THE CONTINUOUS CULTURE OF PLANT CELLS

by

P. J. King

A thesis presented for the degree of Doctor of Philosophy in the University of Leicester.

September, 1973

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To the community - but especially to

my parents, to Jean and to my children.

ACKNOWLEDGMENTS

I am indebted to Professor H. E. Street for the opportunity, for the inspiration and for generous advice and encouragement.

In the initial stages the work relied heavily on the technical skills of Dr. Brian Wilson, who designed and built most of the apparatus used.

I am grateful to Dr. Michael Young and to workers at Unilever Ltd., Sharnbrook, in particular Dr. Laurie Jones and Mr. Ron Kessel, for many helpful discussions.

I wish to thank Valerie Lambert and Eric Singer (laboratory technicians), B. Dobski (engineering), Howard Neale (electronics), Susan Pearcey and Susan Duffey (drawings), whose skilled assistance made the work possible, and Joan Kempston for typing this thesis.

The project was financed by the Science Research Council in cooperation with Unilever Ltd.

Selected aspects of the work described in this thesis have already been published as follows:

Wilson S. B., King P. J. and Street H. E. 1971.
Studies on the growth in culture of plant cells, XII. A versatile system for the large scale batch or continuous culture of plant cell suspensions. J. exp. Bot. 21, 177-207.

Street H. E., King P. J. and Mansfield K. J. 1971.
Growth control in plant cell suspension cultures. pp. 17-40, in "Les Cultures de Tissus de Plantes". Colloques Internationaux du CNRS N°. 193, Paris.

King P. J. and Street H. E. 1973.
Growth patterns in cell cultures. pp. 269-337, in "Plant Tissue and Cell Culture". ed. H. E. Street. Blackwells.

King P. J., Mansfield K. J. and Street H. E. 1973.
Control of growth and metabolism in cultured plant cells. Can. J. Bot. (in press) 51

This thesis is my own work unless otherwise acknowledged and has at no time been submitted for another degree

.....

I certify that this statement is correct

.....

5/9/13

C O N T E N T S

SECTION PAGE

1. GENERAL INTRODUCTION

"..... exciting prospects lie in the possibility that (plant) cells, freed from the restraints of being part of a multicellular, multi-functional organism will, in effect, be a new group of micro-organisms with all the capabilities which this encompasses" (Nickell and Tulecke, 1959).

The earliest suspensions of cultured plant cells capable of repeated subculture (those of Nicotiana tabacum and Tagetes erecta by Muir, 1953) were developed as a source of single viable cells from which single-cell clones could be established (Muir, Hildebrandt and Riker, 1958). Similar suspensions composed of free cells and aggregates were also obtained from cultured carrot root explants (Steward and Shantz, 1955), from normal and tumour tissues of Picea glauca (Reinert, 1956) and from haploid tissue of Antirrhinum majus (Melchers and Bergmann, 1958). Nickell (1956) first demonstrated the feasibility of growing such a cell suspension as a culture of "micro-organisms" using a highly dispersed suspension of cells derived from the hypocotyl of Phaseolus vulgaris. Subsequent studies from his laboratory (Nickell and Tulecke, 1959, Tulecke and Nickell, 1959) described the use of microbial fermentation techniques for studies on growth kinetics, biochemical composition and production of particular metabolites by such plant cell cultures.

Cell suspensions have now been obtained from the tissues of many plant species; particularly well dispersed suspensions in common use (consisting of free cells and small aggregates of cells) are those of Haplopappus gracilis (Eriksson, 1967), Rosa sp. "Pauls Scarlet" (Nash and Davies, 1972) and Acer pseudoplatanus (Street, 1973).

Such plant-cell suspensions have been most commonly maintained as batch cultures: an inoculum of cells is placed into a finite volume of nutrient medium, the cells grow and divide at the expense of the nutrients and, at about the time of nutrient depletion, portions of the population are then used as inocula for further batch cultures. A typical laboratory procedure for the propagation of a cell suspension (Henshaw, Jha, Mehta, Shakeshaft and Street, 1966) involves 70 millilitres of suspension in a 250-millilitre Erlenmeyer flask closed with cotton-wool or alfoil and shaken at 120 rpm on a horizontal rotary shaker; subcultures are made by 1:7 dilution with new medium at three-week intervals.

Since 1960, work involving batch cultures of plant cells in suspension falls into two main categories:

(1) Single-point harvests of cell material (often during the terminal stages of a batch culture) for use in many, varied cell-free biochemical studies of particular cell metabolites (see, for example, Filner, 1965; Graebe and Novelli, 1966; Gamborg and Eveleigh, 1968 and Verma and van Huystee, 1971).

(2) descriptions of growth kinetics and cell metabolism during the term of a batch culture.

The latter studies have demonstrated that balanced growth is rarely achieved in batch cultures of plant cells i.e. that rates of biosynthesis and cell division are rarely constant or equal and, therefore, that the mean composition and metabolic activity of the cells in the population varies with time. For example, the mean weight of DNA, RNA and protein per cell of the population reach peaks at different times (Nash and Davies, 1972) and the specific activity of groups of enzymes involved

in carbohydrate oxidation (Fowler, 1971), the extractable cytokinin activity (Mackenzie and Street, 1972) and rates of accumulation of cellular nitrogen and carbohydrate (Rose, Martin and Clay, 1972) all fluctuate radically during the progress of growth in batch cultures. This phenomenon reflects unfavourably on studies of the type mentioned in (1) above where single-point harvests are made at arbitrary times. Although the complete rationalisation of the growth patterns of plant cells in batch culture is very difficult (and for some purposes may be unecessary), nevertheless when plant cells in culture are to be used, for example, for the study of cytodifferentiation or secondary metabolism, or for the supply of enzymes or substrates for cell-free biochemistry, it is essential to have some prior description of the growth of the cells in realistic terms (by avoiding single-point measurements and by using multiple parameters). It is also essential to appreciate the variations in the metabolic state of the cells that may occur with time and the rapidity with which one state may follow another. This point has been overlooked, particularly in many studies of secondary plant products in tissue culture. A large number of cultures have been examined for the presence of secondary products of pharmaceutical importance and characteristic of the whole plant or a specific organ e.g. seed, tuber or leaf. The cultures have been harvested at an apparently arbitrary time after their inoculation, usually after the phase of most rapid growth. In such cases, the cultures have usually yielded little or none of the desired product (for reviews see: Krikorian and Steward, 1969; Puhan and Martin, 1971; Turner, 1971). Very few systematic studies have been made of secondary product metabolism <u>during the whole term</u> of a batch culture, although in the few such studies reported it has been shown that the secondary

product may only occur in significant concentrations during transient periods of the growth of a batch culture (Kaul and Staba, 1967; Ogutuga and Northcote, 1970; Tabata, Yamamato and Hiraoka, 1971; Nash and Davies, 1972).

The absence of balanced growth in batch cell cultures limits but does not preclude their use for intensive study of particular aspects of cell metabolism. Already important aspects of metabolic regulation in plant cells have been revealed by study of the transient changes themselves and by short-term studies centred upon a particular growth phase of batch cultures. Examples of the latter are studies on glucose transport (Maretzki and Thom, 1972), Shikimic acid metabolism (Gamborg, 1966), amino acid synthesis (Dougall, 1971), arginine catabolism (Maretzki, Thom and Nickell, 1969), the kinetics of ^{14}C- incorporation into RNA from 2-^{14}C-uridine (Cox, Turnock and Street, 1972), and on the incorporation of adenine into adenosine nucleotides (Dorée, Leguay, Terrine, Sadorge, Trapy and Guern, 1971). Furthermore, the study of unbalanced growth has its own intrinsic interest; unbalanced growth is characteristic of primordia (cell division rather than growth) and of cytodifferentiation (growth rather than cell division) (Steward and Mohan Ram, 1961). However, the complexities of the metabolic and environmental fluctuations that occur in batch cultures make for serious difficulties in routinely obtaining populations of cells in an easily reproducible state and in identifying controlling factors.

Alternative cultural procedures have been developed (in particular, by microbiologists) which alleviate some of the problems of batch cultures. They include the techniques of <u>continuous culture</u> and <u>synchronous culture</u>. A continuous culture may be defined as:

> - a suspension of cells of constant volume in which a steady state (a state of balanced growth in a constant environment) is maintained by the continuous inflow of fresh medium and outflow of corresponding volumes of culture.

Continuous cultures of micro-organisms have been in use since ca. 1940, although until the definitive work of Novick and Szilard (1950a) and Monod (1950), which laid out the basic mathematical principles governing the achievement of steady states, they were used simply for producing a continuous supply of cells without the need for routine subculturing. Continuous cultures of micro-organisms are basically of two types:

> (1) a chemostat, in which growth rate and cell density are held constant by a fixed rate of input of a growth-limiting nutrient (described theoretically by Novick and Szilard and by Monod, and later by Herbert, Elsworth and Telling, 1961, and Málek and Fencl, 1966).
>
> (2) a turbidostat, into which fresh medium flows in response to an increase in the turbidity of the culture (described theoretically by Northrop, 1954).

Pirt (1972) assessed the advantages of the chemostat as:

(1) providing a means of fine control of growth rate and, as cells at different growth rates often differ in some metabolic functions, a means of selecting populations of cells in a particular metabolic state.

(2) allowing changes in physical and nutritional conditions to be examined at constant growth rates.

(3) allowing measurable periods of "substrate-limited growth" - a situation unobtainable in batch cultures where the only constant growth state (exponential growth phase) is not substrate-limited.

(4) separating, by means of the steady state, the effects of a

given environment from the effects of the history of the cells.

(5) providing the most rapid and efficient conversion of substrate into biomass.

These properties of the chemostat represent not only a more efficient and less ambiguous situation from which to examine basic functions of plant cells but also a new situation from which to approach, for example, the control of the biosynthesis of useful products and the control of developmental pathways in plants, in particular cytodifferentiation.

Hitherto the emphasis of the limited number of attempts to produce chemostat cultures of plant cells has been almost entirely on biomass production. Tulecke, Taggart and Colavito (1965) described the dilution of 8-litre"phytostat" cultures of Rosa sp. by replacing 1-2 litres of culture with the same volume of fresh medium at approximately daily intervals (a 'semi-continuous' culture). The fresh-weight yield values quoted suggest that some steady states† were achieved. A chemostat culture of Glycine max produced by Miller, Shyluk, Gamborg and Kirkpatrick (1968) by the continuous dosing of a 2-litre culture with fresh medium and the removal of 10-millilitre portions of culture every 20 minutes was used to demonstrate a stabilisation of dry-weight output for a short period. More recently a constant yield of cells from a culture of Haplopappus gracilis

---

† A "steady state" is a theoretical concept probably impossible to achieve in practice due to imperfect culture mixing, mutation and selection, variations in flow rate etc. and the term has only relative significance, particularly in reference to semi-continuous cultures.

was reported by Constabel, Shyluk and Gamborg (1971) using a similar system. Kurz (1971) has cultured suspensions of Glycine max and Triticum monococcum in a continuous-flow chemostat and reported constant dry-weight yields during short dilution periods. Wilson (1971) reported the achievement of populations of constant biomass in continuous cultures of Acer pseudoplatanus.

It was the aim of the work now to be described in this thesis to examine in detail the kinetics of the growth of plant cells in continuous-culture systems and so to lay a secure foundation for future, practical applications of the technique. For the most part the work (Section V. Continuous cultures) takes the form of a systematic test of the"Monod" theory as interpreted by Herbert, Elsworth and Telling (1956) and Málek and Fencl (1966) and was undertaken using as a model system a fine suspension culture of cells derived from cambial tissue of Acer pseudoplatanus L. The feasibility of the turbidostatic culture of plant cell suspensions was also considered. This work was preceded by an examination of the problems of aeration in large suspension cultures (Section III. Aeration of cultures) and of the basic growth patterns of Acer cells in batch culture (Section IV. Growth and cell division in batch cultures).

Synchronous cell cultures are based usually upon batch cultures and as the composition and metabolic activity of the cells changes almost continuously (during each cycle) they may be said to show an extreme of unbalanced growth. In addition the magnitude of the metabolic events during one cycle may be quite different from those in a subsequent cycle. Nevertheless, within the framework of the synchronous cell divisions, cultures showing a high degree of synchrony present a more clearly definable and reproducible system

with which to study cell function than do randomly-dividing batch cultures. Synchronised batch cultures of bacteria, algae, yeast, protozoa and mammalian cells are now used routinely in many laboratories (Padilla, Whitson and Cameron 1969; Mitchison, 1971). Continuous phased (synchronous) cultures of Candida utilis have also been established by repeated 1:1 dilution of growing cultures with fresh medium at a periodicity corresponding to the cell doubling-time (Dawson, 1969).

Description of some of the events of the cell cycle of higher plants has until recently only been achieved by observing naturally-occurring synchrony (Erickson, 1964), by inducing partial synchrony of whole-plant meristems (Mattingly, 1966) or indirectly by selective labelling of asynchronous populations in vivo (Wimber and Quastler, 1963; Phillips and Torrey, 1972). Only very limited synchrony has so far been achieved in plant tissue and cell cultures: Eriksson (1966) produced peaks of mitotic activity in cell cultures of Haplopappus gracilis after release from metabolic blocks; Yeoman (1970) observed peaks of mitotic activity and stepped increases in cell number in tuber explants of Helianthus tuberosus during callus induction; Jouanneau (1971) was able to control peaks of mitotic activity and cell division by the cytokinin-starvation of Nicotiana tabacum. There are other less satisfactory reports of cell synchrony based upon observations of mitotic index alone, for example, by Roberts and Northcote (1970) and by Constabel and Wetter (1972). With the exception of the work of Yeoman and associates (see Yeoman & Aitchison, 1973) none of these studies has progressed far beyond the synchronisation stage to an examination of metabolic events associated with growth and cell division.

The work with synchronous cultures of Acer pseudoplatanus

described in this thesis (Section VI. Synchronous cultures) stems from the observation (Street, 1967) that some step-wise increases in cell number may occur in shake-flask cultures of Acer inoculated at low density with stationary-phase cells. The work was prompted (1) by the development of large-scale batch culture equipment which allowed the continuous (and automatic), aseptic sampling of a single population of cells over a long period; and (2) by the advantages of having to hand a basic description of the cell cycle of Acer cells whilst investigating the activity of the cells as a function of growth rate in continuous cultures.

II. MATERIALS AND METHODS

1. Culture techniques.

The general techniques for the preparation of media and culture glassware and the propagation of the stock suspension cultures of the Acer pseudoplatanus L. cell clone (Plate II.1) were as previously described (Henshaw et al. 1966; Street, 1966).

2. Nutrient medium.

The synthetic culture medium used was essentially as described by Stuart and Street (1969). However, in most 4-litre batch and continuous cultures, ferric chloride was replaced by ferric ethylenediamine tetraacetate, (FeEDTA), sucrose was replaced by glucose and the initial pH of the medium was adjusted to 6·2 instead of 5·2. The inclusion of FeEDTA in media adjusted to pH 6·2 - 6·4 (the pH normally recorded in dividing cultures of Acer cells) was found to increase the yield of cells by ca. 17% (Wilson - unpublished data). The concentration chosen was that used by Givan and Collin (1967). Glucose was found to be an effective substitute for sucrose and was included so that the carbon source might be assayed more specifically.

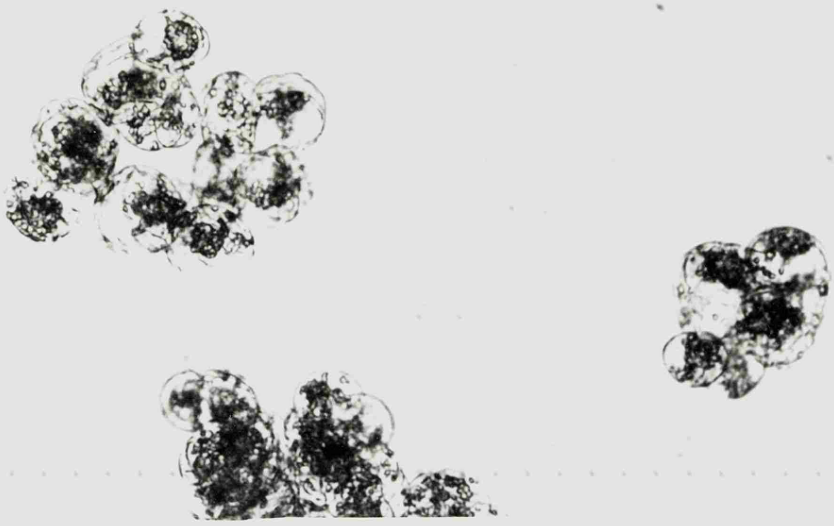
Component	Molarity
KCl	$1{\cdot}0 \times 10^{-2}$
$MgSO_4 \cdot TH_2O$	$1{\cdot}0 \times 10^{-3}$
$NaNO_3$	$7{\cdot}0 \times 10^{-3}$
$NaH_2PO_4 \cdot 2H_2O$	$8{\cdot}3 \times 10^{-4}$
$CaCl_2 \cdot 6H_2O$	$5{\cdot}0 \times 10^{-4}$
$ZnSO_4 \cdot 7H_2O$	$3{\cdot}5 \times 10^{-6}$

Plate II.1. Cell suspension of *Acer pseudoplatanus* L.

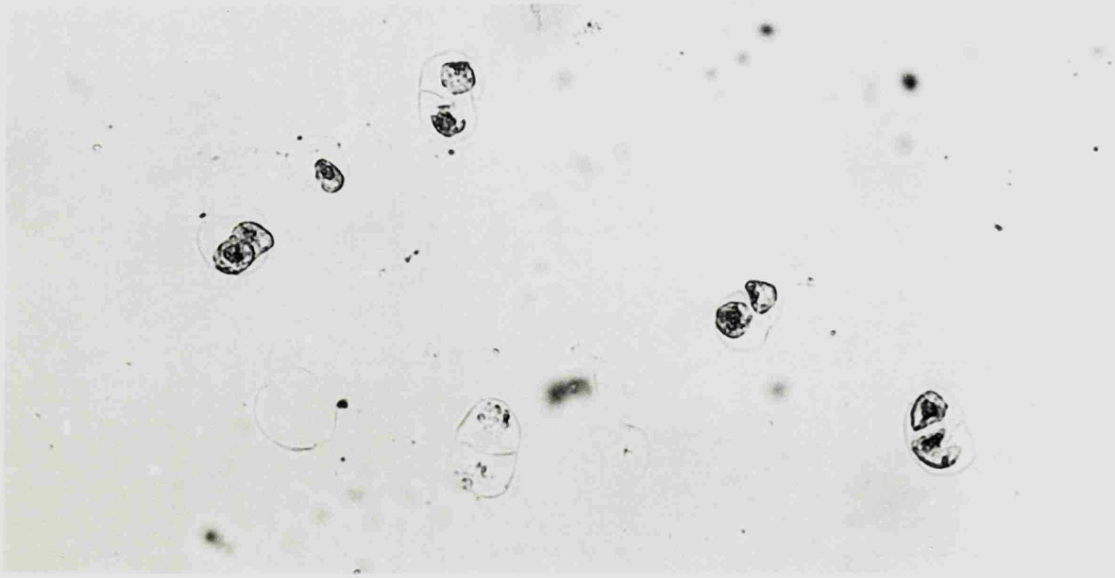
A. Untreated sample of cell suspension from a chemostat culture ($D = 0.150\ day^{-1}$).

B. A sample of the same suspension depicted in A. but after maceration and dilution for cell counting (see II. Materials and Methods. 7).

A



B



100μ

Plate II.1

H_3BO_3	$1{\cdot}6 \times 10^{-5}$
$MnSO_4.4H_2O$	4.5×10^{-7}
$CuSO_4.5H_2O$	1.2×10^{-6}
KI	6.0×10^{-8}
FeEDTA	2.0×10^{-5}
(or $FeCl_3.6H_2O$	0.4×10^{-5})
Thiamine HCl	3.0×10^{-6}
meso-Inositol	5.0×10^{-6}
2,4-D	4.5×10^{-6}
Glucose	1.1×10^{-1}
(or Sucrose	0.59×10^{-1})
Choline chloride	3.6×10^{-6}
Pantothenic acid	5.2×10^{-6}
Cysteine HCl	6.3×10^{-5}
Kinetin	1.2×10^{-6}
Urea	3.3×10^{-3}

3. Large-Scale Culture Apparatus.

The construction and operation of the 4-litre batch and continuous culture systems used in this study have been fully described elsewhere (Wilson, King and Street, 1971). Therefore, to avoid unecessary repetition, a copy of the relevant section of the paper cited above has been bound into the back of this thesis (Appendix 1). Also included (Appendix 2) are brief descriptions of the major modifications made to the design of the apparatus by the present author.

4. Preparation of Large-Scale Culture Vessels and Medium Reservoirs.

Apparatus for 4-litre batch or continuous cultures and associated distilled-water and medium reservoirs were assembled and autoclaved as previously described (Wilson, King and Street, 1971 and Appendix 1). Effective sterilising times at 121°C were found to be:

1. Culture vessels containing 4 l. of medium - 35 mins.
2. Aspirators containing 9 l. of medium - 45 mins.

All cultures and medium reservoirs were inoculated with urea and cell suspension inside a cabinet, which was sterilised by u.v. irradiation and supplied with filtered air (Street, 1973).

Urea was added aseptically to cool, sterile medium in culture vessels and reservoirs via an inoculation port, as a 40% filter-sterilised solution (0·5 ml per litre of medium); (Oxoid, Ltd.).

Culture vessels were normally inoculated with cells by pouring the contents (210 ml) of a stock culture flask through the inoculation port. Two pairs of hands were essential for all inoculations.

5. Medium Flow Rates for Continuous Cultures.

The rate of flow of medium into continuous cultures (F; ml day^{-1}) was regulated by previously-calibrated metering pumps (see Appendix 1). The flow-rate was measured at approximately daily intervals by transferring the contents of the overflow reservoir (the displaced culture volume) to a graduated cylinder. The overflow reservoir was first shaken to re-disperse the settled cells. The flow-rate was calculated from the volume of culture in the cylinder, plus the total volume of samples taken from the culture vessel in the period since the reservoir was last emptied. The fresh weight of the biomass produced by the culture per day was measured by filtering the overflow through

preweighed nylon discs (see Fresh Weight - this Section). A correction was applied to the fresh weight recorded to take account of samples removed from the culture vessel.

Variation in the flow rates recorded (Table II.1.) were largely due to the error involved in relevelling the culture vessel (which contained fixed level-detecting probes) after each occasion when the whole culture vessel was shaken to dislodge cell material thrown up onto the walls. The real flow rate probably did not vary significantly (with the exceptions involving the Delta micro-metering pump noted in Appendix 2). The maximum variation in actual dilution rate, assuming that the culture volume was maintained at 4000 ml ± 100 ml, amounted to less than 3% over a perturbation period of 10 hours. The maximum biomass change during this period would be expected to be less than 1% per hour.

6. Sampling procedure.

The sampling technique for large-scale cultures is described in Appendix 1. The experiments described in this thesis are mainly based upon series of estimations of the biomass and nutrient concentrations in cell cultures over prolonged periods of time (between 30 and 200 days). For this reason, in experiments with non-synchronous batch cultures and continuous cultures, a sampling procedure was established (Fig. II.1.) which (i) could be completed by one operative within three hours and thus allowed time to complete the analysis of samples, (ii) could be repeated at least every 24 hours, (iii) contained the minimum necessary replication, (iv) consumed the minimum volume of culture, and (v) involved the minimum number of manipulations of the culture vessel.

Table II.1. Flow rate variation in representative chemostat cultures.

Culture 78*	Culture 93†
Overflow volume ml day^{-1}	Overflow volume ml day^{-1}
880	630
739	643
738	615
723	714
670	714
764	665
704	615
750	684
895	672
732	676
728	627
Mean 756	659
S.E.M. 24.1	8.0
S.E.M. as % Mean 3.2	1.2

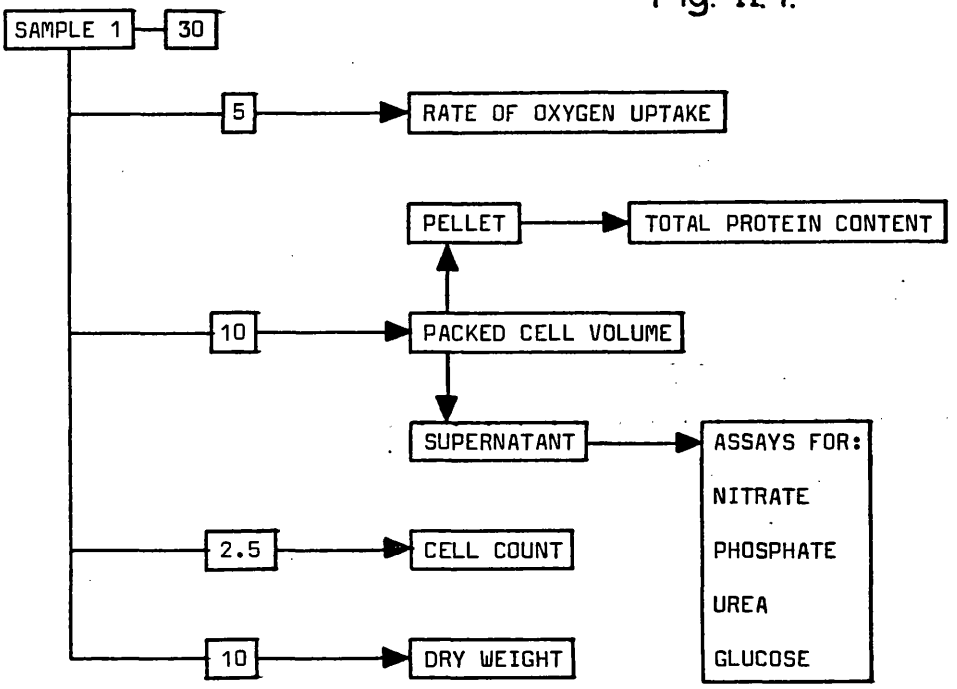
*Culture 78 : 11 estimations over 31 day period using a Delta Micro-metering pump.

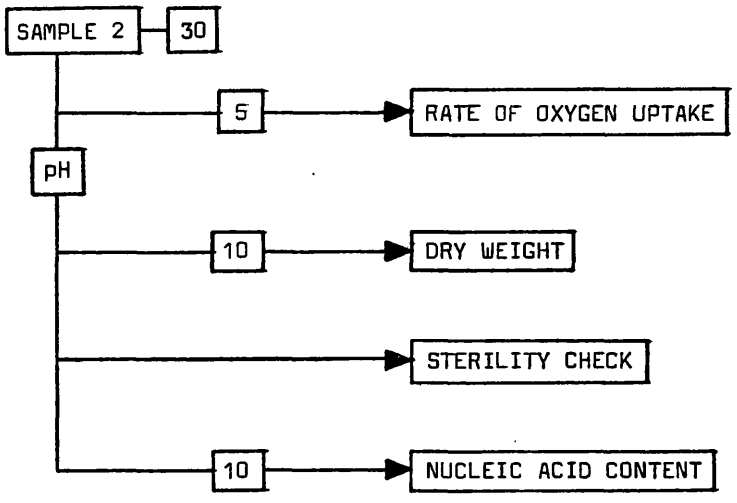
†Culture 93 : 11 estimations over 15 day period using a Hughes Micro-metering pump.

Fig. II.1. Sampling procedure for 4-litre batch and continuous cultures.

Enclosed figures are volumes of culture in millilitres.

Fig. II.1.


SAMPLE 1
30
5
RATE OF OXYGEN UPTAKE
PELLET
TOTAL PROTEIN CONTENT
10
PACKED CELL VOLUME
SUPERNATANT
ASSAYS FOR:
NITRATE
PHOSPHATE
UREA
GLUCOSE
2.5
CELL COUNT
10
DRY WEIGHT


SAMPLE 2
30
5
RATE OF OXYGEN UPTAKE
pH
10
DRY WEIGHT
STERILITY CHECK
10
NUCLEIC ACID CONTENT

The procedure adopted involved the removal of only two samples from the culture vessel. The total volume of the two samples (60 ml) represents only 1·5% of the total volume (4 litres) of the continuous cultures used. Although Table II.2 shows that the perturbation period, i.e. the period before the dilution rate returns to normal, may be 6 hours at the lower end of the range of dilution rates actually imposed on continuous cultures, the effective increase in dilution rate immediately after the sample is removed is less than 2%. Even assuming that the cells do not respond at all during the perturbation period to increased nutrient supply, the wash-out due to the increased dilution rate would be much less than 1% of the biomass per hour.

The effect of withdrawing samples from 4-litre batch cultures was not investigated. However, it has been demonstrated (Short, Brown and Street, 1969) that large volumes of suspension (100 ml - 650 ml) can be withdrawn during the incubation of 4·5-litre cultures of Acer pseudoplatanus L. in spinning bottles without altering the general growth pattern or the yield of cells per unit volume of culture.

Duplicate estimations (one per sample) were made of oxygen uptake rate and dry weight. The duplicates normally agreed within 10% limits. A single estimation of the number of cells present was found to be sufficient (see Cell Number - this Section).

7. Cell number.

The technique used for determining the number of cells per unit volume of culture was a more rapid version of that described by Henshaw, Jha, Mehta, Shakeshaft and Street (1966).

(i) Maceration. a. Continuous cultures and non-synchronous batch cultures.

One volume of culture (normally 2·5 ml) was suspended in four

Table II.2. The effect of sample removal on the effective dilution rate of a 4-litre chemostat culture.

	Before sampling total culture Vol. = 4000 ml		After sampling total culture Vol. = 3940 ml		Effective Perturbation Period	Maximum Increase in Dilution Rate
	D day^{-1}	g h	D day^{-1}	g h	h	%
Minimum*	0.060	276.0	0.061	271.8	6.0	1.7
Maximum*	0.230	72.0	0.232	71.4	1.6	0.9

D = dilution rate

g = calculated mean generation time ($D = 0.69/g$)

* Minimum and maximum refer to the range of dilution rates applied.

volumes of 8% (w/v) chromium trioxide solution in a tube. The tube was then incubated at 70°C in a water bath. The length of the incubation period required to produce sufficient maceration without destruction depended both upon the growth phase of the culture and the concentration of other oxidisable materials (e.g. medium sugars) in the sample. Samples of stationary phase cultures containing expanded, fragile cells and no residual sugars required a very brief incubation. A 10 minute incubation was normally required for batch exponential-phase cells or continuous culture samples. After incubation, the samples were transferred quantitatively to 50 ml polythene bottles (30 mm I.D.) and shaken vigorously using a flask shaker (Baird & Tatlock, Ltd) for up to 5 minutes. Cells macerated in this way were present mostly as pairs and had contracted, stained protoplasts (Plate II.1).

b. Synchronous cultures. Samples of synchronous cultures (5 ml ± 10%) were delivered automatically into tubes (calibrated to 15 ml) which were arranged on a fraction collector (see Appendix 1). The tubes contained 12% (w/v) chromium trioxide solution (5 ml). The volume of the suspension in each tube was made up to 15 ml and the exact sample volume calculated by difference. The tubes were then incubated and shaken as in a. above.

(ii) Counting.

After suitable dilution, samples were pipetted into the wells of specially prepared counting slides. From the dimensions of the slide-well and the diameter of the field of view of the microscope used (magnification x 100), the field volume was calculated to be 0·8 µl. Samples from all cultures were diluted so that 5 - 10 cells were present per field. An estimation of the number of cells per ml of culture was made from the number of cells in 10 randomly selected fields.

(iii) Counting error.

The effectiveness of the maceration technique sometimes varied and, even in the best preparations, the number of cells in some aggregates could not be determined with certainty. For these reasons, 10 estimations of the number of cells in each sample were made i.e. 10 x 10 fields were scored. The mean and standard error of the mean (S.E.M.) were calculated. A cell count was acceptable when the S.E.M. was $< 10\%$ of the mean value. An analysis of the variance of cell number estimations within and between ten separate samples removed from a 4-litre culture (Table II.3.) indicated no significant difference; $F_s \left(\frac{\text{BETWEEN}}{\text{WITHIN}}\right) = 0{\cdot}38$, $F_{.05} \simeq 2{\cdot}0$. In view of this, only single samples were taken for cell counting each time a 4-litre batch or continuous culture was sampled. In experiments with 70 ml shake-flask cultures, a single sample was taken from two combined replicate-cultures.

8. Dry weight.

The cells from culture samples (10 ml) were collected by filtration under vacuum through discs of nylon cloth (75 mm diam., ca. 700 mg dried weight) or glass-fibre filter paper (Whatman GF/C; 25 mm diam., ca. 26 mg dried weight), washed with distilled water and incubated at $80^{\circ}C$ for 12 hours. The discs were cooled inside a dessicator and reweighed.

9. Fresh weight.

Cells were separated from the culture medium by filtration under vacuum through nylon discs of known wet weight. Vacuum was applied until no further filtrate was obtained. The discs were then reweighed.

Table II.3. An analysis of variance of cell number estimations (ITEMS) made on replicate samples (GROUPS) taken from a 4-litre chemostat culture on a single occasion.

	GROUPS (a = 10)										Y_a	S.E.M.(a)
	92	91	85	73	104	91	72	76	73	71	828	3.60
	100	72	63	85	107	88	69	100	83	74	841	
	87	89	77	101	71	108	82	94	82	95	886	3.55
	70	92	87	104	79	67	79	70	84	92	824	
ITEMS	93	104	86	100	104	75	100	70	85	67	884	4.44
(n = 10)	71	92	123	75	58	96	94	106	101	103	919	
	110	55	79	69	80	78	82	87	65	90	795	4.75
	65	83	65	97	72	94	67	100	61	63	767	
	92	66	76	68	66	103	101	70	75	57	774	5.01
	70	101	64	86	119	87	82	88	76	70	843	
n Y	850	845	805	858	860	887	828	861	785	782		
S.E.M. (n)		4.95		4.44		4.00		4.39		4.91		

Source of variation	Degrees of freedom	Variance	Mean Square	F_s Between / Within
Among groups	9	1,110.49	123.39	0.38
Within groups	90	28,927.30	321.41	
Total	99	30,037.79		

$F_{.05\ 9,60} = 2.04$ $F_{.05\ 9,120} = 1.96$

10. Packed-cell volume.

A sample of culture (10 ml) was centrifuged in a graduated glass centrifuge tube at 1000 x g. for 5 minutes. The volume of the cell pellet was expressed as a percentage of the sample volume.

11. Mitotic Indices.

One volume of culture (ca. 5 ml) was added to two volumes of fixative (ethanol : acetic acid, 3:1) in a 25 ml polythene centrifuge tube and incubated at 25°C for 8-12 hrs. The tubes were centrifuged at 1000 x g for 5 minutes and the fixative decanted. The pellet was then spread over a slide and covered with lacto-propionic-orcein (1% w/v orcein in lactic/propionic acid, 1:1). The slide was warmed gently and left for 60 secs. The cell mass was then squashed under a cover-slip using thumb pressure only. The cover-slip was removed and the cells adhering both to the coverslip and slide were washed with absolute ethanol (ca. 1 ml). Both cell squashes were mounted directly in Euparol.

The slides were scanned under oil (x 1250) and 1,000 randomly-selected nuclei examined. The number of mitotic figures noted was expressed as a percentage of the total nuclei scored. The high density of starch grains present in the cells obscured many nuclei. It is likely, therefore, that many of the less distinct phases of mitosis (e.g. early prophase) were underscored. Towards the end of this study it was found that fixation in 65% formic acid solubilised the starch (Bayliss - unpublished method), but the technique made no significant contribution to the results presented here.

12. Estimation of total cell protein.

The cells from a sample of culture (usually the pellet produced

after centrifugation for packed-cell volume determinations) were collected by filtration through glass-fibre filter discs (Whatman GF/C, 2·5 cm diam.). After extraction of free amino acids and phenolics with hot ethanol and hydrolysis in alkaline solution, protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (via Layne, 1957), using bovine serum albumen (BSA) as standard. The BSA standard was made up in 1·0M NaOH and incubated at 100°C for 30 minutes. The reagent blank and all dilutions were prepared with 1·0M NaOH.

Method.

a. Collect cells onto GF/C disc. ca. 50 mg d.wt.

Wash with distilled water. 3 x 5 ml

Extract for 10 min. in boiling 70% ethanol. 2 x 5 ml

Wash with ether. 2 x 5 ml

Dry. Stores discs in tubes at -30°C.

Filtration, washing and extractions were carried out using 2·5 cm stainless-steel Hartley funnels with intermittent agitation.

b. Incubate discs in 1·0M NaOH at 100°C 10 ml
for 30 minutes.

Mix contents of tube and filter directly into tubes using a 2·5 cm Buchner funnel.

c. Filtrate from b. or standard (up to 300 µg protein) 0·2 ml

Alkaline copper reagent (0·2M Na_2CO_3; 5×10^{-4}M $CuSO_4.5H_2O$; 1×10^{-3}M sodium tartrate). 3·0 ml

Mix and leave 10 minutes at 25°C.

Folin-Ciocalteau reagent (BDH Chemicals Ltd., diluted x 2 with water). 0·2 ml

Mix and leave 30 minutes at 25°C.

Read optical density at 750 nm against a reagent blank.

O.D. 1·0 ≡ 300 µg BSA (10 mm. cuvette). The calibration curve was not linear.

13. Estimation of Nucleic Acids.

Lipid-free, TCA-insoluble residues were prepared from culture samples by the method of Holdgate and Goodwin (1965). Nucleic acids were then extracted from the residues by a modified Schmidt-Thannhauser procedure (via Short et al., 1969).

Method

a. Collect cells onto GF/C disc by filtration.	ca. 150 mg. d.wt.
Wash with ice-cold distilled water.	3 x 5 ml
Extract for five minutes each with:	
Boiling 85% methanol	2 x 5 ml
Ice-cold 80% methanol	2 x 5 ml
" " 10% TCA	3 x 5 ml
" " 80% ethanol + sodium acetate	2 x 5 ml
" " 95% ethanol	1 x 5 ml
Chloroform/Ethanol (1:3)	2 x 5 ml
Ether/Ethanol (1:1)	2 x 5 ml
Ether	2 x 5 ml

Filtration and extractions were carried out using a stainless-steel Hartley funnel (5 ml capacity) with intermittent manual agitation. Dry. Store disc in dessicator at -30°C.

b. Incubate discs in 0·3M KOH (10 ml.) at 37°C for 16 hours. Filter.

Wash residue with 0.3M KOH.	2 x 2 ml

Combine filtrate and washings.

c. Cool filtrate from b. to 0-4°C.

Magnesium chloride (0·03M; ice-cold). 1.0 ml

Mix.

Perchloric acid (PCA) (6·0M, ice-cold). 1.5 ml

Mix.

Leave at 0-4°C for 45 minutes.

Centrifuge at 2000 x g for 15 minutes.

Decant supernatant.

d. Read optical density of supernatant from c. at 260 nm and 290 nm against distilled water.

Subtract $(OD_{260}-OD_{290})$.

Compare with the optical densities of a solution of yeast S-RNA, prepared as in b. and c. above.

$OD_{(260-290)}$ 1.0 (10 mm light path) ≡ 75 µg RNA/ml.

e. Incubate residual pellet from c. in 1.0M PCA at 70°C for 20 minutes. 2 x 2 ml

Centrifuge at 2000 x g for 15 minutes.

Combine extracts.

f. Extract from e. or calf-thymus DNA standard.† 2 ml.

Diphenylamine (0.23M in glacial acetic acid). 2 ml

Mix.

Acetaldehyde (0.036M). 0.1 ml

Mix.

Incubate at 30°C for 16 hours.

† DNA standards were made up in 1.0M PCA and incubated as in e. before use. The reagent blank and all dilutions were prepared with 1.0M PCA.

Read optical density at 590 nm and 700 nm against a reagent blank.

Subtract $(OD_{590}-OD_{700})$.

$OD_{(590-700)}$ 1.0 (10 mm light path) ≡ 230 µg DNA.

14. Estimation of Carbon.

In the investigations of the changing distribution of carbon in closed, batch culture systems (Section III) total-cell carbon, cell-wall carbon and total-medium carbon were determined by quantitative oxidation to carbon dioxide; the carbon dioxide was trapped in sodium hydroxide solution and estimated by volumetric analysis (Vogel, 1955). Glucose-carbon was determined by the glucose oxidase/peroxidase system described below (Section II.15). Carbon lost as carbon dioxide during respiration was determined by infra-red gas analysis of the outflowing air stream.

Methods. A. Quantitative oxidation.

a. Sample preparation.

(i) Total cell carbon.

Collect cells onto GF/C disc of known weight.	ca. 25 mg d.wt.
Wash with distilled water	5 x 5 ml
Dry to constant weight.	

(ii) Cell wall carbon.

Autoclave culture sample in glass tube at 121°C for 15 minutes.	ca. 50 mg d.wt.
Filter through GF/C disc of known weight.	
Wash with distilled water.	5 x 5 ml
Dry to constant weight.	

b. Oxidation.

Place dried GF/C discs (bearing residues or blank) or samples of spent medium in combustion-diffusion bottles (Baker, Feinberg and Hill, 1954 - see Fig. II.2A.) together with:

van Slyke-Folch combustion fluid (0.5M chromium trioxide, 6.0M phosphoric acid, 12.0M suphuric acid).	5 ml
Potassium iodate	0.3 g
Diffusion tube containing CO_2-free NaOH (3.0M)	1 ml

Cap bottle tightly.

Autoclave at 121°C for 30 minutes.

Cool slowly.

The average recovery of carbon from known weights of glucose or cellulose oxidised as above was 96.6%.

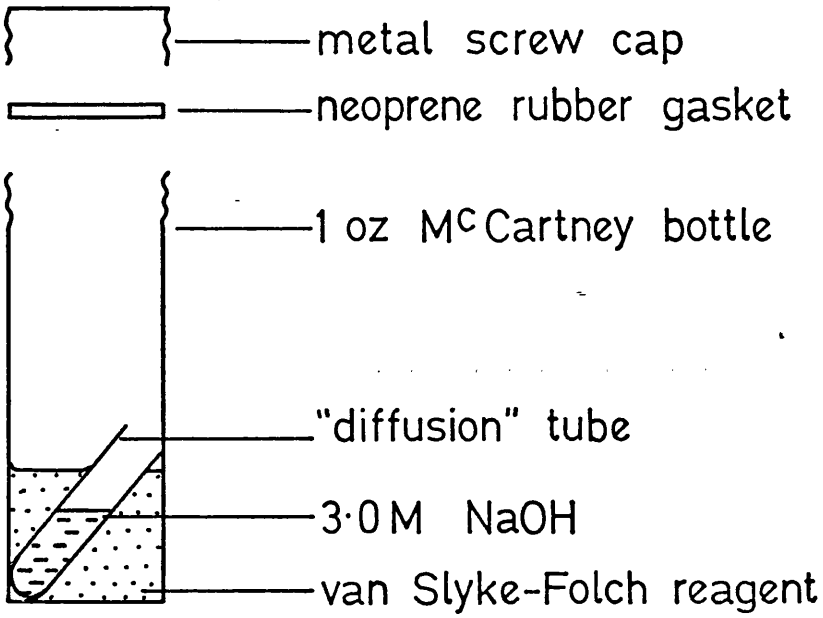
Alternative Oxidation Procedure.

The oxidation of medium samples by the above method, though attempted with cultures 49/50/51, was not satisfactory. The heat liberated when the aqueous samples were mixed with the combustion fluid possibly caused premature oxidation of the sample and loss of CO_2 before the combustion bottle could be capped. Therefore, for cultures 59/60/61, the method of Katz, Abraham and Baker (1954) was used with aqueous samples, as follows:

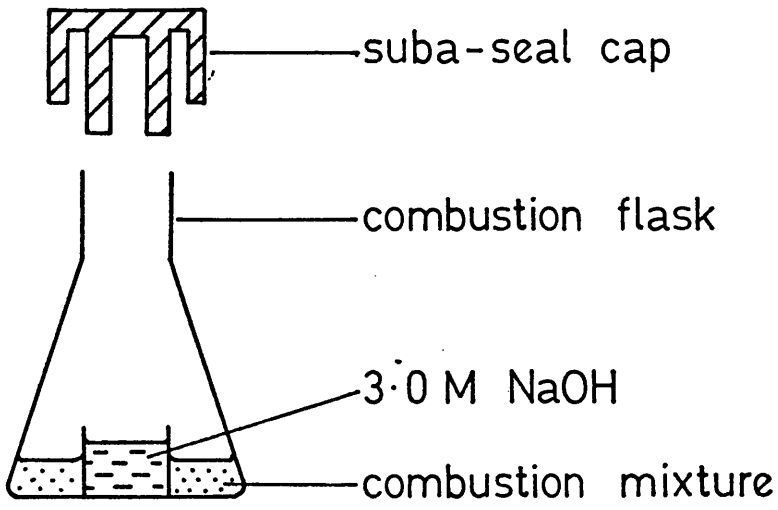
Place potassium persulphate into outer well of combustion vessel (see Fig. II.2B.)	1.5 g
together with:	
Distilled water	15 ml
Sample	ca. 1×10^{-3} g.atoms carbon
Sulphuric acid.	0.1 ml

Fig. II. 2.

A. Combustion - diffusion vessel - dry samples



B. Combustion - diffusion vessel - wet samples



Silver nitrate (0.25M) 3.0 ml

Place CO_2-free NaOH (2.0M) into centre well. 1 ml

Cap flask with Suba-Seal (Jennings, Ltd) and evacuate.

Incubate at 70°C for 2 hours.

Estimate CO_2 recovery by method (ii) below.

The average recovery of carbon from known weights of glucose or cellulose oxidised as above was 98.7%.

c. Volumetric analysis of CO_2 recovery.

The $NaOH/Na_2CO_3$ solution was transferred from the diffusion tube or centre well to a 100 ml. conical flask (final solution volume ca. 25 ml). Two different double-titration procedures were used to estimate the carbonate in the solution:

(i) Cultures 49/50/51

Cool solution to 0-4°C.

Titrate against acid (0.1 MHCl) with phenolphthalein as indicator.

Note burette reading at end-point (pink → colourless).

Continue titration against acid with methyl-orange as indicator.

Note burette reading at end-point (orange → red).

Titration No. 1.

$$NaOH + HCl = NaCl + H_2O$$

$$Na_2CO_3 + HCl = NaCl + NaHCO_3$$

Titration No. 2.

$$NaHCO_3 + HCl = NaCl + CO_2 + H_2O.$$

From titration No. 2 :

Carbon present (g. atoms) = Molarity of acid x V x 10^{-3}, where V = volume of acid required.

The end point of titration No. 2 was not easily recognised, and an alternative procedure was adopted in another experiment.

(ii) Cultures 59/60/61

Heat solution from diffusion tube or centre well to $70^{o}C$.

Add barium chloride (0.05M) slowly until no further precipitate (barium carbonate) was formed.

Cool solution to room temperature.

Titrate against acid (0.1MHCl) with phenolphthalein as indicator.

Note burette reading at end point (pink → colourless).

Continue titration against acid with bromocresol green as indicator.

Note burette reading at end-point (green → yellow).

$$BaCl_2 + Na_2CO_3 = 2NaCl + BaCO_3$$

Titration No. 1.

$$NaOH + HCl = NaCl + H_2O$$

Titration No. 2.

$$BaCO_3 + 2HCl = BaCl_2 + CO_2 + H_2O.$$

From titration No. 2 :

Carbon present (g. atoms) = Molarity of acid $\times \frac{V}{2} \times 10^{-3}$

where V = volume of acid required.

B. Infra-Red Gas Analysis.

Carbon dioxide in the air exhaust of 4-litre batch cultures was estimated using an infra-red gas analyser (Grubb-Parsons Ltd.). The culture exhaust was dried prior to analysis by passing it through 4 silica-gel towers (2 cm x 40 cm). The analyser was zeroed using dry, CO_2-free air, which had passed through 4 silica-gel and 4 soda-lime towers, and it was calibrated using a standard CO_2/air mixture (British Oxygen Company Ltd.).

15. Nutrient analysis.

The supernatant spent-medium produced during the centrifugation of culture samples for packed-cell volume determinations was decanted, filtered through Whatman No. 1 paper and stored at -30°C. Major nutrients were determined colorimetrically in these samples and in samples of input media for continuous cultures as follows:

1. Glucose.

Glucose was assayed by a coupled enzyme system based on the following reactions :

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{GLUCOSE OXIDASE}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{PEROXIDASE}} \text{oxidised chromogen.}$$

The reagents were obtained as a set ("Glucostat" - Worthington Biochemical Comp.) consisting of the lyophilized enzymes and a solid chromogen, O-dianisidine, and were prepared in 0.04M phosphate-buffered glycerol (pH 7.0) according to Washko and Rice (1961). Glucose standards were prepared in a saturated benzoic acid solution. It was essential to deproteinise the medium samples before assay to eliminate interference by peroxidases.

Method.

a. 10 ml. glass centrifuge tube.

Distilled water	3 ml.
Sample or standard (up to 15 µmoles glucose)	0.1 ml
Zinc sulphate (o.15M)	0.5 ml
Barium hydroxide (0.15M)	o.5 ml

Mix.

Centrifuge 1000 x g for 5 minutes.

b. 15 ml wide-bore tube.

Supernatant from a.	0.1 ml
Glucostat reagent	1.0 ml

Mix.

Incubate at $37^{o}C$ for 30 minutes.

Sulphuric acid (6.0M)	2.5 ml

Mix.

Read optical density at 540 nm against a reagent blank.

O.D. 1.0 (10 mm light path) $\equiv$ 0.4 µmoles glucose.

2. Nitrate.

Nitrate was quantitatively reduced to nitrite by reduction with zinc in alkaline solution (Bussey, Young and Sims - unpublished method). The nitrite was then estimated by the diazotisation of sulphanilic acid and the coupling of the resulting compound to N-(1-naphthyl)-ethylene-diamine (NED) to form a stable red azo dye (Montgomery and Dymock, 1961). No nitrite was detectable in unreduced samples of spent medium.

Method.

a. Sample or standard (up to 0.25 µmoles NO_3^-)	1 ml
Ammonium hydroxide (2.2M)	2 ml
Zinc powder	ca. 2 mg

Shake vigorously for 2 minutes.

Filter through Whatman GF/C rapidly.

b. Filtrate from a.	1 ml
Sulphanilic acid (0.115M)	1 ml

Mix.

NED dihydrochloride (2.0×10^{-3}M)	1 ml

Mix.

Leave 15 minutes.

Read optical density at 540 nm against distilled water. Subtract reagent blank value.

Distilled water should be free from traces of nitrite.

$O.D._{540}$ 1.0 (10 mm light path) ≡ 0.083 µmoles NO_3^-.

3. Phosphate.

The medium filtrate was treated with an acid-molybdate reagent and the molybdophosphoric acid formed reduced to molybdenum blue with P-methyl-amino-phenol (metol) (Gomorri, 1942).

Method.

Sample or standard (up to 1.0 µmoles PO_4^{2-})	1 ml
Acid-molybdate (0.02M ammonium molybdate; 2.5M sulphuric acid)	1 ml
Mix.	
Metol reagent (0.1M metol; 0.15M sodium bisulphite)	1 ml
Mix.	

Leave 30 minutes at room temperature.

Centrifuge at 1000 x g for 10 minutes.

Read optical density at 660 nm against a reagent blank.

O.D. 1.0 (10 mm light path) ≡ 0.85 µmoles PO_4^{2-}.

4. Urea.

Urea was hydrolysed enzymically and the ammonium ions produced were reacted with phenol and hypochlorite to form a blue dye (Fawcett and Scott, 1960). No significant concentration of ammonium ions was detectable in non-hydrolysed spent medium samples.

Method.

Sample or standard (up to 0.4 µmoles urea)	0.5 ml
Urease (0.1% w/v)	0.1 ml
Incubate at 37°C for 15 minutes	
Phenol reagent (0.1M phenol; 0.17×10^{-3}M sodium nitro prusside)	5.0 ml
Mix.	
Hypochlorite (o.011M sodium hypochlorite, 0.125M sodium hydroxide)	2.0 ml
Mix.	

Incubate at 50°C for 10 minutes, then at 25°C for 30 minutes.

Read optical density at 570 nm against a reagent blank.

O.D. 1.0 (10 mm light path) ≡ 0.32 µmoles urea.

5. Sucrose.

Sucrose was hydrolysed enzymically and the reducing sugars produced reacted with a copper sulphate reagent (Somogyi, 1952). The reduced copper produced was then estimated by reaction with arseno-molybdate to form molybdenum blue.

Method.

a. Sample or standard (up to 2.5 µmoles sucrose)	2 ml
Acetate buffer (0.1M; pH 4.8)	2 ml
Invertase concentrate	0.1 ml
Toluene	0.1 ml
Incubate at 37°C for 30 minutes.	
b. Solution from a.	1 ml
Copper reagent (0.25M Na_2CO_3; 0.05M sodium potassium tartrate; 0.2M $NaHCO_3$; 1.25M Na_2SO_4; 0.025M $CuSO_4.5H_2O$)	1 ml

Incubate at 100°C for 15 minutes.

Cool in ice-bath.

Arseno-molybdate reagent (0.04M ammonium molybdate; 0.03M sodium arsenate) 1 ml

Mix.

Distilled water 7 ml

Mix.

Read optical density at 525 nm against a reagent blank.

O.D. 1.0 (10 mm light path) ≡ 0.5 μmoles sucrose.

16. The rate of oxygen uptake.

The output of a Clark-type oxygen electrode (Rank Bros., Bottesham, Cambridge) was calibrated using air-saturated distilled water (O_2 saturation concentration at 20°C = 2.4×10^{-4}M). The output was brought to zero by adding sodium dithionite.

As soon as possible after a sample was removed from a culture vessel, a small volume of the suspension (ca. 5 ml.) was pipetted into the electrode chamber. The rate of oxygen consumption by the culture sample (as nmoles O_2 ml^{-1} min^{-1}) was calculated from the linear portion of a chart recording of the change in electrode output. The rate of oxygen consumption by the cells was independent of the concentration of dissolved oxygen when this concentration was above 10% of the saturation value.

The repiration rates of synchronous cultures (Section VI) were determined by conventional Warburg manometry.

17. The concentration of dissolved oxygen.

Dissolved oxygen in 4-litre continuous cultures was measured using

a steam-sterilisable oxygen probe (New Brunswick Scientific Co., Model No. M1016-0200) mounted in a side chamber of the culture vessel (see apparatus). The output of the electrode was amplified using an operational amplifier (Radiospares Ltd. Cat. No. 709) and recorded continuously on a 6-channel chopper-bar galvanometric recorder (LKB Instruments Ltd., Model 6520). The electrode was calibrated *in situ* after autoclaving, but prior to inoculation, by saturating the culture medium with air (100%) or nitrogen (0%). The dissolved oxygen concentration of the culture was then expressed as a percentage of saturation.

18. Oxygen absorption rate.

The maximum rate of oxygen transfer from the gas phase of the aeration stream to the liquid phase of the cultures, at zero oxygen tension under a given set of aeration conditions, was determined by measuring the rate at which sulphite in solution in the culture vessel was irreversibly oxidised in the presence of divalent copper (Cooper, Fernstrom and Miller, 1944) :

$$2SO_3^{2-} + O_2 \longrightarrow 2SO_4^{2-} \qquad 1$$

The procedure followed was based on that of Corman, Tsuchiya, Koepsell, Benedict, Kelley, Feger, Dworschak and Jackson (1957). Copper sulphate was added to distilled water (4 litre) at 25°C in the culture vessel to a concentration of ca. 1.5×10^{-3}M. The magnetic stirrer was started to aid the dissolution of the sulphate. Sodium sulphite was then added to a concentration of ca. 4.0×10^{-3}M. After 10 minutes (the time required for dissolution of the sulphite and removal of the oxygen already present in solution) the stirring speed was adjusted stroboscopically

to a known value and air was passed through the sparger at a known rate. At 15 minute intervals duplicate samples (5 ml) of the sulphite solution were rapidly transferred to tubes containing pellets of solid CO_2. The layer of CO_2 vapour produced above the sample prevented further sulphite oxidation. The sulphite in each sample was estimated immediately by titration against a standard iodine solution (ca. 1.0×10^{-3}M) with starch as indicator :

$$SO_3^{2-} + I_2 + H_2O \longrightarrow SO_4^{2-} + 2H^+ + 2I^- \qquad 2$$

The change in the iodine titer was plotted against time (Fig. II.3.) From equation 2 , the iodine titer is directly proportional to the concentration of sulphite. From equation 1 , the rate of change of sulphite concentration (by oxidation to sulphate) represents twice the rate at which oxygen is absorbed by the solution. Thus the oxygen absorption rate was calculated directly from the change in the iodine titer with time according to the following equation :

$$\text{Oxygen Absorption Rate}\ (\text{nmoles } O_2\ ml^{-1}min^{-1}) = \text{Slope of } I_2 \text{ titer plot} \times \frac{\text{Molarity of } I_2 \text{ solution}}{2} \times \frac{10^6}{\text{sample volume}}$$

19. Sterility Checks.

The contamination risks of continuous cultures are high. The possibility that contaminants might reach an inconspicuous equilibrium density in some steady state cultures of Acer made a routine sterility check essential. The tests applied were as follows:

A. Examination by phase-contrast microscopy (x 1250).

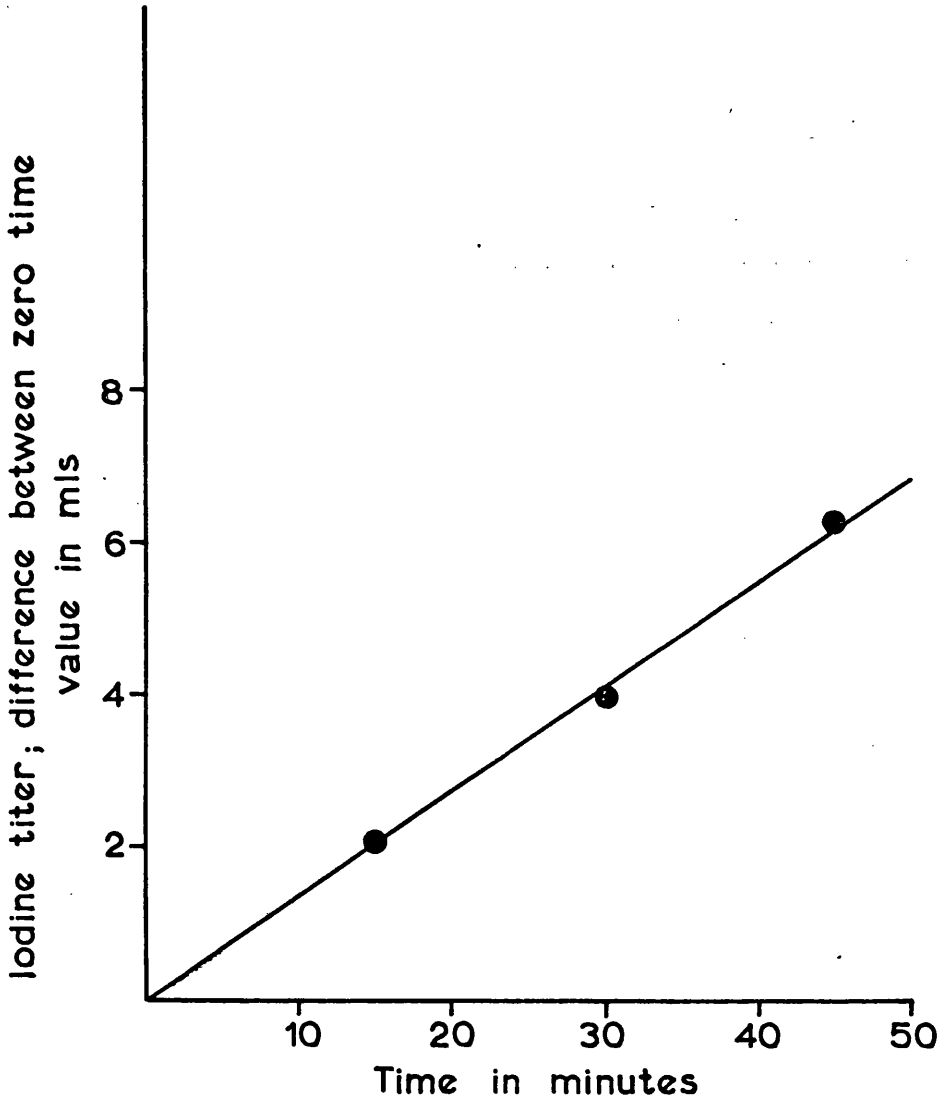
B. Gram-stain of spent medium smears.

Fig. II.3. Increase in iodine titer with time during aeration of a sulphite solution.

The difference between the volume of iodine required to oxidise residual sulphite just prior to aeration (zero time) and that required after increasing periods of aeration is plotted against aeration time.

See II. Materials and Methods.18.

Fig. II.3.


Iodine titer; difference between zero time value in mls
2
4
6
8
10
20
30
40
50
Time in minutes

C. Low-density plating in an enriched medium (see below).

Where the results of a test were inconclusive, the next test in the list was applied. The cultures were considered free of contaminating organisms when no colonies were produced in test C.

Stock cultures were routinely screened at approximately 3-week intervals by plating as follows:

Replicate samples (3) of randomly-selected stock cultures were diluted in sterile medium containing coconut milk (10% v/v), a range of amino acids, giberellic acid (Stuart and Street, 1970) and urea. Replicate volumes (3 x 1 ml) of the diluted culture samples (containing ca. 10,000 cells and 2 mg urea) were pipetted into sterile petri-dishes. Molten agar-medium (10 ml - composition as above + 0.7% Oxoid N°3 agar) was then poured into each plate. The plate was agitated by sliding from side to side to disperse the cell suspension. The plates were incubated at 25°C for about 3 weeks and examined for bacterial colonies.

20. Cell nitrate and α-amino-nitrogen.

a. Nitrate extraction.

Collect cells onto GF/C disc by filtration	ca. 6 mg.dwt
Wash with distilled water	3 x 5 ml
Transfer disc to test tube	
Cover disc with distilled water	5 ml
Incubate 30 mins at 100°C.	
Cool and filter. Store filtrate at -30°C.	

b. Nitrate estimation.

Carry out on filtrate from a. as described in 15.2 above.

c. Amino-nitrogen extraction.

Collect cells onto GF/C disc by filtration	ca. 6 mg.d.wt.
Wash with distilled water	3 x 5 ml
Transfer disc to test tube	
Cover disc with 60% ethanol	5 ml
Incubate 15 mins. at 50°C.	
Cool and filter. Store filtrate at -30°C	

d. Amino-nitrogen estimation.

Amino-nitrogen was estimated by a modification of the ninhydrin method of Yemm and Cocking (1955) using leucine as a standard.

Method

Sample or standard (up to 0.21 µmoles NH_2)	1.0 ml
Citrate buffer (0.2M, pH 4.75)	0.5 ml
Ninhydrin reagent (1.9×10^{-3}M ascorbic acid and 5.2×10^{-2}M ninhydrin in 2-methoxyethanol)	1.2 ml
Incubate 20 minutes at 100°C.	
Cool in iced water 5 minutes.	
60% ethanol	3.0 ml
Mix.	
Leave to stand 15 minutes at 20°C.	
Read optical density at 570 nm against water.	

O.D. 1.0 (10 cm light path) ≡ 0.2 µmoles NH_2.

21. Autoradiography.

Autoradiographs were prepared of cells previously pulse-labelled with ^{3}H-thymidine by using the techniques described by Rogers (1967).

a. Pulse-label

Cell suspensions (20 ml) were shaken at 25°C on a horizontal, rotary shaker at 120 rpm. ^{3}H-thymidine was added to a final activity of 1 µCi ml^{-1} (15 Ci per mMole thymidine).

b. Squash preparation.

After 20 minutes incubation, portions (ca. 5 ml) of the suspension were fixed, stained and spread over microscope slides as described in 11. Mitotic indices above. After squashing, the slides were placed on blocks of solid CO_2 until the cell smear was completely frozen. The cover-slips were flipped off with a razor-blade and the slides transferred rapidly (but gently!) to distilled water at 20°C.

c. Film application.

Rectangular portions of radiograph film (A.R.10, Kodak Ltd) were floated onto the surface of distilled water at 20°C with the emulsion downwards. Microscope slides bearing cell squashes uppermost were dipped into the distilled water below a portion of film and then slowly lifted out of the water so that the film spread itself over the cell squash and onto the slide. The slides were left overnight to dry. The film was then securely attached to the slide by painting around the edges with nail varnish. All manipulations were carried out using a safe-light (Ilford N°.1).

d. Exposure.

The autoradiographs were exposed for 7-10 days at 4°C in a light-tight box containing silica-gel.

e. Development.

Developer (Kodak D19)	8 mins.
Distilled water	1 min.
Fixative (sodium thiosulphate, 30% w/v)	10 mins.
Running tap-water	15 mins.

(all at 18°C)

The preparations were dehydrated through a series of alcohol dilutions to absolute alcohol and mounted in Euparql.

The film above 1000 nuclei was examined for silver grains in excess of the back-ground using oil-immersion.

III AERATION OF CULTURES

1. Introduction.

In much of the work to date involving plant cell suspension cultures, it has generally been assumed that oxygen availability has limited neither growth rate nor yield. Nickell and Tulecke (1960) concluded that the enhanced growth rate of Rosa sp. cells in a carboy vessel supplied with air through a steel tube (both for aeration and agitation), as compared with cultures shaken in Erlenmeyer flasks was a function of enhanced aeration in the carboy vessel. However, no measurement was made of the rate of oxygen supply or consumption. Henshaw et al. (1966) demonstrated that yield in shaken cell cultures of Parthenocissus tricuspidata. Planch. was related to total nitrogen supplied and concluded that the eventual decline in growth rate in these batch cultures was not due to limiting oxygen availability. However in such cultures the onset of linear growth (after the exponential growth phase) might well have been caused by the O_2-demand of the culture reaching the maximum O_2-supply rate set by the aeration conditions. Growth, under these conditions, would have continued at a constant rate (determined by the O_2-supply rate) until an essential nutrient (e.g. nitrate) was depleted. The latter point might well depend upon total nitrate availability. Short, Brown and Street (1969) found that maximum tissue production in 4.5-litre batch cultures spun at 45^{o} in 10-litre bottles depended upon the type of closure employed (normally a cotton-wool bung) and assumed an aeration effect. Recent measurements (Hall, personal communication) indicate that the maximum oxygen availability in such a vessel is 25% of that in a standard shaken culture (70 ml medium in a 250 ml flask shaken at 120 r.p.m.).

Data of Rajasekhar, Edwards, Wilson and Street (1971), from a more definitive study, suggested that oxygen availability was not a factor limiting growth of Acer pseudoplatanus L. cells even at low shaking speeds.

To ensure that oxygen concentration did not become the factor limiting growth in chemostat cultures of Acer cells, factors affecting the rate of oxygen supply to cells in 4-litre batch cultures were first investigated.

2. Theory of aeration.

According to gas $\rightleftharpoons$ liquid absorption theory (Ricica, 1966) the rate of oxygen absorption from a gas of constant O_2- partial-pressure into a liquid at a constant temperature is given by the equation

$$\frac{dc}{dt} = K_L a\ (C_e - C) \quad \text{moles } O_2.l^{-1}.s^{-1} \qquad 1.$$

where C is the concentration of dissolved oxygen at time, t; C_e is the saturation concentration of dissolved oxygen; K_L is a constant related to gas $\rightleftharpoons$ liquid transfer and 'a' is the interfacial gas-liquid contact area. In a culture vessel in which values for the following parameters are selected and fixed,

liquid volume and composition

temperature

agitation speed

pO_2 in air supplied

air flow rate

size of sparger orifice(s),

the term, $K_L a$, is a constant. Thus

$$\frac{dc}{dt} \alpha\ C_e - C \qquad 2.$$

When $C = C_e$ (which is also a constant), the oxygen absorption rate will be zero; when C = 0, the absorption rate is maximal. Further, rearranging equation 1 into the equation of a straight line

$$\frac{dc}{dt} = K_L a.C_e - K_L a.C \qquad 3.$$

the oxygen absorption rate is seen to be inversely related to the dissolved oxygen concentration by a line of slope, $K_L a$. The intercept, $K_L a.C_e$ - the oxygen absorption coefficient, OAC, is numerically equal to the maximum oxygen absorption rate (see Fig.III.1. and Fig. III.3.).

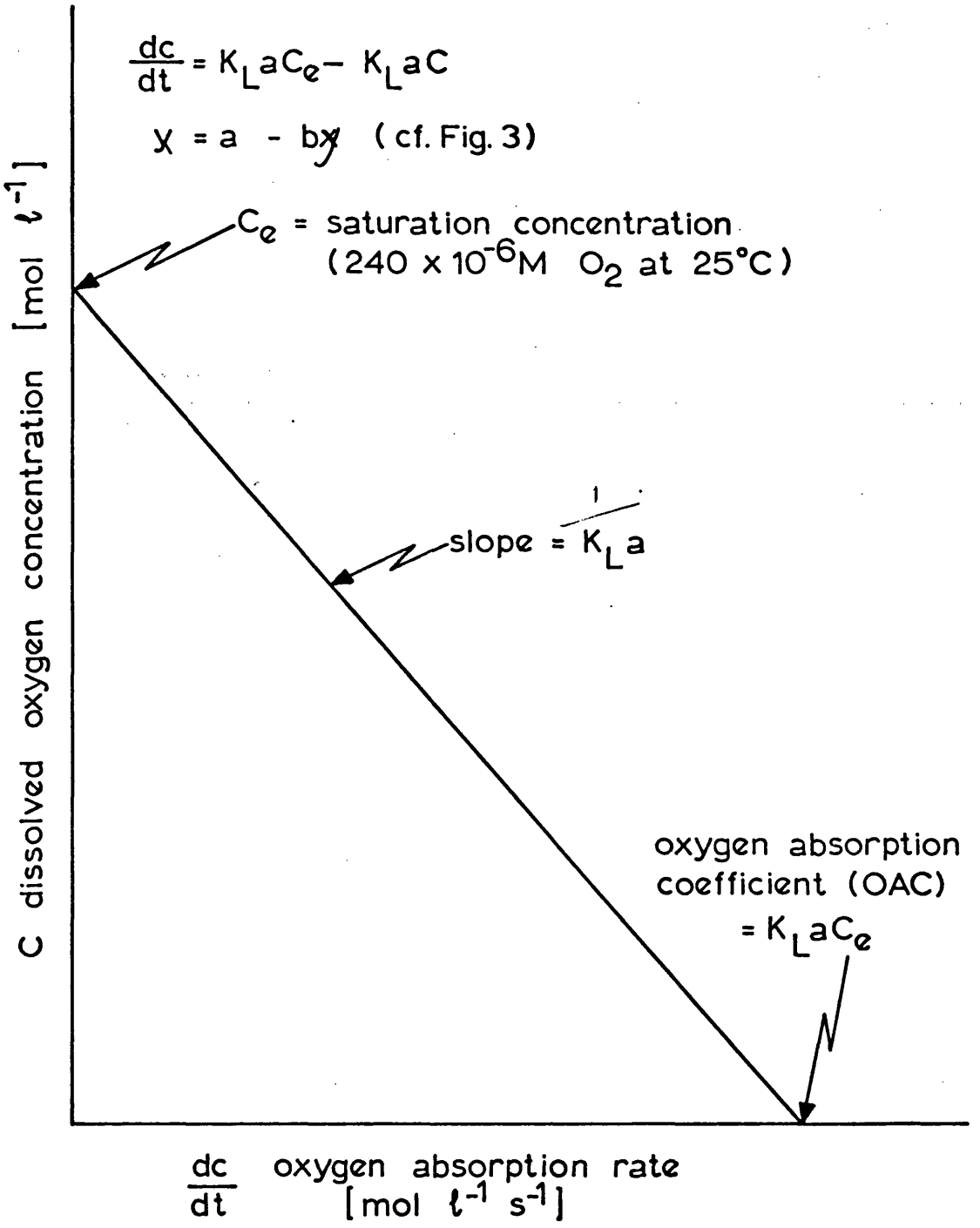
3. Determination of Oxygen Absorption Coefficients.

No reliable steam-sterilizable oxygen electrode was available in the early part of this project. Thus oxygen-limited growth could not be avoided simply by monitoring the dissolved oxygen concentration of the culture and ensuring that the value never approached zero. Neither was it possible to directly determine the critical oxygen tension for the growth of Acer cells. Instead, oxygen limitation was assumed not to occur if the oxygen uptake rate of the culture remained below the OAC for the aeration conditions employed. Whenever possible the aeration conditions were adjusted so that the oxygen uptake rate of a culture was less than 75% of the OAC.

The maximum oxygen absorption rate for a culture under particular sets of aeration conditions (designated OAC in this thesis) in the standard batch culture apparatus (Appendix 1) was measured by the sulphite oxidation technique (see II. Materials and Methods). The '$K_L a$' factor was varied by combinations of stirring speed (260 r.p.m. and 520 r.p.m.), air-flow rate (500 or 1000 ml min^{-1}) and bubble size

Fig. III.1. Theoretical relationship between the rate of absorption (solution) of oxygen into a liquid and the concentration of oxygen dissolved in the liquid.

Fig. III. 1.


$\frac{dc}{dt} = K_L a C_e - K_L a C$
$Y = a - bx$ (cf. Fig. 3)
C_e = saturation concentration
(240 x 10^{-6}M O_2 at 25°C)
slope = $\frac{1}{K_L a}$
oxygen absorption coefficient (OAC) = $K_L a C_e$
C dissolved oxygen concentration [mol l^{-1}]
$\frac{dc}{dt}$ oxygen absorption rate [mol l^{-1} s^{-1}]

(1 mm 1.D. pipe-sparger or Pyrex sinters of different porosity - G_0 is the coarsest). The results presented in Fig.III.2. suggest that:

1. at fixed high or low air-flow rates, higher and more efficient absorption rates (oxygen absorbed as % oxygen supplied) can be obtained by using a finer sinter than a faster stirring speed.

2. at fixed high or low stirring speeds, higher absorption rates can be obtained by increasing the air-flow rate, but similar rates can be obtained more efficiently by using a finer sinter at low air-flow rates.

3. using a fixed sinter grade, higher absorption rates can be obtained by increasing the air-flow rate but less efficiently than by increasing the stirring speed.

4. it is possible to supply oxygen to a 4-litre static culture at a rate similar to the rate of supply in a standard, shaken flask culture. The aeration/agitation required to do this did not produce excessive foaming at the culture surface.

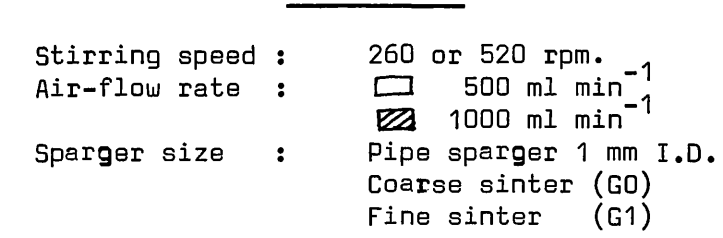
4. Validity of OAC determinations by sulphite oxidation.

The sulphite oxidation technique has been widely used by microbiologists to estimate OAC in large fermentors. The concentration of dissolved oxygen in the sulphite solution is essentially zero and, therefore

$$\frac{dc}{dt} = K_L a \, C_e \qquad 4.$$

Furthermore, the apparent rate of oxygen solution is independent of sulphite or catalyst concentration over wide ranges. However, the rate of sulphite oxidation in standard shake-flask cultures was altered by pH changes (Table III.1.) and the rate of sulphite oxidation

Fig. III.2. Maximum rates of oxygen absorption (oxygen absorption coefficients) into a sulphite solution in a 4-litre batch culture vessel with various combinations of stirring speed, air-flow rates and bubble-size.



Aeration efficiencies (O_2 absorbed as % O_2 supplied) are given at the top of each column.

Fig. III. 2.

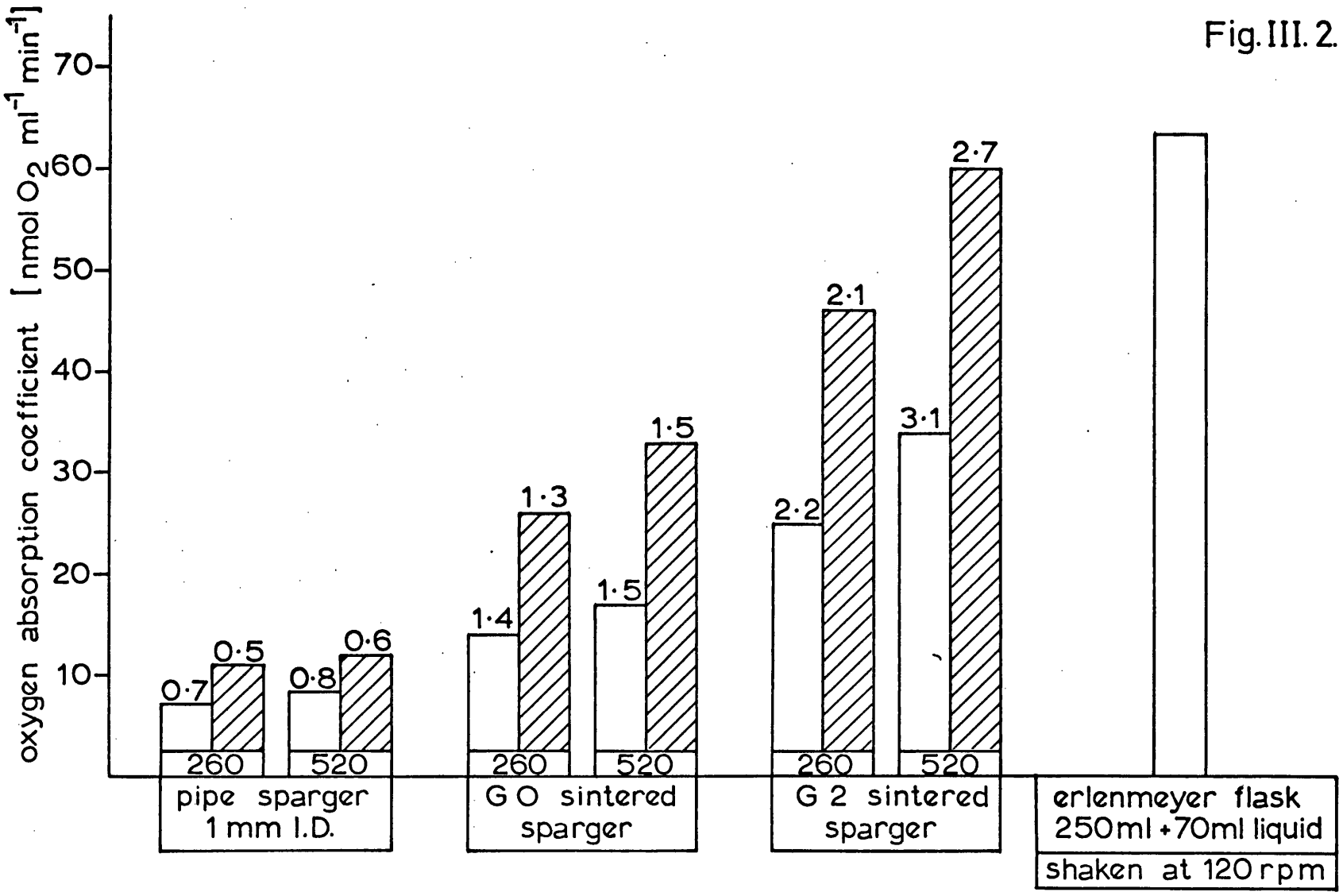

oxygen absorption coefficient [nmol O_2 ml^{-1} min^{-1}]
70
60
50
40
30
20
10
0·7
0·5
0·8
0·6
1·4
1·3
1·5
1·5
2·2
2·1
3·1
2·7
260
520
260
520
260
520
pipe sparger
1 mm I.D.
G O sintered
sparger
G 2 sintered
sparger
erlenmeyer flask
250ml + 70ml liquid
shaken at 120 rpm

Table III.1. Effect of pH on oxygen absorption coefficient determinations by sulphite oxidation.

pH	OAC nmoles O_2 ml^{-1} min^{-1}
3.0	76.2
6.0	63.3
8.0	137.5

70 ml of sulphite solution (c. 8x 10^{-3}M sulphite; 1 x 10^{-3}M $CuSO_4$) was shaken at 120 rpm in a 250 ml flask at 25°C. The pH of the solution (initially pH 6.0) was adjusted as shown.

Six 20millilitre samples from each treatment were titrated against a standard iodine solution (9.7 x 10^{-4}M) at intervals (see 11. Materials and Methods.18).

may also be related to the nature of the catalyst used (Phillips and Johnson, 1956). In some conditions, therefore, the oxidation of sulphite may be rate-limiting and not the rate of oxygen solution. In addition, although the technique may allow comparison of the effectiveness of different aeration conditions, the actual rate of oxygen solution into sulphite is not necessarily equal to the rate into a different solution e.g. a culture medium. The ratio OAC (culture): OAC (sulphite) is close to unity for yeast cultures (Maxon and Johnson, 1953) and 0.5 to 0.7 for Aerobacter cultures (Pirt and Callow, 1958). The validity of the OAC values noted above for Acer cultures (Fig.III.2.) was examined in two ways:

a. Relationship between dissolved oxygen concentration and oxygen uptake rate.

Theoretically, the oxygen demand of cells inoculated into medium saturated with oxygen ($C = C_e$, $dc/dt = 0$) will be met initially from the saturated pool of dissolved oxygen. As the pool declines, $C < C_e$ and oxygen will go into solution from the aeration stream i.e. $dC/dt \neq 0$. The greater the demand, the greater the decline of the pool and thus the greater the solution rate. If the oxygen demand becomes stable, the dissolved oxygen concentration (the pool) will remain unchanged and the oxygen demand will be equivalent to the oxygen solution rate.

Combined data for dissolved oxygen concentration and oxygen uptake rate (both measured polarographically - see II. Materials and Methods) from two nitrate-limited chemostat cultures of Acer cells under the same aeration conditions are shown in Fig. III.3. (cf. Fig. III.1.). During the initial 'growing-up' phase and during a number of steady states, the dissolved oxygen concentration was inversely proportional to the

Fig. III.3. The relationship between dissolved oxygen concentration and oxygen uptake rate during a number of steady states in nitrate-limited chemostat cultures of Acer cells.

Aeration conditions

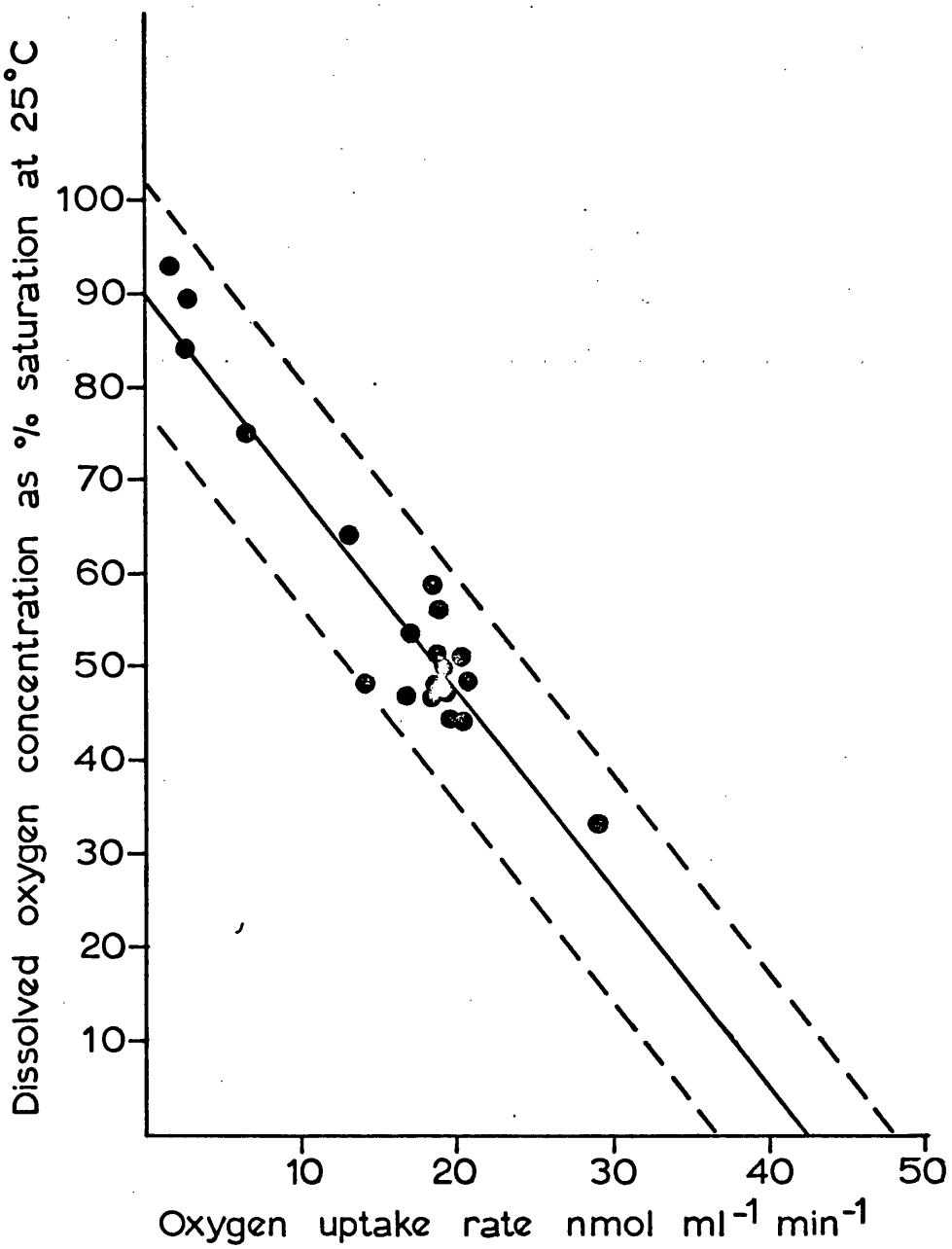
Stirring speed = 520 rpm
Air-flow rate = 500 ml min^{-1}
Sparger size = G2

——— Regression line

----- 95% confidence limits.

(cf. Fig. III.1)

Fig. III. 3.


Dissolved oxygen concentration as % saturation at 25°C
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
Oxygen uptake rate nmol ml-1 min-1

oxygen uptake rate. The extrapolated best-fit line through the data gives a value of 42.2 nmoles O_2 ml^{-1} min^{-1} for the oxygen uptake rate when the dissolved oxygen concentration reaches zero (the maximum solution rate, i.e. the OAC). The OAC estimated by sulphite oxidation for the aeration conditions applied was 34 nmoles O_2 ml^{-1} min^{-1}. Thus:

$$\frac{\text{OAC (culture)}}{\text{OAC (sulphite)}} = 1.24 \pm 0.19$$

b. Relationship between OAC values and maximum oxygen uptake rates in batch cultures.

Six 4-litre batch suspension cultures of Acer cells were established using the standard large-scale batch culture apparatus (Appendix 1). The initial cell density in each culture was between 2.6 and 3.3 x 10^5 cells ml^{-1}. Aeration conditions were chosen (Table III.2.) corresponding to three widely different OAC values previously determined by sulphite oxidation (see Fig. III.2.). The experiment was carried out in two parts on separate occasions using 0.11M or 0.22M glucose medium. The second part of the experiment (at the higher glucose concentration) was carried out (1) to test whether the decline in oxygen uptake rate and growth noted in the two higher OAC cultures (49 and 50) in part 1 (see Fig. III.4. - culture 49) was related to depletion of the carbon source, and (2) to investigate the effect of a major change in medium composition on the OAC. In both parts, measurements were made (at approximately two-day intervals) of cell number, cell dry weight, cell carbon (both cell wall and cytoplasmic fractions), glucose and non-glucose carbon in the medium, oxygen uptake rate and the rate of loss of glucose carbon as CO_2.

Table III.2. Aeration conditions and glucose concentrations employed in oxygen-limitation experiments.

Culture	Sinter porosity	Stirring speed	Air-flow rate	OAC	Glucose concentration
		rpm	ml min^{-1}	nmoles O_2 $ml^{-1}min^{-1}$	M
Experiment 1					
49	G2	520	500	34.0	0.11
50	G2	260	500	25.0	0.11
51	PS	260	500	7.2	0.11
Experiment 2					
59	G2	520	500	34.0	0.22
60	PS	260	500	7.2	0.22
61	G2	260	500	25.0	0.22

Results.

1. In all six cultures, cell number (Fig.III.4.), dry weight (Fig.III.6.), cell carbon and oxygen uptake rate (Fig.III.4.) increased exponentially, at least initially. (Only data for the highest and lowest OAC cultures are illustrated. In general, the four cultures (49,50,59 and 61) with OAC values of 34 or 25 all behaved similarly).

2. At both glucose concentrations, in cultures set to low OAC values (cultures 51 and 60, Fig.III.4.) the oxygen uptake rate of the cells reached a value close to the OAC after 6 days and remained constant thereafter. The ratios of maximum oxygen uptake rate to OAC were 0.93 (culture 51) and 1.05 (culture 60). The rate of CO_2 output were similarly affected (Fig.III.5, lowest curves). (Respiratory quotients of 1.28 (51) and 1.22 (60) calculated from these data during oxygen limited growth may reflect the use of very different detection methods for O_2 and CO_2 and not the use of respiratory substrates other than glucose. Reliable estimations of the CO_2 content of culture air were particularly difficult to obtain).

3. In the high OAC cultures (49 and 59. Fig.III.4.), the oxygen uptake rates continued to increase after day 6 and did not approach so closely the OAC values. The discontinuity of the oxygen uptake rate curve for these two cultures at day 11 is probably related to the depletion of a nutrient. In cultures 50 and 61, at intermediate OAC values (OAC = 1.39 on log. scale in Fig.III.4.), a similar inflection was noted. The nutrient depleted is probably not glucose as ca. 40% of the glucose supplied to cultures 59 and 61 was still present on day 11. (Data from chemostat cultures - Section V - suggest that NO_3^- is the nutrient limiting growth in the standard medium).

Fig. III.4. Increase in cell number and changes of oxygen uptake rate in 4-litre batch suspension-cultures of Acer cells under different aeration conditions and at different (initial) glucose concentrations.

Aeration conditions are given in Table III.2.
Oxygen absorption coefficients (OAC) for each culture were :

Culture	OAC (nmoles o_2 ml^{-1} min^{-1})
49	34.0
51	7.2
59	34.0
60	7.2

OAC values are indicated by dashed line on figures - scale as for oxygen uptake rate.

Predicted growth curves were plotted from daily growth increments given in Table III.4 (see Section III.5a).

● CELL NUMBER

O OXYGEN UPTAKE RATE

Fig. III.4.

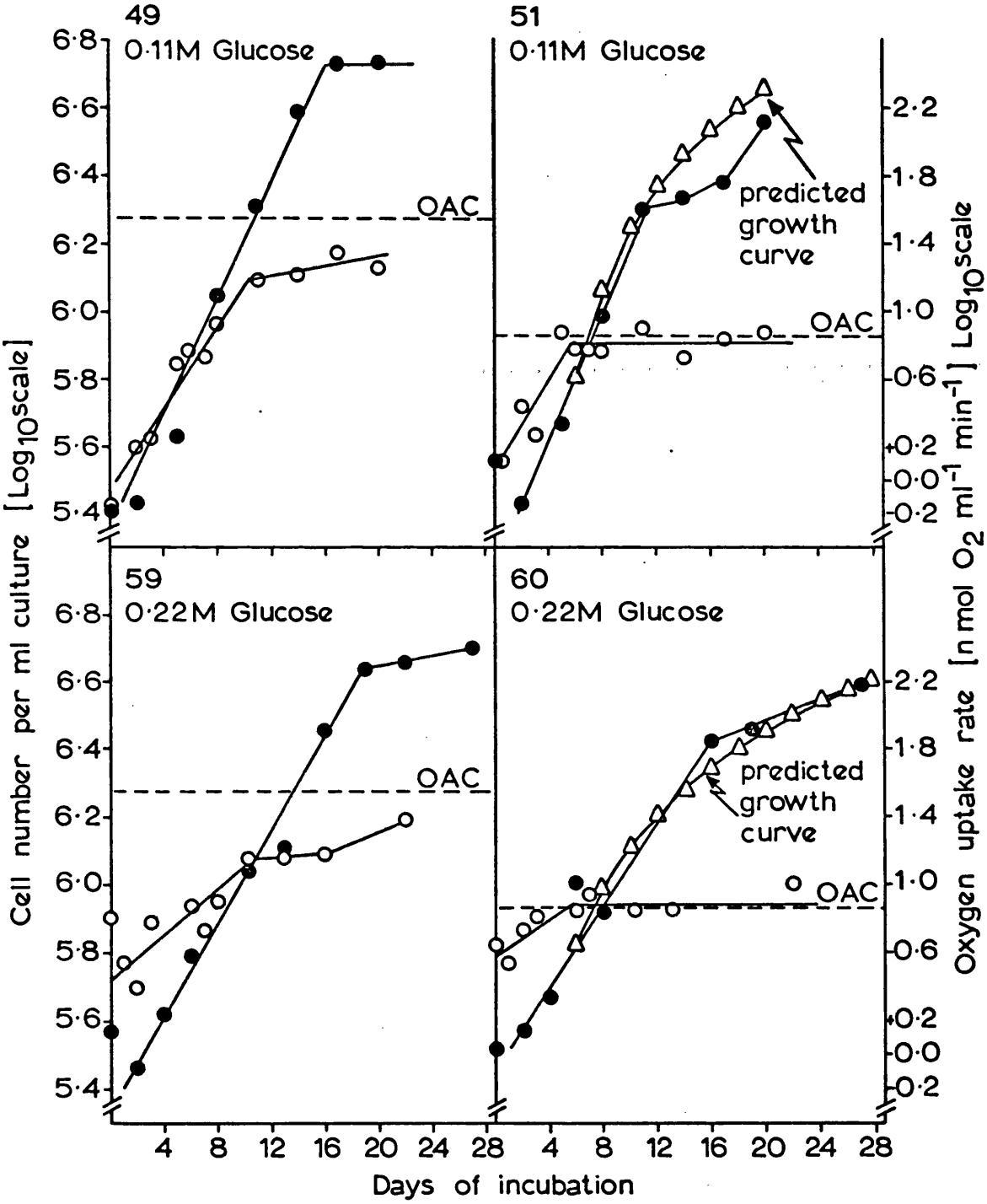

49
0·11M Glucose
51
0·11M Glucose
59
0·22M Glucose
60
0·22M Glucose
OAC
predicted growth curve
6·8
6·6
6·4
6·2
6·0
5·8
5·6
5·4
2·2
1·8
1·4
1·0
0·6
+0·2
0·0
-0·2
4 8 12 16 20 24 28
Days of incubation
Cell number per ml culture [Log10scale]
Oxygen uptake rate [n mol O2 ml-1 min-1] Log10scale

Fig. III.5. The effect of the oxygen absorption coefficient on the rate of loss of carbon as CO_2 from 4-litre batch suspension-cultures of Acer at different (initial) glucose concentrations.

The aeration conditions and oxygen absorption coefficients are given in Table III.2.

The rate of loss of carbon was calculated from the concentration of CO_2 in the air-exhaust of the cultures, as measured by infra-red analysis (see II. Materials and Methods. 14B).

Erratic data suggesting a very excessive recovery of carbon supplied as glucose have been omitted from the data for culture 49 (see also Table III.5 and Appendix 3).

Fig. III.5.

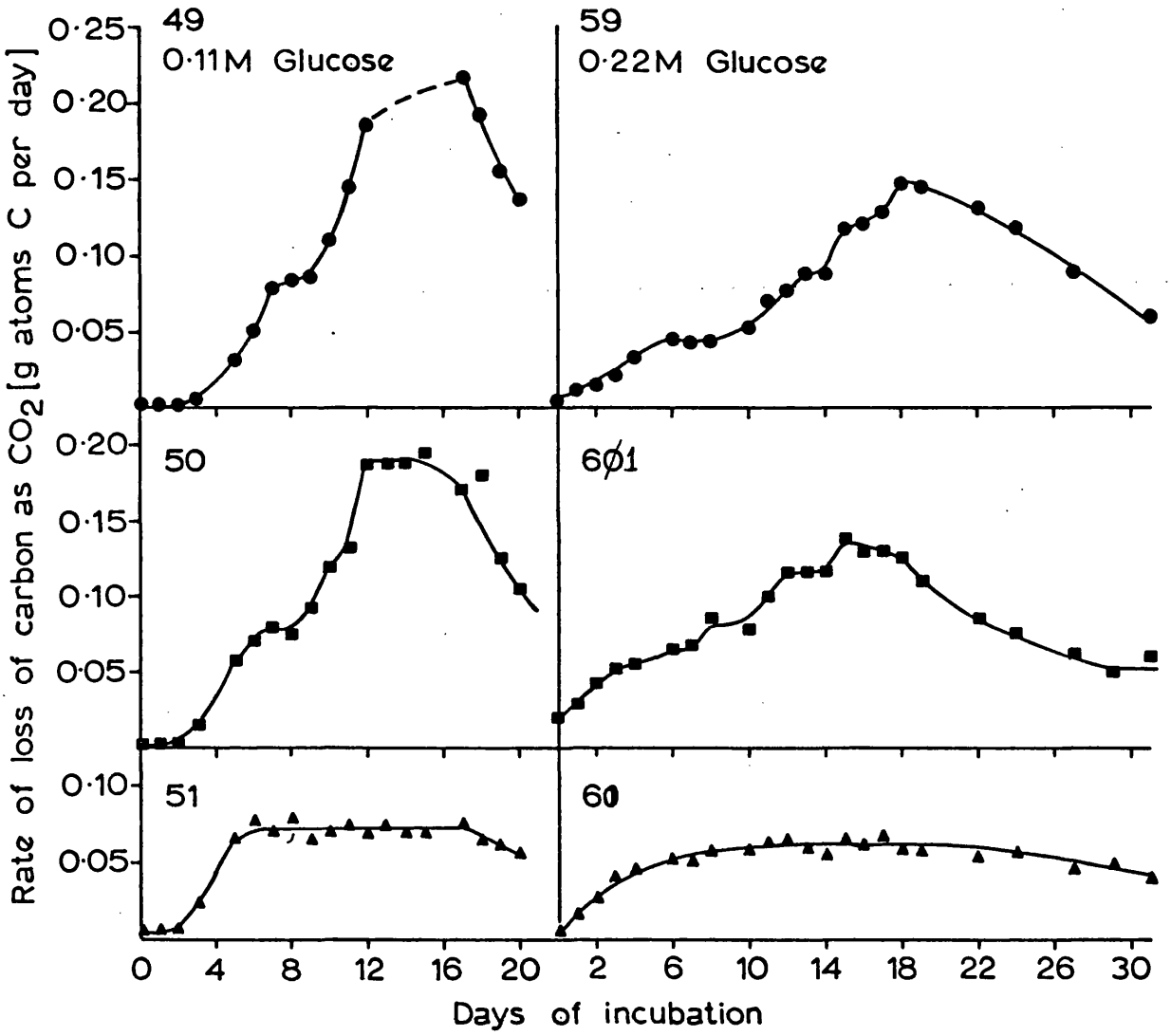
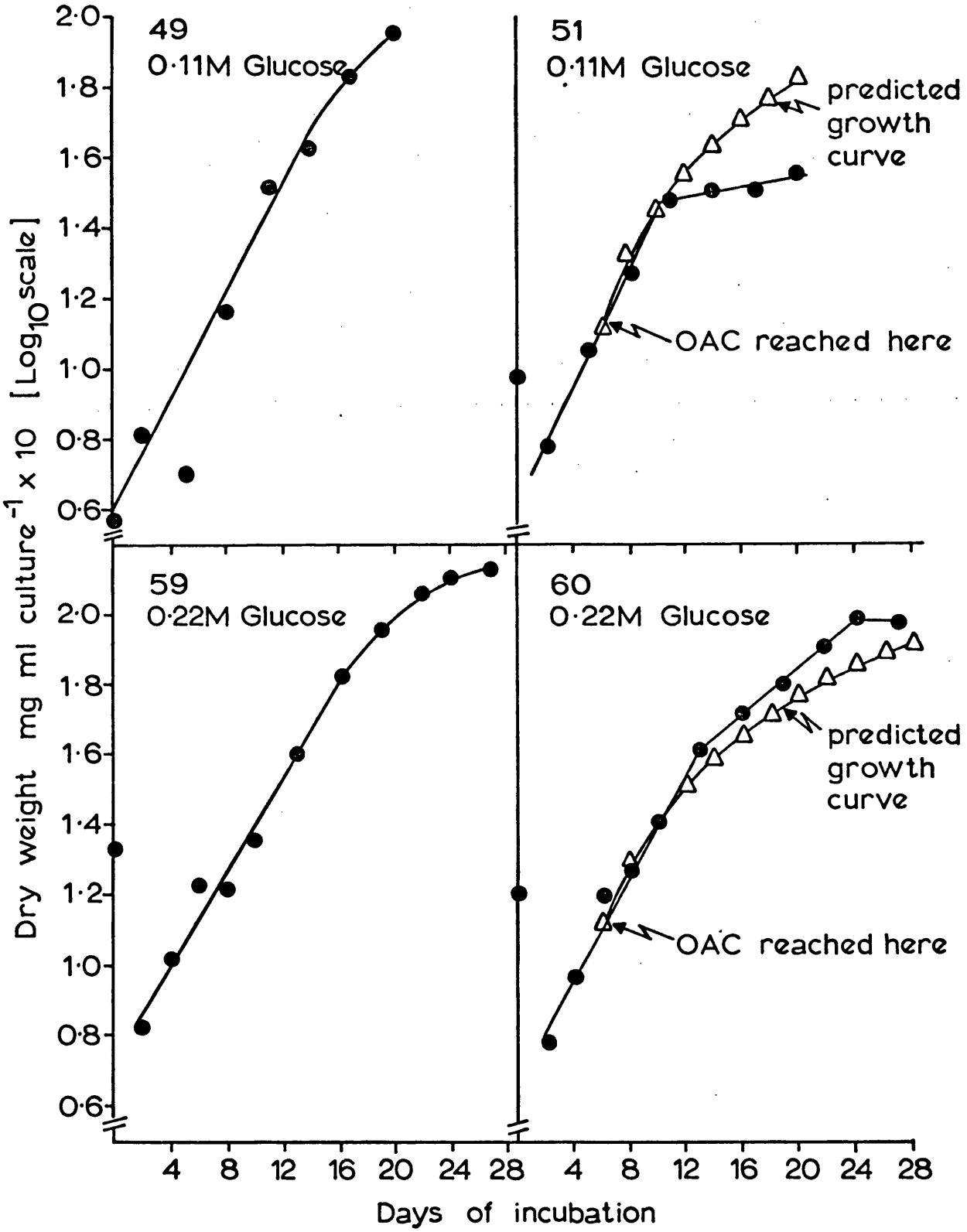

49
0·11M Glucose
59
0·22M Glucose
50
6ø1
51
60
0·25
0·20
0·15
0·10
0·05
0·20
0·15
0·10
0·05
0·10
0·05
Rate of loss of carbon as CO_2 [g atoms C per day]
0 4 8 12 16 20
2 6 10 14 18 22 26 30
Days of incubation

Fig. III.6. Increase in dry weight and changes of oxygen uptake rate in 4-litre batch suspension-cultures of *Acer* cells under different aeration conditions and at different (initial) glucose concentrations.

(See also Table III.2 and the legend to Fig. III.4)

Fig. III.6.


49
0·11M Glucose
51
0·11M Glucose
predicted growth curve
OAC reached here
59
0·22M Glucose
60
0·22M Glucose
predicted growth curve
OAC reached here
Dry weight mg ml culture^{-1} x 10 [Log$_{10}$ scale]
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
4 8 12 16 20 24 28
Days of incubation

It would appear from a. and b. above that "calibration" of the aeration conditions in a culture vessel by the sulphite oxidation technique is a valid means of establishing the upper limit of oxygen availability to batch cultures and of ensuring that oxygen concentration is not a factor limiting growth in chemostat cultures.

5. Oxygen-limited growth in batch cultures.

Data from the experiments described above (Section III.4b.) made possible some general observations on respiration, glucose uptake and carbon economy in Acer batch cultures. The observations were incidental to the main aims of the project and no further experimental work was undertaken.

a. Kinetics of the response to O_2- limitation.

Although oxygen uptake rates in cultures 51 and 60 reached their upper limit on day 6, no response was apparent in the rates of cell division (Fig. III.4.) or dry weight accumulation (Fig. III.6.) until day 11 (culture 51) or day 15/16 (culture 60). At first sight, it might be suggested that, for example, the rate of cell division is largely independent of the respiration rate. However, assuming constant yield relationships between oxygen consumed and biomass (cells or dry weight) produced (see Table III.3.), growth after day 6 (and particularly between days 6 - 11) is generally as might be predicted (though see exceptions discussed below). By integration of the change in the rate of oxygen uptake with time between days 2 - 10 in cultures 49 and 59 (Fig. III.4.), yield coefficients (biomass produced/unit oxygen consumed) were calculated for non-oxygen-limited growth (Table III.3.). Daily growth increments were then calculated for a constant O_2-supply

Table III.3. Product/O_2 yield coefficients during non-oxygen-limited growth at different glucose concentrations.*

	Product per mole O_2 consumed		
Culture	Cell dry weight g	Cell number $\times 10^{-10}$	CO_2 carbon g atoms
49	37.5	2.70	1.28
59	31.5	1.53	1.22

*Period covered was days 2 - 10, Fig. III.4.

Glucose concentrations were: culture 49, 0.11M; culture 59, 0.22M.

Table III.4. Daily growth-increments of oxygen-limited cultures.

	Increment ml^{-1} day^{-1}		
	Number of cells	Dry Weight	Cell Carbon
Culture	$x10^{-6}$	mg	mg
51	0.272	0.377	0.072
60	0.154	0.318	0.147

Data calculated from yield coefficients in Table III.3. for a constant O_2 supply rate of 7.2 nmoles ml^{-1} min^{-1}.

rate of 7.2 nmoles O_2 ml^{-1} min^{-1} (Table III.4.). These accumulating (predicted) growth increments are superimposed on the real data in Fig. III.4. and Fig. III.6.

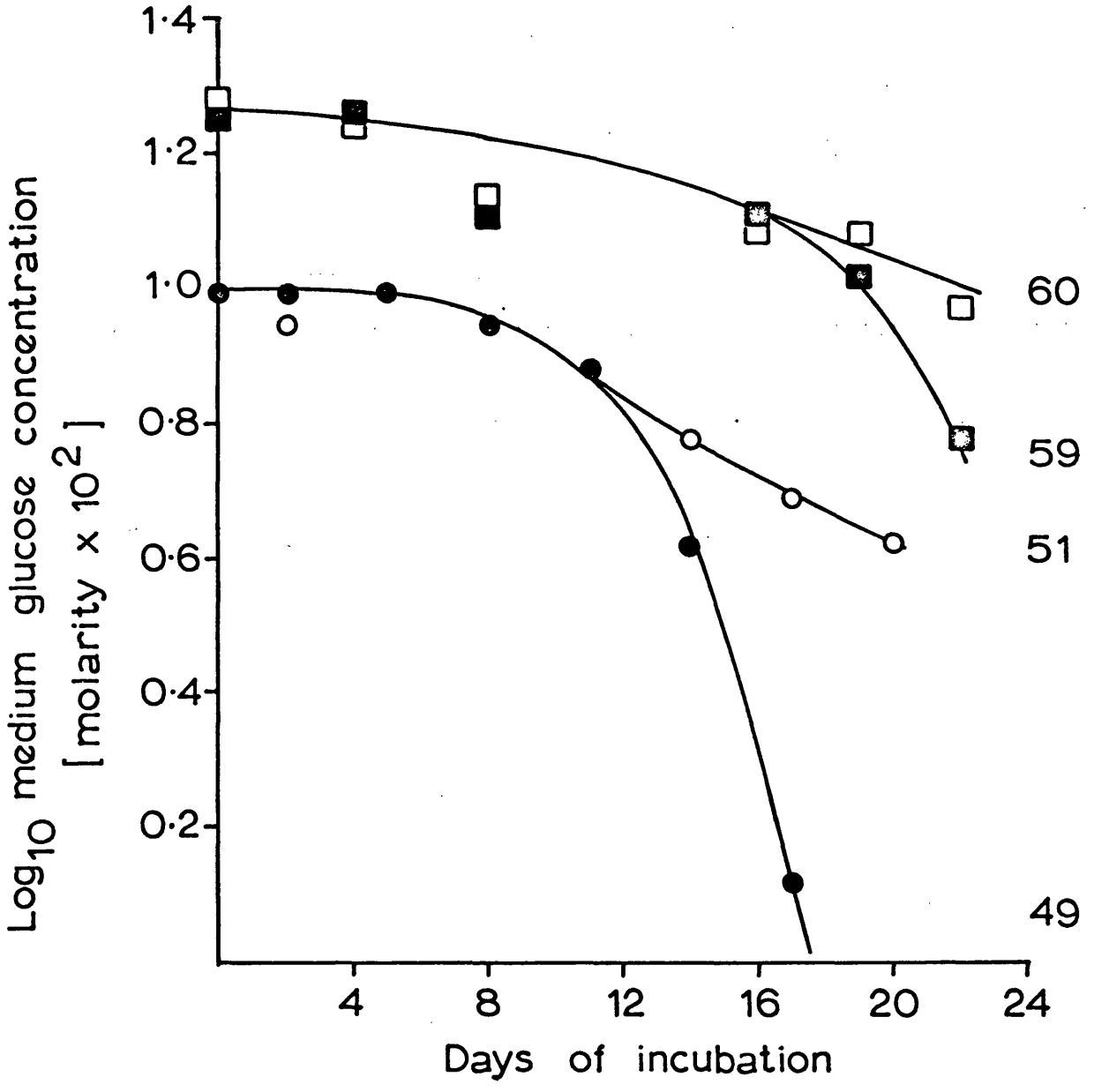
Increase in cell number closely follows the predicted pattern in culture 60 (Fig. III.4.). However, a discrepancy arose with time in culture 51 between predicted and actual values for cell number and especially for dry weight (Fig. III.6.). Less biomass (cells or dry weight) was produced in culture 51 after day 11 than would be expected from the rate of oxygen consumption recorded. It would appear that the yield coefficients have changed at a low dissolved oxygen concentration and a low exogenous glucose concentration. This effect on yield coefficients seen in culture 51 cannot be due solely to low oxygen tension as the same effect is not seen in culture 60 (Figs. III.4 and III.6.), where the O_2-limitation is the same. Neither can the yield changes be due solely to a low exogenous glucose concentration. No effect is seen in culture 49 (Figs. III.4. and III.6.), where with a non-limiting oxygen supply glucose depletion up to day 11 was the same as in culture 51. From day 11 to day 20 in culture 49 glucose depletion was much greater (Fig. III.7.). Furthermore, the discrepancy arises in culture 51 when the medium glucose concentration is still ca. 68% of the initial value (i.e. 0.075M) (Fig. III.7.). In contrast, the medium glucose concentration in culture 60 never dropped below 0.1M.

The biomass/oxygen yield coefficients only changed in the culture in which <u>both</u> the oxygen tension and the glucose concentration were low. To put it another way, growth under oxygen-limited conditions appeared to become dependent upon the exogenous glucose concentration at glucose concentrations below ca. 0.08M. Despite the complications imposed by the non-steady-state conditions in these cultures and the

Fig. III.7. Changes with time in the concentration of glucose in the medium of 4-litre batch suspension-cultures of *Acer* under different aeration conditions and at different (initial) glucose concentrations.

(See also Table III.2 and the legend to Fig. III.4)

Fig. III. 7.


1·4
1·2
1·0
0·8
0·6
0·4
0·2
Log10 medium glucose concentration [molarity x 10^2]
4
8
12
16
20
24
Days of incubation
60
59
51
49

fact that glucose is an immediate respiratory substrate, it may be suggested that the observed effect is due to the existence of two transport systems for glucose into Acer cells. Glucose uptake in all cultures (49, 51, 59 and 60) at least up to day 6 was predominantly via a passive, low-affinity system. Sometime between day 6 and day 11 in culture 49 a high-affinity, respiration-dependent system, which had a similar V_{max} to the low-affinity system, became maximally expressed. The K_m for this system was less than 1.0×10^{-3}M glucose and virtually all the glucose was consumed. However, in culture 51, because of the limitation on oxygen availability, either this high-affinity system was never expressed and the rate of entry via the low-affinity system (which would be saturated at about 0.12M glucose) progressively declined from day 6 as the exogenous glucose concentration fell; or sometime between day 6 and day 11 the proportion of oxygen consumption involved in "maintenance" respiration for the steadily increasing biomass reached a level which curtailed any respiratory activity devoted to transport of the carbon source by the high-affinity system. There is some indication from the data in Fig. III.7. that the rate of glucose uptake by culture 51 steadily declined from about day 10. However, it is interesting to note that, assuming an endogenous respiration rate for dividing Acer cells of 3 nmoles O_2 mg. dry wt.$^{-1}$ min^{-1} (the respiration rate of washed cells in glucose-free medium, Simpkins and Street, 1970), the fixed oxygen supply-rate to culture 51 (7 nmoles O_2 ml^{-1} min^{-1}) would maintain a biomass of 2.3 mg. dry wt. ml^{-1}. This was the biomass reached on day 9 in cultures 51 and 60.

It might be predicted from the scheme above that the rate of glucose uptake in cultures 59 and 60 would be similar so long as the

exogenous glucose concentration remained above ca. 0.12M. Despite the fact that culture 60 suffered the same oxygen limitation as culture 51 by day 6, in cultures 59 and 60 glucose uptake continued at similar rates until day 16 (exogenous glucose concentration = 0.13M), when the rate of uptake by culture 60 was reduced. Growth, however, in culture 60 differed from culture 59, and followed closely the patterns predicted from the oxygen uptake rate. Double transport systems for glucose have been described in higher plant tissues (see for example, Hancock, 1970 - high affinity (HA)K_m = 0.8 x 10^{-3}M, low affinity (LA)K_m = 45 x 10^{-3}M; Maretzki and Thom, 1972 - (HA)K_m = 0.02 x 10^{-3}M, (LA)K_m = 1.4 x 10^{-3}M).

b. Redistribution of carbon during growth in batch cultures.

Redistribution of the glucose - carbon supplied to 4-litre batch cultures of Acer and the overall synthetic efficiency of the culture systems was examined by analysis of five carbon fractions in cultures 49, 51, 59 and 60 :

1. glucose - carbon in the medium.
2. non-glucose - carbon in the medium.
3. cytoplasmic - carbon.
4. cell-wall - carbon.
5. carbon lost as CO_2.

(see II.Materials and Methods - 14.).
Complete tables of the changes in each fraction (as g.atoms C per culture) with time are given in Appendix 3. The average percentage of the total initial carbon accounted for at each sampling time was 93% (the lowest was 85% in culture 59, day 19; the deficits previously described - Street, King and Mansfield, 1971 - were largely eliminated by correction of the CO_2 analysis technique). Table III.5. contains a summary of data

Table III.5. Effect of initial glucose concentration and OAC value on the fate of glucose carbon consumed.

culture	Initial glucose concentration M	OAC	Total glucose carbon consumed g.atoms	Product carbon as a percentage of glucose carbon consumed: Cell carbon Cell wall	Cell carbon Cytoplasm	Cell carbon Total	Medium carbon (non-glucose)	CO_2 carbon	Total
49	0.11	34	2.11	16.0 (0.34)	5.5 (0.11)	21.5 (0.45)	0.5 (0.02)	59.0* (1.24)	81* (1.71)
59	0.22	34	1.98	10.0 (0.20)	17.0 (0.34)	27.0 (0.54)	9.5 (0.19)	43.5 (0.86)	80 (1.58)
51	0.11	7.2	1.48	9.0 (0.14)	2.0 (0.04	11.0 (0.18)	4.0 (0.07	65.5 (0.97)	83 (1.23)
60	0.22	7.2	2.29	10.5 (0.24	10.5 (0.24)	21.0 (0.48)	22.0 (0.50)	37.0 (0.84)	80 (1.82)

After 17 days incubation (0.11M glucose) on 16 days incubation (0.22M glucose).
*The value for CO_2 produced by culture 49 has been adjusted to give a total recovery similar to the other three cultures (see legend to Fig. 111.5). Figures in brackets represent absolute amounts of glucose carbon in each fraction in g atoms C per culture.

from Appendix 3 in which the carbon present in each fraction after a given time is expressed as a percentage of the glucose - carbon consumed. The deficiencies noted above obviously lay in estimations of the products of glucose - carbon assimilation rather than in estimations of the residual glucose, and the average percentage of the glucose - carbon consumed which is accounted for in the products analysed was 81%.

In the standard synthetic medium (0.11M glucose) under non-limiting aeration conditions (Table III.5 - culture 49), the culture converted 21.5% of the consumed carbon to cell material (99% of the glucose supplied was consumed). Of the total cell-carbon (which includes free-sugar pools and stored carbohydrates), 75% was present in the cell wall fraction. A further 0.5% of the total carbon was found in the spent medium, presumably as secreted polysaccharide material (Simpkins and Street, 1970). Cell - carbon was assimilated at the expense of at least 59% of the glucose - carbon lost as carbon dioxide.

c. The effect of glucose concentration and oxygen availability on carbon distribution.

Similar absolute amounts of cell - carbon were produced in cultures 49, 59 and 60. (Table III.5.). As a percentage of the total glucose - carbon consumed, the conversion to cell - carbon in these three cultures was also very similar. This appears so even though oxygen availability to culture 60 was reduced. An explanation for this effect has been discussed above (III.5a.). The distribution of carbon between the two cell fractions estimated was different at high and low glucose concentrations. Cell wall: cytoplasm ratios in cultures 49 and 51 were 3·1 and 3·5 respectively and in cultures 59 and 60, 0·6 and 1·0. This appears to have been mainly due to enhanced uptake of carbon into the "cytoplasm" at the higher glucose

concentration, probably remaining there as free sugar pools and small polysaccharides (see also Table III.6.). The lower cell - wall - carbon fractions in cultures 59 and 60 were balanced by increases in extracellular, medium - carbon, particularly in the low-oxygen culture. An enhanced level of extracellular carbon also appeared in the low-oxygen culture at 0.11M glucose (culture 51). As expected the total conversion of glucose - carbon to cell - carbon in culture 51 was less than in culture 49 (Table III.5.). Furthermore, the cell - carbon in this culture represented a lower percentage of total glucose - carbon metabolised because of the steady loss of carbon as carbon dioxide during respiration. The CO_2/carbon : cell - carbon ratios in cultures 49 and 51 were 2·8 and 5·4 respectively. The CO_2/cell - carbon ratio for culture 60 (1·8) is lower than for culture 51, as would be expected after considerable respiration - independent uptake of glucose in culture 60 (see also Table III.6.). Surprisingly, the CO_2-carbon produced in culture 59 is also low (ratio CO_2/C : cell/C = 1·6) but the oxygen uptake rate of culture 59 (Fig. III.4) was lower than culture 49 at similar biomass levels. This may suggest that enhanced respiration was induced in culture 49 at the (relatively) low glucose concentration (0·11M) and that, in culture 59, the high-affinity respiration - dependent system for glucose uptake suggested in Section III.5a was never fully expressed.

Table III.6. shows more clearly the respiration - independent uptake of glucose by culture 60. Over similar periods of <u>oxygen-limited</u> growth, twice as much cell carbon was formed in culture 60 than in culture 51. The extra carbon in culture 60 was located primarily in the cytoplasm (three times more than in culture 51) though some additional cell wall biosynthesis was indicated.

Table III.6. The effect of glucose concentration on the distribution of carbon during oxygen-limited growth in 4-litre batch cultures.

Culture	Initial glucose conc.(M)	Glucose carbon consumed	Total cell C produced	C incorporated into Cell wall	Cytoplasm
51	0.11	1.20	0.173	0.110	0.062
60	0.22	1.47	0.369	0.187	0.182

Carbon expressed as g atoms per culture during 11 days oxygen-limited growth.

IV. GROWTH AND CELL DIVISION IN BATCH CULTURES

"...'the growth cycle' - a term which ... conveys a quite misleading impression that this sequence is a necessary and inevitable feature of bacterial growth, whereas it is in reality a sequence forced upon the organisms by sequential environmental changes which are inevitable when growth occurs in a closed system. Unfortunately, bacteriologists have been inoculating flasks of culture media for so long now that they have come to regard this as part of the natural order of events, instead of as a convenient but highly artificial experimental procedure". (Herbert, 1961).

1. Introduction.

The batch culture technique involves the isolation of an inoculum of cells in a finite volume of nutrient medium in a system which is closed except for exchange of gases and volatile metabolites with the outside air. Most of the published physiological and biochemical investigations of the growth and cell division of higher plant cells in culture have been carried out using this technique either with suspensions of cells in liquid medium (cell cultures) or with a callus supported on solidified medium (tissue culture). The change in cell number with time in a batch culture of plant cells closely resembles the classical "growth cycle" of bacterial cultures: after inoculation a lag phase may occur during which there is some biosynthesis but no cell division; the number of cells increases exponentially during a period when cell division is most rapid (exponential growth phase); then for a time the growth rate of the culture may decline uniformly (linear phase) and then more and more rapidly (often called the "progressive deceleration phase") until the

terminal phase of the culture (stationary phase) is reached. In this stationary phase no net synthesis of biomass or increase in cell number occurs, but cells may remain viable for many days by utilising intra-cellular reserves or by recycling metabolites from lysed cells. The occurrence and duration of each phase of this "growth cycle" depends upon the cell type (species and strain), the frequency of sub sulture, the initial cell density and the culture medium. For example, cells of Nicotiana tabacum L. var Xanthii in a minimal medium with nitrate as sole nitrogen source (Filner, 1965) or cells of carnation (Engvild, 1972) did not show a lag phase but the cell number increased exponentially for ca. 10 days before the culture rapidly entered stationary phase. However, cell cultures of Rosa sp., grown in a more complex and concentrated medium (Fletcher and Beevers, 1970; Nash and Davies, 1972) or of Acer pseudoplatanus (see below - this section) show a distinct lag phase, a shorter exponential growth phase (the duration of which, however, depends upon the initial cell density) and a gradual decline in the rate of increase of cell number before reaching stationary phase. Other biomass parameters show patterns of change through the growth cycle similar to that of cell number; for example, dry weight (Fletcher and Beevers, 1970; Jones, Barrett and Gopal, 1973); total cell protein, RNA or DNA (Nash and Davies, 1972); oxygen demand (Kessel and Carr, 1972); the total in vitro activities of some enzymes: peroxidases (Veliky, Sandkvist and Martin, 1969; de Jong, Jansen and Olson, 1967), pyruvate kinase and glucose-6-phosphate dehydrogenase (Fowler, 1971); production of total phenolics (Nash and Davies, 1972). However, it is clear from the published data that patterns of growth obtained using many of the parameters quoted above may differ from the pattern revealed by cell-counting in two important ways:

A. the biomass may increase exponentially for a time but each parameter by which it is measured may show a different growth-rate constant, which may be different again to the growth-rate constant obtained by cell-counting.

Mathematically, during exponential growth the number of cells (or the biomass) (x) present after a time (t) is

$$x = x_o \, e^{\mu t} \qquad 1.$$

where x_o is the number of cells (or the biomass) at any time taken as zero and μ is the growth-rate constant. Taking the logarithms of Equation (1) gives an equation of a straight line

$$\ln x = \ln x_o + \mu t \qquad 2.$$

Plotting values of t(abscissa) and of lnx (ordinate), a straight line is obtained for the exponential growth phase of slope = μ. The slope of such a semi-logarithmic plot is also referred to as the specific growth rate - the rate of increase of biomass per unit of biomass concentration. A common method of expressing the specific growth rate of an exponentially-increasing cell population is by the population doubling-time (td) which, from Equation (2), is

$$td = \frac{\ln 2}{\mu} \qquad 3.$$

When μ is obtained by cell-counting, td may represent the mean cell-cycle time (T).

Thus μ(DNA) was greater than μ(dry weight) in cell cultures of Rosa sp (Fletcher and Beever, 1970); μ(dry weight) was less than μ(cell number) in cell cultures of Pogosteman cablin (Jones, Barrett and Gopal, 1973).

B. the times of initiation or suppression of synthesis of particular cell components may be quite different from the initiation or cessation of cell division. Thus the synthesis of RNA by Acer pseudoplatanus cells in culture (Short, Brown and Street, 1969) or Rosa sp. cells (Nash and Davies, 1972) was initiated prior to cell division, occurred at a greater rate than cell number increase and

ceased before cell division ceased. In contrast, the exponential accumulation of phenolic compounds in cultures of Rosa sp. (Nash and Davies, 1972) began long after cell division was initiated, occurred at a higher rate than cell division and continued after cell division had ceased. The rate of DNA synthesis by a cell population might be expected to be closely coupled to the rate of cell division. However, in cell cultures of Rosa sp. (Nash and Davies, 1972) or of Acer (Short, Brown and Street, 1969) DNA synthesis was initiated before cell division occurred and cell division continued after DNA synthesis had virtually ceased. The period during which cytokinesis and DNA synthesis continued uninterrupted and at the same rate was very brief.

As a result of these differences in the rates of cell division and biosynthesis in batch cultures, the mean composition of the cells undergoes continuous change i.e. a state of balanced growth rarely exists. The results of the many, more detailed, metabolic studies of batch cell cultures of a side variety of species in many different media have served to further underline the continuously changing metabolic activity of cells in closed culture systems. These studies include: the accumulation of cellular nitrogen and carbohydrate in Ipomoea sp. (Rose, Martin and Clay, 1972); changes in acetate metabolism in Rosa sp. (Fletcher and Beevers, 1970); ethylene synthesis in Acer (Mackenzie and Street, 1970) and in Haplopappus gracilis (LaRue and Gamborg, 1971); in vitro activity of phenylalanine ammonia-lyase in Glycine max (Hahlbrock, Kuhlen and Lindl, 1971) and in Rosa sp. (Davies, 1971) or of nitrate reductase in Nicotiana tabacum (Filner, 1966) and Acer (Young, 1973); and the relative activities of the pentose-phosphate and Embden-Meyerhof-Parnas pathways

of carbohydrate oxidation in Acer (Fowler, 1971).

The maximum rate of cell division in plant cell cultures varies with the species, strain and the cultural conditions. Population doubling times (td) calculated from cell counts during the exponential growth phase of batch cell cultures (see Equation 3) have been reported as : 48 hours for Nicotiana tabacum (Filner, 1965); 36 hours for Rosa sp. (Nash and Davies, 1972); 24 hours for Phaseolus vulgaris (Liau and Boll, 1971) and 22 hours for Haplopappus gracilis (Eriksson, 1967). These population doubling-times are, in general, longer than cell cycle times reported from studies on whole plant meristems (8-20 hours: Evans and Rees, 1971; Phillips and Torrey, 1972) and are, therefore, likely to be shortened as more optimal cultural conditions are established.

Although the data to be described in this Section illustrate the unbalanced growth occurring in cell cultures of Acer pseudoplatanus (and thus add to the ample data already available for batch cultures of this species - see Short, 1969, Simpkins, 1970; Phillips, 1970; Wilson, 1971) they were obtained primarily to provide information about maximum growth rate, rates of nutrient assimilation, yield coefficients and cell composition to be used as a background to the studies on continuous cultures (Section V) and synchronous cultures (Section VI).

2. Unbalanced growth in batch cultures.

Data presented in Fig. IV.1. shows the accumulation of biomass in a 4-litre batch culture of Acer pseudoplatanus in the modified medium (see II. Materials and Methods.2. and Appendix 1, Fig.11, Plate 1). The culture was initiated at a density of 5×10^4 cells ml^{-1} using cells

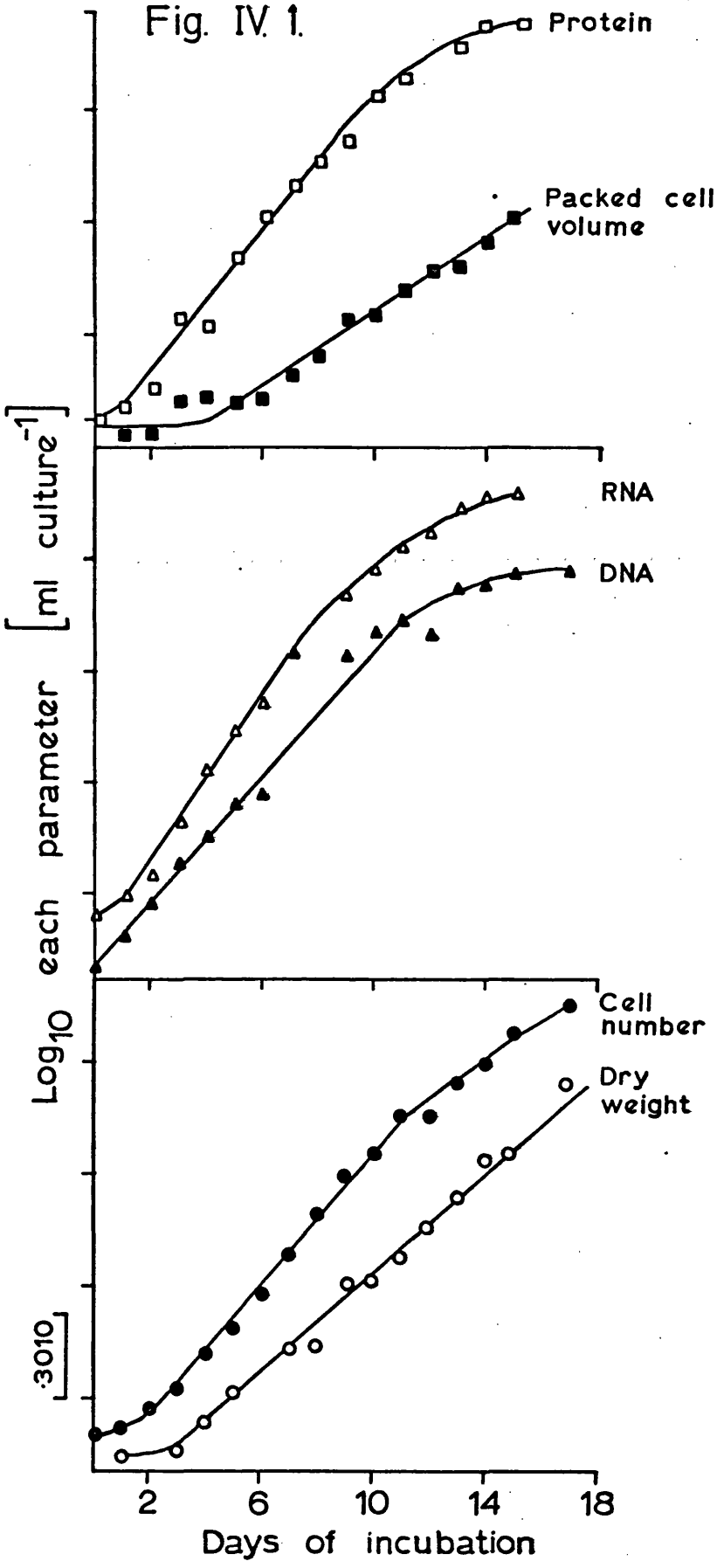
Fig. IV.1. The accumulation of biomass in a 4-litre batch suspension-culture of *Acer* cells.

Semi-logarithmic plot of changes in cell number, cell volume and dry weight, and macromolecules with time.

Aeration conditions	
Stirring speed	= 520 rpm
Air-flow rate	= 500 ml min^{-1}
Sparger	= G2
OAC = 34 (nmoles O_2 $ml^{-1}min^{-1}$)	

These aeration conditions established a rate of oxygen supply which exceeded the oxygen demand of the maximum biomass produced in the basal medium and were used in all other batch and continuous cultures described in this thesis, unless otherwise stated.

Fig. IV. 1.


Protein
Packed cell volume
RNA
DNA
Cell number
Dry weight
Log10 each parameter [ml culture-1]
.3010
2
6
10
14
18
Days of incubation

from a culture in late exponential phase (equivalent to cells on day 19 in Fig. IV.1) and was sampled at 24-hour intervals. Under these conditions there was a lag phase in growth only when measured by packed cell volume or dry weight (c. 2 days) and probably cell number (c. 1 day). However, growth in terms of macromolecule accumulation showed negligible lag. All parameters showed a limited period of exponential increase although the time of the cessation of this exponential growth phase differed with the parameter examined. Rates of cell division and DNA accumulation were similar (td = 58 hours). However, the rate of RNA accumulation (td = 42 hours) was greater than the rate of cell division and rates of accumulation of protein (td = 60 hours), dry weight (td = 83 hours) and packed cell volume (td = 109 hours) were lower than the rate of cell division. There was an eventual gradual decline in growth rate (expressed by all parameters) and all growth had ceased after ca. 22 days of incubation.

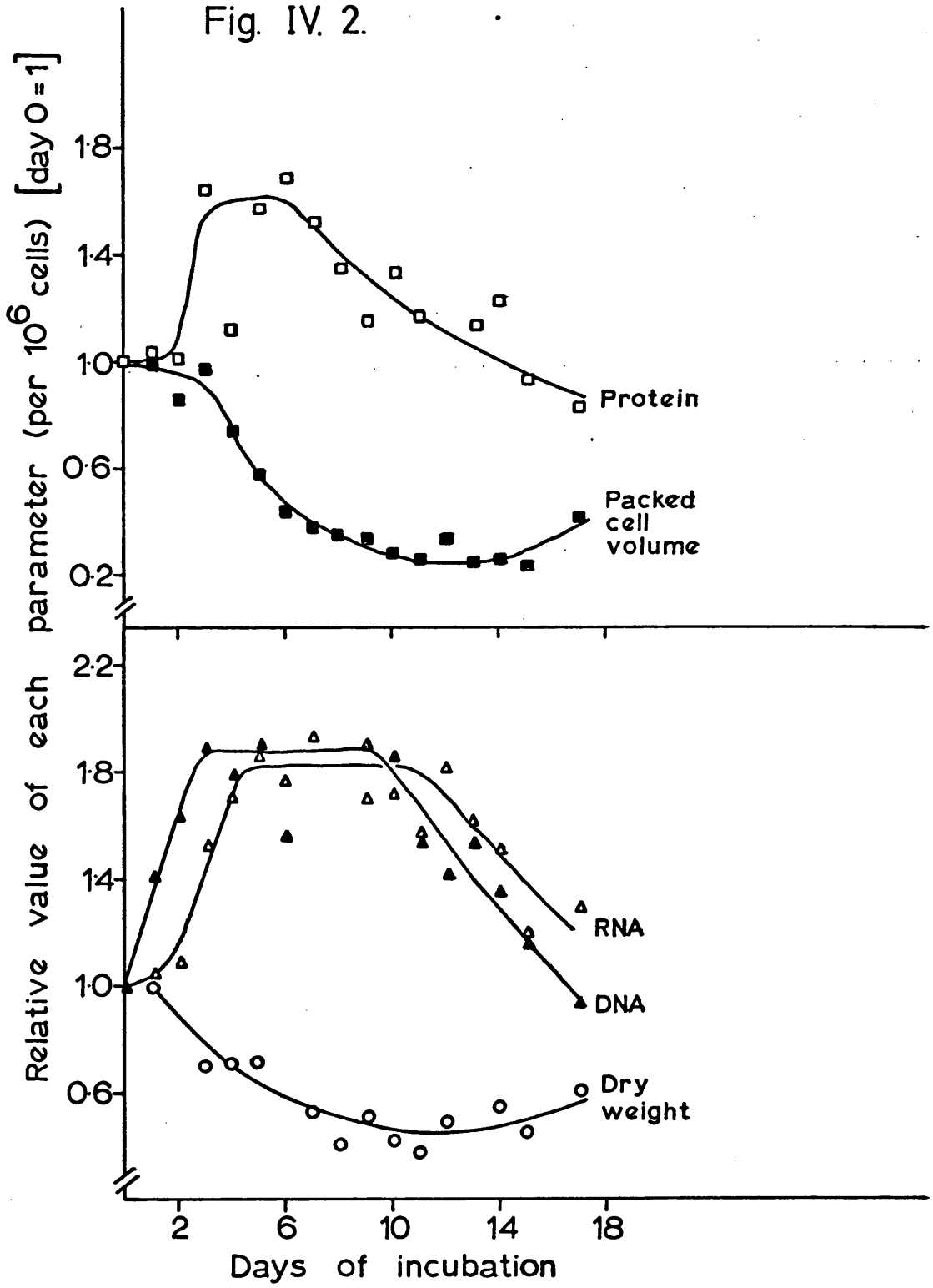
Although the rates of cell division and DNA accumulation during the exponential growth phase were the same, the period during which both cell division and DNA accumulation continued at a constant rate was limited (ca. day 2-8). The apparent, mean DNA content of the cells in the population rose rapidly to 1.8 of the initial value, remained constant for 6 days and then declined more gradually to the initial value. (Fig. IV.2).

The initiation of protein and RNA accumulation prior to the onset of cell division also produced a rapid rise in the mean content per cell of these macromolecules (Fig. IV.2). The slower rate of protein accumulation (relative to cell division) produced a sharp peak in protein content followed by a more gradual decline through the latter part of the exponential growth phase, The higher rate of RNA accumulation

Fig. IV.2. Relative changes in mean size and composition of Acer cells with time in a 4-litre batch suspension-culture.

Values were calculated from the data in Fig. IV.1.

Fig. IV. 2.


Relative value of each parameter (per 10^6 cells) [day 0 = 1]
1·8
1·4
1·0
0·6
0·2
Protein
Packed cell volume
2·2
1·8
1·4
1·0
0·6
RNA
DNA
Dry weight
2
6
10
14
18
Days of incubation

produced a more prolonged period of constant RNA-composition (despite the early departure of RNA accumulation from an exponential) followed by a gradual decline to the initial value. Similar peaks of protein (Givan and Collin, 1967) and RNA (Short, Brown and Street, 1969) have been reported for Acer cells. In addition Sutton-Jones and Street, (1968) report an increase in the density of ribosomes in electromicrographs of Acer cells during lag phase.

Accumulation of biomass dry weight and packed volume was not initiated until after the onset of cell division (Fig. IV.1) but then the biomass increased exponentially at rates less than the rate of cell division. Furthermore, both dry weight and packed cell volume continued to increase exponentially after growth, as expressed by all other parameters measured, had become linear. The result of these trends in the growth of the cell population is a decline in mean cell volume and dry weight during the exponential growth phase with a gradual return to the initial values in early stationary phase. Microscopic examination has revealed a decrease in vacuolar volume and the disappearance of starch grains (a major factor in cell dry weight) during lag phase in Acer cultures, these trends being reversed in early stationary phase (Sutton-Jones and Street, 1968).

A comparison of typical values for cell size and composition of Acer cells in late exponential phase and stationary phase of batch cultures is made in Table V.4.

3. Nutrient consumption.

The consumption by Acer cells of three major components of the medium (C, P and N) and four arbitrarily chosen cations (Ca, Mg, Na and K) in the batch culture described above (Section IV.2) is shown in Fig. IV.3.

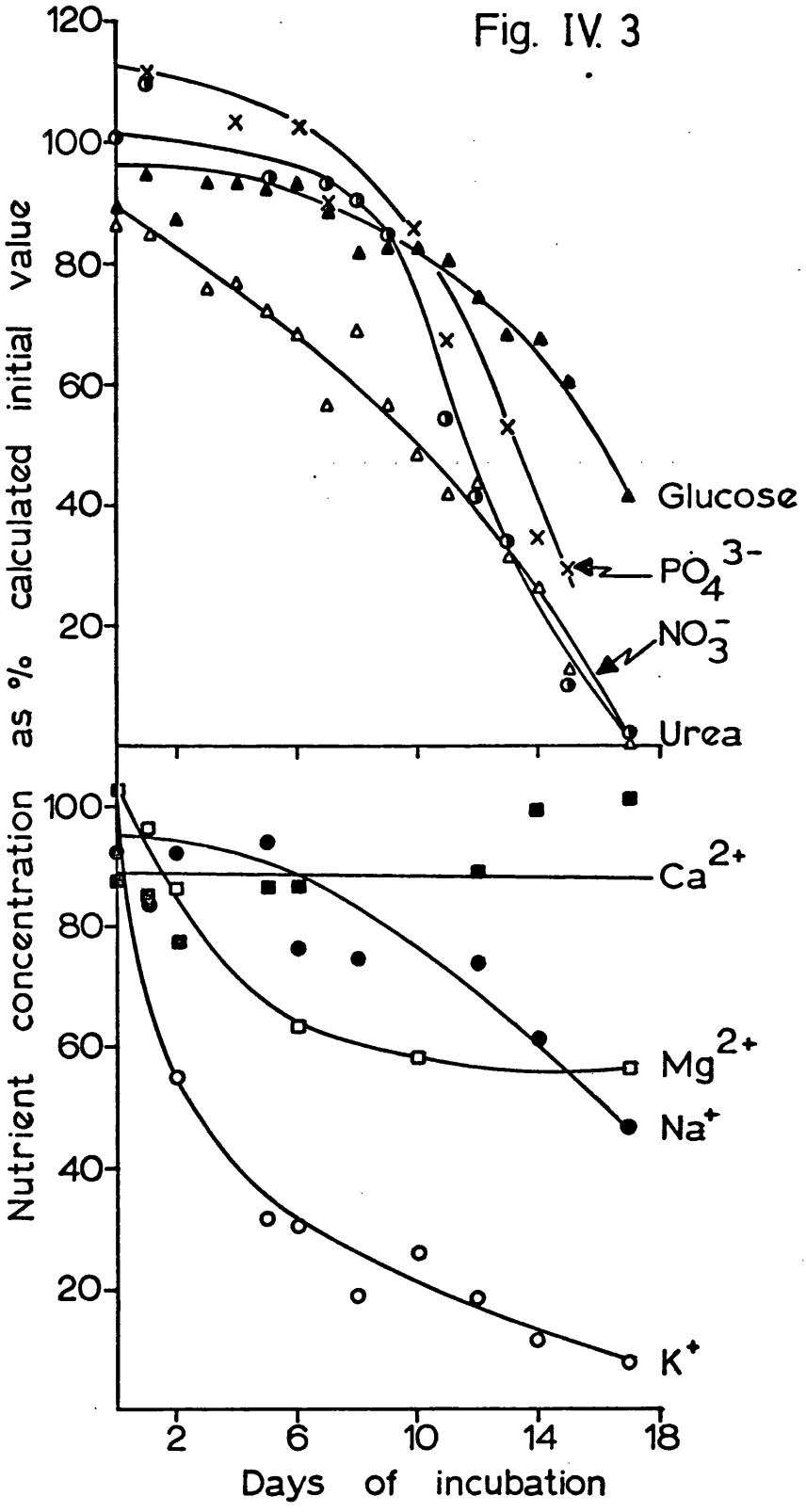
Fig. IV.3. Changes with time in the concentration of nutrients in the medium of a 4-litre batch suspension-culture† of *Acer* cells.

The calculated initial values are given in II. Materials and Methods.2.

The cation analysis was carried out by the analytical section, Unilever Ltd., Sharnbrook.

† See Fig. IV.1. for growth data.

Fig. IV. 3


120
100
80
60
40
20
Nutrient concentration as % calculated initial value
Glucose
PO_4^{3-}
NO_3^-
Urea
100
80
60
40
20
Ca^{2+}
Mg^{2+}
Na^+
K^+
2
6
10
14
18
Days of incubation

The data serve to illustrate the complex, non-steady-state conditions to be expected in a closed culture system. Four of the nutrients analysed (K, urea/N, NO_3/N and P) were depleted to very low levels by ca. day 16 of incubation. Although, in three other similar batch cultures, NO_3^- was depleted 3-4 days before urea depletion (and this coincided with the cessation of exponential increase in cell number), it is not clear which, if any, of the nutrients analysed limits growth in batch cultures of Acer. However, it has been demonstrated (Young, 1973) that an increase in total available nitrogen acheived by increasing the medium NO_3^- concentration produced directly proportional yields of cells and total protein.

4. Rates of cell division.

During the period of the maximum rate of cell division in the batch culture described above (Fig. IV.1), the population doubling time (td), calculated from the semi-logarithmic plot of cell number against time, was 58 hours. Changes in % labelled-metaphases after a ^3H-thymidine pulse suggest that population doubling times calculated from such plots are a measure of the mean cell-cycle time of Acer cell populations in exponential growth (Bayliss and Gould - personal communication). Thus the minimum (mean cell-cycle time of the population described was 58 hours. However, the minimum cell-cycle times of other batch cultures of Acer cells examined in this study were not always in agreement with this value. The frequency distribution of population doubling times for 23 different batch-culture populations of Acer cells is shown in Fig. IV.4. Whilst the distribution shows a distinct peak around 60-70 hours, there is a considerable spread in the mean cycle times recorded. The frequency

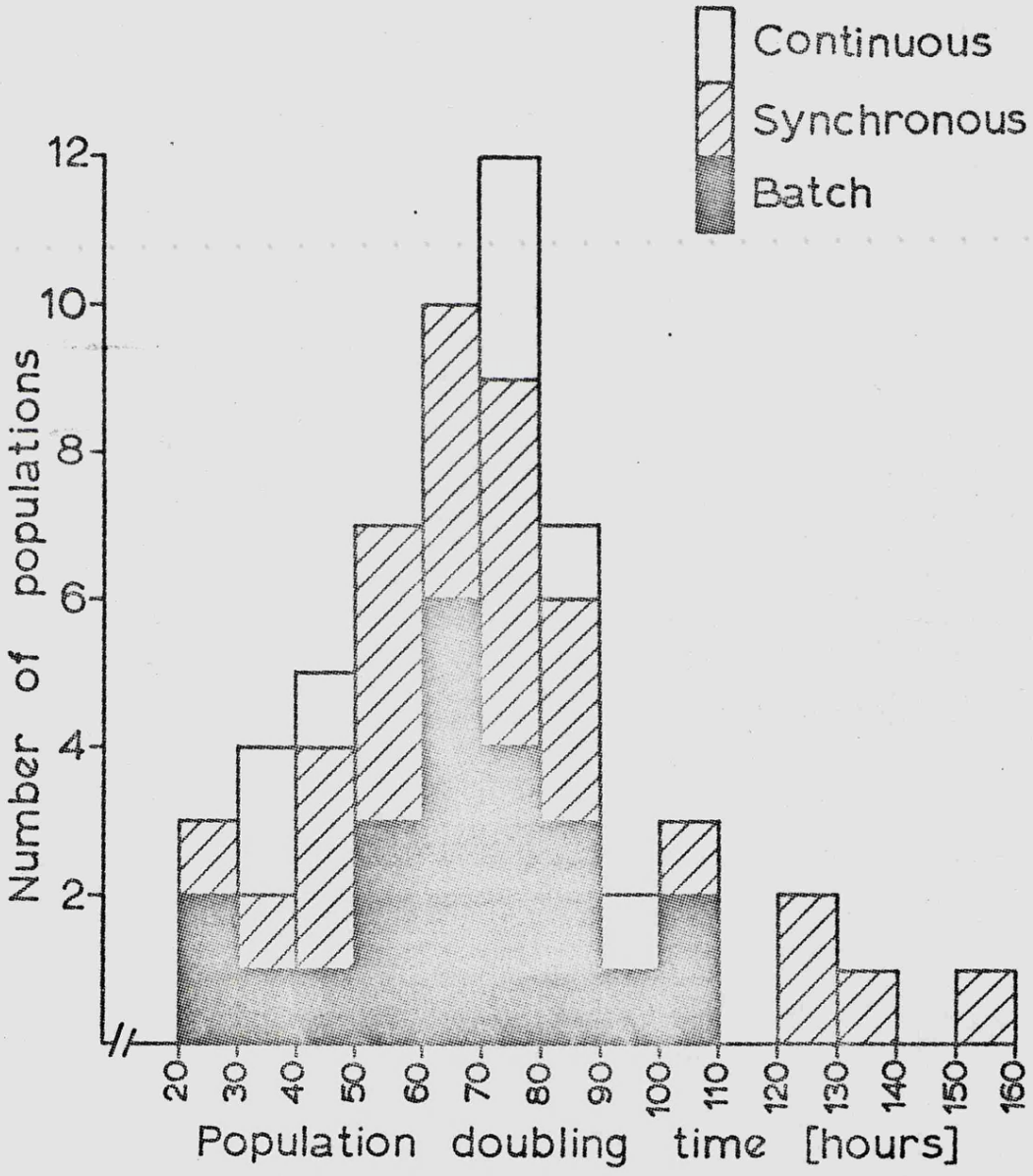
Fig. IV.4. The frequency distribution of population doubling times in suspension cultures of *Acer* cells.

The population doubling times of 23 different batch cultures were calculated using Equation 3 in Section IV.

The 26 cell-cycle times included are taken from the data in Figs. VI.1, VI.2, and VI.12.

The population doubling times quoted for continuous cultures are calculated from Equation 11, Section V and are taken from turbidostat data (Section V.3) and extrapolated chemostat data (Section V.4).

Fig. IV. 4.


Continuous
Synchronous
Batch
Number of populations
12
10
8
6
4
2
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
Population doubling time [hours]

distribution of the duration of 26 cell cycles from synchronised batch cultures of Acer (see Section VI. Figs. VI.1, VI.2 and VI.12) shows both a similar peak and a similar spread (Fig. IV.4). Furthermore, estimations of maximum growth rate in turbidostat cultures (two of which showed very high growth rates at low density - Section V.3) and chemostat cultures (Section V.4) of Acer also fit into this pattern (Fig. IV.4).

5. The effect of 2,4-D concentration on the maximum rate of cell division.

Cells of Acer pseudoplatanus have an essential requirement for an endogenous supply of a plant growth hormone when cultured in the standard, synthetic medium (Section II.2). 2,4-dichlorophenoxyacetic acid (2,4-D) is more effective in this role than either indol-3 yl-acetic acid (IAA), 1-naphthaleneacetic acid (NAA) or kinetin (Simpkins, Collin and Street, 1970). The yield of cells from batch cultures of Acer was shown to be proportional to the initial concentration in the medium of 2,4-D (Street, Collin, Short and Simpkins, 1967) and IAA, gibberellic acid or kinetin (Digby and Wareing, 1966). Similar results are reported for other species: the fresh-weight yield from callus cultures of Nicotiana tabacum and Glycine max is proportional to the concentration of added cytokinins (a fact made use of as a bioassay for cytokinins) (Miller, 1968); the yield of cells in Glycine max cultures is related logarithmically to the initial concentrations of NAA or 2,4-D (Fosket and Torrey, 1969). However, it is not clear from these experiments whether (a) the proportion of the initial cell population participating in cell division is dependent upon hormone concentration, or (b) the yield of cells from a fixed supply of nutrients is enhanced by higher levels of endogenous hormones, or

(c) the pattern of growth of the cells in batch culture is altered e.g. any lag phase occurring is shorter at higher hormone levels, or (d) the specific growth rate of the cells is proportional to hormone concentration. Despite the frequent use of single-point bioassays for the measurement of endogenous levels of growth substances, there is surprisingly little information about the effect of growth-substance concentration on the growth pattern of bioassay cultures, particularly from the point of view of changes in the rates of cell division. One exception is the report by Helgeson, Krueger and Upper, (1969) that the specific growth rate of callus tissue of Nicotiana tabacum was related to the initial concentration of 6 - (γ,γ - dimethylallylamino) purine, although even here growth was measured only by changes in fresh weight. Because of the differential rates of growth and cell division producing states of unbalanced growth, and the apparent variation in cell-cycle times in batch cultures of Acer cells, great interest attaches to the possible affect of 2,4-D concentration on rates of growth and cell division. Both the variations observed in batch culture and the possible affects of the growth regulator have great significance for the efficient control of growth in continuous culture systems and, in addition, the continuous culture systems themselves present a new situation from which to investigate the effect and mode of action of growth regulators. For these reasons a more detailed investigation of the effect of initial 2,4-D concentration on growth rates of Acer cells in batch cultures was undertaken.

a. The standard medium contains 2,4-D at an initial concentration of 4.5×10^{-6} molar. The doubling time for cell number during exponential growth in cultures initiated at this 2,4-D concentration

is normally 60-70 hours (see Fig. IV.1 and Fig. IV.4). In the experiment illustrated in Fig. IV.5, replicate 70-millilitre batch cultures were established in 250-millilitre shake-flasks. Stock cells (subcultured in 4.5×10^{-6}M 2,4-D) were inoculated into a minimal medium† containing <u>either</u> 4.5×10^{-6}M 2,4-D <u>or</u> 9.0×10^{-6}M 2,4-D. At the same time, cells which had already been maintained for at least 10 passages on minimal medium containing 9.0×10^{-6}M 2,4-D were transferred, in identical conditions, once more to 9.0×10^{-6}M 2,4-D. Two flasks from each treatment were harvested at 48-hour intervals, bulked and samples were withdrawn for cell count, dry weight and packed cell volume determinations.

The rates of cell division in the cultures initiated at the higher 2,4-D concentration were significantly greater (in both treatments, td = 43 hours) than that of the culture at the lower concentration (td = 72 hours) (Fig. IV.5A). However, rates of accumulation of dry weight (Fig. IV.5B) and packed cell volume (Fig. IV.5C) in all three treatments were not significantly different. The response of the cells to higher 2,4-D concentration was immediate; exponential growth at a higher specific growth rate occurred in high 2,4-D cultures after a lag phase equal in duration to that of the low 2,4-D cultures. Cells subcultured for many passages in high 2,4-D concentrations continued to show high rates of cell division. (The transfer of such cells to low 2,4-D concentrations was not attempted).

b. The specific growth rate of stock cells subcultured into minimal medium at a range of 2,4-D concentrations was also dependent

---

† The minimal medium lacked kinetin, pantothenic acid, choline chloride and cysteine.

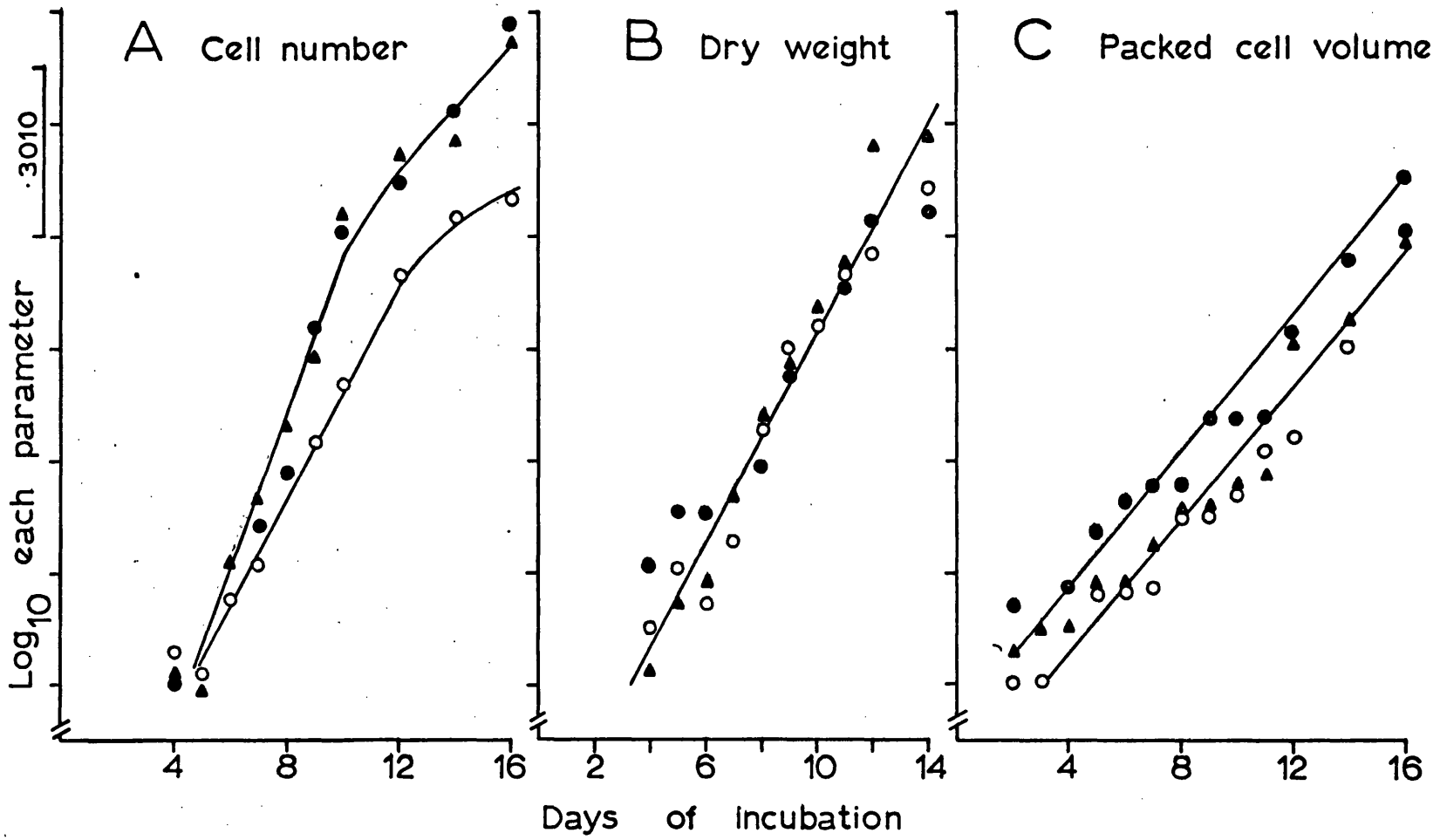
Fig. IV.5. The effect of the initial concentration of 2,4-D on the rate of accumulation of biomass in 70-millilitre batch suspension-cultures of Acer cells.

Semi-logarithmic plot showing changes in cell number, dry weight and packed cell volume.

- o Stock cells (normally subcultured in 4.5×10^{-6}M 2,4-D) reinoculated into medium containing 4.5×10^{-6}M 2,4-D.
- ● Stock cells (normally subcultured in 4.5×10^{-6}M 2,4-D) inoculated into medium containing 9.0×10^{-6}M 2,4-D.
- ▲ Cells (after subculture for $>$ 10 passages in 9.0×10^{-6}M 2.4-D) reinoculated into medium containing 9.0×10^{-6}M 2,4-D.

Specific growth rates were calculated from the regression lines drawn ($p < 0.01$) using Equation 2, Section IV.
A population doubling is indicated against the ordinate.

Fig. IV. 5.


A Cell number
B Dry weight
C Packed cell volume
Log10 each parameter
·3010
4
8
12
16
2
6
10
14
Days of Incubation

upon the level of hormone present (Fig. IV.6). The difference in cell-division rate between cells at 4.5 x and 9.0 x 10^{-6}M 2,4-D (Fig. IV.6A) was very similar to that observed in the first experiment (Fig. IV.5A). However, the cell-division rate was not further increased by raising the 2,4-D concentration to 13.5 x 10^{-6}M; and the rate at 2.25 x 10^{-6}M was not significantly different to the rate at 4.5 x 10^{-6}M 2,4-D (Fig. IV.6B). The specific growth rate in terms of dry-weight accumulation was again more-or-less independent of 2,4-D concentration (Fig. IV.6B). The cell-number and dry-weight of the stationary-phase biomass was very similar in all treatments.

c. In a third experiment (Table IV.1), the rate of cell division at 9.0 x 10^{-6}M 2,4-D (td = 38 hours) was again greater than that at 4.5 x 10^{-6}M (td = 51 hours). The data, although incomplete, suggest that rates of accumulation of total protein and total RNA were also higher. The protein and RNA content of the cells in both treatments was not significantly different (Table IV.1B). This contrasts with the evidence from the first two experiments that the rate of accumulation of total biomass (dry weight) was unaffected by changes in hormone concentration. Unfortunately, the limited data for DNA accumulation are inconclusive. Neither the difference between the DNA content of the two cultures nor the difference between the cell number on day 8 is significant.

d. Evidence was presented in Section IV.4 above (see Fig. IV.4) of apparent random variation in the mean cell-cycle times of Acer batch cultures established in the standard medium. Fig. IV.7A shows the increase in cell number with time in a 3-litre batch culture established by inoculating stock cells into minimal medium at

Fig. IV.6. The effect of the initial concentration of 2,4-D on the specific growth rate (μ) of *Acer* cells in 70-millilitre batch suspension-cultures.

A. Rate of change of cell number (semi-logarithmic plot) at two concentrations of 2,4-D.
B. The influence of 2,4-D concentration on specific growth rate during the exponential growth phase.

Specific growth rates were calculated from the regression lines drawn ($p < 0.01$) using Equation 2, Section IV.

Population doubling times (td) were calculated using Equation 3, Section IV.

Each point on graph A represents the mean of samples taken from two flasks harvested from replicates of each treatment.

Fig. IV.6.

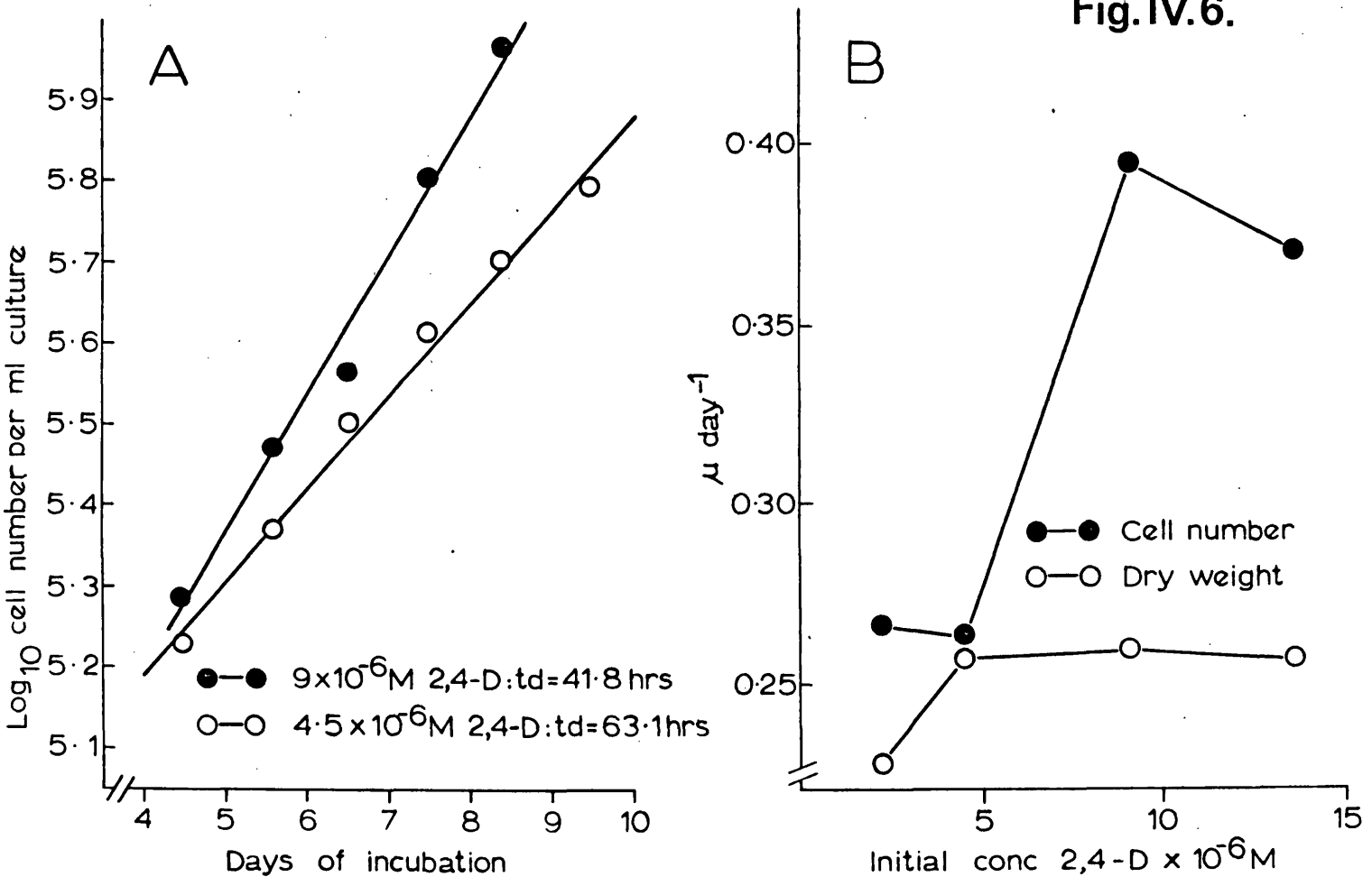

A
B
5·9
5·8
5·7
5·6
5·5
5·4
5·3
5·2
5·1
Log10 cell number per ml culture
4
5
6
7
8
9
10
Days of incubation
9x10-6M 2,4-D:td=41·8 hrs
4·5x10-6M 2,4-D:td=63·1hrs
0·40
0·35
0·30
0·25
μ day-1
Cell number
Dry weight
5
10
15
Initial conc 2,4-D x 10-6M

Table IV.1. The effect of the initial concentration of 2,4-D on rates of cell division and macromolecule accumulation in 70-millilitre batch cultures of Acer.

A	2,4-D Concentration $\times 10^{-6}$ molar	Days of incubation 0	2	4	6	8	10	12	14
Cell number $\times 10^{-6}$ ml^{-1}	4.5	0.24	0.23	0.33	0.50	1.51	2.65		
	9.0	0.22	0.21	0.58	1.12	1.11	2.44		2.47
Total protein μg ml^{-1}	4.5	120	164	168	355		710		
	9.0	134	194	268	400		705		730
Total RNA μg ml^{-1}	4.5			18.5		113.0		92.6	
	9.0			30.0		122.0		105.6	
DNA μg ml^{-1}	4.5					6.3			
	9.0					6.7			

B*	2,4-D Concentration $\times 10^{-6}$	Protein	RNA	DNA
		μg / 10^6 cells		
	4.5	510	56	4.2
	9.0	464	52	6.0

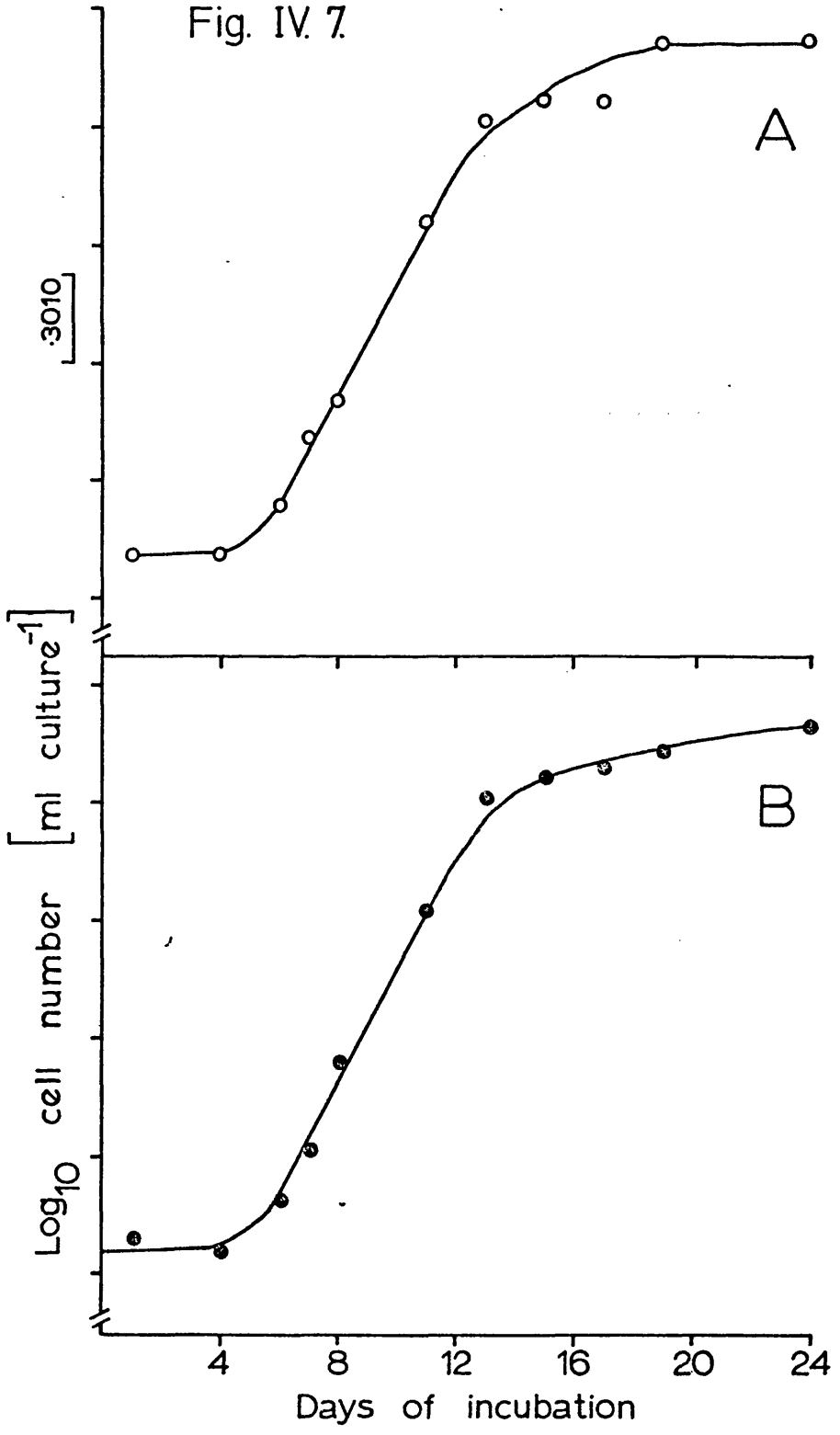
*Values for day 4 (protein and RNA) and day 8 (DNA).

Fig. IV.7. Change in cell number with time in 3-litre batch suspension-cultures of Acer cells initiated at different 2,4-D concentrations.

A. 4.5×10^{-6}M 2,4-D
B. 9.0×10^{-6}M 2,4-D

A population doubling is indicated against the ordinate.

Fig. IV. 7.


A
B
3010
Log10 cell number [ml culture-1]
4
8
12
16
20
24
Days of incubation

4.5×10^{-6}M 2,4-D. The population doubling time (37 hours) in this culture was much less than the mean (60-70 hours). The rate of DNA accumulation was similar to the rate of cell division (td = 44 hours); total RNA accumulated at much the same rate (td = 38 hours). Total dry weight accumulation, although at a rate greater than that recorded for a culture with a rate of cell division closer to the mean (see p. 55), was again less than the rate of increase in cell number. The population doubling time of a second 3-litre batch culture (Fig. IV.7B), which was established at the same time from the same stock cells using minimal medium containing 2,4-D at 9.0×10^{-6} molar, was the same (td = 39 hours). Rates of accumulation of DNA (td = 44 hours), total RNA (td = 47 hours) and dry weight (td = 64 hours) were also very close to the values for the culture established at the lower 2,4-D concentration. The failure of a population of cells, which had spontaneously aquired the capacity for a high rate of cell division, to respond to an increased concentration of 2,4-D was also recorded in a further, separate experiment.

6. Discussion.

The relatively brief but strictly exponential increase in cell number in batch cell cultures of Acer points to a phase of culture growth when a large proportion of the population are engaged in cell division at a similar rate. The autocatalytic synthesis of cell materials (RNA, total and specific proteins, total dry matter, etc.) results in phases of exponential accumulation of each particular cell component. However, in this closed batch-culture situation biosynthesis (= growth) appears to be largely uncoupled from cell division and major changes occur in mean composition and metabolic

activity of the population (see day 2-11 in Fig. IV.2). The fact that the rate of cell division remained constant despite dramatic changes which occurred in cell size and composition suggests that the mechanism regulating the interval between cell divisions in plant cells in discrete and independent of total macromolecular accumulation, cell mass or cell volume. This is also suggested by the alteration in the rate of cell division in Acer cell populations independently of rates of biosynthesis both spontaneously (see Section IV. Fig. IV.12 and Table VI.3) and through the influence of exogenous growth regulators (Fig. IV.6).

It would be unwise (though interesting) to speculate about the nature of the control of cell division in Acer cells from the limited, gross growth-data for the randomly-dividing batch-populations so far available. However, there is a large body of evidence (particularly from work with ciliate protozoa - Mitchison, 1970) for a discrete mechanism for the control of cell division, based either on a labile division-protein accumulating across a cycle or on an initiator-protein accumulating to a point which triggers DNA replication, the interval between S-phase and mitosis being a fixed, causal sequence. More relevant to observations on the growth of Acer cells in batch culture is the evidence obtained from the temporary inhibition of DNA synthesis in some micro-organisms (see e.g. Mitchison, 1970) that the cell cycle consists of two major, fixed sequences of events (the DNA-division cycle and the growth cycle) which may be dissociated to varying degrees. The former includes the initiation and completion of S-phase, mitosis, nuclear division and cell division, whilst the latter would include most of the macromolecular synthesis that occurs. Complete dissociation of such cycles might be said to occur in Acer batch cultures during lag

phase and during early stationary phase, when in the absence of cell division the cells continue to enlarge. The cycles are at least partially dissociated during most, if not all, of the term of a batch culture (i.e. balanced growth rarely occurs) as the cells recover from the imbalance caused by nutrient depletion in the previous passage. In a typical batch culture recovery may never be total as nutrient depletion once more intervenes. It is possible that the extent to which the dissociation alters the size and composition of the cells during the onset of stationary phase might affect the rate at which the DNA-division cycle (the more labile process) proceeds during the recovery period on reinoculation. It is this aspect of the prehistory of cells (i.e. variation in the effects of dissociation in the previous passage, perhaps due to differences in inoculum density or duration of stationary-phase) that may explain the variation in rates of cell division which occurs between Acer batch cultures (Fig. IV.4). The effect of inoculum density and the duration of the stationary phase of the penultimate passage on rates of cell division in batch cultures merits investigation. Assuming that DNA replication is a discrete event in the cell cycle of Acer cells in culture (data in Section VI. Synchronous cultures support this view) then (1) the time lag between the initiation of DNA accumulation and the initiation of cell division in the randomly-dividing batch culture illustrated in Fig. IV.1 and (2) the increase in the mean DNA content per cell (Fig. IV.2) suggests that:

a. the cells in stationary phase (the inoculum cells) are "frozen" in G1.

b. during lag phase each cell goes through a DNA replication step before dividing.

c. during the exponential growth phase the cells spend longer in the 4C state than in the 2C state.

d. the mean cell cycle time (≡ doubling time, td) of the population lengthens prior to stationary phase due to an extension of G1. ((With the data available it is not possible to distinguish between a decline in the rate of cell division due to a gradual increase in the time interval (G1) between mitosis and the next DNA replication in successive cell generations or that due to progressive "drop-out" of cells at a particular event in the cycle occurring in G1)). The mean DNA content of the cells at inoculation (4.5×10^{-12} g $cell^{-1}$) agrees closely with the value (5×10^{-12} g $cell^{-1}$) for the DNA content of 2C cells in synchronous cultures of Acer pseudoplatanus (see Section VI). Furthermore, the expected increase in mean (relative) DNA content per cell in the exponential growth phase of the random cultures, based on the model cell cycles established in Section VI (see Fig. VI.13 and Table VI.5), is 1.7. The calculation assumes (as suggested by the data in Table VI.5) that G2 is constant in duration in cells with different cycle times. The data in Section VI also confirm that cells with cycle times of up to c. 70 hours would spend longer in a 4C than a 2C state, although due to an apparent lag of 10 hours between mitosis and cytokinesis the G2 phase may be only slightly longer than G1.

The data in Section IV.5 suggests that the level of 2,4-D in the culture medium may determine the rate of exponential increase of cell number in Acer cell populations. The effect of 2,4-D on the growth rate of Acer cells is similar to that reported by Helgeson et al (1969) for the influence of cytokinins on the rate of increase in fresh weight of tobacco callus tissue. The effect of the cytokinin on tobacco callus was continuously variable over a concentration-range from 10^{-6}M to

10^{-9}M, whereas the interaction between 2,4-D and Acer cells suggests a threshold effect within a more restricted range of concentrations. The growth of Acer cells at different 2,4-D concentrations would agree also with data of Fosket and Short (1973), Fosket and Torrey (1969), Digby and Wareing (1966) and Street, Collin, Short and Simpkins (1966) in as much as they report a relationship between growth hormone concentration and the cell numbers measured at a single point during batch culture growth-cycles. However, it is clear from the experiments described above that the particular effect of increased 2,4-D concentrations is to bring about an increase in the exponential growth rate of Acer cell populations, which by definition requires the active participation of the majority of the population. Populations at different 2,4-D concentrations had similar lag periods and reached similar final cell numbers. More specifically, increased 2,4-D concentrations appear to have decreased the mean cell-cycle time of the population. Furthermore, the effect appears to be achieved by a shortening of the "DNA-division cycle" rather than the "growth cycle" of the cells; 2,4-D concentration had relatively little influence on rates of biomass accumulation; although there was some evidence (Table IV.1) of enhanced rates of total RNA and protein accumulation. It is not possible from the data available to suggest a mechanism for the action of 2,4-D, nor even to decide whether any specific phase of the cell cycle in involved. However, the unbalanced growth during recovery of the meristematic state at each subculture of Acer cells is remarkably similar to the large differences between rates of cell division and rates of biomass accumulation which occur during the dedifferentiation of plant tissues leading to callus formation (Yeoman, 1970). Furthermore, natural and synthetic auxins (in particular 2,4-D) are effective in inducing callus formation and their

metabolism during callus induction has been investigated. 2,4-D is reported to complex with nuclear proteins (Yamada, Yasuda, Koge and Sekiya, 1971), to induce increased hyperchromicity of DNA (Spæng and Platt, 1973) and to induce RNA polymerase (O'Brien, Jarvis, Cherry and Hanson, 1968). All of these metabolic effects would fit the role proposed for auxins as derepressors at the DNA level and would, therefore, support the proposition that 2,4-D can act as a specific derepressor of portions of the genome coding for specific division - proteins and can thus control the initiation and the duration of the DNA-division cycle. A tentative link between 2,4-D and DNA expression is suggested by the fact that cells in the two populations described above, which divided more rapidly than usual (and which did not respond to increased concentrations of 2,4-D), had a higher DNA-content than usual (Table IV.2). Thus increased 2,4-D concentration appears to have a similar effect on the cell cycle of Acer pseudoplatanus cells as does a spontaneous doubling of their DNA content.

Table IV.2. The DNA-content of populations of Acer pseudoplatanus cells having different growth rates.

Population doubling time (td)	Initial	Mid-Exponential	Stationary Phase
h	µg DNA / 10^6 cells		
58	4.5	8.6	4.1
37	9.0	16.2	10.5
41.5	7.8	15.8	9.4

V. CONTINUOUS CULTURES

".... a method guaranteed to make it difficult to achieve steady-state growth is the usual one of placing some medium in a glass container, inoculating it with micro-organisms and allowing events to run their course". (Herbert, 1961).

1. Introduction

Batch cultures, particularly in so far as balanced growth does not occur, are of limited value for studies of metabolic regulation. Presumably the changing cell composition observed during their transient exponential growth phase (Section IV) would not continue indefinitely if this phase could be extended by preventing the development of growth-limiting conditions. In this way balanced growth might be achieved. This idea has prompted the development of alternative culture systems. An early development was that of semi-continuous cultures, in which portions of a culture in exponential phase are harvested at intervals and replaced by similar volumes of fresh medium. Such cultures have been used widely in research with micro-organisms (see Fencl, 1966), Animal cells (Sinclair, 1966) and with a number of higher plant cell cultures (Tulecke, Taggart and Colavito, 1965: Rosa sp, td* = 88-250 hours; Graebe and Novelli, 1966: Zea mays, td = 300 hours; Veliky and Martin, 1970: Phaseolus vulgaris, td = 63 hours; Verma and van Huystee, 1970,: Arachis hypogaea, td = 120 hours). The technical difficulties of repeated aseptic harvesting and medium additions has led to the development of large-scale culture systems (4-8 litres is usual). The need for a regular supply of cell material for bulk biochemical

*td = population doubling time.

extractions has also prompted the use of larger cultures (Graebe and Novelli, 1966; Verma and van Huystee, 1970). However, semi-continuous culture in a specially-designed, large vessel is no different in effect to rapid, serial subculture of suspensions in small shake-flasks. This latter technique has been used by a number of workers to maintain plant cell cultures in their exponential growth phase (Eriksson, 1965; Doree, Leguay, Terrine, Sadorge, Trapy and Guern, 1971; Liau and Boll, 1971; Henshaw, Jha, Mehta, Shakeshaft and Street, 1966). A variation of this technique, which restricts changes in cell density to within more precisely defined limits, is generation-interval subculture. Wilson (1971) adopted this technique of sub-culturing cells of Acer pseudoplatanus at intervals equal to the cell population doubling time. With the exception of the work of Wilson (1971), it is not clear from the data whether prolonged balanced growth was ever actually achieved in any of these culture systems. Furthermore, a constant maximum growth rate (equivalent to that of the exponential growth phase of a batch culture) may be maintained in a semi-continuous culture by sufficiently rapid harvesting and replenishment of medium, but both the concentrations of cells and nutrients in the culture will oscillate markedly. Thus it is difficult, using such a method, to produce cultures in a steady state, i.e. where balanced growth occurs in an unchanging environment.

Closed-continuous cultures, although extending the exponential growth phase and perhaps leading to more balanced growth (see this Section V.2), can only do so over short periods and do not readily produce steady states of growth. A closed-continuous culture is one in which nutrients, ideally in excess of the culture requirements, are supplied by continuous inflow of fresh medium; this is balanced

by continuous harvesting of spent medium. The cells are separated mechanically from the nutrient flow and are retained in the culture vessel. For a limited period, growth in such a culture may be maximal and not restricted by nutrient availability but inevitably will come to depend upon the rate of supply of a specific nutrient. With an exponentially enlarging population this point is only a short step away from nutrient depletion and the cessation of growth and cell division. Although such systems have been adopted in research and manufacturing processes involving micro-organisms (see Ricica, 1966), little has been achieved with eukaryote cell cultures. However, such a culture system may have value in studies of cytodifferentiation, where it may be important to grow cells under a particular regulated environment and then maintain them for an indefinite period in a non-dividing but viable state. The possibilities of regulating post-division metabolism by the continuous application of different (sequential) nutrient regimes in a controlled environment are very interesting.

The turbidostat is essentially an extension of the semi-continuous culture technique in which harvests of culture and medium additions are automatically linked by a sensitive device measuring increase in biomass. In a turbidostat, the cell population density is monitored in the culture vessel by a physical (e.g. light transmission or scattering) or a physico-chemical method (e.g. pH determination). This information is fed back to a fresh medium-input control system linked to a system for a balancing harvest of culture (the turbidostat is an open culture system - see Ricica, 1966). The medium input system operates each time the population density exceeds a pre-selected value and dilution of the culture reduces the cell density to just below this value. Though termed a 'continuous culture', in practice the input of fresh

medium into a turbidostat is intermittent and hence the cell density and nutrient concentrations are not absolutely constant but oscillate about a mean. However, the sensitivity of the control system can restrict this oscillation and the culture will approach a steady state. For example, in the turbidostat cultures of Acer pseudoplatanus now to be described, each pulse of fresh medium only dilutes the cell population by 2%; the oscillation of the cell population is less than can be detected with the available cell-counting technique (II. Materials and Methods. 7.).

Northrop (1954) showed graphically that microbial growth in a turbidostat in a steady state was a continuation of the exponential growth phase of a batch culture. The relationships now derived are by a simpler approach: during exponential growth, the number of cells (x) † present after a time (t) is

$$x = x_o e^{\mu t} \qquad (1)$$

where x_o is the number of cells at any time taken as zero and μ is a constant that depends on growth rate (growth rate constant ≡ specific growth rate - see Equation (1) Section IV). The derivation of Equation (1) by time gives

$$\frac{1}{x}\cdot\frac{dx}{dt} = \mu \qquad 2$$

or, rearranging

$$\frac{dx}{dt} = \mu x \qquad 3$$

Equation (3) describes the rate of increase in the number of cells in the culture due to cell division.

---

† Symbols used follow convention of Herbert, Elsworth and Telling (1956).

When a culture of constant volume (V) receives a flow of nutrient medium at a constant rate (f) (e.g. millilitres day^{-1}), the fractional rate of replacement of the culture volume is

$$\frac{f}{v} = D \quad day^{-1} \qquad 4$$

D, the dilution rate, or washout rate, is an exponential and the number of non-dividing cells (x) remaining in a constant-volume culture after a time (t) at a dilution rate (D) would be

$$x = x_o e^{-Dt} \qquad 5$$

where x_o is the number of cells at any time taken as zero. The rate of change of cell number due to washout is thus

$$\frac{dx}{dt} = -Dx \qquad 6$$

In a continuous culture of dividing cells, the rate of change of cell number will be the resultant of Equations (3) and (6) thus

$$\frac{dx}{dt} = \mu x \ -Dx \qquad 7$$

If $\mu > D$, dx/dt is positive and the cell density will increase, while if $D > \mu$, dx/dt is negative and the cell density will decrease. In a turbidostat, μ and D are tightly linked by feed-back control of the inflow of fresh medium; as the growth rate increases (to its maximum value), the washout rate is increased by the turbidity control system which responds to increasing biomass. As $\mu = D$, $dx/dt = 0$ and x, the cell density is constant. In a culture in an exponential growth phase which has achieved balanced growth, the specific growth rate, μ, not only describes the average rate of increase in cell number but also describes the average rate of increase of every cell substance. Thus, where y and z represent cell substances

$$\mu = \frac{1}{x} \cdot \frac{dx}{dt} = \frac{1}{y} \cdot \frac{dy}{dt} = \frac{1}{z} \cdot \frac{dz}{dt} \qquad 8$$

Similarly, during washout

$$D = \frac{-1}{x} \cdot \frac{dx}{dt} = \frac{-1}{y} \cdot \frac{dy}{dt} = -\frac{1}{z} \frac{dz}{dt} \qquad 9$$

When $\mu = D$

$$dx/dt = dy/dt = dz/dt = 0 \qquad 10$$

and every biochemical and physical property of the culture should remain constant. In this steady-state, as

$$\mu = D = \frac{f}{v} \qquad 11$$

the growth rate (μ) can be calculated directly from the rate (f) at which fresh medium enters the culture vessel once a density threshold has been established. A turbidostat operates most effectively at low cell densities, when the culture contains an excess of free nutrients (Ricica, 1966). In these conditions, growth rate is independent of cell density (over a defined range) and is at a maximum (μ_{max}) determined only by the chemical and physical quality of the environment and by the associated velocity of metabolic processes in the cells; not by the operation of any substrate limitation (Bryson, 1959). A turbidostat device was first used by Myers and Clark (1944) to regulate the growth of algae, and later by Bryson (1952), Northrop (1954) and Anderson (1956), who worked out the theoretical basis of the technique and applied it to studies of the growth kinetics of antibiotic-resistant bacteria, mutation rates under different growth conditions and adaptation of bacteria to different substrates. The principle has since found far fewer biological uses than the chemostat but has recently been applied to investigations of metabolic oscillations in bacterial cultures (Watson, 1972). A very limited use has been made of turbidostat cultures of mammalian cells (Cooper, Wilson and Burt, 1959). There have been no reports to date of higher plant cells in

turbidostat culture.

The achievement of steady-state growth of higher plant cells in turbidostat culture would (a) provide a source of cell material in an 'extended exponential growth phase' for metabolic studies; (b) allow the direct determination of μ_{max} in a specific set of cultural conditions; (c) make possible the study of the control of rates of growth and cell division by environmental factors (e.g. temperature and light), by specific metabolites (e.g. hormones - see Section IV) and by intrinsic (genetic) factors in a steady state, where growth is not limited by availability of essential nutrients; (d) allow studies of the adaptation of higher plant cells to different nutrients.

Steady states of growth of micro-organisms have also been achieved by continuous dilution of a fixed-volume culture with fresh medium at a chosen rate. Any chosen dilution rate (within certain limits) produces a steady state population (Herbert, Elsworth and Telling, 1956; Málek and Fencl, 1966). An explanation for the self-adjustment of the growth rate of the culture biomass under constant dilution to the point when $\mu = D$, and thus $dx/dt = 0$, - Equation 7, was provided by Monod (1942), who showed that the specific growth rate of a bacterial population is proportional to the concentration of an essential nutrient in the culture vessel. The relationship is approximately expressed by

$$\mu = \mu_{max} \frac{S}{K_s + S} \qquad 12$$

where μ_{max} is the asymptotic value of μ as the nutrient concentration (S) increases, and K_s is a saturation constant equal to S when $\mu = \mu_{max}/2$. Thus in a culture diluted at a rate D, where x (cell density) is

relatively low and S is high (e.g. a newly inoculated culture) and $\mu > D$, the biomass will increase with time. As the nutrient concentration consequently falls, the specific growth rate will decrease until $\mu = D$, after which neither x nor S will change (see Equations 8, 9 and 10) and a steady state is established. Experimental and mathematical proofs exist (Powell, 1965) that starting from non-steady state conditions with a constant dilution rate (where $D < \mu_{max}$)†, a steady state must inevitably be reached. Monod (1950) and Novick and Szilard (1950) derived equations (17 - 20) for such cultures (chemostats) which describe the situation existing once a steady state has been established. These equations are important in that they show that :

1. Steady states are predictable growth states.

2. Unique values of x and S exist for each dilution rate applied using a medium containing a specific concentration of the limiting nutrient and thus that :

3. An established steady state in a chemostat has an in-built self-balancing capacity.

---

† When $D > \mu_{max}$, dx/dt will be negative (Equation 7) and cells will be washed out. No stable state can be reached. Taking the logarithmns of Equation 7 gives

$$\ln x_1 - \ln x_2 = (D - \mu_{max})(t_2 - t_1) \qquad 13$$

Plotting lnx against t will give a line of slope = $(D - \mu_{max})$ from which μ_{max} may be calculated.

These equations rely on the growth rate being a constant fraction, Y, of the substrate utilization rate, i.e. that

$$\frac{dx}{dt} = \mu x = - Y \frac{ds}{dt} \qquad 14$$

where

$$Y = \frac{\text{weight of biomass produced}}{\text{weight of nutrient used}} \qquad 15$$

The rate of change of nutrient concentration in a chemostat is the balance between rates of nutrient input, output and consumption and may be written

$$\frac{ds}{dt} = D_{s_R} - D_s - \frac{\mu x}{Y} \qquad 16$$

where S_R is the concentration of the limiting nutrient in the input medium. Substituting Equation 12 for μ in Equation 7 and 16 gives two equations (17 and 18 respectively) which define completely the behaviour of a chemostat culture:

$$\frac{dx}{dt} = x \left\{ \mu_{max}\left(\frac{S}{K_S + S}\right) - D \right\} \qquad 17$$

$$\frac{ds}{dt} = D \left(S_R - S \right) - \frac{\mu_{max}\, X}{Y} \left(\frac{S}{K_S + S} \right) \qquad 18$$

In Equations 16 and 17 the terms μ_{max}, K_s and Y are constants (assumed). If in addition S_R and D are held constant, the unique values for X and S in the steady state (when dx/dt = ds/dt = 0) are given by

$$S = K_S \left(\frac{D}{\mu max - D}\right) \qquad 19$$

$$X = Y (S_R - S) = Y \left\{ S_R - K_S \left(\frac{D}{\mu max - D}\right) \right\} \qquad 20$$

Despite the many assumptions made in deriving these equations, good agreement has been found between experimental and predicted results in

chemostat cultures of many bacterial species and of some multicellular micro-organisms (Fencl, 1966). However deviations from theory do occur, often when the relationship between biomass and nutrient consumed no longer remains constant, and Y becomes a function of dilution rate (Herbert, 1959, Kubitschek, 1970).

In microbiology, the main advantages of chemostat cultures are found to be:

1. substrate-limited growth in the presence of low levels of critical nutrients;

2. the control of growth rate;

3. the selection of cells in a specific metabolic state.

These advantages appear to offer a number of approaches from which to explore metabolic control and the metabolic potential of higher plant cells. Most attempts hitherto to establish chemostat cultures of higher plant cells have consisted of ad hoc studies of biomass production (Tulecke, Taggart and Colavito, 1965; Miller, Shyluk, Gamborg and Kirkpatrick, 1968; Constabel, Shyluk and Gamborg, 1971; Kurz, 1971). However, more systematic studies on the feasibility of continuous cultures of plant cells have been reported recently (Wilson, 1971; Kessel- personal communication). The experiments now to be described test the 'Monod' theory outlined above as applied to higher plant cells using suspensions of Acer pseudoplatanus L. as a model system. The experiments attempt to answer the following questions:

Turbidostat (Section V.3.)

A. Is it possible to establish unlimited steady states of growth of plant cells by turbidity control?

B. To what extent is the growth rate of a turbidostat culture of A. pseudoplatanus cells in the basal medium maximal and independent of cell density?

Chemostat (Section V.4.)

A. Is it possible to establish steady states of growth of plant cells for long periods by continuous dilution of a non-steady state culture?

B. How far is it possible to produce stable states at different growth rates by selecting different dilution rates?

C. Is there a critical dilution rate above which a stable state is not achieved - but instead cells are washed out of the culture vessel in a predictable way?

D. Does the maximum specific growth rate of A. pseudoplatanus cells calculated from C. above agree with calculations made from batch cultures (Section IV) and turbidostats?

E. What is the growth-limiting nutrient in the basal medium?

F. To what extent do the steady-state concentrations of this nutrient fit the 'Monod' equations (Equations 12 and 19)?

G. Is the relationship between biomass produced and limiting-nutrient consumed constant at different growth rates?

H. Do steady states of A. pseudoplatanus cells respond predictably to perturbations in the concentration of the limiting nutrient in the input medium? (See Equation 20).

I. To what extent do cells in steady states at different growth rates differ in size and composition?

J. Is the growth of A. pseudoplatanus cells in chemostat culture entirely predictable?

There are many technological problems in continuous culture; these problems greatly affected the design and duration of the experiments. The construction and operation of apparatus used in this study is described in Appendices 1 and 2.

2. Closed continuous culture.

The exponential growth phase of a 4-litre culture of Acer cells was extended by the continuous dilution of a closed system. A constant volume of culture was maintained by the continuous outflow of spent medium via a siphon (see Figs. 16 and 17, Appendix 1). The cells were separated from the spent medium by gravity sedimentation in a wide-bore 'stilling' tube, which formed the initial part of the siphon. The fresh-medium flow was begun on day 11. The rates of medium-flow and air-flow into the culture vessel were increased arbitrarily with time in an attempt to keep pace with the needs of the increasing biomass (Table V.1A). In this culture system cells accumulated exponentially for 28 days (c. 6 generations, td $\simeq$ 80 hours) before oxygen availability limited further growth (Fig. V.1.). The specific growth rate (cell number and protein) from day 11 was similar to that normally recorded in batch cultures using the basal medium (cf. Fig. IV.1). However, the specific growth rate (dry weight) was far greater than normal during the initial half of the dilution period (Table V.1B.). The reason for the enhanced non-protein dry weight accumulation is not clear, although it may be due to excessive carbohydrate assimilation in the presence of the high sugar level maintained in the culture vessel (50% of saturation). From day 17 onwards, cell composition varied between relatively narrow limits i.e. a state closer to balanced growth was achieved. (Fig. V.2.). However, there was no clear change in the specific growth rates (e.g. a decline in the rate of cell division) which might indicate an eventual coupling between growth and cell division. The mean values for cell volume and cell protein during this period were lower than for cells late in the exponential growth phase of a batch culture (cf. Fig. IV.2 and Table V.2.).

Table V.1. A. Medium-flow and air-flow rates into a closed-continuous culture system.

Day	Medium-flow rate l day^{-1}	Air flow rate ml min^{-1} (G3 sinter)
0	0.0	500
11	0.2	500
18	0.8	500
21	0.8	500
23	0.8	1000
24	1.8	1000
25	2.0	2000

B. Specific growth rates (μ) and doubling times (td) of biomass in a closed-continuous culture.

Component	μ day^{-1}	td h
Cell number	0.210	80
Protein	0.157	105
Packed cell volume	0.161	103
Dry weight	0.267	62

Fig. V.1. The growth of Acer cells in a 4-litre closed, continuous culture system.

Fresh medium was continuously supplied from day 11.

The rates of medium-flow and air-flow employed are given in Table V.1A.

Fig V 1

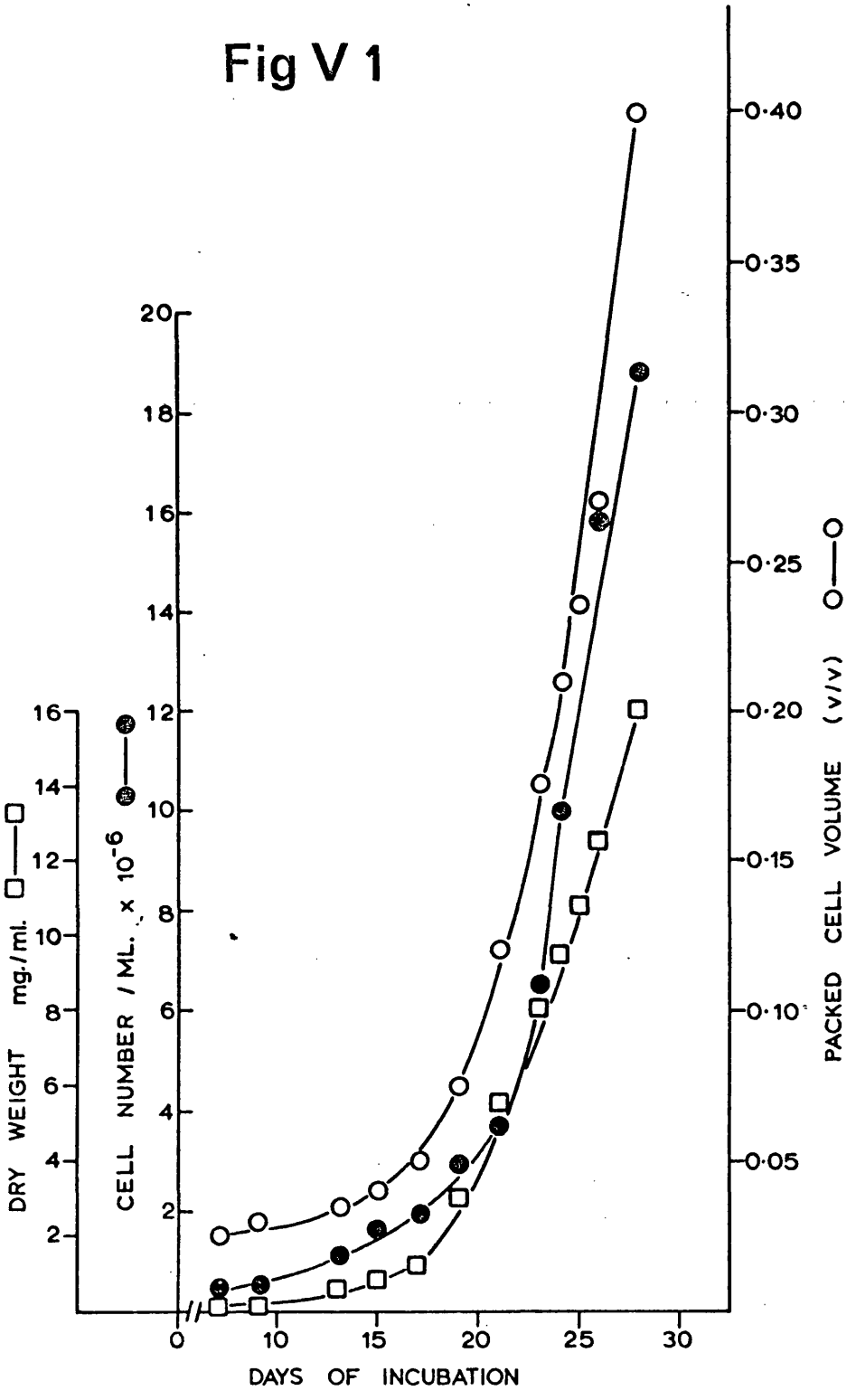

DRY WEIGHT mg./ml. □—□
CELL NUMBER / ML. x 10^{-6} ●—●
PACKED CELL VOLUME (v/v) ○—○
DAYS OF INCUBATION
0
10
15
20
25
30
2
4
6
8
10
12
14
16
18
20
0·05
0·10
0·15
0·20
0·25
0·30
0·35
0·40

Fig. V.2. Changes in the size and composition of Acer cells during prolonged exponential growth in a 4-litre closed, continuous culture system.

Dilution began on day 11. The data were calculated from data given in Fig. V.1. See Table V.1A for further experimental details.

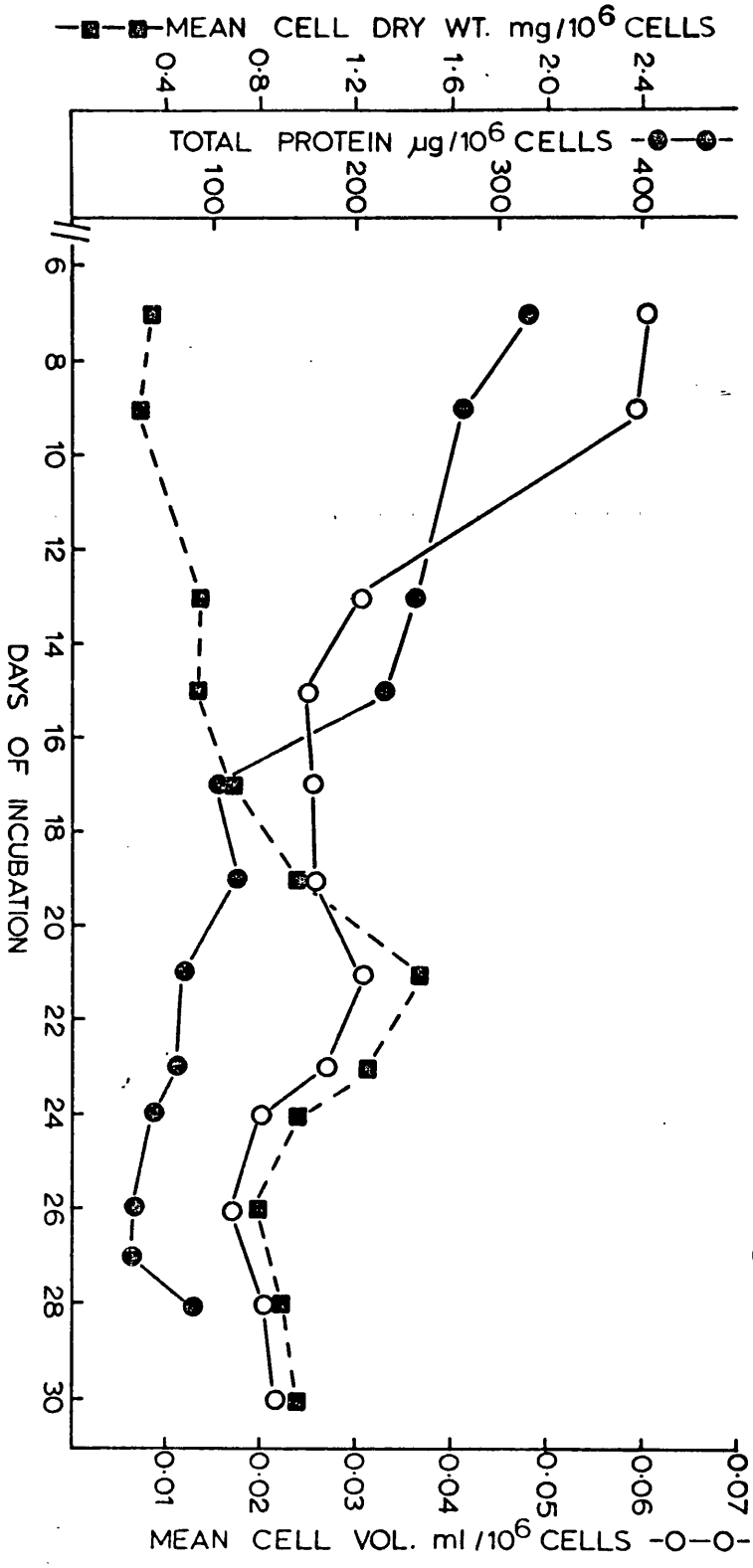

—■—■—MEAN CELL DRY WT. mg/10⁶ CELLS
0·4
0·8
1·2
1·6
2·0
2·4
TOTAL PROTEIN µg/10⁶ CELLS -●—●-
100
200
300
400
6
8
10
12
14
16
18
20
22
24
26
28
30
DAYS OF INCUBATION
0·01
0·02
0·03
0·04
0·05
0·06
0·07
MEAN CELL VOL. ml/10⁶ CELLS -O—O-

Fig V 2

3. Turbidostat cultures.

a. Turbidity monitoring.

There have been several reports of the turbidometric measurement of the growth of plant cell cultures. Eriksson (1965), using a rapidly growing culture of Haplopappus gracilis, demonstrated a relationship between optical density (at 610 nm) and culture dry weight. The growth of cell suspension cultures was also monitored by colorimetry by Dougall (1965) working with Rosa sp. (Paul's Scarlet) and by Bellamy and Bieleski (1966) working with Nicotiana tabacum.

A useful, working relationship was found between the opacity of Acer pseudoplatanus cell suspensions and both their cell density and dry weight (Fig. V.3). Cells from chemostat cultures (see Section V.4), in which steady states at various growth rates had been established, were pumped continuously through a sterile glass cuvette and then returned to the culture vessel (Appendix 1, Fig. 18). The cuvette was held between two cadmium sulphide light-sensitive resistors (Appendix 1, Fig. 22 and Plate 4C and D) which formed the arms of a Wheatstone bridge circuit (Appendix 1 Fig. 23). The bridge circuit was balanced with cell-free culture medium present in the cuvette (zero calibration). The out-of-balance current produced when culture was admitted to the cuvette was recorded on a galvanometric chopper-bar recorder and used as an arbitrary scale of opacity. The relationship between biomass and opacity was not linear (Fig. V.3). Low density cultures in Fig. V.3 represent chemostat cultures of high growth rate; high density cultures were cultures of low growth rate. The non-linearity of the biomass/opacity relationship may be explained by the marked changes in cell size and composition which

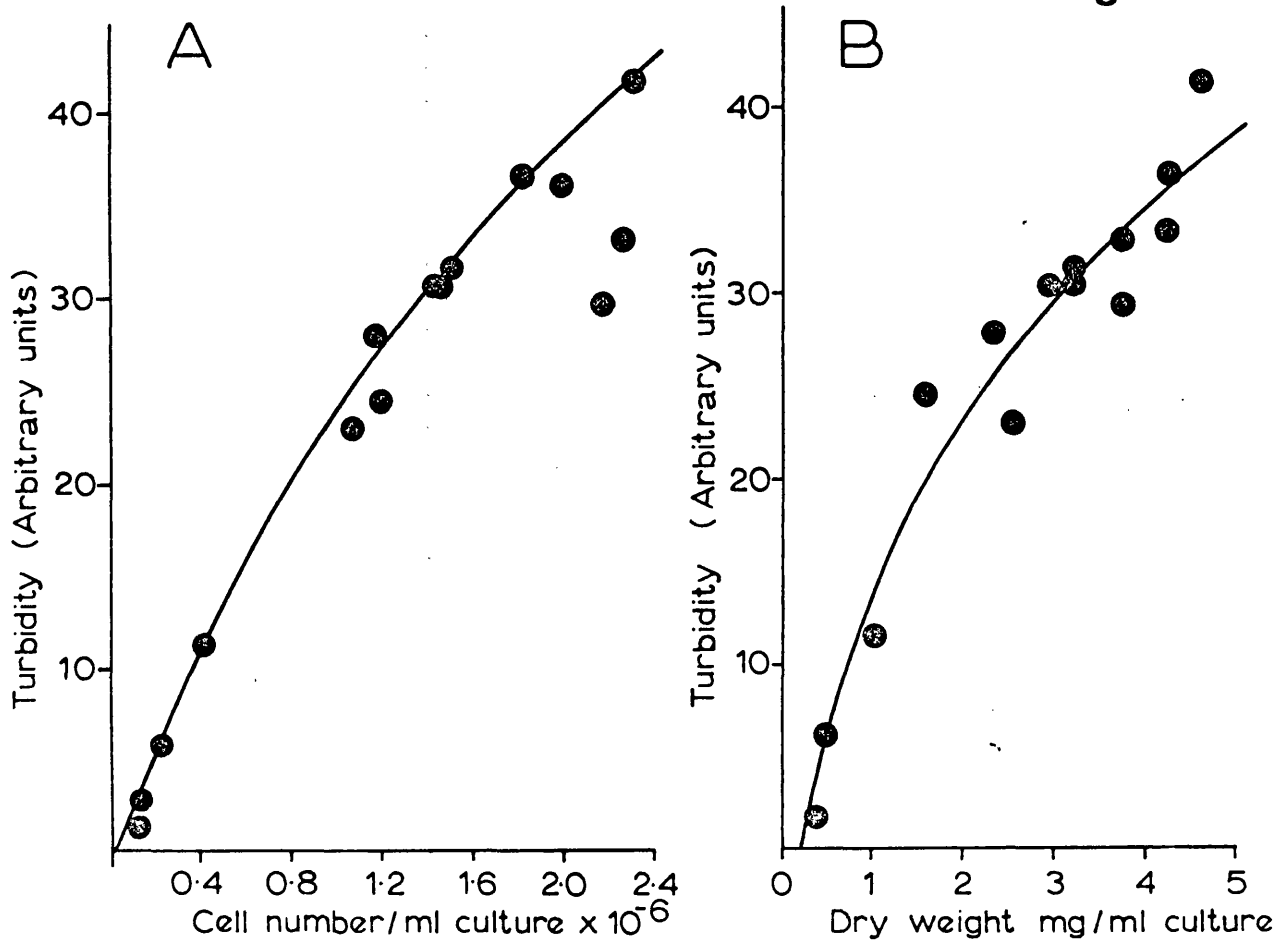
Fig. V.3. The relationship between turbidity and biomass in cell suspensions of Acer.

Stable cultures of different biomass were obtained by selection of a range of dilution rates in chemostat cultures.

The apparatus used for turbidity measurement is described in the text and in Appendix 1.

The electrical output of a Wheatstone bridge circuit, which included the density detectors, was used as a measure of turbidity - see Appendix 1, Fig. 23.

Fig V3


A
B
Turbidity (Arbitrary units)
40
30
20
10
0·4
0·8
1·2
1·6
2·0
2·4
Cell number/ml culture x 10^{-6}
0
1
2
3
4
5
Dry weight mg/ml culture

occur at different growth rates (see Fig. V.22). However, the relationships provided a convenient method for monitoring steady-state biomass in chemostat cultures and proved sufficiently sensitive to permit the turbidimetric control of cell density in a turbidostat system.

b. Turbidity control.

Four-litre turbidostat cultures of Acer cells were established by continuously monitoring culture opacity as described above. The bridge circuit was first balanced at the desired culture density. The out-of-balance current then produced by further growth of the cell suspension was amplified and used to control a solenoid valve (Plate 4B) via a Schmitt trigger circuit (Appendix 1, Fig.20). Each time the solenoid valve fired, fresh medium was admitted into the pumped loop upstream of the pump and the monitoring cuvette (Appendix 1, Fig.21). The solenoid valve was de-energised when the front of fresh medium reached the cuvette. The volume of the tube between the medium-inlet valve and the cuvette determined the pulse volume (usually c. 80 ml, i.e. 2% of the total culture volume). A constant culture-volume was maintained by a second solenoid valve upstream of the medium-inlet valve. This second valve was energised each time contact was made between the culture surface and a suspended stainless-steel probe; a representative sample of culture was then diverted through the valve into an overflow-receiving vessel. Growth rates were calculated from the rates of flow of culture into the receiving vessel (see Equation 11). A more detailed description of the construction and operation of the turbidostat system is given in Appendix 1.

c. Maximum growth rates.

Steady states of biomass and nutrient concentrations were established very soon after the application of density-control. Culture 65 (Fig. V.4.) was inoculated at a density of 0.21 x 10^6 cells ml^{-1}. After six days (zero turbidostat time - Fig. V.4), when the culture density was 0.60 x 10^6 cells ml^{-1}, density-control was applied at a threshold level slightly below that culture density. The system responded by repeated dilution of the culture until the control density was reached. Biomass estimations beginning 30 hours after control was applied show relative stability in all parameters (Fig. V.4A). The growth rate recorded (0.183 day^{-1}, td = 89 hours) was close to the maximum growth rate found in normal batch cultures of Acer at that time (0.226 day^{-1}, td = 73 hours). The steady-state concentrations in the culture vessel of the four major nutrients analysed were very high (at least 60 % of their input concentrations). (Fig. V.5A). When the opacity threshold was stepped up, by adjustment of a potentiometer in the bridge circuit, the culture biomass increased and stabilised at a higher density (Fig. V.4B and Fig. V.5B). The growth rate (0.192 day^{-1}, td = 86 hours) was again close to maximum and nutrient concentrations were still high. The size and composition of the cells under these conditions was close to that expected for cells growing at μmax in batch cultures and close to μmax in chemostats (Table V.2 and Fig. V.22).

d. Limits of operation.

Data from culture 65 suggest that the growth rate of turbidostat cultures of Acer is maximal and independent of cell density and nutrient concentration within a specific range of cell densities.

Fig. V.4. Steady states at high growth rates in a turbidostat culture of *Acer* cells.

At the transition point indicated, the balance point of the density-detection Wheatstone bridge circuit (Appendix 1, Fig. 23) was raised by a potentiometer adjustment.

Fig V 4

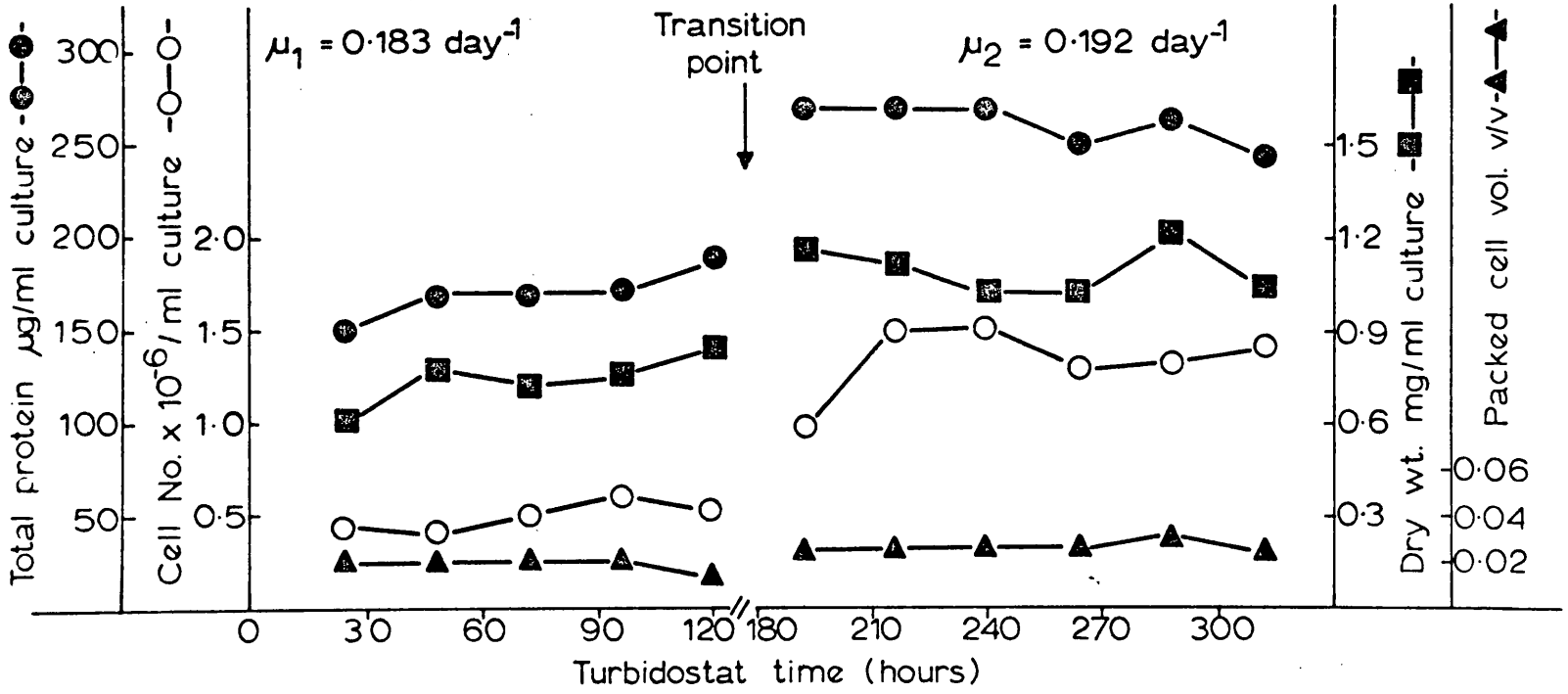

Total protein µg/ml culture
Cell No. x 10⁻⁶/ml culture
Dry wt. mg/ml culture
Packed cell vol. v/v
µ1 = 0·183 day⁻¹
Transition point
µ2 = 0·192 day⁻¹
300
250
200
150
100
50
2·0
1·5
1·0
0·5
1·5
1·2
0·9
0·6
0·3
0·06
0·04
0·02
0
30
60
90
120
180
210
240
270
300
Turbidostat time (hours)

Fig. V.5. The effect of cell density on growth rate and nutrient concentration in turbidostat cultures of Acer cells.

States A and B and the transition between them were as described in Fig. V.4. A further adjustment of State B produced State C.

The concentrations of three nutrients in the spent medium of the culture are listed above each state as a % of their concentration in the input medium.

Fig. V. 5.

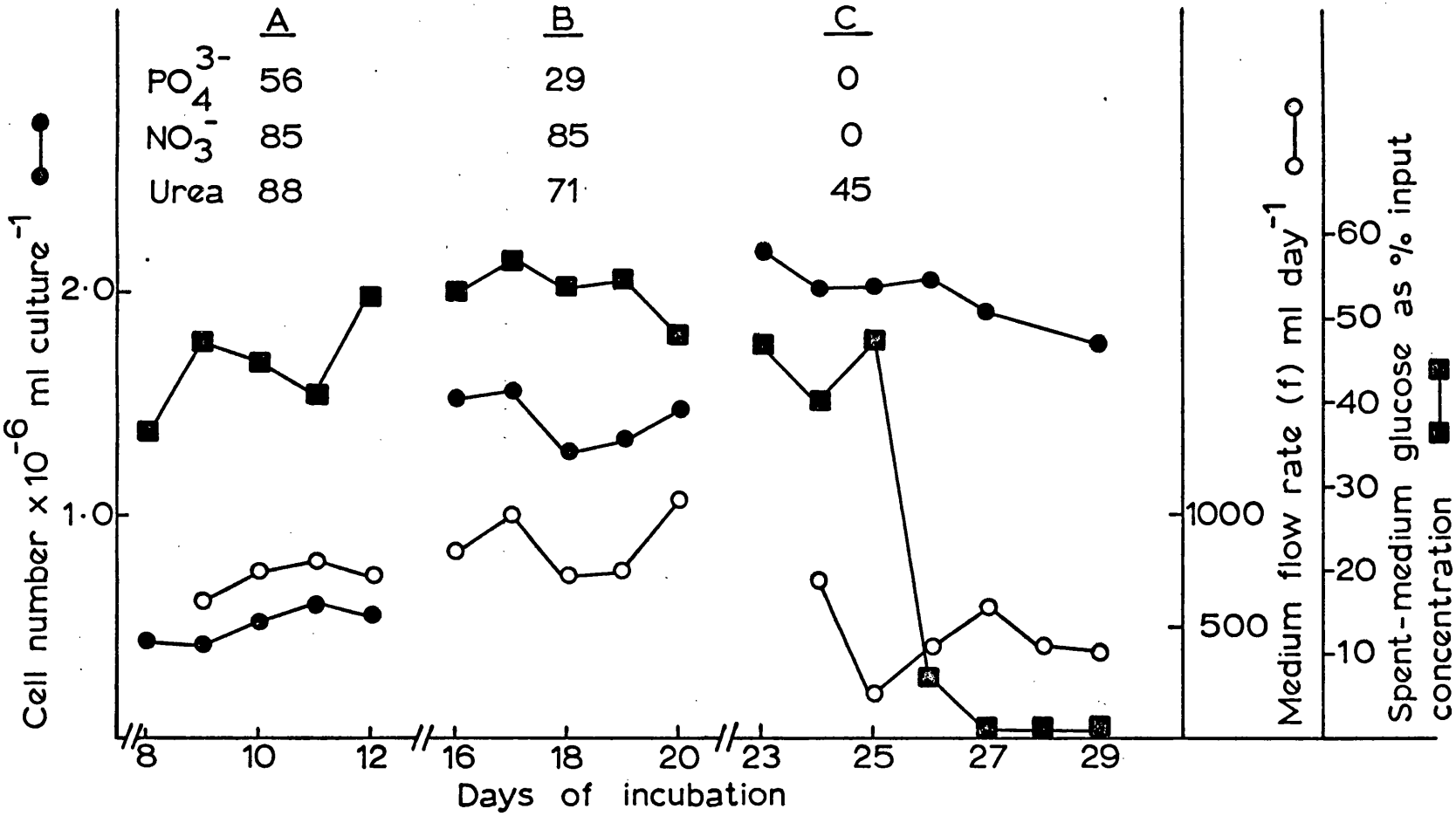

A
B
C
PO_4^{3-} 56 29 0
NO_3^- 85 85 0
Urea 88 71 45
Cell number x 10^{-6} ml culture^{-1}
2·0
1·0
1000
500
Medium flow rate (f) ml day^{-1}
60
50
40
30
20
10
Spent-medium glucose as % input concentration
8
10
12
16
18
20
23
25
27
29
Days of incubation

Table V.2. Comparison of cell size and composition of cells in steady states in turbidostat cultures (A) with cells in chemostat cultures (B), closed-continuous culture systems (C) and batch cultures (D).

	Culture N°	td h	Cell Number $X10^{-6}ml^{-1}$	Protein $\mu g/10^6$cells	Cell Volume $\mu l/10^6$cells	Dry weight $mg/10^6$ cells
A.	40	70	0.750	350	40.0	1.66
	46	83	0.850	298	48.5	1.86
	65	90	0.510	338	37.4	1.49
B.	66	83	1.030	346	37.0	1.36
	54	263	3.770	157	57.2	1.92
C.	57	80	16.300*	50	18.0	0.80
D.	84	58	1.400*	451	35.0	1.50

*The cell number at the point in the exponential growth phase from which the cell composition data were taken.

A second step-up of the opacity threshold in culture 65 resulted in an apparently stable biomass level (Fig. V.5C). However, the rate of flow of medium into the culture dropped markedly to give a new and lower growth rate of 0.10 day^{-1} (td = 166 hours). The concentrations of NO_3^- (by day 24) and of PO_4^{3-} and glucose (by day 27), under these conditions, declined to zero in the culture vessel. In a turbidostat, dilution is entirely dependent upon continued growth (or changes in culture opacity); in this turbidostat culture of Acer cells, growth (and therefore dilution) ceased under these nutrient conditions by day 33. Data for a large step-up of culture density are given in Fig. V.6. The growth rate of the initial low-density culture was 0.236 day^{-1} (td = 70 hours). Dilution restarted after the step-up transition at a rate of 0.096 day^{-1} (td = 150 hours) but eventually ceased on day 20. Although the cell number and cell dry weight increased by 200% to reach the upper threshold level, total culture protein increased by only 50%, presumably due to depletion of nitrogen during the transition. Culture 46 (Fig. V.7) was inoculated at a density of 0.35×10^6 cells ml^{-1} and allowed to grow up as a batch culture for several days. A density threshold was set at the density reached by day 13 (1.5×10^6 cells ml^{-1}). Dilution began immediately at a rate of 0.135 day^{-1} (td = 125 hours) but the flow rate fell gradually from 600 to 230 ml day^{-1} and then dilution ceased. The density threshold was then stepped down. The step-down adjustment of the density-control circuit instigated rapid, continuous dilution until the culture stabilised at a density 50% lower. Intermittent dilution at a rate of 0.20 day^{-1} (td = 83 hours) followed after a short lag. The culture was maintained in this state for 14 days. A high

Figures V.6 and V.7. Transitions between different biomass levels in turbidostat cultures of *Acer* cells.

Biomass adjustments were made as described in Fig. V.4. except that lowering the balance point of the control circuit in culture 46 (Fig. V.7) produced a continual inflow of fresh medium until the cell density had been diluted to below the threshold level.

The time scale (in hours) on the figures represents periods when the cultures were under turbidity control.

Fig V 6

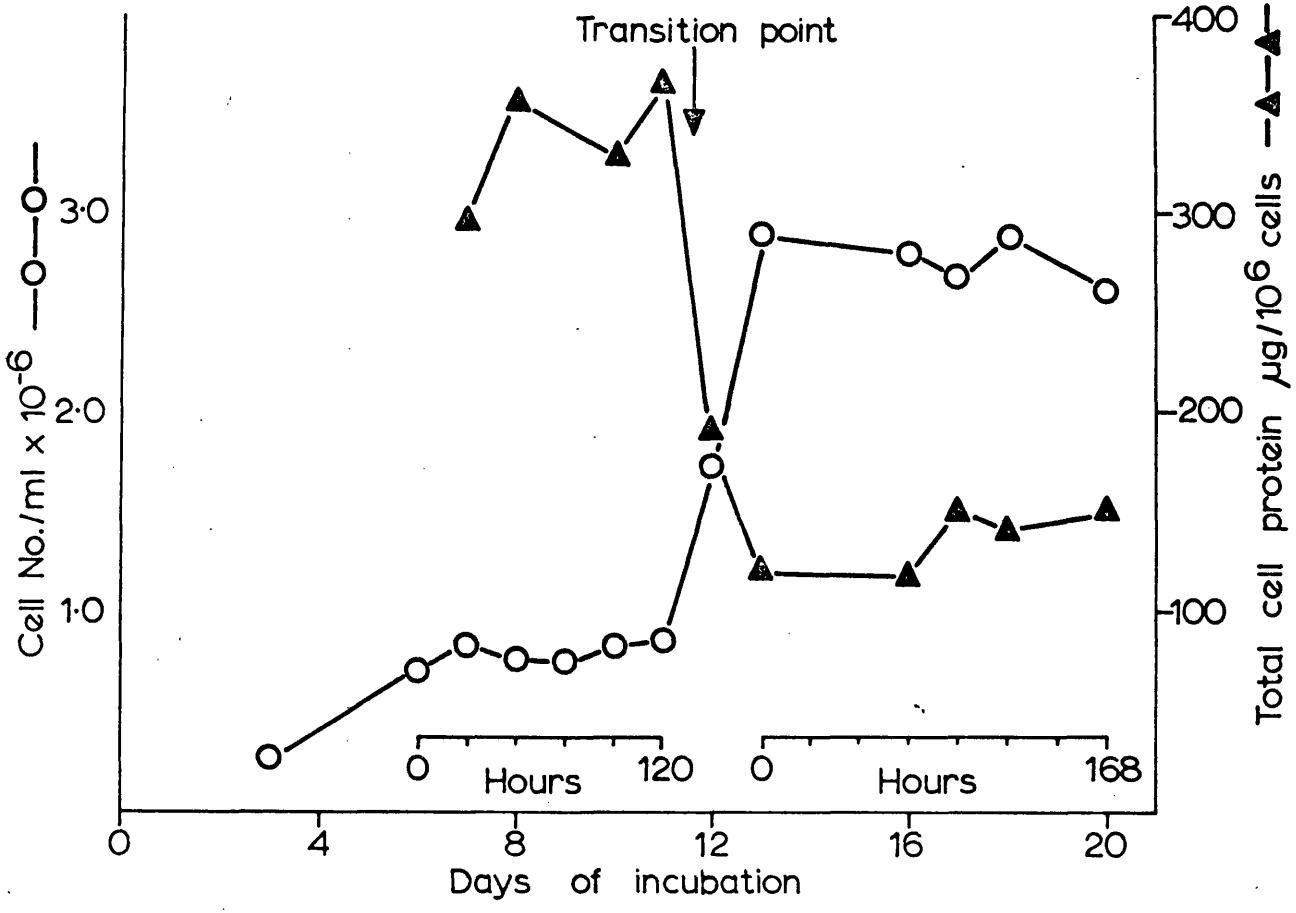
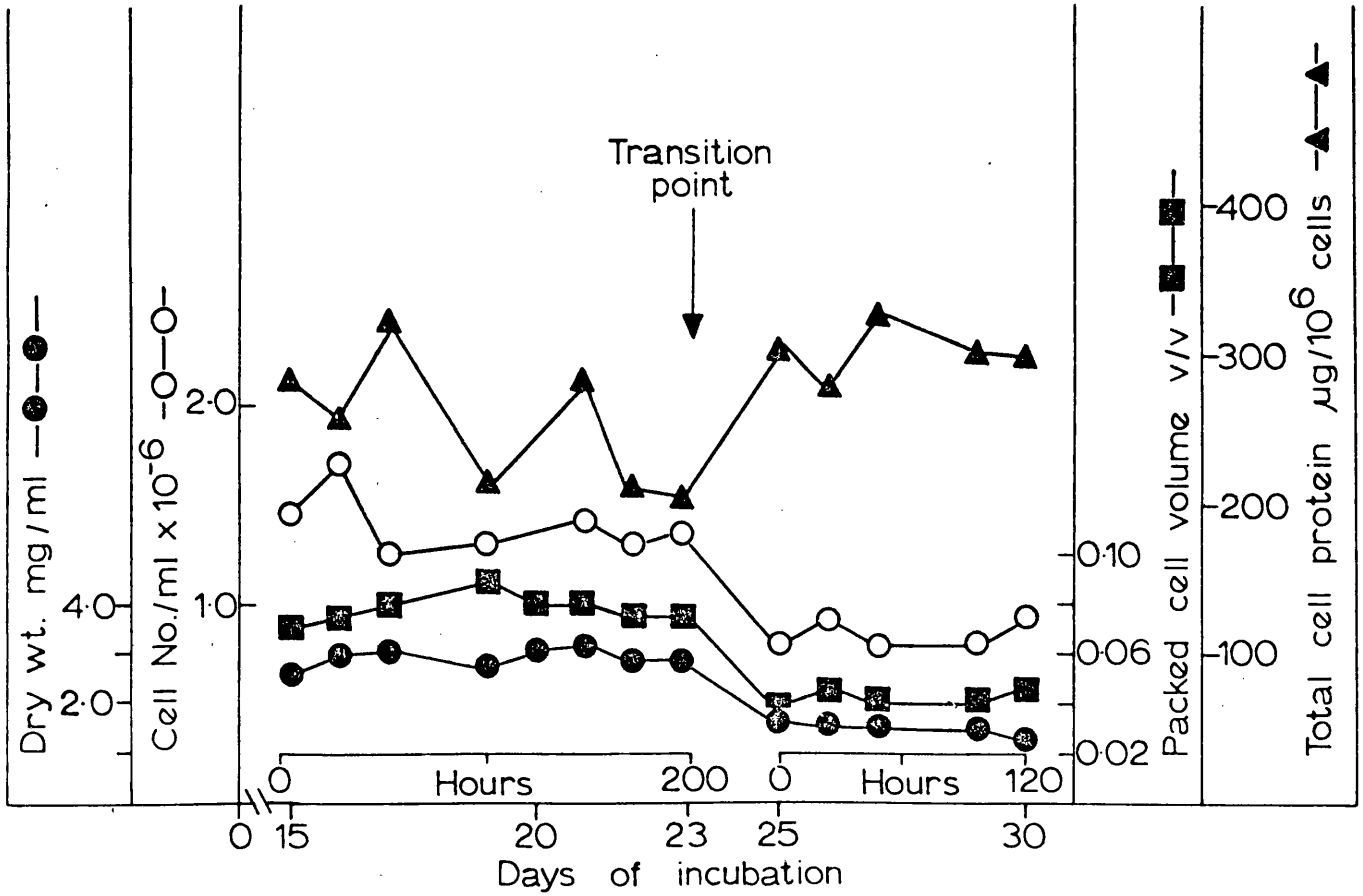

Transition point
Cell No./ml x 10^{-6}
3·0
2·0
1·0
400
300
200
100
Total cell protein µg/10^6 cells
0
Hours
120
0
Hours
168
0
4
8
12
16
20
Days of incubation

Fig V 7


Transition point
Dry wt. mg/ml
Cell No./ml x 10⁻⁶
Packed cell volume v/v
Total cell protein µg/10⁶ cells
4·0
2·0
2·0
1·0
0·10
0·06
0·02
400
300
200
100
0
Hours
200
0
Hours
120
0
15
20
23
25
30
Days of incubation

rate of protein synthesis, stimulated by the step-down dilution, produced a net increase in the protein per cell in the high-growth-rate population.

4. Chemostat cultures

a. Effect of chosen dilution rate

Chemostat theory suggests that a culture of dividing cells diluted at any rate, D (where $D < \mu_{max}$), must inevitably reach a steady state (Monod, 1950; Powell, 1965). This would appear to be so for suspension cultures of Acer pseudoplatanus. A simple test of the theory as applied to Acer is illustrated in Fig. V.8. Culture 54 was established as a 4-litre batch culture, in the apparatus described in Appendix 1, Fig. 18, at a density of 0.3×10^6 cells ml^{-1}. After 13 days incubation, when the culture was still in the exponential growth phase ($\mu_{cell\ number} = 0.219\ day^{-1}$), a continuous flow of fresh medium was admitted to the culture at an arbitrarily chosen rate (f = 260 ml day^{-1}; D = 0.065 day^{-1}; td = 220 hours). A constant culture-volume was maintained as described above (Section V.3). Substitution of $\mu = 0.219\ day^{-1}$ and D = 0.065 day^{-1} in Equation 7. suggests that the cell number should continue to increase exponentially in this culture at a rate of 0.154 day^{-1} until a steady state is established. The rate of increase of cell number in culture 54 between day 13 and day 20 (when the cell number became constant) was 0.151 day^{-1}. Equilibrium levels of packed cell volume and biomass dry-weight were reached by day 25. There was good agreement between the predicted (0.140 day^{-1}) and actual (0.145 day^{-1}) rates of increase of packed cell volume. However, the rate of increase in biomass dry-weight appears to have been enhanced by dilution. The initial rate of dry weight increase

Fig. V.8. The effect of an arbitrarily chosen dilution rate on the biomass and glucose concentration of a 4-litre culture of Acer cells.

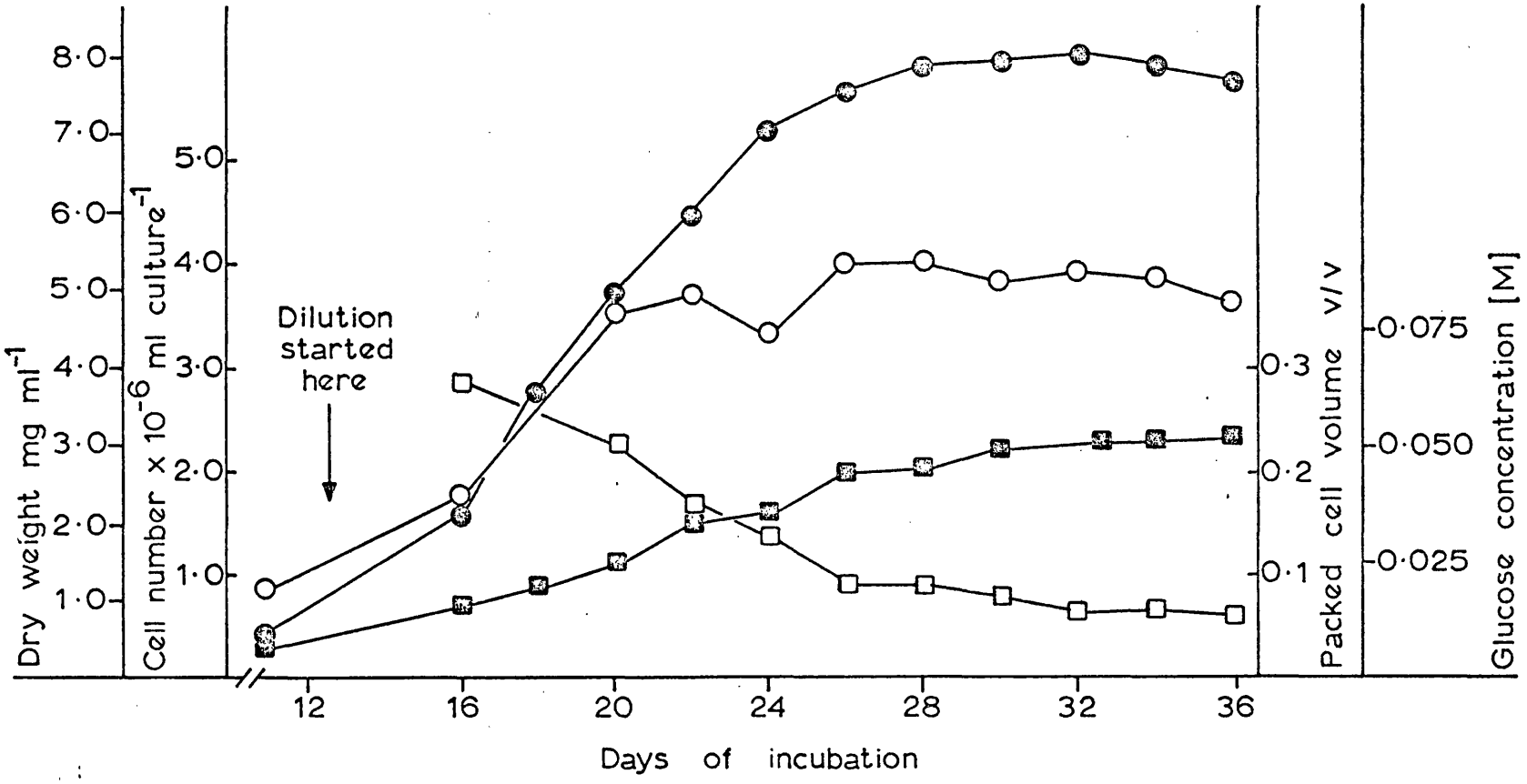
The culture was established in the apparatus described in Appendix 1, Fig. 18.

The dilution rate was 0.065 day^{-1} (flow rate = 260 ml day^{-1}; td = 220 hours).

● Dry weight ■ Packed cell volume

o Cell number □ Glucose concentration.

Fig. V. 8.


Dry weight mg ml^{-1}
Cell number x 10^{-6} ml culture^{-1}
Packed cell volume v/v
Glucose concentration [M]
Dilution started here
Days of incubation
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
5·0
4·0
3·0
2·0
1·0
0·3
0·2
0·1
0·075
0·050
0·025
12
16
20
24
28
32
36

(ca. 0.14 day^{-1}) during the batch-culture phase of culture 54 was, as expected (Section IV), less than the rate of increase in cell number. The rate of dry-weight increase during dilution (0.19 day^{-1}) was, however, about twice that predicted (0.082 day^{-1}), suggesting an apparent increase in μ_{max} (dry weight) to ca. 0.25 day^{-1}. A similar stimulation of biomass dry-weight was noted in two other chemostats (see also Section V.2.).

b. Selected steady states.

Equilibrium biomass-densities were maintained in chemostat cultures of Acer pseudoplatanus for up to 800 hours (ca. 12 cell generations) without perturbation. Selected examples of steady states in three separate chemostat cultures are shown in Figs. V.9, V.10 and V.11. The biomass of the cultures remained constant throughout the dilution periods; lines of best fit through the data all show zero slope. Most of the differences between adjacent readings are not significant at the 5% level. In addition to stability in biomass parameters, several environmental factors did not vary significantly e.g. the concentrations of free nutrients in the cultures (Figs. V.10 and V.11), pH (Fig. V.11) and oxygen tension (Fig. V.11).

c. Range of dilution rates applied.

The Monod equations (see, for example, Equation 12) suggest that steady states of cells at all growth rates below their μ_{max} may be established by selection of an appropriate dilution rate. The steady states of Acer cells shown in Figs. V.9, V.10 and V.11 were at three different growth rates. Furthermore, the biomass densities of these three steady states appear to be inversely proportional to the dilution rates applied. Data in Fig. V.12 show the range of dilution rates

Fig. V.9. Equilibrium biomass levels in a chemostat culture of *Acer* cells.

Dilution rate from zero time = 0.210 day^{-1} (td = 80 h).

The carbon source was sucrose (2%), medium pH 5·2.

See Appendix 1, Fig. 18 for apparatus used.

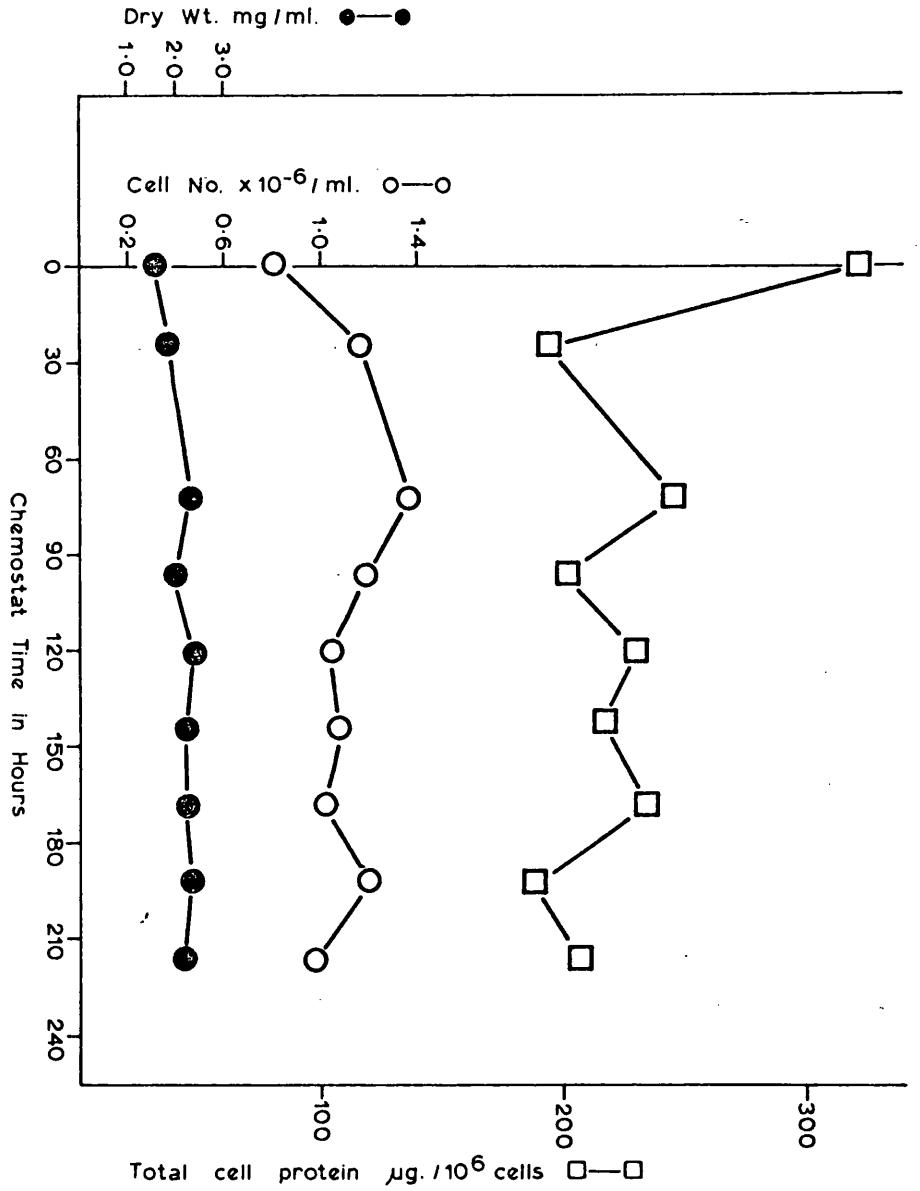

Dry Wt. mg/ml. ●—●
1·0
2·0
3·0
Cell No. x10⁻⁶/ml. O—O
0·2
0·6
1·0
1·4
0
30
60
90
120
150
180
210
240
Chemostat Time in Hours
100
200
300
Total cell protein µg./10⁶ cells □—□

Fig V9

Fig. V.10. Equilibrium biomass levels and glucose concentration in a chemostat culture of Acer cells.

Dilution rate from zero time = 0·185 day^{-1} (td = 88 h).

Carbon source was glucose (2%), medium pH 6·4.

See Appendix 1, Fig. 18 for apparatus used.

● Dry weight ■ Packed cell volume

○ Cell number □ Glucose concentration

▲ Total protein

Dry weight mg ml^{-1}
1·0
2·0
3·0
4·0
Cell number x 10^{-6} ml culture^{-1}
1·0
2·0
3·0
Chemostat time in hours
100
200
300
400
500
0·05
0·10
0·15
Packed cell volume v/v
0·025
0·050
0·075
0·100
Glucose concentration [M]
100
200
300
400
Total cell protein µg 10^{6} cells^{-1}

Fig. V. 10.

Fig. V.11. Equilibrium biomass levels, nutrient concentrations and pH in a chemostat culture of *Acer* cells.

Dilution rate from zero time = 0·194 day^{-1} (td = 86h).

Samples (50 ml) were withdrawn at intervals for biomass measurements, nutrient analysis and respiration rate determinations. Culture opacity and pH were monitored continuously in the culture vessel.

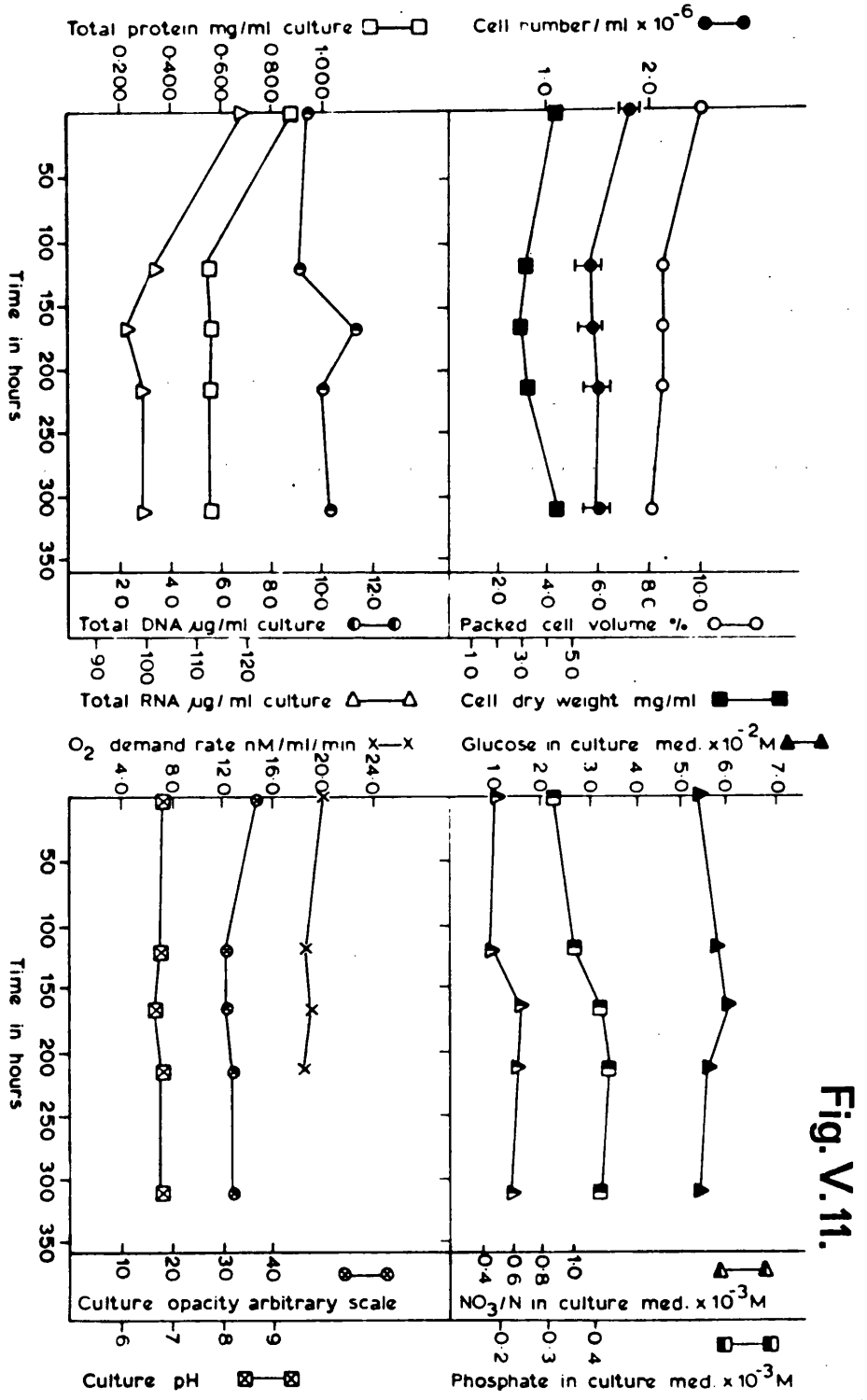

Total protein mg/ml culture
Cell number/ml x 10^-6
Time in hours
Total DNA µg/ml culture
Packed cell volume %
Total RNA µg/ml culture
Cell dry weight mg/ml
O2 demand rate nM/ml/min
Glucose in culture med. x10^-2M
Culture opacity arbitrary scale
NO3/N in culture med. x10^-3M
Culture pH
Phosphate in culture med. x10^-3M

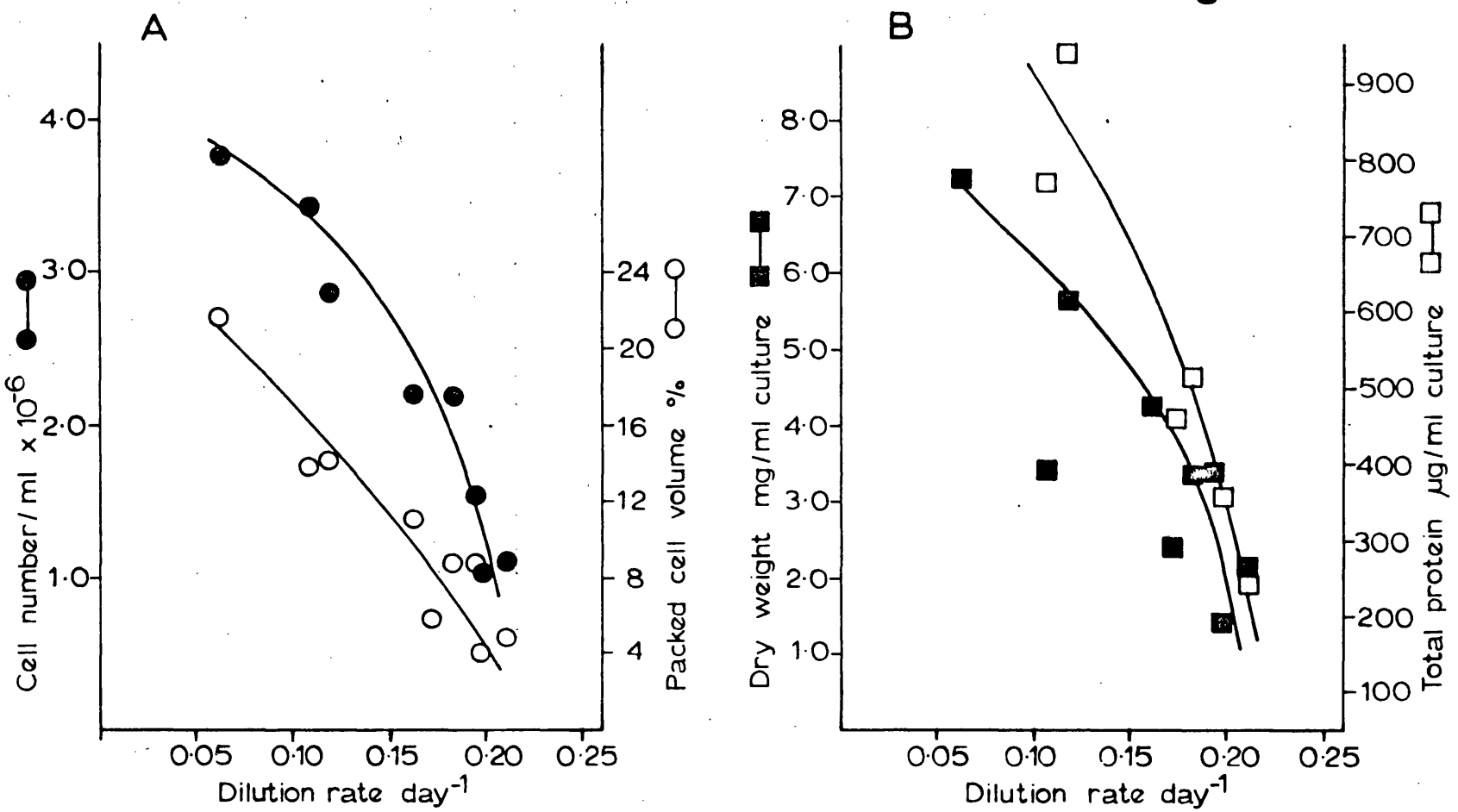
Fig. V.11.

Fig. V.12. Relationship between steady-state biomass and dilution rates in chemostat cultures of Acer cells.

Each point is the mean of values obtained during steady states at the dilution rates indicated.

The standard error of these means were all $< 10\%$ of the mean values.

Fig.V.12.


A
4·0
3·0
2·0
1·0
Cell number / ml x 10⁻⁶
24
20
16
12
8
4
Packed cell volume %
0·05
0·10
0·15
0·20
0·25
Dilution rate day⁻¹
B
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Dry weight mg/ml culture
900
800
700
600
500
400
300
200
100
Total protein µg/ml culture
0·05
0·10
0·15
0·20
0·25
Dilution rate day⁻¹

within which steady states of Acer cells were established. Each point in the curve for each parameter represents the mean of a series of values obtained at intervals during a steady state established at the dilution rate indicated. These data were obtained during nine different steady states (each in excess of 400 hours duration) in five separate chemostat cultures. The uniform trend in biomass density with changing dilution rate suggests that the growth of Acer cells in chemostats may be limited by the concentration of a single nutrient. However, the cells appear to have a relatively low affinity for this nutrient (the saturation coefficient, K_s, must be high relative to the input concentration, S_R - see Equation 20) and/or the yield of biomass per unit of nutrient consumed declines with increased growth rate (the yield coefficient, Y - Equation 20 - is not a constant). [By substitution of hypothetical values of K_S and Y in Equation 20 it may be demonstrated that, where K_S is low and Y is constant, the steady-state biomass density will decline only slightly with increased growth rate until close to μ_{max} when a steep decline in biomass will occur .] The data in Fig. V.12 also suggest that there is an upper limit to dilution rates compatible with stable cultures. Extrapolation of the cell-number curve in Fig. V.12 to zero biomass gives a value of 0.22 day^{-1} for the maximum dilution rate. The Monod equations give this dilution rate (the critical dilution rate, D_C) as

$$D_C = \mu_{max} \left(\frac{S_R}{K_S + S_R} \right) \qquad 21$$

Thus the critical dilution rate is equal to the highest possible value of μ, when the steady-state concentration of the limiting nutrient, S, is equal to its concentration in the inflowing medium, S_R (when consumption

is negligible). If K_S is low relative to S_R, then $D_C \simeq \mu_{max}$. Substitution of $S_R = 7 \times 10^{-3}M \left[NO_3^-\right]$ and $K_S = 0.13 \times 10^{-3}M \left[NO_3^-\right]$ (values obtained with NO_3^- - limited growth - see below, Fig. V.15) in equation 21, gives $\mu_{max} = 0.224\ day^{-1}$. It is interesting to note that extrapolation of the data in Fig. V.12 gives similar values for μ_{max} whichever biomass parameter is chosen.

d. Wash-out.

An equilibrium state should not be reached in cultures diluted above the critical dilution rate; as $D > \mu_{max}$, cells will be continuously washed out of the culture vessel. Furthermore, by manipulation of the basic equation for a steady state (Equation 7), it is possible to predict the washout-rate or conversely to calculate μ_{max} (see Equation 13) for a culture deliberately diluted at a rate above D_C. Data shown in Fig. V.13 were obtained from an Acer chemostat culture diluted at a rate of $0.274\ day^{-1}$. Steady states had previously been established in this culture at growth rates of $0.0625\ day^{-1}$, $0.107\ day^{-1}$ and $0.182\ day^{-1}$ (total steady-state time = 1560 hours) before the critical dilution rate was exceeded. At $D = 0.274\ day^{-1}$, cells were washed outat a rate of $0.045\ day^{-1}$ (Fig. V.13A). Substitution in Equation 13 gives $\mu_{max} = 0.229\ day^{-1}$. However, total biomass (fresh weight - Fig. V.13C - or total protein Fig. V.13A) was washed out more rapidly ($0.070\ day^{-1}$) giving $\mu_{max} = 0.207\ day^{-1}$. The concentration of free glucose in the culture (a non-limiting nutrient - Fig. V.13B) rose rapidly towards saturation. (Unfortunately, no data are available from this culture for the limiting nutrient - NO_3^- - but see Fig. V.14). These wash-out data do not show conclusively that no equilibrium state would be

Fig. V.13. "Wash-out" of Acer cells from a chemostat culture diluted at a rate greater than the critical dilution rate.

The dilution rate was stepped-up from 0·182 day^{-1} to 0·274 day^{-1} at zero time.

A. Decay in cell number (Δ) and protein (▲) per millilitre of culture.

B. Decline in oxygen demand (●) and increase in glucose concentration (o) (input glucose concentration = 0·11M).

C. Decline in the total output of biomass per day (■) and in the daily yield per litre of culture (□) (the latter is equal to the biomass concentration in the culture vessel).

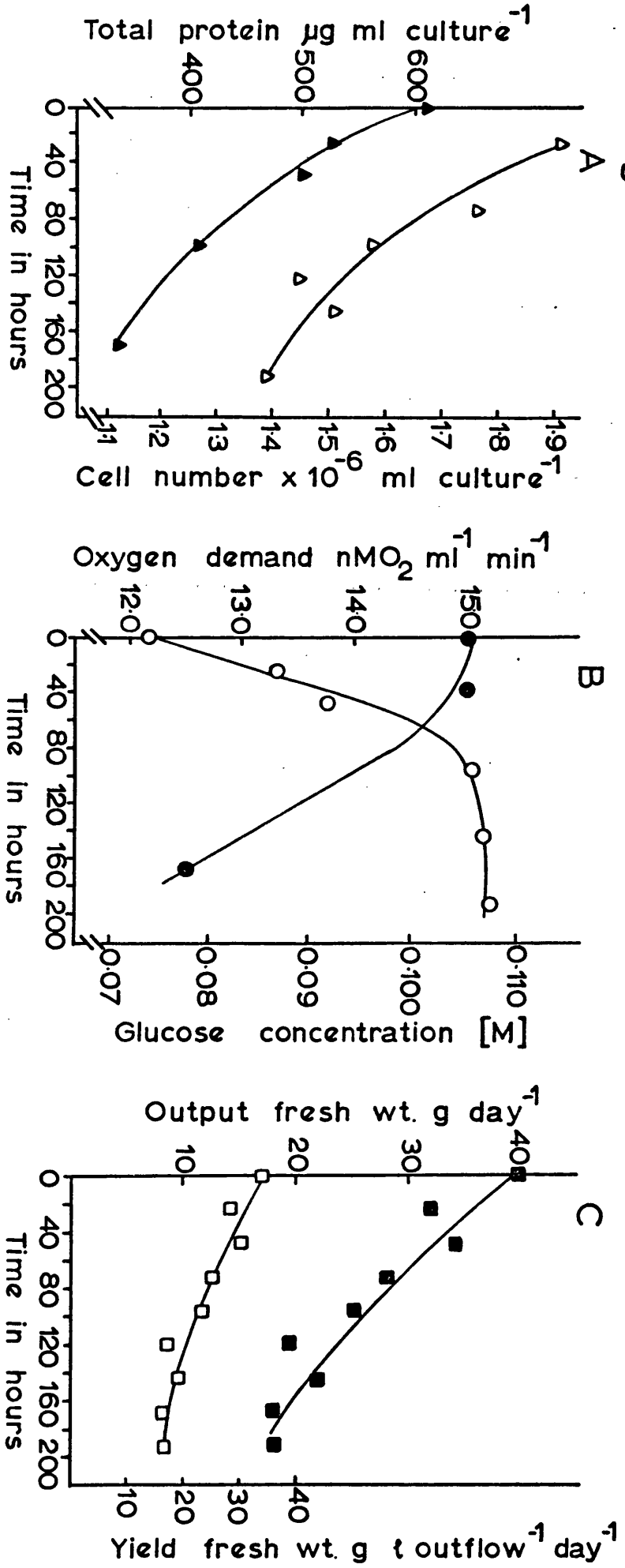

Total protein µg ml culture^{-1}
400
500
600
A
Time in hours
0
40
80
120
160
200
1·1
1·2
1·3
1·4
1·5
1·6
1·7
1·8
1·9
Cell number x 10^{-6} ml culture^{-1}
Oxygen demand nMO_2 ml^{-1} min^{-1}
12·0
13·0
14·0
15·0
B
0·07
0·08
0·09
0·100
0·110
Glucose concentration [M]
Output fresh wt. g day^{-1}
10
20
30
40
C
Yield fresh wt. g t outflow^{-1} day^{-1}

Fig. V. 13.

achieved at this dilution rate. However, the sustained, logarithmic decay of the biomass suggests that the cells in these conditions had achieved a constant (maximum) growth rate, which was independent of cell density.

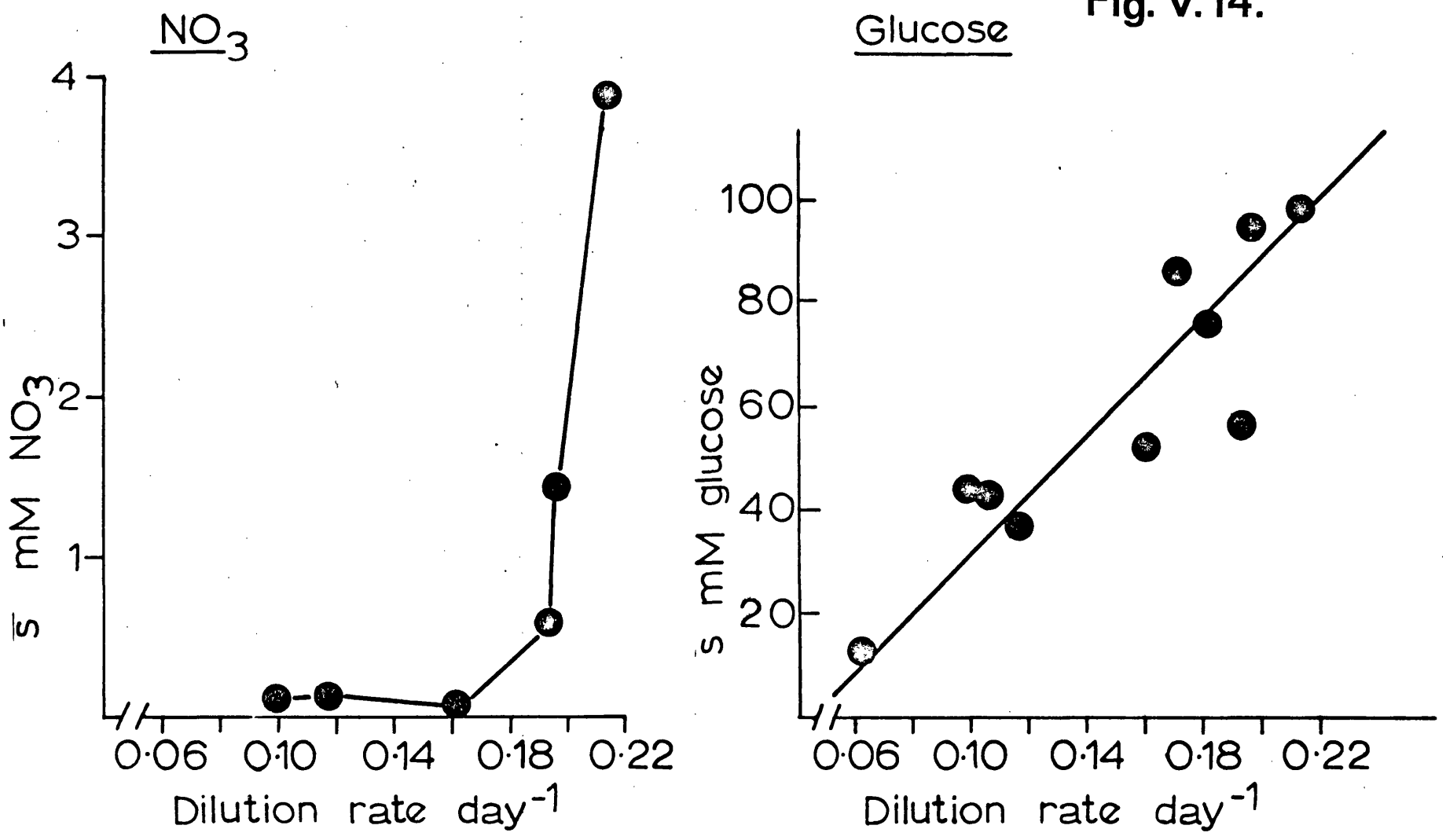
e. The limiting nutrient.

Both the turbidostat and chemostat cultures of Acer described above were established using the slightly modified basal synthetic medium normally used to propagate stock suspensions of Acer cells (see II. Materials and Methods.1.). No attempt was made to reduce the concentration of one specific constituent to the point where it might act as the limiting nutrient required by chemostat theory. However, evidence from batch cultures suggests that nitrate (or total nitrogen) limits cell division in the basal medium. Using this medium, Henshaw et al (1966) showed clearly that the extent of the exponential growth phase (in particular the high rate of cell division) of suspension cultures of Parthenocissus tricuspidata depended upon the initial concentration of nitrate in the medium. Young (1973) reports that the yield of Acer cells in batch culture was predictably enhanced by increasing the initial nitrate concentration in the basal medium. Furthermore, nitrate is depleted from the medium in batch cultures of Acer cells more rapidly than other nutrients assayed (Section IV.3).

Examination of the steady-state concentrations of four major nutrients in chemostat cultures of Acer showed that the relationship between NO_3^- concentration (Fig. V.14A) and dilution rate differed from that of other nutrients e.g. glucose (Fig. V.14B) and urea. At dilution rates below ca. 0.18 day^{-1} the concentration of residual NO_3^- in the culture spent-medium (Fig. V.14A) was very low ($< 0.1 \times 10^{-3}$M, close to the limits of detection by the method used).

Fig. V.14. Steady-state concentrations of nitrate and glucose in chemostat cultures of Acer cells at different dilution rates.

Fig. V.14.


NO3
4
3
2
1
s mM NO3
0·06
0·10
0·14
0·18
0·22
Dilution rate day-1
Glucose
100
80
60
40
20
s mM glucose
0·06
0·10
0·14
0·18
0·22
Dilution rate day-1

As D_c was approached the concentration of NO_3^- rose rapidly towards saturation. However, the steady-state concentrations of glucose (Fig. V.14B), urea and PO_4^{3-} (see for example, Fig. V.11), though low at very low dilution rates, were normally at least 25% of the input concentrations over most of the dilution-rate range examined although data for urea is rather variable. The data in Fig. V.14A for NO_3^- more closely resembles the relationship between the steady-state concentration of a limiting nutrient and dilution rate suggested by Equation 19 than does the data for glucose in Fig. V.14B. Rearranging the data for NO_3^- (Fig. V.14A) and plotting μ (= D) (ordinate) against S (abscissa) produces a Michaelis-Menten-like curve (Fig. V.15A) of the sort expected from Monod's substrate/growth rate equation (Equation 12). Equation 12 may be adjusted to

$$\frac{1}{\mu} = \frac{1}{S} \cdot \frac{K_s}{\mu_{max}} + \frac{1}{\mu_{max}} \qquad 22$$

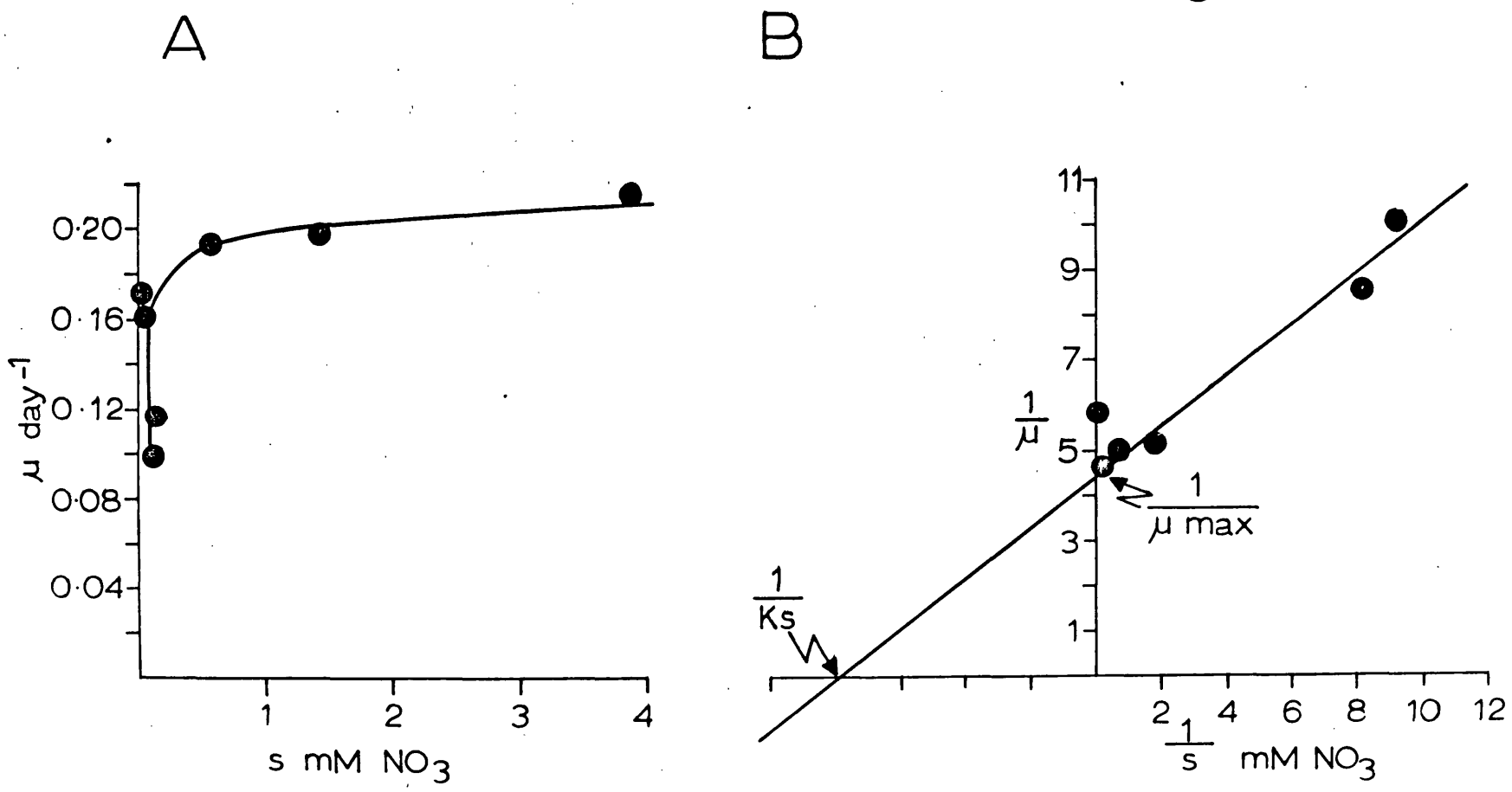
(as suggested by Fencl, 1966) so that values of K_s and μ_{max} may be obtained from a Lineweaver-Burk plot (Fig. V.15B). Although only a limited amount of data was available for this plot, the line of best fit through the data gives $\mu_{max} = 0.225\ day^{-1}$. This agrees with data from standard batch cultures (Section IV), turbidostats (Section V.3) and the extrapolated data for chemostats in Fig. V.12. The saturation coefficient (K_s) for NO_3^- as the limiting nutrient is given as 0.13×10^{-3}M. This value is three orders of magnitude greater than K_s values for amino-nitrogen in bacterial cultures. A similar high value (0.032×10^{-3}M) for K_s was reported by Wilson (1971) for a phosphate-limited chemostat of Acer cells, in contrast to $K_s = 1.5 \times 10^{-6}$ M PO_4^{3-} for bacterial cultures. In general, saturation coefficients

Fig. V.15. Relaticnship between specific growth rate and the steady-state concentration of nitrate in 4-litre chemostat cultures of Acer cells.

The specific growth rates were calculated from the dilution rates applied (Equation 11, Section V).

Values obtained from the Lineweaver-Burk plot (B) were $\mu_{max} = 0{\cdot}225\ day^{-1}$ and $K_s(NO_3) = 0{\cdot}13 \times 10^{-3}M$.

Fig. V. 15.


A
B
0·20
0·16
0·12
0·08
0·04
μ day⁻¹
1
2
3
4
s mM NO3
11
9
7
5
3
1
1/μ
1/Ks
1/μ max
2
4
6
8
10
12
1/s mM NO3

for bacteria are very low (e.g. 2.0×10^{-9}M tryptophan; 1.35×10^{-4}M glycerol - Fencl, 1966), though values for the same substrate may be very different with different bacterial species (e.g. 2.2×10^{-5} - 2.2×10^{-2}M glucose). It is perhaps to be expected that the affinity of plant cells for their substrates will be lower than for micro-organisms which have a higher metabolic rate; rates of uptake and assimilation may in general be lower in higher plant cells than in micro-organisms. However, the low affinities recorded here may also be due to slower interchange of nutrients in the micro-space in the vicinity of the cells due to imperfect culture-mixing and cell aggregation. High affinity (saturation) coefficients make far less efficient production of biomass from continuous cultures (Equation 20); substrate utilization will not be maximal over a wide range of dilution rates. Therefore, it may become important, for economic reasons, to investigate the kinetics of the growth of plant cells in single-stage, recycled continuous cultures or multi-stage continuous cultures.

f. Perturbation of the limiting nutrient.

Confirmation of NO_3^- as the limiting nutrient in the basal medium of Acer cultures was obtained by step-up and step-down perturbations of the concentration of NO_3^- in the inflowing medium at a constant dilution rate. These perturbations also served to test further how far the response of Acer cells to perturbations of their steady-state environment could be predicted by adopting the equations derived by Monod for "ideal", free-cell bacterial populations.

The rate of change of concentration of the limiting nutrient (ds/dt) in a steady state is zero (see Fig. V.11). Thus, Equation 16 may be rewritten

$$DS_R - D_S - \frac{\mu x}{y} = 0 \qquad 23$$

As $\mu = D$ in a steady state (see Equation 7), this equation (23) may be further simplified

$$D(S_R - S) = \frac{\mu x}{y} \qquad 24$$

and so, $x = y\,(S_R - S)$ (Equation 20).

Thus as Y is constant (at least at a constant growth rate) and S, the steady-state concentration of the limiting nutrient, is negligible, the steady-state biamass density, x, should be directly proportional to the concentration of limiting nutrient in the inflowing medium, S_R. At a constant growth-rate, a perturbation of S_R should produce a predictable change in the biomass density. The kinetics of the change from one biomass density to another may also be predicted:

The rate of change of biomass density at a dilution rate, D, and population growth-rate, μ, was given in Equation 7 as

$$dx/dt = \mu x - Dx$$

The rate of increase in biomass due to growth (μx) is directly related to the rate of supply of the limiting nutrient, DS_R; from equation 24,

$$\mu x = D\,(S_R - S)\,Y \qquad 25$$

or, as S is negligible,

$$\mu x = D \cdot S_R \cdot Y \qquad 26$$

Substituting for μx in Equation 7 gives

$$dx/dt = D \cdot S_R \cdot Y - Dx \qquad 27$$

As might be expected, Equation 27 suggests that when S_R is increased dx/dt is positive and x will increase; when S_R is decreased, x will decrease. But it is clear from Equation 26 that the growth rate depends not only upon the rate of supply of nutrient, DS_R, but on the culture

biomass, x, to which the nutrient is supplied. Thus after a switch from one concentration of inflowing nutrient to another ($S_R \rightarrow S_R^1$) at a constant dilution rate, D, dx/dt will vary with x. Integration† of Equation 27 gives

$$\frac{dx}{S_R^1 Y - x} = D \int_o^t dt \qquad 28$$

and thus $$\left[\log(S_R^1 Y - x)\right]_{x_o}^{x} = -Dt \qquad 29$$

$$\log (S_R^1 Y - x) - \log (S_R^1 Y - x_o) = -Dt \qquad 30$$

Then, as in the steady state $x_o = S_R \cdot Y$ (Equation 26)

$$\log \frac{(S_R^1 Y - x)}{S_R^1 Y - S_R Y} = -Dt \qquad 31$$

or $$\frac{S_R^1 Y - x}{Y(S_R^1 - S_R)} = e^{-Dt} \qquad 32$$

Dividing the left-hand side by S_R gives

$$\frac{(Y\, S_R^1/S_R - x/S_R)}{Y(S_R^1/S_R - 1)} = e^{-Dt} \qquad 33$$

and thus

$$x = S_R \left[Y S_R^1/S_R - Y\,(S_R^1/S_R - 1)\, e^{-Dt}\right] \qquad 34$$

Equation 34 gives the change in biomass, x, with time after a switch in the concentration of the limiting nutrient, S_R to S_R^1. (No reference to such an equation was found in the literature).

† I am grateful to Dr. R. Semeonoff for help with this integration.

Figs. V.16, V.17, V.18 and V.19 show data obtained from two chemostat cultures of Acer cells in which steady states were perturbed by a change in the concentration of NO_3^- in the input medium. In each case the new steady-state biomass and the manner in which this would be approached were calculated from Equations 20 and 34 and are indicated on the graphs. In general, the biomass densities reached fit closely those predicted (Table V.3) confirming that nitrate is the limiting nutrient in chemostat cultures supplied with the basal medium. However, the increase in total protein in culture 93 (Fig. V.17) exceeded that predicted by 33%. In the steady state before perturbation, 0.934 moles of protein/N were produced per mole of NO_3^-/N consumed; after the perturbation this yield factor had increased to 1.312. The excess nitrogen was obtained from urea: the steady-state concentration of urea before the perturbation (3.04×10^{-3}M) was 46% of saturation (cf. $0.1 \times 10^{-3}MNO_3^-$ - 1% of saturation) and it was calculated, for the same rate of consumption, that urea would fall to 31% of saturation following the perturbation. However, urea was at less than 11% of saturation after 2000 hours. Furthermore, after the perturbation, 0.8 moles of protein/N were produced per mole TOTAL/N consumed (cf. 0.6 before perturbation). The apparent relationship between urea and nitrate metabolism in these cultures has not been examined further. It is surprising that, in the presence of an alternative nitrogen source (Simpkins, Collin and Street, 1970) nitrate should have appeared to be to limiting nutrient in the basal medium. Yet, whilst in the normal cultural conditions the capacity of the mechanisms for the assimilation of urea and nitrate, or the mechanisms themselves (including the pathways and the end-products of the pathways) may be so different that nitrate

Figures V.16 and V.17. Changes in the biomass (x) of a 4-litre chemostat culture of *Acer* cells after an increase in the concentration of nitrate in the inflowing medium (S_R).

The culture was diluted at a constant rate (0·153 day^{-1}). The yield coefficients (Y) for the first steady state were 0·263 x 10^6 cells per µmole NO_3^- and 0·934 moles protein/N per mole NO_3^-. Protein/N was measured indirectly; all total protein determinations were made using the Folin-Ciocalteau (F-C) reagent (II. Materials and Methods. 12), and converted to protein/N by assuming a nitrogen content of 16% w/w (Long, 1961). To calculate more meaningful yield coefficients for these experiments, the determination of protein/N by the F-C technique was compared with the direct assay of nitrogen by a micro-Kjeldahl method and a correction (x 1·2) applied to the F-C results.

After 420 hours of steady-state growth, the concentration of nitrate in the medium supplied (S_R) was stepped-up directly from 7 x 10^{-3}M to 10·5 x 10^{-3}M.

The expected biomass levels for the new steady state (Equation 20) and the expected rates of change of biomass (Equation 34) were calculated and are indicated on the figures.

Fig. V. 16.

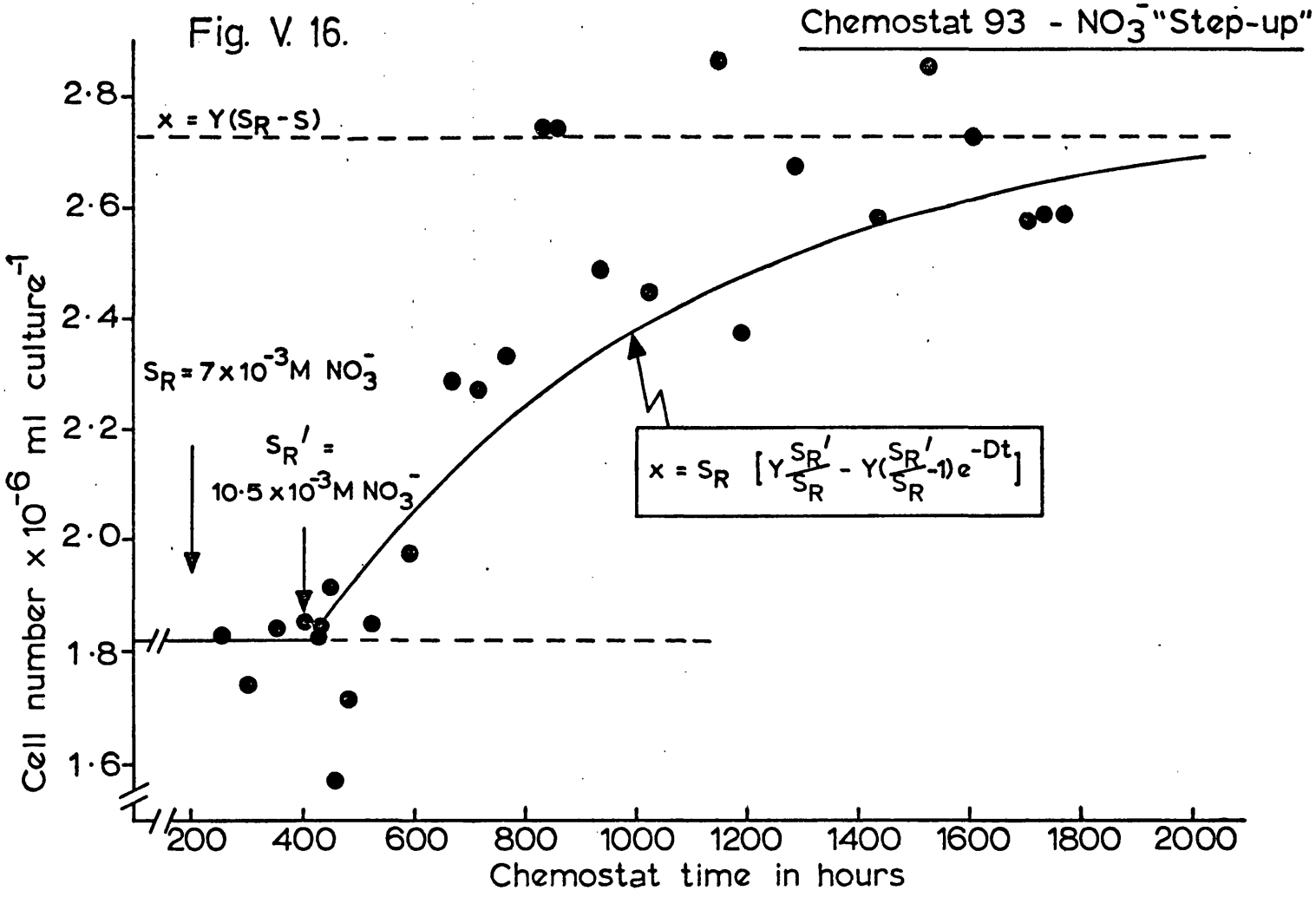
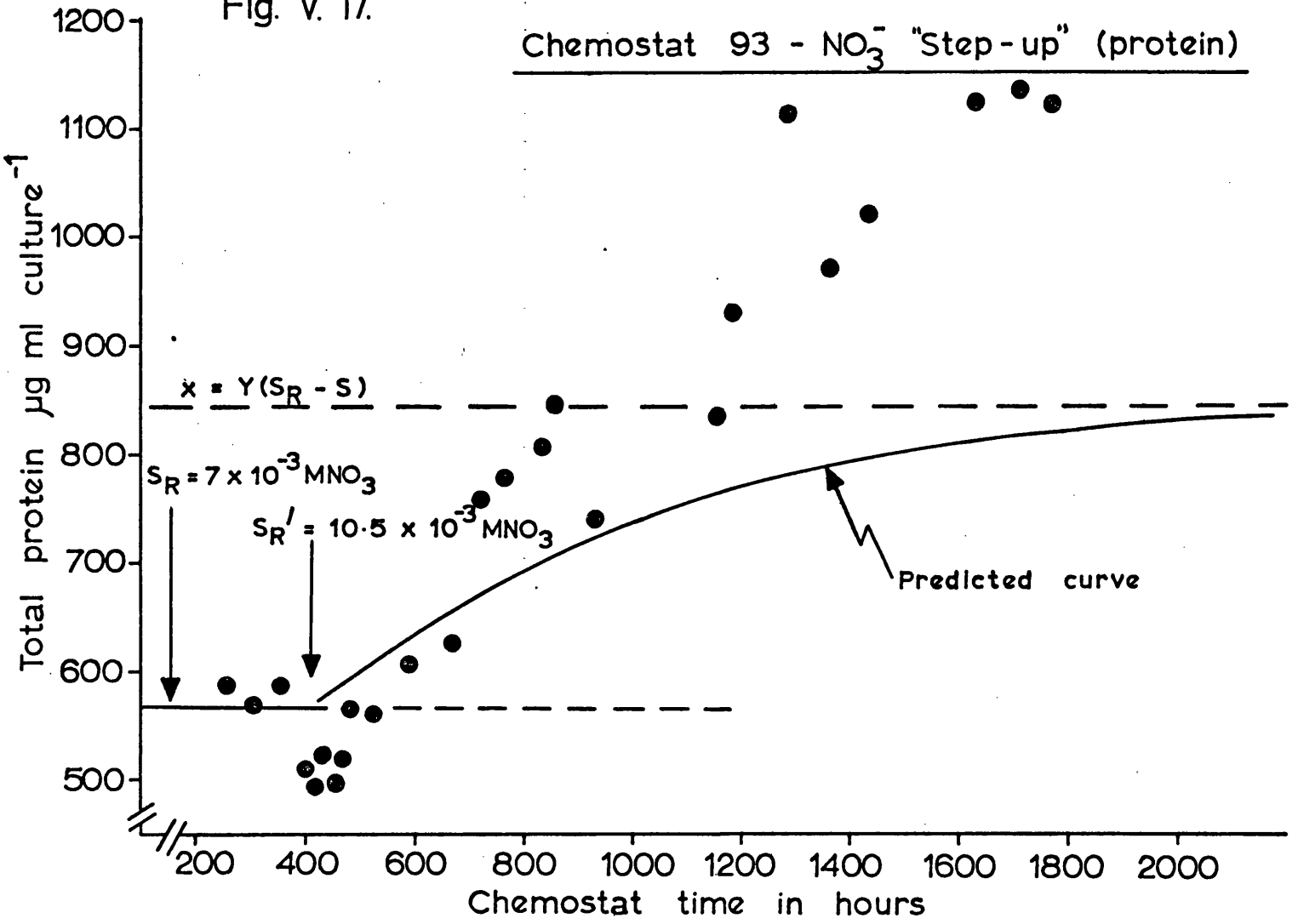

Chemostat 93 - NO_3^- "Step-up"
$x = Y(S_R - S)$
$S_R = 7 \times 10^{-3}$M NO_3^-
$S_R' = 10.5 \times 10^{-3}$M NO_3^-
$x = S_R \left[Y\frac{S_R'}{S_R} - Y\left(\frac{S_R'}{S_R} - 1\right)e^{-Dt} \right]$
Cell number $\times 10^{-6}$ ml culture^{-1}
2·8
2·6
2·4
2·2
2·0
1·8
1·6
200
400
600
800
1000
1200
1400
1600
1800
2000
Chemostat time in hours

Fig. V. 17.


Chemostat 93 - NO_3^- "Step-up" (protein)
Total protein μg ml culture^{-1}
1200
1100
1000
900
800
700
600
500
$x = Y(S_R - S)$
$S_R = 7 \times 10^{-3} MNO_3$
$S_R' = 10{\cdot}5 \times 10^{-3} MNO_3$
Predicted curve
200
400
600
800
1000
1200
1400
1600
1800
2000
Chemostat time in hours

Figures V.18 and V.19. Changes in the biomass (x) of a 4-litre chemostat culture of Acer cells after a decrease in the concentration of nitrate in the inflowing medium (S_R).

The culture was diluted at a constant rate ($0{\cdot}142\ day^{-1}$). The yield coefficients (Y) for the first steady state were $0{\cdot}344 \times 10^6$ cells per μmole NO_3^- and 1·14 moles protein/N per mole NO_3^-.

Protein/N was determined as described in the legend to Figs. V.16 and V.17.

After 360 hours of steady-state growth, the concentration of nitrate in the medium supplied (S_R) was stepped-down directly from $8{\cdot}75 \times 10^{-3}$M to $3{\cdot}5 \times 10^{-3}$M.

The expected biomass levels for the new steady state (Equation 20) and the expected rates of change of biomass (Equation 34) were calculated and are indicated on the figures.

Fig. V. 18.

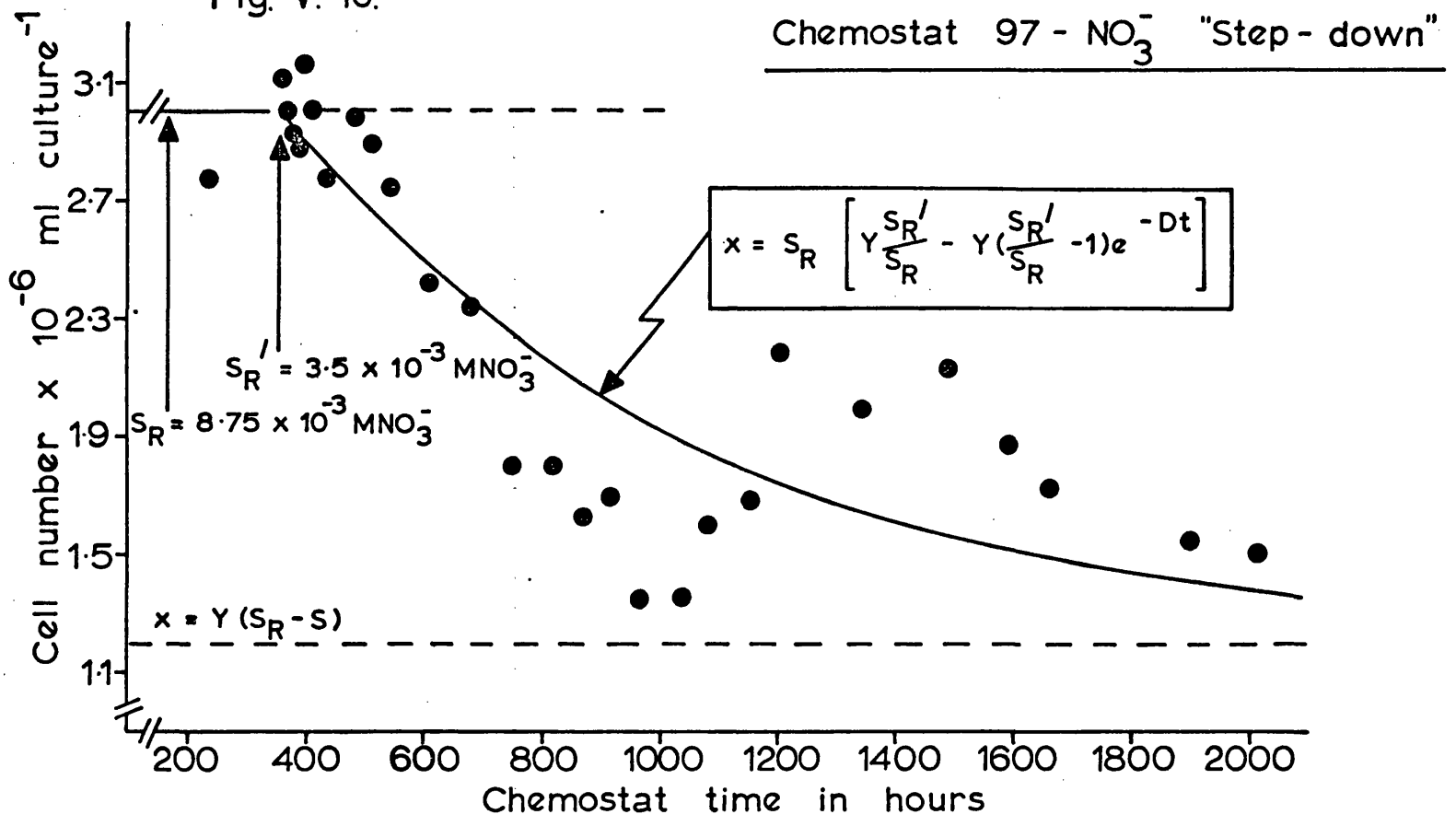

Chemostat 97 - NO_3^- "Step - down"
Cell number x 10^{-6} ml culture^{-1}
3·1
2·7
2·3
1·9
1·5
1·1
$x = S_R \left[Y\frac{S_R'}{S_R} - Y\left(\frac{S_R'}{S_R} - 1\right)e^{-Dt} \right]$
$S_R' = 3{\cdot}5 \times 10^{-3}$ MNO_3^-
$S_R = 8{\cdot}75 \times 10^{-3}$ MNO_3^-
$x = Y(S_R - S)$
200
400
600
800
1000
1200
1400
1600
1800
2000
Chemostat time in hours

Fig. V. 19.

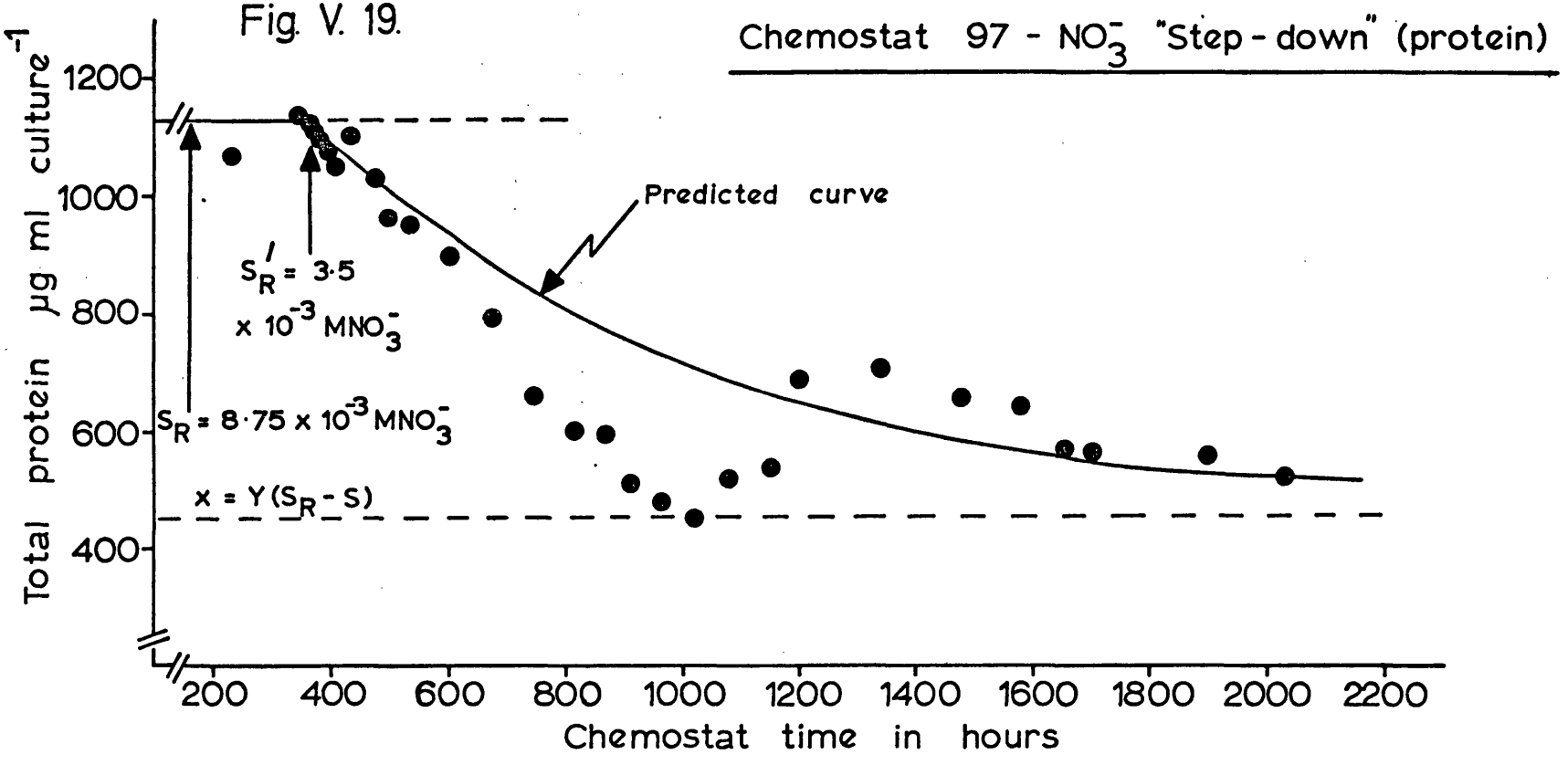

Chemostat 97 - NO_3^- "Step-down" (protein)
Total protein µg ml culture^{-1}
1200
1000
800
600
400
$S_R' = 3.5 \times 10^{-3} MNO_3^-$
Predicted curve
$S_R = 8.75 \times 10^{-3} MNO_3^-$
$x = Y(S_R - S)$
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
Chemostat time in hours

Table V.3. Comparison of actual biomass density (χ_A) and predicted biomass density (χ_p) following a perturbation of nitrate in the input medium.

Culture N°.	χ_A/χ_p FOR EACH BIOMASS PARAMETER					
	CELL NUMBER	DRY WEIGHT	P.C.V.	TOTAL PROTEIN	TOTAL RNA	DNA
93	0.984	1.069	1.019	1.340	1.180	0.898
97	1.199	1.081	0.946	1.072	1.130	1.095

Culture 93 received a "step-up" perturbation and culture 97 a "step-down".

may limit growth independently of urea, it is possible that under changed cultural conditions the balance between the assimilation of the two nitrogen sources (or their end-products) might change. There were considerable changes in cultural conditions following the perturbation referred to above, all related to the increase in biomass: dissolved oxygen concentration fell, pH rose slightly (7.0 to 7.2), the concentrations of the non-limiting nutrients e.g. glucose and phosphate declined. Such a metastable balance between nitrate or total-nitrogen limitation may also explain the occasional cultures whose biomass density was not as expected (see culture 78, Table V.5).

The kinetics of the changes in biomass following perturbation of the two cultures (93 and 97) do not entirely fit the derived equation (Equation 34). In culture 93, although total protein production (Fig. V.17) rose more rapidly than predicted and to a level higher than predicted, the calculated line for the change in cell number lies well within the standard error of the mean of the cell number estimations at most sampling times (Fig. V.16). An oscillation in the biomass of culture 97 (Figs. V.18 amd V.19) following the step-down perturbation makes it difficult to decide whether the data does or does not fit the predicted curve. If the cause of the oscillation was operative at 600-700 hours and produced a trough in the data, then (excluding the trough) the response is as predicted. Alternatively, if the cause operated from 1000 hours and produced the peak, then the biomass density appears to have declined to the predicted level but at a more rapid rate than predicted. The data in Fig. V.20, showing rates of change (semi-logarithmic plots) of biomass and endogenous pools of nitrate and α-amino nitrogen after the perturbation of culture 97, suggest that the latter is the correct interpretation. The "ideal"

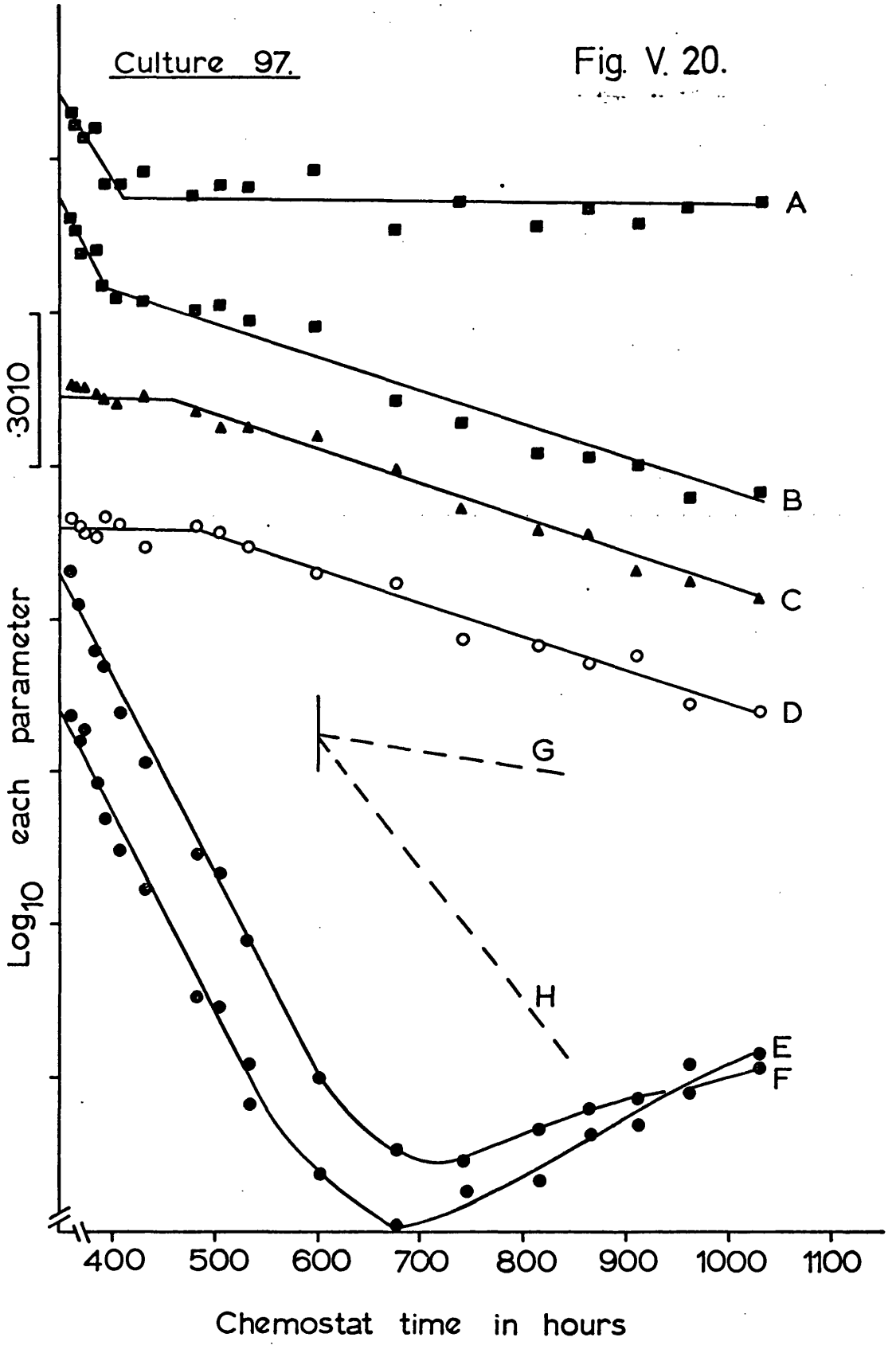
Fig. V.20. Changes in biomass and in endogenous pool-sizes of nitrate and α-amino nitrogen after a step-down in the concentration of nitrate in the medium flowing into a 4-litre chemostat culture of Acer cells.

The step-down in culture 97 is also shown in Figures V.18 and V.19. The nitrate concentration was changed directly from $8{\cdot}75 \times 10^{-3}$M to $3{\cdot}5 \times 10^{-3}$M after 360 hours of steady-state growth.

Semi-logarithmic plots of:

A. Cell α-amino nitrogen (moles per 10^6 cells)
B. cell α-amino nitrogen (moles per ml culture)
C. total cell protein (moles protein/N per ml culture)
D. cell number (10^6 cells per ml culture)
E. cell nitrate (moles per 10^6 cells)
F. cell nitrate (moles per ml culture)
G. expected rate of decrease in biomass (from Equation 34)
H. dilution rate, D, ($x = x_o e^{-Dt}$)

A 50% decay is shown against the ordinate.


Culture 97.
Fig. V. 20.
A
B
C
D
G
H
E
F
.3010
Log10 each parameter
400
500
600
700
800
900
1000
1100
Chemostat time in hours

population of cells for which Equation 34 was derived would have no endogenous storage pools of the limiting nutrient or intermediates and, therefore, the response to nutrient starvation would be immediate. Furthermore, the response to an increasing availability of limiting nutrient as the biomass density declined would be immediate and would not depend upon replenishing pools of precursors or competition between cells for nutrients. However, Acer cells contain pools of free amino-acids and nitrate (Fig. V.20). In the steady-state population of culture 97, prior to the perturbation, the concentrations of the free α-amino (Fig. V.20A) and nitrate (Fig. V.20E) pools were 0.156×10^{-6} moles per 10^6 cells (3.72×10^{-6} moles ml cell volume^{-1}) and 0.645×10^{-6} moles per 10^6 cells (15.35×10^{-6} moles ml cell volume^{-1}) respectively. The exogenous pool of nitrate was very small (equivalent to 0.0129×10^{-6} moles per 10^6 cells) but also constant i.e. the rate of nitrate assimilation = the rate of supply = 1.243×10^{-6} moles ml culture^{-1} day^{-1}. When the rate of supply of nitrate was reduced (by changing S_R) there was no immediate change in the rates of cell division or protein synthesis. The biomass remained constant for a further 150 hours (Fig. V.20C and D). The reduced rate of supply of nitrate to the culture was 0.5×10^{-6} moles ml culture^{-1} day^{-1}. Both the endogenous nitrate and α-amino pools decayed at the rate of 0.15×10^{-6} moles ml culture^{-1} day^{-1} (calculated from the per cell data). The total consumption of nitrogen from the exogenous supply and the endogenous pools was thus ca. 0.8×10^{-6} moles ml culture^{-1} day^{-1} and appeared to be sufficient (at least initially) to support the same steady-state biomass (both in cell number and total protein) at the same growth rate. However, 80 hours after the perturbation the α-amino pool stabilised (Fig. V.20A) thus reducing total nitrogen supply to ca. 0.65×10^{-6} moles ml culture^{-1} day^{-1} and

shortly afterwards the biomass declined due to wash-out of cells growing at a reduced rate. The size of the pool of α-amino nitrogen (amino acids) in the cells thus appears to have closely regulated the rate of protein synthesis (and thus the rate of cell division). The pool declined by only 25%, presumably due to an imbalance between the rate of supply of amino acids from nitrate assimilation and the rate of consumption of amino acids by the pre-existing protein synthesising machinery, before the rate of protein synthesis itself was reduced. The reduced growth rate of the biomass present at the time of the perturbation does not appear to have been linked as tightly to the rate of supply of exogenous nitrate as it was before the perturbation. The decline in biomass density (Fig. V.20D) is greater at all times than that predicted for the new concentration of nitrate supplied. The nitrate pool, having been reduced to 19% of its original level (Fig. V.20E), decayed at a decreasing rate as the biomass-density began to decline. Then, after 700 hours, the pool began to expand again. It appears, therefore, that the capacity for nitrate <u>assimilation</u> had been restricted by the perturbation and that excess nitrate taken up by the cells was accumulating in endogenous pools. It is difficult to be sure that this reduced capacity for nitrate assimilation is sufficient to account for the difference between actual and predicted growth rates. It is also difficult to decide whether the reduced rate of nitrate assimilation is due to repression of nitrate reductase in the presence of low pool levels of nitrate, or whether the nitrogen deficiency produced immediately after the perturbation caused the decay of a significant proportion of the protein synthesising machinery (e.g. the acyl-tRNA synthetases), with the nitrate reductase system then being regulated by feed-back control via the α-amino pool. (The perturbation experiments were not designed

to investigate the regulation of nitrogen assimilation of Acer cells but it is clear that, by also examining the in vitro activities of the enzymes involved, similar perturbations of steady states of higher plant cells might be of great use in such work - see Young, 1973. Especially useful in this context is the facility of estimating the effective in vivo activities of enzymes by calculating rates of consumption of their substrates in the steady state).

It is significant that, although in the steady state prior to perturbation of culture 97 the cells consumed some urea (0.293×10^{-6} moles urea/N 10^6 cells^{-1} day^{-1}), urea was not used as an alternative to nitrate during the perturbation and the exogenous urea concentration rose from 7% to 42% of saturation by 800 hours. The consumption of urea did not change (0.211×10^{-6} moles urea/N 10^6 cells^{-1} day^{-1}). If, as has been suggested (Young, 1973) urease synthesis in Acer cells is subject to negative control by the α-amino pool, in particular by glutamate, then the decline in the α-amino pool after the perturbation might be expected to have increased the rate of urea utilization. However, the α-amino pool remained constant after reaching a level lower than in the previous steady state (Fig. V.20A) and the rates of urea assimilation did not change. Urea is not completely depleted from the basal medium in chemostats of Acer despite the fact that extractable urease activity greatly exceeds the urea assimilation rate in such cultures (Young, 1973). Therefore, it is possible that the generally strict limitation of growth in chemostat cultures of Acer cells by nitrate in the presence of excess urea is due either to a very low substrate affinity of urease or of the transport system for urea or, alternatively, to some form of specific fine control of the activity of urease in vivo. The substantial increase in the rates of protein synthesis and cell division which occurred at

1000 hours (Figs. V.18 and V.19) was accompanied by a decline in the urea concentration in the spent medium from 42% to 7% of saturation within 72 hours. The transient, additional output of protein/N by the culture could be accounted for entirely by the increased urea/N consumption. The transformation of the culture to enhanced urea utilization is analagous to the effect noted in culture 93. The shortness of the period over which the transformation occurred (the reverse process at about 1500 hours was equally as rapid) suggests some short-term modification of the uptake mechanism for urea or of the *in vivo* activity of the urease enzyme. Damped oscillations of chemostat cultures of algae and bacteria after step-function changes in steady-state conditions have been described (Kubitschek, 1970). It remains to be seen whether the oscillation noted in culture 97 is typical of perturbed chemostat cultures of higher plant cells.

g. Yield coefficient.

Equations (19) and (20) suggest that the steady-state concentrations of cells and limiting nutrient depend solely on the values of S_R and D (μ_{max}, K_S and Y being assumed to be constants); steady-state concentrations of cells and nutrient may be predicted for any values of S_R and D once the values of the three growth constants are known. However, the dependence of Y, the yield coefficient, on the dilution rate has been reported for bacterial cultures (Herbert, Elsworth and Telling, 1956; Fencl, 1966), for algae (Foley and Syrett, 1973) for animal cells (Sinclair, 1966) and for *Acer* cells limited by PO_4^{3-} (Wilson, 1971). Data in Fig. V.21 show that *Acer* cells limited by NO_3^- also deviate from the "Monod theory" in this respect; Y decreases with increasing dilution rate both as a function of cell number

(Fig. V.21A) and of total protein (Fig. V.21B). The variation in Y appears to be due to the availability of urea as an additional nitrogen source. A value of ca. 0.175 (10^6 cells μmole nitrate^{-1} was obtained for Y from representative batch cultures of Acer cells by considering only those cells produced by the time that NO_3^- is depleted from the culture medium in the late exponential growth phase (see Section IV). (The batch cultures were initiated using the same modified medium that was used for all of the continuous cultures described; see II. Materials and Methods. 2). This value of Y (marked 'b' on Fig. V.21A) is close to the values obtained in chemostats maintained at growth rates close to μ_{max}. However, a higher value for Y (ca. 0.500) is obtained from batch cultures if the total cell population present when the culture enters stationary phase is considered, It is not unusual for the cell population to double between NO_3^- depletion and the onset of the stationary phase. This value for Y (marked 'a' on Fig. V.21A) is close to the value obtained by extrapolating the data for the variation in Y with dilution rate in chemostats (Fig. V.21A) to zero dilution rate ("no growth" = stationary phase?). A very similar variation in the yield of protein/N from NO_3^- consumed was found both in batch cultures and chemostats (Fig. V.21B). At high growth rates in chemostats and at NO_3^- depletion in batch cultures, Y (moles total protein/N produced per mole NO_3^- consumed) is almost unity, but Y $\simeq$ 2 at low growth rates and in stationary phase cultures.

In the absence of significant pools of NO_3^- and α-amino nitrogen in Acer cells at the time of medium - NO_3^- depletion in batch culture (Young, 1973), continued protein accumulation after NO_3^- depletion can only be at the expense of the alternative nitrogen source in the medium viz. urea. In the modified medium used in all continuous cultures

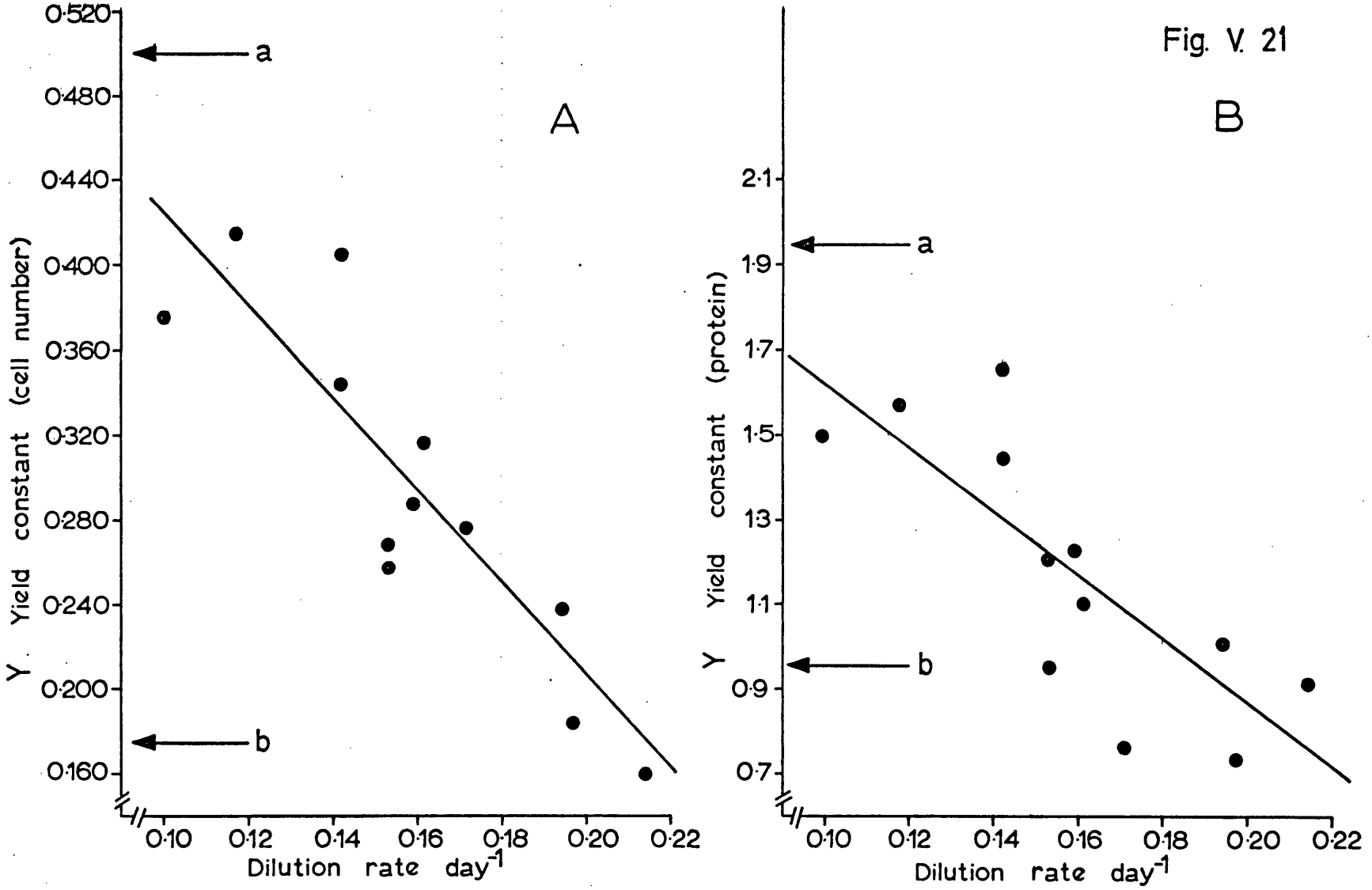
Fig. V.21. The relationship between yield constants (Y) and dilution rate in 4-litre chemostat cultures of Acer cells.

Yield constants expressed as : A. 10^6 cells per µmole NO_3^-

B. moles protein/N per mole NO_3^-.

'a' = the value of Y for batch cultures when the final total cell population is considered; the y-intercepts for the regression lines shown are 0.54 (A) and 2.4 (B).
'b' = the value of Y for batch cultures when only the cell population present at NO_3^- - depletion is considered.

Fig. V. 21


A
B
Y Yield constant (cell number)
Y Yield constant (protein)
0·520
0·480
0·440
0·400
0·360
0·320
0·280
0·240
0·200
0·160
2·1
1·9
1·7
1·5
1·3
1·1
0·9
0·7
a
b
0·10
0·12
0·14
0·16
0·18
0·20
0·22
Dilution rate day^{-1}

described here (but possibly not in the normal culture medium - see Section IV, and II.Materials and Methods.2) up to 50% of the medium urea may remain at the time of NO_3 depletion in batch cultures of Acer. Assimilation of this residual urea together with the small free-nitrogen pools would account for 70-75% of the protein/N accumulating after NO_3^- depletion. The slow uptake of urea from the medium in batch cultures (relative to NO_3^-) may possibly be due to a high K_m for urease or for the urea transport system. If this were so, it would follow that cells with high residence times in chemostats (i.e. at low dilution rates; residence time = 1/D) would utilise more of the available urea than cells with low residence times. The biomass-protein produced per unit of limiting nutrient consumed would increase with decreasing dilution rates despite the fact that the limiting nutrient was itself a nitrogen source.

h. Physiological States.

Acer pseudoplatanus cells in steady states at different growth rates differ significantly in mean size, macromolecular composition and respiration rate. Both cell dry weight and cell volume (Fig. V.22A) decline as growth rate increases, there being a particularly marked drop in cell dry weight close to μ_{max}. However, the QO_2 and RNA content of the cells (Fig. V.22B) rise progressively with increasing growth rate. The protein content of the cells (Fig. V.22B), though highest at growth rates close to μ_{max}, is constant over a range of intermediate growth rates and appears to increase at the lowest growth rates examined. There was an apparent increase in the extractable-DNA per cell with increasing growth rate (Fig. V.23A). Although the data obtained from separate batch cultures was more variable than that from comparable turbidostat or chemostat cultures, values for mean cell

Fig. V.22. The relationship between cell size, composition, respiration rate and dilution rate in 4-litre chemostat cultures of Acer cells.

Steady states at different dilution rates were established with nitrate as the limiting nutrient.

Points in A and B were calculated from the trend lines drawn through the data in Fig. V.12.

● volume ■ total protein

○ dry weight □ respiration rate

◧ total RNA

Fig. V. 22.

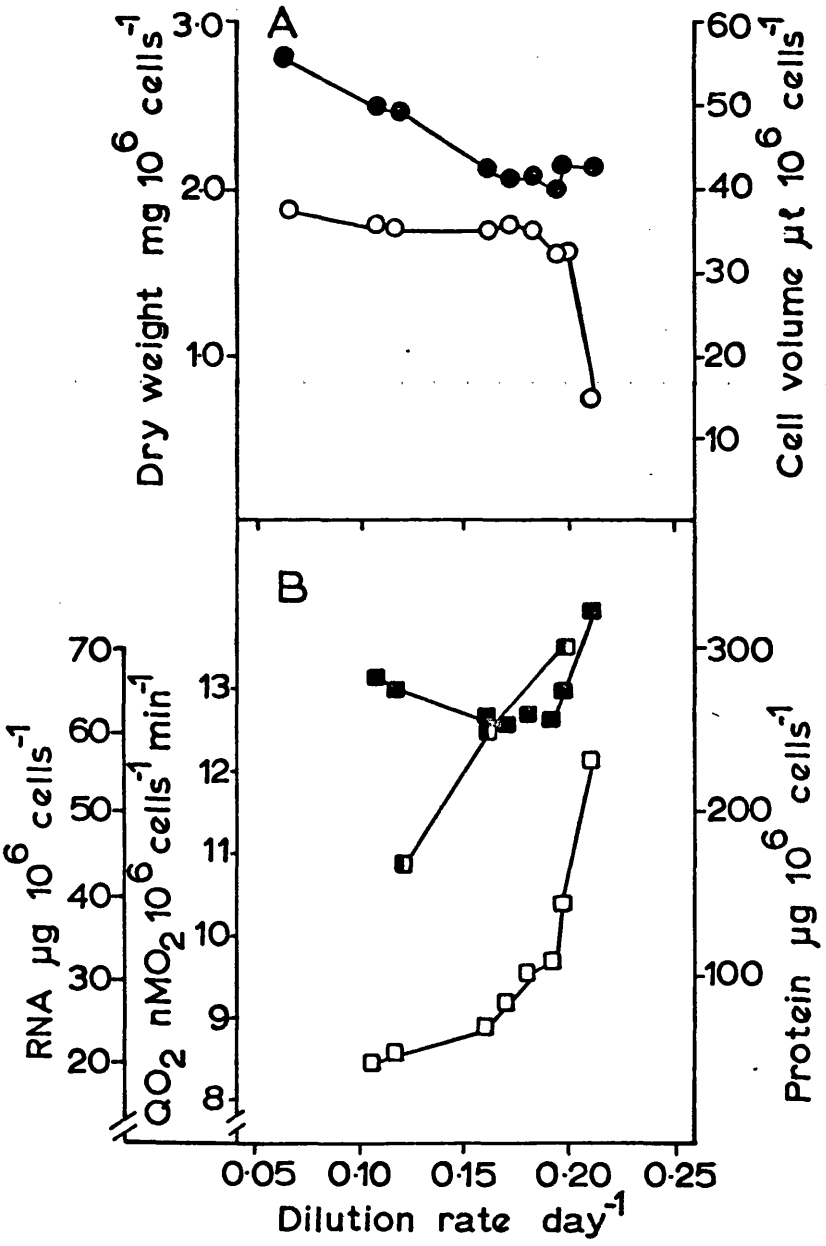

A
B
Dry weight mg 10^6 cells^{-1}
Cell volume µl 10^6 cells^{-1}
RNA µg 10^6 cells^{-1}
QO_2 nMO_2 10^6 cells^{-1} min^{-1}
Protein µg 10^6 cells^{-1}
Dilution rate day^{-1}
3·0
20
10
60
50
40
30
20
10
70
60
50
40
30
20
13
12
11
10
9
8
300
200
100
0·05
0·10
0·15
0·20
0·25

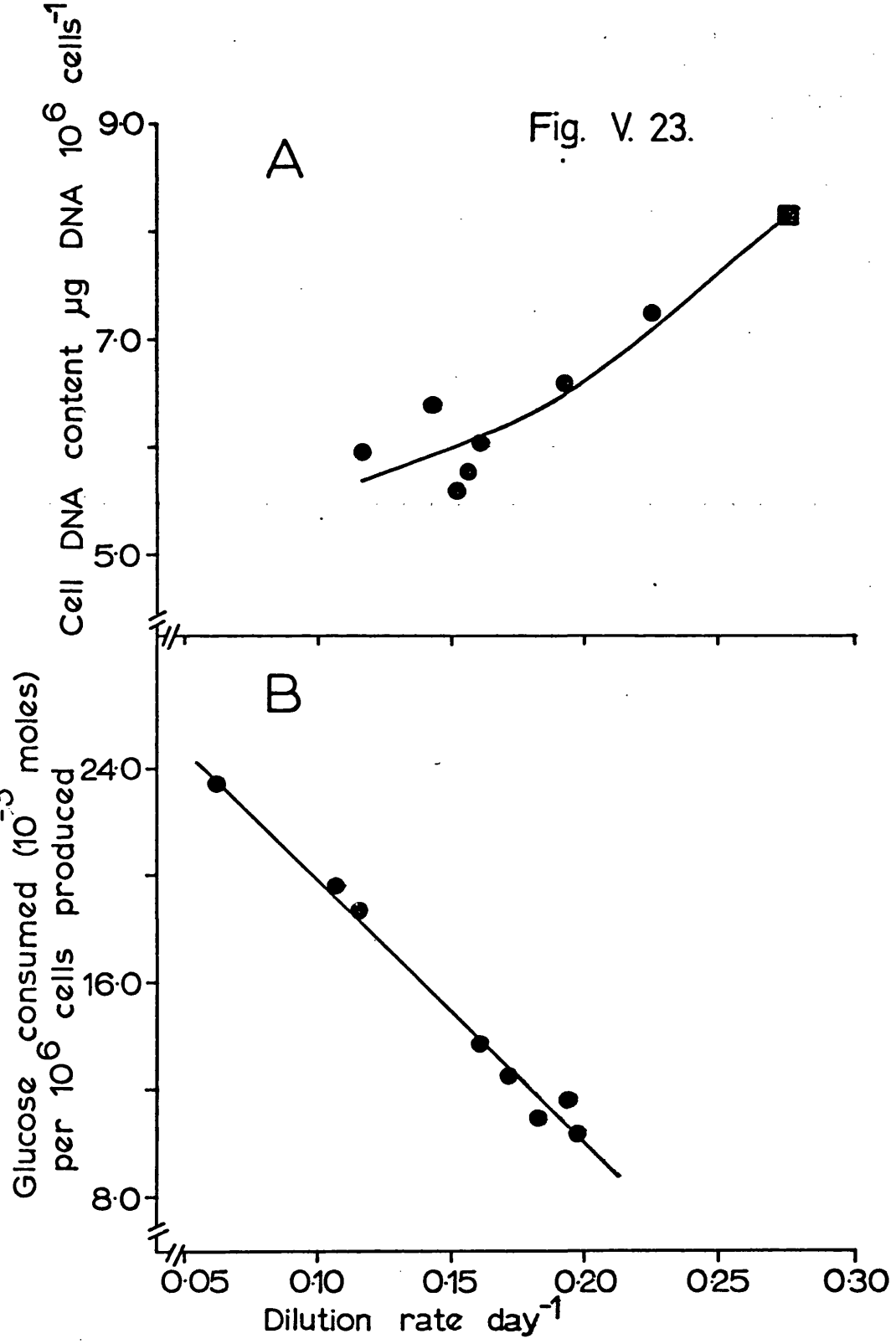
Fig. V.23. The relationship between extractable - DNA content, glucose consumption and dilution rate in 4-litre chemostat cultures of Acer cells.

A. Values obtained from chemostats (●) at different growth rates are compared to the extractable - DNA content (■) of a batch-culture population during exponential growth at a high rate (see Table IV.2).

B. The rate of glucose consumption = $D(S_R-s)$ where D = dilution rate, S_R = the concentration of glucose in the inflowing medium and s = the concentration in the outflowing medium.

The rate of biomass production = Dx, where x = biomass present per unit volume of the culture.

Fig. V. 23.


A
B
Cell DNA content µg DNA 10^6 cells^{-1}
9·0
7·0
5·0
Glucose consumed (10^{-3} moles) per 10^6 cells produced
24·0
16·0
8·0
0·05
0·10
0·15
0·20
0·25
0·30
Dilution rate day^{-1}

size and composition of exponential or stationary phase cells in batch cultures (Section IV and Table V.4) agree, in general, both with the values obtained from turbidostats (≡ batch exponential phase) (see Table V.2) and those indicated by the extrapolation of the chemostat data (Figs. V.22 and V.23A) to μ_{max} (≡ batch exponential phase) or to zero dilution rate (≡ batch stationary phase).

The changes in the respiration rate and RNA content of Acer cells (mainly ribosomal RNA - Cox, 1972) with growth rate (Fig. V.22B), indicate marked differences in the metabolic state of cells at different growth rates. High rates of synthesis of essential cell materials at high growth rates were accomplished by increases in the extent of the synthetic apparatus of the cell (e.g. ribosomal RNA) and in the provision of ATP and reduced co-factors by carbohydrate oxidation. More important, these changes in the synthetic capacity ("the growth rate") of the cells were a direct response to the rate of supply of the limiting nutrient. In contrast, total cell protein (Fig. V.22B) as a primary product of biosynthesis shows more limited change at different growth rates, although the specific activities (in vivo) of key regulatary enzymes might be expected to show significant changes.

Assuming 100% viability of the Acer cell populations at all of the growth rates examined and, therefore, that the population doubling-time, td, is equal to the mean cell-cycle time, T (thus, in the steady state: $D = \mu = \ln 2/T$), the mean cell-cycle times in the separate steady-state populations examined ranged from 72 hours to 263 hours. The decline in the mean DNA content of the cells with increased cell-cycle times (Fig. V.23A) strongly suggests that extension of the cycle time was due primarily to extension of G1 i.e. at low dilution rates the time interval between mitosis and the succeeding DNA replication was larger than at

Table V.4. Cell size and composition of Acer cells in late exponential phase (E) and stationary phase (S) of batch cultures.

		E	S
Cell volume	$\mu l\ 10^6\ cells^{-1}$	30 - 35	50 - 60
Dry weight	$mg\ 10^6\ cells^{-1}$	1.5 - 1.6	2.5 - 3.0
Total protein	$\mu g\ 10^6\ cells^{-1}$	350 - 450	300 - 350
RNA	$\mu g\ 10^6\ cells^{-1}$	90 - 100	50 - 60
DNA	$\mu g\ 10^6\ cells^{-1}$	8.0 - 9.0	4.5 - 5.5
QO_2	(nmoles $O_2 10^6 cells^{-1}\ min^{-1}$)	11 - 14	3 - 5

high dilution rates. The values recorded for mean DNA content of the cells at different growth rates are not incompatible with the view that the changes in the mean cell-cycle time were due entirely to changes in the duration of G1, the durations of mitosis, S-phase and G2 remaining constant. Mitotic indices and ^{3}H- thymidine-labelling indices of chemostat populations of Acer cells at different growth rates show slight, not necessarily significant, increases in the durations of mitosis and S-phase at longer cell-cycle times (see Table V1.4, Section VI). The suggestion here of a greater sensitivity of the time interval between mitosis and S-phase (G1) to environmental changes agrees (i) with the greater spontaneous variability of G1 noted in the synchronous populations examined (see Fig. V1.13, Section VI) and (ii) with the cessation of growth of Acer cells during the G1 interval at the termination of batch cultures (Section IV). It would appear that, whereas the completion of DNA replication and the succeeding mitosis in Acer cells are coupled independently of, for example, the rate of nitrogen assimilation, the interval leading up to DNA replication includes essential biosynthetic events clearly affected, as in the chemostat populations, by the supply of substrates.

Differences in cell volume between high and low growth rates in chemostat populations of Acer cells (Fig. V.22A) reflect closely the changes in cell volume seen during the "growth cycle" of batch cultures of these cells (see Section IV and Table V.4). Acer cells in batch cultures expand (and increase in fresh weight) at the onset of stationary phase when division activity ceases but division potential remains. The coincidence of the cell-expansion phase with loss of cell division activity in batch cultures may seem to offer evidence of the loss of division activity in an increasing proportion of the cell

population at lower dilution rates in chemostat cultures. However, so many changes occur at the termination of batch cell cultures both in cell metabolism and in the culture environment that the exact interdependence of division activity and cell volume is not clear. It is possible that cell expansion is related to (i)the changes in the osmotic potential of the culture medium caused by nutrient assimilation, or (ii) the depletion of one specific nutrient, or (iii) the occurrence of one particular, transient metabolic-state of the cells during the exponential phase, e.g. ethylene production, as has been suggested (Mackenzie and Street, 1970), or (iv) the cessation of cell division during one particular phase of the cell cycle (e.g. in G1 - as in Acer). The first three possibilities could equally as well apply to chemostats as to batch cultures. Work with other species in culture eliminates three of the possibilities. Nash and Davies (1972) found no distinct phases of cell division and cell expansion in cell cultures of Rosa sp. (Paul's Scarlet) despite the fact that other growth-cycle markers were very similar to those for Acer cells: major nutrients (NO_3^-, PO_4^{3-} and sugars) were depleted at similar rates; DNA per cell changes indicate cessation of division in G1; similar metabolic states occur, e.g. ethylene production (La Rue and Gamborg, 1971). In contrast, Fletcher and Beevers (1970) found a marked cell expansion phase in Rosa sp cell cultures grown in different conditions. Thus the presence (or depletion) of one or more specific nutrient(s) may have a controlling affect on cell expansion. Further support for this possibility comes from the work of Simpkins, Collin and Street (1970) who showed that changes in the ratio of growth hormones supplied had a marked affect on cell expansion. The observations in the present study on the relationship between growth rate and cell expansion and

the facility for stabilising populations showing a specific degree of expansion by chemostat culture may allow this feature of cell differentiation to be more easily investigated.

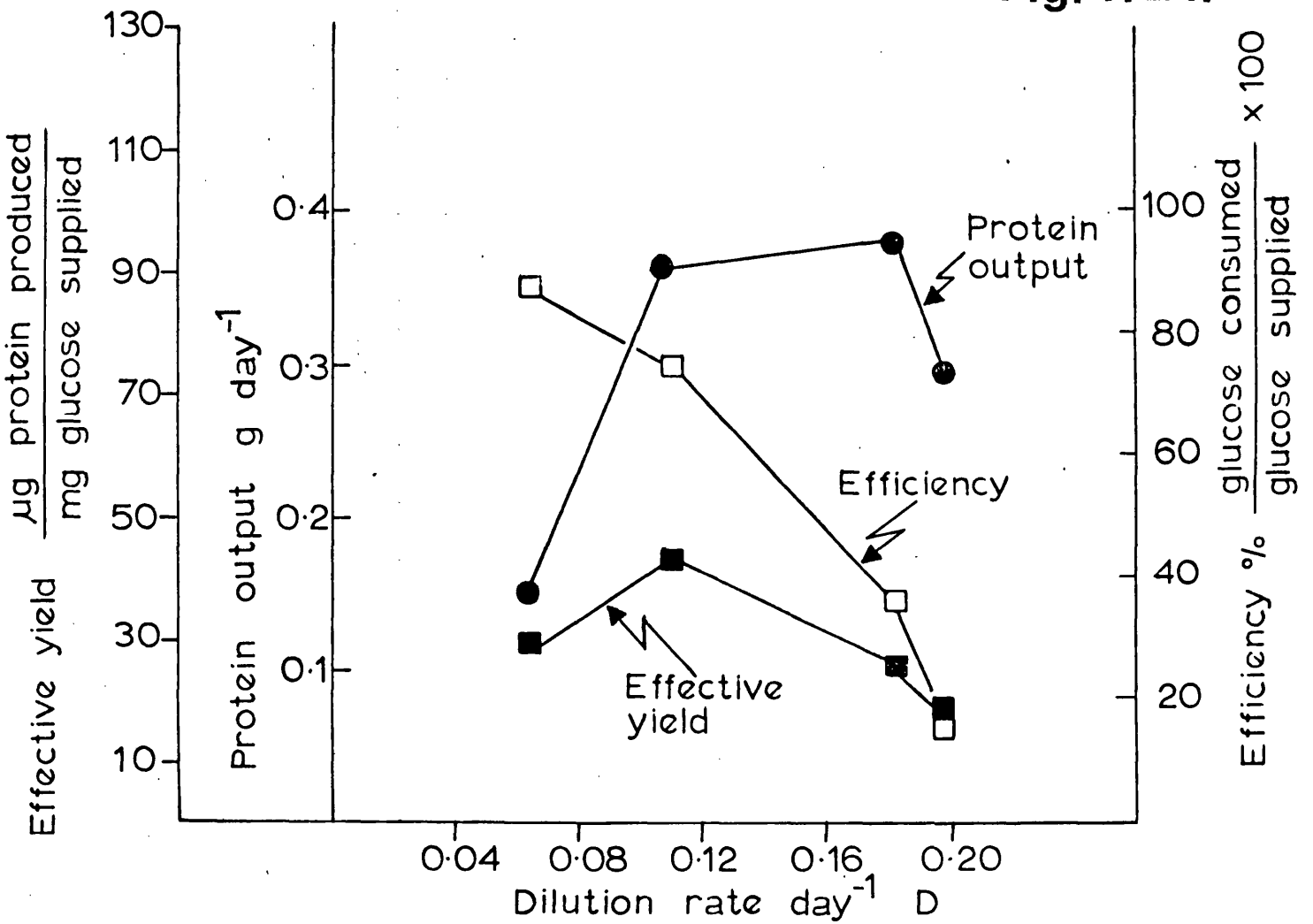
Whereas macromolecular content and the metabolic activity of Acer cells increases with increased growth rate, total cell mass is highest at low growth rates (Fig. V.22A). In view of the evidence for respiration - independent uptake of glucose obtained indirectly from aeration studies (Section III.5) and the changes in steady-state glucose levels with dilution rate (Fig. V.14B), the higher cell mass at lower growth rates is perhaps due to uptake and (probably) condensation of excess glucose. As postulated for urea, high residence times at low dilution rates expose the cells to nutrients, at concentrations above those essential for growth, for which the cells have an unsaturated uptake and assimilatary capacity. In accord with this idea, the amount of glucose consumed per 10^6 cells produced was inversely proportional to dilution rate (Fig. V.23B). Furthermore, the ratio of glucose consumed to oxygen consumed per generation exceeded the stoichiometry required for RQ = 1 except at μ_{max}, the ratio increasing with decreasing dilution rates. If the above interpretation is correct, an inverse relationship would be expected between both (a) the insoluble carbohydrate content of the cells and (b) the amount of secreted polysaccharide (Simpkins and Street, 1970) and the dilution rate.

Changes in cell volume and dry weight similar to those described above, and changes in phenolic compounds (leucoanthocyanins and catechins) with dilution rate have been described for Acer cells in PO_4^{3-}-limited chemostat culture by Wilson (1971). The chemical composition and physiological states of bacterial species were also

found to depend upon growth rate (Herbert, 1961; Kubitschek, 1970). Furthermore, it was demonstrated that the state of the cells was independent of the chemical composition of the culture medium, the identity of the limiting nutrient and even temperature. The value of μ_{max} should (by definition) be independent of the nature of the limiting nutrient in chemostat culture. It would be of great interest to examine whether this is so for Acer cells and how far the metabolic state of the cells at particular growth rates depended upon the identity of the nutrient limiting growth. From the changes in cell metabolism noted for Acer cells at different growth rates, it would appear that examination of plant cells in steady states at contrasting growth rates and, in particular, the transition between two steady states might be a useful way of investigating specific aspects of metabolic regulation e.g. the control of ribosomal-RNA biosynthesis. If, in fact, plant cell populations differing in their metabolism can be selected simply by altering the potentiometer on the pump delivering fresh medium to a chemostat, then the special cultural conditions needed to open up specific metabolic pathways in plant cells in culture which lead to the efficient accumulation of useful products (e.g. secondary metabolites - see Section V.11) may be more easily discovered. This general idea may be illustrated by data relating the yield of cell protein to dilution rate in chemostat cultures of Acer (Fig. V.24). Whilst the absolute output of protein reached a maximum close to D_c (as required by the theory - Fencl, 1966), the efficiency of its production in terms of a major nutrient supplied is greatest at an intermediate dilution rate. However, the production of a specific protein might require a different dilution rate.

Fig. V.24. The relationship between protein output, efficiency, effective yield and dilution rate in 4-litre chemostat cultures of *Acer* cells.

Fig. V. 24.


Effective yield µg protein produced / mg glucose supplied
130
110
90
70
50
30
10
Protein output g day⁻¹
0·4
0·3
0·2
0·1
Efficiency % glucose consumed / glucose supplied x 100
100
80
60
40
20
Protein output
Efficiency
Effective yield
0·04
0·08
0·12
0·16
0·20
Dilution rate day⁻¹ D

i. Anomalous cultures.

Using the same cell line and composition of culture medium, the biomass of a chemostat culture in a steady state should be predictable and reproducible on different occasions. However, the growth of Acer cells in chemostats was not entirely predictable. Two cultures (cultures 78 and 66) responded to dilution in quite a different manner to that already observed for similar dilution rates and contrary to the trends established (see Fig. V.12) by the majority of the data obtained. Table V.5 compares data from one such culture with two separate "normal" cultures. Culture 78 was inoculated at a normal density (ca. 120×10^3 cells ml^{-1}) in the usual culture medium. Services to the culture vessel (e.g. temperature control) were normal. Dilution was begun on day 12 of incubation; from day 18, for 750 hours (≡ 9 cell generations) the biomass was stable. During this period the fresh medium in the reservoir supplying the culture was replenished four times in 9-litre batches. The composition and respiration rate of the cells was as expected (cf. Figs. V22 and V23). However, the steady-state biomass density was approximately double that predicted for that dilution rate. In the absence of any evidence of a persistent (mistaken) change in the cultural conditions likely to have directly produced such an effect, and in view of the evidence that nitrate is normally the limiting nutrient, the enhanced biomass output (in particular cell protein) may be explained by the spontaneous redirection of nitrogen taken up as urea toweards protein synthesis. The yield data for protein/N produced (Table V.5) clearly show the involvement of both nitrogen sources in protein synthesis in culture 78, whereas in culture 90 the data suggest that only one source (presumably nitrate) was involved. Culture 66, after inoculation at ca. 200×10^3 cells ml^{-1}, was maintained in steady-state

Table V.5. Data for steady states obtained at similar dilution rates in chemostat cultures established at different times

Standard error of means and in brackets number of samples examined during the steady state shown against each value. D = dilution rate td = population doubling time.

Culture No.	54	78	90
D Day^{-1}	0.182	0.189	0.194
td (hr)	90	87	85
Biomass of culture			
P.C.V. %	8.7 ± 0.53(10)	13.0 ± 0.35(10)	8.7 ± 0.34(5)
Cell No. x $10^{-6} ml^{-1}$	2.19 ± 0.06(9)	3.62 ± 0.05 (11)	1.53 ± 0.07(5)
Dry Wt. mg ml^{-1}	3.33 ± 0.16(10)	6.20 ± 0.15(11)	3.38 ± 0.27(5)
Total protein µg ml^{-1}	513 ± 34.0(8)	1136 ± 16.0(10)	556 ± 1.9(5)
Total DNA µg ml^{-1}	-	19.3 ± 0.84(10)	10.04 ± 0.35(5)
Total RNA µg ml^{-1}	-	169.1 ± 1.9(10)	103.5 ± 3.7(5)
O_2 Demand nM $ml^{-1}min^{-1}$	12.5 ± 1.0(7)	35.3 ± 2.4(8)	19.0 ± 1.4(5)
Medium constituent levels			
Glucose x 10^{-1}M	0.76 ± 0.02(9)	0.40 ± 0.01(11)	0.56 ± 0.01(5)
Nitrate/N x 10^{-4}M	-	0.36 ± 0.07(10)	5.71 ± 0.48(3)
Urea/N x 10^{-4}M	-	2.64 ± 0.28(11)	5.57 ± 0.31(5)
Phosphate x 10^{-4}M	-	0.21 ± 0.03(11)	2.82 ± 0.21(5)
Yield of protein (moles protein/N per mole nutrient)			
Glucose	0.146	0.161	0.101
Nitrate/N	-	1.63	0.86
Urea/N	-	1.77	0.91
Phosphate/PO_4	-	13.9	9.5

conditions for a total of 400 hours at dilution rates of 0.171 day^{-1} and 0.197 day^{-1}. In these states, biomass densities and cell composition were as expected. However, a further increase in the dilution rate to 0.214 day^{-1} (close to μ_{max}), though it produced the expected decline in biomass within 24 hours, had after 72 hours produced marginal increases in packed cell volume, dry weight and total protein and a marked increase in cell number. For a further 220 hours dilution at this rate, the biomass remained constant but cell volume (19 μl 10^6 $cells^{-1}$), cell protein (260 μg 10^6 $cells^{-1}$) and QO_2 (7.9 nmoles O_2 10^6 $cells^{-1}$ min^{-1}) were all much lower than predicted (cf. Fig. V.22). Furthermore, when the culture was diluted successively at rates of 0.238, 0.279 and 0.307 day^{-1} further slight increases in biomass were recorded at each step. The culture showed signs of wash-out at D = 0.412 day^{-1} (td = 40 hours) but a steady state was finally maintained for 240 hours at D = 0.315 day^{-1} (td = 53 hours) and at a density of 0.8 x 10^6 cells ml^{-1}. The situation in culture 66 resembles that of culture 78 in that a higher-than-expected biomass was obtained at a given dilution rate (especially in terms of cell number and total protein). However, the data from culture 66 also suggest that a change in μ_{max} had occurred (from ca. 0.225 day^{-1} to 0.315 - 0.412 day^{-1}). In view of the high growth rates occasionally recorded in randomly-dividing batch cultures (Section IV) and synchronous cultures (Section VI) such a change was not unexpected.

V1. SYNCHRONOUS CULTURES

"If progress in cell biology is linked to our capacity to control our experimental material, the use of synchronous cultures will help charge the future with discovery". (James, 1966).

1. Introduction

Continuous cultures of dividing cells represent an effective system for investigating, for example, the relationships between populations of cells and their environment, the kinetics of metabolic control or the biosynthesis of specific compounds. However, such cultures contain a mixture of cells randomly distributed through the cell division cycle. Therefore, information obtained using this technique may only reveal a mean of the activities of the individual members of the population and not the real, changing metabolic potential of the cells as they grow and divide. Few techniques are available for describing patterns of growth, biosynthesis and cell division in single living cells (Mitchison, 1971). Since 1953, however, it has been possible to more-or-less synchronise the events of growth and cell division in large populations of cells and thereby amplify the activities of the individual cell to levels within the scope of the usual biochemical techniques (Zeuthen, 1964; Cameron and Padilla, 1966; Padilla, Whitson and Cameron, 1969).

Work with synchronous cultures of bacteria (Donachie and Masters, 1969), algae (Schmidt, 1969), yeast (Williamson, 1966; Mitchison, 1971) protozoa (Padilla, Cameron and Elrod, 1966) and mammalian cells (Petersen, Tobey and Anderson, 1969) has made possible the construction of comparative timetables for the biochemical events separating one generation of cells from its daughter generation. Such work has made

significant contributions to our understanding of gene expression, replication and the control of cell division. Synchronous cultures of myxomycetes and protozoa have also been used in studies of development (Cameron, Padilla and Zimmermann, 1971).

Descriptions of some events in the cell cycle of higher plants have so far been obtained by three types of investigation: (i) the analysis of asynchronous populations of dividing cells by techniques based upon the classical studies of Howard and Pelc (1953), for example, the ^{14}C- and ^{3}H-thymidine double-labelling of cells in root-tip meristems (Wimber and Quastler, 1963); (ii) the exploitation of naturally-occurring synchrony, for example, the investigation of changes in sulphydryl concentrations, in respiration, in enzyme activity and in nucleic acids during synchronous mitoses and meiotic division of spore mother cells in Trillium sp. and Lilium sp. anthers (Erickson, 1964); (iii) the induction of short-lived synchronisation of cell division by chemical treatment of plant meristems, for example, the application of 5-aminouracil to root meristems of Allium cepa and Vicia faba (Mattingley, 1966).

The synchronisation of higher plant cells cultured as suspensions in defined conditions (technically analagous to the successful mass populations of microorganisms) would have many obvious advantages. Such cultures would not only enable a more detailed description of the events of the cell cycle but would also make possible identification of the factors controlling the duration of cycle phases. Identification of those phases most susceptible to changes in extrinsic factors should improve our understanding of the differential rates of growth and cell division which occur in organised plant meristems (Steward and Mohan Ram, 1961; Phillips and Torrey, 1972). The evidence that cytodifferentiation

may be determined during an earlier critical cell cycle and that developmental pathways may only be inducible during specific periods of the cell cycle (Torrey, 1971) suggest that synchronous cultures will be necessary to achieve high levels of cytodifferentiation in tissue and cell cultures (Fosket and Torrey, 1969; Fosket, 1970).

Attempts to induce synchronous cell division in plant cell cultures by the use of metabolic blocks has met with variable success. Neither excess thymidine nor a cold treatment was effective in synchronising cell cultures of Acer pseudoplatanus L. (Roberts and Northcote, 1970). Eriksson (1966), however, induced synchrony in suspension cultures of Haplopappus gracilis (Nutt) Gray by treating dividing cells (mitotic index = 5%) with four different DNA - synthesis inhibitors. A peak of mitotic activity (up to 30% with hydroxyurea) occurred 10-16 hours after removal of the inhibitor. The peak duration was 0.2 cell cycles, compared to an independent estimation of 0.05 cell cycles for the duration of mitosis in the tissue used. Only a single mitotic peak was reported after all treatments, suggesting that the synchrony was limited to a single cell division cycle. Confirmation of the effective synchronisation of the population (even for a single cycle) would require either further peaks of mitotic activity or a rapid reversion to the mitotic index characteristic of the randomly dividing population before treatment. Furthermore, assessment of the degree of synchrony in a population solely from measurement of mitotic index may be misleading. The mitotic index of a randomly dividing population is a function both of the duration of mitosis and the cell cycle time. Any factor which prolongs mitosis or shortens the interval between mitoses will increase the mitotic index without necessarily increasing the degree of cell division synchrony in the

population. A single peak of mitotic index occurs in randomly dividing batch cultures of some species (Torrey, Reinert and Merkel, 1962); Henshaw et al, 1966) when the transition from lag phase (long cycle times) to the exponential growth phase (minimum cycle times) is followed closely by a period of declining rates of cell division (extended cycle times or cycle blocks). The enhancement of such a mitotic index peak by the addition of cytokinins (kinetin or 6-benzylaminopurine) to cultures of Acer pseudoplatanus L. (Roberts and Northcote, 1970) may thus not be a synchronisation effect as claimed. Such data could indicate a reduction in cycle time in the presence of the cytokinins and/or their inhibitory effect on the mitotic apparatus. Evidence exists to support both of these possibilities (Guttman, 1956; Nagl, 1972). The same reservation applies to oscillations of mitotic index induced in phased cultures of Glycine max. Merr. (Constabel and Wetter, 1972). However, when cell cultures of Nicotiana tabacum L. were initiated from stationary phase cells and the additions of 2,4-D and kinetin were delayed for 24-72 hours, up to three successive (but decaying) mitotic-index peaks were observed (Jouanneau, 1971). The repetition of the mitotic peaks after a single induction period and the eventual return to a constant mitotic index characteristic of an exponential population are convincing evidence for synchronisation. Synchronised cell divisions have also been clearly demonstrated during the early stages of callus induction from tuber explants of Helianthus tuberosus L. (Yeoman, 1970). Up to two successive waves of mitoses occurred, followed closely by step-wise increases in cell number (Yeoman and Evans, 1967). Discontinuous DNA synthesis and peaks in activity of enzymes of nucleic acid metabolism were observed during the first division cycle (Yeoman, personal communication).

2. Synchronous growth in cell cultures of *Acer pseudoplatanus* L.

Data indicative of synchronous cell division in *Acer pseudoplatanus* L. cell cultures were obtained (Street, 1967) after the inoculation of stationary phase cells at a density of ca. 3×10^5 cells ml^{-1} into 70 ml (shake-flask) cultures. Counts taken at 8-hour intervals suggest that at least two step-wise increases in cell number occurred with intervening plateaux. Up to 70% of the cells divided, with a cycle time of ca. 70 hours.

The cell division synchrony now to be described was obtained by inoculating stationary phase cells of *A. pseudoplatanus* L. at low density ($20\text{-}140 \times 10^3$ cells ml^{-1}; Table VI.1.) into four litres of medium† in a large-scale batch culture apparatus (Appendix 1). The experiments described were made possible by the development of this apparatus; its use allowed the frequent aseptic withdrawal of samples from the culture (in sufficient amount to allow detailed biochemical and cytological study) without disturbing the growth of the remaining cell population. For frequent overnight sampling the vessel was fitted with an automatic sampling valve (Wilson, King and Street, 1971; Appendix 1, Plate 3.). Low initial cell densities were established so that there might be a measurable period of unrestricted, balanced growth i.e. that the cells of successive generations might have similar compositions and cycle times. The relationship between initial cell density and the synchronisation process was not examined. Synchrony only occurred when stationary phase cells were used as inocula. The inocula used in the experiments described had been in the stationary

---

† Medium as described in II. Materials and Methods, with 2% sucrose, $FeCl_3$ and pH 5.2.

Table VI.1. Initial density and duration of lag phase of synchronised cell cultures of Acer pseudoplatanus L.

Culture No.	Inoculum-days in stationary phase	Initial density 10^3 cells ml^{-1}	Lag phase duration days	Number of synchronous divisions	Remarks
20	14	20	2	5	Good synchrony
62	14	140	7	3	" "
63	14	45	4	4	" "
71	14	30	6	4	Decay to rapid log growth.
73	14	22	7	5	Good synchrony
75	14	38	6	2	Decay to rapid log growth.
77	20	35	11	5	Good synchrony
79	14	13	11	4	50% steps only
80	14	29	>12	0	No division
81	17	22	>12	0	No division
86	22	40	3	0	No synchrony
87	17	22	6	2	Terminated by infection.
89	13	25	8	0	No synchrony

phase for 14-20 days (Table VI.1.). The relationship between the "culture-age" of the inoculum and the incidence of synchrony was not examined.

3. Cell division synchrony.

Semi-logarithmic plots of the changes in cell number with time in four synchronised cultures are shown in Figs. VI.1 and VI.2. Up to 5 steps in cell number were observed. Each step was completed within ca. 0.2 cell cycles. With one exception (the first step in culture 62), the increase in cell number at each step was within the range 62% to 123%, with most values falling between 70% and 95%. There was no progressive decline in the % increase in cell number at each step but a sharp transition from synchronous to asynchronous division (see Section VI.6; Fig. VI.12.). There was considerable variation within each culture in the cycle times (as indicated by the spacing of the vertical lines in Figs. VI.1. and VI.2.).

4. Mitotic synchrony.

Peaks of mitotic activity were recorded in two cultures (cultures 73 and 77, Fig. VI.2.) ca. 10 hours before cytokinesis. The mitotic index peaks (ca. 7.5%) persisted for ca. 0.2 cell cycles and were followed by 'steps-up' in cell number of similar duration when ca. 70% of the cells divided (Fig. VI.3.). Outside of the peaks the mitotic index of the cultures remained at about 3%, even during intervals when no significant change occurred (at the 5% level) in the number of cells.

5. Metabolic changes during the cell cycle.

During two periods of synchronous cell division in 4-litre batch

Fig. VI.1. Cell division synchrony in 4-litre batch cell cultures of Acer pseudoplatanus.

The cultures were established by inoculating stationary phase cells at low density (20 - 140 x 10^3 cells ml^{-1}). The cell number data were obtained from samples (ca. 5 ml) taken by an automatic device at intervals of 3 - 6 hours.

The % increase in cell number at each step is shown against the ordinate. The duration of each step (the cell cycle time) is shown against the abscissa.

Fig. VI.1.

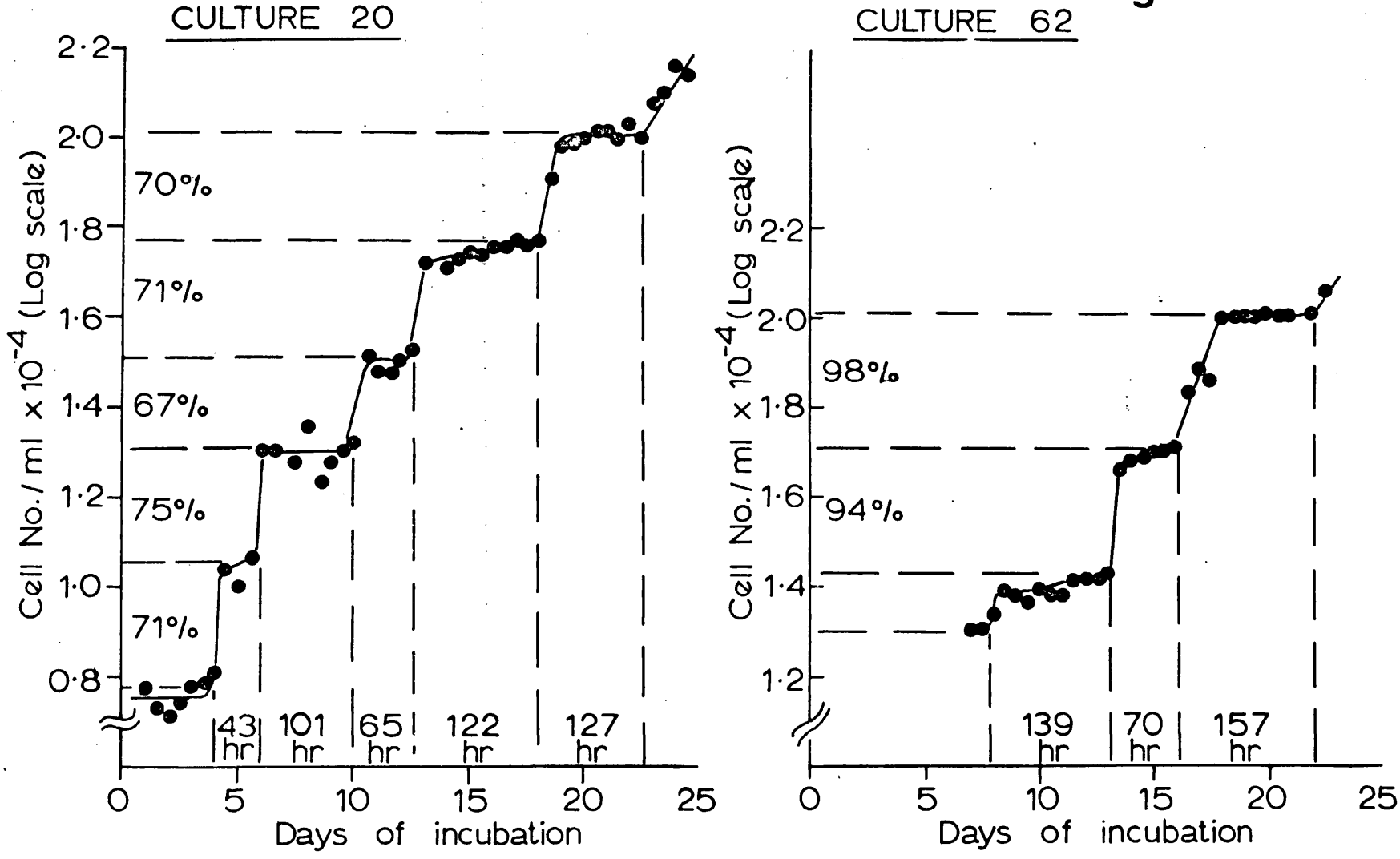
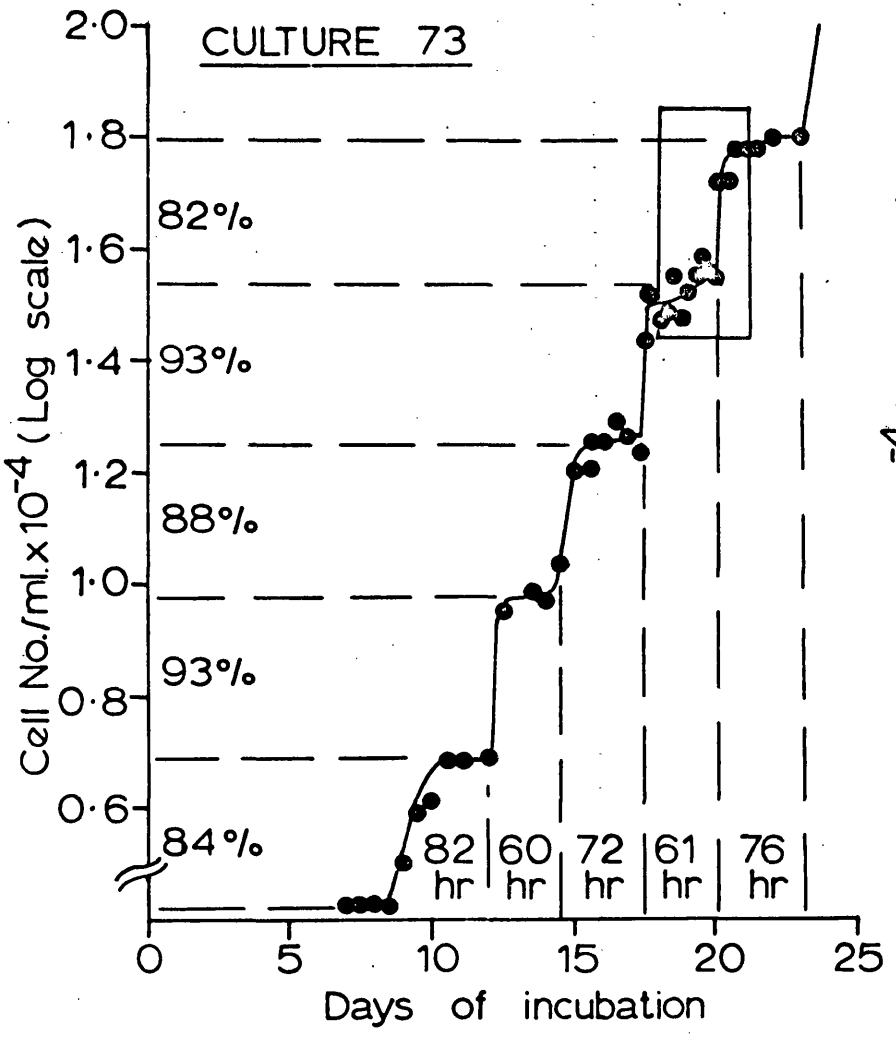

CULTURE 20
Cell No./ml x 10^{-4} (Log scale)
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
70%
71%
67%
75%
71%
43 hr
101 hr
65 hr
122 hr
127 hr
0
5
10
15
20
25
Days of incubation
CULTURE 62
Cell No./ml x 10^{-4} (Log scale)
2·2
2·0
1·8
1·6
1·4
1·2
98%
94%
139 hr
70 hr
157 hr
0
5
10
15
20
25
Days of incubation

Fig. VI.2. Cell division synchrony in 4-litre batch cell cultures of Acer pseudoplatanus.

See legend to Fig. VI.1.

Fig. VI. 2.


CULTURE 73
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
82%
93%
88%
93%
84%
82 hr
60 hr
72 hr
61 hr
76 hr
Cell No./ml. x 10⁻⁴ (Log scale)
0
5
10
15
20
25
Days of incubation

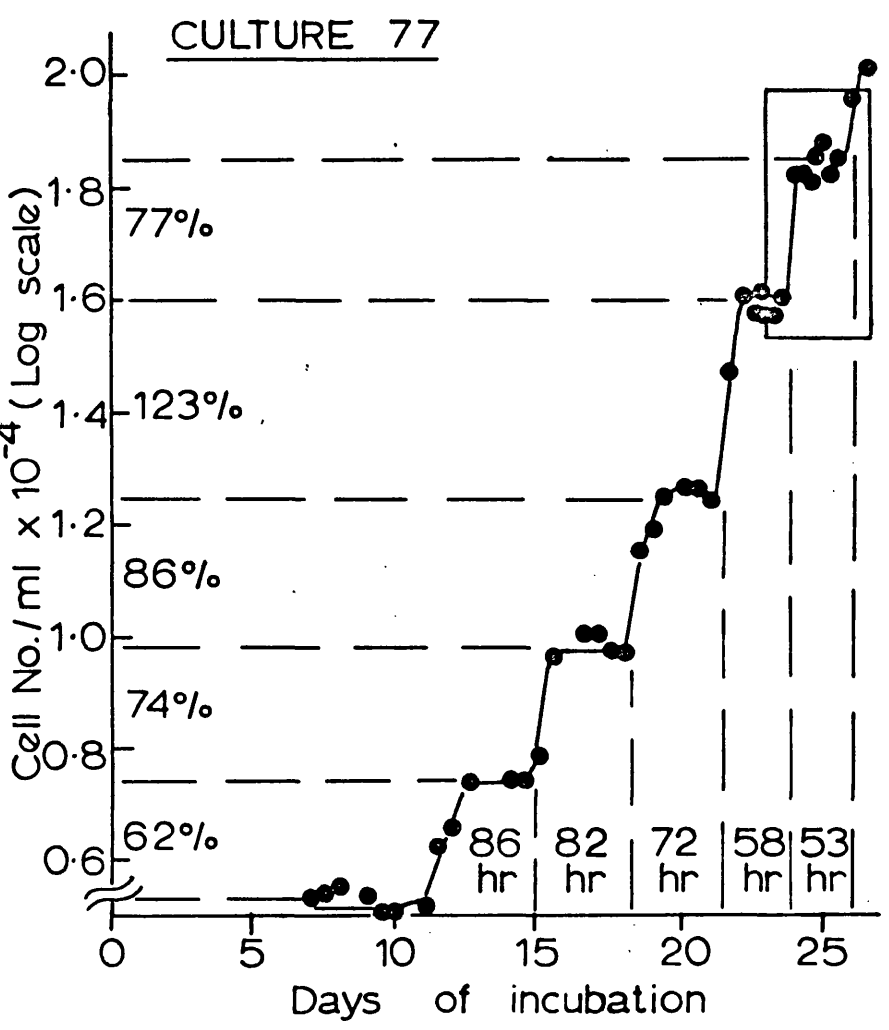

CULTURE 77
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
77%
123%
86%
74%
62%
86 hr
82 hr
72 hr
58 hr
53 hr
Cell No./ml x 1·2 x 10⁻⁴ (Log scale)
0
5
10
15
20
25
Days of incubation

Fig. VI.3. Changes of cell number and mitotic index in synchronised batch cultures of *Acer* cells.

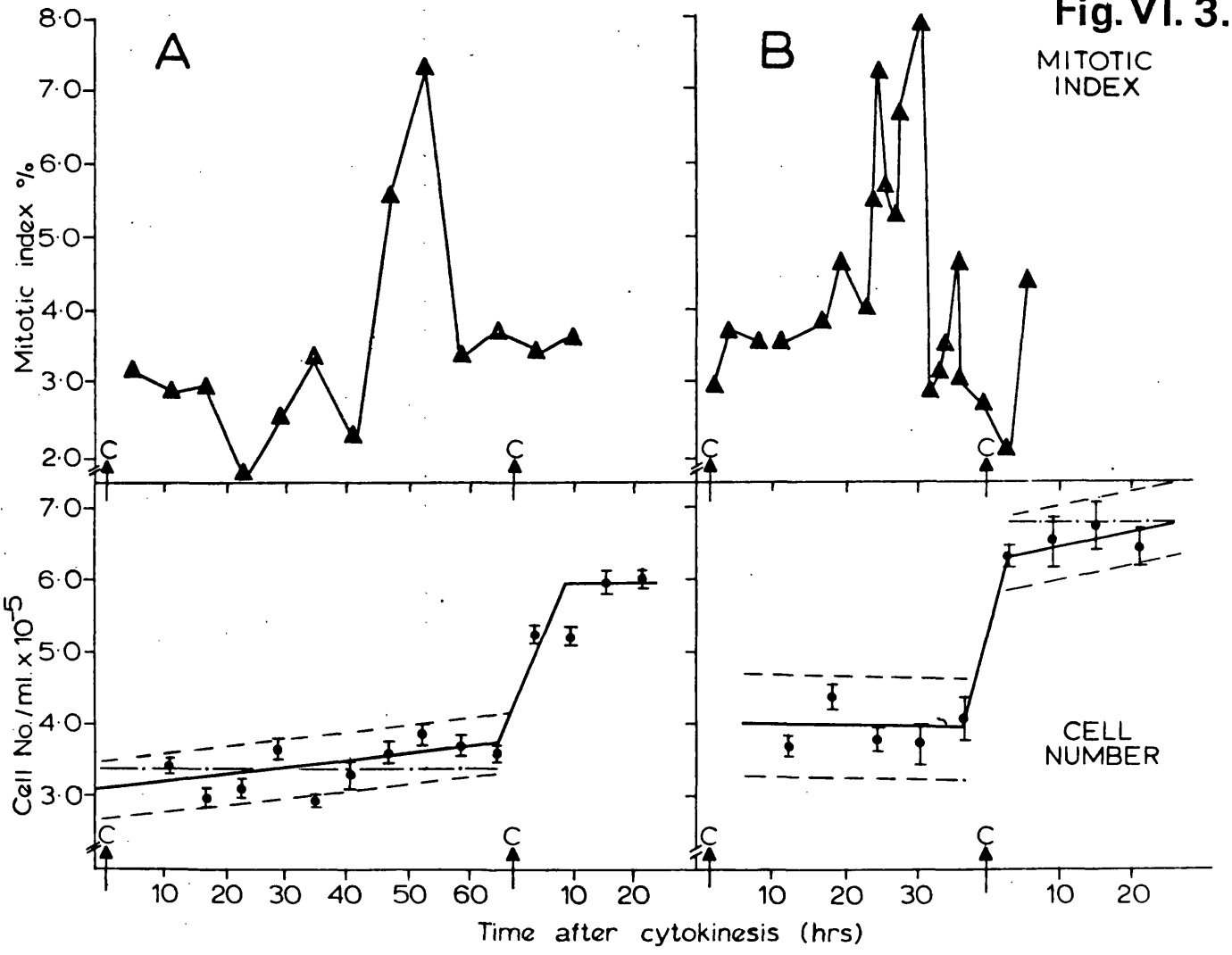
Mitotic indices (the % of cells examined which contained recognisable stages of mitosis) were obtained by examining 1000 nuclei after staining with lactopropionic orcein.

A mean line of zero slope (-·-) falls within the 95% confidence limits (---) of the regression line (-) in both cultures.

The two cell cycles illustrated (A and B) occurred in the intervals marked by the rectangles superimposed on growth data for cultures 73 and 77 (Fig. VI.2) respectively.

C = cytokinesis.

Fig. VI. 3.


A
B
MITOTIC INDEX
CELL NUMBER
Mitotic index %
Cell No./ml. x 10⁻⁵
Time after cytokinesis (hrs)
C
8·0
7·0
6·0
5·0
4·0
3·0
2·0
10
20
30
40
50
60

cultures of Acer a series of large samples (ca. 150 ml) were removed from the cultures at intervals of approximately 6 hours. The culture periods are marked by rectangles on the data for cultures 73 and 77 in Fig. VI.2. Cell counts and mitotic indices recorded during these cycles are shown in Fig. VI.3. The samples were used to investigate changes in respiration rate and the synthesis of DNA, total RNA and both total and specific proteins during the cell cycle. The experiments were carried out with the cooperation of two other workers. With the exception of Fig. VI.14, only data obtained by the present author are illustrated here, though references are made in the discussion (Section VI.7) to the other results obtained.

a. DNA

Aspects of DNA synthesis were examined by direct estimation of extracted DNA (II. Materials and Methods.13.), by thymidine incorporation and by measurement of the in vitro activities of the two enzymes, thymidine kinase(EC.2.7.1.21) and aspartate transcarbamoylase (EC.2.1.3.2). Changes in the amount of DNA extracted from the culture samples are shown in Figs. VI.4A and 5A. In culture 73 (Fig. VI.4.) the duration of the cell cycle examined was ca. 67 hours. The amount of DNA per ml of culture appeared to remain constant for ca. 28 hours after the previous cytokinesis and then increased linearly by ca. 70% during a further 30-hour period. The end of this period coincided approximately with a peak of mitotic activity in the culture (Fig.VI.3A). There was only a relatively small increase in the amount of DNA present during the next 30 hours, until 21 hours into the next cell division cycle. The amount of DNA per cell (Fig. VI.4B), after thus increasing by 70%, remained constant for about 6 hours in the period between mitosis and cytokinesis.

Figures VI.4 and VI.5. Changes in the extractable DNA content of synchronised batch cultures of *Acer* cells.

Fig. VI.4 = Culture 73
Fig. VI.5 = Culture 77

(see growth data in Fig. VI.2)

A. Total DNA per millilitre of culture extracted.

B. Total DNA as a function of the number of cells present in the volume of culture extracted. During the intervals between cytokineses, the cell number was taken to be the mean of the estimates made during that period (see Fig. VI.3).

M indicates the duration of the mitotic index peak.

C indicates the time during which cytokinesis occurred; the arrow indicates the mid-point of the period of most rapid change in cell number.

Cycle A and B, and 'a' and 'b' are referred to in the text.

Fig. VI. 4

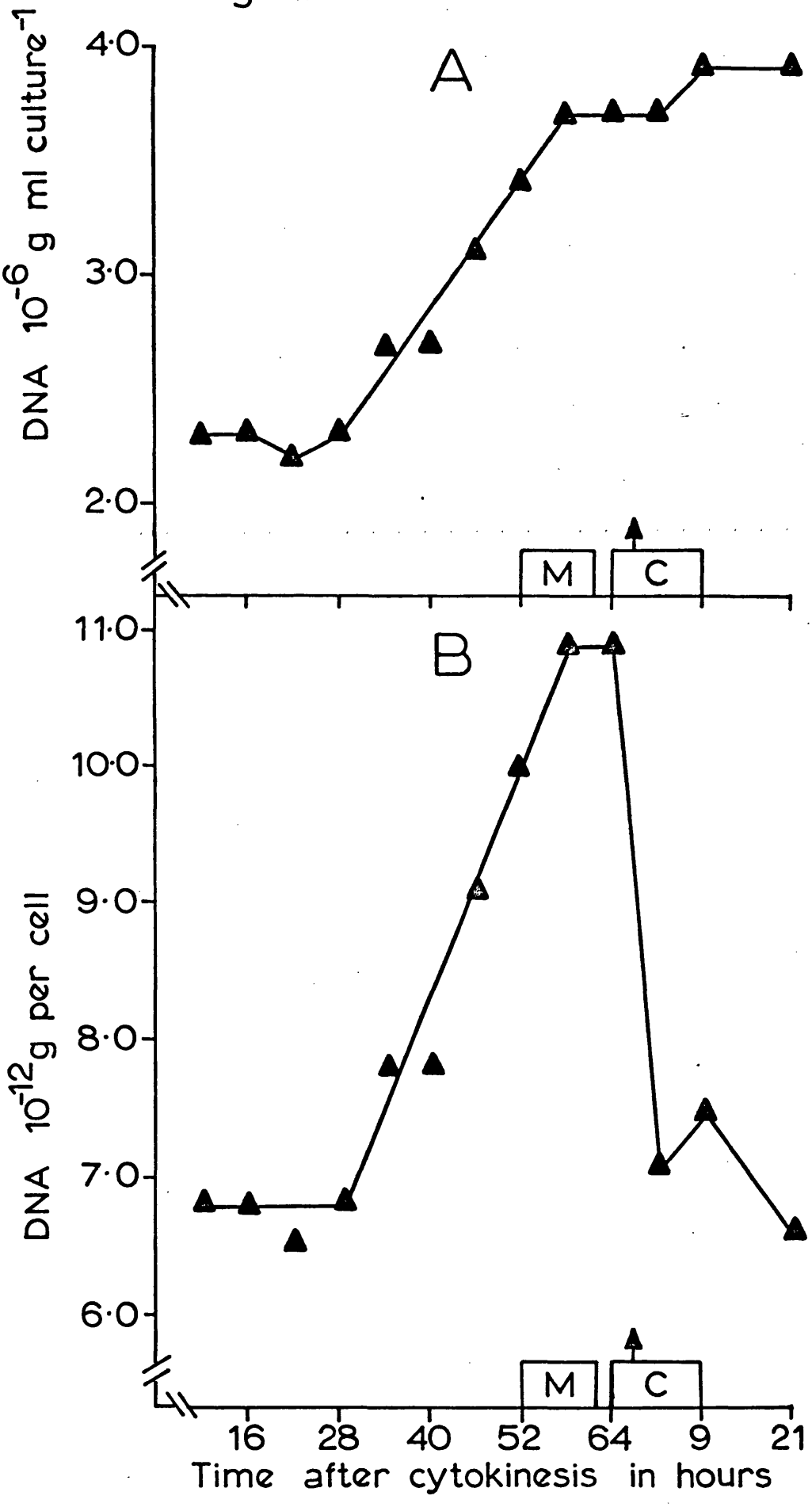
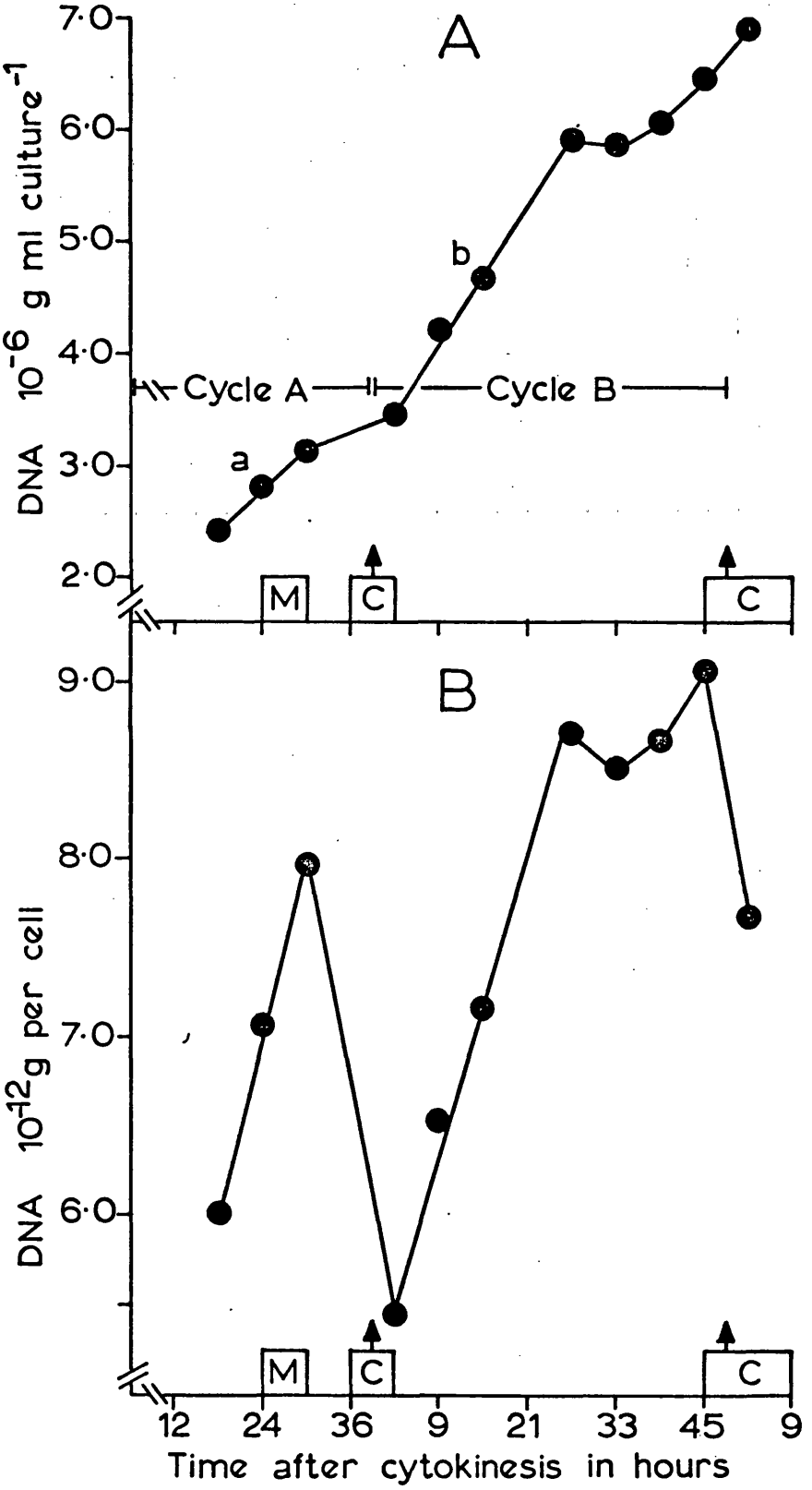

A
B
DNA 10^{-6} g ml culture^{-1}
DNA 10^{-12} g per cell
4·0
3·0
2·0
11·0
10·0
9·0
8·0
7·0
6·0
M
C
M
C
16
28
40
52
64
9
21
Time after cytokinesis in hours

Fig. VI. 5.


A
B
7·0
6·0
5·0
4·0
3·0
2·0
9·0
8·0
7·0
6·0
DNA 10^{-6} g ml culture^{-1}
DNA 10^{-12}g per cell
a
b
Cycle A
Cycle B
M
C
C
M
C
C
12
24
36
9
21
33
45
9
Time after cytokinesis in hours

The original value (ca. 6.7 x 10^{-12}g per cell) was then restored when about 75% of the cells divided.

The two cell division cycles examined in culture 77 (Fig.VI.5.) were both shorter (ca. 40 and ca. 50 hours) than the cycle examined in culture 73. Some changes appeared in the rate of accumulation of DNA (Fig. VI.5A) but these were less marked than in culture 73. In cycle B, a linear increase in DNA per ml of culture (marked 'b') was initiated within a few hours of cytokinesis. The rate of increase was constant for 24 hours by which time the amount of DNA present had increased by 75%. For the following 24 hours (which includes the expected position of the peak of mitotic activity normally associated with the cytokinesis step) the DNA content of the culture was relatively unchanged. However, by the time of the next cytokinesis, the DNA content of the culture was increasing once more. It is possible to interpret the data shown for the latter half of cycle A in a similar way. Furthermore, if it is assumed that the part of the curve marked 'a' (Fig. VI.5A) is the final period of DNA accumulation in cycle A, then the rate of DNA accumulation in cycle B is twice that of cycle A (Table VI.2.). This would be expected if DNA replication during cycle A had doubled the number of independently replicating units (genomes). The specific rate of DNA accumulation (per cell) was the same in cycles A and B (Fig. VI.5B and Table VI.2.). The absolute rate of DNA accumulation in the cycle examined in culture 73 was less than the rate in cycle A, culture 77, but the specific rate (per cell) was very similar (Table VI.2.).

b. RNA

There was an almost continuous rise in the amount of total RNA extracted from samples of both culture 73 and culture 77 during the cell

Table VI.2. Rates of accumulation of DNA in synchronous cultures of Acer pseudoplatanus L.

CULTURE	Rate of accumulation of DNA 10^{-6} g h^{-1} ml^{-1}	10^{-13} g h^{-1} $cell^{-1}$
73	0.047	1.38
77 (cycle A)	0.062	1.56
77 (cycle B)	0.103	1.51

Calculated from Figs. VI.4 and 5.

cycles examined (Fig. VI.6A and Fig. VI.7A). The general pattern of RNA Accumulation may follow that recorded for DNA (Fig. VI.4 and Fig. VI.5) but with somewhat less discontinuity. The data in culture 73 would appear to be an exponential up to the beginning of the second division cycle (Fig. VI.6A and 6B). However, a further possibility (illustrated on the data for culture 77, Fig. VI.7A) is that RNA accumulation is linear for most of the cycle with a rate change midway through the 'interphase' period. Although the maximum RNA content per cell was similar in the two cultures (Fig. VI.6B and Fig. VI.7B), RNA per cell increased by only 60-70% during the cycles examined.

c. Total protein.

The rate of accumulation of total cell protein increased with time during the cell cycles examined in both cultures (Fig. VI.8A and Fig. VI. 9A). However, the increase in rate was not constant, i.e. the data do not fit an exponential. Rather, there appears to be a marked increase in the rate of protein accumulation late in each cycle close to the time of maximum mitotic activity. The rate of protein accumulation at the end of each cycle was approximately double the rate observed initially. The amount of protein per cell increased $\pm$ linearly by ca. 65% during each cell cycle (Fig. VI. 8B and Fig. VI. 9B).

d. Respiration.

For measurement of the rate of oxygen uptake during the cell cycle examined in culture 73, a small volume (ca. 5 ml.) of the main sample removed from the culture vessel was transferred immediately to a Rank oxygen electrode (see II. Materials and Methods. 16.). This procedure was repeated 30 minutes later using a further sample drawn directly from

Figures VI.6 and VI.7. Changes in total RNA content of synchronised batch cultures of *Acer* cells.

Fig. VI.6 = Culture 73
Fig. VI.7 = Culture 77

(see growth data in Fig. VI.2)

A. Total RNA per millilitre of culture extracted.
B. Total RNA as a function of the number of cells present in the volume of culture extracted.

See legend to Fig. VI.4 for further explanation.

Fig. VI. 6

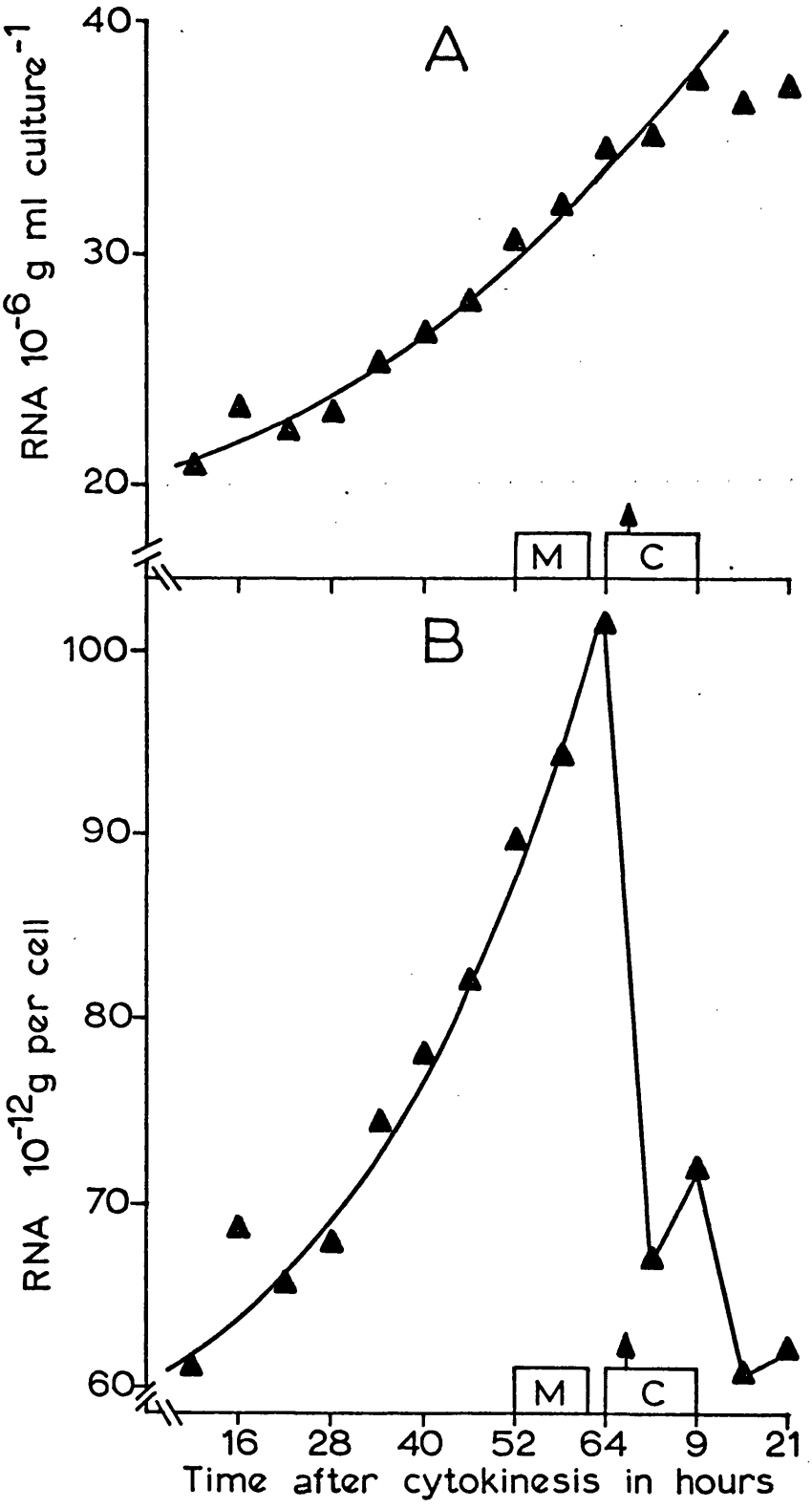
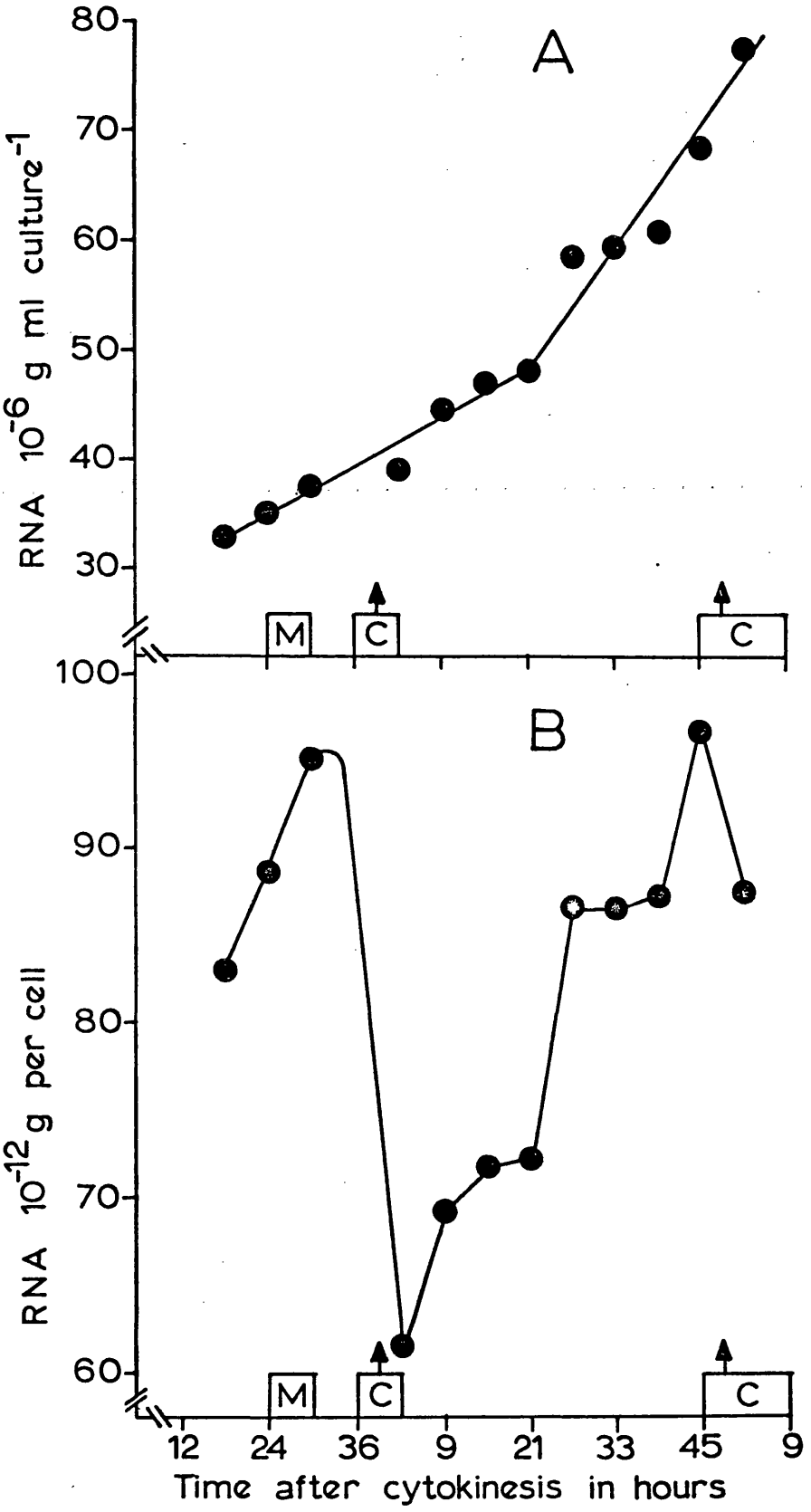

A
B
RNA 10^{-6} g ml culture^{-1}
RNA 10^{-12}g per cell
40
30
20
100
90
80
70
60
M
C
16
28
40
52
64
9
21
Time after cytokinesis in hours

Fig. VI. 7.


A
B
RNA 10^{-6} g ml culture^{-1}
RNA 10^{-12} g per cell
80
70
60
50
40
30
100
90
80
70
60
M
C
C
12
24
36
9
21
33
45
9
Time after cytokinesis in hours

Figures VI.8 and VI.9. Changes in total proteín content of synchronised batch cultures of *Acer* cells.

Fig. VI.8 = Culture 73
Fig. VI.9 = Culture 77

(see growth data in Fig. VI.2)

A. Total protein per millilitre of culture extracted.
B. Total protein as a function of the number of cells present in the volume of culture extracted.

See legend to Fig. VI.4 for further explanation.

Fig. VI. 8

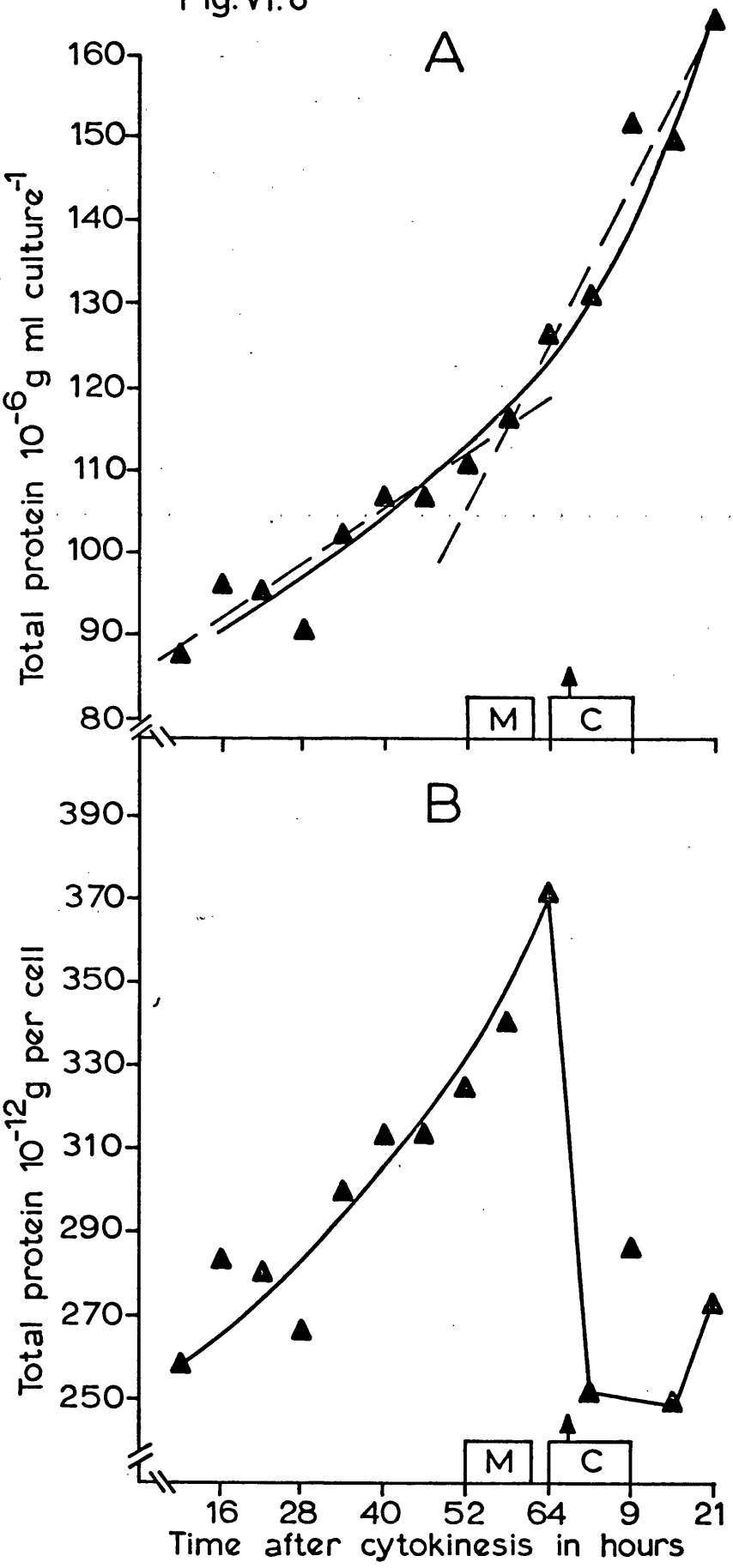
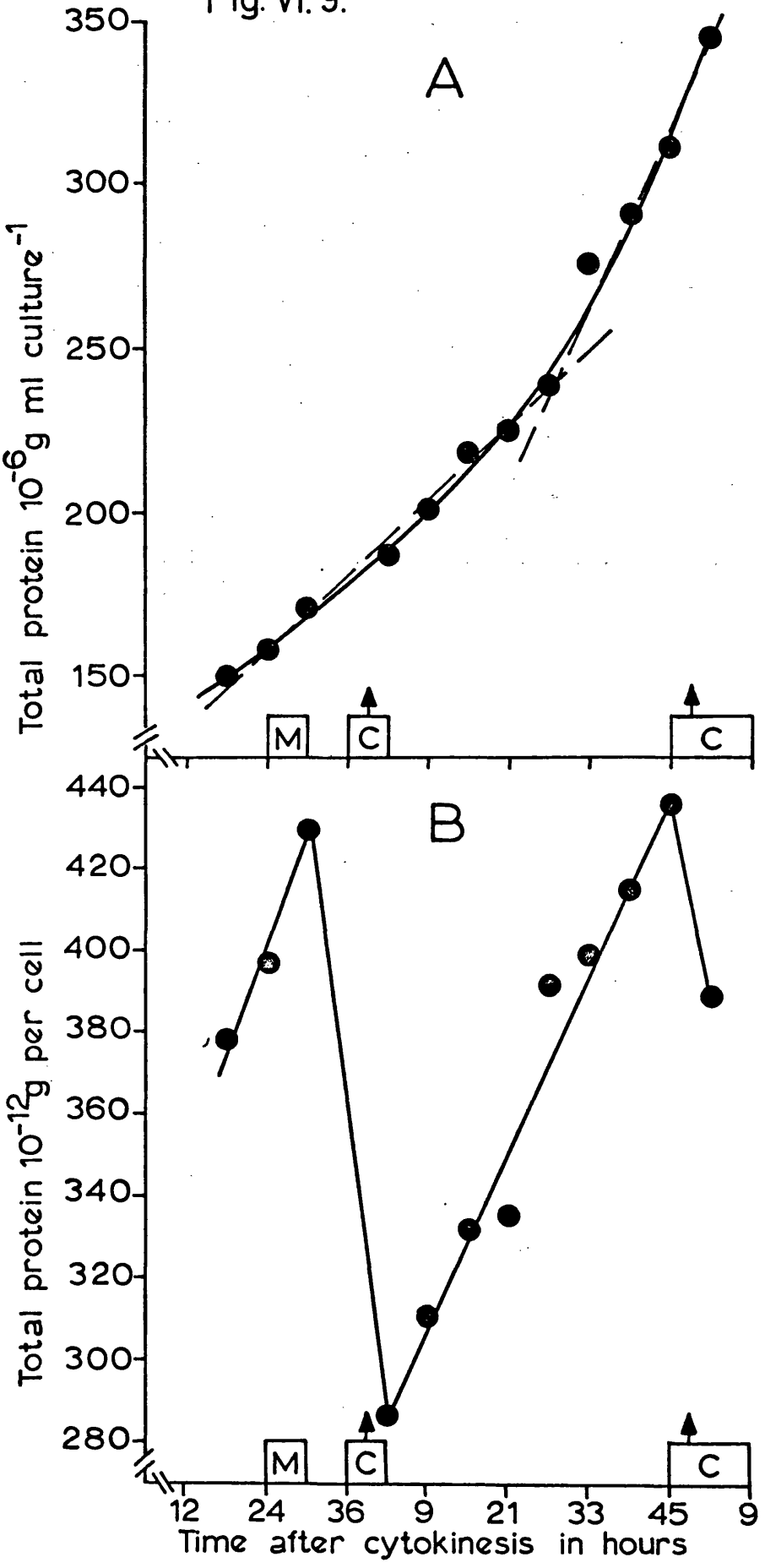

A
B
Total protein 10^{-6}g ml culture^{-1}
Total protein 10^{-12}g per cell
160
150
140
130
120
110
100
90
80
390
370
350
330
310
290
270
250
M
C
16
28
40
52
64
9
21
Time after cytokinesis in hours

Fig. VI. 9.


A
B
Total protein 10^{-6}g ml culture^{-1}
Total protein 10^{-12}g per cell
350
300
250
200
150
440
420
400
380
360
340
320
300
280
M
C
C
M
C
C
12
24
36
9
21
33
45
9
Time after cytokinesis in hours

the culture vessel. The rates of oxygen uptake per ml. of these two samples were usually in close agreement (± 10%). Oxygen uptake rates and rates of carbon dioxide evolution were measured in culture 77 by conventional Warburg manometry at 25°C. Five flasks were set up at each sampling time. A volume of culture (4 ml) was added to four of these flasks and a similar volume of distilled water was added to the fifth. A volume of 10% w/v KOH (0.75 ml) was added to the centre well in two of the flasks which contained culture samples. Changes in the ~~volume of the~~ air pressure in the flasks at constant ~~pressure~~ volume were noted over a 2-hour period.

The results illustrated in Fig. VI.10A and Fig. VI.11A. suggest a progressive, stepped increase in the respiration rate of both cultures across the cycles examined. In general, there appear to be two periods during each cycle when respiration rate per ml of culture changes relatively little. During the intervening periods there are rapid increases in rate. For example, in culture 73 (Fig. VI.10A), in the early part of the cycle the respiration rate increases more and more rapidly to a point 'a', 34 hours after cytokinesis. The rate appears to have declined by the next sample point, producing a small peak of respiratory activity. This process is repeated to a point 'b' at about the time of the next cytokinesis. A marked similarity appears in the data for culture 77 (Fig. VI.11A), where three 'steps' are apparent. Only one 'step' appears as a peak (point 'a'); the others (points 'b') appear as short plateaux.

Expressed on a per cell basis (Fig. VI.10B and Fig. VI.11B), the data tend to suggest that 1. a definite peak in respiratory activity ('a') occurs during the interphase; 2. after a short delay, the respiration rate increases as before; 3. a further peak of activity is reached shortly before cytokinesis (this is more evident in culture 77,

Figures VI.10 and VI.11. Changes in the respiration rate of synchronised batch cultures of Acer cells.

Fig. VI.10 = Culture 73
Fig. VI.11 = Culture 77

(see growth data in Fig. VI.2)

A. Respiration rate per millilitre of culture.
B. Respiration rate as a function of the number of cells present.

Fig. VI.11. • O_2 uptake
o CO_2 evolution

Cycle A and B and the letters a, b, a' and b' are all referred to in the text.

See legend to Fig. VI.4 for further explanation.

Fig. VI. 10

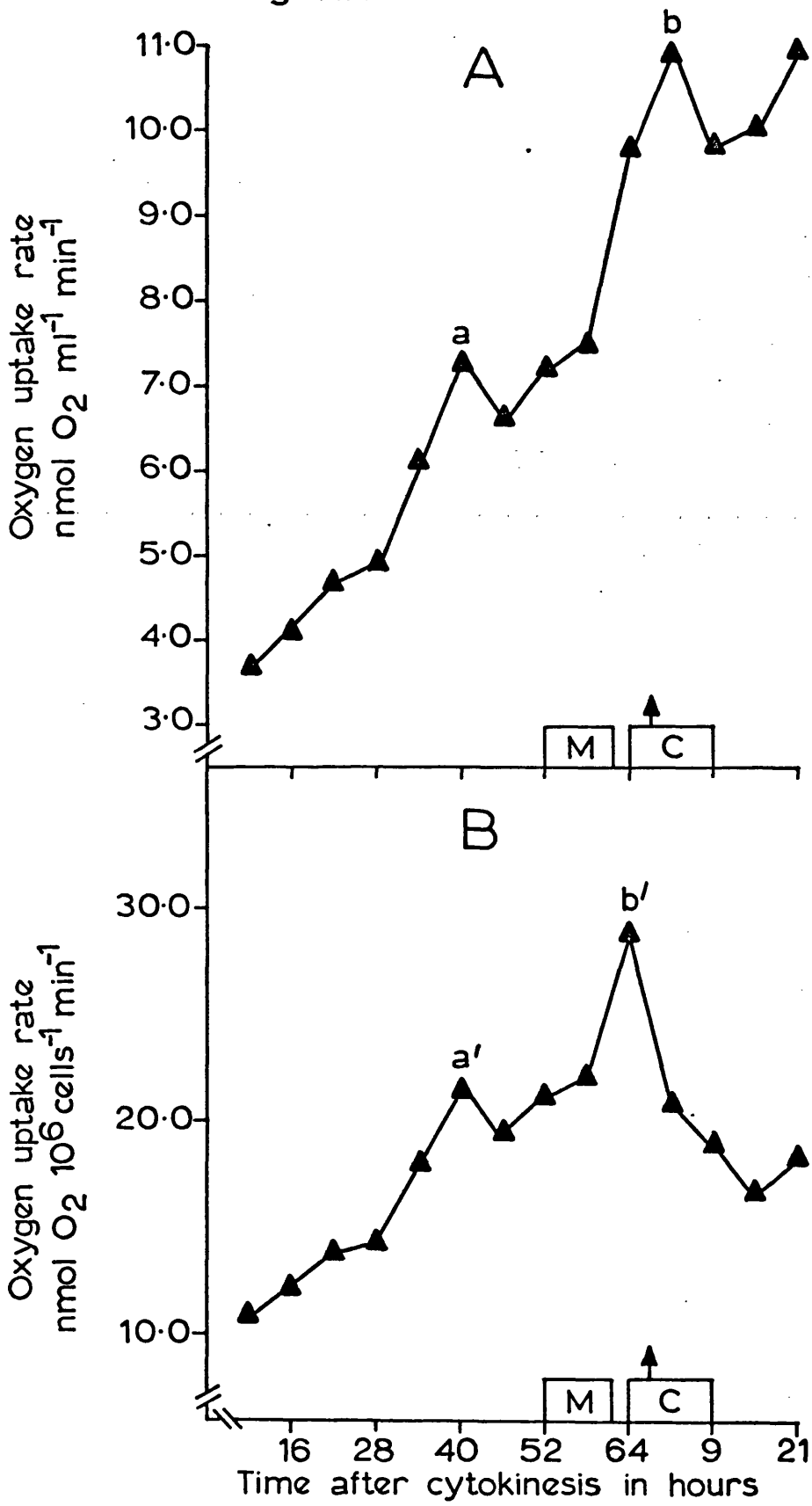

A
B
11·0
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
30·0
20·0
10·0
a
b
a′
b′
M
C
Oxygen uptake rate
nmol O2 ml-1 min-1
Oxygen uptake rate
nmol O2 10^6 cells-1 min-1
16
28
40
52
64
9
21
Time after cytokinesis in hours

Fig. VI. 11.

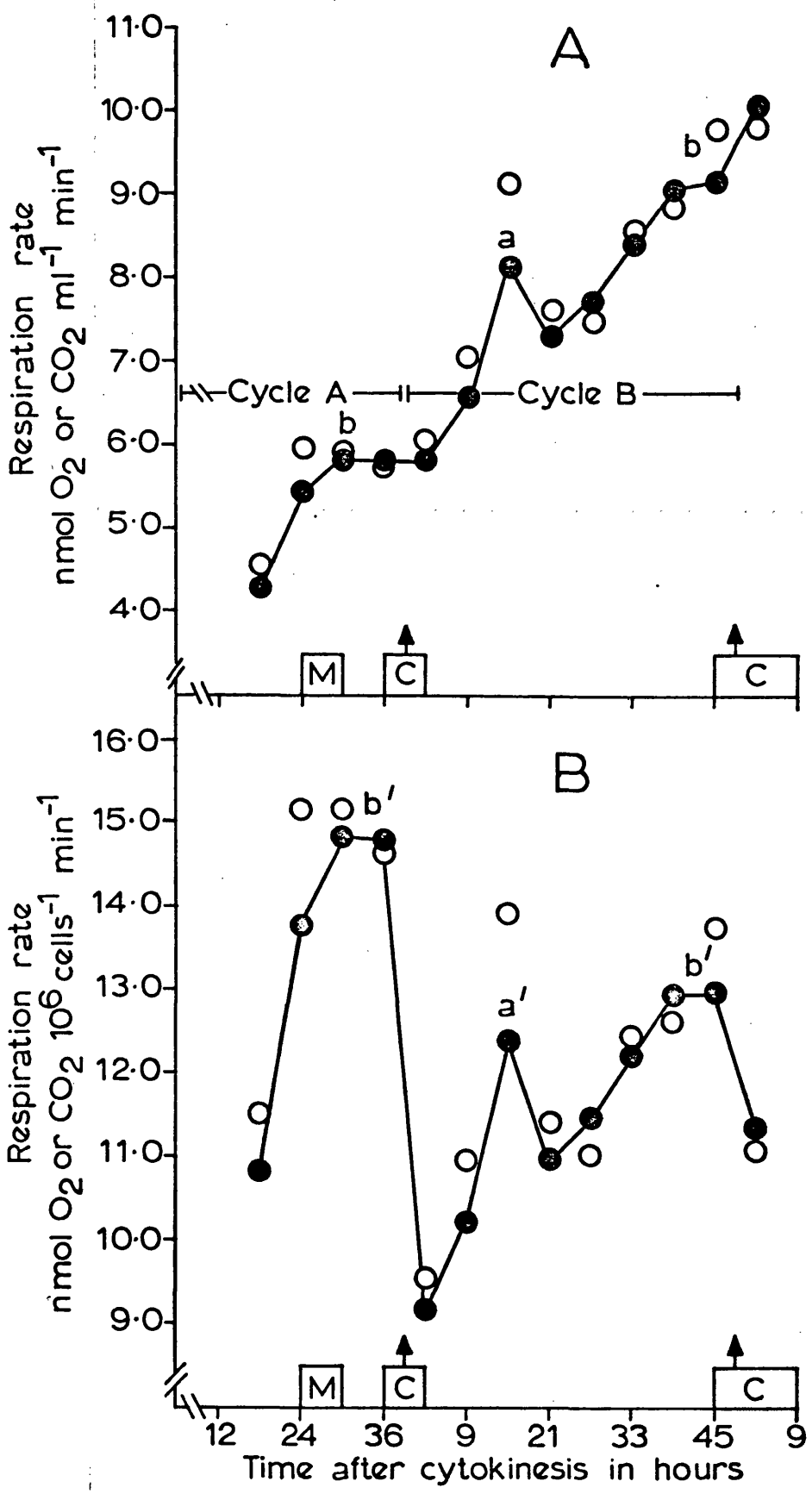

A
B
Respiration rate
nmol O_2 or CO_2 ml^{-1} min^{-1}
nmol O_2 or CO_2 10^6 cells^{-1} min^{-1}
Cycle A
Cycle B
M
C
a
b
a′
b′
Time after cytokinesis in hours

Fig. VI.11B; in culture 73, the second sharp peak produced is an artifact due entirely to a sudden increase in the number of cellular units).

There is some similarity between the two cultures in the timing of the inflexions in the respiration rate curve. In culture 77, the interphase peak ('a') occurs ca. 15 hours after the DNA content of the culture first begins to rise in that cycle and ca. 33 hours before the next cytokinesis step. The corresponding times for culture 73 are 12 hours and 27 hours respectively. Furthermore, the gap between the two inflexions ('a' and 'b') is ca. 30 hours in both cultures.

6. Decay of synchrony.

Up to five cycles of cell division have been observed in synchronous batch cultures of Acer (see Fig. VI.1, Fig. VI.2. and Table VI.1.), each involving at least 70% of the cell population. No consistent trends to shorter cycle times were observed during the progress of growth in these cultures. Neither did the proportion of the population which divided at each step decline with time. A typical culture inoculated at ca. 3×10^4 cells ml^{-1} reached a density of ca. 1×10^6 cells ml^{-1} after 24 days. Then, seemingly at the end of a plateau, the expected step-up in cell number was replaced (rather abruptly) by a more-or-less logarithmic increase in cell number. The cell division rate during this random growth was normally typical of the preceding synchronous cycles and persisted for at least one further generation before the population entered stationary phase.

In two cultures (cultures 71 and 75, Fig. VI.12) abrupt transitions between synchronous and asynchronous division occurred after only 17 and 9 days respectively. In these two cultures the rate of cell division

Fig. VI.12. Rapid transition between synchronous and asynchronous growth in batch cultures of *Acer* cells.

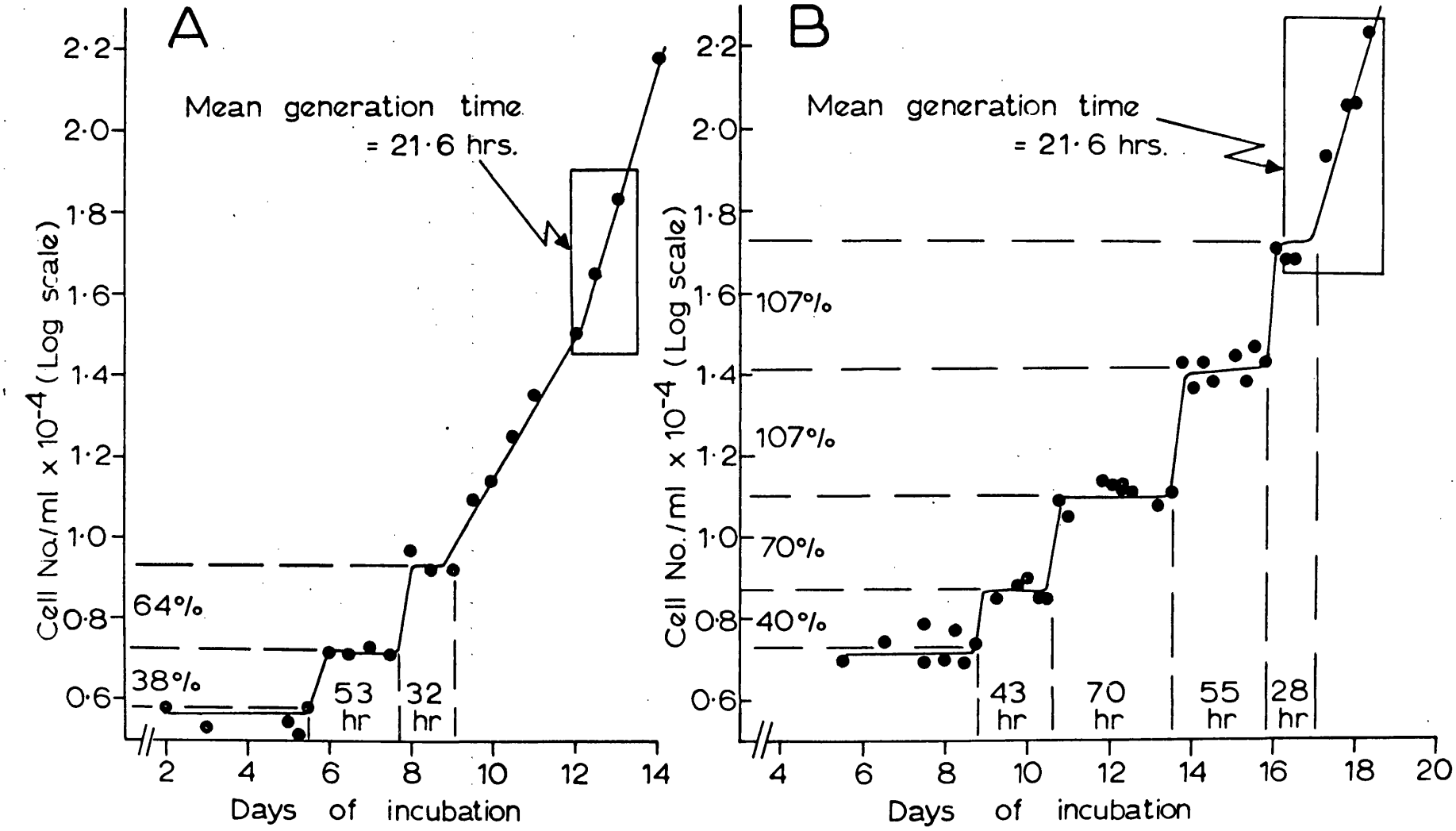
A = Culture 75
B = Culture 71

(see also Table VI.3)

The transitions to exponential growth occurred after 2 steps-up in cell number (A) or 4 steps (B) and were complete within 30 hours of the preceding step.

The % increase in cell number at each step is shown against the ordinate. The duration of each step (the cell cycle time) is shown against the abscissa.

Fig. VI.12.


A
Mean generation time
= 21·6 hrs.
Cell No./ml x 10⁻⁴ (Log scale)
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
64%
38%
53 hr
32 hr
2
4
6
8
10
12
14
Days of incubation
B
Mean generation time
= 21·6 hrs.
107%
107%
70%
40%
43 hr
70 hr
55 hr
28 hr
16
18
20

during the subsequent asynchronous growth was very high. The mean generation time (mean cell-cycle time) of both populations, calculated from the slope of the semi-logarithmic plots (see Section IV), was ca. 22 hours. This is a very much shorter mean cell-cycle time than those recorded in any other synchronous or asynchronous cultures of Acer cells growing in the standard medium. However, rates of accumulation of total cell dry matter, protein, RNA and DNA in both cultures were lower than the rate of cell division (Table VI.3). It was not possible to calculate absolute rates of accumulation in culture 75 (Table VI.3A), but in culture 71 (Table VI.3C) the doubling time for cell dry matter was ca. 40 hours and that for protein, RNA, DNA and respiration rate was ca. 85 hours. There was no increase in packed cell volume in culture 75 during this period of rapid cell division (Table VI.3A). These data, when expressed on a per cell basis (Table VI.3B and 3D), suggest that a significant reduction in cell size and macromolecular composition occurred during these unusual periods of rapid cell division. Cell volume was reduced by ca. 75% of its initial value in culture 75, together with the DNA and RNA content of the cells (Table VI.3B). In culture 71, although the dry weight per cell dropped by less than 30%, the DNA, RNA and protein content and the respiration rate of the cells dropped by more than 50%. The phase of rapid cell division and reduction in cell size was apparently short-lived. In culture 75, although the cell number had only increased to 4.16×10^6 cells ml^{-1} in a further 8 days (Table VI.3A - bottom line), the other parameters showed relatively greater increases. With the exception of the RNA content of the cells, the size and composition of the cells reverted to intermediate values.

Table VI.3. Changes in cell size and composition after a transition from synchronous to asynchronous growth.

Culture 75 : A

Sample time	Cell number	DNA	RNA	PROTEIN	Dry weight	Packed cell vol.
days	$\times 10^{-6}$ ml^{-1}		μg ml^{-1}		mg.ml^{-1}	%
0	0.32	2.72	31.7	212	0.895	2.0
1	0.67	2.55	41.2	281	1.005	1.5
2	1.46	2.78	40.2	336	1.300	2.0
10	4.16	12.96	117.3		8.790	20.0

Culture 75 : B

Sample time	Cell number	DNA	RNA	PROTEIN	Dry weight	Cell volume
days	$\times 10^{-6}$ ml^{-1}		10^{-12} g cell^{-1}		mg/10^6cells	μl/10^6cells
0	0.32	8.5	99.1	664	2.80	63
1	0.67	3.8	61.3	417	1.49	22
2	1.46	1.9	27.5	230	0.89	14
		*(0.225)	(0.278)	(0.360)	(0.319)	(0.223)
10	4.16	3.10	28.2		2.11	48

*Figures in brackets are final composition (after 3 days) as a fraction of the initial value (sample = day 0).
Day = 0 sample was on 12th day of incubation (see Fig. VI.12A).

Table VI.3. continued.

Culture 71 : C

Sample time	Cell number	DNA	RNA	PROTEIN	Dry weight	Oxygen uptake
days	$x10^{-6} ml^{-1}$	$\mu g\ ml^{-1}$			$mg.ml^{-1}$	$nmoles\ ml^{-1} min^{-1}$
0	0.65	3.44	51.4	299	1.11	10.64
1	1.03	3.19	49.5	257	1.62	12.75
2	1.85	3.89	69.1	315	2.15	12.00

Culture 71 : D

Sample time	Cell number	DNA	RNA	PROTEIN	Dry weight	Oxygen uptake
days	$x10^{-6} ml^{-1}$	$10^{-12} g\ cell^{-1}$			$mg/10^6 cells$	$nmoles/10^6\ cells\ min^{-1}$
0	0.65	5.3	79.0	460	1.71	16.4
1	1.03	3.1	48.0	249	1.51	12.4
2	1.85	2.1	37.4	170	1.24	6.9
		*(0.397)	(0.472)	(0.371)	(0.725)	(0.420)

*See Table VI.3B.
Day = 0 sample was on 16th day of incubation (see Fig. VI.12B).

7. Discussion

The data described above (Section VI.3, Figs. VI.1 and VI.2) suggest that persistent, synchronous cell divisions may be induced in large-scale, aseptic populations of Acer cells by a relatively simple starvation and regrowth treatment. If the high degree of synchrony in cell division revealed by cell counting can be shown to extend to some key metabolic events of the cell cycle, then the system described represents a very useful addition to the techniques available for investigating the control of growth and cell division of higher plant cells.

Periodism was observed in some metabolic processes in the cell cycles examined in cultures 73 and 77. There were practical difficulties in demonstrating true periodicity i.e. that events repeated themselves over a number of cell cycles. Because of the limited biomass available and the strenuous sampling schedule required, it was possible in culture 71 only to take samples every 6 hours for 75 hours. This amounted to one cell cycle with an apparent 10-hour overlap. In culture 75, samples were taken every 6 hours for 84 hours, beginning half-way through one cycle, progressing through the next complete cycle and ending 10 hours into a third cycle: total elapsed time = 1.75 cycles.

In culture 73 in the cycle shown in Fig. VI.3, 72% of the population divided within a period equal to 0.149 of the cycle. In culture 77, the corresponding figures are 68% and 0.102. The degree of synchrony of these two populations, using the synchronisation index of Scherbaum (1959), was 0.62 (culture 73) and 0.56 (culture 77). The indices were calculated from the equation:

$$\text{synchronisation index} = \left(\frac{n}{n_o} - 1\right) \cdot \left(1 - \frac{t}{g}\right)$$

where n_o and n are the cell numbers just before and just after a burst of synchronous divisions, t is the time span of the burst and g is the total cycle time. The values obtained should be compared with a theoretical maximum of 1.0 and actual values for other systems ranging between 0.26 and 0.83 (Birnstein, 1960). Although a burst of mitotic activity was recorded in both cultures at about 10 hours before the cell divisions occurred (Fig. VI.3), the peak mitotic index was not as high as might have been expected. Assuming that the mitotic activity indicated by the peak in culture 73 accounts for the subsequent division of the synchronous population, then integration of the change in mitotic index with time under the peak suggests that the average duration of mitosis in the synchronised cells was 1.3 hours. Similarly, the duration of mitosis suggested by the peak for the synchronous population in culture 77 is 0.9 hours. However, the mean value for the duration of mitosis calculated from the mitotic indices of a number of asynchronous populations of Acer in batch and chemostat cultures was 3.14 ± 0.07 hours (Table VI.4). Mitotic figures were found at all sampling times during the cycles examined in the synchronised cultures. The mitotic index rarely fell below 3%. Assuming that these mitotic figures represent the random divisions of a less synchronous proportion of the populations (28% of culture 73 and 32% of culture 77; this possibility is not excluded by the cell count data, Fig. VI.3) then the mean duration of mitosis in these cells would be 3.4 hours in culture 73 and 2.9 hours in culture 77. The data immediately suggest that the Acer strain used in these experiments is a mixture of two cell types (3:1) with different rates for mitosis, and that only the majority type (shorter mitosis) is synchronised by the treatment applied. Although the Acer cultures used have been shown to be a mixture of cells of two different ploidy levels (often in the ratio 3:1)

Table VI.4. The durations of mitosis and S-phase of *Acer pseudoplatanus* cells in chemostat culture, estimated from the mitotic index and ^{3}H-thymidine-labelling index.

td	MI	tm	LI	ts
h	%	h	%	h
66.5	3.2	2.12	11.3	7.55
85.0	4.9	4.19	10.9	9.26
103.0	3.1	3.18		

td = population doubling time (assumed to be equal to the average cell cycle time, T, of the cells).

MI and LT = mitotic and labelling indices.

tm and ts = duration of mitosis and S-phase

Values for tm and ts were calculated from the equations:

$$MI = \frac{tm}{T} \quad \text{and} \quad LI = \frac{ts}{T}$$

equations taken (from Cleaver, 1967)

(Bayliss - personal communication) there is no evidence to suggest that the cells differ in generation time or mitosis. Furthermore, a mixed, asynchronous population of cells, with values for the duration of mitosis as calculated above and in that same ratio, would normally produce an overall mean value for mitosis of 1.9 hours - much less than actually recorded. Another possibility is that nuclear division in cells synchronised by the technique used is shorter than in asynchronous populations, either because the treatment applied itself shortens mitosis or because of some interaction produced between cells when a large proportion of the population are progressing through cycle events simultaneously. There are reports of differences in estimations of cycle-phase durations between synchronous and asynchronous cell populations (Zeuthen, 1963; Rao and Engelberg, 1966). These data may also indicate that the degree of synchrony of nuclear division in the population was less than that apparent for cytokinesis.

There was an unexpectedly low level of prophase figures in the preparations examined from all cultures. The highest prophase:metaphase ratio scored was 1:3, whereas ratios of 3:1 are normal in whole plant meristems (Clowes and Juniper, 1968). The abundance of starch grains in the cells probably obscured some of the less distinct stages of prophase. It is also possible that the presence of kinetin reduced the duration of prophase (Guttmann, 1956). The low prophase counts would not help to explain the unexpectedly low mitotic peaks in the synchronous cultures, but there was a marked difference between the prophase:metaphase ratios in the peaks (1:3) and those during the rest of the cycle (1:64). This suggests either that some of the"non-synchronised" population had actually been blocked in metaphase or that the apparent increased duration of

mitosis in these cells was due to delay in metaphase. Assuming that the metaphase figures scored under the peak in culture 77 correspond with the synchronous proportion of the population, the duration of metaphase in these cells is ca. 30 minutes. This value agrees with published values for metaphase duration (Clowes and Juniper, 1968). However, metaphase in the remaining population, assuming the cells eventually divide, lasts ca. 90 minutes.

Extraction and colorimetric determination of DNA is a relatively insensitive technique for detecting fluctuations in the rate of DNA synthesis in cells. Furthermore, some of the apparent, short-term changes observed in the DNA content of cultures 73 and 77 are clearly due to sampling errors (similar changes are seen in total protein and RNA estimations). However, in both cultures there is an obvious separation in time between DNA synthesis and cell division. In culture 73 (Fig. VI.5) in particular, nuclear and cell division occur during a period of negligible net DNA synthesis. It would appear that DNA replication in the cells in both cultures had been synchronised, though possibly not to the same degree as had mitosis and cytokinesis. The mitoses and cytokenesis of the synchronous population were completed within ca. 0.2 cell cycles. Assuming DNA replication was initiated at random in the whole of this population within a similar period,† then the mean duration of the synthetic period (S-phase) in the population was 14-16 hours (see

---

† The possibility remains that DNA synthesis (as well as other cycle events discussed below) may be more synchronised than cytokinesis. This would require a relatively long S-phase and is considered unlikely, especially in view of the low percentage of cells labelling with ^{3}H-thymidine in random populations.

legend to Fig. VI.13). However, the duration of S-phase in steady-state populations of Acer cells in chemostats was estimated to be 7-10 hours by pulse-labelling with ^{3}H-thymidine (Table VI.4). Although generally lower values for the percentage of cells in S-phase are obtained by labelling than by DNA assay, (Cleaver, 1967; Clowes, 1968), these latter data suggest that initiation of DNA synthesis may have been spread over at least 0.32 cell cycles in the synchronised cultures. If the data given in Table VI.3A and 3C represent a real uncoupling between DNA replication and cell division in Acer cells, it would not be surprising to find a very much lower degree of replication synchrony than cell-division synchrony in the synchronised cultures.

The data in Fig. VI.4 and Fig. VI.5 suggest that the amount of DNA per cell (2C value) of Acer cells in culture is 5-6 x 10^{-12}g (cf. Sections IV and V). This value is low down on the scale for plant cells (Van't Hof and Sparrow, 1963) but as a percentage of cell dry weight (0.2%) is typical (Long, 1961). The minimum mitotic cycle time suggested by the data of Van't Hof and Sparrow (1963) and Evans and Rees (1971) for cells of this DNA content in vivo is only ca. 9.0 hours. The mean cycle times recorded for Acer cells in vitro were often at least eight times longer (see Fig. VI.1, Fig. VI.2 and Section IV). Furthermore, the duration of S-phase in Acer cells in culture (estimated either by labelling or from the synchronous cultures described) does not fit the relationship between S-phase and DNA content described by Van't Hof (1965) and Evans and Rees (1971) for root tip meristems of a large number of species. The rate of DNA synthesis (0.3 - 0.5 x 10^{-12}g h^{-1}, 2C DNA content / duration of S-phase) in the Acer cells was one quarter of that in root tip cells of comparable DNA content. A prolonged S-phase does not entirely account for the greatly extended cycle times in Acer cultures. It would appear, therefore, that

the intervals between cell divisions are far greater than a possible minimum because of delays in a number of cycle phases. Tentative models of the cell cycle of the synchronised populations in cultures 73 and 77 were constructed from the observations discussed above and are presented in Fig. VI.13 and Table VI.5. The durations of S-phase and mitosis in both cultures, and of G1 in culture 77, fit the frequency distribution of lengths of cycle phases in the cells of higher plants compiled by Mitchison (1971). Moreover, the very obvious difference in G1 between the two cultures coincides with the view that G1 is the most variable phase of the cycle (Wimber, 1966; Mitchison, 1971; Phillips and Torrey, 1972). The duration of G2 in both cultures is twice that recorded *in vivo* by pulse labelling techniques with a number of species (Mitchison, 1971). In general, the cycles suggested for *Acer* cells in Fig. VI.13 places them somewhere between the fast-growing root-cap initials of *Zea mays* described in the work of Clowes (1965) and the slow growing cells in the quiescent centre.

During the cell cycles examined in cultures 73 and 77, measurements were made by otherworkers of ^{14}C-thymidine (73) or ^{3}H-thymidine (77) incorporation into a TCA insoluble fraction (DNA) (B. J. Cox), and of the activities of the enzymes : thymidine kinase and aspartate transcarbamoylase (the latter in culture 77 only) (M. W. Fowler). Because of their relevance to this discussion some of the data obtained are presented in Fig. VI.14. There was an obvious periodicity in the rates of thymidine incorporation and in the absolute and specific activities of both enzymes. These data have been interpreted by the present author on the same basis as the rise in DNA content of the cultures, i.e. that initiation of these changes in the synchronised population were spread over 0.2 cell cycles (see legend to Fig. VI.13). The duration of phases of enhanced

Fig. VI.13. Model cell cycles compiled from observations made on two synchronised batch cultures of Acer cells.

KEY C cytokinesis.
SI: initiation of DNA synthesis
SE: end of DNA-accumulation phase in whole population.
Y: proposed completion point for S-phase.
R'-R": period of enhanced respiration.
ATC'-ATC": period of high specific activity of aspartate transcarbamoylase.
TK'-TK": period of high specific activity of thymidine kinase.
$^{3}H'$-$^{3}H"$: period of high rate of ^{3}H-thymidine incorporation.
SDH'-SDH": period of high specific activity of succinate dehydrogenase.
M: peak of mitotic index.
T: cell cycle time.

Calculations of phase durations.

All periodic events in the cell cycles were assumed to be initiated at random among the synchronised population within 0.2 cell cycles, which is the spread noted for mitosis and cytokinesis. Thus, in culture 73, DNA synthesis was initiated in the earliest cells 28 hours after cytokinesis (Fig. VI.4) and in the latest cells 14 hours (0.2 x 67 hours) later i.e. 42 hours after cytokinesis. But as the DNA content of the culture continued to increase until 58 hours after cytokinesis, the approximate duration of S-phase in the latest cells was 16 hours (58 - 42 hours). Other periodic events noted were similarly treated and their calculated durations are indicated in the figure.

All timings were measured from the mid-point of the preceding cytokinesis.

Fig. VI. 13.

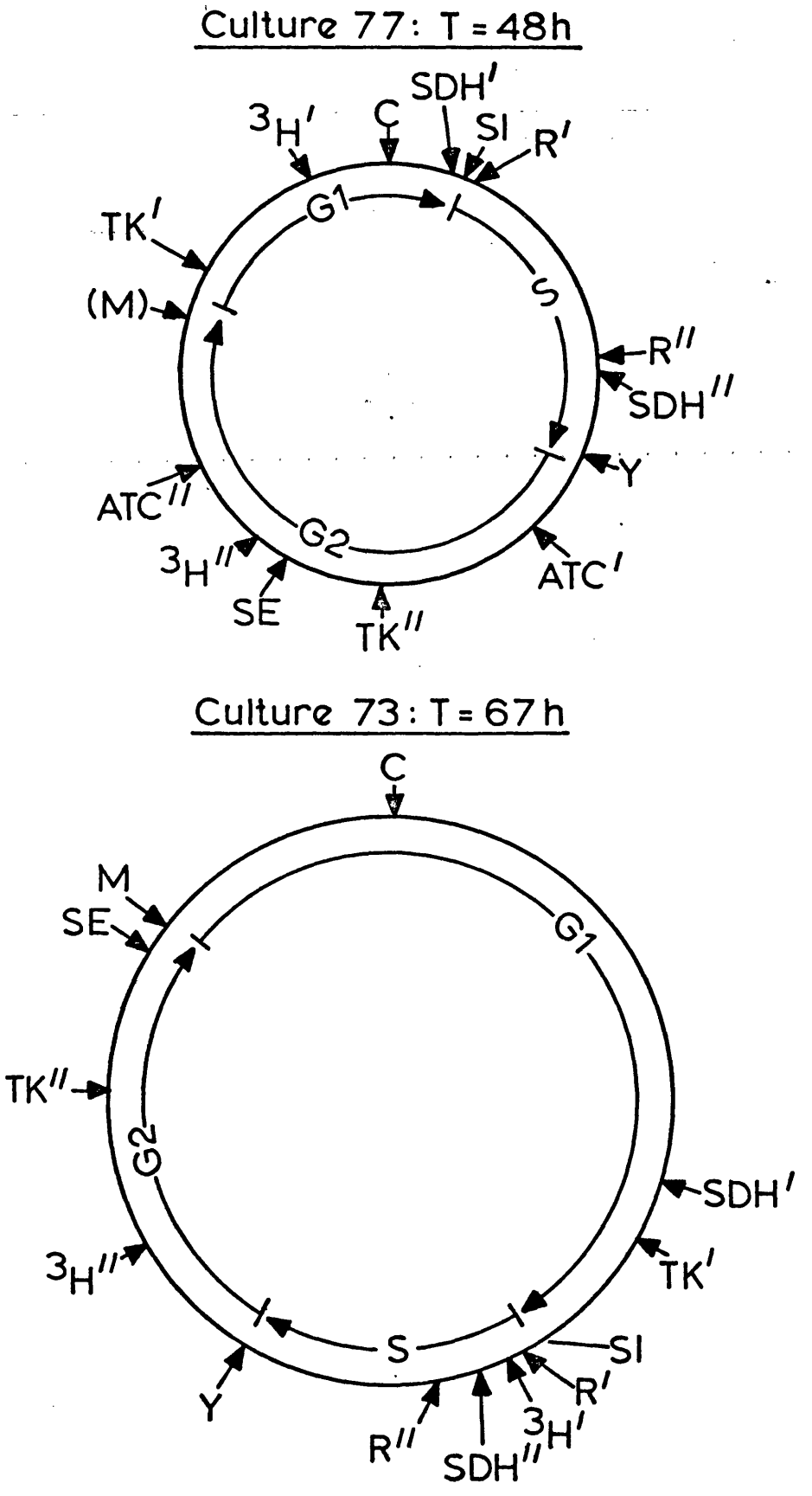

Culture 77 : T = 48h
SDH'
3H'
C
SI
R'
TK'
G1
(M)
S
R''
SDH''
Y
ATC''
G2
ATC'
3H''
SE
TK''
Culture 73 : T = 67h
C
M
SE
G1
TK''
G2
SDH'
3H''
TK'
S
SI
Y
R'
3H'
R''
SDH''

Table VI.5. Estimated duration of cell cycle phases in synchronised cultures of Acer pseudoplatanus. L.

CULTURE No.	Phase duration in hours.				
	G1	S	G2	M	T
73	37	15	14	1.5	67.5
77	13	15	19	1.0	48.0

T = total cycle time

M = duration of mitosis

For method of calculation see legend to Fig. VI.13.

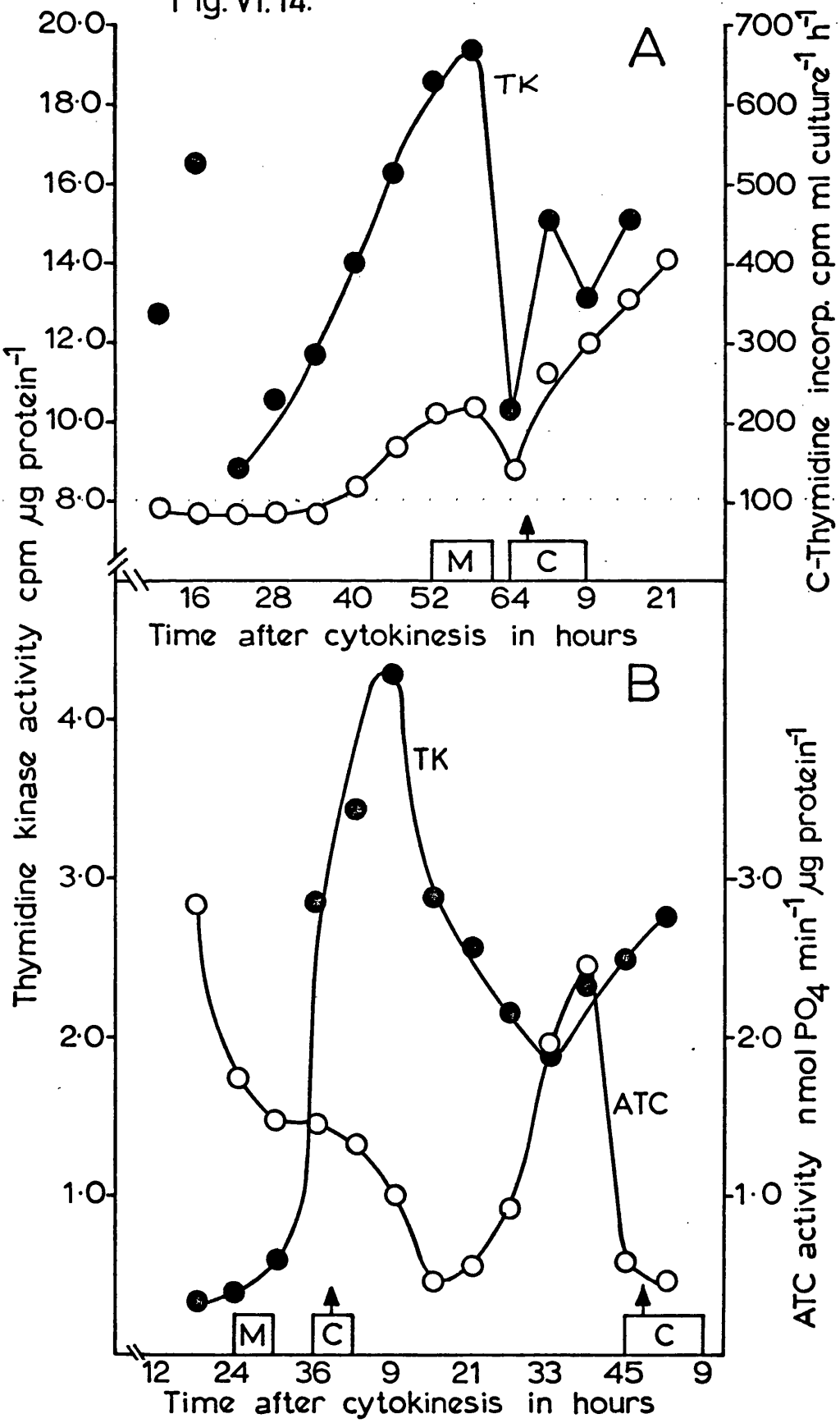
Fig. VI.14. Changes in the activities of thymidine kinase (TK) and aspartate transcarbamoylase (ATC) and in rates of ^{3}H-thymidine incorporation during two cell cycles in synchronised batch cultures of Acer cells.

A = Culture 73
B = Culture 77

Thymidine kinase and aspartate transcarbamoylase were assayed by Dr. M. W. Fowler using the methods of Hiraga, Igarashi and Yura (1967) and Shepherdson and Pardee (1960) respectively.

Rates of incorporation of 2-^{14}C-thymidine were measured by Dr. B. Cox as follows: Aliquots (10 ml) of culture were incubated for 1.0 h with $10^{-4}M$ 2-^{14}C-thymidine (specific activity = 0.5 m Ci/mM) in separate flasks on a rotary shaker at 25°C. Aliquots (2 ml) were then taken and added to ice-cold 10% w/v trichloroacetic acid (TCA) (2 ml) containing x 100 excess of unlabelled thymidine. After 2 hours at 0°C, TCA-insoluble precipitates were collected onto glass-fibre filters, washed, dried and counted in a Beckman LS-100 scintillation spectrometer using Fluoralloy DXA universal scintillation cocktail.

Fig. VI. 14.


A
TK
20·0
18·0
16·0
14·0
12·0
10·0
8·0
700
600
500
400
300
200
100
Thymidine kinase activity cpm µg protein⁻¹
C-Thymidine incorp. cpm ml culture⁻¹ h⁻¹
M
C
16 28 40 52 64 9 21
Time after cytokinesis in hours
B
TK
ATC
4·0
3·0
2·0
1·0
ATC activity n mol PO4 min⁻¹ µg protein⁻¹
M
C
C
12 24 36 9 21 33 45 9
Time after cytokinesis in hours

thymidine incorporation and high specific activities of the enzymes were calculated and are indicated on the model cell cycles in Fig. VI.13. There is good correlation between the period of enhanced thymidine incorporation (H'-H"), the peak in the specific activity of thymidine kinase (TK'-TK") and the proposed S-phase. Thymidine kinase activity apparently increases 6-10 hours before the initiation of DNA replication (in culture 77 this occurs before an intervening cytokinesis). Thymidine incorporation increased much closer to the start of S-phase. Neither thymidine kinase activity nor thymidine incorporation diminished until after the end of the estimated S-phase. The apparent, strict regulation of thymidine kinase such that its activity is restricted to a fraction of the cycle which includes the DNA-replication phase is very surprising. Although the exogenous thymidine supplied is presumably incorporated after phosphorylation by thymidine kinase, neither thymidine nor thymidine kinase are on the normal pathway for DNA synthesis, which goes directly from deoxyuridine monophosphate to thymidine monophosphate (Kihlman, 1966). However, similar peaks of thymidine kinase activity have been demonstrated in synchronised cultures of other eukaryote cells (Mitchison, 1971). There was a sharp peak (0.3 cell cycles) in the apparent activity of aspartate transcarbamoylase (ATC) in culture 77 (ATC'-ATC"). This peak occurred entirely within G2, commencing ca. 2 hours after the end of S-phase. It is interesting to find that this key enzyme in pyrimidine biosynthesis, which is directly involved in the synthesis of uridylic acid (UMP) and hence, by interconversion, in the synthesis of pyrimidine nucleotides, is only active in a short, discreet part of the cycle. Still more interesting is the fact that its activity appears to increase immediately after the S-phase, presumably replenishing precursor pools in preparation for the next

round of replication. It would have been of interest to have assayed the activity of ATC in culture 73 to see whether the change in activity was coupled more closely to the initiation than the cessation of DNA replication. Step-wise (rather than peak) patterns of change in apparent activity of ATC have been demonstrated in some prokaryote and eukaryote cells (Mitchison, 1971). However, the demonstration of end-product inhibition of ATC in plant tissues by pyrimidine nucleoside monophosphates (especially UMP)(Neumann and Jones, 1962) is more in agreement with the peak of ATC activity recorded in Acer cells.

The continuous accumulation of total protein and RNA in the cultures presented few cycle markers. Although the rate of accumulation of both macromolecular fractions approximately doubled during the cycle, neither set of data gave a satisfactory fit to an exponential, most of the increase in rates occurring late in the cycle. It is possible that both RNA and protein were synthesised at a constant rate until a particular point in the cycle, after which the rate doubled (see Figs. VI.7, 8 and 9). Examples of both exponential increase in RNA and protein and a linear pattern with a sudden rate change have been found in synchronised cultures of mammalian cells and lower eukaryotes (Mitchison, 1971). A sharp increase in the rate of protein accumulation towards the end of the cycle might well be associated with mitotic spindle formation. The apparent increase in RNA accumulation (mainly ribosomal RNA) suggests a gene dosage effect; if the rate of ribosomal RNA production is proportional to the number of RNA genes in the cell, then the rate will double during S-phase. The most likely rate change point, however, falls just after the estimated end of S-phase in both cultures (Fig. VI.13). But in culture 77, the rate change point does lie within the phase of high activity of ATC, a possible regulatory enzyme in RNA biosynthesis (Fig. VI.13).

Fig. VI.10. and Fig. VI.11. present convincing evidence for a peak of enhanced respiration ('a') during the interphase in both cultures. A further peak may occur close to cytokinesis but the evidence is not so clear. Assuming that the degree of synchrony of the initiation of this phase of high respiration was similar to that shown for mitosis and cytokinesis, the probable duration of the phase has been calculated (see legend to Fig. VI.13) and is indicated on the model cell cycles in Fig. VI.13 (R'-R"). The apparent activities of two respiratory enzymes were assayed during these experiments (M. W. Fowler). Glucose-6-phosphate dehydrogenase (EC1.1.1.49) activity increased continuously through both cycles. However, the absolute and specific activities of succinate dehydrogenase (EC 1.3.99.1.) showed a marked peak during interphase. The duration of the phase of high activity of the enzyme (SDH'-SDH", Fig. VI.13), estimated by the present author, coincides with the phase of high respiration rate noted above, particularly in culture 77. Furthermore, both of these phases are closely correlated to the initiation and early part of the S-phase. These data indicate some form of regulation of succinate dehydrogenase activity perhaps geared to the provision of large amounts of ATP and thus of the deoxyribonucleoside triphosphates for DNA synthesis.

The great variation in mean cycle times noted in both synchronous (Fig. VI.1) and asynchronous populations (see Section IV) of Acer cells in the standard cultural conditions might be expected for a tissue dividing at rates far below the maximum (p.122), especially if the control mechanism for cell division was only loosely coupled to biosynthesis or was sensitive to specific, small changes in cultural conditions (see Section IV). Viewed in this way, the sudden switch to mean cycle times of only ca. 20 hours in cultures 71 and 75 (Fig. VI.12) is more credible and very

significant. It is clear from data in Section IV that balanced growth is a situation only rarely encountered in batch cultures of Acer cells. (It is surprising that during the synchronised cell cycles examined in cultures 73 and 77 the increase in total macromolecules - 70% - during the cycle matched the proportion of cells which subsequently divided). In view of this apparent independence of the control mechanism for the rate of cell division and that for biosynthesis, it is not surprising to find that accumulation of total cell dry matter, RNA and protein, or the increase in cell volume, lagged behind cell division during the periods of rapid cell division in cultures 71 and 75 (Table VI.3.). However, it is very surprising to find that the rate of DNA accumulation also lagged behind, and that the DNA content of the cells declined by up to 75%. DNA replication and cell division are usually coupled in normal cell cycles, but there is not a universal causal connection between them (Mitchison, 1971). Yet whilst there are many examples of DNA replication without division (see Zeuthen, 1971), instances of division without prior DNA synthesis are very few. They include the reduction of polyploid cells in crown gall tumours of bean stem to diploids (Rasch, Swift and Klein, 1959), or the loose coupling of DNA synthesis to division in Tetrahymena (Jeffrey, Stuart and Frankel, 1970). The phenomena observed in Acer cells could indicate that the clone has developed polyteny by endoreduplication (Sunderland, 1973) and that this process is reversible. The normal chromosome complement of Acer pseudoplatanus L. in vivo is $4n = 52$ (Darlington and Wylie, 1955). Cytological examination of the culture clone used in these experiments (Bayliss, personal communication) normally reveals two modes of chromosome number at 75 and 130. Thus it is also theoretically possible that reduction divisions occurred producing cells close to $2n = 26$. Nuclear densitometry profiles (Gould, personal

communication) showed a reduction both in nuclei of high DNA content and of the number of interclass nuclei during a similar period of rapid cell division in a synchronised Acer culture. The latter suggests a decline in the proportion of nuclei involved in DNA replication at the time of their isolation. Although the occurrence of the periods of rapid cell division are unpredictable, they merit further study, possibly in connection with the effect of 2,4-D discussed in Section IV.

The mechanism of the induction of cell division synchrony in these populations was not directly investigated. However, synchrony was induced only after the inoculation of stationary phase cells into fresh medium (Table VI.1). It may be that during a period of starvation the cells aligned themselves at the same point in the cell cycle and started growth at the same time on reinoculation. Changes in the mean DNA content per cell (and per nucleus) during asynchronous growth of batch cultures of Acer (see Section IV) suggest that cells in stationary phase are predominantly in G1, and that DNA replication precedes cell division when the cells are transferred to fresh medium. There has been no direct identification of the factor, the absence of which prevents the cells completing any essential phases of G1 and initiating S phase. A number of major nutrients in the medium (e.g. nitrogen sources, phosphorus and sugars) are depleted late in the exponential growth phase of Acer batch cultures (see Section IV). Furthermore, nitrogen has been shown to be the factor in the standard medium which limits growth in chemostat cultures of Acer (see Section V) presumably by limiting the rate of synthesis of essential proteins. It would be of great interest to identify the factor(s) in the medium whose depletion apparently halts the majority of a population of Acer cells close to one specific point in the cell cycle. It may also prove useful to compare the effectiveness of the depletion of different

factors in synchronising plant cell cultures.

No correlation was observed (Table VI.1) in Acer cultures between the duration of the starvation period and either the extent of the lag phase (the period from inoculation to the first division) or the degree of synchrony produced. Some cultures (ca. 30% of the total), after an apparently normal starvation period, did not divide, or, if they did divide, they did not do so synchronously. Neither was there a correlation between the initial cell density (Table VI.1) and either the duration of the lag phase or the degree of synchrony. Considering the high degree of synchrony achieved, it is puzzling to find no correlations here. The lag phase (which was generally longer than for normal batch cultures inoculated at densities of ca. 3.0×10^5 cells ml^{-1} with cells eight days into stationary phase - see Section IV) might be expected to be constant if all of the cells were blocked at the same point in the cycle in any stationary phase culture. Superimposed on this one might expect to find a positive correlation to the starvation period (due to the decay of pools, enzymes or"cell-division factors" - see Zeuthen and Rammussen, 1971) or a negative correlation to the initial density (due to a "conditioning" effect - see Stuart and Street, 1969). It is possible that the general variability of cycle times noted above (p.III) during the synchronous divisions extends even to the initial cycle. Thus although there may have been constant periods of recuperation, i.e. true lag phase or G0 periods (Cleaver, 1967), distinct from the events of G1 of the first cycle, the actual duration of the first complete cell cycle in the cultures may have varied considerably. A further possibility is that the percentage viability of cultures in stationary phase may vary. The confidence limits on the cell-counting procedure are such that synchronous cell divisions in a population of less than 30% viability would not register

until beyond the second division.

The "starvation and regrowth" treatment described here appears to be much more simple than that initially described for eukaryote cells (Saccharomyces cerevisiae) by Williamson and Scopes (1961). They found that periodic, controlled exposure to the normal, complex growth medium during starvation on a minimal, salts medium was an essential requirement for synchrony induction. The system used by Yeoman and Evans (1967) with artichoke tuber explants is essentially a starvation and regrowth treatment. However, it is complicated by the fact that the "starvation" period forms part of the sequence of events leading to the differentiation of the storage-organ tissues in the intact plant, and as such is more difficult to investigate. A more straightforward application of the principle has proved successful with Chinese hamster cells (Tobey and Ley, 1970). Isoleucine and glutamine were the components depleted during the starvation period. It is interesting that mitosis may be synchronised in cell cultures of Nicotiana tabacum L. (Jouanneau, 1971) by inoculating either stationary phase or exponential phase cells into medium which temporarily lacks an essential cytokinin. Peaks of mitosis appeared after the addition of cytokinin. The degree of synchrony was related to the duration of cytokinin-starvation. Although cell cultures of Acer pseudoplatanus L. do not require an exogenous cytokinin, it would be useful to know what effect witholding the essential auxin (normally 2,4-D) in the lag phase has on division of synchronised and non-synchronised cultures.

It is possible to propose a number of explanations for the synchronisation of maintained, stationary-phase cultures, based especially on models for the control of cell division which implicate labile cell division factors (proteins) (Smith and Pardee, 1970; Zeuthen, 1971). These now need to be carefully investigated.

VII. SUMMARY AND GENERAL DISCUSSION

The results presented in Section V suggest that steady states of growth of higher plant cells may be established in continuous culture, either as chemostats or turbidostats. Stable states of Acer cells were maintained over a wide range of dilution rates in chemostat cultures. The dependence of steady-state biomass on dilution rate was generally that to be expected from (microbial) chemostat theory (Herbert et al 1956) although relatively large changes in biomass resulted from changes in dilution rate at low growth rates (see Eqn.20 Section V). A critical dilution rate was encountered above which wash-out occurred. The maximum growth rate of Acer cells suggested by the value of the critical dilution rate coincides both with the maximum growth rate most often recorded in the exponential phase of batch cultures of Acer growing in the same medium (Section IV) and with cell-cycle times noted during synchronous growth in batch cultures (Section VI). The response of steady-state populations of Acer cells to changes in the rate of supply of nitrate suggests that, in the standard medium used here for the propagation of Acer cells, nitrate is the growth-limiting nutrient. Despite the presence (and assimilation) of a further nitrogen source (urea) in the medium the relationship between dilution rate (= growth rate) and steady-state concentration of nitrate corresponds with that suggested by Monod (Herbert et al 1956) (see Eqn. 19, Section V). Contrary to the theory, however, the ratio of biomass produced to limiting-nutrient consumed (the yield coefficient, Y) was not a constant; Y decreased with increasing dilution rate as a function of cell number, dry weight and total protein. This variation in Y probably accounts for the deviation from theory observed in the

dependence of biomass on dilution rate and for some of the variation in cell composition (in particular, dry weight and total protein) at different dilution rates.

The size, composition and physiological state of Acer cells appears to depend upon growth rate. The decrease in apparent mean DNA - content of the cell populations with decreased growth rate is considered to be due to the changes in frequency-distribution of cells in the population about the cell cycle resulting from time-extension of G1 and not to an absolute decrease in the basic amount of DNA in the unreplicated genome. There was a marked increase in RNA and respiration rate per cell with increased growth rates, although total protein concentration changed relatively little. The data suggest that both the RNA content and respiration rate of the cells are related to the rate of protein synthesis rather than the protein content of the cell. Similar changes in macromolecular composition of bacteria, yeast and protozoa with growth rate have been obtained (Kubitschek, 1970). However, the decrease in cell volume and dry weight of Acer cells with increased growth rates is the opposite of trends recorded in bacterial chemostats (Kubitschek, 1970).

There appears to have been no significant alteration in the basic growth processes of Acer cells following their transfer from batch to continuous cultures. Maximum growth rates and changes in yield coefficient, physiology and cell composition recorded in batch cultures (see Section IV) all fall within the range of cell states established in chemostat cultures. The state of cells at zero growth and at maximum growth rate in chemostats, suggested by extrapolation of the changes in the cells at different growth rates, agree well with the activity of cells in batch culture during stationary phase and the exponential growth

phase respectively.

The growth of Acer cells in chemostats was not entirely predictable. In one of two cultures in which growth clearly and persistently deviated from the established trends, the deviation was associated with the presence of a secondary nitrogen source (urea). In the other culture, the apparent change in maximum growth rate was probably the result of spontaneous changes in the specific growth rate of the type already observed in batch cultures of Acer cells (see Section IV).

The relationship between biomass and culture opacity, though non-linear for cells at different growth rates, was sufficiently sensitive to permit the turbidometric control of culture density at growth rates close to maximum. Populations of Acer cells at low density were maintained in turbidostat cultures at growth rates close to the maximum suggested by chemostat and batch-culture data. In these steady states, the culture contained an excess of all nutrients analysed and the size and composition of the cells corresponded to that observed at critical dilution rates in chemostat cultures. A critical cell-density ($1.0 - 1.5 \times 10^6$ cells ml^{-1}; ca. 2 mg. dry weight ml^{-1}) exists for Acer cells in the standard medium above which stable, growth-states cannot be achieved in turbidostat culture.

Cell division in batch cultures of Acer was readily synchronised by a simple starvation and regrowth treatment involving unknown components of the standard culture medium (Section VI). When this technique is applied to 4-litre batch cultures, it is possible to follow the growth of the cells through at least five synchronous generations, with an average 85% participation at each cytokinesis. Variation in the cell-cycle time of such populations coincides closely

with the range and frequency of population doubling times (≡ mean cell-cycle times) recorded in randomly-dividing batch cultures. Examination of the interphase of three comparable cell cycles established some basic markers in the cell cycle of Acer cells. DNA synthesis was restricted to a period of ca. 15 hours within the cycle. The time-interval between DNA replication and nuclear division (G2) was relatively constant at ca. 16 hours. Nuclear division (duration = 1 - 2 hours) was separated from cell division by an apparent lag of ca. 10 hours. [Assessment of the occurrence of cytokinesis was based solely upon increases in cell number; the cell-counting procedure probably only resolved cells already separated by a substantial primary wall]. The time interval between nuclear division and the following DNA replication (G1) was most variable (13 - 37 hours). Variability of G1 is also suggested by data obtained from both random batch cultures and continuous cultures. The S-phase or its initiation was coincident with other periodic events in the cycle: peaks of specific activity of thymidine kinase and succinate dehydrogenase; high rates of incorporation of ^{3}H-thymidine into DNA; high respiration rates. In contrast, the highest specific activity of aspartate transcarbamoylase was recorded during G2. The accumulation of total protein and RNA occurred more uniformly across the interphase than the events just listed. It was not clear whether the accumulation of these macromolecules was linear or exponential (a maximum difference of less than 8% would be expected between a linear and an exponential rise over a single generation).

A puzzling feature of the large-scale batch cultures of Acer cells (Section IV) is the variability of specific growth rate between separate cultures. This phenomenon was brought to light by a more precise examination of rates of increase in cell number than has been

attempted before with this species. Reproducibility and precision of the specific-growth-rate analysis was obtained by use of an improved cell-counting procedure, assessment of the confidence limits of this procedure, the initiation of cultures from a low density to extend the exponential growth phase and frequent (at least daily) carefully-timed sampling. Specific growth rate variability also occurs in the other cultural situations examined (synchronous and continuous) and has been confirmed by the data of other workers in the same laboratory (Gould - personal communication). It has, however, not been reported for any other cultures of plant cells. The fact that the most frequently recorded cell-cycle time for Acer cultures in this study (ca. 70 hours) is eight times the value to be expected from in vivo evidence (Evans and Rees, 1971) and that the shortest time (ca. 20 hours) is still twice the expected value suggests not only that the usual cultural conditions are less than optimum for cell division but also that either the inhibition is only loosely applied or that the inhibitory factor itself is subject to random variation. The data obtained does not point to a specific inhibitory factor in the medium but suggests that the total cultural procedure itself may affect the division potential of the cells. The observations that (1) the specific growth rate recorded extrapolates closely to the initiation of division in the batch culture i.e. that the cells were committed to a specific rate of division before inoculation, and that (2) batch cultures produced by inoculating cells from the same stock suspensions into aliquots of the same medium invariably have the same specific growth rate, suggest that random variation in culture procedures (particularly inoculum density and passage duration) during the history of the inoculum used is the factor responsible. It would follow that stabilising the culture conditions (as is effectively done

in a continuous culture) should eliminate this variation. The activity of cells in chemostats was largely predictable and the overall data obtained, which point to a maximum specific growth rate of ca. 70 hours, suggest that continued cell-division favours low rates of cell division. However, in one culture which deviated from the norm, a transformation occurred during continuous culture which suggested that continued cell division favoured lower cell-cycle times. In this connection the data from synchronous cultures are also somewhat contradictory. Although trends towards shorter cycle times were recorded in three cultures, two of which decayed rapidly to exponential growth with mean cell-cycle times of ca. 20 hours, in other cultures no similar trend was observed. Thus it is not altogether clear whether prolonged periods of stationary-phase necessarily favour short cycle-times on reinoculation (the converse of the chemostat situation). Data were obtained which suggested that high maximum growth rates were associated with increases in absolute DNA content of the cells and that an increase in the initial concentration of 2,4-D in the culture medium increased the rate of cell division in cultures which would otherwise have divided at the lowest rate (i.e. td $\simeq$ 70 hours). Further investigation of this phenomenon might increase our understanding of the control of the division of plant cells and might best be tackled using cultures in prolonged steady-states in a turbidostat system.

A comparison of the size and composition of Acer cells after several generations in continuous culture with the changes in size and composition during the progress of growth in batch cultures confirms the view that the changes in batch culture are due to the transformation from a non-dividing state to a dividing state (and back again) which occurs at each subculture. Thus steady states in continuous culture represent an

extended exponential growth phase. However, as Pirt (1972) points out: "the (exponential growth phase)... refers to the period of growth at a constant maximum rate which occurs in a batch culture while there is excess nutrients; this is only one extreme case of the constant conditions possible in a chemostat". It is this fine control and flexibility of the chemostat, permitting the selection and stabilisation of a specific growth-rate and thus the selection of cells in a particular metabolic state which is its prime attraction. For example, using the information obtained for Acer cells, in studies of RNA synthesis cells with low ribosomal-RNA content could be maintained in that state by applying low dilution rates; such dilution rates would also produce cells of low QO_2 for use in studies of carbohydrate oxidation. It should also be possible to select dilution rates to give cells with a particular pool-size of an individual metabolite or a particular specific activity of a given enzyme. Because of the equilibrium between input-nutrients and output-biomass which constitutes the steady state, rates of utilisation of individual nutrients are constant over measurable periods of time and thus the in vivo activities of relevant enzymes may be calculated. Comparing this information with in vitro enzyme activities may be of use in studies of enzyme regulation. Again, in chemostat culture, adaptation to selected nutrient regimes may be more efficiently monitored than in batch cultures; it would be possible, for example, to switch a chemostat culture of plant cells from a nitrogen-limited population growing in an excess of carbohydrate to a carbohydrate-limited population growing in excess nitrogen and to investigate the transition between the two states.

One exciting possibility which arises from the successful propagation of higher plant cells in continuous culture is that the

specific environmental conditions required to switch on in the cells pathways leading to the biosynthesis of plant products of nutritional or pharmaceutical importance to the community may be more efficiently investigated. Many compounds with modern medicinal uses are characteristic of specific intact tissues or organs of whole plants (Turner, 1971). However, so far attempts to produce such compounds in economic amounts in morphogenetically-undifferentiated cell or tissue cultures have failed. In most cases no trace of the compound could be detected in the cultures until organogenesis was initiated. For example, tropane alkaloids could only be detected in cell suspensions of Atropa belladonna after the initiation of root primordia in cell aggregates (Thomas and Street, 1970); nicotine synthesis in tobacco callus cultures was promoted by kinetin only as a function of the number of shoot-buds initiated (Tabata, Yamamoto, Hiraoka, Marumoto and Konoshima, 1971). It appears, therefore, very difficult to induce isolated, cultured cells to carry on aspects of secondary metabolism that they exhibit when part of the intact plant without further knowledge of the microenvironment that they experience in association with other cells of the plant (Krikorian and Steward, 1969; Street, 1973). However, some secondary products (many with pharmaceutical uses) have been detected in apparently undifferentiated batch suspension cultures, e.g. alkaloids, antibiotics, diosgenin, cardenolides (Puhan and Martin, 1971; Turner, 1971). It is very significant that, in a few, detailed studies of product accumulation during the whole of the growth cycle of the batch cultures involved, rates of accumulation depended upon the growth phase (= physiological state) of the cultures. It might perhaps have been anticipated from observations of the whole plant that an increase in concentration of

secondary products would often be associated with the decline of cell division; this is so for the accumulation of phenolics in rose cell-cultures (Nash and Davies, 1972), of caffeine in cultures of Camellia sinensis (Ogutuga and Northcote, 1970) and of chlorophyll in cell cultures of Atropa belladonna (Davey, Fowler and Street, 1971). In contrast, the concentration of visnagin (a physiologically-active furanochromone) in cell cultures of Ammi visnaga (Kaul and Staba, 1967) was greatest (0.3% of the dry weight) during a transient period of the most rapid growth phase; the concentration fell rapidly as growth ceased. Seeds of A. visnaga (the usual source) contain only 0.06% of visnagin; multi-litre suspension cultures were shown to produce 62.7 mg. visnagin l^{-1} day^{-1}. Thus stabilisation of the optimum growth state in a chemostat or turbidostat culture may help to produce such compounds in useful amounts. At the very least, the steady state is a much more sensible starting point for the examination of biosynthetic pathways and the further improvement of yields.

Two further aspects of economic botany might benefit from the application of continuous-culture technology: (1) the synthesis of useful products not detectable in the whole plant but which are unique to cells in culture (Butcher and Conolly, 1971), and (2) the use of cultured cells as agents in biotransformations (Furuya, Hirotani and Kawaguchi, 1971). The most exciting biotransformation of them all - photosynthesis - has been demonstrated only to a very limited extent in callus cultures of plant cells (Chandler, Tandeau de Marsac and de Kouchkovsky, 1972). However, the existence of chlorophyllous cell-suspensions (Davey et al, 1971) is a step towards fully-autotrophic cell cultures. The development and exploitation of such cultures will perhaps demand the controlled facilities of continuous cultures,

probably in the form of a closed continuous system in which photosynthetic cells may be maintained indefinitely in a non-dividing state by a controlled nutrient supply.

Novick and Szilard (1950a) and Bryson (1959) developed the use of the chemostat and turbidostat for selection of mutant cell lines occurring in bacterial populations. The processes of mutagenesis and selection of cell variants from cultures of higher plant cells using plating techniques have already produced clones with distinct biochemical characteristics (Eriksson, 1967; Davey et al, 1971; Mansfield, 1973). Successful continuous cultures of plant cells present a further way in which selection of variants having less exacting nutrient requirements (e.g. independence of exogenous hormone supply) or more efficient secondary metabolism may be achieved under controlled conditions. Continuous cultures may well be the most efficient way to characterise homozygous mutant cell-lines produced by mutagenic treatment of haploid cell-cultures (Sunderland, 1971) which are now becoming available.

A major feature of plant cell cultures is the ease with which (in a number of cases) developmental pathways may be initiated by defined nutrient alterations. Such treatments lead to the differentiation of specific cell types (Fosket and Torrey, 1969), of plant organs (Street, 1969) and of functional embryo-like structures (Thomas, Konar and Street, 1972). So far, however, in investigations of this kind it has only been possible to refer back to the initial concentration in batch culture of the initiating factor. Little or no information has been obtained about sequential changes in the cells and their culture environment as organisation proceeds or about the differential rates of cell division and cell differentiation that so obviously occur.

This unsatisfactory situation can be remedied in part by use of large-scale batch cultures (Kessel and Carr, 1972). It would be further improved by the use of synchronous cell-cultures, or steady states in which the kinetics of changes in rates of cell division and cell differentiation might be followed in a way analagous to the studies of slime-mould differentiation (Garrod and Ashworth, 1973) and bacterial sporulation (Dawes and Mandelstam, 1969). Two related problems, the loss of embryogenic potential (Smith, 1973) and the cytological stability of cell cultures (Bayliss, personal communication) might also yield to a kinetic treatment using steady states.

However, despite the many potential applications of the continuous culture of higher plant cells development of the technique, as described in this thesis, is in reality still very limited. The growth rate of plant cells is such that the establishment of steady states and transitions between steady states are time-consuming processes; with sterile cultures this is likely to lead to a high failure rate. Steady state growth of Acer pseudoplatanus cells is possible, but this species as now in culture differentiates neither morphogenetically nor biochemically in any useful way. It must therefore be an essential feature of future work to explore whether steady-state cultures, and cytodifferentiation and morphogenetic expression under controlled conditions, can be achieved with species of economic importance.

APPENDIX 1. CONSTRUCTIONAL AND OPERATIONAL DETAILS OF THE CULTURE SYSTEMS.

Reprinted from: Wilson S.B., King P.J. and Street H.E., 1971; Journal of Experimental Botany, 22 , 177 - 207 .

Basic 4L batch culture unit

The culture vessel is either a Quickfit 5 l culture vessel (FV5L) or a wide neck reaction vessel (FR5LF) (Fig. 11, Plates 1 and 2). A stainless-steel (Type B.S.EN 58A) cone (Plate 2c) was sealed with epoxy adhesive (Ciba ARL Ltd., Duxford, Cambridge) to the inside centre of the culture vessel (FV5L) as a base bearing for the magnetic stirrer. A glass cone of similar form was worked from the bottom itself in the case of the reaction vessel (FR5LF). This ability of the vessel (FR5LF) to be modified by glass working has led to its exclusive use in the more elaborate culture systems to be described later.

Closure of the culture vessel in each case is by a Quickfit reaction flask lid (MAF 3/52) with five ports, one of which is a B34/35 socket essential for rapid introduction of the inoculum of cell suspension. This lid is secured by a JC 100F clip.

The stirrer (Plate 2F) is a $\frac{1}{4}$-in (6 mm) diameter stainless-steel rod terminating (*a*) at its

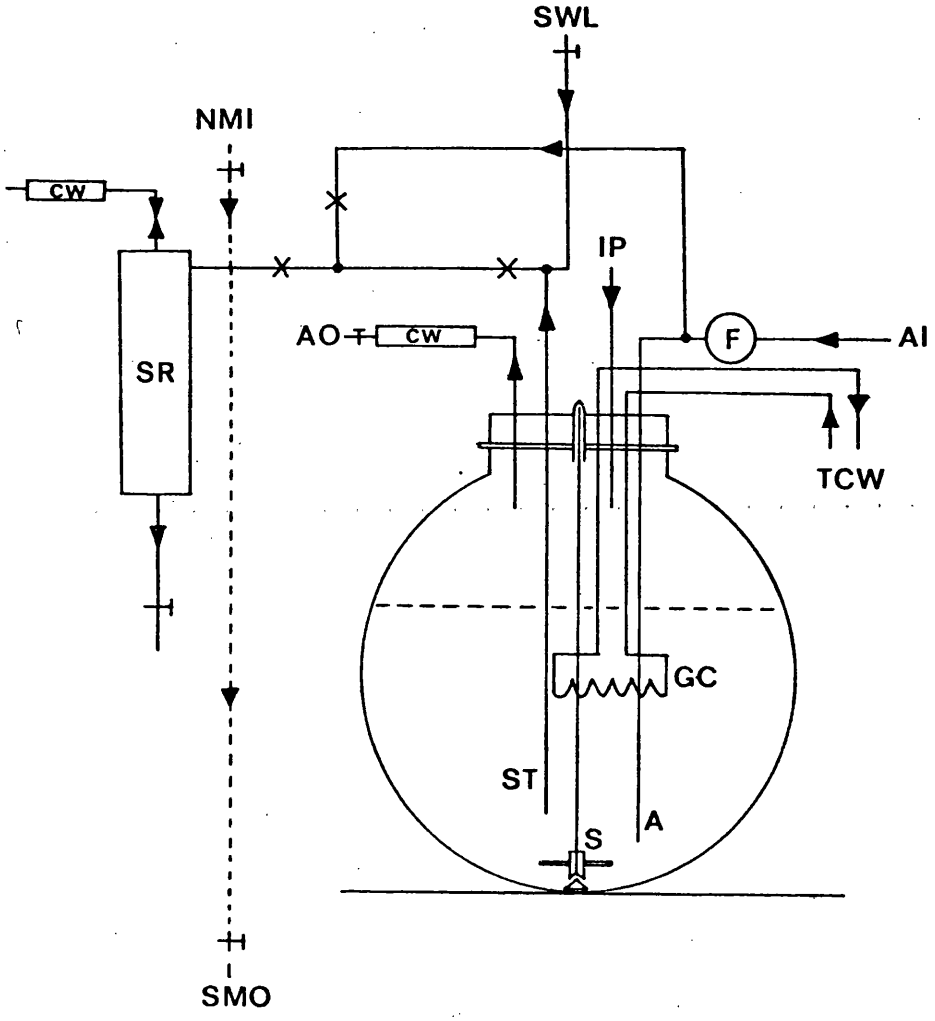


FIG. 11. Flow diagram for the basic batch culture unit (solid lines) and for intermittent medium or culture removal (vertical broken line). Arrows indicate directions of flow: X indicates a screw or spring clip on a silicone rubber tubing line; -•- indicates a T-piece junction; ⊣⊢ indicates a glass tap; A = aerator; AI = air inlet; AO = air outlet; CW = non-absorbent cotton wool filter; F = miniature air line filter; GC = glass coil; IP = inoculation port; NMI = new medium input; S = stirrer; SMO = stale medium or culture outlet; SR = sample receiver; ST = sample tube; SWL = sterile water line; TCW = temperature-controlling water supply.

apex in a small PTFE cap which fits into a sealed glass tube (8·3 mm, I.D.) extending from the centre vertical port (B 24/29 socket) (Plate 2E) of the reactor flask lid, and (*b*) at its base in a machined PTFE lower section, countersunk to fit the cone at the base of the culture vessel and carrying horizontally a 6-cm PTFE-coated magnetic bar (R. W. Jennings & Co. Ltd., East Bridgford, Nottingham, or Fisons Scientific Apparatus Ltd., Loughborough). The drive for the stirrer is a magnetic stirrer plate (A. Gallenkamp Ltd.) which supports the culture vessel directly (vessel FV5L) or via a cork ring (vessel FR5LF). In the experiments

reported in this paper the stirring rate has, unless otherwise stated, been 250–60 rpm (measured by a stroboscope).

The temperature-regulating glass coil is connected to a water circulating system by a special adaptor (Plate 2A) carried on the B34/35 joint of the port also used for introduction of the inoculum of cell suspension. Water is circulated through the spiral glass coil from a large temperature-controlled bath to maintain the culture at 25 °C. A Cadet domestic central heating pump (SMC, Ltd., Bridgwater, Somerset) and an appropriate manifold enable up to nine culture systems to be simultaneously controlled in temperature.

The remaining three ports of the culture vessel lid are used:

(*a*) *For aeration.* This was achieved using the piped compressed air derived from a dry cylinder compresser with after-cooler. The pressure of this supply was reduced by a Norgen precision pressure controller with condensate trap and carbon filter (C. A. Norgen, Ltd., West Bromwich, Staffs.), and the flow to each vessel controlled and measured by a flowstat controller fitted to an appropriate GAP flowmeter (G. A. Platon, Ltd., Basingstoke, Hants) and rendered sterile by passage through two Microflow miniature line filters (Microflow Ltd., Fleet, Hants) in series. The aeration rate was standardized at 500 ml min^{-1} except in the experiment described under closed continuous culture system (Fig. 7). Air was admitted to each culture via a 1 mm I.D. glass jet or, in later experiments, via a no. 2 porosity sintered aerator (Pyrex no. 3830/04).

Although difficulty was not encountered with foaming at the air flow rate and stirring speeds generally used, in certain experiments 1 ml of silicone MS Anti-foam A (Hopkin & Williams, Ltd.) was added to the 4 l of medium in the culture vessel before autoclaving. It had previously been shown with shake cultures that this antifoam can be present at 2 ml l^{-1} without a deleterious effect on culture growth.

(*b*) *For the air outlet cotton wool filter.* By combining aerator and air outlet by means of an adaptor (Plate 2C) this second port can be used to insert a thermometer or pH electrode (Plate 1). Water condensation in the cotton wool filters of the air outlet lines was prevented by heating tapes (Electrothermal Ltd., London; electrotrace HC. 101) supplied with 60 V from a suitable transformer.

(*c*) *For sampling.* The sample (*c.* 35 ml) is forced into the sample receiver (Plates 1 and 2D) by temporary closure of the air outlet line, leading to development of positive pressure in the culture vessel. The sample is removed by opening the tap of the sample receiver (the tap is always opened in the same direction). The sample receiver and sample lines are then washed with sterile water and finally cleared of liquid by air from the aeration line (Fig. 11). The glass sample arms and sample receivers used to collect samples of culture for cell counts were coated with silicone 'Repelcote' (Hopkin and Williams, Ltd., Chadwell Heath, Essex).

The culture unit is assembled, charged with 4 l of culture medium (minus urea), and sterilized by autoclaving at 121 °C for times pre-determined to be effective for the particular volume of medium by the use of Browne Autoclave Control tubes (A. Brown Ltd., Leicester). All open connectors are plugged with non-absorbent cotton wool and wrapped in aluminium foil; air inlet and sample lines are clipped off at the ports of the vessel lid and the air outlet tap is left open. After cooling, the urea solution is added aseptically via the inoculation port as a 40 per cent filter-sterilized solution (Oxoid, Ltd.). Junctions which have to be made or remade in flow lines to supply sterile wash water to sampler systems, or sterile medium, or to harvest the cell suspension or excess medium, were by means of glass cone and socket tubing connectors (B14/23 or B10/19 cones) which are greased before being plugged and wrapped. When connections are to be made, the joints are unwrapped, brought together, and then flamed with a portable blowlamp. The joints were secured with stainless steel springs (Plate 2B).

Silicone rubber tubing (quality TC 156) of Esco (Rubber) Ltd., London, was used throughout. All vessels and glass lines (8 mm O.D.) were of borosilicate glass. Borosilicate glass taps were of high vacuum type. All ground glass joints and taps were greased with high vacuum silicone grease (Edwards High Vacuum Ltd., Crawley, Sussex).

Cultures were initiated in aseptic transfer rooms, normally by introducing 200 ml of a 21-day-old stock culture into the 4 l of medium in the culture vessel.

Assembly for automatic sampling

The assembly (Plate 3A) incorporates a needle valve, the design of which was developed from that described by Miller *et al.* (1968). This valve is constructed from stainless steel

(Plate 3c, Fig. 12) and is fitted through a ground circular hole (1 in (2·54 cm) diameter) in the side of the culture vessel. The valve has a zero dead volume and an orifice of 5 mm diameter to prevent it becoming blocked by cell material. The plunger is hollow to allow wash liquid to clean the rear of the valve seat after each sampling. The conical plunger tip slots into a silicone rubber seat and back leakage is prevented by two silicone rubber 'O'-rings.

The flow diagram (Fig. 13) shows that the sample enters the needle valve unit when this is opened by a flexible cable from the sample solenoid (Davenset Type 4/A1/P1; Partridge, Wilson & Co. Ltd., Leicester). The sample then flows to the sample volume detector (Plate 3B) (the air space volume of the detector is linked directly to the air space of the culture vessel).

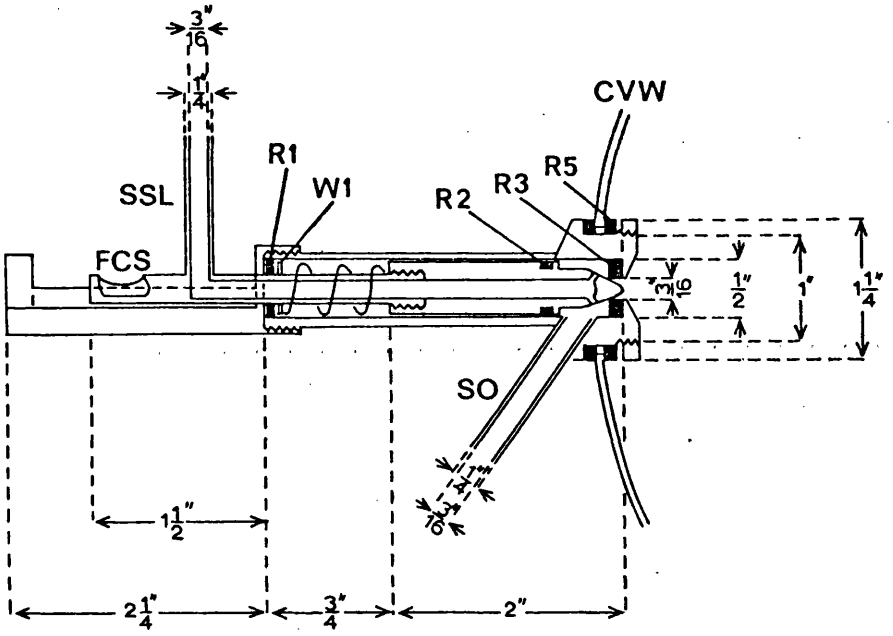


FIG. 12. Vertical section through needle valve of automatic sampling assembly (see also Plate 3A and C). Constructed of stainless steel EN58A.

Key: CVW = culture vessel wall; FCS = flexible cable socket; R 1, 2, 3, 5 = silicone rubber 'O' rings'; SO = sample outlet; SSL = sterile saline line; W1 = stainless steel washer.

The detector is set to collect a sample of pre-determined volume and when this is achieved an electrical signal from the detector permits the needle valve to close by its return spring. The sample is then delivered to a collection tube containing a measured volume of killing reagent (an equal volume of an 8 per cent aqueous solution of chromium trioxide if the sample is for cell counting) by opening of the outlet solenoid valve (Davenset Solenoid Type 461/9, Plates 3A and 4B) for a pre-set time. As this valve closes, the stop on the turntable is released by the latch solenoid operating a latching device (Fig. 14). The gravity-driven turntable (36 tube capacity) now moves forward bringing into position one of the empty tubes which alternate with the sample collecting tubes. As this happens the wash solenoid valve opens releasing sterile saline (0·005 per cent) via the hollow needle valve plunger, washing out the needle valve unit until the saline collecting in the volume detector again operates the outlet solenoid valve. The emptying sequence on this occasion incorporates a terminal sterile air pulse which frees the needle valve unit and volume detector of any residual liquid. The above sequence of operations is initiated by a multiset time clock (Venner, Ltd., New Malden, Surrey) which allows samples to be taken at any time intervals between 30 min and 24 h. The volume of the sample does not deviate more than ± 5 per cent from the pre-set volume.

The electronic circuit which controls the sampling and washing sequence is shown in

Fig. 15. The transfer of the −15 V supply by the clock from the transformer Tr 1 (isolating transformer T/T5, Radiospares Ltd., London, E.C. 2) to the rest of the circuit initiates a pulse which energizes RL1 (Kew Switch Relays Ltd., London, N.W. 2, Type MK 403 P 12V PC119) thereby opening the needle valve unit. The signal from the volume detector via

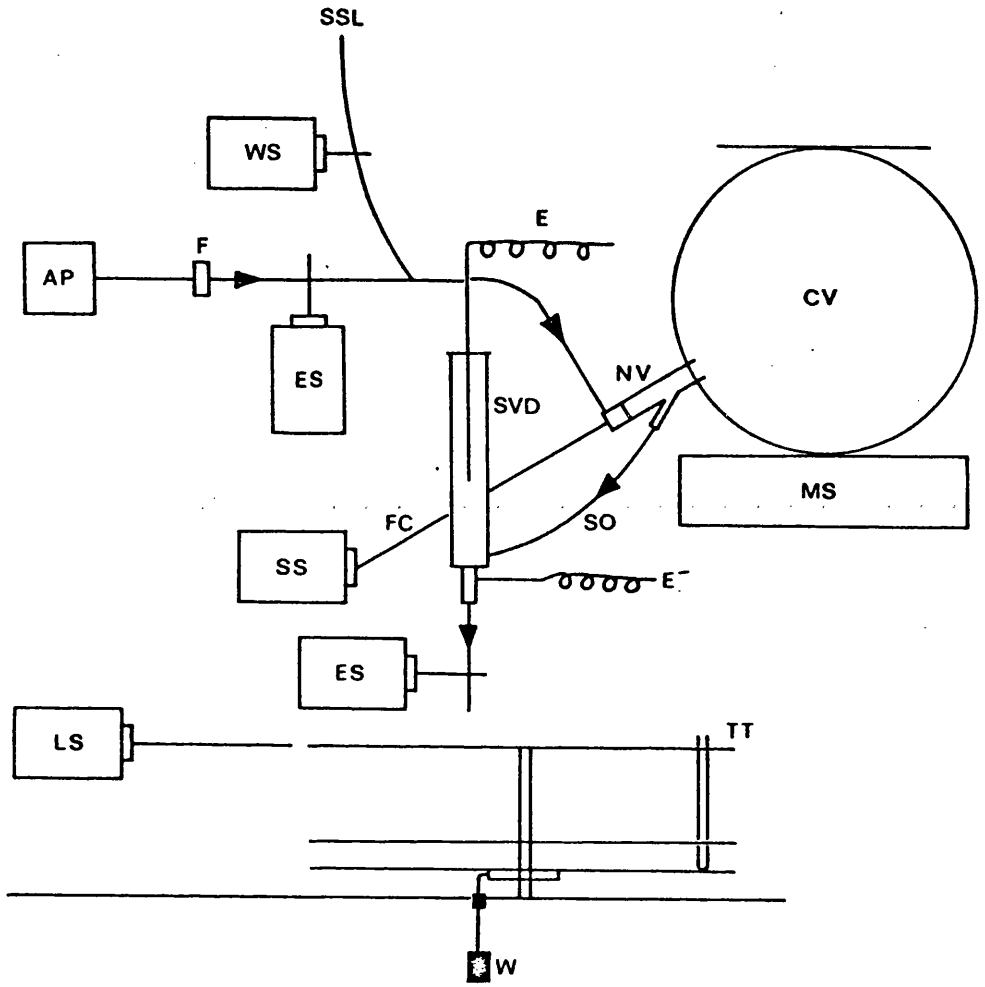


FIG. 13. Flow diagram showing operation of the automatic sampling assembly.

Key: Arrows indicate directions of flow. AP = air pump; CV = culture vessel; E and E' = electrodes of sample volume detector; ES = empty solenoid valve (to simplify layout, this is drawn twice to show its two actions); F = miniature air line filter; FC = flexible cable; LS = latch solenoid valve; MS = magnetic stirrer motor; NV = needle valve unit; SO = sample outlet line from needle valve; SS = sample solenoid; SSL = sterile saline line; SVD = sample volume detector; TT = turntable; W = turntable drive weight; WS = wash solenoid valve.

the Schmitt trigger energizes RL2 and RL3, simultaneously releasing RL1, and thereby permitting the needle valve to close. At the same time the Thyristor TL1 is activated energizing the fail-safe relay which will permit the later use of the wash sequence. RL3 energizes the outlet solenoid and initiates the action of the emptying timer controlled by charging C1 via R9. Linearity of this timing is achieved by feed back through T6 and the length of the ramp can be pre-set by the potentimeter R12. This ramp causes momentary movement of RL4 which de-energizes RL3 and RL5 and thereby closing the outlet solenoid and opening

the wash solenoid until actions of the Schmitt trigger and RL2 again energize RL5, RL3, and RL7 leading to closing of the wash solenoid and opening of the outlet solenoid. RL5 and RL7 are now locked on and a timing sequence involving C1 has again been initiated. A second timer C2 and R10, pre-set by R15, is also started to delay the energizing of RL8 which permits action of the air pump. When the first timer C1/R9 completes the drainage cycle, RL4 is again activated releasing RL3 but since RL5, RL6, and RL7 are all locked on, the

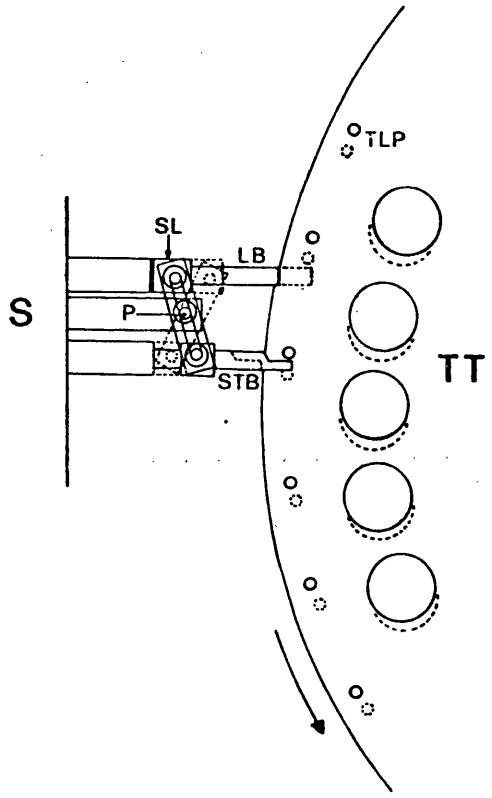


FIG. 14. Turntable and latching device. Direction of rotation of turntable indicated by arrow. Rest position of latching device shown as solid line, energized position as broken line.

LB = latch bar; P = pivot; S = solenoid; SL = slotted link; STB = slave stop bar; TT = turntable; TLP = turntable latch pegs.

wash cycle is not repeated and the de-energizing of RL3 and RL8 only serves to complete the cycle by closing the air and outlet solenoids. The turntable is released each time the emptying sequence is completed. The complete cycle will not repeat until the time clock has restored and subsequently removed the 15 V supply from Tr 1 (the minimum interval for this, using the Multiset timer, is 30 min). The changeover action of the timer also serves to remove the 15 V supply from the relays and this releases them to cancel the sequence. Failure of the 240 V supply does not initiate sampling and failure during the sampling sequence cancels the sequence, all solenoid valves closing. A fresh sample sequence is only initiated following the next signal from the time-clock.

Closed continuous culture system

The flow diagram (Fig. 16) shows the use of culture vessel FV5L in the basic culture unit, the positioning of the special siphon tube unit and a diagram of the special intermediate

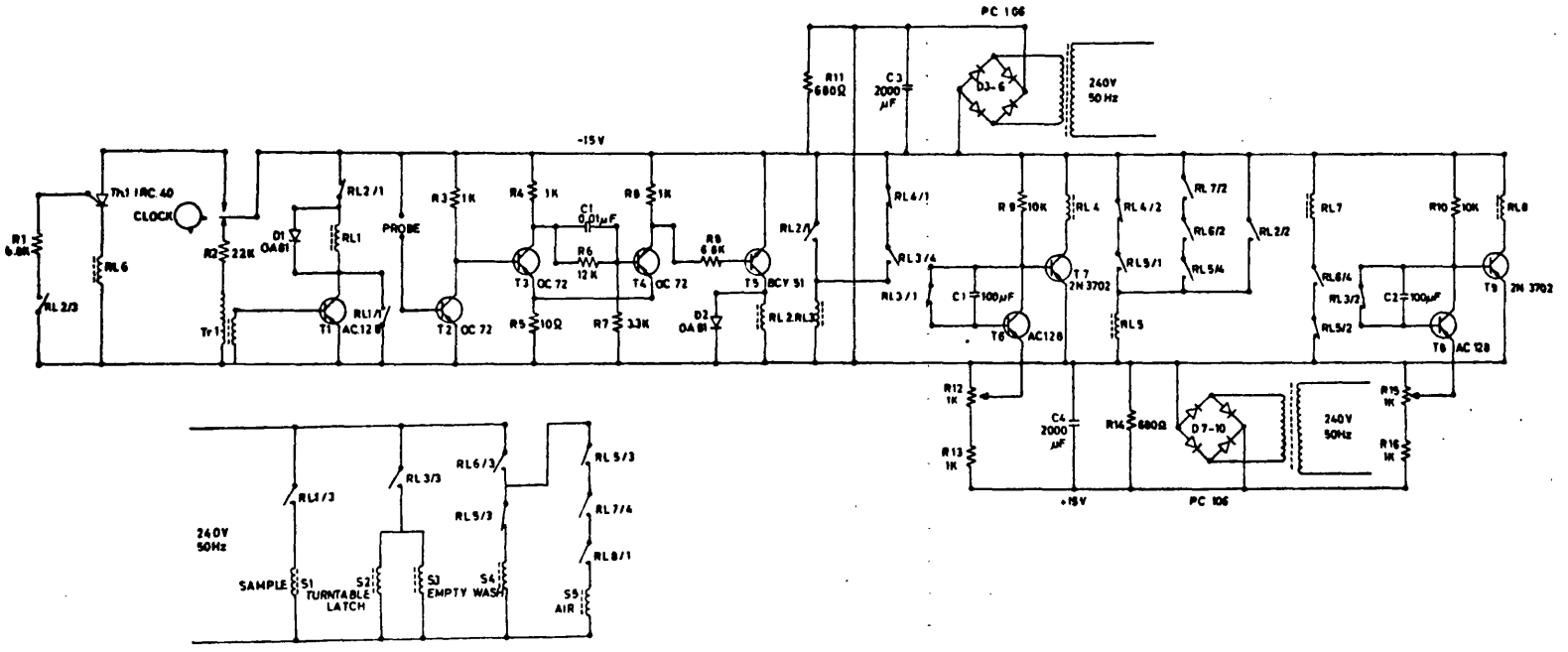


FIG. 15. Circuit diagram of automatic sampler control unit. Resistors (R), capacitors (C), and transistors (T) as specified.

ThI = Thyristor, type RC 40; Clock = Venner Multiset timeclock 240 V 50Hz; TrI = Radiospares transistor transformer T/T5; Probe = Stainless steel electrodes in volume detector unit (Plate 3B); S1 = Sample valve solenoid. Davenset type 4/A1/P1 240 V 50H; S2 = Turntable latch solenoid; S3 = Empty valve solenoid; S4 = Wash valve solenoid; (S2, S3, and S4 Davenset type 4/A1/P1 or 461/9 240 V 50Hz); S5 = Petcraft Aquarium pump 240 V 50Hz; RL 1–8 = Relays 4-pole changeover, contacts as shown. Kewswitch Relays Type MK 403P 12 VDC 119; PC 106 = Packaged DC power supply. Newmarket transistor type PC 106. No load output 15 V DC.

medium reservoir and protective sterilizing filter incorporated when a continuous supply of medium is made available to the culture system (see *Open continuous culture system—chemostat* below). A special siphon tube unit (Fig. 17) fits into a second B34/35 socket replacing the B19 socket available in the basic culture unit for thermometer or electrode. It consists of a 'stilling tube', 2·5 cm I.D. reaching to within 1·5 cm of the base of the vessel and positioned to minimize entry of air bubbles. This 'stilling' tube leads to the siphon and weir and hence to a spent medium receiver, from which medium can be readily drawn off aseptically. The medium is displaced up the stilling tube at a slow rate and hence is free from cells when it moves over the apex of the siphon section. Any air entering the stilling tube is trapped at the apex of the siphon and is drawn off periodically so as not to break the con-

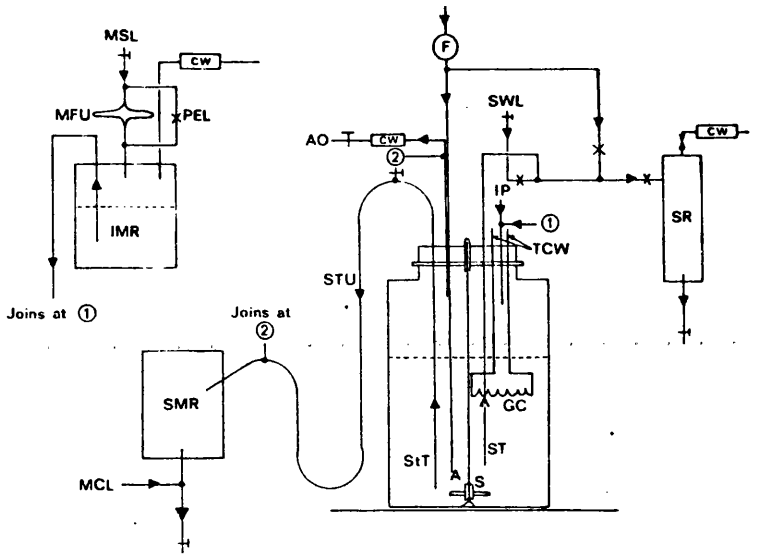


FIG. 16. Flow diagram for closed continuous culture system. Conventions and key as Fig. 11 with the following additions:

IMR = intermediate medium reservoir; MCL = mercuric chloride solution line; MFU = medium filter unit (see Fig. 19); MSL = medium supply line; PEL = pressure equalizing line (open during autoclaving of the filter reservoir unit); SMR = stale medium reservoir; StT = stilling tube; STU = siphon tube unit (see Fig. 17).

tinuity of liquid in the siphon; this same air removal system is used to establish the siphon at the commencement of a culture period. An outlet immediately above the weir of the siphon connects to the air outlet line of the culture vessel to ensure pressure equilibration. Medium from the intermediate medium reservoir is pumped to the medium entry point of the culture vessel by means of the Delta micro-metering pump detailed under *Open continuous culture system—chemostat.*

Open continuous culture system—chemostat

The flow diagram for this system (Fig. 18) shows the following special features:

1. A loop through which the culture is circulated external to the main culture vessel. This is introduced by adding two tubulures (5 mm I.D.) to the culture vessel below the surface of the culture and connecting these by an adequate length of silicone rubber tubing (6·3 mm I.D.). Culture is pumped through the loop, using a flow inducer, Model No. MARK 55 (Watson Marlow, Ltd., Marlow, Bucks.) rotating at 55 rpm. The total length of the rubber tubing must be sufficient to enable the length (7·5 cm) subject to wear by the flow inducer, to be advanced every 14 days. Electrodes can be introduced into the circulation loop and the opacity of the circulating cell suspension can be monitored by the optical monitoring system described under the *Turbidostat* system below.

2. A controlled flow of new sterile medium from a 10 l aspirator via a culture medium filter unit (Fig. 19 and Plate 4A) and an intermediate medium reservoir (Plate 4A). The rate of flow of medium to the culture is regulated by a previously calibrated Delta Micro-Metering variable speed pump with a multi-tube module (Watson-Marlow, Ltd., Marlow, Bucks., Model MHRE 22), using Delta/P adaptors and either Delta 0/1 or 0/2 tube elements. The medium filter unit (Fig. 19) incorporates a 142-mm diameter Gelman Triacetate Metricel GA-8

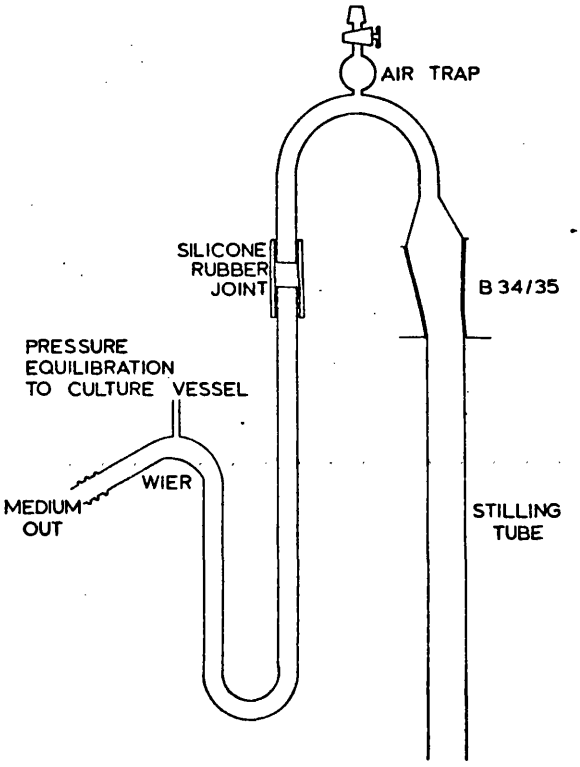


FIG. 17. Siphon tube unit.

filter (mean flow pore size 0·2 μm) supported by a Gelman polypropylene filter and a 1/16th in (1·6 mm) perforated Teflon disc and is constructed from two flat flange joints (Quickfit FG 100). The intermediate-medium reservoir is constructed from a flat-bottomed culture vessel (Quickfit FV 1L) and a multi-socket flat flange lid (Quickfit MAF 2/2) secured by a JC 100F clip. This reservoir remains full of medium because entry from the aspirator is balanced by a head of liquid built up in a tube rising to the level of the aspirator and protected by a cotton wool filter.

3. A culture harvesting system consisting of a constant level device and culture receiving vessel. The constant level device is a vertical side arm (20 mm I.D.) on the culture vessel carrying, via a PTFE stopper, two stainless steel electrodes, one terminating near the base of the side arm and the other terminating at the constant-level height. This side arm is a 'dead leg' in which the culture medium is free from cells. When the level of culture medium reaches the upper electrode, the outlet solenoid valve is opened and cell suspension flows by gravity from a point in the circulation loop upstream of the flow inducer to the culture receiving reservoir until contact between the electrode and the medium is broken. This pulse of culture is of 40–60 ml. The circuit in which a Schmitt trigger utilizes the change in resistance across the electrode to energize, via the relay RL1, the outlet solenoid is shown in Fig. 20. This circuit could also be used in connection with two electrodes in the air space of the main culture vessel to control anti-foam addition.

The culture receiving vessel at present in use is a 2-l Pyrex aspirator bottle (Pyrex no. 1530/08) sealed with a silicone rubber stopper and carrying an upwardly directed glass side

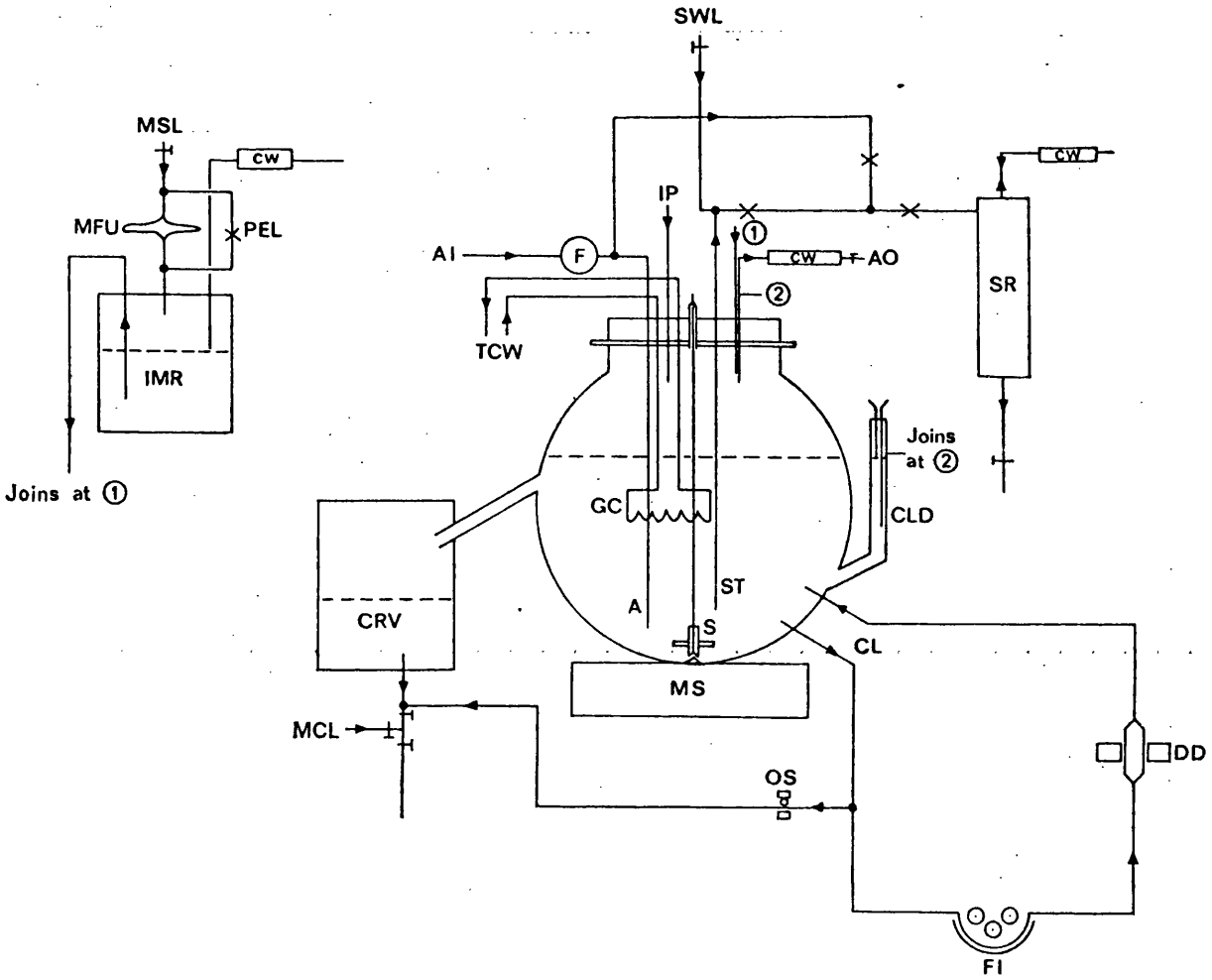


FIG. 18. Flow diagram for open continuous culture system–chemostat. Conventions and key as Fig. 11 and Fig. 16 with the following additions:

CL = circulation line; CLD = constant level device; CRV = culture receiving vessel; DD = density detector; FI = flow inducer; MS = magnetic stirrer motor; OS = outlet solenoid valve.

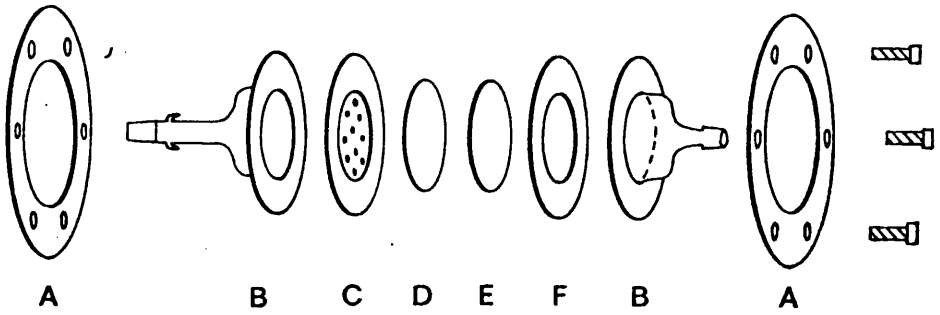


FIG. 19. Culture medium filter unit. Opened out to display components:

Key: A = Aluminium clamp rings; B = 100-mm glass flat flange; C = 1/16 in Teflon support; D = Polypropylene filter; E = Metricel filter; F = Silicone rubber gasket.

arm (120 mm I.D.) which connects to a downward sloping side arm of the same diameter in the side of the culture vessel. This permits back flow of air into the main culture vessel and acts as a safety weir, allowing culture to flow into the receiver should there be a failure of the electronic control of the constant level device. The lower tubulure of the aspirator serves via a glass tap and length of silicone rubber tubing, for the removal of cell suspension for analysis. This exit is a potential site of entry of contaminating organisms. The exit line is therefore connected to a source of mercuric chloride (0·2 per cent) solution and each time cell suspension is withdrawn the line is recharged with mercuric chloride solution. Suspension in this culture receiving vessel is at room temperature and is unstirred. If suspension is required to accumulate prior to analysis, this receiving vessel can be cooled and the suspension stirred by using equipment described under the *Basic batch culture unit.*

Open continuous culture system—turbidostat

The flow diagram for this system is shown in Fig. 21. The system in operation is shown in Plate 4A. New culture medium is admitted to the circulation loop and simultaneously the flow of cell suspension through this loop is interrupted by a medium input solenoid valve controlled by an optical monitoring system consisting of a density detector and an electronic control unit. The density detector is placed downstream of the medium input valve and hence the volume of the pulse of new medium is determined by the volume of the circulating loop between the valve and the density detector. By introducing into this section of the loop a module for insertion of pH and oxygen electrodes and a vertical glass observation chamber, the medium pulse is set at c. 2 per cent (c. 80 ml) of the culture volume. The siting of the electrode module immediately down stream of the medium inlet allows calibration of the electrodes whenever new medium (oxygen saturated and of known pH) is being introduced. The culture harvesting solenoid valve, controlled by the constant level device, is sited upstream of the medium input solenoid valve.

The cell suspension flows through the density detector in a glass cuvette constructed from 1-cm square Pyrex tubing. The density detector, based upon a design by Stanley (1965), comprises two cadmium sulphide light-sensitive resistors (Type ORP12 of Mullard Ltd., Cambridge) attached to screwed adjustment rods, enclosed in side arms and a 6·5 V 0·15 A MES bulb (Radiospares Ltd.) housed with the cuvette in a centre section (Plate 4C, D and Fig. 22). The whole system is constructed out of either Tufnol, Perspex, or Bakelite and made light tight by matt black paint. The light path to one photo-cell is uninterrupted; to the other it is interrupted by the cuvette.

The circuit of the electronic control unit is shown in Fig. 23. A filament transformer (Radiospares Ltd.) provides the 6·3 V DC for the light in the density detector and for a panel indicator light (6·3 V) in series (this panel light indicates function of the light in the density detector and prolongs the life of the bulb). A second 6·3 V winding on the filament transformer, after rectification and stabilization, serves the detection measurement bridge circuit. The two photocells of the density detector with R3, R4, and the density potentiometer R29 form the arms of the bridge circuit. C2 and C3 damp oscillations in optical density in the cuvette caused by the action of the flow inducer and are switched by SW3 to aid in balancing the system. R29 or the screwed adjustment rods carrying the photocells in the density detector are adjusted for the selected cell density and a meter is incorporated to check balance in the bridge circuit. When this balance is disturbed by increase in optical density of the cell suspension by growth, the output of the bridge circuit, amplified by an integrated differential amplifier (model AD 2000, Fenlow Electronics Ltd., Weybridge, Surrey), is used to control a Schmitt trigger circuit, as described under culture harvesting, via the constant level control unit (see *Chemostat system* above). In the turbidostat this circuit first opens the medium input solenoid valve and then closes it when the optical density in the cuvette drops sharply by entry of the front of new medium.

The turbidostat system has so far been based upon control of medium input via optical density, but the density detector is constructed so that one photocell and side arm can be set at 90° to the other arm, thereby permitting control based upon the light scattering properties of the suspension (nephelometry). The electronic control unit has a function switch (SW2) which can be set for control either by optical density or light scattering.

The detection and measuring front of the electronic control circuit has been utilized separately in the Chemostat system to monitor, by a recorder connected across the bridge,

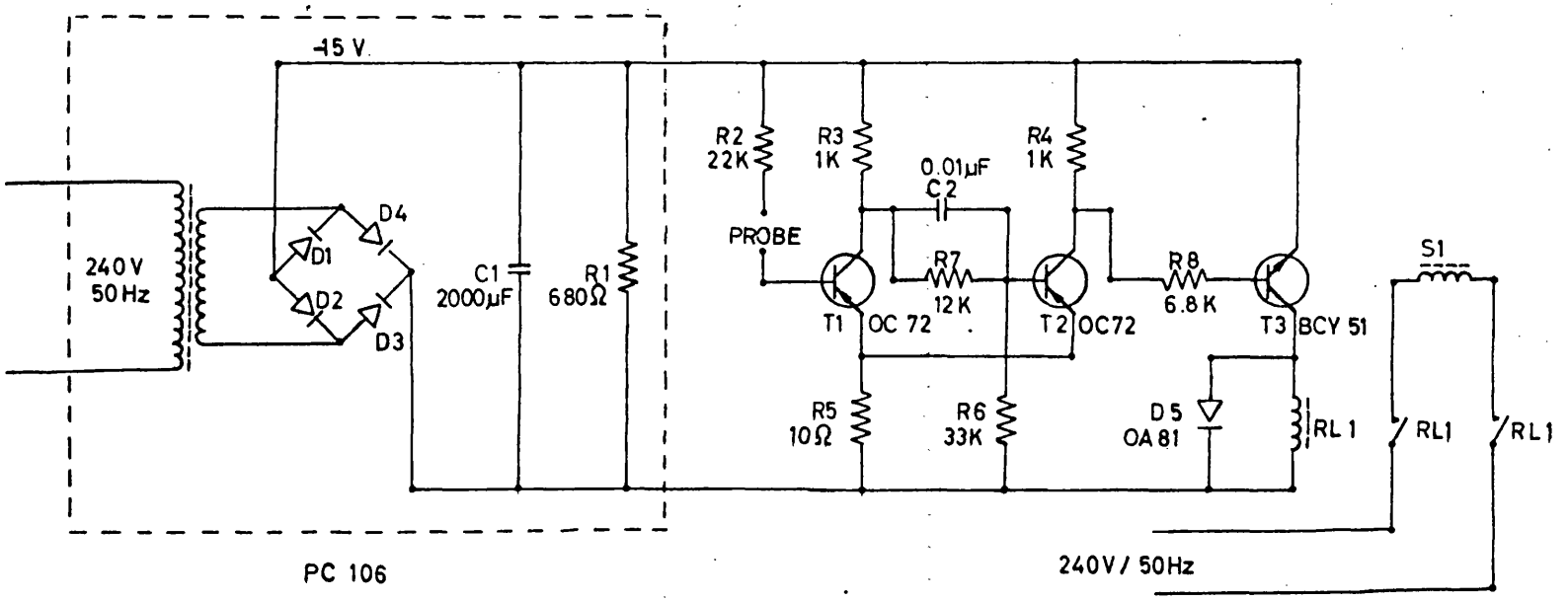


FIG. 20. Circuit diagram of the outlet solenoid control unit. Resistances (R), capacitors (C), transistors (T) and diodes (D) as specified.

PC 106 = packaged DC power supply, Newmarket transistors, type PC 106, 15 V output at zero load; RL1 = pygmy power relay, 2 pole changeover, 12 V D.C., 120 Ω; S1 = solenoid valve Davenset type 4/A1/P1 or 461/9, 240 V 50 H_Z.

the optical densities of the cell suspension established at different rates of continuous medium input.

MATERIALS AND METHODS

Tissue and culture technique

The general techniques for the preparation of media, the propagation of the stock cultures of the *Acer pseudoplatanus* L. cell suspension clone, and measurements of culture growth, were

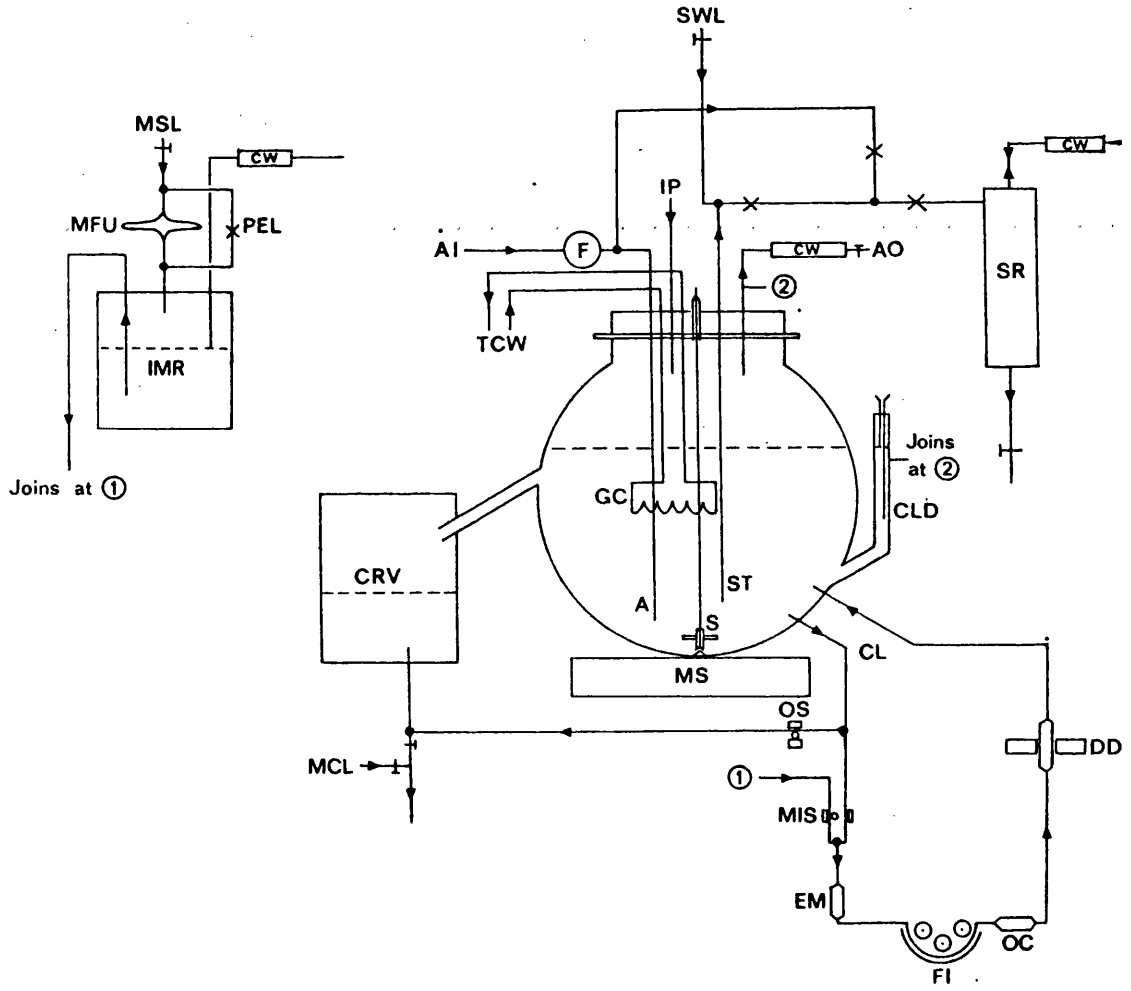


FIG. 21. Flow diagram of open continuous culture system–turbidostat. Conventions and key as Figs. 11, 16, and 18 with the following additions:

EM = electrode module; MIS = medium input solenoid valve; OC = observation chamber.

as previously described (Henshaw *et al.*, 1966). The synthetic culture medium was as described by Stuart and Street (1969), modified by replacement of the ferric chloride by 2×10^{-4} M ferric ethylene diamine-tetraacetate (Givan and Collin, 1967) and adjustment of the initial pH to 6·2. The standard carbon source (2 per cent sucrose) was replaced in certain experiments by 2 per cent glucose.

Analytical methods

Total cell protein was determined by the method of Lowry, Rosenbrough, Farr, and Randell, 1951 (via Layne, 1957)

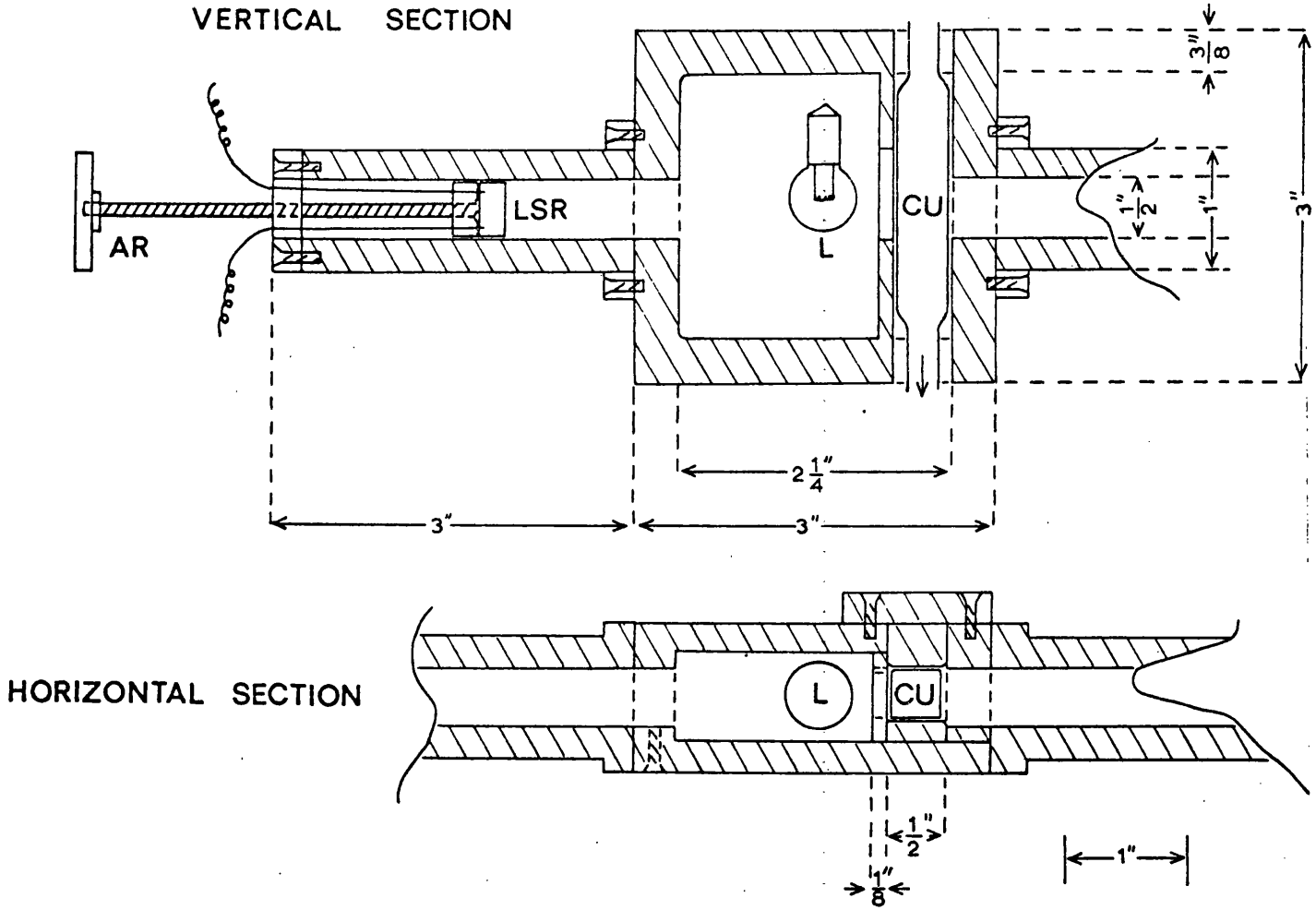


FIG. 22. Constructional drawings of the density detector (see also Plates 4C and D):

Key: AR = screwed adjustment rod; CU = cuvette (flow through cell); L = lamp 6·3 V 0·15 A, MES; LSR = light sensitive resistor; Mullard ORP. 12.

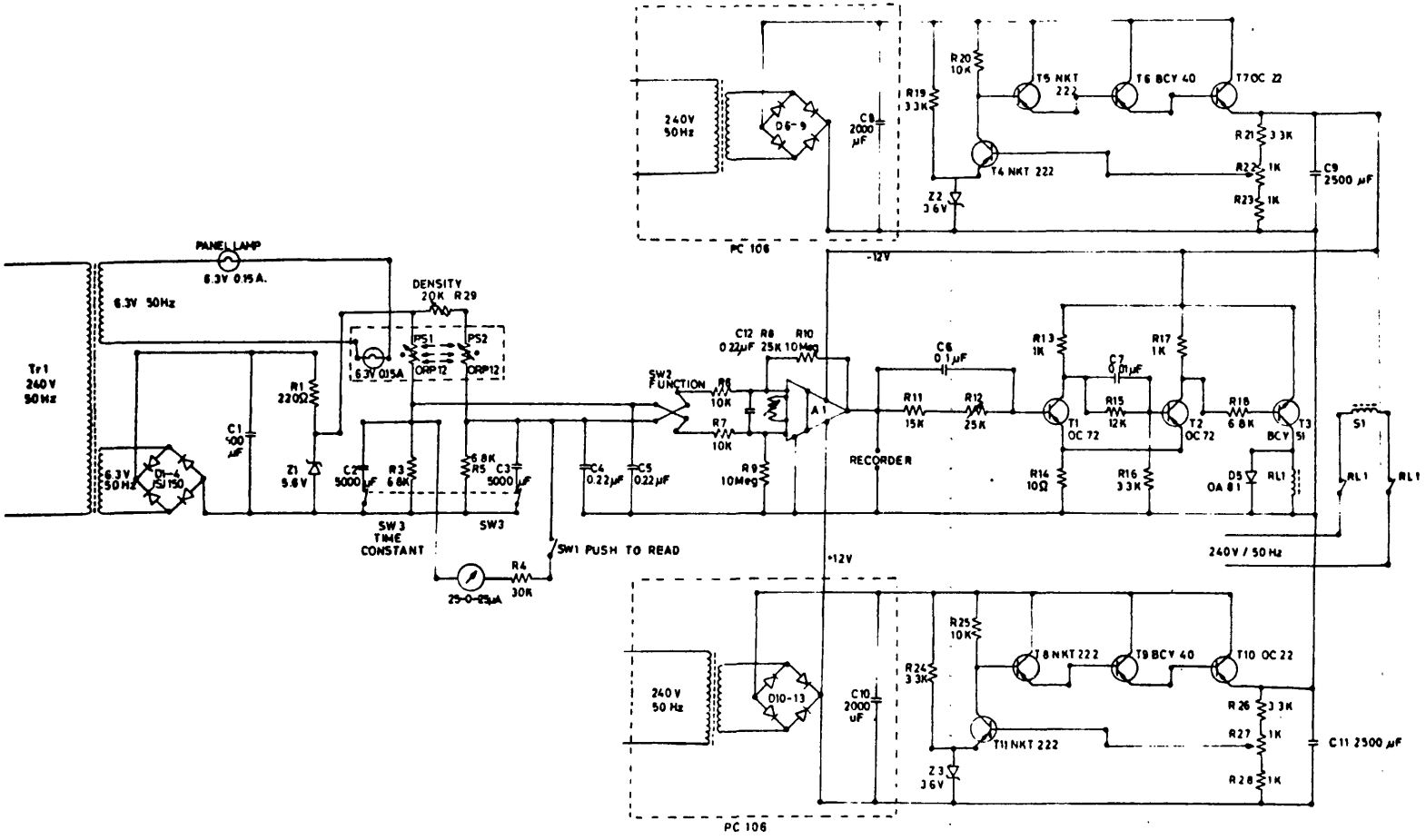


FIG. 23. Circuit diagram of the optical monitoring control unit. Resistances (R), capacitors (C), transistors (T), diodes and zenner diodes (D or Z) and switches (SW) as specified.

PC 106 = packaged DC power supply, Newmarket transistors. Type PC 106, 15 V output at zero load; Tr1 = Radiospares 6·3 V Hygrade filament transformer; PS 1 & 2 = photocells, Mullard ORP. 12 in density detector unit (Fig. 18) with one 6·3 V, 0·15 A lamp; A1 = Fenton AD 2000 amplifier; RL1 = Pygmy power relay, 2 pole change over 12 V DC., 120 Ω; S1 = Medium inlet valve solenoid. Davenset Type 4/A1/P1 or 461/9. 240 V 50 H_Z.

Glucose present in filtered, deproteinized samples of spent medium was estimated enzymatically (Washko and Rice, 1961) using the 'Glucostat' reagent (Worthington Biochemical Corporation, Freehold, N.J.).

Total cell carbon was determined by a wet combustion method in a combined combustion diffusion vessel (Baker, Feinberg and Hill, 1954).

The maximum rate of oxygen transfer from gas to liquid in the culture system (the oxygen absorption coefficient = OAC), was determined for various combinations of air flow rate, sparger type, and stirring speed by measuring the rate at which sodium sulphite solution containing copper ions as catalyst, was oxidized in the vessel (Cooper, Fernstrom, and Miller, 1944). The correlation between oxygen absorption rates in sodium sulphite solution and oxygen availability to the growing cultures has not been determined, although a relationship between these has previously been established for bacterial fermentations (Pirt and Callow, 1958). Further, the maximum oxygen uptake rates recorded in oxygen-limited cultures have been found to agree closely with the predetermined oxygen-absorption coefficients (Fig. 4A).

The rate of oxygen uptake by the cells was determined using a Clarke type oxygen electrode (Rank Bros., Boltesham, Cambridge) as previously described (Rajasekhar, Edwards, Wilson, and Street, 1971).

Plate 1. Basic batch-culture unit.

Key: A = aerator; AO = air outlet; C = flat-flange lid clip; CW = cotton wool filter; F = miniature air line filter; GC = glass coil; IP = inoculation port; S = stirrer; SR = sample receiver; SWL = sterile water line; T = thermometer; TCW = temperature-control water line.

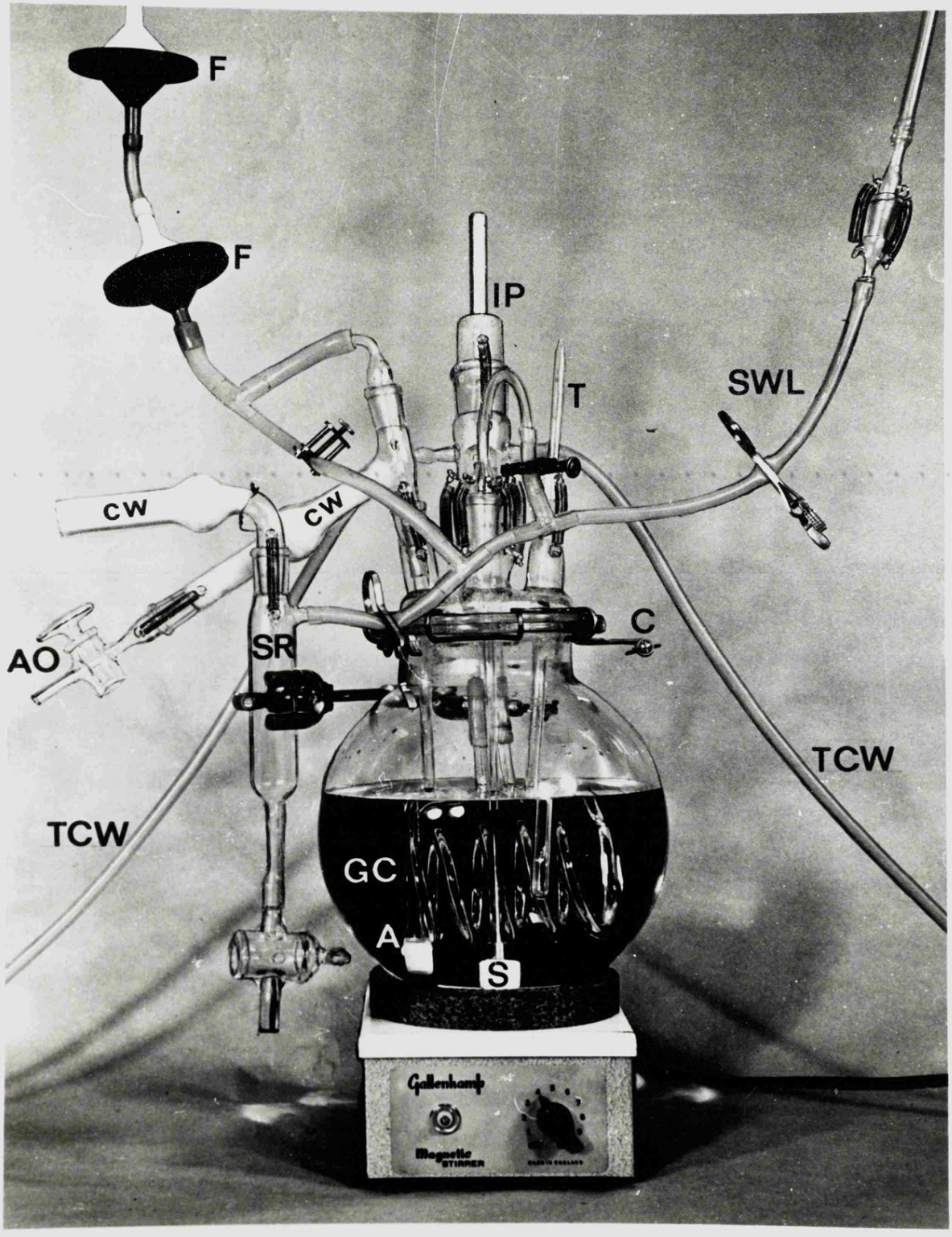

F
F
IP
T
SWL
CW
CW
AO
SR
C
TCW
TCW
GC
A
S
Gallenkamp
Magnetic
STIRRER

Plate 1

Plate 2. Components used in the basic batch-culture unit.

A = temperature-regulating glass coil with special adapter; B = tubing connector (B14/23); C = sintered aerator combined with air outlet adapter; D = sampling tube (left) and sample receiver with air filter (right); E = B24/29 cone and sealed glass tube which is the top bearing of the stirrer; F = stainless steel stirrer rod with PTFE cap, PTFE lower section and magnetic bar; G = stainless steel cone used with culture vessel (Quickfit FV5L).

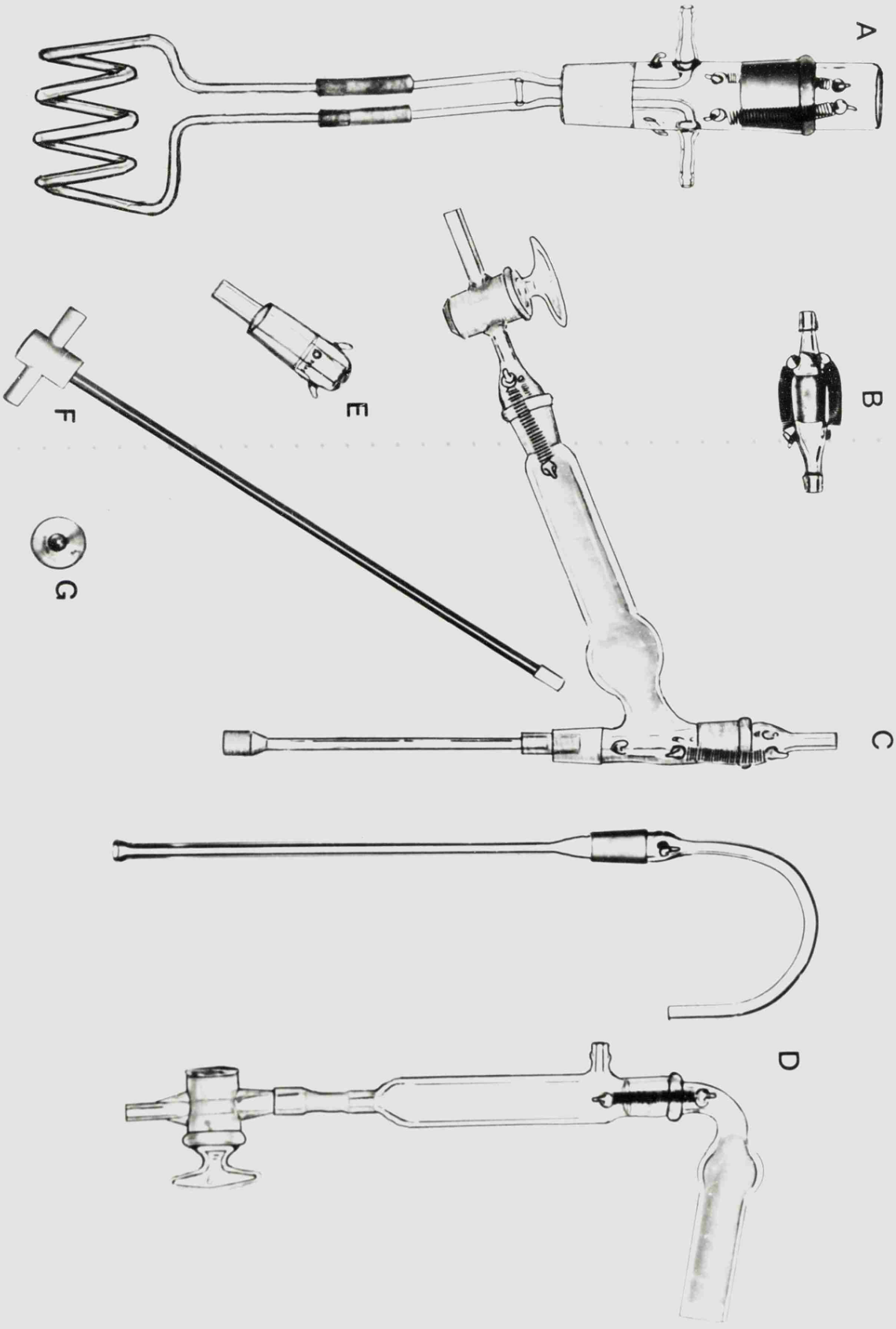


Plate 2

Plate 3. Assembly for automatic sampling.

A. View of complete assembly.

Key: AP = air pump; ES = empty solenoid valve; LD = latching device; NV = needle valve (automatic sampling); SO = sample outlet line from needle valve; SR = sample receiver (manual sampling); SSL = sterile saline supply line; SVD = sample volume detector electrode; TCW = temperature control unit; TT = turntable; WS = wash solenoid valve.

B. Sample volume detector.
E and E' = electrodes; TC = teflon cones.

C. Exploded view of stainless-steel valve unit. See Fig. 12 for assembly of individual components and for positioning and identity of labelled parts.

FC = Flexible cable (see FCS Fig. 12).

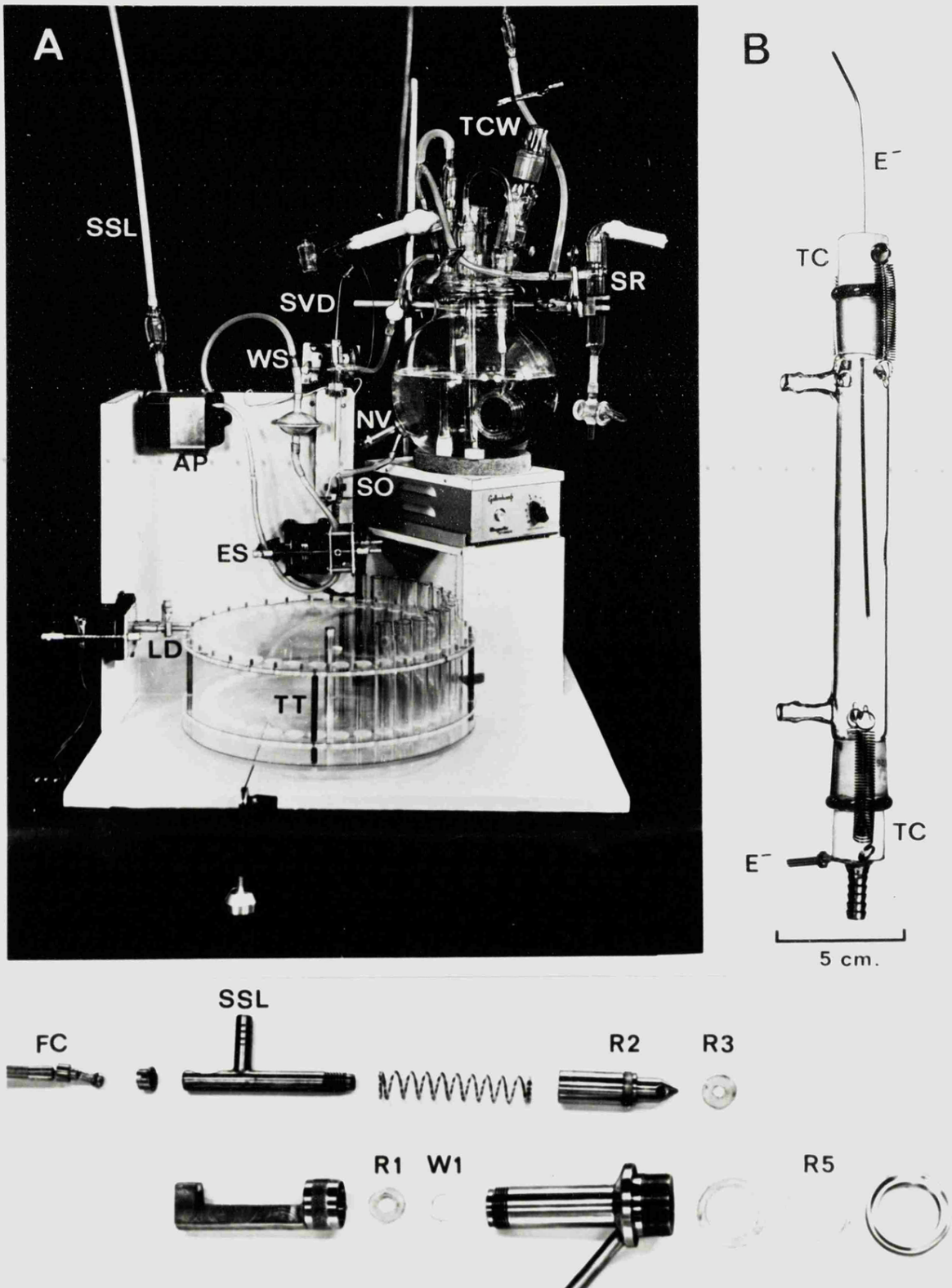

A
TCW
SSL
SR
SVD
WS
NV
AP
SO
ES
LD
TT
B
E⁻
TC
TC
E⁻
5 cm.
SSL
FC
R2
R3
R1
W1
R5
SO
C
5 cm.

Plate 3

Plate 4. Open continuous culture system - turbidostat.

A. Complete assembly.

Key: CL = exit and entry tubulures of circulation loop; CLD = constant level device; CRV = culture receiving vessel; DD = density detector; FI = flow inducer; IMR = intermediate medium reservoir; MCR = mercuric chloride reservoir; MFU = medium filter unit; MIS = medium inlet solenoid valve; MR = medium reservoir; OC = observation chamber; OMC = optical monitoring control unit; OS = outlet solenoid valve; OSC = outlet solenoid control unit; WR = sterile water reservoir.

B. Medium inlet solenoid valve in closed position.

Key: CL = circulation loop; MI = medium inlet line.

C. Density detector with front plate and right hand light sensitive resistor removed (D) (see also Fig. 22).

Key: AR = screwed adjustment rod; CU = cuvette (flow through cell); L = lamp.

D. Right hand light sensitive resistor (LSR) and screwed adjustment rod (AR).

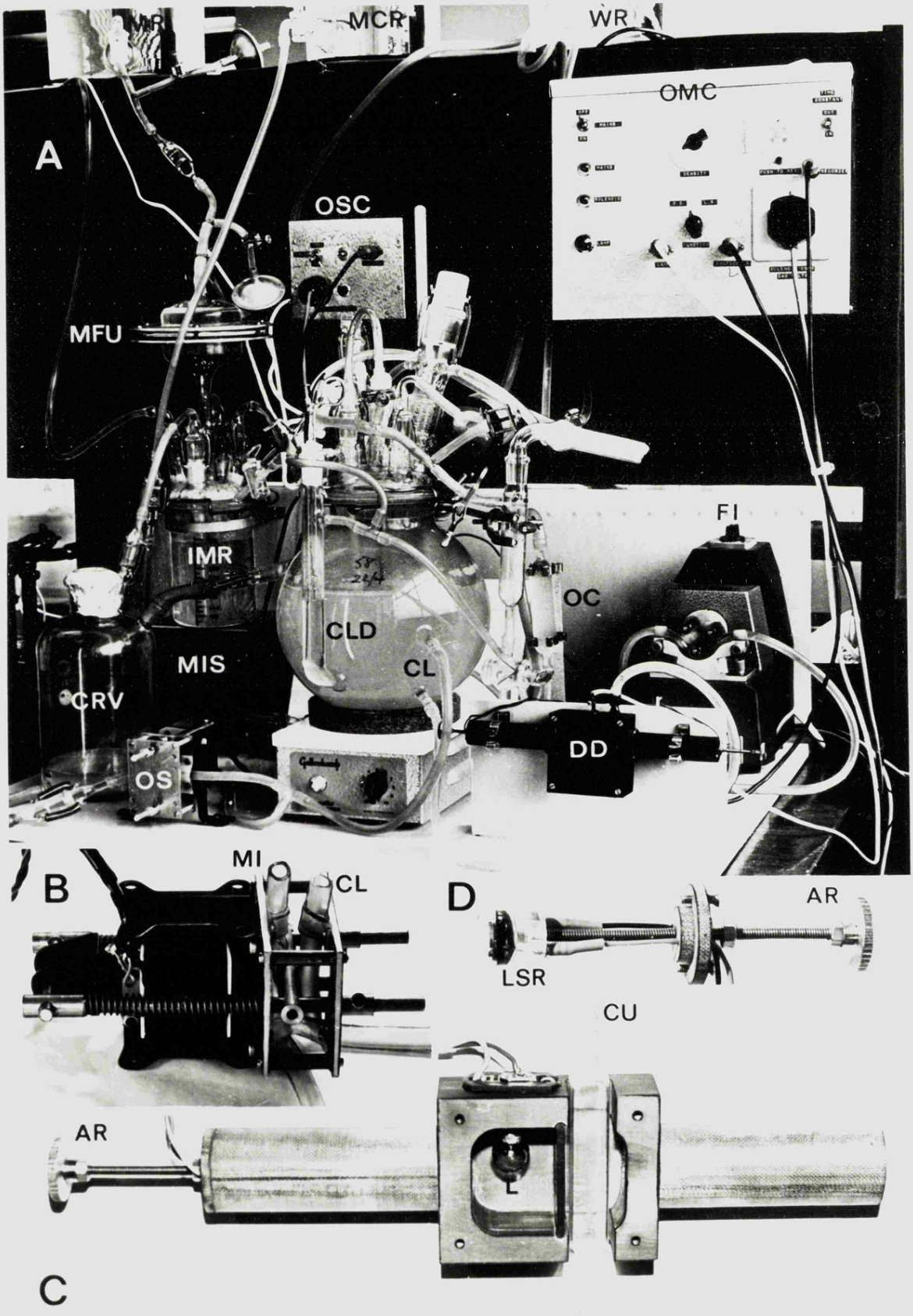

A
MR
MCR
WR
OMC
OSC
MFU
IMR
CLD
MIS
CRV
OS
CL
OC
FI
DD
B
MI
CL
D
AR
LSR
CU
AR
L
C

Plate 4

APPENDIX 2. Modifications to original designs.

1. Stirring.

More effective, tightly-coupled magnetic stirring was achieved by using a double-bar follower (Figs. A and B) based on a design by Veliky and Martin (1970) and a faster, more powerful drive unit (Künkel stirrer, Scientific Instruments Ltd) (cf. Plate 2F).
A teflon block (1) (40 x 25 x 8 mm) was machined to take an inverted, teflon cup (2) (25 x 15 mm OD) which was fitted over a glass peg (3) (30 mm high) formed on the bottom of the culture vessel. Two teflon-coated bar magnets (4) (45 mm) were fitted in parallel through the teflon block.

2. Heat sink and condensers.

In order to maintain culture temperatures at 25°C despite the fluctuating ambient temperature (which exceeded 25°C in the summer) and the heat output of the magnetic stirrers it was necessary to introduce a heat-sink into the temperature-control unit. A copper coil (6' x ¼") placed inside the main water bath was fed with water at 15°C via a closed loop from a subsidiary bath. The second bath was cooled using an immersed refrigeration-coil ("Minichill"; G. A. Bird Ltd.). The whole system was balanced at 25°C.

To reduce culture evaporation, water from the refrigerated bath was also fed to condensers (Fig. C.1) fitted between the culture lid (2) and the air-outlet filter (3) (cf. Plate 2C) on some chemostats and synchronous batch cultures.

3. Medium supply.

The medium filter unit (Fig. 19) proved unreliable over extended culture periods and was omitted. The peristaltic dosing pump on all chemostats was replaced by a stainless-steel pump chamber operated

by a ceramic piston and one-way ball-valves (Metering Pumps Ltd). The inclusion of a condenser in the air-outlet system required the resiting of the medium input line. A third tube (Fig. D and E2) carrying medium was introduced through the adaptor carrying the temperature-control flow lines (cf. Plate 2A). In chemostat experiments requiring a switch between media of different composition, the medium supply system was duplicated as far as the pump head (see Fig. 18). A Y-piece introduced upstream of the pump head linked the two systems. The replacement volume beyond the Y-piece was ca. 50 millilitres.

4. Turbidostat pulses.

To record the distribution of medium pulses into turbidostat cultures a micro-switch, activated by the medium inlet solenoid valve (see Fig. 21), was connected across the event marker circuit of a recorder. The number of pulses was also recorded on an impulse counter connected across the output from the Schmitt trigger circuit of the optical monitoring control unit (Fig. 23).

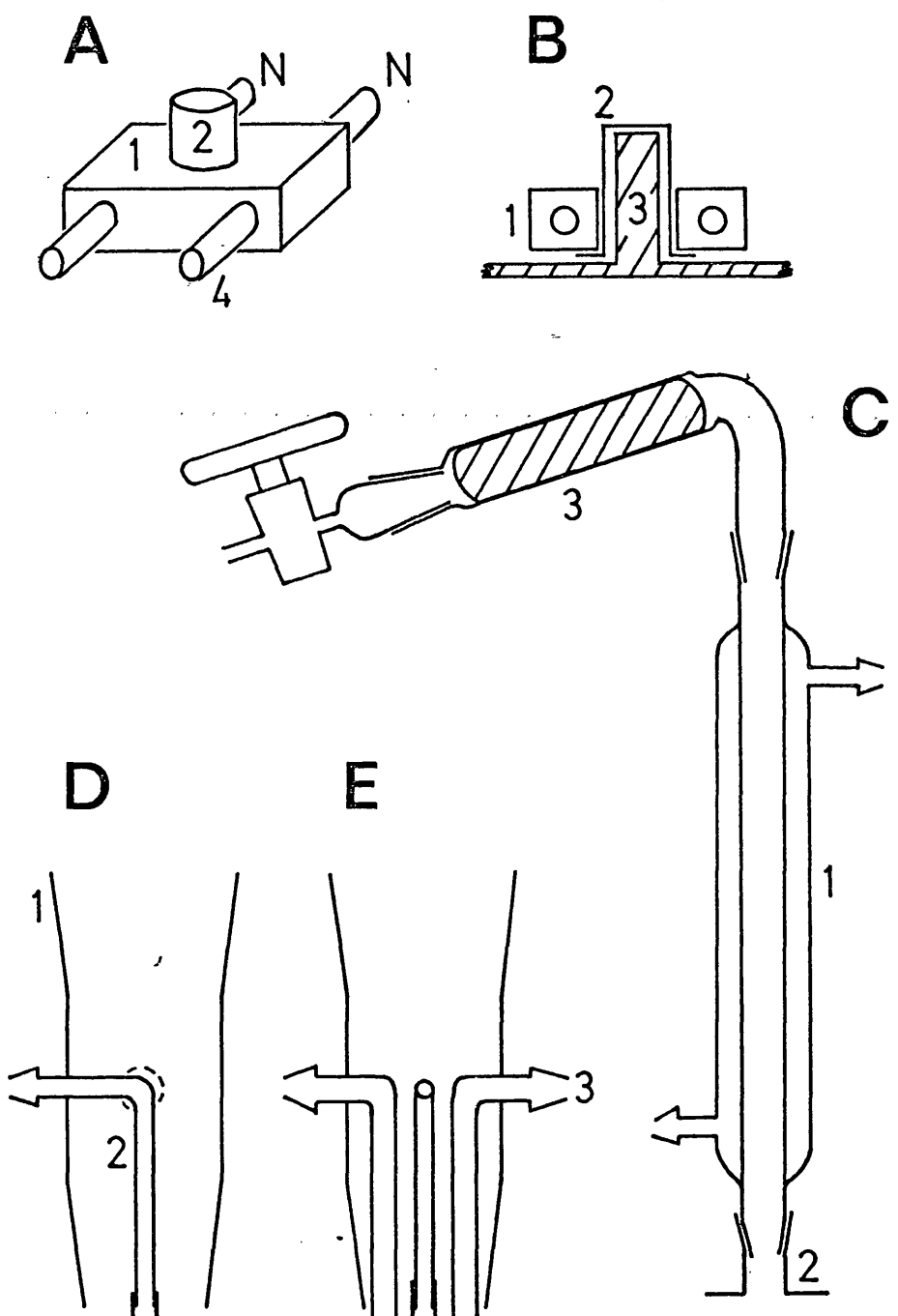
5. Probe inserts.

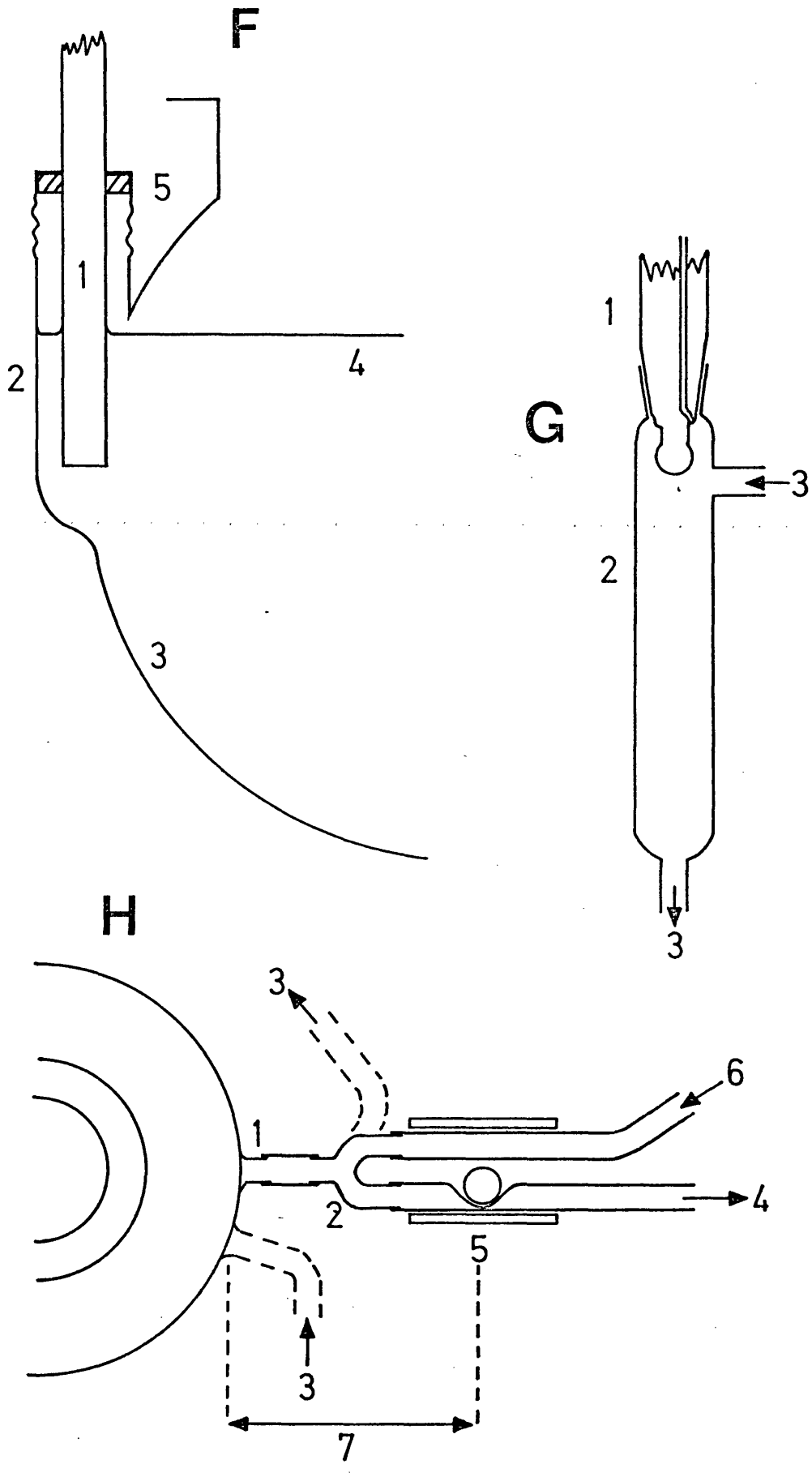
A stainless-steel dissolved-oxygen probe (Fig. F.1) (see II. Materials and Methods. 17) was mounted in a wide side-chamber (2) blown into the wall of the culture vessel (3). The probe was sealed into the chamber by a silicone-rubber 'O'-ring in a Quickfit screwed-cap (5) and projected 60 mm. below the culture surface (4). A steam-sterilizable pH electrode (Activion Ltd.) (Fig. G.1) was mounted through the top of a sample-receiver (2) (see Plate 2D). The sample-receiver was connected into the culture circulation loop (3).

6. Overflow system.

An alternative overflow system was devised which eliminated the pumped loop. A single outlet from the culture vessel (Fig. H.1) was

connected to a short Y-piece (2), the arms of which were connected (using silicone rubber tubing) to the overflow-receiving vessel (4) and, via a sterilising filter, to an air supply (6). (In the previous design, the second arm of the Y-piece was connected to the pumped loop (3)). The connections to the Y-piece passed through a solenoid valve so that the overflow tube was normally-closed and the air-line normally-open. The air flow (ca. 200 ml min^{-1}) served to prevent cells collecting in the outlet tube (1). When the solenoid valve was energised, by a contact between level-detecting probes and the culture surface, the air flow (6) was pinched off and culture flowed into the overflow vessel. The volume of the tube between culture and solenoid valve (7) must be less than the overflow-pulse volume. This overflow system was devised for use with small volume chemostats (500 ml) and for cultures of cells damaged by passage through a pumped loop.


A
N
N
1
2
4
B
2
1
3
C
3
1
2
D
1
2
E
3


F
5
1
2
4
3
G
1
3
2
3
H
3
6
1
4
2
5
3
7

APPENDIX 3. Carbon balance sheet for 4-litre batch cultures of Acer cells at different initial glucose concentrations and aeration conditions (see Table III.2).

Culture	Time (days)	Carbon in each fraction : g.atoms/culture					
		Glucose	Total cell	Cell-wall	Non-glucose medium	CO_2	TOTAL
49	0	2.44	0.038	0.013	0.407	0.000	2.898
	2	2.47	0.049	0.025	0.401	0.005	2.950
	5	2.39	0.035	0.031	ND	0.042	2.498*
	8	1.97	0.090	0.060	0.158	0.200	2.478
	11	1.66	0.208	0.101	ND	0.515	2.484*
	14	0.85	0.284	0.194	0.173	1.193	2.694
	17	0.30	0.445	0.344	0.020	2.120	3.229†
51	0	2.55	0.029	0.017	0.223	0.000	2.819
	2	2.21	0.041	0.025	0.449	0.010	2.735
	5	2.34	0.028	0.032	0.204	0.118	2.822
	8	1.98	0.097	0.062	ND	0.332	2.471*
	11	1.70	0.205	0.136	0.188	0.546	2.875
	14	1.32	0.182	0.117	0.182	0.759	2.560
	17	1.07	0.184	0.144	0.070	0.973	2.441
59	0	4.47	0.088	0.042	0.147	0.000	4.747
	4	4.39	0.100	0.028	0.314	0.062	4.894
	8	2.82	0.207	0.049	1.057	0.230	4.363
	16	2.49	0.548	0.209	0.190	0.860	4.297
60	0	4.85	0.088	0.029	0.109	0.000	5.076
	4	4.27	0.117	0.049	0.230	0.105	4.771
	8	3.26	0.241	0.070	1.075	0.308	4.954
	16	2.56	0.489	0.249	0.509	0.849	4.656

*Data incomplete.

†Excessive recovery apparently due to an unusually high CO_2 measurement - see Fig. III.5. and Table III.5.

ND = No data obtained.

IX. BIBLIOGRAPHY

Anderson, P. A. (1956). Rev. Scient. Instrum. 27, 48.

Baker, N., Feinberg, H. and Hill, R. (1954). Analyt. Chem. 26, 1504.

Bayliss, M. W. Univ. of Leicester, U.K.

Bellamy, A. and Bieleski, R. (1966). Aust. J. biol. Sci. 19, 23.

Bernstein, E. (1960). Science, NY. 131, 1528.

Bryson, V. (1952). Science, NY. 116, 48.

Bryson, V. (1959). In VIIth Int. Cong. for Microbiology, Stockholm. ed. G. Tunevall. pp. 371-380.

Butcher, D. N. and Connolly, J. D. (1971). J. exp. Bot. 22, 314.

Cameron, I. L. and Padilla, G. M. (1966). eds. "Cell Synchrony - studies in biosynthetic regulation". Acad. Press.

Cameron, I. L., Padilla, G. M. and Zimmermann, A. (1971). eds. "Developmental Aspects of the Cell Cycle". Acad. Press.

Chandler, M. T., Tandeau de Marsac, N. and de Kouchkovsky, Y. (1972). Can. J. Bot. 50, 2265.

Cleaver, J. (1967). "Thymidine metabolism and cell kinetics". Amst. - N. Holland Co.

Clowes, F. A. L. (1965). New Phytol. 64, 355.

Clowes, F. A. L. (1968). New Phytol. 67, 631.

Clowes, F. A. L. and Juniper, B. E. (1968). "Plant Cells". Blackwells.

Constabel, F., Shyluk, J. and Gamborg, O. (1971). Planta 96, 306

Constabel, F. and Wetter, L. R. (1972). 49, Suppl., 31.

Cooper, C. M., Fernstrom, G. A. and Miller, S.A. (1944). Ind. Engng. Chem. 36, 504.

Cooper, P. D., Wilson, J. M. and Burt, A. M. (1959). J. gen. Microbiol. 21, 702.

Corman, J., Tsuchiya, M. M., Koepsell, H. J., Benedict, R. G., Kelley, S. E., Feger, V. H., Dworschak, R. G. and Jackson, R. W. (1957). Appl. Microbiol. 5, 313.

Cox, B. J. (1972). Ph.D. thesis, Univ. of Leicester, U.K.

Cox, B. J., Turnock, G. and Street, H. E. (1972). J. exp. Bot. 24, 159.

Darlington, C. D. and Wylie, A. P. (1955). "Chromosome Atlas of Flowering Plants". Allen and Unwin.

Davey, M. R., Fowler, M. W. and Street, H. E. (1971). Phytochemistry 10, 2559

Davies, M. (1971). Pl. Physiol. Lancaster 47, 38.

Dawes, I. W. and Mandelstam, J. (1969). In "Continuous Cultivation of Microorganisms" eds. I. Malek, K. Beran, Z. Fencl, V. Munk, J. Ricica and H. Smrchova. Acad. Press. pp. 157-162.

Dawson, P. S. S. (1969), ibid, pp. 71-85.

de Jong, D., Jansen, E. and Olsen, A. (1967). Expl. Cell Res. 47, 139.

Digby, J. and Wareing, P. F. (1966). J. exp. Bot. 17, 718.

Donachie, W. and Masters, N. (1969). In "The Cell Cycle:gene-enzyme interactions". eds. G. Padilla, G. Whitson and I. L. Cameron. Acad. Press. pp. 37-76.

Doree, M., Leguay, J. J., Terrine, C., Sadorge, P., Trapy, F. and Guern, J. (1971). Colloques Internationaux CNRS N°. 193. Les Cultures de tissus de Plantes. pp. 345-365.

Dougall, D. K. (1965). Pl. Physiol. Lancaster 40, 891.

Dougall, D. K. (1971). Colloques Internationaux CNRS N°. 193. Les Cultures de tissus de Plantes. pp. 367-371.

Engvild, K. C. (1972). Physiologia Pl. 26, 62.

Erickson, R. O. (1964). In "Synchrony in Cell Division and Growth". ed. E. Zeuthen. Interscience. pp. 11-37.

Eriksson, T. (1965). Physiologia Pl. 18, 976.

Eriksson, T. (1966). ibid 19, 900.

Eriksson, T. (1967). ibid 20, 348.

Eriksson, T. (1967a). ibid 20, 507.

Evans, G. M. and Rees, H. (1971). Nature, Lond. 233, 350.

Fawcett, J. K. and Scott, J. E. (1960). J. clin. Path. 13, 156.

Fencl, Z. (1966). In "Theoretical and Methodological Basis of Continuous Culture of Microorganisms". eds. I. Malek and Z. Fencl. Acad. Press. pp. 67-156.

Filner, P. (1965). Expl. Cell Res. 39, 33.

Filner, P. (1966). Biochim. biophys. Acta. 118, 299.

Fletcher, J. S. and Beevers, H. (1970). Pl. Physiol. Lancaster 45, 765.

Foley, M. A. and Syrett, P. J. (1973). Univ. College, Swansea, U.K.

Fosket, D. E. (1970). Pl. Physiol. Lancaster 46, 64.

Fosket, D. E. amd Torrey, J. (1969) Pl. Physiol. Lancaster 44, 871.

Fosket, D. E. and Short, K. C. (1973). Physiol. Plant. 28, 14.

Fowler, M. W. Univ. of Sheffield, U. K.

Fowler, M. W. (1971). J. exp. Bot. 22, 715.

Furuya, T., Hirotani, M. and Kawaguchi, K. (1971). Phytochemistry 10, 1013.

Gamborg, O. L. (1966). Can. J. Biochem. 44, 791.

Gamborg, O. L. and Eveleigh, D. E. (1968). Can. J. Biochem. 46, 417.

Garrod, D. and Ashworth, J. M. (1973). Symp. Soc. gen. Microbiol. 13, in press.

Givan, C. V. and Collin, H. A. (1967). J. exp. Bot. 18, 321.

Gomori, G. (1942). J. Lab. & Clin. Med. 27, 955.

Gould, A. Univ. of Leicester, U. K.

Graebe, J. and Novelli, G. (1966). Expl. Cell Res. 41, 509.

Guttman, R. (1956). Chromosoma 8, 341.

Hahlbrock, K. Kuhlen, E. and Lindl, T. (1971). Planta 99, 311.

Hall, J. The Polytechnic, Leicester, U. K.

Hancock, J. G. (1970). Can. J. Bot. 48, 1515.

Helgeson, J. P., Krueger, S. M. and Upper, C. D. (1969). Pl. Physiol. Lancaster. 44, 193.

Henshaw, G. G., Jha, K. K., Mehta, A. R., Shakeshaft, D. J. and Street, H. E. (1966). J. exp. Bot. 17, 362.

Herbert, D. (1959). In VIIth Int. Cong. of Microbiology, Stockholm. ed. G. Tunevall. pp. 381-396.

Herbert, D. (1961). Symp. Soc. gen. Microbiol. 11, 391.

Herbert, D., Elsworth, R. and Telling, R. (1956). J. gen. Microbiol. 14, 601.

Hiraga, S., Igarashi, K. and Yura, T. (1967). Biochim. biophys. Acta. 145, 41.

Holdgate, D. P. and Goodwin, T. W. (1965). Phytochemistry 4, 831.

Howard, A. and Pelc, S. R. (1953). Heredity, Lond. 6 Suppl. 261.

James, T. W. (1966). In "Cell Synchrony - studies in biosynthetic regulation". eds. I. Cameron and G. Padilla. Acad. Press. pp. 1-13.

Mansfield, K. J. (1973). M.Sc. thesis. Univ. of Sheffield, U.K.

Maretzki, A., Thom, M. and Nickell, L. G. (1969). Phytochemistry 8, 811.

Maretzki, A., and Thom. M. (1972). Biochem. biophys. Res. Commun. 47, 44.

Mattingly, E. (1966). Expl. Cell Res. 42, 274.

Maxon, W. D. and Johnson, M. J. (1953). Industr. Engng. Chem. 45, 2554.

Melchers, G. and Bergmann, L. (1958). Ber. dt. bot. Ges. 71, 459.

Miller, C. O. (1968). In "Biochemistry and Physiology of Plant Growth Substances". eds. F. Wightman and G. Setterfield, Runge Press. pp. 33-45.

Miller, R., Shyluk, J., Gamborg, O. and Kirkpatrick, J. (1968). Science, NY. 159, 540.

Mitchison, J. M. (1971). "The Biology of the Cell Cycle". Cam. Univ. Press.

Monod, J. (1942). Recherches sur la Croissance Bacterienne". Masson, Paris.

Monod, J. (1950). Ann. Inst. Pasteur, Paris. 79, 390.

Montgomery, H. A. and Dymock, J. F. (1961). Analyst 86, 414.

Muir, W. H. (1953). Ph.D. thesis. Univ. of Wisconsin, U.S.A.

Muir, W. H., Hildebrandt, A. and Riker, A. (1958). Am. J. Bot. 45, 589.

Myers, J. and Clark, L. B. (1944). J. gen. Physiol. 28, 103.

Nagl, W. (1972). Am. J. Bot. 59, 346.

Nash, D. and Davies, M. (1972). J. exp. Bot. 23, 75.

Neumann, J. and Jones, M. E. (1962). Nature, Lond. 195, 709.

Nickell, L. G. (1956). Proc. natn. Acad. Sci. U.S.A. 42, 848.

Nickell, L. G. and Tulecke, W. (1959). 136th Symp. Am. Chem. Soc., New Jersey, U.S.A.

Nickell, L. G. and Tulecke, W. (1960). J. biochem. microbiol. Technol. Eng. 2, 287.

Northrop, J. H. (1954). J. gen. Physiol. 38, 105.

Novick, A. and Szilard, L. (1950). Science, NY. 112, 715.

Novick, A. and Szilard, L. (1950a). Proc. natn. Acad. Sci. U.S.A. 36, 708.

O'Brien, T. J., Jarvis, B. C., Cherry, J. H. and Hanson, J. B. (1968). In "Biochemistry and Physiology of Plant Growth Substances". eds. F. Wightman and G. Setterfield. Runge Press. pp. 747-759.

Ogutuga, D. B. and Northcote, D. H. (1970). J. exp. Bot. 21, 258.

Padilla, G., Cameron, I. L. and Elrod, L. H. (1966), in Cell Synchrony-studies in biosynthetic regulation". eds. I. Cameron and G. Padilla. Acad. Press. pps. 269-288.

Padilla, G.,Whitson, G. and Cameron, I. L. (1969). eds. "The Cell Cycle: gene-enzyme interactions". Acad. Press.

Petersen, D., Tobey, R. and Anderson, E. (1969). In "The Cell Cycle: gene-enzyme interactions". eds. G. Padilla, G. Whitson and I. L. Cameron. Acad. Press. pp. 311-359.

Phillips, D. H. and Johnson, M. J. (1956). Abstr. Pap. Amer. Chem. Soc. 130th Meeting. p.23.

Phillips, H. L. (1970). Ph.D. thesis. Univ. of Birmingham, U.K.

Phillips, H. L. and Torrey, J. G. (1972). Am. J. Bot. 59, 183.

Pirt, S. J. (1972). J. appl. Chem. Biotechnol. 22, 55.

Pirt, S. J. and Callow, D. S. (1958). J. appl. Bact. 21, 206.

Powell, E. O. (1965). Lab. Pract. 14, 1145.

Puhan, Z. and Martin, S. M. (1971). Prog. Ind. Microbiol. 9, 13.

Rajasekhar, E. W., Edwards, M., Wilson, S. B. and Street, H. E. (1971). J. exp. Bot. 22, 107.

Rao, P. N. and Engelberg, J. (1966). In "Cell Synchrony - studies in biosynthetic regulation". eds. I. Cameron and G. Padilla. Acad. Press. pp. 332-352.

Rasch, E., Swift, H. and Klein, R. M. (1959). J. biophys. biochem. Cytol. 6, 11.

Reinert, J. (1956). Science, NY. 123, 457.

Ricica, J. (1966). In "Theoretical and Methodological Basis of Continuous Cultures of Microorganisms". eds. I. Malek and Z. Fencl. Acad. Press. pp. 31-66: Continuous systems. pp. 236-240: Theory of aeration.

Roberts, K. and Northcote, D. H. (1970). J. Cell Sci. 6, 299.

Rogers, A. W. (1967). "Techniques of Autoradiography". Elsevier Pub. Co.

Rose, D. Martin, S. M. and Clay, P. P. S. (1972). Can. J. Bot. 50, 1301.

Scherbaum, O. H. (1959). J. Protozool. 6 Suppl., 17.

Schmidt, R. (1969). In "The Cell Cycle: gene-enzyme interactions". eds. G. Padilla, G. Whitson and I. L. Cameron. Acad. Press. pp. 159-177.

Shepherdson, M. and Pardee, A. B. (1960). J. biol. Chem. 235, 3233.

Short, K. C. (1969). Ph.D. thesis, Univ. College, Swansea, U.K.

Short, K. C. Brown, E. G. and Street, H. E. (1969). J. exp. Bot. 20, 579.

Simpkins, I. (1970). Ph.D. thesis, Univ. College, Swansea, U.K.

Simpkins, I., Collin, H. A. and Street, H. E. (1970). Physiologia Pl. 23, 385.

Simpkins, I. and Street, H. E. (1970). J. exp. Bot. 21, 170.

Sinclair, R. (1966). Expl. Cell Res. 41, 20.

Smith, H. S. and Pardee, A. B. (1970). J. Bact. 101, 901.

Smith, S. M. (1973). Ph.D. thesis. Univ. of Leicester, U.K.

Somogyi, N. (1952). J. biol. Chem. 195, 19.

Spang, H. A. and Platt, R. S. (1972). Physiologia Pl. 27, 321.

Stanley, P. E. (1965). Ph.D. thesis, Univ. of Bristol, U.K.

Steward, F. C. and Shantz, E. M. (1955). In "The Chemistry and Mode of Action of Plant Growth Substances". eds. R. L. Wain and F. Wightman. Butterworth. pp. 165-186.

Steward, F. C. and Mohan Ram, H. Y. (1961). Advan. Morphogenesis 1, 189.

Street, H. E. (1966). In "Cells and Tissues in Culture - methods, biology and physiology". Vol. 3 ed. E. N. Wilmer. Acad. Press. pp. 631-689.

Street, H. E. (1967). "Les Cultures de Tissus de Plantes". CNRS. Paris. pp. 177-193.

Street, H. E. (1969). In "Plant Physiology - a treatise". Vol. 5B. ed. F. C. Steward. Acad. Press. pp. 3-324.

Street, H. E. (1973). In "Biosynthesis and its Control in Plants". ed. B. V. Milborrow. Acad. Press. pp. 93-125.

Street, H. E. (1973a). ed. "Plant Tissue and Cell Culture". Blackwells.

Street, H. E., Collin, H. A., Short, K. C. and Simpkins, I. (1968). In "Biochemistry and Physiology of Plant Growth Substances". eds. F. Wightman and G. Setterfield. Runge Press. pp. 489-504.

Street, H. E., King, P. J. and Mansfield, K. J. (1971). Colloques Internationaux CNRS. N°, 193. Les Cultures de Tissus de Plantes. pp. 17-40.

Stuart, R. and Street, H. E. (1969). J. exp. Bot. 20, 556.

Sunderland, N. (1971). Sci. Prog. Oxf. 59, 521.

Sunderland, N. (1973). In "Plant Tissue and Cell Culture". ed. H. E. Street. Blackwells. pp. 161-189.

Sutton-Jones, B. and Street, H. E. (1968). J. exp. Bot. 19, 114.

Tabata, M., Yamamoto, H. and Hiraoka, N. (1971). Colloques Internationaux. CNRS N°. 193. Les Cultures de Tissus de Plantes. pp. 390-402.

Tabata, M., Yamamoto, H., Hiraoka, N., Marumoto, Y. and Konoshima, M. (1971). Phytochemistry 10, 723.

Thomas, E. and Street, H. E. (1970). Ann. Bot. 34, 657.

Thomas, E., Konar, R. N. and Street, H. E. (1972). J. Cell Sci. 11, 95.

Tobey, R. A. and Ley, K. D. (1970). J. Cell Biol. 46, 151.

Torrey, J. G. (1971). Colloques Internationaux CNRS N°. 193. Les Cultures de Tissus de Plantes. pp. 177-186.

Torrey, J. G., Reinert, J. and Merkel, N. (1962). Am. J. Bot. 49, 420.

Tulecke, W. and Nickell, L. G. (1959). Science, NY. 130, 863.

Tulecke W., Taggart, R. and Colavito, L. (1965). Cont. Boyce. Thomp. Inst. P1. Res. 23, 33.

Turner, T. D. (1971). Pharm. J. 206, 341.

Van't Hof, J. (1965). Expl. Cell Res. 39, 48.

Van't Hof, J. and Sparrow, A. H. (1963). Proc. natn. Acad. Sci. U.S.A. 49, 897.

Veliky, I., Sandkvist, A. and Martin, S. (1969). Biotechnol. Bioeng. 11, 1247.

Veliky, I. and Martin, S. (1970). Can. J. Microbiol. 16, 223.

Verma, D. and van Huystee, R. (1970). Can. J. Bot. 48, 429.

Verma, D. and van Huystee, R. (1971). Expl. Cell Res. 69, 402.

Vogel, A. I. (1955). "Quantitative Inorganic Analysis". Longmans.

Washko, M. E. and Rice, E. W. (1961). Clin. Chem. 7, 542.

Watson, T. G. (1972). J. appl. Chem. Biotechnol. 22, 237.

Williamson, D. H. (1966). In "Cell Synchrony- Studies in biosynthetic regulation". eds. I. Cameron and G. Padilla. Acad. Press. pp. 81-101.

Williamson, D. H. and Scoper, A. W. (1961). Symp. Soc. gen. Microbiol. 11, 217.

Wilson, G. (1971). Ph.D. thesis, Univ. of Birmingham, U.K.

Wilson, S. B., King, P. J. and Street, H. E. (1971). J. exp. Bot. 22, 177.

Wimber, D. E. (1966). Am. J. Bot. 53, 21.

Wimber, D. E. and Quastler, H. (1963). Expl. Cell. Res. 30, 8.

Yamada, Y., Yasuda, T., Koge, M. and Sekiya, J. (1971). Colloques Internationaux CNRS, N°. 193. Les Cultures de Tissus de Plantes. pp. 137-153.

Yemm, E. W. and Cocking, E. C. (1955). The Analyst 80, 1948.

Yeoman, M. M. (1970). Int. Rev. Cytology 29, 383.

Yeoman, M.M. and Evans, P. K. (1967). Ann. Bot. 31, 323.

Yeoman, M.M. and Aitchison, P. A. (1973), in "Plant Tissue and Cell Culture". ed. H. E. Street. Blackwells. pp. 240-268.

Young, M. (1973). J. exp. Bot. 24, in press.

Zeuthen, E. (1963). In "Cell Growth and Cell Division". ed. R. J. C. Harris. Acad. Press. pp. 1-7.

Zeuthen, E. (1964). ed. "Synchrony in Cell Division and Growth". Interscience.

Zeuthen, E. (1971). Adv. Cell Biol. 2, 43.

Zeuthen, E. and Ramussen, L. (1971). In "Research in Protozoology". Vol. 4. ed. T. T. Chen. Pergamon.

THE CONTINUOUS CULTURE OF PLANT CELLS

Ph.D. Thesis, 1973. P. J. King

Abstract

Steady states of growth were established in continuous culture, both as chemostats and turbidostats, using a cell suspension of *Acer pseudoplatanus* L. Stable states were maintained over a wide range of dilution rates in chemostat cultures. The relationships between steady-state biomass, the concentration of nitrate (the limiting nutrient) and dilution rate were generally that to be expected from chemostat theory although the yield coefficient was not a constant. The size, composition and physiological state of *Acer* cells depended upon growth rate. There was no significant alteration in the basic growth processes of *Acer* cells transferred from batch to continuous cultures.

The growth of *Acer* cells in chemostats was not entirely predictable. Furthermore, the specific growth rate of batch cultures of *Acer* cells was found to vary (mean doubling time = 70 hours; minimum = 20 hours). The rate of cell division in batch cultures of *Acer* cells was increased by increasing the initial concentration of 2,4-dichlorophenoxyacetic acid.

Cell division in batch cultures of *Acer* was readily synchronised by a simple starvation and regrowth treatment. It was possible to follow the growth of cultures through up to five synchronous cell generations, with an average 85% participation at each cytokinesis. Some basic markers in the cell cycle of *Acer* cells were established

and model cell cycles constructed. DNA synthesis was restricted to a period of ca. 15 hours. G2 was relatively constant at ca. 16 hours. Nuclear division (1-2 hours) was separated from cell division by a 10-hour lag. G1 was most variable (13-37 hours) producing variation in cell-cycle times coincident with that of specific growth rate in random cultures. S-phase or its initiation was coincident with other periodic, metabolic events.