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Biosynthesis Of Nucleotides And Amino Acids During The Vegetative Stage Of The Cellular Slime Mold Dictyostelium Discoideum.

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BIOSYNTHESIS OF NUCLEOTIDES AND AMINO ACIDS DURING THE VEGETATIVE STAGE OF THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

by

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INTRODUCTION

The cellular slime mold Dictyostelium discoideum, which is a member of the group Acrasiales of the class Mycetozoa, was first introduced into the literature in 1935 by K. B. Raper. Research on the slime mold has increased continually since then and the organism has gained prominence as a model for cytodifferentiation. Although the work presented in this dissertation is concerned directly only with the vegetative stage of the life cycle of Dictyostelium discoideum, its possible importance to the rest of the life cycle and theories concerning it will be discussed.

Life Cycle and Theories

The vegetative stage begins when spores germinate producing single independent amoebae. They feed on available nutrients, usually bacteria, and divide, producing two independent daughter cells. When the food supply is exhausted, the cells aggregate into a cell mass which becomes a migrating slug. When the slug ceases movement, it differentiates into the mature fruiting body, a mass of encapsulated spores on a thin stalk supported by a basal disc (Bonner, 1967).

At the onset of aggregation each cell is equally capable of differentiating into a stalk cell, a spore cell or a basal disc

cell. There are specific carbohydrate products which are formed during the differentiation process, which are associated with one or more cell types and which are considered to be the principal biochemical products of differentiation (Ceccarini, 1967; Sussman and Sussman, 1969; Wright, 1966). The appearance and disappearance of enzymes associated with the formation of these products and their precursors as well as enzymes associated with related events have been the subject of research which has produced two major conflicting theories of differentiation in the cellular slime mold.

Maurice and Raquel Sussman began laying the foundation for their "developmental program" theory by observing the response of the accumulation of UDP-galactose polysaccharide transferase to actinomycin D and cycloheximide (Sussman and Sussman, 1965). The enzyme is responsible for the incorporation of galactose into a mucopolysaccharide which is found in spores of the mature fruiting body. The Sussmans were able to define an eight hour period of transcription for the enzyme and an eight hour period of translation for the enzyme. The two periods had a temporally specific relationship to each other (Sussman and Sussman, 1965). Temporally specific periods of transcription and translation were found for several other enzymes, including UDP-glucose pyrophosphorylase, trehalose-6-phosphate synthetase (Roth, Ashworth and Sussman, 1968), alkaline phosphatase (Loomis, 1969), beta-glucosidase-2 (Coston and Loomis, 1969), and alpha-mannosidase (Loomis, 1970).

The theory based on these results is that the events of differentiation are regulated by a "developmental program" of selective gene expression. The control in the program exists at the nucleic acid level and includes temporally and quantitatively specific translation of the message (Sussman and Sussman, 1969). There is also specific disappearance of some enzymatic activities by degradation of the enzyme or specific extrusion of it into the medium (Sussman and Lovgren, 1965).

In the theory developed by Barbara Wright (1963, 1968, and 1970) and published most recently by Wright and Gustafson (1972), metabolite flux is the key to differentiation. Theirs is a computerized kinetic model in which the controlling factors in many enzymatic reactions are the substrate and effectors for that reaction, rather than the level of the enzyme itself (Wright, 1968). The model includes a "metabolic map, metabolite concentrations, enzyme kinetic expressions and constants, enzyme activation functions and time functions giving independent metabolite concentrations" (Wright and Gustafson, 1972). Their model supposes that gluconeogenesis and glycolysis are insignificant at critical times in differentiation (Wright et al., 1973).

The major point of difference between these theories is the level at which control of differentiation is exerted. Sussman maintains that it is at the level of transcription and translation while Wright maintains that it is at the substrate level.

Francis (1969) has attempted to show that neither of these theories fully explains the events of differentiation, but that they may be integrated into a model which will account for all events of differentiation. He proposes that the state of a cell consists of the "gene product units" in it. A gene product unit consists of a unit of metabolic action containing the gene, messenger RNA, the enzyme or protein formed, and the product of the enzymatic reaction associated with it. One gene product unit can influence another at many levels, by induction, repression, masking of messenger RNA, allosteric or competitive inhibition of the enzyme or by affecting availability of the substrate. The timing sequence of differentiation may be described in terms of gene product units ordered with respect to one another in time. A number of simple and complex timing sequences may be described, including transcriptional and metabolite regulation of slime mold differentiation (Francis, 1969).

Nutrition

The most satisfactory food supply for the growth of slime mold amoebae in quantity is living (pre-grown) bacteria, usually E. coli or A. aerogenes. When grown on living bacterial suspensions in liquid shaker culture, the generation time for amoebae is 2.9 hours (Hohl and Raper, 1963a). Amoebae may also be grown on dead bacteria, but the generation time increases significantly.

A number of synthetic or non-particulate food supplies have been devised for slime mold amoebae. A strain of P. pallidum has been found which can grow on tryptose and serum albumin with a generation time of 4.5 hours (Hohl and Raper, 1963c). Similar P. pallidum strains have been grown on more detailed synthetic media (Hohl and Raper, 1963c). The media contain mixtures of amino acids, vitamins, bases, salts and serum albumin and dextrose. The authors were able to use these media to study the growth requirements of the amoebae, but the generation times were so long that no proper logarithmic phase of growth could be detected. Only riboflavine and a carbon source were identified as absolute growth requirements. A supplement of six amino acids, glycine, methionine, lysine, tyrosine, isoleucine and tryptophan, was found to be a strong stimulator of growth. The absence of any one of the six caused a marked decrease in the growth of the amoebae. The six amino acids can not, however, be called absolute growth requirements (Hohl and Raper, 1963c).

Normally D. discoideum will not grow well on a non-particulate food supply, but an axenic mutant has been isolated which can grow on peptone, yeast extract and salts (Watts and Ashworth, 1970). The medium contains no added carbohydrate, although some uptake of carbohydrate from the medium (from the yeast extract) can be detected in the cells. The generation time of the axenic mutant on this medium is 8-9 hours.

The exact chemical composition of the minimal medium for growth of the cellular slime mold is far from being known. With its elucidation will come much information on biosynthetic pathways present or absent in the vegetative stage. Conversely, the identification of pathways present in the vegetative stage may be very useful in determining absolute growth requirements.

Cellular Composition

By the end of the vegetative stage, amoebae have accumulated cellular constituents which will provide all of the energy and building materials needed for the differentiation process. The cells are essentially in a starvation situation for the rest of the life cycle (Bonner, 1967).

If the amoebae are grown on bacteria, the composition of the cells just prior to aggregation is as follows: 48% of the dry weight is protein (White and Sussman, 1961), 18% of the dry weight is nucleic acid (R. Sussman, 1967) and 7% of the dry weight is carbohydrate (White and Sussman, 1961). The amount of glycogen can be varied by growing the cells in axenic culture in the presence of varying amounts of added sugars (Weeks and Ashworth, 1972).

By the end of differentiation the dry weight per cell may fall to 40-60% of its original value (White and Sussman, 1961). RNA per cell also falls to about 40-60% of its original value (White and Sussman, 1961). During the logarithmic growth phase there is

little loss of ribosomal RNA, but almost all of the ribosomes from the growth phase have been turned over by the end of differentiation (Cocucci and Sussman, 1970). Some messenger RNA's synthesized during differentiation are reported to survive for up to eight hours before translation (Sussman, 1966b).

Total protein per cell falls to about 40% of its original value (White and Sussman, 1961). Protein is the principal source of energy during differentiation, and it is utilized in a somewhat specific manner (Wright and Anderson, 1960a). All of the protein fractions examined were turning over, although there was a net loss of each. The amino acid levels began to decrease first, reaching about 30% of their original level in the cells. Then the amount of ethanol-soluble protein per cell decreased, reaching 60% of its original value. Finally ethanol-insoluble protein decreased to about 80% of its original level. While these classes of protein were not identified with respect to specific roles in metabolism, their fates imply that the ethanol-soluble protein is "expendable" in the survival of the cells (Wright and Anderson, 1960a).

The percent of dry weight which is carbohydrate doubled over the course of differentiation, rising from 7% of dry weight to 14%, although this involved little or no conversion of protein to carbohydrate (White and Sussman, 1961). The differentiation of the slime mold involves the production of polysaccharides (White and Sussman, 1963a, b) which are associated with one or more cell types in the fruiting body, and which might be considered to be the

principal biochemical products of differentiation (Wright, 1965). The conservation of the carbohydrate fraction with respect to the loss of other cellular fractions is probably due to the production of these important polysaccharides.

Nucleotide and Amino Acid Studies

Precursor incorporation studies are commonly used to study cellular changes such as total RNA metabolism. A number of these studies have been done on the slime mold.

RNA turnover during differentiation has been studied with ^{3}H-uridine (Inselburg and Sussman, 1967) and ^{3}H-uracil (Pannbacker, 1966). Both investigators found that RNA turnover continues at a fairly constant rate throughout differentiation and that there are no observable changes in general RNA metabolism during this time. ^{3}H-uridine incorporation into ribosomal RNA of amoebae (Cocucci and Sussman, 1970) has shown that there was almost no loss of counts from ribosomal RNA during two cell divisions of a mutant of D. discoideum, indicating considerable conservation of RNA during the growth stage. At least 75% of the ribosomes formed during growth had been replaced with new ribosomes by the end of the differentiation phase.

The adenine nucleotides of the nucleotide pools have been studied with regard to the energy state of the slime mold cells at different times in the life cycle (Rutherford and Wright, 1971;

Wooley and Jones, 1970). Krichevsky and Love (1968) examined the accumulation of mononucleotides and amino acids in the soluble pool of washed amoebae in suspension. Their results linked the accumulation of the metabolites with the loss from amoebae of unnecessary RNA and protein at the time of transition from the vegetative stage to aggregation. When the loss of macromolecules is prevented, and the materials are retained in the cells and broken down to nucleotides and amino acids, a stimulation of morphogenesis is observed.

Another investigation of this phenomenon (Lee, 1972) revealed that the loss of macromolecules reported by Krichevsky and Love was due to lysis of amoebae during the harvesting procedure employed. Washed amoebae lost large amounts of nucleotides and amino acids into the suspending medium at the same transition time and the investigator suggested that it was due to the absence of effective means of retaining these metabolites in the cell. Further, the loss of metabolites was suggested as a key signal in the initiation of differentiation, since differentiation could be prevented by addition of a mixture of glucose, amino acids and vitamins. The decision to differentiate or proliferate is probably based on the presence or absence of a "balanced diet" of essential nutrients within the cell.

While these investigations may hint at the presence or absence of biosynthetic pathways for nucleotides, no complete study on the entry of mononucleotides into the mononucleotide pools of the cellular slime mold has been conducted. It has been assumed

that mononucleotides entered the pool by breakdown of RNA from ingested bacteria and from the slime mold itself, and by absorption from the external medium. The incorporation studies which used labeled bases (Firtel, Jacobson and Lodish, 1972; Pannbacker, 1966) proved that the amoebae are capable of the type of reactions seen in E. coli in which purine and pyrimidine bases are reacted with phosphoribosylpyrophosphate to produce nucleotides (Kornberg et al., 1955; Lieberman et al., 1955). No consideration has been given to the possibility of de novo biosynthesis of nucleotides in any stage of the life cycle.

Fewer studies of protein metabolism have been done, and there are only a few incorporation studies found in the literature. Wright and Anderson (1960a, b) followed incorporation and loss of ^{35}S-methionine in slime mold protein and amino acid pools during differentiation. They found the pattern of protein utilization which has already been discussed. They also found evidence that the amino acid pools may be divided and localized within the amoebae so that some are more accessible to exchange with external amino acids than others.

Wright and Anderson's data demonstrate considerable inhibition of exchange of external methionine with internal pools by dinitrophenol (DNP). This seems to imply an active process of some sort which would act to conserve the amino acids, keeping them within the cell. However, K. C. Lee (1972), in a study of the permeability of amoebae to amino acids, has shown that the amoebae

lose metabolites into the medium just prior to aggregation and that the uptake of amino acids is passive, i.e., not inhibited by DNP.

Protein turnover continues throughout the differentiation process, decreasing slightly toward the end of the development phase (Wright and Anderson, 1960b). Amino acid oxidation is the principal source of energy in the differentiating cells. The enzymes involved in the degradation of amino acids and proteins have been studied by Firtel and Brackenbury (1972). Some of these enzymes appear to be temporally regulated and others are present for the entire life cycle. Lactate dehydrogenase and Krebs cycle related enzymes such as aspartate transaminase and glutamate dehydrogenase fall into the latter category. The authors noted the fact that Dictyostelium discoideum can grow in axenic culture on proteose peptone and yeast extract without added carbohydrate. They suggest the possibility that these cells maintain very high levels of protein catabolizing enzymes at all stages of differentiation. De novo synthesis of amino acids by the slime mold had not been considered in the literature and protein degradation is considered to be the only source of amino acids for the amoebae.

Problem Presentation

Amino acids and nucleotides play a number of roles in the metabolism of Dictyostelium discoideum. They may be obtained from

degradation products of their macromolecules, the supplied nutrients (in the growth stage), interconversion and de novo biosynthesis. This last possible source has never been directly investigated in the slime mold. There are many questions which such a study might attempt to answer. Do the amoebae synthesize these compounds de novo? If so, is this ability maintained throughout the life cycle? How great is the contribution of biosynthesis to the pools of nucleotides and amino acids compared to other sources? Do biosynthetic pathways include those for purines, pyrimidines and all amino acids or only a few of these? How might the presence of such pathways affect reinterpretation of past results and the design of future research on the metabolism of Dictyostelium discoideum?

With these questions in mind a study of the possible biosynthesis of nucleotides and amino acids by the cellular slime mold was begun. Some of the proposed questions are answered and possible approaches to others are suggested.

Experimental Design

One of the most ubiquitous compounds in the culture of most organisms is CO_2 or HCO_3^-. It is formed in many metabolic reactions and fixed in many others.

E. coli fix CO_2 in the biosynthesis of purines, pyrimidines and the following amino acids: aspartic acid, methionine, threonine, isoleucine, lysine, glutamic acid, proline, arginine (Roberts et al., 1955). The CO_2 derived carbons in these compounds do not

make their way into glucose under rapid growth conditions in the presence of added carbohydrate. The label found in E. coli would be found almost exclusively in protein, nucleic acid and the monomer pools (Roberts, 1955). There is very little gluconeogenesis via the glycolytic pathway in rapidly dividing amoebae (Cleland and Coe, 1969), so any $^{14}CO_2$ fixed by the amoebae would most likely be found in the protein and nucleic acid fractions. For this reason $^{14}CO_2$ was chosen as a useful label with which to study general nucleotide and amino acid synthesis without involving gluconeogenesis in the slime mold amoebae.

In each of the experiments presented three cultures of amoebae of D. discoideum were grown on killed bacteria with label provided in a different manner in each culture. The amoebae were grown on dead bacteria because the generation time of amoebae on synthetic media is too long to allow the media to be used in these experiments, and because previous experimental results from amoebae grown on living bacteria were not easily interpreted due to the presence of two metabolic systems. In one culture the amoebae were supplied with $^{14}CO_2$ alone to determine whether they did fix CO_2 and in what fractions the fixed label would be found. Another culture was provided with bacteria grown on $^{14}CO_2$ and then killed. The distribution of fixed label in the bacteria was determined and the distribution of label incorporated by amoebae from the bacteria was determined. The comparison of these two cultures (the labeled bacteria and the amoebae grown on them) gave an indication of the

contribution of bacterial constituents (amino acids and nucleotides) to amoebal macromolecules. The third amoebal culture was provided with labeled bacteria and $^{14}CO_2$. The results from this culture and from the comparison of the other two amoebal cultures gave an indication of the similarity and/or difference of distribution of label incorporated from the two different sources.

MATERIALS AND METHODS

Chemicals

$Ba^{14}CO_3$, 30 microcuries/micromole, was obtained from Isotopes, Inc., Westwood, N. J. Bacto non-nutrient agar was obtained from Difco Laboratories, Detroit, Michigan. Triton-X-100, Scintillation Grade, PPO and POPOP phosphors were obtained from ICN Chemical and Radioisotope Division, Irvine, California. Toluene was obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey and Bio-rad AGX8 ion exchange resin was obtained from Bio-Rad Laboratories, Richmond, California.

Organisms and Culture Conditions

Dictyostelium discoideum was obtained from Turtox Products, General Biological Supply House, Chicago, Illinois, and was grown in association with E. coli B_{s-1} as described by Sussman (1966a). Stock cultures were maintained on agar slants. Working cultures were developed by spreading agar plates with a few drops of a suspension of one bacterial slant in 5 ml distilled water and with spores transferred by wire loop from stock or other working cultures. A sterile glass rod was used to spread the plates. The plates were incubated at room temperature or at 22° C in the Hotpac room until

fruiting bodies formed. They were then sealed with tape to prevent evaporation from the agar and stored in the refrigerator.

Liquid shaker cultures of D. discoideum amoebae were grown as follows: 400 ml shaker cultures of E. coli B_{s-1} were harvested and washed one time with distilled water. The cells were then suspended in approximately 25 ml sterile modified Hohl and Raper salts (Hohl and Raper, 1963a) in a sterile beaker. They were boiled on a heating magnetic stirrer for 4-5 minutes, then irradiated with ultraviolet light for 30 minutes. Finally they were suspended in 100-200 ml sterile H & R salts at an O.D. of a 1:5 dilution of 0.6-0.78 (650 millimicrons). Spores were harvested from a plate, washed four times with distilled water to remove any bacteria and inhibitors of germination, and added to the bacterial suspensions to a concentration of approximately 2 X 10^3/ml.

These cultures were placed on a rotary shaker at 22°C in a Hotpac room for two and a half to three and a half days. At the end of that time the suspensions which had been very cloudy due to suspended bacteria had cleared considerably. The amoebae were harvested in an International Type SB Centrifuge at 400 RCF and washed with distilled water until the washes were clear of bacteria.

E. coli B_{s-1}, an ultraviolet sensitive strain, was obtained from the laboratory of Roy Curtiss, III, Oak Ridge, Tennessee. It was grown and maintained on agar slants. Large quantities of bacteria were obtained by growing the E. coli B_{s-1} in 400 ml liquid cultures. One slant was used to innoculate the sterile medium,

which was composed of 5 grams of mannitol and 1 X Langford salts (Carlson and Brown, 1961) per liter. The flask containing the culture medium and innoculum was set on a rotary shaker at medium speed at 37°C for 24 hours. The cells were harvested on an RC-2 Servall centrifuge at 13200 RCF and washed once with distilled water.

$^{14}CO_2$ Incorporation Studies

As several experiments involving $^{14}CO_2$ were performed, a method of handling the $^{14}CO_2$ was devised to minimize spills and errors. A glass petri dish was divided in half with a "dam" or ridge of Lubriseal. About 6 ml of 6 N H_2SO_4 and a magnetic stirring bar were placed on one side of the dam and a measured amount of $Ba^{14}CO_3$ was placed on the other. The dish was put at the bottom of a vacuum desiccator and the desiccator plate was replaced. Thirty-five to forty 2 cm^2 pieces of Whatman #1 filter paper were dipped into a solution of saturated $Ba(OH)_2$ and placed in small plastic vials on the plate. The desiccator was sealed and evacuated and placed on a magnetic stirrer. The stirring bar was used to break the Lubriseal dam, allowing the acid to flow over the $Ba^{14}CO_3$, releasing the $^{14}CO_2$. The $^{14}CO_2$ was then trapped on the papers in the carbonate form. When the papers had dried the desiccator was reevacuated through 0.2 N NaOH to trap any $^{14}CO_2$ still in the gaseous phase. The papers containing the $Ba^{14}CO_3$ were used to provide $^{14}CO_2$ for labeling of amoebae and bacteria.

These $Ba^{14}CO_3$ papers were used to add $^{14}CO_2$ to the experimental flasks in experiments 2 through 6 (see next section). The actual amounts of radioactivity added from each of the papers were not determined, although the amounts were considered to be of the same order of magnitude. Experiment 1 was the last experiment chronologically and as the last of the $Ba^{14}CO_3$ papers was used to prepare the labeled bacteria, an amount of undiluted $Ba^{14}CO_3$ was used to generate $^{14}CO_2$ for the growth of the amoebae. The $Ba^{14}CO_3$ used was equivalent to the amounts previously used for the preparation of 10-20 papers, and the specific activity of the $^{14}CO_2$ in the atmosphere in which amoebae were grown in experiment 1 was, consequently, several-fold higher than that in all of the other experimental flasks containing $^{14}CO_2$.

Preparation of Labeled and Unlabeled *E. coli* B_{s-1}

Three 400 ml bacterial cultures were grown for each experiment. One was grown as previously described in a one liter Erlenmeyer flask stoppered with a cotton plug. It became the killed, unlabeled bacteria. Two other cultures were grown in round-bottom flasks connected by a Y-tube with ground-glass fittings. See figure 1 for the design of the apparatus. The Y-tube had a stopcock in the third arm through which the $^{14}CO_2$ was added in the following manner.

One of the $Ba^{14}CO_3$ papers was placed in a small (125 ml) suction flask. The flask was stoppered and connected to a soda

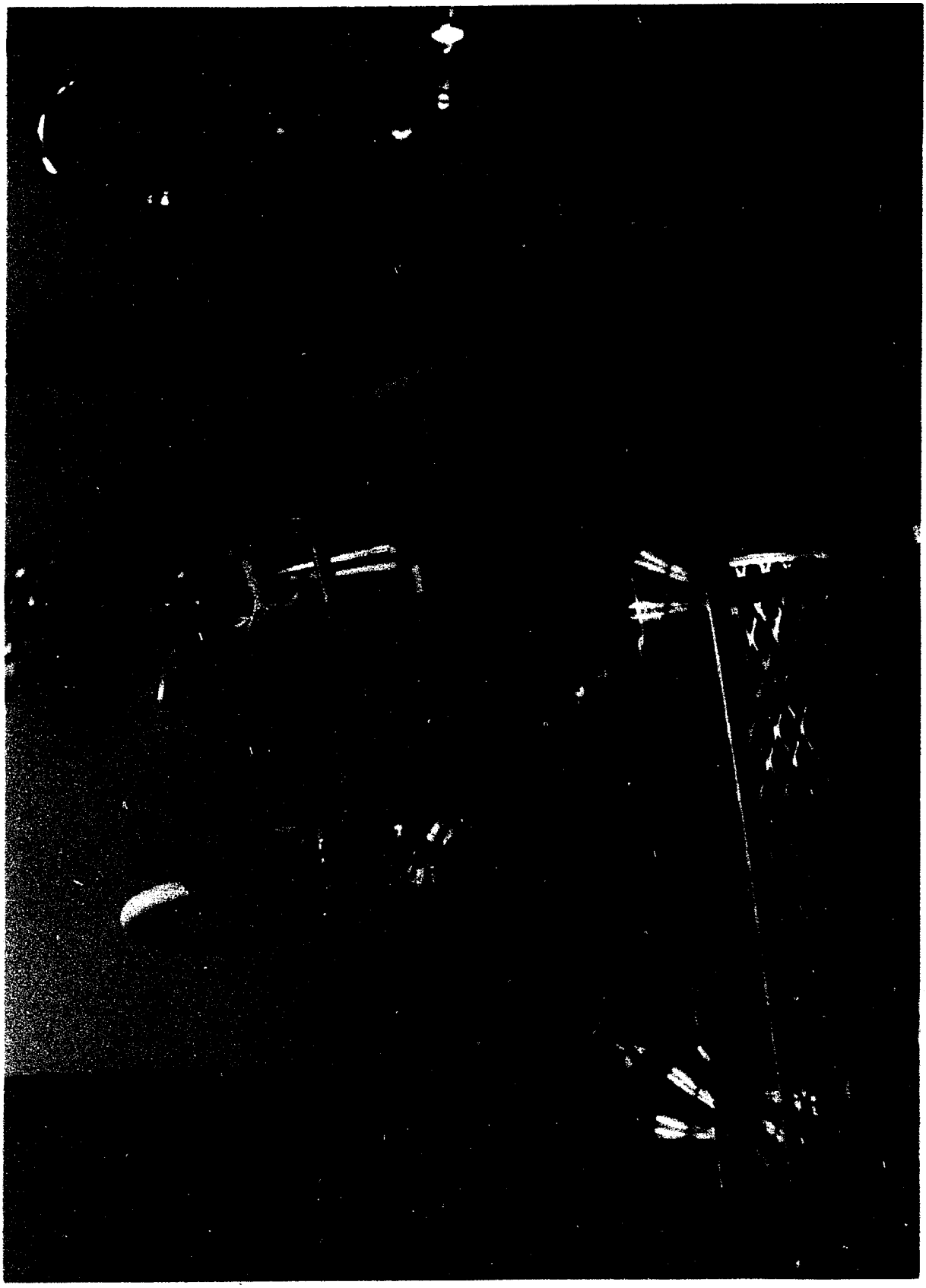
lime trap and then to a vacuum water line. This apparatus was then connected to the round-bottom flasks through the Y-tube. The whole system was evacuated and then closed with a clamp at B and at A (figure 1). The stopcock was closed, the vacuum line disconnected and clamp A removed. Two ml of 6 N H_2SO_4 were added to the system with a syringe through the tube just between clamp B and the stopper of the suction flask. The suction flask was tapped gently to make sure that the paper was saturated with acid and all $^{14}CO_2$ had been released. The $^{14}CO_2$ was pulled into the round-bottom flasks by allowing a little air in through clamp B and then opening the stopcock briefly. The soda lime trap was intended to keep the $^{12}CO_2$ in the air let in to a minimum. The procedure was continued until the pressure in the system was near room pressure. The stopcock was closed, clamps C and B put into place. The sealed round-bottom flasks were removed from the system and put, with the Erlenmeyer flask, on the rotary shaker in a Hotpac room. The cultures were shaken at medium speed for 24 hours at 37°C, harvested and treated as previously described.

Preparation of Labeled Amoebae

The procedures for growth of amoebae on killed bacteria and for addition of $^{14}CO_2$ to the cultures have been described. Three amoebal cultures, each with a different source of carbon-14, were grown for each experiment. A 100-200 ml culture of amoebae was grown on ^{14}C-labeled, killed *E. coli* B_{s-1} in an Erlenmeyer flask

Figure. $^{14}CO_2$ generating apparatus.

A 2 cm^2 piece of filter paper containing $Ba^{14}CO_3$ is placed in a 125 ml suction flask (1) and the flask is stoppered and connected through a soda lime trap (2) to the vacuum water line (3). The flask is also connected to the round bottom flasks (4) through a Y tube (5). The whole system is evacuated and then closed with clamps at A and B. The stopcock (6) is closed, the vacuum line disconnected and the clamp at A removed. Two ml of 6 M H_2SO_4 are added to the system with a syringe through the rubber tube between the clamp at B and the stopper in the suction flask. The $^{14}CO_2$ is pulled into the round bottom flasks by briefly opening the clamp at B and the stopcock alternately. When the pressure in the system was normal (atmospheric pressure), the stopcock was closed and the clamps at C and B were put into place. The round-bottom flasks, connected and sealed, were removed from the system.



sealed with a rubber stopper. Two 100-200 ml cultures of amoebae were grown in 500 ml round-bottom flasks to which $^{14}CO_2$ was added as previously described. One flask contained ^{14}C-labeled, killed E. coli B_{s-1} and $^{14}CO_2$, the other unlabeled, killed E. coli B_{s-1} and $^{14}CO_2$. The three cultures were set on a rotary shaker at 22°C in the Hotpac room. The cultures required two and a half to three and a half days to clear. They were harvested as described and aliquots of all cultures, supernatants and resuspended pellets were saved for counting. The pellets were suspended in 3-5 ml sterile water and subjected to chemical fractionation.

Fractionation Procedure

The fractionation procedure described by Hanawalt (1959) was modified slightly for these experiments.

Duplicate 0.75 ml aliquots of each sample to be analyzed were placed in 12 ml Pyrex centrifuge tubes. 0.5 ml 1 N perchloric acid (PCA) was added to each tube and the tubes were set in ice for 30 minutes. PCA was used where TCA was originally called for because the ions could be precipitated out easily with KOH to allow fractions to be chromatographed. The tubes were then centrifuged, the supernatant fractions decanted and designated as the "monomer" or "PCA-soluble" fractions (Hanawalt, 1959). The walls of the tubes were wiped clean of liquid in this and every subsequent step.

One ml of 50% ethanol was added to each pellet and the tubes were left at room temperature for 30 minutes. Samples were

centrifuged and the supernatant fractions decanted and designated as "lipid" or "ethanol-soluble" fractions (Hanawalt, 1959).

0.5 ml 1 N KOH was added to the pellets and the samples were held in a water bath at 37°C for 2 hours, except in experiment 1, in which the time was three hours (see below). The tubes were plunged directly into ice to stop hydrolysis and 0.75 ml ice cold 1 N PCA was added to each tube. The tubes were centrifuged and supernatant fractions were designated as "RNA" or "hot KOH-soluble" fractions (Hanawalt, 1959).

Examination of the chromatographic separations of the samples hydrolyzed for 2 hours, which had been followed at 260 and 280 millimicrons, revealed that the AMP peak was very small and was surrounded by small peaks of adenine oligonucleotides (Lane and Butler, 1959). The radioactivity in the fractions of the two hour hydrolysates was examined and it indicated that much of the adenine was contained in the general area of the AMP peak. Samples hydrolyzed for 3 hours exhibited AMP peaks of expected size.

1.6 ml of 0.5 N PCA were added to the pellets from the KOH hydrolysates and the samples were held in a boiling water bath for 30 minutes. The samples were spun down and the supernatant fractions were designated as "DNA" or "hot PCA-soluble" fractions (Hanawalt, 1959).

0.75 ml of 1 N KOH was added to the pellets from the previous step and the whole pellet was suspended in this volume. This fraction was counted intact as the "protein" fraction (Hanawalt, 1959).

Counting Procedure

Counting solution tT76 was used for preparation of the counting emulsions of samples and sample fractions (Patterson and Greene, 1965). The solution is 7 parts toluene plus phosphor (4 g/l PPO, 100 mg/l POPOP) to 6 parts Triton-X-100. Samples vials were counted in the Nuclear-Chicago Mark I Scintillation Counter. The counter was set up according to the channels ratio - external standard method of counting. Standard curves were made in each experiment to set up a channels ratio versus efficiency curve. The efficiency of counting was determined and was used to calculate the DPM's in the total fraction of each sample.

The amount of label found in the cold PCA fraction plus the amount of label in the alcohol-soluble fraction was always less than 8% of the total incorporation of carbon-14 by any culture and was considered insignificant with regard to this analysis. Therefore, in discussions of the "distribution patterns" of carbon-14 incorporation of CO_2 derived carbons, the "monomer" and "lipid" fractions will not be included, and discussion will be limited to protein and nucleic acid macromolecules. The KOH hydrolysates and the hot PCA hydrolysates will be treated together as the "nucleic acid fractions" in these discussions (see Fractionation Procedure).

Protein Determination

Protein was determined by the Lowry method (Lowry et al., 1951).

Nucleic Acid Column Chromatography

The polyethyleneimine (PEI)-cellulose method of separation of nucleotides as described by Randerath and Randerath (1964a, b) was unsatisfactory. Bio-Rad AGX8 ion exchange resin was used as the resin for separating the nucleotides and was prepared according to the directions from Bio-Rad Laboratories (1972). A Pasteur pipette (0.5 cm diameter) was plugged loosely with a small wad of cotton near the end and filled with resin up to about one centimeter from the top (total column length, 8½ cm). The draining end of the pipette was linked by thin plastic tubing to a flow cell in the DB Spectrophotometer. The column was washed with water until the 280 millimicron reading of the wash from the column indicated that all unassociated formate had been washed off of the column.

A standard solution of the 3'-mononucleotides was separated on the column. The order of elution of the nucleotides was CMP, AMP, GMP and UMP. A 1-1.5 ml sample (a neutralized aliquot of an RNA hydrolysate) was put on the column. The gradient of formic acid used to elute the mononucleotide from the column was a modification of a gradient for the separation of ribonucleotides devised by G. L. Carlson (Carnegie Institution, 1963).

The elutant from the column went through the flow cell and was read at 280 millimicrons until the first peak had passed and then at 260 millimicrons for the duration of the chromatogram. When the column was finished, the individual fractions were combined

according to peak and read at 260 and 280 millimicrons in quartz cells. A formic acid gradient was run through a column to which no sample had been added and the "blank" fractions were collected and read at 280 and 260 millimicrons. These absorbancies were subtracted from the absorbancies of the corresponding fractions of sample separations to correct for the contribution of formic acid to the observed absorbancies. The 280/260 ratio and the corrected absorbancies were used to identify and quantitate the nucleotides in each combined fraction. The total volume of each combined fraction was measured and each was then transferred to a scintillation vial and counted according to the counting procedure. The specific activity of each nucleotide was determined from the absorbancies of column fractions and the DPM's from the counting procedure.

RESULTS

Liquid cultures of amoebae of Dictyostelium discoideum were grown to the end of the vegetative stage on different sources of carbon-14: 1) killed ^{14}C-labeled E. coli B_{s-1}, 2) killed ^{14}C-labeled E. coli B_{s-1} plus $^{14}CO_2$, and 3) $^{14}CO_2$ alone. In two experiments a control culture of killed, unlabeled bacteria was exposed to the same $^{14}CO_2$ labeled gaseous phase as two of the amoebae cultures (2 and 3 above). Analysis of these controls was used to determine whether any incorporation observed in the three amoebal cultures was due to further incorporation by survivors in the suspension of killed bacteria.

Aliquots of all of the harvested cultures of amoebae and bacteria were analyzed according to the fractionation and counting procedures in the Materials and Methods section. Six experiments were performed under the conditions described above. The data on the individual experiments are contained in the Appendix.

Table A contains the percents of incorporation of carbon-14 into the nucleic acid and protein fractions found in the E. coli B_{s-1} labeled with $^{14}CO_2$ and in the amoebal cultures grown on the three sources of carbon-14. The data here and in other tables are expressed as means ± the standard error of the mean, except as noted.

Table A.

Distribution of carbon-14 in nucleic acid and protein fractions of *Escherichia coli* B_{s-1} and *Dictyostelium discoideum*.

Amoebae and bacteria were grown on the source of carbon-14 listed plus other essential nutrients. They were harvested and analyzed according to the Materials and Methods section. Only label incorporated into macromolecules was considered in these experiments.

Organism Source of Label	Fraction Nucleic Acid* (% of label ± S.E.M.)	 Protein* (% of label ± S.E.M.)
Escherichia coli B_{s-1}		
$^{14}CO_2$	70.1 ± 3.9	29.9 ± 3.9
Dictyostelium discoideum		
Mean	54.4 ± 0.6	45.6 ± 0.6
^{14}C-labeled bacteria	54.8 ± 6.3	45.2 ± 6.3
^{14}C-labeled bacteria + $^{14}CO_2$	56.7 ± 6.8	43.3 ± 6.8
$^{14}CO_2$	51.7 ± 5.5	48.3 ± 5.5

* Each percentage is the mean of six experiments ± the standard error of the mean except the mean under *D. discoideum* which is the mean of eighteen experiments ± S.E.M.

In the E. coli B_{s-1} cells, a mean of 70.1% ± 3.9 of the label incorporated from $^{14}CO_2$ into macromolecules was found in the nucleic acid fractions and a mean of 29.9% ± 3.9 of that label was found in the protein fractions. The mean distribution of the label in macromolecules of the amoebae in all labeling experiments was 54.4% ± 0.6 in the nucleic acid fractions and 45.6% ± 0.6 in the protein fraction. The difference between the mean distribution patterns of all bacterial and all amoebal incorporation experiments is significant to the P = 0.02 level. The distribution of the label resulting from the different sources of label in the amoebal cultures will be discussed separately.

Amoebae Grown on ^{14}C-labeled Bacteria

In the amoebae grown on labeled E. coli B_{s-1}, 54.8% ± 6.3 of the label incorporated into macromolecules was found in the nucleic acid fractions and 45.2% ± 6.3 of the label incorporated into macromolecules was found in the protein fraction (see Table A). Table B contains a comparison of the analyses of amoebae grown on ^{14}C-labeled bacteria and the analyses of the ^{14}C-labeled bacteria on which the amoebae were grown.

The protein content of the two organisms is approximately 48% of their dry weights (Roberts et al., 1955; White and Sussman, 1961). The amoebae grown on labeled bacteria contained 21% ± 4 as much protein as did the bacteria on which they were grown. The total

Table B.

Ratios of analyses of *E. coli* B_{s-1} grown on $^{14}CO_2$ and analyses of amoebae of *D. discoideum* grown on the ^{14}C-labeled *E. coli* B_{s-1}.

Comparison	Ratio ± S.E.M.*
$\frac{\text{Total DPM in amoebae}}{\text{Total DPM in bacteria}}$	.19 ± .02
$\frac{\text{Total mg protein in amoebae}}{\text{Total mg protein in bacteria}}$	.21 ± .04
$\frac{\text{Total DPM in amoebal protein}}{\text{Total DPM in bacterial protein}}$	.28 ± .03
$\frac{\text{Specific activity of amoebal protein}}{\text{Specific activity of bacterial protein}}$	1.49 ± .24
$\frac{\text{Total DPM in amoebal nucleic acids}}{\text{Total DPM in bacterial nucleic acids}}$	.14 ± .01
$\frac{\text{Specific activity of amoebal ribonucleotides}}{\text{Specific activity of bacterial ribonucleotides}}$	1.19 ± .18

* The first five ratios are arithmetic means of six experiments ± standard error of the mean. The last ratio is the arithmetic mean of three experiments.

radioactivity found in the amoebal protein was 28% ± 3 as much as the total radioactivity found in the bacterial protein. The ratio of the specific activities of the amoebal protein fraction to the bacterial protein fraction is 1.49 ± .24.

If the molar proportions of amino acids in bacterial and amoebal protein were the same, and if bacterial amino acids were the only source of amino acids for synthesizing amoebal protein during growth, then the proportion of bacterial protein label recovered in amoebal protein should be the same as the proportion (by weight) of amoebal protein to bacterial protein, and the ratio of specific activities of the proteins should be 1.0. The data suggest, however, that the proportion of bacterial protein label recovered in amoebal protein (28% ± 3) is greater than the percent of protein found in amoebae versus bacteria (21% ± 4). These figures are significantly different to the P = 0.20 level. The ratio of the specific activities of the two proteins (1.49 ± .24) tends to confirm the significance of this difference and the indication that amino acids which are labeled by $^{14}CO_2$ in biosynthesis are found in greater proportion in amoebal protein than they are in bacterial protein. In other words, it appears that when amoebae grow on E. coli, there is selective utilization of bacterial amino acids for amoebal protein building blocks.

The nucleic acid contents of E. coli and amoebae are not the same, as 25% of the dry weight of the bacteria is nucleic acid (Roberts et al., 1955) and 18% of the dry weight of amoebae is

nucleic acid (R. Sussman, 1967). The total radioactivity recovered in the amoebal nucleic acids was 14% ± 1 of the total radioactivity found in the bacterial nucleic acids (Table B). On a weight basis the amoebae contain 18/25ths or 72% as much nucleic acid as the bacteria, and the two organisms contain about the same amount of protein per unit of dry weight, so for every unit of dry weight of protein incorporated into the amoebae from bacterial protein, .72 unit of dry weight of bacterial nucleic acid would be incorporated. The amoebae contained 21% as much protein as the bacteria and they should have contained 72% as much nucleic acid as protein or (.72 X .21 =) 15% of the bacterial nucleic acid. This estimate of the incorporation of bacterial nucleic acid into amoebae on a weight basis is in good agreement with the observed recovery of 14% ± 1 as much radioactivity in amoebal nucleic acids as was originally found in bacterial nucleic acids. The proportions of label recovered in nucleic acid versus protein or 14% ± 1 versus 28% ± 3 are significantly different to the P = 0.005 level.

Both purines and pyrimidines are labeled in de novo synthesis by E. coli in the presence of $^{14}CO_2$. Labeling of pyrimidines is 1.8 times greater than labeling of purines due to the incorporation of the C-4 of aspartate into the base (Roberts et al., 1955). Nucleic acids which contain a greater proportion of pyrimidines than purines will have a higher specific activity when synthesized in the presence of $^{14}CO_2$ than nucleic acids with a greater proportion of purines. Table C contains the mole percents of purines and pyrimidines in RNA

Table C.

Mole percents of bases of RNA from *E. coli** and amoebae of *D. discoideum***.

Organism	Base			
	C	A	G	U
E. coli	20.00	26.15	32.30	21.53
D. discoideum	20.40	24.10	22.00	33.60

* Roberts et al., 1955.

** Pannbacker, 1966; Firtel, Jacobson and Lodish, 1972.

of E. coli and amoebae of Dictyostelium discoideum. The difference in base composition of the RNA in the two organisms is such that RNA hydrolysates of Dictyostelium discoideum should have a specific activity 1.1 times greater than the specific activity of the hydrolysates of bacterial RNA.

Table B gives a value of 1.19 ± .18 for the ratio of the specific activity of amoebal nucleotides to the specific activity of bacterial nucleotides. This figure may be in error due to problems in the procedure. It is, nevertheless, in apparent agreement with the ratio of specific activities of amoebal nucleotides versus bacterial nucleotides calculated from base compositions.

In summary, the amoebae fed on ^{14}C-labeled bacteria incorporate label into their nucleic acids and proteins. Analysis of the data suggests that the difference in specific activity and labeling of amoebal and bacterial nucleic acids and proteins is largely due to difference in base composition of nucleic acids and amino acid composition of proteins in the two organisms. If the amoebae had degraded the labeled bacterial constituents to CO_2 and then labeled amoebal protein and nucleic acid by fixation of $^{14}CO_2$, the specific activities of the nucleotides and protein of the amoebae would probably be much lower than the specific activity of bacterial nucleotides and protein because of dilution of the label.

Amoebae Grown on $^{14}CO_2$

Amoebae which have been grown on unlabeled bacteria in the

presence of $^{14}CO_2$ also incorporate carbon-14 into nucleic acid and protein. The distribution of the label in these amoebae, which is shown in Table A, is not significantly different from the distribution of label in amoebae grown on labeled bacteria or from the mean distribution of label in all amoebal cultures. The nucleic acid fractions contain 51.7% ± 5.5 of the label incorporated into macromolecules, and the protein fraction contains 48.3% ± 5.5 of the label incorporated into macromolecules.

One possible explanation for the incorporation of label by these amoebae was that there were a few surviving bacteria in the food supply which multiplied, fixing $^{14}CO_2$ in their amino acids and nucleotides, and were then consumed by the amoebae. Controls were run to determine the possible contribution of surviving bacteria to observed incorporation.

Table D contains comparisons of the analyses of amoebae grown on killed, unlabeled bacteria in the presence of $^{14}CO_2$ and of samples of the same bacterial suspension on which the amoebae were grown, incubated for the same time and with the same labeled atmosphere as the amoebal cultures (the data in Table D are not means, but individual determinations). The specific activity of the added $^{14}CO_2$ was much higher in experiment one than in experiment two.

The amoebae analyzed in experiment 1 contained only 10.3% as much protein as did the bacteria on which they were grown, but they incorporated six times as much label as did the bacteria. In the

Table D.

Total DPM incorporated from $^{14}CO_2$ by killed, unlabeled *E. coli* B_{s-1} and by amoebae of *D. discoideum* grown on killed, unlabeled *E. coli* B_{s-1}.***

Experiment Fraction	Killed, unlabeled *E. coli* B_{s-1}	Amoebae grown on killed, unlabeled *E. coli* B_{s-1}
Experiment 1*		
Nucleic acids	11,100	53,900
Protein	2,110	18,500
Total	13,210	72,400
Experiment 2**		
Nucleic acids	5,280	10,550
Protein	3,520	13,640
Total	8,800	24,190

* In experiment one, the bacteria contained 104 mg protein total and the amoebae contained 9.7 mg protein total.

** In experiment two, the bacteria contained 71 mg protein total and the amoebae contained 25.2 mg protein total.

*** The data in this table are individual determinations.

second experiment, with the $^{14}CO_2$ of lower specific activity, the amoebae contained 59% as much protein as did the bacteria on which they were grown, but they incorporated almost three times as much label as did the bacteria. The results from these two controls indicate that only a small percentage of the incorporation seen in the amoebae grown on killed, unlabeled bacteria in the presence of $^{14}CO_2$ could be accounted for by the fixation of label by a few surviving bacteria and then consumption of them by the amoebae. The rest is due to fixation of $^{14}CO_2$ by the amoebae.

Table D shows that the recovery of label in amoebae due to incorporation of $^{14}CO_2$ was higher in experiment 1 than in experiment 2. Additional data contained in the Appendix shows that the total DPM's recovered in amoebae exposed to $^{14}CO_2$ and in the medium from which these cells were separated were higher in experiment 1 than in any of the other experiments.

Experiment 1 is different from the other five experiments considered here in that the labeled CO_2 which was added to the amoebal flasks was liberated from a different source of $Ba^{14}CO_3$ than that used for experiments 2 through 6. The specific activity of the $^{14}CO_2$ in the amoebal flasks in experiment 1 was, consequently, several-fold higher than it was in the other 5 experiments, as was the total amount of radioactivity recovered in these cells and medium.

Nevertheless, the paths of incorporation of $^{14}CO_2$ by the amoebae is the same in all six experiments. This is demonstrated

by the similar proportions of radioactivity recovered in the cells versus the media in all of the experiments and under the three conditions of labeling. The following figures are derived from the data in the Appendix. Each mean is the mean of the six experiments ± the standard error of mean.

In the flasks of amoebae grown on labeled bacteria, 50.7% ± 4.4 of the label recovered in the liquid phase was found within the amoebae and the remainder was found in the medium. In experiment 1 this figure was 48.2%, very close to the mean. In the flasks of amoebae grown on $^{14}CO_2$ and cold bacteria, 12.1% ± 0.9 of the recovered label was found in the amoebal cells and the rest was found in the medium. (If some mechanism other than $^{14}CO_2$ fixation by amoebae were occurring in this flask, such as fixation by bacteria and consumption of bacteria by amoebae, the amount of label recovered in the amoebae would be expected to be higher than 12.1%.) In experiment 1 this figure was 11.0%, also very close to the mean. In the flasks of amoebae grown on both sources of label, 29.5% ± 1.5 of the recovered label was found in the cells and the rest in the medium. In experiment 1 this figure was 10.6%. This figure is lower than the mean, probably due to the greater amount of radioactivity in the $^{14}CO_2$ compared with that in the ^{14}C-labeled bacteria in experiment 1.

Thus, despite the fact that the total radioactivity recovered from the medium and cells exposed to $^{14}CO_2$ in experiment 1 is greater than in experiments 2 through 6, the phenomena occurring in all six

experiments are the same. The results from experiment 1 most clearly demonstrate fixation of $^{14}CO_2$ into nucleic acid and protein by the amoebae, confirming the observations in the corresponding cultures in experiments 2 through 6.

In summary, the amoebae can fix CO_2 in the biosynthesis of amino acids and nucleotides, the amount of label fixed from $^{14}CO_2$ being dependent on the specific activity of it.

Amoebae Grown on ^{14}C-labeled Bacteria and $^{14}CO_2$

The distribution of label in amoebae grown on both sources of carbon-14 is contained in Table A. In these cells a mean of 56.7% ± 6.8 of the label incorporated into macromolecules was found in the nucleic acid fractions and a mean of 43.3% ± 6.8 of the label was found in the protein fraction. This distribution of label is not significantly different from those found in the amoebae grown on one source of label or from the mean distribution pattern.

The incorporation of labeled bacterial constituents and the direct fixation of $^{14}CO_2$ are responsible for all of the incorporation of the label seen in these cells. The exact contribution of each source of label is not known because the specific activity and the amount of the labeled CO_2 added was not determined and the amino acid composition of the amoebal protein is not known.

Nevertheless, the contribution of labeled CO_2 or bacteria can be estimated from the results of the amoebae grown on one source of

label. The specific activity of the protein of the amoebae grown on both sources of label (subscript - $Am_{CO_2 + bact}$) should relate to the specific activities of the protein of the amoebae grown on only one source of label (subscripts - Am_{CO_2} and Am_{bact}) in the following manner:

$$S.A._{Am_{CO_2 + bact}} = S.A._{Am_{CO_2}} (mg\ P_{Am_{CO_2}} / mg\ P_{Am_{CO_2 + bact}}) +$$

$$S.A._{Am_{bact}} (mg\ P_{Am_{bact}} / mg\ P_{Am_{CO_2 + bact}}).$$

This relationship would hold within each experiment, and the right side should equal the left side. When this calculation is done on the results from the six experiments, it is found that the right side is equal to 98% ± 14.3 of the left.

In a similar calculation with the specific activities of the RNA fractions, the right side was equal to 103% ± 26.3 of the left side.

The label incorporated into amoebae grown on both sources of label can be fully accounted for by the entry of labeled CO_2 and bacterial constituents into the protein and nucleic acids of the amoebae. In addition the distribution of the label in these amoebae indicates that the precursors from the two sources (bacteria and de novo synthesis) feed into the same precursor pools and that they are probably identical chemical species. The result is that the distribution of label in the amoebae grown under all three conditions is the same.

In summary, the amoebae appear to possess biosynthetic pathways for amino acids and nucleotides which involve fixation of CO_2. These amino acids and nucleotides are used by the amoebae in the same manner as amino acids and nucleotides prelabeled with $^{14}CO_2$ by bacteria.

Nucleotide Studies

An analysis of the RNA hydrolysates was done to determine the extent of labeling of nucleotides in the various labeled amoebal cultures. In four experiments measured volumes of the "hot KOH-soluble" fraction of the bacterial and amoebal cultures were subjected to ion exchange column chromatographic separation as described in the Materials and Methods section. Table E contains the specific activities of the RNA hydrolysates of bacteria and amoebae from four experiments.

The specific activities of the RNA's of the amoebae grown on labeled bacteria correspond closely to the specific activities of the RNA's of the bacteria on which they were grown. The correspondence of specific activities indicates that the major source of nucleotides for amoebal RNA and probably DNA is the degradation of bacterial nucleic acids. If, for instance, the amoebae degraded the labeled bacterial constituents to CO_2 and then labeled amoebal protein and nucleic acid by fixation of $^{14}CO_2$, the specific activity of the RNA would be much lower than the specific activity of bacterial RNA because of dilution of the label.

Table E.

Specific activities of RNA hydrolysates.

Aliquots of neutralized RNA hydrolysates were subjected to ion-exchange column chromatographic separation as described in the Materials and Methods section. The fractions were counted as described and the results of the separation and counting procedures were used to calculate these specific activities in DPM per micro-mole of nucleotides. The specific activity of the total hydrolysate was determined according to the molar proportion of each nucleotide in the RNA of the organism and its measured specific activity. The values are not means, but are determinations for individual experiments.

Organism Source of Label	Experiment 1	2	3	4
E. coli B_{s-1}				
$^{14}CO_2$	415	1390	3410	
D. discoideum				
^{14}C-labeled bacteria	385	1685	4900	6390
^{14}C-labeled bacteria + $^{14}CO_2$	4125	1410	5260	6280
$^{14}CO_2$	3750	285	240	98

The specific activities of the RNA of the amoebae grown on unlabeled bacteria and $^{14}CO_2$ in Table E demonstrate the fixation of $^{14}CO_2$ into nucleotides by the amoebae. The low specific activities of the RNA hydrolysates of amoebae grown on $^{14}CO_2$ in experiments 2, 3 and 4 compared to that in experiment 1 are due to the difference in the specific activity of the $^{14}CO_2$ added to experiment 1 versus that added to experiments 2 through 6.

These specific activities are also low because the contribution of de novo biosynthesis to the nucleotide pool is small and any label incorporated in this manner will be diluted out by the unlabeled precursors derived from bacteria. Only in experiment 1, in which the specific activity of the $^{14}CO_2$ is very high, does the label incorporated from fixation of $^{14}CO_2$ make a significant contribution to the specific activity of the nucleotides.

In the nucleotides of the amoebae grown on both sources of label the effects of the two routes of entry of label are additive. The relationship of the specific activities of the nucleotides is the same as that for the specific activities of the protein fractions found in the equation on page 40.

Table F contains the specific activities of the individual nucleotides separated by the column. The specific activities of the individual nucleotides of the amoebae grown on labeled bacteria correspond closely to the specific activities of the nucleotides of the bacteria on which the amoebae were grown. This again confirms the indication that most of the label in the amoebal RNA is due to

Table F.

Specific activities of individual nucleotides of *E. coli* B_{s-1} and amoebae of *D. discoideum*.**

Experiment		Organism + Source of Label			
		E. coli B_{s-1} + $^{14}CO_2$	Labeled Bacteria	*D. discoideum* + Labeled Bacteria + $^{14}CO_2$	$^{14}CO_2$
1	CMP	618	619	4599	4916 (10)*
	AMP	426	397	4516	4188 (105)
	GMP	398	368	2358	2167 (50)
	UMP	242	248	4698	3746 (0)
2	CMP	1999	2797	2073	342
	AMP	1325	1433	1409	357
	GMP	1055	1049	1221	179
	UMP	1425	1601	1131	270
3	CMP	4507	5639	6864	215
	AMP	3097	3824	4382	263
	GMP	3251	4466	4251	140
	UMP	3003	5492	5564	307
4	CMP		9846	6862	69
	AMP		6723	5958	125
	GMP		5190	6310	65
	UMP		4803	6119	116

*Figures in parentheses represent specific activities of nucleotides of killed, unlabeled bacteria exposed to same $^{14}CO_2$ as amoebae grown on killed, unlabeled bacteria in experiment 1, followed by same extraction and hydrolytic procedures.

**Data are for individual determinations.

incorporation of whole nucleotides, rather than to catabolism of bacterial label to CO_2 and then fixation of the labeled CO_2 by the amoebae.

The incorporation of $^{14}CO_2$ into the nucleotides of amoebae, shown in the last column, demonstrates significant incorporation into both purine and pyrimidine nucleotides by the amoebae. The control figures for incorporation of label by killed, unlabeled bacteria in experiment 1 are listed beside the figures for the amoebae incorporation (in parentheses). This is confirmation of previous findings that the incorporation figures shown for $^{14}CO_2$ grown amoebae in this and other tables could not be due to incorporation by bacteria surviving heat and ultraviolet treatment.

In summary, the column chromatographic separation of the RNA hydrolysates and calculation of specific activities of RNA and of nucleotides of amoebae has shown clearly that the amoebae contain the biosynthetic pathways for purine and pyrimidine nucleotides. These pathways are not the only source of the nucleotides. The degradation of bacterial nucleic acids also provides nucleotides for the synthesis of amoebal nucleic acids.

DISCUSSION

The amoebae of the cellular slime mold Dictyostelium discoideum are capable of de novo biosynthesis of purine and pyrimidine nucleotides and of some amino acids. The presence of purine and pyrimidine pathways is most clearly demonstrated in Table F by the specific activities of amoebae grown on killed, unlabeled bacteria and $^{14}CO_2$ (last column). There is significant incorporation of label from CO_2 into purine and pyrimidine nucleotides in these labeled amoebae, especially in experiment 1. The presence of the amino acid biosynthetic pathways is demonstrated by fixation of $^{14}CO_2$ into protein in the same labeled amoebae (unlabeled bacteria + $^{14}CO_2$). The specific amino acid pathways present in the amoebae have not been identified beyond the speculation that many of them require the fixation of CO_2.

These pathways are not the major source of nucleotides and amino acids during growth of amoebae on bacteria. The specific activity of the protein and nucleic acids of amoebae grown on labeled bacteria correspond to the specific activities of the protein and nucleic acids of the bacteria on which they were grown, as shown in Table B, Tables E and F and the Appendix. The increase in specific activity of the RNA hydrolysates of amoebae grown on labeled bacteria over those of the bacteria is most probably largely due to the

difference in base composition of the two organisms (Table C). Determination of the amino acid composition of the amoebal protein should reveal a difference from the amino acid composition of bacterial protein which would account for most of the difference in the specific activities of the two organisms.

In experiments 2 through 6 the specific activities of the protein and nucleic acid fractions of amoebae grown on ^{14}C-labeled bacteria and $^{14}CO_2$ correspond closely to those of the amoebae grown on ^{14}C-labeled bacteria alone, indicating that the major source of the label found in the constituents of the amoebae labeled by ^{14}C-labeled bacteria and $^{14}CO_2$ was the constituents of the labeled bacteria. In experiment 1 the specific activities of the $^{14}CO_2$ used for labeling bacteria and amoebae were not of the same order of magnitude, the latter being several fold higher. The result of this difference was that the incorporation of carbon-14 in the amoebae from $^{14}CO_2$ was nearly ten fold higher than incorporation in amoebae from labeled bacterial constituents. The specific activity of the protein and nucleic acid of amoebae grown on labeled bacteria plus $^{14}CO_2$ corresponded closely to that of the amoebae grown on $^{14}CO_2$ alone because of the dominance of the specific activity of the $^{14}CO_2$ over that of the ^{14}C-labeled bacteria in the flask.

Nevertheless, it is difficult to say precisely to what extent the amoebae are capable of synthesizing nucleotides and amino acids, since, under the conditions employed in these

experiments, the amoebae use amino acids and nucleotides obtained primarily from bacterial constituents. The growing amoebae contain sufficient proteolytic and nucleolytic enzymes to break down bacterial protein and nucleic acids (Sussman and Sussman, 1969). Enzymes involved in the further catabolism of amino acids have been demonstrated in the differentiating slime mold (Firtel and Brakenbury, 1972) and the authors suggest that the slime mold maintains high levels of these catabolic enzymes at all stages of its life cycle.

The presence of biosynthetic pathways for amino acids and nucleotides is unnecessary during these growth conditions, and since the enzymes necessary for utilization of protein are present in all stages of the life cycle, the amino acid pathways at least are probably unnecessary throughout the life cycle. This is similar to the situation of glycolytic enzymes in the amoebae.

The enzymes of the glycolytic pathway exist in the differentiating slime mold at a time when protein degradation is the major source of energy and glucose equivalents are being used for the production of polysaccharides (Cleland and Coe, 1968). There is not much gluconeogenesis either, since the concentration of one enzyme in the pathway, fructose diphosphatase, is sufficiently low so that the pathway is almost blocked (Cleland and Coe, 1968). The authors found, however, that some 2% of the alkali-insoluble carbohydrate formed in the sorocarp was derived from aspartate, and that the major pathway implicated was glucogenesis via the glycolytic

pathway (Cleland and Coe, 1969). The low levels of de novo synthesis of amino acids and nucleotides indicated in these experiments may be due to a similar limitation by low activity of one enzyme in the pathway. On the other hand, the enzymes may be inducible, rising in activity during differentiation, but low during growth.

The existence of the biosynthetic pathways brings up the question of whether nucleotides and amino acids are partial or total requirements for growth or whether the amoebae can synthesize them in sufficient quantity and at a sufficient rate to supply all of the needs of the growing amoebae. The simplest medium for this slime mold defined so far is that devised by Watts and Ashworth (1970) of peptone, yeast extract and salts.

Strains of D. discoideum able to grow on this medium divide much more slowly than wild type D. discoideum grown on bacteria. Indications from these experiments are that the biosynthetic pathways of amoebae contribute a minor proportion of the amino acids and nucleotides to the precursor pools of amoebae. As in the case of P. pallidum (Hohl and Raper, 1963c), a strain of D. discoideum able to grow without some or all amino acids and/or nucleotides would probably grow so slowly that no logarithmic phase could be detected. A short generation time is one of the most useful aspects of microorganisms when used as a research tool, but a chemically defined medium and a strain of D. discoideum capable of growing on it would assist biochemical and genetic analyses of the organism (Ashworth, 1971).

Even though these biosynthetic pathways may not be necessary to the slime mold in the strict sense of being the sole source of amino acids and nucleotides in the cells, other roles for them may be suggested by examining the literature. At the onset of aggregation, when amoebae have used up all food, metabolites such as nucleotides and amino acids are released into the medium (Lee, 1972). The loss of metabolites places the cells in a condition of internal as well as external starvation. The cells lose metabolites into extracellular medium at this critical transition period possibly because they do not have any active means of retaining some of them (Lee, 1972). Lee has suggested that proliferation or differentiation of cells depends on the "availability of a balanced diet of nutrients at sufficient concentrations to counterbalance losses due to leakage". If the cells retain the biosynthetic pathways for amino acids and nucleotides at this time, then the pathways cannot be very active or the cells would never begin the aggregation process.

Krichevsky and Love (1968) found that mononucleotides have the ability to stimulate the morphogenetic process while amino acids do not. The presence of nucleotide biosynthetic pathways at this point might play a role in stimulating morphogenesis. The other intracellular source of mononucleotides is from the degradation of RNA. Sussman and Sussman (1969) have shown that the RNA for the synthesis of a number of enzymes in the differentiation process is synthesized several hours before it is translated. Any messenger

RNA present at the end of the vegetative stage may be involved in the synthesis of enzymes needed later on in the cycle. Indeed, Pannbacker (1966) has suggested that differentiation may proceed with very little RNA synthesis beyond the aggregation stage. This being the case, the biosynthesis of mononucleotides may be important in synthesizing this messenger RNA and in blocking the need for breakdown of existing messenger and ribosomes for resynthesis and metabolic purposes.

While no direct search for biosynthetic pathways of nucleotides and amino acids has been conducted, there is some evidence in the literature of other means of entry of nucleotides into nucleotide pools in the slime mold. Rutherford and Wright (1971) measured the incorporation of labeled phosphate and labeled adenine into adenine nucleotides. They observed incorporation of labeled phosphate into AMP as well as ADP and ATP. The labeling of AMP might be due to biosynthesis of nucleotides or to biosynthesis of phosphoribosylpyrophosphate and incorporation of free bases into the nucleotides. They also observed incorporation of labeled adenine into all adenine nucleotides. This confirms the possibility of salvage-like reactions found in E. coli (Roberts et al., 1955) and in mammalian cells (Kornberg et al., 1955; Lieberman et al., 1955). Other RNA studies have involved the incorporation of labeled uracil into RNA (Pannbacker, 1966; Sussman, 1967). These studies involving incorporation of both purine and pyrimidine bases into RNA of the slime mold agree with isotopic competition studies on

E. coli which show that free purine and free pyrimidine bases can compete with $^{14}CO_2$ for incorporation into nucleotides and RNA (Roberts et al., 1955).

R. Sussman (1967) has studied the RNA metabolism of another cellular slime mold, Polysphondelium pallidum. She found that during differentiation the net RNA content of the organism fell to about 60% of its initial value, but the specific activity of the ^{14}C-uracil labeled RNA fell to 57% of its original value. Had there been no resynthesis of RNA, the specific activity of the RNA remaining at the end of the cycle should have been 100% of the specific activity of the RNA present after the vegetative stage. The author suggested that this dilution of the isotope was due to new synthesis of RNA. This must involve recycling of labeled nucleotides and de novo synthesis of unlabeled nucleotides. She indicated that there was considerable conservation of nucleotides, but her data cannot be explained without some de novo synthesis of the precursors.

The possibility should also be investigated in Dictyostelium discoideum. Several attempts at calculation of rates of RNA synthesis by labeled precursor incorporation have been made (Inselberg and Sussman, 1967; Pannbacker, 1966). Specific activities of precursor pools must be known for such calculations to be accurate, and de novo synthesis of nucleotides would act to continually dilute the specific activity of these pools. It should be accounted for in the calculations.

Wright and Anderson (1960a, b) carried out ^{35}S-methionine incorporation studies on the amino acids and protein pools of the cellular slime mold. At no time in their considerations did they mention de novo biosynthesis of amino acids or of methionine, even though they found that there was a lack of total equilibration between externally labeled methionine molecules and internal methionine molecules. They supposed that this was due to catabolism of endogenous protein by the slime mold and to localization of endogenous pools, but it might also be due to the biosynthesis of methionine in the slime mold.

During differentiation, the slime mold cell mass is constantly losing metabolites and macromolecules. The ability to interconvert and synthesize amino acids and nucleotides during this time would be a most useful part of metabolism. The biochemical events of the differentiating slime mold have been roughly divided into two classes (Hames, Weeks and Ashworth, 1972). There are those "that are necessarily involved with the characteristic progressive changes in morphology that define the differentiation of the cells", and there are those "necessary to maintain the vital functions of the cells despite the changing environment and interactions imposed on the cells by the morphogenesis of which they are part". The pathways under consideration in these studies would fall into the latter category.

SUMMARY

The amoebae of Dictyostelium discoideum incorporate bacterial nucleotides and amino acids into their own nucleic acids and protein. They will also fix CO_2 in the de novo biosynthesis of nucleotides and amino acids.

The presence of biosynthetic pathways specific for purines and pyrimidines has been demonstrated. The specific biosynthetic pathways for amino acids present in the slime mold have not been identified, although the data on fixation of CO_2 into protein indicates that they include amino acids which require CO_2 fixation during de novo biosynthesis. The amino acids synthesized de novo by amoebae using CO_2 derived carbons are found in amoebal protein in the same proportions as amino acids containing CO_2 derived carbons which are synthesized de novo by bacteria.

Under the growth conditions employed here bacterial amino acids and nucleotides appear to be the major source of precursors for amoebal protein and nucleic acids and the contribution of the biosynthetic pathways to the precursor pools is minor. The pathways might prove more important under other growth conditions.

APPENDIX

The data presented for each experiment show the distribution of the total label recovered in each of the bacterial and amoebal flasks. The numbers represent the total label recovered in the flask, the portion of that label which was recovered in the harvested cells and in the suspending medium. The label recovered in the cells is categorized into the amount of label recovered in each fraction as described in the Materials and Methods section. The numbers in parentheses are the specific activities of the appropriate fractions, DPM/micromole of nucleotide (from the column chromatographic separations) and DPM/milligram of protein (determined by the Lowry method). The numbers listed for the *E. coli* B_{s-1} are the analyses of the bacteria fed to the amoebae.

Experiment 1

	Organism + Source of Label			
	E. coli B_{s-1} + $^{14}CO_2$	Labeled Bacteria	*D. discoideum* + Labeled Bacteria + $^{14}CO_2$	$^{14}CO_2$
Total DPM recovered		31,140	1,248,900	900,720
Total DPM in medium		16,150	1,116,090	823,230
Total DPM in cells	125,450	14,990	132,810	77,490
DPM in fractions				
Monomer + lipid	4,870	530	5,700	5,100
RNA + DNA	103,180 (415)	11,440 (385)	101,640 (4,125)	53,890 (3,750)
Protein	17,400 (170)	3,020 (310)	25,470 (2,625)	18,500 (2,500)
Total mg protein in cells	104	9.7	9.7	7.4

Experiment 2

	Organism + Source of Label			
	E. coli B s-1 + $^{14}CO_2$	Labeled Bacteria	*D. discoideum* + Labeled Bacteria + $^{14}CO_2$	$^{14}CO_2$
Total DPM recovered		119,140	301,260	209,900
Total DPM in medium		49,740	234,020	183,380
Total DPM in cells	296,860	69,400	67,240	26,520
DPM in fractions				
Monomer + lipid	15,840	2,690	2,850	2,330
RNA + DNA	179,540 (1,390)	35,250 (1,685)	31,710 (1,410)	10,550 (285)
Protein	101,480 (1,430)	31,460 (1,248)	32,680 (1,650)	13,640 (530)
Total mg protein in cells	71	25.2	19.8	25.8

Experiment 3

	Organism + Source of Label			
	E. coli B_{s-1} + $^{14}CO_2$	Labeled Bacteria	*D. discoideum* + Labeled Bacteria + $^{14}CO_2$	$^{14}CO_2$
Total DPM recovered		400,760	537,120	67,590
Total DPM in medium		147,710	251,680	50,930
Total DPM in cells	1,301,350	253,050	285,440	16,660
DPM in fractions				
Monomer + lipid	80,870	13,330	10,160	670
RNA + DNA	841,170 (3,410)	94,490 (4,900)	103,900 (5,260)	6,860 (240)
Protein	379,310 (3,030	145,230 (6,790)	171,380 (7,200)	9,130 (415)
Total mg protein in cells	125	21.4	23.8	22.0

Experiment 4

	Organism + Source of Label			
	E. coli B_{s-1} + $^{14}CO_2$	Labeled Bacteria	*D. discoideum* + Labeled Bacteria + $^{14}CO_2$	$^{14}CO_2$
Total DPM recovered		1,093,260	1,175,930	158,060
Total DPM in medium		674,180	744,390	145,050
Total DPM in cells	2,302,040	419,080	431,540	13,010
DPM in fractions				
Monomer + lipid	135,400	13,530	20,030	660
RNA + DNA	1,351,820	177,460 (6,390)	202,810 (6,280)	5,160 (98)
Protein	814,820 (6,790)	228,090 (11,130)	208,700 (10,760)	7,190 (310)
Total mg protein in cells	120	20.5	19.4	23.2

Experiment 5

	Organism + Source of Label			
	E. coli B s-1 + $^{14}CO_2$	Labeled Bacteria	*D. discoideum* + Labeled Bacteria + $^{14}CO_2$	$^{14}CO_2$
Total DPM recovered		121,360	219,850	19,560
Total DPM in medium		54,380	121,350	17,580
Total DPM in cells	308,960	66,980	98,500	1,980
DPM in fractions				
Monomer + lipid	9,180	4,590	4,470	150
RNA + DNA	197,240	37,600	52,980	970
Protein	102,540 (1,280)	24,790 (1,130)	41,050 (1,345)	860 (80)
Total mg protein in cells	80	22.0	30.5	11

Experiment 6

	Organism + Source of Label			
	E. coli B_{s-1} + $^{14}CO_2$	Labeled Bacteria	*D. discoideum* + Labeled Bacteria + $^{14}CO_2$	$^{14}CO_2$
Total DPM recovered		925,780	1,064,410	130,150
Total DPM in medium		545,580	965,360	122,140
Total DPM in cells	2,129,050	380,200	103,050	8,010
DPM in fractions				
Monomer + lipid	79,750	19,280	5,900	450
RNA + DNA	1,519,700	194,210	65,700	4,130
Protein	529,600 (5,040)	166,710 (7,580)	31,450 (5,420)	3,430 (820)
Total mg protein in cells	105	22	5.8	4.2

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