UTILIZATION OF FREE AND COMBINED AMINO ACIDS BY ACTIVATED SLUDGE

Prepared by

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Funded by a Public Health Service Research Grant
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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INTRODUCTION

The removal of nitrogen from waste waters is of primary concern in waste treatment especially where receiving waters are slow moving and warm. In such situations where algal populations can build to sufficient concentration to cause taste and odor problems, the concentration of nitrogen delivered by waste streams can be the growth triggering or growth limiting element for these nuisance algal blooms. Inhabitants of urban and resort areas adjacent to lakes have become increasingly aware of the effect of waste streams in such quiescent waters and expect that control agencies will require treatment removal methods that will alleviate the eutrophication problems brought about by nitrogen and phosphorus entering into lakes from waste water and treatment plant effluent streams. These problems need attention on all phases of the nitrogen cycle from degradation of protein material to reincorporation into living matter. Research has been underway on the various aspects of utilization or disposal of ammonia, nitrites, and nitrates as well as on the algal metabolic pathways and on means of limiting growth.

A major problem with conventional waste treatment plants is that, although nitrogen is converted from one form to another in passage through the plant, much of the incoming nitrogen remains in the effluent of the plant in soluble forms such as nitrates or in escape of volatile suspended solid material.

Desirable means of removal of nitrogen are (a) by incorporation into volatile suspended solids materials and complete elimination of these solids from the liquid fraction, or (b) by exhaust of the nitrogen into the atmosphere as nitrogen gas.

The rate of incorporation of nitrogen into volatile solids is of concern to the engineer who must design treatment plants so that adequate contact time is available for satisfactory nitrogen removal. This rate of incorporation is dependent upon the type of compound in which the nitrogen is bound at the start of the process and upon the metabolic pathways utilized in converting the nitrogen

to a form which can be separated from the liquid stream. Municipal sewage streams have been recognized as containing a large variety of compounds ranging from the very simple to the complex and varying as much as degradability.

Studies on variation in degradability have been made for many of the saccharide compounds and have shown the influence of chemical bonding on the ease of biological utilization. For example, the monomers and dimers of glucose have been noted for their rapid uptake into biological systems while such long chain polymers of glucose as cellulose have been cause for concern because of the slow rate of utilization in aerobic treatment systems.

The bonding of nitrogen into monomers, dimers, and longer units is important as well in the bioutilization of waste streams. The research reported herein is concerned with utilization of nitrogen in the form of amino acids both singly and bound together in peptides and in proteins. Such proteinaceous materials comprise the major nitrogen fraction in domestic sewage and, hence, studies on optimum conditions required for high rate biological utilization of proteins can be useful in the design of more efficient biological treatment facilities for the removal of nitrogen from waste waters.

This report is divided into several sections covering the various aspects of the research effort. The study was concerned first with the utilization of individual amino acids as separate entities, then as components of di- and tripeptides, and finally as components of keratins--a protein type very resistant to degradation.

Complementary sections of the report describe several phases of the overall study which were inaugerated as auxiliary investigations for obtaining and presenting necessary information for the project. These auxiliary topics covered (a) modification of Burton's DNA test for use with the mixed cultures of activated sludge. (DNA was used as a measure of new cell production) (b) development of a graphical digital computer program for evaluating and presenting Warburg respirometer data and (c) development of a KWIC program for retrieval and storage of literature pertinent to the project.

THE UTILIZATION OF FREE AMINO ACIDS

The smallest integral molecular units of the simple proteins are the amino

acids. Thus, the initial studies on this project were conducted on the utilization of these primary units of protein structure. These studies were an expansion of earlier research at the University of Wisconsin (1) and covered several of the aspects of amino acid utilization for such compounds as glycine, glutamic acid, tryptophan, arginine, leucine, cystine, methionine, alanine, and phenylalanine. These amino acids are available to microorganisms as sources of energy (The free energies of the amino acids are listed in Appendix IV), and as components in the synthesis of new cellular material. The pathways of amino acid disappearance in activated sludge cultures were traced by following amino acid depletion, changes in supernatant conductivity, nitrate, nitrite, and ammonia concentration, pH, solids, oxygen uptake, CO_2 production and alkalinity.

Thus, the pathway of amino acid oxidation:

$$C_wH_xO_yN_z + nO_2 \longrightarrow wCO_2 + zNH_3 + \left(\frac{x-3z}{2}\right)(H_2O)$$

and further:

$$NH_3 + O_2 \longrightarrow HNO_2 + H_2O$$

$$HNO_2 + 1/2O_2 \longrightarrow HNO_3$$

could be traced by following amino acid depletion (removal of $C_wH_xO_yN_z$), oxygen uptake (a measure of oxygen utilization in the several equations), and then tracing NH_3, NO_2^-, NO_3^- and pH changes. Conductivity was used to measure variations in ion concentration. Carbon dioxide evolution was indicated by following gas volume changes on the Warburg respirometer without the absorption of CO_2 in potassium hydroxide and by following alkalinity and free CO_2 changes. As is noted later in the appendix of this report the problem of calculating and plotting up the large quantity of Warburg respirometer data was alleviated by using a plotting program on the IBM 709 digital computer (2).

The purpose of this portion of the study was to show that:

1. The amino acids serve as a source of energy and of constituent material for synthesis of new cellular material.
2. The enzymes and permease sites used for amino acid oxidation and incorporation into the cell mass are inducible in an activated sludge culture and hence the utilization of amino acids is accelerated by acclimation of the culture to an amino acid substrate.

3. The enzyme and permease systems for amino acid utilization are stereospecific and unnatural isomer utilization can be limited by racemase activity converting the D to the L form of the amino acids prior to further utilization.
4. The incorporation of combined amino acids is related to the number of peptide linkages and the restriction to usage increases with increase in the number of peptide linkages in the test compound.
5. The utilization of compounds is related to the energy available in the compound.

The research on the availability of the amino acids as sources of energy and of constituent material for synthesis has been reported previously in the literature (2) and is developed in the section on use of the DNA test for measuring growth. The advantages of acclimation for single amino acids also was covered previously but in this report research on the effects of acclimation has been expanded for the incorporation of peptides and is developed later.

Of interest is the effect of isomerism on the utilization of amino acids and peptides. These studies--oxygen uptake, amino acid depletion, nitrate production--all have shown that isomerism is very important in the utilization of both amino acids and peptides.

Isomeric Effects on Amino Acid Utilization

The D-isomers of the amino acids are the unnatural form and are converted to the L form by the bacteria before they are utilized in the cell's machinery. The utilization of the amino acids, as measured by oxygen uptake, is usually limited by the racemase activity; this limiting effect is progressively more pronounced as the size of the amino acid increases. As shown in the bar graphs in Figure 1 the racemase does not limit oxygen uptake for alanine, the smallest isomeric amino acid. The metabolic use of both D and L alanine is in agreement with this. Alanine (3) appears in the D form in the cell walls of both gram positive and gram negative bacteria. Also D alanine appears to have a special role in biological metabolism (4). Note that for tryptophan, however, the oxygen uptake for the D isomer is only 38% of that of the L form. These data have been plotted in Figure 2 to give a relationship between the number of carbons in the molecule and the restriction placed on oxygen uptake by the necessary racemase activity. These data were obtained from cultures acclimated to DL amino acids for a period

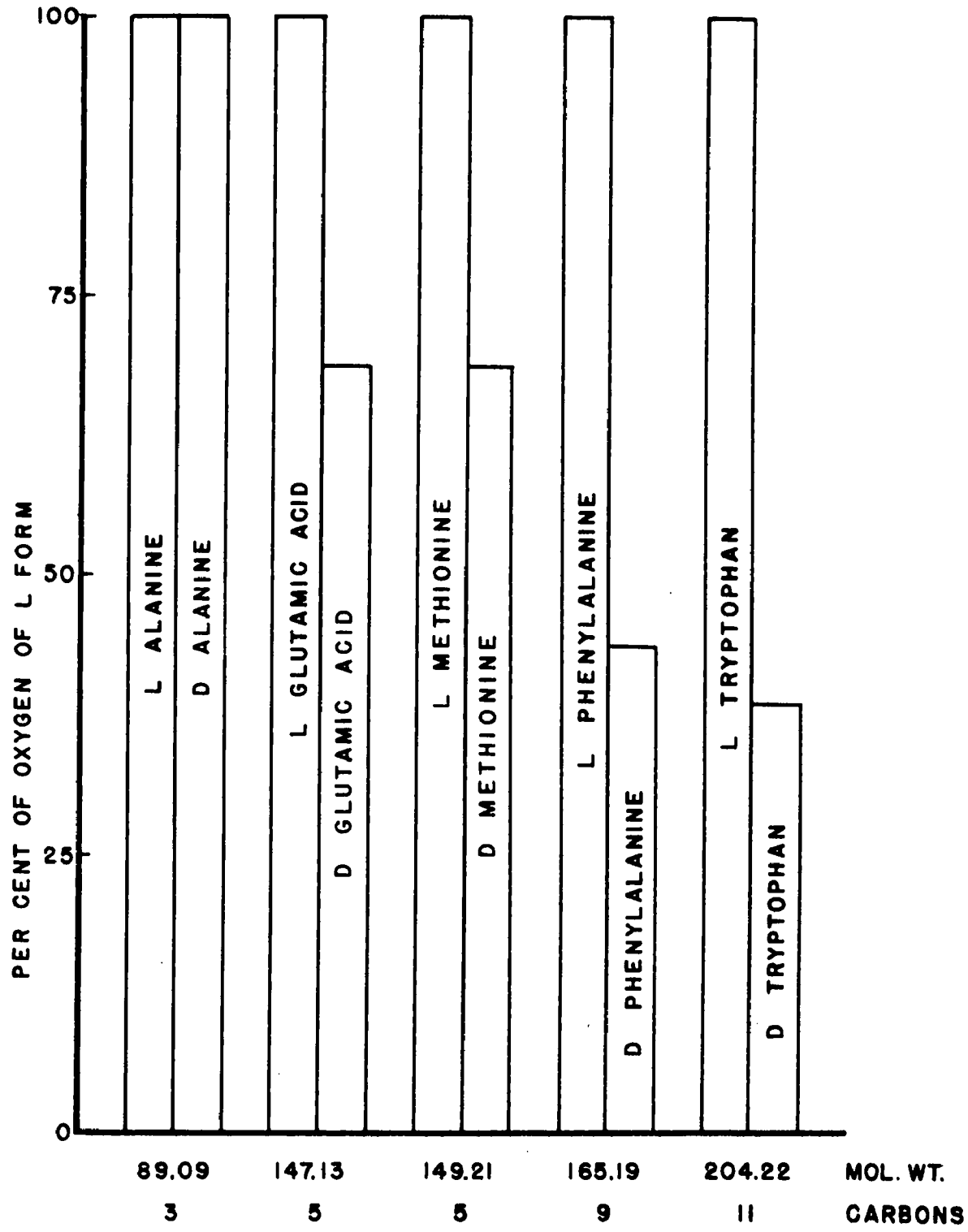


FIG. 1 EFFECT OF MOLECULAR SIZE ON UTILIZATION OF D-ISOMER OF AMINO ACIDS

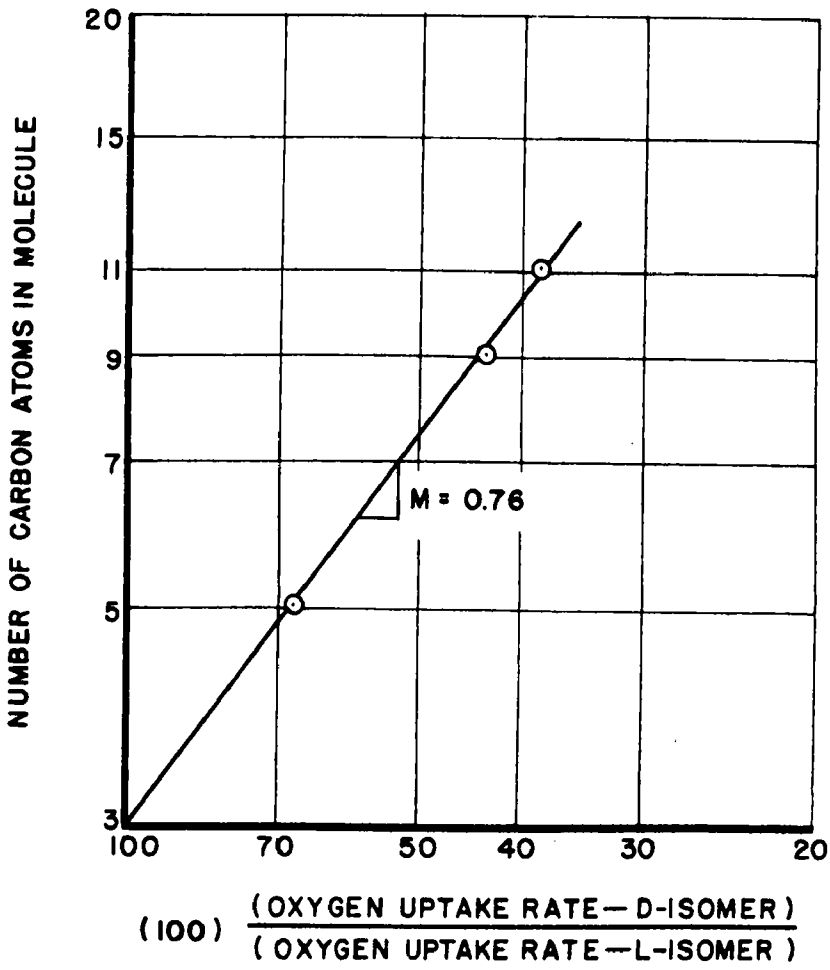


FIG. 2 EFFECT OF MOLECULAR SIZE ON UTILIZATION RATE OF D-ISOMERS OF AMINO ACIDS AT 20°C

of several weeks so that acclimation was accomplished for both the D and L isomers.

From Figure 2 an equation can be established for D isomer utilization by activated sludge. If $X = (100)\ \frac{\text{(oxygen uptake rate - D-isomer)}}{\text{(oxygen uptake rate - L-isomer)}}$

and C = number of carbon atoms in the amino acid molecule.

Then: $\log X = 2.362 - 0.76\ (\log C)$ -1-

or: $X = \frac{231}{C^{0.76}}$ -2-

Thus the effect of isomerism on oxygen uptake at 20°C can be predicted for an amino acid by using the number of carbons in the molecule to give the relative uptake rate for the D isomer. As will be developed later this same equation applies as well to the utilization of D isomers in peptides.

The depletion studies on amino acid substrates correlate with the oxygen uptake results obtained with the Warburg respirometers. The area of the spot appearing with ninhydrin reagent on paper chromatograms is proportional to the concentration of amino acid remaining in the supernatant of the activated sludge test culture; thus these spot areas can be used as a measure of the depletion of amino acid substrates with time (5).

As shown in typical depletion studies in Figure 3 and 4, the D isomer is depleted at a much slower rate than the L isomer for both tryptophan and phenylalanine. Note that in both cases a considerable fraction of the D isomer remains even after twenty-four hours aeration time after the batch feeding.

Respirometer studies have been executed for many amino acids at several different temperatures. The graphical display of these data is available at the University of Washington. The D isomer effect noted above appears to be temperature dependent as well for many of the amino acids studied. The data obtained indicate that the racemase activity is sufficiently temperature sensitive so that the ratio of D-isomer uptake to L isomer uptake increases as the temperature is raised from 10°C to 40°C.

Laboratory Procedures

The test procedures used in this study were taken insofar as practicable from Standard Methods (3). Paper chromatography, Warburg respirometer tests, free amino acid depletion, activated sludge culture techniques were as reported

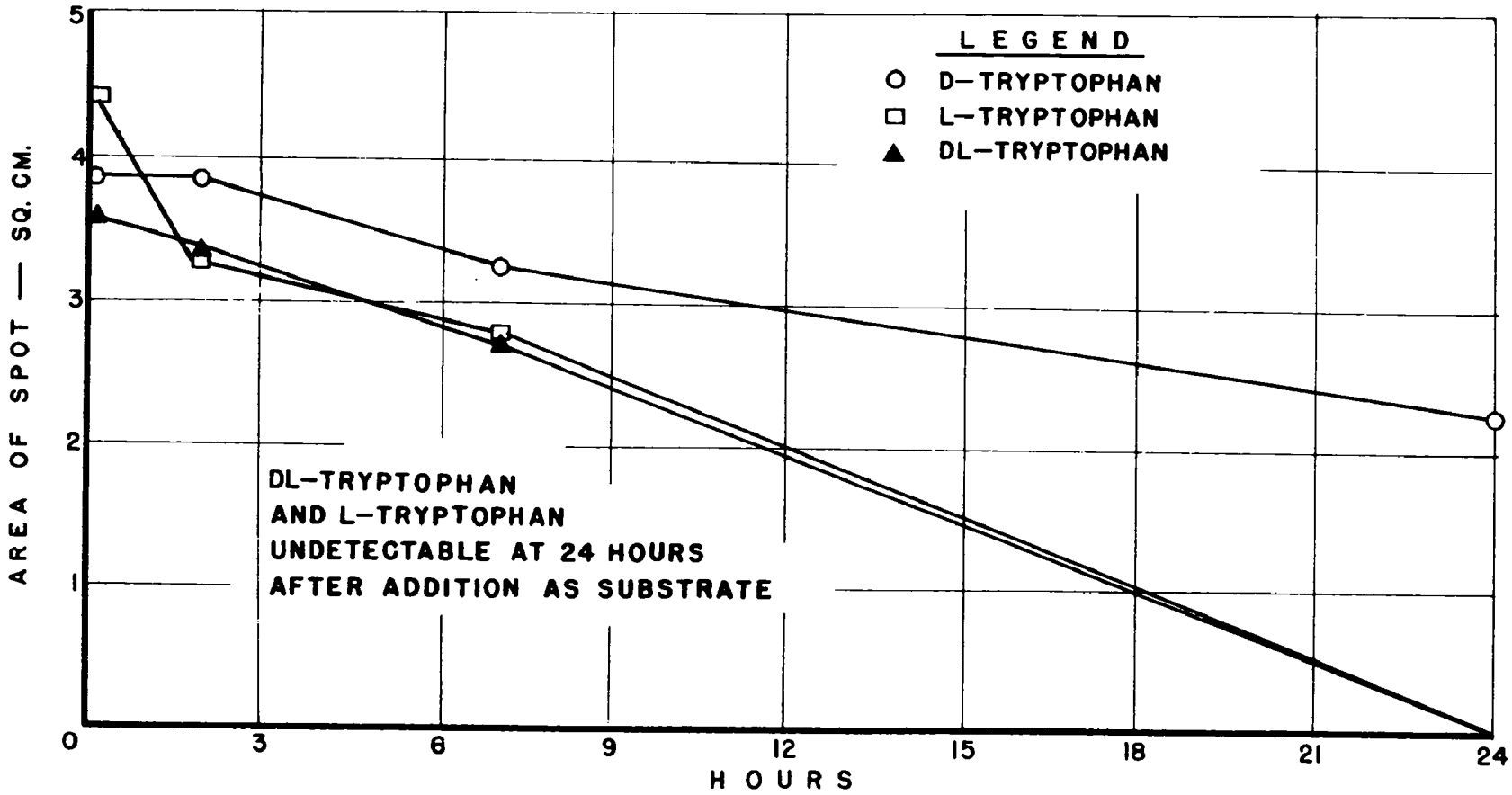


FIG. 3 SUPERNATATANT CONCENTRATIONS OF TRYPTOPHAN ISOMERS AFTER BATCH FEEDING

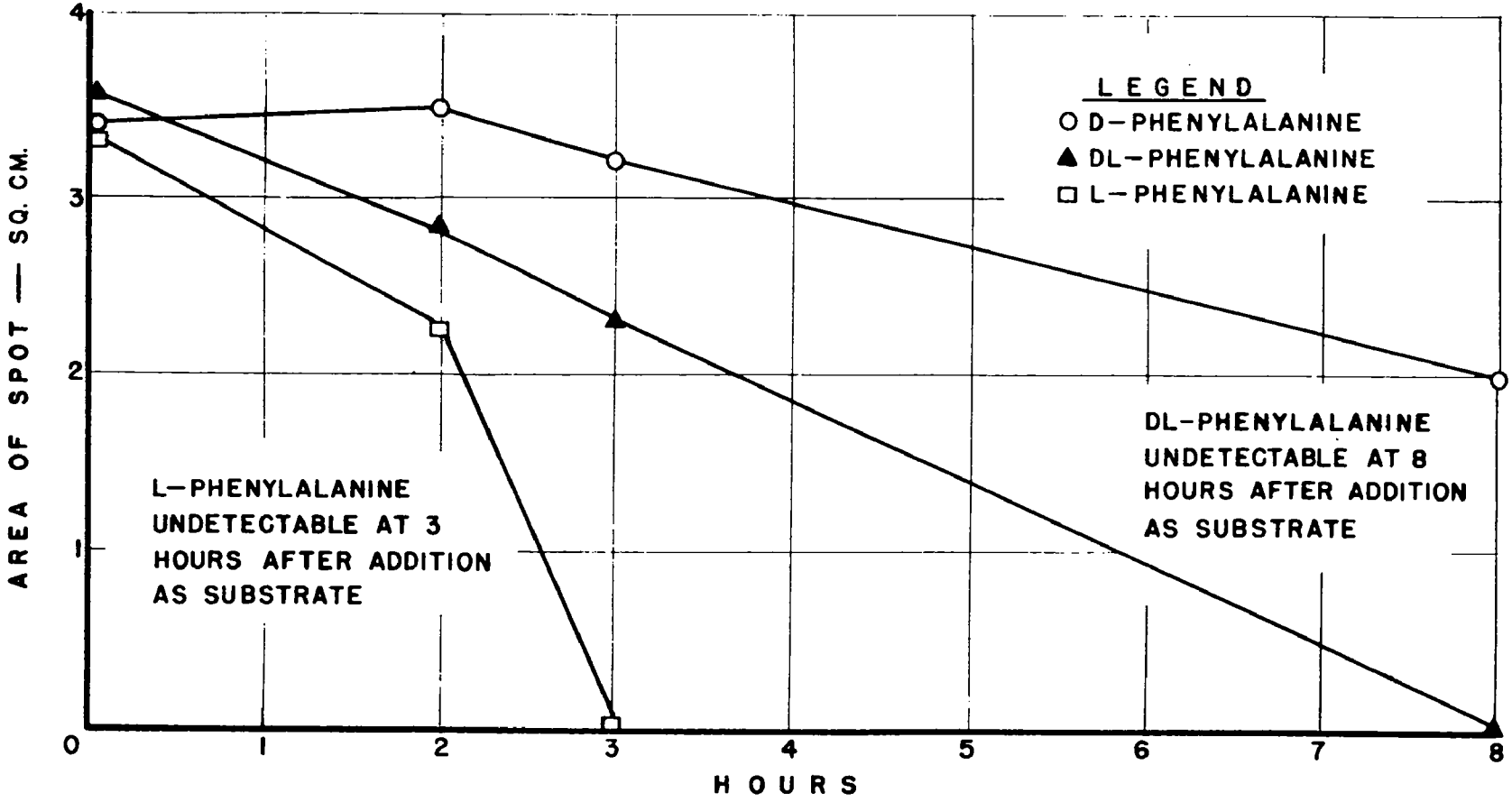


FIG. 4 SUPERNATATANT CONCENTRATIONS OF PHENYLALANINE ISOMERS AFTER BATCH FEEDING

in previous publications (1,4). Other special tests used are incorporated below in the body of the report.

THE UTILIZATION OF PEPTIDES

Following the studies on the free amino acids the next logical research area was the effect of the peptide bond on the utilization of amino acids. General evaluation of oxygen uptake rates for a large group of peptides was carried out but special emphasis was given to glycyl, leucyl, and alanyl peptides. These studies are related below.

The literature contains little information on the metabolism of peptides. Peptides are formed by the condensation of the carboxyl group of one amino acid with the amino group of another to form a peptide bond. Simple proteins are long chains of amino acids linked together in peptide bonds. Protein synthesis occurs on messenger and transfer RNA molecules associated with ribosome particles (6). In a review of Jacob and Monod's theory on genetic regulatory mechanisms (7), Stent (8) presents some of the current theories on the process of protein synthesis. Howe (9) discusses the peptide's importance in synthesis as an intermediate between the free amino acids and proteins. The overall dehydration reaction for peptide and protein formation is

$$n(RCHNH_2COOH) \longrightarrow RCHNH_2CO - (NHCHRCO)\ (n-2) - NHCHRCOOH + (n-1)\ H_2O$$

where R varies with each combining amino acid.

Evidently, the primary atom of a peptide bond is the oxygen atom. Combinations of electron acceptors with the oxygen weaken the carbon-oxygen bond and strengthen the carbon-nitrogen bond. The hydrogen bonding between peptide bonds produces an appreciable degree of stiffening of the bond itself (10).

The catabolic utilization or incorporation of peptides by microorganisms such as the bacteria may follow several pathways. Like the single amino acids, the smaller peptides may be incorporated directly into the interior of the bacterial cell. Following entry into the interior of the cell the peptide may be utilized in the synthesis of new protein material, catabolized for energy production and, sometimes, stored in pools within the cell (5).

Other peptides may not be able to pass directly into the cell's interior and, hence, must await biochemical modification before being utilized inside the

cell. In such cases, processes such as hydrolysis of the peptide can limit the rate of utilization of the peptide.

The effects of peptide bonding on oxygen utilization rates were studied for a variety of compounds. Studies with peptides of glycine provide some interesting information on the controlling oxidation rates for monomers and peptides of glycine. For activated sludge cultures acclimated to the glycine monomer, the peptide bond controlled the oxidation rate of the glycine as evidenced in the table below.

TABLE 1: THE EFFECT OF ACCLIMATION ON UTILIZATION OF GLYCINE PEPTIDES

Test No.	Culture Acclimated To	Test Run On	O_2 Uptake % of Uptake Rate on Acc. Substrate	Temp. °C
6-1	Glycine	Glycyl-Glycine	64	20
7-1	Glycine	Glycyl-Glycyl-Glycine	66	20
7-3	Glycine	Glycyl-Glycyl-Glycyl-Glycine	51	20
19-0	Glycyl-Glycine	Glycine	100	20
19-3	Glycyl-Glycine	Glycyl-Glycyl-Glycine	100	20

However, the table shows also that after the culture has been acclimated to glycyl peptides, the oxygen utilization rates of glycyl peptides are no longer retarded by the peptide bond.

Effect of Temperature on Oxidation with Glycine Substrates

Figure 5 indicates the effect of temperature on oxygen uptakes with glycine substrates. The apparent energy of activation, μ, lowers slightly as the peptide bonding linkages are increased. Whereas, μ is about 8600 cal/mole with glycine, tetra-glycine showed an apparent energy of activation of about 7000 cal/mole. These values were determined from the modified Arrhenius equation:

$$\log \frac{K_2}{K_1} = \frac{\mu}{2.303R} \left[\frac{T_2-T_1}{T_1\ T_2}\right]$$

where: R is the universal gas constant (1.987 cal/degree) and the temperatures T, are in degrees K. This gradual diminution in energy of activation corresponds with the free energy in the glycine substrates since glycine substrate added had

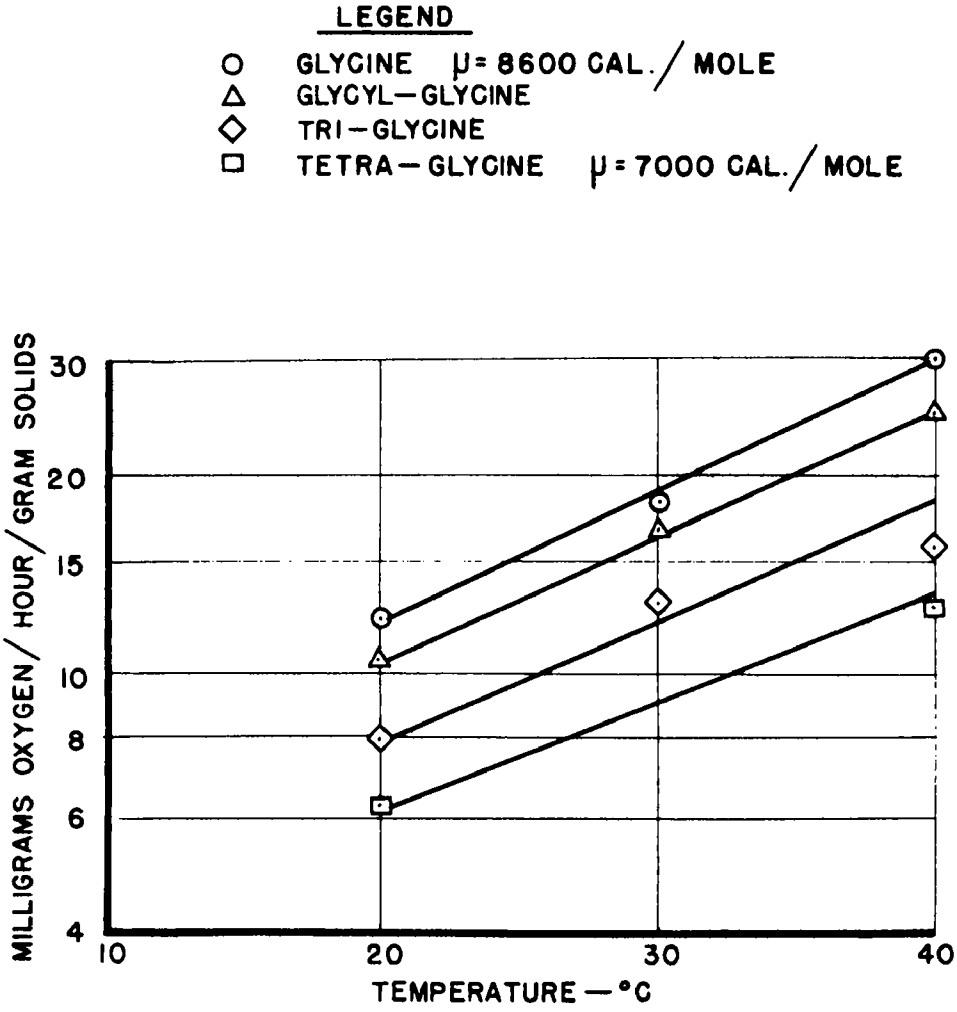


FIG. 5 EFFECT OF TEMPERATURE ON OXYGEN UPTAKE WITH GLYCINE SUBSTRATES

503 cal/liter, glycyl-glycine 442.6 cal/liter, glycyl-glycyl-glycine 397 and glycyl-glycyl-glycyl-glycine 369.2 cal/liter. Thus the energy of activation can be correlated approximately with the energy available in the glycine peptide for this particular series of tests.

Isomeric Effects on Peptides

As noted previously with the single amino acids, the D isomer restricts the utilization of peptides at 20°C. This depression of oxygen uptake was noted for all D isomers tested except alanine. The effect is vividly evident in the display of peptides of leucine and glycine in Figure 6. Note that uptake rates in this case are independent of molecular arrangement (i.e. whether the peptide is leucyl glycine or glycyl-leucine) but are significantly different for the D isomer of leucine.

Especially important is the fact that the utilization of the D isomer peptide follows the same equation as that developed for the utilization of D isomers of the free amino acids.

$$X = \frac{231}{C^{0.76}}$$

where: C is, in this case, the number of carbon atoms in the D isomer of the dipeptide, D-leucine.

Now this equation holds for D leucyl glycine but it holds as well for D leucyl glycyl glycine. Thus, the ratio of utilization for

$$\frac{\text{D leucyl glycine}}{\text{L leucyl glycine}} = \frac{\text{D leucyl glycyl glycine}}{\text{L leucyl glycyl glycine}} = 0.6$$

since: $C^{0.76} = {}_6^{0.76}$ in both cases.

STUDIES ON ALANYL PEPTIDES

For this portion of the study three dipeptides, glycyl DL-alanine, DL alanyl glycine, and DL alanyl DL phenylalanine, were chosen and experiments were carried out with these peptides as nutrient sources for activated sludge cultures.

The purpose of this peptide study was to investigate: (1) the significance of the location of the peptide bond for these peptides, (2) possible differences in utilization due to the presence of a peptide bond as compared with previous findings made with free amino acids, and (3) effects of temperature variations

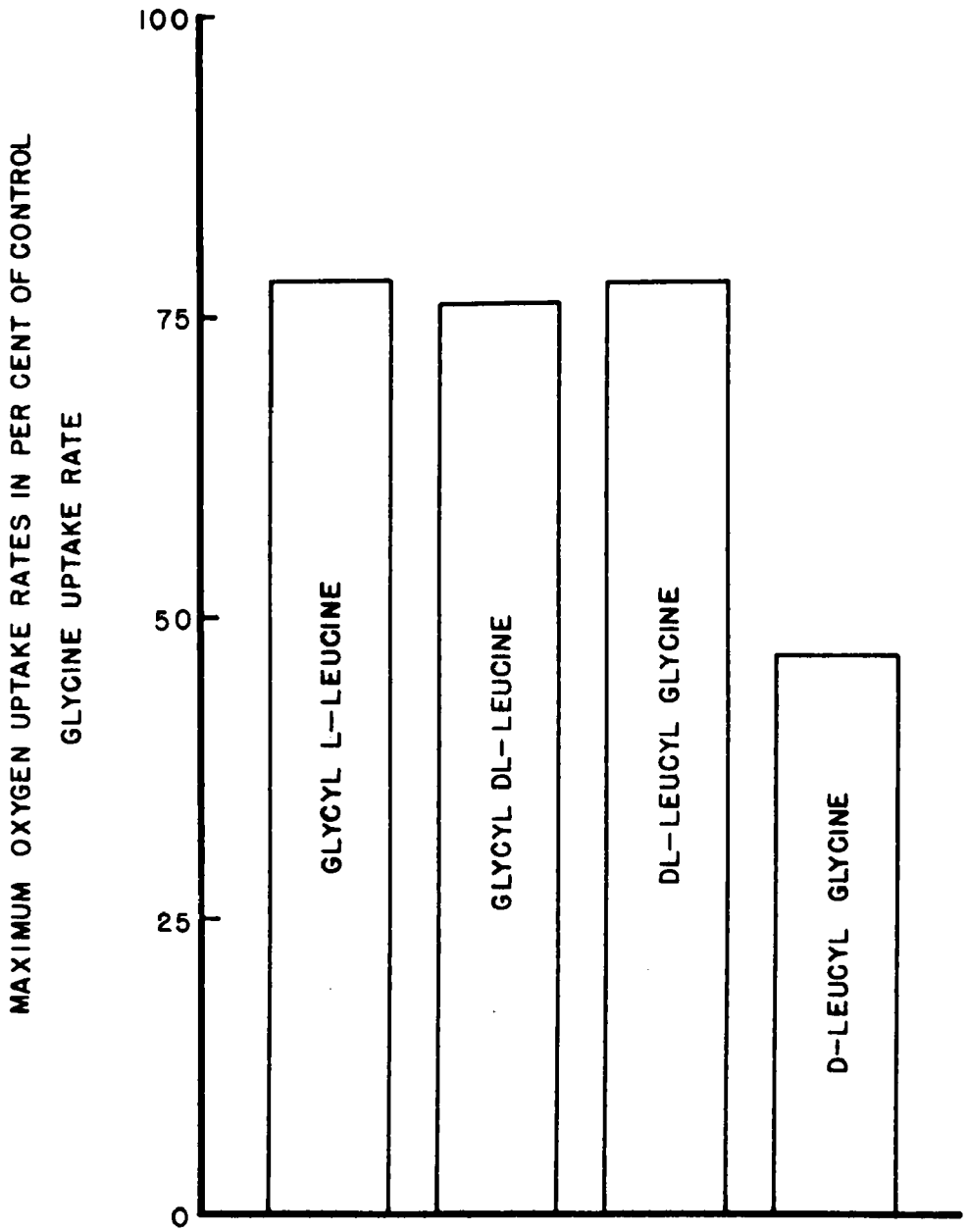


FIG. 6 EFFECT OF STEREOISOMERISM AND AMINO ACID SEQUENCE ON LEUCYL PEPTIDE UTILIZATION RATES AT 20°C

on the peptide utilization. Also, the effects of varying the concentrations of an inorganic salt, sodium chloride, was studied.

In these studies, as before, aliquots of activated sludge were grown on a batch fill and draw basis with a single peptide (or Bacto Nutrient Broth for control cultures) as the nitrogen source supplied in the nutrient substrate. The cultures, after at least one week of acclimation to the substrate, were subjected to the following studies: (1) measurement of oxygen uptake using the Warburg respirometer, (2) measurements of suspended solids to indicate cell mass changes over a given period, (3) concurrent chromatographic studies to show depletion of the peptide in the liquid supernatant of the activated sludge, (4) measurements of pH, alkalinity, nitrates, and ammonia concentrations to give an indication of breakdown or synthesis of the peptide.

Comparison of Alanyl Glycine and Glycyl Alanine

The oxygen uptake for glycyl DL alanine was almost double that for DL alanyl glycine in cultures acclimated to glycyl DL alanine, DL alanyl glycine, or DL alanyl DL phenylalanine (64). This confirms results obtained earlier where oxygen uptake rates for glycyl DL alanine were double those for DL alanyl glycine for cultures acclimated to either glycine or alanine. Figures 7 to 10 show the curves for oxygen uptake plotted against time.

As shown on Figures 8 and 9, when the endogenous oxygen uptake is subtracted from total uptake the net uptake curve becomes horizontal as the peptide supply becomes depleted. This peptide depletion is confirmed by chromatographic studies which are plotted on Figure 11.

Oxygen uptake rates are plotted against free energy on Figure 12 (See Table 2).

TABLE 2A: FREE ENERGY AND OXYGEN UPTAKE RATES FOR AMINO ACIDS

Substrate	Oxygen Uptake Rate at 20°C µl/hr/mg Solids	Free Energy* Calories/Liter
glycine	8.5	593
DL alanine	10	502.31
DL phenylalanine	21	161.74
DL glutamic acid	6.9	594.32

*See Appendix IV

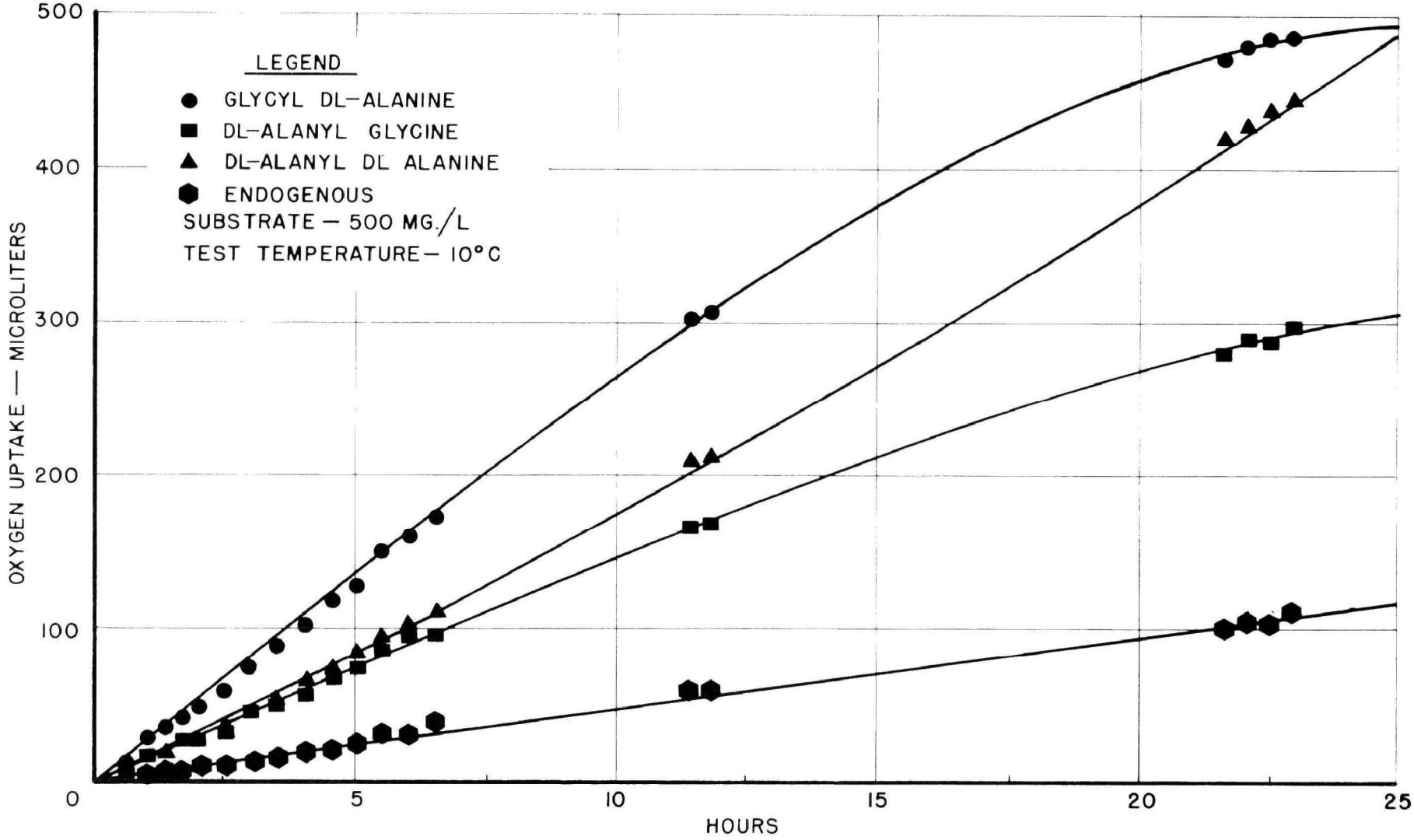


FIG. 7 OXYGEN UPTAKE FOR CULTURE ACCLIMATED TO GLYCYL DL-ALANINE

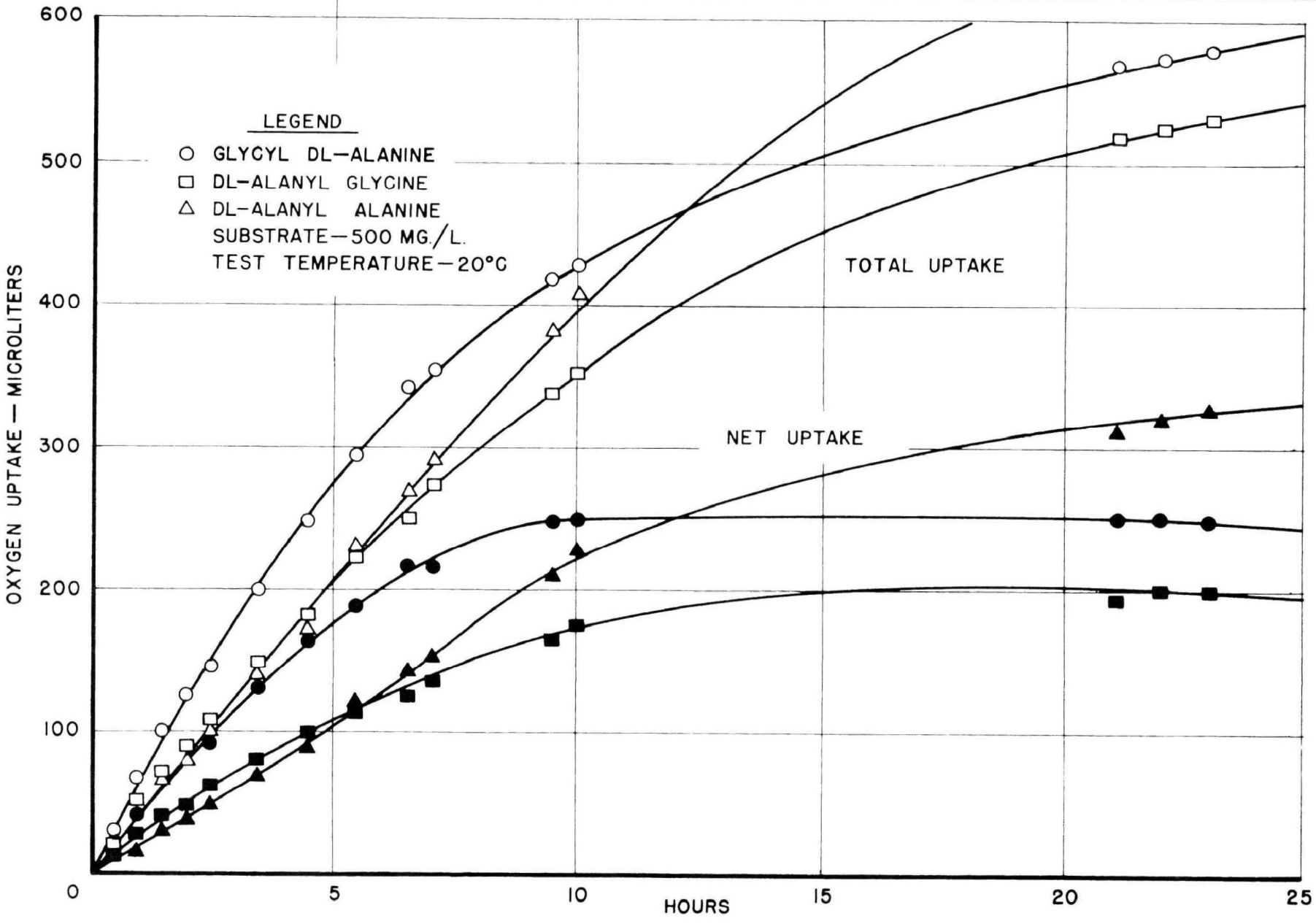


FIG. 8 OXYGEN UPTAKE FOR CULTURE ACCLIMATED TO GLYCYL DL-ALANINE

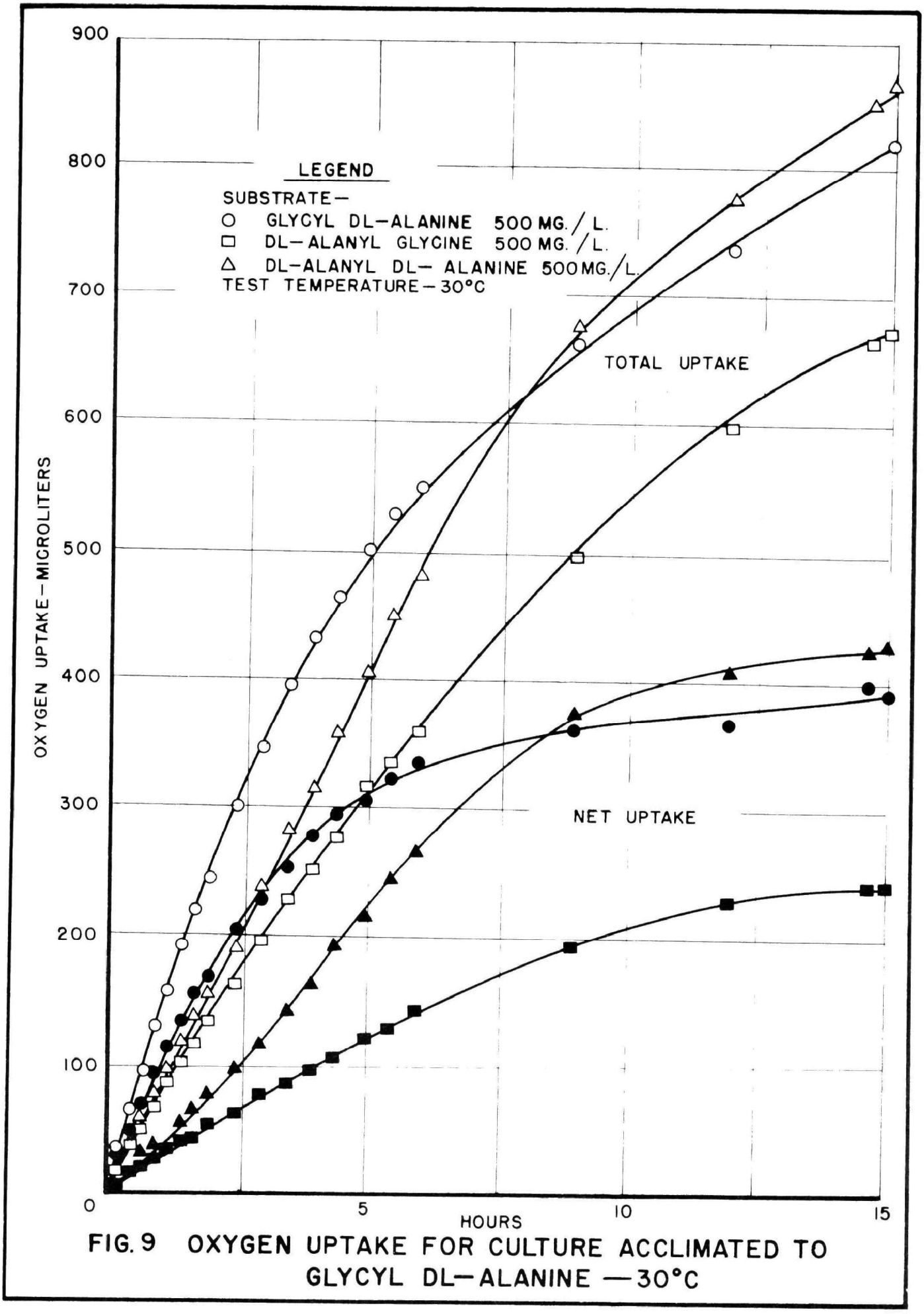
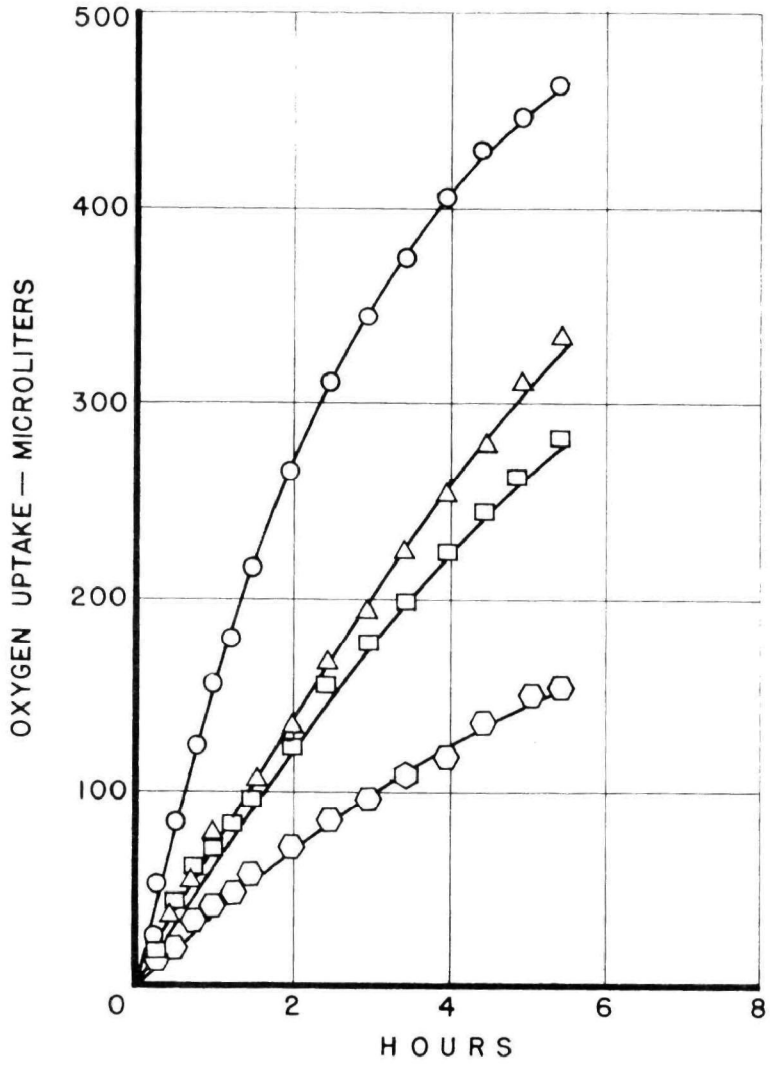


FIG. 9 OXYGEN UPTAKE FOR CULTURE ACCLIMATED TO GLYCYL DL—ALANINE —30°C



LEGEND

○ GLYCYL DL-ALANINE
□ DL-ALANYL GLYCINE
△ DL-ALANYL DL ALANINE
⬡ ENDOGENOUS
TEST TEMPERATURE — 35° C
SUBSTRATE — 500 MG./L.

FIG.10 OXYGEN UPTAKE FOR CULTURE ACCLIMATED TO GLYCYL DL-ALANINE 35°C

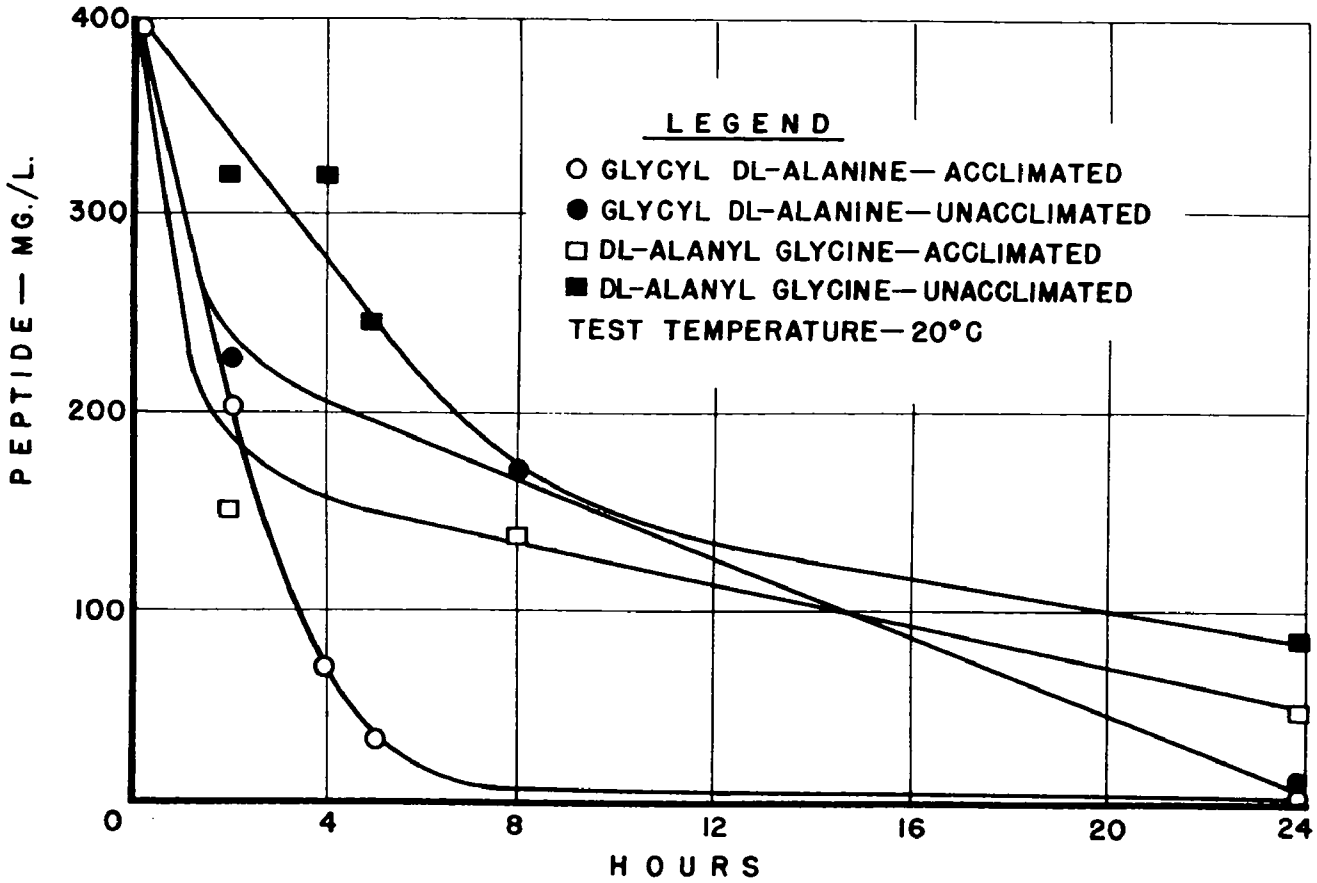


FIG. 11 PEPTIDE DEPLETION WITH TIME

TABLE 2B: FREE ENERGY AND OXYGEN UPTAKE RATES FOR PEPTIDES

Substrate	Oxygen Uptake Rate at 20°C µl/hr/mg solids	Free Energy* Calories/liter
glycyl DL alanine	13.02-13.1	399.63
DL alanyl glycine	7.09-9.12	399.63
DL alanyl DL phenylalanine	10.79-20.6	175.96

*See Appendix IV

The oxygen uptake rates for free amino acid substrate cultures, when plotted against the free energy of the amino acid used, fall along a straight line such that the approximate oxygen uptake could be determined from the free energy by the equation:

$$Y = 26.5 - 0.0325\ F$$

where: F is the free energy in the substrate in calories per liter

and: Y is the oxygen uptake rate in microliters per hour per mg sludge solids.

For sludges not acclimated to the peptide, DL alanyl DL alanine and DL alanyl glycine, a second line can be approximated. The spread of data points is not sufficient to write an equation for the line of best fit. The line CD as drawn on Figure 12 has the equation:

$$Y = 15.4 - 0.018\ F$$

which indicates a curve with about half the slope and initial value of the acclimated cultures. When the culture has been acclimated to DL alanyl Dl alanine, the oxygen uptake rate fits the "acclimated" curve. Acclimation, however, did not have this effect on the utilization of DL alanyl glycine. Note also that glycyl DL alanine was utilized as well as the constituent amino acids even prior to acclimation. This evidence indicates that some reaction, such as hydrolysis to constituent amino acids, is evidently limiting utilization of the peptide sequence DL alanyl glycine but is not a limiting condition when the constituent acids are in the sequence glycyl DL alanine.

It is quite interesting that sequential arrangement could influence oxygen uptake even with the smallest possible length of peptide chain.

Figures 8 to 11 give further evidence of differences in the utilization of glycyl DL alanine and DL alanyl glycine. After eight hours an acclimated culture of glycyl DL alanine had essentially depleted its supply of peptide while a DL

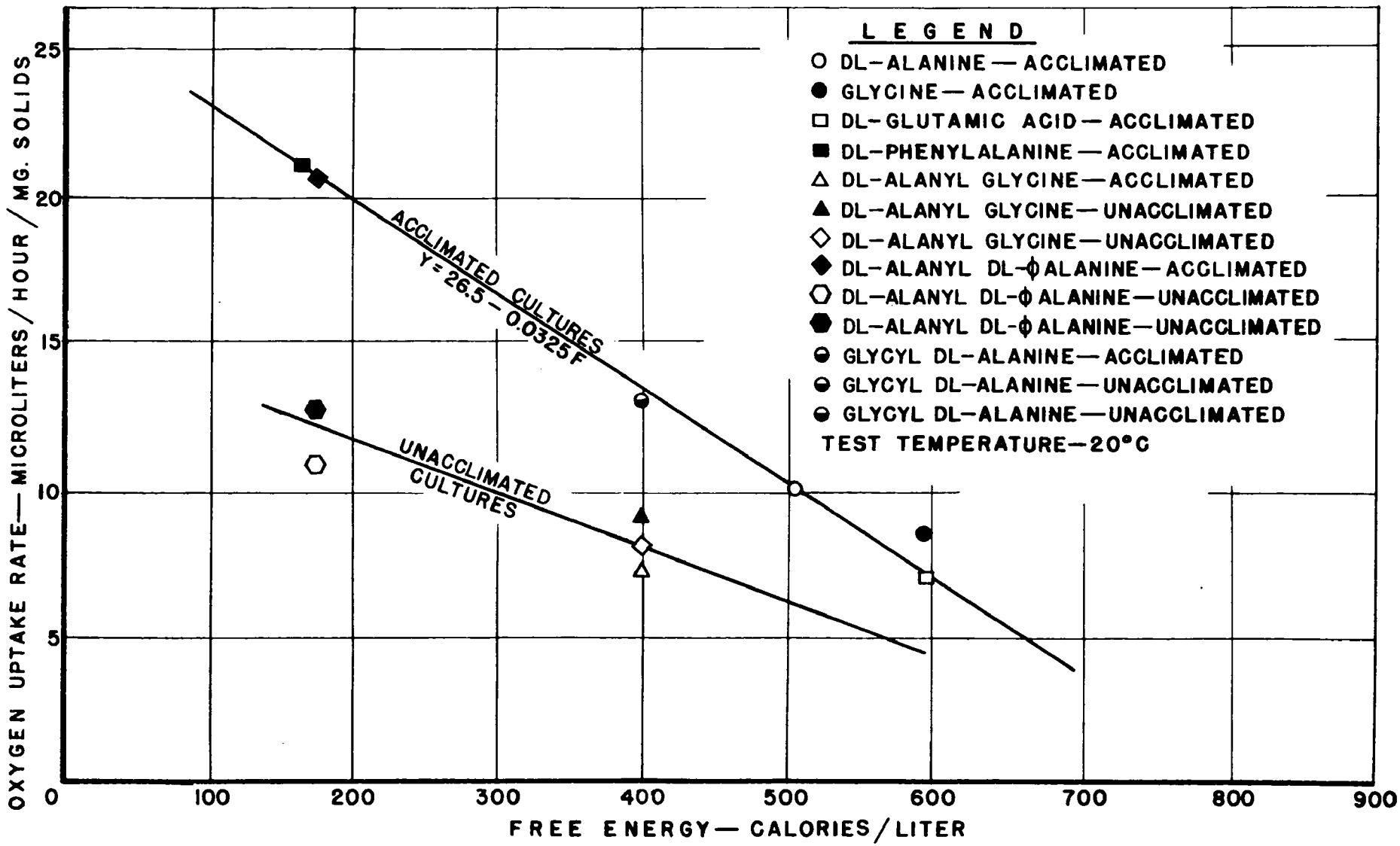


FIG. 12 FREE ENERGY VS. OXYGEN UPTAKE RATE

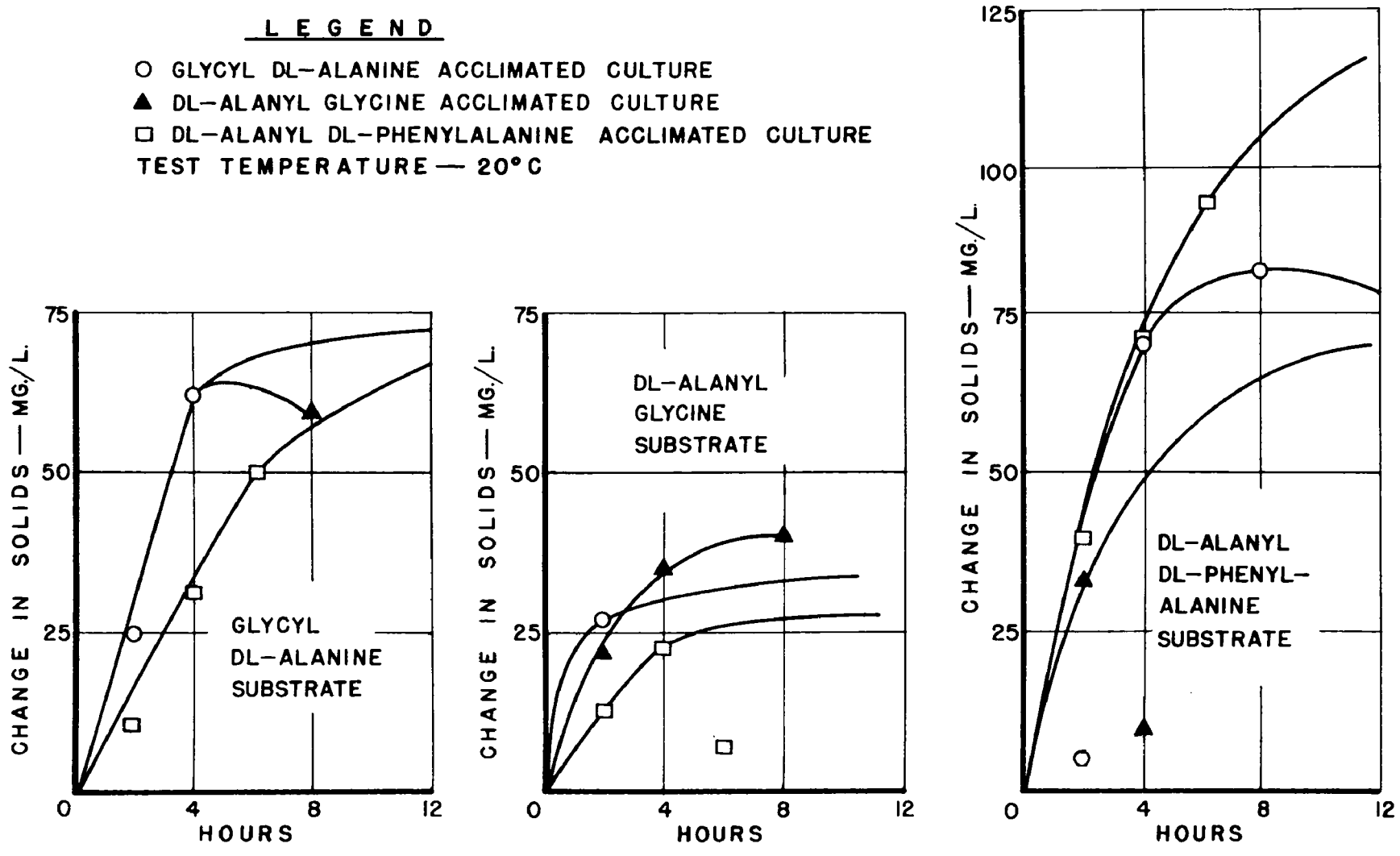


FIG.13 CHANGE IN VOLATILE SUSPENDED SOLIDS WITH ALANYL PEPTIDE SUBSTRATES

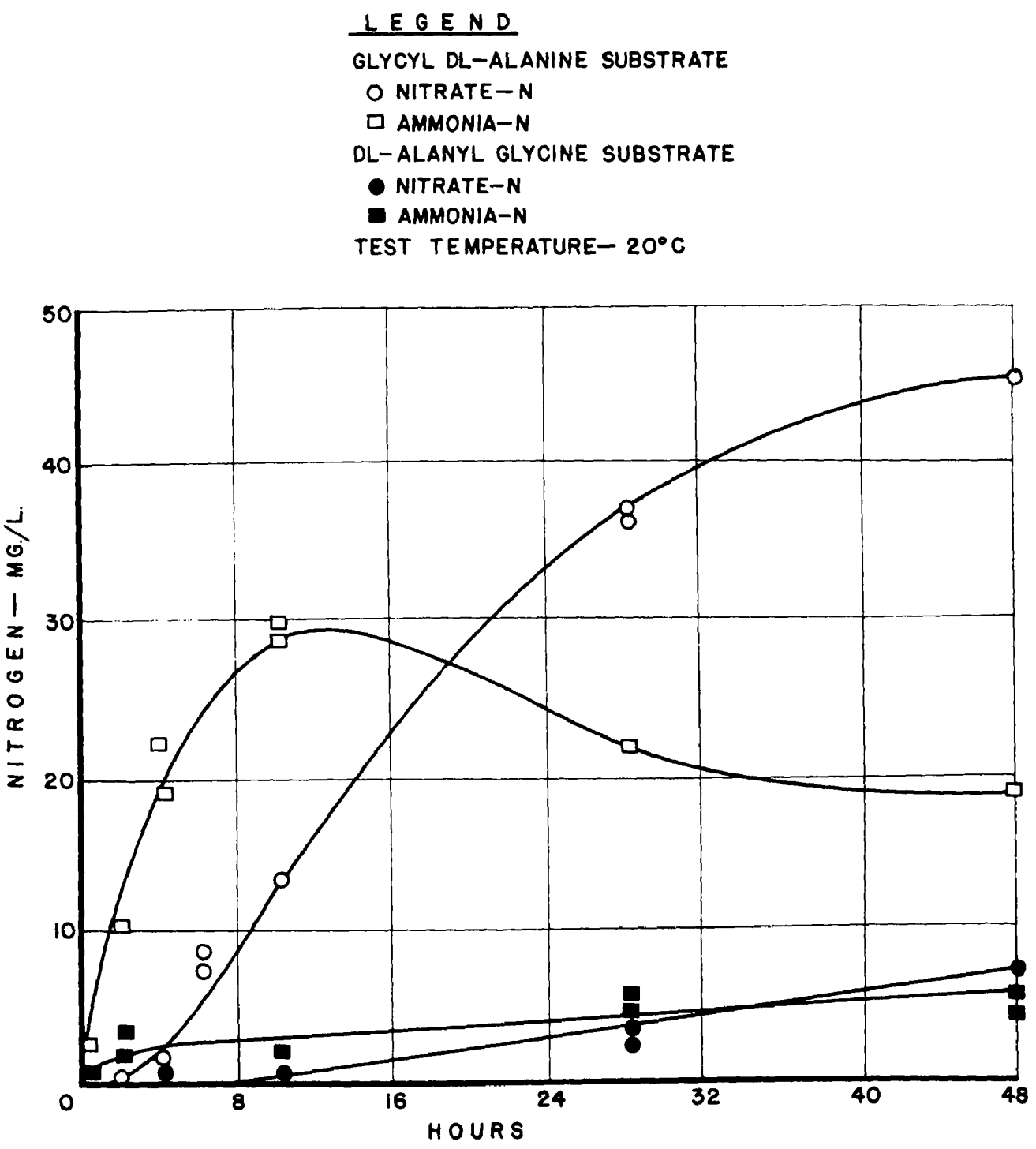


FIG. 14 AMMONIA-NITROGEN AND NITRATE-NITROGEN VS. TIME

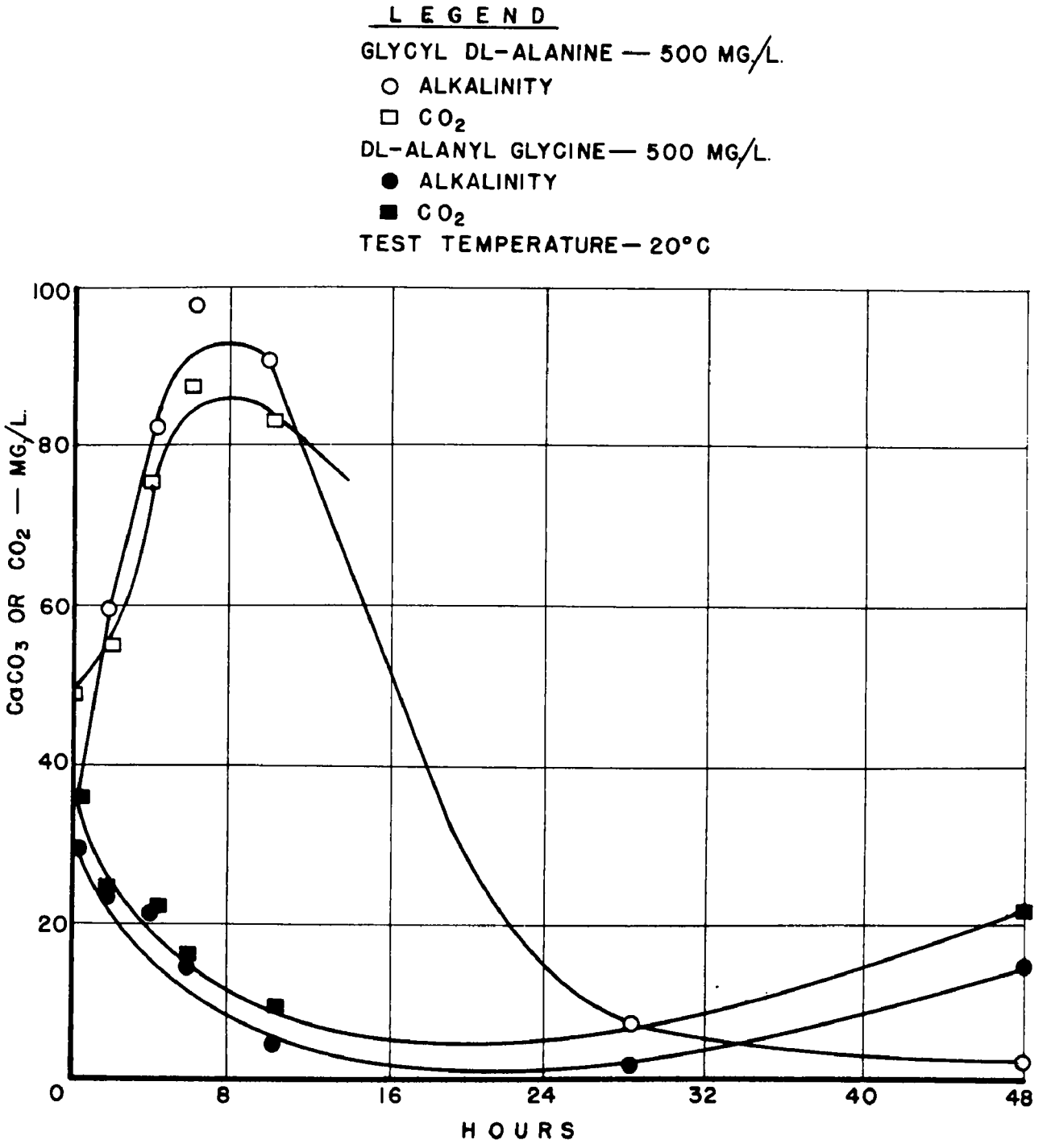


FIG. 15 ALKALINITY AND CARBON DIOXIDE VS. TIME

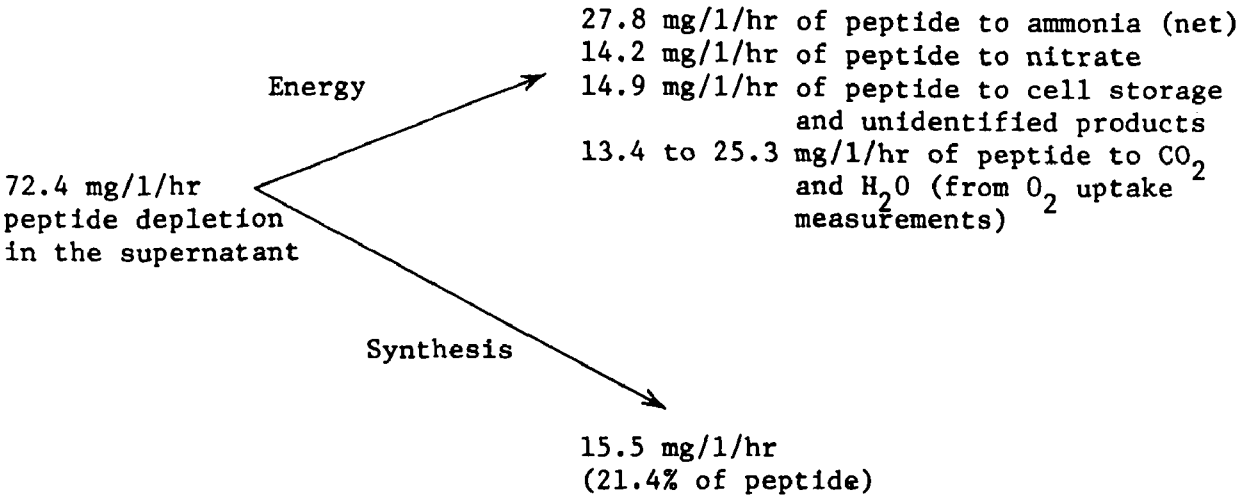
alanyl glycine culture had not fully utilized its supply in 24 hours as shown in Figure 8.

From Figure 9 it is apparent that the cell synthesis is twice as great for glycyl DL alanine as for DL alanyl glycine. At the same time, Figure 10 and 11 respectively show a significant production of ammonia and carbon dioxide for the glycyl DL alanine culture while the DL alanyl glycine culture shows quantities which were hardly measurable.

The following equations can be derived by comparing peptide depletion with ammonia and nitrate production and oxygen uptake rates over a given period of time.

Glycyl DL Alanine

A. Initial reaction rates (until the 500 mg/l of peptide is depleted in the supernatant - 6.9 hours).



B. Reactions 6.9 to 25 hours after feeding.

Cell storage and unidentified products + NH_3 + cell material →

77.4 mg/l as peptide

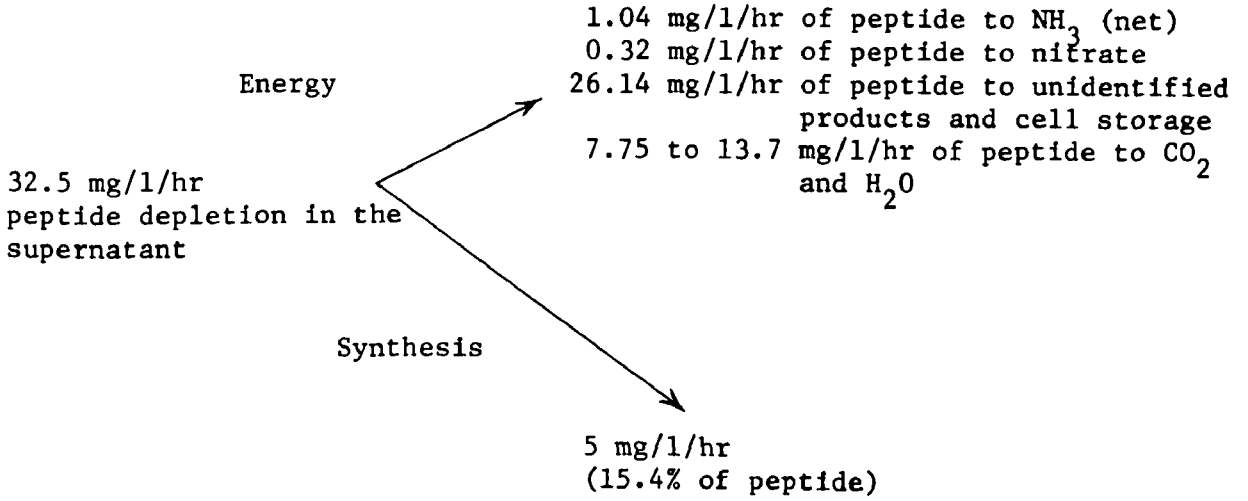
43.6 mg/l as peptide

→ NO_3

240 mg/l as peptide

DL Alanyl Glycine

A. Initial reaction rates (first 10 hours).



B. Reactions 10 to 24 hours after feeding.

175 mg/l of peptide $\longrightarrow$ 66 mg/l of peptide + 2 mg/l of peptide to NH_3 (net)

+ 2.5 mg/l of peptide + 104.5 mg/l of peptide to unidentified products and cell storage

From the preceding equations it is seen that glycyl DL alanine is depleted from the supernatant in 6.9 hours with ammonia and carbon dioxide as the initial products. The pH increased from 6.5 to 7.9 during this same period. After the peptide was depleted in the supernatant, the ammonia and carbon dioxide concentrations were reduced and nitrates continued to increase. The increase in nitrates was greater than the decrease in ammonia and it is therefore assumed that the cell storage proposed above, plus material from volatile solids utilization, were sources of the resulting nitrate production after the peptide was depleted. The pH decreased to 5.9 by 24 hours.

The DL alanyl glycine was not utilized as completely as was glycyl DL alanine. Sixty-five percent of the peptide was removed from the supernatant in 10 hours and 87% after 24 hours. Trace amounts of ammonia and nitrate were measured in the supernatant of the culture. Although oxygen uptake measurements indicated possible CO_2 production, the alkalinity measurements showed a reduction in CO_2. The pH increased from 6.7 at the time of feeding to 7.7 after 6 hours and de-

creased to 5.9 after 24 hours. A possible explaination of the results obtained for the DL alanyl glycine culture might be that the culture is tying up the peptide as unidentified products and cell storage, but it is not able to degrade the peptide to NH_3 and CO_2 readily. Therefore, the ammonia production is low and alkalinity is being removed from the supernatant by the culture as a carbon source. Further verification of these hypotheses awaits additional studies in this area. Cell synthesis was indicated by solid studies results. It is possible that the culture was producing a product from the peptide which was poisonous or inhibitory to further metabolism by the culture and thus resulted in a reduction in peptide depletion rates and a minimum of deamination.

Temperature Effects on Peptide Utilization

A ten degree centigrade increase in temperature should approximately double the oxygen uptake rate. Plotting temperature on an arithmetic scale vs. oxygen uptake rate on a log scale should give a straight line variation in the range of 10° to 30°C. In some cases there was a reduction in oxygen uptake rates at 35°C, probably due to the population of the particular culture.

Temperatures in the range of 30°C are approaching the thermal death point of protozoa and if the protozoan concentration of the culture were high the curve would tend to break over as shown on Figures 16 to 18. On the other hand, bacteria have a thermal death point which is much higher, above 42°C, and if the protozoan population is low a curve similar to the left hand curves on Figure 16 would be more likely where the straight line relationship holds up to a temperature of 35°C. K_2/K_1 values for a 10°C increase in temperature were equal to 2 ± 0.2 for oxygen uptake rates obtained in this investigation. Taking the values from a typical curve the following value for μ was obtained.

For glycyl DL alanine:

$K_1 = 26.2$

$K_2 = 52.4$ $\qquad T_1 = 10°C$

$K_2/K_1 = 2$ $\qquad T_2 = 20°C$

$\mu = 11{,}400$ calories/mole

This is within the values suggested in the literature (10,000 to 20,000 calories/mole). However, this value may change from day to day with variations in the population of the culture within the above range.

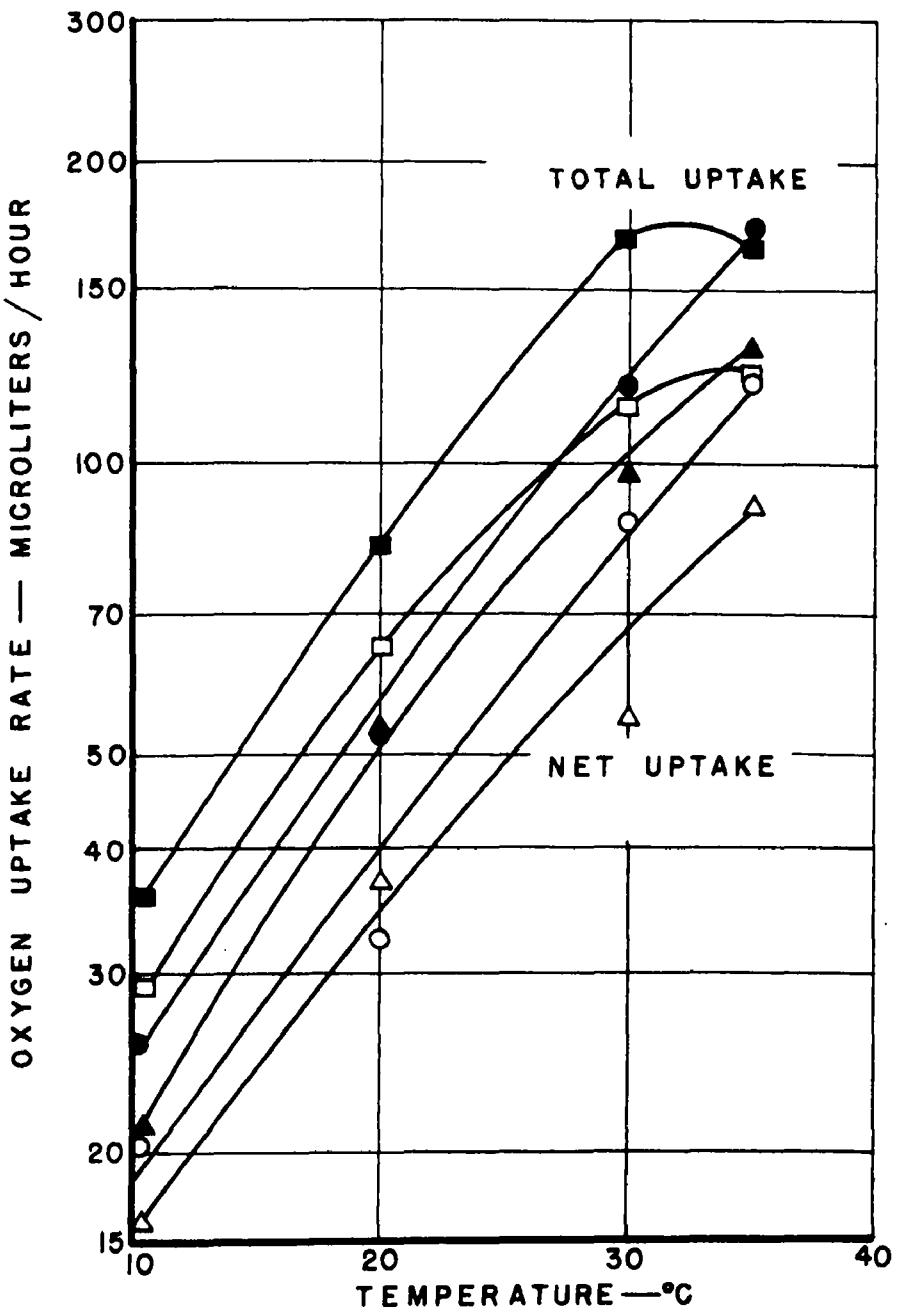


FIG.16 OXYGEN UPTAKE RATES VS. TEMPERATURE FOR GLYCYL DL-ALANINE

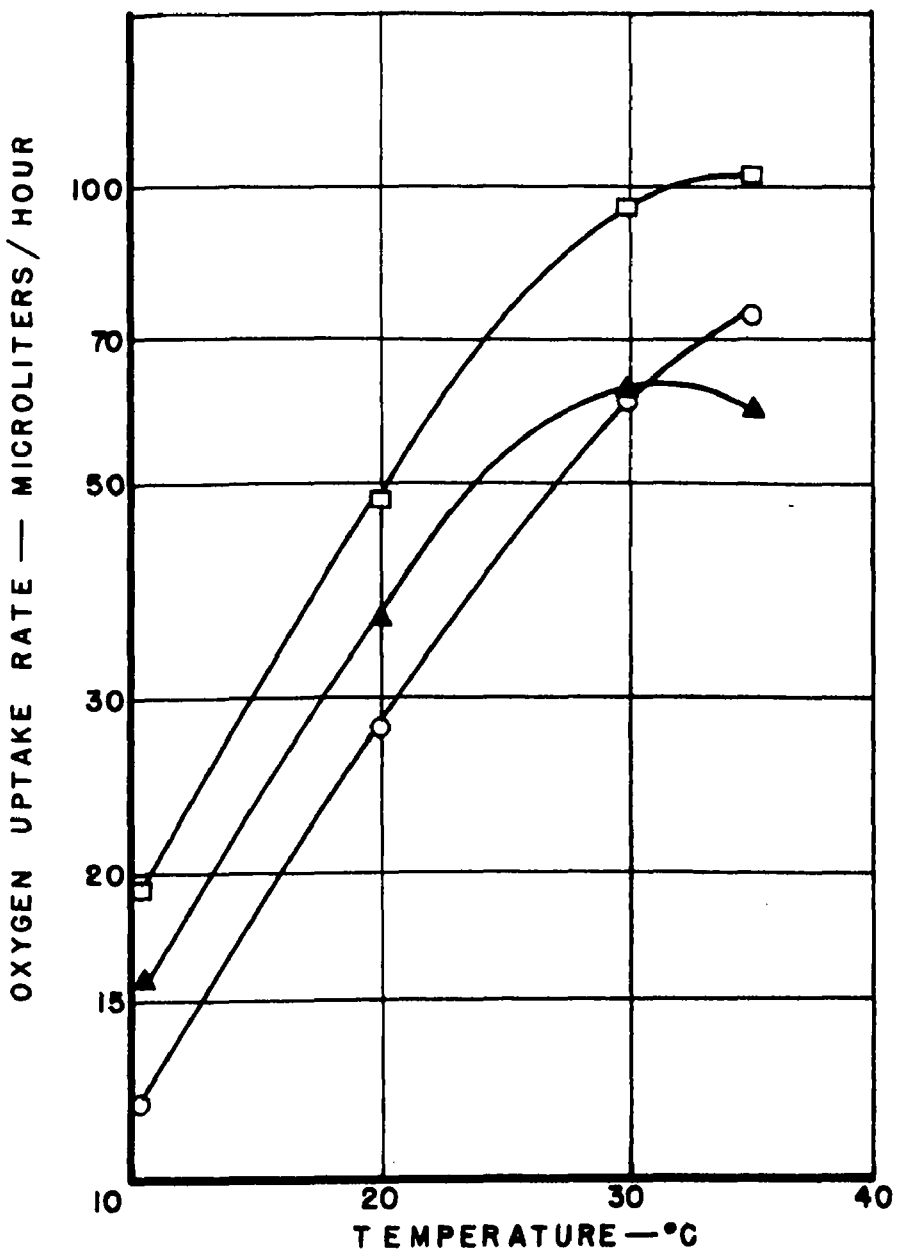


FIG.17 TOTAL OXYGEN UPTAKE RATES VS. TEMPERATURE FOR DL-ALANYL GLYCINE

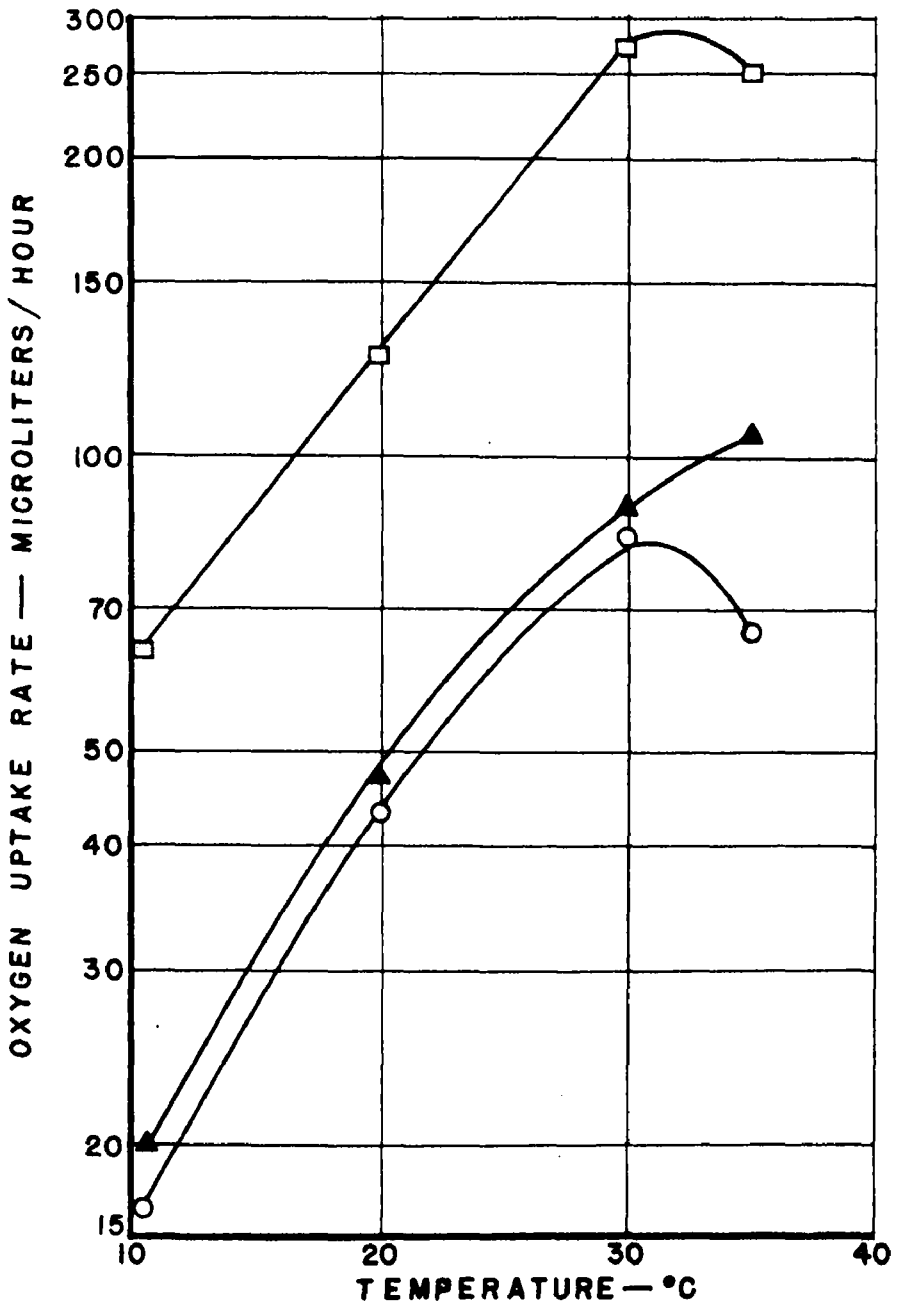


FIG. 18 TOTAL OXYGEN UPTAKE RATES VS. TEMPERATURE FOR DL-ALANYL DL-PHENYLALANINE

APPLICATION OF A DNA TEST FOR DETERMINING CELL GROWTH ON AMINO ACID SUBSTRATES

Commonly used methods for direct measurement of growth in activated sludge cultures are based on the determination of the dry weight of cell mass or the assay of one of their elementary constituents such as carbon or nitrogen. Other methods are based on the determination of the rate of respiration or, in the case of unicellular pure cultures, viable or plate counts can be used. These conventional methods present difficulty in interpretation as applied to mixed cultures because:

a) An increase in dry weight or in carbon or nitrogen content does not necessarily reflect an increase in population size of microorganisms. For instance, the formation of extracellular capsular material or intracellular food reserves may cause a considerable increase in dry weight without an increase in the number of living organisms.

b) An increase in respiration rates may correlate closely with the rate of substrate utilization by a culture, but, like methods based on determination of the dry weight, does not give information on the increase in population size.

c) The viable count methods, although tedious to perform, is a direct method of enumeration of viable population size. However, the tremendous dilution ratios involved render its accuracy doubtful. In addition, the application of this method to mixed cultures is questionable.

In view of the limitations and disadvantages inherent in conventional methods, it is of importance in the field of biological waste treatment to develop a method capable of reflecting the population size and activity of a mixed culture.

A brief survey in the structure and composition of living cells indicates that all cells share a common general chemical composition, the most fundamental feature of which is the invariable presence of three types of complex organic macromolecules: proteins, deoxyribonucleic acid and the ribonucleic acid. Deoxyribonucleic acids (DNA) are the genetic material of the cell (11); that is, the hereditary information needed to specify and direct the growth and reproduction of the cell is contained in the structure of DNA molecules and is passed from generation to generation in this form. Aside from its genetic role in living systems, bacteria and other microorganisms are characterized by a high DNA

content in comparison with higher organisms. In addition, studies on the DNA content of microorganisms show that the DNA in the cells is constant, with the exception of the period just prior to cell division (12,13). These striking facts, as Vendrely (14) stressed, are of great interest for the biologist from the theoretical as well as the practical point of view. The discovery of the constancy of the amount of DNA per nucleus in all tissue of the same animal or in cells of the same microorganism, and the fact that the sperm contains half of the DNA content of somatic cells is confirmation of the theory that DNA is an important component of the gene. From the practical point of view, the constant amount of DNA per nucleus can serve as a measure of cell multiplication and as a standard of reference in the expression of biological changes in living systems.

The purpose of this research phase was to develop a suitable method of DNA determination in activated sludge cultures and to evaluate growth and activity of the culture by virtue of DNA determinations.

Deoxyribonucleic Acid and its Determination

The nucleic acids are polymeric molecules of great size with molecular weights reaching several million. They are complex polymers, made up of repeating non-identical units known as nucleotides. Each nucleotide is composed of a ring shaped nitrogenous base, a sugar and a phosphate group, all linked together. The individual nucleotides are joined through their sugar and phosphate group in the following manner (15):

```
   base                base                base
    |                   |                   |
- sugar - phosphate - sugar - phosphate - sugar - phosphate
```

There are two types of nucleic acids, the ribonucleic acids (RNA) and deoxyribonucleic acids (DNA), which differ from each other in the nature of the base and sugar components of their nucleotide sub-units. In RNA, the bases are adenine, guanine, cytosine and uracil; the sugar in ribose. DNA also contains adenine, guanine and cytosine but has thymine instead of uracil as the fourth base constituent. In addition, the DNA nucleotides differ from those of RNA in that, instead of the sugar ribose, they contain its reduced derivative, deoxyribose.

DNA itself has a unique macromolecular structure. Watson and Crick (16) proposed that two long chain molecules are coiled around one another to form a double helix which is held together by very weak hydrogen bonds formed between

their bases. The configuration of the bases is thought to be such that the base adenine can bind only with the base thymine in the opposite chain, and cytosine can bind only with guanine. Thus, a DNA macromolecule is thought to be composed of two complementary DNA chains (16,17).

The determination of nucleic acids requires, first, isolation of the nucleic acids from cells and then the actual determination. Nucleic acids are quite easy to detect cytochemically because all three of their essential components, phosphoric acid, pentose, and the purine or pyrimidine bases lend themselves well to that purpose (18). Quantitative determinations of the nucleic acids are based usually on (a) their phosphorus content, after separation of RNA and DNA or (b) the ribose of RNA and the deoxyribose of DNA or (c) the ultraviolet absorption of the purine and pyrimidine components in the region of 270 to 350 mμ (14). The last two methods are more specific than the first; the method using the color reaction of the sugars of nucleic acids is perhaps the most widely used and the better tested method. Of the color reactions available for DNA, the most commonly used method is the reaction with diphenylamine in a mixture of acetic and sulfuric acids at 100°C (19). However, this method, prior to the modification by Burton in 1956, involved substantial error which could arise from incomplete separation of DNA from interfering cellular constituents such as glucose, glycogen, monosaccharides, proteins, or amino acids (20).

Convenient and reliable methods for determining the nucleic acid content of cells were not available until Schmidt and Thannhauser (21) and Schneider (22) published their procedure of nucleic acids isolation in 1945.

Deoxyribonucleic Acid in Microorganisms

Microorganisms present a special problem in the isolation and determination of their constituent DNA because microorganisms, in mixed culture, vary greatly in the ease with which their cell walls can be ruptured, in their content of capsular polysaccharides which are difficult to separate from DNA, and in the association of DNA to protein which influences the ease of DNA purification (23).

Several procedures have been used for DNA isolation from microorganisms. Vendrely (24) and Burton (20,25) have successfully applied Schneider's method (22) to microorganisms. Bacteria are particularly rich in nucleic acids. The nucleic acids may account for as much as 15% of the dry weight (26). Table 3 below gives nucleic acid concentrations isolated from several cultures by Ven-

drely (24). The ratio of RNA to DNA is usually in the range of 2:1 to 3:1. Little information is available on the DNA content of higher protists; but the concentration is believed to be lower than for the bacteria. Scherbaum,(27) using the Schmidt - Thannhauser procedure (21) and ultra-violet spectrophotometry, concluded that a normal culture of Tetrahymena pyriformis contained 6% RNA and 0.33% DNA on a dry weight basis.

Aside from the high content of nucleic acids in microorganisms, a study on the dynamics of the nucleic acid content in microorganisms showed that the total nucleic acid content undergoes considerable variation and is closely connected with the physiological state of the culture, i.e., with its age, activity and growth. Investigators (12,13) have noted that young cultures are always characterized by higher nucleic acid contents than old cultures and, in the process of aging, there is always a regular decrease in the nucleic acid content. However, for DNA alone, all results have shown that the DNA content in cells of microorganisms is constant, with the exception of the period just prior to cell division. Thus, the DNA concentration can be used as a measure of cell concentration in a volatile solids mass. The DNA content of the nucleus, therefore, is a useful tool in measuring activated sludge cell concentrations.

TABLE 3: NUCLEIC ACID CONTENT OF MICROORGANISMS (24)

Microorganisms	Total	Percent Dry Weight	
		RNA	DNA
Stapholococcus albus	11.57	8.75	2.83
Escherichia coli			
Various strains	13.90	9.73	4.17
	13.12	8.72	4.40
	14.67	10.43	4.24
	12.37	8.59	3.78
	13.98	10.09	3.89
Bacillus aertryaclce	8.40	5.40	3.00
	10.64	7.00	3.64
Yeast (baker's)	4.26	3.95	0.31

Activated Sludge Growth

The use of the term "growth" without clear definition has been a source of confusion in studies on activated sludge cultures. Growth in activated sludge cultures means synthesis of protoplasm and hence cell multiplication of microorganisms due to the utilization of organic substrate. However, because there has been no suitable and efficient growth parameter to show actual increase in the population of microorganisms, growth has been conventionally defined as an increase in biological mass and the solids concentration has been used widely as a growth parameter. Many workers have realized the limitation of this assumption. Gaudy and Engelbrecht (28) used volatile suspended solids, protein, carbohydrate content and substrate utilization as parameters of activated sludge growth. They concluded that, although synthesis is measured usually as sludge production (mass) and interpreted as an increase in microbial population, such assumptions should be used with caution. Heukelekian and Manganelli (29) stated that the increase in the quantity of sludge in the activated sludge process is a result of a combination of the following influences: (a) incorporation in the floc of resistant and unavailable organic materials (b) residual available and oxidizable organic materials removed from the sewage and which, because of the shortness of sludge age, have not been fully oxidized but which could be oxidized further if longer periods of aeration were available, and (c) the increase in the bacterial protoplasm resulting from the oxidation of the portion of available food materials removed from the sewage.

McCabe (30) noted that a crude estimate of the active biological solids can be obtained from the volatile suspended solids content, but gradual buildup of inert organic materials frequently interferes with this estimate.

Thus, growth parameters in use cannot accurately determine the concentration of active viable cells in activated sludge nor can they indicate the genesis of new cells. The studies herein presented on DNA were predicated on the idea that DNA concentration determinations could provide a measure of the cell concentration as well as follow the generation of new cells or the gradual depletion of existing activated sludge populations.

Extraction and Determination of DNA From Activated Sludge Cultures

Schneider and other investigators (23,31,32) have described the isolation of DNA from selected groups of microorganisms. The procedures involved are not

complicated, no special equipment is required and successive extractions are possible from the same sample. Schneider's procedure, with some modification, was used in this study. Detailed procedures of the DNA determination are presented below:

I. Materials:
(a) Standard Deoxyribonucleic Acid (DNA) Na salt, A grade, extracted from salmon sperm as prepared by the California Corporation for Biochemical Research.
(b) Diphenylamine (Fisher certified reagent grade)
(c) Acetic Acid (glacial)
(d) Aqueous acetaldehyde
(e) Perchloric acid, 0.5 and 1.0N
(f) Concentrated sulfuric acid (reagent grade)

II. Materials:
(a) Safety centrifuge (Fisher 1725 RPM)
(b) 12 ml graduated conical centrifuge tubes
(c) 70°C water bath
(d) Ice bath
(e) 20 ml test tubes
(f) Spectrophotometer (Spectronic 20)

III. Diphenylamine Reagent -- prepared by dissolving 1.5 grams of Fisher certified reagent grade diphenylamine in 100 ml of glacial acetic acid and adding 1.5 ml of concentrated sulfuric acid. This preparation is stored in the dark in a brown bottle. On the day it is to be used, 0.1 ml of aqueous acetaldehyde (15 mg/ml) is added for each 20 ml of reagent required. Acetic acid must be glacial or redistilled since low grade acetic acid may cause the development of a blue color in the reagent which interferes with the colorimetric estimation of DNA. The stored reagent should be transparent.

IV. Preparation of DNA Standard Solution -- DNA used was a highly polymerized sodium salt, A grade, extracted from salmon sperm. The stock solution was 0.1 mg DNA standard/ml of distilled water. From the stock solution, desired concentrations of working solutions were prepared by mixing a measured volume of the stock solution, which was adjusted to 1.25 ml with distilled water, with 1.25 ml of 1 N perchloric acid to give a final 0.5 N perchloric acid solution. After heating for 10 minutes at 70°C two volumes (5 ml) of diphenylamine reagent were added. The total volume (7.5 ml) of solution was then incubated at room temperature (25 to 35°C) for twenty hours. The incubation

temperature is not critical provided the constancy of temperature can be maintained. The intensity of the developed blue color was measured with a spectrophotometer at 600 mμ.

V. DNA Determination Procedure --

(1) Centrifuge a 10 ml aliquot of sample for 10 minutes. Carefully waste clear supernatant and save centrifuged solids in the bottom.

(2) Add 2.5 ml cold 0.25 N perchloric acid. Place in an ice bath for 20 minutes.

(3) Centrifuge for 10 minutes. Waste supernatant, add 2.5 ml of 0.5 N perchloric acid and start extraction. Let stand for 10 minutes in 70°C water bath with occasional stirring of solids.

(4) Centrifuge for 10 minutes and save the supernatant (first extraction), re-extract solids with another 2.5 ml of 0.5 N perchloric acid.

(5) Centrifuge for 10 minutes and save the supernatant (second extraction).

(6) A series of extractions can be obtained by the same procedure to find the total DNA in the sample. In the case of activated sludge cultures, three successive extractions gave nearly 100% of the extractable DNA.

(7) The supernatants from each extraction are retained for the DNA determination. To conserve reagents, smaller aliquots of the total extraction mixture may be used.

(8) Add two volumes of diphenylamine reagent to one volume of the extraction mixture aliquot. Incubate at room temperature for 17 to 20 hours.

(9) The characteristic reaction of diphenylamine to DNA is the development of a blue color. The optical density or percentage transmission is measured against a blank (2.5 ml of 0.5 N perchloric acid plus 5 ml of the diphenylamine reagent) using a spectrophotometer at a wavelength of 600 millimicrons. The value obtained is compared with the prepared DNA standard curve. A known concentration of DNA standard is tested as a reference for each sample set run.

(10) Calculations:

$$\text{DNA mg/l} = \frac{\text{(mg of DNA read from standard curve) (x3)}}{\text{(ml of sample used)}}$$

Preliminary Tests of DNA Procedure

Because DNA determinations on heterogeneous biological systems such as acti-

vated sludge are a relatively new procedure, preliminary tests were carried out to determine:

(1) The applicability of Schneider's and Burton's methods to activated sludge cultures.

(2) The required number of extractions to obtain complete extraction of detectable DNA from activated sludge.

(3) The range of DNA standard concentrations that obey Beer's Law.

(4) The suitable sample sizes and concentrations.

Burton (20) applied Schneider's DNA extraction method to various strains of Escherichia coli and obtained the results shown in Table 4.

TABLE 4: EXTRACTION OF DNA FROM BACTERIA (19)

Conditions	Extraction Time	Vol. ml	% of DNA Extracted				
			1st Extract	2nd Extract	3rd Extract	Total	1st Two Extracts
Experiment I							
0.5 N $HClO_4$ @ 70°C	10	10	63	27	6.4	96.4	90
	20	10	88	8	1.7	97.7	96
	30	10	88	8	1.7	97.7	96
1 N $HClO_4$ @ 70°C	10	10	48	38	10	97.0	86
	20	10	52	33	10	95.0	85
5% Trichloracetic	5	10	68	23	5.5	96.5	91
	10	10	84	11	2.7	97.7	95
Experiment II							
0.5 N $HClO_4$ @ 70°C	10	10	45	46	7.2	97.2	91
	15	10	71	25	1.4	97.4	96

Table 5 shows the results obtained with varying solids concentrations of activated sludge. From the results shown in the table it was concluded that for a suspended solids concentration of 1000 mg/l, three successive extractions with 0.5 N perchloric acid would obtain nearly 100% of the extractable DNA.

The DNA standard curve was prepared from the standard stock solution. The working standards were adjusted to 2.5 ml with distilled water and 1 N perchloric acid to a final concentration of 0.5 N perchloric acid and heated in a water bath

TABLE 5: EXTRACTION OF DNA FROM ACTIVATED SLUDGE CULTURES USING 0.5 N $HClO_4$ FOR 10 MINUTES AT 70°C

Cultures	% of DNA Extracted				
	Supernatant	1st Extract	2nd Extract	3rd Extract	1st Two Extracts
I Alanine Substrate					
S.S. 200 mg/1	0	96.2	3.8	0	100
500	0	93.5	6.5	0	100
750	0	94.3	5.7	0	100
1000	0	68.5	30.2	1.3	98.7
2000	0	69.7	25.3	5.0	95.0
II Glycine Substrate					
S.S. 200	7.1	21.2	71.7	0	92.9
500	0	13.3	86.7	0	100
1000	0	49.0	49.0	2.0	98.0
2000	1.9	19.2	67.3	11.1	86.5
III Nutrient Broth					
S.S. 200	0	64.0	36.0	0	100
500	0	52.0	41.0	7.0	93.0
750	0	54.3	42.3	3.4	96.6
1000	0	20.5	67.3	12.2	87.8
2000	0.3	34.7	56.8	7.9	91.5

for 10 minutes at 70°C. After adding two volumes (5 ml) of diphenylamine reagent, the total volume of 7.5 ml was then stored at room temperature for 20 hours.

The absorption spectra of the DNA standards in the visible light range (λ = 350 to 750 millimicrons) were scanned by a Perkin-Elmer Automatic Recording Spectrophotometer. The peak of absorbance, as shown in Figure 19, occurred at a wave length of 600 millimicrons. The intensities of the blue color, developed in the reaction of diphenylamine with DNA were then measured on a Spectronic 20 at the 600 millimicron wave length. The results indicated that, in the DNA concentration range of 0.01 mg/7.5 ml to 0.10 mg/7.5 ml (1.3 to 13.3 mg/1), the relationship of percentage transmission (% T) to DNA concentration obeyed Beer's Law as shown in Figure 20.

SAMPLE – DNA
ORIGIN – DNA STD. SOLUTION
SOLVENT – $HClO_4$ + REAGENT
SPECTROPHOTOMETER – PERKIN-ELMER
CONCENTRATION – 0.01 0.125 MG. DNA

CELL PATH – 10 MM.
REFERENCE – BLANK, $HClO_4$ + REAGENT
SCAN SPEED – RAPID
SLIT – 25
OPERATOR – F.L.
WAVELENGTH – VISIBLE RANGE

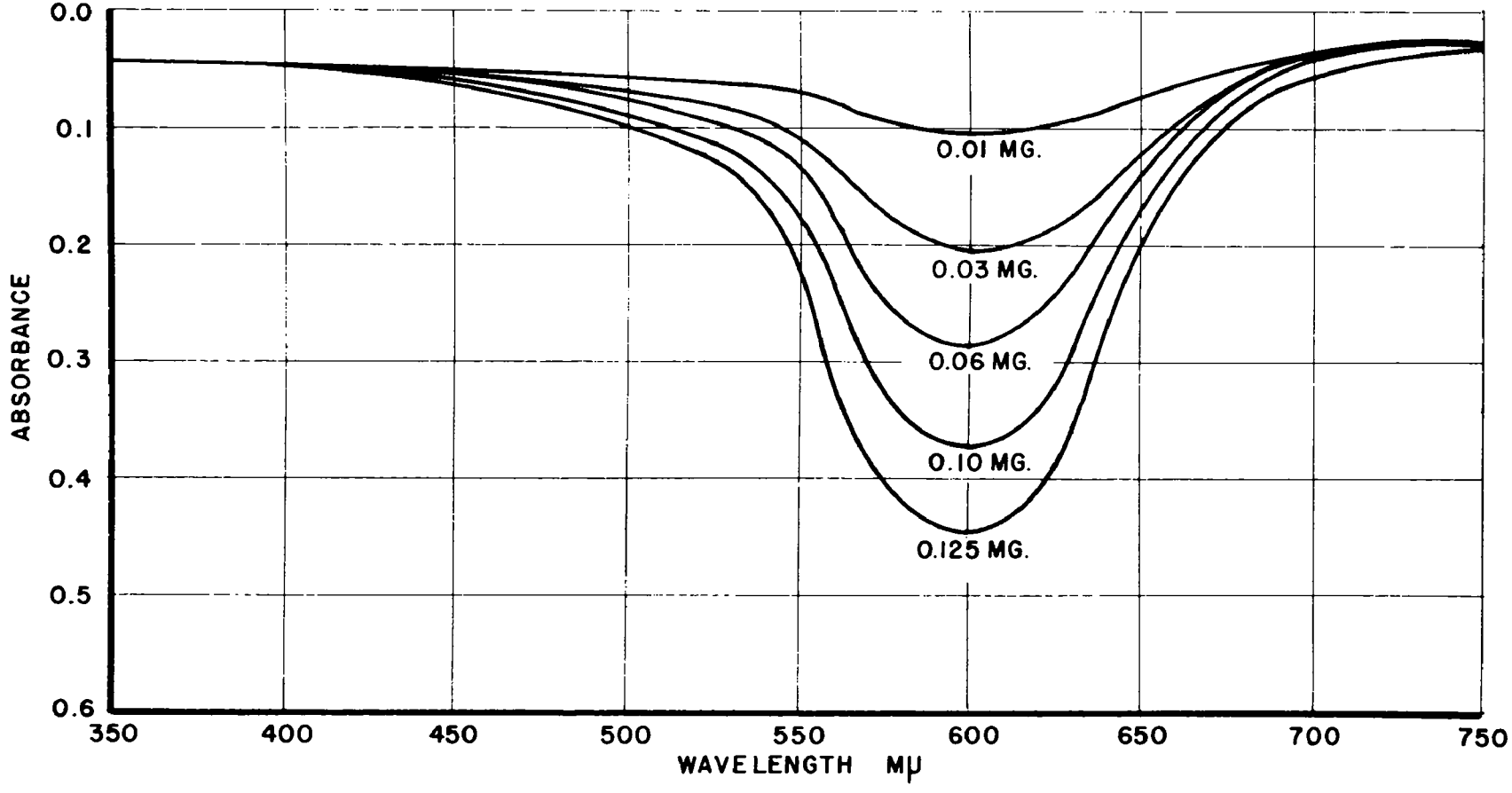


FIG.19 DNA ABSORPTIVE SPECTRA

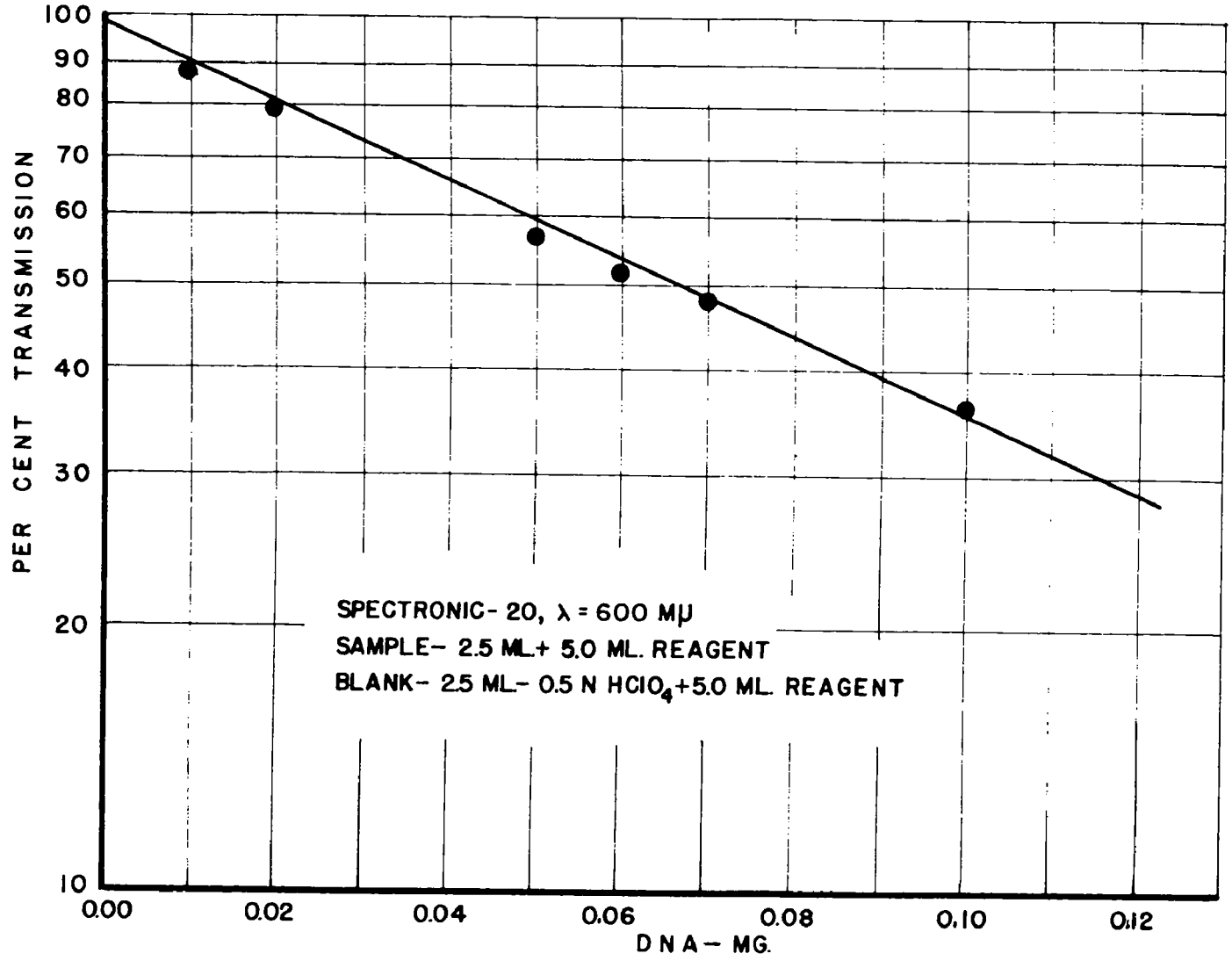


FIG. 20 DNA STANDARD CURVE

To utilize the most accurate portion of the standard curve (% T from 20 to 80%) the sample should contain 0.02 to 0.16 mg of DNA. Therefore, sample sizes were increased to 10 ml to better fit this range for the standard curve and for activated sludge cultures of approximately 1000 mg/l suspended solids concentrations.

Toxicity tests, using copper sulfate and sodium cyanide, were carried out to evaluate the activity of activated sludge cultures and the inhibitory effects of these bacteriocides on sludge growth. In toxicity tests, the experimental procedure followed that used in growth studies, except for the addition of the bacteriocidal agent. Solids, amino acid depletion, oxygen uptake and DNA tests were performed on the activated sludge cultures slugged with bacteriocides. The changes in these measured parameters were compared with results of cultures with normal feedings.

Results of the DNA Studies With Amino Acid Substrates

Data obtained in the study appears in work by Li (33). To facilitate discussion some of the data are shown graphically. Amino acid substrates were developed on a C:N ratio of 5:1. All amino acids used were the L isomers except for DL alanine. These studies have shown that DL alanine is used equally as well as the L isomers alone.

Oxygen uptake studies were used for evaluating the biochemical activity of activated sludge cultures with given substrates. Oxygen uptake tests were run on the Warburg respirometer with four amino acids. To correlate results with growth studies, samples were adjusted to approximately 1000 mg/l suspended solids. Figures 21 through 25 show the uptake curves for L - arginine, DL alanine, L - phenylalanine, and L - glutamic acid.

Because the cultures were acclimated to the desired amino acid and glucose and then subjected to the amino acid as the sole carbon source, differences in oxygen uptake rates were anticipated. However, as shown in Figure 25 no difference was obtained with L - glutamic acid vs. L - glutamic acid and glucose. Figure 26 summarizes the maximum oxygen uptake rates of the four amino acids.

The results obtained in the growth studies appear graphically in Figures 27 through 34. In Figures 27 to 30, the results are shown for a single amino acid as sole carbon source. In Figures 31 through 34 the results are shown for the respective amino acid and glucose as the combined substrate. In both cases, the

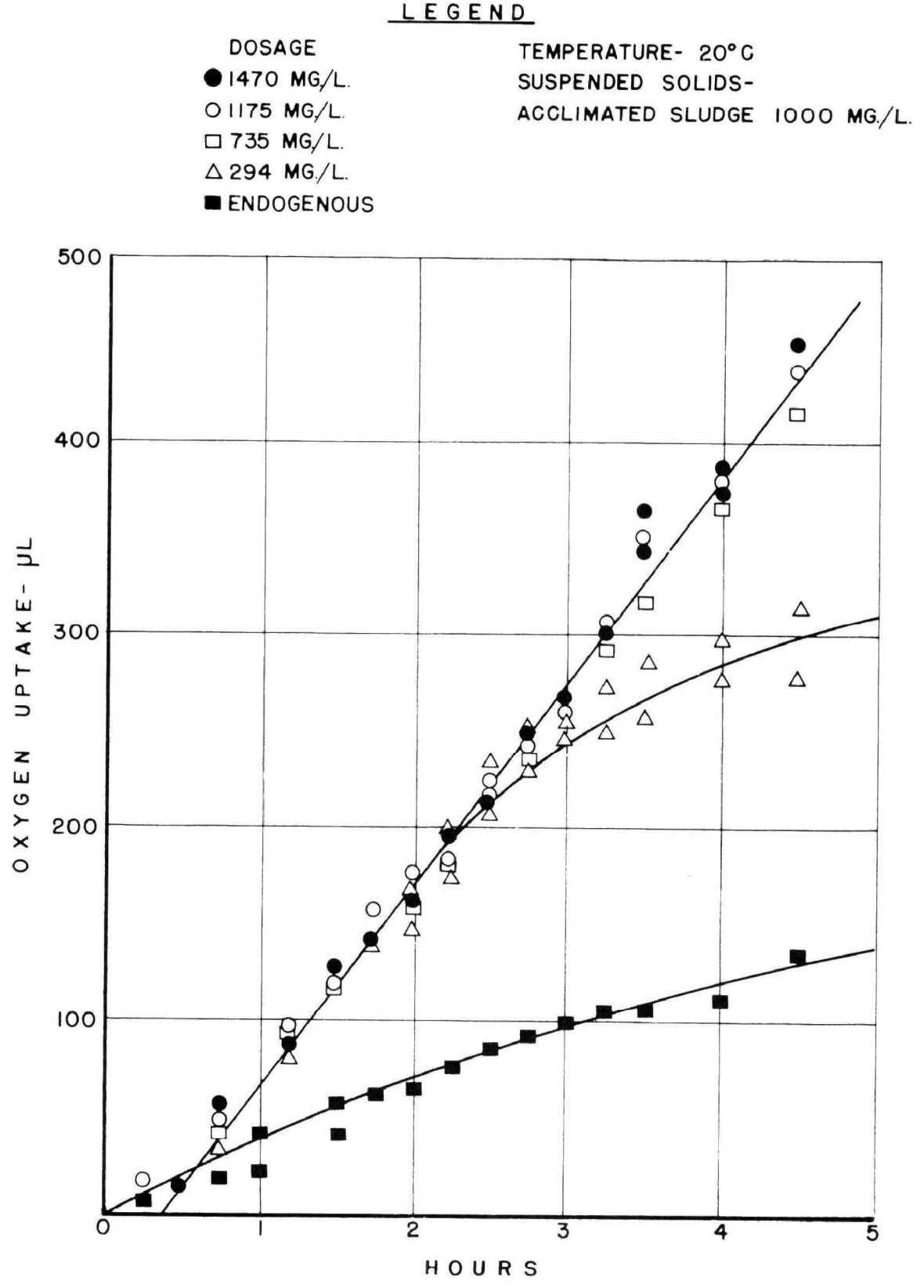


FIG.21 OXYGEN UPTAKE
WITH ARGININE-HCl SUBSTRATE

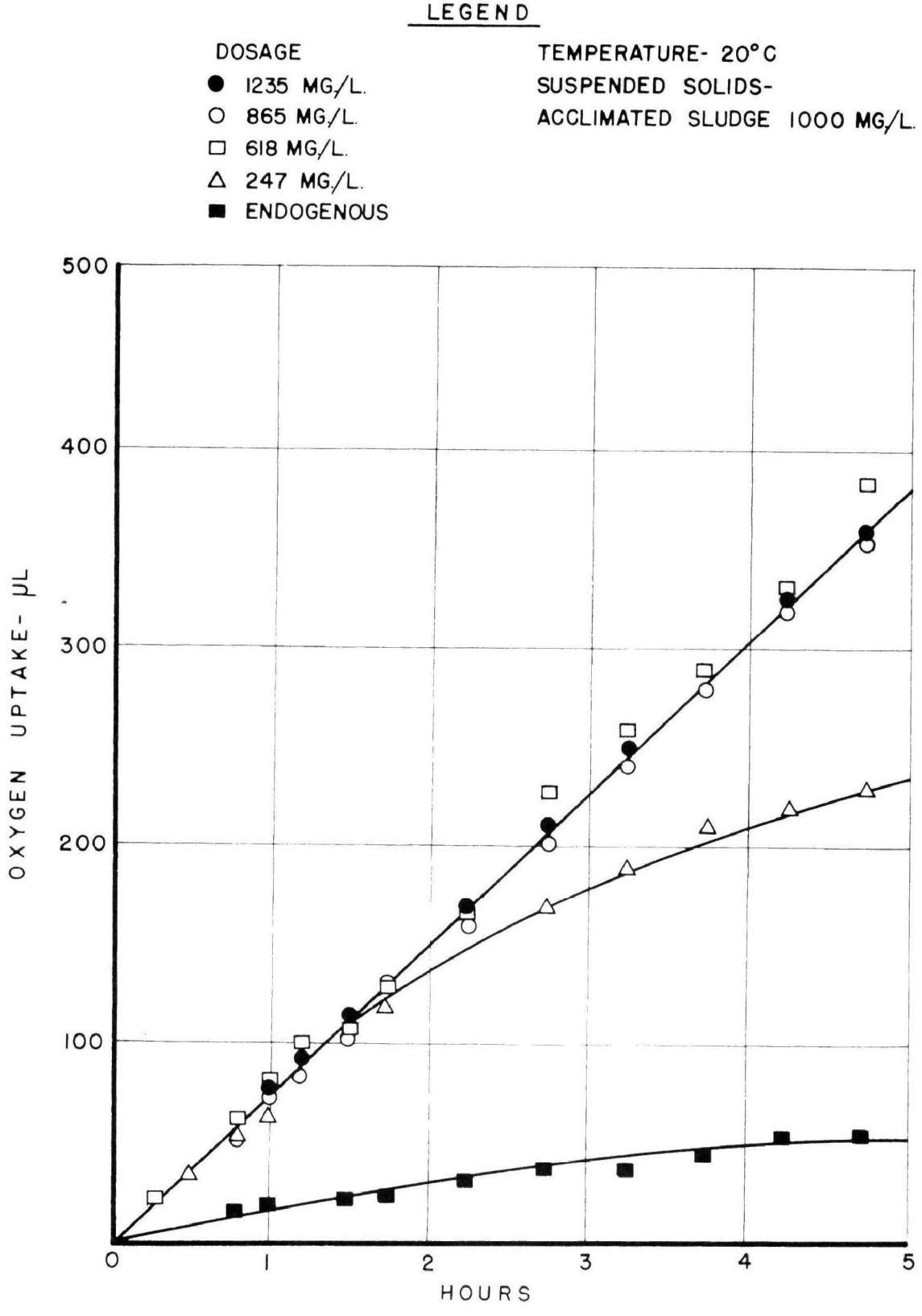


FIG.22 OXYGEN UPTAKE
WITH DL-ALANINE SUBSTRATE

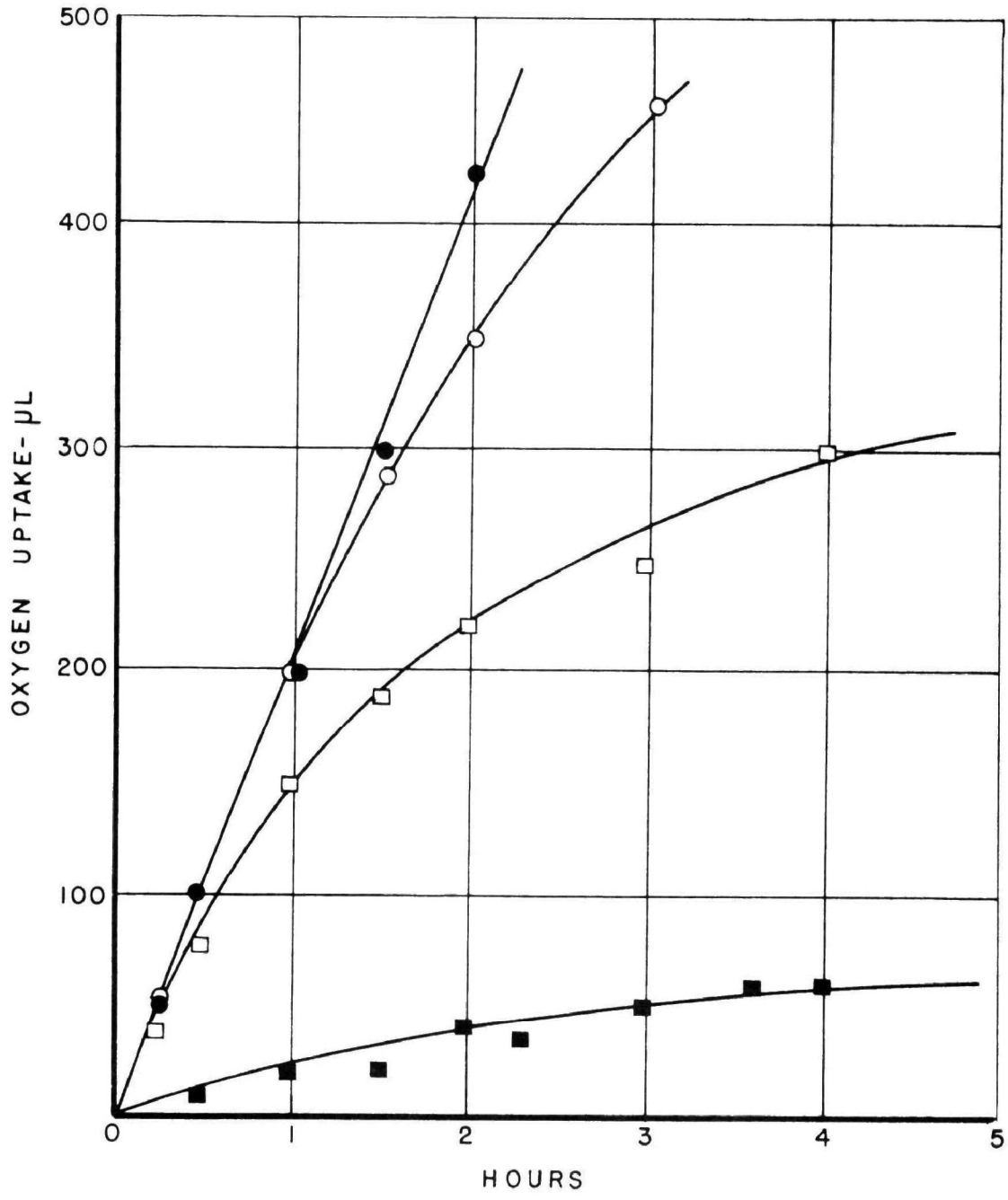


FIG.23 OXYGEN UPTAKE WITH PHENYLALANINE SUBSTRATE

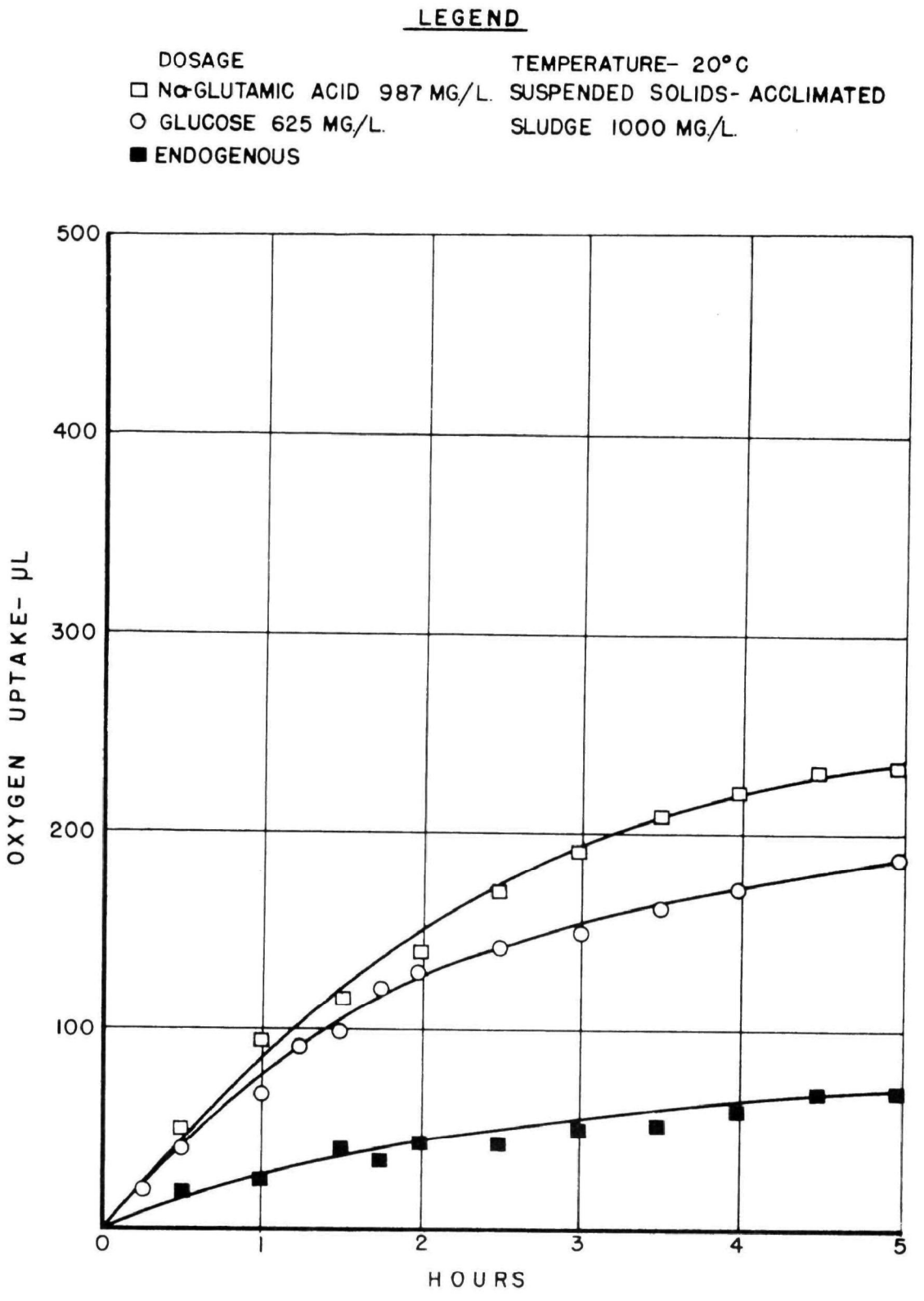


FIG.24 OXYGEN UPTAKE WITH Na-GLUTAMIC ACID AND GLUCOSE SUBSTRATES

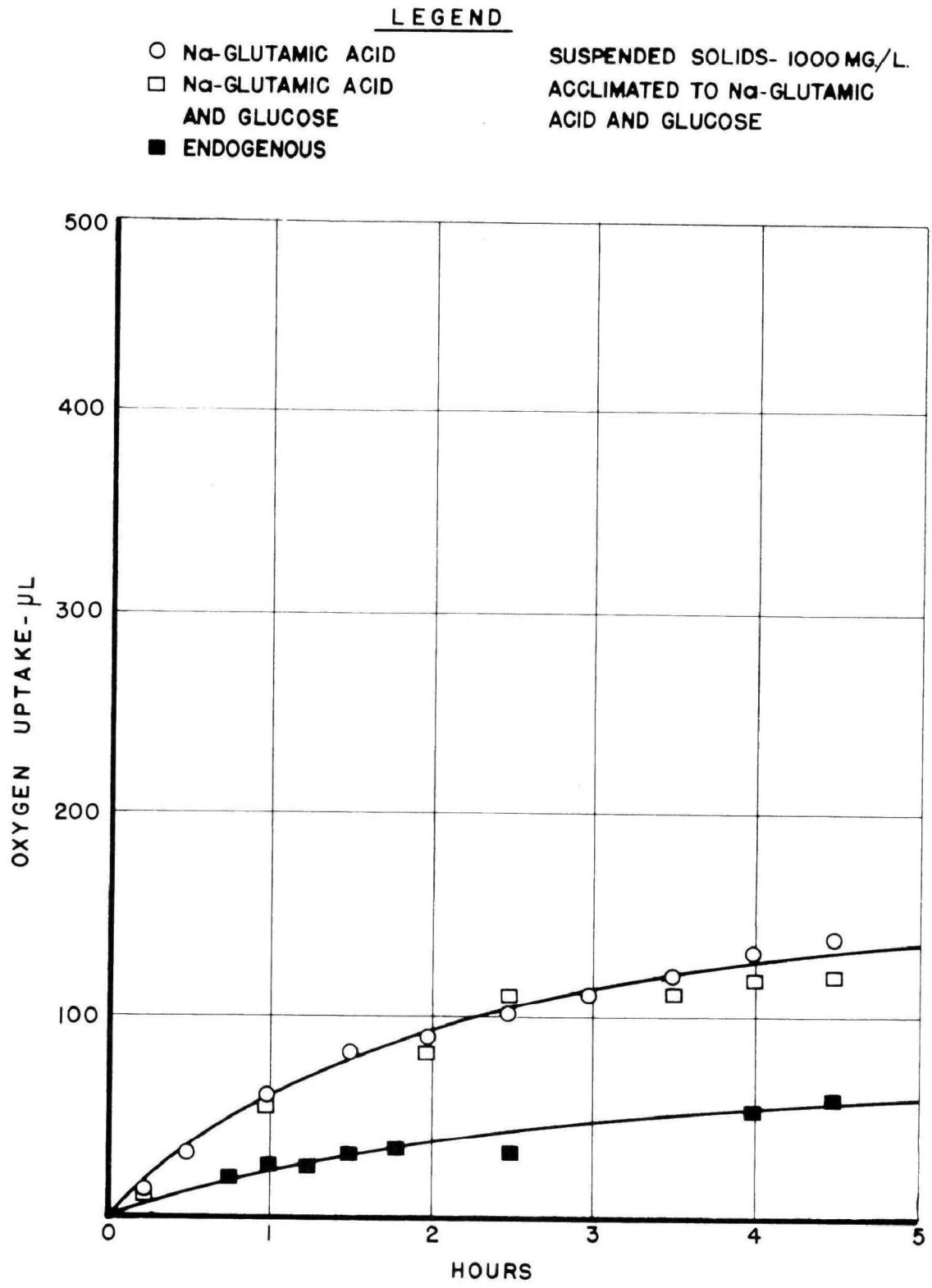


FIG.25 OXYGEN UPTAKE WITH GLUTAMIC ACID AND GLUCOSE

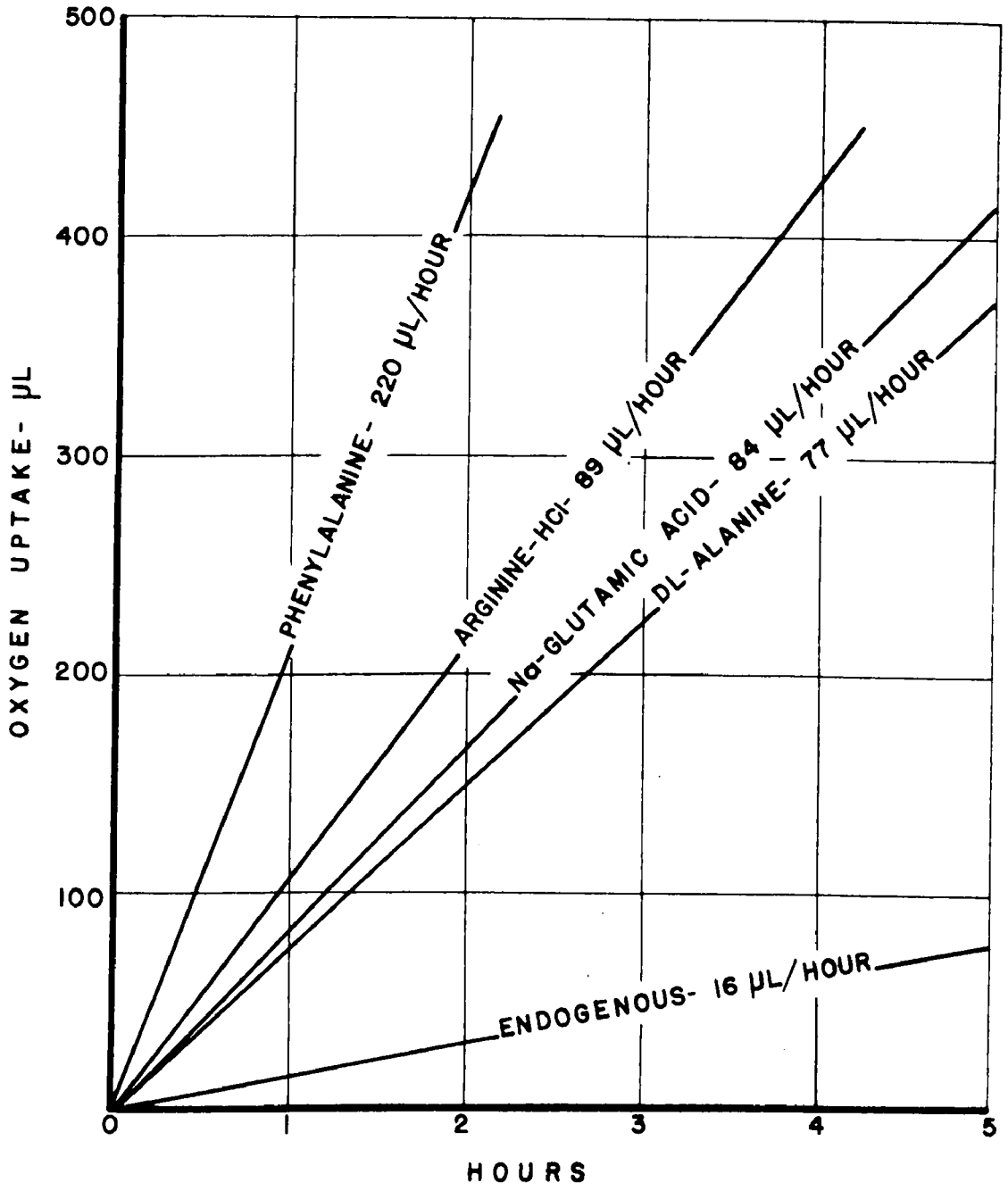


FIG.26 THE MAXIMUM OXYGEN UPTAKE RATE WITH AMINO ACID SUBSTRATES

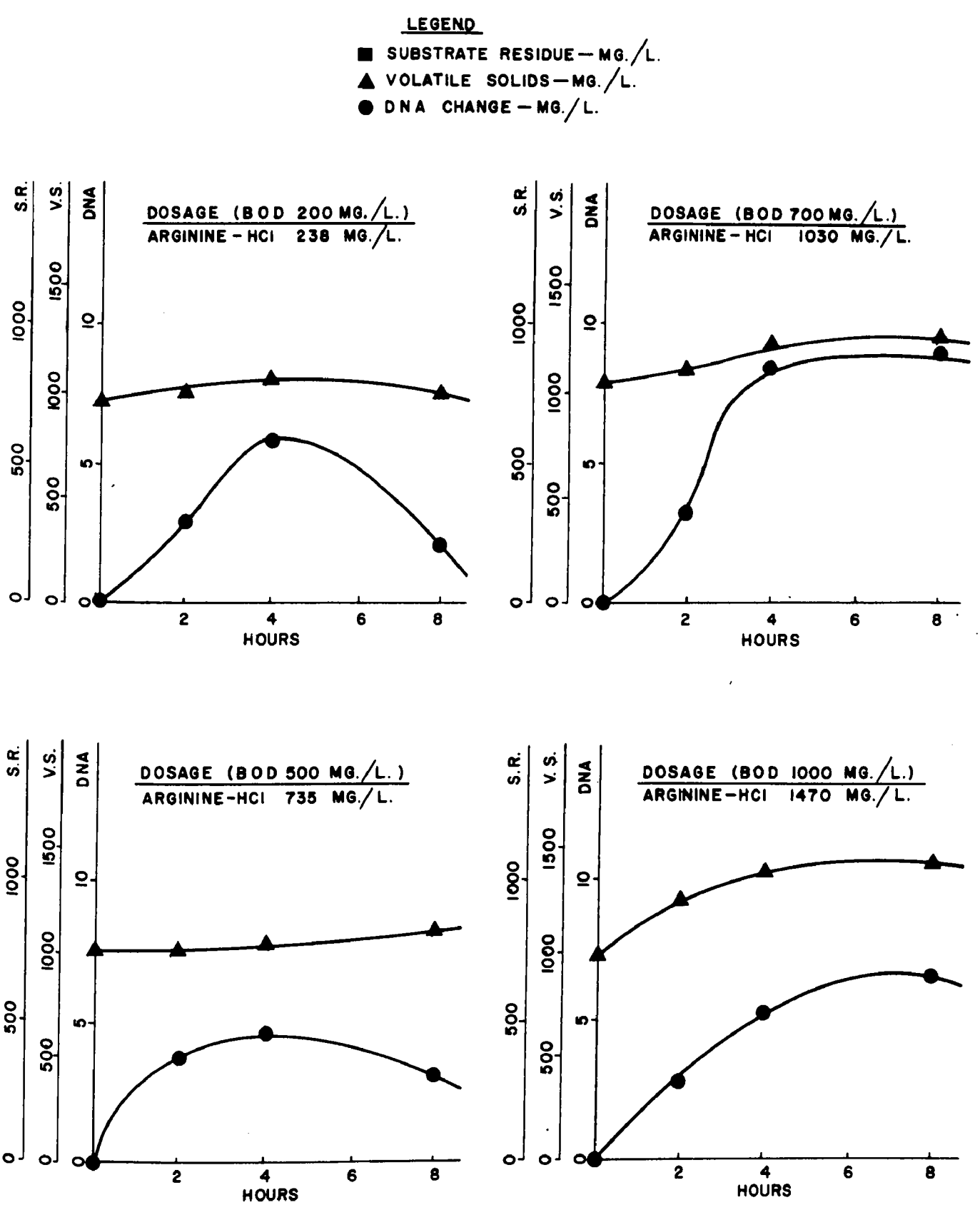


FIG.27 GROWTH STUDY—ARGININE-HCl SUBSTRATE

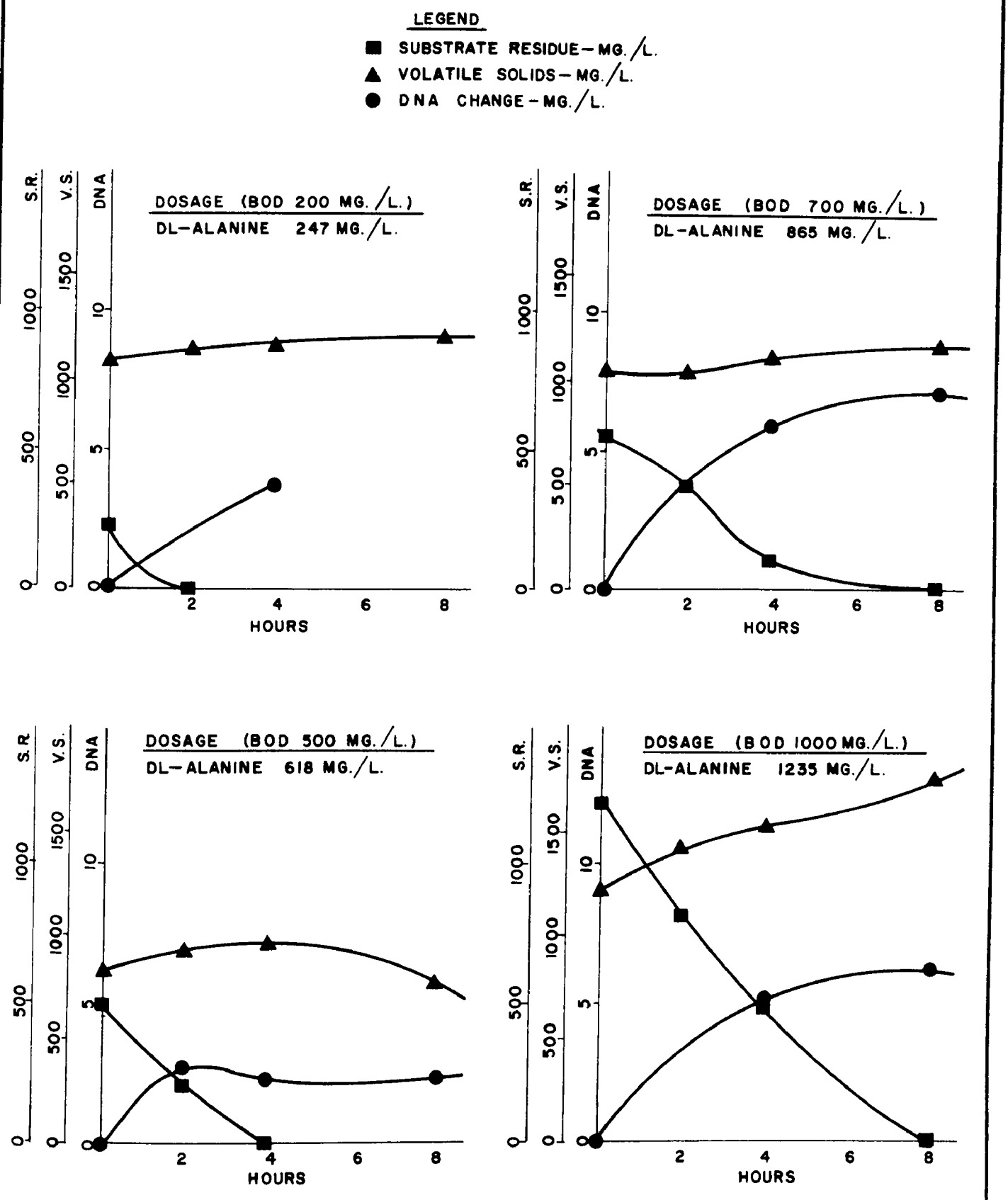


FIG. 28 GROWTH STUDY—DL-ALANINE SUBSTRATE

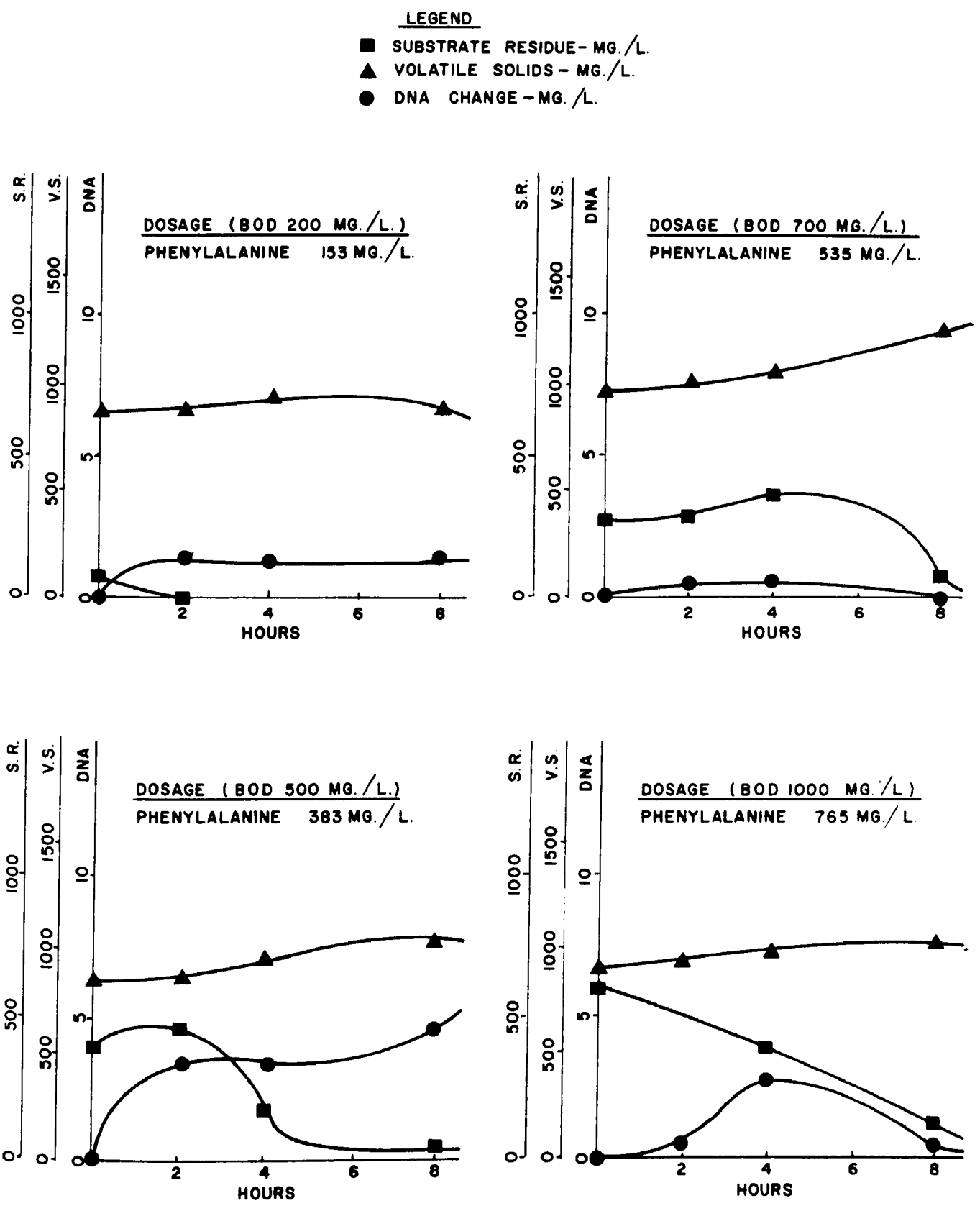


FIG. 29 GROWTH STUDY—PHENYLALANINE SUBSTRATE

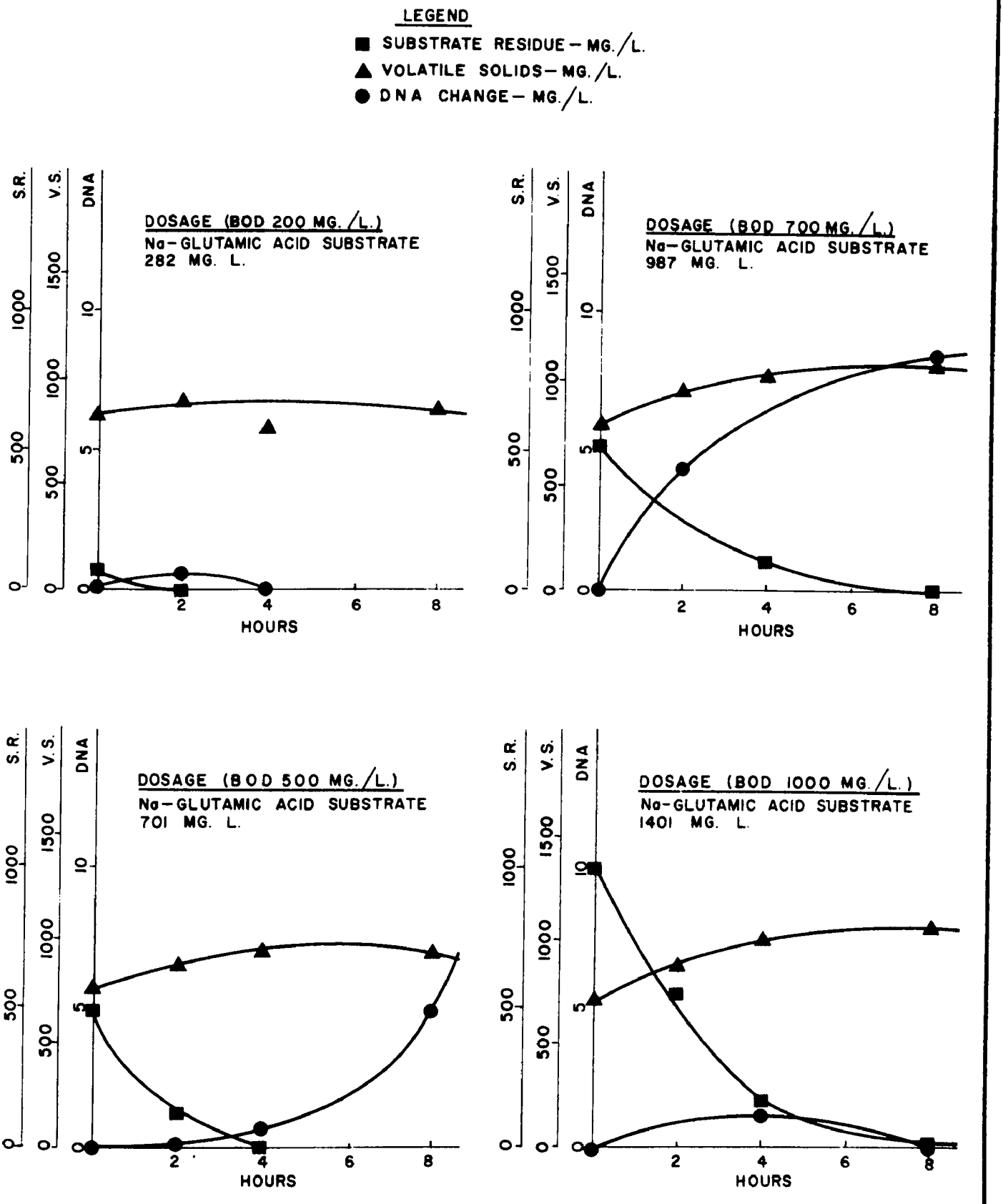


FIG.30 GROWTH STUDY—Na-GLUTAMIC ACID SUBSTRATE

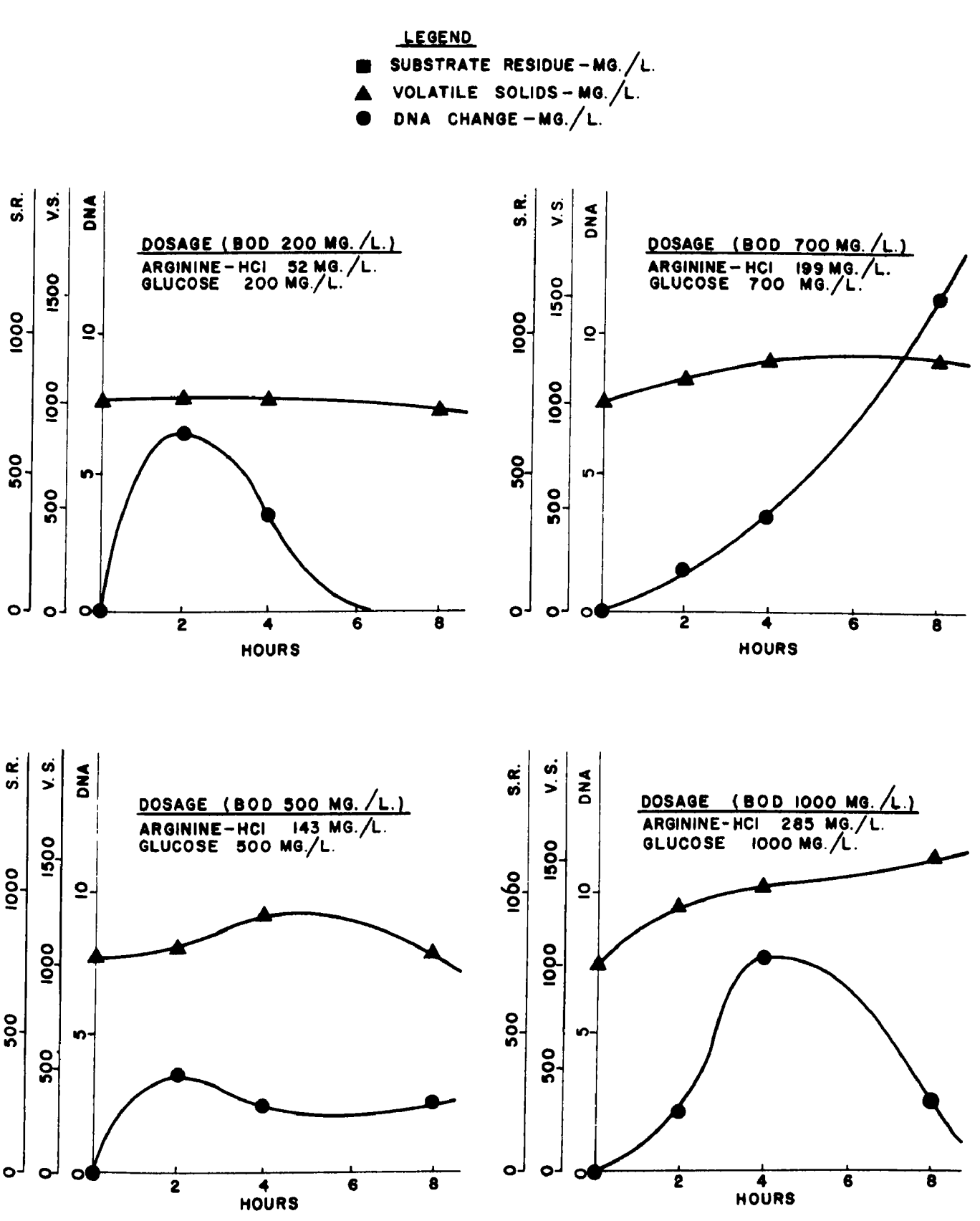


FIG. 31 GROWTH STUDY—ARGININE-HCl AND GLUCOSE COMBINED SUBSTRATE

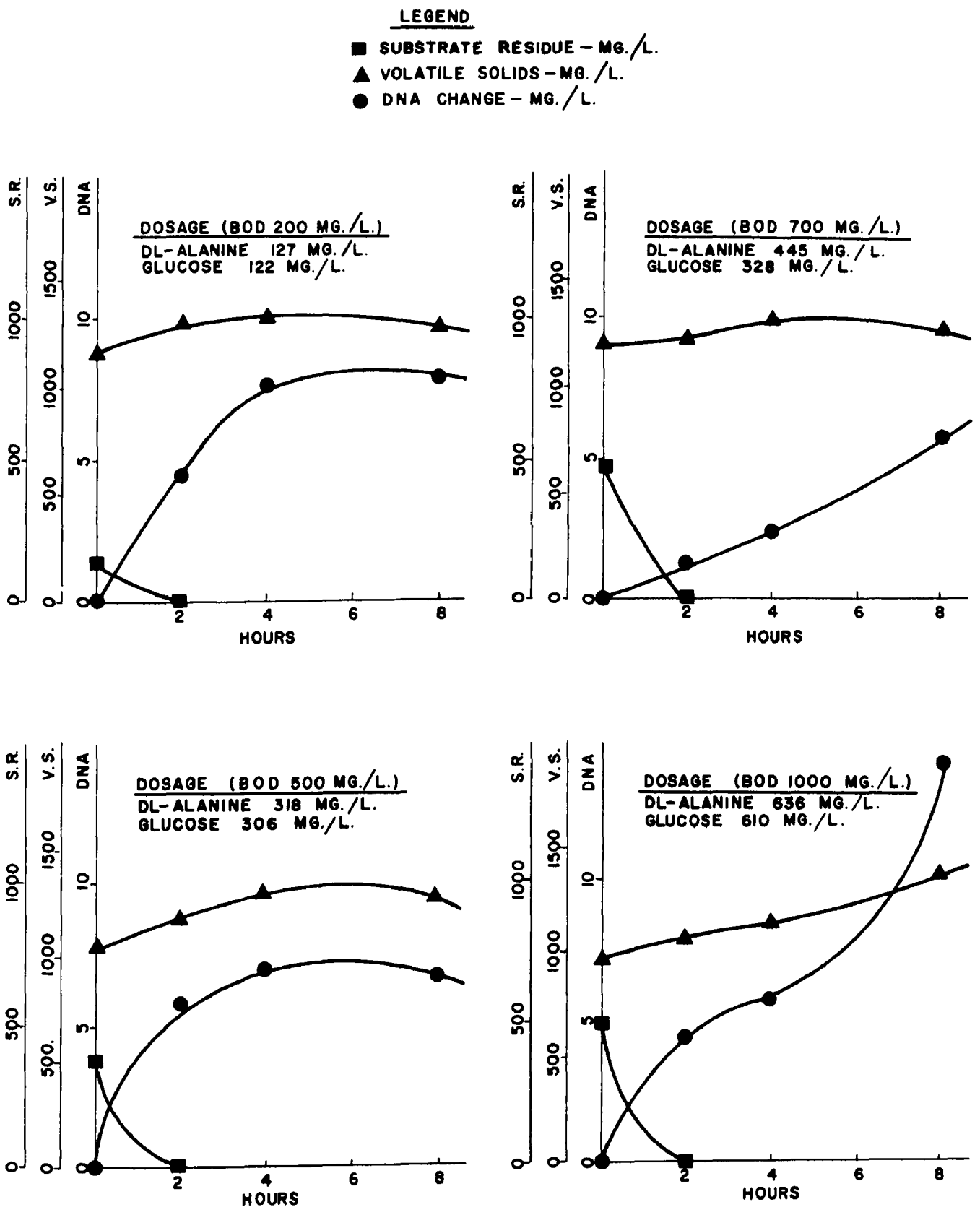


FIG. 32 GROWTH STUDY—DL-ALANINE AND GLUCOSE COMBINED SUBSTRATE

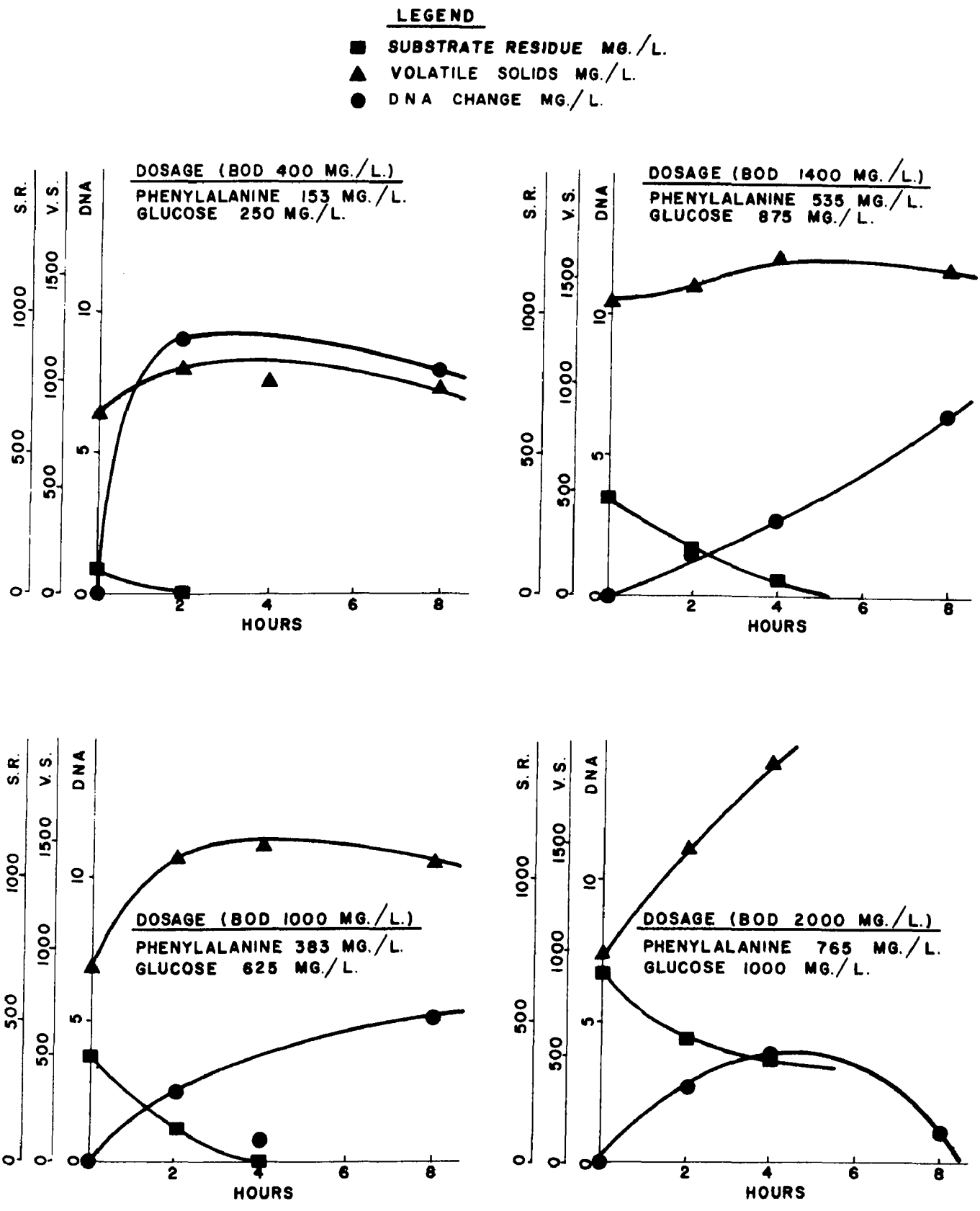


FIG. 33 GROWTH STUDY—PHENYLALANINE AND GLUCOSE COMBINED SUBSTRATE

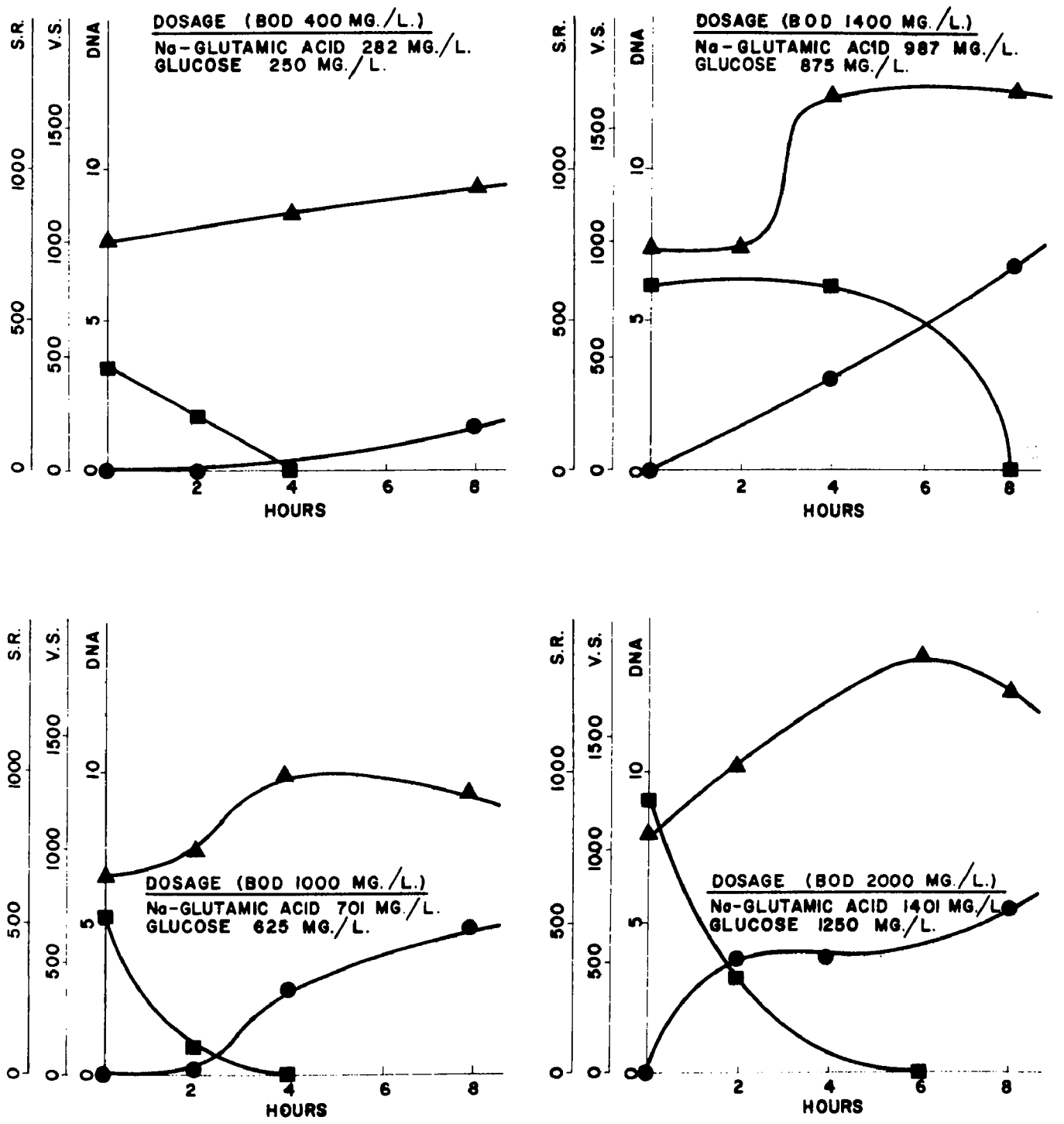


FIG. 34 GROWTH STUDY—Na-GLUTAMIC ACID AND GLUCOSE COMBINED SUBSTRATE

concentrations of substrate have been adjusted to carbon equivalents of 100, 250, 350 and 500 mg/l. For comparison, the three measured growth parameters, suspended volatile solids content, substrate residues, and change in DNA content are plotted on the same figure. As can be noted on the figures, the three measured parameters correlate with one another quite well. In general, increases in both volatile solids and DNA content are seen after the feeding of the substrate. In terms of DNA content, an increase is seen two hours after feeding for the four cultures fed amino acid substrates. However, the ultimate increase and the pattern of increase seem to be dictated by the concentration and type of substrate applied.

Increases in DNA content at eight hours after feeding of single amino acid substrates are shown in Figure 35. Note that there is a given concentration for each substrate at which maximum increase of DNA is obtained and above which DNA increase is depressed. For example, L - phenylalanine produces maximum DNA increase at 400 mg/l whereas L arginine - HCl, DL alanine and Na - L glutamic acid, as a group show the maximum DNA increase at a substrate concentration nearly 1000 mg/l. It is quite interesting to point out that alanine, arginine, and glutamic acid are aliphatic acids and phenylalanine is an aromatic acid. When the optimum concentration of 400 or 1000 mg/l is exceeded, decreases in DNA production are observed. This decrease is considered as due to the inhibitory effect of the high concentration of substrate.

In Figure 36, DNA increases are plotted against the nitrogen content of the substrate. This figure indicates that the DNA increase is related to the nitrogen content of the substrate; however, it is seen also that the addition of glucose to a single amino acid substrate promotes DNA production.

Growth has frequently been defined as an increase in suspended volatile solids content. Experimental results in this study as plotted in Figures 37 and 38 show that the increase in volatile solids correlate with an increase in substrate concentration rather than an increase in DNA content. The experimental evidence showed that in an unsteady-state situation of organic substrate, increase in volatile solids is mainly the result of the accumulation of organic compounds rather than an increase in active solids. This fact suggests that in an unsteady-state situation, volatile solids content is not an efficient growth parameter.

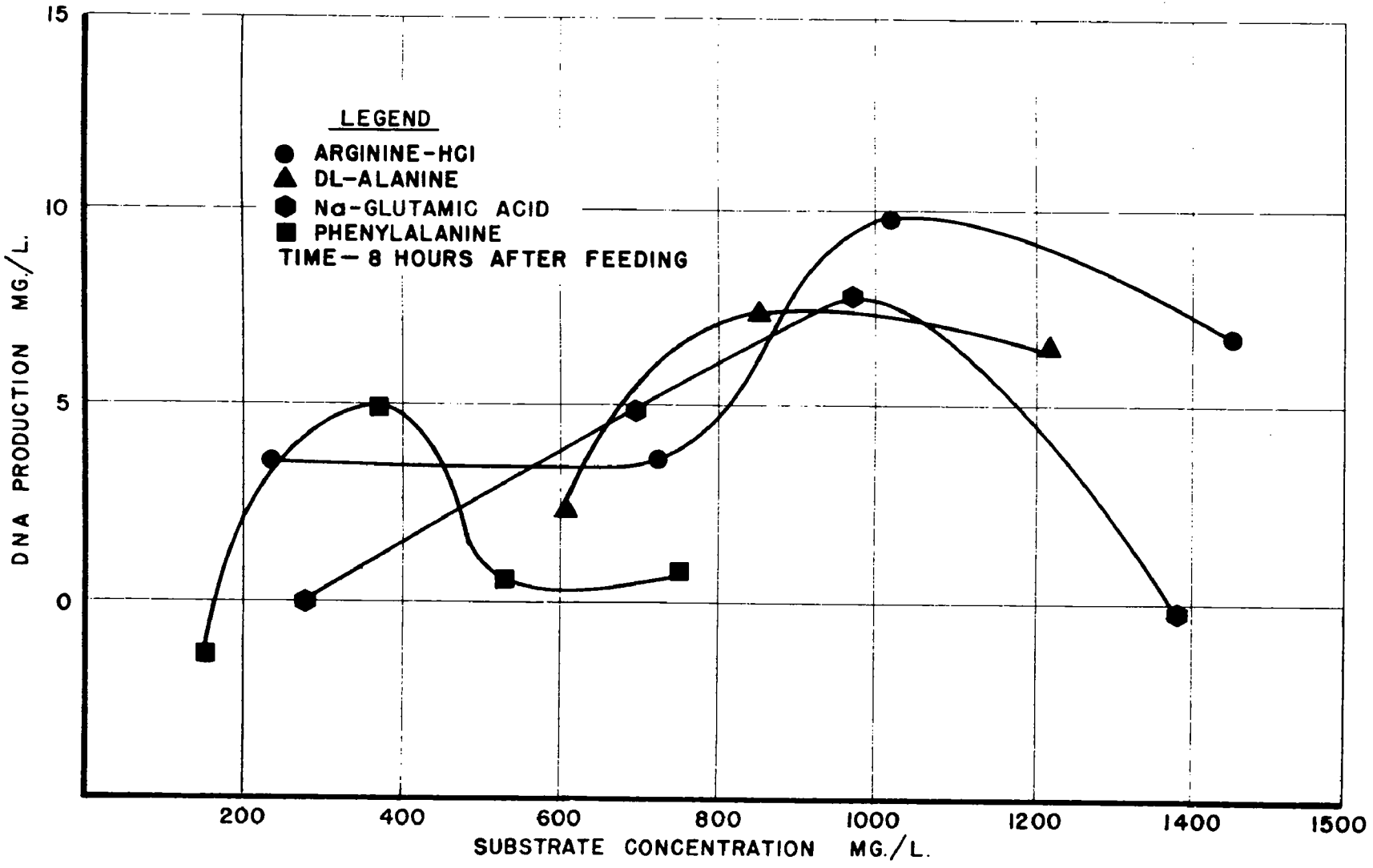
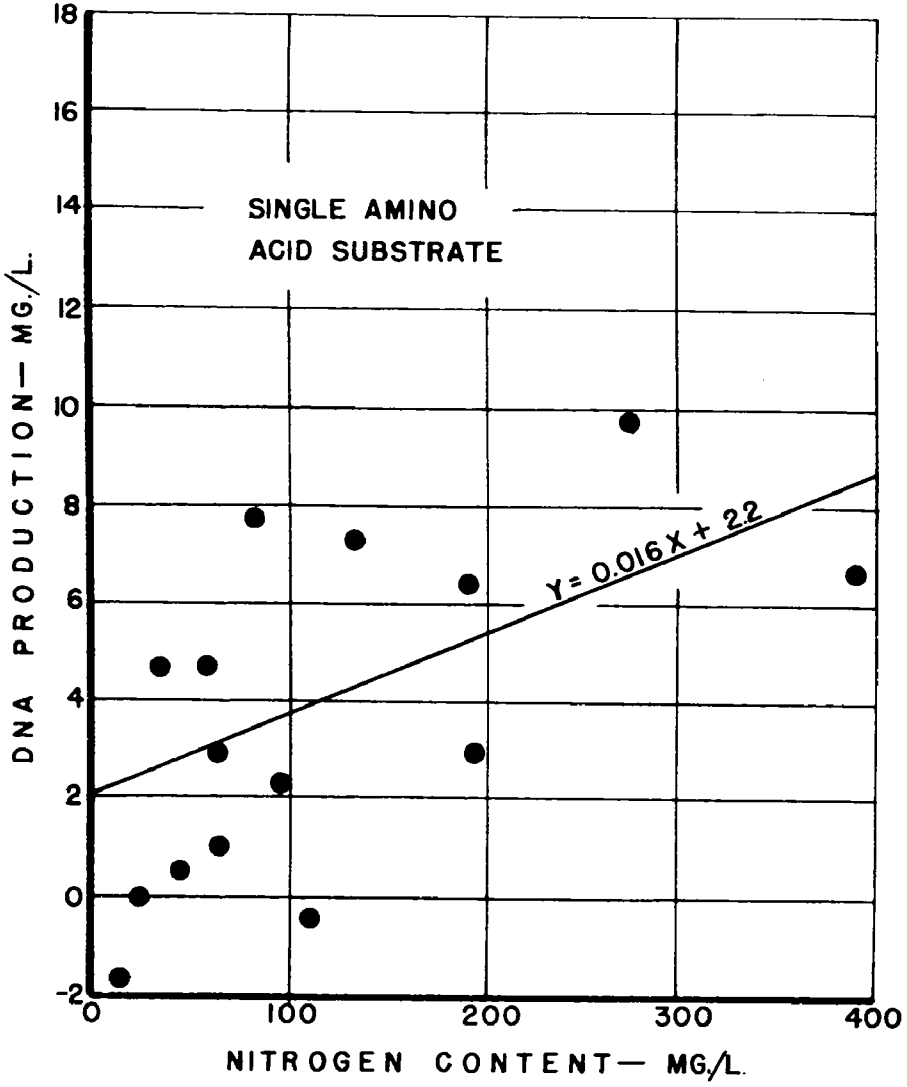


FIG. 35 DNA PRODUCTION VS. AMINO ACID SUBSTRATE CONCENTRATIONS



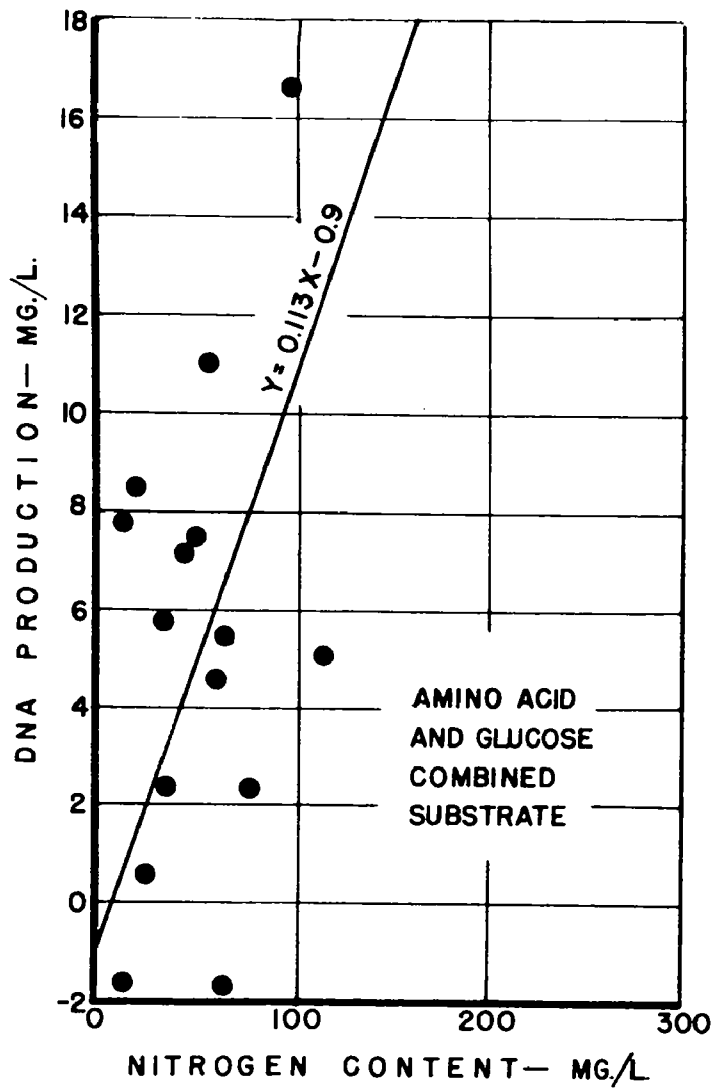


FIG. 36 DNA PRODUCTION VS. NITROGEN CONTENT OF SUBSTRATE

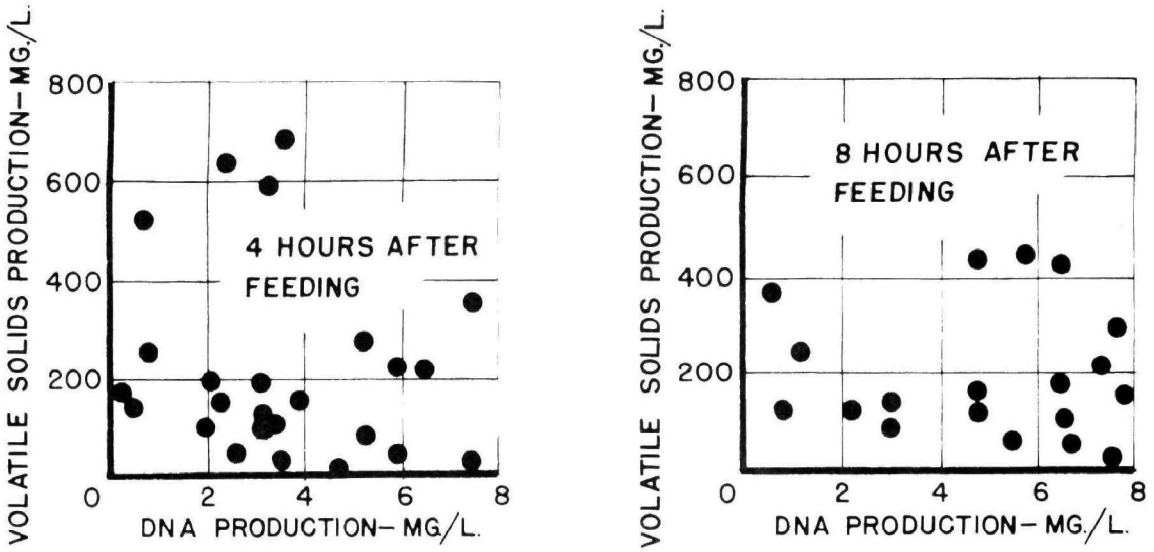


FIG. 37 VOLATILE SOLIDS PRODUCTION VS. DNA PRODUCTION

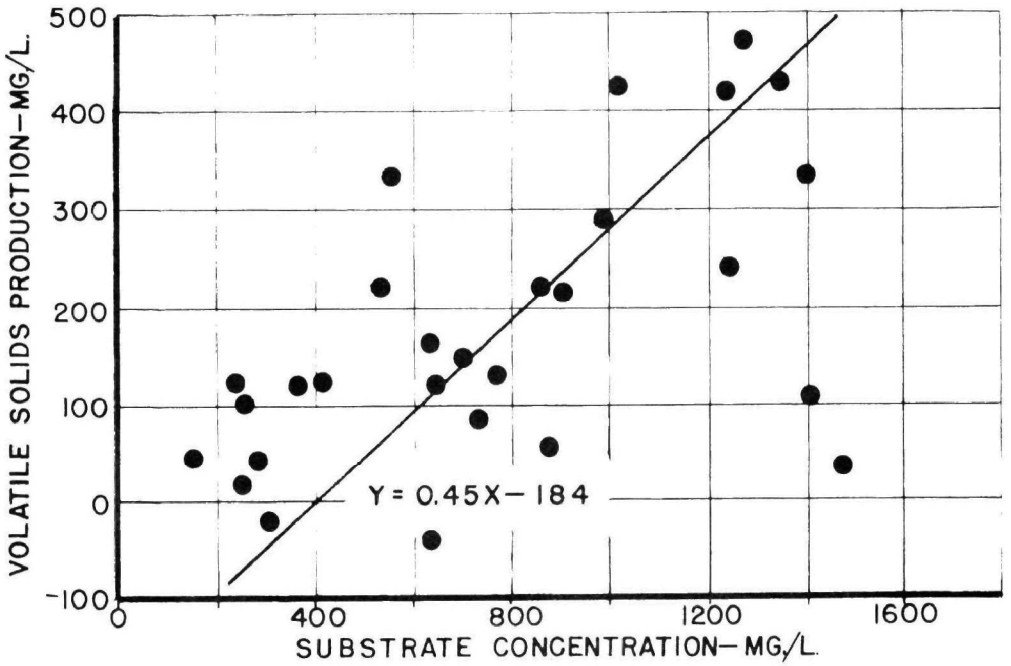


FIG. 38 VOLATILE SOLIDS PRODUCTION VS. SUBSTRATE CONCENTRATION

Field Studies on DNA Concentrations of Activated Sludge

The amount of DNA in activated sludge plants is of interest from the practical point of view because it represents the active portion of the sludge and could function as an index indicating the activity and potential purification ability of the sludge.

Activated sludge samples were collected in the Seattle Metropolitan Waste Treatment Plant, the Safeway Bellevue Milk Waste Treatment Plant and the Holiday Inn Waste Treatment Plant. The samples, after addition of perchloric acid to a final concentration of 0.25 N were brought back to the laboratory and immediately subjected to DNA and solids tests. The results of these field tests are compiled in Table 6 below.

TABLE 6: DNA CONTENT OF VARIOUS ACTIVATED SLUDGES

Samples	DNA (Average) mg/1000 mg Volatile Solids	Sludge Type
Lake City Municipal Waste Treatment Plant	8	Biosorption
Bellevue Milk Waste Treatment Plant	17	Extended Aeration
Holiday Inn Waste Treatment Plant	11	Extended Aeration
Arginine - HCl Cultures	13 - 23	Laboratory Cultivated
DL Alanine Cultures	12 - 37	Laboratory Cultivated
L - Phenylalanine Cultures	18 - 33	Laboratory Cultivated
Na - L - Glutamic Acid Cultures	23 - 32	Laboratory Cultivated

Laboratory sludge cultures grow in a more regulated environment than actual field cultures. Thus, as would be expected, the average DNA content in laboratory cultures is higher than in cultures from treatment plant installations. The laboratory saturation concentration of DNA appears to be about 37 mg per 1000 mg of suspended volatile solids.

Effects of Toxic Compounds on DNA Production

Several methods have been used to evaluate the acitivity of activated sludge

processes. Among them the use of activated sludge oxygen requirements is perhaps the most widely used method. In general, the purpose of these methods is to determine the rate of metabolism of the culture. Because the metabolism of any organism is directed primarily toward assuring its growth and reproduction, DNA tests could be a direct method to evaluate the activity of a culture.

The validity of this concept was tested experimentally by noting the effect of bacteriocides. Copper as copper sulfate and cyanide as sodium cyanide were used as the bacteriocidal agents in this study.

An examination of the literature on the toxic effects of bacteriocides on activated sludge provides rather wide and diverse information on the toxic effects of various substances on waste treatment. From laboratory experience, Oeming et al (34) reported that 0.5 mg/l $CuSO_4$ as copper was toxic to all microorganisms and 0.1 mg/l had a toxic effect on most bacteria. In addition, they concluded that waste water must not contain more than 0.1 mg/l of copper if the water is to be treated biologically. Working with activated sludge, Ridenour (35) observed a decrease in nitrate formation and ammonia reduction as a result of 2 to 3 mg/l of cyanide in the form of NaCN. He stated, however, that at no time was nitrification completely disrupted even when an amount as high as 40 mg/l was added. Oeming (34) and Siebert (36) found that cyanides from metal plating wastes were toxic to biological activity in concentrations as low as 1.0 mg/l. According to Ridenour et al (35) sludge has a tolerance to 3 mg/l of Na CN when first introduced, but that a build-up in tolerance was noticed after a few days until a large daily increment could be absorbed.

The critical limits of toxicity set by the different investigators cited above clearly show the wide range of diversity in the results shown. These conflicting reports, according to Moulton et al (37) can be explained by the fact that each investigator used different test parameters, experimental conditions, and experimental units and methods.

In viewing these reported facts, oxygen uptake tests were carried out with a Warburg Respirometer as screening tests to determine critical limits of two bacteriocides. In Figures 39 and 40, the oxygen uptake curves for $CuSO_4$ and Na CN slugs are shown. In the case of $CuSO_4$, the inhibitory effect became clear at a concentration of 5 mg/l. Between 5 to 10 mg/l, the inhibitory effects remained about the same. Exceeding this limit, an evident lowering in the maximum oxygen uptake rate is observed. In the case of sodium cyanide, no clear effects

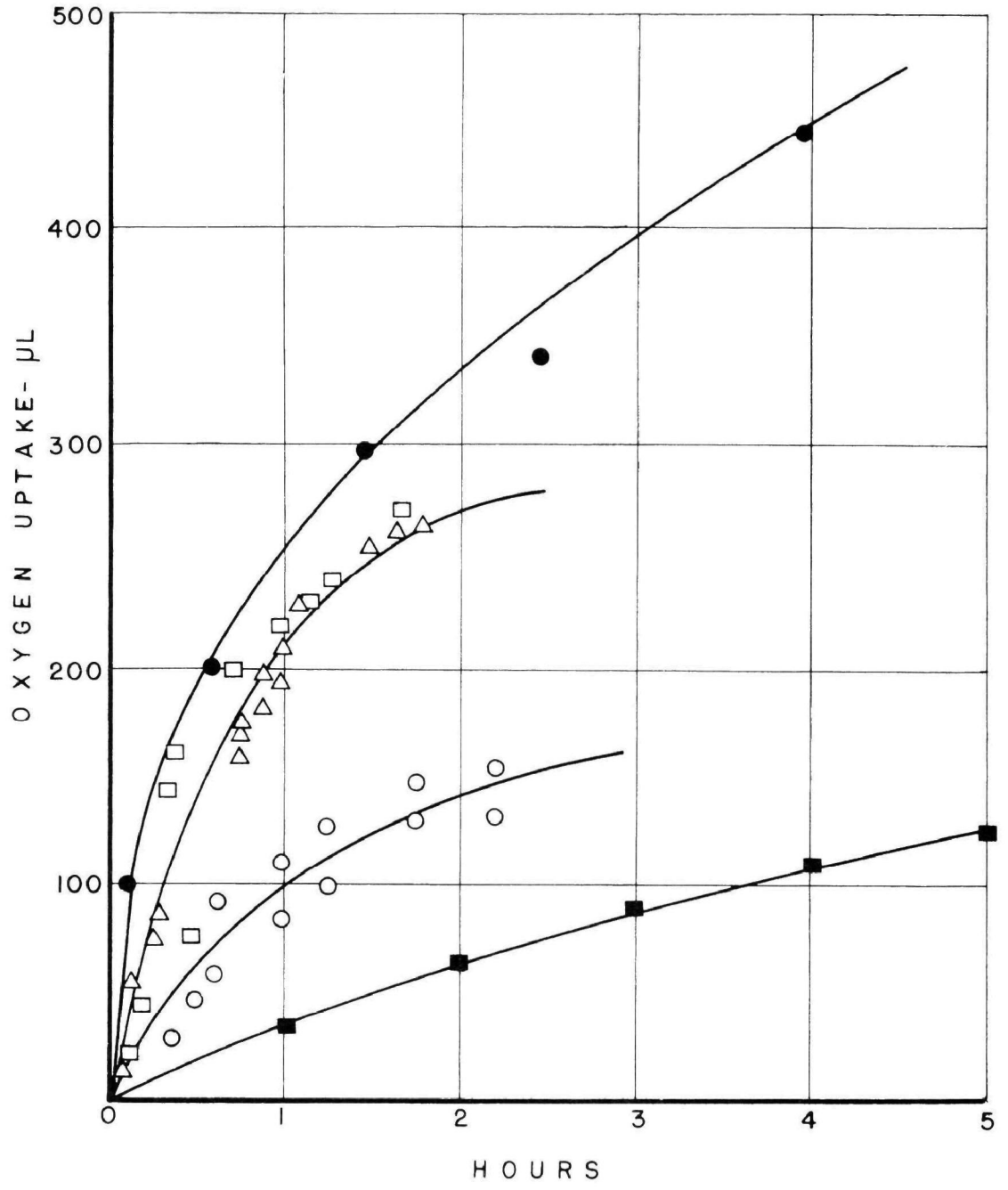


FIG. 39 OXYGEN UPTAKE TOXICITY TEST WITH $CuSO_4$
DL-ALANINE SUBSTRATE- 618 MG/L

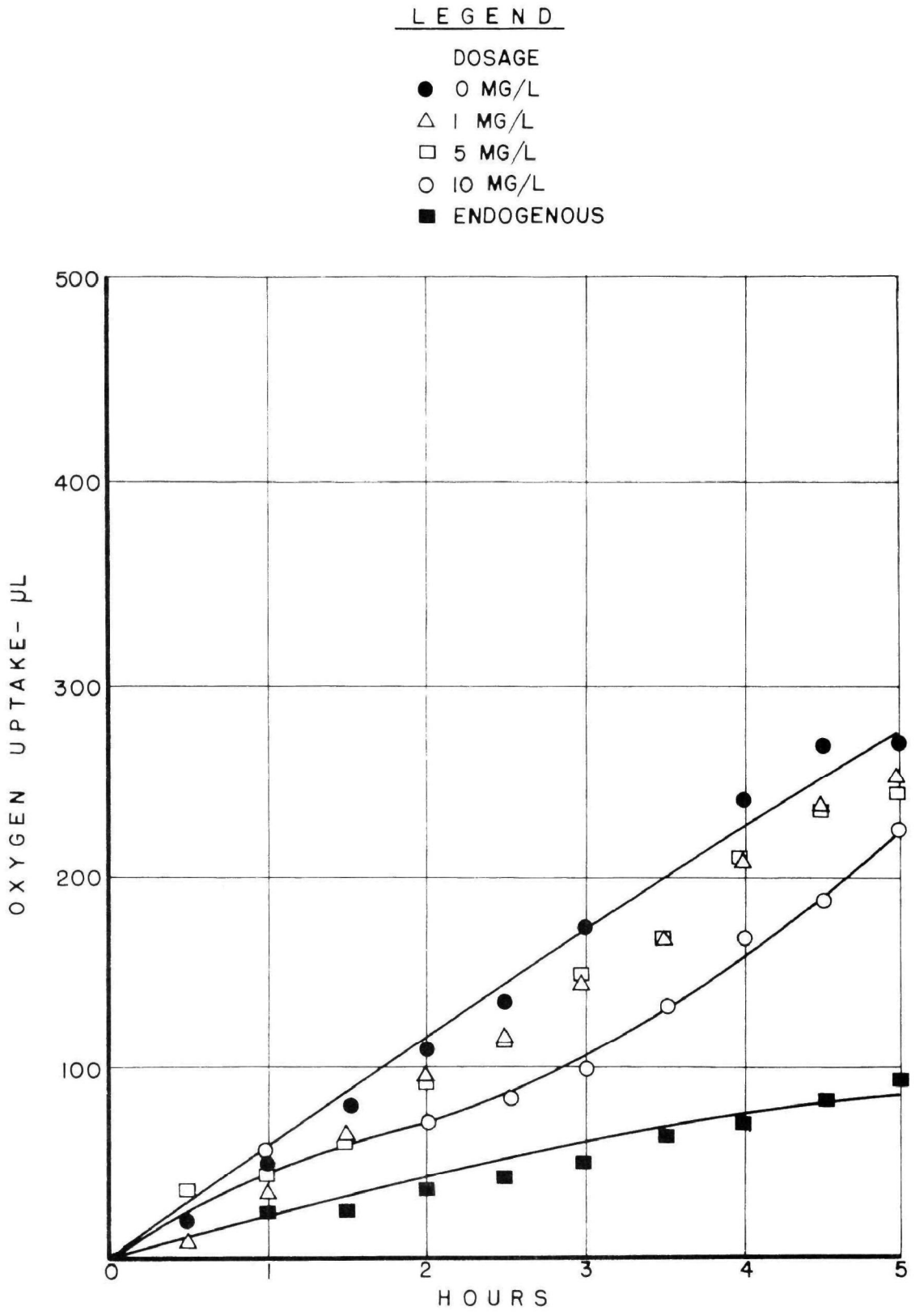


FIG.40 OXYGEN UPTAKE TOXICITY TEST WITH NaCN
ARGININE-HCl SUBSTRATE- 1000 MG/L

are detectable in the dosage range of 1 to 5 mg/l. The critical limit in this case is found at 10 mg/l.

With this background information on the toxic effects on activated sludge, growth studies were initiated. Figure 41 shows the changes of DNA content at different $CuSO_4$ levels and in Figure 42 the results are shown using NaCN as the bacteriocide. From these figures, two varied toxic effects on activated sludge cultures can be shown. The shock dosage of $CuSO_4$ seems to affect respiratory activity of the culture, whereas NaCN appears to inhibit the reproduction activity directly.

Discussion

The purpose of this portion of the study was to develop a valid and efficient test procedure for the determination of growth and activity of activated sludge.

The composition of activated sludge suggests that it can be divided into active and inactive portions. The absorptive power of activated sludge floc surface and successive biochemical transformations of absorbed organic materials through microbial activities are responsible stabilizing processes for the treatment of wastes. The bacteria and protozoa of the floc are the responsible agents for biological waste treatment and, thus, the overall efficiency or treatment capabilities of an activated sludge process could be evaluated from the size of the active microbial population responsible for treatment.

The content of active solids or microorganisms in activated sludge and their pattern of growth are significant because they serve as an index to the potential treatment capabilities of the sludge and, as well, as an index to the biodegradability of a given waste.

Traditionally, the fraction of active solids in activated sludge has been indicated by the volatile suspended solids content and growth by increases in the volatile suspended solids. Volatile solids are actually a mixture of microorganisms and non-living organics. In a steady state condition, where the composition and concentration of organic waste do not fluctuate greatly, a constant relationship could exist for a period of time between active and volatile solids. However, in unsteady state, where composition and concentration of waste vary greatly, the volatile solids fraction of the sludge will vary greatly so that volatile solids cannot be assumed to hold any specific relationship to active

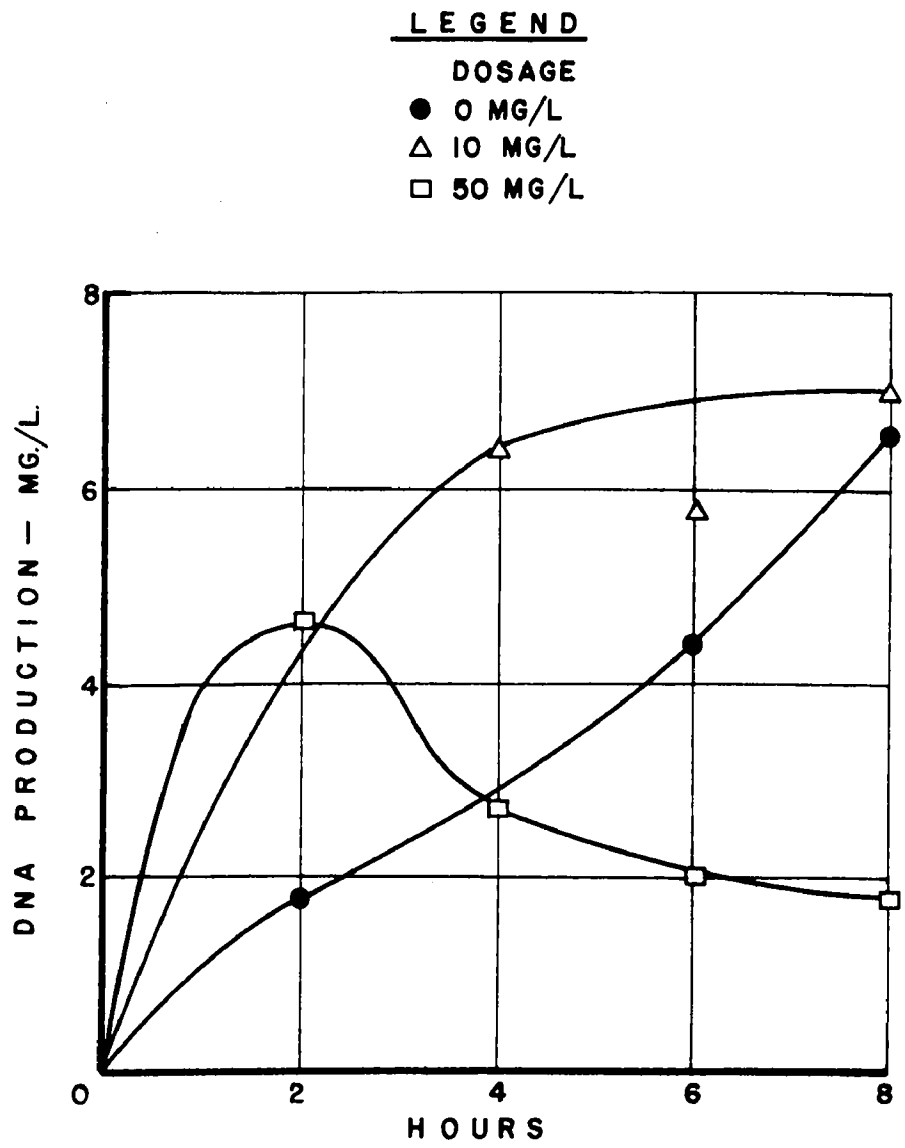


FIG. 41 GROWTH STUDY—TOXICITY TEST WITH $CuSO_4$

DL-ALANINE SUBSTRATE— 618 MG/L

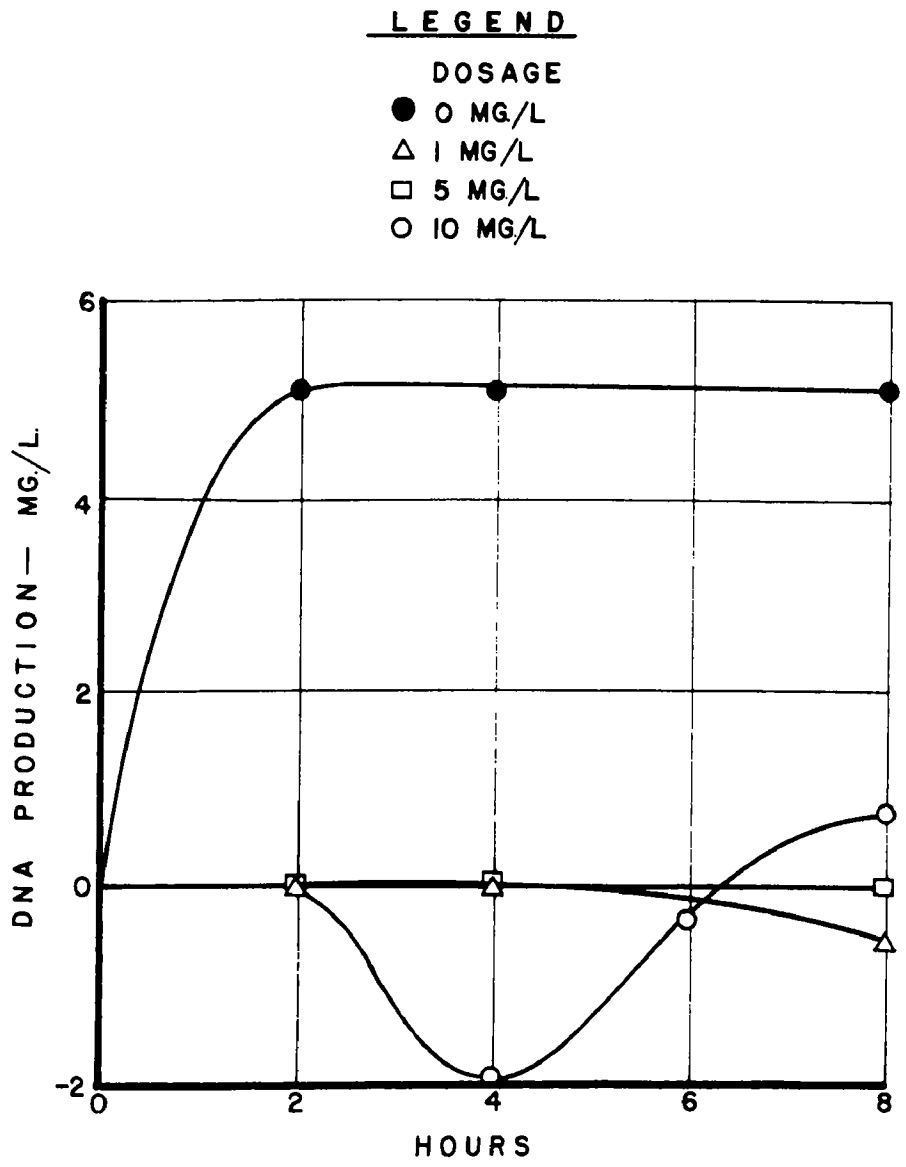


FIG. 42 GROWTH STUDY—TOXICITY TEST WITH NaCN
ARGININE-HCl SUBSTRATE—1000 MG/L

microorganisms. Many investigators have realized this problem in attempting to relate volatile solids to microbial populations. For this reason a new parameter was needed to indicate populations of microorganisms.

In the biological sense, growth is an orderly increase of all the components of an organism. In all cellular organisms, cell multiplication is a consequence of growth. The definition of growth thus suggests one of the direct measurements of growth in a biological system could be based on the assay of one of the fundamental cellular materials shared by all cells. Recent studies in biology have indicated that deoxyribonucleic acid is one of the fundamental and invariable components of living cells. The characteristics of DNA, i.e., as the invariable component of cells and the fact that the amount of DNA per nucleus is constant, bear practical significance because the assay of DNA content in a biological system directly reflects the population size and can be a growth parameter.

The determination of DNA on microbial cultures requires two procedures, i.e., isolation of DNA in the pure state and then the qualitative and quantitative determination of DNA. Because microorganisms vary greatly in the ease of cell wall lysis and in their content of capsular polysaccharides which interfere with DNA determination, there was no procedure available for the isolation of DNA from a diverse group of microorganisms. In this study, Schneider's procedure of DNA isolation was adapted with a slight modification because the method has been applied successfully to several typical activated sludge bacteria such as Escherichia coli and Staphylococcus albus. The simplicity of the procedure and equipment involved, and the successive steps of extraction possible on the same sample are advantages of this procedure.

As for the method of DNA determination, the diphenylamine reaction discovered by Dische in 1933 and modified by Burton (19) in 1955 was adopted. Burton stated his modified method is more sensitive and specific than the original method, and less susceptible to interferences by other compounds. He concluded that approximately 700 mg/l of each of the following substances gave no detectable color development: sucrose, glucose, inositol, ascorbic acid, bovine plasma, glutathione, cystine hydrochloride, tryptophan, glycine, histidine hydrochloride, potassium gluconate, adenine sulfate, uric acid, adenosine-5' phosphate, creatine hydrate and chloral hydrate. The low susceptivity of this method to diverse groups of organic compounds presents advantages if it is to apply in activated sludge cultures where varied groups of organics are present.

Although voluminous studies are available in the fields of biochemistry and microbiology pertaining to the DNA determination on pure cultures, no account is available for the DNA determination on mixed aerobic cultures. The first phase in this study was therefore devoted to the applicability of Schneider and Burton's method to activated sludge cultures. As shown in Figure 43, the absorption spectra of the DNA standard solution and the solution extracted from activated sludge using Schneider's method shows identical absorptive characteristics.

The oxygen uptake tests using four amino acids as sole substrate for acclimated activated sludge cultures all resulted in increases in the maximum oxygen uptake rate. These increases due to the feeding of substrate suggested that the four substrates used in this study were all biodegradable. The growth resulting from the biological oxidation of the substrate was then evaluated by the increase in DNA content.

From the results several items could be cited. They are:

1) DNA increases were seen for all four amino acid substrates.
2) The extent and duration of DNA increase, or in other words, growth phase, depends on the types of amino acids and concentration of the feeding.
3) Although an increase in DNA content and hence growth was seen for a sole amino acid substrate, the presence of glucose in addition to the amino acid accelerated the growth.

Furthermore, from Figure 35, it is seen that there are two optimum dosages for the varied amino acids to give the maximum DNA increase. On the basis of chemical structure, the aliphatic amino acids, arginine-HCl, DL-alanine, Na-glutamic acid, as a group show a peak at a certain dosage; and phenylalanine, an aromatic acid, shows a different peak at another dosage. Mills and Stack (38) and other investigators (39,40) found that biological oxidation of pure compounds was related to the chain structure, molecular size, and the functional group present in the molecule. Figure 35 also implied that the aliphatic group is more amenable to biodegradation than the aromatic group.

The DNA test as an activity index of activated sludge was tested by means of artificial slugging of bacteriocidal agents, $CuSO_4$ and NaCN.

Copper and cyanide are classified as enzyme inhibitors that damage the cell by inhibiting the action of its enzymes. Copper and other heavy metals combine with the sulfhydryl (SH) groups of important cell components. Since the SH groups

SAMPLE- DNA , ACTIVATED SLUDGE EXTRACT
ORIGIN - DNA STD. SOLUTION AND ACTIVATED SLUDGE
SOLVENT - $HClO_4$ + REAGENT
SPECTROPHOTOMETER - PERKIN-ELMER
CELL PATH - 10 MM.

REFERENCE - BLANK , $HClO_4$ + REAGENT
SCAN SPEED - RAPID
SPLIT - 25
OPERATOR - F.L.
WAVELENGTH - VISIBLE RANGE

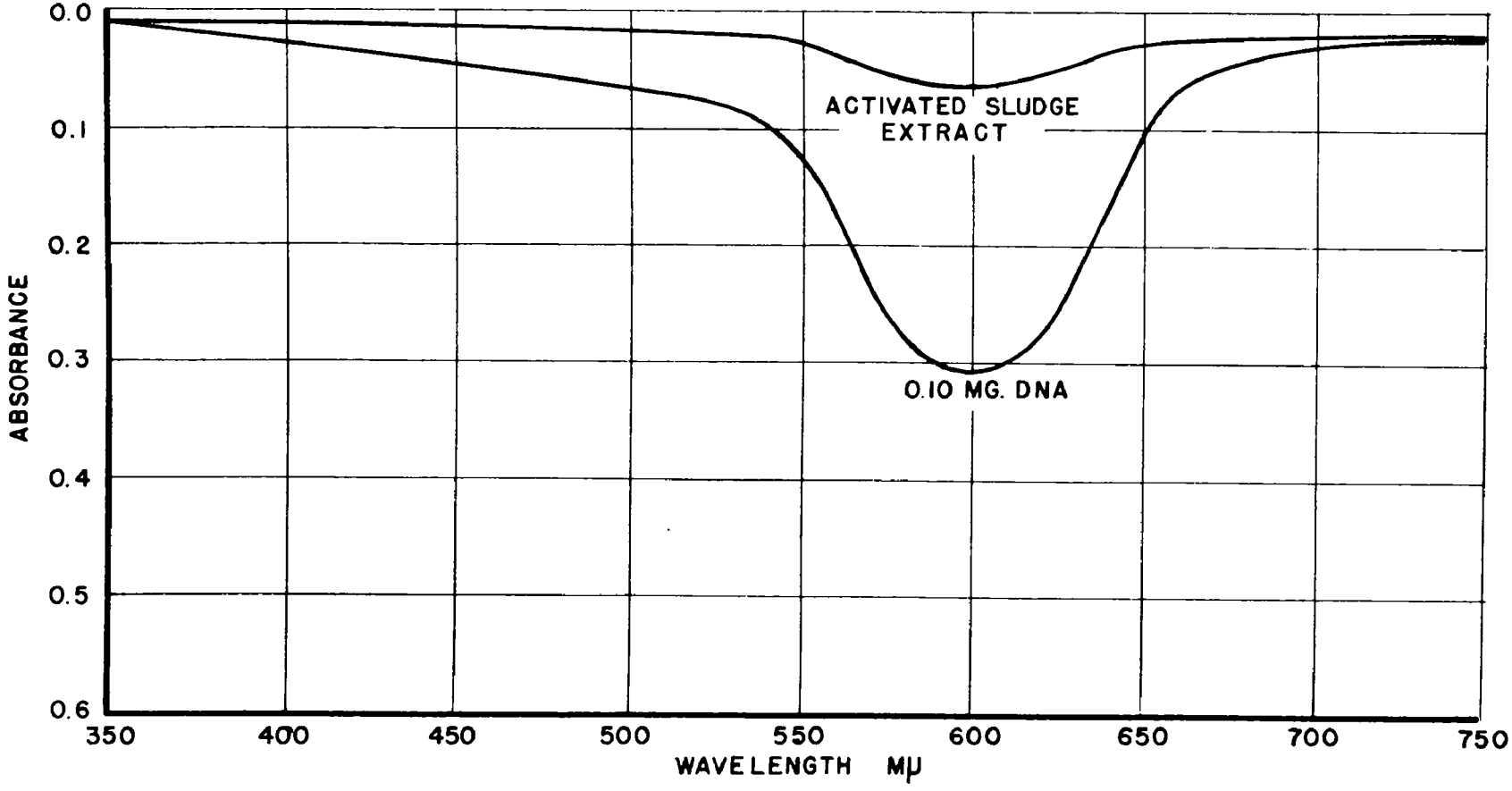


FIG. 43 ABSORPTIVE SPECTRA OF DNA STANDARD AND ACTIVATED SLUDGE EXTRACT

of many enzymes play an important role in catalysis, the effect of heavy metals is to cause a general dearrangement of metabolism. The toxic effect of copper, although it is very powerful, can, within limits, be reversed by the addition of chelating agents such as proteins and amino acids. A more specific group of enzyme inhibitors are compounds such as cyanide, which owe their toxic effects to their ability to combine with and inactivate the terminal cytochrome oxidase of aerobic organisms (15).

Thus, according to their mechanisms of toxic action, copper was expected to block the general metabolic activity and cyanide the respiratory activity of activated sludge cultures. Within the range of tested concentrations, the differences in their theoretical toxic action were not evident in this study. In the case of $CuSO_4$, the critical limits of the toxic effect appeared at about 50 mg/1, which was almost fifty times as much as that quoted in the literature. This high critical limit was believed to be caused by the chelating effect of amino acids which were used as substrates in this study. Although the inhibitory effect on respiratory activity of activated cultures was not evident at the tested concentration range of sodium cyanide, the inhibitory effect of DNA production or growth was evident, as shown in Figure 42.

Summary on Use of the DNA Test

Having applied the Schneider and Burton methods of DNA isolation and determination to activated sludge cultures and having evaluated the DNA content of the culture as a growth and activity parameter, the following conclusions were established.

1) The required steps to isolate all the detectable DNA from an activated sludge culture depend on the solids content of the sample. In a sample of 10 ml with a suspended solids content of 1000 mg/1, three serial steps of DNA extraction by perchloric acid give nearly complete extraction of detectable DNA from the sample (c.f. Table 5).

2) Because of the highly polymerized state of DNA solutions, the workable concentration of DNA stock solution was found about 0.1 mg/ml. It is recommended that working standard solutions be prepared from this stock solution.

3) The absorption spectrum of the blue colored mixture of DNA and disphenylamine reagent showed the maximum absorbance at a wavelength of 600 millimicrons. The maximum sensitivity in spectrophotometry is, therefore, obtainable at this

wavelength (Figure 4).

4) The DNA standard solution obeys Beer's Law in the concentration range of 0.01 mg/7.5 ml to 0.10 mg/ml (1.3 to 13.3 mg/l). Experience showed that a sample size of 10 ml with a suspended solids content of 1000 mg/l gave a DNA content within this range (Figure 5).

5) With experience in running the DNA test procedures, the accuracy of the test was found to be $\pm$0.002 mg/10 ml sample for a 95% confidence interval.

UTILIZATION OF THE KERATIN HAIR-PROTEIN RESISTANT TO BIOLOGICAL DEGRADATION

All proteins share one common structural feature, their amino acid content. A special group of protective proteins, the keratins, have reached a degree of differentiation which confers a number of characteristics not present in other proteins. Among these special properties are their remarkable insolubility and resistance to biodegradation. Thus, these keratins present an interesting removal problem in waste streams from slaughter houses, tanneries, glue manufacturing operations, and meat packing plants where hair, feathers and epidermal layers are a significant part of the waste constituents. Keratins present a number of problems in waste treatment facilities ranging from the clogging of distribution devices to flotation in activated sludge tanks and buildings and interference with digester performance (41). These investigations have shown that with appropriate treatment these insoluble keratins may be transformed into a soluble material accessible to bacterial enzyme systems and, thus, to biological degradation.

This portion of the Amino Acid Study was concerned primarily with the degradation of resistant proteins and, specifically, with the bioutilization of hair especially as concerned with the pretreatment necessary for facilitating biological attack and with the factors affecting the biodegradation of keratins such as hair. Different methods for dissolving hair were investigated and microbiological behavior was analyzed when dissolved keratin was the sole carbon and energy source. The results obtained indicated that the methods developed provide a good, feasible means of utilizing keratins biologically.

The term, "keratin" is applicable to proteins which exhibit characteristics of insolubility in hot water, organic solvents, dilute bases and acids, and resistance to digestion by pepsin and trypsin. It could also be defined as a protein stabilized by disulfide linkages. This definition will be developed more

fully later.

Keratins are found in nature as an evolutionary development of epithelial tissue for protective body functions including acting as a water barrier. They assume, in mammals, the form of hair, wool, hoofs, horn and epidermis. In birds they appear, as well, as feathers, and, in reptiles, as scales.

As shown in Table 7, the chemical composition of keratin varies widely. Also, the molecular structure presents different arrangements in response to peculiar physiological functions. Two types of keratin have been described. This distinction between α and β keratin stems primarily from the diffraction pattern obtained by x-ray analysis. This technique yields information on the arrangement of atoms in the crystalline regions.

All mammalian tissues yield an α pattern. Feather keratin and reptile scales, on the other hand, yield a very distinct β pattern. In addition, mammalian keratin of the α type may assume the β configuration as a type of stereoisomer. In some cases, such as in the hair cuticle, an amorphous highly cross-linked keratin is found (42).

TABLE 7: PARTIAL COMPOSITION OF SOME KERATINS (42)

(grams of component from 100 grams of dry keratin)

Keratin Source Type	Hair Human Hard	Horn Cattle Hard	Feather Chicken Hard	Epidermis Human Soft
Sulfur	5.0 - 5.24	3.7 - 3.9	2.9	1.9
Nitrogen, Total	15.5 - 16.9	14.8 - 16.9	15.0 - 16.2	14.2 - 15.5
Glycine	4.1 - 4.2	9.6	7.2	6.0
Alanine	2.8	2.5	5.4	---
Glutamic Acid	13.6 - 14.2	13.8	9.0 - 9.7	9.1
Arginine	8.9 - 10.8	6.8 - 10.7	6.5 - 7.5	5.9 - 11.7
Cystine	16.6 - 18.0	10.5 - 15.7	6.8 - 8.2	2.3 - 3.8
Methionine	0.7 - 1.0	0.5 - 2.2	0.4 - 0.5	1.0 - 2.5

"Keratinization may be considered as a specific form of cell differentiation in which metabolically highly active epithelial cells pass through cytomorphic and physiologic changes while they reach the terminal stage and become filled with a resistant and considerably insoluble horny material". (43)

The horny cells resulting from the keratinization process may be of a single

type or a complex type depending on the character of the germinative cells. Germinative cells of some epithelia such as epidermis, nail, claw, hoof, horn, beak or keratinized tooth, give rise only to a single type of specialized cell and eventually to a single type of structure. Germinative cells from hair, wool, quills and feathers give rise to different specialized cell types which eventually produce complex structures of horny cells.

Certain epithelial cells produce exclusively cytoplasmic granules of an amorphous material, others produce fibrils or both fibrils and granules. Cells producing cytoplasmic granules are usually responsible for the formation of amorphous keratin. A good example is the cell of the cuticular line of the hair. The cells producing cytoplasmic fibrils are usually responsible for the formation of fibrous keratin as found in the cortical line of the hair. A third type cell producing both cytoplasmic fibrils and granules is characteristic of the epidermis (43).

Formation of Keratin

Biochemically, the production of the terminal keratin, either amorphous or fibrous, proceeds in the same fashion as in the synthesis of other proteins.

The mitochondrion is the organ of respiration in the cell with the citric acid cycle enzymes located in the matrix or soluble inner portion and the cytochromes located in the membranes in the form of molecular assemblies (44); therefore, the mitochondrion is the site, as well, of the synthesis of the energy carrier, adenosine triphosphate (ATP).

It now appears clear that proteins are polypeptides containing peptide bonds formed by the reaction:

$$R'CH(NH_2)COOH + R''CH(NH_2)COOH + \text{Energy} \longrightarrow R'CH(NH_2)CONHCH(R'')COOH + H_2O$$

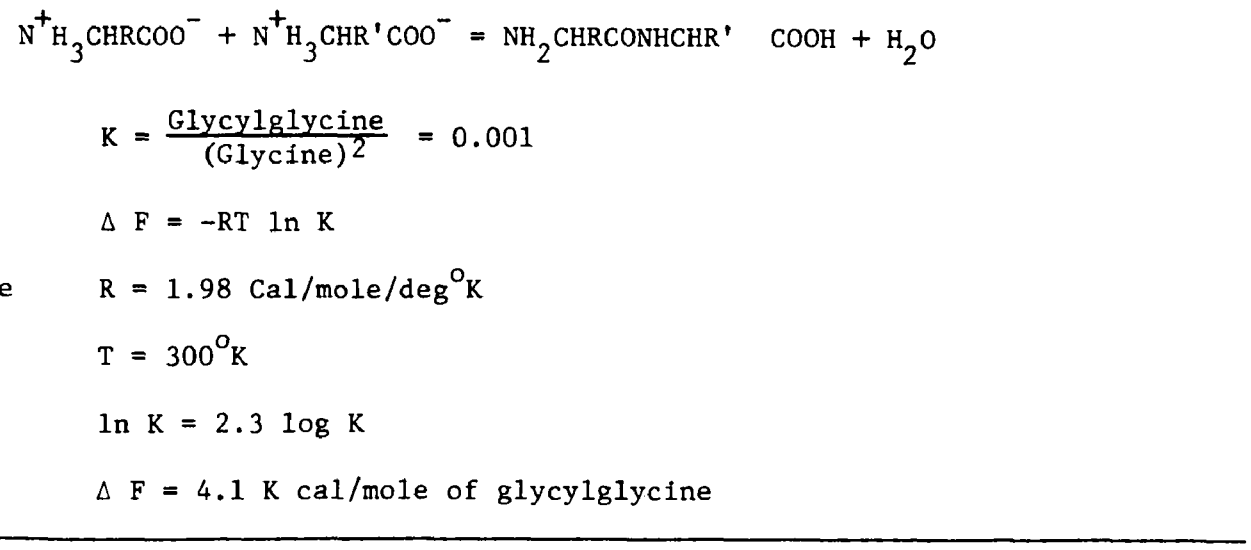
where the equilibrium would be favored in the direction of synthesis by the formation of insoluble products as is the case with keratin.

From thermodynamic data on reactants yielding peptides the free energy necessary for the synthesis of a peptide bond from unmodified amino acids has been determined to be in the range of 2000 - 4000 cal/mole as shown in Table 8.

From the equilibrium constant it can be shown that, at equilibrium, 99% of the material is on the hydrolysis side of the equation. Obviously a continuous supply of energy is necessary.

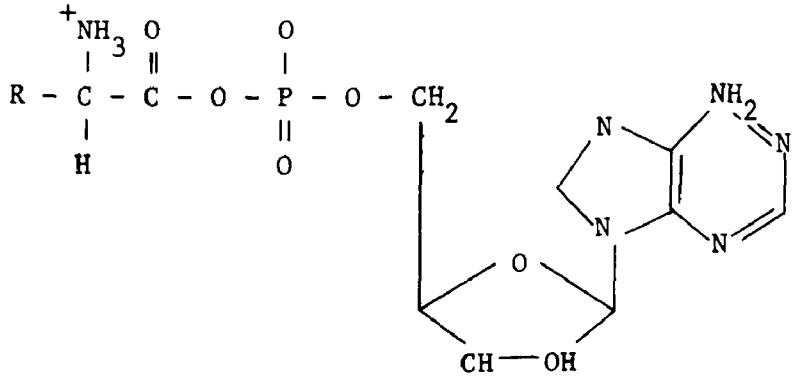
The incorporation of labelled amino acids into microsomes proceeds only in

TABLE 8: THERMODYNAMIC DATA ON FORMATION OF GLYCYLGLYCINE (46)



the presence of ATP suggesting that an activated amino acid is probably an intermediate in the synthesis process and is assembled in a stepwise addition to a growing chain (45). The initial step is the formation of amino acyl AMP.

5' AMINOACYL PHOSPHORYL ADENOSINE DERIVATIVE



$$ATP + AA + E \xrightarrow{Mg^{++}} E\ [\text{Amino Acyl - AMP }] + PP$$

The same enzyme that catalyzes the above reaction catalyzes the transfer of amino acid residues to the acceptor or soluble RNA.

$$E\ [\text{Amino-Acyl - AMP}] + S - RNA \longrightarrow \text{Amino-Acyl - RNA} + E + AMP$$

where PP = pyrophosphate

AMP = adenosine monophosphate

E = Aminoacyl - RNA synthetases

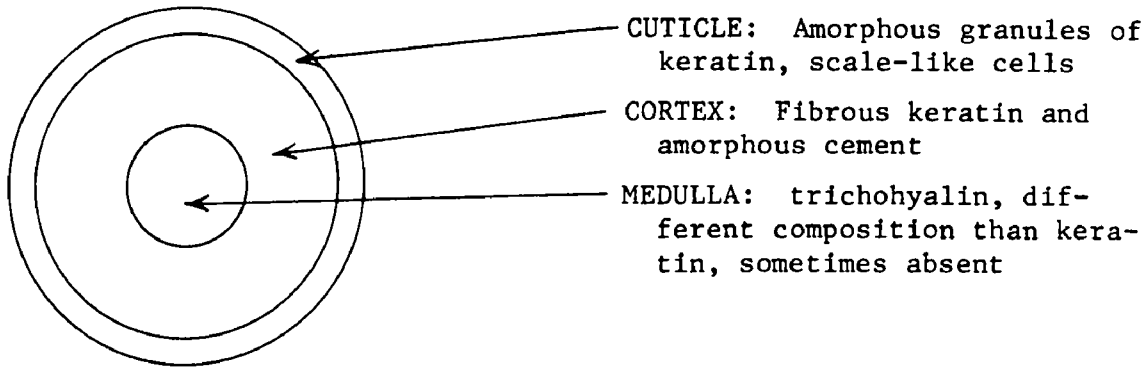
The S - RNA transfers the amino-acyl residues to the ribosomes where the final steps of protein synthesis take place. This final assembly of amino acids takes place in two steps according to Haurowitz, a) Formation of definite polypeptide sequences on a template and b) folding of a polypeptide to form a three dimensional molecule (46).

Morphological Aspects of Hair

Because hair was the primary keratin used in this study a few morphological characteristics should be considered. The wool research workers, mainly from Australia, have provided much of the information available.

In a cross section of hair, three zones can be distinguished: the cortex, the cuticle, and the medulla. (See Figure 44)

FIGURE 44: CROSS SECTION OF HAIR



The association of sulfur rich and sulfur poor constituents in different ratios appear to be an essential factor determining the nature of the final keratin, its stability and its chemical properties (42).

Human hair is fully stabilized at a level about one-third of the total length above the bulb up to the epidermal surface. This keratinization zone up from the bulb can be divided into two zones: the lower zone B where synthesis and orientation is complete but structural stability is poor and the upper zone A which is an area of structural stability (see Figure 45).

Perhaps the most important chemical feature of the keratinization process

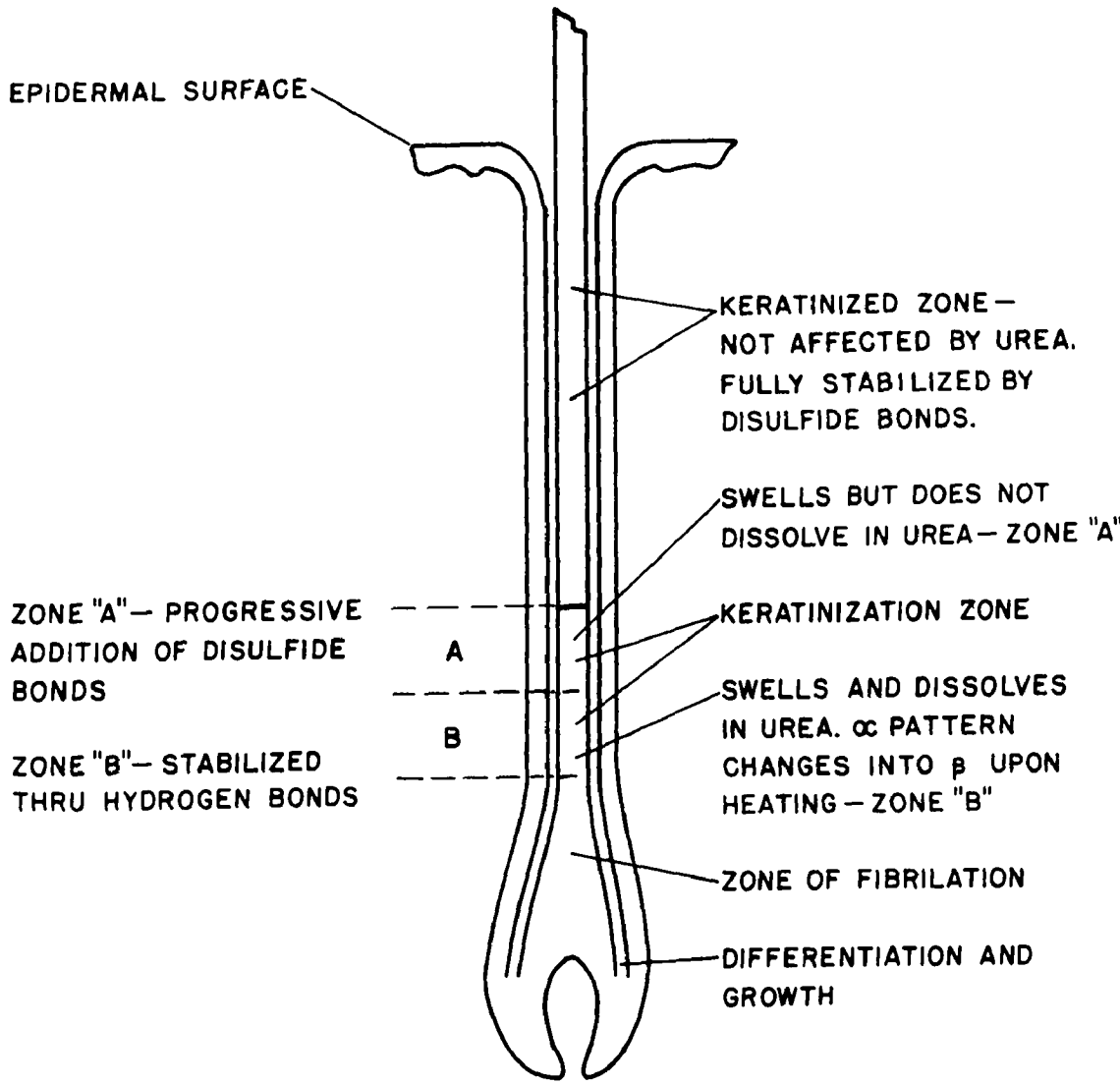


FIG. 45 HAIR STRUCTURE

is the fact that the keratinizing layers give a positive reaction for thiol (SH) groups and that this reaction disappears as the tissues harden. The reaction involved is the oxidation of SH groups to produce cystine bridges.

$$2 - SH + O \longrightarrow -S-S- + H_2O$$

In Figure 45, zone B is stabilized primarily through hydrogen bonding but zone A is consolidated as well by the progressive introduction of cystine bridges.

Frazer (47) showed that wool keratin consists of a matrix (α component) of very high sulfur content but with no particular orientation of the fibers, and regularly oriented fiber embedded on it. The peptide chains of the matrix contain approximately 24% cystine with an average molecular weight of 25,000 to 28,000 (42).

Structural Features of Hair

Keratin fibers of hair are extensible. Ashbury and Street (48) discovered that stretched hair gave a β x-ray pattern, while unstretched hair gave an α pattern. From these observations they inferred that the structure was developed by successive foldings of the structure. They further assumed that the α form is half the length of the β form (42).

Many attempts were made to find a structure that would account for the characteristic x-ray pattern of the α form. Pauling (49) formulated the α helix based on geometrical requirements deduced from the known structures of small peptides. Later it was postulated that the α helices were tilted to form a super helix or a coiled coil with a radius of 10 A°. Six helices would twist around a seventh straight α helix (50).

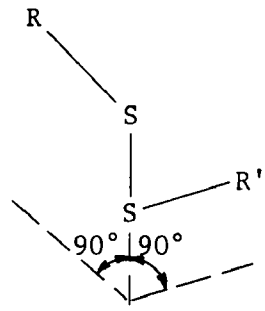
The actual cross section of the elementary filaments of hair keratin is of the order of 60 A°. What is visible in the light microscope of the order of 2000 - 10,000 A° is a fibril composed of a large number of filaments (42).

Experiments with radioactive labelled materials indicate that the contribution of sulfur to the hair comes from methionine and not cystine in the diet and enters directly into the keratinization zone. No radioactivity is noted in the bulb. This evidence is taken to mean that sulfur enters at the level of the keratinization zone through the walls of the follicle (42).

The primary structure for structural bondings in the keratins are covalent cross linkages with bond energies greater than 35 kilocalories per mole. The disulfide bond between cysteine residues forms a dihedral angle of 90°; rotation

is hindered around it. The disulfide bond possesses a bond energy of 60 kilocalories per mole (50). (See Figure 46)

FIGURE 46: THE DISULFIDE BOND



Secondary structural effects from regular and periodic foldings occur because of the formation of hydrogen bonds. Hydrogen bonds possess a bond energy of the order of 6 to 8 kilocalories per mole in vacuo and 1.5 kilocalories per mole in aqueous solution (51). They are caused primarily by the electrostatic interactions of two electronegative ions, such as of oxygen, nitrogen or sulfur, separated by a hydrogen atom. An electronegative atom covalently bonded to a hydrogen localizes an electron pair between the hydrogen atom and the electronegative atom; this allows a large portion of the electropositive hydrogen atom to interact with neighboring electronegative atoms.

$$-C=O\text{----------}H-N\langle$$

Another type of bond, the hydrophobic bond, results when apolar side chains from alanine, leucine, isoleucine, valine, phenylalanine, proline, tyrosine and methionine come together. When this happens, water is excluded and a hydrophobic region is created. The formation of hydrophobic bonds is an exergonic process of about - 0.7 kilocalories per mole due to the combination of a slight endothermic process with a high negative entropy. Because of its endothermicity, hydrophobic bonds become more stable with temperature up to about 60°C. The strength of a hydrophobic bond lies mainly in the reorganization of the water molecules around it which become more hydrogen bonded (45) (See Figure 47). The importance of hydrophobic bonds in protein stability has not been completely elucidated.

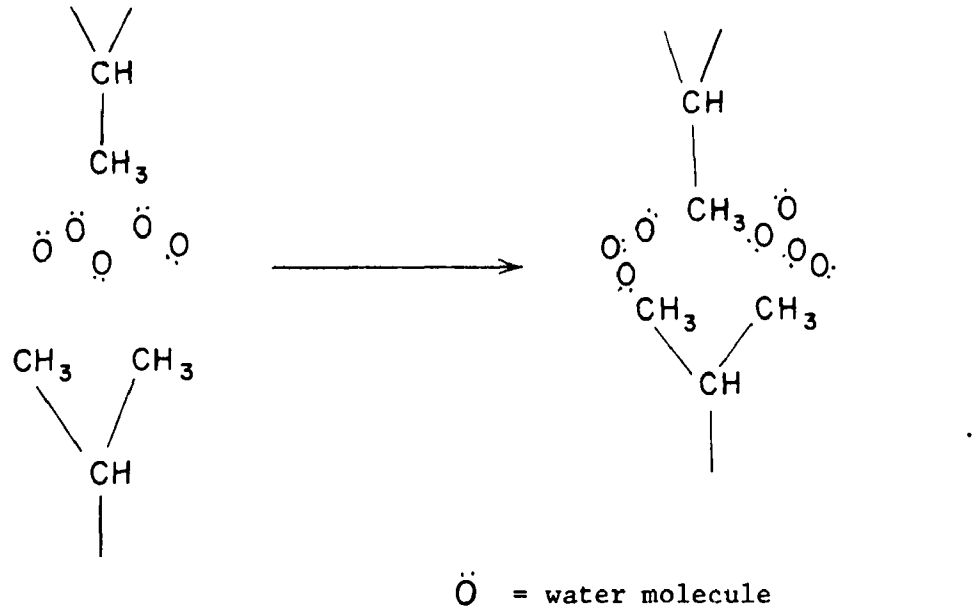
Dissolution of the Hydrogen Bond

The disulfide bonds react through three different mechanisms which have been studied: oxidation, reduction and cleavage.

Alexander and Earland (52) obtained soluble derivatives from keratins by oxi-

dizing the disulfide with peracetic acid. After oxidation, keratins are readily soluble in dilute alkalis; the addition of ammonium sulfate or acid brings about

FIGURE 47: HYDROPHOBIC BOND BETWEEN TWO ISOLATED SIDE CHAINS SHOWING EXCLUSION OF WATER (45)



Ö = water molecule

a partial precipitation. The precipitated material, α keratose, has a low sulfur content and a molecular weight of 50,000. The portion remaining, the γ keratose has a higher sulfur content and a lower molecular weight of about 3000 (52). In general, the oxidation of the disulfide bond gives two molecules of sulfonic acid.

$$RSSR + 6(O) \longrightarrow 2R - SH$$

One cleavage reaction occurs typically in keratins using sodium sulfite.

$$RSSR + NA_2SO_3 \rightleftharpoons RSNa + RSSO\ Na$$

been used (42). The reductive process produces two moles of thiol from the disulfide bond.

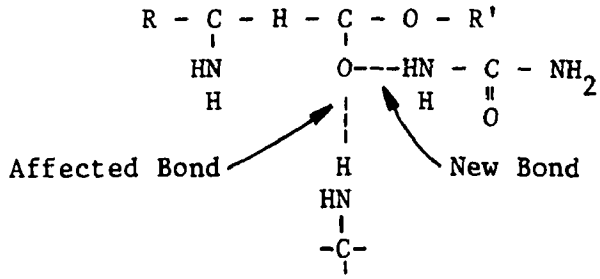
$$RSSR \longrightarrow 2R - SH$$

The reduction of the disulfide bond has been carried out at high pH values using thioglycollic or mercaptoacetic acid. Also sulfides at high pH values have The sulfite cleaves the disulfide bond to produce one S - H group. This is a reversible reaction requiring a high concentration of sulfite and a low concentra-

tion of RS^-. The concentration of $R - S^-$ can be kept low by keeping the reaction at a low pH, thereby keeping the SH group unionized. Also the SH group can be removed as a heavy metal mercaptide.

Urea is the most widely used reagent for rupturing the hydrogen bond. The action of urea involves interference with the internal bonding of the molecule as shown in Figure 48 below.

FIGURE 48: UREA BREAKING A HYDROGEN BOND



The effect of urea is quite dramatic in structures where hydrogen bonding is the primary stabilization factor.

The zone of fibrilar differentiation and growth and the lower zone of keratinization are completely dissolved by an 8M solution of urea, but the dissolving action of urea stops when the S - S bonds take over the primary stabilization role. (Refer to Figure 45) The addition of urea to a protein unfolds the molecule exposing the disulfide groups thereby facilitating any reaction involving them.

Enzymatic action on unmodified keratins is restricted to the cell membranes and the intracellular cement; the keratin itself is invulnerable to proteolytic enzymes.

Experiments with pronase indicate that enzymatic activity is definitely stopped at the fully keratinized zones with less than 20% of the fiber solubilized. The percentage is not any better than the results obtained with other proteases, in spite of the fact that pronase has exo- and endo- peptidase activity together with a very broad specificity spectrum (53).

The reagent used for dissolving the keratin should be able to reach and cleave the disulfide bonds. At the same time, the entrance of water into the hydrogen bonded material breaks a large number of hydrogen bonds disrupting the original structure and favoring enzymatic action.

Theoretical Considerations

The chemical properties of the hair cell membrane care are complimentary to the keratinized protein. When keratin is dissolved through oxidation, reduction or cleavage, the insoluble residue is found to be cell membranes and cement (42). Appropriate treatment for solubilization must take this relationship into consideration. Bacteria require modification of the keratinaceous material because this is the fraction resistant to protease activity.

As previously stated, the insolubility characteristics of the keratins depend largely on the covalent disulfide cross linkages. The importance of the covalent cross linkages in protein behavior can be anticipated by an analysis of their influence in the denaturation process.

The standard free energy of denaturation, ΔF, is, primarily, the sum of the free energy involved in unfolding the helical backbone in the absence of side chain interactions, ΔFu, plus the free energy due to the presence of covalent cross linkages in the crystalline phase, ΔF_x. Other interactions, for the sake of simplicity, are not taken into consideration (45).

$$\Delta F = \Delta F_u + \Delta F_x \qquad -1-$$

$$\Delta F_x = \Delta H_x - T\Delta S_x \qquad -2-$$

If covalent cross linkages are introduced the entropy, ΔS_x, decreases. Assuming no enthalpy contribution to ΔF_x then $\Delta H_x = 0$ so $\Delta F_x = -T\Delta S_x$ where the value of the entropy change, ΔS_x, is given by Flory (45) as

$$\Delta S = \frac{-3R\phi}{4} (\ln n' + 3) \qquad -3-$$

where ϕ = number of polypeptides in a cross sectional area

n' = number of statistical elements between cross links

Even when the maximum number of hydrogen bonds are formed in a polypeptide, four CO and four α amino groups cannot form hydrogen bonds. These end effects will alter the value of ΔF_u by the quantity C

Then $$\Delta F_u = n\Delta F_{residue} + C \qquad -4-$$

where n = number of peptide units

$\Delta F_{residue}$ = free energy change per residue

$$= \Delta H^\circ_{residue} - T\Delta S^\circ_{residue} \qquad -5-$$

$$C = -4\Delta H^\circ_{residue} + T\Delta S^\circ_{residue} \qquad -6-$$

Now combining (4), (5) and (6)

$$\Delta F_u = (n-4)\Delta H^\circ_{residue} - T\,(n-1)\Delta S^\circ_{residue} \qquad -7-$$

or $$\Delta F_D = (n-4)\Delta H^\circ_{residue} - T\,(n-1)\Delta S^\circ_{residue} - T\Delta S^\circ_x \qquad -8-$$

The transition temperature, T_r, is the temperature at which the peptide helix melts or $\Delta F_D = 0$.

$$T_r = \frac{(n-4)\Delta H^\circ_{residue}}{(n-1)\Delta S^\circ_{residue} + \Delta S^\circ_x} \qquad -9-$$

Since ΔS°_x is negative (entropy decreases with increased S-S bonds), an increase in the covalent cross links leads to an increased temperature of transition. That is, cross linkages, such as S-S bridges, stabilize the protein in its native state.

The resultant product from the cleavage of the disulfide bonds is readily hydrolyzed by proteolytic enzymes (54). The pathway for keratin utilization in nature usually involves the cleavage of S - S bonds.

An outstanding example is represented by insects such as the cloth moth which possess, in their digestive tract, some type of sulfhydryl compounds which effect the reduction of the disulfide bond thereby allowing the utilization of wool as food. It is quite well established that microbial solubilization of wool is effected through a joint action of disulfide reducing agents and proteolytic agents (55). Streptomyces fradie solubilize 80 to 90% of wool and feather keratin after 4 days of incubation (56).

The successful utilization of a substrate by a microorganism depends on its ability to convey the substrate through energy and synthesis pathways. Microbial systems will provide their energy through the conveyance of the molecules to the TCA or Krebs cycle for the production of ATP which will store the energy necessary for protein synthesis and other endothermic reactions in the cell. Schematically, modified keratins can be hydrolyzed by proteolytic enzymes to their component amino acids. Pyruvate, acetyl Co-A, α -ketoglutaric and oxaloacetic acid provide the prime matter for the functioning of the TCA cycle. The main link between carbohydrate and amino acid metabolism is provided by the reduction amination of α -keloglutaric acid into glutamic acid where ammonia is the nitrogen source and NADPH is the reducing agent.

Once ammonia is converted to the amino N, the transamination process can transfer it to other carbon skeletons. Obviously this reaction represents a most important pathway for the formation and deactivation of amino acids.

For average values, protein can be considered to be 50% carbon and 16% nitro-

gen (55). For an activated sludge culture, nutritionally balanced feed with a C:N ratio of 5:1 is appropriate (1). Besides the conversion of amino acids in the TCA cycle, intermediates provide N as NH_3 which is thought to be 100% available for bacterial utilization (57). Since the proper amount and ratios of required elements to sustain microbial life are contained in keratin, activated sludge cultures should be able to utilize keratinaceous material as a carbon and energy source.

The basic problems considered in this research effort were the elucidation of the behavior of organisms utilizing keratinaceous material and the extent of biodegradation that could take place. Improvement of the rate and extent of microbial action were considered as areas of interest in practical applications and in the overall evaluation of the problem of protein utilization. The initial phase of this research was concerned with the problem of insolubility of the keratins and with the search, then, for an appropriate keratin solvent.

Experimental Tests on Dissolution of Hair

The study of keratins in this research was concerned mainly with the keratin of human hair. Preparation of the cut hair prior to use in the experiment consisted of triple washing with distilled water in an erlenmeyer flask followed by three vigorous rinses with 50% solution of benzene in 95% ethanol. The hair was then air dried under a hood and stored in a closed container.

A solution of hair was prepared by placing 416 mg of cleaned hair in 20 ml of 0.5 N NaOH and leaving in a covered beaker overnight. The mixture was then placed in a 50°C water bath until complete dissolution was evident. After dissolution, the mixture was neutralized to pH7 with 1 N H_2SO_4 and then was ready to use as a feed for activated sludge cultures. Supplementation of the keratin with glucose and nutrient broth was used when desired.

The above methods for dissolving hair were determined after a series of tests indicated that these procedures were the best for the results desired. The several determining tests are listed below.

The following series of tests were performed in 50 ml pyrex test tubes using reagents in the proportion of 20ml reagent to 0.5 gm of hair.

1. Storage for 10 days at room temperature
   A. Acids
      50% (18N) H_2SO_4

25% (9N) H_2SO_4

ION HCl

Concentrated (12N) HCl

18 N H_3PO_4

B. Bases

0.2 N, 0.5 N, 1 N, 2 N, 3 N NaOH

0.5 N, 1 N KOH

0.5 N $Ca(OH)_2$

0.5 N $Ba(OH)_2$

C. Combined Treatment

Reducing agents:

1 M Thioglycollic acid in 0.1 N NaOH

Na_2SO_3(1 gm/gm of hair) in 0.2 N NaOH

Hydrogen bond breakers:

0.5 N NaOH in a saturated solution of urea

Na_2SO_3(1 gm/gm of hair) in 0.25 N NaOH in a saturated solution of urea

2. At 35°C for 50 hours:

50% H_2SO_4

0.5 N NaOH

1 M Thioglycollic acid in 0.1 N NaOH

Na_2SO_3 (1 gm/gm of hair) in 0.2 N NaOH

Estimation of dissolution was made by pouring the tube contents into a 4 inch petri dish set over a white surface and comparing with 2 blanks; one containing only distilled water, the other containing 0.5 gm of hair.

Analytical Methods

Protein determination followed the method given by Lowry (58). This method consists of the reduction of Folin's phenol reagent (phosphomolybdic acid) by alkaline copper treated protein. The intensity of the blue color developed is measured at 750 mμ and the protein concentration calculated from a previously established standard curve. Experiments were done in triplicate with sample size adjusted to approximately 80 μg of protein.

Oxygen uptake, suspended solids, and DNA determinations were as previously decribed. COD determinations followed Standard Methods (59).

Paper chromatography was used for determination of hydrolytic produsts using descending chromatography on 8" x 17" Whatman No. 1 paper and a solvent system of butanol : acetic acid " water in a 4:1:1 ratio. A platinum loop was used to apply the spot. Spot locations were obtained colorimetrically by spraying a solution of 0.2% ninhydrin in water saturated n-butanol and dried in a 100°C oven for three minutes (60).

Results of Test Procedures

1. Of all the agents used, sodium hydroxide was the best solvent for human hair. Optimal concentrations were between 0.5 N and 1 N.
2. As a general rule, the application of heat favors the dissolution process.
3. The ratio of 0.5 grams of hair to 20 ml of reagents was satisfactory.
4. The dissolution reaction consumes 5.4 milliequivalents of sodium hydroxide per gram of hair.
5. Neutralization of the mixture can be accomplished without precipitation of the proteins. A light smell of sulfur develops during neutralization. Below pH5 proteins begin to precipitate, but a fraction remains in solution.
6. Treatment with 0.5 N NaOH partially hydrolyzes hair. Hydrolysis seems to be produced in the less keratinized portion of the hair. When purified keratin was used, the same treatment did not liberate hydrolysis products detectable by paper chromatographic techniques used.
7. Biological utilization of hair occurs at a slow rate as shown by Warburg respirometer studies. The maximum initial oxygen uptake rate was 31 microliters per hour followed by a rate of 11 microliters per hour. The material oxidized in 44 hours represented 44.8% of the calculated COD. The oxidation curve appears to follow a zero order rather than a first order curve.
8. The best efficiency obtained for removal of keratin derived protein from the culture supernatant was 76%. The mechanism of removal appears to be one of adsorption and metabolism of adsorbed products. Addition of supplements such as glucose and nutrient broth did not improve the rate or efficiency of the utilization process.
9. A rate increase in oxygen uptake of 45% occurred with acclimating of the culture to keratin.

10. Net DNA production in the activated sludge solids was negligible through the first 30 hours of the experiment and thereafter increased slightly indicating some increase in number of cells.
11. Protein content of the supernatant generally followed a downward trend, except at six hours when an increase was detected. This corresponded to a decrease in suspended solids at six hours.
12. Boiling the feed mixture for six hours under reflux prior to feeding did not significantly improve the rate of oxidation and no appreciable change in the U. V. spectra was obtained.
13. No net oxygen uptake was obtained from a culture fed undissolved hair.
14. The net solids yield at the end of 44 hours was approximately 57.6% of the dry weight of substrate fed.
15. Powdered purified keratin was brought into solution at a concentration of 1.25 gm/L in a KH_2PO_4 + NaOH buffer at pH 8.5 and was available as a substrate to acclimated activated sludge cultures.
16. Activated sludge cultures acclimated to keratin retained floc formation and settling properties and microscopic examination did not reveal appearance changes in the culture. However, excessive foaming required addition to Dow Corning Antifoam B and mineral oil to keep foam from overflowing the culture containers.

Discussion

Several interesting points should be presented on the dissolution of hair. For example, the outer membrane of hair seems to have selective permeability to Na^+ ions as against K^+, Ca^{++} and Ba^{++} ions. The selective permeability of biological membranes is a well established fact. Hendricks showed that the outer membrane of frog skin was impermeable to K^+ and permeable to Na^+ ions (61). Also it is well established that the inner structure of hair is unavailable to molecules larger than propanol (62). The experimental results suggest that the size and nature of the anionic component of the base plays a significant role in reaching the internal structure of the keratin.

The action of NaOH on hair may be a cleavage reaction of the disulfide bond and formation of a sodium salt upon ionization of the free carboxyl and amide groups in the keratin molecule.

The reaction of the disulfide bond with metal ions seems to proceed with a direct attack of the metal on the disulfide (45).

$$2\ RSSR + 2\ M^{+} \rightleftharpoons 2\ \begin{matrix} RS - SR^{+} \\ | \\ M \end{matrix}$$

$$2\ \begin{matrix} RSSR^{+} \\ | \\ M \end{matrix} + 2\ OH^{-} \rightleftharpoons 2\ RSM + 2\ RSOH$$

$$2\ RSOH \rightleftharpoons RSO_2H + RSH$$

$$RSH + M^{+} \rightleftharpoons RSM + H^{+}$$

As the concentration of NaOH is increased, a point is reached where the concentration of Na^{+} is high enough to cause a salting out effect because of the competition between the proteins and the salt ions for the molecules of water.

The absence of hydrolysis products from the solubilized pure keratin in contrast with the hydrolysis products of natural hair with dissolution by 0.5 N NaOH indicate that possibly a less stabilized fraction of the hair was being hydrolyzed.

Figure 49 indicates the effect of several chemicals on dissolution of hair. Long digestion periods with 50% H_2SO_4 can accomplish dissolution, but at that concentration, a considerable amount of base is necessary for neutralization. Another inconvenience of treatment with H_2SO_4 is that, when the reaction is accelerated with heat, a powdery humin precipitate appears.

Thio glycollic acid in alkaline solution at 35°C is a satisfactory treatment for solubilizing hair but presents the problems of strong mercaptide odor production plus the release of hydrogen sulfide at low pH.

Sulfitolysis at 35°C in 0.2 N NaOH is satisfactory. Since a smaller amount of base is required this treatment might be considered if the incoming waste can be or is heated.

Figure 50 shows that the best concentration of sodium hydroxide for dissolution lies between 0.5 N and 1 N. The existence of an optimum between these concentrations was not pursued.

In the biological degradation of complex molecules like the keratins, the extreme specificity of the proteases must be considered. This specificity may vary from a single absolute type in which there exists one and only one substrate for the enzyme to a relative type in which a specific linkage is attacked by the enzyme.

Several factors will affect substrate specificity such as the structure,

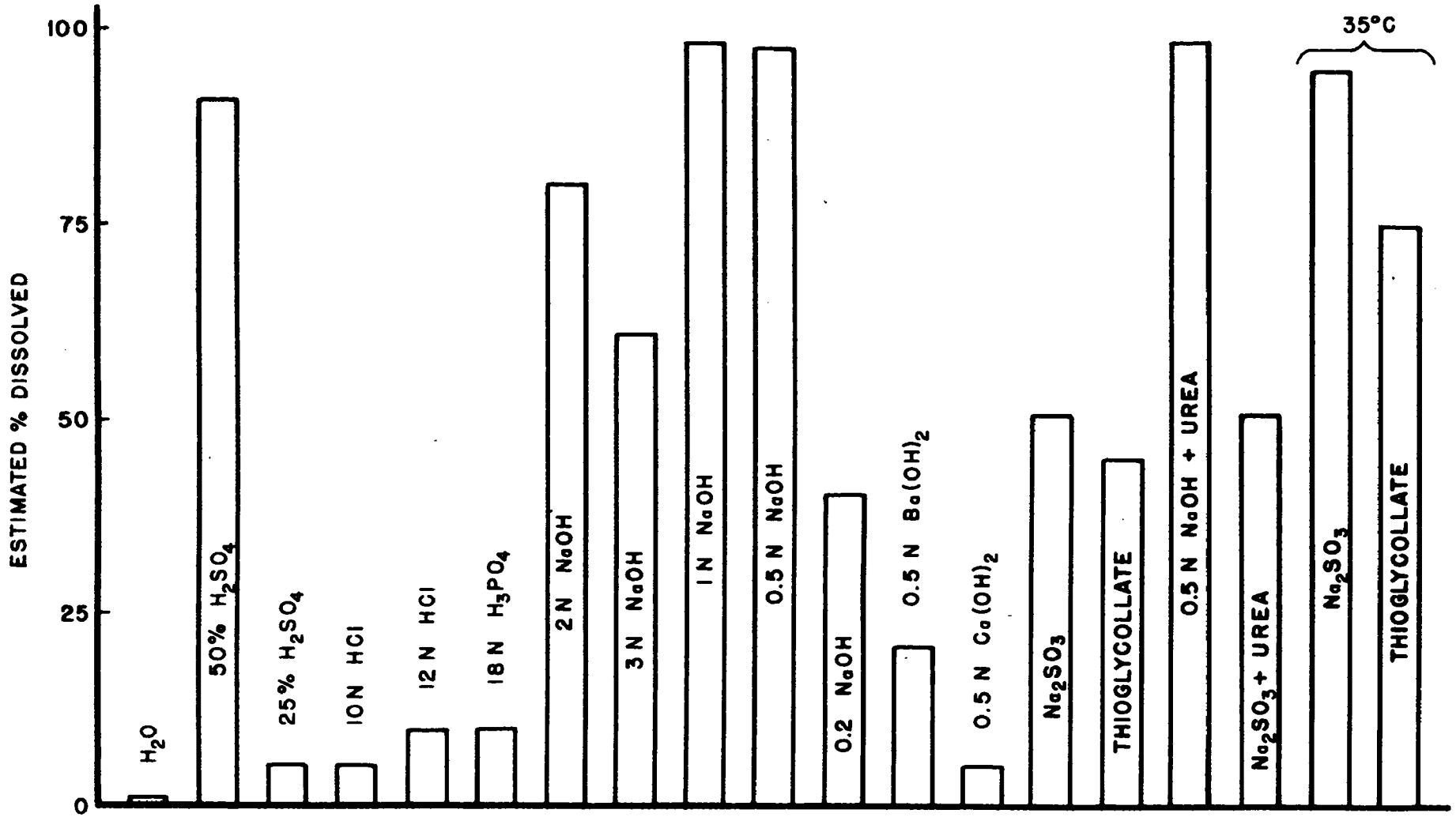


FIG. 49 RELATIVE EFFECT OF VARIOUS SOLVENTS ON DISSOLUTION OF HAIR

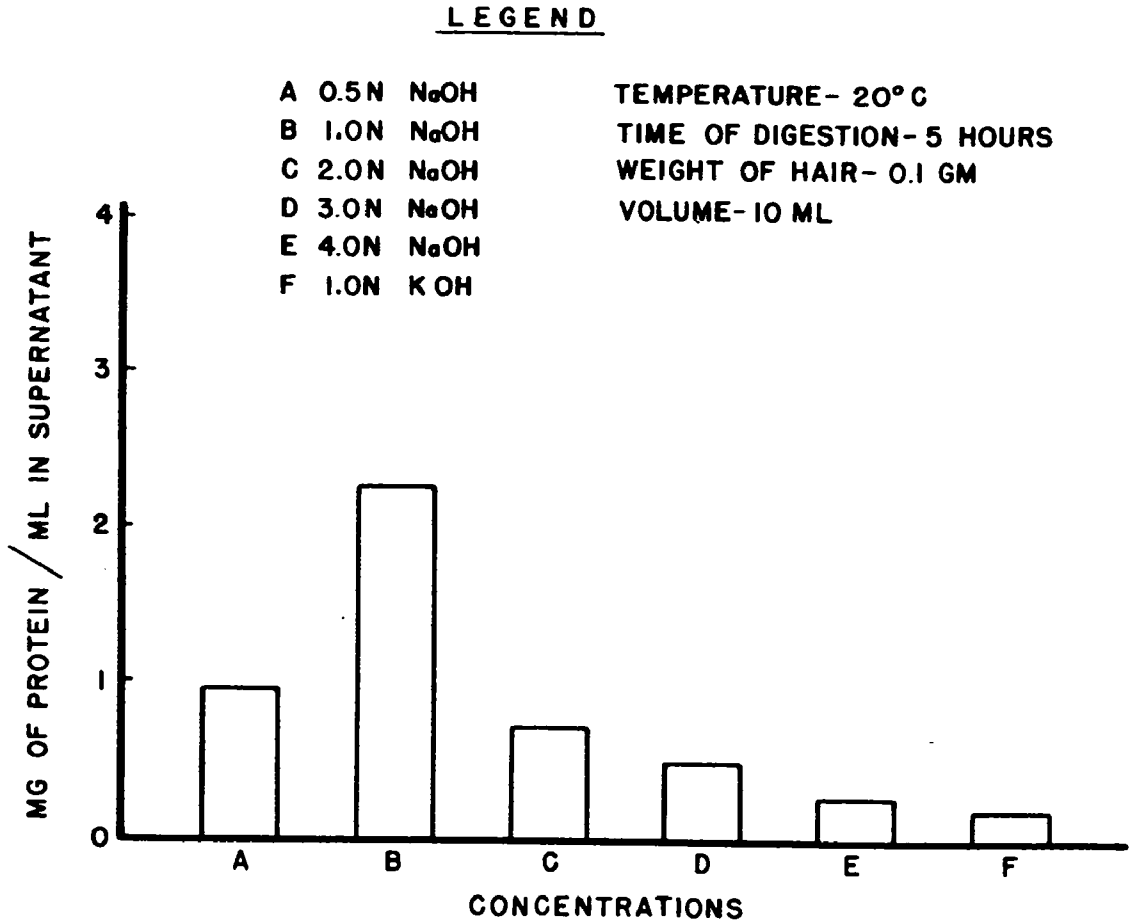
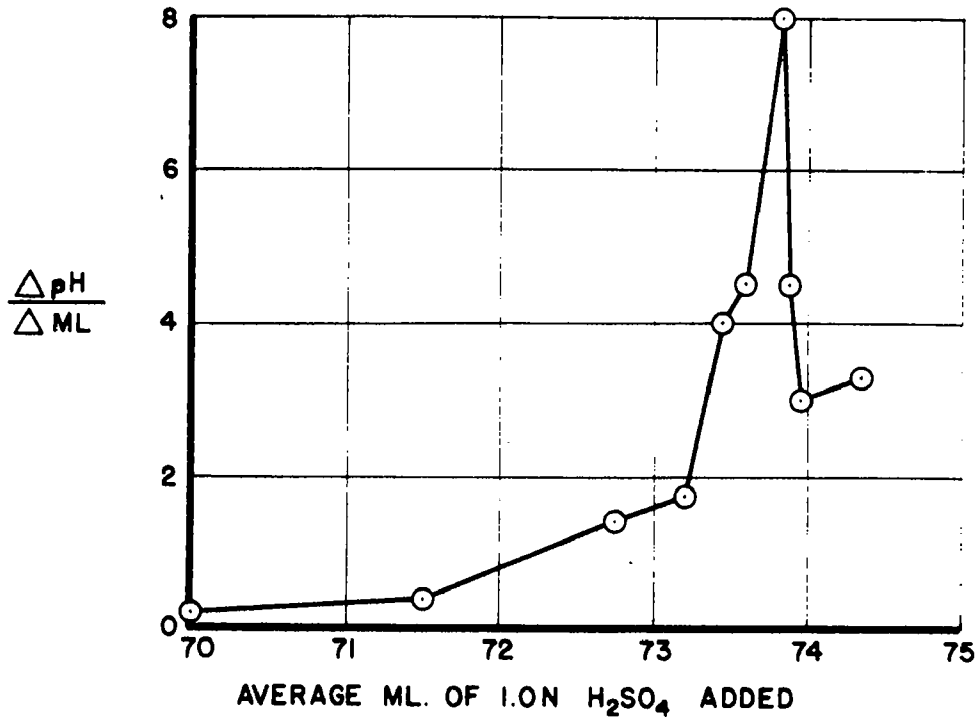


FIG. 50 EFFECT OF DIFFERENT CONCENTRATIONS OF SODIUM HYDROXIDE UPON DISSOLUTION OF HAIR

0.215 GM. OF HAIR DISSOLVED IN 75 ML. OF 1.0 N SODIUM HYDROXIDE.

TEMPERATURE 20° C



EQUIVALENCE POINT— 73.82 MEQ OF SULFURIC ACID ADDED.

1.18 MEQ OF NaOH CONSUMED IN THE REACTION.

FIG. 51 TITRATION OF A SOLUTION OF HAIR

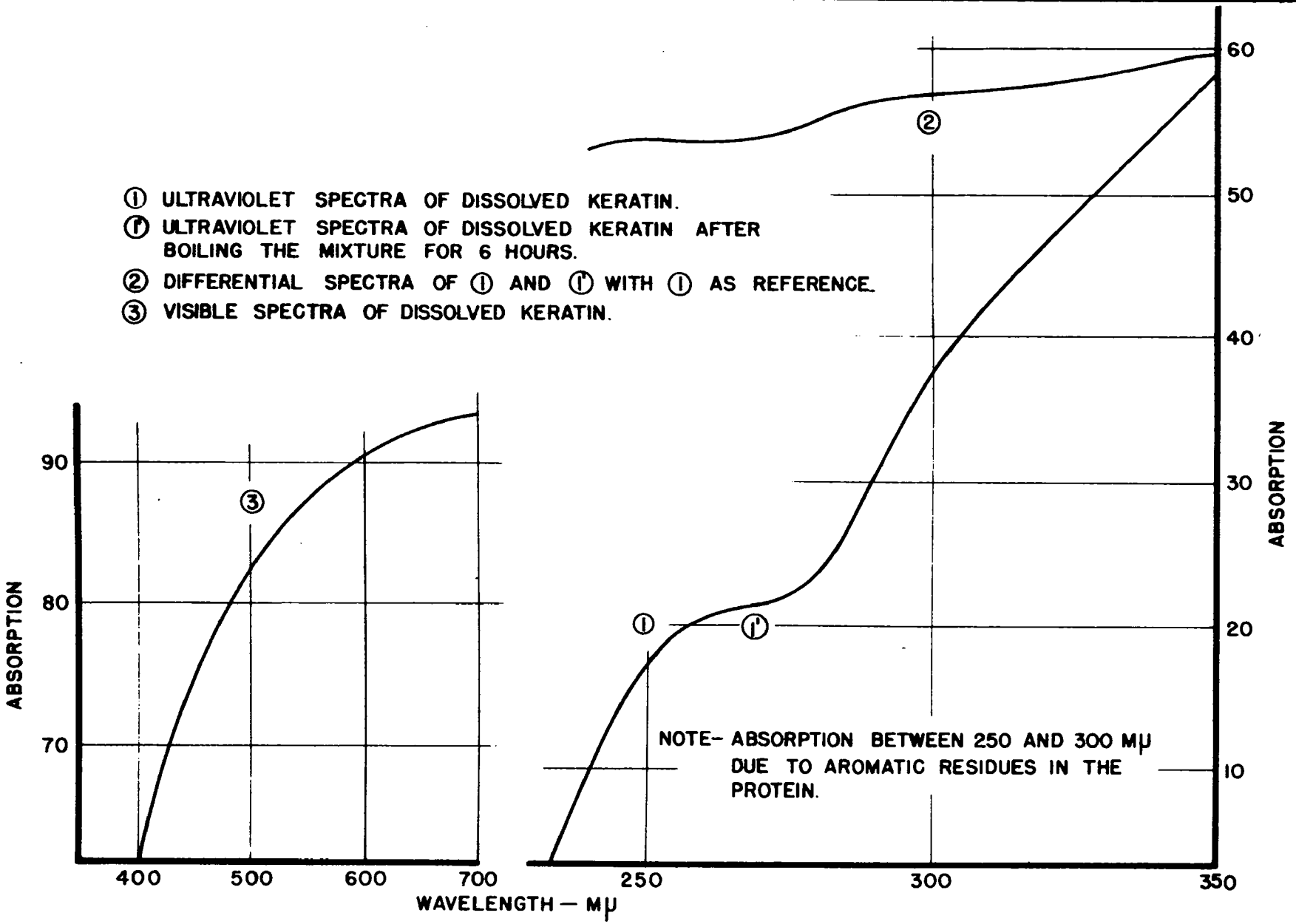


FIG. 52 ABSORPTION SPECTRA OF KERATIN

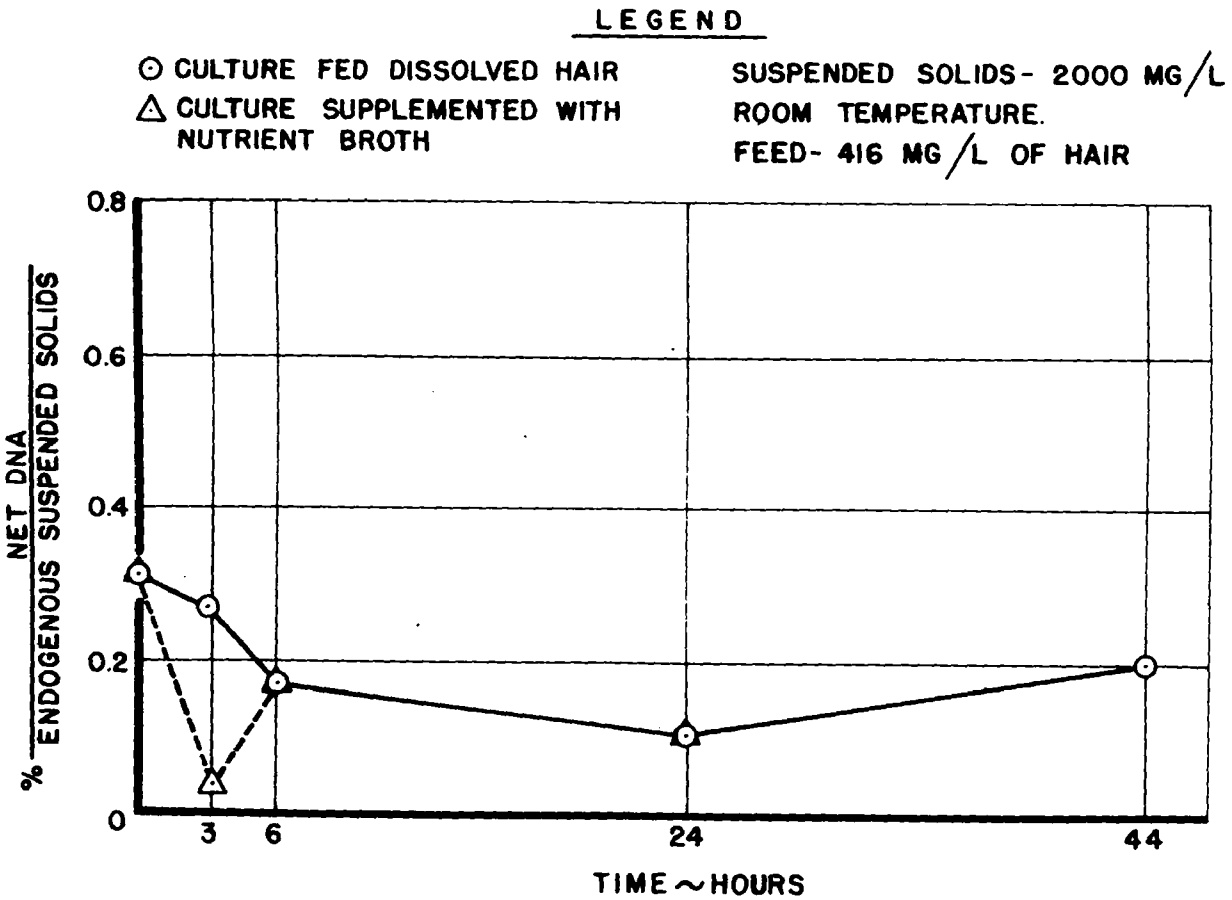


FIG. 53 NET DNA CONTENT OF ACTIVATED SLUDGE SOLIDS

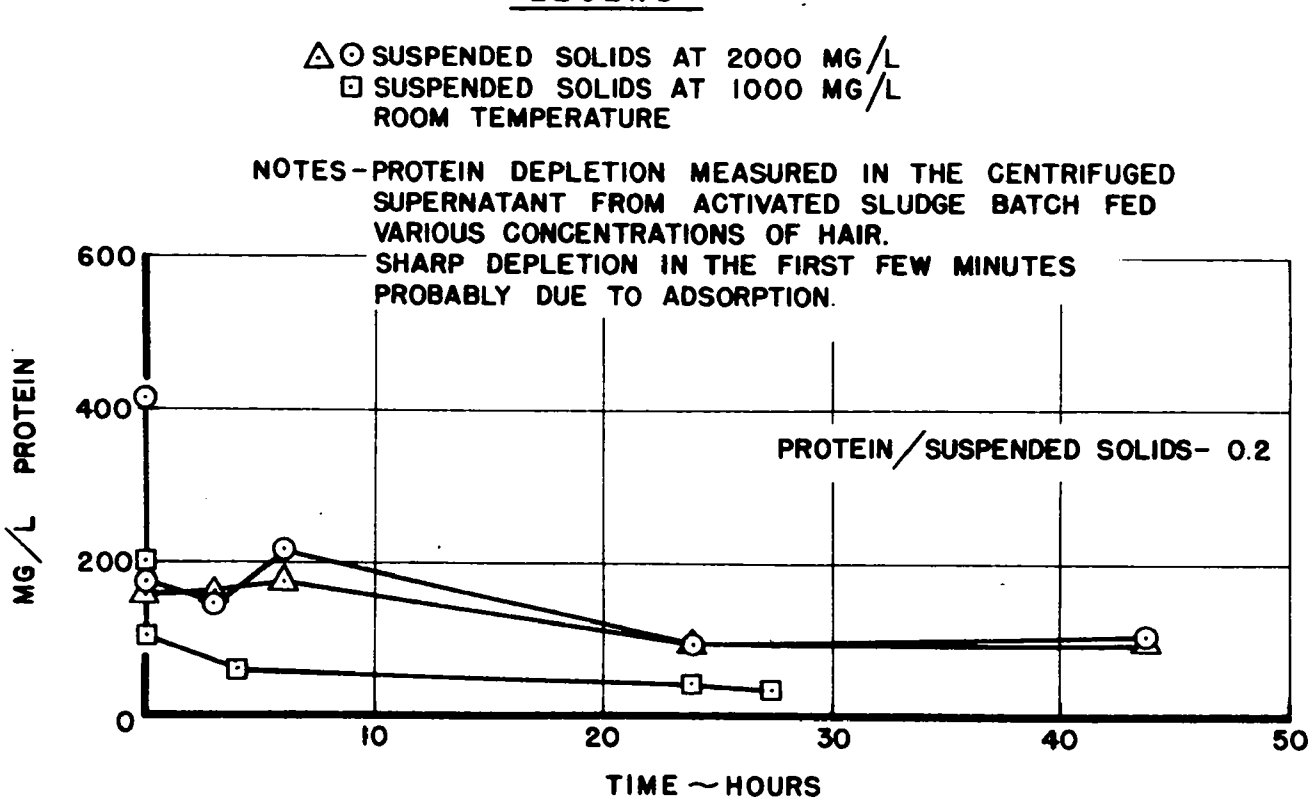


FIG. 54 PROTEIN DEPLETION

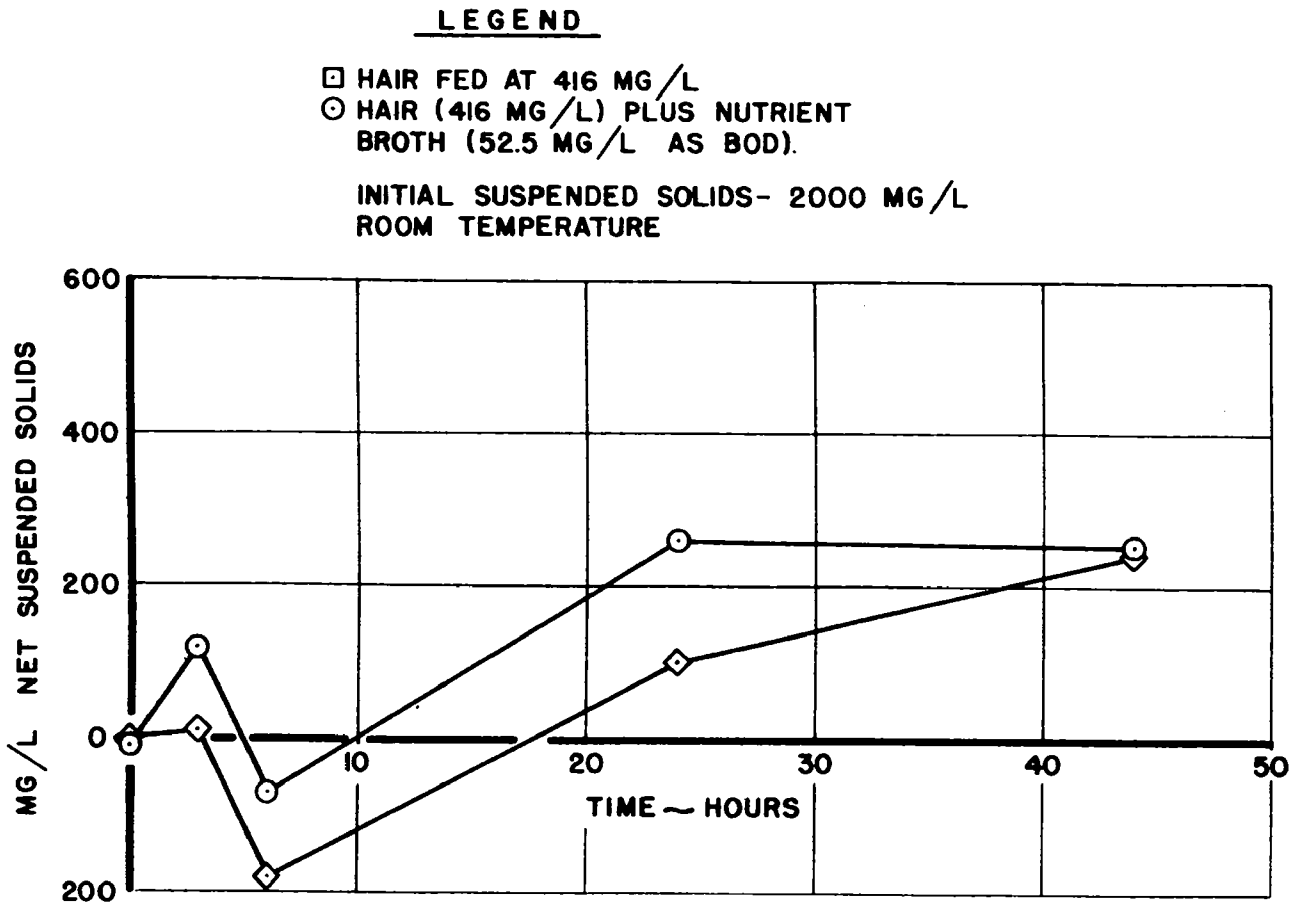


FIG. 55 VARIATION IN SUSPENDED SOLIDS

(CHANGE IN ACTIVATED SLUDGE SOLIDS AFTER BATCH FEEDING OF HAIR AND HAIR WITH NUTRIENT BROTH.)

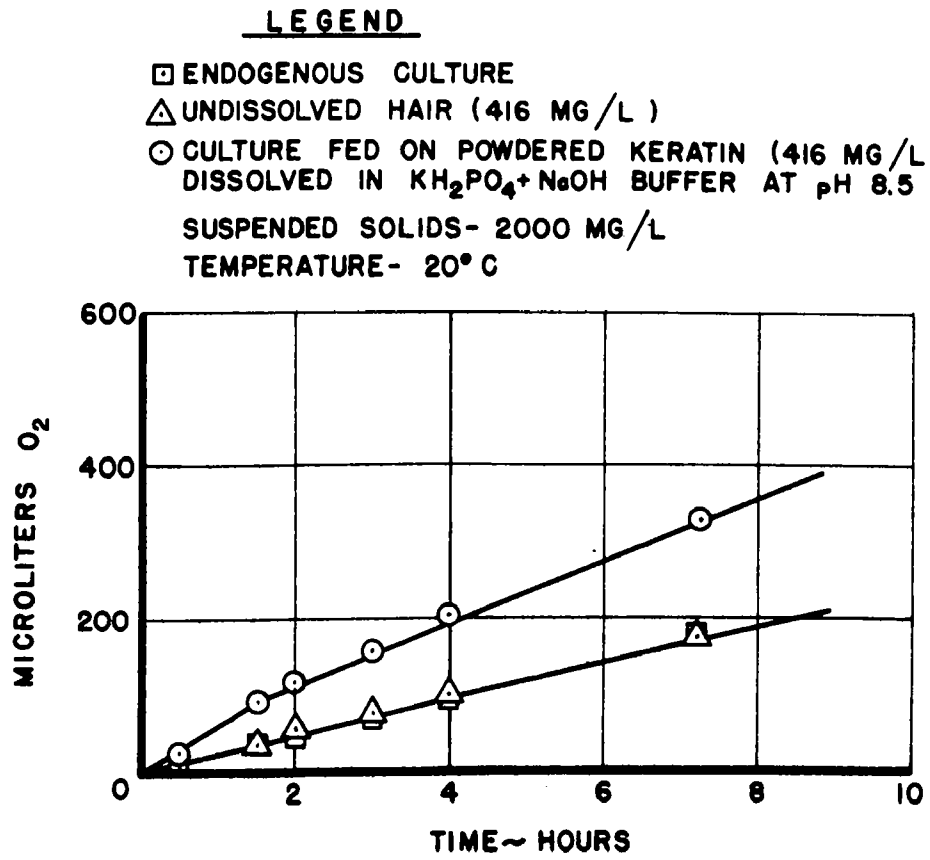


FIG.56 OXYGEN UPTAKE OF A CULTURE FED UNDISSOLVED HAIR

spatial configuration and polar groups of the substrate. The nature of the enzyme itself, its structure, the prosthetic group, the metals involved, all these are of extreme importance on enzyme specificity. It follows, therefore, that a polypeptide or protein will be hydrolyzed at definite loci in the chain.

Chemical treatment disrupts a number of disulfide and hydrogen bonds and diminishes electrostatic and hydrophobic attractions. As well, the protein can be assumed to change from the helical to a random coiled form.

Because the hydrolysis of the peptide bond is an exergonic process favored by the ionization of the amino acids involved, bacteria are not thermodynamically hindered from effecting the hydrolysis. Thus, the slow rate of oxidation of the keratins cannot be explained by lack of energy. The keratin supplemented with glycose and nutrient broth -- both substrates readily available as energy sources -- did not change significantly in rate of utilization.

The highly complex keratin molecule must be reduced to simpler building blocks before it is readily accessible as a bacterial food source. Thus either physical or chemical transformations will be required as pretreatment or exoenzymes will have to hydrolyze a sufficient number of bonds to form small peptides or free amino acids that can travel to the interior of the bacterial cell and be incorporated in the cell's biochemical activities.

The highly specific enzymatic degradation process has been shown by this research to be accelerated by acclimating the activated sludge culture to the keratin substrate thus building up the microbiological populations and enzyme systems utilized in the keratin degradation process. The parameters measured indicate that the respiration and growth of the cells occurs at a low rate which can be attributed to the difficulties incurred by the bacterial cell in hydrolyzing and utilizing the substrate.

The proteolysis of the oxidized high sulfur component of wool (approximately 30% of the dry weight) is about 3.2 times faster than that of the low sulfur component (approximately 60% of the dry weight) and this latter fraction reaches only 26% of the total hydrolizable material in six hours. This difference in proteolysis has been explained as due to the structural difference of the two components (53).

In analyzing the kinetics of the reaction of the biological degradation of hair the rate expressions of Henri (50) are

$$[E] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [E\cdot S] \xrightarrow{k_3} [P] + [E] \qquad -1-$$

where [E] is the concentration of enzyme

[E·S] is the concentration of enzyme-substrate complex

[P] is concentration of products

and the k's are the various reaction rates shown

Now $K_{eg} = \frac{k_2}{k_1} = \frac{[E][S]}{[E\cdot S]}$ for the equilibrium between reactants and the enzyme intermediate. The total enzyme concentration is

$$[E_T] = [E] + [E\cdot S] \qquad -2-$$

and

$$[E_T] = \left[1 + \frac{K_{eg}}{S}\right] [E\cdot S] \qquad -3-$$

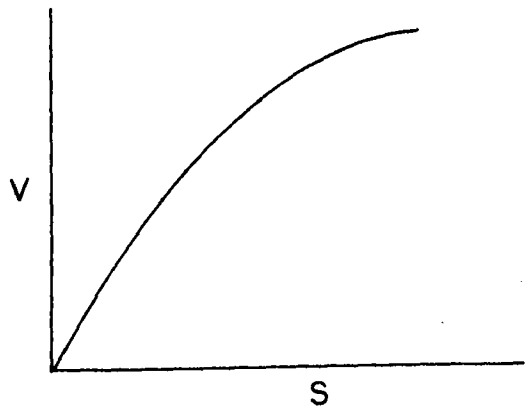
The initial rate of product formation is given by

$$V = \frac{d[P]}{dt} = k_3 [E\cdot S] \qquad -4-$$

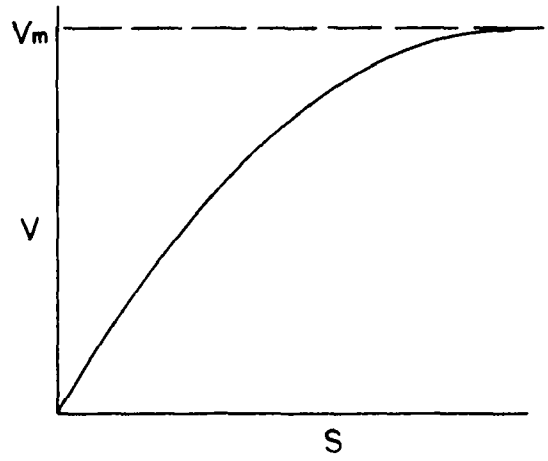
or

$$V = \frac{k_3 E_T S}{S + K_{eg}} \qquad -5-$$

A plot of V (velocity of reaction) versus S (substrate concentration) will yield a curve



For large values of S, equation -5- for V indicates that the reaction rate will be insensitive to substrate concentration. That is, when $S >> K_{eg}$, equation -5- yields $V \cong k_3 E_T$ -6-



Therefore, the maximum reaction, V_m, for a given total enzyme concentration, E_T, should, under these conditions, be dependent only on the equilibrium constant, k_3. This is why the rate of oxygen utilization of a culture fed a simple fully utilizable substrate which is present in excess proceeds in a straight line fashion. Under this condition, the rate of substrate depletion is expressed by $\frac{-ds}{dt} = k_r$, k_r being the rate constant.

The equilibrium constant, k_3, and hence the rate constant, k_r, appear to be a function of the substrate characteristics and will remain constant only when those characteristics are invarient with time in the period where $S>>K_{eg}$.

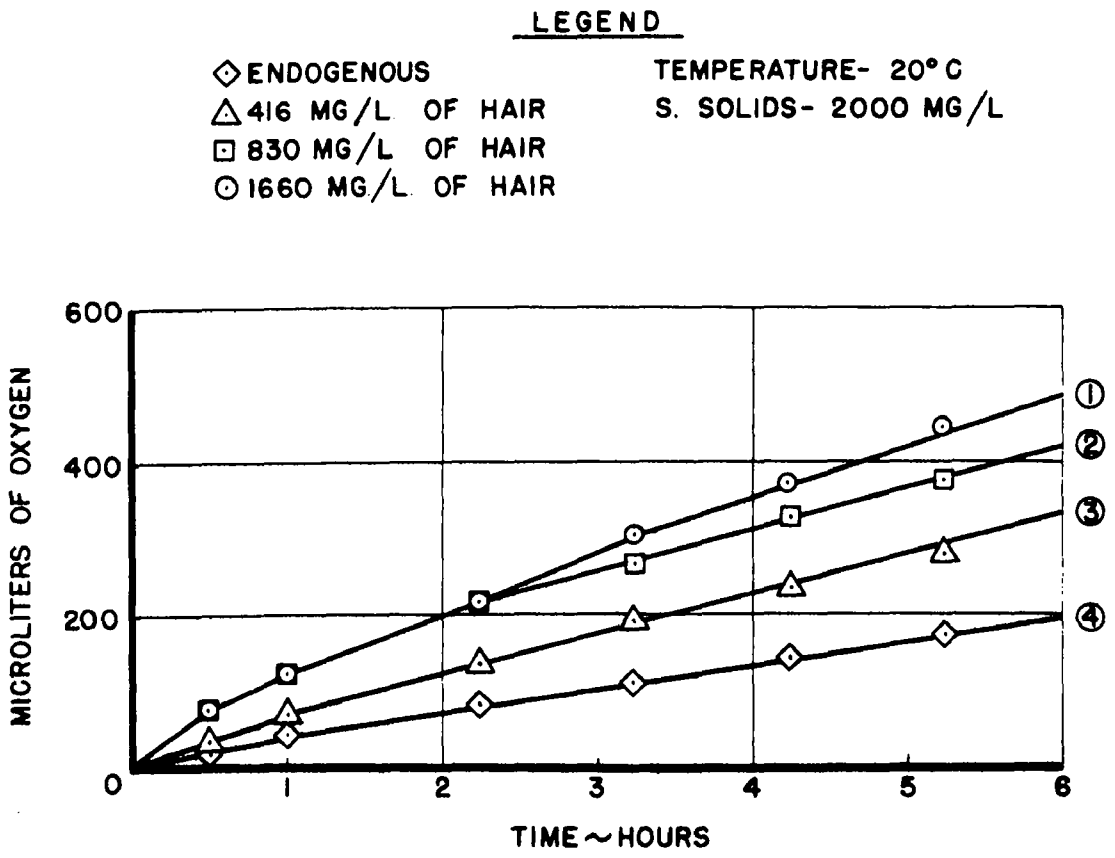
In the case of keratin, however, the substrate is highly complex and is characteristics are not apt to remain constant throughout the reaction. The curves in Figure 57 indicate a time variant equilibrium coefficient as shown by the change in slope of the oxygen uptake curve at 2 1/4 hours elapsed time in batch feeding of keratin. Such a slope change may indicate a change in vulnerability of the keratin.

The oxygen uptake rates shown in Figure 58 show that with the long time experiment, the condition of $S>>K_{eg}$ prevailed probably because of the low concentration of E·S.

If the substrate concentration, S, is very small when compared with K_{eg}, the rate of reaction or enzyme activity is proportional to the substrate concentration.

From -5- $V = \frac{k_3 E_T S}{K_{eg}}$ -7-

which plots as a straight line with slope = $\frac{k_3 E_T}{K_{eg}}$



FROM ZERO TO 2¼ HOURS CURVES ① AND ② FALL ON THE SAME SLOPE, WHICH CURVE ① KEPT UNTIL 3¼ HOURS. AFTER 3¼ HOURS CURVES ① AND ② TAKE THE SAME SLOPE AS ③.

FIG.57 EFFECT OF INCREASING CONCENTRATIONS OF SUBSTRATE ON OXYGEN UPTAKE

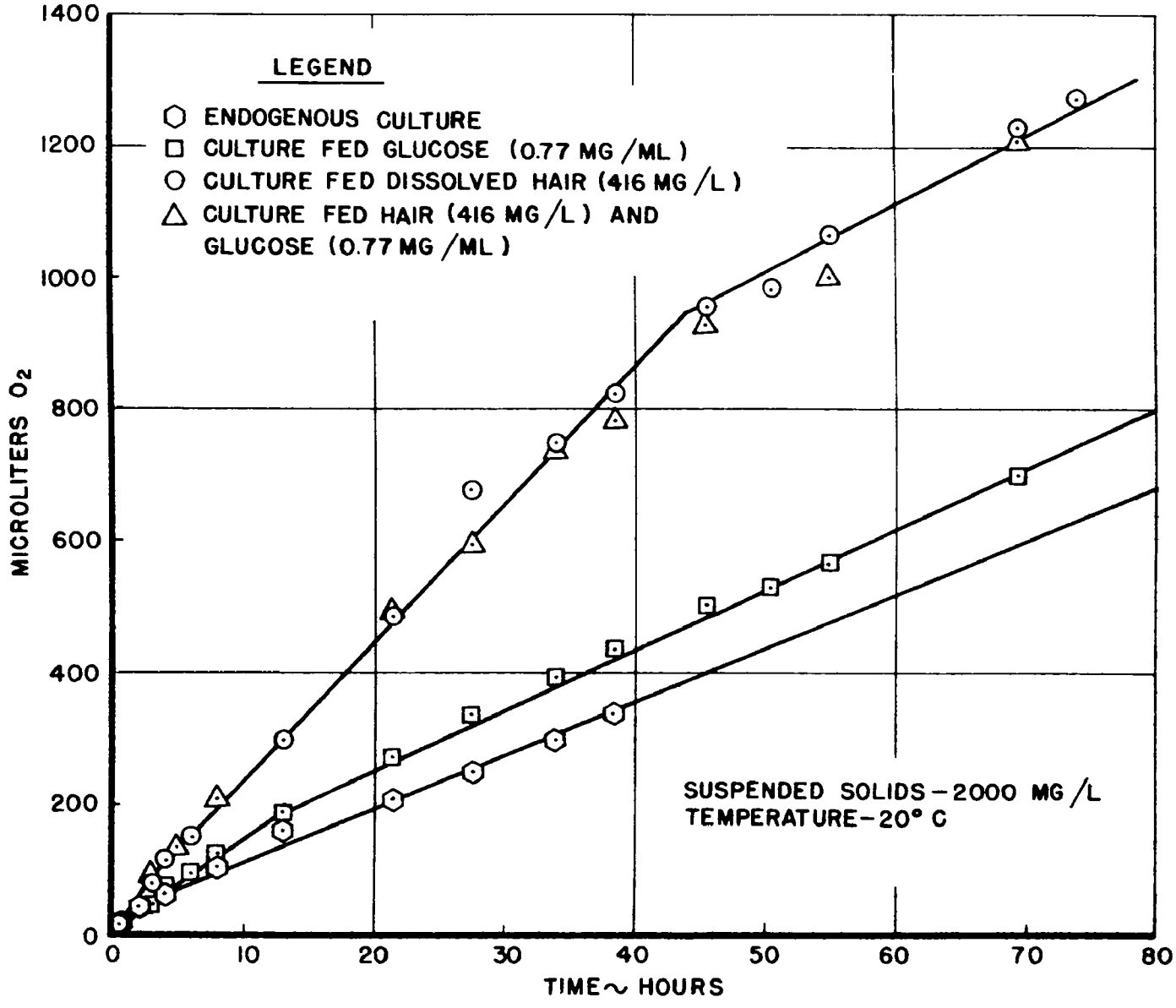


FIG.58 OXYGEN UPTAKE OF A CULTURE FED DISSOLVED HAIR AFFECT OF SUPPLEMENTATION WITH GLUCOSE

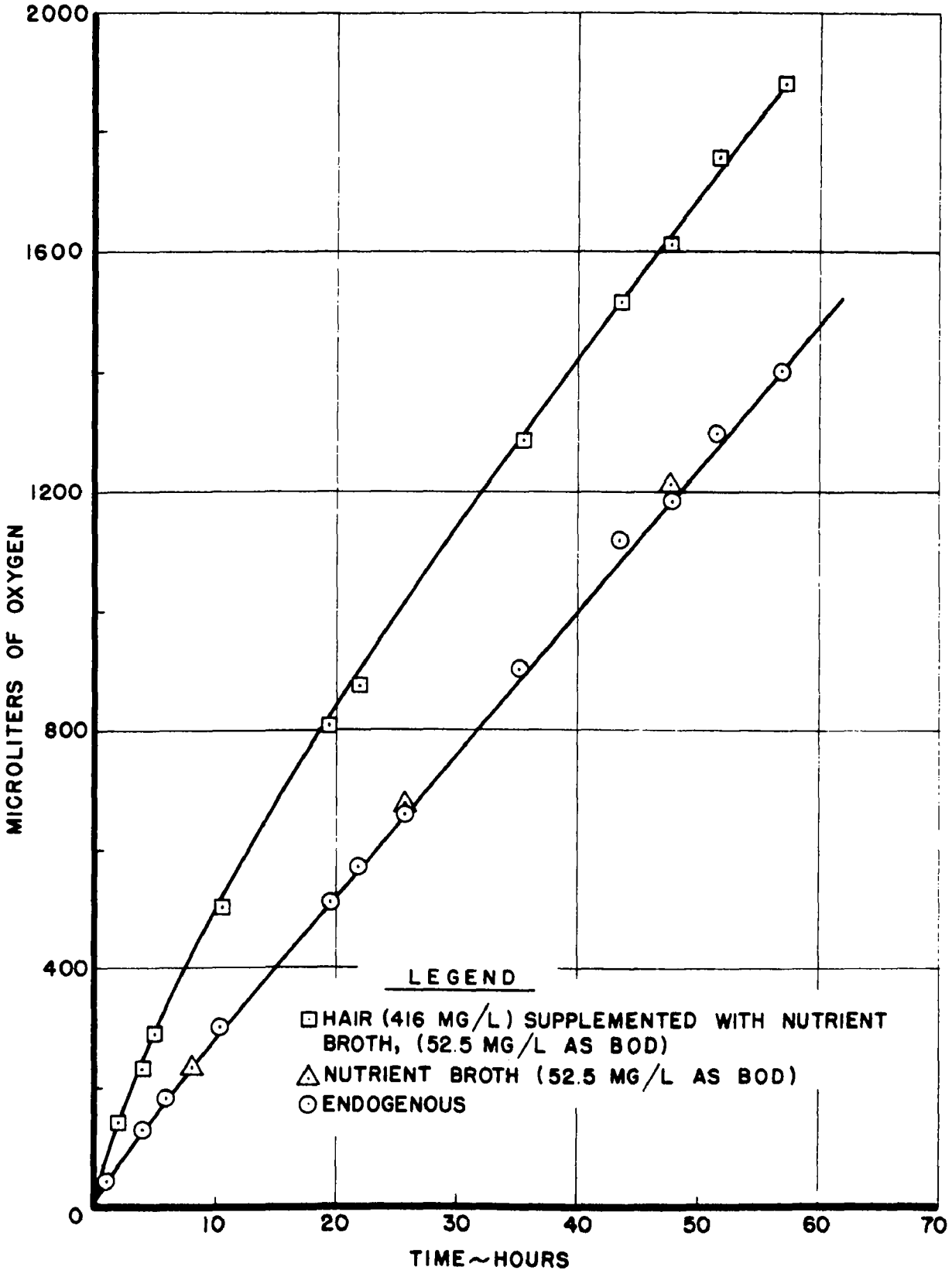


FIG.59 EFFECT OF SUPPLEMENTATION OF KERATINS WITH NUTRIENT BROTH ON OXYGEN UPTAKE

LEGEND

⊙ ENDOGENOUS (ACCLIMATED AND UNACCLIMATED)
△ UNACCLIMATED CULTURE
⊡ ACCLIMATED CULTURE
⬡ ACCLIMATED CULTURE (FEED WAS BOILED 6 HOURS)

FEED- 416 MG/L OF KERATIN
TEMPERATURE- 20°C
SUSP. SOLIDS- 2000 MG/L ACTIVATED SLUDGE SOLIDS

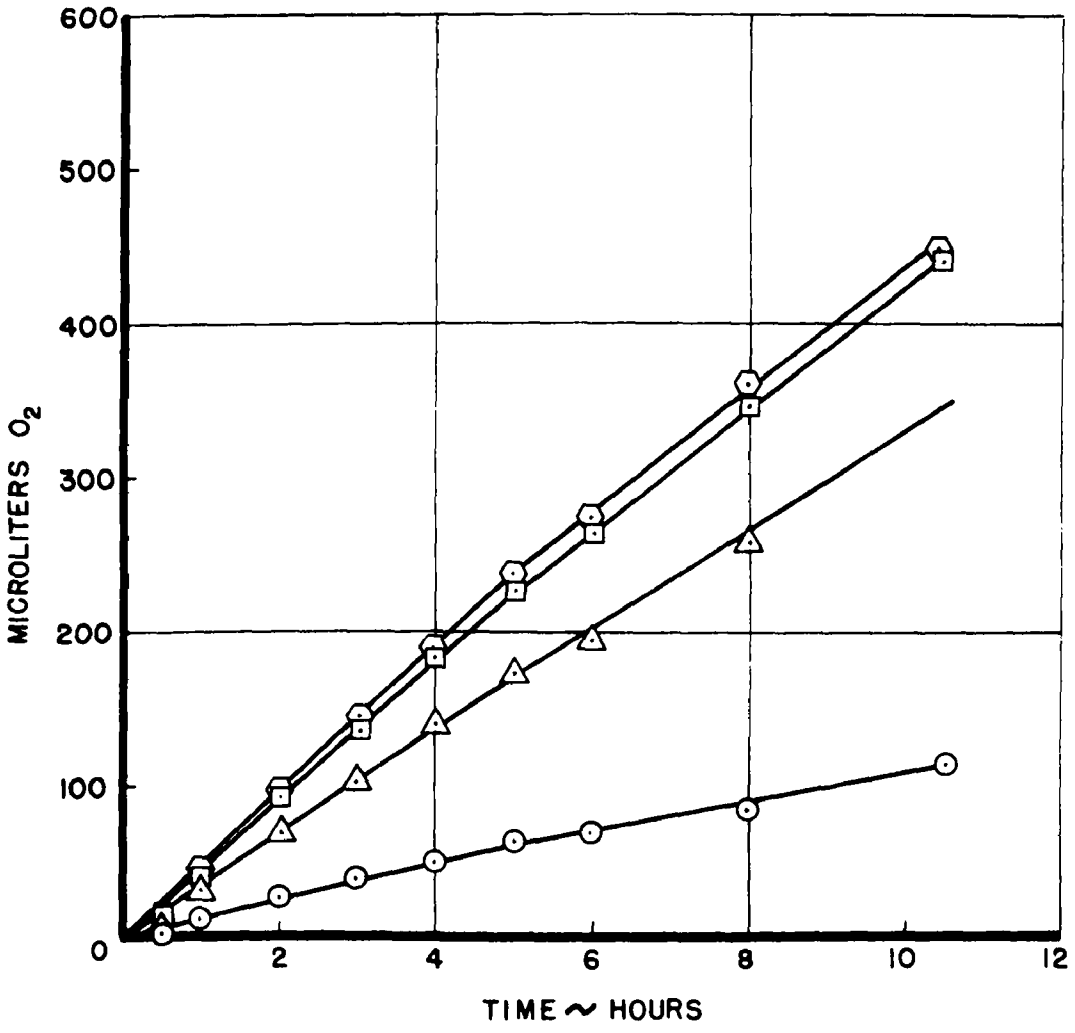
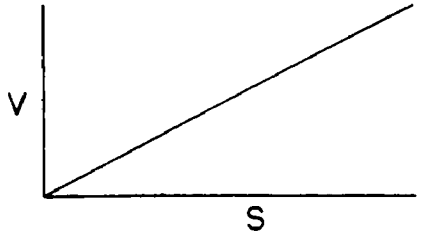


FIG.60 ACCLIMATION EFFECT ON OXYGEN UPTAKE



The final stage in the oxidation scheme of keratin is that of a rate of reaction limited by the amount of substrate present. The oxygen uptake of this period must be reflected by a first order reaction curve,

$$O_2 = Ae^{-kt} \qquad -8-$$

where O_2 = oxygen remaining at time t

A = total oxygen available or remaining at time t = 0

The fact that no first order reaction curve is evident from the experiments suggests that the reaction stops before substrate concentration was noticeable as the limiting factor. Two factors may help to account for the incompleteness of the reaction: toxic products might have accumulated to concentrations sufficient to stop the reaction or the remaining fraction of substrate was fully unutilizable. It may be also that this first order portion of the curve occurred in a time interval not perceptible in the time units measured.

One other important aspect needs consideration. The dissolution of the hair was done in a highly alkaline medium and amino acids racemize at high pH values, the natural L form being converted to the D form. Bacterial systems are highly sensitive to isometric configurations and it has been established that, excepting alanine, the utilization of the D amino acid occurs at a slower rate than for the L form (1). The organisms must change the racemized amino acid back to the L form before it can be utilized. The production of the responsible enzymes, the racemases, could well limit the reaction although racemic mixtures have been shown to be utilized at rates near that of the L form.

Even when the aeration solids yield was high, no significant increase in DNA was obtained which indicates a slow rate of cell division. The net increase in mass may be due to cell volume growth or to accumulation of cell storage material, slime, and debris incorporation in the floc.

SUMMARY AND CONCLUSIONS

The utilization of compounds by mixed biological cultures is influenced by a variety of factors. In this study the central theme has been the consideration of some of these factors that affect the utilization of amino acids by activated sludge systems. Experimental data has been developed on the effects of temperature, stereo-isomerism, peptide linkage sequence and length. In the pursuance of these studies it has been necessary to develop or adapt experimental techniques to reflect or present the desired information. Thus, digital computer programs were designed to present experimental data graphically as well as to store pertinent abstracts in a literature retrieval system. A DNA test was adapted for use with activated sludge cultures and chemical procedures were developed for dissolving keratins prior to use as a substrate.

On the basis of these and other experimental determinations, a number of conclusions can be drawn on the utilization of free and combined amino acids by activated sludge cultures. The summary points outlined below indicate that enzyme systems for incorporation and oxidation of amino acids are increasingly stereo specific with molecular size, are temperature sensitive, increasingly hindered by increasing lengths of peptide chains and, in some cases, are responsive to amino acid sequence in the peptide chain. Further, new cell production, as indicated by DNA, does not directly follow cell mass increases of activated sludge.

In keratins such as hair the complex nature of the incorporated mechanisms thwarting biological breakdown of the compound offer a real challenge to those interested in providing biological decomposition of these compounds in the relatively short time interval used in contemporary waste treatment facilities.

As might be expected the biological utilization of a compound is dependent on the available free energy stored in the compound and in the relative ease with which this energy can be released from the compound.

Some of the other salient observations and conclusions from this research are listed below.

1. D-isomers of amino acids and peptides are not utilized by activated sludge cultures as well as is the natural isomer. The isomeric effect increases with molecular size such that

$$x = \frac{231}{C^{0.76}}$$

where $x = (100) \frac{\text{D isomer oxygen uptake}}{\text{L isomer oxygen uptake}}$

and C = no. of carbon atoms in the D isomer

2. Where acclimation is effective, oxygen uptake rates for amino acids and peptides are related to the free energy in the substrate. The data in this study seemed to fit the straight line:

$$Y = 26.5 - 0.0325F$$

where F = free energy in the substrate in calories per liter

Y = oxygen uptake rate in microliters per hour per milligram sludge solids

3. Special peptide studies with glycyl DL alanine and DL alanyl glycine indicated that, for these two peptides, the sequence of the amino acids in the peptide is very important in their utilizability. While the glycyl DL alanine was essentially removed from the supernatant in 8 hours, DL alanyl glycine was still present after 24 hours. Uptake rates, CO_2 and NH_3 production, were all significantly higher for glycyl DL alanine than for DL alanyl glycine.

4. For temperatures in the range of 10 to 30°C, with a 10°C change in temperature, k_2/k_1 ratios were approximately 2 ± 0.2 for oxygen uptake rates for glycyl DL alanine, DL alanyl glycine and DL alanyl DL phenylalanine. A typical experimental value for the apparent free energy, μ, was 11,400 calories/mole for glycyl DL alanine.

   For temperatures variations above 30°C there was some variation in doubling of oxygen uptake with a 10°C temperature rise because of differences in culture populations. If the activated sludge culture was comprised largely of bacteria, the doubling effect could be expected up to 35°C. However, if a large protozoan population were present temperatures in the 30°C range would be approaching the thermal death point of the protozoa and hence oxygen uptake rates would tend to be depressed above 30°C.

5. The Schneider and Burton method of DNA isolation and determination has been applied to activated sludge cultures. Recommended test conditions are listed under the "Summary on Use of the DNA Test" page 36 .

6. DNA tests on acclimated activated sludge cultures using the amino acids, arginine, alanine, glutamic acid, and phenylalanine, as substrates show-

ed increases in DNA content after feeding. Thus, all four amino acids are an adequate substrate for production of new cells if other environmental conditions are not limiting.

a. The pattern of ultimate increase in DNA content depends on the type and concentrations of amino acids.

b. In terms of DNA change at eight hours after feeding, the aliphatic amino acids (arginine-HCl, DL alanine, and Na-glutamic acid) showed maximum DNA increase at a substrate concentration of 1000 mg/l as a group, whereas phenylalanine, an aromatic amino acid, showed maximum DNA increase at the much lower concentration of 400 mg/l. This experimental evidence suggests that amino acid structure could have a general effect on new cell production with the aliphatic group in one category and the aromatic group in another (See Figure 35).

c. Exceeding these optimum concentrations, a decrease in DNA production indicates the inhibitory effect of overdosage. Also, it should be noted that activated sludge cultures are more tolerant of higher dosages of aliphatic amino acids than of the aromatic acid.

d. Although activated sludge can utilize the four tested amino acids as both carbon and nitrogen sources and can use them for new cell production, the addition of glucose to the substrate increases DNA production (Figure 36).

7. In sanitary engineering reports, growth frequently has been defined as an increase in suspended volatile solids. For the unsteady state phase of batch culture feeding the experimental results in this study showed that the increase in volatile solids correlated with an increase in substrate concentration rather than an increase in DNA content. This experimental evidence from batch cultures indicates that initial unsteady state volatile solids increases result from organic compound accumulation rather than increase in active solids; thus, in this activity phase volatile solids concentration increases would not be a reliable growth parameter.

8. The keratin studies showed that hair can be dissolved at room temperature with a solution of 0.5N to 1N sodium hydroxide. The dissolved keratin is then available for biological degradation in an activated sludge system

with approximately 75% of the material fed removed from solution. The sludge yield is about 58% of the dry weight fed. However, the net DNA content of the activated sludge solids were approximately constant throughout the experiment.

9. Warburg respirometer studies with the dissolved hair as the activated sludge substrate gave zero order reaction rate curves. At the end of 44 hours the material oxidized accounted for 45% of the COD fed. The best efficiency obtained for removal of keratin derived protein was 76%. The addition of substrate supplements such as glucose and nutrient broth did not improve the rate or efficiency of the utilization process.

RECOMMENDATIONS FOR FUTURE WORK

Further research should be concerned with the behavior of other keratins where the difference in composition and structure might affect dissolution and biological utilization. Preliminary work on sea gull feathers show them to be insoluble in 0.5 N NaOH at room temperature until the oily film, which renders them impermeable, is removed with detergent or solvents such as benzene or carbon tetrachloride.

Further study should be given to the possibility of dissolving keratin through biological action because biological dissolution may provide a product more amenable to degradation than chemically dissolved keratin.

The tendency of keratin to foam under aeration may have a practical application in foam fractionation procedures for the removal of dissolved organics in advanced waste treatment. Recirculation of the foamed mixture may enhance the utilization of refractory fractions from the waste or the keratin itself by increasing the detention time in the system.

Further work is needed too on the relationships between cell production and substrate oxidation versus the free energy and molecular configuration and complexity of the substrate. From the indications obtained in this study some underlying biochemical mechanisms should soon become apparent that would be of value in waste treatment system development.

ACKNOWLEDGEMENTS

Information presented in several sections of the report are a contribution from research assistants at the University of Washington. Mr. John Osborn's research was on the utilization of alanyl-glycine and glycyl-alanine, George Capestany developed the keratin studies and the section on the DNA test resulted from the efforts of Feng Li. Tom Shen adapted the KWIC program to the literature of this study. Charles Bowers was consultant and programmer on the graphical display of Warburg respirometer data and William Fung was laboratory assistant for the initial Warburg studies. The author wishes to express his sincere appreciation to these co-authors and investigators.

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APPENDIX I

GRAPHICAL PRESENTATION OF DATA BY DIGITAL COMPUTER

Data Processing and Data Display Systems

Electronic data processing has been incorporated as a beneficial tool in many professional fields in the past decade. Most procedures utilized have been limited to computing, tabulating and summarizing data.

Developments in the past three or four years have shown the feasibility and economy of programming data for automatic graphic display. The processes utilized can make the necessary computations to convert raw to finished data and then present the desired information graphically. This graphical data display can be applied to present scales or plot sizes or, as developed for this Warburg data presentation, the computer program can automatically select the scale dimensions and the size of drawing which most appropriately fit the computed data. Computer systems are especially valuable where large volumes of reiterative data are processed and where many graphical displays are desired.

Applications of computer displays are found in many fields such as presentation of isobars by the U. S. Weather Bureau, population distributions by age groups as prepared by the U. S. Census Bureau, isodose plots indicating the distribution of radioactive iodine in radiotracer thyroid studies or displays of radiation intensities in the earth's atmosphere as relayed by the Tiros satellite.

Several of the electronic data processing programs have been applied to areas of civil engineering and related fields such as in regional planning, transportation and population studies (65). Data displays are available in mapping outputs or in bar graph array outputs. These bar graph arrays may appear with different symbols to show confidence or distribution limits. Machine mapping outputs appear as a series of numbers or symbols. This background of data can be overlaid with a location map transparency by means of check points printed on the machine output and photographed to give a map with the pertinent data appearing in the appropriate map areas. Such processing systems have been used in urban planning studies for plotting housing and population trends. Also, mapping can be photographed from an image displayed on a cathode ray tube. This type of projection has been used to indicate traffic patterns out of Chicago. The desired data is printed as short vectors and the intensity of traffic is indicated by the concentration of vectors

along the arterials emanating from the city center. Other slower processes involve the transferring of data to an inking head that may be directed to any spot on a plane surface by use of either punch card or taped information.

A formidable amount of computations for the Warburg respirometer studies in this project made electronic data processing a realistic means of obtaining the desired data and for graphical presentation of the data. Further, because the various Warburg runs were interrelated it was desirable to be able to compare graphically the results from different runs on a single graphical display.

Because none of the available programs were satisfactory for the processing of data obtained from the Warburg respirometer, it was necessary to develop a new program and display techniques that would provide the necessary flexibility in presentation and the means of computing and comparing the raw input data.

The requirements imposed on the program display were that the computer system present from the raw data the quantity of oxygen utilized as a function of time, the quantity of oxygen utilized per unit of cell mass per unit of time, the relative rates of oxygen used for each substrate expressed in percent and the change in the respiration quotient with elapsed time fater substrate addition to the culture. Also, it was desirable to compare data from several different Warburg runs on a single plot.

Dimensioning demands were placed on the computer program because the time and oxygen uptake scales varied with each batch of data. Also, the areal size of the graphical display was left as a variable with several print-out sizes available to the selection of the programmer.

Program Format and Presentation

The Warburg Respirometer Program as developed for this study is actually a system of subprograms with one main or controlling program. The base program was written in FORTRAN (Formula Translation System) language with the subroutines written in FAP (Formula Assembly Program). These programs have been described previously in the literature as formats for the IBM 709 computer system (66). No tapes, other than standard input and output units, are required. The present format for the computer program now determines from the respirometer manometer readings, the quantity of oxygen utilized per unit of time, the quantity of oxygen used per unit weight of solids per unit of time, and the respirometer quotient for each test run. Also, the program will express oxygen uptake of up to ten

substrates as percentages of any given substrate used as the control or 100 percent value. The program will search the raw data, find the tests and test cases required for any graphical display, perform the desired calculations and then print and plot the results. The program can be expanded by adding subroutines to furnish any other desired calculations or displays from the Warburg data.

The input for the program falls into three major classifications, standard data, test data and plot requests.

Standard Data: The standard data consists of the K-factors for each flask and the abbreviations for the cultures and substrates used on the test data cards.

Test Data: The test data contains two types of cards, test cards and case cards. The test cards contain milli-grams of solids, culture name, number of observations and the time value for each observation. The case cards contain the substrate name, flask number and a respirometer reading fro each observation. Also, the case cards indicate whether the case is a control case and whether the Warburg flask contains potassium hydroxide.

Plot Requests: The plot requests define the type of calculation to be performed, the size of the plot, and the cases to be used. A maximum of ten cases can be plotted together.

The output is both printed in tabular form and plotted. The plot may be varied in size from one to nine pages. Any cases not printed by the computer system are listed in the output.

The actual program used is presented in the appendix. Also representative samples of the output data and graphical display are presented below and in the appendix. The total machine output is on file at the University of Washington, Department of Civil Engineering.

Results of the Warburg Respirometer Studies

The computer system display program developed especially for Warburg respirometer studies permits a convenient means of rapidly computing and displaying the respirometer data. The display program accepts a wide range of values for time and oxygen uptake. The computer automatically set the range of values on the ordinate and abscissa to best fit the data to any desired size of graph up to 99 inches in length. The data plots shown were printed on a preset printout 2 pages long or 15 inches by 22 inches in size.

Summary:

A survey of existing electronic graphical display techniques indicated the necessity of developing a special program for presenting information from respirometer tests. The program designed for the Warburg respirometer is capable of calculating the desired data, presenting this calculated data in tabular form and then displaying the data graphically. The computer system program is flexible in that dimensions of the areal display and of its coordinates can be varied to fit the range of values fed to the program. Also, subroutines can be added to the program which can present additional calculations or graphical presentations as desired by the programmer.

The tabulations and graphical displays in this Warburg respirometer program present the total volume of oxygen used per increment of elapsed time, the volume of oxygen used per unit weight of suspended solids per increment of elapsed time, the respiration quotients and a percentage comparison basis of oxygen uptake rates with any designated control oxygen uptake rate. Other determinations and displays can be added to the output at the discretion of the programmer.

APPENDIX II

KWIC LITERATURE RETRIEVAL SYSTEM

The KWIC (Keyword in Context) Literature Retrieval Program as herein presented is based on formats presented in IBM general information manuals (67 through 71). This program may be applied to either the title, the abstract or the entire text of an article. In this research project the program was restricted to KWIC indexing of titles (72).

To produce a KWIC index of titles, bibliographical entries consisting of at least title, author and literature source are recorded in machine readable form, such as punched cards or paper tape, so that processing can produce a printed index with its related bibliographical data.

The KWIC deck produced contains on each card a portion of a title so chosen that an imbedded keyword starts at column number 25. This deck, consisting of one card for each keyword found in the titles, may be sorted with either a standard tape sort program for a large deck of cards or an EAM sorter for a small deck of cards. The sorting can then produce the KWIC index with keywords aligned on a particular column in printout in alphabetical sequence. Each such contains as well an appropriate "eleven character index word" as a controlling function.

After standard tape or EAM sorting, the program will produce a bibliography listing the articles alphabetically by author and by title. Each entry in the author index and in the title index carries the "eleven character index word".

The "index word" is developed by the IBM computer from the input deck of author, title and source cards. The first six characters of the "index word" are the first four letters of the senior author's last name and his first two initials. The next two characters are the last two digits of the year of publication. The last three letters are first letters of the first three significant words in the title. For example:

1. Author card: BROWN, J P JONES A B
2. Title card: FACTORS AFFECTING THE EFFICIENCY OF TREATMENT PROCESSES
3. Source card: JWPCF - 1960, 29, 12, 1341-55

The "eleven character index word" is then "BROWJP60FAE". This "index word" is inserted in columns 61-71 of every output card.

Keywords to be used in the program indexing may be determined by either ex-

clusion of inclusion in an internally stored word list. For this project, "exclusion criteria" were used whereby a list of unimportant words was prepared. These words such as prepositions, articles, and conjunctions, were then eliminated from the sort and listing process for the key words in the output of the KWIC program. All other words in article titles are listed as subject words in the KWIC output.

Input Deck Preparation

1. Bibliography (Master Record Format)

The input for the KWIC program normally consists of a set of references each of which is processed independently. The format of a single reference is described below.

Each card punched for a given reference has an information field (columns 1-60) which contains the information on the literature reference; a major control column (Column 73) and a minor control column (Column 74) used for sequencing within a particular type of cards. In addition, each card contains the literature identification number (Column 77-80) which is used as an accession number and for bookkeeping purposes. For example, the literature identification number provides a quick means of re-establishing order if card mixups occur. Also, this number can be used to check the completeness of a collection of cards.

The content of the control columns identifies the type of cards according to the following code:

Card Type	Major Control Column Contents	Minor Control Column Contents
Author Card	1	punch 1-9
Title Card	2	punch 1-9
Source Card	3	punch 1-9

Each reference listing as a separate item in itself should consist of at least one of each of the legal type of card in the order author, title, and source.

2. Author Card (Major Control Code 1)

This card carries the author's name, then a space and then the author's initials. If there are several authors, either a separate card is made for each, or as many as three authors are recorded on one card in column 1-18, 21-38, and 41-58, two columns remaining blank between authors. Corporate names are treated as authors if no author is given. When several cards are required to record one

author or corporate entry, the information on the second card is indented four spaces (to column 5) to indicate that it is a continuation of the previous information.

```
1                    21                   41
JONES A B            SMITH C D            NEWTON E P
```

3. Title Card (Major Control Code 2)

The title of the reference item is punched in the information fields of as many title cards as are necessary to completely contain the title. No words should be omitted or abbreviated except in accordance with conventions or established rules as set up in the write up of the KWIC program. Individual words should not be split between information fields of consecutive cards as this would cause them to be interpreted as separate words. When additional cards, beyond the first card, are used, the title continuation is indented two spaces on each card following the initial card.

Card 1:

```
1                                                                  60
USE OF MICROORGANISMS FOR THE QUANTITATIVE DETERMINATION OF
```

Card 2:

```
1 3                                                                60
AMINO ACIDS IN NATURAL MATERIALS
```

4. Source Card (Major Control Code 3)

The source card contains the name and location of the publisher for books or the journal title, volume, and pages, and the date of publication in accordance with conventional library practice. The date field is the last two columns of the information field (columns 90 and 60) and normally contains the last two digits of the year of the title. The date field information is used in processing the cards.

```
1                                                                  60
JWPCF, 1962, 26, 152-162                                         6 2
```

5. Word List

The word list cards contain a single information field (Columns 1-72). The

field begins with Column 1 on the first card of the set and ends with Column 72 on the last card so that no indented spaces are required if several cards are used. The word list itself consists of a series of word entries separated by a comma and a space. The word list is terminated by using a period after the last word.

A typical exclusion list, which was used for title words to be omitted from the KWIC critical word listing, would be as follows:

```
1                                                                    72
ON, IN, BEFORE, AFTER, OF, UNDER, THAT, ITS, NOT, SOME,
1                                                                    72
UPON, BY CERTAIN, A, AN, THE, WILL, DOES.
```

APPENDIX III

EFFECTS OF CHLORIDES ON ACTIVATED SLUDGE CULTURES

Variation in salt concentrations in a nutrient may affect the rate of nutrient utilization by activated sludge. Studies in which various concentrations of chloride were added to cultures of microorganisms are shown in Table 9. The studies reported in Table 9 indicate that the investigators are not in agreement over maximum permissible chloride concentrations. This may be due to differences in laboratory method. Iandolo used a pure culture and controlled pH quite closely. Sachiko used a mixed culture and found that fungi tended to take over and bacteria were reduced at chloride concentrations above 5000 ppm.

Normally concentrations as high as 5000 ppm would not be of concern in the treatment of domestic sewage. Klein (73) reports the following chloride ion concentrations for domestic sewage:

Weak Sewage	70 ppm Cl^-
Medium Sewage	100 ppm Cl^-
Strong Sewage	up to 500 ppm Cl^-

The Public Health Service suggests a maximum of 250 ppm Cl^- for drinking water (74), but some communities have been known to use water with chloride concentrations as high as 2000 ppm (75). From this information it seem safe to assume that a domestic sewage, even under the worst conditions, should not have a chloride concentration much greater than 2000 ppm. However, industrial wastes and sea water infiltration might increase this value considerably. Sea water has a chloride concentration of about 20,000 mg/l (73) and infiltration might give a sewage with chloride concentrations greater than 5000 mg/l. According to Sachiko, chloride concentrations as high as 5000 mg/l would be harmful to the activated sludge culture. Therefore, a study of chloride ion concentration effects on activated sludge might be of value.

In this investigation volatile suspended solid growth and oxygen uptake were studied at various chloride concentrations and an attempt was made to determine maximum permissible chloride ion concentrations.

Chloride Study Results

Solid growth studies showed a stimulation in growth rates with chloride con-

TABLE 9: CHLORIDE EFFECT ON MICROORGANISMS

Organism	Concentration of Cl^-	Comment	Reference
Sewage Bacteria	4000 ppm	microorganism affected	Sachiko (76)
Sewage Bacteria	5000 ppm	reduction in microorganisms. Increase in fungi (raw sewage fed as nutrient source)	Sachiko (76)
Sewage Bacteria	5800 ppm	adequate treatment to sewage if organic loading is closely controlled	Stewart (77)
Staphylococcus aureus	5160 ppm in distilled water	80% Staph. died in 15 minutes 100% in one hour (addition of small amount of nutrient kept Staph.alive at least 24 hours at 37°C)	Parfentjev (78)
Staphylococcus aureus	$\leq$ 24250 ppm	growth rates stimulated at pH 7.0 to 7.5 at 37°C	Iandolo (79)
Staphylococcus aureus	> 24250 ppm	rapid decrease in population	Iandolo (79)

centrations up to 2250 mg/l as shown on Figure 61. Good growth was obtained for concentrations as high as 4500 mg/l. Higher concentrations, 5000 mg/l and greater, resulted in excessive foaming and bulking of the culture making solid determinations unreliable. Values for these higher concentrations are given by Osborn (64) but are not plotted.

Warburg studies showed oxygen uptake without inhibition for chloride concentrations of 2430 mg/l and less, these values are plotted on Figure 62. A culture containing 6075 mg/l of chloride had some inhibition for the first ten hours but seemed to acclimate and at 20 hours the oxygen uptake was as high as for a culture containing no chloride. For the culture containing 12,150 mg/l of chloride there

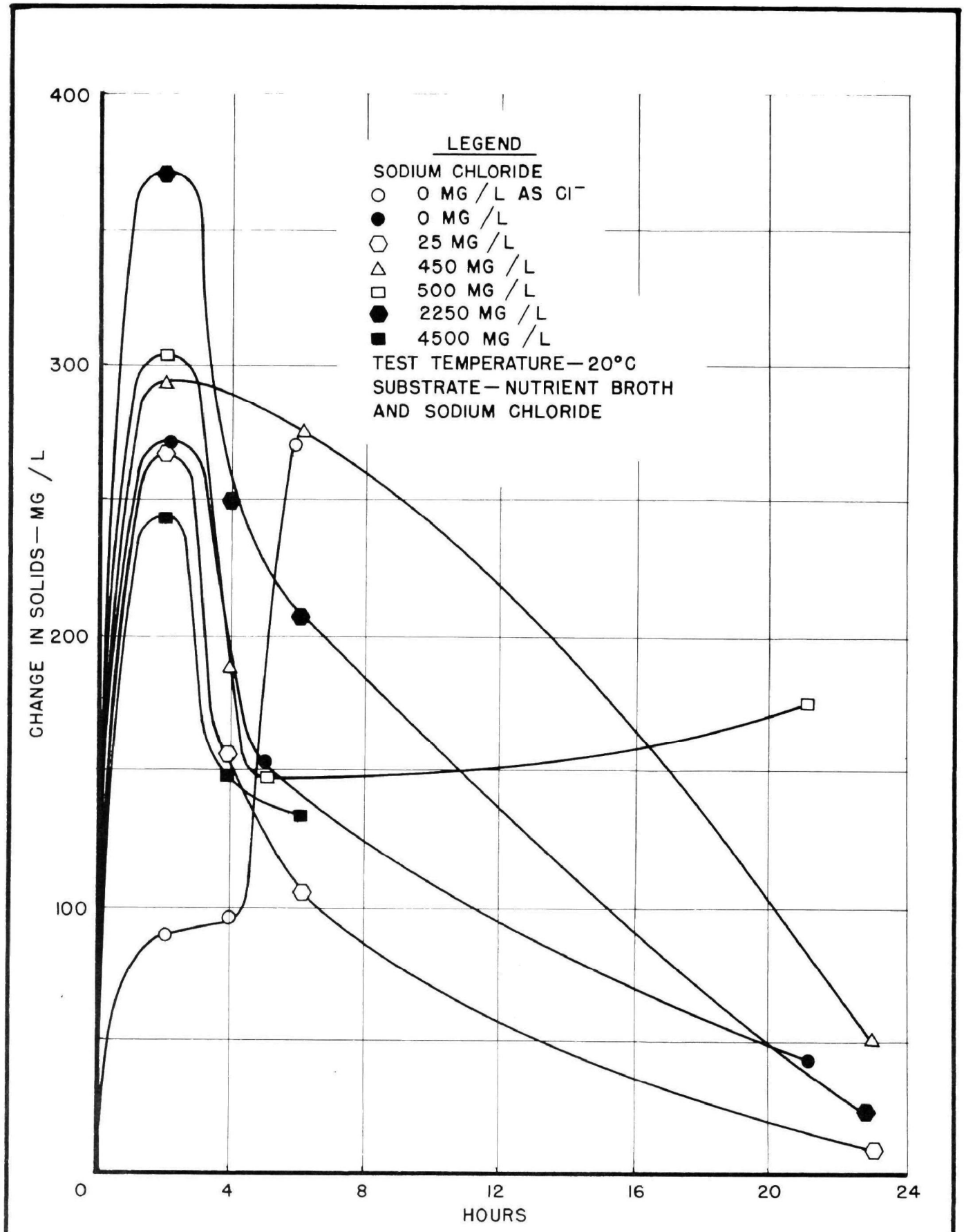

FIG.61 GROWTH IN VOLATILE SUSPENDED SOLIDS

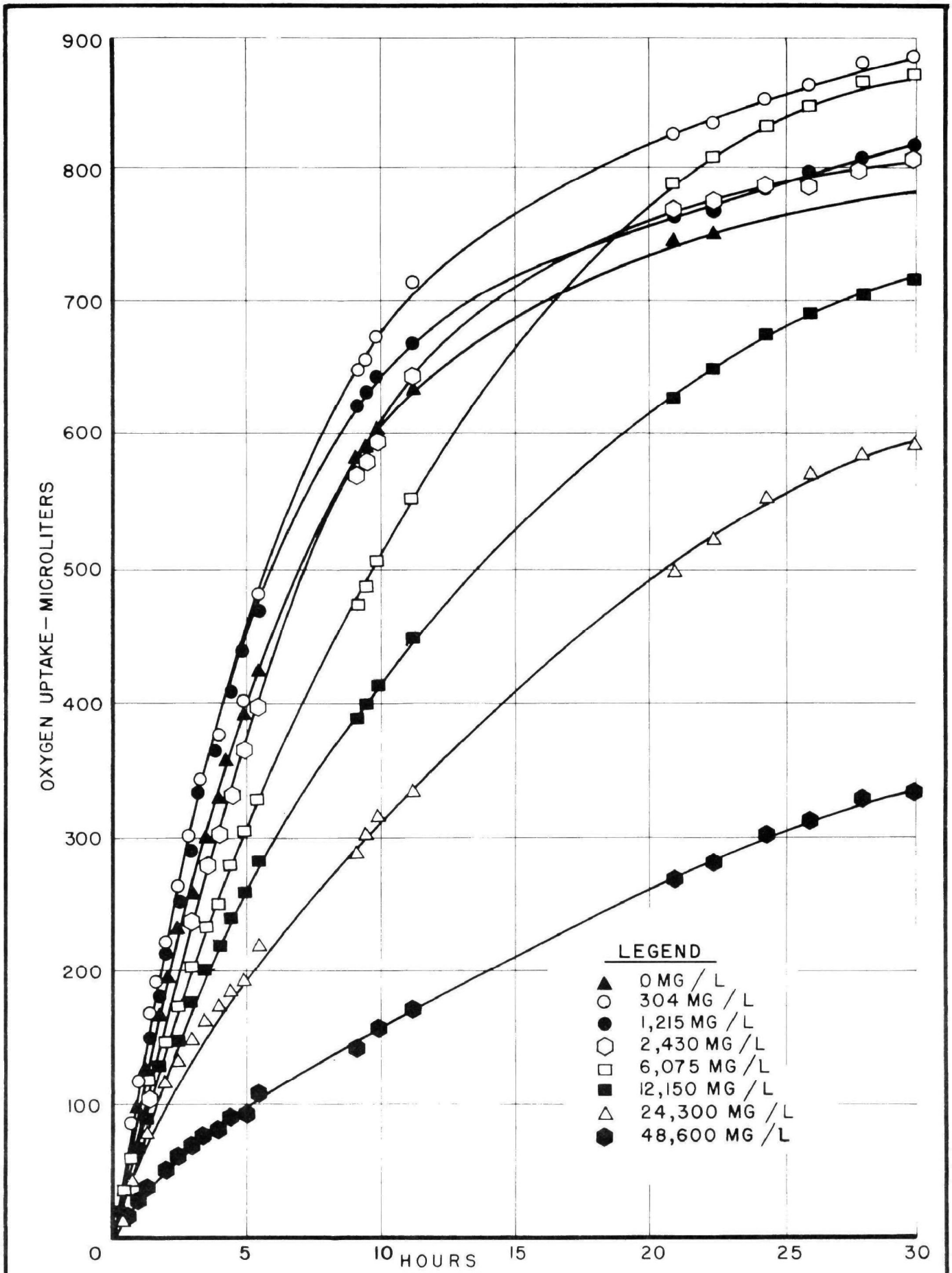

FIG. 62

OXYGEN UPTAKE WITH VARIATION IN CHLORIDE CONCENTRATION

was definite inhibition, although not excessive. Higher concentrations showed increasing degrees of inhibition.

These results might be compared with those found in the literature. According to Sachiko (76) the culture is adversely affected at a chloride concentration of 5000 mg/1. This is substantiated by the foaming and bulking experienced at similar concentrations in this investigation. Iandolo, however, reports good growth at chloride concentrations as high as 24,250 mg/1 if pH is maintained in a range of 7 to 7.5. This might also be reasonable since oxygen uptake results show good uptake for a culture containing 24,300 mg/1 of chloride even though there is inhibition.

A review of the results of this investigation combined with data from the literature might lead to the following conclusions:

Maximum chloride concentration for unbuffered cultures is 5000 mg/1. If buffers and conditioners are used to reduce foaming and bulking chloride concentrations as high as 24,300 mg/1 might be allowed without harm to the culture.

APPENDIX IV

CHEMICAL DATA ON PEPTIDES AND AMINO ACIDS

TABLE 10: ENTROPIES, HEATS OF FORMATION, AND FREE ENERGIES OF FORMATION @ 298.1°C (80)

Cpd	Entropy[a]	Entropy[a] Formation	Heat of Combustion[b]	Heat of Formation[b]	Free Energy of Formation[b]
L-Alanine	31.6	-153.7	387,210	-134,600	-88,780
DL-Alanine	31.6	-153.7	386,620	-135,190	-89,380
L-Asparagine	41.7	-208.0	460,850	-189,360	-127,360
L-Asparagine-H_2O	51.0	-254.4	458,130	-260,390	-184,560
L-Aspartic Acid	41.5	-194.2	382,720	-233,330	-175,440
L-Cysteine	40.6	-152.3	532,200	-127,880	-82,480
L-Cystine	68.5	-286.1	997,700	-251,920	-166,630
Glycine	26.1	-126.6	232,600	-126,660	-88,920
L-Glutamic Acid	45.7	-222.6	537,450	-271,160	-174,800
Hippuric Acid	57.1	-192.1	1,007,860	-147,710	-90,440
L-Leucine	49.5	-233.6	856,090	-153,390	-83,750
D-Leucine	49.5	-233.6	856,110	-153,360	-83,720
DL-Leucine	49.5	-233.6	855,320	-154,160	-84,520
DL-Leucylglycine	67.2	-312.8	1,093,330	-207,100	-113,850
L-Tyrosine	53.0	-227.4	1,058,450	-165,430	97,640
Carbon Dioxide (Gas)				-94,240	-94,100
Water (Liquid)				-68,310	-56,720

[a]In entropy units, calories per degree per mole.

[b]Calories per mole.

Peptide	Molecular Weight	$-\Delta H_r$ k cal/mole	$-\Delta H_f$ k cal/mole[a]	S	ΔS	F_f° k cal/mole[b]	Free Energy of Hydrolysis
DL-Alanyl glycine	146.2	625.9	185.8	51.0	-231.3	-116.85	-3730
Glycyl glycine	132.1	471.4	178.0	45.4	-204.4	-117.10	-3230
Hippuric Acid	179.2	1007.8	145.9	57.2	-192.0	-88.63	-2260
DL-Leucyl glycine	188.2	1093.4	205.4	67.2	- 12.8	-112.13	-2960

[a]Heat of formation of water taken as -68,317 calories and of CO_2 from graphite as -94.030 calories.

[b]From classic relation: $\Delta F_f^\circ = \Delta H_f - T \Delta s$

Glycyl-L-Leucine bond = 2110±50

Glycyl-ϕ Ala " = 2550±50

Tyrosine-glycine " = 1550±100

"The energy functions of the peptide bond constitute a key to the solution of many problems of protein synthesis. It is evident from the table above that hydrolysis of these peptides proceeds spontaneously, with a net loss of approximately 3,000 calories per mole".