Regulation of Amino Acid Metabolism in the Cellular Slime Mould *Dictyostelium discoideum*

by

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Thesis submitted to the
University of Glasgow
for the degree of
Doctor of Philosophy

Department of Biochemistry

March 1984.

In the name of

ALLAH,

Most Gracious, Most Merciful.

To my

PARENTS

BHAI JAN AND BHABI

for their affection and

financial and moral support

and

To my WIFE.

ACKNOWLEDGEMENTS

I wish to express my thanks to everyone who assisted me during the course of my work, especially I am grateful to:

Professor R.M.S. Smellie for the opportunity to carry out this research and for making the facilities of the department available.

Dr. Ian D. Hamilton for his guidance, constant encouragement, constructive comments, unending patience and friendly support at various stages of this work, including the careful reading of this manuscript.

Dr. L.M. Fixter, Dr. W.H. Holms, Professor C.A. Fewson and to all my colleagues in laboratory C24 for critical discussions and their suggestions.

I am grateful to Mrs. M. McKenzie for efficiently and patiently typing the manuscript. Finally, I would like to thank my wife for her patience and encouragement.

ABBREVIATIONS

Abbreviations used in this thesis follow the Biochemical Journal Instructions to authors, (Biochem. J. (1981) 193, 1-27) with the following additions:

ALM	axenic liquid medium
ALM_g	glucose supplemented ALM
ATCC	American type culture collection
DNME	diazoacetyl norleucine methyl ester
EDTA	ethylene diamine tetra acetic acid
MES	2 (N-morpholino) ethane sulphonic acid
PMS	phenyl methosulphate
POP	2,4-diphenyl oxazole
Tris	tris (hydroxymethyl) methylamine
TLCK	tosyl lysyl chloromethyl ketone
GLDH	Glutamate dehydrogenase
UDPG	uridine diphosphate glucose

CONTENTS

	page
DEDICATION	i
ACKNOWLEDGEMENTS	ii
ABBREVIATIONS	iii
CONTENTS	iv
SUMMARY	xvii
INTRODUCTION	
1. Cellular slime moulds	1
2. Growth and differentiation of D. discoideum	1
2.1. Growth conditions	6
3. Metabolic changes during differentiation	8
3.1. Breakdown of amino acids	10
4. Influence of differentiated cell types on metabolism	12
5. Carbohydrate metabolism in D. discoideum	13
6. Protein metabolism in D. discoideum	18
6.1. Proteinases and peptidases	18
6.1.1. Proteinases	18
6.1.1.2. Developmental significance of proteinase activity	20
6.1.2. Peptidases	21
6.2. Enzymes metabolising specific amino acids	21
6.3 Enzymes for the final stage of amino acid breakdown	24
6.4 Other aspects of amino acid metabolism in slime moulds	25
6.5 Role of amino acids in differentiation	25
7. Polyamines and their metabolism in D. discoideum	27
8. Transport system in D. discoideum	28
9. Aims of this research	31

METHODS

1. Materials

1.1. Commercial sources 32

2. Organism 33

3. Maintenance of stock cultures 33

3.1. *D. discoideum* Ax2 33

3.2. *E. coli* B/r 33

4. Preparation of media and buffers 34

4.1. Nutrient agar 34

4.2. Nutrient broth 34

4.3. Cooked meat medium 35

4.4. Axenic liquid medium (ALM) 35

4.5 Phosphate buffer 36

5. Sterilization 36

5.1. Autoclaving 36

5.2. Filtration 37

6. Measurement of cell density 37

7. Measurement of pH 37

7.1. Media and buffer preparation 37

7.2. pH values of culture samples 38

7.3. pH control equipment 38

8. Preparation of inocula 38

9. Growth conditions in liquid culture 40

9.1. Cultures of small volumes 40

9.2. Cultures of large volumes 41

9.3. pH controlled cultures 41

9.4. Processing of samples from growing cultures 42

9.4.1. Amino acid analyses 42

9.4.2. Ammonia estimation 42

9.4.2.1. Chemical method 42

9.4.2.2. Enzymic method 42

9.4.3. Protein estimation 43

10. Experiments using washed cell suspensions 43

10.1. Preparation of washed cell suspensions 43

10.2. Measurement of ammonia production 43

10.2.1. Incubation conditions 43

10.2.2. Samples for ammonia estimation 44

10.3. Measurement of oxygen uptake 44

10.3.1. Apparatus used 44

10.3.2. Estimation method 44

10.4. Measurement of ^{14}C- carbon dioxide production 45

10.4.1. Incubation conditions 45

10.4.2. Measurement of radioactivity 46

11. Analytical techniques and their quantification 47

11.1. Ammonia estimation 47

11.1.1. Chemical method 47

11.1.1.1. Assay procedure 48

11.1.1.2. Interference with ammonia estimation 48

11.1.2. Enzymic method 49

11.1.2.1. Assay procedure 49

11.2. Calibration of oxygen electrode and oxygen uptake measurements 51

11.3. Scintillation spectrometry and calculation of substrate utilization 52

11.4. Protein estimation 53

11.5. Amino acid analysis 53

11.5.1. Method 53

11.5.2. Quantification of analyses 54

11.6. Measurement of osmotic strength 55

12. Measurement of enzyme activity 55

12.1. Preparation of cell extract 55

12.2. Ornithine aminotransferase 58

12.2.1. Assay conditions 58

12.2.2. Assay reliability 59

12.3. Threonine deaminase 59

12.3.1. Assay conditions 59

12.3.2. Assay reliability 61

12.4. Tyrosine transaminase 61

12.4.1. Assay conditions 61

12.4.2. Assay reliability 64

12.5. Glutamate dehydrogenase 64

12.5.1. NAD linked glutamate dehydrogenase 66

12.5.1.1. Assay conditions 66

12.5.1.2. Assay reliability 66

12.5.2. NADP linked glutamate dehydrogenase 66

12.5.1.1. Assay conditions 66

12.5.2.2. Assay reliability 68

12.6. Malic enzyme 68

12.6.1. Assay conditions 68

12.6.2. Assay reliability 70

12.7. Lysine dependent oxygen uptake activity 70

12.7.1. Assay conditions 70

12.7.2. Assay reliability 72

13. Glassware 72

14. Statistical methods 72

Results

1. Growth of D. discoideum Ax2 in liquid culture. 74

1.1. Growth in Axenic liquid medium (AIM). 74

2.	Influence of ammonia and pH on growth in ALM.	77
	2.1. Influence of ammonia on growth.	77
	2.2. Influence of pH on growth.	77
	2.2.1. Attempts to stabilise pH in growth medium.	81
	2.2.1.1. Influence of buffer concentration.	81
	2.2.1.2. pH controlled experiments.	87
3.	Influence of carbohydrate on growth in ALM.	93
	3.1. Glucose	93
	3.2. Fructose	93
	3.3. Galactose	93
4.	Influence of pyruvate on growth in ALM.	97
5.	Ammonia estimation during growth.	97
6.	Changes in amino acid concentrations in the medium during the growth of D. discoideum Ax2	104
7.	Washed cell experiments.	107
	7.1. ALM cells.	107
	7.1.1. Effect of ALM and individual amino acids on ammonia production.	107
	7.1.2. Influence of amino acid concentration on ammonia production.	111
	7.1.3. Effect of nitrogen free metabolites on the rate of ammonia production by cell suspensions.	113
	7.1.3.1. Effect of glucose.	113
	7.1.3.2. Influence of other metabolites.	113
	7.2. ALM_g cells.	117
	7.2.1. Effect of ALM and individual amino acids.	117
	7.2.2. Effect of nitrogen free metabolites on the rate of ammonia production by ALM_g cell suspension.	121
	7.2.2.1. Effect of glucose.	121
	7.2.2.2. Influence of other matabolites.	121

8. Oxygen uptake by D. dicsoideum Ax2 cells. 126

8.1. Effect of ALM on the rate of oxygen uptake by washed cell suspensions. 126

8.2. Influence of amino acids and other metabolites on the rate of oxygen uptake by cells grown in ALM. 126

8.3. Oxygen uptake by ALM_g cells. 129

9. Catabolism of radiolabelled amino acids. 135

9.1. Catabolism of amino acids by ALM and ALM_g cells. 135

9.2. Effect of glucose on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM. 135

9.3. Effect of the addition of metabolites on ^{14}C- carbon dioxide production from arginine catabolismby ALM and ALM_g cells. 138

9.4. Effect of glucose concentration on carbon dioxide production from amino acid catabolism by ALM cells. 138

9.5. Effect of glucose concentration on its own catabolism. 142

9.6. Effect of pyruvate, galactose and phosphate concentration on ^{14}C- carbon dioxide production from amino acids. 142

9.7. Effect of glucose concentration on ^{14}C- carbon dioxide production from amino acids by ALM_g cells. 147

9.8. Effect of pyruvate, galactose and phosphate concentration on the production of carbon dioxide from amino acids by ALM_g cells. 147

9.9. Effect of 2-deoxy D-glucose on carbon dioxide production from arginine by ALM cells. 147

10. Effect of osmotic strength on amino acid catabolism. 153

10.1. Effect of osmotic strength on the rate of ^{14}C- carbon dioxide production from arginine catabolism by ALM cells. 153

11. Effect of growth conditions on the specific activity of amino acid metabolising enzymes. 155

DISCUSSION

1. Growth of D. discoideum 157

1.1. Growth in axenic liquid medium 158

1.1.1. Effect of nutrient concentration 159

1.1.2. Effect of pH 159

1.1.3. Effect of ammonia 160

1.2. Growth conditions in axenic liquid medium 160

1.3. Influence of carbohydrates on growth in axenic liquid medium 162

1.3.1. Glucose 162

1.3.2 Fructose 162

1.3.3 Galactose 162

2. Nature of the organism used in these studies 163

3. Metabolism of ALM in washed cell suspensions 165

4. Metabolism of individual amino acids in washed cell suspensions 167

4.1. Influence of metabolites on amino acid utilization 168

4.1.1. Influence of glucose 169

4.1.2 Effect of other metabolites 170

5. Effect of metabolite concentration on amino acid utilization 172

5.1. ALM grown cells 172

5.2. ALM_g grown cells 175

6. Enzyme activities under different growth conditions 177

6.1. Ornithine aminotransferase 177

6.2. Threonine deaminase 178

6.3. Tyrosine transaminase 178

6.4. Glutamate dehydrogenase 179

6.5. Malic enzyme 180

6.6. Lysine dependent oxygen uptake activity 181

7. Enzyme activity changes 183

8. General conclusion and future prospectus. 184

REFERENCES. 185

APPENDIX 192

Figure

1. Life cycle of D. discoideum 2

2. pH controlled experiment set up 39

3. Effect of additives on osmolarity 57

4. Ornithine amino transferase 60
   a. Time course of glutamate semialdehyde formation 60
   b. Effect of extract volume on glutamate semialdehyde formation 60
   c. Effect of cell density on glutamate semialdehyde formation 60

5. 2-oxobutyrate standard curve 62

6. Threonine deaminase 63
   a. Time course of 2-oxobutyrate formation 63
   b. Effect of extract volume on 2-oxobutyrate formation 63
   c. Effect of cell density on 2-oxobutyrate formation 63

7. Tyrosine transaminase 65
   a. Time course of p-hydroxyphenylpyruvate formation 65
   b. Effect of extract volume on p-hydroxyphenyl-pyruvate formation 65
   c. Effect of cell density on p-hydroxyphenylpyruvate formation 65

8. NAD linked glutamate dehydrogenase 67
   a. Effect of extract volume on NADH production 67
   b. Effect of cell density on NADH production 67

9. NADP linked glutamate dehydrogenase 69
   a. Effect of extract volume on NADPH production 69
   b. Effect of cell density on NADPH production 69

10. Malic enzyme 71
   a. Effect of extract volume on $NADPH_2$ production 71
   b. Effect of cell density on $NADPH_2$ production 71

11. Lysine dependent oxygen uptake activity 73
   a. Effect of extract volume on oxygen consumption 73
   b. Effect of cell density on oxygen consumption 73

12. Growth of D. discoideum Ax2 in ALM and ALM_g pH6.7 75

13. Effect of concentration of ALM pH6.7, on the growth of D. discoideum Ax2 76

14. Effect of ammonium chloride concentration on the growth of D. discoideum Ax2 in ALM pH6.7. 78

15. Effect of ammonium chloride concentration, on the growth of D. discoideum Ax2 in ALM with pH maintained at 6.7. 79

16. Effect of ammonium chloride concentration on the growth of D. discoideum Ax2 in ALM_g pH6.7. 80

17. Effect of pH on the growth of D. discoideum Ax2 in ALM 82

18. Effect of pH on the growth of D. discoideum Ax2 in ALM_g 83

19. Growth of D. discoideum Ax2 in ALM prepared at pH6.7 at different concentrations of phosphate buffer. 84

20. Growth of D. discoideum Ax2 in ALM, prepared at pH6.7 with MES added at different concentrations 85

21. Growth of D. discoideum Ax2 in ALM, prepared at pH6.7 with phosphate and MES buffers added at different concentrations. 86

22. Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.7 88

23. Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.0 89

24. Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.2 90

25. Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.5 91

26. Growth of D. discoideum Ax2 in ALM and ALM_g pH6.2 at different glucose concentrations 94

27. Growth of D. discoideum Ax2 in ALM and $ALM_{fructose}$ with pH maintained at 6.2 95

28. Growth of D. discoideum Ax2 in ALM and $ALM_{galactose}$ with pH maintained at 6.2 96

29. Growth of D. discoideum Ax2 in ALM and $ALM_{pyruvate}$ with pH maintained at 6.2 98

30 Ammonia production during the growth of D. discoideum Ax2 in ALM pH6.2 at different concentrations of glucose. 100

31. Ammonia production during the growth of D. discoideum Ax2 in ALM and $ALM_{Fructose}$ with pH maintained at 6.2 101

32. Effect of incubation time and cell density on the endogenous production of ammonia by washed cell suspensions of D. discoideum Ax2 108

33. Effect of ALM on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 109

34. Effect of amino acid concentrations on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 in the presence of individual amino acids 112

35. Effect of incubation time and cell density on the endogenous production of ammonia by washed cell cell suspensions of D. discoideum Ax2 after growth in ALM_g pH6.2 119

36. Effect of ALM on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 after growth in ALM_g pH6.2 120

37. Rate of ^{14}C- carbon dioxide production from amino acids by D. discoideum Ax2 cells grown in ALM pH6.2. 136

38. Effect of metabolites on the utilization of arginine by ALM and ALM_g cells 140

39. Effect of glucose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2 141

40. Effect of glucose concentration on its own catabolism by D. discoideum Ax2 cells grown in ALM pH6.2 143

41. Effect of pyruvate concenration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2 144

42. Effect of galactose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2 145

43. Effect of phosphate concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2 146

44. Effect of glucose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2 148

45. Effect of pyruvate concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2 149

46. Effect of galactose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2 150

47. Effect of phosphate concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2 151

48. Effect of concentration of 2-deoxy D-glucose on the utilization of arginine by D. discoideum Ax2 cells grown in ALM pH6.2. 152

49. Effect of osmotic strength on the utilization of arginine by D. discoideum Ax2 cells grown in ALM pH6.2. 154

50. Effect of glucose concentration on the "medium clearance" by D. discoideum cells grown in ALM pH6.2. 176

Table

		Page
1.	Interference of components with ammonia estimation.	50
2.	Media for the preparation of extracts of D. discoideum for enzyme assays.	56
3.	Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.7, 6.5, 6.2 and 6.0.	92
4.	Ammonia production during the growth, at fixed pH, of D. discoideum Ax2 in supplemented ALM.	99
5.	Ammonia production during the growth of D. discoideum Ax2 in ALM pH6.2 at different concentrations of glucose.	102
6.	Changes in amino acid concentrations (mM) during growth of D. discoideum Ax2 in ALM pH6.2.	105
7.	Changes in amino acid concentrations (mM) during growth of D. discoideum Ax2 in ALM_g pH6.2.	106
8.	Rate of ammonia production by washed cell suspension of D. discoideum Ax2, grown in ALM, in the presence of individual amino acids.	110
9.	Effect of glucose on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2.	114
10a.	Effect of metabolites on the ALM stimulated rate of ammonia production by washed cell suspensions of D. discoideum Ax2.	115
10b.	Effect of metabolites on the endogenous rate of ammonia production by washed cell suspensions of D. discoideum Ax2.	116
11.	Effect of metabolites on the rate of ammonia production, stimulated by amino acid, in cell suspensions of D. discoideum Ax2.	118
12.	Effect of individual amino acids on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2.	122
13.	Effect of glucose on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2.	123
14.	Effect of metabolites on the endogenous rate of ammonia production by washed cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2.	124
15.	Effect of metabolies on the rate of ammonia production stimulated by amino acids, in cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2.	125
16.	Effect of ALM on the rate of oxygen uptake by washed cell suspensions of D. discoideum Ax2 grown in ALM pH6.2.	127

17. Effect of individual amino acids on the rate of oxygen uptake by washed cell suspension of D. discoideum Ax2 grown in ALM pH6.2. 128

18a. Effect of metabolites on the rate of oxygen uptake in the presence of arginine by washed cell suspensions of D. discoideum Ax2 after growth in ALM pH6.2. 130

18b. Effect of metabolites on the rate of oxygen uptake in the presence of lysine by washed cell suspensions of D. discoideum Ax2 after growth in ALM pH6.2. 131

18c. Effect of metabolites on the rate of oxygen uptake by washed cell suspension of D. discoideum Ax2 after growth in ALM pH6.2. 132

19. Effect of individual amino acids on the rate of oxygen uptake by washed cell suspension of D. discoideum Ax2 grown in ALM_g pH6.2. 133

20. Effect of metabolites on the rate of oxygen uptake by washed cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2. 134

21. Catabolism of radiolabelled amino acids by D. discoideum Ax2 cells grown in ALM and ALM_g pH6.2. 137

22. Effect of glucose on catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2. 139

23. Effect of growth conditions on the specific activity of amino acid metabolising enzymes of D. discoideum Ax2. 156

SUMMARY

The thesis begins with a description of cellular slime moulds and briefly reviews growth and differentiation of D. discoideum and the changes occuring during differentiation. Proteins are a major nutrient during growth and differentiation of D. discoideum Ax2. The proteinases and peptidases involved in the breakdown of protein and peptides, and some amino acid metabolising enzymes are described. The effects of glucose and amino acids on differentiation are discusssed and the introduction concludes with a brief description of transport systems used by the slime mould namely pinocytosis and phagocytosis.

The aims of the experimental work were four fold.

A) to develop and define different growth systems where the rates of amino acid utilization by the cells were different.

B) to determine if rates of amino acid utilization could be altered by growth conditions.

C) to measure the rates of amino acid utilization and the effect of metabolites on the utilization of amino acids.

D) to determine if rates of amino acid metabolism were related to the levels of amino acid metabolising enzymes in the cells.

Axenic liquid medium (ALM), either with or without glucose, at 86 mM, was used to grow the cells. The following results were obtained.

1) In the absence of glucose.

a) During growth of the cells, the pH of the medium increased. The yield of the cells obtained increased as the initial pH of the medium was decreased within the pH range 6.0 - 7.0.

b) Growth of the cells was limited by pH change of the medium. With the pH of the culture maintained at 6.2 the cells grew to a

density of 6.6×10^6 cells/ml. In the absence of pH control, the cell density reached 2.8×10^6 cells/ml.

c) Ammonia was produced during growth. An ammonia concentration of 19 mM was achieved during growth to a density of 6.6×10^6 cells/ml. Cultures growing to a lower cell density produce proportionally less ammonia.

2. In the presence of glucose.

a) The yield of the cells increased to 1.2×10^7 cells/ml and the pH change associated with growth was reduced. The final density was much less susceptable to the pH at which the medium was prepared (routinely 6.2).

b) Control of medium pH at 6.2 marginally increased the final cell density to 1.5×10^7 cells/ml.

c) During growth ammonia production was reduced to about half the value obtained in the absence of glucose.

3. Fructose, added to ALM produced similar results to glucose with respect to growth yield, pH change and ammonia production.

4. Galactose, added to ALM reduced the growth rate and diminished the yield to values less than the control value. Galactose had little effect on ammonia production.

5. Pyruvate, added to ALM had a small effect. It increased the yield by 25% and decreased the ammonia production by 20%.

6. The addition of ammonium chloride (up to 20 mM), sodium and potassium phosphate (up to 28.76 mM) and 2(N-morpholino) ethane sulphonic acid (up to 20 mM) all reduced the growth rate and the yield of the cells.

7. In washed cell suspensions.

a) Glucose, added to the cell suspensions, reduced ammonia production to an extent comparable to its effect during growth.

b) By using the techniques of ammonia estimation, amino acid analysis, radiolabelled substrate utilization and oxygen uptake, amino acid breakdown was measured. In general, cells grown in the absence of glucose had a greater ability to catabolise amino acids.

c) Among the individual amino acids, whose rates of degradation were measured, arginine was the most rapidly catabolised followed by lysine and tyrosine. The pattern was independent of the medium in which the cells were grown.

d) The rate of amino acid catabolism, measured by ammonia production or ^{14}C- carbon dioxide production, was reduced by the addition of a range of compounds to washed cell suspensions.

e) In washed cell suspensions prepared from cells grown in the absence of glucose, glucose at 10 and 20 mM stimulated the breakdown of extracellular amino acids. A similar effect was produced by 2 deoxy D-glucose and trehalose but not by other additives. These results are discussed in terms of stimulation of uptake but reduction in metabolism.

f) Galactose, pyruvate, phosphate, and glucose in ALM_g cells and glucose and 2 deoxy D-glucose above 20 mM in ALM cells, all reduced amino acid metabolism. The data are considered in relation to metabolic and osmotic effects on the cells.

8. The activities of several amino acid catabolising enzymes were measured in cells grown in the presence and absence of glucose. Cells grown in the absence of glucose possessed higher levels of activity and this correlates with their increased ability to catabolise amino acids.

INTRODUCTION

1. Cellular slime moulds

The Cellular Slime Moulds or Acrasiales stand at the boundary between the true protista and the metazoa and metaphyta (Sussman 1965). They are usually grouped within the myxomycophyta with the myxomycetes or true slime moulds (Keeton 1972), although they differ from the myxomycetes in certain important particulars. Unlike the myxomycetes, the acrasiae neither exhibit a swimming stage, nor do they fuse at any time to form a true plasmodium (Raper 1956). Because the cellular slime moulds undergo morphogenesis independently of growth, they have proved to be an ideal system for the study of the development of a eukaryote. Analysis of the growth phase of the organism has been much more limited.

2. Growth and differentiation of D. discoideum.

The cellular slime mould Dictyostelium discoideum NC4 isolated from forest soil by K.B. Raper in 1935, is by far the most studied and the best known of the cellular slime moulds. The spores of this strain of D. discoideum are elliptical in shape and vary considerably in size, being approximately 6 to 9 μ long and 2.5 to 3.5 μ in diameter (Bonner 1967). In the laboratory, optimum germination of the spores occurs between pH6 and 7 in potassium phosphate buffer and can be promoted by heat shock at 45^{o}C for 30 min (Cotter and Raper 1968a), and by the loss of the germination inhibitor Discadenine (Abe et al., 1976) from the spores. Germination occurs in three well-defined stages: activation of dormant spores, swelling of activated spores and emergence of myxamoebae from swollen spores (Cotter and Raper 1968b). Emergence of the amoebae occurs a few hours after the spores have been placed in a favourable environment.

The life cycle of the cellular slime mould D. discoideum (Figure 1) can be divided into two mutually exclusive phases (Raper 1935), the

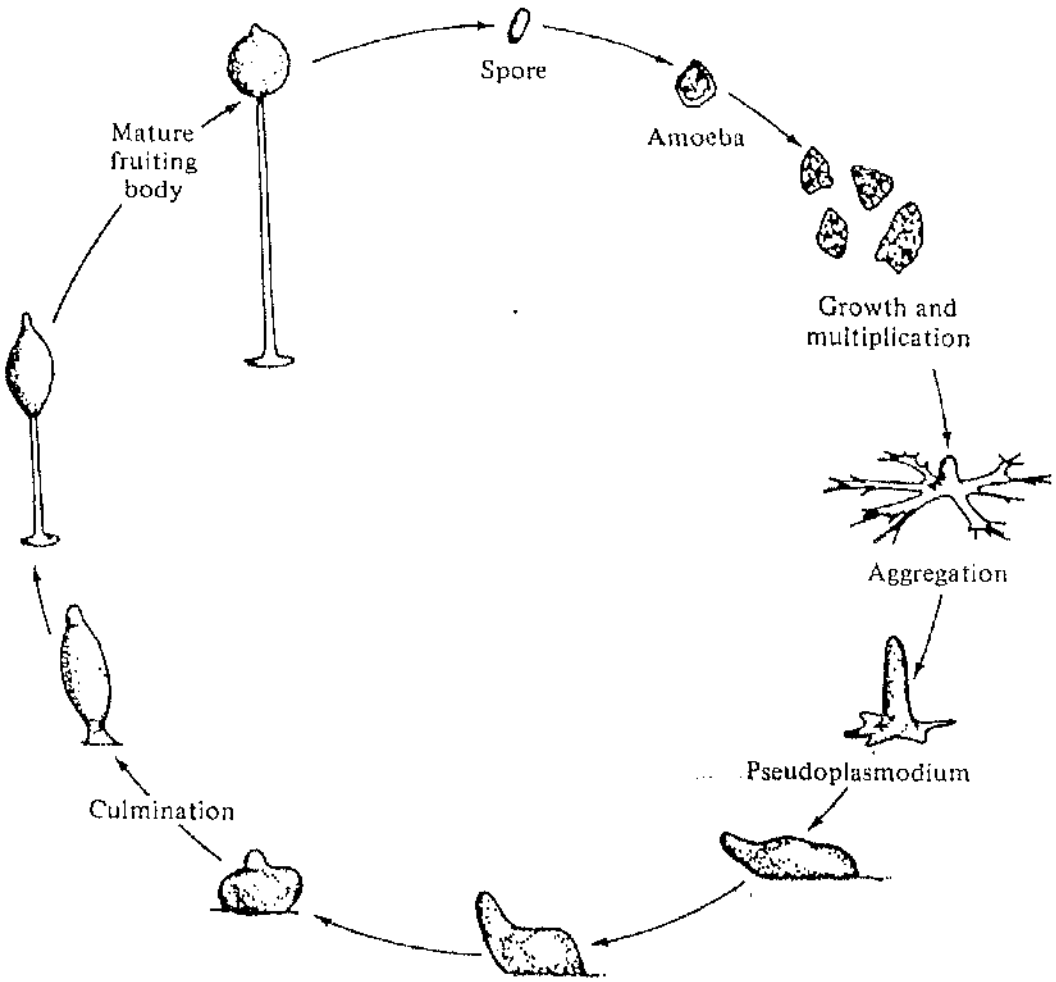


Figure 1: Life cycle of *Dictyostelium discoideum*.

(after Ashworth and Dee (1975)).

growth or vegetative phase when the cells exist as free living amoebae, followed by the differentiation or development phase in which the cells form a multicellular aggregate which progressively integrates to form a final fruiting structure consisting of supportive tissue (stalk) and reproductive elements (spores).

During growth, which occurs successfully between 20-25^{o}C (Cotter and Raper 1968a), the single celled myxamoebae use bacteria as a food source. When one amoeba has eaten about 1000 bacteria, it divides by binary fission into two daughter amoebae which proceed to eat more bacteria and so on. The organism is at all times strictly aerobic, hence growth and development occur only in the presence of oxygen (Gregg 1950).

During the growth phase of the life cycle, the feeding amoebae disperse to find new nutrients. They are aided in their search for bacteria as food because they are chemotactically attracted to folic acid (Pan et al., 1972) and related compounds while growing and these compounds appear to be excreted by bacteria (Ashworth and Dee 1975).

As with a number of organisms (Witkin and Rosenberg 1970; Schaeffer et al., 1965; Haber et al., 1975) exhaustion of the food supply of D. discoideum initiates the differentiation process. The initial phase of differentiation is aggregation and occurs in response to the excretion of a chemotactic agent or acrasin by some of the amoebae. The chemical agent, acrasin, responsible for aggregation has been identified as cyclic AMP in D. discoideum (Konijn et al., 1967). After a certain period of starvation, amoebae synthesize cyclic AMP (Konijn et al., 1967; Bonner et al., 1969) and emit pulses of the cyclic nucleotide with a periodicity of a few minutes (Gerisch and Wick 1975). Cyclic AMP pulses have been shown to regulate cell differentiation to aggregation competence (Darmon et al., 1975; Gerisch et al., 1975a) chemotactic

movements (Robertson et al., 1972), the level of various enzymes (Klein and Darmon 1975; Gerisch et al., 1975b) and cell surface membrane components (Gerisch et al., 1975 a, b). The rhythmic production of cyclic AMP is due to periodic oscillation of adenylate cyclase activity (Roos et al., 1977; Klein et al., 1977). Cellular responses to cyclic AMP are mediated by surface membrane receptors (Malchow and Gerisch 1974) whose primary effect appears to be to increase the intracellular concentration of cyclic GMP and through it to promote chemotaxis and phosphodiestrase production (Van Haastert et al.,1982). When amoebae are stimulated by a pulse of cyclic AMP, they relay this pulse (Schaffer 1962; Robertson et al., 1972; Roos et al., 1975; Shaffer 1975). Relay involves a transient activation of adenylate cyclase (Roos and Gerisch 1976), a brief increase in intracellular cyclic AMP concentration, and the excretion of the newly synthesized cyclic nucleotide (Shaffer 1975; Roos et al., 1975). Such a relay mechanism permits the coordinated aggregation of a relatively large cell population. The amoebae which are now mutually attractive, gather together to form streams of amoebae which flow towards an aggregation centre to form a tissue like aggregate of cells (Bonner 1967). Cyclic AMP is also involved in differentiation (Bonner 1967). The presence of cyclic AMP at high levels during differentiation promotes the formation of stalk cells (Bonner 1970; Chia 1975; Town et al., 1976) and immunological staining of the slugs has shown that the concentration of cyclic AMP is higher in the front prestalk region of the slug than in the prespore region (Pan et al., 1974). The inference would be that cyclic AMP promotes stalk cell formation but there is also considerable evidence that cyclic AMP can promote spore formation (Town et al., 1976; Ishida 1980; Kay 1982). Cyclic AMP appear therefore to promote differentiation with other morphogens such as differentiation inducing

factor(DIF) (Town and Stanford 1979) promoting stalk cell formation (Gross et al., 1981).

After aggregation the multicellular organism forms a migrating pseudoplasmodium or slug whose movement is controlled by its apical tip. The tip can sense and respond to horizontal, unidirectional light and to temperature gradients (Raper, 1940; Bonner, et al., 1950; Poff et al., 1973; Poff and Loomis, 1973; Poff and Skokut 1977). As a pseudoplasmodium, the organism can migrate over a considerable distance in a search for more advantageous environments. This multicellular stage thus ensures distribution over a much more considerable area than could be achieved by individual cells (Bonner et al., 1950; Loomis 1975).

The duration of slug migration is influenced by a number of parameters including light and ionic strength (Newell et al., 1969). Migration is also prolonged by the presence of ammonia and it has been shown that removal of ammonia either by enzymic fixation into glutamate or by charcoal absorption, reduces the migration period. It has been suggested that the presence of ammonia prevents differentiation and maintains migration. Ammonia consequently acts as an antagonist of cyclic AMP (Schindler and Sussman 1977) and it has been demonstrated that the presence of these two mutually antagonistic molecules influences the appearance of a number of developmentally important proteins and markers (Kay 1979).

At the end of migration, the pseudoplasmodium rounds up. The anterior cells from the slug begin to form the stalk portion of the fruiting body. The other cells, which were at the back of the slug rise up the stalk and eventually become spores. When all spores are formed, fruiting body construction is finished and the differentiation phase of the life cycle is completed (Bonner 1967; Loomis 1975). When conditions alter, the spores from the fruiting body disperse, germinate and so start the process once again.

2.1. Growth conditions.

Most of the work carried out to date using D. discoideum has been to study differentiation and development. One of the reasons for the lack of study on growth is that D. discoideum uses bacteria as food for growth (Bonner 1967). The analysis of any biochemical changes occuring in the amoebae are complicated by the bacterial food supply and its digestion in the phagocytic vacuoles of the amoebae. The measurement of DNA content of the amoebae of the cellular slime mould D. discoideum Ax2 grown on bacteria as a food and in axenic medium shows twice the amount of DNA contents in bacterially grown amoebae (Leach and Ashworth 1972) and is a good example of this complicatiion.

Several attempts has been made to develop more defined media for the growth of D. discoideum. Sussman and Sussman (1967) were the first to describe such a medium for D. discoideum. The medium was complex containing liver extract and foetal calf serum in addition to inorganic salts, peptones and yeast extract. Watts and Ashworth (1970) eliminated the most complex components, foetal calf serum and liver extract, by subculturing the cells and gradually reducing the amount of these components in the medium. The medium developed by Watts and Ashworth is called axenic liquid medium (ALM). These studies did not produce a medium for the growth of D. discoideum NC4 but selected mutant strains of the organism that were able to grow in a nutritionally poor environment containing bacteriological peptone, yeast extract, glucose and inorganic salts. The mutants selected, Ax2 and Ax3, are both double mutants with mutations in linkage groups II and III of the D. discoideum chromosomes (William, Kessin and Newell 1974a,b). Strain Ax2 of D. discoideum was used in this study.

Glucose is not an essential component of the medium, since the myxamoebae grow with almost the same growth rate in the medium lacking

glucose. This medium, without added glucose, is not, however, entirely free of carbohydrates and a very small uptake of carbohydrate can be detected during growth of the cells (Ashworth and Watts 1970).

Similar modified media for the growth of D. discoideum, consisting of salts, vitamins, peptones and glucose have been described (Coccuci and Sussman, 1970, Marin,1976).

Recently a number of attempts have been made to define rather more effectively the nutritional requirements of D. discoideum and to devise a completely defined medium that would permit the growth of the organism. Watts and Guest (1975) determined vitamin requirements of D. discoideum. They found that lipoate, folate, thiamine and biotin were essential nutrients for the growth of D. discoideum but pantothenate, nicotinamide, cobalamine, ubiquinone and vitamin K were not essential.

Watts (1977) in a later study confirmed these vitamin requirements and added riboflavin to the nutritional requirements. He also developed a defined medium containing a mixture of 20 L-amino acids, B vitamins, inorganic salts, soluble starch and detergent Tween 80. Tween 80, which is not an essential nutrient, causes starch precipitation and thus stimulates the growth of the myxamoebae. The unique feature of myxamoebal growth in this defined medium is its complete dependence on the presence of a precipitate of starch.

Rahmsdorf (1977) described a defined synthetic medium for the growth of the strain AX 2 of D. discoideum. It contains 20 amino acids, 11 vitamins and glucose. In addition salts and trace elements are supplied.

Franke and Kessin (1977) also described a defined minimal medium for growth of the axenic strain Ax3 of D. discoideum. The medium contains glucose, a number of amino acids whose concentrations in the medium are optimised and a series of vitamins, minerals and trace elements.

Both these media described by Rahmsdorf and Franke and Kessin for the strains Ax2 and Ax3 of D. discoideum require the addition of sugar to produce any growth.

So for the growth of the slime mould D. discoideum, a medium containing many amino acids, vitamins, minerals and carbohydrates, usually glucose, is required and it is possible to select several different media, each of which allows growth to proceed. However, axenic liquid medium (ALM) described by Watts and Ashworth (1970) was considered the medium most suitable for this study since it allows growth both in the presence and in the absence of added glucose.

3. Metabolic changes during development.

Since development is initiated by the removal of exogenous nutrients, the cells must use their endogenous reserves to provide metabolites and energy during differentiation. Endogeneous reserves consist of carbohydrate, largely as glycogen, and protein. Considerable changes occur to both these reserves during differentiation (Gregg et al., 1954; Gregg and Bronsweig 1956; Wright and Anderson 1960; White and Sussman 1961, 1963; Sussman and Sussman 1969; Mizukami and Iwabuchi 1970).

Carbohydrate is not used as the major source of energy in this system. After growth on bacteria, amoebae, on starvation contain about 0.5 mg carbohydrate per 10^8 cells and this decreases by 20% to leave about 0.4 mg/10^8 cells by the end of differentiation (White and Sussman 1961). Although limited changes in amount occur, the carbohydrate is by no means inert and considerable changes in composition take place. During the preculmination stage, glycogen is degraded (White and Sussman 1963a) and replaced by trehalose, the storage carbohydrate of the spore (Clegg and Filosa 1961; Ceccarini and Filosa 1965), mucopolysaccharide,

a component of spore coat (White and Sussman 1963b) and cellulose, which is present in the stalk wall and in the spore coat (Ward and Wright 1965). There is a balance between breakdown and synthesis of carbohydrate with glycogen being converted to other products. It seems reasonable to conclude that cells, after growth on bacteria, contain sufficient carbohydrate to meet their synthetic needs during differentiation.

It would, however, be incorrect to say that breakdown cannot occur. After growth on bacteria there is little breakdown because the cells need the carbohydrate as such. In situations where the cells contain markedly different amount of carbohydrate from their requirements for differentiation, changes in the amount of carbohydrate do occur (Hames and Ashworth 1974). Cells with excessive amounts of carbohydrate degrade it to CO_2 while cells with inadequate amounts carry out gluconeogenesis to increase the available supply of glucose; a process shown to occur only to a very limited extent after growth on bacteria (Cleland and Coe 1968).

The situation with regard to protein is quite different. Considerable breakdown of protein occurs (Gregg et al., 1954; Gregg and Bronsweig 1956; Wright and Anderson 1960; White and Sussman 1961; Wright 1964; Cleland and Coe 1968; Sussman and Sussman 1969; Mizukani and Iwabuchi 1970) with a significant fraction of the amino acids produced being degraded to ammonia (Sussman et al., 1977) and carbon dioxide (Hames and Ashworth 1974). As a consequence, during differentiation the protein content of the cells decreases from 5.0 mg/10^8 cells to 2.5 mg/10^8 cells and the decrease is accompanied by a corresponding production of ammonia (White and Sussman 1961). The loss of protein and production of ammonia occurs continuously during differentiation (White and Sussman 1961; Hames and Ashworth 1974) and the evidence suggests that breakdown decreases

gradually until the late stages of differentiation when spores are being produced. The protein breakdown is essential for differentiation to proceed, is suggested by the inhibition of differentiation by the proteinase inhibitor chloroquine (Fong and Bonner 1979). However this inhibition is not totally specific for proteinase and other reasons for its influence on differentiation are possible (North et al., 1982).

It has, in the past, been considered that protein breakdown increased at preculmination (Gregg et al., 1950; Wright and Anderson 1960; Pong and Loomis 1971) but this conclusion was based on the assessment of carbohydrate synthesis and protein breakdown using dry weight of the multicellular mass as a reference (Gregg et al., 1954). Since dry weight/cell decreases during differentiation, erroneously high values for both these processes were obtained as differentiation proceeded. It now seems clear, if cell number rather than cell mass is used as a reference, that no net carbohydrate synthesis is needed and indeed, does not occur (Cleland and Coe 1968) and that protein breakdown does not increase at preculmination.

3.1. Breakdown of amino acids.

It is difficult to assess the breakdown of amino acids during differentiation because of the lack of work in this area. There are two ways in which metabolism can be assessed, either using indirect measurements or direct measurements of amino acid utlization.

Indirect measurements can be based on two types of data. Loss of protein and consequent production of ammonia can both give a measure of the extent of amino acid breakdown. Neither is an ideal method because they both assume that amino acids produced by protein breakdown are degraded to CO_2 and that after loss of ammonia, no intermediates build up in the cell. On the basis of these measurements the breakdown of

amino acids decreases during differentiation (based on protein) or stays constant (based on ammonia). These methods also assume that no other source of ammonia exists and the known decrease in RNA content could supply ammonia (Hames and Ashworth 1974).

Since it is assumed that protein and amino acids are responsible for the bulk of the energy production, they should also account for the bulk of the oxygen consumption. Oxygen utilization therefore provides a second assay for amino acid degradation. On this basis amino acid utilization decreases as differentiation proceeds (Liddel and Wright 1961). The major drawback to this method of estimation is that non protein material will also consume oxygen as a result of its degradation.

Observations have also been made on the level of amino acid pools in cells during differentiation (Wright and Anderson 1961; Hames and Ashworth 1974) and have been found to decrease quite rapidly early in differentiation. These data have been considered to represent the oxidation of amino acid pools early in differentiation with the later oxidation being restricted by the supply available from protein breakdown (Liddel and Wright 1961).

All of these data considered together suggests that amino acids are broken down at a rate which decreases as differentiation proceeds until at the spore stage metabolism is essentially zero.

Unfortunately this conclusion cannot be rationalised with the measurements made on amino acid pool levels of some individual amino acids and on their rates of metabolism. Measurements of the pool size of glutamate, aspartate and alanine have shown a gradual increase in pool size as differentiation proceeds (Kelly et al., 1979) and the metabolism of glutamate, measured in vivo, increases 7 fold during differentiation (Wright and Bard 1963). The pool size of amino acids in spores are also high, but metabolism does not occur until germination (Ennis 1981). There is therefore no clear picture of amino acid metabolism and its

changes during differentiation with the data that is available providing rather inconsistent hypotheses.

4. Influence of differentiated cell type on metabolism.

The slime mould produces two distinct cell types on differentiation and these differ in many ways. Two of which relate to the metabolism discussed above.

Stalk cells only produce cellulose and consequently their carbohydrate metabolism is different but more importantly, they also degrade all their protein during differentiation (Gregg et al., 1954). In both these respects they differ from spores. As differentiation proceeds, therefore protein and amino acid metabolism diverges in the two cell types and this clearly complicates further any analysis that may be made of protein and amino acid breakdown.

5. Carbohydrate metabolism.

A little breakdown but a considerable rearrangement of carbohydrate components takes place during differentiation in D. discoideum. Glycogen, for example, is degraded and converted to mucopolysaccharide (a polymer in the spore coat), trehalose (storage disaccharide in spores) and cellulose (which is present in the cell wall material of both stalk cells and spores). These changes in composition can be observed at specific times during differentiation. At the same time as these changes in carbohydrate composition are occurring, there are changes observed in the activity of a number of enzymes of carbohydrate metabolism which are essential for the production of new carbohydrate components (Loomis 1975). UDP-glucose pyrophosphorylase (the enzyme used for the synthesis of UDPG and also for the biosynthesis of mucopolysaccharide and cellulose) and trehalose 6-phosphate synthetase (an enzyme used for trehalose synthesis) for example, accummulate at specific times, before the appearance of the end product of their metabolism.

These two enzymes of carbohydrate metabolism have mechanisms for controlling their concentration in cells which may be different because neither has been entirely satisfactorily explained. The evidence for the control of the level of UDPG pyrophosphorylase is more substantial.

UDPG pyrophosphorylase is an enzyme present at low levels during the vegetative stage but which increases as much as 10 fold, during differentiation (Newell and Sussman 1969). The increase in activity is prevented by the addition of actinomycin D and cycloheximide to the cells suggesting that de novo synthesis is responsible for the increased activity and this has been confirmed by measurement of rates of protein synthesis and degradation which have shown that the enzyme is not subject to turnover during its accumulation or at any earlier period of

differentiation and that the rate of change of activity is related to the rate at which de novo synthesis of the enzyme protein occurs. (Franke and Sussman 1971). Recently two different isoenzymes of UDPG pyrophosphorylase activity have been demonstrated with the increase in activity, observed late in differentiaion, being due to the accumulation of a new, developmental specific, species of enzyme (Fishel et al., 1982). Interestingly this most recent work demonstrated that mRNA for the developmental specific isoenzyme present in vegetative cells is apparently not translated. The reason why translation does not occur is not known. In this case the change in enzyme activity depends on a change in the rate of protein synthesis but is not clear if this change can be explained solely by a change in the synthesis of mRNA.

Trehalose 6-phosphate synthetase accumulates during differentiation (Roth and Sussman 1968). The enzyme appear$4h after the initiation of development and its activity reaches a maximum in 16h and then starts to decline. Exposure of cells to the drugs, cycloheximide and actinomycin D prevent the change in activity and suggests that prior protein and RNA synthesis is required for enzyme accumulation.

However Killick and Wright (1972) have shown that the enzyme is present in early differentiation (2h starved amoebae) but is assayed at a low level because it is masked by a specific inhibitor. Precipitation of the enzyme protein by $(NH_4)_2\ SO_4$ followed by solubilization of the precipitated protein in MES-thioglycollate buffer increase the activity of the enzyme 170 fold. These studies suggest that the enzyme is controlled at activity level rather than by synthesis. No attempt had been made to measure the rate of synthesis of this enzyme to provide evidence as described for UDP-glucose pyrophosphorylase. Furthermore Sargent and Wright (1971) studied trehalose synthesis at various stages of differentiation and reported a 100 fold increase in trehalose

synthesis at late culmination (20h). This increase in synthesis, studied in vivo, begins several hours after the increase in enzyme activity, as measured in vitro, occurs. A second control system must be responsible for the lack of trehalose synthesis and could be due to unavailability of adequate supplies of substrate for the enzyme.

A large number of enzymic activities (Loomis 1975) have been shown to increase during differentiation and, if the increase is prevented by addition of cycloheximide or actinomycin D, it has been assumed that the increase is due to de novo synthesis of enzyme protein. However, in only very few cases, such as UDPG pyrophosphorylase, has a change in activity been directly related to a change in rate of protein synthesis. In some cases, such as suggested for T6P synthetase, other mechanisms can operate to control the level of activity and the operation of such mechanisms may be important.

The analysis of trehalose synthesis has also shown that the presence of enzyme activity in an in vitro experiment does not guarantee that the enzyme is active in the cell.

Another enzyme β.N-acetylglucosaminidase - although not directly involved in carbohydrate metabolism, shows yet another mechanism of control. The activity of the enzyme increases dramatically early in differentiation only if the slime mould cells have been grown in association with bacteria on an agar surface and have a low level of activity at the start of differentiation. Growth of the cells in liquid media results in a higher activity at zero time which decreases during the early stages of differentiation largely due to loss of activity by excretion from the cells (Ashworth and Quance 1972). In this case the change in activity appeared to be more related to growth conditions rather than differentiation with excretion playing a regulatory role.

Related to the low level of carbohydrate beakdown, it has been found that the activities of the enzymes of the pathways by which glucose is degraded are also low.

The glycolytic enzymes have all been shown to be present but at low specific activity. Their levels do not alter during differentiation and evidence suggests that the glycolytic pathway catabolises very little glucose (Cleland and Coe 1968). Under conditions where glucose breakdown does occur (Hames and Ashworth, 1974) enzyme activities have not been assayed.

The activities required for operation of the pentose phosphate pathway have also all been found (Edmondson and Ashworth, 1972, Thomas, 1979) but again at low activity. The activities of glucose 6-phosphate dehydrogenase and ribose 5-phosphate isomerase are higher than the others but the dehydrogenase has been shown to catalyse a non-equilibrium reaction suggesting that it is the control reaction of the pathway (Thomas 1979). It is known that oxidative pentose phosphate metabolism serves as a major source of NADPH (McLean 1958). Thus the increase in NADPH levels during development in D. discoideum could be due to enhanced activity of this pathway (Wright and Wasserman 1964). Evidence for the operation of a pentose phosphate pathway in this organism comes from the glucose labelling studies by Wright and Bloom (1961), which demonstrated that during development, the $6\text{-}^{14}CO_2/1\text{-}^{14}CO_2$ ratio from [6-^{14}C] glucose and [1-^{14}C] glucose decreases and is minimal during sorocarp construction, suggesting increased pentose phosphate pathway operation during differentiation.

It has therefore been shown that in carbohydrate metabolism a number of events take place and enzyme activities alter. In some cases such as UDPG pyrophosphorylase and T6P synthetase it is easy to see a

relationship between an enzyme activity change and the accumulation of an end product. In other cases although the mechanism of control is not clear e.g. β.N-acetylglucosaminidase, however, there is no obvious reason for, or result from, a change in the activity of an enzyme and the changes may simply be dependent on metabolic changes occurring in the cells or changes in the nature of the cells. While some of these are changes related to the development of the organism others could equally be a response to altered cellular metabolism rather than an essential component in development. Such changes are also interesting because they demonstrate the operation of metabolic controls as a result of altered metabolism.

6. Protein metabolism in D. discoideum.

Protein breakdown is important for energy production during differentiation in D. discoideum and its inhibition by Chloroquine prevents differentiation (Fong and Bonner 1979). However protein metabolism compared to that of carbohydrates, is difficult to examine because of the complexity of the components, the numerous routes of metabolism and the number of enzyme activites involved.

Furthermore, the breakdown will not necessarily result in massive changes in enzyme activites during differentiation because these activities are also present and operating during growth. Less massive changes in activity, changes in specificity and changes in the utilization of individual amino acids could occur as a result of changes in the nature of metabolism and a number of these changes have been observed in D. discoideum.

The breakdown of protein can be considered as a process that occurs in several stages and can be subdivided into particular regions.

i) proteinases and peptidases

ii) Enzymes, metabolizing specific amino acids

iii) Enzymes for the final stage of breakdown.

These regions will be considered separately.

6.1. Proteinases and peptidases:

Proteinases and peptidases are used for the breakdown of proteins and peptides to amino acids.

6.1.1. Proteinases:

Proteinase activity in D. discoideum was first reported by Sussman and Sussman (1969) who demonstrated this activity by using the chromogenic substrate azoalbumin. The activity, which was present at a constant level at all stages throughout the morphogenetic sequence, was

an acid proteinase. Weiner and Ashworth (1970) reported that this acid proteinase activity was associated with lyosomes of myxamoebae and showed that it hydrolyses haemoglobin at acid pHs with optimum activity at pH2 or below.

Proteinase activity is due to the presence of more than one enzyme. Fong and Rutherford (1978) and Fong and Bonner (1979) reported the presence of two types of proteinase. One was active on protein substrates at pH2.75 and was sensitive to DNME and partially inhibited by pepstatin. The other was active on the peptide analogue substrates α-N-benzoyl-DL- arginine-4-nitroanilide (Bz-Arg-pNA), α-N-benzoyl-DL-arginine-2-naphylamide (Bz-Arg-2NAP) as well as proteins at pH5.5 and was inhibited by chloroquine, leupeptin, iodoacetate, iodoacetamide and TLCK. On the basis of the sensitivity of these enzymes to inhibitors (Barrett and Heath 1977), it was concluded that these proteinases were similar to Cathepsin D and Cathepsin B like activities respectively. The Cathepsin B activity observed is similar to proteinase I; a proteinase activity which was reported to be present in myxamoebae of D. discoideum (Gustafson and Thon 1979) and is inhibited by chloroquine (North et al., 1982).

A fuller analysis of the spectrum of proteinase activities has been carried out by North and Harwood (1979) using electrophoresis on polyacrylamide gels containing denatured haemoglobin. They found that the extracts of amoebae showed eight bands of proteinases active below pH5 which could be divided into two groups according to inhibitor sensitivity. The four most mobile enzymes were found to be sensitive to a number of cysteine proteinase inhibitors and so considered to be responsible for the Cathepsin B activity while the other enzymes were sensitive only to mercuric chloride, a feature of Cathepsin D like proteinases.

One of these Cathepsin D like activities, proteinase E, which constitutes the major portion of the activity has been purified and shown

to have a molecular weight of 53,000 and an optimum pH activity of 2 to 3.5 (North and Whyte 1981).

So at acid pH values, proteins in D. discoideum are broken down by two families of proteinase activities which on the basis of specificity and inhibitor studies can be considered as similar to Cathepsin B and Cathepsin D like activities.

6.1.1.1. Developmental significance of proteinase activity.

North and Harwood (1979) who described eight proteinase bands in the extracts of myxamoebae also studied changes in proteinase activity during differentiation. They found that fruiting body formation is accompanied by the appearance of a new proteinase band.

At a slightly less specific level, changes in Cathepsin like proteinase activities have been observed during differentiation (Fong and Rutherford 1978). Both Cathepsin B and Cathepsin D activities decreased during differentiation but the pattern of decrease was different for the two activities. There was a gradual decrease in Cathepsin D activity during differentiation but a sharp decrease between the aggregation and migrating pseudoplasmodia stages was detected for Cathepsin B activity. These workers also studied proteinase activity differences, by using a microdissection technique with fluorometric assays to detect activity in different differentiating cell types. They found no difference in Cathepsin D activity either between prestalk and prespore cells or between stalk and spore cells at the slug and the sorocarp stage respectively. However, Cathepsin B activity was slightly higher in the prestalk cells than the prespore cells, and was 5 fold higher in stalk cells than in spores (Fong and Rutherford 1978).

So, although one new proteinase activity has been reported to be produced during differentiation, in general proteinase activity

decreases. The decrease is most marked in spore differentiation where Cathepsin B activity decreases markedly. The decrease in this activity is much less in stalk forming cells and this is not unexpected since these cells breakdown almost all their protein during differentiation.

6.1.2. Peptidases.

The study of peptidases of the cellular slime moulds is limited. Firtel and Brackenbury (1972) have described a leucine aminopeptidase in D. discoideum. Its activity increases approximately three fold during early differentiation and reaches maximum at 18 h. The increase in enzyme activity was found to be sensitive to actinomycin D and cycloheximide suggesting that prior RNA and protein synthesis are required for the increase in activity.

To date no carboxypeptidase activity has been defined, although North (1982) has reported that the hydrolysis of chromogenic peptide substrates that he observes, could be caused by carboxypeptidase activity.

So the study of proteinases and peptidases in D. discoideum has revealed a number of activities that could contribute to the breakdown of proteins. Although a number of proteinases have been described, only one peptidase has been identified. It seems likely that additional peptidases would be present since no peptidase activity in vegetative cells has yet been reported.

6.2. Enzymes metabolising specific amino acids.

A number of specific amino acid metabolising enzymes have been studied in D. discoideum.

The first amino acid metabolising enzyme shown to be dependent on development was tyrosine transaminase, studied by Pong and Loomis (1971). They demonstrated that the enzyme which is specific for L-

tyrosine, increased in activity 8 h after the initiation of development. The increase can be prevented by cycloheximide and actinomycin D suggesting de novo synthesis was required for the increase. This increase in activity, after a certain period of starvation, suggested an increase in the utilization of tyrosine by the organism during development.

Pong and Loomis (1973) have also described two distinct threonine deaminase enzymes in the cells of D. discoideum. One of these isozymes, the vegetative threonine deaminase, is sensitive to feed back inhibiton by isoleucine and, as a consequence, is considered a biosynthetic enzyme. The significance of this activity is uncertain and indeed difficult to accept, since isoleucine, whose synthesis it promotes, is an essential amino acid in this organism (Marin 1976).

The second, differentiation enzyme, is insensitive to isoleucine and catalyses the breakdown of serine and threonine and is thus involved in catabolism. While the isoleucine sensitive enzyme is synthesised in growing cells but is inactivated shortly after initiation of development, the isoleucine insensitive enzyme accumulates during the aggregation and pseudoplasmodial stages of development. As with many other enzymes, treatment with cycloheximide or actinomycin D shows that the accumulation of differentiation specific threonine deaminase requires prior protein and RNA synthesis.

Firtel and Brackenbury (1972) have demonstrated activities of a number of other amino acid metabolising enzymes in differentiating slime moulds. One of which, aminopeptidase, has already been discussed. Of the other activities only alanine transaminase, which is specific for alanine metabolism, increases in activity. The increase, which is sensitive to inhibition by cycloheximide and actynomycin D, is developmentally regulated, with the increase in activity occuring after the initiation of differentiation. Mutants, blocked at an early stage

of differentiation do not accumulate the activity. The specific activities of aspartate transaminase and glutamate dehydrogenase were also measured. The activities, however, remain constant throughout the development of D. discoideum and do not appear to be developmentally regulated.

The glutamate dehydrogenase, whose activity does not increase during differentiation, occurs as two isozymes, and both enzymes are subjected to activity controls (Langridge et al., 1977). One enzyme, which was detected in the crude mitochondrial fraction utilizes both NAD^+ and $NADP^+$ as coenzyme while the other extramitochondrial enzyme is NAD^+ specific. The activity of the mitochondrial enzyme is stimulated by both AMP and ADP but inhibited by ATP. While the activity of the extramitochondrial enzyme is found to be sensitive to modulation by a number of intermediates in carbohydrate metabolism and is inhibited by ADP, ATP, GTP and CTP. Both the enzymes are controlled at activity level.

The enzyme ornithine decarboxylase has rather unusual control. Harris and North (1982) have shown that the activity of this enzyme is controlled by the osmotic strength of the environment. Exponentially growing cells of D. discoideum showed changes in ornithine decarboxylase activity upon external osmotic perturbation. The cells, transferred to hypotonic NaCl solution, showed higher enzyme activity when compared to cells transferred to hypertonic solution. The change in activity was reversible and the response to osmotic changes occured rapidly within a few minutes. The physiological significance of this observation is not obvious.

There are therefore reports of a number of amino acid metabolising enzymes in D. discoideum. Some of these enzymes change in amount during differentiation while others do not but are controlled by regulation of activity

Of those that change in amount there is no defined time at which they all increase or decrease. If it is assumed that increase in amount occurs in response to increased metabolic requirement, there would appear to be considerable changes occuring in the nature of amino acids being degraded as differentiaion proceeds and a more systematic approach to amino acid metabolism is needed to complement the observations made during development.

6.3. Enzymes for the final stages of amino acid breakdown.

Several enzymes of central metabolism have been examined in D. discoideum. Of these only one, malic enzyme, appears to play a significant metabolic role in amino acid breakdown.

Malic enzyme which is one of the major enzymes involved in metabolising tricarboxylic acid compounds, utilizes malate as a substrate and functions in the direction of pyruvate and hence acetyl CoA formation (Kelleher et al., 1979). The activity of malic enzyme increases rapidly during the first 4 h after the onset of differentiation and the partially purified enzyme from the culmination stage of differentiation is allosteric and positively affected by aspartate and glutamate.

The enzyme is important in amino acid breakdown because the amino acids produced from Dictyostelium proteins during differentiation, oversupply TCA cycle intermediates with respect to available acetyl CoA. Malic enzyme activity compensate the balance of substrates for the TCA cycle by decarboxylating malate to pyruvate and hence to acetyl CoA. The presence of glutamate and aspartate as effectors clearly promote the enzyme in this role.

Of the other enzymes examined in central metabolism, neither lactate dehydrogenase (Firtel and Brackenbury 1972), which is not involved in amino acid metabolism, nor malate dehydrogenase (Emyanitoff and Kelly 1982), which is only involved as an enzyme of the TCA cycle, change in activity during differentiation. Although both have been

shown to be subjected to modulation of activity by inhibitors, none of these effects are physiologically significant.

So there are clearly differences in the mechanisms that control enzyme activities. Some enzymes change in activity while the others change in amount. In summary the studies carried out on enzymes at all levels of amino acid metabolism show that regulation of activity occurs but that the mechanism by which it is achieved is variable.

6.4. Other aspects of amino acid metabolism in slime moulds.

Amino acids act not only as substrates for energy production in slime moulds cells. They are also involved in protein synthesis and if present in sufficient quantities will allow growth, or at least protein synthesis to occur. They are also involved in the synthesis of polyamines and as such are important in aspects of nucleic acid metabolism. Finally, amino acids are nutrients that must be absorbed from the environment and uptake from media, especially defined media, is important. These other aspects of amino acidsand their metabolism and their effect on differentiation and on polyamine production are considered next, followed by a section describing amino acid uptake in D. discoideum.

6.5. Role of amino acids in differentiation.

Amino acids play an important role in the control of differentiation was first documented by the study of Marin (1976) who showed that cell differentiation can be inhibited by a mixture of amino acids. He reported that a part of this mixture could be substituted by glucose which probably acts as a precursor of the synthesis of 'conditionally essential' amino acids. The other essential amino acids were always required for the inhibition of development. Lee (1972),

Loomis (1975) and Marin (1976) have all reported that glucose on its own at concentrations sufficient to promote cell growth in nutritional medium, has no, or only a slight, effect on the differentiation process. Rickenberg et al., (1975) have data which contradict these results and claims that such concentrations of glucose and other metabolizable sugars, do inhibit development and apparently do so by blocking the rise in cellular and extra cellular cyclic AMP which occurs in differentiating amoebae (Bonner et al.,1969).

Darmon and Klein (1978) attempted to clarify this situation and undertook an extensive analysis of the effects of amino acids and glucose on both the aggregation and differentiation of the cells and on the biochemical events involved in cyclic AMP pulse generation. They concluded that a complete mixture of amino acids inhibits development and showed that amino acids act by preventing the increase in adenylate cyclase activity which occurs after 2 hours of starvation (Klein 1977). Concentration of glucose equivalent to that present in the growth medium (86 mM) was found to have no effect on cell differentiation. At higher concentration (220 mM) glucose did inhibit the development of aggregation competence and this inhibition was associated with low levels of cyclic AMP production by the amoebae (Rahmsdorf et al., 1976).

Thus amino acids regulate differentiation through their ability to control adenylate cyclase activity. Glucose, however, may produce the same effect but only at a very high concentration.

7. Polyamines and their metabolism in D. Discoideum.

The polyamines, putrescine (1, 4 diaminobutane), spermidine and spermine have been found in a wide variety of organisms. Putrescine is synthesised by decarboxylation of ornithine which in turn is derived from arginine. Spermidine and spermine are produced by addition of an amino propyl group, derived from methionine, to putrescine. The polyamines are therefore synthesized from amino acids.

In the cellular slime mould putrescine is the principal polyamine present with spermidine and spermine only detectable by sensitive assay methods (Turner and North 1977). 1,3 diamino propane also occurs but has only been found in two species of slime mould namely D. discoideum and D. mucoroides (North and Murray 1980). The level of 1,3 diaminopropane can be increased by the presence of the compound in the growth medium. Under these conditions it replaces putrescine (North and Turner 1977).

The metabolic role of polyamines is not well understood. They are believed to be involved in membrane structure stability and to be associated with the structure of DNA and RNA. In this latter regard the decrease in polyamine concentrations in cells as a consequence of differentiation (Turner and North 1977) appears related to the fall in nucleic acid content with differentiation (Hames and Ashworth 1974).

Although polyamines are lost during differentiation, synthesis continues (Turner and North 1977) but the cellular content of polyamines and their rate of synthesis represents a small proportion of the amino acid metabolism of the organism.

8. Transport systems in D. discoideum.

During both, vegetative and differentiation phases of the life cycle of D. discoideum the cells are able to take up a variety of materials including small molecules such as nutrients and inhibitors. The organism achieves this by pinocytosis and phagocytosis (Ryter and de Chasteller 1977). During phagocytosis extracellular particles are enclosed by the cell membrane and subsequently internalised. In pinocytosis the process is similar but the new vesicles are smaller and contain no particles (Dayson et al., 1978).

Vogel et al., (1980) have described independent binding sites on the surface of D. discoideum that are characterised by non specific and specific receptors. Non specific binding sites bind substrates like bacteria, erythrocytes and latex beads with the surface of the cells. While for a specific binding the substrate should have a glucose residue on the surface and E. coli B/r for example, is phagocytosed after binding to this specific system on the the cell surface. The phagocytic capacity of the cell increases during starvation and is maximal at 90 min of starvation but then declines as the digestive system becomes autophagic (Ryter and de Chasteller 1977). These autophagic vacuoles persist throughout development and can be found at the culmination stage (George et al., 1972) where they are more prominent in prestalk cells than in prespore cells (Maeda and Takeuchi 1969; Yamamoto et al., 1981).

Pinocytosis, where the vesicles formed are much smaller and are used for the uptake of soluble materials into the cell, has not been extensively examined in D. discoideum. The process would appear to be more important in axenic strains since these organisms obtain all nutrients in a soluble form, the growth medium, and the data on the sensitivity of axenic cells to ω-amino carboxylic acids suggests that

these cells have a greater pinocytic rate than non axenic cells (North and Williams 1978).

There is evidence that two pinocytic systems occur in D. discoideum (Rossomando et al., 1981). One of these activities occurs in cells, irrespective of the extracellular environment. A basal system has been observed, by measurement of uptake of FITC-dextran, in cells suspended in buffer and is independent of the amino acid analogue, hadacidin. The second system measured by the same assay system, is stimulated by the presence of nutrients. It is of similar magnititude to the basal system but is inhibited by hadacidin. Both systems were inhibited by the energy poison azide. This work, carried out on strain Ax3, is interesting because the nutrient stimulated pinocytosis might represent the additional pinocytic activity needed to allow axenic growth.

Several attempts have been made to examine the uptake of soluble materials into D. discoidum. The results obtained indicate that the bulk of such uptake is by pinocytosis with little evidence for direct uptake by passive diffusion and no evidence for active transport as occurs in bacteria (Murray, 1972).

The first analysis of uptake of D. discoideum was made by Lee (1972) who proposed that the uptake of inulin was achieved by pinocytosis since its uptake could be prevented by addition of 2,4 dinitrophenol and by low temperature. Amino acid uptake was less sensitive to inhibition, either by inhibitor or temperature, and it was proposed that uptake of these compounds occured by passive diffusion. Subsequent studies have not provided support for uptake by passive diffusion.

Examination of the uptake of several small molecules, similar to amino acids, has suggested that pinocytosis is part of the uptake mechanism i.e. ω-amino carboxylic acids are taken up by cells (North and Williams, 1978). Uptake is prevented by 2,4 dinitrophenol and azide,

suggesting a requirement for energy, and by cycloheximide. Although the data are not presented, similar results are reported for amino acids such as lysine suggesting that all these molecules require energy linked pinocytosis for uptake. North (1983) reported the uptake of radiolabelled solutes by D. discoideum amoebae under starvation conditions and showed that the uptake of inulin is proportional to its extracellular concentration and is energy dependent. Uptake of β-alanine, glucose, protein hydrolysate and Uracil occur at a similar rate to inulin. Pinocytic activity he reported to be present in starving cells was sensitive to developmental inhibitors ω- amino carboxylic acids chloroquine and KCl. Putrescine uptake has also been examined in some detail (Turner et al., 1979). As with amino acids putrescine uptake is energy dependent and is considerably reduced at low temperature. In the case of putrescine, uptake at low concentrations has an associated affinity constant and maximum velocity suggesting the involvement of a specific transport system in the membrane. At higher concentrations, the uptake becomes non saturable but exchange at high concentrations can occur which again suggest specific membrane exchange. In none of the cases described does any accumulation above the concentration in the medium occur. Amino acids (Lee, 1972) and ε-aminocaproic acid (North and Williams, 1978) have been shown to equilibrate and although putrescine is taken up to high concentration, only a small part of the total is likely to constitute a soluble pool (Turner et al., 1979).

Uptake of these molecules therefore occurs by pinocytosis, which is energy dependent, with membrane binding and transport systems promoting different rates of equilibration into cells from pinocytic vesicles.

9. AIMS OF THIS RESEARCH.

D. discoideum has two mutually exclusive phases in its life cycle, namely the growth or vegetative phase followed by the developmental phase (Raper 1935). To date most of the studies carried out on this organism have been on the developmental phase because it represents a very good system to analyse changes that occur during differentiation. The growth phase has been much less studied partly because the organism uses bacteria as a food source and partly because other eukaryotic cells are more easily studied and analysed.

Nevertheless, the study of the growth phase is important because there is evidence to suggest that both the rate of growth of the cells and the environment in which they have been grown are important for the subsequent developmental phase. Furthermore, there are some similarities between the growth and the developmental phase of D. discoideum. In particular during differentiation cellular protein decreases by almost 50% with the nitrogen being released as ammonia (Wright and Anderson 1960, White and Sussman 1961;). Clearly amino acids derived from protein provide a major energy source during differentiation. During growth, amino acids, although in this case derived from bacterial protein or from growth medium components, provide the major energy supply, with again the release of a considerable quantity of ammonia (Ashworth and Watts 1970).

Therefore a study of cellular metabolism, particularly amino acid metabolism, during growth may be useful in helping to understand aspects of amino acid and protein metabolism that occur during differentiation and it was intended in this study to look at the utilization of amino acids during growth under different growth conditions, and then to examine the mechanisms which regulate amino acid utilization and the amount and activity of amino acid metabolising enzymes in the cells.

METHODS

1. MATERIALS

1.1. Commercial sources

All reagents were the best grade which could be obtained commercially. With the exceptions of the compounds listed below, reagents were obtained from British Drug Houses Ltd., Poole, Dorset BH12 4NN.

Bovine serum albumin (fraction V): Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex BN22 9AG.

Glutamate dehydrogenase, NAD, NADP, NADH, dithiothreitol (Cleland's reagent): Boehringer Corp., Ltd., Lewes, East Sussex BN 7 1LG.

Bactopeptone, Agar, Yeast extract: Difco Laboratories, Detroit, Michigan, U.S.A.

Lemco beef extract: Oxoid, Oxo Ltd., London.

All amino acids(L-form): SAS & Sons Ltd; London WC1B 4DF.

tris (hydroxymethyl) methylamine (Tris), o -aminobenzaldehyde, pyruvate, 2-(N-morpholino) ethane sulphonic acid, pyridoxal phosphate: Sigma Chemical Co; Poole, Dorset BH17 7NH.

L[U-C^{14}]Arginine (344 mCi/mmol), L[U-C^{14}] Lysine (336 mCi/mmol), L[U-C^{14}] Threonine (228 m Ci/mmol), L[U-C^{14}] Tyrosine (509 mCi/mmol), L[U-C^{14}] Glutamic acid (285 mCi/mmol), D[U-C^{14}] Glucose (268 mCi/mmol): Radiochemical Centre Amersham (Amersham, Buckinghamshire, England, HP7 9LL).

2. Organism

The organism used throughout this study was Dictyostelium discoideum Ax2 (Watts and Ashworth 1972). The Strain Ax2 (ATCC-24397) was isolated by J.M. Ashworth as a derivative of the wild type cellular slime mould Dictyostelium discoideum NC4 (ATCC 24697) (Raper 1935). The strain Ax2 is characterised by its ability to grow in nutrient media in the absence of bacteria.

Escherichia coli B/r was used as a food bacterium for the cellular slime mould and was obtained from J.T. Bonner.

3. Maintenance of stock cultures

3.1. Dictyostelium discoideum Ax2: Stock cultures of D. discoideum were maintained at 4°C as fruiting bodies on the surface of nutrient agar plates. Fresh cultures were obtained by transferring spores from a stock culture plate to a fresh nutrient agar plate, adding a portion of a nutrient broth culture of Escherichia coli B/r and incubating at 23°C. After approximately 40 h the slime mould cultures became starved, aggregated and formed fruiting bodies. After an additional 2 days fruiting body formation was complete and the plates were stored at 4°C until used.

For longer term storage, spores were kept in silica gel as described by Reinhardt (1966). At intervals a sample from the silica gel stock was used to prepare fresh stock culture plates of the organism.

3.2. Escherichia coli B/r: E. coli B/r was kept at 4°C in cooked meat medium. At intervals the cooked meat cultures were used to inoculate 10 ml samples of nutrient broth medium in 25 ml Universal bottles and grown at 23°C for 48 h. Cultures were stored at 4°C until required for preparation of slime mould stock cultures.

4. Preparation of media and buffers

The following growth media and solutions were used routinely for maintenance and growth of organisms and preparation of washed cell suspensions.

4.1. Nutrient Agar: Nutrient agar was prepared as described by Bonner (1967). It contains

Bactopeptone	10 g
Glucose	10 g
Na_2HPO_4	0.96 g
KH_2PO_4	1.45 g
Agar	20 g

All components except agar were dissolved in distilled water, adjusted to pH 6.4 and made to 1 litre using distilled water. Agar was then added and the medium sterilised by autoclaving at 15 PSI. The medium was dispensed into 13 cm diameter plastic petri dishes with approximately 25 ml/dish. After the agar had solidified the plates were stored at $4^{o}C$ until required.

4.2. Nutrient broth

Nutrient broth was prepared from Oxoid dehydrated granules. It contains in 1 litre of distilled water

Lemco beef extract	1 g
Yeast extract	2 g
Peptone	5 g
Sodium chloride	5 g
final pH 7.4	

Nutrient broth was dispensed 10 ml into universal bottles, sterilised by autoclaving at 15 PSI and stored at room temperature.

4.3. Cooked meat medium

Cooked meat medium was prepared from Oxoid dehydrated material. One tablet was soaked in 10 ml distilled water for 15 min. in a universal container and sterilised by autoclaving at 15 PSI. The medium was stored at room temperature until used.

4.4. Axenic liquid medium (ALM)

Axenic liquid medium was prepared as described by Watts and Ashworth (1970). It contains

Bactopeptone	14.3 g
Yeast extract	6.16 g
Na_2HPO_4	0.52 g
KH_2PO_4	0.48 g

pH was adjusted as required using NaOH and distilled water added to make to 1 litre. The medium was dispensed as required and sterilised by autoclaving at 5 PSI.

As described in the literature ALM is prepared at pH6.7 and the medium prepared at pH 6.7 was used for a lot of early experiments described in this thesis. We found however that, when prepared at this pH value, the pH of the medium fell as a consequence of autoclaving. The decrease in pH was especially noticable when glucose was added to the medium. As a result of this observation and observations reported in this thesis, supplements to ALM were added after sterilisation. In addition, the pH of ALM was adjusted to pH6.2 before autoclaving for media used for preparation of cells for washed cell studies and enzyme activity measurements.

For the media where supplements were added after sterilisation, the volume was made to 900 ml instead of 1 litre by adding distilled water. This medium which was $10/_9$ ALM was dispensed into flasks.

The supplements were prepared in distilled water at 10 times the required final concentration, adjusted to pH6.2 if necessary and sterilised. Supplements were then added to $10/_9$ ALM to the required concentration. If no supplementation was required, sterilised distilled water was added to bring the medium to the correct final volume.

In this thesis supplemented ALM is provided with a suffix. For glucose as a supplement ALM_g is used. Other supplemented media are provided with a full suffix.

4.5. Phosphate buffer

7.19 mM phosphate buffer was used throughout the work to wash cells. It was prepared as

Na_2HPO_4	0.52 g
KH_2PO_4	0.48 g

adjusted to pH6.2 with NaOH and made to 1 litre with distilled water.

5. Sterilisation

Sterilisation of the media was carried out using one of the two procedures.

5.1. Autoclaving

Heat stable media were sterilised in a pressure chamber (Manlove Alliott, Nottingham, England) using steam supplied by a speedlec-electrode boiler (Bastian and Allen, Harrow, England). The conditions for sterilisation had been determined using thermocouples in the solutions (Fewson, unpublished results). Both the pressure (5 and 15 PSI) and the time of autoclaving depended on the nature and volume of the solutions being sterilised. On all occasions, the effectiveness of sterilisation was checked using Browne steriliser control tubes-type one, black spot (Brown Ltd., Leicester, England).

5.2. Filtration

Media or media components, which could not be safely sterilised by heat were sterilised by filtration using sterifil filter holders of 250 ml capacity fitted with a 0.22 μ pore size millipore filter (Millipore Corp. Massachussetts, U.S.A.). Once assembled the unit was sterilised by autoclaving at 15PSI. After filtration solutions were aseptically transferred to sterile bottles.

6. Measurement of cell density

The density of D. discoideum cells was determined using a haemocytometer (improved Neubauer pattern) supplied by Hawksley U.K. Ltd. If posible at least 200 cells were counted to reduce the counting error. At densities below 10^5 cells/ml it was not always possible to count enough cells to fulfil these criteria of low error levels and consequently cell density measurements below 10^5/ml are less reliable than higher values.

7. Measurement of pH

The pH of solutions and media were measured under several different conditions and different equipment was used. At all times pH values were calibrated against solutions of known pH value (BDH chemicals Ltd.).

7.1. Media and buffer preparation

The pH of these solutions was measured using a direct reading pH meter (Model 7010; E.I.L. Ltd., Cumbernauld, Glasgow G67 1AG) connected to a combination glass/reference electrode (type 224; Probion Ltd., Glenrothes, Fife KY6 3AE). pH adjustment was carried out using stock solutions of sodium hydroxide or hydrochloric acid.

7.2. pH value of culture samples

The pH values of small volumes ($<$2ml) were measured with a pH meter (Model 2320; E.I.L. Ltd.,) fitted with an assembly containing a microelectrode (Type 257; Probion Ltd.).

7.3. pH control equipment

Experiments with pH control were monitored for pH value by using an autoclaveable glass combination electrode (Probion, type 703) which was inserted into the growth medium before sterilisation. The electrode was connected to a pH controller (Model 9150 E.I.L Chertsy, El. Surrey, England) which caused the addition of alkali or acid to the growth medium if the pH value fell below or rose above a preset value. Since pH of the media, in these experiments always rose with growth, to achieve pH control 0.2 N HCl was added by switching on a Watson Marlow Flow Inducer (MHRE 200) fitted with 0.8 μ inner diameter silicon tubing. A reservoir containing 0.2N HCl was prepared and connected to the growth flask using the same piece of 0.8 μ silicone tubing before sterilisation. A set up of the apparatus is provided in Figure 2.

To ensure that the pH electrode and controller did not drift during a growth experiment, the pH of a sample of the medium was checked at intervals by comparing its value with that of a buffer of known pH value.

8. Preparation of inocula

Inocula of D. discoideum cells for growth in ALM were always prepared from spores by growth through 2 passages of ALM. The pH, to which ALM was adjusted before sterilisation, is specified in the figure legends.

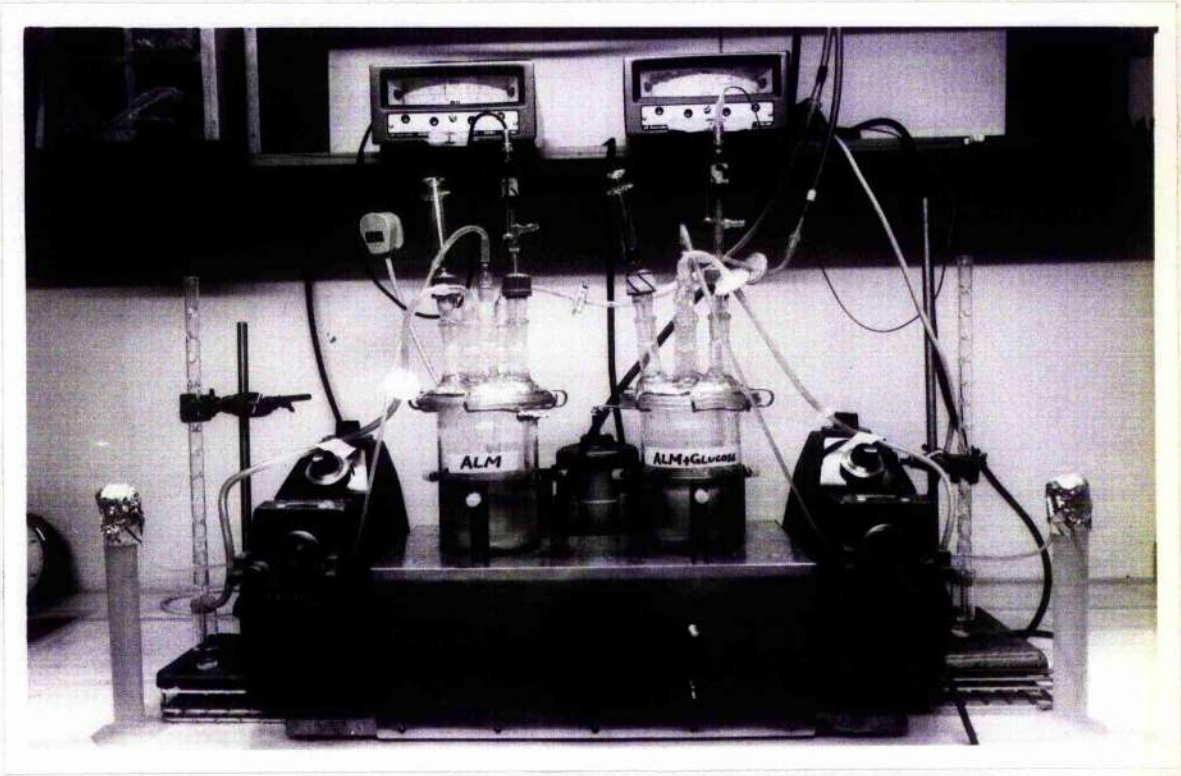


Figure 2: pH controlled experiment set up

For the first passage, spores from a petri dish culture, stored at $4^{o}C$, were transferred aseptically to a 250 ml erlenmeyer flask containing 50 ml of sterilised ALM. The flask was placed on an orbital shaker at $23^{o}C$.

After about 10 days, when the cell density had reached 10^{6} cells/ml and the pH was still less than 7, 1 ml from the first passage was transferred to a similar flask containing the same medium. After a further 3 days the cells had again grown to a density of 10^{6} cells/ml and were used as inocula for growth experiments.

Similarly cells were grown on supplemented ALM before inoculation into supplemented media. In those media in which the generation time was longer than on ALM, the cell density of inocula was maintained and the time scale altered as necessary.

To monitor growth of these cultures, samples were taken at intervals to measure cell density and culture pH as described previously.

9. Growth conditions in liquid culture

Cells were grown in liquid cultures in several different vessels depending on the volume of culture being used. All growth was carried out in a culture room maintained at $23^{o}C$ with cultures inoculated at a density of $5x10^{4}$ cells/ml.

9.1. Cultures of small volume

Cultures of 50 ml liquid medium were grown in 250 ml Erlenmeyer flasks fitted with polystyrene bungs. The flasks were grown on a rotary shaker (Mk,V.Orbital Shaker, L.H. Engineering Co.Ltd., Stoke Poges) moving at about 180 oscillations min^{-1} with no aeration other than diffusion through the polystyrene bung.

9.2. Cultures of large volume

Cultures of 100 ml or greater were grown using the apparatus described by Harvey et al., (1968). Volumes of 100 ml were grown in 500 ml Erlenmeyer flasks and those of 1 litre up to 1.5 litres in 2 litre flat bottomed side arm flasks. Initially these cultures were aerated at 100ml/min. from a compressed air supply but aeration was found to be unnecessary and diffusion through polystyrene bungs was sufficient to permit full growth rate to high cell densities. Samples were removed aseptically from these cultures for the measurement of growth, pH and changes in metabolite concentrations.

9.3 pH controlled cultures

Experiments which required pH control were carried out in 1 litre culture vessels (Jobling Glass) fitted with 5-socket flat flange lids (Figure 2). Each flask contained 500 ml of medium and was connected, using 0.8 μ diameter silicon tubing, to a reservoir containing 0.2 N HCl before autoclaving. The culture was stirred using the apparatus of Harvey et al., (1968) but in this case, forced aeration from a source of compressed air, at 100 ml/min. was provided. Air was filtered before entry and on exit using 37 mm Bacterial Air Vent No. 4210 filters (Gelman Filtration Division). pH was maintained as described in section 6.3.

Inoculation of the medium and sampling of the growing culture were both achieved by using a sterile syringe connected to a needle which passed through a suba-seal stopper (Gallenkamp, East Kilbride) and which ended below the level of medium in the flask. Samples were taken at intervals during growth to monitor the growth and metabolism of the culture.

9.4. Processing of samples from growing cultures

Samples removed from cultures were used to measure culture cell density and pH immediately. Samples for other estimations were treated and stored as follows:

9.4.1. Amino acid analaysis

Samples were stored at -20°C after centrifugation at 12,000 g for 10 min. and 4°C in an MSE high speed 18 centrifuge to remove cells. No chemical treatment was carried out before storage.

9.4.2 Ammonia estimation

The treatment of samples for ammonia estimations depended on the method by which the analyses were to be performed.

9.4.2.1. Chemical method

A 2 ml sample from the culture was chilled, and 2 ml of 0.1N hydrochloric acid added. After centrifugation at 12,000 g for 10 min. at 4°C in an MSE high speed 18 centrifuge, the supernatant was decanted and stored at -20°C.

9.4.2.2. Enzymic method

When this method was used, the sample was kept on ice, an equal volume of chilled 10% trichloroacetic acid added and the mixture centrifuged at 12,000 g for 10 min at 4°C in an MSE high speed 18 centrifuge. A 3 ml sample of supernatant was taken and neutralised (pH 7-7.5) by adding 3 ml 2M potassium bicarbonate and centrifuged again as described above. The sample was then stored at -20°C until assayed.

9.4.3 Protein estimation

Estimation of cellular protein was influenced by components of the growth medium and these were removed before storage. A 2ml portion of the culture was centrifuged at 12,000 g for 10 min. at 4°C in an MSE high speed 18 centrifuge. The pellet was resuspended in 7.19 mM phosphate buffer pH6.2 and centrifuged once more as described above. The pellet of the cells was finally resuspended in 2 ml 7.19 mM phosphate buffer pH6.2 and stored at -20°C until assayed.

10. Experiments using washed cell suspensions

10.1 Preparation of washed cell suspensions

To prepare washed cell suspensions, the cells of D. discoideum were grown at 23°C as described in section 9.2, in 2 litre flat bottomed flasks containing 1.5 litre medium (either ALM or ALMg adjusted to pH6.2). The cells were grown until they reached a density of 10^6 cells/ml. At this density the pH had increased to 6.6 - 6.7 after growth on ALM and to 6.4 - 6.5 after growth in the glucose supplemented medium. The cells were then harvested by centrifugation at 900 g and 4°C for 15 min, washed twice by resuspension in 7.19 mM phosphate buffer pH6.2 and centrifugation at 900 g and 4°C for 15 min. Finally, the cells were resuspended to ten times the required cell density in 7.19 mM phosphate buffer pH6.2 and stored on ice until required. An aliquot was removed for protein estimation and stored at -20°C until assayed.

10.2. Measurement of ammonia production

10.2.1. Incubation conditions

Incubations were carried out in 50 ml Erlenmeyer flasks with polystyrene bung closures. Stock solutions of all the components to be

added to the incubations were prepared individually at high concentration in 7.19 mM phosphate buffer pH6.2, added to the incubation flasks and made to a total volume of 9 ml with 7.19 mM phosphate buffer pH6.2. Then 1 ml of cell suspension was added to each flask and the flasks incubated at 23°C on an orbital shaker (Luckham Ltd.) at 150 rpm.

10.2.2 Samples for ammonia estimation

Samples were taken at intervals over 100 min. for ammonia estimation. The samples of volume 0.5 ml, were added to 0.5 ml 0.1N HCl to stop further ammonia production and to ensure fixation of the ammonia already present, mixed and stored on ice. A batch of samples was then centrifuged at 2000 g and room temperature for 2 min. in an Eppendorf centrifuge 3200 in 2 ml plastic centrifuge tubes. The supernatants were stored in plastic vials at -20°C until assayed for ammonia using the chemical method described in Section 11.1.1.1.

10.3. Measurement of oxygen uptake

10.3.1. Apparatus used

Two Clark type oxygen electrodes (Rank Brothers, Bottisham, Cambridge CB5 9DA) were used to measure oxygen utilisation of the cells. The electrodes were maintained at 27°C by a circotherm unit (Shandon-London) which circulated water from an adjacent water bath through the elctrode jackets. The electrodes produce a millivolt output dependent on the activity of oxygen in the solution and this out-put was measured on a Servoscribe chart recorder (Smith Industries, Ltd. Wembley, England).

10.3.2. Estimation method

Phosphate buffer (7.19 mM, pH6.2) was maintained at 27°C in the

water bath, and was air saturated by stirring the solution vigorously with a magnetic stirring bar and magnetic drive assembly using the apparatus described by Harvey et al., (1968).

The phosphate buffer and cell suspension were pipetted into the incubation vessel and the perspex disc carefully screwed down into position so that no air bubbles were trapped above the incubation mixture. The endogeneous rate of oxygen uptake was recorded for at least 5 min. Additions were then made through the small hole in the perspex disc using a Hamilton syringe to make a total volume of 3 ml and the rate of oxygen consumption measured as a change in the millivolt output of the electrode assembly.

10.4 Measurement of ^{14}C - Carbon dioxide production

10.4.1. Incubation conditions

Incubations were carried out in 10 ml Erlenmeyer flasks fitted with a central well using a modification of the procedure of Wright and Bard (1963).

The central well contained 150 µl 14N sodium hydroxide. With the exception of the compound whose metabolism was being measured, stock solutions of all the components to be added to the incubation were prepared at high concentration in 7.19 mM phosphate buffer pH6.2 and added in a total volume of 100µl, to the flask, 100µl of substrate (0.25 uCi), at 10 times the required final concentration, in 7.19 mM phosphate buffer pH6.2, was added, followed by 0.8ml of washed cell suspension in 7.19 mM phosphate buffer pH6.2 to give a final volume of 1.0 ml with a cell density of 10^7 cells/flask. The flasks were then sealed with subaseal stoppers and incubated on an orbital shaker (Luckham Ltd.) at 150 rpm at 23°C for 90 minutes. After 90 minutes the reaction was terminated by the injection of 250 µl of 5N sulphuric acid

through the subaseal stopper. The flasks were replaced on the shaker for an additional 90 minutes to ensure that any bicarbonate in the acidified incubation was released and trapped in the alkali in the central well.

10.4.2. Measurement of radioactivity

After incubation, the ^{14}C-carbon dioxide produced was assayed in duplicate by transferring 50 µl aliquots of the alkali from the central well of the flask into plastic scintillation vials containing 10 ml of liquid scintillation fluid (1 litre toluene, 300 ml ethanol, 5 g 2,4 diphenyl oxazole (POP)). Similar aliquots were taken from the acidified incubation mixture to measure the total radioactivity still present. Samples were counted on an SL 30 Liquid Scintillation Spectrometer (Intertechnique, 78370 Plaisir, France), and the conversion of substrate to carbon dioxide calculated.

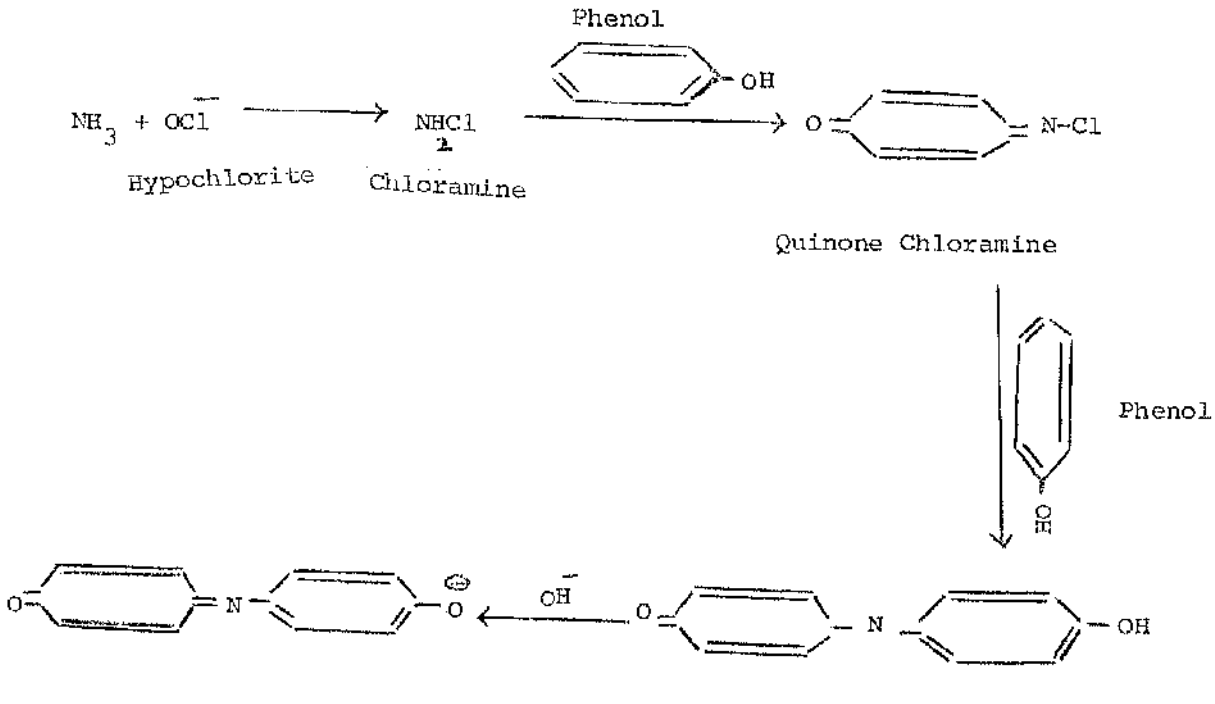
Since all the calculations of substrate utilization were dependent on ratio measurments, cpm values were used directly without correction for efficiency. To ensure the efficiency of counting was constant under the conditions used for counting, vials were set up and counted both before and after the addition of either 14N NaOH or a mixture of buffer and 5N H_2SO_4. No significant effect of alkali or acid addition was observed when up to 100 µl of alkali or acid was added.

11. Analytical techniques and their quantification

11.1. Ammonia estimation

11.1.1. Chemical method

The phenol/hypochlorite method described by Henry (1966) was used to estimate ammonia in the samples. In this method ammonia and hypochlorite react to form chloramine which combines with 2 moles of phenol to form yellow indophenol. The reaction is promoted by the presence of the catalyst sodium nitroprusside. In alkaline solution indophenol dissociates to produce a blue colour which is measured at 630nm.



dissociated in alkaline medium (blue)

associated Indophenol (yellow)

11.1.1.1. Assay procedure

Two reagents were prepared as described below

Solution I

Phenol	10 g
Sodium nitroprusside	0.05 g

dissolved in 1 litre distilled water

Solution II

NaOH	5 g
Sodium hypochlorite	0.42 g

dissolved in 1 litre distilled water, and stored at $4^{o}C$.

Samples were removed from the freezer, thawed, mixed and dilutions made in distilled water. Then to 0.1 ml sample, 2.5 ml solution I was added, mixed thoroughly and 2.5 ml of solution II added. The assay was again mixed thoroughly, incubated at $27^{o}C$ for 40 min. and read at 630nm on SP-30 spectrophotometer (Pye Unicam Ltd., Cambridge, England).

A standard curve was constructed using NH_4Cl, covering the range of 0-200nmol/assay.

11.1.1.2. Interference with ammonia estimation

Some of the components present in the extracts in which ammonia was being measured, were found to alter the optical density change produced by the ammonia standards (Table 1).

The effect varied depending on the component present and was particularly marked if ammonia concentrations in ALM were being measured. To compensate for this effect standard curves were made in the presence of all the components that were present in the incubation or growth medium. For example, to measure ammonia concentration during

growth in ALM, a standard curve for ammonia was prepared in ALM. In this way any component which interfered with the assay also interfered with the standards.

On a few occasions internal compensation for standards was not carried out and in these cases the measured ammonia concentration was corrected arithmetically for any alterations caused by components of the incubation mixture.

11.1.2. Enzymic method

The method of Kun and Kearney (1974) was also used to estimate ammonia in some samples. This assay depends on the reductive amination of 2-oxoglutarate catalyzed by the enzyme glutamate dehydrogenase.

$$NADH + NH_4 + \text{2-Oxoglutarate} \xrightarrow{GLDH} \text{L-Glutamate} + NAD^+ + H_2O.$$

The decrease of NADH, as measured by the change of extinction at 340nm, is a measure of the amount of ammonia present.

11.1.2.1. Assay procedure

The assay were carried out using an assay cocktail containing

tris-buffer pH8	200 mM
2-Oxoglutarate	20 mM
NADH	0.64 mM
glutamate dehydrogenase	18u/ml

Samples were removed from the freezer, thawed, mixed and appropriate dilutions made in phosphate buffer. 500 µl of diluted sample was taken and 500 µl of the 'assay cocktail' described above added. The assay was mixed well and incubated at 27°C for 30 min.

Table 1. Interference of components with ammonia estimation

2.0 mM ammonium chloride and components (amino acids 5 mM; Glucose, Pyruvate and 2-oxoglutarate 20 mM) prepared in 7.19 mM phosphate buffer pH6.2 were incubated with solution I and solution II (methods 11.1.1.1.) and their optical density (OD) measured at 630 nm on SP-30 spectrophotometer. The components were also incubated without the standard and optical density ($\acute{OD}$) measured. $\triangle$OD was calculated

$$\triangle OD = OD - \acute{OD}$$

Components added to the assay	ΔOD from 200 nmol ammonia	% of control
none	1.49	100
glucose	1.50	100.6
pyruvate	1.04	69.8
2-oxoglutarate	1.32	88.6
arginine	1.49	100
tyrosine	1.37	91.9
threonine	1.39	93.3
ALM	0.639	42.9

Samples were read at 340 nm on an SP-800 or SP-30 spectrophotometer. A standard curve covering the range 0——150 nmol/assay was constructed by using ammonium chloride as standard.

11.2 Calibration of oxygen electrode and oxygen uptake measurements

The procedure of Robinson and Cooper (1970) was used to calibrate the electrodes. In this method oxygen is consumed in the stoichiometric reoxidation of phenazine methosulphate (PMS) after it has been reduced by NADH.

$$NADH + H^+ + PMS \longrightarrow NAD^+ + PMSH_2$$

$$PMSH_2 + O_2 \longrightarrow PMS + H_2O_2$$

Hydrogen peroxide formed in the reaction is broken down by including catalase in the reaction mixture

$$H_2O_2 \longrightarrow H_2O + {}^1/_2O_2$$

The net reaction then becomes

$$NADH + H^+ + {}^1/_2O_2 \longrightarrow NAD^+ + H_2O$$

The calibration is carried out by adding aliquots of an NADH solution, whose concentration has been accurately measured spectrophotometrically, to an air saturated assay and measuring the oxygen consumed.

The total oxygen in aqueous solution was also calculated using oxygen solubility at the temperature of assay and found to agree with the measured dissolved oxygen. A value of 248 nmol oxygen/ml was found for an oxygen saturation solution at $27^{\circ}C$.

11.3 Scintillation spectrometry and calculation of substrate utilization

The utilization of substrate was assessed as a fraction of the total radioactivity added to the flask by measuring the radioactivity that was converted to carbon dioxide and consequently was trapped in sodium hydroxide in the central well.

To measure $^{14}CO_2$ produced 2 samples each of 50 µls were taken from the central well and counted. The total radioactivity (X) in central well was assessed as

$$X = \frac{150 \times \text{cpm from central well}}{50} \text{ cpm}$$

Similarly 2 x 50µl samples from the acidified compartment were counted and total radioactivity (Y) in the main compartment of the flask calculated using

$$Y = \frac{1250 \times \text{cpm from acidified material}}{50} \text{ cpm}$$

% metabolism of substrate to carbon dioxide

$$= \frac{X}{X+Y} \times 100\%$$

Since this percentage metabolism occurred during a defined assay time from a known initial substrate concentration, the actual rate of substrate utilization (Z) is given by

$$Z = \left(\frac{X}{X+Y}\right) \times \text{Substrate concentration } (\mu M) \times \frac{60}{\text{Time of assay (min)}} \text{ nmol/h/flask}$$

In calculating substrate utilization in this way two assumptions are made namely, that substrate molecules are broken down completely to carbon dioxide with no accumulation of intermediates, and that the efficiency of counting is independent of the presence of acid or alkali in the sample being counted

11.4 Protein estimation

Protein was estimated by a modification to the method of Lowry et al., (1951).

Samples were thawed, mixed and diluted in 7.19 mM phosphate buffer pH6.2. Portions of diluted samples and bovine serum albumin standards were mixed with an equal volume of 1N NaOH and left to digest overnight at 37°C. The following day, 0.2ml aliquots of the digest were taken, 1 ml of alkaline copper solution (0.01% $CuSO_4 . 5H_2O$ and 0.02% sodium potassium tartrate in 2% sodium carbonate) added, followed after 10 min. at room temperature, by 0.1 ml of 1N Folin reagent. The assays were incubated for a further 30 min. at room temperature, then read at 750 nm on SP-30 spectrophotometer. A standard curve was constructed using bovine serum albumin covering the range 0—50 µg/assay.

11.5. Amino acid analysis

Amino acid analysis was carried out using an LKB 4400 amino acid analyser.

11.5.1. Method

Samples, taken during growth, for amino acid analysis were thawed, thoroughly mixed and an aliquot of 250 µl taken, added to 250 µl 10 mM norleucine (as internal standard) and diluted to 5 ml with distilled water.

250 µl of each of the diluted, norleucine containing samples, were mixed with 250µl aristar HCl in pyrex tube. The tube was drawn, evacuated to remove O_2 and sealed under vacuum. Samples were hydrolysed by placing the tubes in a heating block (Griffin, hot block unit 3) at 110°C for 6 h and were then chilled before opening.

Hydrochloric acid was removed from the samples by lyophylisation over sodium hydroxide and sulphuric acid, 1.25 ml distilled water added to the dried samples and lyophylisation repeated. Samples were then stored dry at -20°C until needed.

For assay, samples were thawed, dissolved in 1.25 ml 0.2 N sodium acetate buffer pH2.2 and 50 µl analysed on the LKB 4400 amino acid analyser using a 3 sodium acetate buffer system described by LKB (LKB protein chemistry note No.10).

11.5.2. Quantitation of analyses

Analyses were quantified by comparing data from samples, with data obtained from standard amino acid mixtures (LKB product supplemented with norleucine). Standards were run with every 3 samples and average values, from all standards, used to assess the quantities of each amino acid present in the individual samples.

Three types of control were considered:

a) a norleucine standard which was not hydrolysed at 110°C for 6 h, was compared with a hydrolysed sample in both the presence and absence of 86 mM glucose.

b) a standard amino acid mixture was also not hydrolysed and compared with a hydrolysed sample in the presence and absence of glucose.

c) Samples of culture medium were hydrolysed for varying times up to 24 h in 6 N HCl at 110°C.

11.6. Measurement of osmotic strength

Osmotic strength of solutions were measured using an osmometer (Advanced Osmometer 3D, Advanced Instrument Inc.) which depended on the depression of the freezing point of aqueous solutions.

The machine was calibrated using a standard sodium chloride solution and all measurments were related to the standard solution. As expected osmolarity was related to concentration, with ionisable compounds giving higher osmotic values than non ionisable compounds, when prepared at the same concentrations (Figure 3).

12. Measurement of enzyme activity

12.1. Preparation of cell extracts

Enzyme assays were carried out on extracts prepared from logarithmically growing cells of D. discoideum. In all cases cells were grown in 2 litre flat bottomed flasks containing 1.5 litres of culture medium as described in methods 9.1. Cells were harvested and washed as for preparation of washed cell suspensions (see methods 10.1) except that the final resuspension of the cells was in the medium appropriate for the enzyme being assayed (Table 2).

To prepare extracts, 5 ml of a cell suspension was subjected to ultrasonic irradiation using a Dawe Soniprobe (Type 1130A; Dawe Instruments Ltd., London) at $0^{\circ}C$. Disruption was effected at a current of 3A for 3 periods of 15 seconds alternated with cooling periods of 30 seconds. The homogenate was then checked microscopically to ensure disruption was complete and an aliquot removed for protein estimation (Section 10.4). The remainder of the homogenate was centrifuged at 12,000 g for 15 min. at $4^{\circ}C$ in an MSE high speed 18 centrifuge to remove debris and the supernatant stored on ice until used to measure enzyme activity in the extract.

Table 2: Media for the preparation of extracts of D. discoideum for enzyme assays

Enzymes	Extract preparation media
Ornithine aminotransferase (ECNo.2.6.1.13)	100 mM Tris HCl pH8.0 (Jenkins and Tsai 1970)
Threonine deaminase (ECNo.4.2.1.16)	20 µg/ml Pyridoxal phosphate (Loomis 1970)
Tyrosine transaminase (ECNo.2.6.1.5)	Distilled water (Diamondstone 1966)
Glutamate dehydrogenase (ECNo.1.4.1.2)	100 mM Tris HCl pH8.0 (Langridge et al., 1977)
Malic enzyme (ECNo.1.1.1.40)	50 mM Tris HCl pH8.1 (Kelleher et al., 1979)
Lysine dependent oxygen uptake activity	100 mM Tris acetate pH8.0 (Nakazana 1971)

Figure 3: Effect of additives on osmolarity

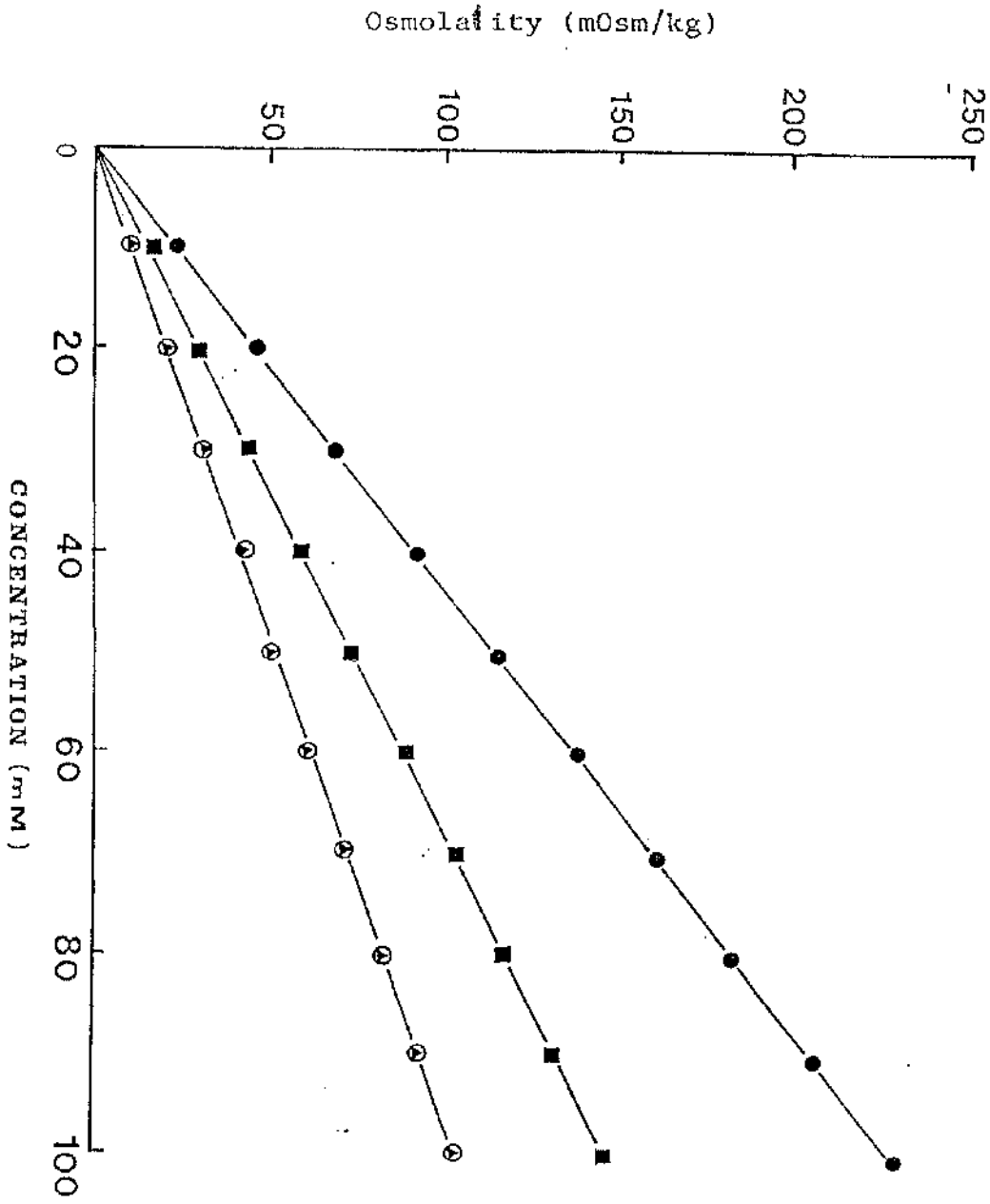
Dilutions of standard solutions of additives were prepared in phosphate buffer pH6.2. Osmotic strength of the dilutions was measured using osmometer (methods 11.6). After subtracting osmotic strength of the dilutions buffer the values were plotted against concentrations.

○ : Glucose

▲ : Galactose

● : Phosphate

□ : Pyruvate


Osmolality (mOsm/kg)
50
100
150
200
250
0
20
40
60
80
100
CONCENTRATION (mM)

12.2. Ornithine amino transferase

Ornithine aminotransferase activity was determined as described by Jenkins and Tsai (1970).

L-Ornithine + 2-oxoglutarate ⟶ L-glutamic semialdehyde + glutamic acid

Transamination of the amino group from ornithine to 2-oxoglutarate proceeds virtually to completion due to the spontaneous cyclization of the product, L-glutamic semialdehyde to Δ^1-pyrroline 5-carboxylate. This latter compound reacts with o-aminobenzaldehyde to form a deep yellow dehydroquinazolium derivitive, which is measured at 440 nm.

12.2.1. Assay condition

The standard Ornithine aminotransferase assay contained, in a final volume of 1.0 ml, 10 mM Sodium pyrophosphate, 20 mM L-Ornithine-HCl, 3.5 mM 2-oxoglutaric acid, 0.8 µg/ml pyridoxal phosphate; adjusted pH 8.0.

The reaction was initiated by addition of extract, routinely 400 µl, prepared from 10^8 cells/ml in 100 mM Tris-HCl pH8, and carried out at 37°C for 60 minutes. The enzyme reaction was then stopped by addition of 1 ml of 10% (w/v) trichloroacetic acid, followed by the addition of 2 ml of 5% (w/v) o-amino benzaldehyde solution in 95% (v/v) ethanol. The colour was developed by incubation for 3-5 minutes at 37°C. After centrifugation at 12,000 g for 15 minutes, the absorbance was determined at 440 nm on SP-30 spectrophotometer and the number of enzyme units present calculated by assuming a molar extinction coefficient for the dehydroquinazolium derivative of 2.7×10^3 M^{-1} cm^{-1} (Jenkins and Tsai 1970). Enzyme specific activity is expressed as µmol of substrate transformed/min/mg of cellular protein in the crude cell extract.

11.2.2. Assay reliability

The assay for ornithine aminotransferase was linear over a period of at least 90 min.and to a final product concentration of 3.9 µmol/assay (Figure 4a). To ensure linearity of the assay, activity was measured over a period of 60 min. and it was ensured that the amount of product formed never exceeded 2.5 µmol/assay. Under these conditions the assay was linear with respect to volume of extract added up to 400 µl (Figure 4b) and with cell density up to 10^8 cells/ml (Figure 4c).

12.3. Threonine deaminase

Threonine deaminase activity was determined as described by Loomis (1970)

$$\text{L-Threonine} \longrightarrow \text{2-oxobutyrate} + NH_3$$

The enzyme was assayed by measuring the amount of 2-oxobutyrate formed as a phenyl hydrazone derivative.

12.3.1. Assay conditions

The standard Threonine deaminase assay contained, in a total volume of 0.5 ml , 10 mM Tris-HCl pH.8.5; 50 mM L-Threonine adjusted to pH8.5 and routinely 100 µl cell extract, prepared from 10^8 cells/ml in 20 µg/ml pyridoxal phosphate. The addition of extract initiated the reaction which was carried out at 25°C for 60 min; the ketone product was converted to the phenylhydrazone by addition of 0.9 ml of a solution containing 0.34 mg/ml 2,4 dinitrophenylhydrazine in 1.2 N HCl and incubating at 25°C for 20 min. The colour was then developed by adding 1.7 ml of 2.5 N NaOH and incubating for 10 min. at 25°C, and the optical density was determined at 520 nm on an SP-30 spectrophotometer.

Figure 4. Ornithine aminotransferase

Cells were grown in ALM, washed and resuspended in 100 mM Tris-HCl pH8.0 and extract prepared as described in methods 12.1. Assays, modified as described below, were carried out as in methods 12.2.1.

4a) Time course of glutamate semialdehyde formation

Assays were set up, initiated by addition of 400 µl extract prepared at 10^8 cells/ml and incubated at 37°C for different times. In the control assay at zero time, acid was added before extract.

4b) Effect of extract volume on glutamate semialdehyde formation

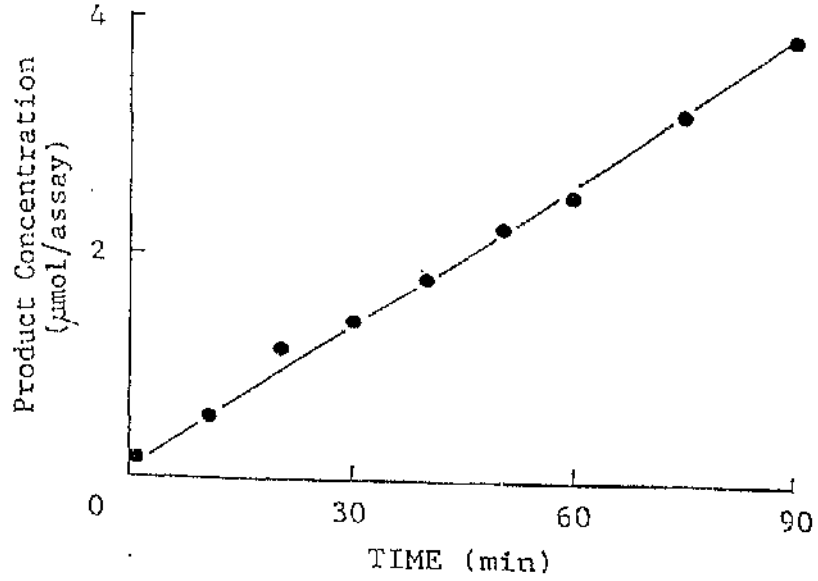
Standard assays were set up and initiated by adding different volumes of extract prepared at 10^8 cells/ml. Where necessary 100 mM Tris-Hcl pH8.0 was added in place of extract to make the assay volume to 1 ml. Assays were incubated for 60 minutes at 37°C. Control assays, set up with no substrate, were otherwise identical.

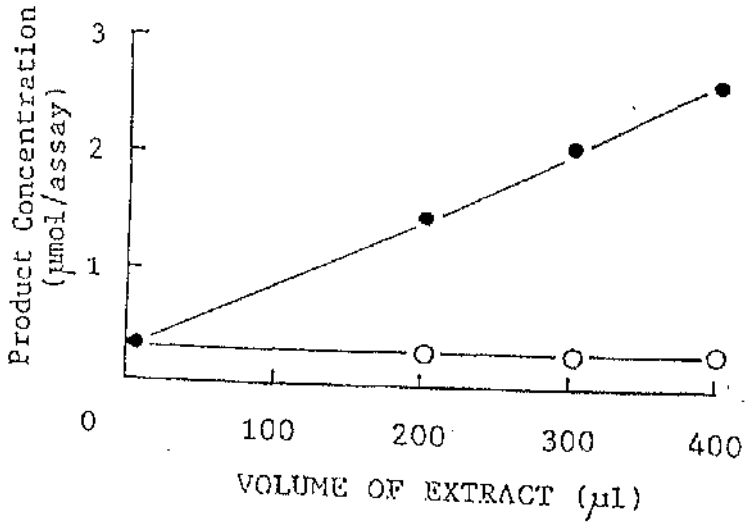
4c) Effect of cell density on glutamate semialdehyde formation

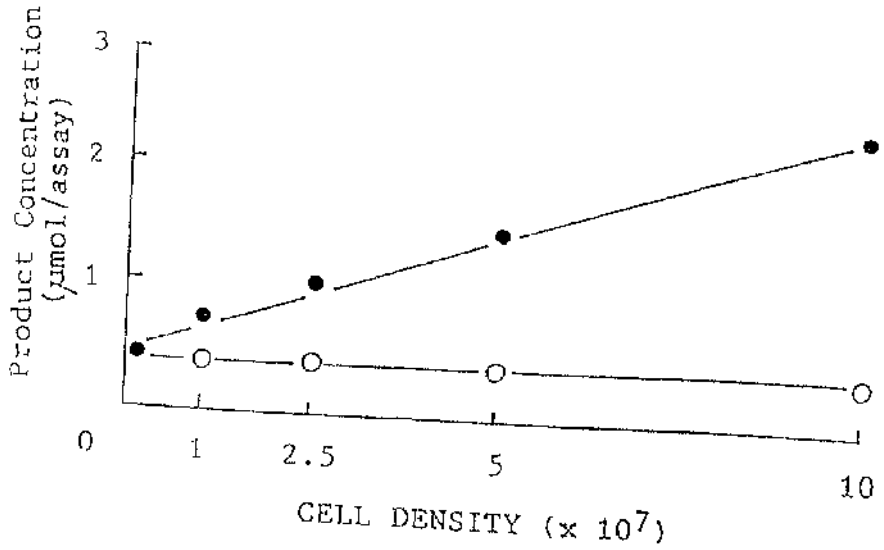
Standard assays were set up and initiated by adding 400 µl of extracts prepared from different cell densities and incubated at 37°C for 60 minutes. As with 4b control assay contained all components except substrate.

● : Substrate added

O : No substrate


Product Concentration
(µmol/assay)
4
2
0
30
60
90
TIME (min)


Product Concentration
(µmol/assay)
3
2
1
0
100
200
300
400
VOLUME OF EXTRACT (µl)


Product Concentration
(µmol/assay)
3
2
1
0
1
2.5
5
10
CELL DENSITY (x 10^7)

The amount of 2-oxobutyrate produced in the assay was calculated by using a 2-oxobutyrate standard curve covering the range 0-2 μmol/assay (Figure 5).

12.3.2 Assay reliability

Threonine deaminase assay was found to be linear over a period of 120 min. and to a final product concentration of 1.1 μmol/assay (Figure 6a). The activity of the enzyme was measured over a period of 60 min. with the amount to product formation never exceeding 0.6 μmol/assay.

Under these conditions, the assay was linear with respect to, volume of extract added up to 200 μl (Figure 6b) and cell density up to 10^8 cells/ml (Figure 6c).

12.4. Tyrosine transaminase

Tyrosine transaminase activity was determined by a modification of the method of Diamondstone (1966).

Tyrosine + 2-oxoglutarate ⟶ p-hydroxyphenyl pyruvate + glutamic acid.

p-hydroxyphenyl pyruvate is measured by its conversion to p-hydroxybenzaldehyde which absorbs at 331 nm.

12.4.1. Assay conditions

The standard reaction mixture, in a total volume of 1.75 ml, contained 5.6 mM L-Tyrosine; 120 mM Tris-HCl pH8.5; 0.057 mM Pyridoxal phosphate and routinely 100 μl cell extract prepared from 10^8 cells/ml in distilled water. The reaction was initiated by addition of 8.57 mM 2-oxoglutarate. After incubating at 32°C for 15 min, the

Figure 5: 2-oxobutyrate standard curve

Different concentrations of 2-oxobutyrate were prepared in 10 mM Tris HCl pH8.5. To 0.5 ml of each concentration 0.9 ml of a solution containing 0.34 mg/ml 2,4 dinitrophenylhydrazine in 1.2 M HCl was added and the assay incubated at 25°C for 20 min. The colour was then developed by adding 1.7 ml 2.5 N NaOH and incubating for a further 10 min. at 25°C (methods 12.3.1.). Optical density was measured at 520 nm.

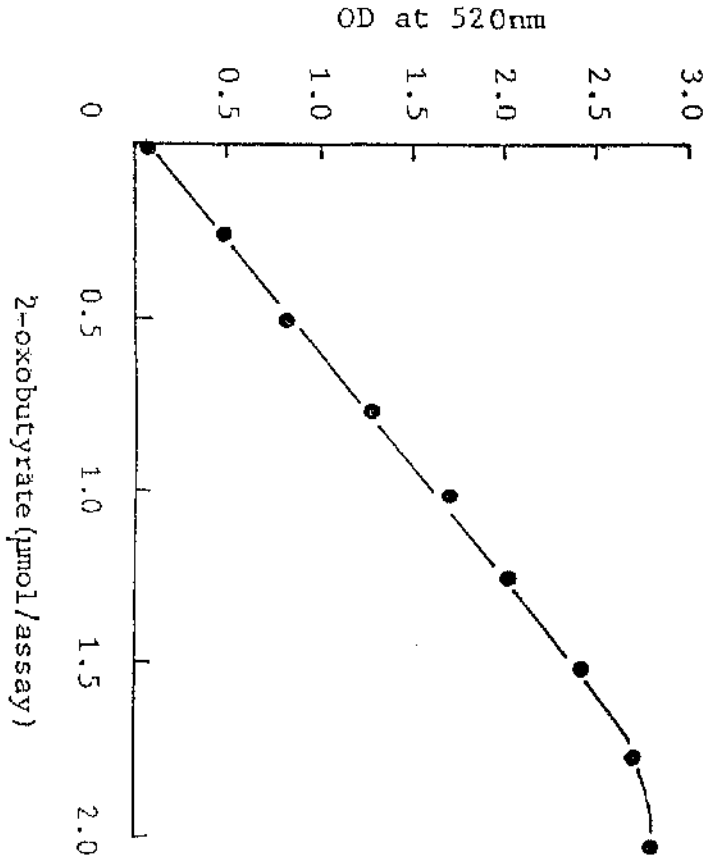

OD at 520nm
0
0.5
1.0
1.5
2.0
2.5
3.0
2-oxobutyrate (µmol/assay)
0.5
1.0
1.5
2.0

Figure 6 Threonine deaminase

Cells were grown in ALM, washed and resuspended in 20 µg/ml pyridoxal phosphate and cell extract prepared as described in methods 12.1. Assays, modified as described below, were carried out as in methods 12.3.1.

6a) Time course of 2-oxobutyrate formation

Assays were set up, initiated by addition of 100 µl extract prepared at 10^8 cells/ml, and incubated at 25°C for different times. In the control assays at zero time, acid was added before extract.

6b) Effect of extract volume on 2-oxobutyrate formation

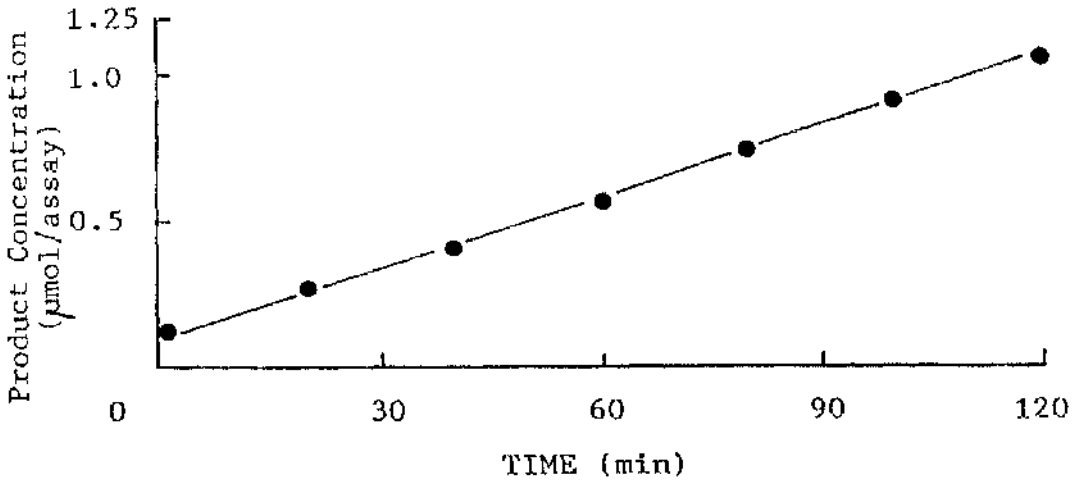
Assays were set up and initiated by adding different volumes of extract prepared at 10^8 cells/ml. Where necessary 20 µg/ml pyrodoxal phosphate was added in place of extract. The assay volume was made to 0.5 ml by adding distilled water and incubated at 25°C for 60 minutes. Control assays, set up with no substrate, were otherwise identical.

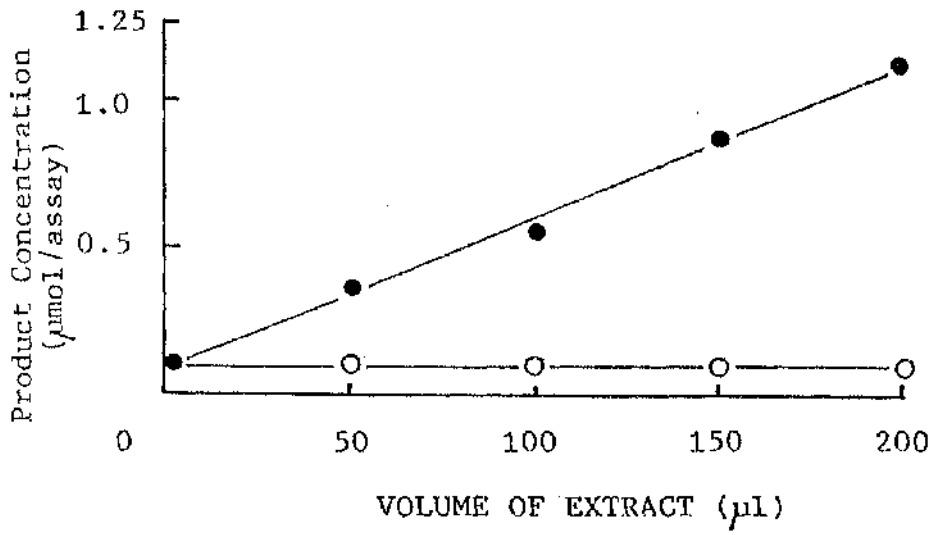
6c) Effect of cell density on 2-oxobutyrate formation

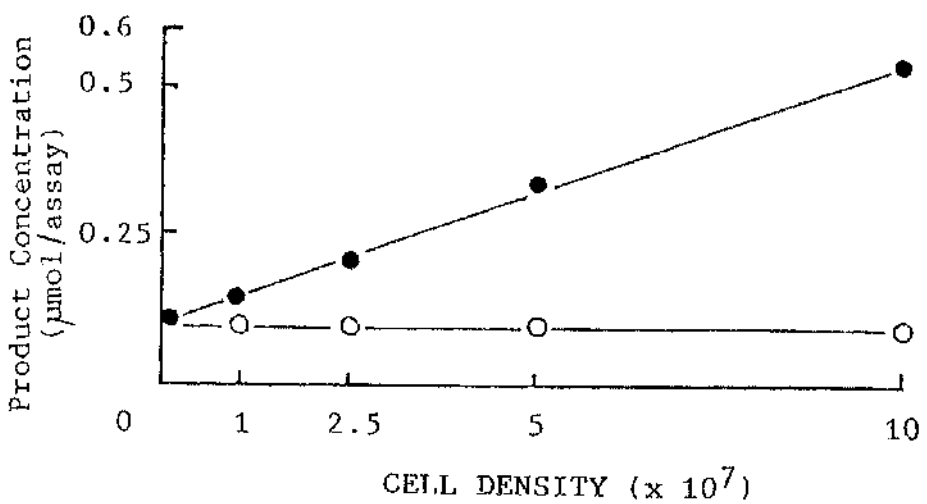
Standard assays were set up and initiated by adding 100 µl of extracts prepared from different cell densities and incubated at 25°C for 60 minutes. As in 6b control assays contained all components except substrate

● : Substrate added

○ : No substrate


Product Concentration (µmol/assay)
1.25
1.0
0.5
0
30
60
90
120
TIME (min)


Product Concentration (µmol/assay)
1.25
1.0
0.5
0
50
100
150
200
VOLUME OF EXTRACT (µl)


Product Concentration (µmol/assay)
0.6
0.5
0.25
0
1
2.5
5
10
CELL DENSITY (x 10^7)

reaction was stopped by addition of 0.1 ml of 10 N NaOH and conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde allowed to proceed for 30 min at 25°C. The reaction mixture was clarified by centrifugation at 12,000 g for 15 min. and the optical density of the supernatant determined at 331 nm on SP-30 spectrophotometer.

The number of enzyme units present were calculated by using a molar extinction coefficient for p-hydroxybenzaldehyde of $19.9 \times 10^{3} M^{-1} cm^{-1}$ (Diamondstone 1966).

12.4.2. Assay Reliability

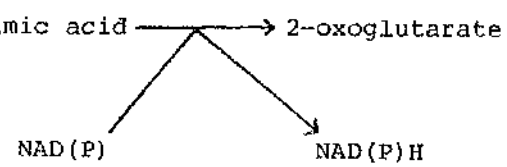
The assay for tyrosine transaminase was linear over a period of at least 30 min. and to a final product concentration of 0.224 µmol/assay (Figure 7a)

To ensure the linearity of assay, the activity was measured over the period of 15 min. with the amount of product formation never exceeding 0.13 µmol/assay (Figure 7a).

Under these conditions, the assay was linear with respect to volume of extract added up to 200 µl (Figure 7b) and with cell density up to 10^{8} cells/ml (Figure 7c).

12.5. Glutamate dehydrogenase

Glutamate dehydrogenase activity was determined as described by Langridge et al., (1977)



The appearance of NADH/NADPH was measured at 340 nm on SP-800 spectrophotometer at 25°C.

Figure 7. Tyrosine transaminase

Cells were grown in ALM, washed and resuspended in distilled water and cell extract prepared as described in methods 12.1. Assays, modified as described below, were carried out as in methods 12.4.1.

7a) Time course of p-hydroxyphenylpyruvate formation

Assays were set up, initiated by addition of 100 µl cell extract prepared at 10^8 cells/ml and incubated at 32°C for different times. In the control assays at zero time, NaOH was added before extract.

7b) Effect of extract volume on p-hydroxyphenylpyruvate formation

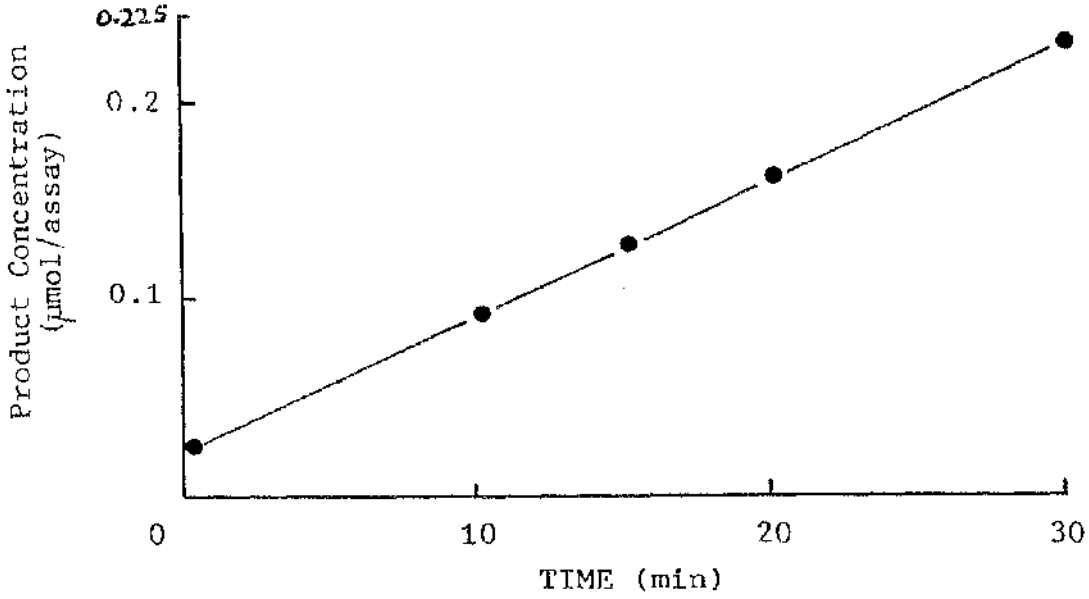
Standard assays were set up and initiated by adding different volumes of extract prepared at 10^8 cells/ml. Where necessary distilled water was added in place of extract to make a final assay volume 1.75 ml. Assays were incubated for 15 minutes at 32°C. Control assays, set up with no substrate, were otherwise identical.

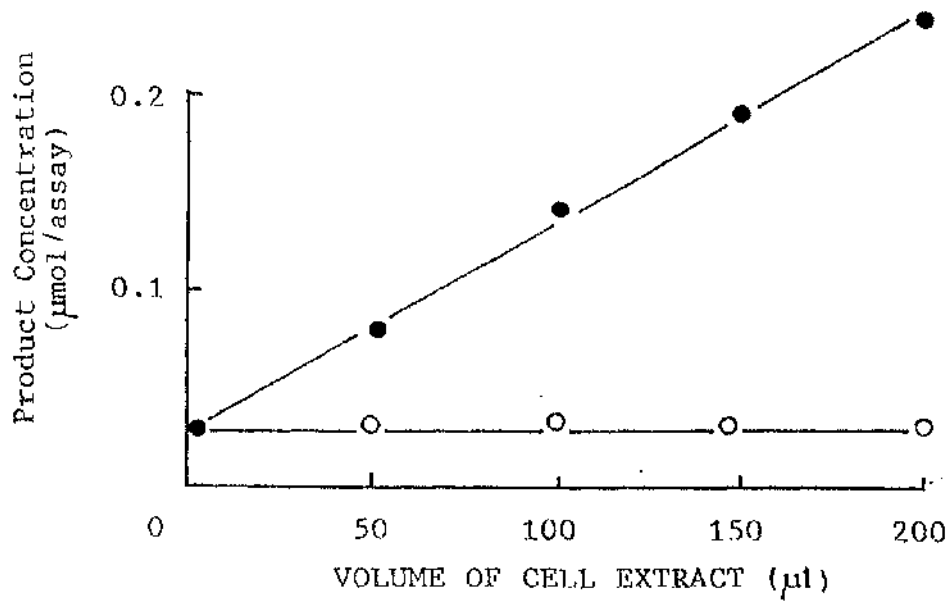
7c) Effect of cell density on p-hydroxyphenylpyruvate formation

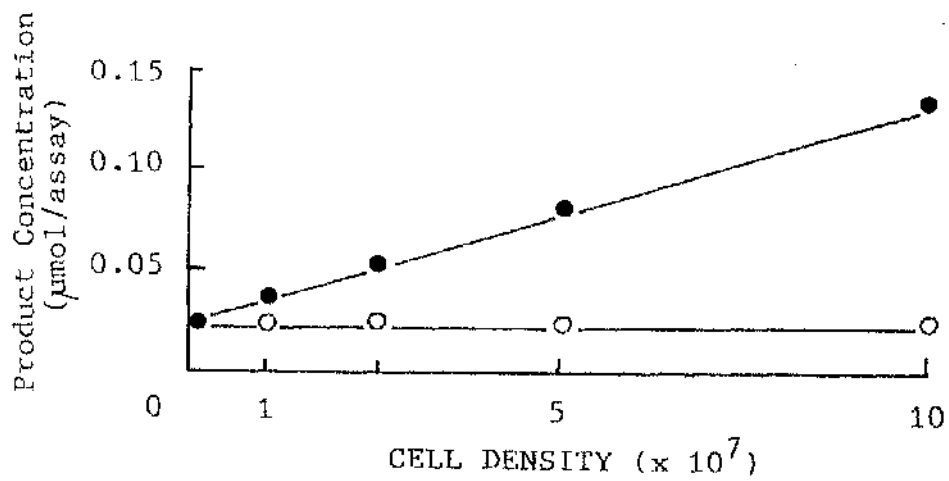
Standard assays were set up and initiated by adding 100 µl of extract prepared from different cell densities and incubated at 32°C for 15 min. As in 7b control assays contained all components except substrate.

● : Substrate added

O : No substrate


0.225
0.2
0.1
Product Concentration (µmol/assay)
0
10
20
30
TIME (min)


0.2
0.1
Product Concentration (µmol/assay)
0
50
100
150
200
VOLUME OF CELL EXTRACT (µl)


0.15
0.10
0.05
Product Concentration (µmol/assay)
0
1
5
10
CELL DENSITY (x 10^7)

12.5.1. NAD linked glutamate dehydrogenase

12.5.1.1. Assay conditions

The standard reaction mixture contained, 10 mM Tris-HCl, pH8.2, 0.3 mM NAD^+, 0.08 mM NADH (this gives an OD of 0.5 at 340 nm) to which 0.1 ml cell extract was added. NADH appearance was recorded and 10 mM Glutamic acid added to make final assay volume 1.0 ml. NADH appearance was recorded and glutamate dehydrogenase activity calculated using 6.22×10^3 M^{-1} cm^{-1} as molar extinction coefficient of NADH. Confirmation of the value of the molar extinction coefficient, under the conditions of assay, were verified using NADH as a standard. The presence of NADH in the assay was to allow measurement, under glutamate dehydrogenase assay conditions, of NADH oxidase activity. NADH oxidase was present at only small amounts in the extract.

The difference in the rate of NADH production, between the assays carried out in the presence and absence of L-glutamate was used to give a value for the activity of NAD linked L-glutamate dehydrogenase.

12.5.1.2. Assay reliability

The assay for NAD linked glutamate dehydrogenase was linear with respect to volume of extract added up to 500 µl with a product concentration 19.2 nmol/min/assay (Figure 8a) and cell density $1x10^8$ cells/ml which gives a product concentration 5 nmol/min/assay (Figure 8b). To ensure the linearity of assay, the activity was measured with 100 µl cell extract of $1x10^8$ cells/ml at a product concentration never exceeding 5 nmol/min/assay.

12.5.2. NADP linked glutamate dehydrogenase

12.5.2.1. Assay conditions

The standard reaction mixture contained 10 mM Tris-HCl pH8.2, 0.3 mM NADP, 0.08 mM NADPH (this gives an OD of 0.5 at 340 nm) to which 0.5

Figure 8. NAD linked glutamate dehydrogenase

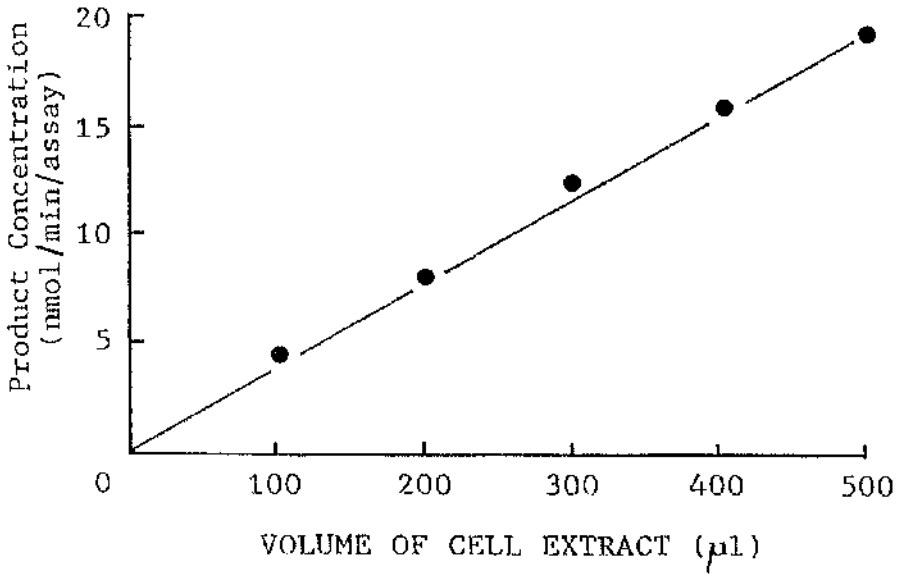
Cells were grown in ALM, washed and resuspended in 100 mM Tris-HCl pH8.0 and cell extract prepared as described in section 12.1. Assays were carried out at 25^{o}C as described below and the optical density at 340 nm was used to record the formation of NADH in the assays.

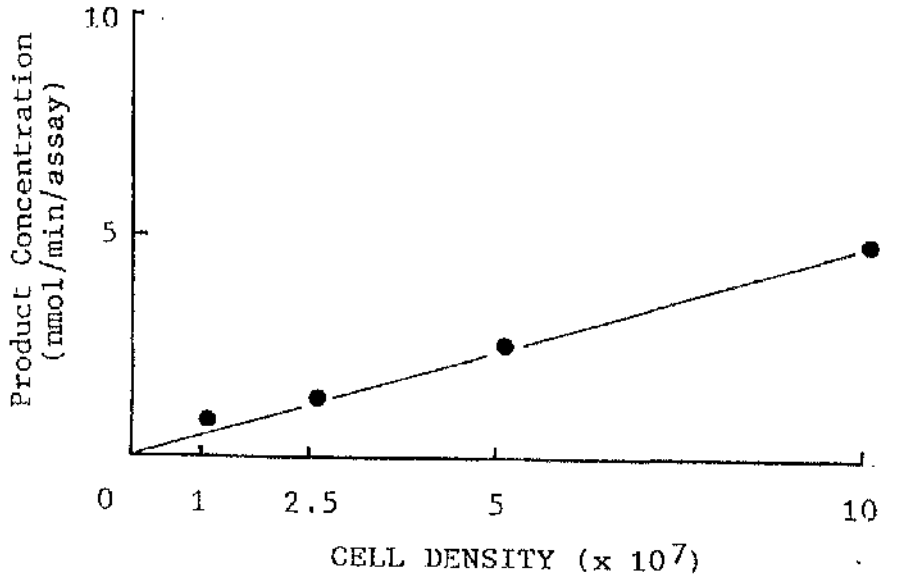
8a) Effect of extract volume on NADH production

Assays were carried out with different volumes of extract prepared from a cell suspension of 10^{8} cells/ml and NADH production recorded. For control assay, equivalent volumes of extracts were added along with assay mixture without the addition of substrate.

8b) Effect on cell density on NADH production

100 µl of cell extract, prepared from cells, suspended in 100 mM Tris HCl pH8.0 at different cell densities, were added in standard assays and NADH production recorded. As with 8a control assays were carried out without the addition of substrate.


Product Concentration
(nmol/min/assay)
20
15
10
5
0
100
200
300
400
500
VOLUME OF CELL EXTRACT (µl)


Product Concentration
(nmol/min/assay)
10
5
0
1
2.5
5
10
CELL DENSITY (x 10⁷)

ml cell extract was added. NADPH appearance was recorded and 10 mM glutamic acid added to make final assay volume 1.0 ml. NADPH appearance was recorded at 340 nm and glutamate dehydrogenase activity calculated using 6.22×10^3 M^{-1} cm^{-1} as molar extinction coefficient of NADPH. Confirmation of the value of the molar extinction coefficient, under the conditions of assay, were verified using NADPH as a standard. The presence of NADPH in the assay was to allow measurement, under glutamate dehydrogenase assay conditions of NADPH oxidase activity. NADPH oxidase was present at only small amounts in the extract.

The difference in the rate of NADPH production, between the assay carried out in the presence and absence of L-glutamate was used to give a value for the activity of NADP Linked L-glutamate dehydrogenase.

12.5.2.2. Assay reliability

The assay of NADP linked glutamate dehydrogenase was linear with respect to volume of extract added, up to 600 µl with a product concentration 3.8 nmol/min/assay (Figure 9a) and cell density 1×10^8 cells/ml which gives a product concentration 3.2 nmol/min/assay (Figure 9b). To ensure the linearity of assay, the activity was measured using 500 µl of cell extract prepared at 10^8 cells/ml.

12.6. Malic enzyme

Malic enzyme activity was measured by the method described by Kelleher et al., 1979. The enzyme activity is assayed at 25°C in the direction of malate decarboxylation using appearance of NADPH at 340 nm as a measure of activity.

12.6.1. Assay conditions

The standard reaction mixture in 50 mM Tris-Hcl pH8.1 contained 10 mM $MgCl_2$, 15 mM malate, 1 mM dithiothreitol, 1 mM EDTA and 0.1 mM

Figure 9. NADP linked glutamate dehydrogenase

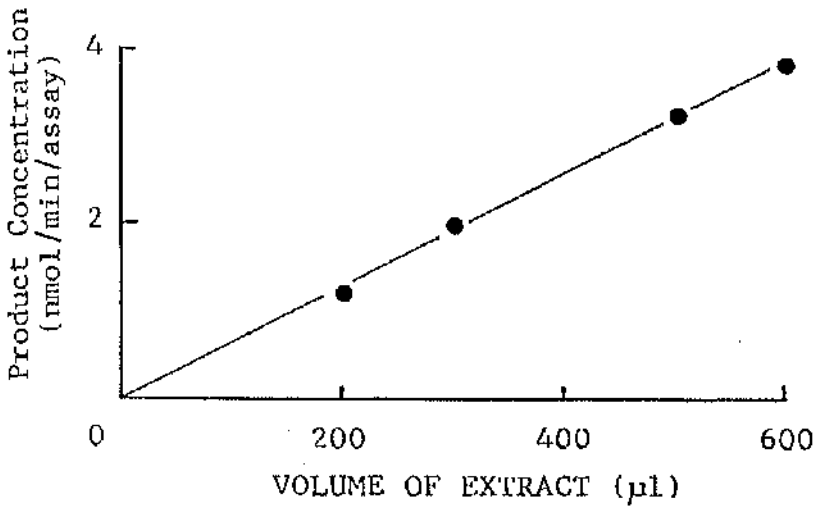
Cells were grown in ALM, washed and resuspended in 100 mM Tris-HCl pH8.0 and cell extract prepared as described in methods 12.1. Assays were carried out at 25^{o}C as described below and the optical density at 340 nm was used to record the formation of NADPH in the assays.

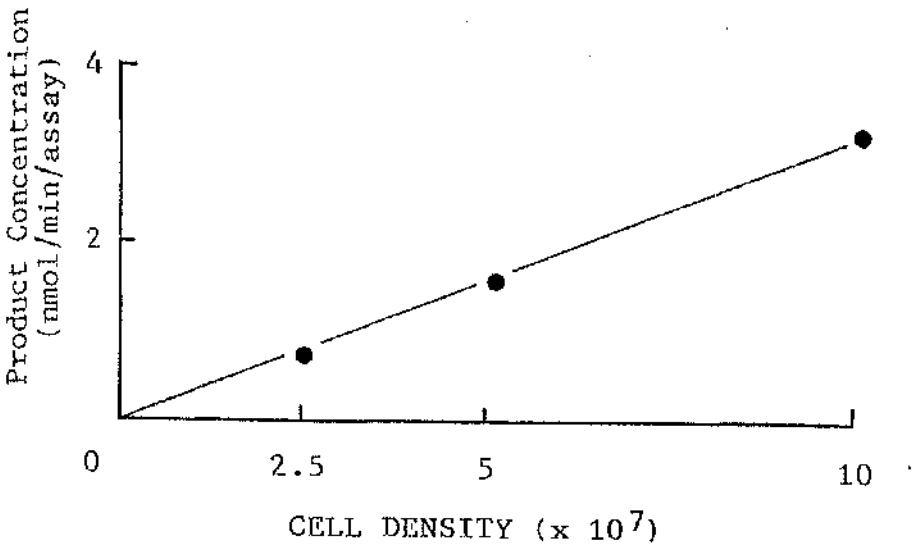
9a) Effect of extract volume on NADPH production

Assays were carried out with different volumes of extract prepared from a cell suspension of 10^{8} cells/ml and NADPH production recorded. For control assay, equivalent volumes of extracts were added along with assay mixture but without the addition of substrate.

9b) Effect of cell density on NADPH production

100 µl of cell extract, prepared from cells, suspended in 100 mM Tris-HCl pH8.0, at different cell densities, were added in standard assays and NADPH production recorded. As with 9a control assays were carried out without the addition of substrate.


Product Concentration
(nmol/min/assay)
4
2
0
200
400
600
VOLUME OF EXTRACT (µl)


Product Concentration
(nmol/min/assay)
4
2
0
2.5
5
10
CELL DENSITY (x 10^7)

NADP. 0.1 ml cell extract was added to the reaction mixture and the total volume adjusted to 1 ml using 50 mM Tris-HCl pH8.1, $NADPH_2$ production was recorded at 340 nm on SP-800 spectrophotometer. The enzyme activity was calculated using 6.22×10^3 $M^{-1}cm^{-1}$ as molar extinction coefficient of $NADPH_2$.

12.6.2. Assay reliability

The assay for malic enzyme was linear with respect to volume of extract added up to 120 µl with product concentration up to 9.8 nmol/min/assay (Figure 10a), and cell density up to 1×10^8 cells/ml. At these upper limits the assay gives a product concentration of 16 nmol/min/assay (Figure 10b). To ensure the linearity of assay, the activity was measured with 100 µl cell extract of 5×10^7 cells/ml to a product concentration never exceeding 8.0 nmol/min/assay.

12.7. Lysine dependent oxygen uptake activity

The activity was measured by the method described by Nakazawa (1971)

$$\text{L-Lysine} + O_2 \longrightarrow \delta\text{-Amino n-valeramide} + CO_2 + H_2O.$$

The assay depends on measuring the rate of oxygen uptake by means of the polarographic technique described in methods 10.3.2. but at a temperature of 34°C.

12.7.1. Assay conditions

The assay system contains in a final volume of 2.7 ml, 2.1 ml of 100 mM Tris-acetate buffer pH8 and 0.5 ml cell extract. The endogenous rate of oxygen uptake was recorded for at least 5 min. The reaction was then initiated by the addition of 0.1 ml of 1M L-lysine through the small

Figure 10. Malic enzyme

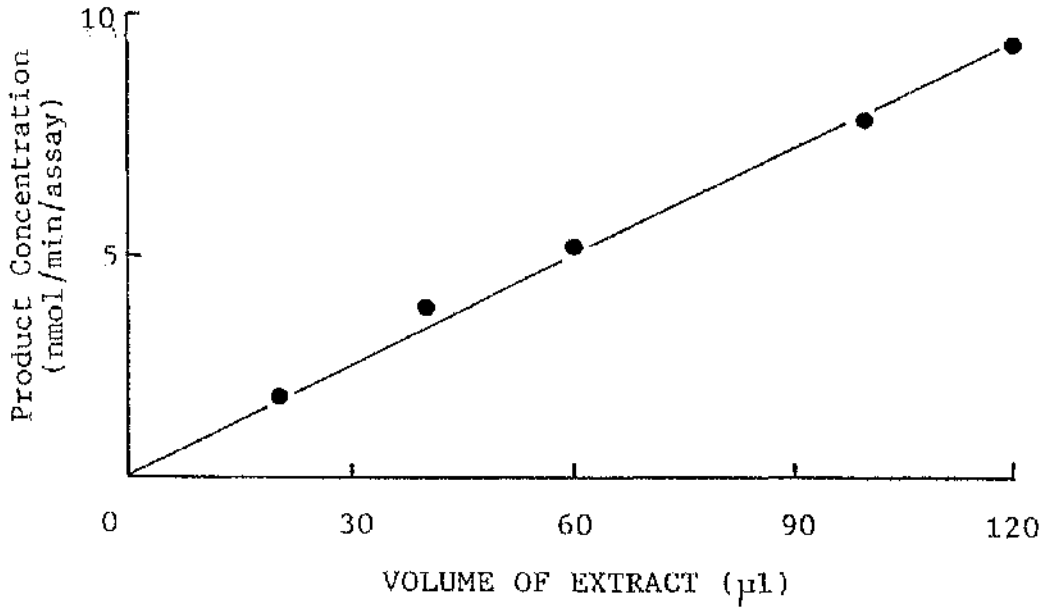
Cells were grown in ALM, washed and resuspended in 50 mM Tris-HCl pH8.1 and cell extract prepared as described in methods 12.1. Assays were carried out at 25°C as described below and the optical density at 340 nm was used to record the formation of $NADPH_2$ in the assays.

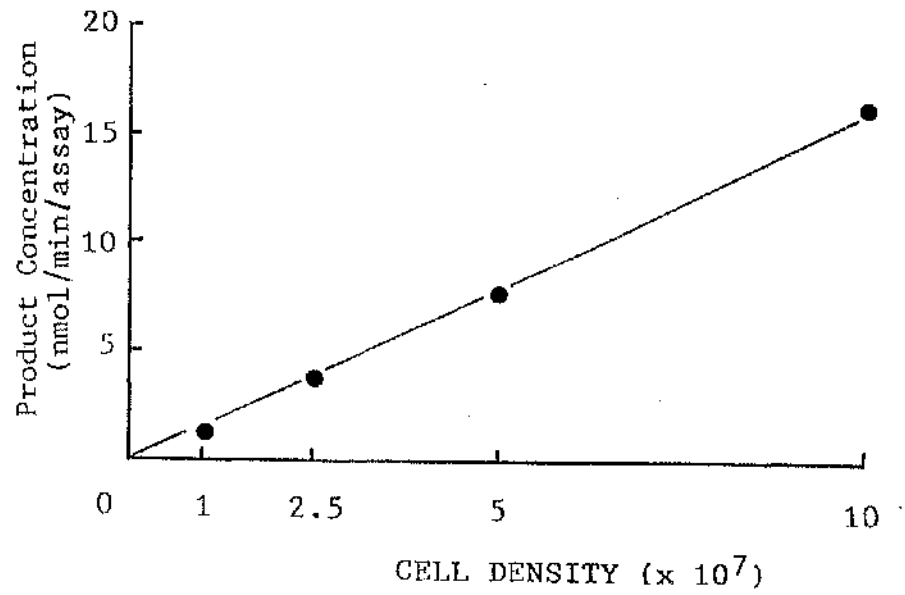
10a) Effect of extract volume on $NADPH_2$ production

Assays were carried out with different volumes of extract prepared from a cell suspension of $5x10^7$ cells/ml and $NADPH_2$ production recorded. For control assays, equivalent volumes of extracts were added along with assay mixture without the addition of substrate.

10b) Effect of cell density on $NADPH_2$ production

100 µl of cell extract, prepared from cells suspended in 50 mM Tris-Hcl pH8.1 at different cell densities, were added in standard assays and $NADPH_2$ production recorded. As with 10a control assays were carried out without the addition of substrate.


Product Concentration
(nmol/min/assay)
10
5
0
30
60
90
120
VOLUME OF EXTRACT (µl)


Product Concentration
(nmol/min/assay)
20
15
10
5
0
1
2.5
5
10
CELL DENSITY (x 10^7)

hole in the perspex disc using a Hamilton syringe to make a total volume of 2.7 ml. The rate of oxygen consumption was measured for another 5 min. and calculated as described in methods 11.2.

12.7.2. Assay reliability

The assay for lysine dependent oxygen uptake activity was linear with respect to volume of extract, up to 500 µl, and cell density up to $1x10^8$/ml. The maximum rate of oxygen uptake measured was 530 nmol/min/assay (Figure 11a). To ensure the linearity of assay, oxygen uptake was measured with 500 µl cell extract of $5x10^7$ cell/ml which gives oxygen uptake never exceeding 290 nmol/min/assay (Figure 11b).

13. Glassware

All growth flasks were cleaned by autoclaving in 1% w/v Hemosol solution (Meinecke & Co., Baltimore, U.S.A.). The flasks were thoroughly rinsed with tap water followed by distilled water and then dried in an oven. All other glassware was only immersed in Hemosol overnight and then rinsed with tap and distilled water and dried.

All pipettes were cleaned after soaking in "Kirbychlor" disinfectant solution (H. & T. Kirby & Co., Ltd., Mildenhall, Suffolk). They were then steeped in Hemosol solution overnight and then rinsed thoroughly in tap water followed by deionized water. They were dried in an electrically heated pipette drier. All pipettes used were plugged with cotton wool.

14. Statistical methods

Means and standard deviations were calculated using the formula given by Baily (1976).

Figure 11. Lysine dependent oxygen uptake activity

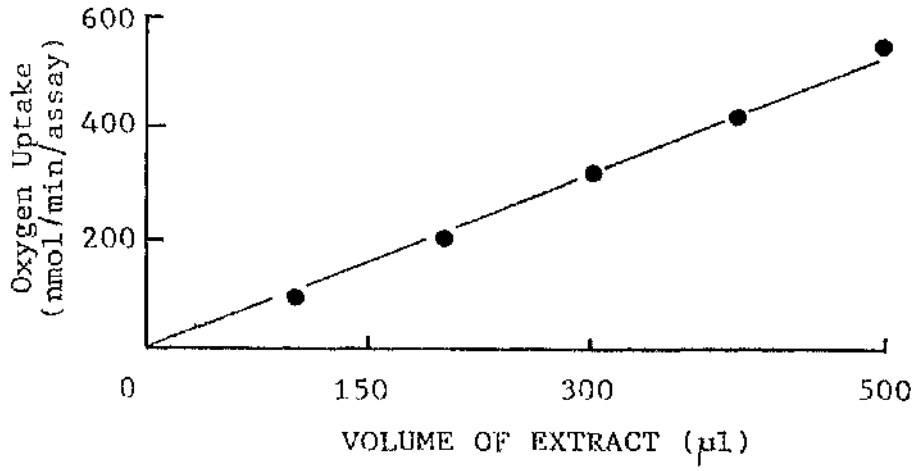
Cells were grown in ALM, washed and resuspended in 100 mM Tris-Acetate pH8.0 and cell extract prepared as described in methods 12.1 Assays were carried out as described below and the rate of oxygen uptake measured by means of the polarographic technique described in methods 10.3.2. at 34°C.

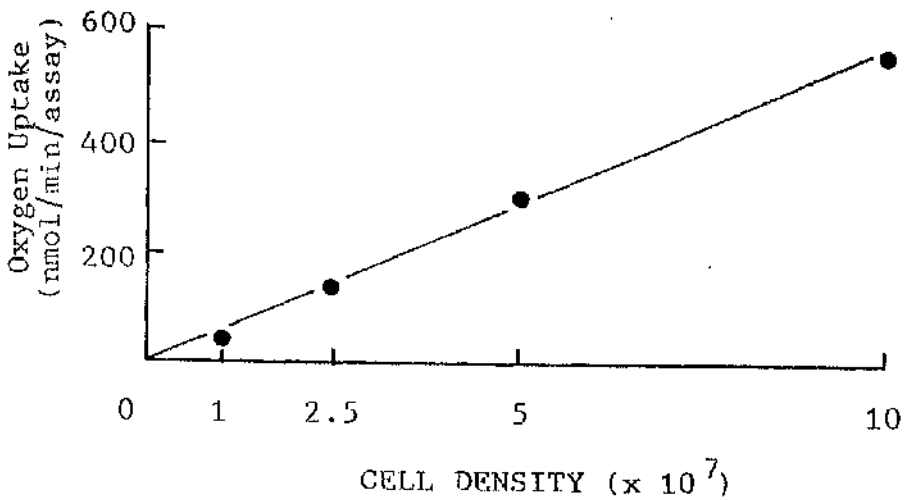
11a) Effect of extract volume on oxygen consumption

Assays were carried out with different volumes of extract prepared from a cell suspension of 10^8 cells/ml and assays carried out as described in Section 11.7.1. For control assays no substrate was added.

11b) Effect of cell density on oxygen consumption

500 µl of cell extract, prepared from cells, suspended in 100 mM TRis-Acetate pH8.0 at different cell densities, were added in standard assays and the rate of oxygen consumption recorded at 34°C. As with 11a control assays were carried out without the addition of substrate.


Oxygen Uptake
(nmol/min/assay)
600
400
200
0
150
300
500
VOLUME OF EXTRACT (µl)


Oxygen Uptake
(nmol/min/assay)
600
400
200
0
1
2.5
5
10
CELL DENSITY (x 10 7)

RESULTS

1. Growth of D. discoideum Ax2 in liquid culture.

1.1. Growth in axenic liquid medium (ALM).

Initial experiments were carried out in ALM which was adjusted to pH6.7 before sterilisation. When this medium was inoculated with D. discoideum Ax2 the cells grew to a density of 2.8×10^6 cells/ml with a growth rate (μ) of $0.037h^{-1}$ (Figure 12). Addition of glucose (86 mM) to ALM increased the final cell density to 1.2×10^7 cells/ml but reduced the growth rate (μ) to $0.032h^{-1}$ (Figure 12).

During growth there were considerable changes in pH. Autoclaving reduced the pH of the medium from 6.7 to 6.5. The pH value increased with growth to a value of 7.4 at the stationary phase in the absence of glucose and to 6.9 in the presence of glucose (Figure 12). After the cells had reached stationary phase, the pH continued to rise.

In ALM the final cell density achieved was proportional to the concentration of nutrients supplied . Changes in the concentration of nutrients had however other effects on the culture. Increasing the concentration of nutrients had the effect of slightly decreasing the growth rate and had an influence on the final pH of the culture. Cultures at low nutrient concentration (0.5 ALM) rose to a pH value of 7.7-7.8 by stationary phase while those at a higher concentration (1.5 ALM) only rose to a pH of 7.4 (Figure 13).

Under these conditions the pH value at the end of growth was changing rapidly and its value cannot be guaranteed to better than 0.1 pH unit.

Figure 12: Growth of D. discoideum Ax2 in ALM and ALM_g pH6.7.

D. discoideum Ax2 was grown in ALM and ALM_g prepared at pH6.7, using inocula grown through 2 passages of equivalent media, and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken from the growth flasks at intervals. pH and cell density were estimated as described in methods 6 and 7.

● : ALM

○ : ALM_g

	ALM	ALM_g
maximum cell density	$2.8x10^6$	$1.2x10^7$
growth rate (h^{-1})	0.037	0.032
pH at the end of growth	7.4	6.9

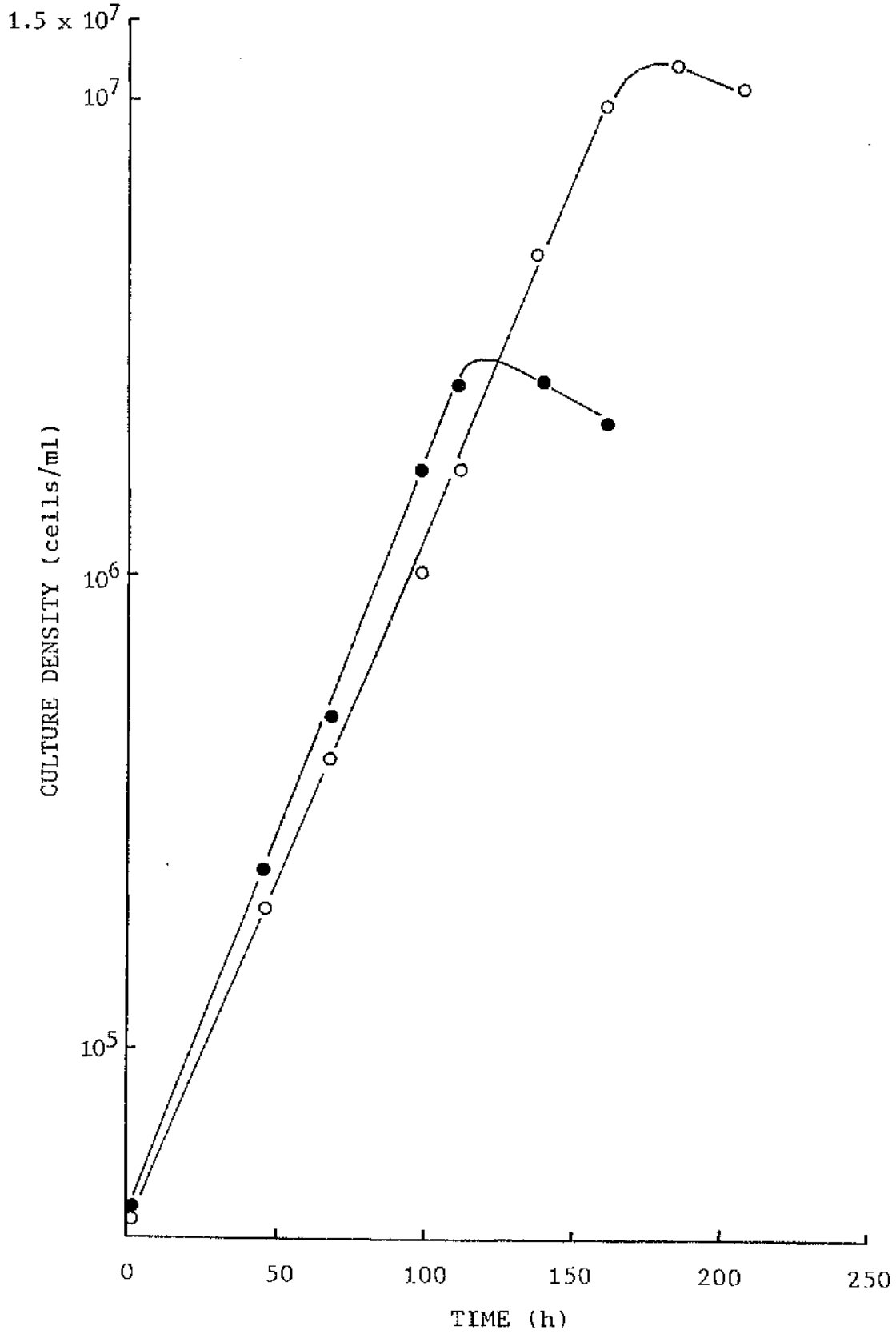

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
250
TIME (h)

Figure 13: Effect of concentration of ALM pH6.7, on the growth of D. discoideum Ax2.

D. discoideum Ax2 was grown in ALM of different concentrations at pH6.7, using an inoculum grown through 2 passages of ALM pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals. pH and cell density were estimated (methods 6, 7).

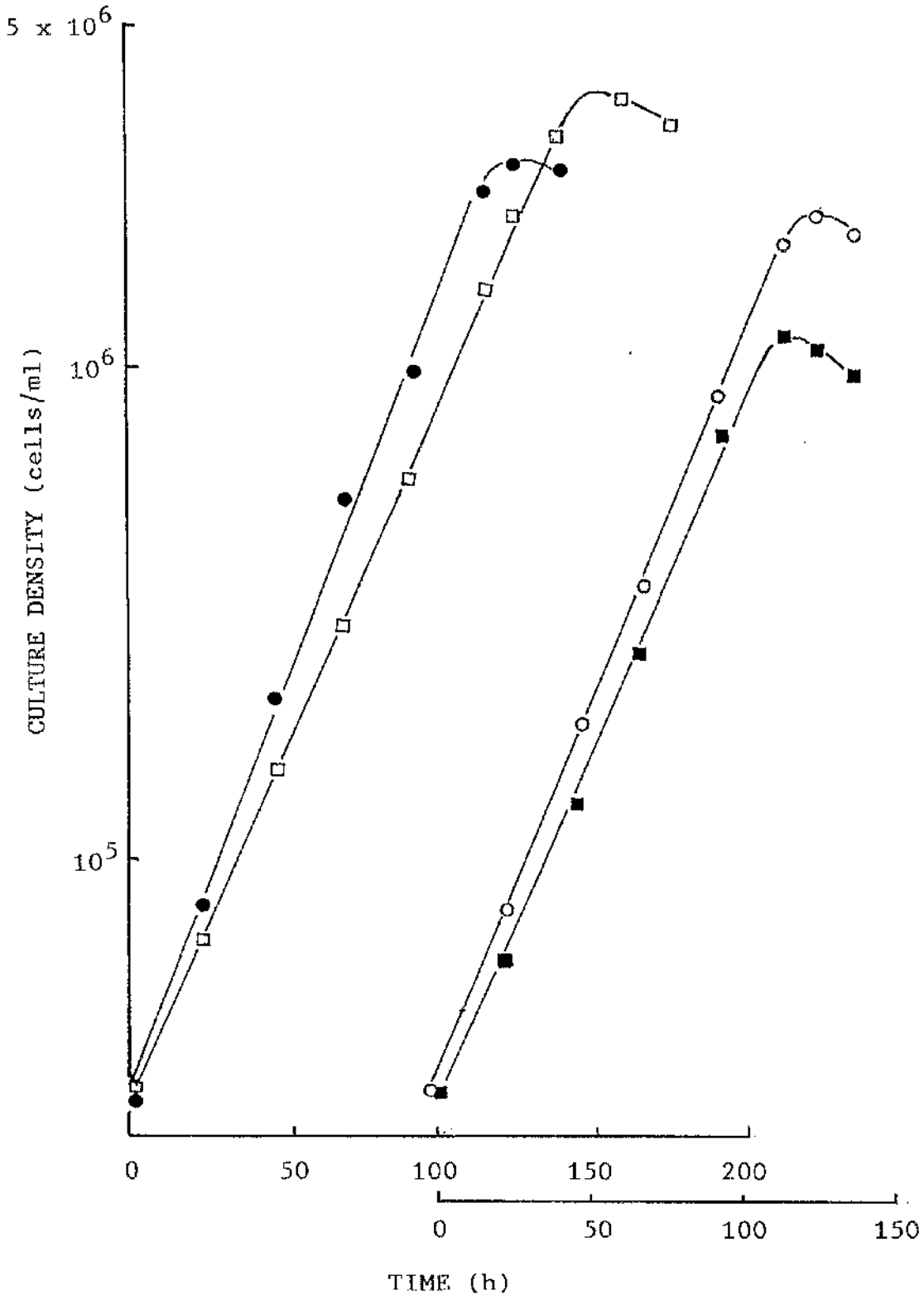
● : ALM (0.5)

□ : ALM (0.75)

○ : ALM (1.0) normal

■ : AJM (1.5)

	ALM concentration			
	0.5	0.75	1.75	1.5
maximum cell density	$1.1x10^6$	$2x10^6$	$2.6x10^6$	$3.5x10^6$
growth rate (h^{-1})	0.035	0.037	0.037	0.029
pH at the end of growth	7.8	7.7	7.5	7.4


5 x 10^6
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
0
50
100
150
TIME (h)

2. Influence of ammonia and pH on growth in ALM.

Two factors which are known to alter during growth are the concentrations of ammonia and hydrogen ions. The influence of both of these on growth of the cells was examined.

2.1. Influence of ammonia on growth.

Cultures grown in the presence of ammonium chloride were altered with respect to both final cell density and growth rate. At 10 mM, the highest concentration examined without pH control, the final cell density decreased from 2.8×10^6 to 1.3×10^6 cells/ml (a decrease of 54%) while the growth rate (μ) decreased from $0.035 h^{-1}$ to $0.029 h^{-1}$ (Figure 14). Some changes in the pH at the end of growth were also observed but their significance is hard to assess. Similar experiments carried out under pH controlled (6.7) conditions with ammonium chloride added up to 20 mM gave similar effects (Figure 15). Cell density decreased from 4.2×10^6 cells/ml to 6×10^5 cells/ml at 20 mM ammonium chloride added in ALM with growth rate (μ) reduced to $0.024\ h^{-1}$.

Effects of the same type were observed when ammonium chloride was added to cultures growing in the presence of glucose. The presence of 10 mM ammonium chloride decreased yield by only 27% and reduced growth rate rate (μ) from $0.032 h^{-1}$ to $0.027 h^{-1}$ (Figure 16).

2.2. Influence of pH on growth.

Since pH changes occur during growth, experiments were carried out to examine the influence that these changes have on growth rate and yield of cells. To achieve this, cells were grown in ALM with starting pH value of 6.0, 6.5, 6.7 and 7.0.

As the pH of the culture was increased, the yield of cells obtained decreased, especially when a pH value above 6.7 was used. The growth

Figure 14: Effect of ammonium chloride concentration on the growth of D. discoideum Ax2 in ALM pH6.7.

D. discoideum Ax2 was grown in ALM pH6.7 at different concentrations of NH_4Cl using an inoculum grown through 2 passages of ALM and inoculated to give an initial cell density of 5×10^4 cells/ml. Samples were taken at intervals. pH and cell density were estimated as described in methods 6 and 7.

● : ALM

Δ : ALM + 5 mM NH_4Cl

O : ALM x 10 mM NH_4Cl

	ALM	ALM+5 mM NH_4Cl	ALM+10 mM NH_4Cl
maximum cell density	2.8×10^6	1.9×10^6	1.3×10^6
growth rate (h^{-1})	0.035	0.032	0.029
pH at the end of growth	7.4	7.7	7.8

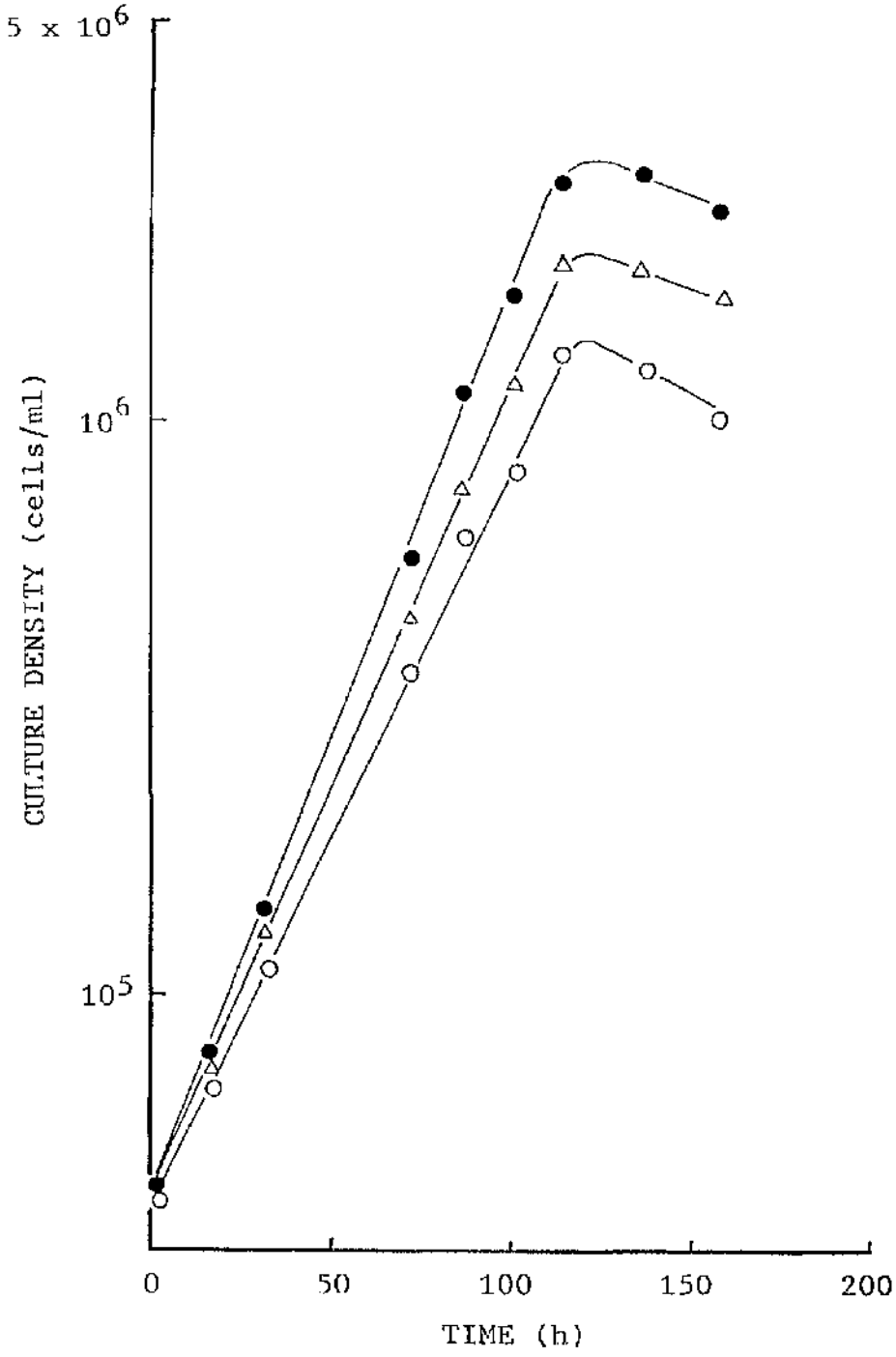

5 x 10^6
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
TIME (h)

Figure 15: Effect of ammonium chloride concentration, on the growth of D. discoideum Ax2 in ALM with pH maintained at 6.7.

D. discoideum Ax2 was grown in ALM with pH maintained at 6.7 with different concentrations of NH_4Cl, using an inoculum grown through 2 passages of ALM pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals to estimate pH and cell denisty (methods 6 and 7).

● : ALM

○ : ALM + 5 mM NH_4Cl

□ : ALM + 10 mM NH_4Cl

■ : ALM + 20 mM NH_4Cl

	ALM	ALM+5mM NH_4Cl	ALM+10mM NH_4Cl	ALM+20mM NH_4Cl
Maximum cell density	$4.2x10^6$	$2.9x10^6$	$2x10^6$	$6x10^5$
Growth rate (h^{-1})	0.035	0.032	0.029	0.024

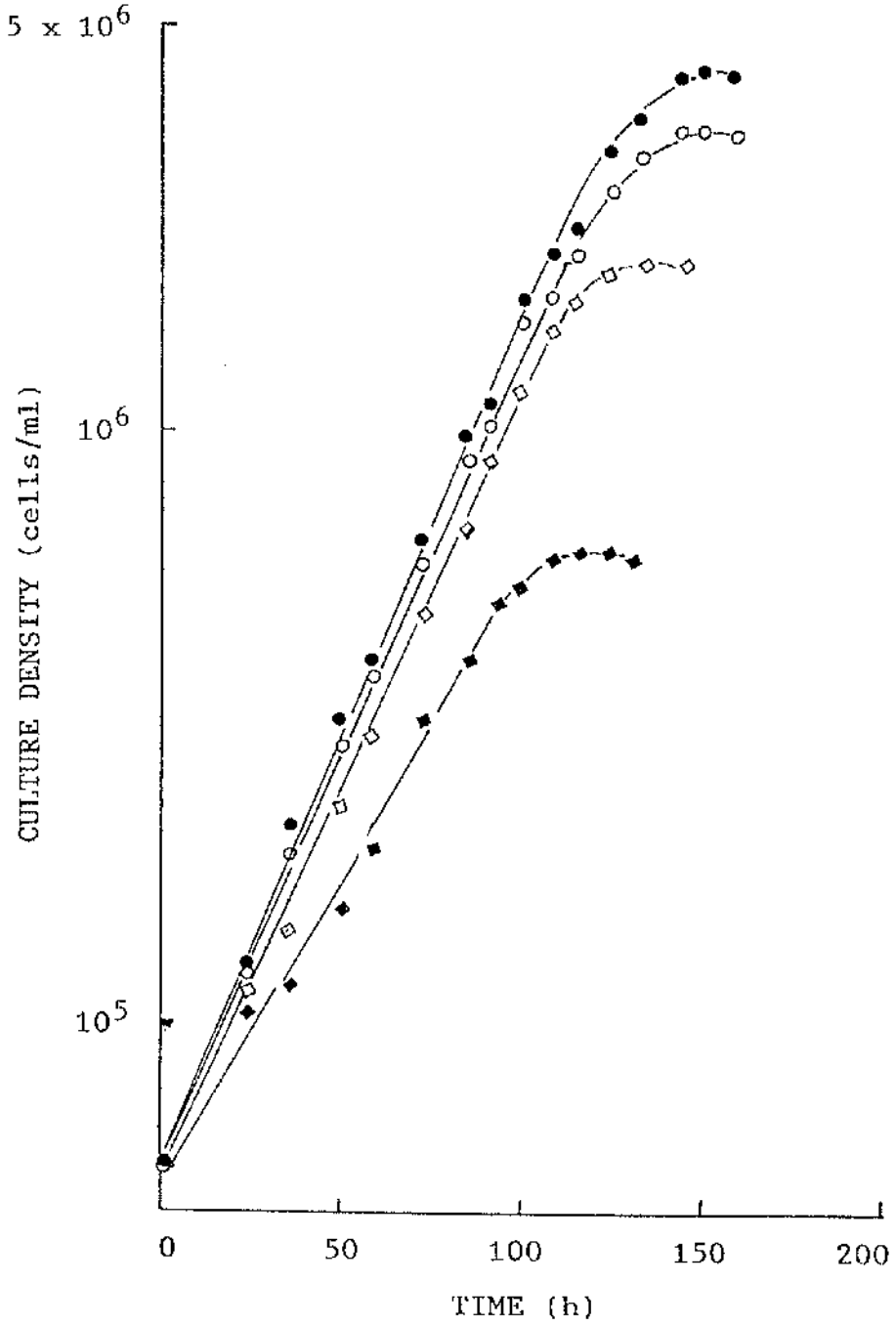

5 x 10^6
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
TIME (h)

Figure 16: Effect of ammonium chloride concentrations on the growth of D. discoideum Ax2 in ALM_g pH6.7.

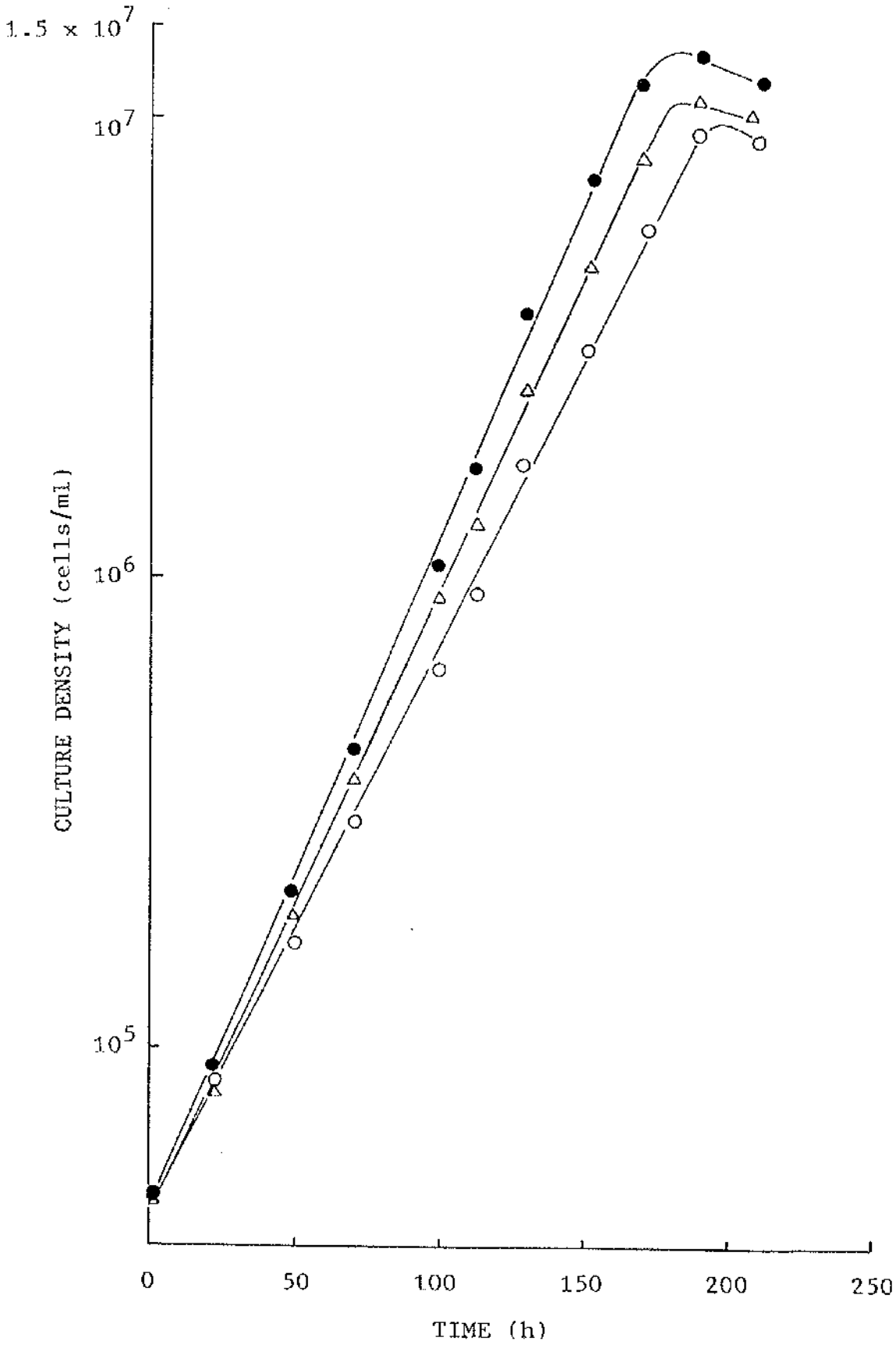
D. discoideum Ax2 was grown in ALM_g pH6.7 at different concentrations of NH_4Cl, using an inoculum grown through 2 passages of ALM_g pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals. pH and cell density were estimated (methods 6 and 7).

● : ALM_g

Δ : ALM_g+5mM NH_4Cl

O : ALM_g+10mM NH_4Cl

NH_4Cl	ALM_g	ALM_g+5mM NH_4Cl	ALM_g+10mM NH_4Cl
Maximum cell density	$1.3x10^7$	$1.1x10^7$	$9.5x10^6$
Growth rate (h^{-1})	0.032	0.029	0.027
pH at the end of growth	7.0	7.4	7.6


1.5 × 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
250
TIME (h)

rate (μ) of the cells also decreased. As might be expected, the final pH was related to initial pH but the values obtained could not be relied upon as being of significance because of the rate at which they were changing. In all cases the pH at stationary phase was 7.3 or above (Figure 17). With glucose present in media at different initial pH values, a similar effect was noted. As before, the cell yield was particularly depressed by growth at an initial pH of 7.0 and in this case the final pH was 7.5 (Figure 18).

2.2.1. Attempts to stabilise pH in growth medium.

2.2.1.1. Influence of buffer concentration.

To reduce the pH changes occuring during growth, increased concentrations of buffer were added to growth media. Both phosphate and MES [(2-(N-morpholino) ethane sulphonic acid] at pH6.7 were used. In the presence of either of these buffers, the change in pH during growth was reduced (Figure 19, 20). The addition of 28.76 mM phosphate buffer reduced the pH value at the end of growth from 7.4 to 7.0. Similarly 20 mM MES reduced the pH value to 7.1 at the end of growth.

Unfortunately the addition of these buffers reduced both the cellular growth rate and final yield. In the case of phosphate (28.76 mM) the growth rate (μ) decreased from $0.039h^{-1}$ to 0.027^{-1} with yield decreased by 30% (Figure 19). The MES buffer produced a similar effect, decreasing the growth rate (μ) to $0.029h^{-1}$ and the yield by 10% (Figure 20). The presence of both phosphate at 28.76 mM and MES at 20 mM provided very satisfactory pH control with the pH value at the end of growth being 6.7 (Figure 21). Unfortunately the presence of both buffers together greatly influence cell density and growth rate

Figure 17: Effect of pH on the growth of D. discoideum Ax2 in ALM.

D. discoideum Ax2 was grown in ALM, prepared at different pH values, using inoculum grown through 2 passages of ALM pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals. pH and cell density were estimated as described in methods 6 and 7.

● :ALM pH6

□ :ALM pH6.5

○ :ALM pH6.7

■ :ALM pH7

	Initial pH of ALM			
	6.0	6.5	6.7	7.0
maximum cell density	$3.6x10^6$	$3x10^6$	$2.7x10^6$	$1.5x10^6$
growth rate (h^{-1})	0.037	0.032	0.032	0.027
pH at the end of growth	7.3	7.5	7.6	7.6

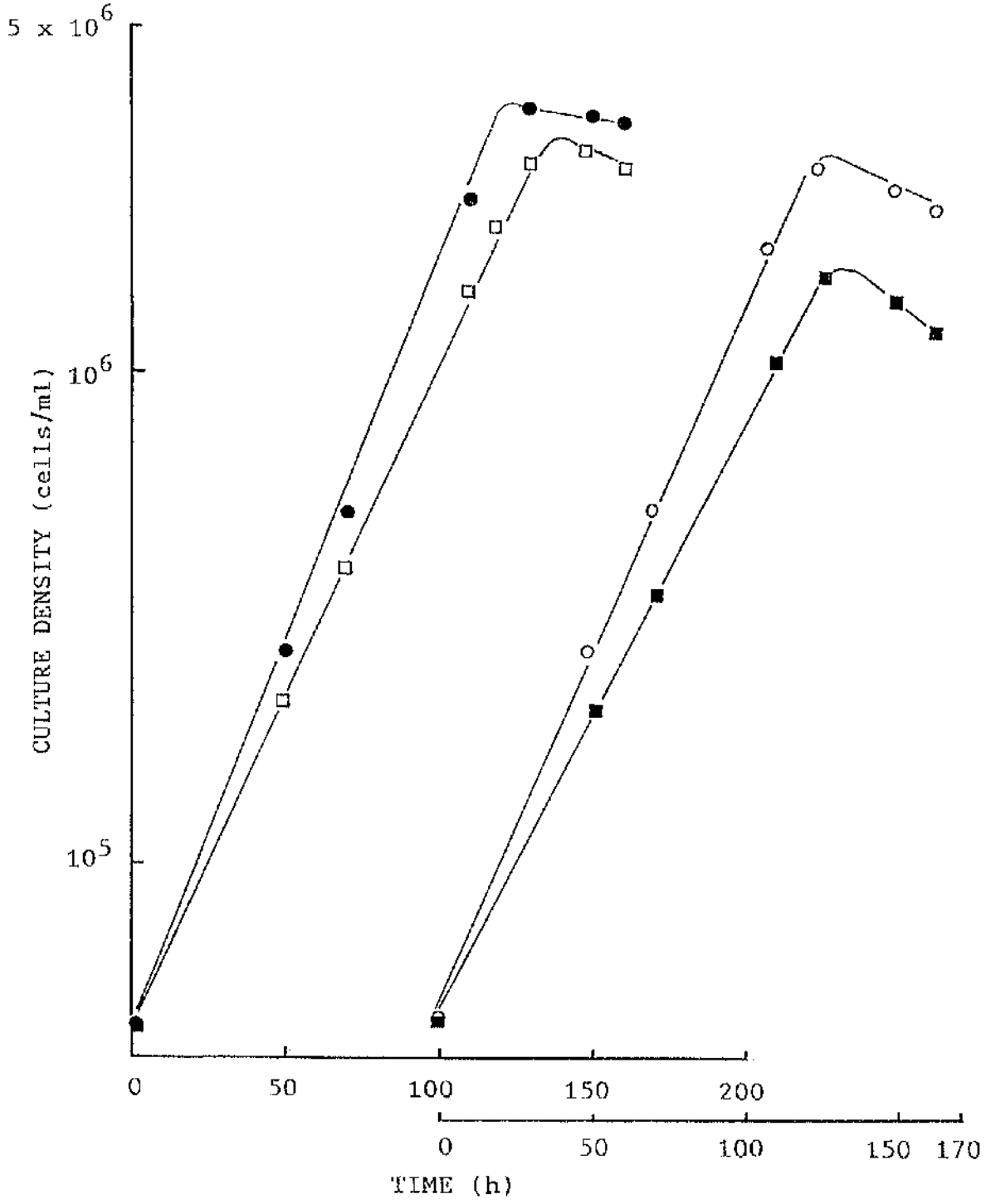

5 x 10^6
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
0
50
100
150
170
TIME (h)

Figure 18: Effect of pH on the growth of *D. discoideum* Ax2 in ALM_g

D. discoideum Ax2 was grown in ALM_g, prepared at different pH values, using an inoculum grown through 2 passages of ALM_g pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals. pH and cell density were estimated (methods 6, 7)

● : ALM_g pH6.0

□ : ALM_g pH6.5

○ : ALM_g pH6.7

■ : ALM_g pH7.0

	Initial pH of ALM_g			
	6.0	6.5	6.7	7.0
maximum cell density	$1.3x10^7$	$1.3x10^7$	$1.1x10^7$	8.10^6
growth rate (h^{-1})	0.029	0.029	0.027	0.026
pH at the end of growth	7	7.3	7.3	7.5

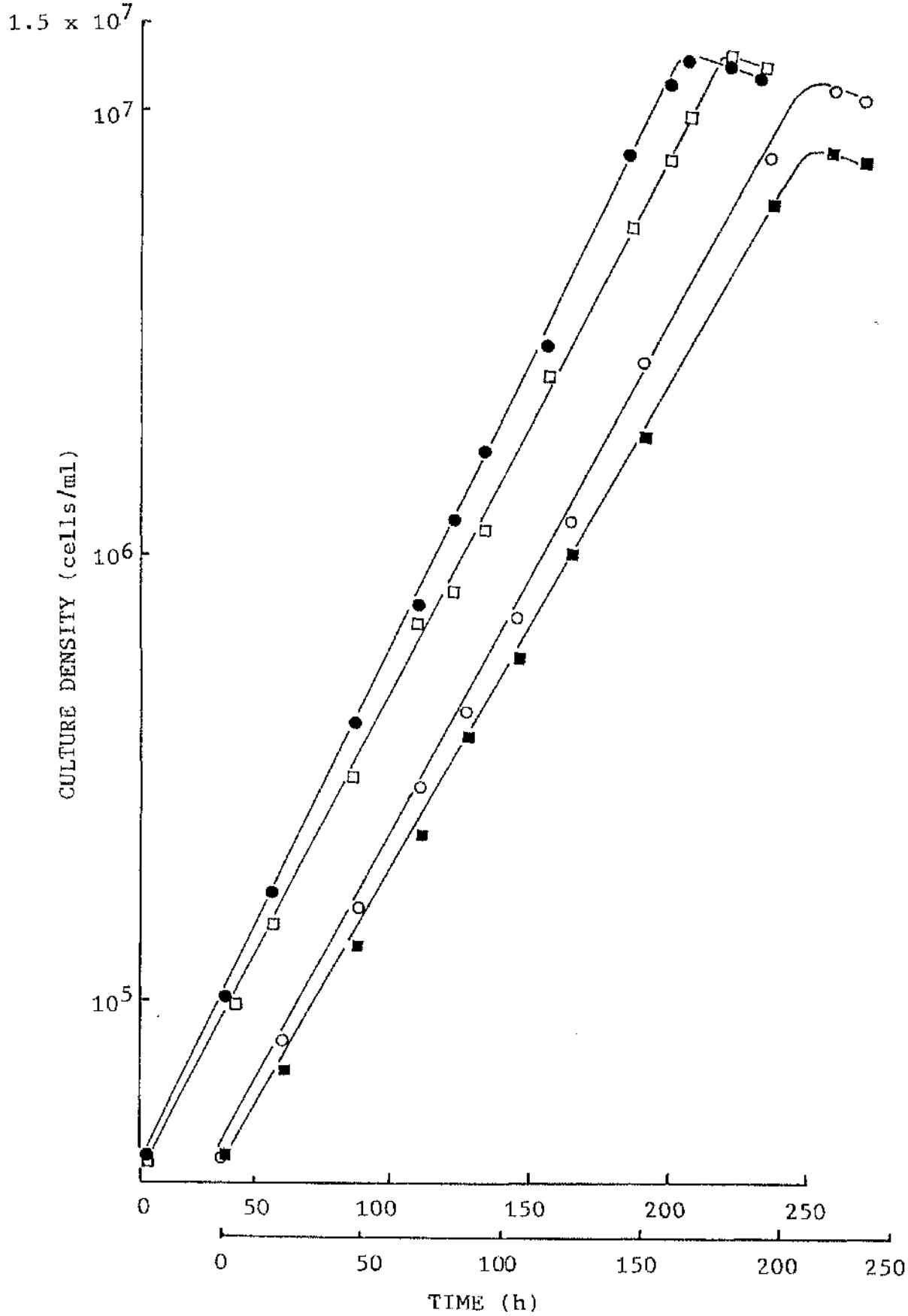

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
250
0
50
100
150
200
250
TIME (h)

Figure 19: Growth of D. discoideum Ax2 in ALM, prepared at pH6.7 at different concentrations of phosphate buffer.

D. discoideum Ax2 was grown in ALM pH6.7 at different phosphate concentrations using an inoculum grown through 2 passages of ALM pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals and pH and cell density estimated as described in methods 6 and 7.

● : ALM

○ : ALM + 14.38 mM PO_4

□ : ALM + 28.76 mM PO_4

	ALM	ALM+14.38 mM PO_4	ALM+28.76 mM PO_4
maximum cell density	$3x10^6$	$2.4x10^6$	$2.1x10^6$
growth rate (h^{-1})	0.039	0.035	0.027
pH at the end of growth	7.4	7.2	7.0

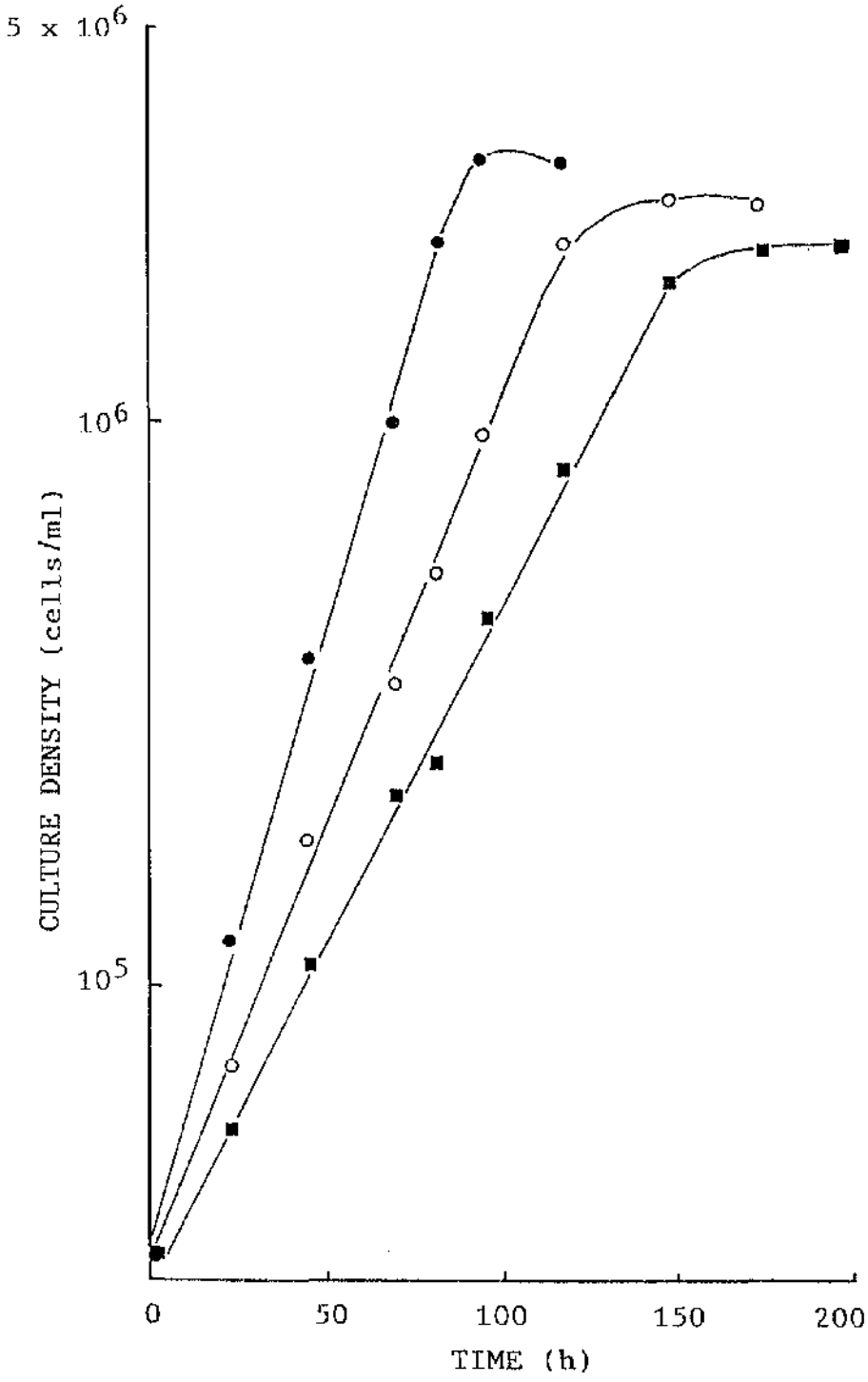

5 x 10^6
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
TIME (h)

Figure 20: Growth of D. discoideum Ax2 in ALM, prepared at pH6.7 with MES added at different concentrations.

D. discoideum Ax2 was grown in ALM pH6.7 at different concentrations of MES [2-(N-morpholino) ethane sulphonic acid] using an inoculum grown through 2 passages of ALM pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals and pH and cell density estimated (methods 6, 7).

● : ALM

○ : ALM + 10mM MES

□ : ALM + 20 mM MES

	ALM	ALM + 10 mM MES	ALM + 20 mM MES
maximum cell density	$2.9x10^6$	$2.8x10^6$	$2.6x10^6$
growth rate (h^{-1})	0.039	0.037	0.029
pH at the end of growth	7.4	7.3	7.2

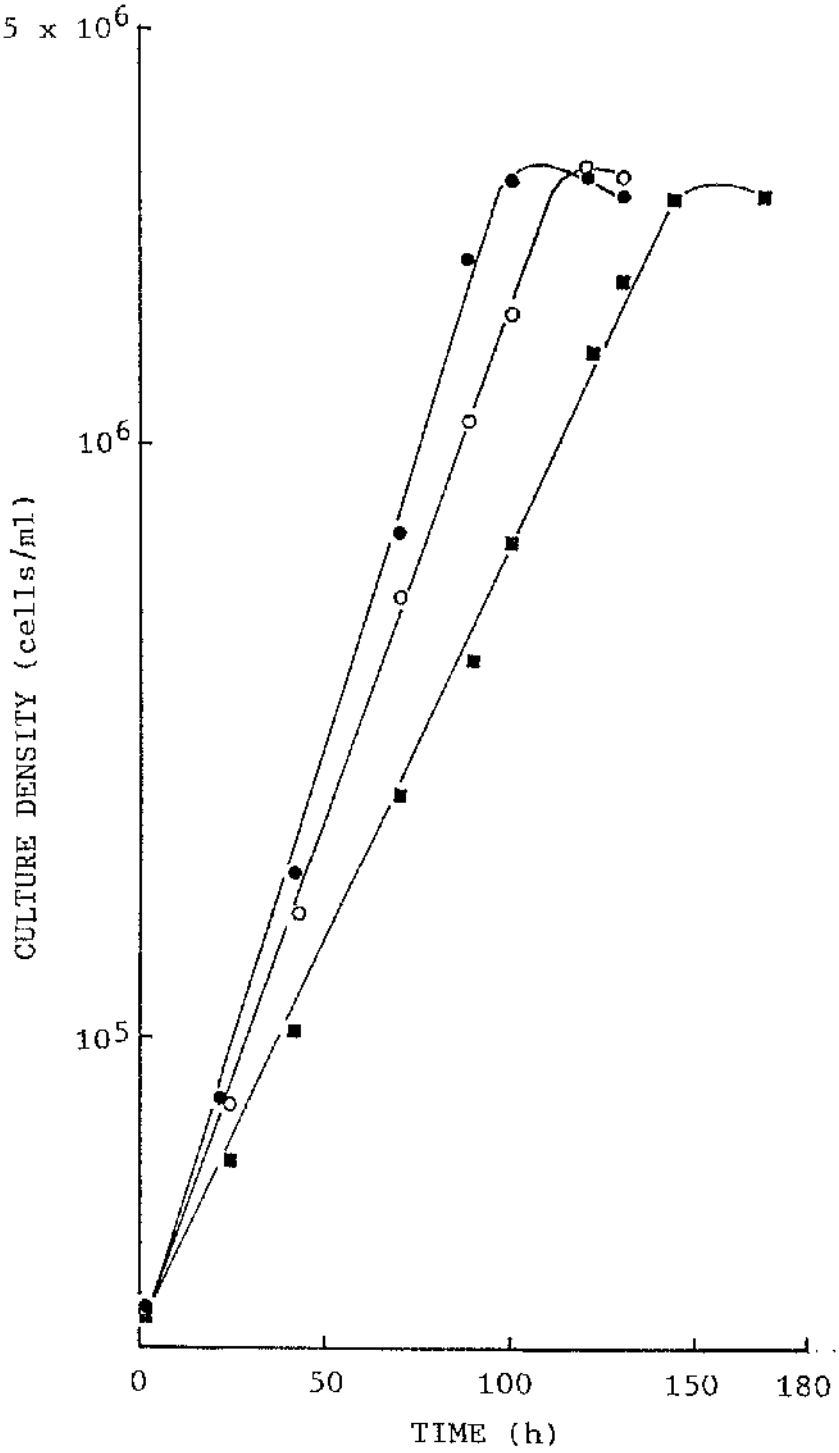

5 x 10^6
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
180
TIME (h)

Figure 21: Growth of D. discoideum Ax2 in ALM, prepared at pH6.7 with phosphate and MES buffers added at different concentrations.

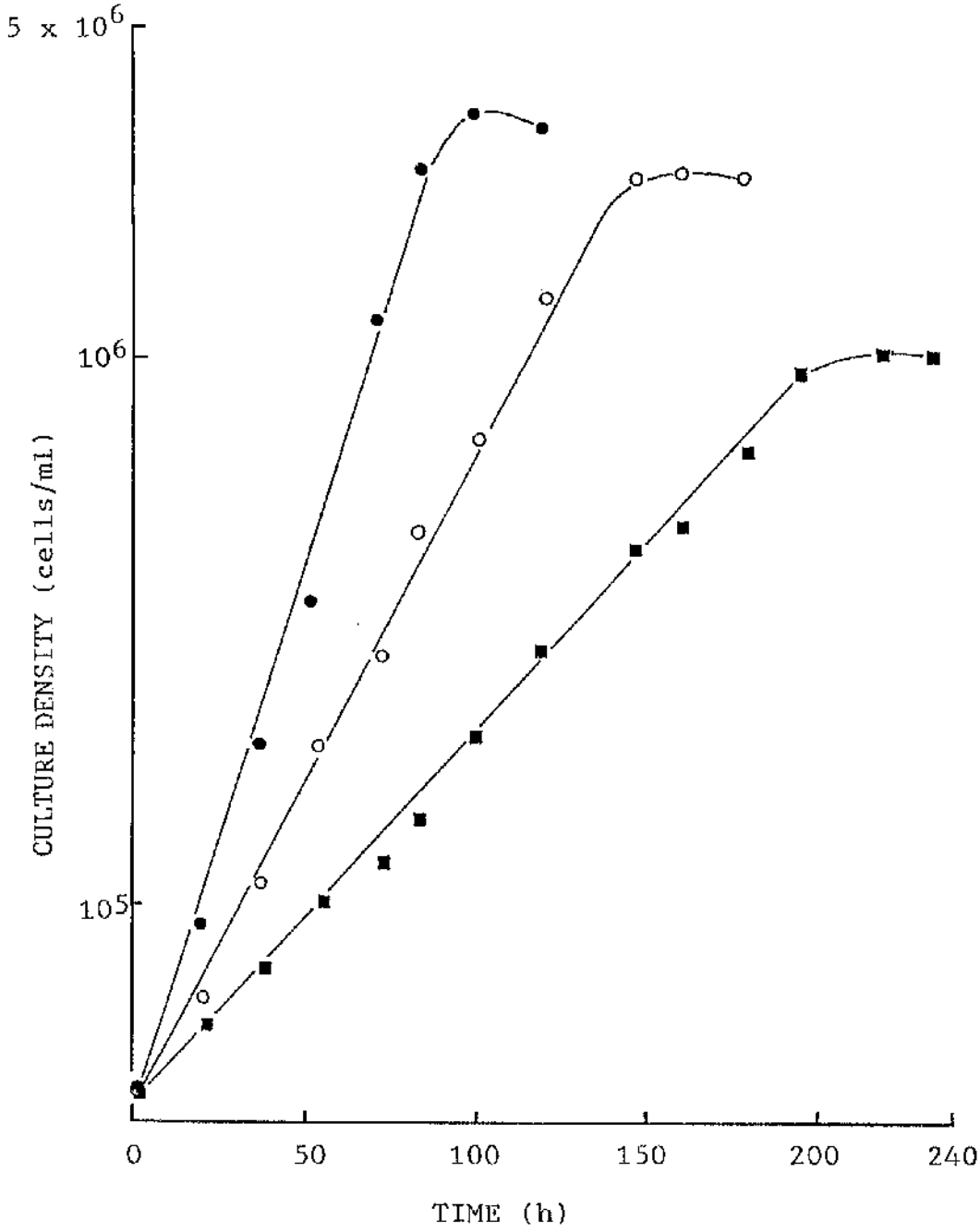
D. discoideum Ax2 was grown in ALM with buffers (MES & PO_4) added at different concentrations and pH set at 6.7, using an inoculum grown through 2 passages of ALM pH6.7 and inoculated to give an initial cell density of $5x10^4$ cells/ml. Samples were taken at intervals and pH and cell density estimated as described in methods 6 and 7.

● : ALM

○ : ALM + 14.38 mM PO_4 + 10 mM MES

□ : ALM + 28.76 mM PO_4 + 20 mM MES

	ALM	ALM+14.38 mMM PO_4+10 mM MES	ALM+28.76 mM PO_4+20 mM MES
maximum cell density	$2.8x10^6$	$2.1x10^6$	$1x10^6$
growth rate (h^{-1})	0.039	0.027	0.018
pH at the end of growth	7.5	6.9	6.7


5 x 10^6
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
240
TIME (h)

The growth rate (μ) decreased to $0.018h^{-1}$ from a control value $0.039h^{-1}$ while the growth yield was decreased to 36% of the control value.

2.2.1.2 pH controlled experiments.

To examine the influence of pH on growth, in the absence of pH change, cultures were grown at several pH values where pH was maintained by addition of acid. Experiments were carried out at pH values of 6.0, 6.2, 6.5, and 6.7 in both the presence and absence of added glucose (Figure 22, 23, 24, 25 and Table 3).

At all pH values tested the growth rate of the cells was independent of pH but the yield obtained was greatly increased by growth at lower pH values. At pH6.2, for example, the final cell density achieved was 6.6×10^6 cells/ml-an increase of 38% on the yield obtained at pH6.7.

A similar but much less pronounced effect was produced when glucose, at 86 mM, was present at these pH values. Again the growth rate was constant ($0.029h^{-1}$), but the yield at low pH values was 15% greater than that observed at pH6.7 (Table 3).

Figure 22. Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.7.

D. discoideum Ax2 was grown in ALM and ALM_g prepared at pH6.7, using inocula grown through 2 passages of equivalent media and inoculated to give an initial cell density of $5x10^4$ cells/ml. pH was maintained at 6.7 (methods 7.3) Samples were taken at intervals to estimate pH and cell density as described in methods 6 and 7.

● : ALM

○ : ALM_g

	ALM	ALM_g
maximum cell density	$4.8x10^6$	$1.3x10^7$
growth rate (h^{-1})	0.035	0.029

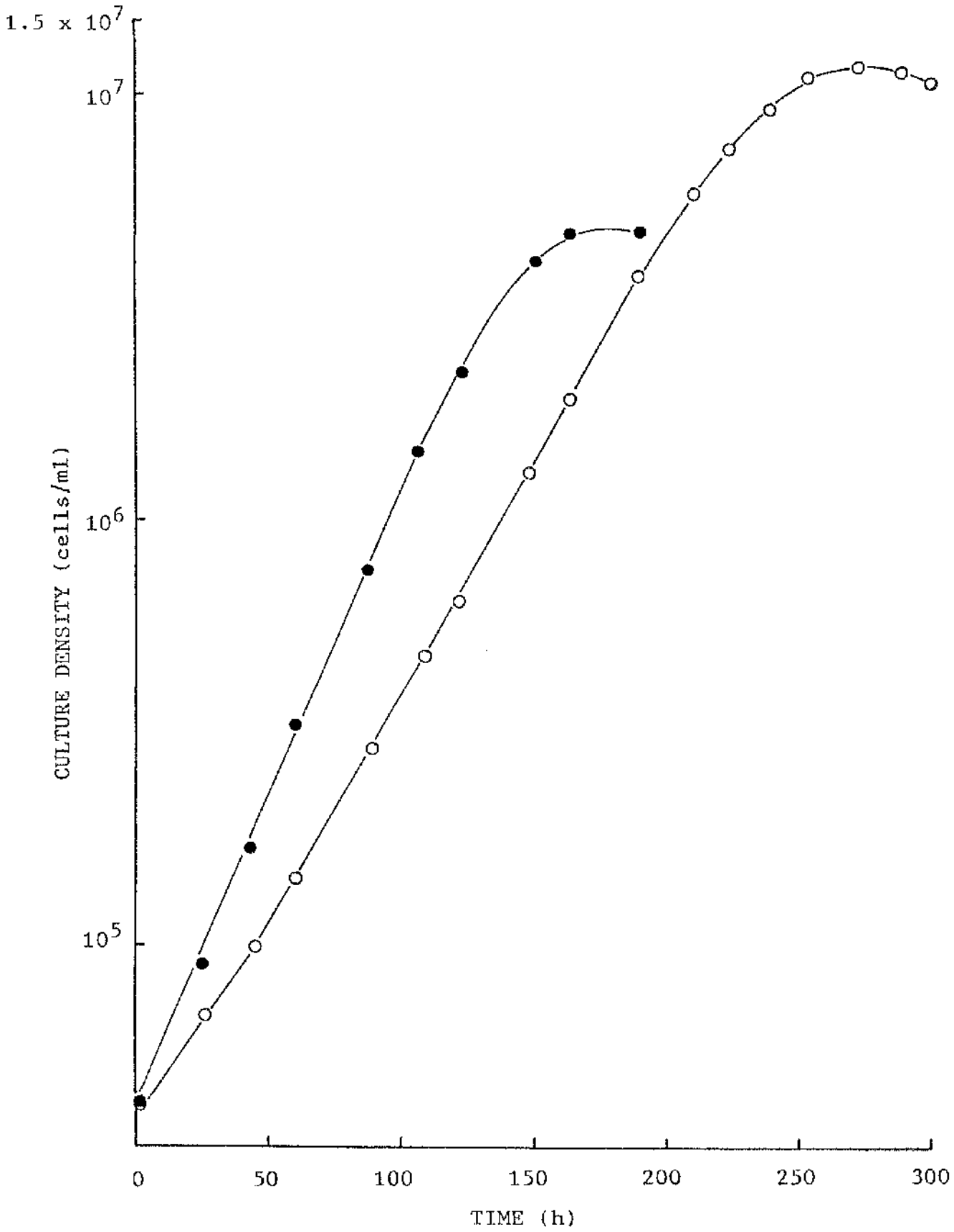

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
250
300
TIME (h)

Figure 23. Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.0.

D. discoideum Ax2 was grown in ALM and ALM_g prepared at pH6.0, using inocula grown through 2 passages of equivalent media and inoculated to give an initial cell density of $5x10^4$ cells/ml. pH was maintained at 6.0 as described in methods 7.3. Samples were taken at intervals to estimate pH and cell density (methods 6, 7).

● :ALM

O :ALM_g

	ALM	ALM_g
cell density	$6.8x10^6$	$1.5x10^7$
growth rate (h^{-1})	0.035	0.029

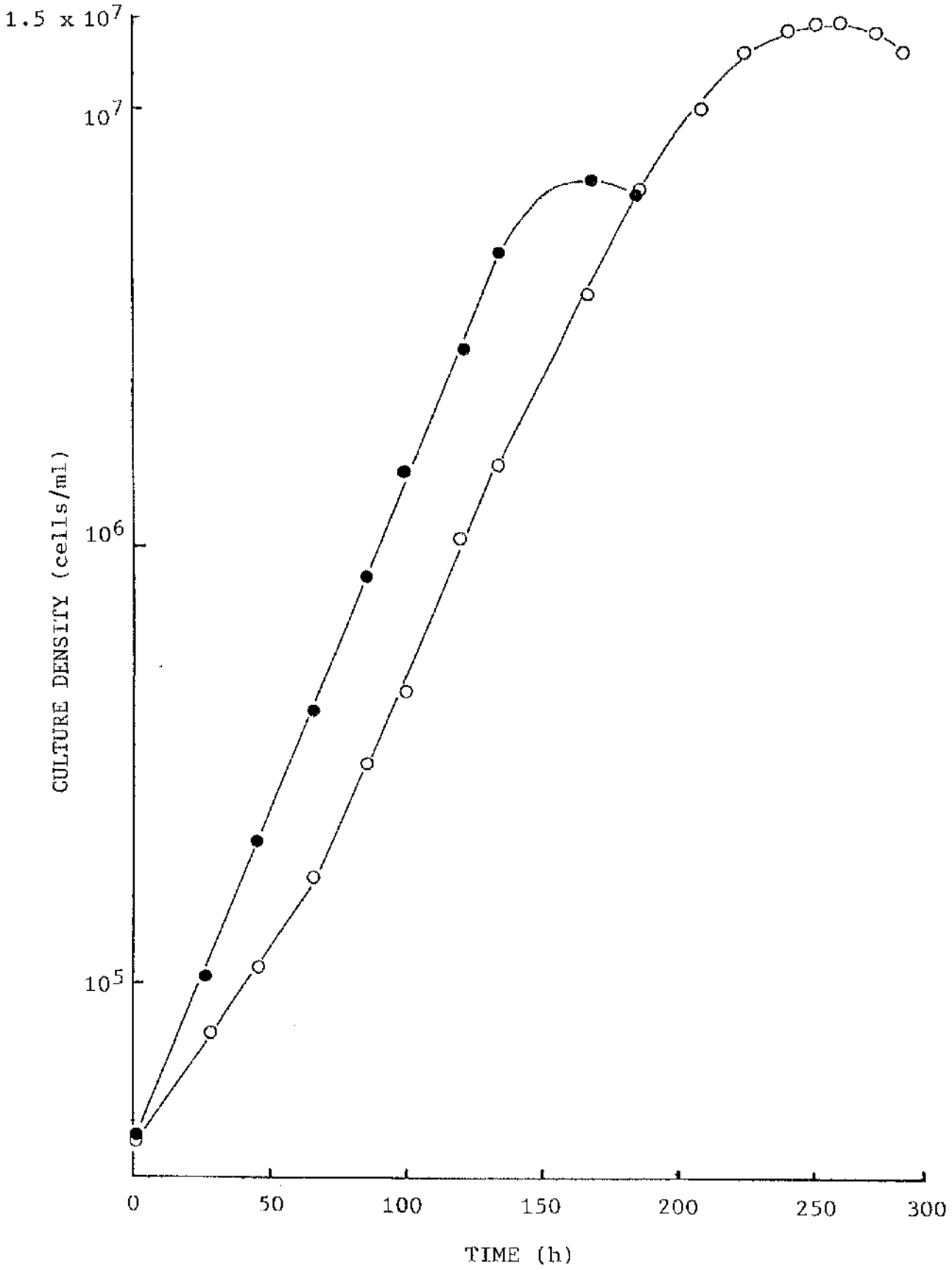

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
250
300
TIME (h)

Figure 24. Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.2.

D. discoideum AX2 was grown in ALM and ALM_g prepared at pH6.2, using inocula grown through 2 passages of equivalent media and inoculated to give an initial cell density of $5x10^4$ cells/ml. pH was maintained at 6.2 (methods 7.3). Samples were taken at intervals to estimate pH and cell density as described in methods 6 and 7.

● : ALM

○ : ALM_g

	ALM	ALM_g
cell density	$6.6x10^6$	$1.5x10^7$
growth rate (h^{-1})	0.035	0.029

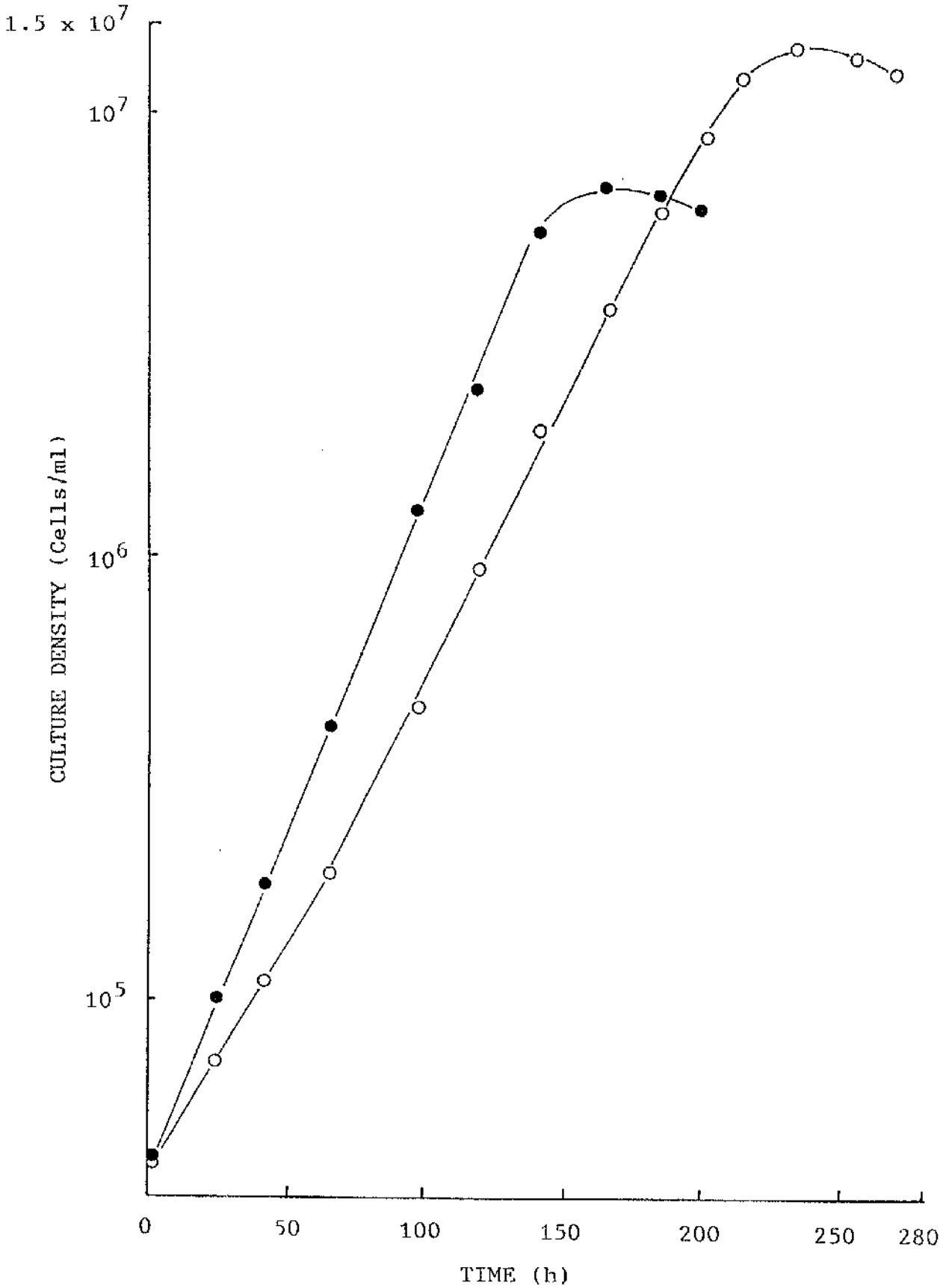

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (Cells/ml)
0
50
100
150
200
250
280
TIME (h)

Figure 25. Growth of *D. discoideum* Ax2 in ALM and ALM_g with pH maintained at 6.5.

D. discoideum Ax2 was grown in ALM and ALM_g prepared at pH6.5, using inocula grown through 2 passages of equivalent media and inoculated to give an initial cell denisty of $5x10^4$ cells/ml. pH was maintained at 6.5. (methods 7.3). Samples were taken at intervals to estimate pH and cell density as described in methods 6 and 7.

● : ALM

O : ALM_g

	ALM	ALM_g
cell density	$6x10^6$	$1.4x10^7$
growth rate (h^{-1})	0.035	0.029

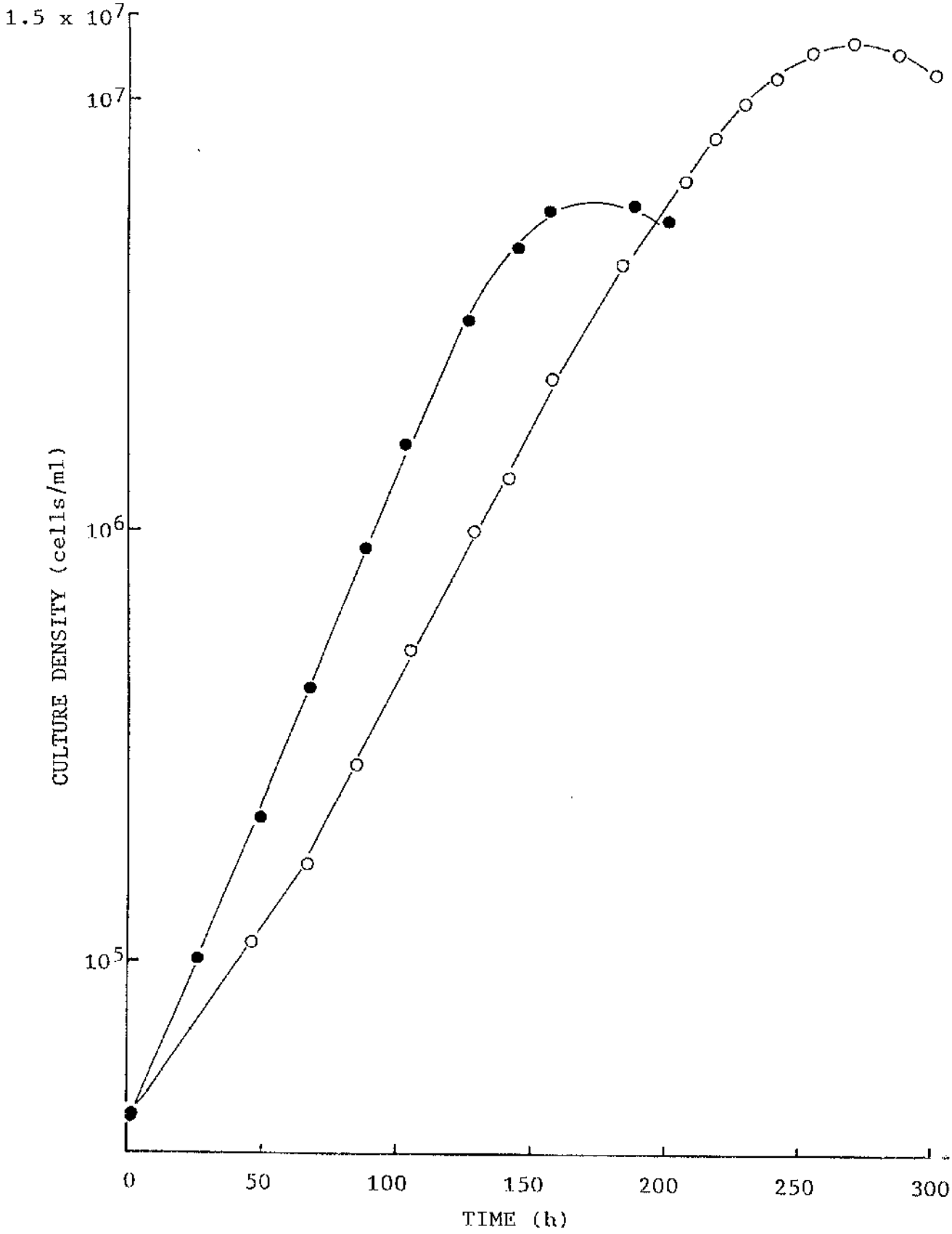

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
250
300
TIME (h)

Table 3: Growth of D. discoideum Ax2 in ALM and ALM_g with pH maintained at 6.7, 6.5, 6.2 and 6.0.

Culture pH	ALM		ALM_g	
	Cell Density	Growth Rate (h^{-1})	Cell Density	Growth Rate (h^{-1})
6.7	4.8×10^6	0.035	1.3×10^7	0.029
6.5	6×10^6	0.035	1.4×10^7	0.029
6.2	6.6×10^6	0.035	1.5×10^7	0.029
6.0	6.8×10^6	0.035	1.5×10^7	0.029

Data in this table summarise the information from growth experiments shown in Figure 22, 23, 24 and 25.

3. Influence of carbohydrates on growth in ALM.

3.1. Glucose: Glucose, at 86 mM, is usually added to ALM for the growth of cells. Its presence profoundly alters not only the growth rate and yield of the cells but also the nature of the culture in other less obvious ways. The presence of 86 mM glucose in ALM increases the cell yield 4 fold. This increase is considerably reduced when the pH of the culture is controlled. With pH maintained at 6.2 the increase in yield is only 2 fold (Figure 24). However, the reason for this is not an alteration of yield in the presence of glucose but an effect on the culture growing without glucose supplementation.

In the absence of pH control, but with the medium prepared at pH6.2 to reduce the pH value at the end of growth, glucose influences the growth of the cells in 3 ways. It increases the density of the cells at stationary phase, decreases the growth rate of the cells and reduces the change in pH that occurs during growth. All of these effects are dependent on the concentration of glucose added to the medium. Within the concentration range tested, all of these effects increase as glucose concentration increases (Figure 26).

3.2. Fructose: The addition of fructose to the growth medium has an effect similar to glucose. In the presence of 86 mM fructose, with pH controlled at 6.2, the cell yield is increased 2 fold and the growth rate is decreased to $0.029h^{-1}$ (Figure 27).

3.3. Galactose: Galactose produced quite different results to glucose and fructose when added to ALM at 86 mM with the pH controlled at 6.2. It decreased the growth rate (μ) to $0.023h^{-1}$, a lower rate of growth than that produced by the presence of the other sugars but did not increase cell yield. In the presence of galactose the cell density is reduced to 34% of the value obtained in the absence of galactose (Figure 28).

Figure 26. Growth of D. discoideum Ax2 in ALM and ALM_g pH6.2 at different glucose concentrations.

D. discoideum Ax2 was grown in ALM pH6.2 at different glucose concentrations. The inoculum for the ALM culture was grown through 2 passagesof ALM pH6.2 whereas the inoculum for the glucose supplemented cultures was grown through 2 passages of ALM + 86 mM glucose, at pH6.2. In both cases the initial cell density was $5x10^4$ cells/ml.
Samples were taken at intervals to estimate pH and cell density as described in methods 6 and 7.

● : ALM

□ : ALM + 17.2 mM Glucose

○ : ALM 43 mM Glucose

■ : ALM + 86 mM Glucose

	ALM	ALM + 17.2 mM Glucose	ALM + 43 mM Glucose	ALM + 86 mM Glucose
cell density	$2.8x10^6$	$5.1x10^6$	$7.8x10^6$	$1.2x10^7$
growth rate (h^{-1})	0.039	0.035	0.035	0.029
pH at the end of growth	7.4	7.4	7.2	7.0

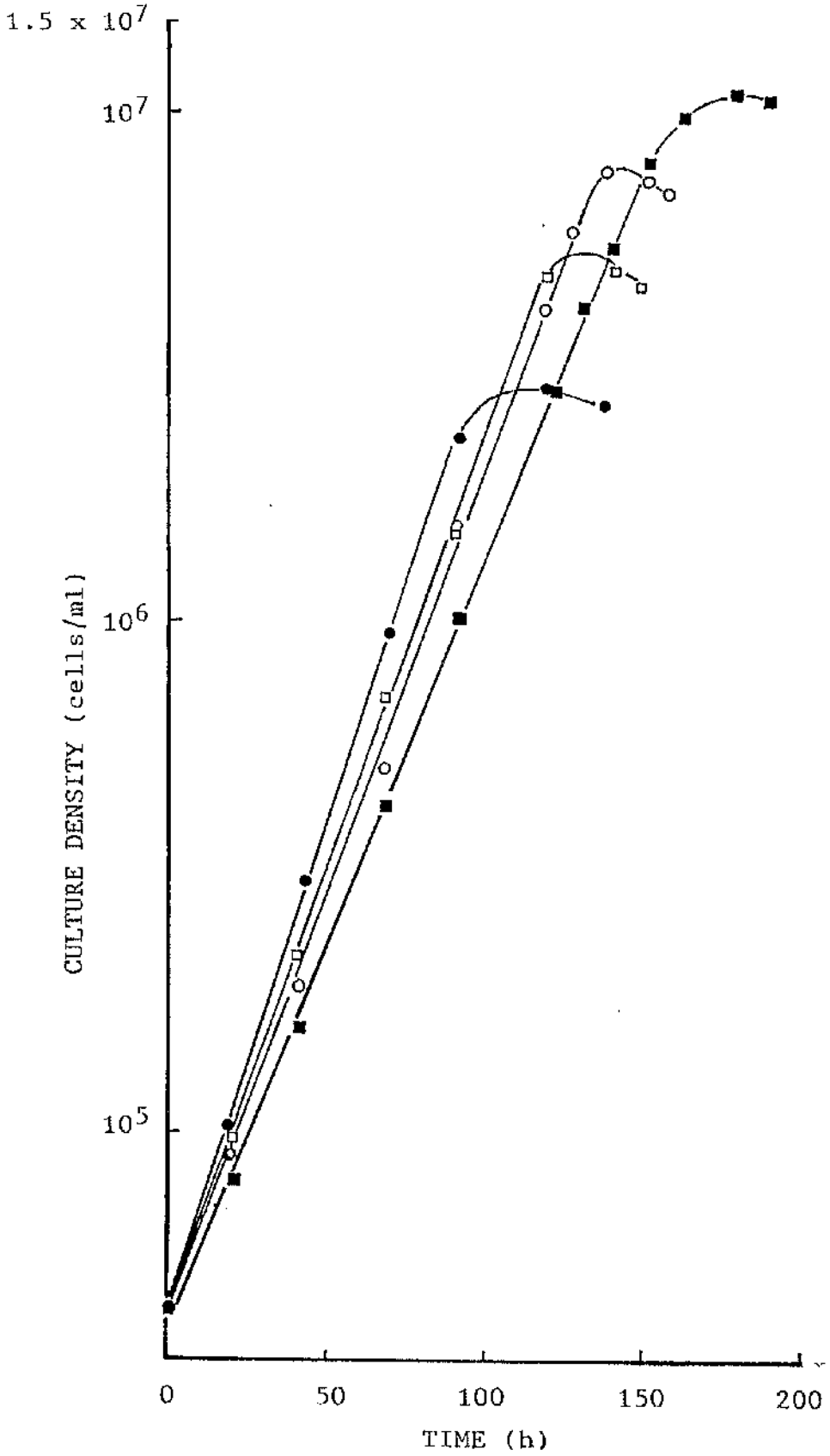

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
TIME (h)

Figure 27. Growth of D. discoideum Ax2 in ALM and $ALM_{fructose}$ with pH maintained at 6.2.

D. discoideum Ax2 was grown in ALM and ALM fructose prepared at pH6.2, using inocula grown through 2 passages of equivalent media at pH6.2 and inoculated to give an initial cell density of $5x10^4$ cells/ml. pH was maintained as described in methods 7.3. Samples were taken at intervals to estimate pH and cell density (methods 6 and 7).

●: ALM

O: $ALM_{fructose}$ (ALM + 86 mM fructose).

	ALM	$ALM_{fructose}$
cell density	$6x10^6$	$1.4x10^7$
growth rate (h^{-1})	0.037	0.029

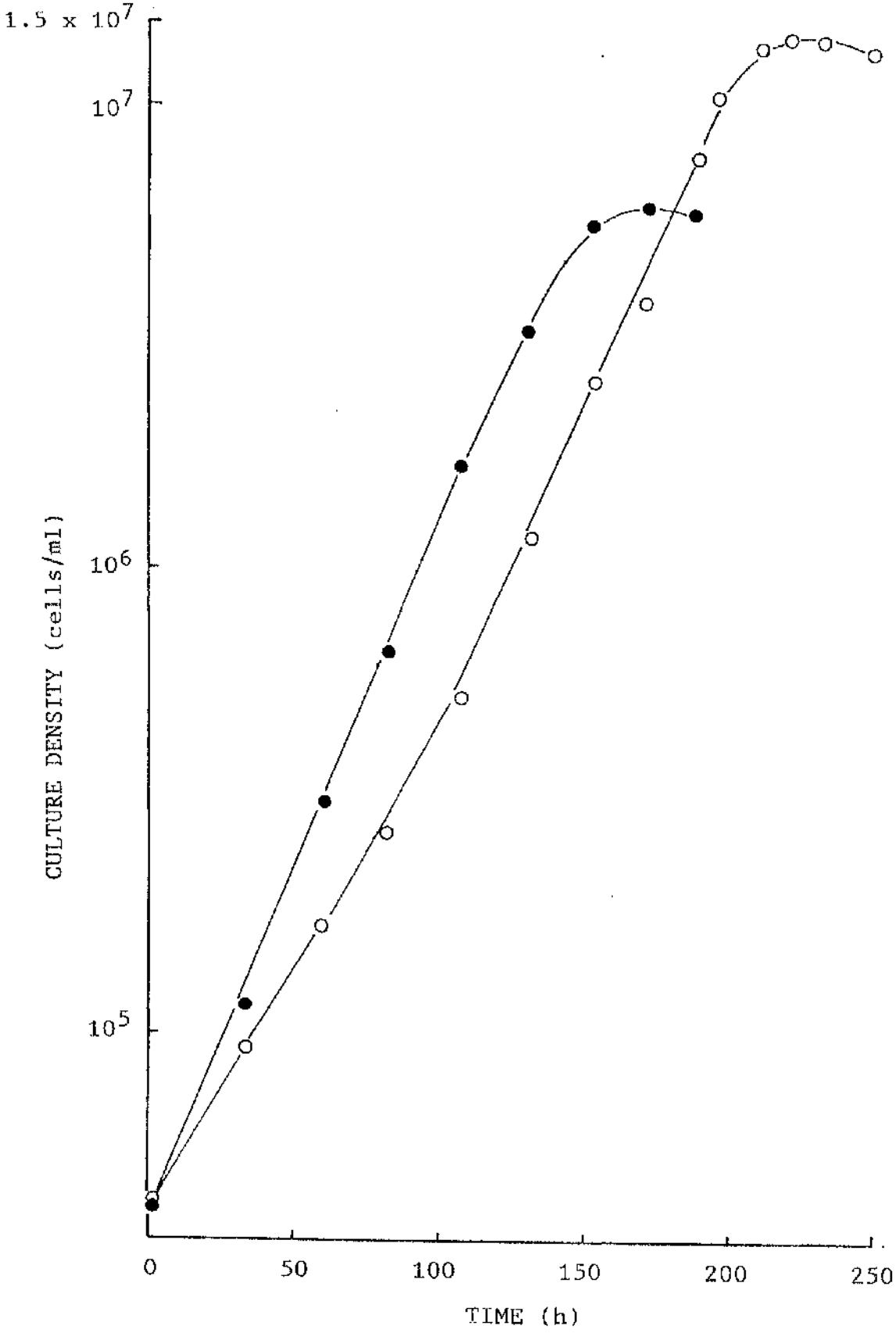

1.5 x 10^7
10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
250
TIME (h)

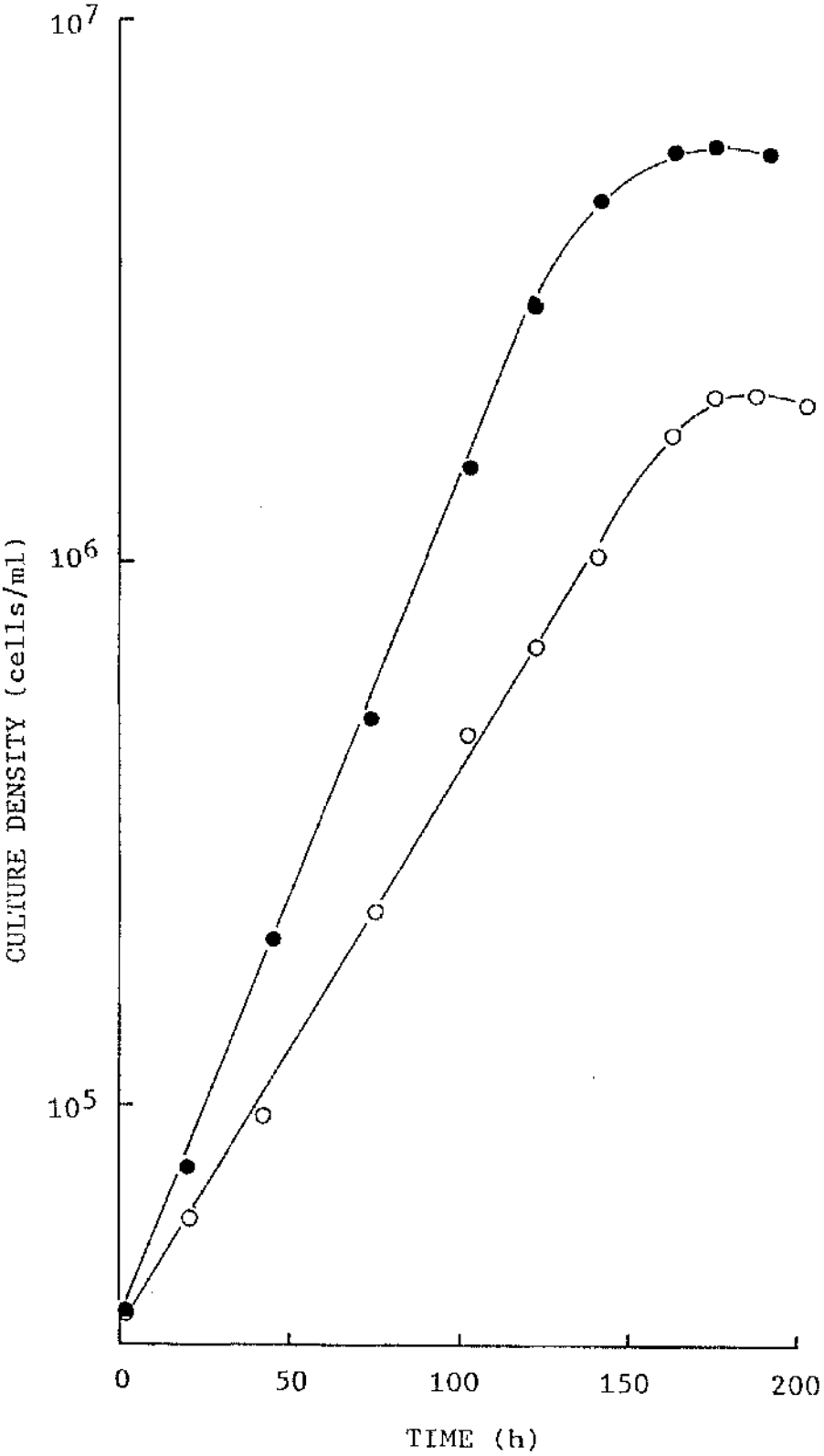
Figure 28. Growth of D. discoideum Ax2 in ALM and $ALM_{galactose}$ with pH maintained at 6.2.

D. discoideum Ax2 was grown in ALM and $ALM_{galactose}$ prepared at pH 6.2, using inocula grown through 2 passages of equivalent media and inoculated to give an initial cell density of $5x10^4$ cells/ml. pH was maintained at 6.2 (methods 7.3). Samples were taken at intervals to estimate pH and cell density as described in methods 6 and 7.

● : ALM

○ : $ALM_{galactose}$ (ALM + 86 mM galactose)

	ALM	$ALM_{galactose}$
cell density	$5.8x10^6$	$2x10^6$
growth rate (h^{-1})	0.037	0.023


10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
TIME (h)

4. Influence of pyruvate on growth in ALM.

The addition of pyruvate to the growth medium slightly increased the cell yield. In the presence of 20 mM pyruvate, with pH maintained at 6.2, the cell density at the end of growth was 25% higher than the control. Pyruvate, however, slightly reduced the growth rate (u) to $0.032h^{-1}$ (Figure 29).

5. Ammonia estimation during growth.

The concentration of ammonia in growing cultures were estimated using samples taken from the cultures that had been grown at constant pH values. In all cases the concentration of ammonia in the cultures increased with growth.

By the end of growth in ALM at different pH values, the amount of ammonia produced varied with pH. Ammonia concentrations at stationary phase were 20, 19, 17.5 and 14 mM at pH values of 6.0, 6.2 6.5 and 6.7 respectively (Table 4). When this data was rationalised to a common cell density value of 6×10^6 cells/ml the concentration of ammonia was 17.6 ± 0.34 mM (Table 4). A similar flask allowed to grow on ALM in the absence of pH control from an initial pH value of 6.2 to a final value of 7.4, produced 8.0 mM ammonia in achieving a cell density of 2.8×10^6 cells/ml (Figure 30, Table 5). When converted to a cell density of 6×10^6 cells/ml, this is equivalent to an ammonia concentration of 17.1 mM. Ammonia production was reduced with the increase in glucose concentration added in ALM under the same conditions and only 8.0 mM ammonia was produced at an equivalent cell density of 6×10^6 cells/ml at 86 mM glucose added in the medium (Figure 30, Table 5).

The presence of either glucose or fructose in the medium reduced the amount of ammonia produced. At a cell density of 6×10^6 cells/ml

Figure 29: Growth of D. discoideum Ax2 in ALM and $ALM_{pyruvate}$ with pH maintained at 6.2.

D. discoideum Ax2 was grown in ALM and $ALM_{pyruvate}$ prepared at pH6.2, using inocula grown through 2 passages of equivalent media and inoculated to give an initial cell density of 5 x 10^4 cells/ml. pH was maintained at 6.2 (methods 7.3). Samples were taken at intervals to estimate pH and cell density as described in methods 6 and 7.

● : ALM

O : $ALM_{pyruvate}$ (ALM + 20 mM pyruvate)

	ALM	$ALM_{pyruvate}$
cell density	$5.6x10^6$	$7x10^6$
growth rate (H^{-1})	0.037	0.032

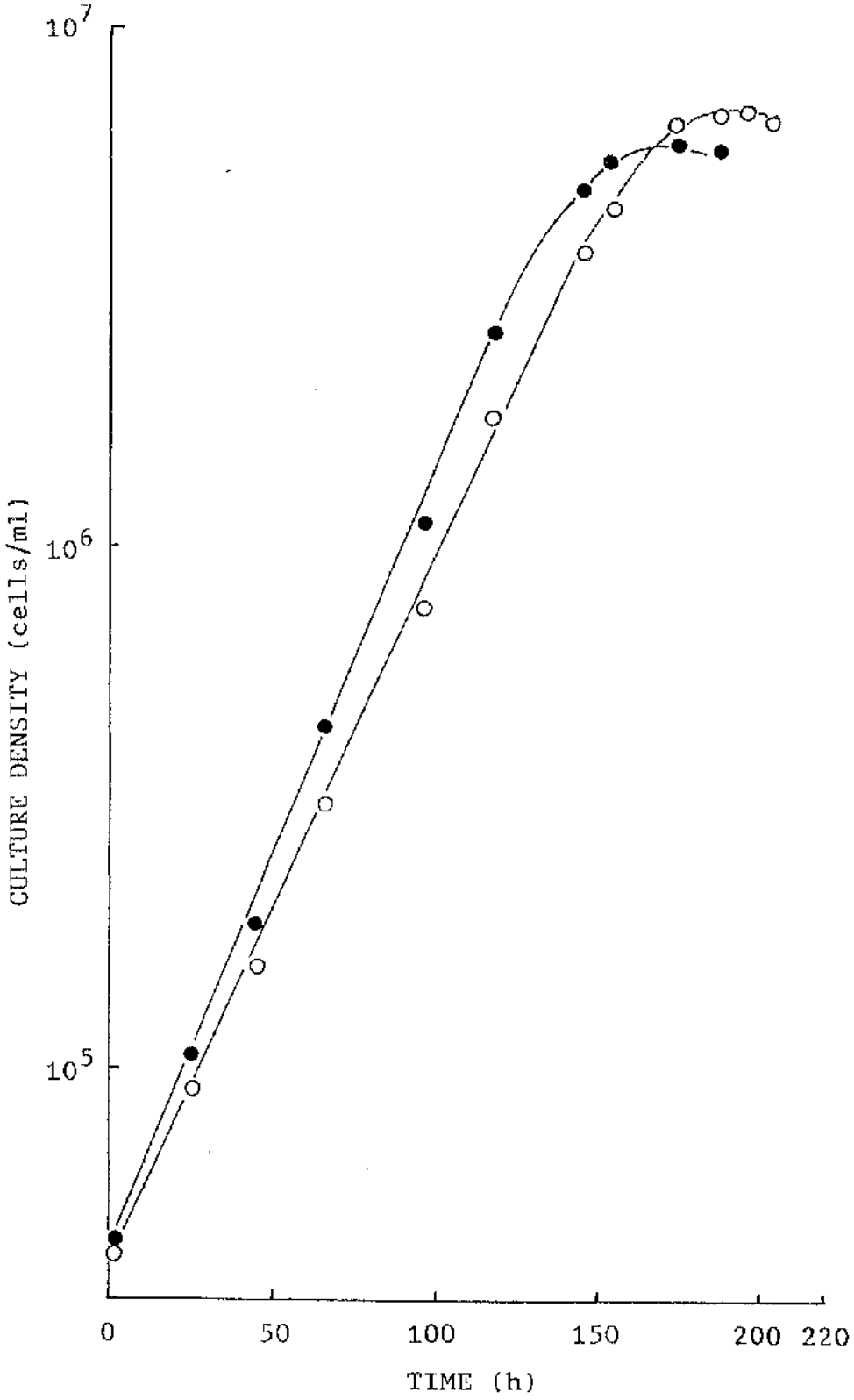

10^7
10^6
10^5
CULTURE DENSITY (cells/ml)
0
50
100
150
200
220
TIME (h)

Table 4: Ammonia production during the growth, at fixed pH, of D. discoideum Ax2 in supplemented ALM.

Samples were taken during the growth of D. discoideum Ax2 in ALM with supplements added and pH maintained at fixed values as described in growth experiments shown in Figures 22, 23, 24, 25, 27, 28 and 29. After HCl treatment and centrifugation (methods 9.4.2.), the samples were stored at -20°C. Ammonia assays were carried out using the phenol/hypochlorite method (methods 11.1.1.1.) and ammonia concentration plotted against time (see Figure 31). Ammonia production at the end of growth was determined from the graph and the concentration at a cell density of 6×10^6 cells/ml calculated.

Samples from the experiment described in Figure 24 were also assayed for ammonia by the enzymic method described in methods 11.1.2.

	Culture pH	Supplement concentration	Final cell density (cells/ml)	Ammonia concentration at the end of growth (mM)	Ammonia concentration at $6x10^6$ cells/ml
ALM	6.0	-	$6.8x10^6$	20.0	17.6
ALM	6.2	-	$6.6x10^6$	19.0	17.2
				19.0 *	17.2 *
ALM	6.5	-	$6.0x10^6$	17.5	17.5
ALM	6.7	-	$4.8x10^6$	14.0	17.5
ALM	6.2		$6.0x10^6$	18.1	18.1
ALM	6.2		$5.8x10^6$	17.0	17.5
ALM	6.2		$5.6x10^6$	16.5	17.7
ALM	6.0	glucose (86 mM)	$1.5x10^7$	22.0	8.8
ALM	6.2	glucose (86 mM)	$1.5x10^7$	21.0	8.4
				20.0 *	8.0 *
ALM	6.5	glucose (86 mM)	$1.4x10^7$	20.0	8.5
ALM	6.7	glucose (86 mM)	$1.3x10^7$	19.0	8.8
ALM	6.2	fructose (86 mM)	$1.4x10^7$	19.0	8.1
ALM	6.2	galactose(86 mM)	$2.0x10^6$	6.0	18.0
ALM	6.2	pyruvate (20mM)	$7.0x10_6$	16.5	14.1

* - assay by enzymic method

Figure 30: Ammonia production during the growth of *D. discoideum* Ax2 in ALM pH6.2 at different concentrations of glucose.

Samples were taken during the growth of *D. discoideum* Ax2 in ALM pH6.2, at different glucose concentrations (growth experiment Figure 26). After HCl treatment and centrifugation (methods 9.4.2.1.), the samples were stored at -20°C. Ammonia assays were carried out, using the phenol/hypochlorite method as described in methods 11.1.1.1.

● : ALM

○ : ALM + 17.2 mM glucose

■ : ALM + 43 mM glucose

▲ : ALM 86 mM glucose

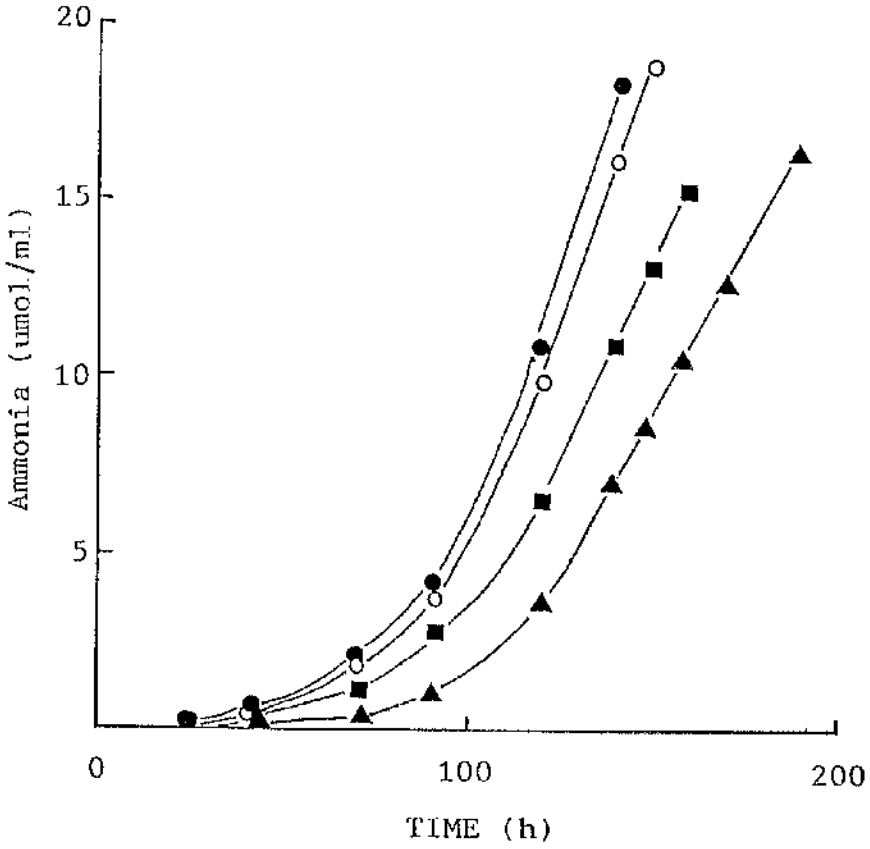

20
15
10
5
0
100
200
Ammonia (umol/ml)
TIME (h)

Figure 31: Ammonia production during the growth of D. discoideum Ax2 in ALM and $ALM_{fructose}$ with pH maintained at 6.2.

Samples were taken during the growth of D. discoideum Ax2 in ALM and $ALM_{fructose}$ with pH maintained at 6.2 as shown in Figure 27. After HCl treatment and centrifugation (methods 9.4.2.1.), the samples were stored at -20°C. Ammonia assays were carried out, using the phenol/hypochlorite method as described in methods 11.1.1.1.

● : ALM

O : $ALM_{fructose}$

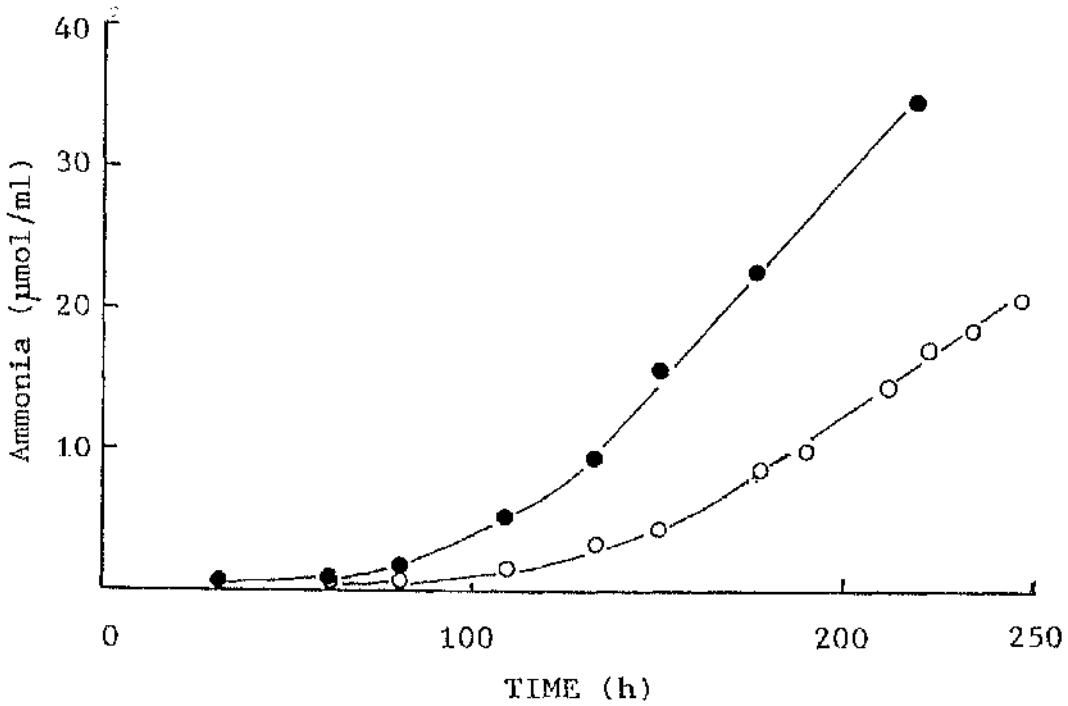

40
30
20
10
Ammonia (μmol/ml)
0
100
200
250
TIME (h)

Table 5: Ammonia production during the growth of *D. discoideum* Ax2 in ALM pH6.2 at different concentrations of glucose.

Samples were taken during the growth of *D. discoideum* Ax2 in ALM pH6.2 at different glucose concentations (growth experiment Figure 26). After HCl treatment and centrifugation (methods 9.4.2.1.), the samples were stored at -20°C. Ammonia assays were carried out using the phenol/hypochlorite method as described in methods 11.1.1.1. and ammonia concentration plotted against time. The concentration of ammonia at the end of growth was determined from this graph and concentration corresponding to a cell denisty of $6x10^6$ cells/ml calculated.

	Supplement concentration	Final cell density (cells/ml)	Ammonia concentration at the end of growth(mM)	Ammonia production at $6x10^6$ cells/ml
ALM pH6.2	-	$2.8x10^6$	8.0	17.1
ALM pH6.2	glucose (17.2 mM)	$5.1x10^6$	12.5	14.7
ALM pH6.2	glucose (43 mM)	$7.8x10^6$	14.0	11.5
ALM pH6.2	glucose (86 mM)	$1.2x10^7$	16.0	8.0

the concentration of ammonia in the glucose containing media was 8.6 ± 0.17 mM and in the presence of fructose, at pH6.2, was 8.1 mM (Table 4). By the end of growth in the presence of these two sugars the ammonia concentration had increased to 20.5 ± 1.2 mM and 19 mM respectively (Table 4).

Because the assay being used to measure ammonia appeared to be influenced by the composition of the samples (see methods 11.1.1.2) independent measurements of ammonia were carried out, on samples from the experiment shown in Figure 24, using an enzymic assay. Essentially the same results were obtained using this method (Table 4).

Galactose present in the medium had little effect on ammonia production. After growth, in the presence of galactose, which only reached a density of 2 x 10^6 cells/ml (Figure 28) the concentration of ammonia was 6 mM. When the data was rationalised to a density of 6 x 10^6 cells/ml, the concentration of ammonia produced was 18 mM and is close to the amount of ammonia produced by the control (Table 4).

Pyruvate added in the medium slightly reduced ammonia production. At maximum cell density, in the absence of pyruvate (5.6x10^6 cells/ml) 16.5 mM ammonia was produced which is equivalent to an ammonia concentration of 17.7 mM when converted to a cell density of 6 x 10^6 cells/ml. Whereas at an equivalent cell density(6 x 10^6 cells/ml), in the presence of pyruvate 14.1 mM ammonia was produced (Table 4).

In all growth experiments, whether in the presence of carbohydrate/pyruvate or not, ammonia production continued after the end of growth. As far as can be estimated the rate of ammonia production was fixed at the same rate as the maximum rate achieved by logarithmically growing cultures. A concentration of 30 mM was measured in an ALM culture after it had been in stationary phase for 35 h.

6. Changes in amino acid concentrations in the medium during the growth of *D. discoideum* Ax2.

Measurements of amino acid concentrations in samples taken during the growth of *D. discoideum* Ax2 in ALM and ALM_g pH6.2 did not show much change in either medium (Table 6, 7). Since ammonia production must be generated from some medium source, it is surprising that the amino acid content does not decline with growth. It appears however that, if anything, the concentration of amino acid increased. This is probably not a real effect but due to evaporation of liquid from the media with growth.

Based on the concentration of amino acid when cells reach a density of 10^6 cells/ml in both media, there is no change in amino acid concentration during growth until cells are harvested for analyses in washed cell suspensions.

Table 6: Changes in amino acid concentration (mM) during the growth of *D. discoideum* Ax2 in ALM pH6.2.

Samples were taken during the growth of *D. discoideum* Ax2 in ALM pH6.2 (experiment Figure 24) and stored at -20°C after centrifugation (methods 9.4.1.). Samples at inoculation (o time, culture density 5×10^4 cells/ml), 94 h (culture density 1.3×10^6 cells/ml) and at the end of growth (165 h, culture density 6.6×10^6 cells/ml) were used for amino acid analysis, using an LKB 4400 amino acid analyser as described in methods 11.5.1. All other samples, taken during growth, were also analysed and gave similar amino acid concentration but the data from these analyses are not shown.

Amino Acids	Amino Acid Concentration (mM)		
	o time	94 h	165 h
Alanine	15.60	15.38	15.54
Arginine	6.13	6.41	6.42
Aspartate	8.02	7.80	7.89
Glutamate	13.83	14.05	14.38
Glycine	36.97	38.60	40.22
Histidine	1.72	1.69	1.69
Isoleucine	2.47	2.58	2.73
Leucine	5.53	5.30	5.24
Lysine	5.40	5.51	5.43
Methionine	1.08	1.06	1.02
Phenylalanine	2.54	2.51	2.54
Serine	6.37	6.18	6.08
Threonine	3.51	3.49	3.52
Tyrosine	0.87	0.82	0.87
Valine	4.07	3.96	4.03

Table 7: Changes in amino acid concentrations (mM) during the growth of D. discoideum Ax2 in ALM_g pH6.2.

Samples were taken during the growth of D. discoideum Ax2 in ALM_g pH6.2, (experiment Figure 24) and stored at -20°C after centrifugation (methods 9.4.1.). Samples at inoculation (o time, culture density $5x10^4$ cells/ml), 130 h (culture density 1.2×10^6 cells/ml) and at the end of growth (268 h, culture density 1.5×10^7 cells/ml) were used for amino acid analysis using an LKB4400 amino acid analyser as described in methods 11.5.1.

Analyses on other samples were also carried out but data is not shown.

Amino Acids	Amino Acid Concentration (mM)		
	O time	130 h	268 h
Alanine	16.22	18.27	17.51
Arginine	5.77	6.39	6.36
Aspartate	8.64	9.75	8.93
Glutamate	14.20	13.83	15.34
Glycine	40.54	46.26	43.51
Histidine	1.06	1.11	0.87
Isoleucine	2.90	3.01	2.76
Leucine	5.59	5.96	5.44
Lysine	6.02	5.95	4.11
Methionine	0.84	0.92	0.63
Phenylalanine	2.62	3.00	2.72
Serine	5.71	6.55	5.88
Threonine	3.40	4.19	3.46
Tyrosine	0.89	1.04	0.93
Valine	5.77	6.39	6.36

7. Washed cell experiments

7.1. ALM cells.

ALM is a complex medium and a number of changes take place simultaneously with growth. To simplify the study of the rate of amino acid metabolism, cell suspensions prepared in 7.19 mM phosphate buffer (phosphate concentration in ALM) pH6.2, after growing the cells in bulk under defined conditions, were used to assess the rate of amino acid metabolism. Ammonia production was initially used as an indication of metabolism.

The production of ammonia in these cell suspensions, in the absence of extracellular nutrients, was proportional to both the time of incubation (Figure 32a) and to the cell density (Figure 32b). Linearity of ammonia production with time was also found when ammonia production was stimulated by adding ALM to the cell suspensions (Figure 33).

7.1.1. Effect of ALM and individual amino acids on ammonia production.

The amount of ammonia produced by the cells in washed cell suspension in phosphate buffer (endogenous rate) was stimulated by the addition of ALM to the incubation.

The cells' endogenous rate of ammonia production was 8.0 nmol/min/mg protein in phosphate buffer and this was increased to 56 nmol/min/mg protein when ALM was added to the cell suspension (Table 8).

Individual amino acids added to the cell suspensions also stimulated ammonia production. Addition of arginine, lysine, tyrosine and threonine stimulated the production of ammonia , in particular (Table 8).

Amino acids which stimulated ammonia production were selected for more detailed examination. Glutamic acid was also examined further.

Figure 32: Effect of incubation time and cell density on the endogenous production of ammonia by washed cell suspensions of D. discoideum Ax2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. from the cells grown in ALM at pH6.2. The suspension was diluted in 7.19 mM phosphate buffer pH6.2 to the densities shown and incubated with shaking at 23°C (methods 10.2.1.1.). Samples were taken at intervals for ammonia estimation during the incubation and stored at -20°C after HCl treatment and centrifugation (methods 10.2.2.).

Ammonia was estimated by the phenol/hypochlorite method as described in methods 11.1.1.1. (Figure 32-a)

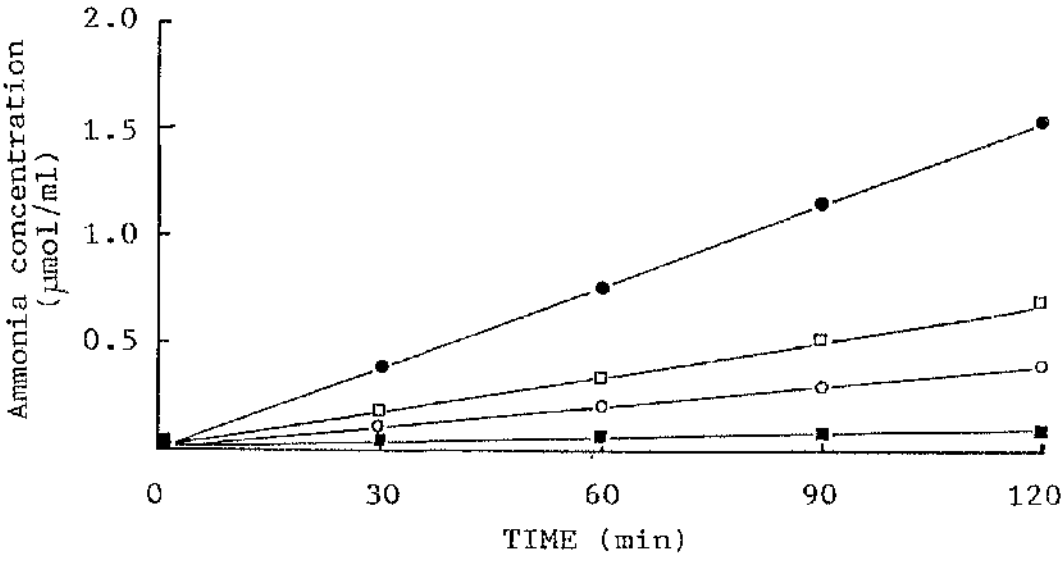
● : 2×10^7 cells/ml

□ : 1×10^7 cells/ml

○ : 5×10^6 cells/ml

■ : 1×10^6 cells/ml

The rate of ammonia production at each cell density was assessed from the gradient of the lines on graph (a) and plotted against cell density (Figure 32-b).


Ammonia concentration
(μmol/ml)
2.0
1.5
1.0
0.5
0
30
60
90
120
TIME (min)

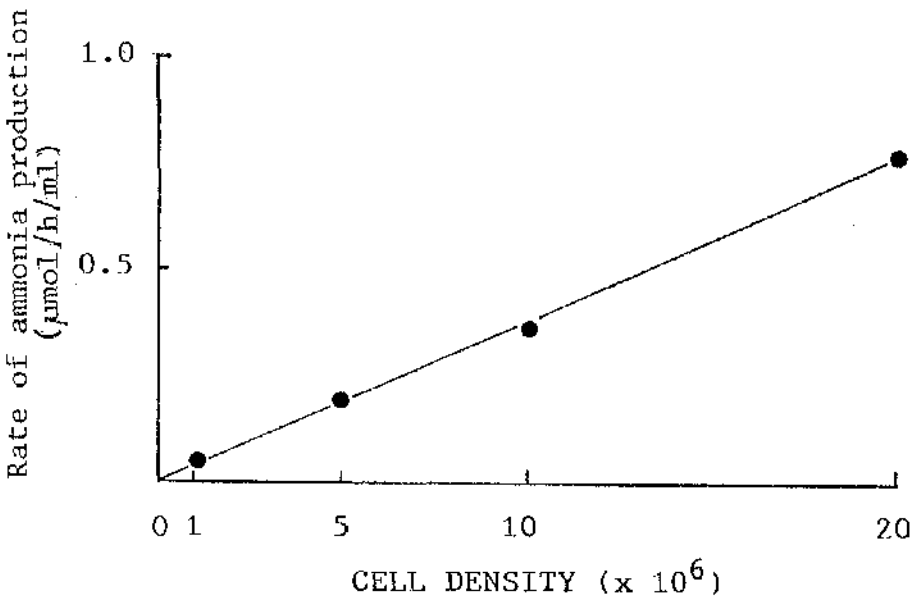

Rate of ammonia production
(μmol/h/ml)
1.0
0.5
0 1
5
10
20
CELL DENSITY (x 10^6)

Figure 33: Effect of ALM on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1, after growth in ALM pH6.2. Cells were incubated at 10^7 cells/ml in 7.19 mM phosphate buffer pH6.2 and in ALM pH6.2 (methods 10.2.1.). Samples were taken at intervals and after HCl treatment and centrifugation stored at -20°C (methods 10.2.2.). Ammonia assays were carried out, using the phenol/hypochlorite method as described in methods 11.1.1.1

● : ALM

○ : phosphate buffer

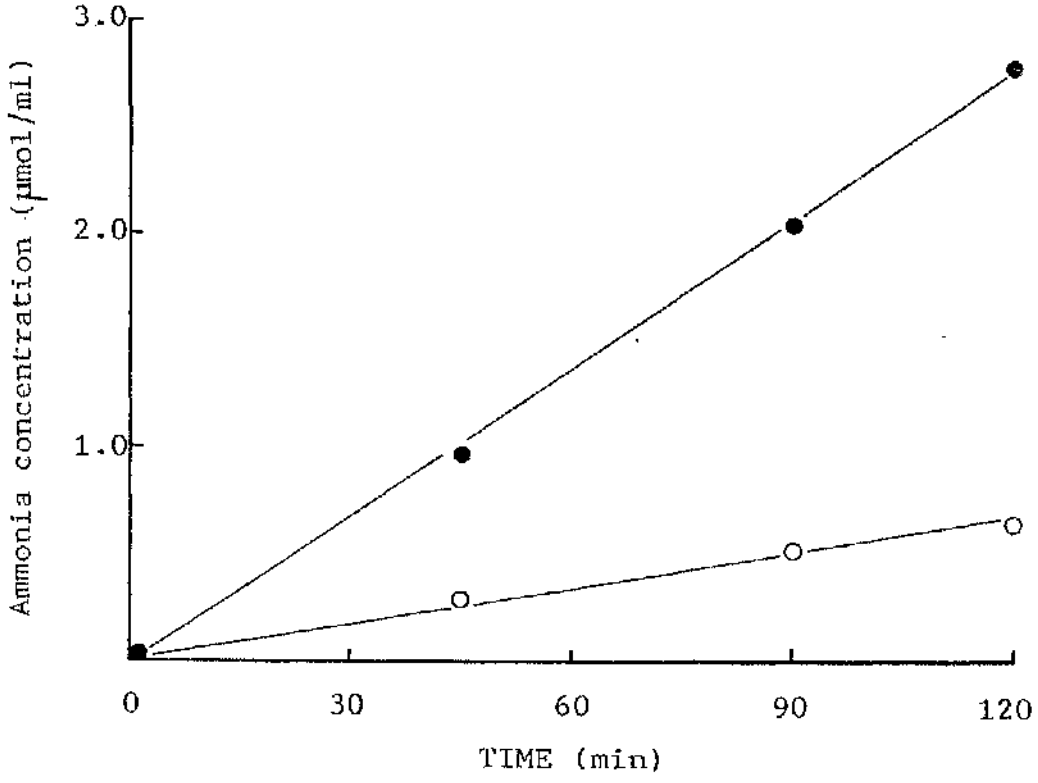

3.0
2.0
1.0
Ammonia concentration (μmol/ml)
0
30
60
90
120
TIME (min)

Table 8: Rate of ammonia production by washed cell suspension of D. discoideum Ax2, grown in ALM, in the presence of individual amino acids.

A washed cell suspension of D. discoideum Ax2 was prepared, as described in methods 10.1. from cells grown in ALM at pH6.2. The suspension was incubated, in 7.19 mM phosphate buffer pH6.2 with individual amino acids added to 5 mM. Samples were taken at intervals during the 100 min incubation and after HCl treatment and centrifugation, stored at -20^{0}C (methods 10.2.2.). Ammonia was estimated using the phenol/hypochlorite method as described in methods 11.1.1.1.
For each amino acid, the concentration of ammonia in the samples was graphed against time and the slope used to estimate the rate of ammonia production.

The results shown are the means of three independent experiments.

AMINO ACID	Rate of ammonia production (nmol/min/mg protein)
alanine	8.2
aspartic acid	11
arginine	19
glutamic acid	11
glycine	12.6
histidine	13.7
hydroxyproline	8
isoleucine	10.4
leucine	10
lysine	15
methionine	8
proline	11.5
phenylalanine	13.4
serine	13
threonine	15
tyrosine	14
valine	8.2
buffer	8
ALM	56

7.1.2. Influence of amino acid concentration on ammonia production.

The rate of ammonia production increased above the endogenous level, as the concentration of the amino acid added to the cell suspension was increased. There was, however no single pattern that related changes in ammonia production to concentration for each of the amino acids studied.

With each amino acid, and at all concentrations examined, the rate of ammonia production was linear with time and the individual points on Figure 34 represent rates of ammonia production measured over a period of 100 min.

In general, the rate of production of ammonia increased with concentration up to 10 mM. Further increase in concentration above 10 mM had lesser and more variable effects on rate of ammonia production. With two of the amino acids examined, tyrosine and arginine, there was a further increase in rate of ammonia formation. With other amino acids, ammonia production was constant.

As a result of these studies, 5mM was chosen as a standard concentration for further examination since it provided a clearly marked increase in ammonia production without oversaturating the system and as a consequence, masking any influence of experimentally made changes on utilization of amino acids or on ammonia production. The single exception to this pattern of ammonia production was lysine which provided almost maximal ammonia release at 1 mM. Nevertheless 5 mm was also selected as a suitable concentrations for further study of lysine metabolism since it was considered sensible to maintain the same molar concentration of all the amino acids until more information was available on their utilization.

All subsequent experiments on ammonia production in the presence of amino acids were carried out at an amino acid concentration of 5 mM.

Figure 34: Effect of amino acid concentration on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 in the presence of individual amino acids.

A washed cell suspension of D. discoideum Ax2 was prepared, as described in methods 10.1, after growth in ALM pH6.2. Cells were incubated at a density of 10^7 cells/ml for 100 min. in 7.19 mM phosphate buffer pH6.2 with individual amino acids added at different concentrations. Samples were taken at intervals over 100 mins., ammonia estimated (methods 11.1.1.1.) and the rate of ammonia production assessed using the slope of the graph of ammonia concentration against time for each condition.

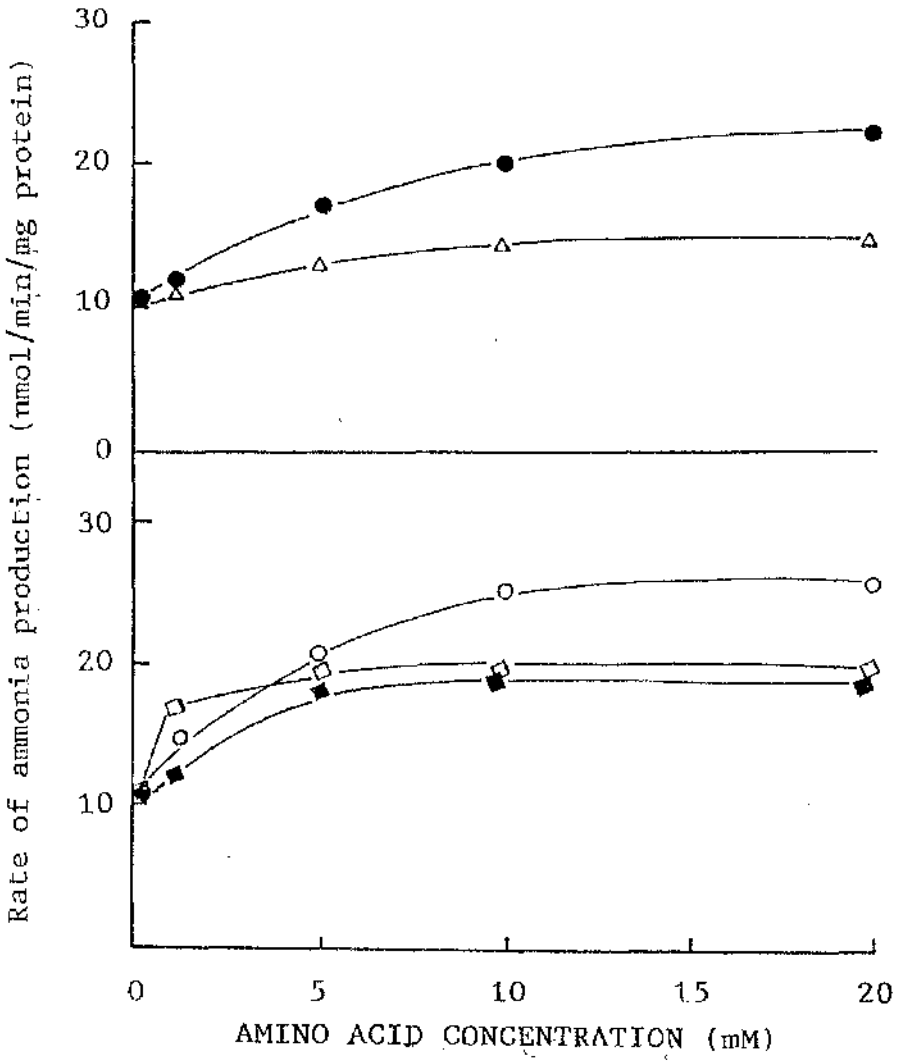
△ : glutamic acid

● : tyrosine

○ : arginine

◇ : lysine

◆ : threonine


30
20
10
0
30
20
10
Rate of ammonia production (nmol/min/mg protein)
0
5
10
15
20
AMINO ACID CONCENTRATION (mM)

7.1.3. Effect of nitrogen free metabolites on the rate of ammonia production by cell suspensions.

7.1.3.1. Effect of glucose: In both the presence and the absence of an exogenous nitrogen source, the rate of ammonia production was reduced by glucose (Table 9).

In the presence of ALM as an exogenous nitrogen source, the cells produced 53 nmol NH_3/min/mg protein. This rate of ammonia production was reduced to 26 nmol/min/mg protein when 86 mM glucose was added with ALM. Similarly, glucose reduced the endogenous rate of ammonia production from 9nmol/min/mg protein to 4.7 nmol/min/mg protein when added to a cell suspension in the absence of any exogenous nitrogen source.

7.1.3.2. Influence of other metabolites.

A range of metabolites, in addition to glucose were added to cell suspensions in both the presence and absence of exogenous sources of ammonia. To accentuate the effect produced, 86 mM concentrations of metabolites were provided when exogenous nutrients were present to stimulate ammonia production. In the absence of exogenous ammonia sources, only 20 mM was provided as an additive to ensure that the rate of ammonia production was not too low for reasonable measurements. The results shown in Table 10a and b indicate that the influence of metabolites was different if ammonia production was dependent on exogenous or endogenous nitrogen sources.

In the presence of ALM as an exogenous nitrogen source, the addition of glucose and trehalose reduced ammonia production to 50 and 52% respectively of the control value. The presence of the analogous compounds maltose and glycerol had a lesser effect, reducing ammonia production to only 68% of the control. The addition of the carboxylic

Table 9: Effect of glucose on the rate of ammonia production by washed cell suspensions of *D. discoideum* Ax2.

A washed cell suspension of *D. discoideum* Ax2 was prepared as described in methods 9.1. after growth in ALM pH6.2. The suspension, diluted to a density of 10^7 cells/ml in 7.19 mM phosphate buffer pH6.2, was incubated in the presence (86 mM) and absence of glucose (method 10.2.1.). Samples were taken at intervals during the 100 min. incubation and, after HCl treatment and centrifugation, stored at -20°C (methods 10.2.2.). Ammonia was estimated using the phenol/hypochlorite method as described in methods 11.1.1.1. and concentration plotted against time. Gradient of the line was used to calculate the rate of ammonia production.

The results are the mean of the three separate experiments.

AMMONIA PRODUCTION
nmol/min/mg protein

ALM	53
ALM + glucose	26
buffer	9
buffer + glucose	4.7

Table 10a: Effect of metabolites on the ALM stimulated rate of ammonia production by washed cell suspensions of D. discoideum Ax2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. from cells grown in ALM at pH6.2. The suspension (10^7 cells/ml) was incubated, as described in methods 10.2.1., in ALM pH6.2, in 7.19 mM phosphate buffer pH6.2 and in ALM with metabolites added at 86 mM.

Samples were taken at intervals during the 100 min. incubation and after HCl treatment and centrifugation, stored at -20°C (methods 10.2.2.). Ammonia was estimated using the phenol/hypochlorite method as described in methods 11.1.1.1 and the rate of ammonia production calculated from the slope of the graph of ammonia concentration against time.

The results shown are the mean of three independent experiments.

CONTENTS	Rate of ammonia production (nmol/min/mg protein)	% of ALM
ALM	56	100
buffer	10	18
ALM + glucose	28	50
ALM + maltose	38	68
ALM + glycerol	34	68
ALM + trehalose	29	52
ALM + malate	20	36
ALM + pyruvate	18	32
ALM + 2-oxoglutarate	15	27

Table 10b: Effect of metabolites on the endogenous rate of ammonia production by washed cell suspensions of D. discoideum Ax2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. from the cells grown in ALM at pH6.2. The suspension (10^7 cells/ml) was incubated, as described in methods 10.2.1. in 7.19 mM phosphate buffer pH6.2 with metabolites added at 20 mM. An incubation in ALM was also included.

Samples were taken at intervals during the 100 min. incubation and, after HCl treatment and centrifugation, stored at -20°C (methods 10.2.2.) ammonia was estimated using the phenol/hypochlorite method as described in method 11.1.1.1. and the rate of ammonia production calculated from the slope of the graph of ammonia concentration against time.

The results are the means of three separate experiments.

CONTENTS	Rate of ammonia production (nmol/min/mg protein)	% of buffer
ALM	54	540
buffer	10	100
buffer + glucose	5	50
buffer + maltose	5.4	54
buffer + glycerol	5.2	52
buffer + trehalose	5	50
buffer + malate	5.4	54
buffer + pyruvate	5.2	52
buffer + 2-oxoglutarate	5.0	50

acids, malate, pyruvate and 2-oxoglutarate all reduced ammonia production to 36 - 27% of the control value.

In the absence of an exogenous nitrogen source, all the compounds added to cell suspensions at 20 mM depressed ammonia production by about 50%. These latter values were subject to some error because of the low rate of ammonia production being measured.

Since all of these compounds influence ammonia production when ALM was added, as an exogenous nitrogen source, similar experiments were carried out using individual amino acids and the results shown in Table 11 were obtained. However it is difficult, due to the small changes in ammonia production being measured to place any quantitative significance on the results.

In summary, it would appear that, despite the effort, the only conclusion that can be made was that the presence of the metabolites reduce the extent to which exogenous amino acids could stimulate ammonia production.

7.2. ALM_g cells.

The production of ammonia from cell suspensions prepared from ALM_g cells (cells grown in ALM in the presence of 86 mM glucose), was similar to that obtained from cells grown in the absence of glucose. Ammonia production was proportional to both the time of incubation (Figure 35a) and the cell density (Figure 35b).

Linearity of ammonia production with time was also observed, when the rate of ammonia production was increased by adding nutrients such as ALM to the cell suspension (Figure 36).

7.2.1. Effect of ALM and individual amino acids.

Cells grown in the presence of glucose, produced only 4.2 nmol ammonia/min/mg protein and was increased to 22 nmol/min/mg potein with

Table 11: Effect of metabolites on the rate of ammonia production stimulated by amino acids, in cell suspensions of D. discoideum Ax2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. from cells grown in ALM pH6.2. The suspension was incubated, as described in methods 10.2.1. in 7.19 mM phosphate buffer pH6.2 with an amino acid at 5 mM and metabolites at 20 mM. Samples were taken at intervals during the 100 min incubation and after HCl treatment and centrifugation, stored at -20°C (methods 10.2.2.).

Ammonia was estimated using the phenol/hypochlorite method as described in methods 11.1.1.1. Ammonia concentration was plotted against time and the slope of the line used to calculate the rate of ammonia production. For each amino acid, the rate of ammonia production in the presence of amino acids, but no other additive, was taken as 100% and other values in the presence of additives calculated as % of this value.

Relative rate of ammonia production from added amino acids by ALM washed cell suspensions

ADDITIONS	buffer	arginine	glutamic acid	lysine	threonine	tyrosine
None	100	100	100	100	100	100
glucose	50	81	59	72	63	72
maltose	55	86	71	75	65	71
trehalose	50	81	58	74	60	76
glycerol	53	86	61	78	78	79
malate	52	75	61	65	58	65
pyruvate	51	73	57	63	57	61
2-oxo glutarate	50	70	54	63	59	59

Figure 35: Effect of incubation time and cell density on the endogenous production of ammonia by washed cell suspensions of D. discoideum Ax2 after growth in ALM_g pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. from cells grown in ALM_g at pH6.2. The suspension was diluted in 7.19 mM phosphate buffer pH6.2 to the densities shown and incubated with shaking at 23°C as described in methods 10.2.1. Samples were taken at intervals for ammonia estimation during the incubation and stored at -20°C after HCl treatment and centrifugation (methods 10.2.2.). Ammonia was estimated by the phenol/hypochlorite method as described in methods 11.1.1.1. (Figure 35a).

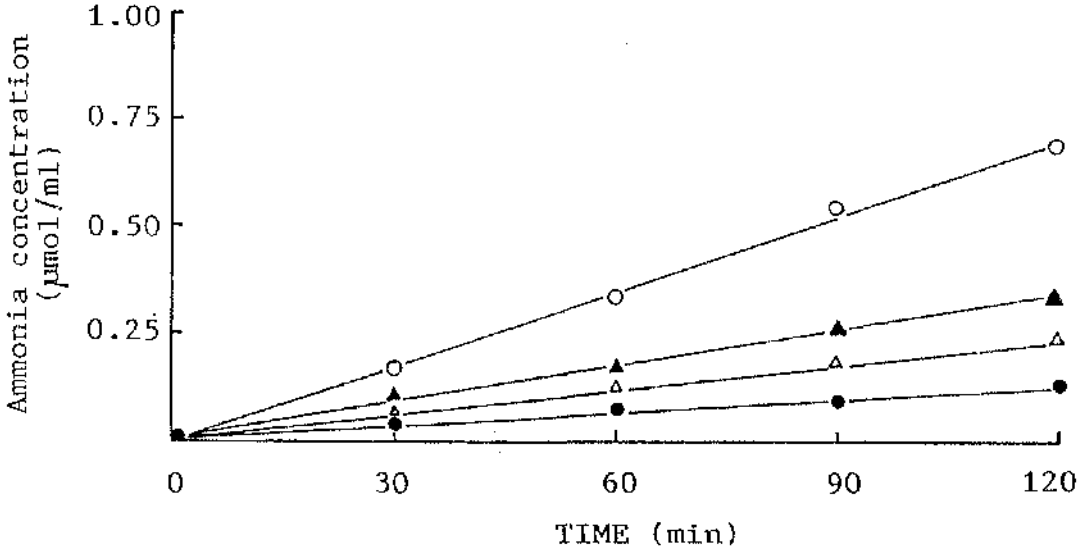
○ : 2×10^7 cells/ml

▲ : 1×10^7 cells/ml

△ : 5×10^6 cells/ml

● : 1×10^6 cells/ml

The rate of ammonia production at each cell density was assessed from the gradient of the lines on graph (a) and plotted against cell density (Figure 35b).


Ammonia concentration (μmol/ml)
1.00
0.75
0.50
0.25
0
30
60
90
120
TIME (min)

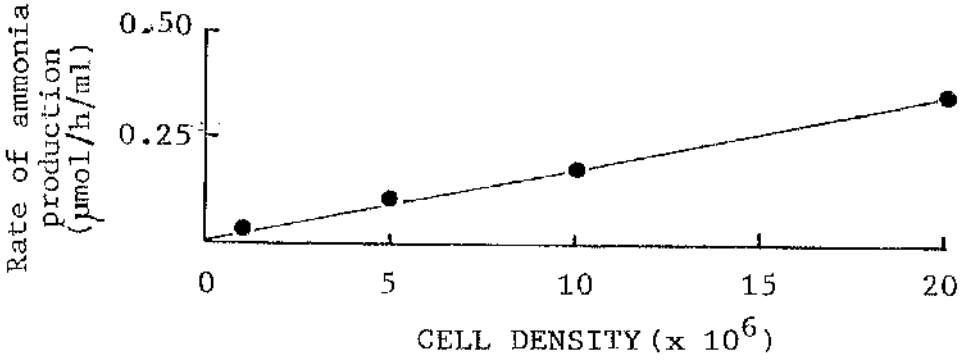
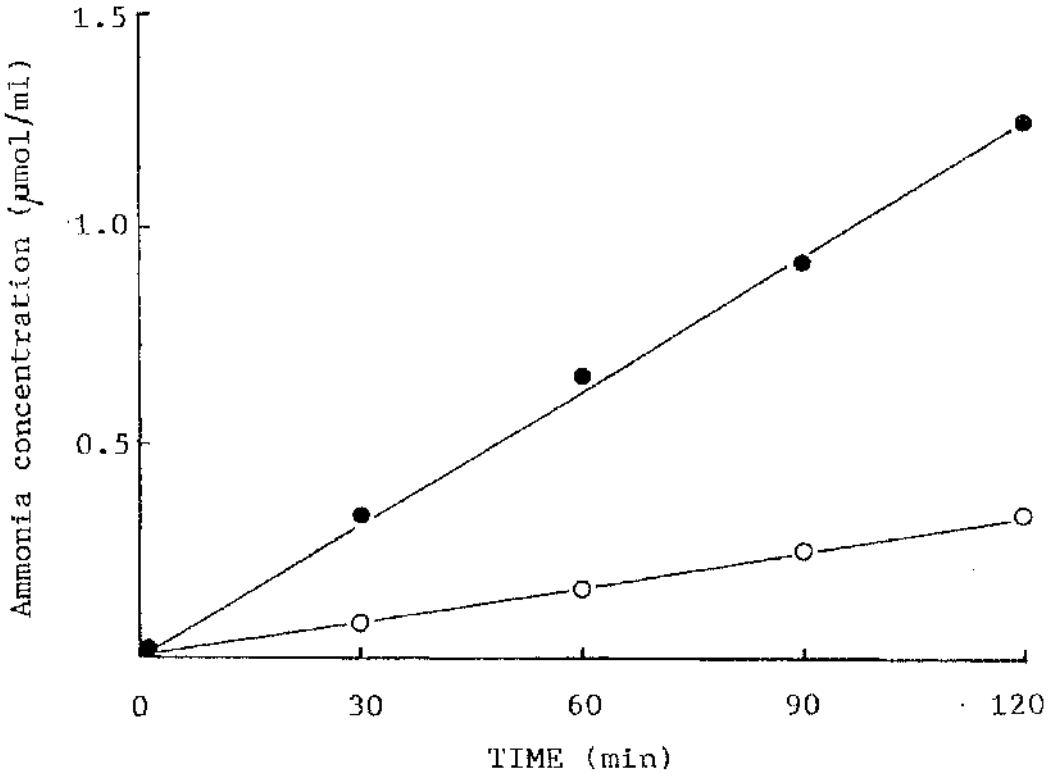

Rate of ammonia production (μmol/h/ml)
0.50
0.25
0
5
10
15
20
CELL DENSITY (x 10^6)

Figure 36: Effect of ALM on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 after growth in ALM_g pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. Cells were incubated at 10^7 cells/ml in 7.19 mM phosphate buffer and in ALM pH6.2 with shaking at 23°C as described in methods 10.2.1. Samples were taken at intervals, for ammonia estimation, during the incubation and stored at -20°C after HCl treatment and centrifugation (methods 10.2.2.). Ammonia was estimated by the phenol/hypochlorite method as described in methods 11.1.1.1.

● : ALM

○ : phosphate buffer


Ammonia concentration (μmol/ml)
1.5
1.0
0.5
0
30
60
90
120
TIME (min)

the addition of ALM to the cell suspension (Table 12). This compares with values of 9 nmol/min/mg protein and 53 nmol/min/mg protein respectively for ALM cells (Table 9). Individual amino acids, selected from the experiments carried out using ALM cells, also stimulated the rate of ammonia production by ALM_g cells. As with ALM cells, the stimulation of ammonia production was greater when arginine and lysine were added, although stimulation was also observed with threonine, glutamic acid and tyrosine (Table 12). In all cases the rates of production of ammonia were lower than those obtained from equivalent experiments using cells grown in the absence of glucose

7.2.2. Effect of nitrogen free metabolites on the rate of ammonia production by ALM_g cell suspension.

7.2.2.1. Effect of glucose: Glucose (86 mM) added to the cell suspension reduced the endogenous rate of ammonia production from 4.2 nmol/min/mg protein to 2.0 nmol/min/mg protein. In the presence of ALM, cells produced 21 nmol ammonia/min/mg protein which was reduced to 9.8 nmol/min/mg protein by adding 86 mM glucose to the cell suspension (Table 13).

Overall, glucose had a similar effect on the rate of ammonia production when added to ALM or ALM_g cells.

7.2.2.2. Influence of other metabolites.

Metabolites, other than glucose, also reduced ammonia production by ALM_g cells. The endogenous rate of ammonia production was reduced by all the metabolites at 20 mM to 51 - 56% of the control value (Table 14).

As with ALM cells, an attempt was made to look at the influence of additives on the ability of exogenous amino acids to stimulate ammonia production by the cells. Inhibition of stimulation was obtained in all cases but quantitatively it is difficult to draw a firm conclusion because of the small changes in ammonia production (Table 15).

Table 12: Effect of individual amino acids on the rate of ammonia production by washed cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. from cells grown in ALM_g pH6.2. The suspension was incubated in 7.19 mM phosphate buffer pH6.2. with individual amino acids added at 5 mM (methods 10.2.1).

Samples were taken at intervals for ammonia estimation during the 100 min. incubation and stored at -20°C after HCl treatment and centrifugation (methods 10.2.2.). Ammonia was estimated by the phenol/hypochlorite method as described in methods 11.1.1.1. and its concentration graphed against time to allow assessment of the rate of ammonia production.

The results shown are the means of three separate experiments.

Amino Acid	Rate of NH_3 production nmol/min/mg protein
arginine	10
glutamic acid	4.5
lysine	10
threonine	7.5
tyrosine	7.3
buffer	4.2
ALM	22

Table 13: Effect of glucose on the rate of ammonia production by washed cell suspensions of *D. discoideum* Ax2 grown in ALM_g pH6.2.

A washed cell suspension of *D. discoideum* Ax2 was prepared, as described in methods 10.1. after growth in ALM_g pH6.2. The suspension diluted to a density of 10^7 cells/ml in 7.19 mM phosphate buffer was incubated (methods 10.2.1.) in the presence (86 mM) and absence of glucose. Samples were taken at intervals for ammonia estimation during the 100 min. incubation and stored at -20°C after HCl treatment and centrifugation (methods 10.2.2.). Ammonia was estimated by the phenol/hypochlorite method as described in methods 11.1.1.1. and its concentration graphed against time to allow assessment of the rate of ammonia production.

The results shown are the means of three independent experiments.

	Ammonia production nmol/min/mg protein
ALM	22
ALM + glucose	9.8
buffer	4.2
buffer + glucose	2.0

Table 14: Effect of metabolites on the endogenous rate of ammonia production by washed cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared, as described in methods 10.1. from cells grown in ALM_g pH6.2. The suspension was incubated (methods 10.2.1.) in 7.19 mM phosphate buffer with metabolites added at 20 mM. An incubation in ALM was also carried out.

Samples were taken at intervals for ammonia estimation during the 100 min. incubation and stored at -20°C after HCl treatment and centrifugation (methods 10.2.2.). Ammonia was estimated using the phenol/hypochlorite method as described in methods 11.1.1.1. and its concentration graphed against time to allow assessment of the rate of ammonia production.

The results shown are the means of three separate experiments.

CONTENTS	Rate of ammonia production nmol/min/mg protein	% of Buffer
ALM	22	565
buffer	3.9	100
buffer + glucose	2.0	51
buffer + maltose	2.1	54
buffer + glycerol	2.2	56
buffer + trehalose	2.0	51
buffer + malate	2.1	54
buffer + pyruvate	2.2	56
buffer + 2-oxoglutarate	2	51

Table 15: Effect of metabolites on the rate of ammonia production stimulated by amino acids, in cell suspensions of D. discoideum Ax2 grown in ALM_g pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. from the cells grown in ALM_g at pH6.2. The suspension was incubated, as described in methods 10.2.1. in 7.19 mM phosphate buffer pH6.2 with an amino acid at 5 mM and metabolites at 20 mM.

Samples were taken at intervals during the 100 min. incubation and, after HCl treatment and centrifugation stored at -20°C (methods 10.2.2.).

Ammonia was estimated using the phenol/hypochlorite method as described in methods 11.1.1.1. Ammonia concentration was plotted against time and the slope of the line used to calculate the rate of ammonia production. Considering 100% rate of ammonia production when no metabolite was added as a control, other values were calculated as % age of the control.

Relative rate of ammonia production from added amino acids by ALM_g washed cell suspensions

ADDITIONS	buffer	arginine	glutamic acid	lysine	threonine	tyrosine
None	100	100	100	100	100	100
glucose	52	85	64	77	72	75
maltose	57	90	71	78	67	77
trehalose	54	86	62	78	69	78
glycerol	68	89	65	82	79	79
malate	57	73	63	68	72	77
pyruvate	62	68	65	75	73	62
2-oxo glutarate	60	67	69	72	69	67

8. Oxygen uptake by D. discoideum Ax2 cells.

8.1.1. Effect of ALM on the rate of oxygen uptake by washed cell suspensions.

Cells, harvested from logarithmic growth in ALM and resuspended in 7.19 mM phosphate buffer, metabolise endogenous reserves and generate a rate of oxygen uptake of 84 nmol/min/mg protein. The addition to the cell suspension, of a complete mixture of nutrients in the form of ALM and so producing conditions identical to growing cells, stimulated the oxygen uptake of the cells by 75% to 147 nmol/min/mg protein (Table 16).

The addition of higher concentrations of ALM, reduced the rate of respiration of the cells. In the presence of four times the normal value for ALM concentration, respiration was reduced to a rate less than that generated endogenously (Table 16).

8.2. Influence of amino acids and other metabolites on the rate of oxygen uptake by cells grown in ALM.

Those amino acids which had been observed to stimulate ammonia production were examined for their ability to influence oxygen uptake by the cell suspensions. No individual amino acid, added at 5 mM, produced a large effect on the rate of oxygen uptake. The addition of glutamic acid, produced the maximum stimulation and increased oxygen uptake by 11% (Table 17).

The amino acids, arginine and lysine, which gave the largest increase in ammonia production by washed cell suspensions (Table 8) were considered in more detail, although neither stimulated the oxygen uptake rate by more than 9% (Table 17). With these amino acids, those compounds, whose influence on the amino acid induced stimulation of ammonia production had been measured, were also examined. Little change was produced by the addition of such compounds to the respiring

Table 16: Effect of ALM on the rate of oxygen uptake by washed cell suspensions of D. discoideum Ax2 after growth in ALM pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. after growth in ALM pH6.2. and resuspended to 10^7 cells/ml in 7.19 mM phosphate buffer pH6.2. Oxygen uptake by cell suspensions was estimated (methods 10.3.2.) at different concentrations of ALM pH6.2.

	Rate of O_2 uptake nmol/min/mg protein
buffer	84
ALM	147
ALM (X2 concn.)	131
ALM (X4 concn.)	75

Table 17: Effect of individual amino acids on the rate of oxygen uptake by washed cell suspensions of D. discoideum Ax2 after growth in ALM pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. after growth in ALM pH6.2 and resuspended to 10^7 cells/ml in 7.19 mM phosphate buffer pH6.2. Oxygen uptake was estimated, as described in methods 10.3.2. with individual amino acids, added at 5 mM, to the cell suspension. The basal rate of oxygen uptake was measured for 5 min before addition of the amino acid.

	Rate of O_2 uptake nmol/min/mg protein
ALM	156
buffer	89
buffer + arginine	97
buffer + lysine	94
buffer + glutamic acid	99
buffer + threonine	90
buffer + tyrosine	93

cells, either in the presence or absence of the amino acid (Table 18 a,b,c). In some cases the rate of respiration was greater in the presence of these compounds alone than that in the presence of these compounds and the amino acids (Table 18 a,b,c).

At no time, did the addition of amino acid, at 5 mM, or of a second compound at 20 mM, with an amino acid cause a reduction in the rate of respiration.

8.3. Oxygen uptake by ALM_g cells.

Measurements of oxygen uptake by washed cell suspensions prepared from ALM_g grown cultures gave very similar results to those found with ALM grown cells.

The basal rate of oxygen uptake was 81 nmol/min/mg protien for ALM_g cells (Table 19) compared to a value of 84 nmol/min/mg protein for ALM cells (Table 16). Furthermore, the addition of ALM to a suspension of ALM_g grown cells, stimulated oxygen uptake to 147 nmol/min/mg protein (Table 19) - exactly the same value as that found for ALM grown cells.

The influence of either the amino acids or the nitrogen free compounds on respiration of ALM_g cells was minimal with no significant changes in the rate of oxygen uptake being detected after addition of these compounds (Table 19, 20).

Table 18a: Effect metabolites on the rate of oxygen uptake in the presence of arginine by washed cell suspensions of D. discoideum Ax2 after growth in ALM pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. after growth in ALM pH6.2. and resuspended to $1x10^7$ cells/ml in 7.19 mM phosphate buffer pH6.2. Oxygen uptake was estimated as described in methods 10.3.2. with arginine, at 5 mM, and metabolites, at 20 mM, added to the cell suspension.

In all cases the basal rate of uptake was measured for 5 min before addition of arginine. Metabolites were added after a further 5 min incubation period.

	Rate of O_2 uptake nmol/min/mg/protein
ALM	150
buffer	81
buffer + arginine	85
buffer + arg. + glucose	84
buffer + arg. + maltose	84
buffer + arg. + trehalose	83
buffer + arg. + glycerol	91
buffer + arg. + malate	97
buffer + arg. + pyruvate	83
buffer + arg. + 2-oxoglutarate	95

Table 18b: Effect of metabolites on the rate of oxygen uptake in the presence of lysine by washed cell suspensions of D. discoideum Ax2 after growth in ALM pH6.2.

A washed cell suspension of D. discoideum Ax2 was prepared as described in methods 10.1. after growth in ALM pH6.2 and resuspended to $1x10^7$ cells/ml in phosphate buffer pH6.2. Oxygen uptake was estimated as described in methods 10.3.2. with lysine, at 5 mM, and metabolites, at 20 mM added to the cell suspension .

In all cases the basal rate of oxygen uptake was measured for 5 min before addition of lysine. Metabolites were added after a further 5 min incubation period.

	Rate of O_2 uptake nmol/min/mg protein
ALM	156
buffer	90
buffer + lysine	100
buffer + lys. + glucose	101
buffer + lys. + maltose	106
buffer + lys. + trehalose	101
buffer + lys. + glycerol	102
buffer + lys. + malate	103
buffer + lys. + pyruvate	100
buffer + lys. + 2-oxoglutarate	104

Table 18c: Effect of metabolites on the rate of oxygen uptake by washed cell suspension of *D. discoideum* Ax2 after growth in ALM pH6.2.

A washed cell suspension of *D. discoideum* Ax2 was prepared as described in methods 10.1. after growth in ALM pH6.2 and resuspended to $1x10^7$ cells/ml in phosphate buffer pH6.2. Oxygen uptake was estimated as described in methods 10.3.2. with nitrogen free metabolites added at 20 mM, to the cell suspension after the basal level had been verified over a 5 min period.

	Rate of O_2 uptake nmol/min/mg protein
ALM	156
buffer	90
buffer + glucose	94
buffer + trehalose	96
buffer + glycerol	102
buffer + malate	96
buffer + pyruvate	113
buffer + 2-oxoglutarate	98

Table 19: Effect of individual amino acids on the rate of oxygen uptake by washed cell suspension of *D. discoideum* Ax2 grown in ALM_g pH6.2.

A washed cell suspension of *D. discoideum* Ax2 was prepared as described in methods 10.1. after growth in ALM_g pH6.2 and resuspended to $1x10^7$ cells/ml in phosphate buffer pH6.2. Oxygen uptake was estimated as described in methods 10.3.2. with individual amino acids added at 5 mM to the cell suspension. The basal rate of oxygen uptake was measured for 5 min before addition of the amino acid.

	Rate of uptake nmol/min/mg protein
ALM_{base}	147
ALM_{glc}	148
buffer	81
buffer + arginine	82
buffer + lysine	81
buffer + glutamic acid	82
buffer + threonine	81
buffer + tyrosine	81

Table 20: Effect of metabolites on the rate of oxygen uptake by washed cell suspension of *D. discoideum* Ax2 grown in ALM_g pH6.2.

A washed cell suspension of *D. discoideum* Ax2 was prepared as described in methods 10.1. after growth in ALM_g pH6.2. and resuspended to $1x10^7$ cells/ml in 7.19 mM phosphate buffer pH6.2. Oxygen uptake was estimated as described in methods 10.3.2. with nitrogen free metabolites added at 20 mM to the cell suspension. The basal rate of oxygen uptake was measured for 5 min before the addition of the metabolites.

	Rate of O_2 uptake nmol/min/mg protein
ALM_{base}	147
ALM_{glc}	148
buffer	81
buffer + glucose	83
buffer + trehalose	86
buffer + glycerol	85
buffer + malate	81
buffer + pyruvate	89
buffer + 2-oxoglutarate	86

9. Catabolism of radiolabelled amino acids.

The rate of amino acids utilization by D. discoideum Ax2 was measured using ^{14}C- carbon dioxide produced by the catabolism of uniformly labelled amino acids as a tool. Cell suspensions prepared as described in methods 10.1 were added to individual amino acids in 10 ml erlenmeyer flasks fitted with a central well (methods 10.4.1.) and ^{14}C-carbon dioxide production by the catabolism of amino acids was measured (methods 10.4.2).

Under the conditions chosen ^{14}C- carbon dioxide production was linear over 150 minutes with all five selected amino acids (Figure 37) but an incubation time of 90 min. was selected and rarely exceeded in the experiments using radioactivity.

9.1. Catabolism of amino acids by ALM and ALM_g cells.

Cells grown in ALM in the presence (ALM_g cells) and absence (ALM cells) of glucose showed different capacities for carbon dioxide formation from the radiolabelled amino acids studied. The rate of ^{14}C- carbon dioxide production by ALM cells is about 200% of that found from ALM_g cells and arginine and lysine added to the cell suspension generate more ^{14}C- carbon dioxide by catabolism than other amino acids studied (Table 21).

9.2. Effect of glucose on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM.

On the basis of ^{14}C- carbon dioxide production, the rate of catabolism of all the amino acids tested was decreased with glucose, at a concentration of 86 mM, added to amino acid catabolising cell suspensions. On the other hand, if glucose was added at a concentration

Figure 37: Rate of ^{14}C carbon dioxide production from amino acids by D. discoideum Ax2 cells grown in ALM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer after growth in ALM as described in methods 10.1. Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi). Cell suspension was added at a cell density of 10^7 cells/flask to initiate the catabolism of the amino acid and incubated at 23°C with shaking as described in methods 10.4.1. After defined periods of incubation the reaction in an individual flask was stopped by injecting 250 ul of 5N sulphuric acid and the flasks retained on the shaker for an additional 90 minutes. ^{14}C carbon dioxide production was measured as described in methods 10.4.2. and the rate of breakdown of amino acid calculated. Series of experiments were carried out, under identical conditions for each of the individual amino acids.

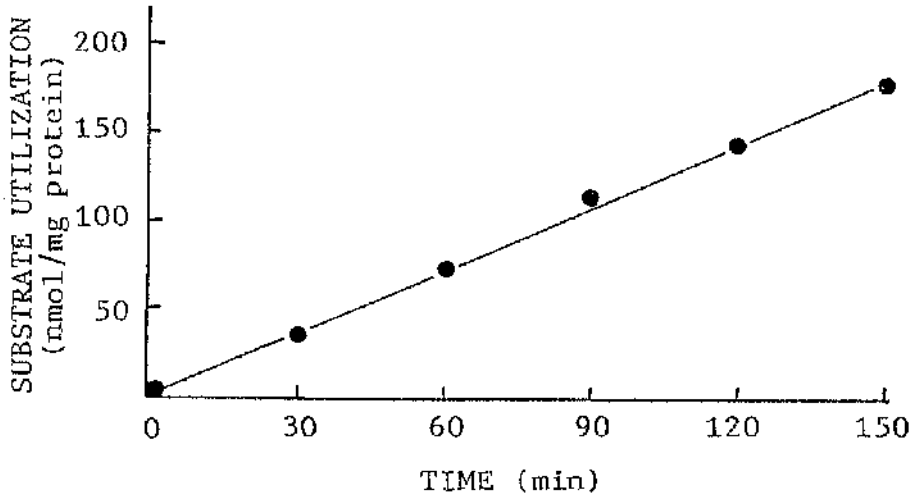
● : arginine (Figure 37a)

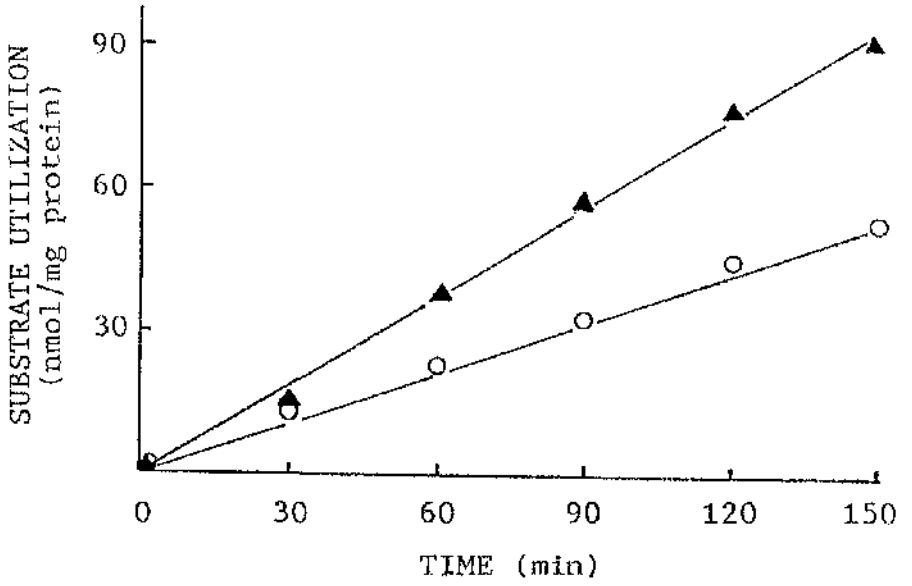
▲ : lysine (Figure 37b)

O : tyrosine (Figure 37b)

◆ : glutamic acid (Figure 37c)

◇ : threonine (Figure 37c)


SUBSTRATE UTILIZATION
(nmol/mg protein)
200
150
100
50
0
30
60
90
120
150
TIME (min)


SUBSTRATE UTILIZATION
(nmol/mg protein)
90
60
30
0
30
60
90
120
150
TIME (min)

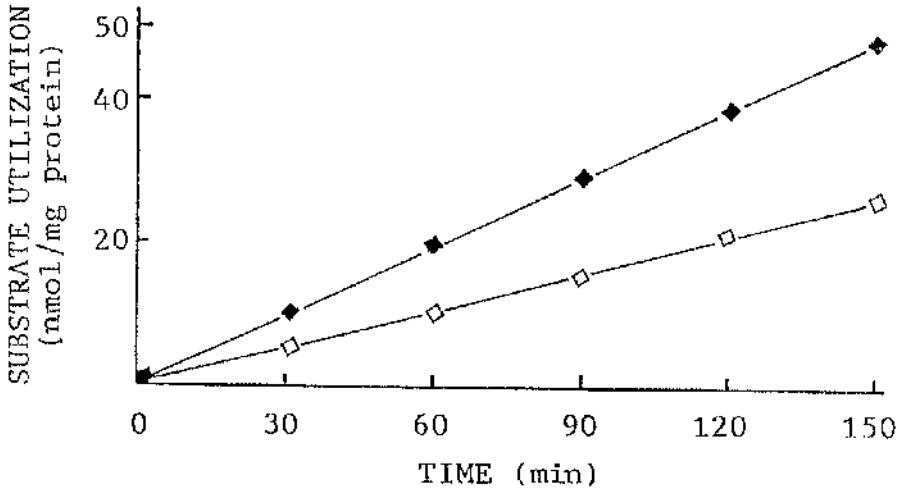

SUBSTRATE UTILIZATION
(nmol/mg protein)
50
40
20
0
30
60
90
120
150
TIME (min)

Table 21: Catabolism of radiolabelled amino acid by D. discoideum Ax2 cells grown in ALM and ALM_g pH6.2

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM (methods 10.1). A series of flasks was set up containing 5 µmol of one of the radiolabelled amino acids listed opposite with 0.25 µCi/flask. Cell suspension was added to each flask at a density of 10^7 cells/flask, to initiate the catabolism of the amino acids, and incubated at 23°C as described in methods 10.4.1. An identical series of flasks was set up under the same conditions using a cell suspension prepared after growth in ALM_g.

^{14}C carbon dioxide production was measured (methods 10.4.2.) and the rate of carbon dioxide production calculated. Assuming that any amino acid catabolised is completely converted to carbon dioxide, the rate of amino acid catabolism was calculated (methods 11.3.).

The results are the means of three independent experiments.

	ALM cells		ALM_g cells	
AMINO ACIDS	Rate of CO_2 production nmol/h/mg protein	Rate of amino acid catabolism nmol/h/mg protein	Rate of CO_2 production nmol/h/mg protein	Rate of amino acid catabolism nmol/h/mg protein
arginine L[U-C^{14}]	456	76	222	37
lysine L[U-C^{14}]	240	40	126	21
tyrosine L[U-C^{14}]	207	23	117	13
glutamic acid L[U-C^{14}]	95	19	50	10
threonine L[U-C^{14}]	36	9	20	5

of 20 mM, the rate of ^{14}C- carbon dioxide production from each of the amino acids was stimulated (Table 22).

This result is quite different to that obtained when glucose was added to amino acid metabolising suspensions and ammonia was used as a measure of amino acid utilization. Measurements of ammonia production in these experiments had demonstrated that the rate of ammonia production decreased as the concentration of glucose added to the cell suspension was increased and that at both 20 mM and 86 mM glucose added, ammonia production was reduced as compared to the control (Table 22).

9.3. Effect of the addition of metabolites on ^{14}C carbon dioxide production from arginine catabolism by ALM and ALM_g cells.

Different compounds, added at 20 mM to the suspension of ALM cells, in addition to arginine altered the rate of carbon dioxide production from the amino acid. The addition of either glucose or trehaolose slightly stimulated the rate of carbon dioxide formation whereas the addition of either malate or pyruvate reduced it.

When ALM_g cells were used in similar experiments, carbon dioxide production from arginine was not affected by the addition of either glucose or trehalose but malate or pyruvate still reduced carbon dioxide production (Figure 38).

9.4. Effect of glucose concentration on carbon dioxide production from amino acid catabolism by ALM cells.

Glucose influences $^{14}CO_2$ production from radiolabelled amino acids. A concentration profile with different concentrations of glucose added to the cell suspensions along with individual amino acids confirmed that low concentrations of glucose (10 mM and 20 mM) stimulate carbon dioxide production from all five amino acids (Figure 39).

Table 22: Effect of glucose on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM (methods 10.1). Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and glucose at the concentrations shown. The cell suspension was added to each flask to give a density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.) and the rate of amino acid utilization calculated (methods 11.3). Samples were also taken from the flasks for ammonia estimation, centrifuged at 2000 g for 2 min. in an eppendorf centrifuge 3200 in 2 ml plastic centrifuge tubes and the supernatant used for ammonia estimation using the phenol/hypochlorite method as described in methods 11.1.1.1.

Amino Acid and Glucose	Basal Rate of Ammonia Production nmol/h/mg Protein	Amino Acid Activated Rate of Ammonia Production nmol/h/mg Protein	Rate of ammonia production due to added amino acid nmol/h/mg Protein	Rate of Amino Acid utilisation based on ammonia production nmol/h/mg Protein	Rate of Amino Acid utilisation based on radioactivity data nmol/h/mg Protein
Arginine					
+ 0 Glucose	660	1260	600	150	79
+ 20 mM Glucose	600	840	240	60	86
+ 86 mM Glucose	480	660	180	45	56
Lysine					
+ 0 Glucose	600	1140	540	270	37
+ 20 mM Glucose	420	900	480	240	42
+ 86 mM Glucose	300	720	420	210	27
Glutamic Acid					
+ 0 Glucose	600	720	120	120	17
+ 20 mM Glucose	420	480	60	60	20
+ 86 mM Glucose	360	360	-	-	11
Tyrosine					
+ 0 Glucose	600	840	240	240	20
+ 20 mM Glucose	480	660	180	180	23
+ 86 mM Glucose	420	540	120	120	11
Threonine					
+ 0 Glucose	540	660	120	120	8
+ 20 mM Glucose	420	540	120	120	9
+ 86 mM Glucose	360	420	60	60	5

Figure 38: Effect of metabolites on the utilization of arginine by ALM and ALM_g cells.

Cell suspensions of D. discoideum Ax2 were prepared in phosphate buffer pH6.2 after growth in ALM and ALM_g as described in methods 10.1. Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and metabolites at 20 mM concentration. Each cell suspension was added in a separate set of flasks to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.), the rate of the amino acid utilization calculated (methods 11.3.) and graphed against the metabolites in a histogram.

arginine

arginine + glucose

arginine + trehalose

arginine + malate

arginine + pyruvate

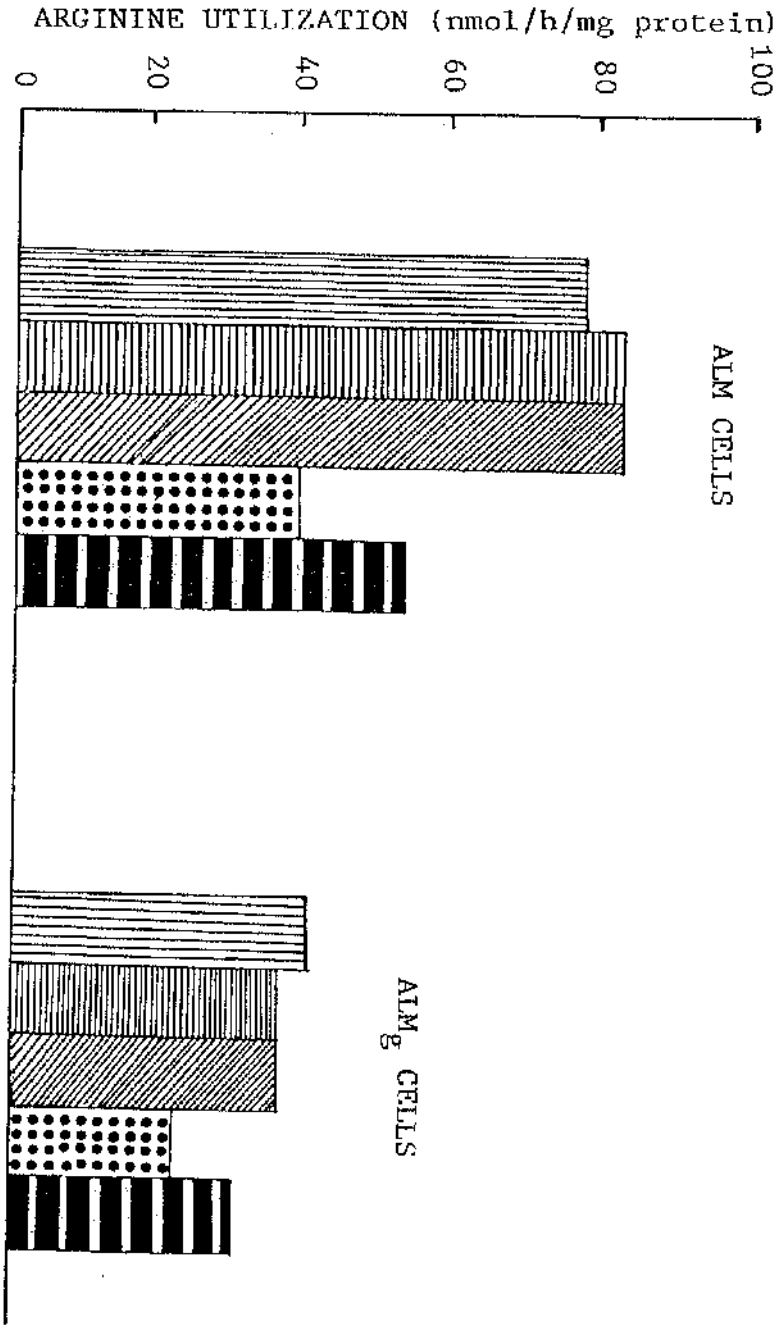

ARGININE UTILIZATION (nmol/h/mg protein)
0
20
40
60
80
100
ALM CELLS
ALM$_g$ CELLS

Figure 39: Effect of glucose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM as described in methods 10.1. Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and glucose at different concentrations as required. Cell suspension was added to give a density 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.), the rate of amino acid utilization calculated (methods 11.3) and graphed against sugar concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

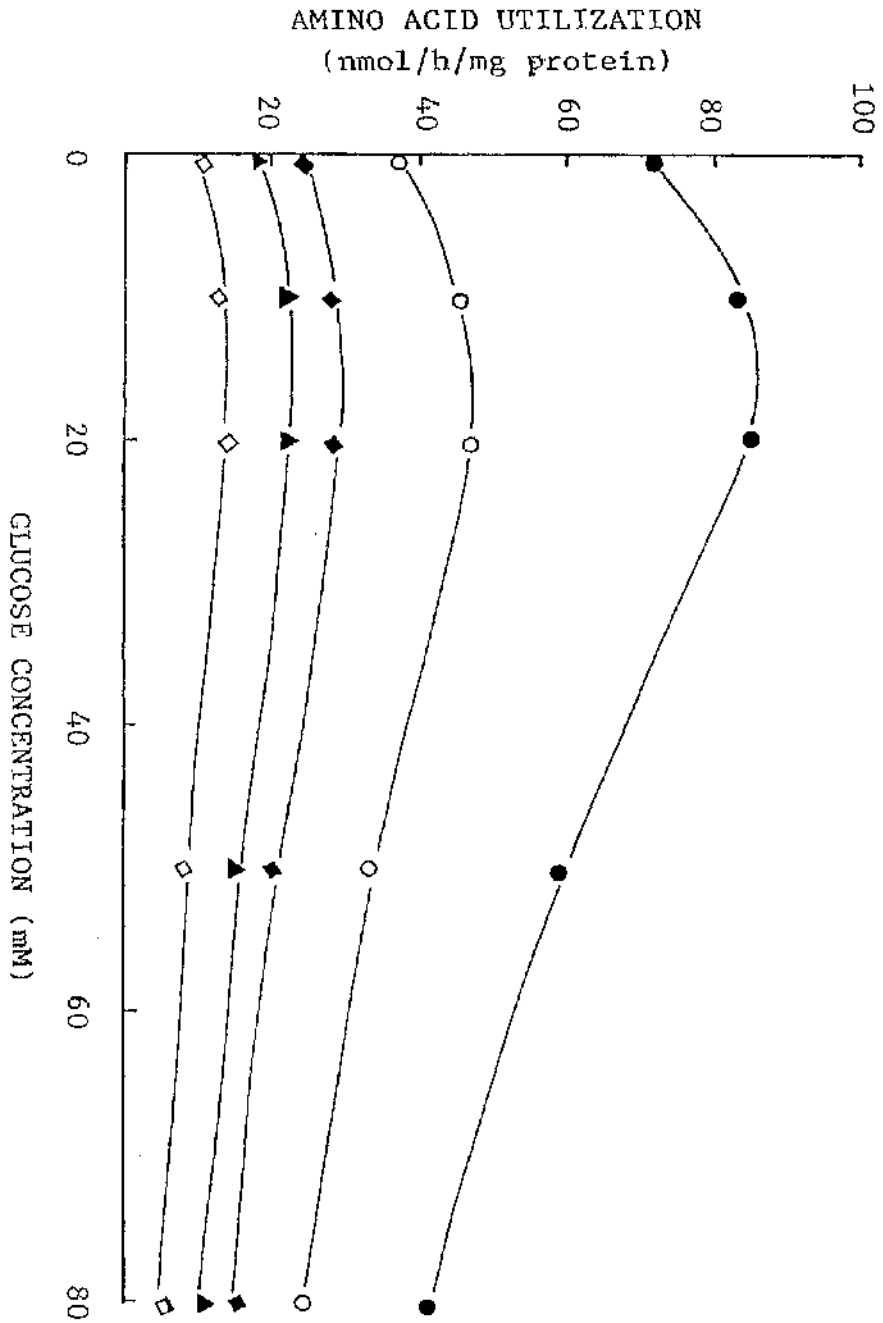
● : arginine

O : lysine

◆ : tyrosine

▲ : glutamic acid

◇ : threonine


AMINO ACID UTILIZATION
(nmol/h/mg protein)
20
40
60
80
100
0
20
40
60
80
GLUCOSE CONCENTRATION (mM)

In all cases the rate of carbon dioxide production was decreased gradually as the concentration of glucose was increased above 20 mM.

9.5. Effect of glucose concentration on its own catabolism.

Different concentrations of glucose added to suspension of ALM cells along with a constant amount of D[U-^{14}C] glucose showed that $^{14}CO_2$ production from glucose increased as the glucose concentration increased (Figure 40). At low concentration of glucose the rate of increase in $^{14}CO_2$ production with concentration was greater than at higher concentration (an increase of 9%/mM between 10 mM and 20 mM compared to an increase of only 0.17%/mM between 50 mM and 80 mM).

9.6. Effect of pyruvate, galactose and phosphate concentration on ^{14}C carbon dioxide production from amino acids.

The influence of several metabolites, namely pyruvate, galactose and phosphate on the rate of carbon dioxide production from exogenous amino acids was examined in cells grown in ALM.

At high (above 20 mM) concentrations of pyruvate, the rate of carbon dioxide production from all the amino acids was decreased in a similar way to that observed with added glucose. However, with the addition of pyruvate at 10 mM and 20 mM, no stimulation in carbon dioxide production was observed. Carbon dioxide production, therefore decreased continuously with increasing pyruvate concentration (Figure 41).

Similar results were obtained when galactose or phosphate buffer pH6.2 were added to amino acid metabolising cell suspensions (Figure 42, 43). The only difference observed was that the rate of decrease in carbon dioxide production was less dependent on galactose concentration than with the other compounds (Figure 42). In all cases, the pattern of carbon dioxide production was the same for all five maino acids with the different added compounds, although, as previously observed, the extent to which each amino acid was metabolised varied.

Figure 40: Effect of glucose concentration on its own catabolism by D. discoideum Ax2 cells grown in ALM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM (methods 10.1). Flasks were set up containing 0.25 μCi radiolabelled glucose at different glucose concentrations. Cell suspension was added to give a density of 10^7 cells/flask to initiate the catabolism of glucose and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.), rate of glucose catabolism calculated (methods 11.3) and graphed against glucose concentration.

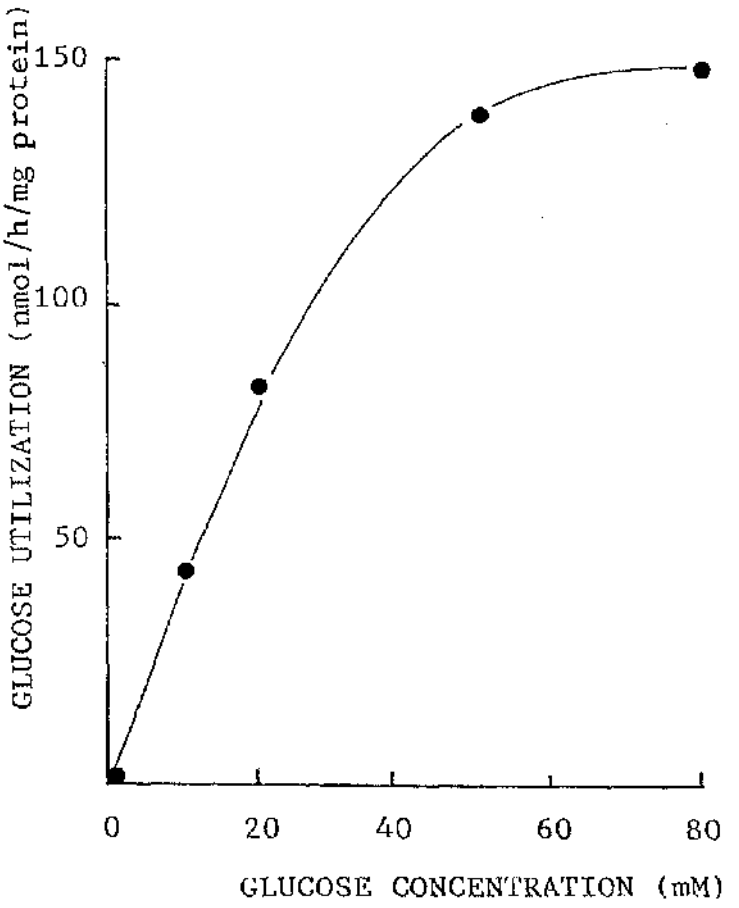

GLUCOSE UTILIZATION (nmol/h/mg protein)
150
100
50
0
20
40
60
80
GLUCOSE CONCENTRATION (mM)

Figure 41: Effect of pyruvate concentration on the catabolism of amino acids by D. discoideum cells grown in AIM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in AIM (methods 10.1). Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and pyruvate at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C-carbon dioxide production was measured (methods 10.4.2.), the rate of amino acid utilization calculated (methods 11.3) and graphed against pyruvate concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

● : arginine

○ : lysine

◆ : tyrosine

▲ : glutamic acid

◇ : threonine

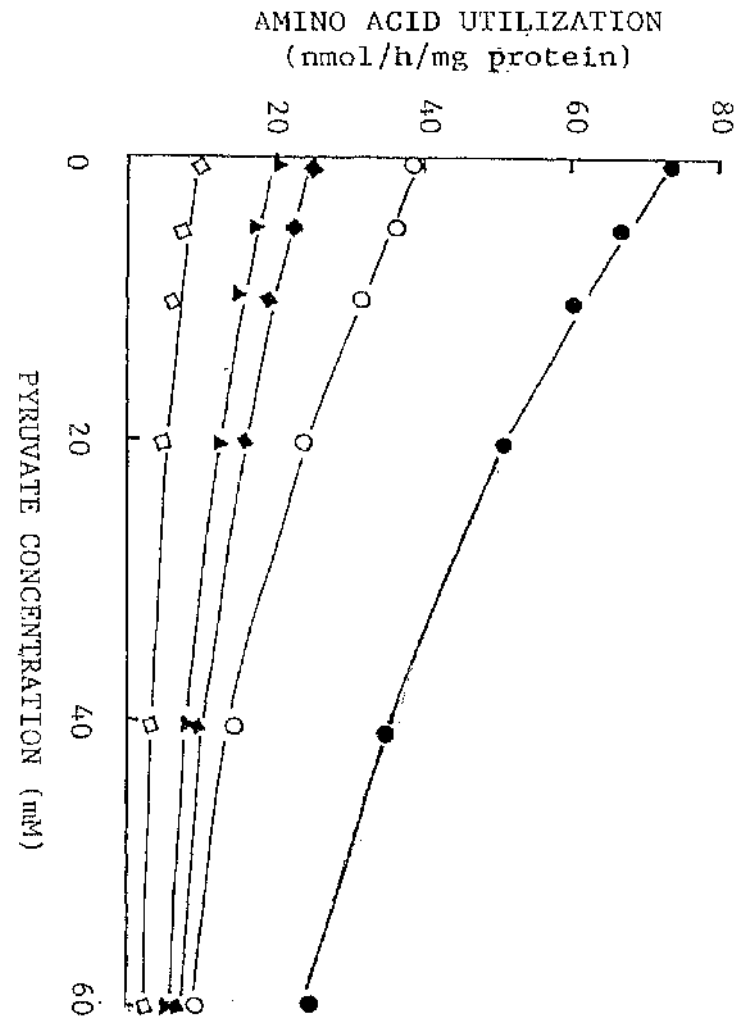

AMINO ACID UTILIZATION
(nmol/h/mg protein)
20
40
60
80
0
20
40
60
PYRUVATE CONCENTRATION (mM)

Figure 42: Effect of galactose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM as described in methods 10.1. Flasks were set up containing 5 μmol radiolabelled amino acid (0.25 μCi) and galactose at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C-carbon dioxide production was measured (methods 10.4.2.), rate of amino acid utilization calculated (methods 11.3) and graphed against galactose concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

● : arginine

O : lysine

◆ : tyrosine

▲ : glutamic acid

◇ : threonine

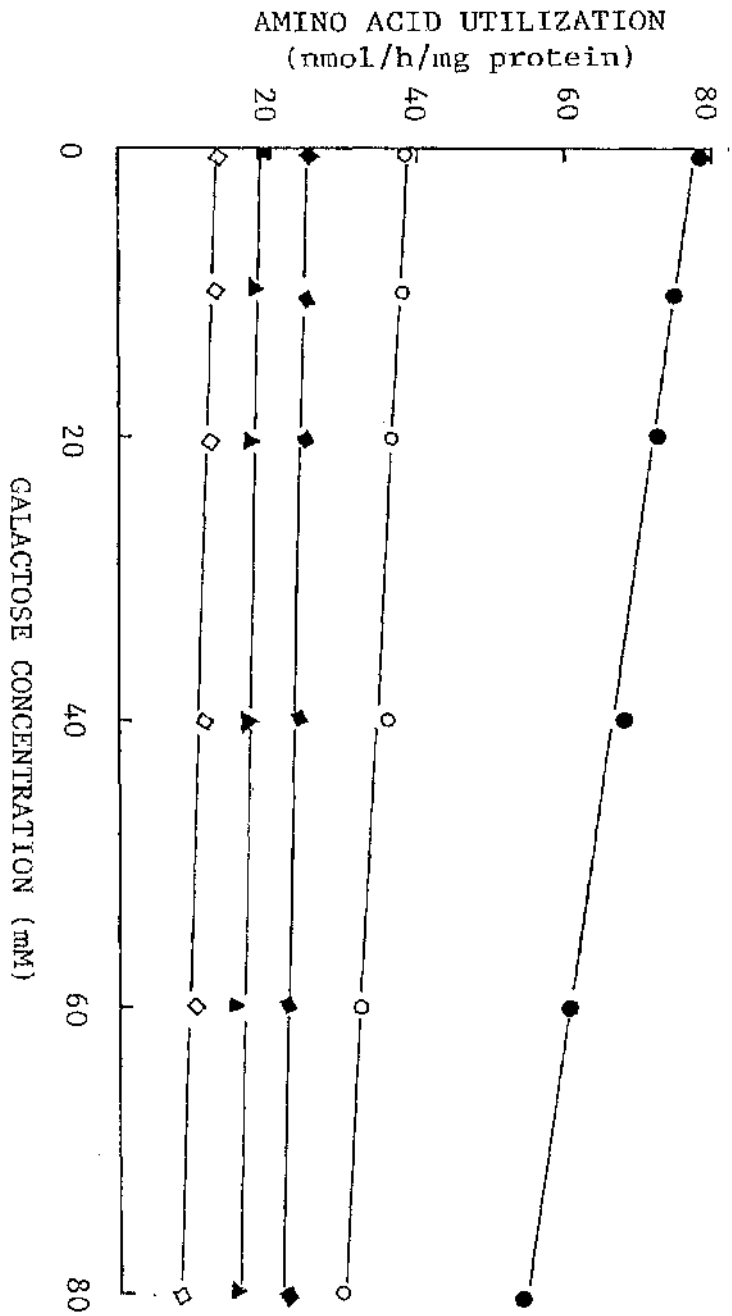

AMINO ACID UTILIZATION
(nmol/h/mg protein)
20
40
60
80
0
20
40
60
80
GALACTOSE CONCENTRATION (mM)

Figure 43: Effect of phosphate concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM as described in methods 10.1. Flasks were set up containing 5 µmol radiolabelled amino acid (0.025 µCi) and phosphate at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.), the rate of amino acid utilization calculated (methods 11.3) and graphed against phosphate concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

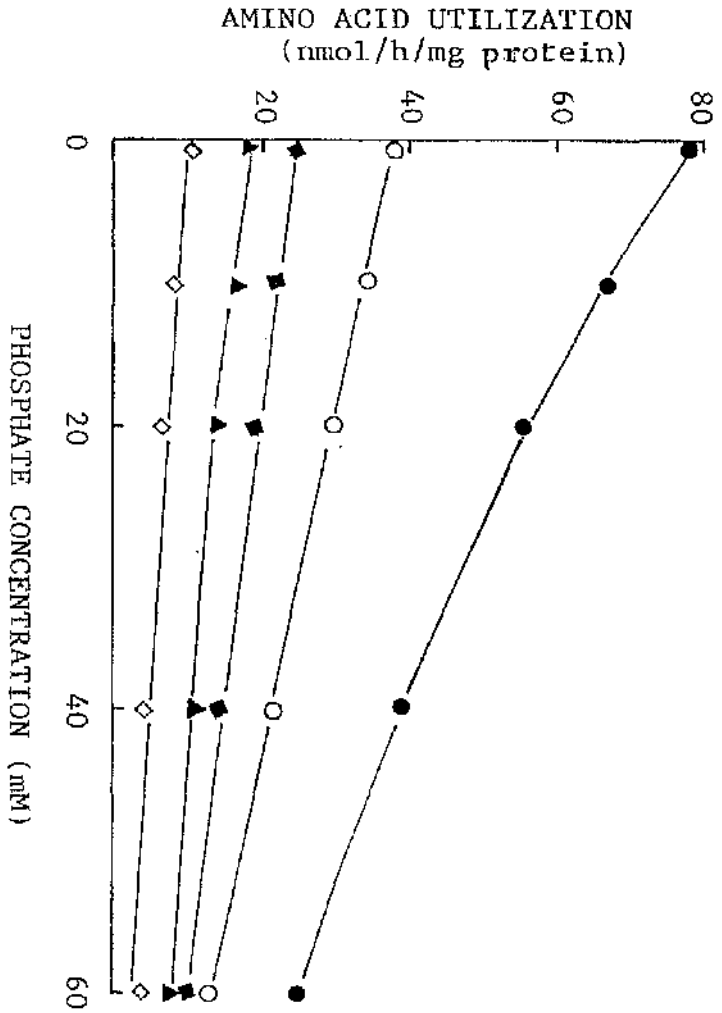
● : arginine

O : lysine

◆ : tyrosine

▲ : glutamic acid

◇ : threonine


AMINO ACID UTILIZATION
(nmol/h/mg protein)
20
40
60
80
0
20
40
60
PHOSPHATE CONCENTRATION (mM)

9.7. Effect of glucose concentration on ^{14}C carbon dioxide production from amino acids by ALM_g cells.

Like ALM cells, the production of $^{14}CO_2$ from amino acids by AIM_g cells was decreased with increasing glucose concentration. There was however, no stimulation in carbon dioxide production with ALM_g cells at low concentration (10 and 20 mM) of glucose (Figure 44) as observed with ALM cells.

9.8. Effect of pyruvate, galactose and phosphate concentration on the production of carbon dioxide from amino acids by ALM_g cells.

As with ALM cells, the addition of any one of pyruvate, galactose or phosphate to ALM_g cells produced a decrease in carbon dioxide production (Figure 45, 46, 47). The decrease in carbon dioxide production was related to the concentration of the compound added to the incubation mixture.

A similar pattern was obtained for all three compounds on carbon dioxide production for all five of the amino acids used. The only difference, between the three compounds examined was that, as with ALM cells, galactose produced a smaller decrease in carbon dioxide production with increasing galactose concentration (Figure 46).

9.9. Effect of 2-deoxy D-glucose on carbon dioxide production from arginine by ALM cells.

The addition of 2-deoxy D-glucose to ALM cell suspensions metabolising uniformly labelled arginine has a similar influence on carbon dioxide production to that produced by glucose. The addition of either glucose or 2-deoxy D-glucose at 20 mM stimulates the breakdown of extracellular arginine to carbon dioxide. At higher concentrations both compounds produce a reduction in carbon dioxide production but 2-deoxy D-glucose has less effect than that produced by glucose (Figure 48).

Figure 44: Effect of glucose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM_g (methods 10.1). Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and glucose at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.), the rate of amino acid utilization calculated (methods 11.3) and graphed against glucose concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

● : arginine

○ : lysine

◆ : tyrosine

▲ : glutamic acid

◇ : threonine

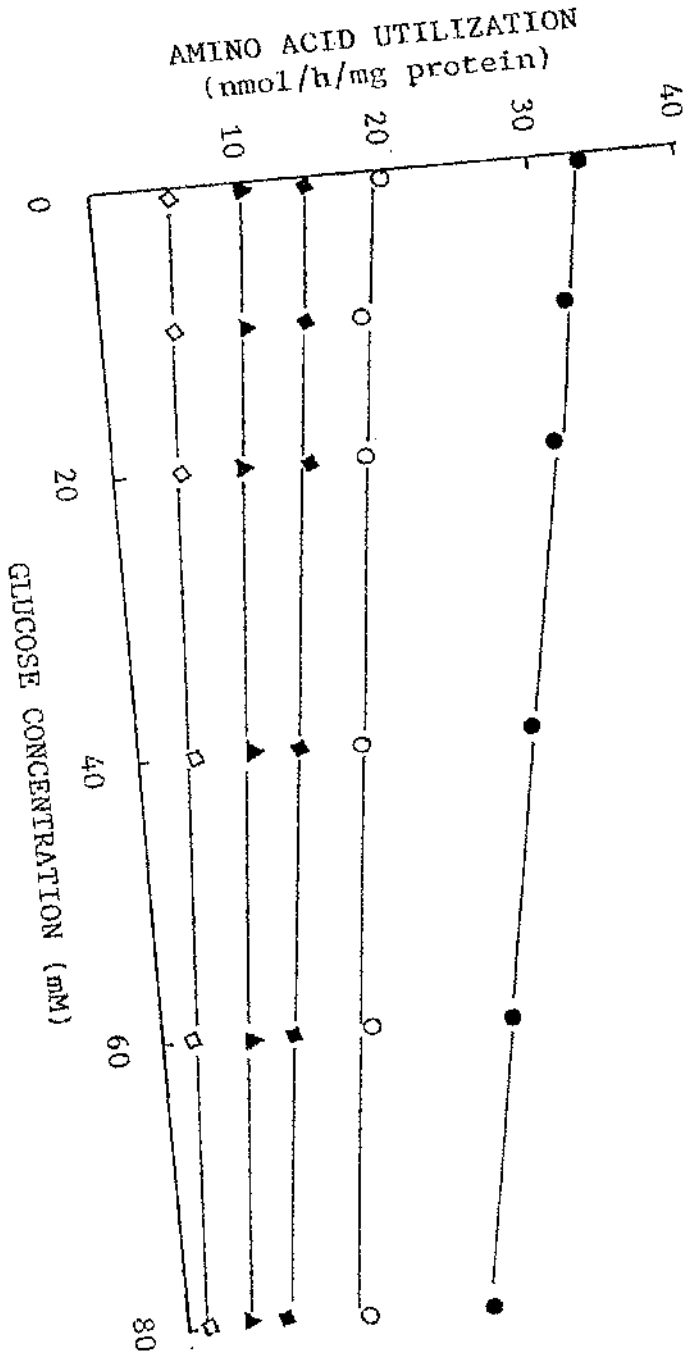

AMINO ACID UTILIZATION
(nmol/h/mg protein)
0
10
20
30
40
GLUCOSE CONCENTRATION (mM)
0
20
40
60
80

Figure 45: Effect of pyruvate concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2..

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM_g as described in methods 10.1. Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and pyruvate at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.), the rate of amino acid utilization calculated (methods 11.3) and graphed against pyruvate concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

●: arginine

O: lysine

◆: tyrosine

▲: gluthamic acid

◇: threonine

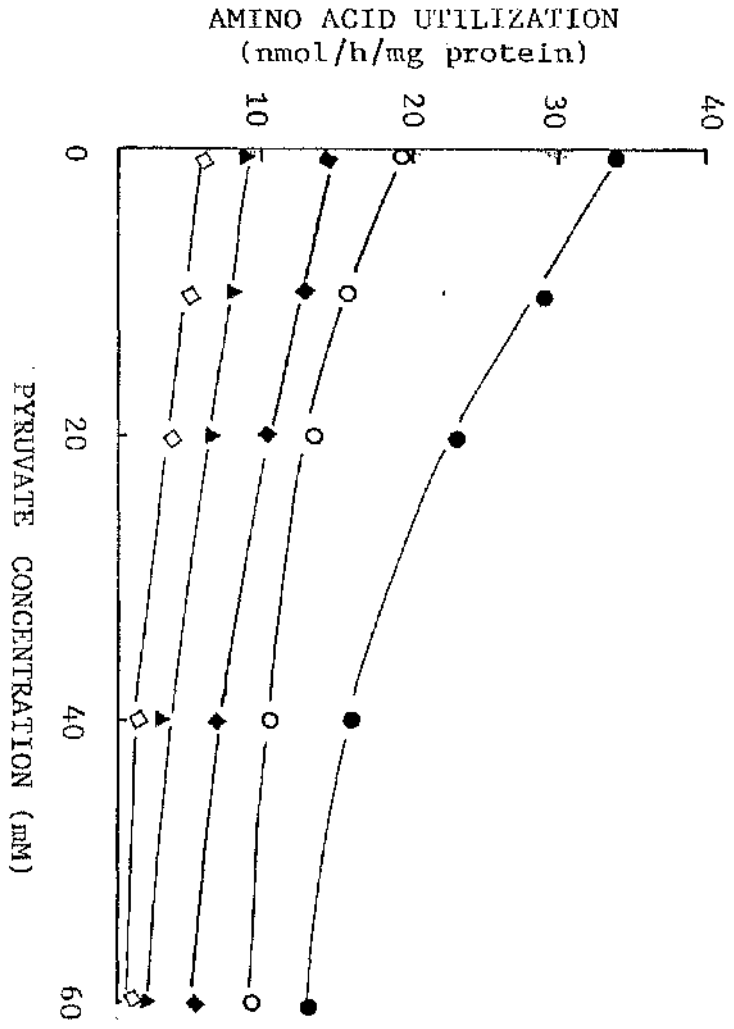

AMINO ACID UTILIZATION
(nmol/h/mg protein)
10
20
30
40
0
20
40
60
PYRUVATE CONCENTRATION (mM)

Figure 46: Effect of galactose concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM_g (methods 10.1). Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and galactose at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C-carbon dioxide production was measured (methods 10.4.2.), the rate of amino acid utilization calculated (methods 11.3) and graphed against galactose concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

● : arginine

○ : lysine

◆ : tyrosine

▲ : glutamic acid

◇ : threonine

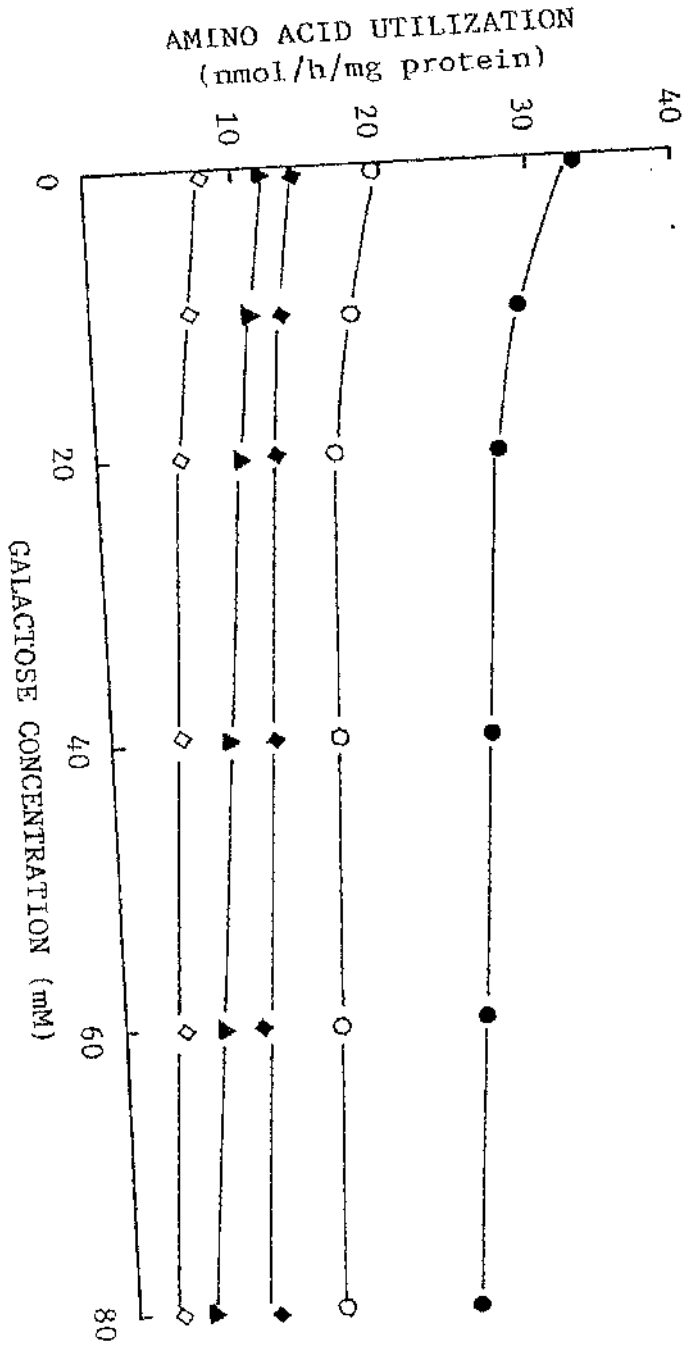

AMINO ACID UTILIZATION
(nmol./h/mg protein)
0
10
20
30
40
GALACTOSE CONCENTRATION (mM)
0
20
40
60
80

Figure 47: Effect of phosphate concentration on the catabolism of amino acids by D. discoideum Ax2 cells grown in ALM_g pH6.2..

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM (methods 10.1). Flasks were set up containing 5 µmol radiolabelled amino acid (0.25 µCi) and phosphate at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of amino acid and incubated at 23°C as described in methods 10.4.1. ^{14}C-carbon dioxide production was measured (methods 10.4.2.), the rate of amino acid utilization calculated (methods 11.3) and graphed against phosphate concentration.

Identical series of experiments were carried out for each of the individual amino acids examined.

●: arginine

O: lysine

◆: tyrosine

▲: glutamic acid

◇: threonine

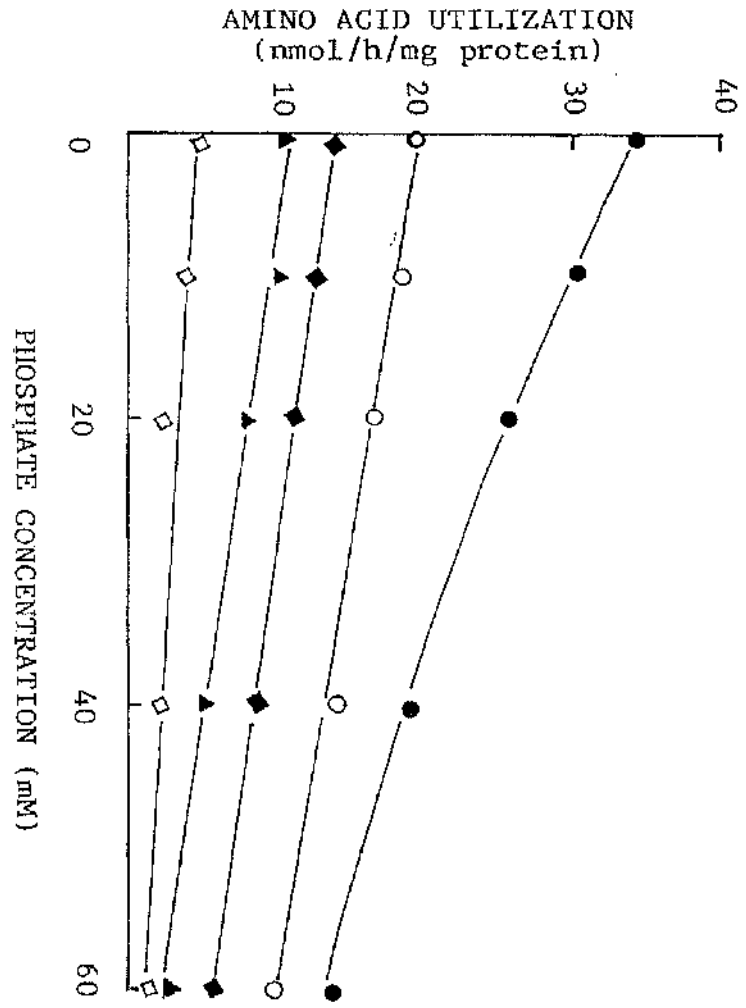
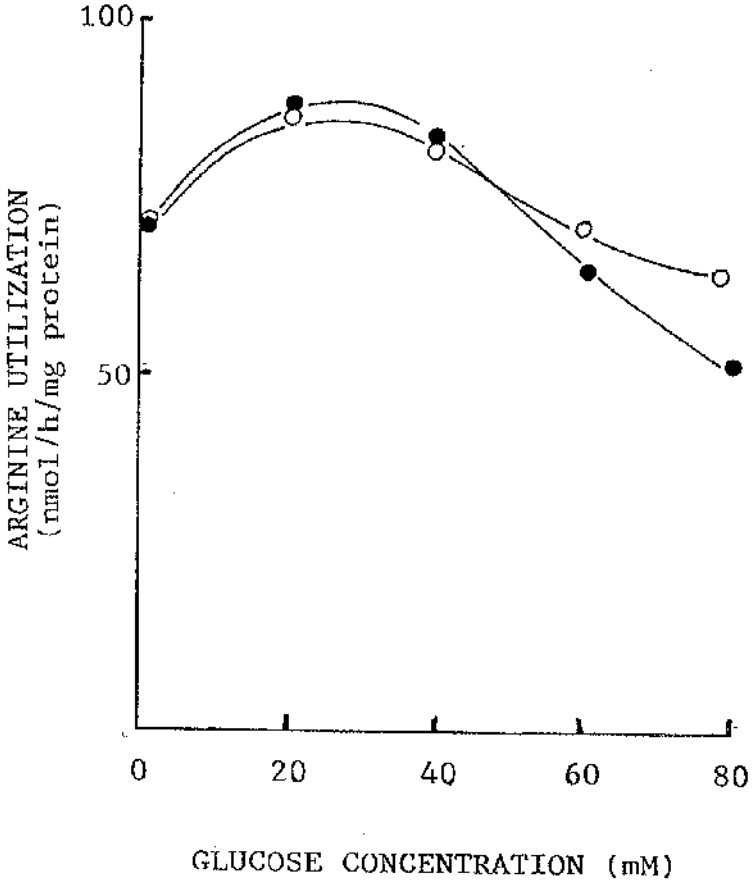

AMINO ACID UTILIZATION
(nmol/h/mg protein)
10
20
30
40
0
20
40
60
PHOSPHATE CONCENTRATION (mM)

Figure 48: Effect of concentration of 2- deoxy D-glucose on the utilization of arginine by D. discoideum Ax2 cells grown in ALM pH6.2.

A cell suspension of D. discoideum Ax2 was prepared in phosphate buffer pH6.2 after growth in ALM as described in methods 10.1. Flasks were set up containing 5 µmol radiolabelled arginine (0.25 µCi) and 2-deoxy D-glucose at different concentrations as required. Cell suspension was added to give a cell density of 10^7 cells/flask to initiate the catabolism of arginine and incubated at 23°C as described in methods 10.4.1. ^{14}C- carbon dioxide production was measured (methods 10.4.2.) and rate of amino acid utilization calculated (methods 11.3).
The results of an identical series of experiments using glucose are also shown.

●: glucose

O: 2-deoxy D-glucose


100
50
ARGININE UTILIZATION
(nmol/h/mg protein)
0
20
40
60
80
GLUCOSE CONCENTRATION (mM)

10. Effect of osmotic strength on amino acid catabolism.

10.1. Effect of osmotic strength on the rate of ^{14}C carbon dioxide production from arginine catabolism by ALM cells.

The addition of glucose or galactose to the cell suspensions causes a change in osmotic strength of the suspension medium that is different from the change produced by addition of equimolar amounts of pyruvate or phosphate (because the latter are ionisable).

To assess the influence that osmotic strength may have on carbon dioxide production, direct measurements of osmotic strength changes were made and the data from Figures 39, 41, 42, 43 for ^{14}C carbon dioxide production from uniformly labelled arginine replotted against the change in osmotic strength. The influence of pyruvate and phosphate concentrations relate closely when assessed on an osmotic basis but galactose is still less able on an osmotic basis to reduce carbon dioxide production from arginine. Glucose still produces a stimulation in carbon dioxide production at low concentrations (Figure 49).

Figure 49: Effect of osmotic strength on the utilization of arginine by D discoideum AX2 cells grown in ALM pH6.2.

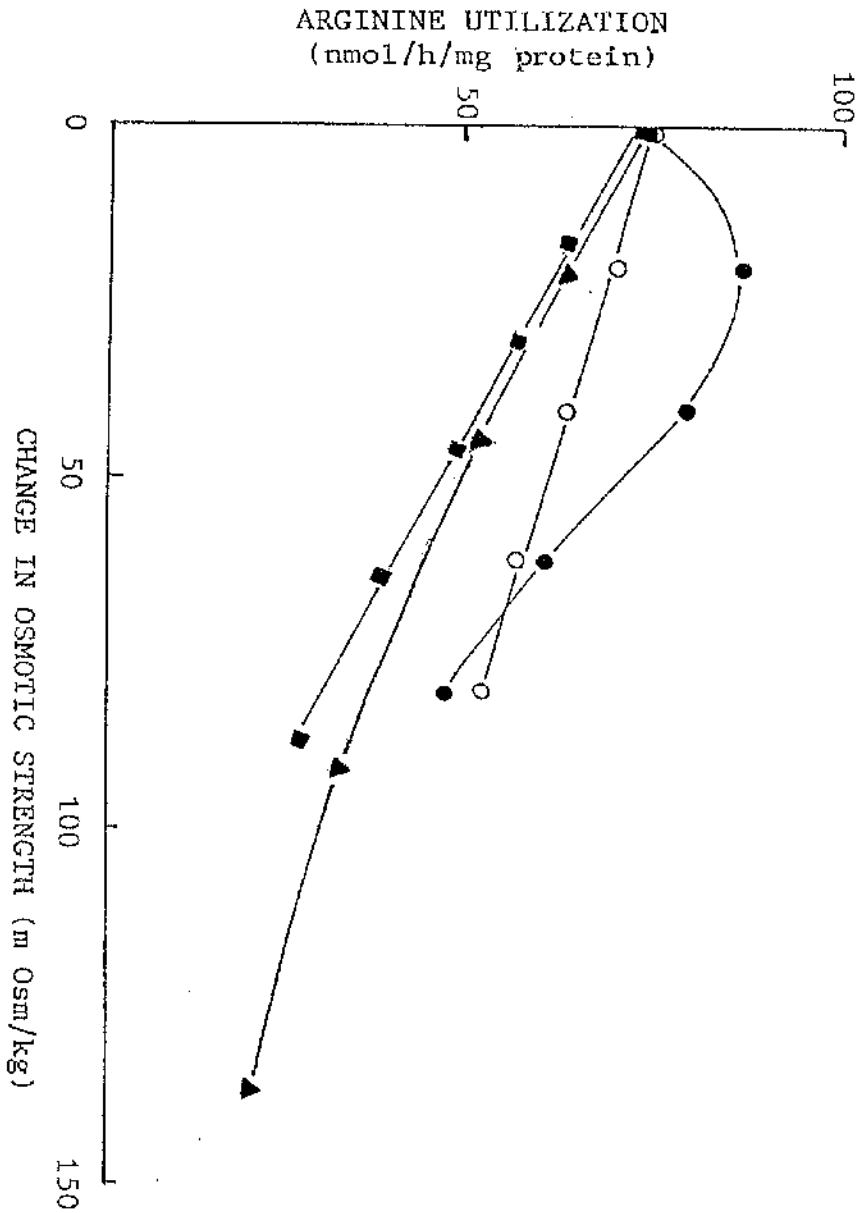
Osmotic strength of different concentrations of glucose, pyruvate, galactose and phosphate buffer used in experiment Figures 39, 41, 42, 43 was measured (methods 11.6) and the data for arginine catabolism in the above mentioned experiments was redrawn against the change in osmotic strength.

●: glucose

○: galactose

■: pyruvate

▲: phosphate


ARGININE UTILIZATION
(nmol/h/mg protein)
0
50
100
CHANGE IN OSMOTIC STRENGTH (m Osm/kg)
50
100
150

11. Effect of growth conditions on the specific activity of amino acid metabolising enzymes.

Cells grown in ALM in the presence of glucose (ALM_g cells) contained lower specific activities of enzymes involved in amino acid metabolism than ALM cells (cells grown in the absence of glucose). The specific activity of five of the seven enzymes measured was reduced by growth in the presence of glucose to about 50% of the control value (Table 23).

Of the other two enzymes, malic enzyme was reduced only to 78% of the control value while the lysine dependent oxygen uptake activity was reduced to 7.6% of the control value (Table 23).

NADP linked glutamate dehydrogenase was found to be 7 - 9% of NAD linked glutamate dehydrogenase after growth under both conditions. The activity of NAD and NADP linked glutamate dehydrogenase in case of ALM_g cells was 40 - 50% of the ALM cells- the level of reduction being similar to that found for other enzymes.

Table 23: Effect of growth conditions on the specific activity of amino acid metabolising enzymes of *D. discoideum* Ax2.

The cells of *D. discoideum* Ax2 were grown under two different growth conditions i.e. in ALM and ALM_g, as described in methods 9.1. Cells were then harvested and washed as for washed cell suspension preparation (methods 10.1.) except that the final resuspension of the cells was in the medium appropriate for the enzyme being assayed (Table 2). Cell extracts were prepared (methods 12.1.) and enzyme activity estimated as described under assay conditions of each enzyme.

Enzyme	Growth Medium	Specific Activity nmol/min/mg Protein 1	2	3	Mean	% of Control
Ornithine Aminotransferase	ALM	15.1	13.0	14.2	14.1	100
	ALM_g	8.65	7.50	8.10	8.1	57
Threonine Deaminase	ALM	32.6	29.9	32.8	31.8	100
	ALM_g	17.2	14.8	18.2	16.7	52.4
Tyrosine Transaminase	ALM	15.2	16.9	16.2	16.1	100
	ALM_g	8.9	6.9	7.5	7.76	48.2
Glutamate Dehydrogenase NAD Linked	ALM	14.92	15.36	15.43	15.24	100
	ALM_g	5.18	6.87	5.99	6.0	39.4
Glutamate Dehydrogenase NAD Linked	ALM	1.37	1.00	0.899	1.09	100
	ALM_g	0.562	0.490	0.557	0.536	49.2
Malic Enzyme	ALM	7.90	6.85	7.54	7.43	100
	ALM_g	6.27	5.38	5.81	5.82	78
Lysine Dependent Oxygen uptake Activity	ALM	10.16	8.89	9.50	9.52	100
	ALM_g	0.651	0.77	0.77	0.72	7.6

DISCUSSION

1. Growth of D. Discoideum

In order to look at amino acid metabolism and its regulation during growth, the work was split into two stages. Firstly to obtain a good growth system which provided suitable conditions for the analysis of amino acid utilization. Secondly to develop and use methods which allowed amino acid utilization and changes in amino acid metabolism to be measured.

A strain Ax2 derived from D. discoideum NC4 was used for the growth studies because it can grow on a defined medium and is adapted for growth in axenic liquid medium (ALM) (Watts and Ashworth 1970). This axenic liquid medium is suitable for these studies because the cells can grow in both the presence and absence of added glucose and thus provide two separate systems for analysis. In other, more defined media, glucose is a necessary component for growth of the cells. During growth in ALM, amino acids must first be deaminated to provide a suitable substrate for energy production (Ashworth and Watts 1970). So, the rate of ammonia production during growth is a measure of rate of amino acid oxidation. Based on this fact Ashworth and Watts (1970) have shown that less ammonia is produced during growth in ALM in the presence of glucose than in its absence (see Table) and consequently amino acid utilization is reduced by glucose in the medium.

Glucose added to medium	Ammonia production μmol/ml culture	Culture density cells/ml
86 mM Glucose	3.28 $\pm$ 0.03	3.6×10^6
None	8.70 $\pm$ 0.67	3.4×10^6
16 mM Glucose	6.44 $\pm$ 0.06	3.8×10^6
None	9.32 $\pm$ 0.22	4.2×10^6

Glucose shows a similar effect during early differentiation and according to Murphy and Klein (1979) the presence of glucose in combination with essential amino acids inhibits cell differentiation and is accompanied by production of lower levels of ammonia than seen by the cells incubated with only essential amino acids.

We have confirmed these observations during growth in ALM in the presence and absence of glucose and have shown that ammonia levels are reduced by addition of glucose (Figure 30, Table 4,5)

Glucose added to medium	Ammonia production umol/6x10^{6}cells/ml
86 mM Glucose	8.6$\pm$0.17
None	17.6$\pm$0.34

Cellular growth in axenic liquid medium (ALM) not only results in ammonia production but also causes the pH of the medium to increase (Figure 12). In glucose supplemented medium growth yield is significantly increased but glucose also helps to minimise the increase in pH that occurs with growth (Figure 12). Glucose seems to promote an increase in cell density in two ways i) by its own utilization by the cells (glucose concentration decreases from 86 mM to 69 mM (data not shown) by the end of growth) ii) by reducing the pH change with growth because of reduced ammonia production from amino acid utilization and glucose being converted to CO_2.

As pH and ammonia changes are higher in ALM, this medium was looked at in more detail to determine if these changes were likely to alter either the amino acid metabolism or the growth of the cells to an extent that could influence the reproducibility of the harvested cells.

1.1. Growth in axenic liquid medium (ALM)

After giving a certain growth yield, the cells stop growing. The cessation of growth could be due either to exhaustion of nutrients or to changes in the medium caused by growth, the most significant of which appear to be pH and ammonia concentration.

1.1.1. Effect of nutrient concentration

The results show that cellular yield is proportional to the concentration of nutrients as ALM. Higher concentrations of ALM increase the final cell density but reduce the growth rate (μ). By the end of growth, however pH increases at all ALM concentrations to as high a value as that produced during growth in normal nutrient concentration of the medium (Figure 13). So it is difficult to say that cessation of growth is due to nutrient depletion and not due to pH alteration. As far as individual amino acids are concerned, amino acid analyses show little change in their concentration by the end of growth (Table 6, 7)

The changes in concentration, measured are too small to detect reliably but all the amino acids, present initially, are still present when growth stops and so seem unlikely to be the cause of the end of growth.

1.1.2. Effect of pH

pH seems to have some effect on growth of D. discoideum Ax2 as the inocula which were allowed to grow to a pH value of 7 or above did not always grow properly on subculturing. A series of experiments carried out with varying starting pH of the medium from 6.0 - 7.0 showed that there is more cell yield with lower starting pH (Figure 17) but by the end of growth in all cases, the pH had gone beyond 7.0.

Certain attempts to stabilise pH, by using higher concentrations of buffer, did help to reduce the changes in pH but cell density and growth rate (μ) are also decreased (Figure 19, 20, 21).

A series of experiments carried out with pH maintained at 6.0, 6.2, 6.5 and 6.7 by addition of acid showed that pH did affect growth. There is an increase of 71% of the yield when pH is maintained at 6.7 during growth in ALM (Figure 22). An increase in cellular yield is also

obtained as pH is maintained at 6.5, 6.2 and 6.0 respectively (Figure 23, 24, 25). These results show that, in the absence of pH control, the cessation of growth is not due to depletion of nutrients, unless it is a pH related depletion, and that cessation of growth could be due to an increase in pH.

1.1.3. Effect of ammonia

In D. discoideum, amoebae, growing either upon a bacterial associate or in axenic medium, protein degradation occurs and ammonia is a major catabolic product (Schindler and Sussman 1977). Hence ammonia is another factor which could cause the termination of growth. During growth in ALM, the amount of ammonia produced is independent of the pH of the medium and 17.6 $\pm$ 0.34 mM ammonia is produced when calculated on the basis of $6x10^6$ cells/ml (Table 4) Growth experiments, either without pH control (Figure 14) or with pH maintained at 6.7 (Figure 15), with ammonium chloride added at concentration up to 20 mM, showed that the presence of ammonia inhibited growth and reduced growth yield. Although there is a 83% reduced cellular yield under pH controlled conditions in the presence of 20 mM NH_4Cl but growth still occurs in the presence of ammonia above 20 mM (Figure 15). Hence, complete cessation of growth is not due to the ammonia produced in the medium by cells during growth under non-pH controlled conditions.

1.2. Growth conditions in axenic liquid medium

In practical terms it proved impossible to grow all cultures with pH control to allow optimal growth, and it is clear that under non-pH controlled conditions changes in the medium are occuring that could influence the nature of the cells.

In addition there are other possible factors that could restrict growth, such as the extracellular inhibitor described by Yarger et al., (1974), which appears when amoebae of D. discoideum reach stationary phase in liquid nutrient medium and inhibits cell multiplication when added to logarithemically multiplying cultures. According to Hanish (1975) accumulation of an inhibitor starts during the logarithmic phase and cessation of cell multiplication and final cell concentration are controlled by the accumulation of this inhibitor to an effective level. Although Ferguson and Soll (1976) contradicted these findings and could find no evidence to support Hanish's conclusion. The possibility of factors being produced during growth to regulate further growth cannot be ignored.

Because of all these changes observed and reported to occur during growth, ALM is considered a suitable medium for the studies, provided it is prepared at pH6.2 and grown up to a density of 10^6 cells/ml before harvesting for study in washed cell suspensions. At this cell density all amino acids measured are in excess and pH of ALM was 6.5 - 6.6. When 86 mM glucose is present in the medium (ALM_g), the pH only rises to 6.3 - 6.4 by this cell density. Ammonia produced at 10^6 cells/ml in ALM and ALM_g is 2 mM and 1 mM which are 12% of the values at stationary phase.

Hence pH6.2 was selected as a standard pH condition for the preparation of ALM for production of inocula and for the subsequent growth of D. discoideum to provide logarithmically growing cells for further metabolic studies. Inocula prepared in this medium gave much more reliable results when subcultured into fresh media.

1.3. Influence of carbohydrates on growth in axenic liquid medium

1.3.1. Glucose

As previously described glucose added in the medium increases cell yield, helps to keep pH low (Figure 12) and reduces ammonia production during growth of D. discoideum (Table 4, 5, Figure 30). The effects of glucose are consistant with those reported by Watts and Ashworth (1970) and are due to its utilization by the cells with consequently lower utilization of amino acids. Rickenberg et al., 1975 have also reported that glucose is metabolised by D. discoideum and enhances vegative growth when added to the growth medium. It also inhibits development.

All the changes observed when glucose is present in ALM, are dependent on glucose concentrations added to the medium and increase with the increase in glucose concentration in the medium (Figure 26, 30 Table 5).

1.3.2. Fructose

Fructose, added in ALM, gives an effect similar to glucose. Cell yield is increased and ammonia production is reduced (Figure 27, 31, Table 4). These facts suggest that fructose is utilized by the cells with consequently lower utilization of amino acids and less ammonia production. Watts and Ashworth (1970) reported similar effect of fructose on growth yield when added in axenic liquid medium.

1.3.3. Galactose

Galactose gives different results to glucose and fructose. The cell yield is 66% reduced by the addition of galactose to the medium compared to ALM alone (Figure 28) and growth rate (μ) is reduced to 0.023 h^{-1}. There is no difference in the amount of ammonia produced at the same cell density (Table 4). Watts and Ashworth (1970) have

reported a similar inhibitory effect of galactose on growth and Rickenberg et al., (1975) have demonstrated that Strain Ax2 of D. discoideum grown in HL5 supplemented with galactose, exhibits a longer generation time and lower final cell yield than cells grown in the same medium with either glucose or no carbohydrate supplemented. Although De Meglio and Friedman (1978) confirmed these findings with respect to growth using strain A3 and demonstrated that the cells could metabolise galactose but the evidence they give is not good enough and is incomplete to draw a conclusion that galactose is utilized by the cells.

Therefore, on the basis of growth of D. discoideum in ALM supplemented with different carbohydrates, and ammonia production during its growth in the supplemented medium, it can be concluded that glucose and fructose are metabolised by D. discoideum and so reduce amino acid utilisation whereas galactose is not utilized.

2. Nature of the organism used in these studies

As a consequence of these growth experiments, it is apparent that there are discrepancies between some of the data presented here and the data reported in the literature for D. discoideum Ax2. The most obvious of these discrepancies is the growth rate (μ) of the organism in ALM in the presence of glucose. Typically in the presence of glucose, D. discoideum Ax2 should grow at a specific growth rate of 0.081 h^{-1} (doubling time 8.5 h) but in the experiments reported here, the growth rate is 0.032 h^{-1} (doubling time 22 h). This, a significant, difference was noticed from the start of the work. It was assumed, however, that the slow growth rate was due to the differences found with the irreproducibility of inocula, but this is clearly not the case. It follows that the organism used for this study is not strain Ax2 but either an unrelated axenic strain of uncertain parentage or a variant

derived from Ax2. None of the work carried out can distinguish between these two possibilities.

The other data from growth experiments suggest that the organism is similar to strain Ax2 (Figures 13, 22, 23, 24, 25, 27, 28, Table 4). Fructose, added to ALM gives an effect similar to glucose; galactose not only reduces growth rate but also reduces the yield below that of the control in the absence of glucose. It could be significant, however, that all these compounds and indeed a number of other compounds (phosphate buffer (Figure 19), MES buffer (Figure 20), ammonium chloride (Figure 14, 15, 16) and some others the data on which are not presented) which were added during growth caused a reduction in the growth rate. According to Dr. Watts (private communication) and information from the literature (Watts and Ashworth 1970) the growth rate of Ax2 should not be influenced by the addition of some of these compounds.

It would, therefore, appear to be a feature of this strain that growth rate is reduced by additions to the basic growth medium (ALM) and this may be because the membrane or uptake processes are sensitive to osmotic strength changes in the medium. No facts other than an osmolarity change to the medium, appears to be common to the range of compounds which reduce the rate of growth of the cells.

In conclusion, it would appear that the strain used for these studies was not D. discoideum Ax2 but a variant derived from Ax2 whose ability to absorb nutrients is more than usually sensitive to alterations in the osmotic strength of the growth medium.

3. Metabolism of ALM in washed cell suspensions

ALM is a complex medium and a number of changes take place simultaneously with growth. Although ammonia production during growth suggested that amino acid catabolism was lower in the presence of glucose (Table 4, Figure 30), direct assay of amino acids during growth does not provide an adequate measure of utilization (Table 6 and 7). In an attempt to simplify this study and to assess the rate of metabolism of individual amino acids, cell suspensions, prepared in 7.19 mM phosphate buffer pH6.2 after growing the cells in bulk under defined conditions, were used.

In the absence of extracellular nutrients, washed cell preparations utilize endogenous reserves of amino acids and ammonia is produced. The endogenous rate of ammonia production in ALM_g cells is lower by about 50% of that observed with ALM cells (Table 9, 13). Addition of ALM, a rich source of nutrients, to the cell suspensions stimulates ammonia production. When added to ALM cells these nutrients increase the rate of ammonia production about 6 fold (9.0 to 53 nmol/min/mg protein) presumably because of the increased availability of amino acids for metabolism (Table 9). Under the same conditions ammonia production by ALM_g cells is also increased about 6 fold (4.2 to 22 nmol/min/mg protein) (Table 13). So after addition to ALM, the rate of ammonia production in ALM_g cells is still about 50% of that found in ALM cells.

In contrast, the basal rates of oxygen uptake by ALM and ALM_g cells are the same and are stimulated equally by the addition of ALM to the cell suspensions (Table 16, 19). These results are rather difficult to interpret.

At the basal level the lower rate of ammonia production by ALM_g cells but equal rate of oxygen uptake by both types of cells can be explained by assuming that endogenous glucose or glycogen is used along

with amino acid reserves. The reduced rate of ammonia production in ALM_g cells is not due to reduced metabolism in general but to amino acid catabolism being reduced because these cells contain large amounts of glucose which contributes, to overall metabolism, to a greater extent than occurs in ALM cells.

When ALM is added to the cell suspension, of both types, there is an equal increase in oxygen uptake which suggests that there is still an equal rate of metabolism in both types of cells. The fact that a greater stimulation in ammonia production is generated in ALM cells, than in ALM_g cells, without a corresponding increase in oxygen uptake is difficult to explain. Two explanations seem feasible.

The first explanation is that oxygen uptake measures total catabolism of all compounds and that the rate of deamination is greater than the rate of catabolism. The higher ammonia output, in ALM cells, simply reflects a higher capacity in these cells to partially metabolise amino acids by deamination. This explanation is not entirely satisfactory because it would represent massive loss of amino acids to the cells, but with regard to partial metabolism, it is supported by some measurements made using individual amino acids as supplements to washed cell suspensions (Table 22).

A second explanation is that the presence of a balanced mixture of amino acids stiumulates cellular metabolism and increases the breakdown of endogenous, non ammonia containing reserves to compensate for a shortfall in metabolism from amino acid breakdown. Such a mechanism would not continue for long but may be used while the cells adapt to the new metabolic balance.

4. Metabolism of individual amino acids in washed cell suspensions

Individual amino acids, added to cell suspensions, are able to stimulate the rate of ammonia production. The degree of stimulation is dependent on the amino acid added and, as the addition has little effect on oxygen uptake rates (Tables 17, 19), is due to added amino acid replacing endogenous metabolite breakdown. Arginine and lysine, in particular, cause large stimulations to ammonia release (Tables 8, 12). Although part of this increase may be due to the high nitrogen content of these amino acids, metabolism of arginine and lysine, measured by radiolabelled carbon dioxide production from uniformly labelled substrate, is still high compared to other amino acids (Table 21). The other amino acids have a smaller influence on ammonia production but, when measured by ^{14}C- carbon dioxide production from radiolabelled amino acid, are still metabolised (Tables 8, 12, 21).

As observed with addition of ALM to cell suspensions, individual amino acids added to ALM cells are catabolised at a greater rate than observed in ALM_g cells (Table 21). In both cell types, arginine and lysine are catabolised at a greater rate, so the pattern of ability to use amino acids is similar in the two types of cells but the overall rate of metabolism of each amino acid in ALM_g cells is only about 50% of the rate in ALM cells, so the ability of cells to catabolise individual amino acids is similar to their ability to catabolise the components of ALM.

In general, the data from these experiments where ALM or individual amino acids are added to suspensions of cells grown in the presence and absence of glucose, show that growth in the presence of glucose reduces the capacity of the cells to catabolise amino acids.

4.1. Influence of metabolites on amino acid utilization

A considerable effort was put into a series of experiments designed to try to measure the rates of utilization of amino acids in cells after addition of nutritional supplements to the cell suspension, using both ammonia and ^{14}C- carbon dioxide production rates as measures of metabolism.

There are, however, considerable discrepancies in the measurements of rates of metabolism depending on the method of estimation used (see Table 22). For example, the rate of lysine catabolism based on increased ammonia production is 270 nmol/h/mg protein whereas the rate calculated from ^{14}C- carbon dioxide production is only 37 nmol/h/mg protein. In calculating these values, it is assumed that the increase in ammonia production, on addition of lysine, is due only to loss of all the ammonia from lysine and that production of ^{14}C- carbon dioxide is due to complete catabolism of lysine to CO_2 and H_2O. This discrepancy clearly means, as would be expected, that these assumptions are not true. Lysine is not fully catabolised to carbon dioxide but will be used to replenish many of the catabolic pools in the cell. This represents partial metabolism of the amino acid and consequently the measurement of CO_2 production is an underestimate of the rate of lysine metabolism. Similarly ammonia is not only derived from lysine but also from the other catabolites and its value must represent the sum of ammonia from all sources. Its quantitative assessment is influenced by the fact that the basal rate of ammonia production is high and in some cases an amino acid does not stimulate its ammonia production to above the basal level. There must therefore be some error involved in the measurement of ammonia production. Furthermore, assays of amino acid metabolism, in the presence of metabolites which influence metabolic rates, will influence the basal rate of ammonia production and so introduce another variable that is hard to assess accurately (Tables 11,15,22)

The problems in assessment of rates of metabolism make it essential that assumptions are made about the measurements before any interpretation of data under different conditions, is carried out. These assumptions are that, irrespective of conditions, the measurement of CO_2 derived from a radiolabelled amino acid represents a constant fraction of that amino acid's metabolism and is therefore a good measure of breakdown of that amino acid. A further assumption is that ammonia production is a measure of the total catabolism of amino acids in the system and can only in exceptional cases be used to measure metabolism of individual amino acids. These assumptions are considered to apply in the analysis of changes in metabolism that follow.

Considerably more effort was made to analyse the influence of glucose and it will be considered as a model additive before looking at evidence for other compounds.

4.1.1. Influence of glucose

In washed cell suspension experiments glucose, at 86 mM, has a similar effect on metabolism to its influence during growth. The presence of glucose depresses the rate of ammonia production from cells irrespective as to the nature of the cells (ALM or ALM_g) or to the presence of extracellular nutrients (Table 9, 13). The reduction in the rate of ammonia production from extracellular nutrients is slightly greater for ALM_g cells (54.5%) than ALM cells (51%) and could be due to ALM_g cells being more able to catabolise glucose.

Glucose has a similar effect on the ability of the cells to catabolise individual amino acids. For all the amino acids examined, glucose, at 86 mM, reduces the rate of ^{14}C- carbon dioxide production from radiolabelled amino acids by the cells. It also reduces ammonia

production from amino acids (Table 22), but it is difficult to assess quantitatively because of changes produced in the basal rate of ammonia production and could be complicated by glucose metabolism generating acceptors for ammonia in transamination reactions.

The data suggest that glucose reduces the metabolism of all amino acids to an equivalent amount (Table 22) and if extrapolated to growing cells, suggests that during growth, the presence of glucose reduces the rate of catabolism of all amino acids.

4.1.2. Effect of other metabolites

A range of metabolites, added to cell suspensions, in the presence or the absence of any exogenous nutrient supply (Table 10 a & b) affect ammonia production and is qualitatively similar to the effect of glucose. Addition of carboxylic acids, in particular, have a greater affect on ammonia production than carbohydrates and could be explained either by utilization of these metabolites by the cells or because they act as acceptors for ammonia. Qualitatively these metabolites have similar effects on ammonia production by individual amino acids added to cell suspensions (Table 11) but the changes are so small that it is difficult to draw any firm conclusions from the data. The only conclusion that can be made is, the general one, that the presence of a metabolite reduces the extent to which exogenous amino acids stimulate ammonia production. While it might be possible to infer that ammonia production from arginine demonstrates similar differential inhibition by additives to ALM, the same cannot be said, with confidence, to any other amino acid.

However, the reduced ^{14}C- carbon dioxide production from uniformly labelled arginine added to the cell suspension along with carbohydrates and carboxylic acids (Figure 38) does indicate that these

additives cause a reduction in arginine utilization by the cells and other experiments (Figure 39, 41, 42, 43) have shown that these effects apply to all the amino acids looked at in detail. Using ^{14}C-carbon dioxide production data, cells grown in the presence of glucose show similar effects due to metabolites at higher concentration on the metabolism of amino acids but quantitatively there are lower rates of metabolism in ALM_g cells, demonstrating once again the reduced capacity of the cells for amino acid metabolism (Figure 44, 45, 46, 47).

In all these studies, little change was measured in the rate of oxygen uptake by cell suspensions. The addition of amino acids therefore merely replaces endogenous metabolites but does not alter the total rate of metabolism. Similarly the addition of other metabolites, either in the presence or absence of added amino acids, does not alter greatly the rate of oxygen uptake (Tables 17, 18a, b, c, 19, 20) and so these must also replace endogenous metabolites in the metabolism of the cells. The metabolism of all these compounds may be quite small since the changes observed in amino acid utilization by ^{14}C- carbon dioxide measurement and ammonia production at these concentrations of additive are small.

In summary, the addition of a range of metabolites therefore depresses amino acid catabolism to similar extents in ALM and ALM_g cells with the only difference being the capacity of the cells to degrade amino acids. There must therefore be controls on the rate of amino acid utilization by these compounds which appear to influence the rates of utilization of several amino acids to similar extents. This would suggest a common factor in this regulation.

5. Effect of metabolite concentration on amino acid utilization

5.1. ALM grown cells

It was noticed that glucose and trehalose, at 20 mM, stimulate ^{14}C- carbon dioxide production from uniformly labelled arginine, when added to the cells grown in ALM (Figure 38), while at a higher concentration (86 mM), glucose reduces ^{14}C- carbon dioxide production. Ammonia production is reduced at both 20 mM and 86 mM glucose (Table 22).

To examine this further, a complete range of glucose concentrations were tested and show a stimulation in ^{14}C- carbon dioxide production at lower concentration (10 mM and 20 mM) which thereafter decreases as glucose concentration increases (Figure 39). The stimulation in ^{14}C- carbon dioxide from added amino acids at low glucose concentrations may be explained by considering the work by Lee (1972) since his work is not inconsistent with the work I have done. Lee reported that the presence of glucose stimulated the utilization of the amino acids lysine and glutamic acid in D. discoideum and attributes this increased utilization to increased uptake, and consequently increased metabolic pools, of the added amino acid. Assuming that the same is the case with other amino acids, glucose, in this system, stimulates the uptake of labelled amino acid and so increases the amount of that amino acid inside the cell. The rest of the amino acid pool, which is derived from endogenous reserves, will remain the same. Metabolism of the added amino acid in proportion to other amino acids is increased due to the higher pool of this amino acid and consequently ^{14}C- carbon dioxide production is stimulated.

In bacteria, active transport of amino acids is usually dependent on the availability of an energy source, e.g. glucose (Gale and Folkes, 1967). However in this work in D. discoideum glucose does not seem to

be associated with energy mediated uptake of amino acids, since 2-deoxy D-glucose, a non metabolisable glucose analogue also stimulates ^{14}C-carbon dioxide production from uniformly labelled arginine at 10 mM and 20 mM when added to the cell suspension which reduces thereafter with the increase in concentration (Figure 48).

Glucose has another effect. It is cometabolised when present with amino acids. As a consequence, as glucose concentration increases, the rate of metabolism of all amino acids, including the added amino acid, decreases. Since only the extracellular amino acid is stimulated by glucose, the catabolism of the other amino acids and so ammonia production decreases as glucose concentration increases.

However, for the added amino acid, the stimulation due to the glucose effect on uptake and the reduction due to metabolism compete and it is only above 20 mM that the decrease in the rate of metabolism of the added amino acid becomes dominant.

It is not clear if glucose metabolism is the only factor decreasing the utilization of amino acids. Other metabolites namely pyruvate, galactose, phosphate and 2-deoxy-D-glucose all decrease ^{14}C- carbon dioxide production as their concentration increases (Figures 41, 42, 43, 48). There is a smaller decrease in ^{14}C- carbon dioxide production with increasing galactose concentration as compared to glucose (Figure 39, 42). Pyruvate, however gives a greater effect than glucose with ^{14}C- carbon dioxide production being reduced at a higher rate with the increase in concentration (Figure 39, 41).

Galactose could be little metabolised. It inhibits growth and has no effect on ammonia production (Figure 28, Table 4) so its metabolism is not great. Pyruvate which slightly increases growth yield and reduces ammonia production (Figure 29, Table 4) seems to be metabolised.

However, on the basis of data from the growth experiments neither galactose nor pyruvate are metabolised as much as glucose. Phosphate is not metabolised and reduced ^{14}C- carbon dioxide production from uniformly labelled amino acids (Figure 43) and 2-deoxy D-glucose, at higher concentrations, also reduces ^{14}C- carbon dioxide production from radiolabelled amino acids arginine (Figure 48). The effect of these last two compounds on ^{14}C- carbon dioxide production cannot be due to their metabolism by the cells.

The concentration of the metabolites added are such as to produce significant osmotic changes in the medium and these might contribute to the rates of amino acid utilisation. There are reports of osmotically induced changes in cellular slime moulds. Harris and North (1982) described the activity of the enzyme, ornithine decarboxylase, which is sensitive to change in the osmolarity of the environment. When the osmolarity was reduced, Harris and North measured high enzyme activity. During differentiation of D. discoideum, raising the osmotic strength to about 150 m Osmol completely inhibits cAMP production in response to cAMP pulses (Darmon and Klein 1978). Toama and Raper (1967) also reported that increasing osmotic pressure in Polyspondylium pallidum, induces microcyst formation instead of aggregation.

Therefore, since osmotic pressure exerted by the metabolites could affect the utilization of amino acids (Figure 49), the decreased rate of ^{14}C- carbon dioxide production, as concentration of metabolites such as glucose and pyruvate increases, is partly because of their utilization by the cells and partly because of the osmotic effect of these metabolites. Whereas the reduced rate of ^{14}C- carbon dioxide production by galactose, phosphate and 2-deoxy D-glucose seem all due to the osmotic effect of these additives on the cells.

Since all metabolites have a similar effect on the utilization of all amino acids, there must be a common feature responsible for this effect. It is not likely to be an enzymic effect because all amino acids, have different metabolic pathways. However it could be the effect of the osmotic pressure of these metabolites on the uptake system of the cells such as pinocytosis. The uptake of the medium by the cells, calculated on the basis of 'medium clearance', decreases as the concentration of glucose increases in the medium (Figure 50) and provides support for this hypothesis.

5.2. ALMg grown cells

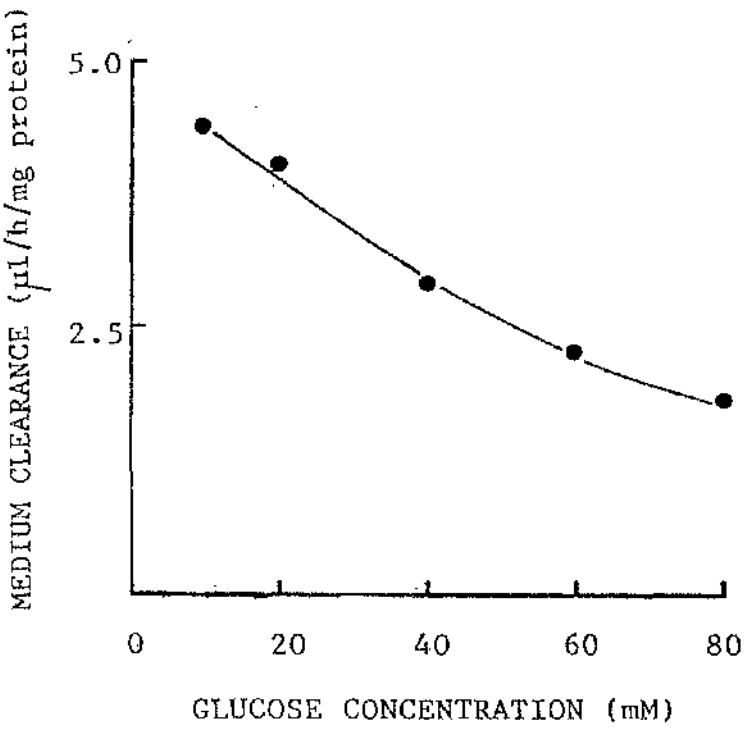
ALMg cells do not show a similar effect to ALM cells. Since in ALMg cells increasing concentration of all the metabolites added, including glucose, decreases the rate of ^{14}C-carbon dioxide production. According to Lee (1972) presence of glucose stimulates the utilization of amino acid. The lack of stimulation in ^{14}C-carbon dioxide production in the presence of low concentration of glucose in ALMg cells can be explained by a combination of a reduced ability to catabolise amino acids (Table 21), a greater endogenous rate of metabolism from non amino acid sources and perhaps a greater ability to catabolise glucose. As a result the reduction due to metabolism, both endogeneous and from added glucose, is greater and so the stimulation is not observed at the concentrations of glucose used.

Figure 50: Effect of glucose concentration on the medium clearance by D. discoideum Ax2 cells grown in ALM pH6.2.

Medium clearance measures the minimum volume of medium that must be endocytosed by the cells to explain the observed rate of metabolism. As such it is a measure of the rate of pinocytosis. Medium clearance was calculated as

$$\frac{\text{Glucose catabolised at given glucose concentration}}{\text{Glucose concentration}}$$

using the data from figure 40 and graphed against glucose concentration. The figure demonstrates the influence of glucose concentration on the rate of pinocytosis (as measured by medium clearance).


MEDIUM CLEARANCE (µl/h/mg protein)
5.0
2.5
0
20
40
60
80
GLUCOSE CONCENTRATION (mM)

6. Enzyme activities under different growth conditions

Although the results of direct amino acid measurement by amino acid analyses are difficult to assess, ammonia estimations, oxygen uptake and ^{14}C-carbon dioxide production from radiolabelled amino acids by the cells, all suggest that there is considerable amino acid utilization by D. discoideum Ax2 during growth. Furthermore different rates of ammonia and ^{14}C-carbon dioxide production by ALM and ALM_g cells indicate changed levels of catabolism by two different types of cells and could indicate the presence of different levels of amino acid metabolising enzymes.

Six enzymes were selected for enzyme activity measurements mainly because these enzymes are important for the metabolism of those amino acids which were studied in detail. They are either directly involved in the metabolism of these amino acids like tyrosine transaminase and threonine deaminase or play an important role in the general metabolism of many amino acids e.g. malic enzyme and glutamate dehydrogenase. Tyrosine transaminase (Pong and Loomis Jr. 1971), threonine deaminase (Pong and Loomis Jr. 1973), glutamate dehydrogenase (Langridge et al., 1977) and malic enzyme (Kelleher et al., 1979) have already been reported in D. discoideum.

6.1. Ornithine aminotransferase

Ornithine aminotransferase is involved in the catabolism of arginine (see appendix). It is present in gram positive bacteria and yeast, in fungi, green algae, protozoa, higher plants and animals (Scher and Vogal 1957; Roberts 1954). It is a mitochondrial enzyme (Peraino and Pitot 1963) which catalyses the interconversion of L-ornithine and 2-oxoglutarate to produce glutamic γ-semialdehyde and glutamate (Strecker 1965; Peraino and Pitot 1963). Hence ornithine aminotransferase is

involved in the catabolism of arginine which is one of the amino acids actively used by D. discoideum Ax2 during growth and indeed most actively catabolised in washed cell suspensions. Therefore, this enzyme was selected for enzyme activity measurements to study the effect of changing growth conditions on its activity. Ornithine aminotransferase activity, under the same assay conditions, was found to be 43% greater in ALM cells than ALM_g cells (Table 23). So the level of activity is related to cellular catabolism of arginine.

6.2. Tyrosine transaminase

Tyrosine transaminase is the enzyme which catalyses the first reaction in the pathway by which tyrosine is degraded to acetate and fumarate and so to CO_2 (see appendix). It catalyses the rate limiting step in tyrosine degradation in higher organisms (Tomkins et al., 1969). The enzyme in D. discoideum accumulates during development to a specific activity more than 3 fold greater than in growing cells (Pong and Loomis Jr. 1971). The increase in activity after 8h of development may suggest increased utilization of tyrosine by D. discoideum at that stage of development.

Studies described earlier, based on ammonia and ^{14}C-carbon dioxide production from tyrosine, indicate that tyrosine can be metabolised more rapidly by ALM than ALMg cells. The rate of tyrosine metabolism is related to levels of tyrosine transaminase activity since enzyme activity is 52% greater in ALM cells than in ALMg cells (Table 23).

6.3. Threonine deaminase

Threonine deaminase is the first enzyme involved in the catabolism of threonine and L-threonine is converted to 2-oxobutyrate and ammonia. 2-oxobutyrate is further catabolised to succinyl CoA which enters TCA cycle for further metabolism (see appendix).

Two distinct threonine deaminases have been described in Escherichia coli; one is biodegradative and the other is biosynthetic (Umbarger and Brown 1957). Pong and Loomis Jr. (1973) have also described two threonine deaminases in D. discoideum. The first enzyme, which is sensitive to feed back inhibition by isoleucine, is synthesised in growing cells and is inactivated after the initiation of development. The second enzyme, which is isoleucine insensitive, accumulates during the aggregation and pseudoplasmodial stage of development.

Since the experiments measured threonine deaminase under growing conditions, the biosynthetic enzyme was expected. However, when activity was assayed in the presence of either of the inhibitors (20 mM isoleucine or 10 mM leucine) no difference in threonine deaminase activity was detected. The activity reported here is insensitive to inhibition and corresponds to the degradative threonine deaminase activity.

As has already been observed with the other enzymes of amino acid metabolism the activity of threonine deaminase is higher in ALM cells by 48% (Table 23) showing greater ability in these cells to catabolise threonine.

6.4. Glutamate dehydrogenase

Glutamic acid has a central position in the metabolism of amino acids since amino acids are catabolised through transamination with 2-oxoglutarate to produce glutamate. Glutamate dehydrogenase then regenerates 2-oxoglutarate and releases ammonia. An alternative pathway proposed by Vender et al., (1965) for release of ammonia, uses transamination with oxaloacetate to form aspartate, which is converted by an enzyme aspartase, to fumarate and ammonia is lost. This alternative

pathway appears to exist in bacteria (Vender et al., 1965) but in D. discoideum glutamate dehydrogenase probably is the enzyme which is involved in release of ammonia from amino groups. This enzyme has been found in many organisms and tissues (Fahien et al., 1965; Corman et al., 1967; Doherty 1970) and has been reported by Langridge et al., (1977) to be present as 2 activities in D. discoideum. One of these activities is mitochondrial and NADP linked while the other is extramitochondrial and NAD linked.

Enzyme activity, of both enzymes changes with growth conditions, with approximately 55% more activity of both enzymes being assayed in ALM extracts compared to ALMg extracts (Table 23). This activity is related to the ability of these differently grown cell types to catabolise glutamate and is also related to their ability to produce ammonia when a range of amino acids, either individual or all together, are added.

6.5. Malic Enzyme

For the complete oxidation of amino acids via the tricarboxylic acid cycle, Goldstein and Newsholme (1976) described a pathway to pyruvate from tricarboxylic acid cycle intermediate for generation of acetyl CoA. The major enzymes involved in metabolising tricarboxylic acid compounds to produce precursors of acetyl CoA are phosphoenol pyruvate carboxykinase, which utilizes oxaloacetate as substrate and malic enzyme which utilizes malate. Although both of these reactions are known to function in vivo in the direction of acetyl CoA formation, Kelleher et al., (1979) gave isotopic evidence that malate rather than oxaloacetate is the tricarboxylic acid compound used in D. discoideum and hence, that malic enzyme was the important enzyme.

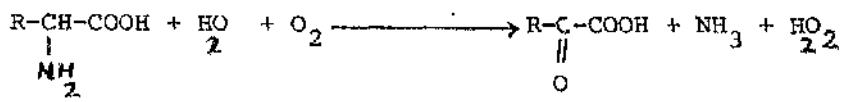
In the experiments described, malic enzyme may be required for amino acid breakdown, but the presence of glucose, which provides an

additional source of acetyl CoA, should reduce the role of malic enzyme. Based on this logic, malic enzyme activity was measured and was found to be lower in the presence of glucose (ALMg cells), although the activity was only reduced by 22% (Table 23). Once again enzyme activity appears to be related to its required activity in the cells.

6.6. Lysine dependent oxygen uptake activity

It was difficult to select a typical or appropriate enzyme activity of lysine, since nothing is known about the route by which lysine is catabolised in D. discoideum and there are many potential degradative pathways (White, Handler and Smith 1978) (see appendix). Lysine is the amino acid which appears to be most rapidly deaminated and since deamination can occur by amino acid oxidase reactions as well as by glutamate dehydrogenase, lysine was used as a substrate for amino acid dependent oxygen uptake activity by oxygen electrode measurements.

The reaction measured would be



Lysine dependent oxygen uptake activity is very low in ALMg cells being only 7.6% of the activity found in ALM cells (Table 23), and would suggest that cells have a very reduced ability to oxidise amino acids by this method while growing in the presence of glucose.

While it is impossible to assess the extent to which this type of activity participates in release of ammonia, its change in activity with growth conditions suggest it may be worth further study.

There are two qualifications that should be made on these measurements of lysine dependent oxygen uptake activities. Firstly the substrate used was L-lysine but the oxidase activity may be able or indeed prefer to use the D- form of the substrate and be used to breakdown D-lysine. As such the activity is not relevant to normal lysine breakdown. Secondly, lysine oxygenase which has been reported to be present in Pseudomonas (Nakazawa, T 1971) and converts L-lysine to δ-amino valeramide which by an other enzyme δ-amino valeramidase, is converted to δ-aminovalerate and ammonia (see appendix), may also be responsible for the observed activity. While these qualifications may alter the significance of the results, they do not reduce its potential interest.

7. Enzyme activity changes

With the exception of the lysine dependent oxygen uptake activity, the changes in enzyme activities measured in the cells grown in two different ways are not large. Those enzymes directly involved in metabolism of amino acids are reduced in activity by 50% and malic enzyme which is less directly involved, by only 20%. Since the cells grow at different growth rates as well as using amino acids at a different rate, there are many possible explanations of these changes.

However, since the ability of the cells to catabolise individual amino acids to carbon dioxide is reduced by 50% by growth in the presence of glucose, and cells growing in the presence of glucose catabolise amino acids at 50% of the glucose free rate (by overall ammonia production measurements) it is tempting to speculate that decreased activity of these enzymes is related to the fact that in the growing cells amino acids are being catabolised at decreased rates. Since most of the activites are reduced in similar proportions, a general control mechanism similar to catabolite repression may be involved. Rickenberg (1974) has reported that growth of bacteria on an effective source of carbon inhibits the formation of cAMP and hence of proteins regulated by catabolite repression. However this conclusion is speculative because it is not even possible to establish that controls on synthesis occur. Similar results could be produced by controls on the rate of enzyme degradation as well as synthesis and no distinction can be made between these possibilities on the basis of these results. There does, however appear to be control in these cells of the level of enzyme activities present under different growth conditions and represents the operation of a 'rough' level of control of metabolism.

8. General conclusion and future prospectus

The initial aim of the project was to ascertain whether the growth of D. discoideum could be used to provide useful information about regulation of protein and more directly amino acid degradation during differentiation.

The answer would appear to be that the system is not suitable for two reasons. Firstly the sort of changes in enzyme activity, are small, - less than occur during differentiation - and consequently are difficult to assess accurately. Activities may be altered to a greater extent if high levels of catabolism of individual amino acids are induced in growing cells but these compounds may for other reasons influence growth rates, and so make interpretation complex. Secondly, the system of analysing growth and getting good values for rates of utilization of particular compounds is difficult to quantify because rates of utilization appear to be dependent on rates of uptake, rates of deamination and partial metabolism. If the data presented here are considered a typical example, it is very difficult to interpret the data obtained in anything other than a very general way.

However, the work could usefully be extended on the following lines.

1) Amino acids are oxidised to provide energy and in D. discoideum it will be interesting to look at the general amino acid oxidases in the cells grown under different growth conditions.

2) Arginine proved to be the most actively utilized amino acid and detailed study of the enzymes of its catabolism i.e. ornithine amino transferase and the enzymes of urea cycle will be of interest to study.

3) Metabolism is dependent on the uptake system such as pinocytosis and it will be useful to study nutrient uptake system in D. discoideum cells grown under different growth conditions using non metabolisable substances.

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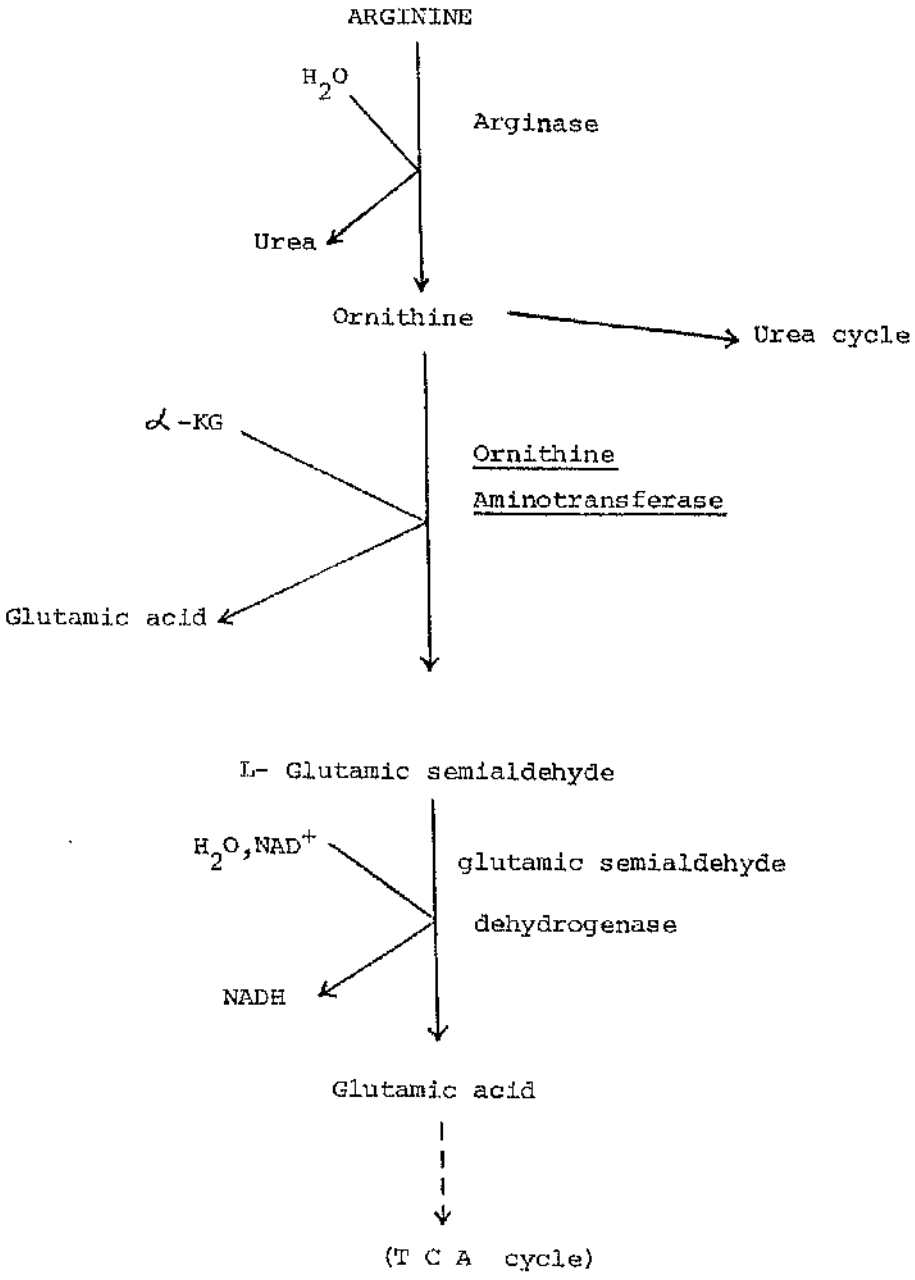
APPENDIX

METABOLIC PATHWAYS

ARGININE



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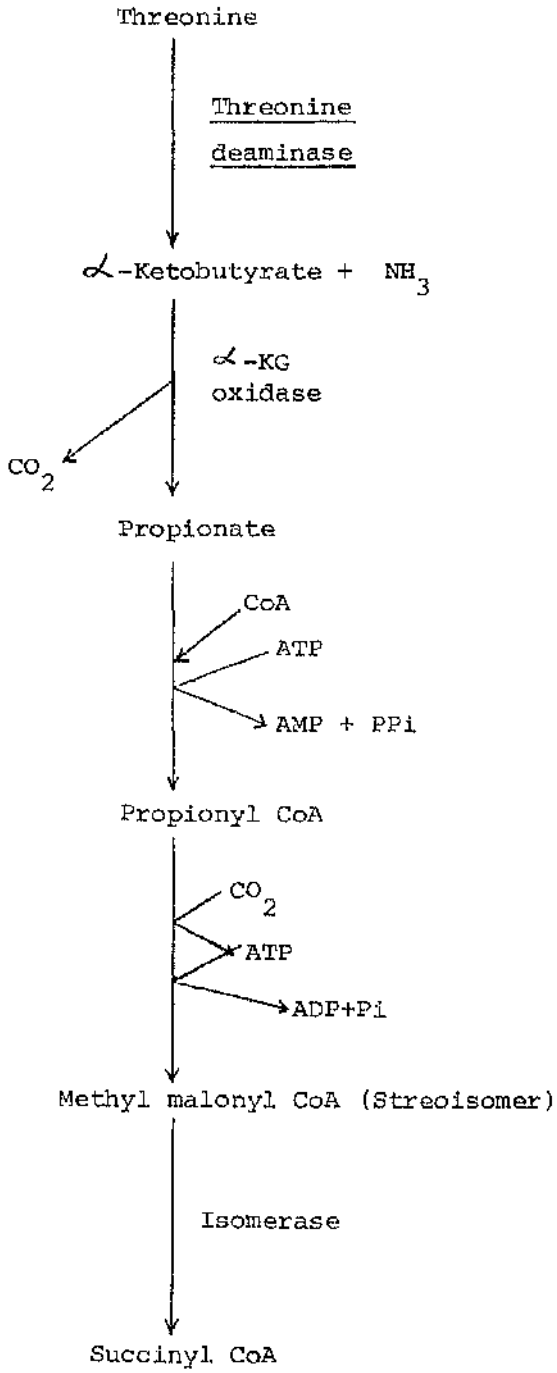
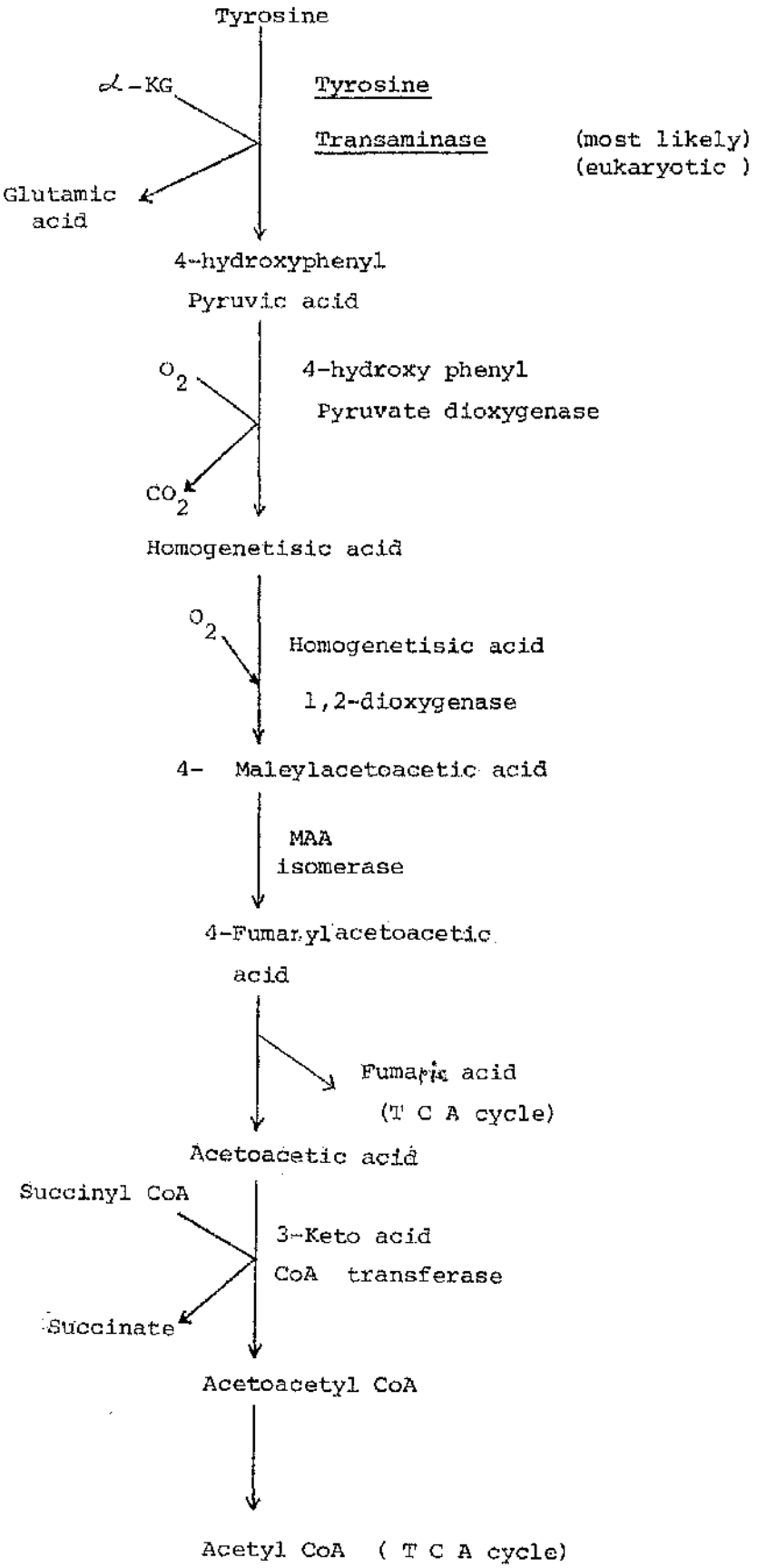
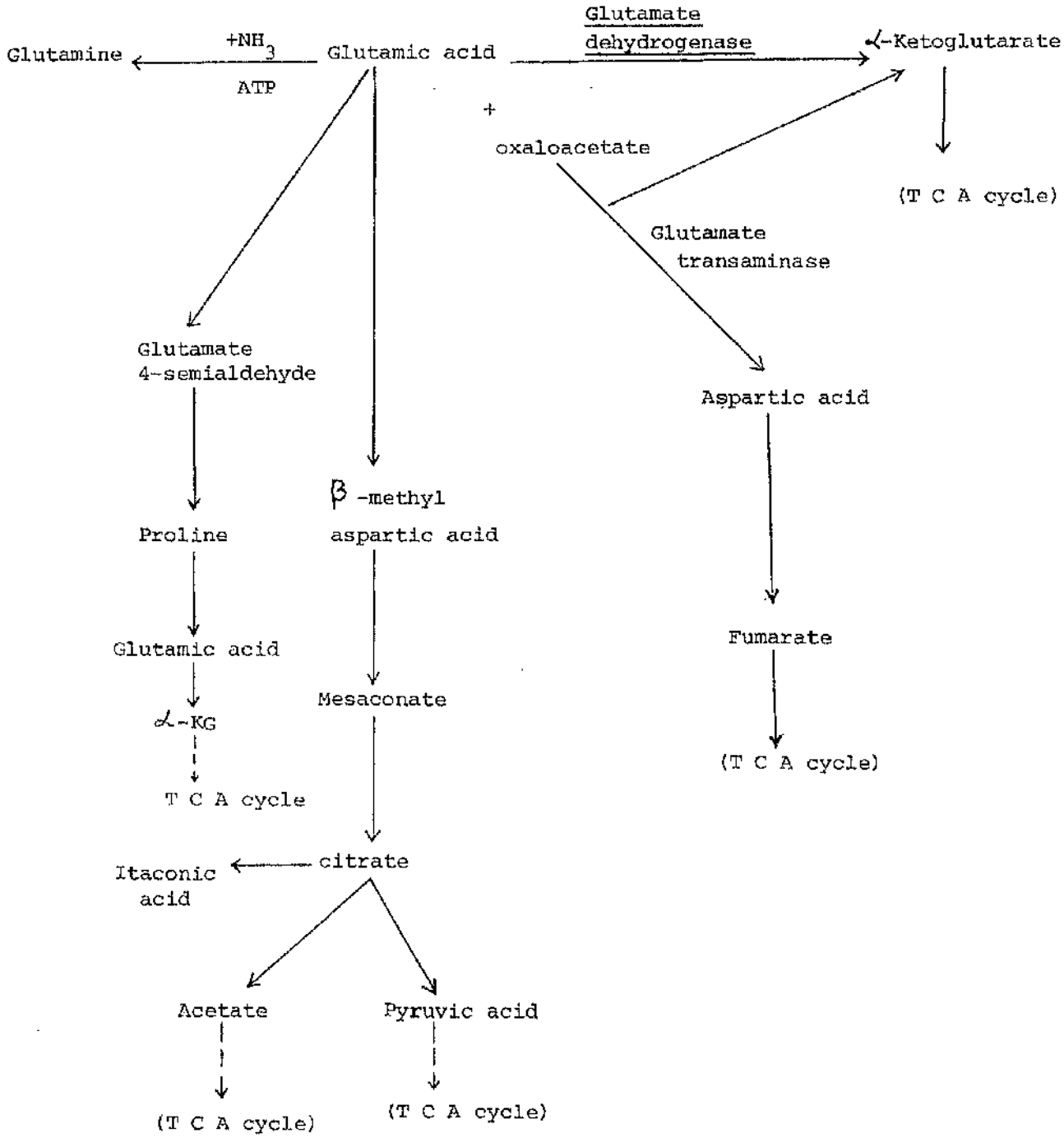
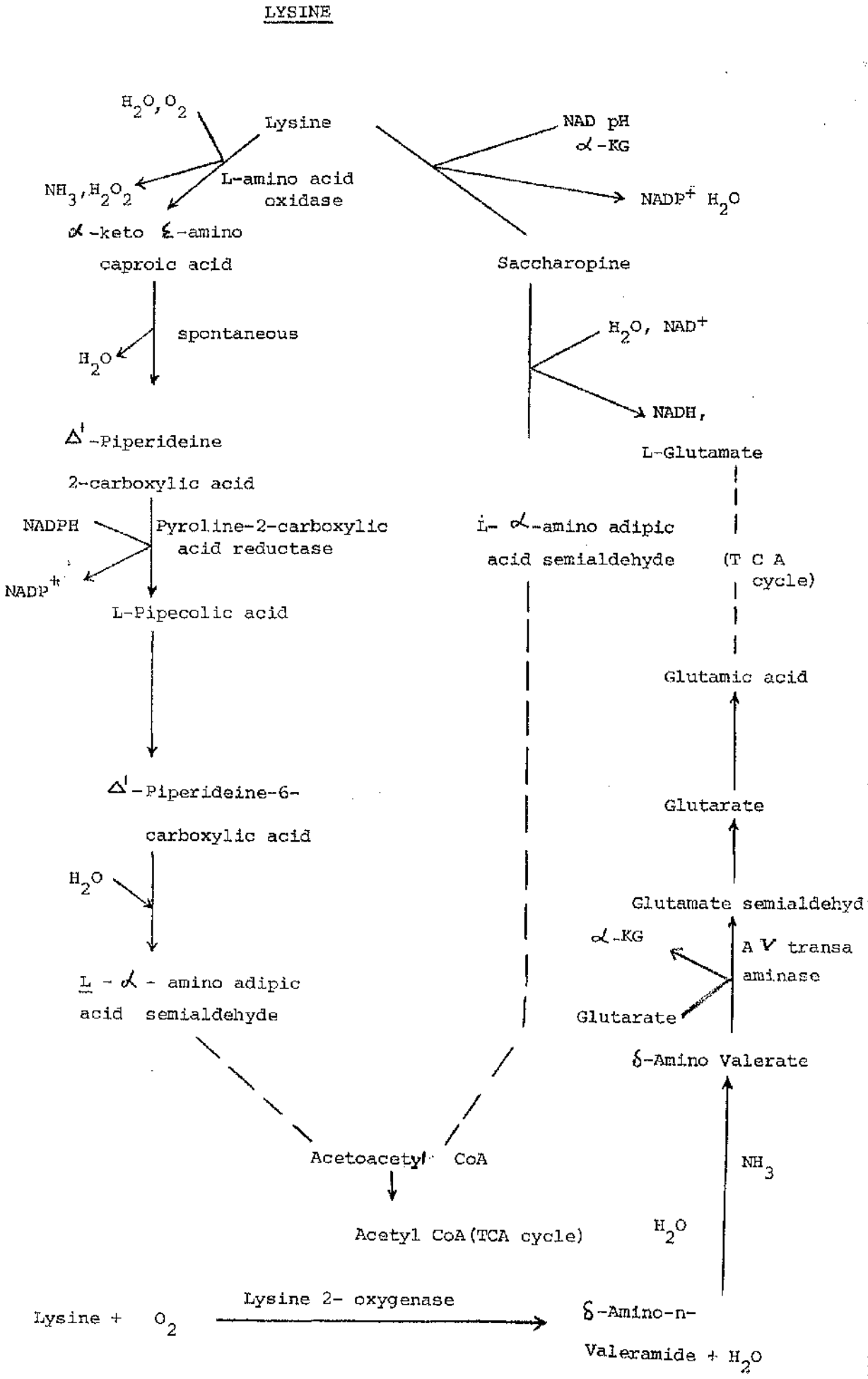

Threonine
Threonine deaminase
α-Ketobutyrate + NH3
α-KG oxidase
CO2
Propionate
CoA
ATP
AMP + PPi
Propionyl CoA
CO2
ATP
ADP+Pi
Methyl malonyl CoA (Streoisomer)
Isomerase
Succinyl CoA
(T C A cycle)

TYROSINE


Tyrosine
α-KG
Tyrosine
Transaminase
(most likely)
(eukaryotic)
Glutamic acid
4-hydroxyphenyl
Pyruvic acid
O_2
4-hydroxy phenyl
Pyruvate dioxygenase
CO_2
Homogenetisic acid
O_2
Homogenetisic acid
1,2-dioxygenase
4- Maleylacetoacetic acid
MAA
isomerase
4-Fumarylacetoacetic
acid
Fumaric acid
(T C A cycle)
Acetoacetic acid
Succinyl CoA
3-Keto acid
CoA transferase
Succinate
Acetoacetyl CoA
Acetyl CoA (T C A cycle)

Glutamic acid




LYSINE
H_2O, O_2
Lysine
NH_3, H_2O_2
L-amino acid oxidase
α-keto ε-amino caproic acid
spontaneous
H_2O
Δ¹-Piperideine 2-carboxylic acid
NADPH
Pyroline-2-carboxylic acid reductase
$NADP^+$
L-Pipecolic acid
Δ¹-Piperideine-6-carboxylic acid
H_2O
L - α - amino adipic acid semialdehyde
NAD pH
α-KG
$NADP^+$ H_2O
Saccharopine
H_2O, NAD^+
NADH,
L-Glutamate
L- α-amino adipic acid semialdehyde
(T C A cycle)
Glutamic acid
Glutarate
Glutamate semialdehyd
α-KG
A V transa aminase
Glutarate
δ-Amino Valerate
Acetoacetyl CoA
Acetyl CoA(TCA cycle)
NH_3
H_2O
Lysine + O_2
Lysine 2- oxygenase
δ-Amino-n-Valeramide + H_2O

The following books/journals were used to draw these metabolic pathways.

1) An introduction to metabolic pathways Dagley, S. and Nicholson, D.E. (ed). Blackwell Scientific Publications, Oxford, Edinburgh (1970).

2) Biochemistry
Lehninger, A.C. (ed).
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3) Reitz, M.S. and Rodwell, V.W.
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4) Methods in Enzymology Vol. XVll A.&B. Tabor, H. and Tabor, C.w. (eds). Academic Press, New York and London (1970).