THE EFFECTS OF EIGHT NON-PHYSIOLOGIC ZWITTERIONIC BUFFERS ON GROWTH OF CHLORELLA PYRENOIDOSA, NEUROSPORA CRASSA AND SACCHAROMYCES ELLIPSOIDEUS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
HANSA VIRAVAIDHYA

1968

ABSTRACT

THE EFFECTS OF EIGHT NON-PHYSIOLOGIC ZWITTERIONIC BUFFERS ON GROWTH OF CHLORELLA PYRENOIDOSA, NEUROSPORA CRASSA AND SACCHAROMYCES ELLIPSOIDEUS

by Hansa Viravaidhya

The purpose of this thesis was to investigate the effects of eight recently introduced buffers on the growth of the unicellular alga, Chlorella pyrenoidosa, an albino strain of Neurospora crassa, and a wild type strain of Saccharomyces ellipsoideus. The buffers chosen for this study were: N,N-bis(hydroxyethyl)glycine (bicine), tris-(hydroxymethyl)methylglycine (tricine), N-hydroxyethyl-piperazineethanesulfonic acid (HEPES), N-hydroxyethyl-piperazine-N'-propanesulfonic acid (HEPPS), morpholine-N-ethanesulfonic acid (MES), morpholine-N-propanesulfonic acid (MOPS), tris(hydroxymethyl)methylaminoethanesulfonic acid (TES), and tris(hydroxymethyl)methylaminopropanesulfonic acid (TAPS). The following aspects of the use of the buffers were investigated:

(1) compatibility of the buffers with the culture media

(2) buffering effectiveness, that is the abilities of the buffers to resist pH changes associated with growth of the organisms

(3) the practical question of optimal concentrations for growth

(4) possible metabolism of the buffers as evidenced by growth stimulation, growth inhibition, or buffer disappearance.

Since there would be no point in using these buffers in growth media outside their buffering ranges, all tests were conducted at or near pKa of the buffer employed. Consequently the pH's of the media were widely different and frequently unfavorable for the test organism. Under unfavorable conditions the factors limiting growth may well differ from those limiting more active growth. Therefore the effects of buffers described herein should not be overgeneralized.

Four aspects of the growth processes were investigated: (1) extent of growth, (2) morphology of the organism, (3) final pH of the growth medium, and (4) approximate amounts of buffers remaining in the medium after growth of the organisms. In no instance was there a large enough disappearance of buffer to be detected by the very rough methods employed. In only one instance a gross morphological change resulted from the addition of a buffer: Neurospora mycelium grown on media containing MOPS became largely

conidial. Effects of the buffers on growth and on pH of the medium can only be appreciated fully by perusal of the graphs in the body of the thesis but may be roughly summarized as follows:

Neurospora crassa

Bicine was slightly inhibitory above 0.01 M, decreasing both the rate of growth and the extent of growth.

Tricine had little effect on the rate of growth, perhaps stimulating growth slightly at the early stages. The highest concentrations (0.05 M) may have slightly decreased the amount of growth obtained in 72 hours.

HEPES increased the rate of growth at all concentrations up to 0.2 M.

HEPPS had little effect on growth at concentrations up to 0.05 M.

MES slowed initial growth rate at all concentrations tried; at 0.05 M the rate was about 50% of the control. However at all concentrations MES increased the extent of the growth achieved after 72 hours.

MOPS also slightly inhibited the rate of growth but somewhat increased the extent of growth after 72 hours.

TAPS had little effect on either the rate of growth or the extent of growth below 0.1 M but inhibited both above that concentration.

TES increased both the rate and extent of growth slightly at all concentrations up to 0.2 M.

Saccharomyces ellipsoideus

Bicine was strongly inhibitory at concentration of 0.05 M.

Tricine increased the rate and extent of growth very markedly at all concentrations up to 0.2 M. At 0.05 M the final growth was about 60% above the control without tricine.

HEPES greatly decreased the rate of growth especially in the early stages but permitted somewhat greater final growth.

HEPPS was strongly inhibitory especially to initial growth. However at 0.05 M the final growth probably would have in time exceeded the control.

MES decreased the rate of growth somewhat, especially in the very early stages but at all concentrations up to 0.2 M permitted greater total growth.

MOPS also decreased the initial rate of growth but significantly increased the final growth, especially at 0.05 M (about 35% above control).

TAPS was very inhibitory to initial growth but permitted somewhat more final growth than the control at the concentration 0.1 M and lower.

TES inhibited initial growth very severely. Indeed this buffer introduced a lag, the duration of which was roughly proportional to the amount of buffer. Once growth had started it proceeded to almost the same rate as in the control. At all buffer concentrations the final growth exceeded that of the control.

Chlorella pyrenoidosa

The effects of buffers on Chlorella growth must be interpreted in light of the unfavorable effects of higher pHs on the growth of this organism. The buffers employed at low pHs, e.g. MES at pH 6.1, had little influence on growth which was excellent with or without the buffer. However most of these buffers employed at unfavorable pHs between 7.0 and 8.0, were quite effective in overcoming the adverse effects of these higher pH's. Notable exceptions were HEPES and HEPPS which were neither inhibitory nor stimulatory.

THE EFFECTS OF EIGHT NON-PHYSIOLOGIC ZWITTERIONIC BUFFERS ON GROWTH OF *CHLORELLA PYRENOIDOSA*, *NEUROSPORA CRASSA* AND *SACCHAROMYCES ELLIPSOIDEUS*

By

Hansa Viravaidhya

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1968

ACKNOWLEDGMENTS

The author wishes to express her deepest gratitude to Dr. Norman E. Good, her thesis advisor, for the freedom he permitted, the guidance he provided and his encouragement during the course of this work.

This investigation was supported by a National Science Foundation grant GB 4568 to Dr. Norman E. Good. The studies were also made possible by a Fulbright Hays Foundation grant to the author.

Sincere thanks are also due to Dr. Edison R. Fowlks for his interest, assistance and counsel and for supplying the yeast strain and the medium here employed.

The interest and helpful suggestions of Dr. G. Douglas Winget, Miss Lidia Sicari and of other persons are deeply appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THE EFFECTS OF EIGHT NON-PHYSIOLOGIC ZWITTERIONIC BUFFERS ON GROWTH OF CHLORELLA PYRENOIDOSA, NEUROSPORA CRASSA AND SACCHAROMYCES ELLIPSOIDEUS

INTRODUCTION

The application of cell culture techniques in studying fundamental problems in many fields of experimental biology has increased almost exponentially in the past few years. The growth of cells under standardized conditions is frequently essential to their use in many types of experiments. Adequate control of the pH of such cultures poses a number of technical problems; differential uptake of cations such as the ammonium ion vs anions such as the nitrate ion, or excretion of organic anions (acids) can produce major pH shift in the medium. In an attempt to simplify the control of pH, a number of compounds that have the appropriate pKa's and are compatible with other ingredients of conventional media should be tested for their ability to replace the traditional unsatisfactory buffers.

Relatively few weak acids with dissociation constant between 10^{-6} to 10^{-9} are known, if we rule out the obviously unsatisfactory phenolic compounds. Only metaboric acid, barbituric acid, carbonic acid, and biphosphate ion and a

few unusable substances such as arsenate have acid strengths lying in this range. Consequently, acid-salt buffer systems suitable for the regulation of pH in the physiologically important range pH 7 to 9 are not numerous.

Furthermore, most of these buffer solutions are incompatible in one way or another with physiological media. Weak acids, or salts such as acid phosphate and carbonate tend to precipitate calcium or to form undissociated compounds. Phosphate has buffering capacity below pH 7.5. It is either a substrate or an inhibitor in many systems. The usefulness of barbital (veronal) buffer is limited not only by the low solubility of barbituric acid but also by a chemical inhibition of certain enzyme system. This buffer has a satisfactory buffering capacity between pH 7 to pH 9.4 [1]. The antiseptic properties of phenols usually prohibit the use of these substances for pH control in physiological media, while the well-known tendency of borate buffer of Sörensen (covering the range around pH 8) to complex organic substances introduces certain disadvantages which severely limit its practical use. It cannot be used with many compounds containing two or more hydroxyl groups, such as polyhydric alcohols especially carbohydrates.

A means of avoiding these difficulties has been sought through the use of nitrogen bases and their salts in the hope that these buffers would be capable of

regulating pH in the range 7 to 9 and, at the same time would be compatible with most physiological fluids. Buffer solutions consisting of ammonium salts with added alkali are not highly stable. Imidazole (glyoxaline) and substituted imidazoles have been suggested as buffers by Kirby and Neuberger [2] and Mertz and Owen [3]. The mixtures of imidazole and hydrochloric acid cover the pH range of 6.2-7.8. Although this substance is compatible with calcium, its relatively high cost has in the past greatly limited its use. It is also rather reactive and unstable.

Triethanolamine is a base of about the desired strength [4,5] and triethanolamine-HCl buffers are accordingly useful for pH control in the range 7 to 9 [6]. This amine is a liquid of very high boiling point, however, and a pure product is often difficult to obtain [7]. It too lacks something in stability especially to oxidants.

In 1946, Gomori [8] proposed three buffer systems suitable for pH control in the range 6.5 to 9.7. One of these is a liquid, 2,4,6-trimethylpyridine (collidine), and the other two are solids at room temperature; they are 2-amino-2-(hydroxymethyl)-1,3-propanediol or tris(hydroxmethyl)aminomethane, widely known to biochemists as "tris," and the closely related substance, 2-amino-2-methyl-1,3-propanediol. Collidine and tris were found to be satisfactory in studies of the oxygen uptake of rat kidney slices. Furthermore, they showed no inhibitory action on many enzymes.

The third amine mentioned did not inhibit alkaline phosphatase at pH 9.1.

These three compounds possess some of the characteristics of ideal physiological buffer substances. They are readily soluble in water, do not precipitate calcium salts, and are low in price. Their solutions have been found to be stable at room temperature for periods of more than three months when protected from contamination with atmospheric carbon dioxide.

Ever since their first introduction into biochemistry by Gomori [9] amine buffers, most notably tris, have been favored by enzymologists. Lately tris was also found useful in fish industry. It is most suitable to stabilize pH and control carbon dioxide accumulation during transportation of fish [10].

Tris is now widely used in medical research studies. The use of carbonate and lactate salts of tris in the treatment of metabolic acidosis has been suggested recently [11]. The substance has been utilized successfully in studies of blood coagulation [12] and in colorimetric investigations of the hydrolysis of adenosine triphosphate [13] and of poly-L-lysine [14]. The complex formed by silver ion and tris serves as a titrant for the determination of sulfhydryl groups in proteins [15]. However tris has a poor buffering capacity below pH 7.5. Moreover it is a primary aliphatic amine of considerable reactivity and it can be inhibitory.

The most common buffers used in complex synthetic media have been phosphate, tartrate and citrate. These substances are utilized by many if not most organisms. It was found that the pH of such media often decrease during autoclaving which suggests some degree of reactivity at high temperature (observation of the author).

The change in pH's of medium during growth of the organisms is largely due to the utilization of nitrogen sources added to culture medium. For instance, when ammonium salt is used in a medium there is a decline in pH after a certain incubating period. Uptake of nitrate from a medium will cause the pH to rise. But when amino acids are employed as nitrogen source there is no change in pH because of this kind of differential ion uptake.

Recently, zwitterionic hydrogen ion buffers covering the pKa 6.15-8.35 have been developed and tested [16]. There is a paucity of data on the effects of these buffers on growth of various organisms widely cultivated for scientific studies. The effects of the substances on growth of microorganisms have not been reported. It is the purpose of this thesis to investigate the effects of the buffers supplied to the conventional media on growth of Chlorella pyrenoidosa, Neurospora crassa and Saccharomyces ellipsoideus.

The organisms were grown in the slightly modified media in the presence of buffers at different concentrations. Growth responses in all treatments were observed at

various intervals. Growth curves were established and compared with the controls. Titration of the culture solutions before and after growth was carried out to see if major pH changes were associated with major disappearance or conversion of the buffers. In addition an experiment performed in an attempt to determine whether or not the great stimulation of Chlorella growth noted with tricine was in any way related to the nature of the nitrogen source-nitrate, ammonium, or peptone.

Some physical properties of the buffers especially their pKa's were determined and pKa's are regularly given here in order to provide more understanding of the reason for selecting particular pH's in this study.

REVIEW OF THE LITERATURE

Though recently numerous studies have been conducted to evaluate the affects of the amine buffers in medical research, the reports on the effects of these substances on growth of organisms are inadequate. At the moment it is feasible to discuss some papers that have been written on the responses of microorganisms to various amines including a few buffers. These reports are considered to be at least indirectly relevant to the matters explored in this thesis. There do not seem to be any studies bearing directly on the growth effects or metabolism of non-physiological zwitterionic amines such as described below.

Dimond and Peltier [17] using Penicillium notatum in liquid cultures studied the relation between pH of the medium and time of incubation as it is effected by varying the source of nitrogen or carbon. With amino acids the pH curve varied according to the source of carbon. They concluded that by supplying the proper nutrients the pH of the medium can be controlled during growth.

In 1953 Morton and MacMillan [18] reported on their classic study on the assimilation of ammonium salts and nitrate by Scopulariopsis brevicaulis. The failure of ammonium sulfate to be completely assimilated was due to a

drop in pH to an inhibitory level. Various organic acids added to the medium acted as buffers to prevent this pH drop and allowed complete utilization of ammonium sulfate. Under corresponding favorable conditions ammonium nitrogen was assimilated faster than nitrate nitrogen. When ammonium and nitrate were available in the medium simultaneously, as with ammonium nitrate, the ammonium was preferentially assimilated until it dropped to a very low level and then the nitrate was assimilated. This was believed to be due to the ammonium ion blocking the reduction of nitrate to nitrite.

Elizarova [19] used Pseudomonas pyocyanea to study various buffer systems (phosphate buffer, tris buffer, and polyelectrolyte buffers or ion exchangers) in a medium containing glutamic acid as a sole carbon and nitrogen source. A 0.5 M phosphate buffer of pH 7.0 and carboxylic acid resin Amberlite IRC-50 (20 gram per 100 c.c. of the medium) used as a mixture of the acid and salt forms, maintain a constant pH. The required concentration of the buffer for optimal growth conditions varied with the amino acid used. Tris buffer did not maintain a constant pH and inhibited bacterial growth.

Neurospora

The ascomycete, Neurospora sp. has been used extensively in investigations of inheritance and mechanisms of

biosynthesis. Westergaard and Mitchell [20] provided evidence that sexual reproduction in this organism is favored by a relatively high sugar supply in proper combination with certain nitrogenous compounds rather than by starvation with respect to the carbon source. A synthetic medium has been devised for promotion of the sexual cycle in Neurospora. The pH 6.5 of the medium was found suitable for this study.

The interrelations in trace-element metabolism and in heavy metal toxicities in Neurospora crassa was studied by Sivarama Sastry [21]. The toxicities of Co, Ni and Zn to the growth of the fungus was observed as a function of the concentration of magnesium supplied. The extent of toxicity of all heavy metals was considerably enhanced by decreasing the normally high magnesium concentration in the medium to a minimal value for optimal growth. Reversal of heavy metal toxicities was possible with Fe as well as Mg. The antagonism between Fe and the toxic metals was competitive, at high Mg concentration, for Co and Ni toxicities only. With minimal Mg supplied a much higher ratio of Fe to toxic metal was necessary in all cases to achieve normal growth. Correlation between mycelial accumulation of the toxic metal and the growth of the mold was investigated under various conditions. In the absence of a counteracting metal there was a direct relation between these two parameters. The reversal of metal toxicities by

Mg is due to suppression of the uptake of toxic metal. Such a control of heavy metal uptake does not occur when Fe is the counteracting metal.

Barbara Weiss and Turian [22] studied conidiation in Neurospora crassa. They found that two forms of the growth of wild-type Neurospora crassa occur. One form completely lacks conidia and carotenoids. The other exhibits enhanced conidiation and pigmentation; it was produced by altering the nitrogen source of the growth medium. Studies of the oxidative and glycolytic metabolism of these two forms showed that the nonconidiating cultures had a significantly greater glycolytic activity than the conidiating cultures, as measured by the production of ethyl alcohol, the presence of ethanol dehydrogenase and pyruvate decarboxylase, and the response of the two culture types to glycolysis inhibitors.

Conidiation occurred when glycolysis was inhibited. Oxidative metabolism in normal conidiating cultures was suppressed by interfering with conidiation. The relative activities of the fermentative and oxidative pathways in the cell probably regulated both conidiation and pigment synthesis.

Conidiating cultures had a very high activity of NADP nucleotidase, while purely mycelial cultures were devoided of it.

Bianchi and Turian [23] reported the effect of nitrogen source and cysteine on the morphology, conidiation, and cell wall fraction of conidial and aconidial Neurospora crassa. Growth of the wild-type, a fluffy mutant, and a "clock" mutant of Neurospora crassa in a medium with NH_4^+ as the nitrogen source produced a colony which was less dense, had thicker hyphae which branched less and had thinner cell walls than the same cultures when grown in a similar medium with NO_3^- instead of NH_4^+ as the nitrogen source. Wild type cultures produced conidia on the nitrate medium but not on the ammonium medium, were more dense and colonial and produced greater amounts of NaOH-soluble cell wall materials. The fluffy mutant and "clock" mutant did not produce conidia but produced more cell wall materials on nitrate medium.

Cysteine added to the ammonium and nitrate media reduced the growth rate, increased branching and increased the amount of cell wall materials.

Yeast

Domnas [24] studied the uptake of amide and amide-like compounds by yeast. He found that the cell of Candida utilis and Saccharomyces cerevisae take up allantoin, allantoic acid, acetamide and, at a lesser rates, urea, butyramide and propionamide. These two yeast strains possess allantoicase and glyoxylurase, but not urease. C^{14} uptake

is stimulated by the presence of biotin or pantothenic acid, in which case urea is not accumulated, but rapidly metabolized as demonstrated by $C^{14}O_2$ evolution. The addition of urea to the medium increases the contents of intracellular lysine, arginine and, to a lesser extent, of serine, glutamic acid, proline and valine.

In 1966 Ter-Karapetyan and Indzhikyan [25] reported their investigation on the utilization of monoaminocarboxylic acids by yeast as the function of the amino group position. Seven Candida strains utilized glucose more rapidly than α- or β-alanine in media containing NH_4^+, but did use these amino acids as carbon sources. Other amino acids with terminal amino groups promoted a more marked glucose and nitrogen utilization, as well as more marked increase in biomass synthesis. In C. gulliemondii γ-amino-N-butyric acid was the most effective amino acid, and was the best carbon source. There was no amino acid synthesis in the growth medium using the α-isomer.

The study of amino acid transport and acid-base balance in yeast by Eddy and Indge [26] indicated that in the presence of glucose aspartic and glutamic acids stimulated uptake of K^+ 200 to 300%, glutamine and asparagine 100%, any one of eight other neutral amino acids no more than 20%. Lysine, arginine and ornithine depressed K^+ uptake. Lysine stimulated K^+ exit from cells 200-300% while glycine and aspartic acid had no effect. However in the

absence of glucose, glycine induced a loss of K^+. Uptake of lysine was linear with time and proceeded for several minutes before there was a marked effect on the entry and exit of K^+. The loss of K^+ induced by lysine was approximately equal to the amount of lysine taken up and the same applied to the induced uptake of K^+ which occurred with aspartic acid. It is suggested that the entry of lysine or aspartic acid into the cell only indirectly effects the movements of K^+, which appears to be especially sensitive to changes of acidity within the cell and to be regulated so as to maintain this at about pH 6.5.

Maw and Coyne [27] found that S-ethyl-L-cysteine added to a suspension of Saccharomyces cerevisiae in a glucose-containing medium is taken up by the cells, the sulfur of the amino acid being rapidly excreted into the medium almost entirely as a single metabolite. This metabolite was isolated and identified as the corresponding α-hydroxy derivative, ethylmercapto lactic acid, by mass spectrometry and by comparison with the authentic compound using chromatography and paper electrophoresis. Under similar experimental conditions, S-methyl-L-cysteine, L-methionine and L-ethionine were also found to give rise to their corresponding α-hydroxy analogs, which also are released into the medium.

Rothstein [28] used Triethylamine (TEA) in combination with organic acids, and tris(hydroxymethyl)amino

methane in a study of metabolism and ion transport in yeast. He pointed out that TEA^+ was a relatively inert cation with respect to the yeast cells. Its use in buffers has allowed investigation of the effect of extracellular pH on functions of metabolism and ion transport. Furthermore, it has provided a baseline for studying the role of extracellular cations, especially K^+. In a continuous flow system (cell column), TEA salts allowed the maintenance of K^+-free solutions despite leakage of K^+ from the cells. The same situation can be obtained in cell suspensions by the use of a TEA^+-cation exchange resin. Tris has also been useful in studies in alkaline pH range, but this substance may not be entirely inert with respect to cellular function.

Algae

In 1962 Elizabeth Birdsey and Victoria Lynch [29] studied the utilization of nitrogen compounds by unicellular algae. They reported that eight species of the phylum Chlorophyta employed urea as a sole nitrogen source; five also utilized uric acid and xanthine. The Cyanophyta, Rhodophyta and Euglenophyta studied did not grow on these three nitrogen sources, although Anacystis nidulans decomposed uric acid to allantoin. None of the algae tested utilized either allantoin or creatinine.

Godnev, Lyakhnovich and Syusyukin [30] studied the effect of sodium chloride on Chlorella growth and on accumulation of chlorophyll. Chlorella terricola with initial density of 1.3 million cells per c.c. of medium and with initial pH 4.82 was grown in the presence of 0.025-3.0% NaCl. Concentration of 0.75, 1.5, and 3.0% NaCl delayed growth and accumulation of chlorophyll. Best growth was obtained at 0.1% NaCl and with illumination with fluorescent lamps for 6 hours each day while best chlorophyll accumulation occurred with illumination for 14 hours each day.

McLachlan's [31] investigation of the effect of tris(hydroxymethyl)aminomethane on growth of Haematococcus pluvialis indicated that, in a complete synthetic culture medium (ASMG), tris inhibited the growth of the alga. Calcium ions antagonized the inhibitory effect of tris, but other cations either alone or with calcium has no such effect. Tolerance towards tris was increased in the presence of 5 mM Ca when the initial pH of the medium was increased from 7.0 to 8.0. However changes in pH had no effect on tolerance to the buffer in the medium containing the usual much lower concentration of calcium. Difference in the reactions of other organism in tris containing media were reviewed. It was suggested that tris forms complexes with calcium and also with trace metals. (However, Good et al [16] did not find such complexes.) It was reported

further that most toxic effects of tris were due to an antagonism of cations, especially potassium, and to a lesser extent magnesium, and it was possible to reverse tris toxicity by adding an excess of these cations. Toxicity was more commonly noted in the growth of freshwater algae, and in general, growth of marine algae was not inhibited by tris probably because of the high concentration of cations in seawater. Toxicity due to factors other than interference with the uptake of cations was observed.

In 1966 Tageeva and Korshunova, and Mikhnevich [32] reported the dependence of some growth and development characteristics of Chlorella pyrenoidosa Pr 82T on the type of nitrogen nutrition and nature of source of illumination. They found that urea compared with nitrate nitrogen was the more efficient nitrogen source with respect to the development and accumulation of biomass of a Chlorella suspension. With urea nitrogen, a dense structural organization of the protoplast was produced. Under conditions of intense cultivation, light was one of the most important factors regulating the development of the suspension, and light sources emitting large amounts of red light were best for obtaining highly productive culture.

Syrett and Morris [33] studied the inhibition of nitrate assimilation by ammonium ion in Chlorella. They concluded that Chlorella vulgaris growing with ammonium nitrate as nitrogen source preferentially assimilates

ammonium ion. Nitrate assimilation ceases completely when ammonium ion is added and recommences as soon as the ammonium ion has disappeared. Ammonium ion does not inhibit nitrate reduction by cells unable to assimilate ammonium because they lack a carbon source. Thus the inhibition is not due to ammonium ion per se but is connected with its assimilation. The inhibition is not thought to result from competition for reduced pyridine nuclectide because nitrate reductase of Chlorella is specific for DPN while glutamic dehydrogenase is specific for TPN. Nitrite addition also inhibits nitrate assimilation completely but ammonium ion only partially inhibits nitrate assimilation. It is probably that amino acids formed by amination reactions using the ammonium ion as nitrogen source specifically repress the induction of nitrate reductase [34].

MATERIALS AND METHODS

Buffers

Eight zwitterionic buffers prepared by Dr. Norman E. Good were used in this study. The table below gives their names, concentrations used for each organism under study, pH's used (near pKa, except with TAPS) and pKa's.

Preparation of Buffer Solutions

One molar solution of each buffer was prepared for each experiment. Purified (twice recrystallized) substances were used for the preparations. Calculated volumes of these solutions were added into each flask containing medium. The volume in each container was made up to the required concentration. The buffer was added to the medium either before or after autoclaving at 115°C for 15 minutes as noted.

Maintenance of cultures

Initial sub-cultures were made from agar slant cultures. Chlorella pyrenoidosa obtained from Indiana Culture Collection was maintained in 30 ml liquid medium [35] on a shaker under 15 hours light and 9 hours darkness at room temperature. Neurospora crassa obtained from Fungal Genetics Stock Center, Department of Biological

TABLE 1

PHYSICAL PROPERTIES OF EIGHT BUFFERS USED

Full Name	Proposed Name	Test Organism	Concentration Used (molar)	pH used	pKa of Buffer
N,N-Bis(hydroxyethyl)glycine	Bicine	Chlorella	0.05, 0.1, 0.2	8.0	8.35
		Neurospora	0.01, 0.025, 0.05		
		yeast	0.05, 0.1, 0.2		
Tris(hydroxymethyl)methylglycine	Tricine	Chlorella 1*	0.05, 0.1, 0.2	8.0	8.15
		Chlorella 2*	0.01, 0.025, 0.05		
		Chlorella 3*	0.001, 0.0025		
			0.005, 0.01		
N-hydroxyethylpiperazine-N'-ethanesulfonic acid	HEPES	Chlorella	0.005, 0.01	7.4	7.55
			0.025, 0.05		
		Neurospora	0.05, 0.01, 0.02		
		yeast	0.05, 0.1, 0.2		
N-hydroxyethylpiperazine-N'-propanesulfonic acid	HEPPS	Chlorella	0.05, 0.1, 0.2	8.0	8.0
		Neurospora	0.01, 0.025, 0.05		
		yeast	0.05, 0.1, 0.2		

*1, 2, 3 = experiment 1, 2 and 3

TABLE 1--Continued

Full Name	Proposed Name	Test Organism	Concentration Used (molar)	pH used	pKa of Buffer
morpholine-N-ethanesulfonic acid	MES	Chlorella	0.01, 0.025, 0.05	6.1	6.15
		Neurospora	0.01, 0.025, 0.05		
		yeast	0.05, 0.1, 0.2		
morpholine-N-propanesulfonic acid	MOPS	Chlorella	0.05, 0.1, 0.2	7.0	7.15
		Neurospora	0.01, 0.025, 0.05		
		yeast	0.05, 0.1, 0.2		
Tris(hydroxymethyl)methyl-aminopropanesulfonic acid	TAPS	Chlorella	0.05, 0.1, 0.2	8.0	8.6
		Neurospora	0.05, 0.1, 0.2		
		yeast	0.05, 0.1, 0.2		
Tris(hydroxymethyl)methyl-aminoethanesulfonic acid	TES	Chlorella	0.01, 0.025, 0.05	7.4	7.5
		Neurospora	0.05, 0.1, 0.2		
		yeast	0.05, 0.1, 0.2		

Science, Dartmouth College, Hanover, New Hampshire, was maintained on agar slant medium [20], and kept in a cold room at <5°C. Saccharomyces ellipsoideus obtained from Ohio State University Stock Culture Collection was maintained on agar slant medium (recommended by Gutz) and kept in cold at 5°C. All cultures employed in this study were transferred to fresh media every three weeks.

Method of Growth and Measurement

Culture container

Modified 125 ml Erlenmeyer flasks (Figure 1) were used for growing Chlorella pyrenoidosa and Saccharomyces ellipsoideus. These flasks permitted frequent convenient turbidity measurement without risk of contaminating the medium. For culturing of Neurospora crassa ordinary 125 ml Erlenmeyer flasks were employed. All flasks were plugged with cotton wool.

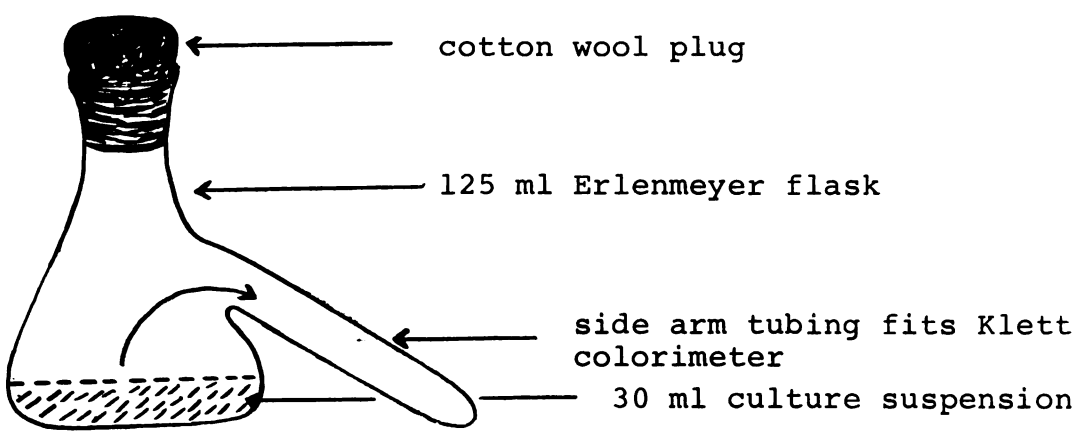

Figure 1. Modified 125 ml Erlenmeyer flask.

A) Chlorella pyrenoidosa

Experiment 1

Medium Employed [35]:

Bristol's solution (as modified by H. C. Bold Bull. Torrey Bot. Club 76:101-108, 1949).

Six stock solutions, 400 ml in volume, were employed. Each contains one of the following salts in the amounts listed:

$NaNO_3$	10.0	g
$CaCl_2$	1.0	g
$MgSO_4 \cdot 7H_2O$	3.0	g
K_2HPO_4	3.0	g
KH_2PO_4	7.0	g
NaCl	1.0	g

10 ml of each stock solution were added to 938 ml of glass distilled water. To this added a drop (from a 5 ml pipette) of 1.0% $FeCl_3$ solution.

The minor elements [36] were combined in one solution of 500 ml in volume. The following salts were employed:

H_3BO_3	1.430	g
$MnCl_2 \cdot 4H_2O$	0.905	g
$ZnCl_2$	0.055	g
$CuCl_2 \cdot 2H_2O$	0.025	g
$Na_2MoO_4 \cdot 2H_2O$	0.0125	g

Two ml of this minor element solution was added to each liter of culture solution.

The pH of freshly prepared medium was 6.4-6.45. The medium was adjusted to a pH near the pKa of the buffer being used by addition of 6 N NaOH or 6 N HCl.

Sterilization and Cleaning

All flasks and pipettes were washed in tap water and detergent, rinsed with running tap water several times. Distilled water was used for the final rinse.

All media were sterilized at 115°C for 15 minutes.

Experimental Procedure

Inoculum for shake culture study was prepared by growing Chlorella pyrenoidosa in 30 ml of the usual liquid medium (pH 6.4) in the modified 125 ml flask. The suspension was left on shaker under intermittent illumination and at room temperature (23-29°C). The optical density of the suspension was measured every day in a Klett colorimeter with a blue filter (maximum about 420 mμ). For all experiments suspensions giving a 0.2 optical density reading were used as inoculum. One ml of the suspension was inoculated to each flask from a sterile 10 ml graduated pipette.

To prepare for the buffer tests the following Table 2 was designed. Duplicate experiments were performed. Moreover the buffer was added both before and after autoclaving to investigate the compatibility of the buffer to the complex synthetic medium at high temperatures.

TABLE 2

SOLUTION FOR BUFFER TEST

	Molarity of Buffer			
	0.00	0.05	0.10	0.20
1.0 M buffer (ml)	-	1.5	3.0	6.0
medium (ml)	23	23	23	23
inoculum (ml)	1.0	1.0	1.0	1.0
distilled water (ml)	6.0	4.5	3.0	-
final volume (ml)	30.0	30.0	30.0	30.0

The mixture contained in 125 ml conical flask (Figure 1) and plugged with cotton wool was autoclaved at 115°C for 15 minutes then cooled overnight.

The alga was grown in the medium on a shaker under intermittent fluorescent light at room temperature. Illumination was for 15 hours followed by 9 hours of darkness. No CO_2 was added other than that contributed by the laboratory air. Growth was estimated turbidimetrically every day using a Klett colorimeter with a blue filter. Growth in most cases was also assessed after seven days as volume of packed cells in cytocrits and at that time the pHs of all suspensions were determined.

To check for disappearance of the buffer, another set of treatments was performed by preparing a mixture as

indicated in Table 2 excepted that 1 ml of glass distilled water was added instead of inoculum and 10 ml from each concentration was titrated with NaOH after growth period. Ten ml of each cell suspension was centrifuged and the aliquot was titrated with NaOH to determine residual buffer. A difference in the concentration of buffer remaining in the culture solutions with and without cells would indicate metabolism of buffer by the cells.

In studies of the relationship between optical densities in the blue region and volumes of cells of the algae culture, suspension of optical densities of 0.2, 0.4, 0.6, and 0.8 were prepared and 1 ml of each suspension was centrifuged into a calibrated capillary tube at about 5000 x g for 10 minutes. Each determination was repeated 3 times and the average cell volume taken (see Table 10 at the end of the results section). All data reporting the growth of alga and yeast are presented as the optical densities of the cell suspensions. However these data can be converted into the actual volumes of cells by use of Table 10. Growth curves were established for the organism when grown in the medium with buffer and without the buffer. Averages were taken from two flasks. In the case of high temperature buffer-medium compatibility study, results are reported in Table 9.

The buffers used in experiment 1 were bicine, tricine, HEPPS, MOPS and TAPS. The concentration studied were 0.05 M, 0.10 M and 0.20 M.

Experiment 2

The results obtained from experiment 1 indicated that at high concentrations of the buffers (except TAPS) decreased growth was observed. Therefore a second experiment was designed to investigate the effects of the remaining buffers; HEPES, MES, TES at lower concentrations; 0.01 M, 0.025 M, and 0.05 M. Special experiments were also performed to explore further the stimulatory effects of tricine (experiment 3).

The same medium [35] was employed for the tests of HEPES, MES and TES. Table 3 explains the method of preparing the media.

TABLE 3

MIXTURE FOR GROWING CHLORELLA WITH HEPES, MES, TES AND TRICINE

	Molarity of Buffer			
	0.00	0.01	0.025	0.05
1.0 M buffer (ml)	-	0.30	0.75	1.5
medium (ml)	27.5	27.5	27.5	27.5
inoculum (ml)	1.0	1.0	1.0	1.0
distilled water (ml)	1.5	1.2	0.75	-
final volume (ml)	30.0	30.0	30.0	30.0

Experiment 3

Effects of tricine were investigated in the usual medium and also in a number of other media with different amounts of nitrogen and different nitrogen sources.

I) Experiment to observe the effect of different amounts of nitrate nitrogen in the usual medium.

The media for growing Chlorella pyrenoidosa in the further studies of tricine was designed as follows, and Table 3 was used for preparing the mixture in each culture container:

treatment 1: usual medium + tricine

treatment 2: usual medium without $NaNO_3$ + tricine

treatment 3: usual medium with double amount of $NaNO_3$ but no tricine added

II) Experiment to study the growth responses to various nitrogen sources supplied to the usual medium in the presence of tricine.

In this experiment effects of various concentrations of tricine were investigated, i.e., 0.001 M, 0.0025 M, 0.005 M and 0.01 M. The following Table 4 was used for preparation of the media studied.

Three types of stock media were prepared for the experiment II:

a) usual medium

b) usual medium with the supplement of peptone (5.0 g/liter), sodium nitrate was omitted

c) usual medium with the NH_4NO_3 (0.0015 M) added as nitrogen source instead of $NaNO_3$.

The experiment procedure for experiment 3 was exactly the same as explained previously in the first experiment. Results are reported in the same fashion.

After the test period all alga cultures described were checked microscopically to determine:

a) morphological changes (if any)

b) contamination (if any)

TABLE 4

MIXTURE FOR GROWING CHLORELLA PYRENOIDOSA IN VERY LOW CONCENTRATIONS OF TRICINE

	Molarity of Buffer				
	0.00	0.001	0.0025	0.005	0.01
1.0 M buffer (ml)	-	0.03	0.075	0.15	0.30
medium (ml)	27.5	27.5	27.5	27.5	27.5
inoculum (ml)	1.0	1.0	1.0	1.0	1.0
distilled water (ml)	1.5	1.47	1.425	1.35	1.2
final volume (ml)	30.0	30.0	30.0	30.0	30.0

B) Yeast

The culture of Saccharomyces ellipsoideus was obtained from the Ohio State University Stock Culture Collection.

Yeast was grown in a synthetic medium recommended by Dr. Herbert Gutz, Division of Biology, Southwest Center for Advanced Studies, Dallas, Texas.

Gutz minimal medium was composed of the following per liter:

Salts:

KH_2PO_4	1	g
$MgSO_4 \cdot 7H_2O$	500	g
NaCl	100	mg
$CaCl_2 \cdot 2H_2O$	106	mg
$(NH_4)_2SO_4$	5	g

Trace elements:

H_3BO_3	0.50	mg
$CuSO_4 \cdot 5H_2O$	0.04	mg
KI	0.10	mg
$FeCl_3 \cdot 6H_2O$	0.20	mg
$MnSO_4 \cdot 4H_2O$	0.53	mg
$H_2MoO_4 \cdot H_2O$	0.16	mg
$ZnSO_4 \cdot 7H_2O$	0.40	mg

Vitamins:

Ca-pantothenate	0.10	mg
Nicotinic acid	1.0	mg
meso-inositol	1.0	mg
Biotin	0.001	mg

All these media were autoclaved for 15 minutes at 115°C and kept in a cold room (<5°C). The medium was solidified with 15 grams agar when required for maintenance of the culture.

Experimental Procedure

Yeast was cultured in a similar manner to Chlorella pyrenoidosa explained above. The only difference was that yeast was grown on a temperature-controlled shaker at 32-34°C in the dark.

Growth was observed by measuring turbidity at 6, 12, 18, 24, 36, 42 and 48 hours. Determination of pHs after growth, titration of culture solution before and after growth, centrifugation of culture suspension, and the study of compatibility were performed by the same procedures as described for Chlorella pyrenoidosa.

Average data from duplicate experiments are reported.

C) Neurospora crassa

The culture of Neurospora crassa #16, albino, was obtained from Mr. William N. Ogata, Fungal Genetic Stock Center, Dartmouth College, Hanover, New Hampshire. This culture grows well on minimal media, that is on the media consisting of mineral salts, nitrogen and carbon sources and biotin.

The following synthetic medium [20] was employed:

For each 3 liters of medium:

Sucrose (instead of glucose)	30	g
$NaH_2PO_4 .H_2O$	6	g
Potassium tartrate	15	g
$MgSO_4 .7H_2O$	1.5	g
$CaCl_2 .2H_2O$	0.4	g
NaCl	0.3	g
Biotin	15	micrograms
NH_4Cl	1.6	g
trace elements [37]	3	ml

Trace elements were combined in one stock solution. The following ingredients per 100 ml of solution were prepared:

Boric acid	1	mg
$CuCl_2 .2H_2O$	10	mg
$FeCl_3$	20	mg
$MnCl_2$	2	mg
$Na_2MoO_4 .2H_2O$	2	mg
$ZnCl_2$	200	mg

The solution was made up to the volumes with glass distilled water. The pH of the medium as made up without additional buffers was 5.6.

For maintenance of the culture the medium was solidified with 15 grams agar per liter.

Experimental procedure

To prepare the inoculum for buffer testing the fungus was grown on 50 ml solidified medium in 300 ml

conical flasks plugged with cotton wool. The culture was incubated in the dark at room temperature for 72 hours. Into the culture, 60 ml sterile glass distilled water was added. The culture was hand shaken a few times to obtain a uniform suspension of conidia. One ml of the suspension was inoculated to each flask containing 29 ml mixture.

The pHs of buffer solutions and medium were raised (or lowered with 6 N HCl) with 6 N NaOH before autoclaving. All treatments were performed in duplicate and also two parallel experiments were done. The buffer solution was added into the medium in each flask both before and after autoclaving to observe the compatibility of the buffer to the complex medium.

The following Tables 5 and 6 were designed for the preparation of medium in this study:

TABLE 5

MIXTURE FOR THE STUDY OF THE EFFECT OF HEPES, TES AND TAPS

	Molarity of Buffer			
	0.0	0.05	0.1	0.2
1.0 M buffer (ml)	-	1.5	3.0	6.0
medium (ml)	23.0	23.0	23.0	23.0
inoculum (ml)	1.0	1.0	1.0	1.0
distilled water (ml)	6.0	4.5	3.0	-
final volume (ml)	30.0	30.0	30.0	30.0

TABLE 6

MIXTURE FOR THE STUDY OF THE EFFECT OF BICINE, TRICINE, HEPPS, MOPS, AND MES

	Molarity of Buffer			
	0.00	0.01	0.025	0.05
1.0 M buffer (ml)	-	0.30	0.75	1.50
medium (ml)	27.5	27.5	27.5	27.5
inoculum (ml)	1.0	1.0	1.0	1.0
distilled water (ml)	1.5	1.2	0.75	-
final volume (ml)	30.0	30.0	30.0	30.0

Neurospora cultures were grown on the temperature controlled shaker at 32-34°C in the dark. Growth was measured as dry weights at 24, 48 and 72 hour intervals. Dry weights were obtained by filtering off the fungus mycelia (or conidia in some cases) on a Whatman #1 filter paper with the aid of suction. The residue was dried at 100°C in an oven for 24 hours. The dried mycelium was weighed quickly to avoid weight increase by moisture uptake. The filtrates from 24 and 48 hour cultures were saved for pH determination. In case of 72 hour culture the filtrates were measured for pH changes and were titrated with NaOH to estimate the amount of buffer remained in the culture solution.

RESULTS

Neurospora crassa

Perhaps because the Neurospora medium was already somewhat buffered with tartrate, few of the buffers discussed herein resulted in any marked improvement of growth. Moreover since the method of sacrificing each culture in order to determine the progress of growth is inherently much less reproducible than the turbidimetric studies used with the alga and yeast, there is some question as to the significance of many of the small effects noted.

All concentrations of bicine used reduced both the rate of growth and the extent of the final growth. With all concentrations of bicine the pH dropped from the initial 8.0 to about 6.1 after 72 hours. This suggests that bicine does not buffer adequately when used in this medium. The following pHs were recorded at three different test periods and buffer concentrations:

	control	0.01 M	0.025 M	0.05 M
24 hours	6.0	6.4	6.8	7.15
48 hours	6.0	6.1	6.32	6.6
72 hours	5.9	6.0	6.1	6.12

It should be noted that the buffering capacity of bicine is almost completely exhausted at pH 7.0 or lower.

In contrast tricine seemed to stimulate growth in the early phases. However growth measured at 72 hours was probably slightly less than that of the control (Figure 3). Decrease of the pH from 8.0 to pH 6.1 was observed after the end of test period.

	control	0.01 M	0.025 M	0.05 M
24 hours	6.48	6.6	7.1	7.4
48 hours	6.3	6.4	6.75	7.0
72 hours	6.0	6.1	6.1	6.65

Again it should be noted that the buffering capacity of tricine is also exhausted at about pH 7.0.

With 0.2 M HEPES, Neurospora grew somewhat better than with the lower concentrations (0.1 M, 0.05 M). These latter concentrations were no better or worse than the control. The pH dropped from 7.4 to pH 6.85 when 0.2 M HEPES was present in the medium (Figure 4). Final pHs of 6.7 and 6.41 were recorded at the end of test period when 0.1 M and 0.05 M HEPES were added to the medium respectively.

HEPPS was neither inhibitory nor stimulatory at the concentrations used (Figure 5). Nor was it very effective in stabilizing the pH when present in the medium. The pHs after growth at each interval were reported as follows:

	control	0.01 M	0.025 M	0.05 M
24 hours	6.36	6.6	6.95	7.33
48 hours	6.3	6.05	6.12	6.55
72 hours	5.93	5.9	6.1	6.45

Growth in the presence of MOPS (Figure 6) was slightly slower than in the absence of MOPS but the final growth was somewhat greater. Furthermore in the presence of MOPS growth was probably still going on at the 72 hour harvesting time. However at 48 and 72 hours, 0.05 M MOPS resulted in conidial growth.

MOPS did not stabilize the pH of the medium. The pHs observed at different test periods and buffer concentrations were as follows:

	control	0.01 M	0.025 M	0.05 M
24 hours	5.55	4.85	5.15	5.55
48 hours	5.6	4.9	5.2	5.61
72 hours	5.48	4.04	5.12	5.55

The pHs observed, as shown, did not correspond with the strength of the buffer present. From these observations one might suspect participation of MOPS in the metabolic processes of the fungus.

MES (Figure 7) reduced the rate of growth at all concentrations tested but permitted appreciably more total growth. The higher the concentration of buffer the greater the reduction in the rate of growth of the fungus. The decreasing pHs observed at different time and various concentrations of buffer were as follows:

	control	0.01 M	0.025 M	0.05 M
24 hours	4.38	4.83	5.58	5.83
48 hours	4.36	4.83	5.51	5.76
72 hours	4.32	4.84	5.48	5.71

A drop of pH from 6.1 to pH 4.32 was recorded for the control medium. Thus it may be seen that MES is fairly effective in stabilizing the pH of the media.

The results observed from the experiment with TAPS (Figure 8) showed that at 24 and 48 hours, 0.05 M and 0.1 M the buffer did not affect the fungal growth. From the figure it can be seen that growth was inhibited when 0.2 M TAPS was present in the medium.

Unlike HEPES and unlike MOPS, TAPS seems to be quite effective in stabilizing the pH of the medium.

The pHs observed as shown below were recorded after each growth period:

	control	0.05 M	0.1 M	0.2 M
24 hours	6.2	7.2	7.55	7.70
48 hours	6.15	7.2	7.47	7.76
72 hours	6.05	7.02	7.2	7.54

Mycelial dry weights determined from experiments with TES (Figure 9) show that in the presence of the buffer growth was slightly enhanced. Best growth was observed from 0.1 M treatment. Decrease of pH was also observed in all cases although not as marked as the case of MOPS. The following pHs were recorded.

	control	0.05 M	0.1 M	0.2 M
24 hours	5.89	6.52	6.88	7.15
48 hours	5.76	6.25	6.39	7.07
72 hours	5.66	5.82	6.06	6.61

Yeast

Medium buffered with various concentrations of bicine at pH 8.0 completely inhibited growth of Saccharomyces ellipsoideus. The yeast grew normally in control medium at the same initial pH. Table 7 shows the average optical density turbidity measured at various intervals. A markedly decreased pH was observed in the control culture.

Growth curves are not plotted in this case. The data present in Table 7 are self-explanatory.

TABLE 7

LACK OF GROWTH (OD_{420}) OF SACCHAROMYCES ELLIPSOIDEUS WITH BICINE

	Molarity of Bicine			
Time (hours)	control	0.05 M	0.1 M	0.2 M
0	.028	.021	.023	.020
6	.056	.021	.023	.020
12	.290	.021	.023	.020
18	.330	.021	.023	.020
24	.402	.021	.023	.020
36	.442	.021	.023	.020
48	.480	.021	.023	.020
pH	2.72	7.60	7.67	7.78

In the presence of tricine (Figure 10) growth was improved. The greatest improvement was at the lower concentration tested (0.05 M). Optical density measured from medium with no tricine added showed that maximum growth was reached at 18 hours, but in the presence of tricine growth continued for about 36 hours. The buffer was not very effective in maintaining the pH but the pH did drop more slowly when higher amounts of buffer were present. From the curves it is clear that no lag in growth occurred even though the initial pH of the medium was unfavorably high (8.0).

HEPES (Figure 11) improved the final yield of yeast but prolonged the lag period and somewhat slowed the rate of growth. When the buffer was present at 0.05 M satisfactory growth was obtained but at higher concentrations the slower rate of growth more than outweighed the slight increase in total growth. Decreasing pH's were observed as recorded on the figure.

All concentrations of HEPPS (Figure 12) decreased the rate of growth very markedly.

A slight increase lag period was observed when yeast was grown in a medium containing MES at initial pH 6.1 (Figure 13) but the final growth achieved was significantly increased. Satisfactory, improved growth of yeast occurred in the medium with all concentrations of MES.

MOPS (Figure 14) behaved in almost exactly the same manner as MES.

It was found that media buffered with TAPS (Figure 15) induced a long lag in the onset of growth. The higher the concentration of buffer the longer the lag period. However satisfactory growth occurred in all treatments in the presence of TAPS after 48 hours except at the highest concentration (0.2 M). A marked drop in the pH of the medium was also recorded in this experiment. However it should be noted that the initial pH was far below the pKa of the buffer therefore the buffering capacity was understandably low.

A similar effect was observed when yeast was grown in the medium containing TES (Figure 16) at the initial pH of 7.4. There was a long lag period, the duration of which was roughly proportional to the concentration of the buffer. However once growth had started it was at the same rate as the control. Final growth was considerably greater than in the control. Decreasing pHs were observed but they did not reach the low value observed in the absence of the buffer.

Chlorella

Before discussing the effects of the buffers on the growth of Chlorella it is well to consider the influence of the pH on the growth of the alga in the control

media. For the reason given earlier in this thesis, namely the need to employ the buffers in their buffering ranges, the pHs of the media were in most cases unfavorably high. Indeed any initial pH above about 7.0 introduced a lag period in the control and often seriously inhibited growth. This may be related in some manner to the fact that the pH of the media regularly increased during growth probably because the nitrogen was nitrate ion.

The sulfonic acid buffers, HEPES (Figure 23), HEPPS (Figure 24), MES (Figure 25), and TES (Figure 28) had little effect on the growth lag but, with exception of MES, somewhat increased the growth rate. All were quite effective in stabilizing the pH. Two other sulfonic acid buffers MOPS (Figure 26) and TAPS (Figure 27) gave very marked stimulation of growth rates and may have reduced growth lags somewhat. These too were very effective in stabilizing the pH. Bicine (Figure 17) and tricine (Figure 18) were exceedingly stimulatory except at high concentrations. Maximum growth was obtained with tricine at 0.01 M or 0.025 M regardless of whether the nitrogen source was nitrate ion, ammonium ion (Figure 22) or peptone (Figure 21). Growth on tricine as sole nitrogen source was significant but poor (Figure 20). No growth of the alga occurred when double amount of nitrate nitrogen

was added into the usual medium in the absence of tricine (Table 8).

TABLE 8

EFFECT OF DOUBLE CONCENTRATION OF NITRATE ON GROWTH OF CHLORELLA PYRENOIDOSA IN THE ABSENCE OF TRICINE IN THE USUAL MEDIUM

time (day)	flask number		
	1	2	3
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	$5x10^{-3}$	$5x10^{-3}$	$5x10^{-3}$
7	$5x10^{-3}$	$5x10^{-3}$	$5x10^{-3}$
pH after 7 days	7.95	7.98	7.95

Compatibility of the Buffers with the Media

All of the data presented in this thesis represent experiments in which the buffers and the other components of the media were autoclaved separately. However some of the buffers were compatible with the media at autoclave temperatures and some were not. All buffers except HEPPS and TAPS were compatible with the minimal

medium used for growing Chlorella, or at least were compatible to the extent that growth was the same whether or not the buffers were autoclaved separately. On the basis of the Neurospora studies one would have said that all of the buffers were compatible with the medium. However this seems doubtful since both HEPPS and MOPS yielded inhibitory substances when they were autoclaved with the yeast medium which was rather similar to the Neurospora medium, or at least there was less growth of the yeast when these buffers were autoclaved with the medium. Yeast may simply be a more sensitive indicator of inhibitory substances (see Table 9).

TABLE 9

COMPATIBILITY OF EIGHT BUFFERS TO SYNTHETIC MEDIA FOR GROWING CHLORELLA PYRENOIDOSA, NEUROSPORA CRASSA AND SACCHAROMYCES ELLIPSOIDEUS

+ indicates that the rate of growth was the same whether or not the buffer and medium were autoclaved together, - indicates that the growth was substantially less if they were autoclaved together.

Test Organism	bicine	tricine	HEPES	HEPPS	MES	MOPS	TAPS	TES
Chlorella pyrenoidosa	+	+	+	-	+	+	-	+
Neurospora crassa	+	+	+	+	+	+	+	+
Saccharomyces ellipsoideus	?	+	+	-	+	-	+	+

Relationships between the Optical Densities of the Chlorella or Yeast Suspensions and the Actual Cell Densities

The optical densities as measured in these studies were not strictly proportional to the volume of cell material in the cultures. The reason for this departure from Beer's Law is not known. The following Table 10 may be used to relate the observed optical density to the volume of cells present.

TABLE 10

Relation of O.D. to packed cell volume

	$O.D._{420}$	cell volume percent of medium
yeast	0.2	0.8
	0.4	1.1
	0.6	1.8
	0.8	2.4
chlorella	0.2	0.2
	0.4	0.3
	0.6	0.4
	0.8	0.5

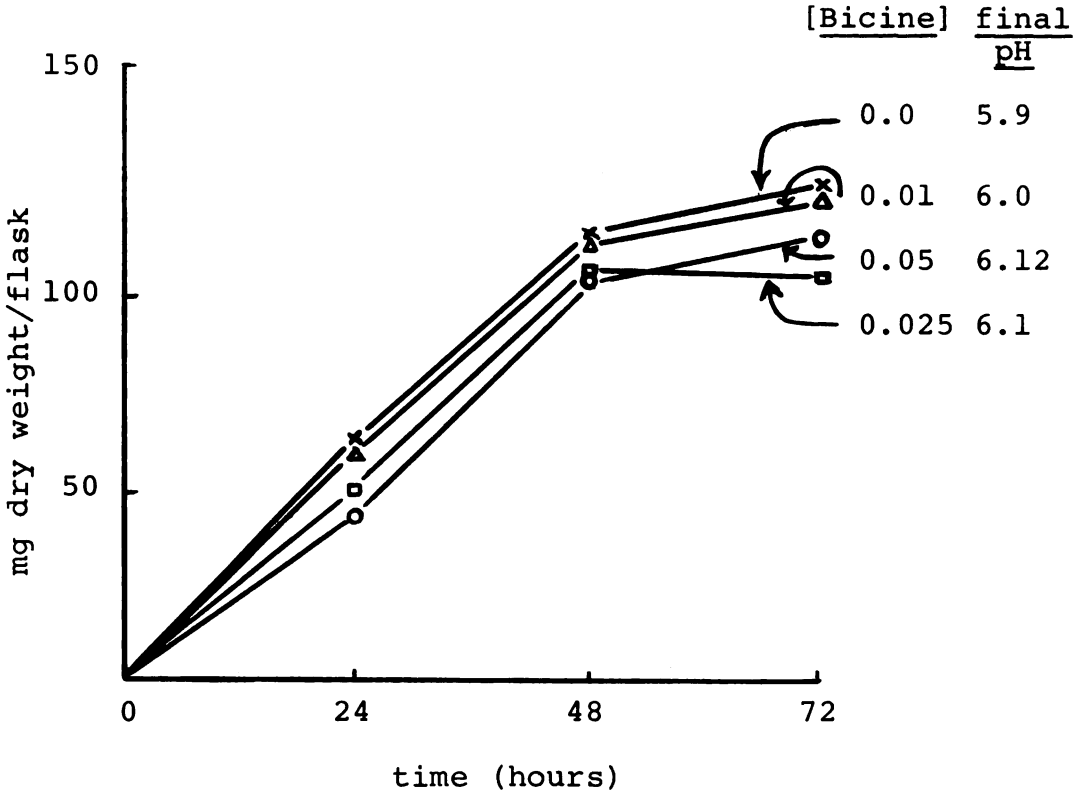

Figure 2. Growth of N. crassa with bicine, initial pH 8.0

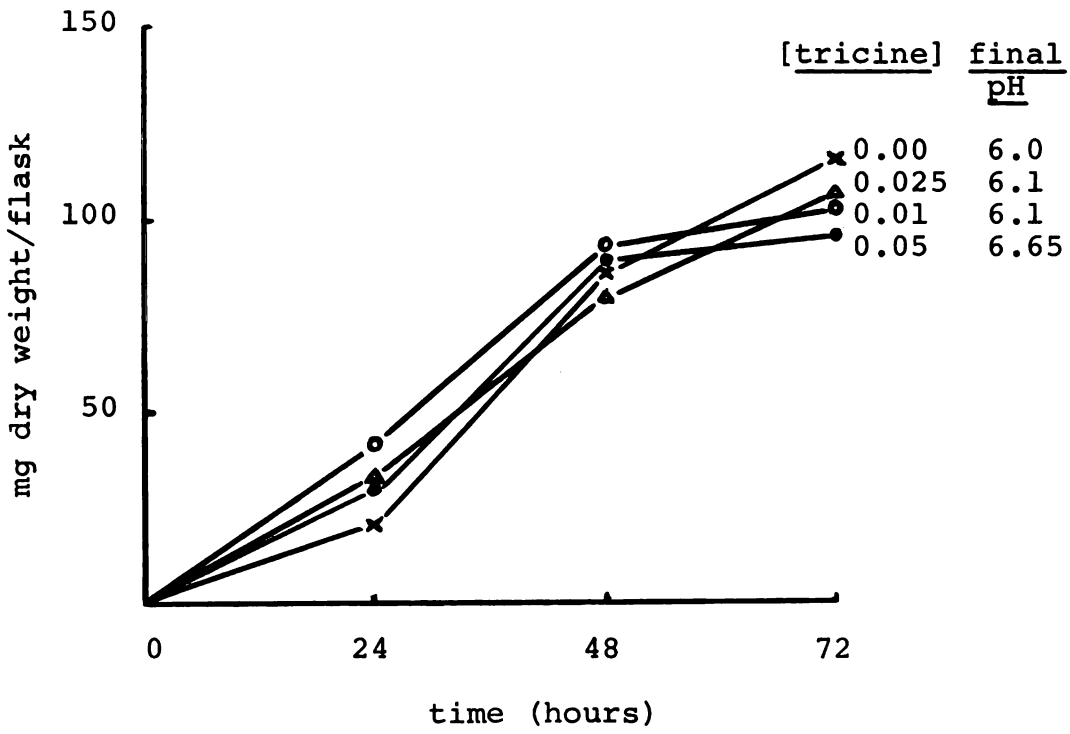

Figure 3. Growth of N. crassa with tricine, initial pH 8.0

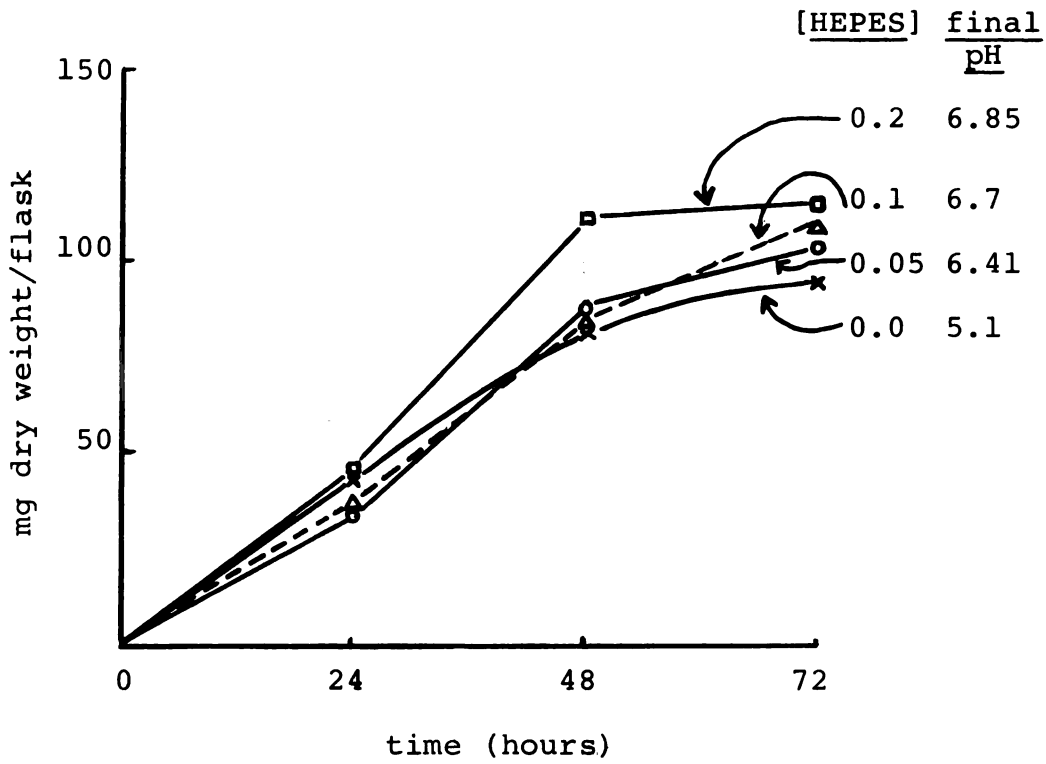

Figure 4. Growth of N. crassa with HEPES, initial pH 7.4

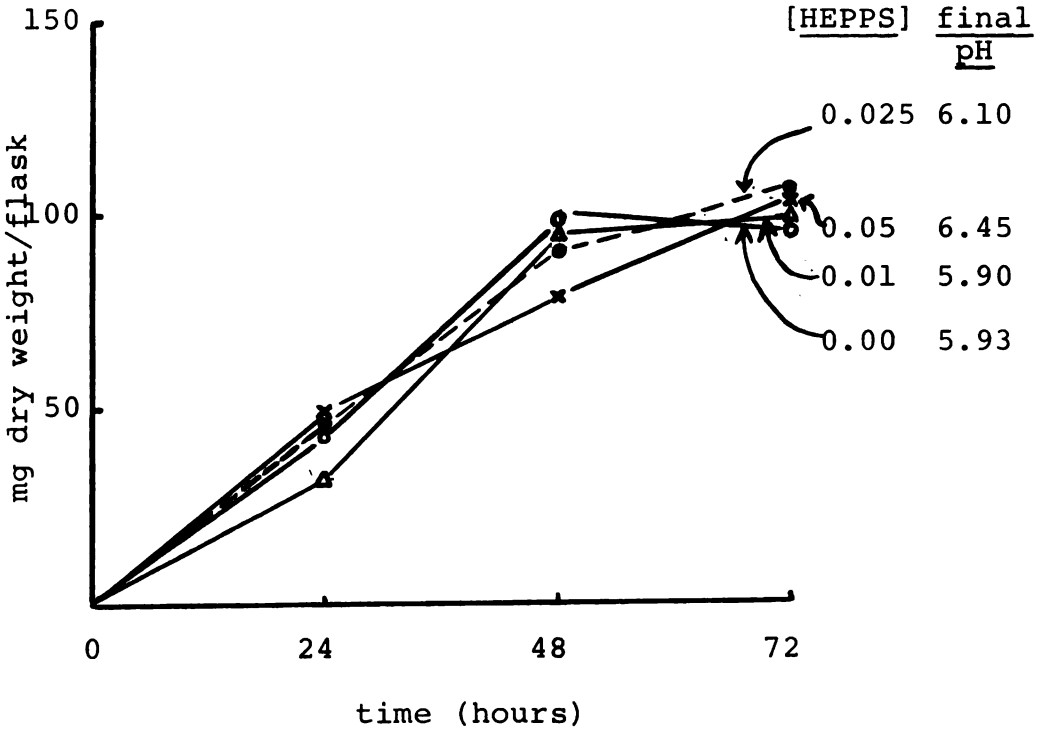

Figure 5. Growth of N. crassa with HEPPS, initial pH 8.0

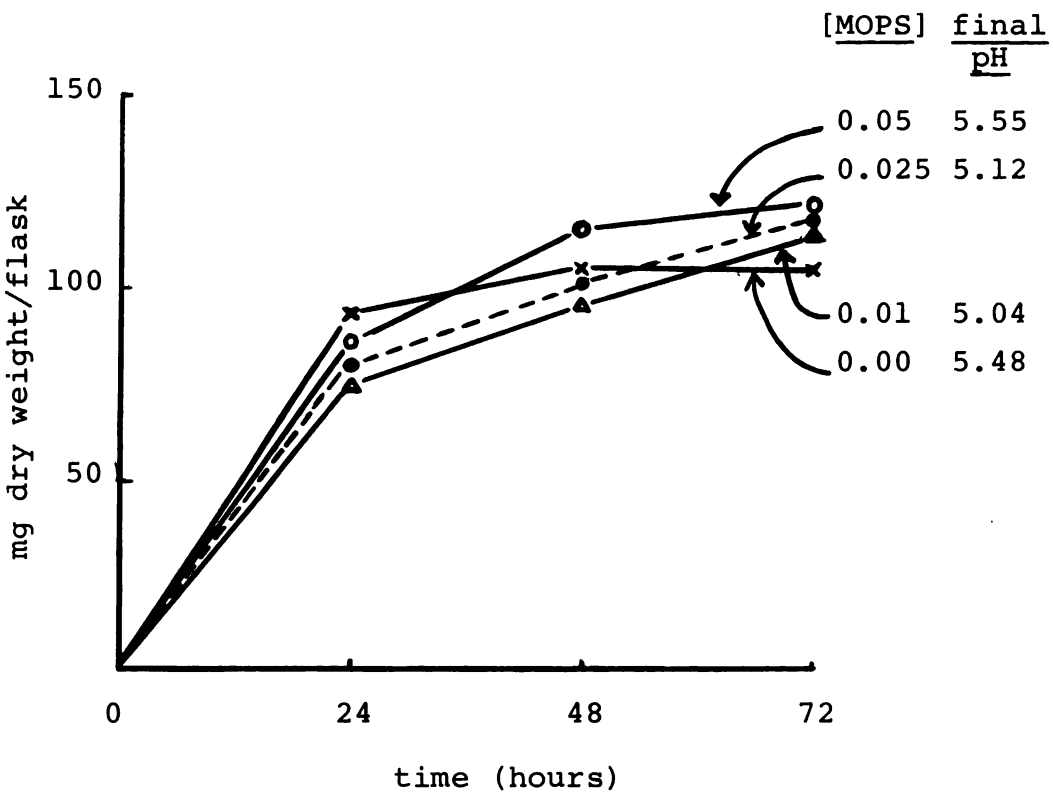

Figure 6. Growth of N. crassa with MOPS, initial pH 7.0

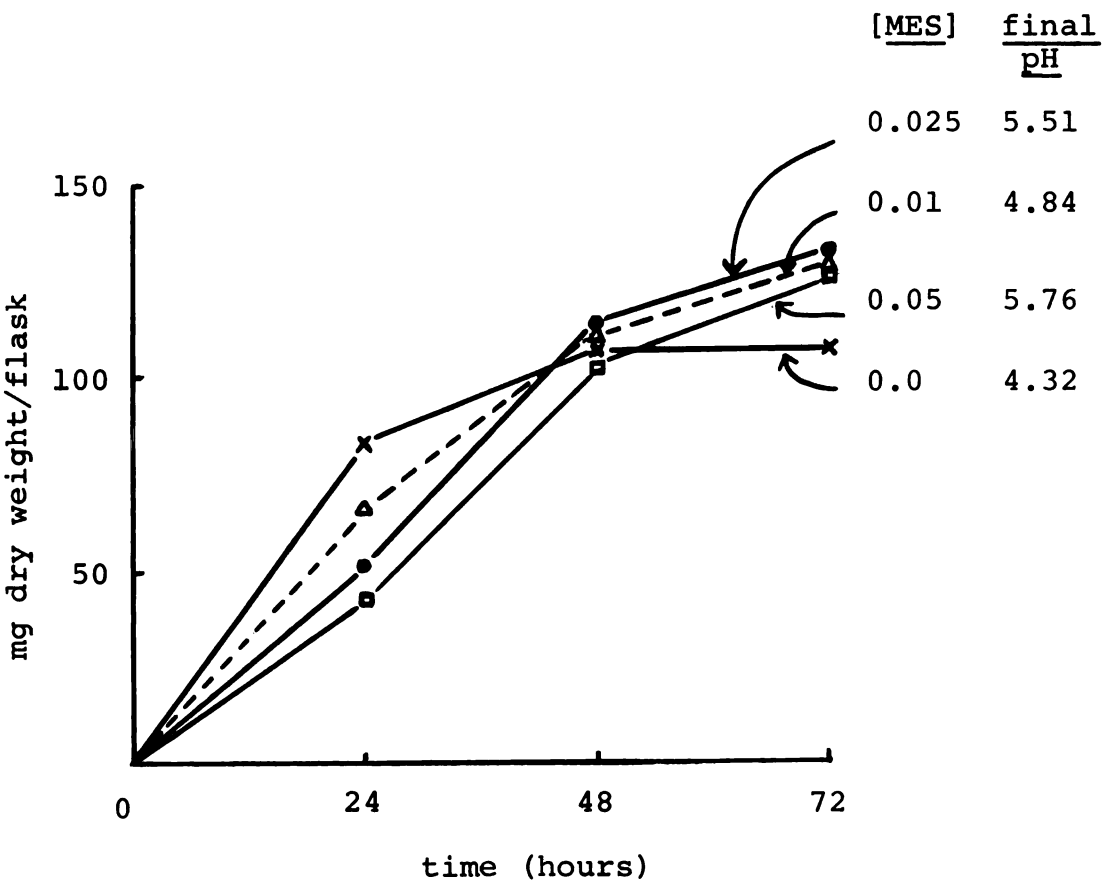

Figure 7. Growth of N. crassa with MES, initial pH 6.1

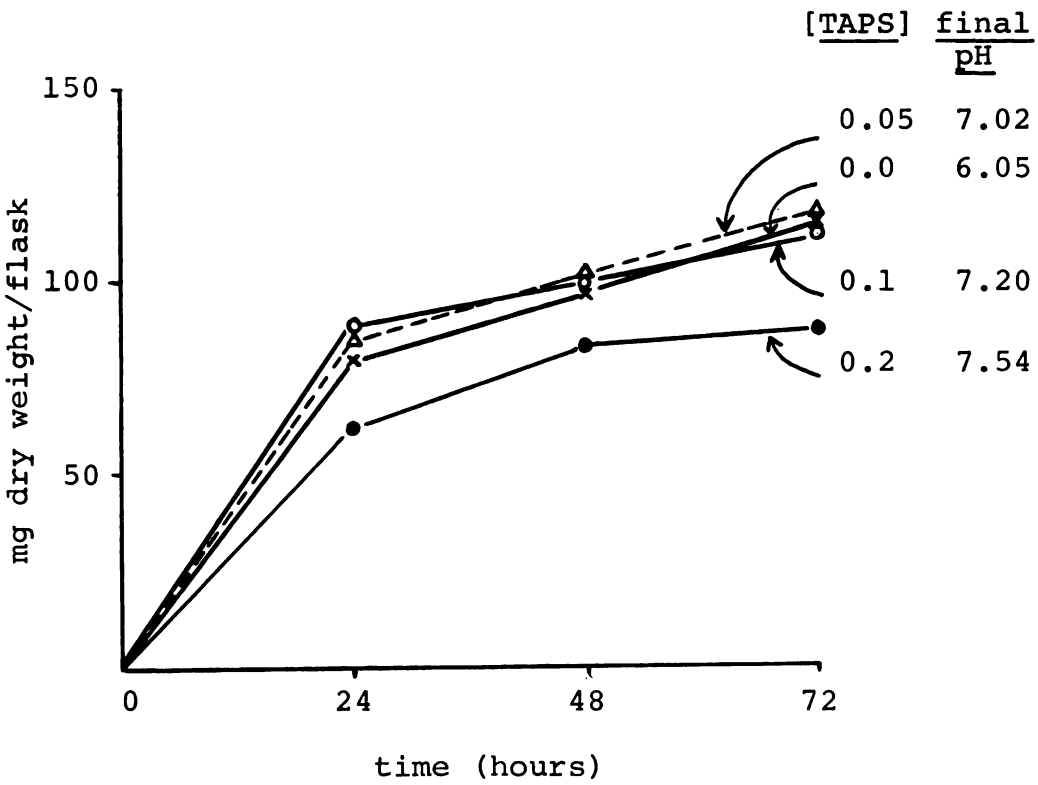

Figure 8. Growth of N. crassa with TAPS, initial pH 8.0

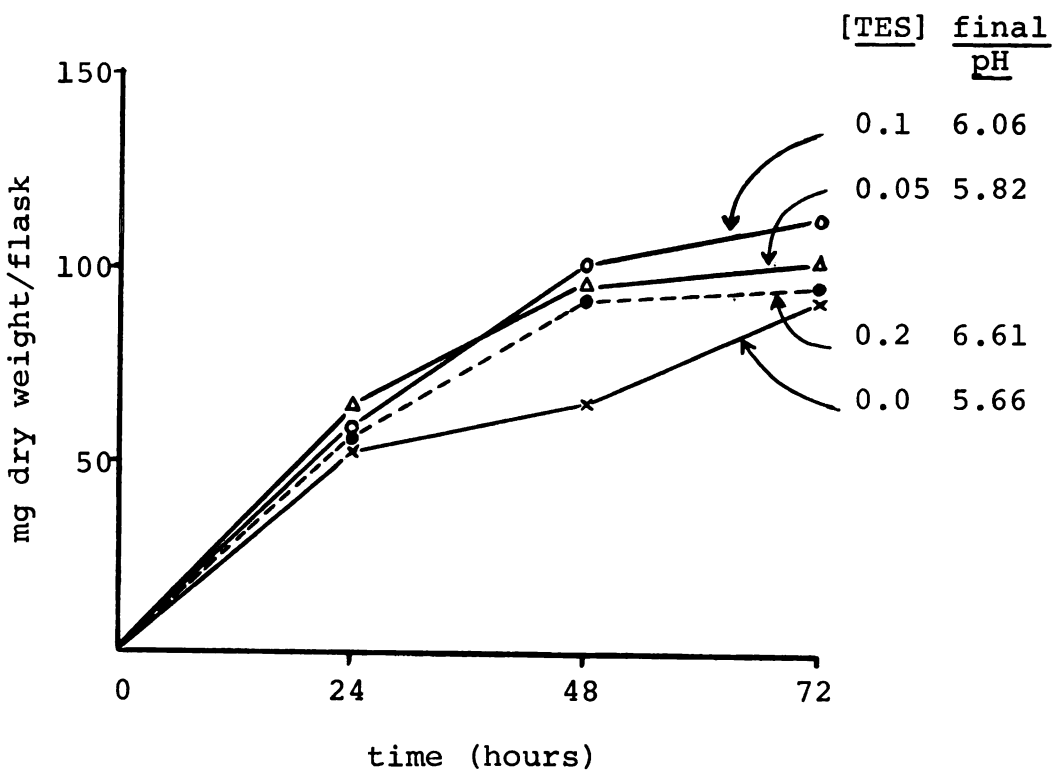

Figure 9. Growth of N. crassa with TES, initial pH 7.4

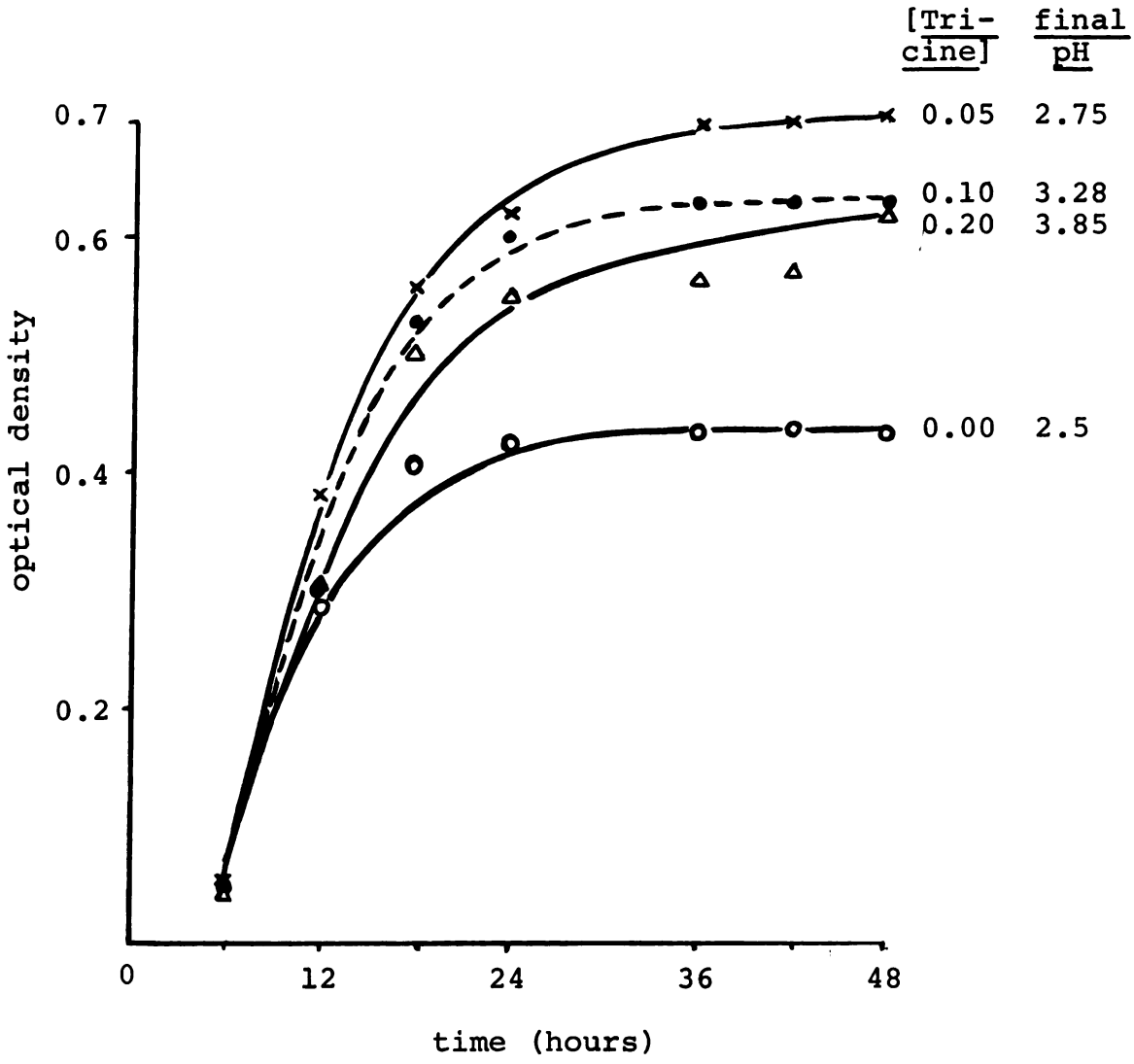

Figure 10. Growth of S. ellipsoideus with tricine, initial pH 8.0

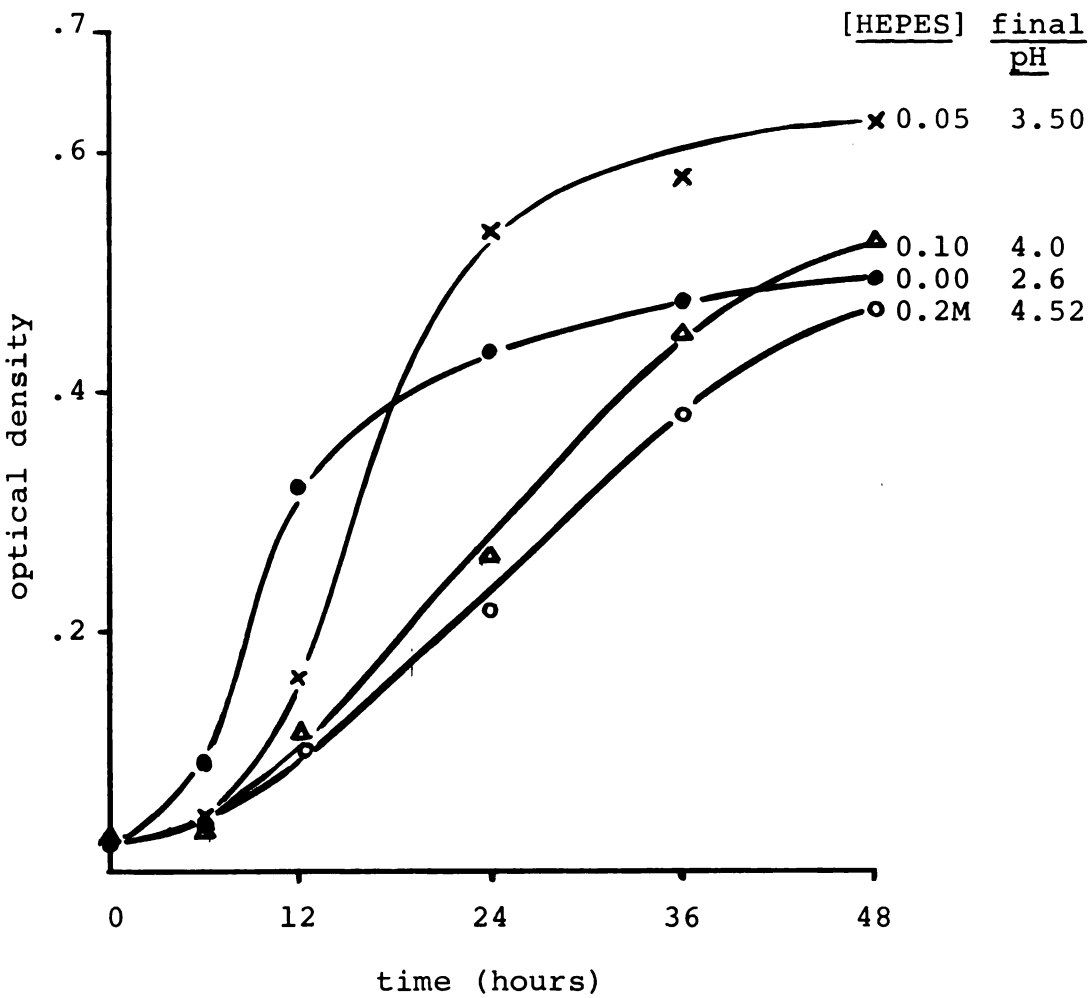

Figure 11. Growth of S. ellipsoideus with HEPES, initial pH 7.4

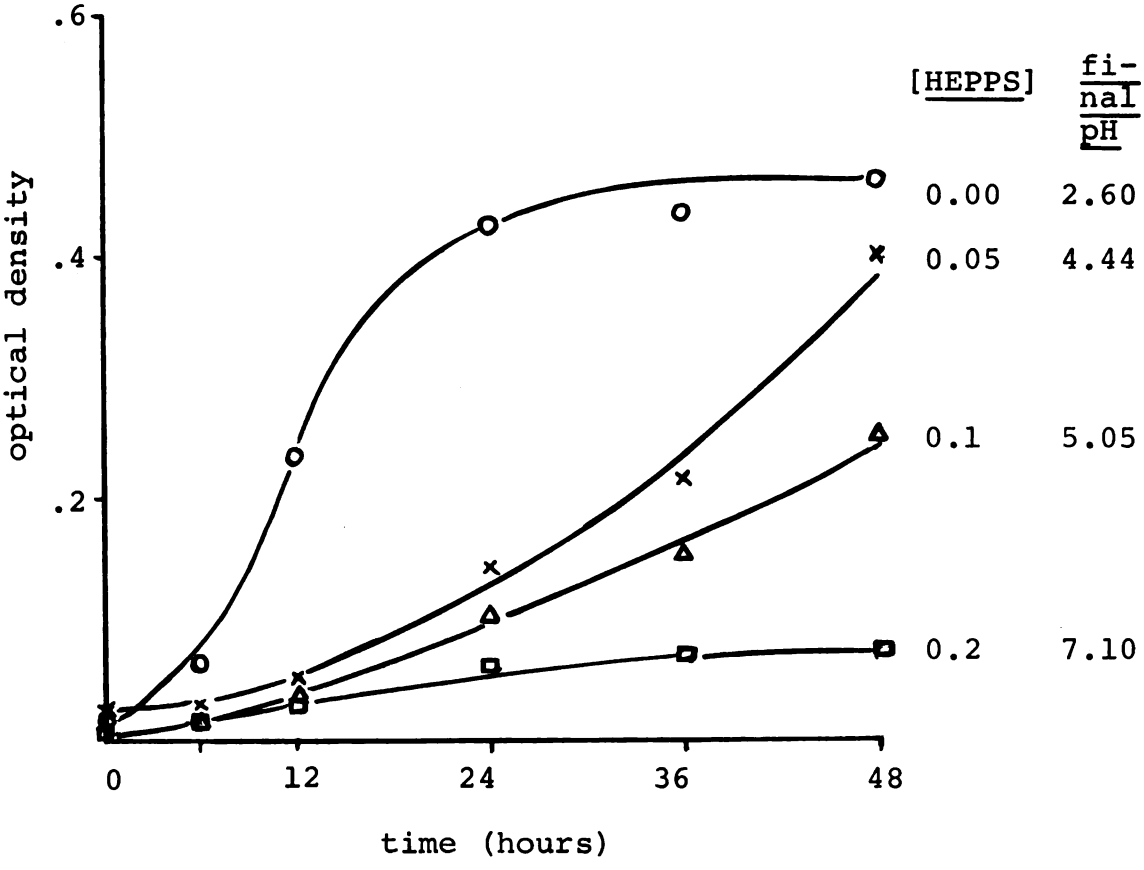

Figure 12. Growth of S. ellipsoideus with HEPPS, initial pH 8.0

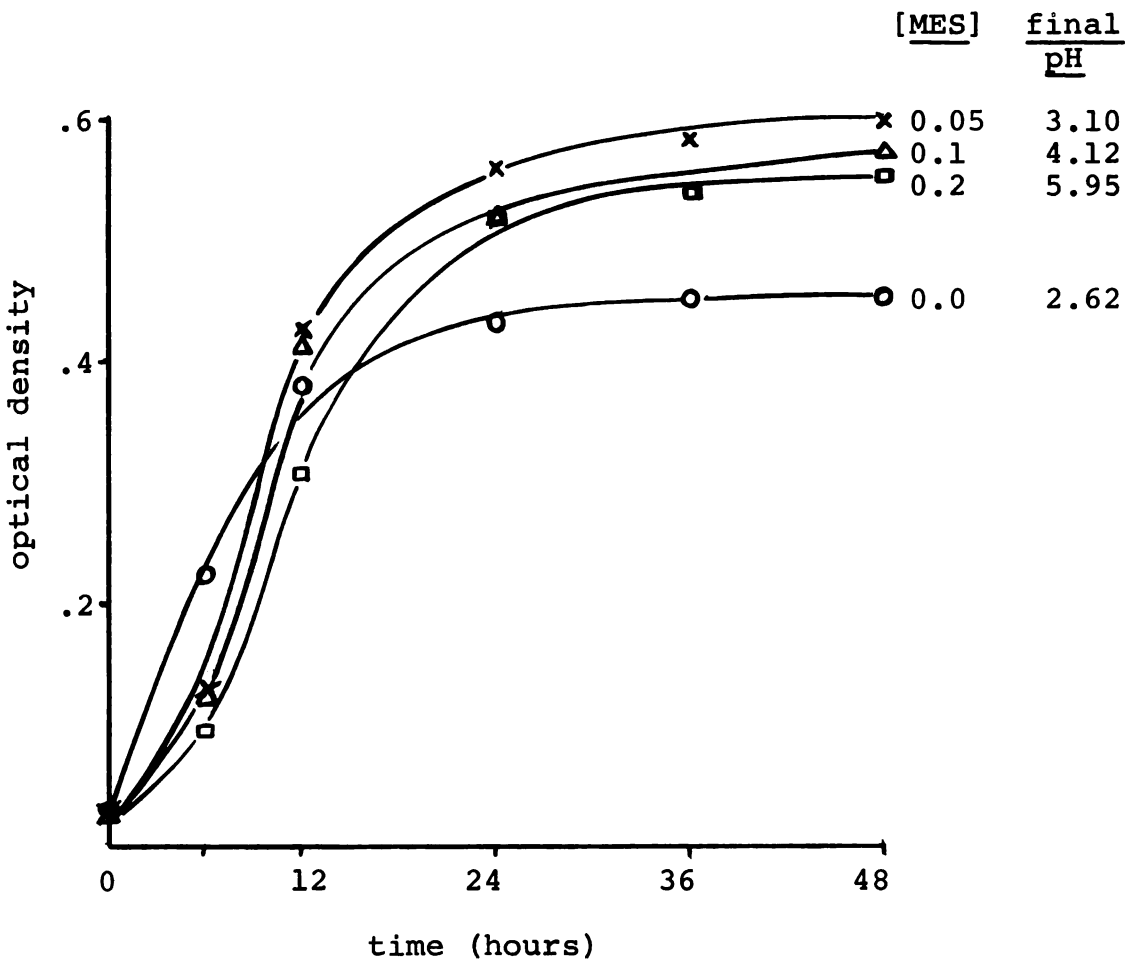

Figure 13. Growth of S. ellipsoideus with MES, initial pH 6.1

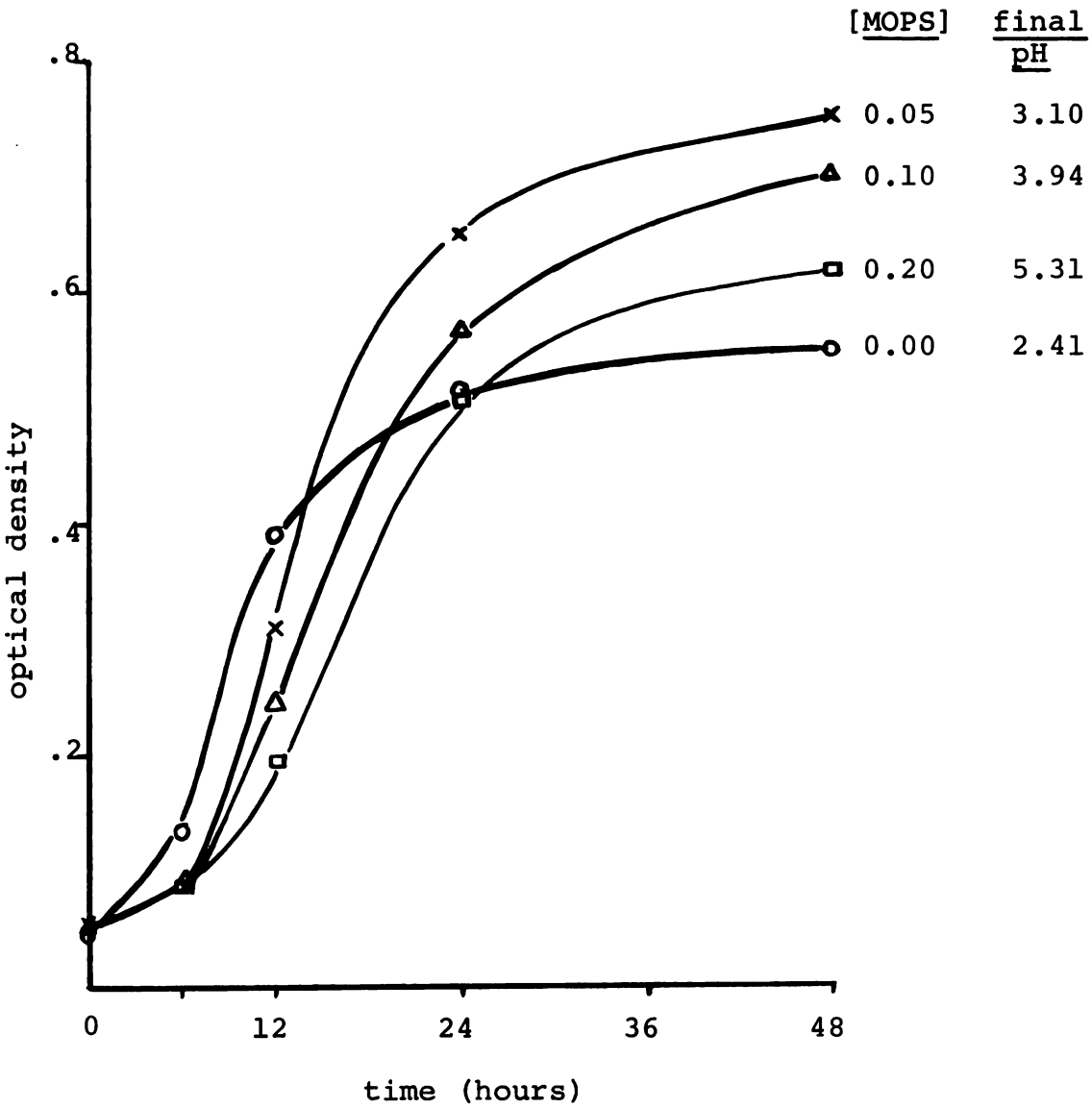

Figure 14. Growth of S. ellipsoideus with MOPS, initial pH 7.0

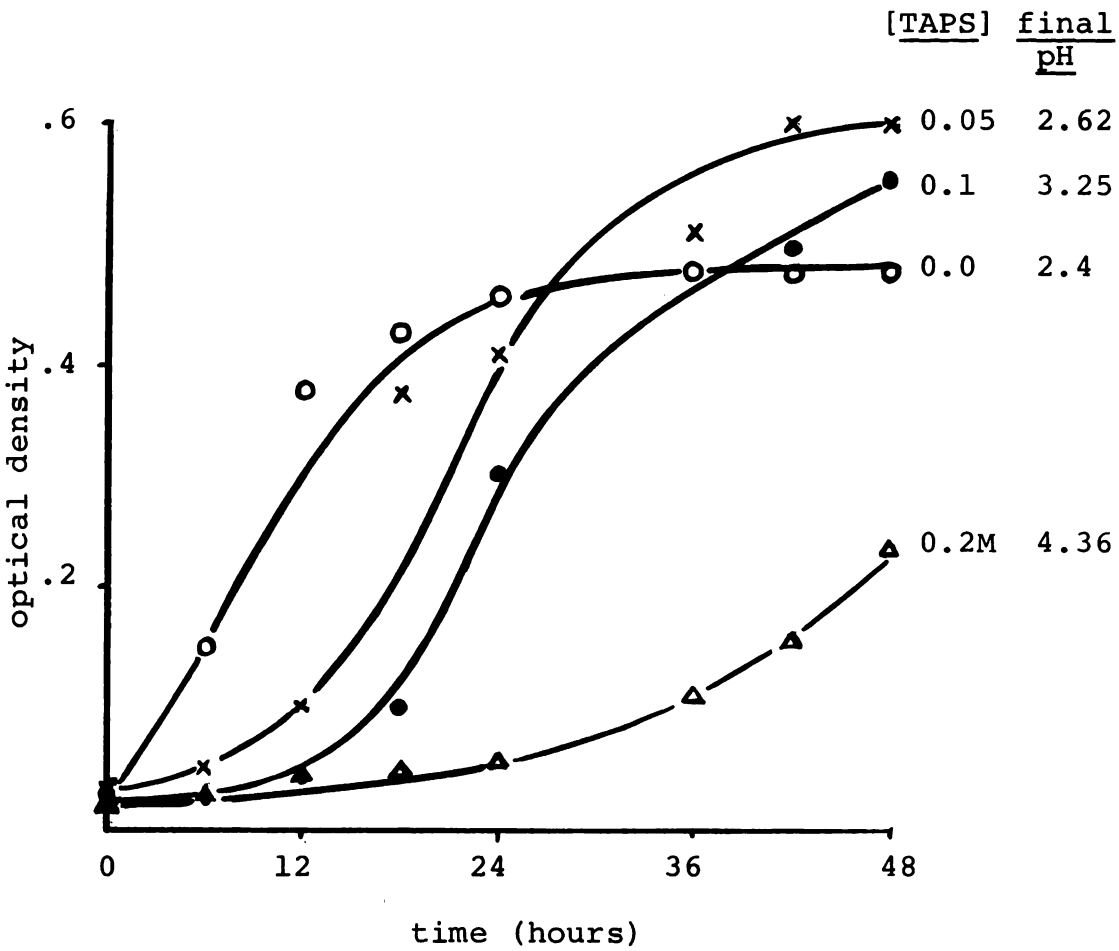

Figure 15. Growth of S. ellipsoideus with TAPS, initial pH 8.0

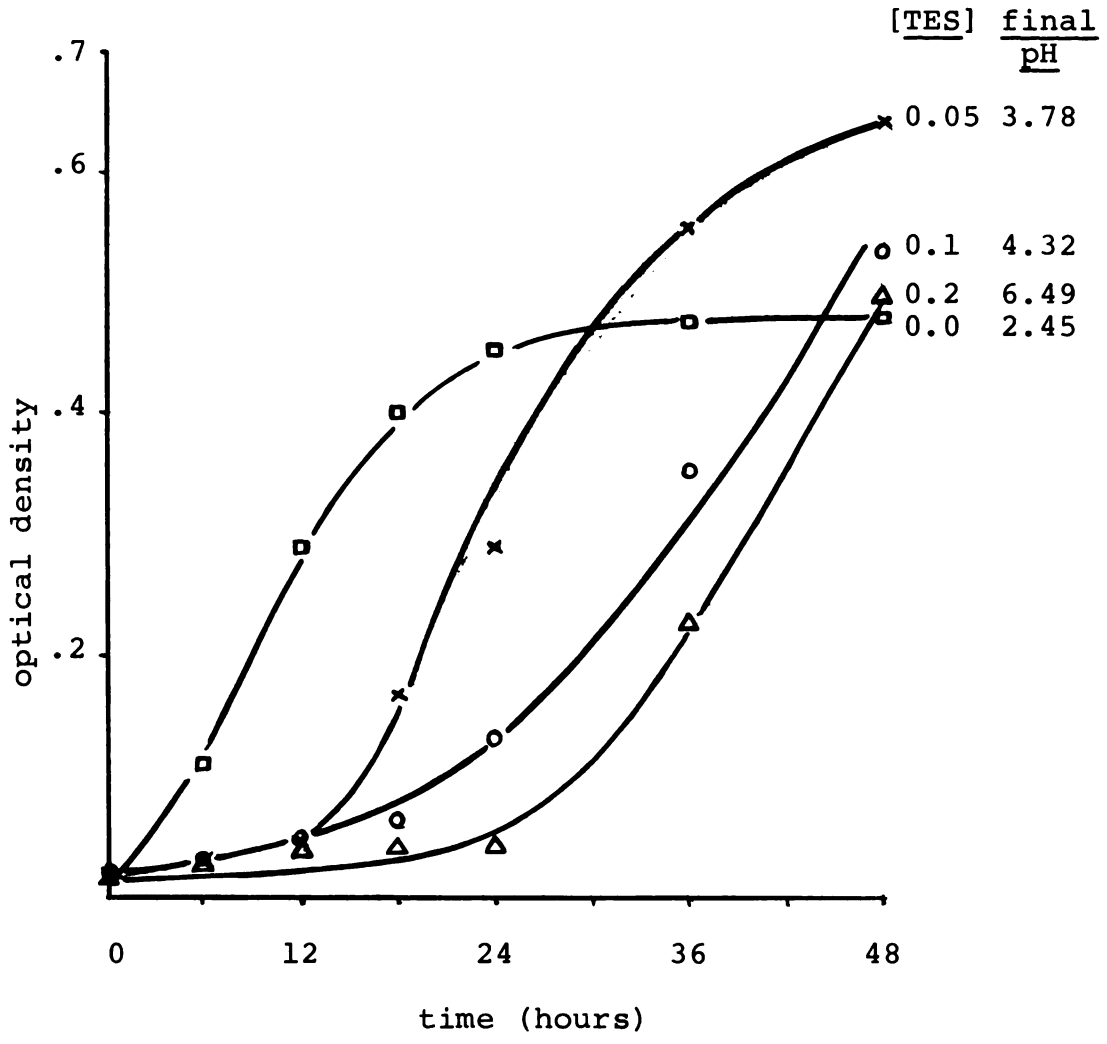

Figure 16. Growth of S. ellipsoideus with TES, initial pH 7.4

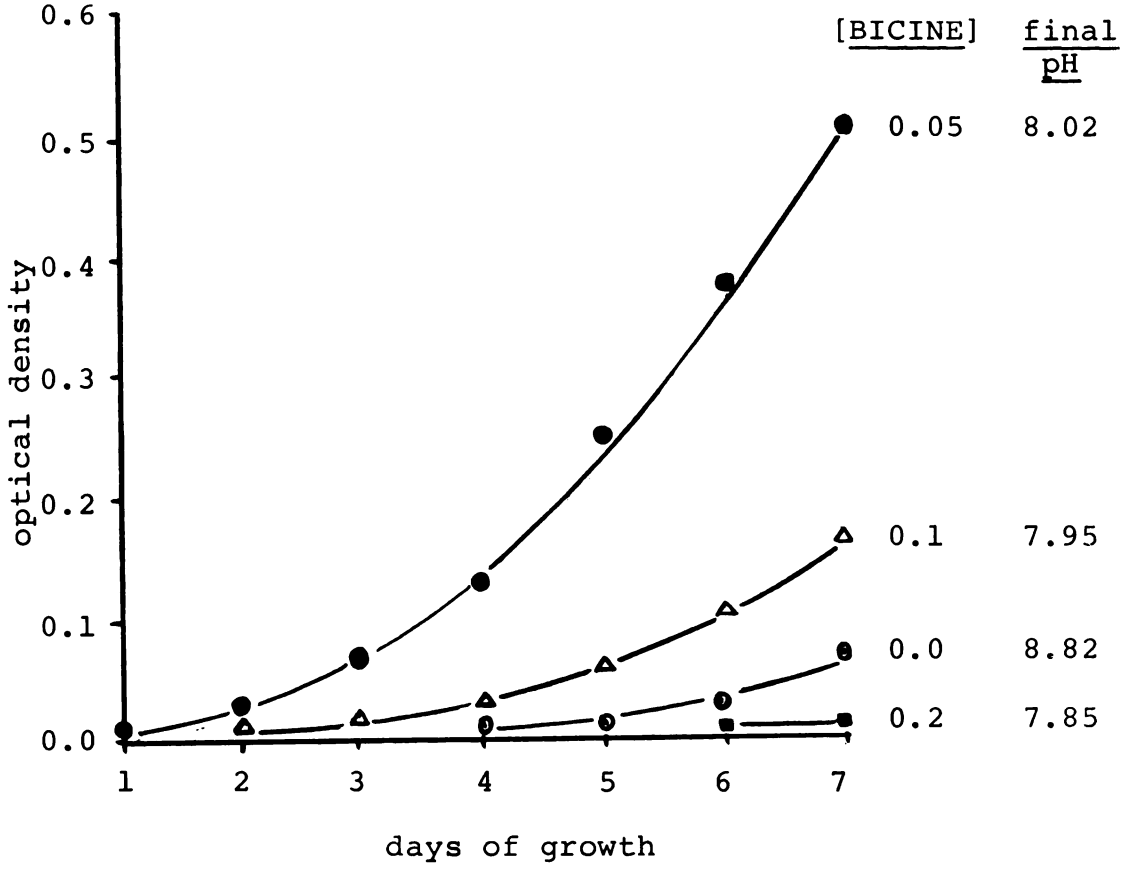

Figure 17. Growth of Chlorella pyrenoidosa with the presence of bicine, initial pH 8.0

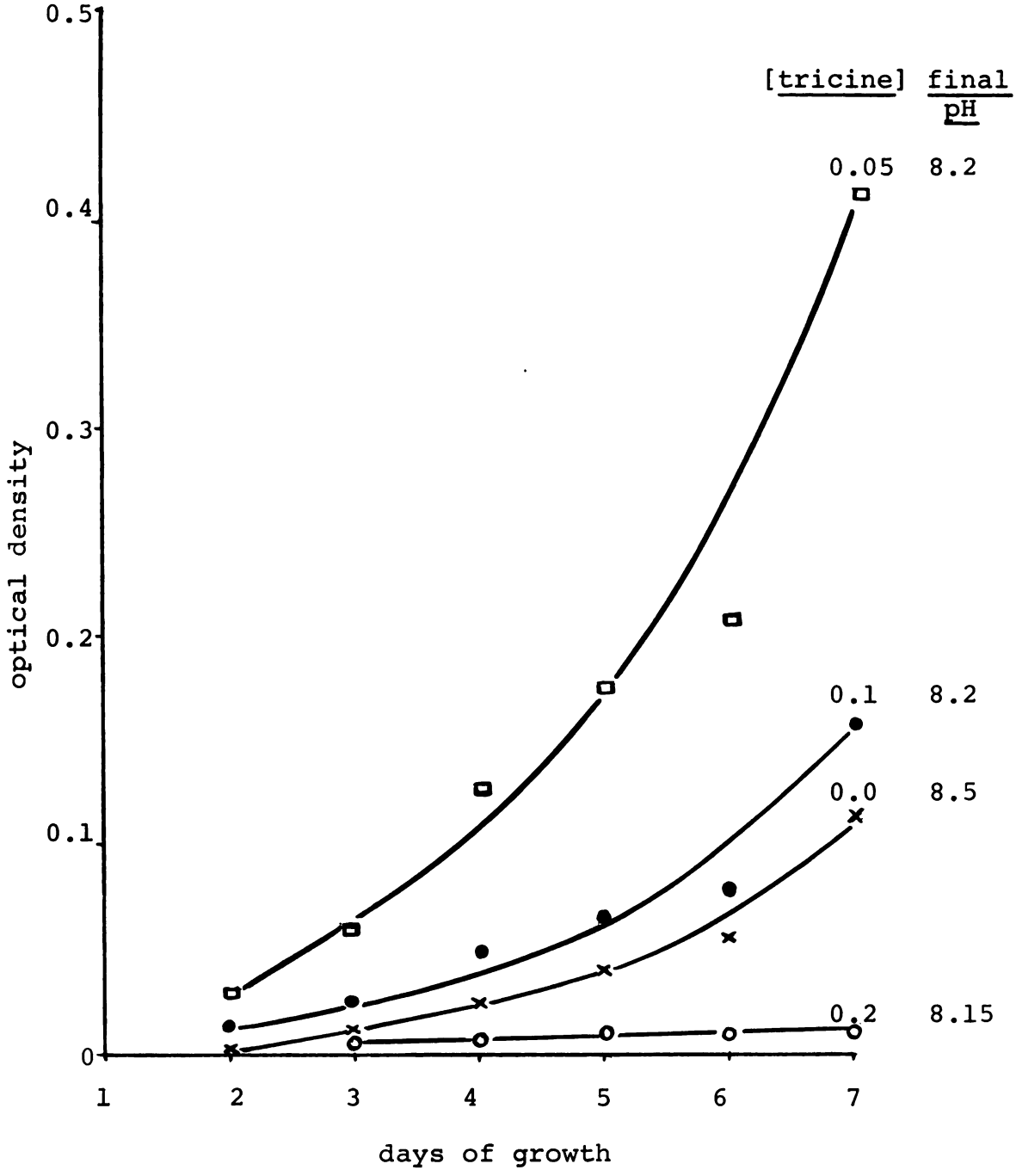

Figure 18. Experiment 1: Growth of Chlorella pyrenoidosa in usual medium with tricine, initial pH 8.0

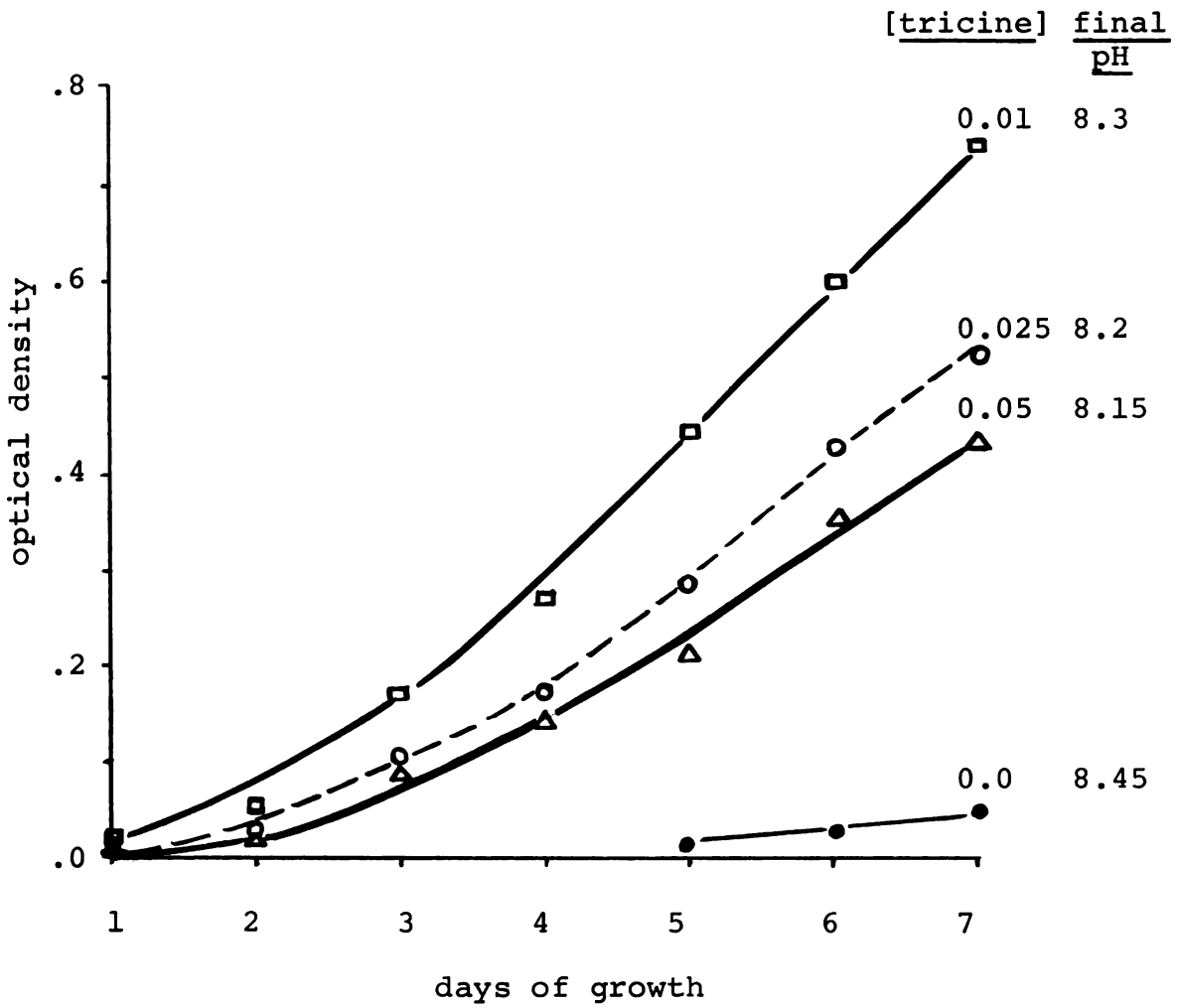

Figure 19. Experiment 2: Growth of Chlorella pyrenoidosa in usual medium + tricine, initial pH 8.0

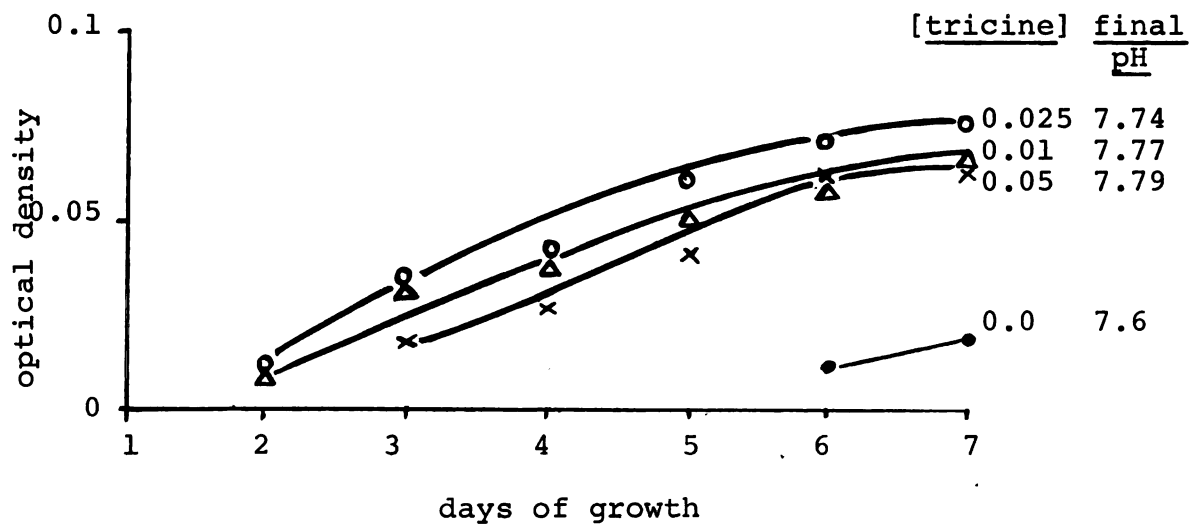

Figure 20. Experiment 2: Growth of Chlorella pyrenoidosa in usual medium (without $NaNO_3$) + tricine, initial pH 8.0

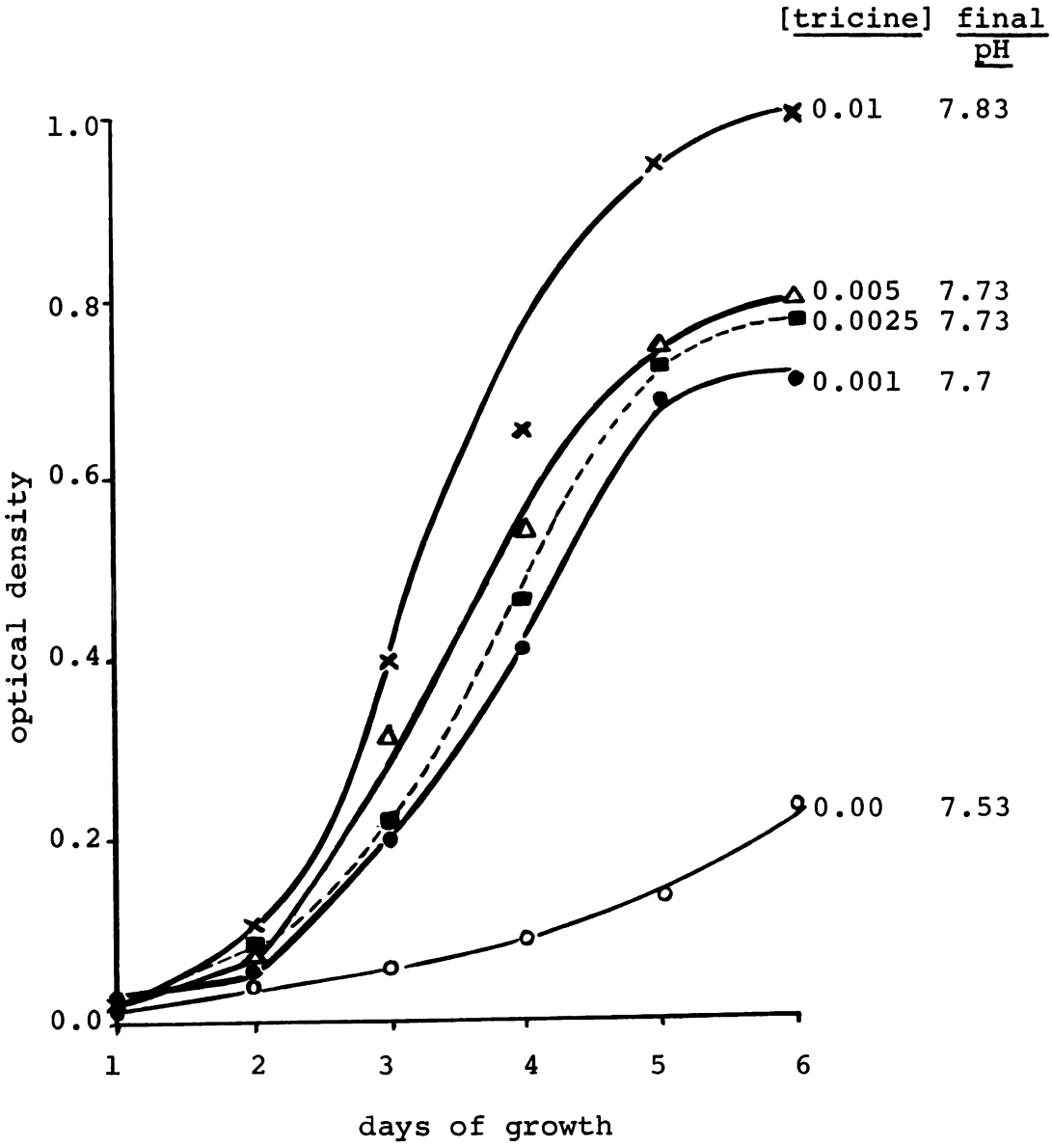

Figure 21. Experiment 3: Growth of Chlorella pyrenoidosa in the presence of peptone + tricine, initial pH 8.0

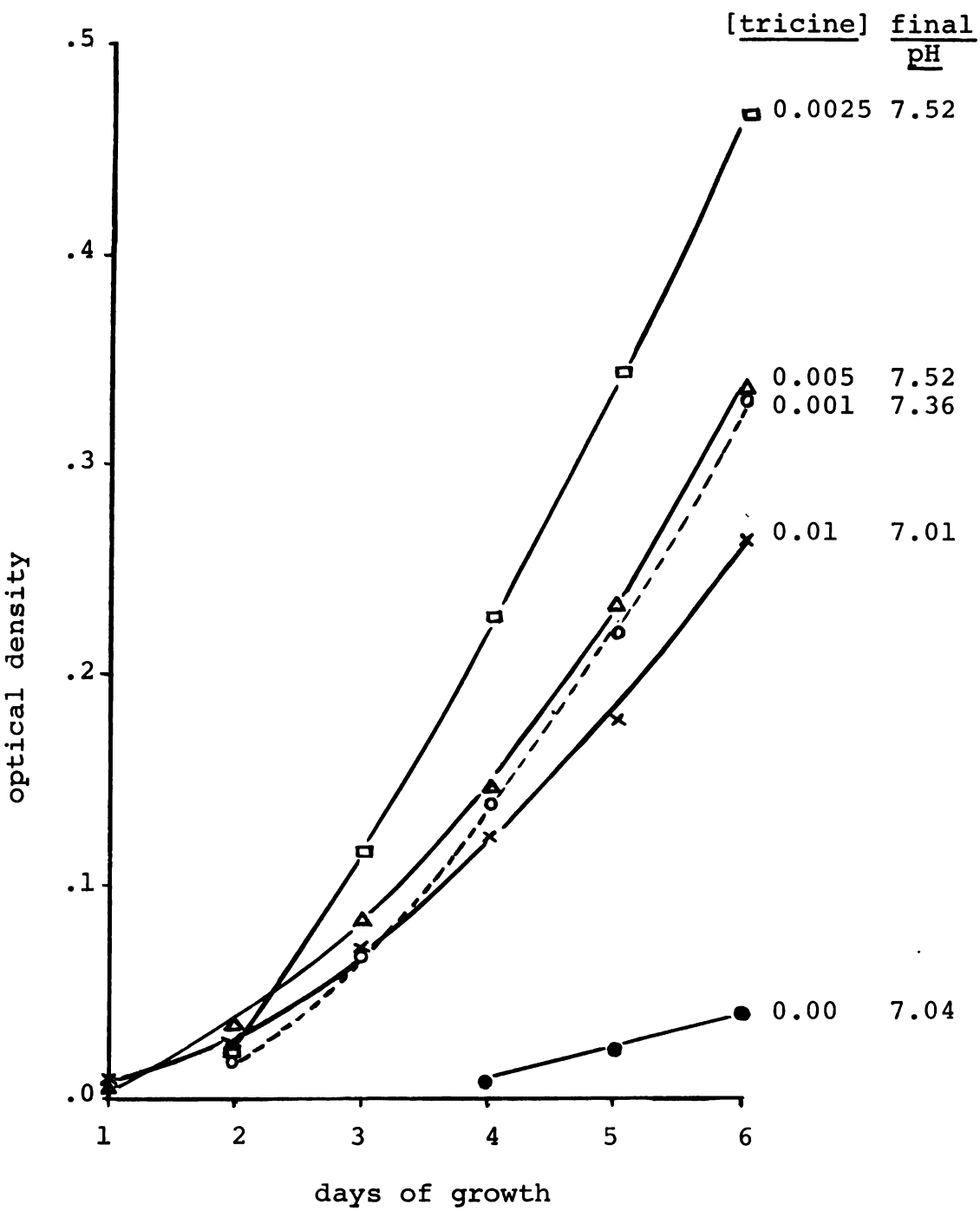

Figure 22. Experiment 3: Growth of Chlorella pyrenoidosa in usual medium supplied with NH_4NO_3 (not $NaNO_3$) and in the presence of tricine

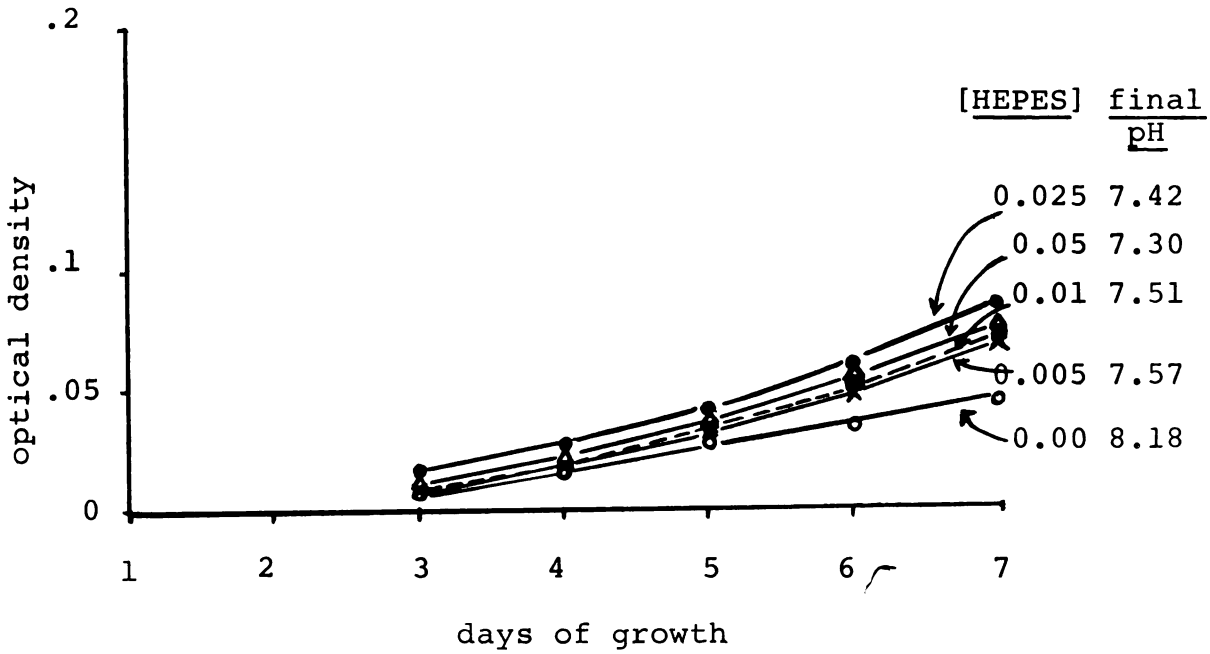

Figure 23. Growth of Chlorella pyrenoidosa with HEPES, initial pH 7.4

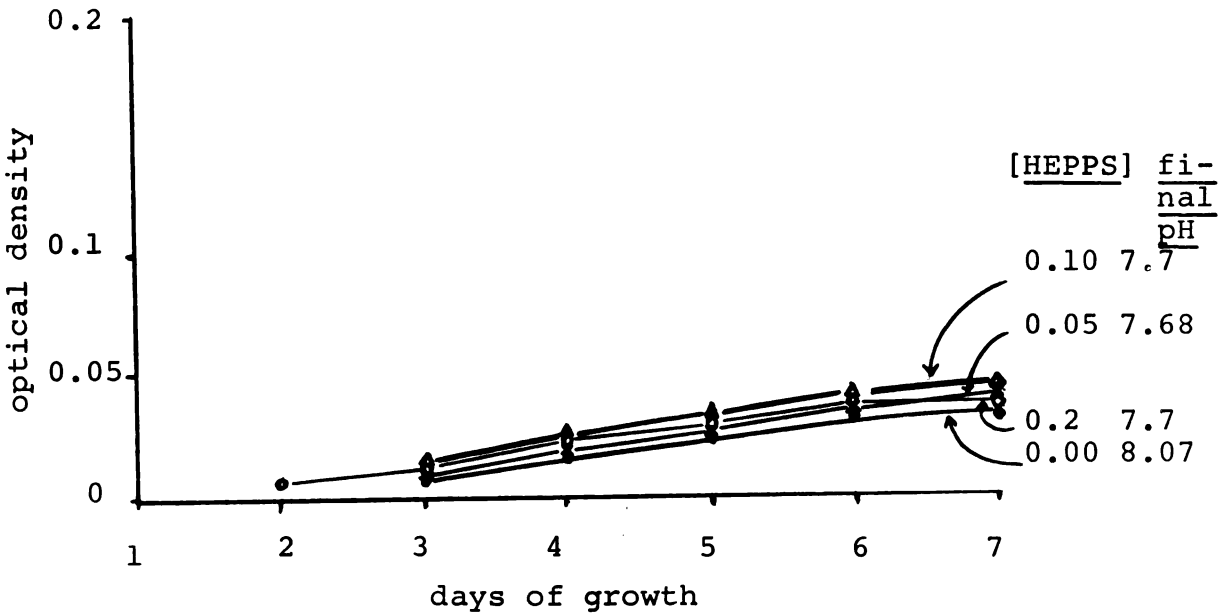

Figure 24. Growth of Chlorella pyrenoidosa with HEPPS, initial pH 8.0

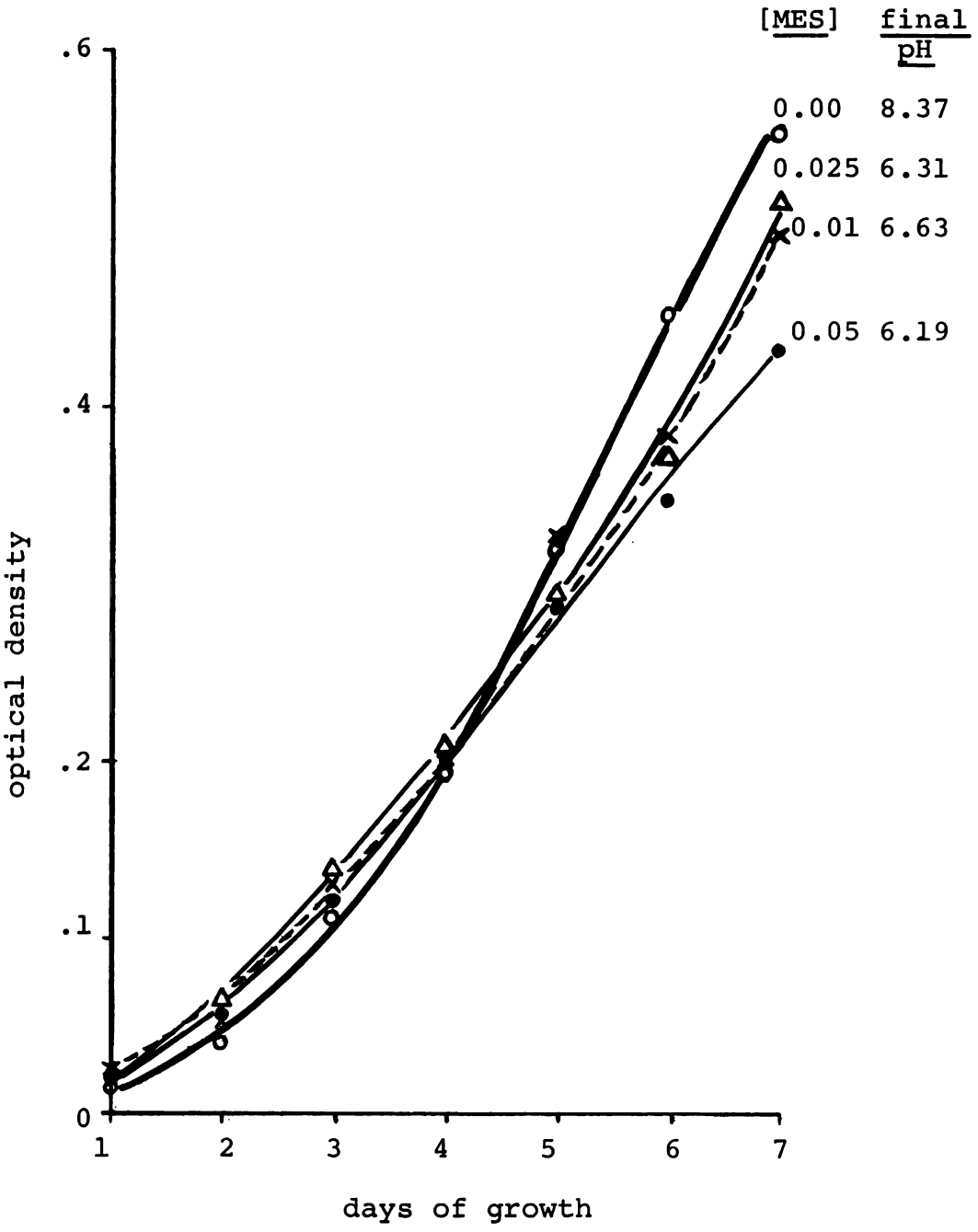

Figure 25. Growth of Chlorella pyrenoidosa with MES, initial pH 6.1

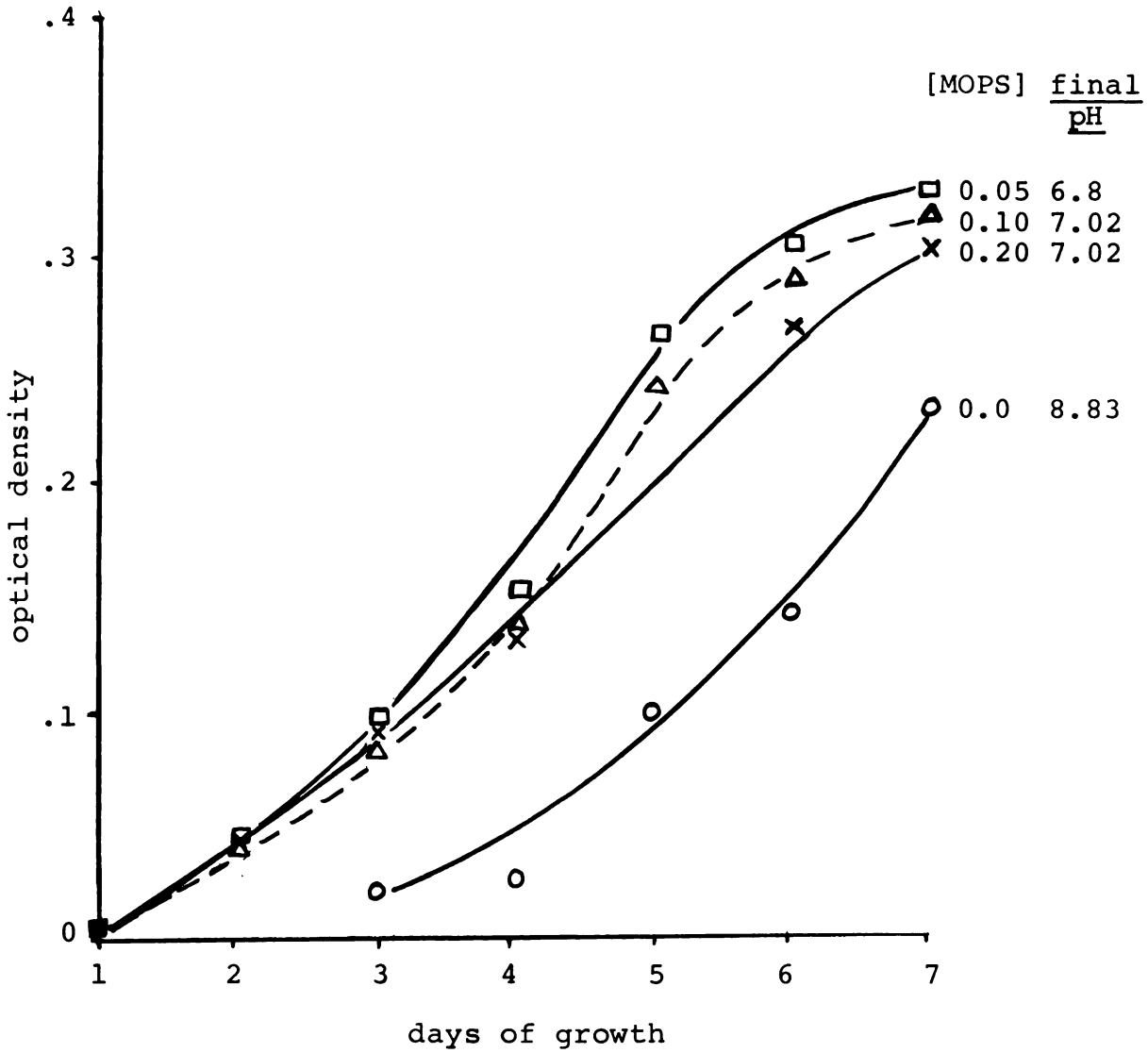

Figure 26. Growth of Chlorella pyrenoidosa with MOPS, initial pH 7.0

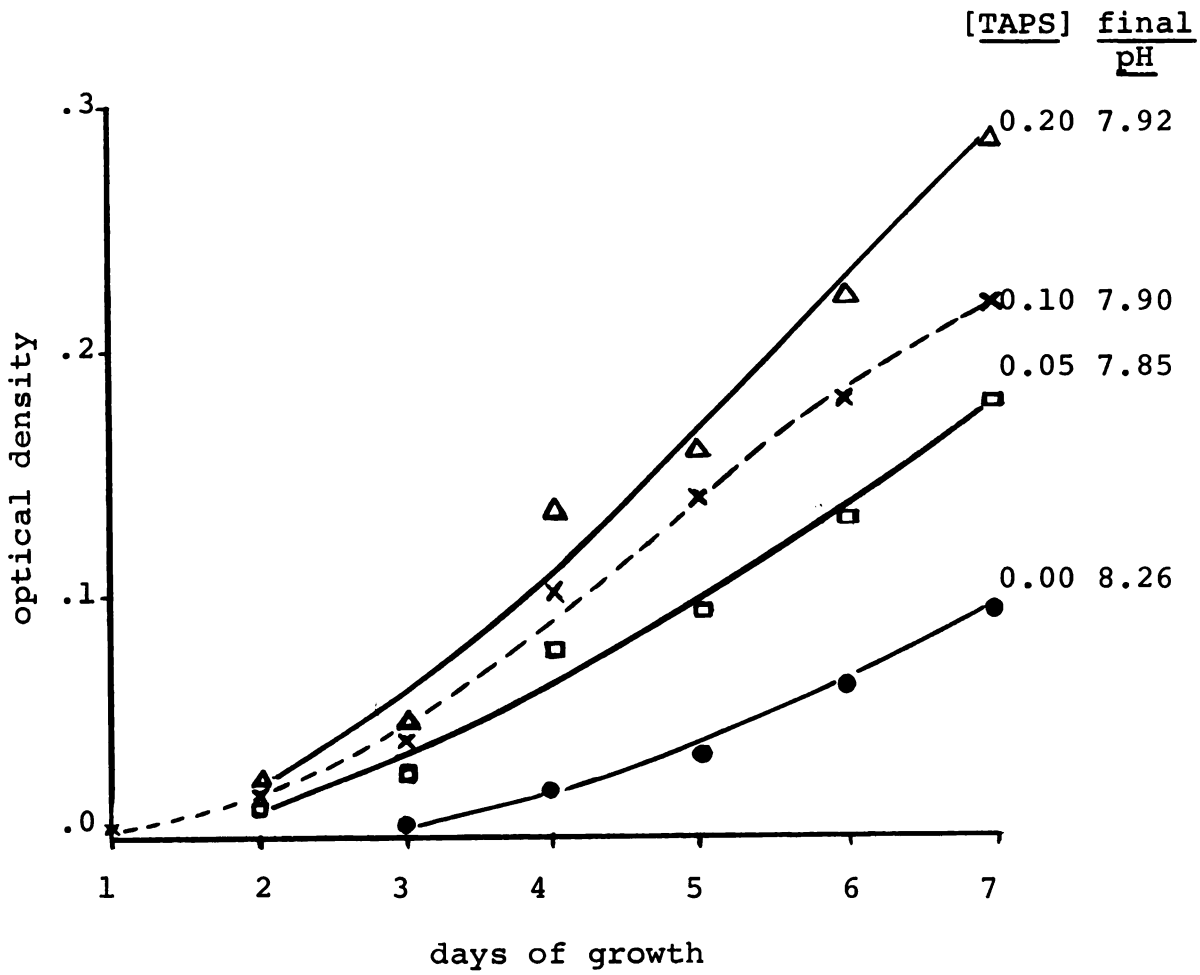

Figure 27. Growth of Chlorella pyrenoidosa with TAPS, initial pH 8.0

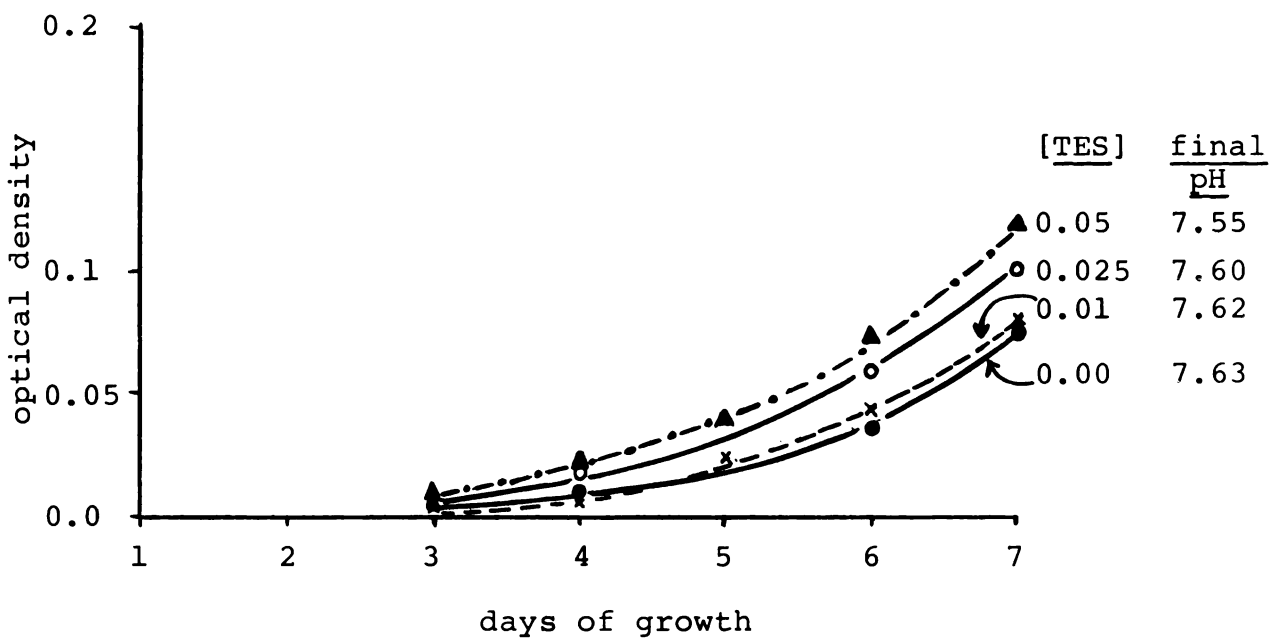

Figure 28. Growth of Chlorella pyrenoidosa with TES, initial pH 7.4

DISCUSSION

Neurospora seems to generally tolerant of all the buffers employed. Moreover the medium used for the Neurospora studies was already somewhat buffered with tartrate. Nevertheless the buffers did hold the pH at higher levels than the control medium. Since the initial pH's and often the final pH's were much higher than in the medium without greatly changing the rate of growth, we must conclude that Neurospora as grown by us is also rather indifferent to pH of the medium.

In contrast the growth of the yeast was very much affected by the nature of the buffers employed. This may be in part because the yeast is more sensitive to pH, starting growth somewhat slowly at the higher pH's. In addition the yeast medium, unlike the Neurospora medium, was poorly buffered and the yeast seemed to produce an inordinate amount of acid. Consequently the yeast tended to terminate its growth when it had acidified its own medium to about pH 2.5. For this reason in the presence of almost any of the buffers we increased the total amount of growth simply by delaying the acidification process--that is by permitting more growth before the organism inhibited itself with its own acid production.

However many of the effects of these buffers cannot be attributed in any way to their buffering action. Bicine was extremely inhibitory at all concentrations while HEPES, HEPPS, TAPS and TES all caused characteristic lags in growth (often not associated with any inhibition once growth had started). These lags are not at all understood. They do not seem to be dependent on either the initial pH or the remaining buffering capacity of the particular buffer employed.

The buffers can be divided into three classes in respect to their effects on Chlorella growth. The substituted glycines, bicine and tricine, cause very large increases in the otherwise rather unsatisfactory rate of growth. HEPES, HEPPS and MES have little effect on growth or on the lag periods. MOPS, TAPS and TES are stimulatory but less so than tricine and bicine. None of these effects can be related in any obvious way to the pH stabilization afforded by the buffers. Nor does the tricine effect seem to depend on the nitrogen source, although tricine + peptone did give higher rate of growth than tricine + sodium nitrate or tricine + ammonium nitrate.

These studies, especially the Chlorella experiments have raised more questions than they have answered. It is true that we have discovered some excellent media for growing Chlorella but we have, as yet, no idea why these are better. It should be remembered that the

experiments did not employ air enriched with CO_2 nor was air of any kind bubbled through the growth media. Therefore the carbon source (in this case CO_2) may have been seriously limiting. Also as mentioned before, the initial pH's employed were probably too high in many cases, especially in view of the fact that they rose even more during growth. Certainly the Chlorella experiments should be repeated with a CO_2 enriched atmosphere and/or with media supplemented with sugars. Another obvious but not particularly easy experiment is to use radioactive buffers as a sensitive test for buffer metabolism since either release of radioactive CO_2 or incorporation of radioactivity into the organism would prove that the buffer was being metabolized. The approximate titration procedures employed in this study could be refined to a point where even small changes in the concentration of buffer in the media could be detected. Our crude estimates only preclude very large changes in buffer concentration and much smaller amounts of buffer metabolism might account for observed growth stimulations. Few of the Chlorella experiments were carried on to the point where growth was beginning to slow down. Therefore these experiments gave little indication of the maximum growth potential of the system. Longer experiments to determine the influence of these buffers on this aspect of growth should be undertaken.

A further and testably unresolved question remains regarding the effects of these buffers on yeast growth. To what extent do the cells of yeast respond to these buffers by producing yet more acid and what is the nature of the acids produced? This is a question in which could be readily answered by well known procedures involving titrations, extractions, ion exchanges and various kinds of chromatography.

REFERENCES

[1]Quastel, J. H. and A. H. M. Wheatley, Proc. Roy. Soc. B 112:60 (1932).

[2]Kirby, A. H. and M. Neuberger, Biochem. J. 28:1251 (1938).

[3]Mertz, E. T. and C. A. Owen, Proc. Soc. Exptl. Biol. Med. 43:204 (1940).

[4]Hall, N. F. and M. R. Sprinkle, J. Am. Chem. Soc. 54:3469 (1932).

[5]Bates, R. G. and G. F. Allen, J. Research. Natle. Bur. Standards, 64 (A):343 (1960).

[6]Aloisi, M., Ann. Univ. Ferrara, 8:Pt. 2, 101 (1960).

[7]Bates, R. G. and G. Schwarzenbach, Helv. Chim. Acta. 37:1437 (1954).

[8]Gomori, G., Proc. Soc. Exptl. Biol. Med. 62:33 (1946).

[9]Gomori, G., Proc. Soc. Exptl. Biol. Med. 68:354-358 (1948).

[10]McFarland, W. N. and K. S. Norris, Ann. N. Y. Acad. of Science 92:446-456 (1961).

[11]Peirce, E. C., II, A. M. A. Arch. Surg. 80:693 (1960).

[12]Stormorken, H. and T. F. Newcomb, Scand. J. Clin. Lab. Invest 8:237 (1956).

[13]Podolsky, R. J. and M. F. Morales, J. Biol. Chem. 218:945 (1956).

[14]Sturtevant, J. M., J. Am. Chem. Soc. 77:1495 (1955).

[15]Benesch, R. E. and R. Benesch, J. Am. Chem. Soc. 77:2749 (1955).

[16]Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, Seikichi Isawa, and Raizada. M. M. Singh., Biochemistry 5:467-477 (1966).

[17]Dimond, A. E. and G. L. Peltier, Amer. J. Bot. 32:46-50 (1945).

[18]Morton, A. G. and A. MacMillan, J. Exptl. Bot. 5:232-252 (1953).

[19]Elizarova, T. N., Mikrobiologiya, 36 (1):38-45 (1967).

[20]Westergaard, M. and H. K. Mitchell, Am. J. Bot. 34:573 (1947).

[21]Sastry, Sivarama K., Biochem J. 85:486-91 (1962).

[22]Weiss, B. and G. Turian, J. Gen. Microbiol. 44 (3):407-418 (1966).

[23]Bianchi, D. E. and G. Turian, Z. Allg Mikrobiol. 7(4):257-262 (1967).

[24]Domnas, A., J. Biochem. (Tokyo). 52:149-154 (1962).

[25]Ter. Karapetyan, M. A. and S. M. Indzhikyan Dokl. Akad. Nauk. Arm. SSR. 43(27):117-123 (1966) (Abstract).

[26]Eddy, A. A. and K. J. Indge, Biochem. J. 85:35-36 (1962).

[27]Maw, G. A. and Cecil M. Coyne, Arch. Biochem. Biophys. 117 (3):499-504 (1966).

[28]Rothstein, A., Ann. N. Y. Acad. of Sci. 92:470-477 (1961).

[29]Birdsey, Elizabeth. C. and V. H. Lynch, Science 137:763-764 (1962).

[30]Godnev, T. N., Ya. P. Lyakhnovich, and V. A. Syusyukin, Fiziol. i Biokhim, Rast, Inst. Biol. Akad. Nauk. Belorussk. SSR, 3-11 (1962) (Abstract).

[31]McLachlan, J., Can. J. Bot. 41:35-40 (1963).

[32]Tageeva, S. V., V. S. Korshunova, and M. L. Mikhnevich Fiziol, Rast. 13(6):985-966 (1966).

[33]Syrett, P. J. and I. Morris, Biochim Biophys. Acta. 67:566-675 (1963).

[34]Filner, P., Biochim Acta. 118:299 (1966).

[35]Bold, H. D., Bull. Torrey Bot. Club. 76:101-108 (1949).

[36]Trelease and Trelease, American J. Bot. 22:520-542 (1935).

[37]Beadle, G. W. and E. L. Tatum, A.J.B. 32:678 (1945).