KINETICS OF CO_2 PRODUCTION BY INTACT BACTERIAL RESTING CELL SUSPENSIONS

BY

Christopher W. Kay

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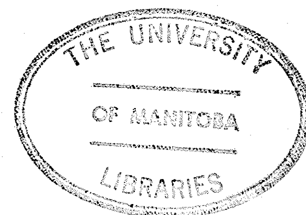

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FOR LYNDA

ABBREVIATIONS

BOD	Biological oxygen demand
DO	Dissolved oxygen
LAS	Linear alkylbenzene sulphonate
CTP	Cytosine triphosphate
ITP	Inosine triphosphate
E. coli	*Escherichia coli*
B. coli	*Bacterium coli* (*Escherichia coli*)
P. aeruginosa	*Pseudomonas aeruginosa*
P. rettgeri	*Proteus rettgeri*

A B S T R A C T

ABSTRACT

A novel infrared gas analysis method was developed and tested for its ability to determine the kinetics of CO_2 liberation by whole bacterial cell preparations. Use of the system allowed the measurement of initial rates of CO_2 evolution by resting cell suspensions and developed some heretofore unrecognized properties of these preparations.

An initial lag before the onset of maximum CO_2 liberation, undetectable by usual manometric techniques, has serious implications in the accuracy of some kinetic constants previously reported.

Resting cell preparations evolved CO_2 in response to exogenous substrate in a manner which could be described by Michaelis-Menten kinetics for single enzymes.

A number of organic compounds including amino acids, organic acids, and fatty acids were examined kinetically. For 26 substrates tested, the V_{max} for CO_2 liberation ranged from 0-182 n moles min^{-1} mg^{-1} dry wt of cells. The K_m for six of the substrates ranged from 0.5-16.7 mM.

Variations in the kinetics of CO_2 production by resting cell preparations as a function of temperature, pH, bacterial growth conditions, age of cell suspensions

and substrate concentration stress the need for system standardization.

Kinetics constants for four partially purified commercial decarboxylating enzyme preparations were determined using this method and the values compared favourably with those derived by other techniques. The method may be useful as a general technique and as a screening test to recognize biologically intractable compounds.

An evaluation was made of the stability of and the methods available to standardize mixed resting cell suspensions of sewage lagoon organisms. Static storage in the cold was found to best preserve cell activity. No satisfactory single method was found to determine active biomass. Exogenous oxygen consumption in the presence of the multicomponent substrate, casamino acids, was proposed as the best single approximation of active biomass.

A model lagoon was evaluated for its ability to provide a stable source of mixed microbial cultures. The model lagoon was found to successfully mimick an actual operating lagoon, but proved unreliable as a source of mixed populations.

The parameters, requisite in generating stable multicultures in a chemostat, were described. A stable mixed population was generated by supplying a multi-

substrate nutrient supply at a dilution rate of 0.06 hr^{-1} to the chemostat. A novel device was designed to eliminate "grow-back" in the chemostat. The chemostat was looked upon optimistically as a laboratory source of mixed cultures.

A C K N O W L E D G E M E N T S

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

The regions of the earth that support life are known as the biosphere. Living matter is created from mineral matter and the maintenance of life on earth is absolutely dependent on the cyclic turnover of certain key elements that enter into the composition of all living organisms.

In addition to the necessary elementary constituents, the maintenance of life on earth requires a steady input of energy from an external source - the sun. This solar energy is trapped and converted to chemical bond energy by the photosynthetic organisms, principally green plants. The chemical bond energy accumulation in the various organic constituents of green plants in turn furnishes, either directly or indirectly, the energy for all non-photosynthetic forms of life.

Plants assimilate the constituent elements of their living matter in mineral form and convert them into the diverse organic constituents of plant tissues. In order to be made available for a fresh cycle of growth, the organically combined elements must eventually be converted into inorganic form. This is the process known as mineralization. The mineralization of organic compounds occurs through combustion (forest fires, and burning of

organic fuels by man), through the respiratory activities of animals and plants, and, most importantly, through the decomposition of plants, animals, and their organic by-products by microorganisms. It is estimated that over 90 per cent of the CO_2 production in the biosphere results from the activity of bacteria and fungi.

The relatively enormous catalytic power of micro-organisms contributes to the major role they play in the chemical transformation occurring on the earth's surface. Because of their small size, bacteria and fungi possess a large surface-volume ratio which permits a rapid exchange of substrates and waste products between the cells and their environment. An even more important factor influencing the chemical role that microorganisms play in nature is their rapid rate of reproduction in favourable environments.

Every organic compound that occurs naturally is decomposed by some microorganism. This fact explains the rarity of undecomposed organic matter on the earth's surface. Except under special conditions, any organic compound that is no longer part of a living organism is avidly consumed and rapidly mineralized by the ever-present microorganisms in the biosphere.

Although there are some microorganisms that are extremely versatile in their ability to attack different organic compounds, the metabolic versatility of the

microbial world as a whole is not primarily a reflection of the metabolic versatility of its individual members. Even the most omnivorous bacteria known, the Pseudomonads, can metabolize not more than two hundred different naturally occurring organic substances. Clearly, therefore, a single bacterial species is only a limited agent of mineralization. Highly specialized physiological groups of microorganisms play important roles in the mineralization of specific classes of organic compounds. Cytophaga, for example, are especially adapted to the decomposition of cellulose.

The turnover of all the elements that enter into the composition of living matter constitutes the cycle of matter. Every biologically important element can be considered to pass through a continuous cycle from the non-living environment to living matter and back to the non-living environment.

The integration of the various processes that constitute the cycle of matter results is a more or less balanced production and consumption of the biologically important materials in the biosphere as it exists at present.

The appearance of man as a member of the community of living organisms has not altered the total balance of the cycle of matter on earth. His activities, however, resulted in local or temporary changes in his environment that are comparable in scale and speed to those brought about by major geological upheavals in the history of the

earth. It is not through his own metabolism that man has temporarily modified the delicate equilibrium of the biosphere, for the respiration of the entire human population is negligible compared with that of other animals and microbes. However, through agriculture, through the mining and burning of fuel deposits, and through the local pollution of waters with industrial wastes he has greatly affected the distribution and growth of other living forms.

In recent years much interest has been expressed regarding man's involvement in the natural processes. Of particular concern is the introduction into the natural milieu of synthetically produced compounds which are recalcitrant to microbial attack. Thus it has been of prime urgency to develop methods to assess the impact of environmental assault. Numerous attempts have been made to develop and standardize methods for evaluating biodegradability and for assessing the natural impact of toxic substances. Eutrophication processes have been investigated by estimating nutrient balances in whole lake studies. New methodologies are being devised and old ones are being improved.

The need for a general method to rapidly and precisely assay physiological activity provided the impetus to initiate this investigation. Since ultimately all organic matter is oxidized to CO_2, the possibility

of employing the kinetics of carbon dioxide production by bacterial suspensions as an index of mineralization appeared attractive.

PART I MICROFLORA FROM THE CHARLESWOOD SEWAGE LAGOON: STABILITY AND STANDARDIZATION

H I S T O R I C A L

HISTORICAL

The survival of bacteria stored in aqueous suspensions has been a topic of numerous studies, many at the beginning of this century. In 1917 Shearer (190) reported that physiological saline was more toxic than distilled water to meningococci. Winslow and Falk (228), in 1923, observed that '*B. coli*' survived longer in physiological saline than in distilled water and that the pH optima for survival were different in the two media. Cook and Willis (45), in 1958, showed that washed buffered populations of *E. coli* maintained higher viabilities at room temperature compared with unwashed or unbuffered aqueous suspensions. An important aspect of the study of washed bacterial cell suspensions is endogenous metabolism, which may be defined as the total metabolic reactions that occur in the cell when it is held in the absence of compounds or elements which may serve as specific exogenous substrates. It must also be recognized that the reactions characteristic of endogenous metabolism may continue in the presence of exogenous substrates.

Microorganisms are able to survive for considerable periods during starvation and consequently must maintain soluble constituents, often against considerable concentration gradients. The regulation of the cytoplasmic

osmotic pressure and pH value appear to be highly selective processes since quantities of some cell substances, e.g. RNA from bacteria and yeasts and also smaller moities, such as amino acids and bases of RNA, ribose and inorganic phosphate, are able to diffuse into the suspending fluid without appreciable loss of viability (21, 49, 50, 84, 180, 204).

The concept of energy of maintenance is now well established and supported by much experimental evidence (128, 132, 161). In the absence of growth, energy is required for; osmotic regulation, maintenance of intracellular pH, motility, turnover of proteins and nucleic acids, and for such specialized phenomena as sporulation, encystment and luminescence. In the absence of exogenous sources of energy these requirements must be met from endogenous sources. Dawes and Ribbons (49, 51) reviewed various aspects of the endogenous metabolism of microorganisms. A clear distinction can be made between compounds which serve as specialized reserves of carbon and energy (e.g. polysaccharides, poly-β-hydroxybutyrate, glycogen and lipids) and those that are essentially basal components of the cell (e.g. proteins and RNA), but which, under certain conditions of starvation, may be degraded to provide energy.

There is ample evidence that certain microorganisms, of which *Pseudomonas aeruginosa* is a good example (154), do not synthesize any specific reserve compound and are

therefore entirely dependent on protein and RNA degradation for their endogenous metabolism and energy of maintenance.

The fundamental properties of endogenous metabolism of bacteria were first investigated by Callow in 1924 (33). But it was not until 1936 that Barker (10) discovered the phenomenon of oxidative assimilation, where a portion of the substrate is oxidized to provide energy for the assimilation of the remainder. This phenomenon assumes considerable importance for endogenous metabolism since the assimilated material becomes a potential substrate for endogenous respiration. Oxidative assimilation was subsequently shown to be a common occurrence with numerous organisms (37, 38).

The concomitant oxidation and assimilation of external energy sources (substrates) has been extensively studied (12, 37, 38). The conclusion of these studies was that oxidation was always greater than assimilation in washed suspensions of non-proliferating cells. Seigel (192) found, in proliferating cells, that not only was assimilation greater than oxidation but, over the whole growth range, the ratio of assimilated to total removed carbon was constant. This was later confirmed by Mackechnie (127) who found the ratio to be more exacting when based on the number of electrons assimilated to the total utilized. Subsequently it was discovered that the same ratio holds for growing heterogenous populations

metabolizing complex substrates (30, 60, 103, 135, 188, 189).

Another finding, first recognized by Cook and Stephenson (46) in 1928, was that endogenous respiration may remain constant while the number of viable cells changed profoundly. The significance of this was not fully realized until Halvorson and Spiegelman (90) and Mandelstam (129) discovered that adaptive enzymes could be synthesized at the expense of endogenous amino acids and this was dependent on protein synthesis (130). In nitrogen-free medium enzyme induction (β-galactosidase) continued for 19 hours but diminished after this period. During the same period cellular ATP levels increased slightly. Storage of washed aerated suspensions at 37°C did not reduce the ability of the bacteria to form β-galactosidase in the presence of the inducer and a suitable nitrogen source such as peptone (203). Starvation did not affect the mechanism of enzyme formation, but only depleted the potential substrates.

In summary the rationale for using washed suspensions to study metabolic activity is based on the following previously tested premises; 1) oxidation of organic compounds exceeds assimilation in non-proliferating cells, 2) in growing cells of both pure and mixed cultures the ratio of the number of electrons assimilated to the total utilized is a constant over the whole growth range even with complex substrates, 3) oxidative assimilation is a wide-spread phenomena, 4) microorganisms

require an energy of maintenance which is derived from both endogenous and exogenous energy sources, 5) endogenous respiration may remain constant while viability decreases, 6) adaptive enzymes can be synthesized at the expense of amino acid pools and depends on protein turnover, and, starvation does not affect the mechanism for enzyme formation, 7) resting cell suspensions can remain viable and active for long periods of time. Krebs (38) reported a suspension of vegetative cells of *Clostridium* in acetate buffer which remained active for one month.

It is inevitable that the supply of endogenous energy reserves will become exhausted and cell deterioration will lead to death. The survival time will vary for different bacterial species and it will be affected by, the rate of basal metabolism for the organism, the type and quantity of energy reserve, and the environmental conditions to which the organism is exposed at the time of environmental stress. Table 1 is an attempt to summarize the half-life starvation time of different microorganisms but direct comparisons are difficult to make because of the different histories and experimental objectives of various investigators. The table does serve to show the wide range of survival time for different organisms. It is apparent from the table that organisms indigenous to soil and aquatic environments which are characterized by slow growth rates experience the longest survival times. This

TABLE 1

Comparison of half-life starvation times of bacteria indigenous to different habitats.

Organism	Habitat	50% Survival time (hr)	Reference
Sphaerotilus discophorus	Running water containing sewage	12	202
Streptococcus mitis	Human saliva; sputum; feces	22	218
Streptococcus lactis	Milk; dairy products	30	183
Escherichia coli	Lower intestine of warm-blooded animals	36	52
Aerobacter aerogenes	Soil; water; dairy products	45	205
Azotobacter agilis	Soil; water	50	195
Sarcina lutea	Soil; water; skin	65	28
Streptococcus faecalis	Feces of warm-blooded animals	75	81
Pseudomonas aeruginosa	Soil; water	84	39
Micrococcus halodenitrificans	Natural and artificial brines	110	193
Salmonella enteritidis	Man; animals	144	58
Nocardia corallina	Soil	480	177
Vibrio sp.	Marine	960	155
Arthrobacter sp.	Soil	1680	238

is due to the stringent control over the rate at which endogenous reserves are depleted. Thus, organisms with a high endogenous rate, such as *E. coli*, show a greater loss of viability during starvation (i.e., in resting cell suspension) than do the autocthanous organisms which may possess mechanisms for reducing the rate of endogenous metabolism under starvation conditions (63). Aquatic and marine bacteria whose natural milieu is a dilute nutrient condition (about 5-10 mg L^{-1} organic matter for marine bacteria) experience generation times as long as 200 hours (108). Intuitively one would expect the endogenous rate of such organisms to be low and their survival time lengthy.

Although washed bacterial suspensions have an early history of use, the term "resting organism" was first applied by Quastel and Whetham (169) to describe an organism which

> "...had been grown for two days in tryptic broth [Cole and Onslow, 1916], separated by centrifuging, thoroughly washed with normal saline, made up to a thick emulsion with saline, and finally very well aerated, which serves to produce a fairly homogeneous emulsion and to remove any traces of easily oxidisable material".

There is practically no formal literature dealing with the properties of resting cell suspensions. However, there seems to be an unquestioned acceptance of these preparations by workers using them as a tool to investigate intermediary metabolism. With 30 years of retrospect Quastel (170) in 1959 wrote:

> "There was much antagonism, at first, particularly among the orthodox bacteriologists, to the introduction of the term "resting cells" or even "non-proliferating cells". The criticisms were rarely expressed other than verbally, but there was an opinion that "resting cells" were "dying cells" whose reactions represented post-mortem phenomena that had little to do with the phenomena of the growing cells... the so-called biological criticisms were soon silenced by observations that the reactions of resting cells were indeed representative of normal cell life and were basal to our understanding of normal cell metabolism and growth. Nearly 30 years have now passed since the introduction of the resting cell technique. The papers being published at the present time, in the field of microbial chemistry, that utilize resting cells or washed suspensions of cells, used under conditions where there

> is but little or no proliferation, are as great as at any time in the history of this subject. The shortcomings and difficulties inherent in the technique are known, but they are surpassed by the advantages of working with a method that is showing itself most fruitful....".

The introduction of the manometric method by Warburg (223) in 1926 was essentially the beginning of the use of washed bacterial suspensions to study biochemical pathways. Washing removes residual nutrients and renders the cells non-proliferating or resting. Umbreit _et al_ (216) listed the following advantages for washed bacterial cells; 1) various bacteria present a wide variety of enzymes for study, 2) organisms can be grown readily under reproducible conditions, 3) cells give uniform suspensions that can be pipetted accurately, 4) most washed cells can be stored for some time at refrigerator temperatures without appreciable loss of activity, 5) respiration remains linear with time, 6) bacteria are extremely active per unit of material and 7) gas diffusion into the cell is not a limiting property. Stanier (199) used resting cell suspensions to formulate the principles of the 'simultaneous adaptation, or sequential induction technique' as a method to elucidate metabolic pathways. The principle has been applied widely to many problems of inter-

mediate metabolism and has been especially fruitful in the area of aromatic decomposition (16, 41, 179, 200, 210, 236). Further applications of the use of resting cells have included studies of accumulation of metabolic end products (15), and the use of mixed resting cell suspensions to study biodegradation.

The microbiology of sewage lagoons was investigated by Ishague (105) who utilized resting cell suspensions to study the physiological activity of microorganisms indigenous to a lagoon stabilizing domestic wastes. He suggested that, while the biotic types of the heterogenous population may vary, the physiological activity of the indigenous population remained reasonably constant attributable to the uniform gross chemical homogeneity of the raw sewage which generated mixed cultures of sewage lagoon bacteria; the BOD loading enriched the population for specific physiological types. He therefore concluded that lagoons offered.... "an excellent and readily attainable source of a mixed bacterial population expressing a vast physiological spectrum which can be used to measure the biodegradability potential of both simple and complex natural products or products of the the laboratory". His proposal was to prepare a resting cell suspension of lagoon organisms, challenge the suspension with an organic compound being appraised for its biodegradability potential, and measure residual substrate at time intervals. In a series of reports,

the facile application of resting cell suspensions of lagoon bacteria demonstrated the biodegradability of several natural compounds, some common organophosphorus insecticides, and a number of LAS-type and nonionic detergents under a wide variety of environmental conditions (86, 87, 88, 89).

The length of time over which the resting cell suspensions of lagoon organisms could remain physiologically active has not been established. They are not always used immediately after they are prepared; the manner of storage prior to use to obtain a minimal loss of activity has not been determined. Halvorson and Ishaque concluded that, where organic compounds were degraded slowly or where lengthy lag phases for enzyme induction were required, a danger existed whereby the resting cell suspension might deteriorate and become inactive before the complete utilization of the substrate (105), i.e., accurate initial degradation rates could not be calculated because the substrate removal rate was uncorrected for loss of cell viability. The following figures illustrate the biodegradability of Liqui-nox, a commercial biodegradable detergent, by summer populations of lagoon organisms.

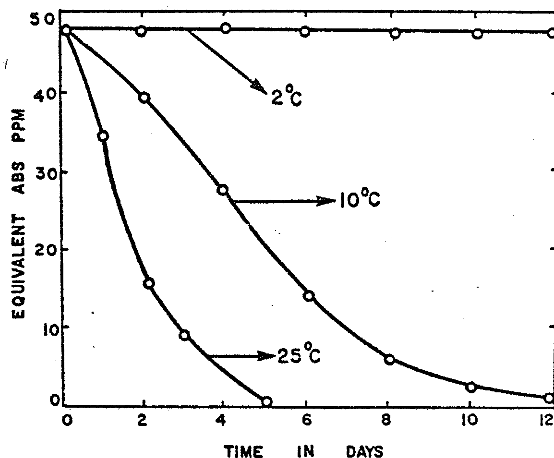

FIG. 1. Use of Liqui-nox by summer populations of lagoon bacteria as a function of temperature.

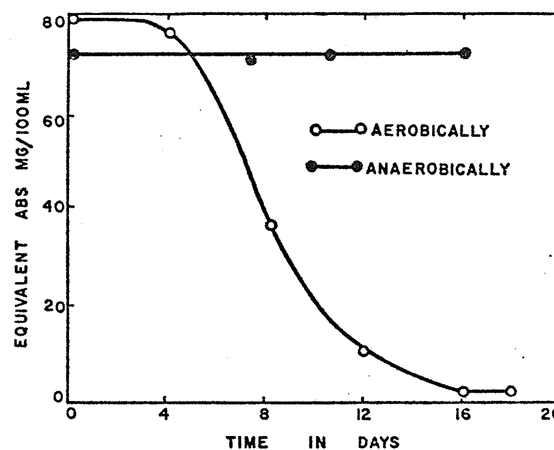

FIG. 2. Use of Liqui-nox by summer populations of lagoon bacteria as a function of aeration.

Figures 1 and 2 show the effect of temperature and aeration on the removal of the detergent respectively. (The four-day period evidenced in Figure 2 caused by the sixteen-fold increase in detergent concentration is probably attributable to impairment of the function of the cell membrane which is known to be rich in lipid and, therefore, sensitive to the presence of the detergent). Of major significance is the lengthy time period over which these experiments were carried out. Finally, rate reactions generated by cell suspensions prepared from different samples of lagoon water are not directly comparable because the suspensions will contain variations in numbers of organisms, i.e., degradation rates from different cell suspensions are uncorrected for differences in biomass. It is to these problems that Part I of this dissertation is addressed.

MATERIALS AND METHODS

MATERIALS AND METHODS

Preparation of Mixed Microbial Resting Cell Suspensions of Sewage Lagoon Bacteria

The 'Charleswood Lagoon' is a conventional dual-cell domestic wastes sewage lagoon with a design loading capacity of 80 lb Biochemical Oxygen Demand (BOD) located in a geographic region experiencing warm summers and severe winters. The bacterial population generated in the lagoon by the influent raw sewage is subject to seasonal change. A complete description of the lagoon along with geographic data and the *modus operandi* have been published (87).

The mixed microflora present in a summer sample of lagoon water was recovered by differential centrifugation. About 200 liters of primary effluent was collected from the lagoon in 18-liter carboys and the total microflora were recovered by centrifugation using a steam-driven high-speed Sharples Super centrifuge (Pennsalt Chemicals Corp., Warminster, Pa.) equipped with a standard clarifier rotor operating at 40 psi steam pressure (ca. 40,000 rpm). The pellet of cell material was suspended in 50 mM phosphate buffer, pH 7.0. Algae and heavy debris were removed from the suspension by slow speed centrifugation at 1000 x g(15 min) in a refrigerated

RC2-B Sorvall centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The supernatant containing the bacteria was decanted and retained; the pellet resuspended in fresh buffer and centrifuged once more at 1000 x g to recover any entrapped bacteria. The supernatant fractions were combined and centrifuged at 16,000 x g to recover the cells. The pellet was washed several times in phosphate buffer and resuspended in the same buffer with the final volume made up to 200 ml; 1 ml of the suspension contained the equivalent of the total bacteria in 1 liter of lagoon water, thusly. The final preparation was a glistening white suspension containing 3×10^9 cells ml^{-1} determined by means of the Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.).

Storage Properties of Resting Cell Suspensions of Sewage Lagoon Bacteria

i) Effect of storage on retention of physiological activity.

To determine conditions for the storage of resting cell suspensions (RCS) of lagoon bacteria which would minimize losses of physiological activity, cells were stored under a variety of conditions. Oxygen uptake determined by means of manometry (to be described) was used to measure the response of the cells to different storage conditions. Substrates chosen to monitor cell

stability were; glucose, acetate, lactate, succinate, cellobiose, and vitamin-free casamino acids. The cells were allowed to respire under static conditions in the cold; storage was under normal laboratory refrigeration conditions (10-12°C). Their ability to oxidize the substrates was then determined after selected time periods. The length of time over which storage was allowed to occur was sufficient to ensure severe environmental stress in order to recognize the most resistant fraction of the population.

The effect of agitation on retention of activity was also determined. The cells were agitated in the cold by means of a magnetic stirrer and were then challenged with the substrates as before.

To determine the effect of storage under extreme conditions, the two series of experiments were repeated, but this time the cells were allowed to respire at ambient temperature (23°C).

ii) 'Adjuvant' effect of substrates on physiological activity.

To determine whether adjuvant substrate protected specific physiological groups within the mixed population, substrate was provided to RCS during storage; acetate and casamino acids were chosen for this experiment. To a separate flask of stirred RCS was added, to a final concentration of 0.5 mg ml^{-1}, acetate or casamino acids, and

the cells were allowed to respire at ambient temperature for 120 hr, a period known to be just sufficient to deplete completely the previously added adjuvant substrate. The ability of the RCS to oxidize the homologous substrate was then determined manometrically at the end of the storage period. Endogenous controls were included.

Manometric Determination of Oxygen Consumption

Oxygen uptake was measured by standard manometric techniques (216) in a Bronwill Warburg instrument (Bronwill Scientific Inc., Rochester, N.Y.). Air was the gas phase, and CO_2 was adsorbed on a small fluted filter paper soaked with 0.2 ml 20% KOH and placed in the centre well. The main compartment contained 2 ml of cell suspension while the side-arm contained 0.5 ml substrate; the amount of substrate used was known to be at substrate saturation. The total fluid volume was brought to 3.2 ml with 50 mM potassium phosphate buffer, pH 7.0. Shaking rate was set at 60 oscillations min^{-1} and the temperature was regulated to $30^{o}C \pm 0.01^{o}C$. The system was equilibrated for 15 min before substrate addition. Corrections were made for endogenous respiration and the cumulative oxygen uptake that occurred over a 1 hr period was recorded.

RESULTS

RESULTS

Effect of storage conditions on retention of activity

The exogenous oxygen uptake by RCS of the lagoon bacteria after the various storage conditions is summarized in Tables 2 and 3. The data are expressed both as the quantity of exogenous O_2 consumed (in μl) over 1 hr, and as a percentage of the initial oxygen uptake rate. It should be made abundantly clear that the values are directly comparable since the same preparation was used in all of these experiments, i.e., all biomass variation has been removed.

The most stable condition was to store the cells in a static condition in the cold. After seven days as shown in Table 2, greater than 75% of the initial activity was retained for every substrate. After 15 days storage under static conditions in the cold, a minimum of about 60% of the activity remained, with the exception of glucose where, remarkably, the preparation failed to show any loss of activity. The physiological group of organisms metabolizing glucose appear to be extremely stable. Agitation, however, caused the cells to lose their activity rapidly. After six days of stirring in the cold, only the glucose- and cellobiose-metabolizing group of organisms retained high activity; all of the other groups showed substantial deterioration. After

TABLE 2.

Exogenous oxygen uptake by resting cell suspensions of sewage lagoon bacteria after various time and agitation treatments when respired in the cold.

Treatment	Storage time, days	Substrate					
		Casamino-acids	Acetate	Lactate	Succinate	Cello-biose	Glucose
initial	0	100* (94)**	100 (110)	100 (178)	100 (38)	100 (21)	100 (73)
static	7	86.2 (81)	81.8 (90)	75.8 (135)	134 (51)	119 (25)	108 (79)
	15	60.6 (57)	57.2 (63)	ND***	68.4 (26)	ND	105 (77)
stirred	6	31.9 (30)	13.6 (15)	16.3 (29)	18.4 (7)	61.9 (13)	118 (86)
	14	0 (0)	5.5 (6)	11.3 (20)	ND	38.9 (8)	118 (86)

*Expressed as % initial activity

**Exogenous oxygen consumption (μl)

***Not determined

14 days, only the glucose-metabolizers remain metabolically active.

When the cells were stored at ambient temperature, as shown in Table 3, the same trend was observed; the stirred cells deteriorated more rapidly than did those that had been held static and much more rapidly than in the cold (compare values with Table 2). The data also show, once again, that the glucose-metabolizing fraction of the population is the most stable one.

The 'adjuvant' effect

The exogenous respiration by preparations stored at ambient room temperature in the presence of the homologous substrate is shown in Table 4. The homologous substrate is very effective in prolonging activity even under stressed conditions. At the end of the storage period, more than two-thirds of the activity had been retained, whereas without the adjuvant, the remaining activity at best was 10% of the initial value.

TABLE 3.

Exogenous oxygen uptake by resting cell suspensions of sewage lagoon bacteria when stored under extreme conditions at ambient temperature (23°C).

Treatment	Storage time, days	Substrate					
		Casamino-acids	Acetate	Lactate	Succinate	Cello-biase	Glucose
initial	0	100* (108)**	100 (156)	100 (260)	100 (86)	100 (56)	100 (108)
static	13	16.7 (18)	0 (0)	42.6 (120)	46.5 (40)	0 (0)	55.6 (60)
stirred	9	16.7 (18)	6.4 (10)	3.8 (10)	0 (0)	0 (0)	0 (0)

*Expressed as % initial activity

**Exogenous oxygen consumption (μl)

TABLE 4.

Effect of adjuvant substrate on retention of initial respiration of sewage lagoon bacteria after storage for 120 hr. Cells were stirred at ambient temperature.

	Substrate	
Storage conditions	Casamino acids	Acetate
-	100+ (141)*	100 (179)
Adjuvant present	66 (93)	79 (141)
Adjuvant absent	10 (14)	6 (10)

*Exogenous oxygen uptake (μl)

+% Initial activity

D I S C U S S I O N

DISCUSSION

Six substrates were selected for study to reflect the different but recognizable physiological groups of organisms indigenous to sewage lagoons; lactate and acetate are common end-products of anaerobic metabolism; glucose and cellobiose are products of cellulose decomposition which is present in the lagoon in abundant supply; succinate is a common intermediate of aerobic metabolism available because of transport difficulties to only a fraction of the population; casamino acids represent the protein component of the BOD of raw sewage and also provide a diverse multicomponent substrate.

The experimental plan was to determine the effect of prolonged storage on the physiological activity of resting cell suspensions. Two approaches were possible. First, an exhaustive but systematic study could be made on the manner in which each parameter (temperature; agitation; time) affected cells under storage; this would have required obtaining lagoon water samples at different times to prepare the suspensions. Because there was no procedure to correct for differences in bacterial numbers in the different preparations, the oxidation rates for different substrates would not be directly comparable. Secondly, a more limited number of tests could be performed on cells obtained from the same manageable volume of lagoon water. This would only

detect relevant trends in response to the different treatments, but would avoid biomass variations. The latter approach was chosen.

Agitation was shown to increase the rate of decay of RCS, probably by aiding the depletion of energy reserves of the cell. Stokes and Parson (202) found a similar result; suspensions of poly-β-hydroxybutyrate-rich pure cultures of Sphaerotilus discophorus were more resistant to death when held stationary in comparison to those that were shaken.

Complex changes occur when resting cell suspensions are stored for long periods. In part, the varying responses of the stored mixed cultures may be explained in terms of lysis of some cells with subsequent regrowth (cryptic growth). However, there was little pattern to the events which ensued and only a decrease in total activity could be predicted with certainty.

It should be noted that the adjuvant effect of storage in the presence of substrate is an encouraging factor in favour of the use of resting cell suspensions to investigate organic decomposition. However this finding is contrary to the effect, described by Strange et al (204), where viability of a suspension of Aerobacter aerogenes was decreased in the presence of glucose. Notwithstanding this loss of viability, 60% of the added glucose was metabolized. Postgate and Hunter (166) also reported substrate-accelerated death.

However, there are numerous reports indicating that washed bacterial suspensions are able to effectively metabolize exogenously supplied substrate as well as endogenously derived materials (12, 28, 51, 87, 146). Strange (203) demonstrated that starved cells could synthesize β-galactosidase when supplied with inducer. More recently Calcott and Postgate (31, 32) have determined that "substrate (lactose)-accelerated death" is not a true loss of cell viability but simply non-division on the recovery medium. They found suppression of growth could be alleviated by incorporating 3'-5'-cyclic adenosine monophosphate (cAMP) into the medium and normal growth was restored.

It is possible to correct for loss of activity by generating an 'initial activity constant' k_A:

$$k_A = \frac{R_i}{R_t}$$

where R_i is the initial rate of reaction and R_t is the rate of reaction at any specific time.

Any value >1 would indicate cell decay. From Table 4, the k_A value for the physiological groups metabolizing casamino acids and acetate is 1.52 and 1.27 after 120 hr respectively. This shows that the physiological groups are decaying at different rates, even when protected with adjuvant. From Table 3, the k_A for casamino acid breakdown by cells stored under static conditions works out to 1.16 and 1.65 for storage times of 7 and 15 days respectively. This shows that any defined single physiological

group of cells in the mixed population is not decaying at a constant rate and no single activity constant is suitable to correct for cell deterioration.

The use of mixed resting cell suspensions to evaluate substrate removal in a laboratory procedure may not be directly comparable to a laboratory procedure. Predation can enhance the physiological state of a bacterial population and, therefore, its ability to effectively utilize substrate (109, 207). This factor is necessarily eliminated when resting cell suspensions are used. The laboratory use of mixed resting cell suspensions is confined mainly to their usefulness to predict potential biodegradability. For comparative purposes this must have a quantitative basis so it is significant that the system should be closely regulated and not subject to unpredictable changes.

Concerning the Question of the Standardization of Active Biomass

The bacterial population in the Charleswood lagoon fluctuates because of variations in lagoon BOD loading and the population is markedly affected both qualitatively and quantitatively by climate. For comparative purposes it is essential to define an activity standard by which to evaluate the relative changes in resting cell suspensions

prepared from different samples of lagoon water. To exploit mixed microbial populations as means of measuring biodegradability potential an activity standard based upon 'active biomass', i.e., total number of viable cells, is essential. Halvorson et al (87) were able to quantitatively describe some stabilization processes as a function of seasonal change by measuring bacterial numbers in lagoon water with the Coulter Counter. Some of their data is presented in the following figure.*

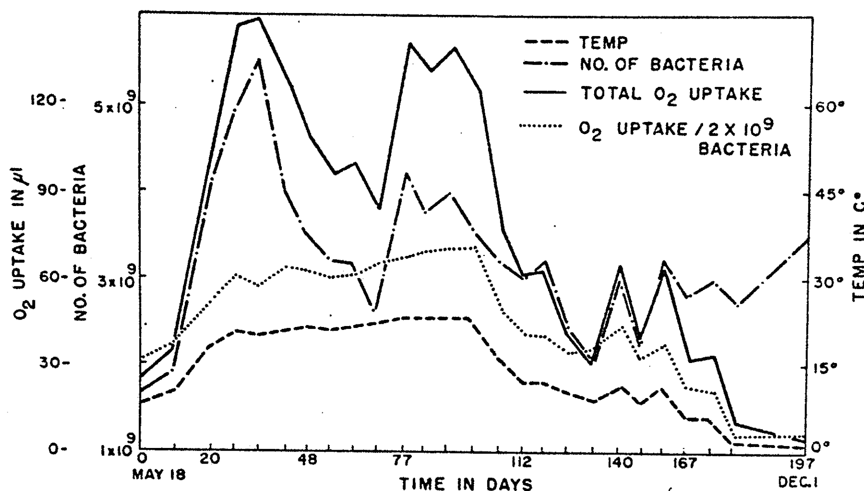

FIG. 3*b*. Oxidation of acetate by lagoon bacteria as a function of seasonal change. Lagoon samples were taken roughly at weekly intervals. Each Warburg vessel contained: 0.2 ml of 20% KOH in the center well and 0.4 mg of substrate, 0.05 *M* phosphate buffer, pH 7.0, to a final volume of 3.2 ml and the entire bacterial population recovered from a 2-liter sample of lagoon water in the main compartment. Oxidation was determined at a temperature identical with that of the lagoon at the time of sampling. The results are expressed as (*a*) — total oxygen uptake in 2 hours by the recovered bacteria and (*b*) · · · oxygen uptake in 2 hours by 2 × 10^9 cells.

It shows that the lagoon population fluctuates extensively over the summer. But when the counts are normalized with the Coulter Counter, the rate of acetate oxidation is clearly temperature-dependent. The Coulter Counter is an

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*From Halvorson et al (87).

electronic particle-counter and cannot distinguish between viable and non-viable cells nor can it distinguish between bacteria and clay particles; problems not experienced by Halvorson et al (87). It is apparent that the Coulter Counter is not a suitable instrument to use to define an activity standard.

Active biomass is an extremely difficult parameter to determine. Numerous techniques have been proposed but no single technique provides a satisfactory estimate of its magnitude. An apparently direct method involves the determination of the number of active cells per unit volume by the standard plate count (198). With this method the only data obtained are for those cells capable of forming colonies on agar media under the given conditions. Monod (147) pointed out that many organisms are extremely sensitive to sudden change in environment and often perish during and after transfer to a new environment. The accuracy of the data depends on the assumption that one viable cell forms one colony which is true only when the cells do not clump, and the cell suspensions used for plating are sufficiently dilute. The procedure is tedious, long incubation times may be required, and regardless of the choice of plating parameters, only a fraction of the viable population can ever be estimated in the case of heterogeneous populations.

Cellular components which have been used to measure active biomass fall into two groups: storage

materials (polysaccharides; poly-β-hydroxybutyrate; glycogen) and basal materials (nucleic acids; proteins). The cellular contents of the storage materials and nucleic acids vary widely with change in the growth rate and physico-chemical factors of the environment (53, 96). Herbert (96) suggested that of all of the cell constituents protein content per cell varies least from cell to cell or from one growth rate to another. Monod (147) considered organic nitrogen to be a satisfactory indicator of protein content and viable biomass. The measurement of chemical constituents specific to the procaryotes such as 2,6-diaminopimelic acid and muramic acid (111) and lipopolysaccharide (221) has been used to determine biomass in complex microbial populations. However, variations in the content of these components in individual bacterial types and cumbersome assay techniques mitigate against their use to standardize mixed microbial population activity. Moreover, none of the methods involving quantitation of cellular components distinguished between viable and nonviable cells and are applicable only in situations where dead cells comprise a negligible fraction of the total population.

There are a number of reports indicating that cellular enzymic content bears a linear relationship to active biomass. Such enzymes as the "glucose enzymes" (197), nitratase (163), tetrathionase (162), amino acid decarboxylase (72), and galactozymase (196) increase in

activity with biomass yielding an S-shaped curve similar to the growth curve. Postgate and Hunter (165) discovered that glycerol dehydrogenase activity declined linearly with viability in a starved bacterial culture. They concluded that this parallelism could imply that the relevant enzymic activities remained unchanged until the organism died at which time they became negligibly small. Hershey and Bronfenbrennar (98) observed that enzymic activity of bacterial protoplasm per unit of dry weight was the same during the phase of adjustment and exponential growth. Cohn (42) showed that the differential rate of synthesis of β-galactosidase remained constant during the growth cycle. Kotze (114) has discussed in depth the methods of determination of dehydrogenases and various other enzymes in which the light-absorbing properties of the pyridine system are utilized. Decolorization of methylene blue has been used to assay dehydrogenase activity in a simple colorimetric method (53). The reduction of the colorless 2,3,5-triphenyltetrazolium chloride (TTC) to the red tetraphenylformozan through the catalytic action of the dehydrogenases was recognized by Mattson _et al_ (133). Lenhard (121) adopted dehydrogenase activity, assayed by TTC reduction, as a measure of "general biological activity". Ghosh (78) outlined the thermodynamic and biochemical bases of the dehydrogenase test and found that TTC activity was highly correlated to biomass as measured by dry weight. How-

ever, assuming that a single enzyme or class of enzymes can be considered as being 'universal', it is readily conceivable that active biomass can be correlated with enzyme content only where viability is required to maintain the enzyme function. Klapeijk et al (112) found that toxic substances such as cyanide and zinc could reduce the oxygen uptake of activated sludge without affecting the dehydrogenase activity as measured by a TTC-test.

The pattern of respiration is a general phenomenon recognizable in fungi (19), algae (115), yeasts (59), and bacteria (33, 46, 49, 50, 51, 124, 128, 132). The endogenous rate of a biological culture under conditions of non-proliferation has been used as a measure of active biomass (60). The technique appears to have a questionable basis because energy reserves responsible for endogenous respiration are depleted over time without necessarily concomitant loss of viability.

Bashucky (11) used oxygen uptake to standardize active biomass of Charleswood lagoon organisms to study the rate of breakdown of organic compounds under different environmental conditions. An example of his data is presented in Table 5 which generates rate constants for substrate removal corrected for fluctuations in biomass. The trials represent cell suspensions prepared from lagoon water taken at different times during one winter season. A good correlation was found between substrate utilization and active biomass (determined as exogenous

TABLE 5.

Rates of substrate removal by psychrotrophic lagoon bacteria corrected for fluctuations in biomass.

Substrate	Trial	A Substrate removal rate at 2°C ($mg\ hr^{-1}$)	B Manometric exogenous oxygen uptake at 15°C ($\mu l\ O_2\ hr^{-1}\ ml^{-1}$)*	A/B Rate constant ($\mu g\ hr^{-1}/A_{ca}$)**	Average rate constant ±standard deviation
ACETATE	1	2.56	50	51	
	2	17.00	172	99	72 ± 20.3
	3	11.10	114	97	
	4	2.43	46	53	
	5	6.58	108	61	
PROPIONATE	1	1.96	50	39	
	2	6.25	172	36	
	3	5.0	114	43	41 ± 2.7
	4	1.90	46	41	
	5	6.67	150	44	
	6	1.65	40	42	
BUTYRATE	1	1.43	50	29	
	2	8.33	172	48	38 ± 11.0
	3	5.26	114	46	
	4	1.25	46	27	

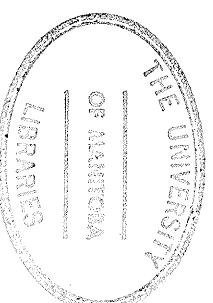
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Table 5. Continued

Substrate	Trial	A Substrate removal rate at 2°C (mg hr^{-1})	B Manometric exogenous oxygen uptake at 15°C (µl O_2 hr^{-1} ml^{-1})*	A/B Rate constant (µg hr^{-1}/A_{ca})**	Average rate constant ±standard deviation
ETHANOL	1	0.91	50	18	
	2	4.00	172	23	
	3	2.78	114	24	23 ± 3.1
	4	1.16	46	25	
	5	2.90	108	26	
BENZOATE	1	0.69	50	14	
	2	2.94	172	17	16 ± 1.7
	3	2.00	114	18	
	4	1.92	118	16	
PHENOL	1	0.05	50	1.0	
	2	0.26	172	1.5	
	3	0.18	114	1.6	1.45 ± 0.31
	4	0.36	215	1.7	

*Oxygen uptake for 1.0 ml of a 1000 x concentration cell suspension of sewage lagoon bacteria metabolizing 0.8 ml of a 0.75% vitamin-free casamino acids solution.

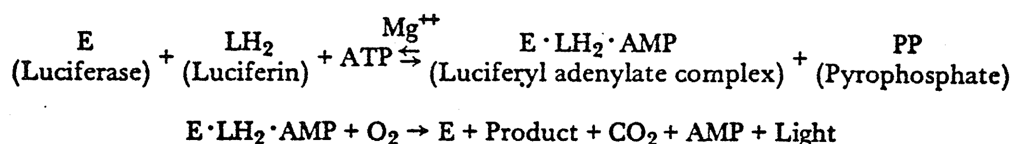
**A_{ca} is defined as an exogenous oxygen uptake of 1.0 µl O_2 hr^{-1} ml^{-1} of cell suspension.

! From Bashucky, (11).

oxygen uptake in the presence of the multiple substrate casamino acids). The standard deviation of the average rate constant is respectable considering the substrate removal rate is uncorrected for loss of activity of cells over time, and this would not be expected to be identical in different trials. The success of the method was attributed to the use of a multicomponent substrate which is better able to encompass the physiological activity spectrum of the composite microbial population. Blok (17) has described the respirometric action of a mixed population on a mixed substrate as unique and one that cannot be described by parameters from mono cultures or single substrates. The monitoring of a common component (oxygen uptake) of microbial catabolism in response to a multiple stimulus (mixed substrate) appears to have meritorious advantage over the estimation of any one single measurement parameter for determining the physiological activity of a mixed microbial population.

The cellular adenosine-5'-triphosphate (ATP) content of intact cells has been a widely used modern method for estimating viable biomass. The method is based on quantitation of light energy generated from the luciferin-luciferase reaction which is ATP-dependent; ATP being extracted from and only associated with viable cells. McElroy (136) first recognized the involvement of ATP in bioluminescence in the firefly. He proposed the following equation to describe the relationship of

ATP to the other components of the firefly reaction.



This reaction yields one quantum of light at its pH optimum of 7.2 - 7.4 (176) and is inactivated by acid pH and by temperatures above 35°C (176). Light emission is also modified by ionic strength of the medium (4).

The firefly luciferin-luciferase reaction, which has been thoroughly investigated and described by McElroy and Strehler (138), was adapted to the quantitative assay of adenosine triphosphate (ATP) as an indicator of biomass in seawater samples by Holm-Hansen and Booth (100). This work was further extended by Hamilton and Holm-Hansen (91) who reported an average content of 1.5×10^{-9} μg ATP/cell for pelagic bacteria. D'Eustachio and Johnson (54) further reported on the constancy of the amount of ATP per unit biomass in microorganisms. These and other findings have led to the development of a luminescence photometer for microbial detection and for measurement of active biomass. Particularly active in this field were the Dupont group (7) who have actively promoted the measurement of ATP as an indicator of biomass.

Numerous reports are available describing various methods for estimation of ATP by the firefly luminescence reaction. Many of these methods incorporate the use of a liquid scintillation counter (1, 4, 43, 66, 101, 126, 187, 201). However, notwithstanding the favourable reception accorded the method by many investigators, there are some situations which still require clarification. The reliability and usefulness of this method for the estimation of biomass depend on the following assumptions:

1) ATP is a constituent of all living cells. There is no evidence in the literature contrary to this assumption, but Strange, Wade and Dark (205) found no direct relationship to exist between ATP concentration and viability, or survival prospects, of a bacterial population in a buffered suspension. They did find that the main factor affecting the ATP pool in resting cells of *Aerobacter aerogenes* was the oxygen tension of the suspending medium. Cole, Wimpenny and Hughes (43) found rapid reduction in the measured ATP pool of *Escherichia coli* during handling of the culture. Holms, Hamilton and Robertson reported that the ATP pool of *E. coli* is characteristic of the energy source (101). Forrest (66) found wide deviations in the relation between concentration of organisms and ATP pool size in *Streptococcus faecalis*. It seems that the behaviour of the ATP pool may be more complex then would be suggested by a simple steady-state balance between well-regulated rates of

production and utilization for synthetic reactions as formerly proposed by Krebs (117).

2) Non-living material will not contain any ATP. The validity of this assumption is normally tested by inactivating the microorganisms by heat, repeated freezing or metabolic poisons. Negligible ATP is detected in the remaining particulate matter. However one point which is frequently neglected is the contribution to light production by other nucleoside triphosphates. Holm-Hansen and Booth (100) found that both CTP and ITP stimulated light production to the same extent as an equivalent weight of ATP. They estimated the contribution to light production of nucleoside triphosphates other than ATP in intact cells to be in the range 5%-35% of the total.

Other workers have attempted to apply the determination of ATP content to estimate active biomass of mixed populations. Paterson, Brezonik and Putnam (159) devised a rapid and sensitive technique for determining ATP levels in activated sludge. They reported a relatively constant ATP pool under endogenous conditions and the pool responded rapidly to changes in the metabolic activity of the activated sludge. No general relationship was found between viable biomass concentration and the sludge dry weight. However Blok (18) was more critical of this application of the ATP measurement and stated that the technique cannot be used for plant

design and plant control. Also he further states that it is not clear what is the correlation between specific biodegradation rate and the ATP concentration. Forsberg and Lam (67) have cautioned against the use of ATP to measure microbial biomass in rumen contents because of the variations in efficiency of extraction of ATP from rumen contents and differences in the concentration of ATP in rumen microbes. ATP content of nine rumen bacterial isolates varied from 1.1 μg/mg dry weight to 17.6 μg/mg dry weight. They were unable to determine whether ATP reflects the overall metabolic activity of the rumen microbiota. King and White (111) stated that rapid changes in a constant number of cells are readily induced by changes in the physiological state of the organisms. Although ATP is found only in viable cells, as a single measurement parameter, ATP content is difficult to apply to the measurement of active biomass of a mixed bacterial microflora due to fluctuations.

Other problems associated with the method are largely of a technical nature. For example, loss of ATP during the extraction procedure has been overcome by improved killing methods, and extraction solvents and procedures have been improved. A unified method, however, has not been accepted.

The above discussion was not intended to mitigate the usefulness of the ATP assay but to point out some of

its short comings and that the technique must be carefully applied. Now that commercial photometers are available and with the advent of computer-assisted analysis of the adenosine triphosphate data (64) the assay of ATP will find more applications.

At the start of this study it was proposed to use ATP content as an indicator to standardize active biomass. Much time and effort was expended to develop a method which adopted the Packard-Tricarb Spectrometer Model 3325/AES (Packard Instrument Co. Inc., Downersville, Ill.) liquid scintillation counter. The physical design of the instrument made the determination somewhat cumbersome, and meticulous care had to be taken during the assay procedure to obtain reproducibility. It was found that the response of crude firefly extract to pure ATP was consistent but tremendous variations were evident when the technique was applied to extracted ATP from replicate samples of lagoon cell suspensions. With mixed cultures no correlation could be found between the amount of cell material and the ATP content of the preparation.

Since it is known that the Coulter Counter method and the oxygen uptake method have been successfully applied to lagoon water samples to correct for differences in bacterial numbers and since we have recognized that it is not advisable to perform experiments over a time period that would permit cell death to occur because

different physiological groups lose their activity at different rates, there was no longer any great urgency to be able to distinguish between viable and nonviable cells. Accordingly, the ATP method was abandoned. It may be of interest to point out that after 100 years of intensive study there is still no acceptable method to determine with accuracy the total number of viable bacteria in soil.

SUMMARY

1. The overall activity of a mixed microbial resting cell suspension of sewage lagoon organisms is best preserved by storage at refrigeration temperatures without agitation. The cumulative effects of increased agitation and increased temperature result in increased rates of cell decay.
2. The diverse physiological types in the suspension react independently, in an unpredictable manner, to stress conditions.
3. The presence of exogenous substrate in a resting cell suspension acts as an adjuvant to prolong physiological activity.
4. Correction factors for cell decay during substrate utilization, become very large when suspensions are used over long time periods, and accuracy suffers accordingly.

5. No single measurement parameter was found to provide a satisfactory evaluation of active biomass of a mixed population. Exogenous multisubstrate respiration was found to best correlate physiological activity to substrate removal.

6. It is recommended that subsequent measurement techniques should implement the initial rate of reaction to avoid the difficulties associated with cell decay.

PART II ALTERNATE SOURCES OF MIXED MICROBIAL POPULATIONS

PART IIA The Model Lagoon as a Source of Mixed Cultures

I N T R O D U C T I O N

INTRODUCTION

The Charleswood Sewage Lagoon is located at approximately 50°N latitude in a geographic region with warm summers and severe winters. The regional climate is described as 'mid-cont ntal'. The average temperature ranges from -17.7^{o}C in January the coldest month, to 20.0^{o}C in July the warmest month (105). The temperature of the lagoon remains below 10°C for approximately two-thirds of the year and is ice-bound for about five months out of each one year period.

The climatic constraints were a serious problem in using the lagoon as a reliable source of mixed cultures with constant physiological properties. In the summertime bright sunny days and appropriate nutrient or feeding conditions caused frequent algal and *Daphnia* blooms. During these times, physiological activity of the resting cell suspension was found to be impaired. The winter ice-cover created anoxic conditions very quickly and completely excluded the aerobic microflora from developing during this portion of the climatic cycle. Collecting samples during the inclement winter weather presented a special challenge. Transition periods during the change-over from summer to winter made lagoon water unsuitable as a source of organisms for several months during the year.

Seasonal changes were responsible, in part, for population changes resulting in a selection of different physiological groups with undetermined characteristics. This was a major concern. It was assumed that summer and winter populations once generated under the stable seasonal conditions were remaining reasonably constant, and though the microbial types would vary, their physiological properties would not vary appreciably. Bashucky (11), however, showed that LAS-type detergents were utilized by summer populations but not by winter populations indicating there were recognizable population differencesin the summer and winter preparations.

Furthermore, the Charleswood Lagoon was being used as an experimental site by the City of Winnipeg Water, Wastes and Disposal Division, where the feasibility of using lagoons in a severe winter climate was being appraised. At times during the study, many unexpected engineering operational changes, unbeknownst to us, were a constant source of annoyance.

To avoid the problems of seasonal change and an unpredictable or unreliable source of mixed cultures, we attempted to find an alternate source of mixed microbial populations. Part II deals with this search.

H I S T O R I C A L

HISTORICAL

Rudd (181) constructed a model lagoon and evaluated its operational characteristics. Based on tests for biochemical oxygen demand (BOD), chemical oxygen demand (COD) and suspended solids (SS) removal, the model lagoon was judged to be a successful secondary treatment system which surpassed the efficiency of the Charleswood lagoon.

Rudd expressed the opinion that such a model system could be a valuable asset in determining the biodegradability of the many new synthetic compounds which were being introduced daily into the environment. Also, he states, the model lagoon could offer the opportunity to intensively study the ecology of microbial processes in sewage treatment and provide information to better integrate design and function of waste stabilization processes.

Rudd and Hamilton (182) studied the effect of temperature on the biodegradation of trisodium nitrilotriacetate (Na_3NTA) in the model lagoon. They were able to conclude that, since the model lagoon had been shown to successfully mimick an actual operating lagoon, NTA loading would be unlikely to affect the normal operation and efficiency of a lagoon sewage treatment system. They further showed that the NTA

removal rate would be expected to be severely limited under actual lagoon conditions due to climatic constraints of the geographic region.

The model lagoon was viewed as a potential source of a mixed population.

MATERIALS AND METHODS

MATERIALS AND METHODS

Description of the Model Lagoon

The dimensional and operational characteristics of the model lagoon have been presented by Rudd (181). Briefly, the system was a dual cell arrangement, the cells being in series. Each cell was constructed of 0.95 cm-thick plate glass glued together with Silicone Seal (Canadian General Electric). Internal dimensions of each cell were 115.6 cm x 76.2 cm x 45.7 cm which gave a working volume of 270 liters per cell when in operation. Each cell was surrounded by an outer jacket of plate glass. Insulation was provided by 1.25 cm of Zonalite insulation (Grace Construction Materials, Winnipeg, Manitoba).

Aeration was provided by an air line regulated at 21 Kp. Air was introduced to a series of 15 needle valves located along a distribution arm suspended above each cell. Each valve supplied air to a 0.08 cm I.D. tygon tube delivery line the opening of which terminated along the mid-line at the bottom of the tank. Air flow was adequate to maintain about 2 mg dissolved oxygen at 25°C.

Temperature control was provided by circulating water through 0.32 cm I.D. glass coils placed along the inside walls of the tank at levels of 2.5 cm, 10 cm, 17.5 cm and 25 cm below the surface of the fluid.

Temperature-regulated water was supplied by the water bath of a Precision Refrigerated Warburg apparatus (Precision Scientific Co., Chicago, Illinois); the water being pumped through the glass coil by means of a small pump.

Raw Sewage Feed

Raw sewage was obtained from the Charleswood Lagoon. The raw sewage was collected in 20 liter plastic containers and stored at -20°C until needed. Every second day, 40 liters of frozen sewage was quick-thawed and added to a reservoir which was maintained at 4°C. The cooled raw sewage was used as the feed source for the model lagoon without further processing.

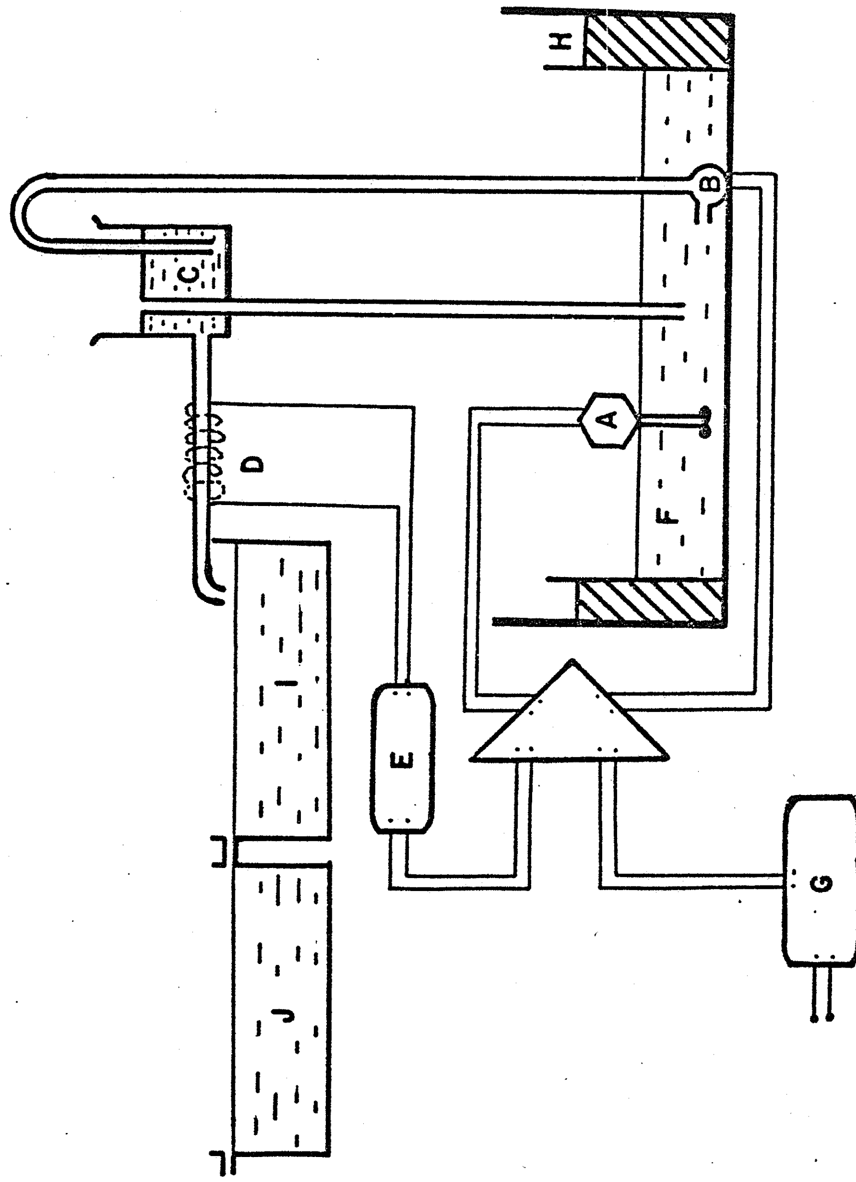
An alternate source of raw sewage was the Canadian Forces Base (North Site), Winnipeg, Manitoba, which operates its own trickling filter plant. The raw sewage was similar to that of the Charleswood Lagoon in that both systems handle domestic wastes exclusively. The raw sewage from the Canadian Forces Base was handled in the same manner as described for the Charleswood Lagoon.

Electronic Control of the Automatic Feed Supply

To simulate the actual operation of the Charleswood lagoon, raw sewage was supplied to the primary cell in a pulsed manner. The feeding schedule was electronically controlled to provide a constant volume of well mixed sewage at prescribed times. The feeding assembly is schematically depicted in Diagram 1. The timing device was set to begin delivery by activating the electric stirrer (A) to vigorously mix the raw sewage in the reservoir (F). Following 2 minutes of mixing the immersion pump (B) was automatically started by means of a delay-relay unit (E) and raw sewage was circulated through the constant-head device (C) with the overflow being returned to the reservoir. Thirty seconds later, solenoid (D) (controlled by the delay-relay unit), opened for a sufficient period of time to deliver 365 ml of mixed sewage from the constant-head device to the primary cell (I). The solenoid, stirrer, and immersion pump then switched off, and sewage remaining in the constant-head device syphoned back into the reservoir. This process was repeated every 30 minutes. The pulsed-feeding program gave a theoretical retention time of 30 days.

DIAGRAM 1. Model lagoon setup with pulse-feeding system

A - Electric stirrer
B - Immersion pump
C - Constant-head device
D - Solenoid switch
E - Delay-relay unit
F - Reservoir
G - Electronic timer
H - Coolant
I - Primary cell
J - Secondary cell


H
B
C
A
D
F
I
E
J
G

Performance of the Model Lagoon

The model lagoon performance was monitored by measuring biochemical oxygen demand (BOD) (corrected for nitrification) of raw sewage, primary, and secondary cell samples according to the procedures prescribed in Standard Methods (198). Total bacterial numbers were determined by the spread plate method, where 0.2 ml aliquots of decimal dilutions were surface plated onto Trypticase Soy Agar (BBL) plates. Replicate plates were incubated for 4 days at 20°C. Temperature, pH, and dissolved oxygen (D.O.) were also monitored.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Rudd (181) found that the model lagoon successfully mimicked the Charleswood Lagoon in operating characteristics; efficient BOD removal being achieved. But Rudd experienced technical difficulties in its operation that were not resolved. To simulate the retention time of the Charleswood Lagoon while working within manageable volumes, he chose to continuously pump raw sewage into the model lagoon at slow rates through small-bore tubing. Sedimenting suspended solids caused frequent blockage of the delivery tubes which disrupted the operation of the model lagoon. Our modification, i.e., pulsed-feed addition of raw sewage into the primary cell, has been described. It also served to more closely simulate the actual operation of the Charleswood Lagoon which is fed on an intermitent basis.

The model lagoon was operated for a total of 120 days. The first 21 days employed the Charleswood site as the source of raw sewage. For the remainder of the experimental period the Armed Forces Base site was used as the raw sewage source. The model lagoon was found to effectively remove BOD. In the primary cell there was an average removal of 93% BOD and there was a further 4% removal by the secondary cell (Table 6).

TABLE 6.

B.O.D. of raw sewage, primary, and secondary cell samples and % removal by the model lagoon.

B.O.D.* mg/ℓ			% Removal	
Raw	Primary	Secondary	Primary	Secondary
360	25	10	93.1	97.2
360	26	9	92.8	97.5
330	19	10	94.2	97.0
355	18	8	94.9	97.7
335	23	9	93.1	97.3
330	20	10	93.9	97.0
345* ± 14.8	21.8 ± 3.3	9.3 ± 0.8	93.7 ± 0.8	97.3 ± 0.3

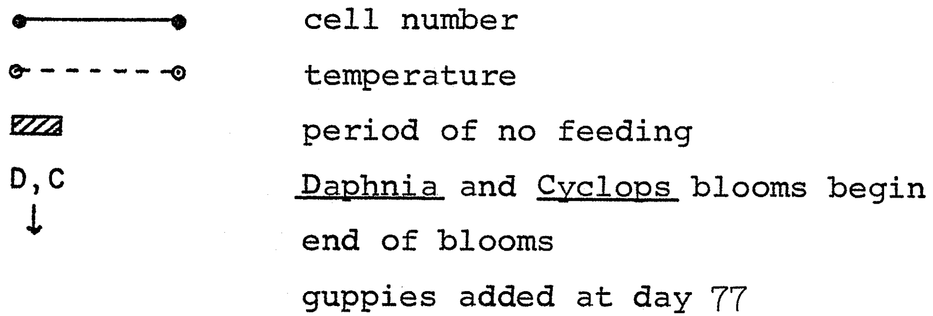
*Determined, prior to freezing, each time a new batch of raw sewage was obtained from source.

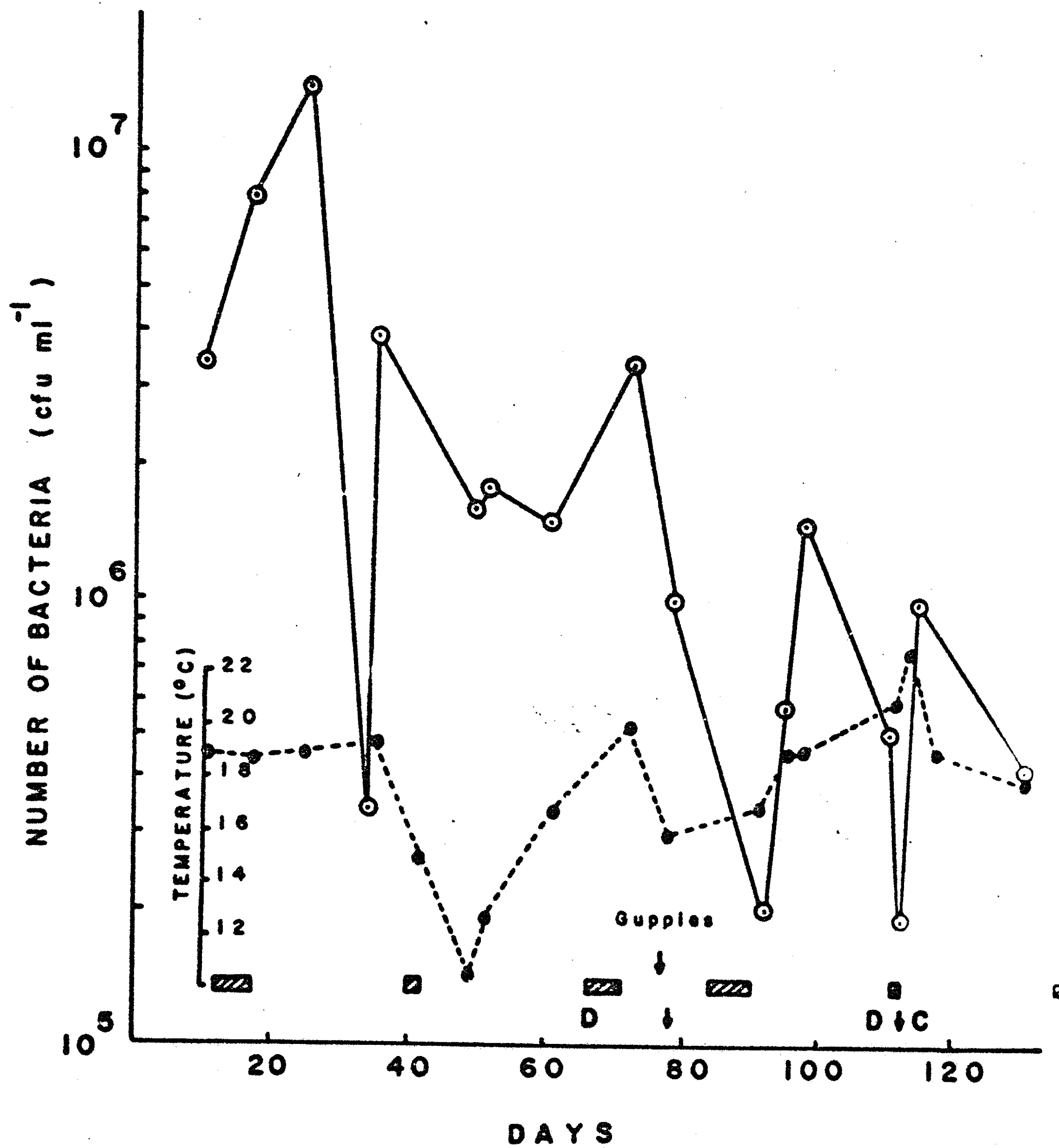
**Average ± standard deviation.

BOD, however, is a crude parameter and is not necessarily a reflection of physiological activity. BOD can be removed by both biological uptake of organic compounds with subsequent sedimentation of the microflora; or it can be removed by physical removal of sedimentable organic particulates without bacterial physiological intervention. The extent to which either of these mechanisms was operating in the model lagoon was not determined either by Rudd or in this study. That substantial amounts of sediment had collected in the bottom of the tanks was obvious, and it would also be likely that the benthic population played a substantial role in BOD removal, especially after the accumulated sediment became appreciable.

The most serious drawback to the operation of the model lagoon was the frequent occurrence of zooplankton of *Daphnia* and *Cyclops* which caused major reductions in bacterial numbers. The periods of bloom occurring in the primary tank are shown in Figure 1 . Good BOD removal continued during periods of low bacterial cell count when a predator-prey relationship was involved. Rudd and Hamilton (182) found that the rates of NTA breakdown in the model lagoon remained high during periods of *Daphnia* grazing. Javornicky and Prokesova (109), and Straskrabova and Legner (207) have also reported that predation can enhance the physiological state of a bacterial population and, therefore, its ability to effectively utilize substrate. The addition of guppies

FIGURE 1. Variation of total bacterial cell numbers in the model lagoon.




NUMBER OF BACTERIA (cfu ml⁻¹)
10⁷
10⁶
10⁵
TEMPERATURE (°C)
22
20
18
16
14
12
Guppies
D
D
C
20
40
60
80
100
120
DAYS

(a zooplankton predator) to the tank did not prevent further blooms and no effective solution to the problem was found.

A number of mechanical failures occurred over the 120 day period. These are indicated as periods of 'no-feed' on the data sheet of Figure 1 . A drastic decline in bacterial numbers tracked the 'no-feed' periods which were of durations as long as six days.

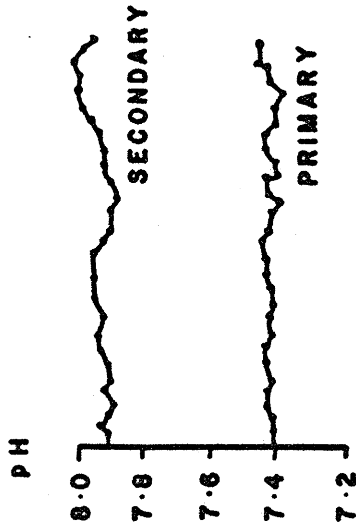
Temperature variations occurred in spite of the attempt to control it. (Figure 2) The model lagoon was in operation during the winter months. It was located in a room-site below large windows which were poorly sealed. The method of temperature regulation was not adequate to cope with the cold draughts entering the room through the windows. Dissolved oxygen was a mirror image of the temperature data, but no other correlations were apparent. In particular, total bacterial cell number bore no relation to temperature fluctuations.

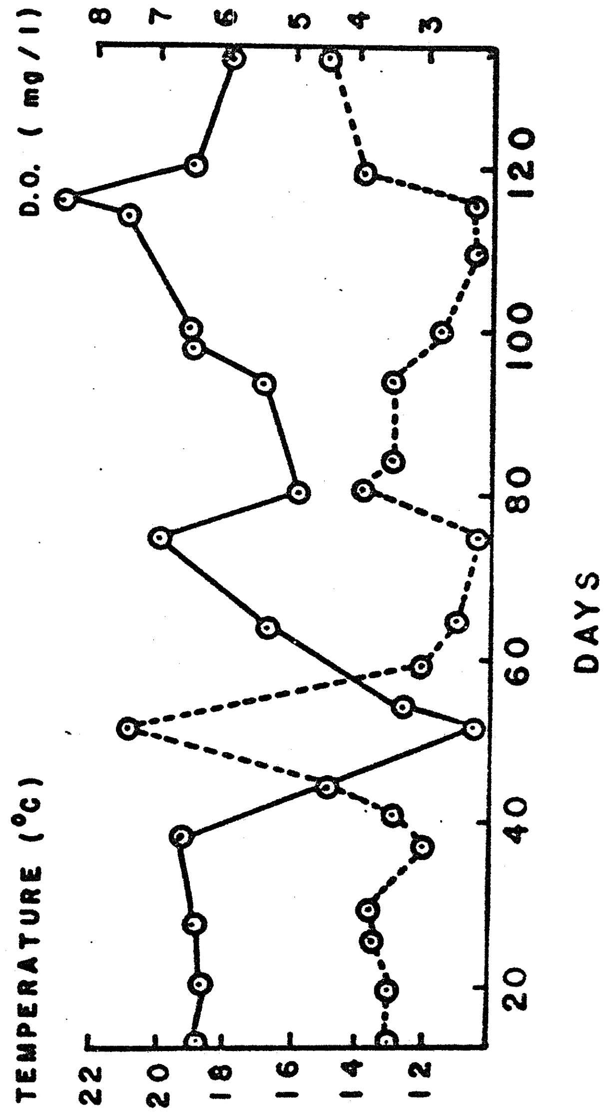
There was a good correlation between total cell count and availability of nutrient, and between total cell numbers and the presence of predatory zooplankton. Figure 1 clearly shows that when the bacterial nutrient supply was limited, there was a drop in cell count. A similar response was found during zooplankton blooms. The zooplankton blooms could be relieved by starving the model lagoon, following which total cell count recovered when feeding was resumed. The large variation

FIGURE 2. Variation in temperature, pH and dissolved oxygen (D O) in the model lagoon.

o————o temperature.

o- - - - - - - -o dissolved oxygen (D O)


PH
8.0
7.8
7.6
7.4
7.2
SECONDARY
PRIMARY


TEMPERATURE (°C)
D.O. (mg / l)
DAYS
22
20
18
16
14
12
8
7
6
5
4
3
20
40
60
80
100
120

in total cell count may partially be reflected in the occurrence of zooplankton blooms, but variations in the raw sewage strength (shown in Table 6) is a more likely causative factor. The immediate response to removal of nutrient supply was a reduction of the total cell count. Over the course of the experiments there was a change of two orders of magnitude in the total bacterial count and a stable bacterial count was never obtained.

The model lagoon may provide a convenient means to study biodegradation and the effects of physico-chemical factors and predation on substrate removal but it was judged as a poor source of mixed microbial populations for more refined work.

PART II ALTERNATE SOURCES OF MIXED MICROBIAL POPULATIONS

PART IIB The Chemostat as a Source of Mixed Cultures

I N T R O D U C T I O N

INTRODUCTION

Since the model lagoon proved unsuccessful for the generation of stable mixed populations, it was thought that the desired results might be obtained in the laboratory by the use of a chemostat, that is, to maintain a mixed population in a constant 'steady state' condition in continuous culture by limiting a growth component.

HISTORICAL

HISTORICAL

The theory of continuous culture is well established. An excellent treatment of pure continuous cultures has been provided by Herbert, Ellsworth and Telling (97).

Briefly, in a perfectly mixed vessel with sterile feed solution being added at a constant rate to a fixed vessel-volume, mass balance considerations give the equations:

$$\frac{dx}{dt} = \mu x - Dx \qquad (1)$$

where x = mass concentration of organisms

D = dilution rate

μ = specific growth rate

That is, the change in the concentration of cells in the reactor is determined by the differential rate at which cells are formed and at which cells leave the vessel in the effluent. At steady state $dx/dt = 0$ and the specific growth rate (μ) is equal to the dilution rate (D).

The temporal change in substrate concentration is determined by the differential rate of input concentration, substrate utilization by the organisms for growth,

and the rate at which substrate is leaving the vessel in the effluent. Mathematically this is:

$$\frac{dS}{dt} = DS_o - \frac{ux}{y} - DS \qquad (2)$$

where S = substrate concentration in the vessel and effluent

S_o = substrate concentration in the feed

y = a yield constant

0 = dilution rate

Again at steady state ds/dt = o and growth rate at dilution rate D is limited by the residual substrate (S_o-S).

The specific growth rate is a function of the limiting substrate concentration and is approximated by:

$$\mu = \mu_{max} \left(\frac{S}{K+S}\right) \qquad (3)$$

where S is as defined above

μ_{max} is the maximum growth rate

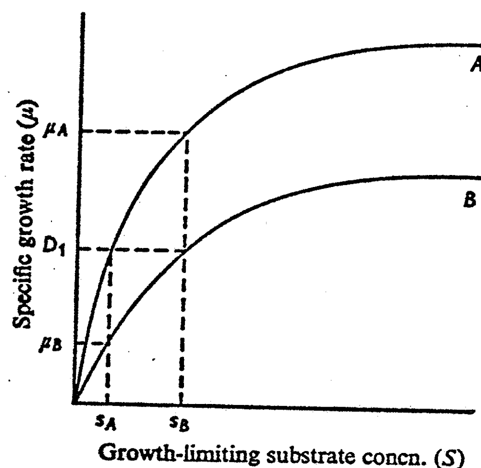
K is the saturation constant numerically equal to the extracellular concentration of growth-limiting substrate at 0.5 μ_{max}.

Equation (3) is established empirically.

The fate of a second organism present in the reaction vessel will be influenced by many factors (e.g. culture pH, temperature, nutritional requirements of the organism and composition of the medium), but assuming that (a) the dilution

rate is constant, (b) the culture is in steady state at the moment the second organism is introduced, (c) growth rate is limited by the deficiency of a single nutrient which is essential for growth of both organisms, (d) there is no interaction between the organisms other than competition for growth-limiting nutrient, (e) the culture is perfectly mixed and (f) growth rates of the organisms adjust themselves to changes in substrate without appreciable lag, then theory predicts that the second organism must either be washed from the growth vessel or completely replace the original organism.

To illustrate this point consider the following figure,



which illustrates the theoretical saturation curves for two organisms (A and B) growing in chemostat culture with the same nutrient limitation. Assuming the saturation curves to be those which would occur if the organisms were grown separately, but under identical conditions at a dilution rate D_1, then the extracellular concentration of limiting nutrient in the culture of A would be S_A, whereas that in the culture of B would be S_B. If a small number of A-type organisms were now transferred to the chemostat containing a steady state population of B organisms, then (assuming no lag period) the A-type organisms would begin to grow at a rate μ_A (since the growth-limiting concentration would be SB). Because μ_A is greater than D_1, the concentration of A-type organisms in the culture would increase. Their growth would cause the growth-limiting substrate concentration to decrease towards the value S_A; at which concentration B-type organisms could grow only at the rate μ_B. Since μ_B is less than the dilution rate this organism would be completely washed from the culture vessel. That is organism A would replace organism B.

The theory for the above discussion has been developed by Powell (167) and Rennesboog-Squilbin (174) to account for the competition between a contaminant or a mutant strain and an established chemostat culture.

Implicit in the theoretical analyses of completely mixed systems is the assumption that cultures (pure or mixed) reach a stable equilibrium, regulated by the dilution rate. However, for a steady state to be established the environment must remain unaltered with time, and the organisms in the environment must not interact with one another.

Meers (139) has described a situation in which two organisms present together in a chemostat were able to alternate dominance in response to a change in the dilution rate. Theory explains this by requiring that the saturation curves of each organism must interconnect at one point. Chian and Mateles (36) studied the competition for two substrates in a chemostat containing pure and mixed cultures of two organisms. Generally, above certain dilution rates only one substrate was used by the pure cultures. In mixed cultures, however, the populations of both species were maintained indefinitely in proportions which varied with the dilution rate and the kinetics of substrate uptake was different from that observed in the pure cultures. For such a situation to exist, one must conclude that each species had a competitive advantage over the other for one of the growth-limiting substrates.

Taylor and Williams (212) have given an elegant mathematical treatment on the coexistence of competing species under continuous-flow conditions. But their practical example was confined to a treatment of only two

species with the implication that the extension to more species was simply a matter of more complicated mathematical manoeuvers.

The theoretical considerations of Taylor and Williams led to the conclusion that to sustain a mixed population of a number of species in a chemostat-type continuous-flow system it is necessary that there are at least as many independent growth-limiting substrates as there are species. The term 'substrate' was used in a very general sense and included; energy sources such as organic material or light; a major carbon, nitrogen or phosphorus source, or an organic or inorganic trace nutrient. On this basis the mathematical treatment is reduced to "something" is limiting growth of one or more species in the mixed microbial population. To formulate the mathematical model Taylor and Williams made a number of simplifying assumptions. In particular interaction was deleted from the treatment and the attainment of a steady state was assumed. Also the model does not allow for effects of population density on growth rate. Meers and Tempest (141) have proposed that extracellular growth-promoting metabolites tend to increase the growth rate with increasing population density.

Unfortunately, some of the theory for mixed cultures has been more concerned with mathematics than with reality. Enrichment would tend to reduce heterogeneity of a natural population. However, other factors

must be combatting enrichment since there is a wide diversity of species in nature. Fluctuations seem to be a necessary factor in optimizing multiculture systems. Oscillations of the component populations serve to sub-optimize the behaviour of the individual species which would allow the establishment of other species to varied extents. This is a frequently observed phenomenon (27, 36, 75). Ghosh (78) pointed out that fluctuations at steady state concentrations with mixed cultures may be expected to be more at lower dilution rates because of the increased degree of heterogeneity. Further, Aldridge and Pye (3) reported that the behaviour of cell populations is a function of individual cells and mutual reactions. They showed that metabolic synchronization was density-dependent. This led them to the hypothesis that the behaviour of the total cell population could be unrelated to the sum of the behaviours displayed by individual cells, i.e. a biochemical synergism exists.

The present laboratory use of the chemostat is to provide a highly selective environment to create and sustain pure culture by closely regulating the selection parameters: - limiting substrate, dilution rate, etc.

It appears that a successful approach to the use of chemostats to generate "stable" mixed cultures should de-emphasize stringent control. With this situation, conditions would be suboptimal for enrichment to occur and minor fluctuations would allow for the coexistence of

competing species by causing a shift in the dominance from one species to another. Ghosh and Pohland (78) have referred to the phenomenon of "shifts" in the species composition and the physiological capability of dominant cultures in response to shifts in the rate of hydraulic dilution as "...an intrinsic property of heterogenous microbial populations grown in completely-mixed continuous flow reactors".

Of course the addition of a further variable to an already complex situation would pose formidable problems for any investigation.

The chemostat approach to describing mixed culture systems is an infant science and Meers (140) concluded a lengthy and extensive review of the growth of bacteria in mixed culture with,

> "In conclusion, it is not difficult to argue the case for further work that will lead to an understanding of the ways in which bacterial species interact. What is now required is that the work done in this area be of the highest quality so that future speculations may be based on data of undoubted soundness".

It appeared probable that a stable mixed population could be established in a chemostat at steady state if supplied with the proper conditions. Present knowledge dictated that for every species established at steady state there must be a growth-limiting substrate specific to that organism. Therefore it seemed that a multisubstrate

nutrient supply would be required. The following section is an account of the conditions employed to realize a stable mixed culture in a chemostat.

MATERIALS AND METHODS

MATERIALS AND METHODS

Growth of Mixed Cultures in a Chemostat

i) Construction of the chemostat (Plate 1)

A one-liter, water-jacketed Bellco jar (Bellco Glass, Vineland, N.J.) equipped with inlet and outlet ports and a magnet stirring assembly was used as the cell reservoir (Plate 1). The glass dome was replaced by a plastic disk equipped with port holes for sampling or for probes; these were plugged with ribber stoppers if not required. Dilution rate was controlled by regulating the inflow of sterile medium with a Watson-Marlow (Fred A. Dungey, Agincourt, Ontario) flow inducer, type MHRE 7. Medium was transferred to the cell reservoir through 2.5 mm I.D. silicone tubing from a 20-liter carboy containing sterile medium. The silicone tubing terminated in a length of stainless steel tubing (200 mm x 2.5 mm I.D. x 4.5 mm O.D.) which entered the cell reservoir through a latex rubber stopper. Three hollow core, 10 ohm, 12 watt resistors (Brown Devil type 1506) wired in parallel were placed over the stainless steel tubing (Plate 2). Power was supplied to the resistor network from a type 167M25, 75 VA transformer (Hammond Manufacturing Ltd., Guelph, Ontario). Power dissipated by the resistor network heated the medium as it passed through the steel tube. This

PLATE 1. Arrangement of the chemostat componentry.

A - Reaction vessel

B - Flow inducer

C - Nutrient reservoir

D - Inlet heater assembly

E - Heater power supply

F - Power regulating rheostat

G - Circulating water bath

H - Ice container

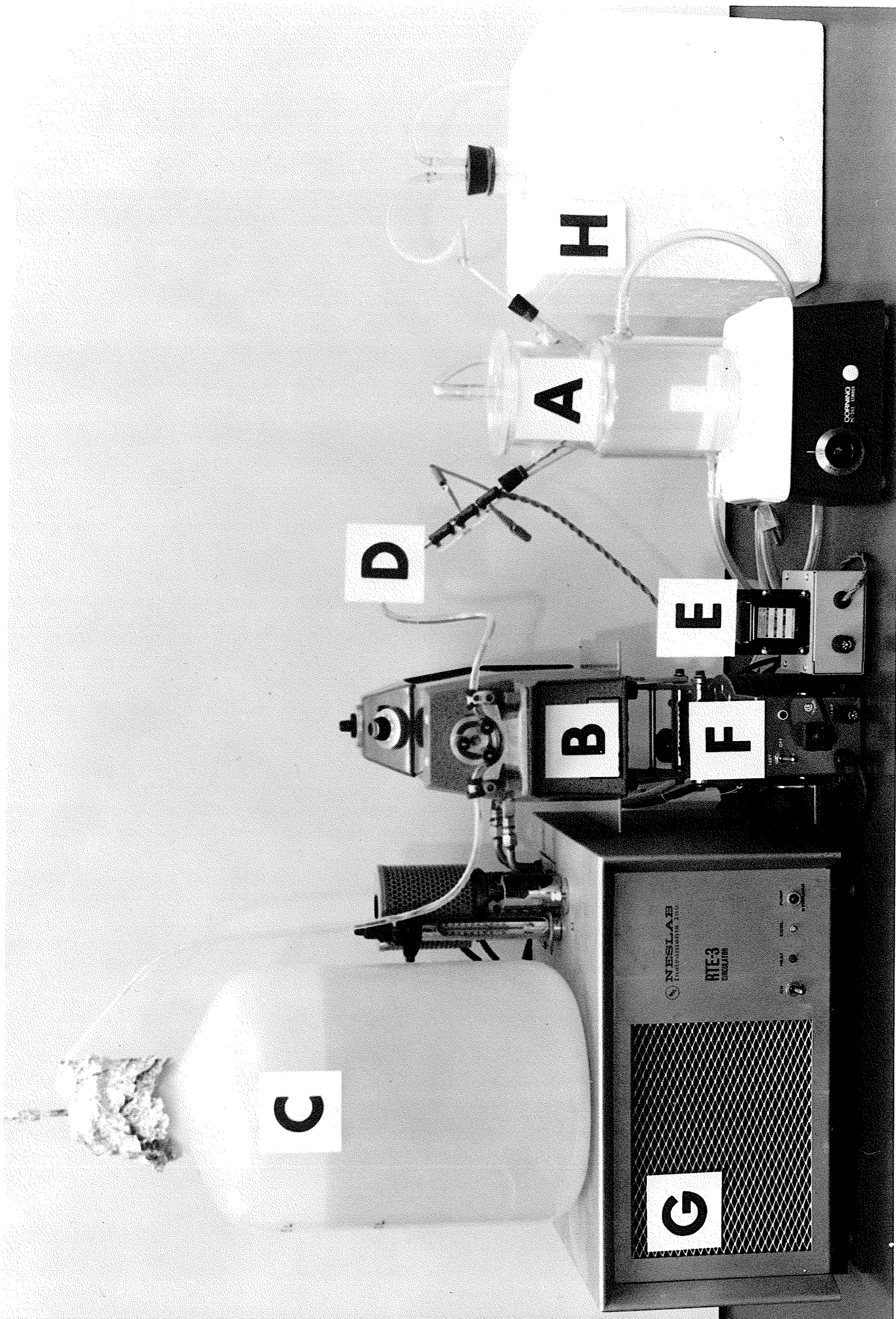
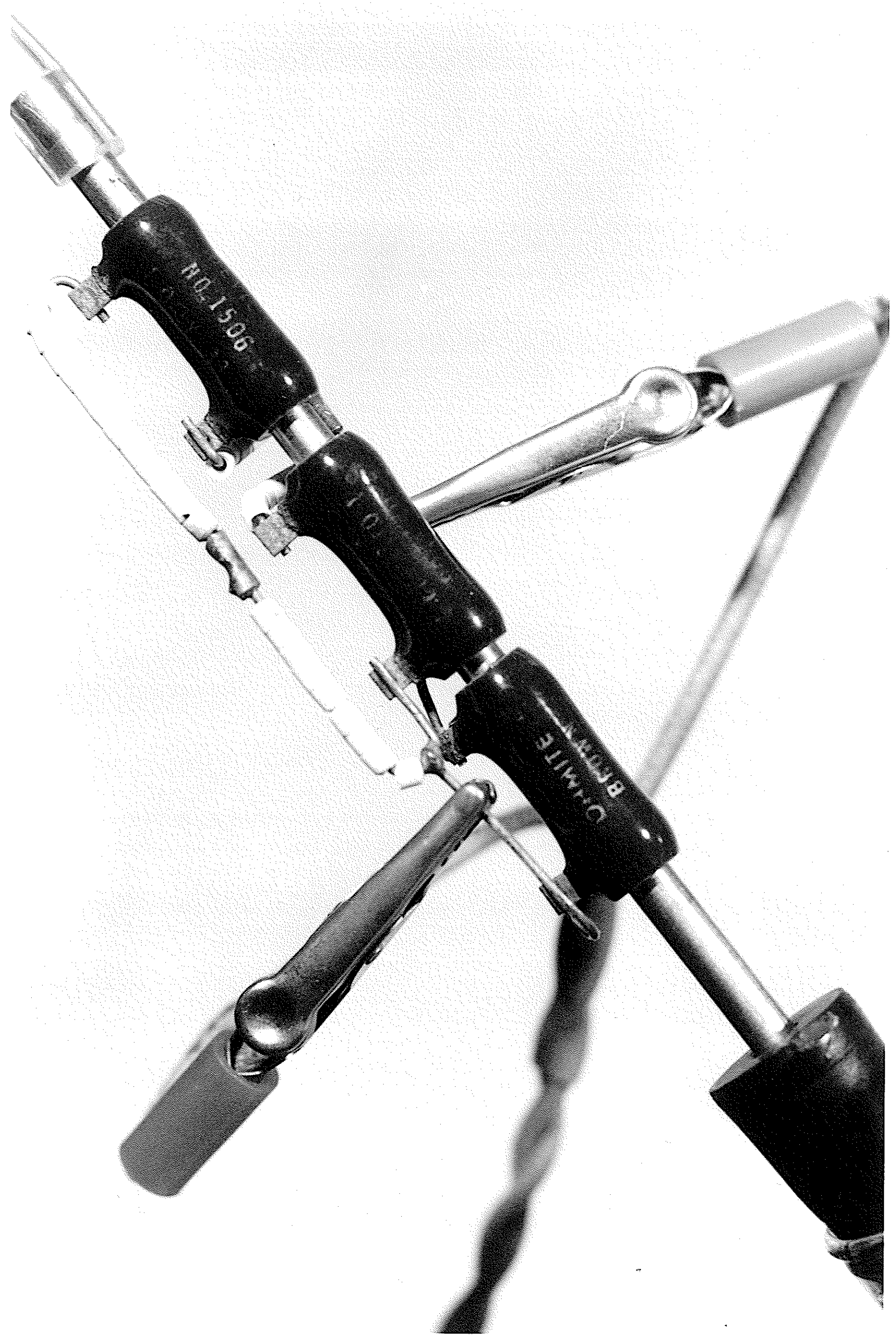

A
B
C
D
E
F
G
H
NESLAB
RTE-3

PLATE 2. Detail of resistor-network at inlet to reaction vessel, showing detachable power supply.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arrangement prevented "growback" of microorganisms in the supply line. Temperature in the supply line was controlled by varying the voltage to the primary of the transformer with a type 3PN1010 variable transformer (Staco Inc., Dayton, Ohio). The temperature of the medium emerging from the steel tubing was calibrated against variable transformer setting (read as percent of mains input) for each dilution rate. The temperature of the emerging medium was regulated to $60^{O}C$.

The temperature of the cell reservoir was controlled to $25^{O}C \pm 0{,}05^{O}C$ by a Neslab (Neslab Instruments Inc.) model RTE-3 circulating water bath.

Volume in the cell reservoir was maintained by exhausting excess materials through an outlet port which was also a common outlet for forced aeration. The excess cells and medium were collected in a 2-liter reservoir contained in an icebox.

Air pressure was maintained at 14 K_p by a type CGA540 Purox air regulator (Union Carbide Canada Ltd., Toronto) and flow rate was adjusted to give a gentle rate of removal of excess cell materials and medium. The rate of air-flow was dictated by the dilution rate. Agitation was effected by magnetic stirring.

The whole assembly was sterilized as a unit. Cotton filters were installed on the air inlet and alligator clips were installed on the power supply leads for dis-

connection during sterilization of the unit.

The cell material collected in the ice-packed overflow reservoir was used to prepare resting cell suspensions by the method previously described.

ii) Calibration of medium temperature at cell reservoir inlet

Medium entering the cell reservoir passed through a heated steel tube, described above. The temperature of the medium as it emerged from the steel tube and dropped into the cell reservoir was regulated, prior to setting up the whole assembly, by monitoring with a YSI model 42 SC Tele-Thermometer (Yellowsprings Instrument Co., Yellowsprings, Ohio) while adjusting the input voltage of the power supply. To prevent growback of bacteria in the inlet line it was found necessary to maintain the temperature $60^{o}C$; a temperature above $70^{o}C$ caused boiling to occur inside the steel tube since heat dissipation from the centre of the tube was not as great as that from either end.

iii) Maintenance of dilution rate

Dilution rate in the chemostat was controlled by regulating the rate of flow of medium into the fixed-volume cell reservoir. The volume of the cell reservoir was 800 ml and could be changed by adjusting the outlet port. The medium reservoir (20-liter carboy) was set up to allow gravity feed of medium into the cell reservoir.

The rate of flow of medium was regulated by adjusting the speed of the Watson-Marlow flow inducer.

iv) Feed medium

Peptonized milk (Difco) at a concentration of 1 gm L^{-1} in 50 mM phosphate buffer, pH 7.0, was used as the feed medium. The BOD-equivalent is ~ 350 mg L^{-1}. Twenty-liter Nalgene reservoirs containing 18 liters of medium were sterilized at 121°C for 55 minutes.

v) Selection of chemostat operating parameters

The following parameters were used.

a) temperature - 25°C ± 0.05°C
b) flow rate of medium - 48 ± 0.1 ml hr^{-1}
c) volume of cell material in reservoir-800 ml
d) dilution rate - 0.06 hr^{-1}
e) pH - 7.0
f) stirring rate - 600 rpm.

vi) Chemostat start up

The chemostat reaction vessel was allowed to fill to a predetermined volume with nutrient from an attached reservoir. In-flow of nutrient was stopped and and the volume in the reaction vessel was inoculated with 1 ml of raw sewage. Two days were allowed for acclimation, after which the nutrient influx was resumed. Growback into the nutrient reservoir was avoided during acclimation by maintaining a high temperature in the inlet of the nutrient supply line.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

The theoretical considerations necessary to the establishment of a steady-state multixenic chemostat predicted dim prospects of success. Nonetheless it was decided to proceed on a purely empirical basis to determine how successful this approach might be.

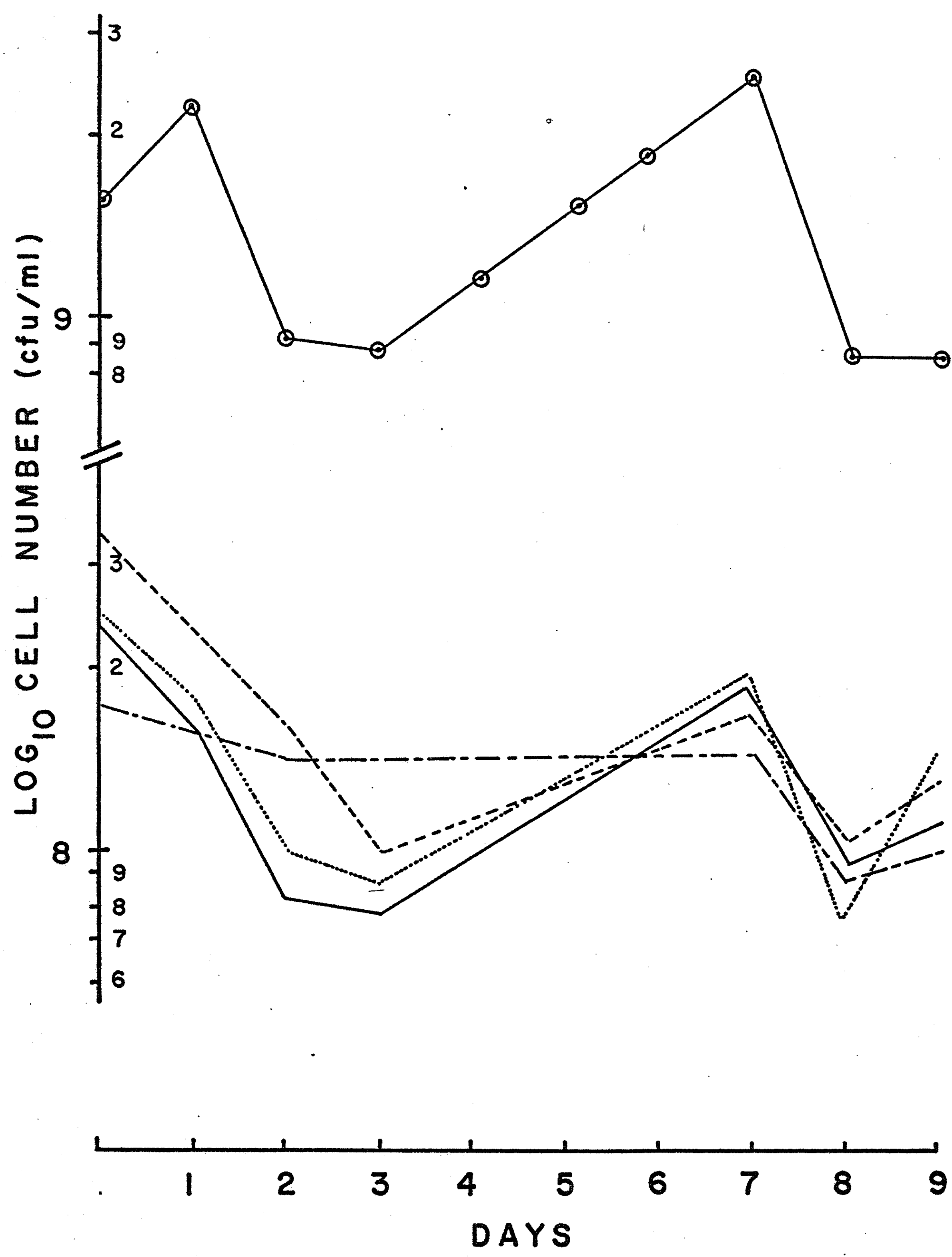
The most difficult problem associated with chemostat-generated populations, a problem frequently encountered in chemostat culture, was that of 'growback' of bacteria along the feed line into the nutrient reservoir. This inevitably resulted in a catastrophic collapse of the fine balance between competing species in the reaction vessel. A heating device designed, fabricated, and then applied to the influent nutrient supply line was remarkably successful in eliminating the problem; the device is shown in detail in Plate 2.

Peptonized milk (0.1%) was arbitrarily chosen as the multicomponent nutrient supply for the chemostat generation of mixed bacterial populations. The choice of peptonized milk was based chiefly on the knowledge that it is increasingly being recognized as a superior standardized medium for the enumeration of soil bacteria. With peptonized milk as the nutrient source a multixenic culture developed. The mixed population comprised of at least five different physiological types (chromogenically

distinct on peptonized milk agar) with one dominant culture exceeding by one order of magnitude four minor cultures which were present in approximately equal numbers. The homeostatic condition was maintained for a five-week period. Figure 3 shows the relationship of the five established cultural types. It is notable that the fluctiations were of a lesser amplitude than those of the model lagoon (Fig. 1).

The low dilution rate of 0.06 hr^{-1} was arbitrarily chosen to maximize heterogeneity of the chemostat population. The effect of altering the dilution rate on the microbial types that will become established has not been explored. The possibility exists that other multicomponent nutrient sources in combination with other dilution rates might be more suitable for the steady-state cultivation of mixed cultures in the chemostat. Rerie (175) compared peptonized milk medium to a synthetic sewage medium and a chemically-defined medium for the generation of mixed cultures using the previously described chemostat. His results showed that the peptonized milk medium was the superior medium of the three for the generation of the most stable steady-state chemostat mixed culture. Moffatt (146) showed that, to a limited extent, it was possible by enrichment to regulate the physiological properties of the organisms growing in association in the chemostat. Clearly, many parameters are open to further investigation.

FIGURE 3. Enumeration of chemostat major bacterial types during a 9 day period. A dominant culture and four minor components of the stable mixed population is shown. The bacterial types were recognized on the basis of chromogenicity.

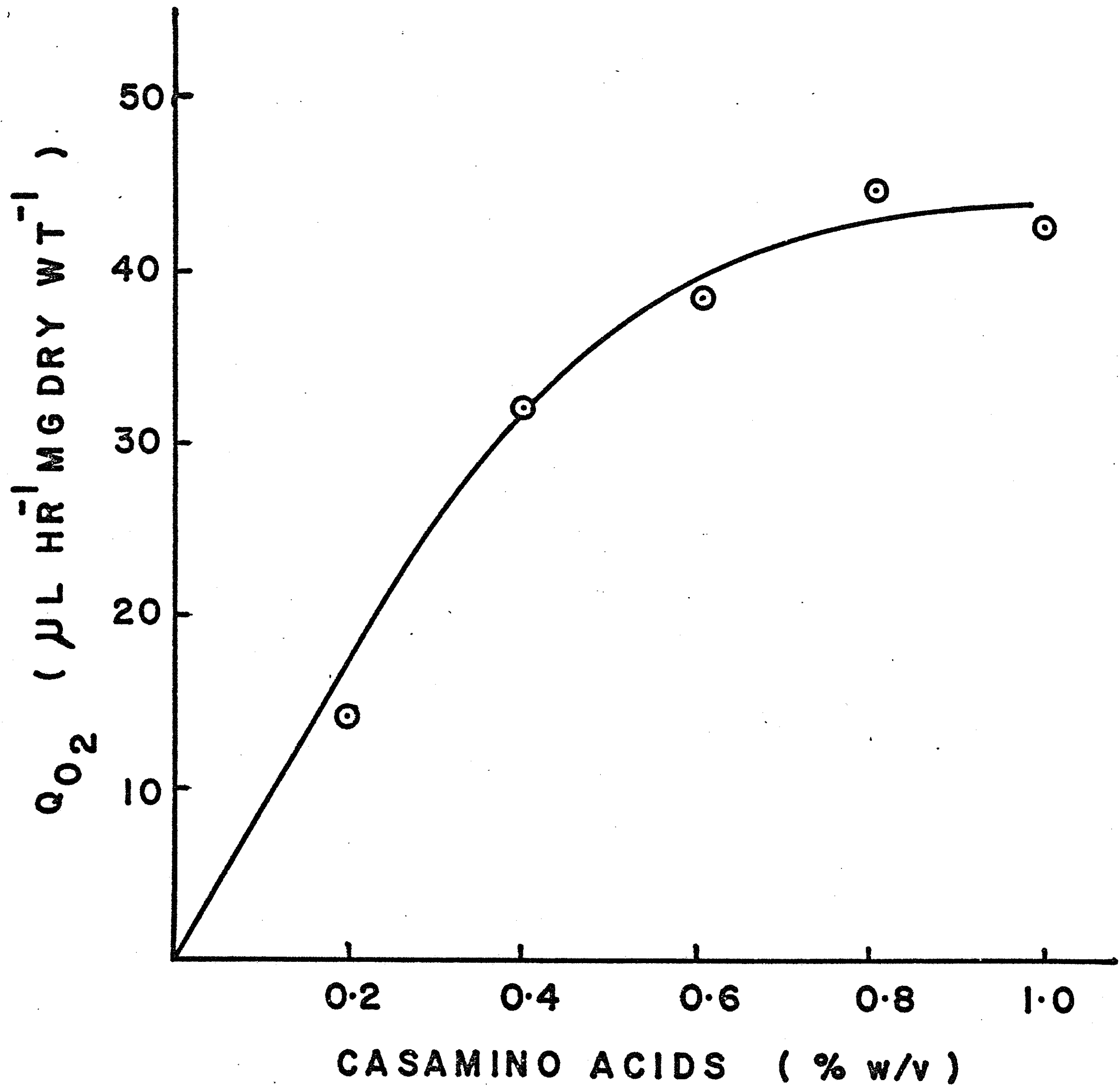

3
2
9
9
8
3
2
8
9
8
7
6
LOG10 CELL NUMBER (cfu/ml)
1
2
3
4
5
6
7
8
9
DAYS

The relevance of mixed culture phenomena to various areas of microbiology has been the subject of a number of reviews (27, 140, 174). Nevertheless, very little is known about fundamental characteristics of mixtures of microorganisms, basic research is needed on growth rates, survival, population dynamics, physiology and ecology. Complex mixed culture systems present formidable problems for research because of the inter-association of a multiple of parameters. Hopefully the use of continuous culture will contribute to this difficult area of research.

The original intent in setting up the chemostat to provide a laboratory source of mixed populations was to compare the activity of its mixed population to that of the sewage lagoon organisms.

On a dry weight basis the chemostat population was more active than the lagoon preparation with the same substrate as measured by oxygen uptake. Figure 4 shows the respiration of a resting cell suspension of the chemostat mixed population in the presence of saturating casamino acids; at substrate-saturation the QO_2 ($\mu l\ hr^{-1}$ mg dry wt^{-1}) was about 40. Since enzyme amount is directly related to catalytic activity, the maximum rate of oxygen uptake is a measure of viable biomass.

FIGURE 4. Respiration of a resting cell preparation of the chemostat mixed population in the presence of saturating casamino acids.



50
40
30
20
10
Q_{O_2} (μL HR^{-1} MG DRY WT^{-1})
0·2
0·4
0·6
0·8
1·0
CASAMINO ACIDS (% w/v)

A lagoon resting cell suspension prepared according to the procedure outlined in Section I routinely exhibited a QO_2 of about 10 under the same conditions. Thus the chemostat was capable of producing a mixed culture suspension with about the same catalytic properties as the lagoon preparation, but the rate is somewhat higher. A direct comparison is difficult to make since the measure of specific activity is based on dry weight which in the case of the lagoon organisms includes non-viable and non-reactive cells as well as inert materials. With suitable manipulation of the nutrient input, dilution rate, and other parameters it should be possible to simulate lagoon mixed culture conditions in the chemostat.

It is regrettable that by the time the method was developed the Charleswood lagoon, which was an experimental pilot project, had been phased out of operation by the City of Winnipeg. The principle reasons for discontinuing the lagoon process were mainly cost-related but sludge build-up and odour problems also affected the decision. Since the lagoon was no longer available as a source of mixed cultures a critical comparative study could not be made.

SUMMARY

SUMMARY

1. A model lagoon was contrived and found to mimick the Charleswood lagoon in efficiency of BOD removal. Albeit the operating characteristics were similar to an actual operating lagoon, as a stable source of mixed microbial populations the model lagoon was judged to be unreliable.

2. A chemostat culture effectively provided a continuous laboratory source of mixed bacterial cultures with 0.1% peptonized milk as the nutrient source at a dilution rate of 0.06 hr^{-1}. The culture was operated for a total of five weeks. At least five recognizably distinct bacterial types were established with one dominant type; the degree of steady-state in the culture being only cursorily examined. The chemostat method of generating laboratory sources of mixed cultures was viewed upon optimistically.

   The physiological activity of the chemostat preparation was found to be considerably higher on a dry weight basis than mixed cultures from the Charleswood lagoon but it was not possible to carry out critical comparisons.

PART III. KINETICS OF CO_2 LIBERATION BY INTACT BACTERIAL CELL SUSPENSIONS: QUANTITATION BY INFRARED ANALYSIS

H I S T O R I C A L

HISTORICAL

Nickerson (153) stated:

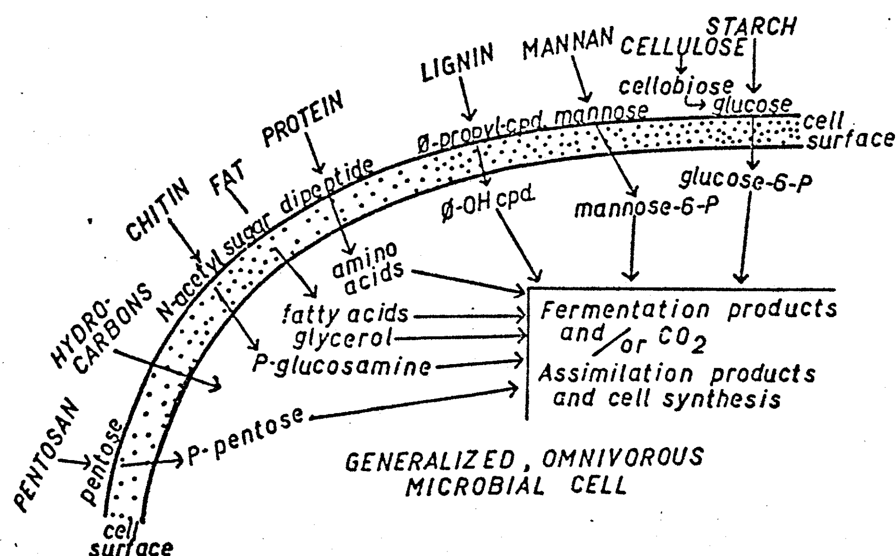
"as more is learned about the decomposition of natural materials by microorganisms, which are the chief agents for the decomposition of carbon compounds in nature, it becomes apparent that the generalization can be made that for every carbon compound formed in nature there exists some microbial agency for its decomposition. And, as more is learned about the biochemical mechanisms involved in the decomposition of a wide variety of substrates, there is emerging the generalization that the initial stage of attack on a carbon compound, no matter how complex, converts it into a substance that feeds into one of relatively few metabolic throughways common to all cells".

Nickerson went on to make liberal use of biochemical flow sheets or metabolic maps as guides for the 'type pathways' exhibited by certain microorganisms in their transformation of carbon compounds. He clearly showed that many of the pathway of organic carbon oxidation were common to many organisms. And he concluded that in the ultimate analysis, the mechanism of microbial decomposition of a carbon compound

is that of enzymic action.

However many of the enzyme systems depicted do not occur in every microorganism. This idea is implicit in Nickerson's presentation, wherein he speaks of many special transformations carried out by particular organisms.

For this reason a mixed microbial population is a versatile enzymic collection. Depicted below is Nickerson's representation of this situation as the "generalized, omnivorous microbial cell", which is able to metabolize complex organic compounds through a series of intermediates, of decreasing simplicity; finally to small metabolites common to all cells and then to carbon dioxide.



GENERALIZED, OMNIVOROUS MICROBIAL CELL

Gas exchange has been considered a fundamental characteristic of life (164, 214). Whitkamp (229) shows that in particular evolution of CO_2 demonstrates highly significant positive linear relationships between microbial activity and mineralization. For over half a century manometric methods for estimating exchange of gases have been used in the study of both chemical and biological reactions. The type of instrument which has met with widest use is commonly called the Warburg constant volume respirometer, which was adapted and modified by Warburg (223) in 1926 from a design previously described by Barcroft and Haldane (9) in 1902. Although most commonly applied to the measurement of oxygen uptake the instrument has numerous other uses. Carbon dioxide has been frequently used as a monitor of physiological activity. Tolbert (215) analyzed respiratory patterns in human subjects by measuring $^{14}CO_2$, $\%CO_2$ and ^{14}C - specific activity in the breath after ingestion of radioactive compounds. Carbon dioxide was measured by infrared gas analysis and an ion-chamber was used to detect $^{14}CO_2$. Zwarun (239) measured $^{14}CO_2$ production while studying the effects of osmotic stabilizers on bacteria in the blood. Williams (226) studies dynamic aspects of the TCA cycle in isolated mitochondria, using an ion chamber to monitor, continuously, the output of $^{14}CO_2$ from metabolizing suspensions. Rates of CO_2

evolution from the forest floor were measured by Reiners (173) to estimate summed energy release as a measure of heterotrophic activity. Wang (220) introduced the radio-respirometric method and successfully elucidated some important biochemical pathways by measuring the $^{14}CO_2$ liberated from specifically labelled organic compounds. Satake and Saijo (184) used infrared analysis to measure concentrations of dissolved CO_2 in acid lakes in a study of CO_2 fixation.

The concentration of carbonate in the mixed layer of the sea is diminishing with time as fossil CO_2 is absorbed. The build up of fossil CO_2 in the atmosphere is due to the exponential increase in the rate of consumption of fossil fuels. Under-saturation of carbonate, in the mixed layer, would tend to dissolve calcareous organisms and the coral reefs. Fairhall (65) proposed monitoring atmospheric CO_2 build-up to avoid these disasterous consequences.

Alford (6) used infrared analysis and a silastic rubber probe to show that CO_2 partial pressure in fermentor exhaust gases is the same as that in the medium. Neal and Jones (152) used an infrared gas analyzer to make Warburg-type CO_2 measurements with yeast. They were able to overcome the complications imposed by two gas exchange systems since the infrared gas analyzer responds specifically to CO_2.

This parameter has been used only infrequently as an indicator of biological activity in microbiological studies.

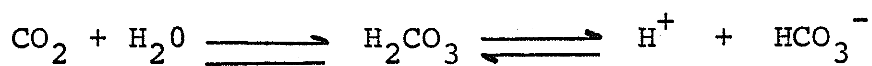
Carbon dioxide production has been proposed as a criterion of biodegradability. Biodegradation of non-ionic surface active agents has been studied using this method (125, 211, 213). Dieldrin, one of the most persistent hydrocarbon insecticides, was degraded by a fungus, Trichoderma konigi. Bixby (13) measured 3.1% conversion of ^{14}C-dieldrin to $^{14}CO_2$ in 17 days. Domsch (56) measured, simultaneously, radioactive and total CO_2 evolved from ^{14}C-supplemented soil samples. ^{14}C-Benomyl, a fungicide, was added to the soil. Inhibition of glucose oxidation and enhanced $^{14}CO_2$ production was observed; 8.7×10^{-3}g of Benomyl was added to the soil sample and 3.6% was oxidized to CO_2, in the presence of glucose, in 24 hr.

To circumvent the problems associated with employing mixed microbial suspensions from the Charleswood lagoon for long term experiments a method was required to determine initial reaction rates of substrate catabolism. The technical problems of analysis would be overcome if a general indicator of biological activity were used. Since carbon dioxide is a product of organic carbon metabolism, and since many studies had utilized CO_2 liberation to determine physiological activity it was thought that rapid determination of this

parameter would provide useful information about bacterial catabolic activity. Part III of this presentation outlines the methodology developed to measure CO_2 liberation by intact bacterial cells.

Theory of CO_2 Measurement

The solubility of carbon dioxide in pure water is essentially no different from the solubility of other gases. Carbon dioxide forms carbonic acid which dissociates to form H^+ and HCO_3^- according to:



$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

However 99% of carbon dioxide in solution is in the form of dissolved carbon dioxide and less than 1% exists as H_2CO_3, H^+ or HCO_3^- (216). In the absence of materials that can combine with the acid, the solubility is comparable to that of any other gas. Higher Van der Waals forces results in a greater deviation from the ideal gas laws, and dissolving CO_2 in pure water also generates H^+ which causes the pH to decrease with increased CO_2 pressure.

The solubility of carbon dioxide in pure water decreases with increasing temperature as indicated in the Table on page 118. The presence of salts, etc., in solution has little effect on the solubility of carbon dioxide, within physiological concentrations, providing that these do not combine with the carbonic acid. The effect of some salts is shown in the Table on page 118.

The Solubility of Carbon Dioxide in Pure Water

Data in terms of α = ml.CO_2/ml. water or μl.CO_2/μl. water at one atmosphere

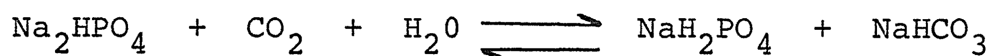
Temp. °C.	(1)	(2)	(3)
0	1.713		
10	1.194	1.194	
15	1.019	1.019	1.014
20	0.878	0.878	
25	0.759		0.756
30	0.665	0.66	
35	0.592		
40	0.530	0.53	

The Influence of Salts and Other Materials upon the Solubility of Carbon Dioxide

Material	At 15°C (α values)			At 25°C (α values)		
	0.5 M	1.0 M	2.0 M	0.5 M	1.0 M	2.0 M
None	1.014	--	--	0.756	--	--
HCl	0.989	0.974	0.948	0.738	0.732	0.728
1/2 H_2SO_4	0.965	0.927	0.867	0.727	0.705	0.669
HNO_3	1.022	1.029	1.043	0.770	0.781	0.803
KCl	0.925	1.850	--	0.695	0.641	--
NH_4Cl	--	--	--	0.720	0.692	0.648
Glycerol	--	0.934	--	--	--	--

*From Umbreit (216).

When buffers are present they may react with CO_2. For example:



$$Na_2HPO_4 + CO_2 + H_2O \rightleftharpoons NaH_2PO_4 + NaHCO_3$$

hence some of the CO_2 produced may be retained. Johnson (216) has suggested a useful method of correcting for CO_2 retention. The effective value of the CO_2 solubility constant (α^1) is related to the actual α value by

$$\frac{\alpha^1}{\alpha} = \frac{(HCO_3^-) + (CO_2)}{(CO_2)} = [\text{antilog } (pH-6.317)] + 1$$

The figure on page 120 shows the relation between the ratio of apparent to real solubility of CO_2 with increasing pH. At values above pH 7 the retention correction becomes very large and the CO_2 measurement suffers accordingly.

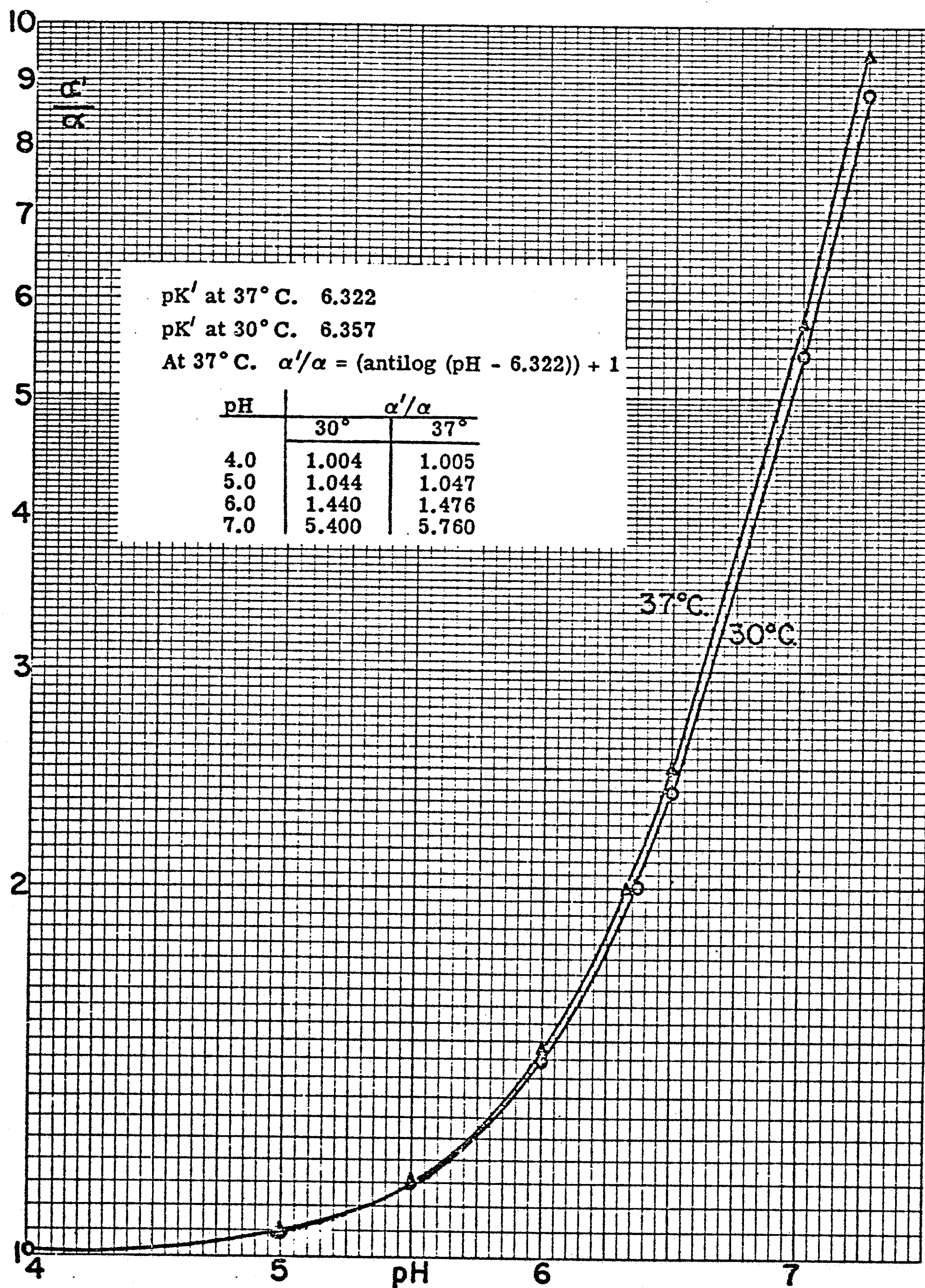
At a given temperature and pH, the quantity of gas in the liquid phase (Q_L) is dependent on the solubility of CO_2 and the partial pressure of CO_2 such that:

$$Q_L = \frac{V_L \ \alpha \ p(CO_2)}{P_o}$$

where, V_L is the liquid phase volume, α is the solubility of CO_2, $p(CO_2)$ is the partial pressure of CO_2, and P_o is the atmosphere pressure.

In the gas phase:

$$Q_G = V_G . \frac{273}{T} . p \frac{(CO_2)}{P_o}$$



pH	α'/α 30°	α'/α 37°
4.0	1.004	1.005
5.0	1.044	1.047
6.0	1.440	1.476
7.0	5.400	5.760

where Q_G is the quantity of gas in the gas phase of volume, V_G, and T is the absolute temperature.

The ratio Q_L/Q_G reduces to $\frac{V_L \alpha T}{273 V_G}$

With increasing temperature α decreases and the main effect on CO_2 solubility is exerted by pH.

Thus by selecting the appropriate ratio of V_L to V_G the error due to CO_2 retention could be determined. As an example consider a 2-ml suspension at 25°C and at pH 7.0. From page 120, the apparent solubility of CO_2 at pH 7 is 4.08 ml/ml; T is 298°K, V_L is 2 ml. Assuming a 5% loss is tolerable then;

$$Q_L/Q_G = 5/100 = \frac{(2)(4.08)(2.98)}{273 V_G}$$

$$V_G = 180 \text{ ml}$$

Increasing the gas phase volume would result in lowered solubility losses but would decrease the sensitivity of the measurement since CO_2 concentration in the gas phase would be decreased.

Gas phase pCO_2 is equilibrated with dissolved CO_2 in the culture liquid (6, 106). The time required to reach this equilibrium is affected by pH, temperature, agitation speed, viscosity and carbonic anhydrase.

Equilibration time is retarded by increasing pH and viscosity, and decreasing temperature and agitation speed. To measure CO_2 evolution rates from bacterial cells it is necessary that the rate of liberation of

CO_2 not be mass-transfer limited. This limitation can be eliminated by providing sufficient agitation to overcome mass transfer so that bacterial cell-generated CO_2 is the limiting step.

Application of Kinetics for Quantitative Analysis

Kinetic measurements offer real or potential advantages over other more conventional analytical methods. One of the stronger cases for the analytical application of kinetics lies in the use of enzymes, which have many useful and important analytical purposes. Their inherent specificity for specific reactions or groups of reactions make them useful as selective reagents for organic analysis. Determination of the concentration or activities of the enzymes themselves is important in many areas including fundamental enzymology and clinical diagnosis of disease. The rates of many enzyme reactions are changed markedly by the addition of small concentrations of other species such as metallic anions. As such, the enzyme reaction can act as a sensitive monitor for the concentration of these inhibitors or activators.

A practical situation for the use of enzymic kinetic analysis is the estimation of galactose in the presence of glucose in human blood. In the disease

galactosemia relatively large concentrations of galactose are present in the blood of the affected individual (22, 57). The relative activity of galactose oxidase is 0.000001% that of galactose. Thus a simple method is available to differentiate galactose from glucose while the reduction methods in common usage would not.

Guilbault (85) has given an extensive listing of applications of enzymes for selective organic analyses including carbohydrates, amino acids, organic acids, alcohols, esters and a few inorganic species. Hess (99), Bodansky (20) and Wilkinson (225) have listed and discussed a large number of enzymes which may have diagnostic significance. Bodansky emphasized the need for automatic instrumentation to handle the increasing number of analyses being performed.

Frequently the same functional group on different organic molecules will undergo a characteristic reaction with different rates. These differences in reaction rates have been utilized in the selective determinations of mixtures of compounds. Rechnitz (171, 172) has listed source material for a number of applications of differential kinetics.

Catalytic oxidation-reduction reactions and ligand exchange reactions are another area where recent developments in the application of kinetics in analysis is in use. An extensive listing of such reactions and analytical procedures have been presented by Yatsimirskii

(237), and Margerum and Steinhaus (131).

There are at least four distinctly different approaches to the measurement and processing of rate data to yield quantitative results. Blaedel and Hicks (14) discussed two approaches which they called the constant-time and variable-time methods. Two other approaches, referred to by Pardue (157) are the slope and signal-stat methods.

A. Constant Time

In the constant time method the total change in signal, monitored over a constant, predetermined time interval, is measured and related to concentration. The time interval may begin at the start of the reaction or at some time after the initiation of the reaction. However, the same time interval is selected for all samples to be run in a given series. Usually it is assumed that the measured signal is a linear function of concentration of the chemical species being monitored and that the reaction rate is proportional to the concentration of the sought-for constituent. Under these conditions the measured signal is a linear function of the rate-determining species. In the ideal case, when the measurement is completed near zero reaction time before the concentrations of the reacting species have changed significantly, the measured signal is proportional to concentration.

The proportionality between measured signal and concentration is a real advantage and one of the reasons this has been the most widely used measurement approach. A second advantage is the measurement time is the same for all samples, independent of concentration. This is significant in the design of control equipment for automatic handling of reagents and samples.

The basic disadvantage of the constant-time approach is the fact that the linear relation between concentration and measured signal is valid only for linear response curves. To use this approach effectively it is necessary that the kinetics of the reaction and the response characteristics of the signal system combine to yield a linear response curve. Non-linear calibration curves will require complex computational equipment for direct presentation of the concentration data. Another disadvantage of this method is that as concentration decreases, the measured signal decreases, usually resulting in a decreased signal-to-noise ratio and a resultant increase in relative error.

B. Variable time

In the variable time method, the time required for the signal to change over a predetermined interval is measured. The signal interval may be measured from zero time or may be selected at some other level. In

practice, the latter approach has the advantage of permitting the reaction mixture to become completely mixed and to reach temperature equilibrium before the measurement is begun. The increased time is inversely related to the concentration of the rate-limiting species, being inversely proportional to it when the measurement is completed before the concentrations of reactants have changed significantly. This reciprocal relationship has made the variable-time approach less attractive than the constant-time method as a kinetic method.

However, there are some attractive features. The reciprocal relationship is independent of the shape of the response curve and is valid for both linear and non-linear curves. Furthermore, non-idealities in signal system response introduce no errors or non-linearities in calibration curves so long as they are reproducible. This means that a wide range of detector systems is applicable to a variety of types of kinetic systems with minimal modification.

Computational equipment for direct presentation of the concentration data involves the use of a reciprocal time computer which, unlike the constant time method, is applicable to any combination of kinetic and signal systems. The variable time method has the additional advantage that the signal interval is constant regardless of concentration, resulting in a

constant signal-to-noise ratio for all concentrations and a constant contribution to relative error.

The varying measurement time for different concentrations presents a minor disadvantage, particularly in programming reagent and sample-handling equipment in automated analysis.

C. Slope method

In the slope method the derivative of the response curve is computed and related to concentration. Theoretically the slope can be measured at zero reaction time but in practice it is desirable to measure the slope after the reaction mixture is thoroughly mixed but before concentrations of reactants have changed significantly. Under these conditions the slope is proportional to the rate-limiting species. This relationship is valid for both linear and non-linear response curves. However, in the latter case the slope must be evaluated at the same absolute value of output signal if the proportionality constant relating concentration and measured slope is to be the same from one sample concentration to another. In the case of linear slopes this approach can be very fast, yielding complete results in a few seconds measurement time independent of the concentration level.

The major limitation of this method is that unless the signal noise is very low or the readout voltage is very well filtered, the noise component of the signal is amplified to intolerable levels by the differentiator.

D. Signal-Stat Method

In the signal-stat method some reagent is added at a rate sufficient to maintain the monitored signal constant. If one of the reactants is being monitored by the signal system, then the reactant is replaced as it is consumed by the reaction.

The familiar pH stat is a special case of this general approach in which the hydrogen ion concentration is monitored potentiometrically. Acid or base is added to maintain a preselected pH. Reaction rate is equated with the rate of addition of acid or base.

The principal advantage of this method is that the concentration of the chemical species being detected is maintained constant. This is particularly important if the reaction rate is dependent on this species.

The method has the disadvantage that a reagent must be added to the reaction mixture. If a solution is added, then the reaction mixture is continuously diluted and a correction factor must be applied.

Of all the above approaches, the slope method best lends itself to the measurement of initial rates of reaction since it can be extrapolated to zero reaction

time. The easily determined slope of the detector response is proportional to the rate-limiting species, and this relationship is valid for both linear and non-linear response curves. This measurement approach was chosen as the best suited method applicable to the measurement of CO_2 liberation rates by bacterial cells.

MATERIALS AND METHODS

MATERIALS AND METHODS

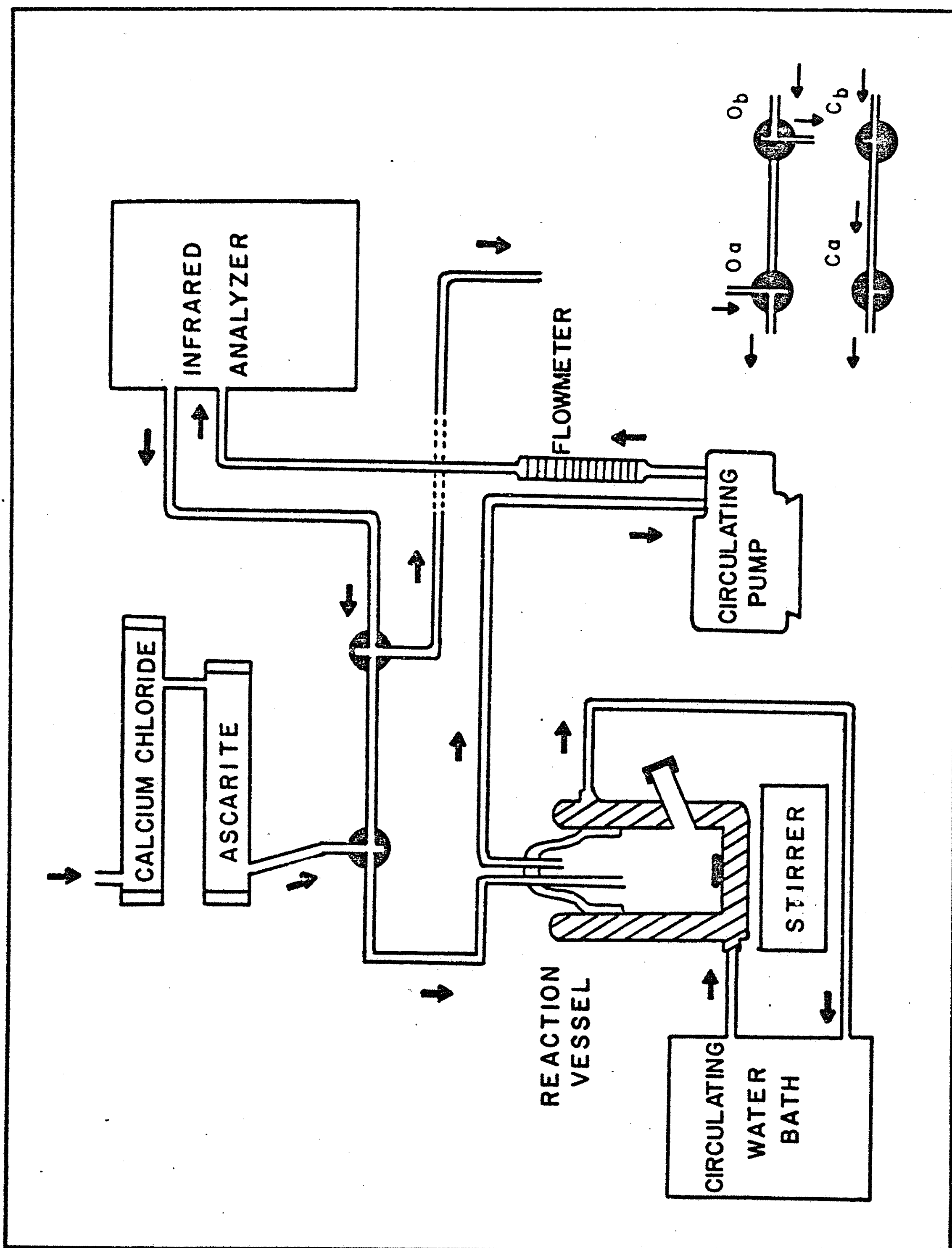
Measurement of CO_2 by Infrared Gas Analysis

a) Description of the system

Carbon dioxide evolved from metabolically active cultures was monitored in the gas phase of the closed circuit apparatus depicted in diagram 2. The bacterial cell suspension (2 ml) was contained in a 25 ml water-jacketed reaction vessel equipped with a 29/32 ground glass joint with inlet and outlet ports and serum-stoppered side-arm. The cell suspension was stirred at 750 rpm by a magnetic stirrer. Temperature was controlled to $\pm$ 0.05^{o}C by a Neslab model RTE-3 circulating water bath (Neslab Instruments, Inc., Portsmouth, N.H.). Gases were circulated through the system at 1.4 L min^{-1} by a model 7071 Masterflex pump (Cole-Parmer Instrument Co., Chicago, Ill.). Carbon dioxide in the gas stream was monitored by a Beckman model 215 - B infrared gas analyzer (Beckman Instruments, Inc., Fullerton, Ca.).

b) Assay of CO_2 by infrared gas analysis

The Beckman 215-B infrared gas analyzer was equipped with reference and sample cells with an optical path-length of 13½ inches. Two measurement ranges were provided, 0-250 ppm and 0-600 ppm.



INFRARED ANALYZER
FLOWMETER
CIRCULATING PUMP
CALCIUM CHLORIDE
ASCARITE
REACTION VESSEL
STIRRER
CIRCULATING WATER BATH
Oa
Ob
Ca
Cb

Calibration gases were, pure nitrogen for zero calibration, and 365 ± 10 ppm and 210 ± 10 ppm (Union Carbide Canada Ltd., Toronto, Ontario) for upscale and downscale calibration respectively. All calibrations were carried out at atmospheric pressure.

The reference cell, filled with nitrogen, was capped off and the nitrogen was replaced every hour during operation. Calibration was checked periodically but remained remarkably constant.

c) <u>Measurement of CO_2 liberated by resting cell suspensions</u>

Two ml of cell suspension in the reaction vessel was purged with dry CO_2-free air to remove dissolved CO_2. The air was made dry and CO_2-free by passing it through Drierite (W.A. Hammond, Xenia, Ohio) and Ascarite (Arthur A. Thomas Co., Philadelphia, Pa.). During the flushing period the gases were passed through the entire system and exhausted (stopcock arrangement O_a and O_b, Diag. 2). The stopcocks were then closed (stopcock arrangement C_a and C_b, Diag. 2) forming a closed circuit. The endogenous rate of CO_2 production was determined for the suspension. The substrate, usually contained in a 100 μl volume, was introduced, by syringe, through the rubber -stoppered side-arm to start the reaction. Substrates which were difficult to solubilize or which were only slightly soluble in water were either added directly to the cell suspension

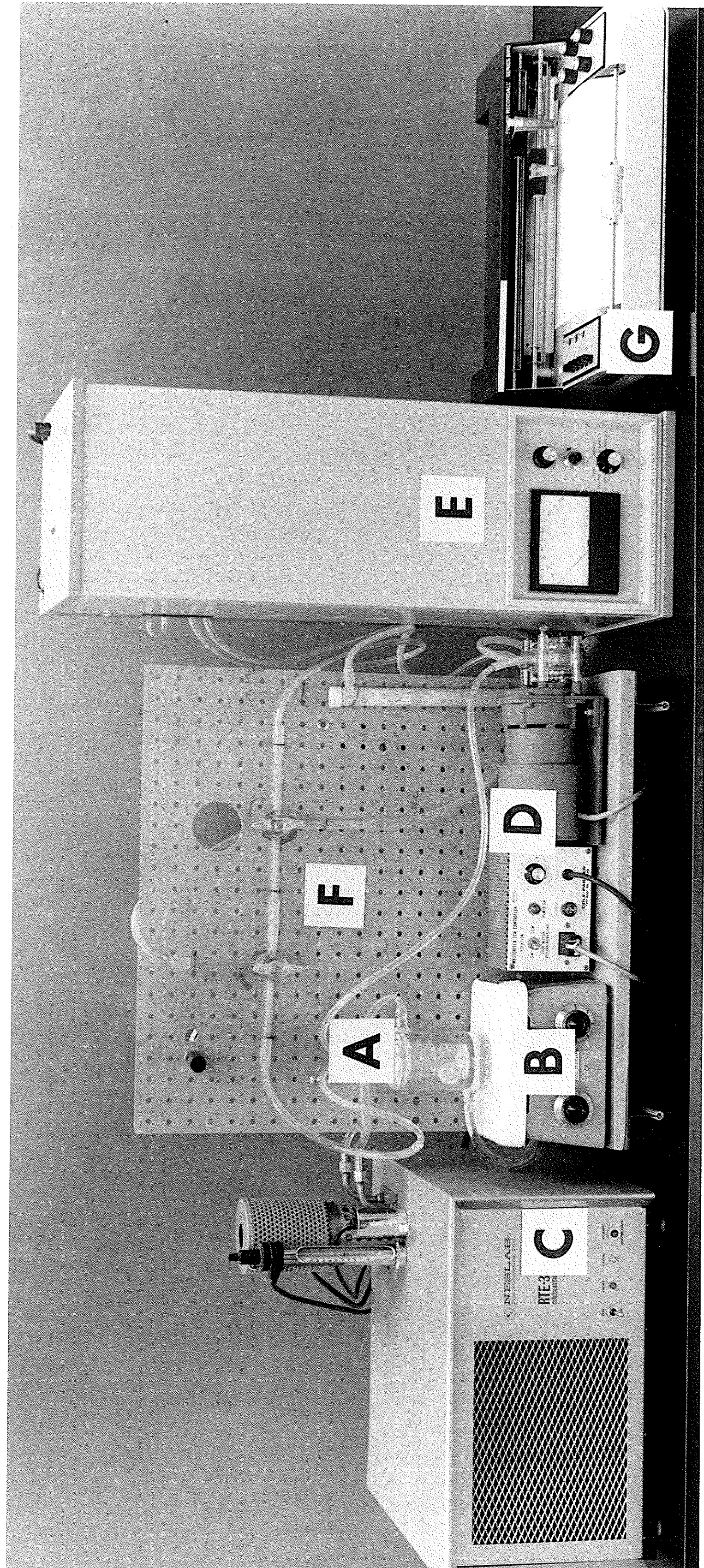
or prepared in a 1 ml volume and added to 1 ml of a 2x cell suspension.

The physical arrangement of the system allowed for both continuous and pulsed monitoring of a reaction. For systems that evolved CO_2 at a rate greater than 10 n moles $min^{-1}mg^{-1}$ dry wt^{-1} the continuous mode was used. The concentration of accumulated CO_2 in the closed system was measured directly and the display on a recorder represented the velocity-time relationship of the reaction. CO_2 was alternately accumulated and evacuated from the system until the rate of liberation of CO_2 was constant.

When the reaction rate was too slow to be measured continuously, the evolved CO_2 was allowed to accumulate in the reaction vessel for a sufficient period of time to give a measurable concentration in the gas phase. The accumulated CO_2 was then expelled from the reaction vessel with the stopcocks in the open position. CO_2 was determined by measuring the area under the peak on the recorder chart when the gas was rapidly flushed out of the circuit. A correction was made for endogenous CO_2 liberated during the accumulation period.

The entire apparatus as it has been described is shown in Plate 3.

PLATE 3. Component arrangement of the infrared gas analysis apparatus.


A
B
C
D
E
F
G
NESLAB
RTE-3

d) Determination of system volume

The volume of the system was about 230 ml (somewhat greater than the theoretical 180 ml volume for maximum 5% error, See page 121) and varied only when the silicone tubing of the Masterflex pump was changed. The volume of the closed circuit was calibrated by first filling the entire system with atmospheric air and determining the CO_2 concentration. The system was then filled with dry CO_2-free air. A small volume of atmospheric air was introduced into the system, by gas-tight syringe, through the rubber-stoppered side-arm and the CO_2 concentration was noted.

The system volume was calculated from the relationship; the ratio of the known volume of injected air to the system volume is equal to the ratio of the respective concentrations.

e) Cleaning the reaction vessel

The used cell suspension was removed from the reaction vessel by aspiration. The reaction vessel was then flushed with phosphate buffer from a wash bottle and aspirated dry several times to ensure complete removal of substrate and cell material.

Effect of agitation rate on CO_2 mass transfer

To determine the influence of agitation speed of the Masterflex stirrer on the rate of liberation of CO_2 into the gas phase the rate of CO_2 evolution was determined at various agitation speeds. Rate determinations were measured in the agitation range of 125 rpm - 900 rpm. The CO_2 was liberated from resting cell suspensions of *Pseudomonas aeruginosa* (to be described) at 30°C, pH 7.0 with 50 mM pyruvate as the substrate.

Pure Cultures

In this study four pure cultures were used; *Pseudomonas aeruginosa* (ATCC 7700), *Escherichia coli* (ATCC 8739) and *Proteus rettgeri* (Department of Microbiology culture collection, isolate #72). All were obtained from the Department of Microbiology, University of Manitoba, culture collection and each was maintained on trypticase soy agar (BBL) after resuscitation from the lyophilized state. The organisms were grown on Trypticase Soy Broth (BBL) unless otherwise stated. *E. coli* K_{12} was obtained from the Genetic Stock Control Center, Yale School of Medicine, New Haven, Conn., and cultured as above.

Growth of Cultures

Colonies of the pure culture were transferred from trypticase soy agar plates into 100 ml of trypticase soy broth. The broth culture was grown at 28°C on a rotary shaker (175 rpm) to a heavy turbidity. This starter culture was used to inoculate 900 ml of trypticase soy broth in a 2-liter Erlenmeyer flask. The culture was allowed to grow to the middle of its exponential phase of growth (estimated by Klett-Summerson colorimeter at 660 nm) with shaking at 175 rpm at 28°C. All pure cultures were grown in a similar manner, unless otherwise specified.

Preparation of Resting Cell Suspensions

Cells were harvested from the growth medium by centrifugation at 16,000 x g in a refrigerated (4°C) Sorvall RC-2B centrifuge. The suspension was washed and resuspended in 50 mM potassium phosphate buffer, pH 7.0, then stirred overnight at 22°C to effect removal of residual substrate. The suspension was again washed and resuspended in the buffer to a final density of 5 mg dry wt ml^{-1} (approximately 10 x growth density).

Effect of pH and Temperature on CO_2 Liberation

To determine the effect of pH on the rate of liberation of CO_2 a resting cell suspension of *P. aeruginosa* was incubated with 50 mM pyruvate in 10 mM phosphate buffer. The pH was varied from 4-8 and the CO_2 produced from pyruvate was measured. Temperature was maintained at 30°C. The pH of the suspension was checked before and after the reaction. The effect of temperature was determined by incubating the resting cell suspension in 20 mM pyruvate at pH 7.0, at the test temperature. The temperature of the water-jacketed reaction vessel was varied from 5°C to 50°C and the CO_2 evolved from the pyruvate was measured.

Confirmation that CO_2 Evolves from a Biological Reaction

To ensure that the CO_2 evolved from a suspension metabolizing an organic substrate was strictly biological in origin, the metabolic inhibitors sodium azide and mercuric chloride were added to the *Pseudomonas* suspension. Sodium azide (5, 10, and 25 mM final conc.) was pre-incubated with the suspension during the flushing period before substrate (pyruvate) was added; mercuric chloride (10 mM final conc.) was added directly to a suspension actively producing CO_2 from pyruvate.

Determination of Acetate and Phenol by Gas Chromatography

Residual acetate and phenol were determined quantitatively by gas-solid chromatography on a Varian Aerograph model 2100 gas chromatograph (Varian Aerograph, Walnut Creek, Cal.), equipped with a flame ionization detector. The column was a 1.83 m x 3 mm I.D. glass "U" column packed with Chromosorb 102, 80/100 mesh (Johns Manville, Celite Div., Denver, Co.). Chromosorb 102 is a high surface-area solid adsorbent which permits direct analysis of aqueous organic solutions.

Operational parameters for acetate and phenol determination were:

	Acetate	Phenol
Column temperature (^{o}C)	195	220
Injection temperature (^{o}C)	220	240
Detector temperature (^{o}C)	220	240
Attenuation	64	16
Range (amp/mv)	10^{-12}	10^{-11}
Air flow (ml/min)	300	300
Hydrogen flow (ml/min)	35	35
Carrier gas (N_2) flow (ml/min)	31	65

Calibration curves were constructed for each compound by relating the amount of compound to total area under the resultant peak on the recorder measured by a disc-type integrator.

R E S U L T S

RESULTS

Standardization of the CO_2 Measurement Apparatus

The Beckman 215B infrared gas analyzer was calibrated by introducing an analyzed standard gas (CO_2 in nitrogen) into the sample cell (with nitrogen in the reference cell) and adjusting the gain setting so that the meter deflection corresponded to that supplied by the manufacturer's calibration chart.

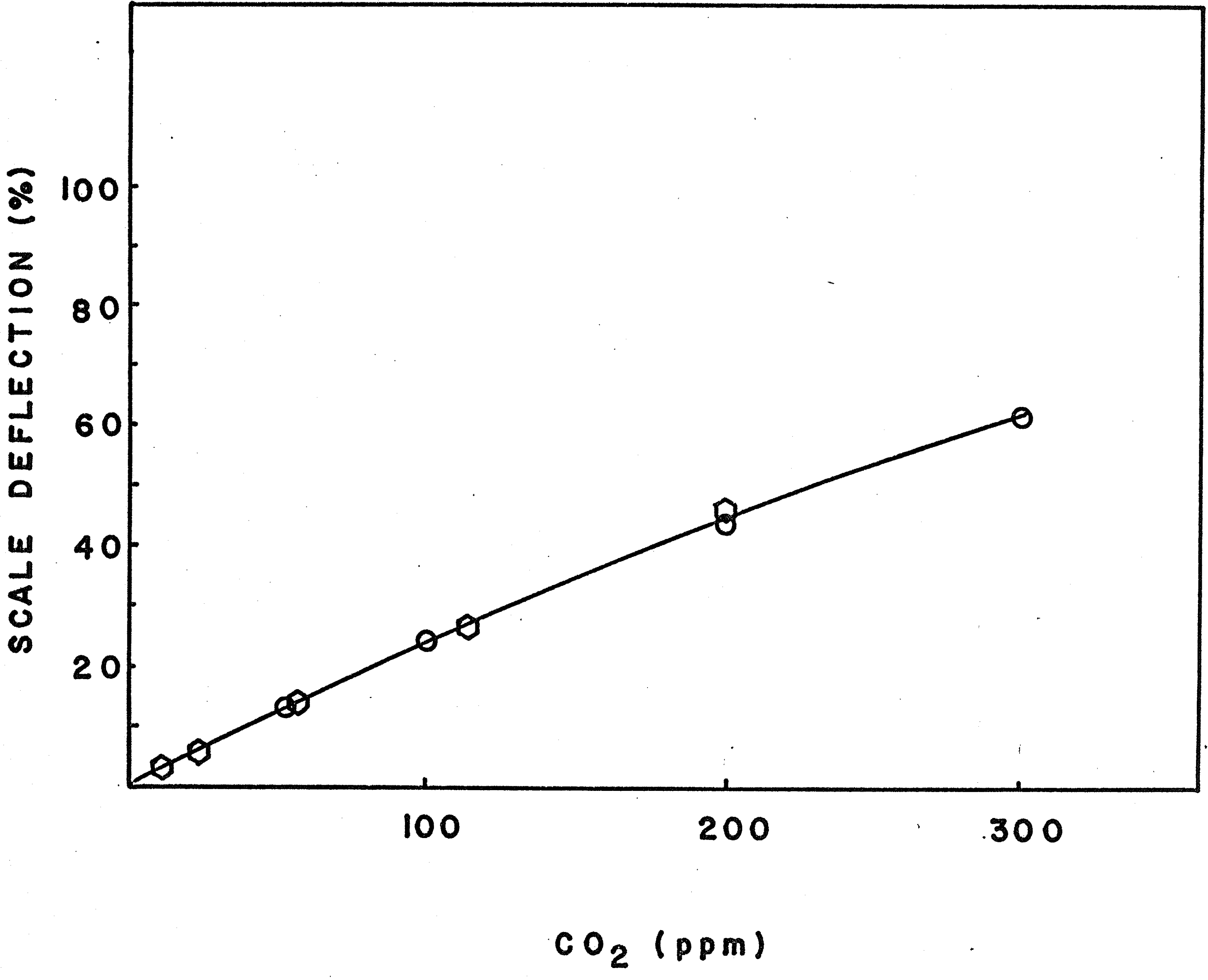
A further check was made by liberating CO_2 from a solution of sodium carbonate by acidification with 2N sulphuric acid. The reaction vessel, which was in the closed circuit mode, had been previously evacuated and refilled with CO_2-free air. Figure 5 shows that this method of calibration is in excellent agreement with that of the standard gas.

When carbon dioxide was purged out of the reaction vessel, using CO_2-free air as the sweep gas, the area under the peak on the recorder chart of the recorder (Recordall Series 5000, dual pen; Fisher Sci.), was used to measure the CO_2 evolved. Peak area was measured by a disc integrator and Figure 6 shows the linear relation between integrator units and amount of CO_2.

FIGURE 5. Calibration curve for Beckman 215 infrared gas analyzer.

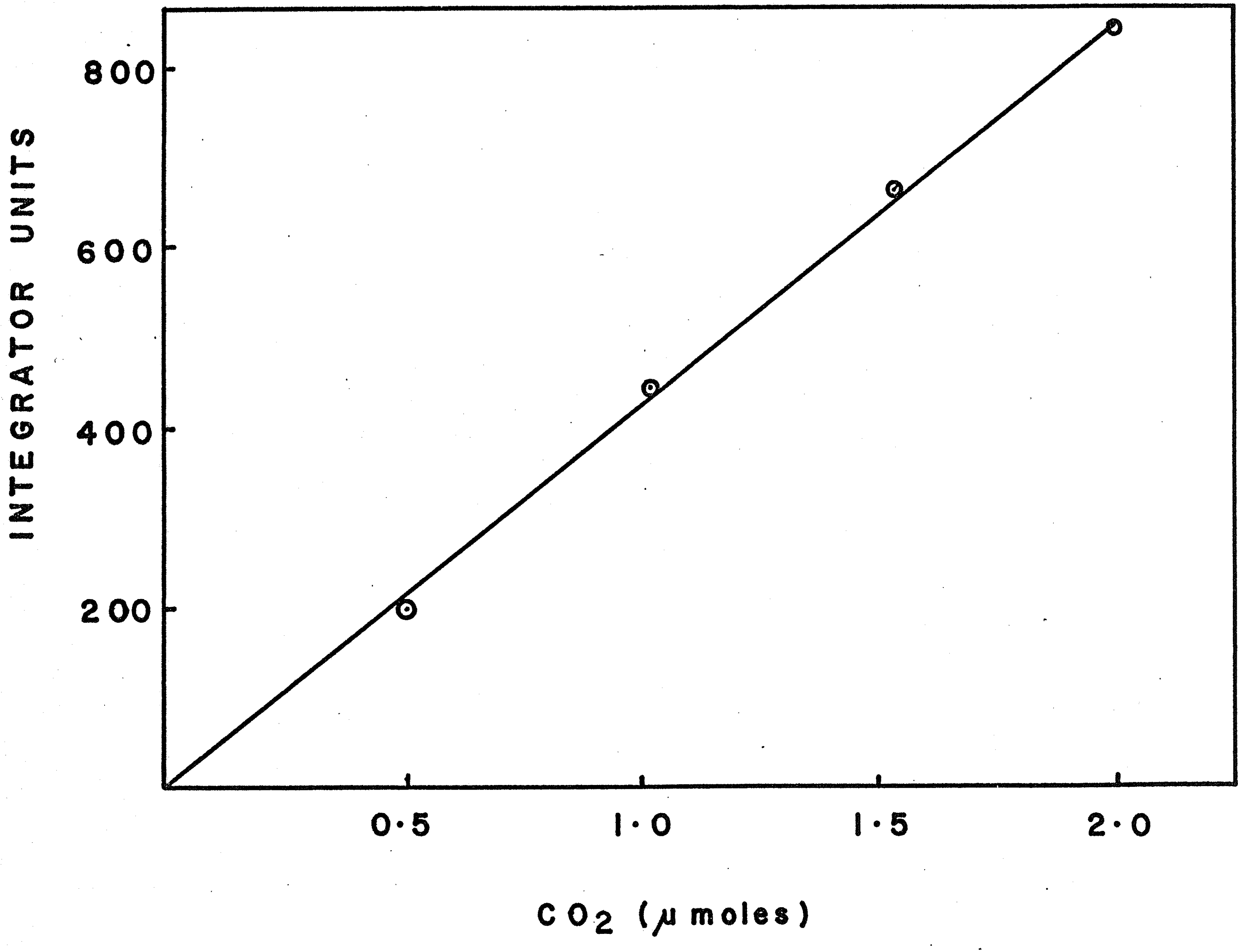
O Standard analyzed gas

O CO_2 liberated from Na_2CO_3



SCALE DEFLECTION (%)
100
80
60
40
20
100
200
300
CO_2 (ppm)

FIGURE 6. Calibration curve showing the relationship between peak area in integrator units and amount of CO_2 (μMoles).


INTEGRATOR UNITS
800
600
400
200
0·5
1·0
1·5
2·0
CO_2 (μ moles)

The Effect of Agitation on Evolution of CO_2

Carbon dioxide evolved from an agitation system is affected by agitation speed. The rate of liberation of CO_2 from a suspension of *Pseudomonas aeruginosa* metabolizing 50 mM pyruvate at 30°C and pH 7.0 was directly proportional to the stirring rate up to 500 rpm, for the system described, as indicated by Figure 7 . Above 500 rpm the rate of liberation is not affected by agitation speed.

Liberation of CO_2 by Whole Cell Preparations

Effect of Amount of Cell Material

The rate of liberation of CO_2 at substrate-saturation, by a *P. aeruginosa* RCS is directly proportional to the amount of cell material present. As shown in Figure 8 this relationship is linear up to at least 10 mg dry weight of cell material. CO_2 evolution was determined when 50 mM pyruvate was supplied as exogenous substrate, temperature was 30°C and pH was 7.0.

Effect of Temperature on CO_2 Evolution

Temperature markedly affects the rate of CO_2 evolution by resting cells from exogenously supplied substrate. Figure 9 shows the effect temperature has on the rate at which a resting cell preparation of

FIGURE 7. Effect of agitation speed on the rate of CO_2-liberation by resting cell suspensions of *Pseudomonas aeruginosa*. Exogenous substrate was 50 mM pyruvate; conditions were 30°C and pH 7.0.

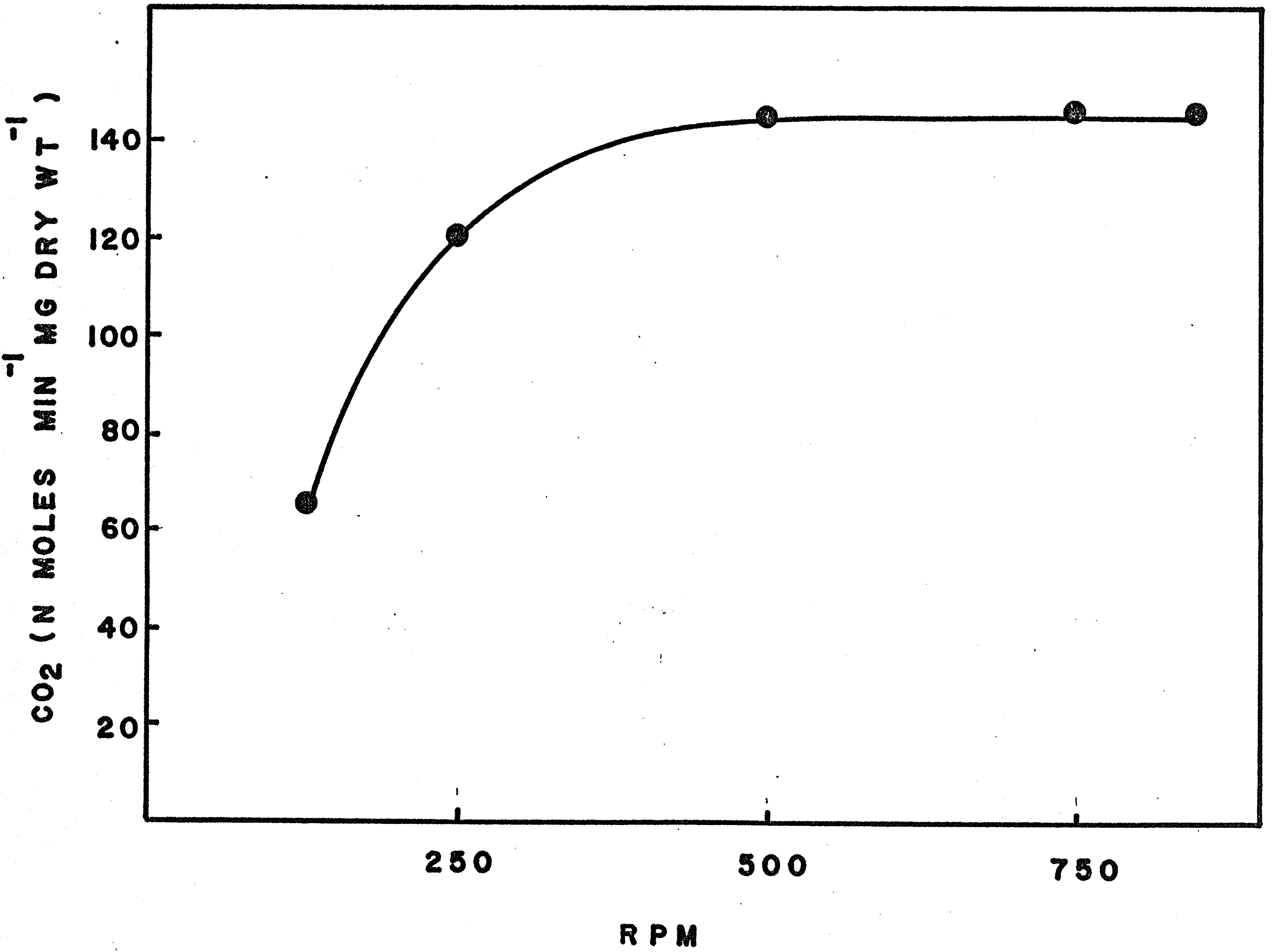
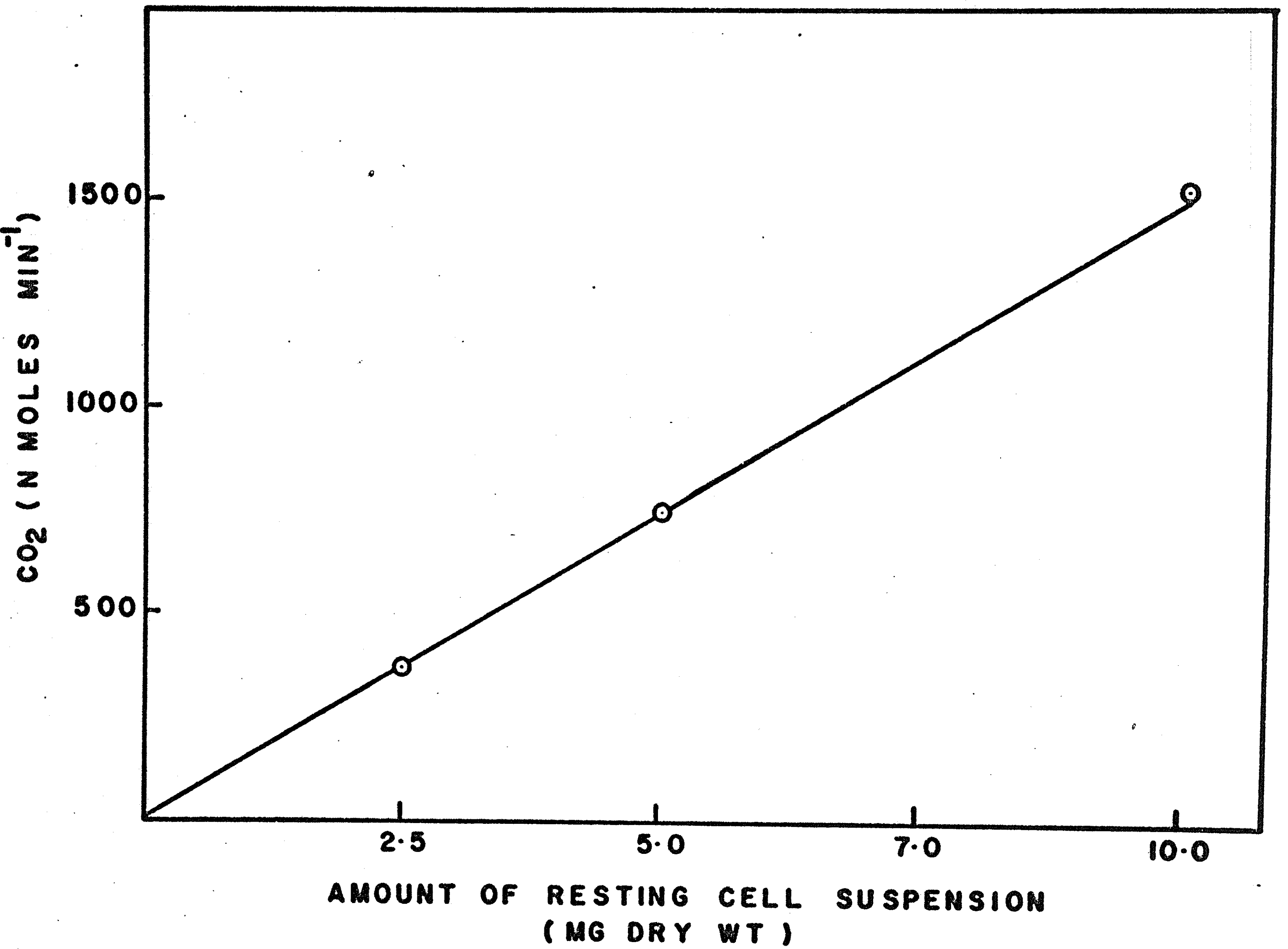
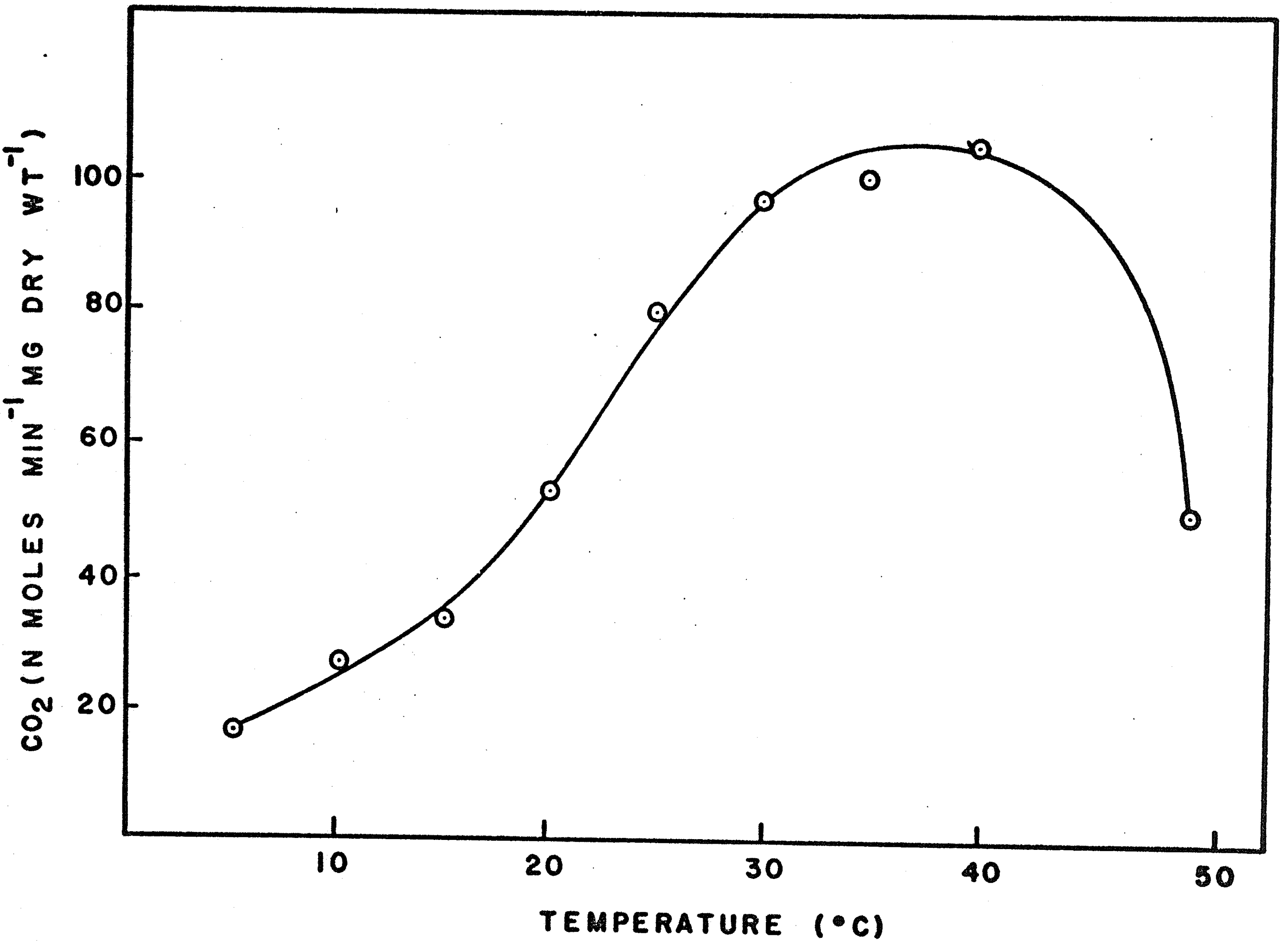

CO2 (N MOLES MIN-1 MG DRY WT-1)
140
120
100
80
60
40
20
250
500
750
RPM

FIGURE 8. Relationship between the rate of CO_2 liberation and the amount of cell material (mg dry weight). *P. aeruginosa* metabolizing 50 mM pyruvate at 30°C and pH 7.0.



CO_2 (N MOLES MIN^{-1})
1500
1000
500
2·5
5·0
7·0
10·0
AMOUNT OF RESTING CELL SUSPENSION
(MG DRY WT)

FIGURE 9. Effect of temperature on the rate of CO_2 production by resting cell suspensions of P. aeruginosa. Suspension fluid was pH 7.0.



CO_2 (N MOLES MIN^{-1} MG DRY WT^{-1})
100
80
60
40
20
10
20
30
40
50
TEMPERATURE (°C)

Pseudomonas aeruginosa liberated CO_2 from pyruvate at pH 7.0. An optimum occurred between 30°C to 40°C with a rapid drop-off on either side.

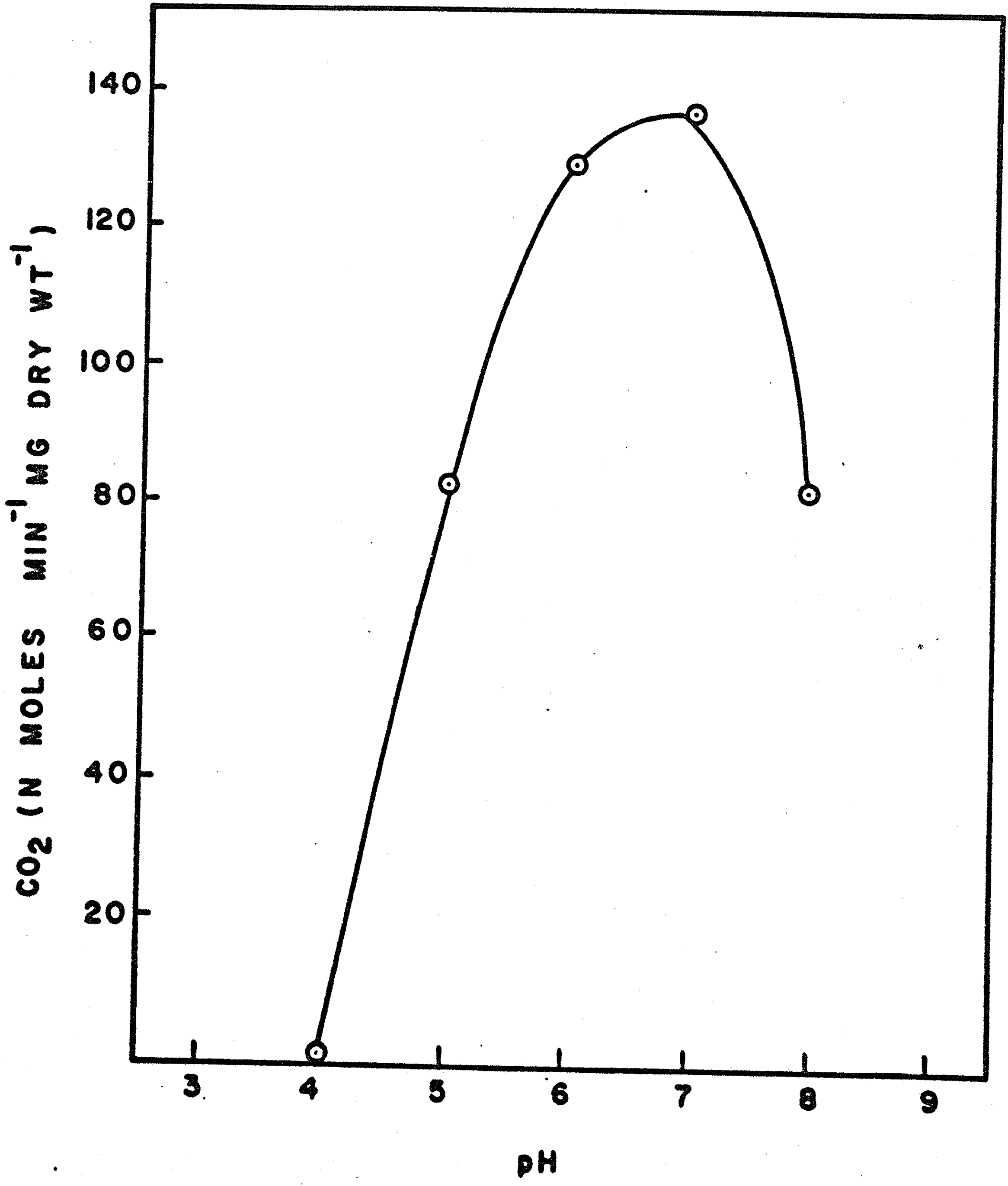
Effect of pH

The influence of pH on the rate of liberation of CO_2 by the resting cell suspension was much more dramatic than the temperature effect. With pyruvate as substrate and at 30°C, exogenous CO_2 liberation was shown to be sharply sensitive to pH of the suspending fluid. Figure 10 shows that the pH optimum for exogenous CO_2 liberation was 6.5.

Effect of Sodium Azide and Mercuric Chloride on CO_2 Production

To ensure that the carbon dioxide evolved was produced by the resting cell suspension the metabolic inhibitors sodium azide and mercuric chloride were added to the suspension and the effect on the rate of CO_2 production was observed. Sodium azide was added in varying amounts and the suspension was preincubated for 10 minutes prior to addition of substrate. Table 6 shows that the rate of CO_2 evolution by *P. aeruginosa* metabolizing pyruvate decreased as the concentration by sodium azide increased. There was 65% inhibition of 25 mM sodium azide. Similarly mercuric chloride (10 mM) added to a suspension metabolizing pyruvate initiated

FIGURE 10. Effect of pH on rate of CO_2 production by resting cell suspensions of P. aeruginosa at 30°C with pyruvate as substrate.



140
120
100
80
60
40
20
CO_2 (N MOLES MIN^{-1} MG DRY WT^{-1})
3
4
5
6
7
8
9
pH

TABLE 6.

Effect of inhibitors on CO_2 liberation by resting cell suspensions of *Pseudomonas aeruginosa* respiring pyruvate at 30°C, pH 7.0.

Inhibitor	Final conc. (mM)	% Inhibition
NaN_3	0	-
	5	36
	25	63
$HgCl_2$	0	-
	10	100

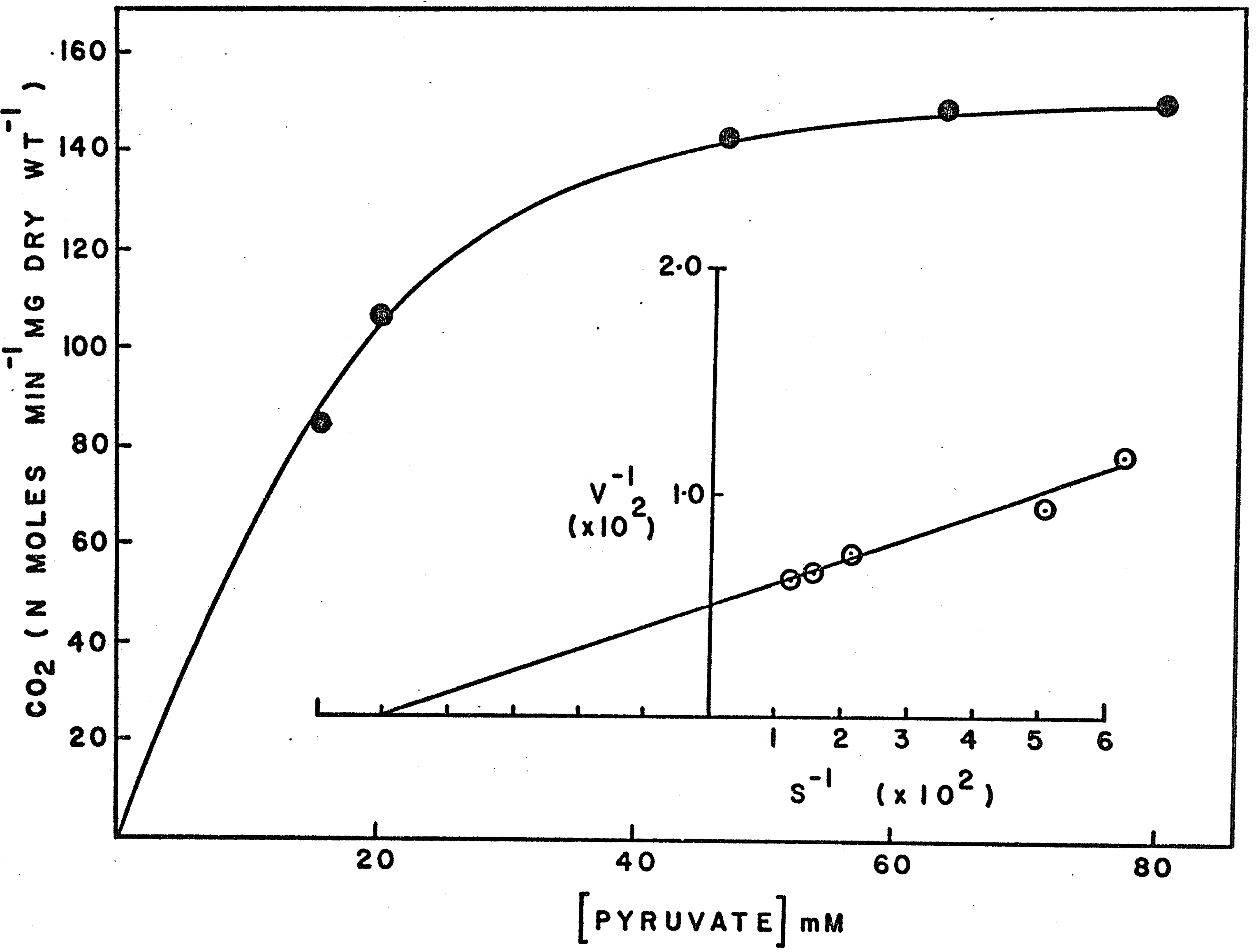
an immediate cessation of CO_2 production by the resting cell suspension.

Effect of Substrate on CO_2 Liberation

The rate of CO_2 evolution by intact bacterial resting cell suspensions responded to increased exogenous substrate by exhibiting saturation kinetics. The kinetics of the rate reaction were describable in terms of the Michaelis-Menten equation. Initial velocities of CO_2 production were plotted against exogenous substrate concentration and the resultant curve was a rectangular hyperbola. The curves were replotted by the method of Lineweaver and Burk (123) for simple graphical interpretation. Representative examples of the response are depicted for resting cell suspensions of *P. aeruginosa* at 30°C and pH 7.0 for pyruvate (Figure 11), L-serine (Figure 12), L-alanine (Figure 13), L-glutamate (Figure 14), L-aspartate (Figure 15) and L-asparagine (Figure 16). The kinetic constants for each of these substrates are listed in Table 7. The V_{max} is the macimum velocity of CO_2 production attained under the conditions, and K_{cat} is the substrate concentration at which the velocity of CO_2 production is half-maximal. It is similar to the apparent K_m of Michaelis and Menten.

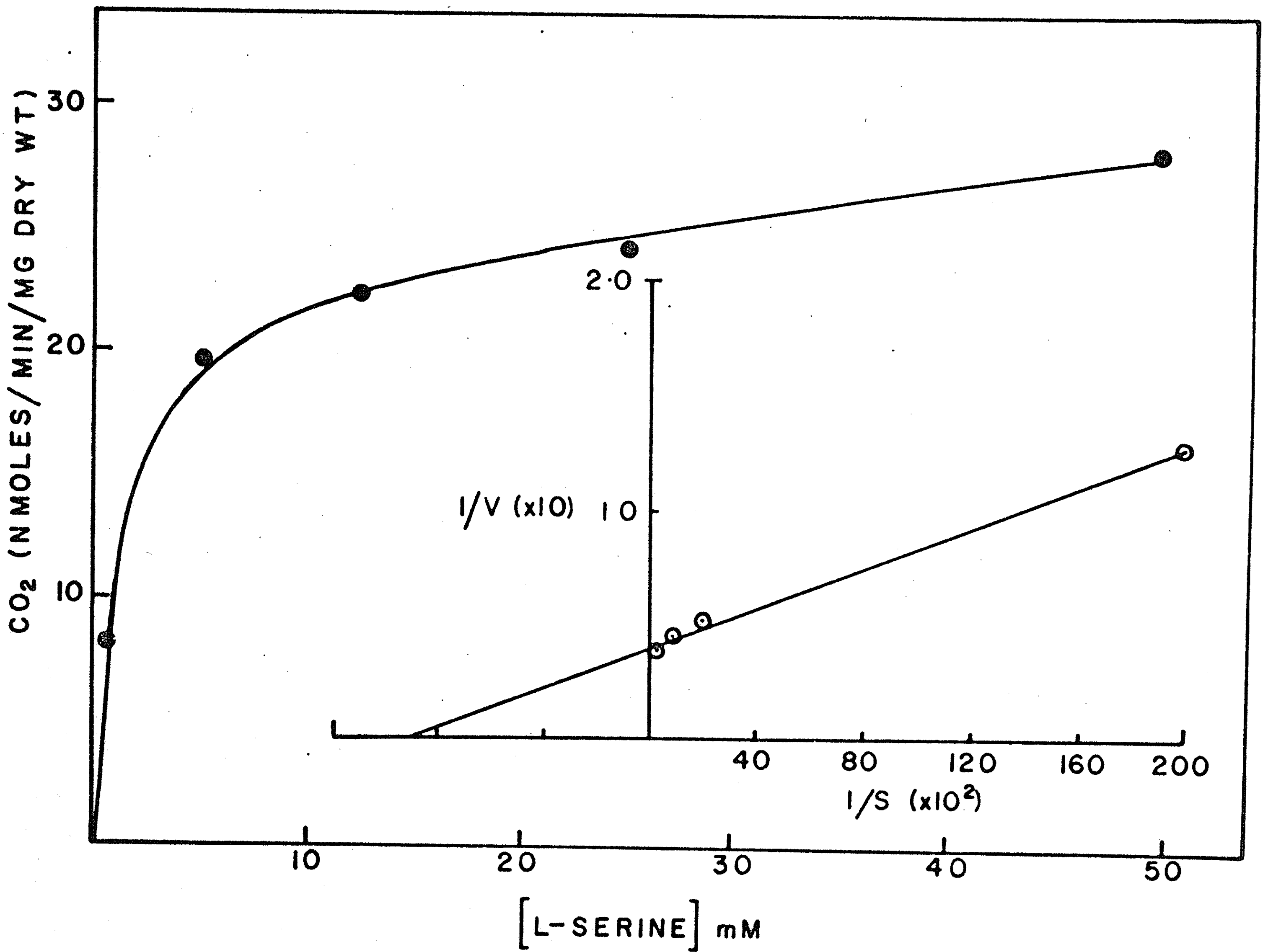
Table 8 lists 26 organic compounds tested for CO_2 release by a suspension of *P. aeruginosa* at 30°C and pH 7.0. The response to the substrates elicited two orders

FIGURE 11. Effect of pyruvate concentration on the rate of CO_2 production by resting cell suspensions of *P. aeruginosa* at $30^{\circ}C$ and pH 7.0. Inset shows double reciprocal replot.



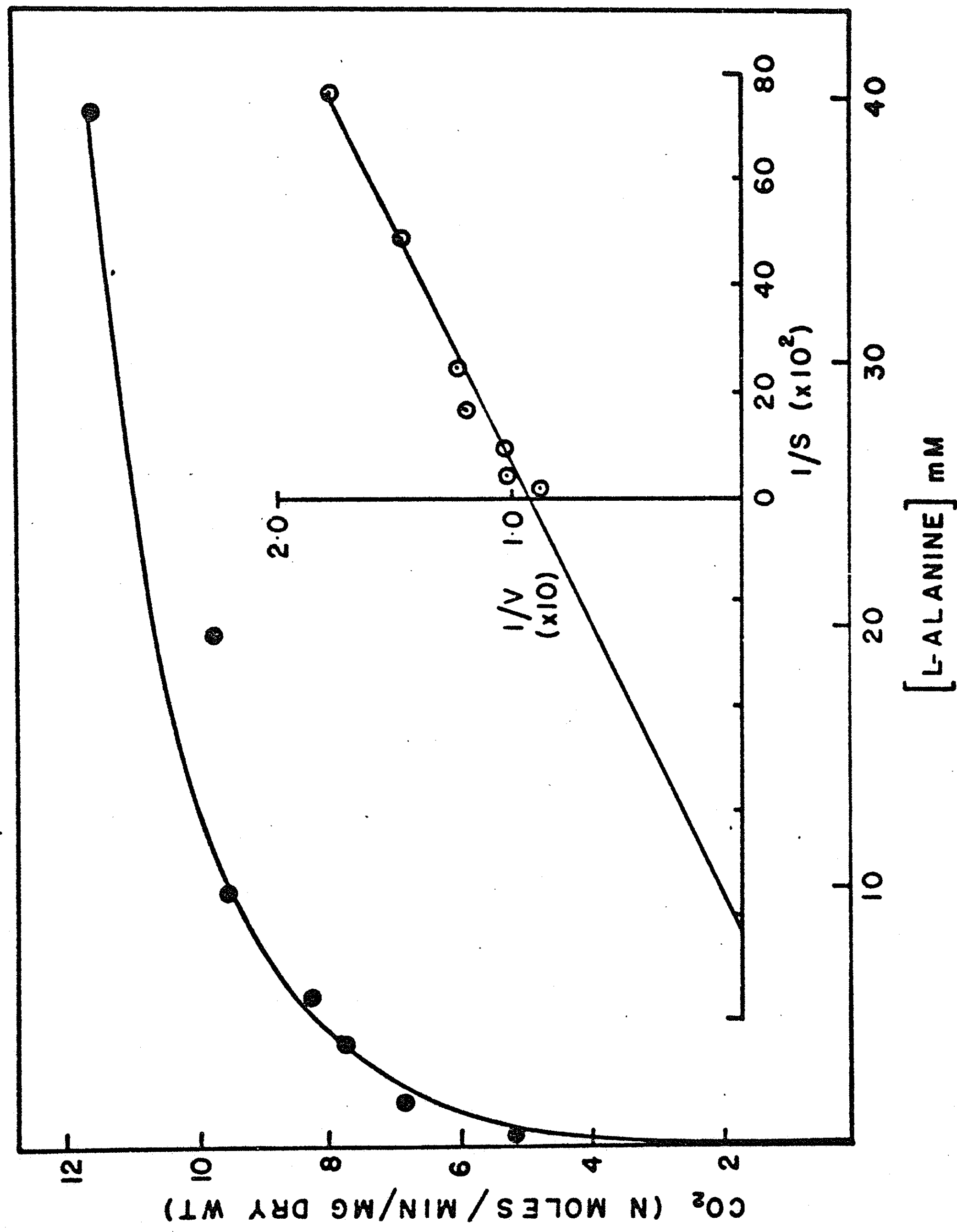
CO_2 (N MOLES MIN^{-1} MG DRY WT^{-1})
160
140
120
100
80
60
40
20
20
40
60
80
[PYRUVATE] mM
V^{-1} (x10^2)
2·0
1·0
1
2
3
4
5
6
S^{-1} (x10^2)

FIGURE 12. Effect of L-serine concentration. Conditions same as Fig. 11. Inset shows double reciprocal replot.



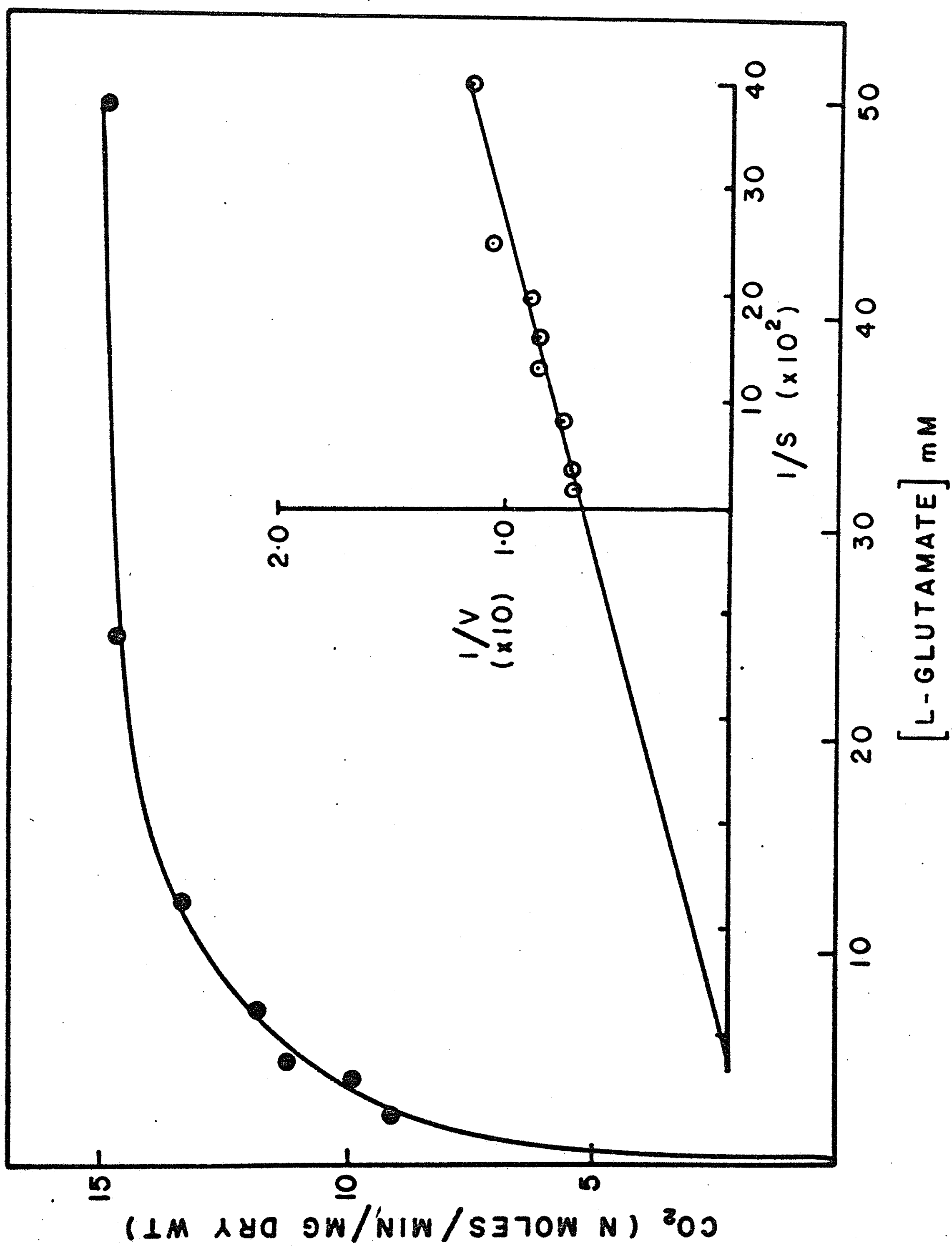
CO_2 (NMOLES/MIN/MG DRY WT)
30
20
10
2.0
1.0
1/V (x10)
40
80
120
160
200
1/S (x10^2)
10
20
30
40
50
[L-SERINE] mM

FIGURE 13. Effect of L-alanine concentration. Conditions same as Fig. 11. Inset shown double reciprocal replot.



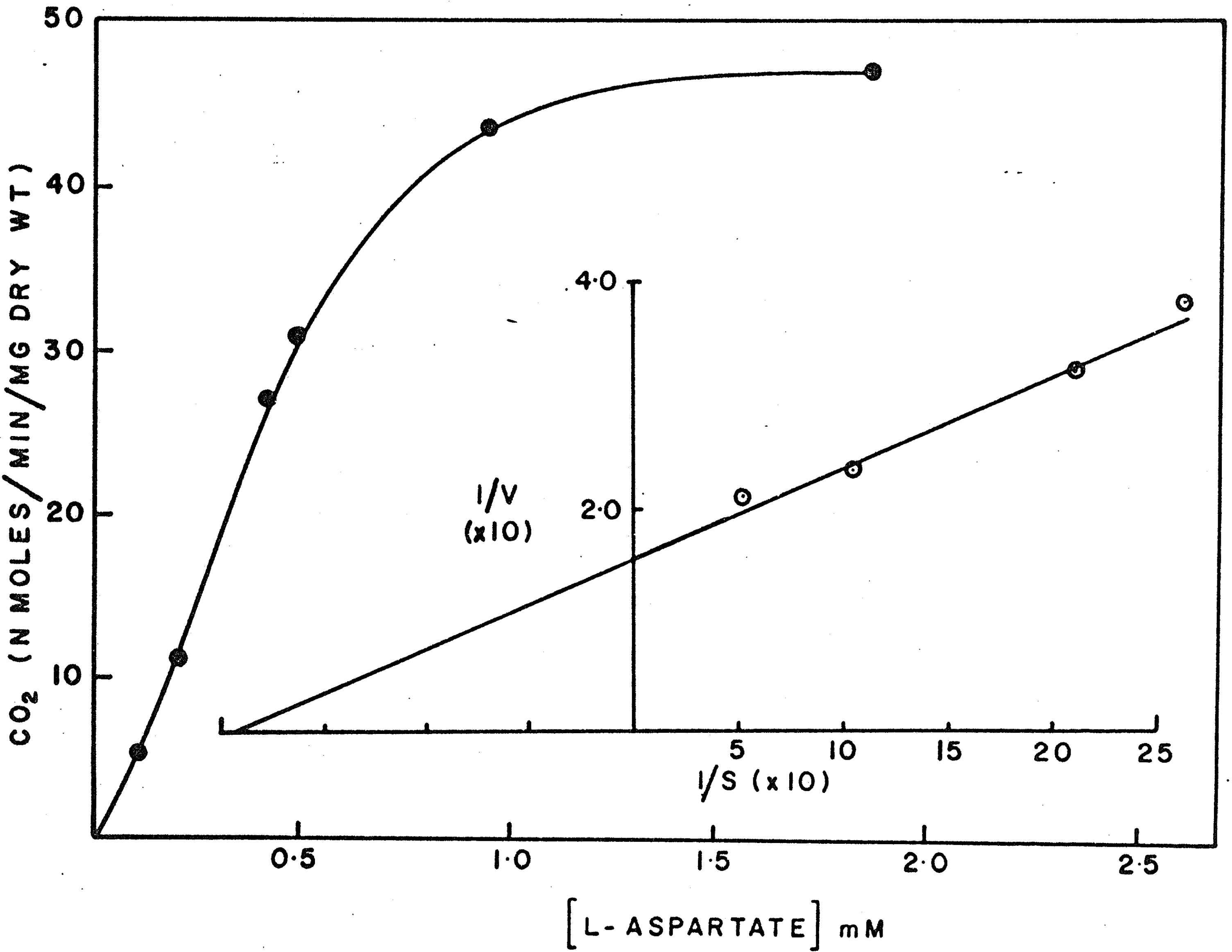
CO_2 (N MOLES / MIN/MG DRY WT)
[L-ALANINE] mM
1/S (x10^2)
1/V (x10)

FIGURE 14. Effect of L-glutamate concentration. Conditions same as Fig. 11. Inset shown double reciprocal replot.



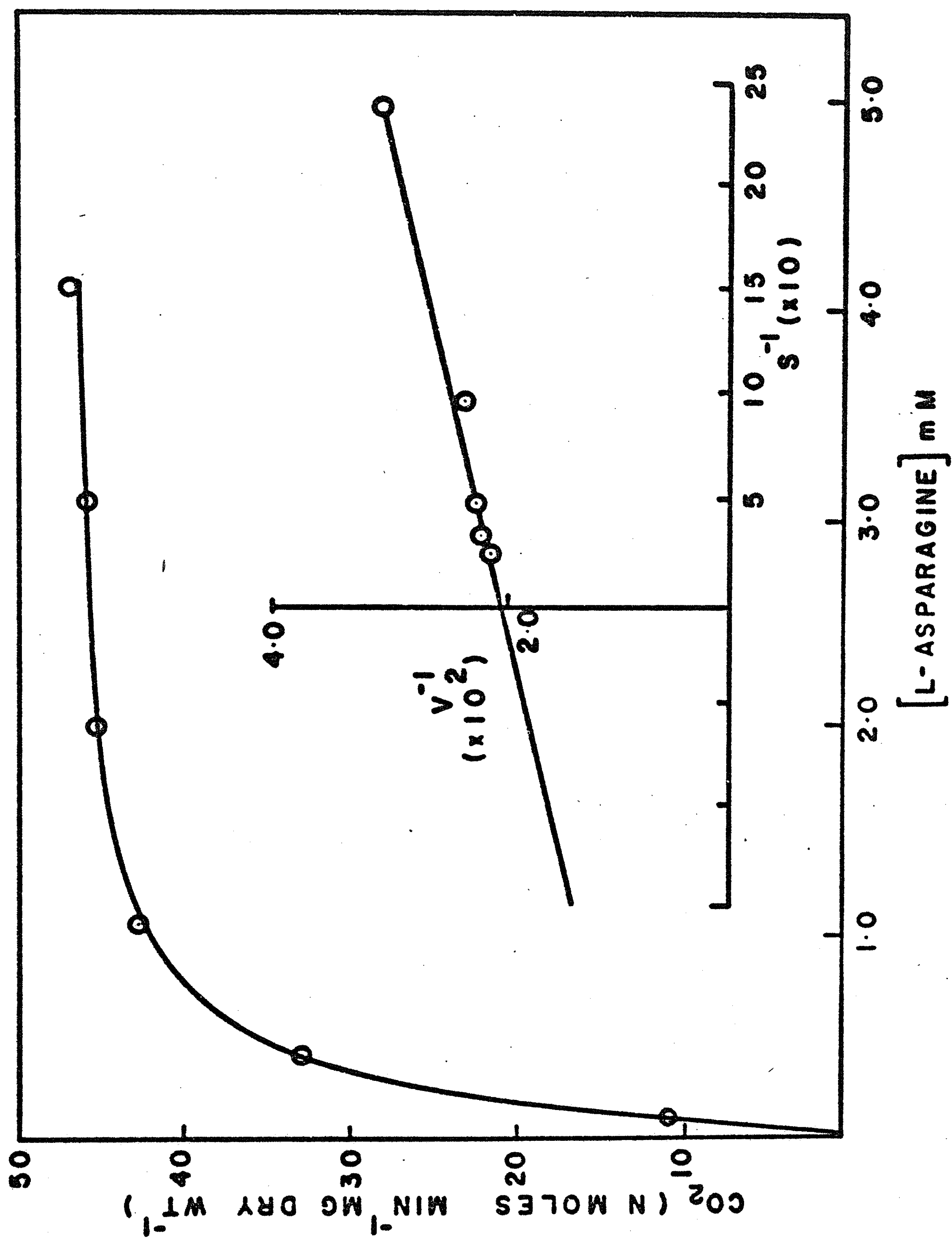
CO_2 (N MOLES/MIN/MG DRY WT)
15
10
5
10
20
30
40
50
[L-GLUTAMATE] mM
1/V (x10)
2·0
1·0
1/S (x10²)
10
20
30
40

FIGURE 15. Effect of L-aspartate concentration. Conditions same as Fig. 11. Inset shown double reciprocal replot.



50
40
30
20
10
CO_2 (N MOLES/MIN/MG DRY WT)
4·0
2·0
1/V (x10)
5
10
15
20
25
1/S (x10)
0·5
1·0
1·5
2·0
2·5
[L-ASPARTATE] mM

FIGURE 16. Effect of L-asparagine concentration. Conditions same as Fig. 11. Inset shows double reciprocal replot.



50
40
30
20
10
CO_2 (N MOLES MIN^{-1} MG DRY WT^{-1})
1·0
2·0
3·0
4·0
5·0
[L-ASPARAGINE] mM
5
10
15
20
25
S^{-1} (x10)
2·0
4·0
V^{-1}
(x10^2)

TABLE 7

Summary of kinetics constants* for *Pseudomonas aeruginosa*

Substrate	V_{CO_2} (n moles min^{-1}mg dry wt^{-1})	K_{cat}(mM)
L-aspartate	60.3	0.5
L-glutamate	15.4	2.0
L-asparagine	49.6	0.2
L-alanine	11.2	1.6
L-serine	26.3	1.8
Pyruvate	181.6	16.7

*Temperature, 30°C, pH, 7.0

TABLE 8.

V_{max} of CO_2 production by a resting cell suspension of *Pseudomonas aeruginosa* at pH 7.0 and 30°C.

Substrate	Conc. (mM)	Length of lag phase	CO_2 production (n Moles min^{-1} mg dry wt $cells^{-1}$
L-alanine	40	12 min	11
L-asparagine	4	10 min	49
L-aspartate	2	8 min	60
L-glutamate	50	12 min	15
L-leucine	50	⩾15 min	12
L-lysine	50	⩾60 min	28
L-proline	50	⩾60 min	94
L-serine	50	15 min	26
L-tryptophan	25	8 min	7
L-tyrosine	1	N.A.**	0
L-threonine	50	10 min	10
glycerol	50	N.A.**	0
glucose	10	N.A.**	0
pyruvate	100	30 min	182
succinate	10	10 min	50
oxalate	50	⩾15 min	10
citrate	50	N.A.**	0
acetate	50	5 min	3
propionate	50	⩾ 4 hr	⩾ 2
butyrate	50	N.A.**	-5
valerate	50	N.A.**	-5
benzoate	50	N.A.**	-5
palmitate	50	⩾ 4 hr	⩾ 13*
stearate	50	⩾ 4 hr	⩾ 13*
myristic acid	50	⩾ 4 hr	⩾ 8*
n-hexane	50	⩾ 4 hr	⩾ 5*

*Saturating substrate concentration not determined.

**Not applicable.

of magnitude range in the rate of CO_2 production (0-182 n moles min^{-1} mg^{-1} dry wt^{-1}). Some substrates; L-tyrosine, glycerol, glucose and citrate, showed no change over the endogenous CO_2 rate, while others; butyrate, valerate and benzoate, were inhibitory at the concentration tested. The concentration of substrate used was either saturating or, in those cases with a lengthy lag phase, arbitrarily selected to give a response.

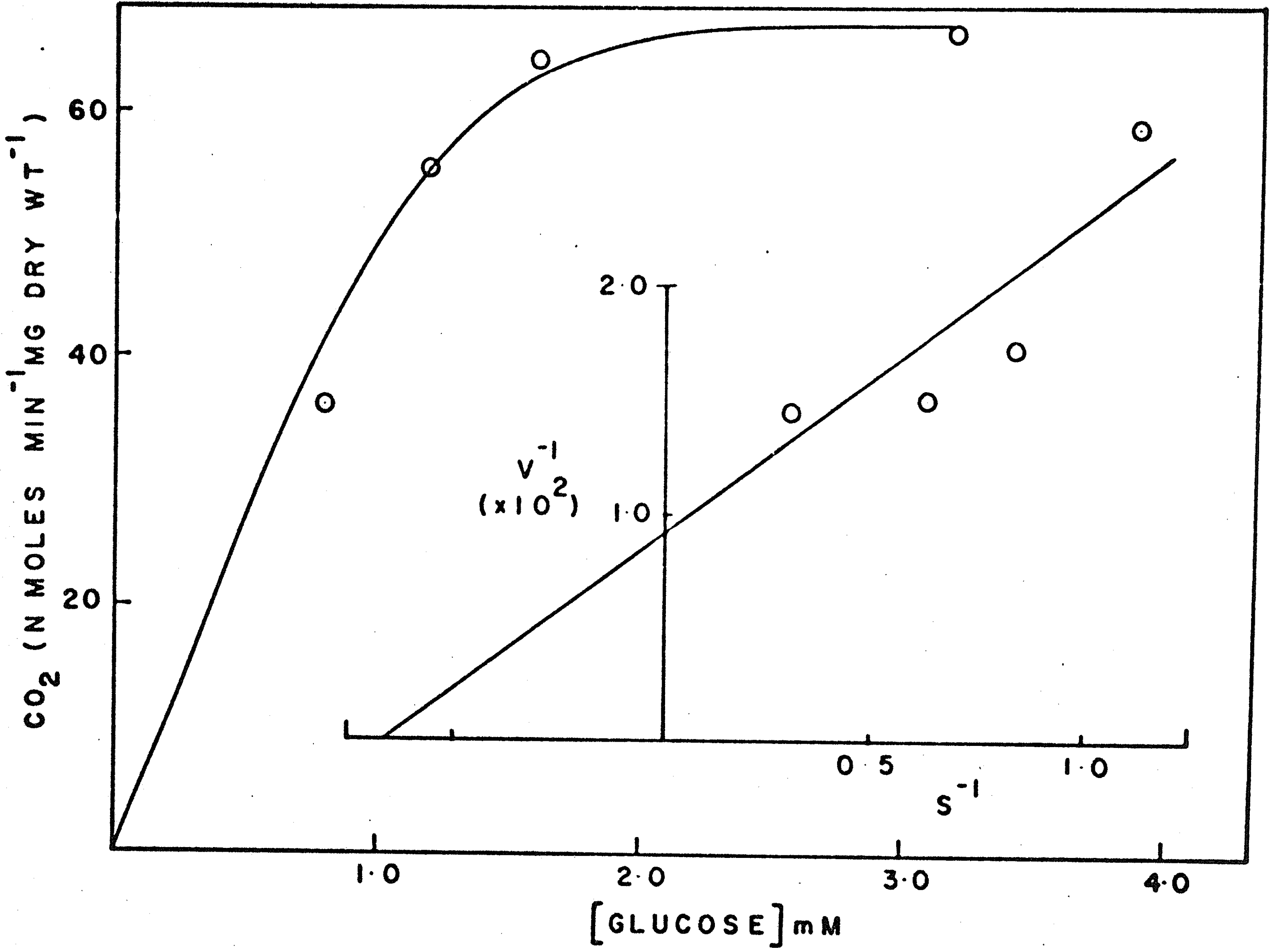
Liberation of CO_2 by *Escherichia coli*

A similar response was demonstrated by *E. coli* metabolizing glucose, at 30°C and pH 7 (Figure 17). CO_2 liberation from acetate by the *E. coli* resting cell suspension under two different growth conditions is shown in Figure 18, with the double reciprocal plots in Figure 19.

Liberation of CO_2 by *Proteus rettgeri*

A study of the effect of temperature on the maximum velocity of CO_2 liberation by *Proteus rettgeri* was made. As expected, there was a increase in maximum velocity of CO_2 production as the temperature was increased. Figure 20 shows this response. Also observed was a change in the K constant. Figure 21 depicts the increasing value of K as temperature was decreased.

FIGURE 17. Effect of glucose concentration on the rate of CO_2 production by resting cell preparations of _E_. _coli_. Conditions were $30^{o}C$ and pH 7.0. Inset shown double reciprocal replot.



60
40
20
CO_2 (N MOLES MIN^{-1}MG^{-1} DRY WT^{-1})
1·0
2·0
3·0
4·0
[GLUCOSE] mM
V^{-1} ($\times 10^2$)
2·0
1·0
0·5
1·0
S^{-1}

FIGURE 18. Effect of acetate concentration on CO_2 production by resting cells of *E. coli* previously grown in medium containing acetate and no glucose, or in medium containing glucose and no acetate. Conditions were 30°C and pH 7.0.

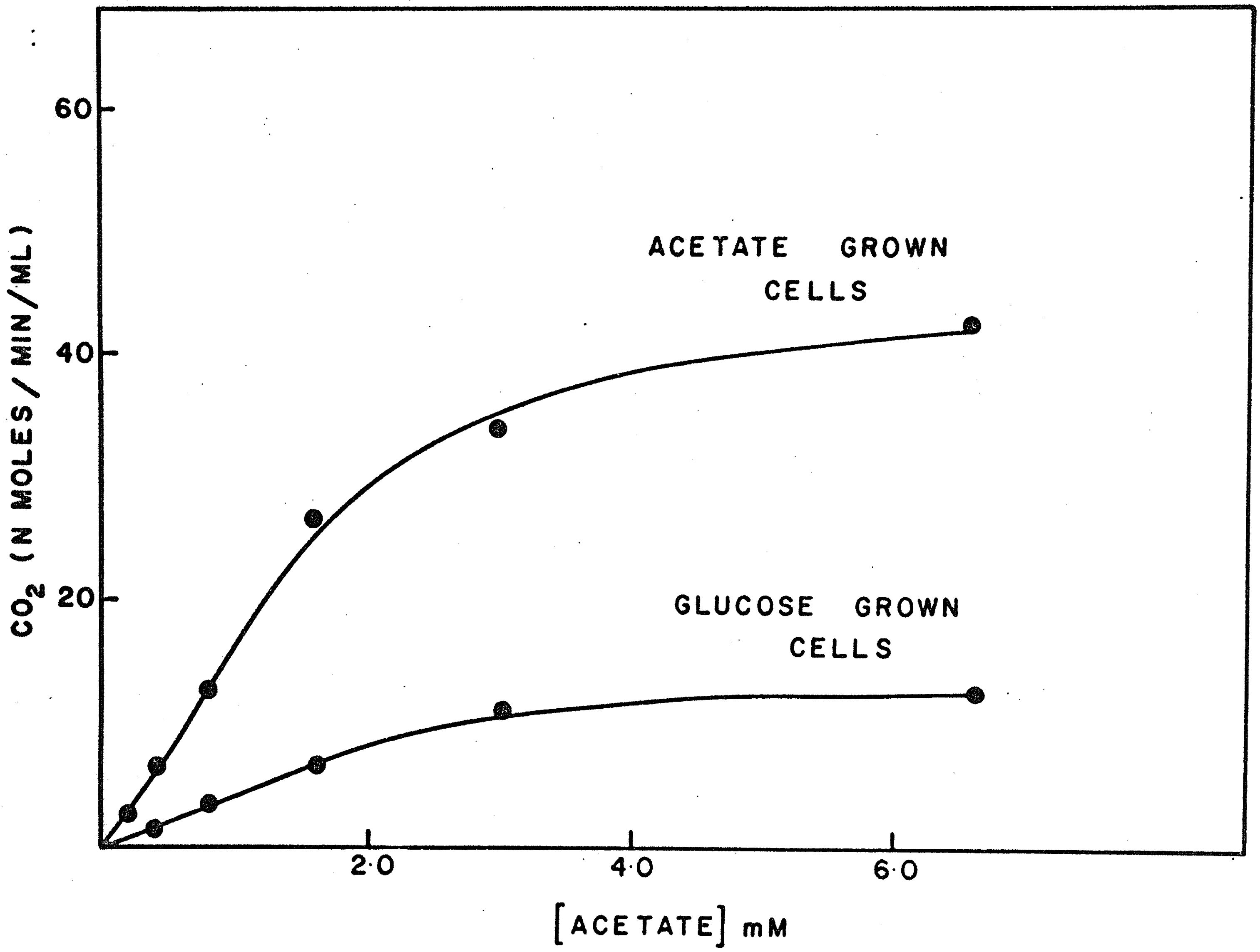

60
40
20
CO_2 (N MOLES / MIN / ML)
ACETATE GROWN
CELLS
GLUCOSE GROWN
CELLS
2.0
4.0
6.0
[ACETATE] mM

FIGURE 19. Fig. 18 replotted in Lineweaver-Burk fashion to enhance the effect on V_{max} and K_m.

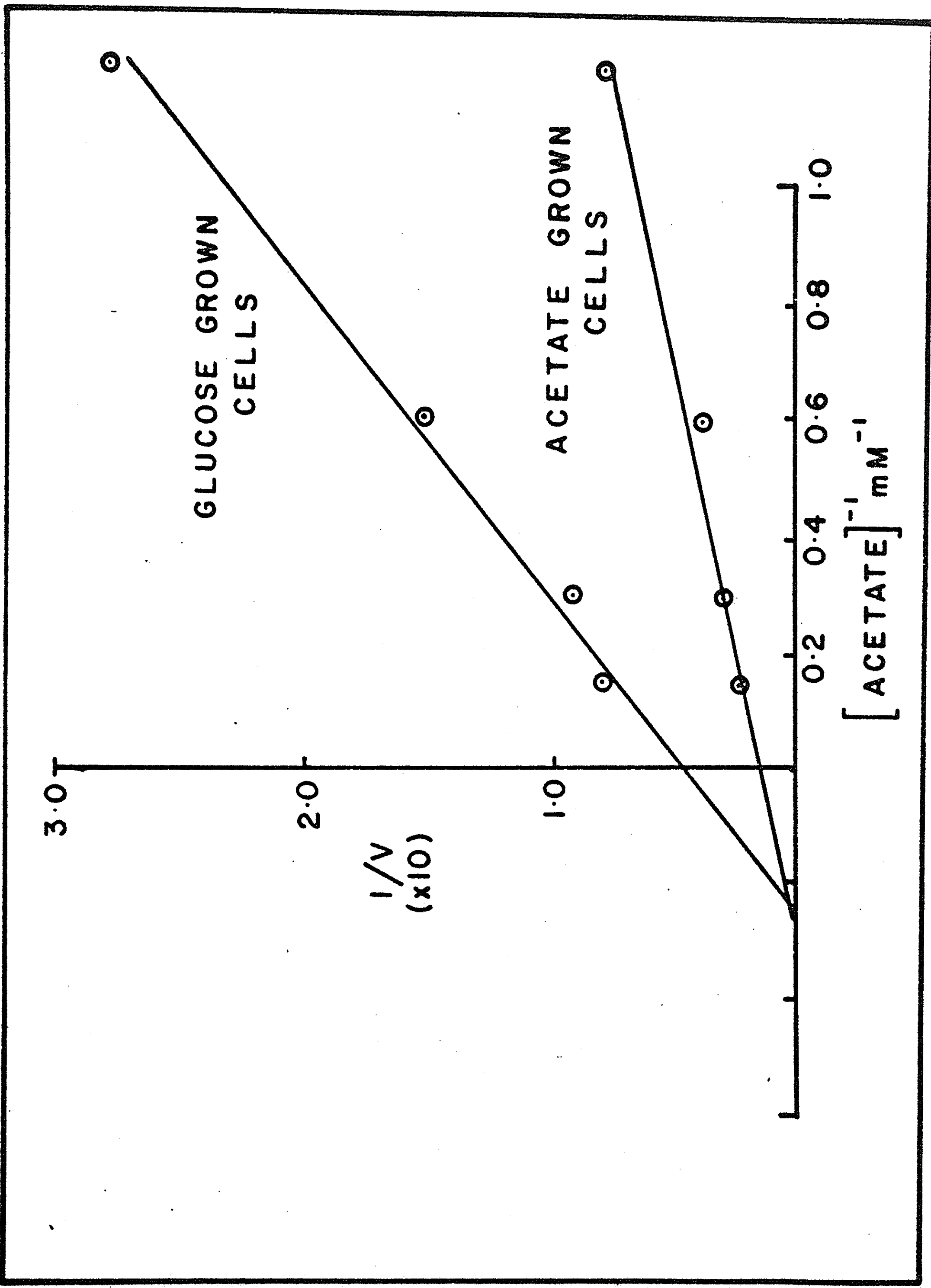

GLUCOSE GROWN CELLS
ACETATE GROWN CELLS
1/V (x10)
3·0
2·0
1·0
0·2
0·4
0·6
0·8
1·0
[ACETATE]$^{-1}$ mM^{-1}

FIGURE 20. Rate of production of CO_2 by resting cell suspensions of *Proteus rettgeri* showing the effect of glucose concentration at $10^{o}C$, $20^{o}C$ and $30^{o}C$. Suspension was at pH 7.0.

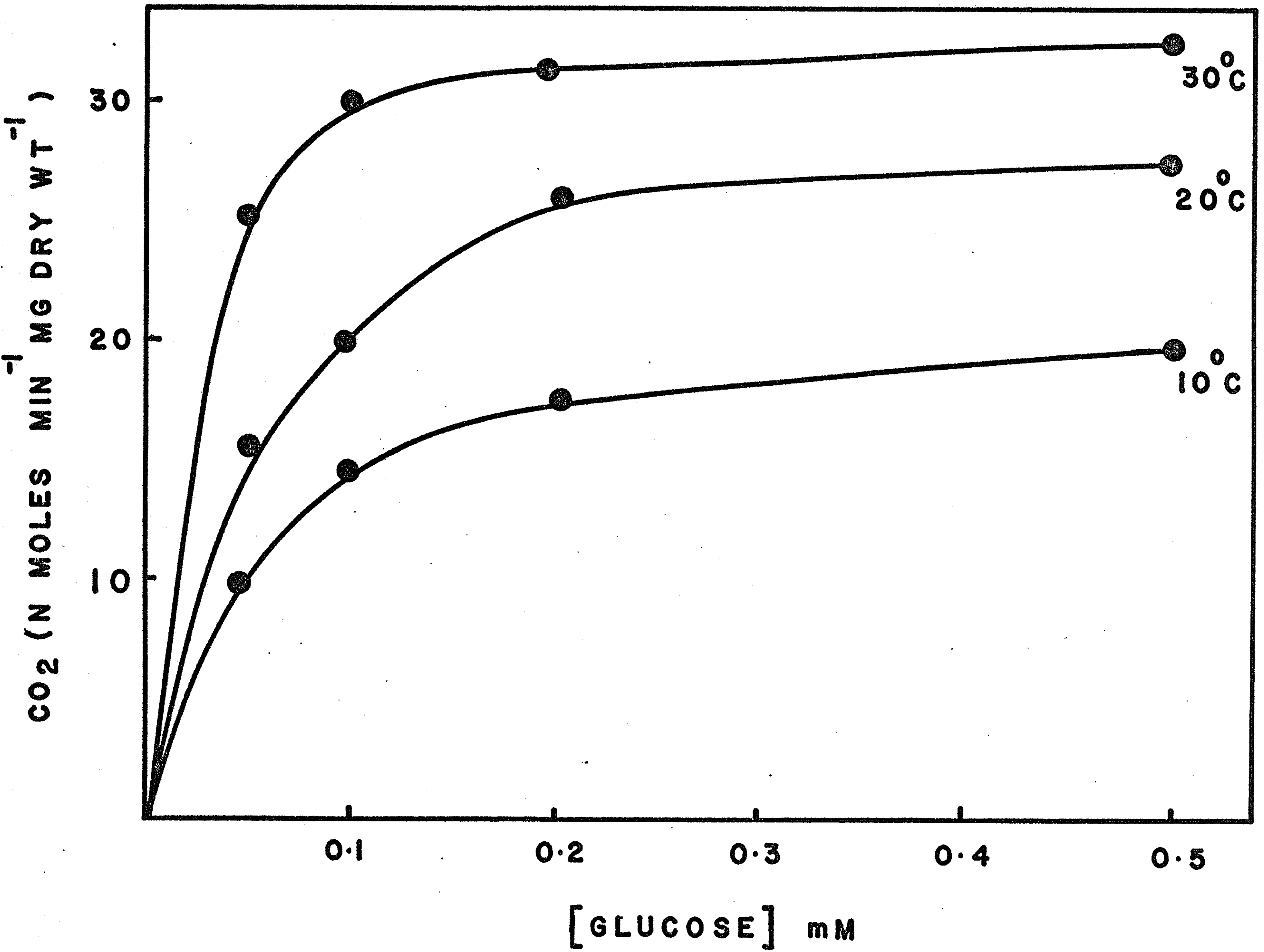
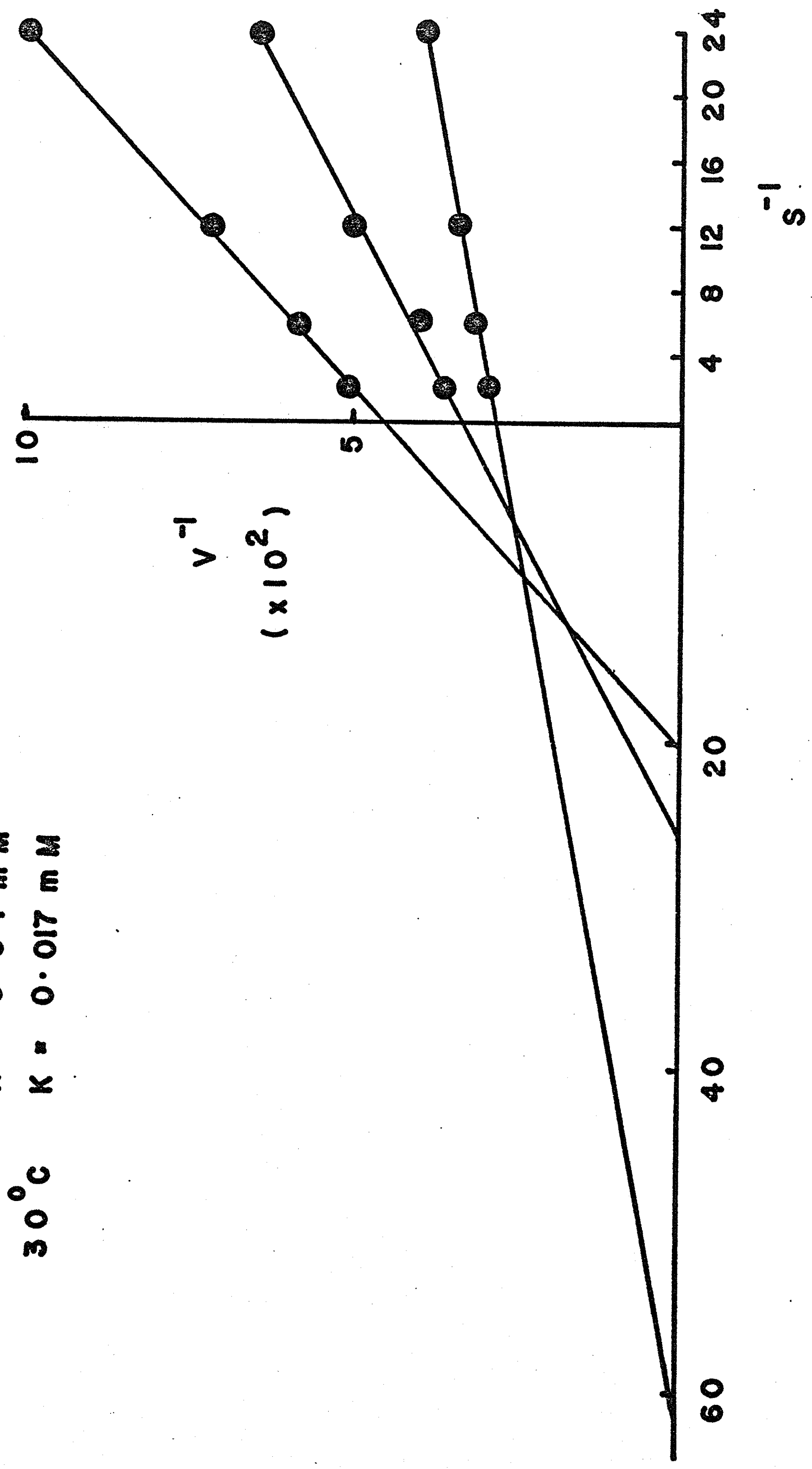

30°C
20°C
10°C
30
20
10
CO2 (N MOLES MIN-1 MG DRY WT-1)
0·1
0·2
0·3
0·4
0·5
[GLUCOSE] mM

FIGURE 21. Fig. 20 replotted by method of Lineweaver-Burk to emphasize the effect on V_{max} and K



10°C K = 0·05 mM
20°C K = 0·04 mM
30°C K = 0·017 mM
V⁻¹ (x10²)
S⁻¹
10
5
4 8 12 16 20 24
20
40
60

Lag Before Onset of Maximum Velocity of CO_2 Production

With *P. aeruginosa* preparations and all substrates tested there was always a lag period before maximum velocity of CO_2 production was reached. Figure 22 and Table 8 give some examples of this lag period. The lag time varied from 5 minutes in the case of acetate to 30 minutes in the case of pyruvate. In some cases lag periods of many hours were observed and these preparations were not used to provide quantitative information, but the results are listed in Table 8 for comparison. The lag was not evident with *E. coli* nor *P. rettgeri* metabolizing glucose.

Failure To Produce CO_2 From an Organic Compound

P. aeruginosa with some substrates; e.g. glucose, glycerol, citrate and L-tyrosine was unable to elicit a rate of CO_2 production over and above the endogenous rate. The fate of these compounds was not studied further but some speculations are detailed in the discussion section.

One case was studied in detail - the fate of phenol when added to a resting cell suspension of *P. aeruginosa*. Figure 23 indicates that for a 12 hour period phenol remained unchanged at its initial concentration in the supernatant. After this time it began to gradually diminish in concentration and was undetectable 40 hours after addition. During the initial lag period;

FIGURE 22. Time required to reach maximum rate of CO_2 production by resting cell suspensions of _P_. _aeruginosa_ metabolizing L-aspartate, succinate, L-serine, L-glutamate and L-alanine at 30°C and pH 7.0.

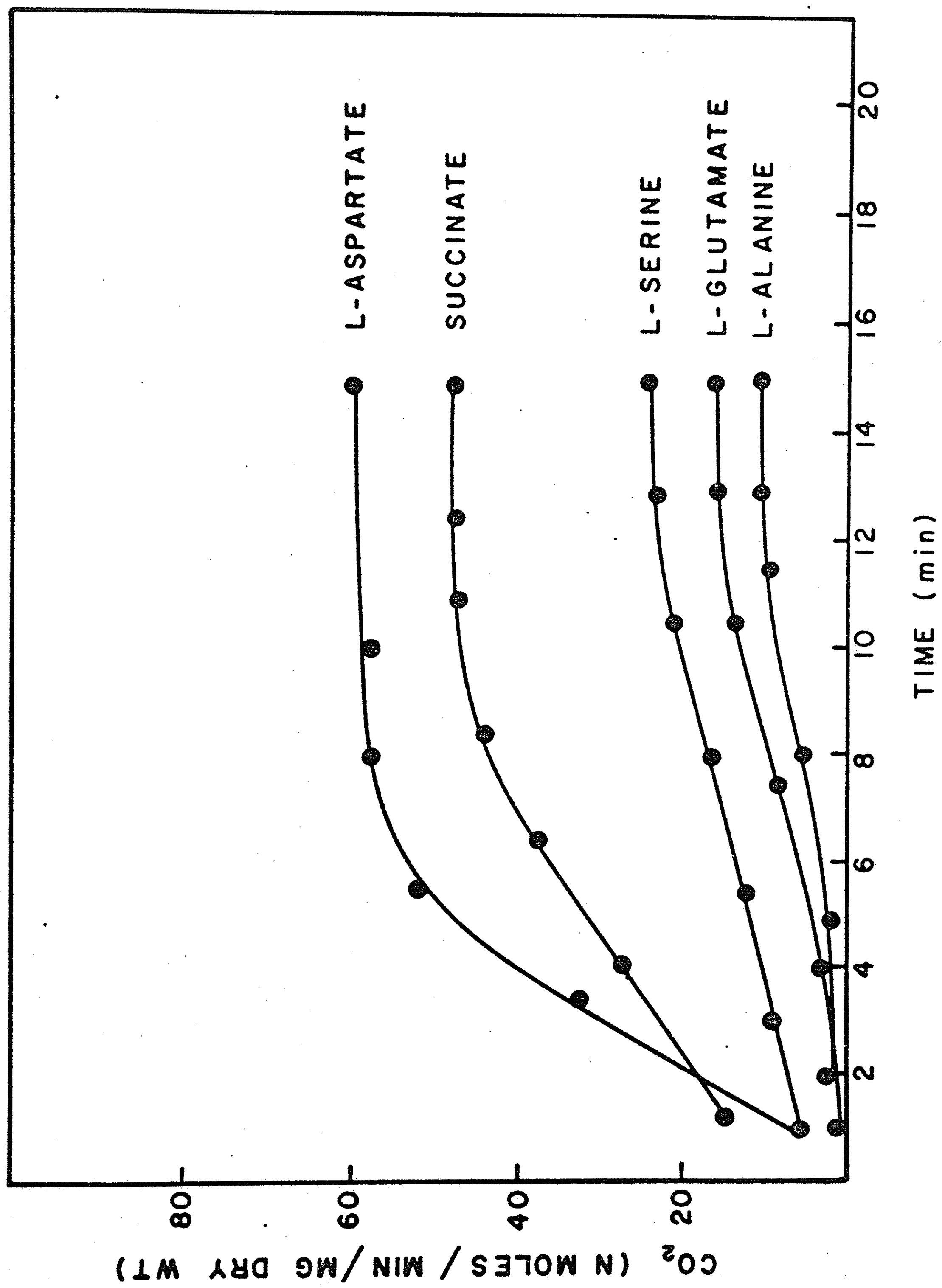
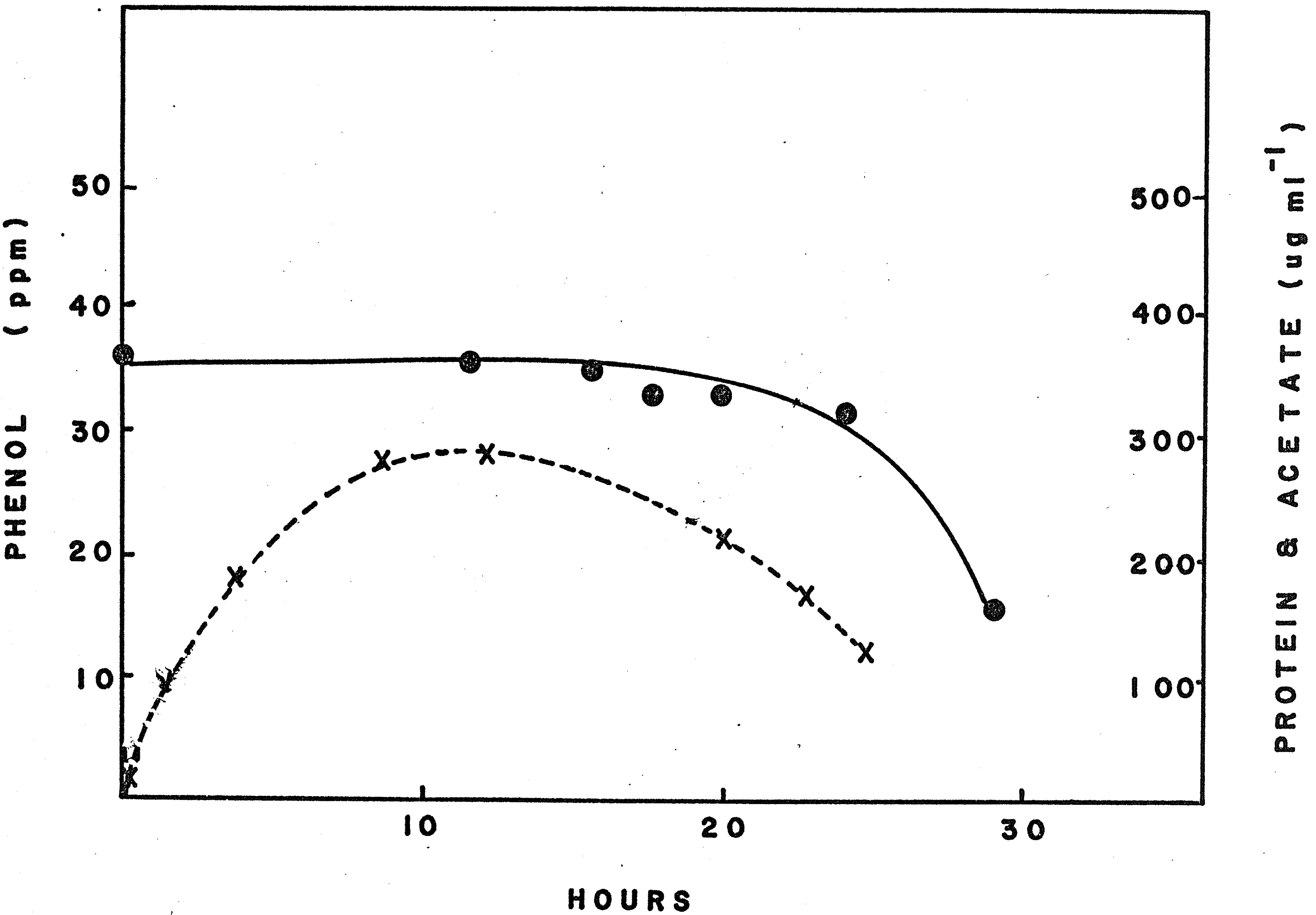

L-ASPARTATE
SUCCINATE
L-SERINE
L-GLUTAMATE
L-ALANINE
TIME (min)
CO_2 (N MOLES / MIN / MG DRY WT)
0
2
4
6
8
10
12
14
16
18
20
20
40
60
80

FIGURE 23. Uptake of phenol (————) by resting cell suspensions of *P. aeruginosa* at 30°C and pH 7.0. The appearance in the supernatant of acetate (-------) is also shown.


PHENOL (ppm)
10
20
30
40
50
HOURS
10
20
30
PROTEIN & ACETATE (ug ml^{-1})
100
200
300
400
500

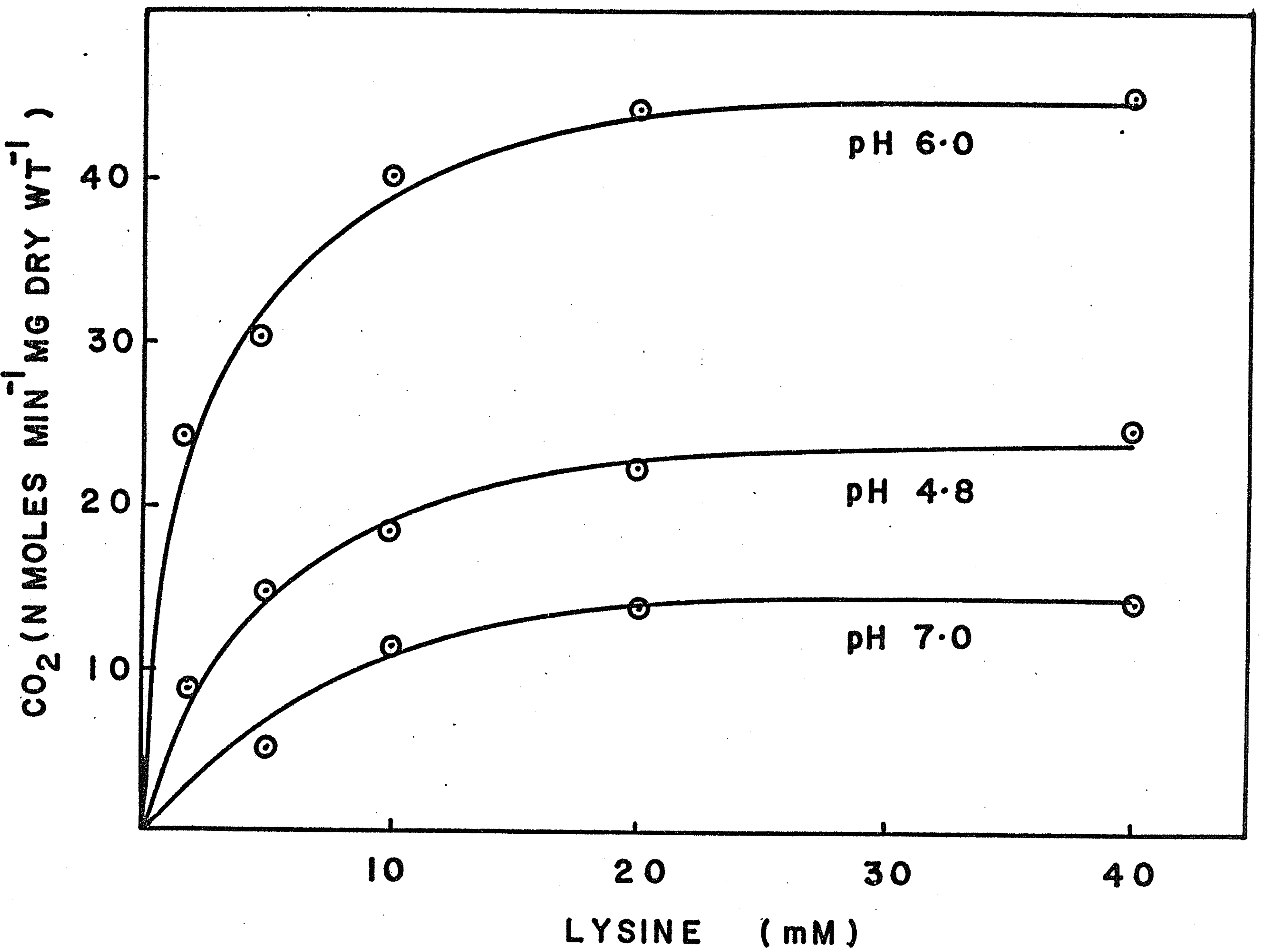
acetate began to appear in the supernatant. Later, coincident with the removal of phenol, there was a concomitant removal of the acetate.

During the entire period from addition to complete removal of phenol there was no detectable change in the rate of CO_2 production compared to that in a parallel run without phenol.

Decarboxylation of Arginine and Lysine by Resting Cell Suspension of *E. coli*

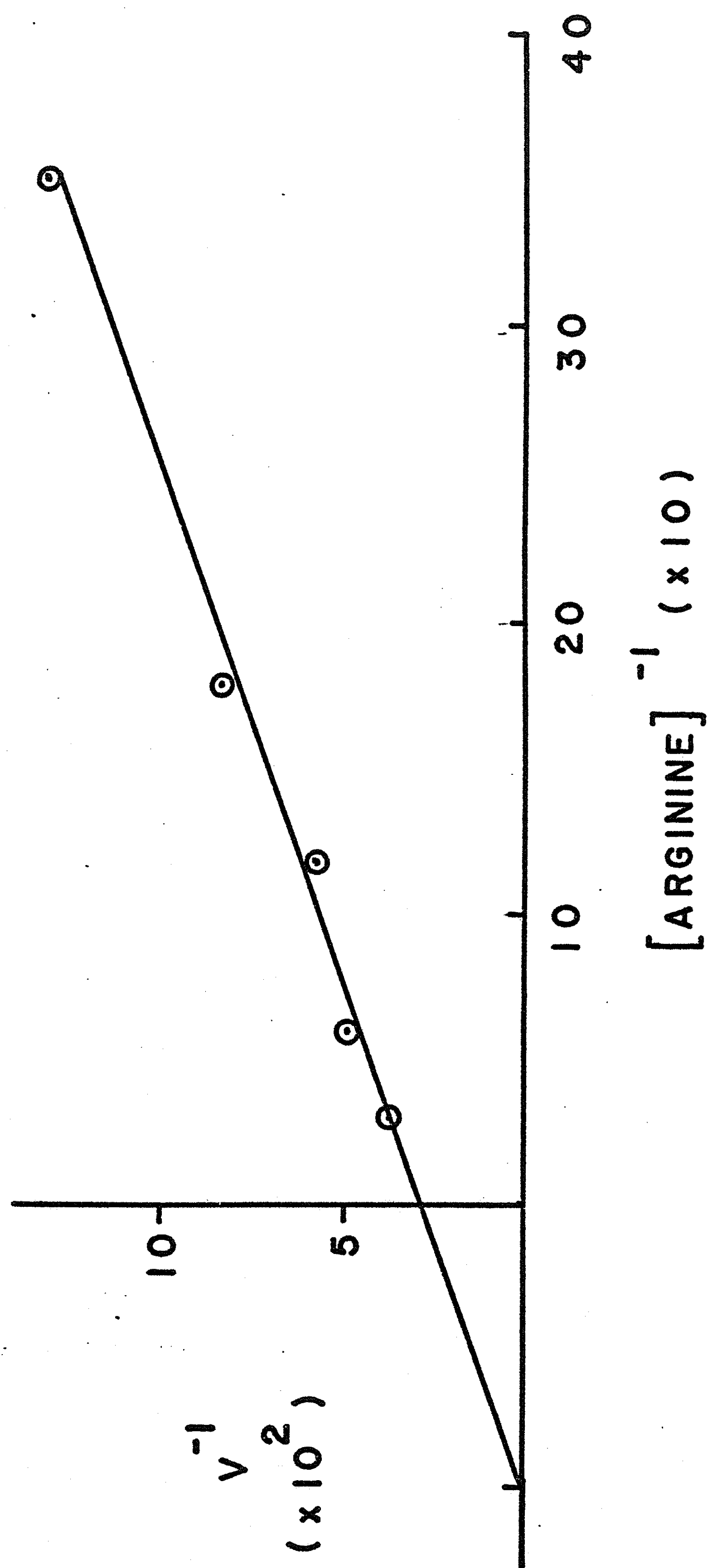
E. coli resting cell suspensions responded to an increase in the concentration of exogenously supplied L-lysine and arginine by producing CO_2 as shown in Figures 24 and 25. The rate of production of CO_2 was critically governed by the pH of the suspending fluid. For lysine the maximum rate of CO_2 production was found to occur at pH 6.0; when arginine was the substrate the optimum pH was 4.8. No exogenous CO_2 was detected at pH 7.

FIGURE 24. Effect of lysine concentration on the rate of CO_2 production by resting cell suspensions of <u>E</u>. <u>coli</u> at 30°C and pH 4.8, 6.0 and 7.0.



pH 6·0
pH 4·8
pH 7·0
CO_2 (N MOLES MIN^{-1} MG DRY WT^{-1})
10
20
30
40
LYSINE (mM)
10
20
30
40

FIGURE 25. Effect of arginine concentration on the rate of CO_2 production by *E. coli* resting cell suspensions at $30^{\circ}C$ and pH 4.8.



V⁻¹ (x10²)
10
5
10
20
30
40
[ARGININE]⁻¹ (x10)

D I S C U S S I O N

DISCUSSION

Parsons and Strickland (158) were the first to explore the feasibility of applying a kinetic model to study the rate reactions of nutrient uptake in whole cells. The technique has been refined by Wright and Hobbie (235) and is now widely used. These workers were studying the utilization rates of organic compounds by the heterotrophic microflora of natural aquatic systems. The technique involves the addition of radioactive compounds to mixed microflora in the natural state and measuring the incorporation or respiration of the radioactive substrate. It is assumed in these studies that enzymes in the intact cell obey the same laws of kinetics as do purified enzymes. In reality, the reaction rates of the intact cell will be regulated by a single rate-limiting reaction and it is generally believed that this reaction is the 'transport' or permease step. Regardless of whether this is true, a plot of rate of incorporation against time gives a hyperbolic function which can be described in terms of the Michaelis-Menton equation, as for single enzymes.

Hamilton and Preslan (92) used continuous culture to study the trophodynamics of a pelagic phagotrophic

protozoan and its prey, a marine bacterium. They were able to apply Michaelis-Menten kinetics to describe the system and stated that K is a measure of the sensitivity of an organism for its substrate.

Curds and Cockburn (47) described a monoxenic protozoan feeding on a bacterium in a chemostat, in terms of saturation kinetics and similarly to Hamilton and Preslan indicated the K measures the affinity of the protozoan for its substrate.

The rate of production of carbon dioxide by resting cell suspensions of bacteria is dependent on environmental conditions (Figures 9 and 10) and on exogenous substrate concentration. This is exemplified in Figures 11 to 16 for *Pseudomonas aeruginosa*, Figures 17 - 19, and 24 and 25 for *Escherichia coli*, and Figures 20 and 21 for *Proteus rettgeri*. In each of these cases the rate of production of CO_2 by the resting cell population is dependent on the concentration of exogenously supplied substrate and can be described by the Michaelis-Menten equation. Table 7 summarizes some kinetic constants. The maximum velocity has been normalized to a per mg dry weight basis. The substrate concentration, at which the velocity of CO_2 production is half-maximal (K_{cat}) is similar to the Michaelis constant (K_m). K_{cat} is a constant only under rigidly controlled conditions and is a measure of the affinity of the catabolic machinery of the bacterial cell

for the substrate. As such this may make the evaluation of K_{cat} a better measurement than uptake of substrate, for determining the adaptability of a bacterial species, or a mixed population, to a particular substrate, since it is the overall effect on metabolism, rather than any one component, that is important from the point of view of cell survival. The phenomenon of active transport is well established and it is a general property of bacterial cells to transport substrate across concentration gradients. But, if two or more species are competing for the same substrate, the species with the most active transport system has an advantage only if its overall metabolism of the substrate (a property measured by CO_2 production) is greater than that of the other competing species. That is to say a bacterial species having a low K_{cat} and a high V_{max} for a substrate may have a competitive advantage.

The assay procedure described satisfies the requirements for studying the kinetics of the oxidation of organic compounds to carbon dioxide by intact bacterial cells in resting cell suspension. The rate of CO_2 production is substrate concentration-dependent and is influenced by temperature and pH. The data can be approximated to an adsorption isotherm similar to that described by Michaelis and Menton for simple enzymic systems. Sodium azide and mercuric chloride inhibited the rate of CO_2 production thus ensuring that the carbon dioxide was solely of biological origin.

The apparent K_m which was determined for a number of substrates has the dimensions of substrate concentration and is the substrate concentration at which the rate of CO_2 production proceeds at one-half maximum velocity. It might more aptly be termed a catabolic constant (K_{cat}) since it is a measure of the ability of an enzyme or a series of enzymes to generate CO_2 in the presence of the substrate.

Although the kinetics of CO_2 production by resting cell suspensions were heretofore unreported, it is not surprising that this relationship exists. Longmuir (124) reported that oxygen is consumed by aerobic bacteria in resting cell suspension in a fashion that can be adequately described by the Michaelis-Menten equation. He also reported that this is a general phenomenon. Gale (73) has documented extensively the production of CO_2 by cell-free extracts containing amino acid decarboxylases, all of which exhibit first order reaction kinetics. Many other reports of decarboxylase activity by purified enzymes or cell-free extracts are available (82, 168, 170, 191).

Washed bacterial cell suspensions evolve carbon dioxide endogenously at a rate determined by the prevailing nutritional status and environmental conditions. Exogenously supplied organic compounds may or may not effect this rate of CO_2 production even though these compounds are assimilated by the cells and catabolized. Therefore, notwithstanding the fact that assimilated

organic substrates may eventually be completely metabolized and emerge from the bacterial cell as CO_2 the rate at which this process proceeds is not in every case distinguishable from the endogenous rate of CO_2 production.

It is significant that, in those cases where CO_2 production rate is dependent on substrate concentration, the rate of CO_2 evolution can be described in terms of Michaelis-Menten kinetics for a single enzyme. This observation and the following theoretical evaluation provide an interpretation of the phenomenon of substrate dependent CO_2 production by washed cell suspensions.

SUMMARY

SUMMARY

1) A methodology was developed and described applicable to the investigation of the kinetics of CO_2 production by washed cell suspensions and purified enzymes. The method is rapid, sensitive, specific and subject to few interferences.

2) The rate of carbon dioxide production by whole cell preparations is substrate concentration-dependent in those cases where CO_2 is evolved at a rate greater than the endogenous rate. The dependence of the rate of CO_2 production on substrate concentration can be described, mathematically, in terms of the Michaelis-Menten equation for single enzymes.

3) Carbon dioxide production above the endogenous rate in response to catabolized substrate is not a general phenomenon and some substrates, known to be metabolized, do not evoke a detectable increase in the rate of CO_2 production by washed cell preparations.

4) With respect to carbon dioxide production, washed whole cell preparations appear to behave in a manner similar to crude enzyme extracts in their response to a substrate which can be decarboxylated directly by a decarboxylating enzyme present in the cell in sufficient quantity.

GENERAL DISCUSSION

GENERAL DISCUSSION

The response of living matter to stimuli can be extremely specific and the use of the biological indicator often is without equal in qualitative assay methods. On the other hand the value of the biological assay as a sound quantitative method is not always appreciated.

The most urgent problem facing microbial ecologists today is the measurement of environmental changes of unnatural origin. Much effort has been expended in elucidating the nature of the natural cycles: the nitrogen, carbon, phosphorous and sulphur cycles, energy transfer and photosynthesis, in the biosphere. Little progress has been made in the area of biodeterioration of natural and synthetic products. Study methods are limited and their application results only in a scratching of the surface of the immensely complex problem. Slow progress in the study of natural phenomena is a result mainly of lack of adequate methodology. Pollution of the environment is a serious problem and the impact of these man-made imbalances may have disasterous consequences. Fairhall (65) has pointed out that the burning of fossil fuels is proceeding at an exponential rate and the increase in atmospheric carbon dioxide may cause a shift in the bicarbonate - CO_2 equilibria, which could result in the dissolution of the calcareous shells of sea inhabitants.

Ozone is produced in the atmosphere by photolysis of oxygen at 185 nm Absorption of ultraviolet radiation in the 220 to 300 nm range causes decomposition of the ozone. The resulting equilibrium layer of ozone, about the earth's surface, serves to filter-out a large portion of the harmful U.V. irradiation from the sun. Changes of these equilibria are manifestly disadvantageous. Over-population and under-productivity are leading to food shortages. This problem is aggrandized by depletion of natural reserves and destruction of renewable resources by chemical pollution, most notably methyl mercury contamination and eutrophication of fresh waters.

The first step in combatting pollution - a necessary evil of modern technology, is the recognition of the problem. The second step requires the ability to assay environmental perturbations and to make predictions based on those measurements.

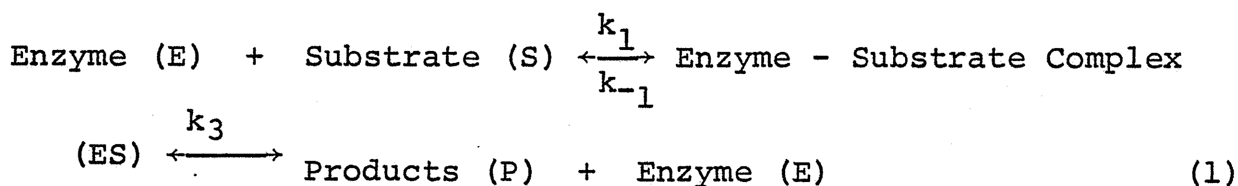
The purpose of the present work was to develop a methodology sufficiently general but sensitive and precise enough to monitor perturbations of the biological milieu. The initial concept was to simply measure the biodegradability of organic materials by measuring the amount of carbon dioxide released from them. Subsequently it was discovered that the evolution of CO_2 from bacterial suspensions, responding to different substrate concentrations, could be described by an equation similar to that proposed by Michaelis and Menten (144) to quantitate the

rate of enzymic action and later used by Monod (147) to describe bacterial growth and also by Parsons and Strickland (158) to measure uptake of organic material by heterotrophs.

Measurement of the rate of CO_2 evolution is a common parameter in physiological studies and has found numerous applications, but except in the case of the amino acid decarboxylases, no literature data are available which specifically relate the velocity of CO_2 evolution by bacterial suspensions to substrate concentration. It was of interest to determine whether this was a general phenomenon and if it had practical applications as an indicator of biological activity.

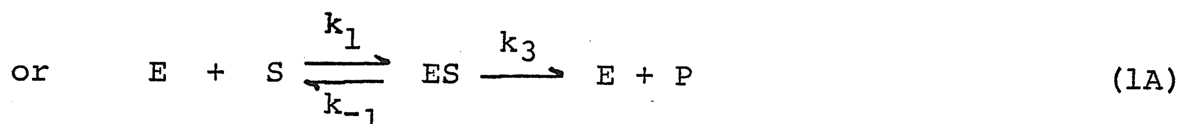
Kinetics of Single Enzymic Reaction

From the mechanism of enzymic reaction (Equations 1 and 1A) proposed by Brown (25), Henry (95) derived the following equation (Eq. 2) for the velocity of the reaction by applying the concept of the law of mass action, which is applicable mainly to a thermodynamically closed system:



$$\text{Enzyme (E)} + \text{Substrate (S)} \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} \text{Enzyme - Substrate Complex}$$

$$\text{(ES)} \overset{k_3}{\longleftrightarrow} \text{Products (P)} + \text{Enzyme (E)} \qquad (1)$$



or $$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (1A)$$

$$V = \frac{k_3 \, X_o^E \, X_t^{sE}}{K + X_t^{sE}} \quad (2)$$

where $$= \frac{dX_t^{sE}}{dt} \quad (3)$$

k_3 = constant

X_t^{sE} = concentration of substrate of the enzyme at time t

k = constant

X_o^E = initial concentration of enzyme, E

Brown's equation was given sound experimental validation by Michaelis and Menten (144) who presented a slightly different version of Equation 2:

$$V = \frac{V^m \, X_t^{sE}}{K_m + X_t^{sE}} \quad (4)$$

It should be noted that, when all the enzyme is complexed, the velocity, V_m would be maximum, V^m, so that

$$k_3 \, X_o^E = V^m \quad (5)$$

In equation 4, K_m is the Michaelis constant for the substrate. It was shown by Michaelis and Menten (144) that $K_m = (k_{-1} + k_3)/k_1$. Equation 4 was derived with

the assumption that the enzyme-substrate complex remains in true equilibrium with the enzyme and substrate, which is contrary to the condition of dynamic equilibrium that exists in an open system such as that of a cell. However, application of the concept of dynamic equilibrium by Briggs and Haldane (23) led to a rate expression identical to Equation 4. The Michaelis-Menten equation is simply the rate expression for a single and isolated enzymic reaction.

The problem of modelling the behaviour of a network of more than 2000 enzymes in a cell is overwhelming and the prospects of arriving at an expression for the overall rate of the system from such an analysis is very dim. However, some progress can be made if some simplifying assumptions are made. The model, built with the assumption, would be acceptable only if it receives experimental support with regard to its closeness of fit to reality and its capability of simulation of prototype systems.

Kinetics of Multi-enzyme Systems

In a metabolic system having a series of reactions as shown in Figure 26 the rate of initial reaction is evidently dependent on the nutrient concentration provided in the medium; the subsequent steps in the pathway depend upon the preceding reaction for their

FIGURE 26. Theoretical Michaelis curves of the component enzymes of an enzyme sequence. *

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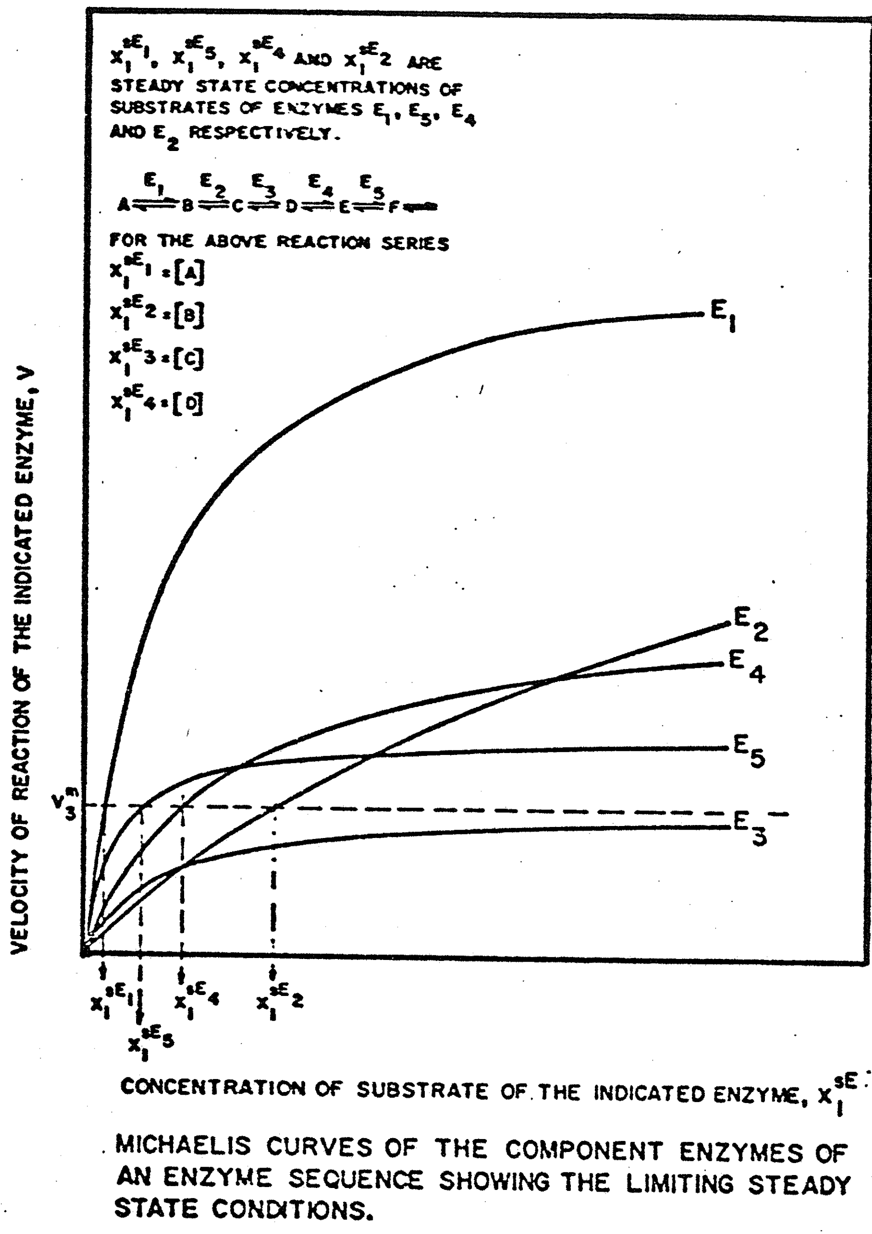
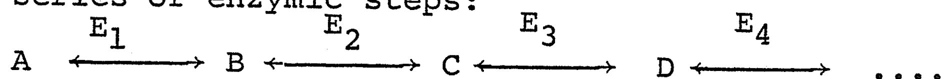
from Ghosh (77)



MICHAELIS CURVES OF THE COMPONENT ENZYMES OF AN ENZYME SEQUENCE SHOWING THE LIMITING STEADY STATE CONDITIONS.

substrate. When the system of enzymes has settled down to a steady state defined by a particular set of environmental conditions, the concentrations of the intermediates are small and remain constant to correspond to the total flow of metabolites. The result is that, at steady state, all the reactions must proceed at the same rate. Dixon and Webb (55) and Hearon (94) have concluded that the velocity of a metabolic line is determined by the slowest enzymic step in the sequence. In a series of enzymic steps:



if, for any given environment, E_3 is slower than E_1 and E_4 then intermediate C would accumulate. Accumulation and increase of concentration of C would tend to accelerate conversion of C to D according to the Michaelis-Menten equation and decelerate E_2 by increasing its rate of back reaction. This leads to a process of equalization of velocities, a conclusion which is compatible with cellular regulatory mechanisms. The rise in concentration of intermediate C would bring into action the negative feedback mechanisms of retro-inhibition and/or catabolite repression to control the velocity of E_1 or E_2 or both by inhibiting their activity and/or adjusting the intracellular concentration of these enzymes. Thus, a key enzyme of the sequence would set the pace for the sequence, and the other enzymes

would merely keep up with it. This is the "pacemaker" concept of Krebs and Kornberg (118). Hearon (94) earlier arrived at the same conclusion and further pointed out that the environment affects the velocity of a metabolic route only indirectly to the extent that it determines the slowest enzyme of the sequence. Thus the key enzymatic step may shift from one position in the enzyme system to another with a shift in the environmental makeup.

The different enzymes of the network will have different substrate concentration curves as described by Michaelis-Menten equation and which depend on K_m, V^M and the amount of each enzyme present as illustrated in Figure 26. The heights of the curves depend on the concentration of the particular enzyme, which can be changed with a change of environment. For any given environment, the enzyme with the lowest V^M will become the limiting or "pacemaker" enzyme, and its maximum velocity will become the velocity of all other enzyme reactions in the chain. Thus, for the enzyme system represented in Figure 26 no matter how much the substrate concentration may be increased, the velocity of the metabolic line cannot rise above the maximum velocity of enzyme E_3. As a consequence, the substrate concentration of each enzyme will adjust itself to that point on its Michaelis curve which gives this rate.

Burton (29) and Dean and Hinshelwood (53) pointed out that the concept of a "pacemaker" enzyme should be used with caution because theoretically it can be shown that the overall rate of a system of several consecutive reversible reactions depends on the rate constants of each step. This objection is valid where the reactions are purely chemical and where no control is exerted over the activity and/or quantity of the catalysts. It should be recognized that in a biochemical system the activity and quantity of the enzymes can be controlled by the genes (107); therefore, it is possible that the overall rate is controlled by one "pacemaker" enzyme.

The essence of the above discussion is that, if the "pacemaker" concept is correct, then the velocity of substrate assimilation *via* an enzymic pathway would be given by the Michaelis-Menten equation of the "pacemaker" enzyme.

After an extensive review of the information accumulated on the subject of enzyme mechanisms and activity, Pardee (156) concluded that the concept of adjustment of all enzyme reactions to keep pace with the slowest reaction is tenable in view of the capacity of the cell to regulate the synthesis and activities of all enzymes.

The above theoretical treatment is congruent with the empirical observation that the physiological response

(as measured by carbon dioxide evolution) of a bacterial resting cell suspension is a function of the environmental conditions and can be described in terms of the Michaelis-Menten equation. Longmuir (124) examined the respiration rates of a number of bacteria in resting cell suspension and found that the respiration rate is related to the oxygen concentration by the Michaelis-Menten equation. It was further established that the K_m determined was dependent on environmental conditions and was related to the size of the organism.

In view of the above theoretical discussion it is tempting to speculate that the slowest step (the pacemaker) in the conversion of substrate to CO_2 by a resting cell suspension, is the decarboxylating enzyme of the substrate itself, or of a subsequent product, since if the slowest step were any other previous step in the sequence, the rate of production of CO_2 would not be influenced by other substrate concentration. This explanation adequately provides for those substrates which do not elicit evolution of CO_2 above the endogenous rate. In these cases the rate of substrate catabolism is controlled by a step which does not have CO_2 as an immediate product.

The most convincing support for this interpretation is provided by the data for the decarboxylation of

L-lysine and L-arginine by washed suspensions of E. coli. In both cases the suspension of whole cells evoked a response similar to that of the purified enzyme in that the pH optimum and K_m were the same (Figures 24 and 25). Similarly a washed suspension of P. aeruginosa exhibited the same properties as a crude enzyme extract of pyruvate decarboxylase. However in this case the pH optima were not comparable (Figures 10, 27).

This interpretation, admittedly, may be far too simplistic as an approach to describe a very complex system. Regardless of whatever explanation may suffice to adequately enucleate the CO_2 production response of washed cell suspensions to substrate concentration this phenomenon is specific and seems to be related to a particular decarboxylase reaction. Thus it is fair to conclude that there would be little value in attempting to employ rate of CO_2 production by resting cells as an indication of catabolic activity.

The methodology developed to investigate the phenomenon of CO_2 production by resting cells may have application in those instances which deal specifically with changes in carbon dioxide concentration. To mention a few: photosynthesis; CO_2 assimilation by autotrophs; metabolic studies involving physiological effects of toxic materials, as a bioassay for substrates of decarboxylases or for assaying the enzyme, and as a taxonomic technique for differentiating bacterial strains on the basis of specific decarboxylases.

The technique also shows promise for use in screening synthetic organic compounds in order to determine if they are biologically intractable. This application would be similar to the presumptive coliform test which is currently a standard test to recognize potentially non-potable waters.

ADDENDUM

ADDENDUM

Note on the Application of Infrared Gas Analysis for the Assay of Decarboxylating Enzymes

Decarboxylating enzymes, particularly the amino acid decarboxylases, are widely distributed in nature (264). Gale and co-workers in a series of fundamental papers dating back to 1940 (247, 248, 249, 250, 251, 252) established the existence of six inducible bacterial enzymes catalyzing the α-decarboxylation of amino acids. The arginine, lysine and ornithine decarboxylases have proved to be of particularly significant value in the differentiation of the Enterobacteriaceae (242, 243) and other gram-negative bacteria such as Aeromonas, Pseudomonas alcaligenes and Moraxella (261). Møller (257) first developed the detection procedures for these enzymes. Several useful modifications have been introduced by Falkow (244), Johnson, Kunz, Baron and Ewing (255), Elstrom (240), Fay and Barry (246), and Goldschmidt, Lockhart and Perry (253).

These procedures are all based on the same principle: development of an alkaline reaction in broth medium by accumulation of amines or diamines. However, in some organisms the alkalization of the medium may either be counteracted and masked by vigorous acid pro-

duction from carbohydrates, or simulated by ammonia production from proteins. Clean-cut proof of decarboxylation in these cases may be questionable.

Zolg and Ottow (264) have developed a method utilizing thin-layer chromatographic techniques to identify the amine end-products of decarboxylation. They were able to identify a number of weak amino acid-decarboxylating bacterial strains previously reported as negative. The method is relatively sensitive but cumbersome.

Gas chromatographic procedures have been developed (256) to provide rapid detection of the amine end-products of the decarboxylation reaction. Again technical difficulties prevail.

The sensitive, rapid and specific detection of carbon dioxide by the infrared gas analytical method previously described provides a simple assay for decarboxylation reactions. Shown in Figs. 24 and 25 are the kinetic constants for lysine and arginine decarboxylation by intact *E. coli* resting cell suspensions. The constants are in close agreement with those presented by Gale (250) for *E. coli* cells under similar conditions, and by Najjar (258) for cell-free extracts.

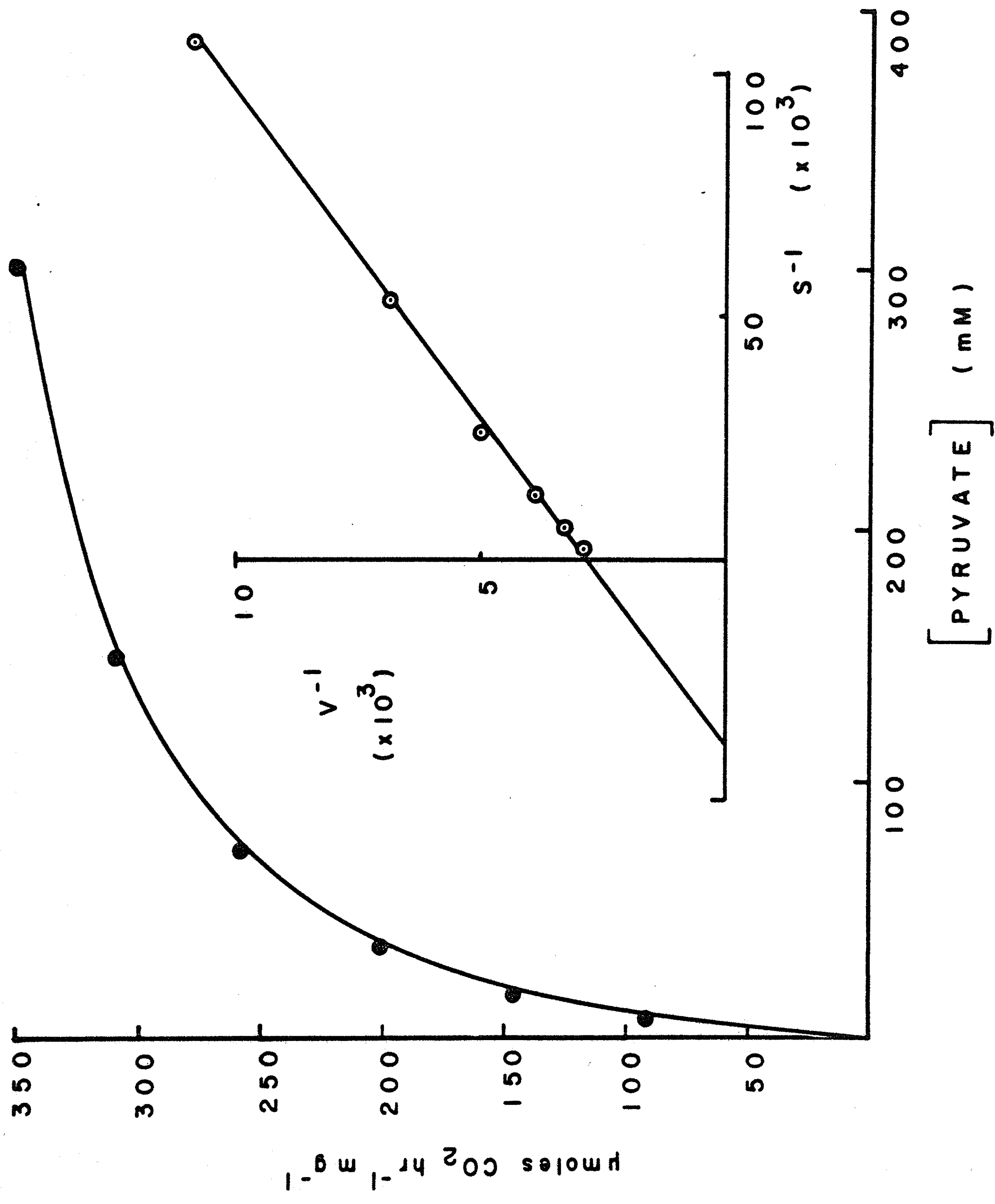
To further exemplify the use of the apparatus to measure CO_2 liberation, four commercially-available crude decarboxylating enzyme preparations were assayed for activity.

Pyruvate decarboxylase (4.1.1.1.) a Sigma Chemical Co. preparation extracted from yeast was suspended in 50 mM citrate buffer, pH 6.0, and assayed at 30°C with increasing pyruvate concentrations by the method described in section III. Fig. 27 shows the saturation curve with the double reciprocal replot inset. The K_m for the reaction, under the specified conditions, was 26.7 mM which compares favourably with that obtained by Green, Herbert and Subrahmanyan (254) for fresh brewer's ale yeast (26 mM) under similar conditions. Pyruvate decarboxylase from Rhodspseudomonas palustris was reported by, Qadri and Hoare (260) to be half-saturating at 12.2 mM, at pH 6.25 and 30°C in citrate buffer.

Oxalate decarboxylase (4.1.1.2.) a partly purified extract from the wood-rotting fungus, Collyvia veltipes (Sigma) was assayed at pH 3.0 and 30°C in the citric acid-phosphate buffer of McIlvaine. The saturation curve and linear replot shown in Fig. 28 indicate a K_m of 1.33 mM. Shimazono and Hayaishi (262) found the K_m to be 2.05 under these conditions.

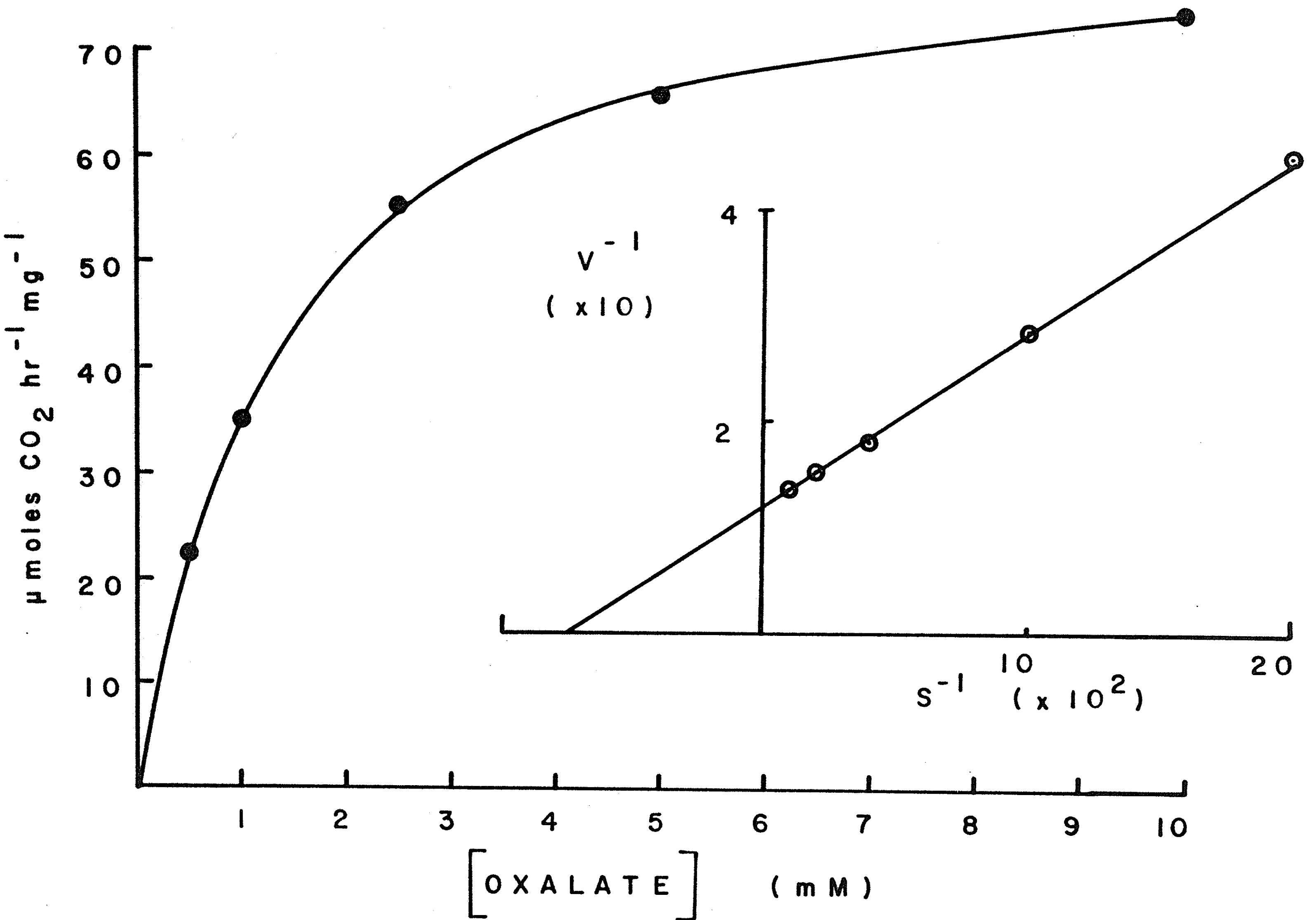
Tyrosine decarboxylase (4.1.1.25) a crude acetone powder obtained from Streptococcus faecalis (Sigma) was assayed at 37°C in McIlvaine's citric acid-phosphate buffer (pH 5.5). Saturation could not be achieved with the insufficiently soluble tyrosine. Fig. 29 displays the initial segment of the saturation curve. The K_m, 1.12 mM, was determined by replotting by the Lineweaver-

FIGURE 27. The kinetics of CO_2 production by a crude extract of pyruvate decarboxylase from yeast. Conditions were 50 mM citrate buffer, pH 6.0, and 30°C. The Km was found to be 26.7 mM with pyruvate. Inset is the Lineweaver-Burk replot.



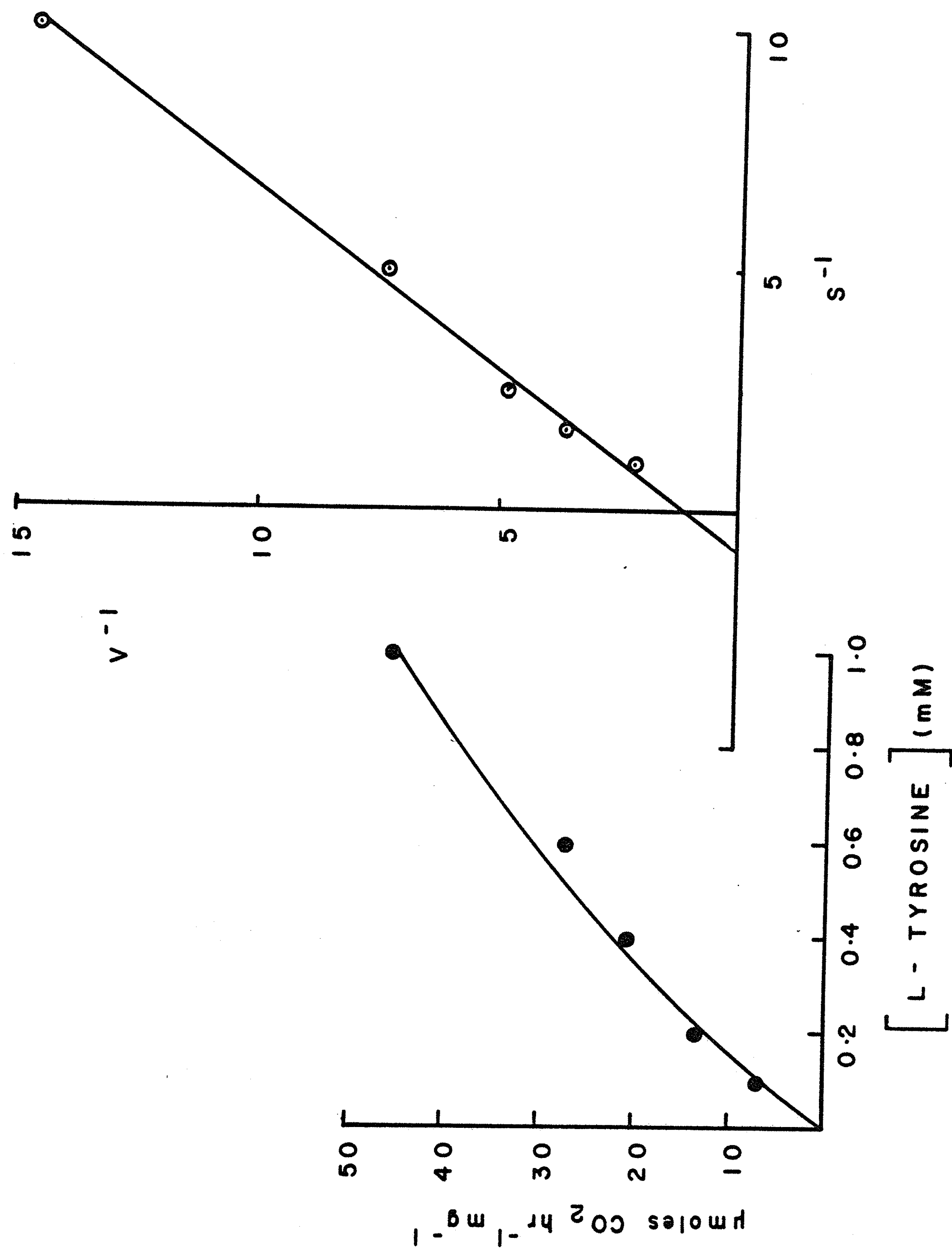
µmoles CO2 hr-1 mg-1
350
300
250
200
150
100
50
100
200
300
400
[PYRUVATE] (mM)
V-1
(x103)
10
5
S-1 (x103)
50
100

FIGURE 28. The kinetics of CO_2 production by a partly purified extract of oxalate decarboxylase from Collyvia veltipes. Conditions were; 30°C and pH 3.0 in McIlvaine's citric acid-phosphate buffer. The Km with oxalate was found to be 1.3 mM. Inset is the Lineweaver-Burk replot.



μ moles CO_2 hr^{-1} mg^{-1}
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
[OXALATE] (mM)
V^{-1}
(x10)
4
2
10
20
S^{-1} (x 10^2)

FIGURE 29. The kinetics of CO_2 production by a crude acetone powder from *Streptococcus faecalis*. Enzyme was suspended in McIlvaine's citric acid-phosphate buffer at pH 5.5 and $37^{\circ}C$. The Km with tyrosine was found to be 1.1 mM, determined from the double reciprocal plots.



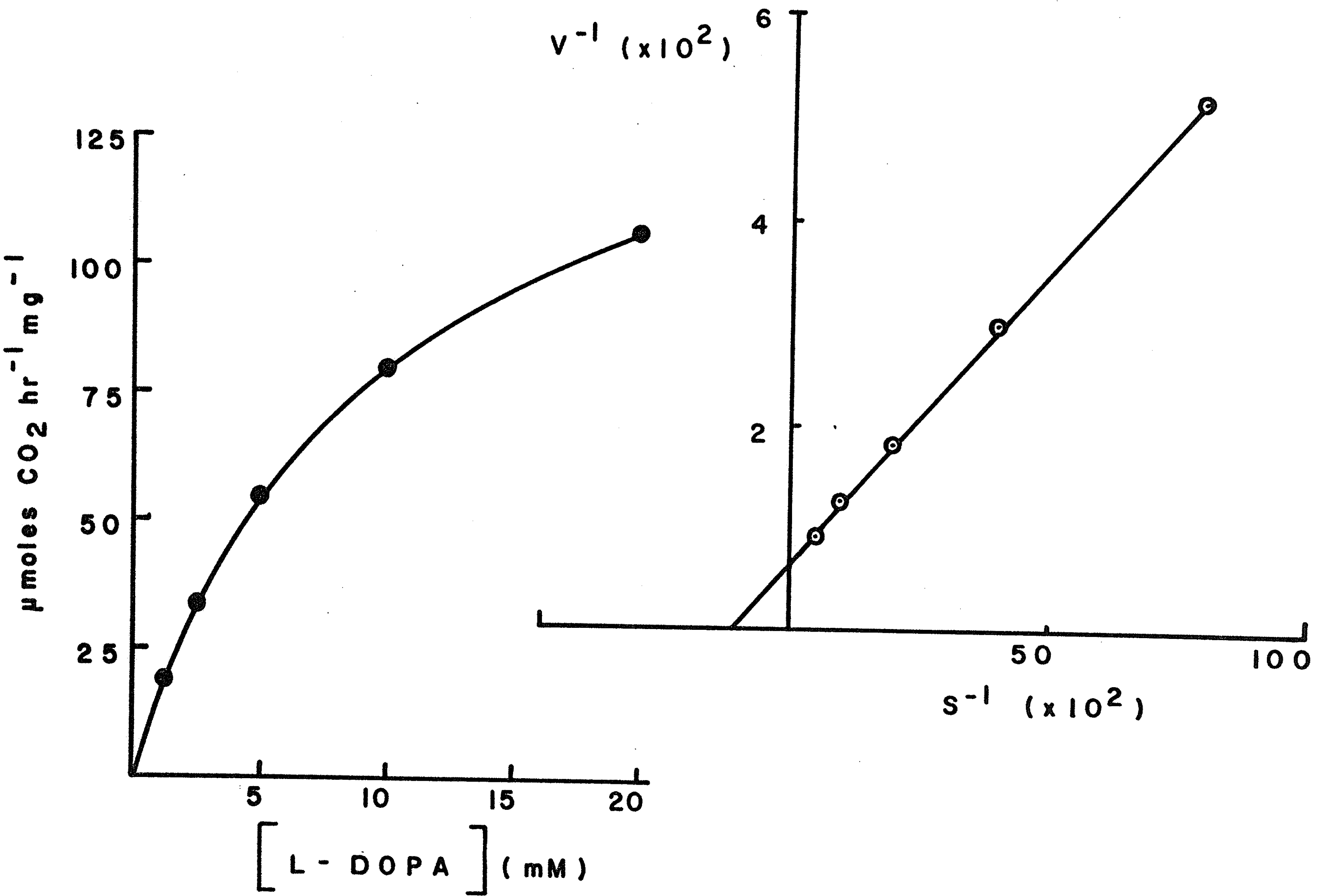
V -1
15
10
5
S -1
5
10
μmoles CO2 hr-1 mg-1
50
40
30
20
10
[L - TYROSINE] (mM)
0·2
0·4
0·6
0·8
1·0

Burk method. Tyrosine decarboxylase also decarboxylates 3, 4-dihydroxy-L-phenylalanine (L-DOPA) and, under the same conditions, displays the kinetic characteristics shown in Fig. 30. The K_m for L-DOPA was determined to be 8.36 mM. Epps (241) reported a K_m of 2.3 mM for L-DOPA as an alternate substrate for tyrosine decarboxylase under the same conditions. However the CO_2 liberation was determined by the manometric method. We have shown (unpublished data) that the maximum rate of CO_2 production by the Warburg method for this system is limited by the shaking rate. Increasing the shaking rate increased the V_{max} and the K_m increased to 5.7 mM.

Urease activity is routinely determined in the clinical bacteriological laboratory for differentiation and determination of the Enterobacteriaceae. Proteus, Klebsiella and also the non-Enterobacteriaceae Yersinia, give typical positive results. Vuye and Pijck (263) made a critical survey of the available methodology for determining this characteristic. They found that detection and intensity of urease activity varies greatly as a function of the media and techniques used.

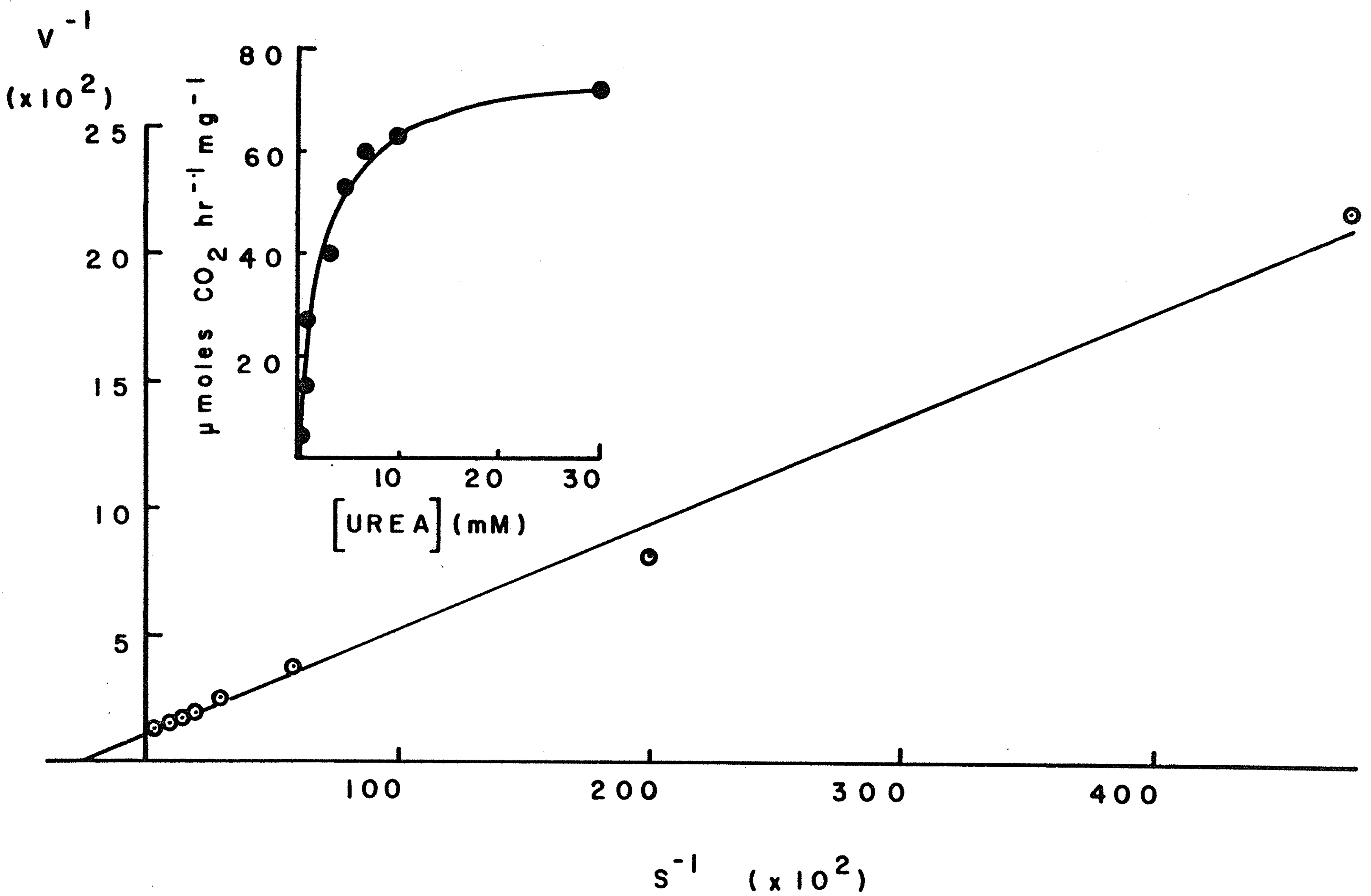
A crude preparation of urease obtained from Jack bean powder (Canalia ensiformis) (Mann Research Co.) was assayed by the infrared gas analysis method. Optimum activity was found at 25°C with the enzyme suspended in HEPES buffer pH 7.55 (Fig. 31). The K_m was found to be 4.12 mM. Peterson et al (259) reported a Km of 10.5 mM

FIGURE 30. Km for L-DOPA as substrate for tyrosine decarboxylase was found to be 8.4 mM. Conditions are the same as for Fig. 29.



μmoles CO_2 hr^{-1} mg^{-1}
125
100
75
50
25
5
10
15
20
[L - DOPA] (mM)
V^{-1} (x 10^2)
6
4
2
50
100
S^{-1} (x 10^2)

FIGURE 31. The kinetics of CO_2 production by a preparation of Jack bean urease (<u>Canalia ensiformis</u>). Conditions were 25°C, pH 7.6 in HEPES buffer. The Km was found to be 4.2 mM. Inset shows the Lineweaver-Burk replot.



V^{-1} (x10^2)
25
20
15
10
5
µmoles CO$_2$ hr^{-1} mg^{-1}
80
60
40
20
10
20
30
[UREA] (mM)
100
200
300
400
S^{-1} (x 10^2)

when the crude Jack bean powder was suspended in phosphate buffer, pH 7.0, at 25°C. However, phosphate is an activator (245) which could account for the larger K_m value.

The measurement of the kinetics CO_2 production by the apparatus described provides excellent agreement with conventional measurement techniques. As it is presently arranged the system can easily detect a difference of 1 μg of CO_2 in one minute. The sensitivity can be increased 100-fold by scale attentuation and by using longer time periods. The infrared gas analysis method avoids the time consuming preparation required of gas chromatographic methods and should provide a routine method for assaying decarboxylase activity.

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