PHYSIOLOGICAL CHARACTERISTICS OF *ESCHERICHIA COLI* IN VARIOUS STATES OF BALANCED GROWTH

by

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TABLE OF CONTENTS

"The experimental method rarely leads astray and then only those who do not use it well... The charm of our studies, the enchantment of science, is that everywhere and always we can give the justification of our principles and the proof of our discoveries." Happy are those who can experience "the serene peace of laboratories and libraries!" There it is that humanity grows, becomes stronger and better. There it learns to read the works of nature, symbols of progress and of universal harmony; whereas the works of man are too often those of fanaticism and destruction."

-Louis Pasteur

INTRODUCTION

Even when subjected to the closest scrutiny by means of the best optical or electronic equipment, bacteria appear as rather simple structures. Compared with higher plants and animals, they certainly do not seem to be as highly differentiated and complex. Nevertheless, as pointed out by Van Niel (1949), "they display an astonishing diversity of function which in many respects they share with the trees, oysters, butterflies, and elephants." The amazing feature of growth in a microbial culture, as in all living systems, is the fact that it involves the manufacture and orderly arrangement of many structural and functional components which characterize the organism in such a way as to duplicate accurately an existing pattern. Detecting the procedure by which this is accomplished remains one of the most significant problems in biology, and the problem is fundamentally as complex in the "simple" bacteria as in the higher forms. On the other hand, the comparative ease of handling microorganisms, their rapid growth, and the opportunity for rigorously controlling various environmental factors all combine to render them favorable material for experimental studies of the basic mechanism of the growth process (Van Niel, 1949).

The environment surrounding a cell is composed of two major groups of factors: 1) physical or physico-chemical factors, for which the relatively few experiments so far conducted have shown little or no effect on general cell physiology, and 2) the chemical environment, the importance of which has become increasingly apparent to the bacterial physiologist. Variations in cellular physiology due to changes in environment result from

the fact that the genotype of a cell controls the range of alteration possible within that cell, while the environment determines which of these phenotypic expressions will be manifested. This condition has prompted Herbert (1961) to say..."It is virtually meaningless to speak of the chemical composition of a microorganism without at the same time specifying the environmental conditions that produced it." Nearly all the early studies on the kinetics of growth in relation to environment were carried out in complex media in which many of the constituents were present in varying amounts (Rahn, 1932), and as late as 1946 a comprehensive review of microbial physiology (Porter, 1946) failed to indicate the importance of environment as an aspect of qualitative as well as quantitative microbial chemistry.

Monod (1942) provided one of the first clear pictures of the relationship between growing bacteria and their nutritional environment. He determined the effect of variation in the concentration of a single essential nutrient, and established that for a given carbon source, the rate of growth is independent of the substrate concentration beyond a critical level, whereas the total yield is strictly proportional to it. Some years later Schaechter, Maaløe and Kjeldgaard (1958) observed that the size and the chemical composition of bacterial cells vary as a result of changes in the environment, and that the variations can be expressed in simple terms as exponential functions of the growth rate at a given temperature. Such investigations have indicated that the effect on the composition of microorganisms due to change in the chemical environment may be either qualitative (e.g., the production in some environments of cell components, such as inducible enzymes, which are completely absent

in other environments) or quantitative (the production of more or less of a cell component which is invariably present).

If bacteria are placed in a suitable medium and grown under appropriate conditions, and the number of cells in the population is plotted against time, one obtains the well known sigmoid growth curve. This plot is called a growth cycle, a term of questionable propriety. Unfortunately bacteriologists have been inoculating flasks of culture media for so long that they have come to regard this as part of the natural order, instead of as a convenient but highly artificial experimental procedure. The shape of the growth curve represents a sequence of metabolic states forced upon the cell by sequential environmental changes which occur whenever growth takes place in a closed system. Of the various portions of this curve, the region of particular interest in this work probably occurs between what are commonly known as the logarithmic and the stationary phase of growth. This area is marked by cellular change and is appropriately termed the transition stage.

There is another procedure used to grow bacteria, termed continuous culture (see Appendix), whereby cells may be grown at a steady state - i.e., in balanced growth. This system has been used by various investigators to study such physiological events as growth limitation (Ecker and Schaechter, 1963), rates of mutation (Novick and Szilard, 1950), and macromolecular synthesis (Kjeldgaard, Maaløe and Schaechter, 1958; Schaechter, et al., 1958). When bacteria are grown in a balanced system the growth rate may lie between that of cells growing in the logarithmic and of those in the stationary phases of growth. Thus it is as though a moment of time from the transition phase is extended, and the cells continue

to grow in that particular physiological state. This procedure provides the experimentor with a "natural" growth system and has been used to obtain the results reported in this dissertation. Several problems that have proven very difficult to study using the classical batch culture techniques are being investigated by means of these newer methods. Monod (1950) pointed out that continuous systems are ideal for the study of the kinetics of cell syntheses. Using a continuous system, Schaechter, et al. (1958) were able to show that the number of nuclei/cell and the content of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were exponential functions of the cellular growth rate. They also suggested that the rate of protein synthesis per unit of ribonucleic acid is the same at all growth rates. Kjeldgaard (1961) investigated the macromolecular changes which occur within bacterial cells during transition between steady states, and demonstrated a dependency of the rate of protein synthesis on the RNA content of the cell. Growth rate changes were achieved by changing the composition of the medium which was supplied to the cells. By enriching the medium the cells grew faster and underwent a "shift up" in growth rate. A "shift down" in growth rate was achieved by removing the cells from an enriched medium and placing them in a minimal environment.

In many such investigations on the effect of growth rate and environment on bacteria, growth rate changes were achieved by changing the composition of the medium which was supplied to the cells, with the tacit assumption that any resulting changes in cell composition were attributable to rate differences rather than to the nature of the medium. However, Holme (1957) in using continuous culture to demonstrate a relationship between growth rate and glycogen synthesis, noted that the nature of the

rate-limiting factor had a profound influence on the chemical composition of the cells. Further, investigators working with batch cultures (e.g., Ecker and Lockhart, 1961b) have found that during the transition between logarithmic and stationary phases, when the cells are most nearly analogous to a population in a balanced growth system, metabolic events and cell composition are critically dependent on the nature of the growth-limiting factor. It therefore seems of interest to discover to what extent the characteristic physiological state of a population of cells in balanced growth is a function only of growth rate, and to what extent it depends on the specific identity of the rate-limiting factor in its nutritional environment. An investigation of both the qualitative and quantitative aspects of these problems constitutes the essence of the work reported here.

EXPERIMENTAL PROCEDURES

Materials

Organism

The organism used in these studies was a substrain of Escherichia coli derived from E. coli K12 by a process of orthoselection and designated G6 by Baarda (1962). Stock cultures were maintained at 5 C on slants of minimal agar (Davis and Mingioli, 1950) and transferred semiannually.

Chemicals and culture media

The organism was cultivated under experimental conditions in a synthetic glucose-salts medium containing: $K_2HPO_4 \cdot 3H_2O$, 0.7%; KH_2PO_4, 0.3%; $MgSO_4 \cdot 7H_2O$, 0.01%; deionized water and varying amounts of $(NH_4)_2SO_4$ and glucose to attain the growth-limiting or nonlimiting concentrations desired. Two media were used, one with glucose in excess such that the limiting substrate was nitrogen, and one with a change in the ratio of the limiting substrates such that glucose became the growth limiting factor. The concentrations of the limiting nutrients were chosen so that there was enough excess non-limiting substrate to have allowed growth of one additional generation beyond that permitted by the limiting nutrient. The actual amounts of glucose and $(NH_4)_2SO_4$ which could be used to obtain the desired limiting concentrations was determined from the data of Baarda (1962). Carbon limited systems contained 500 μg glucose/ml and 400 μg $(NH_4)_2SO_4$/ml, the nitrogen limited cultures contained 4000 μg glucose/ml and 400 μg $(NH_4)_2SO_4$/ml.

Crystalline bovine serum albumin was obtained from the Armour Lab-

oratories, Kankakee, Ill. The merthiolate was produced by Eli Lilly and Co., Indianapolis, Ind. and the tris (hydroxymethyl) aminomethane (tris) was purchased from the Sigma Chemical Co., St. Louis, Mo. Anthrone and acetaldehyde were purchased from Eastman Organic Chemicals, Rochester, N.Y. Fisher Scientific Co., Fairlawn, N. J. produced the Folin phenol reagent, while the various stains used were obtained from E. Merck AG, Darmstadt, Germany. The diethyl ether, glucose, alcohols, diphenylamine, acetone and sodium barbital were purchased from the Mallinckrodt Chemical Works, St. Louis, Mo. Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), glycine, triphenyl tetrazolium chloride (TTC), salmon sperm deoxyribonucleic acid (DNA) and ninhydrin were obtained from Nutritional Biochemicals, Cleveland, Ohio.

Reduced glutathione was obtained from Mann Research Chemicals, New York, N.Y., and ethylene glycol monomethyl ether (methyl cellosolve) was purchased from Union Carbide Chemical Co., New York, N.Y. Heterogeneous compounds such as dehydrated agars, prepared media, beef extract and Freund's incomplete adjuvant were products of the Difco Laboratories, Detroit, Mich.

The materials for the disc-electrophoresis (Canalco, Bethesda, Md.) procedure were generously supplied by Dr. Merlin L. Kaeberle, Iowa State University, Ames, Ia.

All inorganic chemicals were of reagent grade or better quality.

Cellulose polyacetate strips were obtained from the Gelman Instrument Co., Ann Arbor, Mich.

Equipment

Cultures were grown in a continuous culture system described in detail in the Appendix. A small intermittent flow Beckman solution metering pump, model 746, (Beckman Instruments, Inc., Fullerton, Cal.) was used to supply fresh medium to the growth vessel. In order to achieve a continuous flow of medium to the cells, rather than an intermittent addition due to the pump action, it was necessary to prepare a capillary delivery tube drawn out to a fine tip which increased the pressure required to force medium into the culture vessel.

The pump was sterilized prior to each use by dismantling and placing the parts in 70% ethanol overnight. When the pump was reassembled several hundred ml of 70% ethanol was run through the pump before media. The first 100 ml of medium to pass through the pump was discarded. Samples of the media incubated for 36 hr at 37 C indicated that the system was sterile. Aeration of the cultures was accomplished by means of an aeration control apparatus described by Ecker and Lockhart (1961a).

Two spectrophotometers were used throughout these studies. A Beckman DB spectrophotometer with a Beckman laboratory potentiometric recorder was used for all readings in the ultraviolet range and to follow the enzymatic breakdown of glucose-6-phosphate (G-6-P). All other optical density measurements were made using a Coleman Model 14 Universal spectrophotometer, Coleman Instruments Co., Maywood, Ill. The Coleman spectrophotometer was modified by the addition of a 2 x 10 mm slit between the terminal collimating system and the sample chamber in order to reduce the spectral width of the beam and diminish stray light (Ecker, 1961).

A Beckman Model IR-7 infrared spectrophotometer was used to obtain

spectra of cells in the infrared region, and a Beckman expanded scale pH meter was used in determining pH values.

High speed centrifugation was accomplished with a Servall SS - 1 centrifuge (Ivan Servall, Inc., Norwalk, Conn.) while heavier material was centrifuged in a Servall type M centrifuge.

Sonic disruption of cells was performed in a Raytheon 10 Kc Sonic Oscillator, Raytheon Manufacturing Co., Waltham, Mass.

Filtration was accomplished by means of a 47 mm GS millipore filter obtained from the Millipore Filter Corp., Watertown, Mass.

The electrophoretic procedures utilized an electrophoresis chamber and a type 2541 Shandon power supply obtained from the Shandon Scientific Co. Ltd., London, England. Photographs were prepared by means of a Durst 35 mm camera.

The taking of photographs of the material prepared by electrophoresis was facilitated through the use of a view box constructed according to the design recommended by Crowle (1961). The view box provided indirect lighting at the field of view against a black background, intensifying the objects to be photographed.

Cell free extracts

A modification of the procedure of Lengyd, Speger and Ochoa (1961) was used to disrupt the bacteria. Cells from 1 liter of culture were washed in cold distilled water, then suspended in 15 ml 0.02 M tris (hydroxymethyl) aminomethane buffer, pH 7.25 or 8, containing 0.005 M $MgCl_2$ and 0.075 M NaCl. Prior to disruption 2 μ moles of reduced glutathione/ml was mixed with the suspension. The cells were sonically

disrupted for 13 min at a power output of 1.35 amps with tap water (13 - 15 C) as a coolant. The extracts were centrifuged in the cold at 27,000 x g for 45 min. The supernatant was removed and dialyzed at 0 C for 24 hr against 4 liters of 0.02 M tris-HCl buffer with an ionic strength of 0.05 at pH 7.25 or pH 8. The protein content of the cell free extracts was determined by the Folin phenol method (Lowry, et al., 1951) and the individual concentrations adjusted to 3.47 mg/ml. The extracts were separated into several aliquots and frozen at -20 C. The individual extract samples were thawed as required for analysis.

Methods

Culture systems

All cultures were grown in a balanced system by means of a continuous culture apparatus described in the Appendix. The cells were grown at 37 C in a one liter, straight-walled Pyrex bottle containing 500 ml of culture medium.

The medium was prepared in 10 liter amounts and sterilized by filtration through a 0.22 μ Millipore filter. The pH of the medium was between 6.9 and 7.1 when completed. The 10 liter flask containing the reservoir medium was cooled to 4 C before use and maintained its sterile condition for over 3 weeks at room temperature.

Inocula were 20 ml volumes of A broth (minimal glucose-salts broth with 0.05% sodium citrate added) inoculated from a stock slant and incubated without aeration at 37 C for 18 hours. After incubation the cells were separated by centrifugation at 1600 x g for 20 min and the supernatant discarded. The cells were resuspended in 2 ml of pH 7.0 Sorenson's

phosphate buffer, and the suspension was then added to the culture vessel. The cultures were aerated through Pyrex fritted glass dispersion tubes of coarse porosity. Sterilization of the air was accomplished by passage through a sterile glass wool filter. The air flow metering device and culture vessel have been previously described (Ecker and Lockhart, 1959; Ecker and Lockhart, 1961a). Oxygen absorption rates were determined by a modification of the sulfite reduction techniques described by Ecker and Lockhart (1959). Aeration rates were always maintained at an oxygen absorption rate of not less than 40 m moles 0_2/L/hr. This rate will support a population of E. coli up to 1 x 10^{11} cells/ml of medium (Ecker and Lockhart, 1961a). The maximum population achieved in any experiment reported here was never more than 8% of this value.

When the cell population in the culture vessel reached an optical density of about 0.90 at 525 mμ, and while the cells were still in the logarithmic phase of growth, after about 8 hr, dilution of the culture was started so that the cells would achieve a state of balanced growth.

The desired rates of growth in generation/hr were achieved by adjusting the rate of fresh medium imput according to the formula:

$$R = \frac{W}{V} \qquad (1)$$

where R is the growth rate, V is the volume of medium in the culture vessel, and W is the overflow from the culture system in ml/hr. Four rates of growth were chosen between 0.5 and 0.1 gen/hr, as flow rates greater than these led to a wash out of the culture and flow rates lower made sample collection difficult. The actual rate of medium exchange was determined by measuring the effluent from the culture, and the growth

rates were determined by applying this value to formula (1).

As indicated in Table 1, cells were grown at 4 rates of growth. Each limiting substrate was used at all growth rates, making a total of 8 different culture conditions.

Cultures will be designated according to both the limiting nutrient and rate of growth as follows: C5 indicates a carbon limited culture grown at 0.5 gen/hr, N1 indicates a nitrogen limited culture grown at 0.1 gen/hr, the other cultures were designated C3, C2, C1, N5, N3, and N2.

It required 15-20 hours after the first addition of fresh medium to establish a balanced state in the culture vessel. Once such a balanced growth system was established, the optical density at 525 mμ remained within $\pm$ 1 O.D. unit for over 50 hours. After the cells had been in continuous growth for 24 hr, samples were collected by means of an adaptor which was placed in the overflow line. Samples for chemical and other analysis were collected in 10 ml amounts in an ice bath at -2 C. The samples were centrifuged in the cold at 1800 x g for 20 min and the supernatant was removed. If the cells were not used immediately they were frozen at -20 C until needed. It has been demonstrated that such storage procedures do not affect the analytical determinations (Ecker and Lockhart, 1961b). When the cells were to be used they were resuspended in 5 ml Sorenson's phosphate buffer, pH7, and centrifuged for 20 min at 1800 x g. Samples of the culture supernatant were held at -20 C until analyzed.

The number of bacteria in each culture was determined by plate count. A standard 10 fold dilution series was made of the bacteria to be counted. A 0.1 ml sample of three dilutions, either 10^{-5} to 10^{-7} or 10^{-6} to 10^{-8},

Table 1. The growth rates and the nutrient available to the cultures at the indicated rates of growth as determined by the dilution factor

gen/hr (R)	hr/gen (T)	Overflow ml/hr	Available nutrient mg/hr			
			Carbon limited		Nitrogen limited	
			Carbon	Nitrogen	Carbon	Nitrogen
0.5	1.97	250	125	100	1000	100
0.38	2.63	190	95	76	760	76
0.23	4.34	115	57.5	46	460	46
0.1	10.00	50	25	20	200	20

was spread on 4 agar plates containing commercial EMB agar with the agar concentration increased to 1.5%. The plates were incubated for 24 hr, and the colonies were counted.

Biochemical procedures

Substrate The residual substrate in the culture effluent was determined in order to confirm the growth limiting conditions. Ammonia nitrogen was determined by the method of Niss (1957) as adapted to this culture system by Ecker and Lockhart (1961b). All dilutions were made with distilled water and optical densities were determined at 615 mμ against a distilled water-reagent blank. A standard curve was prepared using $(NH_4)_2SO_4$ and recorded as μg ammonia nitrogen/ml. The amount of nitrogen in the samples was determined by comparison with the standard curve.

Carbohydrate Total carbohydrate determinations were made by the anthrone method of Loewus (1952) as modified by Ecker and Lockhart (1961b). The optical densities at 560 mμ were taken against a distilled water-reagent blank. A standard curve was prepared using glucose as a standard. The amount of carbohydrate in the samples was determined as μg glucose by comparison with the standard curve.

Protein Total protein was determined by two methods. The first (Folin phenol or phenol protein) was based upon the method of Lowry, et al. (1951) as modified by Ecker and Lockhart (1961b). Cells from 10 ml of culture were resuspended in distilled water to approximately 10^9 cells/ml. To 0.5 ml of this cell suspension, or to 0.5 ml of cell free extract, 0.3 ml of 4.0 N NaOH was added. This was heated in a boiling water bath for 4 min, cooled rapidly, and 0.2 ml of 4.0 N HCl added. This sample was then treated according to the method of Lowry, et al. (1951). A standard of crystalline bovine serum albumin containing 0.01 mg protein was included with each test series, and the results were expressed as mg serum albumin/ml of original culture.

Total protein was also determined by a procedure based on the biuret method of Layne (1957). A 20 ml sample of culture containing 2×10^9 to 8×10^9 cells was centrifuged as described. To the sedimented cells was added 2 ml of 1.0 N CO_2 - free NaOH, the cells resuspended, and heated for 4 min in a boiling water bath. The samples were cooled and 4 ml of biuret reagent added. The color was allowed to develop for at least 15 min at room temperature and the optical density was taken at 550 mμ against a 1 N NaOH blank treated as a sample. A curve of various concentrations of bovine serum albumin was included with each set of samples

and the results were expressed in terms of bovine serum albumin protein.

Nucleic acid The total extractable nucleic acid in the cells was determined by a modification of the technique of Ogur and Rosen (1950) designed to apply to microbial cells. A 10 ml sample of cells was centrifuged and the supernatant discarded. The cells were resuspended in 0.5 ml of distilled water and 4 ml of 0.5 N $HClO_4$ was added to the suspension. The cell suspension was heated in a water bath for 30 min at 70 C, after which the sediment was separated by centrifugation and the optical density of the supernatant was determined at both 260 mμ and 280 mμ, using 1 x 1 cm matched silica cuvettes. The blank consisted of 0.5 N perchloric acid. The concentration of nucleic acid was found by using a nomograph based on the data given by Warburg and Christian (1942).

A modification of the procedure outlined by Schaechter, et al. (1958) was used to determine DNA. This procedure is based on the diphenylamine reaction of Burton (1956). To 1 ml of the perchloric acid extract prepared for total nucleic acid determination was added 4 ml of diphenylamine reagent. The mixture was allowed to stand at 30 C for 16 hr. The optical density was determined at 630 mμ against a water-reagent blank. The amount of DNA/sample was determined by comparison with a standard DNA curve prepared from salmon sperm DNA.

Ribose nucleic acid (RNA) was determined by subtracting the value obtained for DNA from the total nucleic acid.

Triphenyl tetrazolium chloride reduction The ability of the cells to reduce triphenyl tetrazolium chloride (TTC) was determined by a procedure adapted from Kopper (1952). Throughout the procedure until the time of incubation the cells were kept chilled below -2 C. Samples kept

at this temperature showed no change in reducing ability for up to 6 hrs. A 10 ml sample of the cold cell suspension was centrifuged at 3100 x g for 10 min. The supernatant was discarded and 2 ml of Sorenson's phosphate buffer pH 7 was added to the sample. The cells were then incubated at 37 C for 3 min and 1 ml of freshly prepared 0.1% TTC was added. The samples were again incubated at 37 C for 15 min and 7 ml of acetone was added. The reduced TTC was extracted from the cells by vigorous shaking, and the samples were centrifuged for 5 min at 2100 x g to remove the cellular debris. The optical density of the samples was determined at 450 mμ against a blank containing buffer, TTC, and acetone. A standard curve of reduced TTC was prepared by reducing a known amount of TTC with a few crystals of sodium hydrosulfite (Kun and Abood, 1949). Several amounts of TTC between 15 and 300 μg were reduced and a standard curve was constructed (Figure 1). Optical density readings were made at 3 wave lengths, (420, 430, and 450 mμ) in order to determine which wave length was optimum (Figure 1).

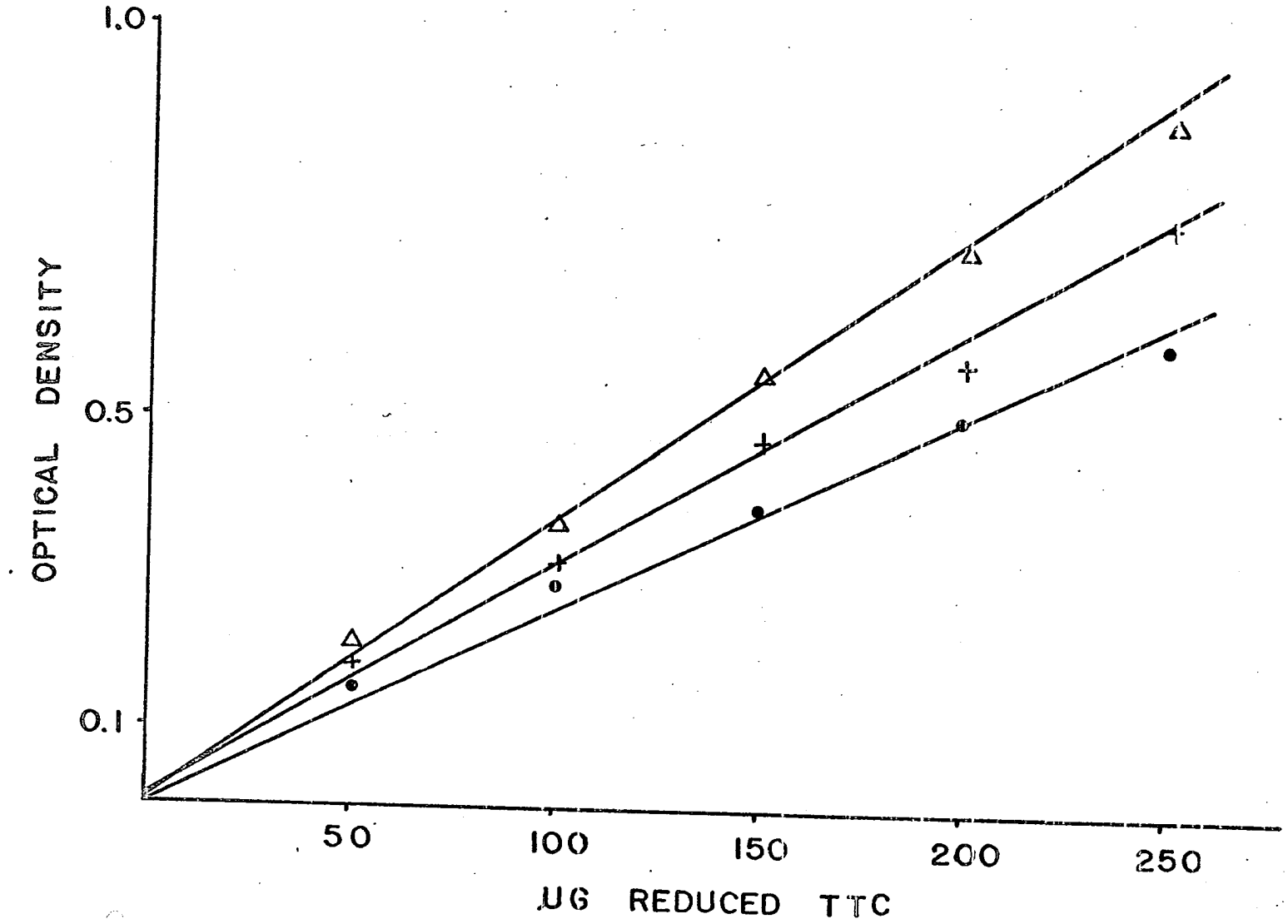
Glycogen The determination of bacterial glycogen was accomplished using a modification of the methods of Good, Kramer and Symogyi (1933), and Palmstierna (1956). Cells were removed from the culture and quickly cooled to -4 C. Such samples can be held at 0 C for 3 days without loss of glycogen (Palmstierna and Eliasson, 1956). A 10 ml sample of cells was centrifuged for 12 min at 2500 x g. The supernatant was decanted, and 0.2 ml of diethyl ether was added to the cells. The ether-cell mixture was boiled for 10 sec to extract the cellular lipids which otherwise would precipitate with glycogen on the addition of alcohol. The glycogen was extracted from the cells by adding 0.2 ml of 30% potassium hydroxide and

Figure 1. Standard curves for triphenyl tetrazolium chloride reduction determinations

Solid circles - optical density changes at 420 mμ

Crosses - optical density changes at 430 mμ

Triangles - optical density changes at 450 mμ


1.0
0.5
0.1
OPTICAL DENSITY
50
100
150
200
250
UG REDUCED TTC

boiling the mixture for 30 min. Two drops of 0.1% sodium sulfate was added to the extract followed by the addition of 0.5 ml ethanol. The suspension was boiled for 30 seconds and allowed to cool to room temperature. The extracted glycogen precipitated as a white floc upon cooling. The samples were centrifuged for 20 min at 3100 x g and the supernatants were discarded.

In order to determine the glycogen present in the sample it was necessary to hydrolyze the precipitate and determine the glucose present in the hydrolysate. Two ml of 0.6 N HCl were added to the residue and refluxed for 3 hr. The sample was then cooled and the volume brought to 2.5 ml with distilled water. The glycogen was then estimated as glucose by the anthrone procedure, and the results expressed at μg glucose.

Amino acids Cells from 10 ml of culture which were to be used in determining free amino acids were resuspended in 3 ml distilled water and immersed in boiling water for 10 min, then centrifuged (Ribbons and Dawes, 1963). Amino acids were estimated by the method of Yemm and Cocking (1955). To 1 ml of the cell extract was added 0.5 ml of 0.2 M citrate buffer, pH 5, and 1.2 ml of a potassium cyanide-ninhydrin-methyl cellosolve solution. The samples were placed in boiling water for 15 min. The samples were quickly cooled and brought to a convenient volume by adding 4 ml of 60% ethanol to each sample. The optical density was determined at 570 mμ against a buffer-reagent blank which had been treated as the samples. Two standard curves were prepared: (a) used 0.1 to 3.5 μg amino acid nitrogen/ml obtained from a stock glycine solution, and (b) contained 0.3 to 10 μg ammonia nitrogen/ml. The distilled water used to prepare the reagents used in this test was passed through a column of

Permutit to remove any trace of ammonia. All values were corrected for the presence of ammonia, which was estimated independently by the procedure of Niss (1957).

Glucose-6-phosphate dehydrogenase The determination of glucose-6-phosphate dehydrogenase (G-6-P DH) activity followed the procedure outlined by Scott and Cohen (1953). Cell free extracts containing 3.47 mg protein/ml at pH 8 were thawed just prior to use.

The enzyme reaction mixture contained, in 3 ml; 0.1 M tris buffer, pH 8.5, 1.85 ml; NADP, 0.4 μ moles; G-6-P, 2 μ moles; and $MgCl_2$, 3×10^{-3} M, 0.2 ml. The reaction was started by adding 0.05 ml of cell free extract to the mixture contained in a 1 x 1 cm silica cuvette. The change in absorbancy was measured against a reference cell containing the same reagents, except that water was substituted for enzyme. The principle of this assay depended on measuring the rate of increase in absorbance at 340 mμ due to the reduction of NADP which occurred during the process of the enzymatic reaction (Noltmann and Kuby, 1963).

Each reaction was allowed to proceed at 25 C for 10 min or until the transmission was less than 15%. The enzymatic activity was calculated as defined by Löhr and Waller (1963), "The unit of enzyme activity ... is the amount of enzyme in 1 ml sample which at 25 C in a 3 ml assay mixture changes the optical density of NADPH (reduced nicotinamide adenine dinucleotide phosphate) at 340 mμ by 0.001 in 1 min."

Immunologic studies

Antigens for immunologic use were prepared from cells C5, C1, N5 and N1. A preservative, 1:10,000 merthiolate, was added to each of the

cell free extracts prepared from these cells in tris buffer at pH 7. The stock merthiolate solution was made by adding 1.4 g borax and 1 g merthiolate to 100 ml distilled water.

Each extract was then mixed with an equal volume of Freund's incomplete adjuvant. A homogeneous suspension was prepared by vigorous mixing just prior to use. This material served as an antigen in the preparation of antibody to the cell extract. The serological reactions used to demonstrate antibody formation were carried out with the same cell extracts without Freund's adjuvant.

Antibodies to the cell extracts were prepared by injecting two 1.5 - 2 Kg rabbits with 1 ml of each antigen. The intradermal injections were spaced so that there were 12 - 15 injection sites on the dorsal surface of each animal. A second dose of 0.5 ml antigen-adjuvant mixture was given adjacent to the sites of the first injection 16 days after the first dose had been given.

Two weeks following the second injection, 5 ml of blood was obtained from each animal by cardiac puncture and the serum was titered using the capillary tube precipitin technique of Kabat and Mayer (1961). A 2 cm column of antiserum was drawn into a 1.5 mm capillary tube followed by a 2 cm column of cell free extract. The tubes were placed upright with the antiserum end down and the tips were sealed with 1:1 mixture of petroleum jelly and paraffin. The tubes were held for 48 hours at room temperature. Frequent observation during this period of time established that the titers in all tubes were less than 1:10.

Thirty days following the first injections the animals were again vaccinated. Each animal was injected subcutaneously with 1 ml of cell

extract without adjuvant. A fourth series of immunizations (1 ml cell extract without adjuvant given subcutaneously) was administered at 46 days, and 7 days later the animals were bled by cardiac puncture.

Twenty ml of blood was obtained from each animal and allowed to stand at room temperature for 6 hours. The blood was then placed in the cold (4 C) for 12 hours, centrifuged and the serum removed. To the sera thus obtained was added 1:10,000 merthiolate. The sera were then stored at 2 C until used.

Difco's purified agar was found to give the best results in those immunological reactions based on gel diffusion. Agar concentrations both at 1.7% and 0.85% as recommended by Campbell, et al. (1963) were used. A 1:10,000 dilution of merthiolate was added to all agar preparations as a preservative. A phosphate buffer at pH 7.4 with an ionic strength of 0.05 as described by Crowle (1961), or a barbital buffer at pH 8.2 with an ionic strength of 0.1, also described by Crowle (1961), was used in the preparation of the agar.

A procedure modified from the technique of Ouchterlony (1949, 1962) was used to demonstrate the antigenic relationships between all extracts. A 100 x 82 mm glass photographic plate was cleaned with 2% potassium bichromate in concentrated sulfuric acid and thoroughly rinsed in distilled water. The cleaned plates were then stored in alcohol until used (Hirschfeld, 1960).

When a test was to be performed, 10 - 15 ml of hot 0.85% agar prepared in buffer was added to the plates. The agar was allowed to solidify while the plates rested on a flat surface. Once the agar had hardened, an appropriate arrangement of circular holes was cut into the agar using

a cork boring device. The patterns and sizes were taken from dies 6865 A and 6866 A of the LKB Instrument Co., Stockholm, Sweden. A diagonal pattern with 3 mm wells was prepared. The distance between the edge of a well in the central row and the edge of the nearest well in the outer row was 5.2 mm. The outside rows measured 10 mm from center to center (Figure 2). This pattern allowed the simultaneous analysis of all sera-cell combinations. In order to achieve a more critical analysis of individual cell-sera reactions two smaller patterns designed for one serum and 2 cell extracts, or the reverse, were prepared. The first was a triangular arrangement of two 3 mm wells separated by 5.2 mm from a third central reactant well and 4.9 mm from each other (Figure 2). The second individual arrangement was a modification of the triangular design arranged to allow the reactants a larger diffusion path combined with the shorter one of 5.2 mm. This particular pattern (Figure 2) also allowed an incident angle change between reactants such that three different angles of contact between antigen and antibody could be observed. All five wells were 3 mm in diameter with the separating dimensions indicated in Figure 2.

The reactants to be placed in the wells were mixed with an equal volume of cooled (45 C) 0.85% agar and the wells were charged. The plates were placed on a flat surface in a humid atmosphere at room temperature and were observed over a 60 hr period. No precipitin lines were noted to first appear, then dissolve with the passage of time. The lines were examined by means of a 2 power hand lens. The lines were made visible through the use of a viewing box constructed as indicated in the "materials" section. The indirect light from such an apparatus served to illuminate the precipitin lines formed in the agar according to the principle of

Figure 2. Agar well patterns used in the gel diffusion studies of cell extracts

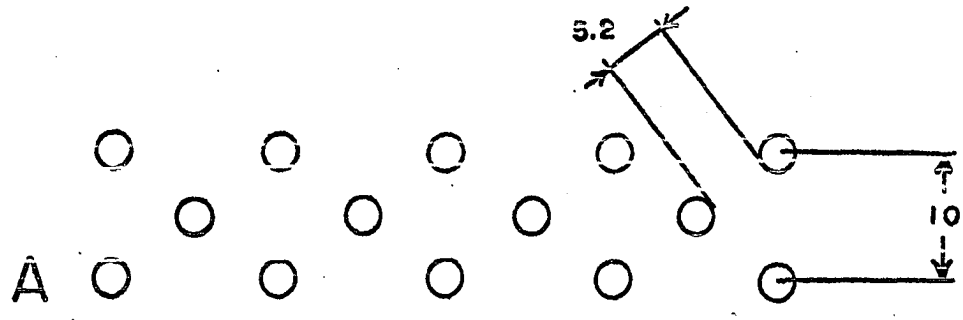
A - Diagonal pattern allowing the simultaneous use of all sera and cells being studied

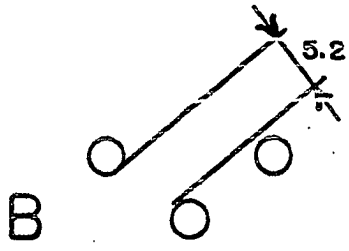
B - A triangular pattern used for careful analysis of one set of reactants

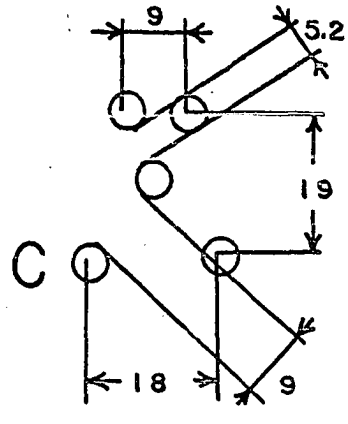
C - A pattern designed to provide a change in the angle of incidence between antigen and antibody

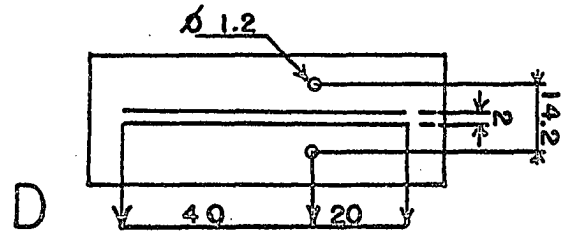
D - A pattern used in immunoelectrophoresis

Dimensions are in millimeters


A
5.2
10


B
5.2


C
9
5.2
19
18
9


D
Ø 1.2
2
14.2
40
20

dark field microscopy.

Photographs were made of all lines in the agar using a Durst 35 mm camera. Prints were made which gave an overall 3X magnification of the original precipitin lines and these photographs were used to compare the results of the tests.

After the precipitin lines had been photographed the wells were filled with distilled water and a piece of E & D #613 filter paper was placed on the agar surface and the slides were left exposed to the atmosphere until dry. The plates were then washed and stained by a procedure adapted from Crowle (1961) and Hirschfeld (1960). The slides were immersed in 0.85% NaCl for 24 hours to remove the unbound protein, and were then placed in 1% acetic acid for 12 - 24 hours to remove the sodium chloride and acidify or fix the bands for staining. Several changes of wash solution were made during these procedures. The plates were dried as before and stained.

Staining for protein was accomplished by using amido black 10B; lipid stains oil Red O and sudan black B were used; alcian blue and the p-Phenylenediamine oxidation reaction (Crowle, 1961) were used to stain for polysaccharide. Following the staining-destaining procedure the slides were washed for 3 min in acidified 1% glycerol in order to prevent the agar from cracking, and the slides were air dried.

In order to increase the resolution of precipitin bands in the antigen system the technique of immunoelectrophoresis was performed (Grabar, 1958). In this procedure the agar, sera, cell extracts and buffers were the same as those described for the Ouchterlony technique except that a barbital buffer, pH 8.2, was used as well as phosphate

buffer.

A 1.7% agar undercoating was prepared on each plate by placing 10 - 12 drops of the very hot agar on one end of the plate and drawing this agar across the plate with a strip of filter paper cut to the width of the plate. This lower gel was then allowed to harden before 15 ml of the 0.85% agar was added (Campbell, et al., 1963). Once the upper agar had gelled an appropriate pattern was cut into the agar as in Figure 2.

The agar was removed from the 1.5 mm sample wells and the wells were charged with extract that had been diluted 1:1 with 0.85% agar. The plates were placed in the electrophorsis chamber (the electrical connection between the buffer and the plates was provided by two pieces of cellulose sponge) and 100 V (6.1 V/cm) was applied for 90 min.

Upon completion of electrophoresis the plates were removed from the chamber; the agar was aspirated from the central trough using a beveled 16 gauge needle attached to the laboratory vacuum system, and the trough was charged with serum which had been mixed with an equal part 0.85% agar. The plates were placed in a humid atmosphere at room temperature for 36 - 48 hours. The precipitin bands which developed were photographed, and the plates were stained and dried as described for the Ouchterlony procedure.

Electrophoresis of the cell free extracts on Whatman 3 mm paper was performed according to the procedure outlined by Kabat and Mayer (1961). An aliquot of 50 lambda of each of the 4 extracts was added to the paper in a 2.5 cm band with a Lang-Levy pipette. The paper strips were placed in the chamber and a low voltage (10 volts/strip) was applied for 4 hours. The strips were removed, dried and stained with bromphenol blue to

identify the protein components.

In order to increase the resolution of the electrophoretic procedure an agar gel support medium was used (Matson, 1962). A 0.5% agar gel was prepared on a 10 x 8 cm photographic plate. The samples were applied to the agar by imbedding a small 12 x 1.5 mm strip of highly absorbent filter paper impregnated with the sample into the agar with the long axis of the strip perpendicular to the intended direction of current flow. The plates were placed in the electrophoresis chamber and the current (4 to 12 ma) was applied until separation was achieved (4 to 8 hr). The plates were removed and dried using an infrared heat lamp (Zak and Sun, 1958), then stained following the methods of Crowle (1961) as shown in Table 2.

Electrophoresis was also conducted on cellulose polyacetate strips. The samples were applied to the strips with a capillary tube and the strips were placed in the chamber. The strips were subjected to 12 volts/cm for 90 min, then removed and dried between filter paper strips. The protein, lipid, and polysaccharide separations which occurred were determined by staining the polyacetate strips in the appropriate stains for 10 - 15 min. Table 2 contains a list of the stains used.

A series of buffers (Miller and Golder, 1950) ranging in pH between 5 and 8.6, which were used with each electrophoretic support, are listed in Table 16.

The disc electrophoresis procedure followed exactly the outline of Davis (1960). Twenty-five lambda of each cell extract was used in this procedure and the gel cells were subjected to 4 mills for 50 min.

Table 2. Stains used to identify the migrating components of cell extracts subjected to electrophoresis in agar gel and on cellulose polyacetate. Staining procedures are reported by Crowle (1961)

Cell component stained	Stain	Destaining solution
Lipid	Sudan Black B, saturated soln.	50% ethyl alcohol
	Oil Red O, Saturated Soln.	50% ethyl alcohol
	Nile Blue A, .001%	1% H_2SO_4
Polysaccharide	Basic Fuchsin .004%	70% ethyl alcohol
	Alcian Blue .001%	1% acetic acid
Protein	Ponceau 2R 0.2%	5% acetic acid
	Azocarmine B .0005%	2% acetic acid
	Light Green SF 0.2%	5% acetic acid
	Amido Black .1%	Acetic acid-Methanol soln.
	Nigrosin .001%	2% acetic acid

Infrared analysis

Cells that had been frozen at -20 C were prepared for infrared spectroanalysis by thawing and washing twice with distilled water at 4 C. The washed cells were then placed in small weighing bottles and dried over $CaCl_2$ at 4 C for 24 hrs. The cells were then placed in a desiccator over $CaCl_2$ and held at room temperature until used.

A mull, containing approximately 99% purified KBr, was prepared with each cell sample (Ford and Wilkinson, 1954). The samples were then placed in the Beckman IR-7 and the absorption spectrum between 700^{cm-1} and 2000^{cm-1} was determined.[1] The spectra which were obtained from the procedure were analyzed for both qualitative and quantitative relationships.

[1]The actual operation of the IR-7 equipment and preparation of the infrared spectra was done by Miss Evelyn Conrad of the Ames Laboratory for Atomic Research.

OBSERVATIONS

Culture and Cells

Residual substrate

One of the greatest advantages afforded by the use of continuous culture is the precise control it allows the investigator over the conditions of growth. Through the use of relatively simple calculations one is able to determine the exact quantities of nutrients available to the cell and the proportions of these nutrients utilized during any given period of time. However, to facilitate calculation of substrate utilization it was necessary to determine the amount of available substrate remaining in the culture vessel during balanced growth conditions.

A sample of culture effluent was freed of cells by centrifugation and the residual substrate nitrogen determined by the method of Niss (1957) while the residual glucose was estimated with anthrone (Loewus, 1952). The results of these analyses (Table 3) indicate that a large excess of non-limiting nutrient and only a small amount of limiting substrate is present in the cultures during balanced growth.

While it is apparent that all cultures limited by carbon availability have approximately the same amount of residual glucose, in nitrogen limited culture there is an increase in the amount of available supernatant nitrogen with an increase in growth rate. A possible explanation for these results is given by Schulze and Lipe (1964). Under balanced growth conditions the growth rate is equal to the dilution rate as long as balance is maintained (i.e., dilution rate, D, is equal to the specific growth rate, μ, see Appendix).

Table 3. Determination of substrate concentrations in the culture effluent of both nitrogen and glucose limited cultures

Culture[a]	Residual nitrogen[b] µg/ml	Residual glucose µg/ml
N1	0	> 1200
N2	0	> 1200
N3	57.6	> 1200
N5	102.4	> 1200
C1	> 250	21
C2	> 300	21
C3	> 300	21
C5	> 300	21

[a]Cultures are designated by limiting substrate and growth rate e.g., N1 corresponds to a nitrogen limited culture grown at 0.1 gen/hr.

[b]Figures reported for residual nitrogen and residual glucose are the modal values obtained for 8 determinations obtained during the course of 3 separate cultures at each growth rate.

This provides a convenient means of establishing the relationship between substrate concentration and µ. Decreasing the feed rate results in a decrease of D = µ and this produces a decrease in substrate concentration due to the increased contact time between substrate and cells. There is an indication of this relationship in the available nitrogen content of carbon limited cultures (Table 3) although it is not as pronounced as in the nitrogen limited cells. The reason for this relationship not being seen in the available carbon in carbon limited cells is not understood. It is possible that the residual glucose values represent a total

utilization of glucose by the cells, and the small amount seen in the culture supernatant is a polysaccharide material excreted by the cells (such as was found by Baarda, 1962). Table 4 shows these relationships between residual substrates and growth rate, and indicates a 96% utilization of the available carbon by glucose limited cells. The available limiting nutrient was determined by multiplying the amount of nutrient/ml by the flow rate. The amount of limiting nutrient utilized by the cells was determined by subtracting the amount of nutrient lost per hour in the effluent from the total nutrient available during that period of time.

The number of cells

The most useful means of expressing bacterial characteristics is on a per-cell basis. Methods of comparison based on total culture volume or optical density relationships are not as revealing as when the figures are converted to cellular values. To facilitate calculation of values on a per-cell basis, a standard plate count was made of the bacteria in each growth condition; the results are summarized in Table 5.

Apparently the number of cells/ml at a given growth rate is not greatly affected by the limiting substrate, but the numbers are markedly affected by the rate at which the cells are grown. There are approximately 5 times as many cells/ml in cultures grown at low growth rates as in the cultures at high rates of growth.

The obvious question which arose from the fact that determinations of cell numbers showed a linear relationship between numbers and rate of growth was how the cell sizes compare at different growth rates. This question was answered by two approaches. The first method was to count

Table 4. The available limiting nutrient, the amount used, and the per cent of available nutrient utilized by cells in balanced growth

Growth rate gen/hr	Carbon limited cultures			Nitrogen limited cultures		
	mg glucose available/hr	mg glucose utilized/hr	% glucose utilized	mg nitrogen available/hr	mg nitrogen utilized/hr	% nitrogen utilized
0.50	125.0	119.8	96	100	74.4	74
0.38	95.0	91.1	96	76	65.9	86
0.23	57.5	55.1	96	46	46.0	100
0.10	25.0	24.0	96	20	20.0	100

Table 5. Number of cells/ml of culture effluent

Culture	Limiting substrate	Growth rate gen/hr	Bacteria/ml (x 10^8)
C1	Glucose	0.1	10.0
C2	Glucose	0.23	4.3
C3	Glucose	0.38	3.3
C5	Glucose	0.5	2.0
N1	Nitrogen	0.1	12.0
N2	Nitrogen	0.23	6.6
N3	Nitrogen	0.38	3.4
N5	Nitrogen	0.5	2.1

the cells/ml from different cell suspensions diluted to a constant optical density (Table 6).

The results in Table 6 suggest two relationships between cell size and growth conditions. It is apparent that for a given cellular mass there are more cells/ml in a culture grown at slow rates than at high growth rates. Table 6 indicates that there are 4 - 6 times as many cells in a low rate culture per unit of bacterial mass. This can only be explained if the cells are smaller in size. The second relationship which appears to exist is between the limiting substrate and the cell size. There are more cells per unit of bacterial mass at each growth rate under carbon limited conditions than in nitrogen limited cultures (Table 6), a feature which appears to be most significant at low growth rates.

The second approach used to indicate the relative size between cells

Table 6. The number of cells/ml in suspensions adjusted to an optical density of 0.60 at 525 mμ

Carbon limited cultures	Cells/ml x 10^8	Nitrogen limited cultures	Cells/ml x 10^8
C1	15	N1	5.9
C2	4.5	N2	2.6
C3	3.3	N3	2.2
C5	2.0	N5	1.2

grown at various rates was to photograph cells from each growth rate. Figure 3 is a photograph of cells grown at a high and a low growth rate under conditions of nitrogen limitation. A simple staining procedure using crystal violet was used to stain the cells on a microscope slide and the cells were photographed using a Durst 35 mm camera.

It is obvious from Figure 3 that cells grown at 0.1 gen/hr are smaller and more pleomorphic than those grown at 0.5 gen/hr, a finding which supports the results of Herbert (1959).

Biochemical

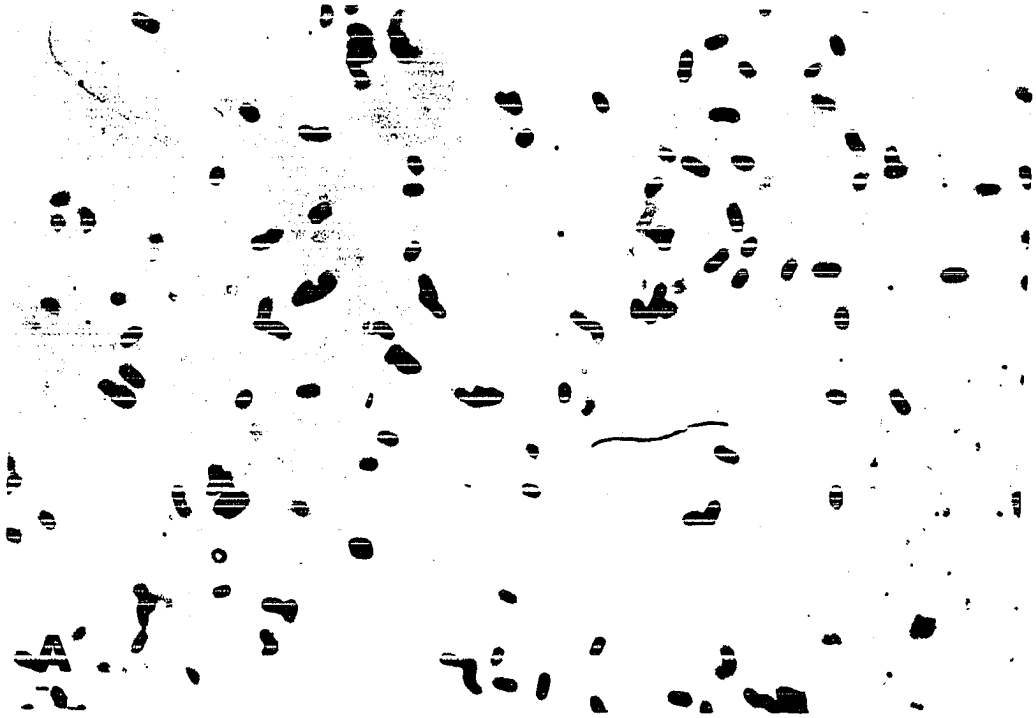
General

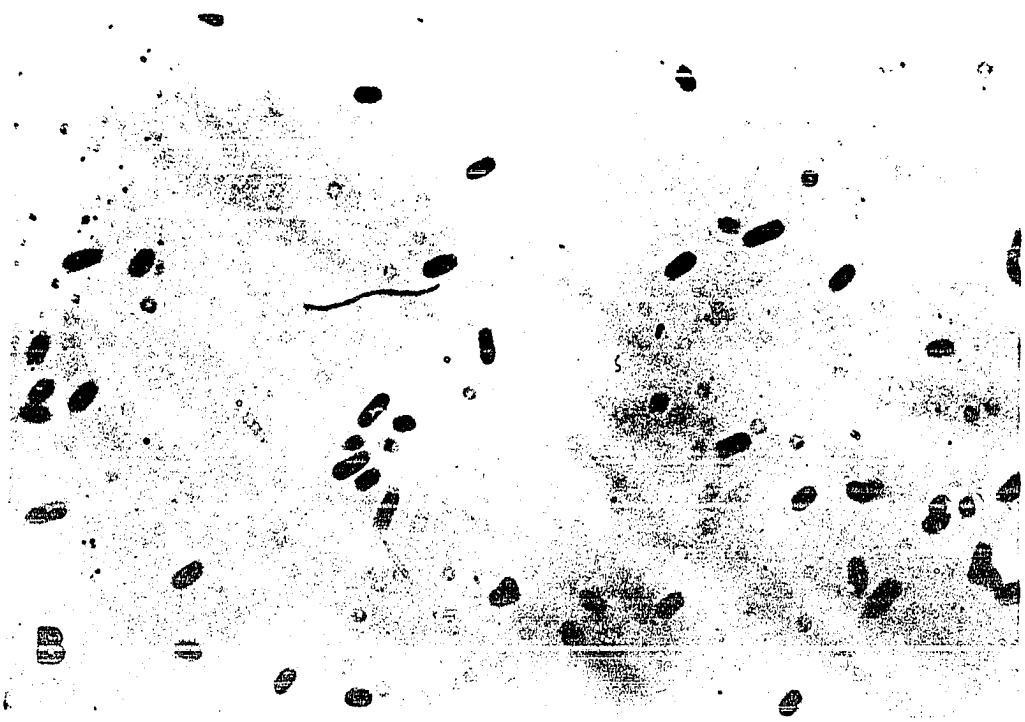
The biochemical procedures were done in order to quantitatively characterize cellular components. The choice of procedures was based on several factors: 1) the data reported in the current literature which have been used to characterize cells from their component parts, 2) those cellular functions and components thought to vary with growth rate which

Figure 3. Photograph of Escherichia coli K12/G6 grown in continuous culture (magnified 3400X)

A - Nitrogen limited cells grown at 0.1 generations/hr

B - Nitrogen limited cells grown at 0.5 generations/hr




B

are particularly amenable to study in a balanced growth system, and 3) a determination of the cellular components known to be directly affected by the nature of the limiting substrate.

Protein

The protein content of the cells was determined by two different methods, biuret and phenol, as described in the "methods" section. The use of two methods was prompted by the results of Baarda and Lockhart (1962) who reported a change in the biuret/phenol ratio associated with growth limitations when cells were grown in batch culture. The results of these experiments are given in Table 7.

From these results it can be seen that there is a definite increase in the amount of protein per cell with an increase in the rate at which cells are grown. This increase appears to be linear, as shown in Figure 4, and supports the data on cell size determinations.

This figure also indicates that the rate of increase in protein/cell in both the carbon limited and nitrogen limited cultures is the same. However, there is a greater difference in protein/cell between the nitrogen and carbon limited cultures demonstrated by the phenol method than by means of the biuret.

From the data in Table 7 it is apparent that there is no change in the ratio between biuret protein, which is an indication of the peptide bond content of the cells, and the phenol protein, largely due to the presence of cyclic amino acids, associated with the limiting substrate or rate of growth. This differs from the data reported for batch culture.

Table 7. The biuret and phenol protein content of cells grown at a steady state, showing the biuret/phenol ratio, and the efficiency of substrate utilization

Sample	Phenol protein[a]		Biuret protein		Ratio:	
	mg/ml culture	mg/cell x 10^{-11}	mg/ml culture	mg/cell x 10^{-11}	Biuret / Phenol	mg protein formed / mg substrate used
C1	0.0878	8.78	0.0805	8.05	0.916	0.16
C2	0.068	15.8	0.0687	15.9	1.0	0.16
C3	0.078	23.6	0.0647	19.6	0.83	0.16
C5	0.065	32.5	0.0910	45.5	1.2	0.16
N1	0.1672	13.9	0.1387	11.58	0.83	0.41
N2	0.204	30.9	0.154	23.4	0.75	0.41
N3	0.151	44.4	0.1285	37.8	0.83	0.41
N5	0.1345	64.0	0.1525	72.6	1.13	0.41

[a]Figures reported for phenol and biuret protein represent the modal value obtained from 6 determinations from each of 4 separate cultures at each growth rate. These values were within 2 mg/cell x 10^{-11} of the mean.

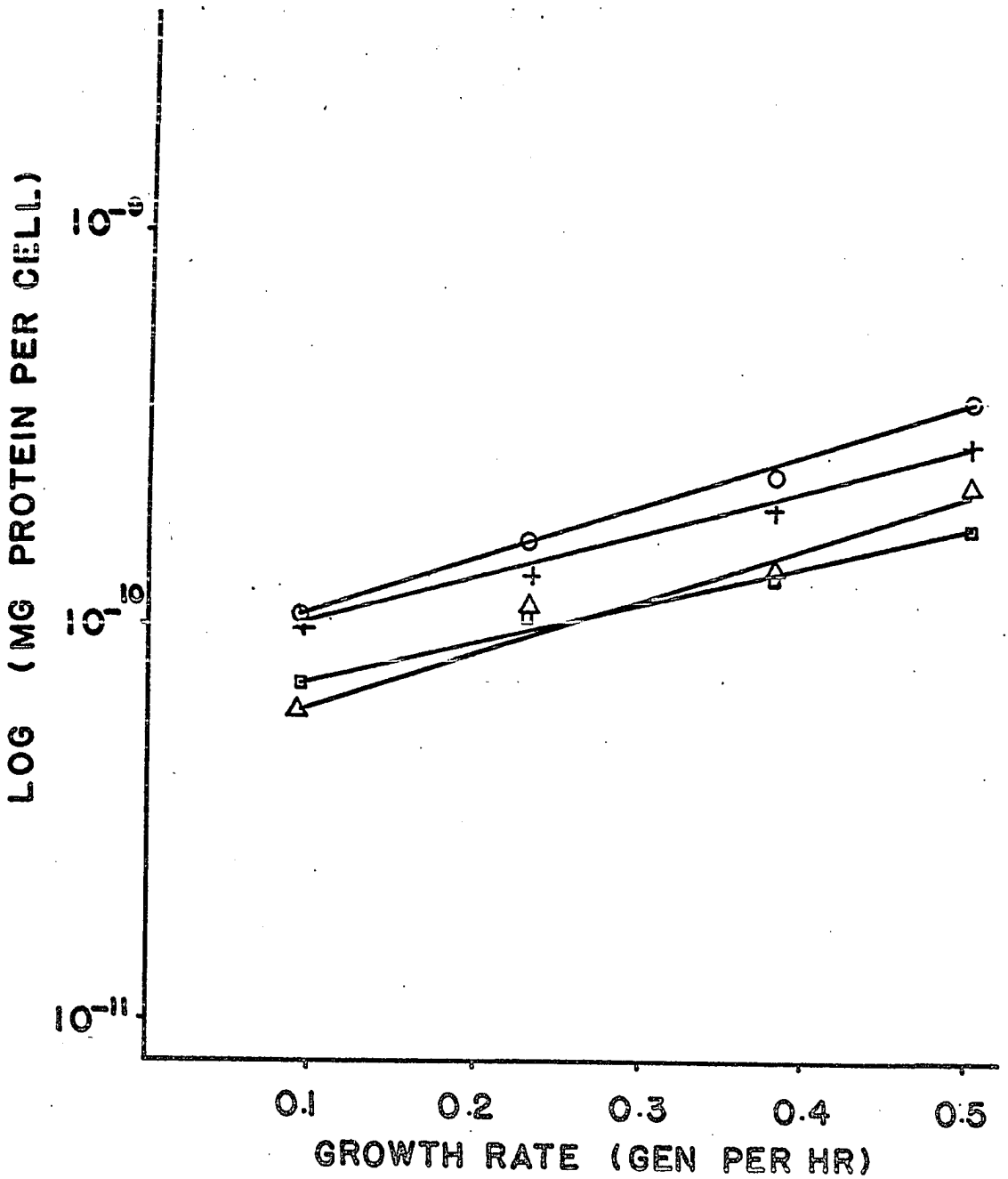
Figure 4. Protein/ml as determined by the phenol and biuret procedures

Circles - phenol protein of nitrogen limited cells

Squares - phenol protein of carbon limited cells

Crosses - biuret protein of nitrogen limited cells

Triangles - biuret protein of carbon limited cells


LOG (MG PROTEIN PER CELL)
10^{-9}
10^{-10}
10^{-11}
0.1
0.2
0.3
0.4
0.5
GROWTH RATE (GEN PER HR)

Nucleic acids

The results of the nucleic acid determinations (Figure 5) are in accord with the hypothesis of Kjeldgaard et al. (1958) that there is an increase in the nucleic acid concentration per cell with an increase in the rate at which the cells grow. As indicated by these data there is a linear relationship between the rate of growth and cellular nucleic acid content. The total nucleic acid can be separated into its component ribonucleic and deoxyribonucleic acids. Schaechter, et al. (1958) demonstrated that cells which are multiplying at a high rate have more nuclei/cell than those which are growing at a lower rate. They also demonstrated that there is a corresponding increase in the DNA/cell in rapidly growing cultures. These data are supported by the findings presented in Figure 5. However, it is apparent that the primary increase in nucleic acid associated with rate is due to the RNA component.

Whole cell reducing activity

An indication of the rate of cellular respiration is given by the cells' ability to metabolically reduce an oxidized indicator substance. The reduction of triphenyl tetrazolium chloride was carried out by cells which had been removed from the presence of growth substrate, and therefore represents the "endogenous" capacity of the cell to carry on metabolic process due to the physiological state imposed upon it by its prior environmental circumstances.

Table 8 suggests that there are two features of environment which markedly affect the cell's capacity to function upon removal from that environment, the growth limiting substrate and the rate of cellular

Figure 5. The total nucleic acid,[a] deoxy[b] and ribonucleic acids in cells grown with either substrate carbon or substrate nitrogen as limiting growth factors

Squares - total nucleic acid, nitrogen limiting

Circles - total nucleic acid, carbon limiting

Triangles - RNA, nitrogen limiting

Crosses - RNA, carbon limiting

Solid circles - DNA, nitrogen limiting

Solid squares - DNA, carbon limited

---

[a]Total nucleic acid was determined from 3 samples at each growth rate obtained from 2 different cultures. The value reported is the mode from all results and is within 0.2 gm/cell x 10^{-14} of the mean.

[b]The values reported for DNA are the modal values obtained for 3 determinations obtained during the course of 3 separate cultures at each growth rate, and agree within 0.01 gm/cell x 10^{-15} with the most extreme values obtained.

LOG (GRAMS PER CELL)
10⁻¹³
10⁻¹⁴
10⁻¹⁵
GROWTH RATE (GEN PER HR)
0.1
0.2
0.3
0.4
0.5

Table 8. The reduction of triphenyl tetrazolium chloride by cells which have been removed from their growth environment

Sample	Mg TTC reduced/10 ml culture[a]	µg TTC reduced/cell
C1	$\leq$ 10	$< 3 \times 10^{-10}$
C2	32	7.1×10^{-9}
C3	66	2.1×10^{-8}
C5	93	4.65×10^{-8}
N1	94	1.62×10^{-8}
N2	171	6.57×10^{-8}
N3	240	1.08×10^{-7}
N5	275	2.27×10^{-7}

[a]The figure reported for TTC reduction is the modal value of 15 determinations on samples taken from two cultures at each rate. The value reported is within 5 µg TTC reduced/10 ml culture of the maximum and minimum values obtained.

growth. Nitrogen limited cells reduce more tetrazolium than carbon limited cultures, a feature which appears to be more important than the rate of growth, although it is apparent that the greater the rate at which a cell was carrying out metabolic process before removal from its environment, the greater is its ability to continue these same metabolic sequences.

Glycogen

It would seem reasonable that where the endogenous metabolic rate is high the cell must be provided with a large amount of a reserve energy source. It has been shown that E. coli is capable of storing a poly-

glucose, called glycogen for simplicity, and that this glycogen serves as a reserve energy depot for use by the cell when exogenous energy sources are not available (Wilkinson, 1963). These facts prompted the determination of cellular glycogen in the carbon and nitrogen limited cultures (Table 9).

The data reported in Table 9 agree well with the reducing activity of the cells as indicated by the reduction of TTC. However, the relationship between bacterial mass and the glycogen content appears to be affected more by the rate at which the cells are grown than by the limiting substrate. Both carbon and nitrogen limited bacteria grown at a fast rate contain more glycogen than when grown at a slower rate.

Free amino acids

There appears to be some correlation between the rate of growth and the free amino acid content of bacteria, as shown in Table 10. These results indicate that there is more free amino acid/cell in cultures growing at fast rates than in those at low rates. These results also show that there is greater efficiency on the part of the cell in the production of free amino acid from limiting substrate in the faster growing cells, although these figures might be interpreted as indicating that the cell places more of its available nutrient into free amino acid production at high rates than at low.

Hydrogen ion concentration

The pH of the supernatant was determined for each culture and is reported in Table 11.

Table 9. The glycogen[a] content of cells grown in continuous cultures limited by carbon or nitrogen showing the yield of glycogen per unit of substrate consumed

Sample[b]	µg/10 ml culture	µg/cell x 10^{-7}[c]	Mg limiting substrate utilized / µg glycogen formed
C1	235	0.80	21.2
C2	645	1.5	12.9
C3	497	3.02	10.0
C5	885	4.43	5.66
N1	> 3000	> 2.49	> 1.33
N2	2510	3.8	1.59
N3	2000	5.87	1.72
N5	1530	7.61	1.80

[a]The determination of glycogen was in terms of glucose (refer to "methods").

[b]Cultures are designated by limiting substrate and growth rate; e.g., N1 corresponds to a nitrogen limited culture grown at 0.1 gen/hr.

[c]Figures reported are the modal value obtained for 12 determinations obtained during the course of 2 separate cultures at each growth rate. This value is within 0.2 µg/cell x 10^{-7} of the mean.

Enzyme activity

The method of determination and calculation of glucose-6-phosphate dehydrogenase activity is indicated in the "methods" section. The results of these determinations are shown in Table 12 and Figure 6. It appears that the limiting substrate is of great importance to the activity of this enzyme as there is considerably more activity in the nitrogen limited cultures than in carbon limited cells.

Table 10. The free amino acid content of Escherichia coli K12G6 grown at high and low rates under condition of carbon or nitrogen limitation

Sample	free amino acid[a] μg/cell × 10^{-9}	μg free amino acid / Mg substrate utilized
C1	0.915	2.7
C2	4.1	3.8
C3	6.7	4.6
C5	10.7	4.6
N1	2.68	8.0
N2	6.17	10.4
N3	9.0	10.8
N5	17.0	11.7

[a]Figures reported are the modal value obtained for 8 determinations obtained during the course of 2 separate cultures at each growth rate, the mode is within 0.3 μg/cell × 10^{-9} of the mean.

Table 11. pH of culture supernatant from cells in balanced growth under carbon and nitrogen limiting conditions

Carbon limited growth rate gen/hr	pH	Nitrogen limited growth rate gen/hr	pH
0.1	6.97	0.1	6.19
0.23	6.95	0.23	6.39
0.38	6.95	0.38	6.65
0.5	6.93	0.5	6.75

Table 12. The activity of glucose-6-phosphate dehydrogenose in *E. coli* K12/G6 grown at two different growth rates in both carbon and nitrogen limited cultures

Sample	Units of enzyme activity/ml[a]
C1	792
C5	540
N1	1840
N5	2060

[a] Figures are from 4 separate determinations of enzyme activity in cell extracts prepared from a single culture at each growth rate. No deviation in value was found in the 4 resulting values.

It should also be pointed out that these relationships are on the basis of protein concentration because of the experimental difficulty in determining the number of cells used in each enzyme preparation. However, there are approximately five times as many cells per unit of protein (Table 6) in the slow rate cells as the high rate cells. It follows then that if these data were reported on a per-cell basis the greatest enzyme activity would be in the cells grown at the higher rates. Although the data reported are for the extreme growth rates, the enzyme activity in the cells at the intermediate growth rates (0.23 and 0.38 gen/hr) was also determined. These values were found to be between those obtained for the maximum and minimum growth rates.

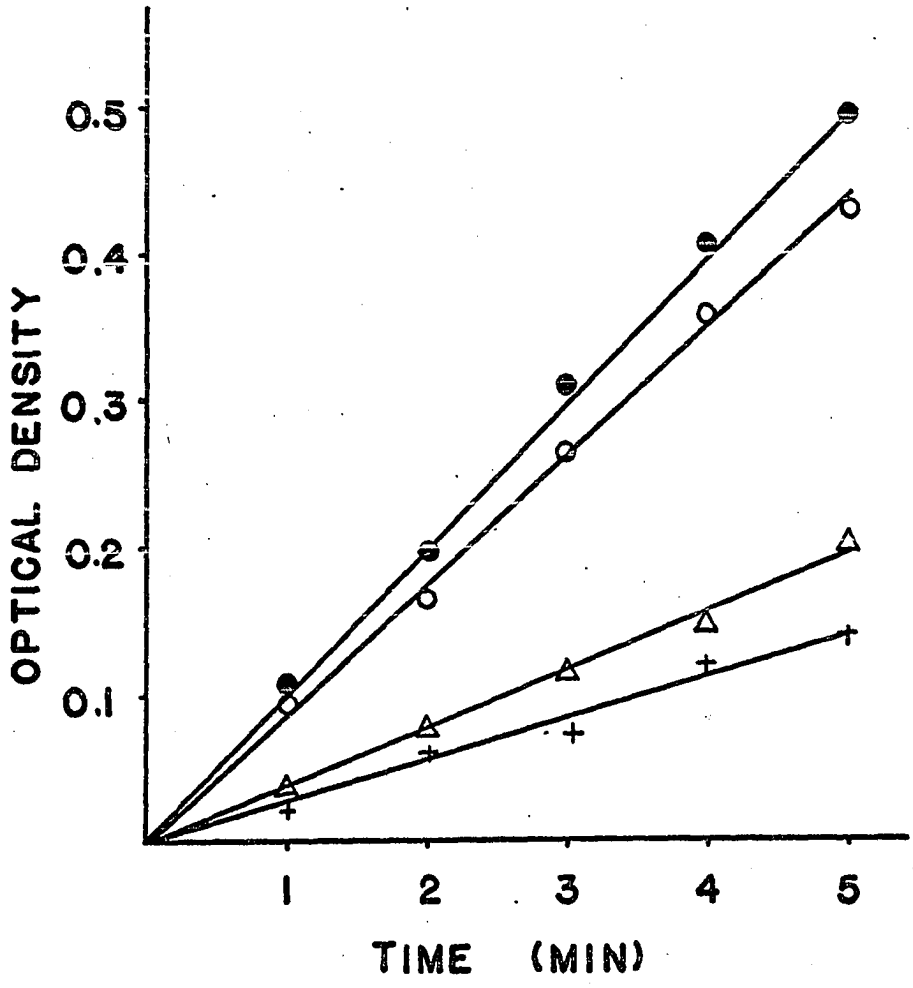
Figure 6. The rate of change in optical density due to the NADP-H formed from NADP by the enzymatic breakdown of glucose-6-phosphate by glucose-6-phosphate dehydrogenase

Solid circles - activity due to the enzymes in nitrogen limited cells grown at 0.1 gen/hr

Open circles - activity due to the enzymes in nitrogen limited cells grown at 0.5 gen/hr

Crosses - activity due to the enzymes in carbon limited cells grown at 0.1 gen/hr

Triangles - activity due to the enzymes in carbon limited cells grown at 0.5 gen/hr


OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
TIME (MIN)

Immunologic Studies

General

The biochemical determinations are particularly useful in that they give an indication of the quantitative relationships between cell components, and suggest the kinetic relationships between cells grown under different environmental circumstances. However, these procedures were not sufficient to demonstrate the nature of the changes which occurred in cells subjected to alteration in their growth environment. Therefore, three methods were chosen to help elucidate the qualitative physiological alterations occurring in cells as their growth environment is altered. These procedures based on immunology, electrophoresis, and infrared spectrometry were selected for use on the basis of their availability and feasibility and the nature of the relationship they are capable of demonstrating.

Gel diffusion

The precipitin band patterns obtained in the Ouchterlony procedure as described under "methods" were photographed and appear along with a line drawing of the bands as seen under the original experimental conditions as Figure 7. Table 13 indicates the relationship between the cell extracts and the precipitin bands as determined from the data in Figure 7. In the experiments reported here the cell extracts were placed in the two outside upper wells and the serum (a combined serum was used, composed of equal parts of the individual serums from rabbits immunized with the 4 cell types) was placed in the center well.

It is obvious from Table 13 and Figure 7 that there are some distinct

Table 13. The precipitin bands which occurred during the Ouchterlony procedure showing the relationship between the bands and the culture conditions

	Precipitin bands					
	A	B	C	D	E	F
Cell extracts N5[a]			X	X		
N1		X	X	X	X	
C5	X	X	X		X	
C1		X	X		X	X

[a]N5 Nitrogen limited cultures grown at 0.5 gen/hr.
N1 Nitrogen limited cultures grown at 0.1 gen/hr.
C5 Carbon limited cultures grown at 0.5 gen/hr.
C1 Carbon limited cultures grown at 0.1 gen/hr.

differences in the precipitin band patterns obtained by testing the various cell extracts against a combined serum.

There are bands of identity between cell extracts N5, N1, C5 and C1 and also between N1, C5 and C1, and bands of partial identity between N5 and N1. Two individual bands representing cell extracts C5 and C1 are also visible.

The Ouchterlony procedure is very sensitive and is capable of determining quantities of protein as low as 6 μg/ml (Marrack, 1963). However, it is recognized that the bands which occur in double diffusion tests of necessity represent the minimum number of precipitating systems present. This limitation suggested that another procedure be used which could increase the resolution of the antigen-antibody systems. It is well known that the process of immunoelectrophoresis greatly increases the

Figure 7. Photographs and line drawings of precipitin bands obtained by a double diffusion in agar gel of the cell free extracts (upper two wells) against a combined antiserum (lower well)

A - cell extracts N1 and N5, left to right

B - cell extracts C5 and N5, left to right

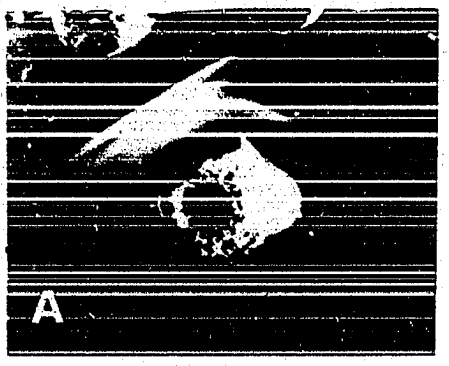
C - cell extracts C1 and N5, left to right

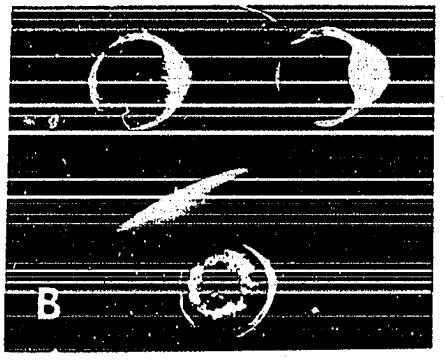
D - cell extracts C5 and N1, left to right

E - cell extracts C1 and N1, left to right

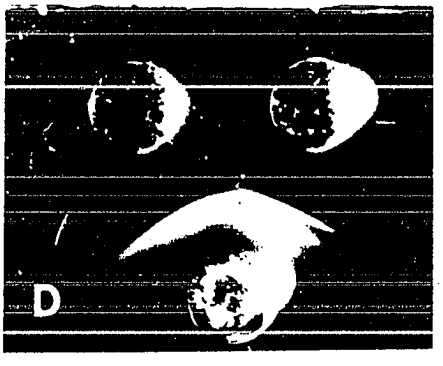
F - cell extracts C1 and C5, left to right

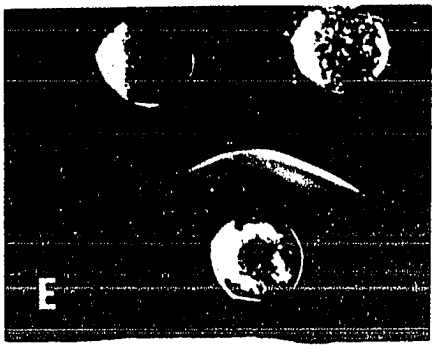
The cell extracts N5 was obtained from nitrogen limited cells grown at 0.5 gen/hr, N1 from nitrogen limited cells grown at 0.1 gen/hr, C5 from carbon limited cells grown at 0.5 gen/hr, and C1 from carbon limited cells grown at 0.1 gen/hr

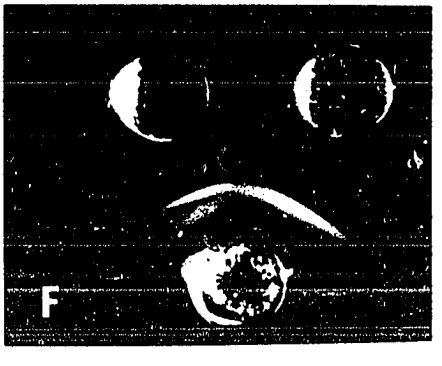

A


B


C


D


E


F

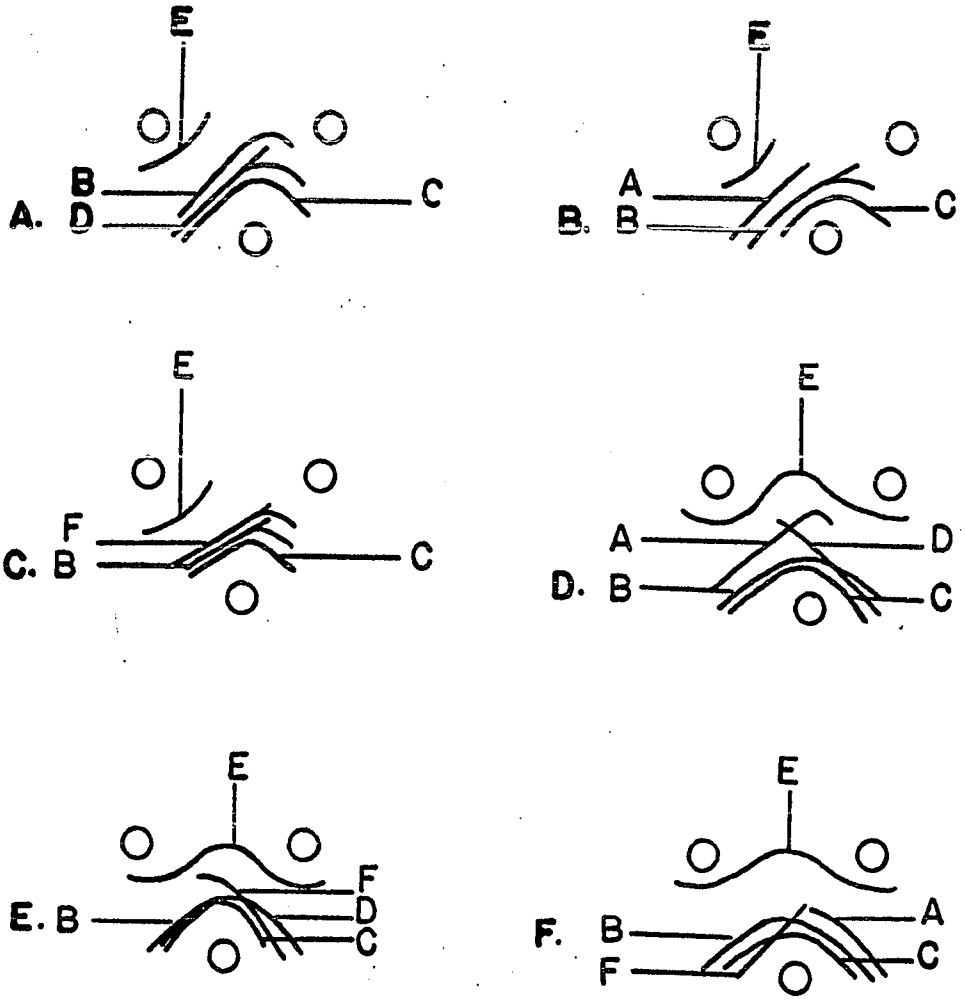


Figure 7. (Continued)

sensitivity in analysis of complex antigen mixtures (Grabar, 1958). This increased resolution was exemplified in these experiments by an increase in the discernible precipitin bands from 6 by means of the double diffusion technique to 22 in the immunoelectrophoretic procedure (Figures 8, 9 and 10). These figures contain photographs of the precipitin bands which occurred in the immunoelectrophoretic procedure and line drawings of the bands observed in the original work. The line drawings were prepared by making transparent plastic overlays of the photographed patterns. The relationships between the patterns were then made by comparison with the overlays. Each band in the line drawings was labelled with a letter of the alphabet. The same letter has been used to identify identical bands in each reaction and in all three figures.

Tables 14 and 15 are tabular summaries of the precipitin bands found in the homologous antiserum vs cell extract and combined antisera vs cell extract represented in Figures 8 and 9, respectively.

Tables 14 and 15 indicate the following relationships among the cell free extracts demonstrated by the precipitin bands found in the electrophoretic procedure: Bands G and H are related to the limiting substrate, both occurring in the nitrogen limited cultures; while bands P and A' are also governed by the limiting substrate, occurring in the cultures limited in their available glucose. Bands K and N are indicative of cells grown at high rates and are independent of limiting substrate, while bands B and E are found in cells grown at low rates and are also independent of the limiting nutrient. There are two common bands A and D, while a variety of individual bands occur with each cell extract.

The extracts which were separated using barbital buffer (Figure 10)

Table 14. A comparison of precipitin bands as they occur in Figure 8, showing the relationship between the common antigenic components of the 4 cell free extracts as compared against their homologous antiserum

Cell extracts[a]	Common bands	Substrate bands[b]	Rate bands[c]	Unique bands
N5	A	G,H	K,N	R,S
N1	A,D	G,H	B,E	C,F,I,J
C5	A,D	P	K,N	I,L,M,O,Q
C1	A,D	P	B,E	T,U

[a]Cell extract N5 is from nitrogen limited cells grown at 0.5 gen/hr, N1 is from nitrogen limited cells grown at 0.1 gen/hr, C5 is from carbon limited cells grown at 0.5 gen/hr and C1 is from carbon limited cells grown at 0.1 gen/hr.

[b]Substrate bands represent those bands common to cells grown under the same substrate limiting conditions.

[c]Rate bands represent those bands common to cells grown at the same rate.

Table 15. A comparison of precipitin bands as they occur in Figure 9, showing the relationship between the common antigenic components of the 4 cell free extracts as compared against a combined antiserum

Cell extracts[a]	Common bands	Substrate bands	Rate bands	Unique bands
N5	A	G,H	K,N	S
N1	A,D	G,H	B,E	C,F,I,Q
C5	A,D	P,A'	K,N	I,M,O,Q
C1	A,D	P,A'	B,E	T

[a]The designation of cell extracts and the significance of the column headings is given in Table 14.

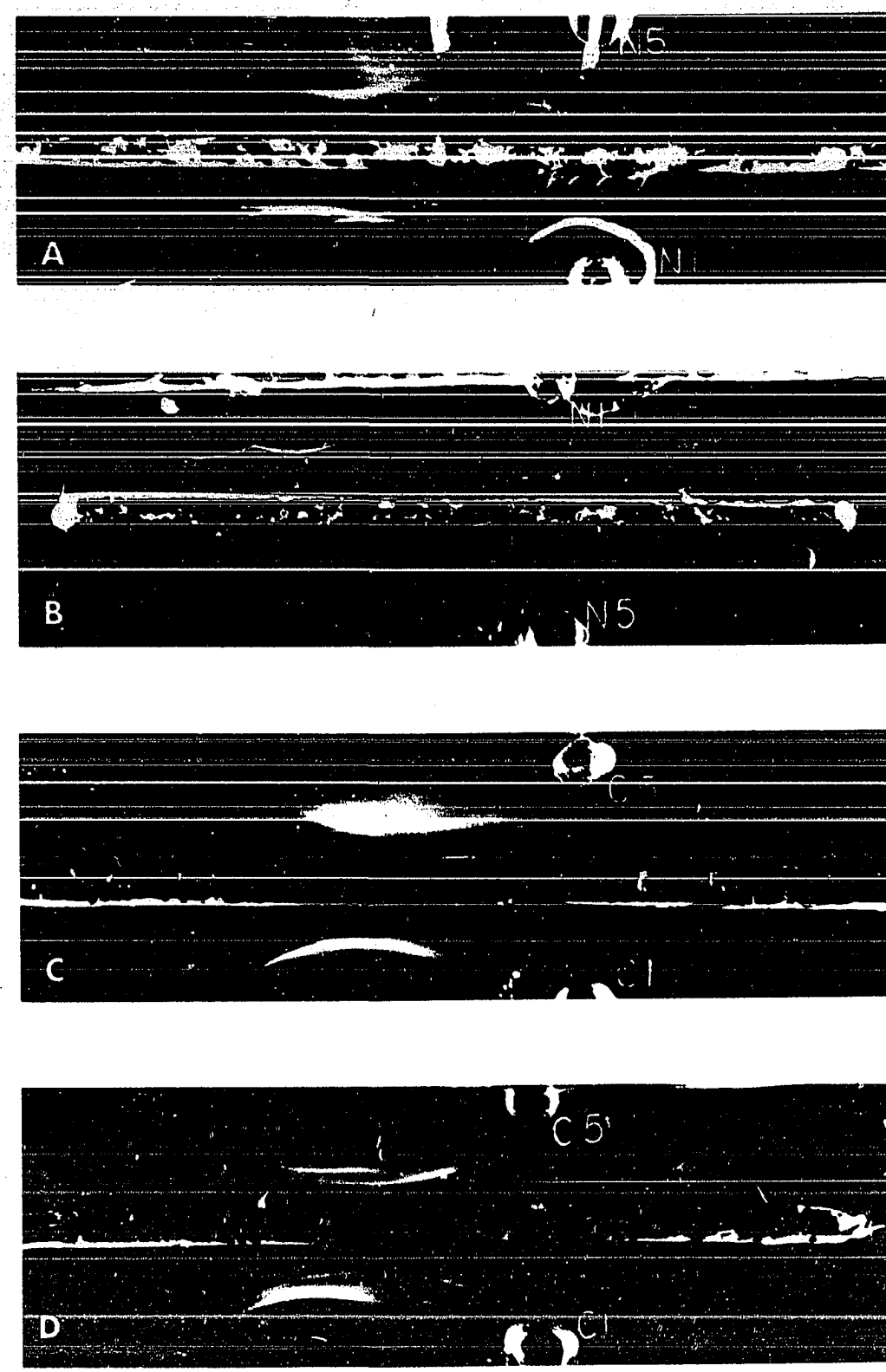
Figure 8. Photographs and line drawings of precipitin bands obtained by means of immunoelectrophoresis. These patterns represent the interactions between cell free extracts and the homologous antiserum prepared against each extract. The agar gel was prepared with phosphate buffer at pH 7.24 and an ionic strength of 0.05.

A - cell extracts N5 (top) and N1 (bottom) against antiserum prepared with cell extract N5.

B - cell extracts N1 (top) and N5 (bottom) against antiserum prepared with cell extract N1.

C - cell extracts C5 (top) and C1 (bottom) against antiserum prepared with cell extract C5.

D - cell extracts C5 (top) and C1 (bottom) against antiserum prepared with cell extract C1.


A
B
C
D

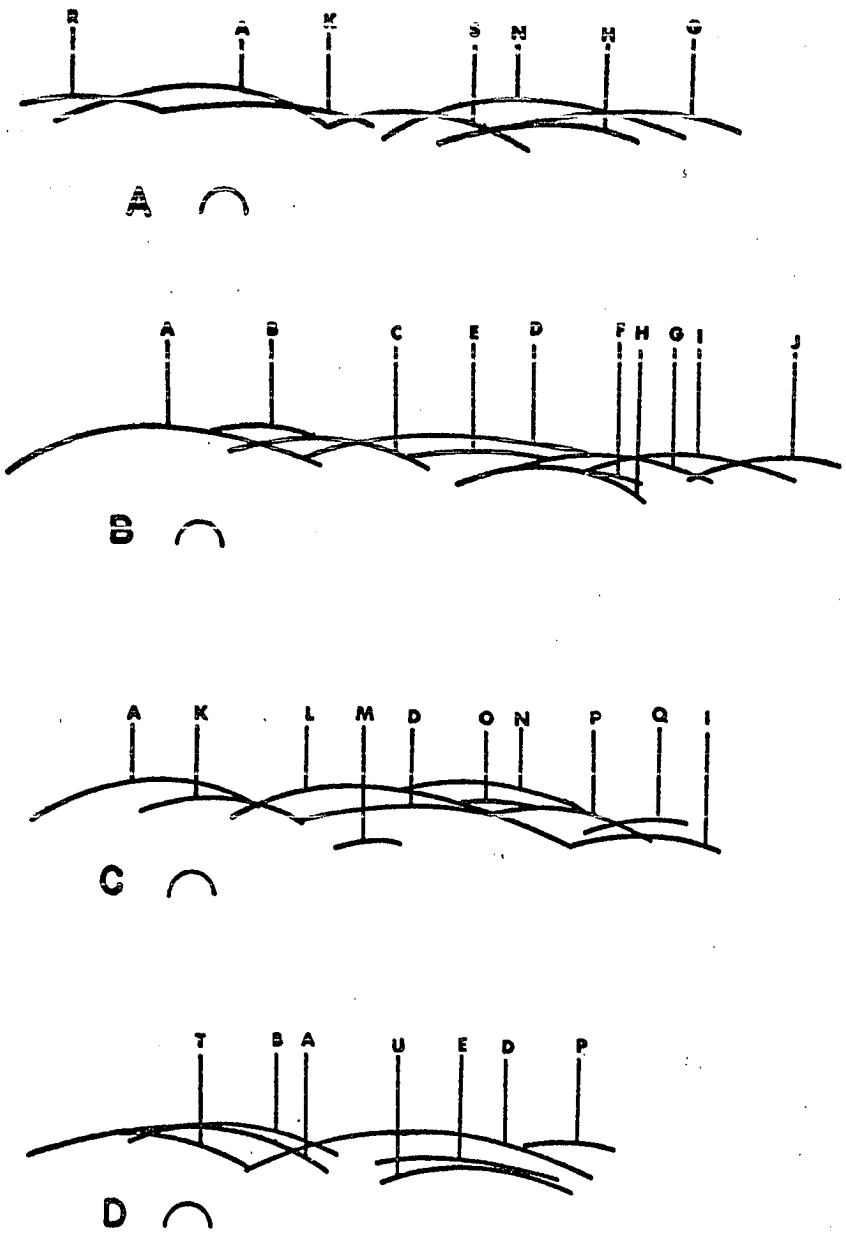


Figure 8. (Continued)

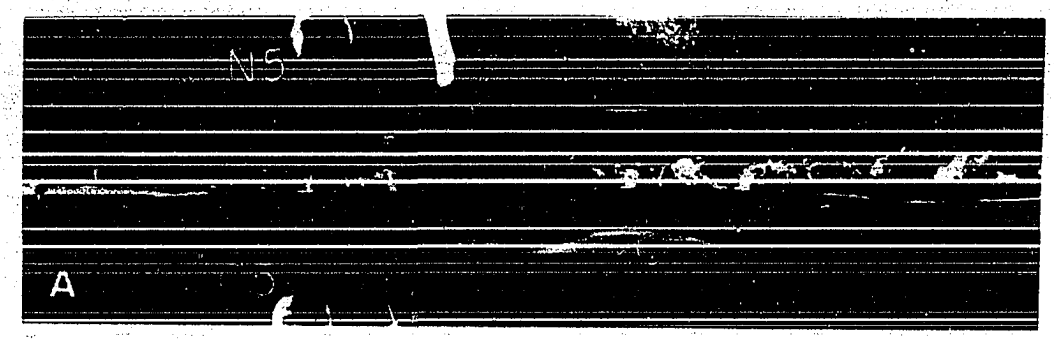
Figure 9. Photographs and line drawings of immunoelectrophoretic patterns obtained from the interaction of cell free extracts and the combined antiserum prepared through the use of those extracts. The reaction was carried out at pH 7.24 in 0.85% agar gel prepared with phosphate buffer with an ionic strength of 0.05

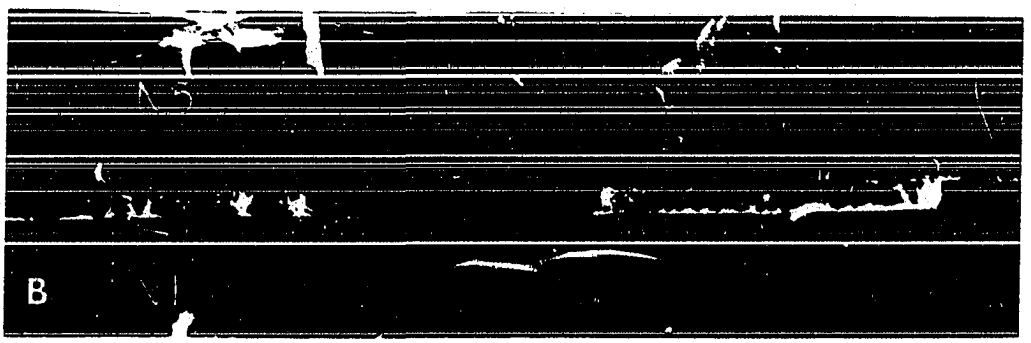
A - cell extracts N5 (top) and C5 (bottom) against a combined antiserum

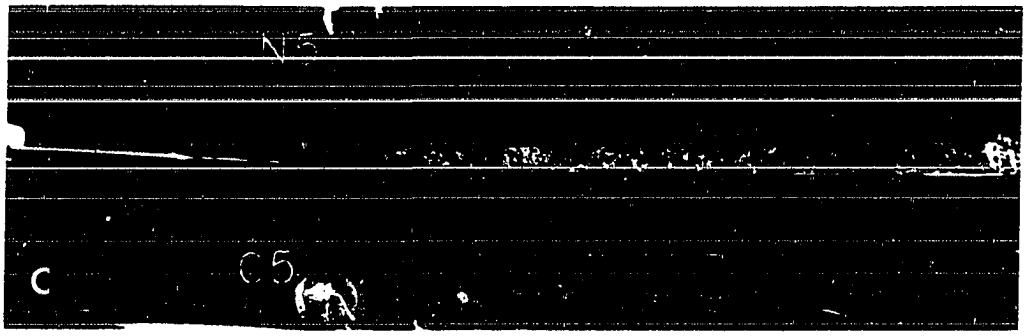
B - cell extracts N5 (top) and N1 (bottom) against a combined antiserum

C - cell extracts N5 (top) and C5 (bottom) against a combined antiserum

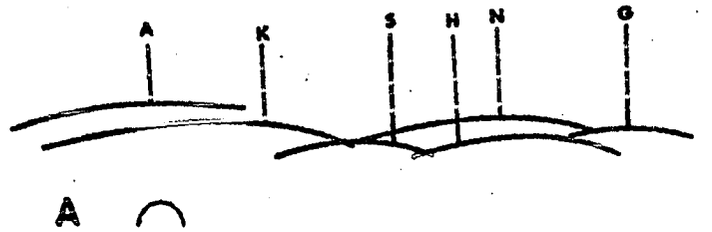
D - cell extracts C5 (top) and C1 (bottom) against a combined antiserum


N-5
A

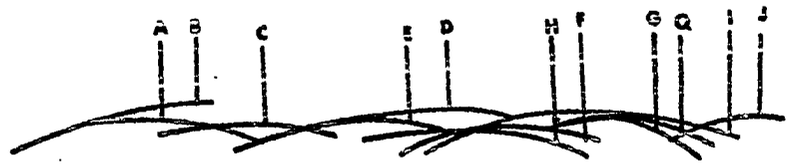

N-5
B


N-5
C
C-5

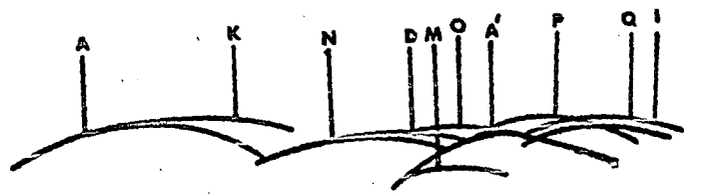

C-5
D
C-1



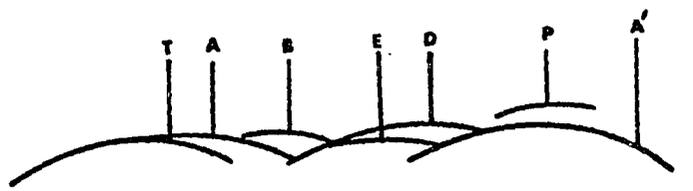
A



B



C



D

Figure 9. (Continued)

Figure 10. Photographs and line drawings of immunoelectrophoretic patterns obtained from the interaction of cell free extracts and the homologous antiserum prepared through the use of these extracts. The reaction was carried out at pH 8.1 in 0.85% agar gel prepared with barbital buffer with an ionic strength of 0.1

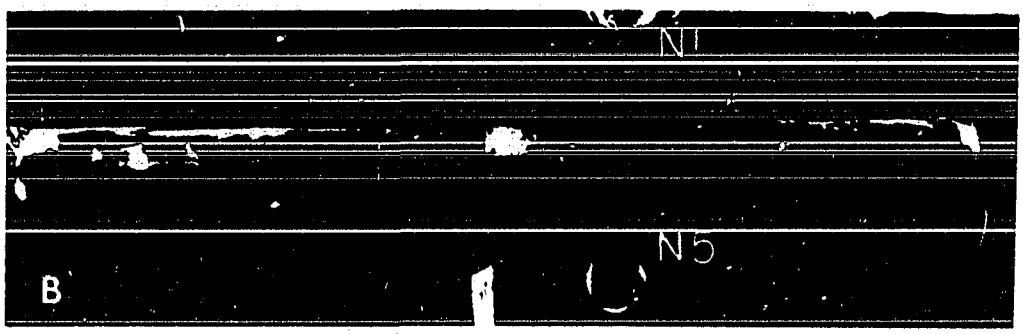
A - extracts N5 (top) and N1 (bottom) against antiserum prepared with cell extract N5

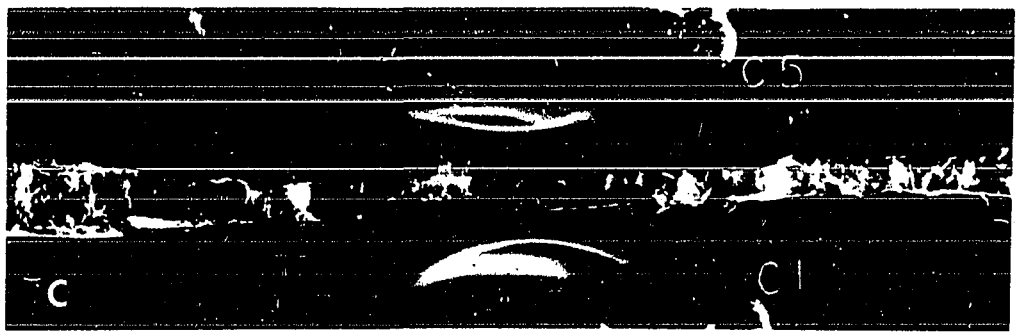
B - extracts N5 (top) and N1 (bottom) against antiserum prepared with cell extract N1

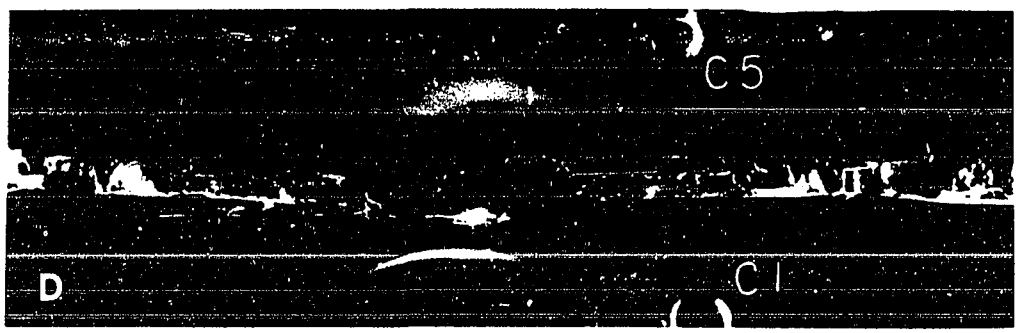
C - extracts C5 (top) and C1 (bottom) against antiserum prepared with cell extract C5

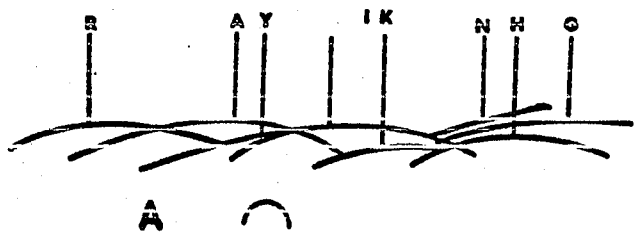
D - extracts C5 (top) and C1 (bottom) against antiserum prepared with cell extract C1


N5
A
N1

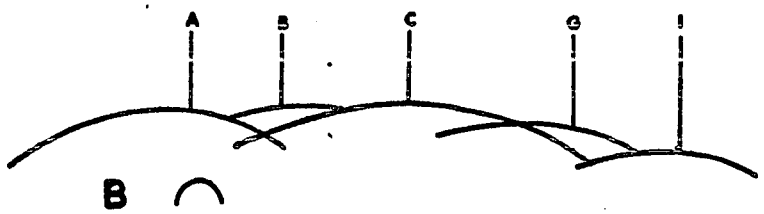

N1
N5
B


C5
C
C1

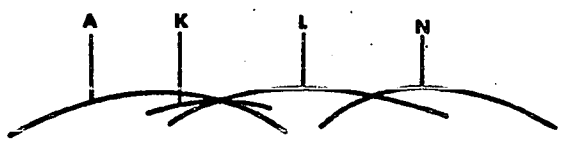

C5
D
C1



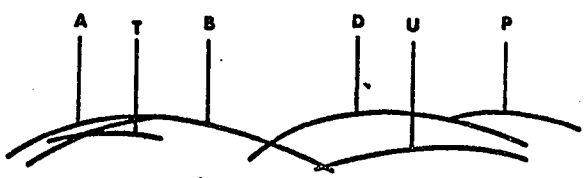
A



B



C



D

Figure 10. (Continued)

had essentially the same bands as those separated in phosphate buffer. However, the bands were not as well separated and in general there are fewer bands than in the phosphate buffered agar.

All of the bands formed in the gel diffusion procedures appear to be due to antiserum interacting with a protein or nucleoprotein cellular material as indicated by negative results when the bands were stained for polysaccharide and lipid. Amido Black gave the best results of all protein stains used and was reproducible. However, other protein stains were useful and produced acceptable results.

In general the immunologic studies demonstrated a qualitative difference between the cells grown as indicated.

Electrophoresis

The results of the electrophoretic procedures were varied and of little value in demonstrating qualitative relationships between the cell free extracts. The use of paper as a support medium proved unsatisfactory because of slow migration and lack of separation. An agar support medium was chosen in order to improve the separation of the macromolecular components of the cell, and because of the immunoelectrophoretic studies which were also conducted in agar gel. Table 16 gives the results of the gel electrophoresis experiments using a graded series of buffers with a pH range between 5 and 8.6. These buffers were used in the electrophoresis of cell free extracts on paper and cellulose polyacetate as well as agar gel.

Under most circumstances there was only one large continuous band appearing on the slides with little or no separation. This band began

Table 16. Results of agar-gel electrophoresis of disrupted cells grown in balanced growth at 0.5 or 0.1 gen/hr with either glucose or nitrogen as limiting substrate

Buffer	pH	Ionic strength	Current (ma)	Separation time (hr)	Migration (cm)
Acetate	5	0.1	12	4	2
Phosphate	6	0.1	12	4	2.5
Phosphate	7	0.1	9	4	2
Phosphate	7	0.1	12	4	2
Phosphate	7	0.05	6	4	2.4
Tris 0.2M	7.25	0.1	9	6	1.5
Tris 0.02M	7.25	0.05	9	6	4.9
Tris 0.02M	7.25	0.1	8	8	3.5
Veronal	8	0.05	4	6	2.2
Barbital	8.6	0.05	9	4	2
Triscitrate	8.6	0.05	10	4	2.5

at the origin and migrated without separation through the agar and stained as a uniform broad strip. Where separation occurred there were only two bands, a single broad strip beginning at the origin and migrating for some distance and a second band separated by about 2 mm from the first and migrating in front of it. It appeared that maximum separation was obtained at about pH 7 and the best migration occurred when the ionic strength was 0.05. The best buffer conditions were achieved with tris, which caused less agar opacity and with which the agar remained most firmly attached to the glass during the staining procedures. The greatest

separation was 4.9 cm in 6 hr, obtained with a discontinuous buffer system, i.e., 0.02 M tris-HCl pH 7.25, μ = 0.05 buffer, used to prepare the agar, and 0.02 M tris-HCl pH 7.25, μ = 0.1 buffer in the buffer tanks. The stains for polysaccharide and lipid were negative except for material at the origin.

The disc electrophoretic procedure provided the best electrophoretic method for separation of protein components. Twelve separate bands were obtained using this procedure; however, the bands appeared to be the same for each cell extract.

Infrared

The use of infrared spectra as a possible means of demonstrating qualitative difference between the cells was suggested by the large amount of current literature indicating the use of this procedure for the identification and classification of various bacterial species.

It is self evident that the infrared spectrum of a mixture of compounds such as occurs in any biological specimen, is essentially that of the sum of the spectra of the individual components. For this reason it is extremely difficult to state that any given absorption band is due to a single molecule or a group of well defined molecules. However, whenever bacteria are examined by infrared the resulting spectrum is basically the same regardless of the species used. This pattern has several well defined absorption bands that have been well characterized. The major bands as indicated in Figure 11 fall at 1075, 1150, 1238, 1395, 1460, 1530 and 1655 cm^{-1}. Those peaks at the lower wave lengths, e.g., 1655 and 1530 cm^{-1}, are largely due to the CONH vibrations of the peptide band and are

representative of the polypeptide concentration in the cells (Levi, Matheson and Thatcher, 1956). The band at 1460 cm^{-1} is largely due to the cellular fat concentrations while the band at 1395 cm^{-1} is one primarily due to polysaccharide (carboxylate ion) (Rideal and Adams, 1957). The band at 1238 cm^{-1} can be traced to the P=O band and the vibration of phosphorous within its band structure (Levine, Stevenson and Bordner, 1953a). Logically then, the cellular nucleic acids are largely responsible for bands appearing in this area. Cellular carbohydrates are primarily responsible for the 1150 cm^{-1} band (Levine, et al., 1953c) while the large deep band between 1125 and 1000 cm^{-1} is due to C-O-C vibrational modes, C-O stretching vibrations and OH deformations (Norris, 1959), which indicates that this band is due primarily to polysaccharide as well as some nucleic acid.

An analysis for the qualitative differences among the cells subjected to infrared investigation indicated that in nearly all respects the cells are chemically the same (Figure 11). Earlier work by Greenstreet and Norris (1957) indicated that bacteria from numerous genera give the same major absorption pattern and differences between the genera were based primarily upon small irregularities of pattern. Figure 11 shows that each cell population has a characteristic and irregular pattern at wave numbers below 1000 cm^{-1}; however, Fraser (1953) indicated that in such complex systems as biological materials, alterations of pattern in this frequency range are without significance. There are only three notable qualitative differences observed in the 4 patterns in Figure 11. The first is a marked shoulder in the absorption pattern between 1136 cm^{-1} and 1200 cm^{-1} which corresponds to the growth rate differences of the

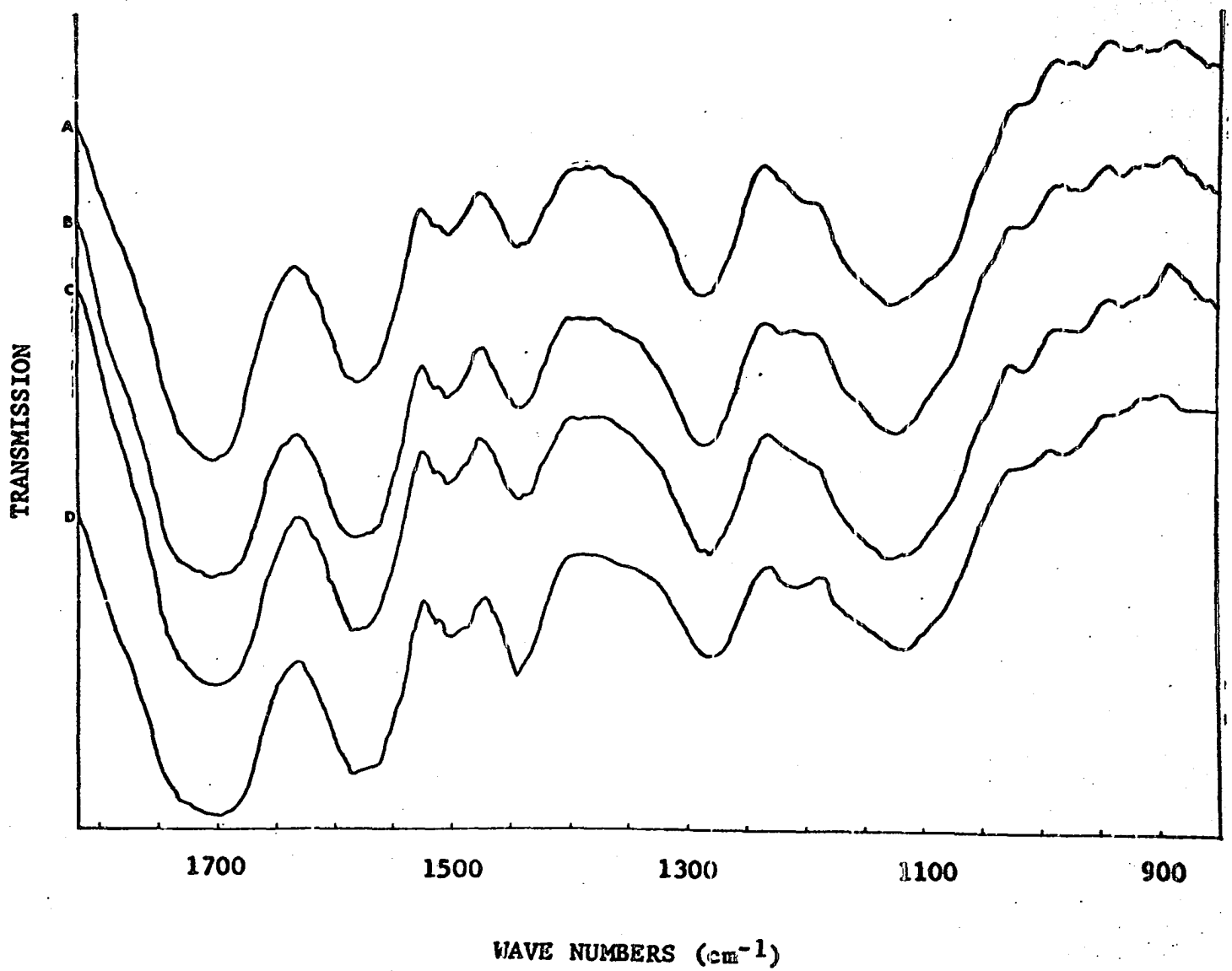
Figure 11. Infrared absorption spectra of *E. coli* K 12 G6 between 850 and 1820 cm^{-1}

A - nitrogen limited cultures grown at 0.5 gen/hr

B - nitrogen limited cultures grown at 0.1 gen/hr

C - carbon limited cultures grown at 0.5 gen/hr

D - carbon limited cultures grown at 0.1 gen/hr


A
B
C
D
TRANSMISSION
1700
1500
1300
1100
900
WAVE NUMBERS (cm-1)

cells. This shoulder appears in those cells grown at slow growth rates but is not found at the higher rates. Absorption in this area is largely due to C=O bonding and according to Levine et al. (1953a) represents cellular carbohydrate. The second notable difference in the patterns is also a function of growth rate; this is a small increase in absorbance at 1388 cm^{-1} in those cells grown at the higher growth rates. The small shoulder found at 1420 cm^{-1} in cells with a limited nitrogen source is the only indication that there is a qualitative difference induced in cells through change in their limiting substrate.

When the infrared patterns were subjected to a quantitative analysis by means of base line ratios (Wright, 1941) it became apparent that the patterns, although grossly similar, were indeed different suggesting that each cell type produces its own characteristic pattern which is dependent both upon its substrate ratio and rate of growth.

The absorbence at the desired frequencies was measured and the quantitative relationships between the various bands was found by dividing these values by the absorbence at 1530 cm^{-1} which is largely due to polypeptide, and thereby provides an internal standard for each cell group (Levi, et al., 1956).

Table 17 indicates the results of this analysis and shows that there is a direct correlation between growth rate and the cellular content of nucleic acid and polysaccharide. These results confirm the information obtained through chemical analysis reported in Figure 5 and Table 9.

Table 17. The absorbence in the infrared region of dried whole cells and the ratio of these absorbence values to the absorbence at 1530 cm^{-1}

Sample	Absorbence 1530 cm^{-1}	1240 cm^{-1}	1150 cm^{-1}	1075 cm^{-1}	OD 1240[a] / OD 1530	OD 1150[b] / OD 1530	OD 1075[c] / OD 1530
N5	0.699	0.456	0.292	0.516	0.652	0.418	0.738
N1	1.09	0.620	0.367	0.638	0.565	0.335	0.581
C5	0.959	0.602	0.387	0.678	0.627	0.403	0.707
C1	1.00	0.523	0.377	0.553	0.523	0.377	0.553

[a]Representative of nucleic acid.

[b]Representative of carbohydrate.

[c]Representative of polysaccharide.

DISCUSSION

Growth Control in Continuous Culture

The bacterial cell, though complex, is an assemblage of distinct and often separable chemical entities. Recent advances in biochemistry have led to the isolation and partial identification of enzymes and enzyme systems which can make proteins, DNA and RNA, and, as stated by Neidhardt (1963), "this success underlines the essential biochemical autonomy of these processes." Within the complexity of the whole cell the syntheses of the individual components can occur independently of each other for a period of time. However, in any environment which supports balanced growth, a bacterial cell produces each of its chemical components in predictable and unvarying proportions to the other components. It is thus apparent that the formation of each cellular component is governed by controls which coordinate synthesis and thereby insure the orderliness on which balanced growth depends. It is also obvious that these controls do not commit the cells to a rigid pattern of biosynthesis; rather they permit selective expansion of different functions in different environments (Neidhardt, 1963).

The problem which has been attacked in this thesis is one of determining the factors most responsible for the physiological state of bacteria at any given time. The specific problem revolves around a three part question: 1) is the growth rate alone responsible for the physiological state, 2) is the external environment, apart from its recognized control of growth rate, responsible for the physiological state or, 3) are both the growth rate and the environment of the cell responsible for the

physiological state of the organism, and if this is so to what extent is each factor responsible? For years investigators have used both chemical and biological procedures to establish the physiological state of Escherichia coli under a myriad of experimental conditions. The work undertaken here is an extension of much that has gone before in an attempt to demonstrate qualitative as well as quantitative differences in cells grown under set environmental circumstances.

One of the obvious problems which arises in any culture system where cells are grown continuously over a long period of time is the nature and extent of the genetic change occurring in the cell population. In any growing population of bacteria, mutants are likely to arise which are better fitted to the environment than the parent type. In balanced growth systems, this would consist of an ability to grow faster than the parental strain at low concentrations of the limiting factor. Demerec, et al. (1955) showed that under nitrogen limiting conditions mutation rates were proportional to growth rates in the regions of rapid growth but constant at lower growth rates (a feature probably related to the increased synthesis of DNA/cell at higher rates of growth). Northrop (1957) developed equations describing the changes that occur when spontaneous mutants are generated in a growing culture with growth rates the same as the parent strain, so that there will be no selection for or against it by the washout rate. Using these formulae, he then showed that with Bacillus megaterium the relative abundance of the mutant increased linearly with time, and in the cases where the mutant strain had a growth rate lower than the parent strain, the number remained constant following an initial rise.

Frequent samplings of the cultures of E. coli K12/G6 used in these

experiments indicated that there were no changes in morphological or clonal characteristics indicative of mutation, although it is reasonable to assume that during the course of some experiments mutants did arise which may have been able to grow at rates significantly higher than the parental strain. However, due to the relatively short duration of total culture time and because of the high initial populations in the cultures, it is unlikely that the effects of such mutant strains could be noted.

Control of Cell Size

Schaechter, et al. (1958) reported that the number of nuclei/cell, the amount of RNA/cell and the DNA/cell were all exponential functions of growth rate, and Herbert (1959) referred to the "striking" alteration in cell size due to growth rate changes. Herbert noted that cells grown at high rates were much larger microscopically and had a much greater mean cell mass, a feature confirmed by Ecker and Schaechter (1963) in their study of the ribosomal composition of Salmonella typhimurium. Herbert also noted that this size increase was mainly in cell length (Figure 3 suggests the same relationship in E. coli) and that Staphylococcus aureus showed little such size increase with growth rate, although the increase in RNA and DNA was similar to that of other species studied. Stevenson (1962) reported a 6 to 7 fold increase in the size of an Arthrobacter species during logarithmic growth. Citing the work of Schaechter, et al. (1958) and Neidhardt and Magasanik (1960), who obtained cell size increase of only 2 to 3 fold over a limited growth range, he suggested that such a large size increase was peculiar to Arthrobacter and represented the exception rather than the rule. However, from my

results (which show a 4 - 6 fold increase in cell size for *E. coli* K12/G6 when the growth rate is increased from 0.1 to 0.5 gen/hr) it would appear that this magnitude of cell size increase obtained by increasing growth rate is general, and not restricted to a single organism as suggested by Stevenson.

Although growth rate appears to have greater effect on cell size than other parameters which have been investigated, there seems also to be a size differential among cells grown at the same growth rate but under different substrate limiting conditions. The data show a 2 to 2.5 fold increase in the size of organisms grown on nitrogen limited medium over those cells grown with carbon as the limiting substrate. This substrate-cell size effect was noted also by Ecker and Schaechter (1963), who found that cells limited in their available nitrogen were much larger than those limited with glucose and suggested that the increase in the size of the nitrogen limited cells was probably due to accumulation of some form of polysaccharide. However, the data reported here indicate that not only are nitrogen limited cells larger because they contain more polysaccharide per cell but because they also contain more nucleic acid and protein per cell. Thus it appears that the increased size of the nitrogen limited cells may well be due to some overall metabolic relationships associated with excess carbon in the medium, which affect many aspects of the cell's physiological constitution.

Growth Rate Control of Protein Synthesis

The values obtained for the rate of protein synthesis are in agreement with previously reported experiments dealing with the problem of protein

and nucleic acid synthesis (Neidhardt and Fraenkel, 1961; Kjeldgaard, 1961; Kjeldgaard et al., 1958). There appears to be a direct correlation between the growth rate of the cells and the rate of protein synthesis. This is further borne out in experiments on nucleic acids, where Schaechter, et al. (1958) showed that the rate of protein synthesis per unit RNA is nearly the same at all growth rates, while the amount of RNA/cell increases with rate of growth. These observations were further supported by Nakada and Magasanik (1964), who demonstrated that the overall rate of protein synthesis is determined by the rate at which protein forming machinery (ribosomes and soluble RNA) functions. Furthermore, the amount of any one protein made per cell is in direct relation to the proportion of its messenger RNA to the total messenger RNA of the cell.

There is a point of interest in that the protein formed per unit of substrate utilized (the yield, Y, see Appendix) is considerably lower in carbon limited systems than for nitrogen limited cells. It has been pointed out by Ecker and Lockhart (1961b) that whereas nitrogen is used by the cells solely to form protoplasmic building blocks, E. coli uses its available carbon for at least three general functions: 1) mass synthesis, 2) cell division, and 3) a rather poorly defined function reflected in the cells' ability to continue the uptake of substrate carbon after growth and division have stopped (Lockhart and Powelson, 1954), termed "energy of maintenance" by Rahn (1932). It is not possible to determine from the data the proportion of glucose going toward each of these cell functions; however, this appears to be a reasonable explanation for the discrepancy in protein yield during the two growth conditions.

As discussed earlier (methods), it was hoped that a difference in

the ratio between the phenol and biuret protein values could be demonstrated for cells grown with carbon vs nitrogen limitation. Such a difference has been reported when these cells are grown in batch culture (Baarda, 1962), but does not appear in the data reported here. Because of the narrow range of values obtained in the protein determinations and because the normal distribution of values overlaps, it is felt that the use of the phenol and biuret protein determinations as well as the biuret/phenol ratio is unwarranted as a criterion for distinguishing between cells grown at high and low rates and between carbon and nitrogen limited cultures grown at a steady state. However, it is perhaps unnecessary to caution against drawing analogies too closely between the data obtained for batch culture and those for continuous culture. The phenol procedure for protein determination shows a greater difference in the protein per cell between carbon and nitrogen limited cultures than does the biuret method. This may suggest that when available carbon is in short supply, economy demands that the cells produce as few cyclic compounds as possible - though the chemical basis for the phenol determination is sufficiently complex to suggest caution in any such interpretation.

Environment-Associated Changes in Nucleic Acid

It appears that there is no unique nucleic acid level which is characteristic of a "growing" cell as compared to that of a "resting" cell. As pointed out by Kjeldgaard and Kurland (1963), at each growth rate the cells are characterized by a well defined macromolecular composition. Thus while the ratio of protein to DNA remains constant, the ratio of RNA to DNA increases with increasing growth rate. While the data of Caldwell

and Hinshelwood, (1950) and Caldwell, Machor, and Hinshelwood (1950) indicate that there is a correlation between growth rate and RNA content of bacteria, they maintained that the DNA content of cells is constant and does not vary with growth rate. However, the data obtained in the studies reported here indicate that there is a correlation between growth rate and DNA content of the cell, as there is a significant increase in total DNA/cell when cells are grown at high growth rates as compared to those grown at a lower rate. These data agree with the findings on Salmonella typhimurium by Schaechter, et al. (1958).

An exponential increase in RNA was noted with an increase in growth rate. Recent studies by Kjeldgaard and Kurland (1963) and Rosset (1964) have demonstrated that the cell is capable of regulating independently its synthesis of the component parts of RNA, i.e., messenger, ribosomal and transfer RNA. Ecker and Schaechter (1963) and Kjeldgaard (1961) have shown that the ribosomal component increases in direct proportion to growth rate and can be extrapolated to a zero rate where cells may contain only one ribosome, or possibly even none at all. Nakada and Magasanik (1964) have shown that the messenger fraction increases in direct proportion to the cellular protein. It is thus clear that although nucleic acid shows a direct relationship to growth rate, the individual RNA components are regulated differentially by the cell with the primary relationship between RNA increase and growth rate due to the ribosomal RNA component.

Much of the work done in balanced growth has been with complex media, so that rates of growth can be increased simply by increasing the nutritional value of the medium supplied to the cells. In these systems it is

virtually impossible to separate the effect on the cell of changes in growth rate from the effect of changes in the medium. Because the use of large numbers of such complex media provided a linear relationship between growth and RNA, Schaechter, et al. (1958) implied that the sole factor controlling the rate of RNA production was growth rate, and that the nutrients provided to the cell were without effect on nucleic acid formation, a concept which has remained current in scientific thought. However, from the present data it is apparent that an alteration in the medium may have a greater significance to the cell than simply providing a change in the rate at which growth is permitted. When the effects of growth rate and substrate are separated it appears that, in addition to growth rate effects, there is a more subtle change due to the nature of the limiting substrate. It was demonstrated that the ratio of RNA in nitrogen limited cells to that in carbon limited cultures decreases with an increase in growth rate from about 2.36 at 0.1 gen/hr to 1.29 at 0.5 gen/hr. This suggests that growth rate is not solely responsible for the rate of RNA synthesis and that some care must be taken in experimental design so that rate constants and other mathematical functions of RNA synthesis are not based entirely on the cellular growth rate.

Amino Acid Control in Biosynthesis

The present data rather strongly support the idea that there is a correlation between the free amino acid content of cells and the rate at which RNA is synthesized. It has been known for some time (Schaechter, et al., 1958; Kjeldgaard, 1961; Maaløe, 1960) that at growth rates higher than 0.6 gen/hr the rate of growth is proportional to the RNA concentration.

However, at growth rates below 0.6 gen/hr, Magasanik, Magasanik, and Neidhardt (1959) demonstrated that the rate of growth decreased without a corresponding change in the RNA. At the same time they noted a significant correlation between amino acid and rate of growth below 0.6 gen/hr, and postulated that at low growth rates amino acids are the rate limiting parameters.

Several lines of evidence point to the possibility that the rate of RNA synthesis is controlled by the concentration of free amino acid in the cell. It is well known that the composition of the growth medium affects the RNA content of the cell (Magasanik et al., 1959), suggesting that the rate of RNA synthesis is sensitive to the concentration of some intermediary product or products of metabolism. The behavior of cellular growth in relation to free amino acid concentration suggests amino acid as a possibility. Cells supplied with energy, pentose, and nucleotides can not synthesize nucleic acid unless amino acids are also present (Gale, 1962). Pardee and Prestidge (1956) demonstrated that RNA synthesis in the presence of chloramphenicol, i.e., without protein synthesis, depends on the presence of all 20 amino acids normally found in cell protein. In staphylococci, the concentrations of chloramphenicol which limit protein synthesis give rise to an enhanced rate of RNA synthesis, suggesting that an increase in cellular amino acid stimulates an increased synthesis of RNA, or acts as a metabolic trigger for such synthesis. Two recent reports lend strong support to the theory of amino acid control of RNA synthesis. Gross, et al. (1963) used purified preparations of RNA polymerase and found that the uncharged transfer RNA (without amino acid) markedly inhibits the transcription of various native DNAs. In contrast

they also demonstrated that transfer RNA molecules which had complete mixtures of amino acids available were at least 3 times less effective as inhibitors. Balassa (1964), using a sporogenous and an asporogenous strain of Bacillus subtilus, demonstrated that RNA synthesis is dependent on the amino acid supply, and concluded that the rate of RNA synthesis during sporulation is controlled by the supply of amino acids. A functional scheme of amino acid control of growth rate has been prepared by Gross, et al. (1963).

There are two obvious conclusions that can be drawn from the close relationship observed in the present experiments between the free cellular amino acid and the RNA content of the cells: 1) nitrogen limited cells have from 1.5 - 2 times as much amino acid as do the carbon limited cells (these relationships can be directly correlated with the greater amount of RNA in cells grown at higher growth rates), 2) the slope of the curves representing amino acid concentrations/cell are the same as those representing RNA/cell, showing a decreasing value of the ratio between nitrogen limited and carbon limited cells as growth rate increases. These data in general support the concept of amino acid control of RNA synthesis, although there could be alternative explanations for these results.

The data do not provide an explanation as to why the amino acid content varies with rate and substrate limitation, but due to the apparent overall control of growth rate exerted by amino acid, and because growth rate is a primary survival factor in the microbial world, it seems reasonable to assume that the cell at all times synthesizes amino acids at the maximum rate allowed by its genetic endowment and the environmental circumstances.

Carbohydrate Metabolism and Metabolic Control

Carbohydrates serve as a major source of carbon and energy for growth of heterotrophic microorganisms. Thus in understanding the chemical basis of biological processes a knowledge of carbohydrates, their chemical properties and transformations, and the pathways of carbon and energy liberation assumes importance, as does control of the reaction routes and formations of the biosynthetic precursors. Glucose, because of its wide distribution and importance in metabolism, has served as the initial substrate for most studies of carbohydrate metabolism in microorganisms (Gunsalus, Horecker and Wood, 1955). Three sets of experiments dealing with the metabolism of glucose (TTC reduction, glycogen synthesis, and the activity of glucose-6-phosphate dehydrogenase) gave results which shed light on the interactions of carbohydrate metabolism in terms of cellular growth rate and substrate limitation.

The experiments on TTC reduction show that the substrate had more effect on the cell's ability to carry out endogenous respiration than did the rate at which the cells were grown, although a relationship between growth rate and reducing activity is also apparent. The influence of the limiting substrate appears to indicate that the cell is better equipped with an available energy reserve to carry out endogenous respiration when removed from the growth medium if it is grown in excess glucose. This would indicate storage of carbon compounds available for metabolic use by the cell. In glucose limited medium there is less carbon stored and therefore less available for use in the endogenous state. However, if one considers the relationship of the endogenous activity of the cells on

a per cell basis, it appears that rate of growth has a greater influence on the endogenous activity of the cells than does the medium in which they were grown. This is in agreement with the results of the glycogen experiments.

Ribbons and Dawes (1963) reported that the endogenous respiration rates of E. coli varied with the phase of growth from which the cells were harvested and could be correlated with the cellular carbohydrate. They also demonstrated that this carbohydrate (glycogen) was the major endogenous energy source of E. coli. The data of Ribbons and Dawes, obtained using batch culture techniques, have been extended by means of continuous culture, which shows a 3 to 4 fold increase in the cellular glycogen concentration with a rate increase from 0.1 to 0.5 gen/hr. Furthermore, there is a relationship between the limiting substrate and cellular glycogen content. Cells grown on a nitrogen limited medium (with excess glucose) accumulated approximately 2 times the glycogen found in carbon limited cells. Holm (1959) showed that when cells were grown in batch culture, glycogen accumulated to the greatest extent in nitrogen limited cultures. Further work along these lines has led Ribbons and Dawes (1963) to suggest that glycogen synthesis is inhibited by NH_4^+, a view consistent with the data reported here.

Holm (1962) pointed out that when factors other than the energy source (such as nitrogen) are limiting growth, the yield calculations are very simple. This is so because in nitrogen limited systems practically all the nitrogen is assimilated (except, as discussed under "observations", when the dilution rate is proximate to the critical or washout value). Such yield calculations for glycogen synthesis from the present data show

a four fold greater efficiency in the production of glycogen by cells growing at high rates than at low rates. There could be several explanations for this relationship; it is possible that the decreased efficiency in production at low rates of growth represents either failure to synthesize, or a breakdown of glycogen by the cells in order to maintain a higher growth rate. Another possible explanation for the apparent difference in efficiency of glycogen production at different growth rates relates to the work of Ecker and Lockhart (1961c). Working with E. coli in batch culture, they derived an equation which defines the relationship between initial substrate concentration and total growth, N max, as

$$N_{max} = K'C_o^s \qquad (2)$$

where C_o is the initial concentration of limiting nutrient and where K' is the theoretical population produced from a single unit of nutrient. This constant in general reflects the efficiency of growth at low maximal population and probably finds its nearest counterpart with continuous culture in the function, Y, (see Appendix) which may be defined as the efficiency of producing cells from substrate. The constant, s, then indicates the nature of change or decrease from this efficiency as the maximal population increases. The existence of such a decrease in the efficiency of conversion of metabolite to protoplasmic component was recognized some years ago by Rahn (1932) who defined "energy of maintenance" and more recently by Mallette (1963), who has presented an excellent paper on the subject of maintenance energy, and by Marr, Nilson and Clark (1963) who demonstrated that if the growth rate is limited by the steady state concentration of the carbon source a portion of the metabolism of the

carbon source is diverted from the synthesis of cell protoplasm to maintaining cellular integrity. It therefore appears possible that the reduced efficiency of glycogen production with a decrease in growth rate in glucose limited cultures is due to the increased demand by the cell population for larger and larger amounts of energy directed toward cellular upkeep. This also appears to be a condition predictable through the use of equation (2) and a continuous culture demonstration of the function s. The fact that this change in efficiency of glycogen production is not seen in nitrogen limited cultures could be due to the presence of excess carbon over and above that required for growth, division, and cell maintenance.

Cohen (1951b) studied the metabolism of glucose in bacteriophage infected E. coli and noted a change in the ratio of carbon metabolized by means of the Embden-Meyerhoff pathway vs that metabolized by means of the hexosemonophosphate (C-1) pathway following virus infection. Scott and Cohen (1953) later found that up to 40 per cent of the glucose metabolized by E. coli under normal conditions was through the pentose (C-1) cycle. The first reaction leading into the hexosemonophosphate pathway involves a two-step (Cori and Lipmann, 1952) conversion of glucose-6-phosphate to 6-phosphogluconic acid by means of the enzyme, glucose-6-phosphate dehydrogenase (G-6-P DH), first demonstrated by Warburg, Christian, and Griese (1935). When E. coli K12/G6 grown under the conditions used throughout these experiments was analyzed for the activity of this enzyme, the results showed a much greater enzyme activity in cells grown with excess glucose (nitrogen limiting) and in cells which were grown at the higher growth rates.

Clifton (1963) pointed out that the nature of the growth medium and culture conditions influence the subsequent metabolic activity of the cells, and that there are definite regulatory mechanisms within the cells which control the nature of their respiratory and assimilatory activity. These metabolic regulations are demonstrated by the following: Cohen (1951a) reported that under oxidative conditions, resting cells of E. coli metabolized glucose by means of the Embden-Meyerhoff pathway, while rapidly growing cells used chiefly the C-1 pathway; Heath and Koffler (1956) showed that with the fungus Penicillium chrysogenum the fraction of glucose oxidized by the C-1 pathway increased with the rate of growth. Using E. coli, Allen and Powelson (1958) showed that a shift from the glucose C-1 preferential pathway toward the Embden-Meyerhoff pathway occurred at the onset of the stationary phase of growth, a point which is at best poorly defined in terms of the physiological growth curve. They further demonstrated that this change in metabolic pathways was not related to cell division and suggested that such shifts are related to growth rate per se. My data confirm the theory that there is an increase in the activity of the C-1 pathway with an increase in growth rate. This might have been expected from the data on nucleic acid synthesis, because the hexose monophosphate pathway can supply pentose units (such as ribose) which are an essential part of the nucleotides found in nucleic acids. Thus, an active C-1 pathway may be related to growth through the demand for synthesis of nucleic acids.

An explanation for the much greater activity of G-6-P DH found in nitrogen limited cells may be found by examining the results of investigation of the "glucose effect" (the ability of glucose to inhibit the

synthesis of certain enzymes). Magasanik (1961) pointed out that this effect might more properly be called catabolite repression because of the relationship between some catabolic products of glucose metabolism and the enzymes they inhibit. Polakis, Bartley and Meek (1964) demonstrated that in Saccharomyces there is an inverse relationship between glucose concentration and the concentration of enzymes associated primarily with oxidative respiration, and Magasanik, et al. (1959) reported that glucose represses enzymes required for oxidation of pyruvate and α-ketoglutarate. Similar inhibition of the synthesis of β-galactosidase has been extensively studied by Cohn and Horibata (1959a, 1959b). A possible explanation for these effects has been indicated by the work of Potter and Niemeyer (1959), who showed in rat brain that both NADP and NADPH would inhibit glycolysis through the Embden-Meyerhoff pathway. Neidhardt (1960) summarized the observed facts in suggesting that the rapid rate of glucose metabolism leads to a high intracellular concentration of catabolic repressor which then regulates the synthesis of "inducible" catabolic enzymes. Based on these experimental findings it seems reasonable to suggest that the increased activity of G-6-P DH in nitrogen limited cultures is due to a catabolic repression of some enzymes necessary for maximal function of the Embden-Meyerhoff system. This repression increases the demand on the hexose monophosphate pathway, resulting in a corresponding increase in the enzymatic activities of these pathways and an increased repression of the Embden-Meyerhoff pathway. These results further suggest that the shift in glucose metabolism seen under batch culture conditions may be caused by a decrease in growth rate due either to the exhaustion of some known or unknown essential metabolite, or to

the depletion of available glucose resulting in a decreased repression of the enzymes of the Embden-Meyerhoff pathway.

Infrared Analysis of Cell Composition

The decision to do infrared (IR) analysis of the bacterial cells was made after it was found that the only qualitative differences shown between cells grown at different rates or with two different limiting substrates were those demonstrated immunologically. It was hoped that additional evidence of qualitative relationships between cultures could be obtained through this procedure; as was indicated under "observations", the results did not prove to be as meaningful as desired. It is apparent however, that a change in the rate of growth of the cells does give rise to some unique absorption bands, and that the limiting substrate has an influence on the qualitative nature of the absorption spectra.

Since Bolduan (1952) proposed the possibility, there have been many attempts to demonstrate species as well as generic differences among several genera of microorganisms through the use of infrared spectra made from whole cells or cell extracts (Iwahara, 1960; Levi, et al. 1956; Scopes, 1962; Randall, Smith and Nangester (1952); Stevenson and Bolduan, 1952). By analysis of the patterns obtained using a number of genera, Thomas and Greenstreet (1954) were able to distinguish between major taxa; however, the separation of species within a genus was based upon differences no greater, and in many cases not as great, as those found using the same organism and varying its environment. It follows then that the separation of strains within a species would be even more tenuous. Nevertheless, most of the reports concerning bacterial identification

by this method are favorable and indicate a general acceptance of the procedure.

In nearly all the work in this area prior to 1957 the differentiation of bacterial genera and species was based on personal judgment as to the significance of very weak absorption bands or on examination of a restricted spectral region. This led Greenstreet and Norris (1957) to point out that the efforts to distinguish between bacterial species based on absorption spectra were the first recorded instances in which conclusions were based on such small spectral differences.

Many of the variables affecting growth control which make this type of analysis extremely difficult have been demonstrated. Greenstreet and Norris were able to get spectral changes by changing the rate at which the bacteria were aerated during growth, the depth of the medium used, and the time of incubation of cultures, while Scopes (1962) pointed out the necessity of a constant method of species preparation and Kenner (1958) discussed the effects of changing other variables such as pH, temperature and available carbohydrates. Microbiologists in general are perhaps not as consciously aware of the ability of the bacterium to change its physiological state to match its environment as is necessary when considering problems of composition and taxonomy. The ease with which the IR spectrum of a bacterial species can be varied by external factors is an indication of the extreme sensitivity of bacteria to changes in their environment.

The work reported here leads to serious doubt as to the actual feasibility of reliable taxonomic distinctions through the use of IR spectra, and it is my conclusion that work thus far reported which bases

differences between species or strains within a genus upon IR spectra is probably overly optomistic, and, except in some rather peculiar circumstances, such differentiation can not be made accurately or with any degree of reproducibility. Apparently pre-formed opinion as to the organization of species based upon classical taxonomy has played a major role in interpretation of the spectra thus far reported. To be of real significance in the separation of genera; IR spectra must show a definite generic relationship occurring within all species of that genus and transcending individual species differences. Only then will such analysis be definitive in distinguishing between species and genera, as yet no one has demonstrated such relationships. The magnitude of the IR changes obtained with genetically identical cells, grown with all factors controlled in an identical manner, and differing only in their rate of growth or limiting substrate, is considerably greater than the magnitude of such changes reported for strains of a species or the species of a genus. These results demonstrate that extremely precise metabolic control is necessary if meaningful results are to be obtained in terms of taxonomic relationships.

Infrared has been put to advantageous use in the study of several bacterial compounds such as exotoxin (Levi et al. 1956), glycogen (Levine et al. 1953b), and extracts of tubercle bacilli (Randall and Smith, 1953); while Mohr, Carter and Cochran (1962) suggested that IR would probably be of greater service to microbiology if used in association with physiological and biochemical studies than as a means of species differentiation. In addition the results reported here point out that this technique may be much more useful for extended examination of

one species than for comparative purposes. The data obtained by such procedures fully support the results obtained biochemically.

Antigenic Composition

The antigenic relationships obtained through the use of the Ouchterlony and immunoelectrophoretic (IEA) procedure are of particular significance to this study as they demonstrate the major qualitative differences found between cells grown at two different growth rates or under different conditions of nutrient limitation. A review by Lacey (1961) covers the most commonly known non-genetic antigenic variations occurring in microorganisms, and discusses the influence of environment on the development of surface antigens.

In many organisms the environment has an important influence in determining which of an array of alternative antigens will be formed in a given cell. For example, strains of Bordetella pertussis have been shown by Lacey (1960) to exist in three phases depending on the concentration of particular mineral salts in the medium, and in Pasteurella pestis (Pirt, Thackery, and Harris-Smith 1961) the various antigenic types which develop are dependent on the incubation temperature and pH of the medium. In some cases the environment may actually induce an antigenic change, as occurs in the induction of enzyme synthesis, while at other times the environment may simply select one antigenic component in favor of another.

Many of the internal as well as external antigenic components are discussed in some detail by Lennox (1960), who points out that there are numerous antigenic components within the bacterial cell. With the excep-

tion of the studies dealing with induced enzyme synthesis, there are to my knowledge no references which discuss the changes in the internal cellular antigens of bacteria resulting from environmental change. Beale and Wilkinson (1961) state that variation in antigenic specificity is inevitably the result of alteration in the synthetic mechanisms of the cell. The loss or gain of an antigen may be caused by the loss or gain of the ability to synthesize a complete polysaccharide or polypeptide molecule, or it may simply involve one particular antigenic determinant on the molecule. Wright (1963) has pointed out that in intact cells, enzymes are frequently operating far below their potential activity or are present in reduced amounts because of a substrate limitation. It is quite apparent that, through the process of sequential enzyme induction or repression, a single substrate may bring about a change in a large number of antigenic components.

The change in enzymes within a cell in a non-genetic (physiological) manner has been termed "biochemical differentiation" by Mandelstam (1960). However, it is perhaps more appropriate to refer to non-genetic change in highly specialized organisms (such as E. coli) as modulation, for it is doubtful that differentiation in the classical sense occurs in this single-celled form of life. Modulation may be defined as those reversible cellular changes which are brought about by alteration in the environment of the cell. In circumstances where non-genetic variations occur, the activity of the corresponding gene may in effect be completely suppressed even though synthesis of a large number of complete molecules of antigen may still be occurring. Antigens of differentiated tissues probably exist in such a potential form in the early stages of embryonic

development (Ebert, 1954), hence to some extent the process of differentiation can be viewed as a progressive antigenic modulation.

The agents which Lacey (1961) reports as being responsible for inducing changes in the cellular antigenic structure include a wide variety of physical and chemical substances, many of which are known also to influence the production of enzymes: toxin, sporulation, etc. Lacey discusses two types of antigenic change. The first is where the amount of antigen clearly varies continuously with continuous environmental changes such as occur in modulations of O, capsular, and slime antigens as well as the widely recognized Vi antigen which varies continuously with temperature (Nicolle, Jude, and Diverneau, 1953); the second type of antigenic variation is one in which the antigenic changes occur abruptly in an all-or-none way in spite of gradual change in the environment. This group of antigenic changes can be represented by the modulations of flagellar antigens and cell wall antigens when these appear as reversible L-form <——> bacillary or whole cell <——> spheroplast changes. This second type of antigenic change may be operative (even if the rate of antigen synthesis varies continuously with environmental change) when the antigen appears or disappears suddenly because either a threshold concentration is required for immunologic detection or because the environmental conditions have not been held sufficiently close to those needed to detect a transitional or intermediate state.

Data from the agar gel double diffusion technique showed several precipitin bands which represent unique antigens within the cell. The location of the bands and their general appearance allow some speculation as to the nature of the antigens forming them. The relative position of

the common band (C in Figure 7) which is found in all cells indicates the similarity of the antigen concentration in the cell free extracts. All extracts contained the same amount of protein and with the exception of extracts from nitrogen limited low growth rate cells, N1, the common precipitin band occurs the same distance from the center well in all cases. It appears as though this antigen may be present in a slightly higher concentration in N1 than in the other cell extracts.

A great deal of the literature pertaining to the antigenic characteristics of cells makes no mention of careful metabolic control. Several studies of the antigenic relationships between various species and even different genera of organisms have been made without regard to the growth physiology of the cells. It is apparent from the work reported here that both parameters studied, i.e., changes in limiting substrate or an alteration of growth rate, affect the antigenic composition of the cells. Precipitin bands which are common to substrate limitation, both carbon and nitrogen, as well as bands unique to high and low growth rates, have been found in the IEA procedures. The reasons for such antigenic changes are undoubtedly related to the organism's effort to achieve a maximum rate of growth under the environmental conditions imposed upon it. Because all of the antigenic differences between the 4 groups of cells were in protein or nucleoprotein components, and because the cell wall and membrane debris were removed prior to use of the cell extract, it would appear that it is a change in the enzymatic components of the cell that provides the antigens unique to this particular system. These antigens, i.e., enzymes, reflect the cell's efforts to meet the demands of the environment in which it is growing. For this reason it is possible that a particular

antigen can be demonstrated in one cell and not another because of quantitative rather than qualitative differences. As an example, under conditions of nitrogen limitation precipitin bands G and H (Figure 8) are present, but are not found when cellular growth is limited by glucose. These bands may be due to an enzyme suppressed under conditions of nitrogen excess but necessary to the cell for maximal growth when growth occurs with excess glucose. However, regardless of the metabolic reasons involved, this represents an actual antigenic difference between carbon and nitrogen limited cells and represents a true change in cellular composition due to changes in growth environment. This system serves as an excellent example of metabolic control by means of environmental regulation. Thus changes (within the genetic capabilities of the cell) are brought about by changes within the cell's environment. The bands, such as K and N, which represent a change in growth rate by the cell probably arise because of an influence similar to that discussed for substrate dependent bands. However, it is possible that in the situations where precipitin bands are unique to cultures growing at high rates, their absence at the lower growth rate represents a quantitative reduction of an enzyme below the threshold of antigenicity even though its presence may still be felt by the cell. But, since it would be difficult to explain bands unique to low growth rates on this basis, it seems likely that such bands both in high and low rate growth do represent true antigenic modulations of the cells in response to environmental stimuli.

Modulations of antigens as a function of environment have received even less attention by authors of scientific articles than non-genetic variation in general. The influence of environment, apart from its

relation to enzyme induction, is considered by many microbiologists only as a promoter of life or death. It has become customary to regard antigens as having a one-to-one correspondence with genes, maintained under all environmental conditions. Consequently antigenic variation is often discussed as though it were exclusively a manifestation of genomic variation occurring spontaneously, induced by mutagens, or following genetic exchange. That this is far from the truth can be seen from the data.

The practical importance of non-genetic variation seems obvious. Such variation comes into play in identification of organisms and antibodies, preparation of vaccines and antisera, and in taxonomy. It is also possible that the occurrence of inducible antigens will also provide opportunities for chemotherapy aimed at modification of virulence rather than stasis or death. For the organism, antigenic plasticity, which will almost certainly have considerable survival value, seems to assure it a lasting place in the struggle for life.

SUMMARY

1. Escherichia coli K12/G6 was grown in a balanced state by means of a continuous culture system. The cultures were limited in their growth by the concentration of either the substrate nitrogen or substrate carbon source, and were cultivated at 4 different rates of growth. Through the use of biochemical, physical, and immunologic techniques, qualitative and quantitative determinations were made of the physiological characteristics of each of the resulting cell populations. The primary aim was to separate the effect on the cells of growth rate from the effects due to substrate changes.

2. The number of cells/ml decreased with an increase in growth rate, while the cell size appeared to change with both the growth rate and medium composition. There was a 6 - 7 fold increase in the size of cells grown at 0.5 gen/hr in a given medium compared to those grown at 0.1 gen/hr. The cell size was 2 - 2.5 greater in nitrogen limited cells than in carbon limited cultures grown at a given rate. The increase in size due to growth rate appears to be a general phenomenon found among all bacilli thus far studied. The increase in size due to the change in the glucose/nitrogen ratio was apparently not due only to an accumulation of glycogen by the cells grown in excess glucose but results from an increase in each of the measured cellular components, suggesting that there is a basic metabolic alteration in cells shifted from glucose to nitrogen limitation affecting all, or nearly all, the major components of the cell.

3. The rate at which protein is synthesized increases in a linear fashion with the rate at which the cells are grown. The yield of protein

per unit of substrate utilized was considerably lower in the carbon limited cultures than in the nitrogen limited cells. This probably represents the multiple uses to which the cells put their carbon supply as opposed to the more limited function of nitrogen within the cell.

4. Data on the nucleic acids supports the concept that at each rate of growth, cells are characterized by a unique macromolecular composition. There is a significant increase in the DNA/cell with an increase in growth rate, and the increase in RNA/cell occurs exponentially. However, in addition to the rate-associated RNA increase there is an effect on RNA synthesis brought about by a change in the environmental substrate relationships. This effect is noted as a decrease (as growth rate increases) in the ratio between the RNA of cells grown under nitrogen limited conditions and the RNA of cells grown in carbon limited cultures. This effect, although more subtle than the rate limited relationships, suggests that care should be exercised in making calculations of nucleic acid formation based on rate alone.

5. There is a strong correlation between the free amino acid content of the cells and the rate at which the cells are grown. The rate at which the values changed paralleled those of the RNA changes, suggesting a further relationship between these two factors. The data in general support the concept of amino acid control of RNA synthesis, as proposed by other investigators.

6. The endogenous whole cell reducing capacity was greater for cells grown at high rates of growth than at low rates. In addition, cells which had an excess glucose supply in the medium, and presumably a greater internal store of glycogen, had a greater endogenous metabolic rate than did those cells grown in glucose limited medium.

7. There are two relationships between environment and glucose accumulation by the cells. Cells grown at high growth rates accumulated glycogen at a greater rate than those at low growth rates, and those cells with an excess of glucose in their environment produced glycogen at greater rates than those where the carbon supply was limited. The data are consistent with, and support the concept of, suppression of glycogen synthesis by the ammonium ion.

8. There is considerably greater efficiency in the production of glycogen by cells growing at fast rates than at the low rates. This is suggested to represent a maintenance energy requirement by the cells. Such a decrease in substrate-product efficiency (yield) can be predicted in batch culture by a mathematical relationship which is discussed, and the data concerning glycogen synthesis may well represent a demonstration of the functions involved.

9. The activity of glucose-6-phosphate dehydrogenase was shown to be increased under conditions of carbon excess and when growth rates were high. This is felt to represent a repression by glucose of the enzymes in the Embden-Meyerhoff pathway. The increased demand by the cell for pentose sugars when growth rates are high indicates that this metabolic shift to C-1 preferential glucose degradation may be a fundamental link in the cellular control of macromolecular synthesis. These data are considered to confirm the reported suggestions that growth rate is significant in determining the pathways of glucose metabolism.

10. Through the use of infrared spectrophotometry it was possible to demonstrate both qualitative and quantitative cellular adjustments to changes in growth condition. It is suggested that infrared data should

be interpreted with great caution when used for taxonomic purposes.

11. It was possible to demonstrate both substrate and growth rate associated antigens by the technique of immunoelectrophoresis. These methods are considered to show a true qualitative change within the cells due to alterations in the growth environment of the cells. Possible explanations for the appearance and disappearance of intracellular antigens due to non genetic influences are discussed.

The data reported here suggest that there is no simple answer to the problems of environment and growth relationships. It is apparent that there is an intricate balance which depends on a complex intracellular response to environment, yet which determines the physiological manifestation to be made by the cell. Above all this work has shown that the concentration of, and the ratio among, the nutrients in the culture medium will have a great influence in determining the apparent physiological phenotype of the cell. This is in direct contrast to that which has been reported earlier, "...Media which give identical growth rates produce identical physiological states, regardless of the actual constituents of the media" (Schaechter, et al., 1958).

The questions remaining to be answered concerning environmental control of, and environmental effect on growth are manifold, as suggested by Neidhardt (1963): "Not a single mechanism has been established as physiologically responsible for integration of the overall rate of synthesis of one class of macromolecules with another... The requirements of amino acids for RNA synthesis comes close to explaining how RNA might be made at a rate exactly appropriate for protein synthesis, and the requirement of protein synthesis to initiate DNA replication comes close

to explaining how a new cell is guaranteed a proper endowment of DNA. Even these mechanisms, though they may be in essence correct, lack a factual explanation as to many of the biochemical details."

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APPENDIX

Continuous Culture

The art of growing microorganisms in a continuous culture had its beginnings when Felton and Dougherty (1924) used such a system to study the virulence of organisms as a function of growth conditions. Until about 10 years ago, reports of the use of this system occurred only occasionally. However, recent years have seen a marked increase in the number of investigators who have turned to a balanced growth system to facilitate their research efforts. Primarily such reports have dealt with the use of continuous culture in the solution of biological problems (James, 1961; Holm, 1962), although the mathematical (Teissier, 1936; Moser, 1958; Schulze and Lipe, 1964) and engineering (Butlin, 1958) aspects also have been rather thoroughly investigated.

A continuous culture system is just one of several methods of achieving balanced growth in microbial cultures. When cells are in balanced growth they are defined as being in a state where every measurable cellular component is changing at the same rate, that is, the molecular components of the cells are being synthesized in balance with each other in an unchanging ratio. The simplest procedure used to achieve balanced growth is the continuous culture system which consists in general of a growing microbial culture, fed with a continuous flow of liquid medium at a constant rate. The culture volume is kept constant by removing the bacterial suspension at the same rate as fresh medium enters the culture.

The increase of the microbial mass in any exponentially growing

culture may be expressed (Herbert, 1959) by:

$$\frac{dx}{dt} = \mu x \tag{1}$$

where x is the dry weight of organisms per unit volume at time t, and μ is the specific growth rate. If the flow of fresh medium fed into a continuous culture is called f, the dilution rate, D, will be f/v, where v is the culture volume. In this case the expression for the increase in bacterial mass in the growth vessel is:

$$\frac{dx}{dt} = \mu x - Dx \tag{2}$$

Equilibrium in the growth vessel will be obtained if $\mu = D$, i.e., the flow rate is equal to the specific growth rate. In such instances the flow rate expresses the rate of growth of the cells in the system. Obviously D cannot exceed the value of μ if steady state is to be maintained. At $D > \mu_m$ (the maximum growth rate for a given set of conditions), dx/dt is negative and the cell concentration in the reactor will therefore decrease progressively with time until eventually no cells are left (Schulze and Lipe, 1964). Just as there are rates of dilution which will result in this washout of the culture, there are no doubt limits as to how slowly an organism will grow before some mechanism is actuated that causes the organism to go into a lag phase.

Monod (1942) demonstrated a simple relationship between the specific growth rate and the concentration of an essential growth substrate. The specific growth rate, μ, is proportional to the substrate concentration when the latter is low but reaches a limiting saturation value at high

substrate concentrations, according to the equation:

$$\mu = \mu_m \left(\frac{s}{K_s+s}\right) \tag{3}$$

where s is the substrate concentration, μ_m is the growth rate constant (i.e., the maximum value of μ at saturation levels of substrate) and K_s is a saturation constant numerically equal to the substrate concentration at which $\mu = 1/2\ \mu_m$. It is thus apparent from equation (3) that exponential growth can occur at specific growth rates having any value between zero and μ_m, provided the substrate concentration can be held constant at the appropriate value (Herbert, Elsworth, and Telling, 1956). It is notable that equation (3) is equivalent to the Michaelis-Menten equation used in enzyme kineties. It has been suggested by Schulze and Lipe (1964) that the bacterial cell can therefore be considered as an enzyme molecule reacting with the substrate, s.

Monod (1942) suggested that if one decreases the concentration of essential nutrient in the medium until it limits the population density, it is then possible to determine the "yield constant" which usually is expressed as grams of organisms formed per gram of nutrient consumed. Under these conditions the growth rate is a constant fraction, Y, of the rate of utilization of substrate (Herbert, 1958):

$$\frac{dx}{dt} = -Y \frac{ds}{dt} \tag{4}$$

Then over any given period of growth:

$$\frac{\text{weight of bacteria}}{\text{weight of substrate used}} = Y.$$

If the values μ_m, K_s, and Y, are known, a complete quantitative description of balanced growth can be given (Herbert et al., 1956).

By utilizing the fact that the growth rate in a bacterial culture may be limited by reducing the concentration of one of the essential nutrients in the medium, it is possible to achieve equilibrium over a wide range of growth rates. While the concentration of one of the essential nutrients is low all others are kept in excess. The nutrient present in low concentration is called the limiting or controlling factor. The concentration of this factor is almost instantaneously reduced when the medium is mixed with the culture, and reaches a level which limits the growth rate (Perret, 1957). It can be shown that this system tends to attain a self stabilizing equilibrium where the growth rate is determined by the flow-rate of the incoming medium and the bacterial mass is determined by the concentration of the limiting factor. A variety of substances can be used as the controlling growth factor in such a system. The substances need only be required by the organism and also have a region of concentration where growth rate is concentration dependent.

In equilibrium, the synthetic rate of every component of the cell is equal to the dilution rate, which in turn can be regulated at will to any value that does not exceed the maximum value of μ, that is, the maximum synthetic capacity of the bacterium.

It is thus apparent that the continuous culture method provides definite advantages in the study of synthetic functions of bacteria, since determinations of the rates of various processes are reduced to measurements of steady state concentrations.

Experimentally there are two systems which have offered a large

measure of success; these differ in the method by which the growth in the culture vessel is maintained. In the internally controlled system (Meyers and Clark, 1944) the cell population is adjusted automatically through the use of a photocell placed in, or adjacent to, the culture vessel and connected to a relay controlling a valve in the fresh medium supply line. A continuous reading of the optical density in the culture vessel is made by the photocell. When the cell density increases above a pre-set limit the photo-cell actuates the relay opening the fresh medium supply valve and additional medium is added to the culture vessel. The additional medium decreases the concentration of cells in the culture vessel until the lowest acceptable optical density is reached. The relay is then actuated to shut off the incoming supply of fresh medium. In reality growth in this system is semi-continuous due to the intermittent addition of fresh medium, although such growth may continue over a long period of time.

The second type of apparatus, termed a "chemostat" by Novick and Szilard (1950), operates as an externally controlled system. The rate at which the fresh medium is added to the culture vessel is fixed manually at a predetermined value and allowed to run into the culture vessel continuously. The growth rate of the cells is adjusted to the rate of input, as the volume of fluid in the culture vessel is maintained constant by an overflow device.

There is no essential difference between the two systems. It has been pointed out (Powell, 1956) that with the Chemostat the experimentor fixes the flow rate, and therefore the growth rate, while the population level adjusts to the input nutrilite concentration. In the internally

controlled system the experimentor selects the population density and the flow rate adjusts itself to the growth rate as it, in turn, is a function of the medium. The only question an investigator need decide is which method will offer the best control for the system he wishes to investigate. The virtue of the internally controlled system is that it allows steady-state growth at (or very close to) the maximum growth rate, μ_m, and in effect allows μ_m to be measured directly. The advantages of the chemostat lie in the possibility of studying growth at very low substrate concentrations and over a wide range of growth rates.

There are many advantages to the use of continuous culture systems under a variety of experimental conditions. The fact that a growing population is held at constant size over a long period of time is one advantage over classical methods. A second is that the concentrations of all chemical substances in the growth tube remain constant, and by virtue of external control the variation of this growth rate becomes an important experimental parameter. The chemostat can be used for accurate measurements of mutational rates in bacteria, (Novick and Szilard, 1950) where the usual methods encounter statistical difficulties that lead to a serious lack of precision. The use of continuous culture in the study of problems in microbial physiology and biochemistry is increasing. Such studies as enzyme induction kinetics (Novick and Weiner, 1957) and the rate of production of metabolic end products and toxins by bacteria, as well as the studies of nucleic acids and proteins previously mentioned (see text) are becoming more frequent. Several newer applications have led into the study of mixed bacterial populations (Zabrzycki and Spaulding, 1958) and the complex relationships found among ruminal flora (Quinn, 1962; Quinn,

Burroughs and Christiansen, 1962).

The continuous culture apparatus used to achieve balanced growth in the present studies is presented schematically in Figure 12. The system was externally controlled by means of solution metering pump B (Figure 12), which pumped medium from the reservoir A, through a 37 C pre-warm C, and into the growth vessel D, maintained at a constant temperature by a 37 C water bath. The culture was aerated with a laboratory air supply fed into an air flow meter I, (Ecker and Lockhart, 1961), through an air humidifier J, and an air sterilizing filter L, (Figure 12). The medium in the culture vessel was maintained at a constant level by means of a vacuum aspirator tube E, which removed excess culture as new medium entered the culture vessel. The culture and incoming medium were mixed thoroughly by the action of the air sparger. (A drop of dye was homogeneously distributed throughout the culture vessel within 3 seconds.)

In conclusion, it would appear meaningful to make a brief statement concerning the relationship between continuous and batch culture. Perhaps no comparisons between the two systems may be made, in the sense that bacteria grown in batch culture are subjected to a continuously changing environment due to metabolic processes occurring as the cells grow, while the cells in balanced growth are in a constant environment not subject to gross change. It may well be that there is no point in time when the two systems are entirely equivalent in terms of environmental conditions and cellular physiology. In terms of growth rate, however, it would appear that a valid comparison can be made. In a batch culture, the growth rate decreases from its highest point to zero as the concentration

of limiting substrate falls to zero; for each point in time on the batch growth curve there is a corresponding point at which a continuous culture may be operated at the same substrate concentration and growth rate (Herbert, 1958).

It is difficult to obtain batch cultured cells which are not either actively dividing or stationary since the transition from one to the other occurs over a comparatively short time interval. The advantages of continuous culture techniques are that both these extreme cell types, and an infinite number of intermediate gradations between them, may be isolated at will for study. With these techniques the changes in the physiological state of a population which are the usual consequences of growth are avoided, and except for mechanical difficulties it might be expected that a population could be kept growing indefinitely.

Figure 12. Schematic diagram of the continuous culture apparatus used in these studies

A - media reservoir

B - pump

C - pre warm

D - culture vessel

E - aspiration tube

F - culture collection vessel

G - vacuum source

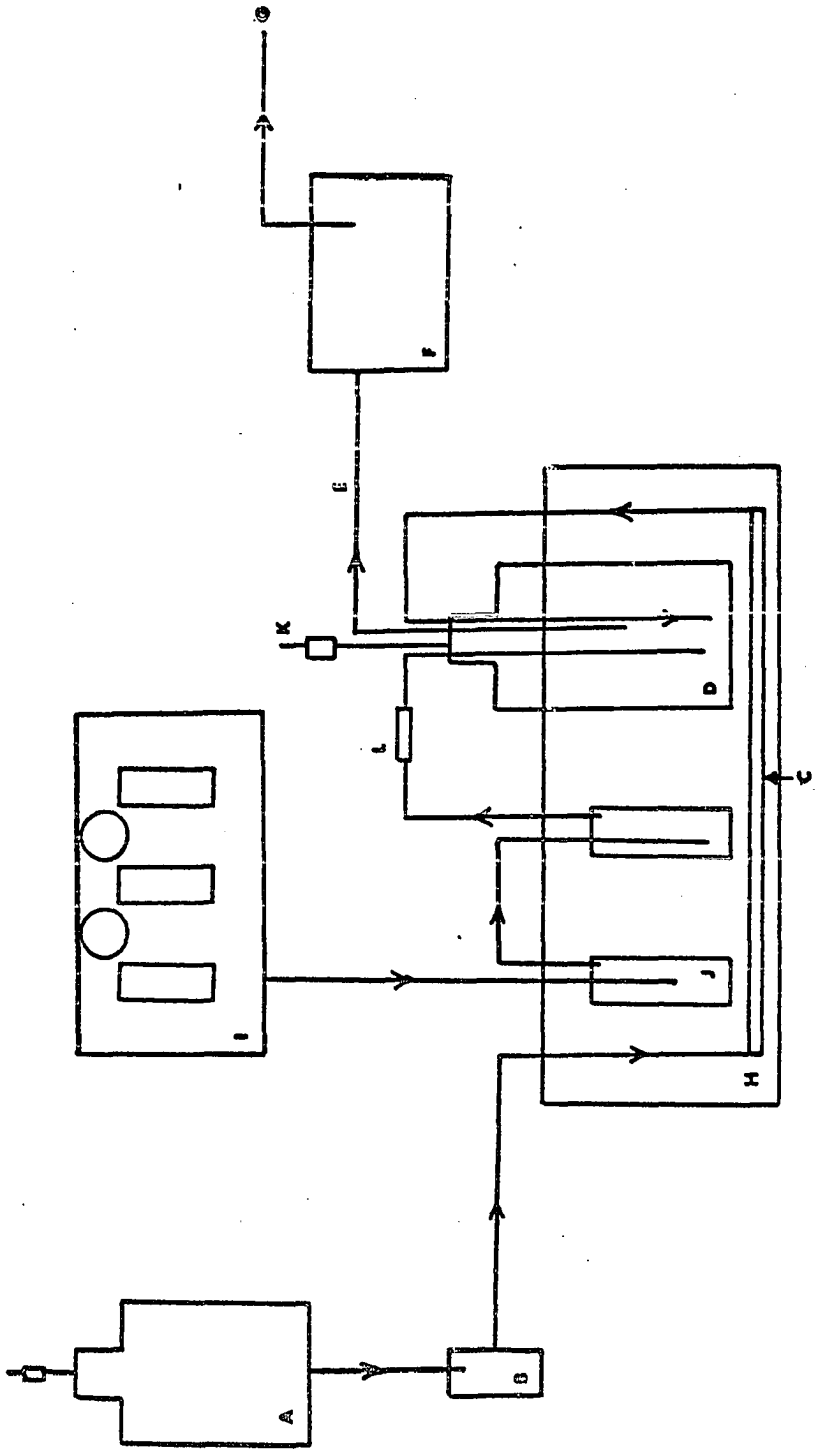
H - water bath

I - air flow meter

J - air humidifier

K - filtered fresh air inlet

L - air line filter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