins but using medium to which 0·002 ml. egg white/ml. had been added to bind any traces of biotin in the medium (Broquist & Snell, 1948). Broquist & Snell (1951) reported a relationship between a requirement for oleate and biotin in some lactic acid bacteria. In the present experiments a requirement for one of these substances was determined in the presence of the other.

RESULTS

Patterns of vitamin requirements of species have proved useful in supporting the classification of the lactobacilli by other criteria (Rogosa & Sharpe, 1959), and this study of the vitamin and amino acid requirements of the leuconostocs was made to see whether in this genus also the results would assist in the definition of species.

Amino acid requirements

The amino acid requirements observed are summarized in Table 3. As with many other properties of the leuconostocs the amino acid requirements were found to be variable between different strains of any species. Only valine and glutamic acid were required by all strains, and methionine was markedly stimulatory for most. No requirement for alanine was observed for any strain. Each of the other 14 amino acids were individually required by, or stimulatory to, some strains.

Table 4 gives the numbers of amino acids required by strains. It shows that the genus can be divided into two groups: (1) the dextran-forming strains of groups IV, V and VI, where each strain requires a few (up to 8) amino acids; (2) strains not forming dextran where each strain requires a larger number (>8) of amino acids. On the basis of the numbers of amino acids needed by each strain, groups VI and III are different. Previously they have been put together in one species but they can be separated on the basis of their amino acid requirements.

Cultures of groups I and VII were slow to grow in the medium used and the final turbidity was low in some instances, even after prolonged incubation (7–10 days).

Growth factor requirements

The growth factor requirements are shown in Table 5. Only five group VII strains are included because the other 11 strains examined did not grow. The group VII strains for which results are given were slow to grow in the medium B; why more strains grew in medium A than in medium B is not clear, since medium A contained amino acids whereas medium B contained enzymic digest and acid-hydrolysed casein

as nitrogen source and cultures would be expected to grow better in the latter medium. Media A and B were not satisfactory for this group of leuconostocs; further work on the nutrition of species is reported elsewhere (Garvie & Mabbitt, 1967).

Table 3. *Amino acid requirements of strains of Leuconostocs. Medium A was used and amino acids omitted singly.*

Amino acid omitted	*L. cremoris* Group I	*L. lactis* Group II	*L. paramesenteroides* Group III	*L. dextranicum* Group IV	*L. dextranicum* Group V	*L. mesenteroides* Group VI	*L. oenos* Group VII
	No. of strains showing a requirement						
Alanine	–	–	–	–	–	–	–
Arginine	3 (2)	+	3 (3)	(1)	–	–	+
Aspartic acid	1	–	1 (3)	–	–	–	(3)
Cystine	2 (1)	+	4 (1)	1	–	5 (1)	+
Glutamic acid	+	+	+	+	+	+	+
Glycine	–	–	(4)	–	(1)	–	2 (1)
Histidine	–	1(2)	3 (2)	1 (1)	1 (1)	2 (1)	6 (3)
Isoleucine	+	+	+	1 (2)	2 (1)	5 (2)	+
Leucine	1	(1)	(3)	(1)	(1)	(2)	1 (3)
Lysine	3 (1)	–	–	(1)	1	–	–
Methionine	3 (2)	1 (2)	(6)	(3)	1 (3)	(9)	4 (5)
Phenylalanine	(2)	1 (2)	2 (2)	–	(1)	–	7 (2)
Proline	–	(2)	(1)	(1)	–	–	–
Serine	–	–	2 (3)	1	–	–	2 (5)
Threonine	(2)	–	4 (1)	–	–	–	1 (1)
Tryptophan	2 (1)	(3)	3 (3)	–	2 (1)	(7)	7 (2)
Tyrosone	(2)	–	(3)	(1)	(2)	–	+
Valine	+	+	+	+	+	+	+
No. of strains examined	5	3	6	3	4	10	9

–, no strains require compound; +, all strains require compound.

* Figures in parentheses are the number of strains to which the compound was stimulatory; figures not in parentheses the number which required the compound.

Table 4. *Numbers of amino acids essential or markedly stimulatory to culture (basal medium A)*

Species	No. of strains examined	No. of amino acids required by strains														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	16–18
L. mesenteroides, Group VI	10	.	.	3	1	1	2	1	2	.	.	.	.	.	.	.
L. dextranicum, Group V	4	.	.	.	1	1	1	.	.	.	.	1	.	.	.	.
Group IV	3	.	.	.	.	1	.	1	.	1	.	.	.	.	.	.
L. paramesenteroides Group III	6	.	.	.	.	.	.	.	1	.	.	.	1	1	2	.
L. lactis, Group II	3	.	.	.	.	.	.	.	.	1	1	1	.	.	.	.
L. cremoris, Group I	5	.	.	.	.	.	1	1	.	1	1	1	.	.	.	.
L. oenos, Group VII	9	.	.	.	.	.	.	.	.	.	1	3	2	3	.	.

The growth factor requirements do not give a clear-cut division into groups; however, the results do support the classification based on other characteristics. Only a few strains of group VI, but most strains of groups IV and V, require riboflavin, which was also required by all the strains which do not form dextran. Groups II and III are

separated by their requirement for folic acid. Group I requires more vitamins than any other species. In common with group VII, group I strains did not grow when adenine, guanine, xanthine and uracil were omitted together from the medium.

Table 5. *The influence of vitamins, purines, uracil and oleate on the growth of leuconostocs*

Medium B was used and various components omitted. No strains showed a requirement for guanine, adenine or xanthine when these were omitted separately. No strains required vitamin B_{12} (cobalamin) or *p*-aminobenzoic acid. All strains required nicotinic acid + thiamine + pantothetnic acid + biotin.

Figures in parantheses give the numbers of strains which showed slight growth only. Figures not in parentheses give the number of strains which showed a requirement for the given compound.

	L. cremoris Group I	*L. lactis* Group II	*L. paramesenteroides* Group III	*L. dextranicum* Group IV	*L. dextranicum* Group V	*L. mesenteroides* Group VI	*L. oenos* Group VII
Substances omitted	No. of strains showing growth requirement						
Uracil	+	−	(2)	−	1	−	1
Guanine + adenine + xanthine + uracil	+	−	6 (1)	1	1	−	+
Riboflavin	+	+	+	4	8	3	+
Pyridoxal	+	−	4 (1)	3	6 (2)	4 (3)	−
Folic acid	+	−	+	3	8	9	+
Tween 80	1 (3)	1	4	1	3	2	3
No. of strains examined	6	6	13	5	11	19	5

+, all strains required compound; −, no strains required compound.

Table 6. *The growth of Leuconostoc dextranicum* NCDO *517 in basal medium B minus adenine, guanine, xanthine and uracil and with these bases added in various admixtures*

Concentration of each base 5 μg./ml. medium Substances added	Growth*
Medium B minus 4 bases	+ + + +
Guanine	−
Adenine	+ + +
Uracil	+ + + +
Xanthine	+ +
Guanine + uracil	+
Guanine + adenine	+
Guanine + xanthine	±
Adenine + xanthine	+ + +
Uracil + xanthine	+ + + +
Adenine + uracil + xanthine	+ + + +
Guanine + uracil + xanthine	±
Guanine + adenine + xanthine	±
Guanine + adenine + uracil	+ + + +
Guanine + adenine + uracil + xanthine i.e. complete medium B	+ + + +

* Growth. −, no growth; ±, very slight growth; +, + +, + + +, + + + +, increasing turbidity in the culture.

The purine and pyrimidine requirements of Leuconostoc dextranicum NCDO 517

Leuconostoc dextranicum NCDO 517 grew when adenine, guanine, xanthine and uracil were all omitted together from the medium. When each substance was omitted singly growth failed when either adenine or uracil was absent. Media were prepared without any of these bases and the effect of adding them separately and in different mixtures is shown in Table 6. At the concentrations used, guanine, and to a lesser extent xanthine, were inhibitory. The inhibition was overcome by the addition of uracil+adenine. Of the other strains examined, those which failed to grow when uracil alone was absent from the medium did not grow when adenine, guanine and xanthine were also omitted.

DISCUSSION

The present results and those previously reported in the literature do not show wide differences, and those there are may well be due to variations in technique. The results reported by Prouty (1961) show the greatest differences from the present observations. He studied strains of *Leuconostoc citrovorum* and *L. dextranicum* isolated from dairy starter and cultured butter-milk, and found that the number of amino acids required for both species varied from 7 to 15. *L. dextranicum* forms dextran and Prouty's results, if compared with Table 4, are more in keeping with a non-dextran former than with a dextran former. Prouty pointed out the discrepancy between the numbers of amino acids required and the numbers required by strains studied earlier by Dunn *et al.* (1947). While Dunn *et al.* (1947) called three of their strains *L. citrovorum* it is doubtful whether any of the leuconostocs used were unable to form dextran. Of those called *L. citrovorum*, strain ATCC 8081 is a pediococcus and two, ATCC 797 (NCDO 824) and ATCC 8082 (NCDO 812), both form dextran. The fourth strain ATCC 7013 is not in the NCDO, but Camien *et al.* (1947) pointed out that ATCC 7013, together with ATCC 797 and ATCC 8358 (*L. dextranicum*), have sugar fermentation patterns characteristic of *L. mesenteroides*.

Garvie (1960) divided the genus *Leuconostoc* into six groups and these divisions are supported by the present results on the amino acid and growth factor requirements. These six groups were placed in four species designated *Leuconostoc cremoris* (group I), *L. lactis* (group II), *L. dextranicum* (group IV) and *L. mesenteroides* (groups III, V and VI). The additional information now available indicates that the demarcation into species can be improved, and it is suggested that the genus be divided into six species, the four named above and two additional ones for which the names *L. paramesenteroides* and *L. oenos* are proposed.

Leuconostoc cremoris (group I) remains unaltered. Whittenbury (1966) found that some strains included in this species gave rise to 'sucrose-fermenting mutants', but these mutants were in other respects identical with the parent culture. He goes on to state that 'the sucrose-fermenting mutants could be regarded as non-dextran forming variants of *L. dextranicum*'. However, there are other differences between *L. cremoris* and *L. dextranicum* than the ability to ferment sucrose and to form dextran. The 'sucrose-forming mutants' of *L. cremoris* would only be confused with *L. dextranicum* if the division into species were based on the minimal tests suggested by Hucker & Pederson (1931), i.e. the fermentation of arabinose, xylose and sucrose.

Leuconostoc lactis (group II). This group, also, remains unaltered. Whittenbury (1966) found this species to be slow growing. However, in this laboratory *L. lactis*

has not been observed to be slow growing and is more active than either *L. cremoris* or *L. oenos.*

Leuconostoc paramesenteroides (group III). Previously this group was regarded as a non-dextran forming variant of *L. mesenteroides.* The differences in carbohydrate fermentation were not considered significant in view of the claims that dextran formation was a variable characteristic (Pederson & Albury, 1955). Other workers have been unable to confirm these observations (Whittenbury, 1966). The differences in the numbers of amino acids required by dextran and non-dextran formers, together with the difference in riboflavin and folic acid dependence, suggest that group III is not a variant of the dextran-forming *L. mesenteroides.* With this information available, other differences between groups III and VI become more significant. Generally the dextran-forming strains give a gassy acid clot in yeast glucose litmus milk while the non-dextran formers often fail to clot the milk and if they do it is seldom gassy, indicating less vigorous growth. The non-dextran formers fail to hydrolyse salicin and generally fail to hydrolyse aesculin, while the dextran formers generally hydrolyse both glucosides. A new species is proposed on the evidence now available, with NCDO 803 as type strain. Details of properties of 17 strains of this species (other than amino acid and growth factor requirements) were given earlier (Garvie, 1960).

Leuconostoc dextranicum (groups IV and V). Earlier Garvie (1960) suggested that group V should be placed with *L. mesenteroides* rather than *L. dextranicum,* following the classification of Hucker & Pederson that only strains failing to ferment arabinose and xylose were placed in *L. dextranicum.* The species described in *Bergey's Manual* (1957) do not include strains which form dextran and which ferment xylose but not arabinose. Ørla-Jensen (1942) included such strains in *Betacoccus bovis* and excluded them from *B. arabinosaceus.* The vitamin and amino acid requirements of the cultures suggest that this is a more satisfactory division, and therefore that the Ørla-Jensen classification should be retained.

Leuconostocs mesenteroides (group VI). This species previously included strains with diverse properties and if both groups V and III are placed elsewhere a more clearly defined species is left.

Leuconostoc oenos. This species is described by Garvie (1967). It is considered that it resembles most closely *L. paramesenteroides,* but the differences separating the species are considered significant and both species should be recognized.

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The Action of Cephaloridine with Cloxacillin or Methicillin against β-Lactamase-Producing Gram-negative Bacteria

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SUMMARY

Cephaloridine has a broad spectrum of antibacterial activity, but certain Gram-negative organisms (e.g. *Aerobacter aerogenes*, *Proteus morgani*) are resistant by reason of their ability to destroy the antibiotic. Methicillin and cloxacillin do not inhibit Gram-negative organisms but are resistant to the β-lactamases which some strains produce and can protect cephaloridine from destruction by such enzymes. This protection effect towards cephaloridine has been demonstrated *in vitro* and on experimentally infected mice. *In vitro*, a relatively small proportion of the penicillin exerted a maximal protecting effect towards cephalosporin and enhanced its bactericidal action against the Gram-negative organisms which produce β-lactamase. The clinical use of a combination of cloxacillin or methicillin with cephaloridine for the treatment of intractable systemic infections with β-lactamase-producing Gram-negative organisms is suggested, even though laboratory tests may show the organisms to be resistant to both cephaloridine and the penicillins when they are tested separately.

INTRODUCTION

Certain Gram-negative organisms such as strains of *Aerobacter aerogenes* and *Proteus morgani* (Muggleton, O'Callaghan & Stevens, 1964) are not susceptible to the antibacterial action of cephaloridine (Ceporin; Glaxo Laboratories, Ltd.). Further investigation has shown that many of these organisms are able to decompose cephaloridine to a substance which has no antibacterial activity, due to destruction of the β-lactam ring. Fleming, Goldner & Glass (1963) described a cephalosporinase able to decompose cephalosporin C; they suggested that this was a β-lactamase, capable of opening the 4-membered ring. Ayliffe (1965), Hamilton-Miller, Smith & Knox (1965) and Sabath, Jago & Abraham (1965) described cephalosporinases from several Gram-negative species. These β-lactamases from Gram-negative organisms can decompose some penicillins, such as benzylpenicillin and ampicillin but not others, such as cloxacillin or methicillin. Cloxacillin and methicillin have no activity against the β-lactamase-producing Gram-negative organisms but protect other penicillins against decomposition by the enzymes (Hamilton-Miller, Smith & Knox, 1964; Sutherland & Batchelor, 1964). Hamilton-Miller *et al.* (1965) and Sabath, *et al.* (1965) extended this observation by showing that the presence of a non-susceptible penicillin would protect susceptible penicillins and cephaloridine from destruction by β-lactamases.

The activity of an antibiotic against an organism intrinsically sensitive to it will be decreased when the organism is able to destroy the substance. When destruction is

rapid, complete insensitivity will result. Inhibition of the destructive ability of the organism may render it sensitive and treatment with the antibiotic may then be effective.

We have investigated the ability of cloxacillin and methicillin to protect cephaloridine against the destructive action of Gram-negative bacterial strains which produce β-lactamase. Our experiments were made with suspensions of living organisms rather than with killed organisms or isolated enzyme systems since we wished to reproduce the conditions which might apply *in vivo*. Thus enzyme induction could take place in our test system and multiplication of organisms would occur at antibiotic concentrations below the minimum inhibitory concentration, as they would in an infected animal. In this way the feasibility of improving the treatment of infections due to β-lactamase-producing Gram-negative organisms with cephaloridine could be assessed.

METHODS

Assay methods. The antibiotic activity of cephaloridine, alone or in the presence of cloxacillin or methicillin, was determined by large plate bioassay with *Escherichia coli* 573 as test organism. This organism is insensitive to the two penicillins used. Difco tryptose agar (with the addition of a 1·25 % w/v, solution of tetrazolium chloride) was seeded with 0·01 % (v/v) of an overnight broth culture. Cloxacillin and methicillin were assayed with *Staphylococcus aureus* NCTC 7447 in the same medium; this organism is sensitive to cephaloridine, so that the penicillins could not be assayed when mixed with cephaloridine.

Estimation of cephaloridine-inactivating activity. We compared the abilities of a large number of organisms to destroy the antibacterial activity of cephaloridine at a concentration 250 μg./ml. Organisms from static broth cultures of various organisms grown at 37° for 48 hr were collected by centrifugation and then resuspended in one-tenth of their original volume of fresh nutrient broth. The suspensions were diluted in doubling series in 2 ml. nutrient broth in tubes; to each tube was added 2 ml. antibiotic solution, consisting of cephaloridine alone or with cephaloridine+inhibitor under test, so that each tube in a series contained 4 ml. organism suspension at a dilution up to 1/400,000, together with 250 μg./ml. of each substance being tested. Except where otherwise stated, the antibiotics were kept at these concentrations. A final tube in any series contained the test solution only without organisms. The tubes were incubated at 37° for 2 hr and the residual cephaloridine was estimated by plate bioassay. From the assay results we calculated the dilution of organism suspension which destroyed 50 % (usually 125 μg/ml.) of the cephaloridine. We then calculated the amount of antibiotic that would theoretically have been destroyed by 1 ml. of the undiluted suspension of organisms. The results (Tables 1, 2, 3 and 5) are expressed in these terms. The method is similar to that used by Chang & Weinstein (1963), but is more quantitative.

Most of the work was done with two organisms; one called *Aerobacter aerogenes* P99 has been subsequently identified by the Salmonella Reference Laboratory, Central Public Health Laboratory, Colindale, as *Enterobacter cloacae* and was given to us by Dr P. C. Fleming (Hospital for Sick Children, Toronto); the other was a strain of *Proteus morgani*, NCTC 235. A 48 hr culture of *P. morgani* contained about 5×10^9 viable organisms and the *A. aerogenes* about 2×10^{10} viable organisms. Both organisms tended to vary from day to day both in sensitivity to cephaloridine

and in ability to destroy it; therefore the activity of any bacterial suspension against any substrate was always compared with its activity against cephaloridine in the same experiment. Accurate quantitative comparisons between different organisms were difficult under the test conditions used because, for example, *A. aerogenes* grew faster than *P. morgani* during the 2 hr period of incubation.

Measurement of minimum inhibitory concentrations. Tube dilution tests (doubling dilutions) in nutrient broth inoculated with 0·05 ml. of a 1/100 dilution of an overnight broth culture of the test organisms were used. Tests were read after incubation for 24 hr at 37°.

Estimation of bactericidal action. Tubes of nutrient broth containing antibiotic mixtures were inoculated with 1 % (v/v) of a 1/100 dilution of an overnight broth culture of *Aerobacter aerogenes* or *Proteus morgani.* The tubes were incubated at 37° and small samples were withdrawn at intervals. Tenfold serial dilutions of the samples were plated in nutrient on agar in Petri dishes which were then incubated overnight at 37°. From the colony counts after incubation, the percentage kill of the organisms was calculated for each time of sampling, based on the viable count at 'Omin.'.

Estimation of serum binding. The extent to which the antibiotic substances were serum bound was estimated by an ultrafiltration method. Solutions of antibiotic (50 μg./ml.) were prepared in undiluted horse serum (Burroughs Wellcome, no. 2). Part of each solution was ultrafiltered under 10 lb./sq. in. pressure through Visking tubing. The filtrate collected was assayed and the amount which remained bound to the serum protein thus calculated.

Protection tests on experimentally infected mice. Groups of 5 to 8 mice were challenged with 5×10^8 organisms of *Proteus morgani* NCTC 235 in saline suspension prepared from overnight nutrient agar cultures. The groups were treated with serial doubling dilutions of cephaloridine, methicillin or cloxacillin by subcutaneous injection in 0·2 ml. of saline solution each animal being treated at 0·5, 4, 8, 24 and 32 hr after challenge; the highest dose was 400 mg./kg. Cephaloridine was also given with cloxacillin or methicillin at 200 mg./kg. dose of each, or 400 mg./kg. dose of total antibiotic. A similar experiment was done with another virulent strain of *P. morgani,* P47. From the numbers of mice that survived on the fifth post-challenge day, the median effective doses (ED50) were calculated. It was not possible to do protection tests with *Aerobacter aerogenes* P99 since this strain was not virulent to mice.

RESULTS

Ability of the test organisms to destroy different substrates

Suspensions of *Aerobacter aerogenes* P99 and *Proteus morgani* NCTC 235 were incubated with different penicillins and cephalosporins; the results (Table 1) indicated that these organisms were much more able to destroy the antibacterial activity of the cephalosporins than of the penicillins, the *A. aerogenes* strain being much more active than the *P. morgani* strain.

Effect of cloxacillin or methicillin with cephaloridine in vitro

The proportion of cloxacillin or methicillin required to protect cephaloridine 250 μg./ml. was determined. We estimated the amount of inactivation of cephaloridine which occurred when various amounts of the penicillins from 250 μg. to 4 μg./ml. were mixed with cephaloridine 250 μg./ml. The tests were made in 0·01 M-phosphate buffer

(pH 7·0) and in whole horse serum with *Aerobacter aerogenes* P99. The results (Table 2) indicated that methicillin and cloxacillin had a strong protective effect on the cephaloridine. Cloxacillin was more effective in phosphate buffer. When tested in serum, however, methicillin and cloxacillin were almost equal in effect, a probable result of the greater degree of serum-protein binding of the cloxacillin. In experiments on the serum binding of the penicillins, we found that of a 50 μg./ml. solution in whole serum, 51 % of cloxacillin, but only 5·4 % of methicillin, was bound.

Table 1. *Destruction of penicillins and cephalosporins by Aerobacter aerogenes and Proteus morgani*

Substrate	Amount (μg.) destroyed in 2 hr by 1 ml. of suspension of: *A. aerogenes* P99	*P. morgani* NCTC 235
Penicillin G	33,300	25,400
Ampicillin	2,300	Nil
Cloxacillin	Nil	Nil
Methicillin	Nil	Nil
Cephalosporin C	> 1,000,000	104,500
Cephalothin	500,000	40,000
Cephaloridine	> 1,000,000	74,200

Table 2. *Effect of concentrations of cloxacillin or methicillin on the inactivation of cephaloridine by Aerobacter aerogenes in phosphate buffer (pH 7·0) or in horse serum*

Concentrations of antibiotics (μg./ml.)		Cephaloridine destroyed (μg.) in 2 hr by 1 ml. of suspension of *A. aerogenes*	
Cephaloridine	Cloxacillin	in pH 7·0 buffer	in horse serum
250	—	5,324,800	1,600,000
250	250	984	11,864
250	62	1,252	77,312
250	31	3,584	150,528
250	16	39,424	Not tested
250	8	77,312	149,504
250	4	111,616	589,824
	Methicillin		
250	250	36,864	14,646
250	62	71,168	76,800
250	31	63,232	256,000
250	16	274,432	Not tested
250	8	131,072	424,960
250	4	305,152	219,477

A similar experiment was done in phosphate buffer only with *Proteus morgani* NCTC 235. This organism was much weaker than *Aerobacter aerogenes* P99 in its inactivating action (Table 1). Only a very small proportion of cloxacillin or methicillin was required to protect the cephaloridine; cloxacillin, 5 μg./ml. was able completely to protect cephaloridine 250 μg./ml. from destruction by *P. morgani*. Without cloxacillin, 96,000 μg. of cephaloridine were destroyed under the same test conditions.

Infrared spectroscopy of samples of cephaloridine after inactivation showed no evidence of a β-lactam ring. Samples were centrifuged and freeze dried for this test.

Nuclear magnetic resonance measurements agreed with this and the loss of antibacterial activity was thought to be due most likely to the action of a β-lactamase which opened the β-lactam ring.

Experiments with other organisms, mainly strains of Aerobacter and Proteus freshly isolated from hospital patients, showed that organisms which were resistant to cephaloridine were, in general, able to inactivate cephaloridine rapidly, although there are exceptions. Sensitive strains of *Escherichia coli* and *Proteus* sp. were unable, under the conditions of the test, to inactivate cephaloridine. Examples are given in Table 3.

Table 3. *Ability of various strains of Gram-negative organisms to destroy cephaloridine and the effect of cloxacillin*

Organism	Strain code	Cephaloridine (m.i.c. μg./ml.)	Cephaloridine (μg./ml.) destroyed by 1 ml. suspension when treated: alone	in presence of cloxacillin (1:1)
Aerobacter aerogenes	P 99	> 500	1,344,000	8,830
	30*	250	9,090	Nil
	44*	250	655,400	25,408
Proteus morgani	NCTC 235	250	33,790	Nil
	47†‡	500	250,770	Nil
	1†‡	125	37,630	Nil
	2†‡	125	39,930	Nil
	3*†	125	79,870	Nil
P. vulgaris	43*†	250	253,950	5,630
P. rettgeri	19*†	250	327,680	29,950
Escherichia coli	573	8	Nil	Nil
P. mirabilis	431‡	16	Nil	Nil
	2*‡	8	Nil	Nil
	14*‡	8	Nil	Nil
Klebsiella pneumoniae	LAUSANNE	250	Nil	Nil
P. pyocyanea (aeruginosa)	NCTC 8203	> 4,000	Nil	Nil

* Given by Dr K. B. Rogers, (Birmingham).
† Given by the late Professor Mary Barber.
‡ Identified for us by the late Professor Mary Barber.

In vitro antibacterial activity of cephaloridine alone and with cloxacillin or methicillin

Minimum inhibitory concentrations (m.i.c.) of cephaloridine in various proportions with cloxacillin or methicillin were measured for several strains of *Aerobacter aerogenes*, *Proteus morgani* and one strain of *Klebsiella pneumoniae*. The results (Table 4), indicated that cloxacillin or methicillin, added to cephaloridine, considerably increased its activity against β-lactamase-producing Gram-negative organisms. Cloxacillin was more effective than methicillin. With *Klebsiella pneumoniae* (LAUSANNE), cloxacillin with cephaloridine was not more inhibitory than cephaloridine alone. This strain does not destroy cephaloridine (Table 3) and must therefore be relatively resistant to it by some other mechanism.

The bactericidal action of cephaloridine with cloxacillin or methicillin

Cephaloridine is bactericidal to many organisms at concentrations very close to the m.i.c. value (O'Callaghan & Marshall, 1965). We tested the bactericidal action of

cephaloridine alone and in 1+1 mixtures with cloxacillin and methicillin against *Aerobacter aerogenes* P 99 and *Proteus morgani* NCTC 235. The results (Figs. 1, 2) showed that, whilst none of the antibiotics was bactericidal at the concentrations tested when used alone, a mixture of cephaloridine with either cloxacillin or methicillin killed the

Table 4. *Effects of cloxacillin or methicillin on the minimum inhibitory concentrations of cephaloridine against β-lactamase-producing Gram-negative bacteria*

	Minimum inhibitory concentration of cephaloridine (μg./ml.) in mixtures				
	Proportion of cephaloridine:cloxacillin				
Test organism	8:8	8:4	8:2	8:1	8:0
Proteus morgani 63/63	16	32	62	125	500
P. morgani 487	32	32	125	125	1000
P. morgani NCTC 235	16	16	32	61	500
Aerobacter aerogenes P 99	125	125	125	250	1000
A. aerogenes 30	8	8	16	16	62
Klebsiella pneumoniae (LAUSANNE)*	125	nt	nt	nt	125
	Proportion of cephaloridine to methicillin				
	8:8	8:4	8:2	8:1	8:0
P. morgani 63/63	62	125	125	125	500
P. morgani 487	125	250	250	250	1000
A. aerogenes P 99	250	500	500	2000	2000
A. aerogenes 30	62	62	125	125	250

nt = not tested, * = non-β-lactamase producing organism.

organisms rapidly. Thus, for example, the m.i.c. for *P. morgani* was 500 μg./ml. with cephaloridine and 1000 μg./ml. with cloxacillin and the cultures grew unimpeded with either at 31 μg./ml. but a mixture of both at 16 μg./ml. killed more than 90% of the organisms in 3 hr; there was no recovery at 24 hr. With the highly resistant strain of *A. aerogenes*, the effect was even more marked. The culture grew normally in 250 μg./ml. of either cloxacillin or cephaloridine alone, but together at 125 μg./ml. of each there was a rapid kill without any recovery at 24 hr. Similar results were obtained with methicillin in place of cloxacillin, but the rate of kill was somewhat slower with these mixtures than with cephaloridine+cloxacillin.

Effect of cloxacillin and methicillin on the β-lactamase production of Gram-negative bacteria

Aerobacter aerogenes P 99 and *Proteus morgani* NCTC 235 were grown for four serial transfers in broth containing cloxacillin 250 μg./ml. About half the cloxacillin remained after 24 hr incubation in the supernatant broth; this was separated from the organisms by centrifuging. Organisms from the cultures at each transfer were tested for ability to destroy cephaloridine as described before. Organisms from parallel cultures grown in the absence of cloxacillin were tested as controls. No cloxacillin was added when the organisms were tested. The *A. aerogenes* organisms grown in the presence of cloxacillin were much less able to destroy cephaloridine than were the control organisms grown without it. The decrease in activity was 10- to 50-fold, which

was well outside the range of the variation of the controls although we could not rule out the possibility that some remaining cloxacillin was being transferred with the organisms. *Aerobacter aerogenes* organisms were, however, still capable of producing β-lactamase after four serial subcultures in the presence of methicillin (Table 5).

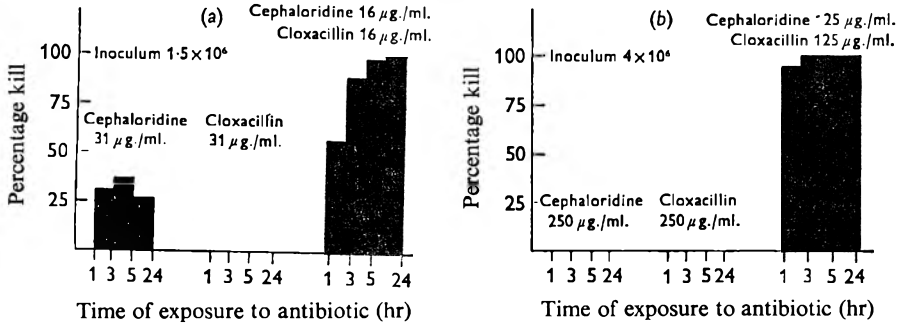

Fig. 1. Bactericidal effects of cephaloridine and cloxacillin, separately and combined; (*a*) *P. morgani* NCTC 235; (*b*) *A. aerogenes* P 99.

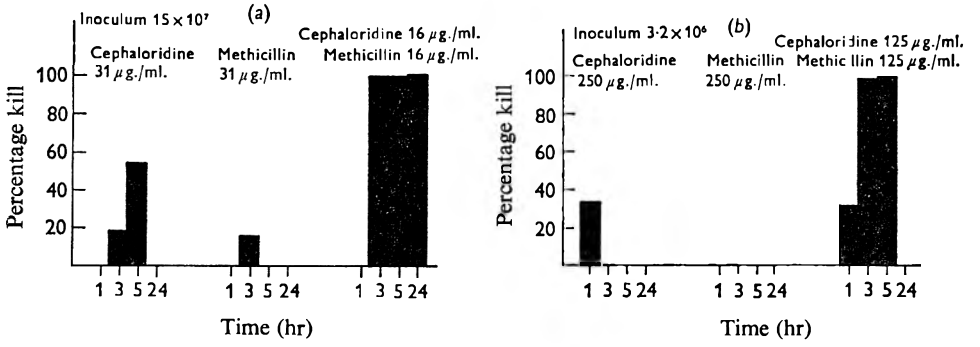

Fig. 2. Bactericidal effects of cephaloridine and methicillin, separately and combined; (*a*) *P. morgani* NCTC 235; (*b*) *A. aerogenes* P99.

Proteus morgani organisms grown in serial subculture in medium containing cloxacillin 250 μg./ml. were also almost entirely unable to destroy cephaloridine, with a decrease of over 500-fold in β-lactomase activity over the controls. Their destructive ability was restored in full by one further subculture in the absence of the penicillin. A similar experiment with methicillin was not possible with this strain since it did not grow in the presence of methicillin at concentrations above 32 μg./ml.

Resistance development

Proteus morgani NCTC 235 was serially subcultivated in cephaloridine alone or in 1+1 mixture with cloxacillin (Table 6). The strain of *P. morgani* used was more sensitive (m.i.c. 31 μg./ml.) to cephaloridine than we had seen previously. Over sixteen transfers in the presence of cephaloridine alone, it increased slowly in resistance to an m.i.c. of 1000 μg./ml. In the parallel experiment with a 1+1 mixture of cephaloridine+cloxacillin, the strain was initially sensitive to cephaloridine 2 μg./ml. (in the presence of 2 μg./ml. also of cloxacillin). During subcultivation, resistance in-

creased slowly in stepwise amounts at a rate similar to that with cephaloridine alone. After the sixteen subcultivations in the mixture the culture was still sixteen times more sensitive to cephaloridine in the 1+1 mixture with cloxacillin than it was when it had been subcultivated in cephaloridine alone. It thus appeared that whereas the resistance of the culture had increased during subcultivation in the cephaloridine+cloxacillin mixture, the cephaloridine-destroying enzyme could still be inhibited with cloxacillin.

Table 5. *Comparison of β-lactamase produced by organisms grown in the presence or absence of cloxacillin*

	Amount of cephaloridine in μg. destroyed in 2 hr by 1 ml. suspension of			
	Aerobacter aerogenes P 99		*Proteus morgani* NCTC 235	
Transfer no.	Cloxacillin culture	Normal culture	Cloxacillin culture	Normal culture
1	160,800	3,584,000	Nil	92,200
2	138,200	7,680,000	Nil	215,000
3	56,300	6,451,000	500	286,700
4	256,000	7,885,000	Nil	770,000
	Subculturing continued but no cloxacillin now added			
5	> 2,000,000	13,517,000	778,200	635,400
6	> 2,000,000	9,523,000	491,520	573,400
	Methicillin culture	Normal culture	Methicillin culture	Normal culture
1	1,254,400	1,984,000	–	–
2	6,225,900	5,785,600	–	–
3	1,939,200	800,000	–	–
4	1,984,000	729,600	–	–
	Subculturing continued but no methicillin now added			
5	1,817,600	3,174,400	–	–
6	1,299,200	1,049,600	–	–

– = not tested

Table 6. *Resistance development of Proteus morgani* NCTC *235 to cephaloridine and to cephaloridine+cloxacillin, 1+1*

	M.i.c. (μg./ml.) at transfer				
Antibiotic	1	4	8	12	16
Cephaloridine	31	62	125	125	1000
Cephaloridine+ cloxacillin, 1+1 (total wt.)	4	8	31	31	62

Protection tests on experimentally infected mice

With *Proteus morgani* NCTC 235, the minimum lethal dose was about 1×10^8 organisms when given intraperitoneally. A challenge of 5×10^8 organisms killed the mice in 24 hr, before which time large numbers of organisms were recovered from the heart blood. Serum taken from such mice 6 hr after giving the challenge dose rapidly inactivated substantial amounts of cephaloridine. The results from different protection tests with the cephaloridine+cloxacillin or +methicillin mixtures varied from experi-

ment to experiment, possibly because the amounts of β-lactamase produced by the challenge organisms *in vivo* differed from test to test. Nevertheless, the mixtures of cephaloridine with either methicillin or cloxacillin were much more effective in protecting the mice than could be explained by a simple additive effect. Typical results are shown in Table 7. Statistical analysis of the results from the experiment summarized in Table 7, using logit transformation, showed that with *P. morgani* NCTC 235, the ED50 dose of 30·5 mg./kg. of cephaloridine:cloxacillin had 95 % confidence limits of 14–58 while the ED50 of 24·5 for the cephaloridine+methicillin had limits of 12·0–48·5. The improvements in performance over the antibiotic components tested separately was thus highly significant.

Table 7. *Tests on experimentally infected mice with cephaloridine alone and with cloxacillin or methicillin*

	ED50 dose of total antibiotic in mg./kg.	
Treatment	*Proteus morgani* NCTC 235	*P. morgani* P 47
Cephaloridine alone	> 400	About 150
Cloxacillin alone	> 400	> 400
Cephaloridine+cloxacillin (1+1)	30·5	About 44
Cephaloridine alone	> 400	About 150
Methicillin alone	> 400	> 400
Cephaloridine+methicillin (1+1)	24·5	About 65

Table 8. *Protection tests with Proteus morgani* NCTC *235*

The effect of varying the proportions of the antibiotic mixtures

	ED50 dose (mg./kg.)	
Treatment	Total antibiotic	Cephaloridine
Cephaloridine alone	100	100
Cloxacillin alone	> 100	—
Cephaloridine+cloxacillin (1+1)	16·6	8·8
Cephaloridine+cloxacillin (1+4)	15·0	3·0
Cephaloridine alone	35	35
Methicillin alone	44	—
Cephaloridine+methicillin (2+1)	14·4	9·6
Cephaloridine+methicillin (1+1)	> 12·5	> 6·0
Cephaloridine+methicillin (1+2)	15·0	3·0

(*Note.* The two parts of the experiment, with cloxacillin and methicillin, respectively, in the above table were done on different occasions.)

It was possible that a 1+1 mixture of cephaloridine with cloxacillin was not optimal for protecting the animals. An experiment with the same conditions as before, to compare various proportions of the antibiotics, was done with groups of eight mice. The results are given in Table 8. The results showed that whilst variation of the proportions of cephaloridine to cloxacillin from 1+1 to 1+4 affected the amount of cephaloridine needed to protect the animals, the total weight of antibiotic mixture required remained constant. A similar effect was seen with cephaloridine+methicillin mixtures. The proportions of the components of the mixture thus did not appear to be critical.

DISCUSSION

Cephaloridine has a broad spectrum of antibacterial activity and many common Gram-negative bacteria are sensitive to it. Some however are resistant, and in many instances this can be related to the ability of the organisms to inactivate the antibiotic. There seems little doubt that the mechanism of inactivation is the destruction of the β-lactam ring by a β-lactamase. This supposition is consistent with our observations in which we have seen alterations in the infrared spectrum and nuclear magnetic resonance of the inactivated antibiotic, which are indicative of destruction of the β-lactam ring, and also with the observations of other workers referred to earlier.

Cloxacillin and methicillin are known to possess a greater affinity for β-lactamase from Gram-negative organisms than other penicillins and cephalosporins. These compounds are not inactivated by the enzyme, despite which they have no activity against the organisms which produce it. In our work, we have found that mixtures of methicillin or cloxacillin with cephaloridine are able to protect the cephalosporin from inactivation by β-lactamase-producing Gram-negative organisms and the mixtures are then active inhibitors of the bacteria. In contrast, we have found that the addition of methicillin or cloxacillin does not increase the activity of cephaloridine against Gram-negative organisms which do not produce β-lactamase, a finding which adds support to the enzyme inhibition hypothesis. Our *in vitro* studies showed that when cephaloridine was protected from enzyme attack by admixture with the penicillins, it was bactericidal. Organisms such as *Proteus morgani* which were not killed by either the penicillins or by cephaloridine were killed rapidly by concentrations of the two substances together which could be reached *in vivo* at a site of infection.

In our *in vitro* tests, we found that the cephaloridine + methicillin mixtures were rather less active than the cephaloridine + cloxacillin ones when these were tested in broth. When tested in serum, however, cloxacillin was slightly less efficient in its protective activity so that methicillin became equally effective. We believe this to be due to the fact that cloxacillin is more highly serum-bound than is methicillin; when the mixtures were tested *in vivo* this hypothesis was upheld by the finding that the two penicillins were of approximately equal effect.

Despite the promising findings from our *in vitro* experiments, the possibility remained that the protective phenomenon would not work in infected animals. Appropriate concentrations of cephaloridine + the protective penicillin would have to be present at the infection site at the same time and this may be difficult to achieve. Our tests on experimentally infected mice have shown, however, that in the type of infection we used, cephaloridine was made effective by the concurrent administration of the β-lactamase-inhibiting penicillin. Moreover, the proportions of the cephaloridine and inhibitor administered were not very critical.

Repeated daily transfers in broth containing just subinhibitory concentrations of cephaloridine alone or of cephaloridine + penicillin mixtures resulted in the development of some intrinsic resistance to the cephaloridine. This was apparently not due to increased production of β-lactamase, since the effect of cloxacillin on the minimum inhibitory concentration of cephaloridine remained in the same proportion after sixteen subcultures as it had been at the first subculture. This is in agreement with the results of Barber & Waterworth (1964) who showed that when strains of *Proteus mirabilis* sensitive to cephaloridine were exposed to this antibiotic in serial subculture, a slow

stepwise increase in resistance occurred. These organisms had not, however, acquired resistance by developing an enzyme able to destroy cephaloridine.

With *Pseudomonas pyocyanea* (*aeruginosa*), an organism resistant to cephaloridine, Sabath, Jago & Abraham (1965) showed that strain NCTC 8203 produced a cell-bound inducible β-lactamase which was inhibited by methicillin and cloxacillin. In experiments in our laboratory with 45 strains of *P. pyocyanea* isolated from clinical cases, we have found by our *in vitro* method that organisms of these strains only slowly inactivate very small amounts of cephaloridine, particularly when they have not previously been exposed to a substance such as a penicillin which could cause β-lactamase induction. It therefore seems to us that the mechanism of resistance in this case is much less dependent on a β-lactamase, and that the organisms themselves are intrinsically far more resistant. It is possible, however, that in conditions where very high concentrations of antibiotics can be achieved, e.g. in the urine, treatment of *P. pyocyanea* infections with such synergistic mixtures may be successful. One case has been reported by Shirley & Moore (1965) using a mixture of benzylpenicillin and methicillin. Sabath, Elder, McCall, Steigbigel & Finland (1966) have reported the successful treatment of human urinary tract infections due to *P. pyocyanea*, and also to a Proteus species and *Escherichia coli*, by using mixtures of cloxacillin and ampicillin. Cloxacillin exerts a similar protective effect with ampicillin as it does with cephaloridine. The protective effects of cloxacillin and methicillin apply only to β-lactamase-producing Gram-negative organisms. The action of β-lactamase produced by penicillin-resistant *Staphylococcus aureus* is unimpaired by either cloxacillin or methicillin, despite the resistance of these penicillins themselves to the action of the enzyme.

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