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ADENOSINE TRIPHOSPHATE (ATP) IN GRASSLAND SOIL: ITS RELATIONSHIP TO MICROBIAL BIOMASS AND ACTIVITY

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ABSTRACT

Measurements of adenosine triphosphate (ATP) concentration were employed as a basis for estimating microbial abundance (biomass) and respiratory activity. ATP levels were closely correlated with numbers and carbon contents of viable cells (bacteria) and with rates of O_2 uptake (fungi) for pure cultures of soil microorganisms grown in liquid media. Also, for soil samples incubated in the laboratory, ATP concentrations quantitatively reflected changes in numbers of viable bacteria and actinomycetes.

A study was made of the relationship of ATP levels to microbial abundance and activity in soil samples collected between June and September 1971 at the irrigated and nonirrigated sectors of the Environmental Stress Area of the Pawnee site. Log of ATP concentration was highly correlated with log of oxygen uptake rate (measured in laboratory) and with log of numbers of viable bacteria, actinomycetes, and fungi. ATP concentration was utilized to calculate total dry weight and carbon content of the viable microflora. The possibility of using ATP and respiratory values to calculate microbial turnover times in grassland soil was explored.

CHAPTER 1

INTRODUCTION

The soil microflora plays a major role in the transformation of energy and nutrients in terrestial ecosystems. To characterize quantitatively the microbial contribution to these processes, information is needed on the abundance and activity of the soil microbiota.

Microbial abundance in soil is customarily given in terms of numbers of viable cells or of biomass (from estimates of total cellular material) per weight of soil or per area of soil surface to a given depth. The dilution plate count technique is commonly utilized for estimating viable cells, while the direct microscopic count technique is employed for estimating total cellular material. However, both procedures are time-consuming and subject to large experimental errors. Also, the data obtained are often difficult to interpret in terms of ecosystem processes.

Because of these problems associated with the use of the classical techniques for measuring microbial abundance, attempts have been made to find more satisfactory methods. Chemical procedures based on the measurement of a specific cell constituent would theoretically suffice to estimate microbial numbers and biomass if two requirements are met: (1) the concentration of the particular material is constant in relation to the mass of the cell, and (2) the material is unstable and rapidly degraded upon the death of the cell. Recently, Levin and co-workers (1964) introduced the use of adenosine triphosphate (ATP) measurements for the quantitative detection and counting of bacteria.

The present study was designed to assess the feasibility of using ATP concentration as an index of microbial abundance and activity in soil. The specific objectives were: (1) to develop a method of measuring the concentrations of microbial ATP in soil; (2) to determine for axenic cultures of microorganisms the quantitative relationship of ATP to other cellular constituents and to cell viability; (3) to determine the quantitative relationship in soil of ATP content to microbial abundance; (4) to determine the suitability of ATP measurements for following microbial population changes in the soil of a grassland ecosystem during a growing season, and; (5) to determine the quantitative relationship of ATP concentrations to respiration in grassland soil samples.

CHAPTER II

LITERATURE REVIEW

Introduction

Microbial abundance in soil is customarily given in terms of numbers of viable cells per weight of soil or per area of soil surface to a given depth. Viable cells are usually counted by the classical dilution plate technique. Abundance may also be expressed on a biomass basis, that is, weight of living cells per weight of soil or per area of soil surface, and is commonly estimated from direct microscopic cell counts in the case of bacteria or, for fungi, from measurements of mycelium lengths. The volume of the cellular material is calculated, and from a knowledge of the specific gravity of the tissue, an estimate is made of the cell weight (wet or dry). Specific gravity values are usually determined for cells grown in pure culture, and values of 1 to 1.5 are used for biomass calculations (Gray and Williams, 1971).

Underestimations of viable cell numbers in soil from plate counts are likely to result because of the following: (1) not all viable organisms will produce colonies on a single medium or under a particular set of incubation conditions; (2) cells remain clumped together or attached to soil particles; (3) cells are adsorbed to pipet walls; (4) cells are killed in the dilution medium; and (5) some spores fail to germinate. Total counts obtained by direct microscopic count tend to result in overestimations of the true microbial biomass because of the difficulties in differentiating living cells from dead cells and even bits of organic debris.

In theory, biomass values and counts of viable cells are related. However, because of the limitations of the methods employed in making these determinations, it is not possible to calculate an accurate biomass for microorganisms in soil on the basis of viable cell counts. According to Parkinson, et al. (1971), the plate count technique is useful as a basis of biomass determinations if the propagules of the organisms being examined are of roughly uniform size and weight. Thus it can provide useful information for some bacteria and yeasts, but it is less useful for actinomycetes and filamentous fungi; it is not possible to distinguish colonies arising from hyphae as opposed to those developing from spores.

Recently a number of chemical techniques have been employed to estimate microbial abundance in soil. The literature concerning these methods will be reviewed with particular attention given to use of ATP measurements as indicators of microbial biomass in pure culture and in complex natural media. The review will consider the principle of the bioluminescence assay for ATP, factors influencing cellular ATP levels, and the employment of the ATP assay in different fields of study.

Chemical Methods Other than the ATP Assay for the Determination of Microbial Biomass in Natural Ecosystems

A number of chemical methods have been utilized to estimate total microbial biomass in water, sewage, and soil samples containing mixed populations of microorganisms.

Measurement of microbial cell constituents in soil

Jenkinson (1966) proposed a method for estimating the total biomass of the soil microflora. In a soil sterilized with chloroform and then reinoculated with nonsterile soil,

a small fraction of the organic matter was rapidly converted to carbon dioxide. Jenkinson considered this portion, which was 2.3 to 3.4% of the soil carbon, to have been derived from recently killed microorganisms, and therefore contained the carbon of the microbial biomass. This technique has yet to be applied to other situations.

Muramic acid, a cell constituent of bacteria has been utilized by Millar and Casida (1970) as an index of bacterial biomass. Muramic acid concentrations ranging from 0 to 150 μg/g dry weight of soil were obtained for 33 soil samples; the values were much higher than could be accounted for on the basis of platable bacterial populations in the soil. Their results suggest that there are many more muramic acid-containing bacteria in soil than found on dilution plates. Alternatively, bacteria in soil may have thicker cell walls and thus contain more muramic acid, or dead cells may contribute to the muramic acid pool in soil. Failure to distinguish between these possibilities prevents the adoption of this method for accurate measurement of the bacterial biomass in soil.

Steubing (1970) found that the chlorophyll content of acid forest soil was correlated with the number of algae (obtained by plate count method). According to Paul and Tu (1965), it may be possible to relate amino acid levels and numbers of microorganisms in soil. Among the amino acids, diaminopimelic acid (DAP) has become of special interest since it is not found in higher plants but is a constituent of the cell wall of some bacteria, especially of sporeforming strains (Meister, 1965). Shazly and Hungate (1966) utilized DAP measurements to estimate rumen bacterial growth. Steubing (1970b) developed a method for extracting and measuring soil DAP. She found that DAP concentrations were related to bacterial masses at different depths in soil.

Measurement of microbial cell constituents in water and sewage samples

The relative biomass of heterotrophic microorganisms on suspended matter in sea water was calculated by Seki (1970), based on the uptake of C^{14}-labeled glucose by the microorganisms in sea water and the glucose uptake rate of a pure marine Vibrio sp.

Holm-Hansen (1969) found algal biomass estimations in ocean profiles based on chlorophyll measurements in excellent agreement with estimations by direct examination.

Attempts to measure active cell mass in activated sludge by methods more specific than the total suspended solids or volatile suspended solids techniques have been reviewed by Weddle and Jenkins (1971). Measurements of deoxyribonucleic acid (DNA) have been proposed for the estimate of microbial biomass in sludge (Southwest Missouri State College, 1971). However, Holm-Hansen (1969) found large amounts of DNA in the detrital fraction in ocean water profiles, so that its usefulness as an indicator of microbial biomass may be limited.

Determination of Microbial Biomass on the Basis of ATP Measurements

Most chemical methods for determining microbial biomass utilize organic compounds unique to specific groups of microorganisms. ATP is found in all living organisms (Huennekens and Whitely, 1960) and is not conserved in nonviable cells, nor is it associated with nonliving materials (Holm-Hansen and Booth, 1966; Hamilton and Holm-Hansen, 1967; Doxtader, 1969). Hence, it might be expected that ATP concentration would be related to the amount of microbial tissue in a system.

The bioluminescence assay for measuring ATP concentration

Enzymatic reactions involving light production and ATP. ATP can be quantitatively measured using the firefly bioluminescent assay (McElroy, 1947). The simplified reaction steps of the luciferin-luciferase enzyme system resulting in bioluminescence (Strehler, 1965; Seliger and McElroy, 1965; McElroy, et al., 1969) are:

1) $LH_2 + ATP + E \xrightarrow{Mg^{++}} E \cdot LH_2\text{-}AMP + PP$

2) $E \cdot LH_2\text{-}AMP + O_2 \longrightarrow E + \text{product} + CO_2 + AMP + \text{light}$

where LH_2 is luciferin, E is luciferase, PP is pyrophosphate, and AMP is adenylic acid. The first step is the initial activation, involving the formation of an enzyme-bound luciferyl adenylate and pyrophosphate. This enzyme complex reacts with molecular oxygen to produce light (step 2). A detailed organic mechanism by which a molecule of D(-)luciferyl adenylate combines with molecular oxygen to give a product molecule in an excited electronic state which emits light on its return to ground state has been proposed (McElroy, et al., 1969).

One quantum of light is emitted for each luciferin molecule (Seliger and McElroy, 1959). Also, the total light output is directly proportional to the amounts of ATP and luciferin present. Hence, when all the other components of the enzyme system are in excess, the reaction becomes quantitatively specific for ATP.

According to Seliger and McElroy (1965), ATP is the only nucleoside triphosphate that will function in the production of light. Deoxyadenosine triphosphate is completely inactive. In crude extracts, adenosine diphosphate (ADP) will function for light emission due to the presence of myokinase. Also, in the presence of ADP and active transphosphorylases, a number of other nucleotide triphosphates will support luminescence. Holm-Hansen and Booth (1966)

found ADP, cytidine-5-triphosphate (CTP) and inosine-5-triphosphate (ITP) effected light emission. The light intensity from the addition of ADP resulted in less than 1% of that from an equivalent amount of ATP. However, both CTP and ITP stimulated light production equivalent to ATP response. Iwamura and coworkers (1963) found a ratio of ATP to GTP and UTP to be 4.5: 1 as an average of all the stages in the life cycle of Chlorella ellipsoidea. Holm-Hansen and Booth (1966) gave an estimate of about 5-35% error in the determination of ATP as a result of the participation of other nucleoside triphosphates in the reaction.

Methods of measuring light. Two methods of quantitating light production have been used. In one, the intensity of the initial light flash produced upon mixing of sample and enzyme is measured with a photometer (Shropshire and Gettens, 1966; Holmsen, et al., 1966; Lyman and DeVincenzo, 1967; Rasmussen and Nielsen, 1968; Klofat, et al., 1969; Tsujinomoto, et al., 1970 and Johnson, et al., 1970). In the other method, a quantum counter such as a liquid scintillation spectrometer is used to measure the number of photons produced (Tal, et al., 1964; Addanki, et al., 1966; Aledort, et al., 1966; Stanley and Williams, 1969; Dufresne and Gitelman, 1970; Schram, 1970; and Tsujinomoto, et al., 1970). With both methods light measured is proportional to ATP concentration.

Medium and temperature effects on the bioluminescent reaction. Aledort and coworkers (1966) studied the ionic effects on bioluminescence and found light emission rates were reduced linearly with increasing concentrations of several cations in the following order: $Ca^{++} > K^{+} > Na^{+} > Rb^{+} > Li^{+} >$ choline^{++}; while anion inhibition occurred in

the following order: $I^- > H_2PO_4^- > Br^- > ClO_3^- > Cl^- > Fl^-$ $HCO_3^- > COOCH_3 \cdot H_2O^-$. Van Dyke (1969) found chloride ion (20-200 mM NaCl) markedly reduced the amount of light detected. To alleviate the problem of variable ionic composition, interfering ions have been diluted out (Van Dyke, 1969; Patterson, et al., 1970), or removed by cation exchange resin (Harris, et al., 1971) or by Sephadex G-10 column (Van Dyke, 1969). McElroy and Strehler (1949) concluded from their studies that a pH of 7.5 and a temperature of approximately 25 C are optimal for measuring luminescence. Chappelle and Levin (1968) reported that over the temperature range of 21 to 27 C there was little change in ATP response. The bioluminescent reaction was inactivated at pH values below 5.5 (Seliger and McElroy, 1960) and by temperatures above 35 C (McElroy and Strehler, 1949).

Purification of enzyme. To improve the sensitivity and reproducibility of the ATP assay, gel filtration of the luciferin-luciferase extracts from firefly tails has been suggested (Nielsen and Rasmussen, 1968; Cortenbosch and Schram, 1971). This procedure eliminates kinase and contaminating ATP in the crude extracts.

Techniques for the extraction of ATP from microbial cells and complex environments

Holm-Hansen and Booth (1966) evaluated the use of perchloric acid, boiling ethanol, boiling water, and boiling trishydroxymethylaminomethane (Tris) buffer for killing and extraction of ATP from algal cells. The last three solvents all gave satisfactory results, although boiling Tris buffer (0.02 M, pH 7.75) was chosen because of its simplicity and reproducibility. Perchloric acid extraction was not satisfcatory because the neutralized solution was found to be strongly inhibitory to the luciferase reaction.

To allow for the detection of only bacterial ATP in urine specimens, nonbacterial ATP was removed by using octylphenoxypolyethoxyethanol to lyse leukocytes, erythrocytes and tissue cells; apyrase was used to hydrolyze the released ATP and any soluble ATP excreted in the urine (Picciolo, et al., 1971). After boiling the sample to destroy apyrase activity, perchloric acid (1 N) was used to extract ATP from bacterial cells, KOH was used to neutralize the solution, which was brought to a final pH 7.4 with buffer. Contrary to the findings of Holm-Hansen and Booth (1966), no inhibition of the luciferase system was noted.

Chappelle and Levin (1968) used a solution containing 0.1 M Tris, 0.01 M potassium arsenate, 0.01 M EDTA, and 6% n-butanol for extracting ATP from bacterial cells. Boiling Tris buffer was found satisfactory for ATP extraction from marine bacterial cells (Hamilton and Holm-Hansen, 1967) and from activated sludge (Patterson, et al., 1970).

Lee, et al. (1971a), analyzed lake sediments and found ATP recoveries ranging from 20 to 85% when they used cold H_2SO_4 as an extracting solution. The extracts were passed through a cation exchange resin to remove interfering substances. These workers found dilute $HClO_4$, neutral dimethylsulfoxide (DMSO) and acidic DMSO comparable to the H_2SO_4 and butanol to be the most efficient extractants of ATP from soil and litter with 82-92% recovery.

Physiological and environmental factors influencing cellular ATP levels

The use of ATP as an index of microbial biomass depends, in part, on the assumption that ATP is present as a relatively constant component of the biomass of diverse microorganisms. A number of investigators have studied ATP levels in diverse organisms growing under varying conditions.

Effect of growth stage. Forrest (1965) studied the ATP pool of Streptococcus faecalis throughout its growth cycle. The level of ATP rose throughout the lag phase to a maximum of 5.5-6.5 µg ATP/mg cells at the point where exponential growth began, then fell steadily to 3 µg ATP/mg as growth proceeded.

In studies of three bacterial species D'Eustachio and Levin (1967) found a relatively constant level of 1.54×10^{-10} µg ATP/cell throughout all phases of growth. Lee and his associates (1971b) using Aerobacter aerogenes observed a marked increase in ATP content to 40×10^{-10} µg/cell during the late lag-early log phase followed by a rapid decline and stabilizing at 5×10^{-10} µg/cell in the stationary phase. This is in agreement with earlier work with a number of marine bacteria in which it was shown that the ATP content in chemostat-grown cells ranged from $5\text{-}65 \times 10^{-10}$ µg ATP/cell (Hamilton and Holm-Hansen, 1967). However, when the amount of ATP was expressed as a fraction of cellular constituents, microbial cells of different species were found to contain a fairly constant amount of ATP. Hamilton and Holm-Hansen (1967) reported an average ATP content of 0.4% of cell carbon for the bacteria they examined.

Holm-Hansen (1970) measured the content of ATP relative to cell size and organic carbon in 30 different algal cultures representing 7 phyla. He showed that there was considerable constancy of ATP levels in algae ranging in size from less than 1 pg C/cell to 215,000 pg/cell and reported an average ATP to C ratio of 0.0035, which is close to the ratio reported for bacteria (Hamilton and Holm-Hansen, 1967; Kelly and Syrett, 1956; Cole, et al., 1967; and Forrest, 1965) and for fungi (Dawes and Large, 1970).

Effect of oxygen tension and solute concentration. Knowles and Smith (1970) working with Azotobacter vinelandii grown under aerobic conditions found 3.97 µg ATP/mg dry weight; in the absence of oxygen the value fell to about 1/4 of the aerobic level. Cole, et al. (1967), had earlier observed under conditions of glucose limitation, the mean ATP concentration in Escherichia coli was 3.46 µg/mg dry weight under aerobic conditions and 2.24 µg/mg dry weight under anaerobic conditions. Strange, et al. (1963), similarly found the ATP content of freshly gathered and washed A. aerogenes varied with oxygen tension, the ATP value of 1.0 µg/mg dry weight under anaerobic conditions was half that under aerobic conditions. In addition Strange, et al. (1963), found that ATP content varied with solute concentration.

Effect of starvation, nitrogen and phosphorus deficiency. ATP concentrations in starved marine bacterial cells were one-fifth of those found in cells grown in a nutrient broth (Hamilton and Holm-Hansen, 1967).

Holm-Hansen (1970) showed that for algae under conditions of extreme nitrogen or phosphorus deficiency, the cellular ATP levels were 20 to 50% less than those found in cells grown in a complete medium.

A fresh water green alga Selenastrum capricornutum was found to contain 3.4, 3.1 and 1.4 µg ATP/mg dry weight of living biomass under phosphorus-rich, balanced and phosphorus deficient medium conditions, respectively (Lee, et al., 1971). For increased accuracy in algal dominated microbial systems known to be phosphorus deficient, Lee, et al. (1971), suggested that the lower ATP/living biomass value be used in calculating algal biomass.

Relationship of ATP concentration to microbial abundance in various materials

The feasibility of using ATP as an index of microbial biomass or numbers has been examined by several workers in studies of human disease, aerospace water systems, exobiology, food, activated sludge, marine waters and sediments, lake sediments, and soil.

Medical microbiology. Levin and coworkers (1964) utilized the ATP firefly luminescent assay for detection of bacteria, and for determining ATP levels in cancerous and noncancerous tissues of mouse and monkey and animal cells infected with adenovirus. Their findings indicated a possible method for studying the kinetics of virus infection, cancer development and other disease mechanisms. Picciolo and associates (1971) developed an automated luciferase assay of bacteria in urine which would facilitate the diagnosis of urinary-tract infection.

Water systems and exobiology. In a study of the ATP assay for the rapid detection of microorganisms in aerospace water systems, as few as 100-300 bacterial cells were detected (Levin, et al., 1968). The possibility of detecting extraterrestrial life based on ATP determination has been suggested (MacLeod, et al., 1969; Butenko, et al., 1970) and would depend on the similarity of the biochemistry of extraterrestrial life to that on earth (Levin, 1964).

Food Microbiology. D'Eustachio, et al. (1968), found instrumental ATP counts were comparable to plate counts when contaminated samples of food and milk were analyzed. The utility of ATP determination as a means of detecting bacterial contamination in foods depends on the efficiency

with which bacterial ATP can be separated from nonbacterial ATP derived from the original tissue and/or on their relative levels. In studies of ATP levels in contaminated food, Sharpe and coworkers (1970) observed that intrinsic ATP decreased during incubation while bacterial ATP increased. They concluded that at sufficiently high levels of contamination, ATP concentrations could be related to bacterial numbers.

Activated sludge. In activated sludge, ATP was found to be constant at 10^{-8} to 10^{-9} µg ATP/viable cell over the growth rate range of 0.03-6.4 day^{-1} (Weddle and Jenkins, 1971). Patterson, et al. (1969), working with activated sludge found ATP levels of 0.2-0.3 µg ATP/g sludge; ATP content reflected toxicity of pH and mercury to activated sludge cultures, and the ATP pool responded quickly to changes in metabolic activity of activity sludge. The studies of Patterson, et al., indicated the usefulness of ATP as a biomass indicator, an estimator of toxic stress on microbial systems, and an activity parameter for activated sludge operations.

Oceans. In studies of phytoplankton production and food chain dynamics in marine water, the use of ATP measurements for biomass estimations have been employed. Measurements of ATP concentrations in ocean water samples coupled with laboratory data on the ATP content of a variety of marine microorganisms permitted estimates of the distribution of living cells at different depths in the ocean (Holm-Hansen and Booth, 1966; Hamilton and Holm-Hansen, 1967; Holm-Hansen, 1969). Hamilton and Holm-Hansen (1967) concluded that ATP data provided a useful estimate of heterotrophic biomass in the oceans.

Marine and lake sediments. ATP has been extracted from marine and lake sediments and evaluated as an indicator of biomass. Ernst (1970) used ATP levels in marine sediment to calculate carbon content of biomass and concluded that the carbon content of living matter amounted to 0.13-1.0 of total carbon content in marine sediments. Lee and coworkers (1971) found ATP (calculated from plate count data) constituted a minor fraction. Their data suggested that ATP measurements may be utilized to measure the presence of microorganisms which do not develop on conventional plate count media and to estimate the relative amounts of C, N, P and S associated with living organisms of a system.

Soils. MacLeod, et al. (1969), found ATP levels of twenty-five soil samples to range from 0.8 to 130 x 10^{-2} µg/g soil; this variation was much greater than could be accounted for on the basis of count of viable bacteria. These workers concluded that under the conditions used, the firefly assay did not provide an accurate index of bacterial population levels in soils, but the procedure was capable of detecting ATP in a variety of soil types, i.e., arctic tundra, Chilean dessert, lateritic and podzolic soils. They did not take into consideration the contribution of non-bacterial sources to the soil ATP pool. Doxtader (1969b) working with pure cultures of bacteria inoculated into sterilized soil found a close correlation between bacterial numbers estimated from dilution plates and ATP concentrations. However, corresponding relationship for fungal species were more variable. The ATP procedure appeared to be satisfactory for estimating biomass of soil bacteria but seemed unable to measure accurately the fungal component of the soil microflora. This shortcoming would limit the usefulness of the ATP method in determining the biomass of mixed microbial populations found under natural conditions.

Conclusions from Literature Review

Among the chemical methods employed in the determination of microbial abundance in natural environments, the procedure based on the measurement of ATP would seem to hold considerable promise. There are a number of important properties of ATP in this regard: (1) ATP is present in all living cells; (2) ATP is degraded rapidly upon death of cells; and (3) ATP can be sensitively and specifically measured using the bioluminescent assay. A major disadvantage would be the difficulty in determining the fraction of soil ATP derived from each microbial group.

The utilization of ATP measurements as quantitative indices of microbial biomass and activity in soil requires: (1) development of a procedure for measuring microbial ATP in soil; (2) quantitation of the relationship of ATP to other cell constituents, to cell viability, and to respiration for soil microorganisms; and, (3) quantitation of the relationship in soil of ATP to microbial abundance and to microbial respiratory activity. Also, the problem of the possible contribution of non-microbial sources to the soil ATP pool would have to be considered.

CHAPTER III

MATERIALS AND METHODS

Description of Study Site

The study area is located on the Pawnee Site, U. S. International Biological Program Grassland Biome study. This site is on the Central Plains Experimental Range (Agricultural Research Service, USDA) and adjacent areas of the Pawnee National Grassland (Forest Service, USDA). The site was established in 1968 to serve as the intensive study area for the Grassland Biome. Study plots are located in Weld County, Colorado, 40 miles N. E. of Fort Collins in Section 15 and 23, Township 10N., Range 66W. Sections 15 and 23 were designated for study purposes of all major trophic levels in a short grass ecosystem.

Isolation of Microorganisms from Grassland Soil Samples

As a preliminary step in determining the suitability of using ATP measurements for following microbial abundance and activity in soil, microbial strains were isolated from grassland soil. Soil samples of the Ascalon series were obtained from the Pawnee Site, U. S. International Biological Program, Grassland Biome. The tops of the grass were cut off and discarded together with the roots and other plant debris. The soil was crushed, mixed, and sieved through a 20-mesh sieve. Ten grams of soil was added to 90 ml sterile, distilled water, and serial dilutions were made. One-ml aliquots of suitable dilutions were transferred to

petri plates (five replicate plates per dilution). Modified soil extract agar (Bunt and Rovira, 1955) was used for counting bacteria and actinomycetes. For counting fungi, Martin's medium (Martin, 1950) was employed. The plates were incubated for 5 days at 30 C at which time colony counts were made. Bacterial, actinomycete, and fungal colonies from plates of appropriate dilution were transferred to agar slants. The cultures were purified by repeated streaking on agar plates, using modified soil extract agar for bacteria, Jensen's medium (Jensen, 1930) for actinomycetes, and Martin's medium for fungi. Gram-stained preparations of the bacterial cultures were examined microscopically. The bacterial isolates were maintained on agar slants of nutrient agar with 1% yeast extract added; actinomycete isolation agar was used for actinomycetes and potato dextrose agar for fungi. The agar media and yeast extract were products of the Difco Company.

Liquid Culture Media and Growth Conditions

Bacterial cultures

A loopful of culture grown on nutrient agar (Difco) was transferred to 100 ml nutrient broth (Difco) with 0.1% yeast extract contained in a 250-ml Erlenmeyer flask. This starter culture was incubated for 12-16 hrs on a rotary shaker at approximately 30 C. An aliquot of the culture was added to fresh liquid medium contained in a 500-ml Erlenmeyer flask (10% starter culture) and incubated at 28-30 C on a rotary shaker. Samples were withdrawn periodically and analyzed for numbers of viable cells by the plate count method, turbidity, and cellular ATP, carbon content. Culture turbidity was measured at 660 nm utilizing an Evelyn colorimeter.

Actinomycete cultures

A loopful of culture grown on a slant of Difco Actinomycete Isolation Agar was streaked on an agar plate of the same medium and incubated for 5-10 days at 30 C. Three to five ml of sterile Tween-80 solution (3 drops Tween-80 in 300-ml distilled water) were added to the agar plate. The actinomycete growth on the agar plate was scraped off and transferred to 50 ml Actinomycete Isolation Broth (2.0 g sodium caseinate, 0.1 g asparagine, 4.0 g sodium propionate, 0.5 g dipotassium phosphate, 0.1 g magnesium sulfate, 0.001 g ferrous sulfate and 1 l distilled water) in a 100-ml Erlenmeyer flask or 250-ml in a 500-ml flask. After 4 days of incubation at 28-30 C on a rotary shaker, 10 ml of the starter culture was added to 100-ml Actinomycete Isolation for ATP content and respiration (O_2 uptake) rate. Culture liquid was filtered through a Millipore filter (0.45 u pore size, 25 mm diameter) prior to ATP analysis.

Fungal cultures

Approximately 4 ml sterile Tween-80 solution (3 drops Tween-80 in 300 ml distilled water) was added to a 6-day old culture on Difco Potato Dextrose Agar slant. The culture was scraped off, transferred to 100 ml NPY broth (8.0 g Difco Nutrient Broth, 5.0 g Difco Peptone and 2.5 g Difco Yeast Extract in one l distilled water) medium and incubated for 2 days at 30 C on a rotary shaker. This starter culture and 10 ml Tween-80 solution were added to 400 ml fresh liquid medium. Two-ml aliquots of this culture were dispensed into test tubes containing 3 ml NPY broth. At each sampling period, 12 test tubes were withdrawn from the shaker, 4 were taken for ATP determinations, 4 for respiration rate measurements and 4 for dry weight analyses. The samples were filtered on Millipore filters (0.45 u pore size, 25 mm diameter) prior to ATP and dry weight analyses.

Counts of Viable Microbial Cells

Liquid cultures

Aliquots of bacterial culture liquid were serially diluted in 1% peptone solution. One-ml aliquots of suitable dilutions were transferred to petri plates of Difco Plate Count Agar. Four replicate plates were used per dilution. The plates were incubated at 30 C for 5-7 days and the number of colonies per plate counted.

Soil cultures

Counts of viable microbial cells in soil were determined using the plate count method. Ten grams of soil were added to 90 ml sterile, distilled water, from which serial dilutions were prepared. One-ml aliquots of suitable dilutions were transferred to petri plates, using five replicate plates per dilution. Difco Plate Count Agar was used for counting bacteria, Difco Actinomycete Isolation Agar for counting actinomycetes, and Martin's medium for counting fungi. Each plate containing between 20 and 300 colonies was counted after 5-7 days of incubation at 30 C. Microbial counts were expressed on the basis of oven-dry soil.

Bioluminescence Measurement of ATP

ATP was quantitatively measured by use of a firefly luciferin-luciferase enzyme system. In the presence of ATP, the luciferase system emits light, the amount being directly proportional to the level of ATP.

Light production was measured using a Nuclear Chicago Mark I liquid scintillation counter. The machine settings were as follows:

Channel A	A500
Background Subtract	Off
Start	One cycle to begin count and switched to auto recycle for 3 consecutive 0.1 min counts
Stop	Time
Print	Scaler A
Display	A channel
Ratios	Off
Scaler A	1 M
Minutes	1
Operation	Manual

Mode setting at back of machine was switched from Coincidence-Sum to Singles MPT 1.

Each aliquot of ATP standard or test solution was made to 3 ml with Tris buffer (0.02 M, pH 7.4) and placed in a vial. Counting with the scintillation instrument was started exactly 30 sec after the addition of 0.1 ml of the firefly enzyme (prepared as indicated below) to the otherwise complete assay mixture. Counts were taken for three 0.1-min intervals. ATP levels in units of ng or pmoles were plotted against instrument counts (total counts for three 0.1-min intervals).

The luciferin-luciferase mixture was prepared by adding 10 ml of cold, distilled water to 250 mg arsenate-buffered, powdered firefly lantern extract (Sigma Chemical Co., St. Louis, Mo.). The mixture was allowed to stand for 4 hr at 4 C after which it was centrifuged for 15 min at 12,100 x g at 0 C using a Sorvall Superspeed RC-2 Automatic Refrigerated Centrifuge. Removal of particulate material by centrifugation resulted in better reproducibility in the assay. Two-ml aliquots were frozen immediately. The enzyme-containing mixture was thawed and kept cold in an ice bath while in use.

A stock solution of 0.2 M Tris buffer was made by dissolving 6.05 g Trishydroxymethylaminomethane in powder form in 250 ml distilled water and adjusting the pH to 7.4 with 2 N H_2SO_4. Twenty ml of 0.5 M $MgSO_4$ was added to the Tris buffer to produce a concentration of 0.04 $MgSO_4$. The stock solution was stored in a refrigerator. A working buffer solution was prepared by diluting the stock solution 1:10 with distilled water. This diluted buffer was then stored in a refrigerator.

ATP standard stock solution (10^{-4} M) was prepared by dissolving 6.4 mg of the disodium dihydrate of Adenosine Triphosphate (Nutritional Biochemical Company) in 100 ml 0.02 M Tris buffer. Aliquots of the standard were frozen. A working standard solution was made each day by diluting the stock solution to 10^{-7} M.

Evaluation of ATP-extracting Procedures

Various procedures for extracting ATP were evaluated by analyzing ATP-containing solutions, microbial cells, and soil.

Measurement of ATP standard solution

To evaluate the efficiency of three possible ATP extracting solutions, the recovery of ATP in these solutions was measured. In one method, modified from the procedure of Holm-Hansen and Booth (1966), 3 ml of boiling Tris buffer (pH 7.4, 0.02 M) was mixed with 1 ml of ATP standard solution (10^{-7} M) contained in a 10-ml beaker and then placed in a boiling water bath for 5 min. The extract was made up to 5 ml with cold Tris buffer (pH 7.4, 0.02 M). In the second method, altered version of technique given in the Du Pont luminescence biometer manual (1969), 1 ml butanol was added to 1 ml standard ATP-containing solution in a 30-ml

centrifuge tube and the mixture shaken for 1 min on a Vortex mixer (Super-mixer, Lab-Line Co.). Then 8 ml octanol was added, the tube closed with a rubber stopper, the mixture shaken for 10 sec and then centrifuged at 1085 x g in a Sorvall Superspeed Centrifuge for 10 min. The butanol-octanol layer was discarded and aliquots of the aqueous layer were diluted to 5 ml with Tris buffer. For the third extracting procedure, modified from the method given by Cole and Ross (1966), 3 ml of ATP standard solution was mixed with 3 ml 80% formic acid and filtered through Whatman No. 43 filter paper. Then 80% ethanol was used to wash the filter and to make the filtrate to a volume of 25 ml. An aliquot was evaporated to dryness under a stream of air by use of a N-Evap apparatus (Organomation Assoc.) and the residue dissolved in Tris buffer (pH 7.4, 0.02 M). Aliquots of the diluted ATP-containing solutions obtained by the three procedures were analyzed for ATP.

Extraction of ATP from microbial cells

To extract bacterial and fungal ATP, cells were collected on Millipore membrane filters (0.45 μ pore size, 25 mm diameter). The filters were then boiled in Tris buffer (pH 7.4, 0.02 M) or mixed with butanol-octanol solution after the addition of the Tris buffer. For the analysis of bacteria and fungi grown in liquid media, 1.0 to 5.0 ml of culture fluid was filtered through the membrane. Unfiltered culture liquid (0.5 to 1.0 ml) containing bacterial cells was also extracted. A modified version of the boiling Tris method of Holm-Hansen and Booth (1966) for extracting ATP was utilized. Bacterial cells from culture liquid were collected on a Millipore filter; each filter was then placed in a 10-ml beaker. Boiling Tris buffer (4 ml) was added, and the beaker was placed in a boiling water bath for 5 min. The filter was

separated from the extract and extracted once more using 0.5 ml Tris buffer in a beaker placed in a boiling water bath for 2 min. The extracts were combined, made to 5 ml in a volumetric flask, and stored at -20 C.

Extraction of ATP from soil

Several methods for extracting ATP from microbial populations in soil were tried in order to find a simple and efficient assay procedure.

One-gram quantities of soil were extracted directly using boiling Tris buffer (pH 7.4, 0.02 M). The procedure was the same as that used for extracting ATP from unfiltered microbial cells.

Extraction of soil ATP with butanol-octanol solution. In method I, 1 ml butanol was added to 0.1 g soil contained in a 30-ml centrifuge tube, and the tube was mixed for 1 min on a Vortex mixer (Super-mixer, Lab-Line Co.). One ml of Tris buffer (pH 7.4, 0.02 M) was added and the suspension again mixed for 1 min. Then 8 ml octanol was added; the mixture was shaken for 10 sec, centrifuged in a Sorvall Superspeed Centrifuge at 1085 x g for 10 min, and the butanol-octanol layer was removed. Aliquots of the aqueous layer were removed for ATP analysis.

In Method II, 90 ml Tris buffer (pH 7.4, 0.02 M) was added to 10.0 g soil in a 150-ml beaker. After 1 min of continuous stirring with a magnetic stirrer, three 1-ml aliquots were withdrawn from the 3-cm depth of the suspension with a wide-tipped Schwarz/Mann Biopette automatic pipet and placed in a 30-ml centrifuge tube containing 3 ml butanol. The centrifuge tube was mixed for 1 min on the Vortex mixer. Twenty ml octanol was added and the tube mixed manually (20 sec) after being closed with a rubber stopper. The mixture was then centrifuged at 1085 x g for

10 min in a Sorvall Superspeed Centrifuge. The butanol-octanol layer was discarded, and 1 ml of the Tris layer was removed and made to 10 ml with the Tris buffer solution. Aliquots of this solution were used for ATP determinations.

For Method III, 1.0 g soil was placed in a 30-ml centrifuge tube and 5 ml Tris buffer (pH 7.4, 0.02 M) was added. The tube was shaken for 1 min on a Vortex mixer. Then 15 ml octanol was added, and the tube was covered, shaken, and centrifuged as in Method II. A 0.5-ml aliquot of the Tris layer was made to 10 ml volume with the Tris buffer solution prior to ATP analysis. The rest of the Tris layer was placed in a vial and kept in the freezer for possible later use.

Determination of Cellular Carbon

For determination of "total" cellular carbon, 10-ml samples of bacterial culture liquids were centrifuged for 10 min at 17,300 x g in the Sorvall Superspeed centrifuge. The cells were washed twice with 0.02 M phosphate buffer (pH 7.0), centrifuged again, resuspended in water, made to 10 ml with distilled water and kept frozen until analyses were performed.

A modification of the method of Lu and coworkers (1959) was used to measure the readily oxidizable organic carbon of microbial tissue ("total" carbon). To an aliquot of cell suspension (made to 4 ml with distilled water), was added 8 ml of potassium dichromate-sulfuric acid solution (1.27 g $K_2Cr_2O_7$ in 200 ml 96% H_2SO_4). After letting the reaction mixture stand for 30 min, measurements were made on an Evelyn colorimeter at 620 nm against a reagent blank consisting of distilled water and the potassium

dichromate-sulfuric acid solution. A standard carbon solution was prepared by dissolving 0.12 g sucrose in 100 ml distilled water. To convert the results to µg C, the sucrose values were multiplied by 0.4206.

Liquid Culture Respiration Studies

Oxygen consumption rates of fungal and actinomycete isolates were determined with a Gilson Differential Respirometer Model GR 20 in a constant temperature room set at 25 C. The respirometer water bath temperature was 25 C. Warburg flasks with 15 cm^3 capacity were used as thermobarometer and sample flasks. The reference flask (250 ml capacity) contained 20 ml sterile distilled water. Each of four thermobarometer flasks contained 1.0 ml of 10% KOH in the sidearm and 5-7 ml sterile NPY broth for fungi or actinomycete isolation broth for actinomycetes. The sample flasks (4 replicates) contained 5-7 ml liquid culture and 1 ml of 10% KOH in the sidearm. All flasks were equilibrated for 30 min in the water bath (instrument controls were in open position for the first 15 min and then closed for the remaining 15 min) prior to taking readings. The flasks were shaken throughout the equilibration period and during the time when respiration measurements were made. Readings were taken every 10 min over a 1 hr period. To calculate respiration rate in µl O_2 consumed/hr, the initial zero time micromanometer was subtracted from the 60-min reading of the sample flasks and corrected for thermobarometer changes. A thermobarometer correction was made for every respirometer run using the average difference between 60-min thermobarometer micromanometer reading and zero time thermobarometer reading.

In one experiment, fungal isolate F-1 was incubated for 48 hr in test tubes on a shaker at 27-30 C. At frequent intervals, tube contents were transferred to respiration flasks, 2 ml of 10% glucose were added to 5 ml liquid culture and oxygen uptake rates were determined.

Field Study During a Plant Growth Season

The field plots used in studies of ATP concentrations and respiration rates in soil over a plant growth season were part of the system stress study area of 15 ha. The plots are located on a sandy loam soil in the Ascalon series at the Pawnee site. The stress study area is rectangular in shape with the long axis oriented east and west and consists of 2 replicate plots which is further divided into 4 treatment plots. The topography is uniformly level except for the northwest corner of plot 1 which slopes to the north. Two subplots of 4.64 m^2 (50 ft^2) each were chosen, one in the irrigated treatment plot (designated E) and another in the nonirrigated treatment plot (designated D) located in replicate plot 1.

A Cobra pneumatic hammer (Atlas Copco Co., Denver, Colorado) was used to collect 5-cm diameter soil cores, 1 m in length. A small hand-operated jack was used in extracting the cores from the ground. Each soil core was divided into 6 sections: 0-3 cm, 3-6 cm, 6-10 cm, 15-20 cm, 30-40 cm, 60-70 cm. Two composite samples, each consisting of 12 separate cores, were taken at each field treatment. The soil samples were placed in plastic bags and taken to the laboratory. Plant materials (tops, crowns and root segments visible to the eye were removed by hand from the soil samples. Samples were crushed, mixed in a shell blender and subsamples removed for analysis.

Soil Respiration Studies

The Gilson respirometer and experimental conditions found optimum for soil oxygen uptake in previous studies (J. W. Nyhan, personal communication) were used in the soil respiration studies. Warburg flasks with 130-cm^3 capacity were used for thermobarometer and sample flasks. The reference flask, 250 ml capacity, contained 20 ml sterile distilled water. Two thermobarometer flasks, each containing 3.0 ml sterile distilled water and 1.0 ml 10% KOH in the sidearm, were used for each respirometer run. Triplicate sample flasks each containing 1.0 ml of 10% KOH in the sidearm and 30.0 g soil were utilized. Three soil treatments were employed. Some soils were unamended (field moisture level); some were amended with sterile distilled water (to bring soil moisture to 25% on weight basis) and some with water and glucose (2.0 ml of 4% glucose solution added to soil and when necessary, sufficient distilled water to bring soil moisture to 25%). All flasks were equilibrated for 45 min in the water bath at 25 C (instrument controls were in open position for the first 15 min and then in closed setting for the remaining 30 min). Readings were taken every 15 min over an hour period, except for the highly active 0-3 cm depth soil samples amended with glucose and water, which were read over a 30 min period.

Respiration rates were calculated in terms of µl O_2 consumed/hr/30 g dry soil by subtracting the initial zero-time micromanometer reading from the 60-min reading of the sample flasks and correcting for thermobarometer changes. However, for the highly active 0-3 cm depth soil samples, the respiration rate was calculated by subtracting the initial micromanometer reading from the 30-min reading and multiplying the difference by 2, followed by corrections for

thermobarometer changes. A thermobarometer correction was made for every respirometer run by using the average difference between the final thermobarometer micromanometer reading and the initial thermobarometer reading.

Soil Water Determination

Soil water content was determined by the gravimetric procedure described by Gardner (1965). ATP concentration, oxygen uptake rates and numbers of viable microbial cells were expressed on the basis of oven-dry soil weight.

CHAPTER IV

RESULTS AND DISCUSSION

Studies with Microbial Isolates Grown in Liquid Cultures

Isolation of microbial strains from the soil

As a first step in determining the relationship of ATP levels to microbial abundance, bacterial, actinomycete and fungal strains were isolated from grassland soil (Ascalon series). A plate count of the soil taken from the 0-10 cm depth yielded the following microbial numbers (per gram of soil): 56.6×10^5 bacteria, 21.2×10^5 actinomycetes and 70.4×10^3 fungi. The isolates included 4 bacterial, 8 actinomycete, and 5 fungal strains.

Development of procedures for the extraction and measurement of microbial ATP

ATP analytical procedure. ATP was quantitatively measured by use of a firefly luciferin-luciferase enzyme system. Two approaches to measuring light production from this system have been employed. One method measures the initial light flash when ATP and the enzyme extract are mixed together. The other procedure measures the light produced over a definite time interval after adding the enzyme to the otherwise complete assay mixture. The latter method was followed using a liquid scintillation spectrometer to measure light produced during the 18-sec interval beginning 30 sec after addition of the enzyme.

The standard procedure consisted of adding 0.1 ml of firefly enzyme preparation to 3 ml of sample or standard in Tris buffer and measuring the resulting light emission. A typical standard calibration curve relating light emission and ATP concentration for a range of 2.5 to 15 pmoles (1.6 to 9.6 ng) is shown in Fig. 1. As little as 1 pmole ATP was detected. In many instances, great sensitivity of the method would allow one to dilute samples to minimize or exclude possible inhibitory substances.

Regression analysis of the relationship between ATP concentration and light emission gave an equation of $Y = 6.9 + 6.8X$, where Y is light emitted (10^4 counts/18 sec) and X is ATP content (pmoles). Typically, an r^2 value of 0.98 was obtained. The linearity of the standard curve was reproducible but the intercept and slope varied for different batches of firefly extracts.

Effect of bacterial cell extracts on ATP determination. To determine the influence of bacterial cells on the quantitation of ATP by the luciferin-luciferase reaction, three sets of ATP standards were prepared and analyzed. A standard curve covering the range of 2.5 to 10 pmoles (1.6 to 6.4 ng ATP) was prepared according to the standard procedure. To another set of standards, 0.05-ml aliquots of bacterial isolate B-5 cell extract were added. The cell extract was prepared by boiling an aliquot of an 18-hr liquid culture of isolate B-5 in Tris buffer. A third calibration curve was prepared by adding 5 pmoles ATP to varying amounts of bacterial cell extract. The results are shown in Fig. 2. The regression equations had highly significant r values of 0.99 ($n = 10$). The regression equations (Y = light emitted [10^4 counts/18 sec] and X = pmoles ATP for the first two equations and X = ml cell extract for the third equation) were as follows:

Fig. 1. A standard calibration curve relating light emission and ATP concentration.

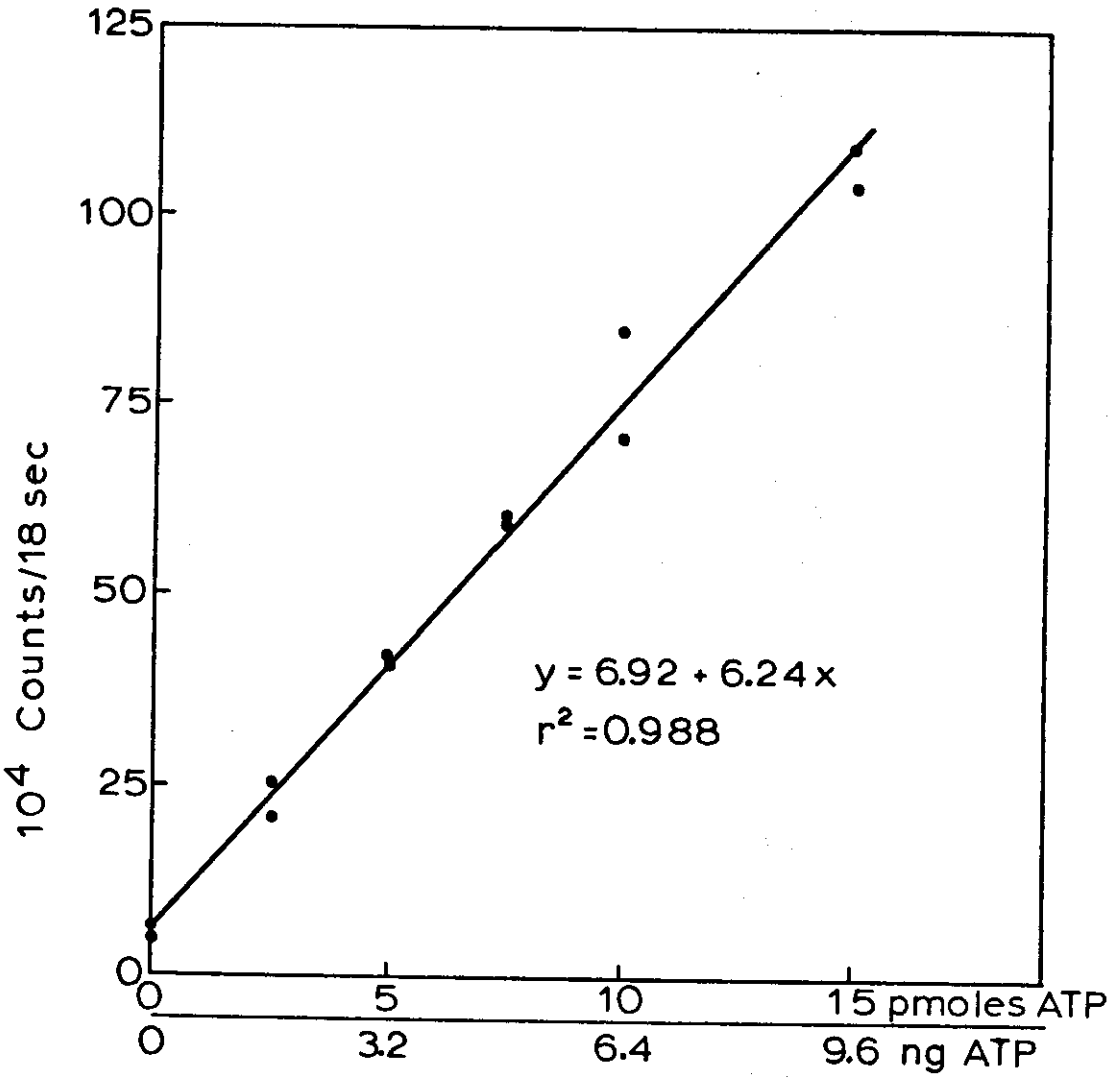
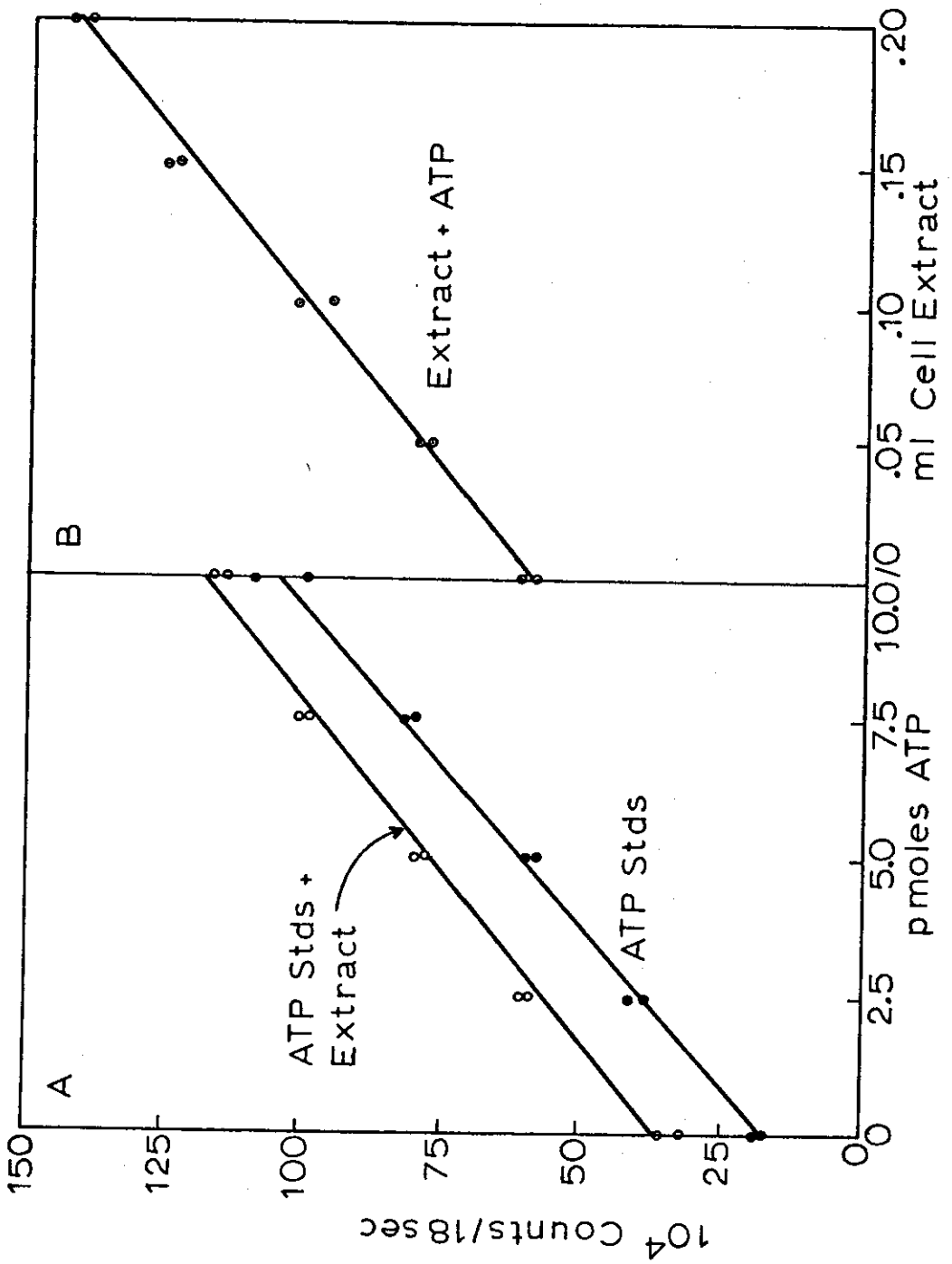

125
100
75
50
25
0
10^4 Counts/18 sec
y = 6.92 + 6.24x
r^2 = 0.988
0
5
10
15 pmoles ATP
0
3.2
6.4
9.6 ng ATP

Fig. 2. Relationship between light emission and ATP concentration with and without internal standard of bacterial extract containing ATP.

A. ATP standards and ATP standards plus 0.05 ml cell extract.
B. ATP standard (5 pmoles) plus different amounts of cell extract.


A
B
ATP Stds + Extract
ATP Stds
Extract + ATP
10^4 Counts/18 sec
150
125
100
75
50
25
0
2.5
5.0
7.5
10.0/0
pmoles ATP
.05
.10
.15
.20
ml Cell Extract

ATP standard curve

$Y = 18.2 + 8.7X$

ATP standards + 0.05 ml cell extract

$Y = 36.8 + 8.1X$

Cell extract + 5 pmoles ATP

$Y = 57.7 + 425.9X$

Thus, the response of the bioluminescent assay to cell extract was in proportion to the ATP content of the extract. No evidence of suppression of ATP-induced light emission was evident.

Effect of three extracting procedures on ATP measurements. Three possible solutions for the extraction of ATP from cells were evaluated by measuring the recovery of known amounts of ATP. In the first procedure, boiling Tris buffer was added to a volume of ATP standard solution; the mixture was then boiled, made up to volume with cold Tris buffer and analyzed for ATP. For the second extraction method, butanol was added to a volume of ATP solution; the mixture was shaken, mixed with octanol, shaken again, and then centrifuged. The butanol-octanol layer was discarded, and aliquots of the aqueous layer were removed, diluted with Tris buffer and analyzed for ATP. In the third method, formic acid was mixed with ATP-containing solution; the clear solution was filtered through a medium-grade filter paper (as would be required for the analysis of cells and soil). Ethanol was used to wash the filter and to make the filtrate to 25 ml. An aliquot was evaporated to dryness; the residue was then dissolved in Tris buffer and analyzed for ATP. With all three extracting solutions, ATP levels were read from a standard curve prepared as previously described. Recovery of ATP from the extracting solutions was calculated by dividing the measured ATP concentration by the amount of ATP originally added to the extracting solutions. The highest

recoveries of ATP were obtained with Tris buffer and butanol-octanol (Table 1). Therefore boiling Tris buffer and butanol-octanol were tested for their ability to extract ATP from cells and soil.

Table 1. Analysis of ATP in three extraction solutions.

Extractant	(a) ATP added	(b) ATP detected*	(a)/(b) x 100
	---------ng ATP----------		
Boiling Tris buffer (pH 7.4, 0.02 M)	64.0	59.0	92
Butanol-octanol	64.0	56.4	88
Formic acid-ethanol	192.0	143.7	75

* Values are means of 8 determinations (2 extractions and 4 determinations per extract).

ATP concentrations in fungi and actinomycetes

As a preliminary step in determining the applicability of the ATP assay to the measurement of microbial tissue in soil, the microbial strains isolated from grassland soil were grown in pure culture and analyzed for ATP content.

Each actinomycete strain was inoculated into 250 ml Actinomycete Isolation Broth in a 500-ml Erlenmeyer flask and incubated for 5 days. The fungal isolates were grown for 3 days in 500-ml Erlenmeyer flasks containing 250 ml NPY medium. Aliquots from the actinomycete and fungal cultures were filtered on Millipore filters; the ATP content and dry weight of the filtered material was then determined (Table 2). Where comparisons are possible, ATP concentrations were higher for the butanol-octanol extractant than for the Tris buffer method. ATP levels among the fungal and

Table 2. ATP contents of fungal and actinomycete isolates.

Isolate*	ATP extracted by	
	Boiling Tris buffer	Butanol-octanol
	µg ATP/g cells**	
F-1	89.87	1306.92
F-2	--	885.45
F-3	--	1858.82
F-4	94.72	--
F-6	523.31	879.02
A-1	323.39	--
A-2	301.27	--
A-3	400.64	--
A-8	640.12	--
A-9	728.99	--
A-7	974.03	1268.19
A-10	222.52	573.93
A-11	211.55	987.42

* F = fungus; A = actinomycete.

** Dry weight.

actinomycete strains ranged from 90 to 974 µg/g dry weight (Tris buffer extraction) and 574 to 1858 µg/g dry weight (butanol-octanol extraction). Macgregor (1970) measured the ATP content of species of Fusarium, Humicola, Aspergillus, an unidentified phycomycete and Nocardia; he reported values of 55 to 235 µg/g weight. The mean ATP concentration reported by Ausmus (1972) for fungal and actinomycete tissue was much lower, 1.7×10^{-2} µg/g (it could not be determined if values were on a wet- or dry-weight basis). The difference among ATP values in these various studies may likely result from variations in extracting procedures and to differences in cultures ages. Macgregor extracted with boiling Tris buffer while Ausmus utilized dilute sulfuric acid. Neither of these workers indicated the ages of culture material taken for analysis.

Relationship of ATP levels to growth in bacterial cultures

The relationships of ATP concentrations to cell numbers in cultures of bacterial isolates B-5, B-6, B-7 and B-10 grown in a liquid medium (nutrient broth with yeast extract) were determined. As shown in Figure 3, for the bacterial isolate B-5, changes in ATP concentrations closely paralleled fluctuations in numbers of viable cells obtained by the plate count procedure. An average of 5.7×10^{-8} µg ATP/cell was found, with a range of $1.6 - 8.3 \times 10^{-8}$ µg. Similar results were obtained with isolate B-10 (Figure 4). Here, the average ATP content per cell was 1.0×10^{-9} µg, with a range of $0.4 - 2.1 \times 10^{-9}$ µg.

The other two bacterial isolates exhibited greater fluctuations in cellular ATP levels. For isolate B-6, the cellular level of ATP was high in the early stage (Figure 5) while for isolate B-7 (Figure 6) it reached a maximum in the later part of the growth phase (Figure 6). The range of bacterial ATP (µg/cell) for isolate B-6 was

Fig. 3. ATP content and numbers of viable cells in cultures of bacterial isolate B-5.

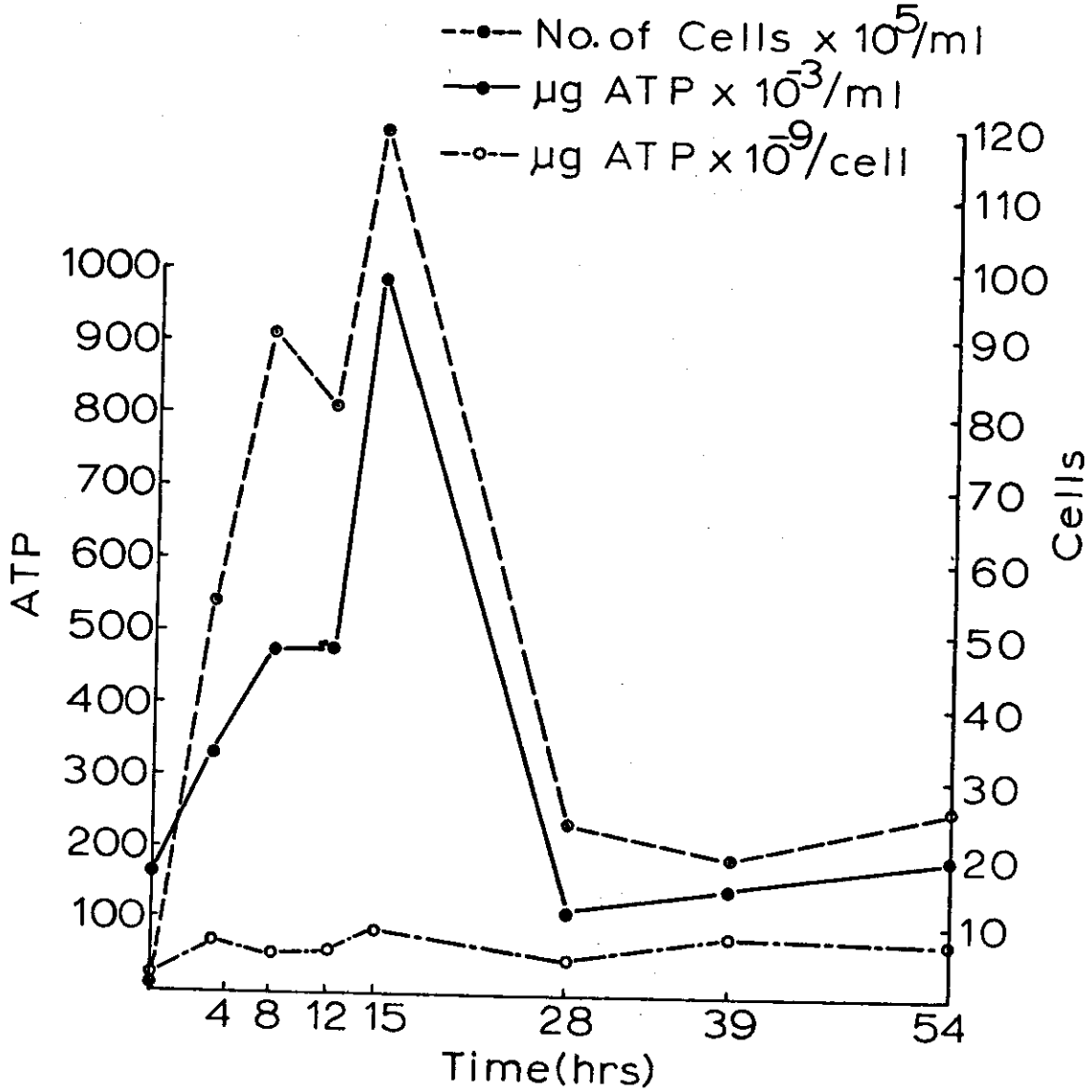

No. of Cells x 10^5/ml
µg ATP x 10^-3/ml
µg ATP x 10^-9/cell
ATP
Cells
1000
900
800
700
600
500
400
300
200
100
120
110
100
90
80
70
60
50
40
30
20
10
4 8 12 15 28 39 54
Time(hrs)

Fig. 4. ATP content and numbers of viable cells in cultures of bacterial isolate B-10.

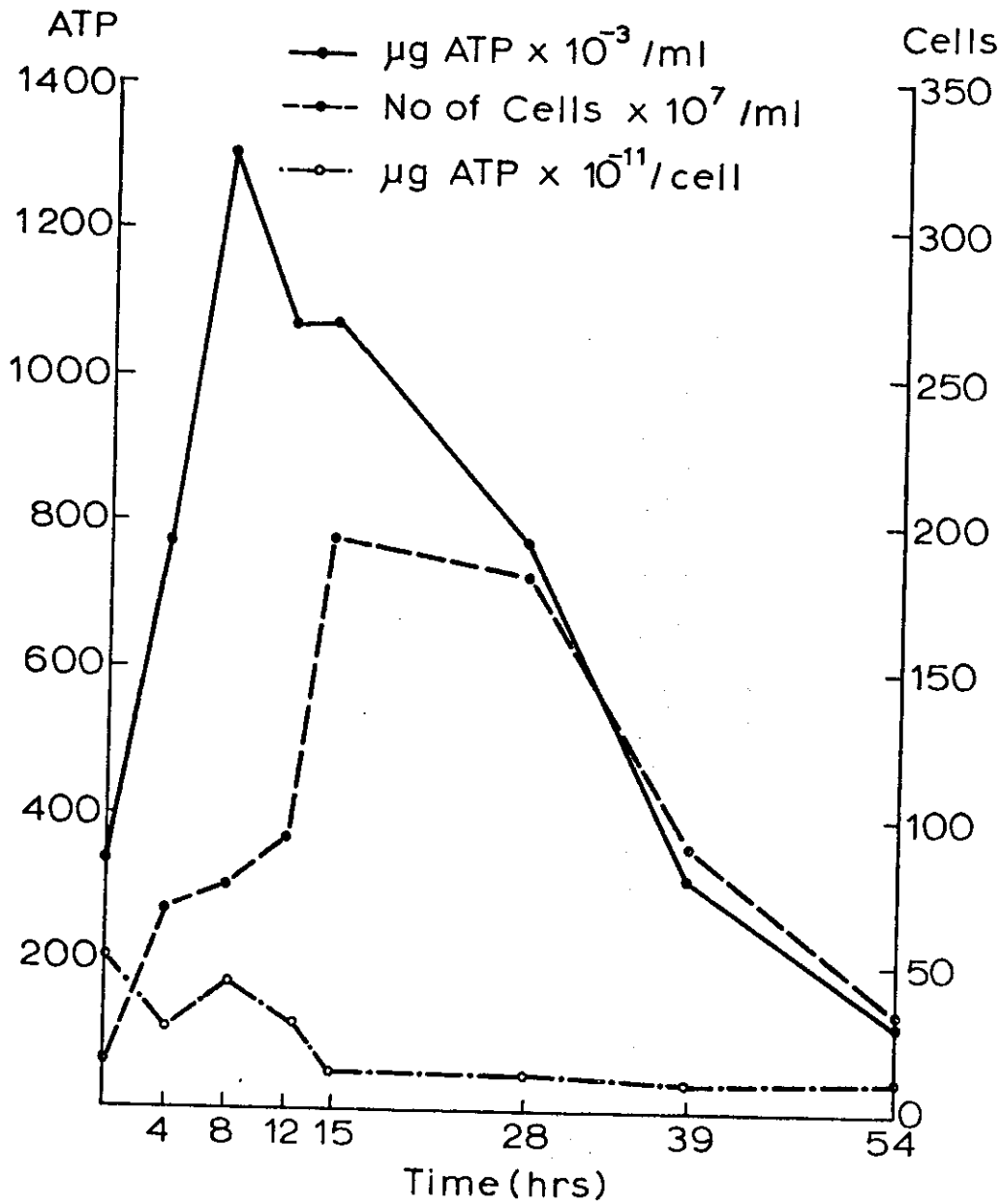

ATP
Cells
μg ATP x 10⁻³ /ml
No of Cells x 10⁷ /ml
μg ATP x 10⁻¹¹/cell
1400
1200
1000
800
600
400
200
350
300
250
200
150
100
50
0
4
8
12
15
28
39
54
Time (hrs)

Fig. 5. ATP content and numbers of viable cells in cultures of bacterial isolate B-6.

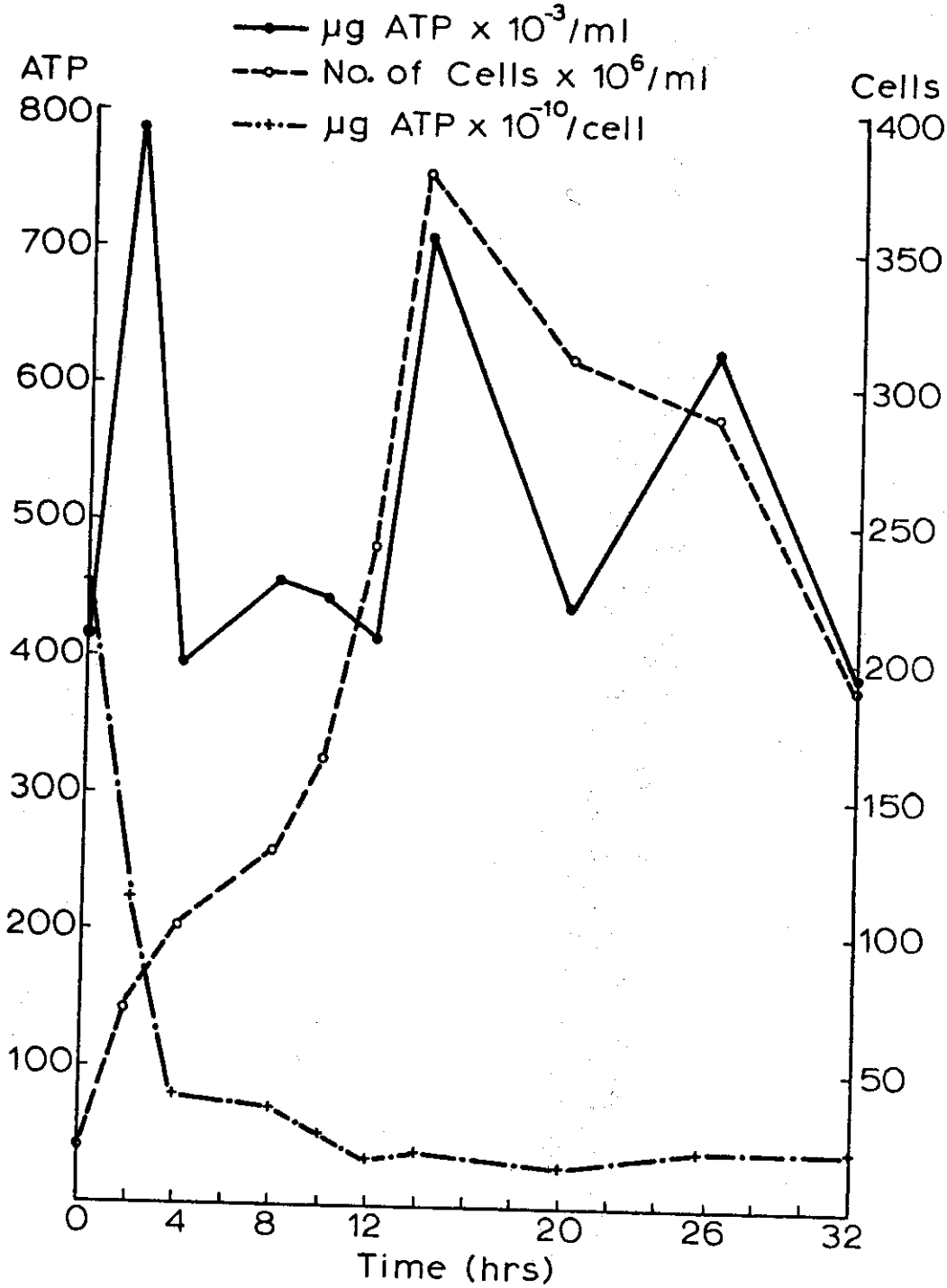
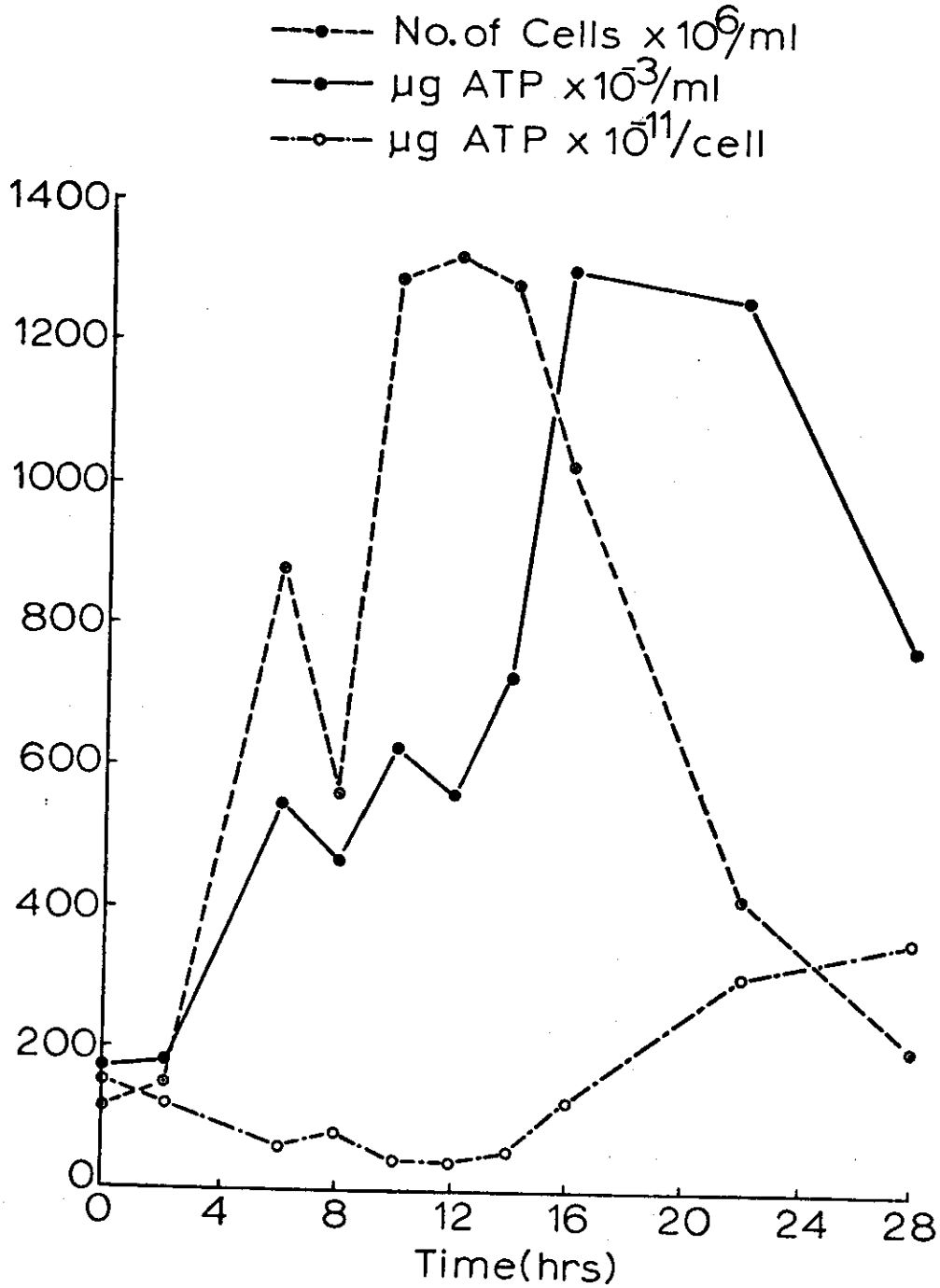

μg ATP x 10^{-3}/ml
No. of Cells x 10^{6}/ml
μg ATP x 10^{-10}/cell
ATP
Cells
800
700
600
500
400
300
200
100
400
350
300
250
200
150
100
50
0
4
8
12
20
26
32
Time (hrs)

Fig. 6. ATP content and viable numbers of cells in cultures of bacterial isolate B-7.


No. of Cells x 10^6/ml
µg ATP x 10^-3/ml
µg ATP x 10^-11/cell
1400
1200
1000
800
600
400
200
0
0
4
8
12
16
20
24
28
Time(hrs)

$0.1 - 2.3 \times 10^{-8}$, with an average of 0.5×10^{-8}, and for isolate B-7 was $0.4 - 3.7 \times 10^{-9}$, with an average of 1.4×10^{-9}.

Cole and his associates (1967) have shown that for E. coli, the rate of ATP formation can equal the rate of growth increase in weight as exemplified by bacterial isolates B-5 and B-10. Cole, et al. (1967), also found that the rate of ATP formation can be greater than the rate of growth; this relationship occurs when growth is not limited by energy production but by a rate-limiting step in biosynthesis that is not energy-dependent. Such may be the situation for isolate B-6. On the otherhand, with isolate B-7, there appears to have been an under-production of ATP in relation to biomass synthesis during the early growth phase, followed by ATP overproduction.

For the 4 bacterial isolates, the average ATP concentration per bacterial cell and cell sizes increased as follows: B-5 > B-6 > B-7 > B-10. The ATP concentration (in 10^{-9} µg) per cell were 57.4, 5.3, 1.4 and 1.0, respectively. The cells of isolate B-5 were approximately 10 times larger than those of isolate B-6. A mean ATP content of 2.5×10^{-9} µg/cell is obtained for the three bacterial isolates B-5, B-6, and B-7; this is in agreement with values reported for bacteria by other workers (Hamilton and Holm-Hansen, 1967; Picciolo, et al., 1971).

For some isolates, ATP levels in the early stages of growth were more than 10 times higher than during the late, less active growth stage. In general, however, these high levels associated with rapid growth are short lived, and the ATP concentration per cell dropped to relatively constant levels during the stationary phase. This is consistent with findings of other workers (Lee, et al., 1971; Hamilton and Holm-Hansen, 1967).

Bacterial isolates B-5, B-10, B-7, and B-6 were grown in nutrient broth with yeast extract, and at frequent intervals analyses were made for cellular carbon and ATP and for culture liquid turbidity (optical density). The results are shown in Figures 7, 8, 9 and 10. The ratio of ATP concentration to carbon content (ATP/C, both in µg) for isolate B-5 ranged from 0.0005 to 0.0063 with an average value of 0.0026; for isolate B-10, the ratio ranged from 0.0040 to 0.0135 with an average value of 0.0074; for isolate B-7, the ratio ranged from 0.0019 to 0.0043 with an average value of 0.0032; and for isolate B-6, the ratio ranged from 0.0002 to 0.0068 with mean value of 0.0028. The overall average ATP/C ratio for the 4 isolates was 0.0040. Hamilton and Holm-Hansen (1967) reported ATP/C ratios of 0.0030 to 0.0100 in marine bacteria.

The quantitative relationship between ATP concentration and other cellular characteristics was examined by means of correlation and regression analyses (Table 3). For bacterial isolate B-5, ATP was directly correlated with viable numbers as determined by the plate count procedure. For isolates B-10 and B-7, there was a direct linear relationship between the log of ATP content and the log of numbers of viable cells, which is consistent with the results of D'Eustachio, et al. (1968). ATP levels were linearly related to cellular carbon concentrations (isolates B-5, B-10 and B-7). In the case of bacterial isolate B-7, ATP content was significantly correlated with cell density ($r = 0.842$) at 1% level; this relationship did not hold for the other isolates, however. For isolate B-6, the relationships of ATP to other cell characteristics were more variable.

The significant correlations among ATP concentrations, numbers of viable cells, and carbon levels for three bacterial isolates grown in pure culture suggest that ATP

Fig. 7. Cellular ATP and carbon levels and optical densities for cultures of bacterial isolate B-5.

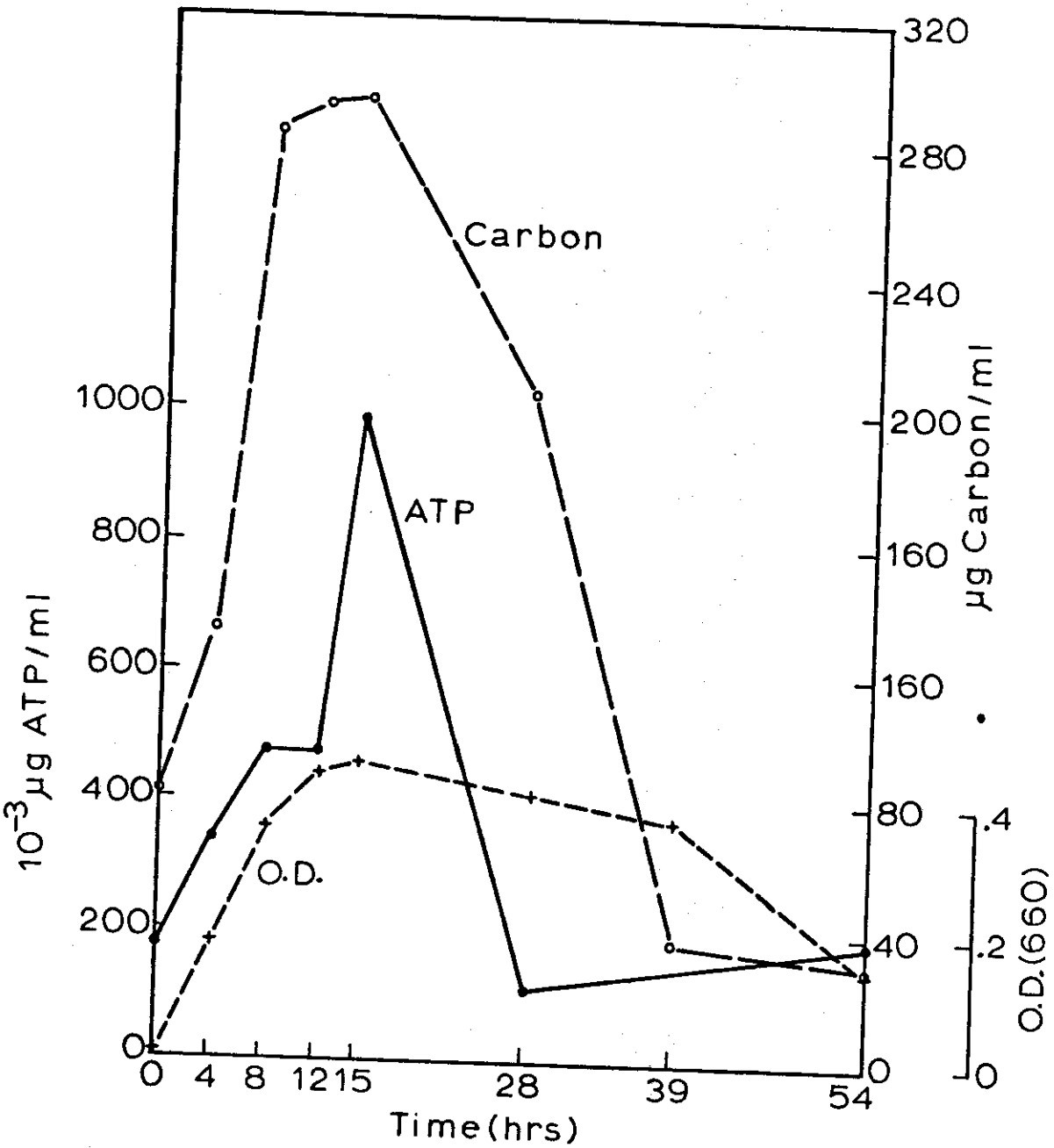

Carbon
ATP
O.D.
10^{-3}µg ATP/ml
µg Carbon/ml
O.D.(660)
Time(hrs)
0
200
400
600
800
1000
40
80
160
160
200
240
280
320
.2
.4
0 4 8 12 15 28 39 54

Fig. 8. Cellular ATP and carbon levels and optical densities for cultures of bacterial isolate B-10.

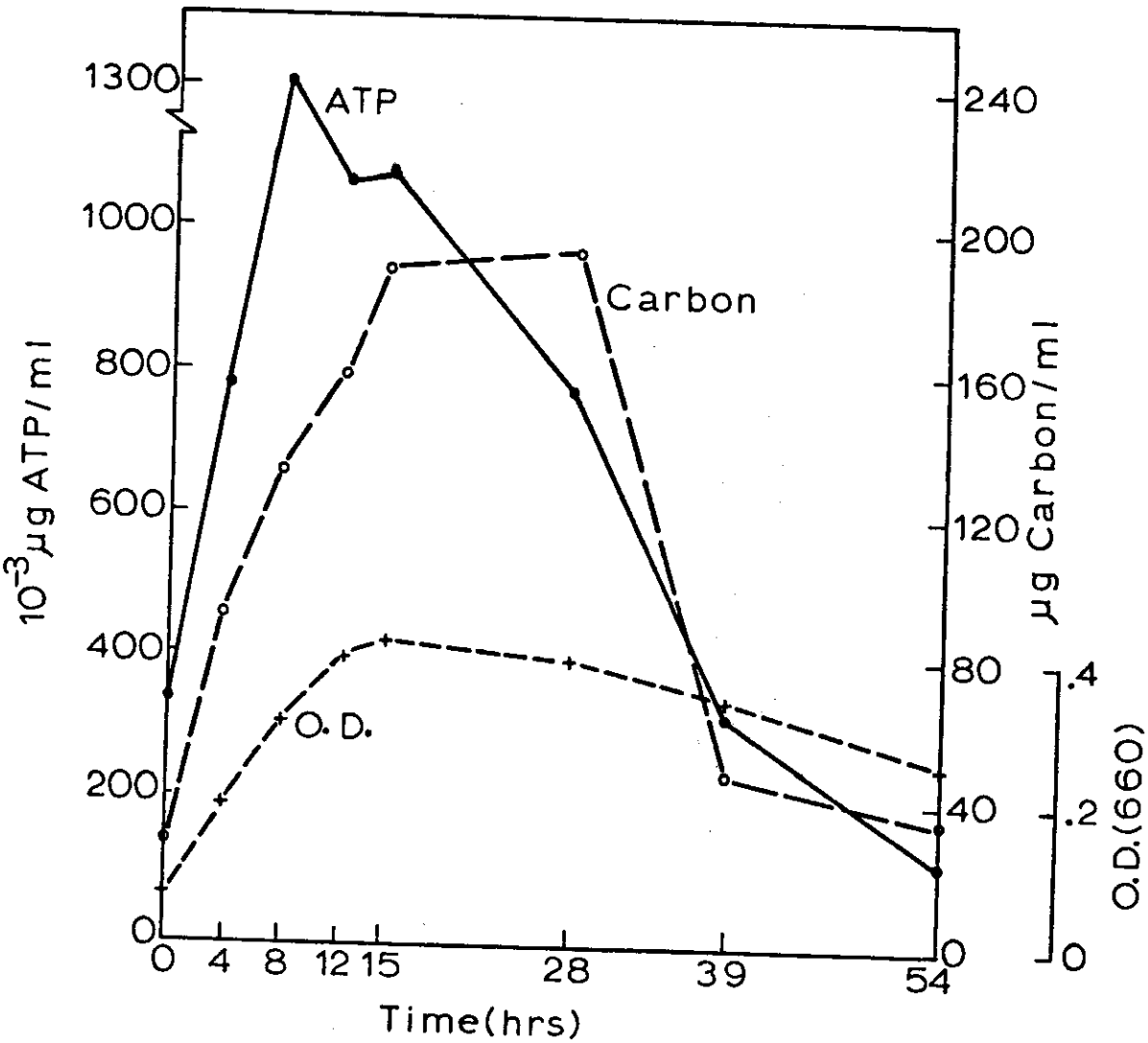

ATP
Carbon
O. D.
1300
1000
800
600
400
200
0
240
200
160
120
80
40
0
.4
.2
0
0 4 8 12 15 28 39 54
Time(hrs)
$10^{-3}\mu g$ ATP/ml
μg Carbon/ml
O.D.(660)

Fig. 9. Cellular ATP and carbon levels and optical densities for cultures of bacterial isolate B-7.

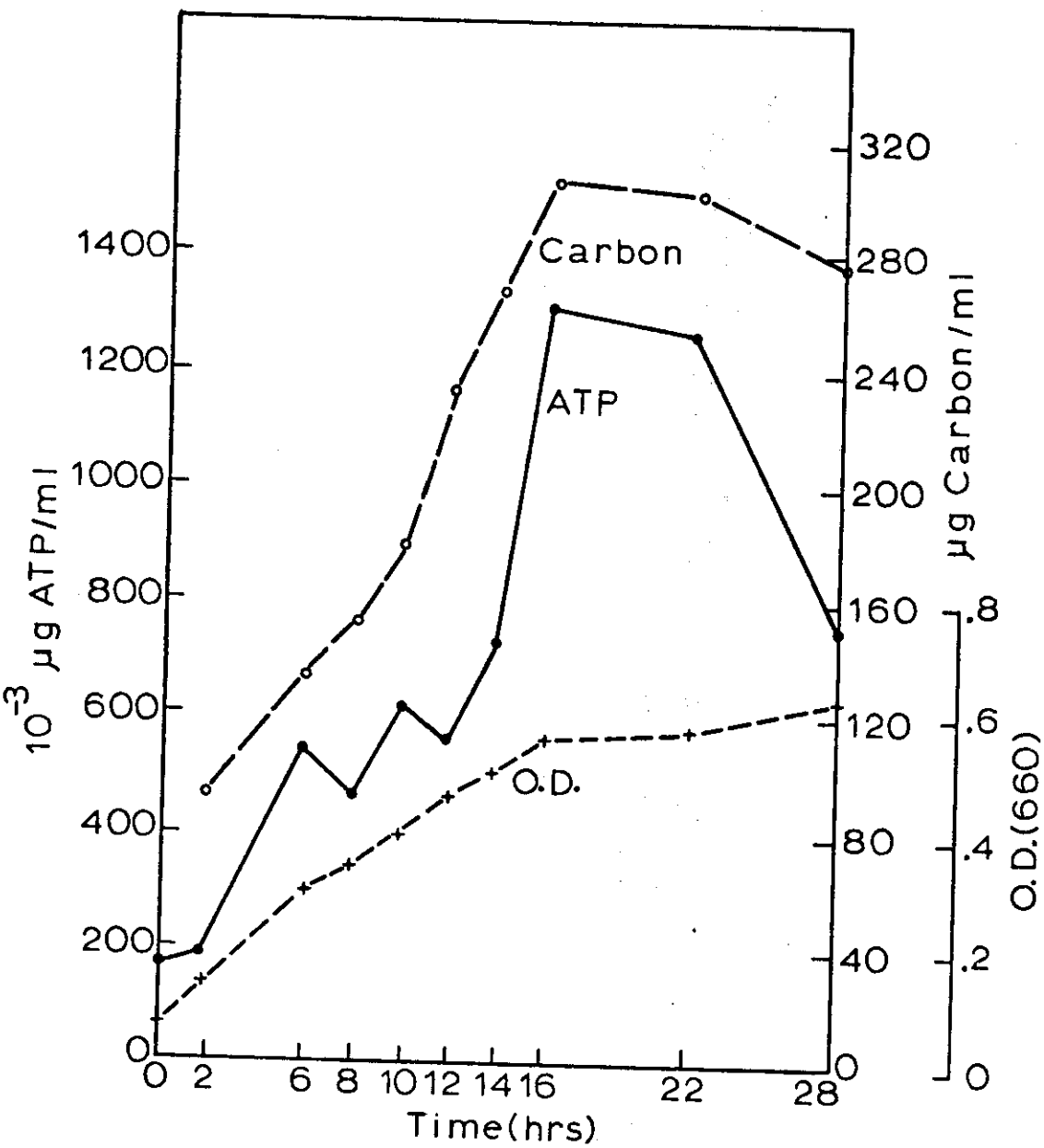

Carbon
ATP
O.D.
10^{-3} µg ATP/ml
µg Carbon/ml
O.D.(660)
Time(hrs)
0
200
400
600
800
1000
1200
1400
40
80
120
160
240
280
320
.2
.4
.6
.8
2
6
8
10
12
14
16
22
28

Fig. 10. Cellular ATP and carbon levels and optical densities for cultures of bacterial isolate B-6.

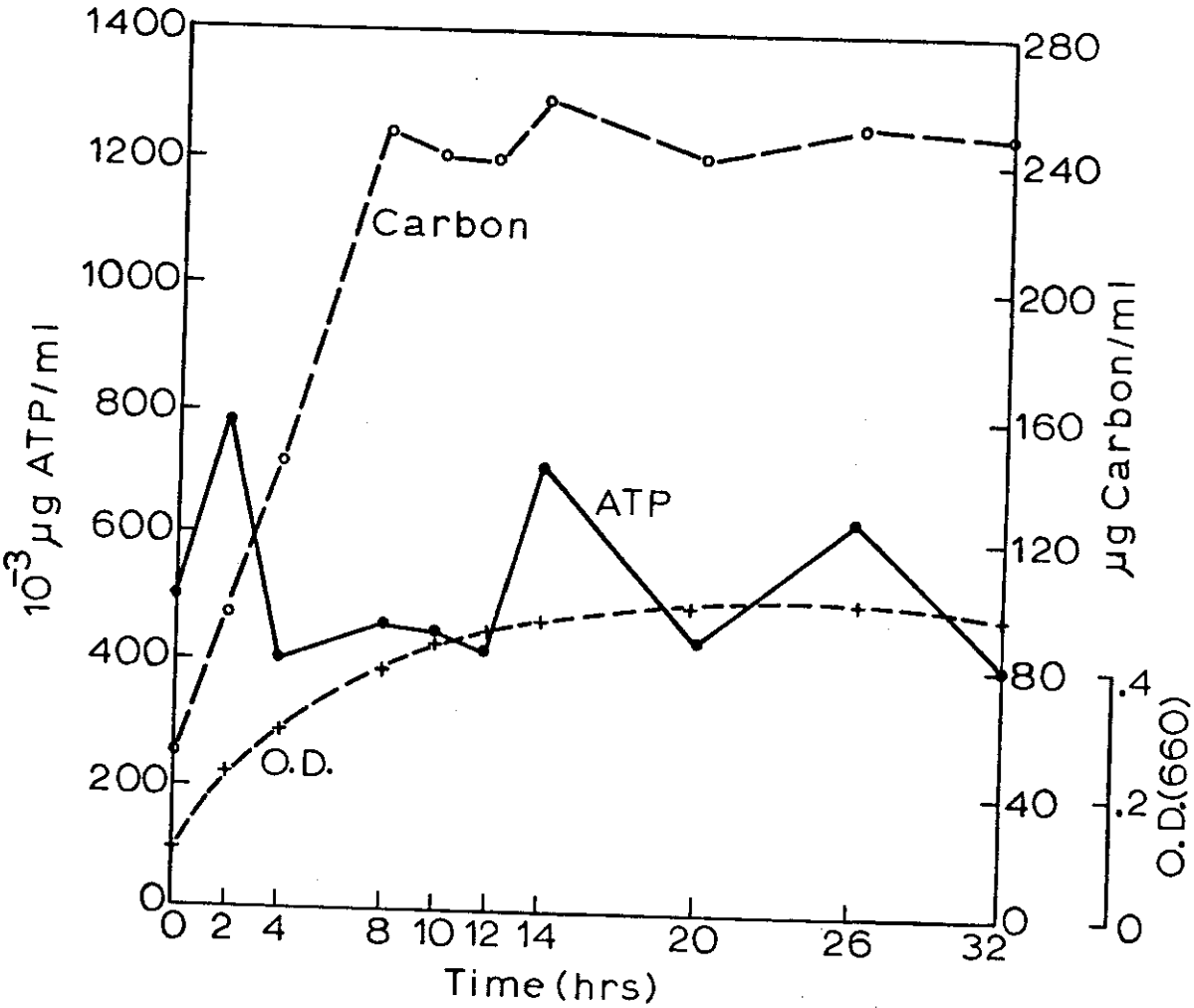

1400
1200
1000
800
600
400
200
0
280
240
200
160
120
80
40
0
.4
.2
0
Carbon
ATP
O.D.
10^{-3} µg ATP/ml
µg Carbon/ml
O.D.(660)
0 2 4 8 10 12 14 20 26 32
Time (hrs)

Table 3. Correlation coefficients (r) and regression equations relating ATP concentrations to cell counts and carbon contents of three bacterial isolates.

Regression equation[a]	X	r[b]	n
	Isolate B-5		
$Y = 23.39 + 6.51X$	10^{-5} x cells/ml	0.923**	8
$Y = 69.38 + 0.28X$	µg carbon/ml	0.714*	8
	Isolate B-10		
$Y = 1.60 + 0.62X$	$\log_{10}$ (10^{-7} x cells/ml)	$0.639^{0.1}$	8
$Y = 15.38 + 0.13X$	µg carbon/ml	0.795*	8
	Isolate B-7		
$Y = 1.97 + 0.45X$	$\log_{10}$ (10^{-7} x cells/ml)	$0.618^{0.1}$	10
$Y = -142.07 + 3.99X$	µg carbon/ml	0.871**	9

[a] $Y = 10^{-3}$ µg ATP/ml when $X = 10^{-5}$ x cells/ml or when X = µg carbon/ml but $Y = \log_{10}$ (10^{-3} µg ATP/ml) when X = $\log_{10}$ (cells/ml).

[b] Levels of significance: ** significant at 1%
* significant at 5%
0.1 significant at 10%

content is relatively constant across the greater portions of growth cycles of the bacterial isolates. From a knowledge of cellular ATP levels in a bacterial culture, one could calculate cell numbers and amounts of biomass carbon.

Relationship of ATP to weight and respiration for actinomycete and fungal cultures

A study was made of the relationships among cellular ATP, respiration and weight for actinomycete and fungal cultures grown in liquid medium. For actinomycete isolate A-11 the ratio of ATP to respiration rate varied from 1.8 to 5.5 at different incubation times (Table 4). The ratio of ATP to respiration rate for four fungal cultures ranged from 12.6 to 18.8, while ATP/dry weight ratios were equally as variable, ranging from 885 to 1860 (Table 5).

Table 4. ATP content and respiration rate of actinomycete isolate A-11 in liquid medium.*

Incubation time	ATP	Respiration rate	ATP/Resp. rate
days	ng/5 ml	µl O_2/hr/5 ml	ng/µl O_2/hr
1	342	128	2.7
2	540	297	1.8
3	2080	356	5.5

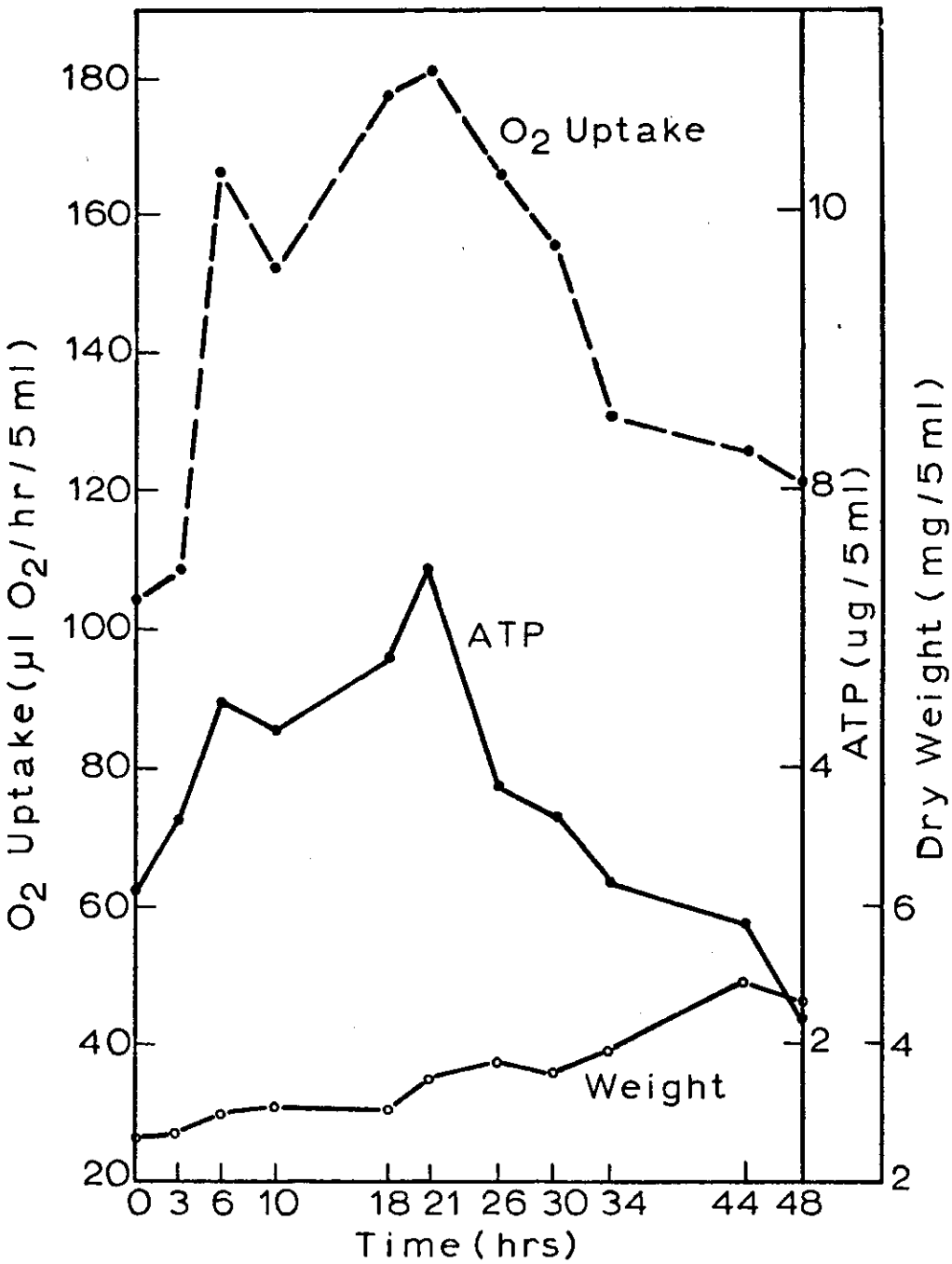
* Actinomycete isolation broth.

A time-course study was made of the growth of fungal isolate F-1 in NPY medium. ATP concentrations and oxygen consumption rates were directly correlated (Figure 11). The regression equation relating oxygen consumption rates and cellular ATP levels is described by: Y = 54.80 +

Table 5. ATP levels, respiration rates and weights of four fungal isolates in NPY broth.

Isolate	ATP	Respiration rate	Dry weight	ATP/ Respiration rate	ATP/ Dry weight
	ng/5 ml	µl O_2/hr/5 ml	mg/5 ml	ng/µl O_2/hr	ng/mg
F-1	1699	134	1.3	12.6	1310
F-2	974	51	1.1	18.8	885
F-3	3346	140	1.8	23.8	1860
F-6	1257	69	1.5	18.2	898

Fig. 11. ATP content, respiration rate and dry weight of fungal isolate F-1 during growth in NPY medium.


180
160
140
120
100
80
60
40
20
O2 Uptake
ATP
Weight
10
8
4
2
6
4
2
O2 Uptake (µl O2/hr/5 ml)
ATP (ug/5 ml)
Dry Weight (mg/5 ml)
0 3 6 10 18 21 26 30 34 44 48
Time (hrs)

23.90X, where Y = µl O_2 consumed/hr/5 ml culture and X = µg ATP/5 ml culture (r = 0.803 at 1% level of significance). Both ATP levels and oxygen consumption rates showed curvilinear relationships with respect to fungal weight. Dry weight accounted for only 58% of variability in oxygen consumption and 49% for variation in ATP content. Cellular ATP levels were found to be linearly correlated with oxygen consumption rates, an important measure of microbial activity. Thus, at least for this one fungal strain, oxygen uptake rates may be estimated from ATP measurements.

ATP in Soil (Laboratory Studies)

Extraction of soil ATP and bacterial ATP added to soil

To assess the efficiency of the boiling Tris buffer and octanol-butanol procedures for extracting microbial ATP from soil, bacterial cells and soil were extracted by the two techniques. Analyses of both kinds of samples indicated that higher ATP levels were found by the butanol-octanol method than by the boiling Tris procedure (Table 6). With the butanol-octanol procedure, total quantities of ATP in extracts of cells plus soil were not significantly different (at the 1% level) from the sum of the ATP extracted from cells alone and from soil alone. The low ATP values obtained with boiling Tris buffer may be the result of difficulty in maintaining the required high temperature conditions to ensure rapid extraction and inactivation of phosphatase enzymes (Beutler and Baluda, 1964). Patterson, et al. (1970), found that temperature is critical in ATP extraction, that a reduction in temperature of the Tris buffer of a few degrees below boiling (100 C) yielded much lower amounts of extracted ATP (a difference of about 100 mg ATP). The temperature of boiling Tris buffer at 1,524 m (altitude of Fort Collins) above sea level is 94-96 C.

Table 6. Extraction of bacterial and soil ATP.

Sample*	Extracting solution	
	Tris buffer (pH 7.4)	Butanol-octanol
	ng ATP**	
Cells	22.8	475.9
Cells + soil	35.4	595.7
Soil	18.9	107.8

* 0.5 ml of an 18-hr culture of a soil bacterium growing in nutrient broth and 1.0 g soil.

** Values are means of 16 determinations (4 extractions per sample and 4 determinations per extraction).

The results in Table 6 also indicate that the ATP in bacterial cells added to soil prior to extraction was efficiently recovered. In the case of boiling Tris buffer, 85% of the added bacterial ATP was recovered and with butanol-octanol, 100% of the ATP was measured. By adding bacterial cells containing ATP, the difficulty of assessing possible chemical and enzymatic degradation of unprotected ATP between the time of ATP addition to the soil and subsequent extraction, was avoided. When a solution of ATP was added to soil only 60% of the ATP was recovered. The low recovery may have resulted from decomposition of the free ATP by chemical or enzymatic processes or by adsorption on to soil particles.

Effect of storage time on soil ATP levels

Experiments were conducted to determine the effect on ATP levels of storage time for soil samples kept in the

refrigerator (5 C) and for extracts in the freezer (-20 C). The soil used was collected from a depth of 0-15 cm from the nonirrigated plot of the systems stress study area at the Pawnee site.

Storage of soil for 24 hr at 5 C. ATP extraction method II (butanol-octanol extractant) was used. Duplicate dilutions of soil in Tris buffer were prepared; four 5-ml aliquots were taken and extracted. Four ATP determinations were made on each extract. The mean level of ATP in unstored soil was 257.3 ng/g, while for soil stored in the refrigerator for 24 hr the mean ATP concentration was 284.4 ng/g (Appendix Table 1). No significant difference in ATP contents of soil before and after storage at 5 C was noted (as shown by F-test, at 1% level of significance in Appendix Table 2).

Storage of soil longer than 24 hr at 5 C. Soil samples were amended to field capacity with a solution containing 1% glucose and 0.1% ammonium phosphate and incubated at 30 C for 2 days. Five-gram quantities of the incubated samples were mixed with 45-ml quantities of Tris buffer. Two 3-ml aliquots of the suspension were extracted. Extracts were combined and diluted tenfold, and 6 ATP determinations were made. Soil ATP levels did not change significantly from the 1st to the 5th day of incubation of the soil (Table 7). At 16 days of storage only 77% of the one-day level of ATP was measured, and at 29 days an even more drastic loss occurred. At that time, the ATP concentration was only 8.8% of that in the soil stored for one day. Thus it appears that such soil samples can be stored at 5 C for up to 5 days without adverse effects on ATP content.

Table 7. Effect of storing soil at 5 C on ATP content.

Length of storage	ATP concentration*
days	ng/g soil
1	5145.8a
3	5028.6a
5	4720.1ab
16	3961.2 b
29	452.8 c

* Each ATP value is the mean of 6 determinations. Means having different letters following them are significantly different at the 5% level (Duncan's Multiple Range Test).

Storage of soil extracts at -20 C. Soil (10 g), amended with glucose and ammonium phosphate and incubated for 2 days, was mixed with 90 ml Tris buffer. Twenty-five 3-ml aliquots of the soil suspension were extracted with butanol-octanol; extracts was pooled and diluted tenfold. Ten-ml quantities were placed in vials and kept at -20 C in a freezer. At each sampling time, one vial of extract was thawed; 6 ATP determinations were made on the contents of each vial. No significant differences in ATP concentrations were found for extracts stored from 0 to 30 days at -20 C (Table 8). However, at 116 days of storage, there was a decrease in ATP concentration (significant at 5% level). These results indicate that such soil extracts can be safely stored for up to 30 days at -20 C without loss of ATP content.

Table 8. Effect of storage time at -20 C on ATP content of soil extract.

Length of storage	ATP concentration*
days	ng/g soil
0	1010.6a
2	1085.9a
4	953.8a
6	990.8a
17	918.1a
30	940.3a
116	625.6b

* ATP values are means of 6 replicate determinations. Means having different letters following them are significantly different at the 5% level (Duncan's Multiple Range Test).

These findings on the stability of ATP in soil samples and in soil extracts are important since in the analysis of large numbers of soil samples storage of soil or extracts may be necessary.

Use of ATP measurement to follow microbial population changes in soil

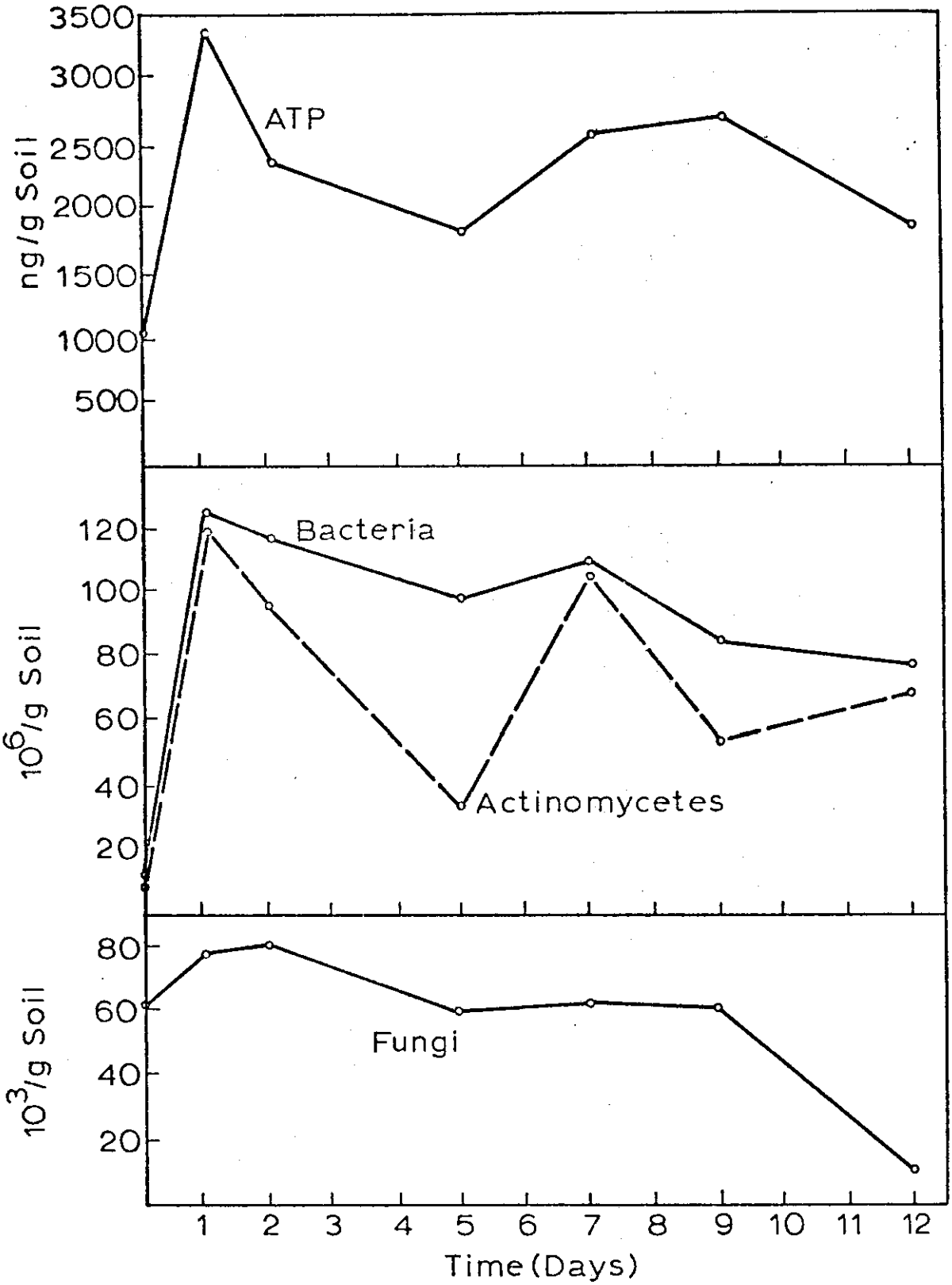
An experiment was conducted to determine the suitability of ATP measurements for following microbial population changes in soil. Grassland soil samples were brought to the laboratory and adjusted to field capacity water level by the

addition of a solution containing 1% glucose and 0.1% ammonium phosphate (17.84 g of solution was added to 400 g soil with initial water content of 8.71% thereby increasing soil moisture level to 13.17%). The soil samples were incubated at 30 C and at frequent intervals ATP levels were measured and compared to counts of viable microorganisms obtained by the plate count technique. The numbers of viable bacteria and actinomycetes closely paralleled ATP levels in the soil samples (Figure 12). The correlation coefficient of ATP levels with bacterial numbers alone was 0.79 and that of ATP concentrations and actinomycete numbers alone was 0.85. Both correlation coefficients were significant at 5% level. No significant correlation was found between ATP concentrations and fungal numbers. Interestingly, the significant correlation between ATP levels and numbers of viable bacteria exists not only in pure cultures but also in mixed microbial populations such as found in soil. These results suggest that ATP measurements can be used to indicate bacterial population changes in soil.

Effect of removal of roots from soil on ATP levels

An experiment was conducted to determine in an indirect way the possible root contribution to the measured ATP pool in soil. Soil containing appreciable root biomass was collected from the Pawnee grassland site. Roots visible to the naked eye and under a low power stereoscopic microscope were removed from the soil samples with a pair of forceps. The process of root removal under the microscope required 45 min. Controls consisted of soil samples with and without large roots placed on the microscope stage for a similar length of time. A significant difference

Fig. 12. ATP levels and microbial numbers in soil over a 12-day incubation period.


3500
3000
2500
2000
1500
1000
500
ng/g Soil
ATP
120
100
80
60
40
20
10^6/g Soil
Bacteria
Actinomycetes
80
60
40
20
10^3/g Soil
Fungi
1
2
3
4
5
6
7
8
9
10
11
12
Time (Days)

was apparent in the ATP content of soil with roots (unexposed to the heating and drying caused by the microscope light) and soil treated to remove roots (exposed to microscope light) (Table 9). The ATP levels in soil samples exposed to the light (resulting in soil dessication) were significantly lower than that found in the unexposed soil. Among the treatments involving soil exposure to light there was no significant difference in ATP concentration. The presence or absence of roots neither increased nor decreased soil ATP concentration; thus it appears that root contribution to ATP extracted from soil is insignificant under these conditions.

Table 9. Effect of removing plant roots on soil ATP levels.

Treatment	ng ATP/g soil*
Soil and roots	485.7a
Soil and roots**	286.4b
Soil minus big roots**	329.4b
Soil minus big and small roots**	357.8b

* Each value is the mean of 16 determinations (4 extractions per sample and 4 determinations per extraction). Means having different letters following them are significantly different at 5% level (Duncan's Multiple Range Test).

** Soil exposed to microscope light.

Further studies are required using other means of determining the extent of root contribution to the measured ATP pool in soil. One approach to this problem is by the

use of aseptic plants grown in sterile soil. In this way ATP associated with the root cells can be measured apart from the ATP of the rhizoplane microbial population.

Preliminary studies on the extraction and determination of ATP levels in field soil samples

General procedures were tested in an effort to find a simple and efficient method to extract ATP from soil. With Method I, in which 0.1 g soil was extracted with butanol, Tris buffer, and octanol, much variation in ATP values was found, probably because small amounts of soil were used. Other procedures for ATP extraction using larger quantities of soil were evaluated.

With Method II, Tris buffer was added to 10 g soil (from nonirrigated plot at 3-6 cm depth) in a beaker and mixed with a magnetic stirrer. Aliquots of the suspension were treated with butanol-octanol; the mixture was centrifuged, and the butanol-octanol layer was discarded. As shown in Tables 10 and 11, there was a significant difference in ATP concentrations in soil among composites and among extractions per dilution at the 5% and 1% level of significance, respectively. No difference was noted between dilutions per composite. Using the Chi square test to determine the number of composites needed for future sampling, both at the 5 and 10% levels of significance, it was calculated that 2 composites were required. To calculate the number of determinations needed per extract, two extreme cases based on 4 replicate determinations were used. One with a coefficient of variation of 41% gave a calculated value of 13 determinations. The other with a low coefficient of variation of 4.6% gave a calculated value of 1 determination per extract.

Table 10. ATP concentration using Method II extraction of soil.

Composite*	ng ATP/g soil**
1	474.1
2	350.5
3	349.3
4	364.4

* 6 soil cores (6.5 cm diameter) per composite.
** ATP value is the mean of 16 determinations (2 dilutions per composite, 2 extractions per dilution and 4 determinations per extraction).

Table 11. Analysis of variance of results using Method II extraction of soil.

Source of variation	df	Mean square	F
Composites	3	3.53	11.39*
Dilutions per composite	4	0.31	0.23
Extractions per dilution	8	1.33	3.59**
Total	63		

* Significantly different at 5% level.
** Significantly different at 1% level.

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Extractions per dilution	8	1.33	3.59**
Total	63		

* Significantly different at 5% level.
** Significantly different at 1% level.

Because of the high variation noted in Method II, another method was tested. For Method III, 1.0 g soil was mixed with Tris buffer, butanol, and octanol. Methods II and III were used to analyze 12 soil cores (6.5 cm in diameter) from the 0-3 cm depth of the nonirrigated site. For Method II, two 1:10 soil-Tris dilutions and 4 extracts per dilution were employed. Eight extractions were made using Method III. Four ATP determinations were made on each extract. No significant difference was found between the means of ATP levels obtained by the two methods (Table 12). However, Method III had a smaller coefficient of variation than Method II. For both methods, there was a difference among extracts significant at 1% level. With the use of the Chi square test, the number of extracts needed for Method III was determined to be 2 at either 5 or 10% level of significance. For Method II, based on the results obtained from one dilution, 2 extracts were required but based on the other dilution, 26 extracts were calculated to be needed. The numbers of determinations per extract was calculated to be between 1 and 3 for Method III and 1 to 17 for Method II.

Table 12. Comparison of the two methods of extracting soil ATP.

Method	ng ATP/g soil*	C.V.
II	606.2	28
III	554.3	19

* ATP value is mean of 32 determinations.

To confirm these results, 8 soil samples were extracted employing Method III. The soil was taken at a depth of 0-3 cm from the nonirrigated site. Four ATP determinations were made per extract. The summary statistics of Method III (second run) is given in Table 13. The overall coefficient of variation is similar to that found in the preceding run. Three extracts per soil sample were required according to the Chi square test, while the number of determinations per extract was calculated to be 2.4 and 3.2 at 5% and 10% level of significance, respectively.

Based on these findings Method III was chosen for extracting ATP from soil samples in the field studies. The analytical scheme involved the use of 2 composites (12 cores per composite), 4 extractions per composite, and 4 determinations per extract, a total of 32 ATP measurements to characterize each field treatment site.

Field Study of Soil ATP Relationships

A study was conducted during the plant-growing season on soils at the Pawnee site to determine (a) the relationship of ATP levels to microbial abundance, (b) the suitability of ATP measurements for following microbial population changes, and (c) the relationship of ATP concentration to soil respiration.

From nonirrigated and irrigated treatment plots in the systems stress study area, soil cores were collected to a depth of 70 cm once each month from June through September, 1971. Composite core samples were analyzed for ATP, moisture content, and respiratory activity. Soil

Table 13. Summary statistics of results obtained using Method III.

Extract No.	Statistical parameter			No. of determinations per extract at level of significance of*	
	Mean	S.D.	C.V.	0.05	0.10
	- ng ATP/g soil -				
1	478.5	28.0	5.8	0.08	0.06
2	415.7	32.2	7.7	0.05	0.04
3	459.2	79.4	17.2	0.97	0.78
4	476.9	80.0	16.8	0.99	0.79
5	352.8	32.1	9.1	0.04	0.03
6	438.2	50.4	11.5	2.31	1.84
7	323.8	47.1	17.0	1.95	1.56
8	369.0	49.1	13.3	2.04	1.63
Overall	414.3	73.4	17.7		

* Calculated by Chi square test.

respiration was determined by measuring oxygen consumption rates with a Gilson differential respirometer. Three soil amendments were employed in the respiration analyses to determine the effect of added moisture and carbonaceous substrate on microbial activity: (1) unamended soil; (2) water added to bring soil moisture content to 25%; and (3) glucose plus water to bring soil moisture to 25%.

ATP concentrations, moisture contents, and respiration rates for individual composites are listed in Appendix Tables 3-11. Soil ATP concentrations and respiration rates (water + glucose amendment) for nonirrigated and irrigated plots sampled in June, July, August, and September are shown in Figures 13-19. No irrigation water was applied to the irrigated treatment plot in August or September. In August, samples were taken only from the nonirrigated plot. Both ATP levels and respiration rates decreased with depth. ATP concentrations were lower in the nonirrigated plots than in the irrigated fields. For the most part, amendments of water and water plus glucose resulted in increased respiration rates for soil samples from both the irrigated and nonirrigated sites. Respiration rates (water + glucose amendment) for the nonirrigated and irrigated soil samples were not significantly different, suggesting that the amendments provided adequate substrate and moisture for the potentially active microflora. ATP levels in the nonirrigated soil samples collected in September were noticeably higher than for those collected in August. Just prior to September sampling, a heavy rainstorm occurred; the resulting increase in soil moisture was likely responsible (directly or indirectly) for higher ATP levels, which reflect greater microbial abundance in the soil samples.

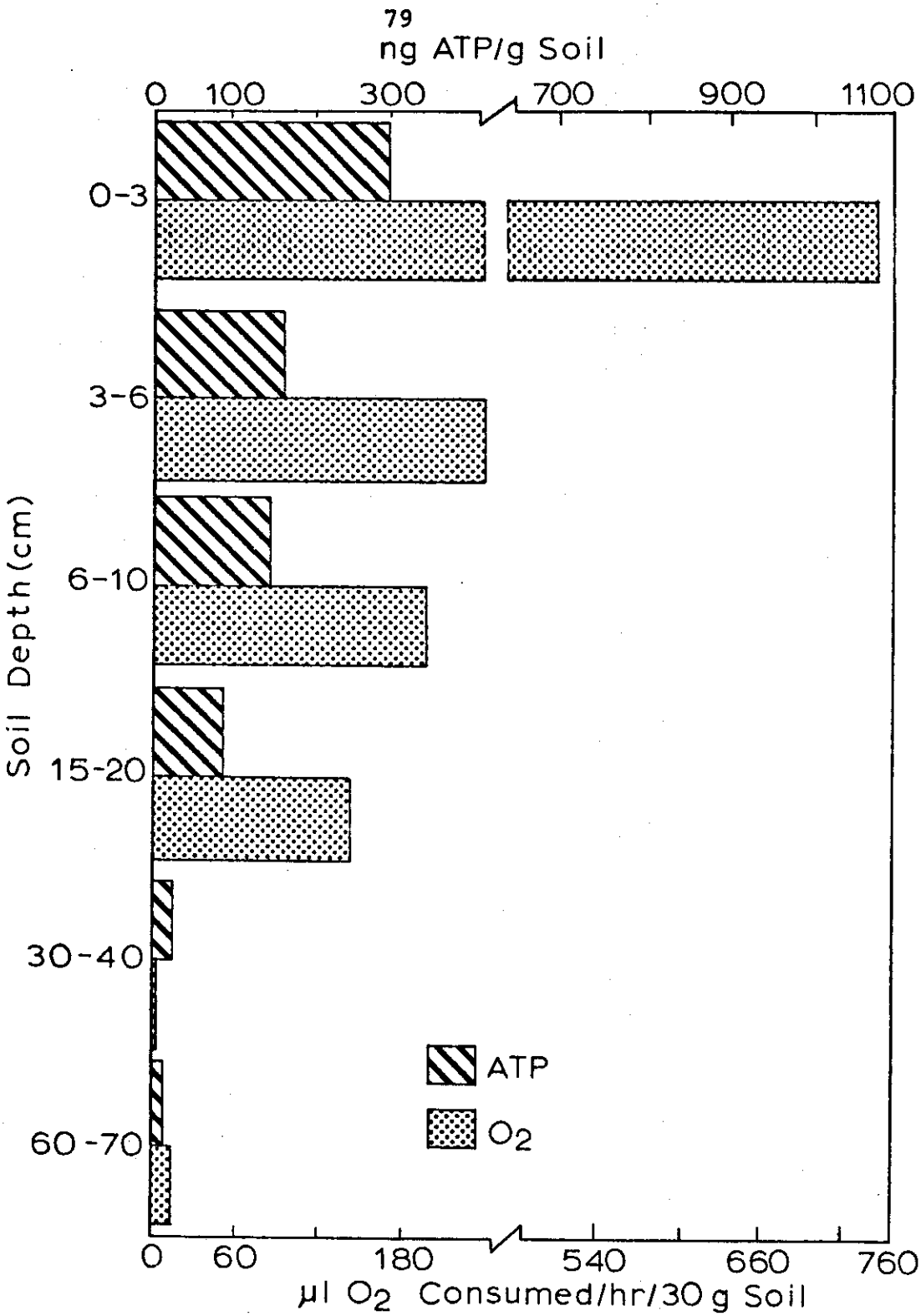

ng ATP/g Soil
0
100
300
700
900
1100
0-3
3-6
6-10
15-20
30-40
60-70
Soil Depth (cm)
ATP
O2
0
60
180
540
660
760
µl O2 Consumed/hr/30 g Soil

Fig. 14. Variation in ATP content and respiration rate (water + glucose amendment) with depth in irrigated soil (June sampling).

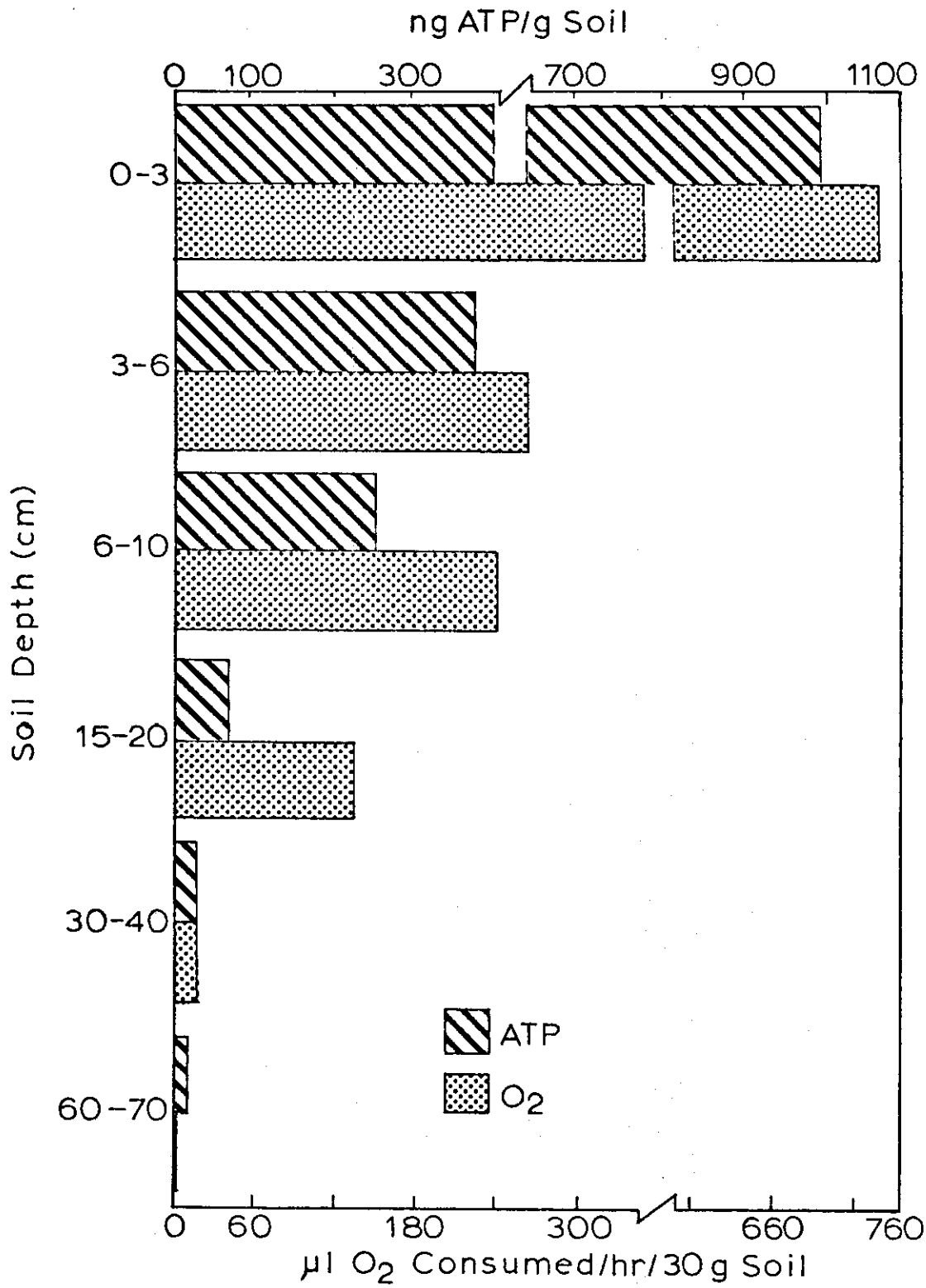

ng ATP/g Soil
0
100
300
700
900
1100
0-3
3-6
6-10
15-20
30-40
60-70
Soil Depth (cm)
ATP
O2
0
60
180
300
660
760
µl O2 Consumed/hr/30 g Soil

Fig. 15. Variation in ATP content and respiration rate (water + glucose amendment) with depth in non-irrigated soil (July sampling).

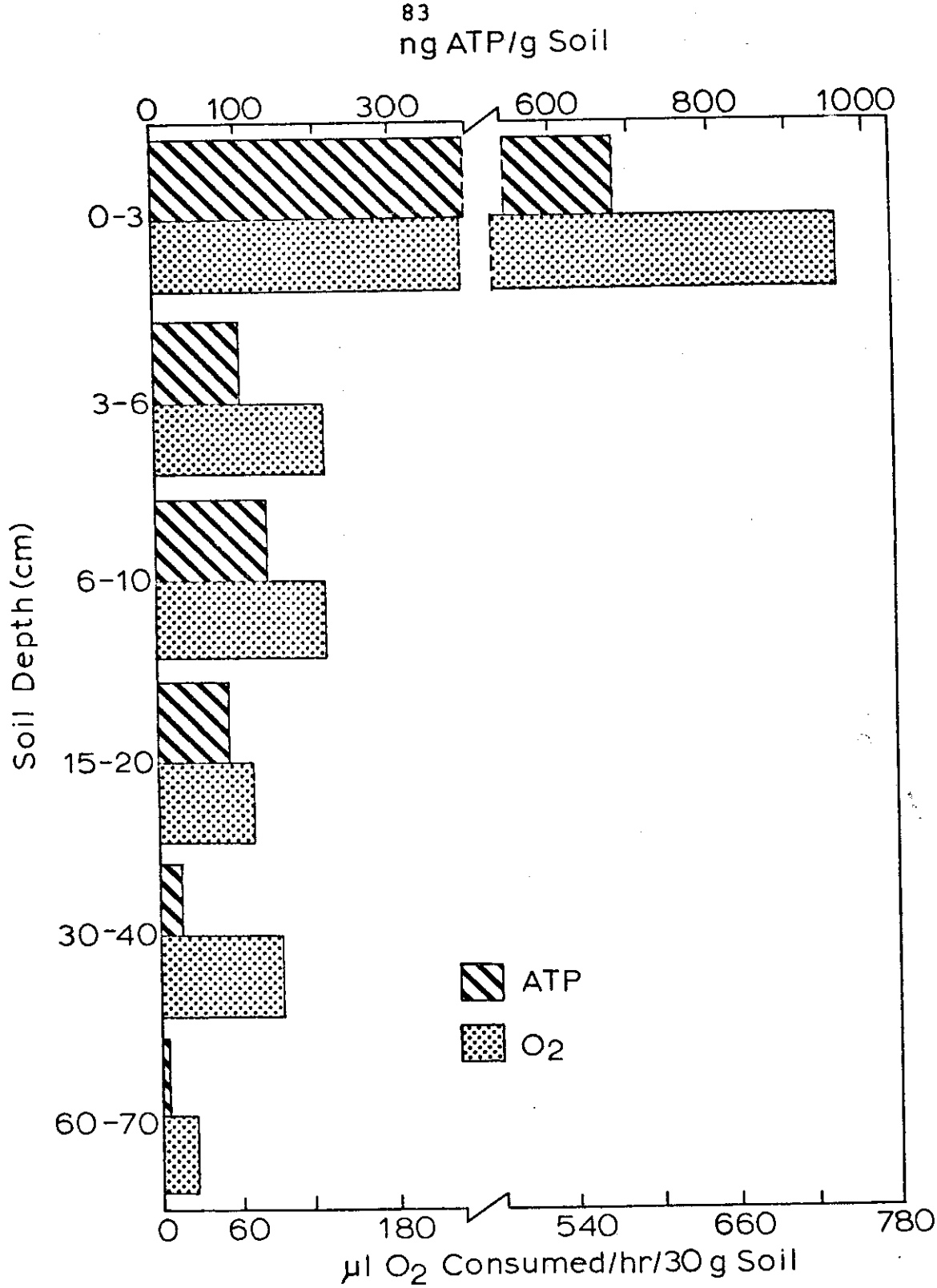

ng ATP/g Soil
0
100
300
600
800
1000
0-3
3-6
6-10
15-20
30-40
60-70
Soil Depth (cm)
ATP
O2
0
60
180
540
660
780
µl O2 Consumed/hr/30 g Soil

Fig. 16. Variation in ATP content and respiration rate (water + glucose amendment) with depth in irrigated soil (July sampling).

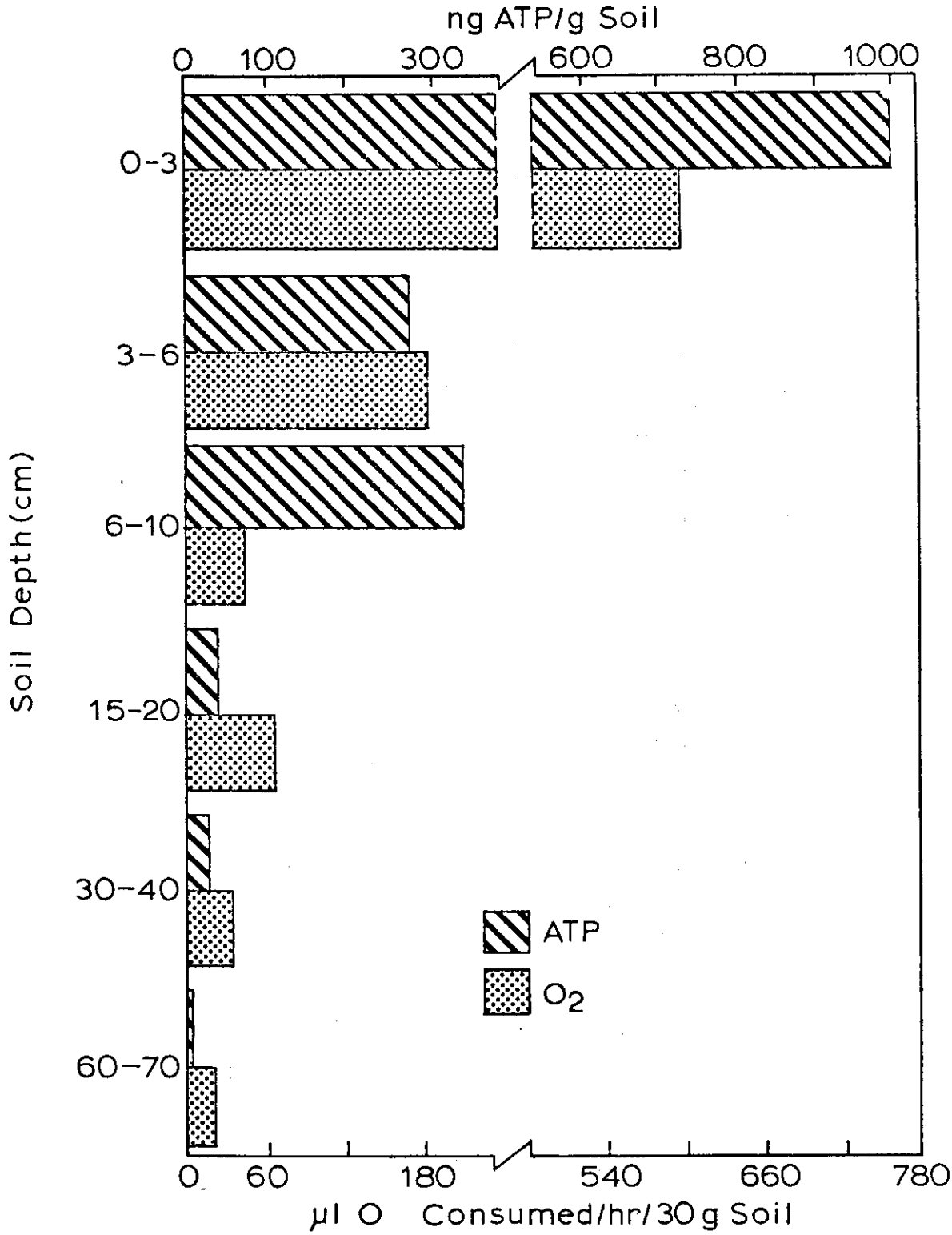

ng ATP/g Soil
0
100
300
600
800
1000
0-3
3-6
6-10
15-20
30-40
60-70
Soil Depth (cm)
ATP
O2
0
60
180
540
660
780
µl O Consumed/hr/30 g Soil

Fig. 17. Variation in ATP content and respiration rate (water + glucose amendment) with depth in non-irrigated soil (August sampling).

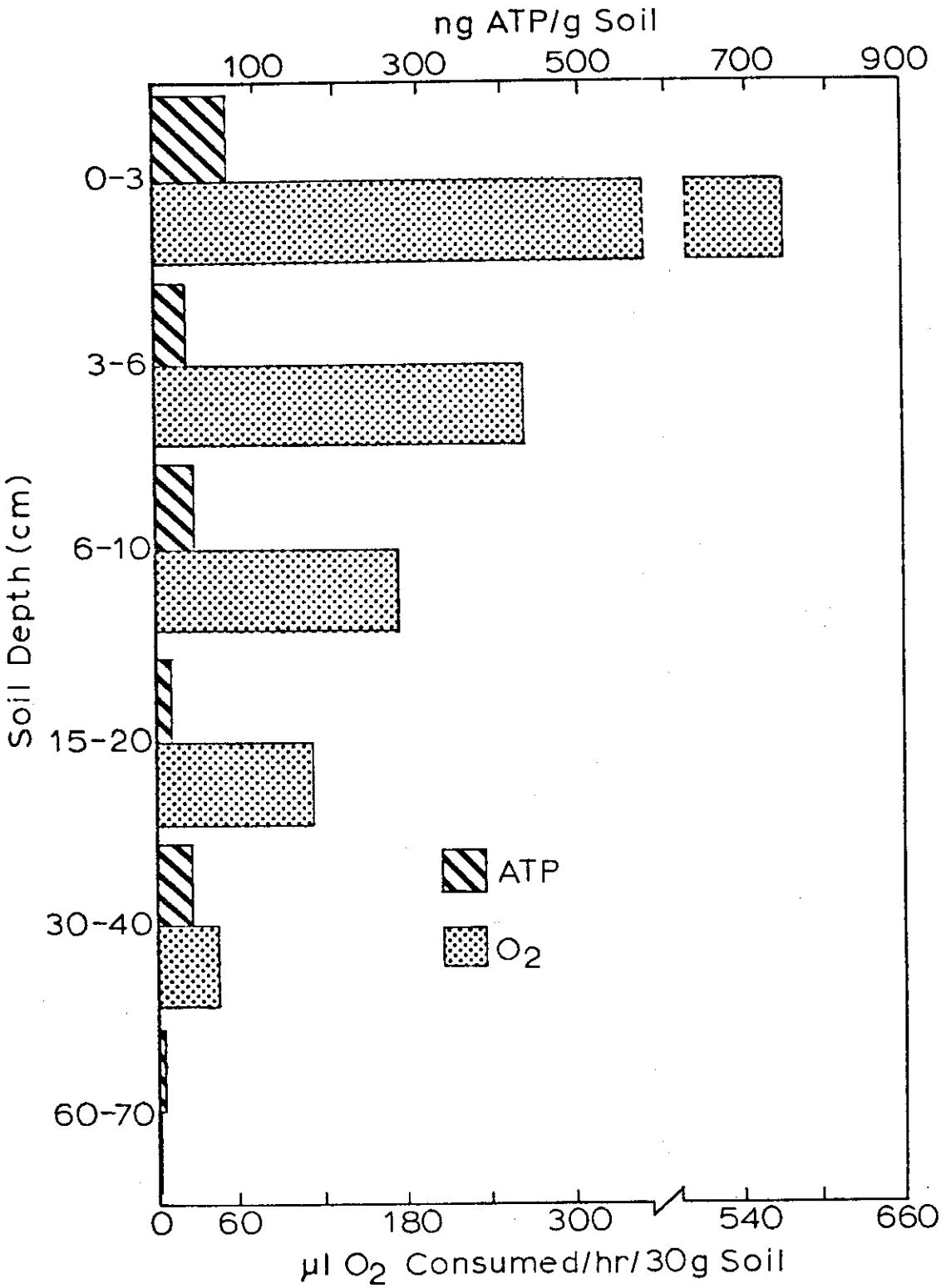

ng ATP/g Soil
100
300
500
700
900
0-3
3-6
6-10
15-20
30-40
60-70
Soil Depth (cm)
ATP
O2
0
60
180
300
540
660
µl O2 Consumed/hr/30g Soil

Fig. 18. Variation in ATP content and respiration rate (water + glucose amendment) with depth in non-irrigated soil (September sampling).

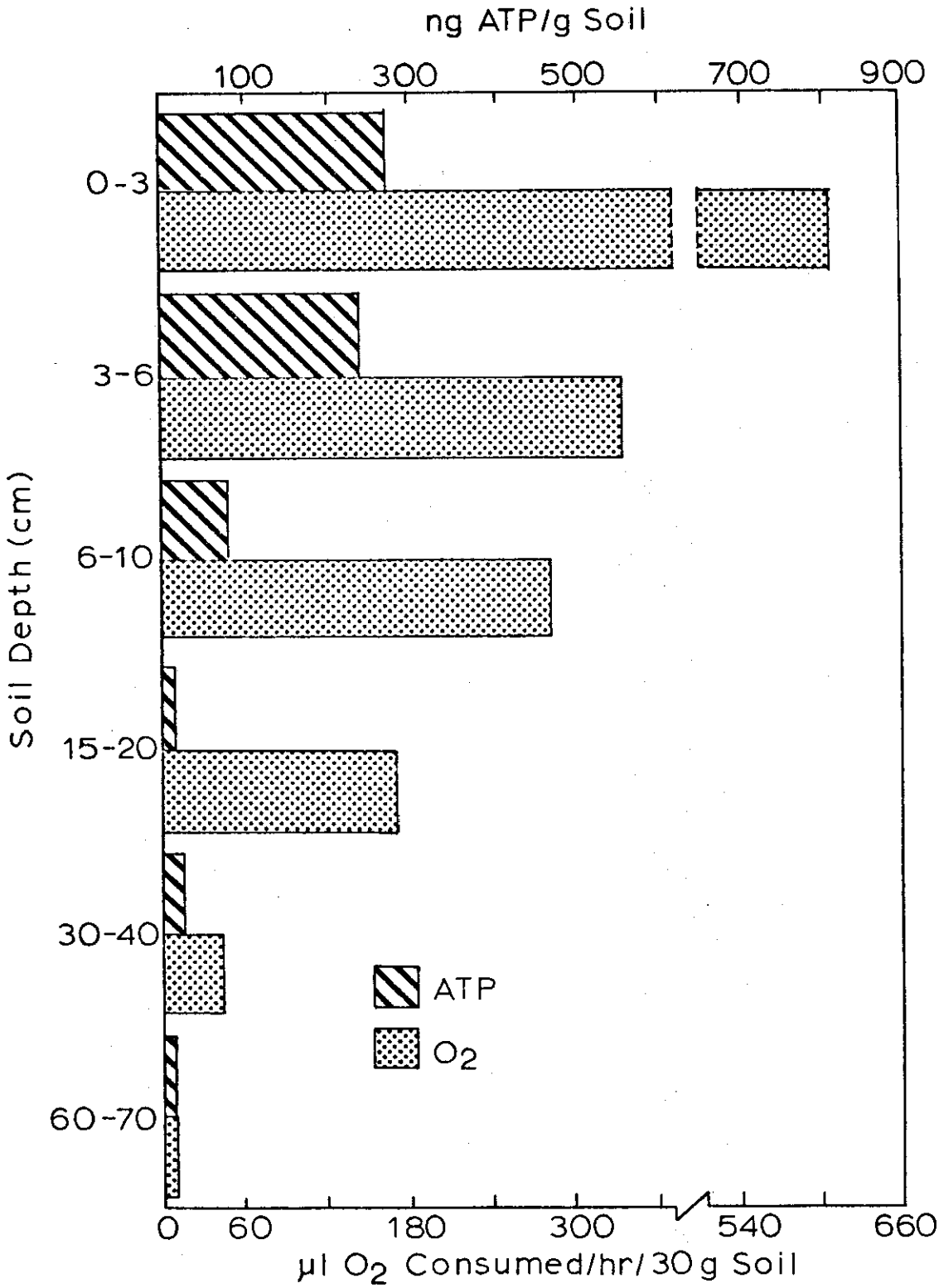
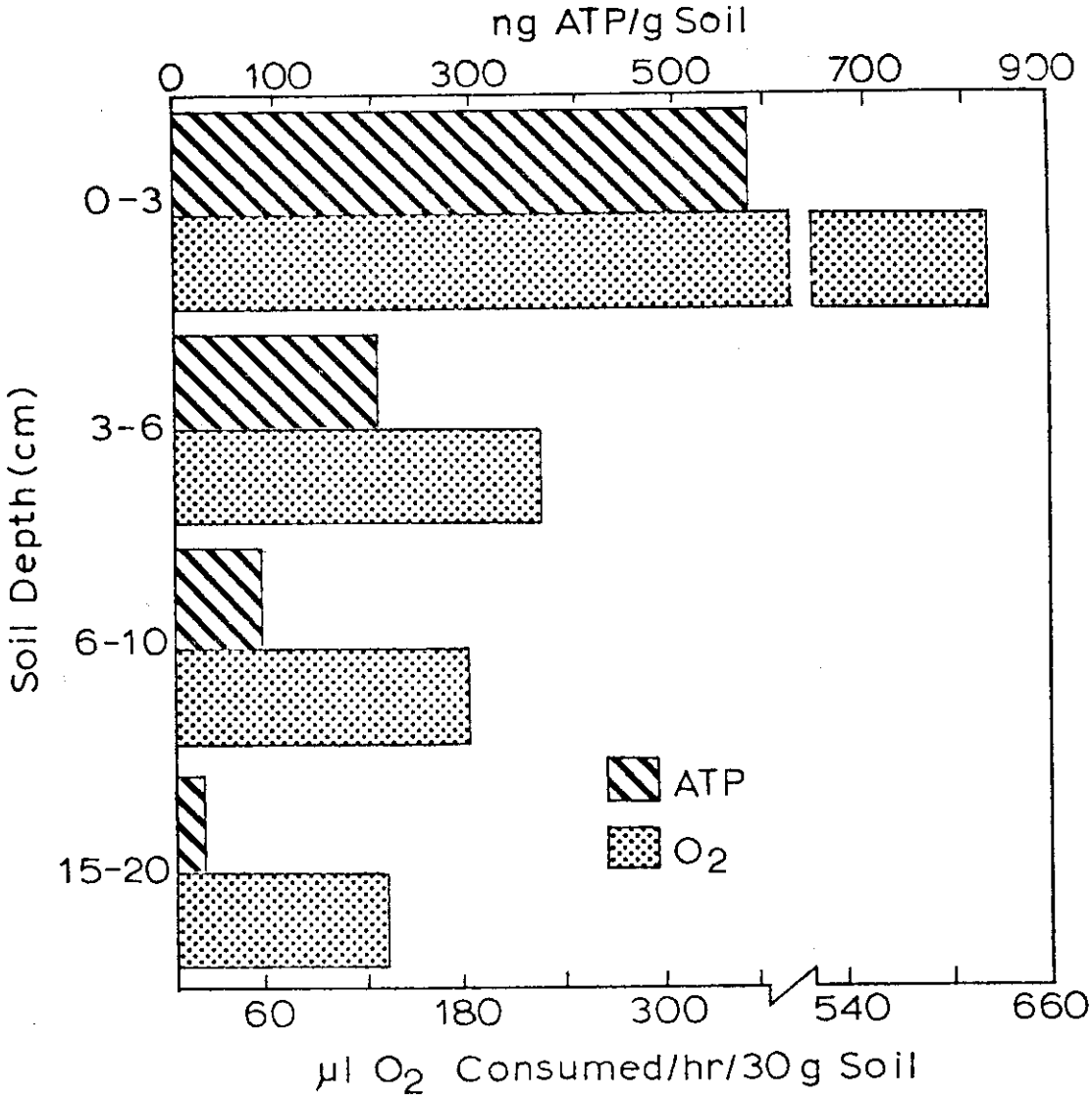

ng ATP/g Soil
100
300
500
700
900
0-3
3-6
6-10
15-20
30-40
60-70
Soil Depth (cm)
ATP
O2
0
60
180
300
540
660
µl O2 Consumed/hr/30g Soil

Fig. 19. Variation in ATP content and respiration rate (water + glucose amendment) with depth in irrigated soil (September sampling).


ng ATP/g Soil
0
100
300
500
700
900
0-3
3-6
6-10
15-20
Soil Depth (cm)
ATP
O2
60
180
300
540
660
µl O2 Consumed/hr/30 g Soil

The data were analyzed by correlation and regression techniques to determine the relationship between respiration rate and ATP content of the soil samples collected each month in nonirrigated and irrigated plots. The regression equations expressed on a log basis take the general form $Y = a + bX$, where Y is $\log_{10}$ µl O_2/30 g soil/hr, X is $\log_{10}$ ng ATP/g soil, a is the intercept and b is the slope. The antilog of this equation is $Y' = a'X'^{b}$, where Y' is µl O_2/30 g soil/hr, X' is ng ATP/g soil, b is a control parameter for the slope of the curve and a' is the proportionality constant between Y' and X'. Log_{10} respiration (with or without soil amendments prior to respiration measurement) was found to be linearly correlated to $\log_{10}$ ATP concentration (Tables 14-17, and Fig. 20), except for $\log_{10}$ respiration rate (unamended soil) and $\log_{10}$ ATP level in the nonirrigated plot, June-August (Table 14).

It is apparent from Figure 20 and the regression equations given in Tables 14, 15 and 17, that over the range of ATP values measured, respiration rates (water and water + glucose amendments) are higher in soil from the nonirrigated field than for that from the irrigated site. Birch (1958) found that rewetting dry soils resulted in greatly increased respiration rates (oxygen consumption) as compared to that of soils maintained at uniform moisture content. The increase in oxygen uptake may be due to (a) the liberation of readily available water-soluble organic matter (Chase and Gray, 1957), which under constant moisture conditions is protected by the clay from microbial attack (Birch and Friend, 1956); (b) high microbial activity associated with the youthful phase of a developing population

Table 14. Correlation coefficients and regression equations relating respiration rates to ATP levels in nonirrigated soil on monthly basis.

Regression equation[a]	Soil amendment	n	r
	June		
Y = 0.48 + 0.25X	none	12	0.308
Y = -0.86 + 1.40X	water	12	0.863**
Y = 0.37 + 0.95X	water + glucose	12	0.837**
	July		
Y = 0.37 + 0.20X	none	12	0.181
Y = 0.19 + 0.78X	water	12	0.764**
Y = 0.78 + 0.69X	water + glucose	12	0.917**
	August		
Y = 0.32 - 0.01X	none	12	0.156
Y = 1.14 + 0.56X	water	12	0.904**
Y = 1.34 + 0.63X	water + glucose	12	0.938**
	September		
Y = 0.93 + 0.29X	none	12	0.599*
Y = 1.26 + 0.35X	water	12	0.826**
Y = 1.39 + 0.48X	water + glucose	12	0.743**

[a] $Y = \log_{10}$ µl O_2 consumed/30 g soil/hr; $X = \log_{10}$ ng ATP/g soil

* significant at 0.05 level
** significant at 0.01 level

Table 15. Correlation coefficients and regression equations relating respiration rates to ATP levels in irrigated soil on monthly basis.

Regression equation[a]	Soil amendment	n	r
	June		
Y = 0.11 + 0.79X	none	12	0.953**
Y = 0.03 + 0.79X	water	12	0.806**
Y = -0.73 + 1.25X	water + glucose	12	0.941**
	July		
Y = -0.18 + 0.78X	none	12	0.751**
Y = -0.45 + 0.82X	water	12	0.691*
Y = -0.18 + 0.91X	water + glucose	12	0.824**
	September		
Y = 0.82 + 0.41X	none	12	0.970**
Y = 1.09 + 0.47X	water	12	0.859**
Y = 1.41 + 0.46X	water + glucose	12	0.917**

[a] $Y = \log_{10}$ µl O_2 consumed/30 g soil/hr; $X = \log_{10}$ ng ATP/g soil

* significant at 0.05 level
** significant at 0.01 level

Table 16. Correlation coefficients and regression equations relating respiration rates to ATP levels in nonirrigated and irrigated soil on monthly basis.

Regression equation[a]	Soil amendment	n	r
	June		
Y = 0.02 + 0.68X	none	24	0.663**
Y = -0.21 + 0.97X	water	24	0.784**
Y = -0.10 + 1.07X	water + glucose	24	0.845**
	July		
Y = -0.04 + 0.57X	none	24	0.497*
Y = -0.09 + 0.78X	water	24	0.666**
Y = 0.32 + 0.79X	water + glucose	24	0.777**
	September		
Y = 0.94 + 0.33X	none	20	0.677**
Y = 1.25 + 0.38X	water	20	0.848**
Y = 1.39 + 0.48X	water + glucose	20	0.771**

[a] $Y = \log_{10}$ µl O_2 consumed/30 g soil/hr; $X = \log_{10}$ ng ATP/g soil

* significant at 0.05 level
** significant at 0.01 level

Table 17. Correlation coefficients and regression equations relating respiration rates and ATP levels in nonirrigated and irrigated soil samples.

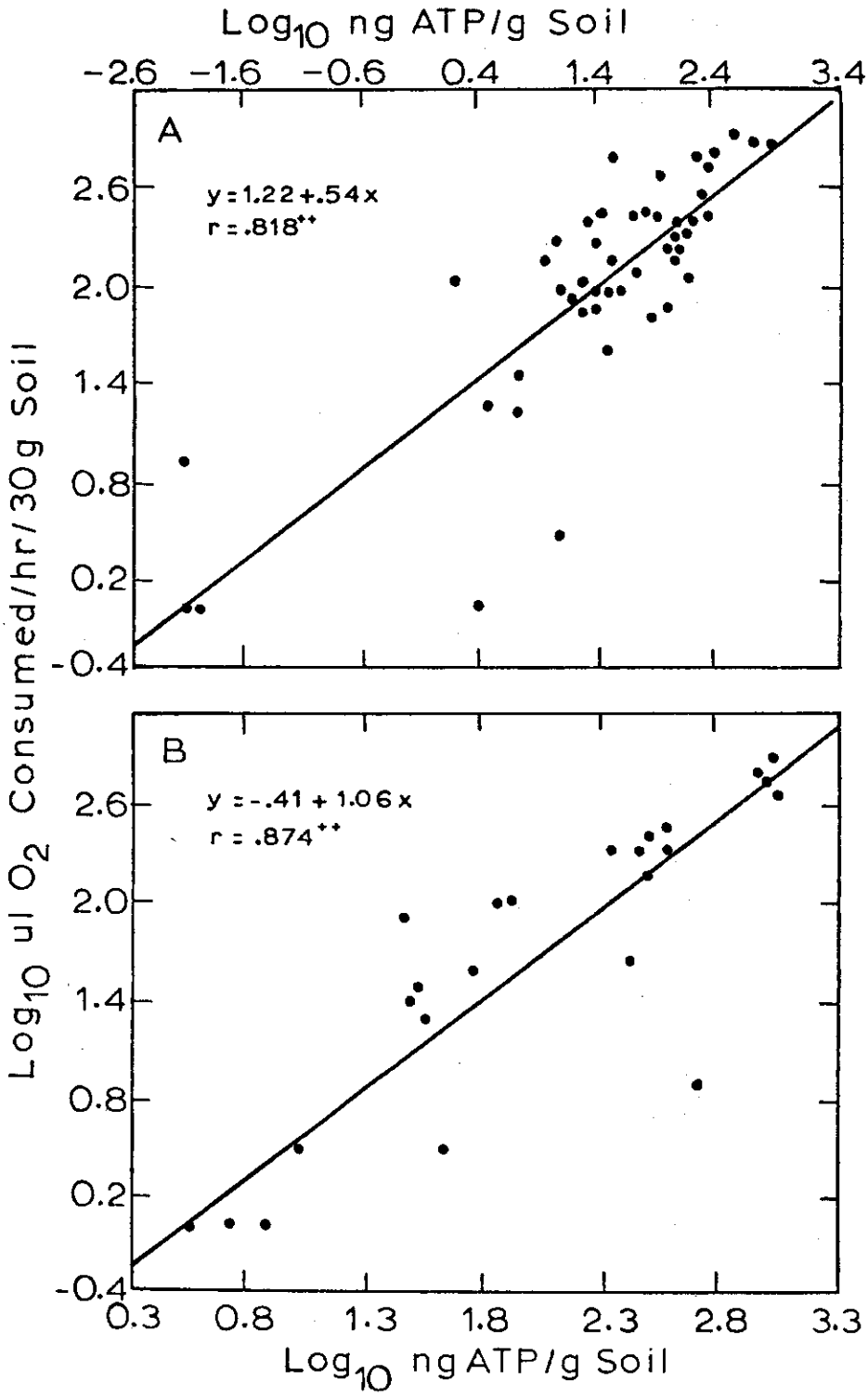
Regression equation[a]	Soil amendment	n	r
	Nonirrigated and irrigated (all dates)		
Y = 0.37 + 0.43X	none	80	0.524**
Y = 0.78 + 0.50X	water	80	0.639**
Y = 0.99 + 0.58X	water + glucose	80	0.729**
	Nonirrigated (all dates)		
Y = 0.43 + 0.22X	none	48	0.336*
Y = 0.95 + 0.49X	water	48	0.720**
Y = 1.22 + 0.54X	water + glucose	48	0.818**
	Irrigated (all dates)		
Y = -0.06 + 0.80X	none	24	0.808**
Y = -0.25 + 0.82X	water	24	0.718**
Y = -0.41 + 1.06X	water + glucose	24	0.874**

[a] $Y = \log_{10}$ µl O_2 consumed/30 g soil/hr; $X = \log_{10}$ ng ATP/g soil

* significant at 0.05 level
** significant at 0.01 level

Fig. 20. Relationship of soil ATP concentration and respiration rate (water + glucose amendment); Y = $\log_{10}$ µl O_2 consumed/hr/30 g soil; X = $\log_{10}$ ng ATP/g soil.

A. Nonirrigated soil samples (June through September).
B. Irrigated soil samples (June and July).


Log10 ng ATP/g Soil
-2.6 -1.6 -0.6 0.4 1.4 2.4 3.4
A
y = 1.22 + .54x
r = .818++
2.6
2.0
1.4
0.8
0.2
-0.4
Log10 ul O2 Consumed/hr/30g Soil
B
y = -.41 + 1.06x
r = .874++
2.6
2.0
1.4
0.8
0.2
-0.4
0.3 0.8 1.3 1.8 2.3 2.8 3.3
Log10 ng ATP/g Soil

(Birch, 1959a); or (c) the increase in surface area of organic matter exposed to decomposition following moistening of the soil (Birch, 1959b).

In this study, $\log_{10}$ ATP concentrations are linearly correlated with $\log_{10}$ oxygen uptake rates in grassland soil. Ausmus (1972) has reported that $\log_{10}$ ATP content was linearly correlated with carbon dioxide evolution from litter and soil in a forest ecosystem. Measurements of respiration (oxygen consumption and carbon dioxide evolution) have been one of the earliest and still most frequently used indices of total microbial activity in soil (Clark and Paul, 1970). The results of the present study suggest the potential of using ATP measurements to estimate microbial respiration and, hence, general microbial activity in the soil.

To relate ATP levels to microbial abundance, soil samples taken in July were analyzed for numbers of viable cells using the plate count procedures and for ATP concentrations. Results of these analyses for nonirrigated and irrigated plots are shown in Table 18 and in Appendix Tables 12 and 13.

Generally soil samples from the irrigated plot contained higher levels of ATP and microbial cells than did those from the nonirrigated field. Microbial numbers, ATP levels, and respiration rates tended to decline with soil depth in both field treatments, which may have reflected substrate distribution. Bartos (1971) reported that on the Pawnee site, 60% of root weight occurred in the upper 10 cm of the soil profile, with about 75% in the upper 20 cm. The roots and aboveground litter are major sources of carbonaceous substrate for microbial populations. Lauenroth and Sims (1973) found higher aboveground and belowground

Table 18. ATP levels, respiration rates, microbial numbers, and water contents of soil at different depths in nonirrigated and irrigated plots (July sampling).*

Depth	Soil water	ATP	Respiration rate for soil receiving			Plate counts		
			Nothing	Water	Water + glucose	Bacteria	Actinomycetes	Fungi
cm	%	ng/g soil	---ul O_2/30 g soil/hr ----			--10^5/g soil------		10^3/g soil
			Nonirrigated field					
0-3	4.86	683.96	112.8	363.4	738.6	72.8	65.3	52.8
3-6	1.93	104.10	1.0	100.2	210.4	46.5	54.3	35.6
6-10	2.67	143.72	1.0	62.9	132.1	42.8	46.4	15.4
15-20	5.19	91.95	2.0	6.2	66.6	37.5	32.3	8.2
30-40	5.31	29.62	19.6	47.9	88.7	18.3	16.7	6.2
60-70	5.95	4.90	7.6	9.4	17.6	14.4	12.6	4.7
			Irrigated field					
0-3	17.76	1007.59	393.5	355.2	587.0	521.5	383.8	114.3
3-6	13.11	290.73	110.2	104.5	187.0	385.7	336.1	94.7
6-10	14.68	363.04	26.9	7.2	29.0	160.0	190.7	77.6
15-20	17.23	41.40	9.7	14.0	63.7	133.4	112.7	12.5
30-40	15.61	32.20	10.2	13.7	25.0	77.4	61.2	7.4
60-70	9.52	4.30	7.2	1.0	1.0	30.3	20.6	3.6

* Values are means of two composites at each depth (for each composite there were 2 determinations for soil moisture, 16 for ATP, 3 for respiration, and 5 replicate plates for microbial counts).

plant yield at the irrigated site than in the nonirrigated plot (1971 season). Considering the higher levels of carbonaceous substrate and water in soil at the irrigated plot, it is not surprising that microbial counts and soil ATP concentrations were higher in this plot as compared to the nonirrigated one.

Log_{10} ATP concentration was found to be linearly correlated with log_{10} microbial counts for soils from both nonirrigated and irrigated plots as illustrated in Figures 21 and 22. The b values (b is the slope of the line in the log form of the equation) for bacterial and actinomycete counts were larger than that for fungal counts because of the smaller number of fungi in the soil samples. For soils from the nonirrigated plot, higher b values were obtained for bacterial and actinomycete counts than for those from the irrigated field. This means that for a given number of bacterial or actinomycete cells the level of ATP is higher in the soils of the nonirrigated plot than in those of the irrigated one. Two possibilities may account for this finding. Not all microbes were counted in the nonirrigated samples. Alternatively, not all of the ATP was measured in the irrigated samples, resulting in lower ATP levels as compared to that from nonirrigated soil. Both explanations seem unlikely. Conklin and Macgreagor (1972) found that soil moisture and texture seemed to have little effect on the recoverability of ATP from desert soils, using the butanol-octanol as extracting solution.

The close correlation between soil ATP concentrations and numbers of viable cells as estimated from plate counts is surprising. One might expect there would be poor agreement between the two since the plate count is believed to determine only a segment of the total microbial community while the ATP method presumably measures the presence of all living cells. The explanation for this finding is unclear,

Fig. 21. Scattergrams and linear regression equations for mean soil ATP levels and microbial numbers in the nonirrigated plot (July sampling). Correlation coefficients are significant at .01 level.

A. $Y = \log_{10}$ ng ATP/g soil; $X = \log_{10}$ bacterial nos. x 10^{-5}/g soil.

B. $Y = \log_{10}$ ng ATP/g soil; $X = \log_{10}$ fungal nos. x 10^{-3}/g soil.

C. $Y = \log_{10}$ ng ATP/g soil; $X = \log_{10}$ actinomycete nos. x 10^{-5}/g soil.

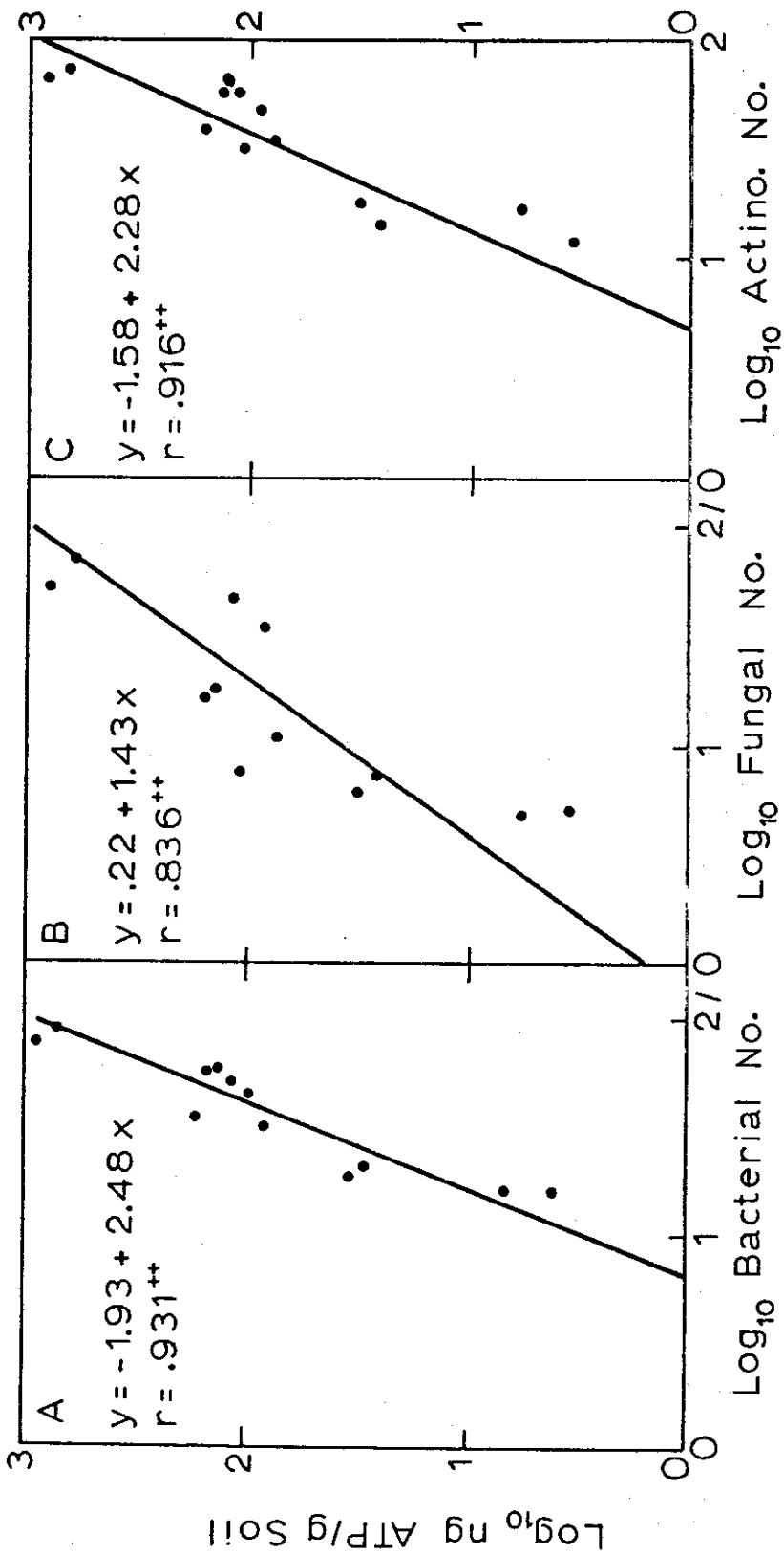
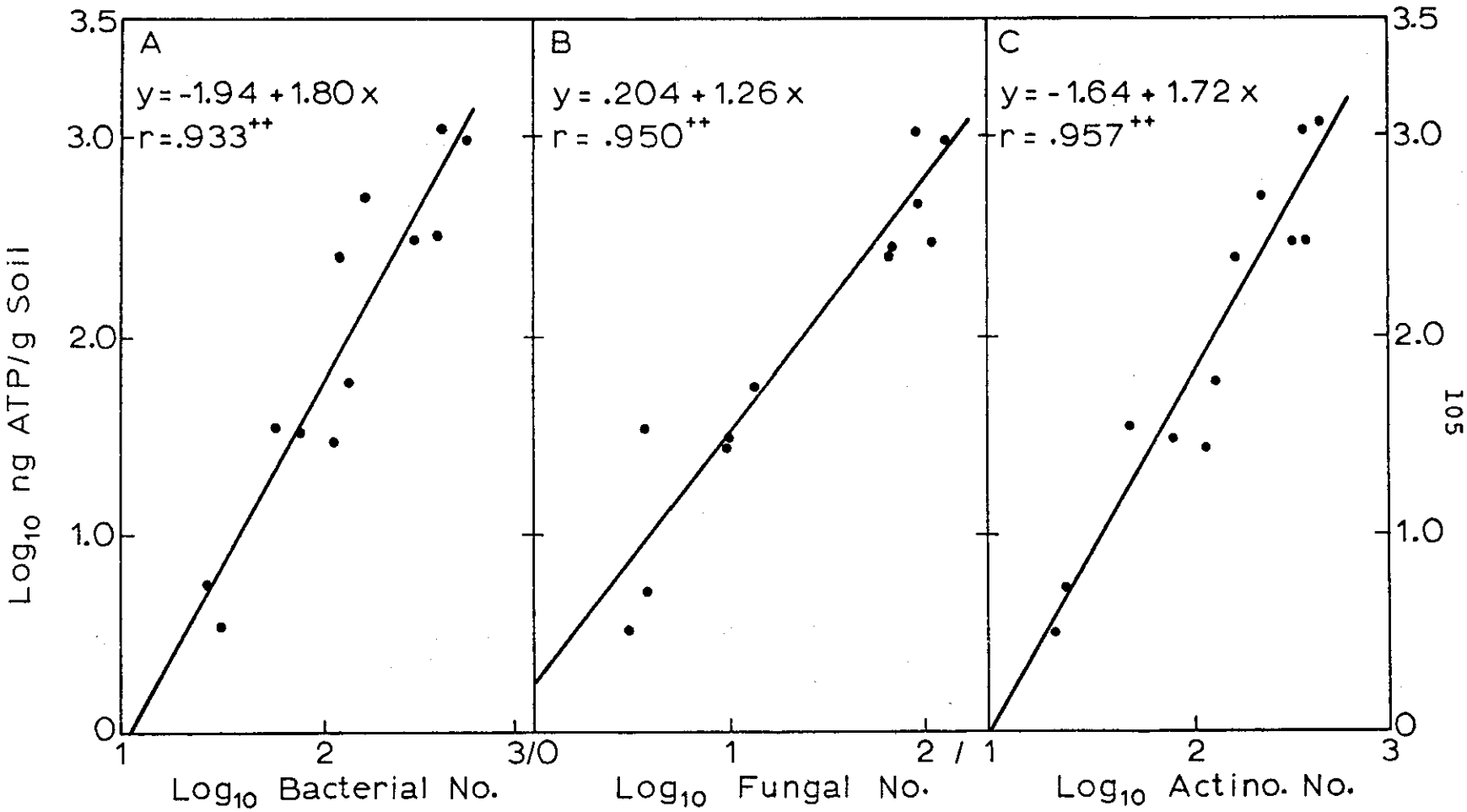

A
y = -1.93 + 2.48 x
r = .931++
B
y = .22 + 1.43 x
r = .836++
C
y = -1.58 + 2.28 x
r = .916++
Log10 ng ATP/g soil
Log10 Bacterial No.
Log10 Fungal No.
Log10 Actino. No.
0
1
2
3

Fig. 22. Scattergrams and linear regression equations for mean soil ATP levels and microbial numbers in the irrigated plot (July sampling). Correlation coefficients are significant at .01 level.

A. $Y = \log_{10}$ ng ATP/g soil; $X = \log_{10}$ bacterial nos. x 10^{-5}/g soil.

B. $Y = \log_{10}$ ng ATP/g soil; $X = \log_{10}$ fungal nos. x 10^{-3}/g soil.

C. $Y = \log_{10}$ ng ATP/g soil; $X = \log_{10}$ actinomycete nos. x 10^{-5}/g soil.


A
y = -1.94 + 1.80 x
r = .933++
B
y = .204 + 1.26 x
r = .950++
C
y = - 1.64 + 1.72 x
r = .957++
Log10 ng ATP/g Soil
Log10 Bacterial No.
Log10 Fungal No.
Log10 Actino. No.
3.5
3.0
2.0
1.0
0
1
2
3/0
1
2 / 1
2
3
105

but may indicate that the plate counts values are in fact reasonable indices of the total viable microflora in soil or, at least, are indicative of a relatively constant fraction of the total microbial biomass.

The relationship of respiration rates (soils receiving different amendments) to microbial counts and to ATP levels was also examined. Significant linear correlations were found between $\log_{10}$ ATP respiration rates and $\log_{10}$ microbial numbers and $\log_{10}$ ATP content except in nonirrigated soil samples where no significant linear correlation was found between respiration rate (unamended, variable soil moisture) and microbial numbers or ATP levels (Tables 19 and 20). The scatter diagrams in Figures 23 and 24 illustrate the linear relationship between $\log_{10}$ respiration rate (water + glucose amendment) and $\log_{10}$ microbial numbers, and $\log_{10}$ ATP concentration for soils from both nonirrigated and irrigated plots. Respiration rates of soils amended with water and with water + glucose were higher than those in nonamended soils.

Microbial numbers and respiration in soil are dependent upon the amount of available energy-yielding material. It is reasonable to expect some degree of relationship between the numbers of viable microbial cells obtained by plate count (representing a proportion of the total actively metabolizing microbial population) and the results of their activities. Gray and Wallace (1957) found a direct relationship between bacterial counts and carbon dioxide evolution in field soil. Witkamp (1966) similarly found such a relationship to hold for a litter environment. He reported positive correlations between mean annual carbon dioxide production and combined estimate for microbial population (sum of bacterial counts plus 153 times fungal counts).

Table 19. Correlation coefficients and regression equations relating respiration rates for different soil amendments to ATP levels and microbial numbers in non-irrigated soil (July sampling).

Regression equation	X	r
	No amendment	
$Y = 0.37 + 0.25X$	$\log_{10}$ bacteria x 10^{-5}/g soil	0.083
$Y = 0.41 + 0.30X$	$\log_{10}$ fungi x 10^{-3}/g soil	0.152
$Y = 1.07 + 0.21X$	$\log_{10}$ actinomycetes x 10^{-5}/g soil	0.076
$Y = 0.37 + 0.20X$	$\log_{10}$ ng ATP/g soil	0.181
	Water added	
$Y = -1.58 + 2.07X$	$\log_{10}$ bacteria x 10^{-5}/g soil	0.701*
$Y = 0.03 + 1.40X$	$\log_{10}$ fungi x 10^{-3}/g soil	0.806**
$Y = -1.15 + 1.84X$	$\log_{10}$ actinomycetes x 10^{-5}/g soil	0.727**
$Y = 0.19 + 0.78X$	$\log_{10}$ ng ATP/g soil	0.764**
	Water + glucose added	
$Y = -0.55 + 1.71X$	$\log_{10}$ bacteria x 10^{-5}/g soil	0.856**
$Y = 0.76 + 1.14X$	$\log_{10}$ fungi x 10^{-3}/g soil	0.888**
$Y = -0.32 + 1.58X$	$\log_{10}$ actinomycetes x 10^{-5}/g soil	0.847**
$Y = 0.78 + 0.69X$	$\log_{10}$ ng ATP/g soil	0.917**

$Y = \log_{10}$ respiration in µl O_2 consumed/hr/30 g soil

* significant at 0.05 level

** significant at 0.01 level

Table 20. Correlation coefficients and regression equations relating respiration rates at different soil amendments to ATP levels and microbial numbers in irrigated soil (July sampling).

Regression equation	X	r
	No amendment	
$Y = -2.02 + 1.56X$	$\log_{10}$ bacteria x 10^{-5}/g soil	0.775**
$Y = -0.20 + 1.11X$	$\log_{10}$ fungi x 10^{-3}/g soil	0.808**
$Y = -1.54 + 1.38X$	$\log_{10}$ actinomycetes x 10^{-5}/g soil	0.738**
$Y = -0.18 + 0.78X$	$\log_{10}$ ATP in ng/g soil	0.751**
	Water added	
$Y = -3.00 + 1.92X$	$\log_{10}$ bacteria x 10^{-5}/g soil	0.840**
$Y = -0.37 + 1.09X$	$\log_{10}$ fungi x 10^{-3}/g soil	0.698*
$Y = -2.33 + 1.67X$	$\log_{10}$ actinomycetes x 10^{-5}/g soil	0.783**
$Y = -0.45 + 0.82X$	$\log_{10}$ ATP in ng/g soil	0.691*
	Water + glucose added	
$Y = -2.64 + 1.96X$	$\log_{10}$ bacteria x 10^{-5}/g soil	0.923**
$Y = -0.08 + 1.09X$	$\log_{10}$ fungi x 10^{-3}/g soil	0.749**
$Y = -2.09 + 1.76X$	$\log_{10}$ actinomycetes x 10^{-5}/g soil	0.893**
$Y = 0.18 + 0.91X$	$\log_{10}$ ATP in ng/g soil	0.824**

Y = $\log_{10}$ respiration in µl O_2/hr/30 g soil

* significant at 0.05 level
** significant at 0.01 level

Fig. 23. Scattergrams and regression equations of mean respiration rate (water + glucose amendment) and ATP levels and microbial numbers for soils from the nonirrigated plot (significant at .01 level).

A. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ ng ATP/g soil.

B. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ bacterial no. x 10^{-5}/g soil.

C. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ fungal no. x 10^{-3}/g soil.

D. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ actinomycete no. x 10^{-5}/g soil.

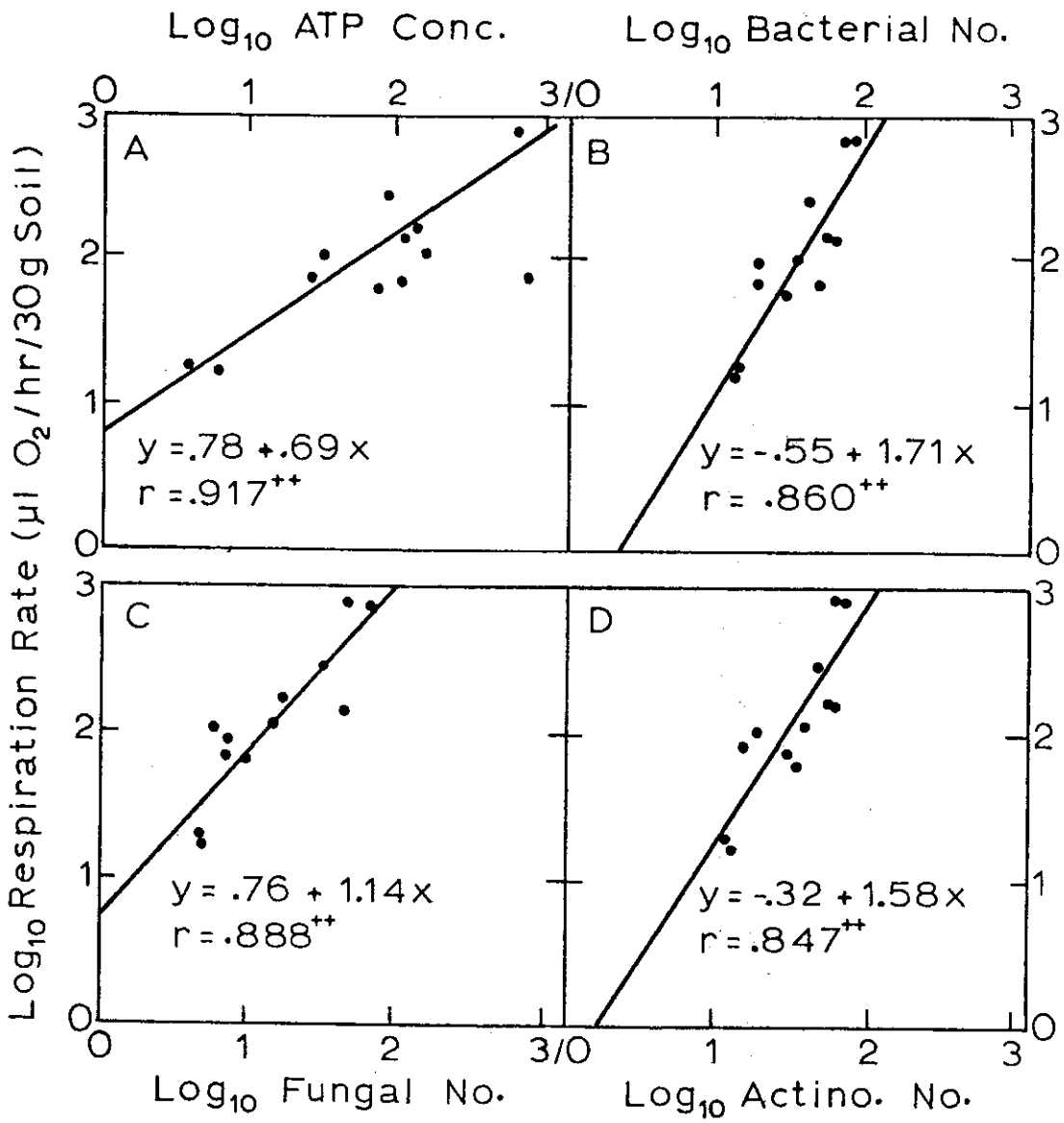

Log10 ATP Conc.
Log10 Bacterial No.
A
y =.78 +.69 x
r =.917++
B
y = -.55 + 1.71 x
r = .860++
C
y = .76 + 1.14 x
r = .888++
D
y = -.32 + 1.58 x
r = .847++
Log10 Respiration Rate (µl O2 / hr / 30 g Soil)
Log10 Fungal No.
Log10 Actino. No.

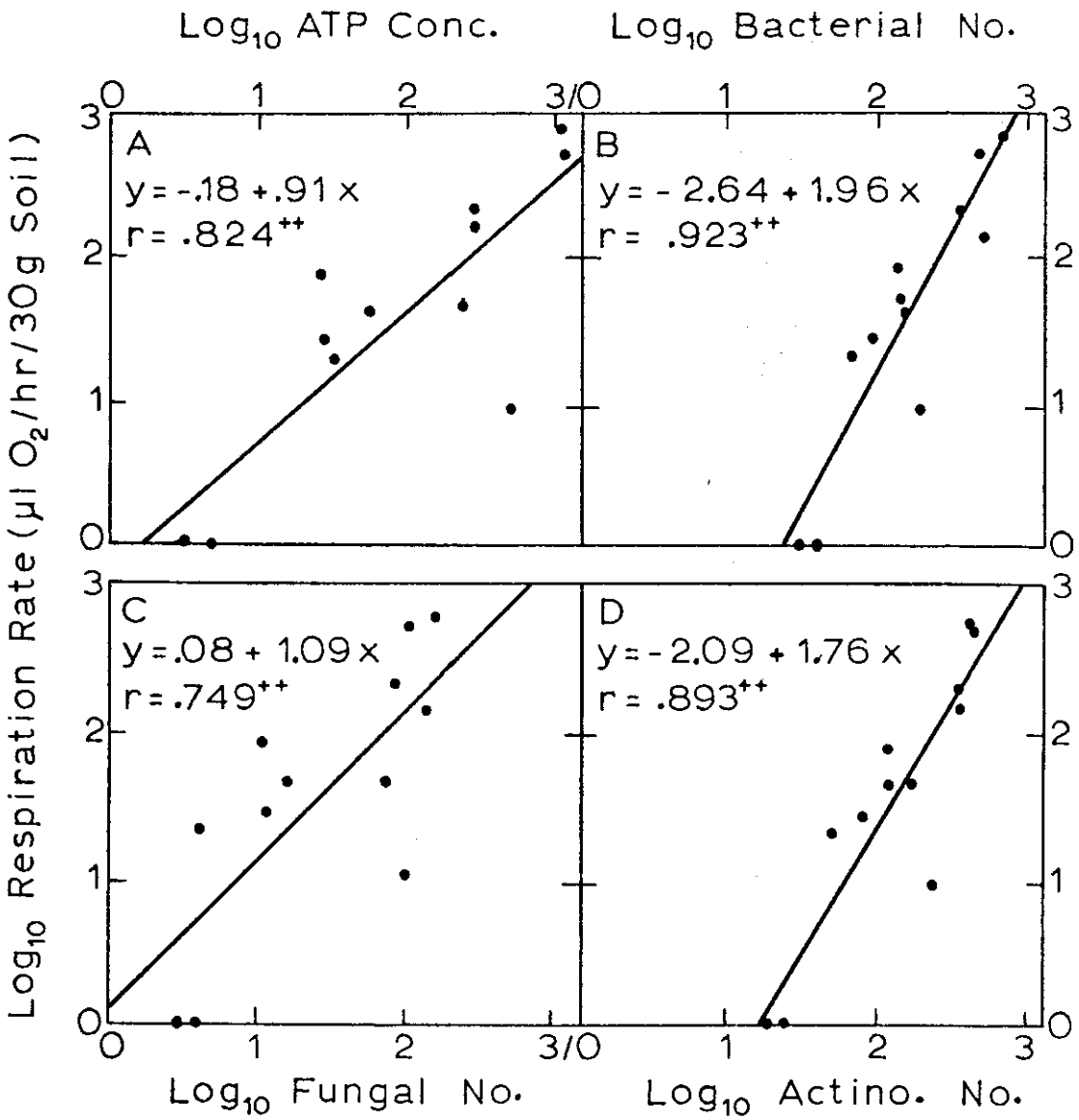
Fig. 24. Scattergrams and regression equations of mean respiration rate (water + glucose amendment) and ATP levels and microbial numbers for soils from the irrigated plot (significant at .01 level).

A. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ ng ATP/g soil.

B. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ bacterial no. x 10^{-5}/g soil.

C. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ fungal no. x 10^{-3}/g soil.

D. $Y = \log_{10}$ µl O_2 consumed/hr/30 g soil;
$X = \log_{10}$ actinomycete no. x 10^{-5}/g soil.


Log10 ATP Conc.
Log10 Bacterial No.
A
y = -.18 + .91 x
r = .824++
B
y = - 2.64 + 1.96 x
r = .923++
C
y = .08 + 1.09 x
r = .749++
D
y = - 2.09 + 1.76 x
r = .893++
Log10 Respiration Rate (µl O2/hr/30g Soil)
Log10 Fungal No.
Log10 Actino. No.

The STAT 38R (originally $BMDO_2R$) multiple regression computer program (of the Colorado State University Computer Center) was utilized to determine which variables accounted for the variance in ATP levels and respiration rates. With stepwise multiple regression one can examine the change in the importance of each variable as additional X variables are added to the equation in successive steps. At each step a coefficient of determination (R^2), a partial regression coefficient ($b_{y\ 1,2,3...k}$), and a partial correlation coefficient (R) may be calculated for each variable. R^2 is the percentage of sum of squares of deviations of Y for its mean that is attributable to the regression (R^2 here measures the proportion of total variation about mean Y explained by regression as a percentage). The partial regression coefficient of variable X_1 is the simple regression coefficient of the residual value of Y or X_1, when all other X variables are held constant at their respective means. The sequential F value can be used to test whether the last variable in the regression contributed significantly in reducing the amount of unexplained variation. With the partial F value each variable can be tested as if it were the last entering the regression thereby allowing one to determine whether the variable contributed a significant decrease in the residual sum of squares (a criterion by which the variable is retained in or removed from the equation). The overall F test is used for determining the statistical significance of the regression model at each step. The exact level of significance is included in the computer output and is one of the criteria used for choosing the best regression model.

The dependent and independent variables were transformed into $\log_{10}$ forms before statistical analyses were performed. $\log_{10}$ ATP content and $\log_{10}$ respiration rate (water amendment and water + glucose amendment) were positively correlated with $\log_{10}$ microbial numbers and negatively correlated with $\log_{10}$ depth for soils from the nonirrigated plot (Table 21) and the irrigated plot (Table 22). In addition, in the irrigated field, $\log_{10}$ ATP and $\log_{10}$ respiration rate (water + glucose amendment) were positively correlated with $\log_{10}$ percent soil water. $\log_{10}$ respiration rate (unamended) was positively correlated with $\log_{10}$ microbial numbers and negatively correlated with soil depth only in soils from the irrigated plot (Table 21). $\log_{10}$ microbial numbers were negatively correlated with $\log_{10}$ depth for soils from both field treatments. The decrease in microbial numbers with depth is consistent with the distribution of available organic carbon but may also be related to other soil factors such as water and oxygen. The significant positive correlations among the microbial counts (between fungal counts and bacterial counts or actinomycete counts, and between bacterial and actinomycete counts) are presumably the result of similar reactions by these groups of organisms to conditions in the soil (available organic carbon, moisture content and composition of soil atmosphere).

In the stepwise multiple regression analyses, the following independent variables were used: bacterial, fungal and actinomycete counts, soil depth, and percent soil water. For soils from the nonirrigated field, $\log_{10}$ percent soil water accounted for 32% for the variance in respiration rate (no amendment), $\log_{10}$ fungal counts contributed 23%, $\log_{10}$ actinomycete counts added 18% and $\log_{10}$ bacterial numbers added 12% (Table 23). $\log_{10}$

Table 21. Simple linear correlation coefficients for correlations between soil depth, ATP levels, respiration rates, microbial numbers, and soil water in non-irrigated plot of July sampling (d.f. = 10 in all cases).

Variable	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9
1 Log_{10} depth	1.000	-0.929**	-0.186	-0.820**	-0.939**	-0.935**	-0.972**	-0.945**	0.404
2 Log_{10} ATP		1.000	0.181	0.764**	0.917**	0.931**	0.836**	0.916**	-0.249
3 Log_{10} AM_1[a]			1.000	0.284	0.346	0.083	0.152	-0.076	0.565
4 Log_{10} AM_2[a]				1.000	0.900**	0.701*	0.806**	0.727**	-0.452
5 Log_{10} AM_3[a]					1.000	0.856**	0.888**	0.847**	-0.275
6 Log_{10} bacteria						1.000	0.878	0.964**	-0.364
7 Log_{10} fungi							1.000	0.914**	-0.476
8 Log_{10} actinomycetes								1.000	-0.487
9 Log_{10} % soil water									1.000

[a] AM_1 Respiration soil amendment$_1$: None
AM_2 Respiration soil amendment$_2$: Water
AM_3 Respiration soil amendment$_3$: Water + glucose

* significant at 0.05 level
** significant at 0.01 level

Table 22. Simple linear correlation coefficients for correlations between soil depth, ATP levels, respiration rates, microbial numbers, and soil water in irrigated plot of July sampling (d.f. = 10 in all cases).

Variable	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9
1 Log_{10} depth	1.000	-0.951**	-0.812**	-0.821**	-0.874**	-0.963**	-0.931**	-0.946**	-0.560
2 Log_{10} ATP		1.000	0.751**	0.691*	0.824**	0.933**	0.951**	0.957**	0.617*
3 Log_{10} AM_1[a]			1.000	0.684*	0.591*	0.775**	0.808**	0.738**	0.191
4 Log_{10} AM_2[a]				1.000	0.902**	0.840**	0.698*	0.783**	0.509
5 Log_{10} AM_3[a]					1.000	0.923	0.749**	0.893**	0.766**
6 Log_{10} bacteria						1.000	0.921**	0.980**	0.627*
7 Log_{10} fungi							1.000	0.950**	0.440
8 Log_{10} actinomycetes								1.000	0.627*
9 Log_{10} % soil water									1.000

[a] AM_1 Respiration soil amendment$_1$: None
AM_2 Respiration soil amendment$_2$: Water
AM_3 Respiration soil amendment$_3$: Water + glucose

* significant at 0.05 level
** significant at 0.01 level

soil depth was the most important single factor accounting for 67% and 88% of the variance in respiration (water amendment) and respiration (water + glucose amendment), respectively. The contribution by the other variables was small due to their close correlation with $\log_{10}$ soil depth.

In the regression equations for irrigated soils shown in Table 24, $\log_{10}$ soil depth and $\log_{10}$ percent soil water together accounted for 76% of the variance in $\log_{10}$ respiration rate (no amendment). The insignificant contributions of $\log_{10}$ microbial counts are evident in the low partial F-values and slight increase in the standard error of estimate when these variables were added. $\log_{10}$ bacterial numbers accounted for 70% of the variability in respiration (water amendment) while $\log_{10}$ of both bacterial and fungal counts accounted for 92% of variability in respiration rate (water + glucose amendment) in irrigated soil samples (Table 24). The regression models indicate the dependency of respiration rate, i.e., oxygen consumption, on microbial counts and soil factors which affect microbial numbers, such as moisture and depth.

Bacterial numbers accounted for 87% of the variability in $\log_{10}$ ATP concentration in soils from the nonirrigated plot while $\log_{10}$ soil depth contributed another 2%, $\log_{10}$ fungal counts added 6%, $\log_{10}$ percent soil water added only 1% and $\log_{10}$ actinomycetes added nothing (Table 25). When the partial F-values and standard error of estimation are considered along with the contribution to the explained variance, only $\log_{10}$ bacterial numbers, $\log_{10}$ soil depth and $\log_{10}$ fungal numbers need be included in the prediction equation.

Table 23. Results of stepwise multiple linear regression analyses with independent variables listed in order of entry into equation, partial F-value, coefficient of determination (R^2), equations predicting $\log_{10}$ µl O_2 consumed/hr/30 g soil (RESP) for different soil amendments, significance level of overall F-ratio and standard error of the estimate in $\log_{10}$ µl O_2 consumed/hr/30 g soil (S.E.E.) in nonirrigated soil.

Independent variable in order of entry	Partial F-value	R^2	Prediction equation	Significance level of overall F-ratio	S.E.E.
No amendment					
$\log_{10}$ % soil water (W)	4.68	0.32	RESP = -0.55 + 2.18W	0.05565	0.68
$\log_{10}$ fungi x 10^{-3} (F)	4.59	0.55	RESP = -2.35 + 3.18W + 1.06F	0.02778	0.58
$\log_{10}$ actinomycetes x 10^{-5} (A)	5.47	0.73	RESP = 0.26 + 2.91W + 2.88F - 3.00A	0.01123	0.48
$\log_{10}$ bacteria x 10^{-5} (B)	5.20	0.85	RESP = 0.37 + 2.17W + 2.85F - 7.10A + 4.30B	0.00565	0.39
$\log_{10}$ soil depth (D)	2.62	0.89	RESP = 7.17 + 1.80W + 0.99F - 7.52A + 3.14B - 1.96D	0.00708	0.35
Water added					
$\log_{10}$ soil depth (D)	20.57	0.67	RESP = 2.70 - 1.01D	0.00108	0.42
$\log_{10}$ bacteria x 10^{-5} (B)	1.07	0.71	RESP = 5.53 - 1.62D - 1.44B	0.00395	0.42
$\log_{10}$ % soil water (W)	0.44	0.72	RESP = 5.66 - 1.54D - 1.40B - 0.47W	0.01277	0.44
$\log_{10}$ actinomycetes x 10^{-5} (A)	0.33	0.74	RESP = 6.65 - 1.73D - 0.41B - 0.73W - 1.42A	0.03399	0.46
$\log_{10}$ fungi x 10^{-5} (F)	0.16	0.74	RESP = 8.60 - 2.34D - 0.76B - 0.86W - 1.32A - 0.75F	0.08116	0.49

Table 23. (Continued)

Independent variable in order of entry	Partial F-value	R^2	Prediction equation	Significance level of overall F-ratio	S.E.E.
			Water + glucose added		
Log_{10} soil depth (D)	74.19	0.88	RESP = 2.94 - 0.85D	0.00001	0.19
Log_{10} actinomycetes x 10^{-5} (A)	1.34	0.90	RESP = 4.35 - 1.18D - 0.71A	0.00004	0.18
Log_{10} fungi x 10^{-3} (F)	1.16	0.91	RESP = 5.62 - 1.62D - 0.76A - 0.63F	0.00016	0.18
Log_{10} % soil water (W)	0.06	0.91	RESP = 5.31 - 1.56D - 0.69A - 0.55F + 0.09W	0.00090	0.19
Log_{10} bacteria x 10^{-5} (B)	0.06	0.91	RESP = 5.54 - 1.63D - 0.48A - 0.62F + 0.12W - 0.27B	0.00413	0.21

* Critical F-value (1,9, 0.90) = 3.36.

Table 24. Results of stepwise multiple linear regression analyses with independent variables listed in order of entry into equation, partial F-value, coefficient of determination (R^2), equations predicting $\log_{10}$ µl O_2 consumed/hr/30g soil (RESP) for different soil amendments, significance level of overall F-ratio and standard error of the estimate in $\log_{10}$ µl O_2 consumed/hr/30g soil (S.E.E.) in irrigated soil.

Independent variable in order of entry	Partial F-value	R^2	Prediction equation	Significance level of overall F-ratio	S.E.E.
			No amendment		
$\log_{10}$ soil depth in cm (D)	19.34	0.66	RESP = 2.65 - 1.23D	0.00134	0.53
$\log_{10}$ % soil water (W)	3.80	0.76	RESP = 7.07 - 1.56D - 3.52W	0.00161	0.47
$\log_{10}$ bacteria x 10^{-5}/g soil (B)	0.40	0.77	RESP = 5.16 - 0.98D - 4.03W + 0.87B	0.00602	0.49
$\log_{10}$ actinomycetes x 10^{-5}/g soil (A)	0.25	0.78	RESP = 5.03 - 1.00D - 3.97W + 1.70B - 0.84A	0.01870	0.51
$\log_{10}$ fungi x 10^{-3}/g soil (F)	0.53	0.80	RESP = 3.34 - 0.58D - 2.63W + 2.45B - 2.34A + 0.88F	0.04259	0.53
			Water added		
$\log_{10}$ bacteria x 10^{-5}/g soil (B)	23.89	0.70	RESP = -3.00 + 1.92B	0.00063	0.57
$\log_{10}$ actinomycetes x 10^{-5}/g soil (A)	1.44	0.74	RESP = -3.41 + 4.20B - 2.17A	0.00211	0.55
$\log_{10}$ fungi x 10^{-3}/g soil (F)	0.25	0.75	RESP = -3.95 + 4.03B - 1.44A -0.44F	0.00816	0.58
$\log_{10}$ soil depth in cm (D)	0.38	0.77	RESP = -1.17 + 2.89B - 0.95A -0.78F - 0.84D	0.02281	0.60
$\log_{10}$ % soil water (W)	0.26	0.78	RESP = 0.23 + 2.69B - 0.08A - 1.24F - 0.96D - 1.74W	0.05622	0.64

Table 24. (Continued)

Independent variable in order of entry	Partial F-value	R^2	Prediction equation	Significance level of overall F-ratio	S.E.E.
			Water + glucose added		
Log_{10} bacteria x 10^{-5}/g soil (B)	57.74	0.85	RESP = -2.64 + 1.96B	0.00002	0.37
Log_{10} fungi x 10^{-3}/g soil (F)	7.34	0.92	RESP = -4.09 + 3.24B - 0.96F	0.00001	0.29
Log_{10} % soil water (W)	2.51	0.94	RESP = -5.36 + 2.63B - 0.69F	0.00003	0.27
Log_{10} soil depth in cm (D)	0.41	0.94	RESP = -4.06 + 2.26B - 0.79F + 1.95W - 0.38D	0.00021	0.28
Log_{10} actinomycetes x 10^{-5}/g soil (A)	0.80	0.95	RESP = -3.13 + 1.35B - 1.24F + 1.24W - 0.58D + 1.29A	0.00084	0.28

* Critical F value (1, 9, 0.90) = 3.36

Table 25. Results of stepwise multiple linear regression analyses with independent variables listed in order of entry into equation, partial F-value, coefficient of multiple determination (R^2), equations predicting $\log_{10}$ ng ATP/g soil, significance level of overall F-ratio and standard error of the estimate in $\log_{10}$ ng ATP/g soil (S.E.E.) in nonirrigated and irrigated soil.

Independent variable in order of entry	Partial F-value	R^2	Prediction equation	Significance level of overall F-ratio	S.E.E.
			Nonirrigated field		
$\log_{10}$ bacteria x 10^{-5}/g soil (B)	65.12	0.87	ATP = -1.92 + 2.48B	0.00001	0.27
$\log_{10}$ soil depth in cm (D)	2.25	0.89	ATP = 0.41 + 1.33B - 0.56D	0.00004	0.25
$\log_{10}$ fungi x 10^{-3}/g soil (F)	8.98	0.95	ATP = 5.36 + 0.60B - 2.16D -1.87F	0.00002	0.18
$\log_{10}$ actinomycete x 10^{-3}/g soil (A)	0.58	0.95	ATP = 5.29 + 0.08B - 2.17D - 2.02F + 0.68A	0.00009	0.19
$\log_{10}$ bacteria x 10^{-5}/g soil (B)	0.01	0.95	ATP = 5.38 - 2.19D - 2.04F + 0.73A	0.00001	0.18
$\log_{10}$ % soil water (W)	0.93	0.96	ATP = 4.22 - 1.93D - 1.77F + 0.99A + 0.33W	0.00006	0.18
$\log_{10}$ bacteria x 10^{-5}/g soil (B)	0.03	0.96	ATP = 4.37 - 1.98D - 1.81F + .12A + 0.34W - 0.17B	0.00042	0.19
			Irrigated field		
$\log_{10}$ actinomycetes x 10^{-5}/g soil (A)	108.88	0.92	ATP = -1.64 + 1.72A	0.00000	0.25
$\log_{10}$ soil depth in cm (D)	2.79	0.94	ATP = 0.58 + 0.98A - 0.64D	0.00000	0.23
$\log_{10}$ bacteria x 10^{-5}/g soil (B)	2.56	0.95	ATP = 2.27 + 1.88A - 1.02D - 1.46B	0.00001	0.22
$\log_{10}$ % soil water (W)	0.65	0.96	ATP = 1.92 + 1.82A - 1.09D - 1.61B + 0.75W	0.00008	0.22
$\log_{10}$ fungi x 10^{-3}/g soil (F)	2.20	0.97	ATP = 0.59 + 0.63A - 0.76D - 1.00B + 1.76W + 0.69F	0.00022	0.20

* Critical F-value (1,9, 0.90) = 3.36.

For soils from the irrigated plot, $\log_{10}$ actinomycetes accounted for 92% of variability in $\log_{10}$ ATP (Table 25). The other variables added from 1-2% to the explained variance and did not really provide a significant decrease in the residual sum of squares as shown by their partial F-values which were below the critical F-value. The contribution of other variables was small due to their close correlations with $\log_{10}$ actinomycete counts (Table 22), and the variance explained by these variables is already accounted for by $\log_{10}$ actinomycete in this model. By means of the multiple regression analyses, the five variables were rated in order of their importance to soil ATP. The results are encouraging in that they reflect the dependency of soil ATP on microbial plate counts. This is consistent with the hypothesis that ATP from soil is extracted mainly from microorganisms.

General Discussion

Use of ATP measurements as indicators of microbial abundance

The present study has shown the utility of the ATP assay as a basis for estimating microbial abundance in soil. A quantitative relationship was found between ATP levels and counts of bacteria and actinomycetes in grassland soil samples provided with moisture and substrate and incubated under laboratory conditions. Similarly, in soil samples freshly collected from the field, a close correlation was observed between ATP content and microbial (bacterial, actinomycete, and fungal) numbers.

For bacterial strains isolated from the Pawnee grassland site, the average carbon to ATP ratio was 250. Other workers have reported average ratios of 250 for bacteria (Forest, 1965; Cole, et al., 1967; Hamilton and

Holm-Hansen, 1967), 226 for fungi (Dawes and Large, 1970), and 286 for algae (Holm-Hansen, 1970). If it is assumed that the average C/ATP ratio for microbial tissue in soil is 250, then total microbial cellular carbon can be calculated from measurements of ATP concentration. Total microbial cellular dry weight can also be derived, if the average carbon content for microflora in soil is taken to be 50% (Spector, 1956; Alexander, 1961).

The ATP assay has several advantages over the conventional methods employed to determine microbial abundance in soil. Results are more readily available, i.e., the waiting period involved in the incubation of plates in the dilution plate technique is avoided. The results are more reproducible, and the ATP assay can be more easily standardized between laboratories and researchers. Great variation in results are common from plate count determinations because of differences in operator techniques, media, and incubation conditions.

It was assumed in the present study that the ATP extracted from soil samples was solely from microorganisms. The contribution of nonmicrobial forms, e.g., plant roots and small soil animals, to the ATP pool was not measured directly. However, all roots visible to the naked eye were removed from soil samples prior to ATP extraction and analysis. Also, similar relationships between ATP levels and microbial numbers were found in soil samples directly from the field and in air-dried samples which would not be expected to contain plant roots or sizable populations of animals.

From liquid culture studies an average bacterial cell was found to contain 2.5×10^{-9} µg of ATP. With this value and a knowledge of the number of bacterial cells in a

soil sample (obtained by the plate count procedure), the amount of ATP attributable to bacteria can be estimated. Bacterial ATP was calculated to represent 11.4% of total ATP extracted from soil samples supplied with adequate substrate and moisture and incubated in the laboratory. For samples collected in July from the 0-3 cm depth of the irrigated field, bacterial ATP constituted 12.9% of total soil ATP by this calculation.

It is important to note that it is not possible to calculate solely from ATP concentration numbers of bacteria (or of any other group) in a soil sample which has a mixed microbial population. The relative contribution of various microbial groups to the soil ATP level cannot be ascertained without additional information. Doxtader (1969) has proposed separating microbial types using membrane filters prior to ATP extraction as an aid in dealing with this problem.

Use of ATP measurements to predict microbial respiration rates

Soil ATP measurements can be utilized to calculate respiration rates from regression equations developed in this study. The following regression equation was found to apply to irrigated soil samples: $\log Y = \log (-0.41) + 1.06 \log X$, where Y is respiration rate in µl O_2 consumed/30 g soil/hr and X is ATP concentration in ng/g soil. By taking the antilogarithms and considering 1.06 equal to 1.00, the equation takes the form of $Y = 0.39X$. For nonirrigated soil samples the logarithmic equation takes the form of $\log Y = \log 1.22 + 0.54 \log X$ and has the form of $Y = 16.6\ X^{1/2}$ when the antilogarithms are taken. The differences in the two equations for irrigated and nonirrigated soil may be due to variations in availability of moisture and substrate in the soils. The equations reflect the fact that for a given amount of ATP respiratory activity in

nonirrigated soil samples amended with water was greater than for similarly treated irrigated samples. A possible explanation for this observation is that upon wetting of dry soils (moisture was applied prior to taking respiration measurements) readily utilizable substrate was released and immediately consumed by the microflora, resulting in rapid oxygen uptake. Such substrate may have already been consumed in the soil of the irrigated site where moisture was not a limiting factor.

Results of the field study are consistent with those obtained in laboratory studies using pure cultures. ATP levels followed changes in oxygen consumption rates of several actinomycete and fungal isolates grown in liquid cultures, but the two parameters were not closely correlated. A time course study of the growth of one fungal strain showed a significant linear correlation between ATP content and oxygen uptake rate.

It should be pointed out that respiration rates of soil samples were determined under optimal conditions and over short periods of time. Adequate moisture, readily available carbonaceous substrate and a suitable temperature (25 C) were provided. Respiration rates measured directly in the field would not be expected to hold the same relationship to ATP. The effect of fluctuating environmental conditions such as temperature, moisture and soil fertility status on the ATP-soil respiration relationship needs to be investigated.

Use of ATP values in computer simulation models of microbial activity in a grassland ecosystem

The ATP method has the advantage of integrating microbial abundance and activity and has the potential for the

estimation of total microbial biomass and respiratory activity in soil. ATP measurements would seem to be useful in efforts to model the flow of energy and nutrients in the Grassland Biome Program, as, for example, in the ELM mathematical model (Anway, et al., 1972) which has been developed to describe carbon, energy and nutrient flow.

In the decomposer section of the ELM model, decomposition is represented by the flow of material from litter, feces, and belowground-dead compartments to the microbial compartment. The flow rates are controlled by temperature and moisture and by the initial nitrogen content of the material. Microbial biomass increases through the assimilation of a fixed proportion of decomposed material and decreases through energy losses in maintenance processes. Respiration is represented by flows from the microbial compartment to atmospheric carbon dioxide.

For ecosystem modeling purposes, ATP measurements should be useful for following changes in the microbial segment of the decomposer group in response to perturbations such as rainfall. However, from ATP data, little can be said concerning the contribution of individual groups of organisms to the total decomposition process.

As previously considered, ATP values can be quantitatively related to soil respiratory rates as measured in the laboratory. Further studies are needed to determine the relationship of soil ATP concentrations to carbon dioxide evolution measurements in the field under fluctuating moisture and temperature conditions. On the basis of findings reported in this study, it would seem worthwhile to investigate the use of ATP measurements for the prediction of decomposition rates under field conditions.

Estimates of total microbial biomasses (dry weights) in soil samples were calculated from ATP concentrations and are presented in Table 26. Here it was assumed that the average ATP/C ratio for microbial tissue was 1:250 and that an average cell contained 50% carbon. These biomass estimates are lower than the combined values for bacterial and fungal biomass reported by Doxtader (1969b) who used direct microscopic techniques on samples of the same Ascalon sandy loam soil (Table 27).

Since the two kinds of analyses were made on soil samples which were collected from different areas of the Pawnee site and during different years, it is difficult to compare the results. However, two points are apparent. First, much higher biomass values were obtained by the direct microscopic procedures than were calculated from ATP concentrations. Second, the direct methods did not indicate as large a change in microbial biomass with depth as did the ATP assay. The higher values obtained with the microscopic method, may result from the inclusion of dead cells and organic debris along with living cells in the estimate of biomass by this procedure.

ATP measurements can be utilized to assess rates of immobilization of nutrient elements such as nitrogen, phosphorus and sulfur in soil by using the ratios of ATP to these elements in microbial cell material. Thus ATP values may also be used in the nutrient flow section of the ELM model. Lee, et al. (1971), obtained ATP/C/N/P/S ratios of 1:250:42:8.6:2.6 by averaging data cited by other workers (Spector, 1956; Strickland, et al., 1969). A more extensive study of the relationship of ATP to other cellular constituents for diverse soil microorganisms under various environmental conditions is needed. It is generally assumed that these ratios are the same for cells grown in liquid culture and in soil. This assumption needs to be tested.

Table 26. Microbial biomass as calculated from ATP measurements on soil from nonirrigated and irrigated sites at various sampling times.

Soil depth	Sampling time (1971)			
	June	July	August	September
cm	----------g/m^2*----------			
		Nonirrigated		
15	16	24	4	13
30	23	31	6	15
		Irrigated		
15	23	39	--	--
30	29	43	--	--

* Dry weight to indicated depth.

Table 27. Combined bacterial and fungal biomass in nonirrigated soil at various sampling times (Doxtader, 1969b).

Soil depth	Sampling time (1969)		
	July	August	September
cm	----------g/m^2*----------		
15	57	36	30
30	82	61	51

*Dry weight to indicated depth, determined by direct microscopic techniques.

Based on the average ratios of ATP to chemical elements present in microbial tissue which are cited by Lee, et al. (1971), the following amounts of nutrients were calculated to be tied up in microbial tissue in the grassland soil during the sampling period (in g/m^2 to 30 cm soil depth): 2.8 to 15 of carbon, 0.47 to 2.6 of nitrogen, 0.10 to 0.53 of phosphorus, and 0.03 to 0.16 of sulfur.

A regression equation relating ATP concentration to respiration rate was utilized to calculate average microbial growth rate in grassland soil. The average ATP content in 0-10 cm depth of nonirrigated soil samples in July was 284 ng/g soil. From the regression equation derived from soil samples of the nonirrigated site (all dates, soil samples amended with water prior to measuring respiration rate) of $Y = 0.95 + 0.49X$ (where Y is $\log_{10}$ µl O_2 consumed/30 g soil/hr and X is $\log_{10}$ ng ATP/g soil), the calculated average oxygen consumption rate is 5.0 µl/hr/g soil. The rate of carbon dioxide evolution was estimated to be 6.86 µg CO_2/hr/g soil by using an assumed respiratory quotient of 0.7 (calculated from values given by Klein, 1972) From the average ATP concentration, the microbial biomass (dry weight) was calculated to be 0.14 mg/g soil. Thus 1 mg of microbial tissue would evolve 0.0484 mg CO_2/hr, or 0.0132 mg C/hr.

It is generally assumed that in soil the amount of evolved CO_2-C represents 80% of the total microbially metabolized carbon, the remaining 20% contributing to an increase in biomass or excretion of metabolites (Alexander, 1961). However, assimilation values of as much as 60% have been reported (Payne, 1970). In the ELM model, it is assumed that when availability of material for growth

exceeds the maintenance energy requirement, approximately 20% of the metabolized carbon is fixed into microbial biomass. With this value the maximum increase in microbial biomass would then be 0.0066 mg/hr (assuming cells are composed of 50% C on a dry weight basis). That is, one unit weight of microbial biomass will double every 150 hr or 6.3 days. During a growing season of 200 days, this would allow a maximum of 32 generations. Table 28 summarizes estimates of microbial growth rates in soil derived by various techniques (table modified from Gray and Williams, 1971b). As can be seen, a wide range of generation times has been reported, from the very short doubling time of 16 hr to as long as 1,200 hr.

Sims and Singh (1971) estimate that over a 200-day growing season at the Pawnee site, a total of 587 g dry matter/m^2 was decomposed (ungrazed treatment), representing an energy supply of 2634 kcal/m^2. The average microbial biomass (dry weight) in the nonirrigated soil samples was calculated to be 18.7 g/m^2. Using the value of 5.5 kcal for the energy required for the production of 1 g of microbial biomass (Clark and Paul, 1970), we find that the substrate is sufficient to support about 25 generations. However, not all of this substrate energy would have been available to microorganisms as animals active in the decomposition process would be expected to utilize some of it. This figure of 25 generations per season is not too different from the calculated number of generations (32) using ATP values. The energy content of the material undergoing decomposition was estimated for the year 1969, while the data in this study were collected in 1971.

Table 28. Microbial growth rates in soil (modified from Gray and Williams, 1971b).

Microbial type	Soil environment	Basis of calculation	Generation time in hr	Reference
Mixed bacteria	Grassland	Energy input	1,200	Babiuk & Paul (1970)
Mixed bacteria	Agricultural	CO_2 output	28.5	Gray & Williams (1971)
Mixed bacteria	Deciduous forest	Energy input	16.0	" "
Nitrosomonas	Agricultural	Metabolite production in perfused soil	38-100	Morrill & Davison (1962)
Nitrobacter	Agricultural	" "	21-58	" "
Mixed microorganisms	Grassland	ATP and O_2 uptake	150	This study

Interestingly, the calculated number of microbial generations (25 or 32) is about the same as the number of rainfall events (29) with a median value of 6 mm (F. Smith, personal communication) within the 200-day growing season of 1971. It may be speculated that the effect of the water amendment on respiration rate of the nonirrigated soil samples is comparable to that of a rainfall event on respiration rate in the field.

CHAPTER V

SUMMARY

A technique was developed to determine ATP concentrations in microbial cells and in soil. A liquid scintillation spectrometer was used to quantify light production from a firefly luminescent assay system, the amount of light emitted being proportional to the quantity of ATP present. An aqueous-butanol-octanol solvent mixture was found to be the most efficient extracting agent for ATP among several reagents tested. No changes in ATP levels were detected in soil stored at 5 C for 5 days or in soil extracts stored at -20 C for 30 days.

Microbial isolates from grassland soil were used to study the relationship of ATP concentration to cellular carbon, cell viability, and respiration rate. In the case of one bacterial isolate (B-5), ATP concentration was linearly correlated with counts of viable cells obtained by the plate count method ($r = + 0.92^{**}$). For the other isolates (B-7 and B-10), log of ATP content was linearly correlated to log of cell numbers. In the case of isolate B-7, ATP level was directly related to cell density (turbidity) in the culture liquid. However, this relationship did not hold for the other isolates. For three bacterial isolates (B-5, B-7, and B-10), ATP concentrations correlated significantly with cellular carbon levels. The results obtained using isolate B-6 were more variable. For 3 of the soil strains, the average ratio of cellular carbon to ATP was 250.

Among various fungal and actinomycete isolates grown in liquid cultures, cellular ATP levels generally followed changes in oxygen consumption rates, but were not closely correlated. However, ATP concentrations and oxygen uptake rates were directly related ($r = + 0.80^{**}$) for a growing culture of one fungal strain.

Grassland soil samples were amended with glucose and ammonium phosphate and sufficient water to bring the soil to field capacity and incubated in the laboratory. ATP levels were measured at frequent intervals and compared to counts of viable microbial cells obtained by the plate count technique. The numbers of bacteria and actinomycetes closely paralleled changes in ATP levels in the samples, with r values of $+ 0.79^{*}$ and $+ 0.85^{*}$, respectively. No significant linear correlation was found between soil ATP content and fungal numbers.

To determine the relationship of ATP concentrations to respiration rates in soil samples collected from the Pawnee grassland during the course of a plant-growing season, nonirrigated and irrigated fields were sampled to depths of 70 cm at monthly intervals from June through September. Three soil treatments were employed in respiration analyses to determine the effect of added moisture and carbonaceous substrate on microbial activity: (1) unamended soil; (2) water added to bring soil water content to 25%. For the most part, the addition of water or water plus glucose resulted in increased respiration rates for soil samples from both nonirrigated and irrigated fields. ATP levels were higher in the samples of the irrigated plot than in those of the nonirrigated one. Log of ATP content was linearly correlated with log of oxygen consumption

rate of soils (with or without amendments) for both nonirrigated and irrigated soils, except in the case of unamended soil from the nonirrigated field.

Soil samples collected from the field in July were analyzed for numbers of viable microorganisms in addition to ATP levels and respiration rates. Higher microbial counts were found in soils from the irrigated plot than in those from the nonirrigated one. Log of ATP content was linearly correlated with log of bacterial, actinomycete, and fungal numbers in soil from both field treatments. Log of respiration rate was also linearly correlated with log of microbial counts in soil. Multiple regression analysis of the data indicated that bacterial and fungal numbers and depth of soil accounted for 95% of the variance in ATP concentrations in soils from the nonirrigated plot while actinomycete numbers accounted for 92% of that in soils from the irrigated field.

The close correlations between ATP concentrations and numbers of viable microbial cells not only for liquid cultures and for soil samples incubated in the laboratory but also for soil freshly collected from the field, suggest that both techniques measure a constant proportion of the total mass of viable cellular material.

Values for microbial cellular dry weight and carbon were calculated from measurements of ATP concentration in grassland soil samples, using a ratio of ATP to carbon of 0.0040 and an average cell carbon content of 50%. From regression equations, ATP determinations were also employed to calculate respiration rates.

The possibility of using ATP and respiratory values to calculate microbial turn-over times was explored. Since ATP concentrations closely correlated with microbial numbers and respiratory activity in liquid cultures and in soil samples, the results of this study indicate that ATP measurements are useful as quantitative indices of microbial abundance and activity in soil.

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A P P E N D I C E S

APPENDICES TABLE OF CONTENTS

Appendix Table 1. Effect on ATP concentration of storing soil for 24 hr at 5 C.

Soil dilution	Extract of soil dilution	ATP determination	ng ATP/g soil
		Before refrigeration	
1	1	1	281.6
		2	179.2
		3	179.2
		4	204.8
	2	1	217.6
		2	230.4
		3	256.0
		4	224.0
2	1	1	371.2
		2	371.2
		3	268.8
		4	371.2
	2	1	230.4
		2	230.4
		3	231.6
		4	268.8
			$\bar{X}$ = 257.3
		After refrigeration	
1	1	1	358.4
		2	281.6
		3	332.8
		4	358.4
	2	1	281.6
		2	256.0
		3	288.0
		4	288.0
2	1	1	256.0
		2	294.4
		3	288.0
		4	307.2
	2	1	345.6
		2	166.4
		3	217.6
		4	230.4
			$\bar{X}$ = 284.4

Appendix Table 2. Analysis of variance of ATP concentrations in soil before and after storage at 5 C for 24 hr.

Source of variation	df	Mean square	F	
Between treatments	1	5886.125	1.75	N.S.
Within treatment	30	3362.5943		
Total	31			

N.S. Non-significant.

Appendix Table 3. Soil ATP levels at different depths in nonirrigated and irrigated plots (June sampling).

(cm)	number	Mean	S.D.	C.V.
		Nonirrigated		
0-3	1	210.33	104.63	49.74
	2	366.61	113.43	30.94
3-6	1	168.00	38.71	23.04
	2	146.41	38.08	26.01
6-10	1	130.24	18.19	13.97
	2	152.90	22.35	14.62
15-20	1	65.53	11.26	17.19
	2	111.28	21.58	19.39
30-40	1	19.61	4.08	20.80
	2	27.95	3.78	13.53
60-70	1	12.88	2.45	19.03
	2	6.30	2.01	32.03
		Irrigated		
0-3	1	1063.32	163.56	15.38
	2	945.90	152.05	16.07
3-6	1	358.86	86.89	24.21
	2	395.91	61.52	16.49
6-10	1	294.02	99.90	33.97
	2	224.99	78.86	35.05
15-20	1	70.39	22.28	31.65
	2	80.96	25.35	31.31
30-40	1	32.35	4.86	15.04
	2	39.48	3.64	9.22
60-70	1	10.00	2.62	26.19
	2	7.16	2.83	39.59

Appendix Table 4. Soil ATP levels at different depths in nonirrigated and irrigated plots (July sampling).

Depth (cm)	Composite number	ng ATP/g soil		
		Mean	S.D.	C.V.
		Nonirrigated		
0-3	1	754.47	96.34	12.76
	2	613.45	76.83	12.52
3-6	1	120.67	17.99	14.91
	2	87.54	22.57	25.78
6-10	1	156.50	35.91	22.94
	2	130.94	22.74	17.36
15-20	1	106.56	17.05	16.00
	2	77.34	17.41	22.51
30-40	1	27.21	4.72	17.37
	2	32.02	3.21	10.02
60-70	1	6.19	2.43	39.27
	2	3.62	1.17	32.32
		Irrigated		
0-3	1	1051.49	198.38	18.86
	2	963.69	188.93	19.60
3-6	1	294.70	46.50	15.78
	2	286.76	65.52	22.84
6-10	1	479.75	109.91	22.91
	2	246.33	45.07	18.29
15-20	1	55.93	15.75	28.16
	2	26.88	18.84	70.09
30-40	1	30.24	2.87	9.51
	2	34.16	4.84	14.18
60-70	1	5.22	3.90	74.62
	2	3.39	2.75	80.90

Appendix Table 5. Soil ATP levels at different depths in nonirrigated plot (August sampling).

Depth (cm)	Composite number	ng ATP/g soil		
		Mean	S.D.	C.V.
0-3	1	95.65	34.56	36.13
	2	42.80	12.79	29.89
3-6	1	32.38	10.71	33.07
	2	22.70	7.27	32.04
6-10	1	37.94	7.60	20.03
	2	25.34	25.62	101.10
15-20	1	15.29	5.16	33.78
	2	2.00	2.15	107.25
30-40	1	32.46	4.84	14.92
	2	44.75	4.23	9.45

Appendix Table 6. ATP levels at different depths in non-irrigated and irrigated plots (September sampling).

Depth (cm)	Composite number	ng ATP/g soil		
		Mean	S.D.	C.V.
		Nonirrigated		
0-3	1	257.57	40.17	15.59
	2	276.35	13.29	4.80
3-6	1	254.28	18.36	7.22
	2	214.88	35.30	16.43
6-10	1	65.57	14.91	22.74
	2	81.86	14.07	17.18
15-20	1	10.74	8.35	77.76
	2	12.55	5.41	43.15
30-40	1	21.05	4.50	21.37
	2	16.36	5.33	28.22
60-70	1	0.00	0.00	0.00
	2	2.76	2.25	81.64
		Irrigated		
0-3	1	521.01	50.48	9.69
	2	650.38	148.74	22.86
3-6	1	179.31	17.67	9.85
	2	227.30	19.78	8.70
6-10	1	95.66	13.86	14.48
	2	68.01	15.02	22.09
15-20	1	23.32	10.19	43.71
	2	29.38	6.27	21.34

Appendix Table 7. Soil water content at different depths for nonirrigated and irrigated plots in June through September.

Month	Depth	Nonirrigated			Irrigated*		
		C_1	C_2	Mean	C_1	C_2	Mean
	cm	-------%--------			--------%---------		
June	0-3	0.90	0.54	0.72	14.92	15.20	15.06
	3-6	0.88	0.70	0.79	11.59	10.70	11.14
	6-10	1.88	0.98	1.43	8.80	8.62	8.71
	15-20	4.31	3.42	3.87	10.48	8.94	9.71
	30-40	6.49	6.67	6.58	7.88	12.95	10.42
	60-70	3.86	4.55	4.20	7.68	9.44	8.56
July	0-3	4.83	4.89	4.86	17.37	18.03	17.76
	3-6	1.75	2.11	1.93	13.46	12.77	13.11
	6-10	2.33	3.01	2.67	14.82	14.55	14.68
	15-20	4.75	5.63	5.19	16.76	17.70	17.23
	30-40	5.28	5.34	5.31	15.46	15.76	15.61
	60-70	8.49	3.42	5.95	9.23	9.82	9.52
August	0-3	1.43	1.72	1.58	-	-	-
	3-6	2.27	2.08	2.18	-	-	-
	6-10	2.84	2.58	2.71	-	-	-
	15-20	4.75	5.03	4.89	-	-	-
	30-40	4.58	4.32	4.45	-	-	-
	50-60	4.01	3.82	3.92	-	-	-
September	0-3	7.00	5.99	6.49	3.68	3.85	3.76
	3-6	8.03	7.18	7.61	5.00	4.99	4.99
	6-10	6.91	7.38	7.14	5.22	6.01	5.62
	15-20	5.74	5.69	5.71	7.22	8.24	7.73
	30-40	5.17	6.29	5.78	-	-	-
	60-70	3.53	4.15	3.84	-	-	-

* Analyses not made in irrigated plot during August period and lowest depths in September period.

Appendix Table 8. Mean soil respiration rates at different amendments and different depths in nonirrigated and irrigated plots (June sampling).

Depth	Composite number	Soil amendment		
		None	Water	Water + glucose
cm		-------µl O_2/30 g soil/hr----------		
		Nonirrigated		
0-3	1	5.7	366.6	664.6
	2	41.6	445.1	823.1
3-6	1	6.4	141.2	243.8
	2	7.4	145.0	249.4
6-10	1	3.6	64.7	166.3
	2	6.7	75.2	224.0
15-20	1	21.3	71.4	127.8
	2	20.0	106.3	158.1
30-40	1	35.2	74.3	110.2
	2	5.7	70.4	102.1
60-70	1	12.8	2.8	3.5
	2	1.0	0.3	29.0
		Irrigated		
0-3	1	341.2	298.9	768.7
	2	386.0	303.8	704.4
3-6	1	95.2	91.1	226.4
	2	124.1	143.1	296.2
6-10	1	130.1	118.8	253.3
	2	127.7	106.5	227.3
15-20	1	35.9	43.0	106.7
	2	50.7	36.4	106.3
30-40	1	15.6	6.2	32.9
	2	12.6	1.5	3.8
60-70	1	5.1	13.0	4.0
	2	15.6	18.0	1.0

Appendix Table 9. Mean soil respiration rates at different amendments and different depths in non-irrigated and irrigated plots (July sampling).

Depth	Composite number	Soil amendment		
		None	Water	Water + glucose
cm		----------µl O_2/30 g soil/hr----------		
		Nonirrigated		
0-3	1	103.7	342.9	746.4
	2	122.0	383.8	730.7
3-6	1	1.0	89.6	143.5
	2	1.0	110.7	277.2
6-10	1	1.0	48.0	109.0
	2	1.0	77.8	155.2
15-20	1	3.8	10.1	71.7
	2	1.0	21.2	61.5
30-40	1	25.9	43.5	77.6
	2	13.4	52.3	99.8
60-70	1	7.5	0.9	16.6
	2	7.6	17.8	18.5
		Irrigated		
0-3	1	376.3	344.5	534.5
	2	410.7	366.0	639.7
3-6	1	73.4	105.3	154.6
	2	146.9	103.7	219.5
6-10	1	40.7	1.0	10.0
	2	13.1	13.8	48.0
15-20	1	18.4	3.2	44.0
	2	1.0	24.7	83.4
30-40	1	19.5	25.8	28.6
	2	1.0	1.6	21.4
60-70	1	5.0	1.0	1.0
	2	9.4	1.0	1.0

Appendix Table 10. Mean soil respiration rates at different amendments and different depths in nonirrigated plots (August sampling).

Depth	Composite number	Soil amendment		
		None	Water	Water + glucose
cm		---------µl O_2/30 g soil/hr---------		
0-3	1	1.0	252.1	498.8
	2	1.0	388.2	627.1
3-6	1	1.0	170.5	285.1
	2	1.0	164.5	233.2
6-10	1	1.0	77.9	147.5
	2	0.7	72.5	186.1
15-20	1	1.0	10.1	99.7
	2	2.5	49.1	115.5
30-40	1	1.0	45.8	45.1
	2	1.0	58.5	97.9
60-70	1	1.0	1.0	1.0
	2	1.0	1.0	1.0

Appendix Table 11. Mean soil respiration rates at different amendments and different depths in nonirrigated and irrigated plots (September sampling).

Depth	Composite number	Soil amendment		
		None	Water	Water + glucose
cm		---------μl O_2/30 g soil/hr---------		
		Nonirrigated		
0-3	1	42.7	166.3	556.3
	2	80.8	261.0	649.6
3-6	1	99.9	77.4	292.7
	2	83.1	92.8	366.1
6-10	1	80.4	117.3	274.5
	2	70.4	138.4	286.3
15-20	1	11.1	62.1	151.2
	2	1.9	73.3	186.8
30-40	1	12.7	9.8	76.6
	2	3.2	47.7	78.7
60-70	1	8.3	5.2	9.5
	2	8.7	12.0	1.0
		Irrigated		
0-3	1	97.1	364.6	687.3
	2	82.3	322.2	545.7
3-6	1	48.4	91.6	228.1
	2	73.3	89.1	213.7
6-10	1	51.3	98.8	179.4
	2	37.9	94.0	177.7
15-20	1	23.9	67.4	135.6
	2	25.2	66.3	128.4

Appendix Table 12. Microbial numbers at different soil depths in irrigated plot (July sampling).

Depth (cm)	Composite number	Mean	S.D.	C.V.
		--Bacteria (10^5/g soil)-		
0-3	1	459.8	87.7	19.07
	2	583.2	77.4	13.27
3-6	1	432.2	54.6	12.63
	2	339.3	54.1	15.94
6-10	1	179.6	34.8	19.38
	2	139.7	24.2	17.32
15-20	1	144.6	21.8	15.08
	2	122.2	6.6	5.40
30-40	1	88.2	7.0	7.94
	2	66.5	9.5	14.28
60-70	1	28.9	4.8	16.61
	2	31.7	8.8	27.76
		--Fungi (10^3/g soil)--		
0-3	1	95.4	58.7	61.53
	2	133.2	16.4	12.31
3-6	1	116.0	29.8	25.69
	2	73.4	10.8	14.71
6-10	1	88.7	19.5	21.98
	2	66.5	8.6	12.93
15-20	1	15.0	2.0	13.33
	2	9.9	1.6	16.16
30-40	1	10.8	2.7	25.00
	2	4.0	2.2	55.00
60-70	1	4.0	2.1	52.50
	2	3.2	1.5	46.88
		--Actinomycetes (10^5/g soil)--		
0-3	1	409.0	85.3	20.85
	2	358.7	73.0	20.35
3-6	1	344.3	72.8	21.14
	2	327.9	107.7	32.84
6-10	1	217.4	33.0	15.21
	2	164.1	11.9	7.25
15-20	1	119.4	6.6	5.53
	2	106.0	12.1	11.42
30-40	1	75.9	8.4	11.07
	2	46.5	11.2	24.09
60-70	1	22.2	9.4	42.34
	2	18.8	4.2	22.34

Appendix Table 13. Microbial numbers at different soil depths in nonirrigated plot (July sampling).

Depth (cm)	Composite number	Mean	S.D.	C.V.
		--Bacteria (10^5/g soil)--		
0-3	1	67.2	10.0	14.88
	2	78.4	15.2	19.39
3-6	1	51.5	12.2	23.68
	2	41.5	3.6	8.67
6-10	1	33.0	21.6	65.45
	2	52.6	4.2	8.17
15-20	1	46.2	11.3	24.46
	2	28.8	17.0	59.03
30-40	1	19.0	1.7	9.95
	2	17.6	5.8	32.95
60-70	1	14.8	1.9	12.84
	2	13.9	1.2	8.63
		--Fungi (10^5/g soil)--		
0-3	1	45.8	6.0	13.10
	2	59.7	4.1	6.87
3-6	1	40.9	2.6	6.36
	2	30.2	2.2	7.28
6-10	1	14.7	1.0	6.80
	2	16.1	2.5	15.53
15-20	1	6.9	0.9	13.04
	2	9.5	0.6	6.32
30-40	1	6.9	1.7	24.64
	2	5.6	1.2	21.43
60-70	1	4.7	0.8	17.02
	2	4.8	0.9	18.75
		--Actinomycetes (10^5/g soil)--		
0-3	1	63.0	10.7	16.98
	2	67.5	14.4	21.33
3-6	1	61.7	6.7	14.26
	2	47.0	9.4	20.00
6-10	1	37.5	7.4	19.73
	2	55.2	3.6	6.52
15-20	1	31.5	4.1	13.02
	2	33.1	8.6	25.98
30-40	1	15.3	3.2	20.92
	2	18.1	2.3	12.71
60-70	1	13.3	1.4	10.52
	2	11.9	2.0	16.81